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PHARMACOLOGICALLY INDUCED LONG-TERM ENHANCEMENT IN THE HIPPOCAMPUS IN VIVO

By

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A dissertation submitted for the degree of Doctor of Philosophy of the University of Dublin, Trinity College, Dublin 2, Ireland.
This research was conducted in the Department of Pharmacology & Therapeutics in the Faculty of Health Sciences.

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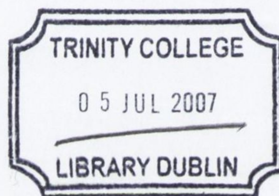
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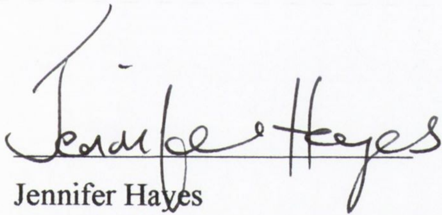

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Abbreviations

ABT 418	(S)-3-methyl-5-(1-methyl-2-pyrrolidinyl) isoxazole
Aβ	Amyloid β
AC	Adenylyl cyclase
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AF-DX 116	(11-[2-[(diethylamino)methyl]-1-piperidinyl] acetyl-5, 11-dihydro-6H-pyrido-[2, 3b] [1, 4] benzodiazepine-6-one)
AMPA	α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid
BDNF	Brain-derived neurotrophic factor
BIBN-99	(5,11-dihydro-8-chloro-11-[[4-[3-[(2,2-dimethyl-1 oxopentyl)ethylamino]propyl]-1-piperidinyl]acetyl]-6H-pyrido[2,3 b][1,4]benzodiazepin-6-one)
BIM	Bisindolylmaleimide I hydrochloride
CA3	Cornus ammonis area 3
CaMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic-adenosine 3', 5'-cyclic monophosphate
CCh	Carbachol
cGMP	Cyclic guanosine monophosphate
ChAT	Choline acetyltransferase
CNS	Central nervous system
CREB	cAMP responsive element-binding protein
DAG	Diacylglycerol
4-DAMP	4-diphenylacetoxy-N-methylpiperedine methiodide
D-AP5	D-(-)-2-amino-5-phosphonopentanoic acid
dBB	Diagonal band of Broca
DHPG	Dihydroxyphenylglycol
DNMTP	Delayed non-matching to position performance task
e-LTP	early-LTP

EPSP	Excitatory postsynaptic potential
GABA	γ-amino-butyric acid
Gal	Galanin
Gall.	Gallamine
GTS-21	3-(2,4-dimethoxybenzylidene)-anabaseine dihydrochloride
HFS	High frequency stimulation
i.c.v.	Intra-cerebroventricular
IP_3	Inositol 1,4,5-triphosphate
LTD	Long-term depression
LTE	Long-term enhancement
LTP	Long-term potentiation
L-LTP	late-LTP
LTPm	Muscarinic-LTP
M35	Galanin (1-13)-Bradykinin (2-9) amide
M40	galanin(1-13)-Pro-Pro-Ala-Leu-Ala-Leu-Ala amide
mACh	Muscarinic acetylcholine
MAPK	mitogen activated protein kinase
MEC	Mecamylamine
Meth.	Methoctramine
mGlu	Metabotropic glutamate receptor
MLA	Methyllycaconitine
MRI	Magnetic resonance imaging
MSDB	Medial septum and diagonal band of Broca
nACh	Nicotinic acetylcholine
Nico.	Nicotine
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKM	Protein kinase M

PLC	Phospholipase C
Rp-cAMPS	Rp-Cyclic 3', 5'- hydrogen phosphorothioate adenosine triethylammonium salt
sAHP	Slow afterhyperpolarisation
S.E.M.	Standard error of the mean
TZTP	3-(3-S-n-pentyl-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine
VDCCs	Voltage dependent calcium channels
YM796	(S)-(-)-2,8-dimethyl-3-methylene-1-oxa-8-azaspiro [4,5] decane-L-tartarate monohydrate
ZIP	Myr-Ser-Ile-Tyr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys-Leu-OH (PKC/Mzeta pseudosubstrate peptide inhibitor)

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Summary

The present study investigated the mechanisms underlying a pharmacologically induced long-term enhancement (LTE) of fast excitatory synaptic transmission in the CA1 region of the rat hippocampus in vivo. The role of cholinergic, glutamatergic and galaninergic receptors in the LTE induced by methoctramine, an M_2 muscarinic acetylcholine (mACh) receptor antagonist, was investigated. Furthermore, the requirement for certain kinases in the induction and maintenance-expression of methoctramine LTE was assessed. Finally the ability of the Alzheimer's disease (AD) associated protein; amyloid β (Aβ), to inhibit the LTE was evaluated.

Experiments were carried out on adult male Wistar rats under urethane anesthesia. Electrodes were implanted in the CA1 region of the hippocampus and the excitatory postsynaptic potential (EPSP) amplitude was measured. Drugs were delivered either via a cannula into the lateral cerebral ventricle (i.c.v.) or systemically. Methoctramine, induced a rapid (generally <8 min onset) and persistent (>2hrs) enhancement of synaptic transmission. Consistent with this, another M_2 preferring mACh receptor antagonist, gallamine, also enhanced synaptic transmission. In contrast BIBN-99, which is also an M_2 mACh receptor antagonist failed to affect synaptic transmission, indicating that not all M_2 mACh receptor antagonists share the ability to induce an (LTE).

The role of a variety of cholinergic and glutamatergic receptors in methoctramine LTE was further investigated. A broad spectrum nicotinic ACh (nACh) receptor antagonist, mecamylamine, did not significantly affect methoctramine LTE. However, an α7 nACh receptor antagonist, methyllycaconitine, appeared to delay the onset of methoctramine-induced LTE but did not block methoctramine LTE. These results suggest that nACh receptor activation is not necessary for methoctramine LTE. Pre-treatment with the competitive N-methyl-D-aspartate (NMDA) receptor antagonist d-AP5 (D-(-)-2-amino-5-phosphonopentanoic acid) or the non-competitive antagonist memantine did not block methoctramine-induced LTE, which suggests that methoctramine LTE is NMDA receptor-independent. The group I metabotropic glutamate (mGlu) receptor antagonist

LY367385 at a dose that blocks mGlu1 receptors did not block methoctramine LTE, which suggests that methoctramine LTE is mGlu1 receptor-independent.

The role of the galaninergic sytem in methoctramine LTE was also assessed. The application of exogenous galanin increased the initial enhancement induced by methoctramine. Pre-treatment with the galanin receptor antagonist M35 strongly inhibited methoctramine LTE, which suggests that methoctramine LTE requires the action of endogenous galanin at its receptors.

The role of protein kinase C (PKC) and protein kinase A (PKA) in methoctramine LTE was also studied. The PKCzeta and protein kinase M (PKM)zeta pseudosubstrate inhibitor (ZIP) blocked methoctramine induced LTE. This suggests that methoctramine-induced LTE involves the activation of the atypical PKC/Mzeta isoform. Pre-treatment with the PKA inhibitor Rp-cAMPs (Rp-cyclic 3',5'- hydrogen phosphorothioate adenosine) blocked methoctramine LTE, which implies that the induction of methoctramine LTE is PKA dependent. In contrast, the maintenance of methoctramine LTE appears to be independent of these kinases as application of the PKA inhibitor or PKC/Mzeta inhibitor 30 min after the induction of methoctramine LTE did not persistently reverse the maintenance of LTE. Pre-treatment with Aβ at a dose that blocks high frequency stimulation (HFS) induced long-term potentiation (LTP) did not affect methoctramine LTE, which suggests that methoctramine LTE is insensitive to inhibition by Aβ.

The finding that methoctramine-induced LTE is NMDA receptor-independent and also insensitive to inhibition by Aβ suggests that the mechanisms underlying methoctramine LTE are at least partially distinct from those underlying NMDA receptor-dependent LTP induced by standard HFS protocols at CA1 synapses. The finding that galanin facilitated, whereas a galanin receptor antagonist reduced methoctramine LTE suggests that the galaninergic system is an effective modulator of cholinergic mediated changes in synaptic transmission in the hippocampus. Therefore, the role of galanin receptor activation in this and other forms of hippocampal plasticity warrants further study.

I. Introduction

1.1 The hippocampal formation

1.1.1 Anatomy of the hippocampus

The hippocampal formation is located in the medial temporal lobe and it is comprised of the dentate gyrus, hippocampus proper, subicular complex and the entorhinal cortex. The hippocampus proper is divided into three areas; cornus ammonis area 3 (CA3), CA2 (usually treated as part of CA3) and CA1, which are collectively referred to as Ammon's horn. The fields of the hippocampal formation are linked by three mainly unidirectional connections, which are usually referred to as the trisynaptic pathway (Amaral and Witter, 1989; Witter et al., 2000). The main input of the trisynaptic pathway are the granule cells of the dentate gyrus, which receives its major input from the entorhinal cortex via the perforant pathway. The dentate gyrus sends information to the CA3 area via the mossy fibres. The mossy fibres also project back to the granule cells and thus form a recurrent network (Lisman and Otmakhova, 2001). The pyramidal cells of the CA3 provide the major input to the CA1 field of the hippocampus via the Schaffer collaterals. Similar to the dentate the CA3 neurons also have projections that feedback and connect with other CA3 neurons (Amaral and Witter, 1989; Lisman and Otmakhova, 2001). The CA1 region completes the trisynaptic pathway and sends information back to the entorhinal cortex by a direct projection to the entorhinal cortex. In addition, the CA1 also projects to the subiculum, which also projects to the entorhinal cortex (Amaral and Witter, 1989; Buzsaki, 1989; Freund and Buzsaki, 1996). The hippocampus proper (CA1 and CA3) has a lamellar organisation and these lamellae are referred to as the alveus, stratum oriens, stratum pyramidale, stratum radiatum and also the stratum lacunosum-moleculare (Andersen et al., 1969; Freund and Buzsaki, 1996). The cell bodies of the pyramidal cells of the Schaffer collateral projection are located in the stratum pyramidale and their dendrites extend to the stratum oriens and stratum radiatum where they are referred to as basal and apical dendrites, respectively.

1.1.2 The role of the hippocampus in learning and memory

Medial temporal lobe resection caused an immediate and profound impairment of memory in a patient referred to as H.M., and thus suggested that this area of the brain is involved in memory (Scoville and Milner, 1957). H.M. underwent this radical and experimental procedure as a last resort to treat his severe epilepsy and as a consequence he developed anterograde amnesia (memory for new information) along with retrograde amnesia (a temporally graded impairment for information learned prior to surgery) (Scoville and Milner, 1957). This case is often quoted as evidence that the hippocampus plays a critical role in memory, however a magnetic resonance imaging (MRI) study of H.M.'s brain demonstrated that in addition to the hippocampus other structures such as the amygdala and entorhinal cortex are also damaged and thus may contribute to the observed impairment in memory (Corkin et al., 1997). Direct evidence for a role of the hippocampus proper in memory was provided by another clinical case, R.B., who sustained a bilateral lesion involving the entire CA1 area and concomitant anterograde amnesia accompanied by little if any retrograde amnesia (Zola-Morgan et al., 1986). Analysis of these clinical cases and the effects of similar lesions induced in animal models suggests that the severity of memory impairments correlates with the extent of the damage to the hippocampal formation (Parkin, 1996; Zola-Morgan et al., 1986; Zola and Squire, 2001). However, more severe memory impairments were produced when the damage was increased to include the adjacent entorhinal and parahippocampal cortices (Jarrard, 1995; Zola-Morgan et al., 1994). Interestingly, lesions of the perirhinal and parahippocampal cortex that spare the amygdala and hippocampal formation produce severe memory impairment (Zola-Morgan et al., 1989). In conclusion, the communication via the bidirectional circuitry between the neocortex, the parahippocampal region and the hippocampus proper appears to play a critical role in memory function (Eichenbaum, 2000; Eichenbaum et al., 1996; Witter et al., 2000).

1.2 The hippocampus and synaptic plasticity

1.2.1 Hippocampal synaptic plasticity and memory

In 1894, Ramon y Cajal suggested that memory is stored as an anatomical change in the synaptic strength of neuronal connections (Ramon y Cajal, 1894). In 1949 Donald Hebb proposed that memory involves the strengthening of synaptic connections, which occurs when a presynaptic neuron repeatedly or persistently takes part in firing the postsynaptic cell (Hebb, 1949). The discovery of hippocampal LTP, whereby brief repetitive activation of the excitatory synapses results in a persistent increase in synaptic strength provided an experimental analogue of Hebb's postulated learning induced changes in synaptic strength (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). Furthermore the properties that Hebb expected to be a feature of an associative memory mechanism are similar to the characteristics of LTP, which include cooperativity, input-specificity, associative interactions, persistence and rapid induction.

LTP involves the presynaptic release of glutamate, which will then excite the postsynaptic cell provided that the afferent stimulation was sufficient to depolarise the postsynaptic membrane. The demonstration of a stimulus intensity threshold for the sufficient depolarization of the postsynaptic cell and thus production of LTP is referred to as cooperativity (Malenka, 2003). The potentiation of the synapses on the postsynaptic cell is input specific, as the increase in synaptic strength at one set of synapses does not occur in other synapses on the same cell, thus the use of a synapse specific mechanism greatly increases the storage capacity of the individual neurons (Martin et al., 2000). The associative property of LTP means that concurrent activation of independent weak and strong synaptic inputs produces LTP of the weak input, where independent tetanisation of the weak input would not result in LTP (Teyler and DiScenna, 1987). This associative property allows the neuron to integrate information that is conveyed by different sets of afferents that synapse on the same postsynaptic cell.

Finally, LTP is generally rapidly induced and can persist for hours to months in experimental models depending on the protocol used to induce it (Abraham, 2003).
A study on the effect of ageing on both LTP and memory observed that the persistence of LTP was statistically correlated with the rate of learning and/or the degree of retention of spatial memory over time, which suggests that both phenomena are linked (Barnes, 1979; Barnes and McNaughton, 1985). Similarly, spatial learning in vivo was previously shown to correlate with LTP of the population spike in slices taken from animals after training (Kleschevnikov and Marchbanks, 1993). The role of LTP in memory is supported by a study where inhibitors of LTP also inhibited hippocampal dependent learning (Morris et al., 1986). LTP and hippocampal memory formation are both believed to involve gene expression, protein synthesis and the formation of new synaptic connections, which is supported by the observation that protein synthesis inhibitors block both phenomena (Lynch, 2004). However, some studies have demonstrated a lack of correlation between the ability to induce LTP and spatial learning. For instance LTP was absent but spatial learning was unimpaired in transgenic mice lacking the AMPA receptor subunit GluR-1 mice (Zamanillo et al., 1999). Similarly, in the rat the antisense knock-down for a presynaptic A-type voltage dependent K^+ channel (Kv1.4) mRNA resulted in normal spatial learning but LTP was eliminated (Meiri et al., 1998).

The initial experimental LTP was induced using highly synchronous activity (Bliss and Lomo, 1973) that has never been observed in vivo, however LTP has also been induced by protocols that mimic theta rhythm, which is the naturally occurring hippocampal activity associated with exploratory behaviour (Diamond et al., 1988). In addition, it has been suggested that a naturally occurring form of hippocampal CA1 activity known as field sharp waves may be sufficient for the induction of a long-term synaptic modification in the CA1 region (Buzsaki, 1989). This seems plausible since sharp field waves represent the summed postsynaptic depolarisation of large numbers of pyramidal cells in the CA1 and subiculum and are assumed to represent synchronous excitation of pyramidal cells by the Schaffer collaterals of the CA3 (Buzsaki, 1989).

Metaplasticity refers to a mechanism whereby synaptic activity that does not alter synaptic strength may lead to a persistent change in the direction or degree of synaptic plasticity in response to subsequent episodes of synaptic activity (Abraham and Bear, 1996; Abraham and Tate, 1997) For instance, in the dentate gyrus LTP induced by weak stimulation was facilitated by prior stimulation (Christie et al., 1995). Therefore, metaplasticity may allow the intergration of a response across temporally spaced episodes of synaptic activity, which would be a valuable feature of an associative memory model. Although the underlying mechanisms of metaplasticity are unknown they may involve an alteration in one or more of the following; NMDA receptor function, Ca^{2+} buffering, the state of kinases or phosphatases and/or the mechanisms involved in protein synthesis (Abraham and Bear, 1996; Abraham and Tate, 1997).

1.2.2 Glutamate receptors and synaptic plasticity

Glutamate binds to ionotropic and metabotropic receptors in the hippocampus.
The metabotropic glutamate receptors comprise of a family of G protein coupled receptors, whereas the ionotropic receptors can be further subdivided into at least two types, termed AMPA and NMDA receptors. The induction of LTP involves the presynaptic release of glutamate along with sufficient depolarization of the postsynaptic membrane. The depolarization of the postsynaptic cell is brought about by a glutamate mediated activation of the postsynaptic α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) receptors and a decrease in GABAergic (γ-amino-butyric acid) inhibition (Collingridge, 2003). Following sufficient depolarization the binding of glutamate promotes the opening of NMDA receptors, which allows Ca^{2+} as well as Na^{+} to enter the dendritic spine. It is widely believed that the influx of calcium into the postsynaptic dendritic spine is the critical event leading to LTP (Bennet, 2000; Bliss and Collingridge, 1993; Lynch, 2004; Malenka and Nicoll, 1999).

NMDA receptors become active only on depolarisation due to a voltage dependent block of the associated ion channel by a magnesium ion. Since the NMDA receptor is dually

regulated by both the ligand and voltage, it acts as a coincidence detector and thus plays a pivotal role in LTP (Platenik et al., 2000). The importance of NMDA receptor activity in LTP and memory is supported by the ability of NMDA receptor antagonists to impair both of these phenomena in vivo (Davis et al., 1992; Morris, 1989). Interestingly, the CA1 region of the hippocampus has also been associated with a NMDA receptor independent form of LTP, which is associated with Ca^{2+} entry via voltage dependent Ca^{2+} channels (VDCCs) (Grover, 1998; Morgan and Teyler, 1999).

AMPA receptors are permeable to Na^+ and K^+ and thereby mediate the major inward current required for synaptic responses, including depolarisation of the postsynaptic cell. AMPA receptors are comprised of subunits GluR1-GluR4. The subunit composition determines the permeability of $Ca^{2+}$and AMPA receptors assembled from GluR2 subunits are impermeable to Ca^{2+} relative to those assembled from GluR1, GluR3 and GluR4 (Lynch, 2004). The GluR2 subunit is constitutively delivered to synapses, whereas the delivery of the GluR1 subunit to the synapse requires HFS or the presence of CaMKII (Song and Huganir, 2002). Intriguingly, prior to LTP many synapses in the CA1 region are characterised by a high level of NMDA receptor activity and a lack of AMPA receptor activity (Lisman, 2003; Malenka and Nicoll, 1999; Song and Huganir, 2002). These synapses are termed 'silent synapses' as they are unable to respond to synaptically released glutamate owing to the voltage dependent block of NMDA receptors by Mg^{2+}. An alternative theory to the 'silent synapse' theory postulates that both receptor types exist at the postsynaptic membrane prior to stimulation but that extrasynaptic glutamate spillover selectively activates NMDA receptors, as NMDA receptors have slow unbinding and desensitisation rates relative to AMPA receptors (Kullmann, 2003). The existence of functional AMPA receptors at the synapse prior to LTP is controversial, however many studies have shown that LTP induction can rapidly enhance AMPA receptor function in the absence of an observable change in NMDA receptor function (Malenka and Nicoll, 1999; Malinow, 2003; Nicoll, 2003). It is believed that trafficking of GluR1 containing AMPA receptors to the postsynaptic membrane or that the modification of the receptor by a kinase underlies this rapid enhancement (Malinow, 2003). Evidence for the trafficking of AMPA receptors to the

postsynaptic membrane was provided by a previous in vitro study using tagged AMPA receptor subunits, which showed that tetanic stimulation induces a rapid delivery of tagged receptors into dendritic spines as well as clusters in dendrites (Shi et al., 1999) and the activation of CaMKII (calcium/calmodulin-dependent protein kinase II) (Hayashi et al., 2000; Poncer et al., 2002) and PKMzeta (Ling et al., 2006) is associated with facilitating this phenomenon. In addition, the phosphorylation of the GluR1 subunit by PKA (Banke et al., 2000) and CaMKII (Barria et al., 1997; Lisman, 2003) is associated with an increase in channel conductance. AMPA receptors appear to be involved in both LTP and memory as drugs known as AMPAkines, which decrease AMPA receptor desensitisation and thus prolong the duration of their activation, enhance both phenomena (Martin et al., 2000).

Presynaptically released glutamate may also activate metabotropic glutamate (mGlu) receptors, which are a class of G protein coupled receptors. The mGlu receptor class can be subdivided into three groups, group I mGlu, group II mGlu and group III mGlu receptors and their activation triggers the production of intracellular second messengers. Most studies have focused on the involvement of group I mGlu receptor in hippocampal LTP. Group I mGlu receptor activation has been shown to induce a slow-onset long-term enhancement of CA1 hippocampal synaptic transmission in vitro (Bortolotto and Collingridge, 1995) and also in vivo (Manahan-Vaughan and Reymann, 1997). Group I mGlu receptor activation has been associated with facilitating hippocampal LTP in vitro (McGuinness et al., 1991) and also in vivo (Riedel et al., 1995a). Conversely group I mGlu receptor antagonists have been shown to inhibit the induction of LTP, however some studies have found that group I mGlu antagonists do not affect LTP (Anwyl, 1999). The role of group I mGlu receptors in spatial learning has also been investigated and group I mGlu receptor antagonists impair spatial learning, whereas group I mGlu receptor agonists applied after learning facilitate recall (Riedel, 1996; Riedel et al., 1995b). The group I mGlu receptor includes two receptor subtypes, the mGlu1a and the mGlu5 receptor subtype. The role of mGlu1a receptors in synaptic plasticity in the CA1 region is complex as many immunocytochemical studies were unable to detect mGlu1a receptors in this region (Baude et al., 1993; Romano et al., 1995). However, mGlu1a

mRNA was detected in CA1 pyramidal cells (Berthele et al., 1998; Shigemoto et al., 1992) and a more recent study revealed low levels of mGlu1 receptors in the CA1 region (Ferraguti et al., 1998). In addition, a previous study found that mGlu1 receptor knock-out mice exhibited deficits in LTP in the CA1 region (Aiba et al., 1994), although another mGlu1 receptor knock-out study failed to find any change in LTP in the CA1 region (Conquet et al., 1994). In contrast to the distribution of mGlu1 receptors in the CA1 the mGlu5 receptor is highly expressed in CA1 pyramidal cells (Romano et al., 1995). Elucidating the role of mGlu5 receptors in hippocampal LTP is also complicated as activation of mGlu5 receptors have been associated with long-term depression (LTD) in the CA1 region in vitro (Huang and Hsu, 2006), which was absent in mGlu5 knock-out mice (Huber et al., 2001). Antagonism of mGlu5 receptors has been associated with inhibition of LTP in the dentate gyrus of freely behaving animals (Naie and Manahan-Vaughan, 2004). In addition, mice lacking mGlu5 receptors were found to have reduced LTP and deficits in spatial learning (Jia et al., 1998; Lu et al., 1997). The inhibition of NMDA receptor activity appeared to underlie the reduction in LTP in mGlu5 receptor knock-out mice as CA1 neurons from these mice showed a complete loss of the NMDA receptor component of LTP, whereas the AMPA receptor component was unaltered (Jia et al., 1998).

1.2.3 Intracellular biochemical events and LTP

It is widely believed that the influx of calcium into the postsynaptic dendritic spine is the critical event leading to LTP (Bennet, 2000; Bliss and Collingridge, 1993; Lynch, 2004; Malenka and Nicoll, 1999). The increase in calcium concentration in the postsynaptic cell is achieved by an influx of Ca^{2+} following activation of NMDA receptors or voltage dependent calcium channels (VDCCs), however Ca^{2+} may also be released from intracellular stores (Malenka, 1991; Morgan and Teyler, 1999; Voronin et al., 1995). Evidence for a role of Ca^{2+} released from intracellular stores in LTP was provided by previous in vitro studies that found LTP in the CA1 region was blocked by thapsigargin, which depletes intracellular Ca^{2+} stores (Harvey and Collingridge, 1992)

and also dantrolene, which antagonises the ryanodine receptor and thus inhibits Ca^{2+} induced Ca^{2+} release (Obenaus et al., 1989). Furthermore the role of each calcium source in the observed LTP appears to depend on the protocol used to induce LTP and a previous study proposed that each Ca^{2+} source appears to be tuned to the differential protocols (Raymond and Redman, 2002). This proposition is supported by the finding that different induction protocols have differential sensitivities to Ca^{2+} buffers (Lisman, 2003). The critical role of Ca^{2+} in LTP is supported by the observation that LTP is blocked by preventing the postsynaptic increase in calcium (Lynch et al., 1983; Mulkey and Malenka, 1992), whereas artificially elevating the postsynaptic calcium concentration using photolysis of caged calcium mimics LTP (Malenka et al., 1988). The transient elevation of Ca^{2+} concentration in the postsynaptic spine can be transduced into a prolonged kinase activity that persists in the absence of elevated Ca^{2+} levels and thus mediates a persistent modification in synaptic strength.

The increase in Ca^{2+} concentration activates the Ca^{2+}/calmodulin-activated adenylyl cyclase (AC) and the concomitant increase in cAMP (cyclic-adenosine monophosphate) activates PKA (Chetkovich and Sweatt, 1993). The activation of PKA has been implicated in numerous mechanisms that modulate/enhance synaptic transmission (Lynch, 2004; Nguyen and Woo, 2003). The role of the cAMP/PKA pathway in synaptic plasticity is supported by the observation that the induction of LTP involves a rise in cAMP (Chetkovich and Sweatt, 1993), activation of PKA (Roberson and Sweatt, 1996) and also phosphorylation of PKA substrates (Blitzer et al., 1998). The cAMP/PKA pathway modulates ion conductance by phosphorylating the GluR1 subunit of AMPA receptors (Roche et al., 1996) and thereby directly increases AMPA receptor function (Banke et al., 2000; Bliss and Collingridge, 1993), which is associated with the induction of LTP (Lee et al., 2000). In addition, the synthesis of additional AMPA receptors, which accompanies LTP, appears to be PKA dependent (Nayak et al., 1998). PKA can also modulate neuronal excitability by phosphorylating K^{+} channels (Hoffman and Johnston, 1998) and Ca^{2+} channels (Gray and Johnston, 1987). Many studies have reported that the cAMP/PKA pathway enables long lasting forms of LTP (Bach et al., 1999; Frey et al., 1993; Nguyen and Kandel, 1997; Silva et al., 1998; Woo et al., 2002)

and transgenic mice in which PKA function is decreased exhibit impaired L-LTP and spatial memory, which suggests that PKA plays a critical role in consolidation (Abel et al., 1997). However studies on the role of the cAMP/PKA pathway in the early phase of LTP or less persistent forms of LTP have yielded variable results (Blitzer et al., 1995; Nguyen and Woo, 2003; Otmakhov and Lisman, 2002; Otmakhova et al., 2000; Winder et al., 1998; Woo et al., 2002; Woo et al., 2003). This lack of consistency may be accounted for by the suggestion that the cAMP/PKA pathway has distinct roles in different forms of plasticity, which in turn have differential sensitivities to pharmacological inhibition or genetic modulation of the cAMP/PKA pathway (Nguyen and Woo, 2003; Otmakhova et al., 2000). The increase in cAMP has also been associated with the activation of the BDNF receptor TrkB and subsequent induction of brain-derived neurotrophic factor (BDNF)-dependent long-lasting potentiation at the Schaffer collaterals (Patterson et al., 2001).

The cAMP/PKA pathway was found to gate the induction of LTP by decreasing phosphatase activity, and thereby enhances the autophosphorylation of CaMKII (Blitzer et al., 1998; Blitzer et al., 1995). Once autophosphorylated CaMKII does not require Ca^{2+} and therefore becomes constitutively active, which implies that this kinase may serve as a reversible molecular switch capable of storing information for long periods of time (Lisman, 1994; Lisman and Fallon, 1999). Importantly an amino acid substitution at Thr-286, which prevents autophosphorylation, was shown to impair memory and also to block LTP (Giese et al., 1998). The role of CaMKII in LTP is supported by the finding that its expression is particularly high in the postsynaptic density, which is an ideal location to respond to changes in Ca^{2+} concentration. Mutant mice lacking αCaMKII and transgenic mice expressing a mutant form of CaMKII show impaired LTP and hippocampal dependent memory (Fukunaga and Miyamoto, 2000; Silva et al., 1992). In addition, LTP was blocked by injecting inhibitors of CaMKII into the postsynaptic cell (Madison et al., 1991), whereas the introduction of an activated Ca^{2+} independent CaMKII into CA1 neurons potentiated synaptic transmission and occluded LTP induced by HFS (Lledo et al., 1995; Pettit et al., 1994). Initially CaMKII was found to modulate LTP by phosphorylating the GluR1 subunit of existing AMPA channels and thereby

increase their conductance (Barria et al., 1997; Lisman, 2003). However, LTP is also believed to involve an increase in AMPA receptor conductance following the insertion of GluR1 containing AMPA receptors into dendritic spines (Shi et al., 2001; Shi et al., 1999) and CaMKII is believed to facilitate this process (Lisman, 2003; Lynch, 2004). The role of CaMKII in AMPA receptor trafficking is supported by the finding that recombinant GluR1 overexpressed in CA1 pyramidal cells can be delivered to synapses after the expression of constitutively active CaMKII (Hayashi et al., 2000). Similarly, the expression of a constitutively active tagged CaMKII was associated with a reduction in the proportion of synapses devoid of AMPA receptors compared with non-infected nearby neurons (Poncer et al., 2002). Therefore, CaMKII may facilitate the insertion of AMPA receptors at synapses that do not express surface synaptic AMPA receptors and thus convert 'silent synapses' into 'non silent synapses' (Lisman, 2003; Malenka and Nicoll, 1999; Song and Huganir, 2002) and/or increase the number of AMPA receptors at synapses that already contain functional AMPA receptors.

