Regulation of proteins involved in synaptic plasticity by Ca^{2+} and cAMP: *novel insights into CA3*

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Declaration

I declare that all the work in this thesis was carried out by myself except where referenced and including LTP studies carried out by Mrs Kara McNair, and that it has not been submitted for any previous higher degree.

Dedication

This PhD is dedicated to my beloved sister, Anaai Abel Alier (1971-1999). Thank you for being my sister. I will always love you. Rest in Peace.

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Abbreviations

AC	Adenylate cyclase
aCSF	Artificial cerebrospinal fluid
AMPA	Alpha amino-3-hyrdroxy-5-methyl-isoxazole-propionate
ANOVA	Analysis of variance
ARC	Activity-regulated cytoskeleton-associated protein
CA	Cornu Ammons
CaMKII	Calcium/Calmodulin-dependent protein kinase II
CAKβ	Cell-adhesion kinase β
cAMP	Cyclic adenosine monophosphate
CD	Central domain
CPE	Cytoplasmic polyadenylation element
CPEB	Cytoplasmic polyadenylation element binding protein
CPSF	Cleavage and polyadenylation specificity
CREs	cAMP response element
CREB	cAMP response element binding protein
CNS	Central nervous systems
CS	Condition stimulus
DAG	Diacylglycerol
DG	Dentate gyrus
ELF4E	Eukaryotic translation initiation factor 4E
E-LTP (LTP1)	Early LTP

FAK	Focal adhesion kinase
FKBP12	FK506-binding protein
FRAP	FKBP12 and rapamycin associated protein
FSH	Follicle stimulating hormone
GABA	Gamma-aminobutyric acid
GEF	Guanine nucleotide exchange factor
GluR	Glutamate receptor
Grb2	Growth factor receptor binding protein 2
HBSS	Hank's balance salt solution
5-HT	5-hydroxytryptamine
HMW	High molecular weight
ir	Immunoreactive
KA	Kainate
I-1	Inhibitor 1
IB	Immunoblot
IGFII	Insulin-like growth factor II
I-LTP (LTP2)	Intermediate LTP
IP	Immunoprecipitation
LTP	Long-term potentiation
LMW	Low molecular weight
L-LTP (LTP3)	Late LTP
MTs	Microtubules
MAP2	Microtubule-associated protein 2

MAPK	Mitogen-activated protein kinase
MF LTP	Mossy fibre LTP
NMDAR	N-methyl-D-aspartate receptor
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
PRD	Proline rich domain
PSD	Post-synaptic density
PBS	Phosphate buffered saline
PBST	PBS and Tween
PC12	Pheochromocytoma cells 12
PIKK	Phosphatidkinositol-related kinases
PKA	Protein kinase A
PKC	Protein kinase C
PKMζ	Protein kinase Mζ
PP1	Protein phosphatases 1
PTK	Protein tyrosine kinases
PyK2	Protein-rich tyrosine kinase 2
SH	Src homology
Sos	Son of sevenless
SPRCs	Synapse-associated polyribosome complexes
STP	Short-term potentiation
TBD	Tubulin binding domain
TGF-β	Transforming growth factor-β

5' TOP	5' Terminal oligo-pyrimidine tract
UTR	Untranslated region

Publications

Alier, K.A. and Morris, B.J., 2002. Regulation of Tyrosine Kinases (Proline- Rich tyrosine kinase and Src) in the Hippocampus. Federation of European Neuroscience Societies (FENS); Abstract number 016.2 page 248.

Alier, K.A. Morris, B.J., 2001. Regulation of microtubule associated protein 2 (MAP2) and calcium-calmodulin dependent protein kinase. British Neuroscience Association (BNA); Abstract number 49.05 page 112.

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Abstract

Elevation of intracellular Ca^{2+} and cAMP are key triggering events leading to hippocampal long-term potentiation (LTP, a form of synaptic plasticity) induction. In this study, the aim was to compare the ability of elevated Ca^{2+} influx (achieved via the Ca^{2+} ionophore, A23187) or increased intracellular cAMP levels (achieved via adenylate cyclase activator, forskolin) to modulate the expression or activation of selected proteins involved in LTP, with emphasis on the CA3 region of the hippocampus. The proteins investigated include: αCaMKII, MAP2, β-activin, Pyk2 and MAPK. They have all been implicated in LTP in CA1 and dentate gyrus (DG) regions of the hippocampus.

To test the hypothesis that Ca^{2+} influx or cAMP elevation might activate Pyk2 and MAPK, the levels of phosphorylated Pyk2 and MAPK were measured in acute rat hippocampal slice preparations after exposure to A23187 (5μM) or forskolin (50μM). Using an immunoprecipitation assay, the levels of phosphorylated Pyk2 were increased in the presence of A23187 with a peak effect around 10 minutes. When the CA1 and CA3 regions of the hippocampus were investigated separately, phosphorylation of Pyk2 was achieved in both regions after exposure to A23187, suggesting a key role for Ca^{2+} in both regions. Staurosporine, a general PKC inhibitor, and chelerythrine, a PKMξ inhibitor, were compared for their ability to attenuate the effect of A23187. Exposure of acute hippocampal slices to chelerythrine or staurosporine prior to A23187 application resulted in the reduced phosphorylation of Pyk2, suggesting that PKMξ and novel PKC may be

involved in Pyk2 activation by Ca^{2+}. Application of forskolin to acute slices resulted in the reduced activation of Pyk2 below basal level, suggesting that cAMP inhibits Pyk2. MAPK was phosphorylated for more than 20 minutes in the presence of either A23187 or forskolin suggesting an important role for Ca^{2+} and cAMP in the activation of MAPK in acute hippocampal slices. However, when CA1 and CA3 regions were investigated separately, A23187 and forskolin only activated MAPK in the CA1 region, with no effect in the CA3 region, hence suggesting a different mode of activation of MAPK in the two regions.

The effect of elevation of intracellular Ca^{2+} and cAMP via A23187 and forskolin respectively, on the expression of αCaMKII, MAP2 and β-activin protein were studied in organotypic slice cultures of rat hippocampus by immunocytochemistry or western blot. The levels of MAP2 expression were increased 4 hours after forskolin treatment, but were unaffected by A23187 treatment. Conversely, the levels of αCaMKII expression were increased 4 hours after A23187 treatment, but were unaffected by forskolin. The regulation of the expression of these proteins was the same in the CA3 region as in the CA1 and dentate gyrus of the hippocampus. While rapamycin reduced the basal levels of MAP2 expression, it did not affect the ability of either forskolin or A23187 to enhance MAP2 or αCaMKII levels. These results suggest that cAMP and Ca^{2+} differentially modulate the expression of these two plasticity-related genes, and that translational enhancement via the mammalian target of rapamycin kinase is not involved in these effects. The expression of β-activin was enhanced in the presence of A23187 or forskolin in both the CA1 and CA3 regions, again suggesting a similar pathway in both regions,

and that both increased intracellular Ca^{2+} and cAMP levels can participate in the regulated expression of this protein.

These results suggest that Ca^{2+} and cAMP mediate distinct components of neurochemical changes that underlie LTP maintenance. The proteins monitored in this study showed clear differences in their response to these two second messengers. However, in general the alterations in protein expression in the CA3 region corresponded with those in the CA1 and DG regions, suggesting that the pathways regulating the expression of a given protein may not be cell-specific.

Chapter 1

Introduction

1.1 Two major forms of memory

Learning and memory are not a unitary process— not a single faculty of the mind— but a family of distinct processes each with its own rules. Learning is the process of acquiring new information about the world and memory is considered to be the process by which knowledge is retained.

Recent studies have shown that memory can be divided into at least two major categories (Polster *et al.*, 1991):

(i) Explicit or declarative memory— conscious recall of knowledge

(ii) Implicit or non declarative memory— non-conscious recall of motor skills

These categories involve different neural circuits (Squire, 1992). Explicit memory uniquely depends on temporal lobe and diencephalic structures e.g. hippocampus, subiculum and entorhinal cortex, while the implicit form is dependent upon the same sensory, motor, or associational pathways used in the expression of the learning process (Bailey *et al.*, 1996). Explicit memory is studied in mammals, while the implicit form can effectively be studied in both non-mammalian vertebrates and higher invertebrates. Both explicit and implicit forms of learning are graded and the duration of memory is related to the number of training trails. There are at least two temporally distinct phases of memory storage:

(i) Short term memory lasting seconds, minutes and hours

(ii) Long term memory lasting days, weeks or years.

Each phase of memory storage employs a cascade of molecular events of molecular events that occur during their consolidation period.

Short-term memory depends on second messenger-mediated covalent modification of proteins which have been previously synthesised and hence modulate the properties of nerve cells and their synaptic connections (Schwartz, *et al.*, 1971; Kandel and Schwartz, 1982; Livingstone, 1985). Acquisition and retention of information for short-term memory, in certain invertebrates (e.g. *Aplysia*, *Hermissenda* and *Drosophila*), does not require the synthesis of new proteins (Schwartz, *et al.*, 1971). The process of acquisition involves the activation of receptor–linked enzymes by neurotransmitters. These enzymes are responsible for the synthesis of intracellular messages which in turn activate protein kinases that phosphorylate substrate proteins required for the plastic neuronal modification (Goelet *et al.*, 1986). The duration of the covalent modification of pre-existing proteins and maintenance of the activity of the enzyme responsible for second messenger synthesis are necessary for the retention process of plastic changes (Goelet *et al.*, 1986). It was proposed that covalent modifications similar to the ones in short-term memory become self-reinforcing for certain instances of long-term memory (Crick, 1984; Lynch and Baudry, 1984; Lisman, 1985). This was not the case, however. Studies have illustrated that in the process of acquisition of information whose memory lasts more than one-day, specific nerve cells need to express genes that are not required in short-term memory (Davis and Squire, 1984).

Goelet *et al.* (1986) concluded that the same modulatory neurotransmitters that induce cytoplasmic messengers during learning in short-term memory are also responsible for the activation of at least three overlapping memory processes, either through the same or additional second messenger systems. Each memory process has its own time course of retention. These overlapping memories include:

(i) Intermediate memory which lasts many hours and is dependent on self–reinforcing covalent modification

(ii) Long-term memory lasting more than one-day is dependent on the induction of new proteins which results from second messengers involved in short-term memory

(iii) Memory lasting weeks and months which is dependent on early regulatory genes whose protein products trigger the maintained expression of late effector genes.

Behavioural studies of learning in vertebrates suggest that memory lasting days or weeks can be disrupted by the inhibition of protein synthesis (Nader *et al.*, 2000; Schafe and LeDoux, 2000). However, at least in some models, vertebrates do not experience any deficit in long-term memory if exposure to the protein synthesis inhibitor is delayed by as little as one-hour after training (Davis and Squire, 1984). Invertebrates have been used to address the question of whether the disruption of protein synthesis by the inhibitors in behavioural studies is due to motivation, motor performance or some other complex brain system. Invertebrates were also used to determine if this protein disruption reflected a fundamental property of long-term information storage in specific nerve cells of the circuit responsible for the modified behaviour. Studies on long-term sensitisation of the

gill-withdrawal reflex in *Aplysia* showed that the basic synaptic connection between sensory and motors neurons is enhanced in both short and long-term sensitisation (Bailey *et al.*, 1996). Long-term facilitation associated with the memory of long-term sensitisation is blocked by both translational and transcriptional inhibitors. This is in contrast to short-term facilitation. The blockage of protein synthesis in *Aplysia* during a two-hour training period blocked the retention of long-term facilitation assayed one-day later; this was similarly observed in equivalent models in vertebrates (Goelet *et al.*, 1986). These studies hence indicate that genes and proteins are necessary for the cellular mechanism underlying long-term memory.

The cortex, amygdala and hippocampus have been implicated in learning. The cortex is widely assumed to store traces of experience underlying both explicit and implicit learning (Martin *et al.*, 2000). In explicit learning, the hippocampus is thought to be involved in the earlier stages of encoding and storage resulting in the eventual consolidation of information in the cortex whereupon the participation of hippocampal formation is no longer required (Martin *et al.*, 2000). The hippocampus is widely thought to be involved in information processing functions related to spatial memory (O'Keefe and Conway, 1978) as well as declarative (Squire, 1992) and episodic memory (Vargha-Khadem *et al.*, 1997). Studies on fear conditioning have implicated the amygdala in many forms of learning. In the classical fear conditioning, the condition stimulus (CS) such as a tone or light, is paired with aversive unconditioned stimulus such as foot shock. After a number of pairings, the CS alone evokes responses such as freezing, increase heart rate, and the potentiation of the startle reflex (Davis *et al.*, 1993; LeDoux, 1995). Lesions of

the lateral amygdala affected the expression of conditioned fear implicating the amygdala as the sole site of long-term storage (LeDoux *et al.*, 1990; Martin *et al.*, 2000). There has been some scepticism over these results since the lateral/basolateral amygdala does not seem to be involved in cognitive/explicit aspects of conditioned fear (Cahill *et al.*, 1999).

1.2 Hippocampus

The hippocampus is a C-shaped structure (Figure 1.1) in the coronal section and is also known as the Ammon's horn (cornu ammonis) (Amaral and Witter, 1989). The hippocampal formation is made up of different cortical regions: dentate gyrus, the hippocampal proper and the subicular complex (made up of subiculum, presubiculum and parasubiculum) (Amaral and Witter, 1989). Transverse (coronal) section reveals that the hippocampus proper has three areas or sectors: CA1, CA2 and CA3 (CA) stands for cornu ammonis. Three layers are identified in the hippocampal cortex (Andersen *et al.*, 1971):

(i) The molecular layer, consisting of interacting axons and dendrites. This synaptic layer is continuous with the molecular layers of the dentate gyrus and neocortex.

(ii) The pyramidal layer which is made up of large neurons, many of them pyramidal in shape. These pyramidal cells are the principal cells of the hippocampus. The dendrites of these principal cells extend into the molecular layer. Schaffer collaterals are branches, which pass through the polymorphic and pyramidal cell layers to synapse in the molecular layer with the dendrites of other pyramidal neurons.

(iii) The polymorphic layer contains axons, dendrites and interneurons.

The dentate gyrus (DG) also has three layers. However, in the DG the pyramidal cells are replaced by a granule cell layer of small neurons, which are the principal cells in the region.

The trisynaptic pathway, a basic circuitry of the hippocampal formation, is series of connections from superficial entorhinal cortex to the hippocampus. These connections are excitatory, largely unidirectional, and dense in some planes of section oriented perpendicular to the long axis of the hippocampus (Andersen *et al.*, 1971, Witter *et al.*, 2000). The trisynaptic pathway is comprised of entorhinal layer II stellate neuronal projections (the perforant pathway) to the apical dendrites of the dentate granule cells. Efferent fibres from the dentate gyrus known as mossy fibres (MF) project into area CA3 where they terminate on the proximal apical dendrites of pyramidal neurons (Figure 1.1). Axons on the CA3 pyramidal cells leave the hippocampus via the alveus and collaterals (Schaffer collaterals) and ascend to cross the cell layer and terminate predominantly on the proximal apical dendrites (stratum radiatum) of CA1 (Amaral and Witter, 1989; Witter *et al.*, 2000). A fourth synapse occurs in the subiculum as a result of CA1 pyramids projection onto subicular cells (Amaral *et al.*, 1991). Subiculum is one of the principal outputs of the hippocampal region with projections to hypothalamus, mammillary nucleus, nucleus accumbens, septum and the parahippocampal cortices (Naber and Witter, 1998). The output of the subiculum can take several paths, with respect to cortical connections (Witter *et al.*, 2000). One output of the subiculum is into the deep layers of entorhinal cortex via the alveus. Ascending collaterals of deep layer

neurons onto the superficial layer of neurons completes a long loop from the entorhinal cortex through the hippocampus. Another pathway is into the adjacent presubiculum. The connection between the presubiculum, parasubiculum and entorhinal cortex complete the entorhinal-hippocampal circuit with a different set of intervening connections. A key anatomical feature of area CA3 is that its pyramidal cells receive the majority of their inputs from other CA3 pyramidal cells (Amaral and Witter, 1989; Amaral *et al.*, 1990). The resulting recurrent network has been extensively explored as a plastic attractor model of the way that the hippocampus stores episodic memory (McNaughton and Morris, 1987; Levy, 1996; Rolls, 1996, 2000). Other anatomical studies indicated that other pathways parallel to the existing system (the trisynapic loop) and these by-pass the dentate gyrus relaying the information directly from the entorhinal cortex to CA1 or CA3 (Amaral and Witter, 1989).

All the major pathways within the hippocampal formation (perforant pathway, mossy fibre and Schaffer collaterals) use the excitatory amino acid glutamate as neurotransmitters (Malenka and Nicoll, 1999). Other neurotransmitters have also been shown to play a role in the hippocampal signalling; these neurotransmitters include, dopamine (Huang and Kandel, 1995; Gurden *et al.*, 1999), 5-HT (Kulla and Manahan-Vaughan, 2002) and acetylcholine (Anagnostaras *et al.*, 2003; Colgin *et al.*, 2003). β-adrenergic agonist has also been shown to play role in hippocampal signalling (Huang and Kandel, 1996; Lin *et al.*, 2003). The excitatory glutamate neurotransmitters act on ionotropic as well as metabotropic glutamate receptors located both pre- and

postsynaptically. The abundance of these receptors in the three regions of the hippocampus will be discussed in detail in the next section.

1.3 Amino acid receptors

Ionotropic glutamate receptors are ligand-gated ion channels, that mediate rapid excitatory neurotransmission in the central nervous system (CNS) and they include N-methyl-D-aspartate (NMDA), γ-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (KA) types of receptor, named according to their prototypical agonists. These receptors play a role in synaptic plasticity (Sommer and Seeburg, 1992; Bliss and Collingridge, 1993; Bettler and Mulle, 1995).

Four closely related subunits of AMPA-selective ionotropic glutamate receptors have been identified using molecular cloning and are termed GluR1, GluR2, GluR3 and GluR4 (Sommer *et al.*, 1991; Gasic and Hollmann, 1992; Hollmann and Heineman, 1994; Wenthold *et al.*, 1996) and this gives rise to two splice variants, flip and flop (Sommer *et al.*, 1990). Each contain 850-950 amino acids and share some 70-80% similarity. In the CA1, AMPARs are composed mainly of GluR1-GluR2 and GluR2-GluR3 heteromers (Wenthold *et al.*, 1996). AMPARs have been shown to contribute to excitatory synaptic transmission in a population of hippocampal and neocortical nonpyramidal, and spinal dorsal horn neurons (McBain and Dingledine, 1993; Itazawa *et al.*, 1997) and are assumed to contribute to long-term modification of synapses and neurological disorders (Pellegrini-Giampietro *et al.*, 1997, Tanaka *et al.*, 2000).

KA receptors (KAR) consist of various combinations of GluR2/6/7 and KA1/2 subunits (Bettler *et al.*, 1990, 1995; Egebjerg *et al.*, 1991; Herb *et al.*, 1992; Paternain *et al.*, 2000). KAR are concentrated in a few specific areas of the CNS, generally complementary to the distribution of NMDA and AMPA receptors (Wisden and Seeburg, 1993; Porter *et al.*, 1997). Radioligand binding studies suggested that kainate binding sites are highly localised on the stratum lucidum in the CA3 region, where the MF terminates (Monaghan and Cotman, 1982). In the hippocampus, CA3 pyramidal cells were stained more densely than CA1 pyramidal cells with high levels of GluR6/7 subunits (Petralia *et al.*, 1996). In situ hybridisation studies revealed that transcripts of most KAR subunits are expressed in the presynaptic dentate gyrus and postsynaptic CA3 pyramidal cells (Wisden and Seeburg, 1993), it seems likely that the MF-CA3 synapse expresses KARs with subunit composition containing at least GluR6 or GluR7 at both pre- and postsynaptic sites. One unique feature of presynaptic KARs is that their activation modulates transmitter release bi-directionally; weak activation enhances glutamate release, while strong activation leads to inhibition (Kamiya, 2002). This mechanism of action might be due to their ionotropic action resulting in axonal depolarisation, which in turn regulates several voltage dependent channels involved in action potential-dependent Ca^{2+} entry processes.

Four subunits of NMDA ionotropic glutamate receptors have been identified are: NR1, NR2A-D, NR3A-B (Seeburg, 1993; Nakanishi and Masu, 1994, Riedel *et al.*, 2003). The NR2 subunits are only 20% homologous to NR1. It is thought that every NMDA receptor

contains both NR1 and NR2 subunits (Seeburg, 1993; Nakanishi and Masu, 1994). Subunits are composed of a pentameric structure based on NR1 combining with NR2A-D or NR3A-B subunits (Ciabarra *et al.*, 1995; Hollmann and Heineman, 1994; Matsuda *et al.*, 2002).

NMDA receptors differ from AMPA and KA receptors because the application of agonist when the cell is polarised does not activate the integral ion channel due to inhibition of the receptor by physiological extracellular concentrations of Mg^{2+}. The Mg^{2+} ion blocks the channel. When the membrane is depolarised, the Mg^{2+} block is removed and the channel can open when agonist is present. The open channel allows influx of Na^{+} and Ca^{2+} with a 10:1 ratio of permeabilities of Ca^{2+}: Na^{+} (Riedel *et al.*, 2003). The NMDA receptor is a novel combination of a ligand-activated and voltage-operated channel, requiring both glutamate and depolarisation for activity. All synapses in the CA1 region of the hippocampus have been shown to contain NMDARs (Watanabe *et al.*, 1998; Racca *et al.*, 2000). All three subunits of NMDARs were also detected in the dentate gyrus as well as neurophil layers of CA3 (Watanabe *et al.*, 1998). The NR1 and NR2 subunits of NMDAR were found in low levels in the stratum lucidum, a mossy fibre-recipient layer of the CA3 subfield (Watanabe *et al.*, 1998) and hence it was concluded that the selective scarcity of NMDARs in this region reflects different synaptic targeting mechanisms (Watanabe *et al.*, 1998). Distribution of NMDA receptors at the Schaffer collateral synapse in the CA1 area differs from that of the AMPA receptors with every Shaffer collateral synapse onto the CA1 pyramidal spines containing NMDA receptors (Racca *et al.*, 2000). AMPA receptors only make up 75-85% of these synapses (Takumi *et al.*,

1999; Racca *et al.*, 2000, Nusser 2000). AMPA and NMDA are co-localised in only 75-85% of these synapses (Takumi *et al.*, 1999; Racca *et al.*, 2000).

Genetic studies have indicated the existence of seven different metabotropic receptors designated mGluR1-8 (Riedel *et al.*, 2003). There are three categories of mGluR members based on their amino acid sequence homology, pharmacological profiles and second messenger coupling. Group I mGluRs (mGluR1 and 5 and their splice variants) stimulate the hydrolysis of inositol-bis-phosphate to inositol-1,4,5-trisphosphate (IP3), this is achieved via activation of phospholipase C (PLC) . PLC also activates diacylglycerol which co-activates protein kinase C while IP3 promotes Ca^{2+} release from internal stores (Riedel *et al.*, 2003). Group II (mGluR4, 6-8, including splice variants) inhibit adenylate cyclase hence reducing intracellular levels of cAMP. Group III regulate glutamate release due to their autoreceptor function. Hippocampal mGluRs are particularly important in mediating memory consolidation. In fear conditioning studies, mGluR5 expression was enhanced in the CA3 after one day post-training, but this enhancement diminish at ten days and was replace by an overexpression of mGluR5 that was strong in the CA1 and somewhat weaker in the dentate gyrus (Casabona *et al.*, 1997; Riedel *et al.*, 2000). These data indicates the temporal involvement of hippocampus in fear conditioning and a strong hint at an early involvement of CA3 followed by a later prolong activation of CA1 (Riedel *et al.*, 1993).

1.4 Synaptic plasticity

1.4.1 General principles

Synaptic plasticity is an activity dependent change in the efficiency of synaptic transmission which requires:

(i) Pre and/or postsynaptic activity

(ii) Biochemical and/or morphological changes in dendritic spines (Halpain, 2000)

These requirements (i and ii) result in changes in synaptic strength. Synaptic plasticity differs when comparing associative and non-associative plasticity (section 1.4.3). There is also a difference in plasticity with respect to protein synthesis dependent versus independent form (section 1.4.7). Synaptic plasticity is thought to underlie higher cognitive functions such as learning and memory. This idea was first refined by Hebb (1949). Hebb proposed that when two interconnected neurons fire at the same time, the synapse between them became stronger and remain so for a considerable time afterwards.

Many different forms of synaptic plasticity exist and they vary in a number of respects:

(i) Duration: from milliseconds to days, months or years

(ii) Location in the brain

(iii) Induction mechanism (NMDA receptor-dependent/independent)

(iv) Expression (maintenance) mechanisms (pre/postsynaptic)

Long-term potentiation (LTP) is a form of synaptic plasticity that is thought to underlie some forms of learning and memory. It was first described by Bliss and Lomo (1973) when they demonstrated that application of a relatively brief burst of high frequency stimulation (HFS) resulted in long lasting enhancement of synaptic responses in the mammalian hippocampus.

The phenomenon of LTP has been shown in different parts of the brain. LTP was shown in kittens' visual striate cortex synapses (Komatsu *et al.*, 1981) as well as in synapses formed by the brachium of the interior colliculus in the medial genticultae nucleus of auditory system (Gerren and Weinberger, 1983). Racine *et al.* (1983) also showed LTP in the synapses in the limbic system. LTP-like mechanisms were also involved in amygdala fear conditioning and learning-related cortical plasticity (Martin *et al.*, 2000). Hence, LTP is not restricted to the hippocampus. However, hippocampal pathways show significantly larger LTP effects than do the non-hippocampal pathways (Bennett, 2000).

LTP was initially associated with learning by Morris *et al.* (1986) through chronic intraventicular infusion of the NMDA antagonist, aminophosphonovaleric acid (AP5) into rats. This caused a selective impairment of place learning, a process thought to be dependent on the hippocampus (O'Keefe and Conway, 1978). However, it was not possible to test whether the NMDA receptor antagonist might be blocking some part of the motor pathway that is uniquely utilised in learning process. Other studies attempted to associate LTP with learning involved stimulation of the pyriform pathway to the dentate gyrus *in vivo* (McNaughton *et al.*, 1986; Castro *et al.*, 1989). These studies concluded that

spatial memory involves at least temporary storage in the fascia dentate through the mechanism of LTP.

Expression of LTP results in the persistent increase (lasting hours, days or weeks) in the size of the synaptic component of the evoked response recorded from individual cells or from population of neurons. LTP is induced in a number of ways, most conveniently by delivering a tetanus (typically a train of 50-100 stimuli at 100 Hz or more) (Bliss and Collingridge, 1993). Modest stimuli which fall within certain critical ranges can also induce LTP examples include theta-burst stimulation (Bliss and Collingridge, 1993).

Long-term depression (LTD) is another form of synaptic plasticity, which results in a lasting decrease in synaptic effectiveness. It is made up of two types:

(i) Heterosynaptic LTD, which can occur at synapses that are inactive, normally during high-frequency stimulation of a converging synaptic input.

(ii) Homosynaptic LTD, which can occur at synapses that are activated, normally at low frequencies (Bear and Abraham, 1996).

Homosynaptic LTD requires NMDA receptor change in post-synaptic Ca^{2+} (Holland and Wagner, 1998; Connor *et al.*, 1999) in CA1 region, while the heterosynaptic LTD has been observed in the DG (Christie and Abraham, 1992; Christie *et al.*, 1995).

1.4.2 Silent Synapses

The concept of 'silent synapse' in the CNS has been around for a few years. Studies by Dimitri Kullmann (Kullmann, 1994) inferred the existence of synapses without AMPA receptors via statistical analysis. This was followed by direct evidence, which showed that the failure rates for synaptic transmission was greater at hyperpolarised potentials (pure AMPA EPSCs, excitatory postsynaptic currents) than at depolarised potentials (mixed AMPA and NMDA EPSCs) (Liao *et al.*, 1995). Liao and co-workers together with other groups went on to show that LTP was associated with the apparent activation of previously silent AMPA synapses via insertion of these receptors into the membrane (Liao *et al.*, 2001; Lu *et al.*, 2001). Studies have shown that early on in the development of the CA1 (Durand *et al.*, 1996) (e.g. postnatal day 1-2), synapses are mainly silent (i.e. lack AMPARs) at resting potentials, but possess functional NMDARs. It seems that LTP-inducing stimuli can 'switch on' these immature synapses so that synaptic transmission can occur at resting membrane potentials. Hence, the concept of silent synapses provides an important impetus for relating AMPA receptor trafficking mechanisms to the expression of LTP as well as spine maturation. In fact, studies have shown that maintenance of spine morphology requires AMPARs (McKinney *et al.*, 1999).

1.4.3 Properties of hippocampal LTP

CA1 LTP is characterised by three basic properties (Malenka and Nicoll, 1999):

(i) Cooperativity— this describes the existence of an intensity threshold for induction, hence LTP cannot be triggered by a 'weak' tetani which activates relatively few afferents (McNaughton *et al.*, 1978)

(ii) Input specificity— LTP specific to those pathways which receive conditioning stimuli (Malenka, 1991)

(iii) Associativity— pairing a weak stimulus with strong input induces LTP in the weak input (Malenka, 1991)

The three properties can be explained on the assumption that potentiation of the synapse is only achievable when it is active at the time when the region of dendrite on which it terminates is sufficiently depolarised.

1.4.4 LTP induction at the Schaffer collateral/CA1 pyramidal cell synapses

The most intensely studied form of LTP is the NMDA receptor dependent LTP in the CA1 region of the hippocampus (Malenka and Nicoll, 1999, Martin *et al.*,2000). LTP induction requires the activation of postsynaptic excitatory amino acid receptors (NMDA and AMPA receptors which are co-localised) by glutamate neurotransmitters. Sufficient activation of AMPA receptors causes depolarisation of the postsynapse, which alleviates

the voltage-dependent Mg^{2+} block of NMDA receptors, allowing influx of Ca^{2+}, which causes the biochemical cascade leading to LTP. Depolarisation of AMPA receptors is limited due to strong inhibitory influences provided by simultaneously released gama aminobutyric acid (GABA) acting on postsynaptic $GABA_A$ and $GABA_B$ receptors. Therefore NMDA can only be activated under strong postsynaptic depolarisation (tetanus or pairing) or when inhibition has fatigued due to GABA acting on inhibitory $GABA_B$ autoreceptors.

The involvement of several amino acid receptor subtypes in the induction of LTP has been determined largely by the use of antagonists (Collingridge *et al.*, 1983). Blockage of LTP induction by NMDA receptor antagonist APV (AP5; D-2-amino-5-phosphonopentanoate) indicates the requirement of NMDA receptors for LTP. DGG (γ-D-glutamylglycine) is a mixed AMPA and NMDA receptors antagonist which also blocks LTP.

$GABA_B$ autoreceptors regulate induction of LTP. Application of $GABA_B$ antagonist (CGP35348) has been shown to block postsynaptic $GABA_B$-mediated IPSC (at 100μM concentration) and at 1mM, it blocks presynaptic $GABA_B$-mediated PPD (paired-pulse depression) and enhances primed-burst stimulation. 1mM CGP35348 blocks induction of primed-burst LTP (Davies *et al.*, 1991).

There also exists an NMDA receptor independent form of LTP that has been studied in the CA1 region of the hippocampus. The NMDA receptor independent form of LTP can

be achieved via bath application of the K^+ channel blocker tetraethylammonium (TEA) (Aniksztejn and Ben-Ari, 1991) or very high frequency stimulation (Grover and Teyler, 1990). Both forms of LTP are dependent on postsynaptic Ca^{2+} influx. Application of nifedipine resulted in the blockage of both NMDA receptor dependent (Grover and Teyler, 1990) and independent (Huang and Kandel, 1995; Powell *et al.*, 1994) forms of LTP in CA1. NMDA dependent form of LTP results in an increase in the quantal AMPA current, together with an increase in the quantal content of this current (Stricker *et al.* 1996). The NMDA independent form of LTP results in no change in the quantal current, the increase in synaptic strength is due to an increase in the quantal content of the AMPA current (Sticker *et al.*, 1999).

Involvement of mGluR in the induction of LTP has been controversial especially in the use of selective mGluR antagonist, α-methyl-4-carboxyphenlglycine (MCPG). MCPG was shown to block the induction of CA1 and mossy fibre LTP (Bashir *et al.*, 1993; Breakwell *et al.*, 1996; Bortolotto and Collingridge, 1999). However, some groups were unable to reproduce these results in the CA1 (Selig *et al.*, 1995; Manzoni *et al.*, 1994) or mossy fibre (Manzoni *et al.*, 1994; Hsia *et al.*, 1995). A 'priming' phenomenon was used to explain the discrepancy of the results (Bortolotto *et al.*, 1994): mGluRs were not involved in the induction of LTP if the hippocampal slice had been 'primed'. Slices could show mGluR-dependent LTP only when they are not 'primed' (i.e. naïve). Primed slices could be rendered naïve by repeated low-frequency stimulation. This scenario indicates that mGluRs act as molecular switches, which once thrown can result in mGluR-independent LTP. In addition, studies have shown that mGluR priming of LTP results

from biochemical cascades triggered by activation of phospholipase C coupled to group I mGluRs in CA1 hippocampal slices (Cohen and Abraham, 1996; Cohen *et al.*, 1998).

1.4.5 Role of Ca^{2+} in the induction of LTP

Ca^{2+} plays an important role in the induction of LTP. Filling of CA1 neurons with calcium chelator EGTA blocked the induction of LTP (Lynch, 1983). Collingridge and co-workers (Collingridge *et al.*, 1983) also showed that the blockade of NMDA receptors with D-APV blocked the induction of LTP and therefore concluded that calcium influx required for the induction of LTP may enter via the NMDA receptor channel. There is considerable evidence that the release of Ca^{2+} from internal stores is also required for the induction of LTP (Harvey and Collingridge, 1992; Matias *et al.*, 2002). Indeed, synaptic activation of NMDA receptors causes the release of calcium from internal stores, 'trigger' Ca^{2+} entering via NMDA channel causes the subsequent release of calcium from internal stores (Alford *et al.*, 1993). Since it is assumed that NMDA receptors are located on dendritic spines (a specialise structure in a mature brain), it is believed that spines may act to localise the Ca^{2+} signal (Connor *et al.*, 1994; Halpain, 2000). Diffusion of calcium is restricted by the spines (Connor *et al.*, 1994; Korkotian and Segal, 2000) but it is not known if this occurs in LTP. However, LTP has been shown to cause increases in spine size as well as spine numbers (Yuste and Bonhoeffer, 2001).

1.4.6 Mossy fibre LTP (MF LTP)

Mossy fibre (MF) LTP is NMDA receptor independent. MF LTP may not be associative (Zalutsky and Nicoll, 1990) nor show cooperativity. MF LTP may involve mGlu receptors (metabotropic glutamate receptors, G-protein coupled receptors). This has been highly controversial with evidence for (Bashir *et al.*, 1993; Conquet *et al.*, 1994) and against (Hsia *et al.*, 1995). The opioid peptide dynorphin has been shown to play an important inhibitory role in the induction of MF LTP via kappa 1 receptors in the guinea pig hippocampus (Salin *et al.*, 1995). Studies have shown a presynaptic mechanism for induction via Ca^{2+} influx through P- or N-type Ca^{2+} channels (Castillo *et al.*, 1994). This causes an enhancement of transmitter release; resulting in a presynaptic potentiation of synaptic transmission via a cAMP-dependent PKA-mediated mechanism (Weisskopf *et al.*, 1994).

1.4.7 Temporal phases of LTP

Research suggests that there are three distinct temporal phases of LTP (Roberson *et al.*, 1996b). These include LTP1 (Early LTP, E-LTP) which lasts for <3h duration and LTP2 (Intermediate LTP, I-LTP), which last 1-6 hours after the initial induction (Morris, 1996). LTP3 (late-LTP) is more stable and lasts for up to 8 hours (Frey *et al.*, 1993) in CA1 region of hippocampal slices and for days in the intact animal (Abraham *et al.*, 1993).

E-LTP can be produced by a single train of presynaptic action potentials and is protein synthesis independent (Bliss and Collingridge, 1993). It results from covalent modifications of pre-existing proteins mediated by the cAMP-dependent protein kinase (PKA) in MF/CA3 synapses (Weisskopf *et al.*, 1994). In the CA1, E-LTP depends on Ca^{2+}/Calmodulin-dependent protein kinase (CaMKII) (Malinow *et al.*, 1989; Ito *et al.*, 1991; Huang and Kandel, 1994; Liu *et al.*, 1999), protein kinase C (PKC) (Akers *et al.*, 1986; Klan *et al.*, 1991) and tyrosine kinases (O'Dell *et al.*, 1991). Blitzer *et al.* (1995) suggested that the E-LTP in CA1 region also involves the cAMP signalling pathway which results in PKA activation. Apart from receptor phosphorylation, PKA also acts to gate LTP by regulating the activity of phoshoprotein phosphatases.

LTP2 is induced by multiple trains and is suppressed by calcineurin in the CA1 region and like E-LTP is gene transcription independent but unlike E-LTP is dependent on protein synthesis (Winder *et al.*, 1998). Calcineurin is a calcium-sensitive serine/threonine phosphatase that is present at high concentrations in the hippocampus and is enriched at the synapses (Kuno *et al.*, 1992). Activated calcineurin can act on protein substrates both directly or indirectly and hence regulate specific cellular functions by dephosphorylating target proteins or modulating an even larger variety of substrates via dephosphorylating inhibitor 1 (I-1). For example, the suppression of phosphatase activity by PKA during LTP2 via phosphorylation of I-1, may simply act to allow a more robust utilisation of mechanism recruited for E-LTP. Activated I-1 causes dephosphorylation of protein phosphatases 1 (PP1), a suppression which requires a

stronger stimulus than the one 100 Hz train necessary to produce E-LTP (Winder *et al.*, 1998).

L-LTP requires repeated trains, activation of PKA and synthesis of new proteins and RNA (Frey *et al.*, 1988; Frey *et al.*, 1993; Huang and Kandel, 1994). The late phases of LTP in MF and Schaffer collateral pathways are similar in outline (Huang *et al.*, 1994). Both pathways use a cAMP-mediated mechanism and are dependent on new RNA and protein synthesis. Use of protein synthesis inhibitor, anisomycin, and mRNA synthesis inhibitor, actinomycin, has indicated that the proteins necessary for the maintenance of LTP over 6 hours is synthesised in the DG from pre-existing mRNA without the involvement of protein in the cell bodies of the afferent fibres (Otani and Abraham, 1989). The synthesis of these proteins was completed within 15 minutes of tetanisation (Otani *et al.*, 1989). The group concluded that there might exist two phases of LTP, one short phase independent of new protein synthesis and a later phase which is dependent on synthesis of proteins. Other studies have shown that shown the involvement of PKA in L-LTP in CA1 region (Frey *et al.*, 1993).

1.5 Protein Kinases

Protein kinases are enzymes that covalently attach phosphate groups to the side chain of serine, threonine and tyrosine, other modification of cellular function include glycosylation (attachment of a sugar unit such as glycan) and ubiquitinoylation (covalent addition of ubiquitin residues to proteins). They are attractive candidates for role in LTP,

due to the fact that phosphorylation represents an effective but reversible means for modulating protein function. Another reason is that many protein kinases are regulated by second messengers.

Kinases which have been shown to play a role in NMDA-dependent LTP include non-receptor tyrosine kinases, protein kinase C (PKC), mitogen activated protein kinases (MAPK), calcium-calmodulin dependent protein kinase II (CaMKII), and cAMP-dependent protein kinase (PKA) (Roberson *et al.*, 1996a).

