THE ROLE OF GALANIN IN SYNAPTIC TRANSMISSION AND PLASTICITY IN THE CA1 AREA OF THE RODENT HIPPOCAMPUS

URANIA COUMIS

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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 Dr. I. R Kearns (Chapter 5).

Urania Coumis

III. Abstract

Galanin is believed to be co-released with acetylcholine by neurones projecting from the medial septum and nucleus of Meynert to the hippocampus in rodents. Galanin inhibits acetylcholine and glutamate release, thereby depressing excess neuronal excitability in the brain. Although this effect established galanin as an endogenous neuroprotective substance, released only during high frequency neuronal firing, it may also explain why it impairs memory and cognition *in vivo*. The sustained increase in glutamatergic synaptic strength following high frequency stimulation of hippocampal neurones, a phenomenon termed long-term potentiation (LTP), has been widely recognised as a model of the synaptic changes that may underlie learning and memory in vertebrates. It may thus be predicted that the physiological action of galanin at the cellular level would be to depress LTP, thereby causing an impairment in mnemonic processes mediated by the hippocampus. Experiments were designed to address aspects of this hypothesis, namely: (1) *in vitro* characterisation of the effect of galanin agonists and antagonists on synaptic transmission and plasticity in the CA1 area of rodent hippocampus and (2) investigation of glutamatergic synaptic plasticity in galanin knockout mice and their wild-type littermates. Exogenous galanin induced a dose-dependent increase in the slope of baseline fEPSPs, which appeared to be dependent on the pathways from CA3 to CA1 being intact, but it did not have any effect on paired-pulse facilitation ratios (PPF) in low concentration. However, in higher concentration, galanin induced a significant decrease in PPF in intact slices. In CA3-hemisected hippocampal slices the aforementioned effects did not occur. The effect of galanin on LTP and long-term depression (LTD) of glutamate mediated synaptic transmission in apical and basal dendrites of CA1 pyramidal neurones were investigated using both intracellular and extracellular recording techniques *in vitro*. LTP induced in either apical or basal dendrites of CA1 pyramidal neurones by different paradigms was significantly inhibited by galanin. Galanin also inhibited LTP in hippocampal slices prepared from wild-type mice. This effect was reversible by the known galanin antagonist, galantide (M15). Galanin did not affect isolated pure NMDA receptor-mediated postsynaptic potentials or the loss of spike frequency adaptation and increase in input resistance

evoked by metabotropic glutamate receptor activation, indicating that its inhibition of LTP was downstream of these receptors. Galanin applied had no effect the expression of LTP indicating that galanin may inhibit LTP by interfering with kinase activity necessary for the induction of LTP, e.g. protein kinase C. Galanin did not affect the induction of LTD. Subsequent studies in the galanin-null transgenic mice yielded no effect on synaptic strength or paired pulse facilitation ratios. Galanin gene deletion caused a significant impairment of LTP, which was only observed in basal dendrites, the magnitude of which increased with age. The underlying molecular mechanism for this impairment might be a significantly faster saturation of synaptic plasticity in the mutant mice *in vivo*, compared to wild-type mice. No effects of galanin were noted in mutant mice. This could suggest a developmental loss of galanin-responsive cells concomitant with global galanin gene deletion.

In summary, galanin seems to have a modulatory effect on excitatory neurotransmitters in the hippocampus, such as glutamate, thereby delaying the neurodegenerative effect of age. The research described in this thesis is deemed of importance in biomedical research of drug therapy for protection against neurodegenerative disease.

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De morbo sacro 14.1 to De morbo sacro 14.15

Εἰδέναι δὲ χρὴ τοὺς ἀνθρώπους, ὅτι ἐξ οὐδενὸς ἡμῖν αἱ ἡδοναὶ γίνονται καὶ αἱ εὐφροσύναι καὶ γέλωτες καὶ παιδιαὶ ἢ ἐντεῦθεν, καὶ λῦπαικαὶ ἀνίαι καὶ δυσφροσύναι καὶ κλαυθμοί. Καὶ τούτῳ φρονεῦμεν μάλιστα καὶ νοεῦμεν καὶ βλέπομεν καὶ ἀκούομεν καὶ γινώσκομεν τά τε αἰσχρὰ καὶ τὰ καλὰ καὶ τὰ κακὰ καὶ ἀγαθὰ καὶ ἡδέα καὶ ἀηδέα, τὰ μὲν νόμῳ διακρίνοντες, τὰ δὲ τῷ ξυμφέροντι αἰσθανόμενοι, τῷ δὲ καὶ τὰς ἡδονὰς καὶ τὰς ἀηδίας τοῖσι καιροῖσι διαγινώσκοντες, καὶ οὐ ταὐτὰ ἀρέσκει ἡμῖν. Τῷ δὲ αὐτῷ τούτῳ καὶ μαινόμεθα καὶ παραφρονέομεν, καὶ δείματα καὶ φόβοι παρίστανται ἡμῖν τὰ μὲν νύκτωρ, τὰδὲ μεθ' ἡμέρην, καὶ ἐνύπνια καὶ πλάνοι ἄκαιροι, καὶ φροντίδες οὐχ ἱκνεύμεναι, καὶ ἀγνωσίη τῶν καθεστεώτων καὶ ἀηθίη καὶ ἀπειρίη. Καὶ ταῦτα πάσχομεν ἀπὸ τοῦ ἐγκεφάλου πάντα, ὅταν οὗτος μὴ ὑγιαίνῃ, ἀλλ' ἢ θερμότερος τῆς φύσιος γένηται ἢ ψυχρότερος ἢ ὑγρότερος ἢ ξηρότερος, ἤ τι ἄλλο πεπόνθῃ πάθος παρὰ τὴν φύσιν ὃ μὴ ἐώθει.

'Men ought to know that from the brain, and from the brain only, arise our pleasures, our joys, laughter, jests, as well as sorrows, pains, griefs and tears. Through it, in particular, we think, see, hear and distinguish the ugly from the beautiful, the bad from the good, the pleasant from the unpleasant...It is the same thing that makes us mad, or delirious, inspires us with dread and fear, whether by night or by day, brings sleeplessness, inopportune mistakes, aimless anxieties, absent – mindedness, and acts that are contrary to habit. These things that we suffer all come from the brain, when it is not healthy...'
attributed to Hippocrates, 600 B.C.

V. Abbreviations

AC	adenylate cyclase
ACh	acetylcholine
AChE	acetylcholinesterase
ACSF	artificial cerebrospinal fluid
A/D	analogue to digital
AD	Alzheimer's disease
AHP	after-hyperpolarization
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP	apolipoprotein
ATP	adenosine 5'-triphosphate
cAMP	cyclic adenosine 5'-monophosphate
CCh	carbachol
C7	galanin-1-13-spantide amide
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

D-AP5	D-2-amino-5-phosphonopentanoate
DC	direct current
DRG	dorsal root ganglion
EGTA	Ethyleneglycol-*bis*(*N*-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
fEPSP	field excitatory postsynaptic potential
GABA	γ-aminobutyric acid
GALR1	galanin receptor 1
GALR2	galanin receptor 2
GALR3	galanin receptor 3
Gal1	galanin gene 1
Gal2	galanin gene 2
Gal3	galanin gene 3
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
Ko	knockout transgenic mouse
LTP	long-term potentiation
M15	galanin-1-13-substance P-5-11 amide, galantide
M32	galanin-1-13-neuropeptide Y
M35	galanin-1-13-bradykinin-2-9 amide
M40	galanin-13-Pro-Ala-Leu-Ala-Leu-Ala amide
mAChR	muscarinic acetylcholine receptor
mGluRs	metabotropic glutamate receptors
mRNA	messenger ribonucleic acid
nAChR	nicotinic acetylcholine receptor
NBQX	6-nitro-7-sulphamoylbenzo[*f*]quinoxaline-2,3-dione
NMDA	*N*-methyl-D-aspartate
NFTs	neurofibrillary tangles
NO	nitric oxide
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

SCH202596	2-[(5-methoxy-3-oxo-1-carboxymethyl)-6-spiro(1,4-cyclohexadienyl)]-(3,S,4S,5S,6S)-6-[(5,7-dichloro-6-methyl-3-oxo-2,3-dihydrobenzo[b]furan-4-yl)oxyl]-3,4,5-trihydroxy-1-cyclohexene-1-methylcarboxylate
s. oriens	*stratum oriens*
s. pyramidale	*stratum pyramidale*
s. radiatum	*stratum radiatum*
S.E.M.	standard error of the mean
SCG	superior cervical ganglion
SFA	spike frequency adaptation
STD	short-term depression
VIP	vasointestinal peptide
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 acquisition and memory formation, relevant to key synaptic mechanisms discussed in this thesis. Most of the experimental results obtained, investigate the role of the neuropeptide galanin on synaptic transmission and plasticity mechanisms in the hippocampus of rodents. Therefore, the first section of this chapter is devoted to discussing recent research following the discovery of galanin. In the second section, the hippocampus and the molecular mechanisms thought to underlie learning and memory in vertebrates are discussed. The contribution of the septohippocampal input, with particular reference to the modulatory role of galanin, is laid out in the context of such hippocampal function. Chapter 1 concludes with a review of transgenic mouse strains with reference to resulting alterations in synaptic transmission affecting learning and memory.

1.1. Galanin

Galanin is a neuropeptide widely expressed in brain and peripheral tissues, thereby exerting a broad range of physiological effects (Kask *et al.*, 1995a-b & 1997). The neuropeptide was first isolated from porcine intestine as a twenty nine amino acid neuropeptide in Victor Mutt's laboratory in Karolinska Institute in Stockholm. Galanin derives its name from its N- and C – terminus residues, glycine and alanine respectively. It has long been known that many biologically active peptides contain a C-terminal amidated structure, like, for example, neuropeptide Y. As such, they make easy targets to proteolytic conversion of their C-terminus amide to the easily extractable by thin-layer chromatography dansyl derivative. This was how galanin was isolated from both porcine intestine and brain tissue extracts (Tatemoto *et al.*, 1983). Following isolation, Victor Mutt's group reported a series of physiological studies, which established the hyperglycaemic action of galanin in the dog. More

importantly, however, Tatemoto *et al.*, 1983 suggested the rat ileum preparation as a suitable pharmacological assay for galanin-like activity.

Molecular biology studies laid down the basis for the production of transgenic mice in which various manipulations of the galanin gene allow the precise characterization of galaninergic physiology. Therefore, a review of the studies on the transcriptional control of the galanin gene is described in paragraph 1.1.1. Moreover, such studies allowed the delineation of the anatomical distribution of galanin in the CNS of vertebrates and this is dealt in more detail in section 1.1.2 and Tables 1.1.1. and 1.1.2.

Galanin possesses a highly conserved N – terminal associated with high biological activity as reported by Lundkvist *et al.*, 1995 and Kakuyama *et al.*, 1997. Pharmacological studies using peptide analogues such as M35, M32 and C7 have led to speculation about multiple galanin receptor subtypes being expressed in neural tissues of vertebrates (Iisma *et al.*, 1999). Since 1994, a total of three G-protein coupled receptors have been cloned, all of which possess significant homology to the rhodopsin family of G-proteins including seven predicted hydrophobic membrane-spanning domains. Expression of at least three different galanin receptor subtypes appears to be as flexible as the regulation of galanin transcription itself and is broadly gender- and tissue-specific. Potent, selective antagonists are yet to be discovered for any of the cloned receptors. A review of galanin receptor subtypes and their pharmacology is presented in sections 1.1.3. and 1.1.4. respectively.

The link between the known physiological actions of galanin and each of the cloned receptors and evidence for additional galanin receptor subtypes are issues under current investigation in this field (Section 1.1.5). More interestingly to this thesis, the role of galanin in modulating learning and memory in vertebrates is laid out in section 1.1.6.

1.1.1. The Galanin Gene

Endogenous galanin is synthesised as the pre-pro-hormone pre-pro-galanin, which comprises a 59 amino acid C-terminal flanking region called Galanin Message Associated Peptide (GMAP), which has no known function as yet (Rökeaus &

Brownstein, 1986; Hökfelt *et al.*, 1992). To accomplish the diverse physiological functions, the transcription of the gene for galanin is highly plastic. Namely, transcription of the galanin gene is controlled by endogenous hormones and neuromodulators e.g. oestrogen in the anterior pituitary and NGF in the rat basal forebrain. Galanin gene transcription also appears to be unpregulated by external stress factors e.g following denervation and injury in the dorsal root and trigeminal ganglia. Investigations by Rökeaus *et al.*, 1998 have led to the discovery that bovine galanin gene induction can be transactivated in human neuroblastoma cells, by cJun/cFos proteins and the PKC activator phorbol ester PMA. Further genetic studies indicated that the functional response to galanin gene enhancers or silencers regulating basal and activity dependent transcription, along with the CRE-like elements are mapped in the promoter region located upstream the galanin gene transcription start site. Although the quality and levels of transcriptional control may vary, the galanin gene promoter region appear to be highly conserved across species (Wynick *et al.*, 1998a-b).

1.1.2. Anatomical Distribution of Galanin

The isolation and synthesis of galanin allowed the development of antibodies towards different parts of the galanin molecule, which have been used to map the distribution of galanin-like immunoreactivity by immunocytochemical methods and to measure the content of galanin protein and mRNA content in several tissue extracts by radioimmunoassay, *in situ* hybridisation and immunocytochemistry, respectively. Tables 1.1.1. and 1.1.2 summarise the results of the above studies. Galaninergic cell bodies and fibre systems are present in the central nervous system in varying degrees. Notable is the presence of galanin-like immunoreactivity in the cell bodies of septal nuclei and in the fimbria of the hippocampus in the rat. A similar distribution has been observed in the monkey and it is summarised in Table 1.1.2 (Melander *et al.*, 1986 a-b & 1987; Langel *et al.*, 1992; Chang *et al.*, 1995). Interestingly, galanin has been found to coexist with some of the classical neurotransmitters such as acetycholine, glutamate, noradrenaline and serotonin (Crawley & Wenk, 1989; Hökfelt *et al.*, 1998, 1999).

Area	Human	Monkey	Pig	Rat	Mouse
Brain	IH	IH	IH/ RIA ISH	IH/ RIA ISH	
Spinal Cord	IH		IH/ RIA ISH	IH	
Respiratory Tract			IH	RIA	
GI tract	IH		IH/ RIA ISH	IH/ RIA	IH
Pancreas			RIA	RIA	
Adrenal Medulla			IH/ RIA ISH		
Urinary Bladder	IH/RIA			IH/RIA	
Genital tract	IH/RIA			IH/RIA	

Table 1.1.1. Anatomical distribution of galanin-like immunoreactivity measured in different areas using immunohistochemistry (IH), radioimmunoactivity (RIA) and *in situ* hibridisation studies (ISH) (Skofitch *et al.*, 1986 a-b; Bartfai *et al.*, 1993).

Area of the Brain	Cell Bodies	Fibre Systems
Nucleus of the Diagonal Band	+++	
Medial Septal Nucleus	+++	++
Nucleus of stria terminalis	+++	+++
Medial preoptic area/nucleus	+++	+++
Amygdaloid nucleus	+++	++
Supraoptic nucleus	+++	
Paraventricular nucleus	+++	++
Preoptic Area		+++
Septohypothalamic nucleus	+++	+++
Dorsomedial hypothalamic nucleus		+++
Median eminence	+++	
Arcuate nucleus	+++	
Locus coeruleus	+++	++
Nucleus of the solitary tract	++	
Caudal spinal trigeminal nucleus		+
Fimbria of the hippocampus	++	++
Pituitary gland	+	++
Dorsal horn of the spinal cord	+	
		+

Table 1.1.2. Galanin-positive cell body and fibre system distribution in the monkey brain (Melander *et al.*, 1985 and 1986a-c; Crawley and Wenk, 1989).

1.1.3. Distribution of Galanin Receptor Subtypes

Table 1.1.3. summarises the anatomical distribution of galanin receptor subtypes.

GALR1

The human galanin receptor type 1 (GALR1) was isolated by the human Bowes melanoma cell line and other sources. GALR1 contains 349 amino acids with the structure of a G-protein coupled receptor (Habert-Ortoli *et al.*, 1994). In amino acid composition GALR1 is most similar to rat GALR1, GALR2 and GALR3, while it retains some homology to human receptors activated by peptides like somatostatin and opioids (Lee *et al.*, 1999). Rat GALR1 was cloned from brain cells and shares the same consensus sequences for N-glycosylation and intracellular phopshorylation with the exception of two additional phospshorylation sites in the human GALR1 C-terminal domain. The mouse GALR1 was mapped to chromosome 18, which coincides with the human gene position. The distribution of GALR1 is particularly prominent in the human fetal brain and gastro-intestinal tract. In the rat, GALR1 is most prominently expressed in the brain and spinal cord. The pattern of rat GAL1 receptor expression shows variability, for example gal1 mRNA is elevated in females compared to males and varies during the estrous cycle (Faure-Virelizier *et al.*, 1998). Human and rat GAL1 receptors share similar binding profiles in [^{125}I] galanin binding assays (Wang *et al.*, 1998a). The salient physiological actions resulting from the activation of GAL1 receptor include the inhibition of neurotransmitter or hormone release in many brain regions with potential to modify memory, feeding, emotion, nociception, gut secretion and motility, glucose/insulin homeostasis and human growth and development (Bartfai *et al.*, 1993). In agreement with the latter role, children with a growth hormone insufficiency phenotype display a common deletion of two megabases from chromosome 18, which results in the absence of the GALR1 (Cody *et al.*, 1997).

GALR2

Originally cloned from the rat, GALR2 is thought to be a G-protein coupled protein consisting of 372 amino acids including consensus sequences for glycosylation and intracellular phosphorylation sites distinct from GALR1 (Smith *et al.*, 1997b). The human GALR2 protein contains 387 amino acids and both rat and human receptors have been mapped to chromosome 17, while the mouse GALR2 maps to chromosome 11 (Pang *et al.*, 1998; Fathi *et al.*, 1998). Preliminary investigations, using RNAase protection, support the idea that GALR2 may be expressed instead of GALR1 in some tissues. For example, in the rat anterior pituitary, GALR2 but no GALR1 is expressed, suggesting that GALR2 receptor may mediate the effects of galanin on pituitary hormone secretion (Fathi *et al.*, 1997). However, there appears to exist confusion between reports as to the distribution of human gal2 mRNA in the brain (Fathi *et al.*, 1997).

The widespread distribution of rat gal2 mRNA suggests numerous physiological consequences of GALR2 signalling, including prolactin release, lactation, growth hormone release, feeding, emotion, memory, nociception, cellular growth, nerve regeneration, pancreatic islet function, cardiovascular tone, peripheral metabolism and reproduction (Smith *et al.*, 1997a-b; Howard *et al.*, 1997;Wang *et al.*, 1997a-b; Fathi *et al.*, 1997).

Interestingly, the gene encoding human GALR2 is associated with two human diseases, hereditary neuralgic amyotrophy and Russel-Silver syndrome, which are characterised in part by short stature, and low birth weight dwarfism respectively, in addition to other developmental defects (Fathi *et al.*, 1998). Galanin null mice, on the other hand, do not seem to suffer from such abnormalities (Wynick *et al.*, 1998b).

Relevant to this thesis is the possible role of galanin receptors in diseases of the nervous system. In this respect, it has been shown that in dementia of the Alzheimer's type (AD) galanin containing fibres hyperinnervate acetylcholine containing neurones of the basal forebrain, prompting speculation that a receptor such as GALR2 might function to promote survival of the ageing population of acetylcholine neurones (Chan-Palay *et al.*, 1988). Consistent with the above concept,

it has been shown that in models of experimental injury galanin and gal2 mRNA levels are upregulated, leaving open the possibility that GALR2 may have a dual role as a survival mediator and autoreceptor (Sten-Shi *et al.*, 1999).

GALR3

The GALR3 was originally cloned from the rat and exists as a protein of 370 residues. GALR3 amino acid composition bears most similarity to the rat GALR2 (Lee *et al.*, 1999; Smith *et al.*, 1998a-b). Human GALR3 was cloned from a human genomic library based on the structural similarity to human GALR1 and GALR2 proteins (Kolakowski *et al.*, 1998). Human GALR3 contains 368 amino acids and exhibits 90% similarity to rat GALR3. They both contain a single consensus sequence for N-glycosylation and multiple intracecellular consensus sites for phosphorylation, the most distinct of which is the C-terminus PKC phosphorylation site. The human and rat GALR3 was mapped to chromosome 22, while the mouse GALR3 was cloned and mapped to chromosome 15 (Kolakowski *et al.*, 1998). Possible effects mediated by GALR3 activation, based on its distribution pattern, include emotion, feeding, pituitary hormone release, nociception and metabolism. The distribution of GALR3 mRNA is widespread in rat, mouse and human in both neural and peripheral tissues as shown in Table 1.1.1.

The three galanin receptor proteins share 83 conserved amino acids and although they are similar to somatostatin receptors (types 4 and 5) and to a nociceptin receptor (ORL1), they form a distinct receptor superfamily.

Table 1.1.3. The distribution of expression of the three galanin receptor subtypes (Mufson *et al.*, 1998; Kolakowski *et al.*, 1998;Wang *et al.*, 1998a; Iisma *et al.*, 1999).

Receptor	Isolated/ Cloned from	Method	Distribution	Coupling	Physiological Effect
Human GAL1	Bowes melanoma cell line	northern blot RT-PCR	foetal brain GI tract	↓ cAMP ↑K^+ CHANNELS ↑ MAP KINASE	growth & development glucose/ insulin homeostasis GI tract secretion & motility memory hormonal homeostasis
Rat GAL1	brain RIN-14b cells	northern blot ISH [^{125}I] binding	hypothalamus amygdala hippocampus thalamus brainstem spinal cord		
Rat GAL2	RNAase protection ISH RT-PCR	Rat cDNA	brain & peripheral tissues apart from the hypothalamus in rat	↑ PLC ↑ IP ↑ $Ca^{2+}{}_I$ ↑ Ca^{2+} - dependent Cl^- channel	Pituitary hormone secretion in rat Prolactin release cell growth nerve regeneration pancreatic islet function cardiovascular tone peripheral metabolism reproduction
GAL3	Human genomic library	Nothern blot RNAase protection	Heart spleen testes hypothalamus pituitary olfactory bulb medulla oblongata caudate putamen	↑K^+ CHANNELS IN *XENOPUS* OOCYTES	glucose/insulin homeostasis in rat and human

1.1.4. Pharmacology of Galanin Receptors

All three galanin receptors possess high affinity for full length and N-terminal fragments of galanin and for chimeric peptides containing the N-terminus from galanin (Smith *et al.*, 1997b; Wang *et al.*, 1997c-e). The structure of galanin analogues is presented in Figure 1.1.*A*. GALR1 and GALR2 receptors share similar pharmacological profiles, while the pharmacology of GALR3 combines the profiles of GALR1 and GALR2 (Figure 1.1.*B*). Human and rat GALR2 appear to share common pharmacological profiles as well (Wang *et al.*, 1997c).

Endogenous Ligands

Structure-activity studies on the galanin peptide demonstrated that the N-terminal 1-16 amino acids are conserved across species and that this peptide fragment is sufficient for receptor recognition (Fisone *et al.*, 1989a). The 17-29 C-terminal fragment is not recognised by hypothalamic, hippocampal, spinal cord and pancreatic receptors and seems to bare no biological effect. Nevertheless, this fragment appears to be required for galanin binding to receptors in smooth muscle, different subtype to neuronal and exocrine tissue (Rossowski *et al.*, 1990). Galanin 1-12 is the shortest form of galanin demonstrating full receptor agonism, but the C-terminal portions of the galanin 1029 polypeptide are arranged by beta helix to protect the peptide configuration from proteolytic cleavage and for receptor subtype recognition. Human galanin is a 30 amino acid long peptide with a free carboxy-terminus because the amide donor glycine present in all other species is replaced by serine (See Figure 1.1). Various other ligands apparent in many anatomical areas of the brain and exerting their effect on varied physiological responses have been reported to act through multiple receptor subtypes and/or second messenger systems (Branchek *et al.*, 2000). Evidence for multiple receptor subtypes in native brain 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 hippocampus (Fisone *et al.*, 1989b). Also there is evidence for an N-terminal preferring receptor in the rat dorsal hippocampus, neocortex and neostriatum (Hedlund *et al.*, 1992). Porcine, rat and human endogenous galanin peptides are laid out in the top panel of Figure 1.1.*A*.

Exogenous Ligands

The NH_2 terminal fragments 1-12,1-15, and 1-16 behave like high affinity agonists to galanin binding sites in the brain in both *in vivo* and *in vitro* experiments. Peptide fragment experiments have shown that for hippocampal galanin receptors the NH2 terminal 1-12 amino acids are sufficient for ligand recognition (Langel & Bartfai., 1998). Another approach to using truncated peptides containing the amino acid sequence necessary and sufficient for galanin receptor recognition has been the design of high affinity chimeric peptides, that share the NH2 terminal 1-13 amino acids with galanin and have different sequences covalently attached to the carboxy group of proline 13, like for example, M15, galanin-1-13-neuropeptide Y (M32), galanin-1-13-bradykinin-2-9 amide (M35), galanin-13-Pro-Ala-Leu-Ala-Leu-Ala amide (M40) and galanin-1-13-spantide amide (C7). Although these compounds appear to act as antagonists to the effects of exogenous galanin application in physiological experiments, like, for example, in the hippocampus and hypothalamus (Kask *et al.*, 1995; Jureus *et al.*, 1997), they act as partial agonists in transfected cell lines expressing 5-500 fold more galanin receptor sites per cell than *in vivo* (Smith *et al.*, 1997b). Their structure is laid out in Figure 1.1.*B*. Recently, small, non-peptide ligands to galanin receptors have been discovered which bind to cloned human GALR1 in human Bowes melanoma cells. One such compound is the fungal metabolite SCH202596.

Activated biochemical pathways

The coupling mechanisms of the three galanin receptors are summarized in Table 1.1.3.

Cloned GAL1 receptor activation leads to a reduction in the intracellular concentration of cAMP, opening of G-protein coupled inwardly rectifying K^+ channels and the pertussis toxin sensitive stimulation of MAP kinase activity (Wang *et al.*, 1998a); this is consistent with coupling to $G_{i/o}$-type G-proteins (Habert-Ortoli *et al.*, 1994; Parker *et al.*, 1995; Burgevin *et al.*, 1995). 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.*, 1997b) 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.*, 1998a). Taken together, it appears likely that GALR1 may act broadly to inhibit neurotransmitter/hormone release.

Activation of GALR2 leads to the stimulation of multiple intracellular events, most of which result from the activation of phospholipase C. This finding is consistent with reports of GALR2 mediating pertussis toxin resistant inositol phosphate hydrolysis, intracellular Ca^{2+} mobilisation and Ca^{2+} dependent Cl^- channel opening (Smith *et al.*, 1997b). Additionally, GALR2 may activate other intracellular pathways depending on the host cell and/or the G-protein mixture in the cell and the species homologue of the receptor (Wang *et al.*, 1997a-d & 1998f). Although functional studies support the existence of native galanin receptors positively coupled to inositol phosphate hydrolysis, a native receptor with a well defined GALR2-like pharmacology has not yet been described. Human and rat GALR3 proteins share many pharmacological profiles in [^{125}I] binding assays and they both mediate a pertussis toxin-sensitive activation of an inward rectifying K^+ current in *Xenopus* oocytes, which is consistent with them acting on $G_{i/o}$ proteins. GALR2 activation appears to produce a variety of cellular responses such as pertussis toxin-resistant inositol phospholipid hydrolysis, calcium mobilisation (Smith *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.*, 1997b) in CHO cells which, taken together, suggest a primary coupling to G-proteins $G_{q/11}$ activating phospholipase C.

A native cell line or tissue model with a distinctive GALR3 pharmacology has not yet been described, but GALR3 activation leads to a hyperpolarising current consistent with a role in secretion e.g. glucose/insulin homeostasis in the pancreas.

The complex binding and functional profiles for galanin and other galanin peptides such as M32, M35 and C7 with a spectrum of antagonist/agonist activity are consistent with the existence of multiple receptor subtypes. 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 voltage-gated Ca^{2+} channels (Palazzi *et al.*, 1991; Kalkbrenner *et al.*, 1995); 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 (Sethy & 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.

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 $G_{i/o}$ (Smith *et al.*, 1998).

A

Agonist Peptides	Amino Acid Composition
Rat/Mouse Galanin	GWTLNSAFYLLGPH AIDNHRSFSDK HGLT
Human Galanin	GWTLNSAFYLLGPH AVGNHRSFSDK NGLTS*
Porcine Galanin	GWTLNSAFYLLGPH AIDNHRSFSDK YGLA
Chimeric Peptides	
M15	GWTLNSAFYLLGP QQFFGLM
M32	GWTLNSAFYLLGP RHYINLITRQRY
M35	GWTLNSAFYLLGP PPGFSPFR
M40	GWTLNSAFYLLGP PPALALA
C7	GWTLNSAFYLLGP rPKPQQwFwLL

B

Ligand	GALR1		GALR2		GALR3	
	Human	Rat	Human	Rat	Human	Rat
Porcine Galanin	9.63	9.49	9.02	8.98	8.01	8.14
Rat Galanin	9.54	9.47	8.79	8.87	7.91	8.48
Human Galanin	9.36	9.22	8.63	8.60	7.16	7.28
M15	9.61	9.19	8.97	9.00	7.40	7.98
M32	9.58	9.17	8.84	9.10	8.22	8.91
M35	9.95	9.49	8.71	8.49	7.84	8.68
M40	8.62	8.17	8.39	8.45	6.54	7.10
C7	9.58	9.55	9.20	9.25	8.09	7.98

Figure 1.1. Galanin receptor ligands and pharmacology. *A*, Peptide analogues used to characterise galanin receptor pharmacology. All galanin homologues shown are conserved in residues 1-14. Human galanin contains 30 amino acids rather than 29 and in terminating with a C-terminal free acid(*) rather than an amide. The chimeric peptides share a common motif in which the biologically active N-terminal region of galanin –1-13 is conserved (adapted from Branchek *et al.*, 2000). *B*, Radioligand binding displacement profile of [^{125}I]Galanin from rat and human GALR1, GALR2 and GALR3 (pK_i values taken from Smith *et al.*, 1998).

1.1.5. The Neuromodulatory Effect of Galanin

The physiological effects of galanin have been studied extensively in experimental paradigms which include gut secretion and motility, cardiovascular and cerebrovascular tone, feeding behaviour, neuroendocrine regulation (Roassmanith *et al.*, 1996), lactation and reproduction, pain (Palkovits *et al.*, 1995), neuroregeneration (Kask *et al.*, 1997), depression and the modulation of acetylcholine release, memory and Alzheimer's disease (McDonald *et al.*, 1997). All the galanin ligands mentioned

in this section are discussed in more detail in section 1.1.4. and Figure 1.1. Additionally, their full, published names are mentioned in the Abbreviations section.

On the cellular level, galanin has been shown to affect motility of smooth muscle and neurotransmitter release, e.g. of insulin, somatostatin, VIP, NO and glutamate (Schmidt *et al.*, 1991; Zini *et al.*, 1993; Niiro *et al.*, 1998; Wang *et al.*, 1998g). As such, the physiological functions of galanin, linked to cognitive and behavioural deficits, include the inhibition of glutamic acid release, the decrease of spinal neurone excitability and the blockade of voltage activated calcium channels. Human endogenous galanin is co-localised with acetylcholine in cholinergic terminals, thereby inhibiting its release in a number of central areas including the basal forebrain Nucleus Basalis of Meynert and stria terminalis (Chan-Pallay *et al.*, 1988; Melander *et al.*, 1989; Mufson *et al.*, 1993 & 1998; Crawley, 1993 & 1996; Bowser *et al.*, 1997; McDonald *et al.*, 1997), striatum (Sebok *et al.*, 1996), nucleus solitarius (Maley, 1996) and tegmental nucleus (Gai *et al.*, 1993). Porcine galanin inhibits neurotransmitter release, inhibits secretion of somatostatin, insulin and pancreatic polypeptide and blocks voltage-gated calcium channels (Haynes, 1986; Tatemoto *et al.*, 1983). Rat galanin inhibits neurotransmitter release, blocks voltage-sensitive calcium channels.

Galanin and Feeding

Intraventricular injection of galanin stimulates food intake in rats, an effect that can be reversed by C7 and M40 (Corwin *et al.*, 1993). Although all receptors for galanin are present in brain regions important in feeding, only gal1 mRNA is upregulated by glucose and fat metabolism inhibitors (Faure-Virelizier *et al.*, 1998). As such, it seems likely that feeding may be influenced by GALR1 or an unknown galanin receptor subtype rather than GALR2 or GALR3 peptides.

Galanin and Pain

Galanin has been studied in many experimental models of pain. In the rat flexor reflex model, which is a measure of motor response to nociceptive stimuli, galanin applied intrathecally facilitates the nociceptive reflex at a low dose, but inhibits it at a high dose (Wiesenefeld-Hallin *et al.*, 1989). The effect is blocked by C35 and M32, while M40 is a partial agonist. In the tail flick and Randall-Selitto tests, intrathecal

application of galanin potentiates the effect of morphine-induced analgesia, while M35 and M15 produce the opposite effect (Reimann *et al.*, 1994).

In all pain models the antagonist behaviour *in vivo* does not agree with their action on cloned receptors. A role for GALR1 in the nociceptive reflex pathway was suggested after it was shown that intrathecal administration of a cell-penetrating peptide nucleic acid (DNA) complementary to GALR1 blocked the inhibitory effect of galanin on the flexor reflex in the rat (Wynick *et al.*, 1998a), but the effect on the chimeric peptides such as M40 and C7 was not reported. Interestingly, after sciatic nerve transection galanin and galanin message associated peptide undergo a long lasting upregulation in dorsal root ganglia. In transgenic mice lacking galanin there is a loss of galanin containing fibres in dorsal root ganglia and, a defective rate of nerve regeneration and the absence of autonomy after sciatic nerve cut, in addition to defects in prolactin secretion and lactotroph function. The aforementioned studies suggest that galanin receptors might be active in hyperalgesic states such as neuropathological pain. However, the roles of the individual receptors have yet to be characterized in detail.

1.1.6. Galanin impairs learning and memory

Intraventricular or intrahippocampal infusion of galanin in rats impairs performance in various learning and memory tasks. An example is the delayed non-matching to position task, in which the effect was reversed by M40 (McDonald *et al.*, 1996). These observations coupled with the finding that galanin may inhibit acetylcholine release have led to the speculation that a galanin antagonist might enhance cognition (Fisone *et al.*, 1987). Further interest in galanin and cognition has been sparked by the observation that galaninergic fibres hyperinnervate to nurture the rapidly decaying cholinergic neurones in Alzheimer's disease (Ögren *et al.*, 1996 & 1998). In a recent study, a combination of the galanin antagonist M35 and the M1 muscarinic receptor agonist TZTP has been shown to improve performance in the delayed non-matching to position task in rats whose performance is compromised by forebrain lesions (McDonald *et al.*, 1998).

It is clear that there is a lack of profile concordance for cloned galanin receptor subtypes with their effects in native systems. The cloned receptors do not readily account for the reported antagonist actions of the chimeric peptides M15, M32, M35, M40 and C7.These apparent discrepancies could be explained by the presence of further unknown galanin receptor subtypes awaiting discovery. Moreover, it might be that peptide tools used for evaluating these receptors are biologically or chemically unstable at the time of the experiment. Finally, it might be that the lack of detectable antagonist activity in the heterologous expression systems is artefactual as a result of high receptor reserve.

