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SYMPOSIUM REPORT

CaMKII phosphorylation of the $GABA_A$ receptor: receptor subtype- and synapse-specific modulation

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As a major inhibitory neurotransmitter, GABA plays a vital role in the brain by controlling the extent of neuronal excitation. This widespread role is reflected by the ubiquitous distribution of $GABA_A$ receptors throughout the central nervous system. To regulate the level of neuronal inhibition requires some endogenous control over the release of GABA and/or its postsynaptic response. In this context, Ca^{2+} ions are often used as primary or secondary messengers frequently resulting in the activation of protein kinases and phosphatases. One such kinase, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), can target the $GABA_A$ receptor to cause its phosphorylation. Evidence is now emerging, which is reviewed here, that $GABA_A$ receptors are indeed substrates for CaMKII and that this covalent modification alters the expression of cell surface receptors and their function. This type of regulation can also feature at inhibitory synapses leading to long-term inhibitory synaptic plasticity. Most recently, CaMKII has now been proposed to differentially phosphorylate particular isoforms of $GABA_A$ receptors in a synapse-specific context.

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Type A γ-aminobutyric acid ($GABA_A$) receptors form part of the Cys-loop ligand-gated ion channel family and are activated by GABA, which is released from inhibitory axon terminals (Fritschy & Brunig, 2003; Jacob *et al.* 2008). They are responsible for controlling neuronal excitability, which they achieve by mediating two forms of inhibition, termed synaptic and tonic (Farrant & Nusser, 2005). The former relies on the rapid activation of $GABA_A$ receptors at inhibitory synapses following the phasic release of GABA, whereas tonic inhibition requires the persistent activation of extrasynaptic $GABA_A$ receptors by low ambient levels of GABA.

The coding and integration of information is a major function of the mammalian central nervous system and this relies heavily on the interplay between synaptic excitation and inhibition (Silberberg *et al.* 2005). Therefore, regulating the efficacy of GABA mediated inhibition, by affecting GABA current amplitude and/or its duration, will have significant consequences for the input-output relationships of neurons.

There are modulators that can change synaptic efficacy by targeting pre- and/or postsynaptic sites. For synaptic inhibition, the action potential driven release of GABA often attains quite high concentrations, sufficient to fully occupy the postsynaptic $GABA_A$ receptors (Grabauskas, 2005; Nusser *et al.* 2001). Thus, under normal physiological conditions, inhibitory postsynaptic current (IPSC) amplitudes may not be increased by modulators unless the number of postsynaptic receptors is also increased. Modulators that affect $GABA_A$ receptor kinetics are more likely to affect the IPSC duration. By contrast, for tonic inhibition, manipulating the current duration is less meaningful due to the persistent nature of the $GABA_A$ receptor activation; however, modulating the tonic current amplitude is feasible as the basal concentrations of GABA activating extrasynaptic receptors will be insufficient to attain full receptor occupancy (Mody, 2001; Farrant & Nusser, 2005; Mortensen & Smart, 2006).

In addition to short-term changes to synaptic inhibition, normal function in the nervous system also requires synaptic transmission to be dynamically regulated over longer periods. Such synaptic plasticity, often

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involving receptor trafficking, is thought to be the cellular and molecular correlate of learning and memory (Lisman *et al.* 2002; Neves *et al.* 2008) as well as being important for the appropriate establishment of synaptic connections during development (Ben Ari *et al.* 2007).

One of the most important groups of modulators that affect $GABA_A$ receptor mediated inhibition are the protein kinases (Moss & Smart, 1996; Brandon *et al.* 2002; Kittler & Moss, 2003). They catalyse phosphorylation by transferring a charged phosphate group from ATP to the substrate protein. Activating protein kinases by intracellular signalling pathways has the ability to directly affect receptor function and/or alter the trafficking of receptors. Here, we concentrate on reviewing the evidence underlying the roles that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has in the modulation of $GABA_A$ receptor function.

The $GABA_A$ receptor: a substrate for CaMKII?

Calcium/calmodulin-dependent protein kinase II is a serine/threonine second messenger-dependent protein kinase that responds to increases in intracellular Ca^{2+} concentrations (Soderling *et al.* 2002; Hudmon & Schulman, 2002; Fink & Meyer, 2002). It is a multifunctional protein that phosphorylates a broad range of substrates many of them involved in synaptic transmission (Colbran, 2004; Schulman, 2004). It is activated by the binding of Ca^{2+}/Calmodulin, which disrupts the association of an auto-inhibitory domain with the catalytic domain thereby enabling the latter to bind to substrates. The regulation of CaMKII activity is complex and is determined by a number of autophosphorylation sites, one of which (Thr^{286}) also acts to disrupt the auto-inhibitory domain and allows Ca^{2+}-independent kinase activity (Hudmon & Schulman, 2002).

CaMKII is well known as an important part of the postsynaptic density of excitatory synapses (Sheng & Hoogenraad, 2007). It can associate with a number of different synaptic scaffolding proteins (Colbran, 2004), which can potentially alter its activity (Strack *et al.* 2000; Robison *et al.* 2005). CaMKII is known to translocate to the postsynaptic density after its activation (Shen & Meyer, 1999; Shen *et al.* 2000; Merrill *et al.* 2005). The ability of CaMKII to regulate excitatory synaptic transmission and also to sustain a persistent level of kinase activity after undergoing autophosphorylation at Thr^{286} has led to its recognition as a key molecule underlying the synaptic plasticity phenomenon of long-term potentiation (LTP) and the cellular mechanisms that underlie learning and memory (Lisman *et al.* 2002; Merrill *et al.* 2005).