1.5.1 Non-receptor tyrosine kinases and LTP

Two families of neuronal protein tyrosine kinases (PTK) have been described (Cantley *et al.*, 1991): receptor and non-receptor PTKs. The receptor PTKs are single transmembrane protein with an intracellular region which often contains more than one copy of the catalytic domain. These are activated by signalling growth factors (Schlessinger and Ullrich, 1992), which bind to the receptor in the extracellular domain. Non-receptor PTKs are intracellular proteins with one catalytic domain, which is normally located near the C-terminus (Girault *et al.* 1999).

The best characterised members of non-receptor PTKs are the Src–related PTKs. Src is the lead member of a family of PTKs with ten members, six of which are located in the brain (Src, Pyk2, FAK, Yes, Lyn, Fyn and Lck) (Ali and Salter, 2001). The primary sequence similarity between PTKs lies in:

(i) The catalytic domain— also referred to as the Src homology 1 (SH1) domain. This domain can be divided into 11 sub-domains that are shared by all the PTKs (Avraham *et al.*, 2000)

(ii) The Src homology 2 (SH2) domain— capable of high affinity binding to phosphotyrosine-containing peptide sequences that promote protein-protein interaction.

(iii) The SH3— binds proline-rich peptide sequence and also promotes protein-protein interaction.

Many neuronal proteins have been identified as the substrates of PTKs, an example of which are glutamate receptors (Moss *et al.*, 1993). The use of PTK inhibitors such as lavendustin A and genistein have implicated these kinases in LTP induction. O'Dell *et al.*, (1991) showed that the application of these inhibitors blocked the induction of LTP. The use of transgenic mice was employed to study further the involvement of particular PTKs in LTP. Fyn-knockout mice showed impairment of both LTP (although strong stimulation resulted in LTP) and spatial learning (water maze) (Grant *et al.*, 1992; Kojima *et al.*, 1997). However, fyn knockouts also showed an abnormal hippocampal homology, thus complicating the interpretation of the analysis.

Focal adhesion kinase (FAK) and Pyk2, like fyn are also a family of non-receptor PTKs with molecular mass between 110-125KDa. This sub-family has a closely related overall structure, which will be discussed in Chapter 3. Two spliced isoforms of Pyk2 have been identified and these are characterised by the presence or absence of an exon that codes for

42 amino acids between the two proline rich sequences of the C-terminal region (Figure 1.2). The unspliced form of Pyk2 is predominant in the brain.

1.5.2 Protein Kinase C

Ca^{2+}/phospholipid-dependent protein kinase or PKC has been implicated in the molecular mechanisms of brain development, synaptic plasticity, epilepsy, ischemia and neuronal cell death (Tanaka and Nishizuka, 1994). PKC is a multigene family of at least ten isoforms, nine of which are present in the brain (α, βI, βII, γ, δ, ε, η, ζ, ι/λ). Nishizuka (1988) has divided the PKC isoform family into three groups:

(i) Conventional— α, βI, βII and γ which are activated by both the lipid second messenger, diacylglycerol (DAG) and Ca^{2+}

(ii) Novel— δ, ε, and η which are activated by DAG, but not Ca^{2+}

(iii) Atypical-- ζ, and ι/λ which are activated by neither DAG nor Ca^{2+} but by an alternative set of lipid second messengers, including arachidonic acid (Nakanish and Exton, 1992).

PKC was the first kinase to be implicated in LTP (Akers *et al.*, 1986) (Figure 1.4). Use of PKC inhibitors resulted in blockage of the induction of LTP; in most studies the inhibitors had no affect on short-term potentiation (STP). This was first suggested by using the inhibitory peptides PKC(19-31) applied directly into the post-synaptic neuron (Malinow *et al.*, 1989; Malenka *et al.*, 1989). Use of PKCγ knockout mice has implicated PKCγ in LTP (Abeliovich *et al.*, 1993). The knockout mice displayed no LTP (or indeed

STP) in response to tetanus, therefore the PKCγ isoform was suggested to have a regulatory role in LTP expression. These studies are complicated by the fact that other PKC isoforms may have compensated for loss of PKCγ. However, in behavioural studies, these mice were shown to have mild memory deficits in contextual learning tasks (Abeliovich *et al.*, 1993).

Recent studies have discovered the role of other PKC isoforms in LTP. Studies of the PKC isoform, PKMζ, in the CA1 region of the hippocampus has implicated the enzyme in both the initial induction and the sustained maintenance phases of LTP (Klann *et al.*, 1993; Sacktor *et al.*, 1993). Autonomous activation of PKC resulting from persistent activation is known to occur during E-LTP (Roberson *et al.*, 1996a). The best characterised mechanism for obtaining autonomous activation of PKC is via proteolytic activation (Inoue *et al.*, 1977). Proteolytic activation of PKC results in cleavage at specific sites between the regulatory domain and catalytic domain (Figure 1.4). This results in a 45-50kDa catalytic fragment, known as PKM (an atypical PKC).

1.5.3 Mitogen-activated protein kinases (MAPK)

MAPK is a prototype for a family of signalling cascades that share the motif of three serially linked kinases regulating each other by sequential phosphorylation. These kinases include MAP kinase kinase kinases (MAPKKK, Raf-1 and B-Raf) which activate the second kinase, a MAP kinase kinase (MAPKK, MEK), which then activates MAPK.

MAPKs are proline-directed serine\threonine kinases of which p44 MAPK (also known as extracellular-signal regulated kinase 1, ERK1) and p42 MAPK (ERK2) isoforms are the best characterised. These isoforms act as critical transducers of growth factors signalling to the nucleus in mammalian cells. MAPKs are abundantly expressed in the neurons of mature central nervous system and thus raising the question of the function of these prototype molecular regulators of cell division and differentiation in non-dividing, terminally differentiated neurons (Fiore *et al.*, 1993).

During the regulation of cell proliferation ERK1/2 are activated via the ubiquitous Raf-1 pathway (Figure 4.4). This pathway is activated by Ras, which is stimulated by growth factor tyrosine kinases, which act via the adaptor protein, Grb2 and Sos. The Grb2 and Sos pathways are also stimulated by PKC, which interacts with either Ras or Raf-1. Activated Raf-1 causes the activation of MEK and consequently ERKs. MAPKactivation can occur independent of PKC (Ebinu *et al.*, 1998) via the second messenger DAG, which can stimulate a family of phorbal ester-binding Ras/Rap guanine nucleotide exchange factor (GEFs) resulting in elevated Ras and Rap activity. The Ras/Raf-1 pathway is inhibited by PKA via inhibition of Raf-1 (Sweatt, 2001). However, PKA can also activate MAPK via the Rap1/B-Raf pathway. MAPK can also be activated by cAMP independently of PKA via cAMP-responsive GEF (de Rooij *et al.*, 1998). Hence the effect of increased cAMP levels on MAPK activity is hard to predict, and may depend on which of these signalling intermediates are present in the cell.

Other effectors of MAPK activity include Ca^{2+}, which enters the cytosol via voltage-gated calcium channels (VGCC) or NMDA receptors. This increase in intracellular Ca^{2+} activates Ras via Sos and Grb2 which in turn activates MEK/MAPK pathway (Mazzucchelli and Brambilla, 2000). Intracellular increases in Ca^{2+} as well as PKC activation have also been shown to cause the activation of Pyk2 that then causes the modulation of ion channel function (NMDAR) and activation of MAPK signalling pathway in PC12 cells (Lev *et al.*, 1995) (Figure 1.7 and Figure 4.4). A potential link between CaMKII and MAPK has been established involving a GTPase activating protein, SynGAP. This involves CaMKII activation of MAPK via inhibition of SynGAP's GTPase-regulating activity (Chen *et al.*, 1998) (Figure 1.7).

Behavioural studies have also shown the importance of MAPK activation for memory processes. Contextual fear conditioning results in the activation of MAPK in the hippocampus (Sweatt, 2001). Application of the NMDA receptor antagonist, MK801, before training of the animals resulted in attenuation of learning and MAPK activation (following assaying of the hippocampus 1 hour after training) and hence indicated a necessity for NMDA receptor activation for learning associated MAPK activation (Sweatt, 2001). Inhibition of MEK, and hence MAPK, also reduces the performance of rats in spatial learning task (Blum *et al.*, 1999; Selcher *et al.*, 1999) consistent with this kinase cascade playing a critical role in learning-related plasticity in the hippocampus. However, it has been suggested that MAPK activation is not important for the expression of MF LTP (Kanterewicz *et al.*, 2000).

Metabotropic glutamate receptor activation as well as activation of muscarinic acetylcholine activation and dopamine (DA) receptors resulted in the activation of hippocampal MAPK (Roberson *et al.*, 1999). This MAPK activation, mediated by metabotropic and muscarinic receptors agonists was blocked by PKC inhibitors (Roberson *et al.*, 1999). Hence, neuromodulatory receptor agonist studies coupled with studies indicating that both PKC and PKA pathways can elicit hippocampal MAPK activation suggest that either (or both) PKA and PKC might be utilised to couple synaptic stimulation to MAPK activation (Sweatt, 2001).

Evidence suggests that one MAPK isoform in particular, the 42kDa MAPK isoform (p42 MAPK), might be a component of the biochemical machinery supporting LTP. Stimulation of NMDA receptors results in activation of p42 MAPK in hippocampus, metabotropic glutamate receptor stimulation also leads to increased p42 MAPK activation in cortical cultures (Bading and Greenberg, 1991; Fiore *et al.*, 1993). However, p44 MAPK must also be activated, based on the other studies discussed in this section. Thus a great deal of evidence supports an important role for MEKs and MAPKs in hippocampal synaptic plasticity, at least in the CA1 and DG regions.

1.5.4 Ca^{2+}/Calmodulin dependent protein kinase II (CaMKII)

CaMKII, a calcium-activated protein kinase, is highly expressed in the brain and enriched at synaptic structures especially the post-synaptic density (PSD). CaMKII makes up ~2% of total protein in the hippocampus and has been shown to be necessary for LTP (Figure

1.5) due to increases in intracellular calcium (Petitt *et al.*, 1994). Calcium binds to a Ca^{2+}-binding protein known as calmodulin. Calmodulin is a small protein consisting of a single polypeptide chain of about 150 amino acids. Calmodulin binds four Ca^{2+} ions with high affinity resulting in a large conformational change to reveal sites that allow it to interact with target proteins which include CaMKII.

The CaMKII enzyme, a heteromultimer consists of two types of individual subunits, 10-12 of which together form one single CaMKII holoenzyme. These two types of subunits are known as α and β (derived from different genes) and can combine in various ratios to give active CaMKII molecules. Each single-subunit is made up of an active site and a calmodulin binding site (Roberson *et al.*, 1996a).

A variety of techniques have been used to study the role of CaMKII in both LTP induction and maintenance, including pharmacological and knockout studies. The use of calmodulin inhibitors (calmidazolium, calmodulin binding peptides) (Malenka *et al.*, 1989) or CaMKII inhibitors such as $CaMKII_{273-302}$ (Malinow *et al.*, 1989) resulted in the inhibition of the induction of LTP in the CA1 region. Mice with targeted deletion of the α-isoform of CaMKII had reduced ability to exhibit LTP in the CA1 region (Silva *et al.*, 1992) and were unable to perform behavioural tasks (water maze) (Silva *et al.*, 1992). Hippocampal slices transfected with vaccina virus, expressed high quantities of CaMKII that resulted in occlusion of LTP in the CA1 region and the authors concluded that CaMKII is both necessary and sufficient to generate LTP (Pettit *et al.*, 1994).

CaMKII activation results in its autophosphorylation at Thr-286 located within the regulatory domain thus converting the enzyme from Ca^{2+}-dependent to a Ca^{2+}-independent (constitutively active) form (Fukunaga and Miyamoto, 2000). This is known to occur during E-LTP, suggesting that CaMKII plays a role in the expression of E-LTP in the CA1 region (Roberson *et al.*, 1996a). The ability of CaMKII both to phosphorylate and enhance the activity of postsynaptic AMPA-type glutamate receptors are two features which suggest that CaMKII activity is particularly important for inducing LTP (McGlade-McCulloh *et al.*, 1993; Pettit *et al.*, 1994; Lledo *et al.*, 1995; Barria *et al.*, 1997). Phosphorylation of AMPA receptors via phosphorylation of the GluR1 subunit by CaMKII during LTP results in an increase in the single-channel conductance of the receptor ion channel (Barria *et al.*, 1997). In addition, CaMKII may be important for translocating AMPA receptors to the synapses (Fukunaga and Miyamoto, 2000). Other substrates of CaMKII include MAP2 which undergoes phosphorylation during LTP induction (Fukunaga *et al.*, 1995). Functions of MAP2 in LTP induction will be discussed later (section 1.12). Induction of LTP in freely moving rats results in a transient increase in CaMKII expression in the DG region of the hippocampus (Thomas *et al.*, 1994), suggesting a role in LTP maintenance in that region.

It is unclear if CaMKII plays a role in synaptic plasticity in the CA3 region of the hippocampus. However, CaMKII is important for NMDAR-dependent LTP in other brain regions such as the visual cortex (Gordon *et al.*, 1996).

1.5.5 Cyclic AMP-dependent protein kinase (PKA) and Adenylate cyclases

Elevation of cAMP is due to the activation of adenylate cyclases. So what are adenylate cyclases? They are enzymes, which convert ATP to cAMP and are activated by a variety of hormones, neurotransmitters and other regulatory molecules.

Nine mammalian adenylate cyclases isotypes (AC1-AC9) have been identified (Krupinski *et al.*, 1989; Bakalyar and Reed, 1990; Feinstein *et al.*, 1991; Gao and Gilman, 1991; Katsushika *et al.*, 1992) and each is distributed and regulated uniquely (Choi *et al.*, 1993; Iyengar , 1993; Sunahara *et al.*, 1996). The mRNAs of AC1 (Xia *et al.*, 1993), AC2 (Furuyama *et al.*, 1993), AC3 (Glatt and Snyder, 1993), AC8 (Cali *et al.*, 1994) and AC9 (Premont *et al.*, 1996) have all been detected in the mammalian hippocampus. AC2 and AC4 have been shown to be localised in the mouse hippocampal formation and colocalised with MAP2 (Baker *et al.*, 1999). They have also been suggested to play a role in certain forms of synaptic plasticity (Baker *et al.*, 1999). Ca^{2+}-stimulated adenylate cyclases (AC1 and AC8) have been shown to play a role in synaptic plasticity in the hippocampus (Choi *et al.*, 1993; Weisskopf *et al.*, 1994; Wu *et al.*, 1995; Villacres *et al.*, 1998), but other isoforms may also be necessary. Elevation of cAMP has been shown to be necessary for MF-LTP (Huang *et al.*, 1994; Weisskopf *et al.*, 1994) as well as long lasting LTP (L-LTP) in area CA1 (Frey *et al.*, 1993). AC1 knockout mice failed to induce MF LTP (Villacres *et al.*, 1998), thus indicating the importance of AC1 in MF LTP.

Earl Sutherland discovered cAMP, a second messenger, in his studies of glycogen metabolism (Sutherland, 1970). Formation of cAMP is a result of metabolism of ATP catalysed by adenylate cyclase (refer to Adenylate cyclase section). The main effector of cAMP is PKA although may other effectors exist. PKA is a holoenzyme which is a heterotetramer comprised of two catalytic units, which have phosphotransferase activity, and two regulatory subunits, that have both a cAMP binding activity and an autoinhibitory domain which blocks catalytic subunit activity (Figure 1.6). In the absence of cAMP, inactive PKA exists as a tetrameric holoenzyme. Upon binding the two molecules of cAMP to each regulatory subunit, a conformational change occurs resulting in dissociation of the catalytic subunits and hence expression of enzymatic activity. (Figure 1.6) (Roberson *et al.*, 1996a).

Levels of cAMP are elevated immediately after LTP inducing stimuli (Chetkovich *et al.*, 1991) and this production of cAMP occurs downstream of the calcium trigger for LTP, as it depends on both extracellular calcium and activation of NMDA receptors (Chetkovich *et al.*, 1991). Calcium activates calmodulin resulting in Ca^{2+}/calmodulin complex which activates the Ca^{2+}/calmodulin-sensitive adenylate cyclase type 1 (AC1) found in the hippocampus (Chetkovich and Sweatt, 1993). Elevation of cAMP immediately after LTP induction, results in activation of PKA (Roberson and Sweatt, 1996).

Studies indicated that activity of PKA was not required to support LTP during the initial phases of LTP and that PKA inhibitors only interfere with a late phase of LTP (L-LTP), which develops after 3-4 hours (Frey *et al.*, 1993; Matthies and Reymann, 1993).

However, work by Blitzer *et al.* (1995) suggested that E-LTP in the CA1 region involved a cAMP signalling pathway which resulted in PKA activation. Thus PKA also acts to gate LTP by regulating the activity of phosphoprotein phosphatases. Blitzer *et al.* (1995) suggested that the gating pathway involves the influx of Ca^{2+} via NMDA receptors, Ca^{2+} then binds to calmodulin and the Ca^{2+}/calmodulin complex activates Ca^{2+}/calmodulin dependent adenylate cyclase which in turn activates PKA. PKA inactivates protein phosphatases resulting in an increased response to protein kinase stimulation. Ablation of gene targeting of a catalytic subunit isoform or a regulatory subunit isoform of PKA produced a selective defect in MF LTP (Huang *et al.*, 1995).

PKA has been linked to changes in gene expression. Transcription of genes is induced by PKA which activates a transcription factor which then interacts with the cAMP response element (CREs) found upstream of certain genes (Montminy and Bilezikjian, 1987). This CRE-binding protein (CREB) is phosphorylated by PKA and the phosphorylated-CREB stimulates transcription of downstream genes (Yamamoto *et al.*, 1988). The hypothesis is that PKA phosphorylates CREB at the early stages of LTP resulting in a cascade of changes in gene expression which eventually produces the L-LTP. Increased phosphorylation of CREB occurs during LTP induction in CA1 and DG regions (Schulz *et al.*, 1999). Studies have shown that MF LTP may be dependent on the phosphorylation of CREB by PKA (Kanterwicz *et al.*, 2000). Also, CREB knockout mice have abnormal LTP and deficiencies in long-term learning tasks (Bourtchuladze *et al.*, 1994). Hence these studies point to a link between PKA and CREB to plasticity in the CA3 region.

1.6 Microtubule-associated protein 2

Microtubules (MTs), components of the cytoskeleton, are thought to be essential for neurite formation and maintenance (Sanchez *et al.*, 2000). The cytoskeleton is a major determinant of neuronal morphology. The main function of MTs are to provide inner scaffolding for the growth of neurites and also act as tracks for transport of organelles between neuronal cell bodies and neurite endings. MTs consist of a core cylinder built from heterodimers of α and β tubulin monomers (Mandelkow *et al.*, 1995; Desai and Mitchison, 1997). Microtubule associated protein 2 (MAP2) binds to tubulin polymers and regulates their functions (Sanchez *et al.*, 2000). MAP2 have been shown to play an important role in the neurite outgrowth and neuronal plasticity (Diaz-Nido *et al.*, 1990; Sheetz *et al.*, 1998; Woolf, 1998).

MAP2 isoforms expressed in neurons are a result of alternative splicing of a pre-mRNA transcribed from a single gene (Shafit-Zagardo and Kalcheva, 1998). There are two groups of MAP2 isoforms in the mammalian brain:

(i) High molecular weight MAP2 (HMW MAP2), and this includes MAP2a and MAP2b with molecular weights of 280 and 270kDa respectively.

(ii) Low molecular weight MAP2 (LMW MAP2), consisting of MAP2c and MAP2d with molecular weights of 70 and 75kDa respectively.

LMW MAP2 contains the N- and C- terminal regions of HMW MAP2 linked together but lacks the central domain (CD) (Figure 1.8) (Kindler *et al.*, 1990). The presence of the

proline-rich region (PRD) just before the tubulin-binding domain in all MAP2 isoforms could result in the regulation of the isoforms (Goode *et al.*, 1997; Preuss *et al.*, 1997). A unique sequence of 31 amino acids in the N-terminal of MAP2 constitutes the binding region of the regulatory subunit RII of the PKA (Obar *et al.*, 1989). The difference between a and b isoforms of MAP2 is due to the presence in MAP2a of an additional sequence of 83 amino acids (Shaft-Zagardo *et al.*, 1997).

The expression of MAP2 occurs primarily in the nervous system (Schoenfeld and Obar, 1994). HMW MAP2 is specifically expressed in neurons (Cacares *et al.*, 1984; Tucker, 1990) while LMW MAP2 is present also in glial cells (Rosser *et al.*, 1997, Matsunaga *et al.*, 1999). While MAP2b is present all through development of the nervous system, MAP2a expression occurs in the adult brain (Sanchez *et al.*, 2000).

The levels of MAP2c protein and mRNA are detectable at early developmental stages, while MAP2d protein is detected in rat brain after postnatal day 5, even though its mRNA is present all through the various developmental stages (Sanchez *et al.*, 2000). This differential expression of MAP2 isoforms may be an indication of the complexity of their regulation at both transcriptional and translational levels. HMW MAP2 has been shown to be located in neuronal cell bodies and dendrites where it associates with MT. It has also been shown to co-localise with actin in the dendritic spines and postsynaptic densities (Cacares *et al.*, 1983, 1984; Langnaese *et al.*, 1996). LMW MAP2 in contrast is widely distributed in every neuronal compartment (Albala *et al.*, 1995).

Several experiments in neuronal cells have implicated MAP2 in neuronal outgrowth and polarity (Ferreira *et al.*, 1989). Suppression of MAP2 expression in neuronal cultures resulted in no neurite outgrowth (Dinsmore and Solomon, 1991; Cacares *et al.*, 1992). Similarly overexpression of MAP2 in cultured non-neuronal cells resulted in outgrowth of cytoplasmic elongations similar to neurites (Boucher *et al.*, 1999).

1.7 Activin/Inhibin

Activin and inhibin belong to the transforming growth factor-β (TGF-β) super-family of molecules and activin has been implicated in nerve cell survival and inhibition of differentiation *in vitro* (Hashimoto *et al.*, 1990; Schubert *et al.*, 1990). Both activin and inhibin are dimeric proteins which were first isolated from the ovary and were able to modulate follicle stimulating hormone (FSH) release from the pituitary in a long loop endocrine fashion (Halvorson and Decherney, 1996; Ling *et al.*, 1986b). Both dimeric proteins are sulphydryl-linked comprising two of three distinct inhibin proteins subunits (α, βA, βB). While inhibins are heterodimers composed of one α-subunit and one β-subunit, resulting in inhibin A (α-βA) or inhibin (α-βB) (Mason *et al.*, 1986), activins are dimers made up of any combination of the β-subunits resulting in activin A (βA-βA), activin AB (βA-βB) and activin B (βB-βB) (Ling *et al.*, 1986a; Vale *et al.*, 1986). Activin produces its effect via binding to a heterotrimeric receptor complex with transmembrane serine/threonine kinase activity (Mathews, 1994). The role of activin β-A in LTP induction will be discussed in section 7.1.

1.8 mRNA localisation in Neurons

Neurons are thought to employ three strategies for the activity-dependent regulation of protein synthesis and targeting. The first strategy focuses on the translation of proteins in the soma from newly transcribed mRNAs e.g. activation of transcription factors such as CREB in response to a particular form of synaptic stimulation (Alberini *et al.*, 1994) results in increased transcription of specific target genes, followed by translation of the corresponding mRNAs. This process is thought to involve the creation of 'tags' at the activated synapses (Frey and Morris, 1997). These 'tags' act to capture the newly synthesised proteins, at the synapse that has been potentiated, as they are transported out along the dendrites. The second strategy also involves activity-dependent gene transcription but involves the transportation of newly transcribed mRNAs to activated synapses where they are thought to be translated. This mechanism was recently described for Arc (activity-regulated cytoskeleton–associated protein), an immediate early gene whose transcription is tightly regulated by synaptic activity (Steward *et al.*, 1998; Steward and Worely, 2002). The third and final strategy for achieving activity-dependent regulation of protein synthesis is via the regulation of mRNA localised at synapses. This will be the focus of some of this investigation.

The discovery of synapse-associated polyribosome complexes (SPRCs) sparked an interest in RNA localisation in neurons. SPRCs are clusters of polyribosomes and associated membranous cisterns that are selectively localised beneath postsynaptic sites in the dendrites of CNS neurons (Steward *et al.*, 1996). It was suggested that a particular

subset of mRNA would be localised in dendrites, enabling a localised synthesis of certain proteins at postsynaptic sites. In situ hybridisation analysis looked at the features of mRNA localisation in the dendrites and the following were ascertained:

(i) The mRNAs in dendrites encode proteins of different functional types and these include MAP2, Arc, and αCaMKII as well as other proteins of unknown function (Steward *et al.*, 1996).

(ii) Different mRNAs are localised in the dendrites of different neuron types e.g. the mRNA of MAP2 and αCaMKII are prominent in the dendrites of neurons in the cortex and hippocampus.

(iii) Different mRNAs are localised in different domains within the dendrites, with mRNA for αCaMKII distributed throughout the dendrites and MAP2 mRNA concentrated in proximal dendrites (Steward *et al.*, 1996).

Recent work has established a direct link between local mRNA translation and synaptic plasticity. As already mentioned above (section 1.6) the NMDA receptor dependent form of LTP results in the elevation of MAP2 mRNA and the corresponding protein. Ouyang *et al.* (1999), showed that tetanic stimulation of the Schaffer collateral pathway in the CA1 region of hippocampus results in the increased expression of αCaMKII protein in the dendrites via dendritic protein synthesis. Anisomycin was used to block the increase, which was detected by both quantitative immunoblot and semi-quantitative immunocytochemistry. L.A. Roberts *et al.* (1996) also showed an increase in the levels of αCaMKII mRNA following induction of LTP in the CA1 neurons in rat hippocampal slices maintained *in vitro*.

The mechanism by which dendritic mRNAs become transitionally active may involve regulated polyadenylation by CPEB (cytoplasmic polyadenylation element binding protein) (Wu *et al.*, 1998). Understanding of the role of CPEB in neurons is based on work in oocyte maturation. The CPE (cytoplasmic polyadenylation element) is bound by CPEB which contains two RNA recognition motifs (RRMs) and a zinc finger- all of which are important for CPE-dependent RNA binding. CPEB is activated by a single phosphorylation event (Wells *et al.*, 2000) and this phosphorylation in the Xenopus is catalysed by serine/threonine kinase, Erg2. A possible mechanism of Erg2 is to recruit or stabilise the binding of CPSF (cleavage and polyadenylation specificity factor) which in turn recruits poly(A) polymerase to the end of the mRNA (Wells *et al.*, 2000). Hence the process of polyadenylation initiates mRNA translation. CPEB is highly expressed in the cell bodies and dendritic layers of the hippocampus (Wu *et al.*, 1998). The 3' untranslated region (UTR) of αCaMKII mRNA contains two CPEs. Therefore, CPEB binds this 3'UTR in a CPE-depedent manner and the 3'UTR can regulate protein translation in a CPE dependent manner in a heterologous system (Wu *et al.*, 1998). However, MAP2 mRNA contains no CPEs, and Erg2 has never been linked to hippocampal plasticity, so firm evidence that this pathway is operative in LTP is lacking.

1.9 The mTOR Kinase

Rapamycin is a lipophilic macrolide, isolated from a strain of Streptomyces hygrosocopicus which is indigenous to Easter Island (Raught *et al.*, 2001). FKBP12

(FK506-binding protein) is a small ubiquitous receptor protein to which rapamycin binds intracellularly in all eukaryotes (Harding *et al.*, 1989). Rapamycin and FKBP12 bind to form a "gain of function" complex which then interacts with specificity to the evolutionarily conserved mammalian target of rapamycin (mTOR) protein resulting in potent inhibition of signalling of targets downstream (Raught *et al.*, 2001). A single mTOR protein has been cloned from several species. mTOR is also known as FRAP (FKBP12 and rapamycin associated protein) or RAPT (rapamycin targets) (Raught *et al.*, 2001). In this thesis, the kinase will be referred to as mTOR.

The TOR protein belongs to a protein family termed phosphatidylinositiol kinase-related kinases (or PIKKs), a large group of signalling molecules (Raught *et al.*, 2001). Rapamycin has been used recently to study a growth factor-regulated signalling pathway that results in enhanced translation of a specific sub-set of mRNAs via mTOR activation. Rapamycin significantly inhibits translation of mRNAs derived from a few genes (Brown and Schreiber, 1996). The class of mRNAs whose translation is inhibited by rapamycin posses a 5' terminal oligo-pyrimidine tract (5' TOP), these mRNAs are present in mammalian cells (Jefferies *et al.*, 1997). Rapamycin sensitive genes include those encoding ribosomal proteins (S3, S6, S14 and S24), translation elongation factors (eEF1A and eEF2), and a secreted peptide growth factor called insulin-like growth factor II (IGFII) (Brown *et al.*, 1996). It is likely that more 5' TOP mRNAs are yet to be identified.

mTOR controls the mammalian translation machinery in response to amino acids and growth factors, via activation of $p70^{S6K}$ protein kinase and via inhibition of the eIF4E (eukaryotic translation initiation factor 4E, directs the translation machinery to the 5' end of the mRNA) inhibitor, 4EBP1 (Figure 1.9). Activation of $p70^{S6K}$ resulting in phosphorylation of the 40S ribosomal protein, S6, ultimately drives translation of 5'TOP mRNAs (Schmelzle and Hall, 2000). Regulation of $p70^{S6K}$ is complex and involves a hierarchical phosphorylation of several sites in $p70^{S6K}$ by different kinases (Schmelzle and Hall, 2000); rapamycin principally affects only one of these sites. Rapamycin inhibits the phosphorylation of S6 kinase at threonine 399 but not at threonine 239 (Khan *et al.*, 2001).

Using the techniques of western blot and immunostaining, the translational machinery mTOR, 4EBP, and eIF4E, were shown to be localised in the soma and dendrites in the hippocampus (Tang *et al.*, 2002). Tang *et al.* (2002) suggested that the localisation of this translational signalling pathway in the postsynaptic sites may provide a mechanism for controlling local protein synthesis at potentiated synapses. The group went on to show that the disruption of this translational signalling pathway with rapamycin inhibited expression of LTP from 2-3 hours after tetanus. Tskas *et al.* (2002) looked at the effect of blockage of phosphorylation of $p70^{S6K}$ by rapamycin and indicated that induction of L-LTP is associated with dendritic activation of translation initiation pathways.

1.10 A23187 and Forskolin

A23187 is a Ca^{2+} ionophore that has been shown to elevate intracellular Ca^{2+} levels at 6μM concentrations in PC12 cells (Lev *et al.*, 1995) and 10μM concentration in hippocampal cells (Siciliano *et al.*, 1996). Forskolin is a naturally occurring diterpene which has been shown to activate adenylate cyclase at 50μM concentration resulting in increased levels of cAMP which then activates PKA (Weisskopf *et al.*, 1994, Huang *et al.*, 1995). Hence, elevation of cAMP via application of forskolin is important in MF LTP and this is achieved by activation of Ca^{2+} /calmodulin sensitive AC (Weisskopf *et al.*, 1994). In the L-LTP, the CA1 and CA3 regions of the hippocampus were shown to require cAMP-mediated mechanism (Huang *et al.*, 1994).

1.11 Aims of the study

Ca^{2+} and cAMP are the two major effectors of NMDA receptor activation with Ca^{2+} entering via activated receptor and cAMP elevation due to Ca^{2+}-sensitive adenylate cyclase activation. LTP2 is dependent on mRNA translation but is independent of *de novo* protein synthesis (Morris, 1996) and its lasts between 1-6 hours. αCaMKII and MAP2 mRNAs are present in neuronal dendrites and cell soma and have been shown to be associated with various forms of neuronal plasticity. Most of the studies have looked at the increased levels of MAP2 mRNA and αCaMKII mRNA which were detected after LTP induction at least in the DG and CA1 regions of the hippocampus, it is not known yet if similar increases are observed in the CA3. In the case of β-activin, increases of the

mRNA encoding β-activin were also detected in the CA1 and DG regions with little known about the CA3. The effect of the two messengers on the activation of Pyk2 (chapter 3) and MAPK (chapter 4), and on the levels of protein expression of αCaMKII (chapter 5), MAP2 (chapter 6) and β-activin (chapter 7) in the acute or organotypic hippocampal slices was investigated. The increase of MAP2, αCaMKII and β-activin proteins were investigated by using A23187 (Ca^{2+} ionophore) and forskolin (adenylate cyclase activator). The effect of inhibition of mTOR on the expression of MAP2 and αCaMKII proteins in organotypic hippocampal slices was also investigated to determine whether the rapamycin-sensitive translational pathway was involved in their expression.

Using the techniques of acute hippocampal slice preparations, the effect of Ca^{2+} and cAMP on the phosphorylation of Pyk2 and MAPK was investigated. Like MAP2, αCaMKII and β-activin, most of the studies on the two enzymes have been carried out in the CA1 and DG with little known about the CA3. In case of Pyk2, inhibitors of novel PKC, staurosporine, and atypical PKC (PKM), chelerythrine were used prior to activation, in order to investigate the role of Pyk2 in LTP maintenance since PKM has been implicated in LTP maintenance (Ling *et al.*, 2002).

Therefore the thesis is set out to answer the following questions:

i. Is PyK2 activation affected by intracellular elevation of Ca^{2+} or cAMP in the hippocampus? If so, is that the case in CA3 region?
ii. Are these changes in Pyk2 affected on the application of PKC inhibitors?

iii. Is MAPK activation affected by increases in the level of intracellular Ca^{2+} or cAMP elevation in the CA3 region?

iv. Are the expressions of αCaMKII, MAP2 and β-activin affected by Ca^{2+} or cAMP intercellular elevation in the CA3 region?

v. Is rapamycin-sensitive pathway involved in the expression of αCaMKII and MAP2?

Figure 1.1: Hippocampal formation of rat brain. The hippocampus is an elongated C-shape structure made up of dentate gyrus, hippocampal proper (CA3, CA2 and CA1, only CA1 and CA3 regions shown) and subicular region. The CA1 region is blown up to show the different laminae while the blown CA3 regions shows its pyramidal cells receiving the majority of their inputs from other CA3 pyramidal cells. In the dentate gyrus the pyramidal layer is replaced with the granular layer. Adapted from Amaral and Witter, 1989.

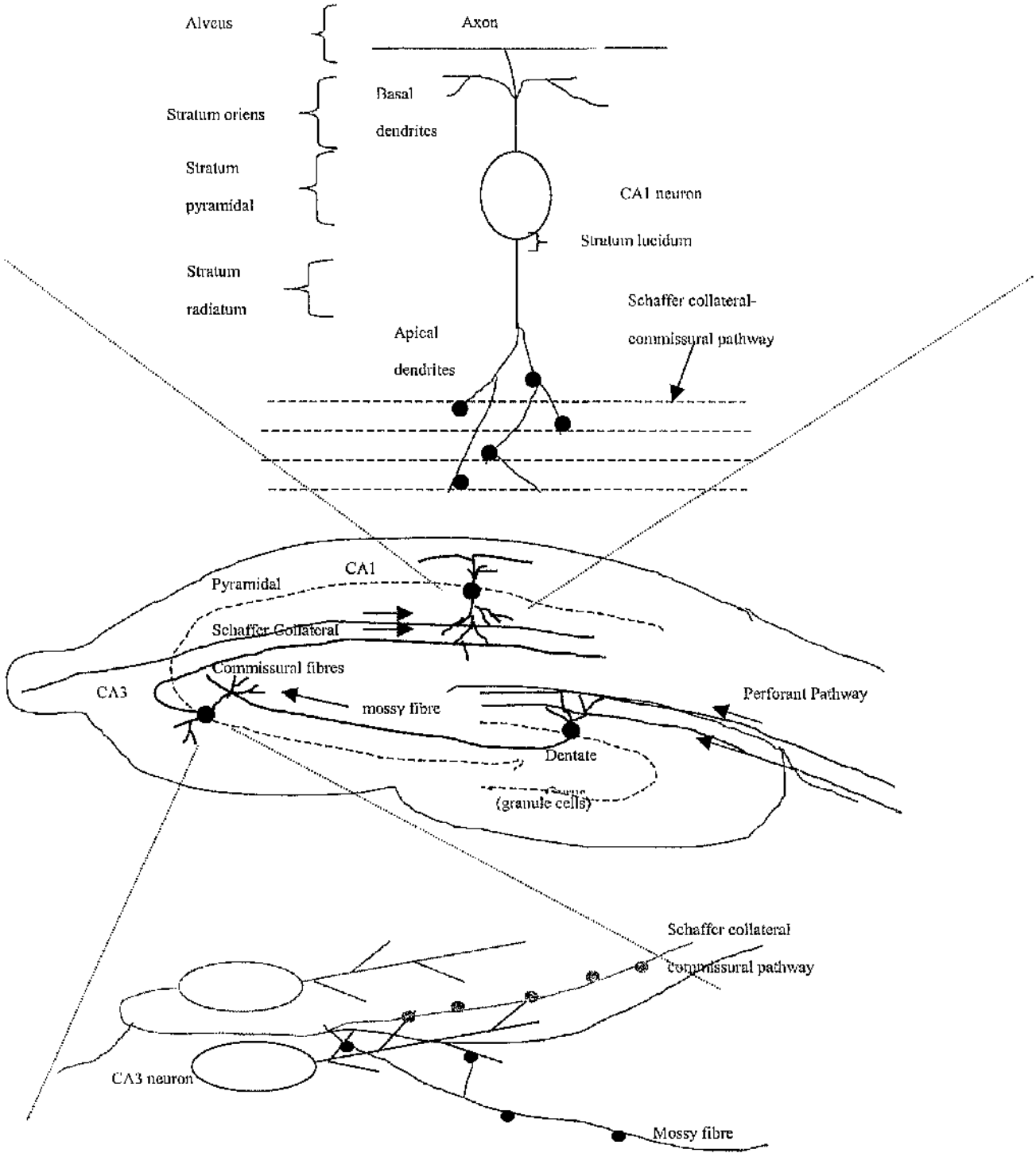
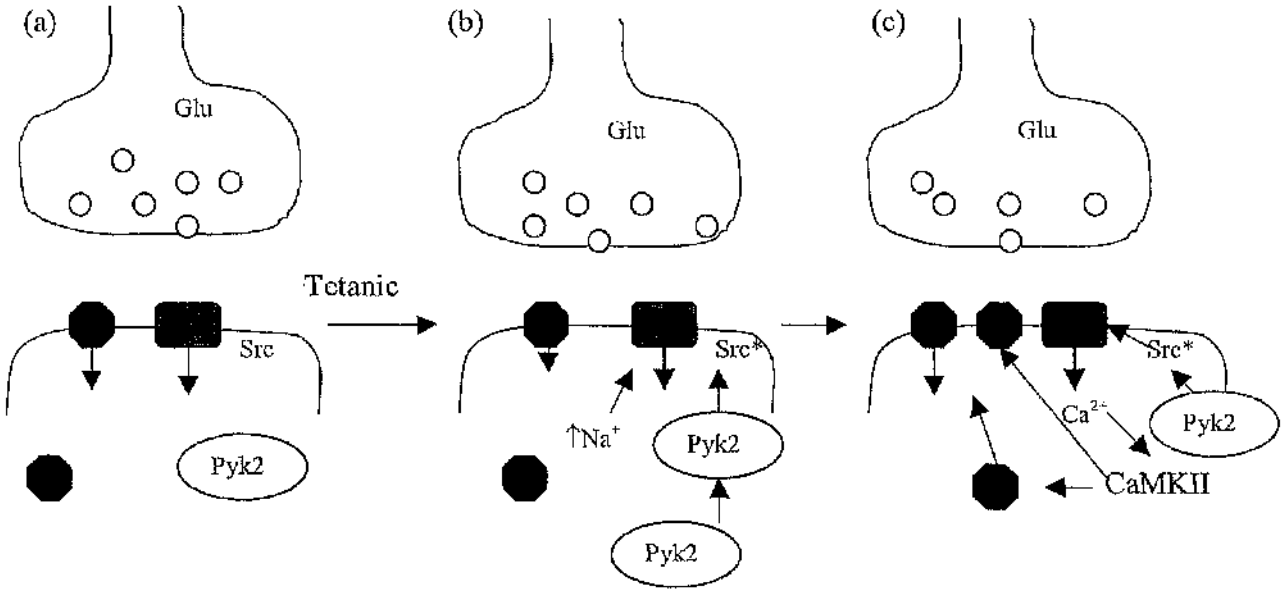

Alveus
Axon
Basal
dendrites
Stratum oriens
Stratum
pyramidal
CA1 neuron
Stratum lucidum
Stratum
radiatum
Schaffer collateral-
commissural pathway
Apical
dendrites
CA1
Pyramidal
Schaffer Collateral
Commissural fibres
CA3
mossy fibre
Perforant Pathway
Dentate
(granule cells)
Schaffer collateral
commissural pathway
CA3 neuron
Mossy fibre

Figure 1.2: The structure of Pyk2. The length of the peptide chain is indicated without the alternatively splice exons, which are represented as boxes with the corresponding number of amino acids inserted below the sequence. The position of the autophosphorylated tyrosine residue is Tyr402. Adapted from Girault *et al.*, 1999.