Other ways to elucidate the role of galanin, apart from pharmacological studies, include antisense techniques, orphan receptor characterisation and continued cloning efforts as well as the use of transgenic animals.

Anatomy of the Galanin Colocalisation with Acetylcholine

As mentioned above, galanin has been localised with and shown to modulate cholinergic transmission in the gastrointestinal tract and hippocampus in rodents and other vertebrates (Larson *et al.*, 1990; Talmage *et al.*, 1992; Bennett *et al.*, 1991; Herzig *et al.*, 1993; Degli-Uberti *et al.*, 1996; Ferris *et al.*, 1999). Galanin colocalises

to 30-35% ChAT positive neurones in the medial septum and diagonal band in the rat (Melander *et al.*, 1985 and 1986a) and mouse. To a great extent these galanin positive neurones project to fibre terminals in the hippocampus (Melander *et al.*, 1986a; Gaykema *et al.*, 1991), as shown in Table 1.1.2. In primates, however, Galanin does not coexist with acetylcholine. Instead, numerous galaninergic interneurones are localised near the cholinergic neurones (Gentleman *et al.*, 1989; Benzing *et al.*, 1993; Mufson *et al.*, 1993). In senile dementia of the Alzheimer's type (AD), these galaninergic interneurones hyperinnervate the decaying cholinergic cell bodies. The axons of the galanin positive neurones are enlarged and show varicosities embracing the cholinergic neurones and dendrites. The increase in galanin expression appears to correlate with the severity of the disease (Chan-Palay *et al.*, 1998; Mufson *et al.*, 1993).

Galanin affects Behaviour by modulating Ach release

Structural findings have led to a number of physiological studies addressing the role of galanin in the modulation of the cholinergic system including its effects on acetylcholine (ACh) release, learning and memory.

Acute, intrahippocampal administration of galanin to target the third ventricle of rodents inhibits scopolamine induced ACh release in a dose-dependent manner and is reversed by co-administration of galanin receptor antagonists M15 and M40 (Verge *et al.*, 1993; Yu, J. *et al.*, 1997). Centrally administered galanin also has inhibitory effects on several tests of learning and memory such as the Morris water maze and those involving conditioning (McDonald *et al.*, 1997; Mastropaolo *et al.*, 1988). These effects are reversed by the antagonist M40. In contrast to these inhibitory actions, Crawley (1996) demonstrated that exogenous galanin has no effect on the increased release of ACh that occurs when a rodent is exposed to a novel environment. Neither of the galanin antagonists have an effect on ACh release or on cognition, in the absence of exogenously administered galanin. Similarly, Sakurai *et al.* (1996) have shown that while exogenous galanin inhibits LTP *in vitro*, M40 has no effect on LTP when applied on its own. However, it is becoming more and more apparent that M15 and M40 ligands may act as partial agonists in some tissues and as full agonists *in vitro* to cloned galanin receptor subtypes (Yu *et al.*, 1997; Gu *et al.*,

1993). Despite the conflicting data, which point to the limitations of existing pharmacological tools in this field, it is possible and quite likely that endogenous galanin may still be released *in vivo* during high frequency firing to regulate ACh release during plasticity, injury or anoxic damage.

The Galanin-Null Mouse

The way galanin null mice were generated is described in more detail in section 2.1.3. The electrophysiological analysis of the galanin-null mice and their wild-type littermates are dealt with in Chapter 5. To date, there is little evidence to support the role of galanin on cell survival, and neuromodulation of the rapidly decaying cholinergic neuronal population in basal forebrain during dementia. However, the importance of galanin on cell survival and regeneration of sensory neurones following injury was discovered by Wynick *et al.* (1998a). Moreover, the role of galanin as a growth factor to the prolacting secreting cells of the pituitary gland has been well documented (Hammond *et al.*, 1996). While *in vitro* techniques and cell lines have an important place in integrated studies of biological systems, homeostatic mechanisms are best studied within the intact organism. The generation of transgenic animals may provide a bridge between molecular and physiological studies investigating the role of galanin in the intact organism, but would shed light on the action of galanin in learning and memory. Wynick *et al.*, (1998b) have generated a mutant mouse lacking a functional galanin gene (a galanin knockout mouse), the analysis of which has allowed the function of galanin to be studied in a manner not previously possible.

The absence of galanin had a marked degradatory effect on prolactin message levels and protein content, both in the pituitary and serum rendering mutant female mice unable to lactate. However, no effect was observed in the pituitary content of GH, TSH, LH and FSH or the hypothalamic content of NPY and glucagon-like peptide-1, peptides involved in the growth and food intake, respectively. The foregoing data provide supporting evidence for the role of galanin as a survival factor to the lactotroph and added to the observations that galanin mutant animals show a long-term impairment in peripheral nerve regeneration following injury.

1.2. The Hippocampus

1.2.1. Historical Perspectives

The earliest reference to the human brain is found in a recovered ancient Egyptian papyrus written in 17th century B.C. and it described the prognosis of head injury. However, written proof on the debates concerning the function of the brain are attributed to the Greek philosophers at 400 B.C. Hippocrates believed that the brain was the seat of consciousness whilst Aristotle opposed this view. Instead, he held that intellectual, perceptual and related functions originated from the heart. Again, Hippocrates was drawing his estute conclusions from observation of patients with neurological disorders, like epilepsy, or head injuries. However, the physiology of the brain remained unexplored and it was not until the nineteenth century that the concept of "functional localisation" was developed by Paul Broca. Since then, the trend has been to describe the brain in ever-smaller functional units. This concept was initially based on the rationale that anatomical segregation confers distinct functions (Finger, 1994).

The hippocampus has proven a challenging area to investigate on both counts: its distinctive shape (in Greek *hippos* means 'horse' and *campos* 'sea monster') and readily identifiable, laminated structure and its fundamental role in certain aspects of learning and memory. The hippocampus is of high interest in biomedical research because it is one of the most susceptible areas affected by pathological conditions, such as epilepsy, ischaemia and anoxia, senile dementia and especially AD. Evidence linking the hippocampus with a role in memory and learning commence as early as 1887, when it was shown that damage to the temporal lobes and the underlying hippocampus in monkeys produced deficits in memory formation and retention (Brown & Schafer, 1888). Twelve years later, Bechterew described a memory deficit in a patient who, upon autopic examination, displayed bilateral softening of the hippocampus and adjoining temporal cortex. 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 aspiration of 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 several years (Scoville, 1954; Scoville & Milner, 1957). Non-declarative or procedural memory tasks, such as learning a motor skill, were unaffected and, therefore, this cognitive process was believed not to be dependent on the hippocampus. These findings led to a plethora of experimental lesion studies conducted mainly on primates and rodents, which confirmed a role for the hippocampus in certain types of memory formation.

1.2.2. Anatomical Structure of the Rodent 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 septotemporal (long) axis, bending in a C-shaped manner from the septal nuclei rostro-dorsally to the temporal lobe caudo-laterally (Figure 1.2.*A*). In the perpendicular transverse (orthogonal) section (Figure 1.2.*B*) the principal pathways can be viewed and have been determined in classical Golgi studies (Lorento de Nó, 1933 & 1934) and degeneration studies. 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 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*.

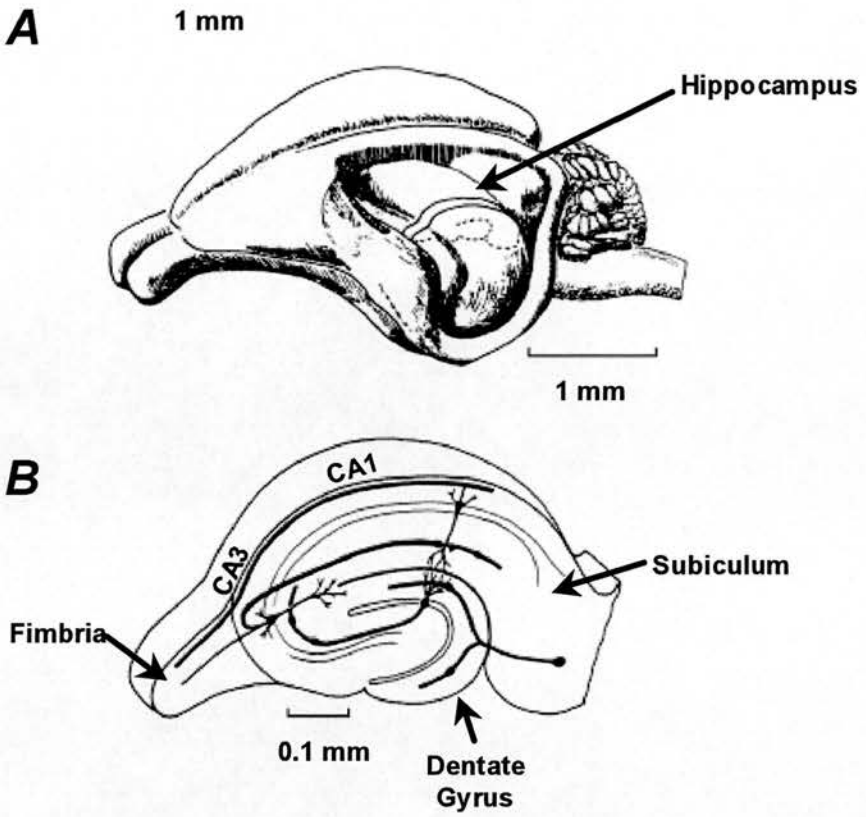


Figure 1.2 The Hippocampus

***A* shows a schematic representation of the rat brain and the position of the left 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.**

Per Andersen and coworkers suggested a lamellar organisation of these pathways, splitting the hippocampus into functional 'strips' that are stacked along the septotemporal axis (1964, 1969 and 1971). It has subsequently been shown that, besides the mossy fibre projection from 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 and Witter, 1989).

The principal cells of the hippocampus are CA1 and CA3 pyramidal glutamatergic cells. Glutamate receptor density is high throughout the structure with autoradiography of NMDA receptor binding highest at the zones connecting granule

cell dendrites and CA1 pyramidal cell dendrites. In addition to the principal cells of the hippocampus, there exist a variety of intrinsic GABAergic interneurons that form extensive connections with the pyramidal and granule cells. 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 1.2.4.

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 1.3.

1.2.3. The Hippocampal Slice

Andersen's idea was influential leading to the development of the transverse hippocampal brain slice preparation introduced in 1971 (Skrede *et al.*, 1971). Most electrophysiological experiments performed on the hippocampus exploit the slice cut at right angles to the orthogonal axis of the hippocampal formation (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 on the anatomy of the hippocampus and its principal connections see Amaral & Witter (1989).

A striking feature of the hippocampus is that it is essentially laminar, each layer containing the same circuitry. Therefore, a thin e.g. 150 - 350 μm hippocampal slice contains all components and appropriately connected associations, so it is thought to be a valid substitute for the whole structure and the regularity of the circuitry facilitates attempts at functional modeling (Suter, Smith & Dudek, 1999). Consequently, the transverse hippocampal slice has proven extremely popular in investigations of excitatory synaptic transmission due to the conservation of the trisynaptic circuitry (see Figure 1.4 overleaf) 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 extracellular milieu. Although 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, recordings may be more stable and longer lasting than during an *in vivo* experiment. A great advantage in using hippocampal slices for studying excitatory synaptic transmission is that animal anaesthesia becomes obsolete. Additional methods to visualize neurons have been devised e.g. intracellular staining with biocytin, lucifer yellow or Nissl staining (Figure 1.3).

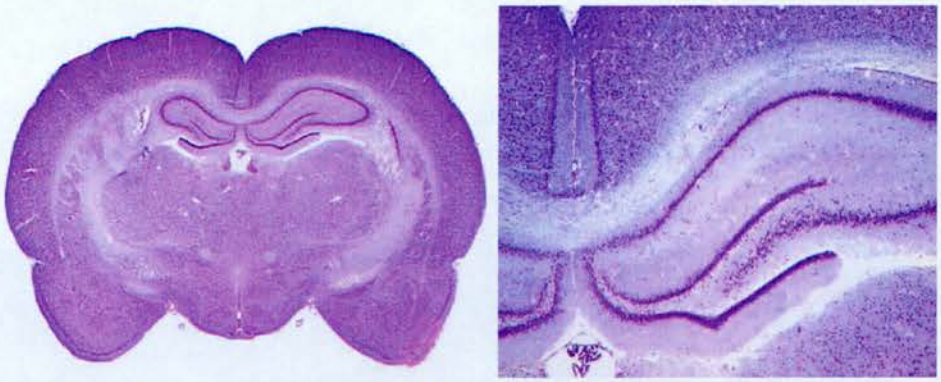


Figure 1.3. A Nissl-stained section of the hippocampal slice.

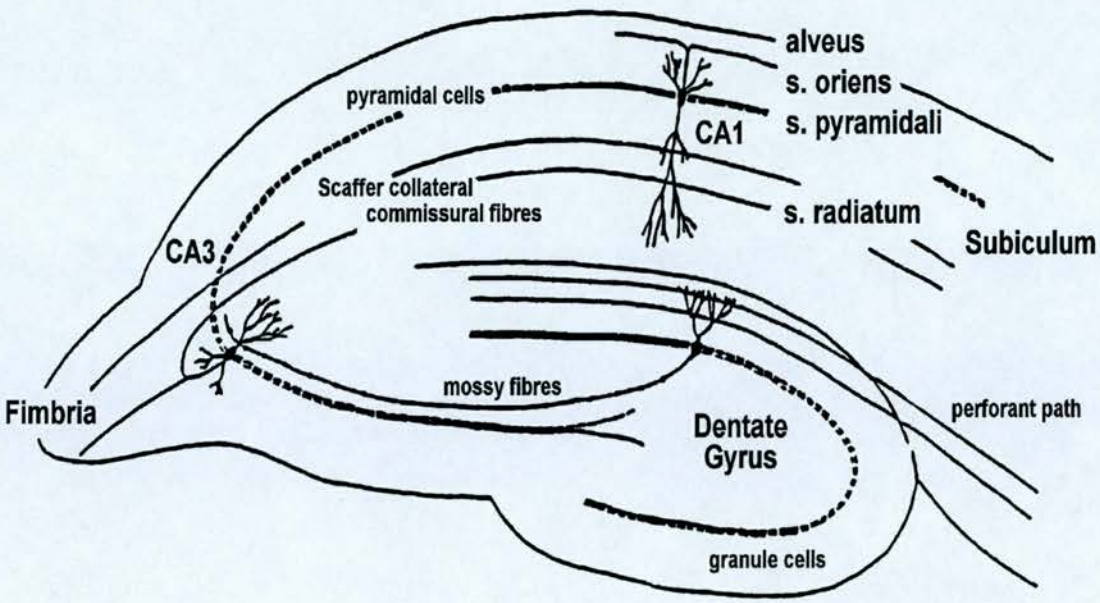


Figure 1.4. Synaptic circuitry of the rat hippocampal slice

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. pyramidale and s. radiatum*).

1.2.4. 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 rapidly curtailed by a biphasic IPSP (Kandel *et al.*, 1961; Alger & Nicoll, 1982b). The EPSP is believed to be mediated by AMPA receptors ($EPSP_A$) (Davies & Collingridge, 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, 1988c; Soltesz *et al.*, 1988; Lambert *et al.*, 1989). Under a variety of conditions, e.g. reduced presynaptic inhibition (Wigström *et al.*, 1986b), 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). However, it was realised that a feedforward inhibitory circuit was also responsible for the IPSP (Alger & Nicoll, 1982b; Scwartzkroin *et al.*, 1983; Buzsaki, 1984b). Accordingly, activation of AMPA and NMDA receptors on GABAergic interneurons were shown to result in the IPSPs observed in CA1 pyramidal neurons (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; Dingledine & Gjerstad, 1980). Feedforward inhibitory circuits also activate a $GABA_B$ receptor-mediated slow $IPSP_B$, which was originally characterised by its similarity to (-)-baclofen-induced hyperpolarisations (i.e. both exhibited similar I-V relationships and dependency on extracellular K^+) and was subsequently confirmed by the inhibition of $IPSP_B$s using selective $GABA_B$ receptor antagonists (Lambert *et al.*, 1989).

Table 1.2.1. shows the principal receptor systems and Figure 1.5 is a schematic representation of the known inhibitory and excitatory receptor systems involved in synaptic transmission and plasticity in the rodent hippocampus.

Receptors	GABA		Ionotropic Glutamate			Metabotropic Glutamate		
	$GABA_A$	$GABA_B$	NMDA	AMPA	Kainate	I	II	III
Subunits	α: 6 γ: 3 β: 4 ρ: 3 δ: 1 ε: 1	c, d, R1a, b R2	NR_{1a-e} NR_{2A-D}	Ca^{2+}permeable GluR 1-4 Ca^{2+}impermeable GluR 2	$GluR_{5-7}$ edited $GluR_{5,6}$ $KA_{1,2}$	$mGlu_{1,5}$	mGlu 2,3	$mGlu_{4-8}$
Signals	Cl^-	$G_{i/o}$ ▼ AC	Ca^{2+} K^+ Na^+	Ca^{2+} K^+ Na^+ Mg^{2+}	K^+ Na^+	$G_{q/11}$ ▲ PLC	$G_{i/o}$ ▼ AC	$G_{i/o}$ ▼ AC
Agonists	GABA Muscimol Zolpidem	Baclofen GABA	NMDA L-Glu L-Asp Glycine Serine	AMPA Kainate Glutamate	Kainate ATPA	DHPG ACPD	LY379 268	L-AP4 ACPD
Antagonists	Bicuculline SR95531 ZK93426 Flumazenil BZDs	CGP55845 A Saclofen	D-AP5 AP7 CPP	CNQX Mg^{2+}	LY382884 NBQX CNQX	AIDA	MCPG	LY34149 5 CPPG

Table 1.2.1. The principal receptor systems involved in hippocampal synaptic transmission and plasticity (Nistry and Constanti, 1979; Bigge, 1999; Botrolotto *et al.*, 1999; Bigge, 1999).

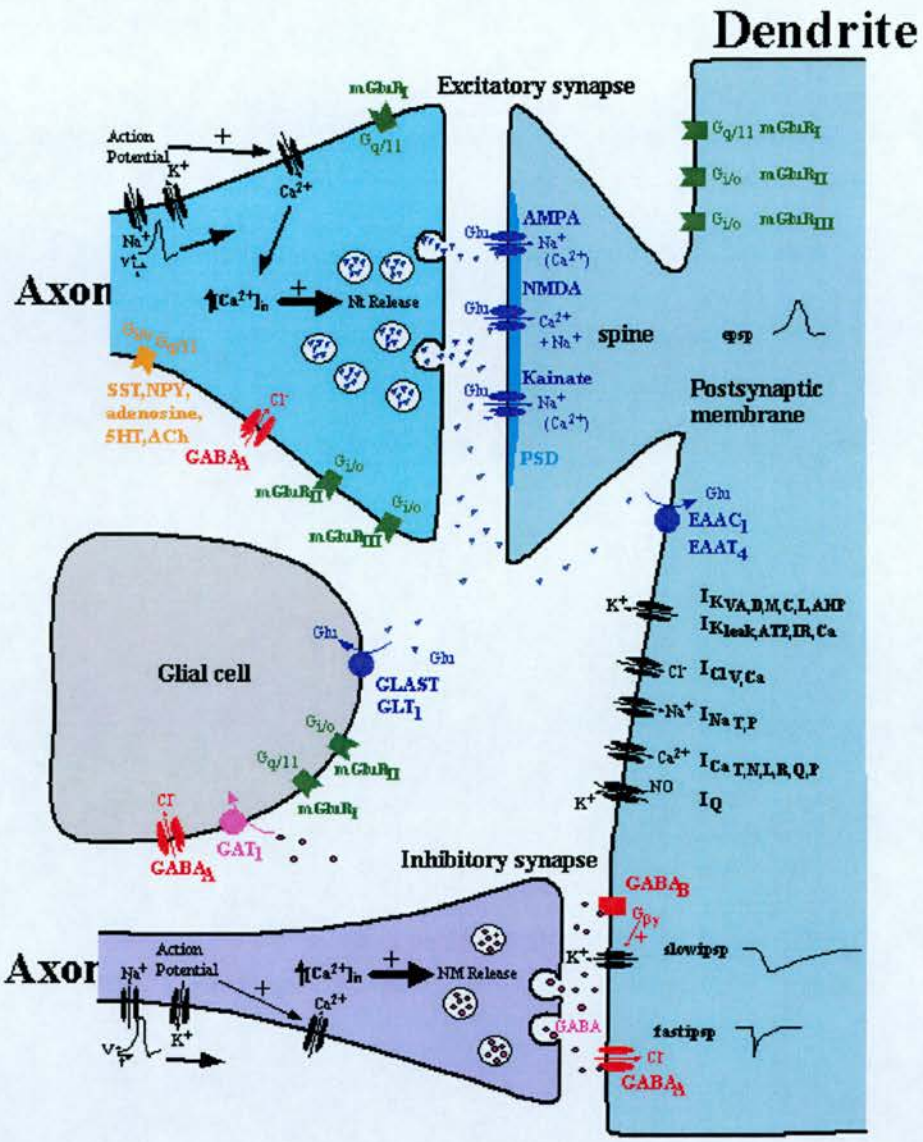


Figure 1.5. Schematic representation of the molecular mechanisms of the principal excitatory and inhibitory receptor systems involved in synaptic transmission and plasticity at the *en passage* synapses of the Schaffer collateral comissural pathway with CA1 apical and basal dendrites (picture obtained from F. Duprat with permission but based on research discussed in Collingridge and Bliss, 1993 and Bortolotto *et al.*, 1999).

1.2.5. Synaptic Plasticity

When the Spanish anatomist Ramon y Cajal gave the Croonian Lecture to the Royal Society in London in 1894, he advocated that the physiological basis of memory lay in the alterations of neuronal connectivity. He put forward the idea that the likely site, where activity of neuronal circuits could encode memory traces, might be between adjacent neurones, the synapses.

Fifty years on, Konorski (1948, summarised in Konorski, 1967) and Hebb (1949) each suggested rules by which these synaptic changes might occur. The timescale and extent of these changes are variable ranging from the permanent gain or loss of synapses to transient fluctuations in the strength of individual synapses, thereby altering the efficacy with which they may interact with the postsynaptic cell. Hebb's rule published in 'The Organisation of Behaviour' in 1949 states that if two neurones are excited at the same time, then any active synapses between them will be strengthened. Hence, Hebbian synapses act as coincidence detectors, equipped to monitor the degree of correlation between firing of pre- and postsynaptic neurones. As such, although the way the hippocampus encodes memory is not precisely known, it is thought to occur via a Hebbian mechanism. Indeed, computer modeling, which retains fidelity to hippocampal cytoarchitecture, suggests that the CA3 region acts as a network, where arbitrary associations between active stimuli are stored as a code of modified strengths. Crucially, in this model the change in synaptic strength that encode the associations are generated by Hebb's rule.

In the 1970s, Kandel and his colleagues established that synapses increase or decrease in efficiency during learning in *Aplysia* and that these changes may be short or long-lived. Two initial discoveries suggested an important role for the hippocampus in memory formation. Firstly, it was found that during recording from hippocampal pyramidal cells in conscious rats during exploratory navigation, the hippocampus could encode information about space (O'Keefe & Dostrovsky, 1971). Secondly, the synapses within the tri-synaptic circuit of the hippocampus undergo long-term potentiation (Bliss & Lømo, 1973), called LTP for short.

Bliss and Lømo discovered, by serendipity, that a high frequency train of action potentials evoked at any of the three major pathways of the hippocampus produced LTP, i.e. the persistent increase in efficacy of synaptic transmission lasting hours *in vitro* and in the anaesthetized animal and days or weeks in the awake, freely moving animal. In addition, a theta burst stimulation protocol (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). Indeed, LTP has been suggested to be the molecular model for learning and memory. This is a widely debated topic, which is further discussed in more detail in paragraph 1.2.6.

In vitro, LTP is typically measured as the sustained rise in the slope and amplitude of the field excitatory postsynaptic potential (fEPSP) mediated principally by ionotropic glutamate receptors in the hippocampus. In the Schaffer collateral commissural pathway, where CA3 cells synapse with CA1 apical and basal dendrites, LTP is Hebbian in nature. This can be explained by the properties of the NMDA receptor and the role of synaptic inhibition in the induction of LTP by tetanic stimulation.

How Receptors mediate coincidence-detection

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, thereby allowing the entry of Ca^{2+} through the NMDA receptor upon the release of glutamate into the synaptic cleft (Mayer *et al.*, 1984). There is little chance of activating the NMDA receptors sufficiently to lift their voltage dependence due to Mg^{2+} blockade by low frequency stimulation (LFS). The frequency dependence of LTP induction is largely attributable to the susceptibility of NMDA receptor-mediated currents to $GABA_B$ receptor mediated 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). That NMDA receptor activation requires both glutamate and sufficient depolarisation of the postsynaptic

membrane to induce LTP, is shown by the finding that the latter process is blocked by properly timed hyperpolarising pulses and facilitated by blockade of postsynaptic inhibition using GABA antagonists. Several reasons may account for the large depolarisation generated by tetanic stimulation as it has the following effects:

(a) it produces sustained glutamate release causing summation of AMPA receptor mediated EPSPs,

(b) high internal Cl^- and external K^+ renders GABAergic inhibition less effective due to prolonged activation of both $GABA_A$ and $GABA_B$ receptors respectively, causing the equilibrium potentials for these ions to shift in depolarising direction and

(c) $GABA_B$ autoreceptors, during HFS, inhibit GABA release when activated by GABA, thereby compromising inhibition further.

Once induced, LTP does not depend on the activation of NMDA receptors any more, as antagonists to the receptor do not affect expression of LTP. The maintenance and expression of LTP may be pre- and/or postsynaptic in origin. Experimental evidence suggests that modifications in either synaptic locus are in a delicate balance, which depends on the protocol of induction and the timescale of the experiment assessing the activity dependent change following induction. For example, protein synthesis inhibitors have been shown to inhibit LTP after 3 hours *in vitro* and both transcription and translation inhibitors *in vivo*. However, these drugs do not affect LTP induced by a less robust HFS protocol.

Thus synaptic potentiation can be temporally categorised into:

(1) short-term potentiation (STP), lasting up to 30 minutes

(2) early-phase LTP (E-LTP), lasting up to 3 hours and

(3) late-phase LTP (L-LTP), lasting up to 8 hours, *in vitro*.

Table 1.2.2. summarises the characteristics of kinases active during STP and E-LTP.

Kinase	LTP Induction Protocol	Activation Timecourse
CaMKII	Theta-burst	Up to 1 hour
PKA	Multiple tetanic	Up to 10 minutes
PKC	Multiple tetanic	Up to 45 minutes
PKM	Single tetanic	Active in 30 minutes
P42 MAPK	Multiple tetanic	Active from 2 minutes
SRC	Multiple tetanic	Active from 1 minute

Table 1.2.2. Temporal characteristics of kinase activation during E-LTP (Soderling & Derkach, 2000).

L-LTP is thought to depend on protein translation and transcription rather than kinase activation. There is no *a priori* reason favouring a common synaptic locus for the latter components. However, a consensus is emerging that NMDA receptor dependent E-LTP in CA3-CA1 synapses embraces a postsynaptic locus (Diamond *et al.*, 1998).

Calcium

Upon activation, the NMDA receptor allows intracellular Ca^{2+} entry (Dani, Jahr & Stevens 1987). Increases in intracellular Ca^{2+} concentration 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 (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 (IP_3)-induced release of Ca^{2+} and ryanodine receptor sensitive calcium-induced Ca^{2+} release from intracellular stores (Obenaus *et al.*, 1989; Harvey & Collingridge, 1992). Inhibitors of these stores also inhibit LTP. Although, LTP in the CA1 area is associative, LTP induction in the synapses of mossy fibres with CA3 pyramidal cells does not depend on NMDA receptor

activation, nor on intracellular calcium elevation in the postsynaptic neuron. LTP there appears to be dependent on increased neurotransmitter release from the presynaptic bouton (Bennett, 2000).

Postsynaptic Modifications: Signal transduction by protein phosphorylation and dephosphorylation

Support for the postsynaptic hypothesis for associative LTP is that synaptic plasticity is expressed via an augmented sensitivity of the postsynaptic membrane brought about by:

(a) an increase in number of receptors

(b) a change in the phosphorylation state of existing receptors, so that they become more sensitive. Experimental evidence suggests that both AMPA and NMDA receptors have consensus sequences encoding phosphorylation sites, acted upon by kinases and phosphatases.

(c) a change in the cytoskeletal systems, brought about by intracellular second messenger systems e.g. of the scaffolding organelle holding the NMDA and AMPA receptors attached at the postsynaptic membrane, postsynaptic density (PSD).

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 (Matthies *et al.*, 1990). 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 that 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).

Several Ca^{2+}-sensitive 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 evidence that implicated protein phosphorylation following LTP induction in the perforant pathway of hippocampal

slices came from Bar *et al.*, in 1980. They observed an enhanced incorporation of [^{32}P] into a protein band with a molecular weight of 50 KDa. If LTP was not induced, then no enhanced incorporation of [^{32}P] was observed. However, it was not until 1981, that it was shown conclusively that E-LTP could be blocked by kinase inhibitors (Lovinger *et al.*, 1981; Reymann *et al.*, 1988a-b; Malinow *et al.*, 1988). In 1989, it was shown that intracellular injection of the non-selective kinase protein inhibitor H-7 into CA1 pyramidal neurons blocks LTP induction (Malinow *et al.*, 1989). Although intracellular injection of H-7 does not block LTP expression following its induction, it does so when applied extracellularly (Malenka *et al.*, 1989), which suggests a presynaptic locus for expression while a postsynaptic locus for LTP induction.

Calcium-dependent Kinases

As mentioned above, LTP induction may be blocked by intracellular calcium chelators and ser/thr protein kinase inhibitors. Therefore, a role in LTP expression of CaMKII and/or PKC becomes plausible.

The first kinase to be implicated in LTP was the Ca^{2+}/phospholipid-dependent protein kinase (PKC) (Bar *et al.*, 1980). 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. Phorbol esters, which are known PKC activators, were found to enhance synaptic transmission in hippocampal slices, in a manner similar to LTP (Routtenberg *et al.*, 1986; Malenka *et al.*, 1986). Furthermore, it was shown that PKC activity was elevated twofold in membranes from hippocampal regions subjected to LTP induction paradigms (Akers *et al.*, 1986). PKC specific inhibitors, e.g. PKC-(19-31), block LTP induction (Malinow *et al.*, 1989). Similarly, antibodies raised against specific PKC isoforms prevent long-term synaptic plasticity (Lopez-Molina *et al.*, 1993; Angestein & Staak, 1997). Active PKC injection to the postsynaptic site potentiates synaptic plasticity (Hu *et al.*, 1987). Though the method of activation during LTP is still under investigation, recent evidence points to the involvement of G-protein coupled receptors to PLC activation e.g. group I metabotropic glutamate (mGluRs)(Otani *et al.*, 1993). Consistent with the above

observation it has been shown recently that mGluRs constitute a requirement for LTP induction (Bortolotto *et al.*, 1994, 1995).

Enhanced activity of PKC in the CA1 area of the hippocampus in transgenic mice has been reported to aid in their performance in spatial learning behavioural tasks (Fordyce *et al.*, 1994).

The Ca^{2+}/calmodulin-dependent protein kinase CaMKII has also been implicated in LTP (Malenka *et al.*, 1989; Malinow *et al.*, 1989; Lledo *et al.*, 1995; Bortolotto & Collingridge, 1998; Fukunaga & Miyamoto, 1999; Malenka & Nicoll, 1999). CaMKII is an oligomeric protein (10-12 subunits) and is contained at high levels in PSD. Its unusual biochemical properties render it capable of sustaining prolonged kinase activity in response to a transient elevation of calcium in the dendritic spine, which persists even after the calcium transient is gone. CaMKII targets the native AMPA receptor following its activation during LTP (Baria *et al.*, 1997a), as shown in isolated PSD studies (Brown & Schulman, 1995) and cultured hippocampal neurons (Tan *et al.*, 1994) and the GluR1 subunit of the AMPA receptor in HEK293 cells (Baria *et al.*, 1997a). All the above observations are corroborated with the use of GluR2 subunit knockout transgenic mice, where LTP is slightly enhanced (Jia *et al.*, 1996). In contrast, in adult GluR1 null transgenic mice, LTP is absent in the CA1 area (Zamanillo *et al.*, 1999). The physiological significance of GluR1 subunit phosphorylation by CamKII in all of the above preparations result in potentiation of AMPA receptor mediated whole-cell current, which could contribute to synaptic potentiation during LTP. Transgenic mice lacking CaMKII show impaired synaptic plasticity, inability to form stable place cells and cognitive performance in learning and memory tasks (Bach *et al.*, 1995; Mayford *et al.*, 1996; Routtenberg *et al.*, 1996). Recent evidence also points out a role for CaMKII in carbachol-induced, theta-like rhythmic activity in the CA1 area of the hippocampus in rats; a process thought to modulate synaptic plasticity during exploratory behaviour *in vivo* (Alberi *et al.*, 2000). Finally, other kinases believed to influence LTP expression are cAMP- and cGMP-dependent protein kinases (PKA and PKG respectively) (Chetkovich *et al.*, 1991) and the SRC family of tyrosine kinases (O'Dell *et al.*, 1991b).

SRC Tyrosine Kinases

NMDA receptors form heteromeric channels, which consist mainly of NR1 and NR2A-D subunits and they form complexes in the PSD with, amongst other proteins, PSD-95, Ca^{2+}-Calmodulin and the tyrosine kinase family members (Kennedy, 1998). Although FYN tyrosine kinases have been shown to be important for LTP expression in the hippocampus (O'Dell *et al.*, 1991b; Grant *et al.*, 1992; Grant & O'Dell, 1994), emphasis has been recently put on SRC family of tyrosine kinases.

Phosphorylation of the NR2A and NR2B subunits of the NMDA receptor result in the alleviation of zinc inhibition and the potentiation of current through the receptor (Zheng *et al.*, 1998). SRC-specific inhibitors prevent LTP induction (Lu *et al.*, 1999). SRC or FYN infusion culminates in the potentiation of AMPA receptor mediated current, which probably results from the enhancement of NMDA receptor function (Yu *et al.*, 1997). The aforementioned findings led to the development of the hypothesis that LTP induction results in the rapid activation of the SRC family of tyrosine kinases, which enhance calcium influx through the NMDA receptor by altering its channel properties, via phosphorylation. This, in turn, results in the activation of calcium-dependent kinases e.g. CaMKII, leading to potentiation of AMPA-receptor-mediated current (Soderling & Derkach, 2000). The action of different kinases reflects the temporal segregation of synaptic potentiation phases (Table 1.2.2.).

The activation of kinases, e.g. CaMKII, is shut down or reversed by protein dephosphorylation due to the action of phosphatases, such as PP1, PP2A and 2B (calcineurin)(Soderling & Derbach, 2000). Stable synaptic potentiation requires the prolonged activation of CaMKII, as mentioned above, which can be maintained by inhibition of such phosphatases (Makhinson *et al.*, 1999).

