MODULATORY MECHANISMS INVOLVED IN THE NEURAL PROCESSES OF LEARNING AND MEMORY IN THE RODENT HIPPOCAMPUS

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II. Declaration

In accordance with Regulation 3.8.7 of the University of Edinburgh, I declare that this thesis has been composed by myself and all the work is my own except certain results which were obtained in conjunction with Ben Davies Ph.D., Robin A. Morton Ph.D., and Urania Coumis B.Sc.

Ian R. Kearns

III. Abstract

The aim of this investigation was to examine the mechanisms implicated in the processes of learning and memory in the rodent hippocampus using electrophysiological recording techniques to monitor CA1 pyramidal cell activity. In the presence of ionotropic glutamate and γ-aminobutyric acid (GABA) receptor antagonists, stimulation of the septohippocampal cholinergic afferents, in *stratum oriens*, produced a slow excitatory postsynaptic potential (EPSP). Subsequent pharmacological classification established that this response was mediated by activation of muscarinic acetylcholine receptors (mAChR) and as such this response was termed an $EPSP_M$.

Specific blockers of voltage-gated Ca^{2+} channels, ω-conotoxin GVIA and ω-agatoxin IVA, revealed that the release of ACh necessary to evoke the $EPSP_M$ was mediated by activation of both N- and P/Q-type Ca^{2+} channels. Blockade of presynaptic 4-AP-sensitive K^+ channels further enhanced the release of ACh.

A previous report had shown that mAChR-mediated synaptic transmission could be modulated by adenosine A_1 receptor activation but had not examined the precise cellular mechanisms underlying this effect. Investigations have revealed that the activation of A_1 receptors inhibited the $EPSP_M$ irrespective of the Ca^{2+} channel supporting this response and that there maybe a partial involvement of 4-AP sensitive K^+ channels. As adenosine A_1 receptors are known to act via the G-protein, $G_{i/o}$, we also investigated the involvement of cAMP in the inhibition of the $EPSP_M$. It was demonstrated that forskolin stimulated increases in cAMP partially occluded and 8-Br cAMP application fully occluded the adenosine A_1 receptor-mediated inhibition of the $EPSP_M$.

Most synapses are under the regulation of a variety of GPCRs. In this respect we also demonstrated that opioid receptor agonists could modulate the $EPSP_M$. Interestingly, it was found that opioid agonists acting at a presynaptic μ-opioid receptor caused an enhancement of the $EPSP_M$ although the mechanism of how this is achieved is unclear.

Another G-protein coupled receptor implicated in cholinergic modulation is the galanin receptor which has been shown previously, and again here, to produce an inhibition of the $EPSP_M$.

This inhibition was absent in transgenic mice lacking the galanin gene. This result may have predicted that these mice might learn better. However the opposite was true. Therefore, we chose to study whether LTP in these animals was impaired since LTP represents a working model of molecular memory at synapses. Consistent with their impaired learning ability it was found that LTP induced at synapses in the *stratum oriens* was decreased in the $Gal^{-/-}$ mice.

To establish whether other proteins of varied function could modulate hippocampal synaptic plasticity, LTP was assessed in other transgenic mice with disruptions to genes found to be expressed predominantly in the hippocampus. $Hpk^{-/-}$ mice, possessing a disruption to the Epstein Barr-inducible G-protein coupled receptor, displayed enhanced LTP whilst other transgenic mice investigated showed no change in LTP. However, although no change in LTP was observed, mice lacking the gene encoding Brain Serine Protease 1 (*BSP1*) displayed increased epileptogenic activity.

In summary, we have demonstrated multiple mechanisms that modulate the cholinergic input into the hippocampus as well as LTP and general excitability of glutamatergic synapses. Therefore, this suggests mechanisms that may play an important role in regulating the processes of memory and learning.

Here, we have discovered new targets with the potential to modulate the processes of memory and learning via action on the cholinergic input or synaptic plasticity in the hippocampus both of which have been extensively implicated in the physiology of memory.

IV. Acknowledgments

Undetaking a Ph.D. is a long and sometimes difficult journey and you take help from wherever you can find it.

Firstly, I thank the Medical Research Council for providing the funding for my research.

Supervisors, although not ever present, are important and therefore I thank Ceri Davies for the support and advice when I needed it.

Lab colleagues and friends are, perhaps, fundamental to your day-to-day existence and are cucial for maintaing your sanity. It is in this respect I would like to thank the people I worked with: Urania, Nick, Stuart and Robin. In addition, Paul, Eilis and Alyson were always there for a good moan or even just a pint and a cigarette. Thanks guys.

Finally, I would like to thank all the people in the department who gave me advice, or even just bothered to stop and have a chat in the corridor. Your destractions were always most welcome (except perhaps when I needed to perfuse a drug).

V. Abbreviations

4-AP	4-aminopyridine
4-DAMP	*N*,*N*-dimethyl-4-piperidinyl diphenylacetate
8-Br cAMP	8-bromoadenosine 3,5-cyclic monophosphate
A/D	analogue to digital
A_1R	adenosine A_1 receptor
A_2R	adenosine A_2 receptor
A_3R	adenosine A_3 receptor
AC	adenylate cyclase
ACh	acetylcholine
AChE	acetylcholinesterase
ACSF	artificial cerebrospinal fluid
AD	Alzheimer's disease
AD	Alzheimer's Disease
AHP	after hyperpolarization
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine 5'-triphosphate
ATX	ω-agatoxin IVA
BSP	brain serine protease
CADO	2-chloroadenosine
cAMP	cyclic adenosine 5'-monophosphate
CCh	carbachol
CCPA	2-chloro-N^6-cyclopentyladenosine
cDNA	coding deoxyribonucleic acid

CGP 40116	D-(*E*)-2-amino-4-methyl-5-phosphono-3-pentanoic acid
CGP 55845A	[1-(S)-3,4-dichlorophenyl)ethyl]amino-2-(S)-hydroxypropyl-*p*-benzyl-phosphonic acid
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CTX	ω-conotoxin GVIA
DAGO	[D-Ala^2, N-Me-Phe^4, Gly^5-ol]-Enkephalin
D-AP5	D-2-amino-5-phosphonopentanoate
DC	direct current
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
DRG	dorsal root ganglion
DTLET	(D-Thr^2)-Leu-enkephalin-Thr
EBI	Epstein-Barr inducible
EGTA	Ethyleneglycol-*bis*(β-aminoethyl)-N,N,N',N'-tetraacetic acid
EPSP	excitatory postsynaptic potential
$EPSP_A$	AMPA receptor-mediated EPSP
$EPSP_M$	muscarinic acetylcholine receptor-mediated EPSP
$EPSP_N$	*N*-methyl-D-aspartate receptor-mediated EPSP
ETZ	etonitazene hydrochloride
fEPSP	field excitatory postsynaptic potential
Forskolin	7β-Acetoxy-8,13-epoxy-1α,6β,9α-trihydroxy-labd-14-ene-11-one
GABA	γ-aminobutanoic acid
GALR1	galanin receptor 1
GALR2	galanin receptor 2
GALR3	galanin receptor 3

GFAP	glial fibrillary acidic protein
G-protein	guanine nucleotide binding protein
I_{AHP}	calcium activated AHP current
$I_{K(LEAK)}$	leak K^+ current
IP_3	inositol 1,3,5-triphosphate
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
$IPSP_A$	$GABA_A$ receptor-mediated IPSP
$IPSP_B$	$GABA_B$ receptor-mediated IPSP
LTP	long term potentiation
mAChR	muscarinic acetylcholine receptor
mRNA	messenger ribonucleic acid
MT-1	muscarinic toxin 1
nAChR	nicotinic acetylcholine receptor
NBQX	6-nitro-7-sulphamoylbenzo[*f*]quinoxaline-2,3-dione
Neo	neomycin
NMDA	*N*-methyl-D-aspartate
PA	plasminogen activator
PH domain	pleckstrin homolog domain
PIP2	phosphatidyl-2-bisphosphate
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PLA_2	phospholipase A_2
PLC	phospholipase C

PLD	phospholipase D
PPD	paired pulse depression
PPF	paired pulse facilitation
PSFV	presynaptic fibre volley
PTP	post tetanic potentiation
PZP	pirenzipine
s. oriens	*stratum oriens*
s. pyramidali	*stratum pyramidali*
s. radiatum	*stratum radiatum*
S.E.M.	standard error of the mean
SCG	superior cercvical ganglion
SFA	spike frequency adaptation
SH domain	src-homology domain
STP	short term depression
THIP	4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol
U-50488	(1S-*trans*)-3,4-Dichloro-N-methyl-N-[2-(2-(1-pyrrolidinyl) cyclohexyl]-benzeneacetamide hydrochloride
V_m	membrane potential
wt	wild type

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CHAPTER 1

GENERAL INTRODUCTION

The aim of this chapter is to present an overview of mechanisms believed to be involved in the processes of learning and memory formation which are relevant to the basic synaptic mechanisms studied in this thesis. As such, I will cover the hippocampus and the molecular mechanisms underlying memory formation and the contribution of the septohippocampal pathway to this important neural process. Each individual chapter will contain a more detailed introduction associated with it focusing on the issue to be addressed.

1.1 The Hippocampus

1.1.1 Historical

In 400 B.C. the great philosophers of Greece debated the function of the brain. Hippocrates believed that the brain was the seat of consciousness whilst Aristotle opposed this view, thinking instead that intellectual, perceptual and related functions originated from the heart. It was Hippocrates that proved to be correct. However, how the brain functioned remained a mystery and it was not until the nineteenth century that the concept of "functional localisation" was asserted by Paul Broca. Since then the trend has been to describe the brain in ever-smaller functional units, initially based on the sound logic that if it looks different then it probably has a different function (Finger, 1994). This assumption was supported when, in 1887, it was shown that damage to the temporal lobes and the underlying hippocampus in monkeys produced deficits in memory and memory formation (Brown & Schafer,

1888). This finding was supported when, 12 years later, Bechterew described a memory deficit in a patient who, upon autopic examination, displayed bilateral softening of the hippocampus and adjoining temporal cortex (Bechterew, 1900). However, it was not until 1953 that interest in the hippocampus was renewed when Case H.M. underwent neurosurgery to alleviate the symptoms of his severe epilepsy by bilateral removal of parts of the temporal lobe. After lesions to the hippocampus and a number of associated brain areas, the patient displayed profound anterograde declarative memory loss as well as some retrograde amnesia extending back for many years (Scoville, 1954; Scoville & Milner, 1957). Non-declarative or procedural memory tasks, such as learning a motor skill, were spared and, therefore, this cognitive process was believed to not be dependent on the hippocampus.

These findings led to a plethora of lesion studies conducted on animals that confirmed a role for the hippocampus in certain types of memory formation.

1.1.2. Anatomical Structure of the Rat Hippocampus

The hippocampal formation comprises four cortical regions including the dentate gyrus, the hippocampus proper (divided into the CA1, CA2 and CA3 subfields, the latter two subfields sharing many connectional characteristics), the subicular complex and the entorhinal cortex. The three dimensional shape of the hippocampal formation appears grossly as an elongated structure with its long axis, also referred to as the septotemporal axis, bending in a C-shaped manner from the septal nuclei rostro-dorsally to the temporal lobe caudo-laterally (fig. 1.1*A*). In the perpendicular transverse section (fig 1.1*B*) the principal pathways can be viewed and have been determined in classical Golgi studies (Ramon y Cajal, 1893; Lorento de No, 1933, 1934) and degeneration studies (Blackstad, 1956). These studies showed the dentate gyrus receiving its major input from the perforant path of the entorhinal cortex. The granule cells of the dentate gyrus, in turn, project mossy fibres that terminate on the dendrites of the CA3 pyramidal cells. These cells project collateralised afferents that terminate within the CA3 region as associational connections and provide the major input to the CA1 field of the hippocampus, termed the

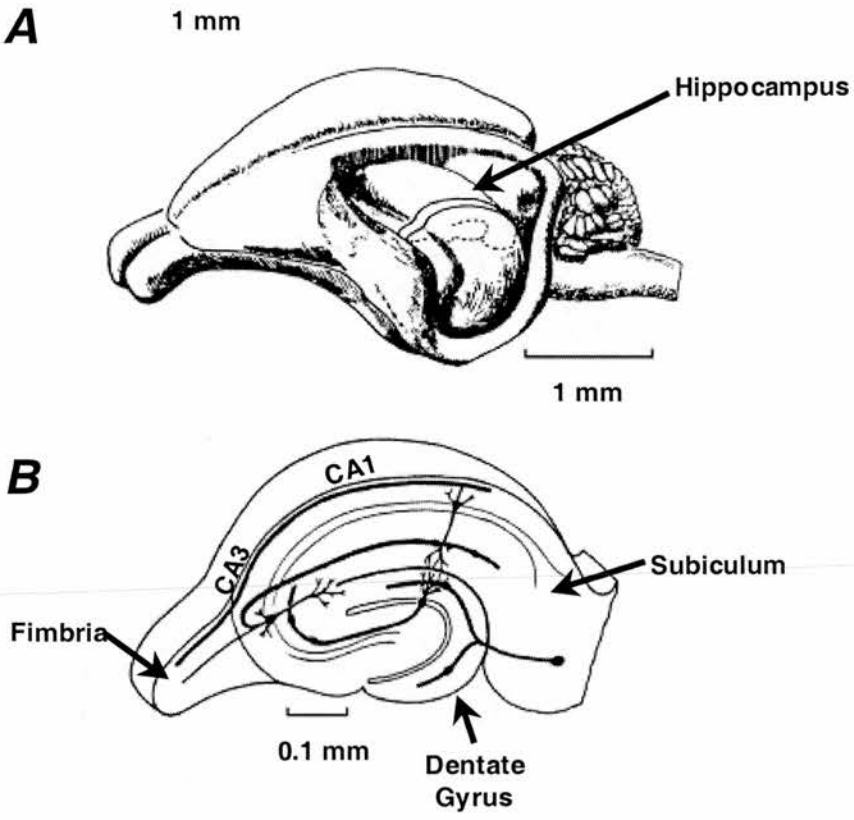


Figure 1.1 Location and general structure of the hippocampus

A shows a schematic representation of the rat brain and the position of the hippocampus within it. *B* illustrates a schematic representation of the transverse hippocampal slice in which the main areas (area CA1, area CA3, dentate gyrus, subiculum and fimbria) are marked. Note the different scales in the two figures.

Schaffer collaterals. The majority of these afferents synapse within the apical dendritic field of the CA1 pyramidal cells in the region known as the *stratum radiatum* (fig. 1.2). Per Anderson and coworkers suggested a lamellar organisation of these pathways, splitting the hippocampus into functional cells that are stacked along the septotemporal axis (1971). However, it has subsequently been shown that, besides the mossy fibre projection form the dentate gyrus, the hippocampal projections are as extensive and highly organised in the septotemporal axis of the hippocampus as in the transverse axis (Amaral & Witter, 1989).

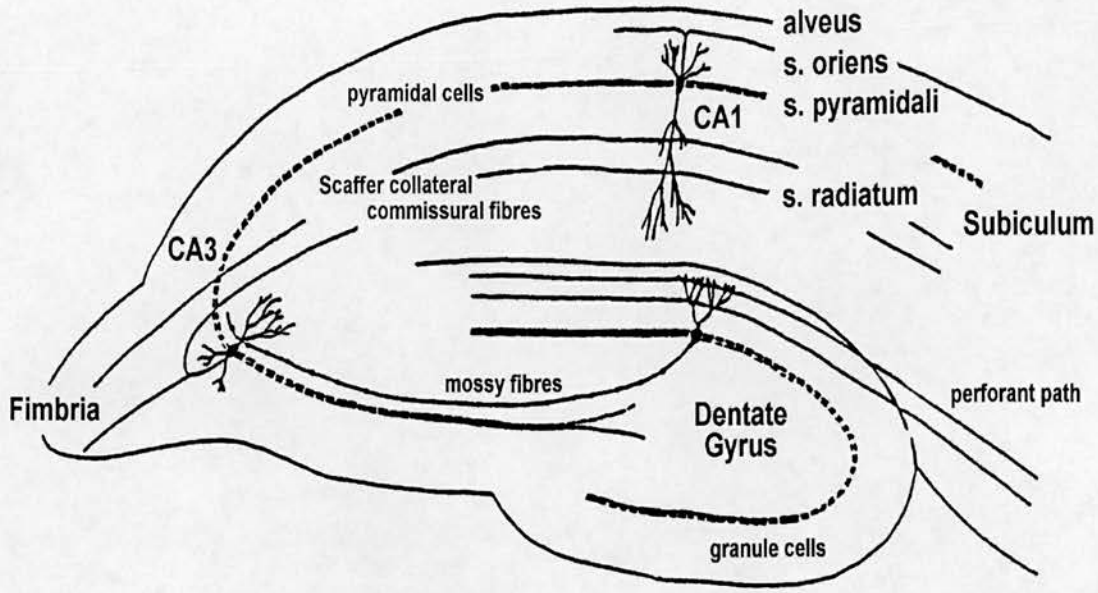


Figure 1.2 Synaptic circuitry of the rat hippocampal slice

A schematic representation of the hippocampal slice illustrating the major excitatory pathways which comprise the trisynaptic circuit. The direction of synaptic connectivity between pathways is: perforant path → mossy fibres → Schaffer collateral-commissural fibres. Note the clear differentiation between laminae (*s. oriens, s. pyramidali and s. radiatum*).

However, most electrophysiological experiments performed on the hippocampus exploit the transverse slice thus severing the septotemporal connections. As the experimental work within this thesis is performed on transverse slices, the connections and synapses of the transverse hippocampal orientation will be focused upon. For a review of the anatomy of the hippocampus and its principal connections see Amaral & Witter (1989).

The transverse hippocampal slice has proven extremely popular in investigations of excitatory synaptic transmission due to the conservation of the tri-synaptic circuitry (see fig. 3.2) and the clear morphological visibility of the different fields that comprise the hippocampus (Yamamoto & McIlwain, 1966; Skrede & Westgaard, 1971; Andersen, 1981). This preparation allows the recording of electrophysiological activity from single cells or populations of cells in a controlled environment. However, interpretation of such data and its relation to *in vivo* activity is subject to

constraints due to the gross severance of convergent extrinsic inputs and intrinsic septotemporal connections.

In addition to the principal cells of the hippocampus, there exist a variety of GABAergic interneurons that form extensive connections with the pyramidal and granule cells. For a review, see Freund & Buzsaki (1996). In conjunction with inhibition provided by pyramidal cell recurrent collaterals, the GABAergic interneurons generate the IPSP component of the pyramidal cell response by feedforward inhibition. This is discussed in greater detail in section 1.1.3.

There are also extrinsic inputs into the hippocampus that utilise different neurotransmitters to exert their effect, including serotonergic afferents from the dorsal raphé nucleus, nor-adrenergic afferents from the locus coeruleus and a heterogeneous population of afferents arising from the medial septum/diagonal band complex of the basal forebrain (Swanson *et al.*, 1982). These latter connections consist of GABAergic, peptidergic (in particular, galaninergic afferents) and cholinergic fibres. The cholinergic pathway has been extensively implicated in memory and learning related behaviour and is discussed in section 1.2.3.

1.1.3. Synaptic Transmission in the Schaffer-Collateral Commissural Pathway

During low frequency (<0.1 Hz) stimulation of the Schaffer-collaterals, the evoked response observed in CA1 pyramidal neurons comprises a fast EPSP followed by a biphasic IPSP (Kandel *et al.*, 1961; Nicoll & Alger, 1981; Alger & Nicoll, 1982b). The EPSP is believed to be mediated by AMPA receptors ($EPSP_A$) (Davies & Collingridge, 1989; Andreasen *et al.*, 1989) whilst the biphasic IPSP is mediated by $GABA_A$ ($IPSP_A$) (Schwartzkroin & Prince, 1980; Alger & Nicoll, 1982a) and $GABA_B$ ($IPSP_B$) receptors (Dutar & Nicoll, 1988b; Soltesz *et al.*, 1988; Lambert *et al.*, 1989). Blocking the $IPSP_A$ using $GABA_A$ receptor antagonists reveals an NMDA receptor-mediated EPSP ($EPSP_N$) (Andreasen *et al.*, 1989). Under a variety of conditions, e.g. reduced presynaptic inhibition (Wigstrom *et al.*, 1986; Dingledine *et al.*, 1986), both $EPSP_A$s and $EPSP_N$s can be evoked. In each case it is believed that it

is the release of L-glutamate from presynaptic terminals that accounts for each response.

Originally, it was thought that the IPSP component of the evoked response was solely caused by recurrent (feedback) inhibition (Kandel *et al.*, 1961; Andersen *et al.*, 1964; Dingledine & Langmoen, 1980; Knowles & Schwartzkroin, 1981). However, it was realised that a feedforward inhibitory circuit was also responsible for the IPSP (Alger & Nicoll, 1982b; Scwartzkroin & Knowles, 1983; Buzski, 1984). Accordingly, activation of AMPA and NMDA receptors on GABAergic interneurons were shown to result in the IPSPs observed in CA1 pyramidal neurons (Davies & Collingridge, 1989; Andreasen *et al.*, 1989; Sah *et al.*, 1990).

The classic, fast hyperpolarising $IPSP_A$, evoked by both recurrent and feedforward circuits, was characterised by its sensitivity to bicuculline and to changes in both intracellular and extracellular Cl^- concentrations (Kandel *et al.*, 1961; Andersen *et al.*, 1964; Allen *et al.*, 1977; Eccles *et al.*, 1977; Dingledine & Langmoen, 1980; Dingledine & Gjerstad, 1980). Feedforward inhibitory circuits also activate a $GABA_B$ receptor-mediated slow $IPSP_B$ (Nicoll & Alger, 1981; Newberry & Nicoll, 1984a), which was originally characterised by its similarity to (-)-baclofen-induced hyperpolarisations (i.e. both exhibited similar I-V relationships and dependency on extracellular K^+) (Newberry & Nicoll 1984b) and was subsequently confirmed by the inhibition of $IPSP_B$s using selective $GABA_B$ receptor antagonists (Lambert *et al.*, 1989).

1.1.4. Synaptic Plasticity

It is far beyond the scope of this thesis to discuss synaptic plasticity and LTP in great depth due to the wealth of literature regarding this subject. A brief outline of the main points about synaptic plasticity will be reviewed here. For a more detailed review see Bliss & Collingridge (1993).

Two initial discoveries suggested an important role for the hippocampus in memory formation. Firstly, it was found that the pyramidal cells could encode information about space (O'Keefe & Dostrovsky, 1971) and, secondly, that the synapses within

the tri-synaptic circuit of the hippocampus undergo long-term potentiation (Bliss & Lømo, 1973).

Bliss and Lømo found that a high frequency train of action potentials evoked at any of the three major pathways of the hippocampus produced LTP i.e. an increase in glutamatergic synaptic strength in that pathway that could last for hours in the anaesthetised animal and days or weeks in the freely moving, awake animal. In addition, a theta burst stimulation pattern (several bursts of 4 shocks at 100 Hz separated by a 200 ms interburst interval) also reliably induced LTP (Larson *et al.*, 1986) mimicking the firing patterns observed in the hippocampus during learning tasks (Otto *et al.*, 1991).

The induction of LTP, produced by tetanic stimulation, can be explained by the properties of the NMDA receptor and the role of synaptic inhibition. It is the voltage-dependent block of the integral channel by Mg^{2+} that allows the NMDA receptor to act as a coincidence detector, i.e. the postsynaptic membrane must be depolarised to remove the Mg^{2+} block allowing the entry of Ca^{2+} through the NMDA receptor upon the release of glutamate into the synaptic cleft. The frequency dependency of LTP induction is largely attributable to the susceptibility of NMDA receptor-mediated currents to $GABA_B$ autoinhibition i.e. during a train of stimuli delivered to the afferents, each subsequent stimulus-evoked release of glutamate produces a larger NMDA receptor-mediated response in the pyramidal cell due to decreased GABA release from interneurons as GABA from the previous stimulus inhibits its own release (Collingridge *et al.*, 1988; Davies *et al.*, 1991).

Upon activation, the NMDA receptor becomes highly permeable to Ca^{2+} (Ascher & Nowak, 1988; Jahr & Stevens 1987). Increases in intracellular Ca^{2+} were shown to be vital in LTP induction (Lynch *et al.*, 1983) as induction is occluded by the intracellular injection of EGTA. This was supported by Ca^{2+} imaging techniques which visualised this calcium elevation in dendritic spines during high frequency stimulation (Regehr & Tank, 1990; Muller & Connor, 1991). The Ca^{2+} increase appeared to last for several seconds and produced Ca^{2+} gradients from spines to dendrites lasting for several minutes. This is unnecessary for LTP induction, which can be induced by Ca^{2+} transients of 3 seconds (Malenka *et al.*, 1992). There is also

evidence to suggest that the Ca^{2+} influx is augmented by calcium-induced Ca^{2+} release and release of Ca^{2+} from intracellular stores (Alford *et al.*, 1993; Obenaus *et al.*, 1989; Harvey & Collingridge, 1992). Inhibitors of these stores also inhibit LTP.

Several Ca^{2+}-sensitve enzymes have been proposed to play a part in converting the induction signal into a persistent change in synaptic efficacy although most interest has focused on protein kinases. The first such kinase to be implicated in LTP was the Ca^{2+}/phospholipid-dependent protein kinase (PKC) (Bar *et al.*, 1984). It is generally thought that PKC activation is not a necessary factor for LTP induction but may be involved in the conversion of short- to long-term potentiation. The Ca^{2+}/calmodulin-dependent protein kinase CaMKII has also been implicated in LTP (Malenka *et al.*, 1989; Malinow *et al.*, 1989) as well as cAMP- and cGMP-dependent protein kinase (PKA and PKG respectively) (Chetkovich *et al.*, 1991) and protein tyrosine kinases (O'Dell *et al.*, 1991a).

The use of protein synthesis inhibitors has demonstrated that protein synthesis from existing mRNA is required for the maintenance of LTP during the first few hours (Krug *et al.*, 1984; Otani *et al.*, 1989; Frey *et al.*, 1989). However, there is also evidence for gene transcription and increased mRNA production after tetanisation (Mackler *et al.*, 1992; Dragunow *et al.*, 1989; Nikolaev *et al.*, 1991). The obvious proteins to upregulate or modify would be ion channels involved in low frequency synaptic transmission. Accordingly, there is evidence for AMPA receptor phosphorylation by protein kinases, particularly PKA, following LTP induction (Reymann *et al.*, 1990; Greengard *et al.*, 1991; Wang *et al.*, 1991). Alternatively, the induction of LTP could alter the ratio of flip:flop AMPA receptors or change the subunit composition resulting in a change in whole cell conductance properties (Sommer *et al.*, 1990). There is some evidence suggesting NMDA receptor function can also be enhanced by protein kinase activity, such as PKC providing a means by which synapses increase their plasticity as well as their efficacy (Kelso *et al.*, 1992).

As well as postsynaptic modifications, presynaptic modifications have been considered as a locus of expression. This involves diffusable retrograde messengers. Two such candidates are arachidonic acid and nitric oxide (NO) (McNaughton, 1982;

Schuman & Madison, 1991; O'Dell *et al.*, 1991b) which could act to increase glutamate release or decrease glutamate uptake into surrounding glia.

1.2. The Septohippocampal Pathway

The septohippocampal pathway is one of the best examples of a central cholinergic pathway and provides most of the cholinergic innervation of the hippocampal formation. It also holds a critical role in the generation and maintenance of various forms of electrical rhythmic activity, e.g. the theta rhythm, implicated in the spatial memory acquisition of the rat (Winson, 1978). The septohippocampal pathway is notably susceptible to the aging process and, furthermore, the target of neurodegenerative diseases such as Parkinson's and Alzheimer's disease where loss of cognitive function, particularly memory, is a major symptom.

1.2.1. Anatomical Structure

The septohippcampal afferents arise from large neurons located in the medial septal nucleus (MS) and the vertical limb of the diagonal bands of Broca (vDBB) (fig. 1.3). These neurons form a continuum of cells that are difficult to subdivide because of the absence of clear histological boundaries. The MS/vDBB receives various inputs from the hippocampal formation, limbic regions and brain stem nuclei (fig. 1.4) and sends efferents to many brain areas inclusing the cingulate cortex, hypothalamus, contralateral diagonal band as well as the hippocampal formation (fig. 1.3) (Dutar *et al.*, 1995).

It has been demonstrated that choline acetyltransferase (ChAT)-positive axons infiltrate the hippocampus at postnatal day 2 in the rat (Hohmann & Ebner, 1985) and the intensity of ChAT staining increases until the third to fifth week (Gould *et al.*, 1991).

The majority of septal neurons innervate the ipsilateral hippocampus via three main routes: the fimbria, the dorsal fornix, and the supracallosal striae (Gage *et al.*, 1984).

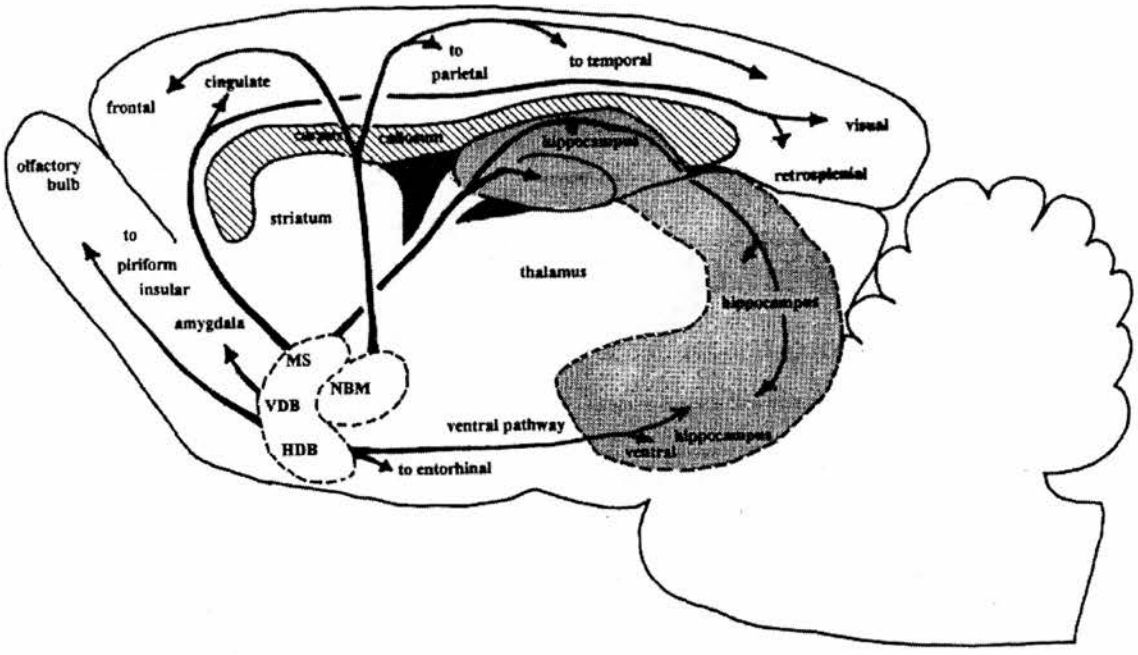


Figure 1.3 **Schematic cross-section of the rat brain showing the major pathways originating from the rat basal forebrain.**

This figure illustrates schematically the major efferent pathways basal forebrain nuclei of the rat, namely the medial septum (MS), the vertical limb of the diagonal bands of Broca (VDB), the horizontal limb of the diagonal bands of Broca (HDB) and the nucleus basalis of Meynert (NBM).

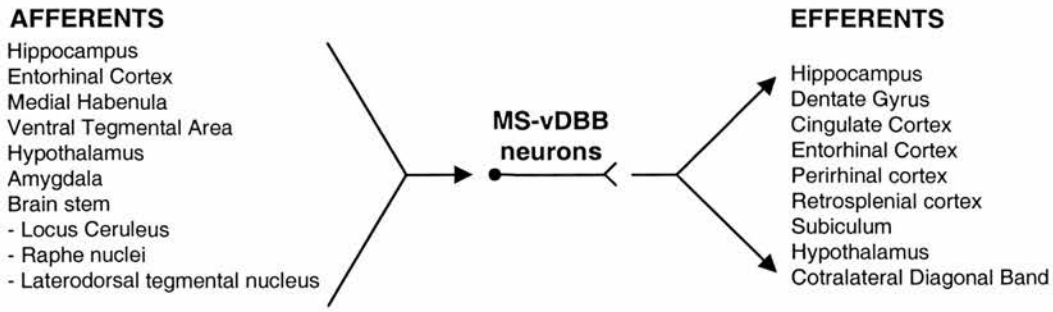


Figure 1.4 **Summary diagram of brain areas projecting (afferents) to the Medial septum/vertical limb of the diagonal bands of Broca (MS/vDBB) and of structures receiving projections (efferents) from this area.**

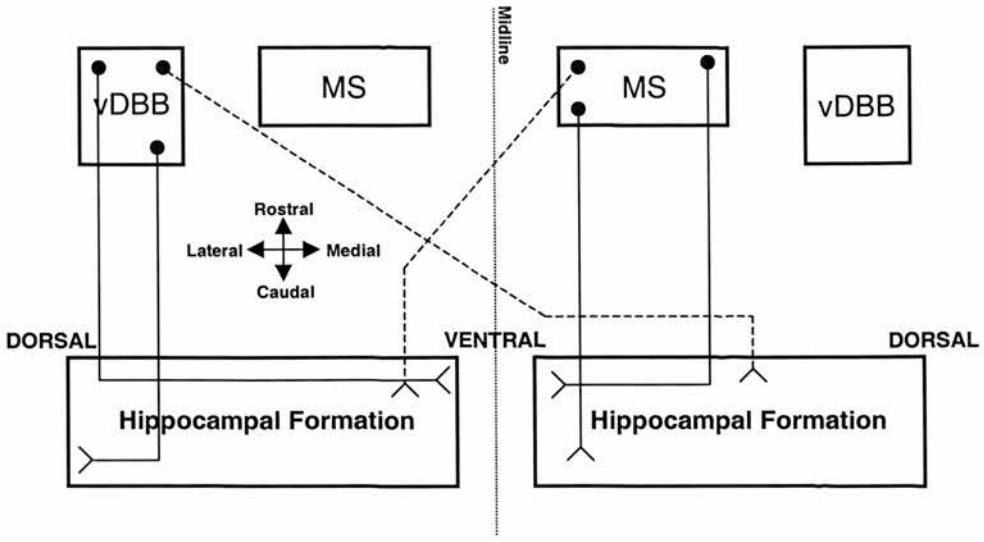


Figure 1.5 **Schematic diagram illustrating the topographical projection of the septohippocampal pathway.**

For ease of illustration the projections for the medial septum (MS) and the projections from the vertical limb of the diagonal bands of Broca (vDBB) have been shown on opposite hemispheres. However, both hemispheres of the brain show the above MS/vDBB projections to the hippocampal formation.

A topographical projection of afferents has been shown, the more laterally the septal neurons are located the more ventrally the axons terminate within the hippocampus and the more rostral neurons project to the rostral parts of the hippocampus (Amaral & Kurz, 1985; Gaykema *et al.*, 1990). In addition, it has been demonstrated that the CA1 pyramidal cells and granule cells of the dentate gyrus receive afferents from vDBB whereas the cells of the ventral hippocampus receive afferents from both the vDBB and the MS (Nyakas *et al.*, 1987) (fig 1.5). Furthermore, two sets of septohippocampal fibres that terminate on the dendrites of pyramidal and granule cells have been described: (1) thick, coarse axons with large terminal boutons present in the *strata oriens*, *radiatum*, *moleculare*, the dentate gyrus and the infragranular zone of the dentate gyrus and (2) thin fibres displaying varicosities in the hippocampal pyramidal cell layer, the dentate granular layer and the middle one-third of the dentate molecular layer (Nyakas *et al.*, 1987; Gaykema *et al.*, 1991).

1.2.2. Distribution of Neurotransmitters and Neuropeptides

Stimulation and lesion studies as well as histochemical detection of the enzymes acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) led to the discovery that a proportion of the septohippocampal pathway was cholinergic.

Lesions of the MS or fimbria/fornix transection resulted in a decrease in ACh, choline and choline uptake in the rat hippocampus paralleled by a decrease in ChAT (Kuhar *et al.*, 1973; Potemska *et al.*, 1975; Sethy *et al.*, 1973). In addition, electrical stimulation of the septum induces an activation of pyramidal cells in the hippocampus and dentate gyrus (Andersen *et al.*, 1971a, 1971b), supported by *in vivo* release of ACh in the hippocampus after medial, but not lateral, septal stimulation (Dudar, 1975; Moroni *et al.*, 1978).

AChE and ChAT have been detected in the rat hippocampus (Fonnum, 1970; Shute & Lewis, 1966) at axon terminals that make synaptic contacts on dendrites of pyramidal and granule cells, mainly on dendritic spines. Immunohistochemical studies have confirmed the presence of ChAT-positive cell bodies of two types: (1) small, round neurons of the medial septal nucleus and (2) larger, fusiform neurons of the vDBB (Mesulam *et al.*, 1983; Amaral & Kurz, 1985; Armstrong *et al.*, 1983). Staining for ChAT revealed preferential distribution of cholinergic terminals in the hippocampal pyramidal and dentate granular layers as well as at layers adjacent to them, with the highest density in the *stratum oriens* (Frotscher & Leranth, 1985; Houser *et al.*, 1983). As well as synapsing with the dendritic spines of pyramidal cells, cholinergic fibres synapse with GABAergic and somatostatin-containing neurons in the *stratum oriens* of CA1 and CA3 subfields (Leranth & Frotscher, 1987).

Histochemical studies have also demonstrated a GABAergic subpopulation of septohippocampal neurons making up 10-30% of the afferents (Kohler *et al.*, 1984). In contrast to the cholinergic afferents, the GABAergic septal neurons terminate mainly on GABAergic interneurons (Freund & Antal, 1988).

However, not all of the neurons of the septohippocampal pathway are cholinergic or GABAergic. As such, it has been demonstrated that medial septal cells contain

neuropeptides such as galanin and *N*-acetyl-aspartyl-glutamate (NAAG) which in some afferents are co-released with ACh (Andersen *et al.*, 1971b; Forloni *et al.*, 1987; Melander *et al.*, 1985).

1.2.3. Role of Septohippocampal System in Learning and Memory

The involvement of the septohippocampal system in the processes of learning memory has been ascertained from several lines of investigation including lesion and pharmacological studies, behavioural performance and behavioural recovery after lesion.

Lesions of the MS or fimbria-fornix led to a deficit in working memory, especially in spatial tasks (Poucet & Herrmann, 1990; Olton *et al.*, 1978). However, the exact way in which the septohippocampal pathway contributes to memory related tasks is complicated by the fact that the lesions also destroy other pathways *en route* to the hippocampus such as the noradrenergic and serotonergic pathways, as well as local neurons in the case of the MS lesion. However, immunotoxic lesioning of rats using the specific cholinergic immunotoxin, 192-IgG saporin, produced long lasting spatial learning impairment after cholinergic denervation (Nilsson *et al.*, 1992).

Microinjection of phenoxybenzamine, an α-noradrenergic receptor antagonist, or biccuculine, a $GABA_A$ receptor antagonist, caused an impairment of working memory (Chirobak *et al.*, 1992; Marighetto *et al.*, 1989) suggesting an involvement of noradrenergic and GABAergic neurotransmission in the functioning of the septohippocampal system. Cholinergic antagonists, such as scopolamine, also impair many types of memory tasks (Bartus *et al.*, 1987). Conversely, systemic administration of cholinergic agonists and drugs that increase hippocampal ACh improve behavioural performances (Levey *et al.*, 1991; Flood *et al.*, 1984) although it is hard to assess the exact contribution of hippocampal cholinergic synapses as the administered drugs could be acting at a number of areas of the brain, not to mention the periphery (Rush & Streit, 1992).

Following learning tasks, increased ChAT and ACh levels have been observed (Matthies *et al.*, 1974; Jaffard *et al.*, 1985) as well as increases in sodium-dependent high affinity choline uptake (HACU) in the rat hippocampus and frontal cortex (Kuhar *et al.*, 1978; Rauca *et al.*, 1980). It has been demonstrated that biochemical markers of septohippocampal cholinergic activity recover back to control levels within 6 weeks after lesions limited to the supracollosal pathway (Gage *et al.*, 1983). In addition, there is a partial recovery of the deficit in passive and active avoidance 10 weeks after fimbria-fornix lesions (Dravid *et al.*, 1986). This evidence demonstrates a capacity of the septohippocampal system to compensate for loss of function. One study shows an upregulation of ACh synthesis and storage in the hippocampus after partial fimbria-fornix lesion (Lapchak *et al.*, 1991) and this recovery is potentiated by various neurotrophic factors, especially NGF.

1.2.4. Involvement in Aging and Disease

The septohippocampal pathway is vulnerable to aging. In the rat, a decrease in the number and size of AChE-positive MS-DBB neurons was observed with age (Biegon *et al.*, 1986; Fischer *et al.*, 1989) whereas only a decrease in the size of AChE-positive cells was seen in the mouse (Hornberger *et al.*, 1985). This appears to correlate with a behavioural deficit (especially in learning and memory tasks) (Fischer *et al.*, 1989). Similar age-related changes seem to occur to the human nucleus basalis of meynert (De Lacalle *et al.*, 1991). In addition to neuronal loss, the sensitivity of hippocampal neurons to cholinergic agonists is decreased both *in vivo* and *in vitro* (Potier *et al.*, 1992, 1993; Lippa *et al.*, 1981; Segal, 1982c). It was also shown that the slow EPSP, due to the evoked release of ACh from septohippocampal neurons, is dramatically depressed in the aged rat (Potier *et al.*, 1992).

Presenile dementia, such as that found in Alzheimer's disease (AD), incorporates many cognitive impairments including memory loss. Since the 1970's, ACh deficits have been implicated in the pathophysiology of AD, e.g. a significant and selective loss of ChAT activity in different parts of AD brain samples (cortex, hippocampus and amygdala) was demonstrated (Bowen *et al.*, 1976, 1979). This was supported by

the observation that neurons in the basal forebrain were selectively degenerated in AD (Whitehouse *et al.*, 1981), explaining the reduced ChAT activity in the cortex and hippocampus. Other studies have demonstrated reductions in ACh levels (Richter *et al.*, 1980), ACh synthesis (Sims *et al.*, 1980) and high affinity choline uptake (Rylett *et al.*, 1983). In addition, an inverse relationship was found to exist between ChAT and AChE activities in the cortex and the number of senile plaques, a pathological marker that is argued to correlate with AD progression (Neary *et al.*, 1986; Perry *et al.*, 1978).

This body of work investigates neural mechanisms that have been reported to play critical roles in memory and learning behaviour. Specifically, the aims of this thesis are to:

1. explore the regulation of the septohippocampal cholinergic input into the hippocampus. This involves the examination of the mechanisms by which ACh release may be controlled and the neurotransmitters/neuromodulators which may exert that control. Studying the complex interactions of different neuromodulators regulating of the cholinergic input may aid in the understanding of how this pathway is involved in memory formation in the rodent hippocampus.
2. investigate possible mechanisms involved in LTP induction. As LTP has been demonstrated to be critical in memory and learning performance, we investigated LTP in a number of transgenic mice possessing mutations in novel genes thought to be expressed predominantly in the hippocampus and particularly in the CA1 pyramidal cell layer. This work may contribute to our better understanding of the molecular mechanisms involved in synaptic plasticity with the long term goal of discovering targets for the possible development of cognitive enhancers.

CHAPTER 2

METHODS AND MATERIALS

2.1 EXPERIMENTAL PREPARATION

2.1.1 General

All experiments were performed using standard intra- and extra-cellular electrophysiological techniques. Synaptic recordings were made from the CA1 region of transverse coronal rat hippocampal slices obtained from 2–5 week old female Cobb-Wistar rats. All recordings from transgenic mice were also made from the CA1 region of transverse coronal mouse hippocampal slices obtained from 1–4 month old male/female C57BL/6 mice.

2.1.2 Preparation of rat hippocampal slices

The rats were cervically dislocated and subsequently decapitated in accordance with UK Home Office guidelines. The brain was removed swiftly and placed immediately in ice cold (0–4°C) artificial cerebrospinal fluid (ACSF). The brain, minus the cerebellum, was hemisected and an agar block fixed to the base of each hemisphere using superglue. The hemisphere was then fixed to a polypropylene block for the subsequent cutting of 400 μm thick transverse coronal slices using a vibroslicer (Campden Instruments, Loughborough, UK). Throughout the slicing procedure the brain and slices were held in a chamber containing ice cold ACSF. The slices were then transferred to a glass petri dish containing ACSF at room temperature (18–24 °C). The hippocampus was cut away from the rest of the brain slice and the CA3 region cut away to eliminate changes in network function that can occur due to epileptiform bursting in area CA3 when picrotoxin is applied to the

slice. Following an incubation period of at least half an hour the resultant CA3-ectomized slices were placed in the recording chamber upon a nylon mesh at the interface of warmed (32 ± 2 °C), perfusing (1–3 ml min^{-1}) ACSF and an oxygen-enriched (95% O_2, 5% CO_2; BOC Medical Gasses, UK), humidified atmosphere (Fig. 2.1). The slices were then allowed to equilibrate in this environment for approximately 1–2 h before any electrophysiological recording was attempted. Surplus slices contained in the petri dishes remained viable for several hours. If required, these slices could be transferred to the recording chamber at a later time.

The standard perfusion medium comprised (in mM): NaCl, 124; KCl, 3; $NaHCO_3$, 26; NaH_2PO_4, 1.25; $CaCl_2$, 2; $MgSO_4$, 1; D-glucose, 10; and was bubbled with 95% O_2, 5% CO_2 to maintain a pH of 7.4–7.5. ACSF was made up using distilled water filtered (10–18 MΩ Cm^{-1}) using the Millipore Milli-Q filter system (Millipore; Molsheim, France) and all chemicals were analaR grade (BDH Chemicals Ltd., Poole, UK).

2.1.3 Preparation of transgenic mouse hippocampal slices

The mouse brains were extracted and maintained as above with the exception that the CA3 region was not removed. Slices were placed in a submerged recording chamber perfused (1–3 ml min^{-1}) with room temperature (18–24 °C) ACSF and held in place by short pieces of silver wire.

2.2 RECORDING SET-UP

2.2.1 Intracellular recording from rat brain slices

The recording chamber and the micromanipulators were mounted on magnetic stands (Narishige, Japan) supported on a steel plate on an anti-vibration table (Ealing, MA, USA). A steel Faraday cage, grounded through the amplifier (Axoclamp-2B, Axon

Instruments, CA, USA), was placed around the recording system in order to isolate it from extraneous electrical noise. All electrical equipment that was fitted with earth leads was secured in a rack and earthed through the mains.

The recording chamber was a modified version of an interface electrophysiological recording chamber designed by (Spencer *et al.*, 1976) (Fig. 2.1). It consisted of an outer water bath with a centrally mounted platform on which the tri-compartmental recording chamber was secured. The outer water bath was partially filled with distilled water and was bubbled with a 95% O_2, 5% CO_2 mixture. The height of the covering lid was adjusted to allow the oxygen-enriched humidified atmosphere to flow over the slices.

ACSF was pumped, by means of a peristaltic pump (Watson-Marlow, England), to a gravity-feed syringe. This fed a constant flow (1–3 ml min^{-1}) of ACSF into the first well of the recording chamber. The ACSF then flowed around the circumference of this well and through a small inlet into the recording chamber proper. Here, it flowed underneath the nylon mesh and was removed through a separate outlet by suction through a syringe needle connected to a water-powered suction pump (Brownall, England). If the recycling of drugs was required, the ACSF would be removed using a multi-channel peristaltic pump (Watson-Marlow 205U, England) where one channel would provided the inflow and another channel would be used to remove the ACSF from the recording chamber. By adjusting the height of the gravity feed and the magnitude of the outlet suction, the level of ACSF in the recording chamber was maintained so that it was in contact with the mesh but did not cover the slices. A heated patch connected to a constant current source (Maplin XG89, Taiwan) was attached to a metal plate in the base of the water bath (Fig 2.1) to maintain the temperature at 32 ± 2 °C.

The slices in the recording chamber were epi-illuminated with a fiber optics system (Nachet, France). All stimulating and recording electrodes were mounted on micromanipulators (Narishige, Japan) which allowed movement in the x, y and z axes. An additional single axis fine movement hydraulic manipulator (Narishige, Japan) was used for the recording electrode to allow increased sensitivity when

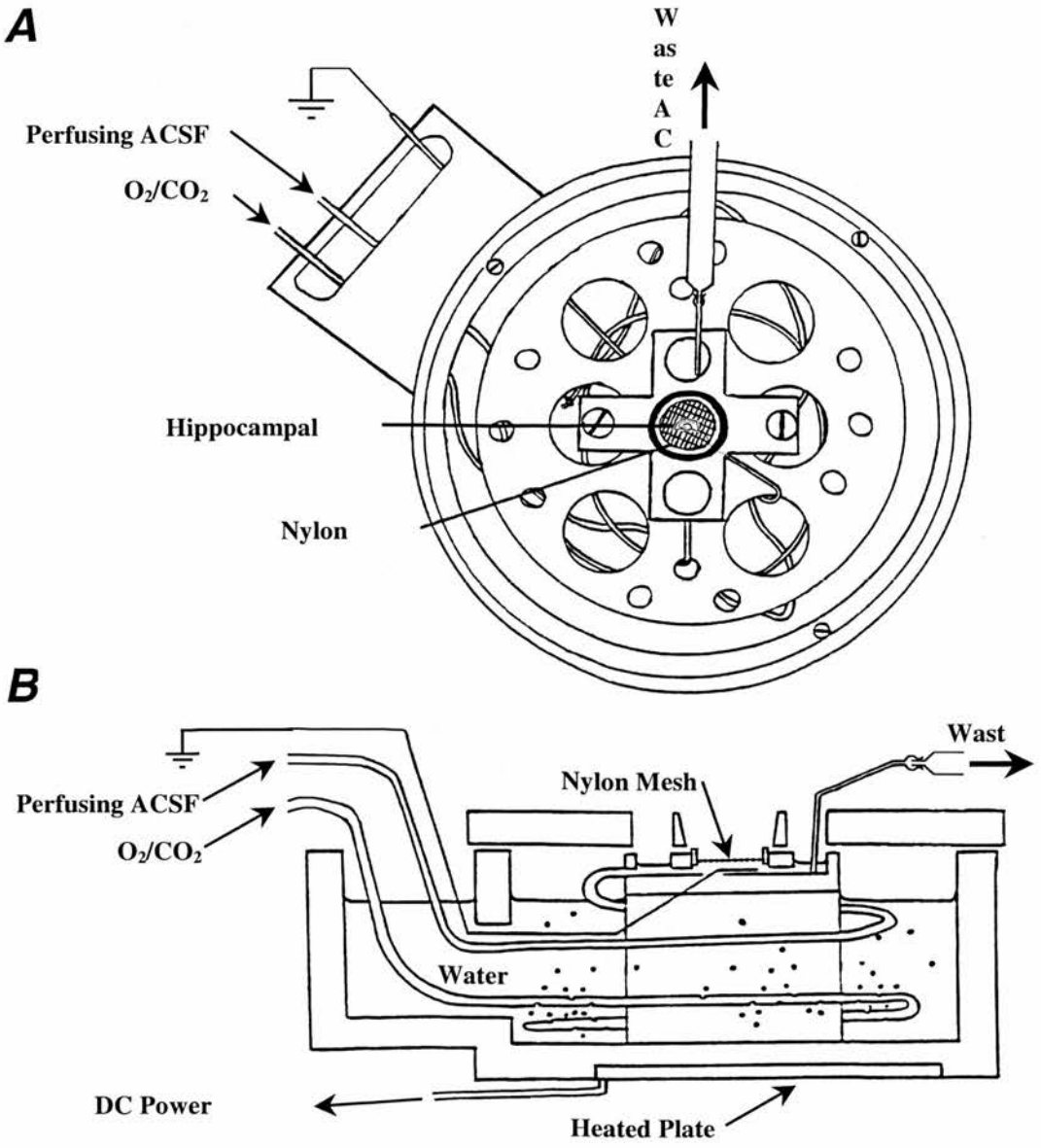


Figure 2.1 The interface recording chamber

This figure shows a schematic representation of the recording chamber as viewed from above (*A*) and from the side (*B*). ACSF, perfused with 95% O_2/5% CO_2 and maintained at 32°C, was passed through tubing immersed in the temperature controlled external water bath and into the interface chamber. The level of ACSF in the interface chamber was maintained just above the level of the nylon mesh which supported the slice. Waste ACSF was drawn off by suction through a hypodermic needle. The humidified atmosphere was maintained by passing 95% O_2/5% CO_2 through distilled water maintained at 32 °C (by a heated plate) to create a fine mist. The reference electrode was grounded through the Axoclamp 2B.

advancing the electrode for the impalement of neurones. To enable the positioning of electrodes in the slice, the slice was viewed using an overhead dissecting microscope (M3C, Leica, England). Recording microelectrodes were placed, at an angle of approximately 45° to the vertical, in either *stratum oriens* (CA1 pyramidal basal-dendritic layer) or *stratum radiatum* (CA1 pyramidal cell apical dendritic layer).

2.2.2 Extracellular recording from transgenic mice

As above except extracellular experiments on transgenic mice were performed using a slightly different set-up as regards the recording chamber itself. This comprised a recording bath mounted on a fixed stage upright microscope (Olympus). ACSF flowed into the slice holding chamber by gravity feed and was removed by a suction needle placed in an adjoining chamber connected to a peristaltic pump (Watson Marlow, UK). ACSF was not recycled.

2.3 RECORDING TECHNIQUES

2.3.1 Recording electrodes

Microelectrodes were pulled from thick walled (internal diameter: 0.69 mm; outer diameter: 1.2 mm) borosillicate glass capillaries with an inner filament (120F-10, Clark Electromedical Instruments, England), on a horizontal Flaming-Brown P-97 micropipette puller (Sutter Instruments Co., USA). Intracellular microelectrodes were back-filled with 2 M potassium methylsulphate (ICN Biomedicals Inc., USA) and had resistances ranging 60–120 MΩ. Extracellular microelectrodes with resistances ranging 1–5 MΩ were back-filled with 2 M sodium chloride.

Recording microelectrodes were then mounted in electrode holders (Clark Electrochemical Instruments, England) allowing the electrolyte solution within the microelctrode to make contact with a silver chloride coated silver wire. The holders were inserted into unity gain head stages (current gain x 0.1 or x 1.0: Axon

Instruments, CA, USA) and connected to an Axoclamp-2B amplifier through the microelectrode 1 (ME1) port for use in "bridge balance" or "discontinuous current clamp" (DCC) modes. A silver-silver chloride bath reference electrode, submerged in the recording chamber, was also connected to the head stage and grounded through the Axoclamp amplifier.

2.3.2 Intracellular recording

Impalement of CA1 pyramidal neurones was achieved by manually advancing the recording microelectrode through the slice using the hydraulic vertical axis manipulator, and intermittently applying a 1–2 ms "buzz" from the Axoclamp-2B. "Buzzing" momentarily increases the capacitance neutralization of the electrode and causes the headstage to oscillate at high frequency. This procedure aids penetration of cells when the electrode is opposed to the cell membrane although the mechanism by which this occurs is unclear. Cell impalement was routinely performed in normal ACSF (with occassional exceptions where pre-incubation of a slice in a particular drug was required) and only after a stable recording was obtained for at least 10 min were drugs administered.

The membrane potential (V_m) of the neurone (as measured by the Axoclamp 2B amplifier) was measured as the potential difference between the microelectrode and the bath reference electrode. Prior to searching for cells, when the microelectrode had been positioned extracellularly in the slice, V_m was set to zero. Immediately following the end of each experiment this reading was checked again and any correction in the recorded membrane potential made.

2.3.3 Current injection

Before searching for cells the resistance of the microelectrode was measured in bridge balance mode. This was done by balancing out the unwanted potential drop across the microelectrode resistance through a differential amplifier incorporated in the Axoclamp 2B amplifier. The mode of recording was then switched to

discontinuous current clamp mode which allowed rapid switching (frequency 3–5 kHz) between current injection and voltage recording. Thus, current was injected during the first 30 % of the cycle and the resulting potential across the microelectrode resistance due to charging continually monitored on an oscilloscope. The capacitance neutralization was increased so that when the cell V_m was sampled, just prior to the next current injection, the potential across the microelectrode tip had fully decayed. Low resistance microelectrodes (60–90 MΩ) allowed the most accurate recording of membrane potential during current injection.

2.3.4 Amplification and Filtering

Synaptic potentials recorded through the ME1 port on the Axoclamp-2B amplifier were amplified 10 fold by an in-built gain. In DCC mode the amplifier set the sample rate (3–5 kHz switching frequency). Signals above 1–3 kHz were filtered, using a low-pass filter, which did not noticeably affect the waveform of the synaptic potentials. Secondary amplification of synaptic responses was provided by variable gain DC amplifiers (Neurolog, Digitimer, England). The output signals were then digitally filtered through a Digidata 1200 interface (Axon Instruments Ltd.) connected to a Dell Dimension P75t IBM personal computer (PC) (Dell, Texas, USA) (Fig. 2.3). Any further filtering was carried out during off-line analysis with Clampfit software (Axon Instruments Ltd.).

2.3.5 Data display and storage

Digitized data were captured and simultaneously viewed using pClamp6 software (Axon Instruments Ltd.) on the PC. Digitized records were stored on the hard disk of the PC for off-line analysis using Clampfit software (Axon Instruments Ltd

The potential across the microelectrode in discontinuous current clamp mode was monitored on an analogue oscilloscope (Phillips, Holland). A continuous chart record or digital tape (DAT) record of the membrane potential of the cell was

captured by a chart recorder (Gould, Ilford, Essex, UK) or DAT recorder (DTR1404, Biologic Scientific Instruments, Claix, France). This provided a means to assess the passive stability of the cell throughout each experiment.

2.3.6 Extracellular recording

Extracellular field potential recordings from the apical and basal dendritic layer of CA1 pyramidal cells were made with respect to the reference electrode and the rising slope of the fEPSP was measured (Fig. 2.4). For experiments investigating the population spike recorded from the cell body region of the CA1 pyramidal cells, the peak amplitude was measured. The population spike is the summation of action potentials firing from a group of pyramidal cells characterised by a sharp negative drop in potential difference followed by a rapid recovery (see fig. 6.1*Ba*).Data were displayed and stored in a similar manner to that used for intracellular recording. For the majority of extracellular LTP experiments, a piece of software called "LTP" was employed which allowed the recording, averaging and on-line analysis of data (written by William Anderson, University of Bristol). In experiments investigating the effect of drugs or the induction of LTP on the slope of the field EPSP, the test field EPSP size was adjusted to approximately 50 % of maximum (maximum being defined as when a population spike begun to appear superimposed on the field EPSP).

2.3.7 Differences Between Intracellular and Extracellular Recording

Extracellular experiments were performed in a submerged chamber which provided more stability during the length of the recording. Additionally, the experiment was conducted at room temperature.

Intracellular experiments were performed in an interface chamber to increase the stability of the recording. The temperature was maintained at 32°C as this is the optimum temperature to maintain stable impalements. The CA3 was removed to

prevent any spontaneous neuronal activity. This region was not removed from transgenic mouse slices as there was little observed spontaneous activity even when NMDA receptor-mediated EPSPs were being recorded. Due to the positioning of the net that weighted down the slice in the chamber, it is likely that the CA3 region was damaged and, thus, the slice behaved similar to a CA3-ectomized slice.

2.4 STIMULATION

The septo-hippocampal cholinergic input or the Schaffer collateral-commissural fiber input to CA1 pyramidal neurones were orthodromically stimulated using bipolar stimulating electrode(s) which were placed on the surface of the slice in *stratum radiatum* and *oriens* respectively. The stimulating electrodes consisted of two 50 μm diameter Formvar insulated nickel-chromium (80 %: 20 %) wires (Advent Research Materials Ltd., England) twisted together and cut at the end to provide a focal stimulation. Stimuli were produced by constant voltage or constant current isolated stimulator boxes (Digitimer, England) in turn stimulated by a 5 V pulse produced by the PC and relayed via the Digidata 1200. In every series of experiments stimuli comprised square-wave pulses (20–200 μs; 0.1–30 V or 0.1–10 mA) delivered homosynaptically at a fixed intensity every 15–30 s for ionotropic glutamate and GABA receptor-mediated responses and every 5–10 min for mAChR-mediated responses.

2.5 SLICE AND CELL SELECTION CRITIERIA

Slices were selected from the ventral region of the hippocampus. Besides possessing a clearer morphology (mainly due to the angle of transection being parallel to the cytoarchetectonic plane of the trisynaptic circuity of the hippocampus), this region of the hippocampus demonstrates cholinergic responses that are inhibited by galanin. In addition, taking slices from this region helped to minimise inter-experimental variability as it is entirely possible that responses will vary depending on whether

slices are taken from the ventral or dorsal region of the hippocampus, eg the inhibition of ACh release by galanin.