PyK2/CAKβ

Figure 1.3: Model for the role of protein kinases Src and Pyk2 (CAKβ) in LTP induction in the CA1 region. a) At rest, the Src and CAKβ are inactive in the post-synapse and the Mg^{2+} inhibits but does not completely block NMDAR currents. b) Tetanic stimulation results in the release of the Mg^{2+} block, causing an increase in the NMDAR currents resulting in the activation of CAKβ (CAKβ*) and Src (Src*) and increase sensitisation of NMDAR to raised in intracellular levels of Na^{+}. c) There is an increased in intracellular Ca^{2+} as a result of upregulation of NMDAR, this increase causes activation of CaMKII and the expression of LTP results from insertion of AMPARs into postsynaptic membrane. Adapted from Ali and Salter, 2001.
Abbreviation: CAKβ- cell adhesion kinase β


(a)
Glu
(b)
Glu
(c)
Glu
Tetanic
Src
Pyk2
↑Na+
Src*
Pyk2
Pyk2
Src*
Ca2+
Pyk2
CaMKII
AMPAR
NMDAR

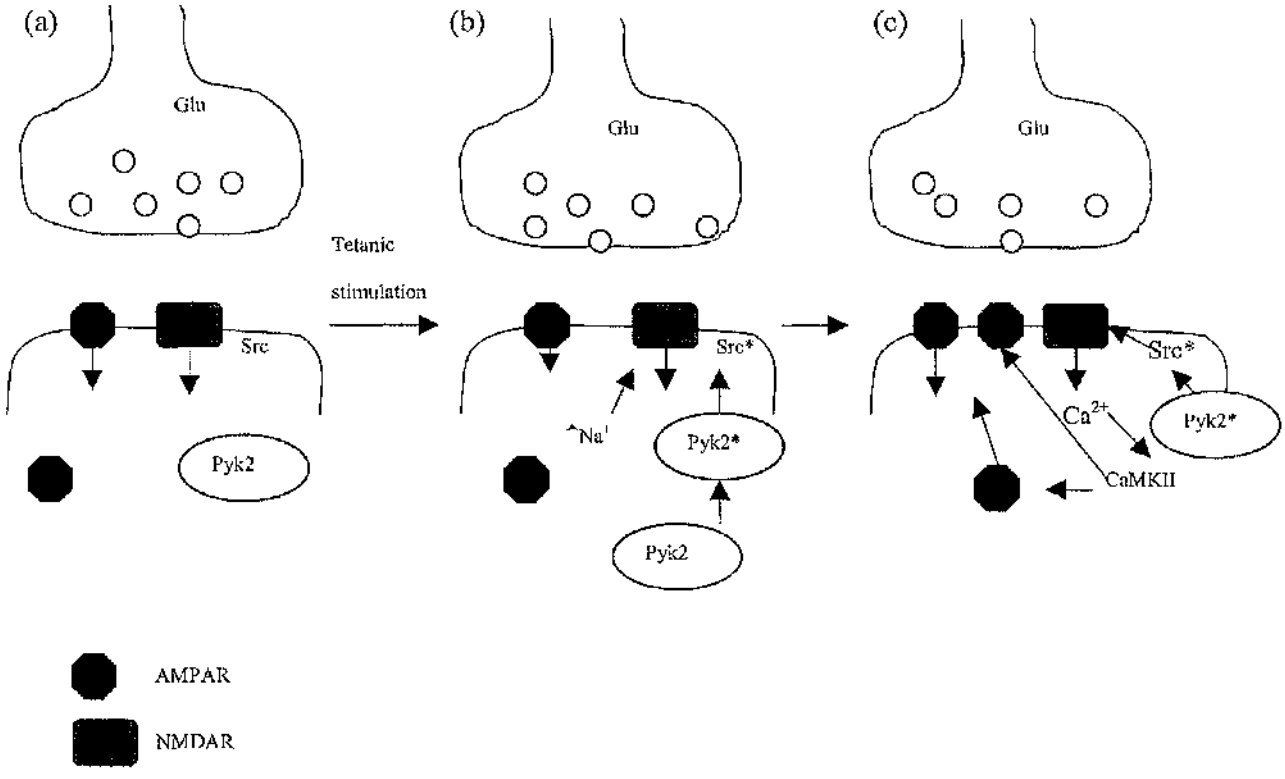
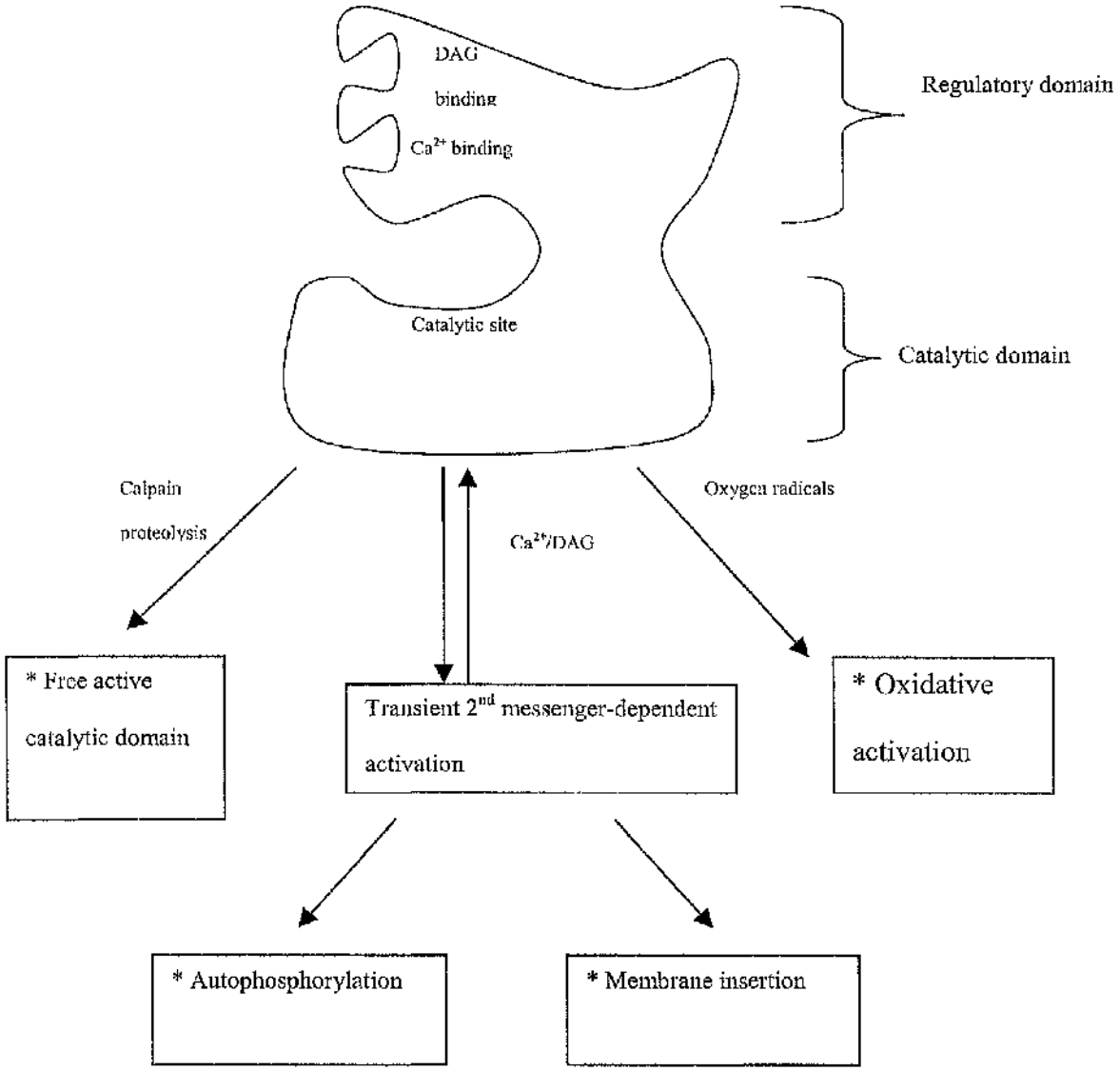
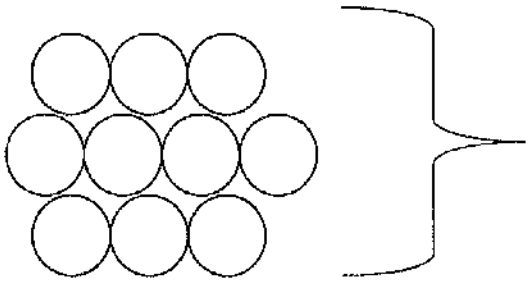

(a)
Glu
(b)
Glu
(c)
Glu
Tetanic
stimulation
Src
Pyk2
↑Na+
Src*
Pyk2*
Pyk2
Src*
Ca2+
Pyk2*
CaMKII
AMPAR
NMDAR

Figure 1.4: Mechanism for activation of PKC during LTP. The diagram shows different 2nd messengers and enymes involved in the activation of the different isoforms of PKC (Adapted from Roberson *et al.*, 1996a).

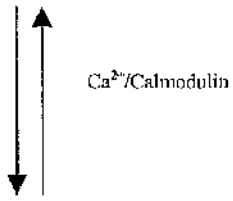


* Autonomous activity

Figure 1.5: Mechanism of CaMKII activation in LTP. The mechanisms of transient and auotphosphorylated activation of CaMKII are highlighted. Adapted from Roberson *et al.*, 1996a.



10-12 subunits each with regulatory and catalytic domains



Transient 2nd messenger dependent activation

* Autophosphorylation

Ca^{2+}-independent form

* Autonomous activity

Figure 1.6: Schematic diagram of the effect of cAMP on PKA. Binding of cAMP to the regulatory subunit results in conformational change that causes the release of the active catalytic subunit. The activated PKA phosphorylates CREB resulting in synthesis of new proteins via activation of late response genes. Abbreviation: CREB-cAMP response element binding protein.

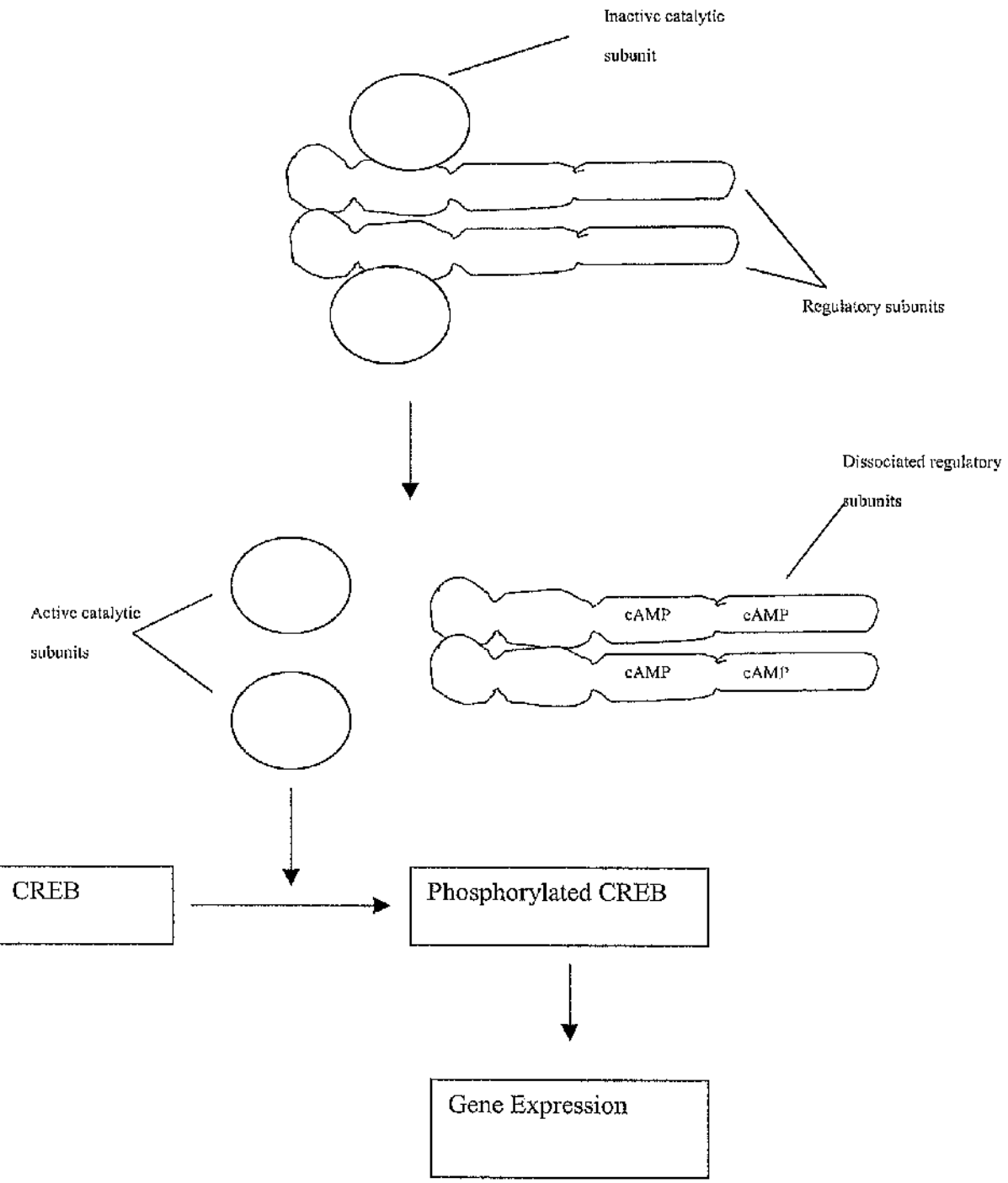

Inactive catalytic
subunit
Regulatory subunits
Dissociated regulatory
subunits
Active catalytic
subunits
cAMP
cAMP
cAMP
cAMP
CREB
Phosphorylated CREB
Gene Expression

Figure 1.7: Schematic diagram summarising the activation of the following protein kinases: Src, PyK2, PKC, CaMKII, PKA and MAPK. The diagram also indicates the substrates of the kinases. Activation of Pyk2 due to increases in intracellular Ca^{2+} results in the activation of Src, which in turn increases potentiation of NMDA receptors. Actiavtion of PKC (due to increases in Ca^{2+} levels or activation of mGluR) also activates Pyk2. CaMKII activation results in phosporylation of the AMPA receptor, while activation of PKA (due to increase levels of cAMP) causes the activation of MAPK. MAPK then activates CREB resulting in gene expression.

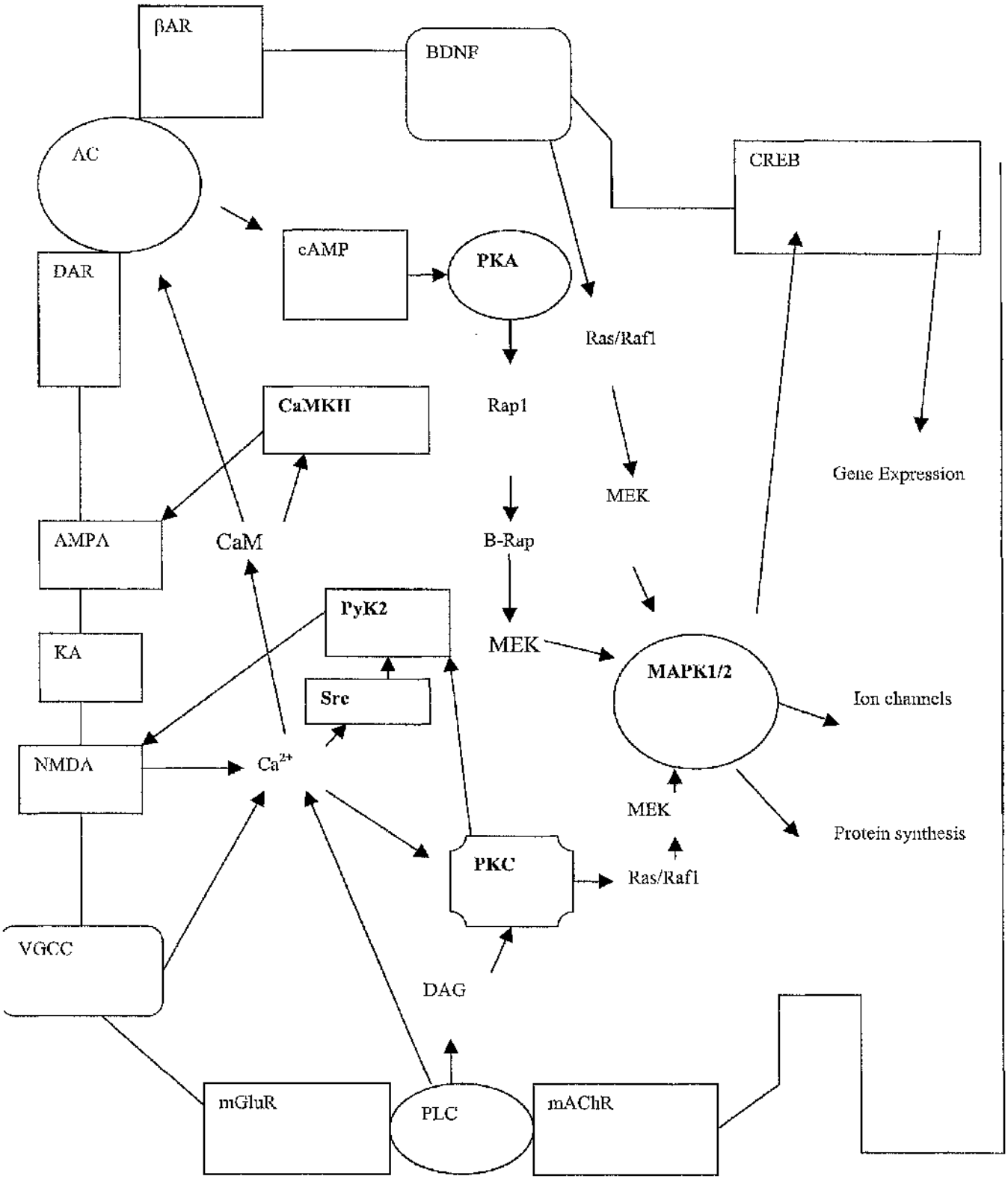

βAR
BDNF
AC
CREB
cAMP
PKA
DAR
Ras/Raf1
CaMKII
Rap1
Gene Expression
MEK
AMPA
CaM
B-Rap
PyK2
MEK
KA
MAPK1/2
Src
Ion channels
NMDA
Ca2+
MEK
Protein synthesis
PKC
Ras/Raf1
VGCC
DAG
mGluR
PLC
mAChR

Figure 1.8: Structure of MAP2 isoforms showing the MAP2 domains. Two groups of MAP2 isoforms exist, the HMW MAP2 which includes MAP2A and MAP2B and LWM MAP2 which includes MAP2C and MAP2D. The LMW MAP2 lacks the central domain (CD) but contains N- and C-terminal regions of HMW MAP2 linked together. Adapted from Sanchez *et al.*, 2000. Abbreviations: RII-regulatory subunit of PKA; TBD-tubulin binding domain; PRD-proline-rich domain; CD-central domain.

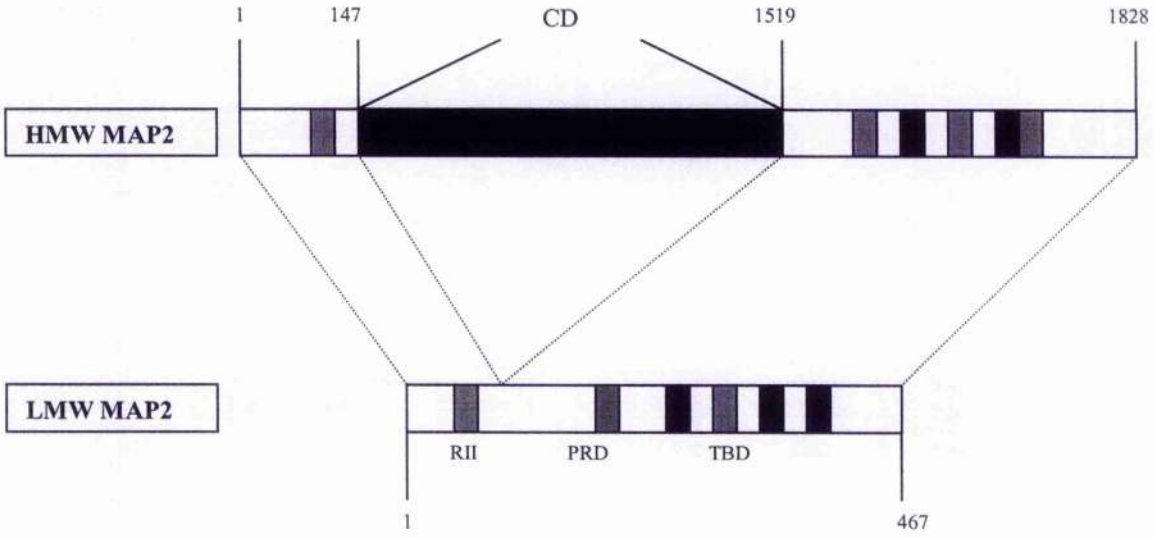
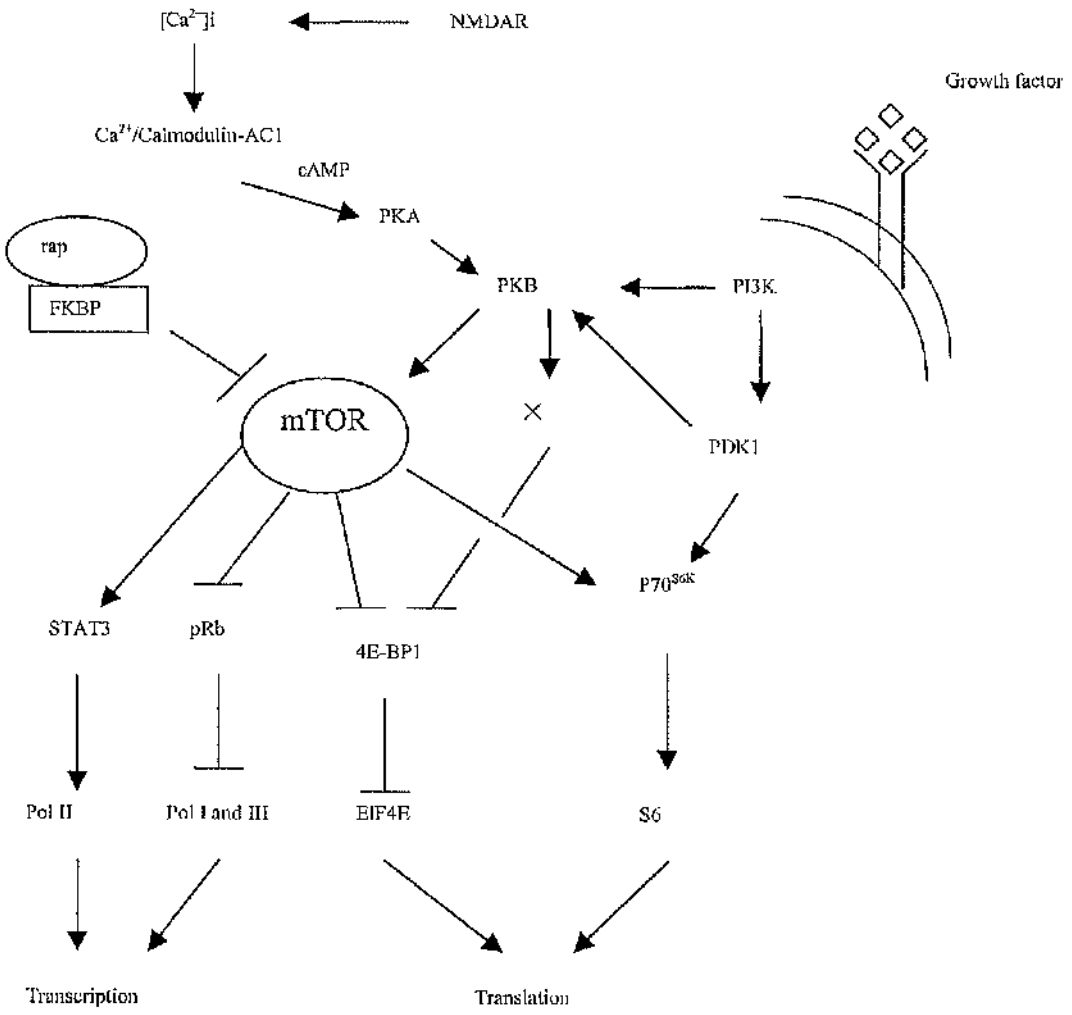

1
147
CD
1519
1828
HMW MAP2
LMW MAP2
RII
PRD
TBD
1
467

Figure 1.9: Model of the effectors of mTOR signalling pathway in mammalian cells. Arrows indicate activation while the bars represent inhibition. mTOR activates the translation machinery, i.e. $p70^{S6K}$ and phosphorylates 4E-BP1 hence allowing translation. Adapted from Schmelzle and Hall, 2000. Abbrevaitions: PDK1-3'-phosphoinositide-dependent protein kinase 1; pRb-retinoblastoma protein; PI3K-phosphotidylinositol-3-kinase; ×- unknown enzyme/protein.


[Ca2+]i
NMDAR
Growth factor
Ca2+/Calmodulin-AC1
cAMP
PKA
rap
FKBP
PKB
PI3K
mTOR
PDK1
P70S6K
STAT3
pRb
4E-BP1
Pol II
Pol I and III
EIF4E
S6
Transcription
Translation

Chapter 2

Methods

2.1 Organotypic Slice Culture

Many different techniques have been tested to maintain explants of nervous tissue in culture. Roller tube technique was one of the most successful procedures (Gahwiler, 1988). This procedure enabled the culturing of slices from different areas of the central nervous system. One characteristic of this procedure was the organotypic organisation of the explant and the monolayer aspect. The monolayer developed over a period of 2-3 weeks, enabling the visualisation of individual cells with phase contrast microscopy. A different and simple method was developed in which the explants were placed on a membrane at the interface between air and medium. This also allowed maintenance of explant of central nervous system and more specifically hippocampal slices in culture. The results obtained with this technique were similar to roller tube technique, but the major advantages of this method were the simplicity, the well preserved organotypic organisation of the tissue and suitability of this technique for the studies of physiological mechanisms occurring during the first day or weeks in culture (Stoppini *et al.*, 1991).

Organotypic slice cultures were prepared according to the method of Stoppini *et al.* (1991). Neonatal, Wistar rats, between ages 6-10 days were injected with a lethal dose of anaesthetic [Euthatal, (Rhone Merieux), 0.2mg/kg]. After decapitation the brain was quickly removed and placed in ice cold HBSS (Hanks' Balanced Salt Solution, GIBCO™) buffer which had been gassed (95% O_2 and 5% CO_2) for 10-15 minutes. The dissection took place in the solution and the hippocampus was placed on the tissue chopper after extraction. The tissue

chopper was set to 250μm and the hippocampus cut transversely. The sliced hippocampus was placed in a fresh ice-cold gassed (95% O_2 and 5% CO_2) HBSS and the slices teased apart using sealed glass micropipettes (Clarke Instruments).

Between 12-16 hippocampal slices were normally obtained. The slices were then placed in another solution of fresh ice-cold gassed HBSS and transferred to the culture room.
In a laminar flow cabinet, the slices were transferred to 6-well plates (Iwaki). 1ml of a Wilde medium [BME (Basal Medium Eagle) 1× liquid with Earle's salt without L-glutamine (88.5%), horse serum heat inactivated (17.7%), glucose (22.5g/L), glutamax™-1 Supplement (0.46%), penicillin/streptomycin solution (5%)] was added to 4 wells each. Inserts of 0.2 μm pore (Falcon) were placed in the 4 wells and placed in the humidified, 37 C, 95 % O_2 and 5% CO_2 incubator for 30 minutes prior to dissection. All the contents of the Wilde medium were purchased from GIBCO™.

Two sterile pastettes (Alpha Laboratories Ltd) were used to transfer the slices onto the inserts. One to extract the 4 hippocampal slices from the oxygenated HBSS and one to remove the excess HBSS on the inserts. This procedure was repeated until all the slices had been placed in the wells (normally 4 slices/well). The 6-well plates with their contents were then placed in the incubator.

The Wilde medium was changed after 24 hours incubation. This involved the use of two sterile needles and syringes. One syringe was used to extract the old medium from the wells while the second syringe was used to add 1ml of the fresh medium. The slices were incubated for 7-8 days changing the medium again after 3-4 days. At the end of 7-8 days the slices were ready for drug treatment and immunocytochemistry or western blot.

2.2 Acute Slice Preparations

An adult rat (weighing 150-200g) was culled by injecting a lethal dose of an anaesthetic (Euthatal, 1.5mg/kg). After decapitation the brain was removed. This involved opening of the skull with a pair of scissors and removal of the brain using a spatula. The brain was hemisected in ice-cold (at 4°C) high Mg^{2+} (10mM) artificial cerebrospinal fluid (aCSF) which was made up of 124mM NaCl, 3mM KCl, 26mM $NaHCO_3$, 1.25mM NaH_2PO_4, 10mM $MgSO_4(H_2O)$, 18.02mM D-glucose and 2mM $CaCl_2$. All the compounds used were purchased from BDH Laboratory Supplies apart from glucose which was purchased from Fisher Scientific.

The hippocampus was removed from the rest of the brain by using spatulas, and using a tissue chopper, 300μm transverse hippocampal slices were obtained. The slices were then placed in a petri dish of fresh ice cold high Mg^{2+} aCSF solution (same composition as above) in a petri dish and allowed to recover for 60 minutes in an gassed chamber (95% O_2 and 5% CO_2) at room temperature. An hour after recovery the temperature was increased to 32°C and the slices were left for a further hour after which the slices were ready for drug treatment.

2.3 Drug Treatment

The drugs used in this set of experiments were: 5μM of A23187 (Sigma), 50μM forskolin (Sigma), 20nM rapamycin (Calbiochem), 1μM chelerythrine (CN bioscience) and 100nM staurosporine (CN bioscience). The stock solution of these drugs was dissolved in dimethyl sulfoxide (DMSO, BDH laboratories Supplies), apart from staurosporine which was dissolved in methanol.

The drugs (A23187, forskolin and rapamycin) were diluted in HBSS, and HBSS with DMSO was used as the control for cultured slices since HBSS was the saline solution. After the incubation, the cultured slices were treated by placing 1.5µl of the drug/control on top of each slice. The procedure was performed in a sterilised laminar flow cabinet. In the case of acute slices, the drugs were diluted in high Mg^{2+} aCSF and high Mg^{2+} aCSF with DMSO was used as control. The slices were then placed in a gassed aCSF solution containing the drugs of interest. High Mg^{2+} was used in this study in order to prevent neurotoxicity as result of NMDA activation.

2.4 Dissection of the CA1 and CA3 sub-regions of acute hippocampal slices

After drug treatment, the acute hippocampal slices were placed in an ice-cold, high Mg^{2+}, gassed aCSF [containing 1mM sodium orthovanadate (Na_3VO_4, Sigma, inhibits protein phosphatases) and 50mM sodium fluoride (NaF, Sigma)]. The CA1 and CA3 were dissected from the rest of the hippocampus using sealed glass micropipettes and carbon steel surgical blades (Swann-Morton), using a dissection microscope (Figure 2.1). CA1 and CA3 sub-regions of acute hippocampal slices were then ready for immunoprecipitation.

2.5 Electrophysiological Recordings

Slices from mice (5-10 weeks old) were prepared and used in electrophysiology according to the method described in the Appendix. After induction and measurement of the late phase of LTP, the slices were quickly frozen in liquid nitrogen and the CA1 region was removed from the rest of the hippocampus. The CA1 region was then thawed and placed in an ice-cold (4°C) RIPA buffer, according the method describe in section 2.4. The CA1 region was then ready for western blot technique (see section 2.9).

2.6 Determination of Protein Concentration

Protein concentrations from cultured or acute slices were initially determined, before performing the technique of Western Blot, by using the BIO-RAD micro protein assay, based on the method of Bradford (1976), with BSA used as a standard. 4µl of the sample was diluted in 400µl of dH_2O and 200µl of diluted BIO-RAD reagent (1:1 dH_2O). The samples were then placed in 96 well plate (Iwaki) and using the Plate Reader Program (Thermo Life Sciences) set at 595nm, the concentrations were measured.

2.7 Immunocytochemistry

The cultured slices were fixed for 30 minutes using 3% buffered formaldehyde (Riedel-de Haen) and then processed for immunocytochemistry as described previously (Morris, 1997). The buffer used was phosphate buffered saline solution (PBS) made up of 130mM NaCl, 8mM Na_2HPO_4, 2mM NaH_2PO_4 and 500ml of distil H_20, pH 7.5. Fixation was followed by 2×5 minutes washes in PBS.

The slice cultures were then blocked in blocking serum [15%of normal goat serum (purchased from Law Hospital, Carluke, Scotland) and 1ml of PBS] for 1hour. Hippocampal slice cultures were left overnight at 4°C in primary antiserum [primary antibody diluted to required concentration, 3% normal goat serum, 0.5% Triton ×100 (Sigma) and 1ml PBS]. After 3×5 minute washes in PBS, the slice cultures were then incubated for 1hour in secondary antiserum [0.5% of biotinylated anti-mouse/anti- rabbit antiserum (Vector Laboratories Ltd), 1.5% of normal goat serum and 1ml PBS]. The slice cultures were then washed 3×5 minutes in PBS and incubated for 1 hour with ABC reagent [2% of solution A, 2% solution B (Vector Laboratories Ltd) and 1ml of PBS]. Following another 3×5 minute washes in PBS the slice cultures were incubated in peroxidase substrate kit (Vector VIP™, catalogue number 4600, Vector Laboratories, consisting 3 drops of reagent 1, 3 drops of reagent 2, 3 drops of reagent 3 and 3 drops of H_2O_2) with 5mls PBS, for between 5-10 minutes. Afterwards the slice cultures were washed for 5 minutes in PBS followed by 5minutes in distilled H_20, and then placed for 30 minutes in 70%, 90%, 100%, 100%, 100% ethanol respectively. Slices were allowed to dry briefly and then placed in histoclear (National Diagnostics) overnight. The following day slices were histomounted using a histomount (National Diagnostics) and coverslipped (using BDH microscope slides and coverglass).

In all experiments, vehicle controls were performed alongside drug treated slices, the control and treated slices were processed identically in the immunocytochemical procedure.

2.8 Immunoprecipitation (IP)

The hippocampal slices (acute slices), CA1 or CA3 sub-regions were placed in 100-200µl of ice-cold (4°C) radioimmunoprecipitation (RIPA) buffer [1M Tris pH 8 (Boehringer Mannheim), 1M NaCl, 0.5% IGEPAL CA-630 (NP40, Sigma), 0.5% deoxycholate (DOC, Sigma), 10% sodium dodecyl sulfate (SDS, Fisher Scientific International Company), 1mM EGTA (Sigma), 1mM EDTA (Sigma), 1mM Na_3VO_4, 1 protease inhibitor cocktail tablet (Roche Diagnostic) and 10 ml of distilled H_2O].

The acute slices were homogenised, using pellet pestle (Sigma), in 100-200µl of RIPA buffer, and underwent 10,000g centrifugation at 4°C for 10 minutes. The supernatant was removed and was centrifuged again at 10,000g, 4°C for 10 minutes. The volume of the resultant supernatant was increased to 500µl, ensuring there was enough volume to incubate in the secondary antibody.

A primary antibody [anti-Pyk2, 1:100 dilution, Table 2.1] was added to the supernatant samples. The samples were placed in a rotator for 2 hours at 4°C. A 50µl proteinG-sepharose (Sigma) was added to the samples which were left for 1 hour at 4°C in the rotator.

After 1 hour, the samples were centrifuged at 400g for 30 seconds. 100µl of the supernatant and the sepharose (the pellet in this case) were kept. The samples were kept at 4°C for the duration of the washes. The sepharose was washed three times in IP washing buffer {100mM (N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]), HEPES (Sigma), 100mM NaCl and 0.5% NP40} and after each wash the solution was centrifuged (400g for 30 seconds) and the IP washing buffer removed. The sepharose was then washed for the last time in IP washing buffer without NP40 and centrifuged (400g for 30 seconds). Once the washing

buffer had been removed, 100µl of 2× NuPAGE ™LDS Sample Buffer (Invitrogen) was added to the sepharose. A reducing agent (1×), NuPAGE ™ Sample reducing agent (10×) purchased from Invitrogen, was also added to the sample (10µl of reducing agent in 100µl of sample solution) and mixed. The reducing agent (which contains β-mercaptoethanol) prevents aggregation due to disulfide formation between newly exposed cysteins of protein samples.

The samples were placed in a heating block set at 70°C for ten minutes, to denature the sample protein. After mixing the sample, it was centrifuge at 400g for 30 seconds. The resultant supernatant was then ready for electrophoresis as explained in the Western Blot protocol (see section 2.9).

2.9 SDS PAGE and Western Blotting

After drug treatments the slices (acute or organotypic cultures) were placed in a RIPA buffer. The slices were homogenised as in the immunoprecipitation protocol (section 2.8). A 1× final concentration of sample buffer and reducing agent were added to the resultant supernatant. Placing them in a heating block at 70°C for 10 minutes denatured the samples.