Further Postsynaptic Modifications

Other mechanisms contributing to E-LTP induction and expression include the 'silent synapse' hypothesis, which states that prior to LTP induction some synapses do not have operative AMPA receptor channels, while following LTP induction they exhibit AMPA receptor mediated currents (Isaac, Nicoll & Malenka, 1995; Liao *et al.*, 1995; Isaac *et al.*, 1998). This might result from the rapid insertion of AMPA

receptors into the postsynaptic membrane from an intracellular pool, which is thought to be protein a phosphorylation-dependent process. Consistent with this hypothesis, postsynaptic infusion of the membrane fusion pathway, such as N-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment protein (SNAP), depress LTP (Lledo *et al.*, 1998). Infusion of SNAP enhances LTP and this enhancement is occluded by prior induction of LTP. NSF and SNAP both bind to the GluR2 subunit and disruption of the formation of this complex results in the rundown of AMPA receptor mediated whole cell current (Noel *et al.*, 1999). Finally, calcium seems to promote exocytocis of dendritic organelles, which is thought to be a CaMKII dependent process (Maletic-Savatic *et al.*, 1998). Hence, there seems to be AMPA receptor translocation to the postsynaptic membrane going on following LTP induction. However, the functionality and precise location of these receptors have yet to be shown. The use of protein synthesis inhibitors has demonstrated that protein synthesis from existing mina is required for the maintenance of L-LTP during the first few hours (Krug *et al.*, 1984; Obtain *et al.*, 1989; Frey *et al.*, 1989). However, there is also evidence for gene transcription and increased mina production after tantalization (Hackler *et al.*, 1992; Dragoon *et al.*, 1989; Nicole *et al.*, 1991). Of particular interest is the involvement of camp-response-element-binding-protein (CREB)-mediated transcription in LTP, as indicated by genetic studies in *Drosophilae* and *Alyssa*. However, it is not clear which kinases are responsible for CREB phosphorylation during LTP (Frank & Greenberg, 1994).

Presynaptic Modifications

As well as postsynaptic modifications, presynaptic modifications have also been considered as a locus of LTP expression. However, a presynaptic hypothesis for LTP expression has to explain how an initial postsynaptic triggering event may cause a sustained increase in glutamate release. This concept originally maintained by Bliss & Collingridge and independently by Kandel, challenges the original concept voiced by Sherrington that synapses are polar and that electrical communication between neighboring neurones is synapse-specific.

One such idea is that this process may involve 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. The functional significance of the retrograde messengers is currently an issue of debate, due to the fact that their effect may not be synapse specific and to the lack of incontrovertible evidence supporting their existence. Another idea implicates presynaptic protein kinase activity in LTP expression in CA3 synapses between pairs of hippocampal neurons. Direct injection of the non-selective kinase blocker H-7 into the presynaptic neurone, inhibits LTP expression probably by restricting glutamate release (Pavlidis *et al.*, 2000).

Long-term Depression

Long-term depression (LTD) is a long-lasting, activity-dependent reduction in glutamatergic synaptic strength most prominent in the hippocampus and cerebellar cortex (Christie *et al.*, 1994, 1996; Goda & Stevens, 1996). In the CA1 area repetitive, low frequency stimulation (1-5 Hz) of a synapse causes a gradual reduction in the slope and amplitude of the fEPSP, which is stable for more than an hour. Such homosynaptic LTD is input-specific and depending on the area of the brain can be induced by a variety of stimulation protocols. For example, in the striatum LTD induction requires tetanic stimulation. Like LTP, LTD has cooperative and associative properties.

LTP and LTD interact (Abbot & Nelson, 2000) and in the CA1 area their induction depends on the activation of NMDA receptors (Dudek & Bear, 1992; Mulkey and Malenka, 1992). There is a wealth of evidence suggesting that both LTP and LTD are mechanistically similar, for example induction of both depends on intracellular calcium levels and blocked by inhibitors of intracellular calcium stores. Hippocampal LTD depends on a critical level of depolarisation partly mediated by NMDA receptors, but is not always inhibited by NMDA receptor inhibitors.

Finally, it has been proposed that the phosphorylation state of CaMKII, which critically depends on intracellular calcium levels, determines synaptic strength (Lisman, 1985; Lisman & Goldring, 1988; Lisman, 1994). If the phosphorylated form predominates then the synapse undergoes LTP, while if the concentration of the dephopshorylated form is higher, LTD is favoured. The phosphorylation state of

CaMKII is determined by calcium dependent phosphatase enzymes e.g. PP1 (Dudek and Bear, 1992; Mulkey and Malenka, 1992).

1.2.6. Role of LTP in Learning and Memory

One of the reasons why hippocampal synaptic plasticity is one of the major research interests of neuroscience is the generally shared belief that the mechanisms underlying plasticity are common to those facilitating learning and memory. Evidence in support of this concept has recently been reviewed by Martin *et al.* (2000).

Although, the fact LTP induction subsides as the animal gets older was already known, no causal relationship was established between them (Barnes, 1979). This came with the discovery that in behavioural tasks assessing memory and learning in rats, e.g. the Morris water maze task, their performance is disrupted by NMDA receptor antagonists, such as D,L-AP5. This observation points to LTP as the underlying mechanism for hippocampus-dependent spatial navigation and learning (Morris *et al.*, 1986). Place learning is a hippocampus dependent process (O'Keefe, 1999). The inactive L-isomer of AP5 did not have any effect on spatial learning in rats tested. As there was no impairment in LTP-independent visual discrimination tasks, the findings established a correlation between LTP and hippocampus-dependent spatial learning. Furthermore, it was found that saturation of LTP in the pyriform pathway to the dentate gyrus *in vivo* impaired spatial learning (McNaughton *et al.*, 1986; Castro *et al.*, 1989).

Targeted knockout mutations are being increasingly used to allow the relationships between learning and cellular processes to be identified. In most, but not all studies, impairment in hippocampal synaptic plasticity is accompanied with cognitive deficits and with a parallel distortion of place cell properties (Tsien, 1996; Rampon et al., 2000). Nevertheless, this experimental approach has been heavily critisiced on the grounds that during the development of transgenic animals any number of secondary alterations may occur as a result of the primary mutation and that these may be responsible for the altered phenotype (Gerlai *et al.*, 1998). Tsien *et al.* surpassed this problem by pioneering a time-dependent targeted mutation occurring in mice after

hippocampal development is completed only in CA1 hippocampal cells. Floxed NMDAR1 mice fail to show NMDAR-dependent LTP in the CA1 accompanied by impaired performance in the watermaze task and coupled with distorted place cell properties in the CA1 area. Further elucidation of the role of the hippocampus in learning and memory will follow if similar strategies are used to target precicely timed gene mutations in other regions, such as the CA3 and entorhinal cortex. The role of transgenic approaches in the development of mouse models of learning and memory will be discussed in paragraph 1.4.

It is noteworthy that there is growing experimental evidence to support the role of LTP-like, plasticity related processes in neuronal growth, development and death in most parts of the mammalian brain. However, as much as synaptic transmission and plasticity depend on glutamatergic excitatory neurotransmission, there are other important neurotransmitter inputs to the hippocampus, which are thought to modulate learning and memory, like acetylcholine (reviewed in Pepeu, 2001) and galanin (Ukai *et al.*, 1995; O'Meara *et al.*, 2000). In the next paragraph, a brief review on the major cholinergic projection, the septohippocampal pathway, to the hippocampus will be presented.

1.3. 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 ageing process and, furthermore, the target of neurodegenerative diseases such and Alzheimer's disease where loss of cognitive function, particularly memory, is a major symptom.

1.3.1. Anatomical Structure

The septohippocampal afferents project from large neurons located in the medial septal nucleus (MS) and the vertical limb of the diagonal bands of Broca (vDBB). These neurons form a continuum of cells that are difficult to subdivide because of the absence of clear histological boundaries.

It has been demonstrated that choline acetyltransferase (ChAT)-positive axons infiltrate the hippocampus at postnatal day 2 in the rat (Hofmann & 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). 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). 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).

1.3.2. 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 (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.*, 1971), supported by *in vivo* release of ACh in the hippocampus after medial, but not lateral, septal stimulation (Dudar, 1975).

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 (Amaral & Kurz, 1985). 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* (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.

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 (Forloni *et al.*, 1987; Melander *et al.*, 1986a-b).

1.3.3. 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 lead to a deficit in working memory, especially in spatial tasks (Olton *et al.*, 1978a-b; Poucet & Herrmann, 1990). 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, produces long lasting spatial learning impairments after cholinergic denervation.

Microinjection of phenoxybenzamine, an α-noradrenergic receptor antagonist, or bicuculine, a $GABA_A$ receptor antagonist, caused an impairment of working memory (Chrobak *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, have long been known to impair many types of memory tasks.

Following learning tasks, increased ChAT and ACh levels have been observed (Jaffard *et al.*, 1980) 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). 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. This evidence demonstrates a capacity of the septohippocampal system to compensate for loss of function.

1.3.4. 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 has been observed with age (Biegon *et al.*, 1986; Fischer *et al.*, 1989) whereas only a decrease in the size of AChE-positive cells is 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 Lacaille *et al.*, 1991). In addition to neuronal loss, the sensitivity of hippocampal neurons to cholinergic agonists is decreased both *in vivo* and *in vitro* (Lippa *et al.*, 1981; Segal, 1982a; Potier *et al.*, 1992). Additionally, it has been 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 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. 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 (Perry *et al.*, 1978).

The development of mouse transgenic models for neurodegenerative diseases has shed some light on the mechanisms underlying the pathology of several diseases especially senile dementia and dementia of the Alzheimer's type. As this thesis will be dealing with the synaptic processes underlying learning and memory in rodents in health and disease and particularly focusing on mouse models of the ageing brain, a brief account is laid out here of research performed on existing mouse models of dementia.

1.4. Transgenic Models of Learning and Memory

In an attempt to link Hebbian LTP to memory formation several approaches have been used in neuroscience research. In section 1.2.6., some of them were described, namely the use of behavioural tasks assessing learning and memory in combination with pharmacological manipulations thought to disrupt synaptic plasticity in the hippocampus *in vitro* (Collingridge *et al.*, 1988; Morris *et al.*, 1986; McNaughton *et al*, 1984). These methods, although innovative at the time, did not account for the effects of non-region specific drug diffusion and drug toxicity, factors which may interfere with learning behaviour. An alternative method is to use genetic models to examine the correlation between LTP and memory. Such models rely on the design and production of mutant animals and the analysis of the impact of the manipulated gene on synaptic plasticity in vitro and on the performance of animals in learning and memory paradigms. When genes resulting in proteins that regulate LTP are aberrant, synaptic plasticity is usually disrupted and this may be accompanied by a concomitant impairment in spatial learning.

How reliable is the correlation between LTP and learning then?

Although this is usually the expected outcome, there are exceptions to the rule e.g. in mice lacking GluR1 subunit of the AMPA receptor complex basal transmission is not affected, but LTP is impaired. Interestingly, these mice do not show any cognitive deficits in spatial learning acquisition (Zamanillo *et al.*, 1999). Another example comes from mutant mice lacking the gene for PSD95 protein, which show enhanced LTP *in vitro*, but impaired performance in the Morris watermaze task (Migaud *et al.*, 1998).

Disadvantages of the genetic approach

The genetic approach may result in the 'gain-of-function' phenotypes in transgenic mice, as many recent reports claim. For example, in GluR2-null mice an enhanced NMDA receptor – independent LTP was observed, coupled with a ninefold increase in kainate induced calcium permeability. These mice showed several behavioural anomalies, probably due to the lack of GluR2 during development (Jia *et al.*, 1996;

Gerlai *et al.*, 1998). In transgenic mice overexpressing the constitutively active form of FYN in the forebrain, LTP was reported to be enhanced and was induced by low threshold theta stimulation (Lu *et al.*, 1999). In addition, GABAergic inhibition was compromised.

Furthermore, the behavioural phenotyping of the cognitive performance of mutant mice mostly consists of the Morris watermaze task, when other tests should be used as well, such as hippocampus-dependent non-spatial tasks e.g. novel object recognition task, contextual fear conditioning and social transfer of food preference.

Differences in the genetic background of mutant mice are not the only reason to account for differences in the behavioural tasks. Other factors include differences in the experimental execution, handling of the animals and the lack of standardized behavioural testing conditions. Moreover, as not every genetic method guarantees molecular specificity, failure to detect a behavioural phenotype may arise from genetic compensation or inherent insensitivity of the behavioural test. Finally, as the traditional knockout approach introduces a null mutation in every tissue throughout development, interpretations of these transgene animals should be done to account for any possible developmental change and genetic compensation at both the synaptic and behavioural level.

Interpreting the Phenotype of Knockout Mice

The genetic approach has proven more specific than the pharmacological approach, especially in cases where the antagonists are not specific to the different receptor subtypes e.g. 5-HT1B knockout mice have revealed a role in the anti inflammatory effects of sumatriptan and the anorectic effects of fenfluramine (Yu *et al.*, 1996; Lucas *et al.*, 1998). However, if the mutated gene is not present throughout development may yield no or unexpected phenotypic changes in the adult animal. The absence of the gene may cause developmental compensation from other genes to make up for the loss and render a viable adult organism. For example, mice bearing null mutation for either eNOS or nNOS do not show impaired LTP. However, double knockouts for both genes show a clear deficit in LTP (Son *et al.*, 1996). Null mutations may affect the expression of neighbouring genes e.g. in the case of pGK NEO promoter, which reduces expression of downstream Hox genes. The genetic

background strain is very important when trying to determine phenotypic characteristics of a knockout mouse strain e.g. 5-HT1B mice have been shown to display differences to alcohol preference as a result of a drift in their genetic background (Phillips *et al.*, 1999). Other factors confounding the interpretation of a knockout phenotype include maternal behaviour (Liu *et al.*, 1997) and pleiotropy e.g. in 5-HT1A receptor knockout mice, it is unclear whether the phenotype of increased anxiety is due to presynaptic, postsynaptic receptors or both.

The conditional knockout

The first region- and cell type-specific knockout was produced by Tsien *et al.*, in 1996. The adult mice did not express the NR1 subunit of the NMDA receptor in CA1 pyramidal neurons and this modification resulted in the complete loss of NMDA currents, STP, LTP and LTD in the CA1 area, while the gross anatomy of the area remained intact. These mice were cognitively impaired in hippocampus dependent tasks assessing both spatial and non-spatial learning (Rampon *et al.*, 2000), but were normal in hippocampus independent tasks, such as cued fear conditioning. Analysis of the conditional knockout showed that the NMDA receptor is crucial for plasticity induction in the CA1 area and for spatial learning and memory. Interestingly, overexpression of NR2B subunit of the NMDA receptor channel in the forebrains of transgenic mice led to an enhancement of LTP and an enhanced performance in six different behavioural tasks, but had no effect on LTD in the CA1 area (Tang *et al.*, 1999). These experiments further strengthen the role of the NMDA receptor as a coincidence detection switch for linking Hebbian LTP and learning and memory in rodents.

The first successful transgenic model of disease

The first successful transgenic mouse model of Alzheimer's disease was developed in 1995 by Dora Games *et al.* (1995). It involved the overexpression of V717F human β-amyloid precursor protein, which rendered a mouse strain with aspects of Alzheimer-type neuropathology. More specifically the mutant mice show an age-dependent development of neuritic plaques, synaptic loss and gliosis. This discovery confirmed the role of V717F β-amyloid precursor protein (PDAPP mice) in the development of hallmark anatomical features of dementia. These mice develop many

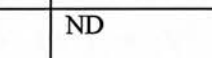
hallmark AD neuropathologies including Aβ deposition into senile plaques in the hippocampus and cortex, neurodegerative changes and astrocytosis, microgliosis and the deposition of acute phase proteins. The brain regions most affected are those areas associated with early and extensive damage in AD. These neuropathological changes in the transgenic mice have been confirmed in at least five generations of animals. The AD symptoms investigated are displayed in a robust manner that increases with age and gene dosage. The model provides evidence that abnormal APP processing and Aβ deposition can result partly in AD like neuropathology and can contribute to a mechanistic understanding of AD as well as providing a useful model for the testing of various therapeutic interventions directed towards specific aspects of the neurodegenerative process (Chen *et al.* 1998). In agreement with the above findings, impaired synaptic plasticity and learning, and impaired histology were observed in hAPP695SWE mice (Chapman *et al.*, 1999).

Finally, Lewis *et al.*, (2001), managed to generate a transgenic mouse model bearing both major hallmarks of AD, i.e. amyloid plaques and neurofibrillary tangles and neurofibrillary tangles celebrating the possibility that genetic manipulation of mice may result in experimentally useful models of neurodegerative disease.

Table 1.4.1 summarises the characteristics of some transgenic mice developed so far.

Mutation	Neuroanatomy	NMDAR properties	Input/Output	PPF	LTP Induction	LTP Maintainance	Reference
NR1 Ko	As Wt	No functional NMDARs	ND	ND	ND	ND	Forrest *et al.*, 1994
NR1 CA1 Ko	As Wt	No functional NMDARs		NA	No LTP	NA	Tsien *et al.*, 1996
NR2A Ko	As Wt	NMDAR current reduced	As Wt	NA	LTP	<Wt	Sakimura *et al.*, 1995
NR2A - C-Terminal	ND	Increased rate of desensitisation	ND	ND	No LTP	NA	Sprengel *et al.*, 1998
NR2B Ko	Impaired whisker patterns	ND	ND	NA	ND	ND	Rostas *et al.*, 1996
NR2C Ko	ND	NA	NA	ND	NA	NA	Ebralidze *et al.*, 1996
GluR1 Ko	ND	As Wt	Somatic <Wt Dendritic >Wt	ND	No LTP	NA	Zamanillo *et al.*, 1999
GluR2 Ko	As Wt	As Wt, AMPAR increased permeability to Ca^{2+}	As Wt	As Wt	2*>Wt not saturable	>Wt	Jia *et al.*, 1996
GluR2 Q/R site	Atrophy basal CA3 dendrites	As Wt	ND	ND	AMPAR LTP	Dep GluR2 edition and Ca^{2+} inlfux	Feldmeyer *et al.*, 1999
GluR5	ND	ND	ND	ND	?	?	Sailer *et al.*, 1999
mGluR1 Ko	ND	As Wt	ND	As Wt			
mGluR1 Ko	As Wt	NA	ND	As Wt	ND CA1 No mossy fibre LTP	NA	Aiba *et al.*, 1994
mGluR2 Ko	As Wt	ND	ND	ND	ND LTP No LTD	ND	Yokoi *et al.*, 1996
mGluR4 Ko	ND	NA	NA	NA	Impaired	Impaired	Pekhletski *et al.*, 1996

Mutation	Neuroanatomy	NMDAR properties	Input/Output	PPF	LTP Induction	LTP Maintainance	Reference
mGluR5	ND	ND	ND	ND	ND in CA1	ND	Lu *et al.*, 1997
mGluR7 Ko	As Wt	NA	?	>Wt	NA	NA	Manahan-Vaughn *et al.*, 1995
PSD-95 truncation	As Wt	As Wt	>Wt	ND	>Wt	>Wt	Migaud *et al.*, 1998
Ras-GRF Ko	As Wt	ND	ND	ND	As Wt	As Wt	Brambilla *et al.*, 1997
Yes Ko	As Wt	ND	ND	As Wt	As Wt	As Wt	Grant *et al.*, 1992
Abl Ko	As Wt	NA	ND	As Wt	As Wt	As Wt	Grant *et al.*, 1992
Fyn Ko	Abnormal cell number in hippocampus	As Wt	ND	As Wt	As Wt	<Wt	Grant *et al.*, 1992
α-CaMKII Ko	As Wt	As Wt	ND	<Wt	No LTP	NA	Silva *et al.*, 1992
α-CaMKII T286D activated form	As Wt	ND	As Wt	As Wt	As Wt	As Wt	Mayford *et al.*, 1996
Inducible aCaMKII T286D-activate form CA1 specific	NA	NA	NA	NA	No LTP induced (10Hz)	NA	Mayford *et al.*, 1996
PKCγKo	ND	As Wt	ND	>Wt	No LTP induced LTP induced if LTD previously induced	NA As Wt LTD precedes LTP induction	Abeliovich *et al.*, 1993
PKA R(AB) inhibitory domain forebrain specific	As Wt	ND	As Wt	As Wt	As Wt (100Hz) No LTP induced (10Hz)	No L-LTP	Abel *et al.*, 1997



Mutation	Neuroanatomy	NMDAR properties	Input/Output	PPF	LTP Induction	LTP Maintainance	Reference
PKA R1b regulatory subunit Ko	As Wt	ND	ND	As Wt	As Wt No mossy fibre LTP induced	As Wt NA in mossy fibre path	Brandon *et al.*, 1995
PKA Cb1 catalytic subunit specific	As Wt	NA	ND	As Wt	As Wt No mossy fibre LTP	As Wt NA in mossy fibre path	Huang *et al.*, 1995
CREB αδ Ko	As Wt	ND	>Wt	As Wt	LTP induced	No L-LTP decays to baseline	Bourtchula-d*zet al.*, 1994
n-NOS Ko	As Wt	ND	ND	As Wt	As Wt	As Wt	Son *et al.*, 1996
e-NOS Ko	ND	ND	ND	ND	As Wt	As Wt	Son *et al.*, 1996
n-NOS/ e-NOS double Ko	As Wt	ND	<Wt	As Wt	<Wt	<Wt	Frey *et al.*, 1996
t-PA KO	As Wt	ND	ND	As Wt	As Wt	Decays after 1 hr	Huang *et al.*, 1996
Steel Ko	As Wt	ND	ND	ND	As Wt	As Wt	Motro *et al.*, 1996
BDNF Ko	As Wt	As Wt	As Wt	As Wt	<Wt	<Wt LTP decays in Ko	Korte *et al.*, 1995
BDNF Ko	ND	ND	ND	<Wt	<Wt	<Wt	Patterson *et al.*, 1996

Mutation	Neuroanatomy	NMDAR properties	Input/Output	PPF	LTP Induction	LTP Maintainance	Reference
Fyn Ko	NA	NA	>Wt	NA	<Wt	NA	Lu et al., 1999
GAP-43 Tg	NA	NA	ND	ND	>Wt	>Wt	Routtenberg *et al.*, 2000

Table 1.4.1. Some Trangenic models developed, where gross anatomy of the hippocampus, synaptic transmission and plasticity in the CA1 area have been characterized (ND = no defference, NA = not analysed).

1.5. Aims

This thesis investigates neuronal mechanisms that may play a critical role in learning and memory. Specifically, the aims are to investigate further the mechanisms underlying LTP induction and maintenance *in vitro,* in the galanin deficient mouse strain with a view to defining the role of galanin (Chapter 4) in synaptic transmission and plasticity in the CA1 area of the hippocampus. Physiological experiments will be reported in the context of anatomical and behavioural studies. Further aims include the investigation of the role of galanin in synaptic transmission and plasticity in the CA1 Schaffer collateral commissural pathway in the rat by way of exogenously applied galanin, the assessment of the effect of the known galanin antagonist, M15, by administering it alone or co-applied with galanin and the comparison of synaptic transmission and plasticity processes in the rat with mutant and wild-type transgenic mice lacking galanin (Chapter 5). This research is considered important for the discovery of neuroprotective drugs and cognitive enhancers.

CHAPTER 2

METHODS & MATERIALS

2.1. Experimental Preparation

2.1.1. General

All experiments were performed using standard intra- (Morton & Davies, 1997) and extracellular electrophysiological recording techniques (Coumis & Davies, 1998). Synaptic recordings were made from the CA1 region of transverse parasaggital rat hippocampal slices obtained from 3-week old female Cobb-Wistar rats. All recordings from transgenic mice were also made from the CA1 region of transverse parasaggital mouse hippocampal slices obtained from male/female 129sv mice (Wynick *et al.*, 1998; O'Meara *et al.*, 2000).

2.1.2. Preparation of rat hippocampal slices

The rats were killed by cervical dislocation and were subsequently decapitated in accordance with U.K. Home Office guidelines (Schedule 1). The brain was rapidly removed 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 slices using a vibroslicer (Campden Instruments, Loughborough, UK). Throughout the slicing procedure the brain and slices were held in a chamber containing ice-cold, oxygenated ACSF. The slices were then transferred to a glass petri dish containing ACSF at room temperature (18–24 °C). A shallow petri dish was preferred than a 200ml beaker/tea strainer design, because the former allows for better oxygenation of the slices without damaging them.

The hippocampus was cut away from the rest of the brain slice and the CA3 region cut away mainly to eliminate changes in network function that can occur due to

epileptiform bursting in that area only when picrotoxin is applied to the slice or otherwise stated.

Following an incubation period of at least an hour, the resultant 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% 0_2, 5% CO_2; BOC Medical Gasses, UK), humidified atmosphere (Fig. 2.1). Slices were transferred using either a wide-bored Pasteur pipette or a wet painter's brush (No3). The slices were then allowed to equilibrate in this environment for approximately one hour before any electrophysiological recording was attempted. For some experiments, a submerged chamber was used allowing the flow of ACSF at approximately 2.5ml/min to allow better optics of the slices. This was achieved by an Olympus microscope and using both X10 and X40 magnifying lenses. 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. Equilibration period before recordings was essential to allow for recovery from excitotoxic amino acids, proteases and potassium ions, which are released because of cell damage during dissection. Slices were viable for up to 18 hours following dissection, as they were prepared from adult rodents.

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% 0_2, 5% CO_2 to maintain a pH of 7.4–7.5. ACSF was made up from solid salts 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

Galanin transgenic mice were obtained by the Wynick group in Bristol and not by myself, as follows:

A mouse 129sv cosmic genomic library was screened using a full-length rat galanin cDNA as a probe under high stringency. A positive/negative selection-targeting vector was constructed with the signal peptide for the coding region for galanin and most of the galanin associated peptide removed. The vector was linearised and electroporated in an E14-embryonic stem cell line. In total, nine clones were identified. The galanin loss of function mutation has been bred to homozygosity in the 129/OlaHsd mouse strain. Galanin levels were measured using radioimmunoassay in several brain regions, stomach and small intestine. Levels in heterozygotes were 50% of wild-type controls while levels in homozygotes were below the limit of detectability in all cases. Results of genotype analysis of live births were in the expected ratio predicted by Mendelian genetics and the sex ratio of homozygote offspring was 1:1 (Wynick *et al.*, 1998; O'Meara *et al.*, 2000).

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 warm (30-32°C) ACSF and held in place by short pieces of interwined 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). 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 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 angle microscope (M3C, Leica, England). Recording microelectrodes were placed, at an approximately 45° to the vertical, in either *stratum oriens* (CA1 pyramidal basal-dendritic layer) or *stratum radiatum* (CA1 pyramidal cell apical dendritic layer). During the acquisition of the experiments contained in this thesis ACSF was not recycled.

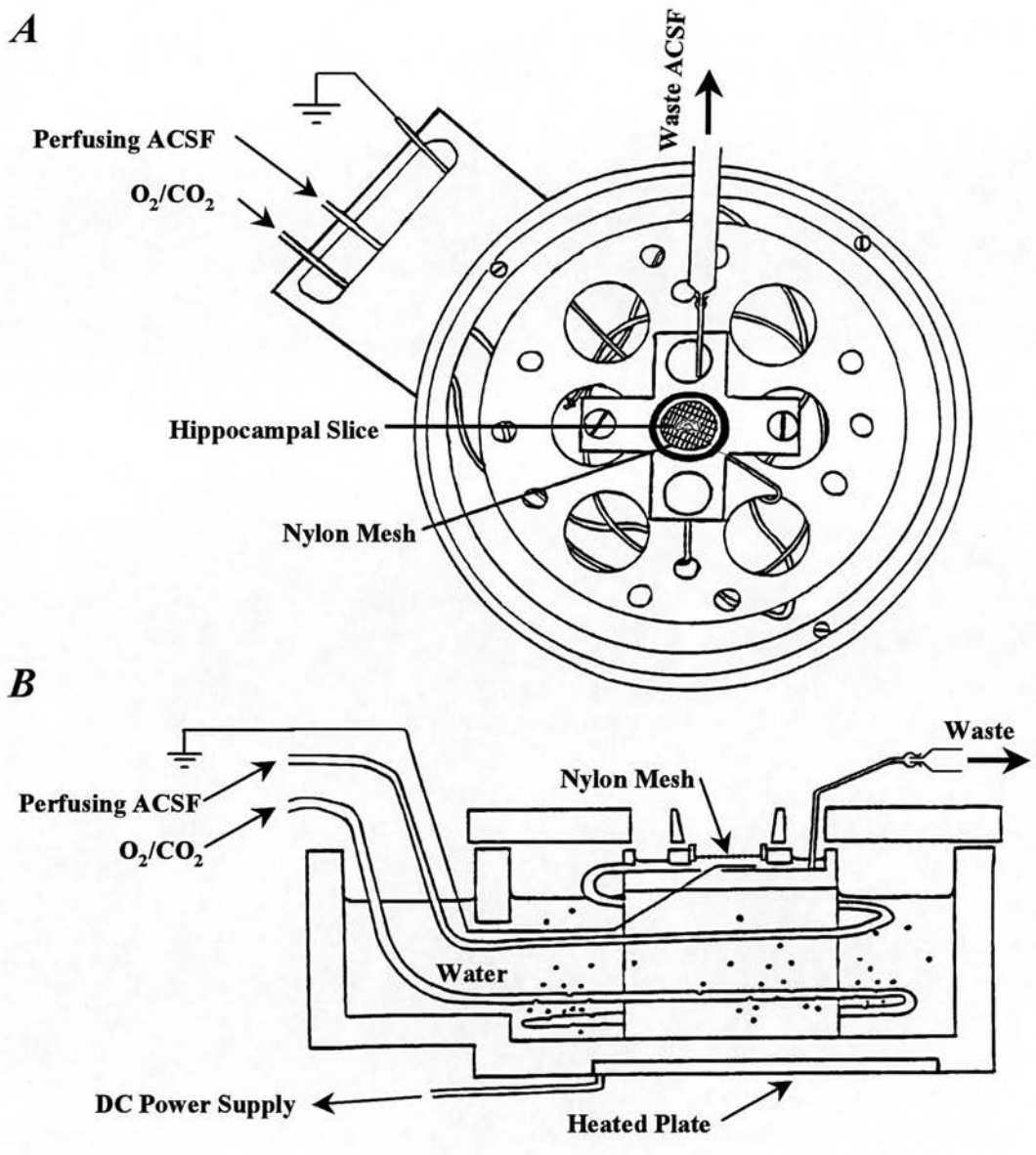


Figure 2.1. The interface recording chamber. This figure shows a schematic representation of the recording chamber as viewed from above (*A*) and from the side (*B*). ACSF, perfused with 95% O_2/5% CO_2 and maintained at 32°C, was passed through tubing immersed in the temperature controlled external 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.

2.2.2. Extracellular recording

Extracellular recordings were done as described above except for that 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; external diameter: 1.2 mm) borosillicate glass capillaries with an inner filament (120F-10, Harvard Apparatus, 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. Thin walled extracellular microelectrodes (internal diameter 0.86mm, external diameter 1.5mm) resistances ranging 1–5 MΩ were back-filled with 2 M sodium chloride (150F-10, Harvard Apparatus, England).

Recording microelectrodes were then mounted in electrode holders (Harvard Apparatus, 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 impalements/recordings was routinely performed in normal ACSF 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). Any further filtering was carried out during off-line analysis with Clampfit software (Axon Instruments Ltd.).

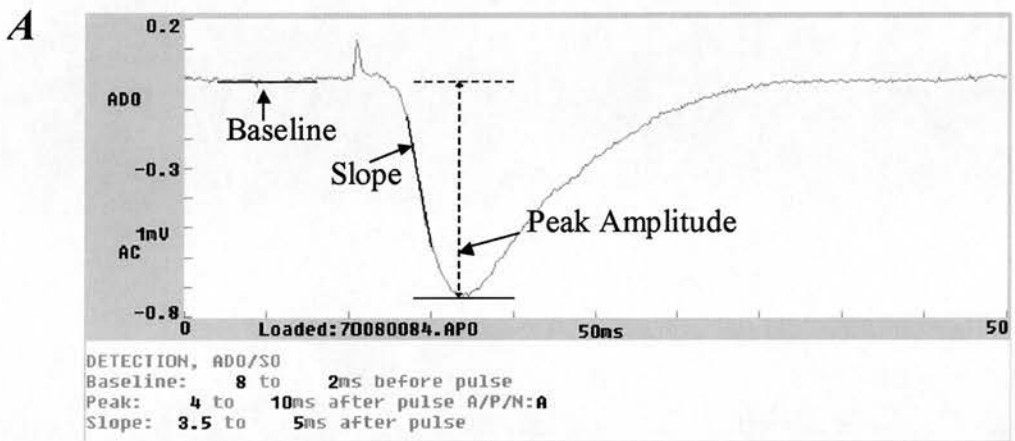
2.3.5. Data display and storage

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 drop 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 and/or peak amplitude of the fEPSP was measured (Figure 2.2). Data were displayed (amplification output gain *500 and lowpass Bessel filtering at 5KHz) 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 (Version

1.14J) was employed which allowed the recording, averaging and on-line analysis of data (written by William Anderson, University of Bristol).



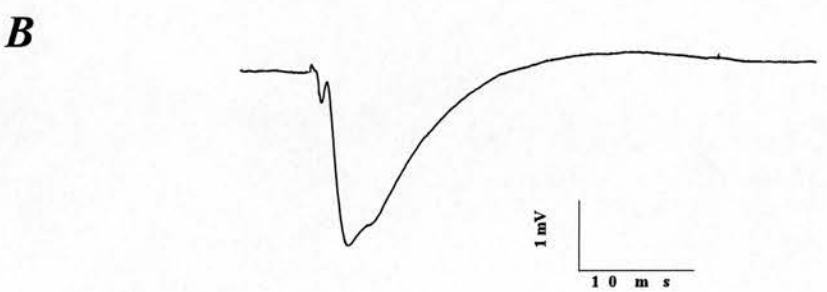


Figure 2.2. fEPSP analysis with the LTP Program. *A*, Prestimulus DC baseline, slope and peak amplitude of a model response. *B*, Representative fEPSP obtained from hippocampal slice resting on an interface chamber. The response is the average of 8 consecutive fEPSP sweeps.

In experiments investigating the effect of drugs or the induction of LTP on the slope of the fEPSP, the test field EPSP size was adjusted to approximately 50 % of maximum (maximum being defined as when a population spike began to appear superimposed on the fEPSP). Slope of fEPSPs was set to measure 20-80% of the peak in all experiments and throughout acquisition as described in Anderson and Collingridge, 2001. *Posthoc* analysis was only carried out when I was not present throughout the experiment and hence I had to re-analyse the experiment to make sure it was done properly.

2.4. Stimulation

The Schaffer collateral-commissural fiber input (or the septohippocampal cholinergic 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 focal stimulation. Stimuli were produced by constant voltage or constant current isolated stimulator boxes (Digitimer, England) in turn stimulated by a 5V pulse produced by the PC and relayed via the Digidata 1200. In every series of experiments stimuli comprised square-wave pulses (200 μs; 0–100 V) 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 & Cell Selection Criteria

For both intra- and extracellular 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.

2.6. Experimental Design

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 by no more than 15 %. At this point, a high frequency LTP induction protocol was delivered to the slice and the recording of EPSPs was continued for 1–4 h depending on the protocol employed. Some control dual pathway experiments were performed in random order, where one pathway was tetanised and one not to ensure that the two pathways were independent.