By comparison, the potential involvement of CaMKII in regulating inhibitory synaptic transmission has received less attention. Such an interaction was evident from early studies that introduced pre-activated CaMKII into neurons via a patch or intracellular recording pipette (Table 1). Pre-activation of the kinase with Ca^{2+} and calmodulin removes the normal reliance of activation on Ca^{2+} influx and Ca^{2+} stores. For both GABA-evoked whole-cell currents in isolated spinal dorsal horn neurons and GABA-mediated evoked IPSPs in CA1 hippocampal pyramidal cells, supplementing the intracellular solution with the α isoform of CaMKII led to the enhancement of current amplitudes (Wang *et al.* 1995). A similar application of pre-activated CaMKII to cerebellar Purkinje cells was also found to enhance GABA-induced whole-cell currents and sIPSCs (Kano *et al.* 1996). In both the hippocampal and cerebellar studies, heat-inactivated CaMKII was ineffective. Whilst demonstrating that the modulation of $GABA_A$ receptors by CaMKII was clearly possible, these early studies did not address whether CaMKII was directly phosphorylating the receptor, or if its effect was mediated by intermediary proteins. It was therefore quite important to know whether the $GABA_A$ receptor was a substrate for CaMKII and this could only be established using biochemical methods.

Biochemical studies of CaMKII phosphorylation of $GABA_A$ receptors

To ascertain whether protein kinases are directly affecting the function and/or trafficking of neurotransmitter receptors requires an unequivocal demonstration that the receptors are themselves phosphorylated by the kinases. Studies of many other proteins have enabled a consensus site for CaMKII to be proposed as: R-X-X-**S/T**, where the phosphorylated serine or threonine residues are N-terminally flanked by two 'recognition neutral' residues (X) that could be any amino acid, and preceded by an arginine (R), with its positively charged side-chain (Kennelly & Krebs, 1991; Pearson & Kemp, 1991; Moss & Smart, 1996). For $GABA_A$ receptors, such consensus sites have been identified within the large intracellular domain residing between transmembrane (M) domains 3 and 4 of receptor β and γ subunits. To determine whether these putative consensus sites were actually phosphorylated by CaMKII, purified glutathione-*S*-transferase (GST) fusion proteins of the intracellular domains for GABA receptor β and $\gamma 2$ subunits were generated (McDonald & Moss, 1994; Brandon *et al.* 2002). These GST fusion proteins were exposed to CaMKII in the presence of ^{32}P-orthophosphoric acid and then subject to two-dimensional phosphopeptide mapping and finally phosphoamino acid analysis (Moss *et al.* 1992). This revealed sites of phosphorylation for CaMKII on the $\beta 1$ fusion protein at Ser^{409} (RRRA**S**QLK) and Ser^{384} (RKPL**S**SRE) (McDonald & Moss, 1994). The latter site is interesting because it departs from the typical consensus site for CaMKII and is also a unique site of

Table 1. Effect of CaMKII on native and recombinant $GABA_A$ receptors

Cells	Receptors	CaMKII prep	Phosphatase inhibitors	Kinase inhibitors	Plasticity	Other effects	Ref
Cerebellar Purkinje neurons	Native	Native, depol. stim.	Calyculin-A (PP1/PP2A)	Staurosporine, KN-62, calmodulin binding domain peptide (CBD), calmidazolium, heat inactivation	sIPSCs, I_{GABA}	Amplitude enhanced	Kano *et al.* 1996
		Preactivated CaMKI			I_{GABA}	Amplitude enhanced	
Dorsal horn spinal neurones	Native	Preactivated CaMKII	Calyculin-A (PP1, PP2A)	Heat inactivation	I_{GABA}	Amplitude increased, Desensitisation reduced	Wang *et al.* 1995
Hippocampal CA1					eIPSPs	Enhanced amplitude	
HEK cells	Recombinant $\alpha1\beta2$, $\alpha1\beta2\gamma2$	Preactivated CaMKII	—	—	I_{GABA}	No modulation	Houston and Smart 2006
	$\alpha1\beta3$, $\alpha1\beta3\gamma2$				I_{GABA}	No modulation	
NG108-15 cells	Recombinant $\alpha1\beta2$, $\alpha1\beta2\gamma2$	Preactivated CaMKII	—	—	I_{GABA}	No modulation	Houston and Smart 2006
	$\alpha1\beta3$, $\alpha1\beta3\gamma2$				I_{GABA}	Enhanced	
Cerebellar granule neurons	Recombinant $\alpha1\beta2$, $\alpha1\beta2\gamma2$	Preactivated CaMKII	—	—	I_{GABA}	No modulation	Houston and Smart 2006
	$\alpha1\beta3$, $\alpha1\beta3\gamma2$				I_{GABA}	Enhanced	
NG108-15 cells	Recombinant $\alpha1\beta3^{S383A}$	Preactivated CaMKII	—	—	I_{GABA}	No modulation	Houston *et al.* 2007
	$\alpha1\beta3^{S383A}\gamma2$				I_{GABA}	Reduced modulation	
	$\alpha1\beta3^{S383A}+\gamma2^{Y365F,Y367F}$				I_{GABA}	No modulation	
Cerebellar granule neurons	Native	Preactivated CaMKII	—	—	I_{GABA}	Enhanced	Houston and Smart 2006
Cerebellar granule neurons	Native $\beta2^{-/-}$	Preactivated CaMKII	—	—	I_{GABA}	Enhanced	Houston and Smart 2006
Cerebellar granule neurons	Native	Preactivated CaMKII	—	—	sIPSCs	Amplitude enhanced, Duration prolonged	Houston *et al.* 2008
Cerebellar granule neurons	Native $\beta2^{-/-}$	Preactivated CaMKII	—	—	sIPSCs	Only duration prolonged	Houston *et al.* 2008
Hippocampal CA1	Native	Native $+Ca^{2+}$/calmodulin		CaMKII autoinhib. pep. (281–301), BAPTA, Vincristine, Cytochalasin D, Colchicine	eIPSCs sIPSCs	Amplitude enhanced, Amplitude & Frequency enhanced	Wei *et al.* 2004