The samples were run in a NuPAGE ™ 12% Bis-Tris precast gels [based upon Bis-Tris-HCl buffered (pH 6.4) polyacrylamide gel] with rainbow markers (Amersham Life Science) for 1 hour at 150V in a NuPAGE ™ MES running buffer. The running buffer contained NuPAGE ™ anti-oxidant (1:400 dilution). Into each well of the gel, 15µl of the sample was placed. After the hour, the samples were transferred to a PVDF membrane (Invitrogen). The process

took place in a 1× transfer buffer (NuPAGE ™ 10× transfer buffer, Invitrogen), 10% methanol and NuPAGE ™ anti-oxidant (1:100) and the voltage was set to 30V.

After 2 hours, the PVDF membrane was removed and rinsed in distilled water. The membrane was "blocked" for 1 hour with continuous shaking in PBS containing 0.05% Tween (Sigma) (PBST) and 5% skimmed milk powder (Amersham Life Science). The membrane was then washed briefly in distilled water before being left overnight on a shaker at 4°C in the 1:1000 dilution primary antibody [same antibodies as in immunocytochemistry, anti-Pyk2 phosphospecific, β-Actin, or anti-MAPK phosphospecific, Table 2.1]. The solution containing the antibody was made up of 3-5% skimmed milk powder in PBST.

The blot was washed the following day for 15 minutes and then 2× 5 minutes in PBST. The membrane was then placed in a secondary antibody, anti-mouse or anti-rabbit (1:1000 dilution, Diagnostic Scotland) for 1 hour on a shaker. The antibody was diluted in 3-5% skimmed milk powder and PBST. After the blot had been washed 3× PBST as above, an ECL detection kit from Amersham Life Science (97.6% solution A and 2.4% solution B) was used to visualise the protein bands. The ECL was placed on the blot and left for 5 minutes with shaking. The blot was again washed three times in PBST. It was then covered in cling film and placed in an x-ray film cassette. In a dark room a sheet of autoradiography film (Hyperfilm™ ECL, Amersham Life Science) was placed on the top of the blot for 1-5 minutes exposure. The film was then placed in a automatic developing machine (Amersham).

2.9.1 Stripping of the PVDF membrane

Once the required bands were obtained the PVDF membrane was placed in a stripping solution [80mM Tris, 2% SDS and 68% β-mercapto-ethanol (Sigma)] for 30 minutes at 60°C. The membrane was then washed in a copious amount of H_2O for 5-10 minutes. The membrane was re-probed with a different primary antibody (β-actin, Sigma) and then processed for immunoblot (section 2.9).

2.10 Image Analysis

For immunocytochemistry the staining was quantified by using "Image" software (W.Rasband, NIH) as described (Simpson and Morris 2000). The measurements were made in the stained cell bodies and dendrites of individual neurons of cultured slices, a circle or square was used to highlight the area of interest and the mean density was measured. An area in the cultured hippocampus with no cell bodies or dendrites was highlighted in order to measure the mean density of the background. Ten areas with cell bodies/dendrites were randomly measured in each region of the hippocampus and the same applied for measurement of the background. Therefore, ten values of the mean density were used per region of the cultured hippocampal slice. The number of slices per animal was 16 and the number of animals used was more than three. In order to avoid bias, the microscope settings were not altered until all the samples had been measured.

In western blot and IP, the bands were quantified also by using the "Image" software. A rectangle highlighted the band of interest and the mean density as well as the area of rectangle were measured. A rectangle of the same size was then used to measure the background over an area with no staining adjacent to the band.

2.11 Statistics

For immunocytochemistry, the mean density of the background was subtracted from the mean density of the cell bodies/dendrites in order to obtain a specific staining of the cell bodies/dendrites. The mean for the ten random values (mean densities of cell bodies/dendrites) was calculated. Once the mean values of more than three slices were calculated, a mean and standard error of the means of these values were calculated using the Minitab statistical package.

The intensity of staining in drug-treated slices was expressed as a percentage of the staining in parallel vehicle-treated slices. The significance was determined using analysis of variance (ANOVA) with post hoc Fisher's test for multiple pairwise comparison and one-sample Wilcoxon signed rank test using Minitab statistical program. The Wilcoxon signed rank test of the median was the preferred choice for determining the significance because the data was non-parametric. In analysis of a non-parametric data, there is no assumption of a specific distribution for the population.

In Western blot and IP, the product of mean density and area of rectangle for the bands was deducted from the product of its corresponding background, in order to obtain the specific integrated band intensity. The mean densities of phosphorylated Pyk2 and phosphorylated MAPK were expressed as the ratios of the corresponding Pyk2 (total) and β-actin respectively. The ratio of drug-treated acute slices was then expressed as a percentage of ratios in parallel vehicle treated acute slices. The significance was determined using one-

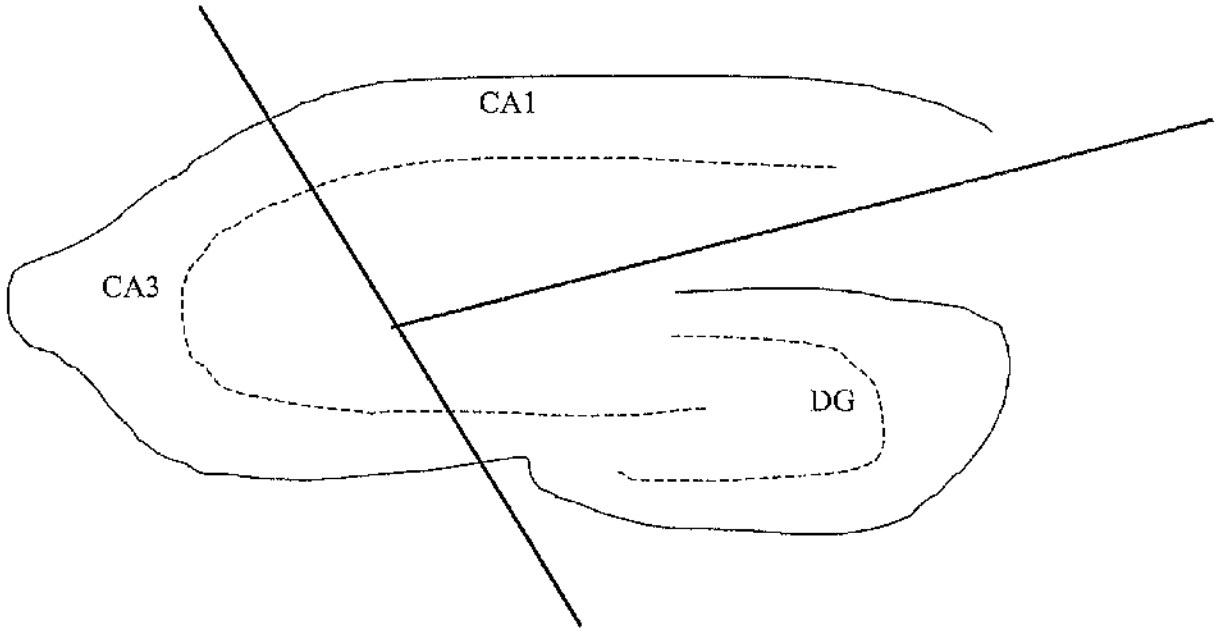
sample Wilcoxon signed rank test of the median using the Minitab statistical program. The data was considered significant if the $P<0.05$.

The histogram of the values for both the cultured and acute slices was derived using the Prism software (GraphPad Prism Project). The mean and standard error of mean of values as well as the 'n' number of experiments were used.

Antibody	Concentration/ Dilultions	Source	Species	Supplier	Catalogue Number
Monoclonal Anti-β-Actin	2.9μg/mL (WB)	Hybridoma produced by fusion of mouse myeloma cells and splenocytes from immunized mouse	Mouse	Sigma	A5441
Anti-human inhibin beta/activin	1:1000, (ICH)	Synthetic peptide corresponding to the 82-114 residues of Beta A subunit of Human inhibin A and activin A	Mouse	Serotec	MCA950S
Anti calmodulin-dependent protein kinase II monoclonal	0.1μg/mL, (WB, ICH)	Partially purified rat CaMKII	Mouse	Chemicon International	MAB3119
Monoclonal anti-MAP2	3.9μg/mL, (WB, ICH)	Same as β-actin	Mouse	Sigma	M4403
Phospho-p44/42 MAP kinase monoclonal antibody	1:1000, (WB)	Mice immunized with synthetic phospho-Thr202 and phospho-Tyr204 peptide corresponding to residues Thr202/Tyr204 of human p44 MAP kinase	Mouse	New England BioLabs	9106L
Pyk2/CAKβ	25μg/mL, (IP)	Generated from rat Pyk2	Mouse	BD Transduction Laboraotries	610548
Polyclonal Anti-Pyk2 [pY^{402}] phosphospecific	0.5μg/mL, (IB)	Chemically synthesized phospho-peptide derived from the region of human Pyk2 that contains tyrosine 402	Rabbit	Biosource International	44-618
v-Src monoclonal antibody	1:1000, (IB)	Immunizing BALB/c mice with purified Src protein and fusing with P3X63 Ag8.653 mycloma cells	Mouse	Oncogene research products	OP07, OP07L, OP07A

Table 2.1: List of antibodies used in this study. Abbreviations: WB- western blot, IP- immunoprecipitation, ICH- immunocytochemistry, IB- immunoblot.

Figure 2.1: Schematic image of the hippocampus showing the DG and hippocampus proper (i.e. CA1 and CA3 regions). The bold lines across the hippocampus indicate the points of dissection of the CA1 and CA3.


CA1
CA3
DG

Chapter 3

Activation of Pyk2

3.1 Introduction

Proline rich tyrosine kinase (Pyk2), which is also referred to as cell-adhesion kinase β (CAKβ), related adhesion focal kinase (RAFTK), Ca^{2+}-dependent tyrosine kinase (CADTK), or focal adhesion kinase 2 (FAK2) (Herzog *et al.,* 1996), is a non-receptor tyrosine kinase that has been implicated in LTP (O'Dell *et al.*, 1991). Pyk2 belongs to a focal adhesion kinase family, which includes focal adhesion kinase (FAK). FAK and Pyk2 exhibit approximately 48% amino acid identity (65% similarity, i.e. high degree of sequence similarity). Both kinases exhibit a similar domain structure: a unique N-terminus, a centrally located protein tyrosine kinase domain and two proline-rich regions at the C-terminus (Avraham *et al.*, 2000), (Figure 3.5). Both tyrosine kinases are highly expressed in the CNS, but are thought to be differentially regulated by neurotransmitters and depolarisation (Siciliano *et al.,* 1996).

There are two isoforms of Pyk2, the alternatively spliced (lacking 42 amino acids in the C-terminal region) isoform (Dikic and Schlessinger, 1998) and the unspliced isoform known as Pyk2-related non-kinase (PRNK). PRNK appears to be predominant in the brain especially in the hippocampus (Xiong *et al.,* 1998) and in fact Pyk2 expression was shown to be abundant in DG, and hippocampal proper (i.e. CA1and CA3) (Menegon *et al.,* 1999).

Reports have indicated that the unspliced Pyk2 is phosphorylated/activated by various stimuli, including raising intracellular Ca^{2+} (Siciliano *et al.*, 1996) indirectly. Stimulation of PKC due to a rise in intracellular Ca^{2+} levels is thought to cause direct activation of Pyk2 (Lev *et al.*, 1995; MacDonald *et al.*, 2001) via phosphorylation. Upon activation, Pyk2 autophosphorylates on tyrosine 402, resulting in a SH2 ligand, which then binds to SH2 domain of Src, thereby activating it (Dikic *et al.*, 1996). Studies have shown an association between Pyk2 and Src (Huang *et al.*, 2001). Lauri *et al.*, 2000 indicated that high frequency stimulation (HFS) enhanced association between Src and Pyk2 in the CA1 region of the hippocampus. Activation of Src by Pyk2 is thought to boost influx of Ca^{2+} through NMDARs and sets in motion the downstream cascade (Huang *et al.*, 2001; Salter 1998). Huang *et al.*, 2001, also showed that Pyk2 acts downstream of Src in the signalling cascade by which tyrosine phosphorylation enhances NMDA receptor function. Src is thought to produce its effect on NMDA by causing the phosphorylation of the tyrosine residue in NR2A and NR2B subunits (Moon *et al.*, 1994).

PKC has been shown to be an upstream regulator of Pyk2 (Huang *et al.*, 1999; Lu *et al.*, 1999; Huang *et al.*, 2001). PKC inhibitors and activators have been shown to inhibit or activate Ca^{2+}-induced activation of Pyk2 (Siciliano *et al.*, 1996). Chelerythrine and staurosporine, PKC inhibitors, have been used to study the effect of PKC on both induction and maintenance of LTP (Muller *et al.*, 1992; Mathies *et al.*, 1991; Denny *et al.*, 1990; Grosshans and Browning 2001; Bortolotto and Collingridge 2000). Staurosporine, a general kinase inhibitor, blocks conventional and novel PKCs, but not atypical PKCs such as PKMξ. Chelerythrine, a highly selective PKC inhibitor, at low concentrations inhibits PKMξ relative to conventional and novel PKCs (Ling *et*

al., 2002). Ling *et al.* (2002) proceeded to show that the maintenance of LTP in the hippocampus requires PKMξ, by blocking expression of CA1 LTP in low concentrations of chelerythrine. Atypical forms of PKC, such as PKMξ (Figure 3.6), are highly activated neither by DAG nor Ca^{2+} directly compared to conventional PKC (refer to section 1.5.2). Naik *et al.,* 2000, showed using immunoblots that PKMξ was located in the DG, CA3 and CA1.

Hence, evidence suggests that PKMζ is particularly important for the expression of LTP at least in the CA1 region, but it remains unclear whether PKMζ is also involved in Pyk2 phosphorylation and activation. Equally, it is not clear if Pyk2 is involved in the plasticity in other areas of the hippocampus other than the CA1 region. Although Pyk2 activation by stimuli that increases intracellular Ca^{2+} has been extensively investigated, very little work has been done on the possible regulation of Pyk2 by cAMP elevations.

The study aims to investigate the following:

(i) The time points at which the phosphorylation of Pyk2 reaches its maximal expression in the acute hippocampus using A23187, Ca^{2+} ionophore

(ii) The effects of A23187 on the phosphorylation of Pyk2 in CA1 and CA3 sub-regions of the hippocampus

(iii) The effect of chelerythrine and staurosporine on the phosphorylation of Pyk2

(iv) The effect of forskolin on the phosphorylation of Pyk2

3.2 Drug treatments

The methods used were in general as described in chapter 2 (Methods) with the following modifications: Once the slices had recovered in the gassed chamber for 2 hours (Acute Slice preparation, section 2.2), chelerythrine (1μM) and staurosporine (100nM) were applied 30 minutes, prior to the activation of the acute slices for 10 minutes with A23187.

After stimulation the slices were placed in an ice-cold RIPA buffer in order to stop the intracellular signalling. The CA1 and CA3 sub-regions of the hippocampus were then dissected out (Figure 2.1, section 2.4). The tissue was then processed for immunoprecipitation as described (section 2.8)

3.3 Results

3.3.1 A23187 increases the phosphorylation of Pyk2 in acute hippocampal slice preparations

A23187 (5μM) increased phosphorylated Pyk2 levels significantly (*P<0.05, n=5) between 5 and 10 minutes by 23±12% and 34±9% (mean increase ± SEM) respectively (Figure 3.1a, upper panel and 3.1b). The levels of phosphorylated Pyk2, recovered back to basal levels within 20minutes (Figure 3.1b). The PVDF membrane was stripped twice. After the first strip, the blot was reprobed with an antibody against total Pyk2 (0.25μg/ml) and the results showed that equal concentrations of Pyk2

protein were loaded into each well of the precast gel (Figure 3.1a, middle panel). Following a second stripping of the membrane blot, it was then reprobed with Src antibody (Oncogene, 0.1μg/ml), Figure 3.1a (lower panel) and this indicated an association between Src and Pyk2. This data was representative of n=3. The Src was associated with all the different levels of phosphorylated Pyk2 (Figure 3.1c).

3.3.2 A23287 Increases the phosphorylation of Pyk2 in both CA1 and CA3 sub-regions of acute hippocampal slice preparations

In Figure 3.2a, upper panel and Figure 3.2b, the levels of phosphorylated Pyk2 were significantly elevated in the two sub-regions of the hippocampus, CA1 and CA3 after A23187 treatment (*P<0.05). The mean levels of phosphorylated Pyk2 increased by 30±11% in CA1 and 83±35% in CA3. On stripping the blot and reprobing with an antibody against total Pyk2, equal concentrations of Pyk2 protein were detected in each lane of the precast gel (Figure 3.2a, lower panel).

3.3.3 Chelerythrine and Staurosporine reduce the phosphorylation of Pyk2 in acute hippocampal slice preparations

Basal levels of phosphorylated Pyk2 was not convincingly altered by chelerythrine (1μM) or by staurosporine (100nM) treatment, 91±2% and 81±11% respectively compared to non-treated vehicle (Figure 3.3a, upper panel, b, upper panel and c). In contrast, both chelerythrine (76±8% reduction, *P<0.05 compared to vehicle) and staurosporine (53±7% reduction) dramatically attenuated the A23187 induced

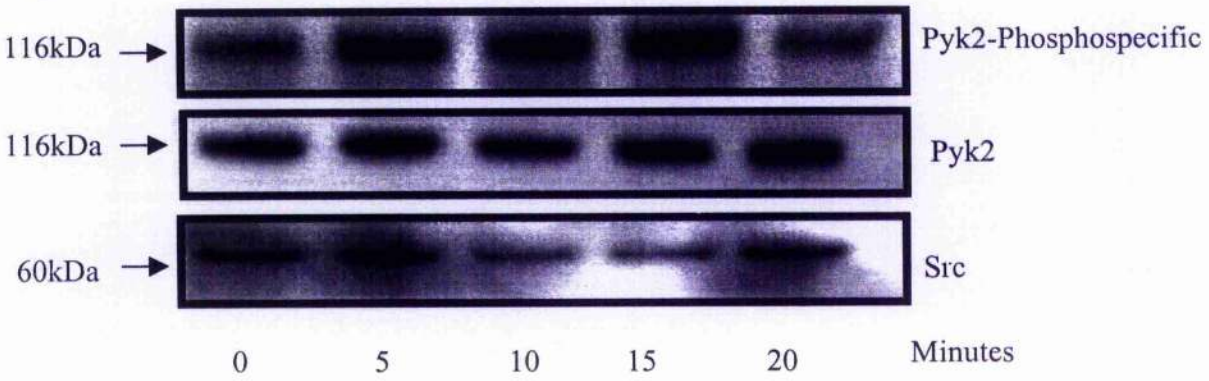
increase in phosphorylated Pyk2 (154±12%, ~P<0.05 compared to vehicle). The blots were stripped and reprobed with antibody for Pyk2, and the results showed that equal Pyk2 concentrations were loaded into each well of the precast gel.

3.3. Forskolin inhibits the phosphorylation of Pyk2 in acute hippocampal slice preparations

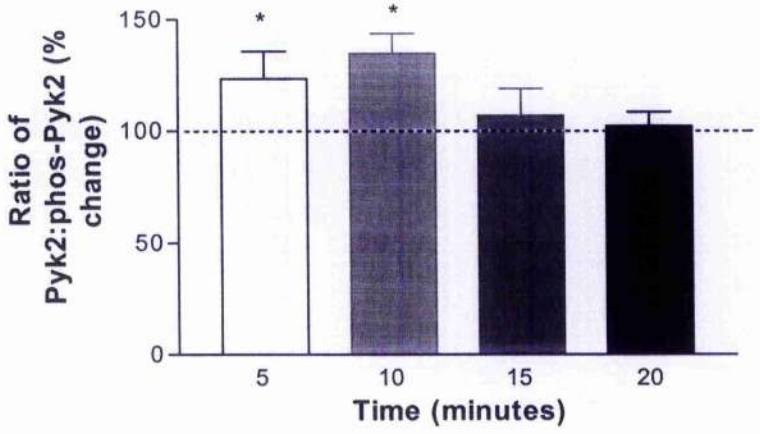
Expression of phosphorylated Pyk2 was significantly reduced (*P<0.05, n=5) by 20±9when acute hippocampal slices were treated with forskolin (50μM). The levels of the phosphorylated Pyk2 seem to recover back to basal levels within 20 minutes. The significant reduction occurred after a 5 minute treatment of the slices with forskolin (Figure 3.4a, upper panel and Figure 3.4b). The membrane blot was stripped and reprobed with total Pyk2 antibody; the result indicated equal application of Pyk2 protein into each well of the precast gel (Figure 3.4a, lower panel).

Figure 3.1: Effect of A23187 (5μM) on the phosphorylation of Pyk2 in an acute hippocampal slice preparation and association between Pyk2 and Src. Immunoprecipitation assay using anti-Pyk2 (total) and blotting with anti-Pyk2 (phoshospecific) antiserum indicated a significant (*P<0.05 versus 100%, n=5, one-sample Wilcoxon signed rank test of the median) increase in the phosphorylation of ir Pyk2 between 5 and 10 minutes (figure a, upper panel and figure b). The blot was stripped twice, once to indicate that the levels of protein in each well of precast gel were of equal concentration (middle panel) and second to indicate an association between Pyk2 and Src (lower panel), figure a and c. Results are expressed as a percentage of the specific signal at t=0.

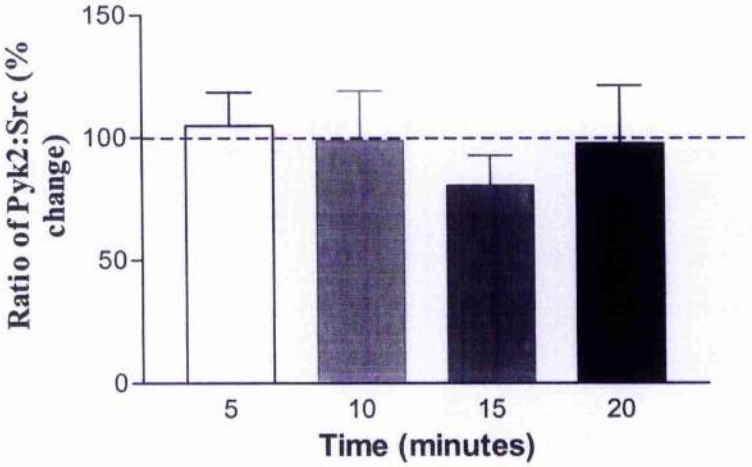
a)


116kDa
Pyk2-Phosphospecific
116kDa
Pyk2
60kDa
Src
0
5
10
15
20
Minutes

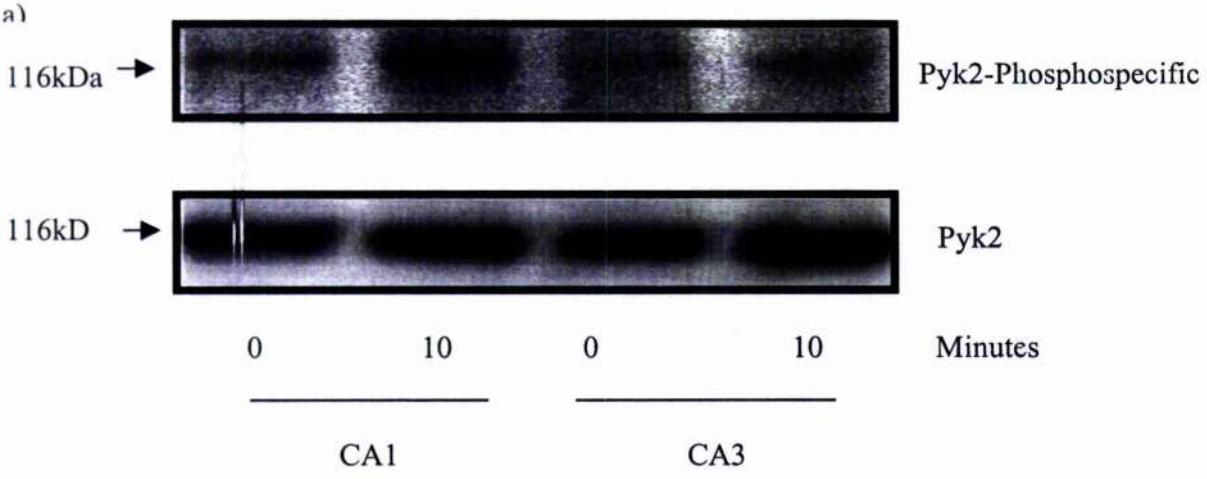
b)


Ratio of Pyk2:phos-Pyk2 (% change)
150
100
50
0
*
*
5
10
15
20
Time (minutes)

c)


Ratio of Pyk2:Src (% change)
150
100
50
0
5
10
15
20
Time (minutes)

a)


116kDa
Pyk2-Phosphospecific
116kD
Pyk2
0
10
0
10
Minutes
CA1
CA3

b)

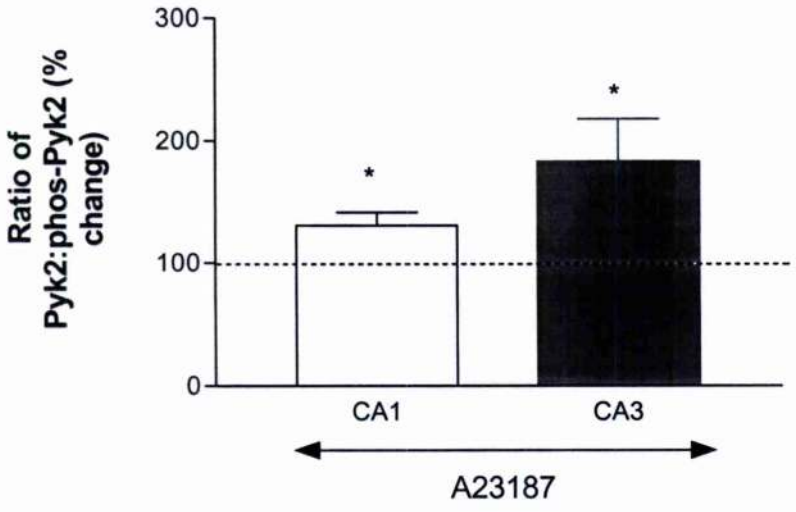
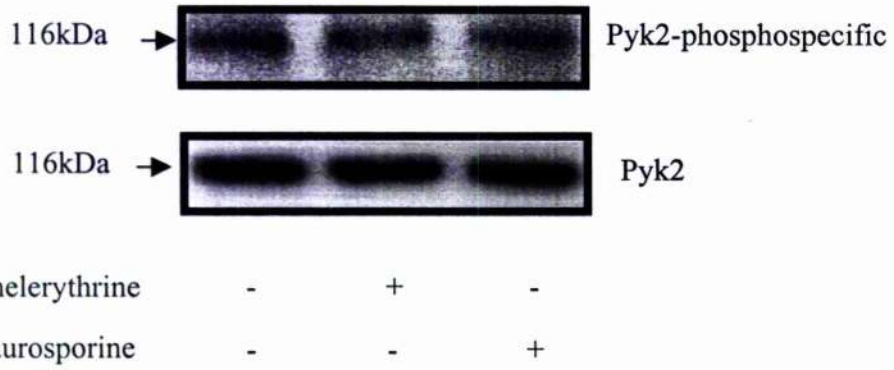
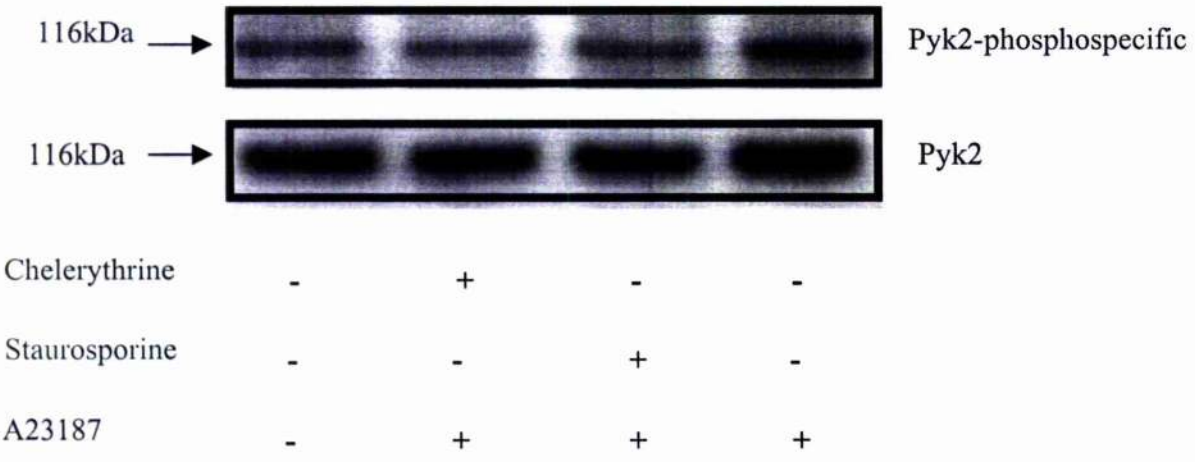

Ratio of Pyk2:phos-Pyk2 (% change)
300
200
100
0
*
*
CA1
CA3
A23187

Figure 3.2: Effect of A23187 (5μM) on the phosphorylation of Pyk2 in both CA1 and CA3 acute slice preparations. Immunoprecipitation assay using anti-Pyk2 (total) and blotting with anti-Pyk2 (phoshospecific) anitserum indicated a significant increase in the levels of phosphorylated ir Pyk2 in both CA1 (*P<0.05 versus 100%, n=5, one-sample Wilcoxon signed rank test of the median) and CA3 (*P<0.05 versus 100%, n=5, one-sample Wilcoxon signed rank test of the median), figure a and b, upper panel. The PVDF membrane was stripped and re-probed with total Pyk2 (figure a, lower panel), which indicated an equal concentration of proteins loaded into each well of precast gel. Results are expressed as a percentage of the specific signal at t=0.

a)


116kDa
Pyk2-phosphospecific
116kDa
Pyk2
Chelerythrine - + -
Staurosporine - - +

b)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Pyk2-phosphospecific
116kDa
Pyk2
Chelerythrine - + - -
Staurosporine - - + -
A23187 - + + +

c)

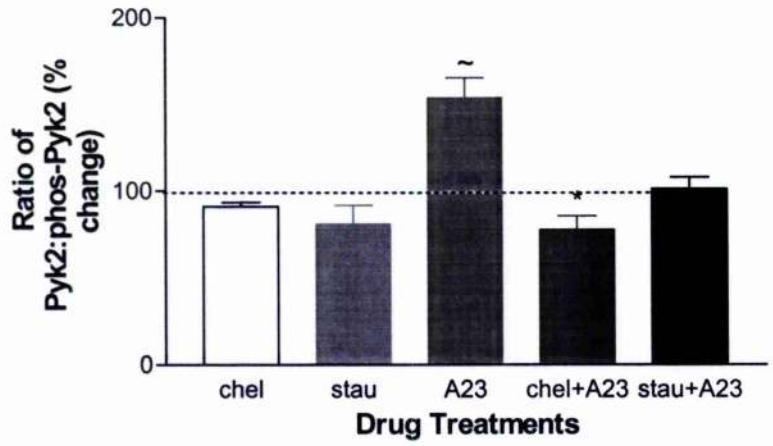
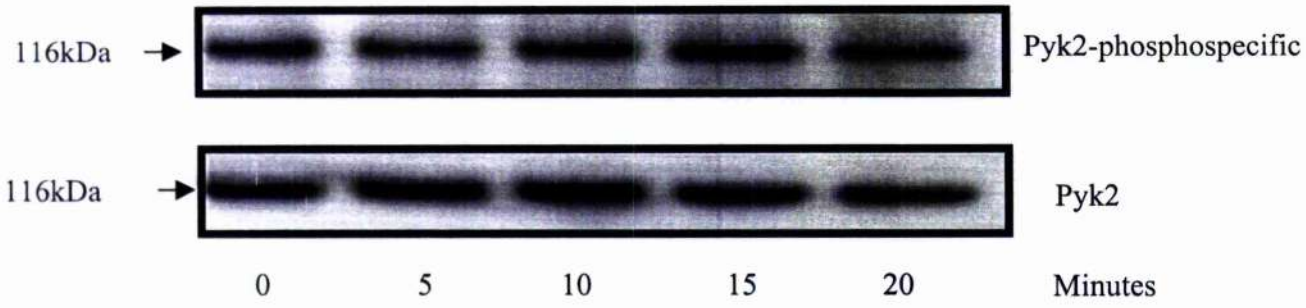

200
100
0
Ratio of Pyk2:phos-Pyk2 (% change)
chel
stau
A23
chel+A23
stau+A23
Drug Treatments

Figure 3.3: Effect of chelerythrine (1μM) and staurosporine (100nM) on the phosphorylation of ir Pyk2 in both A23187 (5μM) and vehicle-treated acute hippocampal slice preparations. Chelerythrine reduced phosphorylation of ir Pyk2 at basal level and significantly in the presence of A23187 (*P<0.05, n=5, one-sample Wilcoxon signed rank test of the median) in hippocampal slices while staurosporine did not have any significant effect on the basal phosphorylation of Pyk2, however it attenuated the A23187 effect (figure a and b, upper panel, and figure c). A23187 significantly elevated phosphorylation of ir Pyk2 (~P<0.05 compared to vehicle, chelerythrine, staurosporine, n=5). Stripping and reprobing the PVDF membrane showed equal concentrations of Pyk2 in each well of the precast gel (figure a and b, lower panel. Results are expressed as a percentage of the specific signal, t=0. Abbreviations: chel- chelerythrine, stau- staurosporine, A23- A23187

a)


116kDa
Pyk2-phosphospecific
116kDa
Pyk2
0
5
10
15
20
Minutes

b)

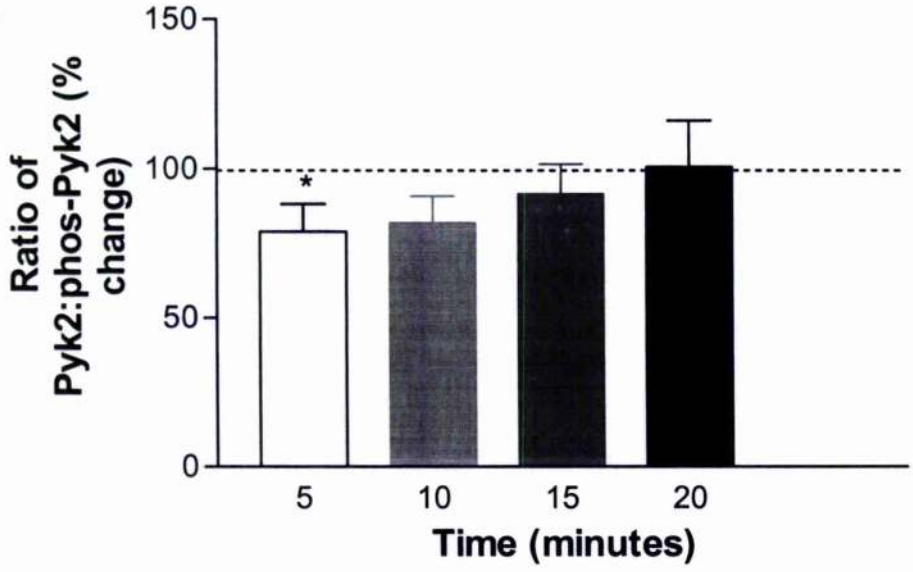

Ratio of Pyk2:phos-Pyk2 (% change)
150
100
50
0
*
5
10
15
20
Time (minutes)

Figure 3.4: Effect of forskolin (50μM) on the phosphorylation of Pyk2 in acute hippocampal slice preparations. Immunoprecipitation assay using anti-Pyk2 (total) and blotting with Pyk2 (phosphospecfic) antiserum indicated that the levels of phosphorylated ir Pyk2 were significantly reduced after 5minutes (*P<0.05 versus 100%, n=5, one-sample Wilcoxon signed rank test of the median), figure a, upper panel and figure b. Stripping the blot and reprobing with total Pyk2 indicated that equal concentrations of the protein in each well of the precast gel. Results are expressed as a percentage of the specific signal at t=0.

3.4 Discussion

Phosphospecific Pyk2 (Shi *et al.*, 2000 and Keely *et al.*, 2000), Pyk2 (Lev *et al.*, 1995; Dikic *et al.*, 1996 and Sasaki *et al.*, 1995), and Src (Bolen *et al.*, 1984 and Cartwright *et al.*, 1985) antibodies had all been previously characterised. Pyk2 was generated from rat Pyk2 and phosphospecific Pyk2 was chemically synthesised in a phospho-peptide derived from the region of human Pyk2 that contains tyrosine 402 (Table 2.1). The Src antibody was obtained from BALB/c mice that have been immunised with purified Src protein. For each antibody, single bands were obtained. Using the rainbow marker, the band obtained when using Pyk2 antibody was approximately 116kDa while that for Src was around 60kDa, hence this supports claims of specificity.

The effect of vehicle treatment over time has not been tested and therefore it remains theoretically possible that effects due to addition of drug/vehicle, might not be due to the drug. However, this is very unlikely, since only small volumes of the drugs were added; in addition forskolin and A23187 affects act in opposite directions, so it is unlikely to be due to vehicle.

3.4.1 A23187

The phosphorylation of Pyk2 was elevated significantly between 5 and 10 minutes by 23% and 34% respectively on the application of A23187 (Lev *et al.*, 1995; Wang and Brecher, 2001), a Ca^{2+} ionophore, on acute hippocampal slices (Figure 3.1). This

suggested that Ca^{2+} plays a role in the phosphorylation of tyrosine kinase, Pyk2. This is in agreement with previous studies which indicated elevation of phosphorylated Pyk2 levels for 15 minutes in PC12 cells on application of 6μM A23187 (Lev *et al.*, 1995). In addition, studies had shown that Pyk2 could be activated by stimuli that increase intracellular Ca^{2+} levels especially depolarisation (Siciliano *et al.*, 1996) in the hippocampus.

The levels of Pyk2 seem to recover back to basal levels within 20 minutes, which could be due to the tyrosine phosphatases (PTPs). In fact PTPs had been shown to be highly expressed in the CNS and their inhibition potentiated NMDA currents (Wang and Salter, 1994).

Phosphorylation of Pyk2 reportedly resulted in the activation of Src (Lev *et al.*, 1995; Girault *et al.*, 1999; Ali and Salter, 2001) and the data obtained here indicated an association between Pyk2 and Src (Figure 3.1b) since Src was immunoprecipitated by Pyk2 antibody. Src had been shown to enhance the potentiation of NMDA receptors (Salter, 1998) via phosphorylation of tyrosine residue in NR2A subunit of NMDA receptor (Salter, 1998; Lau and Huganir, 1995) so it has been suggested that this interaction is crucial of the expression of LTP. However, the results reported here showed no evidence of increased association of Src with Pyk2 after Pyk2 activation. This raises the possibility there is no Src activation by Pyk2 after Ca^{2+} influx alone and that some other signalling pathway activates Src.

Previous studies have shown the expression of Pyk2 in DG and hippocampal proper (i.e. CA3 and CA1) (Menegon *et al.*, 1999). But most of the electrophysiological

studies on tyrosine kinases were carried out in the CA1 region, looking at the role of Pyk2 in LTP (Huang and Hsu, 1999; Lauri *et al.*, 2000). The data in this study showed that there was a significant elevation of phosphorylated Pyk2 levels in both CA1 and CA3 on the application of A23187 for 10 minutes (Figure 3.2). The levels of phosphorylated Pyk2 were elevated by 31% and 83% in CA1 and CA3 regions respectively. This might imply that the intracellular signalling in the CA3 was similar to CA1.