All data obtained from transgenic mice, contained in this thesis, were performed blind to the genotype of the mice and both the design of the experiments and the way data were handled and analysed were discussed and agreed before the start of each study. Care was taken to exclude any data obtained when deviations from the agreed procedure were noted. Some experiments were supervised by a senior member of the group, on the day, without prior warning to ensure that any such deviations did not occur.

A randomnised list of mice to be used was made by the animal house technician and kept in the animal house in a safe place. This list, which comprised interleaved wild-type and transgenic mice for each day, was not given to me until the end of each set of experiments, so that the final stages of analysis could be performed. Each individual experiment was analysed on the day the recording was performed and pooled data were only drawn at end of each set of experiments. Subsequently, the analysis of both pooled and individual experiments included were double-checked by a senior member of the lab group then and also before the data were submitted for publication and/or for thesis write-up. Data were backed up then and copies were kept by me and also by the senior member of the group who supervised the analysis of the data. Should genotyping was needed *posthoc*, to ensure that numbers enlisted corresponded to the correct mouse, the tails of each mouse sacrificed were kept in a labeled eppendorf tube with the date of the experiment at -80°C.

In intracellular recordings, 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 sub-threshold stimulation induced reductions in SFA, were evoked, baseline recordings comprised 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. In the sets of experiments where the effect of drugs was assessed, control and galanin-treated recordings were interleaved in slices obtained from the same rat. If more than one recording of the same treatment was obtained per rat, then data were pooled together, so that final n values corresponded to recording per rat, rather than per slice.

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 fEPSPs). The synaptic responses analyzed were single responses in the case of mAChR-mediated responses. Average responses were used to reduce noise and mean the effects of biological variation for all other types of responses.

The data-handling software package Sigmaplot (Version 2.01, 3.01 & 5.0; Jandel Scientific, USA) was run on an IBM PC (Dell) and was used to generate plots for individual experiments 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. Nissl Staining

Along with electrophysiological recordings, some slices were cut in thinner sections (of 80 μm) and were Nissl-stained to visualise the layered structure of the hippocampus. Following dissection, some slices were stored in 10% formalin and stored at 4°C for up to 4 days, when they were transferred to 20% sucrose solution and stored overnight. The following day they were placed in a shallow petri dish containing 70% alcohol and simultaneously frozen and cut into 50μm sections using a cryostat (Leitz Wetzlar, 1310 Germany). Then slices were washed in Tris-buffered saline (TBS; 0.05M, pH 7.4) and incubated overnight at 4°C with % avidin-biotinylated horseradish peroxidase complex (ABC, Vector Laboratories).
Then sections were mounted on gelatinised slides and left to dry overnight at room temperature and then slides went through a cycle of washes in alcohol of different concentrations to dehydrate and later to remove excess cresyl violet stain, as follows: 90% ethanol, 100% ethanol, xylene, 100% ethanol, 90% ethanol, 70% ethanol, 50% ethanol, distilled water, 50% ethanol, 70% ethanol, 90% ethanol, 100% ethanol, xylene. Slides were then coverslipped and allowed todry overnight. Stained sections were viewed under low power dissection microscope and photographed (see Chapter 1, Huntley & Jones, 1991).

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, de-ionised water, or α-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, Ach and picrotoxin were purchased from Sigma (Poole, UK). 6-nitro-7-sulphamoylbenzo[*f*]quinoxaline-2,3-dione (NBQX), were purchased from Tocris Cookson Ltd. (Bristol, UK). Galanin, galanin receptor active ligands M15, M40 and C7 were purchaced from Sigma. All protein kinase inhibitors, like Calphostin C (PKC inhibitor), KT5823 (PKG inhibitor), K-252b (mainly for inhibition of PKA) and H-7 (non-specific; primarily used to target PKC) were bought by Sigma.

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; Basel, Switzerland)

CHAPTER 3

CHARACTERISATION OF THE EFFECTS OF GALANIN ON BASAL SYNAPTIC TRANSMISSION AND SHORT-TERM SYNAPTIC PLASTICITY IN THE CA1 AREA OF THE HIPPOCAMPUS IN RATS

3.1. Introduction

In this chapter, I show that exogenous galanin application caused an increase in basal synaptic transmission of glutamate receptor mediated fEPSPs recorded in the CA1 of rat hippocampal slices, which appeared to be dose-dependent (Figures 3.1. and 3.2.). The aforementioned effect was not seen when recordings were performed in CA3-ectomised slices (Figure 3.2). Figure 3.3. shows that galanin (100 nM) did not have any significant effect on PPF in slices that retained the CA3 area intact. Because that was the case, I decided to perform a positive control experiment in CA3 containing slices, to show that doubling the concentration of calcium in the external medium did produce a significant reduction in PPF, only at 25 ms interstimulus intensity (Figure 3.4). Perfusion of galanin at the concentration, which causes a significant effect on the slope of the fEPSPs (300 nM) had a similar effect on PPF (Figure 3.5) only apparent at interstimulus interval of 25 ms. In conclusion, galanin may cause an enhancement of synaptic transmission by activating galanin receptors located in presynaptic neurones originating in the CA3 area and which synapse to pyramidal cells in the CA1 area.

3.2. Results

3.2.1. Galanin enhances basal synaptic transmission in the CA1 area of hippocampus in rats

First, I wanted to test the effect of exogenous galanin perfusion on the slope and amplitude of the fEPSPs generated by low frequency stimulation of the *en passage* synapses of the CA3 Schaffer collateral commissural pathway onto basal and apical dendrites in the CA1 area. All drugs were perfused for at least 20 minutes until the slope and amplitude of fEPSPs were stable. Recordings were made for at least 40 minutes following the addition of any drugs.

In Figure 3.1, the pooled data from the complete study are presented where the effect of porcine galanin (100-1 μM) was found to be dose-dependent in *stratum radiatum* and *oriens*. As shown in figure 3.2.*A*, 300nM porcine galanin induced a significant increase in the slope of fEPSPs in eight experiments. More specifically, and measuring the percentage baseline change of the last point of each set of experiments perfusion of 100nM galanin had a non significant effect on the fEPSP slope (103±3% SEM, n=8), whereas 300nM, 1 μM and 3 μM induced an increase in fEPSP slope amounting to145±2, 174±3 and 187±5 % SEM respectively. The same experiment was repeated for field responses obtained during low frequency stimulation of CA3 collateral commissural fibres ending on the basal dendrites of *stratum oriens* and the pooled data are shown in Figure 3.2.*B*. In eight experiments, concentrations of galanin of 300 nM, 1 μM and 3 μM induced increases of fEPSP slope amounting to 147+/-3, 175+/-4 and 210+/-7% SEM respectively.

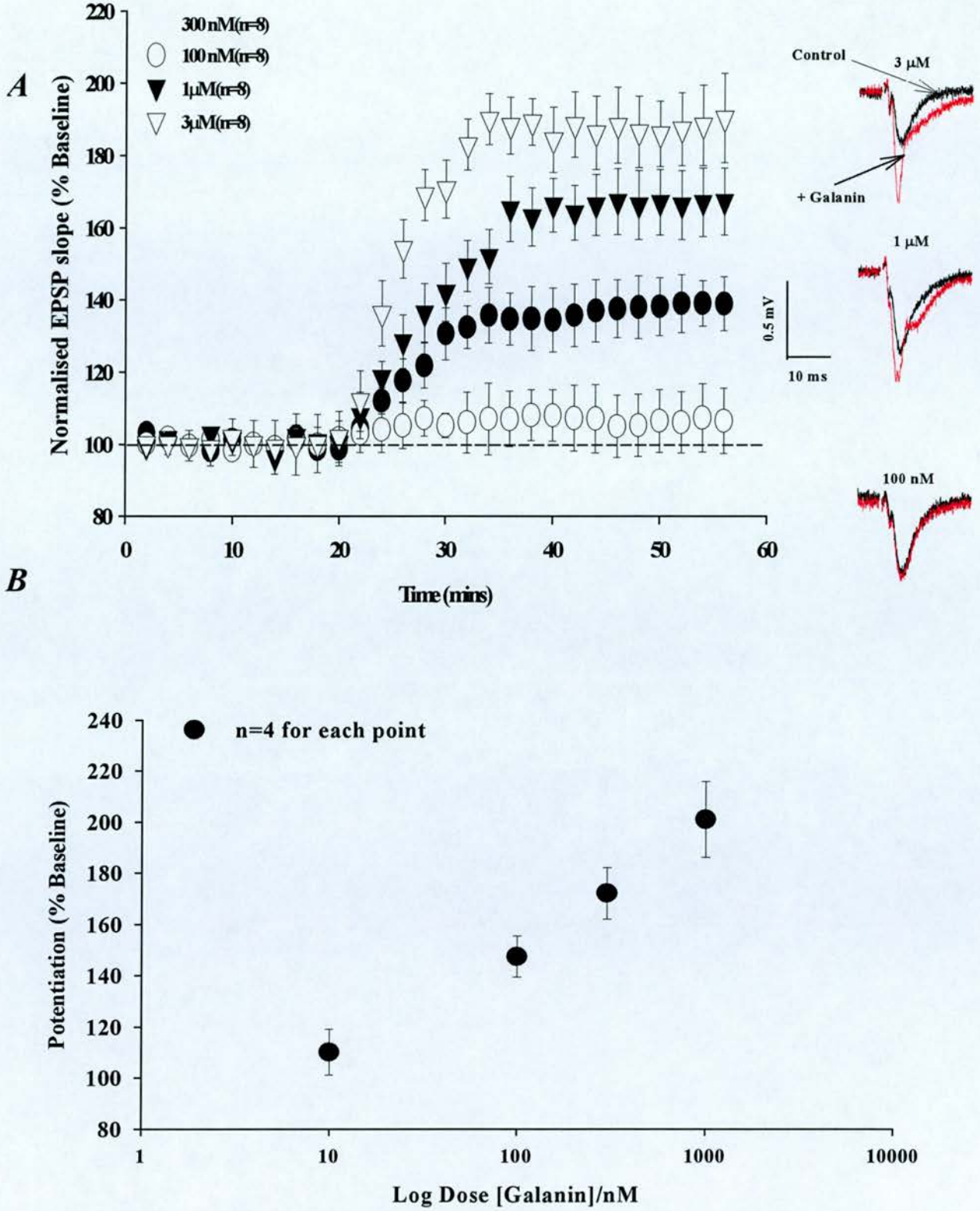


Figure 3.1. Galanin enhances the slope of fEPSPs in a dose-dependent manner in the CA1, where *A*, shows the effect of 100 nM to 3 μM in *stratum radiatum* and *B*, *in stratum oriens*.

Sample traces are shown from a recording in stratum radiatum prior to (black traces) and following the perfusion of galanin (red traces) at the aforementioned concentrations (top right hand panel).

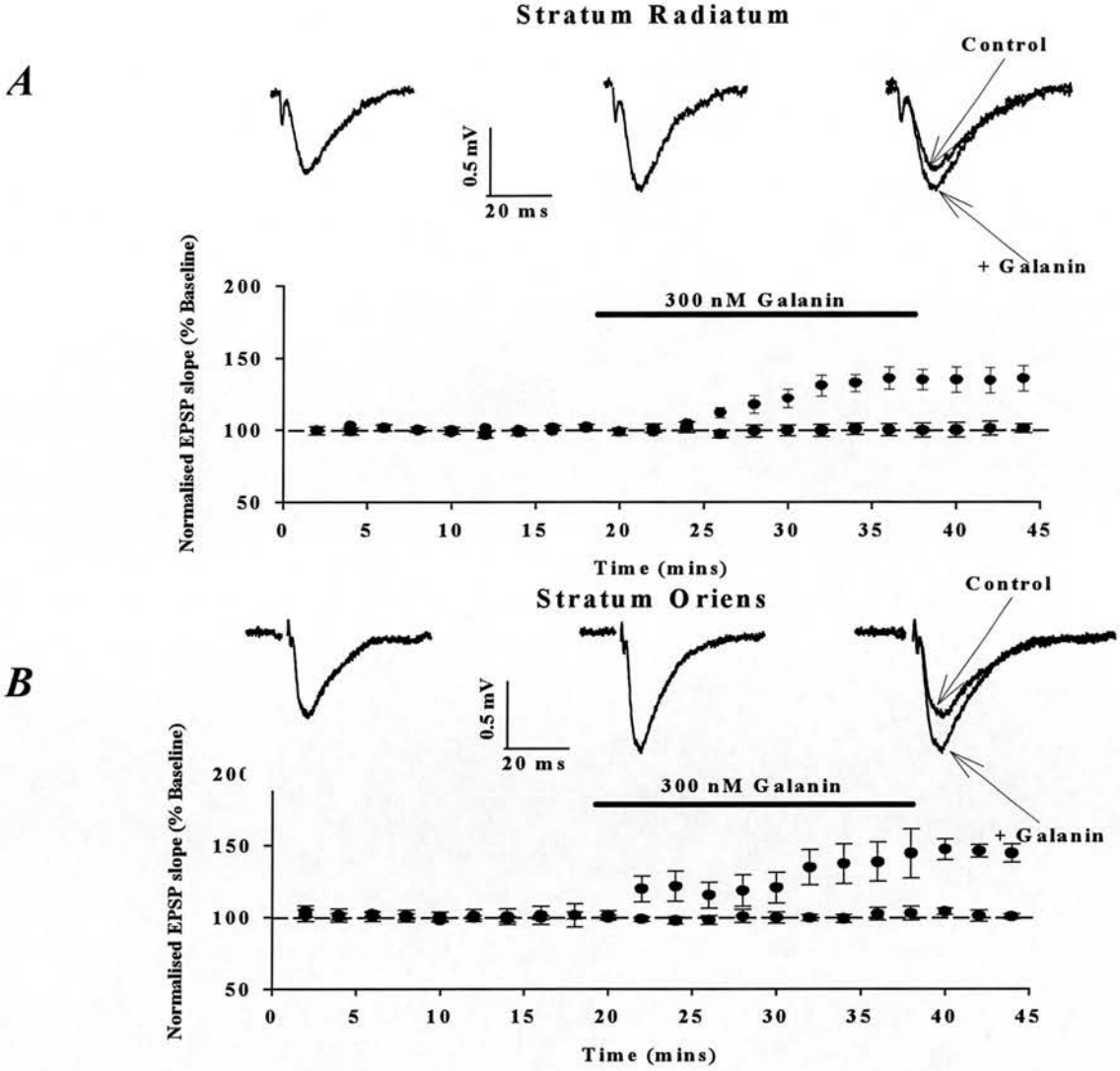


Figure 3.2. High concentration of galanin (300 nM) enhances basal synaptic transmission only in slices with the CA3 intact, the graphs represent pooled data from recordings performed in *stratum radiatum* (panel *A*) or *oriens* (panel *B*), where 300 nM galanin caused a significant increase in the slope of fEPSPs in slices with the CA3 area intact, compared to synaptic transmission in CA3-hemisected slices (n=8,8 for both sets of recordings). The traces are example waveforms (average of 8) recorded from intact slices prior to and following the addition of the neuropeptide.

3.2.2. The effect of galanin on PPF ratio

PPF is measured as the ratio of the rising slope of the second fEPSP to the rising slope of the first fEPSP. PPF is a short lasting presynaptic alteration in synaptic efficacy, where a high and low PPF ratios indicate low and high release probability, respectively (Bekkers & Stevens, 1990; Deupree *et al.*, 1993; Manabe & Nicoll, 1994). The investigation of the effect of galanin on paired-pulse facilitation was the basis of the next experiment, because I wanted to test the hypothesis that galanin at 300 nM caused an enhancement of presynaptic excitability, thereby producing an increase in the slope of fEPSPs in the CA1 and then I wanted to check whether if indeed that was the case this effect depended on the slice being intact or not.

Galanin was dissolved to make 100 mM and 300 mM stock drug aliquots and was kept at - 80°C, with a separate batch of vials, which contained ACSF (vehicle). Stock dilutions were made by a lab partner, who was not involved in my project and were colourcoded by him. I performed this experimental protocol blind. The protocol consisted of acquiring a fEPSP in the CA1 area and obtaining a stable baseline of FEPSPs in response to a single shock at low frequency stimulation (@30 s). Two consecutive shocks at 25 and 50 ms were interleaved for 4 sweeps just before adding the drug, 20 minutes following the addition of the drug and 20 minutes following the start of the washout.

Figure 3.3. summarises the finding of recordings performed in intact slices using 100 nM galanin. Exogenous galanin (100 nM) had no significant effect on the PPF ratios in either interpulse intervals in the CA1 area (n=6; $P>0.05$). Figure 3.4. show the results of a positive control experiment, in which I raised the concentration of external calcium from 2mM to 4mM to check whether indeed that reduced significantly PPF (n = 6, $P<0.05$) and which interstimulus interval (25 ms). Finally, Figure 3.5. contains pooled data in graphical representation from 6 epxeriments in which perfusion of 300 nM galanin caused a siginificant reduction in PPF at 25 ms only in intact slices ($P<0.05$), but not in CA3-hemisected slices (n=6, $P>0.05$).

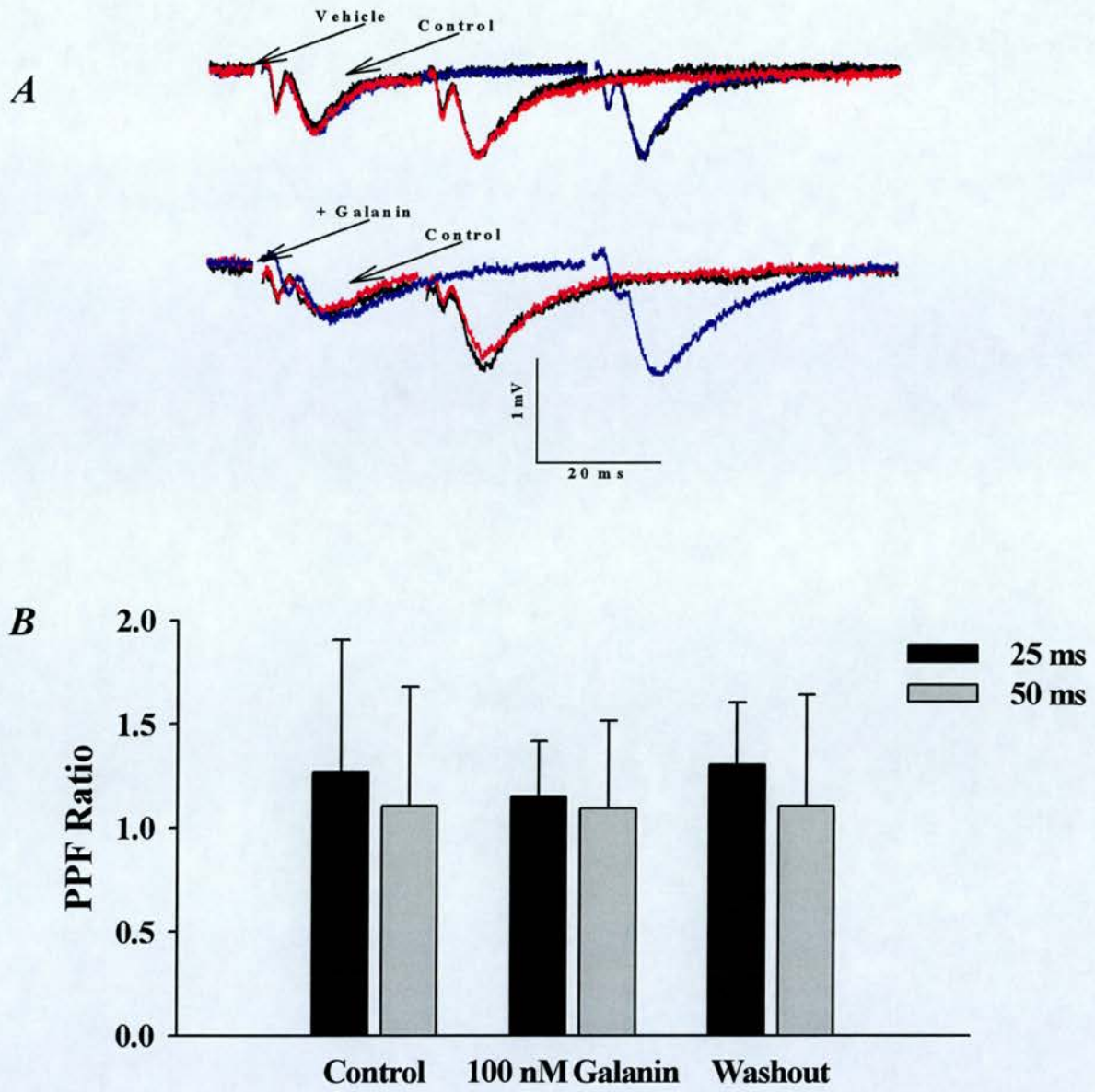


Figure 3.3. Low concentration of galanin (100nM) has no significant effect on paired pulse facilitation in the CA1 area. ***A*****, representative traces of field recordings obtained during control (black traces)/vehicle (red = 25 ms; blue = 50 ms) and galanin-treated (red = 25 ms; blue = 50 ms) conditions, showing the responses following PPF stimulation protocol with interstimulus interval of 25 ms and 50 ms. Each trace is the average of four trials.** ***B*****, Pooled data in graphical format of six recordings obtained from intact hippocampal slices, where black bars denote 25 ms and grey bars 50 ms interstimulus interval. There was no significant difference between the control and galanin-treated groups (n=6; Student's** ***t-test*****, P>0.05). Values are means ±S.E.M. represented by the error bars.**

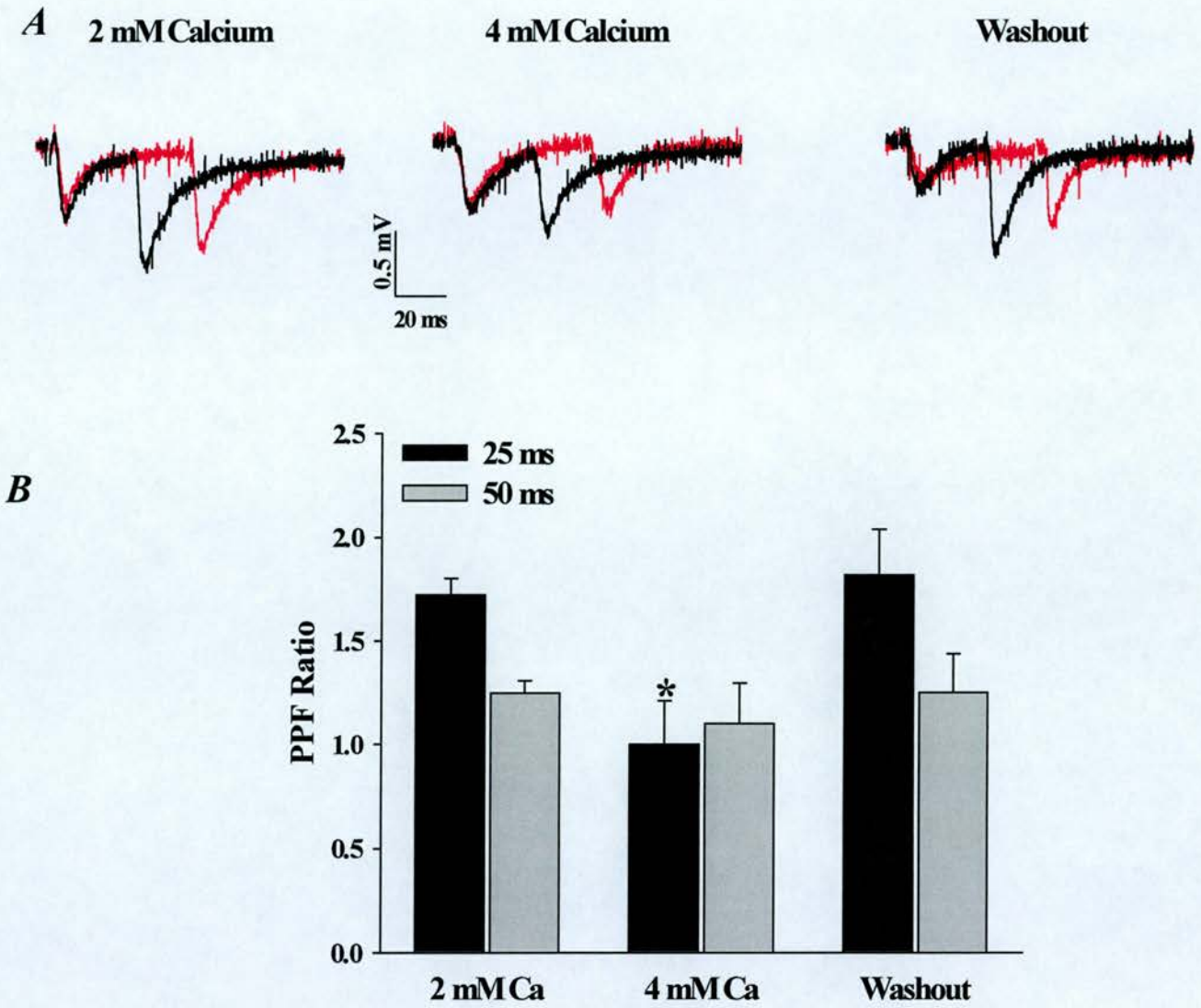


Figure 3.4. High external calcium concentration (4mM) causes a reduction in PPF ratio. *A*, Representative waveforms (average of 4 consecutive trials) for each of the interstimulus intervals tested (black traces = 25 ms and red traces = 50 ms) and B, pooled data for 6 experiments showing that in intact slices alteration of external calcium from 2 mM to 4 mM causes PPF to drop significantly (Student's t-test, $P<0.05$) for the 25 ms interstimulus interval (black bars), but not for the 50 ms interstimulus interval.

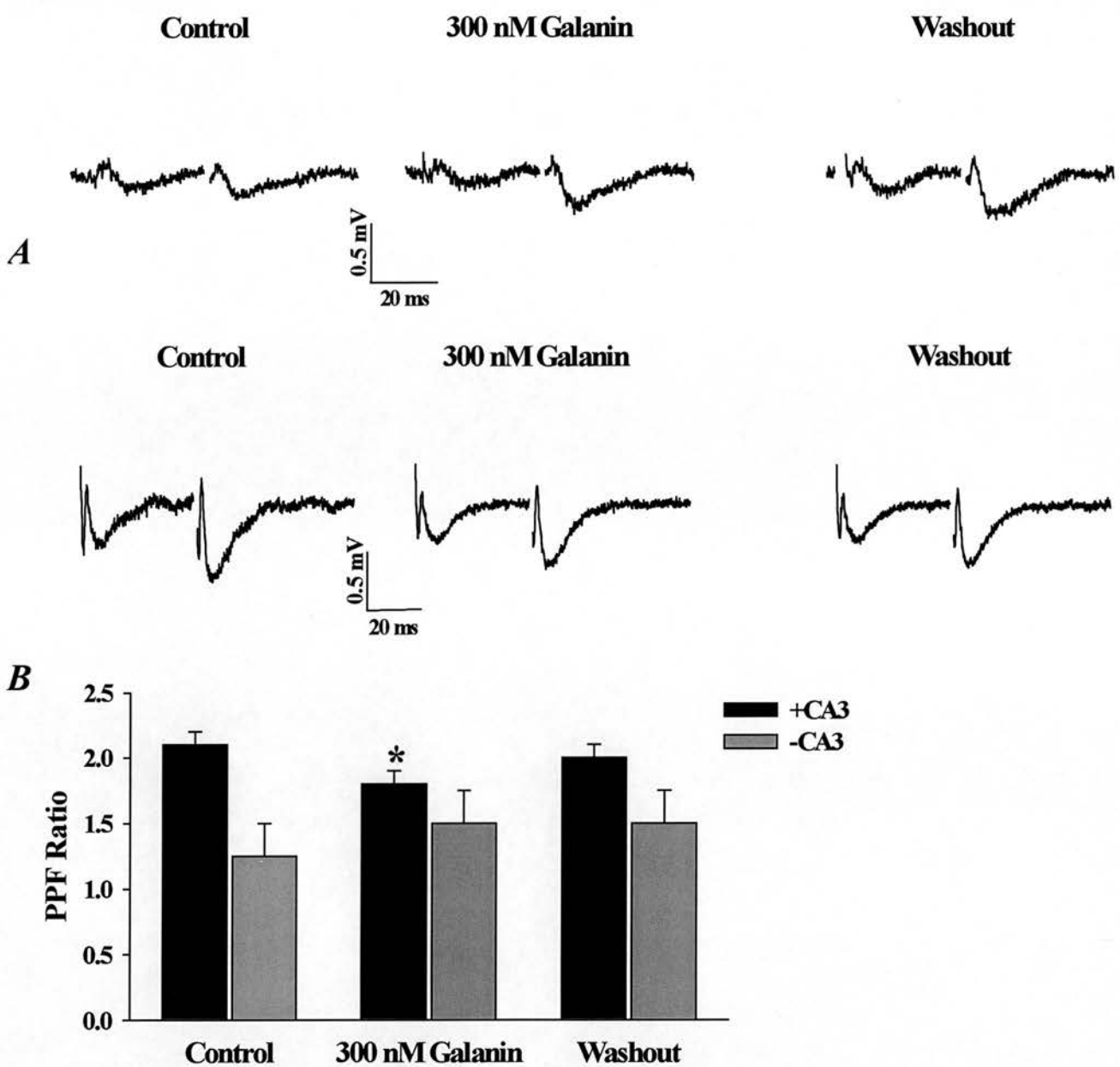


Figure 3.5. High concentration of galanin (300 nM) causes a reduction in PPF ratio. *A*, Representative waveforms (average of 4 consecutive trials) for 25 ms interstimulus interval tested and *B*, pooled data for 6 experiments showing that in intact slices (black bars) galanin causes PPF to drop significantly (Student's t-test, $P<0.05$), but not in CA3-hemisected slices (grey bars).

3.3. Discussion

In this chapter, I demonstrated that, galanin causes a significant increase in the slope of fEPSPs in a dose-dependent manner. Although this effect was seen when galanin was perfused at 300 nM, it was not observed at the lower concentration of 100 nM. Moreover, this effect appeared to be dependent on the CA3 area being intact, because it was not observed in recordings obtained from CA3-hemisected slices.

Interestingly, galanin at a 300 nM concentration also caused a significant reduction in PPF (25 ms interstimulus interval). This leads me to conclude that galanin may act presynaptically to either block potassium channels or activate a voltage-gated calcium channels, thereby causing an alteration in the excitability of neurones projecting from the CA3 area to CA1. This finding does not preclude the dual action of galanin on both pre- and postsynaptic galanin receptors in the hippocampus. A dual differential effect on pre— and postsynaptic sites could explain my finding described in Chapter 4, that galanin at the lower concentration of 100 nM significantly inhibits LTP in the CA1.

The presynaptic modulation of neurotransmitter release by galanin is not a novel physiological observation. Indeed, the way galanin might act to modulate acetylcholine is by acting on presynaptic receptors in cholinergic terminals or postsynaptic receptors on smooth muscle modifying the biochemical cascade initiated by acetylcholine acting through M_1 receptors. M_1 receptors are believed to act via a G-protein coupled receptor to decrease potassium conductance, thereby causing membrane depolarisation in nerve cells (Goyal, 1989). Interestingly, galanin was found to inhibit the twitch contractions of longitudinally and circularly oriented muscle strips mediated by the stimulation of cholinergic neurons in the guinea pig ileum (Botella *et al.*, 1992), but not the contractions mediated by direct stimulation of smooth muscle cells with carbachol. The inhibitory effect of galanin was antagonized by galantide and did not appear to affect other neurotransmitter effects, like of noradrenaline or GABA. As such, it seems that galanin inhibits the motility of guinea pig ileum by inhibiting acetylcholine release from the enteric cholinergic neurons,

probably through the specific receptor located on soma-dendritic regions and nerve terminals of cholinergic neurons (Kuwahara *et al.*, 1988; Akehira *et al.*, 1995; Botella *et al.*, 1995, Kakuyama *et al.*, 1997).

In the pancreas, galanin inhibits insulin release by either activating a potassium channel or inhibiting a voltage sensitive calcium channel, the reverse mechanism of what might be happening in my slices and indeed galanin has been shown to reduce the release of excitatory amino acids in the hippocampus by the same mechanism (Zini *et al.*, 1993). 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.*, 1989). 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 &1991). Finally, a more recent study has shown that a G_i (pertussis-sensitive) coupled galanin receptor (GalR1) mediates the inhibition of acetylcholine release in the rat cerebral cortex, probably by the same mechanism as it does in the gut (Wang *et al.*, 1999; Branchek *et al.*, 2000).

What I show here need not, however, contradict all previous published findings. Indeed, in conditions other than high frequency firing, such in form of short-term plasticity e.g. the PPF paradigm, galanin may act as as a potassium channel blocker presynaptically, thereby altering the K^+/Ca^{2+} dynamics in the terminal and activating voltage-gated Ca^{2+} channels thus increasing glutamate (Zini *et al.*, 1993) or acetylcholine release (Wang *et al.*, 1999; Ögren *et al.*, 1998 & 1999). The enhanced glutamate release activates postsynaptic AMPA receptors, thereby leading to an

increase in postsynaptic excitability that may alleviate the Mg^{2+} blockade of the NMDA receptor.

Although, GALR3 mRNA is not expressed in the hippocampus (Smith *et al.*, 1998; Kolakowski *et al.*, 1998; Wang *et al.*, 1999), a presynaptic action of galanin is further corroborated by the expression of galanin receptors (GALR2) in the presynaptic terminals of cholinergic neurons projecting from the septum to the hippocampus in rodents (Ögren *et al.*, 1998). Although this has not yet been shown, I cannot exclude the possibility that GALR2 is also expressed on the presynaptic terminals of glutamatergic neurons, thereby directly modulating glutamate release (Zini *et al.*, 1993) at high frequency stimulation paradigms.

Exogenously applied galanin inhibits synaptically evoked cholinergic responses in CA1 pyramidal cells according to several reports regarding the inhibition of ACh release by galanin (Dutar *et al.*, 1989; Fisone *et al.*, 1987; Palazzi *et al.*, 1988, 1991). Moreover, it has been proposed that this effect may also be also mediated by a presynaptic galanin receptor. It is likely that this effect is mediated by the $G_{i/o}$ coupled GALR2 as mRNA for this receptor is apparent in the ventral hippocampus where the inhibition of ACh release is observed. GALR2 is also expressed in the dentate gyrus of the dorsal hippocampus and may be responsible for increased ACh release in the awake rat, while GALR1 mRNA is found in hippocampal neurons and the receptor is thought to be expressed postsynaptically (Ögren *et al.*, 1998).

3.5. Future Experiments

To reverse the effects of galanin one may consider using galantide, which has been shown to antagonise galanin in a variety of preparations (Lindskog *et al.*, 1992, Reymann *et al.*, 1994; Takahashi *et al.*, 1994; Mahns & Courtice, 1996; Selve *et al.*, 1996). However, galantide has been known to have a controversial action of, for example in some preparations it has been shown to be a weak agonist of GalR1 (Ceresini *et al.*, 1998; Gu *et al.*, 1993, Wang *et al.*, 1999). It could be useful to repeat some of these experiments using other more selective galanin antagonists to attribute the effects of the neuropeptide to specific receptor subtypes.