Summary of the effects of CaMKII on native neuronal $GABA_A$ receptors (wild-type and a $\beta2$ knock-out), and on recombinant $GABA_A$ receptors. The CaMKII preparations used are either: native, which is then activated by depolarising stimulation (depol. stim.) together in one study, with the intracellular addition of Ca^{2+} and calmodulin, or it is a preactivated form of CaMKII that is perfused internally into the cells. The effect of CaMKII was studied on whole-cell GABA-activated currents (I_{GABA}) or spontaneous (s) or evoked (e) IPSCs, with one study using IPSPs.

Table 2. Sites of phosphorylation for CaMKII on $GABA_A$ receptor β and γ2 subunits

Receptor subunit	CaMKII substrate residues			Reference
α1	n/i	—	—	Churn *et al.* 2002
β1	S384	S409	—	McDonald & Moss, 1994
β2	—	S410	—	
β3	S383	S409	—	
γ2S	—	S348	T350	Houston *et al.* 2007
γ2L	S343	S348	T350	

The sites of CaMKII phosphorylation on $GABA_A$ receptor subunits are shown. The residues (S-serine; T-threonine) are located between the 3rd and 4th transmembrane domains of β and γ2 subunits and have been identified using biochemical analyses of GSK fusion proteins. n/i- residue(s) not identified.

phosphorylation on $GABA_A$ receptors that is reserved for CaMKII. By contrast, Ser^{409} is an established site of phosphorylation for other serine/threonine kinases, such as cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG) and Ca^{2+}-dependent protein kinase (PKC) (Moss & Smart, 1996; Brandon *et al.* 2002). Similar analyses, using fusion proteins based on the large intracellular domains of β2 and β3 subunits, identified only Ser^{410} (RRRA**S**QLK) as the major site of CaMKII phosphorylation in β2 subunits, whilst Ser^{383} (RKQ**S**MPK) and Ser^{409} (RRRS**S**QLK) were substrates for CaMKII in β3 subunits (McDonald & Moss, 1997). As for S^{409} in β1, the homologous Ser^{410} in β2 and Ser409 in β3 are also substrates for other serine/threonine kinases (Moss & Smart, 1996; Brandon *et al.* 2000), but Ser^{383} in β3 subunits, just like Ser^{384} in β1, is a unique site for CaMKII (Table 2).

Biochemical analysis of phosphorylation also revealed that a γ2 fusion protein was phosphorylated by CaMKII at Ser^{348} (IRPR**S**ATIQ) and Thr^{350} (PRSA**T**IQMN) (McDonald & Moss, 1994). The γ2 subunit can be expressed in two forms (short (S) or long (L)) following alternative RNA splicing (Whiting *et al.* 1990). The extra insert of eight amino acids (LLRMF**S**FK) in γ2L contains another consensus sequence for phosphorylation (Moss & Smart, 1996). Indeed, Ser^{343} proved to be phosphorylated by CaMKII in the γ2L form (McDonald & Moss, 1994; Machu *et al.* 1993) and it is also a substrate for PKC (Krishek *et al.* 1994).

Although it is considered that $GABA_A$ receptor β and γ subunits are the major substrates for protein kinase phosphorylation, there is evidence that other subunits may also be phosphorylated. Activation of endogenous CaMKII in a synaptosomal membrane preparation has been shown to increase agonist binding by increasing the number of GABA receptors (Churn & DeLorenzo, 1998). Notably, this group reported that the α1 subunit from rat forebrain can be phosphorylated by CaMKII, but the residue(s) that form the substrate site have yet to be identified (Churn *et al.* 2002). Thus, the major outcome from the biochemical studies is a clear demonstration that the $GABA_A$ receptor is a substrate for CaMKII, and that phosphorylation occurs principally on β and γ2 subunits, and most likely also on the α1 subunit. The identification of these phosphorylated residues posed the inevitable question – what are the effect(s) of CaMKII phosphorylation on $GABA_A$ receptor function and trafficking?