As already mentioned (section 1.6 and 1.8), LTP induction in Schaffer collaterals fibres in the CA1 region is NMDAR dependent while the MF LTP in CA3 region is NMDAR independent, but both forms of LTP require calcium influx. Therefore, it is possible to suggest that although different forms of LTP are activated the CA1 and CA3 regions of hippocampus, they still might share a common intracellular pathway.

3.4.2 Chelerythrine and Staurosporine

Pyk2 is reportedly stimulated by activation of protein kinase C (PKC) (Lev *et al.*, 1995; Siciliano *et al.*, 1996). PKC had been shown to play an important role in LTP (Roberson *et al.*, 1996a). PKMζ, a constitutively active form of an atypical PKC, has been shown to be necessary and sufficient for LTP maintenance in CA1 region of the hippocampus (Ling *et al.*, 2002) when using the PKC inhibitors, chelerythrine and staurosporine. Chelerythrine has been shown to block LTP maintenance at a dose of 0.3μM (Bortolotto and Collingridge, 2000) while staurosporine blocks the induction (Muller *et al.*, 1992). Staurosporine was less effective than chelerythrine in inhibiting

the protein kinase activities of PKCδ and PKMξ when compared to PKCα (McGlynn *et al.*, 1992).

In this study, the role of these two PKC inhibitors on phosphorylation of Pyk2 in acute hippocampal slices was investigated. The concentrations used were those reported to confer specificity (Ling *et al.*, 2002). Chelerythrine had minimal effect on basal levels of phosphorylated Pyk2 (9% reduction), however it attenuated effect of A23187 on phosphorylated Pyk2 (19% reduction) (Figure 3.3). Grosshans and Browning (2001) have previously used chelerythrine (10μM) to block tyrosine phosphorylation (Src phosphorylation) and observed a reduction of 12%. These results might suggest that endogenous phosphorylated Pyk2 was regulated by PKMζ, since application of PKMζ selective inhibitor, chelerythrine, resulted in reduced phosphorylation of Pyk2. Since a similar dose of chelerythrine blocks LTP maintenance it can be suggested that phosphorylated Pyk2 might play a role in the maintenance of synaptic plasticity.

Since the reductions in the levels of activated Pyk2 were in the whole hippocampus, it seems logical to assume that signalling in the three regions of the hippocampus was similar although this has not been shown. Also it was already suggested in this study that the intracellular signalling in CA1 and CA3 regions were similar in case of Pyk2 phosphorylation. Staurosporine did not have any effect on the basal phosphorylated levels of Pyk2. This indicates that the basal levels of phosphorylated Pyk2 are maintained independently of a general PKC inhibitor, staurosporine. However, as with chelerythrine, staurosporine completely blocked the ability of A23187 to elevate Pyk2 phosphorylation (Figure 3.3). This suggests that other form of neuronal PKC

and PKMζ are both required for Pyk2 activation after Ca^{2+} influx. Previous studies have shown that the use of different general PKC inhibitors, RO31-8220 and GF109203X, resulted in the inhibition of Pyk2 phosphorylation in the hippocampus (Siciliano *et al.*, 1996).

3.4.3 Forskolin

The levels of phosphorylated Pyk2 were significantly attenuated (Figure 3.4) when forskolin was applied to acute hippocampal slices. The reduction (21%) occurred 5 minutes after the application of forskolin. Forskolin, a diterpene, directly activates adenlyate cyclase (Seaman and Daly, 1986) producing cAMP and the subsequent activation of PKA. This might indicate that, elevation of cAMP and activation of PKA have a negative effect on Pyk2, which results in reduced phosphorylation of Pyk2 targeted proteins. Studies by Derkindern *et al.* (1996) showed that the level of phosphorylation of FAK, a member of non-receptor tyrosine express in the neurons, were elevated via anadamide (an endogenous ligand for central cannabinoid) release due to depolarisation (Figure 3.7). This ligand inhibited adenylate cyclase thus reducing the levels of cAMP. Hence inhibition of adenylate cyclase might have a positive effect on Pyk2 (i.e. cause phosphorylation of Pyk2) similar to the effect on FAK (Figure 3.7).

3.4.4 Summary

Therefore this data had shown the following:

(i) Intracellular increase in Ca^{2+} between 5 and 10 minutes played a role in the increased phosphorylation of Pyk2 in the acute hippocampus

(ii) The intracellular signalling pathway regulating Pyk2 in the CA3 sub-region of acute hippocampus is similar to the CA1

(iii) Concentration of chelerythrine and staurosporine that reportedly inhibit PKMζ and novel PKC respectively both reduced A23187-induced phosphorylation of Pyk2 in acute hippocampal slices suggesting that parallel PKC pathways may be involved in Pyk2 activation

(iv) Elevation of cAMP levels reduced the phosphorylation of Pyk2 and this might be the first evidence for a cAMP-activated pathway suppressing Pyk2, analogous to that proposed for FAK

Figure 3.5: Schematic presentation of homology comparison of the N-terminus, kinase domain and C-terminus between Pyk2 and FAK. (Adapted from Avraham *et al.*, 2000)

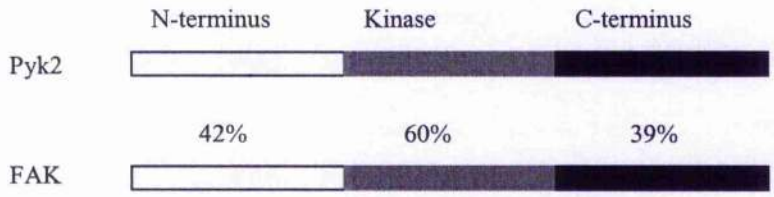

N-terminus
Kinase
C-terminus
Pyk2
42%
60%
39%
FAK

Figure 3.6: Schematic diagram of the major domains of conventional (α, βI, βII and γ), novel (δ, θ, η and ε), and atypical (ζ and ι) PKC Adapted from Naik *et al.*, 2000. The independent catalytic domain of PKC is the PKM.

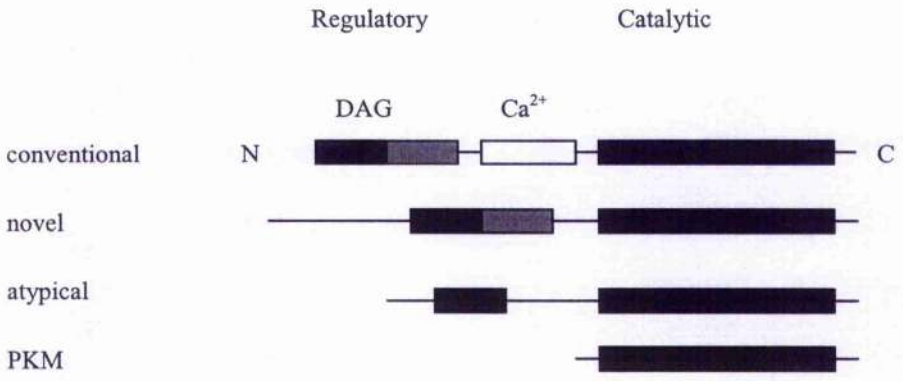
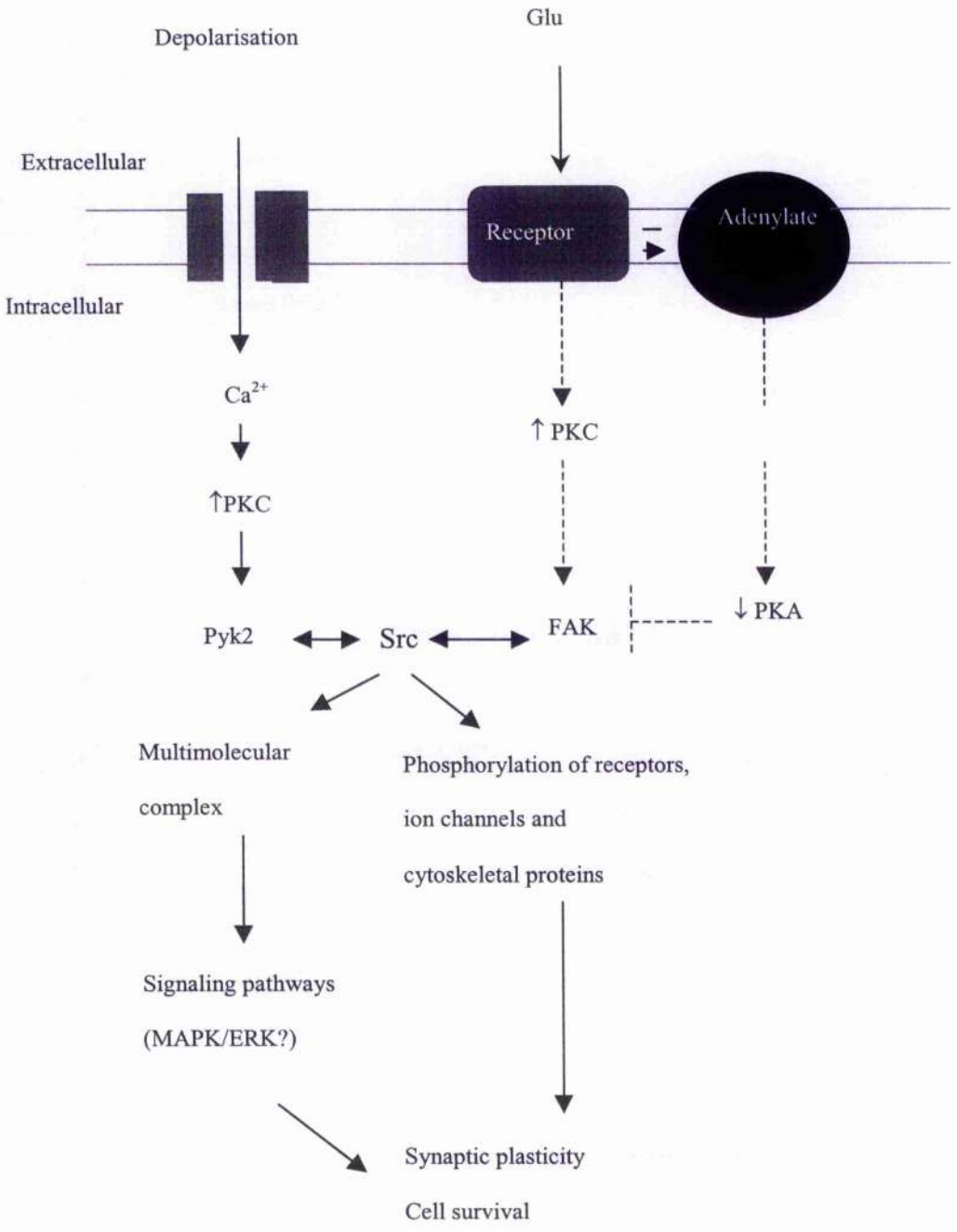

Regulatory
Catalytic
DAG
Ca^{2+}
conventional
N
C
novel
atypical
PKM

Figure 3.7: Schematic diagram indicating the intracellular signalling that regulate the non-receptor tyrosine kinases, Pyk2, Src and FAK^{+} in rat hippocampal slices (Adapted from Girault *et al.*, 1999). A raise in intracellular Ca^{2+} results in the activation of Pyk2 via activation of PKC. FAK is also activated by PKC as well as by cannabinoid CB_1-receptor agonists such as anadamide. The available evidence suggests that the following might occur. Pyk2 interacts with Src and leads to the formation of multiprotein complexes which activates various signalling pathways including MAPK/ERK (mitogen-activated protein kinase/ extracelluar-signal regulated kinase) pathway. Src is also capable of phosphorylating receptors and cytoskeletal proteins. These pathways provide a possible mechanism for regulating synaptic plasticity. Abbreviation: — inhibition.


Depolarisation
Glu
Extracellular
Intracellular
Receptor
Adenylate
Ca^{2+}
↑PKC
↑ PKC
Pyk2
Src
FAK
↓ PKA
Multimolecular
complex
Phosphorylation of receptors,
ion channels and
cytoskeletal proteins
Signaling pathways
(MAPK/ERK?)
Synaptic plasticity
Cell survival

Chapter 4

Activation of MAPK

4.1 Introduction

There has been an increasing interest on the role of MAPKs in the regulation of neuronal functions. MAPKs have been shown to play a vital function in the signalling pathways associated with activity dependent regulation of neuronal function. A number of studies have identified novel pathways that include the incorporation of second-messenger systems such as cAMP/PKA and DAG in the regulation of MAPK signalling (Sweatt, 2001).

MAPKs (p44 and p42) are expressed in abundance in post-mitotic neurons of the developed nervous system. Fiore *et al.* (1993) have shown the expression of MAPK in the dendrites and somas of pyramidal cells of the hippocampus with little to no staining in the non-pyramidal cells. Hence activation of MAPK (especially p42) detected in assays would most likely occur in pyramidal neurons (English and Sweat, 1996).

In the CA1 region, the cAMP pathway has been shown to utilise the MAPK cascade as an obligatory intermediate in regulating CREB (cAMP-response element binding protein), a constitutive transcription factor (Sweatt, 2001) (refer to PKA section). Impey *et al.* (1998) demonstrated CREB phosphorylation in area CA1 in response to LTP-inducing stimulation, an effect that was blocked by MAPK inhibition. In CA1

hippocampal region, activation of cAMP cascade led to a PKA activation of MAPK (Martin *et al.*, 1997; Roberson *et al.*, 1999).

MAPK activation has been shown to play a critical role in LTP induction. Studies have initially focused on NMDA receptor-dependent LTP in area CA1, using hippocampal slices *in vitro* (English and Sweatt, 1996, 1997; Atkins *et al.*, 1998; Impey *et al.*, 1998; Winder *et al.*, 1999). MAPK activation was also shown to be necessary in NMDA receptor-independent LTP (Coogan *et al.*, 1999), LTP in DG *in vitro* (Coogan *et al.*, 1999), and LTP *in vivo* (McGahon *et al.* 1999; Davis *et al.*, 2000). Use of MEK inhibitors, which blocked activation of ERKs, blocked L-LTP, indicating the importance of MAPK activation in L-LTP (English and Sweatt, 1996, 1997; Impey *et al.*, 1998). This effect was not restricted to L-LTP, as E-LTP was also reduced (Sweatt, 2001).

Upon activation after LTP-inducing stimulation, a fraction of MAPK translocates from the cytosol into the nucleus (Davis *et al.*, 2000) where it is thought to alter gene expression by transcriptional control (Impey *et al.*, 1998). CREB phosphorylation is an end product of activation of MAPK, which has been shown to play a role in gene expression (Roberson *et al.*, 1999) in the CA1 region.

The aim of this study was to investigate the effects of Ca^{2+} influx and cAMP elevation on the activation of p42 MAPK in the CA1 and CA3 sub-regions of acute hippocampal slices using western blot techniques (section 3.9).

4.2 Results

Two bands (one for p42 MAPK and second for p44 MAPK) were usually detected when using the phosphospecific MAPK antiserum, but low MW (42KDa) was frequently detectable in this investigation.

4.2. A23187 increases phosphorylation of p42 MAPK

The levels of the phosphorylated p42 MAPK appeared elevated in the presence of 5μM A23187 in acute hippocampal slice preparations. The levels appeared elevated after 10 minute (164±36% of vehicle treated slices, n=4) stimulation of the hippocampal slices and were still appeared elevated after 20 minutes (150±48% of vehicle treated slices, n=4) stimulation (Figure 4.1a, upper panel). However, these effects did not reach statistical significance.

On reprobing of the PVDF membrane with β-actin, the concentration of proteins in each well of the precast gel were found to be of equal concentrations (Figure 4.1a, lower panel).

4.2.2 Forskolin increases phosphorylation of p42 MAPK

On application of forskolin (50μM), the levels of phosphorylation of p42 MAPK were significantly elevated (*P<0.05 versus 100% of vehicle-treated slices) in acute

hippocampal slices (Figure 4.2a, upper panel). The levels were prominent after 5 (165±9% of vehicle treated slices), 10 (172±30% of vehicle treated slices) and 20 minutes (149±21% of vehicle treated slices) after stimulation of the whole hippocampal slice.

The blot was then stripped and reprobed with anti-β-actin antiserum (as an internal standard) (Figure 4.2a, lower panel).

4.2.3 A23187 and forskolin increase phosphorylation p42 MAPK in CA1 but not CA3 sub-regions of the hippocampus

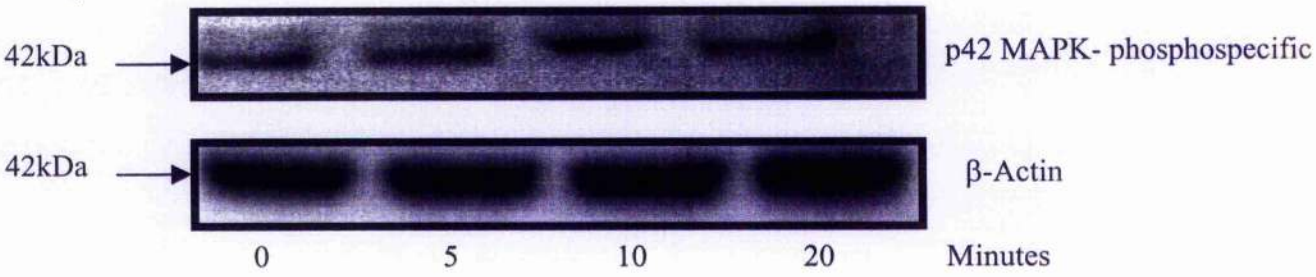
A23187 (5μM) significantly increased the phosphorylation of p42 MAPK in the CA1 region after 10 minutes (*P<0.05, 194±37% of vehicle treated slices) but had no effect on the phosphorylation of p42 MAPK in the CA3 (P>0.05, 116±26% of vehicle treated slices) in acute hippocampal slice preparations (Figure 4.3a, upper panel and Figure 4.3b).

Forskolin (50μM) also significantly increased the phosphorylation of p42 MAPK in the CA1 region of the hippocampus (*P<0.05, 181±61% of vehicle treated slices) but had no effect on the phosphorylation of p42 MAPK in the CA3 sub-region (P>0.05, 78±10% of vehicle treated slices) (Figure 4.3a, upper panel and Figure 4.3b). The blot was then stripped and reprobed with anti-β-actin, which was used as an internal standard (Figure 4.3a, lower panel.

Figure 4.1: Effect of A23817 (5μM) on the phosphorylation of p42 MAPK in acute hippocampal slices. Western blot analysis using anti-MAPK (phosphospecific) indicated an increase in the phosphorylation of p42 MAPK isoform, between 10 and 20 minutes. However, the increase did not reach statistical significance ($P>0.05$) (figure a, upper panel and figure b). The histogram and error bars represent the mean and SEM respectively. Stripping and reprobing of the PVDF membrane with β-actin shows equal concentrations of protein in each well of precast gel (figure a, lower panel). n=4

Abbreviations: SEM- standard error of the mean.

a)


42kDa
p42 MAPK- phosphospecific
42kDa
β-Actin
0
5
10
20
Minutes

b)

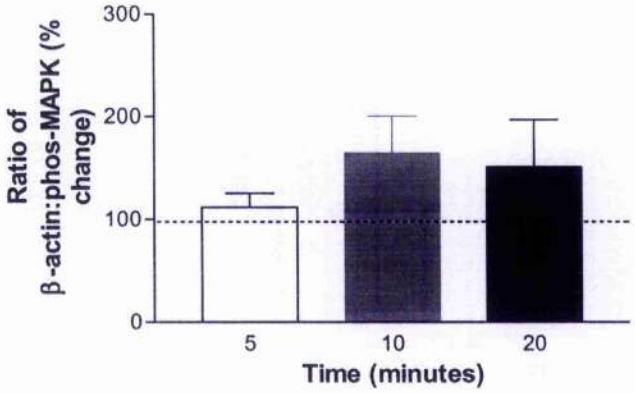
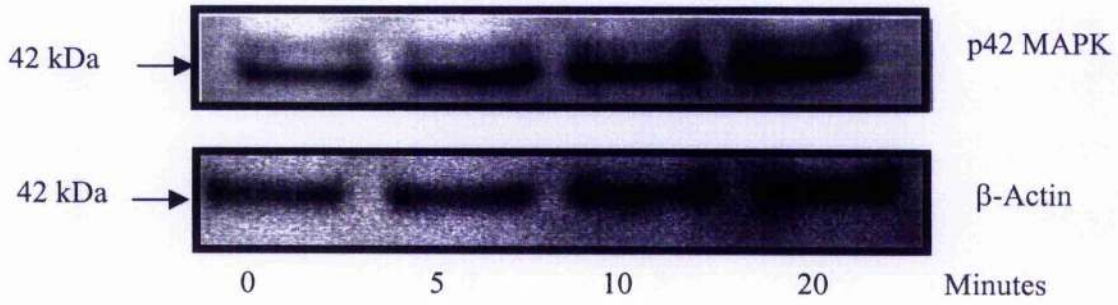

Ratio of β-actin:phos-MAPK (% change)
300
200
100
0
5
10
20
Time (minutes)

Figure 4.2: Effect of forskolin (50μM) on the phosphorylation of p42 MAPK in acute hippocampal slices. Western blot assay using anti-MAPK (phosphospecific) antiserum indicated a significant increase in the phosphorylation of p42 MAPK isoform between 5 and 20 minutes (*P<0.05 versus 100%, n=4, one-sample Wilcoxon signed rank test of the median) (figure a, upper panel and figure b). The histogram and error bars represent the mean and SEM respectively. The blot was then strip and reprobed with anti-β-action antiserum (figure a, lower panel).

a)


42 kDa
p42 MAPK
42 kDa
β-Actin
0
5
10
20
Minutes

b)

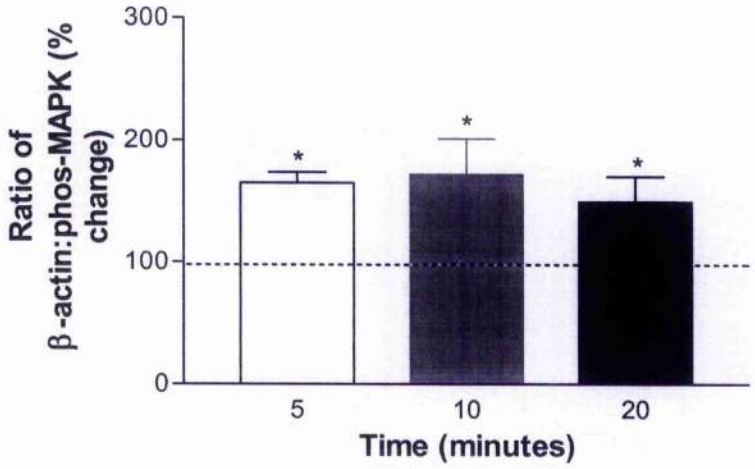
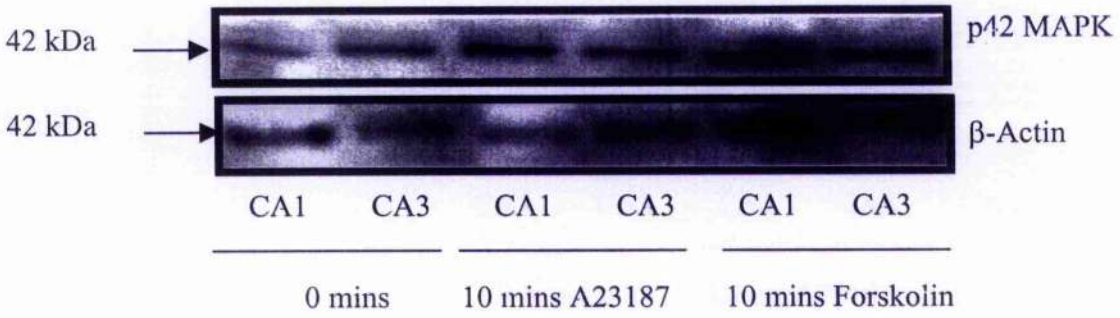
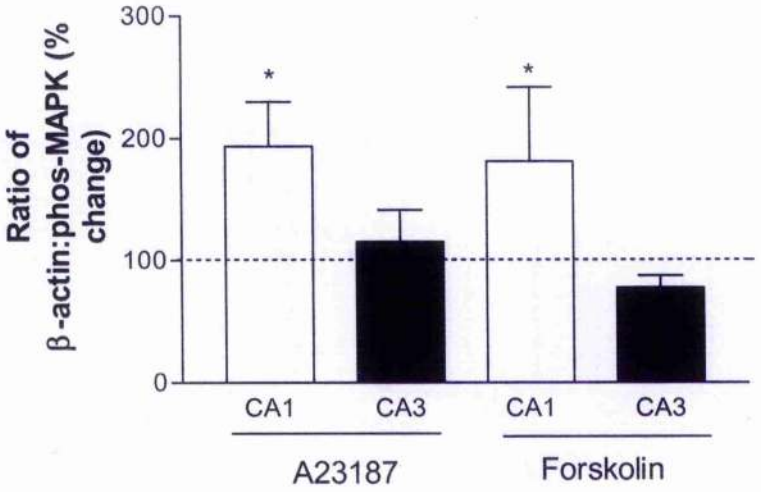

Ratio of β-actin:phos-MAPK (% change)
300
200
100
0
*
*
*
5
10
20
Time (minutes)

Figure 4.3: Effect of A23187 (5μM) and forskolin (50μM) on the phosphorylation of p42 MAPK in the CA1 and CA3 sub-regions of acute hippocampal slices. Western blot analysis using anti-MAPK (phosphospecific) antiserum indicated a significant increase in the phosphorylation of p42 MAPK isoform in the CA1 region (*$P<0.05$ versus 100%, n=4, one-sample Wilcoxon signed rank test of the median) after 10 minute stimulation with either A23187 or forskolin (figure a, upper panel and figure b). However, A23187 or forskolin had no effect on the phosphorylation of p42 MAPK isoform in the CA3 region ($P>0.05$, n=4) (figure a, upper panel and figure b). The blot was stripped and reprobed with anti-β-actin anti-serum (figure a, lower panel). Abbreviations: mins- minutes.

a)


42 kDa
42 kDa
p42 MAPK
β-Actin
CA1
CA3
CA1
CA3
CA1
CA3
0 mins
10 mins A23187
10 mins Forskolin

b)


Ratio of β-actin:phos-MAPK (% change)
300
200
100
0
*
*
CA1
CA3
CA1
CA3
A23187
Forskolin

4.3 Discussions

Phospho-p44/42 MAPK antibody (Marshall *et al.*, 1995; Kang *et al.*, 2001) and β-actin antibody (North *et al.*, 1993) had previously been characterised, and are highly specific for their respective antigen. Phospho-p44/42 MAPK antibody recognises the doubly phosphorylated threonine 202 and tyrosine 204 of p44 and p42 MAPK respectively; while the monoclonal β-actin antibody detects an epitope located on the N-terminal end of β-isoform of actin (Table 2.1).

4.3.1 A23187

The relative weak signal obtained for p44 MAPK precludes any study of its regulation by A23187 and forskolin. Clearly, p42 MAPK is the predominant phosphorylated species in the hippocampus, a result consistent with previous reports (English and Sweatt, 1996, 1997). The levels of phosphorylated p42 MAPK were elevated in the presence of 5μM A23187, a Ca^{2+} ionophore. The elevations occurred between 10 and 20 minutes (Figure 4.1), but were not significant (P=0.1 for both time points). The study was carried out on acute hippocampal slices while most of the studies on the activation of p42 MAPK were performed on the CA1 (English and Sweatt, 1996, 1997) and DG (Coogan *et al.*, 1999) regions of the hippocampus. The MAPK cascade has been shown to be necessary for the NMDA receptor-dependent LTP in area CA1 (English and Sweat, 1997; Atkins *et al.*, 1998) and the DG (Coogan *et al.*, 1999) of the hippocampus. Hence, the Ca^{2+} ionophore would be predicted to elevate the levels of p42 MAPK in the area CA1 and DG. The

effects of A23187 has not been previously investigated in the CA3 sub-region also. Stage two of the study was to investigate the effect of Ca^{2+} influx in both the CA1 and CA3 sub-regions of the hippocampus. A23187 elevated the phosphorylation of p42 MAPK in the CA1 region while phosphorylation of p42 MAPK in the area CA3 was not affected. Hence, the lack of effect of A23187 on the phosphorylation of p42 MAPK in CA3 sub-region might explain the insignificant effect of the Ca^{2+} influx on the whole hippocampus.

In the CA1 area, Ca^{2+} influx was thought to cause activation of p42 MAPK via activation of PKC (English and Sweat, 1996). Therefore, an increase in intracellular Ca^{2+} was thought to activate MAPK via the Ca^{2+}-PKC-Ras/Raf1-MEK-MAPK cascade (Figure 4.4). In the CA3 sub-region of the hippocampus, the regulation of p42 MAPK might follow a different pathway. Kanterewicz *et al.* (2000) had shown using MEK inhibitors that MF-LTP in area CA3 does not require MAPK signalling while LTP in CA1 region requires MAPK signalling. These results support the concept of a differential role for MAPK in the response to Ca^{2+} influx in the CA1 and CA3 regions.

4.3.2 Forskolin

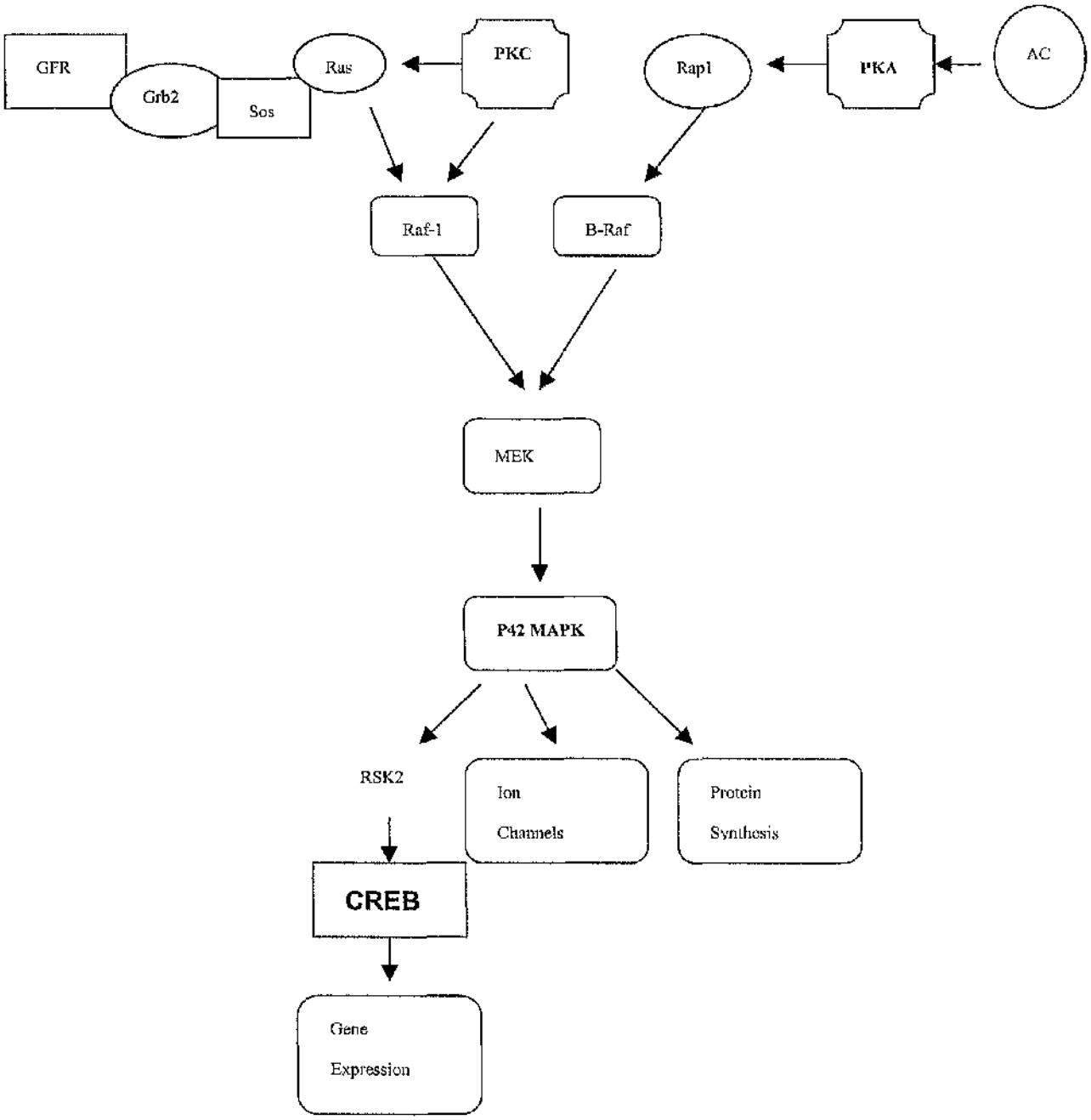
In a second series of studies, the effect of adenylate cyclase activator, forskolin (50μM) on p42 MAPK activation was investigated. Forskolin caused an increased phosphorylation of p42 MAPK between 5 and 20 minutes (Figure 4.2). As with Ca^{2+} influx, cAMP elevations were studied mostly in the CA1 sub-regions (Roberson *et al.*,

1999) of the hippocampus. In contrast, the effect of p42 MAPK activation in the CA3 sub-region remains largely unknown. Forskolin significantly elevated the phosphorylation of p42 MAPK in the CA1 region while the levels of p42 MAPK in area CA3 were unaffected (Figure 4.3). Forskolin induced activation of the PKA signalling cascade has been shown to be coupled to activation of p42/p44 MAPK in the CA1 sub-region (Roberson *et al.*, 1999). Hence cAMP was thought to produce its effect on MAPK via the PKA/Rap1-B/Raf-MEK/MAPK cascade. In area CA3, forskolin at a concentration sufficient to induce MF LTP (Weisskopf *et al.*, 1994) did not seem to have any effect on the phosphorylation of p42 MAPK, again suggesting an alternative pathway to the CA1 sub-region. Kanterewicz *et al.* (2000) showed that MAPK signalling cascade was not necessary for the forskolin-induced potentiation of mossy fibre synapses and hence suggested that activation of p42/p44 MAPK is not necessary for induction of MF LTP. Clearly there is substantial expression of MAPK in the CA3 region, yet it is not activated either by Ca^{2+} or cAMP stimulation. The stimuli that cause MAPK activation in the CA3 region remain to be identified.

4.3.3 Summary

The present data complements previous studies carried out by Kanterewicz *et al.* (2000) in the CA3 region of the hippocampus. In summary, PKA and PKC coupled activation of p42 MAPK was not essential in the CA3. Thus activation of any effectors (such as CREB) of p42 MAPK in the CA3 may occur through an alternative pathway.

Figure 4.4: Upstream regulators and downstream effectors of MAPK cascade (Adapted from Sweat 2001). There are two pathways by which MAPK is activated; the PKC-Raf1-MEK-MAPK signalling and the PKA-B-Raf-MEK signalling. Downstream effects of MAPK activation include gene expression, via activation of CREB, ion channel activation, and protein synthesis. Abbreviations: AC, adenylate cyclase; CREB, cAMP respond element binding protein; GFR, growth factor receptor tyrosine kinase; PKC, protein kinase C, PKA; cAMP-dependent protein kinase; SOS, son of sevenless.


GFR
Grb2
Sos
Ras
PKC
Rap1
PKA
AC
Raf-1
B-Raf
MEK
P42 MAPK
RSK2
Ion
Channels
Protein
Synthesis
CREB
Gene
Expression

Chapter 5

Expression of αCaMKII

5.1 Introduction

Induction of LTP in the CA1 and DG regions of the hippocampus is dependent on activation of NMDA class of glutamate receptors while in the CA3 region; it is primarily dependent on increased cAMP levels (Collingridge and Bliss, 1993). LTP as already mentioned (General Introduction) is divided into three temporal phases - early (dependent on covalent modification of pre-existing proteins), intermediate and late phase (dependent on both transcription and translation). LTP2 appears between 1-6 hours after initial stimulation, depending on the experimental model, is dependent on mRNA translation and *de novo* protein synthesis but independent of gene transcription (Abraham *et al.*, 1993). Thus, it will be of interest to identify the proteins which are synthesised at this time in response to stimulation and the mechanisms which regulate their expression.

In the CNS few mRNA species have been detected in the dendrites as most are restricted to the cell body. mRNA species located in the dendrites are in close proximity to the afferent synapses and therefore are in a position to be affected rapidly by synaptic activity. These plasticity-like dendritic mRNAs include αCaMKII mRNA (Thomas *et al.*, 1994; L.A Roberts. *et al.*, 1996).

Expression of αCaMKII

CaMKII is present in high levels in the forebrain and cerebellum where it makes up ~2% of the total protein in the hippocampus. The kinase can be visualised in the cytosol of neuronal cell bodies (Erondu and Kennedy, 1985; Apperson *et al.*, 1996).

Biochemical studies have shown that persistent activation of CaMKII occurs during LTP and mediates persistent phosphorylation of GluR1 (Barria *et al.*, 1997; Fukunaga *et al.*, 1993; 1995; Lee *et al.*, 2000), implicating CaMKII in LTP maintenance. Maintenance of LTP has been shown by several groups to require *de novo* protein synthesis (Fazeli *et al.*, 1993; Frey *et al.*, 1989; Krug *et al.*, 1984).

A number of studies have reported increased levels of αCaMKII after LTP induction. Most of the studies on the effect of synaptic stimulation on the levels of αCaMKII mRNA (L.A. Roberts *et al.*, 1996, 1998) and hence the corresponding αCaMKII protein have been carried out in the CA1 region (Ouyang *et al.*, 1999) and the DG (Steward and Halpain, 1999). Therefore it is not clear whether increased αCaMKII expression is also a feature of LTP in the CA3 region, where Ca^{2+} influx is relatively less important for plasticity. Equally, the signalling pathways involved in the induction of αCaMKII mRNA following high frequency stimulation are unclear. Therefore in these studies, the effect of stimulation, using A23187 (a calcium ionophore) and forskolin (an adenlyate cyclase activator), on the levels of αCaMKII mRNA have been investigated, since Ca^{2+} and cAMP are major effectors of NMDA receptor activation.

In addition the effect of rapamycin, a protein synthesis inhibitor, on the expression of αCaMKII in both stimulated and non-stimulated organotypic slices was investigated. The major target for rapamycin in cells is FRAP or mTOR (Brown and Schreiber, 1996). Activation of mTOR leads to phosphorylation of the ribosomal protein, S6, resulting in increased translation (Jefferies *et al.*, 1997; Peterson and Schreiber, 1998). Therefore, the study aimed to investigate a possible role for mTOR in the regulation of αCaMKII expression.

5.2 Results

5.2.1 A23187 but not forskolin increases the expression of αCaMKII on hippocampal organotypic slice culture preparations

Neurons in all the sub-regions of the hippocampus were stained with αCaMKII antibody; this staining was prominent in the soma and dendrites (Figure 5.1). Application of 5μM A23187 resulted in a significant increase in the intensity of staining of αCaMKII in the cell bodies (*P<0.05 versus vehicle-100%, n=15, Figure 5.2d, e and f, 5.3a) of hippocampal formation i.e. DG, CA3 and CA1 with no significant difference in the expression of αCaMKII between the sub-regions (One way ANOVA with *post hoc* Fisher test, P=0.71). A significant increase in staining intensity of αCaMKII also occurred in the dendrites (Apical dendrites) of DG, CA3 and CA1 (*P<0.05 versus vehicle-100%, n=10, Figure 5.2d, e and f, 5.4b) upon application of 5μM A23187. Similar to cell bodies, there

was no significant difference in the expression of αCaMKII between hippocampal sub-regions.