CHAPTER 4

THE EFFECTS OF GALANIN ON LONG-TERM SYNAPTIC PLASTICITY IN THE CA1 AREA OF RODENT HIPPOCAMPUS

4.1. Introduction

This section covers the basic information relevant to the rest of the chapter. A more detailed account on the cellular mechanisms of synaptic transmission and plasticity is laid down in Chapter 1 of this thesis. In brief, long-term synaptic plasticity of glutamate-mediated synaptic transmission in the hippocampus is believed to be an important process for learning and memory in vertebrates (Bliss & Collingridge, 1993). In particular, those forms of long-term synaptic plasticity that are NMDA receptor dependent (e.g., LTP and LTD) are thought to be particularly important for these processes. To induce LTP and LTD requires the appropriate integration of GABAergic inhibitory and glutamatergic excitatory synaptic inputs (Davies & Collingridge, 1993).

However, in addition to amino-acid mediated synaptic transmission each area of the brain, which supports LTP or LTD, also receives a variety of non amino-acid mediated synaptic inputs. These may have the capacity to either promote or restrict the induction of long-term synaptic plasticity (Blitzer *et al.*, 1990; Bliss & Collingridge, 1993; Edagawa *et al.*, 2000). Thus, for example, the CA1 region of the rodent hippocampus receives the septohippocampal projection, amongst others, which is a heterogeneous population of afferents capable of releasing neurotransmitters such as ACh, 5-HT and various neuropeptides into the hippocampus (Decker & McGaugh, 1991; Dutar *et al.*, 1995). A detailed account of the anatomy and physiology of the septohippocampal projection is laid out in introductory section 1.3.3.

Each of these afferents specifically targets distinct populations of cell types that differentially influence the behaviour of individual pyramidal cells or interneurones. Consequently, each afferent can directly or indirectly affect synaptic transmission and plasticity in the hippocampal CA1 region. As such, cholinergic innervation through depolarization of pyramidal neurones (Cole & Nicoll, 1983, 1984; Morton & Davies, 1997), activation of intracellular biochemical cascades (Dutar & Nicoll,

1988b) and inhibition of GABA release (Freund & Buszaki, 1996; Manuel & Davies, 1998) can strongly promote the induction of LTP (Auerbach & Segal, 1994; Blitzer *et al.*, 1990). Whilst the effect of the primary neurotransmitters, such as ACh and 5-HT, on LTP have been extensively studied, investigation of the effects of co-released neuropeptides have attracted less attention.

Here, I have attempted to address how galanin, one of these neuropeptides (Tatemoto *et al.*, 1983; Palazzi *et al.*, 1991; Kask *et al.*, 1995), affects LTP and LTD. The reason for choosing this particular twenty-nine amino-acid neuropeptide (Bedecs *et al.*, 1995) is that it is co-localised with ChAT in 30-35% of medial septal and basal forebrain vertical VLDB cholinergic afferents (Melander *et al.*, 1985; 1986; Crawley & Wenk, 1989; Gaykema *et al.*, 1991) innervating the hippocampus. Furthermore, selective lesion of cholinergic afferents and exogenous application of galanin (McDonald *et al.*, 1997) impair spatial learning tasks for which LTP and LTD are believed to form part of the synaptic basis (Bliss & Collingridge, 1993). In summary, what I have found and am presenting in this chapter is that galanin inhibited LTP, but not LTD, irrespective of whether this was induced by tetanic or theta-burst simulation, in both *stratum radiatum* and *oriens* of the area CA1 in the rat hippocampus (Figures 4.2, 4.3, 4.4 and 4.12). The degradatory effect of galanin on tetanically induced LTP was dose-dependent (Figure 4.1) and was reversed by the galanin antagonist, M15 (Figures 4.13 and 4.14). Furthermore, galanin did not affect isolated NMDA EPSPs or Group I metabotropic glutamate receptor effects in the CA1, thereby rendering the probability of the neuropeptide exerting its effect on LTP via the NMDA or mGluR receptor system, slim (Figures 4.5, 4.6, 4.7 and 4.8). However, galanin impaired phorbol ester (PDBu) induced potentiation of synaptic transmission, which suggests that it may act to inhibit LTP by interfering with PKC activity (Figures 4.11). A proportion of these data have been reported previously in abstract form (Coumis & Davies, 1998, 1999; O'Meara *et al.*, 2000; Coumis & Davies, 2002).

4.2. Results

4.2.1. Galanin inhibits tetanus-induced long-term potentiation in *stratum radiatum*

In a first series of experiments, described in more detail in Chapter 3, I attempted to establish whether galanin affected glutamatergic synaptic transmission at apical dendrites in area CA1. Perfusion of galanin (100nM - 1 μM) did not affect baseline fEPSPs *per se* recorded in *stratum radiatum* in response to stimulation in the same dendritic field, in CA3 ectomised rat slices (Figure 3.2, n=8; $P>0.05$). Subsequent afferent tetanization at 100 Hz for 1 s, in the continued presence of galanin at concentrations greater than 10 nM, resulted in a short-lasting potentiation, which decayed back to baseline over the course of the next 20-30 minutes. In contrast, in naïve slices the same tetanization protocol resulted in a robust LTP that was maintained throughout the recording period (Figure 4.1*A*; ANOVA, $P<0.05$). As such, in galanin (100 nM)-treated slices the magnitude of LTP measured 30 minutes post-tetanus was only 110 ± 8 % ($n = 8$) as compared to 152 ± 15 % SEM in control slices ($n = 8$). Figure 4.1. shows the dose-dependent effect of galanin on LTP and Figure 4.2 presents a more detailed account of single experiments and pooled data for the galanin-induced impairment at 100 nM in *stratum radiatum* of the CA1 area.

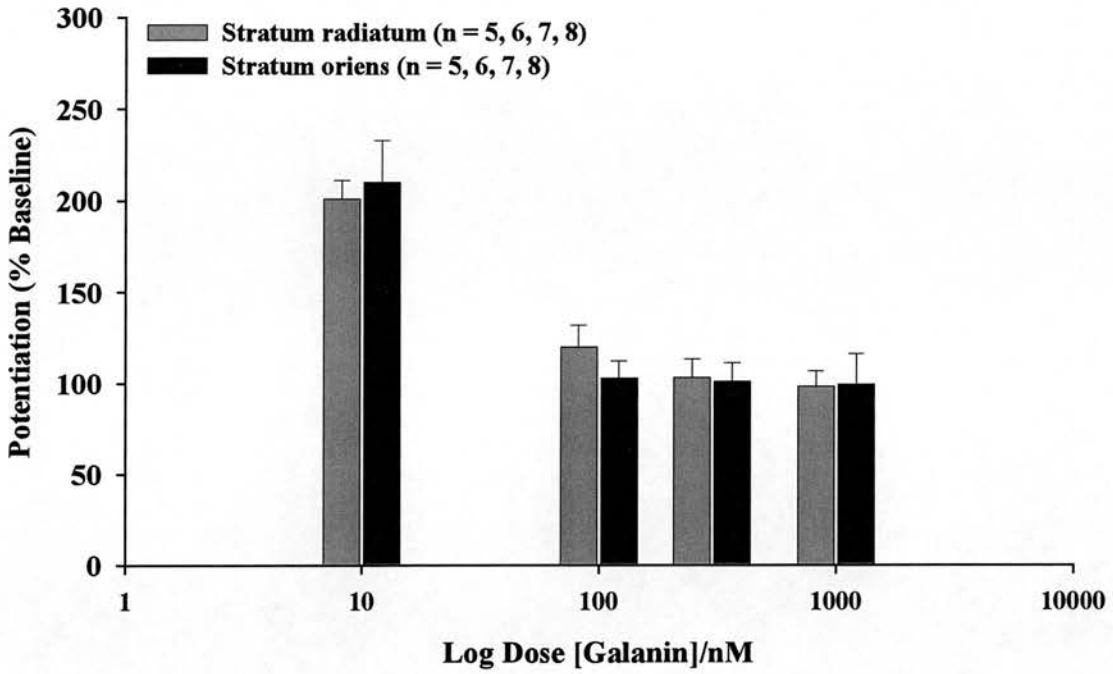


Figure 4.1. The effect of galanin on LTP is dose-dependent regardless of whether recordings were made *stratum radiatum* or *oriens*. This is a summary data for tetanus-induced LTP recordings in the CA1 area following perfusion of 10nM, 100nM, 300nM and 1 μM galanin.

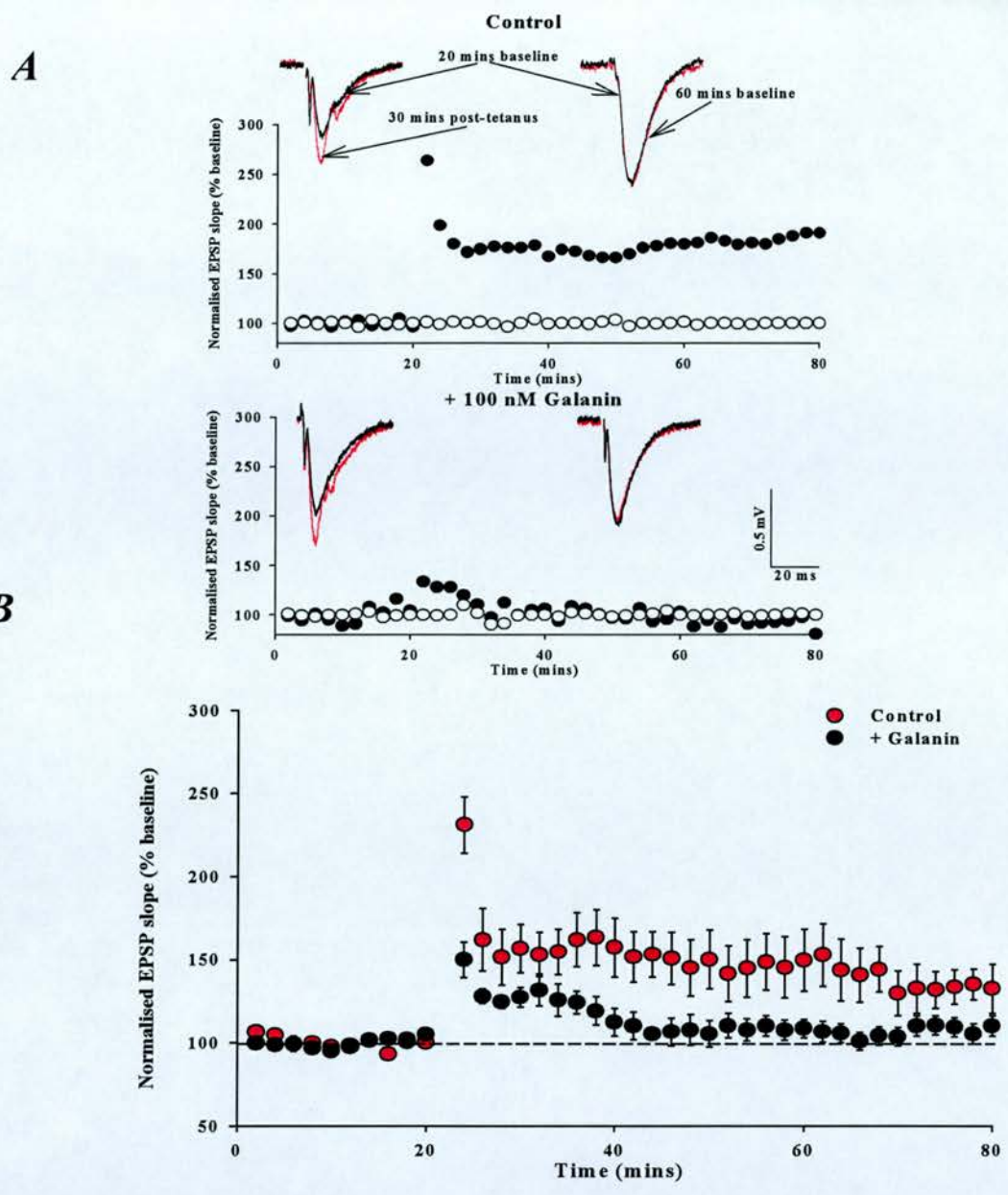


Figure 4.2. Galanin impairs the induction of LTP in *stratum radiatum* of the CA1 area following tetanus stimulation. *A,* Two sets of synaptic traces representing superimposed fEPSPs recorded prior to and 30 minutes following delivery of a 100 Hz for 1 s tetanus in the absence (top) and in the presence of 100 nM galanin (bottom left hand traces). The two graphs are plots of the slope of the fEPSP, normalized with respect to the 20-minute baseline immediately preceding the tetanus, versus time, in the absence (top panel) and presence of 100 nM galanin (bottom panel) obtained from individual dual pathway recordings. Each experiment includes a non-tetanised (open circles) and a tetanised pathway (closed circles). *B,* The graph is a plot of the mean ± S.E.M. of the fEPSP slope, normalized in the same way as the individual experiments shown above, versus time, in the absence (red circles) and presence (black circles) of galanin. Galanin application began 15 minutes prior to tetanization and persisted up until 10 minutes post-tetanus and induced a significant impairment on LTP (ANOVA, $P<0.05$). Each point is the average slope measurement obtained from four successive responses, obtained over a 2-minute period, and has been pooled across 8 experiments in each case.

4.2.2. Galanin inhibits theta-induced long-term potentiation in *stratum radiatum*

Since (1) LTP can be induced by a number of distinct stimulation protocols (Davies & Collingridge, 1993; 1996) and (2) LTP induced by each of these protocols may be differentially influenced by distinct pharmacological treatments (Davies *et al.*, 1991; Seabrook *et al.*, 1997) I chose to perform a second series of experiments to establish the effect of galanin on LTP induced by a theta-burst tetanus (i.e. 5 bursts of four stimuli at 100 Hz delivered at intervals of 200 ms). As with tetanic stimulation, galanin (100 nM) inhibited LTP induced by theta-burst tetanic stimulation in *stratum radiatum* ($n = 8$; Figure 5.1*B*, ANOVA, $P<0.05$). Thus, in galanin-treated slices the magnitude of LTP measured 30 minutes post theta-burst was only 108 ± 9 % ($n = 8$) as compared to 139 ± 11 % in control slices ($n = 8$). See also Figure 4.3.

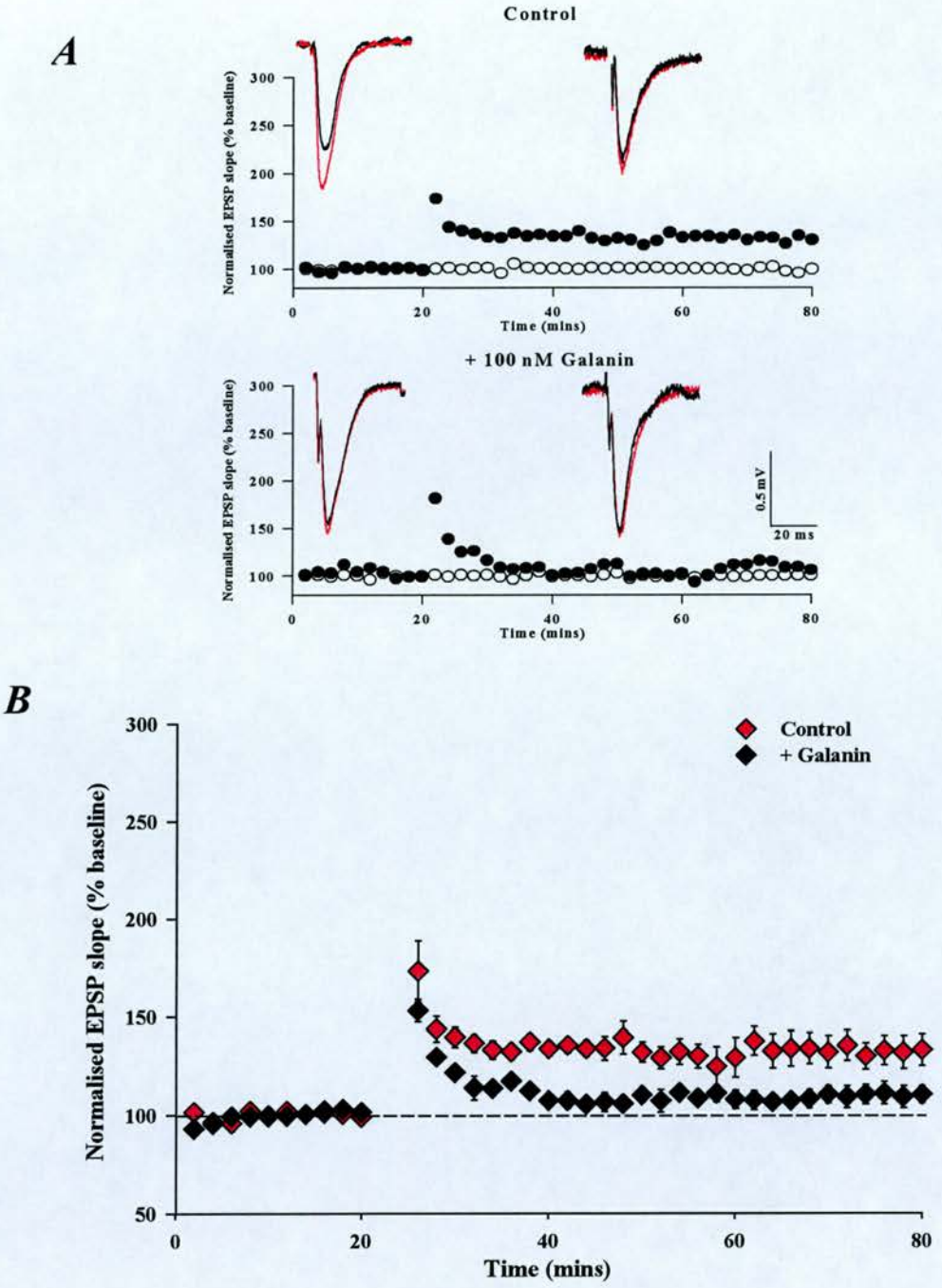


Figure 4.3.Galanin impairs the induction of LTP in *stratum radiatum* of the CA1 area following theta-burst stimulation. This is a replica of Figure 4.1. showing that 100 nM galanin perfusion induces a significant impairment of LTP (ANOVA, P<0.05).

4.2.3. Galanin inhibits long-term potentiation in *stratum oriens*

Galanin receptor binding is generally higher in *stratum oriens* than in *stratum radiatum* (Dutriez *et al.*, 1996). As such, I addressed next whether galanin also inhibited LTP in basal dendrites. As illustrated in Figure 4.2*A*, 100 nM galanin produced an almost identical profile of inhibition of LTP in *stratum oriens* to that in *stratum radiatum*. Thus, for 100 Hz for 1 s tetanic stimulation, the magnitudes of LTP measured 30 minutes post-tetanus in the presence and absence of galanin in *stratum oriens* were 107 ± 9 % (n = 7) and 140 ± 11 % SEM (n = 9) (Figure 4.3, ANOVA, P<0.05), respectively compared with 110 ± 8 % (n = 8) and 152 ± 15 % (n = 8) for *stratum radiatum* (Figure 4.4).

4.2.4. Mechanism by which galanin inhibits long-term potentiation

Thus, galanin was clearly capable of inhibiting LTP; but the mechanism by which this occurred was yet unknown. A striking feature of the short-term potentiation induced by both 100 Hz for 1 s and theta-burst tetanic stimulation in the presence of galanin is its marked similarity to that induced by stimulation paradigms that weakly activate the NMDA receptor system (Abraham & Bear, 1996). As such, I addressed the possibility that galanin may restrict NMDA receptor activation to a level that may be subthreshold for inducing LTP. Initially, I compared the envelopes of synaptic potentials evoked by both tetanic and theta-burst stimulation paradigms in each dendritic field in control medium with those evoked in the presence of galanin. This analysis revealed no substantial differences between any of the pairs of responses compared, indicating that changes in NMDA receptor function were unlikely to account for the inhibition of LTP in either *stratum radiatum* or *oriens* (Figures 4.5 and 4.6).

To further substantiate this, I tested the effect of galanin on isolated NMDA receptor-mediated fEPSPs recorded in the presence of 5 μM NBQX, 50 μM picrotoxin and 1 μM CGP 55845A to block all AMPA/kainate, $GABA_A$ and $GABA_B$ receptor activation, respectively. As illustrated in Figure 4.7, galanin had no significant effect on the peak amplitude (Student's *t-test*, P>0.05) or duration of pure NMDA receptor-mediated fEPSPs. To assess statistical significance on the duration of NMDA receptor mediated fEPSPs the area under each waveform prior to and following the

addition of galanin was measured and the results were averaged to 83 ± 13 and 101 ± 20 for 5 experiments.

Another possibility for why galanin inhibits LTP is that galanin affects the activation of mGluRs since antagonists of these receptors have been shown to limit post-conditioning stimulation potentiation to a short-term potentiation lasting 20-50 mins (Bashir *et al.*, 1993; Breakwell *et al.*, 1996). To test whether galanin inhibited mGluR function I examined whether galanin affected the increase in cell input resistance and reduction in spike frequency adaptation that was induced by the selective mGluR agonist (1S, 3R)-ACPD (10-50 μM) (Breakwell *et al.*, 1996). In all experiments performed, galanin did not significantly affect any of the responses evoked by this agonist (Figure 4.8.*A*; $n = 5$). The way I analysed my results was intially qualitative, by measuring number of spikes prior to and following the addition of galanin for 5 cells (control, *trans*-ACPD, plus 100nM galanin; n=2.9±0.1, 6.6±0.18, 6.7±0.2 SEM). Subsequently, I used a quantitative method, by calculating the interspike intervals (control, *trans*-ACPD, plus 100nM galantine; n=2.5±0.2, 13.9±2.3, 12.9±2.5 SEM). No significant difference was noted in the measurements prior to and following the addition of galanin (Student's *t-test*, P>0.05). See also Figure 4.8.*B* provided to show that under galanin does not affect firing properties of the cell if applied before (1S, 3R)-ACPD (n=4, Student's *t-test*, P>0.05).

As a result of these negative experiments I turned my attention away from the receptor systems involved in the induction of LTP in favour of an investigation of mechanisms further downstream of receptor activation that convert STP into LTP. As such, I initially examined whether galanin was capable of inhibiting the expression of pre-established LTP to enable me to pinpoint the type of biochemical processes that it may be affecting. In two sets of experiments, galanin was found not to affect LTP when applied either 20 or 60 minutes after delivery of a 100 Hz for 1 s tetanus (Figure 4.9). This pointed to a possible interaction between galanin and kinase activation since many kinase inhibitors have been shown to be effective in preventing the conversion of STP into LTP when applied during the period of

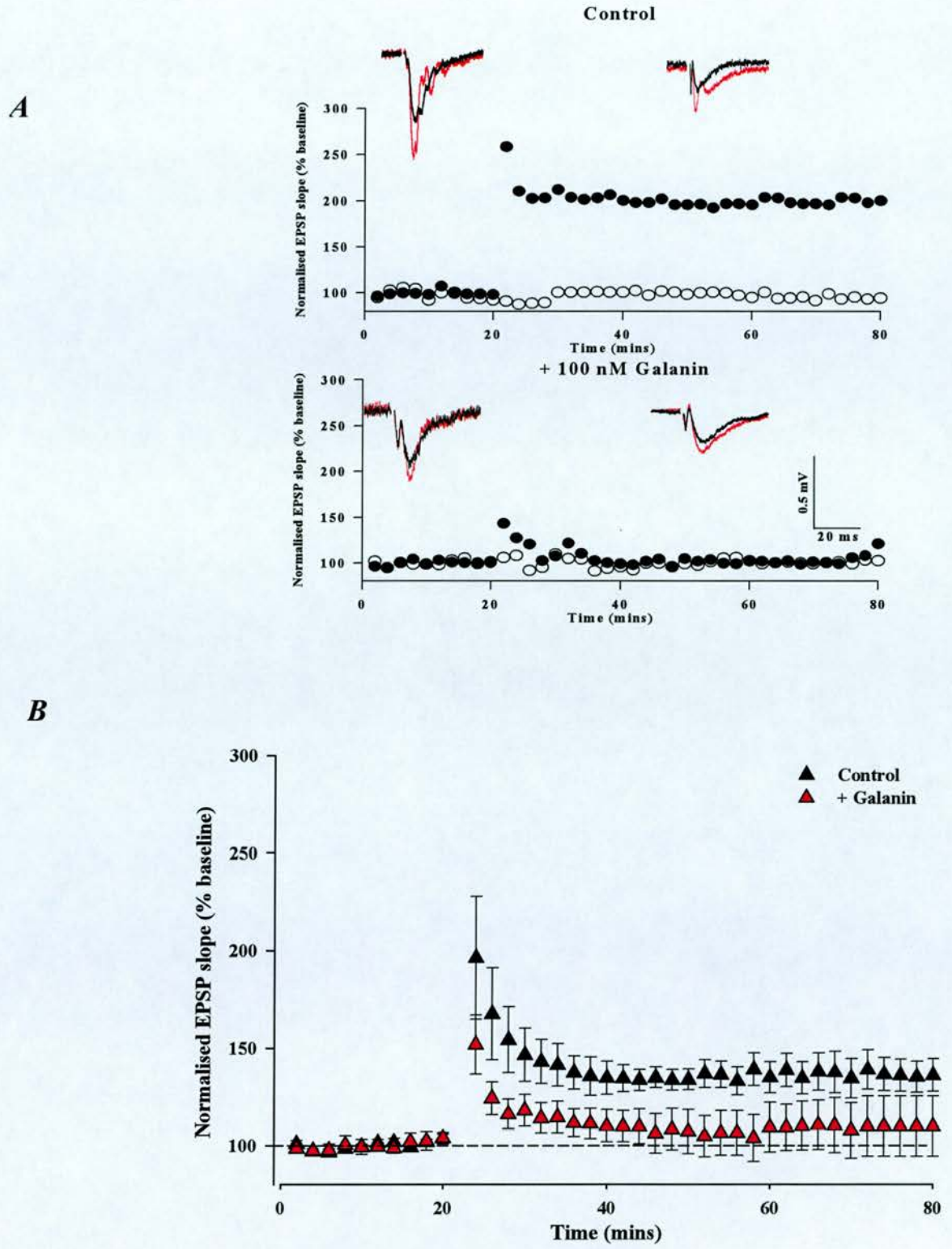


Figure 4.4. Galanin inhibits the induction of tetanus induced LTP in *stratum oriens* of area CA1. This figure is a replica of Figure 4.1. for this experiment showing that 100 nM galanin induced a significant impairment in LTP (ANOVA, P<0.05) in a total of 8 pooled experiments for each control and galanin treated slices.

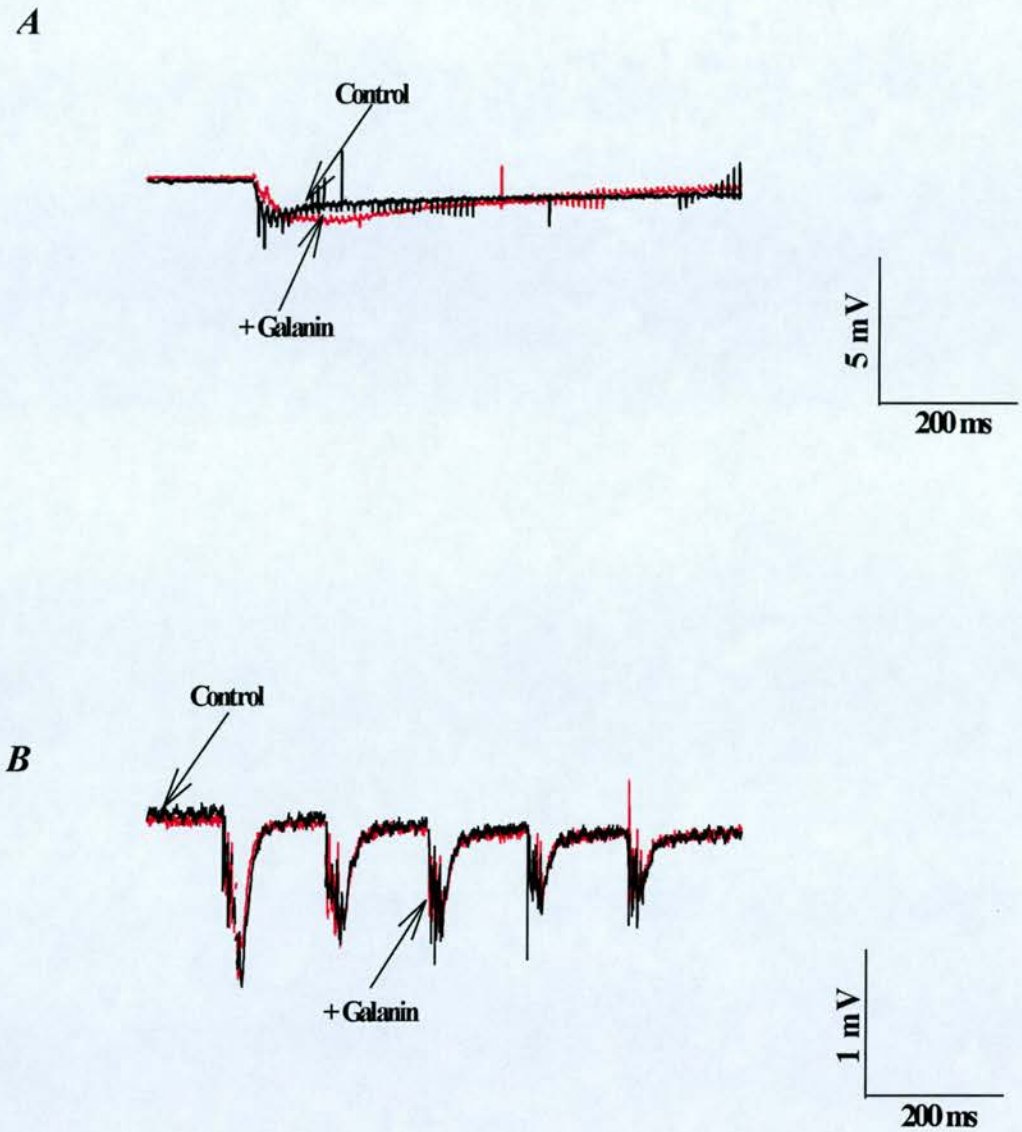


Figure 4.5.Galanin does not affect extracelluraly recorded synaptic responses evoked by either *A*, tetanic or *B*, theta-burst stimulation in *stratum radiatum*. Superimposed traces of the respective envelopes are shown in each case, where black traces denote the responses recorded in control medium, while red traces the responses recorded in 100 nM galanin. Pooled data of 8 traces for each stimulation paradigm show no significant effect of galanin on the area under the waveforms (Students' *t-test*, P>0.05, n=5).

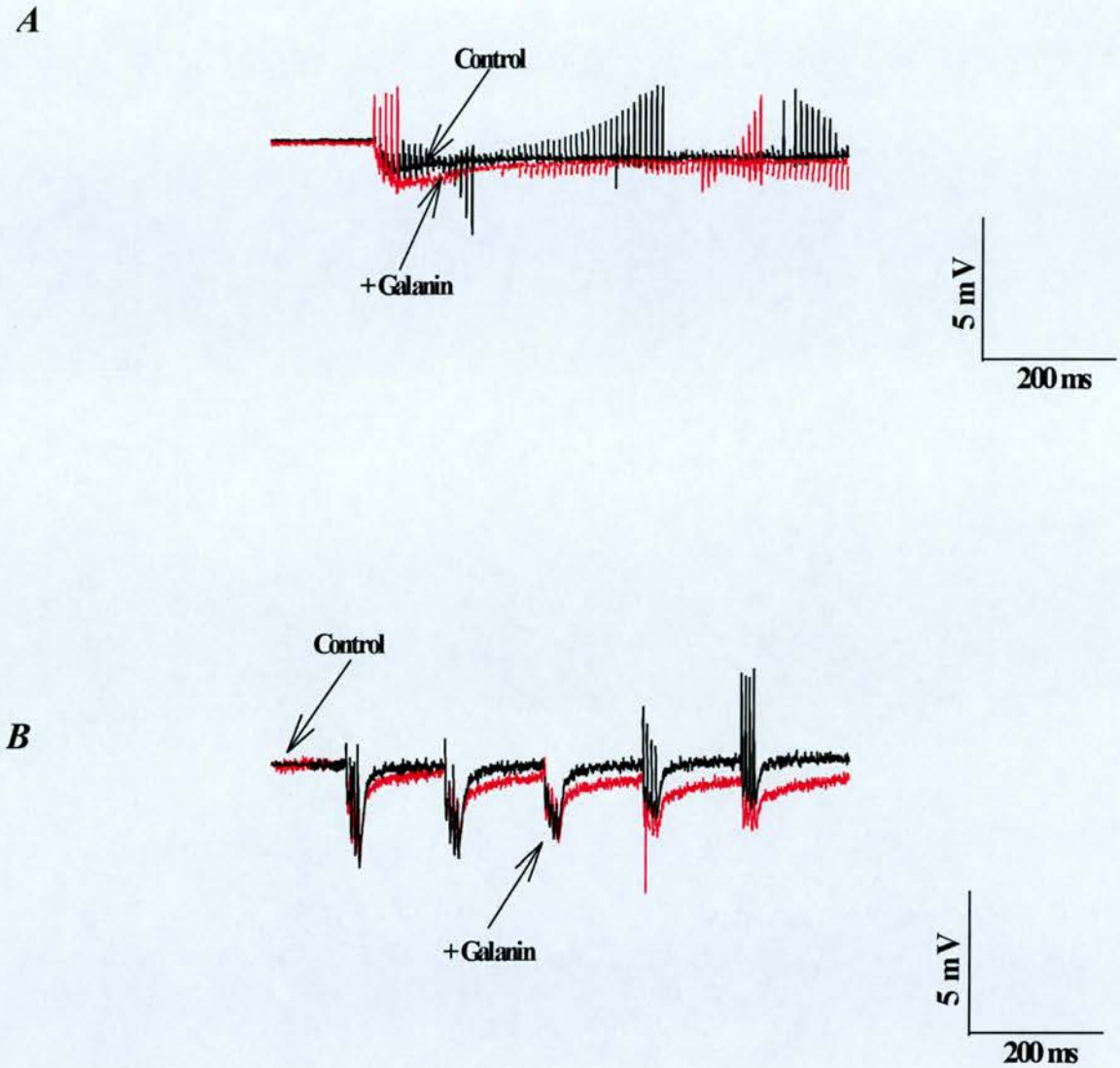


Figure 4.6. Galanin does not affect extracelluraly recorded synaptic responses evoked by either *A*, tetanic or *B*, theta-burst stimulation in *stratum oriens*. Superimposed traces of the respective envelopes are shown in each case, where black traces denote the responses recorded in control medium, while red traces the responses recorded in 100 nM galanin. Pooled data of 8 traces for each stimulation paradigm show no significant effect of galanin on the area under the waveforms (Students' *t-test*, P>0.05, n=5).