For both intra- and extra-cellular recordings slices were chosen that evoked only a single population spike to a low frequency stimulus (0.033 Hz) in standard ACSF. This was taken as an indicator for healthy synaptic inhibition. For intracellular recordings the input resistance of the cell was greater than 30 MΩ measured as described in section 2.6 below. Action potentials overshot 0 mV and the cells exhibited some degree of SFA (an accommodation of action potential firing when a positive current step e.g. + 0.3-0.5 nA, 300ms was applied to the cell). In all experiments care was taken that both the recorded response and the passive membrane properties of intracellularly recorded neurones were stable for a period of 10–15 min before any physiological or pharmacological protocol was employed. All cells were held at a membrane potential of –67 mV throughout the experiment using current injection (except for agonist experiments where changes in the membrane potential were under examination).

2.6 EXPERIMENTAL DESIGN

During the period between stimuli the input resistance and the extent of SFA of each neurone were measured routinely using 300–600 ms long negative and positive current steps (± 0.1–0.4 nA), respectively. In all experiments in which $EPSP_M$s (Fig. 2.4), or sub-threshold stimulation induced reductions in SFA, were evoked, baseline recordings comprised either successive EPSPs which had peak amplitudes that differed by no more than 15 %, or reductions in SFA, that were consistent over 20–30 min, respectively. In all experiments where the effects of drugs on V_m were assessed, stable baseline recordings, where the V_m and input resistance of the cell varied no more than 1 mV and 10 % respectively over a period of 10–30 min, were obtained prior to application of the drug.

In other experiments where it was required to compare the EPSPs evoked in the presence and absence of a drug at the same V_m, DC was injected through the electrode to compensate for any drug-induced changes in membrane potential.

A minimum inter-response interval of 8 min between successive $EPSP_M$s was required to avoid run-down of the response. This placed limitations on this type of experiment, for instance, it was very difficult to construct input/output curves and thus find a 50% maximal response for each experiment as this would take considerable time and given the difficulty of maintaing a viable cell for the length of time it takes to conduct an experiment it was deemed to be unfeasible to adopt this approach. Generally, responses were chosen which exhibited a peak amplitude of approximately 8-10 mV. In addition, it wasn't possible to examine tonicity, i.e. inhibitory neuromodulators released during the first stimulation on an $EPSP_M$ having an effect on the second $EPSP_M$.

For all LTP experiments, 4–8 field EPSPs were averaged together in a two minute period. A 20 minute stable baseline comprised of successive field EPSP slopes that differed no more than 15 %. At this point a tetanus was delivered to the slice and the recording of EPSPs was continued for 1–4 h depending on the LTP induction protocol employed.

2.7 ANALYSIS OF DATA

Analysis of intracellularly recorded responses usually comprised the measurement of the peak amplitude or the slope of the rising phase of the recorded response (extracellular field-EPSPs). The synaptic responses analyzed were averages of four to eight successive responses in the case of ionotropic glutamate and GABA receptor-mediated responses or single responses in the case of mAChR-mediated responses. Average responses were used to reduce noise and mean the effects of biological variation.

The data-handling software package Sigmaplot (Version 3.01; Jandel Scientific, USA) was run on an IBM PC (Dell) and was used to generate single plots and plots of means. Pooled data are presented as means ± standard error of the mean (S.E.M.) and statistical significance was assessed using a paired or unpaired Students *t*-test performed on raw data with $P < 0.05$ being taken as indicating statistical

significance. *n* values refer to the number of times a particular experiment was performed, each in a different slice taken from a different rat or mouse.

2.8 LacZ STAINING

Transgenic mouse hippocampal slices were placed in a frshly prepared fixing solution comprising of 0.2% (v/v) gluteraldehyde, 5 mM EGTA (pH 7.3), 2 mM $MgCl_2$ in a 0.1 M sodium phosphate buffer (pH 7.3). After 7 minutes, the fixed tissue was transferred to a wash solution comprising 2 mM $MgCl_2$, 0.01 % (v/v) deoxycholate and 0.02 % (v/v) Nonidet-P40 in a sodium phosphate buffer. They were left for a further 7 minutes before being transferred to a fresh wash solution. This procedure was repeated until the slices had been washed 3 times at which point the tissue was placed in X-gal stain. The stain comprised 1 mg/ml X-gal (Bethesda Research Labs), 0.212 % (w/v) potassium ferrocyanide (Sigma, UK) and 0.164 % (w/v) potassium ferricyanide (Sigma, UK) in a 0.1 M sodium phosphate buffer. The slices were then left overnight at 37 °C whereupon they were transferred into wash buffer to allow further development of the stain.

2.9 DRUGS

Drugs were stored frozen in stock aliquots (100 µl to 5 ml) of 100 to 10000 times final concentration and dissolved in ACSF, deionised water, 25 mM NaOH, ethanol or Dimethyl sulphoxide (DMSO) (Sigma, UK). All drugs were added to the control ACSF and administered by bath perfusion for at least 15 min to allow their full equilibration within the slice.

Atropine, 8-bromoadenosine 3,5-cyclic monophosphate (8-Br cAMP), 2-chloroadenosine (CADO), carbachol (CCh), physostigmine, picrotoxin and [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-Enkephalin (DAGO) were purchased from Sigma (Poole, UK). 6-nitro-7-sulphamoylbenzo[*f*]quinoxaline-2,3-dione (NBQX), Oxotremorine M, and 4-Ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride (ZD7288)

were purchased from Tocris Cookson Ltd. (Bristol, UK). 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), (4-[[[(3-Chlorophenyl)amino]carbonyl]oxy]-N,N,N-trimethyl-2-butyn-1-aminium chloride) (McN-A-343), Telenzepine Dihydrochloride, 7β-Acetoxy-8,13-epoxy-1α,6β,9α-trihydroxy-labd-14-ene-11-one (Forskolin), 2-[(4-Ethoxyphenyl)methyl]-N,N-diethyl-5-nitro-1H-benzimidazole-1-ethanamide hydrochloride (Etonitazene), (1S-*trans*)-3,4-Dichloro-N-methyl-N-[2-(2-(1-pyrrolidinyl) cyclohexyl]-benzeneacetamide hydrochloride (U-50488) were purchased from Research Biochemicals International (MA, USA).

Muscarinic toxins MT-1, MT-2, MT-3 and calcium channel blockers, ω-conotoxin GVIA and ω-Agatoxin TK were purchased from Alomone (Israel) through distributers TCS Biologicals LTD (Botolph Claydon, UK).

Galanin (rat), ω-agatoxin IVa and (D-Thr^2)-Leu-enkephalin-Thr (DTLET) were purchased from Bachem (USA).

(+)-Himbacine and N-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89) were purchased from Calbiochem (CA, USA).

D-(*E*)-2-Amino-4-methyl-5-phosphono-3-pentanoic acid (CGP 40116) and [1-(*S*)-3,4-dichlorophenyl)ethyl]amino-2-(*S*)-hydroxypropyl-p-benzyl-phosphonic acid (CGP 55845A) were gifts from Ciba-Geigy Ltd. (now Novartis; Basle, Switzerland).. 11-2[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11,-dihydro-6H-pyrido[2,3b] [1,4]benzodiazepin-6-one (AFDX 116) was a gift from Boehringer Ingelheim (Berkshire, UK).

CHAPTER 3

INVESTIGATION OF mAChR SUB-TYPES MEDIATING THE $EPSP_M$

3.1. INTRODUCTION

Within this chapter I investigated the response generated in CA1 pyramidal neurons by ACh release from septohippocampal afferents in the hippocampal slice preparation. As such, a background to ACh receptors, their pharmacology, and the functional consequences of their activation within the CNS will be covered in some detail.

3.1.1. Historical

The bioactive nature of acetylcholine (ACh) was first discovered when adrenal extract reduced blood pressure in rabbits (Hunt & Taveau, 1906). Subsequently, pharmacological studies distinguished the nicotinic and muscarinic actions of ACh (Dale, 1914) and firm evidence that ACh acted as a chemical transmitter was provided in the vagal innervation of the heart (Loewi & Navratil, 1926). Dale and coworkers later proposed that ACh was acting within the sympathetic and parasympathetic nervous systems (Dale, 1934; Dale *et al.*, 1936) and also hypothesised that ACh acted centrally. This theory was based on the presence of acetylcholinesterase (AChE) in the CNS, the synthesis and release of ACh here, and its subsequent central effects and those effects of eserine, an inhibitor of AChE (Dale, 1938; Feldberg, 1945).

However, it was not until Eccles and coworkers (1954; 1956) demonstrated that ACh acts between motor-axon collaterals and Renshaw cells in the spinal cord that the

presence of cholinergic synapses was established. The study of cholinergic mechanisms have been furthered by advances in pharmacological and molecular cloning techniques (Brann *et al.*, 1993) elucidating the presence of multiple receptor subtypes of both nicotinic ACh receptors (nAChRs) and muscarinic ACh receptors (mAChRs) in the central and peripheral nervous system.

Due to the wide range of physiological functions performed by ACh, it is beyond the scope of this body of work to fully explore this subject. Instead, I will focus on the actions of ACh in the brain and specifically the cholinergic neurons of the septohippocampal pathway.

3.1.2. Acetylcholine Receptors

The receptors that mediate nicotinic and muscarinic responses have entirely different structures and functions. nAChRs are ligand-gated ion channel (ionotropic) receptors whereas mAChRs are guanine nucleotide binding (G)-protein coupled (metabotropic) receptors

3.1.2.1. Muscarinic acetylcholine receptors

Muscarinic ACh receptors (mAChRs) belong to the G-protein coupled receptor superfamily (Hall, 1987). Along with other receptors, such as the adenosine receptor, the β-adrenergic receptor and rhodopsin, mAChRs possess a structure characterised by an extracellular N-terminal region, seven transmembrane spanning domains and an intracellular C-terminal region. Initial classification of the mAChR-mediated response was by the antagonistic effect of atropine (Dale, 1914). More recently, pharmacological investigation led to the identification of two subtypes of muscarinic receptor, termed M_1 and M_2, based on their relative sensitivities to pirenzipine (Hammer *et al.*, 1980). This distinction was subsequently clarified when the molecular cloning of both receptors (termed m1 and m2) revealed structural differences (Kubo *et al.*, 1986). In addition, three more muscarinic receptor subtypes

have been identified by homology cloning (termed M_3, M_4 and M_5) (Bonner *et al.*, 1987; Bonner *et al.*, 1988).

Despite this molecular classification the further pharmacological study of these five mAChR subtypes has been hampered by the limited selectivity of the compounds available, typically exhibiting a 10-fold selectivity for one subtype over another. For instance, pirenzipine demonstrates relative selectivity for M_1 mAChR whereas AFDX 116 shows selectivity for M_2 mAChRs (Giachetti *et al.*, 1986). In addition, 4-DAMP possesses moderate selectivity for the M_3 mAChR (Doods *et al.*, 1987). These antagonists have been employed to narrow down the identity of the mAChR mediating a physiological response but fail to conclusively identify the exact subtype (Pitler & Alger, 1990; Mitchelson, 1988). As such, there is a lack of good evidence identifying muscarinic receptor subtypes, particularly M_4 and M_5. As a result there is some controversy over which subtype(s) may mediate the variety of mAChR-mediated effects evoked by either carbachol, an agonist of AChRs, or by synaptically released ACh (Cole & Nicoll, 1984b, Benson *et al.*, 1988; Dutar and Nicoll, 1988a; Pitler & Alger, 1990). Table 3.1 provides a summary of mAChR subtypes and their most selective antagonists.

In addition to the poor selectivity of drugs for mAChRs, the existence of allosteric binding sites further complicates the pharmacology of mAChRs (Tucek & Proska, 1995). Neuromuscular blocking agents, such as gallamine, allosterically inhibit the actions of mAChR agonists at all five mAChRs and particularly the M_2 subtype (Ellis *et al.*, 1991). In contrast, alcuronium can augment, as well as inhibit, the binding of mAChR ligands (Tucek *et al.*, 1990).

Recently, it has been found that the polypeptides comprising the green mamba snake toxin possess varying selectivity and high affinity for specific mAChR subtypes (Jerusalinsky & Harvey, 1994). Eight such polypeptides have been characterised, of which three are commercially available. Muscarinic toxin 1 (MT-1) displays selective agonism for m1 and m4 receptors whereas MT-2 displays agonism at the m1 receptor only (Kornisiuk *et al.*, 1995). MT-3 is a selective antagonist of the m4 receptor with some activity at m1 (Jolkkonen *et al.*, 1994).

Table 3.1. mAChR subtypes: Pharmacology and transduction mechanisms.

mAChR (molecular subtype)	**M_1 (m1)**	**M_2 (m2)**	**M_3 (m3)**	**M_4 (m4)**	**M_5 (m5)**
Selective Antagonists	Pirenzipine Telenzipine	Methoctramine Himbicine AFDX 116 Tripitramiune	4-DAMP Darifenacin	Tropicamide Himbicine	None Known
G-protein Coupling	G_q/G_{11}	G_i/G_o	G_q/G_{11}	G_i/G_o	G_q/G_{11}
Signal Transduction Mechanisms	$\uparrow IP_3/DAG$	$\downarrow$cAMP	$\uparrow IP_3/DAG$	$\downarrow$cAMP	$\uparrow IP_3/DAG$
mAChR Protein/mRNA Expression in Hippocampus †	+++++	+++	++	+++	++
Expression of mAChR Proteins in the Hippocampus ‡	soma & dendrites of pyramidal neurones	Nonpyramidal neurones and in fibers surrounding pyramidal neurones	pyramidal neurones	Nonpyramidal neurones	no data

† (Brann *et al.* 1993); ‡(Levey *et al.* 1995).

The lack of specificity of muscarinic ligands limits the localisation of mAChRs by binding and autoradiographical studies (Cortés & Palacios, 1986; Waelbroeck *et al.*, 1990; Zubieta & Frey, 1993; Levey *et al.*, 1994). However, such studies in concert with *in situ* hybridisation and immunocytochemical analysis have elucidated regional distributions of mAChRs in the CNS, including the subfields of the hippocampus (Buckley *et al.*, 1988; Weiner & Brann, 1989; Levey *et al.*, 1995). All five subtypes of mAChR protein and mRNA have been detected in the hippocampus as well as the cortex, striatum, olfactory tubercle, thalamus and basal forebrain (Brann *et al.*, 1993). m1 predominates in the hippocampus, followed by m2/m4 receptors and then m3/m5 receptors. The cellular distribution of mAChR subtype protein and mRNA is varied within the hippocampus, with m1 and m3 mAChR protein expression prominent in pyramidal neurons whereas m2 and m4 are localised to non-pyramidal neurons and fibre pathways (Levey *et al.*, 1995). Interestingly m2 mAChR proteins are expressed

presynaptically in cholinergic axons, presumably of the septo-hippocampal pathway (Levey *et al.*, 1995). The localisation of m2 mAChR mRNA in the cells of the medial septum supports this conclusion (Buckley *et al.*, 1988; Rouse *et al.*, 1997).

As already stated, mAChRs couple to G-proteins. In general, M_1, M_3 and M_5 mAChRs have been found to exert their effects through the G-protein $G_{q/11}$, to activate phospholipase C (PLC) and thus the inositol triphosphate (IP_3) messenger system whereas M_2 and M_4 mAChRs act via G-protein $G_{i/o}$, leading to decreased adenylate cyclase activity and, thus, reduced intracellular cAMP (Hulme *et al.*, 1990; Felder, 1995). It is clear that the functional mechanisms activated by mAChRs are diverse and could result in numerous physiological effects such as both increased and decreased neurotransmitter release (see Table 3.1).

3.1.2.2. Nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors (nAChRs) are members of the superfamily of ion channel receptors which include $GABA_A$ and glycine receptors. nAChR-mediated responses can be pharmacologically distinguished from mAChR-mediated responses using the competitive antagonist curare (Dale, 1914). A broad classification of nAChRs can be made based on anatomical location and pharmacology: (1) muscle nAChRs, (2) neuronal nAChRs that bind α-bungarotoxin and (3) neuronal nAChRs that do not bind α-bungarotoxin (Lindström *et al.*, 1995).

The structure of the ACh-gated cation channel consists of a pentamer of homologous subunits forming a central ion channel (McCarthy *et al.*, 1986; Hucho *et al.*, 1996). In addition to the subunits which form muscle and ganglionic nAChRs, eight neuronal α subunits and three β subunits have been cloned (Albuquerque *et al.*, 1997) and are proposed to form heterooligomeric complexes with a stoichiometry of 2α, 3β or homomeric complexes of α7 or α9 subunits. Receptors of different subunit composition display different pharmacological and biophysical properties (Deneris *et al.*, 1991; Alkondon & Albuquerque, 1993; McGehee & Role, 1995; Albequerque *et al.*, 1995a). Only α7 and to a lesser extent α9 are found in the rat. α7 nAChR is

thought to be of particular functional importance due to its high Ca^{2+} permeability and presence at presynaptic terminals.

A paucity of selective ligands for the various nAChR subunit assemblies has restricted their functional characterisation within the CNS. However, it is known that nAChRs modulate at least three separate cationic conductances, termed types I-III, (Alkondon & Albuquerque, 1993, Albuquerque *et al.*, 1995a, Albuquerque *et al.*, 1997) that can be distinguished by their channel kinetics, sensitivity to nicotinic receptor agonists and antagonists such as methylcaconitine, β-erythroidine, α-bungarotoxin and mecamylamine (Albuquerque *et al.*, 1995b; Albuquerque *et al.*, 1997).

Nicotinic AchRs are present pre- and post-synaptically. There is evidence to show that nAChRs act as modulators of presynaptic neurotransmitter release and glutamate synaptic transmission in several brain regions, including the hippocampus (Araujo *et al.*, 1988; Gray *et al.*, 1997; Wonnacott, 1997).

3.1.3. Functional Characterisation of Muscarinic ACh Receptor Activation in the CNS

Muscarinic AChR activation in the CNS results in a variety of physiological responses that are both inhibitory and excitatory (Brown *et al.*, 1997). The main effects include (1) the inhibition/activation of specific K^+ conductances, (2) the inhibition/activation of voltage-dependent Ca^{2+} currents, and (3) activation of non-selective cationic currents.

3.1.3.1. Effects on K^+ channels

mAChR-mediated modulation of K^+ channels has been extensively studied in the hippocampus Synaptic activation of mAChRs on CA1 pyramidal cells produces a slow depolarisation accompanied by an increase in cell input resistance (Cole & Nicoll, 1983; 1984a). This response is similar to that seen by the application of carbachol or exogenous ACh on hippocampal or cortical slices (Dodd *et al.*, 1981;

Haas, 1982; Segal, 1982a; Cole & Nicoll, 1983, 1984a & 1984b). Evidence suggests that the depolarisation observed in these studies is due to an apparent inward current (Halliwell & Adams, 1982; Madison *et al.*, 1987; Benson *et al.*, 1988) which represents a decrease in a voltage-independent *leak K^+ current* ($I_{K(LEAK)}$) (Benardo & Prince, 1982b; Madison *et al.*, 1987; Benson *et al.*, 1988).

Another current affected by mAChR activation is the voltage independent and Ca^{2+} dependent K^+ current ($I_{K(Ca)}$) known as the small-K current or IAHP in a variety of brain regions (Madison & Nicoll, 1984; Constanti & Sim, 1987; Lancaster & Adams, 1986; Sah, 1996) whereas the voltage and calcium-dependent current (maxi-K current) I_C apparently is not affected (Madison *et al.*, 1987). Hippocampal CA1 pyramidal cells demonstrate a fast and slow afterhyperpolarisation following an action potential. These responses occur as a result of Ca^{2+} entry during the depolarisation phase and are mediated by I_C and I_{AHP} respectively. During a sustained depolarisation sufficient to generate action potential, the slow AHP following each successive action potential summates due to the slow inactivation of I_{AHP} (>1s) resulting in a longer refractory period between action potentials. This phenomenon is known as Spike Frequency Adaptation (SFA). Carbachol has been shown to consistently inhibit I_{AHP} and thus the slow AHP resulting in decreased SFA (Bernado & Prince, 1982a & 1982b; Cole & Nicoll, 1983; Madison *et al.*, 1987; Dutar & Nicoll, 1988a). Furthermore, the same inhibition of the SFA can be seen after stimulation of cholinergic afferents resulting in synaptic release of ACh in area CA1 of the rat hippocampus (Cole & Nicoll 1983, 1984a; Nicoll, 1985). Taken together, it is evident that evoked synaptic ACh release causes a depolarisation of the membrane potential, accompanied by an increase in cell input resistance due to the inhibition of $I_{K(LEAK)}$ and a decrease in SFA as a result of I_{AHP} inhibition.

Other K^+ channels that have been implicated in mAChR-mediated responses include the *M current* (I_M), a voltage-dependent K^+ current which does not inactivate over time. First described in bullfrog sympathetic neurons (Brown & Adams, 1980; Brown, 1983), I_M was shown to be inhibited by carbachol and other muscarinic agonists in the hippocampus (Halliwell & Adams, 1982; Brown, 1983; Madison *et al.*, 1987; Benson *et al.*, 1988; Dutar & Nicoll, 1988a; Halliwell, 1990). Inhibition of I_M was shown to be 10-fold less sensitive to carbachol agonism than the inhibition of

I_{AHP} (Madison *et al.*, 1987). Consequently, synaptically released ACh did not achieve inhibition of I_M as readily as the inhibition of I_{AHP} or the $I_{K(LEAK)}$. However, in the bullfrog sympathetic ganglion cells, I_M is more readily inhibited than I_{AHP} by mAChR agonism (Pennefather *et al.*, 1985).

There is also evidence for a mAChR-mediated inhibition of a fast, transient, voltage-dependent K^+ conductance called the *A current* (I_A) which acts to increase action potential repolarisation and to slow the firing rate (Nakajima *et al.*, 1986). In neostriatal neurons mAChR activation inhibits the I_A, which at hyperpolarised potentials tends to reduce action potential firing frequency whereas at depolarised potentials mAChR activation results in the complete inactivation of I_A thereby increasing neuronal excitability (Akins *et al.*, 1990).

Two K^+ channels whose activation is potentiated by mAChR agonism in the hippocampus are the *delayed rectifier K^+ current* (I_K) (Zhang *et al.*, 1992) and the *anomalous inward rectifying current* (I_Q), which is mediated by both K^+ and Na^+ ions (Colino & Halliwell, 1993).

3.1.3.2. Effects on Ca^{2+} and nonselective cation channels

There is limited evidence for the involvement of Ca^{2+} channels in mAChR-mediated responses. Inhibition of Ca^{2+} currents by mAChR activation has been observed in area CA3 of hippocampal slice cultures (Gähwiler & Dreifuss, 1982; Gähwiler & Brown, 1987) as well as in the cortex and neostriatum (Misgeld *et al.*, 1986). mAChR activation has been shown to inhibit high voltage activated Ca^{2+} currents in rat embryonic hippocampal neurones (Toselli & Taglietti, 1995) and in excised membranes of hippocampal neurons (Toselli & Taglietti, 1994). In the superior cervical ganglion (SCG) and basal forebrain neurons mAChR-activation inhibits N-type Ca^{2+} channels (Plummer *et al.*, 1991). In the guinea pig hippocampus, one study suggests that mAChR activation at mossy fibre-CA3 synapses results in an increased probability of opening of T- but not N-type Ca^{2+} channels and a decreased probability of opening of L-type Ca^{2+} channels (Fisher & Johnston, 1990). There are also reports of L-type Ca^{2+} channel inhibition in the SCG (Mathie *et al.*, 1992) and

the cerebral cortex (Boess *et al.*, 1990). In contrast, blockade of L-type Ca^{2+} currents inhibited mAChR-mediated membrane depolarisation of CA1 pyramidal cells in the guinea pig hippocampus (Blitzer *et al.*, 1991).

Using mAChR cDNA-transfected neuroblastoma hybrid cells, it has been suggested that m2 and m4 but not m1 and m3 mAChRs mediate the inhibition of an "N" current (Higashida *et al.*, 1990; Brown *et al.*, 1993). The "N" current, although somewhat differing from the current conducted by the N-type Ca^{2+} channel, is likely to correspond to the Ca^{2+} current responsible for neurotransmitter release and therefore it has been suggested that this is how m2/m4 mAChR activation results in presynaptic inhibition of neurotransmitter release (Wu & Saggau, 1997).

Conversely, mAChR activation is reported to activate a low threshold transient (T-type) Ca^{2+} current in interneurons within the CA1 *laculosum-moleculare* region of the hippocampus as well as a novel regenerating current which underlies a plateau potential involving a high voltage-activated Ca^{2+} conductance and a Ca^{2+} activated nonselective cation conductance (Fraser & MacVicar, 1991). mAChR-mediated depolarisation of CA1 and CA3 pyramidal neurons through the activation of nonselective cationic conductances has also been demonstrated in hippocampal slices and slices cultures (Colino & Halliwell, 1993; Caesar *et al.*, 1993; Guérineau *et al.*, 1995).

3.1.3.3. Physiological consequences of mAChR activation on hippocampal function

mAChR activation has been reported to inhibit glutamate and GABA release at synapses in the CA1 (Hounsgaard, 1978; Valentino & Dingledine, 1981; Dutar & Nicoll 1988a; Cohen *et al.*, 1991) and CA3 regions (Williams & Johnston, 1988) of the hippocampus. This presynaptic inhibition can potentially result from K^+ channel activation, Ca^{2+} channel inhibition or through the modulation of the Ca^{2+} dependent release machinery (see Chapter 1). It has been suggested that mAChR-mediated presynaptic inhibition in area CA1 is mainly mediated by the inhibition of voltage-dependent Ca^{2+} channels (Qian & Saggau, 1997).

In addition, cholinergic activity has been implicated in the modulation of synaptic plasticity. mAChR activation enhances long-term potentiation (Blitzer *et al.*, 1990) and has been shown to cause a long lasting potentiation, termed LTP_M, in its own right (Auerbach & Segal, 1996). The role that mAChRs may play in LTP and, consequently, learning/memory has been reviewed in some detail (Krnjevic, 1993; Aigner, 1995; Segal & Auerbach, 1997).

At the level of neural circuitry, carbachol application can induce a theta-like rhythm (Konopacki *et al.*, 1987, Bland *et al.*, 1988) which consists of synchronised membrane potential oscillations and rhythmic action potential firing at a frequency of 4-10 Hz. It is this rhythm that is observed in the EEG of the learning rat learning rat and, as such, disruption of the theta-rhythm impairs performance in spatial memory tasks (Winson, 1978). In addition, mAChR activation can induce a related phenomenon called rhythmic slow activity (RSA). This has been observed in CA3 pyramidal neurons and manifests itself as periodic bursts of 4-8 Hz oscillatory depolarisations (MacVicar & Tse, 1989; Traub *et al.*, 1992).

At high concentrations of carbachol, Ca^{2+}-dependent changes in the action potential waveform have been observed (Figenschou et al., 1996). It is clear that cholinergic action in the hippocampus is complex and important in the functional processing of neural information within the hippocampus.

The aim of this chapter is to characterise the responses obtained from the rat hippocampal slice and, further, investigate the different mAChR subtypes that may be responsible for generating these responses.

3.2. RESULTS

3.2.1. Characterisation of Acetylcholine Receptor Mediated Responses

Brief (approx. 1 min) application of the acetylcholine receptor agonist, 3 μM carbachol (eg. Morton & Davies, 1997), resulted in a slow depolarisation of the membrane potential of sufficient magnitude to fire action potentials in CA1 pyramidal cells (fig. 3.1*A*). Current was injected through the microelectrode to restore the membrane potential to pre-carbachol levels enabling observation that this depolarisation was accompanied by increased cell input resistance and decreased spike frequency adaptation (SFA) (also referred to as Accommodation) in response to a depolarising current step (fig. 3.1 *B&C*). After washout of carbachol, the cell repolarised back to control membrane potential. This was accompanied by a recovery of SFA and input resistance to control levels.

This effect of carbachol could possibly be via an action at synaptic or extrasynaptic sites. To examine responses to synaptically released ACh electrical stimulation of terminals in the *stratum oriens* of the rat hippocampal slice was employed. Initially, a glutamate receptor-mediated excitatory postsynaptic potential (EPSP) followed by a GABA receptor-mediated inhibitory postsynaptic potential (IPSP) were evoked. This response was subsequently blocked using 1 μM CGP-40116, 5 μM NBQX, 50 μM picrotoxin and 1 μM CGP-55845A (eg. Morton & Davies, 1997) which are antagonists of NMDA, AMPA, $GABA_A$ and $GABA_B$ receptors respectively. In the presence of this cocktail of antagonists, spike frequency adaptation in response to a depolarising current step could be inhibited by increasing the stimulation intensity used to evoke the glutamate/GABA EPSP/IPSP complex (fig. 3.2), i.e. the current step preceding the stimulus fired action potentials separated by longer intervals than those evoked by the current step following the stimulus. This compared favorably with the loss of accommodation seen after carbachol application. This block of spike frequency adaptation was inhibited by the application of 1 μM pirenzipine (Segal & Fisher, 1992) (fig. 3.2). Spike frequency adaptation is a robust cellular response to

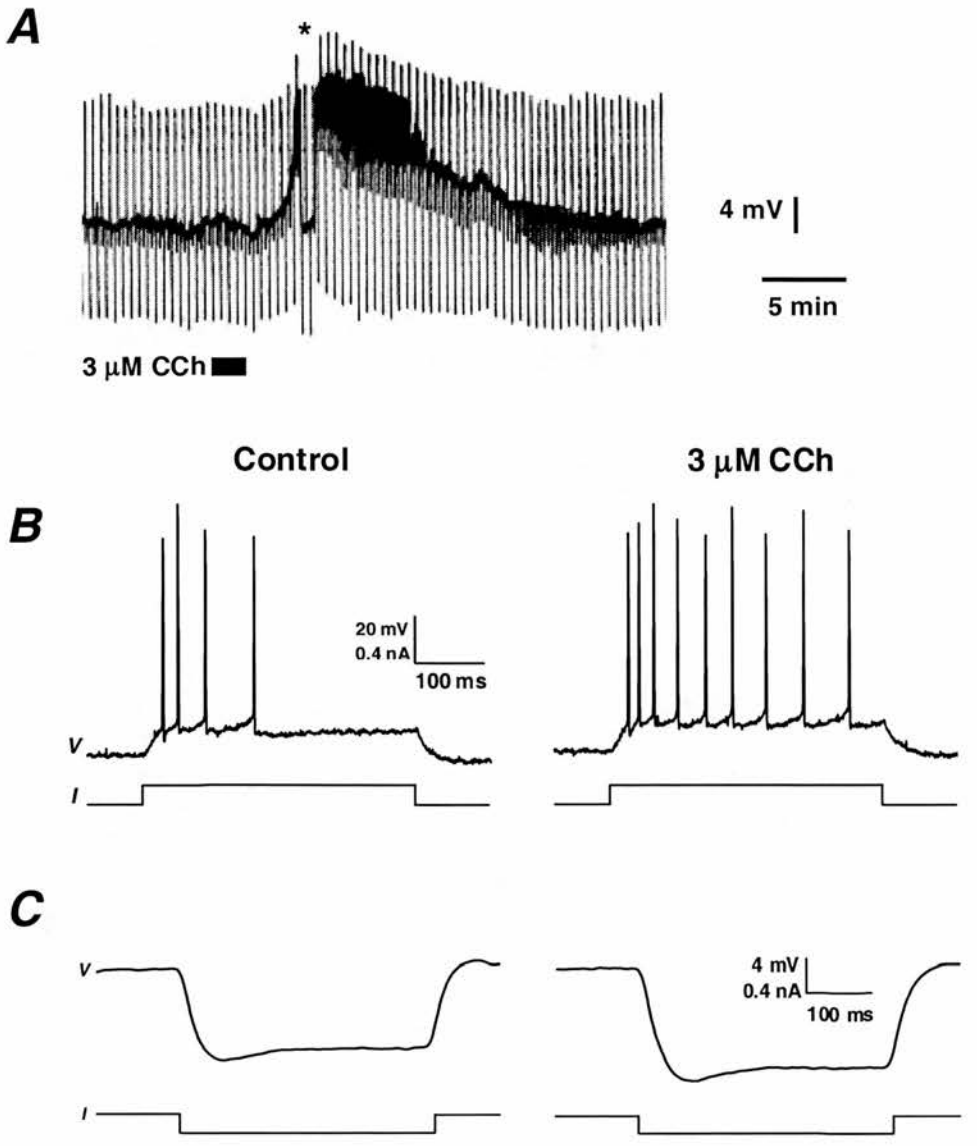


Figure 3.1 The effect of muscarinic ACh receptor activation in a CA1 pyramidal cell.

A shows a chart recording of the membrane potential of a CA1 pyramidal cell, current clamped to –67 mV and its responses to depolarising and hyperpolarising current steps. On application of 3 μM carbachol (CCh) for 1 min, a slow depolarisation of the membrane potential accompanied by spontaneous action potential firing and an increase in cell input resistance was observed. *B* illustrates spike frequency adaptation in response to a depolarising current step and the reduction of SFA in response to CCh. *C* shows the change in membrane potential in response to a hyperpolarising current step before (*left*) and after (*right*) the application of CCh. The input resistance of the cell was 35 MΩ, the resting membrane potential was –67 mV and the spike height was greater than 0 mV. Apparent variations in spike height were due to the sampling rate employed (488 μs sampling interval).

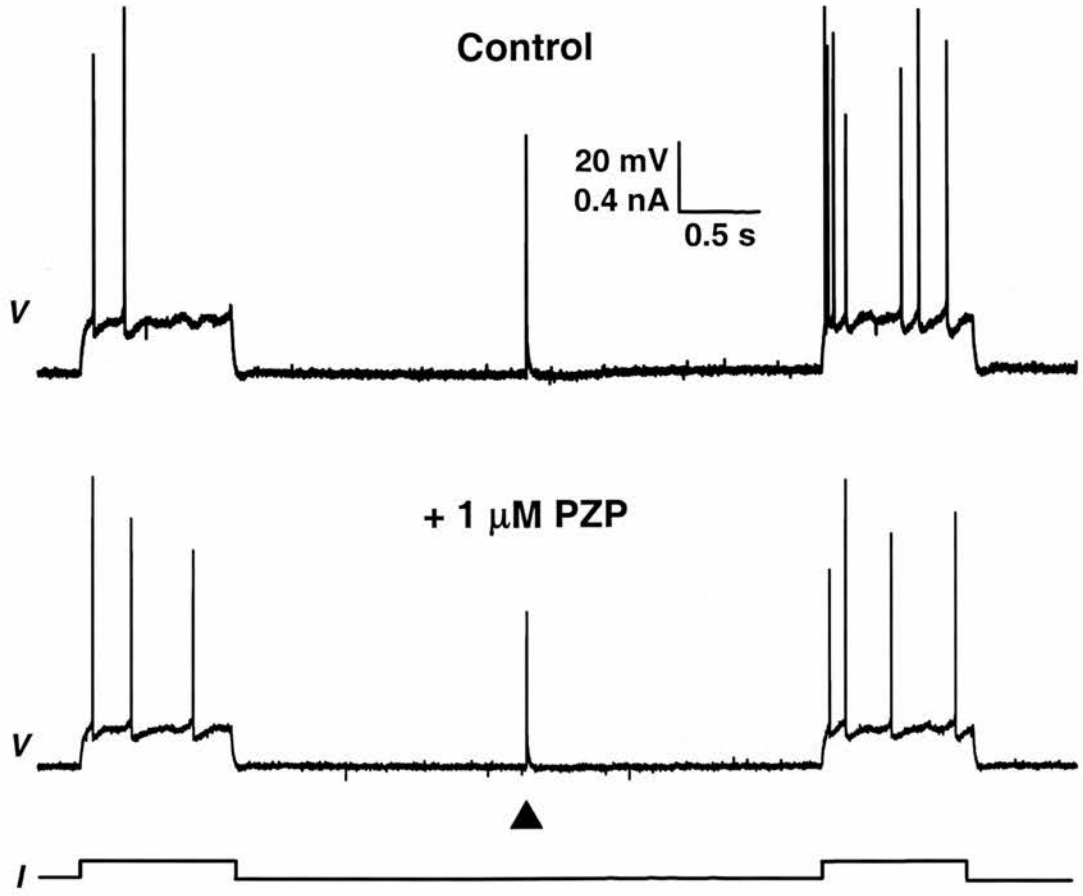


Figure 3.2 The effect of electrically released ACh on a CA1 pyramidal cell.

A shows a synaptic trace (*top*) from a CA1 pyramidal cell and its response to a depolarising current step preceding and following electrical stimulation of the stratum oriens in the presence of 1 μM CGP-40116, 5 μM NBQX, 50 μM picrotoxin and 1 μM CGP-55845A. Note that the stimulation is insufficient to cause depolarisation but does result in a loss of SFA of action potentials during the second current step. The *bottom trace* demonstrates that the loss of SFA of action potential firing during the second depolarising current step is pirenzipine-sensitive and therefore mediated by activation of mAChRs. The input resistance of the cell was 31 MΩ, the resting membrane potential was –70 mV and the spike height was greater than 0 mV. Apparent variations in spike height were due to the sampling rate employed (600 μs sampling interval).

a depolarising current step and the number of action potentials fired during the step is maintained over time as long as the membrane potential of the cell remains constant (this was achieved by adjusting the amount of continuous current injected throughout the experiment or by monitoring the membrane potential and using cells that have a steady membrane potential) (fig. 3.3).

If the stimulation intensity was further increased and/or the number of stimuli increased at a frequency of 20 Hz a slow depolarising EPSP was evoked (fig. 3.4*A*). The peak amplitude of this response could be increased by increasing the intensity of stimulation such that the cell fired intense bursts of action potentials during the response. Sometimes the EPSP depolarised the membrane potential past the action potential firing threshold and into a region in which action potential generation was inactivated.

Occasionally, the EPSP was preceded by an IPSP (fig. 3.4*A*) the nature of which is unclear. However, the EPSP was significantly blocked by 2 μM atropine (eg. Morton & Davies) (21.4 ±7.2% of control, n=6; $p<0.05$) suggesting that it was mediated by muscarinic ACh receptors. As such, this EPSP was termed an $EPSP_M$ (fig 3.4*B*).

Further pharmacological characterisation confirmed that the electrical stimulation of the *stratum oriens* released ACh from cholinergic terminals. Thus, fig. 3.5 shows that 50 μM vesamicol, an inhibitor of the vesicular ACh transporter, attenuated the peak amplitude of the $EPSP_M$, 1 μM eserine, a cholinesterase inhibitor, increased the peak amplitude of the $EPSP_M$ and 50 μM hemicholinium-3, a choline reuptake inhibitor, decreased the $EPSP_M$ (n=3) (eg. Morton & Davies, 1997; Pitler & Alger, 1990).

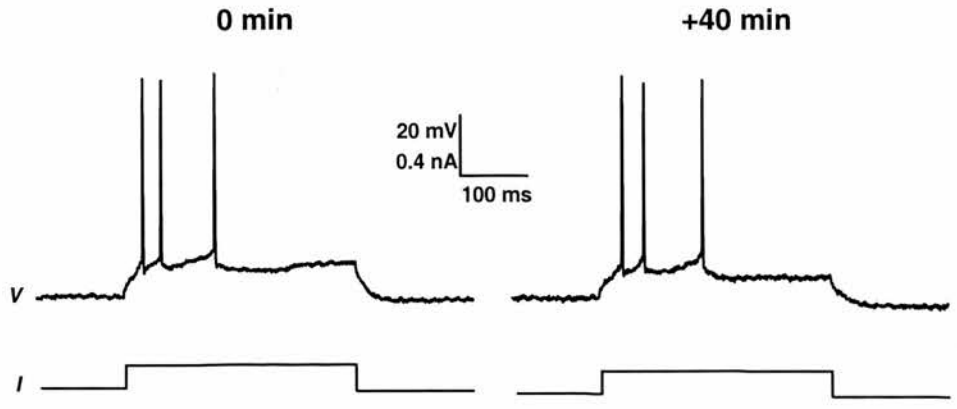


Figure 3.3 Spike Frequency Adaptation is a robust phenomena.

This figures shows intracellular recordings from CA1 pyramidal cells in response to a depolarising current step. The left trace shows the response at 0 min and the right hand trace shows the response at 40 min. Note that the number of spikes are the same and occur at similar points along the current step. The input resistance of this cell was 38 MΩ, the resting membrane potential was –70 mV and the spike heights are greater than 0 mV. The cell was held at –67 mV throughout the experiment.

3.2.2. Determination of the mAChR Subtype Responsible for Mediating the $EPSP_M$

Since it is not known which postsynaptic mAChR is responsible for the $EPSP_M$, I examined the $EPSP_M$ in the presence of different antagonists of muscarinic ACh receptors. Pirenzipine possesses selectivity for M1 receptors and, at 10 μM (Segal & Fisher, 1992), inhibited the $EPSP_M$ to 37.8 ±13.2% of control (n=3; $p<0.05$) (fig. 3.6). However, the M_1/M_4 mAChR antagonist, MT-3, at 100 nM (Jerusalinsky & Harvey, 1994), had no significant effect on the $EPSP_M$ (82 ±19% of control, n=3) (fig. 3.7). Zamifenacin is reported to have selectivity for M_3 receptors

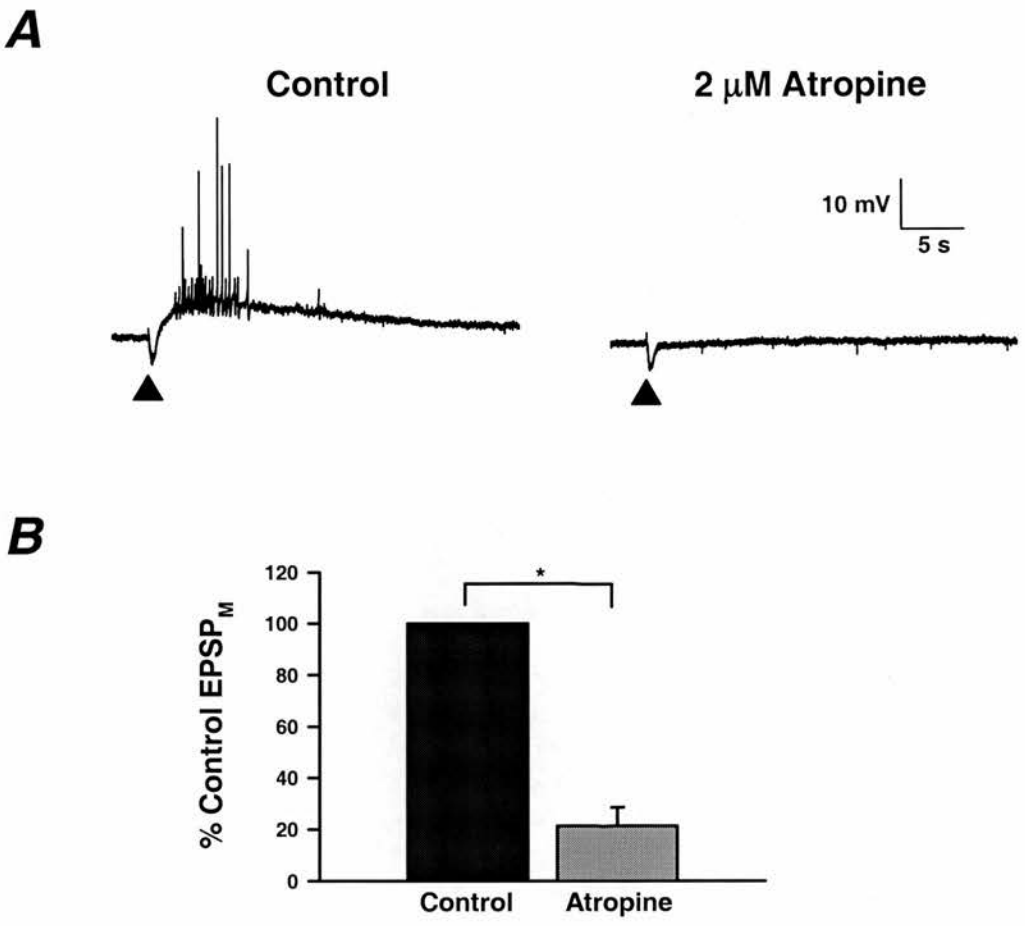


Figure 3.4 Atropine inhibits the synaptically evoked slow depolarisation

A Synaptic traces recorded intracellularly from CA1 pyramidal cells, held at –67 mV, illustrates a synaptically-evoked slow depolarisation in control media (*left*) and the absence of this depolarisation in the presence of 2 μM atropine (*right*). The filled triangles denote the position of the stimulus (10 stimuli at 20 Hz). *B* shows a bar graph illustrating the mean inhibition of the peak amplitude of the slow depolarisation by atropine in six experiments. Note that atropine almost completely blocks the slow depolarisation suggesting the response is mediated primarily by mAChRs and is thus termed an $EPSP_M$. Values are means ± S.E.M. represented by the error bars. The input resistance of the cell was 39 MΩ, the resting membrane potential was –63 mV and the spike height was greater than 0 mV.

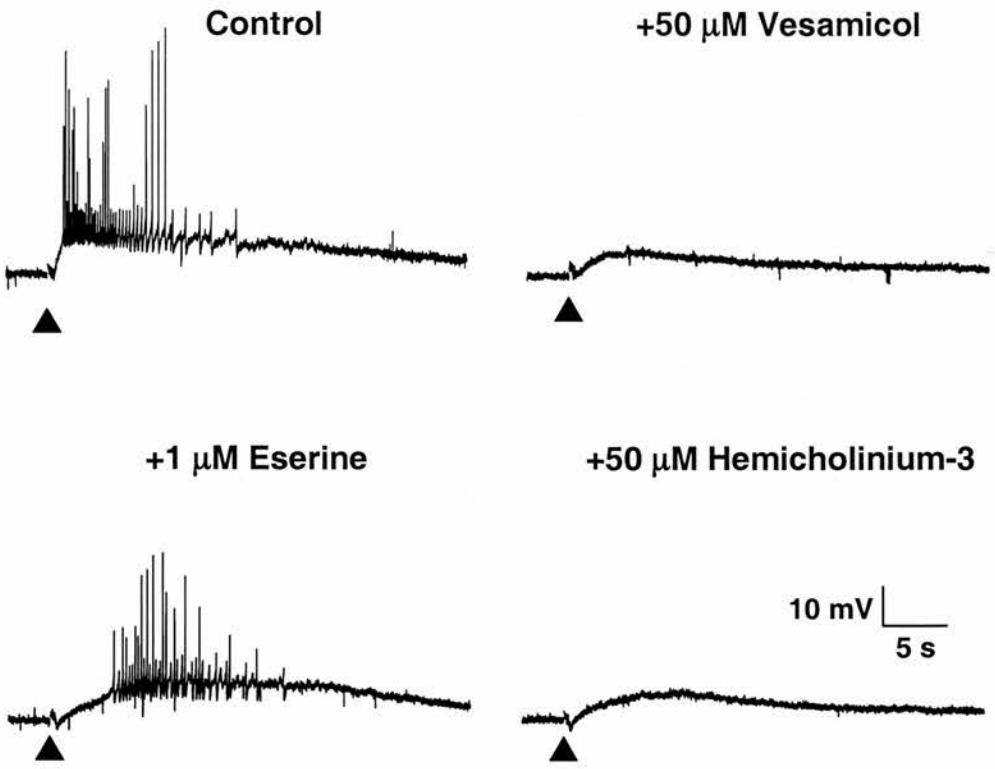


Figure 3.5 **The effect of vesamicol, eserine and hemicholinium-3 on the slow EPSP in CA1 pyramidal cells.**

Synaptic traces recorded intracellularly from CA1 pyramidal cells, held at –67 mV, illustrates the effect of 50 mM vesamicol, 1 mM eserine and 50 mM hemicholinium-3 on the slow EPSP providing evidence that this response is mediated by mAChRs and as such has been termed the $EPSP_M$. The filled triangles denote the position of the stimulus (10 stimuli @ 20 Hz). The input resistance of the cell was 33 MΩ, the resting membrane potential was –65 mV and the spike height was greater than 0 mV.

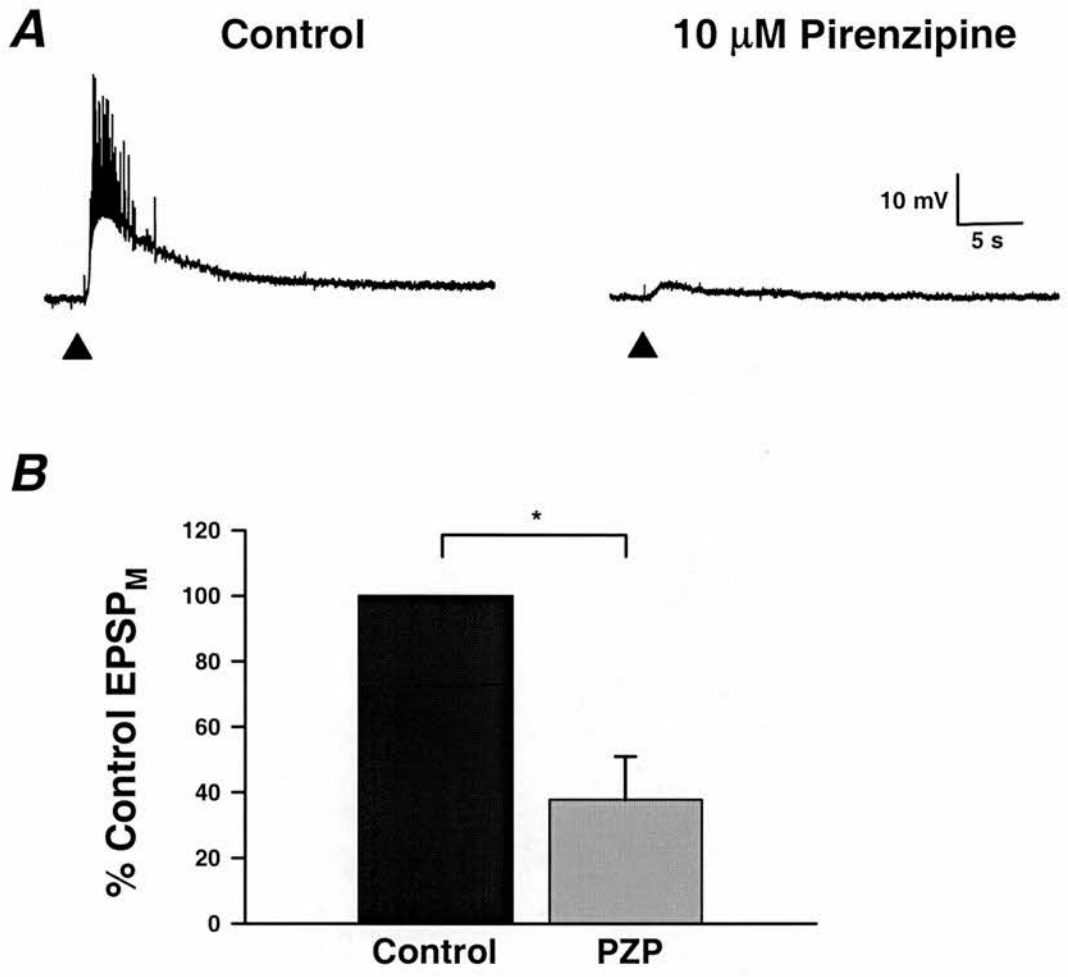


Figure 3.6 The effect of pirenzipine on the $EPSP_M$.

A shows intracellular synaptic traces recorded from CA1 pyramidal cells held at –67 mV before (*left*) and after (*right*) the application of 10 μM pirenzipine. The filled triangles denote the position of the stimulus (1 stimulus). The bar graph in *B* illustrates the mean inhibition of the peak amplitude of the $EPSP_M$ by 10 μM pirenzipine in 3 experiments. Values are means ± S.E.M. represented by the error bars. The input resistance of the cell was 33 MΩ, the resting membrane potential was –64 mV and the spike height was greater than 0 mV.

(Watson *et al.*, 1995) and this was confirmed in the guinea pig ileum preparation (fig. 3.8). However, when applied to hippocampal slices, the effects of zamifenacin were equivocal. In one cell, 1 μM zamifenacin produced a depression of the $EPSP_M$ peak amplitude whereas in another cell 1 μM zamifenacin had no significant effect upon the $EPSP_M$ which was subsequently shown to be sensitive to pirenzipine (fig. 3.9*A*). Upon averaging, no significant effect of zamifenacin upon the $EPSP_M$ peak amplitude was observed (n=5) (fig. 3.9*B*). To rule out the possibility that the magnitude of block exerted by zamifenacin was a function of the control amplitude of the $EPSP_M$, a graph plotting these two parameters against each other was generated, where 100% represents no change from the control amplitude. One high outlier represented an experiment in which the $EPSP_M$ was enhanced by zamifenacin. However, there appeared to be no correlation between the amplitude of the $EPSP_M$ and the magnitude of block by zamifenacin (fig. 3.9*C*).

As a parallel study, antagonists were tested against carbachol-induced activation of postsynaptic mAChRs. Antagonists were applied to slices following carbachol-induced depolarisation of the cell membrane potential. Following depolarisation of the cell using 3 μM carbachol, Zamifenacin was titrated upto a final concentration of 1 μM at which there was clearly no inhibition of the carbachol-induced depolarisation (n=3). In contrast, application of 1 μM pirenzipine readily repolarised the cell (n=3) (fig. 3.10).

To examine the subtype mediating cell depolarisation, a second series of experiments were performed. Toxin from the green mamba snake, *dendroaspis augusticeps*, possesses very high affinity for mAChRs. Subsequent fractionation of this toxin into its constituent polypeptides has led to a series of toxins that display high selectivity for particular subtypes of mAChR (Jerusalinsky & Harvey, 1994). Muscarinic toxin 1 (MT-1) possesses agonist affinity for M_1 and M_4 mAChRs. Application of 100-1000 nM MT-1 (Jerusalinsky & Harvey, 1994) did not produce any effect on the membrane potential, input resistance or spike frequency adaptation (n=3) (fig. 3.11). Subsequent carbachol application resulted in membrane depolarisation. However, in one experiment (see inset in fig. 3.10) 200 nM MT-1 produced a profound depolarisation that was reversed by 10 μM pirenzipine. MT-2 is similar to MT-1 in

that it possesses selective agonism for the M1 mAChR. 400-1000 nM MT-2 (Jerusalinsky & Harvey, 1994) produced no effect on the CA1 pyramidal cell (n=3) (fig.3.12).

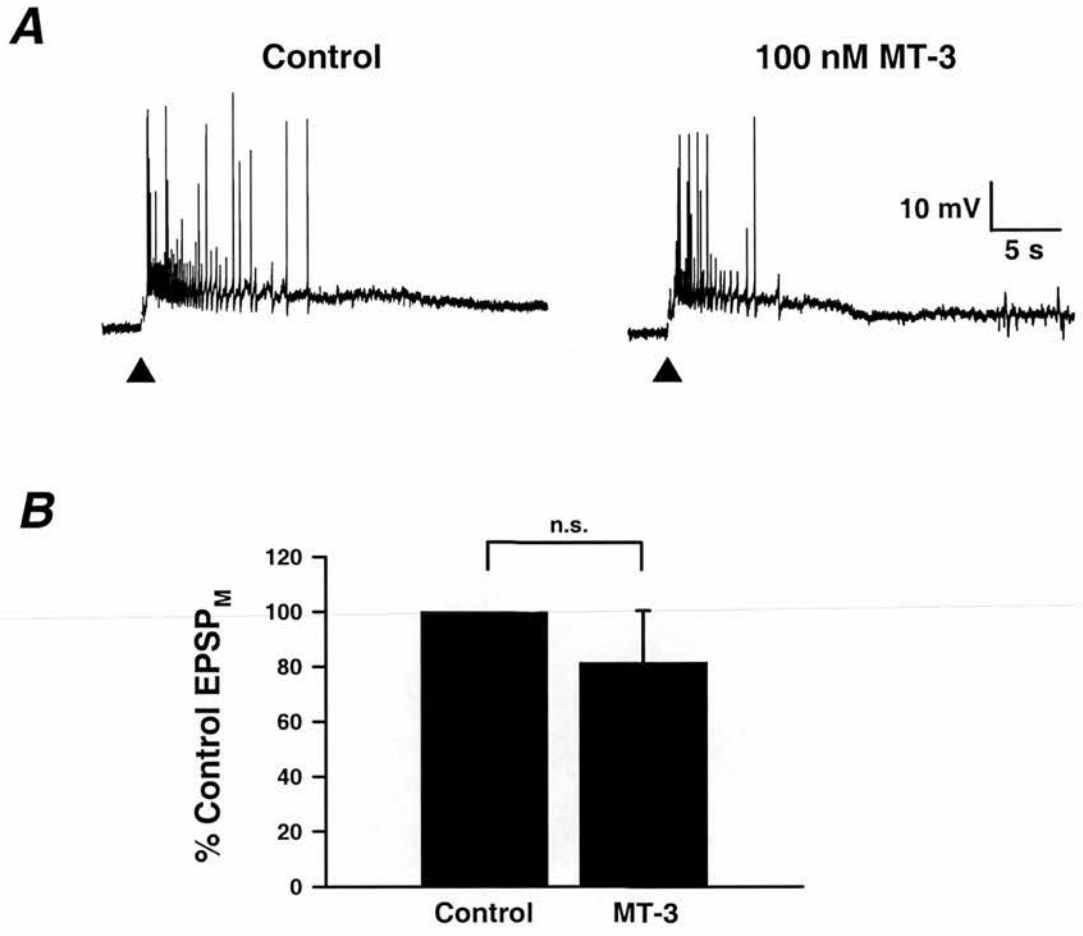
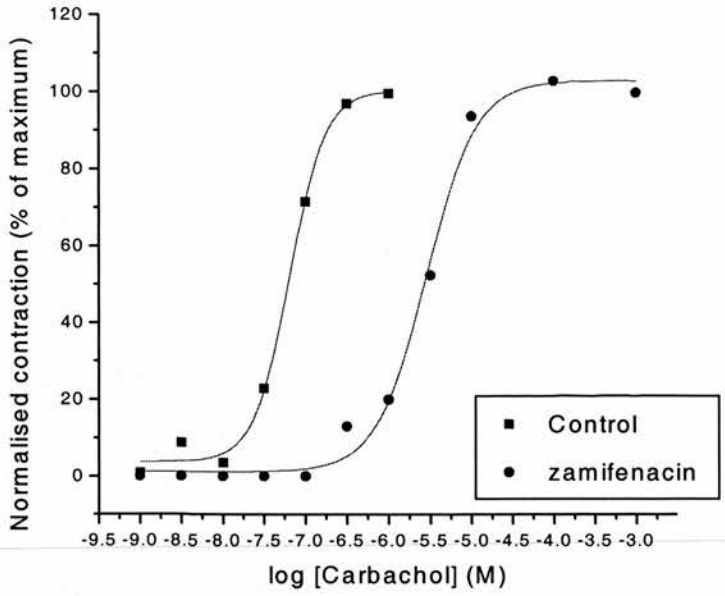


Figure 3.7 The effect of the mucarinic toxin, MT-3, on the $EPSP_M$.

A shows synaptic traces recorded intracellularly from CA1 pyramidal cells before (*left*) and after (*right*) application of 100 nM MT-3. The filled triangles denote the position of the stimulus (10 stimuli at 20 Hz). The bar graph in *B* illustrates the mean effect of 100 nM MT-3 on the peak amplitude of the $EPSP_M$ in 3 experiments. Values are means ± S.E.M. represented by the error bars. The input resistance of the cell was 30 MΩ, the resting membrane potential was –62 mV and the spike height was greater than 0 mV.



$$pA2 = \log[((2818/64.5)-1)/1e-7]$$

$$= 8.64$$

Figure 3.8 Zamifenacin displays antagonist activity in the guinea pig ileum preparation

The graph shows dose response relationships for the carbachol-induced contraction of guinea pig ileum in the presence (closed circles) and absence (closed squares) of 100 nM zamifenacin.

