INVESTIGATION OF THE ROLE OF NICOTINIC ACETYLCHOLINE RECEPTORS IN MODULATING EPILEPTIFORM ACTIVITY

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ABSTRACT

Nicotinic acetylcholine receptors (nAChRs) are ligand–gated ion channels that have been implicated in a variety of brain functions as well as pathological states. In the hippocampus, nAChRs appear to modulate both excitatory and inhibitory circuits. The numerous subunits that make up nAChRs result in a great diversity of functional receptors, equipping them with different pharmacological and biophysical properties. It has recently been found that certain forms of epilepsy may arise from mutation in the genes responsible for encoding of nAChR subunits. Moreover, many reports have shown that high doses of nicotine induce seizures in animals, which are blocked by different nAChR antagonists. However, the mechanism underling the role of nAChRs in patterning epileptiform activity is poorly understood. This project aims to establish the role that nAChRs may play in experimental models of epilepsy and to assess whether pharmacological agents acting at these receptors might represent a novel avenue for developing future anticonvulsants.

To assess the possible modulatory influence of nAChRs on epileptiform activity, a range of nAChR ligands were applied during experimentally induced epileptiform activity in hippocampal slices prepared from wistar rats (2-6 weeks). Extracellular recordings were obtained in the *stratum pyramidale* of the area CA3 (n=280). Initial experiments investigated the effects of nAChR ligands on 4-aminopyridine (4AP)-induced epileptiform activity. Bath application of 4AP (10-50 μM) resulted in the development of spontaneous epileptiform bursting activity in area CA3 (n=250 of 280) occurring regularly at a frequency of 0.4 ± 0.02Hz. Subsequent co-application of the selective nAChR agonists dimethylphenylpiperazinium iodide (DMPP; 0.3-300 μM, n=31 of 37), choline (0.3-10 mM, n=23 of 33) and lobeline (3-30 μM, n=8 of 10) produced sustained and concentration-dependent increases in burst frequency with maximal frequency potentiation of 37 ± 5%, 27 ± 5% and 24 ± 11%,

respectively. The increase in burst frequency induced by nAChR activation was accompanied by a decrease in the duration of individual burst events. These effects were reversed upon subsequent washout of nAChR agonist or upon co-application of selective nAChR antagonists including dihydro-beta-erythroidine (DHBE, 10-30 μM, n=6 of 8), alpha-bungarotoxin (α-Bgt, 100 nM, n=8) and mecamylamine (50-200 μM, n=7 of 9).

To assess whether nAChR activation has a modulatory influence over epileptiform activities more generally we examined the action of DMPP on two additional pharmacological paradigms. Following bath application of bicuculline (20 μM) or by incubating slices in a low magnesium medium, spontaneous intermittent events occurred at a mean frequency of 0.146 ± 0.02Hz and 0.165 ± 0.02Hz, respectively. Subsequent co-application of DMPP (10-30 μM, n=24) produced a significant increase in both bicuculline and low magnesium-induced burst frequency with a mean maximal frequency potentiation of 248 ± 76% and 110 ± 37%, respectively. These effects were reversed upon subsequent washout of DMPP or upon co-application of DHβE (20-40 μM, n=7 of 10). These results suggest that nAChRs may have a general role in regulating a range of pathological neuronal discharges.

The work presented in the rest of the thesis was focused to establish the mechanisms by which nAChRs mediate their pro-epileptogenic actions. To identify possible excitatory circuits involved in nAChRs-induced pro-epileptogenic effects, we investigated the effect of DMPP on evoked glutamatergic synaptic transmission in area CA3. Bath application of DMPP (30 μM) resulted in a sustained and reversible enhancement of glutamate afferent evoked fEPSP amplitude by 15.7 ± 5.1% (mean ± SEM; P=0.007, n=8 of 12) suggesting that glutamatergic transmission is enhanced by nAChR activation in the CA3 region of the hippocampus. In a further set of experiments we investigated the action of DMPP on epileptiform activity when glutamate receptors were blocked to uncover possible contribution of glutamate receptors in the pro-epileptogenic action of nAChRs. In these experiments application

of NMDA receptor blocker CGP40116 (50μM) resulted in a depression of burst frequency but did not affect the ability of subsequent DMPP application to potentiate epileptiform burst frequency. On the other hand, application of the AMPA/Kainate receptor antagonist NBQX (2 μM) completely abolished epileptiform activity suggesting that these receptors are crucial for supporting such activity. Moreover, subsequent co-application of DMPP failed to reintroduce extracellular field activity. However, slices preincubated with a lower concentration of NBQX (0.2 μM), which results in a partial blockade of AMPA/Kainate receptor-mediated synaptic transmission, were still able to exhibit burst frequency potentiation upon DMPP application. These results suggest that NMDA receptor activation is not necessary in the DMPP-induced burst frequency potentiation and when these receptors are blocked DMPP can potentiate epileptiform activity through activation of AMPA/Kainate receptor-mediated circuitry.

Functional nicotinic receptor-mediated responses are most prominently observed in hippocampal interneurones and thus it is likely that the pro-epileptogenic action of nAChRs is mediated in part through GABAergic circuits. However, such an action of nAChRs appears to be independent of fast GABAergic transmission since it is resistant to the blockade of $GABA_A$ receptors. To identify possible contribution of the $GABA_B$ receptor in the nAChR-induced effect a set of experiments was carried out in the presence of $GABA_B$ receptor antagonist CGP55845A. Slices pre-incubated with 1μM CGP55845A were not able to exhibit burst frequency potentiation of 4AP-induced epileptiform activity upon DMPP application (-8.3 ± 7%, n=11). Similarly, in the presence of 1μM CGP55845A, slices exhibited negligible burst frequency potentiation of bicuculline-induced (20μM) epileptiform activity upon DMPP application (27.6 ± 18%, n=9), in comparison to those observed in the absence of CGP55845A (248 ± 76 %, n=14). These data suggests that nAChRs may regulate the excitability of hippocampal networks through $GABA_B$ receptor-mediated mechanisms.

In conclusion, this study demonstrates that nAChRs regulate epileptiform discharges generated by a number of different pharmacological manipulations. The cellular mechanisms generating each pattern of epileptiform activity are quite distinct involving complex interactions between synaptic and non-synaptic elements of different neuronal circuits. Since nAChRs produce a similar phenotype of modulation in each epileptiform model it is possible that nAChRs target a common cellular mechanism that is prevalent in each model and which mediates the increase in burst frequency in these models.

DECLARATION

I declare that all the work presented in this thesis is by my own efforts, with the exception of the results presented in section 6.3 and figure 6.6, which were carried out in conjunction with Leanne Ferrigan. Recordings presented in Figure 3.1 were assisted by Dr Mohammad Javad khoshnood.

Signature:.....

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PUBLICATIONS

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ABBREVIATION

%	percentage
4AP	4-aminopyridine
5-HT	serotonin
α-Bgt	alpha bungarotoxin
μm	micrometre
μM	micromolar
AC	adenylate cyclase
ACh	acetylcholine
AChE	acetylcholinesterase
ACSF	artificial cerebrospinal fluid
AD	Alzheimer's Disease
ADNFLE	Autosomal Dominant Nocturnal Frontal Lobe Epilepsy
AEDs	antiepileptic drugs
AHP	after hyperpolarisation
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolpropinoic acid
APV	D,L-2-amino-5-phosphonovaleric acid
ATP	adenosine triphosphate
BIC	bicuculline
Ca^{2+}	calcium
CAMP	cyclic adenosine 5' monophosphate
CGP 40116	D-(E)-2-amino-4-methyl-5phosphono-3-pentanoic acid
CGP 55845A	3-*N*-[1-(S)-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl-*P*-benzyl-phosphinic acid
ChAT	choline acetyltransferase
Cl^-	chloride
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system

CPP	3-(+-2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid
DHβE	dihydro-β-erythroidine
DMPP	1,1-dimethyl-4-phenyl-piperazium iodine
DMXB	2,4-dimethoxybenzylidene anabaseine
EEG	electroencephalogram
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
fEPSP	field excitatory postsynaptic potential
GAB	glutamic acid decarboxylase
GABA	γ-aminobutyric acid
G-protein	guanine nucleotide binding protein
GAT	GABA transporters
GTP	guanine triphosphate
I_A	fast inactivating A current
I_{AHP}	slow calcium activated K^+ current
$I_{K(LEAK)}$	leak K^+ current
I_M	M current
IP_3	inositol 1,3,5-triphosphate
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
IS cell	Interneurone selective cell
K^+	potassium
LTP	long term potentiation
mAChR	muscarinic acetylcholine receptor
mEPSC	minature spontaneous excitatory postsynaptic current
mGluR	metabotrophic glutamate receptor
mIPSC	minature spontaneous inhibitory postsynaptic current
mins	minutes
mA	miliampere
ms	milisecond(s)

ml	milliliter
mM	millimolar
mV	millivolt
MCC	N-methylcarbamylcholine
MCPG	(S)-α-methyl-4-carboxyphenyglycine
Mg^{2+}	magnesium
MLA	methyllycaconitine
MSN	medial septal nucleus
nM	nanomolar
nAChR	nicotinic acetylcholine receptor
Na^{+}	sodium
NBQX	6-nitro-7-sulphamoybenzo[*f*]quinoxaline-2,3-dione
NE	norepinephrine
NMDA	*N*-methyl-D-aspartate
PDS	paroxysmal depolarisation shift
PI	phosphoinositide
PKC	protein kinase C
PLC	phospholipase C
PNS	peripheral nervous system
S.E.M	standard error of the mean
sIPSC	spontaneous inhibitory postsynaptic currents
SLM	*stratum lacunosum moleculare*
SM	*stratum moleculare*
SO	*stratum oriens*
SOM	somatostatin
SP	*stratum pyramidale*
SR	*stratum radiatium*
TLE	temporal lobe epilepsy
TTX	tetrodotoxin
VDCC	voltage dependent calcium channels

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CHAPTER 1

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

1.1 Epilepsy

Epilepsy is a very common neurological disease which affects more than 0.5% of the world's population (Kaneko et al., 2002). Epilepsy has been defined as an "episodic disorder of the nervous system arising from the excessively synchronous and sustained discharge of a group of neurones"(Jackson, 1890). During epilepsy, brain function is interrupted by usually transient episodes of abnormal neuronal activity. The term epilepsy refers to recurrent and numerous episodes of seizures with a sudden and unpredictable onset. Seizures are characterised by excessive bursts of activity of CNS neurones and are not necessarily epileptic. At the level of the single neurone, epileptic discharges are associated with very rapid bursts of action potentials, which in turn are associated with abrupt depolarisations of the membrane potential (Lockard, 1980). Epilepsies may be caused by many factors: trauma, cerebrovascular disease, tumours, infection, toxic states and genetically inherited factors, e.g., Autosomal Dominant Nocturnal Frontal Lobe Epilepsy (ADNFLE). In about half of the patients, however no specific causative factors are found (idiopathic epilepsy) and the underlying cellular mechanisms are not well understood (Sandercock and Mumford, 1996).

1.1.1 Forms of epilepsy

Epilepsies come in many forms and display a variety of symptoms, which range from vacant stares in absence seizures, to loss of consciousness accompanied by

muscle spasm and thence jerking of the limbs which is characteristics of tonic-clonic seizures. Seizures are initially characterised into two groups depending on whether the abnormal discharge remains localised in a brain region, partial seizures, or whether it spreads rapidly, generalised seizures.

1.1.2 Pathophysiology

Two sets of changes can determine the epileptogenic properties of neuronal tissues: hyperexcitability and hypersynchrony. Abnormal neuronal excitability is thought to occur as a result of disruption of the depolarisation and repolarisation mechanisms of the cell, which is termed the "excitability of neuronal tissue". Aberrant neuronal networks that develop abnormal synchronisation of a group of neurones can result in the development and propagation of an epileptic seizure which is termed the "synchronisation of neuronal tissue" (Engel, 1989).

The excitability of individual neurones is affected by:

- Cell membrane properties (selective ion permeability and ionic pumps).
- Intracellular processes (Intracellular Ca^{2+} changes, receptor functions, transmitter release and ionic channels).
- Structural features of neuronal elements (the cerebral neocortex and the hippocampus are particularly prone to the generation of epilepsy).
- Interneuronal connections (release of neurotransmitter into the synaptic cleft and the postsynaptic membrane, resulting in excitatory or inhibitory postsynaptic potentials).

A hyperexcitability of neurones that results in random firing of cells, by itself, may not lead to propagation of an epileptic seizure. Indeed, patterns of behaviour require a certain degree of synchronisation of firing in a population of neurones for normal brain function. It is only when this synchronisation becomes excessive or uncontrolled that epileptic seizure originates.

In general seizures can be generated in response to a loss of balance between excitatory and inhibitory influences.

1.1.3 Diagnosis

Electroencephalogram (EEG) recording is commonly used in the investigation and diagnosis of epilepsies since different forms of epilepsy exhibit distinct EEG signatures. Bursts of action potentials and synchronisation are particularly prominent during seizures, but they can also be seen at other times, as the "inter-ictal EEG spike" which is often used as a diagnostic for patients likely to suffer epilepsy (Jefferys, 1993).

Interictal discharges are electrical events occurring *in vivo* between seizures (Dichter and Spencer, 1969), which are not especially troublesome in patients but form a useful diagnostic indicator. Ictal discharges refer to the patterns of electrical activity that occur during a seizure. The extent of depolarisation and speed of activity is prolonged and more diverse when compared to interictal events thus producing a full blown seizure. Interictal bursts are approximately 100-200ms in duration and are localised to restricted brain regions, whereas ictal discharges last for seconds to minutes and spread to large regions of the brain.

Interictal-like and ictal-like discharges can also occur *in vitro* in the slice preparation (Schwartzkroin and Prince, 1978). Interictal spikes in the slice preparation are similar to those occurring *in vivo* in their spontaneous periodic occurrence, extracellular field potential, and intracellular bursting, which is correlated with the extracellularly recorded event (Traub and Wong, 1982).

Electrophysiological recordings from hippocampal slices and computer simulation data have revealed that key cellular and synaptic properties in the generation of interictal bursts are intrinsic burst firing and activation of ionotropic glutamate receptors at recurrent synapses between pyramidal cells (Miles et al., 1984; Traub and Wong, 1982; Wong et al., 1986). In contrast, the critical factors that

precipitate seizures and that are involved in the maintenance of ictal discharges remain to be elucidated (Lee et al., 2002).

The background of the spontaneous epileptiform process is a synchronised activity of all neurones in which a paroxysmal depolarisation shift (PDS) develops (Prince, 1968). PDS is a period of spontaneous cellular depolarisation on top of which the cell generally displays spiking activity. PDS represents the cellular correlate of inteictal spikes seen on the electroencephalogram of epileptic patients (Matsumoto and Marsan, 1964) and probably represents the huge EPSP resulting in synchronous activation of recurrent axonal collaterals (Ayala et al., 1973).

1.1.4 Current therapy

Many classes of pharmacological agents are currently available to control epilepsy. For most epilepsy patients, drug intervention is successful in preventing seizures almost continually. Most antiepileptic drugs (AEDs) are thought to operate through two main mechanisms (Jefferys, 1994): (1) suppressing membrane excitability through ion channel blockade, with selectivity either for sodium (e.g. phenytoin and valproate), or calcium (e.g. ethosuximide), or by (2) enhancing GABA-mediated synaptic inhibition (e.g. the benzodiazepines and barbiturates). Also included in this second class of drugs interacting with the GABAergic system there is a range of newer AEDs including vigabatrin (an inhibitor of GABA transaminase), gabapentine (a GABA analogue) and tiagabine (a GABA uptake blocker). Other new drugs such as topiramate appear to have multiple mechanisms of action including Na^+ channel blockade, enhancing GABA actions, blocking AMPA receptors and inhibiting carbonic anhydrase. Moreover, there is a new group of potential anticonvulsants that can reduce excitatory glutamatergic neurotransmission (e.g. MK-801 and phencyclidine) but are not used clinically partially due to adverse psychological effects associated with their use. Other compounds have yet unspecified or multiple actions.

Seizures are well controlled with a single anticonvulsant in most patients with epilepsy. However, approximately 20% of patients with primary generalised

epilepsy and 35% of patients with focal epilepsy have medically intractable seizures (Reutens and Berkovic, 1995; Spencer et al., 1981). Clearly there remains the need to develop new and more effective AEDs. For this, the pharmaceutic industry and academic labs are trying towards investigating novel targets and transmitter systems in order to identify new AEDs. The focus of the current research was to identify the possible role of acetylcholine receptors in epileptogenesis. Indeed, there is some evidence that the therapeutic action of some novel and some classical AEDs, is in part due to their effects on the cholinergic system (Loscher et al., 2003). Identifying new therapeutic agents focusing on these neurotransmitter systems may suggest novel and improved strategies in controlling epileptiform activities based upon pharmacological targeting of defined microcircuits as opposed to generalised suppression of excitability or enhanced inhibition.

1.2 Neuronal receptors and neurotransmitter systems

1.2.1 The cholinergic system

1.2.1.1 Acetylcholine

Acetylcholine (ACh) is a major neurotransmitter in the central and peripheral nervous systems (Reviewed by Caulfield, 1993). Acetylcholine was first described back in 1906 (Hunt and Taveau, 1906) and was the first substance shown to be a neurotransmitter (Dale, 1934; Dale, 1938; Dale et al., 1936). Chemically it is composed of choline and an acetyl group, synthesised in the axon terminal by the enzyme choline acetyltransferase (ChAT) where it is subsequently taken up into vesicles for storage and release. It is released from presynaptic terminals to act on postsynaptic acetylcholine receptors generating an excitatory postsynaptic response that can be detected as excitatory voltage (EPSP) or current

(EPSC) deflections. The response is terminated when acetylcholine is broken down by the enzyme acetylcholinesterase (AChE) back to choline and acetate.

1.2.1.2 Muscarinic acetylcholine receptors (mAChRs)

mAChRs play key roles in regulating the activity of many important functions of the central and peripheral nervous system. Central mAChRs are involved in regulating an extraordinarily large number of cognitive, behavioural, sensory, motor, and autonomic functions (Eglen et al., 1999; Felder et al., 2000). Reduced or increased signalling through distinct mAChR subtypes has been implicated in the pathophysiology of several major diseases of the CNS, including Alzheimer's and Parkinson's disease, depression, schizophrenia, and epilepsy (Eglen et al., 1999; Felder et al., 2000).

mAChRs are members of the superfamily of G protein-coupled receptors and act indirectly through guanine nucleotide-binding proteins. Agonist binding triggers a conformational change, which leads to the activation of specific G-proteins. The activated G-protein then stimulates or inhibits one of many effector systems. The ultimate outcome of agonist binding depends on the type of G-protein activated and the effector system targeted. Due to this additional step of G-protein activation mAChR mediate slower neurotransmission than the nicotinic ligand-gated ion channels.

The muscarinic actions of ACh are mediated by five molecularly distinct mAChR subtypes (M_1-M_5) (Caulfield, 1993; Caulfield and Birdsall, 1998), which are encoded by five distinct mAChR genes (Bonner et al., 1987; Bonner et al., 1988; Kubo et al., 1986). Based on their ability to activate different classes of heterotrimeric G proteins, the five mAChR subtypes can be subdivided into two major functional classes. The M_2 and M_4 receptors selectively couple to G proteins of the G_i family, which leads to the inhibition of the enzyme Adenylate Cyclase and a decrease of the intracellular messenger cyclic adenosine 5' monophosphate (cAMP). The M_1, M_3, and M_5 receptors selectively couple to G

proteins of the G_q class (Caulfield, 1993; Caulfield and Birdsall, 1998) which activates the enzyme Phospholipase C (PLC), leads to release the intracellular messengers inositol triphosphate (IP3) and DAG.

mAChR proteins have differential cellular distributions within individual regions of the brain. In the hippocampus M1 and M3 receptors are expressed in pyramidal neurones and M2 and M4 receptors expressed in non-pyramidal neurones (Levey et al., 1995).

mAChR activation produces a variety of responses in CNS depend on the circuit involved, the neurotransmitter concentration (Segal, 1991), and the location and the subtype of receptor. Muscarinic stimulation causes a sustained depolarisation associated with an increased input resistance. These effects are attributed to a cholinergic-induced depression of four potassium conductances, including the M-current (I_M) (Halliwell and Adams, 1982; Madison et al., 1987), a fast inactivating current (I_A) (Nakajima et al., 1986), a slow Ca^{2+}-activated current (I_{AHP}) (Benardo and Prince, 1982; Madison et al., 1987), and a background leak current (I_{LEAK}) (Madison et al., 1987). Voltage-dependent Ca^{2+} channels are also modulated by cholinergic stimulation (Misgeld et al., 1986; Toselli et al., 1989).