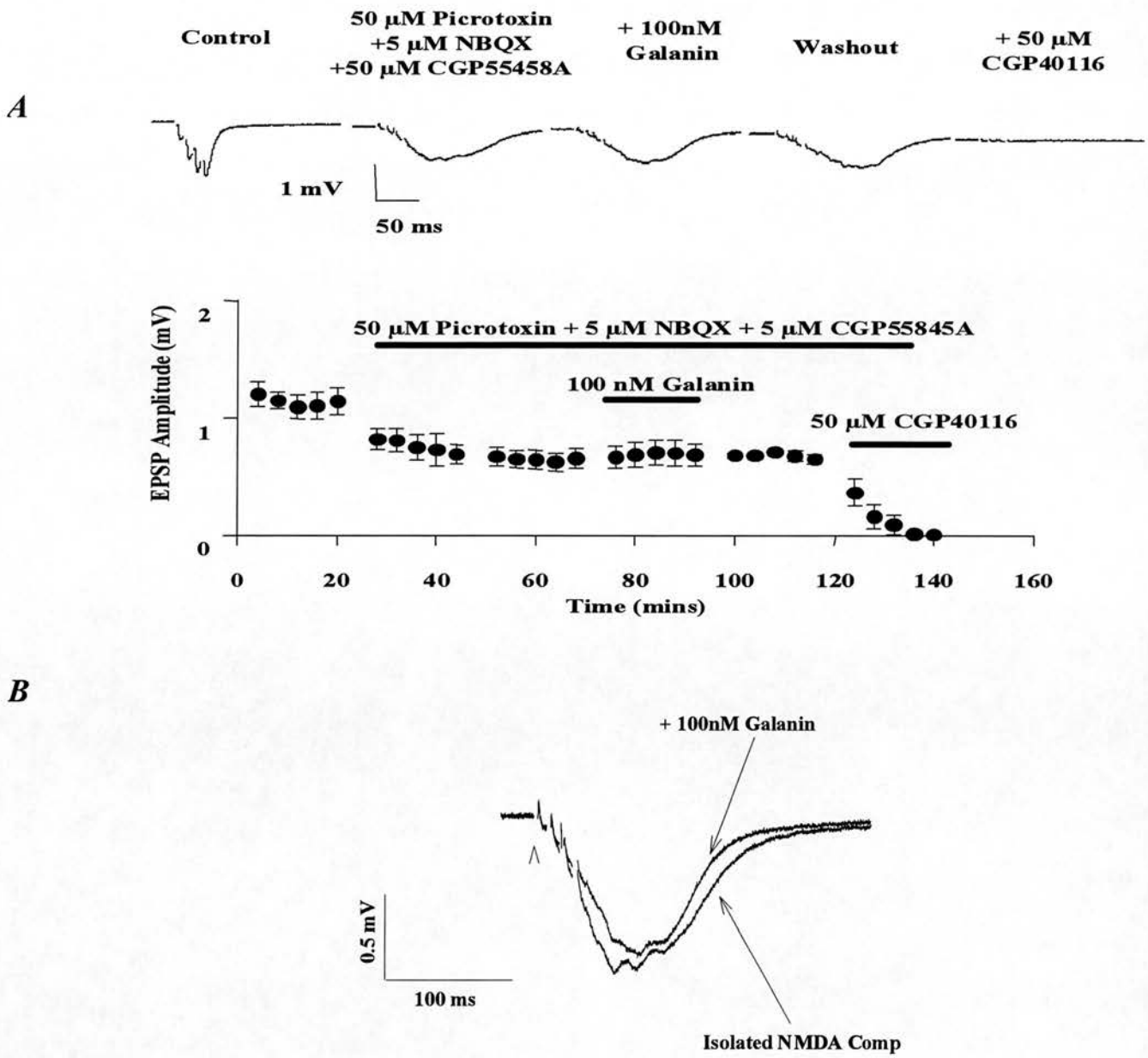


Figure 4.7. Galanin does not affect NMDA receptor function. ***A,*** **shows, from left to right, synaptic traces evoked by a burst of four stimuli delivered at 100 Hz in control medium, in the presence of a cocktail of AMPA, $GABA_A$ and $GABA_B$ receptor antagonists, in the additional presence of 100 nM galanin, following washout of galanin and after subsequent application of the NMDA receptor antagonist CGP 40116. The graph below these traces is a plot of the peak amplitude of the composite fEPSP throughout the period of the experiment. In** ***B,*** **synaptic traces prior to and following application of galanin depicted in** ***A,*** **are shown superimposed. Galanin had no statistically significant effect neither on average peak amplitude nor on duration of NMDA receptor mediated fEPSPs (Student's** ***t-test,*** **P>0.05).**

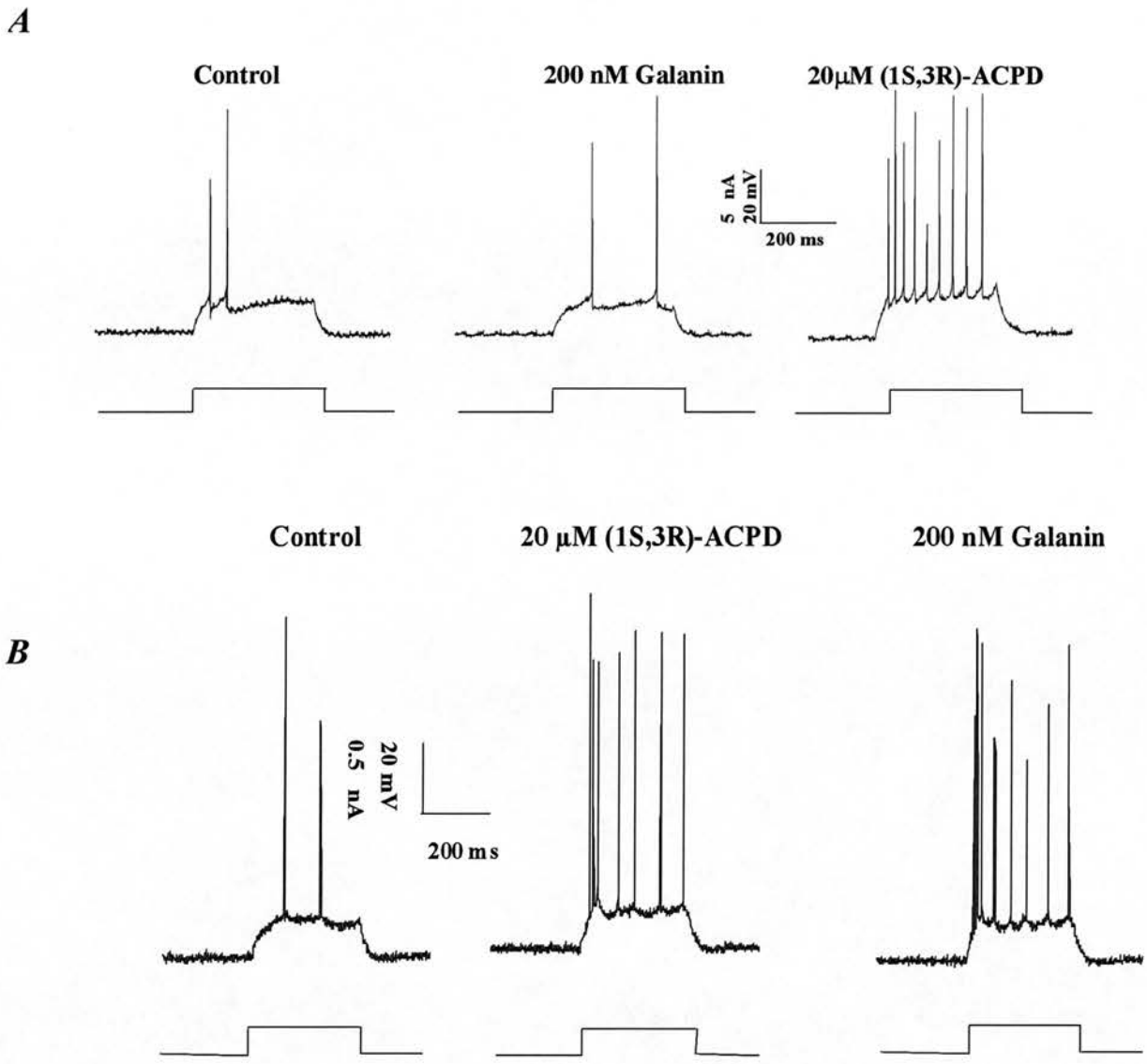


Figure 4.8. Galanin does not affect metabotropic glutamate receptor function. In *A*, traces are intracellular recordings of the membrane potential response of a CA1 pyramidal neurone to intracellular injection of a depolarising current step (+0.3 nA) illustrating the loss of spike frequency adaptation induced by (1S,3R)-ACPD and the lack of effect of galanin on this response. Quantitative measurement of spike frequency adaptation revealed no statistically significant difference prior to and following galanin application (n=5; Student's *t-test*, P>0.05), while SFA was significantly reduced by perfusion (1S,3R)-ACPD (n=5; Student's *t-test*, P<0.01). In *B*, the bottom set of figures is another cell, where the same experiment was repeated, with galanin added before (1S, 3R)-ACDP to ensure that it does not affect the firing of spikes (n=4; Student's *t-test*, P>0.05).

conditioning stimulation but not thereafter (Malinow *et al.*, 1989; Grant & O'Dell, 1994). Indeed, in my own hands, we have been able to demonstrate that in the presence of kinase inhibitors such as 1 μM KT5823 (protein kinase G inhibitor), K252b (PKC inhibitor) and calphostin C (inhibitor of PKC, PKA and myosin light chain kinase) tetanic stimulation that normally induces LTP induced only STP (Figure 4.10).

However, to demonstrate conclusively that galanin is restricting kinase activity required for LTP is a particularly problematic issue to address experimentally since (1) LTP is synapse-specific (Bliss & Collingridge, 1993) and, therefore, biochemical modifications are highly restricted to the small number of synapses undergoing LTP and (2) it is still unclear what the precise biochemical cascades that account for conversion of STP into LTP are. Nevertheless, to establish whether galanin could inhibit electrophysiological consequences of protein kinase activation we examined whether galanin was capable of inhibiting electrophysiological effects of phorbol esters that activate protein kinase C. In eight experiments, a 20 minute application of phorbol dibutyrate (10 μM) potentiated the initial slope of fEPSPs (Malenka *et al.*, 1986) such that the magnitude of the potentiated fEPSP slope amounted to 281 ± 11 % of the baseline control slope. This effect was partially inhibited by 100 nM galanin (n = 8; Figure 4.11), such that the phorbol dibutyrate enhancement in the presence this neuropeptide amounted to only 192 ±17 % of control; representing a 63% reduction in potentiation.

These data pointed towards a negative interaction between galanin and kinase activity as a potential explanation for the restricted expression of LTP in the presence of this neuropeptide. If this hypothesis was correct, it might be expected that galanin should not affect synaptic plasticity that is not highly dependent upon activation of kinase activity, and instead is critically dependent upon phosphatase activity (Bear & Malenka, 1994). As such, I tested next the ability of galanin to restrict the induction of long-term depression in area CA1. In eight experiments, LTD was induced in *stratum oriens* using a low frequency stimulation paradigm comprising 900 shocks delivered at 2 Hz. Galanin (100 nM) did not significantly affect the magnitude or temporal profile of this LTD (Figure 4.12). Thus, the slope of the fEPSP measured 20 minutes after the conditioning stimulation in galanin treated slices was 83 ± 5 %

of control baseline ($n = 8$) as opposed to 81 ± 7 % ($n = 8$) in untreated slices, respectively.

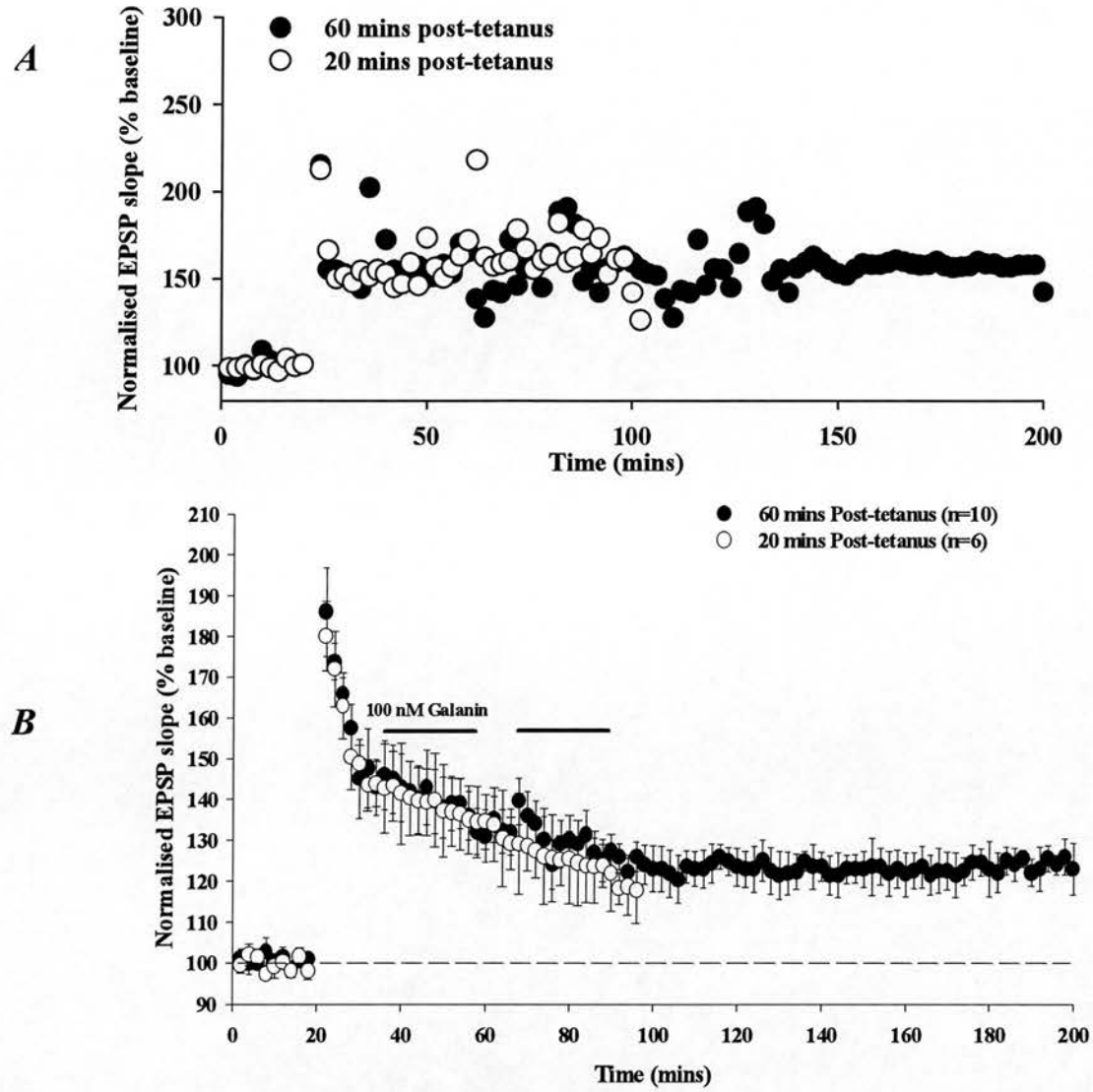


Figure 4.9. Galanin does not affect the maintenance of LTP. ***A,*** **Graphical representation of two individual recordings, where galanin was applied 20 minutes (open circles) and 60 minutes (closed circles) post-tetanus.** ***B,*** **Pooled data of the above experiment showing LTP recorded in** ***stratum radiatum*** **in response to a 100 Hz for 1 s tetanus. The format of the data presented is identical to that in previous figures except that galanin was applied 20 (n=6) and 60 (n=10) minutes after tetanization. Note that LTP persisted for the period of the recording. Synaptic traces are representative example responses taken from one of the pooled experiments illustrating fEPSPs recorded prior to tetanization, post-tetanization just before galanin application and post-tetanization just after termination of the galanin application. No significant difference is observed between the two groups of experiments (ANOVA, P>0.05).**

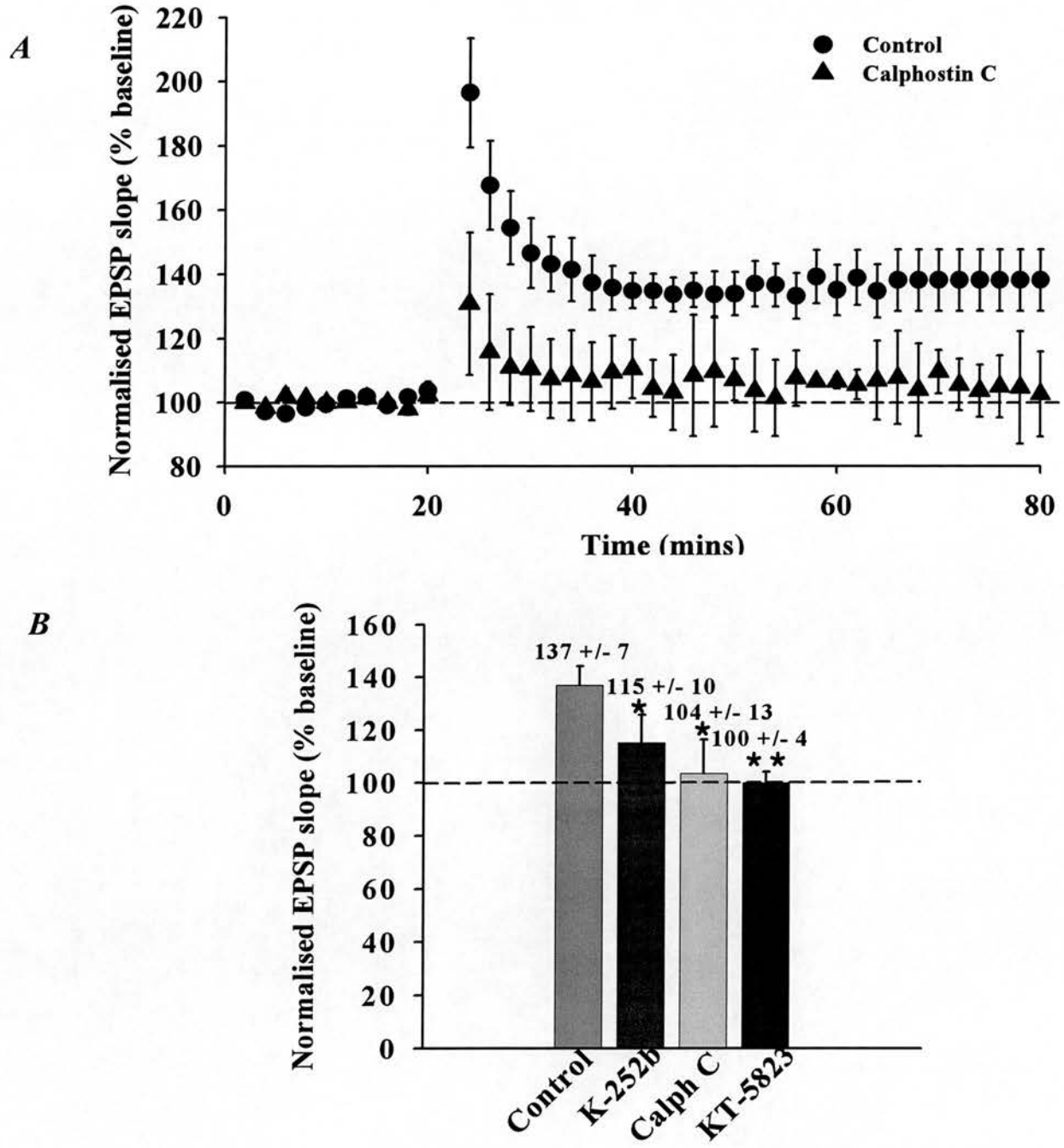


Figure 4.10. Protein kinases are important in long lasting synaptic potentiation. *A* is an LTP graph showing that protein kinase inhibitor Calphostin C prevents the induction of LTP but still permits a short-lasting potentiation similar to that observed in the presence of galanin. In *B*, the bar graph illustrates the magnitude of LTP induced by a 100 Hz for 1 s tetanus in control medium and in the presence of 1 μM of the protein kinase inhibitors K-252b (n=5), calphostin C (n=5) and KT5823 (n=5), 30 minutes post-tetanus (ANOVA, P<0.05, 0.05 and 0.01 respectively).

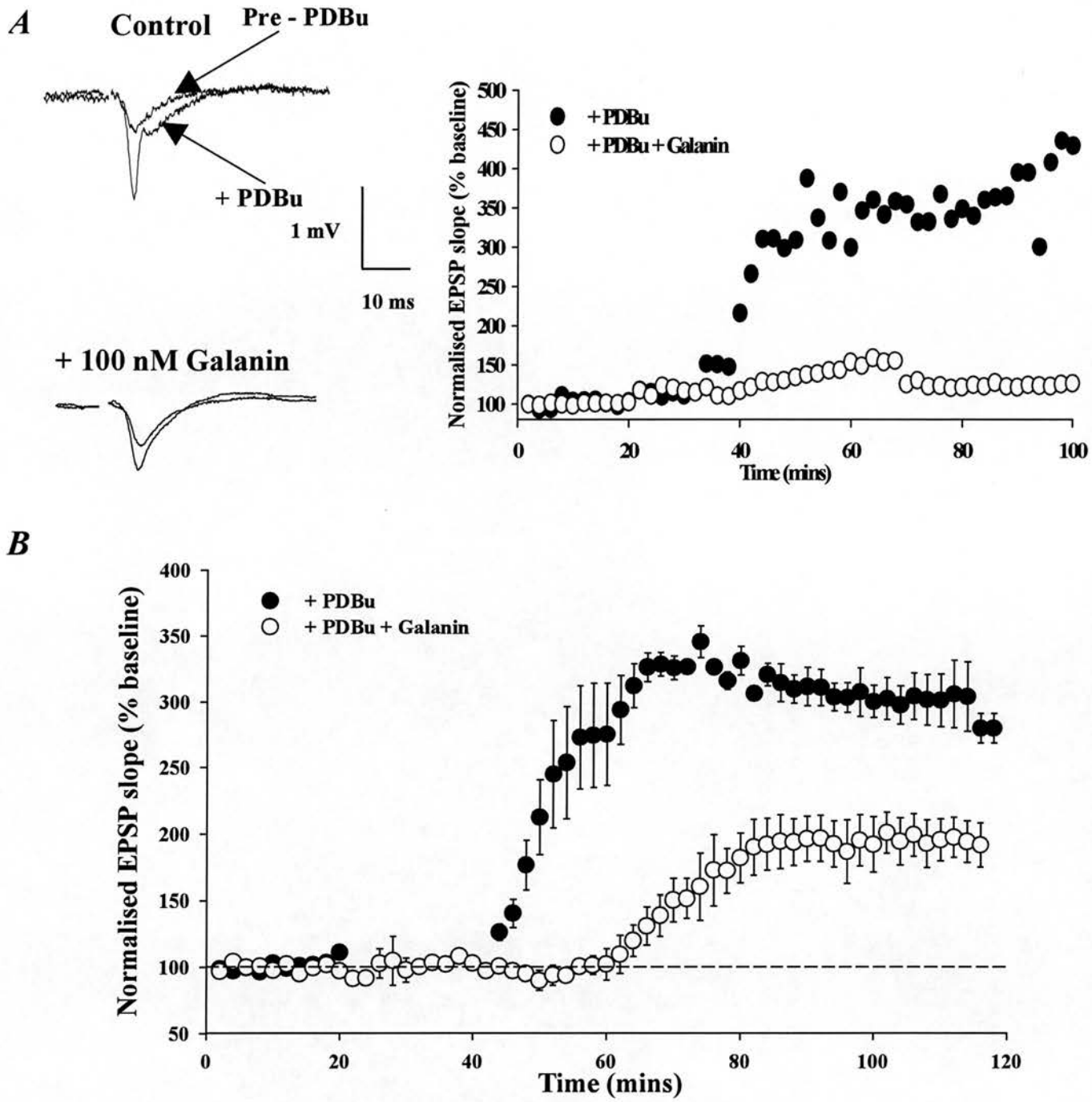


Figure 4.11.Galanin impairs phorbol-dibutyrate induced potentiation of synaptic transmission. In *A*, the superimposed synaptic traces are representative examples of fEPSPs recorded in these two sets of experiments before PDBu application and 30 minutes afterwards (left panel). Graphical representation (right panel) of two individual recordings, where PDBu-induced potentiation (filled circles) is inhibited by coapplication of galanin (open circles). In *B,* the graph shows pooled data in a time-dependent plot of the fEPSP potentiation induced by phorbol-dibutyrate in the presence (n=8) and absence (n=8) of galanin (ANOVA, P<0.05).

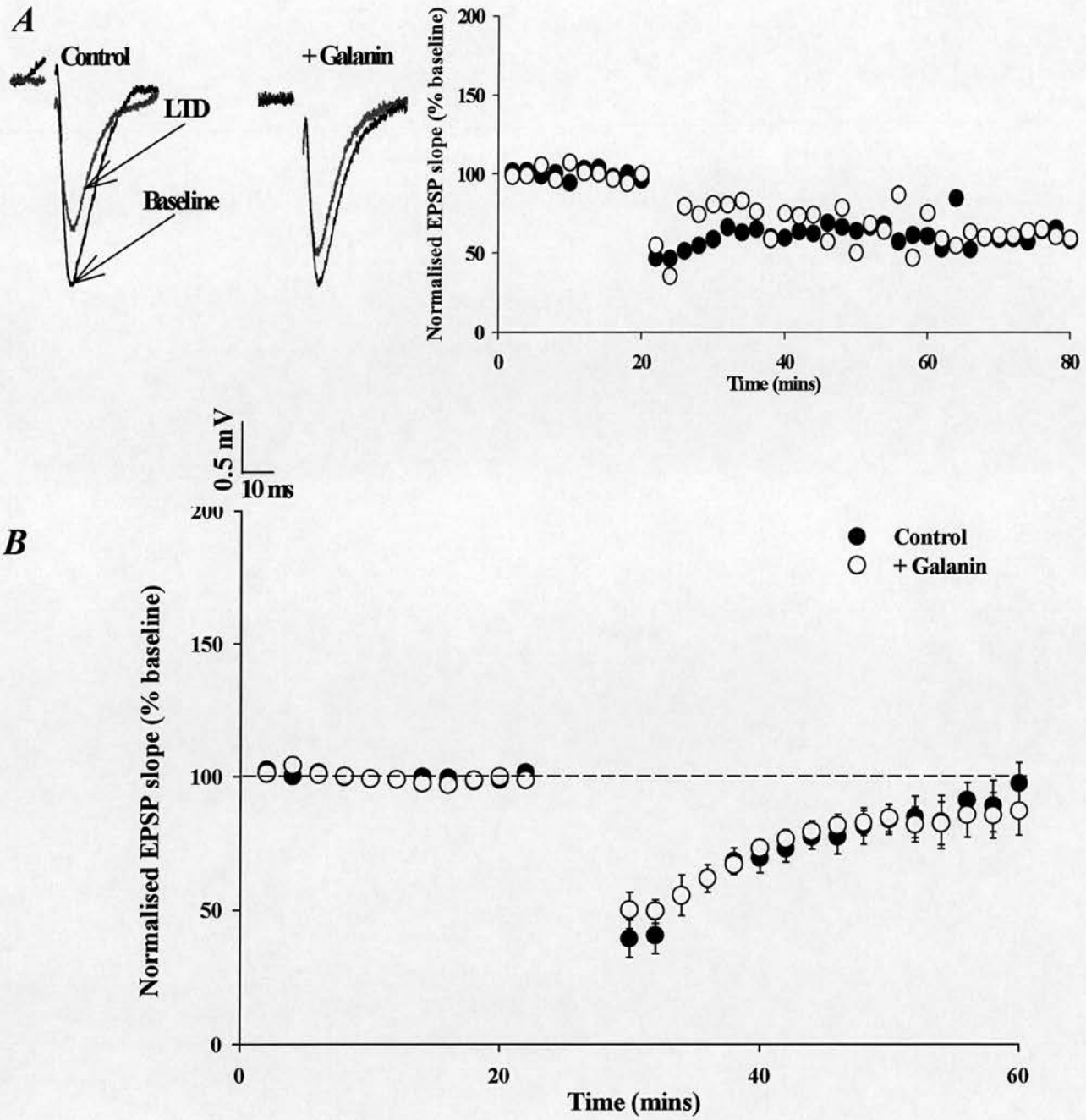


Figure 4.12. Galanin does not affect LTD. *A*, superimposed traces recorded prior to (black traces) and following (blue traces) a 2 Hz for 7.5 min LTD induction protocol in the absence (left panel) and presence (right panel) of galanin. On the right, two individual recordings are shown in graphical representation, where LTD was induced in the absence (filled circles) and the presence (open circles) of galanin. *B*, pooled data are shown in a plot of the mean ± S.E.M. of the slope of the fEPSP in *stratum radiatum*, normalized with respect to the 20 minute baseline immediately preceeding the LTD conditioning stimulation paradigm, *versus* time, in the absence (closed circles) and presence of 100 nM galanin (open circles). Data points have the same meaning as described in previous figures. Synaptic traces correspond to representative field EPSPs recorded prior to and 20 minutes after the period of conditioning stimulation in the presence and absence of galanin. No statistical significance was observed in recordings prior to and following the application of galanin (ANOVA, $P>0.05$).

4.2.5. Galanin fails to impair LTP in the presence of M15

Next, galanin receptor mixed agonist M15 was perfused on hippocampal slices to investigate whether it antagonises the effect of galanin on glutamate receptor mediated synaptic plasticity. As such, in *stratum radiatum* M15 (1 μM) perfused with galanin (100 nM) induced an average LTP 151 ± 14 % SEM (n=6), while M15 perfused on its own 149 ± 10 % SEM (n=6). Both sets of experiments yielded no significant difference between LTP recorded in control conditions (Student's *t-test*, $P>0.05$), while they were statistically significant to LTP induced in the presence of galanin (Student's *t-test*, $P>0.05$). There was no significant difference prior to and following galanin application (ANOVA, $P>0.05$). In Figure 4.13, LTP graphs obtained in the presence of M15 on its own (filled triangles, n=6) or in combination with galanin (open triangles, n=6) are presented, to show that M15 when applied with galanin reverses the degradatory effect of galanin on LTP. Recordings were performed in apical and basal dendrites and data were pooled together. All normalised values following tetanic stimulation were averaged and the results for each drug treatment are presented in graphical format (Figure 4.14). Application of galanin has a statistically significant impairment on LTP compared to control, galantide & galanin or galantide applied on its own (ANOVA, $P<0.05$).

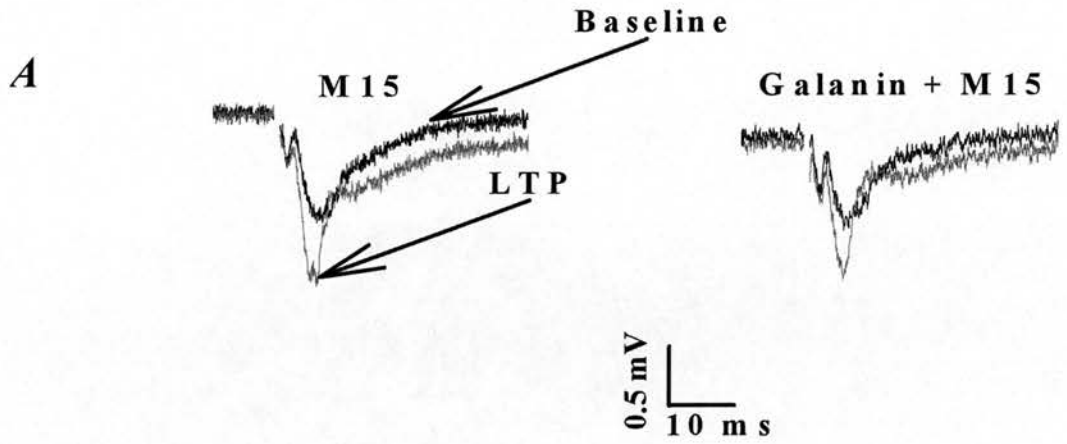
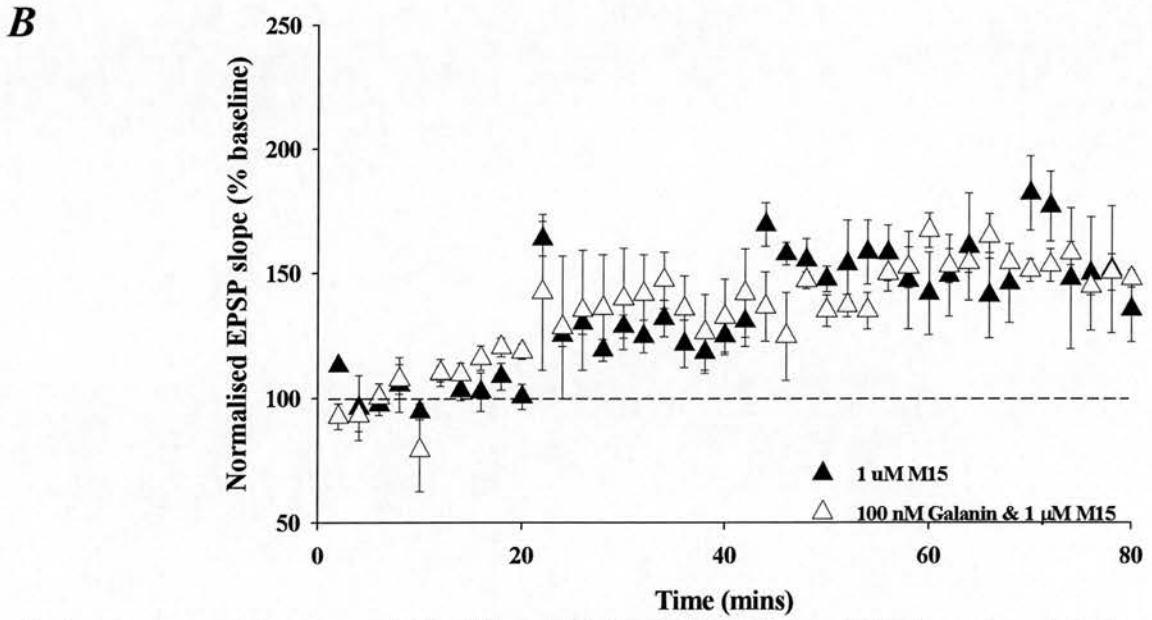


Figure 4.13. M15 blocks galanin-induced LTP impairment (1).



In *A*, superimposed traces are obtained form individual tetanus induced LTP experiments in the CA1 area, where M15 was perfused 10 minutes prior to (black trace, left) and 15 minutes following tetanic stimulation (green trace, left). The second pair of superimposed traces show a baseline trace (black, right) recorded in galanin and M15 compared to a trace following tetanic stimulation (green, right). *B*,Graphical representation of the effects of galanin inhibitor M15 on LTP (n=6, ANOVA, P>0.05). When M15 is coapplied with galanin (n=6), then the impairment induced by galanin on LTP is alleviated (ANOVA, P<0.05).