CaMKII regulation of $GABA_A$ receptor function

The effects of covalent modification of $GABA_A$ receptor structure by protein kinase phosphorylation can range from altered receptor function to the regulation of receptor trafficking (Moss & Smart, 1996; Smart, 1997; Brandon *et al.* 2002; Kittler & Moss, 2003). To understand how phosphorylation affects $GABA_A$ receptors and to determine the importance of the receptor's substrate sites usually requires the use of recombinant receptors expressed in heterologous cell lines. The usual cellular vehicle for such studies is the human embryonic kidney (HEK) cell line, but for CaMKII this proved a poor choice as $\alpha1\beta2$ and $\alpha1\beta3$ $GABA_A$ receptors were unaffected by CaMKII (Houston & Smart, 2006) (Table 1). By contrast, the undifferentiated NG108-15 neuroblastoma cell line, which also lacks endogenous $GABA_A$ receptors (Searles & Singer, 1988; Ishizawa *et al.* 1997; Houston & Smart, 2006), but retains a neuronal lineage unlike HEK cells, supported CaMKII modulation of recombinant $GABA_A$ receptors. In these cells, CaMKII potentiated GABA-activated currents for $\alpha1\beta3$ and $\alpha1\beta3\gamma2$S receptors, but not for $\alpha1\beta2$ and $\alpha1\beta2\gamma2$S $GABA_A$ receptors (Houston & Smart, 2006). The reasons why CaMKII was only able to modulate $GABA_A$ receptors in NG108-15 cells, is unknown, but it could reflect a limited availability of anchoring proteins for CaMKII to bind to $GABA_A$ receptors in HEK cells. To date, little is known about the anchoring of CaMKII to $GABA_A$ receptors.

Interestingly, demonstrating that PKA could modulate $\alpha1\beta2\gamma2$ receptors in HEK cells also proved difficult (McDonald *et al.* 1998) despite PKA modulating IPSCs in neurons that express high levels of the β2 subunit (Poisbeau *et al.* 1999; Nusser *et al.* 1999). In comparison, phosphorylation of β2 subunits at Ser^{410}, the major substrate in this subunit for all Ser/Thr kinases, has been shown to mediate an increase in cell surface receptor number when phosphorylated by PKB in HEK cells and neurons (Wang *et al.* 2003).

A differential regulation of $GABA_A$ receptors by CaMKII was also evident after over-expressing $\alpha1/\beta3$ or $\alpha1/\beta2$ subunits in cultured cerebellar granule cells (Houston & Smart, 2006). The potentiation of GABA currents by CaMKII, apparent with $\beta3$ over-expressing neurons, was significantly reduced when there was a surplus of $\beta2$ subunit-containing receptors. Although $\beta2$ subunits are known to be phosphorylated by CaMKII, the lack of any functional effect suggested that $\beta2$ subunit phosphorylation may have a distinct role. Indeed, the potentiation of GABA currents by CaMKII is unaffected when recording from neurons taken from $\beta2^{-/-}$ mice (Houston & Smart, 2006). Thus, in terms of functional regulation, the major $GABA_A$ receptor target for CaMKII appears to be the $\beta3$ subunit, as $\beta1$ subunits are poorly expressed in these neurons (Laurie *et al.* 1992; Pirker *et al.* 2000).

Sites of action for CaMKII on $GABA_A$ receptors

Identifying the sites for CaMKII phosphorylation on $GABA_A$ receptors does not necessarily indicate that phosphorylation at each of these sites is able to modulate GABA-activated currents. By expressing $\alpha1\beta3$ receptors in NG108-15 cells, and using site-directed mutagenesis of $GABA_A$ receptor $\beta3$ subunits, Ser^{383} was identified as the major site for CaMKII modulation of GABA currents, as phosphorylation of Ser^{409} had no functional consequences (Houston *et al.* 2007). This might explain the lack of effect of CaMKII in modulating $GABA_A$ receptors containing the $\beta2$ subunit, since the homologous residue to Ser^{383} in the $\beta3$ subunit is not phosphorylated by CaMKII.

Mutating both the CaMKII substrates in the $\gamma2S$ subunit, Ser^{348} and Thr^{350}, did not affect the modulation of the $\alpha1\beta3\gamma2S$ receptor. However, co-expressing the mutant $\beta3$ subunit ($\beta3^{S383A}$), which abolished potentiation by CaMKII on $\alpha1\beta3$ receptors, with $\alpha1$ and $\gamma2S^{S348A,T350A}$, reduced, but did not ablate the functional effects of CaMKII (Houston *et al.* 2007). This strongly suggested that other residues must be involved, yet the biochemical analyses had not identified any further CaMKII substrates. This implied that another kinase may be involved. The prime candidate was a tyrosine kinase as this had previously been shown to potentiate GABA currents at $\alpha\beta\gamma$ subunit-containing receptors (Moss *et al.* 1995; Valenzuela *et al.* 1995). Indeed, genistein, a tyrosine kinase inhibitor, reduced CaMKII potentiation of GABA currents at $\alpha1\beta3\gamma2S$ receptors and abolished it at $\alpha1\beta3^{S383A}$ $\gamma2S$. Mutating the sites for tyrosine phosphorylation on the $\gamma2S$ subunits, Y^{365} and Y^{367}, together with the CaMKII site on the $\beta3$ subunit, S^{383}, was entirely sufficient to ablate the effects of CaMKII on GABA-activated currents (Houston *et al.* 2007). These results clearly implicated the tyrosine residues in the regulation of $GABA_A$ receptor function after CaMKII activation. This was emphasised by biochemical analyses demonstrating increased phosphorylation of tyrosine residues 365 and 367 on the $\gamma2$ subunit after CaMKII was activated. Thus tyrosine kinases and CaMKII may interact to modulate $GABA_A$ receptors, with CaMKII possibly activating a tyrosine kinase (Zwick *et al.* 1999; Guo *et al.* 2004; Fan *et al.* 2005).