1.2.1.3 Nicotinic acetylcholine receptors (nAChRs)

General

Nicotinic acetylcholine receptors are cationic channels whose opening is controlled by acetylcholine and nicotinic receptor agonists. They belong to the large family of ligand-gated ion channels that also includes the $GABA_A$, glycine, and 5-HT_3 type receptors. Nicotinic receptors are key molecules in cholinergic transmission at the neuromuscular junction of striated muscles, at the synapse in the autonomous peripheral ganglia, and in several brain areas (Changeux and Edelstein, 1998; Clementi et al., 2000; Gotti et al., 1997; Sargent, 1993). Our

knowledge of neuronal nicotinic receptors started in 1889 with the publication of the famous paper by Langley and Dickinson, who first reported that nicotine could block neuronal transmission in the superior cervical ganglion. The concept of the presence of nicotinic receptors in central nervous system developed in subsequent papers published by Langley in 1905-1906 (Langley, 1905). Until only a few years ago, knowledge of neuronal nicotinic receptors remained confined to the ganglia and more recently neuronal nicotinic receptors have been investigated systematically. Although, much is known about nAChRs in ganglionic transmission, neuromuscular junction and peripheral autonomic system their real function in the brain are still unclear (Reviewed by Clementi et al., 2000).

Nicotinic acetylcholine receptors are distributed widely in human brain and ganglia and form a family of receptors with a variety of different subtypes, each of which has a specific physiology and pharmacology. Recent progress in neurochemical and pharmacological methods indicates that the major effect of nAChRs is often slow neromodulation rather than processing of fast synaptic transmission (Reviewed by Vizi and Lendvai, 1999).

Structure of neuronal nicotinic receptors

The receptor and associated ion channel consists of five subunits (α,β,δ,γ and ε), all of which arrange a large structure inserted into the membrane and surround a central aqueous channel (Figure 1.1-1.2). nAChRs exist as a variety of subtypes due to the diversity of genes encoding acetylcholine nicotinic receptor subunits (Clementi et al., 2000). Earlier binding studies have detected only two subtypes in the brain: (1) a low affinity receptor labelled by α-bungarotoxine, and (2) a high affinity receptor labelled by nicotine or acetylcholine (Clarke et al., 1985; Marks et al., 1986). However, sixteen nAChR subunit genes have so far been cloned (α1 to α9, β1 to β4, δ,γ and ε) (Le Novere and Changeux, 1995). The subunits have several common structural features, a large extracellular N-terminal domain, four putative transmembrane sequences (M1 - M4), a short C-terminal extracellular

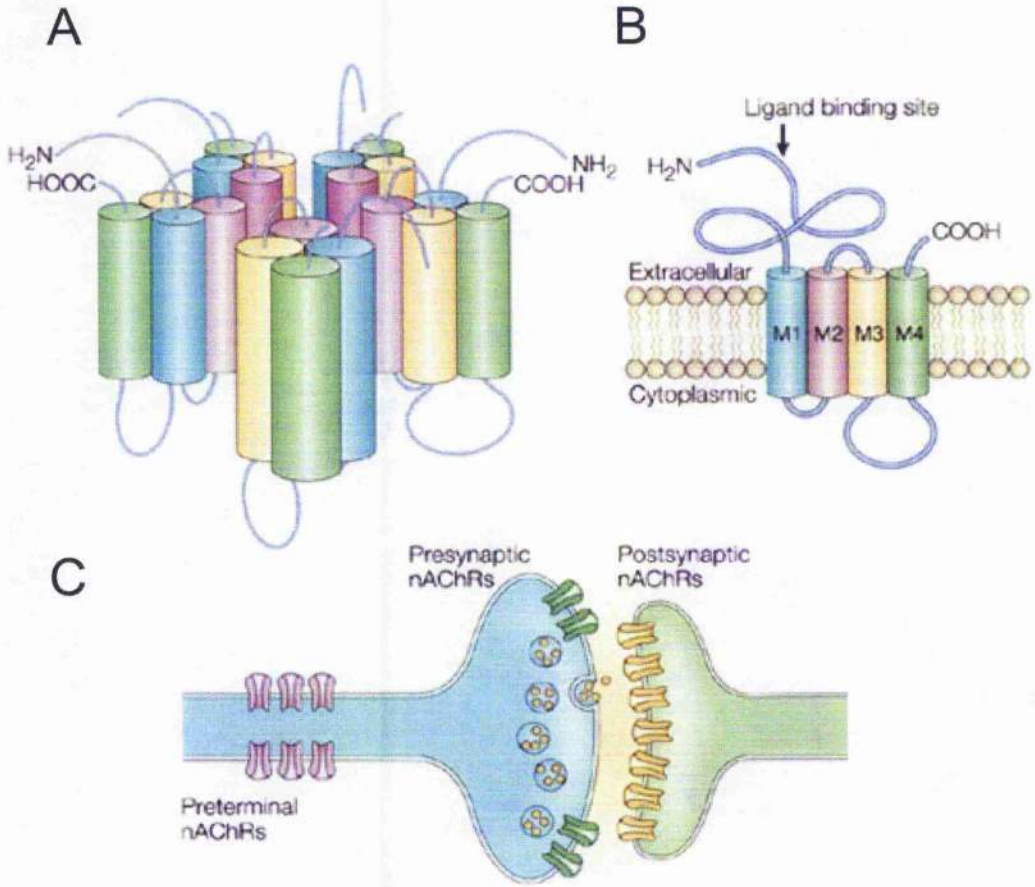

Figure 1.1. *The structure of neuronal nicotinic acetylcholine receptors (nAChRs).* Diagram adapted from Laviolette and Van der kooy 2004. A. Each nAChR is composed of five subunits arranged in either homomeric or heteromeric complexes of α or β subunit arrangements. Different subunit combinations confer unique functional properties to the ubiquitously distributed nAChRs throughout the brain. B. The schematic shows the transmembrane topology of a single nAChR subunit. The transmembrane domains are labelled M1–M4. The larger amino-terminal domain contains the acetylcholine-binding site, whereas the M2 domain determines the ionic selectivity of the receptor and faces the inside of the channel pore. C. nAChRs are located on axons, presynaptic terminals and on all postsynaptic sites (dendrites and soma). This widespread localization confers the receptor with a wide range of functions, influencing neuronal signalling at the pre- and postsynaptic levels.

sequence, and an intracellular loop of varying length, depending on the subunit, joining the third and fourth transmembrane domain which is very important for the regulation of receptor function (Reviewed by Clementi et al., 2000; Laviolette and van der Kooy, 2004) (Figure 1.1 B and 1.2 B).

Nicotinic receptors can be subdivided into three sub-families according to the amino acid sequences of the subunits (Figure 1.2 C): The first subfamily consists of heteromeric muscle nicotinic acetylcholine receptors which have a pentameric subunit composition $(\alpha1)_2\beta1\gamma1\delta1$ in the foetal form and $(\alpha1)_2\beta1\varepsilon1\delta1$ in the mature form. The second one consists of heteromeric neuronal acetylcholine nicotinic receptors, which do not bind α-bungarotoxin. These neuronal nAChRs have a pentameric structure formed from combination of α2,α3,α4,α6 with either β2 or β4 subunits and sometimes also with α5 or β3 subunits. The last subfamily consists of neuronal homoligomeric acetylcholine nicotinic receptors that are formed by the α7 or α8 or α9 subunits and bind α-bungarotoxin.

The α subunits participate in the formation of agonist binding sites and the β subunits are considered structural subunits (Gotti et al., 1997). In the heteromeric muscle and neuronal nAChRs, the acetylcholine binding site is located in the large extracellular N-terminal domain, at the interface between the α and non-α subunit (Clementi et al., 2000). The homoligomeric α7 or α8 receptors have five identical acetylcholine binding sites per receptor molecule, one in each subunit (Changeux and Edelstein, 1998). As indicated earlier, α7,α8 and α9 are the subunits that can form homomeric receptors, whereas α2,α3,α4 and α6 always need to be co-expressed with β2 or β4 in order to form functional channels.

Receptor distribution

Nicotinic receptors are distributed widely in human brain and ganglia and are key molecules in cholinergic transmission at the neuromuscular junction of striated muscles, at the synapse in the autonomous peripheral ganglia and in several brain

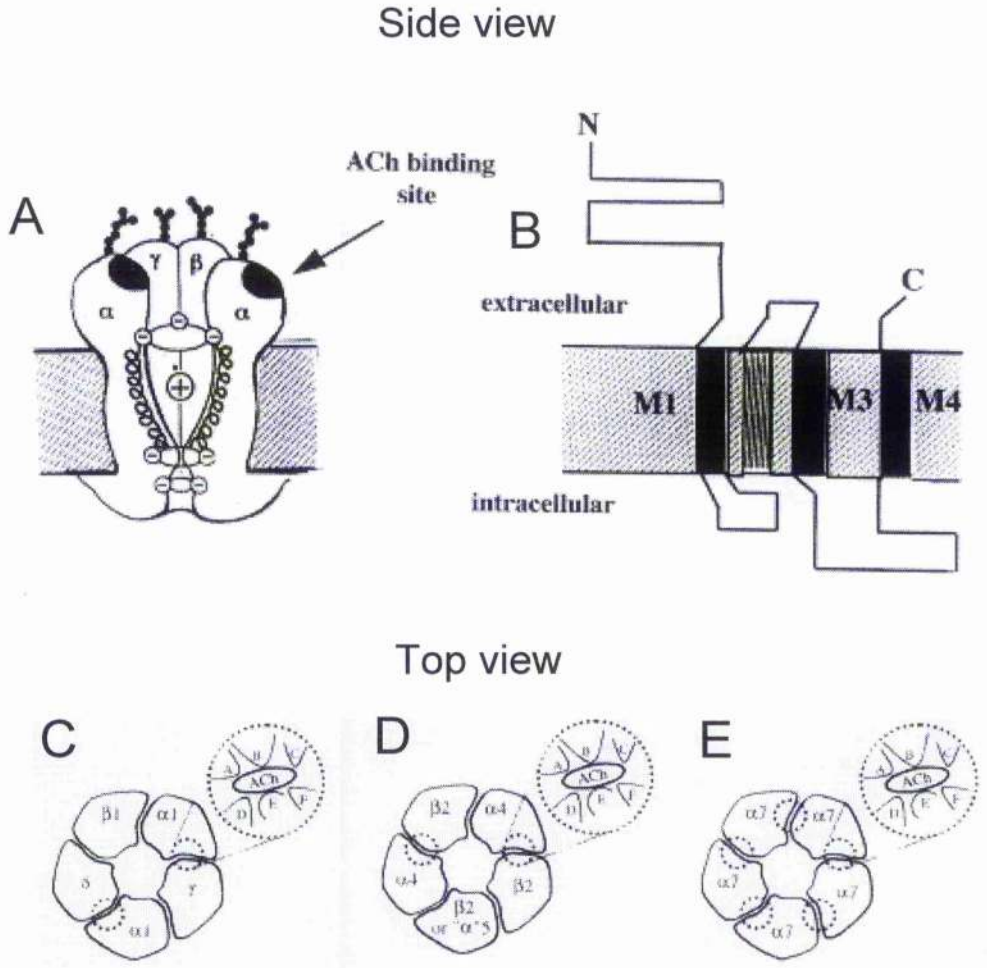

Figure 1.2. *Subunit organization and ligand binding loops for nicotinic receptors.* Diagram adapted from clementi *et al*, 2000, original drawing by Changeux and Edelestein, 1998. (A) Vertical section of the putative transmembrane organisation of the muscle nicotinic acetylcholine receptor. The black areas indicate the acetylcholine binding sites and rings the charged areas that control the channel permeability. (B) Putative transmembrane organisation of the nicotinic acetylcholine receptor subunit. (C) Subunit arrangement of muscle-type receptors. Two agonist binding sites are indicated by the dashed circles. (D) Subunit arrangement of heteropentameric neuronal receptors with 2 or 3 subunit types. Two agonist binding sites are indicated by the dashed circles. The α in quotation marks indicates that, unlike other α subunits, α5 may not directly contribute to agonist binding. (E) Subunit arrangement of homopentameric receptors. Agonist binding sites are indicated by the dashed circles; all five are equivalent, but one is enlarged to show the contributions of loops A–F.

areas (Sargent, 1993). In the CNS, they are mainly located in various cortical areas, the periacqueductal gray matter, the basal ganglia, the thalamus, the hippocampus, the cerebellum and the retina (Reviewed by Clementi et al., 2000). Although the hippocampus contains α3, α4, α5, α7, β2, β3 and β4 subunits (Jones et al., 1999), three subtypes of nAChRs have been shown to be present on the most hippocampal neurones: (1) an α7 bearing nAChR that gives rise to fast-desensitising, α-bungarotoxin–sensitive nicotinic currents (named as type IA current), (2) an α4β2-containing nAChR that subserves slowly desensitising, DHBE-sensitive nicotinic currents (named as type II current), and (3) an α3β4-bearing nAChR that accounts for very slowly desensitising, mecamylamine-sensitive nicotinic currents (named as type III current) (Albuquerque et al., 1997; Albuquerque et al., 1995; Alkondon and Albuquerque, 1993).

Hippocampal nAChRs are expressed on GABAergic inhibitory interneurones (Alkondon et al., 1997; Frazier et al., 1998a; Frazier et al., 1998b; Freedman et al., 1993; Freund and Buzsaki, 1996; Jones and Yakel, 1997; McQuiston and Madison, 1999b), on the excitatory glutamatergic pyramidal cells (Albuquerque et al., 1997; Alkondon et al., 1997; Ji et al., 2001) and also on the granule neurones, so both inhibitory and excitatory synapses are directly modulated by nAChR activity. Nicotinic receptors do not only exist on neuronal cell bodies and dendrites but are also located on axon terminals and involved in modulation of multiple transmitter releases (Wilkie et al., 1996; Wonnacott et al., 1989). Receptors in the presynaptic localisation can function as synaptic or non-synaptic receptors. The term of preterminal receptors has been assigned to nonsynaptic receptors (Wonnacott, 1997). Postsynaptic receptors may also be localised in synaptic and non-synaptic areas of the neurone. However, the postsynaptic responses after activation of these receptors have to be summed at the axon terminal to prompt action potential.

Receptor Subtype	Anatomical Distribution (Ref 1)	Cellular Location * (Refs 1,2,3,4 &5)	Pharmacology (antagonist)	Functional Properties (Ref 6)
α7	Hippocampal dentate granular layer, Hippocampus, Entorhinal cortex, Subiculum	Somata and dendrites, at both synaptic and non-synaptic sites	α-bungarotoxin, MLA	High calcium permeability, Rapidly decaying nicotinic current (type I)
α4β2	Dentate gyrus, Hippocampal pyramidal cell body layer, Subiculum	Somatadendritic regions of various hippocampal areas, at both pre and post-synaptic sites	DHβE	Slowly decaying nicotinic current (type II)
α3β4	Dentate gyrus, Hippocampal pyramidal cell body layer, Subiculum	Somatadendritic regions of various hippocampal areas, at both pre and post-synaptic sites	Mecamylamine	Very slowing nicotinic current (type III)

Table 1.1 *Distribution, pharmacology and functional properties of nicotinic acetylcholine receptor subunits.*
The table summarising the anatomical and cellular distribution, pharmacology and functional properties of nAChR subunits in the rodent hippocampus. * Note, nAChR subunit composition varies in different brain areas; however, at the moment the spatial resolution is still too low for precise subcellular subtype localization. References: 1-(Gotti et al., 1997), 2- (Dominguez del Toro et al., 1994), 3-(Marshall, 1981), 4-(Smolen, 1983), 5-(Brown and Fumagalli, 1977), 6-(Alkondon and Albuquerque, 2003, 2004).

Receptor functions

Although much is known about the role of nAChRs in ganglionic transmission and control the function of the peripheral autonomic system, their true functions in the brain are still unclear. They are known to be involved in various complex cognitive functions, such as attention, learning, memory, control the locomotor activity, pain perception and body temperature regulation (Reviewed by Gotti et al., 1997). The majority of these effects are due to the presynaptic nicotinic receptors that modulate the release of a number of neurotransmitters (Wonnacott, 1997). However, postsynaptic nicotinic receptors also play important roles in controlling of ganglionic transmission and fast ACh-mediated synaptic transmission as reported in the hippocampus and in sensory cortex (Reviewed by Clementi et al., 2000).

Put in the simplest terms, ACh binds nAChRs and ion channel opens for several milliseconds. Then the receptor/channel closes again to the initial state or enters an inactivated phase that is unresponsive to ACh or other agonists (Dani, 2000). The speed of activation, the intensity of the depolarisation, the size of the calcium signal, the rate of desensitisation and recovery and the pharmacology of the ACh response will all depend on the nAChRs subunit composition as well as other local factors (Reviewed by Alkondon and Albuquerque, 2004; Dani, 2000). Different receptor subtypes are involved in different neurone functions. For example, the β2 subtype is important in the control of presynaptic GABA release (Lu et al., 1998) where as dopamine release from brain dopaminergic neurones is partially controlled by an α4–containing subtype (Le Novere and Changeux, 1995). It is also reported that the release of glutamate from glutamatergic inputs to the ventral lateral geniculate nucleus in chick brain slices is mainly controlled by an α7-containing subtype (Guo et al., 1998) while in the hippocampal CA1 region an α3β4 subtype contributed to the modulation of glutamate release onto CA1 *stratum radiatum* interneurones (Alkondon and Albuquerque, 2004; Alkondon et al., 2003). The presence of the α7 subunit greatly increase Ca2+ permeability (Seguela et al., 1993) and thus may be important to trigger intracellular second

messenger systems (Chiodini et al., 1999). Moreover, nicotine and also a novel nicotinic agonist, 2,4-dimethoxybenzylidene anabaseine (DMXB), possibly via the α7 subtype, can induce long-term potentiation in the hippocampus in a micromolar concentration range, possibly due to the high Ca^{2+} permeability of the α7 subunit (Hunter et al., 1994; Mann and Greenfield, 2003).

In the cerebral cortex and in the hippocampus, cholinergic afferents project in a diffuse manner (Mesulam et al., 1983; Schafer et al., 1998; Woolf, 1991). However, in both regions, unlike the glutamatergic and GABAergic afferents, cholinergic fibres form direct synaptic contacts with only a minor fraction of the total number of terminals present (Mrzljak et al., 1995; Umbriaco et al., 1995). These anatomical data are consistent with the physiological observations that a direct nicotinic synaptic transmission has only been demonstrated in a few brain regions (Alkondon et al., 1998; Frazier et al., 1998a; Frazier et al., 1998b). In the hippocampus, fast nicotinic transmission has been found only onto GABAergic interneurones (Alkondon et al., 1997; Frazier et al., 1998a) and not glutamatergic pyramidal cells (Frazier et al., 1998b; Jones and Yakel, 1997), but in developing visual cortex, stimulation has evoked nAChR-mediated synaptic responses in both pyramidal cells and interneurones (Roerig et al., 1997). Because nicotinic synapses are of low density and difficult to detect experimentally, fast nicotinic transmission may be present at low densities in more neuronal area than the few that have been reported (Dani, 2000). Where it has been reported, fast nAChR-mediated transmission is a minor component of the excitatory input, which is overwhelmingly glutamatergic. Therefore, direct nicotinic excitation of a neurone usually dose not predominate, but it could influence the excitability of a group of neurones owing to the broad cholinergic projections into area (Dani, 2000).

nAChR activation is reported to produce a variety of responses in hippocampal interneurones which depend on the type and the location of interneurones and also nAChR subtypes expressed (Reviewed by Alkondon and Albuquerque, 2004). The majority of interneurones show a fast depolarisation mediated by α7 subunit containing receptors (Frazier et al., 1998a; McQuiston and Madison, 1999b).

However McQuiston and Madison also reported a subset of interneurones that showed a fast depolarisation in combination with a slower response. Alkondon (1997) reported similar response in some interneurones but also interneurones which displayed only a slow response probably due to the activation of $\alpha4\beta2$ nAChR. Three different responses modulated by at least three distinct nAChR subtypes have also been reported in a single type of interneurone (e.g. CA1 *stratum radiatum* interneurone) (Alkondon and Albuquerque, 2004). Interneurones showing no nicotinic response at all have also been observed (McQuiston and Madison, 1999b).

It is possible that the difficulty in detecting fast excitatory nicotinic synapses in the brain reflect the participation of neuronal nAChRs in modulation rather than the mediation of synaptic transmission *per se* (Lapchak et al., 1989; Lena et al., 1993; McGehee et al., 1995; McMahon et al., 1994). nAChRs at presynaptic sites can modulate synaptic transmission by regulating the extent of transmitter release (see below).

nAChRs also have roles during development. The density of nAChRs varies during the course of development. In addition, short-term and long-term regulation of nAChR number and function is likely to be important for modulating synaptic efficacy. These regulatory and developmental factors are particularly important when considering the development of epilepsy. Alterations in nAChRs could exert their effects directly and immediately to alter excitability, or there could be indirect and /or developmental consequences of mutations in nAChR genes that subsequently produce the epilepsy (Dani, 2000).