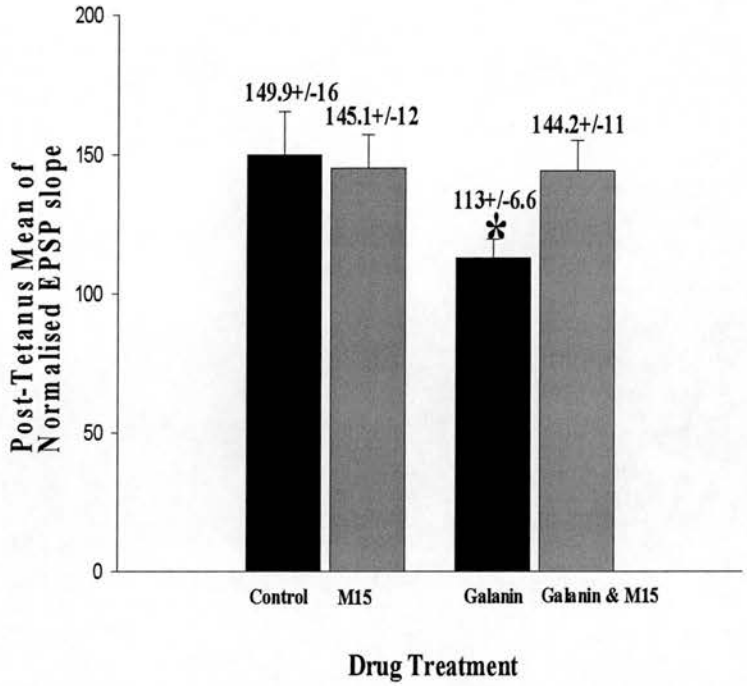


Figure 4.14. M15 blocks galanin-induced LTP impairment (2). Graphical representation of 30 minutes post-tetanus mean of normalised fEPSP slope for control LTP experiments (n=8) compared to LTP experiments obtained in the presence of M15 (n=6) alone, galanin alone (n=8) and a coctail of galanin and M15 (n=6). Addition of M15 reverses the significant impairment induced by galanin (ANOVA, $P<0.05$).

4.3. Discussion

The present data confirm previous findings of Sakurai *et al.* (1996) that galanin restricts the induction of LTP in the apical dendrites of CA1 pyramidal neurones. In addition, here I now showed that galanin inhibits LTP in the basal dendrites of these neurons. Taking Chapters 4 and 5, I show that this inhibitory effect of galanin on LTP is conserved in three species *viz* guinea pig (Sakurai *et al.*, 1996), rat and mouse. Furthermore, this effect is independent of the pattern of afferent stimulation used to induce LTP and is selective for this form of long-term synaptic plasticity in that LTD is not affected by galanin applied during the conditioning period.

Until now, no formal studies have attempted to address the mechanism by which galanin restricts the induction of LTP. In this respect, my study was an innovative way to address the cellular mechanism by which galanin may inhibit learning and memory. Consistent with this behavioural effect of galanin, the observations that galanin (1) does not affect basal synaptic transmission, (2) is effective only when applied during the period of conditioning stimulation and (3) still permits short-term potentiation to be expressed in its presence suggests that its mechanism of action is related to an effect on those cellular processes that are necessary for the induction of LTP and, more specifically, to those processes that are important for the conversion of STP into LTP. Relevant to this point, the activity-dependent alterations in glutamatergic and GABAergic synaptic transmission that are necessary to promote NMDA receptor activation and the induction of LTP during tetanic and theta-burst tetanic stimulation are quite distinct (Davies & Collingridge, 1993, 1996). Illustrating this point, pharmacological agents that modify GABAergic synaptic transmission, such as benzodiazepines or $GABA_B$ receptor antagonists, inhibit theta-burst induced LTP but not tetanus-induced LTP (Davies *et al.*, 1991; Seabrook *et al.*, 1997). Thus, as galanin blocks LTP induced by both conditioning stimuli it would seem unlikely that its mechanism of action is related to subtle modulation of GABAergic or glutamatergic synaptic activity.

Indeed, there is little if any evidence to suggest that galanin directly affects transient activity-dependent changes in either GABAergic or glutamatergic synapses. Instead, galanin is most likely having its inhibitory effect through a robust inhibition of one

or more critical LTP inducing mechanisms that are common to both stimulation protocols.

One such common feature of these different methods of inducing LTP, is that each is critically reliant on activation of NMDA receptors during the period of conditioning stimulation. However, our experiments suggest that galanin does not (1) inhibit glutamate release *per se*, as suggested in one neurochemical study (Zini *et al.*, 1993). or (2) directly antagonize NMDA receptors triggering the induction of LTP by both stimulation protocols (Collingridge *et al.*, 1983; Davies *et al.*, 1991). In addition, whilst antagonism of metabotropic glutamate receptors during both stimulation paradigms leads to the generation of prolonged STP (similar to that observed when galanin is applied during the period of conditioning stimulation) as opposed to maintained LTP, (Bashir *et al.*, 1993; Breakwell *et al.*, 1996) galanin did not appear to act as an antagonist of mGluR Group I function, that may be involved in modulating LTP in the CA1 area via the activation of PLC, which is thought to be the coupling mechanism of GALR2 (Fathi *et al.*, 1997; Wittau *et al.*, 2000)

Given the complexity of LTP and the varied effects of galanin in the CNS (e.g., inhibition of transmitter release, inhibition of adenylate cyclase (Rökeaus, 1987; Kask *et al.*, 1995), it is possible to devise many alternative mechanisms that account for its effect on LTP. For example, galanin may inhibit the intracellular calcium rise that is responsible for triggering changes in intracellular biochemistry that maintain LTP. In this respect, whilst galanin can inhibit intracellular calcium rises through inhibition of voltage-gated calcium currents (Palazzi *et al.*, 1991) these channels have little bearing on the induction of LTP although they have been reported to influence the induction of LTD (Wang *et al.*, 1997a-c). Furthermore, based on our own observations that galanin inhibited LTP without affecting LTD, it seems unlikely that galanin is exerting its inhibitory effect through this mechanism.

Beyond the levels of receptor and intracellular calcium rises, there is a strong consensus that kinase activity is responsible for the conversion of STP into LTP, although the precise sequence of this multi-kinase biochemical cascade has yet to be precisely defined (Malinow *et al.*, 1989; Bliss & Collingridge, 1993; Grant & O'Dell, 1994). Thus, it is conceivable that galanin may restrict the induction of LTP by disturbing this biochemical cascade. Indeed, four lines of evidence reported here,

when taken together, suggest that this may be the case. These are (1) the time points of application of galanin that are effective at inhibiting LTP mirror those for many kinase inhibitors (Malinow *et al.*, 1989; Bliss & Collingridge, 1993; Grant & O'Dell, 1994; Zhuo *et al.*, 1995) (2) the time course of STP induced in the presence of galanin is similar to that observed for many kinase inhibitors (Malinow *et al.*, 1989; Bliss & Collingridge, 1993; Grant & O'Dell, 1994), (3) galanin does not affect long-term synaptic plasticity (LTD) that predominantly depends upon phosphatase, as opposed to kinase activity (Bear & Malenka, 1994) and (4) galanin inhibits synaptic potentiation induced by phorbol esters. Furthermore, galanin (a) restricts protein kinase C-induced protein phosphorylation in the hippocampus (LaPorta *et al.*, 1992) and (b) inhibits adenylate cyclase activity (Kask *et al.*, 1995a-b) potentially reducing protein kinase A activity; both of which may selectively restrict LTP expression. Whatever the mechanism involved, this inhibitory effect of galanin does provide a synaptic correlate for the galanin-induced learning impairments that have been reported in behavioural tests such as the Morris water maze task, delayed non-matching to sample and active avoidance tests (Crawley & Wenk, 1989; Crawley, 1993). It should be pointed out, however, that this is unlikely to be the only mechanism contributing to the galanin-induced impairment of learning since galanin can inhibit septohippocampal cholinergic inputs (Fisone *et al.*, 198; Dutar *et al.*, 1995; Palazzi *et al.*, 1991), which have a major impact on these learning paradigms. Whilst this may have an negative effect on learning in its own right, in terms of LTP this effect will be most evident on associative LTP in which cholinergic and glutamatergic afferents are activated coincidentally (Sokolov *et al.*, 1995). This, however, is unlikely to explain the *in vitro* results presented here where non-associative LTP was examined and which is independent of either muscarinic or nicotinic acetylcholine receptor stimulation. If the galanin mutation does not affect glutamate or GABAergic synaptic transmission in rats and mice that would indicate that the galanin-null knockout mouse exhibits selective modification of the cholinergic system (O'Meara *et al.*, 2000).

4.4. Future Experiments

I was unable to determine the site of action of galanin from my recordings. However, it would be useful to determine whether galanin acts at a pre- or a postsynaptic site to exert its degradatory effect on synaptic plasticity in the CA1 area of rodent hippocampus. Furthermore, as already mentioned earlier in this chapter, galantide is not a very specific antagonist to galanin receptors. Further pharmacological characterisation of galanin receptor mediated physiology hippocampal slices awaits the development of galanin receptor subtype specific agonists and antagonists. *In vitro* experiments are difficult to perform due to the instability of peptide analogues throughout the duration of an experiment. Therefore, non-peptide compounds active on galanin receptors would be favoured, e.g. SCH202596. Finally, new insights into the physiology of galanin and galanin sensitive receptors in the cortex would be shed with a conditional receptor knockout or overexpression animal model rather than a global galanin-null knockout mouse. It remains to be investigated how exogenous galanin affects GABAergic transmission proper in the CA1 area of rodent hippocampus in rats and the galanin null mouse (Coumis and Davies, 2002).

CHAPTER 5
ELECTROPHYSIOLOGICAL CHARACTERISATION OF SYNAPTIC PLASTICITY AND TRANSMISSION IN GALANIN-NULL TRANSGENIC MICE AND WILD-TYPE LITTERMATES

5.1. Introduction

Here, I show that although basal synaptic transmission and short-term synaptic plasticity is not affected by the galanin-null mutation (Figures from 5.1 to 5.6), tetanus-induced LTP is significantly impaired in *stratum oriens*, but not *radiatum* in slices obtained from $Gal^{-/-}$ mice (Figures 5.7 and 5.9). This is impairment is not age-dependent, but appears to depend on the conditioning stimulus, as it does not occur during LTP induced by theta-burst (Figure 5.8). Additionally, synaptic plasticity exhibits a significant impairment following a saturation paradigm as well, which is confined to recordings performed in *stratum oriens* only (Figure 5.10). Finally, exogenous galanin perfusion causes an impairment in tetanus induced LTP and inhibits the cholinergic $EPSP_M$ in wild-type mice, but not in the $Gal^{-/-}$ mice (Figures 5.11 and 5.12). All the results in this chapter were obtained by myself, apart from the intracellular $EPSP_M$ data, which were obtained in conjunction with Dr. I. R. Kearns.

5.2. Results

5.2.1. Global galanin gene deletion does not affect synaptic strength

Synaptic strength of glutamatergic synaptic transmission may be assessed by mapping the size of fEPSPs to the intensity of stimulation used (Skelton *et al.*, 1983). I recorded fEPSPs evoked in response to electrical stimulation. Two groups of mice were used (1) young (3-5 months) and (2) old (11-13 months) and for each age group synaptic transmission was assessed in two areas of the CA1, *stratum radiatum* and *stratum oriens*. The slope of fEPSPs was measured over a range of stimulus intensities from 0 to 80V. There was no significant difference observed in *stratum radiatum* of young (wild-type, $Gal^{-/-}$; n=7,7) or old (wild-type, $Gal^{-/-}$; n=9,12) mouse groups. Similarly, there was no significant difference observed in *stratum oriens*

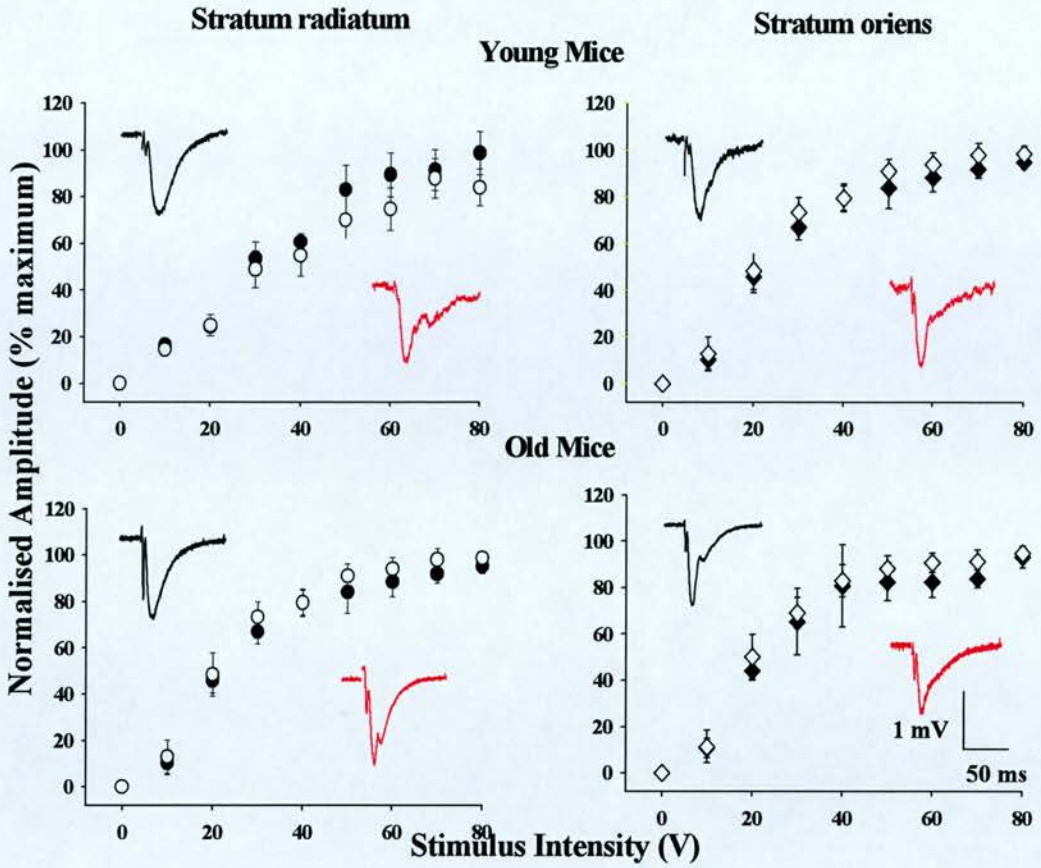


Figure 5.1. Global galanin gene deletion does not affect synaptic strength. Input-output curves featuring the normalised amplitude to percentage maximum of field EPSPs evoked in CA1 area of the hippocampus over a range of stimulus intensities from 0 to 80 V. The experiment was performed in both basal (wild-type Wt, transgenic $Gal^{-/-}$; n=7,7) and apical (Wt, $Gal^{-/-}$ n=9,12) dendrites in young (WT, $Gal^{-/-}$; n=7,7) and old mice (Wt, $Gal^{-/-}$; n=8,6). Paired *Student's t-test* revealed no significant difference between wild-type (closed shapes) and transgenic (open shapes) slices in neither group (Student's *t-test,* P>0.05). Each point is the pooled average of four sweeps. The traces inside each panel depict a synaptic response obtained from a wild-type (black traces) or a $Gal^{-/-}$ (red traces) mouse slice for the respective group, evoked at 40V.

young (wild-type, $Gal^{-/-}$; n=7,7) or old (wild-type, $Gal^{-/-}$; n=8,6) mice. Statistical significance was assessed using *Student's t-test*, $P<0.05$. See Figure 5.1.

5.2.2. Lack of galanin does not affect short-term synaptic plasticity

I used paired pulse facilitation (PPF) as a marker of short-term synaptic plasticity in the CA1 area of the hippocampus. PPF was examined by delivering two consecutive shocks across a range of interpulse intervals between 12.5 and 400 ms. Short-term synaptic plasticity was assessed by measuring the ratio of the initial slope of the fEPSP in response to the second stimulus over that of the field in response to the first stimulus. The pooled average ratios from means of four sweeps for each interpulse interval were plotted against the intervals (Figure 5.2.). In the young group of animals tested in both *stratum radiatum* (wild-type, $Gal^{-/-}$; n=6,8) and *stratum oriens* (wild-type, $Gal^{-/-}$; n=9,7) there was no significant difference between transgenic and wild-type littermates. Similarly, in the old group of animals tested in *stratum radiatum* (wild-type, $Gal^{-/-}$; n=7,7) there was no significant difference at all interpulse intervals tested. During recordings in *stratum oriens*, however, there was a statistical significance when the two pulses were delivered 100 and 200 ms apart. Statistical difference was assessed using *Student's t-test* ($P<0.05$). Figures 5.3 to 5.6 illustrate superimposed traces of individual recordings underoing a paired-pulse facilitation protocol.

5.2.3. The effect of galanin-null mutation on LTP

How does the absence of galanin from birth affect LTP in the CA1 area of mouse slices? This series of experiments is illustrated in Figures 5.7 to 5.9. In panels 5.7*A*, 5.8*A* and 5.9*A* superimposed traces of baseline and post-conditioning stimulation fEPSPs are shown corresponding to individual recordings laid out immediately below.

Similarly to the way LTP experiments were performed as described in Chapter 4, tetanic or theta-burst stimulation was delivered after a 20-minute baseline was obtained. LTP was assessed for at least 60 minutes following the conditioning stimulation in the CA1 area of slices obtained from either young or old mice. As

shown in Figure 5.7*B* (bottom panel), tetanus-induced LTP was significantly impaired in *stratum oriens* of young mice from 172 ± 19 % S.E.M. in slices prepared from

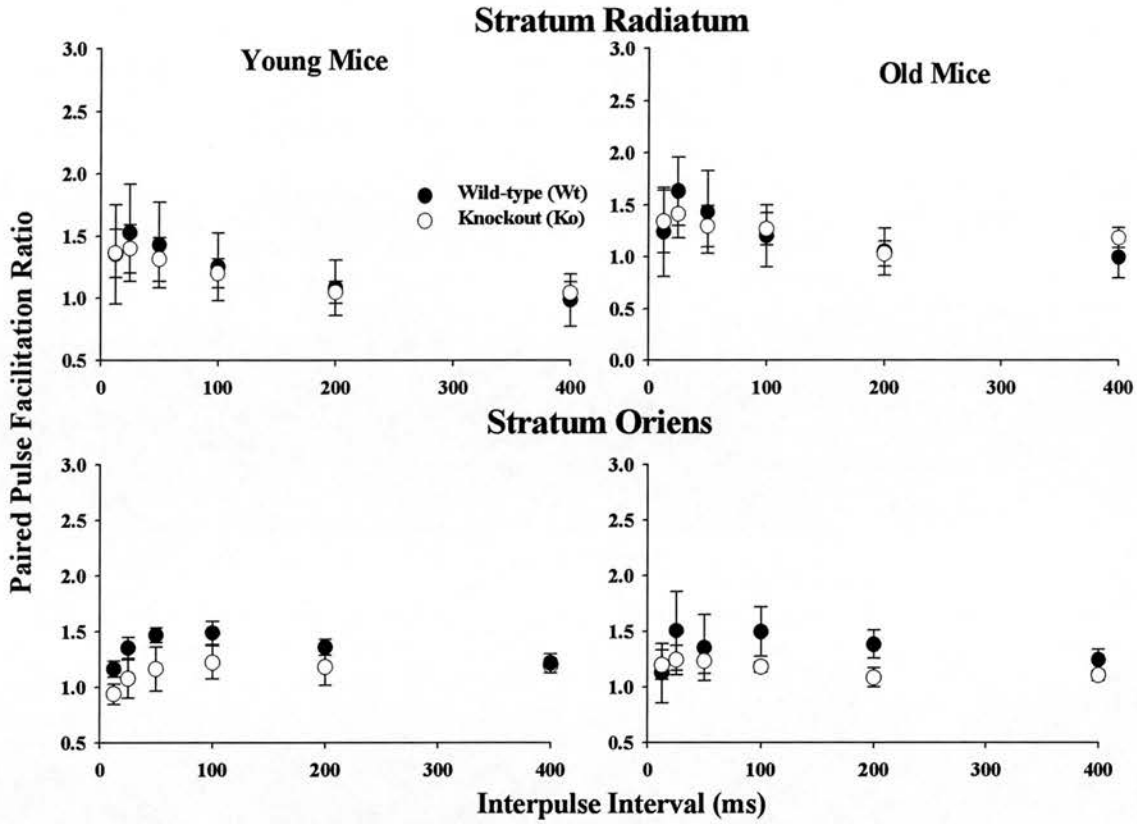


Figure 5.2. Global galanin gene deletion does not significantly alter PPF ratios. PPF experiments were carried out by two simultaneous shocks of identical strength at interpulse intervals between 12.5 and 400 ms, so that the magnitude of the evoked field EPSP slope in response to the first shock (S1) is half maximal. Each point in the above graphs constitutes the PPF ratio, equal to S2/S1, where S2 is the initial slope of fEPSPs in response to the second stimulus and is the pooled average of four consecutive sweeps. Paired *Student's t-test* revealed no significant difference ($P<0.05$) between wild-type (filled circles) and transgenic (open circles) slices when the experiment was performed in *stratum radiatum* in young mice (Wt, $Gal^{-/-}$; n=6,6) and in old mice (Wt, $Gal^{-/-}$; n=7,7) and in *stratum oriens* in young mice (Wt, $Gal^{-/-}$; n=7,7) and old mice (Wt, $Gal^{-/-}$; n=6,6).

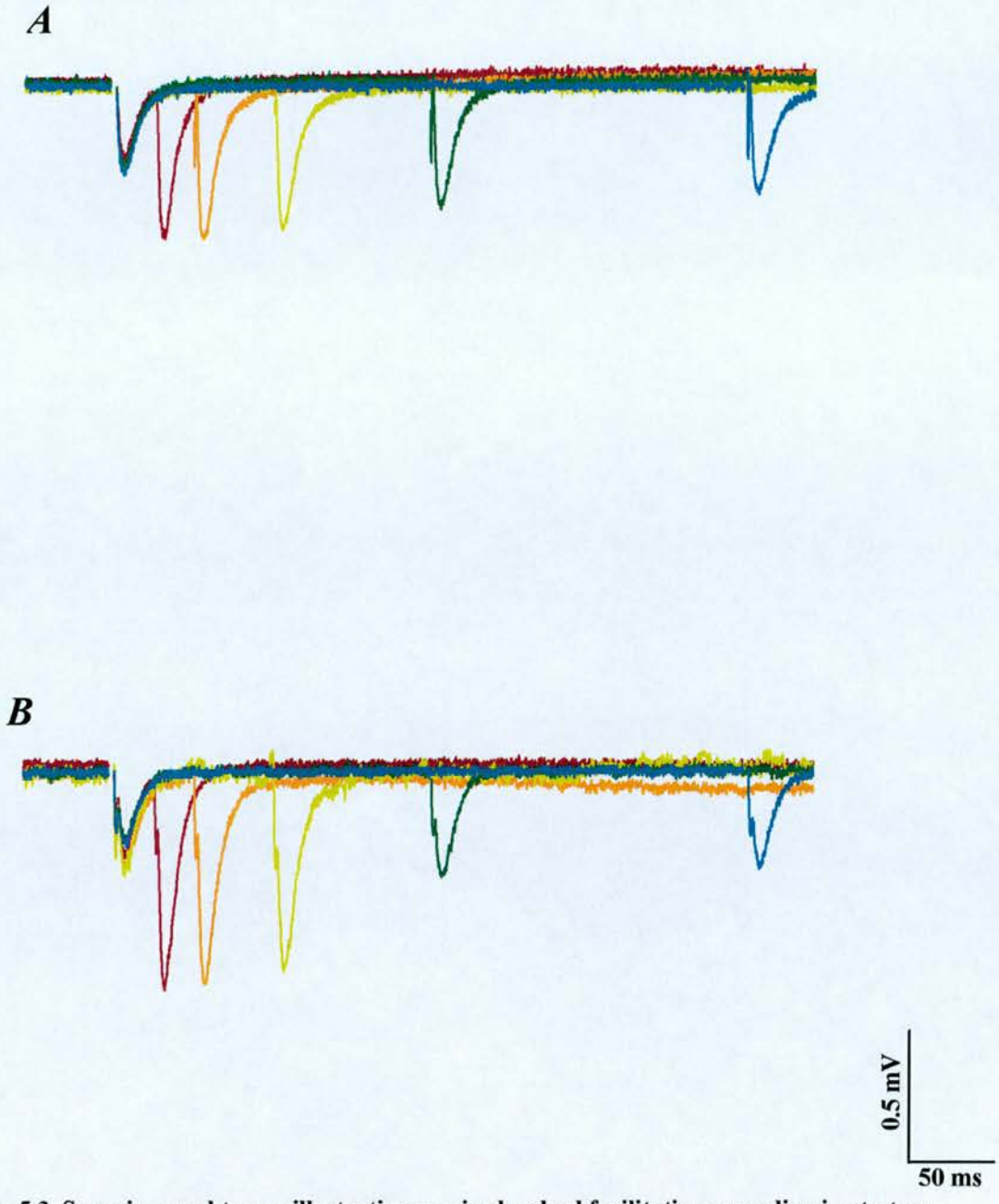


Figure 5.3. Superimposed traces illustrating a paired-pulsed facilitation recording in *stratum radiatum* of young wild-type (*A*) and Gal$^{-/-}$ mice (*B*) at interpulse intervals between 25 and 400 ms.

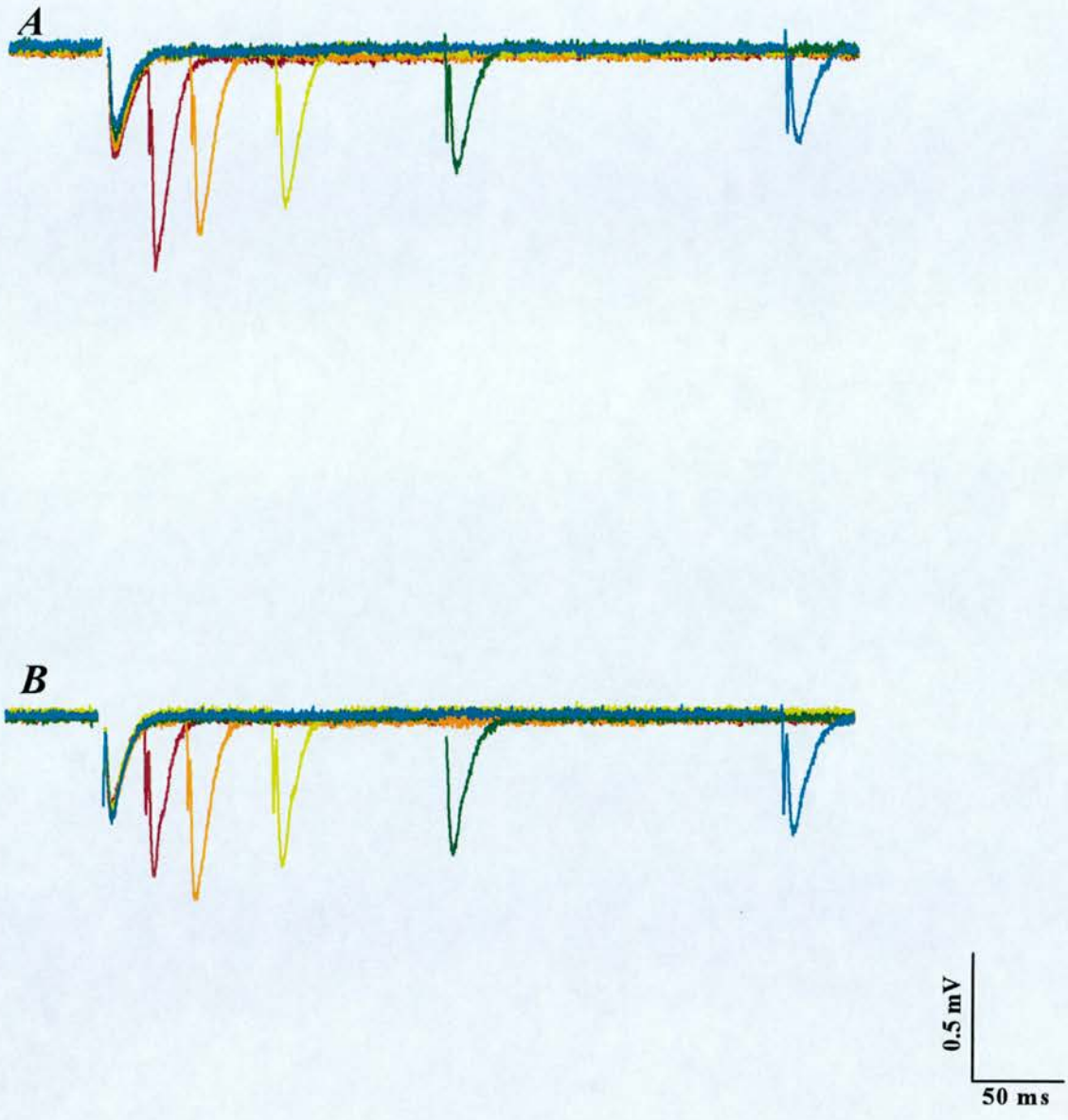


Figure 5.4. Superimposed traces illustrating a paired-pulsed facilitation recording in *stratum oriens* of young wild-type (*A*) and Gal$^{-/-}$ mice (*B*) at interpulse intervals between 25 and 400 ms.

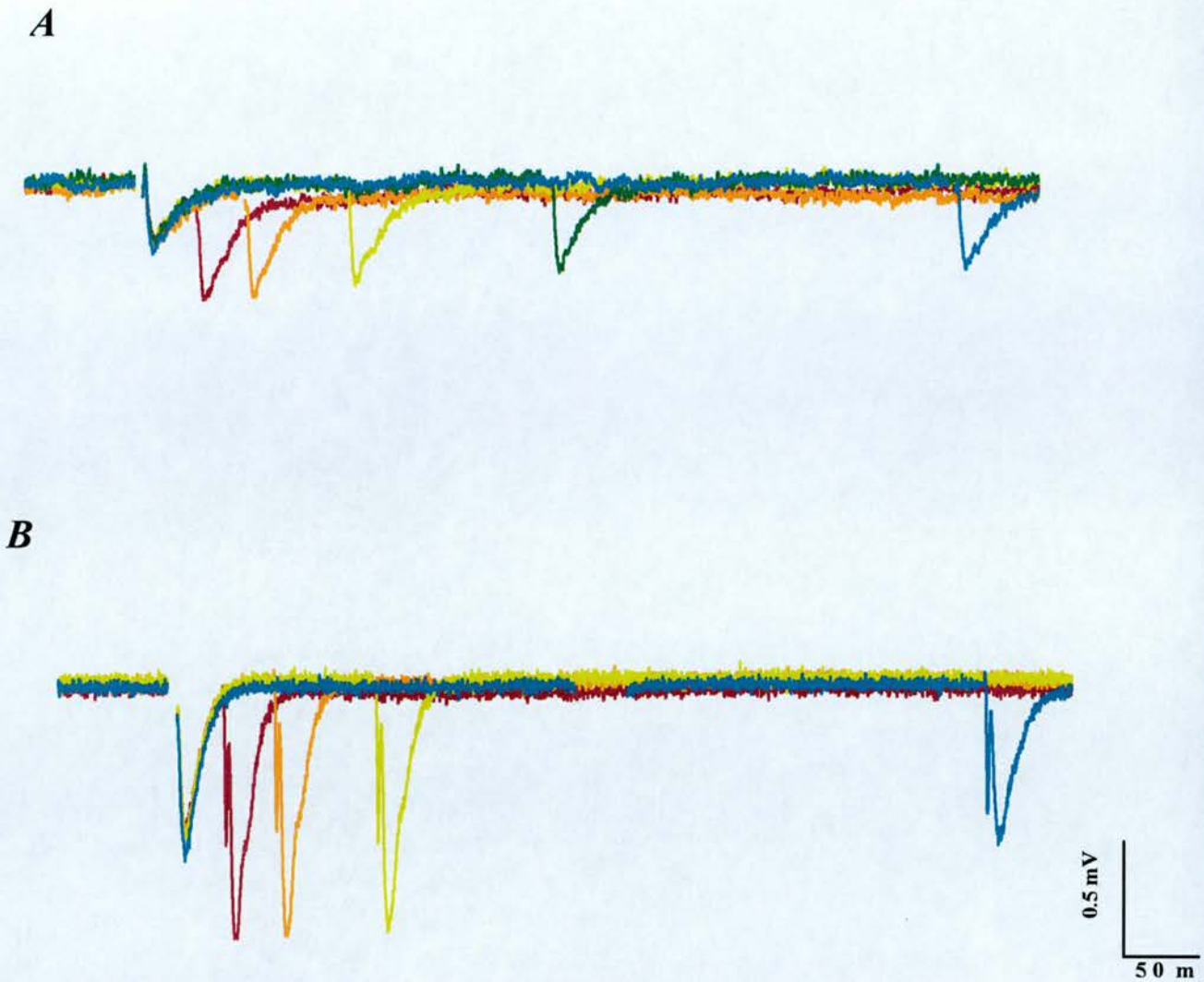


Figure 5.5. Superimposed traces illustrating a paired-pulsed facilitation recording in *stratum radiatum* of old wild-type (*A*) and Gal$^{-/-}$ mice (*B*) at interpulse intervals between 25 and 400 ms.

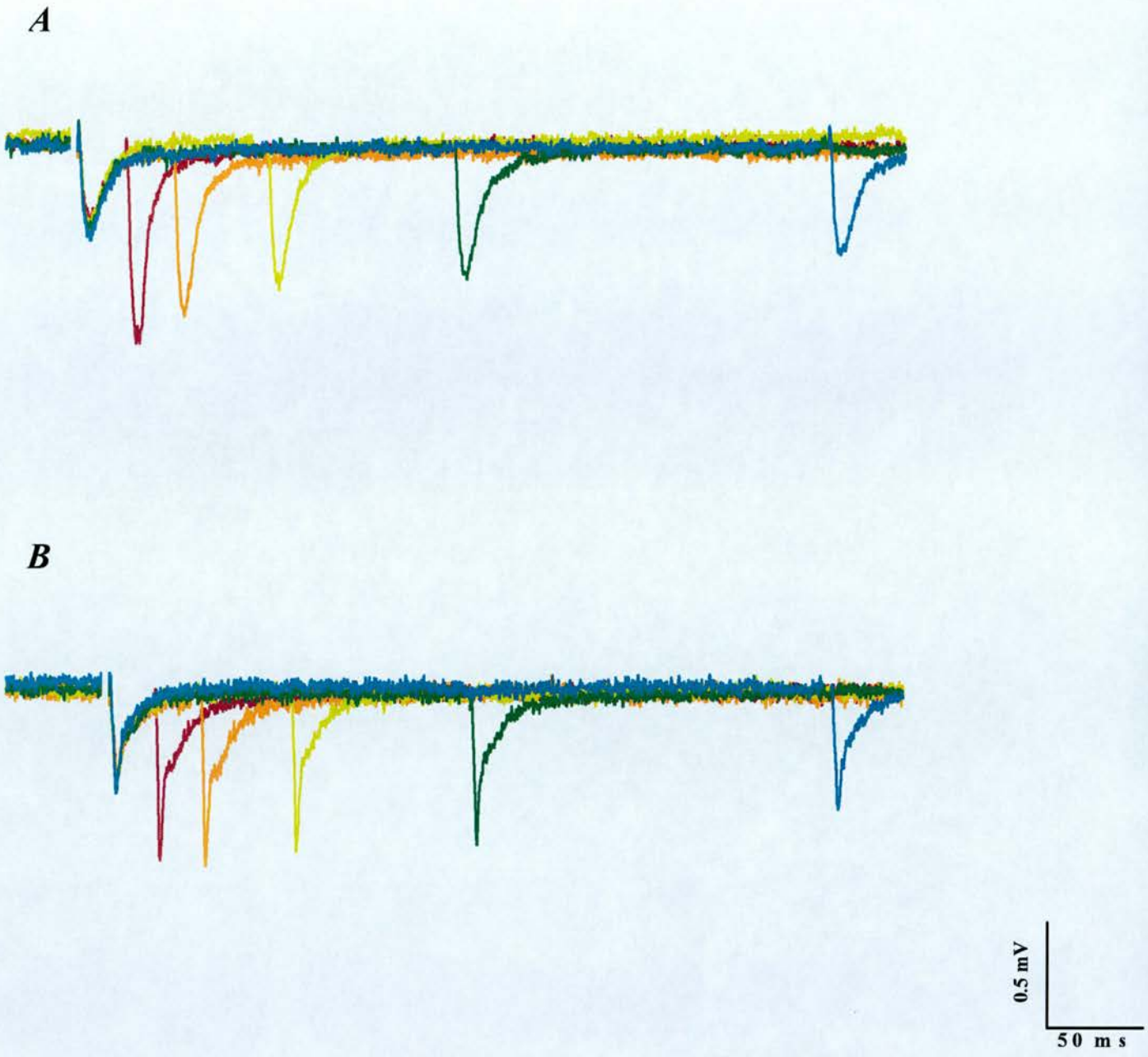


Figure 5.6. Superimposed traces illustrating a paired-pulsed facilitation recording in *stratum oriens* of old wild-type (*A*) and Gal$^{-/-}$ mice (*B*) at interpulse intervals between 25 and 400 ms.

wild-type (n=8) to 134 ± 8 % S.E.M. in slices prepared from transgenic mice (n=8). However, in *stratum radiatum* the values as measured 30 minutes post-tetanus were 152 ± 9 % S.E.M. for wild-type (n=8) and 149 ± 8 % S.E.M. for transgenic mice (n=8). Figure 5.9*B* illustrates that the magnitude of LTP impairment was increased in recordings performed in *stratum oriens* of old mouse slices from 145 ± 11 % S.E.M. (wild-type, n=6) to 119 ± 9 % S.E.M. ($Gal^{-/-}$, n=9), while LTP in *stratum radiatum* was indistinguishable between wild-type (155 ± 6 % S.E.M., n=8) and $Gal^{-/-}$ mice (150 ± 5 % S.E.M., n=8). Theta-burst induced LTP was indistinguishable between genotypes, in both *stratum oriens* (150 ± 5 % S.E.M., 151 ± 4 % S.E.M. for wild-type, $Gal^{-/-}$; n=8,8) and *radiatum* (140 ± 5 % S.E.M., 139 ± 4 % S.E.M. for wild-type, $Gal^{-/-}$; n=8,8) in slices obtained from young mice, as shown in Figure 5.8*B*.