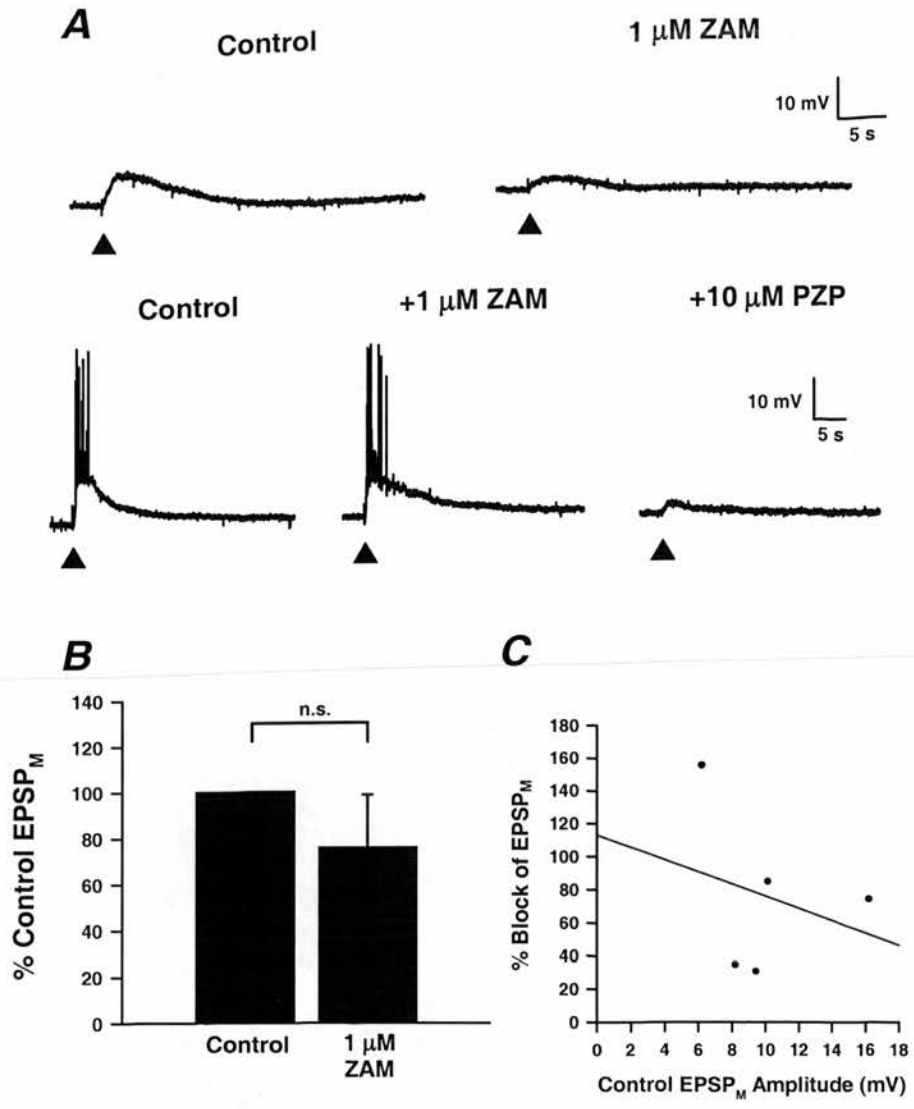


Figure 3.9 The effect of zamifenacin on the $EPSP_M$.

A demonstrates the effect of 1 μM zamifenacin on the $EPSP_M$. The top traces show an inhibitory effect of zamifenacin on the peak amplitude of the $EPSP_M$. The bottom traces illustrate no effect of zamifenacin upon the $EPSP_M$ whereas 10 μM pirenzipine substantially reduced the $EPSP_M$ The filled triangles denote the position of the stimulus (10 stimuli at 20 Hz). The bar graph (*B*) shows the mean effect of 1 μM zamifenacin on the peak amplitude of the $EPSP_M$ in 5 experiments. Values are means ± S.E.M. represented by the error bars. C shows a scattergraph plotting the control $EPSP_M$ peak amplitude against the magnitude of block exerted by zaminfenacin. 100% represents no block of the $EPSP_M$, thus the outlier at the top of the graph represents an experiment in which zamifenacin enhanced the $EPSP_M$ There appears to be no correlation between the two parameters. The input resistance of all cells was greater than 30 MΩ and the spike height was greater than 0 mV.

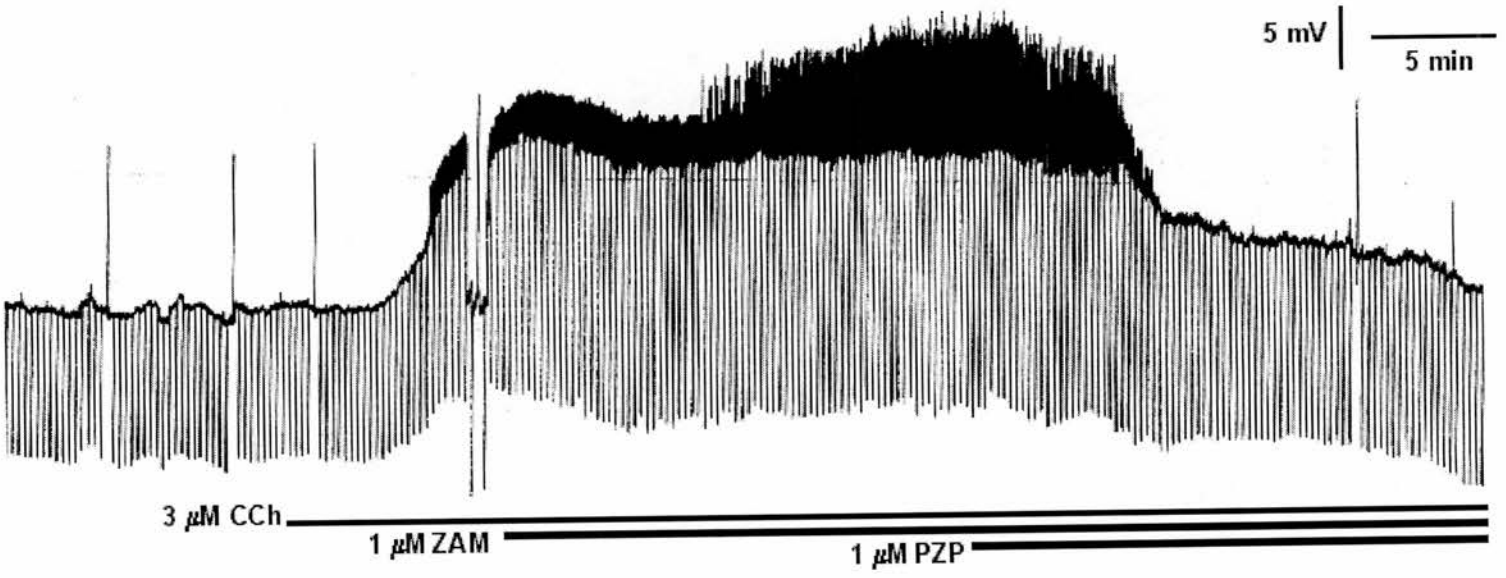


Figure 3.10 The effect of zamifenacin and pirenzipine on the carbachol-induced depolarisation of CA1 pyramidal cells.

A chart recording illustrating the membrane potential and the response to depolarising and hyperpolarising current steps of a CA1 pyramidal cell. 3 μM carbachol (CCh) causes a profound depolarisation of the membrane potential accompanied by increased input resistance. 100–1000 mM zamifenacin (ZAM) has no effect on the magnitude of depolarisation whereas 1 μM pirenzipine (PZP) repolarises the membrane potential although the input resistance is still increased. The input resistance of the cell was 35 MΩ, the resting membrane potential was –63 mV and the spike height was greater than 0 mV

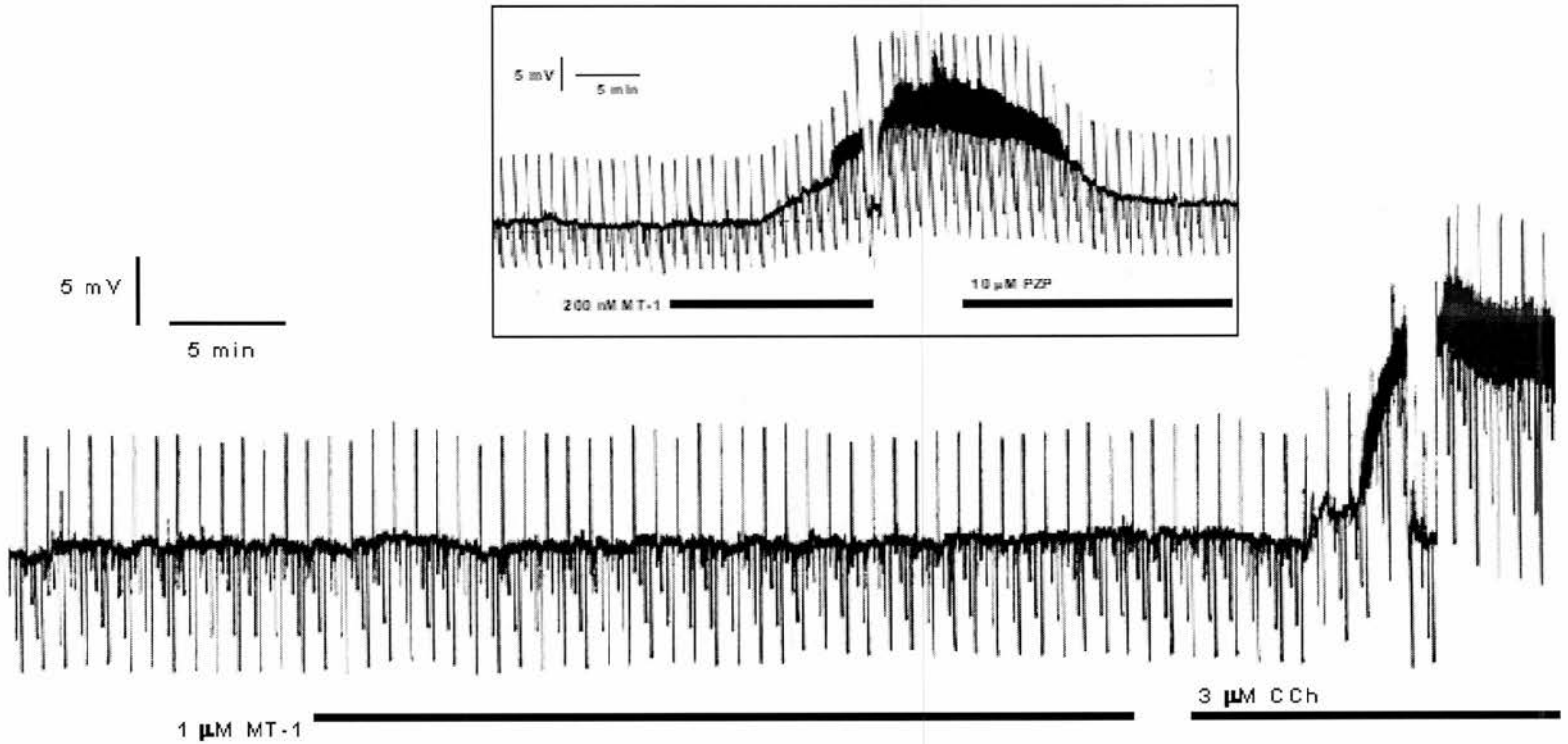


Figure 3.11 The effect of the muscarinic toxin, MT-1, on the CA1 pyramidal cell.

A chart recording illustrating the membrane potential and the response of a CA1 pyramidal cell to depolarising and hyperpolarising current steps. After a 10 min baseline period, 1 µM MT-1 was applied to the slice with no effect on the membrane potential, SFA or input resistance. Subsequent application of 3 µM carbachol resulted in a profound depolarisation. The *inset* illustrates a single experiment where 200 nM MT-1 caused a convincing depolarisation that was fully reversed by 10 µM pirenzipine. The input resistance of the cell was 32 MΩ, the resting membrane potential was –67 mV and the spike height was greater than 0 mV.

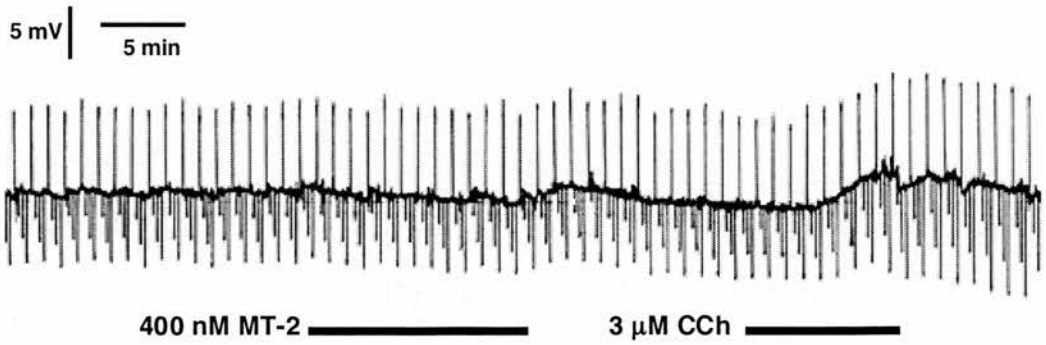


Figure 3.12 The effect of the muscarinic toxin, MT-2, on a CA1 pyramidal cell.

A chart recording illustrating the membrane potential and the response of a CA1 pyramidal cell to depolarising and hyperpolarising current steps. After a 10 min baseline period, 400 nM MT-2 was applied to the slice with no effect on the membrane potential, SFA or input resistance. Subsequent application of 3 μM carbachol resulted in a depolarisation of the membrane potential. The input resistance of the cell was 30 MΩ, the resting membrane potential was –63 mV and the spike height was greater than 0 mV

3.3. Discussion

In this chapter we have examined mAChR mediated synaptic responses in the rat hippocampus. Initially, we demonstrated that bath applied carbachol, a mAChR and nAChR receptor agonist, caused a depolarisation of the membrane potential, decreased spike frequency adaptation and increased cell input resistance. This is consistent with previous reports (Dodd *et al.*, 1981; Haas, 1992; Segal, 1982; Cole & Nicoll 1983, 1984a & 1984b). As this agonist-induced response could be mediated through extrasynaptic mAChRs, electrical stimulation of putative septohippocampal cholinergic terminals was used to demonstrate that a similar slow depolarisation of CA1 pyramidal cells could be evoked. This response was confirmed as mAChR-mediated by the use of muscarinic receptor antagonists. This is consistent with the findings of Cole & Nicoll (1983; 1984). More recently, Robin Morton (1997) demonstrated that these mAChR-mediated synaptic responses could be (1) evoked by a single stimulus (2) evoked in the absence of AChE inhibitors and (3) evoked in isolation from amino acid mediated synaptic transmission by the use of a cocktail of glutamate and GABA receptor antagonists. It was under these conditions that we reproduced the mAChR-mediated synaptic responses.

In addition to confirming that the $EPSP_M$ was indeed mediated by mAChRs, we also demonstrated that inhibitors of choline uptake across the cell membrane and vesicular membrane also inhibited the $EPSP_M$ thus strengthening the evidence for synaptic release of ACh.

Controversy exists concerning which mAChR subtype(s) is responsible for the excitatory responses observed in CA1 pyramidal cells. It is with considerable interest that researchers seek a subtype responsible for the effects of ACh in the hippocampus as this may aid in the development of more efficacious cognitive enhancers for sufferers of AD and other diseases involving cognitive impairment.

Here, we have tested several antagonists with some selectivity for M1 over M3 mAChRs and *vice versa*. Initially, we examined the $EPSP_M$ for sensitivity to M1 mAChRs using pirenzipine. We saw a consistent and significant inhibition. However,

this was unsupported due to the lack of effect of the muscarinic toxin MT-3, an antagonist at M1 and M4 mAChR subtypes, upon the $EPSP_M$.

Zamifenacin was used to examine the involvement of the M3 mAChR in the generation of the $EPSP_M$. However, this antagonist proved ineffective at consistently inhibiting the $EPSP_M$. Furthermore, zamifenacin failed to inhibit the carbachol-induced cell membrane depolarisation, a response that was, however, reversed by pirenzipine.

In addition, the muscarinic receptor selective toxins MT-1 and MT-2, selective agonists of the M1 mAChR, were used to try and mimic carbachol-induced depolarisation. With the exception of one experiment where MT-1 produced a robust cell membrane depolarisation, the muscarinic toxins proved ineffectual at producing depolarisations like those induced by carbachol.

These data argue that it is more likely to be the M1 mAChR that primarily mediates the action of ACh on CA1 pyramidal neurons in the hippocampus although the pharmacological tools available are far from ideal. This is supported by immunocytochemical and *in situ* hybridisation studies which provide evidence for the existence of both M1 and M3 mAChRs on pyramidal neurons whilst M2 and M4 mAChRs reside on non-pyramidal cells suggesting that it is more likely that M1/M3 mAChRs are responsible for the generation of the $EPSP_M$.

The hypothesis that the $EPSP_M$ is mediated by the M1 receptor is supported by previous studies which demonstrated a similar inhibition of the cholinergic response by pirenzipine (Segal, 1984) and, furthermore, demonstrated that AF102B, an agonist with some selectivity for M1 receptors, mimicked the effects of carbachol on pyramidal neurons, e.g. increased the cell input resistance and caused cell membrane depolarisation which was reversed by atropine (Segal & Fisher, 1992).

However, Pitler & Alger (1990) demonstrated that 4-DAMP, an M3 mAChR antagonist, was most effective at blocking the synaptically evoked slow EPSP as well as the carbachol-induced inhibition of the $K^+_{(LEAK)}$ current thought to underlie the slow cholinergic EPSP in the rat hippocampus.

The debate between M1 and M3 mAChRs mediating cholinergic responses in the hippocampus is complicated by possible factors such as inter- and intra-slice

variability coupled with the putative heterogeneity of cholinergic synapses being activated. Hence, a reasonable conclusion is that there may exist synapses that mediate the cholinergic slow EPSP via M1 mAChRs whilst other synapses employ M3 mAChRs and this may vary from animal to animal as well as slice to slice.

It remains unclear which subtype(s) of muscarinic ACh receptor mediate the $EPSP_M$ in the rat hippocampus. This is by no means an exhaustive study and a lot of these experiments need to be repeated. This is especially true for the toxin studies which showed some ambiguous results. To further this study would require a greater supply of the toxins that were available to me at the time and until a range of specific mAChR agonists and antagonists are commercially available and the appropriate studies performed, the M1/M3 story will remain unresolved.

CHAPTER 4

PRESYNAPTIC MECHANISMS INVOLVED IN THE GENERATION AND MODULATION OF THE $EPSP_M$

4.1 Introduction

Another aspect of the generation of electrically-evoked $EPSP_M$s is the presynaptic mechanisms by which ACh is released from the cholinergic terminals in the hippocampus and how release can be modulated by heteroreceptor activation. As such, I have examined the voltage gated Ca^{2+} channels that may be involved in ACh release and, in collaboration with Robin Morton, furthered the investigation of adenosine receptor-mediated presynaptic inhibition of the $EPSP_M$. This involved the study of the mechanisms by which adenosine could exert its effects upon ACh release. Accordingly, this chapter introduction will review voltage-gated Ca^{2+} channels, adenosine receptors, and mechanisms involved in presynaptic inhibition.

4.1.1. Voltage-gated Ca^{2+} channels

Ca^{2+} entry upon depolarisation of presynaptic terminals is an essential link between action potential invasion and neurotransmitter exocytosis (Katz, 1969; Augustine *et al.*, 1987). As such, voltage gated Ca^{2+} channels have become the focus of considerable interest as a critical component of the presynaptic release machinery. Here, I will review the basic structure, subtype classification and implication of voltage gated Ca^{2+} channels in neurotransmitter release in the CNS, with particular focus on the hippocampus.

4.1.1.1 Structure

Ca^{2+} channels have a putative multimeric composition consisting of at least 4 subunits: α1, β, α2 and δ. The α1 subunit is the largest of the four and contains four hydrophobic regions each comprising 6 transmembrane spanning domains. Six different α1 subunits have been cloned. Each demonstrates pore formation, contains the voltage sensor on segment 4 and is responsible for the binding of different pharmacological compounds. In contrast, the much smaller, entirely cytoplasmic, β subunit appears not to form a functional channel but does modify both the pharmacological and biophysical properties of the channel. Four putative β subunits have been identified and it is believed that this subunit contributes to variability between channels that contain the same α1 subunit. In the brain, the α2δ subunits are coded for by a single gene transcript. The α2 subunit is primarily extracellular and is disulphide bonded to the δ subunit that spans the membrane and possesses an intracellular C-terminal domain. These subunits contribute to the properties of the channel by increasing channel expression and modifying the sensitivity of the channel to drugs. For a more in depth review of Ca^{2+} channel structure see De Waard *et al.* (1996).

4.1.1.2. The classification of Ca^{2+} channel subtypes

Traditionally, Ca^{2+} currents have been classified based on voltage dependency into low voltage activated (LVA) or T currents and high voltage activated (HVA) currents (Carbone & Lux, 1984). HVA currents can be further divided into those that are dihydropyridine (DHP)-sensitive (L-type) and non-dihydropyridine-sensitive (Tsien *et al.*, 1988) which became classed as N-type Ca^{2+} channels. These channels showed differing biophysical properties (inactivation kinetics, steady-state inactivation and single-channel conductance) that appear intermediate between L and T type channels (Nowycky *et al.*, 1985; Fox *et al.*, 1987). The discovery of selective toxins that blocked DHP resistant Ca^{2+} currents demonstrated that N-type currents

could be further classified into N-, P- and Q-type (McCleskey *et al.*, 1987; Mintz *et al.*, 1992a).

N-type Ca^{2+} currents are ω-conotoxin GVIA-sensitive and are typically evoked above –30 mV, peak near to –10 mV and partially inactivate during depolarising pulses. However, their inactivation rates vary from less than 100 ms in sympathetic neurons (Plummer & Hess, 1991), to 600 ms in dorsal root ganglia (DRG) (Regan *et al.*, 1991) and 1.5 s in supraoptic neurons (Fisher & Bourque, 1995). This difference in inactivation kinetics may reflect differences in the basal state of the channel (e.g. G-protein regulation, phosphorylation state) between studies rather than differences in the channel protein *per se* although another explanation is that there are multiple forms of N-type Ca^{2+} channels supported by the existence of $\alpha 1_B$ splice variants combining with ancillary channel subunits (De Waard & Campbell, 1995)

P-type Ca^{2+} currents were first described in cerebellar Purkinje cells and showed resistance to DHPs and ω-conotoxin GVIA (Llinas *et al.*, 1989; Regan *et al.*, 1991). These currents are evoked above –50 mV, peak at –30 to –10 mV but differ from N-type currents by their lack of inactivation during 50 ms depolarising pulses. These currents can be inhibited by ω-agatoxin IVA with an IC_{50} of 2-10 nM (Mintz *et al.* 1992a; Mintz *et al.*, 1992b). In other cell types, such as cerebellar granule cells, a Ca^{2+} current was inhibited by a higher concentration (100-200 nM) of agatoxin IVA and thus Q-type currents were described (Zhang *et al.*, 1993). However, due to problems of poor selectivity of toxins for Q-type Ca^{2+} currents and N/P type currents, the biophysical properties of Q-type Ca^{2+} channels remain largely unexplored.

R-type currents have been described by Tsien and coworkers as the residual current left after the blockade of N, L, P and Q currents (Zhang *et al.*, 1993). However, this putative current is poorly defined and it is not clear whether this is a real subclass of Ca^{2+} channel or a mixture of residual currents from incomplete antagonism of N, L, P and Q currents.

4.1.1.3. Voltage-gated Ca^{2+} channels and neurotransmitter release

There exists extensive evidence for the involvement of multiple DHP-resistant HVA Ca^{2+} currents in presynaptic release of various neurotransmitters in the CNS (Luebke *et al.*, 1993; Takahashi & Momiyama, 1993; Castillo *et al.*, 1994; Mintz *et al.*, 1995). In the hippocampus, it has been demonstrated that both N- and Q-type Ca^{2+} channels are involved in glutamate receptor-mediated synaptic transmission at CA3-CA1 synapses in rat slices (Wheeler *et al.*, 1994) as well as synapses in pyramidal cell cultures (Scholz & Miller, 1995; Reuter, 1995). High potassium-evoked release of neurotransmitter from rat brain slices demonstrated an important role for P-type Ca^{2+} channels with N-type channels being partially involved and a lack of involvement of L-type Ca^{2+} channels (Kimura *et al.*, 1995). In addition, it has been observed that the proportions of N-type to Q-type Ca^{2+} channels involved in presynaptic release alters with age in cultured rat hippocampal neurons (Scholz & Miller, 1995) with N-type Ca^{2+} channels being more important at immature synapses and P/Q-type becoming more significant in older cultures. This finding is supported by the findings of Verderio and coworkers (1995).

GABA release from the variety of interneurons within the hippocampus has also been shown to involve Ca^{2+} channels (Poncer *et al.*, 1997). In contrast to glutamate release, it was discovered that neurotransmitter release from interneurons in the *stratum radiatum* was majorly mediated by N-type Ca^{2+} channels whereas interneurons in the *stratum oriens* and *stratum lucidum* utilised P-type Ca^{2+} channels demonstrating that different channel subtypes are employed by different synapses releasing the same neurotransmitter. However, it has been suggested (Dunlap *et al.*, 1995) that there may be spare channels that become active at higher action potential durations or if a subtype of calcium channel is blocked (Wheeler *et al.*, 1996). Furthermore, one study suggests that there is heterogeneity of Ca^{2+} channels at different synapses of the same afferent leading to a possible terminal-specific modulation of synaptic function (Reid *et al.*, 1997).

There have been a couple of studies performed on ACh release in the hippocampus. Firstly, it was demonstrated that ω-conotoxin GVIA completely blocks electrically-

evoked ACh release in the hippocampus (Dutar *et al.*, 1989a). It has been subsequently demonstrated that K^+-evoked [^{3}H]acetylcholine release can be partially inhibited by either blocking N- or Q-type Ca^{2+} channels (Saydoff & Zaczek, 1996), a contradictory result to that of Dutar and coworkers. The lack of effect of verapamil suggested little involvement of L-type Ca^{2+} channels in ACh release.

There is evidence for voltage gated Ca^{2+} channels being a target for neuromodulation of transmitter release by the activation of certain G-protein coupled receptors, e.g. the adenosine receptor. This modulation sometimes involves direct G-protein-mediated modulation and sometimes interaction via intracellular protein kinases (Dolphin, 1996). This is reviewed in more detail within section 4.1.2.4.

4.1.2 Adenosine

4.1.2.1 General

ATP and its precursor, adenosine, act at specific cell surface receptors and are involved in cellular metabolism (Williams, 1987). These receptors are known as purinoreceptors (Burnstock, 1978). ATP has been shown to act as a neurotransmitter at P_2 receptors in the peripheral and central nervous system (Edwards & Gibb, 1993; Fredholm *et al.*, 1994b). In contrast, adenosine is synthesised and released by many cells (Stone *et al.*, 1990) but is not thought to act as a classical neurotransmitter in the CNS but rather as a neuromodulator or local hormone (Dunwiddie, 1985; Snyder, 1985; Daval *et al.*, 1991). Its function within the CNS is predominantly inhibitory resulting in reduced cell firing or excitability and the inhibition of neurotransmitter release. Only adenosine will be reviewed in any depth in this chapter; however, ATP and P_2 receptors have been extensively reviewed in recent publications (Fredholm *et al.*, 1994a, Fredholm *et al.*, 1997).

Adenosine was initially discovered to be physiologically active in studies of the mammalian heart (Drury & Szent-Györgyi, 1929) but it was not until it was demonstrated that caffeine inhibited the action of adenosine that this observation was given credence (De Gubareff & Sleator, 1965). The actions of ATP and adenosine

were subsequently distinguished and the P_1/P_2 receptor classification was established. (Brunstock, 1978). Adenosine was later found to couple to adenylate cyclase (AC). Due to the observations that (1) both inhibition and stimulation of AC was observed and that (2) agonist potencies to adenosine and its related analogues varied, the classification was divided into two subtypes of adenosine receptor, A_1 (A_1R) and A_2 (A_2R) receptors (Londos *et al.*, 1980; Van Calker *et al.*, 1979). Further pharmacological characterisation confirmed and extended this classification by utilising more specific agonists and antagonists (Collis & Hourani, 1993; Dalziel & Westfall, 1994). Ultimately, on the basis of molecular cloning techniques, the existence of the adenosine A_3 receptor was proposed (Caruthers & Fozard, 1993; Collis & Hourani, 1993). The current receptor classification is summarised in Table 4.1.

Table 4.1. Adenosine receptor subtypes: Pharmacology and transduction mechanisms.

Subtype	**A_1**	**A_{2a}**	**A_{2b}**	**A_3**
Selective agonists	CCPA	CGS 21680	none known (NECA)	APNEA
Agonist Potency Ratios	R-PIA>NECA >S-PIA	NECA>R-PIA >S-PIA	NECA>R-PIA >S-PIA	NECA=R-PIA >S-PIA
Antagonists	DPCPX	ZM 241385	none known	I-ABOPX
Signal Transduction Mechanisms	↓AC	↑AC	↑AC	↓AC
G-protein Interaction	G_i/G_o	G_s	G_s	?

Abbreviations: APNEA: N^6-2-(4-amino-3-iodophenyl)-ethyladenosine.

4.1.2.2. Adenosine Receptors

The adenosine receptors are found throughout the CNS as well as in peripheral tissues (Thompson *et al.*, 1992) and belong to the superfamily of G-protein-coupled receptors possessing the characteristic seven transmembrane spanning domains (Olah & Stiles, 1992).

A_1Rs are negatively coupled to AC and therefore inhibit the turnover of intracellular cAMP. A_1Rs can be pharmacologically distinguished from A_2Rs using the specific agonist, 2-chloro-N^6-cyclopentanyladenosine (CCPA) or the specific antagonist, DPCPX which has a 700-fold selectivity for A_1Rs over A_2Rs (Lohse *et al.*, 1987).

A_2R were initially distinguished by their ability to stimulate adenylate cyclase and thus increase the levels of intracellular cAMP (Van Calker *et al.*, 1979). Binding studies have subsequently shown that A_2Rs can be subdivided into the high affinity A_{2a}R and the low affinity A_{2b}R, both of which positively couple to AC via the G-protein, G_s (Daly *et al.*, 1983; Bruns *et al.*, 1986; Ongini & Fredholm, 1996). This was later confirmed by the A_{2a}R selective agonist, CGP 21680 (Jarvis *et al.*, 1989), and the non-xanthine antagonist, ZM 241385 (Poucher *et al.*, 1995).

The A_3R was originally cloned from a cDNA sequence derived from rat striatal cells (Zhou *et al.*, 1992). When expressed in CHO cells, A_3Rs displayed a different rank order of agonist potencies than that of A_1Rs and A_2Rs. Furthermore, this new receptor was resistant to blockade by certain xanthine antagonists. A_3R was found to negatively couple to AC.

4.1.2.3. Adenosine: The Neuromodulator

Although there is little evidence to support a neurotransmitter role for adenosine (Bruns, 1991), adenosine receptors are widely distributed throughout the CNS and adenosine is released from neurons (Bender *et al.*, 1981; Jonzon & Fredholm, 1985; Stone *et al.*, 1990; Mitchell *et al.*, 1993; Fredholm *et al.*, 1994b). Consequently, a neuromodulatory role for adenosine was proposed.

The principle action of adenosine is inhibition of neurotransmitter release at a variety of excitatory and inhibitory synapses (Dunwiddie, 1990). In addition, adenosine receptor-mediated effects acting at postsynaptic sites have been reported, e.g. modulation of postsynaptic K^+ or Ca^{2+} conductances and inhibition of spontaneous neuronal firing. Furthermore, *in vivo and in vitro* studies demonstrate that there is an overall "purinergic inhibitory tone" over neuronal systems that can be alleviated with adenosine antagonists and exacerbated by the inhibition of adenosine breakdown or

uptake (Harms *et al.*, 1978; Jackisch *et al.*, 1984; Pak *et al.*, 1994; Dunwiddie & Diao, 1994).

Although the effects of adenosine are well established the source of adenosine is still the subject of some debate due to its general production as an integral part of the process of cellular metabolism and energy production (Arch & Newsholme, 1978). Stimulation evoked release of adenosine from presynaptic terminals is apparent but is thought to be a carrier-mediated process rather than exocytosis (Sweeney, 1996). Of course, ATP is also expelled from cells where it is catabolised by ecto-5'-nucleotidases into adenosine (Cunha *et al.*, 1998). During periods of hypoxia or if the cell metabolism is compromised, local extracellular concentrations of adenosine are dramatically elevated (Hagberg *et al.*, 1987; Rudolphi *et al.*, 1992). Taken together with the role of adenosine as an inhibitory modulator of synaptic activity, adenosine has been described as a neuro-protective agent or a "retaliatory metabolite" (Newby, 1984).

4.1.2.4. Adenosine receptor-mediated effects in the CNS

Several interlinked effects of adenosine action have been characterised in the hippocampus: (1) the inhibition of spontaneous neuronal firing and epileptic bursting (Phillis *et al.*, 1979; Dunwiddie, 1980; Lee *et al.*, 1984; Schubert & Lee, 1986), (2) modulation of K^+ or Ca^{2+} conductances, and (3) presynaptic modulation of neurotransmitter release.

These effects appear to be mediated by postsynaptic A_1Rs and are thought to involve the activation of postsynaptic K^+ conductances which result in hyperpolarisation and enhancement of I_{AHP} thus reducing postsynaptic excitability. In addition, it has been demonstrated that adenosine receptor activation opens a voltage-insensitive K^+ conductance in the hippocampus (Okado & Ozawa, 1980, Segal, 1982b; Greene & Haas, 1985). This effect appears to be mediated by a pertussis toxin-sensitive G-protein but does not involve cAMP (Trussel & Jackson, 1987). Also, the enhancement of I_{AHP} by adenosine receptor activation has been reported in CA1 pyramidal neurons (Haas & Greene, 1984). One study also provides evidence of an

adenosine receptor-mediated activation of a voltage-dependent Cl^- conductance in cultured hippocampal neurons (Mager *et al.*, 1990).

The inhibition of Ca^{2+} channels by adenosine receptor activation has also been explored in the hippocampus. One Ca^{2+} conductance underlying regenerative Ca^{2+}-dependent action potentials (calcium spikes) is reported to be inhibited by adenosine receptor activation although it is not certain if this effect is direct or due to the activation of a K^+ conductance which in turn inhibits Ca^{2+} influx (Proctor & Dunwiddie, 1983). In addition, adenosine receptor activation has been shown to inhibit N-type Ca^{2+} channels in hippocampal CA3 neurons (Mogul *et al.*, 1993) and mouse motoneurons (Mynlieff & Beam, 1994). Moreover, it has been shown that the blockade of ω-conotoxin GVIA-sensitive Ca^{2+} transients attenuated adenosine A_1 receptor-mediated inhibition of glutamate mediated synaptic transmission in area CA1 of the rat hippocampus (Wu & Saggau, 1994). In cultured DRG neurons, adenosine receptor activation inhibited the duration of Ca^{2+} spikes (Dolphin *et al.*, 1986; MacDonald *et al.*, 1986).

Despite these numerous effects, presynaptic inhibition of transmitter release is considered the best-recognised neuromodulatory action of adenosine. Presynaptic inhibition is a phenomenon originally described in the neuromuscular junction (Ginsborg & Hirst, 1972). Adenosine, acting through presynaptic adenosine receptors, have been reported to inhibit release of both excitatory and inhibitory neurotransmitters including ACh, adrenaline, dopamine, GABA, glutamate and 5-HT (Harms *et al.*, 1978; Harms *et al.*, 1979; Burke & Nadler, 1988, Morton & Davies, 1997). However, this inhibition is not consistent for all brain regions, for example, GABA release is inhibited by adenosine receptor activation in the cortex and striatum but not in the hippocampus (Harms *et al.*, 1979; Hollins & Stone, 1980).

Recently, one study showed that presynaptic adenosine A_1R activation resulted in an inhibition of electrically-evoked release of ACh from cholinergic terminals in the hippocampus (Morton & Davies, 1997) and it was my aim, in conjunction with Robin Morton, to follow up this investigation by exploring the possible mechanisms by which adenosine could bring about this inhibition of release. Mechanisms of

adenosine receptor-mediated presynaptic inhibition remain somewhat controversial and revolve around three likely putative modes of action:

- The activation of a presynaptic K^+ conductance, resulting in a reduction of Ca^{2+} influx upon invasion of an action potential leading to a reduction in transmitter release.
- The inhibition of a presynaptic Ca^{2+} conductance directly and thus a reduction of transmitter release.
- The modulation of the release machinery *per se*, somewhere downstream of Ca^{2+} entry.

The involvement of Ca^{2+} channel in adenosine receptor inhibition of transmitter release has received most attention. In this respect, adenosine was shown to reduce Ca^{2+} influx into presynaptic terminals measured using Ca^{2+}-imaging techniques in the guinea pig hippocampus (Wu & Saggau, 1994) and chick ciliary ganglion (Yawo & Chuhma, 1993). In the hippocampus, it was shown that the Ca^{2+} conductance inhibited by adenosine was of the N-type channel and possibly the Q-type. In other brain areas, such as the rat brainstem, adenosine acting via A_1Rs causes inhibition of presynaptic neurotransmitter release predominantly through the inhibition of an N-type Ca^{2+} conductance (Umemiya & Berger, 1994). A_2Rs function in a somewhat different manner in that their activation can lead to a facilitation of synaptic transmission in area CA3 and the brainstem by potentiation of P-type Ca^{2+} currents (Mogul *et al.*, 1993; Umemiya & Berger, 1994). To date there is a paucity of evidence suggesting an involvement of K^+ channels and/or actions on the presynaptic release machinery *per se* in the inhibition of transmitter release by adenosine. In the latter case, this is probably due to the technical difficulty and lack of pharmacological tools with which to perform these experiments.

4.2 Results

4.2.1. The Role of Voltage-Gated Calcium Channels (VGCCs) in the Generation of $EPSP_M$s

Initially, it was important to assess which Ca^{2+} channels were responsible for the release of ACh mediating the $EPSP_M$. Although it is widely accepted that VGCCs are involved in synaptic transmission, it was necessary to ascertain whether they played a part in the cholinergic responses generated by stimulation of cholinergic presynaptic terminals in the hippocampus. It is feasible that the release machinery was by-passed by the strong stimulation protocol resulting in the direct electrically evoked release of ACh. $CdCl_2$ was employed to non-specifically block Ca^{2+} channels. Fig. 4.1 shows that 0.5 mM $CdCl_2$ caused a total inhibition of the $EPSP_M$ within 30 min (n=3). This effect was accompanied by an increase in cell input resistance (data not shown).

To more specifically identify which Ca^{2+} channels mediated the release of ACh, ω-conotoxin GVIa (CTX), a specific blocker of N-type VGCCs was studied. 300 nM (Zhang *et al.*, 1993) CTX produced a partial inhibition of the $EPSP_M$ (34.8 ±5.74% of control; n=5) reaching steady state within 45 min (fig. 4.2). No changes in spike frequency adaptation (fig. 4.3B) or cell input resistance of a pyramidal cell were observed in the presence of CTX (106±5.7% of control, n=5).

To ascertain whether or not CTX was producing this inhibition via a pre- or post-synaptic locus, it was necessary to examine the effect of the toxin on post-synaptic mAChR activation alone. A 1 min application of 3 μM carbachol produced (1) robust depolarisation of the membrane potential to a point where the pyramidal cell fired trains of action potentials, (2) reduced spike frequency adaptation and (3) increased cell input resistance. In the presence of CTX little difference was observed in all three effects of post-synaptic mAChR activation, suggesting a presynaptic locus for the action of CTX on the $EPSP_M$ (fig. 4.3).

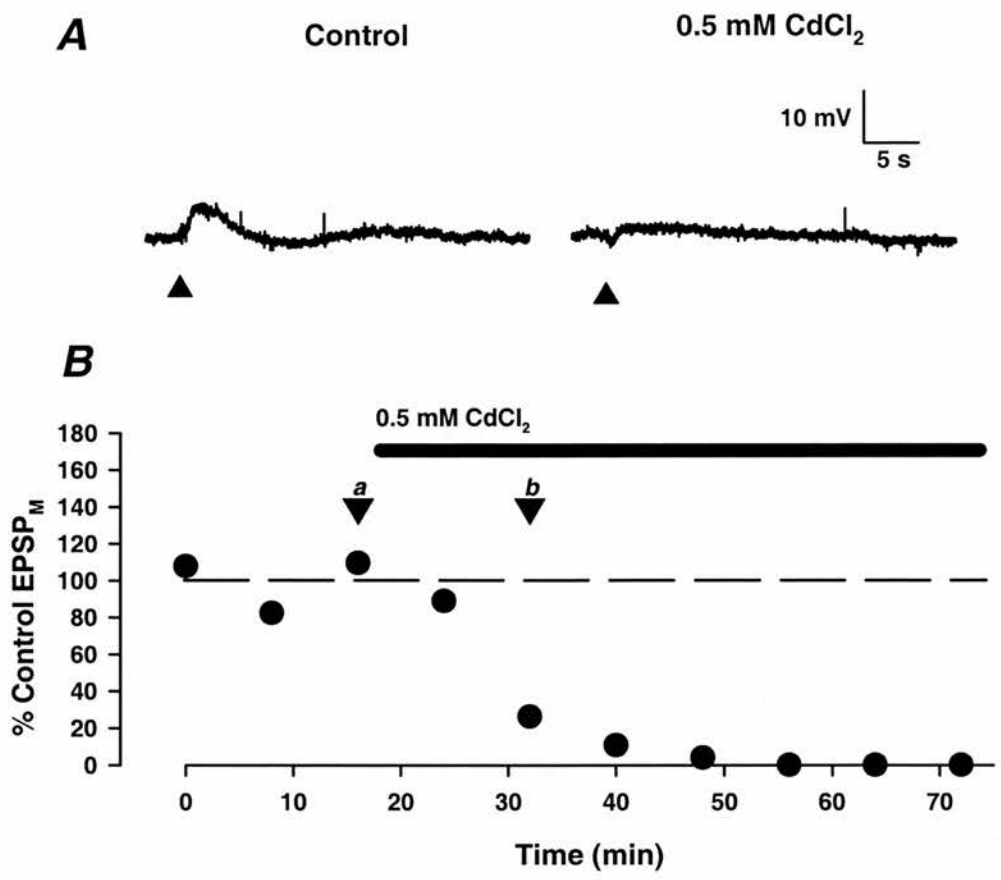


Figure 4.1 **The $EPSP_M$ is fully blocked by $CdCl_2$**

A shows intracellular current clamp recordings of $EPSP_M$s before (*left*) and 16 min after (*right*) the application of 0.5 mM $CdCl_2$. Cell membrane potentials were maintained at –66 mV throughout the experiment. The filled triangle denotes the time of stimulation (10 stimuli at 20 Hz). B shows the $EPSP_M$ peak amplitude expressed as a percentage of control $EPSP_M$ amplitude versus time. Note that $CdCl_2$ produced a rapid and full inhibition of the $EPSP_M$. The input resistance of the cell was 32 MΩ, the resting membrane potential was –63 mV and the spike height was greater than 0 mV

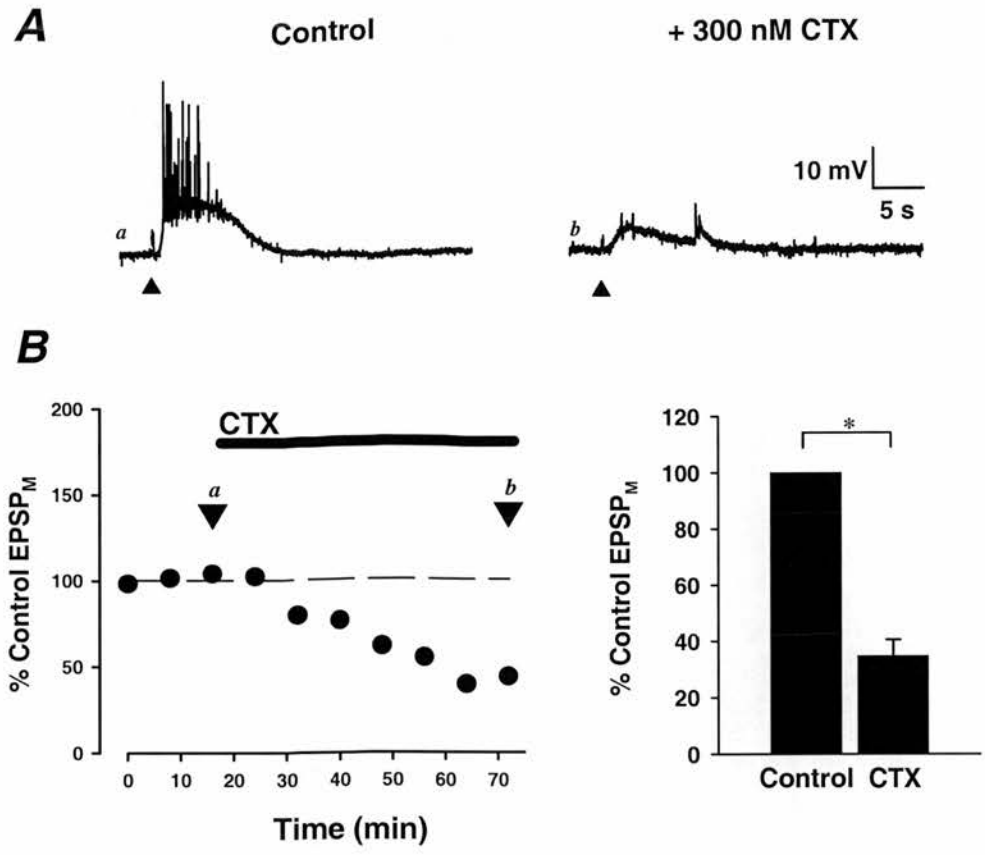


Figure 4.2 300 nM ω-conotoxin GVIa (CTX) partially inhibits the EPSP$_M$.

A shows intracellular current clamp recordings of EPSP$_M$s before (*left*) and after (*right*) the application of 300 nM CTX. Cell membrane potentials were maintained at –68 mV throughout the experiment. The filled triangles denote the position of the stimulus train (10 @ 20 Hz). B demonstrates the inhibition of the EPSP$_M$ expressed as the peak amplitude of the EPSP$_M$ normalised to 3 responses in control media plotted versus time. Bar graph (*right*) illustrates the mean inhibition from 5 experiments ($p<0.05$). Values are means ± S.E.M represented by the error bars. The input resistance of the cell was 42 MΩ, the resting membrane potential was –65 mV and the spike height was greater than 0 mV.

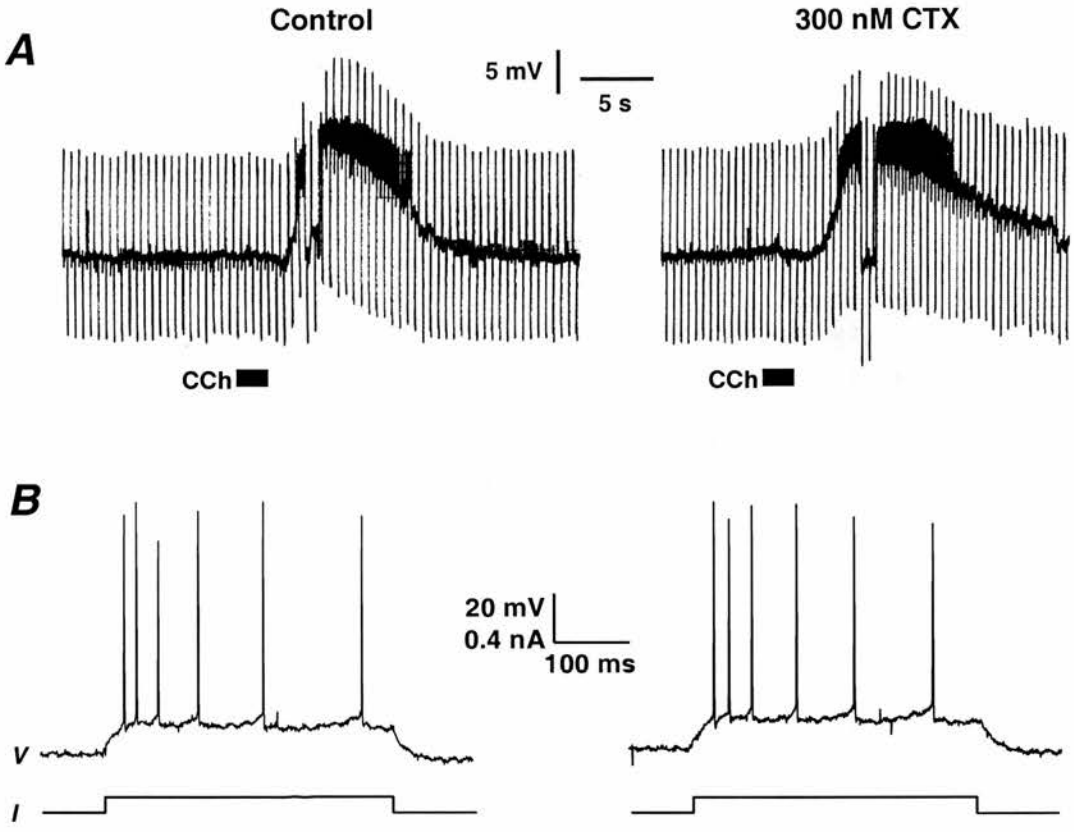


Figure 4.3 **Effects of CTX on spike frequency adaptation and carbachol-induced depolarisation of CA1 pyramidal cells.**

A shows chart recordings of the membrane potential (V_m) of a CA1 pyramidal cell and its response to depolarising and hyperpolarising current steps. On application of 3 μM carbachol (Cch) for 1 minute, a slow depolarisation of V_m accompanied by spontaneous action potential firing and an increase in cell input resistance were observed. These effects of CCh are unchanged in the presence of 300 nM CTX (right). *B* illustrates the response of a cell to a depolarising current step in the absence (*left hand trace*) and presence (*right hand trace*) of 300 nM CTX. The number of action potentials and, therefore, spike frequency adaptation, is unchanged by 300 nM CTX. The input resistance of the cell was 38 MΩ, the resting membrane potential was –66 mV and the spike height was greater than 0 mV. Variations in spike height are due to the sampling rate (488 μs sampling interval).

As CTX only provides a partial blockade of the $EPSP_M$, it was thought that other VGCCs might contribute to the generation of this response. Initially, Agatoxin TK was utilised to block P/Q type calcium channels. At 200 nM, Agatoxin TK produced an inconsistent reduction of the $EPSP_M$ (data not shown). Therefore, the more potent P/Q-type Ca^{2+} channel inhibitor, ω-agatoxin IVa (ATX) was tested. At 400nM, ATX (Mintz *et al.* 1992a; Mintz *et al.*, 1992b) produced significant inhibition of the $EPSP_M$ (47±17% of control; n=2), reaching steady state after 100 min. This effect was accompanied by a small increase in cell input resistance (120±2% of control, n=2) but no apparent change in spike frequency adaptation (fig. 4.4).

4.2.2. Mechanisms of presynaptic adenosine receptor mediated inhibition of the $EPSP_M$.

Since the $EPSP_M$ was dependent upon presynaptic Ca^{2+} channel activation it was hypothesised that broadening of presynaptic action potentials using K^+ channel blockers such as 4-aminopyridine (4-AP) should also enhance the $EPSP_M$. 100 μM 4-AP (Madison *et al.*, 1987) enhanced the $EPSP_M$ peak amplitude by 1171 ±338% of control (n=4). To establish whether this excitatory effect of 4-AP was pre- or post-synaptic I tested whether 4-AP affected carbachol induced depolarisations. It was found that carbachol-induced depolarisation was unaffected by 4-AP (fig. 4.5*C*) as was spike frequency adaptation and cell input resistance (data not shown) which suggests that 4-AP enhanced the $EPSP_M$ by increasing the duration of action potentials and, therefore, increased the influx of Ca^{2+} into the cholinergic terminals. To investigate the role that K^+ channels may play in the modulation of ACh release by adenosine, 4-AP was employed at high concentrations to non-specifically block a range of K^+ channels. At 100 μM 4-AP, the $EPSP_M$ was greatly enhanced. 1 μM CADO (eg Morton & Davies, 1997; Davies *et al.*, 1991), a non-hydrolysable adenosine analogue was subsequently applied. This produced a significant level of inhibition of the $EPSP_M$ (fig. 4.5*A*). However, this inhibition was significantly less (46±12% of control; n=4 ($p<0.05$)) than that observed when CADO alone was applied (17±5% of control; n=9) (fig 4.5*B*).

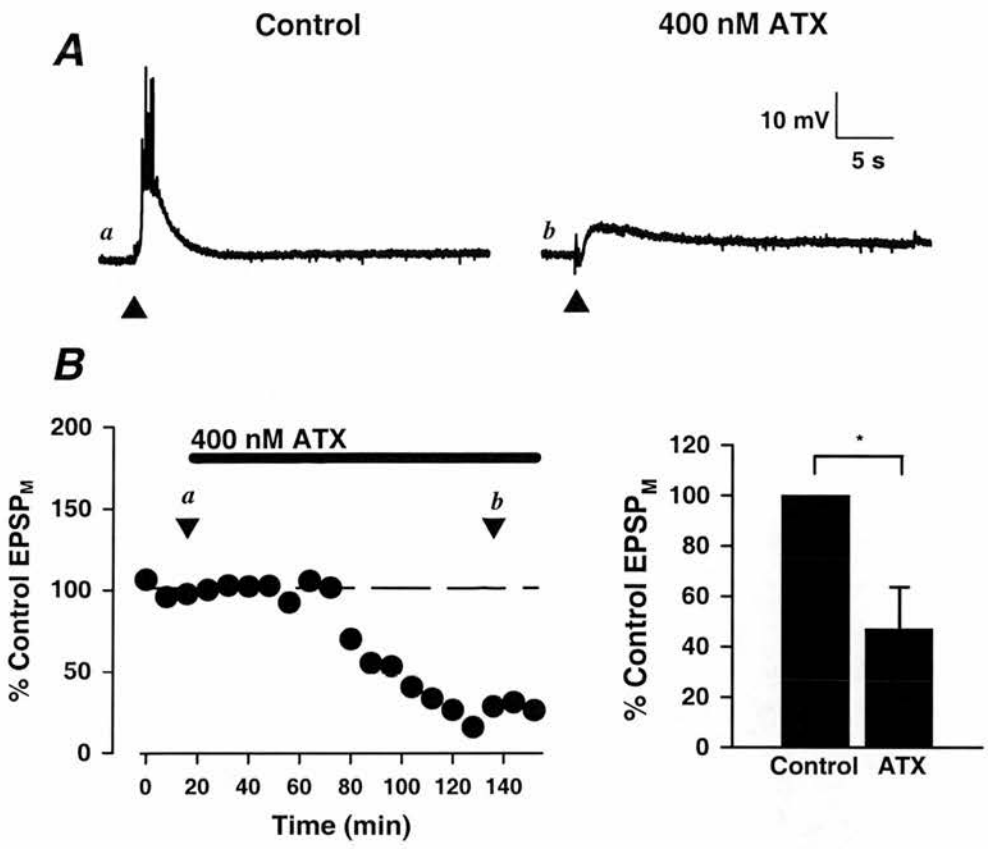


Figure 4.4 400 nM ω-agatoxin IVa partially inhibits the EPSP$_M$

A illustrates intracellular current clamp recordings of EPSP$_M$s in control media (*left*) and after 100 min application of 400 nM ATX (*right*). Cell membrane potentials were maintained at –68 mV throughout the experiment. Filled triangles denote the position of the stimulus (10 @ 20Hz). *B (left)* shows the time course of ATX application and its effect on the peak amplitude of the EPSP$_M$. The bar graph (*right*) shows the mean inhibition of the EPSP$_M$ by ATX for 2 experiments ($P<0.05$). Values are means ± S.E.M represented by the error bars. The input resistance of the cell was 40 MΩ, the resting membrane potential was –63 mV and the spike height was greater than 0 mV.

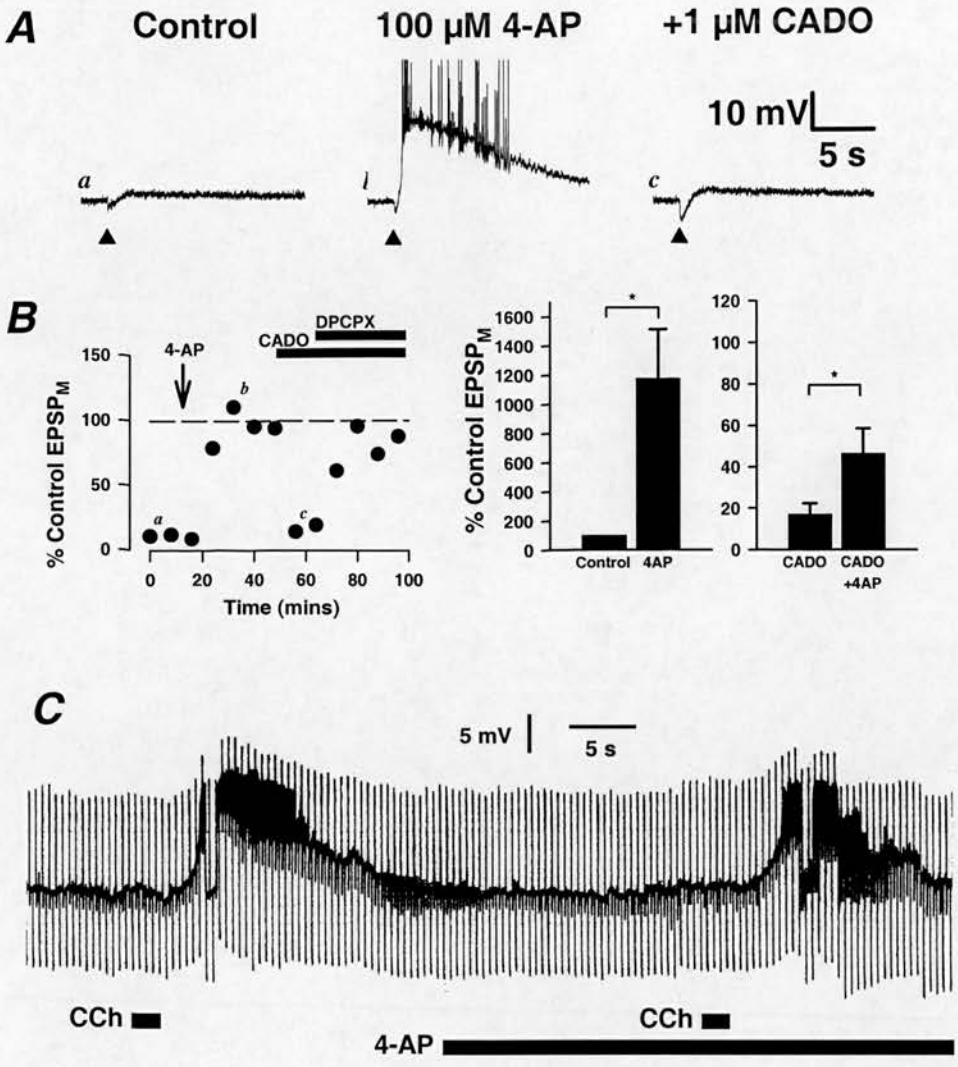


Figure 4.5 **The blockade of 4-AP sensitive K^+ channels partially occludes the inhibition of the $EPSP_M$ by CADO.**

A shows intracellular current clamp recordings of $EPSP_M$s in control media (left), during 100 µM 4-AP application (middle) and after 4-AP + 1 µM CADO application. The cell membrane potential was maintained at –67 mV throughout the experiment. The filled triangles denote the position of the stimulus (10 @ 20Hz). *B* shows a time course graph illustrating the enhancement of the $EPSP_M$ peak amplitude by 4-AP and the subsequent inhibition by CADO and block of CADO-induced inhibition by 200 nM DPCPX. The bar graph (*right*) demonstrates the significant difference ($p<0.05$) in mean levels of (*left*) enhancement of the $EPSP_M$ amplitude by 4-AP and (*right*) CADO induced inhibition, with and without 4-AP present, for 9 and 4 experiments respectively ($p<0.05$). The input resistance of the cell was 37 MΩ, the resting membrane potential was –67 mV and the spike height was greater than 0 mV. Values are means ± S.E.M represented by the error bars.
C shows chart recordings of the membrane potential (V_m) of a CA1 pyramidal and its response to depolarising and hyperpolarising current steps. On application of 3 µM carbachol (Cch) for 1 minute, a slow depolarisation of V_m, accompanied by spontaneous action potential firing, was observed. Note the increase in cell input resistance during carbachol application. These effects of CCh are unchanged in the presence of 100 µM 4-AP (right).