Regulation of inhibitory synaptic receptors by CaMKII

Although preceding studies revealed that CaMKII phosphorylation depended upon the $GABA_A$ receptor subunit composition, and also identified which sites on the receptors were actually phosphorylated by CaMKII, we had little idea as to whether the location of the receptor in the cell membrane (e.g. synaptic or extrasynaptic) was important. This was most simply studied by monitoring GABA-mediated IPSCs in the presence of CaMKII. Any modulation would immediately implicate synaptic receptors as targets for this Ca^{2+}-activated kinase. In cerebellar granule neurons, CaMKII increased the amplitude and prolonged the duration of sIPSCs (Houston *et al.* 2008). By using a $\beta2$ knock-out line, these effects were attributed to specific receptor isoforms with an increased IPSC amplitude associated with $\beta2$ subunit receptors and the prolongation of the sIPSC duration reflecting phosphorylation of $\beta3$ subunit-containing $GABA_A$ receptors (Houston *et al.* 2008). Immunofluorescence of $\beta2$ and $\beta3$ subunits in cerebellar granule neurons suggested that some inhibitory synapses are likely to contain $GABA_A$ receptors with only one or other of the β subunits. This differential localisation of β subunits is also apparent in dentate gyrus granule cells where $\beta2$ subunits are mostly extrasynaptic and $\beta3$ subunits primarily synaptic (Herd *et al.* 2008). Thus CaMKII modulation of $GABA_A$ receptors is not only subtype selective but also appears to be inhibitory synapse specific.

The surprising result from this study was that $\beta2$ subunit-containing receptors were modulated by CaMKII. This was unexpected considering that $\beta2$ subunit receptors expressed in NG108-15 cells remained unaffected by CaMKII. This observation implies that the cytoskeleton, scaffolding proteins and signalling pathways present at inhibitory synapses may be important for CaMKII to modulate $GABA_A$ receptors. It further suggests that $\beta2$ subunit-containing receptors expressed at extrasynaptic locations are not modulated by CaMKII, probably due to a lack of kinase anchoring proteins.

Using non-stationary noise analysis of the sIPSC decays suggested that CaMKII enhanced IPSC amplitudes by increasing the number of open $\beta2$ subunit-containing channels at the peak of the IPSC. This could have occurred

by simply increasing receptor number in the synapse, and/or by increasing the channel open probability. By contrast, for β3 subunit-containing receptors, the change in sIPSC decay rate was a consequence of altered channel kinetics following phosphorylation of the receptor (Houston *et al.* 2008). Recruitment of $GABA_A$ receptors to the cell surface membrane has been suggested in the hippocampus, where IPSC amplitudes were enhanced by promoting internal Ca^{2+} release, or by internally perfusing neurons with Ca^{2+}/calmodulin (Wei *et al.* 2004). The effectiveness of CaMKII inhibitor peptide (281–301) and the blockers of tubulin and actin polymerization in preventing the potentiation of IPSCs implicated CaMKII in the trafficking of synaptic $GABA_A$ receptors.

Ca^{2+}/calmodulin dependent regulation of $GABA_A$ receptors

In addition to CaMKII regulating $GABA_A$ receptors, several studies have also noted that these receptors can be modulated by just changing intracellular Ca^{2+} and calmodulin concentrations (Inoue *et al.* 1986; Taleb *et al.* 1990; Chen *et al.* 1990; Mouginot *et al.* 1991; Stelzer & Shi, 1994; Chen & Wong, 1995; Aguayo *et al.* 1998; Akopian *et al.* 1998; Lu *et al.* 2000; Yu *et al.* 2006), possibly by altering the phosphorylation status of the $GABA_A$ receptor. It is therefore not surprising that many effects have been reported, presumably depending on whether the elevation of internal Ca^{2+} concentration resulted in kinase or phosphatase activation, or simply from a direct action on the $GABA_A$ receptor. Changes to GABA current amplitudes can occur by affecting the entry/exit rates of the receptor into/out of various conformations, such as desensitised states, e.g. high internal Ca^{2+} reduced whole-cell GABA current amplitudes and increased their desensitisation in hippocampal neurons (Mozrzymas & Cherubini, 1998). The potential for Ca^{2+} and calmodulin to act independently of CaMKII can cause difficulties when interpreting the effect of this kinase on $GABA_A$ receptors. Such difficulties can be obviated by pre-activating CaMKII prior to its use. Thus any regulation of synaptic $GABA_A$ receptors is likely to be a function of CaMKII activity, rather than being caused by Ca^{2+} or calmodulin. Furthermore, a direct effect of CaMKII on the phosphorylation status and function of $GABA_A$ receptors can also be inferred by establishing the effectiveness of mutating substrate sites on the receptor in ablating the actions of the kinase (Houston *et al.* 2007).