Interaction between nAChRs and other neuronal transmitters

An important function of neuronal nicotinic acetylcholine receptors in the CNS appears to be their involvement in neurotransmitter release (Wonnacott, 1990). Strong neurochemical evidence indicates that presynaptic nAChRs are involved in the enhanced release of a number of transmitters, including norepinephrine,

dopamine, serotonin, acetylcholine, GABA and glutamate (Gray et al., 1996; Lapchak et al., 1989; Lena et al., 1993; McGehee et al., 1995; Vidal and Changeux, 1993). It is hypothesised that activation of calcium-permeable presynaptic nicotinic receptors enhances transmission directly by elevating presynaptic calcium levels (Gray et al., 1996). According to Vizi and Lendvai (1999), interacellular mechanisms leading to release of transmitters in response to nAChRs stimulation are initiated by axonal firing, or directly induce Na^{-} and Ca^{2+} influx followed by a depolarisation sufficient to activate local voltage–sensitive Ca^{2+} channels, as a result transmitter of vesicular origin will be released (Figure 1.3).

However, some other studies were unable to evoke an increase in presynaptic calcium levels with local application of nicotinic receptor agonists suggesting an indirect synaptic modulation could arise from nicotinic excitation (Vogt and Regehr, 2001).

Moreover, some of the nAChR agonists such as DMPP and lobeline, besides their effects on presynaptic nAChRs, at higher concentrations are able to inhibit the uptake of NE and 5-HT into nerve terminals, thereby their transmitter releasing effects are extended in time and space (Vizi and Lendvai, 1999) (see Figure 1.3). The effect on the uptake process is not being sensitive to nAChR antagonism, but can be prevented by selective uptake blockers or reduced temperature.

Tetrodotoxin (TTX) is a neurotoxin that blocks voltage dependent Na^{+} channels, thereby blocking axonal conduction. It has been suggested by different authors (Clarke and Reuben, 1996; Sacaan et al., 1995), that the sensitivity or resistance to TTX indicates the pre-, or postsynaptic localisation of nAChRs. The TTX-insensitive part of transmitter release evoked by nAChR agonists has been taken as evidence of presynaptic receptor localisation (Wonnacott, 1997). It is likely that TTX reduces that portion of transmitter release in which Na^{+}-influx has been involved. TTX-dependence of nAChR-induced neurotransmitter release can be very different across brain regions, for example, nicotine-induced dopamine

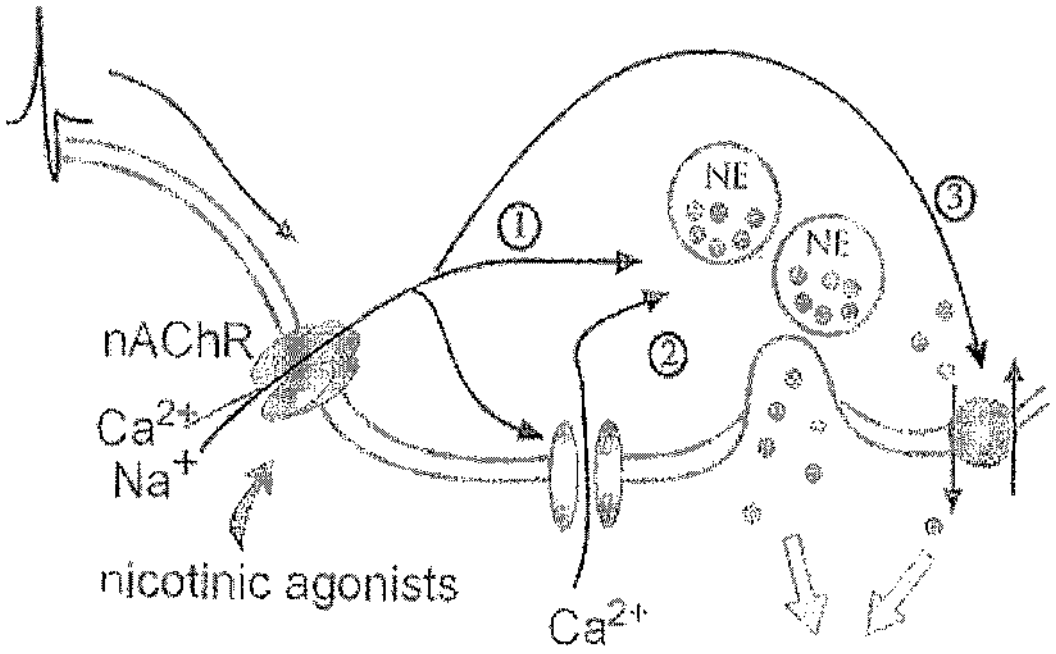

Figure 1.3. *Putative intracellular mechanisms for nicotinic receptor activation-induced release.*
Diagram adapted from Vizi and Iendvai, 1999. (1) Integral ion-channel function - the calcium entry via presynaptic nAChR channel directly triggers exocytosis, (2) activation of voltage-dependent ion channels by the local depolarization (excitatory postsynaptic potentials) due to nAChR activation, calcium enters via VDCCs and induces vesicle fusion (calcium channel blocker-sensitive part of nAChR stimulation-evoked release), (3) reversal of membrane uptake carrier to release NE from the cytoplasm.

release has been found TTX-sensitive in the striatum but not in the cortex using *in vivo* microdialysis (Marshall et al., 1996; 1997). Moreover the hippocampal release of NE evoked by nAChR stimulation was completely inhibited by TTX (Sershen et al., 1997), whereas, in chick sympathetic neurones release of NE and increase in $[Ca^{2+}]_i$ evoked by nAChR stimulation can be still elicited in the presence of TTX (Dolezal et al., 1996). These results can easily be explained by different localisation of presynaptic nAChRs in the very varicose dopaminergic and noradrenergic fibres with a dominance of receptor far from the actual release site or closer (Vizi and Lendvai, 1999).

As mentioned earlier, the release of these transmitters can be modulated by different nAChR subtypes. The release of glutamate is mainly controlled by an α7 or α3β4 - containing subtypes (Alkondon et al., 2003; Guo et al., 1998). However, a study in mice, indicated that the β2 subtype is important in the control of presynaptic GABA release (Poth et al., 1997). It seems likely that presynaptic nAChRs on monoaminergic fibres are composed of α3 or α4 subunits in combination with the β2 subunit (Vizi and Lendvai, 1999). This is supported by the observation that nAChR agonists have no presynaptic effect on transmitter release in knockout mice lacking theβ2 nAChR subunit gene.

1.2.2 The GABAergic system

1.2.2.1 Gamma-aminobutyric acid (GABA)

The neutral amino acid γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in vertebrates as roughly 40% of all cerebral synapses are GABAergic (Reviewed by Ure and Perassolo, 2000). GABA is synthesised from glutamate in neurones expressing the enzyme glutamic acid decarboxylase (GAB). It is released from presynaptic terminals to act on postsynaptic GABA receptors generating an inhibitory postsynaptic response that can be detected as voltage (IPSP) or current (IPSC) deflections. Termination of action is due to uptake into neurones and surrounding glial cells by GABA transporters (GAT1-3)

and the breakdown of GABA by transaminase (Reviewed by Soudijn and van Wijngaarden, 2000).

Early work with the neurotransmitter GABA indicated that it produced inhibitory hyperpolarising postsynaptic responses in neurones (Krnjevic and Schwartz, 1967), which could be blocked by the alkaloid bicuculline (Curtis et al., 1970). During the 1970s Bowery and colleagues showed that GABA could inhibit noradrealine release in the heart (Bowery et al., 1981; Bowery and Hudson, 1979). However this response was not blocked by bicuculline and ultimately lead to the classification of two pharmacologically distinct GABA receptors named $GABA_A$ and $GABA_B$ (Hill and Bowery, 1981). This novel $GABA_B$ receptor was also shown to be present in the brain (Bowery et al., 1980b; Bowery et al., 1987) and is activated by the specific agonist baclofen (Bowery et al., 1980b; Hill and Bowery, 1981). More recently the existence of a third type of GABA receptor has been confirmed (Johnston, 1996). Johnston's original work on GABA analogues identified a GABA receptor insensitive to bicuculline (Johnston et al., 1975). These analogues were later shown not to effect binding of the $GABA_B$ agonist baclofen and as such a third subclass of GABA receptor was suggested and named $GABA_C$ (Drew et al., 1984). Fast inhibitory neurotransmission is mediated by the $GABA_A$ receptor and the $GABA_C$ receptor, both of which are ligand-gated Cl^- channels. Slow inhibitory neurotransmission is mediated by the G protein-coupled $GABA_B$ receptor.

1.2.2.2 $GABA_A$ receptors

$GABA_A$ receptors are ionotropic receptors, which open channels permeable to Cl^- and are blocked by picrotoxin and bicuculline. The action of GABA on ionotropic receptors is both a hyperpolarisation and a reduction of excitation, each of which can be considered inhibitory. $GABA_A$ receptors mediating most inhibitory synaptic transmission in the CNS belong to the ligand-gated ion channel superfamily (Schofield et al., 1987). As with all others members of this superfamily they have a pentameric architecture (Nayeem et al., 1994) with the

exact subunit composition conferring discrete functional characteristics (Reviewed by Sieghart, 1995; Sieghart et al., 1999). Five subunits are arranged within the plasma membrane to form a channel that is mainly selective for chloride and bicarbonate ions (Bormann et al., 1987). The first two receptors subunits identified (α and β) were cloned by Schofield in 1987. Currently, 20 $GABA_A$ receptor subunits have been identified in mammalian tissue, including six α, four β, three Y, one δ, one ε, one π, one θ, and three ρsubunits (Reviewed by Sieghart et al., 1999). At least one α, β and γ subunit is required to form a fully functional channel (Pritchett et al., 1989). The most common formation is two α1 subunits in combination with two β2 subunits and a single γ2 subunit which accounts for approximately 43% of $GABA_A$ receptors (McKernan and Whiting, 1996). A large diversity of $GABA_A$ receptor subtypes are found within the hippocampus (Wisden et al., 1992).

1.2.2.3 $GABA_B$ receptors

$GABA_B$ receptors are metabotropic receptors and belong to the G-protein coupled receptor superfamily (Reviewed by Bowery et al., 2002). The heterodimeric nature of the $GABA_B$ receptor was not initially appreciated when this receptor was first cloned by Kaupmann *et al.* in 1997. Subsequent studies (Marshall et al., 1999) revealed that functionally expressed the $GABA_B$ receptor was not a single protein but instead existed as a heterodimer with the subunits designated $GABA_BR1$ and $GABA_BR2$, neither of which was functional on its own (Reviewed by Bowery et al., 2002). Agonist binding occurs at the $GABA_BR1$ subunit, whereas no ligand binding is detectable on the R2 subunit (Kniazeff et al., 2002). Conversely, the R1 subunit is not able to activate effector systems, whereas the R2 subunit is responsible for G protein coupling (Duthey et al., 2002).

$GABA_B$ receptors are activated by agonists GABA and (-)baclofen (Bowery et al., 1981; Bowery et al., 1980b; Hill and Bowery, 1981) and antagonised by saclofen as well as a range of more recently developed selective and potent antagonists

including CGP55845A (3-*N*-[1-(S)-(3,4-dichlorophenyl)ethyl] amino-2-hydroxypropyl-*P*-benzyl-phosphinic acid) (Davies et al., 1993).

Effector mechanisms associated with neural $GABA_B$ receptors are the adenylate cyclase system and Ca^{2+} and K^+ ion channels (Reviewed by Bowery et al., 2002). When activated, $GABA_B$ receptors open K^+ channels on both the pre and postsynaptic sides of the synapse (Dutar and Nicoll, 1988) and increase K^+ conductance. In the postsynaptic cell this leads to a hyperpolarisation of the membrane potential which has a slower onset and slower decay than the $GABA_A$ response. In the presynaptic terminal, activation of $GABA_B$ receptors reduces transmitter release by an inhibition of Ca^{2+} conductance. It depresses transmitter release of virtually all transmitters investigated including glutamate and GABA. Therefore, effects of $GABA_B$ receptors can be complex and are not always inhibitory (Freund and Buzsaki, 1996). Electrophysiological data indicate that $GABA_B$ receptors are present at both pre- and postsynaptic sites within the hippocampus (Dutar and Nicoll, 1988).

1.2.3 The glutamatergic system

1.2.3.1 Glutamate

L-Glutamate is the major excitatory neurotransmitter within the adult central nervous system and was the first excitatory amino acid to be so recognised (Curtis et al., 1959). It is one of the quantitatively more important neurotransmitters in mammalian CNS (Curtis and Johnston, 1974). Glutamate is synthesised in and released from the presynaptic terminal by Ca^{2+} dependant exocytosis (Nicholls, 1989) and acted on specific glutamate postsynaptic receptors to elicit excitatory postsynaptic responses (EPSPs or EPSCs) (Curtis and Watkins, 1960; 1963). The postsynaptic action of glutamate is terminated by glutamate uptake carriers present in the plasma membrane of glial cells and neurones (Rothman et al., 1987).

Once again, the action of glutamate is on two main types of receptors, ionotropic and metabotropic:

1.2.3.2 Ionotropic glutamate receptors

The ionotropic glutamate receptors consist primarily of three types named after their selective agonists, hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate and *N*-Methyl-D-aspartate (NMDA) receptors (Reviewed by McLennan, 1983; Westbrook, 1994). AMPA and kainate receptors mediate fast EPSPs whereas NMDA receptors mediate slower-rising and slower-decaying EPSPs. AMPA and kainate receptors are often referred to as non-NMDA receptors but in fact represent different classes of receptors with discrete functional, pharmacological properties and distributions. Under normal conditions transmission within the hippocampus is carried out by non-NMDA receptors (Collingridge et al., 1982; 1983).

More recent molecular biological studies of glutamate receptors have revealed that each of these three subgroups is encoded by a number of different genes, including *GluR1-4* for AMPA receptors, *GluR5-7*, *KA1* and *KA2* for kainate receptors and *NMDAR1* and *NMDAR2A-D* (also known as NR1 and NR2A-D) for NMDA receptors (Reviewed by Barnes and Henley, 1992; Hollmann and Heinemann, 1994). Different subunits generated by these genes are of a wide variety and exhibit varying electrophysiological and pharmacological properties, depending upon the combination of subunits expressed (McCormick, 1998). All ionotrophic glutamate receptor subunits differ from the nicotinic acetylcholine receptor subunits in their transmembrane topology by having only three membrane spanning domains.

Activation of excitatory amino acid receptors underlies fast glutamatergic excitatory postsynaptic potentials (EPSPs). The postsynaptic potentials mediated by AMPA and kainate receptors, like those associated with nicotinic channels, are caused by an increase in a mixed cation conductance (mainly Na^+ and K^+, but

sometimes Ca^{2+} as well) such that the reversal potential is approximately 0 mV (Hollmann and Heinemann, 1994). These synaptic potentials have a very short delay from the arrival of the action potentials at the presynaptic terminal, and a rapid rate of rise. The falling phase is much slower, being determined in large part by the membrane properties of the neurone.

In contrast to the fast PSPs mediated by AMPA/kainate receptors, the action of glutamate through NMDA receptors is more complicated. Stimulation of NMDA receptors results in the activation of a voltage-dependent current that is carried not only by Na^+ and K^+ but also importantly by Ca^{2+}. The voltage-dependent nature of this NMDA-mediated current is due to the differential block of the ionic channel by magnesium ions (Mg^{2+}) at more hyperpolarised membrane potentials (Mayer et al., 1984). High calcium permeability of NMDA receptors is important in initiating intracellular processes including long-term metabolic or structural changes.

1.2.3.3 Metabotropic glutamate receptors (mGluRs)

mGluRs represent a family of G protein-coupled receptors, which can trigger long-lasting intracellular processes and 'metabolic' changes and mediate synaptic plasticity (Neugebauer, 2002). Eight mGluR subtypes have been cloned to date and are classified into groups I (mGluRs 1 and 5), II (mGluRs 2 and 3) and III (mGluRs 4, 6, 7, and 8) based on their sequence homology, signal transduction mechanisms, and pharmacological profile (Reviewed by Anwyl, 1999; Gasparini et al., 2002; Schoepp et al., 1999).

A major distinction between group I mGluRs and groups II and III mGluRs is that group I couple through $G_{q/11}$ proteins to the activation of phospholipase C (PLC), resulting in phosphoinositide (PI) hydrolysis, release of calcium from intracellular stores, and protein kinase C (PKC) activation. In contrast, groups II and III mGluRs are negatively coupled to adenylyl cyclase (AC) through G_i/G_o proteins, thereby inhibiting cyclic AMP (cAMP) formation and cAMP-dependent protein

kinase (PKA) activation (Reviewed by Anwyl, 1999; Gasparini et al., 2002; Schoepp et al., 1999). In general, it appears that the predominant effect of group I mGluR activation is enhanced neuronal excitability and synaptic transmission whereas activation of groups II and III typically produces inhibitory effects, although exceptions exist.

Activation of the mGluR has a very diverse range of electrophysiological effects. These include inhibition of potassium and calcium currents, activation of potassium, calcium and non-specific cation currents, mediation of slow excitatory postsynaptic potentials, presynaptic inhibition of transmitter release, potentiation of AMPA and NMDA receptor synaptic responses and involvement in the generation of oscillatory and epileptiform activity (Reviewed by Anwyl, 1999). Members of each mGluR subgroup can presynaptically inhibit excitatory (glutamatergic) as well as inhibitory (GABAergic) synaptic transmission and transmitter release, which appears to be modulated by groups II and III mGluRs. Group I mGluRs can facilitate excitatory and inhibitory synaptic transmission. Importantly, inhibition of GABA release by groups II and III mGluRs might actually increase neuronal excitability through disinhibition whereas facilitation of GABAergic transmission by group I mGluRs would strengthen the inhibitory tone (Reviewed by Neugebauer, 2002).

Although group I mGluRs can be found presynaptically, they are localised primarily on the postsynaptic membrane. Group III mGluRs represent predominantly presynaptic receptors, whereas group II mGluRs have been detected both pre- and postsynaptically (Reviewed by Neugebauer, 2002).

1.3 Neurotransmitters and epilepsy

The normal functioning of the brain rests on maintaining a fine balance between excitation and inhibition. Disruption in this balance within interconnected neuronal networks may lead to a predisposition for the generation of uncontrolled and hypersynchronous discharges as occurs in epilepsies.

1.3.1 GABA

Reduction of inhibition by GABA antagonists generates epileptiform activity both *in vivo* and *in vitro* models raising the possibility that epilepsy can arise from a loss of GABA-mediated inhibition (Ameri et al., 1997; Colom and Saggau, 1994; Gulyas-Kovacs et al., 2002; Herron et al., 1985; Knowles et al., 1987; Kohr and Heinemann, 1990). Decreased concentrations of GABA were found in tissue removed from epileptic patients when compared to normal concentrations determined from non-epileptic patients (Van Gelder et al., 1972). The use of *in vivo* micro-dialysis found lower GABA levels in the epileptic compared with the non- epileptic hippocampus (During and Spencer, 1993). Immunohistochemistry revealed a decreased number of GABA-immunoreactive neurones in epileptic tissue (Ribak et al., 1986). The induction of status epilepticus in animals results in a loss of GABA binding sites in the rat forebrain (Kapur et al., 1994). However, a clear reduction in either GABA concentration or in the activity of GABAergic neurones is not the significant underlying cause that it was initially thought to be. GABAergic inhibition is not impaired in some epilepsy models such as exposure to low magnesium (Tancredi et al., 1990), 4-aminopyridine (Rutecki et al., 1987) and high frequency tetanic stimulation (Higashima, 1988). In the 4AP model, epileptiform activity occurs despite the presence of normal or even enhanced synaptic inhibition (Chesnut and Swann, 1988; Rutecki et al., 1987). A preserved GABAergic inhibitory postsynaptic potential in sclerotic, epileptic hippocampi has also been reported (see Schwartzkroin, 1994 for review). Although some alteration in inhibitory process may cause or result from seizures, the exact nature and extent of this involvement remains unclear.

1.3.2 Glutamate

The alternative explanation of increased excitability is enhanced activity through excitatory circuits, which probably involves glutamate. The role of glutamate as a convulsant and excitotoxic agent is well documented (Reviewed by Ure and Perassolo, 2000). Glutamate is also a precursor of the powerful inhibitor GABA and thus it has been suggested that the glutamate/GABA ratio might exert the most crucial influence in the generation of paroxysmal neuronal discharges (Ure and Perassolo, 2000). A major contention in the theory that enhanced glutamate levels are responsible for seizures are the conflicting reports regarding the concentration of glutamate before and during seizures. In epileptic patients undergoing surgery, an increase in glutamate levels was detected in samples of extracellular fluid extracted by microdialysis (During and Spencer, 1993; Ronne-Engstrom et al., 1992) although no change was apparent in seizures induced in animals by kainate, bicuculline, picrotoxin or electrical stimulation (Lehmann et al., 1985; Obrenovitch et al., 1996). A reduced concentration of glutamate in epileptic foci (Van Gelder et al., 1972) and in CSF from epileptic patients with normal values of other aminoacids (Mutani et al., 1974) has also been reported. Thus it is proposed that seizures and raised glutamate levels are not necessarily related.

Enhanced excitability can be attributed to other mechanisms apart from elevated glutamate levels. An increased density of NMDA and kainate receptors in the entorhinal cortex and hippocampal CA1 region (Geddes et al., 1990; McDonald et al., 1991) and an increase in AMPA receptor density in the dentate gyrus (Hosford et al., 1991) has been shown in tissue removed from epileptic patients.