In contrast 50μM forskolin (Huang *et al.*, 1994) had no significant effect on the expression of αCaMKII in both the soma and dendrites of all the sub-regions of the organotypic hippocampal slice culture (Figure 5.3 and 5.4).

5.2.2 Rapamycin had no effect on the expression of αCAMKII in both vehicle-treated and A23187-treated organotypic slice preparation

Hippocampal slice cultures were pre-treated with rapamycin for 30 minutes before being stimulated with A23187 (5μM) for 4 hours. In the cell bodies the expression of αCaMKII was not significantly affected by pre-treatment of hippocampal slices with 20nM rapamycin (Khan *et al.*, 2001; Jefferies *et al.*, 1997) ($P>0.05$, n=4, Figure 5.5), but A23187 significantly increased the intensity of staining of αCAMKII in both vehicle-treated and rapamycin pre-treated slices (+$P<0.05$ versus corresponding region after pre-treatment alone-ANOVA with *post hoc*, Fisher's test) (Figure 5.5). These increases occurred in the DG, CA3 and CA1. Similar to the cell bodies, pre-treatment of hippocampal slices with rapamycin (20nM) had no significant effect on the expression of αCAMKII (+$P<0.05$ versus corresponding region after pre-treatment alone-ANOVA with *post hoc*, Fisher's test) (Figure 5.6) in the dendrites. The intensity of staining of αCAMKII was significantly increased by A23187 in the dendrites (Figure 5.6) of the CA3 and CA1.

5.2.3 Western blot analysis shows A23187 but not forskolin increased expression of αCaMKII

A23187 (5μM) appeared to increase the expression of αCaMKII in organotypic hippocampal slice preparations as assessed by western blotting (Figure 5.7a, upper panel and Figure 5.7b). The levels of expression appeared to be increased after 4 hour treatment (110±10 of vehicle treated slices, n=4). However, these effects did not reach statistical significance. Forskolin (5μM) did not have any effect on the expression of αCaMKII after 4 hour treatment of organotypic slices as assessed by western blotting.

On reprobing of the PVDF membrane with an antibody to detect β-actin, the levels of β-actin in each pair of control and treatment well of the precast gel were found to be of equal concentration (Figure 5.7a, lower panel).

5.2.4 Tetanus induced LTP elevates the expression of αCaMKII in the CA1 sub-region of acute hippocampal slice preparations

Acute hippocampal slices underwent three tetanic stimulations in stratum radiatum in the CA1 region, followed by a signal stimulus every 30 seconds for 2.5 hours or 3.5 hours (Appendix, Figure 1 and 2). The CA1 was then removed, and prepared for Western blot analysis. The analysis showed that the expression of αCaMKII appeared to be elevated in expression after 3.5 hours (153±15% of vehicle-100%, n=3) (Figure 5.8). Nevertheless, these effects did not reach statistical significance.

Figure 5.1: Image of organotypic slice culture. The image shows the hippocampal formation, DG, CA3 and CA1. The slice has undergone no drug treatment and has been stained to reveal immunoreactivity for αCaMKII. Scale bar: 0.4mm

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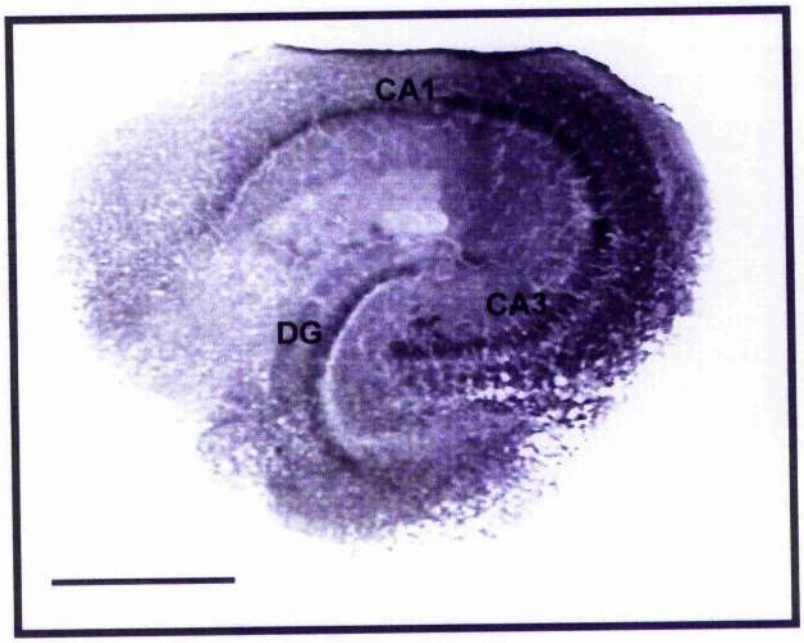

CA1
CA3
DG

Figure 5.2: Effect of A23187 (5μM) on the expression of αCaMKII in cultured hippocampal neurons. Organotypic hippocampal neurons were stained to reveal immunoreactivity for αCaMKII in DG (a, d), CA3 (b, e) and CA1 (c, f). Organotypic slice cultures were treated 4 hours prior to fixation with either vehicle (a, b and c) or A23187 (d, e and f). Note that A23187 increased the staining intensity of αCaMKII in both the cell soma and dendrites of all the hippocampal regions. Arrow head indicates the dendrites (apical dendrites); and the arrows show the somata. Scale bar: 20μm for CA1 and CA3, 12-15μm for DG

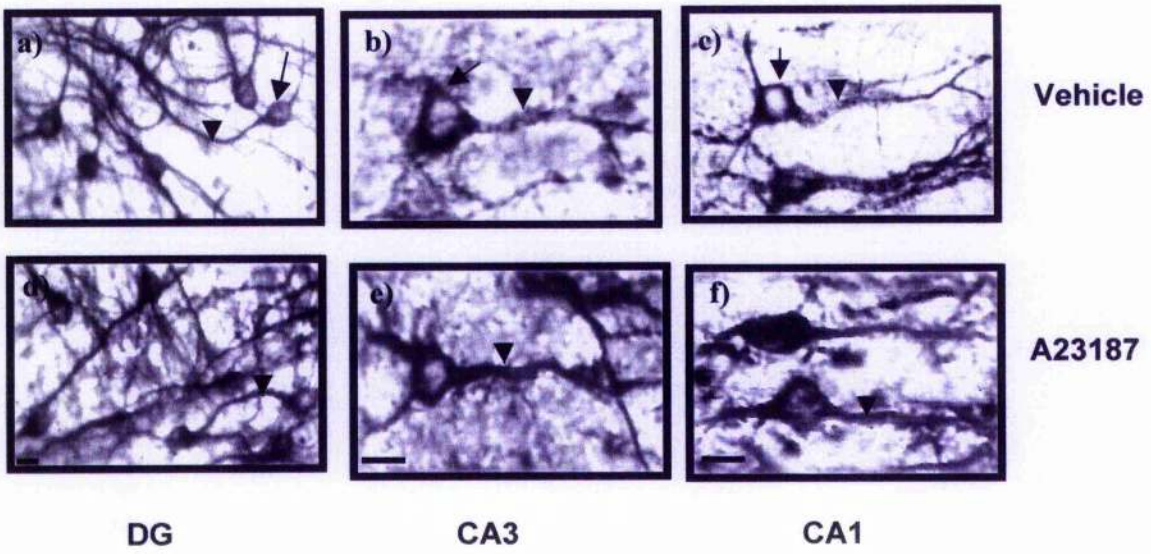
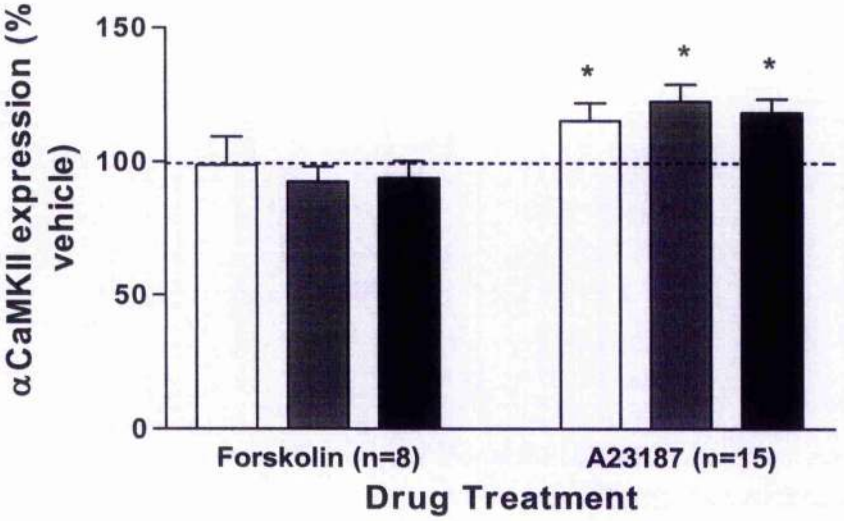

a)
b)
c)
d)
e)
f)
Vehicle
A23187
DG
CA3
CA1

Figure 5.3: Effect of forskolin (50μM) and A23187 (5μM) on the expression of αCaMKII in the soma (a) and apical dendrites (b) of cells of organotypic hippocampal slices cultures. Immunocytochemistry analysis using anti-αCAMKII antiserum indicated a significant increase (*P<0.05 versus vehicle in the same region-100%, one-sample Wilcoxon signed rank test for the median) in the staining intensity of αCaMKII in the presence of A23187 in all the three regions of the hippocampus, DG, CA3 and CA1. Forskolin had no significant effect on the three regions of the hippocampus (P>0.05)

a)


αCaMKII expression (% vehicle)
150
100
50
0
*
*
*
Forskolin (n=8)
A23187 (n=15)
Drug Treatment

b)

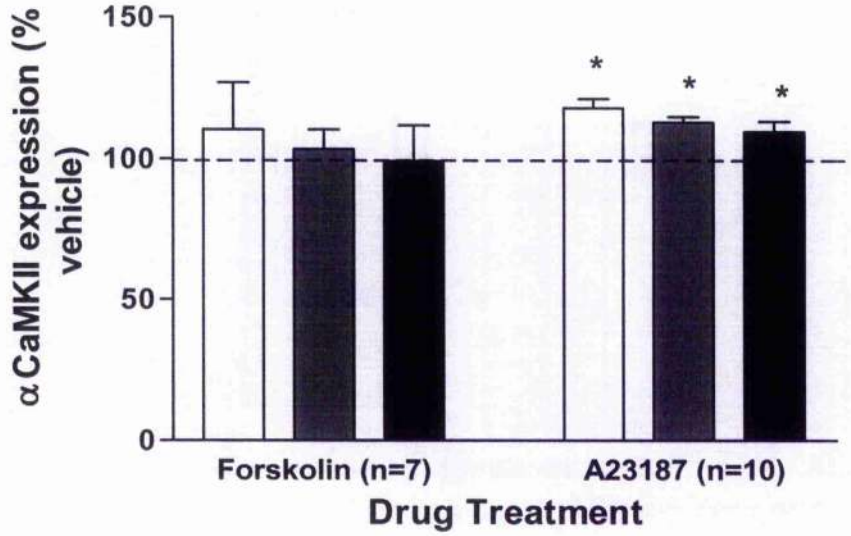
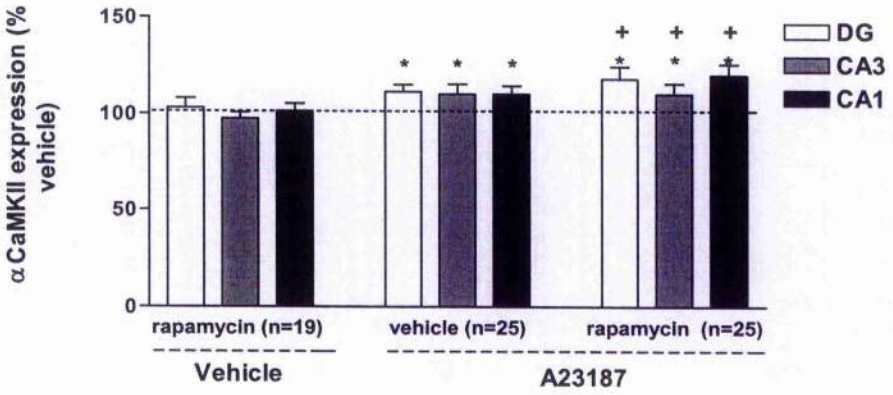

αCaMKII expression (% vehicle)
150
100
50
0
*
*
*
Forskolin (n=7)
A23187 (n=10)
Drug Treatment

Figure 5.4: Effect of rapamycin (20nM) on the expression of αCaMKII in both vehicle-treated and A23187-treated (5μM) soma (a) and apical dendrites (b) of cells in organotypic hippocampal slice cultures. The staining intensity levels of αCaMKII in the presence of A23187 were significantly increased in all three regions of the hippocampus in both vehicle and rapamycin pre-treated slices (*P<0.05 versus corresponding region after vehicle treatment in the absence of rapamycin). The intensity of staining of αCaMKII in presence of rapamycin/A23187 was significantly increased in all three regions of the hippocampus (+P<0.05 versus corresponding region after rapamycin pre-treatment alone – ANOVA with *post hoc* Fisher's test, F=2.15)

a)


αCaMKII expression (% vehicle)
150
100
50
0
DG
CA3
CA1
rapamycin (n=19)
vehicle (n=25)
rapamycin (n=25)
Vehicle
A23187

b)

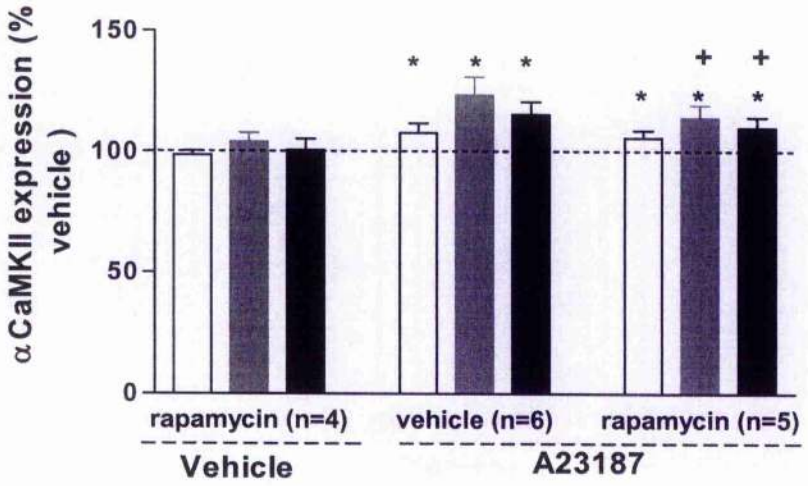
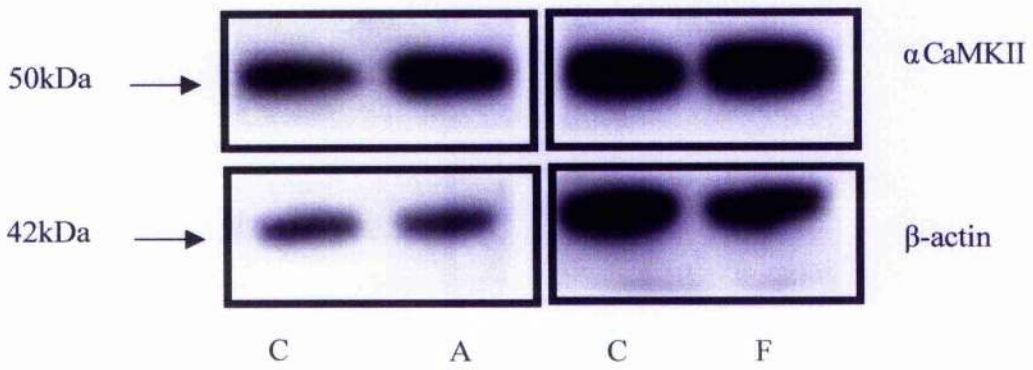

αCaMKII expression (% vehicle)
150
100
50
0
rapamycin (n=4)
vehicle (n=6)
rapamycin (n=5)
Vehicle
A23187

Figure 5.5: Western blot analysis of the effect of forskolin (50μM) and A23187 (5μM) on the expression of αCaMKII in organotypic hippocampal slice culture. Western blot analysis using anti-αCaMKII antiserum indicated no significant change in the levels of αCaMKII in the presence of A23187 (109.58±9.66% of the vehicle-100%, n=4, P>0.05, one sample Wilcoxon signed rank test for median) or forskolin (86.30±11.80%, n=4, P>0.05 one sample Wilcoxon signed rank test for median), figure a, upper panel and figure b. The PVDF membrane was stripped and reprobed with antibody that detects β-actin (figure. a, lower panel). Abbreviations: C- Control, A- A23187, F- Forskolin

a)


50kDa
42kDa
αCaMKII
β-actin
C
A
C
F

b)

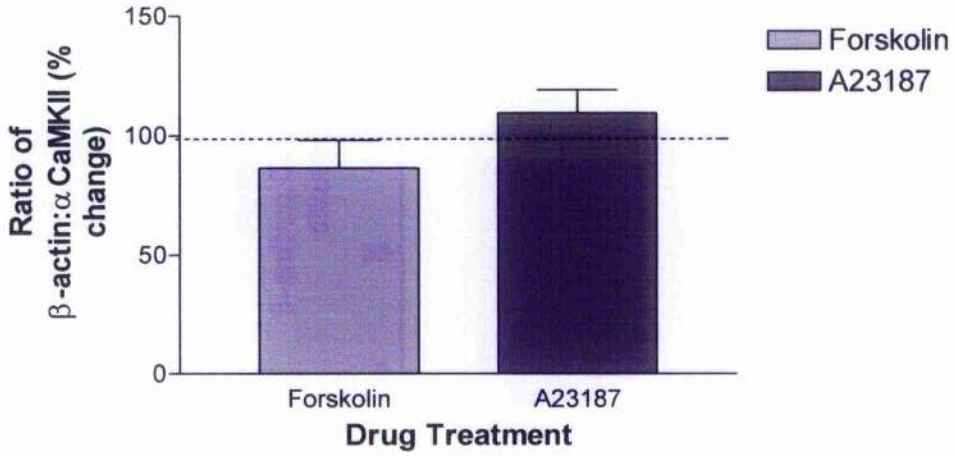
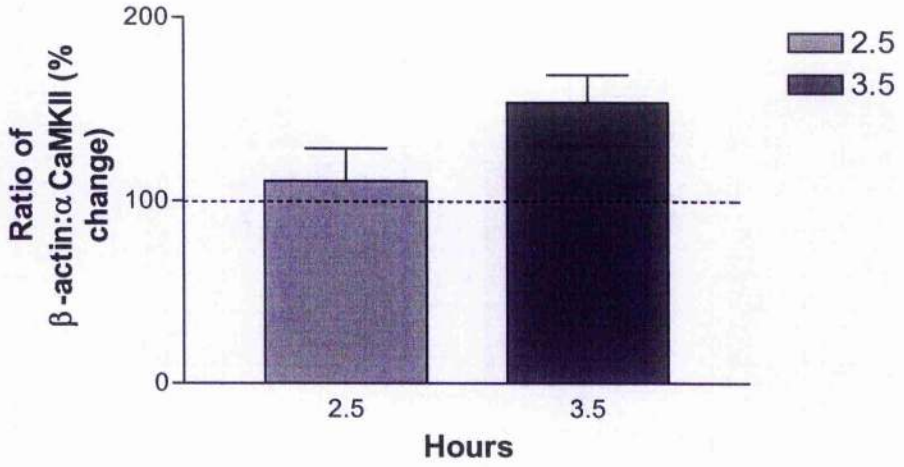

150
100
50
0
Ratio of β-actin:αCaMKII (% change)
Forskolin
A23187
Forskolin
A23187
Drug Treatment

Figure 5.6: Effect of LTP on the expression of αCaMKII in acute hippocampal slices. Western blot assay using anti-αCaMKII antiserum indicated an increased in the levels of αCaMKII after 2.5 hours (111±18, n=3, P>0.05 versus 100%, one-sample Wilcoxon signed rank test of the median) and 3.5 hours (153±15, n=3, P>0.05 versus 100%, one sample Wilcoxon signed rank test of the median).


200
100
0
Ratio of β-actin:αCaMKII (% change)
2.5
3.5
Hours
2.5
3.5

5.3 Discussion

Characterisation of αCaMKII antibody had been previously reported (McKee *et al.*, 1990; Erondu *et al.*, 1985; Kennedy *et al.*, 1983; Hendry *et al.*, 1986). The antibody was shown to identify the purified alpha subunit of CaMKII in rats and to identify a single band of identical molecular weight in gels of rat brain homogenate. In protocols used in these studies, omission of the primary antibody eliminated immunostaining completely. αCaMKII is the major protein found in postsynaptic densities and constitutes some 2% of total hippocampal protein (Erondu *et al.*, 1985; Kelly *et al.*, 1984).

A variety of different experimental approaches have revealed the importance of CaMKII for the expression of synaptic plasticity in the hippocampus (Barria *et al.*, 1997; Fukunaga and Miyamoto, 2000). Increased levels of αCaMKII in the hippocampal CA1 neurons are sufficient to result in a sustained increase in the efficiency of afferent synapses (Pettit *et al.*, 1994)

5.3.1 A23187

Results of these studies showed that an increase in the influx of Ca^{2+}, using 5μM A23187, led to a significant increase in the staining intensity of αCaMKII in somas of DG (15 ±7% increase), CA3 (23±6% increase) and CA1 (18±5% increase), sub-regions of the hippocampus (Figure 5.2 and 5.3a). Ca^{2+} influx also increased αCaMKII immunostaining in dendrites (apical dendrites) of DG (18±3 % increase), CA3 (13±2% increase) and CA1

(10±4% increase) (Figure 5.2 and 5.3b). This suggests that an increase in intracellular concentration of Ca^{2+} plays an important role in the expression of αCaMKII in both soma and apical dendrites of hippocampal sub-regions. This is the first time increased immunostaining of αCaMKII had been shown to be elevated in both soma and dendrites of CA3 sub-region. These results might suggest that the intracellular signalling in the CA3 region is similar to the CA1 and DG regions. As with the DG and CA1 regions, αCaMKII mRNA is present in the dendrites of CA3 pyramidal cells. This may suggest that αCaMKII plays a similar role in relation to plasticity in the CA3 region. Previous data had shown that the αCaMKII mRNA levels are elevated following plasticity in the cell bodies and dendrites in the DG (Johnston and Morris, 1995) and CA1 (Roberts *et al.*, 1996; Ouyang *et al.*, 1997; Ouyang *et al.*, 1999), but no similar changes in αCaMKII mRNA in the CA3 region has been reported. Since, increased synthesis of proteins requires mRNA, it can be assumed that mRNA levels will also be distributed in both the soma and dendrites of CA3 region of the hippocampus.

Maintenance of LTP is said to be dependent on *de novo* protein synthesis (Krug *et al.*, 1984; Barzilai *et al.*, 1989; Bliss and Collingridge, 1993; Huang *et al.*, 1994; Frey *et al.*, 1996), but LTP2, which lasts up to 4 hours after initial induction (Abraham *et al.*, 1993), is not dependent on *de novo* mRNA synthesis (Otani *et al.*, 1989; Bliss and Collingridge, 1993). This is reported to be true for the CA3 region as well as the intensively studied DG and CA1. Therefore the increased αCaMKII expression detected here is consistent with αCaMKII playing a role in LTP maintenance. And, in dendrites the increased immunostaining of αCaMKII might be due to localised mRNA which regulates the

synthesis of the αCaMKII (Morris, 1997) while in the soma increase αCaMKII expression can be attributed to mRNA elevation in the somata. This process of αCaMKII synthesis from dendritic and somata mRNA seems to be applicable to the CA3 region as well as the other hippocampal areas. Thus the αCaMKII synthesised from pre-existing αCaMKII mRNA in the CA3, might be required for the maintenance of LTP during LTP2.

One possible mechanism by which Ca^{2+} influx stimulates αCaMKII synthesis is via phosphorylation of CPEB protein, which is present in the hippocampal dendrites (Wu *et al.*, 1998). Wu *et al.*, (1998) provided evidence that binding of CPEB protein to CPE sites located in the 3'-end of the RNA message for αCaMKII can stimulate its translation rate. CPEB is responsible for cytoplasmic polyadenylation-induced translation. The same method of Ca^{2+} stimulation of hippocampal dendrites might also be applicable to the somas, since CPEB is distributed in the hippocampal neurons (Wu *et al.*., 1998).

5.3.2 Forskolin

Elevation of cAMP levels using forskolin (50μM, adenylate cyclase activator) did not seem to elevate the levels of αCaMKII in either the soma or dendrites of DG, CA1 or CA3 (Figure 5.2 and 5.3a, b). Studies have shown that cAMP is required for Mossy fibre LTP in the CA3 region (Weisskopf *et al.*, 1994; Huang *et al.*, 1994), but this requirement for LTP is achieved via activation of Ca^{2+} sensitive-type I adenylate cyclase (neurospecific) and results in activation of PKA (Villacres *et al.*, 1998). These results

suggest that while elevated cAMP is required to initiate LTP in the CA3 region, it is not sufficient to induce elevated αCaMKII expression.

Western blot analysis suggested that an increase in Ca^{2+} influx might slightly elevate αCaMKII in the organotypic hippocampal slices (Figure 5.5a, upper panel and Figure 5.5b), however these results did not reach statistical significance. An elevation would be expected as a result of stimulation of all the three regions of the hippocampus since immunocytochemistry results indicate an elevation in DG, CA3 and CA1 regions. The lack of a significant change (P=0.091) suggests western analysis may be less sensitive than immunocytochemistry for detecting changes, or may reflect the smaller number of replicates in this experiment.

5.3.3 LTP induction

Studies were also carried out on acute hippocampal slices to investigate the effect of LTP on the expression of αCaMKII. The CA1 region of the hippocampal slices was assayed 2.5 and 3.5 hours after LTP induction, and the results appear to show an increase in the expression of αCaMKII after 3.5 hours. However, the data did not reach statistical significance (Figure 5.6). The levels of αCaMKII appeared to be more elevated after 3.5 hours (53 ±15% increase) compared to 2.5 hours (10±17% increase). Since the immunocytochemistry results suggested a similar intracellular signalling in all three regions of the hippocampus, it can be proposed that the elevations resulting from LTP

induction might also be applicable to CA3 region. The lack of statistical significance probably reflects the small number of replicates in this experiment.

These results are consistent with the general hypothesis that CaMKII is a target for calcium influx through activated NMDA receptors (Kennedy *et al.*, 1983; Kennedy, 1989; L.A. Roberts *et al.*, 1996), and in the CA3 region, there is evidence that Ca^{2+} influx contributes to MF LTP (Weisskopf *et al.*, 1994). It has been suggested that this may occur via Ca^{2+} permeable KA receptors (Huettner, 2001). Induction of αCaMKII may be a consequence of Ca^{2+} influx via this route. Other studies have shown that mossy fibre activity evokes Ca^{2+} release from internal stores via group I mGluRs activation (Kapur *et al.*, 2001), hence in this study the role of group I mGluRs cannot be ruled out.

5.3.4 Rapamycin

The next stage of these studies was to investigate the effect of a protein synthesis inhibitor, rapamycin (20nM), on the immunostaining of αCaMKII in the soma and dendrites of all the three sub-regions of the organotypic hippocampal slices. As mentioned above the major target of rapamycin is mTOR. Tang *et al.*, 2002 indicated the presence of mTOR and other translational signalling components in soma and dendrites in cultured rat hippocampal neurons using immunostaining and western blot analysis. Inhibition of mTOR by rapamycin at a dose of 20nM blocked LTP in CA1 region (Tang *et al.*, 2002). Rapamycin had no effect on basal levels of αCaMKII in soma and dendrites of DG, CA3 and CA1 regions of the hippocampus (Figure 5.4a, b). A23187 increased the

levels of αCaMKII irrespective of pre-treatment of slices with rapamycin, this increased expression occurred in both soma and dendrites of all the regions of the hippocampus. The increased levels of immunostaining of αCaMKII in both the soma and dendrites of the CA3 region of the hippocampus are not blocked by rapamycin, again suggesting a similar intracellular signalling pathway between CA3, DG and CA1. This might suggest that the stimulation of expression of αCaMKII might follow a rapamycin insensitive pathway. There are other pathways leading to increased translational efficiency that do not involve mTOR- for example the $p70^{S6K}$ pathway, and these may be involved. Alternatively, this could be due to pre-translational modification resulting from mRNA stabilisation (Morris, 1997).

5.3.5 Summary

In summary, the data in these studies have implicated Ca^{2+} influx in the increased immunostaining of αCaMKII in all the sub-regions of the organotypic slice culture and this for the first time indicates there might be similar intracellular signalling for the regulation of αCaMKII expression between CA3 and the other regions i.e. DG and CA1. Elevation of cAMP levels in the hippocampus did not have a role in increasing the levels of αCaMKII in both the soma and dendrites of the hippocampus. Despite the reported dependence of LTP2 in CA1 on activation of mTOR, rapamycin did not affect the Ca^{2+}-dependent stimulation of αCaMKII expression, suggesting that other pathways are involved.

Chapter 6

Expression of MAP2

6.1 Introduction

MAP2 belongs to a family of microtubule-associated proteins. MAP2 is expressed in the nervous system and consists of isforms resulting from the alternative splicing of a pre-mRNA transcribed from a single gene (Neve *et al.*, 1986; Shafit-Zagardo and Kalcheva, 1998). There are two isoforms of MAP2 in the mammalian brain as already mentioned (refer to section 1.12). HMW MAP2 and LMW MAP2.

MAP2 mRNA has been associated with various forms of neuronal plasticity (Ferreira *et al.*, 1990; L.A. Roberts *et al.*, 1996). MAP2 cross-links microtubule and facilitates changes in dendritic architecture (Caceres *et al.*, 1988; 1992; Dinsmore *et al.*, 1991) making it an attractive candidate for enabling the synaptic re-modelling associated with LTP.

The I-LTP, which lasts around 3-6 hours (Abraham *et al.*, 1993), is dependent on mRNA translation and is independent of transcription. One of the mRNA species affected during this intermediate phase is the MAP2 mRNA. Levels of MAP2 mRNA have been shown to be elevated after LTP induction, at least in the DG (Thomas *et al.*, 1994; Johnston and Morris, 1994) and CA1 (L.A. Roberts *et al.*, 1998) regions of the hippocampus, but it is not known yet if similar increases are observed in the CA3 region. The induction of LTP

in CA1 and DG is dependent on activation of NMDA receptors while in the CA3 region the induction is dependent on increased cAMP levels (Bliss and Collingridge, 1993). There is evidence to suggest that the increases in MAP2 mRNA levels are the result of post-transcriptional regulation of existing mRNA in dendrites local to the region of stimulation (Morris, 1997). This local increase in mRNA is then translated into elevated levels of MAP2 protein. The mechanisms that trigger the induction of LTP, via glutamatergic activation of NMDA receptors, resulting in enhancement of MAP2 mRNA levels are unclear.

Two major effectors of NMDA receptor activation are Ca^{2+} influx via activated NMDA receptors and cAMP via adenylate cyclase activation. Thus in this study, the effects of increased Ca^{2+} influx or raised cAMP levels on the expression of MAP2 was investigated. The effect of a protein synthesis inhibitor, rapamycin, on the levels of MAP2 in both stimulated and un-stimulated slices was investigated. Rapamycin produces its effect via inhibition of a kinase, mTOR. Therefore, the effect of mTOR on the regulation of MAP2 expression was investigated.

6.2 Results

6.2.1 Forskolin but not A23187 increases the expression of MAP2 in organotypic slice preparation

MAP2 antibody stained the neurons of all the sub-regions of the hippocampus and this staining was prominent in the soma and dendrites (Figure 6.1). Following the treatment of organotypic slices with forskolin (50μM) or A23187 (5μM) for 4 hours (a time point at which protein expression is widely believe to occur, Abraham *et al*, 1993), the slices were fixed and ready for immunocytochemical analysis. Application of 50μM forskolin (Huang *et al.*, 1994) on the organotypic cultures resulted in a significant increase in the intensity of staining of MAP2 in the cell bodies (Figures 6.2d, e, f and 6.3a) of hippocampal formation i.e. DG, CA3 and CA1. This significant increase in the intensity of MAP2 was also produced in the dendrites (Figures 6.2d, e, f and 6.3b) of hippocampal formation. A23187 had no significant effect on the expression of MAP2 in both the soma and dendrites of DG, CA3 and CA1 regions (Figure 6.3a and b).

6.2.2 Rapamycin decreases the basal expression of MAP2 with no obvious inhibitory effects on forskolin

Neurons in the organotypic hippocampal slice cultures were stained with antibody for MAP2, with staining prominent in the soma and dendrites (Figures 6.5a, 6.6a and 6.7a).

Having established the importance of forskolin in the increased expression of MAP2, the effect of rapamycin (20nM) pre-treatment on the expression of MAP2 in both the presence and absence of forskolin was investigated. Slices were pre-treated with rapamycin for 30 minutes prior to application of forskolin. Intensity of staining of MAP2 at basal level was significantly reduced in the soma and apical dendrites after pretreatment of slices with rapamycin in the hippocampal formation i.e. DG (22% and 14% decrease in soma and apical dendrites respectively), CA3 (20% and 5% decrease in soma and apical dendrites respectively) and CA1 (20%and 12% decrease in soma and apical dendrites respectively) (*P<0.05 versus 100% vehicle-treatment of corresponding regions) (Figures 6.5b, 6.6b, 6.7b and 6.8a and b). However, forskolin was still able to increase MAP2 expression in presence of rapamycin (~P<0.05 versus corresponding region after rapamycin pretreatment alone-ANOVA with *post hoc*, Fisher's test) but to lesser extend than in the absence of rapamycin (*P<0.05 versus 100% vehicle-treatment of corresponding regions) (Figure 6.8a and b). These increases of MAP2 expression in presence of forskolin after rapamycin pre-treatment occurred in soma and apical dendrites of hippocampal formation (Figure 6.5c, 6.6c, 6.7c, 6.8a and b). The increases of MAP2 expression in rapamycin/forskolin treated somata was 17% in DG, 9% in CA3 and 14%in CA1 while in the apical dendrites these increases were 17%, 24% and 23% in DG, CA3 and CA1 regions respectively.

6.2.3 Western blot analysis show that forskolin but not A23187 increases the expression of MAP2

After application of either 5μM A23187 or 50μM forskolin for 4 hours, organotypic slice cultures were homogenised in ice-cold RIPA and were thus ready for western blot analysis. The MAP2 antibody recognised three isoforms of MAP2 (MAP2a/b, Mr 280kKDa and MAP2c, Mr 70kDa). Forskolin significantly increase the expression of MAP2 (Mr 280kDa; 217±59% of vehicle, n=5, *P<0.05, one sample Wilcoxon signed rank test for median) (Figure 6.5a, upper panel and 6.5b). Expression of MAP2, Mr 70kDa, was also increased in the presence of forskolin (150±43% of vehicle)(Figure 6.5a, upper panel and 6.5b), but the increase did not reach statistical significance. A23187 had no significant effect on all the isoforms of MAP2.

The PVDF membrane was stripped and reprobed with an antibody that detects β-actin (Figure 6.5a, lower panel).

6.2.4 Tetanus induced LTP dramatically increases the expression of MAP2 in the CA1 sub-region of the acute hippocampal slice preparations.

Three tetanic stimulations were applied in the stratum radiatum of CA1 region of acute hippocampal slices, followed by single stimulus every 30 seconds for 3.5 hours (Appendix, Figure 2). The CA1 region was then removed and prepared for western blot

analysis. The analysis indicated that the expression of MAP2 (280kDa) was elevated substantially after 3.5 hours (1063±515% of vehicle, n=3)(Figure 6.11a, upper panel and Figure 6.11b). MAP2 with molecular weight of 70kDa was not detected.

The blot was then stripped and reprobed using anti-β-actin antiserum which is used as an internal standard (Figure 6.11a, lower panel).

Figure 6.1: Image of organotypic slice culture. The image indicates the three sub-regions of the hippocampus, DG, CA3 and CA1. The slice has undergone no drug treatment and has been stained to reveal immunoreactivity for MAP2. Scale bar: 0.4mm

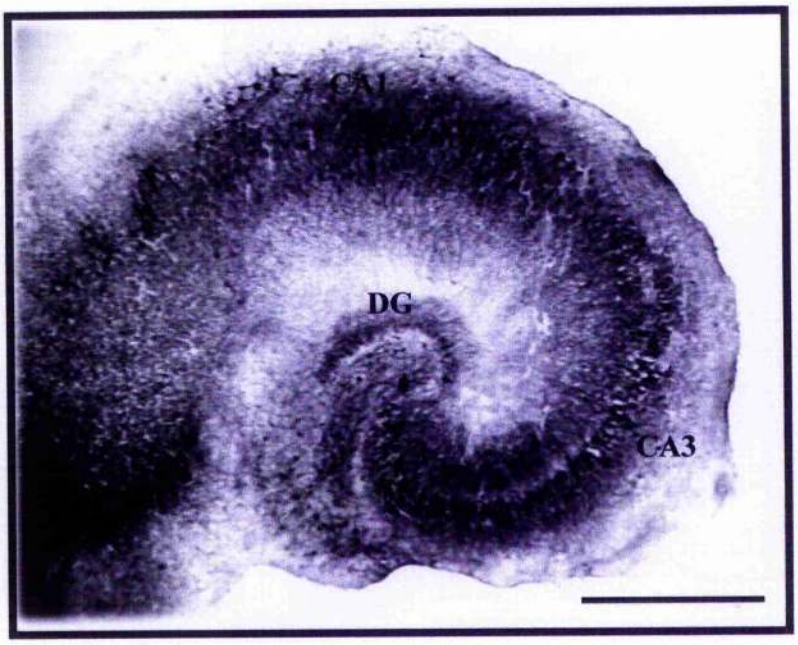

CA1
DG
CA3

Figure 6.2: Effect of forskolin (50μM) on the expression of MAP2 in cultured hippocampal neurons. Organotypic hippocampal neurons were stained to reveal immunoreactivity for MAP2 in DG (a, d), CA3 (b, e) and CA1 (c, f). Organotypic slice cultures were treated 4 hours prior to fixation with either vehicle (a, b and c) or forskolin (d, e and f). Note that forskolin increased the staining intensity of MAP2 in both the cell soma and dendrites of all the hippocampal sub-regions. The arrow heads and arrows show the somata and apical dendrites respectively. Scale bar 20μm for CA1 and CA3, 12-15μm for DG.