Since 4-AP caused a partial occlusion of the depressant action of CADO on the $EPSP_M$ it is possible that CADO may also inhibit Ca^{2+} channel activity. As such, we compared the effects of CADO on the $EPSP_M$ evoked in the presence and absence of ω-conotoxin GVIA. Because CTX only produced a partial inhibition of the $EPSP_M$, it was possible to assess the extent to which CADO could depress the $EPSP_M$ in the absence of functional N-type Ca^{2+} channels. As such, it was demonstrated that the residual $EPSP_M$, subsequent to CTX application, was still inhibited by 1 μM CADO (fig. 4.6*A*). A comparison of the level of CADO inhibition in the absence of CTX (17 ±5% of control; *n*=9) revealed that there was no change in the magnitude of CADO induced depression of the $EPSP_M$ after N-type Ca^{2+} channel blockade (24 ±7% of control; *n*=3) (fig. 4.6*B*) suggesting that adenosine modulation does not exert its actions solely via the N-type Ca^{2+} channel. In a separate series of experiments CADO was also found to depress the $EPSP_M$ when P/Q type Ca^{2+} channels were blocked by 400 nM ATX (*n*=2; data not shown).

As N, P and Q type Ca^{2+} channels appeared not to play a notable role in adenosine modulation of ACh release and 4-AP sensitive K^+ channels only a partial role, it was possible that the major reason for CADO-induced inhibition of the $EPSP_M$ was inhibition of the release machinery *per se*. As such, we next explored whether it was possible to inhibit the CADO-induced depression of the $EPSP_M$ by modifying intracellular signaling mechanisms. In this respect it has been demonstrated that the adenosine A_1 receptors couple to the G-protein, G_i, which negatively regulates adenylate cyclase. Therefore, we focussed our experiments toward modifying intracellular cAMP levels to establish its involvement in the process of presynaptic adenosine modulation of ACh release.

Increasing endogenous cAMP was achieved using 10 μM forskolin. This had no effect on the $EPSP_M$ *per se* (92 ±14% of control) (fig. 4.7) but reduced spike frequency adaptation (data not shown). In the presence of forskolin, CADO induced inhibition of the $EPSP_M$ was significantly attenuated (43 ±3% of control, *n*=4) compared with control media (17 ±5% of control, *n*=9 ($p<0.05$)).

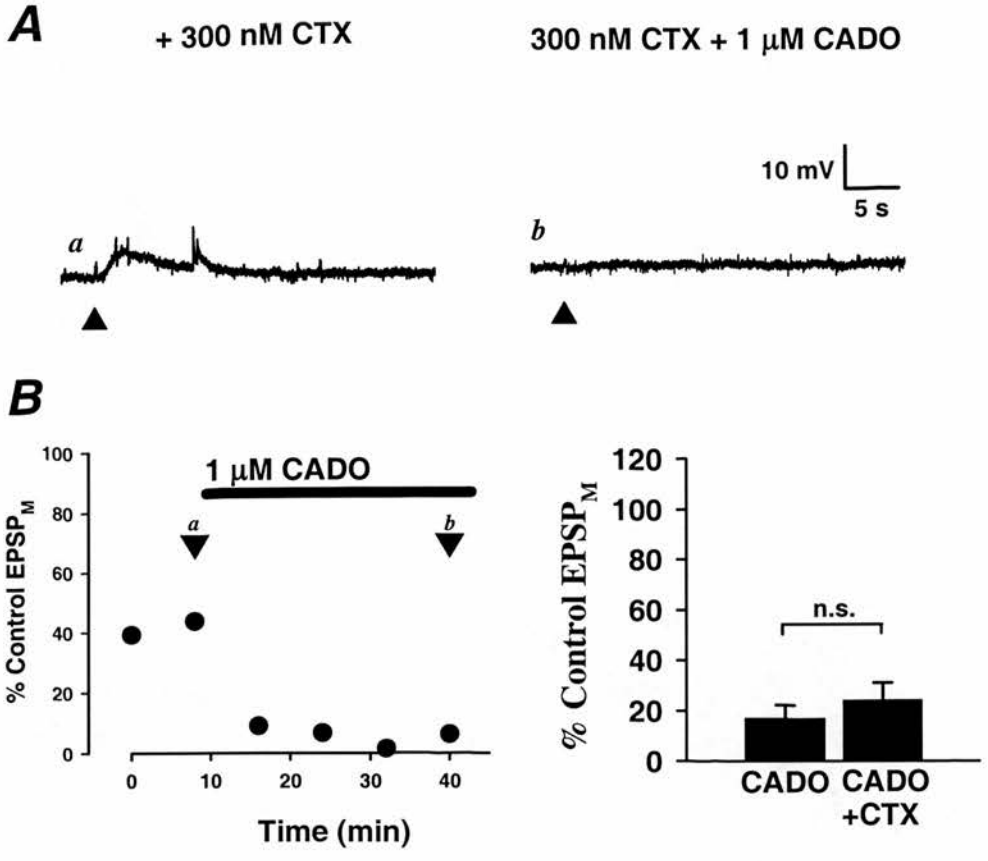


Figure 4.6 **The blockade of N-type Ca^{2+} channels does not affect the inhibition of the $EPSP_M$ by CADO.**

A shows intracellular current clamp recordings of $EPSP_M$ evoked in the presence of CTX before (*left*) and after (*right*) the application of 1 μM CADO. Cell membrane potentials were maintained at –66 mV throughout the experiment. Filled triangles denote position of stimulus (10 @ 20Hz). *B* illustrates a time course graph showing the inhibition of the $EPSP_M$ peak amplitude by CADO. The bar graph (*right*) demonstrates that there was no change in mean CADO induced inhibition, with or without CTX present, for 9 and 3 experiments respectively ($p>0.05$). Values are means ± S.E.M represented by the error bars. The input resistance of the cell was 34 MΩ, the resting membrane potential was –66 mV and the spike height was greater than 0 mV.

To support this result, the non-hydrolysable analogue of cAMP, 8-Br-cAMP, was used to activate processes normally activated by cAMP. As with forskolin, 8-Br-cAMP had no effect on the $EPSP_M$ *per se* (102 ±11% of control, n=5) but succeeded in completely occluding the depressive effect of CADO on the $EPSP_M$. Thus, in the presence of CADO plus 8-Br-cAMP, the peak amplitude of the $EPSP_M$ was 110 ±5% of control, n=4 (fig. 4.8).

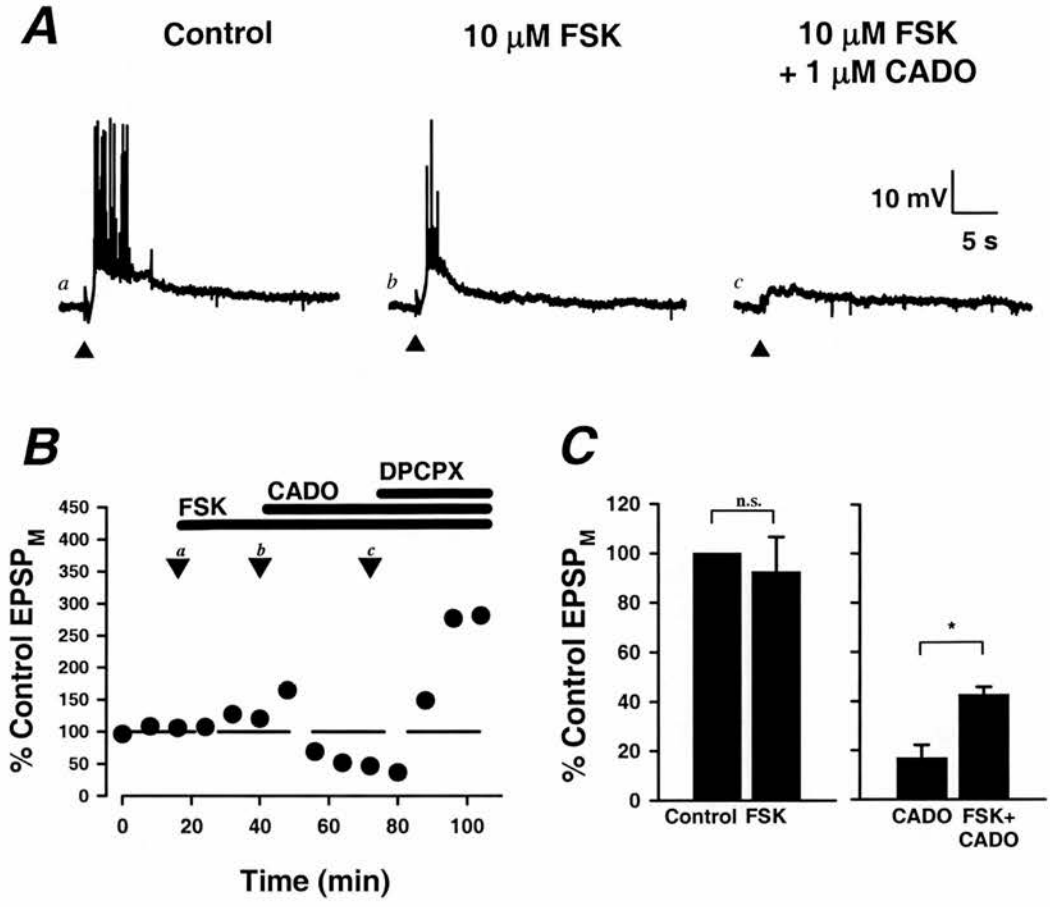


Figure 4.7 **Increasing endogenous intracellular cAMP partially occludes the inhibition of the $EPSP_M$ by CADO.**

A shows intracellular current clamp recordings of $EPSP_M$s in control media (*left*), during 10 μM forskolin application (*middle*) and after 10 μM forskolin plus 1 μM CADO application (*right*). Cell membrane potentials were maintained at –68 mV throughout the experiment. Filled triangles denote the position of the stimulus (10 @ 20Hz). *B* illustrates a time course graph showing the change in $EPSP_M$ peak amplitude following applications of forskolin, CADO and DPCPX. (*C*) Bar graph (*left*) demonstrates that there was no change in mean $EPSP_M$ peak amplitude after forskolin application for 4 experiments ($p>0.05$). The bar graph (*right*) shows that there is a significant reduction in mean CADO induced $EPSP_M$ inhibition in the presence of forskolin $n=9$ and $n=4$ for experiments in the absence of forskolin ($p<0.05$). Values are means ± S.E.M represented by the error bars. The input resistance of the cell was 43 MΩ, the resting membrane potential was –69 mV and the spike height was greater than 0 mV.

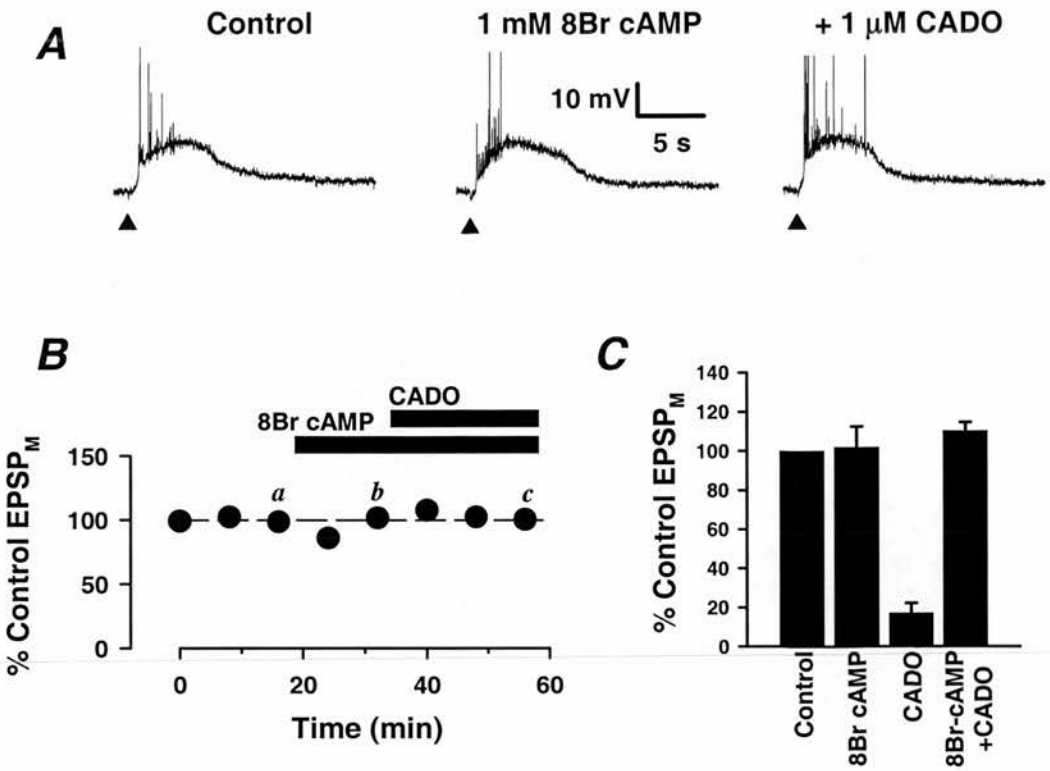


Figure 4.8 8Br-cAMP fully occludes the inhibition of the EPSP$_M$ by CADO.

A shows intracellular current clamp recording of EPSP$_M$s in control media (left), during 1 mM 8Br cAMP application and after 8Br cAMP and 1 μM CADO application. Cell membrane potentials were maintained at –65 mV throughout the experiment. Filled triangles denote the position of the stimulus (single stimulation). *B* illustrates the time course of a typical experiment showing that the EPSP$_M$ peak amplitude is unchanged by 8Br cAMP and that CADO failed to inhibit the response. The bar graph (*C*) demonstrates the lack of effect of 8Br cAMP on the EPSP$_M$ in 5 experiments and the total block of CADO induced inhibition of the EPSP$_M$ by 8-Br-cAMP in 4 experiments. Values are means ± S.E.M represented by the error bars. The input resistance of the cell was 38 MΩ, the resting membrane potential was –65 mV and the spike height was greater than 0 mV.

4.3 Discussion

4.3.1. Voltage-gated Ca^{2+} channels are involved in the generation of $EPSP_M$s

Here, we have demonstrated the involvement of multiple subtypes of Ca^{2+} channel in the generation of cholinergic responses. Furthermore, we purport that the primary site of action of these Ca^{2+} channels is at the presynaptic cholinergic terminal based on the lack of effect that the blockade of these channels has on postsynaptic mAChR-mediated responses.

The $EPSP_M$ was inhibited by 65% in the presence of the N-type channel blocker, ω-conotoxin GVIA, and by 53% in the presence of the P/Q-type Ca^{2+} channel blocker, ω-agatoxin IVA. The fact that the amount of inhibition exerted by both toxins exceeds 100% can be explained by the non-linear relationship between Ca^{2+} influx and neurotransmitter release as measured by the postsynaptic effect of the neurotransmitter. This relationship can be expressed as the following function:

$$Release=[Ca^{2+}]^m$$

Where *Release* refers to postsynaptic potentials, $[Ca^{2+}]$ refers to the intracellular Ca^{2+} concentration (spatially averaged) and m has been found to equal between 3 and 4. Therefore, a small decrease in Ca^{2+} influx can result in a disproportionately larger decrease in release. This relationship is described in more detail by Wu and Saggau (1997). Thus, blocking a proportion of the Ca^{2+} channels mediating Ca^{2+} influx may result in a large percentage decrease in ACh release disproportionate to the percentage of Ca^{2+} channels blocked. Ideally, to assess the proportional contributions of the different Ca^{2+} channel subtypes, increases in intracellular Ca^{2+} should be measured directly using calcium fluorescent imaging.

Whilst there is evidence to support the findings in this chapter in that Saydoff & Zaczek (1996) showed a similar involvement of N- and P/Q-type Ca^{2+} channels in

K^+-evoked ACh release, Dutar and coworkers (1989a) have demonstrated a total block of the cholinergic response by ω-conotoxin GVIA suggesting N-type Ca^{2+} channels alone were involved in mediating the cholinergic response. However, a number of differences exist between the experiments of Dutar and the experiments presented in this chapter. Firstly, Dutar and coworkers evoked cholinergic responses that were not isolated by the use of antagonists of GABA and glutamate receptor-mediated synaptic transmission. Secondly, Dutar *et al.* used eserine to amplify the cholinergic response. This suggests that they are not evoking the same amount of ACh release and thus presynaptic Ca^{2+} influx is lower. It is possible that at lower levels of Ca^{2+} influx, blocking N-type Ca^{2+} is all that is necessary to prevent neurotransmitter release. This infers that P/Q-type Ca^{2+} channels do not become involved in ACh release until higher stimulation intensities. However, it would be interesting to observe the effect of ω-agatoxin IVA on the cholinergic responses in the study of Dutar *et al.* (1989a).

That there exists a heterogeneous involvement of Ca^{2+} channel subtypes modulating ACh release is not unusual. Indeed, Wheeler and colleagues (1994) have demonstrated that glutamate release in the hippocampus required functional N- and Q-type Ca^{2+} channels. This subtype heterogeneity can be explained by either different cholinergic afferents exhibiting different Ca^{2+} channel subtypes allowing for more specific modulation of synaptic function, or each terminal utilising multiple Ca^{2+} channel subtypes.

4.3.2. Mechanisms involved in the presynaptic modulation of ACh release by adenosine A_1 receptor activation

It has previously been demonstrated that adenosine A_1R activation results in a postsynaptic hyperpolarisation of pyramidal cells by G-protein mediated activation of a barium-sensitive K^+ conductance (Thompson *et al.*, 1992). In addition, adenosine A_1R activation inhibits amino acid-mediated excitatory synaptic transmission in the hippocampus (but not GABAergic inhibitory synaptic

transmission). Taken together, these data suggest a powerful inhibitory role for adenosine in the CNS.

Another inhibitory role was reported by Morton & Davies (1997) who demonstrated that presynaptic adenosine A_1R activation mediated a profound inhibition of ACh release from septohippocampal cholinergic terminals in the *stratum oriens* of the hippocampus. Here, we have extended this finding by exploring the possible mechanisms by which adenosine A_1R activation may cause this presynaptic inhibition.

Initially, we demonstrated a partial involvement of 4-AP sensitive K^+ channels in adenosine A_1R-mediated presynaptic inhibition. The blockade of these K^+ channels did not appear to affect postsynaptic mAChR activation suggesting that they resided at a presynaptic locus. These data suggest a possible role for 4-AP sensitive K^+ channels in adenosine receptor modulation. However, the concomitant increase of Ca^{2+} influx due to presynaptic action potential broadening may explain the partial occlusion of A_1R-mediated presynaptic inhibition by 4-AP as this may override the inhibitory mechanisms invoked by A_1 receptor activation.

As we had already established that N- and P/Q-type Ca^{2+} channels are involved in mediating ACh release, it was logical to explore if these voltage-gated Ca^{2+} channels were also the effector site for adenosine A_1R-mediated presynaptic inhibition especially since Ca^{2+} channels are intimately involved in ACh release. However, N-type Ca^{2+} channels proved not to be involved. Preliminary results showed that P/Q-type channels appeared not to play a part in adenosine receptor-mediated presynaptic inhibition of ACh release also, although more experiments need to be conducted to conclusively rule out this site of action. Interestingly, adenosine A_1R activation causes presynaptic inhibition at granule cell to purkinje cell synapses in the cerebellum by the modulation of N-,P- and Q-type Ca^{2+} channels (Dittman & Regehr, 1996). This result is accompanied by numerous reports of different neuromodulators presynaptically inhibiting neurotransmitter release from a variety of different synapses within the CNS by reducing Ca^{2+} influx through voltage-gated Ca^{2+} channels (Qian *et al.*, 1995; Wu & Saggau, 1994, 1995; Mintz *et al.*, 1995). However, this does not exclude the hypothesis that adenosine is acting at presynaptic

sites to cause inhibition of ACh release by another mechanism downstream of Ca^{2+} entry.

It is technically very difficult to examine the functional effect that inhibiting part of the presynaptic release machinery has on a postsynaptic response. Thus, our initial experiments began with the second messenger system by which adenosine A_1Rs may mediate their effect. As A_1 receptors inhibit adenylate cyclase we looked at cAMP. Accordingly, we observed an occlusion of presynaptic inhibition by the use of either 8-Br cAMP, a non-hydrolysable analogue of cAMP, or forskolin, a stimulator of adenylate cyclase. Interestingly, gross increases in cAMP levels appeared not to affect the cholinergic response supported by the preliminary observation that the broad spectrum adenylate cyclase inhibitor, SQ 22536, also proved ineffective at inhibiting the $EPSP_M$. These data demonstrate that cAMP does not play a direct role in mediating either presynaptic ACh release or postsynaptic mAChR-mediated responses as increasing or decreasing basal cAMP levels does not alter the cholinergic response. In contrast, one study demonstrated that an enhancement of adenylate cyclase results in an increase in evoked and spontaneous release of glutamate from Schaffer collateral/commissural terminals in area CA1 of the rat hippocampus (Chavez-Noriega & Stevens, 1994). Taken together, this suggests that the release and action of some neurotransmitters are sensitive to changes in intracellular cAMP whilst other neurotransmitters are relatively insensitive to cAMP and maybe under the control of other second messengers.

These data provide functional confirmation of the accepted notion that adenosine A_1Rs act through $G_{i/o}$ to inhibit adenylate cyclase although the result of increasing or decreasing cAMP will have differing functional consequences on neurotransmitter release depending on the type of synapse and the basal level at which cAMP rests in the presynaptic terminals examined. However, the interpretation of the data is complex as increased cAMP could be having other effects like simply increasing neurotransmitter release machinery directly. This could include adenosine A_1R desensitisation and the phosphorylation of ion channels controlling terminal excitability. Nethertheless, the fact that increased cAMP does not affect the $EPSP_M$ suggests that release cannot be further facilitated by raising intracellular cAMP.

The logical progression of these experiments is to explore the involvement of cAMP-dependent protein kinase A by stimulation of this enzyme to try and occlude adenosine A_1R-mediated inhibition of the cholinergic response. As increased cAMP does not facilitate the evoked cholinergic response then it is unlikely that stimulating PKA activity would increase an $EPSP_M$ unchallenged by adenosine. However, one possible interpretation for this assumes that the basal levels of cAMP or PKA are sufficient to produce maximal facilitation of neurotransmitter release. As such, the use of a PKA stimulator would have no effect on the $EPSP_M$ *per se* but may prevent adenosine A_1R-mediated reductions in cAMP from inhibiting release by artificially maintaining PKA activity. There is recent evidence to suggest that PKA acts to facilitate an early step in the secretory machinery at a protein called SNAP25 (Trudeau *et al.*, 1998). Taken together, a clear path can be described connecting adenosine A_1 receptor activation to fusion and release of neurotransmitter-filled vesicles.

Likewise, another protein kinase, PKC, has been shown to affect adenosine receptor modulation of presynaptic glutamate release in area CA3 of organotypic hippocampal slice cultures. PKC activation reduced the ability of adenosine to cause presynaptic inhibition (Thompson *et al.*, 1992). This study provides another means by which adenosine may exert its presynaptic effects. It is entirely possible that one or more intracellular signaling pathways may be utilised to produce presynaptic inhibition.

CHAPTER 5

MODULATION OF CHOLINERGIC RESPONSES BY OPIOID RECEPTOR ACTIVATION

5.1. Introduction

Other G-protein coupled receptors exist in the hippocampus and some may play a role in the modulation of cholinergic responses. In the previous chapter, we demonstrated that adenosine receptor activation resulted in a depression of mAChR-mediated synaptic responses presumably by inhibition of ACh release. Here we have focused on the effect that opioid receptor activation has upon the generation of $EPSP_M$s in the hippocampus.

5.1.1. General

The opioid receptor was first thought to exist when it was questioned why the analgesic actions of morphine and other related opioids required rigid structure and similar stereochemistry (Beckett & Casey, 1954). It was later thought that more than one type of receptor existed because nalorphine, a synthetic opioid, antagonised the actions of morphine but also acted as an analgesic in its own right. It was concluded that this latter action was mediated by an alternative opioid receptor (Martin, 1967). Pharmacological profiling of agonist activity in the chronic spinal dog preparation lead to the classification of the μ, κ and σ opioid receptors (Martin *et al.*, 1976). Later, the existence of δ-opioid receptors was confirmed by the relative potency of the non-specific opioid antagonist, naloxone, to reverse the endogenous opioid peptide inhibition of nerve-evoked contractions of the mouse vas deferens (Lord *et al.*, 1977).

It is now widely accepted that there are three pharmacologically well defined opioid receptors: κ, δ and μ. A single gene has been cloned for each subtype (Evans *et al.*,

1992; Kieffer *et al.*, 1992; Chen *et al.*, 1993; Minami *et al.*, 1993). However, homology screening of cDNA libraries identified a new opioid receptor, termed theORL$_1$ receptor (opioid receptor-like) (Mollereau *et al.*, 1994).

Cloning has identified the opioid receptors as belonging to the superfamily of G-protein coupled receptors, closely related to the somatostatin receptors (Evans *et al.*, 1992; Kieffer *et al.*, 1992). Extensive structural conformity exists between the three opioid receptor cDNA clones and the predicted proteins are of similar size with a 50-60% homology at the amino acid level.

Table 5.1 Endogenous opioids and their preferred receptors.

Precursor	Endogenous peptide	Receptors	Reference
Pro-opiomelanocortin	β-endorphin	μ and δ-receptor	Kosterlitz & Paterson, 1985
Proenkephalin	[Met]enkephalin [Leu]enkephalin	High affinity at δ and lower at μ	Corbett *et al.*, 1993
Pro-dynorphin	Dynorphin A Dynorphin A(1-8) Dynorphin B α-neoendorphin β-neoendorphin	High affinity for κ but significant affinities for δ and μ also.	Corbett et al, 1993
Pro-nociceptin/OFQ	Nociceptin	ORL$_1$ receptor	Meunier *et al*, 1995
Pro-endomorphin	Endomorphin-1 Endomorphin-2	High affinity for μ receptor only	Zadina *et al*, 1997

All three receptors share consensus N-linked glycosylation sites at the highly divergent N-terminal extracellular domain. The transmembrane domains share broad similarities between receptors especially in the second and third membrane spanning region whereas the C-terminal intracellular domains are quite different. Like other peptidergic receptors, the opioid receptors have a small (approximately 25 amino acid residues) third intracellular loop involved in G-protein binding. The extracellular loops show a lesser degree of homology between receptors compared to the intracellular loops which suggests membrane receptors that exhibit varied ligand binding properties but convergent intracellular signaling mechanisms.

In mammals, the endogenous opioid peptides are mostly derived from four precursors and tend to be active at more than one receptor subtype (see Table 5.1).

5.1.2. μ-Opioid Receptor Subtypes

Two splice variants of the MOR-1 gene, encoding the μ-opioid receptor, have been cloned, one variant having 8 amino acid residues missing from the C-terminal domain. The variants exhibit differences in agonist-induced receptor internalisation but show little variation in ligand binding (Koch *et al.*, 1998). Two pharmacologically defined subtypes of μ-opioid receptor, μ_1 and μ_2, have been postulated on the basis of biphasic radioligand binding characteristics of μ, δ and κ agonists (Wolozin & Pasternak, 1981). Very high affinity binding of each subtype agonist to the putative μ_1-receptor was observed as well as a high affinity binding to the appropriate receptor (μ, δ or κ). Naloxone blocked this very high affinity binding site. More recent studies have failed to confirm this classification. There is evidence, however, for a novel form of the μ-opioid receptor that binds analogues of morphine but not morphine itself. The ability of these morphine analogues to bind to this novel site is not blocked by δ and κ analogues (Rossi *et al.*, 1996). Further evidence comes from the MOR-1 knockout mouse which still shows antinociception by morphine analogues but not morphine (Schuller *et al.*, 1999). Two exons exist in MOR-1. The MOR-1 knockout mouse has a mutation in exon 1. However, another MOR-1 knockout mouse with a disruption to exon 2 was engineered displayed a lack of

antinociception by morphine or any of its analogues. This points toward an alternative transcript of exon 1 of the MOR-1 gene to produce a different μ-receptor.

5.1.3. δ-Opioid Receptor Subtypes

The DOR-1 gene encodes for the δ-receptor and, based on pharmacological investigations, two overlapping subdivisions of the receptor have been proposed (δ_1/δ_2 and δ_{cx}/δ_{cnx}). The best evidence for a δ_1/δ_2 subclassification comes from *in vivo* supraspinal antinociception experiments. It was found that the antinociceptive activity of two different δ-agonists was blocked by two different antagonists (Jiang *et al.*, 1991; Sofuoglu *et al.*, 1993) and showed no cross tolerance (Mattia *et al.*, 1991). δ-opioid receptor-mediated inhibition of adenylate cyclase performed on rat brain membranes reinforced the δ_1/δ_2 classification due to differential antagonism of δ-agonists (Buzas *et al.*, 1994). However, studies on NG108-15 cells (Ho *et al.*, 1997) or the human neuroblastoma cell line, SH-SY5Y (Connor *et al.*, 1997) have failed to find functional evidence for δ-receptor subtypes. The pharmacological properties of the cloned DOR-1 receptor show little evidence of the δ_1/δ_2 subdivision.

Alternatively, δ_{cx} was hypothesised to form a complex with μ or κ-receptors whilst δ_{ncx} did not. The evidence for this came from the observation that sub-antinociceptive doses of agonists at the δ_{cx} receptor potentiated μ-receptor mediated analgesia whereas higher doses acted at the δ_{ncx} receptor producing analgesia in its own right (Rothman *et al.*, 1993). Radioligand binding studies have furthered this classification of the δ_{ncx} receptor into $\delta_{(ncx\text{-}1)}$ and $\delta_{(ncx\text{-}2)}$, although more recently it has been suggested that the $\delta_{(ncx\text{-}1)}$ receptor is synonymous with the δ_1-receptor and δ_{cx} receptor the same as the δ_2 receptor (Xu *et al.*, 1993).

5.1.4. κ-Opioid Receptor Subtypes

Due to the poor selectivity of κ-agonists, receptor subtype identification is more tenuous than those proposed for μ- and δ-opioid receptor. [^{3}H]-ethylketocyclazocine binding in guinea-pig spinal cord demonstrated an heterogeneous population of high-affinity binding sites leading to the first proposal for κ_1-and κ_2-sites distinguished by

their sensitivity to DADLE (Attali *et al.*, 1982). Later, using the specific κ-opioid agonist, U-69593, the κ_1/κ_2 subdivision was affirmed (Zukin *et al.*, 1988).

A further subclassification of the κ_1-site came about when it was shown that dynorphin B and α-neo dynorphin, which both preferentially bind to the proposed κ_{1b} subtype, displaced [^{3}H]-U-69,593 binding in guinea pig brain (Clark *et al.*, 1989). It has also been suggested that a U-50,488 insensitive, κ_3-site, is present and is postulated to be the receptor mediating the antinociceptive effect of nalorphine (Paul *et al.*, 1991). The argument for the subdivision of the κ_1 receptor has been strengthened by the complex binding of [3H]-U69,593 in mouse brain (Lai *et al.*, 1994). κ_2 has also been classified into κ_{2a} and κ_{2b} (Rothman *et al.*,1990), the κ_{2b}-site having a higher affinity for β-endorphin and DADLE. Further subdivisions have been demonstrated in the guinea pig spinal cord (Ni *et al.*, 1995).

Although binding studies have classified a plethora of κ-opioid receptor subtypes, there is little functional pharmacological evidence to support these findings, a problem attributed to the lack of subtype specific antagonists available. Also, one gene each has been identified for the opioid receptor subtypes, κ, δ and μ but it is suggested that the sub-subtypes could be formed by post-translational modifications or from the interactions of the gene products with associated proteins such as RAMPs (McLatchie *et al.*, 1998).

5.1.5. Effector Systems

All of the opioid receptors belong to the G_i/G_o-coupled superfamily of receptors with no observed interaction with G_s and G_q G-proteins. Initially it was thought that only δ and μ opioid receptors acted via $G_{i/o}$ to inhibit adenylate cyclase, activate an inwardly rectifying potassium conductance and also inhibit voltage-dependent calcium conductances (VDCCs) whereas κ receptors only acted via the latter mechanism. However, recent evidence has demonstrated that κ receptors, in some cell types, also act via an inwardly rectifying potassium channel (Grudt & Williams, 1993).

Although it appears that there is convergent signaling of the same downstream effectors, the heterogeneity of G-Protein subunits which can combine to form heteromers allows for subtle differences in downstream effector targets. Besides the direct G-protein βγ or α subunit-mediated cellular response to opioid receptor activation, there are a number of responses with unknown intermediate mechanisms including the activation of PLA_2, PLCβ, MAPkinase.

5.1.5. Opioid receptors in the hippocampus

Histological evidence for the presence of opioid receptors in the hippocampus comes from several lines of investigation. The majority of opioid binding sites (approximately 80%) appear to be either μ- or δ-receptors (Chang *et al.*, 1981). Autoradiographical studies demonstrated that high densities of μ-receptor binding was evident in the CA1-3 cell body region and *stratum oriens* with lower densities of δ-receptors showing a similar distribution (Crain *et al.*, 1986). This distribution pattern was confirmed by mRNA *in situ* hybridisation (Mansour *et al.*, 1994). δ-receptor mRNA was also identified in the granule cells of the dentate gyrus. μ-receptor mRNA was observed in lesser amounts with the exception of certain large cells, expressing high amounts of mRNA, scattered throughout the *stratum oriens, pyramidale* and *radiatum*. δ and μ-receptor mRNA is found mainly in the dorsal hippocampus whilst κ-receptor mRNA was restricted to the temporal hippocampus and localised in the *stratum radiatum*.

The δ-receptor has been examined in greater detail using immunocytochemistry (Commons & Milner, 1997). This determined that δ-receptors were present on many interneurons and non-principal cells, particularly in the *stratum oriens*, with little staining of CA1-3 pyramidal cells. Double-staining techniques localised the δ-receptor with GABA interneurons containing somatostatin. Electron microscopy localised the receptor to the dendrites and spines of pyramidal cells at or near postsynaptic densities as well as on a heterogeneous population of axon terminals and astrocytes. This indicates multiple sites of action of the δ-opioid receptor on a range of cell types. μ-receptors have been shown to co-localise with the δ-receptor

and GABA (Bausch & Chavkin, 1995) and the GABA receptor in the hippocampus and dorsal raphe (Kalyuzhny *et al.*, 1997) suggesting a similarity of distribution of the two subtypes of opioid receptor.

It is generally accepted that opioid receptor activation in the CNS leads to activation of $G_{i/o}$ which results in an inhibitory effect on neurons by decreasing adenylate cylcase activity, inhibiting VDCCs (Childers, 1993; Moises *et al.*, 1994) as well as activating an inwardly rectifying K^+ current (G_{irk}) causes cell hyperpolarisation (Williams *et al.*, 1982; Madison & Nicoll, 1988) and an inhibition of a hyperpolarisation-activated cation conductance (I_h) (Svoboda & Lupica, 1998) also resulting in cell hyperpolarisation. In the hippocampus, pyramidal cell neurotransmission is enhanced by opioid receptor activation, an effect that can be explained by the inhibition of local inhibitory GABAergic interneurons which results in a disinhibition of the pyramidal cells (Zieglgansberger *et al.*, 1979; Lee *et al.*, 1980; Lupica & Dunwiddie, 1991). Recent studies demonstrated that activation of μ-opioid receptors inhibit a subclass of interneurons that terminate on pyramidal cell bodies whereas δ-opioid receptor activation inhibits interneurons that terminate in the dendritic region of the pyramidal cell (Svoboda *et al.*, 1999) suggesting different sites at which modulation of principal neuron excitatory neurotransmission may occur. μ-opioid receptor activation may increase signal output whilst δ-receptor activation may amplify afferent signaling to the pyramidal cell dendrites.

Due to its excitatory effects in the hippocampus, the opioidergic system was examined for a potential role in learning and memory. Dynorphin, injected into the hippocampus, was shown to act as a reinforcer of operant conditioning in behavioural tests (Stevens *et al.*, 1991). In addition, endogenous opioids are only released upon high frequency stimulation (≥10 Hz) (Wagner *et al.*, 1990). It was therefore argued that opioids might play a role in LTP formation, possibly by inhibiting GABA release. This was confirmed by the inhibition of LTP by naloxone (Derrick & Martinez, 1994) at synapses of the CA3 region and dentate gyrus. However contradictory evidence showed no such blockade of mossy fibre LTP (Weisskopf *et al.*, 1993; Salin *et al.*, 1995). This may be, in part, due to the complex circuitry of the CA3 region. Using intracellular voltage-clamp recordings of

characterised synaptic responses it was shown that opioid receptor antagonism does inhibit LTP formation at mossy fibre-CA3 pyramidal cell synapses (Williams & Johnston, 1996).

The septohippocampal cholinergic projection is also implicated in memory processing and could also be a site of action for endogenous opioids. Initial evidence showed that activation of μ-receptors resulted in a decrease of ACh release (Ennis & Wyllie, 1984). However, various responses to different opioid receptor subtypes have been reported in different tissues and/or species. For example, activation of κ-opioid receptors decreases ACh release in the rabbit hippocampus (Jackisch *et al.*, 1986) and guinea pig cortex (Siniscalchi & Bianchi, 1988) but not in rat striatum (Mulder *et al.*, 1984). In addition, it was shown that only μ-opioid receptor activation caused a decrease in high K^+ evoked ACh release (Lapchak *et al.*, 1989) in the rat hippocampus suggesting a potential inhibitory role for opioids in mnemonic processing. However, dynorphin A, an endogenous κ-agonist which demonstrated no effect on ACh release, blocked galanin-induced inhibition of ACh release and reduced the associated impairment of learning and memory in the rat (Hiramatsu *et al.*, 1996). However, κ-agonism did not enhance memory acquisition in the normal rat.

These reports demonstrate that the opioid system has a role to play in learning and memory and, specifically, which receptor subtypes may modulate these processes by regulating cholinergic transmission in the hippocampus. However, to date there has been no studies that have directly examined the consequences of opioid receptor activation on synaptic responses mediated by cholinergic afferents in the hippocampus. Here we have examined the effect of a range of opioid agonists on the mAChR-mediated slow EPSP in the rat hippocampus.

5.2. Results

I have shown in previous chapters that cholinergic neurotransmission in the brain is subject to regulation by adenosine A_1 heteroreceptors. By comparison to amino acid-mediated synaptic transmission it is likely that other receptors also modulate the $EPSP_M$. For the reasons described in the introduction of this chapter we chose to investigate opioid receptors since these are known to couple to $G_{i/o}$ and can inhibit the release of transmitters such as GABA.

In contrast to the inhibition of the $EPSP_M$ by adenosine A_1 receptor activation, DAGO (Zajac *et al.*, 1990) (1 μM), an opioid receptor agonist of moderate selectivity for the μ subtype, produced an enhancement of the $EPSP_M$ (208±35% of control, n=4) within 8 min of being applied (fig. 5.1). The opioid receptor antagonist, naloxone (Zajac *et al.*, 1990) (5 μM), fully and rapidly reversed this excitatory effect (99±8% of control, n=3).

As DAGO does not possess high specificity for the μ subtypes of the opioid receptor and since numerous reports suggest different roles for different opioid receptor subtypes in the regulation of ACh, more subtype specific agonists were tested. 100 nM Etonitazene (May *et al.*, 1999), a potent μ-opioid receptor agonist produced a comparable enhancement of the $EPSP_M$ to that of DAGO (186±35% of control, n=3). In contrast, 1 μM U-50488 (Narita et al., 1999), a κ-opioid receptor agonist, had little effect on the $EPSP_M$ (103±11% of control; n=5). Similarly, the δ-opioid receptor agonist, DTLET (Zajac *et al.*, 1990), had no effect on the $EPSP_M$ at 1 μM (109±16% of control, n=5) (fig. 5.2). None of these agonists produced any changes in cell input resistance, membrane potential or spike frequency adaptation to a depolarising current step. These data suggest that μ-opioid receptor activation alone causes an enhancement of the $EPSP_M$ and that the κ– and δ-opioid receptor subtypes appear not to be majorly involved in the modulation of muscarinic ACh receptor-mediated synaptic responses in the rat hippocampus (fig. 5.3). To establish whether the effect of DAGO was mediated pre- or post-synaptically, the effect of DAGO on carbachol

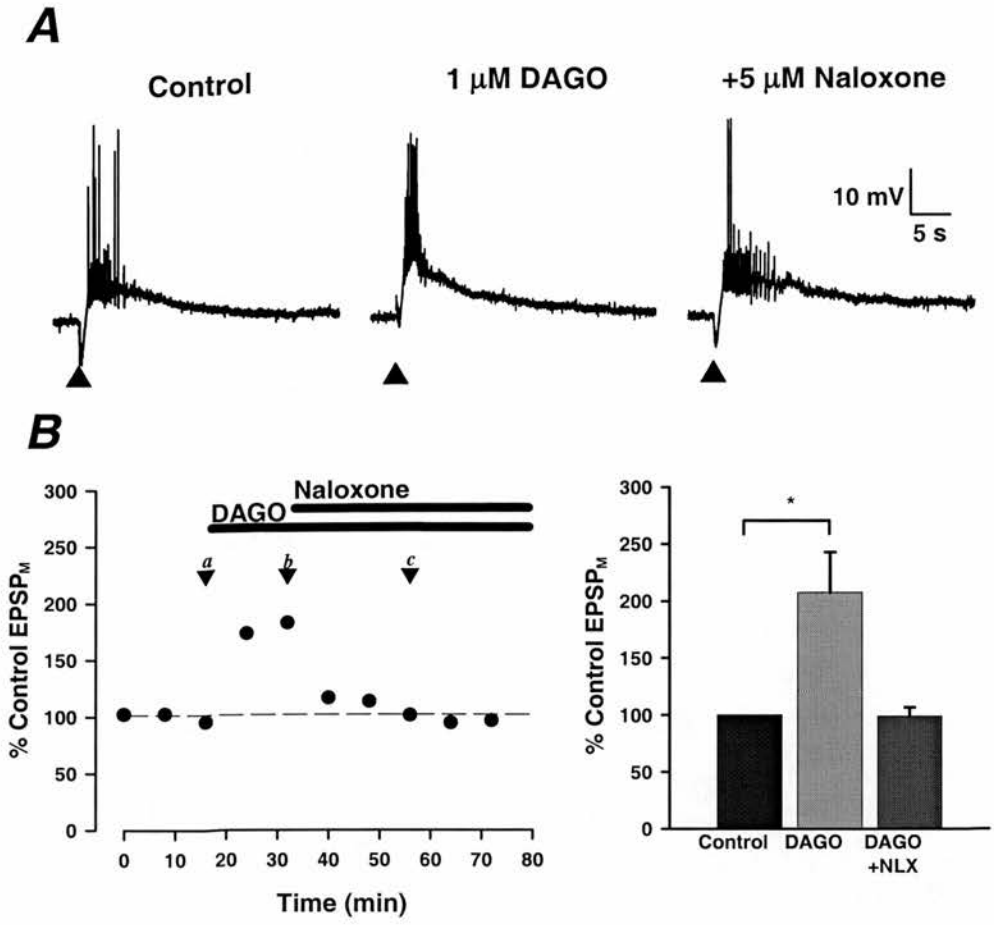


Figure 5.1 Opioid receptor activation causes an enhancement of the $EPSP_M$.

A shows intracellular current clamp recordings of $EPSP_M$s in control media (left), during 1 μM DAGO application (middle) and after subsequent addition of 5 μM Naloxone. Cell membrane potentials were maintained at –67 mV throughout the experiment. Filled triangles denote the position of the stimulus (10 @ 20Hz). *B* illustrates the effect of DAGO and naloxone on the $EPSP_M$ peak amplitude over time. The bar graph (*right*) demonstrates that DAGO caused a mean enhancement of the $EPSP_M$ peak amplitude in 4 experiments (Control vs DAGO, $p<0.05$). Naloxone fully reversed this mean enhancement in 2 experiments (Control vs DAGO+Naloxone, $p>0.05$). Values are means ± S.E.M represented by the error bars. The input resistance of the cell was 40 MΩ, the resting membrane potential was –63 mV and the spike height was greater than 0 mV.

induced cell depolarisation was assessed. 5 μM DAGO had little effect on the magnitude of depolarisation that was induced by carbachol (fig. 5.4*A*). Likewise, the mAChR-induced loss of spike frequency adaptation and increase in input resistance were unaffected by opioid receptor activation (fig. 5.4*B*&*C*). This evidence points towards a presynaptic locus for the action of DAGO on $EPSP_M$ generation. Close analysis of the opioid receptor enhancement of the $EPSP_M$ revealed that opioid receptor activation predominantly increases the peak amplitude of the $EPSP_M$ with little effect on the duration of depolarisation (fig. 5.5). This is in contrast to the effects of the potassium channel blocker 4-AP and the AChE inhibitor, eserine, which produced enhancements of both the peak amplitude and the duration of the $EPSP_M$.

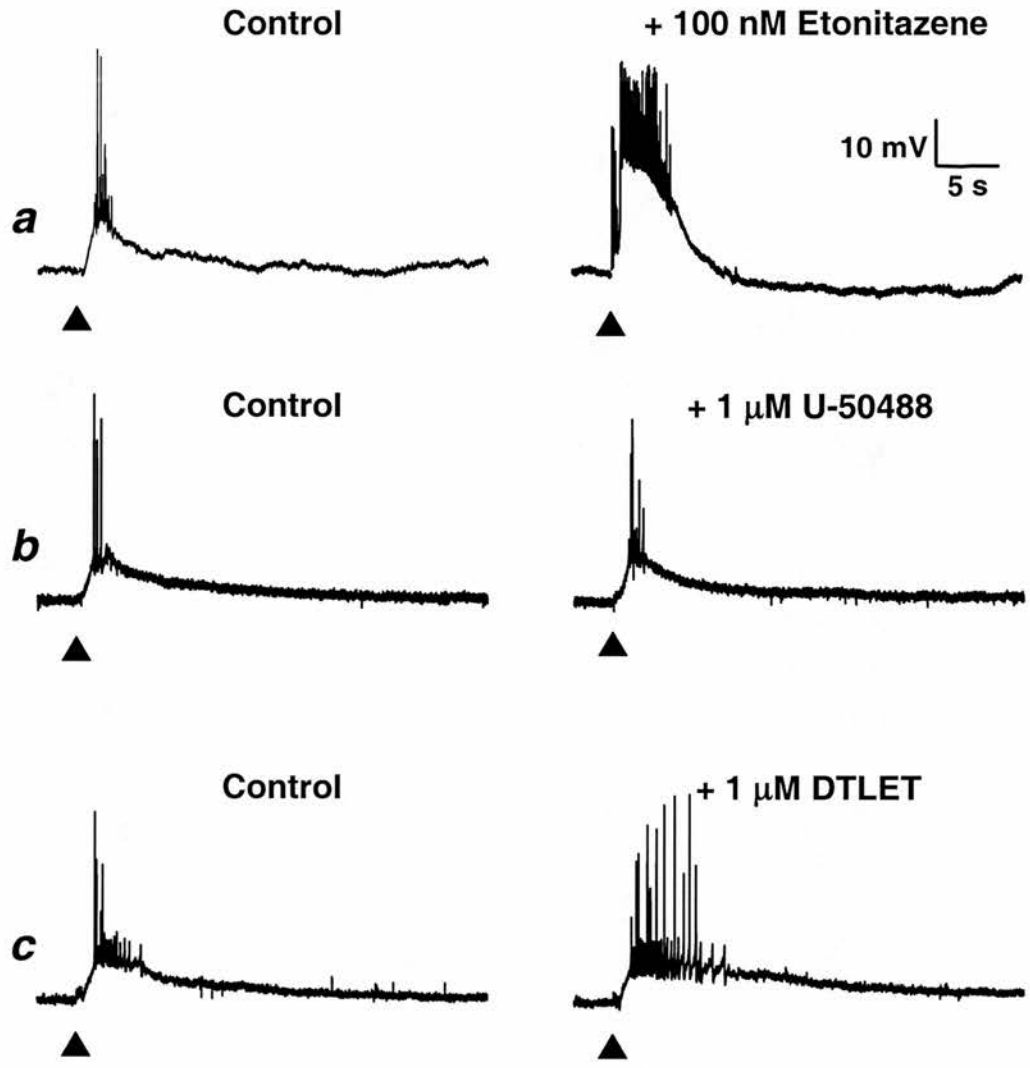


Figure 5.2 **μ-opioid receptor activation alone enhances the $EPSP_M$.**

Intracellular current clamp recordings showing the effect of 100 nM Etonitazene (*a*), 1 μM U-50488 (*b*) and 1 μM DTLET (*c*) on the $EPSP_M$. . Cell membrane potentials were maintained between –66 to -69 mV throughout the experiments. Filled triangles denote the position of the stimulus (10 @ 20Hz). The input resistance of the cells ranged from 33-39 MΩ, the resting membrane potential ranged from –63 to -68 mV and the spike height was greater than 0 mV

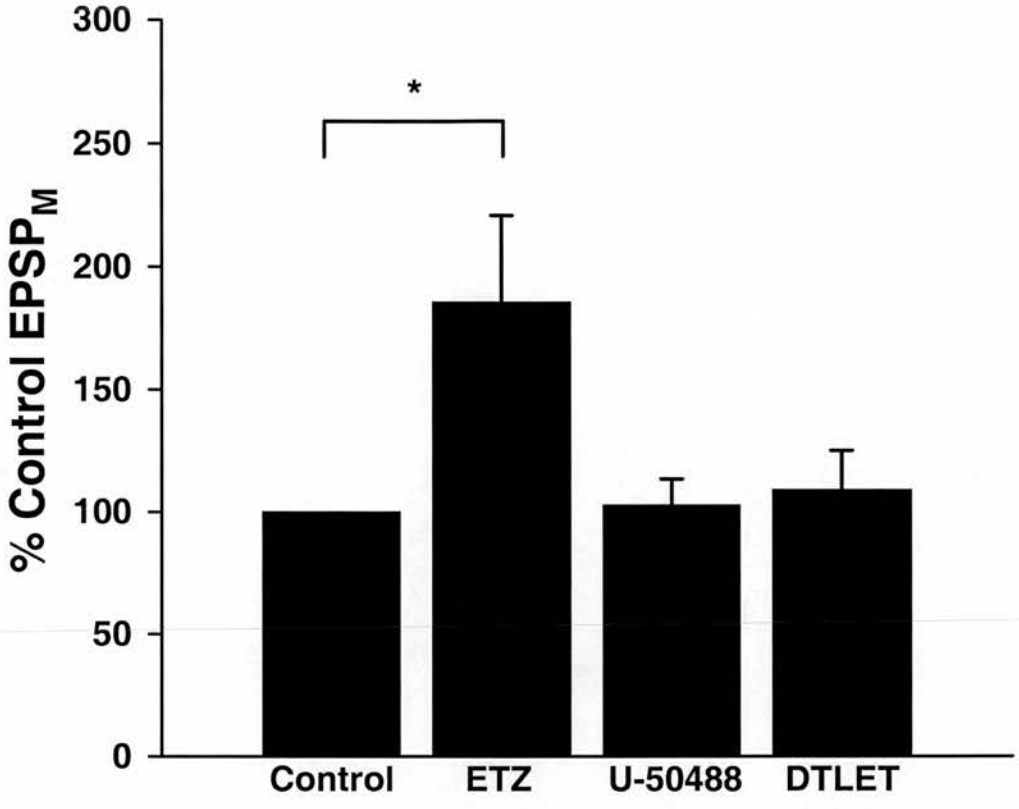


Figure 5.3 μ-opioid receptor activation causes a mean enhancement of the $EPSP_M$.

A bar graph to show the mean effect of three opioid receptor subtype selective agonists, 100 nM etonitazene (ETZ), 1 μM U-50488 and 1 μM DTLET, on the peak amplitude of the $EPSP_M$ in three, five and five experiments respectively (Control vs Etonitazene, $p<0.05$). Values are means ± S.E.M represented by the error bars.

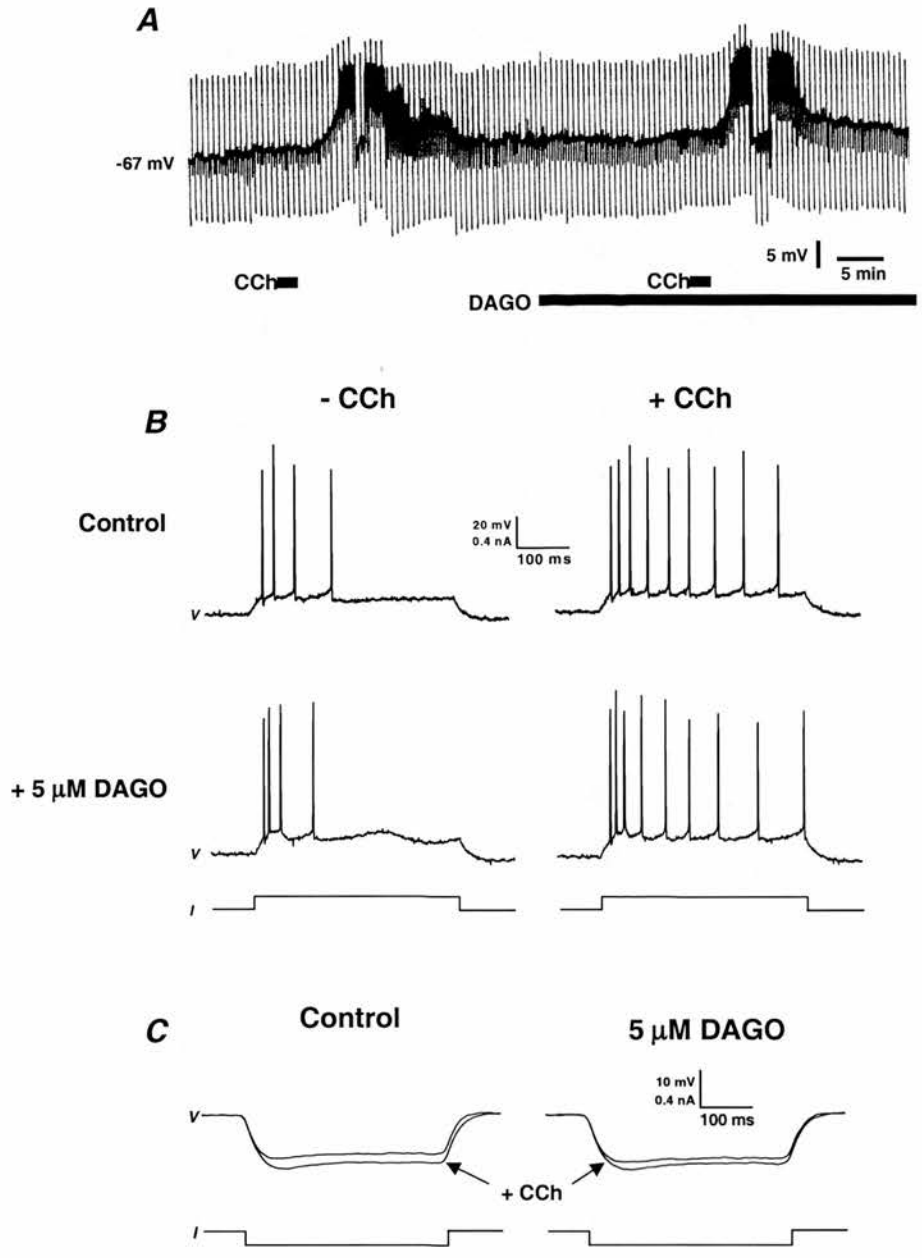


Figure 5.4 **Effects of DAGO on spike frequency adaptation and carbachol-induced depolarisation of CA1 pyramidal cells.**

A shows chart recordings of the membrane potential (V_m) of a CA1 pyramidal neuron and its response to depolarising and hyperpolarising current steps. On application of 3 μM carbachol (CCh) for 1 minute, a large, slow depolarisation of V_m, accompanied by spontaneous action potential firing, was observed. Note the increase in input resistance also. These effects of CCh were unchanged in the presence of 5 μM DAGO (*right*). *B* illustrates the response of a cell, held at a control membrane potential, to a depolarising current step in the absence (*top left*) and presence of carbachol (*top right*). This is repeated (*bottom traces*) in the presence of 5 μM DAGO. Spike frequency adaptation, is unchanged by 5 μM DAGO. *C* shows the response of the cell membrane potential, current clamped back to a control membrane potential, to a hyperpolarising current step in the absence and presence of carbachol (*left*) and in the presence of DAGO in the presence of and absence of carbachol (*right*). The input resistance of the cell was 31 MΩ, the resting membrane potential was –64 mV and the spike height was greater than 0 mV. Apparent variations in spike height is due to the sampling rate (488 μs sampling interval).

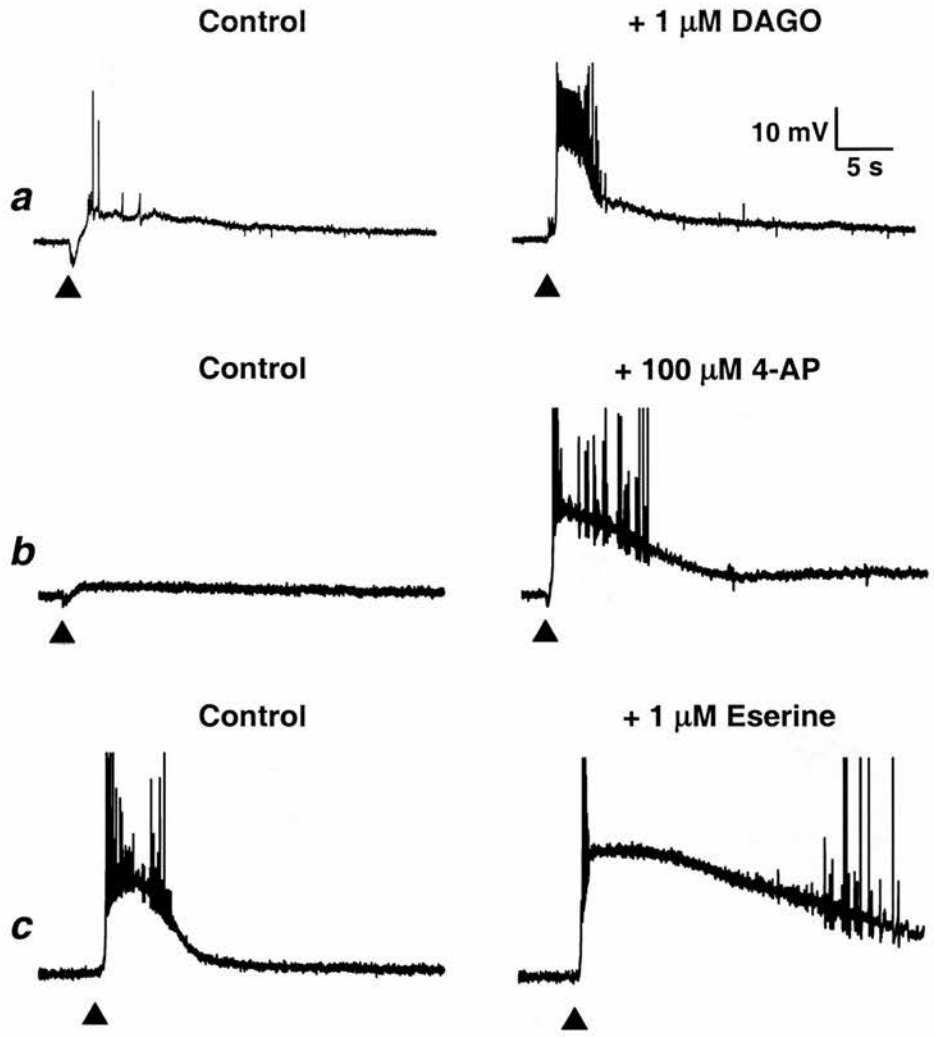


Figure 5.5 **A comparison of the enhancement of the $EPSP_M$ produced by 4-AP, eserine and DAGO.**

Intracellular current clamp recordings illustrating $EPSP_M$s in control media (*left*) and in the presence of 1 μM DAGO (*top right*), 100 μM 4-AP (*middle right*) and 1 μM eserine (*bottom right*). Note the enhancement produced by DAGO appears to be limited to an increase in peak amplitude and not duration. Cells were held between –65 and –68 mV. The filled triangles denote the position of the stimulus (10@20Hz). The input resistance of the cells ranged between 30-40 MΩ, the resting membrane potential ranged between –65 to -68 mV and the spike height was greater than 0 mV.

5.3. Discussion

The evidence presented in this chapter suggests that opioid receptor activation enhances cholinergic synaptic responses. Further investigation of this enhancement pointed towards presynaptic μ-opioid receptor involvement.

In situ hybridisation and autoradiographical studies provide good evidence for the presence of both μ and δ opioid receptor within the *stratum oriens* of the hippocampus (Chang *et al.*, 1981; Crain *et al.*, 1986; Mansour *et al.*, 1994). A similarity of distribution of both δ and μ opioid receptors has been suggested (Bausch & Chavkin, 1995; Kalyuzhny *et al.*, 1997) localising these receptors to dendrites and spines of pyramidal neurons as well as to a heterogeneous population of axon terminals. This evidence supports a presynaptic role for the μ-opioid receptor in cholinergic neurotransmission in the hippocampus.