The increase in Ca^{2+} in the postsynaptic cell stimulates phosphatidylinositol-4,5-bisphosphate (PIP_2) hydrolysis by phospholipase C (PLC), which generates a variety of second messengers, including diacylglycerol (DAG) and inositol 1,4, 5-triphosphate (IP_3). IP_3 releases calcium from intracellular stores, which in conjunction with DAG, activates PKC. PKC was the first kinase to be implicated in LTP and therefore its involvement has been studied extensively. Inhibitors of PKC impair LTP (Hvalby et al., 1994; Lovinger et al., 1987; Malinow et al., 1988) and also impair learning (Nogues, 1997; Paratcha et al., 2000). In addition, PKC knock-outs exhibit diminished LTP (Abeliovich et al., 1993). Conversely intracellular injection of the catalytic subunit of PKC induces synaptic potentiation (Hu et al., 1987) as does the extracellular application of activators of PKC, such as phorbol esters (Goda et al., 1996; Kamal et al., 2003). PKC activity is associated with enhanced neurotransmitter release (Malenka et al., 1986b; Swartz et al., 1993), inhibition of K^+ channels (Baraban et al., 1985; Hoffman and Johnston, 1998), modulation of NMDA receptors (Lan et al., 2001; Lu et al., 2000) and also morphological changes (Pilpel and Segal, 2004). PKC also appears to be involved in the activation of the non-receptor protein tyrosine kinase (Lu et al., 1999),

which is important as src activity accompanies and is necessary for LTP induction in CA1 pyramidal neurons (Lu et al., 1998). The role of the src family in LTP is supported by the finding that transgenic animals lacking the src family member fyn exhibit deficits in LTP (Huang and Hsu, 1999).

PKC is a multigene family and multiple isoforms are transiently activated in the induction of LTP (Hrabetova and Sacktor, 1996; Sacktor et al., 1993). However a single isoform, PKMzeta, exists as an independent catalytic domain of the atypical PKCzeta and is persistently activated during the maintenance phase of LTP (Hernandez et al., 2003). PKMzeta is formed following proteolytic cleavage of the full length PKCzeta atypical isoform (Kishimoto et al., 1983). However, a PKCzeta knock-out mouse, in which the catalytic domain is spared, still expresses neuronal PKMzeta, which indicates that PKMzeta may also be generated by de novo protein synthesis (Hernandez et al., 2003). It appears that particular forms of stimulation may prompt de novo synthesis of PKMzeta as metabolic labelling of PKMzeta showed that tetanic stimulation induces the protein synthesis of PKMzeta using neuronal specific PKMzeta mRNA, which is generated by an internal promoter within the PKCzeta gene (Hernandez et al., 2003). Following synthesis PKMzeta is transported to the synaptodendritic compartments of neurons (Muslimov et al., 2004). PKC/Mzeta inhibitors reversed the maintenance of LTP in the dentate gyrus in vivo (Pastalkova et al., 2006) and in the CA1 in vitro (Ling et al., 2002; Serrano et al., 2005). Furthermore introduction of active PKMzeta into the postsynaptic cell induced synaptic potentiation, which occluded LTP induced by tetanic stimulation (Ling et al., 2002; Pastalkova et al., 2006; Serrano et al., 2005). These findings infer that PKMzeta is necessary and sufficient for the maintenance of LTP. The maintenance of LTP usually refers to a persistent biochemical signal that lasts in a cell and acts on an effector such as a glutamate receptor, which results in the expression of LTP. A recent study found that PKMzeta specifically maintains a long-term increases in active postsynaptic AMPA receptor number (Ling et al., 2006), which was 3-4 fold greater than the increase in AMPA receptor function associated with CaMKII (Poncer et al., 2002). There was no evidence of a conversion of synapses containing NMDA receptors only, 'silent synapses', into 'non-silent' synapses containing NMDA and

AMPA receptors and thus it was concluded that the increase in AMPA receptor function occurred at synapses containing only AMPA receptors (Ling et al., 2006).

The activation of intracellular kinases prompts the activation of transcription factors and thereby initiates the increase in protein synthesis that accompanies LTP. Previous studies have shown that CaMKII and PKA are associated with the activation of CREB (cAMP responsive element-binding protein) (Hu et al., 1999), which is important since CREB activity plays an important role in spatial learning (Bourtchuladze et al., 1994; Pittenger et al., 2002) and also LTP. Mutant mice lacking CREB display LTP that decays faster than usual (Bourtchuladze et al., 1994). Similarly PKA and PKC modulate the activity of the MAPK (mitogen activated protein kinase) signalling pathway (Sweatt, 1999), which is necessary for LTP in the CA1 (English and Sweatt, 1997; Impey et al., 1998). Hippocampal MAPK is also activated by β-adrenergic receptors, mGlu receptors, mACh receptors (Roberson et al., 1999) and also BDNF (Gottschalk et al., 1999). Furthermore MAPK is also involved in the activation of CREB (Impey et al., 1998; Roberson et al., 1999), which suggests that MAPK serves as an integrator of a wide variety of convergent receptor generated messengers allowing for the functional integration of diverse cell surface signals at the level of the cell nucleus (Dineley et al., 2001a).

In conclusion the induction of LTP involves the transient elevation of second messengers, which activates protein kinases (Dineley et al., 2001a). Furthermore the maintenance of LTP is generally divided into two phases; early LTP (E-LTP) and late LTP (L-LTP) (Dineley et al., 2001a; Huang, 1998). E-LTP involves persistent kinase activation and thus it is suggested that autonomously active kinases such as CaMKII and PKMzeta maintain this phase. In contrast L-LTP involves protein synthesis and altered gene expression (Dineley et al., 2001a). The increase in protein synthesis that accompanies LTP is believed to mediate some of the morphological changes such as the increase in the postsynaptic area, spine number and spine area that are associated with LTP (Lynch, 2004; Ma et al., 1999).

1.3 The cholinergic system and the hippocampus

1.3.1 The discovery of ACh and source of hippocampal ACh

Hunt and Taveau 'discovered' ACh 100 years ago, when they first demonstrated its powerful hypotensive action (Hunt and Taveau, 1906). Experimental proof in favour of ACh as a chemical transmitter at the vagal endings in the heart came from Loewi in 1921, although he named this active substance 'vagusstoff' (Loewi, 1921). The release of ACh from the intact cerebral cortex was established by assaying saline that had been exposed to the surface of the cortex (Elliott et al., 1950) and shortly after the excitatory effect of ACh on cortical neurons was demonstrated (Krnjevic and Phillis, 1963; Spehlmann, 1963). Krnjevic and Phillis (1963) were intrigued by the finding that sometimes the application of ACh did not have any perceptible affect, however in these instances ACh was still able to strongly potentiate other forms of excitation, thus ACh is the prototypical neuromodulator (Krnjevic and Phillis, 1963). ACh consists of a choline group, which is available via diet, and an acetyl group, provided by acetyl-Coenzyme A. The synthesis of ACh takes place in the axonal terminals and is catalysed by the cytosolic enzyme choline acetyltransferase (ChAT) (van der Zee and Luiten, 1999).

The hippocampus receives a major cholinergic input from the medial septum and the vertical limb of the diagonal band of Broca (MSDB) via the fimbria-fornix (Krnjevic, 2004; Milner et al., 1983; van der Zee and Luiten, 1999). This projection is comprised of heterogeneous nuclei and also contains GABAergic neurons, which are also known to innervate the hippocampus (Segal and Auerbach, 1997; van der Zee and Luiten, 1999). The septo-hippocampal projection also contains a neuropeptide projection (Senut et al., 1989) and a possible glutamatergic projection (Sotty et al., 2003). ACh is believed to modulate synaptic transmission via activation of metabotropic muscarinic ACh receptors and/or ionotropic nicotinic ACh receptors. The mACh receptor family consists of 5 heterogeneous G protein coupled mACh receptor subtypes (M_1-M_5) and all 5 are expressed in the hippocampus (Caulfield, 1993; Levey et al., 1995). The nACh receptor

family consists of ligand gated ion channels and the nACh receptors are formed by five subunits arranged around a central pore that is perpendicular to the membrane (Pereira et al., 2002). To date nine nACh receptor α subunits (α2-α10) and three nACh receptor β subunits (β2-β4) have been cloned and sequenced from the brain tissue of various animal species (Pereira et al., 2002). Of the neuronal nACh receptors subunits cloned α7, α3β4 and α4β2 nACh receptors are the most abundantly expressed in the hippocampus (Alkondon et al., 2000a).

1.3.2 The release of ACh in the hippocampus

Numerous studies have shown that in awake animals hippocampal ACh increases in a variety of tasks and also in conditions where the environment requires the analysis of novel stimuli that may represent a threat or offer a reward (Fadda et al., 2000; Inglis and Fibiger, 1995; Mark et al., 1996; Thiel et al., 1998), for review see (Pepeu and Giovannini, 2004). The activity of the medial septum is correlated with the release of ACh in the hippocampus as electrical stimulation of the medial septum is known to increase hippocampal ACh release (Dudar, 1977; Smith, 1974) and the infusion of cholinergic agents into the septum also modulates hippocampal ACh release (Elvander et al., 2004). In addition, many neurotransmitters such as dopamine (Imperato et al., 1993), serotonin (Consolo et al., 1994b) and adenosine (Cunha et al., 1995) also augment hippocampal ACh release. The activity of glutamatergic receptors is involved in hippocampal ACh release, as both non-NMDA receptor antagonists and NMDA receptor agonists injected i.c.v. (intra-cerebroventricular) increase the release of ACh from the hippocampus in vivo (Giovannini et al., 1994; Giovannini et al., 1998). Likewise, the activity of GABAergic receptors modulates hippocampal ACh release, as the $GABA_A$ receptor agonist, muscimol, applied intrahippocampally increases the release of hippocampal ACh (Giovannini et al., 1994), which correlates with the identification of $GABA_A$ receptors on cholinergic neurons (Gao et al., 1995). The extracellular concentration of ACh regulates ACh release via presynaptic inhibitory auto-receptors on cholinergic terminals (Bernard et al., 2003; Kitaichi et al., 1999;

Quirion et al., 1995; Rouse et al., 2000; Stillman et al., 1996; Vannucchi and Pepeu, 1995) but also see (Vannucchi and Pepeu, 1995). This correlates with the ability of M_2/M_4 mACh receptor antagonists to increase the level of ACh in the hippocampus in vivo (Carey et al., 2001; Kitaichi et al., 1999; Quirion et al., 1995; Stillman et al., 1996; Stillman et al., 1993; Vannucchi and Pepeu, 1995; Vannucchi et al., 1997). A previous in vitro study proposed that ACh release is mediated primarily by the M_2 mACh receptor in the hippocampus and cerebral cortex, but predominantly by the M_4 mACh receptor in the striatum (Zhang et al., 2002). This proposition is supported by an in vivo study in the hippocampus that found that an M_4 mACh receptor antagonist had a weak effect on hippocampal ACh release relative to the prominent effect of an M_2 mACh antagonist on ACh release (Kitaichi et al., 1999).

Nicotine injected systemically (i.p.) increased the release of hippocampal ACh and this increase was blocked by pretreatment with nACh receptor antagonist, mecamylamine, which suggests that nACh receptors may also play a role in hippocampal ACh release (Tani et al., 1998). Finally galanin, an endogenous neuropeptide, has also been shown to modulate ACh release, however its effect on ACh release is location-specific as galanin has opposite effects on ACh release in the dorsal and ventral hippocampus, where local galanin injections increase and decrease ACh release, respectively (Schott et al., 2000).

1.3.3 The distribution of ACh receptor subtypes in the hippocampus

The M_1 mACh receptor is the most abundant subtype in the hippocampus (Levey, 1993; Levey et al., 1995) and also predominantly expressed at postsynaptic sites (Levey et al., 1995; Rouse et al., 1999). Although M_2 receptors are not as abundant as M_1 receptors they have a higher affinity for ACh (Auerbach and Segal, 1996). Analysis of the hippocampal distribution of M_2 mACh receptors found that they are expressed in a striking network along the stratum oriens/alveus border and also on varicose processes along the pyramidal layer and occasionally in the stratum radiatum (Levey, 1993; Levey et al., 1995). Further investigations of the neurons expressing M_2 mACh receptors

suggest that they are located predominantly on the presynaptic membrane of cholinergic and non-cholinergic cells (Levey et al., 1995; Rouse et al., 2000), where they are believed to function as auto-receptors on cholinergic neurons (Carey et al., 2001; Quirion et al., 1995; Rouse et al., 2000; Rouse et al., 1999; Stillman et al., 1996; Stillman et al., 1993) and also heteroreceptors on both GABAergic (Hajos et al., 1998; Rouse et al., 2000; Rouse et al., 1999) and possibly glutamatergic (Marchi and Raiteri, 1989; Nikbakht and Stone, 1999) neurons. Analysis of the distribution of M_3 mACh receptors shows that they are expressed on pyramidal and non-pyramidal neurons in the stratum oriens and stratum lucidum in CA3 and in the stratum lacunosum-moleculare in the CA1 (Levey et al., 1995). Although mRNA for m5 has been detected in the CA1 pyramidal cells the protein has not been detected in the hippocampus and therefore it appears unlikely that M_5 mACh receptor activation significantly contributes to hippocampal synaptic plasticity (Levey, 1993; Levey et al., 1995). Of the neuronal nACh receptors subunits cloned $\alpha7$, $\alpha3\beta4$ and $\alpha4\beta2$ nACh receptors have been shown to be pre- and post-synaptically expressed in the hippocampus. The $\alpha7$, $\alpha3\beta4$ and $\alpha4\beta2$ nACh receptors are abundantly expressed on the somatodendritic region of hippocampal interneurons (Alkondon and Albuquerque, 2002; Alkondon et al., 2000b).

1.3.4 The role of the cholinergic system in attention, learning and memory

Cholinergic activity facilitates human and animal memory (Aigner, 1995; Blokland, 1995; Deutsch, 1971; Furey et al., 2000; Parent and Baxter, 2004). However, it is unclear if ACh directly enhances memory or indirectly alters attention since sensory stimuli that produce arousal and attention also increase the concentration of extracellular ACh in the hippocampus (Inglis and Fibiger, 1995). Evidence that the cholinergic system is involved in attention was provided by a study with positron emission tomography in humans subjected to a visual working memory task in which systemic application of the acetylcholinesterase (AChE) inhibitor, physostigmine, improved working memory by enhancing selective attention (Furey et al., 2000). This study in

humans concluded that the effect of cholinergic enhancement is not due to a simple increase in alertness or arousal, since physostigmine increased the selectivity of responses to task-relevant stimuli but did not increase the response to all stimuli (Furey et al., 2000). However, this study involving a cholinergic enhancement of selective attention could not ascertain if ACh was having a direct effect on systems that control attention or if the enhancement involved modulation of the effect of the input from the control systems on local neural activity in perceptual areas. Interestingly, a study on the role of ACh in attention did not report a correlation between attention and ACh release (Passetti et al., 2000). In addition, lesions that impair performance on a variety of attentional tasks do not substantially affect memory, however it is difficult to draw conclusions from these studies as there are many varieties of attentional processing and likewise many different kinds of memories (Parent and Baxter, 2004).

The observation that hippocampal ACh release increases during learning and also the finding that the increase in ACh release is positively correlated with performance improvement during the task, suggests that changes in the activity of cholinergic neurons are involved in learning (Fadda et al., 2000). Intriguingly, the cholinergic system is activated in response to novel inputs but not when the stimuli are repeated, leading to habituation (Acquas et al., 1996), which has lead to the proposition that the cholinergic system is activated by tasks that require the analysis of novel stimuli representing a threat or offering a reward and thus supports a role for ACh in the learning of new information (Parent and Baxter, 2004; Pepeu and Giovannini, 2004).

The distinction between attention and memory is difficult and according to Hasslemo and Bower (1993) the conceptual divisions between learning and memory may not be reflected on a physiological level (Hasselmo and Bower, 1993). In addition, it is not established where pure attention ends and learning information begins and consequently it may be more useful to focus on the neuromodulatory effects of ACh. It has been proposed that the cholinergic system regulates the interaction between the cortex and hippocampus and thus mediates a switch from the learning of new information to the retrieval of previously learned information (Hasselmo, 1999). In this context ACh is

believed to enhance learning of new information by inhibiting the intrinsic hippocampal circuitry and thereby ACh prevents interference from previously stored memories whilst simultaneously enhancing encoding by increasing the response to afferent stimulation (Hasselmo, 1999; Hasselmo and Bower, 1993; Hasselmo and Schnell, 1994; Kremin et al., 2006).

1.3.5 The role of the cholinergic receptor subtypes in learning and memory

Evidence for a role of M_1 mACh receptors in learning was provided by a study that reported enhanced performance in an inhibitory avoidance task following intrahippocampal injection of a compound isolated from snake venom, MT2, which is a M_1 receptor selective agonist (Adem and Karlsson, 1997; Ferreira et al., 2003; Jerusalinsky et al., 1993), similar results were obtained with other M_1 receptor agonists (Ruske and White, 1999; Sen and Bhattacharya, 1991). However studies with M_1 mACh receptor knock-out mice did not reveal major cognitive deficits in different hippocampus-dependent learning tasks, which suggest that M_1 mACh receptors play an important role in memory but are not essential (Miyakawa et al., 2001).

Many studies have shown that M_2/M_4 mACh receptor antagonists delivered i.c.v. or systemically facilitate cognition (Aura et al., 1997; Baratti et al., 1993; Carey et al., 2001; Lachowicz et al., 2001; Packard et al., 1990; Sen and Bhattacharya, 1991). However, intrahippocampal injections of methoctramine alone did not enhance working memory (Ohno et al., 1994). Finally, M_2 receptor knock- out mice also exhibit deficits in spatial learning (Seeger et al., 2004), which suggests that the activity of M_2 mACh receptors is required for learning, although pharmacological antagonists of M_2 mACh receptors enhance cognition (Aura et al., 1997; Baratti et al., 1993; Carey et al., 2001; Lachowicz et al., 2001; Packard et al., 1990; Sen and Bhattacharya, 1991).

It appears that $\alpha4\beta2$ and $\alpha7$nACh receptor are involved in cognition since the local hippocampal application of the $\alpha7$nACh receptor antagonist methyllycaconitine or the

α4β2 nACh receptor antagonist dihydro-β-erythroidine impair learning and memory (Bancroft and Levin, 2000; Felix and Levin, 1997; Levin et al., 2002). In addition, learning and memory is impaired by the non-selective nicotinic antagonist mecamylamine (Levin, 1992) Conversely, learning and memory are improved by the α7 nACh receptor agonist 3-(2,4-dimethoxybenzylidene)-anabaseine (GTS-21) (Arendash et al., 1995) or the α4β2 nACh receptor agonist (S)-3-methyl-5-(1-methyl-2-pyrrolidinyl) isoxazole (ABT 418) (Buccafusco et al., 1995).

1.3.6 The cholinergic system and cognitive dysfunction

Many studies have endeavoured to elucidate the function of the cholinergic system in the central nervous system (CNS) as deterioration of the cholinergic system is believed to contribute to the cognitive deficits associated with Alzheimer's disease (AD) and also possibly healthy ageing (Bartus et al., 1982; Mesulam, 1996). The degeneration of the cholinergic system in AD is supported by many studies which found that AD is associated with a dramatic depletion in cholinergic markers (Francis et al., 1999). The lesions associated with AD, the Aβ-containing neuritic plaques and the tau containing neurofibrillary tangles, are present in the cholinergic septal-hippocampal and basal forebrain-neocortical pathways (Whitehouse et al., 1982). In addition, studies have suggested that Aβ may reduce ACh synthesis/release (Hoshi et al., 1997) and/or inhibit the coupling of ACh receptors to G proteins (Kelly et al., 1996). The release of ACh reduces with increased age as the release of ACh is reduced in the cerebral cortex, hippocampus and striatum in aged rats relative to young rats (Wu et al., 1988). Interestingly, the level of hippocampal ACh release appears to correlate with cognitive function as rats that display memory deficits often display correlated decreases in ACh release, whereas aged rats that have unimpaired memory did not exhibit a decrease in ACh release relative to young rats (Quirion et al., 1995). Finally, the use of acetylcholinesterase (AChE) inhibitors such as tacrine, donepezil and rivastigmine, increase the extracellular concentration of endogenous ACh and are associated with improved cognitive function in AD (Francis et al., 1999).

Given that the loss of cholinergic markers is associated with the pathology of AD many studies have endeavoured to reproduce the accompanying cognitive dysfunction by inducing lesions of the cholinergic system in experimental animal models. However, cholinergic lesion studies fail to consistently cause cognitive deficits. It is possible that lesioning induces a reorganization of the brain that can compensate for the loss of the cholinergic system. Alternatively, this lack of consistency could be explained by the use of unselective lesioning agents and/or an inefficient abrogation of the cholinergic system. The importance of effective abrogation of the cholinergic system was highlighted by a previous study with i.c.v. injections of 192IgG-saporin, the most selective procedure for disruption of the cholinergic system, where only very extensive lesions involving >90% of cholinergic neurons reliably resulted in severely impaired performance (Parent and Baxter, 2004; Wrenn and Wiley, 1998).

It appears that additional factors may contribute to the cognitive dysfunction associated with AD such as a reduction in the efficiency of coupling of mACh receptors to effector systems such as PIP_2 turnover (Chouinard et al., 1995; Ladner et al., 1995). It has also been proposed that a disturbance of the GABAergic-cholinergic interaction may underlie AD memory deficits (Araki et al., 1996). This proposition is supported by a previous study that showed selective lesioning of the septal GABAergic projection combined with lesioning of the septal cholinergic system (using 192IgG-saporin) impaired hippocampal-dependent memory, whereas selective lesions of the septo-hippocampal GABA projection or cholinergic projection alone did not impair spatial learning (Pang et al., 2001). In addition, the interaction of the cholinergic septo-hippocampal projection with the peptidergic and putative glutamatergic septo-hippocampal projections may also contribute to the role of ACh in AD (Parent and Baxter, 2004). Evidence for the role of the neuropeptide, galanin, in AD was provided by the finding that galanin and galanin receptors are overexpressed in limbic brain regions of AD patients (Counts et al., 2003).

Originally the neuropathological plaques that accompany AD were believed to mediate the impairment in cognition, however more recently it has been proposed that prior to

the development of plaques, soluble non-fibrillar Aβ initiates a complex cascade of biochemical and cellular changes that culminate in cognitive and memory impairment. This proposition is substantiated by the finding that the level of soluble non-fibrillar Aβ in the brain measured post mortem, correlates with the severity of clinical dementia suffered by patients (Lue et al., 1999; McLean et al., 1999), whereas cognitive deficits correlate poorly with the presence of fibrillar Aβ (Terry, 2000). The deleterious effect of Aβ on hippocampal function was demonstrated by the ability of soluble non-fibrillar Aβ injected i.c.v. prior to HFS to block LTP (Cullen et al., 1997) and likewise impair learning (Flood et al., 1991). Furthermore transgenic mouse models with APP-linked familial AD mutations have an age-dependent impairment of learning and memory and also a deficit in hippocampal LTP prior to Aβ plaque desposition, for review see (Rowan et al., 2003; Selkoe, 2002).

Although AD involves many pathological changes in addition to the loss of the cholinergic system many studies support the view that the cholinergic system plays an important role in modulating learning and memory deficits (Parent and Baxter, 2004; Pepeu and Giovannini, 2004; Steckler et al., 1998). This concept is supported by studies, which found that impaired cognitive functions caused by lesions of the hippocampus are reversed by muscarinic agonists (Fisher et al., 1991; Inagawa, 1994; Yamazaki et al., 1991). The role of the cholinergic system in learning and memory is also supported by the ability of mACh receptor antagonists, such as scopolamine (Blokland, 1995; Steckler et al., 1998) to induce a deficit in learning. Conversely AChE inhibitors ameliorate the scopolamine-induced deficit in learning (Bejar et al., 1999; Braida et al., 1996), which is reminiscent of the ability of AChE inhibitors to improve cognitive deficits associated with AD (Francis et al., 1999). Likewise, enhanced release of endogenous ACh following antagonism of presynaptic inhibitory M_2/M_4 mACh receptors has also been found to ameliorate the cognitive deficits induced by scopolamine (Quirion et al., 1995), brain injury (Pike and Hamm, 1995) and also the cognitive impairment associated with aging and (Quirion et al., 1995; Tombaugh et al., 2002). M_1 mACh agonists have also been reported to reduce the cognitive impairments induced by scopolamine (Iga et al., 1996) and other pharmacological agents (Brandeis et al., 1995). The nACh receptors

also appear to have a significant role in the cognitive dysfunction associated with loss of the cholinergic system, since nicotine reverses memory deficits caused by lesions of the cholinergic system in animals (Decker et al., 1992; Levin et al., 1993; Yamazaki et al., 2002). Similarly, nicotine partially ameliorates the cognitive deficit in patients with Alzheimer's (Newhouse et al., 2004). The activity of α7 nACh receptor is believed to mediate neuroprotection against Aβ- induced neurotoxicity (Kihara et al., 2001; Shaw et al., 2002), which may underlie the effect of nicotine in AD. The role of the cholinergic system in memory deficits is also supported by the suggestion that the alterations in the expression of hippocampal mACh and nACh receptors following prolonged exposure to antipsychotics (Terry et al., 2005; Terry et al., 2006a) underlies the memory impairments associated with long-term use of antipsychotics (Terry et al., 2006b).
In conclusion, it is generally accepted that the cholinergic system is involved in cognition, however an outstanding question is: how does the activation of cholinergic receptors contribute to learning and memory processes?

1.3.7 Muscarinic acetylcholine (mACh) receptors and hippocampal synaptic plasticity

Evidence supporting the hypothesis that the cholinergic system mediates it effects on learning and memory in the hippocampus was provided by a study which showed that spatial discrimination learning selectively increases mACh receptor immunoreactivity in cell bodies of the CA1 region pyramidal neurons (van der Zee and Luiten, 1999). It has been proposed that an LTP-like plasticity underlies learning and memory (Lynch, 2004; Morris et al., 1986) and thus if ACh plays a role in memory it would be expected to induce/modulate this type of plasticity.

The application of the acetylcholinesterase (AChE) inhibitor, physostigmine, increases the extracellular ACh concentration and was previously shown to induce an LTP-like persistent enhancement of the population spike in the hippocampus in vivo (Ito et al., 1988; Levkovitz and Segal, 1994). This finding is consistent with a role for

hippocampal ACh in learning as spatial learning in vivo was previously shown to correlate with LTP of the population spike in slices taken from animals after training (Kleschevnikov and Marchbanks, 1993). A similar enhancement of the population spike was obtained with another AChE inhibitor in vitro (Kojima and Onodera, 1998). An in vitro study found that somatically applied ACh caused a long term increase in glutamatergic synaptic transmission in the CA1 region (de Sevilla et al., 2005).

Antagonists of presynaptic inhibitory M_2/M_4 mACh receptors, 11-[2-[(diethylamino)methyl]-1-piperidinyl] acetyl-5, 11-dihydro-6H-pyrido-[2, 3b] [1, 4] benzodiazepine-6-one (AF-DX116) or methoctramine, which increase the concentration of extracellular ACh, have also been shown to induce a long term increase in synaptic transmission in the CA1 region of the hippocampus in vivo (Li, 2002). This M_2 receptor mediated enhancement of synaptic transmission was muscarinic receptor dependent as it was blocked by pretreatment with an unselective mACh receptor antagonist, scopolamine (Li, 2002). However, the maintenance of methoctramine LTE was not mACh receptor-dependent as scopolamine at a dose that blocked the induction of methoctramine LTE, failed to significantly alter methoctramine LTE when it was applied 30 min after methoctramine (Li, 2002). The M_1 receptor antagonist telenzepine (Galvan et al., 1989) and the M_1/M_3 receptor antagonist 4-diphenylacetoxy-N-methylpiperedine methiodide (4-DAMP) (Dorje et al., 1991) blocked the methoctramine-induced LTE of synaptic transmission (Li, 2002), which suggests that methoctramine LTE is dependent on the activation of non-M_2 mACh receptors. A somewhat similar increase in synaptic transmission was obtained in vitro following bath application of an M_2 receptor antagonist and this enhancement was absent in M_2 knock-out mice (Seeger et al., 2004).

Bath application of a muscarinic agonist, carbachol, was previously shown to induce a slow onset long lasting enhancement of synaptic transmission, which was referred to as muscarinic LTP (LTPm) (Auerbach and Segal, 1996; Seeger et al., 2004; Segal and Auerbach, 1997). This enhancement was found to comprise of a transient and reversible increase in response to NMDA, whereas responses to AMPA increased gradually and

remained potentiated after washout (Auerbach and Segal, 1996; Segal and Auerbach, 1997). LTPm was not associated with a change paired-pulse facilitation or the size of the afferent volley, which suggests a postsynaptic locus of action (Auerbach and Segal, 1996; Yun et al., 2000). Auerbach and Segal (1996) proposed that LTPm is mediated by postsynaptic M_2 receptors. The role of M_2 receptors in LTPm is consistent with the lack of LTPm in slices from M_2 knock-out mice (Seeger et al., 2004).

Establishing the mechanism by which ACh receptor activation induces an increase in synaptic transmission is complex as the effect of ACh on synaptic transmission appears to depend on the exact site of ACh release/application. The location-specific effect of ACh on synaptic transmission was demonstrated by a previous in vitro study in the CA1, which found that ACh applied to the dendrities decreased the magnitude of the locally evoked EPSP via presynaptic inhibition, whereas ACh applied to the cell body layer resulted in an increase and prolongation of EPSPs along with a transient decrease in the recurrent somatic IPSPs (Valentino and Dingledine, 1981).

Distinct pools of receptor subtypes may exert differential and even opposing effects on cellular activity. For instance the activation of presynaptic M_2/M_4 receptors suppresses somatic calcium currents and inhibits transmitter release (Krnjevic, 2004). However, it appears that there are at least two other distinct pools of M_2 receptors located postsynaptically in the hippocampus, which have opposing functions. The activation of postsynaptic M_2 receptors that are positively coupled to inwardly rectifying K^+ channels will decrease the excitability of the postsynaptic cell (Seeger and Alzheimer, 2001), whereas the activation of postsynaptic M_2 receptors may also block an outward K^+ current, and thus increase the excitability of the postsynaptic cell (Dutar and Nicoll, 1988). A similar situation appears to apply to M_1 receptors as the activation of M_1 receptors expressed on the postsynaptic cell are associated with an increase in excitability (de Sevilla et al., 2005; Iga et al., 1996), whereas the activation of M_1 receptors expressed on the presynaptic cell are associated with a decrease in neurotransmitter release (Kremin et al., 2006; Sheridan and Sutor, 1990). It has been suggested that these differential effects of ACh on cellular excitability may achieve a

suppression of presynaptic transmission and thus prevent interference from old memories whilst simultaneously enhancing encoding by increasing postsynaptic activity in response to afferent stimulation (Hasselmo, 1999; Hasselmo and Bower, 1993; Hasselmo and Schnell, 1994; Kremin et al., 2006).