CaMKII and inhibitory synaptic plasticity

Changes to inhibitory transmission that last longer than just a few minutes can be indicative of underlying structural changes at synapses. These could be manifest in the formation of new synapses or long-term changes in the number of receptors expressed at existing synapses; both could also be coupled with chronic alterations to receptor function. All of these changes typically characterise synaptic plasticity (Bliss *et al.* 2003). For inhibitory synapses, there is now increasing evidence of numerous forms of synaptic plasticity (Gaiarsa *et al.* 2002), but one of the earliest recorded examples that involves CaMKII concerns the phenomenon of rebound potentiation, which is expressed at the interneuron–Purkinje cell synapse in the cerebellum and involves an increase in the mean IPSC amplitude (Kano *et al.* 1992).

Rebound potentiation can be induced by stimulating the climbing fibre (CF) input (Kano *et al.* 1992) or by directly depolarizing the Purkinje neuron (Hashimoto *et al.* 1996) to cause internal Ca^{2+} to rise. Notably, whole-cell GABA-activated currents are also enhanced by CF stimulation. The increase in the IPSC amplitude can be blocked by inhibiting the internal Ca^{2+} rise or by inhibiting CaMKII (Kano *et al.* 1996; Hashimoto *et al.* 1996; Kawaguchi & Hirano, 2002). A similar enhancement was observed after just internally perfusing unstimulated Purkinje neurons with CaMKII, and calyculin-A, a protein phosphatase 1 and 2A inhibitor (Kano *et al.* 1996). A variety of inhibitors ranging from KN-62 (CaMKII inhibitor) and staurosproine (non-specific kinase inhibitor) to calmidazolium (calmodulin antagonist) and calmodulin binding domain peptide (dominant-negative peptide), all resulted in a reduced rebound potentiation providing compelling evidence of a role for CaMKII in this form of synaptic plasticity. Delaying the application of KN-62 or calmidazolium until 5 min after the CF stimulation was sufficient to neutralise their inhibitory activity (Kano *et al.* 1996). This suggested that activation of CaMKII was necessary to induce or trigger rebound potentiation, but that its continued activation was unnecessary for its maintenance. However, KN-62 does not block CaMKII autophosphorylation, which may be critical for the maintenance phase of rebound potentiation. In comparison, the regulation of IPSCs by CaMKII recorded in cerebellar granule cells required the binding of both Ca^{2+} and calmodulin to the kinase, but did not require the enzyme to be autophosphorylated (Houston *et al.* 2008).

Rebound potentiation has recently been shown to be dependent on $GABA_A$ receptor binding to $GABA_A$ receptor-associated protein (GABARAP) (Kawaguchi & Hirano, 2007). Tyrosine kinases have also been implicated in the induction of this plasticity (Boxall, 2000; Kawaguchi & Hirano, 2006). As an interaction between CaMKII and tyrosine kinase signalling pathways leading to phosphorylation of $GABA_A$ receptors in NG108-15 cells has been demonstrated (Houston *et al.* 2007), it seems plausible that these two kinases also interact to regulate

the induction of rebound potentiation. Interestingly, the predominant β subunit in Purkinje neurons is the $\beta 2$ subunit (Laurie *et al.* 1992; Pirker *et al.* 2000), which is in accord with the $\beta 2$-dependent CaMKII mediated increase in IPSC amplitudes observed in cerebellar granule cells (Houston *et al.* 2008).

There is growing evidence that inhibitory synaptic plasticity may be important in many different physiological conditions. In hippocampal neurons, it has been reported that NMDA receptor activation, which drives excitatory LTP, simultaneously drives inhibitory potentiation in a form of homeostatic plasticity. This process was dependent on CaMKII, and the trafficking proteins GABARAP and NSF, causing an increase in the number of functional receptors at the cell surface (Marsden *et al.* 2007). Furthermore, a Ca^{2+}-dependent plasticity mechanism involving alterations to $GABA_A$ receptor trafficking is involved in the transition from awake to sleep states in cortical pyramidal neurons (Kurotani *et al.* 2008). It has also been demonstrated that frequency-dependent plasticity of developing inhibitory synapses is dependent on $GABA_B$ receptor activation, which stimulates postsynaptic CaMKII activation (Xu *et al.* 2008). Thus, taken overall, CaMKII not only produces relatively acute changes to inhibitory synaptic transmission, but can also underwrite longer-term inhibitory synaptic plasticity.

Discussion and conclusion

CaMKII is considered to be an important kinase that is associated with the postsynaptic density of excitatory synapses, as well as being a vital component of LTP. The realisation that CaMKII can also modulate the efficacy of synaptic inhibition increases its significance, not least because it can act as a point of convergence for numerous signalling pathways that involve changes to intracellular Ca^{2+}. Thus, CaMKII can affect the function of synaptic $GABA_A$ receptors and may play a key role in homeostatic forms of inhibitory synaptic plasticity.