Although receptor classification is somewhat unclear for opioid receptors in general, there does appear to be reasonable evidence for the existence of two subtypes of μ-opioid receptor. However, the pharmacology of these two binding sites is controversial and, as such, it is beyond the boundaries of this study to implicate a specific μ-opioid receptor subtype in the enhancement of cholinergic neurotransmission.

The activation of μ-opioid receptors has been reported to directly activate the G-protein, $G_{i/o}$, and ultimately resulting in the activation of a variety of intracellular messengers and effector mechanisms. However, it is generally accepted that these receptors mediate an inhibitory effect by decreasing neurotransmitter release and cell hyperpolarisation. In the hippocampus, evidence suggests that opioid receptors inhibit local inhibitory GABA interneurons and, thereby, result in increased excitatory amino acid-mediated synaptic transmission at pyramidal cell synapses. The experiments presented in this chapter are conducted in the presence of $GABA_A$ and $GABA_B$ antagonists thus suggesting that the enhancement of cholinergic

responses is not due to disinhibition of GABAergic interneurons acting to restrict cholinergic synapses.

If opioid receptor activation produces mainly inhibitory effects upon neurotransmission in the hippocampus, then how can it be that cholinergic neurotransmission is enhanced? One possibility is that opioid receptor systems interact with other receptor systems that act to tonically inhibit cholinergic release. As such, the adenosine A_1 or the galanin receptor systems may be inhibited by opioid receptor activation. Interestingly, all these receptor types act via $G_{i/o}$ and constant activation of opioid receptors may possibly utilise G-proteins that ordinarily are mediating adenosine A_1R inhibition or possibly galanin inhibition. Thus, the removal of adenosine A_1R or galanin receptor-mediated inhibition may mask any inhibition resulting from opioid receptor activation. It is also possible that opioid receptor activation results in the inactivation of adenosine or galanin receptors without itself having an inhibitory influence. These theories depend upon the tonic activation of A_1 and galanin receptors or, alternatively, co-activation of these receptors by co-released adenosine or galanin. Laboratory observations using the adenosine A_1 receptor antagonist DPCPX (Morton & Davies, 1997), suggest a tonic inhibition of the $EPSP_M$ whereas no such tonicity has been tested using galanin receptor antagonists. Obvious experiments would be to compare adenosine receptor- or galanin receptor-mediated inhibition of cholinergic responses in the presence and absence of an opioid agonist such as DAGO. Alternatively, the enhancement of cholinergic responses by opioid agonists could be compared in the presence and absence of antagonists of adenosine A_1 or galanin receptors.

Another possible explanation for opioid receptor-mediated enhancement of cholinergic neurotransmission would be an inhibition of acetyl cholinesterase (AChE) thus increasing the amount of ACh present in the synaptic cleft. However, on comparison of the action of eserine, a cholinesterase inhibitor, with the actions of opioid receptor agonists, it is fair to say that each pharmacological agent produces a different type of enhancement of the $EPSP_M$. Thus, whereas eserine causes an increase in peak amplitude and duration of the cholinergic response, opioid receptor activation appears to increase the peak amplitude without affecting the duration of response. This tentatively suggests that the opioid agonists used in these experiments

do not exert their effect by cholinesterase inhibition. To confirm this supposition, a good experiment to perform would be to use ACh to depolarise the cell in the presence and absence of DAGO. Unlike carbachol, ACh is broken down by AChE and should DAGO inhibit AChE activity it should increase the magnitude and duration of the ACh-induced response. It would also be useful to examine the enhancement of the $EPSP_M$ by opioid agonists in the absence and presence of an AChE inhibitor.

Like AChE inhibition, the blockade of 4-AP sensitive K^+ channels resulted in an increased peak amplitude and duration of the $EPSP_M$ contrasting with the enhancement observed in the presence of opioid receptor agonists. Therefore, it is unlikely, but not impossible, that opioid receptors are acting via 4-AP sensitive K^+ to enhance the $EPSP_M$ for example by allowing an increase in presynaptic Ca^{2+} influx.

Evidence does exist to contradict the enhancement of cholinergic transmission in the hippocampus observed here. Thus, high K^+ evoked ACh release is decreased in the rat hippocampus by μ opioid receptor (Lapchak *et al.*, 1989). However, this method of ACh release can not be directly compared with synaptically evoked release as every neuron in the tissue will depolarise, *en masse*, releasing numerous neurotransmitters resulting in unphysiological network activity with unpredictable consequences.

The effect of μ-opioid agonists to enhance of cholinergic transmission would be expected to facilitate memory and learning tasks. This precise hypothesis has not been formally tested. However, as mentioned in the introduction, it was found that κ-opioid agonists did not improve memory acquisition in the normal rat (Hiramatsu *et al.*, 1996) but did reverse a galanin-induced inhibition of ACh release and associated impairment in memory and learning. This suggests opioid receptor activation does play a role in memory and learning in so far as it can compensate for memory deficits caused by decreased cholinergic transmission. In the present study we found that k agonists did not themselves modulate the $EPSP_M$. However, it would be interesting to investigate whether they overcome the galanin-induced blockade of the $EPSP_M$. The fact that κ-agonists do not modulate the $EPSP_M$ might also point to a lack of endogenous galanin inhibitory tone on the $EPSP_M$.

Generally, endogenous opioids are released in the CNS in response to pleasurable stimuli and are heavily implicated in mechanism of addiction but also play a part in the pain response. It makes sense that during pleasurable experiences memory would be facilitated, for example, teaching an animal to perform tasks by positive reinforcement. It is also reasonable to assume that opioids released due to a pain response would enhance memory in order to prevent the same course or action resulting in injury. This could be equated with aversion therapy or negative reinforcement of learning.

Therefore, in addition to the increased excitatory amino acid receptor mediated neurotransmission caused by the inhibition of GABA interneurons by opioids, enhanced cholinergic transmission at septohippocampal terminals could contribute, synergistically, to a generally heightened state of arousal and an improvement in memory acquisition.

CHAPTER 6

MODULATION OF CHOLINERGIC RESPONSES BY GALANIN: IS GALANIN IMPORTANT IN LTP FORMATION?

6.1. Introduction

This chapter aims to investigate the role of galanin in the hippocampus. Galanin has been localised in the hippocampus and has been shown to modulate cholinergic function in the rodent hippocampus. The opportunity to study the function of this neuropeptide in a galanin knockout mouse has allowed the investigation of how galanin affects the modulation of the cholinergic response as well as examining the role galanin may play in LTP. As such, galanin, its receptors, and its function in memory and learning will be reviewed in some detail.

6.1.1. General

Galanin is a peptide comprising 29 amino acids (30 in human) first isolated from the upper small intestine of pigs (Tatemoto *et al.*, 1983). The name is derived from the N- and C-terminal residues, **g**lycine and **alanin**e respectively. The peptide is synthesised as a preprohormone which contains a 59 amino acid C-terminal flanking region called Galanin Message Associated Peptide (GMAP) which, so far, has no known function (Rökaeus & Brownstein, 1986). Both galanin and GMAP appear to belong to a new peptide family. Galanin is involved in the regulation of a variety of physiological processes including feeding, insulin release, lactation, spinal reflex, gut contractility, growth, learning, memory and depression (Bedecs *et al.*, 1995; Kask *et al.*, 1997). Only the role of galanin in cognition will be covered in any depth within this chapter.

Structure-activity studies performed on the galanin peptide demonstrated that the N-terminal 1-16 amino acids are conserved across species and this peptide fragment is sufficient for receptor recognition (Fisone *et al*, 1989b). The 17-29 C-terminal fragment is not recognised by hypothalamic, hippocampal, spinal cord or pancreatic receptors and appears to have no biological effect. However, this fragment may be required for galanin binding to receptors in smooth muscle (Rossowski *et al.*, 1990) suggesting an alternative receptor subtype to that of the neuronal and exocrine tissue. Gal_{1-12} is the shortest form of galanin that demonstrates full receptor agonism but possesses a shorter half life than Gal_{1-29}. Fluorescence studies suggest that the N- and C-terminal portions of the Gal_{1-29} polypeptide line up, via a β bend, to protect sensitive bonds from proteolytic cleavage (Rigler *et al.*, 1991).

6.1.2. Galanin Receptors

Using [^{125}I]-Galanin, autoradiography of brain slices has established the existence of galanin binding sites in the cerebral cortex, amygdala, hypothalamus, thalamus, pons and cerebellum in humans (Johard *et al.*, 1992) and the prefrontal cortex, olfactory bulb, amygdaloidal complex, hippocampus, stria terminalis, nucleus accumbens, hypothalamus, thalamus and superior colliculus in the rat (Skofitsch *et al.*, 1986; Fisone *et al.*, 1989a).

Logic dictates that an endogenous ligand apparent in many anatomical areas and exerting its effects on varied physiological responses must act through multiple receptor subtypes and/or second messenger systems. Evidence for multiple receptor subtypes in native tissue comes from both radioligand and functional studies. For example, Galanin binding sites with a 10-fold difference in affinity for galanin and galanin 1-16 have been reported in the rat ventral hippocampus. (Fisone *et al*, 1989b). Also, there is evidence for an NH_2-terminal preferring receptor in the rat dorsal hippocampus, neocortex and neostriatum (Hedlund *et al.*, 1992).

Native galanin receptors have been reported to activate several second messenger pathways resulting in numerous cellular responses. Some examples include the inhibition of cAMP production (Amiranoff et al., 1991), inhibition of L-type and N-

type Ca^{2+} channels (Kalkbrenner et al., 1995; Palazzi et al., 1991); the activation of ATP-sensitive K^+ channels (Dunne et al., 1989) and inwardly rectifying K^+ channels such as GIRK-1 (Philipson et al., 1995), as well as the stimulation of phosphoinositol breakdown, calcium mobilisation (Sethi & Rozengurt, 1991), phospholipase A_2 (Mulvaney & Parsons, 1995) and MAP kinase (Seufferlein & Rozengurt, 1996).

It is clear that there exists a complexity of physiological responses to galanin binding but also differences in the functional pharmacology of such responses. Galanin reduced forskolin-stimulated cAMP with 250-fold greater potency in rat ventral hippocampus versus dorsal hippocampus suggesting either receptor diversity or differences in signal transduction pathways (Valkna *et al.*, 1995). Other possible explanations could be differences in the number of functioning receptors or the efficiency of receptor/effector coupling in the different regions of the hippocampus. However, the complex binding and functional profiles for galanin and other galanin-related peptides such as M35, M32 and C7 with a spectrum of antagonist/agonist activity are consistent with the existence of multiple receptor subtypes.

To date, three genes encoding galanin receptors have been cloned all of which possess significant homology to the rhodopsin family of G-protein coupled receptors including seven predicted hydrophobic membrane-spanning domains.

The cDNA for the human GALR1 encodes a protein of 349 amino acids (Probst *et al.*, 1992) sharing a high degree of homology to the rat GALR1 receptor (Burgevin *et al.*, 1995) and the mouse GALR1 homolog (Wang *et al.*, 1997). The mRNA distribution of rat GALR1, determined by *in situ* hybridisation, localised peptide expression to the brain including the hypothalamus, amygdala, ventral hippocampus, thalamus, brain stem (medulla oblongata, locus ceruleus, and lateral parabrachial nucleus) and spinal cord (dorsal horn) (Parker *et al.*, 1995; Gustafson *et al.*, 1996; Burgevin *et al.*, 1995) which appears to be in good agreement with [^{125}I]-galanin binding. No expression was detected in peripheral tissue.

Upon agonist binding, cloned GALR1 receptors, expressed in CHO cells, mediated a reduction of forskolin-stimulated cAMP (Parker *et al.*, 1995; Burgevin *et al.*, 1995). This reduction of cAMP was blocked by pertussis toxin (Parker *et al.*, 1995; Smith *et al.*, 1997) supporting the involvement of $G_{i/o}$-type G-proteins. Also, it has been

reported that GALR1 activated an inwardly rectifying K^+ current when GIRKs 1 and 4 were cotransfected into *Xenopus* oocytes (Smith *et al.*, 1998). Taken together, it appears likely that GALR1 may act broadly to inhibit neurotransmitter/hormone release.

GALR2 displays a low degree of amino acid identity with rat and human GALR1 (Smith *et al.*, 1997; Howard *et al.*, 1997; Wang *et al.*, 1997b). Rat GALR2 cDNA encodes a protein of 372 amino acids and shares 85% sequence identity with human GALR2 which contains an extra 15 amino acids on the C-terminus. Unlike GALR1, GALR2 appears to be transcribed in all tissues examined including brain (hypothalamus, hippocampus, amygdala, and pyriform cortex) and peripheral tissues such the vas deferens, prostate, uterus, ovary, stomach, large intestine, dorsal root ganglia and pancreatic cells (Smith *et al.*, 1997; Sten Shi *et al.*, 1997; Howard *et al.*, 1997; Wang *et al.*, 1997b).

GALR2 activation appears to produce a variety of cellular responses such as pertussis toxin-resistant inositol phospholipid hydrolysis, calcium mobilisation (Smith *et al.*, 1997) and arachidonic acid efflux (Walker *et al.*, 1997) in CHO cells as well as activating Ca^{2+}-dependent Cl^- channels in *Xenopus* oocytes. GALR2 activation did not inhibit forskolin-stimulated cAMP (Smith *et al.*, 1997) in CHO cells which, taken together, suggest a primary coupling to G-proteins $G_{q/11}$ activating phospholipase C.

The GALR3 gene was recently described by two groups (Smith *et al.*, 1998; Wang *et al*, 1997c) and is reported by Smith and coworkers to encode a protein of 370 amino acids sharing a sequence identity of 35 % with rat GALR1 and 52% with rat GALR2. The human GALR3 receptor is predicted to contain 368 amino acids with 90% identity to rat GALR3. 83 amino acids are conserved across the three subtypes of galanin receptor. mRNA transcripts have been detected in the heart, spleen and testis. However, low levels of mRNA were detected in discrete regions of the rat CNS with highest levels in the hypothalamus, lower levels in the olfactory bulb, cerebral cortex, medulla oblongata, caudate putamen, cerebellum and spinal cord, and no significant detection in hippocampus or substantia nigra. Both rat and human

GALR3 share a unique consensus phosphorylation site in the third intracellular loop that may be implicated in G-protein-coupling and receptor function.

GALR3 activation in *Xenopus* oocytes open GIRKs in response to applied galanin (Smith *et al.*, 1998). This effect was blocked by pertussis toxin suggesting receptor coupling to $G_{i/o}$ (Yao *et al.*, 1998).

6.1.3. Galanin Involvement in Memory Function

Galanin was first hypothesised to act as an inhibitory neurotransmitter co-released with ACh due to galanin immunoreactivity being present within 50-70% of cholinergic neurons in the medial septal-diagonal band complex of colchicine-treated rats (Melander *et al.*, 1985). Further *in vivo* and *in vitro* studies strengthened this idea that galanin acted on presynaptic cholinergic terminals in the rat ventral hippocampus to inhibit release of ACh and acted at postsynaptic sites to antagonise the actions of ACh (Fisone *et al.*, 1987; Hökfelt *et al.*, 1987, Crawley *et al.*, 1990).

Central administration of galanin results in performance deficits on a wide variety of learning and memory tasks in rodents. These deficits have been reported on delayed non-matching to position in rats (Robinson & Crawley, 1994; McDonald & Crawley, 1996), T-maze delayed alternation (Mastropaolo *et al.*, 1988), star-burst radial maze (Malin *et al.*, 1992), passive avoidance in mice (Ukai *et al.*, 1995), and Morris water maze in rats (Sundström *et al*, 1988). This behavioural effect is consistent with the idea that galanin inhibits cholinergic memory processes. Treatment with a galanin receptor antagonist alone facilitates performance in the Morris water maze (Ögren *et al.*, 1992) suggesting that endogenous galanin may tonically inhibit cholinergic transmission.

Recently, it has been questioned to what extent galanin is co-secreted by cholinergic neurons. Immunolabelling in colchicine-treated rats may have overestimated the levels of coexistence. Using colchicine untreated rats and double *in situ* hybridisation, it was discovered that relatively few ventral hippocampal projecting neurons in the cholinergic basal forebrain expressed galanin mRNA. Basal forebrain neurons projecting to the dorsal hippocampus showed the greatest amount of mRNA

expression (Gaykema *et al.*, 1990; Miller *et al.*, 1998) although still not as much as previously thought. This evidence suggests that galanin is not widely co-released with ACh from cholinergic terminals in the ventral hippocampus and galaninergic fibres may arise from other brain regions such as the locus ceruleus (Melander *et al.*, 1986).

The ventral hippocampus expresses high levels of GALR1 mRNA (Burgevin *et al.*, 1995) and GALR2 is mainly expressed in the granule cell layer of the dentate gyrus in the dorsal hippocampus (Fathi *et al.*, 1997). Galanin inhibition of evoked [^{3}H]-ACh release was only apparent in the ventral, not dorsal, hippocampus (Fisone *et al.*, 1987, 1991). This suggests that the inhibitory effects of galanin on ACh release are primarily mediated through GALR1 in the rat ventral hippocampus. In contrast, it has been reported that [^{3}H]-ACh release in the dorsal hippocampus is increased by galanin perfusion in the awake rat (Schött *et al.*, 1998).

Studies performed on the *in vitro* hippocampal slice preparation showed a galanin receptor-mediated inhibition of long-term potentiation at the Schaffer-collateral-CA1 synapses in the guinea pig hippocampus (Sakurai et al., 1996) although there was no effect in the operant delayed non-matching to sample task, a test of short term memory, in the equivalent region of the rat hippocampus (Robinson & Crawley, 1994). There is no evidence to suggest that galanin causes cognitive impairment in the dorsal hippocampus.

Recent studies from our laboratory have demonstrated that galanin inhibits LTP, but not LTD, induced by theta-burst or tetanic stimulation in the apical and basal dendrites of CA1 pyramidal cells in the rat ventral hippocampus (Coumis & Davies, 1999). Galanin was shown not to affect NMDA receptor- or metabotropic glutamate receptor-mediated responses implying that the effect of galanin upon LTP is downstream of these receptors. It is suggested that galanin may be acting via inhibition of a kinase, possibly protein kinase C.

6.1.4. Electrophysiological Effects of Galanin on Hippocampal Neurons

Previous studies have demonstrated that bath applied galanin resulted in a slight hyperpolarisation of the membrane potential in CA1 pyramidal cells without any changes in input resistance (Dutar *et al.*, 1989b). Galanin did have an effect on electrically evoked responses. Excitatory amino acid mediated neurotransmission at Schaffer collateral-CA1 synapses was slightly reduced by galanin. However, it was the inhibition of an atropine-sensitive long lasting depolarisation by galanin that proved of major interest. Galanin did not show any effect on responses to exogenously applied ACh suggesting a presynaptic inhibition of galanin on ACh-releasing nerve terminals. This result is consistent with several studies of galanin regulation of ACh release and ACh-dependent functions in the hippocampus (Fisone *et al.*, 1987; Palazzi *et al.*, 1988; Palazzi *et al.*, 1991).

6.1.5. A Transgenic Mouse Lacking a Functional Galanin Gene

In this chapter I have had the opportunity, in conjunction with Urania Coumis, to examine LTP and the septohippocampal cholinergic projection in hippocampal slices from mice possessing a null mutation in the galanin gene (Wynick *et al.*, 1998).

There is no evidence to suggest that there is any changes in galanin receptor expression levels in the $Gal^{-/-}$ mice due to the lack of a galanin receptor antisera that would allow immunohistochemical analysis.

However, Ach function was examined and these mice were shown to possess fewer cholinergic markers in the medial septum and vertical diagonal band and an increase in Choline acetyltransferase (ChAT) activity. This implies that the remaining cholinergic neurons have compensated for the developmental loss by upregulating ChAT activity which is supported by the lack of behavioural deficits when mutant mice were tested in the Morris water maze. The loss of cholinergic cells with age is a process that has been reported in the rat (Luine *et al*, 1986; Armstrong *et al.*, 1993). There is no further age-dependent loss of cholinergic neurons in the mutant mice suggesting that the cholinergic neurons lost with age are the same subset of neurons

that are lost due to the galanin gene knockout. However, a decrease of ACh release was observed in aged mutant mice, paralleled by significant deficits in performance in the Morris water maze.

In this chapter, we have examined LTP and other parameters of basal synaptic transmission in wt and galanin knockout mice. Also, a study of the effect of bath applied galanin on cholinergic neurotransmission was completed in the *stratum oriens* of rat slices as well as slices taken from wt and galanin deficient mice.

6.2. Results

Initially, it was necessary to assess the effect of galanin on the $EPSP_M$ generated in the rat hippocampus. Fig 6.1 demonstrated that galanin significantly inhibited the peak amplitude of the $EPSP_M$ to 55 ±6% of control (n=3; $p<0.05$) without affecting spike frequency adaptation or input resistance (n=3; data not shown).

Subsequently, slices prepared from transgenic mice lacking a functional galanin gene and their wild type littermates were used and intracellular recordings were made from CA1 pyramidal cells. Firstly, we examined the general passive cell properties and response to electrically-evoked glutamate and GABA-mediated synaptic transmission (fig. 6.2). Spike frequency adaptation was not significantly affected by the absence of functional galanin (wt, n=5; *Gal*$^{-/-}$, n=7), nor was the input resistance of the cell (wt, 49 ±5 MΩ; n=5 and *Gal*$^{-/-}$, 59 ±2 MΩ; n=7). In addition, no appreciable differences in the glutamate/GABA-mediated EPSP/IPSP were observed between wt (n=5) and *Gal*$^{-/-}$ mice (n=7).

Electrical stimulation of the *stratum oriens* region of mouse hippocampal slices produced, in CA1 pyramidal cells, a mAChR-mediated slow depolarisation which appeared very similar to the $EPSP_M$ recorded from rat hippocampal neurons (fig. 6.3*A*). Both wt (n=3) and *Gal*$^{-/-}$ mice (n=4) exhibited $EPSP_M$s. On application of 400 nM rat galanin (Dutar *et al.*, 1989b), the peak amplitude of the $EPSP_M$ was inhibited by 50 ±3% of control in wt mice (n=3; $p<0.05$). In contrast, 400 nM galanin had no significant effect on the peak amplitude of the $EPSP_M$ in *Gal*$^{-/-}$ mice (94 ±5% of control, n=4; $p>0.05$) (fig. 6.3*B*). A significant difference was observed in the magnitude of inhibition that galanin exerted on the $EPSP_M$ in wt compared with *Gal*$^{-/-}$ mice ($p<0.05$).

Extracellular field recordings were used to examine paired pulse facilitation and long-term potentiation (LTP) of field excitatory postsynaptic potentials (fEPSPs) at CA3-CA1 glutamatergic synapses in the *stratum oriens*. No significant difference in PPF at interpulse intervals of 25 ms and 50 ms was observed between wt and *Gal*$^{-/-}$ mice (fig. 6.4).

LTP induced by tetanic stimulation (100 stimuli @ 100Hz) was examined in the CA3-CA1 synapses of both the *stratum oriens* and the *stratum radiatum* (fig. 6.5). No difference in LTP was observed in the *stratum radiatum* (wt, 143 ±9 of control (*n*=7); *Gal*$^{-/-}$, 155 ±13 (*n*=12)). In contrast, a significant but small deficit in LTP was observed in the *stratum oriens* (wt, 158 ±13 (*n*=8); *Gal*$^{-/-}$, 130 ±8 (*n*=8)). This deficit was apparent from the onset of induction and was maintained as the LTP plateaued.

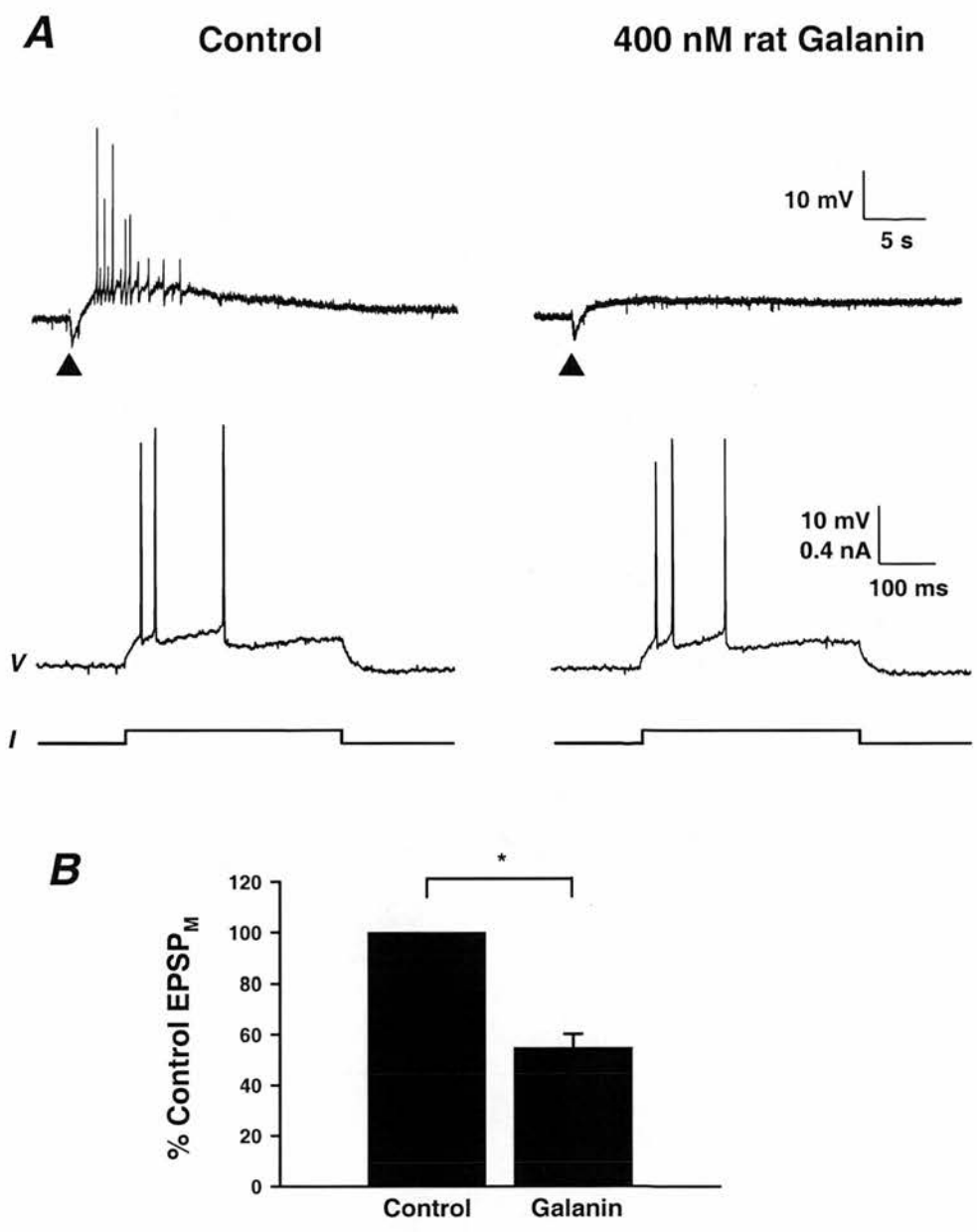


Figure 6.1 Galanin inhibits the EPSP$_M$ in rat CA1 pyramidal cells

A (*top traces*) shows synaptic traces of EPSP$_M$s from CA1 pyramidal cells held at –67 mV before (*left*) and after (*right*) 400 nM rat galanin was applied to the slice. The filled triangles denote the position of the stimulus (10 @ 20Hz). A (*bottom traces*) illustrates the voltage change of the cell in response to a depolarising current step before (*left*) and after (*right*) the application of galanin. Note that spike frequency adaptation of action potential firing is unaltered by galanin. Apparent variations in spike height are due to the sampling rate (sampling interval was 488 μs). *C* shows a bar graph illustrating the mean inhibition of the peak amplitude of the EPSP$_M$ by galanin in 3 experiments. Values are mean ± S.E.M. represented by the error bars. The input resistance of the cell was 34 MΩ, the resting membrane potential was –66 mV and the spike height was greater than 0 mV.

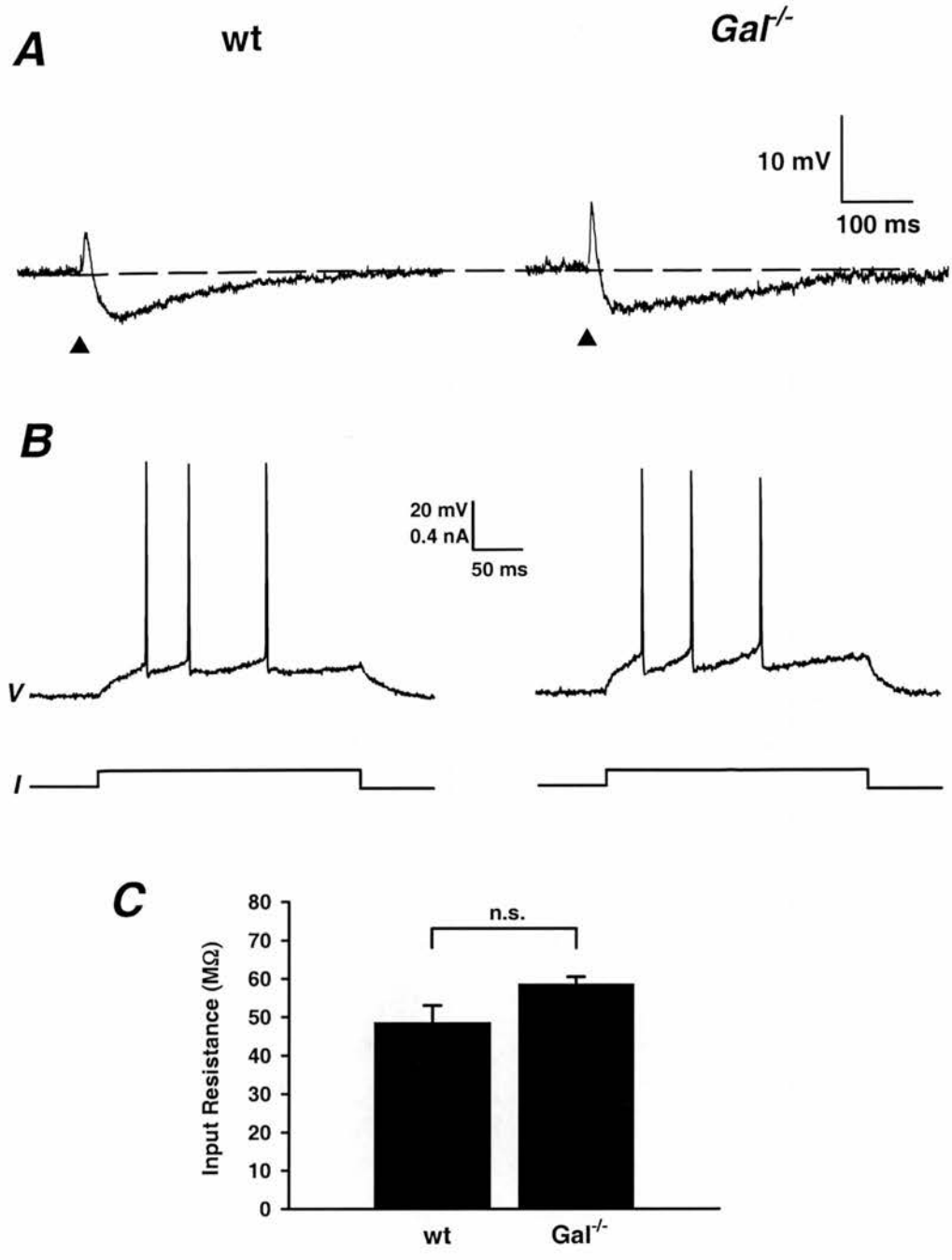


Figure 6.2 **Comparison of amino acid receptor-mediated neurotransmission and passive cell properties between wild type and galanin knockout mice**

A shows synaptic traces illustrating an EPSP/IPSP complex recorded from CA1 pyramidal cells from wild type (*left*) and Gal$^{-/-}$ mouse (*right*) slices. The filled triangles denote the position of the stimulus (single stimulus). *B* illustrates the voltage change of the cell in response to a depolarising current step in wild type (*left*) and Gal$^{-/-}$ mice (*right*). Note that spike frequency adaptation of action potential firing is unaltered by the galanin gene knockout. Apparent variations in spike height are due to the sampling rate (sampling interval was 488 μs). Bar graph (*C*) shows the mean input resistance of CA1 pyramidal cells from wild type and Gal$^{-/-}$ mouse slices in 5 and 7 experiments respectively. Values are mean ± S.E.M. represented by the error bars. The input resistance of the cell was 30 MΩ, the resting membrane potential was –64 mV and the spike height was greater than 0 mV.

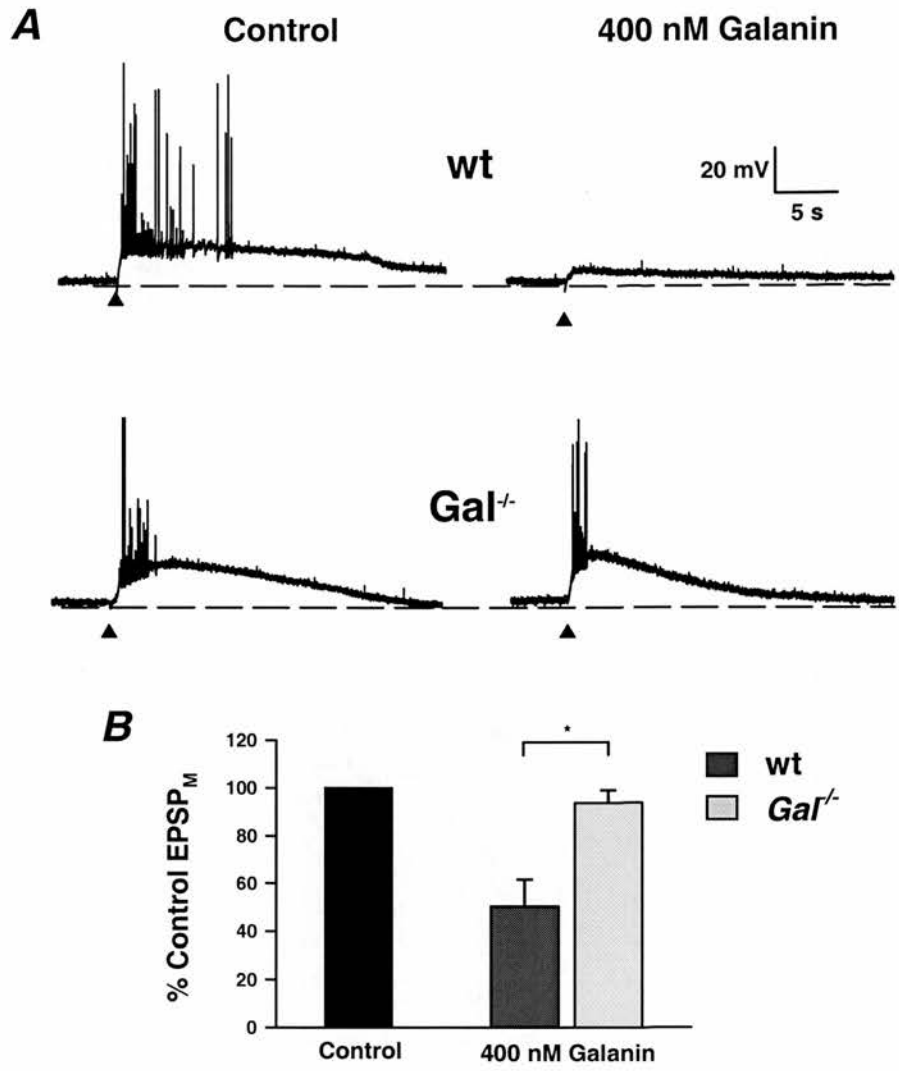


Figure 6.3 **Comparison of cholinergic responses in the CA1 pyramidal cells between wild type and galanin knockout mice.**

A shows synaptic traces of $EPSP_M$s recorded from the CA1 pyramidal cell held between –64 and –69 mV. The *top traces* illustrate the $EPSP_M$ from wild type slices before (*left*) and after (*right*) the application of 400 nM rat galanin. The *bottom traces* show the $EPSP_M$ from $Gal^{-/-}$ slices before (*left*) and after (*right*) the application of galanin. The filled triangles denote the position of the stimulus (single stimulation). Bar graph (*B*) illustrates the effect of galanin on the mean peak amplitude of the $EPSP_M$ in wild type and $Gal^{-/-}$ mice in 3 and 4 experiments (wt vs $Gal^{-/-}$, $p<0.05$) respectively. Values are mean ± S.E.M. represented by the error bars. The input resistance of the cells ranged between 30-33 MΩ, the resting membrane potential ranged between –62 to -66 mV and the spike height was greater than 0 mV.

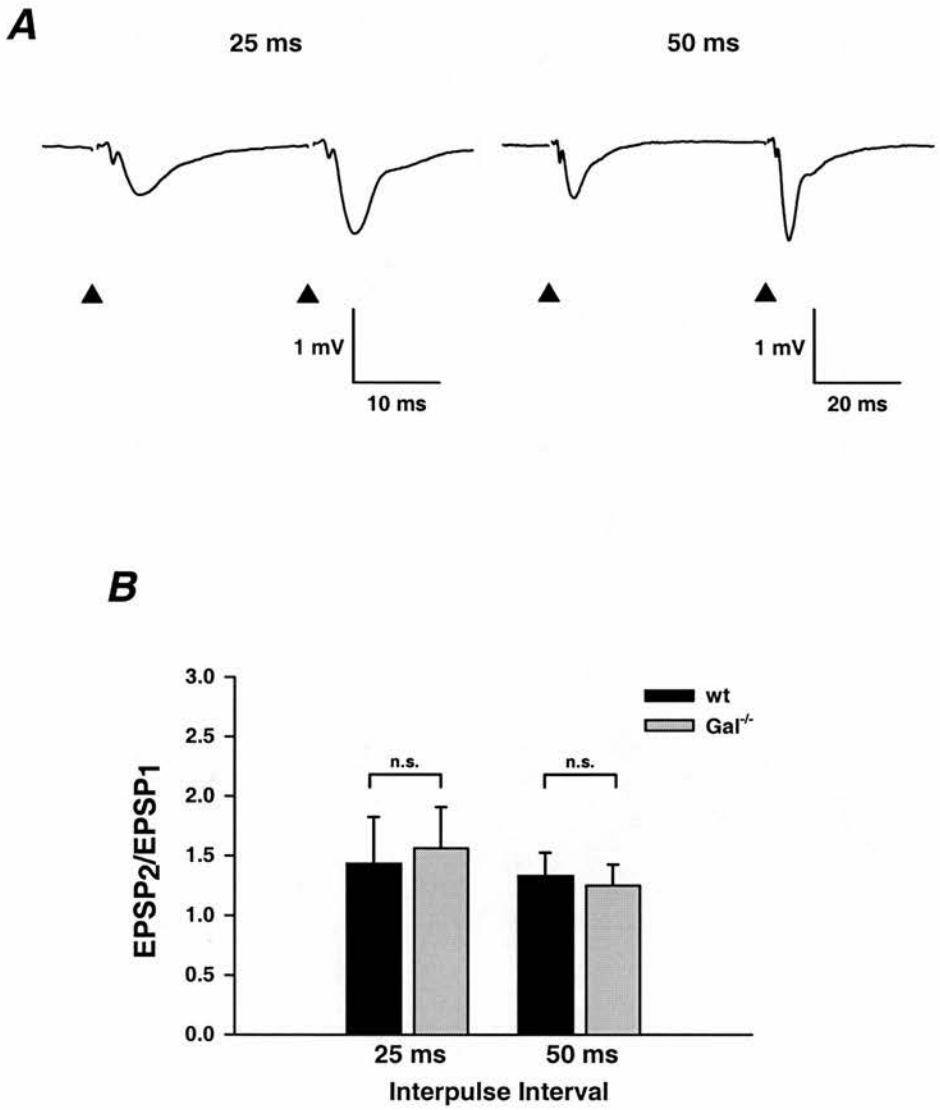


Figure 6.4 PPF are not significantly altered by the galanin gene deletion

The traces in *A* illustrate fEPSPs recorded from CA3-CA1 synapses of hippocampal slices taken from a transgenic mouse. The left hand trace demonstrates PPF with a 25 ms interpulse interval and the right hand trace demonstrates PPF with a 50 ms interpulse interval. Note the facilitation of the second response in each trace. Bar graph *B* shows a bar graph which plots the ratio of the second to the first fEPSP left hand slope for wt and Gal-/- mice at 25 and 50 ms interpulse intervals recorded from the *stratum oriens*. Note that PPF is not significantly altered in *Gal*$^{-/-}$ mice. Values are means ± S.E.M represented by the error bars.

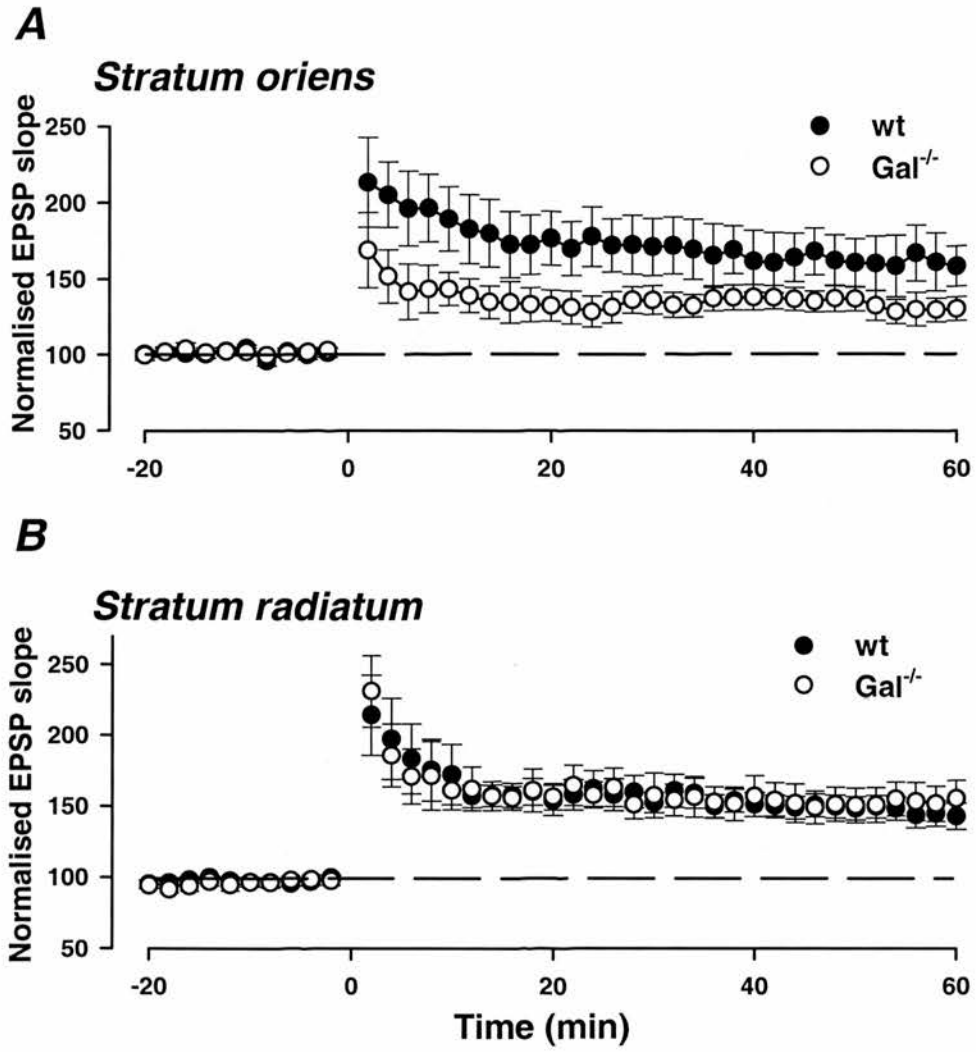


Figure 6.5 The effect of galanin gene knockout upon LTP.

A charts the potentiation of fEPSPs induced by high frequency stimulation recorded in the CA1 *stratum oriens* of slices from *Gal*$^{-/-}$ (open circles; n=8) and control littermate (wt) mice (closed circles; n=8; $p<0.05$). *B* charts the potentiation of fEPSPs induced by high frequency stimulation recorded in the CA1 *stratum radiatum* of slices from *Gal*$^{-/-}$ (open circles; n=12) and control littermate (wt) mice (closed circles; n=7; $p>0.05$). The baseline stimulation intensity was adjusted to evoke a fEPSP with an amplitude equal to 50% of its maximum amplitude (without superimposed population spike) and was not significantly different between *Gal*$^{-/-}$ and wt mice. LTP was induced by high frequency stimulation (100 shocks at 100 Hz) at t=0. Each data point represents the average rising slope of 4-8 responses. The magnitude of LTP was measured 60 min post-tetanus. Note the diminished LTP in the *Gal*$^{-/-}$ group. Values are means ± S.E.M represented by the error bars.

6.3. Discussion

Here, we have demonstrated that exogenously applied galanin inhibits synaptically evoked cholinergic responses in CA1 pyramidal cells. This result is consistent with several reports regarding the inhibition of ACh release by galanin (Dutar *et al.*, 1989; Fisone *et al.*, 1987; Palazzi *et al.*, 1988, 1991). Thus, it has been proposed that this effect is mediated by a presynaptic galanin receptor. Moreover, it is likely that this effect is mediated by the $G_{i/o}$ coupled GALR1 as mRNA for this receptor is apparent in the ventral hippocampus where the inhibition of ACh release is observed. GALR2 is mainly expressed in the dentate gyrus of the dorsal hippocampus and may be responsible for increased ACh release in the awake rat. GALR3 mRNA is not expressed in the hippocampus.

Further investigation showed that the galanin receptor-mediated inhibition of cholinergic responses was apparent in the mouse. In addition, mice lacking the gene for galanin showed a reduced magnitude of inhibition by exogenously applied galanin suggesting that cholinergic terminals are less responsive to galanin. It is unclear whether this lack of galanin-receptor mediated inhibition of cholinergic responses is due to decreased expression of galanin receptors as the GALR expression levels in the galanin knockout mice have not been investigated due to the unavailability of the immunohistochemical tools.

Furthermore, it appears that the galanin mutation does not affect glutamate or GABAergic synaptic transmission indicating that this knockout mouse exhibits selective modification of the cholinergic system. Therefore, it is unlikely that this mutation is causing any major reorganisation of neuronal circuits as tyrosine kinase knockouts do for example.

The proposed unresponsiveness of cholinergic terminals to galanin in the knockout mice could be explained by a downregulation or inactivation of presynaptic galanin receptors. This being the case, one might expect $Gal^{-/-}$ mice to exhibit cholinergic hyper-responsiveness but it is likely that any increase in ACh release, due to reduced galanin receptor mediated inhibition, is compensated for by other mechanisms that can inhibit ACh release or possible postsynaptic mAChR desensitisation. However,

if increased basal cholinergic excitation of hippocampal neurons was apparent then it may result in increased levels of learning and memory in $Gal^{-/-}$ mice.

To explore this hypothesis of increased memory, we examined LTP in brain slices from wild type and transgenic $Gal^{-/-}$ mice. Contrary to expectations, a reduction in tetanus-induced LTP was observed in the *stratum oriens*, but not *stratum radiatum*, of mice lacking the galanin gene. It is possible that this correlates with some kind of alteration to the septohippocampal terminals as the greatest densities of cholinergic terminals are observed in the *stratum oriens*. This result is difficult to interpret but one explanation could be that due to a possible enhancement of the cholinergic input or glutamatergic neurotransmission in the $Gal^{-/-}$ mice, LTP is much closer to saturation prior to brain removal. Consequently, LTP induced in brain slices from $Gal^{-/-}$ mice would appear less than LTP induced in brain slices from wild type mice. Furthermore, LTP may be closer to saturation in the *stratum oriens* than in the *stratum radiatum* due to the greater density of cholinergic terminals in the *stratum oriens*.

However, one study using atropine to block any muscarinic AChR-mediated activity, demonstrated no change in LTP recorded from the *stratum oriens* (Sokolov & Kleschevnikov, 1995). This does not entirely rule out a role for ACh in LTP in the *stratum oriens*. To mimic the putative effect of the galanin deletion in the mouse, wild-type mice could be injected with a cholinergic agonist prior to brain removal. If increased chronic mAChR activity decreased LTP observed in the slices, this would help to support the theory that the absence of functional galanin results in increased mAChR-mediated modulation of LTP.

The impaired ability of the mice to exhibit strong LTP may have behavioural consequences in learning and memory tasks. However, no such deficit was apparent in mutant mice compared with their wild type litter mates at this age. It is possible that the small reduction in LTP is not detrimental to the ability of the mouse to perform such tasks.

From this and previous studies, it is clear that galanin plays a role in memory and learning and facilitates LTP formation. However, in this mutant mouse, the effect of the galanin gene deletion appears ho have been compensated for by other systems,

i.e. a reduction in the number of cholinergic terminals is accompanied by an increase in ChAT activity, resulting in minor phenotypic manifestations (Dawson, Personal communication). One phenotype demonstrated by these mice is a decrease in ACh release in aged mice paralleled by significant deficits in behavioural tests. However, the reasons for this are unclear. It would be interesting to examine LTP in these mice.

CHAPTER 7

OTHER GENES EXPRESSED IN THE HIPPOCAMPUS THAT MAY BE IMPLICATED IN LTP

7.1. Introduction

The opportunity arose to further investigate transgenic mice that had a disrupted gene which was found to be expressed in the hippocampus. Therefore, this chapter explores the electrophysiology of four such transgenic mice, focussing on LTP formation in slices obtained from these mice.

7.1.1. Transgenic Mice by β-*geo* Gene-trap Insertion

The galanin knockout mouse is a transgenic line that has been constructed by identifying a gene product of interest and disrupting the function of the appropriate gene. Another approach to investigating the molecular machinery involved in the formation and/or maintenance of LTP is to employ a random gene-trap insertion method to disrupt potential proteins involved in this phenomenon. This is a process whereby a defective reporter gene, in this case lacZ β-galactosidase-neomycin resistance fusion cassette (β-*geo*) (Friedrich and Soriano, 1991; Skarnes *et al.*, 1995) is randomly inserted into mouse embryonic stem (ES) cells. Successful integration of the cassette into an endogenous gene not only disrupts the function of that gene but also supplies promoter and translation initiation signals lacking from the reporter (Skarnes *et al.*, 1992; Skarnes, 1993) resulting in expression of both neo (G418 resistance) and active β-galactosidase enzyme. These ES cells are subsequently used to generate transgenic mice by blastocyst injection.

A major advantage of this approach is that staining for β-galactosidase activity in transgenic mouse tissue reveals patterns of expression of the endogenous gene rendered dysfunctional by the reporter gene insertion. In this study it was hoped that by looking for β-galactosidase enzyme activity, it would be possible to screen for genes with expression restricted to the hippocampal formation. Three mutants were observed where reporter gene activity was detected within the hippocampus. These were named *obn*, *kin* and *hpk*.

7.1.2. Features of the *obn* Gene

X-Gal staining detected patterns of expression that coincided with the cell bodies of pyramidal neurons of the CA1/CA3 subfields of the hippocampus. However, other brain regions showing expression were layers II and IV of the entorhinal cortex, para-subiculum and subiculum. Expression was also apparent in several other brain regions, most notably in the cortex. Also, a number of organs outwith the central nervous system displayed levels of *obn* expression (V. Wilson *et al.*, 1999).

Obn was screened for homology against a human cDNA library. It was found that there was an 89% homology at the amino acid level between obn and an unknown human cDNA denoted B2-1 (Liu and Pohajdak, 1992). The function of this gene is unclear. However, it does possess 42% homology with a yeast protein, SEC7, required for intracellular protein trafficking (Julius *et al.*, 1984; Tschopp *et al.*, 1984). Further domains conserved between *obn*/B2-1/SEC7 are a guanine nucleotide releasing factor and a mouse *src*-homology domain (SH-3). It is argued that this sub-region is part of a Pleckstrin homology (PH) domain (Haslam *et al.*, 1993) which allows the protein to anchor itself to cellular membranes (Hyvonen *et al.*, 1995) by binding to phosphatidylinositol 4,5, bisphosphate (PIP_2) (Harlan *et al.*, 1994)

7.1.3. Features of the *kin* Gene

In contrast to *obn*, *kin* expression patterns appeared to be displaced within the cell body layer and substantially, but not exclusively, restricted to the CA1-CA3 subfields of the hippocampus. Reporter gene activity was weakly detected in the molecular layer of the dentate gyrus and the lateral septum as well as the amygdala (stronger staining was observed in the interstitial nucleus), striatum and olfactory bulb. Chromogenic staining revealed punctate expression patterns within the axo-dendritic fields of CA1-3. This pattern did not co-localise with glial fibrillary acidic protein (GFAP) antibody labeling for astrocytes, thus excluding astrocytic glia as an obvious origin of kin gene expression. *In situ* hybridisation confirmed the neuronal origin of *kin* expression by identifying CA1-3 pyramidal cell bodies as containing β-*geo* mRNA. However, β-*geo* mRNA was much more widely spread than enzyme activity suggesting that, in many brain regions, the fusion gene is transcribed but not translated.

Striking homology (85% at the nucleotide sequence level) was detected between *kin* cDNA and one form of the alternatively spliced gene encoding a non-receptor tyrosine kinase of the *abl* family, *arg* (Kruh *et al.*, 1986, 1990). This argues that *kin* is the mouse ortholog of *arg*, a kinase that has been previously described but not regionally localised (Perego *et al.*, 1991).

arg is a splice variant whereby an upstream exon is joined to the main body of the transcript coding the conserved kinase domains (Shtivelman *et al.*, 1986; Ben-Neriah *et al.*, 1986; Kruh *et al.*, 1990). The upstream part of the *kin* fusion transcript is homologous to the upstream *arg* exon coding region indicating that the *kin* insertion is located between exons, thus it is feasible that the endogenous gene function has not been disrupted in the *kin* mutant mice.

It has been confirmed (Wang and Kruh, 1996) that type B *abl* cDNA, with which *kin* bears extensive N-terminal homology, is post-translationally myristoylated for transport to the membrane. Thus, it is inferred that this modification directs transport of the *kin* hybrid protein from the cell body to the axo-dendritic region of CA1-3 pyramidal neurons.

7.1.4. Features of the *hpk* Gene

Hpk demonstrates a similar expression pattern to that of *obn*, although, restricted more to the pyramidal cell bodies of the CA1 subfield of the hippocampus and at lower levels of expression. *Hpk* is also expressed in the cortex.

There was a 98% homology (over a 40 nucleotide sequence) with the previously described mouse G-protein coupled receptor, Epstein-Barr Inducible-1 (EBI-1) (Schweickart *et al.*, 1994). It is possible that the fusion protein, in *hpk* mice, might lack the N-terminal signal sequence that allows insertion of the EBI-1-like protein into the cell membrane.

7.1.5. Features of the BSP1 Gene

The serine proteases comprise a large family of endopeptidases that function in a diverse range of physiological processes such as hemostasis, digestion, the immune response and tissue remodeling (Neurath, 1989; Barrett, 1990). Their name is derived from a conserved serine residue which, along with a histidine and aspartate residue, forms the 'catalytic triad' that mediates peptide bond cleavage.

These enzymes have been extensively studied in the periphery. However, their role in the CNS remains largely unexplored although there is evidence to suggest important involvement in brain development and function, specifically the outgrowth and migration of neurons. Cultured neurons overlaid with fibronectin demonstrated regions of proteolysis located around the growth cone (Krystosek & Seeds, 1981a; Pittman, 1985; McGuire & Seeds, 1990) suggesting a clearance of substrate is required for neurite extension.

In support of this involvement in neural growth, other serine proteases, such as thrombin and the plasminogen activators (t-PA and u-PA) have been held to play roles in the adult brain. High levels of PA activity are present in migrating neural crest cells (Valinsky & LeDourain, 1985) and migrating granule cells of the postnatal

cerebellum (Krystosek & Seeds, 1981b). t-PA is thought to convert inactive zymogen plasminogen to active plasmin which can degrade most of the extracellular matrix by either direct means or by the activation of other proteolytic enzymes such as the metalloproteases (Saksela & Rifkin, 1988)

Plasminogen-dependent proteolysis has been observed in the hippocampus and cerebral cortex of adult brains (Soreq & Miskin, 1981; Sappino *et al.*, 1993). In addition, t-PA expression is upregulated following seizure, kindling and LTP in the dentate gyrus (Qian *et al.*, 1993) suggesting an activity dependent increase in proteolysis. t-PA null mutant mice, however, showed no gross morphological abnormalities of the CNS (Carmeliet *et al.*, 1994) but showed an increased level of GABAergic inhibition and deficits in the maintenance of long lasting (Late) LTP in the hippocampus (Frey *et al.*, 1996; Huang *et al.*, 1996). These mice were also resistant to excitatoxic insult (Tsirka *et al.*, 1995, 1996).

Two novel serine proteases have been identified and cloned using degenerate oligonucleotide primers designed on the conserved residue motifs surrounding the catalytic triad of the major (chymotrypsin-type) clan of serine proteases. These gene products were named Brain Serine Protease (BSP) 1 and 2 (Davies *et al.*, 1998). BSP-2 will not be dealt with in this body of work.

The BSP-1 gene encodes for an enzyme that bares greatest similarity with the trypsin subfamily of proteases (40% sequence homology and contains the catalytic triad in the anticipated region). The predicted polypeptide sequence exhibited a trypsin-like cleavage site, suggesting the liberation of the active enzyme from the inactive precursor protein. Northern blot analysis identified BSP-1 in the hippocampus only. Furthermore, in situ hybridisation of BSP1 probes in brain sections localised the BSP1 transcripts to the CA fields, with significant expression in the CA1 and CA3 and low-level expression in the CA2, dentate gyrus and entorhinal cortex.

BSP1 null mutant mice were consequently engineered and, due to evidence supporting the role of serine proteases in synaptic plasticity and BSP1 expression being restricted to the hippocampus, were examined for differences in synaptic transmission and plasticity using *in vitro* extracellular recordings of fEPSPs from the CA1 region of the hippocampus.

7.2. Results

7.2.1. *Obn* Gene Knockout

Stimulation of the Schaffer/collateral commissural pathway of the hippocampus in slices obtained from *obn* mice produced a field EPSP preceded by a presynaptic fibre volley in the *stratum radiatum* dendritic field of the CA1 pyramidal cell. A half maximal response was attained by adjusting the stimulus intensity. Paired pulse stimulation with a 25 and 50 ms interpulse interval, produced facilitation of the second fEPSP. This phenomenon is termed paired pulse facilitation (PPF). The ratio of the rising slope of the second fEPSP to the rising slope of the first fEPSP was used as an index of this facilitation. No significant difference ($p<0.05$) was observed in PPF between wild type (1.77 0.±16 at 25 and 1.88 ±0.33 at 50 ms (n=6)) and *obn* knockout mice (1.72 ±0.1 at 25 ms and 1.71 ±0.13 at 50 ms (n=7)) (fig. 7.1*A*) at either a 25 ms or 50 ms interpulse interval.

Moving the recording electrode to the CA1 pyramidal cell body layer permitted the recording of population spikes. A paired pulse protocol applied at a 15 ms interpulse interval produced a marked depression of the second population spike such that its amplitude was reduced by more than 50%. A ratio of peak amplitude of the second population spike to the first population spike was used a measure of paired-pulse depression in different slices. No significant difference was noted between wt (0.48 ±0.05 (n=16)) and *obn* mutant mice (0.47 ±0.06 (n=6)) (fig. 7.1*Bb*).