Raised NMDA receptor activation may contribute to hippocampal hyperexcitability in some models. Kindling, the repeated stimulation of a pathway at intervals until seizures are initiated, is highly dependent on NMDA receptor activation (Cain et al., 1988; Mody et al., 1988; Robinson, 1991). The main trigger of long-lasting potentiation is the Ca^{2+} influx initiated by glutamate and mediated by NMDA receptors. It has also been reported an increased binding in

the hippocampus (McDonald et al., 1991) and dentate gyrus (Roper et al., 1992) of ligands to NMDA receptors in the cortex removed from patients with temporal epilepsy, as well as an increase in NMDA receptor density (Geddes et al., 1990; McDonald et al., 1991).

Glutamate receptor agonists, NMDA, AMPA and kainate are convulsants producing seizures both *in vivo* (Chiamulera et al., 1992; Koek and Colpaert, 1990) as well as epileptiform activities *in vitro* (Fisher and Alger, 1984). Glutamate receptor antagonists are also good at inhibiting seizures in models where the involvement of either NMDA or non-NMDA receptors is clear. For example, NMDA receptor antagonists totally or partially block epileptiform activity induced by lowering magnesium levels (Gulyas-Kovacs et al., 2002; Horne et al., 1986; Mody et al., 1987; Schneiderman and MacDonald, 1987; Tancredi et al., 1990), while have no or little effect on the epileptiform discharges seen with other models such as $GABA_A$ receptor blockade by bicuculline and picrotoxin (Gulyas-Kovacs et al., 2002; Neuman et al., 1988; Thomson and West, 1986). AMPA/Kainate receptor antagonist significantly affect epileptiform activity in BIC model (Avoli et al., 1993; Gulyas-Kovacs et al., 2002; Perreault and Avoli, 1991; Traub et al., 1993), while have only a minor influence in the generation of epileptiform discharges in low magnesium model (Gulyas-Kovacs et al., 2002). In fact, there are significant alterations in contribution of NMDA and AMPA/Kainate glutamate receptors to the development and maintenance of epileptiform activity in the different convulsants. It has also been reported that 4AP-induced epileptiform activity is mediated through the activation of AMPA/Kainate receptors (Avoli et al., 1993; Perreault and Avoli, 1991). However, the finding of Avoli *et al.*, (1996) and Gulyas-Kovacs *et al.*, (2002) are contrary to this suggesting that in 4AP model, both types of ionotropic excitatory amino acid receptors are overactivated and contribute to seizure initiation and propagation. While data related to the relevance of each of these receptors in focal epileptogenesis are contradictory, there is a noticeable trend towards a strong activation of both NMDA and AMPA/Kainate receptors during epileptiform

activity (Lee and Hablitz, 1991; Siniscalchi et al., 1997; Valenzuela and Benardo, 1995).

Metabotropic glutamate receptors have also been shown to be critically involved in modulating ictal activity during seizures in animal models of epilepsy (Dalby and Thomsen, 1996; Tizzano et al., 1995). mGluR agonists have also been reported to induce epileptiform discharges *in vitro* (Cobb et al., 2000; Merlin et al., 1995; Merlin and Wong, 1997).

1.3.3 Acetylcholine

Acetylcholine may play a role during epilepsy. An increase in ACh synthesising and degrading enzymes in the epileptic cortex of patients undergoing anterior temporal lobectomy has been reported (Kish et al., 1988), but discussion persists as regards the exact significance of such changes (Ure and Perassolo, 2000).

1.3.3.1 mAChRs and epilepsy

In spite of the conflicting data in the literature, there may be only a pro-epileptogenic action for nAChRs (see below), while muscarine receptors may play an excitatory or suppresor role, depending both on the circuits involved and on the neurotransmitter concentration (Segal, 1991). However, it has been reported that endogenous ACh may enhance epileptogenesis in immature hippocampus (Psarropoulou and Dallaire, 1998) and neocortex (Potier and Psarropoulou, 2001; Potier and Psarropoulou, 2004) via activation of muscarinic receptors.

1.3.3.2 nAChRs and epilepsy

Whilst the exact functional significance of different populations of nAChRs in modulating hippocampal dependent physiological processes is unclear there is increasing evidence that nAChRs may also be involved in disease states

(Reviewed by Jones et al., 1999). It has been claimed that neuronal nicotinic receptors are involved in a number of brain diseases such as epilepsy, schizophrenia, dementia and Alzheimer's disease and are currently the subject of intense research interest with respect to their therapeutic potential in these series of neurological and psychiatric conditions (Decker et al., 1998; Elmslie et al., 1997; Freedman et al., 1997; Hodgkiss and Kelly, 2001; James and Nordberg, 1995; Lena and Changeux, 1997; Lena and Changeux, 1998; Marubio et al., 1999; Phillips et al., 1998; Steinlein et al., 1995). Recent studies have indicated that specific nAChR subtypes are selectively responsible for certain brain diseases (Reviewed by Clementi et al., 2000). For example, the number of receptors containing α4 subunit is decreased in Alzheimer's disease (Martin-Ruiz et al., 1999). However, the sensory gating defect in schizophrenia seems to be associated with an abnormal expression of the α7 subunit (Leonard et al., 1996).

The association between a particular form of genetically transmissible epilepsy and mutations in genes coding for nAChR subunits constituted the first demonstration of the contribution of a neurotransmitter-activated receptor in epilepsy and the first identification of the role of neuronal nAChRs in human brain dysfunction (Reviewed by Raggenbass and Bertrand, 2002). This epilepsy was termed *Autosomal Dominant Nocturnal Frontal Lobe Epilepsy* (ADNFLE), because of its autosomal dominant mode of transmission, its occurrence during sleep and its frontal origin. In 1995, Phillips *et al.* found that ADNFLE is a genetic disease whose gene is located in chromosome 20q 13.2-q 13.3, the region that also contains the gene of the α4 nicotinic subunit (Phillips et al., 1995). On the basis of this information Steinlein *et al.* (1995) studied the sequence of the α4 gene and found a mutation that replaces serine with phenylalanine and inserts a leucine, this mutation is present in all affected siblings but not in normal subjects (Steinlein et al., 1995). The serine-to-phenylanaline substitution mainly speeds desensitisation and slows its recovery, and the leucine insertion increases ACh affinity and alters the signal-channel conductance (Dani, 2000). The possible mechanism linking this form of epilepsy is due to a decrease in α4 receptor function that lowers the seizure threshold (Gotti et al., 1997). It is also

hypothesised that decreased nAChR function leads to decrease in GABA release, with an accompanying increase in excitability (Jones et al., 1999). Several other mechanisms have also been proposed to contribute to ADNFLE. For example, mutant nicotinic receptors may display an increased acetylcholine sensitivity with respect to normal receptors (Bertrand et al., 2002; Bertrand et al., 1998). The increase ACh sensitivity may produce a faster response of the cortical or thalamic neurones and thereby unbalance this phase sensitive network. The thalamus and cortex are strongly innervated by cholinergic afferents from the pons and basal forebrain and ACh exert modulatory actions on thalamic and cortical neurones by acting via either nicotinic or muscarinic acetylcholine receptors (McCormick, 1992). Neuronal synchronisation could occur by a small but significant shift between cortical and thalamic activity. Other possibility is that ACh indirectly can act on presynaptic terminals by enhancing other neurotransmitter release and thereby causing the unbalance between excitation and inhibition (Raggenbass and Bertrand, 2002). Therefore, single amino acid changes produce effects that are potent enough presumably to disrupt proper functioning of these receptors and, in certain cases, provoke the unbalance of excitation versus inhibition that is at the origin of epileptic seizures (Dani, 2000; Raggenbass and Bertrand, 2002).

Other epilepsies due to mutations in the genes encoding the α7 and β3 nAChR subunits have also been reported (Durner et al., 1999; Elmslie et al., 1997; Neubauer et al., 1998; Phillips et al., 1998)

In addition to the genetic studies mentioned above reporting an involvement of nAChRs in epilepsy, some *in vivo* studies have also demonstrated that high does of nicotine induce clonic-tonic convulsions in animals after systemic and intracerebral injections (Dixit et al., 1971; Miner et al., 1985). Electrophysiological studies have indicated that nicotine-induced seizures originate in the hippocampus (Floris et al., 1964). Moreover, it has been indicated that mice with large numbers of brain α-bungarotoxin binding sites are more prone to develop seizures in response to nicotine (Marks et al., 1989). Thus, α7-receptor subtype may underlie nicotine-induce seizures because the α7 subunit is

thought to be the major α-bungarotoxin binding site in the mammalian brain (McLane et al., 1992). Nevertheless, the pharmacological mechanisms involved in the convulsive effect of nicotine are poorly understood.

In 1999, Damaj *et al,* extended the pharmacological characterisation of nicotine-induced seizures by examining the role of nAChRs subtypes in mediating this effect after systemic and central administration of several nicotinic agonists in mice (Damaj et al., 1999). In addition, different nAChR antagonists were used in combination with nAChR selective agonists. According to this study, nicotine-induced seizures are centrally mediated and involve the activation of α7 along with other nAChRs subunits. MLA, a α7- antagonist, blocked the effects of nicotine and mecamylamine, a non-competitive antagonist, blocked nicotine-induced bursting more potently than MLA. Although these results did not eliminate the involvement of α4β2- receptors, they suggested that this receptor subtype dose not play a major role in nicotine- induced seizures. This study also suggests that nicotine enhances the release of glutamate either directly or indirectly. Glutamate release in turn stimulates NMDA receptors, thus triggering the cascade of events leading to nitric oxide formation and possibly seizure production (Damaj et al., 1999).

Despite these links to epilepsy, information regarding the role of nAChRs in patterning epileptiform activity is extremely sparse. The aim of this thesis, therefore, was to address this gap in our knowledge by investigating the effects of nAChR ligands on pharmacologically-induced epileptiform activity in rat hippocampal slices.

As all studies were carried out in the hippocampal slice, I will provide a brief account of the hippocampal formation and its importance in epilepsy research.

1.4 The Hippocampus

1.4.1 General

The hippocampus is a region of the temporal lobe, which has long been known to play a crucial role in important brain functions such as learning and memory. In pioneering studies, memory impairment was been reported in patient having undergone a bilateral temporal lobectomy for the treatment of severe epilepsy (Scoville and Milner, 1957) whilst more recent studies have shown similar deficits in patients with bilateral damage restricted to the hippocampus (Zola-Morgan et al., 1986). In the past two decades this brain region became a major focus of neuroscience research. Interest in this structure stems from its importance in normal cognitive functions as well as involvement in some major neurological disorders such as Alzheimer's disease and temporal lobe epilepsy (Robbins and Kumar, 1987).

1.4.2 Anatomy

The hippocampus is a bilateral and banana-shaped structure found within the forebrain located beneath the posterior and temporal neocortex. The hippocampus proper is connected to cortical regions of the brain through the perforant pathway and numerous subcortical regions such as raphe nucleus and medial septum, through the fornix. It is one of a group of structures within the limbic system typically called the *hippocampus formation* that includes the dentate gyrus, the hippocampus, the subiculum and the entorhinal cortex. Division of the hippocampus into the *regio superior* and *regio inferior* was based on differences in cell morphology and fibre projections (Cajal, 1911). However, in 1934, Lorente de Nó divided the hippocampus into four field described as *cornu ammonis* (CA) 1-4 (Figure 1.4) based on the size and appearance of the neurones (Johnston and Amaral, 1998). He also noted a variety of subtle differences in the dendritic organisation of pyramidal cells in different parts of CA3 and CA1 and used these distinctions, in part, to further subdivide these fields into three subareas each

(CA3a,b,c; CA1a,b,c). He also used the term CA4, referred to the region occupied by the polymorphic layer of the dentate gyrus. The CA1 and the CA3 regions constitute most of the hippocampus. In addition to differences in the size of cells in CA3 and CA1, there is a clear-cut connectional difference. The CA3 pyramidal cells receive a mossy fibre input from the dentate gyrus and the CA1 pyramidal cells do not. The CA2 field has been a matter of some controversy. It is a narrow zone of cells interposed between CA3 and CA1 that have large cell bodies like CA3 but do not receive mossy fibre innervation like CA1. In many respects, CA2 resembles a terminal portion of the CA3 field. In other ways, however, CA2 is quiet distinct from either CA3 or CA1 (Amaral and Witter, 1995). The CA2 and CA4 regions are less well defined which leads to these regions being generally ignored.

Both the dentate gyrus and the hippocampus are three-layered cortices (Figure 1.4). The three fundamental layers of the dentate gyrus are the polymorphic layer (the *hilus*) containing the granule cell axons, the granular layer (the *stratum granulosum*) containing the granule cell bodies and the molecular layer (*stratum moleculare*) containing the granule cell dendrites. The dentate granule cell layer, with the local circuitry in the hilus, act as a gate controlling the sensory input to the hippocampus from the entorhinal cortex (Jefferys, 1993).

The hippocampus also consists of a polymorphic layer (the *stratum oriens*), a pyramidal layer (the *stratum pyramidale*) and a molecular layer (the *stratum radiatum* and *stratum lacunosum-moleculare*). The polymorphic layer contains the basal dendrites of the pyramidal cells, the pyramidal layer consists mainly of the pyramidal cell bodies and the molecular layer contains distal apical dendrites. The white coat of the hippocampus is produced by the axons of the pyramidal cells, which are grouped to form the alveus. As described above the pyramidal cell layer has been divided into four regions designated CA1-CA4 (Lorente de No, 1934) based on the size and appearance of the neurones.

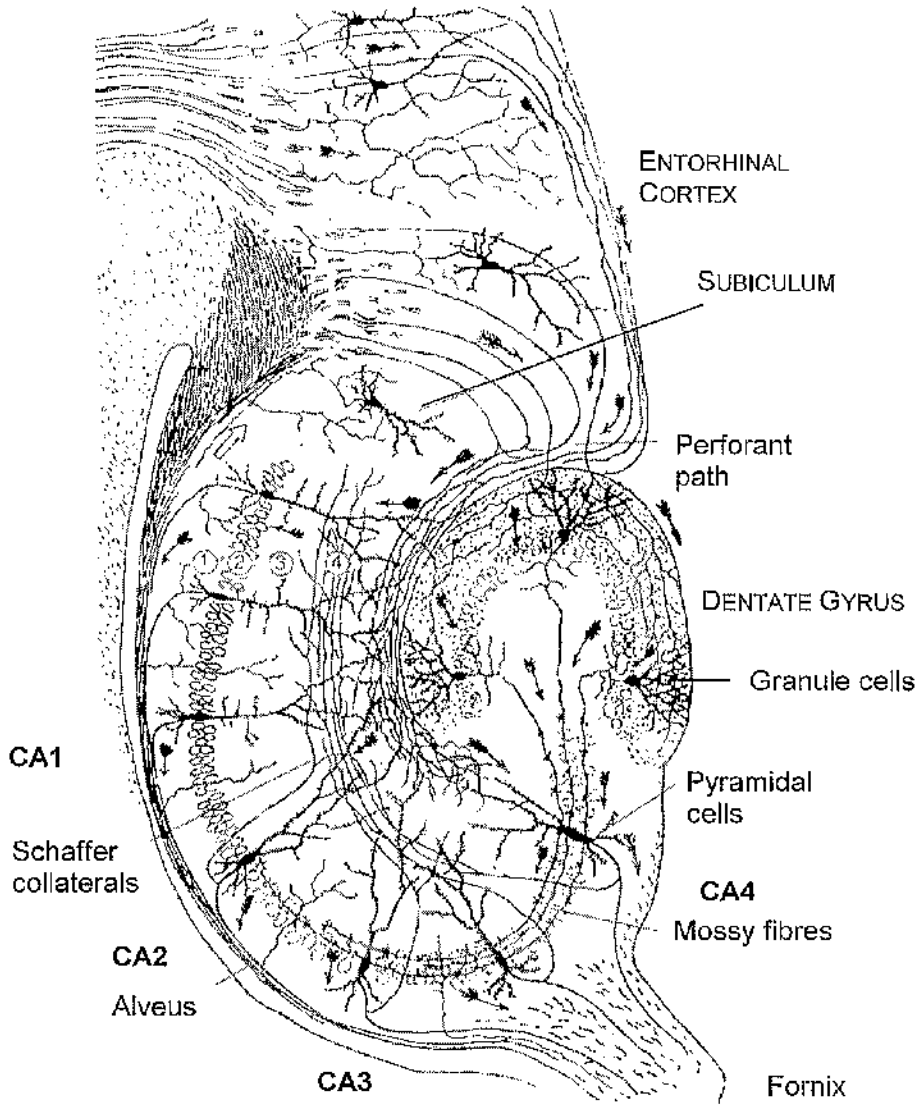

Figure 1.4. *The hippocampal formation.*
Diagram adapted from Brown and Zador, 1990, original drawing by Raymon y Cajal, 1911. Each region of the hippocampal formation is indicated, dentate gyrus, entorhinal cortex, subiculum with the hippocampus proper separated into CA1-CA4 regions. All principal cell types are illustrated and the direction of the arrows represents the flow of information throughout the hippocampal formation. The circled numbers 1-4 indicate the different strata of the hippocampus; 1-*stratum oriens*, 2-*stratum pyramidale*, 3-*stratum radiatum*, 4- *stratum lacunosum-moleculare*. Also shown are the certain excitatory pathways including the Perforant path, the mossy fibers and Schaffer collaterals.

1.4.3 Cytology

1.4.3.1 Principal neurones

The *pyramidal cells* are the principal cells and the most numerous classes of neurones of the hippocampus proper. The cell bodies of the hippocampal pyramidal neurones are arranged, 3-6 cells deep, in the pyramidal cell layer. These neurones have elaborate dendritic trees extending perpendicularly to the cell layer in both directions and are thus considered to be bipolar neurones. The dendrites of pyramidal cells are highly spineous and such spines are known to represent functional microdomains of the cell which receive discrete synaptic inputs. The morphology of pyramidal cells varies gradually from region CA3 to region CA1, the cell bodies become smaller and the apical dendrites longer and more slender (Figure 1.5 B, Brown and Zador, 1990). The apical dendrites are longer than the basal and extended from the apex of the pyramidal cell body toward the center of the hippocasmpus. The basal dendrites extend form the base of the pyramidal cell body fanning out through *stratum oriens*.

The principal cells of the dentate gyrus are the *granule cells*. They have small, spherically shaped cell bodies that are arranged 4-6 cells thick in the granule cell layer. They are the most numerous neuronal type in the hippocampal formation and their soma are densely packed within *stratum granulosum*. They are monopolar with dendrites extending into the molecular layer.