ACh also appears to modulate hippocampal LTP as the muscarinic receptor dependent antagonist, scopolamine, blocks the cholinergic enhancement of hippocampal LTP that occurs during walking in rats (Leung et al., 2003). Establishing the role of ACh in hippocampal LTP is difficult as many investigations have found that mACh receptor antagonists do not effect the induction of tetanic-LTP in the CA1 region (Kikusui et al., 2000; Sokolov and Kleschevnikov, 1995; Tanaka et al., 1989) or in the dentate gyrus (Abe et al., 1994) and the disruption of the cholinergic system does not consistently affect the induction of LTP at the CA1 ex vivo (Jouvenceau et al., 1996; Yamazaki et al., 2002). However, LTP induced by weak conditioning stimulation appears to be cholinergic-dependent (Abe et al., 1994; Huerta and Lisman, 1993; Ovsepian et al., 2004) and mACh receptor activation facilitates LTP induced by tetanic stimulation (Blitzer et al., 1990; Iga et al., 1996; Leung et al., 2003; Shimoshige et al., 1997; Shinoe et al., 2005; Ye et al., 2001). Therefore it is believed that mACh receptors contribute to particular forms of synaptic plasticity but are not essential for all types of synaptic plasticity.

1.3.8 Nicotinic acetylcholine (nACh) receptors and hippocampal synaptic plasticity

Nicotine induces a slowly developing potentiation in the dentate gyrus of the mouse, which is believed to involve activation of α7nACh and α4β2 nACh receptors (Matsuyama and Matsumoto, 2003; Matsuyama et al., 2000). Nicotine has been found to facilitate the induction of LTP (Fujii et al., 1999; Ji et al., 2001; Mann and Greenfield, 2003) and reverse the cholinergic lesion-induced impairment of LTP induction (Yamazaki et al., 2002) in the CA1 region of the hippocampus. Isolation of the nACh receptor sub-types involved in the nicotinic effect on plasticity is complicated as nACh receptors desensitise following activation (Alkondon et al., 1998; Alkondon et al., 1997), which explains the finding that the effect of nACh receptor agonists on LTP are mimicked by α7nACh antagonists (Fujii et al., 2000a). Since nACh receptors are highly expressed on interneurons they are believed to mediate facilitation of LTP through disinhibition of glutamatergic neurons (Frazier et al., 1998; Ji and Dani, 2000; Jones and Yakel, 1997; McQuiston and Madison, 1999), which is substantiated by the finding that nicotine reverses GABAergic inhibition of LTP (Fujii et al., 2000b). However, α7nACh receptors are also expressed presynaptically on glutamatergic neurons and their activation enhances glutamate release (Gray et al., 1996), which could also contribute to the role of nACh receptor activation on plasticity. A direct role for nACh receptors in depolarisation of the postsynaptic pyramidal cell is controversial as the initial evidence from slice recordings did not support the expression of nACh receptors on the postsynaptic membrane of pyramidal neurons (Frazier et al., 1998; Jones and Yakel, 1997; McQuiston and Madison, 1999). However, Ji et al. (2001) demonstrated the presence of nACh on the membrane of postsynaptic pyramidal cells. In addition, they demonstrated that in the CA1 pyramidal cells in vitro the observed facilitation of LTP induction following local application of ACh was predominantly due to α7nACh receptor activation (Ji et al., 2001). They proposed that the activation of α7nACh receptors facilitated the induction of LTP by enhancing depolarisation of the postsynaptic cell and increasing the level of intracellular calcium (Ji et al., 2001). The

proposed role of α7nACh receptors in Ca^{2+} influx correlates with the result of a previous study where α7nACh receptors were found to have a greater relative permeability to Ca^{2+} than NMDA receptors and also other nACh receptors (Seguela et al., 1993).

The effect of nACh receptor activation on LTP also appears to be location specific. It has been suggested that in the CA1 region the activation of nACh receptors on interneurons that directly innervate pyramidal neurons causes inhibition of pyramidal cells, whereas activation of nACh receptors that innervate other interneurons causes disinhibition of pyramidal cells (Ji and Dani, 2000; Yamazaki et al., 2005). This is supported by the finding that in the CA1 there are distinct subsets of interneurons, which are distinguished with regard to nicotinic receptor expression (McQuiston and Madison, 1999). Furthermore the classification of interneurons with regard to nACh receptor expression also correlates strongly with morphological characteristics (McQuiston and Madison, 1999).

1.3.9 ACh and hippocampal neuronal excitability

The ACh mediated increase in excitability in the CA1 region of the hippocampus is associated with membrane depolarization, impairment of adaptation during repetitive spike firing, suppression of slow-afterhyperpolarisation and the appearance of after depolarization (Dutar and Nicoll, 1988; Krnjevic, 2004; Segal, 1988; Segal and Auerbach, 1997). Most of these effects are achieved by the reduction or blockade of several neuronal potassium currents (Cole and Nicoll, 1984; Dutar and Nicoll, 1988; Segal, 1988; Segal and Auerbach, 1997). The first hippocampal membrane current found to be blocked by ACh was the voltage-dependent, Ca^{2+} independent, resting outward rectifying M-current (I_M), however K^+ currents seem to exhibit differential sensitivities to ACh as a previous study found that low concentrations of ACh enhance excitability by preferentially acting on a voltage and Ca^{2+} independent 'leak' current (I_{LEAK}) (Brown et al., 1997; Loffelholz, 1996; Madison et al., 1987). The blockade of M-current (I_M) along with the 'leak' current (I_{LEAK}) results in the suppression of

adaptation, which makes the neuron more excitable and hence more responsive to stimulation, as adaptation involves a decrease in firing during sustained excitatory stimulation (Cole and Nicoll, 1984; Madison et al., 1987; Segal, 1988). Madison et al. (1987) proposed that synaptically released ACh is likely to mediate blockade of the voltage and Ca^{2+} dependent current that underlies the slow afterhyperpolarisation (I_{AHP}). Successive sAHPs result in hyperpolarisation, which normally prevents sustained firing in response to a depolarizing input, therefore blockade of this current by ACh would promote sustained action potential discharge to depolarizing stimuli (Brown et al., 1997; Cole and Nicoll, 1984; Krnjevic, 2004; Madison et al., 1987; Segal, 1988). ACh may also mediate excitatory effects via the blockade of a transient outward, voltage-activated delayed rectifier like K^+ current (I_K) (Brown et al., 1997; Cole and Nicoll, 1984; Krnjevic, 2004; Madison et al., 1987; Segal, 1988).

The closing of K^+ channels may depolarize the membrane and thus ACh may indirectly modulate other ionic conductances such as Na^+, Ca^{2+} Cl^- (Brown et al., 1997; Caulfield, 1993; Felder, 1995; Krnjevic, 2004; Segal and Auerbach, 1997). Indeed the activation of mACh receptors has been associated with increased Ca^{2+} influx through VDCCs in the entorhinal cortex (Klink and Alonso, 1997) and also enhanced Ca^{2+} entry via NMDA receptors (Egorov et al., 1999; Markram and Segal, 1990). M_1 mACh receptor activation has been shown to enhance NMDA receptor activity in the CA1 region of the hippocampus (Marino et al., 1998; Markram and Segal, 1990). Furthermore the selective M_1-toxin antagonist was shown to block a carbachol mediated enhancement of NMDA currents in the CA1 region in vitro (Rouse et al., 1999). The role of the M_1 receptor in the regulation of NMDA receptor currents also correlates with an immunocytochemical study that found the NR1 subunit of the NMDA receptor and the M_1 receptor are co-localised in the rat hippocampus (Rouse et al., 1999). ACh receptors are coupled to G proteins and thus their activation may also generate second messengers and thus may mediate an increase in the release of Ca^{2+} from intracellular stores, which is also associated with an increase in neuronal excitability (Segal and Auerbach, 1997). Although ACh is believed to enhance excitability of the post synaptic neuron a previous study determined that in the CA1 region ACh may also activate the inwardly rectifying

K^+ current and thus decrease glutamatergic synaptic transmission (Seeger and Alzheimer, 2001). Furthermore ACh has also been reported to suppress high voltage gated Ca^{2+} currents, which is believed to underlie the ability of mACh receptor activation to modulate presynaptic neurotransmitter release (Brown et al., 1997; Krnjevic, 2004).

A link between neuronal excitability and learning was provided by a previous study that observed that a reduction in sAHP in the CA1 region of the hippocampus correlates with learning in the Morris water maze as this phenomenon was absent in animals that failed to learn the task (Oh et al., 2003). The dysfunction of the mechanisms underlying neuronal excitability has been associated with aging and aged rats have been shown to express an increase in L-type calcium channels which have been linked to slow afterhyperpolarisation (Marrion and Tavalin, 1998). Dysfunction of sAHP correlates with learning as the CA1 neurons of aged rodents exhibit both a larger sAHP and a depressed membrane excitability that vary inversely with hippocampal-dependent learning (Disterhoft et al., 1996).

1.3.10 Cholinergic signaling mechanisms

ACh stimulates the activation of enzymes that generate second messengers and thereby modulates the activity of intracellular kinases such as PKA and PKC (Loffelholz, 1996), which are involved in synaptic plasticity (Lynch, 2004; Nguyen and Woo, 2003). ACh may increase the intracellular Ca^{2+} concentration by modulating ionic conductance or via the generation of second messengers associated with the release of Ca^{2+} from intracellular stores (Felder, 1995; Segal and Auerbach, 1997) and an increase in intracellular Ca^{2+} is involved in the activation of many kinases (Felder, 1995; Lisman, 2003; Lynch, 2004). The role of kinase activity in mACh receptor mediated enhancement of synaptic transmission has been documented previously (Auerbach and Segal, 1996; Li, 2002).

Agonist stimulation of M_1, M_3 and M_5 receptors stimulate PIP_2 hydrolysis by PLC and generate a variety of second messengers, including diacylglycerol and IP_3. IP_3 releases calcium from intracellular stores, which in conjunction with DAG, activates PKC. The ability of mACh receptors to activate PKC is supported by the finding that PKC activation accounts for many of the effects of muscarinic receptor activation (Cantrell et al., 1996; Malenka et al., 1986a; May et al., 1999; Stratton et al., 1989). Kinase activation may also initiate feedback regulation as the phosphorylation of muscarinic receptors by PKC has been associated with a decrease in mACh receptor function (Hosey, 1992; Jia et al., 1989; Scherer and Nathanson, 1990) and may even prompt mACh receptor internalisation (Jia et al., 1989; Liles et al., 1986; Pediconi and Barrantes, 1995).

The M_2/M_4 mACh receptors are mainly located presynaptically and are negatively coupled to adenylyl cyclase (AC), therefore activation of these receptors results in a decrease in the intracellular cAMP concentration and a subsequent reduction in PKA activity (Caulfield, 1993). Thus antagonism of these receptors has been associated with increased PKA activity. Although M_2/M_4 receptor activation inhibits AC and thus PKA, it has been suggested that the activation of M_1 receptors and the concomitant increase in intracellular Ca^{2+} may also stimulate AC and thus may activate PKA (Caulfield, 1993). The activation of mACh receptors also prolongs the activation of the hippocampal MAPK family (Rosenblum et al., 2000) and this is believed to involve mACh receptors that couple to Gi and Gq proteins (Loffelholz, 1996).

The activation of α7nACh receptors have a greater relative permeability to Ca^{2+} than NMDA receptors and also other nACh receptors (Seguela et al., 1993). Therefore, the activation of α7nACh receptors on the postsynaptic pyramidal cell may facilitate depolarisation and increase the intracellular calcium concentration and thus facilitate the activation of kinases such as, PKA, PKC and CaMKII. In addition, the activation of

nACh receptors was previously found to induce the activation of MAPK (Dajas-Bailador et al., 2002) and phosphatidylinositol3-kinase (Kihara et al., 2001).

1.4 The neuropeptide galanin

Galanin, a 29 amino acid neuropeptide, was originally isolated from the porcine intestine (Bedecs et al., 1995; Tatemoto et al., 1983). However galanin has a widespread distribution in the CNS and in the periphery (Crawley, 1996; Gentleman et al., 1989). Galanin is believed to be co-transported with classical neurotransmitters being present in cholinergic, GABAergic, noradrenergic and serotonergic neurons (Chan-Palay, 1988; Melander et al., 1986; Miller et al., 1997). Endogenous galanin is tonicallly released from the ventral hippocampus of freely behaving rats and enhanced following electrical stimulation of the diagonal band of Broca (Consolo et al., 1994a). To date three galanin (Gal) receptor subtypes Gal-receptor 1, Gal-receptor 2 and Gal-receptor 3 have been identified and cloned (Branchek et al., 2000). Gal-receptor 1 is highly expressed in the ventral hippocampus (Gustafson et al., 1996) and its activation results in a decrease in AC activity as it is coupled to Gi/o protein (Wang et al., 1998). Gal-receptor 2 is expressed mainly in the granule cell layer of the dentate gyrus in both the dorsal and ventral hippocampus (Fathi et al., 1997; O'Donnell et al., 1999). Gal-receptor 2 is coupled to Gi/o (Wang et al., 1998) and also Gq/11proteins, which induces the activation of phospholipase C (Smith et al., 1997), which is required for PKC activation. Gal- receptor 3 is preferentially expressed in the hypothalamic nuclei, pituitary, spinal cord and has also been reported to occur in the pyramidal cell layer of the hippocampus (Kolakowski et al., 1998; Smith et al., 1997). Gal-R3 also couples to Gi/o and therefore inhibits adenylyl cyclase (Kolakowski et al., 1998).

An in vitro study found that galanin inhibits LTP in the CA1 region (Coumis and Davies, 2002; Sakurai et al., 1996). Galanin attenuates (Badie-Mahdavi et al., 2005a; Badie-Mahdavi et al., 2005b), whereas a Gal-receptor 2 agonist fully blocked (Badie-Mahdavi et al., 2005b) LTP induced in the dentate gyrus in vitro. The inhibitory effect of galanin on LTP in vitro is consistent with the results of an in vivo transgenic study which found that LTP in the dentate gyrus was enhanced in galanin knock-out mice and reduced in galanin over-expression mice (Mazarati et al., 2000). In the CA1 region of the

hippocampal slice galanin was found to block the slow cholinergic excitatory EPSP induced by the release of endogenous ACh from the stratum oriens (Dutar et al., 1989).

Galanin has also been shown to modulate ACh release, however its effect on ACh release is location specific as exogenously applied galanin has the opposite effect on ACh release in the dorsal and ventral hippocampus, where local galanin injections increase and decrease ACh release, respectively (Schott et al., 2000). Galanin injected i.c.v. decreases ACh release in the ventral hippocampus without altering ACh release in the dorsal hippocampus (Fisone et al., 1987). In contrast galanin infused into the medial septum increases ACh release in the ventral hippocampus (Elvander et al., 2004), which is consistent with the results of a previous study that found galanin excites neurons from the diagonal band of Broca (dBB) (Jhamandas et al., 2002).

The effect of galanin on learning is somewhat complicated, however the effect appears to depend on site of application, as galanin injected into the ventral hippocampus impairs learning (Ogren et al., 1996; Schott et al., 1998) although a lower dose was also shown to facilitate learning (Ogren et al., 1996). In contrast, galanin injected into the dorsal hippocampus did not significantly affect spatial learning, although it tended to facilitate learning (Ogren et al., 1999). Galanin injected into the medial septum was shown to facilitate spatial learning (Elvander et al., 2004). However, galanin injected i.c.v. impairs spatial learning (Kinney et al., 2003), whereas the galanin antagonist, M35, injected i.c.v., facilitated acquisition in a spatial learning task in the rat (Ogren et al., 1992). This suggests that endogenous galanin plays a role in learning. Interestingly, galanin also appears to antagonise the effects of muscarinic receptor activation as i.c.v. injections of the galanin receptor antagonist, galanin(1-13)-Pro-Pro-Ala-Leu-Ala-Leu-Ala amide (M40), enhanced the ability of the systemically applied muscarinic M_1 agonist, 3-(3-S-n-pentyl-1,2,5-thiadiazol-4-yl)-1,2,5, 6-tetrahydro-1-methylpyridine (TZTP), to ameliorate deficits in a delayed non-matching to position performance task (DNMTP) task, whereas M40 alone had no affect on learning (McDonald et al., 1998).

Finally galanin also acts as an anticonvulsant as galanin overexpressing mice were less susceptible, whereas galanin knock-out mice were more susceptible to seizures induced by perforant path stimulation relative to wild-type mice (Mazarati et al., 2000). The anticonvulsant effect of galanin is associated with the inhibitory effect of galanin on hippocampal glutamate release (Mazarati et al., 2000; Zini et al., 1993a; Zini et al., 1993b).

1.5 Objectives

Deterioration of the cholinergic system is believed to contribute to the cognitive deficits associated with AD and also possibly healthy aging. AChE inhibitors increase the extracellular concentration of endogenous ACh and are associated with improved cognitive function in AD. However, AChE inhibitors would be expected to partly negate their enhancement of the cholinergic system since the increase in ACh will inhibit its own release through the activation of presynaptic inhibitory M_2/M_4 mACh receptors. Agents which antagonise M_2/M_4 mACh receptors increase ACh release and have also been found to enhance cognition and ameliorate cognitive deficits associated with ageing in animal models. Therefore, it has been suggested that M_2/M_4 mACh receptor antagonists may be of potential therapeutic benefit in the treatment of AD.

Previous experiments in this laboratory found that M_2 mACh receptor antagonists, AF-DX116 and methoctramine, injected i.c.v., induced a fast onset and long-term enhancement in synaptic transmission (Li, 2002). The purpose of the present series of experiments was to further investigate the mechanism underlying this LTE of synaptic transmission.

The main objectives of this thesis were as follows:

(1) To compare the effect of different M_2 mACh receptor antagonists on synaptic transmission in the CA1 region of the hippocampus.

(2) To investigate the role of nACh receptor activation in methoctramine LTE.

(3) To investigate the NMDA receptor and group I mGlu receptor dependence of methoctramine LTE.

(4) To evaluate the role of different kinases in the induction, maintenance and expression of methoctramine LTE.

(5) To determine the galanin receptor dependence of methoctramine LTE

(6) To assess the ability of Aβ to inhibit methoctramine LTE.

It is hoped that this work will contribute to the understanding of how the cholinergic system modulates hippocampal function and thus lead to improved pharmacological treatments for central nervous system disorders.

II. Materials and Methods

2.1 Animals

All animals used in this study were male Wistar rats (in-bred strain), Bioresources Unit, Trinity College Dublin) weighing between 250-360g, which corresponds to an age of two to four months. The rats were housed two per cage in a Scantainer (Denmark) and maintained between 19-24 °C under a twelve hour light /dark cycle with free access to food (standard rodent chow) and water at all times.

2.2 Anaesthesia

Animals were weighed before each experiment to determine the dose of anaesthetic required. Anaesthesia was induced using urethane (ethyl carbamate; 1.5 mg/kg i.p.). Fifteen minutes after the first dose the animal was tested for depth of anaesthesia by pinching the animal's paw to determine if there was any muscle reflex. If there was a response to this stimulus a supplemental dose of urethane was delivered. The animal remained immobilised and anaesthetised for at least 4-6 hrs.

2.3 Surgery

The hair over the scalp was removed with a scissors and 2ml of lidocaine hydrochloride with adrenalin (Norocaine) was injected subcutaneously over the skull. A scalpel was used to make a midline incision from between the ears to between the eyes. The periosteum was removed by scraping with a scalpel and any excess tissue was cut away. The skull was dried with tissue paper and the skull plates were revealed.

2.4 Electrode implantation

A monopolar recording electrode and a bipolar stimulating electrode was used for each experiment. The electrodes were made in the laboratory by taking two lengths of Teflon coated tungsten wire (625μm tungsten diameter, 750 μm total external diameter, Advent Research Materials, Ltd.) and the teflon at one of the ends was

removed using a scalpel. Each of the two wires were soldered to an individual pin of a two pin connector and then twisted together using a forceps. The wires were then glued together using cyanoacrylate to make them stronger and they were sealed into place at the point where the wires join the socket with dental (acrylic) cement to ensure a stable connection. The ends of the wires were cut at an angle to expose the tips so that one was marginally below the other. The reference and ground electrodes were made from small stainless steel screws (Plastics 1 Inc, Bilaney, Germany) to which single pins were soldered.

The intersection of bregma with the midline was used as the zero reference point in order to mark out with a compass and waterproof pen the coordinates for the sites of insertion of reference and earth screws and the recording and stimulating electrodes. The recording electrode was positioned 3.4mm posterior to bregma and 2.5mm lateral (right) to the midline. The stimulating electrode was positioned 4.2mm posterior to bregma and 3.8mm lateral (right) to the midline. The reference and ground electrodes were positioned on the opposite hemisphere (left) to that used for electrode implantation. The ground electrode was positioned 7mm posterior to bregma and 5mm lateral to the midline (left). The reference electrode was located 8mm anterior to bregma and 1mm lateral (left) to the midline. Coordinates for the electrode positions were obtained by referring to the rat brain atlas of Paxinos and Watson (1998).

Burr holes were drilled over the electrode implantation sites using drill bits of 1.5mm and 1mm. Care was taken not to disturb the dura matter or cortical hemispheres whilst drilling. The two modified screws designed to act as reference and ground electrodes (as described above) were put in place and secured using a small amount of glue and powder cement. The rat was then placed onto a heating blanket (Harvard Apparatus Homeothermic Blanket Control Unit), which was used to monitor and maintain the rat's temperature between 36.5-37.5 °C throughout the experiment. The rat was placed on the heating blanket for approximately 10min before being placed in the stereotaxic recording apparatus (KOPF Instruments, USA). The dura matter in the electrode holes were pierced using a sharp sterile syringe needle. The holes were kept moist using saline applied by syringe and needle. The recording and stimulating electrodes were lowered into the CA1 region of the dorsal hippocampus until the

desired response was found (as outlined in 2.7). A drop of superglue was applied around the electrodes and the entire assembly was secured using dental cement.

2.5 Intra-cerebroventricular (i.c.v.) cannula implantation and injections

Cannulae were used to deliver drugs to the brain via the lateral cerebral ventricle. The optimal location for the injection into the lateral cerebral ventricle was 0.5mm posterior, 1mm lateral and ventral 4mm from the outer surface of the skull. These coordinates were obtained by referring to the rat brain atlas of Paxinos and Watson (1998) and were determined to be effective by this research group. The external cannulae were constructed using a stainless steel hypodermic needle (22 gauge, 0.7mm outer diameter) which was cut to 12mm in length. The bevelled end of the needle was ground to a length of 1.5mm to reduce the angle of the exposed tip. An internal plug (stylet) was made from 28 gauge (0.36 diameter) stainless steel wire that was kept in the cannula when it was not in use and served to prevent blockages. The internal plug (stylet) protruded by 1mm below the end of the external cannula.

The cannula was implanted above the right lateral ventricle and sealed into place with glue and dental cement 30 minutes before the electrodes were implanted. For intra-cerebroventricular injections the stylet was removed and the drug/vehicle was delivered via a 10 μl Hamilton syringe to which an internal cannula (28 gauge, 0.36mm outer diameter) was attached. The internal cannula was designed so that it protruded by 1mm below the end of the external cannula. The drugs/vehicles were delivered in a 5 μl volume (except for experiments with the ZIP where 7 μl was injected). The drug/vehicle was injected over a 3-4 min period and stimulation was stopped immediately before the start of infusion and resumed immediately after the delivery of the drug/vehicle. The internal cannula was left in place for 5 minutes after the drug/vehicle was injected to allow for diffusion and to avoid reflux. Then the internal cannula was removed carefully over a 5 minute period. Verification of the location of the cannula was carried out post-mortem by the spread of dye (Indian ink) after it was injected i.c.v.

2.6 Recording apparatus

The electrophysiological recording apparatus was surrounded by a Faraday cage to isolate the signal from environmental electrical interference. The Faraday cage and the electrical electrophysiological equipment were grounded to a central point to eliminate electrical interference. The electrophysiological equipment consisted of a constant current isolation unit (Grass Instrument Co. photoelectric stimulus isolation unit) linked to the stimulating output of the recording unit. The evoked response was transmitted via a pre-amplifier (gain 11) to an analogue-to-digital converter (MacLab 2e, Analog Digital Instruments) operated by scope Program versions 3.6 using an Apple Machintosh Power PC 8200/120.

2.7 Location of recording and stimulating electrodes during surgery

The locations of the recording and stimulating electrode are outlined using a schematic diagram of a coronal section of the rat hippocampus (see Fig. 2-2).
The rat was placed in the stereotaxic apparatus and the electrodes were brought to the surface of the dura. This position signified the zero reference point. The depths of the electrodes moving through the cortical and hippocampal layers to the dendrites of the stratum radiatum were monitored as they were lowered through the tissue. Responses (evoked using 0.2 ms duration, 3mA pulse at a frequency of 0.1Hz) were displayed on the computer screen as the electrodes were lowered into place in the CA1 area. Both the cerebral cortex and hippocampal formation possess laminar structures. When a local depolarisation such as an excitatory postsynaptic potential (EPSP) was created, an electron sink was set up along a vertical superficial axis. A phase reversal was encountered when this dipole was crossed; indicating that this was the area generating electromotive forces and the response recorded was not from a distal site by voltage conduction. From this method it was possible to determine which layer the electrodes were in by referring to the electrophysiological criteria determined for each region of the hippocampus as described previously (Leung, 1980). The monopolar recording electrode was placed on the surface of the dura and

the bipolar stimulating electrode was lowered to a depth of 2 mm. The stimulating electrode was then lowered in increments of 200μm to a depth of approximately 2.5mm. The first potential recorded was a small negative response of approximately 10ms duration, which was generated when the stimulating electrode penetrated the alveus. The stimulating electrode was lowered approximately another 200 μm and a larger negative response was seen as the stimulating electrode penetrated the stratum oriens (see Fig. 2-1a). As the stimulating electrode approached the cell body layer the amplitude of the evoked response became smaller and reversed when the stimulating electrode passed through the cell body layer into the dendritic layer of the stratum radiatum (see Fig. 2-1b-c).

The monopolar recording electrode was lowered 2mm from the dura and the positive response increased in amplitude (see Fig. 2-1d). The recording electrode was lowered 500 μm and the response became smaller as the recording electrode penetrated the cell body layer (see Fig. 2-1e). The recording electrode was lowered 200 μm and stimulation was stopped for 5 minutes so as not to over stimulate the brain. Once stimulation was resumed the response was a negative EPSP (see Fig. 2-1f). The EPSP was recorded (and was evoked by a 0.2 ms duration, 2.3mA pulse at a frequency of 0.033 Hz) for 10 minutes. The stimulation electrode was adjusted in increments of 20μm to maximise the response and then the recording electrode was adjusted in increments of 20μm to further maximise the response. Typically the latency of the EPSP was less than 15ms and the maximal amplitude was greater than 2mV.

On average the depth of the stimulating electrode was approximately 2.9mm from the dura, whereas the average depth of the recording electrode was approximately 2.7mm from the dura.

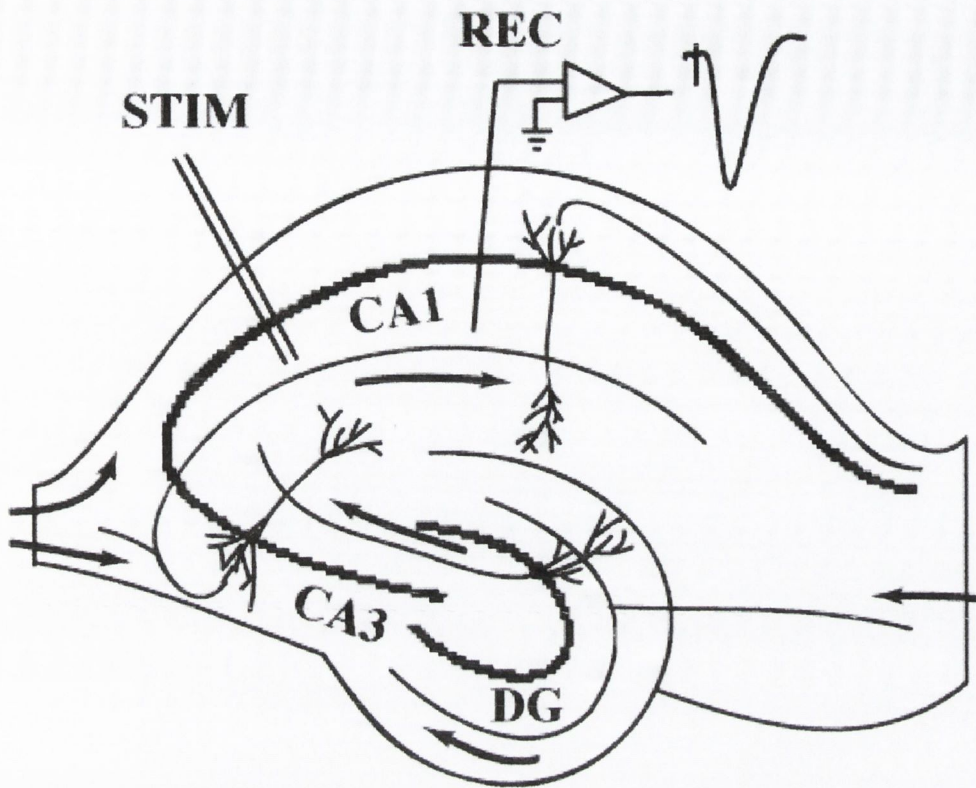

Fig. 2-1. Schematic diagram of a coronal section of the rat hippocampus showing the sites of the recording and stimulating electrodes and a representative trace.

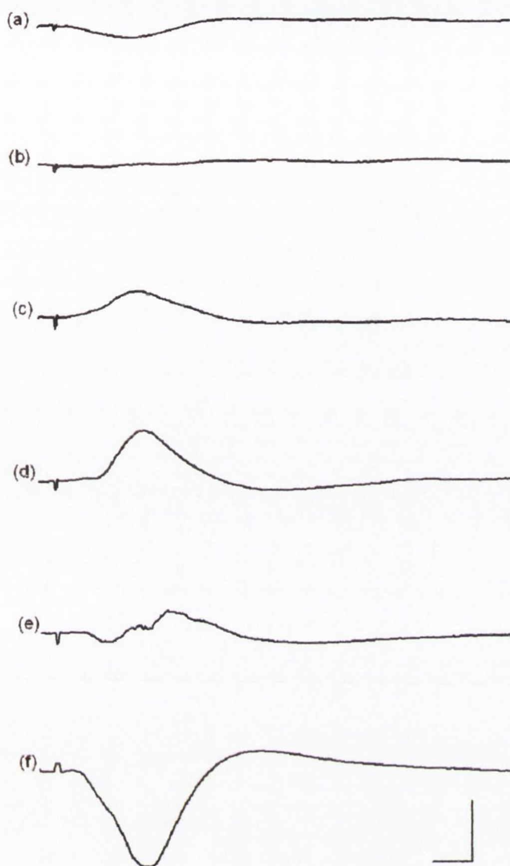

Fig. 2-2. Representative EPSP traces from a single experiment during which the electrodes were lowered into place. Monitoring of the characteristic shape indicated the position of the electrode in the CA1 area of the dorsal hippocampus in the urethane anaesthetised rat. (a) Potential recorded when recording electrode was at the dura and the stimulating electrode was in the stratum oriens. (b) Potential recorded when recording electrode was at the dura and the stimulating electrode was in pyramidal cell body layer. (c) Reversal of potential when the recording electrode was at the dura and the stimulating electrode was in the stratum radiatum. (d) The positive potential increased in amplitude when the recording electrode was lowered 2mm below the dura. (e) The positive potential decreased as the recording electrode entered the cell body layer. (f) The electrodes were adjusted until the EPSP was optimal. Horizontal bar 5ms; vertical bar, 1.0mV.