To establish whether any of the genetic mutations affected LTP recordings from the *stratum radiatum* were made and a tetanus of 100 shocks at 100 Hz was delivered to the Schaffer/collateral commissural pathway. LTP was apparent in seven out of nine mice tested. The potentiation immediately following tetanisation, termed Post-Tetanic Potentiation (PTP), was unchanged between wt and *obn* mice (218 ±14% (n=11) and 232 ±41% of control in wt and *obn* mice respectively (n=7)) (fig. 7.1*C*). Short-Term Potentiation (STP) defined as the potentiation of the left hand slope of

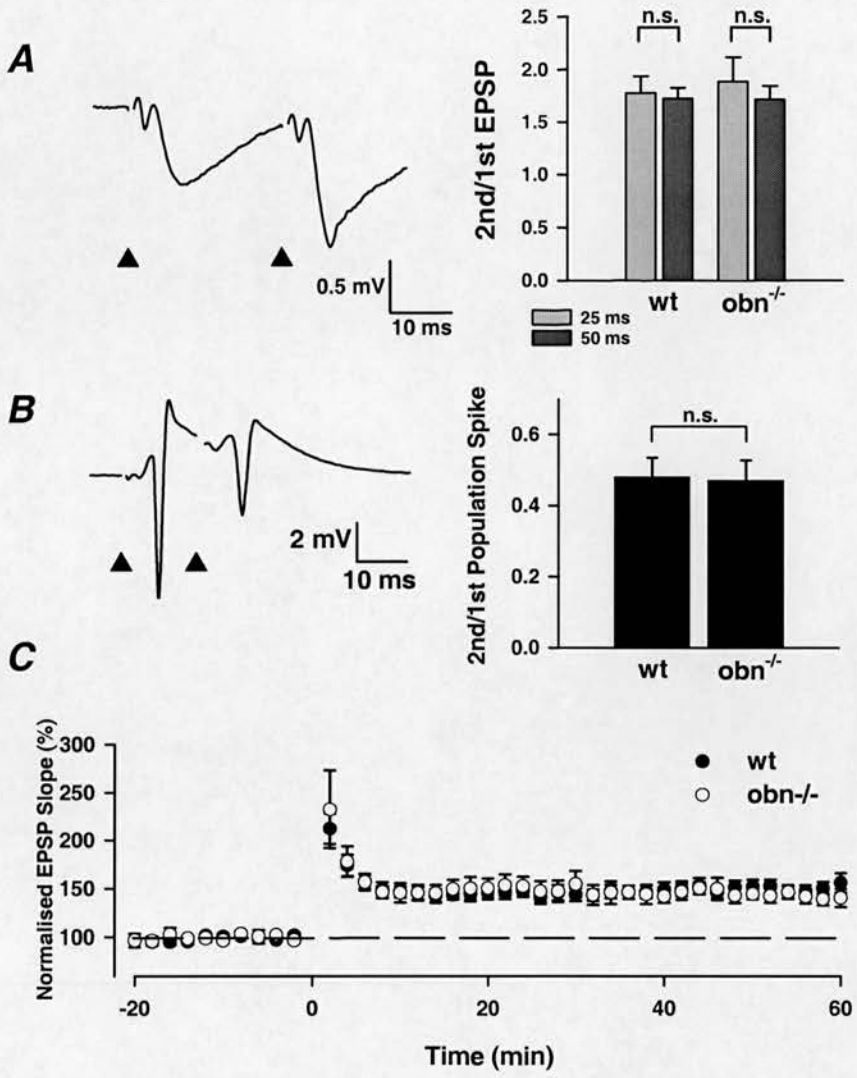


Figure 7.1 **The effect of *obn* gene disruption on basal neurotransmission and synaptic plasticity at glutamatergic synapses in the *stratum radiatum*.**

Aa shows extracellular recordings of field EPSPs from the apical dendritic region of CA1 pyramidal cells evoked by paired pulse stimulation of the Schaffer/collateral commissural pathway. Filled triangles denote the time at which the stimulus was delivered in all figures showing trace recordings. (*Ab*) The bar graph plots the ratio of the left hand slope of the second fEPSP to that of the first fEPSP comparing wt mice with *obn* null mutants at 25 (grey) and 50 ms (black) interpulse intervals. *Ba* shows extracellular recordings of population spikes from the CA1 pyramidal cell body layer evoked by paired pulse stimulation of the Schaffer/collateral commissural pathway. (*Bb*) This bar graph plots the ratio of the second to the first population spike peak amplitude for pooled wt and *obn* data at 15 ms interpulse interval. *C* charts the potentiation of fEPSPs induced by high frequency stimulation (100 shocks @ 100Hz) recorded in the CA1 *stratum radiatum* of slices from *obn*$^{-/-}$ (open circles; n=7) and control littermate (wt) mice (closed circles; n=11; $p>0.05$). The baseline stimulation intensity was adjusted to evoke a fEPSP with an amplitude equal to 50% of its maximum amplitude (without superimposed population spike) and was not significantly different between *obn*$^{-/-}$ and wt groups. LTP was induced by high frequency stimulation (100 shocks at 100 Hz) at t=0. Each data point represents the average rising slope of 4-8 responses. The magnitude of LTP was measured 60 min post-tetanus. Values are means ± S.E.M represented by the error bars.

the field EPSP at 30 min post-tetanus was also comparable in both wt and *obn* mice (148 ±4 % of control in wt (*n*=11), 143 ±10% in *obn* (*n=7)*). Long-Term Potentiation (LTP) as measured 60 min post-tetanus also showed no significant difference between wt (153 ±5% of control (*n*=11)) and *obn* mice (140 ±9% of control (*n*=7)).

7.2.2. *Kin* Gene Knockout

Initially, it was only possible to examine properties of synaptic transmission and LTP in *kin* heterozygous mutants due to the poor survival rate of the *kin* homozygous mutant mice. Paired pulse facilitation, at 25 and 50 ms interpulse intervals, was unaffected in $kin^{+/-}$ mice (1.69 ±0.08 at 25 ms, 1.66 ±0.06 at 50 ms (*n*=9)) compared with wt mice (1.77 ±0.16 at 25 ms, 1.72 ±0.1 at 50 ms (*n*=11)) (fig. 7.2*A*). Paired pulse depression proved not to be significantly affected across a range of interpulse intervals (25-200 ms) employed in $kin^{+/-}$ mice (fig. 7.2*B*). PTP, STP and LTP were comparable between $kin^{+/-}$ (230 ±21% of control, PTP; 152 ±9% of control, STP; 163 ±13% of control, LTP (*n*=7)) and wt mice (218 ±14% of control, PTP; 148 ±4% of control, STP; 153 ±5% of control, LTP (*n*=11)) (fig. 7.2*C*).

The breeding difficulties exhibited by this transgenic mouse were eventually overcome allowing the investigation of $kin^{-/-}$. As with heterozygous mice no significant difference in paired pulse facilitation in the *stratum radiatum* Schaffer/collateral commissural pathway at 25 and 50 ms interpulse intervals ($p>0.05$) was observed between wild-type (1.77 ±0.16 at 25 ms, 1.72 ±0.1 at 50 ms (*n*=11)) and homozygous *kin* mutants (1.87 ±0.08 at 25 ms, 1.7 ±0.02 at 50 ms (*n*=7)) (fig.7.3*A*).

A range of interpulse intervals, from 15 ms to 200 ms, was used to assess paired pulse depression in the CA1 pyramidal cell body layer of $kin^{-/-}$ mice (fig. 7.3*B*). Depression of the second population spike in both wild type and $kin^{-/-}$ mice was only apparent up to an interpulse interval of 25 ms. With longer duration interpulse intervals, facilitation of the second population spike was prevalent. There was a tendency of wt mice (*n*=16) to display larger facilitation at higher interpulse intervals

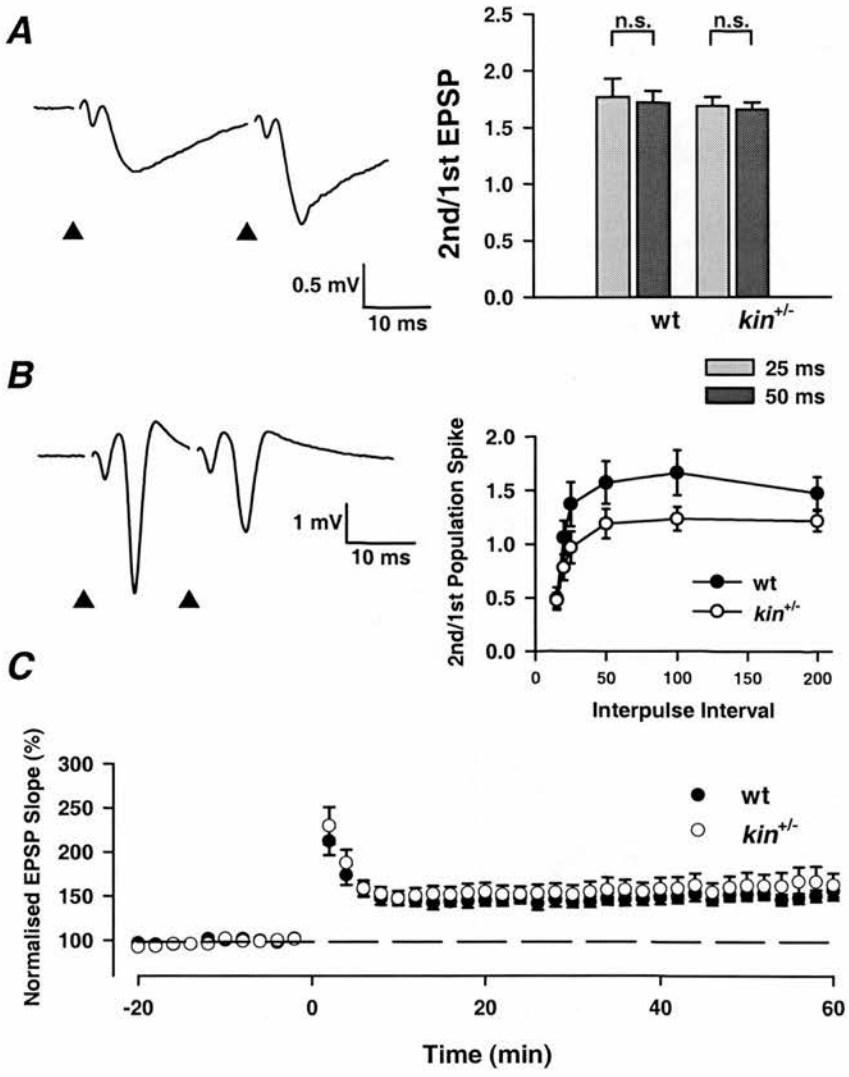


Figure 7.2 **The effect of *kin* heterozygous gene disruption on basal neurotransmission and synaptic plasticity at glutamatergic synapses in the *stratum radiatum***

Aa shows extracellular recordings of field EPSPs from the apical dendritic region of CA1 pyramidal cells evoked by paired pulse stimulation of the Schaffer/collateral commissural pathway. Filled triangles denote the time at which the stimulus was delivered in all figures showing trace recordings. (*Ab*) The bar graph plots the ratio of the left hand slope of the second fEPSP to that of the first fEPSP comparing wt mice with *kin* heterozygous mutants at 25 (grey) and 50 ms (black) interpulse intervals. *Ba* shows extracellular recordings of population spikes from the CA1 pyramidal cell body layer evoked by paired pulse stimulation of the Schaffer/collateral commissural pathway. (*Bb*) The line graph plots the ratio of the peak amplitude of the second to that of the first population spike for pooled wt (*closed circles*) and $kin^{+/-}$ (*open circles*) data against the interpulse interval. *C* charts the potentiation of fEPSPs induced by high frequency stimulation (100 shocks @ 100Hz) recorded in the CA1 *stratum radiatum* of slices from $kin^{+/-}$ (open circles; n=7) and control littermate (wt) mice (closed circles; n=11; $p>0.05$). The baseline stimulation intensity was adjusted to evoke a fEPSP with an amplitude equal to 50% of its maximum amplitude (without superimposed population spike) and was not significantly different between $kin^{-/-}$ and wt mice. LTP was induced by high frequency stimulation (100 shocks at 100 Hz) at t=0. Each data point represents the average rising slope of 4-8 responses. The magnitude of LTP was measured 60 min post-tetanus. Values are means ± S.E.M represented by the error bars.

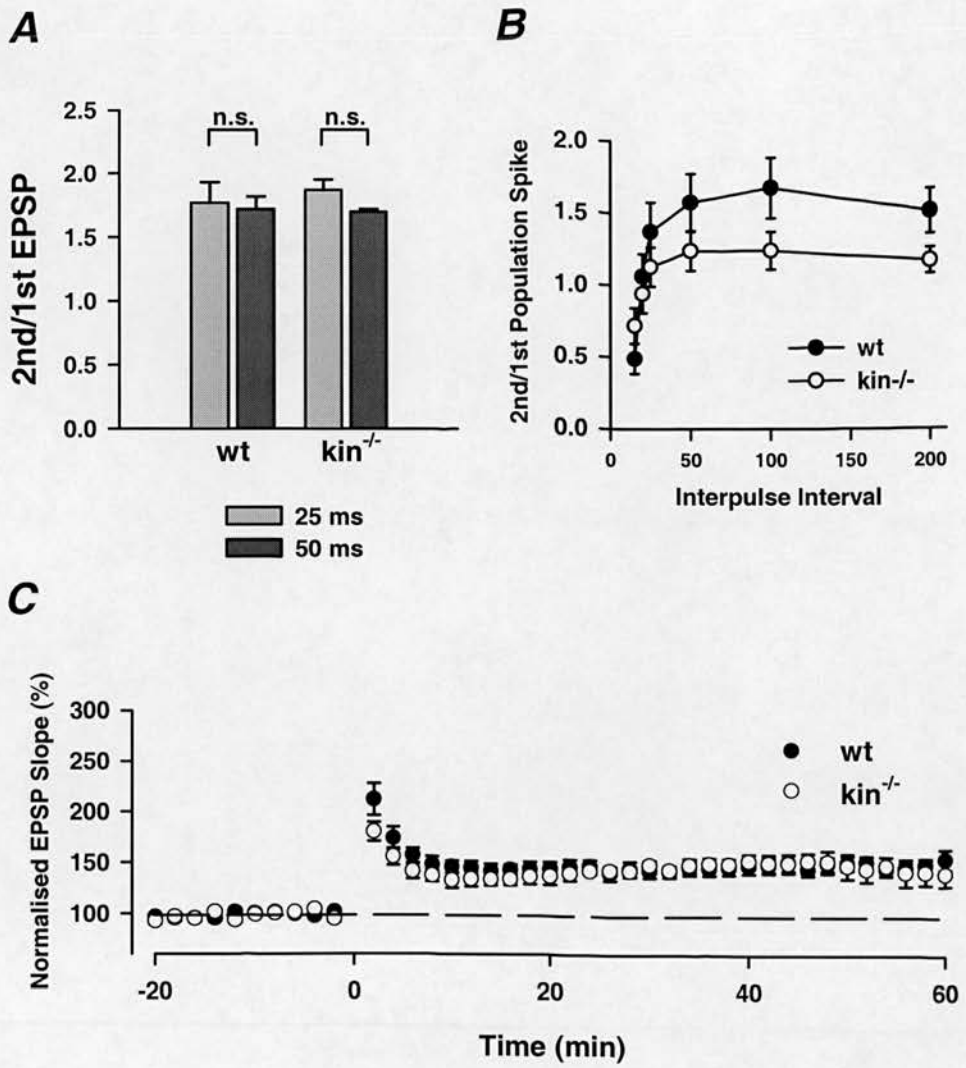


Figure 7.3 **The effect of *kin* homozygous gene disruption on basal neurotransmission and synaptic plasticity at glutamatergic synapses in the *stratum radiatum*.**

Aa shows extracellular recordings of field EPSPs from the apical dendritic region of CA1 pyramidal cells evoked by paired pulse stimulation of the Schaffer/collateral commissural pathway. Filled triangles denote the time at which the stimulus was delivered in all figures showing trace recordings. (*Ab*) The bar graph plots the ratio of the left hand slope of the second fEPSP to that of the first fEPSP comparing wt mice with *kin* null mutants at 25 (grey) and 50 ms (black) interpulse intervals. *Ba* shows extracellular recordings of population spikes from the CA1 pyramidal cell body layer evoked by paired pulse stimulation of the Schaffer/collateral commissural pathway. (*Bb*) The line graph plots the ratio of the peak amplitude of the second to that of the first population spike for pooled wt (closed circles) and *kin* (open circles) data against the interpulse interval. *C* charts the potentiation of fEPSPs induced by high frequency stimulation (100 shocks @ 100Hz) recorded in the CA1 *stratum radiatum* of slices from *kin*$^{-/-}$ (open circles; *n*=7) and control littermate (wt) mice (closed circles; n=11; p>0.05). The baseline stimulation intensity was adjusted to evoke a fEPSP with an amplitude equal to 50% of its maximum amplitude (without superimposed population spike) and was not significantly different between *kin*$^{-/-}$ and wt mice. LTP was induced by high frequency stimulation (100 shocks at 100 Hz) at t=0. Each data point represents the average rising slope of 4-8 responses. The magnitude of LTP was measured 60 min post-tetanus. Note the enhanced PTP in the *kin*$^{-/-}$ group. Values are means ± S.E.M represented by the error bars.

compared with *kin*$^{-/-}$ mice (n=3). However, this proved statistically insignificant ($p>0.05$ for all interpulse intervals). All mice tested demonstrated robust LTP. Short- and Long- Term Potentiation were comparable between wt (148 ±4% of control, STP; 153 ±5% of control, LTP (n=11)) and *kin*$^{-/-}$ mice (144 ±5% of control, STP; 142 ±12% of control, LTP (n=4)). However, PTP in *kin*$^{-/-}$ mice was significantly decreased ($p<0.05$) (wt, 218 ±14% of control (n=11); *kin*$^{-/-}$, 142 ±12% of control (n=4)) (fig. 7.3*C*).

7.2.3. *Hpk* Gene Knockout

Similar to *obn*$^{-/-}$ and *kin*$^{-/-}$, *hpk*$^{-/-}$ mice displayed no significant changes in paired pulse facilitation of fEPSPs recorded from the apical dendritic field of CA1 pyramidal cells (fig. 7.4*A*). At a 25 ms interpulse interval, comparable levels of facilitation were observed in wt (1.77 ±0.16 (n=5)) and *hpk* mutants (1.64 ±0.09 (n=9)). The same was true for an interpulse interval of 50 ms (wt, 1.72 ±0.1 (n=5); *hpk*, 1.73 ±0.08 (n=9)).

Paired pulse depression of population spikes at 15 ms intervals was reduced in *hpk* mice (fig. 7.4*B*) but not significantly (wt, 0.53 ±0.06 (n=16); *hpk*, 0.81 ±0.11 (n=9) ($p>0.05$)). At the 20-25 ms interpulse interval, the depression of the second population spike was no longer apparent and, at longer intervals, small levels of facilitation were evident in both wt and *hpk* mutants.

Out of 12 slices that were tetanised, 10 exhibited LTP. LTP in *hpk* mice was larger than in wt (fig. 7.4*C*). Thus the magnitudes of potentiation observed in wt and *hpk* mice were: during PTP (wt, 218 ±14% of control(n=11); *hpk*, 319 ±15% of control (n=10) ($p<0.05$)), STP (wt, 148 ±4.3% of control(n=11); *hpk*, 185 ±15% of control (n=10)($p<0.05$)) and LTP (wt, 153 ±4.5% of control (n=11); *hpk*, 196 ±17% of control (n=10) ($p<0.05$)).

To further investigate this apparent increase in LTP magnitude observed in *hpk* null mutants, a second group of *hpk* mice were studied to explore whether they exhibited larger NMDA receptor-mediated fEPSPs during low and high frequency stimulation.

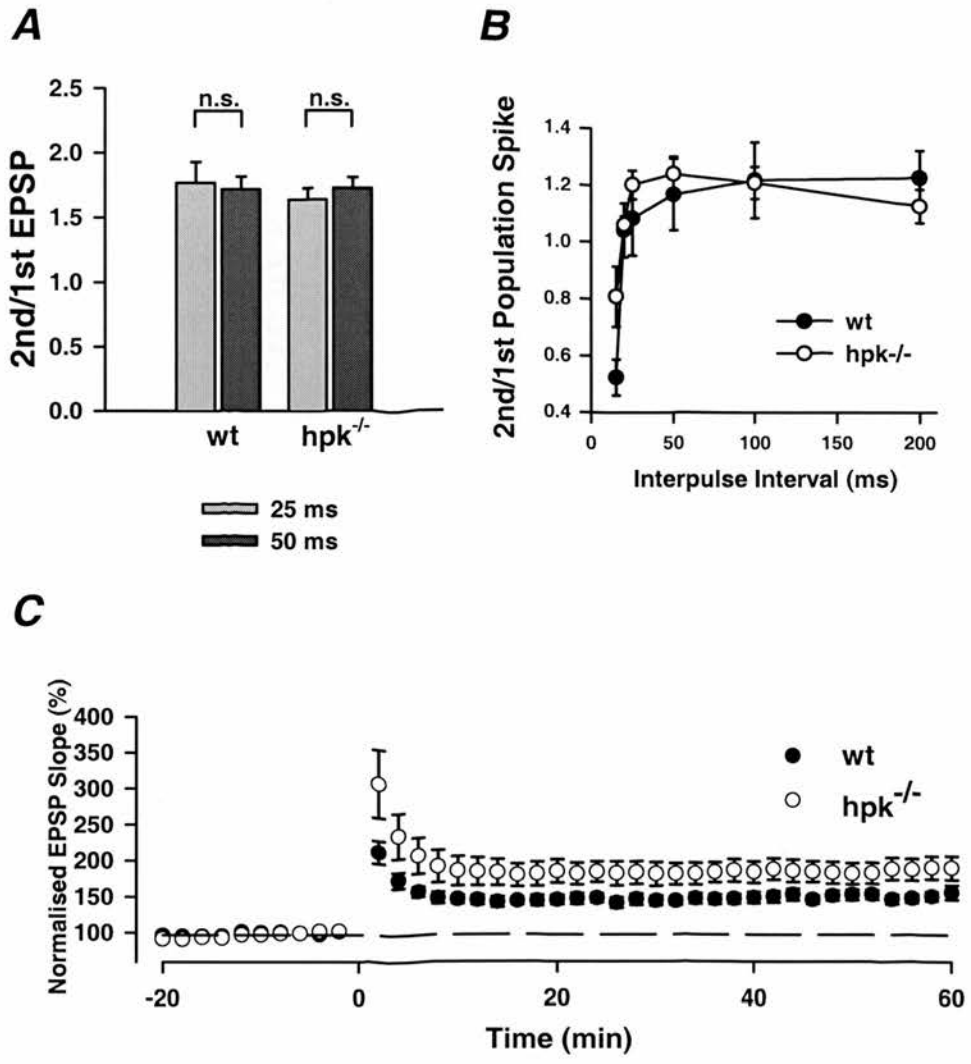


Figure 7.4 **The effect of *hpk* gene disruption on basal neurotransmission and synaptic plasticity at glutamatergic synapses in the *stratum radiatum*.**

Aa shows extracellular recordings of field EPSPs from the apical dendritic region of CA1 pyramidal cells evoked by paired pulse stimulation of the Schaffer/collateral commissural pathway. Filled triangles denote the time at which the stimulus was delivered in all figures showing trace recordings. (*Ab*) The bar graph plots the ratio of the second fEPSP to the first fEPSP left hand slope comparing wt mice with *hpk* null mutants at 25 (grey) and 50 ms (black) interpulse intervals. *Ba* shows extracellular recordings of population spikes from the CA1 pyramidal cell body layer evoked by paired pulse stimulation of the Schaffer/collateral commissural pathway. (*Bb*) This line graph plots the ratio of the second to the first population spike peak amplitude for pooled wt (closed circles) and *hpk* (open circles) data against the interpulse interval. *C* charts the potentiation of fEPSPs induced by high frequency stimulation (100 shocks @ 100Hz) recorded in the CA1 *stratum radiatum* of slices from *hpk*$^{-/-}$ (open circles; n=7) and control littermate (wt) mice (closed circles; n=11; p>0.05). The baseline stimulation intensity was adjusted to evoke a fEPSP with an amplitude equal to 50% of its maximum amplitude (without superimposed population spike) and was not significantly different between *hpk*$^{-/-}$ and wt mice. LTP was induced by high frequency stimulation (100 shocks at 100 Hz) at t=0. Each data point represents the average rising slope of 4-8 responses. The magnitude of LTP was measured 60 min post-tetanus. Note the enhanced LTP in the *hpk*$^{-/-}$ group. Values are means ± S.E.M represented by the error bars.

Fig. 7.5*A* provides an example of an NMDA receptor-mediated fEPSP evoked in the presence of 50 μM picrotoxin, 1 μM CGP-55845A and in the absence of Mg^{2+}. Characteristically, this response was longer in duration (approx. 100 ms) compared to the AMPA receptor-mediated fEPSP (approx. 25 ms). An input/output curve was generated for this response by stepping up the stimulus intensity and measuring the peak amplitude of the resultant evoked presynaptic fibre volley and fEPSP (fig. 7.5*B*). No differences in input/output curves for this component of glutamatergic synaptic transmission for wt and *hpk* mice were observed.

7.2.4. Brain Serine Protease 1 (*BSP1*) Gene Knockout

To investigate the effect of disrupting the function of *BSP1* on basal synaptic transmission in the hippocampus, three parameters were examined: input/output of fEPSPs, paired pulse facilitation of fEPSPs and paired pulse depression of population spikes.

By increasing the amplitude of the stimulus intensity and measuring the evoked presynaptic fibre volley (PSFV) (a measure of the number of axons and synapses recruited by the stimulus) and field EPSP peak amplitude, an input/output curve was constructed (fig. 7.6). At low stimulus strengths, the fEPSP amplitude increased with increased PSFV amplitude. At higher strengths, there was little or no increase in the fEPSP as the maximum peak amplitude of the fEPSP was reached. *BSP1* mutants (n=9) showed no difference in their input/output characteristics compared with wild-type litter mates (n=6).

Paired pulse facilitation in the *stratum radiatum* was examined over a range of interpulse intervals, from 15 to 300 ms for both wt and *BSP1* null mutants. Facilitation was observed over the entire interval range in both groups of mice. Generally, *BSP1* mice exhibited larger facilitation, however, this only proved significant at an interpulse interval of 200 ms (wt, 1.22 ±0.06 (n=6); BSP1, 1.42 42 ±0.09 (n=10) ($p<0.05$)) (fig. 7.7*B*). As this coincided with the time interval when $GABA_B$ receptor-mediated autoinhibition is at a maximum, the decay half times of

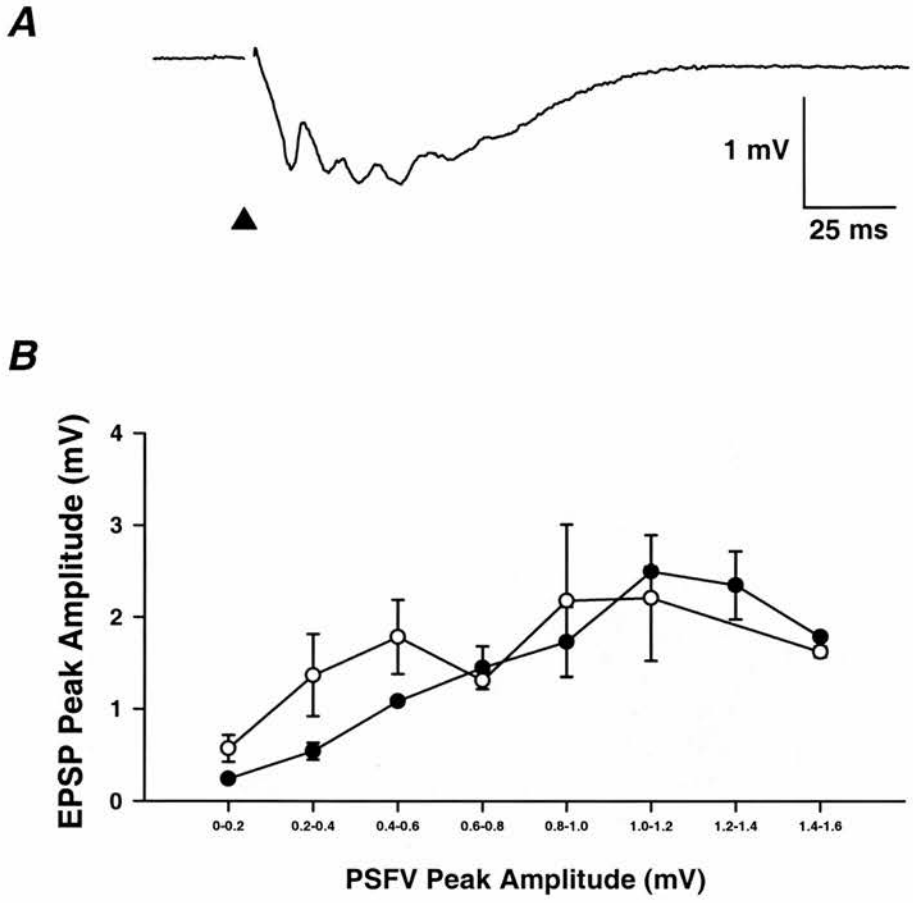


Figure 7.5 NMDA glutamate receptor (NMDAR) mediated field EPSPs in *hpk* and wt mice.

A illustrates a NMDAR-mediated field EPSP recorded from the stratum radiatum of *hpk* mutant mice evoked by stimulation of the Schaffer/collateral commissural pathway in the presence of 2 μM NBQX, 50 μM picrotoxin, 1 μM CGP 55845A and the absence of Mg^{2+}. The filled triangle denotes the time at which the stimulus was delivered. *B* shows an input/output graph plotting NMDAR-mediated fEPSP peak amplitude against presynaptic fibre volley peak amplitude for pooled wt (closed circles) versus *hpk* (open circles) data. Values are means ± S.E.M represented by the error bars.

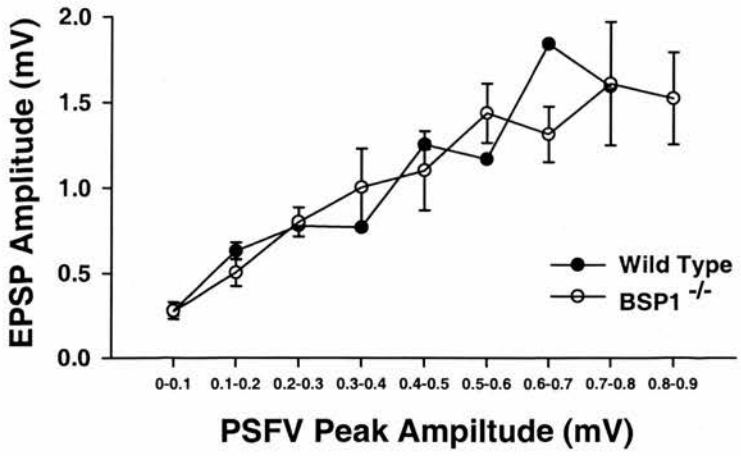


Figure 7.6 **The disruption of the *BSP1* gene has no effect on low frequency basal synaptic transmission in the *stratum radiatum*.**

The line graph plots the field EPSP peak amplitude against the presynaptic fibre volley peak amplitude over a range of stimulus strengths. Closed circles denote wt and open circles denote *BSP1* mutant group data. Values are means ± S.E.M represented by the error bars.

the second fEPSP of the pair were measured and expressed as a ratio of the decay half time of the first fEPSP (fig. 7.7*C*). However, upon examination, no significant difference was detected between the half times for wt (1.01 ±0.1(n=6)) and BSP1 (0.95 ±0.08 (n=10) (p>0.05)) knockout mice. Population spikes were evoked by stimulation of the Schaffer/collateral commissural pathway and recorded from the CA1 pyramidal cell body layer. Two interpulse intervals for PPD were employed, 15 and 200 ms, to allow the investigation of both $GABA_A$ and $GABA_B$ (Nathan *et al.*, 1990; Olpe *et al.*, 1993) neurotransmission during low frequency stimulation in *BSP1* mice. Initially, the most striking observation was that slices from both wt and *BSP1* mutants displayed little or no PPD at either interpulse interval (fig. 7.8*B*). At a 15 ms interval, PPD was unchanged between the two groups of mice (wt, 0.97 ±0.03 (n=6); *BSP1*, 1.05±0.05 (n=9) (p>0.05)). Similarly, at 200 ms interval, PPD was no different in *BSP1* (1.07 ±0.02 (n=9)) compared to wt (1.03 ±0.05 (n=6) (p>0.05)).

Since other protease knockouts have caused changes in inhibitory tone we further explored the possibility that the balance between synaptic excitation and inhibition

might be altered in BSP1 mutant mice by examining the effects of repeated stimulation (30 shocks at 1 Hz) on the population spike. Multiple population spikes, or polyspiking, were observed during the train of stimuli. The amplitudes of the second, third and sometimes fourth spikes were measured, summated and expressed as a percentage of the first population spike and plotted against its position in the train of stimuli (fig. 7.9). Throughout the entire train it was apparent that the *BSP1* mutants exhibited greater amounts of polyspiking than wt mice.

I also examined whether LTP was affected by the BSP knockout. Late-LTP was induced by using four trains of stimuli (100 shocks for 1 s) with an intertrain interval of 5 minutes (fig. 7.10). This protocol successfully produced potentiation lasting for 4 hours at which point there was no significant difference ($p<0.05$) in L-LTP between wt (136 ±15% of control, $n=5$) and *BSP1*$^{-/-}$ (167 ±15% of control, $n=7$) mice.

7.2.5. Examination of Activity-Dependent Gene Expression

The mutant mice used in this body of work all possess the β-galactosidase gene which was inserted into the native gene whose function we were studying. As both the native gene and the β-galactosidase gene are under the control of the same promoter region, increased β-galactosidase expression can be equated with increased native gene expression. Thus we used X-gal staining to visualise expression in hippocampal slices. Using the K^+ channel blocker, 4-AP (100 μM), to induce spontaneous neuronal synaptic activity, we investigated the expression of *obn*, *hpk*, *kin* genes. For all genes, no change in the level or pattern of expression was observed in the hippocampal slice (fig. 7.11).

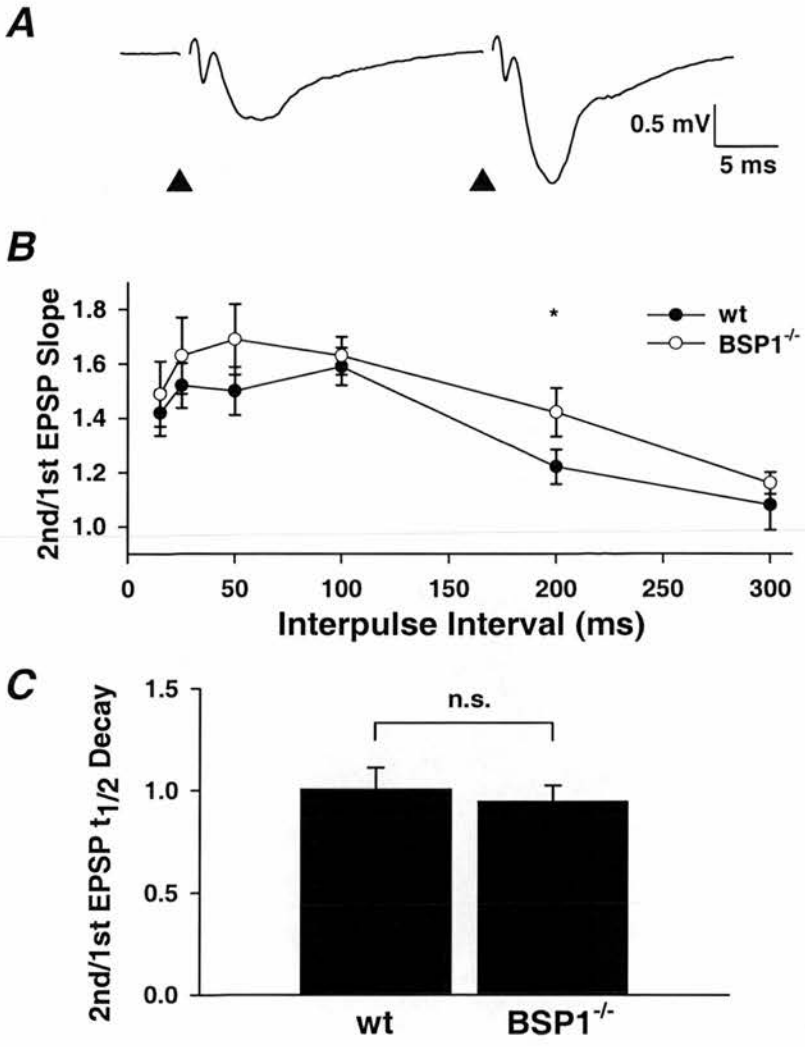


Figure 7.7 The effect of *BSP1* gene disruption on paired pulse facilitation.

A illustrates a recording of fEPSPs from the stratum radiatum of *BSP1* mutant mice, evoked by a paired pulse stimulation of the Schaffer/collateral commissural pathway. (B) A line graph, which plots the ratio of the second to the first fEPSP left hand slope against the interpulse interval. Note the significant difference ($p<0.05$) between wt (closed circles) and *BSP1* (open circles) mice at an interpulse interval of 200 ms. Values are means ± S.E.M represented by the error bars. C shows a bar chart measuring ratio of the second to the first fEPSP half time to decay at an interpulse interval of 200 ms in wt and *BSP1* mice.

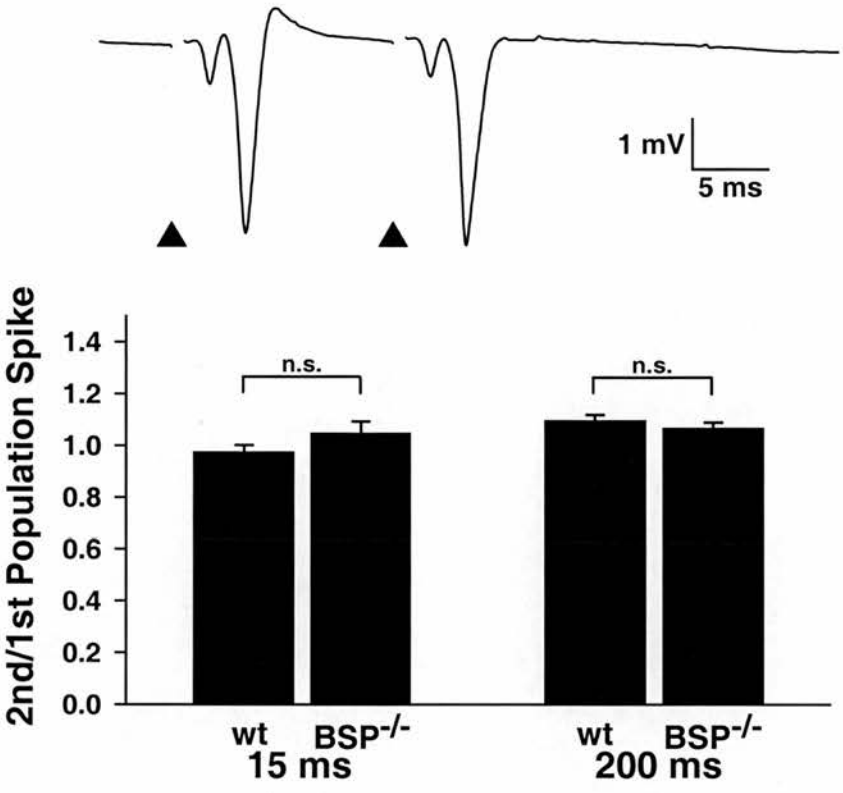


Figure 7.8 **The effect of *BSP1* gene disruption on paired pulse responses from the CA1 pyramidal cell body layer.**

A shows a recording of population spikes from the CA1 pyramidal cell body layer of BSP1 mutant mice, evoked by paired pulse stimulation of the Schaffer/collateral commissural pathway. (B) A bar graph that shows the ratio of the second to the first population spike peak amplitude for wt versus BSP1 groups at 15 ms and 200 ms interpulse intervals. Values are means ± S.E.M represented by the error bars.

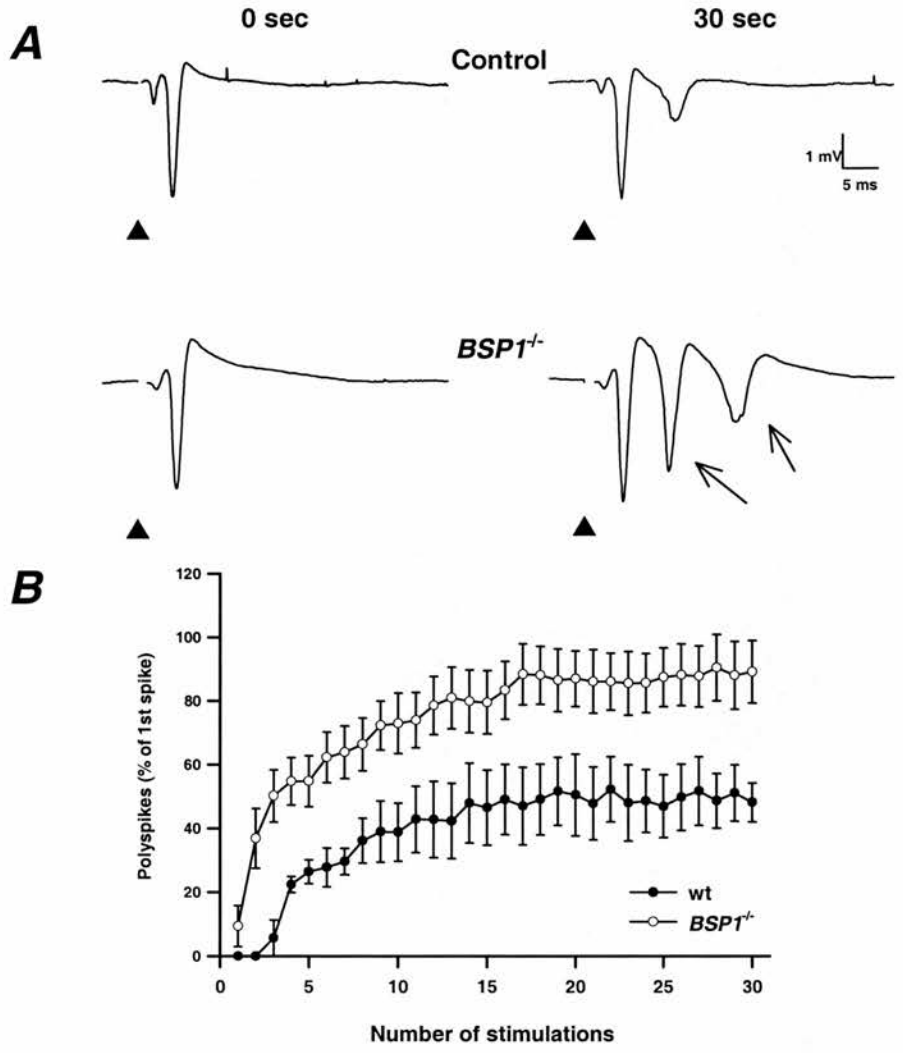


Figure 7.9 BSP1-deficient mice exhibit *in vitro* epileptiform activity.

A Example of field potentials evoked by stimulation of the Schaffer collaterals and recorded in the *stratum pyramidale* of the CA1 area. After repetitive stimulation (1 Hz for 30 s), slices from *BSP1*$^{-/-}$ mice exhibit an increase in polyspiking (i.e. multiple population spikes, *arrow*) in contrast to littermate control mice. *B* shows a time course of the appearance of polyspikes during repetitive stimulation (amplitudes of the secondary spikes expressed as a percentage of the first population spike) in slices from *BSP1*$^{-/-}$ (*open circles*; n=4 animals) and from littermate control mice (*closed symbols*; n=9 animals; $p<0.05$). Values are means ± S.E.M represented by the error bars.

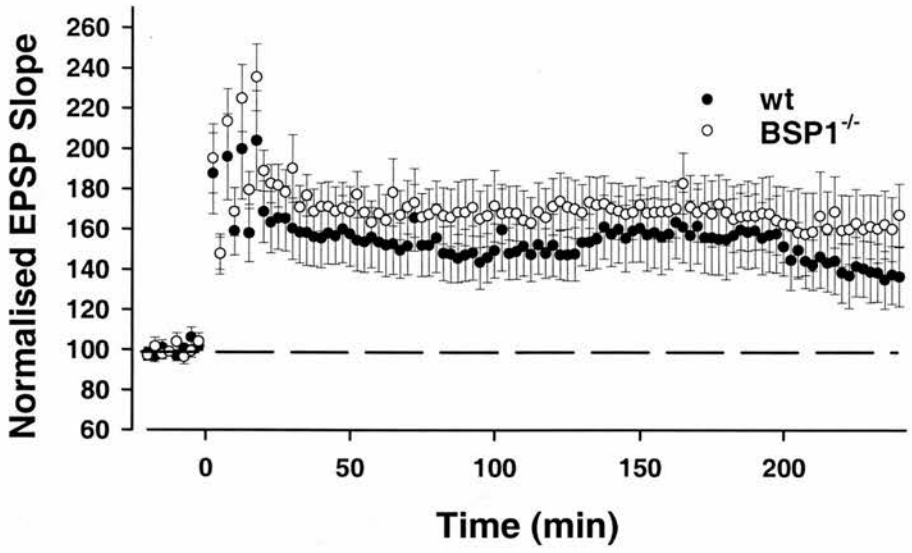


Figure 7.10 The effect of BSP1 gene disruption on Late-LTP

The graph shows the fEPSP slope expressed as a percentage of control responses over time. All data points represent 5-10 averaged fEPSPs. At time 0, 4 trains of stimulation, consisting of 100 shocks at 100 Hz separated by five minutes in between each tetanus, were delivered to the Schaffer/collateral commissural pathway. Values are means ± S.E.M represented by the error bars. Open circles represent wt pooled data and closed circles represent BSP1 pooled data.

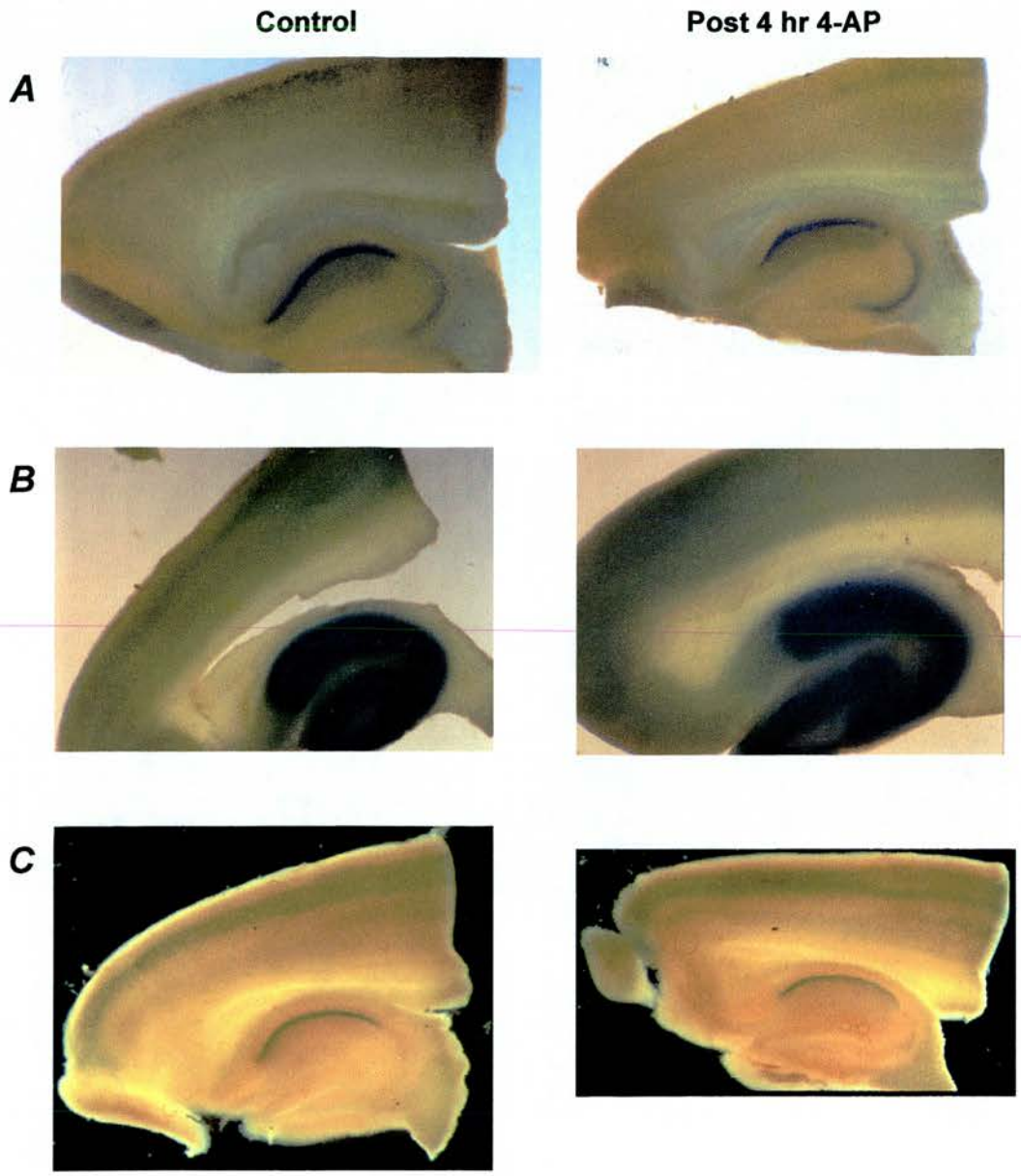


Figure 7.11 ***Obn*$^{-/-}$, *kin*$^{-/-}$, and *hpk*$^{-/-}$ mice demonstrate no change in gene expression following 4-AP exposure.**

This figure shows photographs of transgenic mouse hippocampal slices that have been stained for β-galactosidase activity using X-gal. X-gal staining shows up as dark areas. Note that expression of the β-galactosidase, is largely restricted to the cell body region of the CA1 subfield in *hpk*$^{-/-}$ mice (*C*) and to the CA1 and CA3 cell body regions in *obn*$^{-/-}$ mice (*A*). *Kin*$^{-/-}$ mice (*B*) show an X-gal staining pattern that encompasses the dendritic regions and the cell body regions of the CA fields and the dentate gyrus. The *left hand* photographs demonstrate expression of the transgene in slices perfused with control media whilst the right hand photographs demonstrate expression of the transgene following a 4 hr perfusion of 100 μM 4-AP. Note there is no difference in the levels or patterns of β-galactosidase expression in these three transgenic lines.

7.3. Discussion

The transgenes under investigation in this study were found to have homologues that, in some cases, are characterised to a certain extent.

7.3.1. *Obn*

The *Obn* gene was found to correspond to uncharacterised cDNA denoted B2-1 (Lui & Pohajdak, 1992). This cDNA encodes a protein that bears a strong resemblance to the yeast protein, SEC7. SEC7 appears to be involved in transport between functional compartments of golgi (Achstetter *et al.*, 1988; Franzusoff & Schekman, 1989) and may be a component of non-clathrin coated vesicles that cycle to and from the cell surface (Franzusoff *et al.*, 1992; Kean *et al.*, 1993). *Obn* also shows a pleckstrin homology (PH) domain which is conserved between the homologs identified. PH domains possess the ability to bind βγ subunits of G-proteins (Touhara *et al.*, 1994), phosphatidylinositol 4,5 bisphosphate (Harlan *et al.*, 1994) as well as tether the protein to cellular membranes. This argues that the *obn* gene encodes a membrane associated protein with the capacity to interact with other intracellular second messenger systems whilst such a protein might be expected to affect synaptic plasticity because of the strong involvement of biochemical cascades in this phenomenon. However, this gene appears to play no part in tetanus-induced LTP or basal excitatory amino acid-mediated synaptic transmission in the hippocampus.

7.3.2. *Kin*

The *kin* gene was found to be homologous to the Abelson (*abl*)-related tyrosine kinase, *arg*, a non-receptor tyrosine kinase. *abl*-deficient mice show major deficits in their immune system and die shortly after birth (Schwartzberg *et al.*, 1991; Tybulewicz *et al.*, 1991) whilst no phenotype of *arg*-deficient mice has been reported.

Initially, *kin* appeared to be an ideal candidate to be involved in LTP formation in the hippocampus as other non-receptor tyrosine kinases, such as *fyn*, have been implicated in LTP (Grant *et al.*, 1992; Moon *et al.*, 1994; Rosenblum *et al.*, 1996) and *src* in regulating NMDA receptor function (Lancaster & Rogers, 1998) and $GABA_A$ receptor function (Moss *et al.*, 1995). In addition, the *arg* binding protein, ArgBP1, is highly expressed in the brain.

Because of the selective transmission deficit of the heterozygous transgene, homozygous kin mutant mice were difficult to breed. As such, we examined LTP in the heterozygous *kin* mutants. All parameters tested displayed no phenotypic differences which suggests that the functional disruption of one *kin* gene has no effect upon LTP formation.

Sufficient homozygous *kin* mutant mice were eventually bred. These mice demonstrated little differences in synaptic transmission and LTP formation although there was a small decrease in post-tetanic potentiation. This was not accompanied by a decrease in paired pulse facilitation. The implication of this finding is that the non-receptor tyrosine kinase, *arg*, does not have a direct role in tetanus-induced LTP formation in the hippocampus.

7.3.3. *Hpk*

The hpk gene encodes a protein which is likely to be the mouse homolog of the Epstein-Barr virus (EBV)-inducible G protein-coupled receptor, EBI-1. EBI-1 appears to belong to a subfamily within the G protein-coupled receptor superfamily which also includes C-C chemokine, somatostatin, interleukin-8 (IL8) and neuropeptide Y receptors (Birkenbach *et al.*, 1993). Little is known about this receptor except that its expression is upregulated in cells of the immune system by Epstein-Barr virus (Birkenbach *et al.*, 1993) and Herpes virus infection (Hasegawa *et al.*, 1994) and this is the first time it has been reported that the EBI-1 receptor is expressed in neural tissue (Steel *et al.*, 1998).

Whilst low frequency amino acid-mediated synaptic transmission remains intact in the *hpk* mutant, high frequency stimulation produced significantly larger LTP. There

appeared to be no change in GABAergic neurotransmission as paired pulse depression of population spikes was unchanged and similarly there was no obvious presynaptic enhancement of basal release as determined by changes in paired pulse facilitation of fEPSPs. The next obvious locus of enhancement would be a change in NMDA receptor-mediated neurotransmission. The stimulation/response relationship of NMDA receptor-mediated fEPSPs was unaffected by the *hpk* mutation.

Thus, it remains unclear how the *hpk* gene contributes to an inhibitory modulation of LTP. A simple explanation would involve the EBI-1 receptor acting via a G-protein to inhibit some part of the molecular signaling pathway that is involved in LTP formation downstream of NMDA-receptor activation. However, the possibility that the *hpk* mutation had an effect during the neural development of the mouse cannot be unequivocally ruled out.

7.3.4. *BSP1*

BSP1 appears to be exclusively expressed in the hippocampus and encodes a polypeptide that bears a resemblance to the trypsin subfamily of proteases. Furthermore, the expression pattern of this serine protease centers around the CA1 and CA3 subfields.

Other serine proteases, such as tissue plasminogen activator (t-PA), have been implicated in long lasting (Late) LTP (Qian *et al.*, 1993; Frey *et al.*, 1996; Huang *et al.*, 1996) where t-PA played a facilitatory role. Taken together, this evidence suggests that *BSP1* may also be involved in Late LTP.

The deletion of the *BSP1* gene in mice had no effect of basal transmission properties such as stimulation/response relationships, paired pulse facilitation of fEPSPs and paired pulse effects on population spikes. A stimulation protocol of 4 tetani of 100 shocks for 1 sec at 5 minute intervals was used to induce Late LTP. No differences in LTP magnitude were observed after four hours post-tetanus suggesting that the BSP1 gene product does not play a role in this form of LTP.

One effect of the *BSP1* gene deletion was observed during the repeated low frequency (1 Hz) stimulation of population spike responses. The *BSP1* null mutants displayed a greater propensity towards polyspiking, i.e. multiple action potential firing after a single stimulus, suggesting an increase in excitability of the CA1 pyramidal neurons. Several mechanisms may contribute to this including increased $GABA_B$ autoinhibition and increased $GABA_A$ receptor desensitisation. In support of this increased susceptibility toward epileptogenic activity in the hippocampus, preliminary studies using kainic acid have shown that mice lacking the BSP1 gene are more prone to excitatoxic insult. However, how the precise mechanisms by which this gene contributes to regulating excitability of neurons in the hippocampus remain unclear. It is feasible that this gene is involved in the development of the hippocampus and although no gross morphological changes were observed between wt and *BSP1*$^{-/-}$ mice, a difference at the ultrastructural level is possible. A follow-up study might look at GABAergic function within the hippocampus in more detail.

7.3.5. Activity-dependent gene expression

The K^+ channel blocker, 4-AP, was used to increase spontaneous firing in the hippocampal slice. It was hypothesised that increasing neuronal activity could be used to examine activity dependent increases in gene expression in the mutant mice. This relied on the ability of the β-geo gene insertion to be expressed under the same promoter region as the native gene it had been inserted into. Upon X-gal staining for β-galactosidase expression, no changes in expression levels or patterns were detected for *obn*, *kin*, *hpk* or *BSP1* mutant mice.

The lack of changes in expression levels could be due to the indiscriminate activation of all excitatory, inhibitory and modulatory neurons within the hippocampal circuitry, i.e. inhibitory neurons could be subduing excitatory synaptic transmission. A better method would be to activate a pathway using electrical stimulation and look for increased expression within a population of neurons. If a gene was found to be upregulated then this could be used as a histological tool to determine patterns of synaptic activation in a variety of experimental models.

CHAPTER 8

GENERAL DISCUSSION

The aim of this thesis was to use electrophysiological techniques to examine the mechanisms implicated in the synaptic processes of memory and learning in the rodent hippocampus. In this respect, two of the major mechanisms currently thought to play a role in these functions are the septal cholinergic input to the hippocampus and long-term synaptic plasticity within the tri-synaptic pathway of the hippocampus.

Stimulation of the putative septohippocampal cholinergic terminals produced $EPSP_M$s recorded from CA1 pyramidal cells. An investigation into the identity of postsynaptic mAChR subtype mediating this response proved inconclusive as the pharmacological tools available provided equivocal data. Consequently, it was decided that this study required a more thorough investigation which would be costly and would not necessarily unequivocally resolve the issue.

Therefore, the research focus was shifted to investigating the mechanisms responsible for the release of ACh from cholinergic terminals and how this process is modified by other G protein coupled receptors. As such, we decided to examine the involvement of voltage gated Ca^{2+} channels that are known to be implicated in the neurotransmitter release process. It was discovered that both N-type and P/Q-type Ca^{2+} channels contributed to presynaptic calcium influx at cholinergic terminals, a heterogeneity which is not at all unusual in CNS presynaptic terminals. There are numerous reports of neurotransmitter release involving multiple HVA Ca^{2+} channels in the hippocampus and other brain regions. Also, evidence has demonstrated changes in the proportion of Ca^{2+} channel subtypes at synapses with increasing age. This dynamic heterogeneity may indicate a synapse-specific point of control for neurotransmitter release.

It is logical to assume that a strong excitatory input like the cholinergic pathway should have some kind of inhibitory check to guard against detrimental events such as epileptogenesis. It had previously been demonstrated that adenosine A_1R activation caused a profound presynaptic inhibition of ACh release. To further this investigation, we examined the cellular mechanisms by which adenosine may exert its effect. Our studies determined that there may be a partial involvement of 4-AP sensitive K^+ channels whilst there was no evidence to suggest that a single sub-type of voltage-dependent Ca^{2+} channel mediates the inhibitory effect of A_1R activation.

Moreover, we discovered that increasing the levels of intracellular cAMP by stimulating adenylate cyclase resulted in a significant reduction in the levels of inhibition provided by adenosine receptor activation. Using a non-hydrolysable analogue of cAMP confirmed this result by producing a full blockade of adenosine receptor-mediated inhibition of ACh release. This result is consistent with A_1Rs acting via G_i to affect a reduction in AC activity. The logical progression of these experiments would explore how cAMP may act within the cholinergic terminal to bring about this reduction in release. This would involve looking at PKA and, if possible, the phosphorylation/dephosphorylation of elements of the vesicular release machinery, e.g. SNAP25. Elucidating the mechanisms by which neurotransmitter release is regulated in the physiological state would provide a useful standard by which to compare the same functions within diseased brains to ascertain how neurological illnesses alter brain function at the synaptic level. This could possibly result in the development of some remedial therapy to correct or compensate for any such abberrations. That psychiatric diseases are caused by disturbances in neurotransmitter release is suggested by Selective Serotonin Reuptake Inhibitor therapy in depression, dopamine replacement therapy for the treatment of Parkinson's disease as well as the lack of ACh release in Alzheimer's disease. Although these diseases often result from a progressive neurodegeneration, there may be mechanisms to increase the efficiency of the remaining healthy synapses.

A recent study has shown that the septohippocampal cholinergic pathway atrophies in aged rhesus monkeys (Smith *et al.*, 1999). Furthermore, it was demonstrated that this neuronal atrophy could be reversed by grafting fibroblasts genetically modified to produce and secrete human nerve growth factor (NGF) into a region of the basal

forebrain containing the cell bodies of the cholinergic neurons that project to cortical regions of the brain involved with cognition and memory function. This kind of gene therapy holds promise for the future treatment of neurodegenerative diseases such as presenile dementia. Obviously, the more we understand about the septohippocampal cholinergic pathway the most the future treatment can be refined to produce beneficial reatment with minimal associated side effects.