1.4.3.2 Interneurones

There are also many different morphological types of inhibitory interneurones in the hippocampus and dentate gyrus. Although the interneuron/principal cell ratio in the hippocampus is only 1:10–1:12 (Woodson et al., 1989), interneurones have a much higher structural and functional diversity than pyramidal or granule cells (Freund and Buzsaki, 1996). All hippocampal interneurones are thought to be GABAergic and inhibitory in simple terms. Their diversity is manifested in their

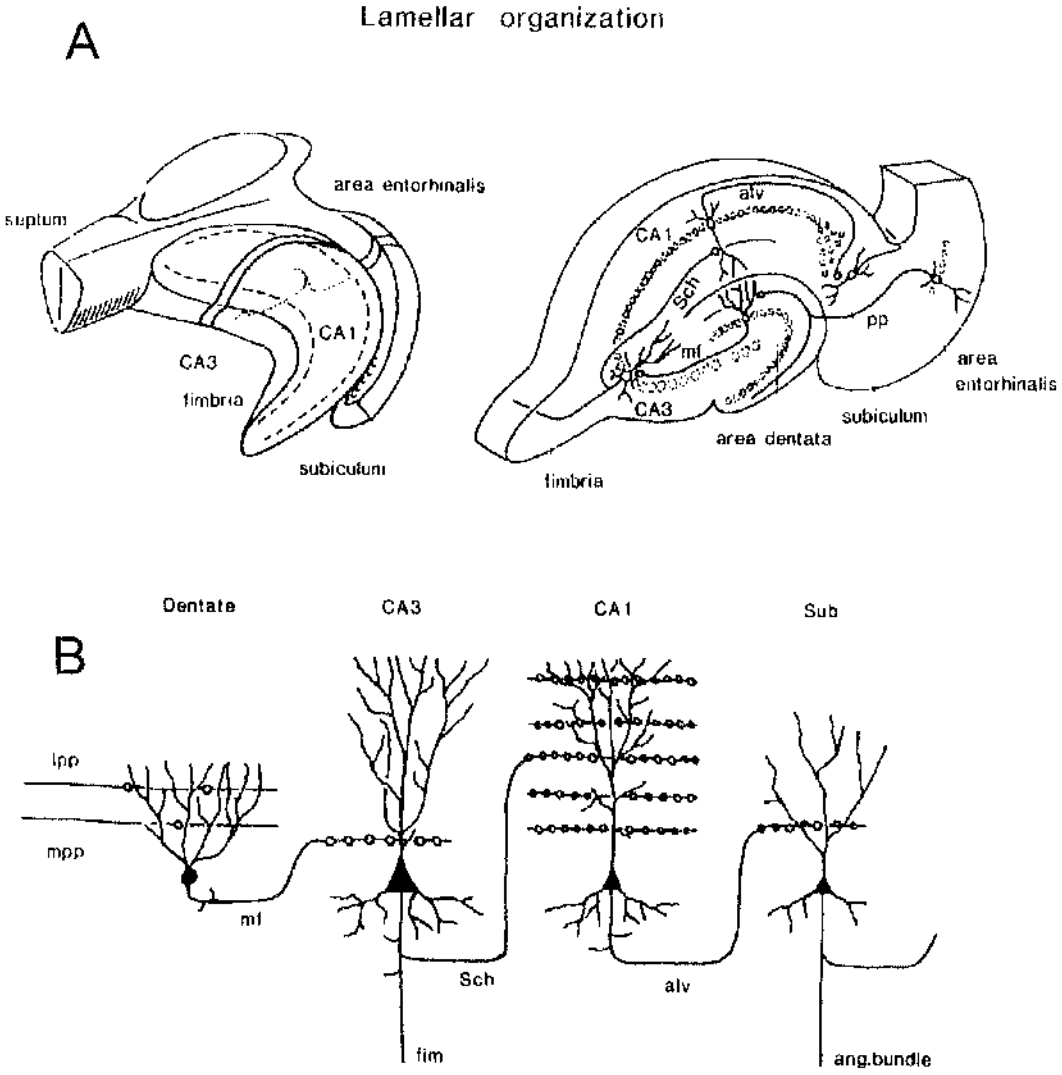

Figure 1.5. *Structure of the transverse hippocampal slice and the trisynaptic circuit.* A. A slice cut perpendicular to the long axis of the hippocampus shows several regions of the hippocampal formation and indicating the principal intrinsic connections. B. Diagram shows typical principal cells within each region of the hippocampus and their connectivity by outlining the three major synapses in the hippocampus. Fibres of the perforant path (pp) synapse onto dentate gyrus granule cells. The granule cells send their major axons to synapse onto the CA3 pyramidal cells via mossy fibres (mf). The CA3 pyramidal cells send a major axon collateral, the Schaffer collaterals (Sch), to synapse onto the CA1 pyramidal cells. To complete the circuit CA1 pyramidal cells send their axons back to the deep layers of the entorhinal cortex partially via the subicular complex. Note that the morphology of pyramidal cells varies gradually from region CA3 to region CA1, the cell bodies become smaller and the apical dendrites longer and more slender.

anatomical, neurochemical, electrophysiological and pharmacological properties (Freund and Buzsaki, 1996).The vast majority of interneurones do indeed have locally restricted target regions, generally lack spines, and are GABAergic (Freund and Buzsaki, 1996). The best studied interneuron is the basket cells which form inhibitory contacts onto CA1 pyramidal cells and receive excitatory input from these same CA1 pyramidal cells (Buhl et al., 1995). These interneurones thus appear to mediate both feedforward and feedback inhibition of pyramidal neurones (Brown and Zador, 1990).

The glutamatergic principal (pyramidal and granule) cells along with the divers population of GABAergic interneurones as well as a poorly defined population of mossy cells and cholinergic interneurones (Frotscher et al., 1986; 2000), make up the hippocampal neuronal network. It is proposed that patterned signalling of the principal cells coupled with changes in synaptic strength known as plasticity at the glutamatergic synapses may underlie hippocampal dependent tasks including adaptive processes such as learning and memory. However the interneurones of the hippocampus are extremely important in that they act to govern principal cell properties such as action potential generation, firing patterns and membrane potential oscillations, while also regulating other interneurones. Overall, it is suggested that they set conditions for synaptic plasticity (Paulsen and Moser, 1998) and have role in the synchronisation and patterning of principal cell activity (Cobb et al., 1995; Freund and Buzsaki, 1996).

1.4.4 Hippocampal connectivity

The major input into the hippocampus is from the entorhinal cortex in the form of the perforent path. The perforant path originates in the superficial layers of medial and lateral entorhinal cortex and terminate in either the hippocampus or dentate gyrus (Lorente de No, 1934; Raisman et al., 1966). The perforent path input to the dentate granule cells is particularly strong. The dentate gyrus in turn sends a projection, the mossy fibre projection, which selectively innervates CA3 pyramidal cells of proximal dendritic sites. This selective input forms the lamina

stratum lucidum. The CA3 pyramidal cells in turn give rise to highly collateralised axons that distribute fibres both within the hippocampus as well as sending fibres to contralateral hippocampus and also to subcortical regions such as the lateral septal nucleus. The projections within hippocampus from CA3 pyramidal cells to the CA1 field are among the most intensively studied pathways in the brain and are known as *the Schaffer collaterals*. Within the CA3 region, CA3 pyramidal cells give rise to a high degree of recurrent (feedback) collaterals, which form what is referred to as *the associational pathway*. It is this high degree of recurrent excitatory connectivity that is suggested to be the basis for the hippocampus to be a particularly seizure prone brain structure (see below). Unlike the CA3 field, pyramidal cells in CA1 do not appear to give rise to a major set of collaterals that distribute within CA1 (Amaral et al., 1991; Tamamaki et al., 1987). The CA1 field rather gives rise to a projection to the entorhinal cortex, by way of the adjacent subiculum (Amaral et al., 1991). A small number of CA1 neurones may project to the contralateral CA1 (van Groen and Wyss, 1990). As the CA1 axons extend in the alveus or in stratum oriens towards the subiculum, occasional collaterals arise and terminate on the dendrites of other CA1 cells (Deuchars and Thomson, 1996), but the massive associational network which is so apparent in CA3 is largely missing in CA1.

The functional organisation of the hippocampus has traditionally been described in terms of the *trisynaptic circuit* (Andersen et al., 1966, Figure 1.5 B). This older view is still useful for introducing the hippocampus. The fibres of the perforant pathway arise from entorhinal cortex form excitatory synapses onto the dendritic spines of the granule cells. The granule cells of the dentate gyrus send their mossy fibre axons to the CA3 region. The pyramidal neurones of the CA3 region send their Schaffer collateral axons to the CA1 region. To complete the circuit CA1 pyramidal cells send their axons back to the entorhinal cortex via the subicular complex. These three synapses define the trisynaptic circuit, which is a purely feed forward network (Brown and Zador, 1990). The circuit described includes a simple sequence of excitatory connections from one region to the next, forming a closed loop that enables unidirectional flow of information. More over, each

region, with the exception of the dentate gyrus, projects not only to the next region in the sequence but also to one or two after it (Brown and Zador, 1990). Thus, multiple closed excitatory loops appear to make up the normal hippocampal circuitry.

1.4.5 Local interactions

In addition to synaptic connections among regions of the hippocampus formation, there are also relatively complex synaptic interactions within each region. These local circuits consist of principal neurones and a diverse array of GABAergic interneurones (Freund and Buzsaki, 1996).

Recurrent axon collaterals of CA3 pyramidal cells make extensive excitatory synapses on the dendrites of neighbouring pyramidal cells (Li et al., 1994) so that when one pyramidal cell fires, its neighbours are powerfully excited (MacVicar and Dudec, 1982; Miles and Wong, 1983). This facilitates the rapid synchronisation of action-potential firing in CA3 neurones that underlies the normal electroencephalographic pattern known as hippocampal sharp wave activity (Buzsaki, 1986) and periodic *in vitro* burst discharges (Traub, 1991). This synchrony is important for activity-dependent modification of synaptic strength (Buzsaki, 1986), but the positive feedback from recurrent collaterals that underlies the synchronisation should produce continuous discharging of all CA3 neurones, such as may occur during human temporal lobe seizures (Jefferys, 1993). But why does this not happen under normal conditions and what process terminates burst activity?

The primary mechanism terminating CA3 bursts are inhibitory conductances. As indicated earlier, the pyramidal neurones of the CA3 region also receive powerful synaptic inhibition. There is both feedback and feedforward synaptic inhibition of the pyramidal neurones in the CA3 region (Brown and Zador, 1990).

Moreover, calcium influx during the burst triggers a potassium current that results in an afterhyperpolarisation that can terminate bursts of action potentials initiated by a depolarising current injection (Wong, 1981). Thus the afterhyperpolarisation, in conjunction with inhibitory postsynaptic conductances, is a logical mechanism for burst termination (Traub, 1991). Some evidence, however, raise the possibility that inhibitory conductances are not the primary mechanism terminating CA3 bursts. According to Staley *et al* (1998), population-burst duration is limited by depletion of the releasable glutamate pool at these recurrent synapses and postsynaptic inhibitory conductances further limit burst duration but are not necessary for burst termination. The interval between bursts *in vitro* depends on the rate of replenishment of releasable glutamate vesicles and the probability of release of those vesicles at recurrent synapses. Therefore presynaptic factors controlling glutamate release at recurrent synapses regulate the probability and duration of synchronous discharges of the CA3 network (Staley et al., 1998).

1.4.6 Input and output pathways

The major inputs to the hippocampus and dentate gyrus arise from the entorhinal cortex, the contralateral hippocampus, and the septal region. There are also important but less numerous projections from several other regions including the brain stem, hypothalamus, thalamus and amygdala (Brown and Zador, 1990).

The entorhinal cortex, a transitional area between the cortex and hippocampus, provides a major sensory input to the hippocampus, dentate gyrus and subiculum via the fibres of the alvear and perforant pathways. The fibres of the perforant pathway arise from stellate and pyramidal cells in layers 2 and 3 of the medial (non-olfactory) and lateral (olfactory) entorhinal cortex (Steward, 1976). They pass through the subicular complex and form excitatory synapses onto the dendritic spines of the granule cells. The entorhinal cortex also receives inputs from many other regions of the brain including the association cortices, several thalamic nuclei, the claustrum, and the amygdala.

The medial septum and diagonal band of Broca provide one of the major subcortical inputs to the hippocampus. Five cell types have been distinguished in these nuclei (Jakab and Leranth, 1995), however the two major types are cholinergic and GABAergic in nature. These neurones have large cell bodies and project via the fimbria-fornix to terminate in all levels of the hippocampus. This projection was first suggested in 1910 (Herrick, 1910) and is now firmly established.

The medial septum provides a major cholinergic and GABAergic input to the hippocampus. The cholinergic afferents of the septo-hippocampal input are thin beaded fibres (Heimrich and Frotscher, 1993) making up 2/3 of all projections. These projections contact both interneurones and principal cells (pyramidal and dentate granule cells) synapsing on dendritic shafts, spines and cell bodies (Heimrich and Frotscher, 1993). The GABAergic afferents are thick in diameter with large varicose swellings and make up only 1/3 of all projections. These GABAergic afferents contact only interneurones (Freund and Antal, 1988).

The cholinergic nature of the septo-hippocampal projection was first demonstrated in 1967 (Lewis and Shute, 1967) and almost a decade later septal stimulation was shown to cause a direct increase in acetylcholine released within the hippocampus (Dudar, 1977; Smith, 1974). The cholinergic component of the septo-hippocampal input has received considerable attention due to its prominent role in cognitive functions, if the input is destroyed hippocampal dependant learning is severely impaired (Dutar et al., 1995; Hasselmo, 1999). The cholinergic input is involved in generation and patterning of theta activity (Cobb et al., 1999) through the action of acetylcholine at principal cells and interneurones. The septal afferents are not the only source of acetylcholine as local cholinergic neurones also exist (Frotscher et al., 1986; 2000).

The hippocampus also has several output pathways and targets. One major output proceeds via the fimbria, which is a sheet composed of the axons of pyramidal cells and cells in the subiculum. These axons then gather to form the fornix, which

crosses the midline of the brain. The other major outputs are to the subiculum and to the deep layers of the entorhinal cortex. Thus the hippocampus projects to many of the same regions that provide its input (Brown and Zador, 1990).

1.4.7 Neurotransmission in the hippocampus

1.4.7.1 Gamma-amino butyric acid (GABA)

GABA is considered the main inhibitory transmitter in the hippocampus (Roberts et al., 1976), which can be released by inhibitory interneurones onto the soma, dendritic regions and axons of pyramidal and granule neurones (Buhl et al., 1994; Freund and Buzsaki, 1996). Although glycine is a prominent inhibitory neurotransmitter in the spinal cord and in some brain regions, it plays little role as a classical neurotransmitter in the hippocampus.

It was once believed that inhibitory synapses were primarily on the cell bodies of pyramidal neurones (Andersen et al., 1964). There is now much evidence that GABAergic synapses occur both on the cell bodies as well as throughout the neurone.

1.4.7.2 Glutamate

Glutamate is the most important excitatory neurotransmitter in the hippocampus (Roberts et al., 1981). Various combinations of AMPA, kainate and NMDA receptors are present at all of the excitatory pathways of hippocampus although there may be variations at individual synapses (Johnston and Amaral, 1998). For example there are fewer NMDA receptors at mossy fibre synapses (Monaghan et al., 1983). Also it has been proposed that some Schaffer collateral synapses contain only NMDA receptors (Isaac et al., 1995; Liao et al., 1995). The metabotropic glutamate receptors are also present at glutamatergic synapses, both at the pre- and postsynaptic side of synapse. They coexist in different

combinations with ionotropic receptors postsynaptically and modulate transmitter release presynaptically (Schoepp and Conn, 1993).

1.4.7.3 Acetylcholine

Acetylcholine (ACh) is widely distributed in the hippocampus, where it exerts a number of neuromodulatory effects. The hippocampus exhibits a particularly high level of nAChR expression on both principal cells and GABAergic interneurones where they are located at presynaptic, postsynaptic and extra-synaptic sites (Fabian-Fine et al., 2001). As such, nAChRs are ideally placed to regulate neuronal excitability within hippocampal circuits. In this respect, nAChRs classically mediate fast acetylcholine-mediated neurotransmission in the hippocampus (Alkondon et al., 1998; Frazier et al., 1998a; 1998b; Jones and Yakel, 1997; McQuiston and Madison, 1999b). In addition, there is now mounting evidence for both excitatory and inhibitory modulatory effects of nAChR activation. Thus, neurochemical evidence indicates that activation of presynaptic nAChRs enhances the release of neurotransmitters such as acetylcholine, GABA and glutamate (Gray et al., 1996; Vogt and Regehr, 2001; Wonnacott et al., 1989). Therefore, both excitatory and inhibitory synapses can be modulated by nAChR activation.

In the hippocampus, fast nAChR-mediated transmission has been identified only onto GABAergic interneurones. nAChR activation produces a variety of responses in interneurones. The majority of interneurones show a fast depolarisation mediated by α7subunit containing receptors (Frazier et al., 1998a; 1998b; McQuiston and Madison, 1999b). However, McQuiston and Madison (1999) also reported a subset of interneurones that showed a fast depolarisation in combination with a slower response and Alkondon (1997) reported seeing similar response in some interneurones but also interneurones which displayed only a slow response probably due to the activation of α4β2 nAChR. Interneurones showing no nicotinic response at all have also been observed (McQuiston and Madison, 1999b).

Functional nAChRs are also present on pyramidal cells (Albuquerque et al., 1997; Alkondon et al., 1997; Ji et al., 2001) yet the production of postsynaptic responses in these cells is the subject of an ongoing dispute. Alkondon (1997) reported that pyramidal cells responded to application of nicotinic agonist with a slow depolarisation, however studies by Frazier (1998b) and McQuiston and Madison (1999b) generally disagree with these findings reporting no response in the majority of pyramidal cells. In another study, properly timed nicotinic activity at pyramidal neurones increased the induction of long-term potentiation via presynaptic and postsynaptic pathways but nAChRs activation evoked currents in these cells were smaller than in interneurones (Ji et al., 2001).

mAChRs have been described at both pre and post synaptic sites in the hippocampus (Williams and Johnston, 1993). Their presynaptic effects are to decrease glutamate and GABA release and thus could be considered inhibitory or excitatory. Their postsynaptic effects are to decrease potassium conductances, the M current, I_M (Halliwell and Adams, 1982) and the calcium activated current, I_{AHP} (Storm, 1990) , which in turn produces a depolarisation of the postsynaptic neurone, making it more likely to fire an action potential (Halliwell and Adams, 1982). This action is decidedly excitatory because not only is the neurone depolarised by the action of ACh on muscarinic receptors but the decrease in the potassium conductance increase the input resistance, making other concomitant excitatory inputs more likely to fire the neurone (Johnston and Amaral, 1998). By Recording directly from interneurones, using the whole cell patch clamp technique, McQuiston and Madison (1999) have recently shown that mAChR activation produces a variety of responses in interneurones including depolarisation, hyperpolarisation, a biphasic response or no response but failed to find a relationship between interneurone subtype and the nature of the pharmacologically induced cholinergic responses (McQuiston and Madison, 1999a). Moreover, such changes are not reported to be associated by a change in input resistance in interneurones. Similar findings have recently been reported in

interneurones in response to physiological stimulation of cholinergic afferents (Ferrigan et al., 2003).

1.4.8 hippocampal slices

Since it was shown that slices of brain tissue could be maintained *in vitro*, slices have been extensively used as *in vitro* models of CNS activity (Yamamoto and McIlwain, 1966). *In vitro* brain slices also have considerable value in pharmacological experiments whereby the physiological actions of neurotransmitters and drugs can be easily investigated. Several aspects of the slice preparation facilitate this type of analysis. Many classical pharmacological techniques (e.g., dose-response curves, tests for competitive and non-competitive antagonisms) can be used to examine drug responses because drugs can be applied and tested in a relatively quantitative manner (Dunwiddie et al., 1983).

Transverse hippocampal slice has been so useful in evaluating synaptic and circuit properties because trisynaptic circuitry is organised approximately in a plain that is perpendicular to the long axis of the hippocampus (Andersen, 1971). This is why organisation of the hippocampus is sometimes termed "Lamellar". This parallel lamellar organisation favours the use of the hippocampus as a slice preparation since each slice containing all the functional areas and can act as a single unit. However, the actual synaptic organisation is not exclusively lamellar. There are, in fact, significant longitudinal connections that are not preserved within thin transverse brain slices (Amaral, 1987).

According to Jefferys (1994), in hippocampal slices seizure-like activity can be induced with ease by a variety of epileptogenic agents, and stable potentials can be recorded over several hours. Moreover, drugs can be used without the restriction of the *Blood Brain Barrier* and systemic metabolism. However, for studies of seizure-like activities, which involve larger areas of the brain, absence of the rest of the brain can be a real problem and influence these studies (Jefferys, 1994).

1.4.9 The hippocampus and epilepsy

Despite practically every part of the brain being able to generate an epileptic seizure, not all parts of brain are equally susceptible to epileptic activity. In general it is the cortex which is most directly implicated, either the neocortex, or older regions such as the hippocampus, entorhinal cortex and piriform cortex. Moreover, the interaction of the cerebral cortex and the thalamus, in conjunction with intrathalamic communication, can generate spike waves similar to those occurring during human absence seizure discharges. Therefore, the structure affected determines the kinds of symptoms, the time course and to some extent the cellular substrate for the epileptic activity (Jefferys, 1993).

The hippocampus is a major site of seizure generation (Schwartzkroin, 1994) and it has the lowest seizure threshold of any brain region (Green 1964). Clinical foci here often present a major challenge to current drug therapies and can result in cognitive impairments. Indeed, epilepsies with a hippocampal foci constitute a significant part of the caseload in surgery for epilepsy (Laidlaw et al., 1993).

In the hippocampus, the CA3 region normally initiates epileptic discharges resembling brief "interictal" electroencephalogram spikes (Jefferys, 1993; Traub, 1991). CA3 susceptibility results from relatively powerful connections between its pyramidal cells (MacVicar and Dudec, 1982; Miles and Wong, 1983), with unitary excitatory postsynaptic potentials (EPSP) up to approximately 1mv between 1% and 2% of pairs of neurones (Jefferys, 1994).

The cells of the CA3 region can produce paroxysms, which transmit to the CA1 region in a number of models in dependent of the dentate gyrus (Schwartzkroin and Prince, 1978). For this reason, the CA3 region has been named the "epileptic pacemaker" for the hippocampus (Lothman and Collins, 1990). Ionic movement through numerous voltage and ligand gated channels can determine the basal level of cell excitability. Transmission between CA3 neurones can be also amplified by "intrinsic burst" driven by dendritic voltage-dependent calcium currents (Wong and Prince, 1978). Even under normal conditions CA3 cells can produce

spontaneously bursting activity which resembles paroxysmal depolarisation shifts (PDS) (Wong and Prince, 1978). As indicated earlier, connections between CA3 cells are numerous and excitatory, thus excitation in one cell can be communicated to the next and so forth. Normally synaptic inhibition contains the spread of excitation through the CA3 network, but when this is blocked, or excitation or excitability is enhanced, activity in one or more neurones causes a chain reaction which recruits all the neurones within five to six synaptic relays, or a few 10s of milliseconds (Jefferys, 1993; Traub, 1991). Recurrent discharges generated by CA3 cells spread to CA1 where ictal events can be generated. Pyramidal CA3 cells may act as interictal spikes pacemakers, because of very high calcium conductance and a profuse disposition of recurrent axonal collaterals, predisposing to PDS (Ure and Perassolo, 2000). Conversely, CA1 region is able to maintain seizures by itself, without previous interictal spikes (Lothman and Collins, 1990).