2.8 Input/Output curves (I/O)

The animal was allowed to recover for a period of one hour post electrode implantation and then an I/O curve was determined. This involved stimulation with a 0.2 ms duration, 1.4-4.5mA pulse at a frequency of 0.033 Hz. The stimulation was applied twice at each intensity using a range of intensities at 0.3mA intervals.

2.9 EPSP Recordings

The population field EPSP was used as a measure of synaptic transmission in the stratum radiatum of the CA1 region by stimulating the Schaffer collaterals /commissural fibers. Test EPSPs were evoked by a single square wave pulse of current at low frequency (0.033 Hz, 0.2ms duration) generated by a constant current isolation unit. The test stimulus intensity was set to evoke responses of 55-60% of maximum EPSP amplitude (typically 2.2-2.8mA). Baseline synaptic transmission was recorded for at least one hour prior to pharmacological intervention.

2.10 Drug Treatment

Peptides were dissolved in milli-Q water. All other drugs were dissolved in distilled water except for LY367385 and BIBN-99, which were dissolved in saline. Drugs were administered via either the intra-peritoneal (i.p.) or intra-cerebroventricle (i.c.v.) routes.

2.11 Data Analysis

EPSP amplitude was taken as a measure of excitatory synaptic transmission. For the purpose of graphical presentation data was expressed in epochs of 5 min and S.E.M. was calculated using Microsoft Excel 6.0. A stable baseline was recorded for 60 min prior to pharmacological intervention. The effect of pharmacological intervention on synaptic transmission was expressed as a percentage of the average EPSP amplitude of the proceeding 60 min (baseline). For the purpose of statistical analysis data was

expressed in epochs of 10 min. Statistical comparison were made between average baseline EPSP ampiltude 10 min prior to drug treatment compared to 10, 60 and 120 min after drug treatment (unless otherwise stated) and means were expressed with S.E.M. Statistical significance of the difference between the means was estimated using two tailed paired and unpaired Student's t-test. Statistical significance was taken at the 95% level (P-values< 0.05). P-values referred to in the graph legend are the result of the comparison of the drug treated group with the vehicle treated group at 120 min after drug/vehicle unless otherwise stated (unpaired t-test). The data were analysed using JMP IN 3.2.1 statistical software on a Mac OS X (version 10.3.9).

2.12 Drugs

D-AP5	D-(-)-2-Amino-5-phosphonopentanoic acid (Tocris)[1]
Amyloid β	Synthetic human amyloid β (1-42) (Bachem)[2]
BIBN-99	(5,11-dihydro-8-chloro-11-[[4-[3-[(2,2-dimethyl-1-oxopentyl)ethylamino]propyl]-1-piperidinyl]acetyl]-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one) (gift from Boeringer Ingelheim, Germany)
BIM	Bisindolylmaleimide I hydrochloride (Calbiochem, CN Biosciencecs (UK) Ltd., Boulevard Industrial Park, Beeston, Nottingham, NG9 2JR UK, cnbiosciences.co.uk)
Dental acrylic liquid and powder	Associated Dental Products Ltd., Purton, Swindon, Wiltshire, SN5 9HT, UK
Galanin (rat)	H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-His-Ala-Ile-Asp-Asn-His-Arg-Ser-Phe-Ser-Asp-Lys-His-Gly-Leu-Thr-NH_2 (Bachem)[2]
Gallamine	N-[2-[2,3-bis(2-diethylaminoethoxy)phenoxy]ethyl]-N-ethyl-ethanamine (Sigma)[3]
M35	Galanin (1-13)-Bradykinin (2-9) amide (Bachem)[2]
Indian ink	Geoge T. Gurr Ltd., London, SW6, UK

LY367385	(S)-(+)-α-Amino-4-carboxy-2-methylbenzeneacetic acid (Tocris)[1]
Mecamylamine	N,2,3,3-Tetramethylbicyclo(2.2.1)heptan-2-amine Hydrochloride (Sigma)[3]
Memantine	3,5-Dimethyl-1-adamantanamine hydrochloride (Sigma)[3]
Methoctramine	N,N'-bis[6-[[(2Methoxphenyl)methyl]amino]hexyl]-1,8-octane diamine tetrahydrochloride (RBI)[4]
Methyllcaconitine Citrate	[1α,4(S),6β,14α,16β]-20-Ethyl-1,6,14,16-tetramethoxy-4-[[[2-(3-methyl-2,5-dioxo-1-pyrrolidinyl)benzoyl]oxy]methyl]-aconitane-7,8-diol citrate (Sigma)[3]
(-)-Nicotine	(−)-Nicotine (+)-bitartrate salt (Sigma)[3]
Lidocaine hydrochloride with adrenaline	Norocaine (Norbrook Labratories Ltd., Newry BT35 6JP, Northern Ireland)
ZIP	Myr-Ser-Ile-Tyr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys-Leu-OH (Biosource International, Inc., 542 Flynn Road, Camarillo, CA 93012, USA).
Rp-cAMPS	Rp-Cyclic 3',5'- hydrogen phosphorothioate adenosine triethylammonium salt (RBI)[4]
Saline	Sodium chloride injection (Baxter Healthcare Ltd., Thetford, Norfolk, England)

Urethane	Ethyl carbamate (Sigma)[3]

Notes

[1]Tocris Cookson Ltd., Northpoint Fourth Way, Avonmouth, Bristol, BS11 8TA UK www.tocris.com).

[2]Bachem, Bubendorf, Switzerland (http://www.bachem.com/)

[3]Sigma, 3050 Spruce Street, Saint Louis, Missouri 63103 USA, (www.sigma-aldrich.com)

[4]RBI-Research Biochemicals International, One Strathmore Road, Natick, MA 01760-2447 USA, (www.Sigma-aldrich.com)

III. Results

3.1 The effect of M_2 mACh receptor antagonists on synaptic transmission

3.1.1 The effect of the methoctramine on synaptic transmission

Previous studies found that methoctramine preferentially binds to the M_2 mACh receptor (Caulfield, 1993; Doods et al., 1993c; Dorje et al., 1991; Waelbroeck et al., 1990). The dose of methoctramine used in the present study was based on a previous study where methoctramine (17nmol/2.5μL) injected i.c.v. was found to induce a long-term enhancement of synaptic transmission (Li, 2002). Methoctramine (34nmol/5μl) injected i.c.v. induced a rapid (<8 min onset) and long-term enhancement (LTE) (>2hrs) of synaptic transmission (128.5±3.4% and 128.6±3.3% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, n=5) (Fig.3-1). The methoctramine-induced LTE was significantly different from the vehicle treated group (n=5, $P<0.05$ at 10 and 120 min after injection, unpaired t-test). The baseline of the vehicle treated control group did not change significantly during the recording period (100.6±2.3% and 99.4±4% at 10 and 120 min after vehicle injection, respectively; $P>0.05$ at 10 and 120 min after vehicle compared to pre-injection baseline, paired t-test, n=5).

3.1.2 The effect of gallamine on synaptic transmission

Gallamine is commonly referred to as a mixed muscarinic and nicotinic ACh receptor antagonist, however there is evidence that it preferentially binds to M_2 muscarinic receptors (Burke, 1986; Price et al., 1986). The doses used in the present study were estimated from a previous study, which observed the behavioural effects of gallamine applied intrahippocampally (Hoss et al., 1990). Gallamine (25nmol) injected i.c.v. induced a long-term enhancement of synaptic transmission, although the enhancement was not significant in the first 10 min (98.6±1.2% and 112.2±2.6 % of baseline at 10 and 120 min after gallamine, respectively; $P>0.05$ and $P<0.05$ at 10 and 120 min after gallamine compared to pre-gallamine baseline, respectively, paired t-test, n=4) (Fig.3-2). The gallamine-induced LTE was significantly different from the vehicle treated group at 120 but not 10 min after gallamine ($P>0.05$ and $P<0.05$ at 10 and 120 min after gallamine compared to the vehicle control group, respectively, unpaired t-test, n=4). The baseline of the vehicle injected control group did not change significantly throughout the recording period (100.6±2.3% and 99.4±4% of baseline at 10 and 120 min after vehicle, respectively; $P>0.05$ at 10 and 120 min after vehicle compared to pre-vehicle baseline, paired t-test, n=5). Higher doses of gallamine (42-56nmol) injected i.c.v. also induced a long-term enhancement of synaptic transmission, although the enhancement was not significant in the first 10 min (96.3±5.7% and 125.3±8.5% of baseline at 10 and 120 min after gallamine, respectively; $P>0.05$ and $P< 0.05$ at 10 and 120 min after gallamine compared to pre-gallamine baseline, respectively, paired t-test, n=5) (Fig.3-2). The gallamine-induced LTE was significantly different from the vehicle treated group at 120 but not 10 min after gallamine ($P>0.05$ and $P<0.05$ at 10 and 120 min after gallamine compared to the vehicle control group, respectively, unpaired t-test, n=5). One animal that received gallamine (56nmol) exhibited behavioural evidence of mild seizure type activity (shivers, jerking and bulging eye movements) towards the end of the recording period; however this activity did not affect the EPSP amplitude. The slightly lower dose of 42nmol did not cause these effects.

3.1.3 The effect BIBN-99 on synaptic transmission

BIBN-99 was previously characterised as a lipophilic and high affinity competitive antagonist selective for the M_2 mACh receptor subtype (Doods et al., 1993a; Doods et al., 1993b). The effect of BIBN-99 injected i.c.v. on synaptic transmission has not previously been reported. In the present study the effect of a range of doses on synaptic transmission was investigated. BIBN-99 (22nmol or 44nmol) injected i.c.v. did not significantly change synaptic transmission (101.4±4.1% and 98.6±7.7% of baseline at 10 and 120 min after BIBN-99 injection, respectively; $P>0.05$ at 10 and 120 min after BIBN-99 compared to pre-BIBN-99 baseline, paired t-test, n=5) (Fig.3-3). The EPSP amplitude of the BIBN-99 injected group was not significantly different from the vehicle pre-treated group ($P>0.05$ at 10 and 120 min after injection compared to the vehicle pre-treated control group, respectively, unpaired t-test, n=5). The baseline of the vehicle treated control group did not change significantly throughout the recording period (101.6±3.9% and 103.8±2.8% of baseline at 10 and 120 min after vehicle, respectively; $P>0.05$ at 10 and 120 min after vehicle compared to pre-vehicle baseline, paired t-test, n=5). In a pilot study, a higher dose of BIBN-99 (66nmol) injected i.c.v. transiently depressed baseline (20%) for approximately 1 hour followed by a return to pre-injection baseline (n=1).

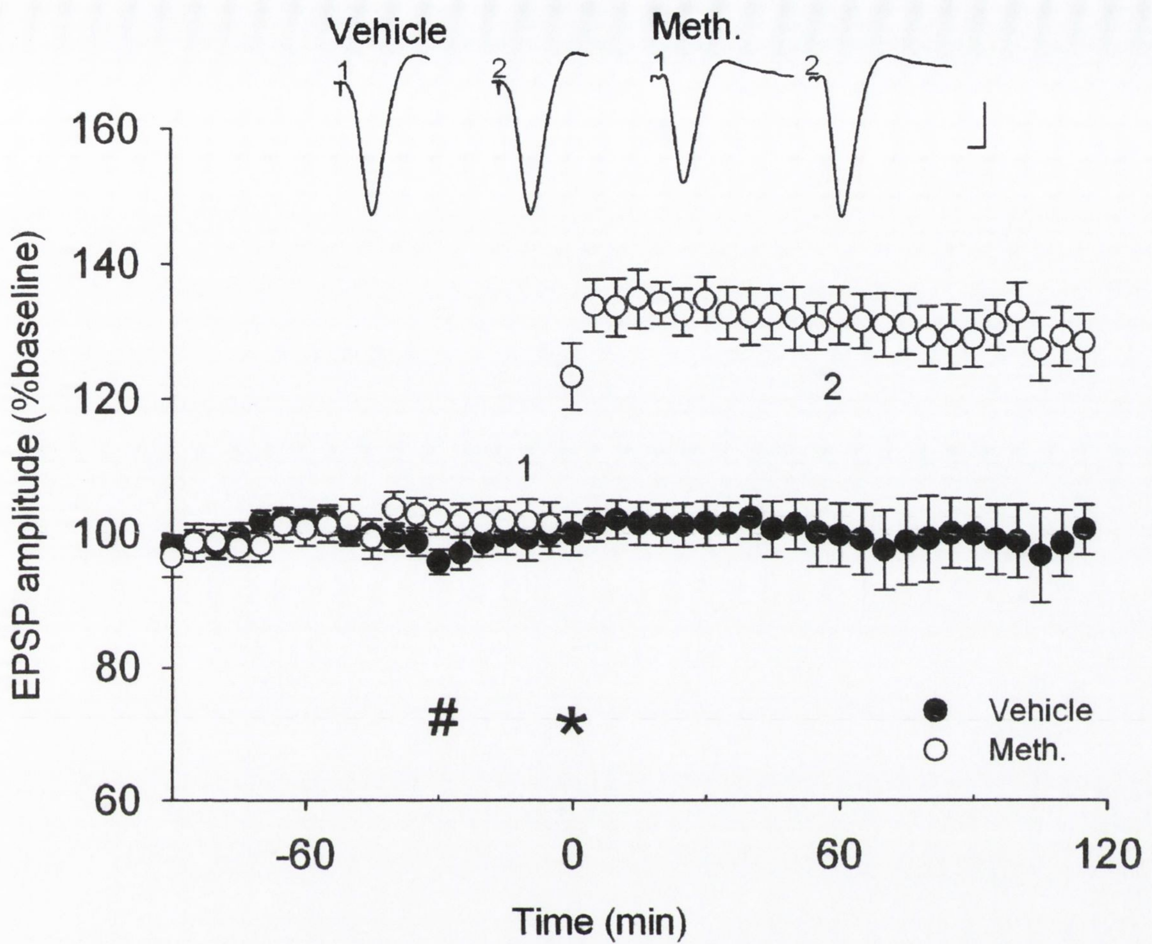

Fig. 3-1. Methoctramine, an antagonist of M_2 mACh receptors, induced a rapid and long-term enhancement of synaptic transmission in the CA1 region of the hippocampus in vivo. Methoctramine (meth., 34nmol) injected i.c.v. significantly enhanced the EPSP amplitude for at least 2hrs, (n=5; ○), $P<0.05$ compared to pre-methoctramine baseline or vehicle i.c.v. (n=5; ●). All animals received a vehicle (water) injection (#) 30 min before vehicle/methoctramine injection (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

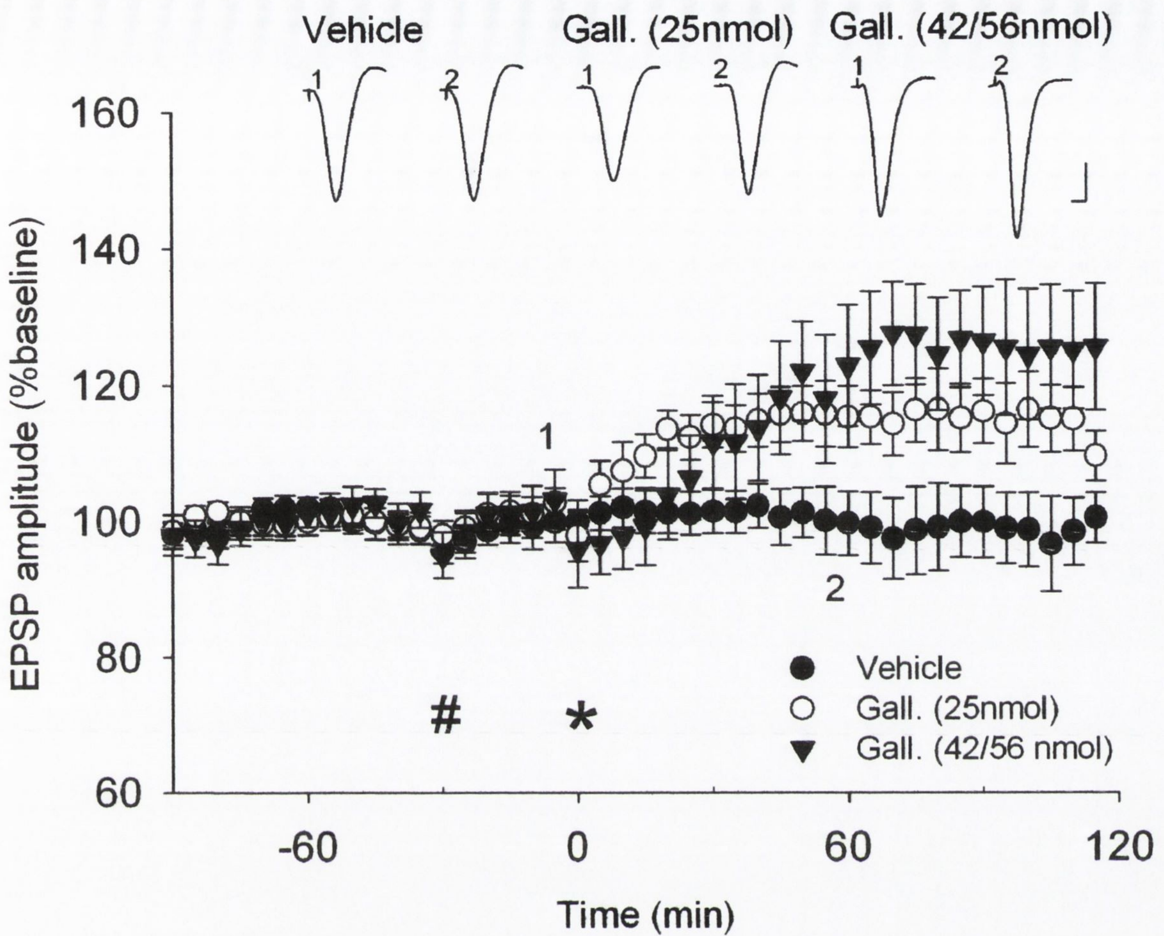

Fig. 3-2. Gallamine, an antagonist of M_2 mACh receptors, induced a slow-onset long-term enhancement of synaptic transmission in the CA1 region of the hippocampus in vivo. Gallamine (gall., 25nmol) injected i.c.v. induced a slow-onset significant enhancement of the EPSP amplitude, (n=4; ○), $P<0.05$ compared to pre-gallamine baseline and vehicle i.c.v. (n=5; ●). Gallamine (42-56nmol) injected i.c.v. induced a slow-onset significant enhancement of the EPSP amplitude, (n=5; ▼), $P<0.05$ compared to pre-gallamine baseline or vehicle i.c.v. (n=5; ●). All animals received a vehicle (water) injection (#) 30 min before vehicle/gallamine injection (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

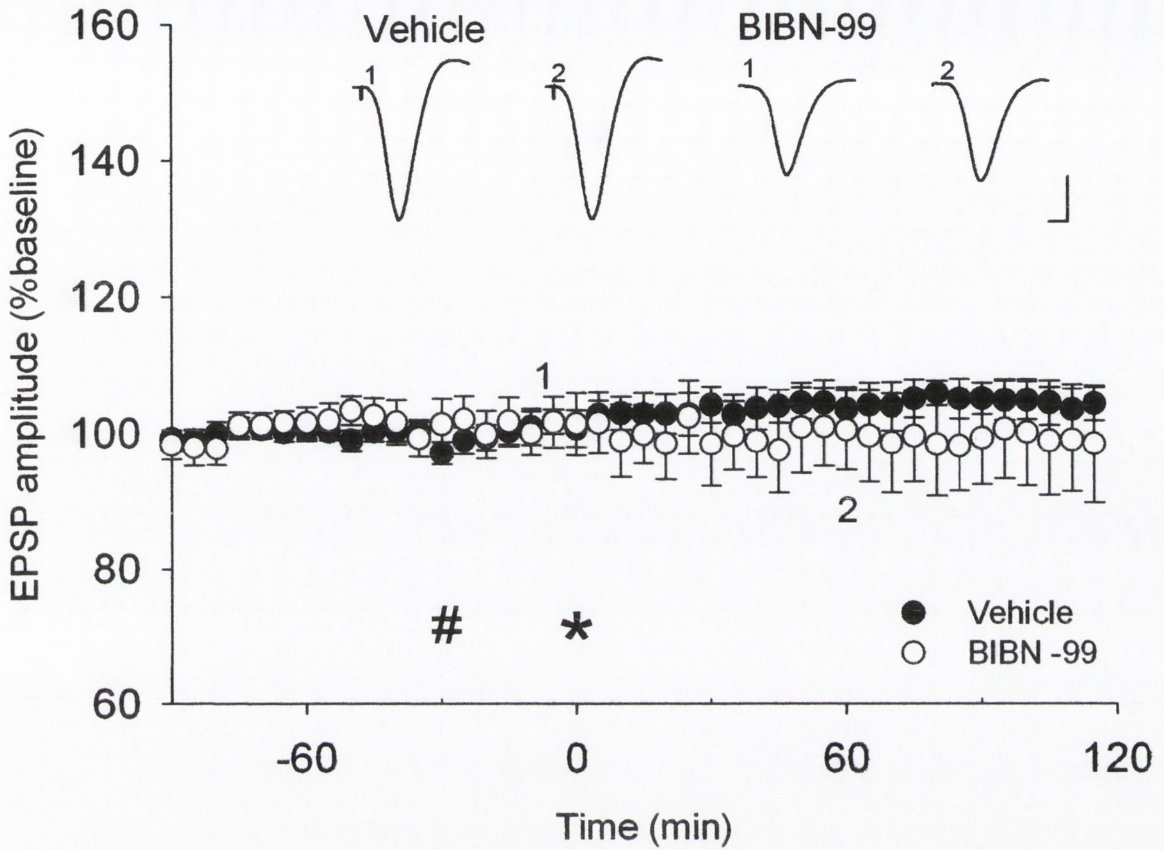

Fig. 3-3. BIBN-99, an antagonist of M_2 mACh receptors, failed to significantly alter synaptic transmission in the CA1 region of the hippocampus in vivo. BIBN-99 (22-44nmol) injected i.c.v. failed to significantly alter EPSP amplitude during the 2 hr recording period, (n=5; ○), P>0.05 compared to pre-BIBN-99 baseline or saline i.c.v. (n=5; ●). All animals received a vehicle (saline) injection (#) 30 min before saline/BIBN-99 injection (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

3.2 The effect of nicotinic receptor agents on methoctramine LTE

3.2.1 The effect of mecamylamine (MEC)

Methoctramine has been reported to increase ACh release in the hippocampus in vivo (Stillman et al., 1993) and therefore the action of ACh at nicotinic ACh receptors may be necessary for the induction of LTE by methoctramine. Mecamylamine blocks most neuronal nACh receptors in the low micromolar range (Wonnacott et al., 1991), however mecamylamine is believed to be somewhat less potent at blocking α7nACh compared to α/β heteromers (Chavez-Noriega et al., 1997). The dose of mecamylamine used in the present study was based on the dose used in a previous study which prevented discernable activation of hippocampal nACh receptors (Tani et al., 1998). Mecamylamine (3mg/kg, i.p.) injected 30 min before methoctramine had no discernible effect on baseline (see table 3.2). Methoctramine subsequently induced a significant LTE although the enhancement in the first 10 min was not statistically significant (122.3±8.8% and 134.6±8.6% of baseline at 10 and 120 min after methoctramine, respectively; $P=0.06$ and $P< 0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, respectively, paired t-test, n=5) (Fig.3-4). The methoctramine-induced LTE in animals pre-treated with mecamylamine was not significantly different from the LTE in the vehicle pre-treated control group ($P>0.05$ at 10 and 120 min after methoctramine compared to the vehicle pre-treated methoctramine group, unpaired t-test, n=5). The LTE of the vehicle pre-treated methoctramine control group was significant (120.6±3.4% and 124.5±1.6% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, n=5).

3.2.2 The effect of methyllycaconitine (MLA)

As mecamylamine is believed to be somewhat less potent at blocking α7nACh compared to α/β heteromers (Chavez-Noriega et al., 1997) it was decided to investigate the effect of applying the α7 nACh receptor antagonist methyllycaconitine. Methyllycaconitine was previously characterised as a selective α7 nACh receptor antagonist (Macallan et al., 1988). The dose of methyllycaconitine used in the present study was based on the dose of methyllycaconitine reported to impair spatial memory (Felix and Levin, 1997). The α7 nicotinic ACh receptor antagonist, methyllycaconitine (50nmol or 65nmol), was injected i.c.v. 30 min before methoctramine and had no discernible effect on baseline transmission (see table 3.2). Methoctramine subsequently induced a significant LTE, although the enhancement in the first 10 min was not statistically significant (107.7±3.1% and 115±3.6% of baseline at 10 and 120 min after methoctramine, respectively; $P>0.05$ and $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, respectively, paired t-test, n=5) (Fig.3-5). The methoctramine-induced LTE in animals pre-treated with methyllycaconitine was significantly reduced compared to LTE in the vehicle pre-treated control group during the initial 10 min but not thereafter ($P<0.05$ and $P>0.05$ at 10 and 120 min after methoctramine compared to LTE of the vehicle pre-treated control group, respectively, unpaired t-test, n=5). The methoctramine LTE of the vehicle pre-treated methoctramine control group was significant (123.5±1.4% and 118±1.8% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared with pre-methoctramine baseline, paired t-test, n=5).

In view of the significant delay in the onset of the methoctramine LTE in the presence of methyllycaconitine it was decided to evaluate the effect of a combination of methyllycaconitine and mecamylamine. The injection of mecamylamine (3mg/kg, i.p.) and methyllycaconitine (65nmol, i.c.v.) at 30 and 25 min before methoctramine, respectively had no discernible effect on baseline transmission (see table 3.2). Methoctramine subsequently induced a significant LTE, although the enhancement in the first 10 min was not statistically significant (109.8±3.8% and 116.3±4.8% of baseline at 10 and 120 min after methoctramine, respectively; $P>0.05$ at 10 min and $P<0.05$ at 120 min compared to pre-methoctramine baseline, paired t-test, n=5) (Fig.3-6). Although the onset of methoctramine-induced LTE in animals pre-treated with both methyllycaconitine and mecamylamine was initially delayed, this reduction was not significant compared to the methoctramine LTE in the vehicle pre-treated control group ($P>0.05$ at 10 and 120 min after methoctramine compared to vehicle pre-treated control group, unpaired t-test, n=5). Methoctramine LTE of the vehicle pre-treated methoctramine group was significant (120.6±3.4% and 124.5±1.6% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t test, n=5).

3.2.3 The effect of nicotine

The dose of nicotine employed in the present study was based on a previous in vivo study, which found that nicotine (3mg/kg, i.p.) caused a long- term enhancement of synaptic transmission in the mouse dentate gyrus (Matsuyama et al., 2000). The baseline of the nicotine pre-treated plus vehicle group did not significantly change (96.2±2%, 94.7±3.4% at 10 and 120 min after nicotine injection, respectively; $P>0.05$ at 10 and 120 min after injection compared to pre-vehicle baseline, paired t-test, n=4). Nicotine (3mg/kg, i.p.) injected 30 min before methoctramine had no discernible effect on baseline transmission (see table 3.2). Methoctramine subsequently induced a significant LTE (131.5±4.5% and 137.1±4% of baseline at 10 and 120 min after

methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, n=5) (Fig.3-7). Nicotine pre-treatment tended to increase methoctramine LTE compared to the methoctramine LTE of the vehicle pre-treated control group, however, the methoctramine LTE was not significantly different between these groups ($P>0.05$ at 10 and 120 min after methoctramine compared to the LTE of vehicle pre-treated control, unpaired t-test, n=5). Methoctramine-induced LTE of the vehicle pre-treated control group was significant (126.8±4.7% and 126.8±3.5% of baseline at 10 and 120 min after methoctramine for the vehicle pre-treated methoctramine control group, n=5). The methoctramine LTE of the vehicle control group was statistically significant ($P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, respectively, paired t-test, n=5). In pilot experiments higher doses of nicotine were also injected systemically, however they were found to cause a transient decrease in synaptic transmission.