It is generally accepted that most synapses in the CNS are under the control of multiple types of G-protein coupled receptors. In this respect we determined that activation of presynaptic μ-opioid receptors produced an enhancement of the $EPSP_M$. It was unclear as to the mechanism by which this was achieved as the accepted action of opioid receptor activation is to inhibit GABAergic interneurons therefore "disinhibiting" excitatory neurons such as the glutamatergic CA1 pyramidal cells. This could not be the case the present study as GABAergic neurotransmission was blocked by antagonists. It is most likely that μ-opioid receptors inhibit the release of other neurotransmitters which negatively regulate the $EPSP_M$. This aside, an enhancement of processes that contribute to memory and learning is a reasonable role that opioids could fulfill. This is because, generally, opioid release is associated with pleasurable or painful stimuli and it is at these times that animals want or need to remember the context in which they experienced this sensory information so that they can repeat or avoid the same situation in the future. Therapeutically this receptor may not provide an optimal drug target because of the widespread distribution of μ-opioid receptors in the CNS as well as the tolerance and dependence effects associated with m-opioid drugs. However, the μ-opioid receptors regulating mAChR-mediated neuromodulation may be associated with other regulatory proteins, e.g. RGS, which may be unique and enable more targeted intervention by small molecule drugs.

In contrast to the opioid-mediated enhancement of the $EPSP_M$, galanin, a neuropeptide linked to ACh release in the hippocampus, was reported to produce an inhibition of ACh release. In this respect, we seized the opportunity to investigate the effect of galanin upon cholinergic transmission in the hippocampus of mice lacking a functional galanin gene. As predicted, galanin produced little inhibition in *Gal*$^{-/-}$

mice. However, the hypothesised improvement in learning that this mutation might be expected to result in was not apparent. In fact, quite surprisingly the opposite was found to be the case. We therefore chose to examine whether there was an impairment in LTP in these animals since LTP represents a working model of molecular memory at synapses. Consistent with their impaired learning ability, a deficit in LTP was evident at CA3-CA1 synapses in the *s. oriens*. This reduction in LTP could be a result of general septohippocampal cholinergic overexcitation within the *s. oriens* so placing LTP much closer to saturation in the $Gal^{-/-}$ mice prior to brain removal. An LTP saturation study would be useful in confirming this theory. Also, it would be interesting to examine the status of LTP in cortical areas which are devoid of ACh function to establish whether these synapses are similarly affected by the galanin gene deletion. Furthermore, the investigation of other learning phenomena that involves hippocampal cholinergic function, e.g. theta and gamma rhythm oscillations, would provide a more in depth insight into how the galanin gene mutation could be affecting learning ability in these mice.

Long-term potentiation is undoubtedly under the control of many regulatory elements which function in concert to fine tune memory formation at the synaptic level. A increasingly useful and recognised method of exploring proteins involved in the expression and control of synaptic plasticity is to screen transgenic mice, produced by random gene trap insertion, for genes that are expressed in the hippocampus. LTP in a number of these transgenic mice was examined and it was found that one line of mice possessing a disruption of to the EBI-1 G-protein coupled receptor demonstrated enhanced LTP. It is unclear how this receptor may contribute to the inhibitory modulation of LTP. A full study would be required to examine this issue further. This may involve the development of specific agonists/antagonists to explore the transduction pathway of this novel G protein-coupled receptor as well as the development of a specific antibody raised against the EBI-1 receptor thus allowing the use of immunohistochemical techniques to more accurately pinpoint the location of this receptor.

It had previously been demonstrated that serine proteases may play a role in the expression of late LTP by facilitating the remodeling of synaptic connections in the hippocampus. However, a transgenic mouse possessing a disrupted gene encoding a

brain serine protease that is uniquely expressed in the hippocampus did not display any change in Late LTP. However, the *BSP1*$^{-/-}$ mice did display increased epileptogenesis demonstrated by an increased number of action potentials fired following a single stimulus at CA3-CA1 synapses. This is likely to result from an increase in GABAergic autoinhibition via presynaptic $GABA_B$ receptors on glutamatergic terminals or a increased desensitisation of postsynaptic $GABA_A$ receptors. Follow-up experiments would explore GABAergic function in *BSP1*$^{-/-}$ in greater detail.

Recently, it has been reported that an adult transgenic mouse which overexpressed the N2RB subunit of the NMDA receptor, a subunit that is associated with young mice, demonstrated enhanced LTP and improvements in a number of behavioural tests designed to measure learning and memory (Tang *et al.*, 1999). Although this report primarily shows the importance of the NMDA subunit composition in synaptic plasticity and memory function, it also points towards a therapeutic target that may be exploited in the future. For instance, increasing the relative proportion of N2RB subunit- to N2RA subunit-containing NMDA receptors in the hippocampi of AD sufferers may compensate for their cognitive impairments.

In order to have any chance of finding an effective treatment for any of the neurological diseases that impair cognition, it is essential that we have a clear understanding of how the numerous elements of the brain act and interact to carry out these higher functions. This thesis just touches on some possible mechanisms that, in time, when put in context with research around the world may provide us with the knowledge to devise more beneficial treatments for a population of patients that, due to a prolonged life expectancy, is inevitably on the increase.

CHAPTER 9

BIBLIOGRAPHY

ACHSTETTER, T., FRANZUSOFF, A., FIELD, C. & SCHECKMAN, R. (1988). SEC7 encodes an unusual, high molecular weight protein required for membrane traffic from the yeast golgi apparatus. *J. Biol. Chem.* **263**, 11711-11717.

AIGNER, T.G. (1995). Pharmacology of memory: cholinergic-glutamatergic interactions. *Current Opinion in Neurobiology* **5,** 155-160.

AKINS, P.T., SURMEIER, D.J. & KITAI, S.T. (1990). Muscarinic modulation of a transient K^+ conductance in rat neostriatal neurones. *Nature* **344,** 240-242.

ALBUQUERQUE, E.X., PEREIRA, E.F.R., ALKONDON, M., SCHRATTENHOLZ, A. & MAELICKE, A. (1997) Nicotinic acetylcholine receptors on hippocampal neurons: distribution on the neuronal surface and modulation of receptor activity. *Journal of Receptor and Signal Transduction Research* **17,** 243-266.

ALBUQUERQUE, E.X., PEREIRA, E.F.R., CASTRO, N.G. & ALKONDON, M. (1995a). Neuronal nicotinic receptors: function, modulation and structure. *Seminars in the Neurosciences* **7,** 91-101.

ALBUQUERQUE, E.X., PEREIRA, E.F.R., CASTRO, N.G., ALKONDON, M., REINHARDT, S., SCHRÖDER, H. & MAELICKE, A. (1995b). Nicotinic receptor function in the mammalian central nervous system. *Annuls of the New York Academy of Sciences* **757,** 48-72.

ALFORD, S., FRENGUELLI, B.G., SCHOFIELD, J.G. & COLLINGRIDGE, G.L. (1993). Characterization of Ca^{2+} signals induced in hippocampal CA1 neurones by the synaptic activation of NMDA receptors. *J. Physiol. (Lond)* **469**, 693-716.

ALGER, B.E. & NICOLL, R.A. (1982a). Pharmacological evidence for two kinds of GABA receptor on rat hippocampal pyramidal cells studied in vitro. *Journal of Physiology (London)* **328,** 125-141.

ALGER, B.E. & NICOLL, R.A. (1982b). Feed-forward dendritic inhibition in rat hippocampal pyramidal cells studied in vitro. *Journal of Physiology (London)* **328,** 105-123.

ALKONDON, M. & ALBUQUERQUE, E.D. (1993). Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons.I. Pharmacological and functional evidence for distinct structural subtypes. *The Journal of Pharmacology and Experimental Therapeutics* **265,** 1455-1473.

ALLEN, G.I., ECCLES, J., NICOLL, R.A., OSHIMA, T. & RUBIA, F.J. (1977). The ionic mechanisms concerned in generating the i.p.s.p.s of hippocampal pyramidal cells. *Proceedings of the Royal Society of London Series B* **198,** 363-384.

AMARAL, D.G. & KURZ, J. (1985). An analysis of the origin of the cholinergic and non-cholinergic septal projections to the hippocampal formation of the rat. *J. Comp. Neurol.* **240**, 37-59.

AMARAL, D.G. & WITTER, M.P. (1989). The three-dimensional organization of the hippocampal

formation: A review of anatomical data. *Neuroscience* **31,** 571-591.

AMIRANOFF, B., LORINET, A.M. & LABURTHE, M. (1991). A clonal rat pancreatic delta cell line (Rin14B) expresses a high number of galanin receptors negatively coupled to a pertussis toxin-sensitive cAMP-production pathway. *Eur. J. Biochem.* **195**, 459-463.

ANDERSEN, P. (1981). Brain slices - a neurobiological tool of increasing usefulness. *Trends in Neuroscience* **4,** 53-56.

ANDERSEN, P., BLISS, T.V.P. & SKREDE, K.K. (1971). Lamellar organization of hippocampal excitatory pathways. *Experimental Brain Research* **13,** 222-238.

ANDERSEN, P., BRULAND, H. & KAADA, B.R. (1961). Activation of the dentate area by septal stimulation. *Acta Physiol. Scand.* **51**, 17-28.

ANDERSEN, P., BRULAND, H. & KAADA, B.R. (1961). Activation of the field CA1 of the hippocampus by septal stimulation. *Acta Physiol. Scand.* **51**, 29-40.

ANDERSEN, P., ECCLES, J.C. & LOYNING, Y. (1964). Pathways of postsynaptic inhibition in the hippocampus. *Journal of Neurophysiology* **27,** 608-619.

ANDREASEN, M., LAMBERT, J.D.C. & JENSEN, M.S. (1989). Effects of new non-N-methyl-D-aspartate antagonists on synaptic transmission in the in vitro rat hippocampus. *Journal of Physiology (London)* **414,** 317-336.

ARAUJO, D.M., LAPCHAK, P.A., COLLIER, B. & QUIRION, R. (1988). Characterization of *N*-[^{3}H]methylcarbamylcholine binding sites and effect of *N*-methylcarbamylcholine on acetylcholine release in rat brain. *Journal of Neurochemistry* **51,** 292-299.

ARCH, J.R. & NEWSHOLME, E.A. (1978). The control of the metabolism and the hormonal role of adenosine. *Essays in Biochemistry* **14,** 82-123.

ARMSTRONG, D.M., SAPER, C.B., LEVEY, A.I., WAINER, B.H. & TERRY, R.D. (1983). Distribution of cholinergic neurons in rat brain: demonstration by the immunocytochemical localisation of choline acetyltransferase. *J. Comp. Neurol.* **216**, 53-68.

ARMSTRONG, D.M., SHEFFIELD, R., BUZSAKI, G., CHEN, K.S., HERSH, L.B., NEARING, B. & GAGE, F.H. (1993). Morphological alterations choline acetyltransferase-positive neurons in the basal forebrain of aged behaviourally characterised Fisher 344 rats. *Neurobiol. Aging* **14**, 457-470.

ASCHER, P. & NOWAK, L. (1988). The role of divalent cations in the N-methyl-D-aspartate responses of mouse central neurones in culture. *Journal of Physiology (London)* **399,** 247-266.

ATTALI, B., GOUARDÈRES, C., MAZARGUIL, H., AUDIGIER, Y. & CROS, J. (1982). Differential interaction of opiates to multiple "kappa" binding sites in the guinea-pig lumbo-sacral spinal cord. *Life Sci.* **31**, 1371-5.

AUERBACH, J.M. & SEGAL, M. (1996). Muscarinic receptors mediating depression and long-term potentiation in rat hippocampus. *Journal of Physiology (London)* **492,** 479-493.

AUGUSTINE G.J., CHARLTON, M.P. & SMITH, S.J. (1987). Calcium action in synaptic transmitter release. *Annu. Rev. Neurosci.* **10**, 633-693.

BARRETT, A.J. (1990). Proteolytic enzymes: serine and cysteine proteases. *Meth. Enzymol.* **224**, 19-32.

BARTUS, R.T., DEAN, R.L. & FLICKER C. (1987). Cholinergic psychopharmacology: an

integration of human and animal research on memory. *Psychopharmacology: the Third Generation of Progress* (Meltzer, H.Y., ed.) pp. 219-232 (*New York: Raven*).

BAUSCH, S.B. & CHAVKIN, C. (1995). Colocalisation of mu and delta opioid receptors with GABA, parvalbumin and a G-protein-coupled inwardly rectifying potassium channel (GIRK1) in the rodent brain. *Analgesia* **1**, 282-285.

BECHTEREW, V. (1900). Demonstration eines gehirns mit zerstörung der vorderen und inneren theile der hirnrinde beider schläfenlappen. *Neurologisches Centralblatt* **19**, 990-991.

BEDECS, K., BERTHOLD, M. & BARTFAI, T. (1995). Galanin: 10 years with a neuroendocrine peptide. *Int. J. Biochem. Bell. Biol.* **27**, 337-349.

BENARDO, L.S. & PRINCE, D.A. (1982a). Cholinergic excitation of mammalian hippocampal cells. *Brain Research* **249,** 315-331.

BENARDO, L.S. & PRINCE, D.A. (1982b). Ionic mechanisms of cholinergic excitation in mammalian hippocampal pyramidal cells. *Brain Research* **249,** 333-344.

BENDER, A.S., WU, P.H. & PHILLIS, J.W. (1981). The rapid uptake of [^{3}H]adenosine by rat cerebral cortical synaptosomes. *Journal of Neurochemistry* **36,** 651-660.

BEN-NERIAH, Y., BERNARDS, A., PASKIND M., DALEY, G. Q. & BALTIMORE, D. (1986). Alternative 5' exons in c-abl RNA. *Cell* **44**, 577-586.

BENSON, D.M., BLITZER, R.D. & LANDAU, E.M. (1988). An analysis of the depolarization produced in guinea-pig hippocampus by cholinergic receptor stimulation. *Journal of Physiology (London)* **404,** 479-496.

BIEGON, A., GREENBERGER, V. & SEGAL, M. (1986). Quantitative histochemistry of brain acetylcholinesterase and learning rate in the aged rat. *Neurobiol. Aging* **7**, 215-217.

BIRKENBACH, M., JOSEFSEN, K., YALAMANCHILI, R., LENOIR, G. & KIEFF, E. (1993). Epstein-Barr virus-induced genes: First lymphocyte-specific G protein-coupled peptide receptors. *J. Virol.* **67**, 2209-2220.

BLACKSTAD, T.W. (1956). Commissural connections of the hippocampal slice of the rat, with special reference to their mode of termination. *Journal of Comparative Neurology* **105,** 417-538.

BLAND, B.H., COLOM, L.V., KONOPACKI, J. & ROTH, S.H. (1988). Intracellular records of carbachol-induced theta rhythm in hippocampal slices. *Brain Research* **447,** 364-368.

BLISS, T.V.P. & COLLINGRIDGE, G.L. (1993). A synaptic model of memory: Long-term potentiation in the hippocampus. *Nature* **361**, 31-39.

BLISS, T.V.P. & LØMO, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the unanaesthetised rabbit following stimulation of the perforant path. *J. Physiol.* **232**, 331-356.

BLITZER, R.D., GIL, O. & LANDAU, E.M. (1990). Cholinergic stimulation enhances long-term potentiation in the CA1 region of rat hippocampus. *Neuroscience Letters* **119,** 207-210.

BLITZER, R.D., GIL, O., OMRI, G. & LANDAU, E.M. (1991). Nifedipine blocks calcium-dependent depolarisation in the guinea pig hippocampus. *Brain research* **542**, 293-299.

BOESS, F.G., BALASUBRAMANIAN, M.K., BRAMMER, M.J. & CAMPBELL, I.C. (1990). Stimulation of muscarinic acetylcholine receptors increases synaptosomal free calcium concentration

by prorein kinase-dependent opening of L-type calcium channels. *Journal of Neurochemistry* **55,** 230-236.

BONNER, T.I., BUCKLEY, N.J., YOUNG, A.C. & BRANN, M.R. (1987). Identification of a family of muscarinic acetylcholine receptor genes. *Science* **237,** 527-532.

BONNER, T.I., YOUNG, A.C., BRANN, M.R. & BUCKLEY, N.J. (1988). Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. *Neuron* **1,** 403-410.

BOWEN, D.M., SMITH, C.B., WHITE, P. & DAVISON, A.N. (1976). Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies. *Brain* **99,** 459-496.

BOWEN, D.M., WHITE, P., SPILLANE, J.A., GOODHARDT, M.J., CURZON, G., IWANGOFF, P., MEIER-RUGE, W. & DAVISON A.N. (1979). Accelerated ageing or selective neuronal loss as an important cause of dementia? *Lancet* **i,** 11-14.

BRANN, M.R., JØRGENSEN, H.B., BURSTEIN, E.S., SPALDING, T.A., ELLIS, J., JONES, S.V.P. & HILL-EUBANKS, D. (1993). Studies of the pharmacology, localization, and structure of muscarinic acetylcholine receptors. *Annuls of the New York Academy of Sciences* **707,** 225-236.

BROWN, D.A. & ADAMS, P.R. (1980). Muscarinic suppression of a novel voltage-sensitive K^+ current in a vertebrate neurone. *Nature* **283,** 673-676.

BROWN, D.A. (1983). Slow cholinergic excitation – a mechanism for increasing neuronal excitability. *Trends in Neurosciences* **6,** 302-307.

BROWN, D.A., ABOGADIE, F.C., ALLEN, T.G.J., BUCKLEY, N.J., CAULFIELD, M.P., DELMAS, P., HALEY, J.E., LAMAS, J.A. & SELYANKO, A.A. (1997). Muscarinic mechanisms in nerve cells. *Life Sciences* **60,** 1137-1144.

BROWN, D.A., HIGASHIDA, H., NODA, M., ISHIZAKA, N., HASHII, M., HOSHI, N., YOKOYAMA, S., FUKUDA, K., KATAYAMA, M., NUKADA, T., KAMEYAMA, K., ROBBINS, J., MARSH, S.J. & SELYANKO, A.A. (1993). Coupling of muscarinic receptor subtypes to ion channels: Experiments on neuroblastoma hybrid cells. *Annuls of the New York Academy of Sciences* **707,** 237-258.

BROWN, S. & SCHAFER, E.A. (1888). An investigation into the functions of the occipital and temporal lobes of the monkey's brain. *Philosophical Transactions of the Royal Society of London (Biology)* **179**, 303-327.

BRUNS, R.F. (1991). Role of adenosine in energy supply/demand balance. *Nucleosides & Nucleotides* **10,** 931-943.

BRUNS, R.F., LU, G.H. & PUGSLEY, T.A. (1986). Characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. *Molecular Pharmacology* **29,** 331-346.

BUCKLEY, N.J., BONNER, T.I. & BRANN, M.R. (1988). Localization of a family of muscarinic receptor mRNAs in rat brain. *Journal of* Neuroscience **8,** 4646-4652.

BURGEVIN, M.C., LOQUET, I., QUARTERONET, D. & HARBERT-ORTOLI, E. (1995). Cloning, pharmacological characterisation and anatomical distribution of a rat cDNA encoding for a galanin receptor. *J. Mol. Neurosci.* **6**, 33-41.

BURKE, S.P. & NADLER, J.V. (1988). Regulation of glutamate and aspartate release from slices of the hippocampal CA1 area: effects of adenosine and baclofen. *Journal of Neurochemistry* **51,** 1541-1551.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptor. *In: Cell membrane receptors for drugs and hormones: A multidisciplinary approach.* Editors: Straub, R.W. & Bolis, L.. Raven Press, New York, 117-118.

BÚZÁS, B., IZENWASSER, S., PORTOGHESE, P.S. & COX, B.M. (1994). Evidence for delta opioid receptor subtypes regulating adenylyl cyclase activity in rat brain. *Life Sci.* **54**, 101-6.

BUZSÁKI, G. (1984). Feed-forward inhibition in the hippocampal formation. *Progress in Neurobiology* **22,** 131-153.

CAESER, M., BROWN, D.A., GÄHWILER, B.H. & KNÖPFEL, T. (1993). Characterization of a calcium-dependent current generating a slow afterdepolarization of CA3 pyramidal cells in rat hippocampal slice cultures. *European Journal of Neuroscience* **5,** 560-569.

CARBONE, E. & LUX, H.D. (1984). A low voltage-activated, fully inactivating Ca^{2+} channel in vertebrate sensory neurones. *Nature* **310**, 501-502

CARMELIET, P., SCHOONJANS, L., KIECKENS, L., REAM, B., DEGEN, J., BRONSON, R., DE VOS, R., VAN DER OORD, J.J., COLLEN, D. & MULLIGAN, R.C. (1994). Physiological consequences of loss of plasminogen activator gene function in mice. *Nature* **368**, 419-424.

CARUTHERS, A.M. & FOZARD, J.R. (1993). Adenosine A_3 receptors: two into one won't go. *Trends in Pharmacological Science* **14,** 290-291.

CASTILLO, P.E., WEISSKOPF, M.G. & NICOLL, R.A. (1994). The role of Ca^{2+} channels in hippocampal mossy fibre synaptic transmission and long-term potentiation. *Neuron* **12**, 261-269.

CHANG, K.-J., HAZUM, E. & CUATRECASAS, P. (1981). Novel opiate binding sites selective for benzomorphan drugs. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4141-4145.

CHAVEZ-NORIEGA, L.E. & STEVENS, C.F. (1994). Increased transmitter release at excitatory synapses produced by direct activation of adenylate cyclase in rat hippocampal slices. *J. Neurosci.* **14**, 310-317.

CHEN, Y., MESTEK, A., LIU, J. & YU, L. (1993). Molecular cloning of a rat kappa opioid receptor reveals sequence similarities to the mu and delta opioid receptors. *Biochem J.* **295 (Pt 3),** 625-8

CHETKOVICH, D.M., GRAY, R., JOHNSTON, D. & SWEATT, J.D. (1991). N-methyl-D-aspartate receptor activation increases cAMP levels and voltage-gated Ca^{2+} channel activity in area CA1 of hippocampus. *Proc. Natl. Acad. Sci. USA* **88**, 6467-71.

CHILDERS, S.R. (1993). Opioid receptor-coupled second messenger systems. *Handbook of Experimental Pharmacology: opioids, Vol. I* (Herz A., ed), pp. 189-216. New York: Springer.

CHIROBAK, J.J. & NAPIER, T.C. (1992). Antagonism of GABAergic transmission within the septum disrupts working/episodic memory in the rat. *Neuroscience* **47**, 833-841.

CLARK, J.A., LIU, L., PRICE, M., HERSH, B., EDELSON, M. & PASTERNAK, G.W. (1989). Kappa opiate receptor multiplicity: evidence for two U50,488-sensitive kappa 1 subtypes and a novel kappa 3 subtype. *J. Pharmacol. Exp. Ther.* **251**, 461-8.

COHEN, G.A., DOZE, V.A. & MADISON, D.V. (1991). Differential modulation of inhibitory transmission in the hippocampus by carbachol, adenosine, seratonin and norepinephrine. *Neuroscience Abstracts* **17,** 1487.

COLE A.E. & NICOLL, R.A. (1984a). Characterisation of a slow cholinergic post-synaptic potential recorded *in vitro* from rat hippocampal pyramidal cells. *Journal of Physiology (London)* **352**, 173-188.

COLE, A.E. & NICOLL, R.A. (1983). Acetylcholine mediates a slow synaptic potential in hippocampal pyramidal cells. *Science* **221**, 1299-1301.

COLE, A.E. & NICOLL, R.A. (1984b). The pharmacology of cholinergic excitatory responses in hippocampal pyramidal cells. *Brain Research* **305,** 283-290.

COLINO, A. & HALLIWELL, J.V. (1993). Carbachol potentiates Q current and activates a calcium-dependent non-specific conductance in rat hippocampus *in vitro*. *European Journal of Neuroscience* **5**, 1198-1209.

COLLINGRIDGE, G.L., HERRON, C.E. & LESTER, R.A.J. (1988). Synaptic activation of N-methyl-D-aspartate receptors in the Schaffer collateral-commissural pathway in rat hippocampus. *J. Physiol. Lond.* **399**, 283-300.

COLLIS, M.G. & HOURANI, M.O. (1993). Adenosine receptor subtypes. *Trends in Pharmacological Sciences* **14,** 360-366.

COMMONS, K.G. & MILNER, T.A. (1997). Localisation of delta opioid receptor immunoreactivity in interneurons and pyramidal cells in the rat hippocampus. *J. Comp. Neurol.* **381**, 373-387.

CONNOR, M.A., KEIR, M.J. & HENDERSON, G. (1997). Delta-opioid receptor mobilization of intracellular calcium in SH-SY5Y cells: lack of evidence for delta-receptor subtypes. *Neuropharmacology* **36**, 125-33.

CONSTANTI, A. & SIM, J.A. (1987). Muscarinic receptors mediating suppression of the M-current in guinea-pig olfactory cortex neurones may be of the M_2-subtype. *British Journal of Pharmacology* **90,** 3-5.

CORBETT, A., ET AL. (1993). Handbook of experimental pharmacology (Vol. 104). (Herz, A., Akil, H. & Simon, E.J., eds), pp. 645-679, Springer-verlag.

CORTÉS, R. & PALACIOS, J.M. (1986). Muscarinic cholinergic receptor subtypes in the rat brain. I. Quantitative autoradiographic studies. *Brain Research* **362,** 227-238.

COUMIS, U. & DAVIES, C.H. (1999) *In preparation.*

CRAIN, B.J., CHANG, K.-J. & McNAMARA, J.O. (1986). Qunatitative autoradiographic analysis of mu and delta opioid binding sites in the rat hippocampal formation. *J. Comp. Neurol.* **246**, 170-180.

CRAWLEY, J. (1990). Coexistence of neuropeptides and "classical" neurotransmitters. Functional interactions between galanin and acetylcholine. *Ann. N.Y. Acad. Sci.* **579**, 233-245.

CUNHA, R.A., SEBASTIÃO, A.M., RIBEIRO, J.A. (1998). Inhibition by ATP of hippocampal transmission requires localised extracellular catabolism by ecto-nucleotidases into adenosine and channeling to adenosine A_1 receptors. *J. Neurosci.* **18**, 1987-1995.

DALE, H.H. (1914). The action of certain esters and ethers of choline, and their relation to muscarine. *The Journal of Pharmacology and Experimental Therapeutics* **6,** 147-190.

DALE, H.H. (1934). Chemical transmission of the effects of nerve impulses. *British Medical Journal* **I,** 835-841.

DALE, H.H. (1938). Acetylcholine as a chemical transmitter of the effects of nerve impulses. *Journal of the Mount Sinai Hospital* **4,** 401-429.

DALE, H.H., FELDBERG, W. & VOGT, M. (1936). Release of acetylcholine at voluntary motor nerve endings. *Journal of Physiology (London)* **86,** 353-380.

DALY, J.W., BUTTS-LAMB, P. & PADGETT, W. (1983). Subclasses of adenosine receptors in the central nervous system: interaction with caffeine and related methylxanthines. *Cell Molecular Neurobiology* **3,** 69-80.

DALZIEL, H.H. & WESTFALL, D.P. (1994). Receptors for adenosine nucleotides and nucleosides: subclassification distribution and molecular characterization. *Pharmacological Reviews* **46,** 449-466.

DAVAL, J., NEHLIG, A. & NICOLAS, F. (1991). Physiological and pharmacological properties of adenosine: therapeutic implications. *Life Sciences* **49,** 1435-1453.

DAVIES, B.J., PICKARD, B.S., STEEL, M., MORRIS, R.G.M. & LATHE, R. (1998). Serine proteases in rodent hippocampus. *J. Biol. Chem.* **273**, 23004-23011.

DAVIES, C.H., STARKEY, S.J., POZZA, M.F. & COLLINGRIDGE, G.L. (1991). GABA autoreceptors regulate the induction of LTP. *Nature* **349**, 609-611.

DAVIES, S.N. & COLLINGRIDGE, G.L. (1989). Role of excitatory amino acid receptors in synaptic transmission in area CA1 of rat hippocampus. *Proceedings of the Royal Society of London Series B* **236,** 373-384.

DE GUBAREFF, T. & SLEATOR, W. (1965). Effects of caffeine on mammalian atrial muscle and its interaction with adenosine and calcium. *The Journal of Pharmacology and Experimental Therapeutics* **148,** 202-214.

DE LACALLE, S., IRAIZOZ, I. & MA GONZALO, L. (1991). Differential changes in cell size and number in topographic subdivisions of human basal nucleus in normal aging. *Neuroscience* **43**, 445-456.

DE WAARD, M. & CAMPBELL, K.P. (1995). Sub-unit regulation of the neuronal alpha 1A Ca^{2+} channel expressed in Xenopus oocytes. *Journal of Physiology* **485**, 619-634.

DE WAARD, M., GURNETT, C.A. & CAMPBELL, K.P. (1996). Structural and functional diversity of voltage-activated calcium channels. *Ionic Channels* (Vol. 4) (Narahashi, T., ed.), pp. 41-87, Plenum Press.

DENERIS, E.S., CONNOLLY, J., ROGERS, S.W. & DUVOISIN, R. (1991). Pharmacology and functional diversity of neuronal nicotinic acetylcholine receptors. *Trends in Pharmacological Sciences* **12,** 34-40.

DERRICK, B.E. & MARTINEZ, J.L. (1994). Opioid receptor activation is one factor underlying the frequency dependence of mossy fibre LTP induction. *J. Neurosci.* **14**, 4359-4367.

DINGLEDINE, R. & GJERSTAD, L. (1980). Reduced inhibition during epileptiform activity in the in vitro hippocampal slice. *Journal of Physiology (London)* **305,** 297-313.

DINGLEDINE, R. & LANGMOEN, I.A. (1980). Conductance changes and inhibitory actions of hippocampal recurrent IPSPs. *Brain Research* **185,** 277-287.

DINGLEDINE, R., HYNES, M.A. & KING, G.L. (1986). Involvement of N-methyl-D-aspartate receptors in epileptiform bursting in the rat hippocampal slice. *Journal of Physiology (London)* **380,** 175-189.

DITTMAN, J.S. & REGEHR, W.G. (1996). Contributions of calcium-dependent and calcium-independent mechanisms to presynaptic inhibition at a cerebellar synapse. *J. Neurosci.* **16**, 1623-33.

DODD, J., DINGLEDINE, R. & KELLY, J.S. (1981). The excitatory action of acetylcholine on hippocampal neurones of the guinea pig and rat maintained in vitro. *Brain Research* **207,** 109-127.

DOLPHIN, A.C., FORDA, S.R. & SCOTT, R.H. (1986). Calcium-dependent currents in cultured rat dorsal root ganglion neurones are inhibited by an adenosine analogue. *Journal of Physiology (London)* **373,** 47-61.

DOODS, H.N., MATHY, M-J., DAVIDESKO, D., VAN CHARLDORP, K.J., DE JONGE, A. & VAN ZWIETEN, P.A. (1987). Selectivity of muscarinic antagonists in redioligand and *in vivo* experiments for the putative M_1, M_2 and M_3 receptors. *The Journal of Pharmacology and Experimental Therapeutics* **242,** 257-262.

DRAGUNOW, M., ABRAHAM, W.C., GOULDING, M., MASON, S.E., ROBERTSON, H.A. & FAULL, R.L. (1989). Long-term potentiation and the induction of c-fos mRNA and proteins in the dentate gyrus of unanesthetized rats. *Neurosci. Lett.* **101**, 274-80.

DRAVID, A.R., JATON, A.L. & VAN DEUSEN, E.B. (1986). Recovery from biochemical and behavioural deficits in adult rats with partial lesions of the fimbria. *Exp. Brain Res.* **13**, *Suppl*, 247-249.

DRURY, A.D. & SZENT-GYORGYI, A. (1929). The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *Journal of Physiology (London)* **68,** 213-237.

DUDAR, J.D (1975). The effects of septal nuclei stimulation on the release of acetylcholine from the rabbit hippocampus. *Brain Res.* **83**, 123-133.

DUNLAP, K., LUEBKE, J.I. & TURNER T.J. (1995). Exocytotic Ca^{2+} channels in mammalian central neurons. *Trends in Neuroscience* **18**, 89-98.

DUNNE, M.J., BULLETT, M.J., LI, G.D., WOLLHEIM, C.B. & PETERSEN, O.H. (1989). Galanin activates nucleotide-dependent K^+ channels in insulin-secreting cells via a pertussis toxin-sensitive G-protein. *EMBO J.* **8**, 413-420.

DUNWIDDIE, T.V. & DIAO, L. (1994). Extracellular adenosine concentrations in hippocampal brain slices and the tonic inhibitory modulation of evoked excitatory responses. *The Journal of Pharmacology and Experimental Therapeutics* **268**, 537-546.

DUNWIDDIE, T.V. (1980). Endogenously released adenosine regulates excitability in the in vitro hippocampus. *Epilepsia* **21,** 541-548.

DUNWIDDIE, T.V. (1985). The physiological role of adenosine in the central nervous system. *International Review of Neurobiology* **27,** 63-139.

DUNWIDDIE, T.V. (1990). Electrophysiological aspects of adenosine receptor function. *Adenosine and Adenosine Receptors.* Editor, Michael Williams. The Human Press Inc. 143-172.

DUTAR, P. & NICOLL, R.A. (1988a). Classification of muscarinic responses in hippocampus in terms of receptor subtypes and second-messenger systems: electrophysiological studies *in vitro*. *The Journal of Neuroscience* **8**, 4214-4224.

DUTAR, P. & NICOLL, R.A. (1988b). Pre- and post synaptic $GABA_B$ receptors in the hippocampus have different pharmacological properties. *Neuron* **1,** 585-591.

DUTAR, P. & NICOLL, R.A. (1988c). A physiological role for $GABA_B$ receptors in the central nervous system. *Nature* **332,** 156-158.

DUTAR, P., BASSANT, M.-H., SENUT, M.-C. & LAMOUR, Y. (1995). The septohippocampal pathway: structure and function of a central cholinergic system. *Physiological reviews* **75**, 393-427.

DUTAR, P., LAMOUR, Y. & NICOLL, R.A. (1989b). Galanin blocks the slow cholinergic EPSP in CA1 pyramidal neurons from ventral hippocampus. *Eur. J. Pharmacol.* **164**, 355-360.

DUTAR, P., RASCOL, O. & LAMOUR, Y. (1989a). ω-conotoxin GVIA blocks synaptic transmission in the CA1 field of the hippocampus. *Eur. J. Pharmacol.* **174**, 261-266.

ECCLES, J.C., ECCLES, R.M. & FATT, P. (1956). Pharmacological investigations on a central synapse operated by acetylcholine. *Journal of Physiology (London)* **131,** 154-169.

ECCLES, J.C., FATT, P. & KOKETSU, K. (1954). Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurones. *Journal of Physiology (London)* **126,** 524-562.

ECCLES, J.C., NICOLL, R.A., OSHIMA, T. & RUBIA, F.J. (1977). The anionic permeability of the inhibitory postsynaptic membrane of hippocampal pyramidal cells. *Proceedings of the Royal Society of London Series B* **198,** 345-361.

EDWARDS, F.A. & GIBB, A.J. (1993). ATP - a fast neurotransmitter. *Federation of European Biochemical Societies Letters* **325,** 86-89.

ELLIS, J;HUYLER, J;BRANN, MR (1991). Allosteric regulation of cloned M1-M5 muscarinic receptor suntypes. *Biochemical Pharmacology* **42,** 1927-1932.

ENNIS, C. & WYLLIE, M.G. (1984). Evidence for functionally distinct μ receptors modulating acetylcholine release. *Neuropeptides* **5**, 109-112.

EVANS, C.J., KEITH, D.E. JR., MORRISON, H., MAGENDZO, K. & EDWARDS, R.H. (1992). Cloning of a delta opioid receptor by functional expression. *Science* **258**, 1952-5.

FATHI, Z., CUNNINGHAM, A.M., IBEN, L.G., BATTAGLINO, P.B., WARD, S.A., NICHOL, K.A., PINE, K.A., WANG, J., GOLDSTEIN, M.E., IISMAA, T.P. & ZIMANYI, I.A. (1997). Cloning, pharmacological characterisation and distribution of a novel galanin receptor. *Mol. Brain Res.* **51**, 49-59.

FELDBERG, W. (1945). Present views on the mode of action of acetylcholine in the central nervous system. *Physiological Reviews* **25,** 596-642.

FELDER, C.C. (1995). Muscarinic acetylcholine receptors: signal transduction through multiple effectors. *FASEB Journal* **9,** 619-625.

FIGENSCHOU, A., HU, G. & STORM, J.F. (1996). Cholinergic modulation of the action potential in rat hippocampal neurons. *European Journal of Neuroscience* **8**, 211-219.

FINGER S. (1994). Origins of neuroscience. A history of explorations into brain functions. pp. 12-17 (Oxford University Press).

FISCHER, W., GAGE, F.H. & BJORKLUND, A. (1989). Degenerative changes in forebrain cholinergic nuclei correlate with cognitive impairments in aged rats. *Eur. J. Neurosci.* **1**, 34-45.

FISHER R. & JOHNSTON D. (1990). Differential modulation of single voltage-gated calcium channels by cholinergic and adrenergic agonists in adult hippocampal neurons. *Journal of Neurophysiology* **64**, 1291-1302.

FISHER, T.E. & BOURQUE, C.W. (1995). Voltage-activated calcium channels in the magnocellular neurosecretory cells of the rat supraoptic nucleus. *Journal of Physiology* **486**, 571-580.

FISONE, G., BARTFAI, T., NILSSON, S. & HÖKFELT, T. (1991). Galanin inhibits the potassium-evoked release of acetylcholine and the muscarinic receptor-mediated stimulation of phosphoinositide

turnover in slices of monkey hippocampus. *Brain Res.* **568**, 279-284.

FISONE, G., BERTHOLD, M., BEDECS, K., UNDEN, A., BARTFAI, T., BERTORELLI, R., CONSOLO, S., CRAWLEY, J., MARTIN, B. & NILSSON, S. (1989b). N-terminal galanin-(1-16) fragment is an agonist at the hippocampal galanin receptor. *Proc. Natl. Acad. Sci. USA* **86**, 9588-9591.

FISONE, G., LANGEL, U., CARLQUIST, M., BERGMAN, T., CONSOLO, S., HOKFELT, T., UNDEN, A., ANDELL, S. & BARTFAI, T. (1989a). Galanin receptor and its ligands in the rat hippocampus. *Eur. J. Biochem.* **181**, 269-276.

FISONE, G., WU, C.F., CONSOLO, S., NORDSTRÖM, Ö., BRYNNE, N., BARTFAI, T., MELANDER, T. & HÖKFELT, T. (1987). Galanin inhibits acetylcholine release in ventral hippocampus of the rat: Histochemical, autoradiographic, in vivo, and in vitro studies. *Proc. Natl. Acad. Sci. USA* **84**, 7339-7343.

FLOOD, J.F., SMITH, G.E. & CHERKIN, A. (1984). Memory retention test performance in mice: improvement by chronic oral cholinergic drug treatment. *Pharmacol. Biochem. Behav.* **20**, 169-173.

FONNUM, F. (1970). Topographical and subcellular localisation of choline acetyltransferase in rat hippocampal region. *J. Neurochem.* **17**, 1029-1037.

FORLONI, G., GRZANNA, R., BLAKELY, R.D. & COYLE, J.T. (1987). Co-localisation of *N*-acetyl-aspartyl-glutamate in central cholinergic, noradrenergic and serotonergic neurons. *Synapse* **1**, 455-460.

FOX, A.P., NOWYCKY, M.C. & TSIEN, R.W. (1987). Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurons. *Journal of Physiology (London)* **394**, 149-172.

FRANZUSOFF, A. & SCHEKMAN, R. (1989). Functional compartments of the yeast golgi apparatus are defined by the sec7 mutation. *EMBO J.* **8**, 2695-2702.

FRANZUSOFF, A., LAUZE, E. & HOWELL, K.E. (1992). Immuno-isolation of sec7p-coated transport vesicles from the yeast secretory pathway. *Nature* **355**, 173-175.

FRASER, D.D. & MACVICAR, B.A. (1991). Low-threshold transient calcium current in rat hippocampal laculosum-moleculare interneurones: kinetics and modulation by neurotransmitters. *Journal of Neuroscience* **11,** 2812-2820.

FREDHOLM, B.B., ABBARACHIO, M.P., BURNSTOCK, G., DALY, J.W., KENDALL-HARDEN, T., JACOBSON, K.A., LEFF, P. & WILLIAMS, M. (1994a). Nomenclature and classification of purinoceptors. *Pharmacological Reviews* **46,** 143-156.

FREDHOLM, B.B., ABBRACCHIO, M.P., BURNSTOCK, G., DUBYAK, G.R., HARDEN, T.K., JACOBSON, K.A., SCHWABE, U. & WILLIAMS, M. (1997). Towards a revised nomenclature for P1 and P2 receptors. *Trends in Pharmacological Sciences* **18,** 79-82.

FREDHOLM, B.B., LINDSTRÖM, K. & WALLMAN-JOHANSSON, A. (1994b). Propentofylline and other adenosine transport inhibitors increase the efflux of adenosine following electrical or metabolic stimulation of rat hippocampal slices. *Journal of Neurochemistry* **62,** 563-573.

FREUND, T.F. & ANTAL, M. (1988). GABA-containing neurons in the septum control inhibitory interneurons in the hippocampus. *Nature* **336**, 170-173.

FREUND, T.F. & BUZSAKI, G. (1996). Interneurons of the hippocampus. *Hippocampus* **6**, 347-470.

FREY, U., KRUG, M., BRÖDEMANN, R., REYMANN, K. & MATTHIES, H. (1989) Long-term

potentiation induced in dendrites separated from rat's CA1 pyramidal somata does not establish a late phase. *Neurosci. Lett.* **97**, 135-9.

FREY, U., MULLER, M. & KUHL, D.(1996). A different form of long-term potentiation revealed in tissue plasminogen activator mutant mice. *J Neurosci.* **16**, 2057-2063.

FRIEDRICH, G. & SORIANO, P. (1991). Promoter traps in embryonic stem cells: A genetic screen to identify and mutate developmental genes in mice. *Genes and Development* **5**, 1513-1523.

FROTSCHER, M. & LERANTH (1985). Cholinergic innervation of the rat hippocampus as revealed by choline acetyltransferase immunocytochemistry: a combined light and electron microscope study. *J. Comp. Neurol.* **239**, 237-246.

GAGE, F.H., BJORKLUND, A. & STENEVI, U. (1983). Reinnervation of the partially deafferented hippocampus by compensatory collateral sprouting from spared cholinergic and noradrenergic afferents. *Brain Res.* **268**, 27-37.

GAGE, F.H., BJORKLUND, A. & STENEVI, U. (1984). Cells of origin of the ventral cholinergic septohippocampal pathway undergoing compensatory collateral sprouting following fimbria-fornix transection. *Neurosci. Lett.* **44**, 211-216.

GÄHWILER, B.H. & BROWN, D.A. (1987). Muscarine affects calcium-currents in rat hippocampal pyramidal cells. *Neuroscience Letters* **76,** 301-306.

GÄHWILER, B.H. & DREIFUSS, J.J. (1982). Multiple actions of acetylcholine on hippocampal pyramidal cells in organotypic explant cultures. *Neuroscience* **7,** 1243-1256.

GAYKEMA, R.P.A., LUITEN, P.G.M., NYAKAS, C & TRABER, J. (1990). Cortical projection patterns of the medial septum-diagonal band complex. *J. Comp. Neurol.* **293**, 103-124.

GAYKEMA, R.P.A., VAN DER KUIL, J., HERSH, L.B. & LUITEN, P.G.M. (1991). Patterns of direct projections from the hippocampus to the medial septum-diagonal band complex: anterograde tracing with phasoleus vulgaris leucoagglutinin combined with immunochemistry of choline acetyltransferase. *Neuroscience* **43**, 349-360.

GIACHETTI, A., MICHELETTI, R. & MONTAGNA, E. (1986). Cardioselective profile of AF-DX-116, a muscarine M2 receptor antagonist. *Life Sciences* **38,** 1663-1672.

GINSBORG, B.L. & HIRST, G.D.S. (1972). The effect of adenosine on the release of the transmitter from the phrenic nerve of the rat. *Journal of Physiology* **224**, 629-645.

GOULD, E., WOOLF, N.J. & BUTCHER, L.L. (1991). Postnatal development of cholinergic neurons in the rat. I. Forebrain. *Brain Res. Bull.* **27**, 767-789.

GRANT, S.G., O'DELL, T.J., KARL, K.A., STEIN, P.L., SORIANO, P. & KANDEL, E.R. (1992). Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. *Science* **258**, 1903-1910.

GRAY, R., RAJAN, A.S., RADCLIFFE, K.A., YAKEHIRO, M. & DANI, J.A. (1997). Hippocampal synaptic transmission enhanced by low concentrations of nicotine. *Nature* **383,** 713-716.

GREENE, R.W. & HAAS, H.L. (1985). Adenosine actions on CA1 pyramidal neurones in rat hippocampal slices. *Journal of Physiology* **366**, 199-127.

GREENGARD, P., JEN, J., NAIRN, A.C. & STEVENS, C.F. (1991). Enhancement of the glutamate response by cAMP-dependent protein kinase in hippocampal neurons. *Science* **253**, 1135-8.

GRUDT, T.J. & WILLIAMS, J.T. (1993). kappa-Opioid receptors also increase potassium conductance. *Proc. Natl. Acad. Sci. U S A* **90**, 11429-32.

GUÉRINEAU, N.C., BOSSU, J.L., GÄHWILER, B.H., GERBER, U. (1995). Activation of a nonselective cationic conductance by metabotropic glutamatergic and muscarinic agonists in CA3 pyramidal neurones of the rat hippocampus. *Journal of Neuroscience* **15,** 4395-4407.

GUSTAFSON, E.L., SMITH K.E., DURKIN, M.M., GERALD, C. & BRANCHEK, T.A. (1996). Distribution of rat galanin receptor mRNA in rat brain. *NeuroReport* **7**, 953-957.

HAAS, H.L. & GREENE, R.W. (1984). Adenosine enhances afterhyperpolarisation and accommodation in hippocampal pyramidal cells. *Pflügers Archives* **16**, 218-222.

HAAS, H.L. (1982). Cholinergic disinhibition in hippocampal slices of the rat. *Brain Research* **233,** 200-204.

HAGBERG, H., ANDERSSON, P., LACAREWICZ, J., JACOBSON, I., BUTCHER, S. & SANDBERG, M. (1987). Extracellular adenosine, inosine, hypoxanthine, and xanthine in relation to tissue nucleosides and purines in rat striatum during transient ischemia. *Journal of Neurochemistry* **49,** 227-231.

HALL, Z.W. (1987). Three of a kind: the β-adrenergic receptor, the muscarinic receptor, and rhodopsin. *Trends in Neurosciences* **10,** 99-101.

HALLIWELL, J.V. & ADAMS, P.R. (1982). Voltage-clamp analysis of muscarinic excitation in hippocampal neurons. *Brain Research* **250,** 71-92.

HALLIWELL, J.V. (1990). Physiological mechanisms of cholinergic action in the hippocampus. *Progress in Brain Research* **84,** 255-272.

HAMMER, R., BERRIE, C.P., BIRDSHALL, N.J.M., BURGEN, A.S.V. & HULME, E.C. (1980). Pirenzipine distinguishes between different subclasses of muscarinic receptors. *Nature* **283,** 90-92.

HARLAN, J.E., HADJUK, P.J., YOON H.S. & FESIK, S.W. (1994). Pleckstrin homology domains bind to phosphatidylinositol-4, 5-bisphosphate. *Nature* **371**, 168-170.

HARMS, H.H., WARDEH, G. & MULDER, A.H. (1978). Adenosine modulates depolarization-induced release of ^{3}H-noradrenaline from slices of rat brain neocortex. *European Journal of Pharmacology* **49,** 305-308.

HARMS, H.H., WARDEH, G. & MULDER, A.H. (1979). Effects of adenosine on depolarization-induced release of various radiolabelled nerotransmitters from slices of rat corpus striatum. *Neuropharmacology* **18,** 577-580.

HARVEY, J. & COLLINGRIDGE, G.L. (1992). Thapsigargin blocks the induction of long-term potentiation in rat hippocampal slices. *Neurosci. Lett.* **139**, 197-200.

HASEGAWA, H., UTSUNOMIYA, Y. YASUKAWA, M., YANAGISAWA, K. & FUJITA, S. (1994). Induction of G protein-coupled peptide receptor EBI-1 by human herpesvirus 6 and 7 infection in CD4plus T cells. *J. Virol.* **68**, 5326-5329.

HASLAM, R.J., KOIDE, H.B. & HEMMINGS, B.A. (1993). Pleckstrin domain homology. *Nature* **363**, 309-310.

HEDLUND, P.B., YANAIHARA, N. & FUXE, K. (1992). Evidence for specific N-terminal galanin fragment binding sites in the rat brain. *Eur. J. Pharmacol.* **224**, 203-205.

HIGASHIDA, H., HASHII, M., FUKUDA, K., CAULFIELD, M.P., NUMA, S. & BROWN, D.A. (1990). Selective coupling of different muscarinic acetylcholine receptors to neuronal calcium currents in DNA-transfected cells. *Proceedings of the National Academy of Sciences* **242,** 68-74.

HIRAMATSU, M., MORI, H., MURASAWA, H. & KAMEYAMA, T. (1996). Improvement by dynorphin A (1-13) of galanin-induced impairment of memory accompanied by blockade of reductions in acetylcholine release in rats. *Br. J. Pharmacol.* **118**, 255-260.

HO, B.Y., HOLMES, B.B., ZHAO, J. & FUJIMOTO, J. (1997). Determination of delta-opioid in NG108-15 cells. *Eur. J. Pharmacol.* **319**, 109-14.

HOHMANN, C.F. & EBNER, F.F. (1985). Development of cholinergic markers in mouse forebrain. I. Choline acetyltransferase enzyme activity and acetylcholinesterase histochemistry. *Dev. Brain Res.* **23**, 225-241.

HÖKFELT, T., MILLHORN, D., SEROOGY, K., TSURUO, Y., CECCATELLI, S., LINDH, B., MEISTER, B., MELANDER, T., SCHALLING, M., BARTFAI, T. & TERENIUS, L. (1987). Coexistence of peptides with classical transmitters. *Experientia* **43**, 768-780.

HOLLINS, C. & STONE, T.W. (1980). Characteristics of the release of adenosine from slices of the rat cerebral cortex. *Journal of Physiology (London)* **303,** 73-82.

HORNBERGER, J.C., BUELL, S.J., FLOOD, D.G., McNEILL, T.H. & COLEMAN, P.D. (1985). Stability of numbers but not size of mouse forebrain cholinergic neurons to 53 months. *Neurobiol. Aging* **6**, 269-275.

HOUNSGAARD, J. (1978). Presynaptic inhibitory action of acetylcholine in area CA1 of the hippocampus. *Experimental Neurology* **62,** 787-797.

HOUSER, C.R., CRAWFORD, G.D., BARBER, R.P., SALVATERRA, P.M. & VAUGHN, J.E. (1983). Organisation and morphological characteristics of cholinergic neurons: an immunohistochemical study with a monoclonal antibody to choline acetyltransferase. *Brain Res.* **266**, 97-199.

HOWARD, A.D., TAN, C., SHIAO, L.L., PALYHA, O.C., MCKEE, K.K., WEINBERG, D.H., FEIGHNER, S.D., CASCIERI, M.A., SMITH, R.G., VAN DER PLOEG, L.H. & SULLIVAN, K.A. (1997). Molecular cloning and characterization of a new receptor for galanin. *FEBS Lett.* **405**, 285-90.

HUANG, Y.-Y., BACH, M.E., LIPP, H.-P., ZHUO, M., WOLFER, D.P., HAWKINS, R., SCHOONJANS, L., KANDEL, E.R., GODFRAIND, J.-M., MULLIGAN, R., COLLEN, D. & CARMELIET, D. (1996). Mice lacking the gene encoding tissue-type plasminogen activator show a selective interference with late-phase long-term potentiation in both Schaffer collateral and mossy fibre pathways. *Proc. Natl. Acad. Sci. USA* **93**, 8699-8704.

HUCHO, F., TSETLIIN, V.I. & MACHOLD, J. (1996). The emerging 3-dimensional structure of a receptor. The Nicotinic acetylcholine receptor. *European Journal of Pharmacology* **239,** 539-557.

HULME, E.C., BIRDSALL, N.J.M. & BUCKLEY, N.J. (1990). Muscarinic receptor subtypes. *Annual Review of Pharmacology and Toxicology* **30,** 633-73.

HUNT, R. & TAVEAU, R.D. (1906). On the physiological action of certain cholin derivatives and new methods for detecting cholin. *British Medical Journal* **II,** 1788-1791.

HYVÖNEN M., MACIAS, M.J., NILGES, M., OSCHKINAT, H., SARASTE, M. & WILMANNS, M. (1995). Structure of the binding site of inositol phosphates in a PH domain. *EMBO J* **14**, 4676-4685.

JACKISCH, R., GEPPERT, M., BRENNER, A.S. & ILLES, P. (1986). Presynaptic opioid receptors modulating acetylcholine release in the hippocampus of the rabbit. *Naunyn-Schmiedeberg's Arch. Pharmac.* **332**, 156-162.

JACKISCH, R., STRITTMATTER, H., KASAKOV, L. & HERTTING, G. (1984). Endogenous adenosine as a modulator of hippocampal acetylcholine release. *Naunyn-Schmiedeberg's Archives of Pharmacology* **327,** 319-325.

JAFFARD, R., GALEY, D., MICHEAU, J. & DURKIN, T. (1985). The cholinergic septo-hippocampal pathway in learning and memory. *Brain Plasticity, Learning and Memory* (Will, B.E., Schmitt, P. & Dalrymple-Alford, J.C., eds.) pp. 167-181 (*New York: Plenum*).

JAHR, C.E. & STEVENS, C.F. (1987). Glutamate activates multiple single channel conductances in hippocampal neurones. *Nature* **325**, 522-525.

JARVIS, M.F. & WILLIAMS, M (1989). Direct autoradiographic localization of adenosine A_2 receptors in the rat brain using the A_2-selective agonist, [^{3}H]CGS 21680. *European Journal of Pharmacology* **168,** 243-248.

JERUSALINSKY, D. & HARVEY, A.L. (1994). Toxins from mamba venoms: small proteins with selectivities for different subtypes of muscarinic acetylcholine receptors. *Trends Pharmacological Sciences* **15**, 424-430.

JIANG, Q., TAKEMORI, A.E., SULTANA, M., PORTOGHESE, P.S., BOWEN, W.D., MOSBERG, H.I. & PORRECA, F. (1991). Differential antagonism of opioid delta antinociception by [D-Ala2,Leu5,Cys6]enkephalin and naltrindole 5'-isothiocyanate: evidence for delta receptor subtypes. *J. Pharmacol. Exp. Ther.* **257**, 1069-75.

JOHARD, H.A., LUNDQUIST, C.T., ROKAEUS, A. & NASSEL, D.R. (1992). Autoradiographic localisation of ^{125}I-galanin binding sites in the blowfly brain. *Regul. Pept.* **42**, 123-134.

JOLKKONEN, M. (1994). A toxin from the green mamba Dendroaspis augusticeps: amino acid sequence and selectivity for muscarinic m4 receptors. *FEBS Lett.* **352**, 91

JONZON, B. & FREDHOLM, B.B. (1985). Release of purines, noradrenaline, and GABA from rat hippocampal slices by field stimulation. *Journal of Neurochemistry* **44,** 217-224.

JULIUS, D., SCHEKMAN, R. & THORNER, J. (1984). Glycosylation and processing of prepro-alpha-factor through the yeast secretory pathway. *Cell* **36**, 309-318.

KALKBRENNER, F., DEGTIAR, V.E., SCHENKER, M., BRENDEL, S., ZOBEL, A., HESCHLER, J., WITTIG, B. & SCHULTZ, G. (1995). Subunit composition of G(o) proteins functionally coupling galanin receptors to voltage-gated calcium channels. *EMBO J.* **14**, 4728-4737.

KALYUZHNY, A.E. & WESSENDORF, M.W. (1997). CNS GABA neurons express the μ-opioid receptor: immunocytochemical studies. *Neuroreport* **8**, 3367-3372.

KANDEL, E.R., SPENCER, W.A. & BRINDLEY, JR.F.J. (1961). Electrophysiology of hippocampal neurones. I. Sequential invasion and synaptic organization. *Journal of Neurophysiology* **24,** 225-242.

KASK, K., BERTHOLD, M. & BARTFAI, T. (1997). Galanin receptors: Involvement in feeding, pain, depression and Alzheimer's disease. *Life Sci.* **60**, 1523-1533.

KATZ, B. (1969). The release of neural transmitter substances. Liverpool: Liverpool UP.

KEAN, L.S., FULLER, R.S. & NICHOLS, J.W. (1993). Retrograde lipid traffic in yeast: Identification of two distinct pathways for internalisation of fluorescent-labeled phosphatidylcholine

from the plasma membrane. *J. Cell Biol.* **123**, 1403-1419.

KELSO, S.R., NELSON, T.E., & LEONARD, J.P. (1992). Protein kinase C-mediated enhancement of NMDA currents by metabotropic glutamate receptors in Xenopus oocytes. *J. Physiol. (Lond)* **449**, 705-18.

KIEFFER, B.L., BEFORT, K., GAVERIAUX RUFF, C. & HIRTH, C.G. (1992). The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. *Proc. Natl. Acad. Sci. U S A* **89**, 12048-52.

KIMURA, M., YAMANISHI, Y., HANADA, T., KAGAYA, T., KUWADA, M., WATANABE, T., KATAYAMA, K., NISHIZAWA, Y. (1995). Involvement of P-type calcium channels in high potassium-elicited release of neurotransmitters from rat brain slices. *Neuroscience* **66**, 609-615.

KNOWLES, W.D. & SCHWARTZKROIN, P.A. (1981). Local circuit synaptic interactions in hippocampal brain slices. *Journal of Neuroscience* **1,** 318-322.

KOCH, T., SCHULZ, S., SCHRÖDER, H., WOLF, R., RAULF, E. & HÖLLT, V. (1998). Carboxyl-terminal splicing of the rat mu opioid receptor modulates agonist-mediated internalization and receptor resensitization. *J. Biol. Chem.* **273**, 13652-7.

KOHLER, C., CHAN-PALAY, V. & WU, J.Y. (1984). Septal neurons containing glutamic acid decarboxylase immunoreactivity project the hippocampal region in the rat. *Anat. Embryol.* **169**, 41-44.

KONOPACKI, J., MACIVER, M.B., BLAND, B.H. & ROTH, S.H. (1987). Carbachol-induced 'theta' activity in hippocampal brain slices. *Brain Research* **405,** 196-198.

KORNISIUK, E., JERUSALINSKY, D., CERVENANSKY, C. & HARVEY, A.L. (1995). Binding of muscarinic toxins MTx1 and MTx2 from the venom of the green mamba Dendroaspis augusticeps to cloned human muscarinic cholinoceptors. *Toxicon* **33**, 11-8.

KOSTERLITZ, H.W. & PATERSON, S.J. (1985). Types of opioid receptors: relation to antinociception. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **308**, 291-7.

KRNJEVIC, K. (1993). Central cholinergic mechanisms and function. *Progress in Brain Research* **98,** 285-292.

KRUG, M., LÖSSNER, B. & OTT, T. (1984). Anisomycin blocks the late phase of long-term potentiation in the dentate gyrus of freely moving rats. *Brain Res. Bull.* **13**, 39-42.

KRUH, G.D., KING, C.R., KRAUS, M.H., POPESCU, N.C., AMSBAUGH, S.C., McBRIDE, W.O. & AARONSON, S.A. (1986). A novel human gene closely related to the abl proto-oncogene. *Science* **234**, 1545-1548.

KRUH, G.D., PEREGO, R., MIKI, T. & AARONSON, S.O. (1990). The complete coding sequence of arg defines the Abelson subfamily of cytoplasmic tyrosine kinases. *Proc. Natl. Acad. Sci. USA* **87**, 5802-5806.

KRYSTOSEK, A. & SEEDS, N.W. (1981a). Plasminogen activator release at the neuronal growth cone. *Science* **213**, 1532-1533.

KRYSTOSEK, A. & SEEDS, N.W. (1981b). Plasminogen activator secretion by granule neurons in cultures of developing cerebellum. *Proc. Natl. Acad. Sci. USA* **78**, 7810-7814.

KUBO, T., FUKUDA, K., MIKAMI, A. MAEDA, A., TAKAHASHI, H., MISHINA, M., HAGA, T., HAGA, K., ICHIYAMA, A., KANGAWA, K., KOJIMA, M., MATSUO, H., HIROSE, T. & NUMA, S. (1986). Cloning, sequencing and expression of complimentary DNA encoding the muscarinic

acetylcholine receptor. *Nature* **323,** 411-416.