When ictal activity encompasses the dentate gyrus, it will increase in CA1, subiculum and entorhinal cortex, which can initiate the discharges via the perforant pathways making epilepsy difficult to arrest (Heinemann et al., 1994). Moreover, other features of the hippocampus, electronic synapses, feedback loops and ephaptic coupling are important in epilepsy and can reinforce the synchronisation of activity (Reviewed by Jefferys, 1995). The anatomical arrangement of neurones into regular sheets provides an ideal substrate for the action of electric fields on membrane potentials. This complex organisation with so much potential for aberrant excitation makes the hippocampus so prone to epileptic tendencies.

1.5 *In vitro* epileptiform models

1.5.1 General

In vitro models of epileptiform activity have been used extensively over the last few decades to investigate the genesis of epilepsy-like behaviours within

hippocampal and neocortical circuits. These models generally involve slices of brain tissue, for example hippocampus (Morris et al., 1996; Voskuyl and Albus, 1985; Watts and Jefferys, 1993), neocortex (Mattia et al., 1993) and amygdala (Arvanov et al., 1995).These preparations can be manipulated by either changing the medium used for perfusion or by the direct application of compounds to the slice. Epileptiform activity is generated by the raising the basal level of excitation or by altering the ion content of the perfusing medium. Epileptiform activity can take the form of spontaneous bursts of population spikes or as multiple waveforms in response to afferent stimulation. As mentioned earlier, epileptiform activity *in vitro* consisting of short periods of defined activity is referred to as inter-ictal due to the resemblance to this activity *in vivo*. Similarly periods of intense activity lasting seconds to minutes is denoted ictal-like activity. Although caution must be exercised when making direct comparisons between situations *in vitro* and *in vivo*, *in vitro* models have been beneficial in understanding the fundamental mechanisms which may underlie the generation of human epilepsy (Jefferys, 1993).

Indeed, *in vivo* models have been used in screening potential anticonvulsant activity of many AEDs. Many commonly used AEDs have been shown to be efficacious in suppressing epileptiform activity in brain slices (Dost and Rundfeldt, 2000; Kapetanovic et al., 1995; Sagratella, 1998; Schneiderman and Schwartzkroin, 1982). Such slice models, whilst reductionism in nature, do therefore represent a good predictive indicator of whether a particular compound or receptor type are likely to be of significance in human epilepsy states.

Moreover, with specific regard to the septo-hippocampal cholinergic system, the morphological and physiological literatures suggest that the human and rodent innervation and cholinergic receptor expression are very similar (Reviewed by Gotti et al., 1997). However, the precise nicotinic receptor distribution in human is still not clear and further studies are needed to complete our understanding of receptor (and particularly receptor subtype) distribution. Knowledge of receptor subtype distribution is important in order to allow the correct correlations to be

made between receptor subtypes and brain functions or pathologies, which would thus assist in the search for the subtype-specific therapeutic agents.

1.5.2 4-aminopyridine- induced epileptiform activity

As a specific blocker of K^+ current (Hermann and Gorman, 1981), 4-aminopyridine (4AP) has been widely used as a pharmacological agent in diverse studies of K^+ channels (Klee et al., 1995; Storm, 1988; Ulbricht and Wagner, 1976), mechanisms of epileptogenesis (Chesnut and Swann, 1988; Galvan et al., 1982; Rutecki et al., 1987; Voskuyl and Albus, 1985) and synaptic plasticity (Andreasen and Lambert, 1999; Buckle and Haas, 1982; Lee et al., 1986). 4AP can induce epileptiform activity *in vitro* (Avoli et al., 1996; Chesnut and Swann, 1988; Galvan et al., 1982; Traub et al., 1995; Watts and Jefferys, 1993) and *in vivo* (Morales-Villagran et al., 1996; Szente and Baranyi, 1987; Szente and Pongracz, 1979) as well as producing clinical seizure in man (Spyker et al., 1980; Thesleff, 1980). Relevant biophysical actions of 4AP include the following: (1) Blockade of transient K^+ currents in postsynaptic soma-dendritic membrane, including the fast A current and the slower "delay" (D) current. The A current is a rapidly activating and deactivating current and involved in controlling excitability via a role in spike repolarisation (Voskuyl and Albus, 1985). Epileptogenic concentrations of 4AP (<75 μM) probably do not block the A current (Storm, 1988; Traub et al., 1995). The D current has a rapid activation but a slow inactivation, which introduces a long delay in firing induced by just threshold depolarisations (Storm, 1988). The D current is more sensitive to 4AP with complete blockage occurring between 30-40 μM (Storm, 1988). (2) An increase in the excitability of axons and presynaptic terminals (Buckle and Haas, 1982; Kocsis et al., 1983), probably also caused by K^+ current blockade which prolongs the action potential in the nerve terminals, allowing a greater calcium influx and thus a larger transmitter release (Molgo et al., 1977). (3) A direct effect on voltage dependent calcium channels could also be an effect of 4AP (Lundh and Thesleff, 1977; Rogawski and Barker, 1983).

Three types of spontaneous, synchronous activity were recorded in the presence of 4AP. The most prevalent is the generation of frequently occurring bursts of short duration which are reminiscent of inter-ictal activity seen *in vivo* (Avoli et al., 1993; Mattia et al., 1994; Morris et al., 1996; Voskuyl and Albus, 1985; Watts and Jefferys, 1993). Less frequent negative potentials which have been proposed to be GABAergic have been found in some instances (Voskuyl and Albus, 1985; Mattia et al., 1994; Michelson and Wong, 1994; Morris et al., 1996; Perreault and Avoli, 1991; 1992; Avoli et al., 1993;). Prolonged seizure-like bursts, ictal activity, lasting up to tens of seconds can also occur (Avoli et al., 1993; 1996; Gean, 1990; Mattia et al., 1993; Watts and Jefferys, 1993).

The involvement of glutamatergic transmission in 4AP-induced epileptiform activity has been investigated using selective NMDA and non-NMDA receptor antagonists. There are some reports showing that interictal and ictal-like activity induced by 4AP are suppressed by non–NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX) (Avoli et al., 1996; Avoli et al., 1993; Gean, 1990; Perreault and Avoli, 1991). NMDA receptor antagonists generally have no or very little effect on 4AP-induced bursts (Avoli et al., 1993; Gean, 1990; Perreault and Avoli, 1992). However, exceptions have been reported. Avoli *et al* (1996) showed that ictal-like events in the entorhinal cortex were abolished by an NMDA receptor antagonist 3-(±-2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid (CPP) (Avoli et al., 1996). This data was further supported by Gulyas-Kovacs *et al* (2002), who showed that another NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid (APV) significantly decreased frequency of 4AP-induced epileptiform activity (Gulyas-Kovacs et al., 2002). Inter-species differences, the cortical area from which the slice originated and different experimental conditions may account for such differences. For example, 4AP-induced ictal-like bursts in rat neocortex were inhibited by non-NMDA receptor antagonist and not affected by a NMDA receptor antagonist, whereas the opposite finding was observed with slices of guinea-pig origin (Mattia et al., 1993).

The disappearance of 4AP bursts in low or zero calcium media also suggests a dependence on synaptic activity for generation (Gean, 1990; Voskuyl and Albus, 1985).

One of the unusual features of 4AP-induced epileptiform activity is that both excitatory and inhibitory synaptic transmission is enhanced (Perreault and Avoli, 1991; Rutecki et al., 1987; Tapia and Sitges, 1982). Unlike other convulsant drugs that act primarily by diminishing the efficiency of GABA-mediated inhibition, the evidence available indicates that 4AP-induced epileptiform activity occur despite the presence of normal or even enhanced synaptic inhibition (Chesnut and Swann, 1988; Rutecki et al., 1987).

1.5.3 Epileptiform activity induced by GABA receptor antagonists

In respect to the action of these compounds epileptiform activity results primarily from reduced GABA mediated inhibition. Reducing the amplitude of $GABA_A$-mediated IPSPs by 20% is sufficient for synchronous discharges in neocortex (Chagnac-Amitai and Connors, 1989). However, the hippocampus is more resistance, requiring a nearly complete block of evoked inhibitory potentials for synchronous bursts (Schneiderman et al., 1992). While much of the early work studied the topical application of these compounds to the neocortex, hippocampus and other structure *in vivo*, most of current understanding of the cellular mechanisms of their actions derives from work on brain slices (Jefferys, 1994). Several *in vitro* models have produced epileptiform activity by using various GABA receptor antagonists, for example picrotoxin (Knowles et al., 1987; Kohr and Heinemann, 1990) and bicuculline (Colom and Saggau, 1994; Gulyas-Kovacs et al., 2002; Herron et al., 1985). The activity found with these disinhibitory models is reminiscent of the interictal spike of the epileptic EEG (Jefferys, 1994).

The synchronisation of neuronal discharges in disinhibited hippocampal slices using GABA receptor antagonists has been studied in some detail. These studies

revealed that hippocampal neurones are required through a network of recurrent excitatory synaptic connections between CA3 pyramidal cells (Miles and Wong, 1987; Traub and Wong, 1982). Recurrent GABA mediated inhibition normally exerts a tight control over the spread of activity via polysynaptic recurrent excitatory pathways between CA3 cells (Miles and Wong, 1987). Disinhibition of hippocampal slices by picrotoxin or bicuculline revealed a polysynaptic connection between cells, which previously were not connected. Bicuculline decreases the amplitude of inhibitory postsynaptic potentials and impairs inhibition, therefore facilitate propagation of excitatory signals through multisynaptic pathways (Gutnick et al., 1982; Miles and Wong, 1987).

Development of synchronised PDSs contribute to burst generation in the spontaneous epileptiform process (Prince, 1968). In bicuculline-containing solutions a regenerative Ca^{2+} spike is suggested to initiate depolarisation and contribute to the PDS formation (de Curtis et al., 1999). The cellular mechanisms involved in the generation of spontaneous epileptiform potentials in bicuculline model are investigated in the pirifom cortex of the *in vitro* isolated guinea-pig brain (de Curtis et al., 1999). In this study, a large plateau potential similar to that observed in neocortical pyramidal neurones bathed in tetrodotoxin and tetraethylammonium (Friedman and Gutnick, 1989) is activated at high threshold during the spontaneous interictal spike when the sodium conductance is blocked in the presence of a calcium chelator. Such a plateau potential was reversibly abolished by a selective blocker of the calcium conductances and by membrane potential hyperpolarisation. The exact threshold of activation of this calcium potential could not be determined.

1.5.4 Low magnesium-induced epileptiform activity

Reduced levels of magnesium have been associated with symptoms of clinical epilepsy (Durlach, 1967). Slice preparations of various central nervous system tissues generate epileptiform activity when perfused with a medium devoid of added magnesium: hippocampus (Mody et al., 1987; Schneiderman and

MacDonald, 1987; Whittington et al., 1995), entorhinal cortex (Jones, 1989), neocortex (Horne et al., 1986) and amygdala (Gean and Shinnick-Gallagher, 1988).

At normal physiological concentrations magnesium exerts a voltage-dependent block on NMDA receptors by binding to a site which is thought to be located within the ion channel (Ascher and Nowak, 1988; Mayer et al., 1984; Nowak et al., 1984). Relief of this block can ensue during periods of depolarisation, allowing the movement of Na^+, K^+ and Ca^{2+} through the receptor channel. The activation of NMDA receptors following removal of magnesium from the bathing medium is considered to be the principal mechanism involved in the generation of epileptiform activity in low magnesium model. Evidence comes from the total or partial reduction of epileptiform activity in the presence of selective NMDA receptor antagonists (Horne et al., 1986; Mody et al., 1987; Schneiderman and MacDonald, 1987; Tancredi et al., 1990).

In general the epileptiform activity instigated by low magnesium medium is inter-ictal-like and characterised by burst of milliseconds (Mody et al., 1987; Tancredi et al., 1990; Whittington et al., 1995). Ictal events with corresponding tonic and clonic periods of activity has also been recorded in hippocampal slices using a low magnesium medium (Anderson et al., 1986).

GABAergic inhibition is still evident during low magnesium epileptiform activity due to the maintained ability to evoke inhibitory postsynaptic potentials (IPSPs) (Tancredi et al., 1990) and thus may act to limit the extent of firing. In other studies inhibition has been found to be reduced in comparison to normal circumstances (el-Beheiry and Puil, 1990; Jefferys, 1994).

1.5.5 Elevated potassium-induced epileptiform activity

The extracellular concentration of potassium rises during seizures induced *in vivo* in cats (Fisher et al., 1976; Moody et al., 1974) and *in vitro* hippocampal slices

(Yaari et al., 1986). Basal extracellular potassium levels are approximately 3 mM. Raising the concentration of potassium in the medium bathing slices to 5-10 mm results in the generation of epileptiform activity *in vitro* (McBain, 1994; Traub and Dingledine, 1990; Traynelis and Dingledine, 1988).

Inter-ictal activity in the CA3 region of hippocampus occur when slices are bathed in a high potassium medium (Korn et al., 1987; Rutecki et al., 1985; Traynelis and Dingledine, 1988). This activity propagates to the CA1 where intense ictal like activity results (Traynelis and Dingledine, 1988). The transition from inter-ictal to ictal-like activity has been suggested to revolve around an increase in potassium concentration (Dichter et al., 1972).

Increased extracellular potassium reduces potassium efflux as a result of a modified concentration gradient (Dietzel et al., 1980) which increase neuronal excitability and increases incidence of spontaneous EPSPs (Jefferys, 1993). Increased potassium levels, paralleled with a decreased potassium driving force, cause a decrease in the amplitude of a potassium mediated after hyperpolarising potential (AHP) and a decrease in the amplitude of GABAergic IPSPs (Korn et al., 1987; McBain, 1994). Inhibition is depressed in this model because the reversal potential for Cl^- shifts towards the resting potential but it is not abolished (Jefferys, 1993).

1.5.6 Low calcium-induced epileptiform activity

Reducing extracellular calcium $[Ca^{2+}]_o$ is a method of inducing epileptiform activity in rat hippocampal brain slices that effectively blocks all chemical synaptic transmission (Haas and Jefferys, 1984; Jefferys and Haas, 1982; Taylor and Dudek, 1982). This model of synchronisation was reported by several laboratories within a few weeks of each other (Haas and Jefferys, 1984; Jefferys and Haas, 1982; Taylor and Dudek, 1982). Hippocampal slices bathed in solutions containing level of $[Ca^{2+}]_o$ low enough (0-0.2 mM) to block synaptic transmission, after a delay of tens of minutes, started to generate synchronous discharges. These

discharges differed from those seen with more commonly used convulsant treatments in originating most easily from the CA1 region. They also differed in their appearance which is more prolonged lasting up to tens of seconds (Jefferys, 1993).

Two non-synaptic mechanisms were at work in the low $[Ca^{2+}]_o$ field bursts. Fast synchronisation which is mediated by electric field effects and slower synchronisation is mediated through fluctuations in $[K^+]$ (Dudek et al., 1986; Haas and Jefferys, 1984; Konnerth et al., 1984). The electric field effects are caused by the extracellular currents generated by the activity of one group of neurones depolarising the membranes of their neighbours enough to change their excitability. This phenomenon has also been termed ephaptic interaction. As with the low magnesium model a reduction in the concentration of divalent cations reduces membrane charge screening and produces high extracellular current densities and thus facilitates membrane depolarisation (Frankenhaeuser and Hodgkin, 1957; McLaughlin et al., 1971). The slower form of non-synaptic synchronisation is due to fluctuations in extracellular ions, specially increased $[K^+]$, result from neuronal activity, and tend to depolarise neighbouring neurones and glia (Konnerth et al., 1984). A reduction in calcium dependent potassium currents which are responsible for after-hyperpolarisation also contribute to low calcium epileptogenesis (Jefferys and Haas, 1982).

The essential condition for this kind of synchronisation is that the neurones need to be close to threshold. Experimentally this is achieved by the low $[Ca^{2+}]_o$, low to normal $[Mg^{2+}]_o$ and moderately high $[K^+]_o$ (Jefferys, 1993). These conditions can arise during seizures (Heinemann et al., 1986), so that non synaptic synchronisation may well have a role in development of seizure rather than the generation of seizure (Jefferys, 1993). One factor in susceptibility of the CA1 to this kind of synchronisation is the tight packing of the pyramidal cell bodies in *stratum pyramidale*; the restricted extracellular space reinforces both the electric and ionic mechanisms (Jefferys, 1993).

1.5.7 Other models

Much of the early experimental works used intact laboratory animals, either under anaesthesia or freely moving, for studies of the epilepsy as a disorder of whole brain. These preparations *in vivo* have largely replaced by preparation *in vitro*, such as the brain slice, for studies at the level of the single cell or of small population of cells and local circuits. However, studies *in vivo* still have important roles for placing more reductionist studies into context. Even the most distinct focal epileptic discharge tends to propagate to other parts of the brain, and indeed may have profound effects on the body as a whole through the motor, autonomic and endocrine systems. *In vivo* methods also are crucial for screening new anticonvulsants and studying their toxicity and pharmacokinetics. They are also essential for studying seizure propagation and generation, the behavioural correlates of seizures, the long term structural and functional consequences of repeated seizures (Jefferys, 1993).

Kindling is a special chronic model of experimental epilepsies which differs from many of the other chronic models in that it does not necessarily involve convulsant drugs (Jefferys, 1993). Its essential feature is the repeated presentation of subconvulsive stimulus that involves activity-dependent changes in neuronal structure and function (Mody, 1993). The phenomenon of kindling in epilepsy was first discovered by accident by researcher Graham Goddard in 1967. Goddard was studying the learning process in rats, and part of his studies included electrical stimulation of the rats' brains at a very low intensity, too low to cause any type of convulsing. What he found was that after a couple of weeks of this treatment, the rats did experience convulsions when the stimulation was applied (Goddard et al., 1969). Goddard and others later demonstrated that it was possible to induce kindling using a chemical (Bell et al., 1992; Goddard and Douglas, 1975) such as pentylenetetrazol, either injected through an implanted electrode, or given systemically. The definitive feature of kindling is that the electrical and behavioural response to these stimuli progressively increases, so that a constant stimulus which initially can be minimal and clearly non-convulsive response

reduces seizure threshold and eventually triggers generalised motor seizures (Jefferys, 1993).

Activation of muscarinic cholinergic receptors produces oscillations in the hippocampal slice that resemble the theta rhythm, but also may produce abnormal synchronous activity that is more characteristic of epileptiform activity. Pilocarpine, a muscarinic agonist, can produce status epilepticus *in vivo* (Cavalheiroet al., 1991, 1996; Turski et al., 1983, 1984; Liu et al., 1994) as well as inducing epileptiform activity *in vitro* (Nagao et al., 1996; Rasmussen et al., 1996; Rutecki and Yang, 1998). When applied to rat hippocampal slices, pilocarpine (10 μM) produced brief interictal-like activity, as well as more prolonged ictal-like activity, which was comparable to epileptiform activity induced by 4AP. However, the interictal activity observed in pilocarpine-treated slices displayed a lower rate of occurrence and a longer duration than in 4AP-bathed slices (Nagao et al., 1996). All types of synchronous epileptiform activity induced by pilocarpine disappear during application of the non-NMDA receptor antagonist (Nagao et al., 1996). However, NMDA receptor antagonists can only reduce epileptiform discharge duration (Nagao and Avoli, 1994). GABA mediated inhibition is decreased in pilocarpine model (Houser and Escaplez, 1996). Moreover, a recent study has revealed that intrinsic bursting in CA1 pyramidal cells is upregulated in the pilocarpine model (Sanabria et al., 2001).

Numerous other compounds, in addition to the ones already mentioned, have been used both *in vivo* and *in vitro* to generate epileptiform activity:

- Kainic acid (Stringer and Sowell, 1994; Westbrook and Lothman, 1983)
- Penicillin (Dichter and Spencer, 1969; Lathers et al., 1993; Wong and Prince, 1979)
- Pentylenetetrazol (Mirski et al., 1994; Stringer, 1994; Stringer and Sowell, 1994)
- Tetanus toxin (Whittington and Jefferys, 1994)

- Trains of electrical stimuli (Anderson et al., 1987; Stasheff et al., 1985)

Clearly there are multiple experimental approaches that could be used to investigate the role of nAChRs in epilepsy states including human and whole animal studies (Damaj et al., 1999; Dixit et al., 1971; Miner et al., 1985) as well as *in vitro* imaging (calcium or voltage sensitive dyes) (Gray et al., 1996; McGehee et al., 1995; Vogt and Regehr, 2001) and biochemical (transmitter release assays) (Gray et al., 1996; Marshall et al., 1997; Sershen et al., 1997; Vidal and Changeux, 1993) approaches. However, I have chosen to adopt (induced *in vitro* slice epileptiform activities monitored by electrophysiological means) was based upon the ease of pharmacological and electrophysiological intervention and thus the ability to investigate underlying synaptic and cellular mechanisms for an electrophysiological perspective.