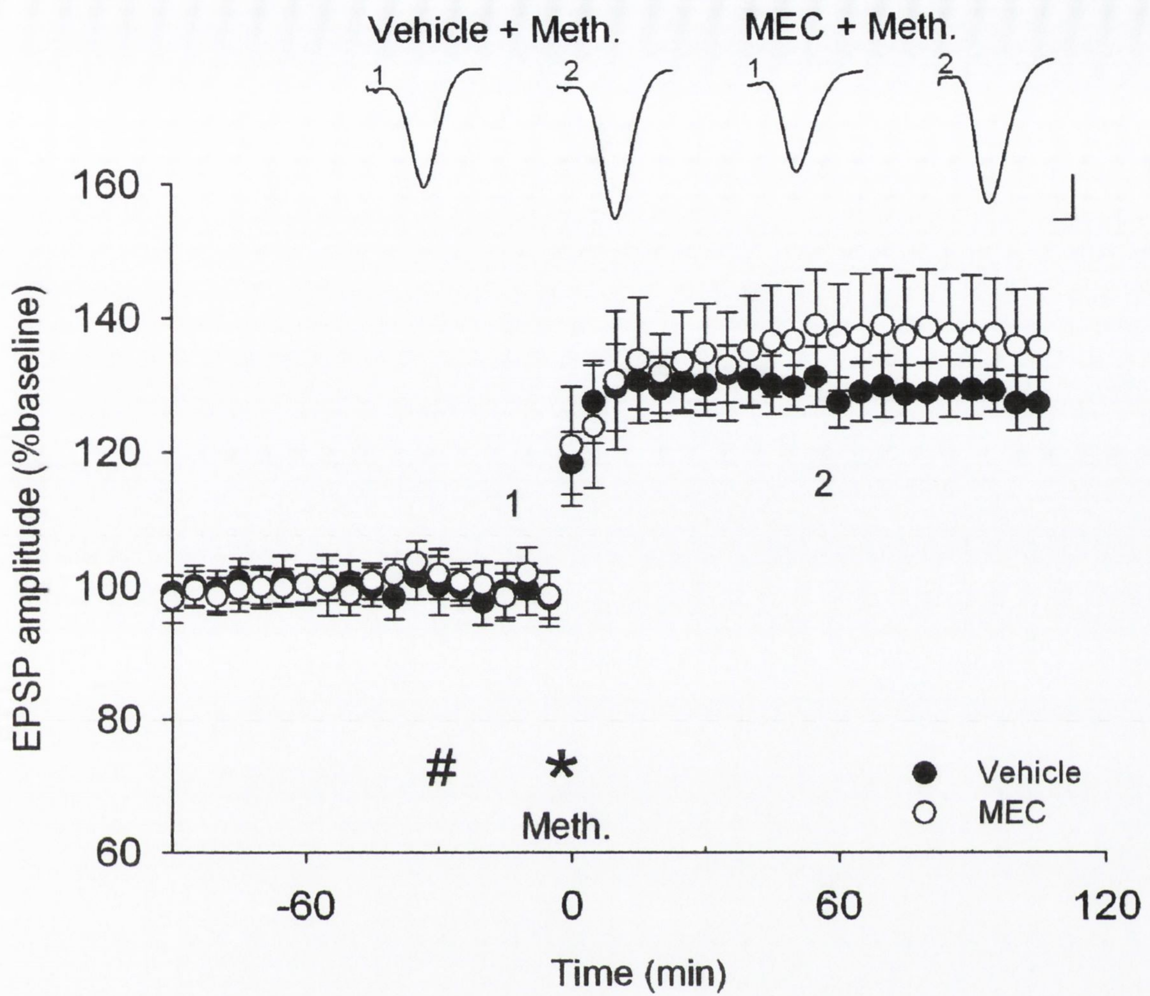

Fig. 3-4. Mecamylamine, a broad spectrum nicotinic receptor antagonist, did not significantly affect LTE. Mecamylamine (MEC, 3mg/kg, i.p.) had no significant effect on methoctramine LTE, (n=5; ○), P>0.05 compared to the vehicle injected control (n=5; ●). Mecamylamine/vehicle (#) was injected 30 min before methoctramine (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

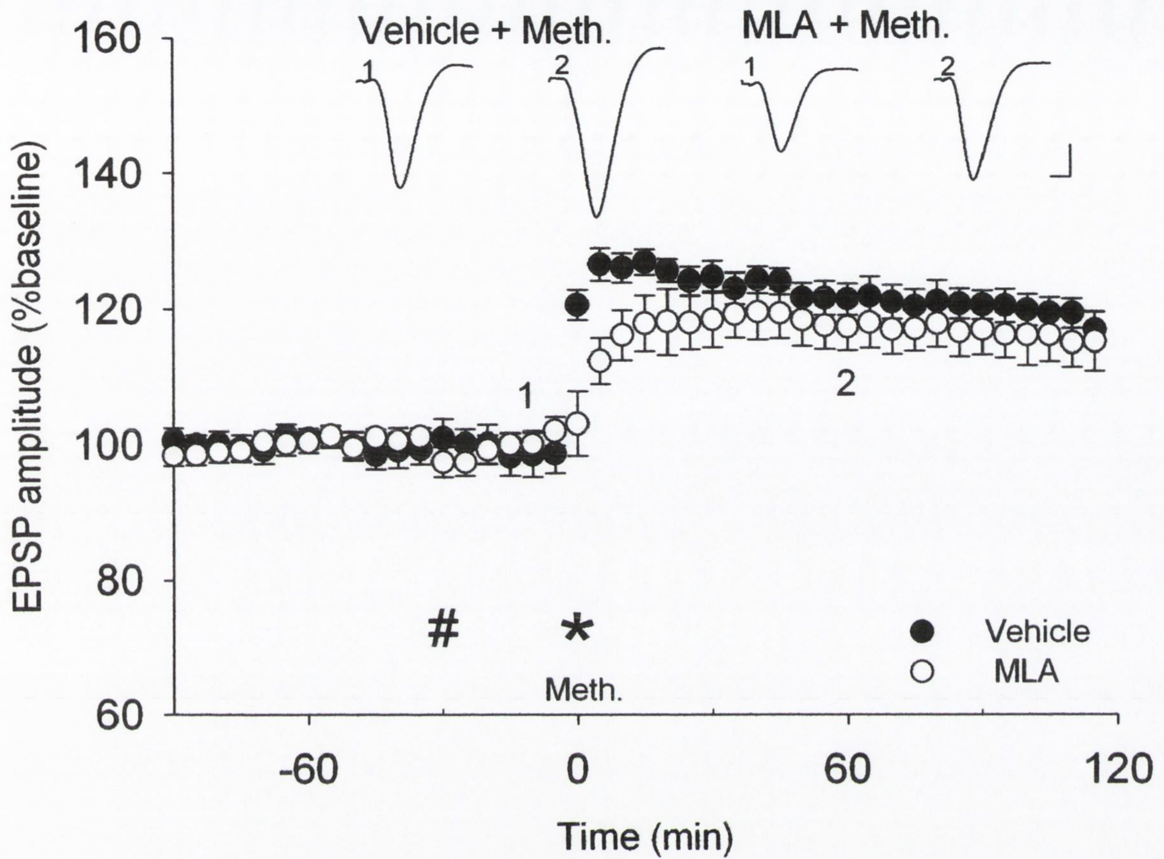

Fig. 3-5. Methyllycaconitine, an inhibitior of α7 nicotinic receptors, delayed the onset but did not block LTE. Methyllycaconitine (MLA, 50-65nmol) injected i.c.v. significantly reduced the initial enhancement of the EPSP amplitude induced by methoctramine, (n=5; ○), $P<0.05$ at 10 min and $P>0.05$ at 120 min compared to the vehicle injected control group (#) (n=5; ●). Methyllycaconitine/vehicle (#) was injected 30 min before methoctramine (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

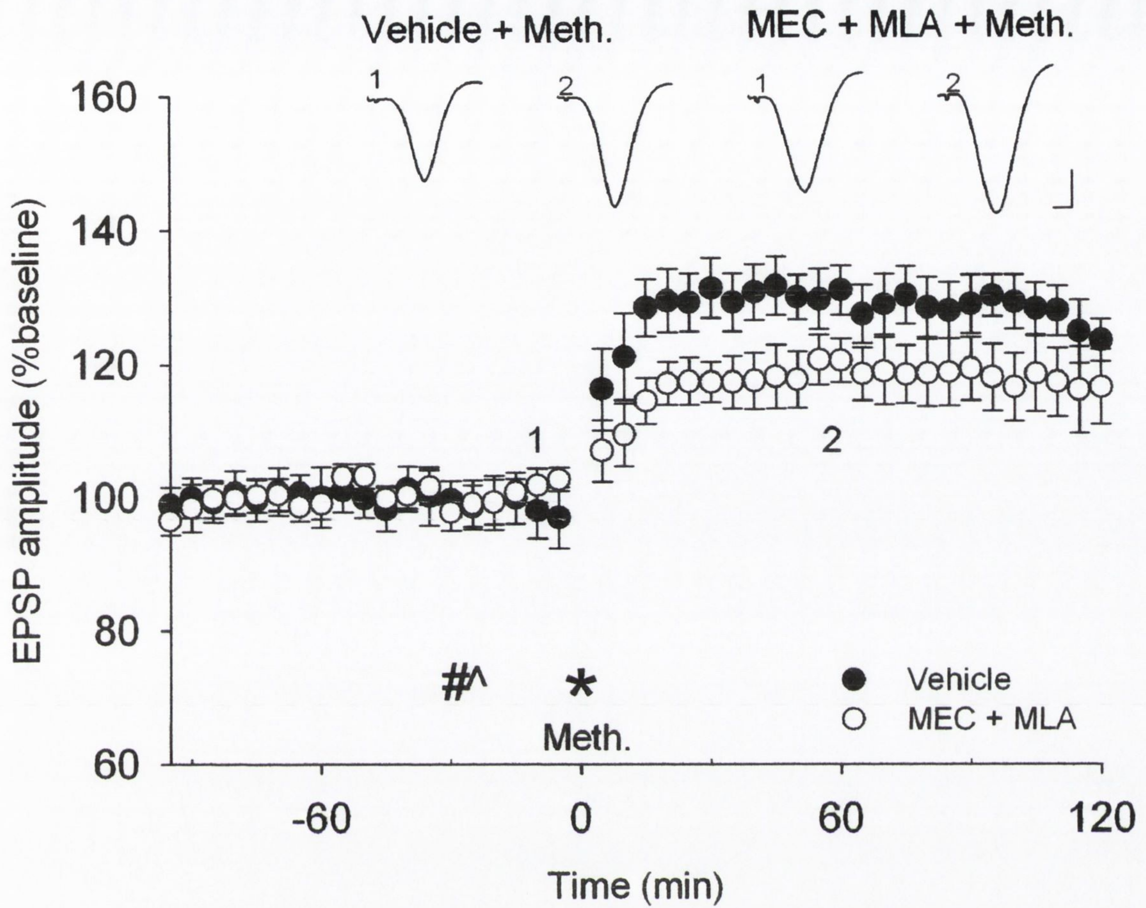

Fig. 3-6. The combination of mecamylamine and methyllycaconitine tended to delay the onset of LTE but failed to significantly affect LTE. Mecamylamine (3mg/kg, i.p.) and methyllycaconitine (65nmol) i.c.v. did not significantly affect methoctramine-induced LTE, (n=5; ○), P>0.05 compared to vehicle injected control (#) (n=5; ●). Mecamylamine/vehicle (#) and methyllycaconitine/vehicle (^) were injected 30 min and 25 min before methoctramine (*), respectively. Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

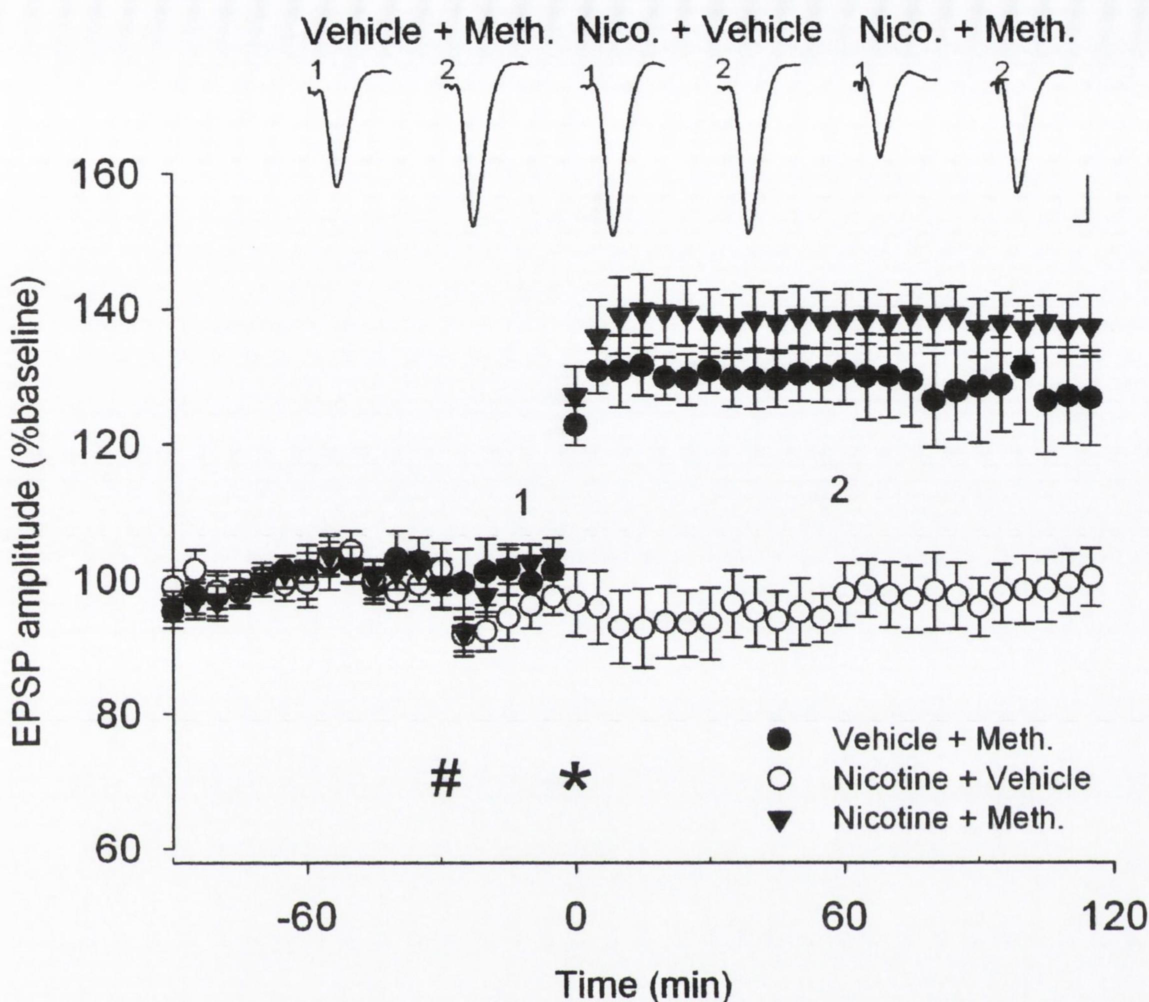

Fig. 3-7. Nicotine, applied systemically, failed to significantly affect LTE.

Nicotine (nico., 3mg, i.p.) did not significantly affect methoctramine-induced LTE, (n=5; ▼), P>0.05 compared to the vehicle injected control (n=5; ●). Nicotine (3mg, i.p.) followed 30 min later by a vehicle injection (i.c.v.) failed to significantly affect the amplitude of the EPSPs, (n=5; ○), P>0.05 compared to pre-nicotine baseline. Nicotine/vehicle (#) was injected 30 min before vehicle/methoctramine (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

3.3 The effect of glutamate receptor antagonists on methoctramine LTE

3.3.1 The effect of the competitive NMDA receptor antagonist, d-AP5

Previous studies found that the competitive NMDA receptor antagonist d-AP5 (0.1μmol) injected i.c.v. blocked HFS induced LTP in the CA1 region of the urethane anaesthetised rat (I. Klyubin, TCD, personal communication), and also in freely behaving rats (Leung and Shen, 1999). D-AP5 (0.1μmol) was injected i.c.v. 15 min before methoctramine and had no discernible effect on baseline (see table 3.2). Methoctramine subsequently induced a significant LTE although the enhancement in the first 10 min was not statistically significant (114±7.2% and 121.5±6.7% of baseline at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, respectively; $P>0.05$ and $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, respectively, paired t-test, n=5) (Fig.3-8). The methoctramine-induced LTE in animals pre-treated with d-AP5 was not significantly different from the methoctramine LTE in the vehicle pre-treated control group ($P>0.05$ at 10 and 120 min after methoctramine compared to the vehicle pre-treated methoctramine control group, respectively, n=5, unpaired t-test). The methoctramine-induced LTE in the vehicle pre-treated methoctramine group was significant (120.2±2.2% and 117.1±1.8% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, n=5).

3.3.2 The effect of the non-competitive NMDA receptor antagonist, memantine

The non-competitive NMDA receptor antagonist, memantine (20mg/kg, i.p.), was previously found to block LTP induced by HFS in the CA1 region of the urethane anaesthetised rat (I. Klyubin, TCD, personal communication). Memantine (20mg/kg, i.p.) was injected 30 min before methoctramine and had no discernible effect on baseline (see table 3.2). Methoctramine subsequently induced a significant LTE (111.8±3.7% and 108.9±1.1% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, n=5) (Fig.3-9). The methoctramine-induced LTE in animals pre-treated with memantine was not significantly different from the LTE in the vehicle pre-treated control group ($P>0.05$ at 10 and 120 min after methoctramine compared to vehicle plus methoctramine group, unpaired t-test, n=5). The methoctramine LTE of the vehicle pre-treated methoctramine control group was significant (115.1±3.8% and 114.2±5.5% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, n=5).

3.3.3 The effect of the group I metabotropic glutamate receptor antagonist, LY367385

The dose of the group I metabotropic glutamate receptor antagonist, LY367385, used in the present study was based on the dose of LY367385 (8-32nmol, i.c.v.) found to inhibit LTP in the dentate gyrus in a previous in vivo study (Naie and Manahan-Vaughan, 2005). LY367385 (29nmol) was injected i.c.v. 30 min before methoctramine and had no discernible effect on baseline (see table 3.2). Methoctramine subsequently induced a significant LTE (132.6±4.2% and 124.5±4.6% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, n=5) (Fig. 3-10). Methoctramine LTE of the LY367385 pre-treated group was not significantly different from the methoctramine LTE of the vehicle pre-treated control group ($P>0.05$ at 10 and 120 min after methoctramine compared to the LTE of vehicle pre-treated control, unpaired t-test, n=5). Methoctramine LTE of the vehicle pre-treated methoctramine group was significant (126.4±4.8% and 129.5±2.5% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, n=5).

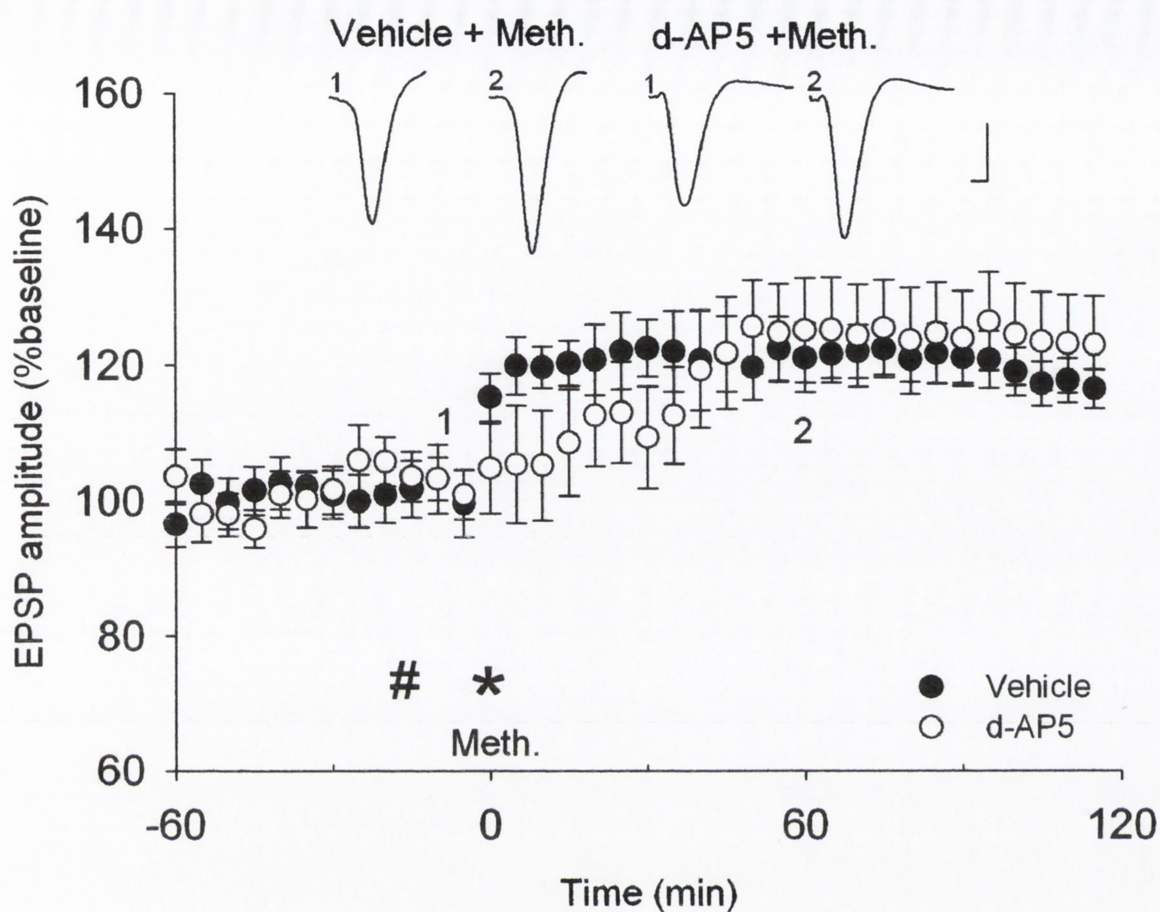

Fig. 3-8. The NMDA receptor antagonist, d-AP5, did not significantly affect LTE. D-AP5 (0.1μmol) injected i.c.v. did not significantly affect methoctramine LTE, (n=5; ○), P>0.05 compared to vehicle injected control (n=5; ●). D-AP5 /vehicle (#) was injected 15 min before methcotramine (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

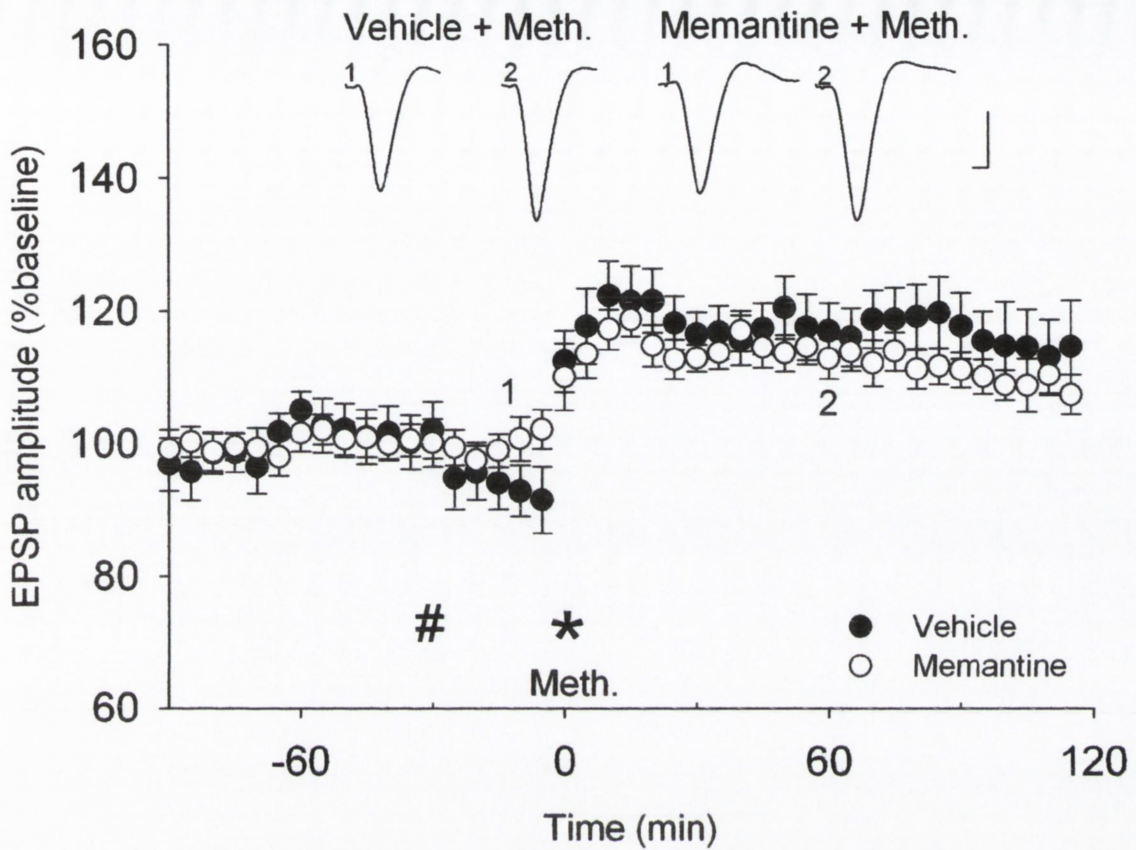

Fig. 3-9. The NMDA receptor antagonist, memantine, did not significantly affect LTE. Memantine (20mg/kg, i.p.) did not significantly affect methoctramine LTE, (n=5; o), P>0.05 compared to vehicle injected control (n=5; •). Memantine/vehicle (#) was injected 30 min before methoctramine (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

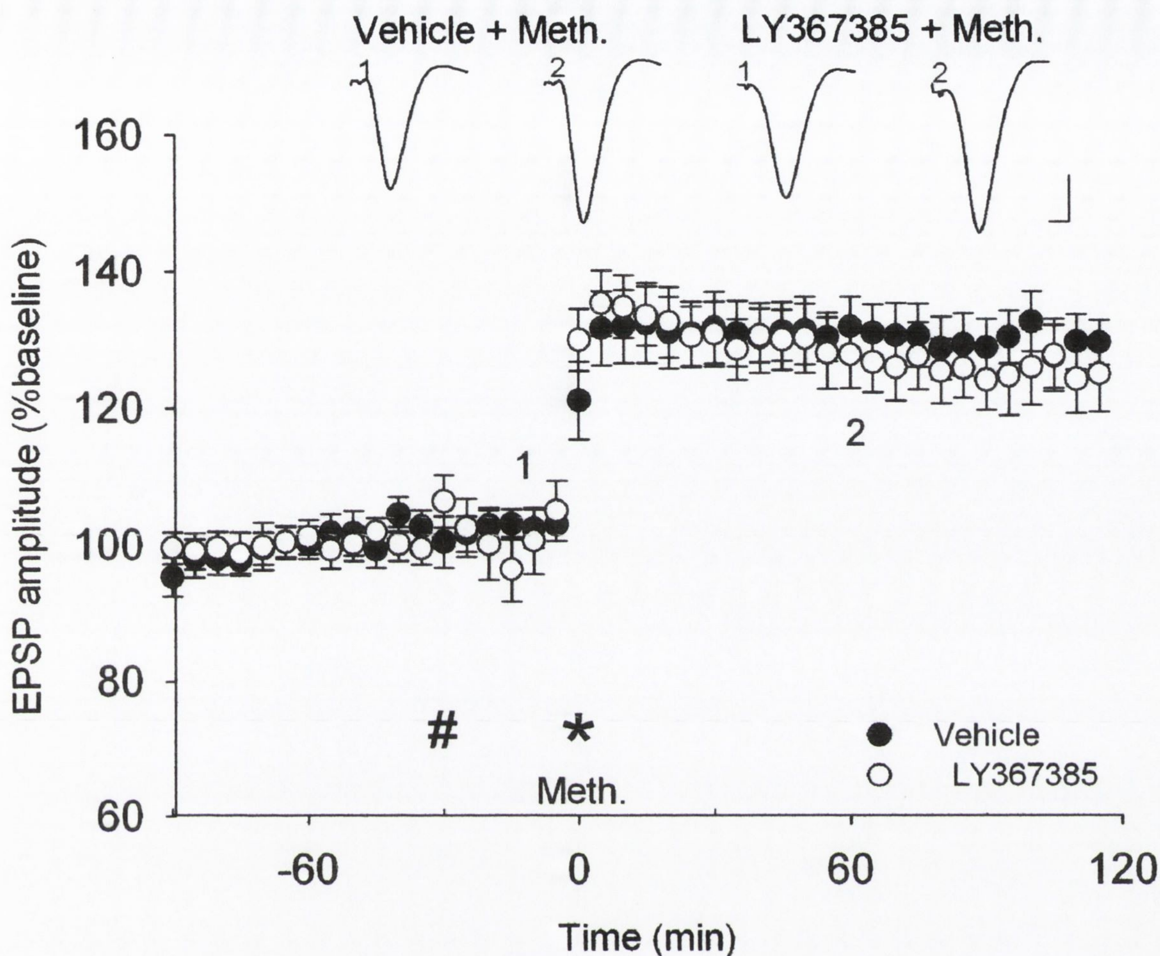

Fig. 3-10. LY367385, a Group I metabotropic receptor antagonist, did not significantly affect LTE. LY367385 (29nmol) injected i.c.v. did not significantly affect methoctramine LTE, (n=5; ○), P>0.05 compared to vehicle injected control (n=5; ●). LY367385/vehicle (#) was injected i.c.v. 30 min before methoctramine (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

3.4 The galaninergic system and methoctramine LTE

3.4.1 The effect of galanin

The doses of galanin used in these experiments were determined from previous studies on the effects of galanin in the hippocampus (Consolo et al., 1991; Consolo et al., 1998). Galanin has been reported to be rapidly cleared (5-20min) from the extracellular space after infusion into the ventral hippocampus (Schott et al., 1998), therefore in the present study galanin was injected either 5 min before methoctramine or at the same time as methoctramine. Pilot studies showed that lower doses of galanin (2.25nmol or 4.5nmol) had no discernible effect on baseline. Galanin (2.25 nmol) injected i.c.v. 5 min before methoctramine (n=3) tended to increase methoctramine LTE but the facilitation was only apparent 20 min after methoctramine (142% of pre injection baseline 20 min after methoctramine) (Fig.3-11A). Galanin (4.5 nmol) was injected i.c.v. 5 min before or co-injected with methoctramine. The subsequent methoctramine-induced LTE was significant (147.8±5.7% and 136.9%±6.8% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, n=5) (Fig.3-11B). Galanin significantly facilitated the initial enhancement induced by methoctramine compared to the vehicle pre-treated methoctramine control group initially but not at later stages ($P<0.05$ and $P>0.05$ at 10 and 120 min after methoctramine compared to the vehicle pre-treated methoctramine control group, respectively, unpaired t-test, n=5). The methoctramine LTE of the vehicle pre-treated methoctramine control group was significant (126.8±4.7% and 126.8±3.5% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, n=5). Galanin (4.5 nmol) i.c.v. applied 5 min after methoctramine (n=2) tended to have no effect on the expression of methoctramine induced LTE (129.7% of pre injection baseline 10 min after galanin and no further enhancement was observed). Higher doses of galanin (6-45nmol) caused a decrease in

baseline (approximately 15%), however, the level of methoctramine LTE appeared to be facilitated ($n=5$).