KUHAR, M.J. & MURRIN, L.C. (1978). Sodium-dependent, high affinity choline uptake. *J. Neurochem.* **30**, 15-21.

KUHAR, M.J., SETHY, V.H., ROTH, R.H. & AGHAJANIAN, G.K. (1973). Choline: selective accumulation by central cholinergic neurons. *J. Neurochem.* **20**, 581-593.

LAI, J., MA, S.W., ZHU, R.H., ROTHMAN, R.B., LENTES, K.U. & PORRECA, F. (1994). Pharmacological characterization of the cloned kappa opioid receptor as a kappa 1b subtype. *Neuroreport* **5**, 2161-4.

LAMBERT, N.A., HARRISON, N.L., KERR, D.I.B., ONG, J., PRAGER, R.H. & TEYLER, T.J. (1989). Blockade of the late IPSP in rat CA1 hippocampal neurons by 2-hydroxy-saclofen. *Neuroscience Letters* **107,** 125-128.

LANCASTER, B. & ADAMS, P.R. (1986). Calcium-dependent current generating the afterhyperpolarization of hippocampal neurons. *Journal of Neurophysiology* **55,** 1268-1282.

LAPCHAK, P.A., ARAUJO, D.M. & COLLIER, B. (1989). Regulation of endogenous acetylcholine release from mammalian brain slices by opiate receptors: hippocampus, striatum and cerebral cortex of guinea-pig and rat. *Neuroscience* **31**, 313-325.

LAPCHAK, P.A., JENDEN, D.J. & HEFTI, F. (1991). Compensatory elevation of acetylcholine synthesis in vivo by cholinergic neurons surviving partial lesions of the septohippocampal pathway. *J. Neurosci.* **11**, 2821-2828.

LARSON, J., WONG, D. & LYNCH, G. (1986). Patterned stimulation at the theta frequency is optimal for the induction of long-term potentiation. *Brain Research* **368**, 347-350.

LEE, H.K., DUNWIDDIE, T.V. & HOFFER, B.J. (1980). Electrophysiological interactions of enkephalins with neuronal circuitry in the rat hippocampus. II. Effects on interneuron excitability. *Brain Res.* **184**, 331-342.

LEE, K.S., SCHUBERT, P. & HEINEMANN, U. (1984). The anticonvulsive action of adenosine: a postsynaptic, dendritic action by a possible endogenous anticonvulsant. *Brain Research* **321,** 160-164.

LERANTH, C. & FROTSCHER, M. (1987). Cholinergic innervation of hippocampal GAD- and somatostatin-immunoreactive commissural neurons. *J. Comp. Neurol.* **261**, 33-47.

LEVEY, A., KONG, R.M. & LIEBERMAN, H.R. (1991). Nimodipine improves spatial working memory and elevates hippocampal acetylcholine in young rats. *Pharmacol. Biochem. Behav.* **39**, 781-786.

LEVEY, A.I., EDMUNDS, S.M., HEILMAN, C.J., DESMOND, T.J. & FREY, K.A. (1994). Localization of muscarinic M_3 receptor binding in the rat brain. *Neuroscience* **63,** 207-221.

LEVEY, A.I., EDMUNDS, S.M., KOLIATSOS, V., WILEY, R.G. & HEILMAN, C.J. (1995). Expression of m1-m4 muscarinic acetylcholine receptor proteins in rat hippocampus and regulation by cholinergic innervation. *Journal of Neuroscience* **15,** 4077-4092.

LINDSTROM, J., ANAND, R., GERZANICH, V., PENG, X., WANG, F. & WELLS, G. (1995). Structure and function of neuronal nicotinic acetylcholine receptors. *Progress in Brain Research* **109,** 125-137.

LIPPA, A.S., CRITCHETT, D.J., EHLERT, F., YAMAMURA, H.I., ENNA, S.J. & BARTUS, R.T. (1981). Age-related alterations in neurotransmitter receptors: an electrophysiological and biochemical

analysis. *Neurobiol. Aging* **2**, 3-8.

LIU, L. & POHAJDAK, B. (1992). Cloning and sequencing of a human cDNA from cytolytic NK/T cells with homology to yeast SEC7. *Biochim. Biophys. Acta.* **1132**, 75-78.

LLINAS, R., SUGIMORI, M., LIN, J.W. & CHERKSEY, B. (1989). Blocking and isolation of a calcium channel from neurons in mammals and cephalopods utilising a toxin fraction (FTX) from funnel-web spider venom. *Proc. Natl. Acad. Sci. USA* **86**, 1689-1693.

LOEWI, O. & NAVRATIL, E. (1926). Über humorale übertragbarkeit der herznervenwirkung. XI. Mitteilung. Über der mechanismus der vaguswirkung von physiostigmin und ergotamin. *Arch. Ges. Physiol.*, **214,** 689-696.

LOHSE, M.J., KLOTZ, K.-N., LINDENBORN-FOTINOS, J., REDDINGTON, M., SCHWABE, U. & OLSSON, R.A. (1987). 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) – a selective high affinity antagonist radioligand for A_1 adenosine receptors. *Naunyn-Schmiedeberg's Archives of Pharmacology* **336,** 204-210.

LONDOS, C., COOPER, D.M.F. & WOLFF, T. (1980). Subclasses of external adenosine receptors. *Proceedings of the National Academy of Sciences (USA)* **77,** 2551-2554.

LORD, J.A., WATERFIELD, A.A., HUGHES, J. & KOSTERLITZ, H.W. (1977). Endogenous opioid peptides: multiple agonists and receptors. *Nature* **267**, 495-9.

LUEBKE, J.I., DUNLAP, K., TURNER, T.J. (1993). Multiple calcium channel types control glutamatergic synaptic transmission in the hippocampus. *Neuron* **11**, 895-902.

LUINE, V.N., RENNER, K.J., HEADY, S. & JONES, K.J. (1986). Age and sex-dependent decreases in ChAT in basal forebrain nuclei. *Neurobiol. Aging* **7**, 193-198.

LUPICA, C.R. & DUNWIDDIE, T.V. (1991). Differential effects of μ- and δ-receptor selective opioid agonists on feedforward and feedback GABAergic inhibition in hippocampal brain slices. *Synapse* **8**, 237-248.

LYNCH, G., KESSLER, M., HALPAIN, S. & BAUDRY, M. (1983). Biochemical effects of high-frequency synaptic activity studied with in vitro slices. *Fed Proc* **42**, 2886-90.

MACDONALD, R.L., SKERRITT, J.H. & WERZ, M.A. (1986). Adenosine agonists reduce voltage-dependent calcium conductance of mouse sensory neurons in cell culture. *Journal of Physiology* **370,** 75-90.

MACKLER, S.A., BROOKS, B.P. & EBERWINE, J.H. (1992). Stimulus-induced coordinate changes in mRNA abundance in single postsynaptic hippocampal CA1 neurons. *Neuron* **9**, 539-48.

MACVICAR, B.A. & TSE, F.W.Y. (1989). Local neuronal circuitry underlying cholinergic rhythmical slow activity in CA3 area of rat hippocampal slices. *Journal of Physiology (London)* **417,** 197-212.

MADISON D.V., LANCASTER, B. & NICOLL, R.A. (1987). Voltage-clamp analysis of cholinergic action in the hippocampus. *Journal of Neuroscience* **7**, 733-741.

MADISON, D.V. & NICOLL, R.A. (1984). Control of the repetitive discharge of rat CA1 pyramidal neurones *in vitro*. *Journal of Physiology (London)* **354,** 319-331.

MADISON, D.V. & NICOLL, R.A. (1988). Enkephalin hyperpolarises interneurones in the rat hippocampus. *J. Physiol. (LOND)* **398**, 123-130.

MAGER, R., FERRONI,S. & SCHUBERT, P. (1990). Adenosine modulates a voltage-dependent chloride conductance in cultured hippocampal neurones. *Brain Research* **532,** 58-62.

MALENKA, R.C., KAUER, J.A., PERKEL, D.J., MAUK, MD.D, KELLY, P.T., NICOLL, R.A. & WAXHAM, M.N. (1989). An essential role for postsynaptic camodulin and protein kinase activity in long-term potentiation. *Nature* **340**, 554-557.

MALENKA, R.C., LANCASTER, B. & ZUCKER, R.S. (1992). Temporal limits on the rise in postsynaptic calcium required for the induction of long-term potentiation. *Neuron* **9**, 121-128.

MALIN, D.H., NOVY, B.J., LETT-BROWN, A.E., PLOTNER, R.E., MAY, B.T., RADULESCU, S.J., CROTHERS, M.K., OSGOOD, L.D. & LAKE, J.R. (1992). Galanin attenuates retention of one-trial reward learning. *Life Sci.* **50**, 939-944.

MALINOW, R., SCHULMAN, H. & TSIEN, R.W. (1989). Inhibition of postsynaptic PKC or CAMKII blocks induction but not expression of LTP. *Science* **245**, 862-866.

MANSOUR, A., FOX, C.A., BURKE, S., MENG, F., THOMPSON, R.C., AKIL, H. & WATSON, S.J. (1994). Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: An in situ hybridisation study. *J. Comp. Neurol.* **350**, 412-438.

MARIGHETTO, A., DURKIN, T., TOUMANE, A., LEBRUN, C. & JAFFARD, R. (1989). Septal alpha-noradrenergic antagonism in vivo blocks the testing-induced activation of septo-hippocampal cholinergic neurones and produces a concomitant deficit in working memory performance of mice. *Pharmacol. Biochem. Behav.* **34**, 553-558.

MARTIN, W., BELL, J., GILBERT, P., SLOAN, J. & THOMPSON J (1976). The effects of naltrexone in the chronic spinal dog and acute spinal cat possible interaction with naturally-occurring morphine-like agonists. *NIDA Res Monogr*, **9**, 27-30.

MASTROPAOLO, J., NADI, N., OSTROWSKI, N.L. & CRAWLEY, J.N. (1988). Galanin antagonises acetylcholine on a memory task in basal forebrain-lesioned rats. *Proc. Natl. Acad. Sci. USA* **85**, 9841-9845.

MATHIE, A., BERNHEIM, L. & HILLE, B. (1992). Inhibition of N- and L-type calcium channels by muscarinic receptor activation in rat sympathetic neurons. *Neuron* **8,** 907-914.

MATTHIES, H., RAUCA, C. & LIEBMANN, H. (1974). Changes in the acetylcholine content of different brain regions of the rat during a learning experiment. *J. Neurochem.* **23**, 1109-1113.

MATTIA, A., VANDERAH, T., MOSBERG, H.I. & PORRECA, F. (1991). Lack of antinociceptive cross-tolerance between [D-Pen2, D-Pen5]enkephalin and [D-Ala2]deltorphin II in mice: evidence for delta receptor subtypes. *J. Pharmacol. Exp. Ther.* **258**, 583-7.

MAY, T., JUILFS, F. & WOLFFGRAMM, J. (1999) Effects of etonitazene consumption and abstinence on the signal transmission of mu-opioid receptors in brain membranes of rats. *Neurosci Lett* **275**, 109-12.

McCARTHY, M.P., EARNEST, J.P., YOUNG, E.F., CHOE, S. & STROUD, R.M. (1986). The molecular neurobiology of the acetylcholine receptor. *Annual Review of Neuroscience* **9,** 383-413.

McCLESKEY, E.W., FOX, A.P., FELDMAN, D.H., CRUZ, L.J., OLIVERA, B.M., TSIEN, R.W. & YOSHIKAMI, D. (1987). Omega-conotoxin: direct and persistent blockade of specific types of calcium channels in neurons but not muscle. *Proc. Natl. Acad. Sci. (USA)* **84**, 4327-4331.

McDONALD, M.P. & CRAWLEY, J.N. (1996). Galanin receptor antagonist M40 blocks galanin-induced choice accuracy deficits on a delayed nonmatching to position task. *Behav. Neurosci.* **110**,

1025-1032.

McGEHEE, D.S. & ROLE, L.W. (1995). Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Annual Review of Physiology* **57,** 521-546.

McGUIRE, P.G. & SEEDS, N.W. (1990). Degradation of underlying extracellular matrix by sensory neurons during neurite outgrowth. *Neuron* **14**, 633-642.

MCLATCHIE, L.M., FRASER, N.J., MAIN, M.J., WISE, A., BROWN, J., THOMPSON, N., SOLARI, R., LEE, M.G. & FOORD, S.M. (1998). RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* **398**, 333-9.

MCNAUGHTON, B.L. (1982). Long-term synaptic enhancement and short-term potentiation in rat fascia dentata act through different mechanisms. *J. Physiol. (Lond)* **324**, 249-62.

MELANDER, T., STAINES, W.A. & ROKAEUS, A. (1986). Galanin-like immunoreactivity in hippocampal afferents in the rat, with special reference for cholinergic and noradrenergic inputs. *Neuroscience* **19**, 223-240.

MELANDER, T., STAINES, W.A., HOKFELT, ROKAEUS, A., ECKENSTEIN, F., SALVATERRA, P.M. & WAINER, B.H. (1985). Galanin-like immunoreactivity in cholinergic neurons of the septum-basal forbrain complex projecting to the hippocampus of the rat. *Brain Res.* **360**, 130-138.

MESULAM, M.M, MUFSON, E.J., WAINER, B.H. & LEVEY, A.I. (1983). Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1-Ch6). *Neuroscience* **10**, 1185-1201.

MEUNIER, J.C., MOLLEREAU, C., TOLL, L., SUAUDEAU, C., MOISAND, C., ALVINERIE, P., BUTOUR, J.L., GUILLEMOT, J.C., FERRARA, P., MONSARRAT, B., ET AL (1995). Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* **377**, 532-5.

MILLER, M.A., KOLB, P.E., PLANAS, B. & RASKIND, M.A. (1998). Few cholinergic neurons in the rat basal forebrain coexpress galanin mRNA. *J. Comp. Neurol.* **391**, 248-258.

MINAMI, M., TOYA, T., KATAO, Y., MAEKAWA, K., NAKAMURA, S., ONOGI, T.,KANEKO, S. & SATOH, M. (1993). Cloning and expression of a cDNA for the rat kappa-opioid receptor. *FEBS Lett.* **329**, 291-5.

MINTZ, I., SABATINI, B. & REGEHR, W. (1995). Calcium control of transmitter release at a cerebellar synapse. *Neuron* **15**, 675-688.

MINTZ, I.M., ADAMS, M.E. & BEAN, B.P. (1992b). P-type calcium channels in rat central and peripheral neurons. *Neuron* **9**, 85-95.

MINTZ, I.M., VENEMA, V.J., SWIDEREK, K.M., LEE, T.D., BEAN, B.P. & ADAMS, M.E.(1992a). P-type calcium channels blocked by the spider toxin omega-Aga-IVA *Nature* **355**, 827-829.

MISGELD, U., CALABRESI, P. & DODT, H.U. (1986). Muscarinic modulation of calcium dependent plateau potentials in rat neostriatal neurons. *Pflügers Archives* **407,** 482-487.

MITCHELL, J.B., LUPICA, C.R. & DUNWIDDIE, T.V. (1993). Activity dependent release of endogenous adenosine modulates synaptic responses in the rat hippocampus. *Journal of Neuroscience* **13**, 3439-3447.

MITCHELSON, F. (1988). Muscarinic receptor differentiation. *Pharmacology & Therapeutics* **37,**

357-423.

MOGUL, D.J., ADAMS, M.E. & FOX, A.P. (1993). Differential activation of adenosine receptors decreases N-type but potentiates P-type Ca^{2+} current in hippocampal CA3 neurons. *Neuron* **10,** 327-334.

MOISES, H.C., RUSIN, K.I. & MACDONALD, R.L. (1994). μ-opioid receptor-mediated reduction of neuronal calcium current occurs via a G_o-type GTP-binding protein. *J. Neurosci.* **14**, 3842-3851.

MOLLEREAU, C., PARMENTIER, M., MAILLEUX, P., BUTOUR, J.L., MOISAND, C., CHALON, P., CAPUT, D., VASSART, G. & MEUNIER, J.C. (1994) ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. *FEBS Lett.* **341**, 33-8.

MOON, I.S., APPERSON, M.L. & KENNEDY, M.B. (1994). The major tyrosine-phosphorylated protein in the post-synaptic density fraction is N-methyl-D-aspartate receptor subunit 2B. *Proc. Natl. Acad. Sci. USA* **91**, 3954-3958.

MORONI, F., MALTHE-SORENSSEN, D., CHENEY, D.L. & COSTA, E. (1978). Modulation of ACh turnover in the septal-hippocampal pathway by electrical stimulation and lesioning. *Brain Res.* **150**, 333-341.

MORTON R.A. & DAVIES C.H. (1997). Regulation of muscarinic acetylcholine receptor-mediated synaptic responses by adenosine receptors in the rat hippocampus. *Journal of Physiology (London)* **502.1**, 75-90.

MULDER, A.H., WARDEH, G., HOGENBOOM, F. & FRANKHUYZEN, A.L. (1984). κ- and δ-opioid receptor agonists differentially inhibit striatal dopamine and acetylcholine release. *Nature* **308**, 278-280.

MULLER, D., BUCHS, P.A., STOPPINI, L. & BODDEKE, H. (1991). Long-term potentiation, protein kinase C, and glutamate receptors. *Mol. Neurobiol.* **5**, 277-88.

MULVANEY, J.M. & PARSONS, R.L. (1995). Arachidonic acid may mediate the galanin-induced hyperpolarisation in parasympathetic neurons from *Necturus maculosus. Neurosci. Lett.* **187**, 95-98.

MYNLIEFF, M. & BEAM, K.G. (1994). Adenosine acting at an A_1 receptor decreases N-type calcium current in mouse motoneurons. *Journal of Neuroscience* **14,** 3628-3634.

NAKAJIMA, Y., NAKAJIMA, S., LEONARD, R.J. & YAMAGUCHI, K. (1986). Acetylcholine raises excitability by inhibiting the fast transient potassium current in cultured hippocampal neurons. *Proceedings of the National Academy of Sciences, USA* **83,** 3022-3026.

NARITA, M., MIZOGUCHI, H., NARITA, M., SORA, I., UHL, G.R. & TSENG, L.F. (1999). Absence of G-protein activation by mu-opioid receptor agonists in the spinal cord of mu-opioid receptor knockout mice. *Br J Pharmacol* **126**, 451-6.

NATHAN, T., JENSEEN, M.S. & LAMBERT, J.D.C. (1990). GABAB receptors play a major role in paired-pulse facilitation in area CA1 of the rat hippocampus. *Brain Res.* **531**, 55-65.

NEARY, D., SNOWDEN, J.S., MANN, D.M., BOWEN, D.M., SIMS, N.R., NORTHEN, B., YATES, P.O. & DAVISON, A.N. (1986). Alzheimer's disease: a correlative study. *J. Neurol. Neurosurg. Psychiatry* **49**, 229-37.

NEURATH, H. (1989). Proteolytic processing and physiological regulation. *Trends Biochem. Sci.* **14**, 268-270.

NEWBERRY, N.R. & NICOLL, R.A. (1984a). A bicuculline-resistant inhibitory postsynaptic

potential in rat hippocampal pyramidal cells in vitro. *Journal of Physiology (London)* **348,** 239-254.

NEWBERRY, N.R. & NICOLL, R.A. (1984b). Direct hyperpolarizing action of baclofen on hippocampal pyramidal cells. *Nature* **308,** 450-452.

NEWBY, A.C. (1984). Adenosine and the concept of 'retaliatory metabolites'. *Trends in Biochemichal Sciences* **9,** 42-44.

NI, Q., XU, H., PARTILLA, J.S., DE COSTA, B.R., RICE, K.C., KAYAKIRI, H. & ROTHMAN, R.B. (1993). Opioid peptide receptor studies. 3. Interaction of opioid peptides and other drugs with four subtypes of the kappa 2 receptor in guinea pig brain. *Peptides* **16**, 1083-95.

NICOLL, R.A. & ALGER, B.E. (1981). Synaptic excitation may activate a calcium-dependent K^+ conductance in hippocampal pyramidal cells. *Science* **212,** 957-959.

NICOLL, R.A. (1985). The septo-hippocampal projection: a model cholinergic pathway. *Trends in Neurosciences* **8,** 533-534.

NIKOLAEV, E., TISCHMEYER, W., KRUG, M., MATTHIES, H. & KACZMAREK, L. (1991). c-fos protooncogene expression in rat hippocampus and entorhinal cortex following tetanic stimulation of the perforant path. *Brain Res.* **560**, 346-9.

NILSSON, O.G., LEANZA, G., ROSENBLAD, C., LAPPI, D.A., WILEY, R.G. & BJORKLUND, A. (1992). Spatial learning impairments in rats with selective immunolesion of the forebrain cholinergic system. *Neuroreport* **3**, 1005-1008.

NOWYCKY, M.B., FOX, A.P. & TSIEN, R.W. (1985). Three types of neuronal Ca^{2+} channel with different calcium agonist sensitivity. *Nature* **316**, 440-446.

NYAKAS, C., LUITEN, P.G.M., SPENCER, D.G. & TRABER, J. (1987). Detailed projections patterns of septal and diagonal band efferents to the hippocampus in the rat with emphasis on innervation of CA1 and dentate gyrus. *Brain Res. Bull.* **18**, 533-545.

O'DELL, T.J., HAWKINS, R.D., KANDEL, E.R. & ARANCIO, O. (1991a). Tests of the roles of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger. *Proc. Natl. Acad. Sci. USA* **88**, 11285-9.

O'DELL, T.J., KANDEL, E.R. & GRANT, S.G. (1991b). Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. *Nature* **353**, 558-60.

O'KEEFE, J. & DOSTROVSKY, J. (1971). The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Res.* **34**, 171-5.

OBENAUS, A., MODY, I. & BAIMBRIDGE, K.G. (1989). Dantrolene-Na (Dantrium) blocks induction of long-term potentiation in hippocampal slices. *Neurosci. Lett.* **98**, 172-8.

ÖGREN, S.O., HÖKFELT, T., KASK, K., LANGEL, Ü. & BARTFAI, T. (1992). Evidence for a role of the neuropeptide galanin in spatial learning. *Neuroscience* **51**, 1-5.

OKADA, Y. & OZAWA, S. (1980). Inhibitory action of adenosine on synaptic transmission in the hippocampus of the guinea pig in vitro. *European Journal of Pharmacology* **68,** 483-492.

OLAH, M.E. & STILES, G.L. (1992). Adenosine receptors. *Annual Review of Pharmacology and Toxicology* **54,** 211-225.

OLPE, H.R., STEINMANN, M.W., FERRAT, T., POZZA, M.F., GREINER, K., BRUGGER, D., FROESTL, W., MICKEL, S.J. & BITTIGER, H. (1993). The actions of orally active GABAB

receptor antagonists on GABAergic transmission in vivo and in vitro. **233**, 179-186.

OLTON, D.S., WALKER, J.A. & GAGE, F.H. (1978). Hippocampal connections and spatial discrimination. *Brain Res.* **139**, 295-308.

ONGINI, E. & FREDHOLM, B.B. (1996). Pharmacology of adenosine A_{2A} receptors. *Trends in Pharmacological Sciences* **17,** 364-372.

OTANI, S. & ABRAHAM, W.C. (1989). Inhibition of protein synthesis in the dentate gyrus, but not the entorhinal cortex, blocks maintenance of long-term potentiation in rats. *Neurosci. Lett.* **106**, 175-80.

OTTO, T., EICHENBAUM, H., WIENER, S.I. & WIBLE, C.G. (1991). Learning-related patterns of CA1 spike trains parallel stimulation parameters optimal for inducing hippocampal long-term potentiation. *Hippocampus* **1**, 181-192.

PAK, M.A., HAAS, H.L., DECKING, U.K.M. & SCHRADER, J. (1994). Inhibition of adenosine kinase increases endogenous adenosine and depresses neuronal activity in hippocampal slices. *Neuropharmacology* **33**, 1049-1053.

PALAZZI, E., FELINSKA, S., ZAMBELLI, M., FISONE, G., BARTFAI, T. & CONSOLO, S. (1991). Galanin reduces carbachol stimulation of phosphoinositide turnover in rat ventral hippocampus by lowering Ca2+ influx through voltage-sensitive Ca2+ channels. *J. Neurochem.* **56**, 739-747.

PALAZZI, E., FISONE, T., HÖKFELT, T., BARTFAI, T. & CONSOLO, S. (1988). Galanin inhibits the muscarinic stimulation of phosphoinositide turnover in rat ventral hippocampus. *Eur. J. Pharmacol.* **148**, 479-480.

PARKER, E.M., IZZARELLI, D.G., NOWAK, H.P., MAHLE, C.D., IBEN, L.G., WANG, J. & GOLDSTEIN M.E. (1995). Cloning and characterisation of the rat GALR1 galanin receptor from Rin14B insulinoma cells. *Brain Res. Mol. Brain Res.* **34**, 179-189.

PAUL, D., PICK, C.G., TIVE, L.A. & PASTERNAK, G.W. (1991). Pharmacological characterization of nalorphine, a kappa 3 analgesic. *J. Pharmacol. Exp. Ther.* **257**, 1-7.

PENNEFATHER, P.B., LANCASTER,B., ADAMS, P.R. & NICOLL, R.A. (1985). Two distinct Ca-dependent K currents in bullfrog sympathetic ganglion cells. *Proceedings of the National Academy of Sciences (USA)* **82,** 3040-3044.

PEREGO, R., RON, D. & KRUH, G.D. (1991). Arg encodes a widely expressed 145 kDa protein-tyrosine kinase. *Oncogene* **6**, 1899-1902.

PERRY, E.K. PERRY, R.H., BLESSED, G. & TOMLINSON, B.E. (1978). Changes in brain cholinesterases in senile dementia of Alzheimer type. *Neuropathol. Appl. Neurobiol.* **4**, 273-7.

PHILIPSON, L.H., KUZNETSOV, A., TOTH, P.T., MURPHY, J.F., SZABO, G., MA, G.H. & MILLER, R.J. (1995). Functional expression of an epitope-tagged G-protein-coupled K+ channel (GIRK1). *J. Biol. Chem.* **270**, 14604-14610.

PHILLIS, J.W., EDSTROM, J.P., KOSTOPOULOS, G.K. & KIRKPATRICK, J.R. (1979). Effects of adenosine and adenine nucleotides on synaptic transmission in the cerebral cortex. *Canadian Journal of Physiology and Pharmacology* **57,** 1289-1312.

PITLER, T.A. & ALGER, B.E. (1990). Activation of a pharmacologically defined M_3 muscarinic acetylcholine receptor depolarizes hippocampal pyramidal cells. *Brain Research* **534**, 257-262.

PITTMAN, R.N. (1985). Release of plasminogen activator and a calcium-dependent metalloprotease from cultured sympathetic and sensory neurons. *Dev. Biol.* **110**, 91-101.

PLUMMER, M.R. & HESS, P. (1991). Reversible uncoupling of inactivation in N-type calcium channels. *Nature* **351**, 657-659.

PLUMMER, M.R., RITTENHOUSE, A., KANEVSKY, M. & HESS, P. (1991). Neurotransmitter modulation of calcium channels in rat sympathetic neurons. *The Journal of Neuroscience* **11,** 2339-2348.

PONCER, J-C., McKINNEY, R.A., GAHWILER, B.H. & THOMPSON, S.M. (1997). Either N- or P-type calcium channels mediate GABA release at distinct hippocampal inhibitory synapses. *Neuron*, 18, 463-472.

POTEMSKA, A., GRADKOWSKA, M. & ODERFELD-NOWAK, B. (1975). Early changes in acetylcholine pools in the hippocampus of the rat brain after septal lesions. *J. Neurochem.* **24**, 787-789.

POTIER, B., LAMOUR, Y. & DUTAR, P. (1993). Age-related alterations in the properties of hippocampal pyramidal neurons among rat strains. *Neurobiol. Aging* **14**, 17-25.

POTIER, B., RASCOL, O., JAZAT, F., LAMOUR, Y. & DUTAR, P. (1992). Alterations in the properties of hippocampal pyramidal neurons in the aged rat. *Neuroscience* **48**, 793-806.

POUCET, B. & HERRMANN, T. (1990). Septum and medial frontal cortex contribution to spatial problem-solving. *Behav. Brain Res.* **37**, 269-280.

POUCHER, S.M., KEDDIE, P., SINGH, P., STOGGALL, S.M., CAULKETT,, P.W.R., JONES, G. & COLLIS, M.G. (1995). The *in vitro* pharmacology of ZM 241385, a potent, non-xanthine, A_{2a} selective adenosine receptor antagonist. *British Journal of Pharmacology* **115,** 1096-1102.

PROBST, W.C., SNYDER, L.A., SCHUSTER, D.I., BROSIUS, J. & SEALFON, S.C. (1992). Sequence alignment of the G-protein coupled receptor superfamily. *DNA Cell Biol.* **b**, 1-20.

PROCTOR, W.R. & DUNWIDDIE, T.V. (1983). Adenosine inhibits calcium spikes in hippocampal pyramidal neurones in vitro. *Neuroscience Letters* **35,** 197-201.

QIAN, J. & SAGGAU, P. (1997). Presynaptic inhibition of synaptic transmission in the rat hippocampus by activation of muscarinic receptors: involvement of presynaptic calcium influx. *British Journal of Pharmacology* **122,** 511-519.

QIAN, J., WU, L.G. & SAGGAU, P. (1995). Presynaptic calcium influx is modulated during muscarinic receptor-induced presynaptic inhibition and LTP at CA3-CA1 synapses in the guinea pig hippocampus. *Soc. Neurosci. Abstr.* **21**, 1096.

QIAN, Z., GILBERT, M.E., COLICOS, M.A., KANDEL, E.R. & KUHL, D. (1993). Tissue-plasminogen activator is induced as an immediate-early gene during seizure, kindling and long-term potentiation. *Nature* **361**, 453-457.

RAMON Y CAJAL, S. (1891). Sur la structure de l'ecorce cerebrale de quelque mammiferes. *La Cellule* **7,** 125-176.

RAUCA, C., KAMMERER, E. & MATTHIES, H. (1980). Choline uptake and permanent memory storage. *Pharmacol. Biochem. Behav.* **13**, 21-25.

REGAN, L. (1991). Voltage-dependent Ca^{2+} currents in purkinje cells from rat cerebellar vermis. *J. Neurosci.* **11**, 2259-2269.

REGAN, L.J., SAH, D.W.Y. & BEAN, B.P. (1991). Ca^{2+} channels in rat central and peripheral neurons: high threshold current resistant to dihydropyridine blockers and omega-conotoxin. *Neuron* **6**, 269-280.

REGEHR, W.G. & TANK, D.W. (1990). Postsynaptic NMDA receptor-mediated calcium accumulation in hippocampal CA1 pyramidal cell dendrites. *Nature* **345**, 807-10.

REID, C.A., CLEMENTS, J.D. & BEKKERS, J.M. (1997). Non-uniform distribution of Ca^{2+} channel subtypes on presynaptic terminals of excitatory synapses in hippocampal cultures. *J. Neurosci.* **17**, 2738-2745.

REUTER, H. (1995). Measurements of exocytosis from single presynaptic nerve terminals reveal heterogeneous inhibition by Ca^{2+}-channel blockers. *Neuron* **14**, 773-779.

RICHTER. J.A., PERRY, E.K. & TOMLINSON, B.E. (1980). Decreased acetylcholine content in Alzheimer's disease. Life Sci. 26, 1683-1689.

RIGLER, R. et al. (1991). Galanin: A new multifunctional peptide in the neuroendocrine system (HÖKFELT, T., BARTFAI, T. , JACOBOWITZ, D.M & OTTOSON, D., eds) pp. 17-25 Macmillan Press.

ROBINSON, J.K. & CRAWLEY, J.N. (1994). Analysis of anatomical sites at which galanin impairs delayed nonmatching to sample. *Behav. Neurosci.* **108**, 941-950.

ROKAEUS, A. & BROWNSTEIN, M.J. (1986). Construction of a porcine adrenal medullary cDNA library and nucleotide sequence analysis of two clones encoding a galanin receptor. *Proc. Natl. Acad. Sci. USA* **83**, 6287-6291.

ROSENBLUM, K., DUDAI, Y. & RICHTER-LEVIN, G. (1996). Long-term potentiation increases tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit 2B in rat dentate gyrus in vivo. *Proc. Natl. Acad. Sci. USA* **93**, 10457-10460.

ROSSI, G.C., BROWN, G.P., LEVENTHAL, L., YANG, K. & PASTERNAK, G.W. (1996). Novel receptor mechanisms for heroin and morphine-6 beta-glucuronide analgesia. *Neurosci. Lett.* **216**, 1-4.

ROSSOWSKI, W.J., ROSSOWSKI, T.M., ZACHARIA, S., ERTAN, A. & COY, D.H. (1990). Galanin binding sites in rat gastric and jejunal smooth muscle membrane preparations. *Peptides* **11**, 333-338.

ROTHMAN, R.B., BRADY, L.S., XU, H. & LONG, J.B. (1993). Chronic intracerebroventricular infusion of the antiopioid peptide, Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH2 (NPFF), downregulates mu opioid binding sites in rat brain. *Peptides* **14**, 1271-7.

ROTHMAN, R.B., BYKOV, V., DE COSTA, B.R., JACOBSON, A.E., RICE, K.C. & BRADY, L.S. (1990). Interaction of endogenous opioid peptides and other drugs with four kappa opioid binding sites in guinea pig brain. *Peptides* **11**, 311-31.

ROUSE, S.T, THOMAS, T.M. & LEVEY, A.I. (1997). Muscarinic acetylcholine receptor subtype, m2: diverse functional implications of differential synaptic localization. *Life Sciences* **60,** 1031-1038.

RUDOLPHI, K.A., SCHUBERT, P., PARKINSON, F.E. & FREDHOLM, B.B. (1992). Neuroprotective role of adenosine in cerebral ischaemia. *Trends in Pharmacological Sciences* **13,** 439-445.

RUSH, D.K. & STREIT, K. (1992). Memory modulation with peripherally acting cholinergic drugs. *Psychopharmacology* **106**, 375-382.

RYLETT, R.J., BALL, M.J. & COLHOUN, E.H. (1983). Evidence for high-affinity choline transport in synaptosomes prepared from hippocampus and neocortex of patients with Alzheimer's disease. Brain Res. 289, 169-175.

SAH, P. (1996). $Ca2^{+}$-activated K^{+} currents in neurones: types, physiological roles and modulation. *Trends in Neurosciences* **19**,150-154.

SAH, P., HESTRIN, S. & NICOLL, R.A. (1990). Properties of excitatory postsynaptic currents recorded in vitro from rat hippocampal interneurones. *Journal of Physiology (London)* **430,** 605-616.

SAKSELA, O. & RIFKIN, D.B. (1988). Cell-associated plasminogen activation: regulation and physiological functions. *Ann. Rev. Cell Biol.* **4**, 93-126.

SAKURAI, E., MAEDA, T., KANEKO, S., AKAIKE, A. & SATOH, M. (1996). Galanin inhibits long-term potentiation at Schaffer collateral-CA1 synapses in guinea pig hippocampal slices. *Neurosci. Lett.* **212**, 21-24.

SALIN, P.A., WEISSKOPF, M.G. & NICOLL, R.A. (1995). A comparison of the role of dynorphin in the hippocampal mossy fibre pathway in guinea pig and rat. *J. Neurosci.* **15**, 6939-6945.

SAPPINO, A-P., MADNAI, R., HUARTE, J., BELIN, D., KISS, J-Z., WOHLWEND, A. & VASSALLI, J-D. (1993). Extracellular proteolysis in the adult murine brain. *J. Clin. Invest.* **92**, 679-685.

SAYDOFF, J.A. & ZACZEK, R. (1996). Blockade of N- and Q-type Ca^{2+} channels inhibit K^{+}-evoked [^{3}H]acetylcholine release in rat hippocampal slices. *Brain Research Bulletin* **40**, 283-286.

SCHOLZ, K.P. & MILLER, R.J. (1995). Developmental changes in presynaptic calcium channels coupled to glutamate release in cultured rat hippocampal neurons. *J Neurosci.* **15**, 4612-4617.

SCHÖTT, P.A., KEHR, J., SANDIN, J. & ÖGREN, S.O. (1998). Galanin differentially modulates spatial learning and *in vivo* acetylcholine release in ventral and dorsal hippocampus of the rat. *Neuroscience.* Submitted.

SCHUBERT, P. & LEE, K.S. (1986). Non-synaptic modulation of repetitive firing by adenosine is antagonized by 4-aminopyridine in a rat hippocampal slice. *Neuroscience Letters* **67,** 334-338.

SCHULLER, A.G., KING, M.A., ZHANG, J. BOLAN, E., PAN, Y.X., MORGAN, D.J., CHANG, A., CZICK, M.E., UNTERWALD, E.M., PASTERNAK, G.W. & PINTAR, J.E. (1999). Retention of heroin and morphine-6 beta-glucuronide analgesia in a new line of mice lacking exon 1 of MOR-1. *Nat. Neurosci.* **2**, 151-6.

SCHUMAN, E.M. & MADISON, D.V. (1991). A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science* **254**, 1503-6.

SCHWARTZBERG, P.L., STALL, A.M., HARDIN, J.D., BOWDISH, K.S., HUMARAN, T., BOAST, S., HARBISON, M.L., ROBERTSON, E.J. & GOFF, S.P. (1991). Mice homozygous for the ablm1 mutation show poor variability and depletion of selected B and T cell populations. *Cell* **65,** 1165-1175.

SCHWARTZKROIN, P.A. & KNOWLES, W.D. (1983). Local interactions in the hippocampus. *Trends in Neurosciences* **6,** 88-92.

SCHWARTZKROIN, P.A. & PRINCE, D.A. (1980). Changes in excitatory and inhibitory synaptic potentials leading to epileptogenic activity. *Brain Research* **183,** 61-76.

SCHWEICKART, V.L., RAPORT, C.J., GODISKA, R., BYERS, M.G., EDDY, R.L., Jr, SHOWS,

T.B. & GRAY, P.W. (1994). Cloning of human and mouse ENI1, a lymphoid-specific G-protein-coupled receptor encoded on human chromosome 17q12-q21.2. *Genomics* **23**, 643-650.

SCOVILLE, W.B. & MILNER, B. (1957). Loss of recent memory after bilateral hippocampal lesions. *Journal of Neurology, Neurosurgery and Psychiatry* **20**, 11-21.

SCOVILLE, W.B. (1954). The limbic lobe in man. *Journal of Neurosurgery* **11**, 64-66.

SEGAL, M. & AUERBACH, J.M. (1997). Muscarinic receptors involved in hippocampal plasticity. *Life Sciences* **60,** 1085-1091.

SEGAL, M. & FISHER, A. (1992). AF102B, a muscarinic M_1 receptor agonist, mimics some effects of acetylcholine on neurons of rat hippocampus slices. *European journal of pharmacology* **220**,103-106.

SEGAL, M. (1982a). Multiple actions of acetylcholine at a muscarinic receptor studied in the rat hippocampal slice. *Brain Research* **246,** 77-87.

SEGAL, M. (1982b). Intracellular analysis of a postsynaptic action of adenosine in the rat hippocampus. *European Journal of Pharmacology* **79,** 193-199.

SEGAL, M. (1982c). Changes in neurotransmitter actions in the aged rat hippocampus. *Neurobiol. Aging* **3**, 121-124.

SEGAL, M. (1988). Synaptic activation of a cholinergic receptor in rat hippocampus. *Brain Research* **452**, 79-86.

SETHI, T. & ROZENGURT, E. (1991). Galanin stimulates Ca^{2+} mobilisation, inositol phosphate accumulation, and clonal growth in small cell lung cancer cells. *Cancer Res.* **51**, 1674-1679.

SETHY, V.H., KUHAR, M.J., ROTH, R.H., VAN WOERT, M.H. & AGHAJANIAN, G.K. (1973). Cholinergic neurones: effects of acute septal lesion on acetylcholine and choline content of rat hippocampus. *Brain Res.* **55**, 481-484.

SEUFFERLEIN, T. & ROZENGURT, E. (1996). Galanin, neurotensin, and phorbol esters rapidly stimulate activation of mitogen-activated protein kinase in small cell lung cancer cells. *Cancer Res.* 56, 5758-5764.

SHTIVELMAN, E., LIFSHITZ, B., GALE, R.P., ROE, B.A. & CANAANI, E. (1986). Alternative splicing of RNAs transcribed from the human abl gene and from the bcr-abl fused gene. *Cell* **47**, 277-284.

SHUTE, C.C.D. & LEWIS, P.R. (1966). Electron microscopy of cholinergic terminals and acetylcholinesterase containing neurones in the hippocampal formation of the rat. *Z. Zellforsch.* **69**, 334-343.

SIMS, S., BOWEN, D.M. & SMITH, C.C.T. et al. (1980). Glucose metabolism and acetylcholine synthesis in relation to neuronal activity in Alzheimer's disease. Lancet i, 333-336.

SINISCALCHI, A. & BIANCHI, C. (1988). Effect of ethylketocyclazocine on acetylcholine release in guinea-pig brain slices. *Pharmac. Res. Commun.* **20**, 73-85.

SKARNES, W.C. (1993). The identification of new genes: Gene trapping in transgenic mice. *Curr. Opin. Biotechnol.* **4**, 684-689.

SKARNES, W.C., AUERBACH, B.A. & JOYNER, A.L. (1992). A gene trap approach in mouse embryonic stem cells: the lacZ reporter is activated by splicing, reflects endogenous gene expression,

and is mutagenic in mice. *Genes Dev.* **6**, 903-918.

SKARNES, W.C., MOSS, J.E., HURTLEY, S.M. & BEDDINGTON, R.S. (1995). Capturing genes encoding membrane and secreted proteins important for mouse development. *Proc. Natl. Acad. Sci. USA* **92**, 6592-6596.

SKOFITSCH, G., SILLS, M.A. & JACOBOWITZ, D.M. (1986). Autoradiographic distribution of 125I-galanin binding sites in the rat central nervous system. *Peptides* **7**, 1029-1042.

SKREDE, K.K. & WESTGAARD, R.H. (1971). The transverse hippocampal slice: a well defined cortical structure maintained in vitro. *Brain Research* **35,** 589-593.

SMITH, D.E., ROBERTS, J., GAGE, F.H. & TUSZYNSKI, M.H. (1999). Age-associated neuronal atrophy occurs in the primate brain and is reversible by growth factor gene therapy. *Proc. Natl. Acad. Sci. USA* **96**, 10893-10898.

SMITH, K.E., FORRAY, C., WALKER, M.W., JONES, K.A., TAMM, J.A., BARD, J., BRANCHEK, T.A., LINEMEYER, D.L. & GERALD, C. (1997). Expression cloning of a rat hypothalamic galanin receptor coupled to phosphoinositide turnover. *J. Biol. Chem.* **272**, 24612-6.

SMITH, K.E., WALKER, M.W., ARTYMYSHYN, R., BARD, J., BOROWSKY, B., TAMM, J.A., YAO, W.J., VAYSSE, P.J., BRANCHEK, T.A., GERALD, C. & JONES, K.A. (1998). Cloned human and rat galanin GALR3 receptors. Pharmacology and activation of G-protein inwardly rectifying K^+ channels. *J. Biol. Chem.* **273**, 23321-6.

SNYDER, S.H. (1985). Adenosine as a neuromodulator. *Annual Review of Neuroscience* **8,** 103-124.

SOFUOGLU, M., PORTOGHESE, P.S., & TAKEMORI, A.E. (1993). 7-Benzylidenenaltrexone (BNTX): a selective delta 1 opioid receptor antagonist in the mouse spinal cord. *Life Sci.* **52**, 769-75.

SOKOLOV, M.V. & KLESCHEVNIKOV, A.M. (1995). Atropine suppresses associative LTP in the CA1 region of rat hippocampal slices. *Brain Res.* **672**, 281-284.

SOLTESZ, I., HABY, M., LERESCHE, N. & CRUNELLI, V. (1988). The $GABA_B$ antagonist phaclofen inhibits the late K-dependent IPSP in cat and rat thalamic and hippocampal neurones. *Brain Research* **448,** 351-354.

SOMMER, B., KEINÄNEN, K., VERDOORN, T.A., WISDEN, W., BURNASHEV, N., HERB, A., KÖHLER, M., TAKAGI, T., SAKMANN, B. & SEEBURG, P.H. (1990). Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. *Science* **249**, 1580-5.

SOREQ, H. & MISKIN R. (1981). Plasminogen activator in the rodent brain. *Brain Res.* **216**, 361-374.

SPENCER, H.J., GRIBKOFF, V.K., COTMAN, C.W. & LYNCH, G.S. (1976). GDEE antagonism of iontophoretic amino acid excitations in the intact hippocampus and in the hippocampal slice preparation. *Brain Res.* **105**, 471-481.

STEEL, M., MOSS, J., CLARK, K.A., KEARNS, I.R., DAVIES, C.H., MORRIS, R.G.M., SKARNES, W.C. & LATHE, R. (1998). Gene-trapping to identify and analyse genes expressed in the mouse hippocampus. *Hippocampus* **8**, 444-457.

STEN SHI, T.J., ZHANG, X., HOLMBERG, K., XU, Z.Q. & HÖKFELT, T. (1997). Expression and regulation of galanin-R2 receptors in rat primary sensory neurons: effect of axotomy and inflammation. *Neurosci. Lett.* **237**, 57-60.

STEVENS, K.E., SHIOTSU, G. & STEIN, L. (1991). Hippocampal μ-receptors mediate opioid

reinforcement in the CA3 region. *Brain Res.* **545**, 8-16.

STONE, T.V., NEWBY, A.C. & LLOYD, G.E. (1990). Adenosine release. *Adenosine and Adenosine Receptors*. Editor, Michael Williams. The Human Press Inc. 143-172.

SUNDSTRÖM, E., ARCHER, T., MELANDER, T. & HÖKFELT, T. (1988). Galanin impairs acquisition but not retrieval of spatial memory in rats studied in the Morris swim maze. *Neurosci. Lett.* **88**, 331-335.

SVOBODA, K.R. & LUPICA, C.R. (1998). Opioid inhibition of hippocampal interneurons via modulation of potassium and hyperpolarisation-activated cation (I_h) currents. *J. Neurosci.* **11**, 7084-7098.

SVOBODA, K.R., ADAMS, C.E. & LUPICA, C.R. (1999). Opioid receptor subtype expression defines morphologically distinct classes of hippocampal interneurons. *J. Neurosci.* **19**, 85-95.

SWANSON, L.W., TEYLER, T.J. & THOMPSON, R.F. (1982). Hippocampal long-term potentiation: mechanisms and implications for memory. *Neurosciences Research Program Bulletin* **20,** 611-770.

SWEENEY, M.I. (1996). Adenosine release and uptake in cerebellar granule neurons both occur via an equilibrative nucleoside carrier that is modulated by G proteins. *Journal of Neurochemistry* **67,** 81-88.

TAKAHASHI T. & MOMIYAMA, A. (1993). Different types of calcium channels mediate central synaptic transmission. *Nature* **366**, 156-158.

TANG, Y.P., SHIMIZU, E., DUBE, G.R., RAMPON, C., KERCHNER, G.A. ZHUO, M., LIU, G. & TSIEN, J.Z. (1999). Genetic enhancements of learning and memory in mice. *Nature* **401**, 63-69.

TATEMOTO, K., ROKAEUS, A., JORNWALL, H., McDONALD, T.J. & MUTT, V. (1983). Galanin, a novel biologically active peptide from porcine intestine. *FEBS Lett.* **164**, 124-128.

THOMPSON, S.M., HAAS, H.L., & GÄHWILER, B.H. (1992). Comparison of the actions of adenosine at pre- and postsynaptic receptors in the rat hippocampus *in vitro*. *Journal of Physiology (LOND)* **451**, 347-363.

TOSELLI, M. & TAGLIETTI, V. (1994). Muscarinic inhibition of high-voltage activated calcium channels in excised membranes of rat hippocampal neurons. *European Biophysics Journal* **22,** 391-398.

TOSELLI, M. & TAGLIETTI, V. (1995). Muscarine inhibits high-threshold calcium currents with two distinct modes in rat embryonic hippocampal neurons. *Journal of Physiology (London)* **483,** 347-365.

TOUHARA, K., INGLESE, J., PITCHER, J.A., SHAW, G. & LEFTKOWITZ, R.J. (1994).Binding of G protein beta/gamma subunits to pleckstrin homology domains. *J. Biol. Chem.* **269**, 10217-10220.

TRAUB, R.D., MILES, R. & BUZSÁKI, G. (1992). Computer simulation of carbachol-driven rhthmic population oscillations in the CA3 region of the *in vitro* rat hippocampus. *Journal of Physiology (London)* **451,** 653-672.

TRUDEAU, L., FANG, Y., & HAYDON, P.G. (1998). Modulation of an early step in the secretory machinery in hippocampal nerve terminals. *Proc. Nat. Acad. Sci. U.S.A.* **95**, 7163-7168.

TRUSSEL, L.O. & JACKSON, M.B. (1987). Dependence of an adenosine-activated potassium current on a GTP-binding protein in mammalian central neurons. *Journal of Neuroscience***7,** 3306-

3316.

TSCHOPP, J., ESMON, P.C. & SCHEKMAN, R. (1984). Defective plasma membrane assembly in yeast secretory mutants. *J Bacteriol.* **160**, 966-970.

TSIEN, R.W., LIPSCOMBE, D., MADISON, D.V., BLEY, K.R. & FOX, A.P. (1988). Multiple types of neuronal calcium channels and their selective modulation. *Trends Neurosci.* **11**, 431-438.

TSIRKA, S.E., GUALANDRIS, A., AMARAL, D.G. & STRICKLAND, S. (1995). Excitotoxin induced neuronal degeneration and seizure are mediated by tissue plasminogen activator. *Nature* **377**, 340-344.

TSIRKA, S.E., ROGOVE, A.D. & STRICKLAND, S. (1996). Neuronal cell death and t-PA. *Nature* **384**, 123-124.

TUCEK, S. & PROSKA, J. (1995). Allosteric modulation of muscarinic acetylcholine receptors. *Trends in Pharmacological Sciences* **16,** 205-212.

TUCEK, S., MUSILKOVA, J., NEDOMA, J., PROSKA, J., SHELKOVNIKOV, S. & VORLICEK, J. (1990). Positive cooperativity in the binding of alcuronium and N-methylscopalamine to muscarinic acetylcholine receptors. *Molecular Pharmacology* **38,** 674-680.

TYBULEWICZ, V.L., CRAWFORD, C.E., JACKSON, P.K., BRONSON, R.T. & MULLIGAN, R.C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. *Cell* **65**, 1153-1163.

UKAI, M., MIURA, M. & KAMEYAMA, T. (1995). Effects of galanin on passive avoidance response, elevated plus-maze learning, and spontaneous alternation performance in mice. *Peptides* **16,** 1283-6.

UMEMIYA, M. & BERGER, A.J. (1994). Activation of adenosine A_1 and A_2 receptors differentially modulates calcium channels and glycinergic synaptic transmission in rat brainstem. *Neuron* **13,** 1439-1446.

VALENTINO, R.J. & DINGLEDINE, R. (1981). Presynaptic inhibitory effect of acetylcholine in the hippocampus. *Journal of Neuroscience* **1,** 784-792.

VALINSKY, J.E. & LeDOURAIN, N.M. (1985) Production of plasminogen activator by cephalic neural crest cells. *EMBO J.* **4**, 1403-1046.

VALKNA, A., JUREUS, A., KARELSON, E., ZILMER, M., BARTFAI, T. & LANGEL, U. (1995). Differential regualtion of adenylate cyclase activity in rat ventral and dorsal hippocampus by rat galanin. *Neurosci. Lett.* **187**, 75-78.

VAN CALKER, D., MULLER, M. & HAMPRECHT, B. (1979). Adenosine regulates, via two different types of receptors, the accumulation of cAMP in cultured brain cells. *Journal of Neurochemistry* **33,** 999-1005.

VERDERIO, C., COCO, S., FUMAGALLI, G. & MATTEOLI, M. (1995). Calcium-dependent glutamate release during neuronal development and synaptogenesis: Different involvement of ω-agatoxin IVA- and ω-conotoxin GVIA-sensitive channels. *Proc. Natl. Acad. Sci. USA* **92**, 6449-6453.

WAELBROECK, M., TASTENOY, M., CAMUS, J. & CHRISTOPHE, J. (1990) Binding of selective antagonists to four muscarinic receptors (M_1 to M_4) in rat forebrain. *Molecular Pharmacology* **38,** 267-273.

WAGNER, J.J., CAUDLE, R.M. & CHAVKIN, C. (1990). Stimulation of endogenous opioid release

displaces mu receptor binding in rat hippocampus. *Neuroscience* **37**, 45-53.

WANG, B. & KRUH, G.D. (1996) Subcellular localisation of the Arg protein tyrosine kinase. *Oncogene* **13**, 193-197.

WANG, L.Y., SALTER, M.W. & MACDONALD, J.F. (1991). Regulation of kainate receptors by cAMP-dependent protein kinase and phosphatases. *Science* **253**, 1132-5.

WANG, S., HASHEMI, T., HE, C., STRADER, C. & BAYNE, M.(1997a). Molecular cloning and pharmacological characterization of a new galanin receptor subtype. *Mol. Pharmacol.* **52**, 337-43.

WANG, S., HE, C., HASHEMI, T. & BAYNE, M. (1997). Cloning and expressional characterisation of a novel galanin receptor. Identification of different pharmacophores within galanin for the three galanin receptor subtypes. *J. Biol. Chem.* **272**, 31949-31952.

WANG, S., HE, C., HASHEMI, T. & BAYNE, M. (1997b). Cloning and expressional characterization of a novel galanin receptor. Identification of different pharmacophores within galanin for the three galanin receptor subtypes. *J. Biol. Chem.* **272**, 31949-52.

WANG, S., HE, C., MAGUIRE, M.T., CLEMMONS, A.L., BURRIER, R.E., GUZZI, M.F., STRADER, C.D., PARKER, E.M. & BAYNE, M.L. (1997c). Genomic organization and functional characterization of the mouse GalR1 galanin receptor. *FEBS Lett.* **411**, 225-30.

WATSON, N., REDDY, H., STEFANICH, E. & EGLEN, R.M. (1995). Characterization of the interaction of zamifenacin at muscarinic receptors in vitro. *Eur. J. Pharm.* **285**, 135-142.

WEINER, D.M. & BRANN, M.R. (1989). Distribution of m1-m5 muscarinic acetylcholine receptor mRNAs in rat brain. *Trends in Pharmacological Science (Suppliment)* **4,** 115.

WEISSKOPF, M.G., ZALUTSKY, R.A. & NICOLL, R.A. (1993). The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fibre synapses and modulates long-term potentiation. *Nature* **362**, 423-427.

WHEELER D.B., RANDALL, A., TSIEN, R.W. (1994). Roles of N-type and Q-type Ca^{2+} channels in supporting hippocampal synaptic transmission. *Science* **264**, 107-111.

WHEELER, D.B., RANDAL, A. & TSIEN, R.W. (1996). Changes in action potential duration alter reliance of excitatory synaptic transmission on multiple types of Ca^{2+} channels in rat hippocampus. *J. Neurosci.* **16**, 2226-2237.

WHITEHOUSE, P.J., PRICE, D.L., CLARK, A.W., COYLE, J.T. & DE LONG, M.R. (1981). Alzheimer's disease: evidence for selective loss of cholinergic neurons in the nucleus basalis. *Ann. Neurol.* **10**, 122-126.

WIGSTROM, H., GUSTAFSSON, B. & HUANG, Y.-Y. (1986). Mode of action of excitatory amino acid receptor antagonists on hippocampal long-lasting potentiation. *Neuroscience* **17,** 1105-1115.

WILLIAMS, J.T., EGAN, T.M. & NORTH, R.A. (1982). Enkephalin opens potassium channels on mammalian central neurones. *Nature* **299**, 74-77.

WILLIAMS, M. (1987). Purine receptors in mammalian tissues: pharmacology and functional significance. *Annual Review of Pharmacology and Toxicology* **27,** 315-345.

WILLIAMS, S. & JOHNSTON, D. (1988). Muscarinic depression of long-term potentiation in CA3 hippocampal-neurons. *Science* **242,** 84-87.

WILLIAMS, S.H. & JOHNSTON, D. (1996). Actions of endogenous opioids on NMDA receptor-

independent long-term potentiation in area CA3 of the hippocampus. *J. Neurosci.* **16**, 3652-3660.

WILSON, V. (1999). *Personal communication.*

WINSON, J. (1978). Loss of hippocampal theta rhythm results in spatial memory deficit in the rat. *Science Wash. DC* **201**, 160-162.

WOLOZIN, B.L. & PASTERNAK, G.W. (1981). Classification of multiple morphine and enkephalin binding sites in the central nervous system. *Proc. Natl. Acad. Sci. U S A* **78**, 6181-5.

WONNACOTT, S. (1997). Presynaptic nicotinic ACh receptors. *Trends in Neurosciences* **20,** 92-98.

WU, L. & SAGGAU, P. (1995). $GABA_B$ receptor-mediated presynaptic inhibition in guinea pig hippocampus is caused by reduction of presynaptic Ca^{2+} influx. *J. Physiol. (LOND)* **485**, 649-657.

WU, L. & SAGGAU, P. (1997). Presynaptic inhibition of elicited neurotransmitter release. *Trends in Neurosciences* **20**, 204-212.

WU, L-G. & SAGGAU, P. (1994). Adenosine inhibits evoked synaptic transmission primarily by reducing presynaptic calcium influx in area CA1 of hippocampus. *Neuron* **12,** 1139-1148.

WYNICK, D., SMALL, C.J., BLOOM, S.R. & PACHNIS, V. (1998). Targetted disruption of the murine galanin gene. *Ann. N Y Acad. Sci.* **863**, 22-47.

XU, H., PARTILLA, J.S., DE COSTA, B.R., RICE, K.C. & ROTHMAN, R.B. (1993). Differential binding of opioid peptides and other drugs to two subtypes of opioid delta ncx binding sites in mouse brain: further evidence for delta receptor heterogeneity. *Peptides* **14**, 893-907.

YAMAMOTO, C. & MCILWAIN, H. (1966). Electrical activities in thin sections from the mammalian brain maintained in chemically-defined media in vitro. *Journal of Neurochemistry* **13,** 153-156.

YAO, W.-J., TAMM, J.A., SMITH, K.E., BARD, J., BRANCHEK, T., GERALD, C. et al. (1998) GALR3 and GALR1 galanin receptors activate GIRK in Xenopus oocytes. *Soc. Neurosci. Abstr.* **24**, 1590.

YAWO, H. & CHUHMA, N. (1993). Preferential inhibition of o-conotoxin-sensitive presynaptic Ca^{2+} channels by adenosine autoreceptors. *Nature* **365,** 256-258.

ZADINA, J.E., HACKLER, L., GE, L.J. & KASTIN, A.J. (1997). A potent and selective endogenous agonist for the mu-opiate receptor. *Nature* **386**, 499-502.

ZAJAC, J.M., BIGEARD, A., DELAY GOYET, P. & ROQUES, B.P. (1990). Affinity states of rat brain opioid receptors in different tissue preparations. *J Neurochem* **54**, 992-9.

ZHANG, J.F. ET AL. (1993). Distinctive pharmacology and kinetics of cloned neuronal Ca^{2+} channels and their possible counterparts in mammalian CNS neurons. *Neuropharmacology* **32**, 1075-1088.

ZHANG, L., WEINER, J.L. & CARLEN, P.L. (1992). Muscarinic potentiation of I_K in hippocampal neurons: electrophysiological characterization of the signal transduction pathway. *Journal of Neuroscience* **12,** 4510-4520.

ZHOU, Q-U., LI, C., OLAH, M.E., JOHNSON, R.A., STILES, G.L. & CIVELLI, O. (1992). Molecular cloning and characterization of an adenosine receptor: The A_3 adenosine receptor. *Proceedings of the National Academy of Sciences* **89,** 7432-7436.

ZIEGLGANSBERGER, W., FRENCH, E.D., SIGGINS, G.R. & BLOOM, F.E. (1979). Opioid peptides may excite hippocampal pyramidal neurons inhibiting adjacent inhibitory interneurons. *Science* **205**, 415-417.

ZUBIETA, J.K. & FREY, K.A. (1993). Autoradiographic mapping of M_3 muscarinic receptors in the rat brain. *The Journal of Pharmacology and Experimental Therapeutics* **264,** 415-422.

ZUKIN, R.S., EGHBALI, M., OLIVE, D., UNTERWALD, E.M. & TEMPEL, A. (1988). Characterization and visualization of rat and guinea pig brain kappa opioid receptors: evidence for kappa 1 and kappa 2 opioid receptors. *Proc. Natl. Acad. Sci. U S A* **85**, 4061-