1.6 Aims

This project aims to establish the role that nicotinic acetylcholine receptors may play in experimental models of epilepsy and to assess whether pharmacological agents acting at these receptors might represent a novel avenue for developing future anticonvulsants. Specific questions to be addressed include:

1. Investigation into the effects of nAChR activation on various experimental models of epilepsies.
2. Identification the subtypes of nAChRs in modulating epileptiform activities.
3. Establish through which circuits nAChR-mediated effects might be occurring.

CHAPTER 2

MATERIAL AND METHODS

CHAPTER 2

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2.1 Slice Preparation

All experiments carried out during the course of this project were approved by the Ethical Review Process Applications panel of the University of Glasgow and were performed in accordance with the UK Animals (Scientific procedures) Act 1986.

Male wistar rats (2-6 weeks) were bred and housed in group cages under controlled environmental conditions (temperature 19-23°C and 12hr light/dark cycles).

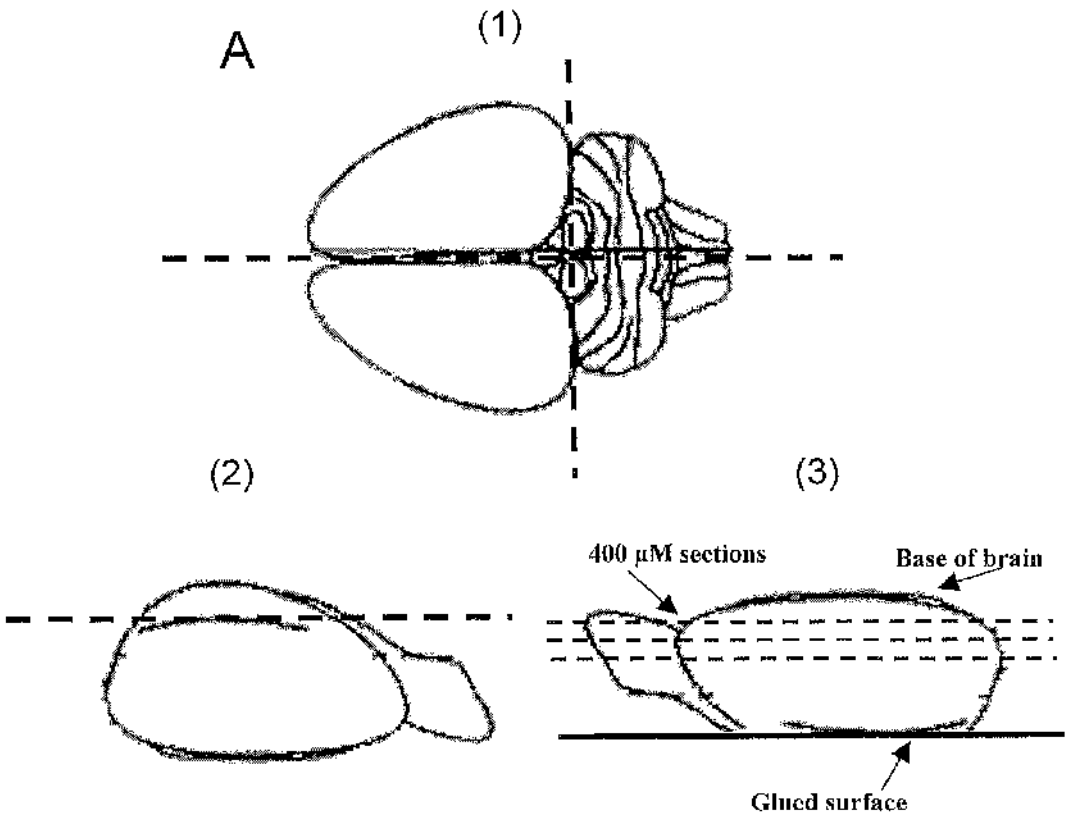
All efforts were made to minimise the number of animals used and their suffering. 2-3 weeks old wistar rats were cervically dislocated and decapitated in compliance with Home Office guidelines on the operation of the Animals (Scientific Procedures) Act 1986. Older rats (4-6 weeks) were terminally anaesthetised with an intraperitoneal injection of Pentobarbitone Sodium (1000mg/kg) and then decapitated, following unconsciousness and lack of paw pitch pain reflexes.

An incision was made along the midline of the head using a scalpel and the skin separated to reveal the skull. Using sharp dissecting scissors the skull was cut down the midline from back to front and the bone folded to each side to expose the brain. The brain, separated from the spinal cord, was carefully removed with a small spatula and placed in a beaker of chilled (0-3°C), oxygenated (95% oxygen-5% carbon dioxide) artificial cerebrospinal fluid (ACSF). The standard perfusion medium (ACSF) comprised (mM): NaCl, 124; KCl, 3; $NaHCO_3$, 26; NaH_2PO_4,

1.25; $CaCl_2$, 2; $MgSO_4$, 1; D-glucose, 10. 5% CO_2 was included to maintain a correct PH of 7.4.

Horizontal orientation was chosen for slice preparation (Figure 2.1). The brain minus the cerebellum was subsequently hemisected and top surface of brain was glued to the stage of a vibrating blade microtome (Leica VT1000, Milton Keynes, U.K.). 400 µm thick transverse horizontal slices were cut using a vibrating microtome (Leica VT1000, Milton Keynes, U.K.). Throughout the slicing procedure the brain and slices were held in a chamber containing chilled (~2-4°C) ACSF. Using a large bore suction pipette, the slices were then transferred to a cold petri dish containing chilled ACSF where the hippocampal region of each slice was dissected free from surrounding brain tissue. Hippocampal slices were maintained in an interface incubation chamber with an oxygen-enriched atmosphere at room temperature (controlled at 26 ± 2°C). Following an incubation period of 1hour the slices were individually transferred to the recording chamber using a large bore suction pipette. Spare slices were stored in the interface incubation chamber where remained viable for up to 12 hours.

For path clamp recordings hippocampal slices were made from male and female Wistar rats (P16-28). Animals were terminally anaesthetised with an intraperitoneal injection of Pentobarbitone Sodium (1000mg/kg). Following unconsciousness and lack of paw pitch pain reflexes, the abdomen and rib cage were opened and a transcardial perfusion was performed. A hypodermic needle (19G 1 ½) was inserted and fixed into the left ventricle and an incision made in the right atrium, the heart was then perfused with 20-60ml of chilled high sucrose ACSF (composition mM: NaCl, 87, KCl, 2.5, $NaHCO_3$, 25, NaH_2PO_4, 1.25, $MgSO_4$, 7, $CaCl_2$, 0.5, glucose, 11, sucrose, 75) in accordance with U.K. home office guidelines. Removal of the brain and preparation of 250µm slices followed an identical procedure to that stated above but in the presence of a high sucrose ACSF solution. Slices were transferred to a submerged holding chamber, heated to 37°C for 1 hour and the high sucrose ACSF was gradually (10% every 15mins) changed to normal ACSF over a period of two hours. Slices were stored in the



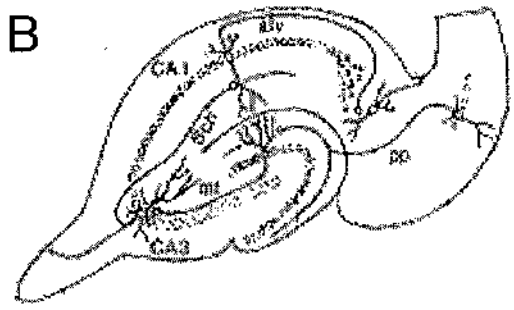


Figure 2.1. *Process and orientation for slice preparation.*
A. Diagram illustrating the direction of slicing. For preparation of slices in a horizontal orientation the cerebellum was firstly separated and the brain then hemisected along the midline (1). The top part of brain was separated (2) and the brain was then inverted and the top surface of brain glued to the stage of a vibrating blade microtome (3). Slices were subsequently cut from the bottom surface of the brain at a thickness of 400µM. Dotted lines indicate the incisions made and the exposed surface of the brain. All procedures were carried out at 2-4ºC to reduce metabolic decay and to firm the brain for care of sectioning. B. Picture of a trimmed hippocampal slice showing several fields of the hippocampal formation and the intrinsic connections.

submerged style holding chamber at room temperature (19-24°C) until needed. They were then individually transferred to a submerged recording chamber using a large bore suction pipette as needed.

2.2 Electrophysiology

2.2.1 Recording set-up

The recording chamber, microscope (Olympus, IMT-2, Japan) and manipulators (Narishige, MC35, Japan) were mounted on a pressure sensitive anti-vibration table (Intracel, UK, Figure 2.2). All other electrical equipment including signal processor (model 440, Brownlee Precision), analogue-to-digital converter (Digidata 1320 series, Axon instruments), humbug noise subtraction device (Quest Scientific) and PC were secured in a racks alongside the Axoclamp 2B current and voltage clamp amplifier (Figure 2.2). A schematic of the experimental apparatus is illustrated in Figure 2.3.

An interface type chamber (Figure 2.4) was chosen for extracellular and intracellular recordings. This consisted of a 35 mm diameter plastic chamber in which the slices were placed on a small 1 cm square pieces of lens tissue at the interface of a warmed ACSF and an oxygen-enriched, humidified atmosphere. The ACSF was heated to approximately 32-34 ^{o}C and bubbled with 95% O_2, 5% CO_2 to provide a humid oxygen enriched atmosphere. Oxygenated ACSF was pumped, by means of a peristaltic pump (Gilson, France), through the central chamber providing a constant flow rate of 1-2 ml.min^{-1}.

Recordings electrodes for extracellular and intracellular experiments were pulled from standard-wall (1.2mm internal-/0.69mm external-diameter) borosilicate glass capillaries on a Brown and Flaming-type horizontal electrode puller model P-87

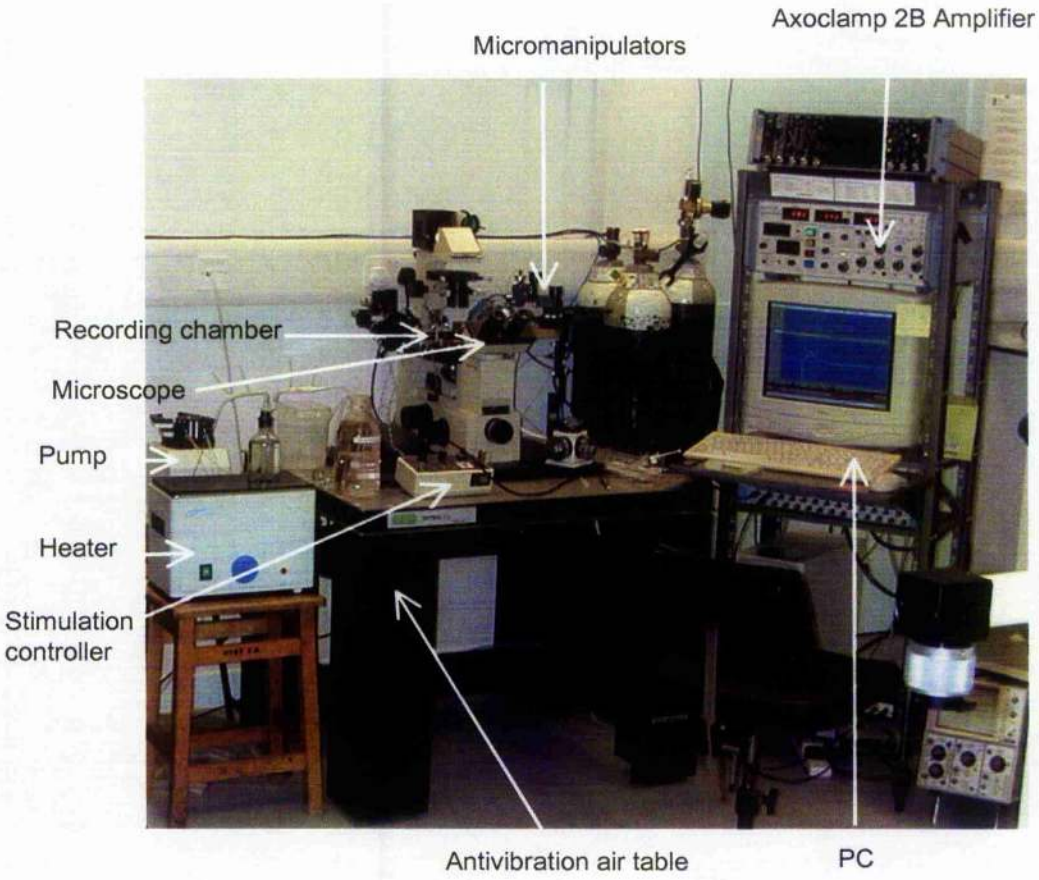


Figure 2.2. *Photograph showing the Recording apparatus.*
Photographic image illustrating manipulators and microscope secured on antivibration table, with all other equipment secured in racks alongside. Recording chamber is mounted under microscope. Micromanipulators are used to position recording electrodes. PC drives experiments and captures data.

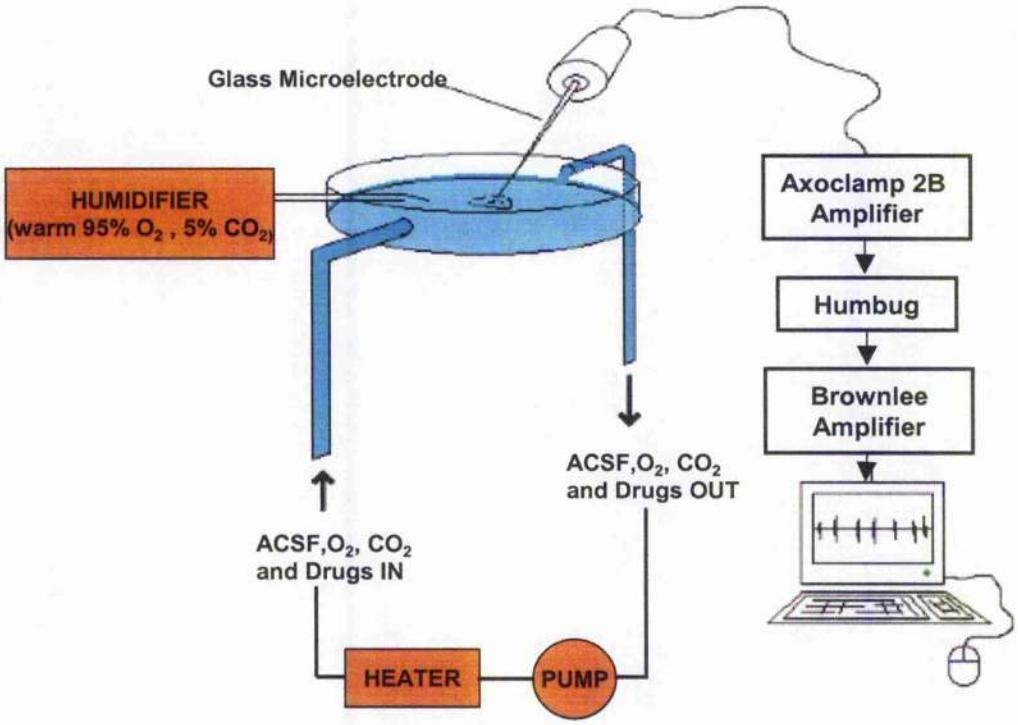


Figure 2.3. *Schematic of the experimental apparatus.*
Flow chart illustrates the apparatus used for electrophysiological recordings. Heated oxygenated ACSF is pumped through the central chamber providing a constant flow rate. As indicated, the system can be operated in a closed loop so that small volumes of drug containing medium can be recirculated if necessary. Hippocampal slices are placed in recording chamber at an ACSF/ humidified oxygen (95% O_2, 5% CO_2) interface and extracellular recordings then made from hippocampal slices using glass microelectrodes. Such field potentials are amplified by an Axoclamp 2B amplifier and a Brownlee 440 signal processor. Extraneous 50Hz line frequency noise was subtracted from traces where necessary using a humbug device. Data is digitised (Digidata 1342) and captured as a continuous data stream on PC for further off-line analysis.

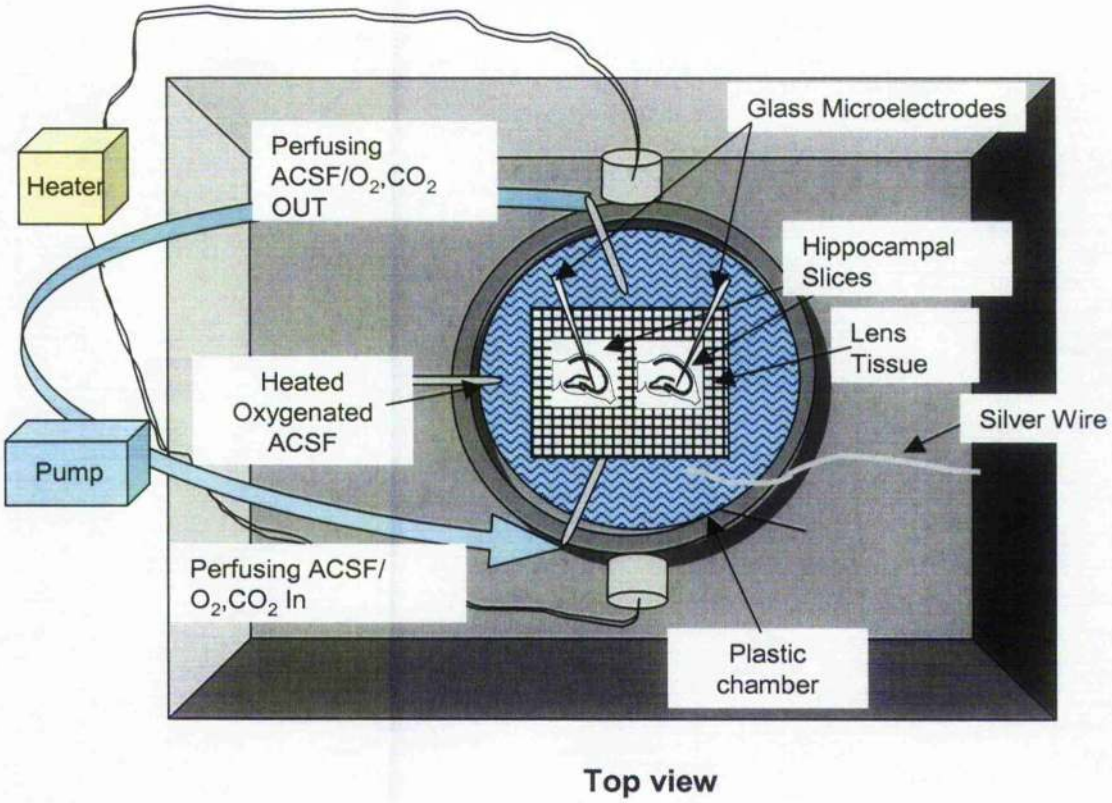


Figure 2.4. *Interface recording chamber.*
Top view of interface recording chamber showing position of hippocampal slices, recording microelectrodes, silver wire (amplifier ground) and lens tissue. Oxygenated, heated ACSF is pumped through the chamber. Two hippocampal slices are transferred to recording chamber and extracellular recordings then obtained from *stratum pyramidale* of area CA3 using glass microelectrodes. Experiments can thus be run in tandem to increase throughout.

(Sutter Instruments, USA). Electrode resistance and filling solutions varied according to the type of experiment performed. Recording electrodes for extracellular recordings had blunt tips and were filled with standard ACSF to a d.c. resistance of around 1-5MΩ. Electrodes for intracellular recordings were filled with 1.5M potassium methyl sulphate and had a d.c. resistance in the range of 80-150MΩ.

Filled recording electrodes were mounted into electrode holders where their filling solution came into contact with silver chloride coated silver wire. The electrode holder was then inserted into a unity gain head stage (current gain 0.1: Axon instruments) and connected to the axoclamp 2B amplifier. In many experiments, two headstages were employed so that it was possible to run experiments on 2 hippocampal slices in parallel (thus increasing the yield of data collection) or to enable simultaneous intra and extracellular recording from an individual slice. A silver chloride bath reference electrode submerged in the recording chamber was also connected to the headstage.

Bipolar stimulation electrodes were constructed from two lengths of 0.05mm diameter Nickel 80% /chromium 20% wire (Advent Research Materials Ltd., England) twisted together and cut at the end to produce a focal stimulation. Stimuli were produced by constant current isolated stimulator boxes (Digitimer Ltd., England), which in turn were triggered by a Master 8 pulse generator (A.M.P.I., Israel).

All stimulating and recording electrodes were mounted on micromanipulators (Narishige MC35, Japan) to allow course and fine movement in all (x,y and z) directional planes.

2.2.2 Recording Technique

2.2.2.1 Extracellular field recordings

These experiments were performed in an interface recording chamber. Standard ACSF was used as the electrode filling solution to achieve a final d.c. resistance between 1-5MΩ.