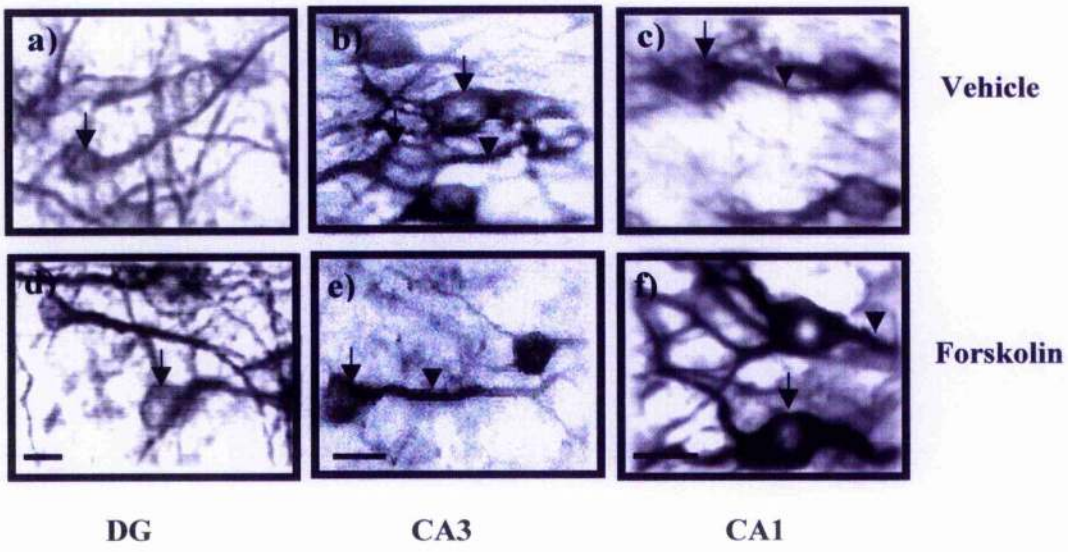
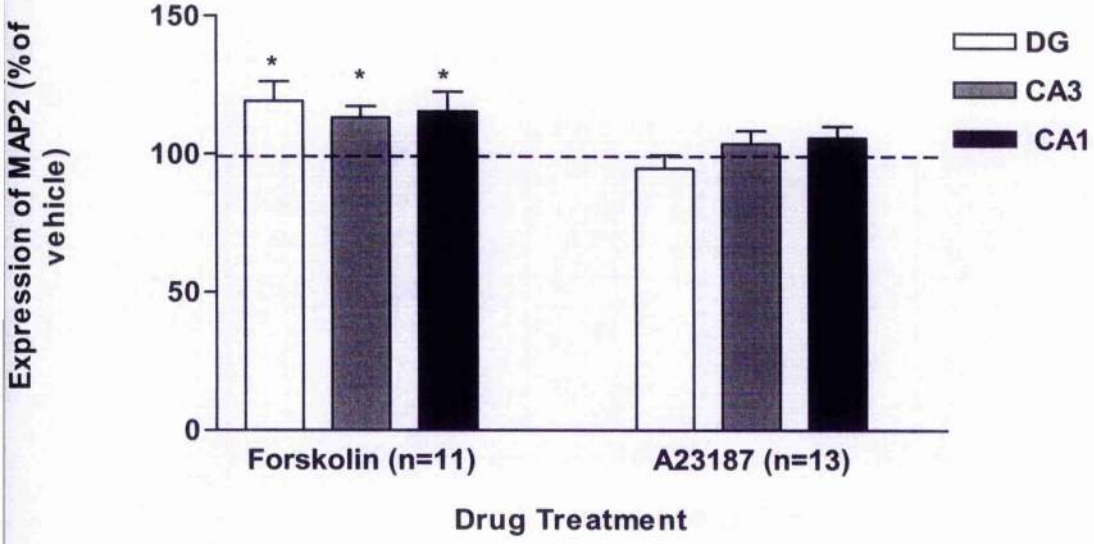

a)
b)
c)
Vehicle
d)
e)
f)
Forskolin
DG
CA3
CA1

Figure 6.3: Effect of forskolin (50μM) and A23187 (5μM) on the expression of MAP2 in the soma and dendrites (apical dendrites) of organotypic hippocampal slice cultures. Immunocytochemical analysis using anti-MAP2 antiserum indicated a significant increase (*P<0.05 versus vehicle-treatment in the same region, one-sample Wilcoxon signed rank test for the median) in the staining intensity of MAP2 in the presence of forskolin in hippocampal formation i.e. DG, CA3 and CA1. A23187 had no significant effect on the three regions of the hippocampus (P>0.05

a)


Expression of MAP2 (% of vehicle)
150
100
50
0
*
*
*
DG
CA3
CA1
Forskolin (n=11)
A23187 (n=13)
Drug Treatment

b)

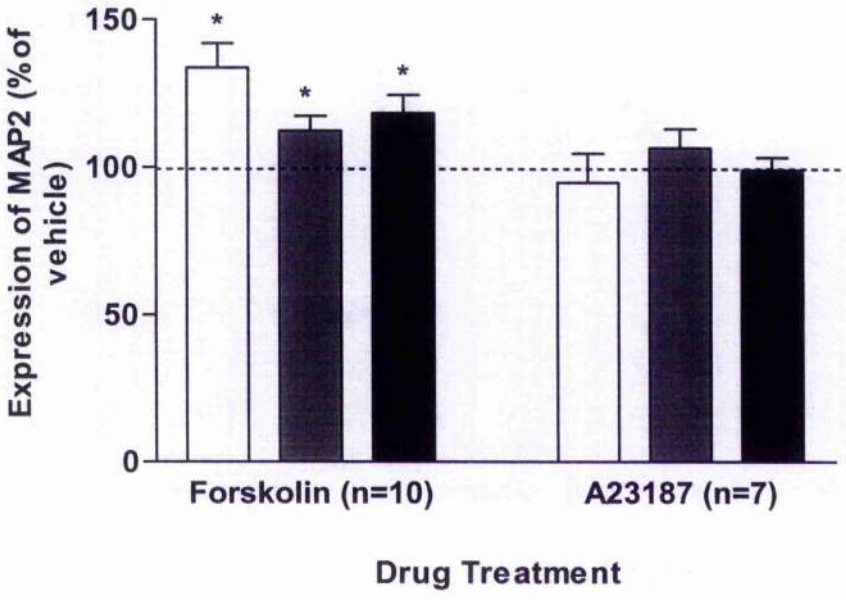
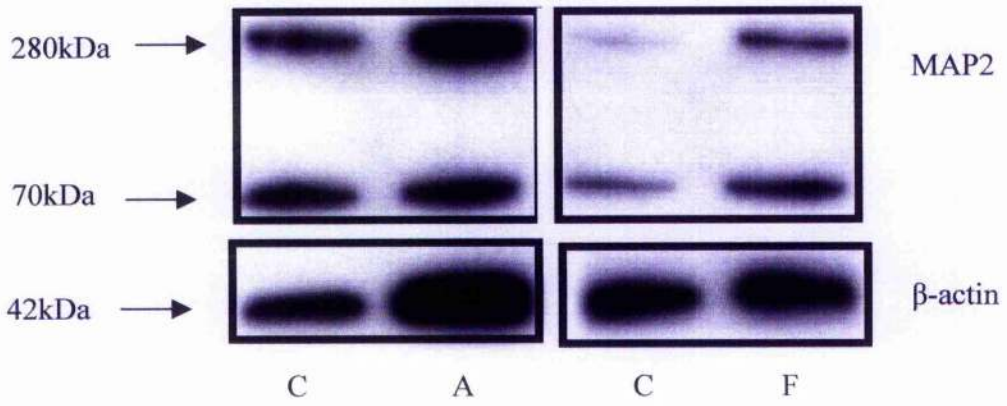

Expression of MAP2 (% of vehicle)
150
100
50
0
*
*
*
Forskolin (n=10)
A23187 (n=7)
Drug Treatment

Figure 6.4: Western blot analysis of the effect of forskolin (50μM) and A23187 (5μM) on the expression of MAP2 in organotypic hippocampal slice culture. Western blot analysis using anti-MAP2 antiserum indicated MAP2 with two different molecular weights (Mr: 280kDa and 70kDa respectively). There was a significant increase in the levels of MAP2 (Mr 280) in the presence of forskolin (216.9±59.10% of the vehicle- 100%, n=5, $P<0.05$, one sample Wilcoxon signed rank test for median) and an increase in the levels of MAP2 (Mr 70) in presence of forskolin (149.20±42.70% of vehicle, n=5, $P>0.05$, one sample Wilcoxon signed rank test for median), figure a, upper panel and figure b. The PVDF membrane was stripped and reprobed with β-actin (fig. a, lower panel) which indicated the concentration of proteins loaded into each well. Abbreviations: C- Control, A- A23187, F- Forskolin

a)


280kDa
70kDa
42kDa
MAP2
β-actin
C
A
C
F

b)

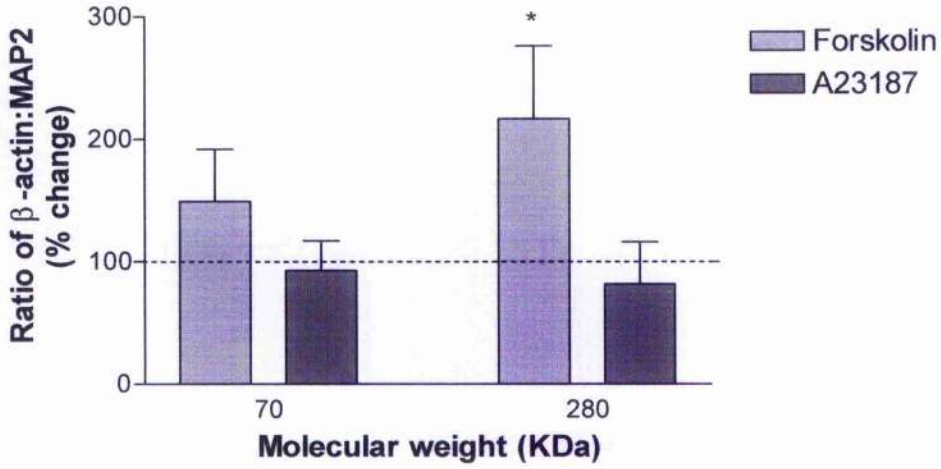
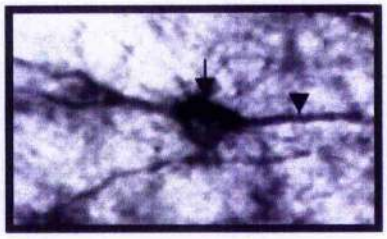
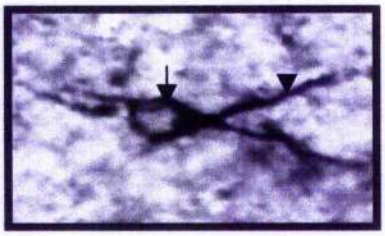

Ratio of β-actin:MAP2 (% change)
300
200
100
0
*
70
280
Molecular weight (KDa)
Forskolin
A23187

Figure 6.5: Effect of rapamycin (20nM) on the expression of MAP2 in DG region of organotypic hippocampal neurons. DG neurons in organotypic cultures were stained to reveal immunoreactivity for MAP2. Organotypic slice cultures were pre-treated for 30 minutes with either rapamycin (b, d) or vehicle (a, c) followed by stimulation with 50μM forskolin (c and d) for 4 hours prior to fixation. Note that rapamycin decreased the staining intensity for MAP2 in both cell soma and the dendritic region at basal levels (b) compared to vehicle (a), but had no effect on increased expression of MAP2 in the presence of forskolin (d). The increased staining intensity in soma and dendrites of both vehicle/forskolin (c) and rapamycin/forskolin (d) were similar. The arrow heads and arrows show the somata and apical dendrites respectively. Scale bar: 12μm.

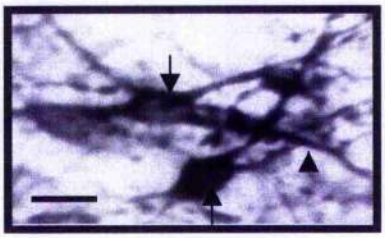
a)



b)



c)



d)

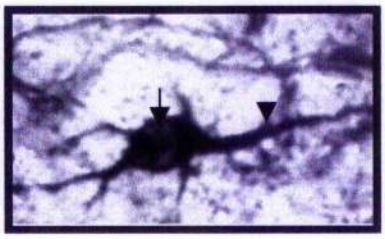
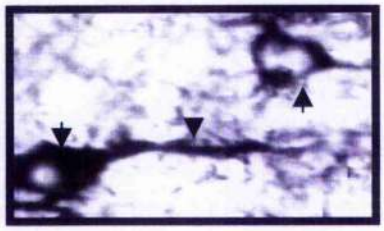
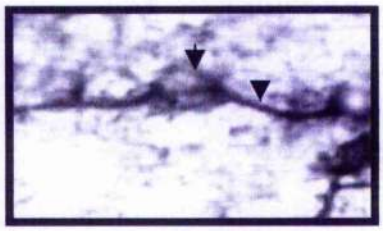


Figure 6.6: Effect of rapamycin (20nM) on the expression of MAP2 in CA3 region of organotypic hippocampal neurons. CA3 neurons in organotypic cultures were stained to reveal immunoreactivity for MAP2. Organotypic slice cultures were pre-treated for 30 minutes with either rapamycin (b, d) or vehicle (a, c) followed by stimulation with 50μM forskolin (c and d) for 4 hours prior to fixation. Note that rapamycin decreased the staining intensity for MAP2 in both cell soma and the dendritic region at basal levels (b) compared to vehicle (a), but had no effect on increased expression of MAP2 in the presence of forskolin (d). The increased staining intensity in soma and dendrites of both vehicle/forskolin (c) and rapamycin/forskolin (d) were similar. The arrow heads and arrows show the somata and apical dendrites respectively. Scale bar: 20μm.

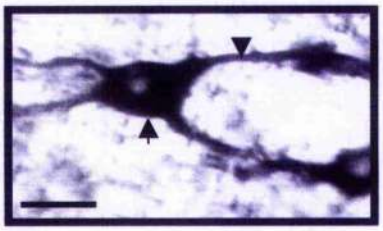
a)



b)



c)



d)

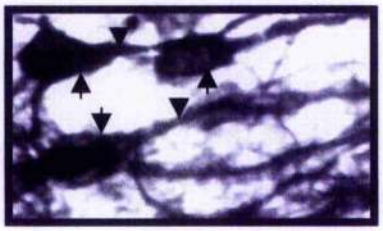
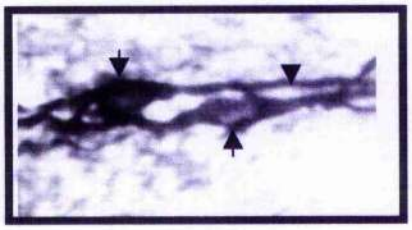
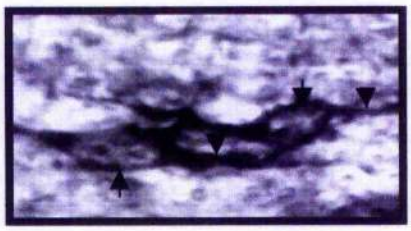


Figure 6.7: Effect of rapamycin (20nM) on the expression of MAP2 in CA1 region of organotypic hippocampal neurons. CA1 neurons in organotypic cultures were stained to reveal immunoreactivity for MAP2. Organotypic slice cultures were pre-treated for 30 minutes with either rapamycin (b, d) or vehicle (a, c) followed by stimulation with 50μM forskolin (c and d) for 4 hours prior to fixation. Note that rapamycin decreased the staining intensity for MAP2 in both cell soma and the dendritic region at basal levels (b) compared to vehicle (a), but had no effect on increased expression of MAP2 in the presence of forskolin (d). The increased staining intensity in soma and dendrites of both vehicle/forskolin (c) and rapamycin/forskolin (d) were similar. The arrow heads and arrows show the somata and apical dendrites respectively. Scale bar: 20μm.

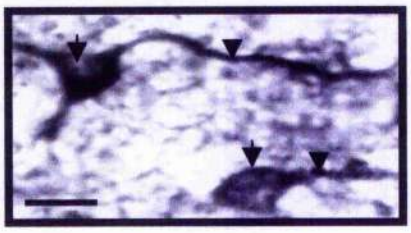
a)



b)



c)



d)

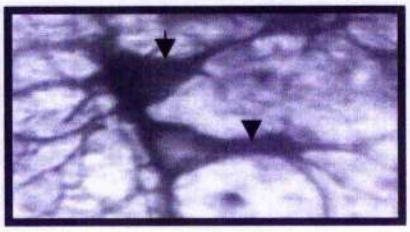


Figure 6.8: Effect of rapamycin (20nM) on the expression of MAP2 in both vehicle treated and forskolin (50μM) treated soma and dendrites (apical) in organotypic hippocampal slice cultures. The staining intensity levels of MAP2 in the presence of forskolin were significantly increased in all three regions of the hippocampus in vehicle pre-treated slices (*P<0.05 versus corresponding region after vehicle treatment in the absence of rapamycin). Intensity of staining of MAP2 was significantly reduced in the presence of rapamycin/vehicle treatment in all three regions (*P<0.05 versus corresponding region after vehicle treatment in the presence of rapamycin). The staining intensity of MAP2 in rapamycin/forskolin treated slices was significantly different from rapamycin/vehicle treated slices in all three regions (~P<0.05 versus corresponding region after rapamycin pre-treatment alone- ANOVA with *post hoc* Fisher's test, F=11.01, P<0.05)

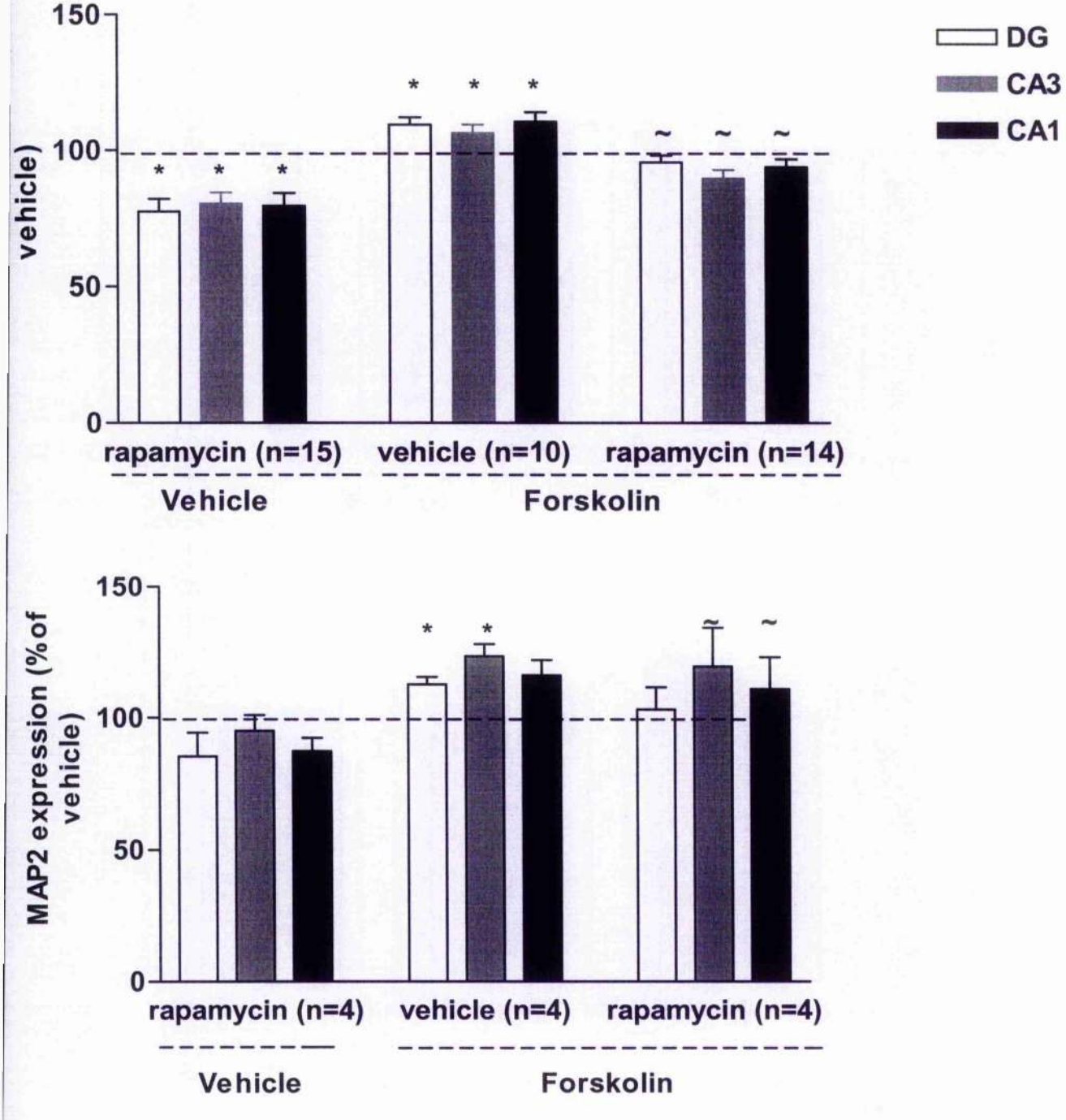
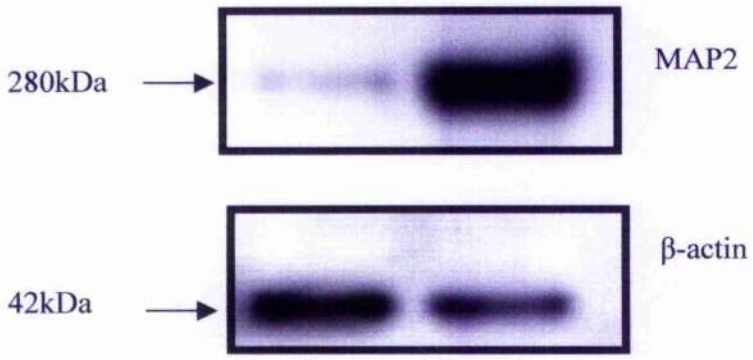
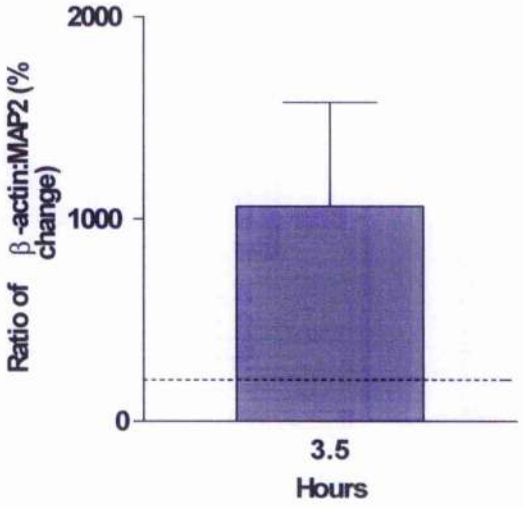

150
100
50
0
vehicle)
DG
CA3
CA1
rapamycin (n=15)
vehicle (n=10)
rapamycin (n=14)
Vehicle
Forskolin
MAP2 expression (% of vehicle)
rapamycin (n=4)
vehicle (n=4)
rapamycin (n=4)
Vehicle
Forskolin

Figure 6.9: Effect of LTP induced in the Schaffer collateral/CA1 synapses on the expression of MAP2 in acute hippocampal slices. Western blot assay using anti-MAP2 antiserum indicated an increased in the levels of MAP2 after 3.5 hours (1063±515%, n=3, $P<0.05$ versus 100%, one sample Wilcoxon signed rank test of the median), figure b and a, upper panel. The PVDF membrane was stripped and re-probed with an antibody to detect β-actin (figure a, lower panel).

a)


280kDa
MAP2
42kDa
β-actin

b)


2000
1000
0
Ratio of β-actin:MAP2 (% change)
3.5
Hours

6.3 Discussion

The antibody for MAP2 has been previously characterised (Woolf *et al.*, 1999; Lim and Halpain, 2000). The antibody recognises both the HMW MAP2a, b and LMW MAP2c isoforms which have a molecular weights (Mr) of 280kDa and 70kDa respectively in gels of rat brain homogenate. In procedures used, the exclusion of primary MAP2 antibody resulted in the elimination of immunostaining of organotypic hippocampal slices, suggesting that the signal derives from the primary antibody. In view of the specificity of this antibody for MAP2, this strongly suggests that the immunohistochemcial staining represents authentic MAP2. MAP2 isoforms have been shown to be localised in neuronal cell bodies and dendrites (Burgoyne and Cumming, 1983; Woolf *et al.*, 1999; Lim and Halpain, 2000).

Prolonged structural changes thought to be associated with LTP might be due to alteration in gene expression (Chang and Greenough, 1984; Lisman and Harris, 1993) and one of the possible candidates includes the MAP2 gene, since altered MAP2 expression has been linked to morphological changes in neurons (section 1.12).

6.3.1 Forskolin

These studies aimed to investigate the effect of Ca^{2+} influx and elevation of cAMP levels on the staining intensity of MAP2. The data indicates that the levels of staining intensity of MAP2 were significantly elevated on the stimulation of organotypic slice cultures with

50μM forskolin (Figure 6.2d, e, f). These increases were prominent in the cell bodies of DG (19±7% increase), CA3 (13±4% increase) and CA1 (15±7% increase) (Figure 6.3a). A similar result was also produced in the apical dendrites of DG (33±8% increase), CA3 (12±5% increase) and CA1 (18±6% increase) (Figure 6.3b). Therefore, the data suggests that the activation of adenylate cyclase (AC) by forskolin elevates cAMP levels which in turn increases the expression of MAP2 in the both the cell bodies and dendrites of DG, CA3 and CA1 regions of the organotypic slice culture. Since the elevation occurred in whole hippocampal formation, it can be assumed that the intracellular pathways that increase MAP2 expression in CA3 region are similar to those in the CA1 and DG. Thus it can be suggested that MAP2 plays a similar role in relation to plasticity in the CA3. It has been shown that LTP induction increases MAP2 expression in the DG and CA1 (Roberts *et al.*, 1998). The concentration of forskolin used here is known to induce LTP in the mossy fibre/CA3 synapses in acutely prepared hippocampal slices (Weisskopf *et al.*, 1994). Assuming the same is true in slice cultures, this is the first evidence that elevated MAP2 expression may be associated with plasticity in this region of the hippocampus.

Previous studies have shown that high frequency stimulation will result in increased levels of MAP2 mRNA in the CA1 (Robert *et al.*, 1998) and DG (Johnston and Morris, 1995; Roberts *et al.*, 1998). The elevated mRNA will presumably be translated into corresponding MAP2 protein. It is therefore likely that the increased MAP2 protein in both the soma and dendrites of CA3 region of the hippocampus results from elevated MAP2 mRNA.

Studies have shown that maintenance of LTP requires *de novo* protein synthesis (Krug *et al.*, 1984; Barzilai *et al.*, 1989; Bliss and Collingridge, 1993; Huang *et al.*, 1994; Frey *et al.*, 1996). However, the I-LTP, which last up to 4 hours after initial induction (Abraham *et al.*, 1993) is not dependent on *de novo* mRNA synthesis (Otani *et al.*, 1989; Bliss and Collingridge, 1993). This can be suggested to be true for CA3 region as well as DG and CA1. Hence the increase in MAP2 expression is consistent with MAP2 playing a role in LTP maintenance. It can also be suggested that the increase in the immunostaining of MAP2 in dendrites might be attributed to localised dendritic mRNA which regulates the synthesis of MAP2 (Morris, 1997), while the increase in immunostaining of MAP2 in the soma might due to mRNA in the soma.

AC2 and AC4, isoforms of adenylate cycalse, have been shown to be expressed in the hippocampus formation and are co-localised with MAP2 (Baker *et al.*, 1999). AC2 and AC4 have been labelled in the dendrites and cell bodies of DG, CA3 and CA1 (Baker *et al.*, 1999). Hence these two isoforms are likely to be involved in this effect. As mentioned above, elevation of cAMP levels as a result of AC activation results in MAP2 elevation. Hence cAMP and PKA might act on the MAP2 mRNA via an unknown mechanism resulting in the elevation of MAP2 in the three regions of the hippocampus. cAMP and PKA have been shown to be essential for polyadenylation and translational activation of c-mos mRNA (Maller and Krebs, 1998) and they are though to produce their effect via phosphorylation of an unknown target protein. This mechanism of activation might also be applicable to MAP2 mRNA.

6.3.2 A23187

A23187 (5μM) had no significant effect on the staining intensity of MAP2 in both the cell bodies and apical dendrites of hippocampal cultures suggesting that Ca^{2+} might not play a role in the expression of MAP2 (Figures 6.3a and b). While MAP2 mRNA levels have been shown to be elevated following NMDA receptor stimulation (Roberts *et al.*, 1998), via high frequency afferent activity, these results suggest that one of the other signalling pathways recruited by NMDA receptor activation is responsible for MAP2 stimulation. The results with forskolin imply that AC activation would be sufficient to induce MAP2 expression. It may be that there are Ca^{2+}-independent routes to AC activation following high frequency afferent activity. Clearly, Ca^{2+} is not the critical signalling intermediate for increased MAP2.

Western blot analyses were used in these studies to investigate the effect of 5μM A23187 and 50μM forskolin on the expression of MAP2. In mammalian brain there are two isoforms of MAP2; a HMW MAP2a/b, Mr 280kDa and LMW MAP2c, Mr 70kDa. Forskolin appeared to increase the levels of ir HMW MAP2 and LMW MAP2 (Figure 6.4a, upper panel and 6.4b). There was a significant increase in ir HMW MAP2 (216±59% increase) but the increase in ir LMW MAP2 were not of statistical significance. These data support the immunocytochemical analyses, suggesting that an increase in cAMP levels appears to be important in the expression of MAP2 in organotypic cultures. The results did not clearly demonstrate a specific effect on either HMW or LMW MAP2.

6.3.3 LTP

Western blot techniques were also used to investigate the effect of LTP in the CA1 region of the hippocampus. The data indicated a significant increase in the levels of HMW MAP2, after 3.5 hour following tetanus stimuli (Figure 6.9a, upper panel and 6.9b). HMW MAP2 was the only isoform detected since MAP2b is present throughout the development of the nervous system (Sanchez *et al.*, 2000) and MAP2a is mostly expressed in the adult brain (Sanchez *et al.*, 2000). LMW MAP2 is expressed at only the early developmental stages (Sanchez *et al.*, 2000). The animals used in these set of LTP experiments were mice whereas neonatal rats are used to prepare the slice cultures. Since it was earlier shown that there was a similarity in the intracellular pathways regulating MAP2 expression in the three regions of the hippocampus, it can be assumed in this case that this increase in MAP2 might also occur in the CA3 region following high frequency stimulation as well as the DG particularly since CA3 LTP is dependent on elevation of cAMP levels.

Despite the fact that whole slices were used while only the CA1 area was stimulated, the induction of MAP2 by high frequency stimulation was greater in this experiment than the induction when the whole slice culture was bathed in forskolin. This might imply a greater sensitivity of the mechanisms regulating MAP2 expression in the mice, or alternatively, it may suggest that other signalling pathways, other than Ca^{2+} or cAMP, also contribute to MAP2 induction. A study investigating the effect of LTP in the CA1

region in slice cultures, or the effect of forskolin in acute adult slices, would address this question.

6.3.4 Rapamycin

Rapamycin, an immunosupresssant, was used to investigate the regulation of MAP2 expression in the three regions of organotypic hippocampal slices. After pre-treatment of the organotypic slices for 30 minutes with 20nM rapamycin, 50μM of forskolin (or vehicle) was applied for 4 hours to stimulate the slices. Rapamycin reduced basal expression of MAP2 in DG, CA1 and CA3 regions in both soma cells and apical dendrites, however forskolin after rapamycin pre-treatment significantly increased the expression of MAP2 in these regions (Figures 6.5c, 6.6c, 6.7c, 6.8a and b). These increases in the expression of MAP2 were less than the effect of forskolin alone in the absence of rapamycin (Figure 6.8a and b)

Again the results highlight the similarity in the signalling pathways between CA1, DG and the CA3 of hippocampal formation in the presence of rapamycin. At basal levels the translation of MAP2 mRNA seems to follow the rapamycin-sensitive pathway, but after pre-treatment with rapamycin, the forskolin induction of MAP2 expression seems to follow a rapamycin-insensitive pathway. Rapamycin as mentioned in the previous chapter (Chapter 5) inhibits the mammalian target of rapamycin (mTOR). mTOR kinase stimulates the translation of specific mRNAs via phosphorylation of $p70^{S6Kinase}$ ($p70^{S6K}$) (Figure 6.10). At basal levels, cAMP activation of PKA might result in PKA activation of PKB (Atk)(Filippa *et al.*, 1999). PKB might then cause the activation of mTOR (Figure

6.10). Therefore, in both the cell bodies and dendrites of DG, CA3 and CA1 sub-regions, pre-treatment with rapamycin will inhibit the PKA-PKB-mTOR-$p70^{S6K}$ pathway. If this pathway is involved in sustaining the basal expression of MAP2, this might result in the decrease intensity of MAP2 in all the sub-regions of the hippocampus. However, mTOR does not seem to be involved in the forskolin-induced elevation of MAP2 expression. This clearly indicates that distinct signalling pathways are involved in controlling basal and plasticity-regulated expression.

During cAMP elevation via forskolin stimulation of AC, activation of PKA might directly activate $p70^{S6K}$ resulting in increased translation (Cass *et al.*, 1999) and hence increase MAP2 levels in both the cell bodies and dendrites. $p70^{S6K}$ has been suggested to be important in LTP maintenance (Tskos *et al.*, 2002). Therefore, two pathways might be involved in regulating the translation of MAP2 mRNA (Figure 6.10): one involving the PKA-PKB-mTOR-$p70^{S6K}$ and PKA-$p70^{S6K}$ with the former pathway being dominant in the regulation of basal MAP2 and the latter pathway dominant in regulation of MAP2 under activated conditions. PKA has been shown to directly activate $p70^{S6K}$ (Cass *et al.*, 1999).

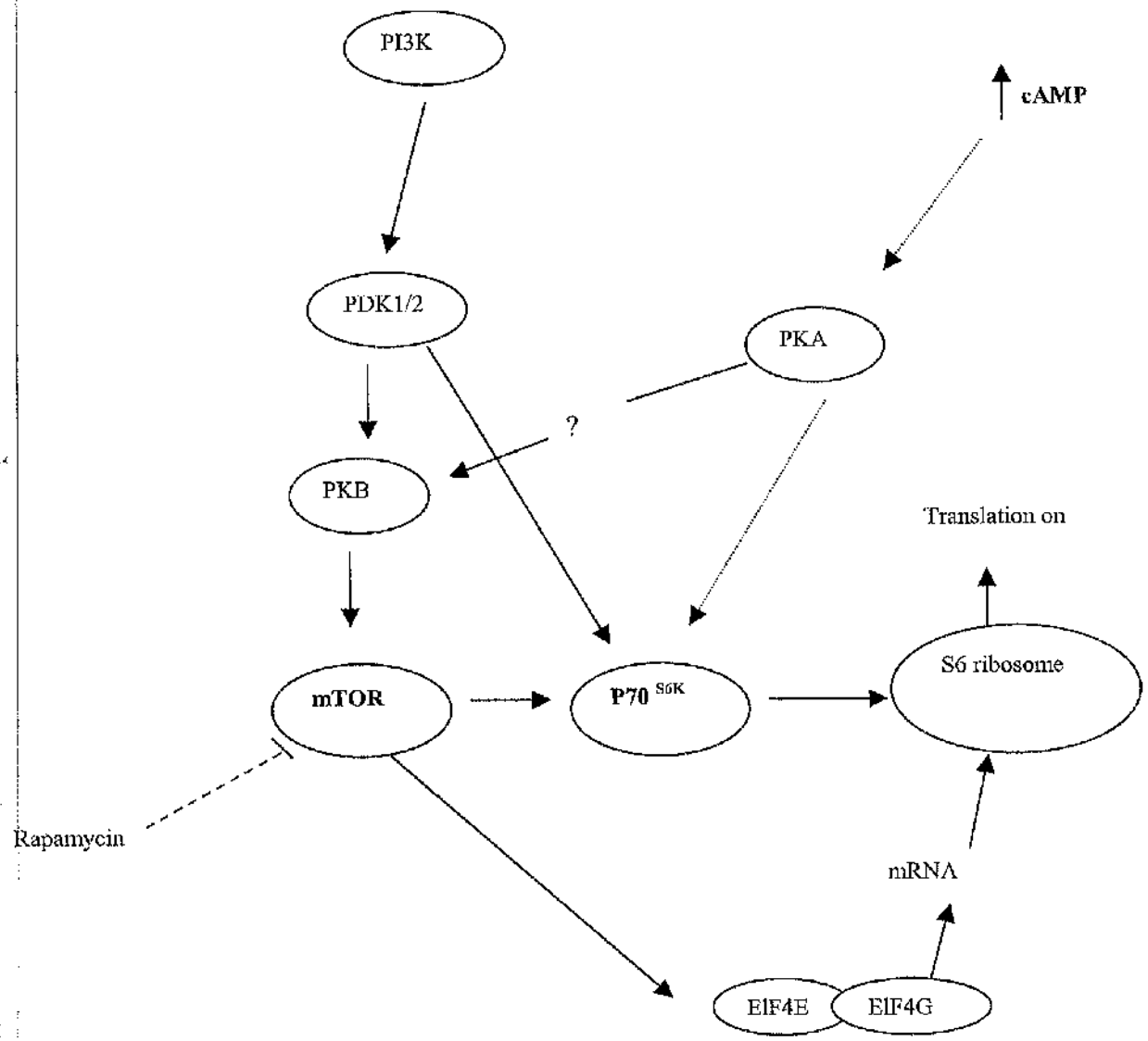
These results suggest that mTOR is not involved in the forskolin–induced elevation in MAP2 expression, despite evidence that rapamycin can reduce the magnitude of LTP (Tang *et al.*, 2002). This may suggest that MAP2 is not one of the key proteins sustaining I-LTP, or alternatively as suggested above, that other pathways apart from Ca^{2+} and

cAMP contribute to the elevation of MAP2 expression during LTP. These hypothetical additional pathways may involve mTOR.

6.3.5 Summary

Even though the mechanism of induction of LTP in CA1 and DG differ from that of CA3, intracellular signalling pathways that lead to the elevation of MAP2 levels appear to be similar in all the three regions of the hippocampus. cAMP elevation plays a more prominent role in the increased levels of MAP2, while Ca^{2+} influx appears to have no effect at least in the organotypic hippocampal cultures. Two pathways seem to determine the expression of MAP2 in organotypic cultures; the rapamycin-sensitive pathway seems to control the basal levels of MAP2 while under activated conditions the rapamycin-insensitive pathway seems to play an important role in increased MAP2 expression.

Figure 6.10: Schematic diagram of the activation of mTOR (FRAP) and its effect on downstream translational factors. mTOR is activated by a number of effectors. The effect of PKB which is activated via PKA might explain the effect of rapamycin inhibition on the basal levels of MAP2. Direct activation of p70 ^{S6K} by PKA might explain the lack of effect of rapamycin on the expression of MAP2 in forskolin-stimulated slices. Abbreviations: PI3K –Phosphoinositol-3-kinase; PDK1/2 –3'-phosphoinositide-dependent protein kinase 1/2; PKB –Protein kinase B; PKA – cAMP-dependent protein kinase.


PI3K
cAMP
PDK1/2
PKA
?
PKB
Translation on
S6 ribosome
mTOR
P70 S6K
Rapamycin
mRNA
EIF4E
EIF4G

Chapter 7

Expression of β-activin

7.1 Introduction

Using substractive hybridisation techniques, the identities of genes that may play a role in activity dependent plasticity in vertebrate brain were determined (Yamagata *et al.*, 1993, 1994). One of the cDNAs identified in differential screen of stimulated rat hippocampus demonstrated close similarity to the terminal 3' sequence of human β-A activin. Studies have also shown that activin β-A mRNA are elevated in the DG during excitatory synaptic input such as high frequency stimulation (HFS) which induces LTP (Andreasson and Worley, 1995; Inokuchi *et al.*, 1996) and this elevation is dependent on NMDA receptor activation. Therefore, activin plays a role in the maintenance of neural plasticity (via enhancement of neuronal survival) in the adult rat brain (Inokuchi *et al.*, 1996) and developmental neuroplasticity (Andreasson and Worley, 1995). The I-LTP lasting 4 hours (Abraham *et al.*, 1991) is dependent on *de novo* protein synthesis and will be of interest to find out if activin β-A expression is elevated at this phase.