Many studies have investigated the effects of internal Ca^{2+} on $GABA_A$ receptor function yielding a spectrum of results ranging from potentiation to inhibition of GABA-activated currents. This variation could well be related to the delicate balance that is struck between the activities of kinases and phosphatases in neurons. Although many studies did not investigate whether or not $GABA_A$ receptors are directly phosphorylated, it is conceivable that CaMKII and calcineurin are prominent key proteins that affect $GABA_A$ receptor function when internal Ca^{2+} levels are perturbed.

When CaMKII has been preactivated and perfused into neurons, both the subunit composition and the location of the $GABA_A$ receptors appear to be important in determining the functional outcome (Fig. 1). For example, it seemed quite clear that $\beta 2$ subunit-containing recombinant receptors were unaffected by CaMKII, in contrast to $\beta 3$ subunit receptors, where GABA currents were potentiated. This distinction was apparent even when recombinant receptors were over-expressed in neurons. Nevertheless, by focusing on synaptic $GABA_A$ receptors the regulation by CaMKII revealed some interesting differences. Firstly, IPSCs mediated by synaptic receptors that contained $\beta 2$ subunits exhibited large amplitude enhancements, whilst IPSCs mediated by $\beta 3$ subunits showed only prolonged durations. It is therefore important to note the cellular context in which the role of CaMKII is studied. The inability of $\beta 2$ subunit containing receptors to be modulated by CaMKII in heterologous expression systems, or in extrasynaptic domains when over-expressed in neurons, suggested that CaMKII can only phosphorylate these receptors at inhibitory synapses. This might indicate that CaMKII can only bind to $\beta 2$ subunit-containing $GABA_A$ receptors at inhibitory synapses, possibly because of the density of anchoring proteins. Although CaMKII can phosphorylate $GABA_A$ receptors, it is also likely that there may be other targets in the postsynaptic density such as anchoring proteins, e.g. gephyrin. How these proteins will be affected by phosphorylation and what the consequences will be for inhibitory synaptic transmission is at present unknown.

A notable feature of CaMKII regulation of synaptic $GABA_A$ receptor function is that the mechanism by which it potentiates IPSCs varies depending on the inhibitory synapse and the isoforms of $GABA_A$ receptor β subunits expressed therein (Fig. 1). At $\beta 2$ subunit-containing synapses, CaMKII increased IPSC amplitudes, which, at synapses where GABA reaches saturating concentrations, is more likely to be mediated through changes to cell surface receptor numbers, whilst at $\beta 3$ subunit-containing synapses, the regulation of IPSCs is mediated by a change to their decay times, probably by receptor phosphorylation changing the kinetic properties of the GABA channels.

Potentially, a change in IPSC decay times at a specific set of inhibitory synapses, compared to a change of amplitude at other synapses, could have diverse consequences for integration of excitatory inputs and thus the overall effect on neuronal excitability. Across the whole brain it is increasingly evident that different $GABA_A$ receptor subtypes have specific physiological roles. Indeed, in keeping with their localisation at specific subsets of synapses, β subunits are thought to play different physiological roles in mice in mediating the sedative and anaesthetic actions of the anaesthetic etomidate (Reynolds *et al.* 2003).

The role of CaMKII in regulating synaptic inhibition by modulating IPSCs in a synapse-specific manner is likely to be important in mature synapses by determining the extent of inhibitory synaptic plasticity, such as

rebound potentiation (Kano *et al.* 1996; Kawaguchi & Hirano, 2002). CaMKII regulation of synaptic $GABA_A$ receptors could also play a significant role during activity-dependent formation of inhibitory synapses, which occurs throughout development (Ben Ari *et al.* 2007). The significance of a signalling mechanism is often best appreciated when it suffers dysfunction. There are several examples where CaMKII signalling is implicated in neurological disorders that affect inhibitory transmission. Notably, the neurodevelopmental disorder, Angelman's syndrome, is associated with low expression of $GABA_A$ receptor $\beta 3$ subunits and it is also linked to disrupted CaMKII signalling (DeLorey *et al.* 1998; Weeber *et al.* 2003). Also of significance is the link between dysfunctional CaMKII signalling and epilepsy, which may be mediated by an interaction with