3.4.2 The effect of the galanin receptor antagonist, M35

M35 is a chimeric peptide [galanin (1-13)-bradykinin (2-9) amide]) (Bartfai et al., 1992) and has equal affinity for Gal-receptor1 and Gal-receptor2 (Fathi et al., 1997). M35 (1nmol) delivered to the ventral hippocampus was previously shown to block the learning impairment caused by galanin (3nmol) delivered to the ventral hippocampus (Schott et al., 2000). The galanin receptor antagonist, M35 (4.5nmol), was injected i.c.v. 5 min before methoctramine. The enhancement was not significant at the initial stage and borderline significant at the end of the recording period ($112 \pm 4.1\%$ and $108.4 \pm 4.2\%$ at 10 and 120 min after methoctramine, respectively; $P>0.05$ and $P=0.05$ at 10 and 120 min after methoctramine compared to pre-injection baseline, respectively, paired t-test, $n=5$) (Fig.3-12). The methoctramine-induced enhancement in animals pre-treated with M35 was significantly different from the LTE in the vehicle pre-treated control group ($P<0.05$ at 10 and 120 min after methoctramine compared to the vehicle pre-treated methoctramine group, unpaired t-test, $n=5$). The methoctramine LTE of the vehicle pre-treated methoctramine control group was significant ($132.8 \pm 4.2\%$ and $130.7 \pm 6.3\%$ of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, $n=5$).

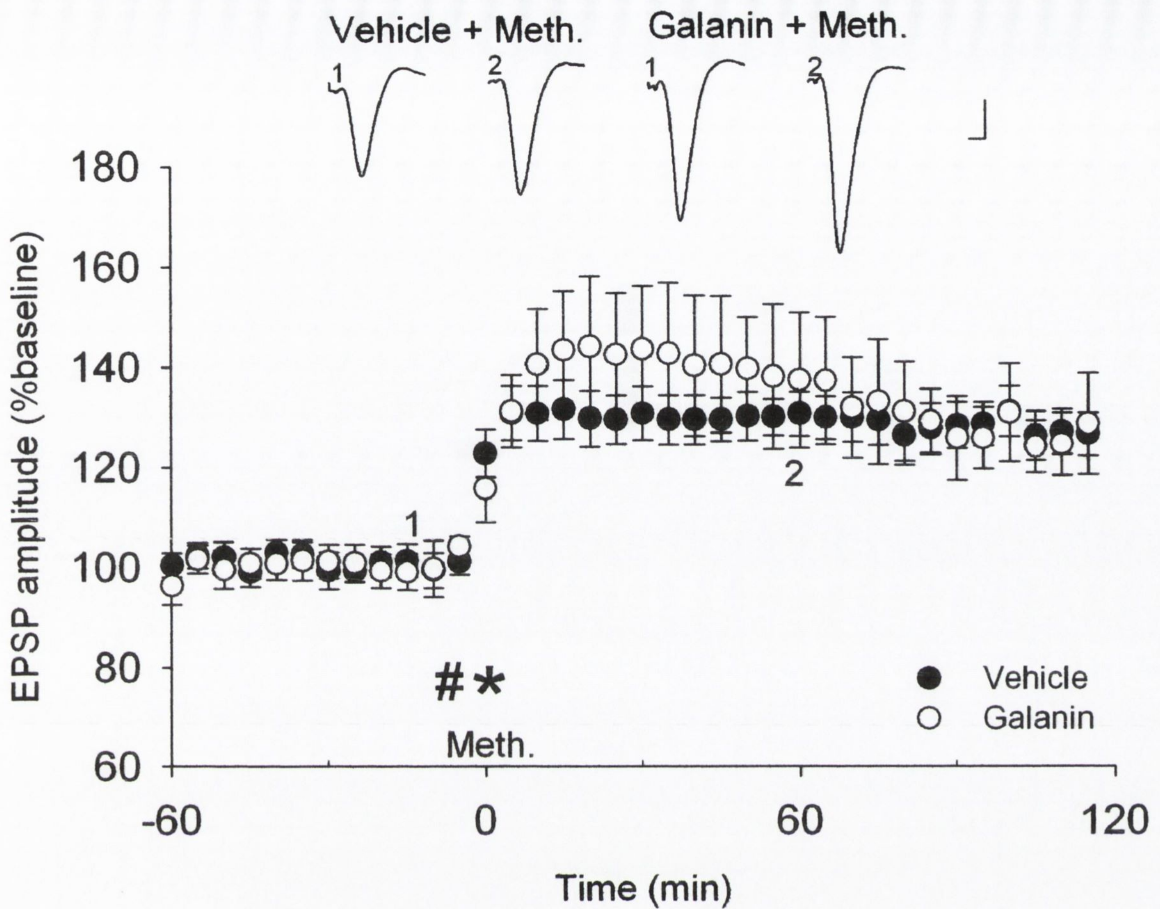

Fig. 3-11A. The neuropeptide galanin (2.25nmol) tended to increase the initial LTE. Galanin (2.25nmol) injected i.c.v. tended to increase the initial enhancement induced by methoctramine (n=3; ○) compared to the vehicle injected control (n=5; ●). Galanin/vehicle (#) was injected 5 min before methoctramine (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

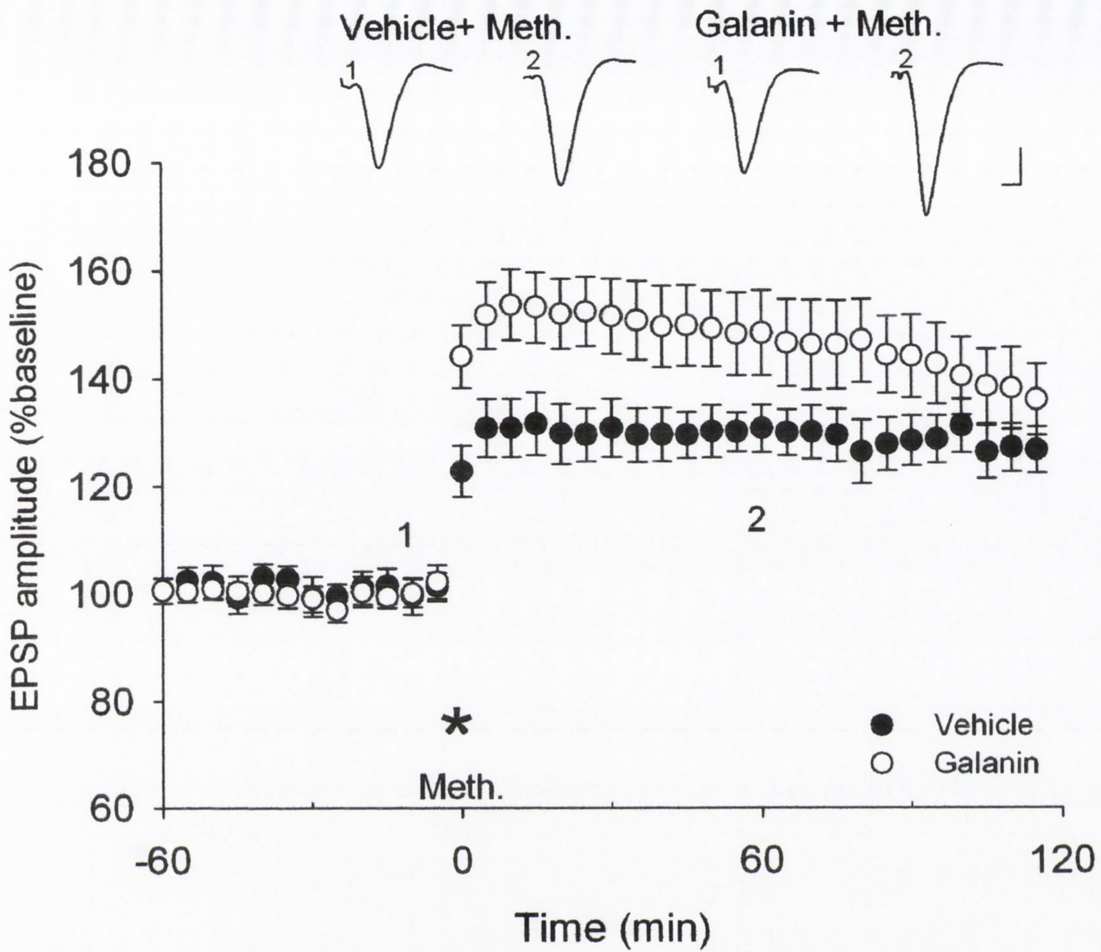

Fig. 3-11B. The neuropeptide galanin (4.5nmol) significantly increased the initial enhancement but did not significantly affect the later stage of the LTE. Galanin (4.5nmol) injected i.c.v. significantly increased the initial enhancement induced by methoctramine (n=5; ○) $P<0.05$ and $P>0.05$ at 10 and 120 min after methoctramine compared to the vehicle injected control (n=5; ●). Galanin/vehicle (#) was injected 5 min before or with methoctramine (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

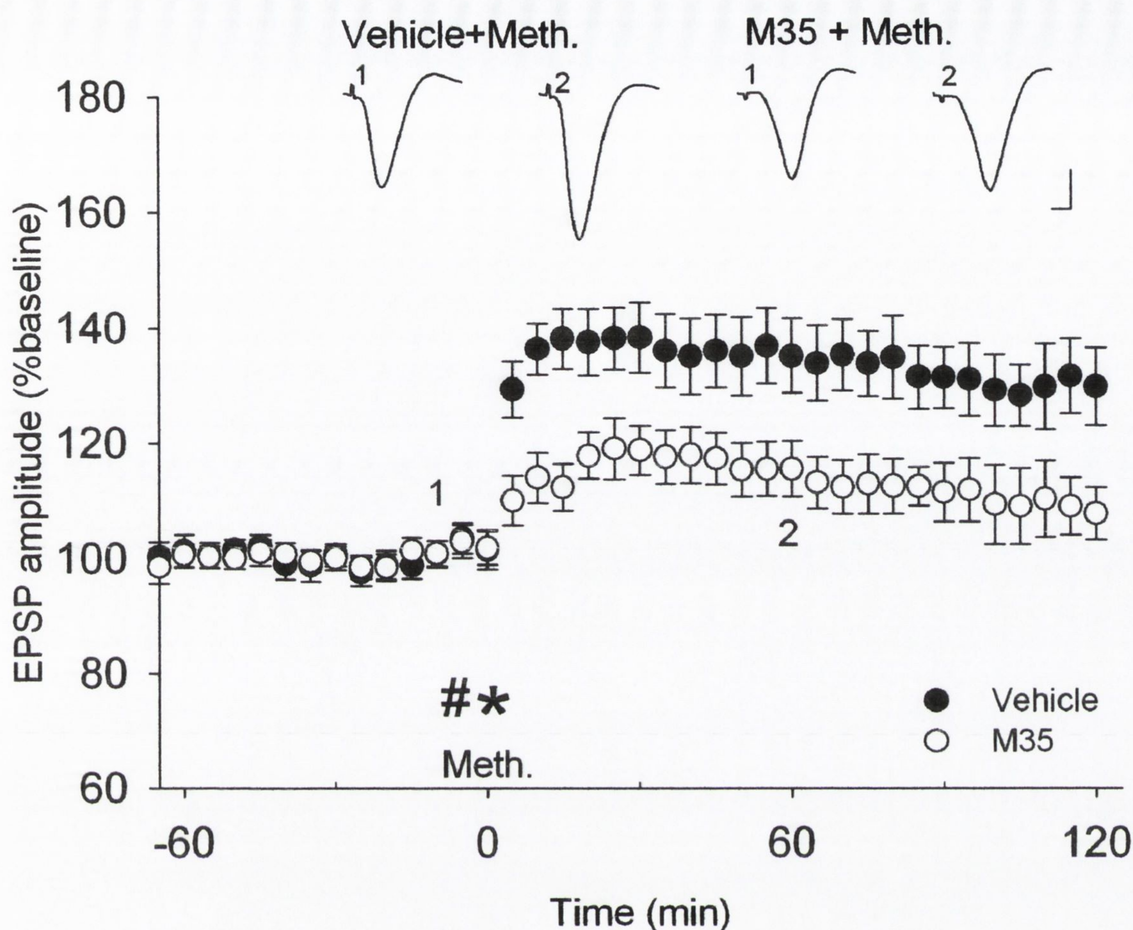

Fig. 3-12. M35, a galanin receptor antagonist, strongly reduced LTE. M35 (4.5nmol) injected i.c.v. significantly diminished methoctramine LTE, (n=5; ○), $P<0.05$ compared to the vehicle injected control (n=5; ●). M35/vehicle (#) was injected 5 min before methoctramine (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

3.5 The effect of kinase inhibitors on methoctramine LTE

3.5.1 The effect of the PKA inhibitor, Rp-cAMPs, on the induction and maintenance-expression of methoctramine LTE

A previous study found that Rp-cAMPs, a selective and potent PKA inhibitor, injected (0.2 μmol) i.c.v. blocked methoctramine LTE (Li, 2002). In the present study Rp-cAMPS (0.2-0.43 μmol) was injected i.c.v. 30 min before methoctramine and did not have a statistically significant effect on baseline synaptic transmission (see table 3.2). Rp-cAMPs blocked the methoctramine-induced LTE (105.4±6.3% and 101.6±9.3% of baseline at 10 and 120 min after methoctramine, respectively; $P>0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, n=5, paired t-test) (Fig.3-13). The methoctramine-induced LTE in animals pre-treated with Rp-cAMPs was significantly different from the LTE in the vehicle pre-treated control group at 120 min but not at 10 min ($P>0.05$ and $P<0.05$ at 10 and 120 min after methoctramine compared to the vehicle pre-treated methoctramine control group, respectively, unpaired t-test, n=5). The methoctramine LTE of the vehicle pre-treated methoctramine control group was significant (119±1.8% and 125±2% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared with pre-methoctramine baseline, paired t-test, n=5).

Rp-cAMPs was applied after the induction of methoctramine LTE to investigate if PKA activity was required for the maintenance-expression of methoctramine LTE. Rp-cAMPS (0.2μmol) injected i.c.v. 30 min after methoctramine transiently (for approximately 40min) reduced methoctramine LTE, the peak inhibition occurring at 20 min after Rp-cAMPs. Thus the reduction was statistically significant at 20 but not at 90min after Rp-cAMPs (113±4.5% and 118.7±4% of baseline at 20 and 90 min after the application of Rp-cAMPS; $P<0.05$ at 20 min and $P>0.05$ and 90 min after Rp-cAMPS compared to pre-Rp-cAMPS baseline, respectively, paired t-test, n=5) (Fig.3-14). Moreover, the methoctramine-induced LTE in the group treated with Rp-cAMPS after

methoctramine was significantly different from the LTE in the vehicle treated group at 20 min but not at 90 min after Rp-cAMPs ($P<0.05$ at 20 min after Rp-cAMPs and $P>0.05$ at 90 min after Rp-cAMPS compared to the vehicle treated group, unpaired t-test, n=5). The methoctramine LTE of the vehicle treated group did not significantly change after vehicle injection (128.3±5.5% and 120.4±4.9% of baseline at 20 and 90 min after vehicle injection; $P>0.05$ at 20 and 90 min after vehicle compared to pre-vehicle baseline, paired t-test, n=5). The methoctramine LTE was significant in both the vehicle and Rp-cAMPS treated groups ($P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, respectively, paired t-test n=5).

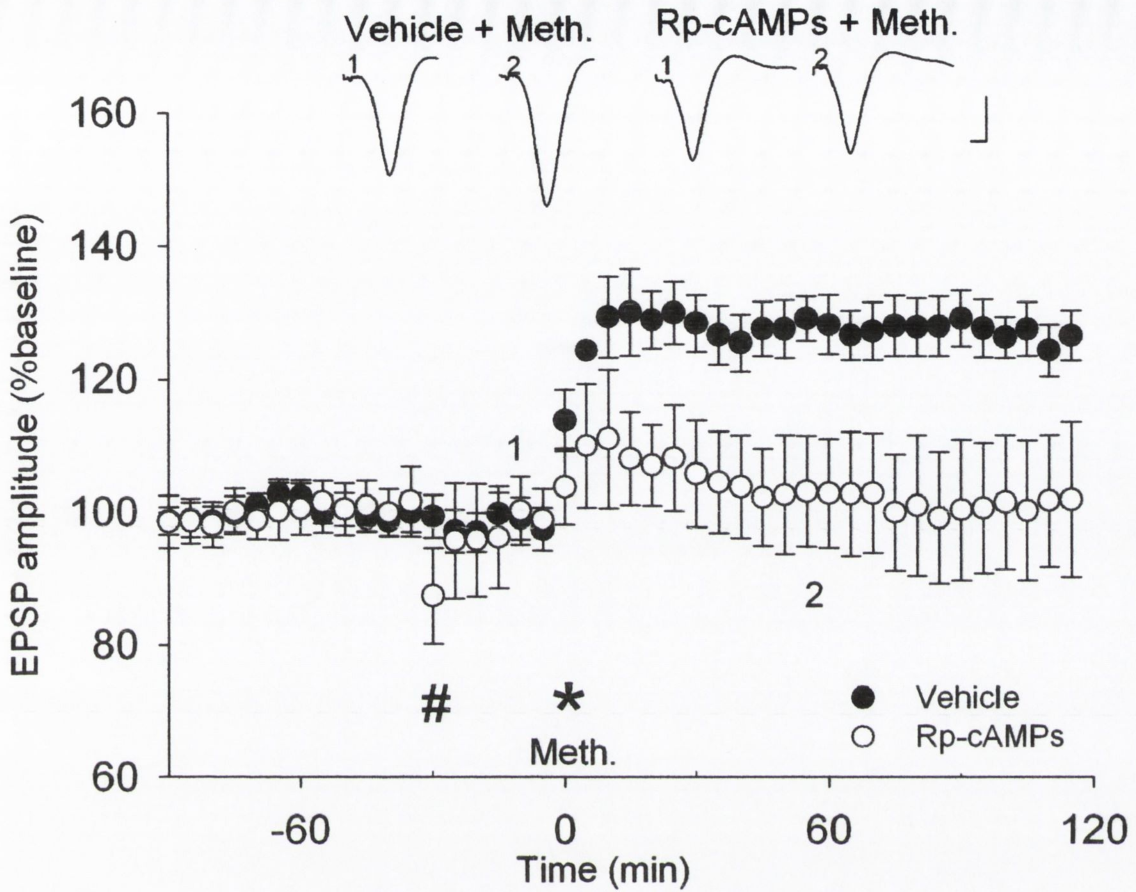

Fig. 3-13. Rp-cAMPs, an inhibitor of PKA, blocked the induction of the LTE. Rp-cAMPs (0.2-0.43µmol) injected i.c.v. significantly inhibited methoctramine-induced LTE, (n=5; ○), P<0.05 compared to the vehicle injected control (n=5; ●). Rp-cAMPs/vehicle (#) was injected 30 min before methoctramine (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

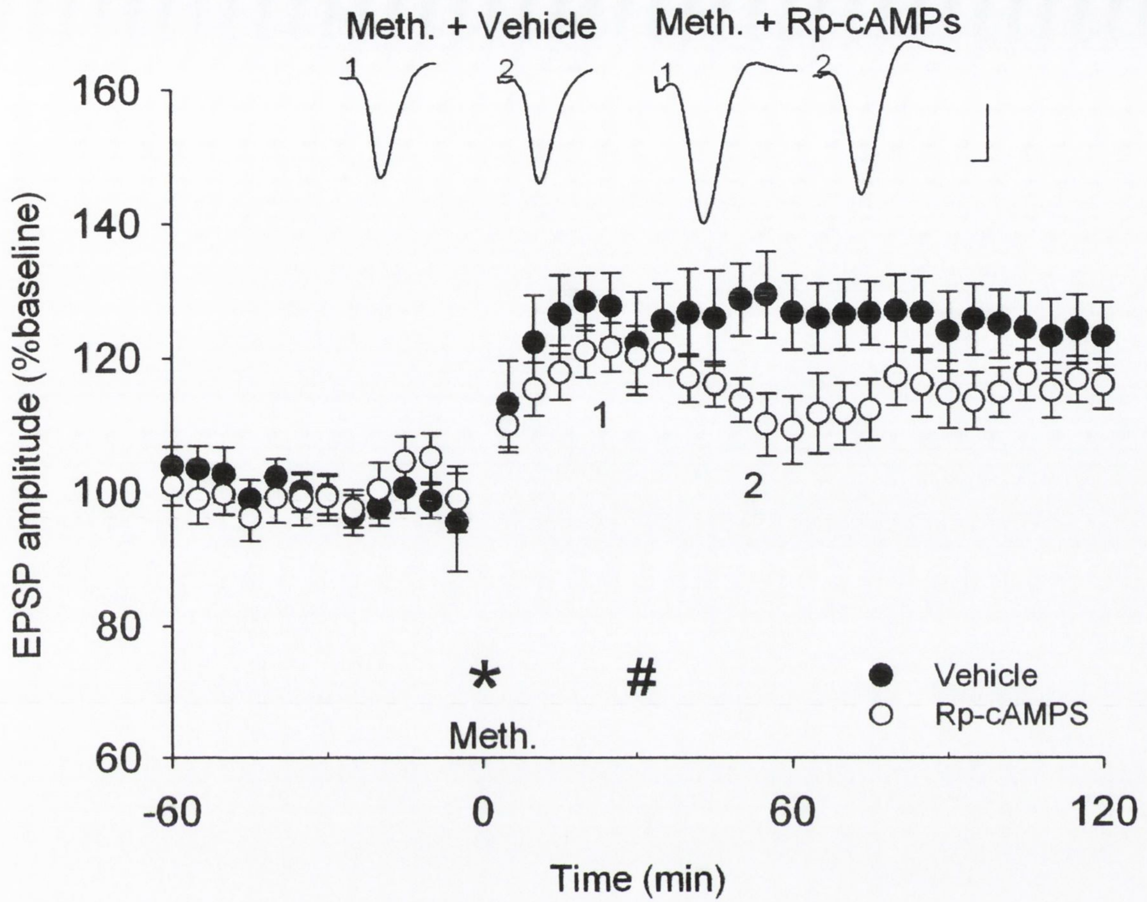

Fig. 3-14. Rp-cAMPS, an inhibitor of PKA, transiently reversed the LTE. RP-cAMPs (0.2µmol) injected i.c.v. transiently reversed methoctramine-induced LTE, (n=5; ○), $P<0.05$ and $P>0.05$ at 20 and 90 min after Rp-cAMPs, respectively, compared to the vehicle injected control, (n=5; ●). Rp-cAMPS/vehicle (#) was injected 30 min after methoctramine (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

3.5.2 The effect of the PKC inhibitor, Bisindolylmaleimide I (BIM), on the induction of methoctramine LTE

Bisindolylmaleimide I (BIM) has been characterised as a selective and potent PKC inhibitor (Toullec et al., 1991). In a previous in vivo study the PKC inhibitor, BIM (89nmol, i.c.v.) blocked methoctramine LTE (Li, 2002). The PKC inhibitor, BIM (89nmol), was injected i.c.v. 30 min before methoctramine and had no discernible effect on baseline (see table 3.2). Methoctramine subsequently induced a significant LTE although the enhancement in the first 10 min was not statistically significant (110.9±5.4% and 113.2±3.8% of baseline at 10 and 120 min after methoctramine, respectively; $P>0.05$ and $P< 0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, respectively, paired t-test, n=5) (Fig.3-15). The methoctramine-induced LTE in animals pre-treated with BIM was not significantly different from the LTE in the vehicle pre-treated control group ($P>0.05$ at 10 and 120 min after methoctramine compared to the vehicle pre-treated methoctramine group, unpaired t-test, n=5). However, from the graph (Fig. 3-15) it is apparent that BIM reduced methoctramine LTE for most of the recording period. For example, the reduction was statistically significant at 60 min after methoctramine (see table 3.2). The methoctramine LTE of the vehicle pre-treated methoctramine control group was significant (121.6±3.2% and 124.9±3.5% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, n=5). The effect of a higher dose of BIM on methoctramine LTE was not evaluated as BIM is not water soluble at higher concentrations.

3.5.3 The effect of the PKC/Mzeta pseudosubstrate myristoylated peptide inhibitor (ZIP), on the induction and maintenance-expression of methoctramine LTE

PKMzeta has been identified as the constitutively active catalytic domain of PKCzeta (Sacktor et al., 1993). Staurosporine is not an effective inhibitor of PKC/Mzeta (Kochs et al., 1993; Ling et al., 2002; McGlynn et al., 1992). Since BIM is an analogue of staurosporine (Toullec et al., 1991) BIM may not inhibit PKC/Mzeta. In contrast, the PKCzeta pseudosubstrate peptide inhibitor (ZIP) has been reported to inhibit PKMzeta (Ling et al., 2002) and therefore is referred to as a PKC/Mzeta inhibitor in the present study. The dose of the ZIP used in the present study was chosen on the basis of a previous study in the dentate gyrus in vivo where ZIP (10nmol, i.h.) reversed the maintenance of LTP (Pastalkova et al., 2006). ZIP (1.75nmol/7μl) had no discernible effect on baseline (see table 3.2). ZIP (1.75nmol/7μl) injected i.c.v. 30 min before methoctramine blocked the methoctramine-induced LTE (103.9±3.4% and 108.4±4.7% of baseline at 10 and 120 min after methoctramine, respectively; $P>0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, n=5) (Fig.3-16). The animals pre-treated with ZIP were also significantly different from the vehicle pre-treated control group ($P<0.05$ at 10 and 120 min after methoctramine compared to the vehicle pre-treated methoctramine group, unpaired t-test, n=5). The methoctramine LTE of the vehicle pre-treated methoctramine control group was significant (118.8±1.7% and 125.3±2.8% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, n=5).

ZIP was previously shown to reverse the maintenance-expression of LTP in the dentate gyrus in vivo (Pastalkova et al., 2006) and in the CA1 in vitro (Ling et al., 2002; Serrano et al., 2005). Therefore, the effect of ZIP on the maintenance-expression of methoctramine LTE was investigated. ZIP (1.75nmol/7μl) was injected i.c.v. 30 min after methoctramine. Methoctramine LTE did not change significantly after the application of the ZIP (123.4±3.5% of baseline at 90 min after the application of ZIP;

P>0.05 compared to pre-ZIP baseline, paired t-test, n=5) (Fig. 3-17). Moreover the methoctramine-induced LTE in the group treated with ZIP was not significantly different from the LTE in the vehicle treated group (P>0.05 of baseline at 90 min after ZIP compared to the vehicle treated group, unpaired t-test, n=5). The methoctramine LTE was significant both in the vehicle and ZIP treated groups (P<0.05 at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, respectively, paired t-test, n=5) (Fig.3-14). Pilot studies indicated that the ZIP (5nmol/5μl) or (2.5nmol/5μl) injected i.c.v. tended to increase synaptic transmission (10-15%, n=2). Therefore, the effects of higher doses of ZIP on methoctramine LTE were not tested.

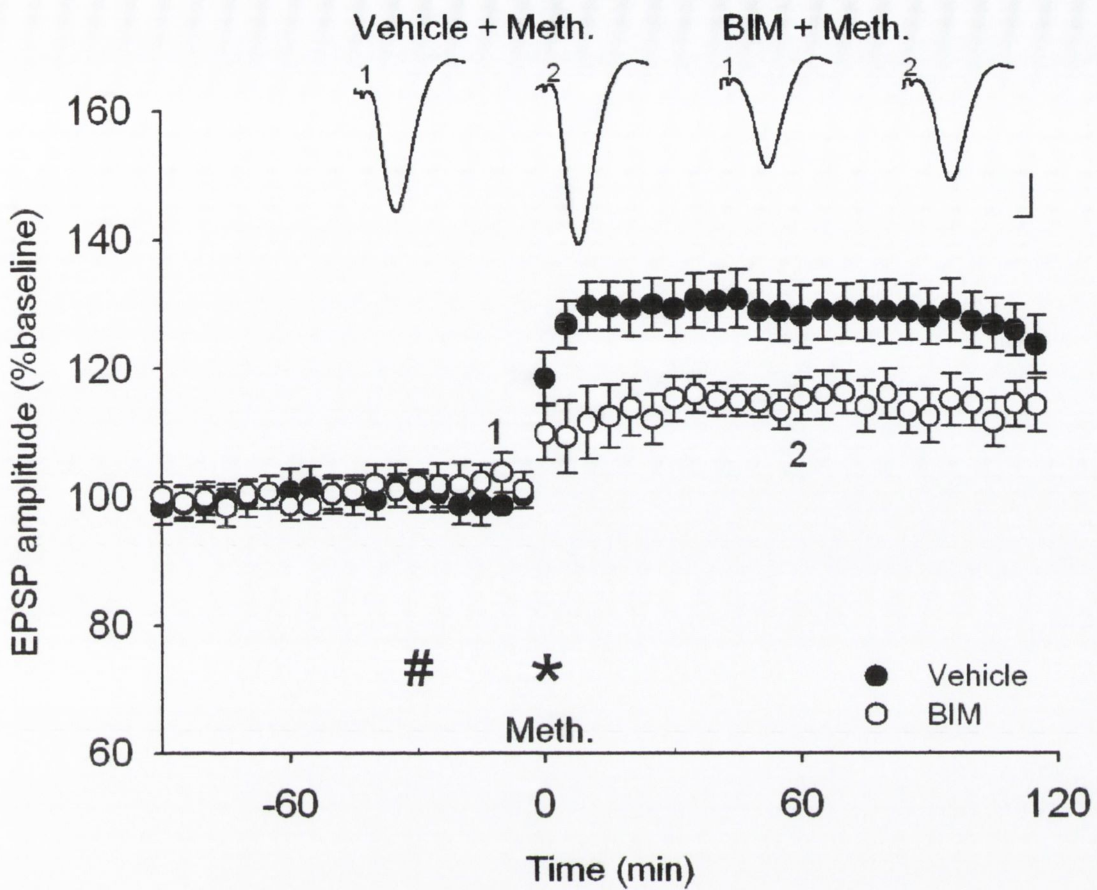

Fig. 3-15. BIM, an inhibitor of PKC, tended to reduce LTE.