In this study, the effect of Ca^{2+} influx and cAMP elevation was investigated in the CA1 and CA3 sub-region of the organotypic hippocampal slice cultures. Elevation of Ca^{2+} and cAMP as already mentioned are major consequences of NMDA receptor activation with Ca^{2+} entering via the activated NMDA receptor and cAMP activated by adenylate cyclase (AC).

7.2 Method

The methods used in general were as described (Chapter 2) with the following modification:

After incubation of organotypic slice cultures, for 4-5 days, the slices were then stimulated for 4 hours with vehicle or A23187 and vehicle or forskolin. The slices were then fixed followed by addition of 3% H_2O_2 (to remove the endogenous peroxidase activity) for ten minutes. After 10 minutes the slices were washed in PBS and the process of immunocytochemistry started (please refer to section 2.7).

7.3 Results

7.3.1 A23187 and forskolin increase the expression of activin β-A in organotypic slice preparations.

The activin/inhibin antibody, which recognises the β-A subunit of both activin and inhibin, was used to stain all the sub-regions of the organotypic slice cultures where the staining was prominent in the CA1 and CA3 regions (Figures 7.1, 7.2 and 7.3). Application of A23187 (5μM) for 4 hours resulted in significant elevation of the staining intensity of activin β-A (the intensity of staining was measured after 4 hours). This

significant increase in staining was in the CA1 (*P<0.05 versus the vehicle treated slices, 142 ±4% of vehicle, n=4)(Figure 7.3c and 7.4) and CA3 (*P<0.05 versus the vehicle treated slices, 123.7±11.9% of vehicle, n=4)(Figure 7.3d, 7.4) of the organotypic slice cultures. The data in this section represents both the cell bodies and apical dendrites of each of the regions investigated i.e. CA1 and CA3.

Forskolin (50μM) produced a significant increase in the staining intensity of activin β-A when applied for 4 hours prior to fixation. This significant increase in activin β-A was intense in the CA1 (*P<0.05 versus vehicle treated slices, 113±5% of vehicle, n=7)(Figure 7.2c, 7.4) and CA3 (*P<0.05 versus vehicle treated slices, 123±6% of vehicle)(Figure 7.2d, 7.4). As with the A23187 data, the data for this study represents both the cell bodies and dendrites of the regions investigated. The significance was determined using the Wilcoxon rank test of the median with the data considered significant when the P<0.05.

The increase in expression of activin β-A after treatment with A23187 was significantly greater than that detected in forskolin-treated slices. However, this was the case only in the CA1 region of the hippocampus (~P<0.05 versus forskolin-treated slices, one way ANOVA with *post hoc* Fisher's test, Figure 7.4), there was no significant difference between the increase in A23187-treated cultured slices and forskolin-treated cultured slices in the CA3 region of the organotypic hippocampal slice culture.

Figure 7.1: Image of an organotypic slice culture. The hippocampal slice was cultured 4-5 days prior to staining with an antibody for the β-A subunit of activin and inhibin protein. The antibody stained specifically the three sub-regions of the hippocampus i.e. DG, CA3 and CA1. Scale bar:0.4mm

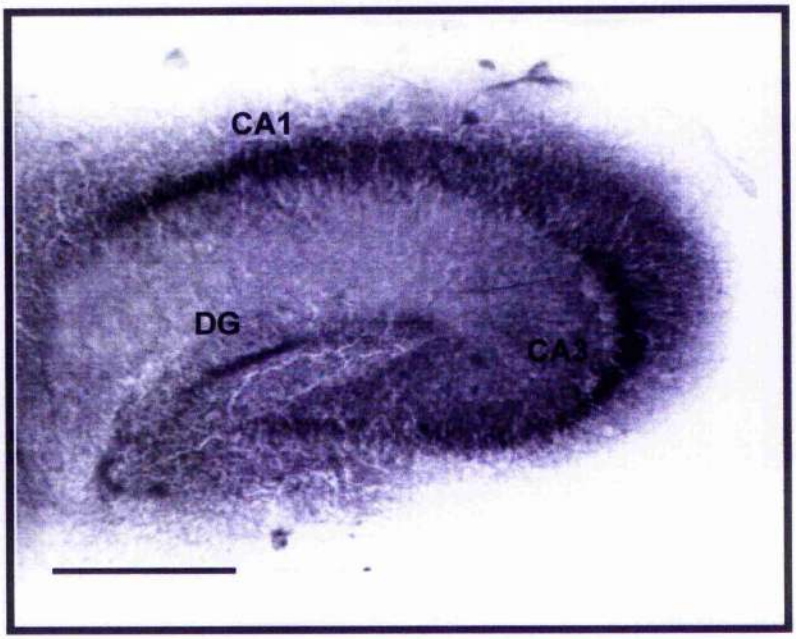

CA1
DG
CA3

Figure 7.2: Effect of 50μM forskolin on the staining intensity of β-activin in organotypic slice cultures. The cultured neurons in the CA1 and CA3 sub-regions were stained with an antibody for β-activin following 4 hour stimulation in vehicle (a, b) and forskolin (c, d). Note the increase in staining in the CA1 (c) and CA3 (d) in the presence of forskolin compare to the vehicle (a, CA1 and b, CA3). Scale bar: 100μm. Abbreviations: SO- Stratum Oriens; SP- Stratum Pyramidal; SR- Stratum Radiatum.

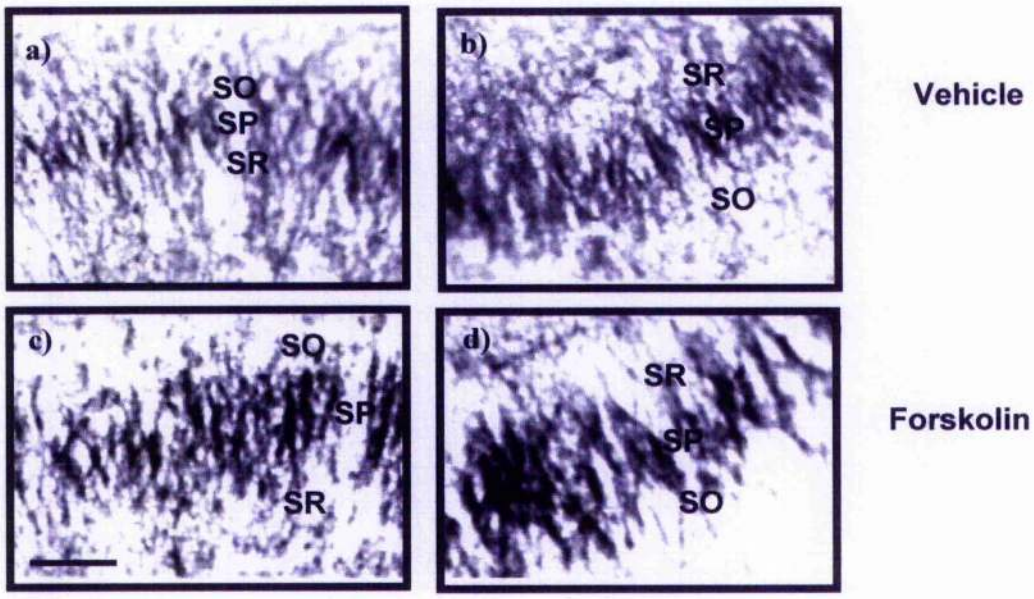

a)
SO
SP
SR
b)
SR
SP
SO
Vehicle
c)
SO
SP
SR
d)
SR
SP
SO
Forskolin
CA1
CA3

Figure 7.3: Effect of 5μM A23187 on the staining intensity of β-activin in organotypic slice cultures. The cultured neurons in the CA1 and CA3 sub-regions were stained with an antibody for β-activin following 4 hour stimulation in vehicle (a, b) and A23187 (c, d). Note the increase in staining in the CA1 (c) and CA3 (d) in the presence of A23187 compare to the vehicle (a, CA1 and b, CA3). Scale bar: 100μm. Abbreviations: SO- Stratum Oriens; SP- Stratum Pyramidal; SR- Stratum Radiatum.

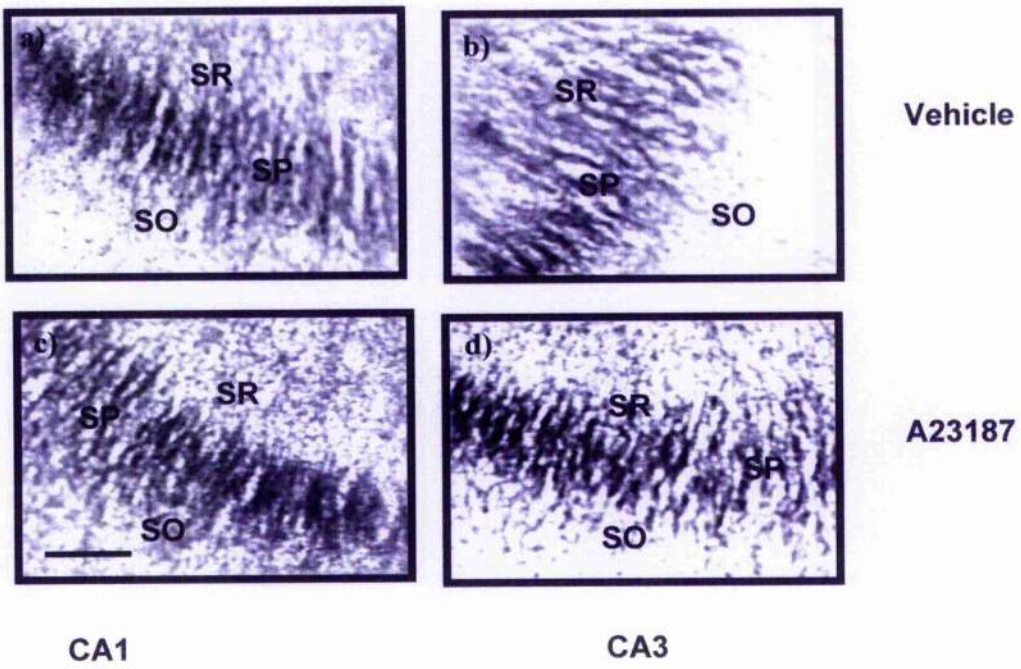
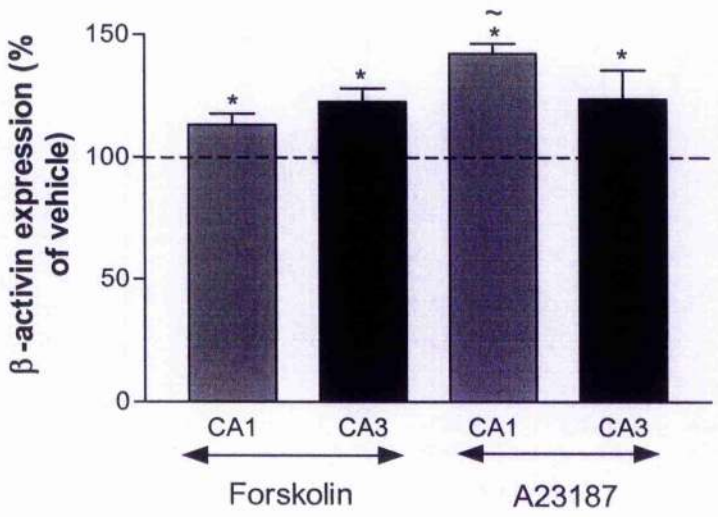

a)
SR
SP
SO
b)
SR
SP
SO
Vehicle
c)
SP
SR
SO
d)
SR
SR
SO
A23187
CA1
CA3

Figure 7.4: Effect of forskolin (50μM) and A23187 (5μM) on the expression of ir β-activin in CA1 and CA3 sub-regions of organotypic hippocampal slices cultures. Immunocytochemistry analysis using anti-activin/inhibin antiserum indicated a significant increase (*P<0.05 versus vehicle treatment in the same region, one-sample Wilcoxon signed rank test for the median) in the staining intensity of activin/inhibin in the presence of A23187 and forskolin in CA1 and CA3 sub-region of the hippocampal neurons. A23187 increased significantly the intensity of staining of activin/inhibin in the CA1 sub-region when compared to forskolin-treated slices (~P<0.05 versus forskolin-treated slices in the same region using one way ANOVA with post hoc Fisher's test).


β -activin expression (% of vehicle)
150
100
50
0
*
*
~
*
*
CA1
CA3
CA1
CA3
Forskolin
A23187

7.4 Discussion

The activin/inhibin β-A antibody used in this study had been previously characterised (Otani *et al.*, 1998) where it was used in the immunohistochemical localisation of activin A in human endometrial tissues during the menstrual cycle and early pregnancy. The antibody recognises the β-A subunit of both activin and inhibin proteins; however inhibin levels have been shown to be up-regulated under ischemic insult (Soriano *et al.*, 2000) hence inhibin is only expressed during pathological responses.

7.4.1 A23187

In these studies, application of the Ca^{2+} ionophore (5μM A23187) caused a significant increase in the staining intensity of activin β-A subunit in the CA1 and CA3 sub-regions of organotypic slice cultures. These immunocytochemical studies suggest that increases in the levels of intracellular Ca^{2+} via calcium entry through the Ca^{2+} pores are important in the elevation of activin β-A subunit. Ca^{2+} influx via NMDA receptors has been shown to elevate the expression of activin β-A mRNA in the DG (Inokuchi *et al.*, 1996). Therefore in these studies, under physiological conditions Ca^{2+} entry via NMDA receptors, at least in CA1, might cause the elevation of activin β-A. LTP induction in the CA3 region is NMDA receptor independent, but pre-synaptic Ca^{2+} influx has been shown to be important in MF LTP. Hence in these studies an increase in intracellular Ca^{2+} in the pre-synapse might cause the elevation of activin β-A levels post-synaptically, via an activation of a process, most likely involving Ca^{2+}/calmodulin-regulated adenylate

cyclase (AC) resulting in the enhancement of evoked transmitter release (Nicoll and Malenka, 1995). This could then increase the levels of cAMP resulting in the activation of PKA-mediated mechanisms (Weisskopf *et al.*, 1994). Thus, the A23187-induced increase in the activation of β-A activin expression in the CA3 region could reflect activation of PKA-dependent processes. However, it is more likely to reflect a direct action of Ca^{2+} influx independent of PKA (General Discussion, section 8.3).

7.4.2 Forskolin

In the presence of the AC activator (50μM forskolin), there was a significant increase in the levels of activin β-A subunit in both CA1 and CA3 regions of the organotypic slice cultures. This again shows the importance of elevation of cAMP, via AC activation, on the increased staining intensity of activin β-A subunit. In the CA1 region, activation of type 1 AC might result in the elevation of cAMP in the post-synapse, the cAMP then activates PKA. The cAMP and PKA might act on the activin β-A mRNA via an unknown mechanism resulting in the elevation of activin β-A levels. Also, the increased cAMP levels due to forskolin might activated the same pathway as the one which results from Ca^{2+} influx i.e. Ca^{2+}/AC/cAMP/PKA mediated pathway.

The increase in the activin β-A subunit immunostaining in the CA1 region was greater in the presence of elevated intracellular calcium levels than following elevation of cAMP levels. This might suggest that Ca^{2+} elevation is more potent in activation of activin β-A subunit than cAMP. However, dose-response studies have not been conducted, hence it is

not known if either A23187 or forskolin effects are maximal – only single concentrations were tested for each drug. Alternatively, Ca^{2+} might be acting independent of activation of AC1 and that this effect is more powerful. These data also indicated that the intracellular signalling in the CA1 and CA3 were similar when the organotypic slice cultures were stimulated by either increased intracellular Ca^{2+} or increase cAMP levels.

Studies have recently shown that the levels of activin β-A mRNA are rapidly and transiently induced in neurons of adult rat brain by excitatory synaptic input (Andreasson and Worley, 1995). In fact, activin β-A mRNA was elevated in the granule cells of the DG (Andreasson and Worley, 1995; Inokuchi *et al.*, 1996). Since there was an increase in the staining intensity of activin β-A in the CA1 and CA3 in this study, it will be logical to assume that these increases are a result of increases in mRNA levels in the CA1 and CA3 similar to those in the DG. This also indicates for the first time that the induction of activin β-A also occurs in the CA1 and CA3.

The organotypic slice cultures were stimulated for 4 hours, which, is time point broadly corresponding to LTP2 (Abraham *et al.*, 1991). LTP2 has been shown to be dependent on *de novo* protein synthesis (Otani *et al.*, 1989; Bliss and Collingridge, 1993). Hence, in this investigation the expression of activin β-A might play a role in the maintenance of LTP in the CA1 and CA3 regions of the hippocampus. In fact, activin β-A has been suggested to play a role in maintenance of LTP in the DG (Inokuchi *et al.*, 1996) and this maintenance is dependent on NMDA receptor activation. Since LTP induction in the CA1 region is NMDA receptor dependent, it can be assumed that activin β-A has a role

in LTP maintenance via NMDA receptor activation while in the CA3 region, activin β-A role in LTP maintenance might be via NMDA receptor independent. It can also be suggested that the increased levels of β-activin protein is a result of the presence of pre-transcribed β-activin mRNAs since LTP2 is independent of transcription. However, this remains to be investigated and the presence of activin mRNA has never been reported.

7.4.3 Summary

In conclusion, activation of activin β-A in the CA1 and CA3 sub-regions of organotypic slice culture are affected by elevation of Ca^{2+} and cAMP. These elevations result in an increase in the expression of activin β-A in both regions of the hippocampus. Thus activin β-A expression can be elevated by an appropriate stimulus in all three regions of the hippocampus. This may suggest that increased activin β-A expression plays a fundamental role in the process of synaptic plasticity, irrespective of the nature of the stimulus that triggers the plasticity.

Chapter 8

General Discussion

8.1 Aims of the Study

The aim of the thesis was to investigate *in vitro* the mechanisms regulating the activation or expression of the following synaptic related proteins in the rat hippocampal slices:

(i) Pyk2 is non-receptor tyrosine kinase which has been shown to cause the activation src which then results in induction of LTP (Huang *et al.*, 2001),

(ii) Src is also a non-receptor tyrosine kinase which induces LTP via potentiation of NMDARs (Huang *et al.*, 2001),

(iii) MAPK plays a critical role in LTP induction via activation of CREB resulting in increase protein synthesis (Kanterewicz *et al.*, 2000),

(iv) αCaMKII is necessary and sufficient for LTP induction, and can, by itself, enhance the efficacy of synaptic transmission (Lisman *et al.*, 2002),

(v) MAP2 is important in prolonged structural changes that are associated with LTP (Sanchez *et al.*, 2000),

(vi) β-activin activation is a result of LTP induction (Inokuchi *et al.*, 1996).

Hence, these proteins have all been implicated in the induction and/or maintenance of LTP in the hippocampus. Two major effectors of NMDA receptor activation, Ca^{2+} and cAMP, are likely to play a major role in the activation of these plasticity-related proteins. Although the elevation of activation or expression of these proteins has been studied extensively in the DG and CA1 prior to this study, little is known about the regulation of

the activation or expression of these proteins in the CA3 region of the hippocampus. The present study provides insight into the regulation of these proteins in CA3 region.

In this study both organotypic hippocampal slice cultures and acute hippocampal slices were employed. The advantages and disadvantages of each technique are listed in table 8.1.

8.2 Effect of A23187 and Forskolin on hippocampus

Forskolin at 50μM concentration has been shown to induce both early and late phases of LTP in the CA3 region, with synaptic efficiency remaining elevated for up to 6 hours (Huang *et al.*, 1994). Forskolin also induces LTP in the CA1 region at this concentration (Huang *et al.*, 1994). However, reportedly only the late phase can be induced by forskolin in the CA1 region (Huang *et al.*, 1994). Forskolin was shown to completely occlude electrical stimulation induction of LTP in the CA3 region, and the action of forskolin was greatly reduced after LTP induced by electrical stimulation (Weisskopf *et al.*, 1994). This suggested that after LTP the action of forskolin and MF LTP interact in acute hippocampal slices. It should be noted that 50μM forskolin does not have any toxic effect as shown by long-term viability of slices after this treatment (Weisskopf *et al.*, 1994; Huang *et al.*, 1994).

A23187 at concentrations of 1-7μM has been widely used to increase the intracellular concentration of Ca^{2+} in cultured cells and tissue slices (Lev *et al.*, 1995). There have been no studies to investigate the effect of A23187 on LTP induction in the hippocampus,

however Cobb and coworkers (unpublished observations) showed depolarisation in the CA1 region of the hippocampus on the addition of A23187. At low concentrations (<7μM), A23187 does not have any toxic effects (Lev *et al.*, 1995; Siciliano *et al.*, 1996). In this study the exposure of organotypic slice cultures to A23187 (5μM) for 4 hours did not affect cell viability as assessed by dendritic MAP2 staining. Therefore the effects of A23187 reported in this thesis are likely to be related to physiological and not pathological responses.

In the CA1 and DG, induction of LTP resulted in the increase influx of Ca^{2+} via activated NMDA receptors (Bliss and Collingridge, 1993) while induction of LTP in CA3 resulted from elevation of cAMP (Weisskopf *et al.*, 1994). For most of the proteins monitored in this study, the mechanisms downstream of these triggers were shown to be similar in CA3, DG and CA1 regions.

8.3 PyK2 phosphorylation

In this study Pyk2 activation, as a result of the elevation of intracellular Ca^{2+} was achieved in both the CA1 and CA3 regions of the hippocampus. This activation of Pyk2 was probably mediated by PKMξ since the activation was blocked by a PKM selective inhibitor, chelerythrine. Increases in intracellular Ca^{2+} have been shown to activate PKC which in turn activates Pyk2 in the hippocampus (Siciliano *et al.*, 1996). Therefore in this study Pyk2 activation in the CA3 region of hippocampus might be achieved via a Ca^{2+} proteolytic activation of PKC resulting in a catalytic fragment, PKMξ. Another might be

that presynaptic Ca^{2+} might play a role, this however, will not involve PKA activation since cAMP was shown to reduce Pyk2 phosphorylation. Since the intracellular signalling leading to Pyk2 activation is similar in both CA1 and CA3, it can be suggested that Pyk2 is involved in the signalling events leading to synaptic plasticity in all regions of the hippocampus.

There was no success with the investigation of phosphorylated Src, because there were problems with obtaining specific staining with the antibody. Thus the probability of an association between Pyk2 and total Src was investigated as an alternative approach. An association between Pyk2 and Src was established, since Src was immunoprecipitated along with Pyk2, the presence of Src suggested an association between the two. However, there was no increase in the association between Pyk2 and after stimulation, hence it raises the possibility that there is no Src activation by Pyk2 after Ca^{2+} influx alone, and that some other signalling pathway activates Src. Most of the studies investigated the Pyk2-Src up-regulation of NMDARs in LTP (Ali and Salter, 2001) and it is worth mentioning at this point that direct contribution of Pyk2 and Src in LTP has not been tested in this study. In fact, direct involvement of the plasticity-related proteins in this study in LTP has not been monitored.

8.3 MAPK Phosphorylation

MAPK activation differed in CA1 and CA3 regions of the hippocampus. Elevation of intracellular Ca^{2+} or cAMP resulted in the activation of MAPK in CA1 with no effect in

CA3. This suggests that there is a differential intracellular signalling in the two regions when p42 MAPK activation is involved. Therefore, Ca^{2+} influx and cAMP elevation in the CA1 region might involve the Ca^{2+}/calmodulin dependent AC1 and might be essential for p42 MAPK activation while in the CA3 region, Ca^{2+} and cAMP are not essential for p42 MAPK activation. So, although cAMP elevation and pre-synaptic Ca^{2+} influx are essential for LTP in the CA3 region (Weisskopf *et al.*, 1994), activation of p42 MAPK might not be necessary for LTP in the CA3 region. Kanterewicz *et al.* (2000) showed that MF LTP in area CA3 did not require MAPK signalling and suggested that cAMP/PKA/Rap1/B-Raf/MEK pathway was involved in the activation of MAPK in the CA1 region. Therefore, this data compliments work done by Kanterewicz *et al.* (2000), demonstrating that forskolin, which has been shown by many groups to induce LTP at the MF-CA3 synapses, fails to activate MAPK.

8.4 Expression of αCaMKII, MAP2 and β-activin

Despite a difference in signalling (MAPK data), there were no differences in the protein induction detected (αCaMKII, MAP2 and β-activin) between the CA3, DG and CA1 regions. However, the data implicated differential expression of αCaMKII and MAP2 with increased Ca^{2+} influx playing an important role in the expression of αCaMKII and the importance of elevated cAMP levels in the expression of MAP2. The expression of β-activin was increased as a result of either an increase in intracellular Ca^{2+} or cAMP elevation.

8.4.1 αCaMKII

A possible mechanism of activation of αCaMKII in the CA3 region might involve the pre-synaptic influx of Ca^{2+} which results in increased glutamate release via Ca^{2+}/calmodulin dependent AC1. However, this might not be the case since cAMP elevations had no effect on αCaMKII expression. Since forskolin can induce elevated protein synthesis-dependent LTP in the CA3 region (Huang *et al.*, 1994), this might suggest that αCaMKII expression is not a key component of LTP2. Alternatively, while it is clear that 50μM forskolin induces LTP in acutely prepared hippocampal slices, this has not been shown for slice cultures. It is conceivable that MF LTP is not induced in these studies, and so a role for αCaMKII in LTP2 cannot be ruled out. However, the results clearly demonstrate that Ca^{2+} influx is more important than cAMP for the regulation of αCaMKII at this time point. While the possibility was not tested in this study, it is conceivable that Pyk2 is involved in the pathways leading to increased αCaMKII expression. A23187, but not forskolin, activated Pyk2 in both CA1 and CA3 regions, and a similar pattern was noted for the induction of αCaMKII. It would be of interest to test the ability of chelerythrine to attenuate the increased expression of αCaMKII after exposure to A23187.

8.4.2 MAP2

The mechanism of activation of MAP2 might involve increased activation of PKA. MAP2 has been shown to be co-localised with AC2 and AC4 in both dendrites and cell

bodies of the hippocampus (Baker *et al.*, 1999). Both Ca^{2+}/calmodulin independent AC2 and AC4 have been linked to certain forms of hippocampal synaptic plasticity (Baker *et al.*, 1999). AC2 is thought to integrate inputs from multiple signalling pathways, while AC4 can act as a coincidence detector of paired Gs and Gi inputs. Hence, it is possible that there exist two (or more) parallel pathways involving cAMP that encode different forms of synaptic plasticity. MAP2, in this case, might be affected by these two types of adenylate cyclases in all the three regions of the hippocampus.

8.4.3 β-activin

Expression of β-activin might involve two independent processes. In the CA3 region, increased pre-synaptic Ca^{2+} influx results in increased transmitter release via activation of AC1. Therefore, in CA3 region, A23187 should always produce same effects as forskolin. However, that is not the case in MAP2 and also as already mentioned above A23187 has not been shown to elevate cAMP levels i.e. increased intracellular Ca^{2+} as result of the ionophore has not been shown to activate AC1 either presynaptically or postsynaptically.

8.4.4 mTOR Kinase and expressions of αCaMKII and MAP2

This study also investigated the affect of mTOR kinase inhibitor on the translation of αCaMKII and MAP2 mRNAs. Rapamycin had no effect on the expression of αCaMKII

or MAP2 (in activated hippocampal slices) in both dendrites and cell bodies of all regions of the hippocampus. Hence, the data suggests that translation of αCaMKII and MAP2 (in activated hippocampal slices) follows a rapamycin-insensitive pathway i.e. mTOR kinase does not play a role in the translation of these two genes. However, basal levels of MAP2 were reduced in both dendrites and cell bodies in the presence of rapamycin, suggesting a rapamycin-sensitive pathway. The effect of rapamycin was similar in all three regions of the hippocampus, thereby re-enforcing the idea that the CA3 intracellular signalling pathway for induction of both αCaMKII and MAP2 is similar to those in CA1 and DG.

8.5 Conclusion

The results of this study indicate that the factors regulating the expression of αCaMKII, MAP2 and β-activin and the phosphorylation of Pyk2 in the CA3 region are similar to those in the intensely studied DG and CA1 regions. However these increases in protein expression/phosphorylation are differentially activated by the second messengers, Ca^{2+} and cAMP (table 8.2). Thus the $Ca^{2+}$and cAMP-dependent pathways activated during synaptic plasticity may serve distinct functions by regulating discrete proteins during the various temporal phases of the response to stimulation. However, MAPK phosphorylation in the CA3 region was not affected by the modulation of Ca^{2+} and cAMP. Hence, it will be of interest to investigate further the stimuli necessary for MAPK activation in the CA3 region.

This general similarity in intracellular signalling is surprising, considering the distinct mechanisms involved in triggering LTP in the CA3 region. However, these are some of the first investigations of proteins changes in the CA3 region after plasticity-related stimulation. It seems likely that a great deal of commonality will be found in the mechanisms of late phase LTP in these regions.

	Advantages	Disadvantages
Organotypic slice cultures	• Tissue-specific cell connection • Preservation of local neuronal circuits with appropriate innervation pattern • Easy application of drugs • Applicable to studies lasting a number of days • Used in studies lasting days	• Slice viability, slices were necrotic after few days. • Enormous variation in terms of cell number and tissue structure • Large number of slices required for statistical significance • Organotypic cultures do not reflect *in vivo* physiology
Acute hippocampal slices	• Neurons, their synapses and part of the circuitry in which they are involved are well preserved and viable and in mature state, so that interpretation of results is directly applicable to the adult brain • Thickness of the slice, allows efficient entry of most pharmacological reagents • Amount of nervous tissue allows for quantitative biochemistry	• Not useful for time periods in excess of 4 hours due to slice deterioration

Table 8.1 List of advantages and disadvantages of both organotypic slices and acute hippocampal slices

	Ca^{2+} ionophore (A23187)				PKA activation (forskolin)				LTP
	hippo	DG	**CA3**	CA1	hippo	DG	**CA3**	CA1	**CA1**
Pyk2 activation		—				—	—	—	—
MAPK activation		—	**NE**			—	**NE**		—
αCaMKII expression					NE	NE	NE	NE	
MAP2 expression	NE	NE	NE	NE					
β-activin expression	—	—			—	—			—

Table 8.2 Summary of the effects of A23187 and forskolin on the activation/expression of plasticity-related proteins. The effects were investigated in both acute (Pyk2 and MAPK activation) and organotypic (αCaMKII, MAP2 and β-activin expression) hippocampal slices. Abbreviations: hippo- hippocampus; NE- no effect

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Appendix

Methods for long term potentiation experiments.

(Author: Kara McNair)

Male ICR mice (5-10wks) were deeply anaesthetised with 100mg/kg intra-peritoneal (i.p.) injection of urethane in sterile saline until paw withdrawal reflexes were completely abolished. Cardiac perfusion was carried out by insertion of a 19G needle into the left ventricle and perforating the right atrium with sharp dissecting scissors. Approximately 60mls of oxygenated (95% O2, 5% CO2), ice cold (2-4°C), modified artificial cerebrospinal fluid (mACSF) of the following composition (in mM) NaCl 87, KCl 2.5, $NaHCO_3$ 25, $MgSO_4$ 0.7, NaH_2PO_4 1.25, D-Glucose 25, Sucrose 75 and $CaCl_2$ 0.5, pH 7.4 – 7.5 was perfused through the heart. Low NaCl containing ACSF was used to reduce passive chloride entry which has previously been suggested to be actively responsible for neurotoxicity during slice preparation. For details regarding the use of the above mACSF see (Mellor & Nicol, 2001; Rasmussen & Aghajanian, 1989). All the above procedures were carried out in accordance with current Home Office legislation. The brain was then rapidly excised and placed in fresh, ice cold, oxygenated mACSF. Horizontal brain slices (400μM thick) were prepared using a VT-1000s vibrating microtome (Leica, Cambridge, UK). Throughout the slicing procedure the brain and slices were held in a chamber containing ice cold mACSF. They were then transferred to a petri dish also containing ice cold mACSF where the hippocampus was dissected free from surrounding brain tissue

and a cut placed through the CA3 region to prevent the occurrence of epileptiform activity. The hippocampal slices were initially stored at 35°C for 30 minutes in approximately 135mls of mACSF, then transferred to room temperature. The mACSF solution was gradually replaced (15mls every 15 minutes) with standard ACSF (sACSF) of the following composition (in mM): NaCl 124, KCl 3, $NaHCO_3$ 26, NaH_2PO_4 1.25, $MgSO_4$ 1, D-Glucose 10 and $CaCl_2$ 2 (pH 7.4 – 7.5). Following complete changeover of ACSF solutions, individual hippocampal slices were held in a submerged recording chamber for 1 hour prior to electrophysiological recordings. The slices were constantly perfused (flow rate 1-2ml/min) with oxygenated, sACSF by means of a gravity fed system. They were maintained at a temperature of 28°C using a TC-20 temperature controller (Npi Electronics, Tamm, Germany).

Hippocampal slices were simultaneously stimulated with two bipolar stimulating electrodes (Nickel80/ chromium20 wire, 50μM thick). Placed field excitatory post synaptic potentials (EPSPs) were recorded using a standard walled glass electrode filled with sACSF. The microelectrodes were pulled on a horizontal Flaming-Brown P-87 micropipette puller (Sutter Instruments Co., USA). Recording electrodes were mounted in electrode holders inserted into a digitimer head stage. The recording chamber and micromanipulators were supported on a steel plate which was itself mounted on an anti-vibration table (Wentworth Laboratories, UK). The recording chamber was a submerged chamber designed in house (IBLS workshop, University of Glasgow) into which a temperature probe (Npi electronic, Tamm, Germany) and a silver/ silver chloride

reference electrode was placed. A rigid plastic mesh was also placed within the recording chamber onto which the slice was placed. All stimulating and recording electrodes were mounted on micromanipulators (Narishige, Japan). To enable the positioning of electrodes in the slice, the slice was viewed using an OPMI1 Zeiss overhead, dissecting microscope.

Synaptic potentials were initially amplified 100 fold using a Neurolog system (Digitimer, England, UK). Secondary 20-fold amplification was carried out using a Brownlee Amplifier (Model 440, Brownlee Precision, CA, USA). Signals above 5kHz were filtered using a low pass filter. Extraneous 50Hz noise was removed using a Hum Bug device (Digitimer, England, UK). Signals were digitised using a Pico42 A-D (analogue – digital) converter and captured on a Viglen Pentium III P.C. using the Bristol LTP software (www.ltp-program.com; Anderson & Collingridge, 2001).

Stimulation of the CA1 pyramidal cell apical dendritic layer (*stratum radiatum*) was carried out using an isolated constant current stimulator (model D52A, Digitimer, UK) (0 – 32mA). The stimulation strength was gradually increased to produce and input/ output curve (I/O curve) measuring EPSP slope output against current input. The current that produced 50% of the maximum EPSP slope was subsequently used in the remainder of the experiment.

The hippocampal slices were stimulated once every 30 seconds and a 30 minute stable baseline recording was obtained. Following this, three tetanic stimulations (100hz, 0.2ms

pulse width, 10 minute intertrain interval) were delivered which induced a potentiation of the field EPSP slope. Stimulations then returned to one every 30 seconds for the duration of the recordings. Long term potentiation of responses was recorded for approximately 2.5 to 3.5 hours. Control slices were maintained in a holding chamber for the corresponding length of time. At the end of the recording, slices were removed and placed on an ice cold microscope slide, where the CA1 region was dissected. This region was then snap frozen in liquid nitrogen and stored at –80°CS for further analysis. Data are expressed as a percentage of baseline EPSP slope response and presented as a mean ± S.E.M. Data was collected using LTP software (Anderson and Collingridge, 2001), and analysed using Microsoft Excel and MicroCal Origin packages. N values refer to the number of times a particular experiment was performed, each in a different slice taken from a different animal.

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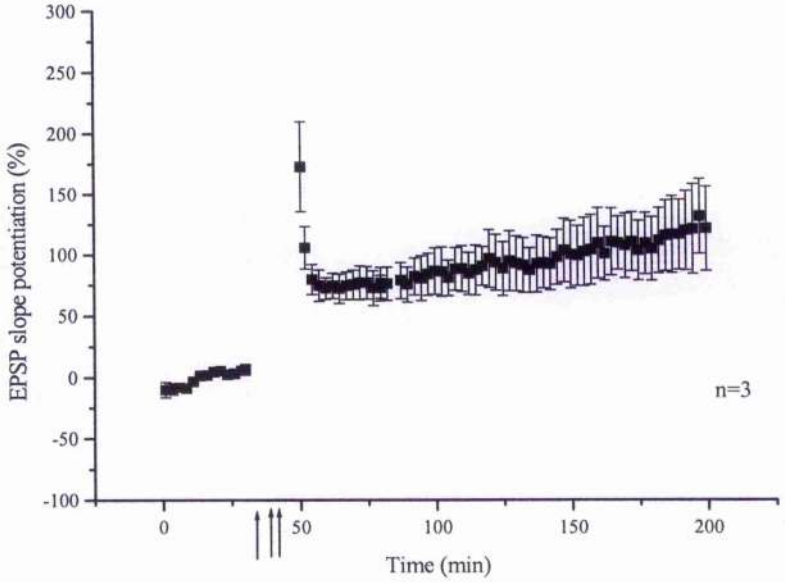

Figure 1: Induction of LTP in the CA1 region of the hippocampus following 3 tetanic stimulations of 100Hz (three arrows). Note the increase in the excitatiory post-syanptic potentiation (e.p.s.p.) after application of tetanus. The potentiation remained elevated for 2.5 hours after LTP induction.

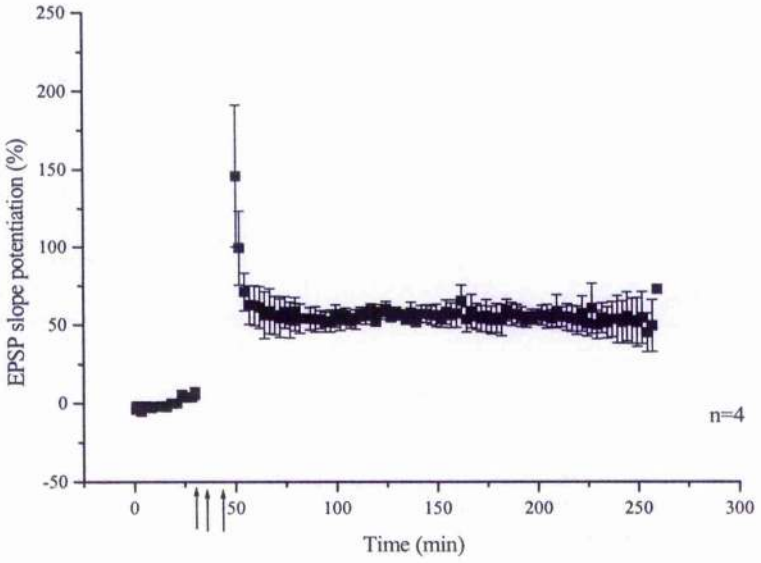

Figure 2: Induction of LTP in the CA1 region of the hippocampus following 3 tetanic stimulations of 100Hz (three arrows). Note the increase in e.p.s.p. after application of tetanus. The potentiation remained elevated for 3.5 hours after LTP induction.