5.2.4. Why does galanin deletion impair LTP in *stratum oriens*?

The final experiment was designed to address the reasons why the absence of galanin caused a significant impairment in LTP restricted to recordings performed in *stratum oriens* in the old animals. One possibility could be that the aforementioned genetic manipulation caused a faster saturation of synaptic plasticity in that area. Therefore, we designed a saturation protocol in which LTP was induced by a series of 100Hz, 1s tetani and each tetanus separated from the previous one by 30 minutes. This was repeated as many times as required to induce saturation of LTP. Saturation was defined as no further potentiation of the initial slope of fEPSPs following a tetanus. For most slices saturation occurred following the fifth tetanus and then stimulus intensity was turned down to obtain pre-LTP baseline sized fEPSPs. Again a baseline was recorded for twenty minutes before a further final tetanisation was delivered. This was used as a check that saturation of glutamate receptor mediated synaptic plasticity had actually occurred. Figure 5.10 shows pooled data in graphical format of recordings from both *stratum radiatum* and *oriens* in young and old animals. As expected saturation occurred to a singificantly greater extent in slices obtained from transgenic mice compared to wild-type littermates.

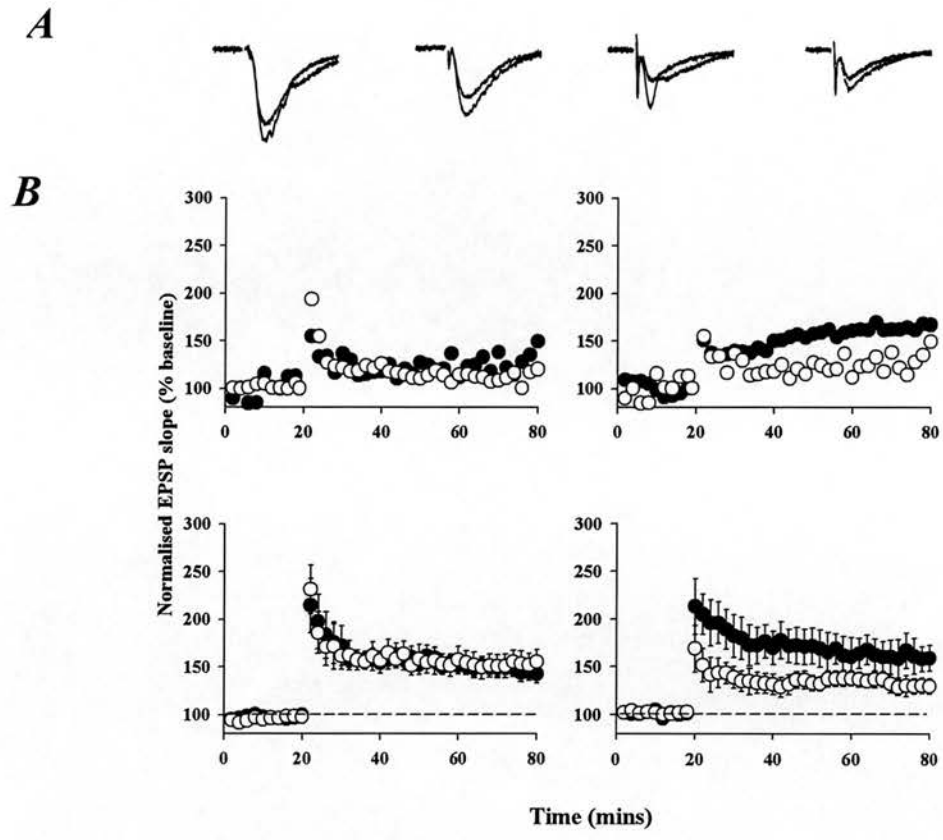


Figure 5.7. Tetanus mediated LTP is significantly impaired in *stratum oriens* of young transgenic mice. LTP experiments were performed as described in Figure 4.1. In *A*, superimposed traces denote control and 30 minutes post-tetanus synaptic response from wild-type (left panels) and Gal$^{-/-}$ mice (right panels). In *B*, individual experiments illustrate LTP in *stratum radiatum* (top left panel) or *oriens* (top right panel) in slices prepared from wild-type (filled circles) or Gal$^{-/-}$ (open circles) mice. Pooled data from young mice are shown in graphical representation, where tetanus induced LTP is indistinguishable between wild-type (filled circles) and Gal$^{-/-}$ mice (open circles) in *stratum radiatum* (bottom left panel), but significantly impaired in *stratum oriens* of Gal$^{-/-}$ mice (bottom right panel). Statistical significance was assessed by Student's *t-test* and ANOVA, P>0.05.

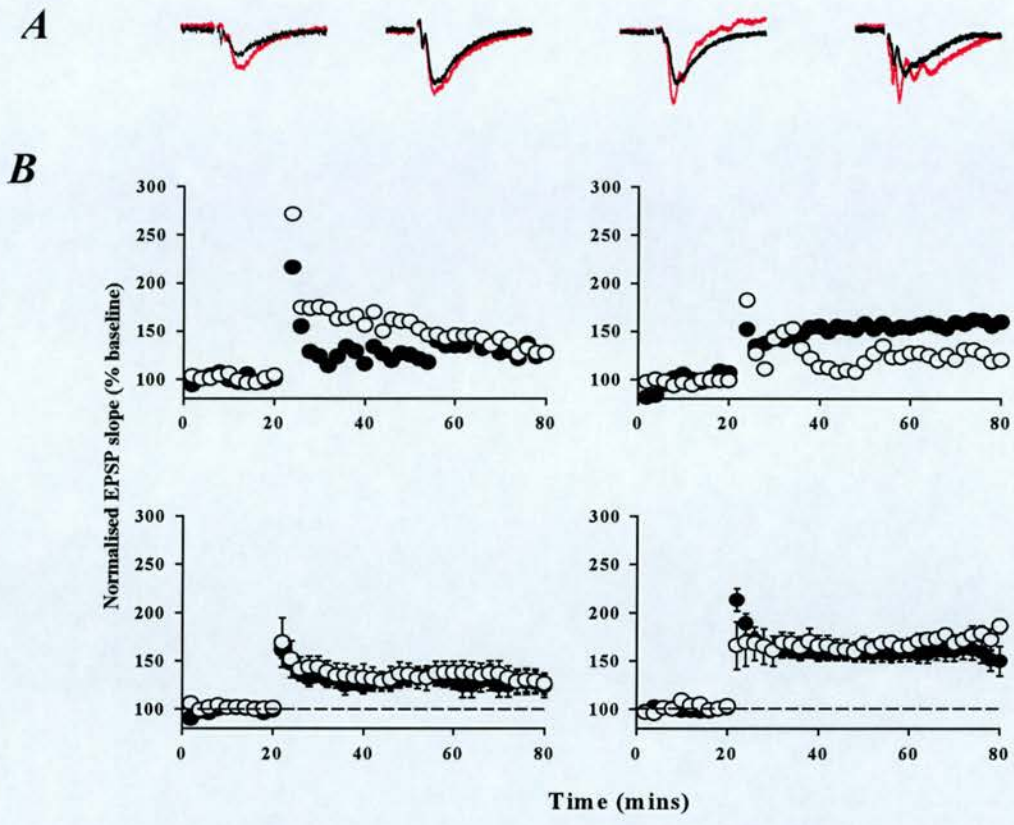


Figure 5.8. Theta-burst mediated LTP is indistinguishable between genotypes in the CA1 area of young transgenic mice. LTP experiments were performed as described in Figure 4.1. In *A*, superimposed traces denote control (black traces) and 30 minutes following theta-burst synaptic responses (red traces) from wild-type (left panels) and Gal$^{-/-}$ mice (right panels). In *B*, individual experiments illustrate LTP in *stratum radiatum* (top left panel) or *oriens* (top right panel) in slices prepared from wild-type (filled circles) or Gal$^{-/-}$ (open circles) mice. Pooled data from young mice are shown in graphical representation, where LTP induced by theta-burst is indistinguishable between wild-type (filled circles) and Gal$^{-/-}$ mice (open circles) in *stratum radiatum* (bottom left panel) or *oriens* (bottom right panel). Statistical significance was assessed by Student's *t-test* and ANOVA, P>0.05.

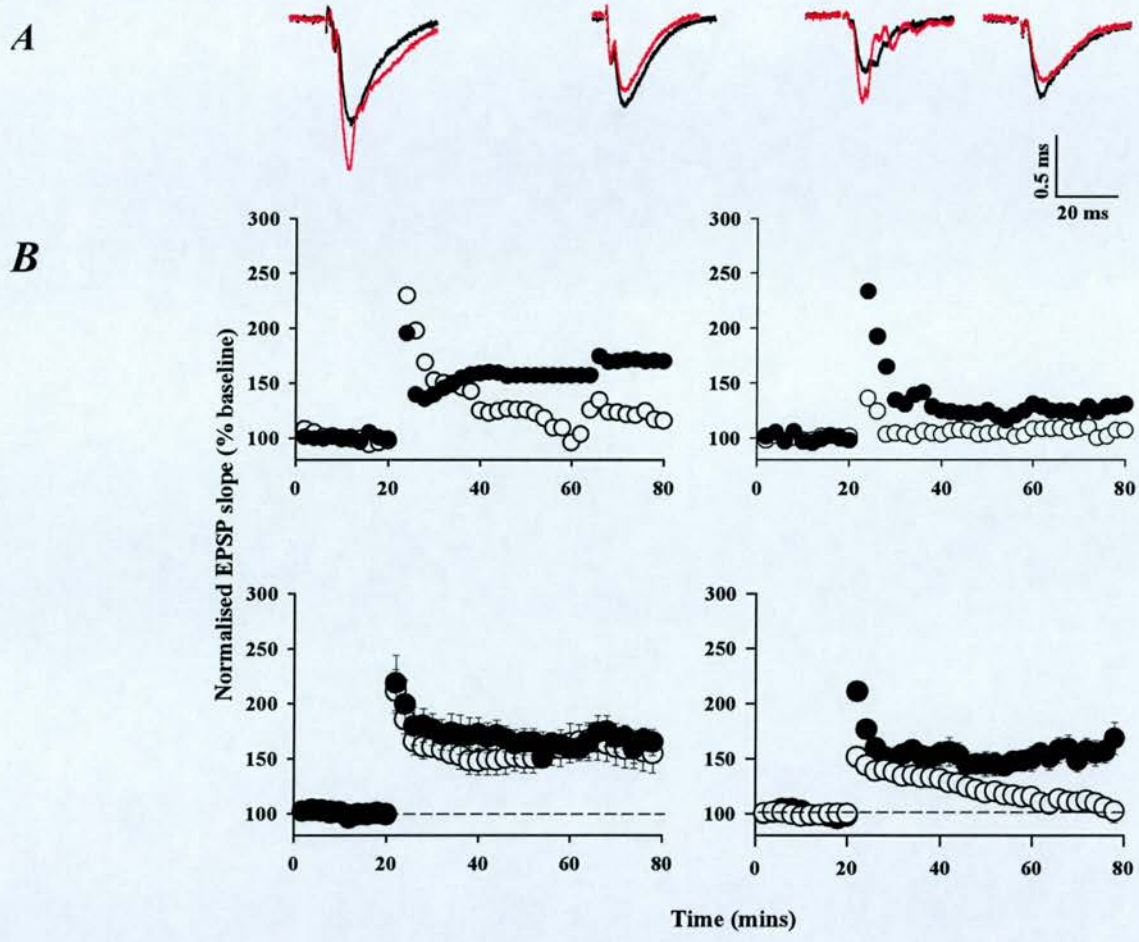


Figure 5.9. Tetanus mediated LTP is significantly impaired in *stratum oriens* of old transgenic mice. LTP experiments were performed as described in Figure 4.1. In *A*, superimposed traces denote control and 30 minutes post-tetanus synaptic response from wild-type (left panels) and $Gal^{-/-}$ mice (right panels). In *B*, individual experiments illustrate LTP in *stratum radiatum* (top left panel) or *oriens* (top right panel) in slices prepared from wild-type (filled circles) or $Gal^{-/-}$ (open circles) mice. Pooled data from young mice are shown in graphical representation, where tetanus induced LTP is indistinguishable between wild-type (filled circles) and $Gal^{-/-}$ mice (open circles) in *stratum radiatum* (bottom left panel), but significantly impaired in *stratum oriens* of $Gal^{-/-}$ mice (bottom right panel). Statistical significance was assessed by Student's *t-test* and ANOVA, P>0.05.

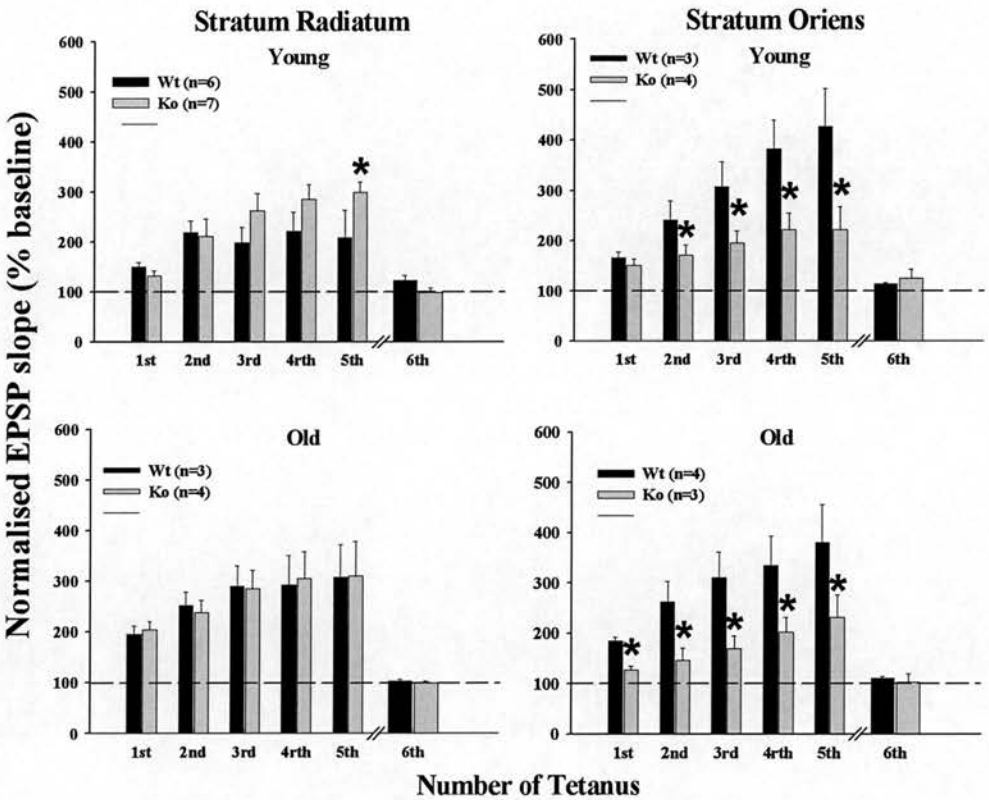


Figure 5.10. Saturation of LTP was tested by repeated tetanic stimulation (100Hz, 1s) every 30 minutes before the next tetanisation. The procedure was reapeated 5 times until synapses did not potentiate any further. Breaks indicate subsequent reduction (LFS, 0.067Hz) so that fEPSP slope was of similar magnitude to baseline values and a new baseline was recorded for 20 minutes before delivery of a final tetanus. The experiment was performed for wild-type and transgenic mouse slices and pooled data are presented in bar charts showing percentage of normalised fEPSP slope 20 minutes following each tetanus for wild-type (grey bars) and transgenic (black bars) slices. Statistical significance is indicated by * Student's *t-test*, ANOVA, $P<0.05$. Values are represented as ± S.E.M.

5.2.5. Exogenous galanin causes LTP impairment only in wild-type mouse slices

Exogenous galanin at a concentration of 100nM was perfused to assess its effects on LTP in both wild-type or $Gal^{-/-}$ mouse slices. Pooled data are illustrated in Figure 5.11. It is noteworthy that galanin did impair LTP induced by 100Hz for 1s significantly in wild-type animals, but did not have any effect on $Gal^{-/-}$ mouse slices. To investigate whether lack of endogenous galanin has any effect on hippocampal synaptic plasticity experiments described in Chapter 4, were repeated in galanin-null mouse slices. As such, 100nM galanin reduced the magnitude of LTP induced by a 100 Hz for 1 s tetanus delivered in both *stratum radiatum* and *oriens*. The magnitudes of LTP measured 30 minutes post-tetanus in the presence and absence of galanin in *stratum radiatum* were 146.9 ± 9.6 % (n = 7) and 149.6 ± 11.6 % SEM (n = 10) (ANOVA, P<0.05) and in *stratum oriens* 137.8 ± 8.7 % (n = 8) and 135.8 ± 8.7 % SEM (n = 6) (ANOVA, P<0.05). The averaged normalised values were 145.5 ± 12.2 % & 135.2±10 % SEM (*stratum radiatum*) for control and 144.7 ± 9.6 % & 143.1 ± 14.5 % SEM (*stratum oriens*) for galanin-treated slices (Student's *t-test*, P<0.05) compared to average baseline values 100.1±3 % and 98.2±4.8 % SEM and 99.27±2.2 % SEM, respectively.

5.2.6. Lack of galanin compromises cholinergic $EPSP_M$ in the CA1 area

The last series of experiments was designed to test the effect of the absence of galanin on cholinergic transmission in the hippocampus assessed by the muscarinic acetylcholine receptor mediated field EPSP ($EPSP_M$).

Electrical stimulation of stratum oriens of mouse hippocampal slices produced in CA1 pyramidal cells a muscarinic acetylcholine receptor mediated slow depolarisation which appeared very similar to the $EPSP_M$ recorded from rat hippocampal neurones (Nicoll & Cole, 1983; Dutar *et al.*, 1987). Figure 4.5. Both wild-type (n=4) and $Gal^{-/-}$ (n=4) mice demonstrated $EPSP_M$s. On application of 400nM exogenous galanin, the peak amplitude of $EPSP_M$ was inhibited by 50±3% of control in wild-type mice (n=4; p<0.05). In contrast, 400nM galanin had no significant effect on the peak amplitude of the $EPSP_M$ in *Gal*$^{-/-}$ mice (94±5% of

control, n=4; $p>0.05$). This significant difference was observed in the magnitude of inhibition that galanin exerted on the $EPSP_M$ in wild-type compared with *Gal*$^{-/-}$ mice ($p<0.05$)(Figure 5.12).

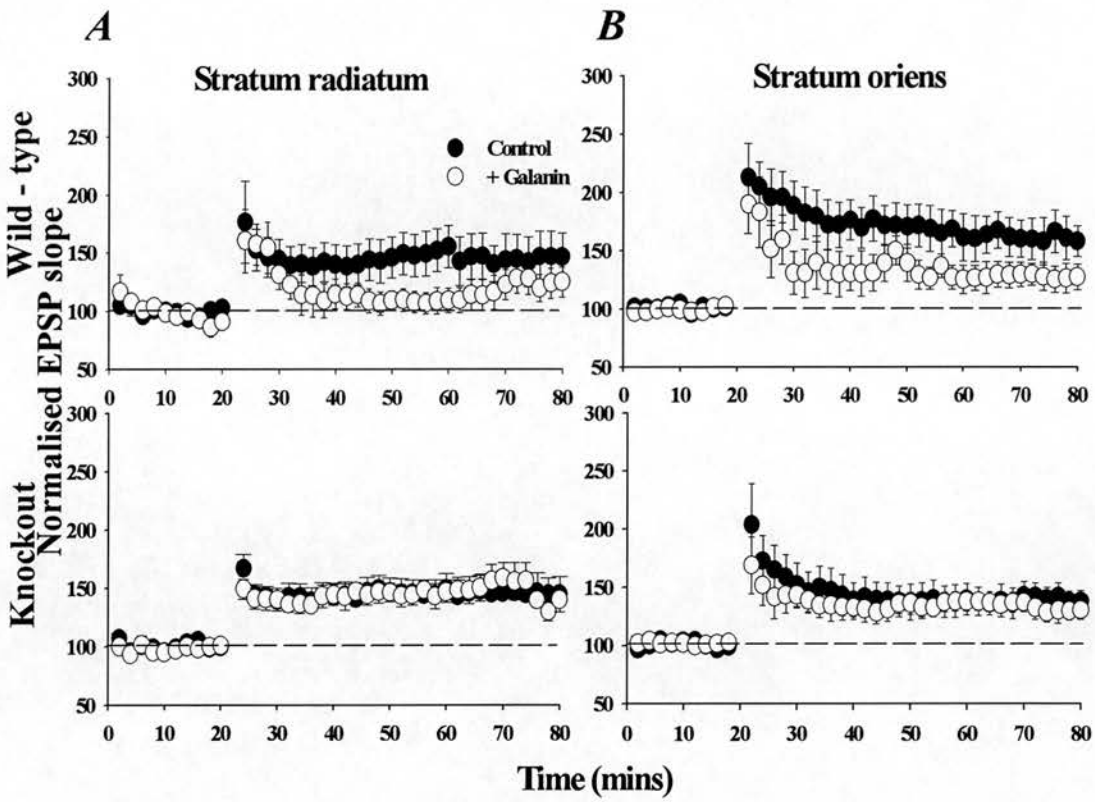


Figure 5.11. Exogenous Galanin fails to affect LTP in galanin null mice. Galanin caused an impairment in LTP induced in slices prepared from young mice. Galanin (100 nM) was perfused for 20 mins before and was terminated 15 mins after high frequency stimulation. *A,* LTP induced in in slices prepared from wild-type animals was diminished in the presence of galanin but unaffected in slices from galanin-null transgenic animals (naive filled circles; Wt, Ko n=12,11) and (galanin-treated open circles; Wt, Ko n=7,7). *B,* The same experiment was repeated in *stratum oriens* (Wt, Ko n=8,8) and (Wt, Ko n=7,7). Statistical significance was assessed by Student's *t-test* at t=30 mins following HFS, P<0.05 (top graphs) and P>0.05 (bottom graphs).

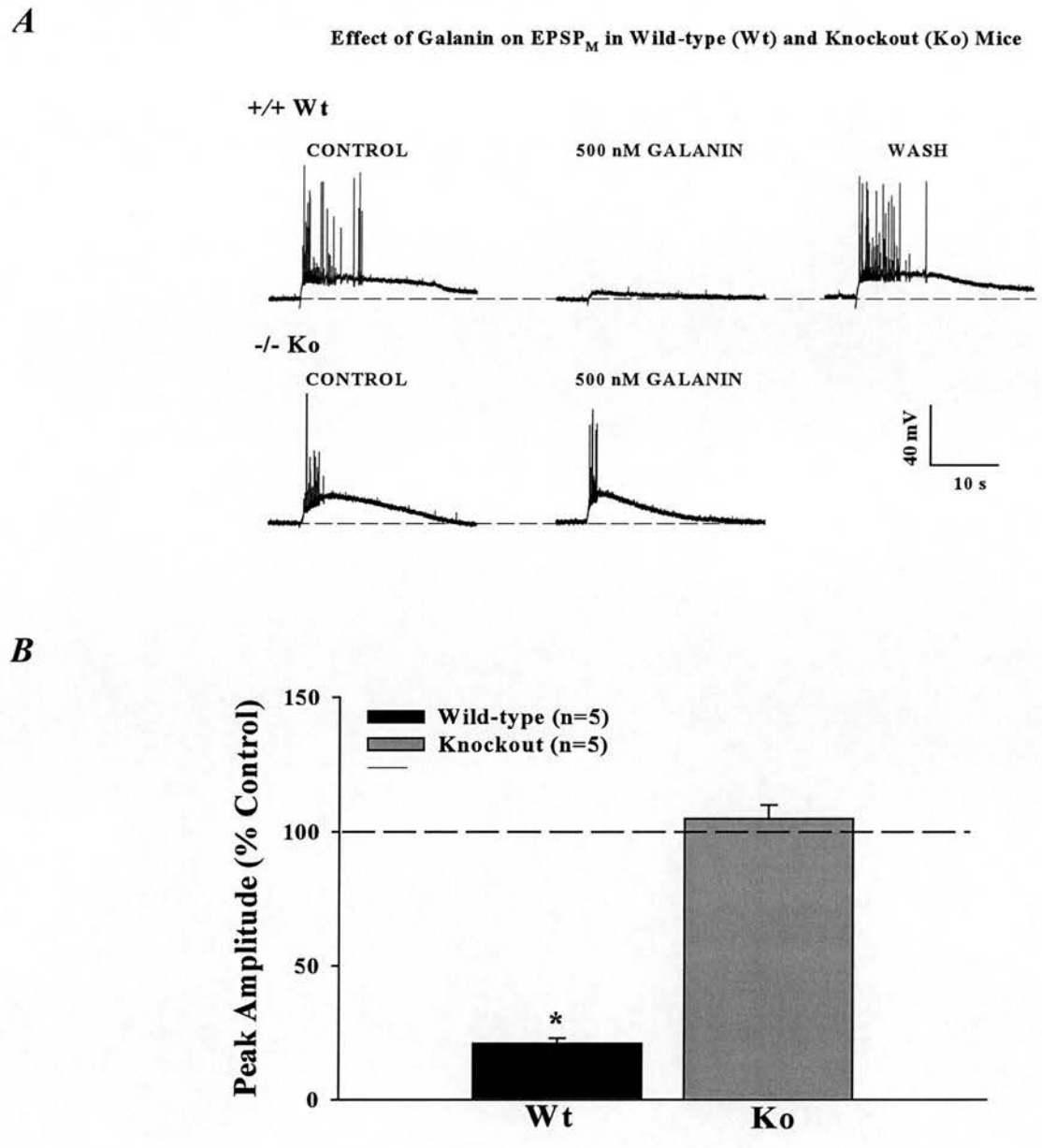


Figure 5.12. The effect of galanin deletion on cholinergic EPSP$_M$s. ***A*** **shows synaptic traces of EPSP$_M$s recorded from the CA1 pyramidal cell held between –64 and –69 mV. The top traces illustrate the EPSP$_M$ from wild type slices before (left) and after (right) the application of 500 nM rat 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. Bar graph (*B*) illustrates the effect of galanin on the mean peak amplitude of the EPSP$_M$ in wild type and Gal$^{-/-}$ mice in 4 and 4 experiments respectively. Values are mean ± S.E.M. represented by the error bars.**

5.4. Discussion

Here, I showed that global galanin deletion does not seem to affect basal glutamate receptor mediated synaptic transmission or short-term synaptic plasticity in the CA1 area of the hippocampus in galanin null mice or wild-type littermates. However, the absence of galanin appears to have a selective, progressive, degradatory effect on glutamate receptor long-term synaptic plasticity restricted to *stratum oriens* from the young to the older animals. This effect seems to stem from the lack of a background, neuroprotective physiological role of galanin. This role may entail a greater susceptibility to saturation of synaptic plasticity in the galanin null mouse slices compared to wild-type littermates. The effect is progressive with age which may reflect the fact that galanin's neuroprotective role may become increasingly more important with age (Lamour *et al.*, 1988; Mufson *et al.*, 1993; White *et al.*, 1994; Tsutsui *et al.*, 1994; Auchus *et al.*, 1994; Krywlowski *et al.*, 1993 & 1994; Ceresini *et al.*, 1994). The region specificity to *stratum oriens* is only expected since there are more galanin sensitive receptor sites in that area and also the cholinergic projection, from which galanin is released, terminates densely in the apical dendrites of the CA1 area (Melander *et al.*, 1985; Fisone *et al.*, 1987; Ögren *et al.*, 1992 & 1998; Xu *et al.*, 1998; Schott *et al.*, 1998). I, therefore, speculate that the role of galanin becomes pivotal in that area of the hippocampus. This conclusion is in agreement with our final finding that exogenous galanin supresses both tetanus induced LTP and the muscarinic receptor mediated cholinergic EPSP in the CA1. This observation provides further evidence that galanin directly affects cholinergic transmission, while its effect on LTP may be a resulting outcome of a compromised cholinergic excitatory neurotransmission, rather than a direct effect of glutamate receptor mediated synaptic plasticity. As such, provides indirect evidence for the excitatory role of cholinergic transmission in the modulation of synaptic plasticity in the CA1 area. These mice show fewer cholinergic markers in the medial septum and vertical diagonal band of Broca and an increase in ChAt activity implying that the remaining cholinergic neurons have compensated for the developmental loss by upregulating ChAT activity. This 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. 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. Also, in this chapter, 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 wild-type and galanin deficient mice.

It appears that the galanin mutation does not affect glutamate synaptic transmission indicating that this knockout mouse exhibits selective modification of the cholinergic system (O'Meara *et al.*, 2000; Coumis and Davies, 2002). Therefore, it is unlikely that this mutation is causing any major reorganisation of neuronal circuits as tyrosine kinase knockouts do for example (Grant & Silva, 1994).

5.5. Future Experiments

The use of galanin antagonists to investigate their effects on synaptic transmission and plasticity in the CA1 area would be a useful method of confirming whether endogenous galanin is tonically released to modulate amino acid and cholinergic neurotransmission in the hippocampus. The most likely explanation of why tetanus induced LTP is significantly impaired in the old animals is that the theta-burst stimulation paradigm was subthreshold for the expression of that impairment. It would be of interest to investigate whether LTP induced by a variety of stimulation paradigms is also impaired in these animals.

CHAPTER 6

CONCLUSION

In this thesis, prior knowledge of the molecular mechanisms of synaptic transmission and plasticity in the CA1 area of the hippocampus is rodents and the methods of investigating those, were utilised to study *in vitro* hippocampal function in rats and galanin-null mice with a view to determining the physiological role of galanin.

In Chapter 3, I showed that galanin causes a dose-dependent increase in the slope of fEPSPs which is dependent on the hippocampal slice being intact. In CA3 ectomised slices, galanin does not have any effect on neither the slope of the fEPSPs nor PPF. However, at high concentrations galanin also significantly reduced PPF in the intact slice. I conclude that galanin may act on presynaptic receptors to enhance presynaptic excitability and that this effect is abolished when the presynaptic input to the CA1 area coming from the CA3 area is cut.

In chapter 4, I attempted to investigate the role of exogenously applied galanin on synaptic transmission and plasticity in the CA1 area in rats. In brief, my results show that galanin, by itself, causes a dose-dependent impairment in LTP induced by traditional high frequency stimulation protocols. This action does not appear to interfere with NMDA, AMPA, GABA or mGlu receptor function, but is probably mediated by distinct galanin receptor sites and is possible the combined effect of a multiple type of galanin sensitive receptors and appears to be reversible by the known galanin antagonist M15.. My data suggest, but do not conclusively prove, that the galanin-induced LTP impairment may be mediated via a PKC dependent intracellular pathway. Galanin does not seem to affect LTD in the CA1 area. (Coumis & Davies, 2002).

The research study described in Chapter 5, involved galanin-null mice and wild-type littermates. The results indicate an age-dependent, progressive impairment in long-term synaptic plasticity, which appears to be specific to *stratum oriens*. This does not appear to be accompanied by any significant difference between wild-types and transgenic mice in the strength of fEPSPs or short-term plasticity, assessed by paired-pulse facilitation. My data support histological studies, which corroborate cholinergic loss in markers and neurotransmitter in the septocholinergic pathway projecting from

MS to the hippocampus and denotes the importance of that pathway to its densest projection in *stratum oriens* (Nyakas *et al.*, 1987; Biasco-Ibanez & Freund, 1995; Morton & Davies, 1997; Xu *et al.*, 1998;O'Meara *et al.*, 2000). My data correlate nicely with behavioural impairment in the standard Morris watermaze task and further confirm the role of galanin as a neuroprotective agent in neuropathological conditions. The lack of galanin seems to cause deterioration of saturarability in synaptic transmission evident in both *stratum oriens* and *radiatum* (O'Meara *et al.*, 2000). However, more detailed *in vitro* studies should be done in conditional, time specific knockout model rather than a mouse strain subjected to global galanin gene deletion. This would achieve the precise delineation of the role of galanin in the hippocampus and would allow correlations to be made with standard models of neurodegerative disease. As such, the galanin-null mouse strain, although it resembles ageing phenotype in disease it fails both to ascribe a specific function to galanin and to resemble any of the hallmarks of a well-defined disease condition. Chapters 4 and 5, in combination, point towards a possible role for galanin largely modulating glutamatergic and/or cholinergic transmission in that area, probably by restricting it during high frequency stimulation, thereby causing a deterioration in glutamate receptor mediated long-term synaptic plasticity *in vivo*. This action may be neuroprotective during pathological conditions. Galanin receptor conditional knockouts and specific galanin receptor subtype drugs will prove useful in determining which receptor is responsible for which effect.

On the whole, the work presented in this thesis is the result of a contemporary trend in biomedical research incorporating the study of transgenic animals at different levels, thereby contributing to a hollistic view of the biology of the hippocampus in health and disease. That would facilitate the discovery and development of therapeutic drugs which could treat central nervous system disorders of old age in humans.

CHAPTER 7

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