BIM (89nmol) injected i.c.v. decreased methoctramine LTE, however this decrease was not significant at 2 hrs, (n=5; o), P>0.05 compared to the vehicle injected control (n=5; •). BIM/vehicle (#) was injected 30 min before methoctramine (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

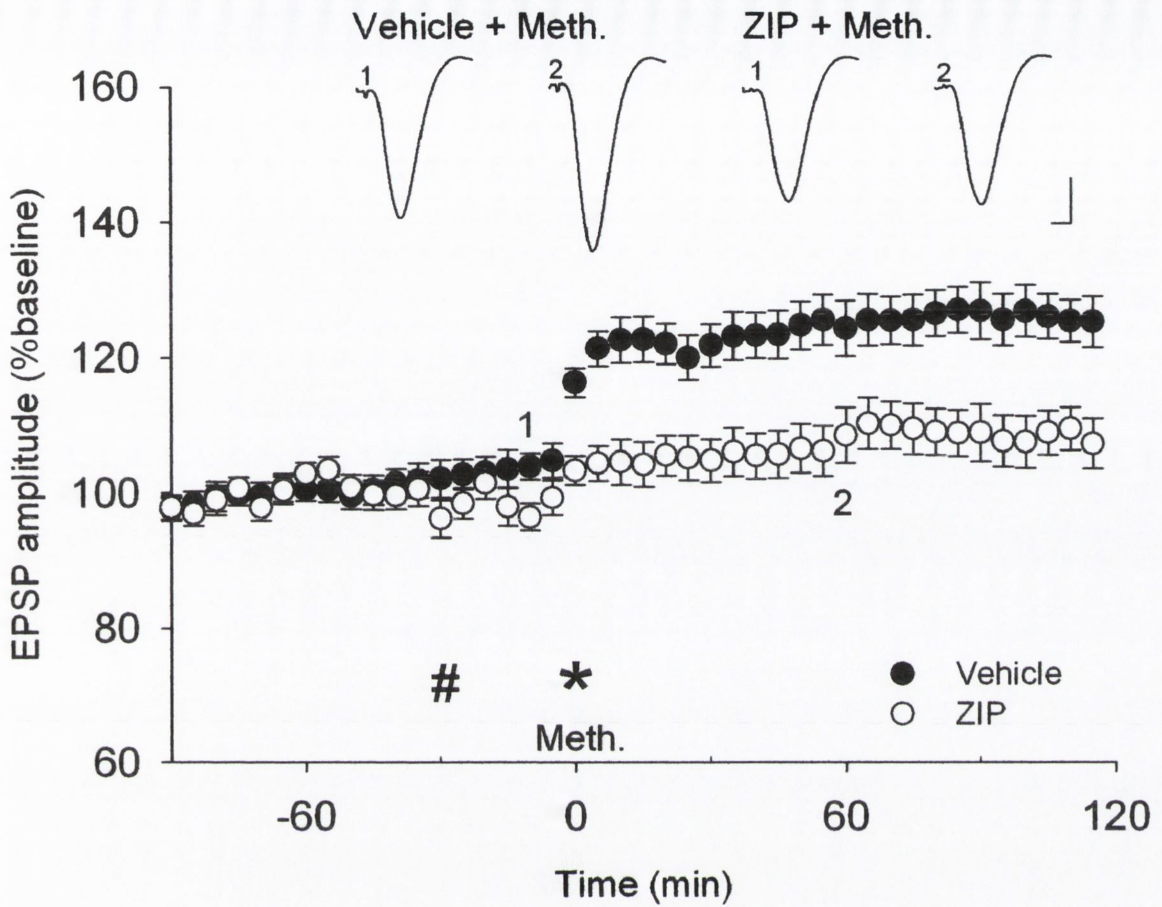

Fig. 3-16. ZIP, an inhibitor of PKC/Mzeta, inhibited LTE induction.

ZIP (1.75nmol) injected i.c.v. significantly inhibited methoctramine LTE (n=5; ○) P<0.05 compared to the vehicle injected control (n=5; ●). ZIP/vehicle (#) was injected 30 min before methoctramine (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

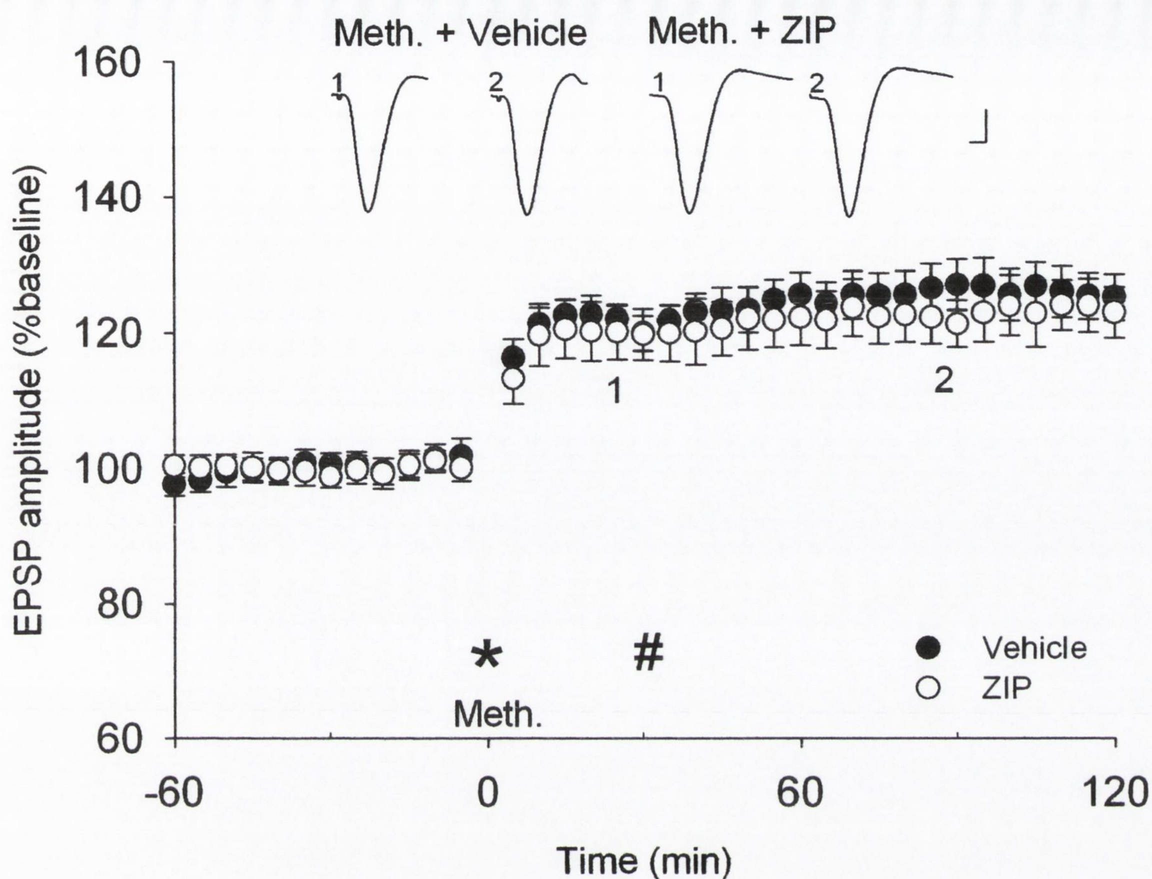

Fig. 3-17. ZIP, an inhibitor of PKC/Mzeta, did not reverse LTE. ZIP (1.75nmol) injected i.c.v. did not significantly affect methoctramine LTE, (n=5; ○), P>0.05 compared to the vehicle injected control (n=5; ●). ZIP/vehicle (#) was injected 30 min after methoctramine (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

3.6 The effect of amyloid β on methoctramine LTE

A dose of Aβ (15pmol) that was previously found to block HFS induced LTP in the CA1 region of the region of the urethane anaesthetised rat (I. Klyubin personal communication) was studied. Aβ (15pmol) was injected i.c.v. 30 min before methoctramine and had no discernible effect on baseline (see table 3.2). Methoctramine subsequently induced a significant LTE (115.4±4.3% and 132.1± 9.9% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, n=5) (Fig. 3-18). Methoctramine LTE of the Aβ pre-treated group was not significantly different from the methoctramine LTE of the vehicle pre-treated control group ($P>0.05$ at 10 and 120 min after methoctramine compared to LTE in the vehicle pre-treated control group, unpaired t-test, n=5). The methoctramine LTE of the vehicle pre-treated methoctramine control group was significant (116.4±5% and 120.3±3.3% of baseline at 10 and 120 min after methoctramine, respectively; $P<0.05$ at 10 and 120 min after methoctramine compared to pre-methoctramine baseline, paired t-test, n=5).

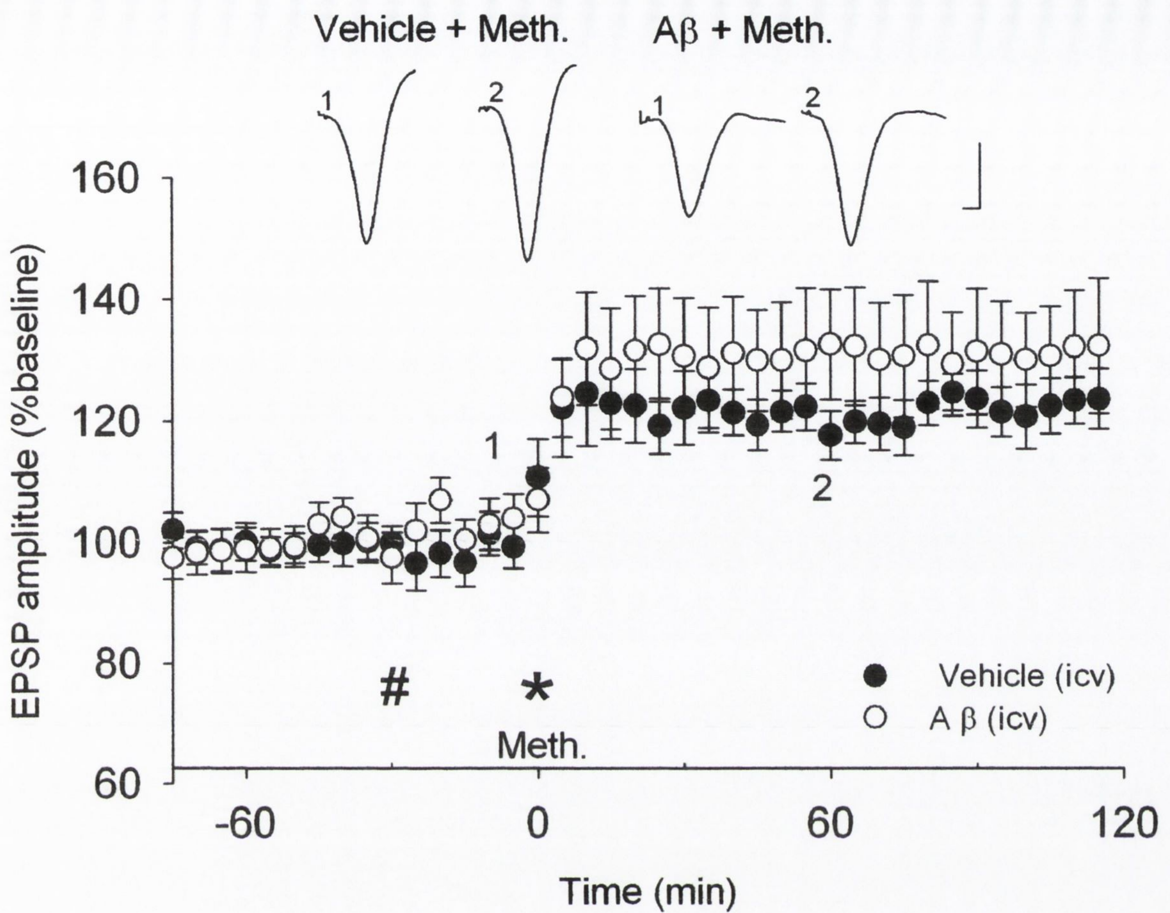

Fig. 3-18. Aβ did not significantly affect LTE.

Aβ (15pmol) did not significantly affect methoctramine LTE, (n=5; ○), P>0.05 compared to vehicle injected control (n=5; ●). Aβ/vehicle (#) was injected i.c.v. 30 min before methoctramine (*). Insets show traces of the field EPSPs recorded at times indicated. Horizontal bar, 5ms; vertical bar, 1.0mV.

Table 3-1 The effect of different M_2 mACh receptor antagonists on synaptic transmission

Drug	Dose	n	Pre-Drug	Post-drug 10min	60min	120min
Methoctramine	34nmol	5	101.5	128.5	131.1	128.6
			±2	±3.4*#	±3.5*#	±3.33*#
Gallamine	25nmol	4	99.2	98.6	115.7	112.2
			±2.3	±1.2*	±3.9*#	±2.6*#
Gallamine	42/56nmol	5	102.3	96.3	120	125.3
			±4.1	±5.7	±7.9	±8.5*#
BIBN-99	22/42nmol	5	100.7	101.4	100.8	98.6
			±3.5	±4.1	±5.8	±7.7

Values are mean ± S.E.M.

* $P<0.05$ compared to interleaved vehicle treated animals (see results) (unpaired t-test)

# $P<0.05$ compared to pre-drug baseline (paired t-test)

Table 3-2 The effect of different drugs on methoctramine LTE

Drug	Dose	n	Pre-Drug	Pre-Meth.	Post-drug 10min	Post-drug 60min	Post-drug 120min
Mecamylamine	3mg/kg	5	102.5± 1.43	100.2 ±2.2	122.3 ±8.8	137.6 ±8#	134.6 ±8.6#
Methyllycaconitine	50/65nmol	5	100.9± 0.3	101± 1.1	107.7 ±3.1*	118.2 ±7.1#	115 ±3.6#
Mecamylamine + Methyllycaconitine	3mg/kg 65nmol	5	100.1± 0.43	102.3± 1.22	109.8 ±3.8	120.5 ±3#	116.3 ±4.8#
Nicotine	3mg/kg	5	101.4 ±1.2	103 ±2.1	131.5 ±4.5#	138.4 ±4.1#	137.1 ±4#
d-AP5	0.1μmol	5	104.2 ±2.8	103.2 ±2.2	114 ±7.2	124.6 ±6.6#	121.5 ±6.7#
Memantine	20mg/kg	5	101.3 ±1.4	101.4± 1.43	111.8 ±3.7#	114.1 ±1.25#	108.9 ±1.1#
LY367385	29nmol	5	99.9 ±1	102.8 ±3.9	132.6 ±4.3#	129.1 ±4.5#	124.5 ±4.6#
Galanin	4.5nmol	5	N/A	N/A	147.8 ±5.7*#	148.6 ±7.1#	136.9 ±6.8#
M35	4.5nmol	5	N/A	N/A	112 ±4.1*	115.5 ±4.4*#	108.4 ±4.2*#
Rp-cAMPs	0.2/0.43 μmol	5	101.1 ±1.7	98.5 ±3.7	105.4 ±6.3	102.7 ±7.31*	101.6 ±9.3*
BIM	89nmol	5	101.5 ±1.9	102.4 ±2.5	110.9 ±5.4	114.2 ±2.7*#	113.2 ±3.8#
ZIP	1.75nmol	5	100.1 ±1.3	97.9 ±3	103.9 ±3.4*	106.4 ±5.4*	108.3 ±4.7*
Aβ	15pmol	5	102.4 ±1.4	103.5 ±1.8	115.4 ±4.3#	130.7 ±9.6#	132.1 ±10#

Values are mean ± S.E.M.

* P<0.05 compared to interleaved methoctramine treated animals (see results) (unpaired t-test)

# P<0.05 compared to pre-methoctramine baseline (paired t-test)

Comparison of pre drug baseline with pre methoctramine baseline was not significant for all groups.

IV. Discussion

4.0 Discussion

In the present study an M_2 mACh receptor antagonist, methoctramine, induced a rapid and persistent (>2hrs) enhancement of synaptic transmission in the CA1 region of the intact hippocampus (methoctramine LTE). Consistent with this, another M_2 preferring mACh receptor antagonist, gallamine, also enhanced synaptic transmission. In contrast BIBN-99, which is also an M_2 mACh receptor antagonist failed to affect synaptic transmission, indicating that not all M_2 receptor antagonists share the ability to induce an LTE.

The role of a variety of cholinergic and glutamatergic receptors in methoctramine LTE was further investigated. A broad spectrum nACh receptor antagonist, mecamylamine, did not significantly affect methoctramine LTE. However, an α7 nACh receptor antagonist, methyllycaconitine, appeared to delay the onset of methoctramine-induced LTE but did not block methoctramine LTE. These results suggest that nACh receptor activation is not necessary for methoctramine LTE. Pre-treatment with the competitive NMDA receptor antagonist d-AP5 or the non-competitive antagonist memantine did not block methoctramine-induced LTE, which suggests that methoctramine LTE is NMDA receptor-independent. The group I mGlu receptor antagonist, LY367385, at a dose that blocks mGlu1 receptors did not block methoctramine LTE, which suggests that methoctramine LTE is mGlu1 receptor-independent.

The role of the galaninergic sytem in methoctramine LTE was also assessed. The application of exogenous galanin increased the initial enhancement induced by methoctramine. Pre-treatment with the galanin receptor antagonist M35 strongly reduced methoctramine LTE, which suggests that methoctramine LTE requires the action of endogenous galanin at its receptors. The role of PKC and PKA in methoctramine LTE was also studied. Pre-treatment with the PKC/Mzeta pseudosubstrate inhibitor (ZIP) blocked methoctramine-induced LTE. This suggests that methoctramine-induced LTE involves the activation of the atypical PKC/Mzeta isoform.

Pre-treatment with the PKA inhibitor Rp-cAMPs blocked methoctramine LTE, which implies that the induction of methoctramine LTE is PKA dependent. In contrast, the maintenance of methoctramine LTE appears to be independent of these kinases as application of the PKA inhibitor or PKC/Mzeta inhibitor 30 min after the induction of methoctramine LTE did not persistently reverse LTE. Finally pre-treatment with Aβ at a dose that blocks HFS induced LTP did not affect methoctramine LTE, which suggests that methoctramine LTE is insensitive to inhibition by Aβ.

4.1 M_2 mACh receptor antagonists and the induction of LTE

4.1.1 The effects of different M_2 mACh receptor antagonists on synaptic transmission in the CA1 region of the hippocampus

In the present study methoctramine, an M_2 mACh receptor antagonist, induced a rapid (generally <8 min onset) and long-term (>2hrs) enhancement of synaptic transmission in the CA1 region of the intact hippocampus, which was referred to as methoctramine LTE. Gallamine, an M_2 preferring mACh receptor antagonist, also enhanced synaptic transmission. However, the onset of the gallamine-induced enhancement was slower (20-30min) compared to methoctramine (8min). In contrast, BIBN-99, which is also an M_2 mACh receptor antagonist failed to affect synaptic transmission

The ability of methoctramine to induce an LTE is consistent with a previous study, which demonstrated that methoctramine delivered i.c.v. persistently enhanced synaptic transmission in vivo (Li, 2002). The methoctramine LTE was proposed to involve inhibition of M_2 receptors as methoctramine was previously shown to preferentially bind to the M_2 mACh receptor (Caulfield, 1993; Doods et al., 1993c; Dorje et al., 1991; Waelbroeck et al., 1990) and was mimicked by another M_2 mACh receptor antagonist AF-DX 116 (Li, 2002).

The neuromuscular blocker gallamine is usually referred to as a mixed mACh and nACh receptor antagonist, which preferentially binds to M_2 mACh receptors (Burke, 1986; Price et al., 1986; Tucek and Proska, 1995). Gallamine delivered i.c.v. induced an LTE of synaptic transmission, which supports the hypothesis that methoctramine LTE is mediated by antagonism of M_2 mACh receptors. Gallamine was previously reported to induce a rapid and significant increase in synaptic transmission in vitro, which appeared to be M_2 mACh specific as it was absent in M_2 knock-out mice (Seeger et al., 2004). The ability of gallamine applied to the bath to induce a rapid increase in synaptic transmission suggests that the slow-onset of the gallamine-induced LTE in the present

study is a result of delayed diffusion of gallamine from the lateral ventricle. Indeed, the highly polar character of gallamine (Bowan and Rand, 1970) is likely to delay the diffusion of gallamine from the lateral ventricle, which could account for the slow-onset (20-30min) of the gallamine-induced LTE relative to the fast onset (8min) of the methoctramine-induced LTE. In addition, the molecular weight of methoctramine is approximately one fifth that of gallamine, which may also contribute to the slow onset of gallamine-induced LTE. The slow-onset of the gallamine-induced LTE relative to the fast onset of the methoctramine-induced LTE may also be explained by the finding that these two compounds have different mechanisms of inhibition, since methoctramine is a competitive inhibitor (Rosini et al., 1999), whereas gallamine is believed to be both a competitive and allosteric inhibitor (Burke, 1986; Tucek and Proska, 1995).

In the present study BIBN-99 failed to affect synaptic transmission and the effect of a range of doses was tested (22nmol-66nmol). BIBN-99 is a high affinity competitive M_2 mACh receptor antagonist (Doods et al., 1993a; Doods et al., 1993b), which would be expected to diffuse readily from the lateral ventricle as it is a lipophilic compound (Doods et al., 1993a; Doods et al., 1993b). The effect of BIBN-99 on baseline synaptic transmission has not previously been reported, however BIBN-99 ameliorates the deficit in theta burst LTP in aged impaired rats but does not enhance the level of LTP induced by theta burst in young rats (Tombaugh et al., 2002). In the present study the lack of effect of BIBN-99 on synaptic transmission compared to the effect of methoctramine and gallamine is surprising and indicates that not all M_2 mACh receptor antagonists share the ability to induce an LTE. Overall, the induction of an LTE by methoctramine and gallamine in the present study and by AF-DX 116 in a previous study (Li, 2002) strongly supports the hypothesis that M_2 mACh receptor antagonism is responsible for their ability to induce an LTE.

4.1.2 Putative mechanisms underlying M_2 mACh receptor antagonist-induced LTE

The present study found that M_2 antagonists, methoctramine and gallamine, are capable of inducing a long-term enhancement of synaptic plasticity. Presynaptic M_2 receptors suppress somatic calcium currents and thus inhibit release, therefore it is possible that an increase in transmitter release is required for the induction of methoctramine LTE (Krnjevic, 2004). Since the M_2 mACh receptor can act as an auto-receptor and heteroreceptor the modulation of ACh, GABA and glutamate release along with their respective receptors may be involved in methoctramine LTE (Freund, 2003; Hajos et al., 1998; Marchi and Raiteri, 1989; Nikbakht and Stone, 1999; Quirion et al., 1995; Rouse et al., 2000; Stillman et al., 1996). However, it appears that there are at least two other distinct functions of M_2 receptors located postsynaptically in the hippocampus. Thus, blockade of postsynaptic M_2 receptors that are positively coupled to an inwardly rectifying K^+ current could contribute to the LTE induced by M_2 mACh receptor antagonists as the activation of these postsynaptic M_2 receptors is associated with inhibition of glutamatergic transmission in the CA1 (Seeger and Alzheimer, 2001). In contrast, the activation of postsynaptic M_2 receptors may also block an outward K^+ current, and thus increase the excitability of the postsynaptic cell (Dutar and Nicoll, 1988; Muller and Misgeld, 1986). However, inhibition of this particular pool of postsynatic M_2 mACh receptors would be expected to oppose the induction of an LTE by an M_2 mACh receptor antagonist.

The ability of methoctramine and other M_2 receptor antagonists to enhance synaptic transmission in vivo is in contrast with previous in vitro studies that reported cholinergic drugs induce an enhancement of synaptic transmission via activation of M_2 mACh receptors (Auerbach and Segal, 1996; Segal and Auerbach, 1997). A previous in vitro study reported a slow-onset persistent potentiation induced by an mACh receptor agonist carbachol (LTPm), which was blocked by an M_2 receptor antagonist (methoctramine) but not M_1/M_3 receptor antagonists (Auerbach and Segal, 1996). Consistent with this,

LTPm was absent in M_2 knock-out mice (Seeger et al., 2004). This in vitro M_2 mACh receptor activation-dependent increase in synaptic transmission is difficult to reconcile with the present study, where an M_2 mACh receptor antagonist methoctramine induced a long-term enhancement of glutamatergic synaptic transmission. This suggests that the in vitro and in vivo mediated increase in synaptic transmission involves recruitment of different pools of M_2 mACh receptors, which is consistent with the proposition that presynaptic and postsynaptic M_2 mACh receptors can have opposing functions in the hippocampus. It is plausible that the relative muscarinic receptor subtype selectivity of a particular drug and/or differences between the experimental techniques employed determine which of the M_2 receptor mediated functions will predominate.

The activity of the cholinergic system is altered by the preparation of the in vitro hippocampal slice, as this involves severing of the cholinergic inputs to the hippocampus and therefore the basal activation of M_2 receptors by endogenous ACh would be expected to be greatly diminished relative to in vivo experiments. In addition, in vitro investigations often require the addition of bicuculline to the bath and this compound has recently been shown to alter the expression of mACh receptors on the neuronal membrane (Schneider and Rodriguez de Lores Arnaiz, 2006). The discrepancies between in vitro and in vivo results may also be explained by exogenous stimulation of mACh receptors using carbachol as opposed to an indirectly mediated stimulation of endogenous mACh receptors using methoctramine. Methoctramine may stimulate mACh receptors by amplifying the release of endogenous ACh and therefore bring about a more physiological pattern of mACh receptor stimulation compared to carbachol. It is important to take into account that carbachol may transiently reduce ACh release by activating presynaptic inhibitory receptors. Previous studies reported that in vitro somatically applied ACh (de Sevilla et al., 2005) and in vivo systemic/localised application of physostigmine (Ito et al., 1988; Levkovitz and Segal, 1994) persistently enhanced hippocampal synaptic transmission. These methods increased the local ACh concentration and therefore may be analogous to methoctramine. However, these methods would be expected to partly negate their enhancement of the cholinergic system

since ACh will inhibit its own release through the activation of presynaptic inhibitory receptors, unlike methoctramine.

Many studies suggest that M_2 receptor activation enhances synaptic transmission by directly increasing the excitability of the postsynaptic cell (Auerbach and Segal, 1996; Dutar and Nicoll, 1988; Segal and Auerbach, 1997). However, M_2 receptor mediated disinhibition may regulate LTP induced by theta burst conditioning stimulation since it was strongly reduced in M_2 knock-out mice and pretreatment with a $GABA_A$ receptor antagonist, bicuculline, restored STP and significantly abrogated the inhibition of LTP (Seeger et al., 2004). In addition, whole-cell recordings from CA1 pyramidal cells demonstrated a much stronger disinhibition of GABAergic than glutamatergic transmission in M_2 knock-out mice, which was particularly prominent during stimulus trains (Seeger et al., 2004). The results of this in vitro study suggest that endogenously released ACh regulates hippocampal plasticity primarily via the activation of M_2 receptors located on interneurons, which leads to suppressed $GABA_A$ receptor mediated transmission and thus disinhibition of pyramidal cells (Seeger et al., 2004).

The M_2 mACh receptor antagonist methoctramine is unlikely to mediate an LTE of synaptic transmission via an increase in GABA release as the activation of interneurons that directly inhibit the pyramidal cells would be expected to result in a decrease in transmission (Gulyas et al., 1993; Miles et al., 1996). However, an increase in GABA release can cause an increase in ACh release since activation of $GABA_A$ receptors located presynaptically (Gao et al., 1995) was previously shown to increase ACh release in vivo (Giovannini et al., 1994). In addition, inhibitory systems are complex and it is possible that enhanced GABA release could promote inhibition of interneurons that inhibit pyramidal cells, which would disinhibit pyramidal cells and thus increase their excitability (Freund, 2003; Hajos et al., 1998). Finally ACh may indirectly modulate GABAergic transmission as the activation of mACh receptors has been shown to increase the release of endocannabinoids (Fukudome et al., 2004; Kim et al., 2002), which are associated with the suppression of GABAergic inhibition and subsequent disinhibition of pyramidal cells (Kim et al., 2002).

A previous in vivo study showed that pre-treatment with either the M_1 receptor antagonist telenzepine (Galvan et al., 1989) or the M_1/M_3 receptor antagonist 4-DAMP (Dorje et al., 1991) blocked methoctramine induced LTE (Li, 2002), which suggests that methoctramine LTE is dependent on activation of non-M_2 mACh receptors. The apparent role of M_1 mACh receptors in methoctramine LTE is consistent with a previous in vitro study where somatically applied ACh (de Sevilla et al., 2005) induced a long term increase in glutamatergic transmission, which was blocked by the M_1 mACh receptor antagonist, pirezepine. It is unlikely that the M_1 receptor mACh activation alone explains methoctramine LTE since the i.c.v. application of a M_1 receptor mACh receptor agonist, (S)-(-)-2,8-dimethyl-3-methylene-1-oxa-8-azaspiro [4,5] decane-L-tartarate monohydrate (YM 796), failed to induce a persistent enhancement of synaptic transmission (Li, 2002). This suggests that M_1 receptor mACh receptor activity may be necessary but that M_1 receptor activity alone does not account for the induction of LTE by methoctramine. The putative role of M_3 mACh receptor activation in methoctramine-induced LTE is consistent with a previous study where M_3 receptor activation was found to increase the release of Ca^{2+} from intracellular stores (Wakamori et al., 1993). However, M_3 mACh receptors were found to underlie the carbachol (high concentration) mediated inhibition of synaptic transmission in the hippocampus (Auerbach and Segal, 1996). Finally, it appears that methoctramine LTE requires transient mACh receptor activation as it was previously found that a non-specific mACh receptor antagonist, scopolamine, at a dose that blocked the induction of methoctramine LTE failed to significantly alter methoctramine LTE when it was applied 30 min after methoctramine (Li, 2002).

4.2 The role of nACh receptors in methoctramine LTE

A methoctramine-induced increase in hippocampal ACh release (Stillman et al., 1996) may activate nACh receptors and thus it is possible that nACh receptor activation contributes to methoctramine LTE. Mecamylamine injected systemically before methoctramine did not significantly affect methoctramine LTE. In contrast, methyllycaconitine, the α7nACh receptor antagonist, delayed the onset of methoctramine LTE. The dose of mecamylamine used in the present study was based on the dose used in a previous study, which prevented activation of hippocampal nACh receptors (Tani et al., 1998), however another study showed that mecamylamine is less potent at blocking α7nACh compared to α/β heteromers (Chavez-Noriega et al., 1997). Therefore, both mecamylamine and methyllycaconitine were applied to determine if a more complete inhibition of nACh receptors might completely block methoctramine LTE. However, the combination did not block methoctramine LTE.

The activation of α7nACh may contribute to the initial enhancement induced by methoctramine by indirectly disinhibiting pyramidal cells (Frazier et al., 1998; Ji and Dani, 2000; Jones and Yakel, 1997; McQuiston and Madison, 1999) or by directly increasing glutamate release (Gray et al., 1996). Alternatively, the activation of α7nACh receptors on the postsynaptic pyramidal cell may facilitate depolarisation and increase the intracellular calcium concentration, which could also contribute to the initial enhancement induced by methoctramine. The putative role of nACh receptors in Ca^{2+} influx correlates with a previous study where α7nACh receptors were found to have a greater relative permeability to Ca^{2+} than NMDA receptors and also other nACh receptors (Seguela et al., 1993). The activation of nACh receptors was previously found to stimulate MAPK and phosphatidylinositol3-kinase (Dineley et al., 2001b; Kihara et al., 2001; Wang et al., 2001) and activation of these kinases may also contribute to the onset of methoctramine LTE.