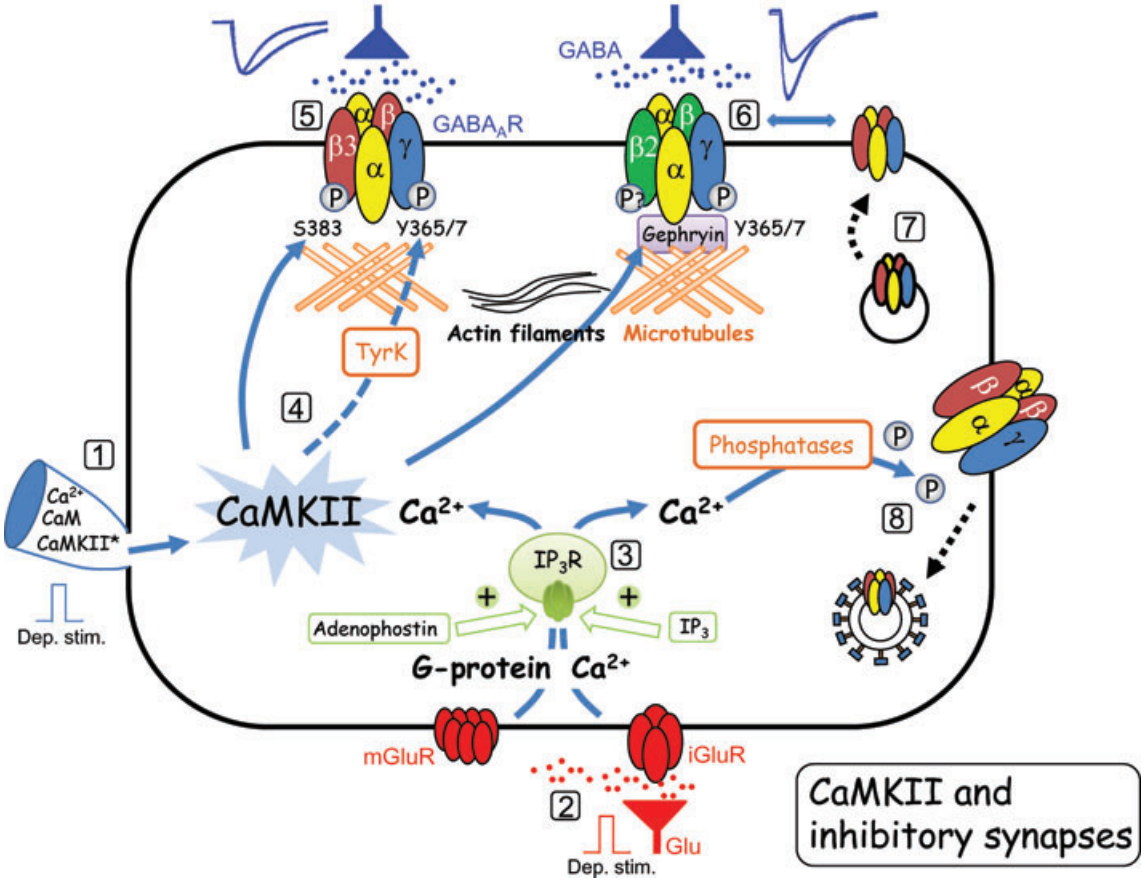

Figure 1. Modulation of GABAA receptors by CaMKII: a working model
This schematic diagram is based on a number of studies (see main text) that have investigated the actions of CaMKII at inhibitory GABAergic synapses and at recombinant $GABA_A$ receptors in heterologous expression systems. It shows a hypothetical model that integrates the main observations to explain how CaMKII may regulate $GABA_A$ receptors in neurons. Specifically, direct stimulation of the neuron via voltage steps delivered by the patch electrode (1) or by stimulating a glutamatergic afferent input (red, 2), can cause an increase in internal Ca^{2+}, either directly via ionotropic glutamate receptors (iGluR), or by metabotropic glutamate receptors (mGluR) resulting in the release of Ca^{2+} from internal stores (3). This release can also be achieved by using IP_3 receptor agonists, adenophostin or IP_3 (3). The rise in internal Ca^{2+}, with calmodulin, activates CaMKII (4). CaMKII activation can also be achieved by internal delivery of Ca^{2+} and calmodulin via the patch pipette (1) or native CaMKII activation can simply be by-passed by internally perfusing with pre-activated CaMKII (CaMKII*) (1). The activation of CaMKII has two major effects on synaptic GABAA receptors (blue, 5, 6). It is proposed that by phosphorylating the receptor β and γ subunits at specific residues that also involves a tyrosine kinase (TryK; dashed arrow as mechanism is unclear), GABAergic IPSCs are either simply prolonged in duration at $\beta 3$ subunit-containing receptors (5) or increased in amplitude with unchanged kinetics at $\beta 2$ subunit-containing receptors (6). The simplest explanation for the increased amplitude of IPSCs at the latter synapses is an increase in trafficking of receptors from secretory vesicles to the cell surface involving both actin filament and microtubule polymerization (7). Several studies also report that a rise in internal Ca^{2+} can cause a reduction in GABA synaptic and whole-cell currents. This may conceivably occur by the activation of Ca^{2+} dependent phosphatases (calcineurin) perhaps causing clarithin-dependent endocytosis of GABAA receptors (8) and/or by direct effects on receptor kinetics.

$GABA_A$ receptors (Churn *et al.* 2000; Singleton *et al.* 2005).

The implications of CaMKII differentially regulating inhibitory transmission by phosphorylation of $GABA_A$ receptor subtypes are potentially of some significance, particularly given that these receptors are targeted to specific locations within neurons, where they can undertake different functional roles (Nyíri *et al.* 2001; Brünig *et al.* 2002; Reynolds *et al.* 2003; Herd *et al.* 2008). Finally, CaMKII is notable in having the capacity to act as a point of signalling convergence for homeostatic synaptic plasticity whereby excitatory transmission, which involves Ca^{2+} influx via either AMPA or NMDA receptors, can enable glutamate to exert some control over the post-synaptic sensitivity of neurons to GABA and thus the efficacy of synaptic inhibition.

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