A previous in vivo study in the CA1 found that methyllycaconitine significantly reduced LTP (Freir and Herron, 2003). In the present study methyllycaconitine delayed the onset of methoctramine LTE, whereas in the previous in vivo study on LTP in the CA1 the reduction in LTP was maximal 40 min after HFS (Freir and Herron, 2003). Comparison of the role of α7nACh receptors in LTP induced by HFS and methoctramine-induced LTE in the CA1 region in vivo suggests that α7nACh receptors are differentially recruited by both of these protocols. The present study also found that the NMDA receptor antagonist memantine had no effect on methoctramine LTE. Memantine was reported to antagonise α7nACh receptors more potently than NMDA receptors (Aracava et al., 2005) and therefore, its lack of effect on methoctramine LTE is inconsistent with the effect of methyllycaconitine on methoctramine LTE. Although this lack of consistency is difficult to explain it is possible that the local application of methyllycaconitine inhibited a local population of α7nACh receptors that are involved in methoctramine LTE more effectively than memantine. To further investigate the role of nACh receptors in methoctramine LTE nicotine was injected systemically prior to methoctramine. Nicotine pre-treatment tended to augment methoctramine LTE; however this increase was not statistically significant.

In the present study an α7nACh receptor antagonist delayed the onset of methoctramie LTE but did not block methoctramine LTE. In contrast, pre-treatment with an M_1/M_3 mACh receptor antagonists blocked methoctramine LTE (Li, 2002). This suggests that methoctramine LTE involves a predominant activation of M_1/M_3 mACh receptors over nACh receptors.

4.3 The role of glutamate receptors in methoctramine LTE

4.3.1 The role of NMDA receptor activation

NMDA receptor activity requires coincident depolarisation of the postsynaptic cell and glutamate binding (Collingridge, 2003; Platenik et al., 2000). Previous studies have shown that ACh can inhibit several currents including voltage gated Ca^{2+} dependent potassium channels, which underlies the slow afterhyperpolarisation (I_{AHP}) and also the voltage dependent Ca^{2+} independent M current (I_M) (Dutar and Nicoll, 1988; Egorov et al., 1999; Madison et al., 1987; Segal and Auerbach, 1997). Blockade of these conductances can augment firing and calcium responses and thus lead to depolarisation of the postsynaptic cell, which could facilitate activation of NMDA receptors and VDCCs (Jerusalinsky et al., 1997). Indeed the activation of mACh receptors has been associated with increased Ca^{2+} entry via NMDA receptors (Egorov et al., 1999; Marino et al., 1998; Markram and Segal, 1992).

The present study found that the NMDA receptor competitive antagonist d-AP5 and the non-competitive NMDA receptor antagonist memantine failed to block methoctramine LTE. The NMDA receptor antagonist, d-AP5 but not memantine, tended to reduce the initial enhancement induced by methoctramine, which suggests that NMDA receptor activation may be necessary for the initial enhancement but is not required for methoctramine LTE. The doses of d-AP5 and memantine used in the present study were previously shown to block LTP induced by HFS (I. Klyubin, TCD, personal communication). Therefore, the finding that NMDA antagonists do not block methoctramine LTE suggests that the mechanism underlying methoctramine LTE is at least partially distinct from the mechanism involved in LTP induced by HFS. The activation of mACh receptors was previously reported to increase Ca^{2+} influx through VDCCs (Klink and Alonso, 1997), however VDCCs do not appear to be involved in methoctramine LTE as pre-treatment with the VDCC inhibitor, mibefradil, did not significantly affect methoctramine LTE (Li, 2002).

The NMDA receptor-independence of methoctramine LTE is consistent with a previous in vitro study, which found that somatically applied ACh induced an NMDA receptor-independent enhancement of transmission at CA1 glutamatergic synapses (de Sevilla et al., 2005). This previous in vitro ACh-mediated enhancement was shown to involve a postsynaptic muscarinic mechanism that appeared to be mediated by calcium released from the endoplasmic reticulum (de Sevilla et al., 2005). Methoctramine LTE is blocked by M_1/M_3 receptors (Li, 2002), which are predominantly expressed on the postsynaptic membrane. Therefore, methoctramine induced LTE may also involve the release of Ca^{2+} from intracellular stores following activation of postsynaptic mACh receptor. This mechanism would be consistent with previous investigations where mACh receptor activation was shown to initiate the release of calcium from intracellular stores via activation of second messenger systems (Berridge and Irvine, 1989; Markram and Segal, 1992).

A previous in vitro study found that endogenously released ACh facilitated LTP independently of NMDA receptor activation by inhibiting GABAergic neurons that modulate pyramidal neurons (Ye et al., 2001). Similarly, methoctramine LTE may involve an NMDA receptor independent decrease in GABAergic transmission.

4.3.2 The role of group I metabotropic glutamate receptor activation

There is evidence that M_2 mACh receptor antagonism may increase the release of glutamate (Marchi and Raiteri, 1989; Nikbakht and Stone, 1999) and thus the activation of metabotropic glutamate receptors may also contribute to methoctramine LTE. A previous study demonstrated that the activation of group I metabotropic glutamate receptors mediates a slow-onset potentiation in the CA1 region in vivo (Manahan-Vaughan and Reymann, 1997). In addition, the activation of both group I mGlu receptors and mACh receptors is associated with the recruitment of PKC (Angenstein et al., 1999; Cantrell et al., 1996; Codazzi et al., 2006; Malenka et al., 1986a; Stratton et al., 1989). Since methoctramine LTE requires the activation of PKC (see 3.5.2 and 3.5.3 in

the present study), it is possible that methoctramine recruits mGlu receptors along with mACh receptors in order to achieve the required PKC activation. Methoctramine LTE may also involve a release of Ca^{2+} from intracellular stores and this may also involve group I mGlu activation as a previous in vitro study showed that a group I mGlu antagonist, LY367385, blocked the increase in intracellular calcium and the direct depolarization of CA1 hippocampal neurons induced by the group I mGlu receptor agonist, DHPG (Mannaioni et al., 2001).

In the present study LY367385 at a dose that blocks mGlu1 receptors did not affect methoctramine LTE, which suggests that the activation of mGlu1 receptors is not involved in methoctramine LTE. The application of a similar dose of LY367385 was previously shown to inhibit LTP in the dentate gyrus of freely behaving animals (Naie and Manahan-Vaughan, 2005). The present study provides evidence that methoctramine LTE is mGlu1 receptor-independent. LY367385 is more selective at mGlu1a receptors relative to mGlu5 receptors (Clark et al., 1997) and there are several other mGlu receptor subtypes, thus this study does not completely dismiss a role for mGlu receptor activation in methoctramine LTE.

4.4 The role of the galaninergic system in methoctramine LTE

In the present study the galanin receptor antagonist, M35, strongly inhibited methoctramine LTE, whereas exogenously applied galanin increased the initial enhancement induced by methoctramine. M35 is a chimeric peptide [galanin (1-13)-bradykinin (2-9) amide]) (Bartfai et al., 1992) and has an equal affinity for Gal-receptor1 and Gal-receptor2 (Fathi et al., 1997). M35 (1nmol) delivered to the ventral hippocampus was previously shown to block the learning impairment caused by galanin (3nmol) delivered to the ventral hippocampus (Schott et al., 2000). Given the selectivity of M35 for galanin receptors it seems likely, that M35 inhibits methoctramine LTE by blocking the action of tonically released endogenous galanin and/or galanin released by methoctramine.

Galanin is believed to be co-transported with classical neurotransmitters, being present in cholinergic, GABAergic, noradrenergic and serotonergic neurons (Chan-Palay, 1988; Melander et al., 1986; Miller et al., 1997). Methoctramine is thought to act primarily as an antagonist of M_2/M_4 receptors, which are presynaptic inhibitory auto-receptors on cholinergic neurons (Kitaichi et al., 1999; Quirion et al., 1995; Stillman et al., 1996; Stillman et al., 1993; Vannucchi and Pepeu, 1995; Vannucchi et al., 1997) and heteroreceptors on GABAergic neurons (Freund, 2003; Hajos et al., 1998; Rouse et al., 2000; Rouse et al., 1999) in the hippocampus. Therefore, it is possible that methoctramine directly increased the release of galanin from cholinergic and GABAergic neurons in the hippocampus. Endogenous galanin is tonicallly released from the ventral hippocampus of freely behaving rats and enhanced following electrical stimulation of the diagonal band of Broca (dBB) (Consolo et al., 1994a), which may be due to the co-release of galanin from septo-hippocampal cholinergic and GABAergic neurons. Therefore, it is also possible that methoctramine initiates galanin release by activating the diagonal band of Broca.

Galanin was found to increase the initial enhancement induced by methoctramine and this increase appeared to depend on the dose of galanin applied. The ability of galanin to augment methoctramine LTE is consistent with the finding that M35 reduced methoctramine LTE. However, M35 reduced the methoctramine LTE throughout the 2 hr recording period, whereas exogenously applied galanin increased (for approximately 50 min) the initial enhancement induced by methoctramine. These results suggest that the activation of galanin receptors is necessary for methoctramine LTE, and that increased galanin receptor activity during the induction (via exogenously applied galanin) can further facilitate the initial enhancement induced by methoctramine. Although galanin is cleared rapidly from the extracellular space (Schott et al., 1998) it is unlikely that rapid clearance explains the transient effect of exogenously applied galanin as pilot studies indicated that galanin injected after methoctramine did not seem to affect the methoctramine-induced LTE. The lack of effect of galanin applied after methoctramine suggests that galanin acts on the induction but not the maintenance of methoctramine LTE.

It is possible that galanin modulates methoctramine LTE indirectly via another neurotransmitter as galanin administered i.c.v. induced long term changes (greater than 3 hours) in the levels of serotonin and noradrenaline in the ventral hippocampus and also modulated the activity of the raphe nucleus and locus coeruleus (Kehr et al., 2001; Ogren et al., 1999; Xu et al., 1999). Galanin can also reduce the release of dopamine from the forebrain (Ericson et al., 1999). The role of noradrenaline may be particularly relevant since virtually all galanin-containing fibers in the dorsal hippocampus are identical to noradrenergic terminals originating in the locus coeruleus (Xu et al., 1998).

The ability of galanin to enhance methoctramine LTE contrasts with in vitro findings in the rat, mouse and guinea pig transverse hippocampal slice where galanin inhibited LTP (Coumis and Davies, 2002; Sakurai et al., 1996). Galanin's inhibition of LTP was found to be independent of NMDA receptor and metabotropic glutamate receptor function, indicating that galanin acts downstream of glutamate receptor activation, possibly at the level of kinase regulation to prevent the establishment of LTP. Although both

methoctramine LTE and HFS induced LTP seem to depend on activation of kinases (Li, 2002) it is highly likely that they both utilise different signalling mechanisms to achieve kinase activation and in turn that galanin modulates these signalling mechanisms differentially. The results of the present study also contrast with another in vitro study where galanin was found to block the slow cholinergic EPSP induced by release of endogenous ACh from the stratum oriens, as recorded intracellularly in the CA1 neurons of the ventral hippocampal slice (Dutar et al., 1989). Therefore, the results of the present study suggest that in the hippocampus galanin has a facilitatory effect on methoctramine LTE independent of its inhibitory effect on LTP and the cholinergic EPSP. Alternatively, the lack of conformity between the previous in vitro and present in vivo results may be accounted for by differences between the slice preparation and the intact hippocampus as slice recording often entails removal of the medial septal region. Removal of the medial septum could alter the effect of galanin on the hippocampus since galanin can increase ACh release in the ventral hippocampus following its infusion into the medial septum (Elvander et al., 2004). Intriguingly, the enhanced release of ACh following infusion of galanin into the medial septum decayed substantially with time (Elvander et al., 2004), which is reminiscent of galanin's transient augmentation of methoctramine LTE in the present study. Somewhat similarly, galanin administered i.c.v. was previously shown to stimulate the cGMP pathway in the rat ventral hippocampus in vivo, via galanin receptors located outside the hippocampus (Consolo et al., 1998). Previous studies have found that galanin has the opposite effect on ACh release in the dorsal and ventral hippocampus, as local galanin injections increase and decrease ACh release, respectively (Schott et al., 2000). The location dependent nature of the hippocampal response to galanin may account for the differential effects of galanin on hippocampal plasticity in vitro and in the present study.

Galanin and galanin receptors are over-expressed in limbic brain regions of AD patients (Counts et al., 2003). Since galanin inhibits LTP (Coumis and Davies, 2002; Sakurai et al., 1996) and the release of ACh (Schott et al., 2000), the increase in the galaninergic system that accompanies AD has been proposed to play a deletrious role in AD. However, galanin infused into medial septum increases ACh release in the ventral

hippocampus (Elvander et al., 2004) and excites cholinergic neurons of the dBB (Jhamandas et al., 2002). Therefore, it has been suggested that galanin may play a compensatory role in AD by augmenting the release of ACh from the remaining cholinergic neurons, which would delay the progression of AD pathology linked to the decrease in cholinergic tone (Counts et al., 2003; Jhamandas et al., 2002). In the present study galanin facilitated and M35 blocked a cholinergic mediated enhancement in synaptic transmission. These findings are consistent with the proposition that the observed increase in the galaninergic system that accompanies AD may be part of a homeostatic mechanism that compensates for the decrease in cholinergic tone.

4.5 The role of protein kinase activation in methoctramine LTE

4.5.1 The role of PKA activation

In the present study the PKA inhibitor, Rp-cAMPs, blocked methoctramine induced LTE. This suggests that a methoctramine induced increase in cAMP and subsequent PKA activity is necessary for methoctramine LTE. The M_2 mACh receptor is mainly located presynaptically and negatively coupled to adenylyl cyclase (Caulfield, 1993), therefore antagonism of this receptor by methoctramine may cause an increase in the intracellular cAMP concentration and subsequent activation of PKA. Since methoctramine increases ACh release (Stillman et al., 1993), it may indirectly activate other cholinergic receptors, which have also been shown to modulate the cAMP/PKA pathway (Caulfield, 1993; Felder, 1995) and thereby may also contribute to the induction of methoctramine LTE. M_2 mACh are heteroreceptors, thus M_2 mACh antagonists may induce an increase in GABA and glutamate release along with activation of their respective receptors, which may also be involved in methoctramine LTE (Freund, 2003; Hajos et al., 1998; Marchi and Raiteri, 1989; Nikbakht and Stone, 1999; Quirion et al., 1995; Rouse et al., 2000; Stillman et al., 1996). Therefore, the activation of the cAMP/PKA pathway by a variety of cholinergic and non-cholinergic receptors could contribute to methoctramine LTE.

The PKA inhibitor, Rp-cAMPS, was also applied after the induction of methoctramine LTE to investigate if a persistent enhancement of PKA activity is necessary for the maintenance-expression of methoctramine LTE. This was investigated using the dose of Rp-cAMPs that blocked the induction of methoctramine LTE but that did not significantly affect baseline transmission. Rp-cAMPs applied 30 min after methoctramine transiently reduced LTE for approximately 40 min but did not persistently reverse the LTE. The reduction in LTE was maximal and statistically significant at 20 min after Rp-cAMPs. The transient nature of the reduction indicates that the maintenance of methoctramine LTE is independent of PKA activation.

Alternatively, the dose of Rp-cAMPS used may not have been sufficient to strongly inhibit PKA activity. The maintenance of LTP usually refers to a persistent biochemical signal that lasts in a cell, whereas the expression of LTP refers to the action of the persistent biochemical signal upon an effector such as a glutamate receptor (Sweatt, 1999). Therefore, the inhibition of maintenance should be irreversible. In contrast it has been suggested that the inhibition of the expression of an LTP should be reversible as the LTP will recover following removal of the inhibitor as the persistent biochemical process that maintains expression is unaffected (Sweatt, 1999). Therefore, the finding that Rp-cAMPs applied after the induction of methoctramine LTE transiently reduced LTE suggests that PKA may be involved in the expression of methoctramine-induced LTE.

Many studies have reported that the cAMP/PKA pathway is necessary for long lasting forms of LTP (Abel et al., 1997; Bach et al., 1999; Frey et al., 1993; Nguyen and Kandel, 1997; Silva et al., 1998; Woo et al., 2002), whereas studies on the role of the cAMP/PKA pathway in the early phase of LTP or less persistent forms of LTP have yielded variable results (Blitzer et al., 1995; Nguyen and Woo, 2003; Otmakhov and Lisman, 2002; Otmakhova et al., 2000; Winder et al., 1998; Woo et al., 2002; Woo et al., 2003). The ability of Rp-cAMPS to block the induction but not persistently reverse the maintenance of methoctramine LTE suggests that PKA is not required for the duration of methoctramine LTE. The cAMP/PKA pathway was previously shown to gate the induction of LTP by decreasing phosphatase activity, which triggers the autophosphorylation of CaMKII (Blitzer et al., 1998; Blitzer et al., 1995). Therefore, the cAMP/PKA pathway may play an essential but brief role during the induction of methoctramine LTE by triggering the autophosphorylation of CaMKII. The idea that activation of PKA plays a brief role during the induction of methoctramine LTE is consistent with a previous in vitro investigation of LTP, where PKA was found to be activated at 2 and 10 min after the delivery of HFS in the CA1 area of the hippocampal slice (Roberson and Sweatt, 1996).

4.5.2 The role of PKC activation

In the present study the PKC inhibitor, BIM, decreased methoctramine-induced LTE over the 2 hr recording period. Although this decrease was not statistically significant at the final time point it was significant at 60 min and therefore considered significant overall. The finding that BIM decreased methoctramine LTE is somewhat consistent with a previous in vivo study where BIM blocked methoctramine LTE (Li, 2002). A role of PKC in methoctramine LTE is consistent with the idea that methoctramine may increase the release of ACh to initiate LTE, since PKC activation can account for many of the effects of muscarinic receptor activation (Cantrell et al., 1996; Malenka et al., 1986a; May et al., 1999; Stratton et al., 1989). However, M_2 mACh are heteroreceptors, which suggests that increased GABA and glutamate release along with the activation of their respective receptors may also be involved in methoctramine LTE (Freund, 2003; Hajos et al., 1998; Marchi and Raiteri, 1989; Nikbakht and Stone, 1999; Quirion et al., 1995; Rouse et al., 2000; Stillman et al., 1996). The stimulation of glutamatergic receptors is associated with activating PKC; therefore methoctramine LTE may recruit PKC via the activation of glutamatergic receptors and/or mACh receptors. The activation of M_1, M_3 and M_5 mACh receptors and also glutamatergic receptors stimulates PIP_2 hydrolysis by PLC, which generates a variety of second messengers, including IP_3 and diacylglycerol. IP_3 releases calcium from intracellular stores, which in conjunction with diacylglycerol, displaces the inhibitory regulatory subunit and thus activates PKC.

PKC is a multigene family and multiple isoforms are transiently activated in the induction of LTP (Hrabetova and Sacktor, 1996; Sacktor et al., 1993). However a single isoform, PKMzeta, exists as an independent catalytic domain of the atypical PKCzeta isoform and is persistently activated during the maintenance phase of LTP (Hernandez et al., 2003). As the PKMzeta isoform lacks a regulatory subunit its activation does not require the presence of diacylglycerol or IP_3. PKMzeta is formed following proteolytic cleavage of the full length PKCzeta atypical isoform (Kishimoto et al., 1983). However,

particular forms of stimulation may prompt de novo protein synthesis of PKMzeta as metabolic labelling of PKMzeta found that tetanic stimulation induces the synthesis of PKMzeta using neuronal specific PKMzeta mRNA, which is generated by an internal promoter within the PKCzeta gene (Hernandez et al., 2003).

The PKCzeta pseudosubstrate peptide inhibitor (ZIP) employed in the present study has also been reported to also inhibit PKMzeta (Ling et al., 2002) and therefore is referred to as an PKC/Mzeta inhibitor in the present study. In this present study ZIP blocked methoctramine LTE. The essential role of PKC/Mzeta in methoctramine LTE is in contrast with a previous in vitro study in the CA1 where ZIP, applied to the bath 120 min before HFS, did not block the initial 60 min of LTP induced by tetanic stimulation (Serrano et al., 2005). A recent in vivo study in the dentate gyrus reported that ZIP applied 1 hr after HFS rapidly reversed LTP (Pastalkova et al., 2006), however the effect of ZIP on the induction of LTP in vivo was not reported. The finding that ZIP blocks the induction of methoctramine-induced LTE suggests that methocramine-induced LTE recruits PKC/Mzeta during the induction, whereas LTP induced by HFS appears to recruit PKC/Mzeta 60 min post the induction of LTP. The present experiments on methoctramine LTE were carried out in the intact hippocampus, whereas the previous studies on the effect of ZIP on the induction of LTP by HFS were undertaken in the in vitro slice; therefore differences between the two preparations may influence the recruitment of PKC/Mzeta. Indeed there is evidence to suggest that differences between the in vivo and in vitro preparation influence the sensitivity of PKC/Mzeta to inhibition as ZIP required 2 hours to reverse LTP of the EPSP recorded in the CA1 region in vitro (Serrano et al., 2005), whereas ZIP reversed LTP recorded in the dentate gyrus in vivo in 15 minutes (Pastalkova et al., 2006).

ZIP reversed LTP maintenance-expression in the CA1 region in vitro (Ling et al., 2006; Serrano et al., 2005) and in the dentate gyrus in vivo (Pastalkova et al., 2006) and therefore, it was decided to evaluate the effect of ZIP applied after methoctramine. The application of ZIP (at a dose that blocked the induction of LTE) 30 minutes after methoctramine did not reverse methoctramine LTE. This finding suggests that

PKC/Mzeta is not involved in the maintenance-expression of methoctramine LTE and is in contrast with the role of PKC/Mzeta in the maintenance-expression of LTP induced by HFS in the dentate in vivo (Pastalkova et al., 2006) and in the CA1 in vitro (Ling et al., 2002; Serrano et al., 2005).

In the present study BIM decreased, whereas ZIP completely blocked the induction of LTE by methoctramine, which suggests that at the dose used BIM does not effectively inhibit PKC/Mzeta. Previous studies found that a compound that is structurally related to BIM, staurosporine (Toullec et al., 1991), is not an effective inhibitor of atypical PKC/Mzeta isoforms (Kochs et al., 1993; Ling et al., 2002; McGlynn et al., 1992). This suggests that BIM may not inhibit the atypical PKC/Mzeta isoforms. However, the results of the present study suggest that BIM may partially inhibit the PKC/Mzeta isoform as this would explain the ability of BIM to decrease methoctramine LTE. Alternatively, the finding that BIM reduced methoctramine LTE may indicate that methoctramine LTE also involves the activation of typical PKC isoforms which are readily inhibited by BIM. Finally, the block of methoctramine-induced LTE by ZIP does not distinguish between the involvement of PKMzeta and PKCzeta in methoctramine LTE.

4.6 Amyloid β-protein (Aβ) does not block methoctramine LTE

Soluble non-fibrillar Aβ has been proposed to initiate a complex cascade of biochemical and cellular changes that culminate in cognitive impairment (Hardy and Selkoe, 2002; Selkoe, 2002; Selkoe and Schenk, 2003). This proposition is supported by the finding that soluble non-fibrillar Aβ injected i.c.v. prior to HFS blocks LTP (Cullen et al., 1997) and likewise impairs learning (Flood et al., 1991). In the present experiments i.c.v. application of soluble non-fibrillar Aβ at a dose which was previously shown to block HFS induced LTP (I. Klyubin, TCD, personal communication), failed to block methoctramine induced LTE. The concentration of soluble non-fibrillar Aβ injected in the present study is believed to reflect the level of soluble non-fibrillar Aβ present in the brain of pre-dementia patients with AD (Walsh et al., 2000). Since the dose of Aβ used in the present study was previously shown to block LTP the lack of effect of Aβ on methoctramine LTE in the present study suggests that the mechanisms underlying methoctramine LTE and LTP induced by HFS are at least partially distinct.

Methoctramine LTE was blocked by M_1/M_3 receptor antagonists, which suggests that methoctramine LTE involves the activation of M_1/M_3 mACh receptors (Li, 2002). A previous in vitro study found that Aβ impairs muscarinic receptor coupling to G proteins (Kelly et al., 1996). Therefore, the lack of effect of Aβ on methoctramine LTE suggests that the dose of Aβ used in the present in vivo study does not significantly impair the coupling of mACh receptors to G proteins. A previous in vitro study showed that the treatment of cultured hippocampal neurons with Aβ results in inhibition of PKA and a concomitant decrease in glutamate stimulated phosphorylation of CREB (Vitolo et al., 2002). Aβ inhibited LTP in the hippocampal slice and this inhibition was reversed by agents that enhance the cAMP/PKA pathway, which suggests that the Aβ mediated inhibition of LTP involves abrogation of the cAMP/PKA pathway (Gong et al., 2004; Vitolo et al., 2002). In the present study the PKA inhibitor, Rp-cAMPs, blocked methoctramine LTE, which suggests that methoctramine LTE is PKA-dependent. The

lack of effect of Aβ on methoctramine LTE suggests that Aβ did not sufficiently inhibit the cAMP/PKA relative to Rp-cAMPs.

The mechanism underlying methoctramine LTE appears to be insensitive to the deletrious effects of Aβ, however it is also possible that methoctramine may oppose the adverse effects of Aβ pre-treatment. Interestingly, a previous in vitro study showed that co-administration of Aβ with an AChE inhibitor prevented the Aβ induced suppression of LTP of the population spike in the CA1 region and AChE inhibitors alone did not significantly affect baseline or the magnitude of LTP (Ye and Qiao, 1999). The mechanisms underlying methoctramine LTE and LTP appear to be distinct; however this previous in vitro study suggests that enhanced cholinergic transmission at the time of Aβ application may actually compensate for the adverse effects of Aβ on the hippocampus. Methoctramine has been previously shown to increase the release of ACh in the hippocampus in vivo (Stillman et al., 1996) and therefore might overcome the inhibition of LTP by Aβ.

V. Conclusion

5.0 Conclusion

A previous study found that M_2 mACh receptor antagonists, AF-DX116 and methoctramine, injected i.c.v. induced a fast onset and long-term enhancement in synaptic transmission (Li, 2002). Likewise, in the present study the M_2 mACh receptor antagonist, methoctramine, induced a fast (generally <8 min onset) and persistent (>2hrs) enhancement of synaptic transmission in the CA1 region of the intact hippocampus (methoctramine-induced LTE). Consistent with this, another M_2 preferring mACh receptor antagonist, gallamine, also enhanced synaptic transmission. However, the onset of the gallamine-induced enhancement was slower (20-30min) compared to methoctramine (8 min). In contrast, BIBN-99, which is also an M_2 mACh receptor antagonist failed to affect synaptic transmission, indicating that not all M_2 mACh receptor antagonists share the ability to induce an LTE.

The role of a variety of cholinergic and glutamatergic receptors in methoctramine LTE were investigated further. A broad spectrum nACh receptor antagonist, mecamylamine, did not significantly affect methoctramine LTE. However, an $\alpha7$ nACh receptor antagonist, methyllycaconitine, appeared to delay the onset of methoctramine-induced LTE but did not block methoctramine LTE. These results suggest that nACh receptor activation is not necessary for methoctramine LTE. Pre-treatment with the competitive NMDA receptor antagonist d-AP5 or the non-competitive antagonist memantine (at doses that blocked LTP induced by HFS) did not block methoctramine-induced LTE, which suggests that methoctramine LTE is NMDA receptor-independent. The group I mGlu receptor antagonist, LY367385, at a dose that blocks mGlu1 receptors failed to block methoctramine LTE, which suggests that methoctramine LTE is mGlu1 receptor-independent.

The role of the galaninergic sytem in methoctramine LTE was also assessed. The application of exogenous galanin increased the initial enhancement induced by methoctramine, whereas pre-treatment with the galanin receptor antagonist M35 blocked

methoctramine LTE. These results suggest that the activation of galanin receptors is necessary for methoctramine LTE, and that increased galanin receptor activity during the induction (via exogenously applied galanin) can further facilitate the initial enhancement induced by methoctramine. Interestingly, pilot studies indicated that galanin injected after methoctramine did not seem to affect the methoctramine-induced LTE. The apparent lack of effect of galanin applied after methoctramine suggests that galanin acts on the induction but not the maintenance-expression of methoctramine LTE.

The role of PKC and PKA in the induction and maintenance-expression of methoctramine LTE was also studied. Pre-treatment with the PKC/Mzeta pseudosubstrate inhibitor (ZIP) blocked methoctramine-induced LTE. Thus methoctramine-induced LTE appears to involve the activation of the atypical PKC/Mzeta isoform. The block of methoctramine-induced LTE by ZIP does not distinguish between the involvement of PKMzeta or PKCzeta in methoctramine LTE. Pre-treatment with the PKA inhibitor Rp-cAMPs blocked methoctramine LTE, which implies that the induction of methoctramine LTE is PKA-dependent. In contrast, the maintenance of methoctramine LTE appears to be independent of these kinases as application of the PKA inhibitor or PKC/Mzeta inhibitor 30 min after the induction of methoctramine LTE did not persistently reverse LTE. Pre-treatment with Aβ at a dose that blocks HFS induced LTP did not affect methoctramine LTE, which suggests that methoctramine LTE is insensitive to inhibition by Aβ.

The finding that methoctramine-induced LTE is NMDA receptor-independent and also insensitive to inhibition by Aβ (at doses that block LTP induced by HFS) suggests that at least some of the mechanisms underlying methoctramine LTE are distinct from those underlying NMDA receptor-dependent LTP induced by standard HFS protocols at CA1 synapses. In the present study antagonists of nACh receptors, NMDA receptors and a group I mGlu receptor antagonist did not block methoctramine LTE. Since, a previous study found that methoctramine LTE was blocked by M_1/M_3 receptor antagonists (Li, 2002), the present study is consistent with the proposal that methoctramine LTE is predominantly dependent on mACh receptor activation.

In the present study galanin facilitated the initial enhancement induced by methoctramine, whereas the galanin receptor antagonist M35 strongly inhibited methoctramine-induced LTE. Therefore, this study suggests that the galaninergic system is an effective modulator of a cholinergic mediated increase in synaptic transmission in the hippocampus. Pilot studies suggested that galanin acts on the induction but not the maintenance-expression of methoctramine LTE and thus it would be interesting to extend these results. Analysis of the effect of M35 on the maintenance-expression of methoctramine LTE would help to clarify if the maintenance-expression of methoctramine LTE is galanin receptor-dependent. The effect of galanin and M35 on methoctramine LTE suggests that the role of galanin in this and other forms of hippocampal plasticity warrants further study.

VI. References

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