Spontaneous epileptiform activity recordings:

In order to measure synchronous neuronal discharges that characterise epileptiform activities, the majority of recordings were carried out using the extracellular configuration (Figure 2.5). Following an equilibration period of 1 hour, two slices were transferred to the recording chamber where the slices were allowed to a further equilibration period of at least 15 minutes before recording commenced. Simultaneous extracellular recordings were then made from area CA3 of each hippocampal slice.

The recording electrodes were placed on the surface of each hippocampal slice, close to *stratum pyramidale* within area CA3 (usually CA3c). A microscope slide was subsequently placed over the recording chamber to maintain a high humidity. Synchronous activity within the slice was manifested extracellularly as field potentials (positive and negative deflections) capable of being detected by electrodes in contact with the external space (Figure 2.5). Such field potentials were first amplified 10 times by an Axoclamp 2B amplifier operated in bridge mode, then a further 200 times by a Brownlee 440 signal processor. The DC recordings were then filtered (5 to 1000Hz band pass) and extraneous 50Hz line frequency noise was subtracted using a Humbug device (Quest Scientific, Canada). The processed signal was fed through a Digidata 1320A analogue-to-digital converter operating at 0.5-2 kHz, and thence to a PC for capture on hard-disk using pClamp8.0 software (Axon Inst., Union City, CA, USA).

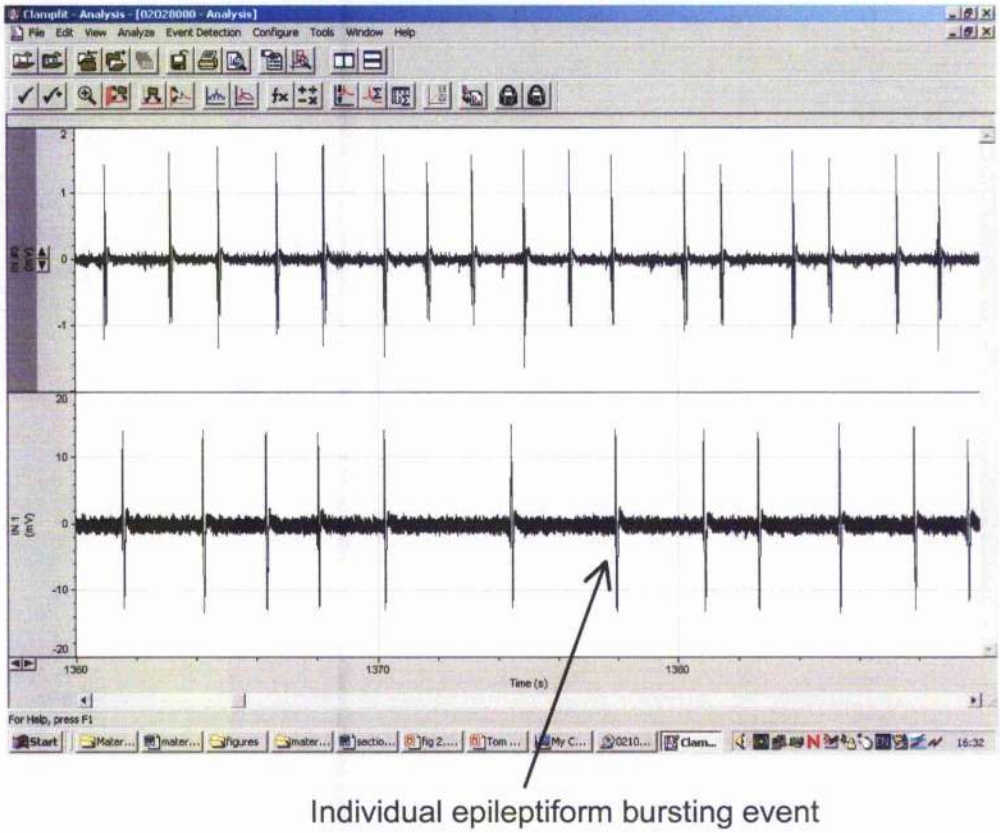


Figure 2.5. *Examples of extracellular field potentials recordings.*

PC screen image showing simultaneous recording from two channels, each representing activity in different hippocampal slice. Note that both traces show intermittent bursting responses which are recorded in chart recorder mode for subsequent off-line analysis.

Three different convulsant models were used in this study: 4-aminopyridine (4AP), bicuculline and low magnesium ACSF. Spontaneous synchronous epileptiform activity was induced via the addition of 4AP (10-50 μM) or bicuculline (20 μM) to the perfusion medium, and the burst frequency then allowed to stabilise (~ 60 minutes). In the bicuculline experiments, the level of KCl in the standard medium was raised from 3 to 5mM in accordance with previously described methods (Ives and Jefferys, 1990). In the low Mg^{++} experiments, $MgSO_4$ was simply omitted from the perfusing ACSF medium (Gulyas-Kovacs et al., 2002).

Evoked responses recordings:

Extracellular recordings were carried out in both the CA3 and CA1 region of the hippocampus. The 1-5MΩ recording electrodes were placed on the surface of the slice in the *stratum radiatum* of the CA3 and CA1 regions. Stimuli were delivered of 20 second intervals via the bipolar stimulation electrode positioned within the hilus or Schaffer collaterals pathways and resulting field EPSPs were recorded. The stimulus comprised of a square wave pulses of 20μs duration and 0-30mA constant current amplitude. The stimulus intensity was adjusted to evoke a response of half of maximum response value for each experiment (range 1.2-2.5 mA).

2.2.2.2 Intracellular current clamp recordings

In a minority of slices intracellular recordings were performed simultaneously with extracellular recordings. For intracellular recording, electrodes were filled with 1.5M potassium methylsulphate (filtered using 0.2μm syringe filter) to achieve a final d.c. resistance between 50 – 180MΩ. Electrodes were pulled to provide a fire tip to permit entry into pyramidal neurones with minimal injury discharge.

Intracellular recordings were made from cells in *stratum pyramidale* close to the extracellular recording electrode. Putative pyramidal cells within area CA3 of the hippocampus were identified and differentiated from fast spiking interneurones based upon their characteristic properties including a pronounced spike frequency adaptation and relative lack of very fast afterhyperpolarisations (Buhl et al.,1994).

Impalement of pyramidal neurones was achieved by advancing the recording electrode through the *stratum pyramidale* using a Narishige (MC35, Japan) water hydraulic drive in two axes. Negative current pulses (0.1nA) were applied through the recording electrode so that any increase in voltage deflection gave an indication that the electrode tip was approaching a cell membrane. Subsequent application of an oscillatory current "buzz" was then used to facilitate penetration of the electrode tip. Following successful impalement spontaneous epileptiform activity at the single cell level was monitored under current clamp bridge mode. Signals for intracellular recordings were typically amplified x 100 and DC recordings low pass filtered (Brownlee 440, 8-pole bessel) at 2KHz and sampled at 5-10 KHz for subsequent off-line analysis.

2.2.2.3. Patch clamp whole-cell recordings

Patch clamp experiments were performed with the assistance of Leanne Ferrigan. Slices were transferred from the holding chamber to a submerged recording chamber mounted on the stage of an Olympus BX50WI upright microscope. Electrodes were back filled with a intra-pipette solution, composition (mM): $KmeSO_4$, 122.5, KCl, 17.5, NaCl, 9, $MgCl_2$, 1, EGTA, 0.2, GTP, 0.3, ATP, 3 and 0.5% Neurobiotin or CsCl, 135, $MgCl_2$, 2, HEPES, 10 and 0.5% Neurobiotin. (Filtered using 0.2μm syringe filter) to achieve a final DC resistance between 6-10MΩ. Whole-cell voltage clamp recordings were obtained from CA1

interneurones under visual control using infrared differential interference contrast (DIC) optics and the blow and seal patch-clamp technique.

Healthy target cells were chosen on a shiny, smooth appearance of the cell surface. Slight positive pressure was applied to the pipette interior while advancing through the slice towards the chosen target cell so that the pipette solution streamed outwards maintaining a clean electrode tip and cleaning the surface of the target cell. The electrode tip was slowly manoeuvred to the cell surface while recording current steps applied through the electrode. Once the electrode tip touches the cell membrane the positive pressure was immediately released to form a low resistance seal, characterised by a reduced current trace. At this point slight negative pressure was applied to increase the seal resistance until a high resistance gigaohm seal was achieved. Subsequent hyperpolarisation of the electrode to –70mV and application of short suction pulses ruptured the membrane patch to achieve the whole cell patch-clamp formation. Voltage clamp configuration was chosen for the duration of the experiment and spontaneous inhibitory postsynaptic currents (IPSC) were recorded from CA1 interneurones within *stratum radiatum* and *stratum oriens*.

2.3 Data Display and Storage

All digitised data captured directly onto DAT tape (DTR-1404; Biologic Scientific Instruments, Claix, France) and/or onto a PC hard disk using pClamp8.0 software (Axon Instruments, CA., U.S.A.) for further analysis.

2.4 Data Analysis

All analysis was carried out off-line using pClamp8 (Axon Instruments), Origin 6 (Microcal, MA. U.S.A.) and Mini Analysis version 5.6.28 (Synaposoft Inc. GA,

USA) software packages. Instantaneous frequency was calculated at a given time point as the inverse of the preceding inter-event interval. The data were then represented in the scatter plots in which time point of a given experiment was plotted against the inverse of the preceding inter-event interval ('frequency'). In order to not bias any change, which occurred within clusters individual events were considered independently. As the basal frequency of bursting under control conditions varied considerably from slice to slice (Figure 2.5), all data within a given experiment were normalised to the mean activity recorded in the presence of each convulsant alone (i.e., mean burst frequency induced by 4AP, BIC or low magnesium) to enable comparison and pooling of data between individual experiments (control; 100%). For example when calculating mean values for the frequency of bursting, the average number of spikes over a 10 minutes period before (-10 to 0 mins of pre-drug control period), during (10 to 20 mins post-drug application) and following wash out of drugs (20-30 mins following commencement of washout) was measured and normalised to mean control values. Thus all data were expressed as a percentage change from the pre-drug control value and presented as means ± standard error of the mean (S.E.M.). Statistical significance determined using Wilcoxon matched pairs test, paired Students *t*-tests, analysis of variants (ANOVAs) and subsequent post hoc tests (Student-Newman-keuls and tukeys) were performed using Graph Pad InStat version 3.05 for windows (GraphPad Software, San Diego California, USA). Probability values of less than 0.05 being taken as indicating statistical significance. *n* values refer to the number of times a particular experiment was repeated.

2.5 Drugs and Chemicals

4-aminopyridine (4AP), choline, dimethylphenyl-piperanzinium (DMPP), N-methylcarbamylcholine (MCC), (±) epibatidine, cytosine, atropine, dihydro-β-erythroidine (DHβE), α-bungarotoxin (α-Bgt), methyllycaconitine (MLA) and

mecamylamine, all purchased from Sigma-Aldrich Company Ltd (Poole, England); Bicuculline, baclofen and 6-nitro-7sulphamoylbenzo[*f*]quinoxaline-2,3-dione (NBQX) purchased from Research Biochemicals International (Natick, MA, USA.); and Nicotine purchased from BDH chemicals Ltd (Poole, England.). Lobeline and (S)-α-methyl-4-carboxyphenyglycine (MCPG) were purchased from Tocris Cookson (Bristol, U.K.).

D-(*E*)-2-Amino-4-methyl-5-phosphono-3-pentanoic acid (CGP 40116), [1-(*S*)-3,4-dichlorophenyl)ethyl]amino-2-(*S*)-hydroxypropyl-p-benzyl-phosphoic acid (CGP 55845A) were gifts from Dr Kumlesh Dev, Novartis Pharmaceuticals, Basel, Switzerland.

All drugs were dissolved in deionised water and stored as frozen 1ml aliquots of stock solutions 100 to 1000 times the final concentration. Drug application (final dilution) was via perfusion medium for a period not less than 15 minutes.

CHAPTER 3

REGULATION OF 4-AMINOPYRIDINE-INDUCED EPILEPTIFORM ACTIVITY BY nAChR ACTIVATION

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REGULATION OF 4-AMINOPYRIDINE-INDUCED EPILEPTIFORM ACTIVITY BY nAChR ACTIVATION

3.1 Introduction

The 4-Aminopyridine (4AP) model of epileptiform activity was initially chosen for this study since it gives rise to rather stereotyped and reliable responses that are stable over prolonged periods. We also become interested in 4AP as our main experimental model of focal epilepsy for since its action as a convulsant is well described. First, 4AP can induce epileptiform activity *in vivo* (Morales-Villagran et al., 1996; Szente and Baranyi, 1987; Szente and Pongracz, 1979) and *in vitro* (Avoli et al., 1996; Chesnut and Swann, 1988; Galvan et al., 1982; Traub et al., 1995; Watts and Jefferys, 1993) when injected or applied in low concentrations. 4AP is also reported to produce clinical seizures in man (Spyker et al., 1980; Thesleff, 1980). Second, unlike other convulsant drugs that act primarily by diminishing the efficiency of GABA-mediated inhibition (Avoli et al., 1988), the evidence available indicates that 4AP-induced epileptiform discharges occur despite the presence of normal and even enhanced synaptic inhibition (Chesnut and Swann, 1988; Rutecki et al., 1987). 4AP may therefore provide a suitable model to investigate the pathophysiological mechanisms involved in the generation of epileptiform activity in conditions where synaptic inhibition is preserved. Third, 4AP is known primarily as a blocker of K^+ currents, the fast A current and the slower D current (for review see Rudy 1988), although other evidence suggests it might affect Ca^{2+} currents as well (Rogawski and Barker, 1983; Segal and Barker, 1986). In the somato-dendrite region, these effects of 4AP on

intrinsic conductance depolarise the cell so membrane potential is become closer to action potential threshold and reduce the latency of action potential generation (Rudy, 1988), whereas at presynaptic terminals, the result is a facilitation of neurotransmitter release (Thesleff, 1980) that can enhance both excitatory and inhibitory synaptic transmission (Kita et al., 1985; Perreault and Avoli, 1991; Rutecki et al., 1987; Thesleff, 1980)).

Three types of burst characteristics have been documented to result from the use of 4AP. The most prevalent is the generation of frequently occurring bursts of short duration, namely interictal activity (Watts and Jefferys, 1993; Mattia et al., 1994; Avoli et al., 1996; Morris et al., 1996). Less frequent negative potentials, which have been proposed to be GABAergic have been found in some instances (Michelson and Wong, 1991; Perreault and Avoli, 1991,1992; Mattia et al., 1994). Prolonged seizure-like bursts, ictal activity, can also occur (Gean et al., 1990; Mattia et al., 1993; Watts and Jefferys, 1993; Avoli et al., 1996; Morris et al., 1996).

The specific aims of this initial result chapter were to:

1. Identify whether nAChR ligands modulate 4AP-induced epileptiform bursting.
2. Identify the subtypes of nAChRs involved in modulating epileptiform activity.
3. Identify the site of generation of epileptiform activity produced by 4AP.

3.2 Characteristics of 4AP-induced epileptiform activity

Extracellular recordings were obtained from the cell body layer of area CA3 in hippocampal slices prepared from 2-6 week old Wistar rats (n=280). Recordings under control (drug free) conditions revealed the absence of any detectable spontaneous extracellular field events in all slices tested (n=280). Subsequent application of the potassium channel blocker and convulsant 4-aminopyridine (10-50

μM) resulted in the gradual appearance of intermittent large amplitude (0.5-4mV peak-to-peak; mean=1.78 ± 0.2mV) rhythmical field potentials (Figure 3.1 A and Figure 3.2 A) that eventually settled into a regular frequency of occurrence that persisted for long periods in the majority of slices tested (n=250 of 280). This intermittent bursts activity was reminiscent of inter-ictal activity seen *in vivo* and was similar to those reported previously (Voskuyl and Albus, 1985).

Individual interictal-like (hereafter referred to as interictal) events comprised of (1) an initial negative potential followed by (2) a slow positive potential and then (3) a slow negative potential and lasted in the range of 200-400ms (mean burst duration=257.2 ± 15.2ms; Figure 3.1 Bi). On top of this characteristic triphasic waveform however was a higher frequency oscillatory component (Figure 3.1 Bii) with voltage fluctuation in the range of 82 to 175Hz (mean 129 ± 9Hz). Simultaneous intracellular recording from CA3 pyramidal cells close to the extracellular recording electrode revealed the intracellular correlate of individual spontaneous field events to comprise of a burst of action potentials on top of a slower depolarising waveform (Figure 3.1 A-B, n=6). Closer scrutiny of such traces showed an association between the timing of action potentials within individual bursts and the higher frequency oscillatory activity nested within each individual field event (see stippled lines in Figure 3.1 Bii). As the 4AP washed into the bath, the instantaneous burst frequency increased to eventually become very stable and uniform over long periods (Figure 3.2 A). At a concentration of 20μM 4AP, individual epileptiform bursts occurred every 2.6 ± 0.1s (n=26, range = 1.5–3.3s), a mean instantaneous frequency of 0.4 ± 0.02Hz.

Upon washout of 4AP the spontaneous field potentials steadily decreased in frequency although complete abolition of spontaneous events was never achieved even after prolonged (up to 200 minutes) washout (n=5, Figure 3.2 B).

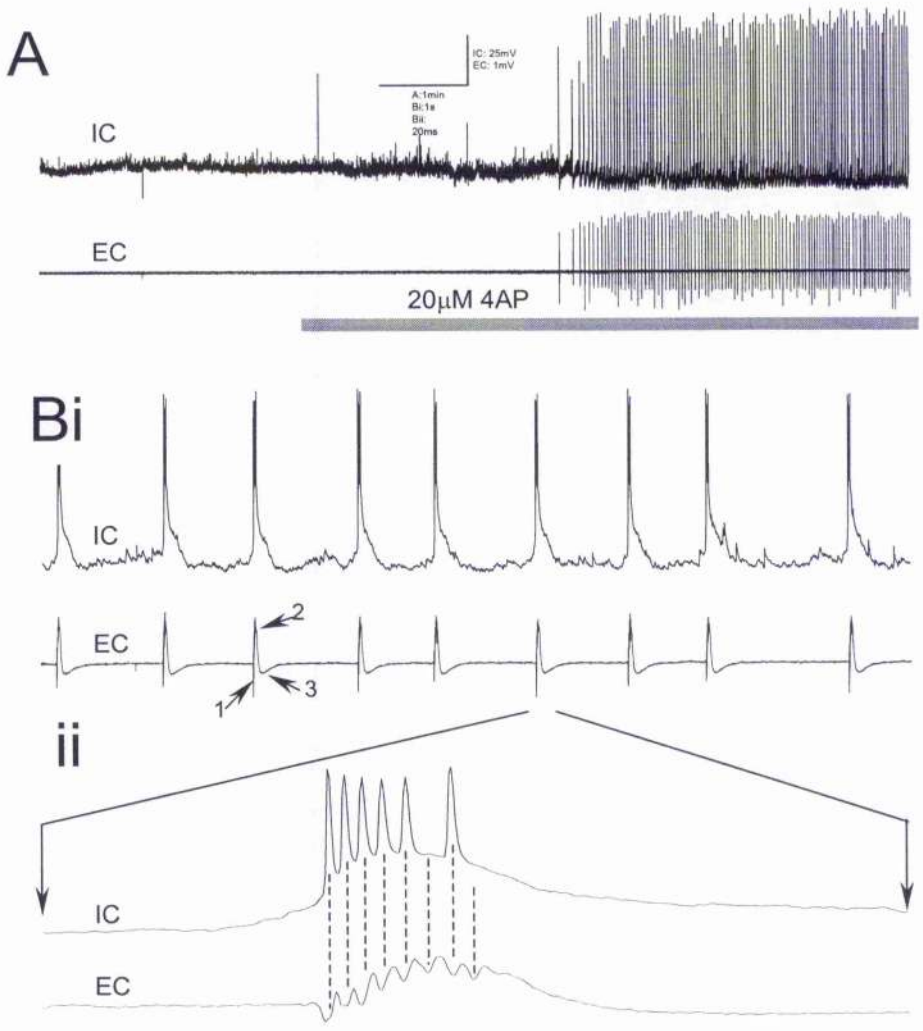

Figure 3.1. *4-aminopyridine-induced epileptiform bursting activity in area CA3 of the rat hippocampal slice.*

A. Intracellular (IC) recording from a putative CA3 pyramidal cell and simultaneous extracellular (EC) field potential recording upon bath application of the convulsant compound 4AP (20μM) as indicated by the solid bar. Note that under control conditions the CA3 pyramidal cell and extracellular field response displays little or no spontaneous activity.Following 4AP application both traces become dominated by an intermittent and coherent bursting response. *Bi.* Expanded traces from the same slice showing individual bursting responses both at the level of the single cell and the population response. For the extracellular trace, arrows indicate the characteristic (1) fast negative potential followed by a (2) slow positive potential and (3) slow negative potential. *Bii* Further expansion of an individual burst event reveals coherent high frequency oscillatory activity within each burst as indicated by the stippled lines. Scales vertical IC = 25mV, IC = 1mV; horizontal A, 1min, Bi, 1s, Bii, 20ms.

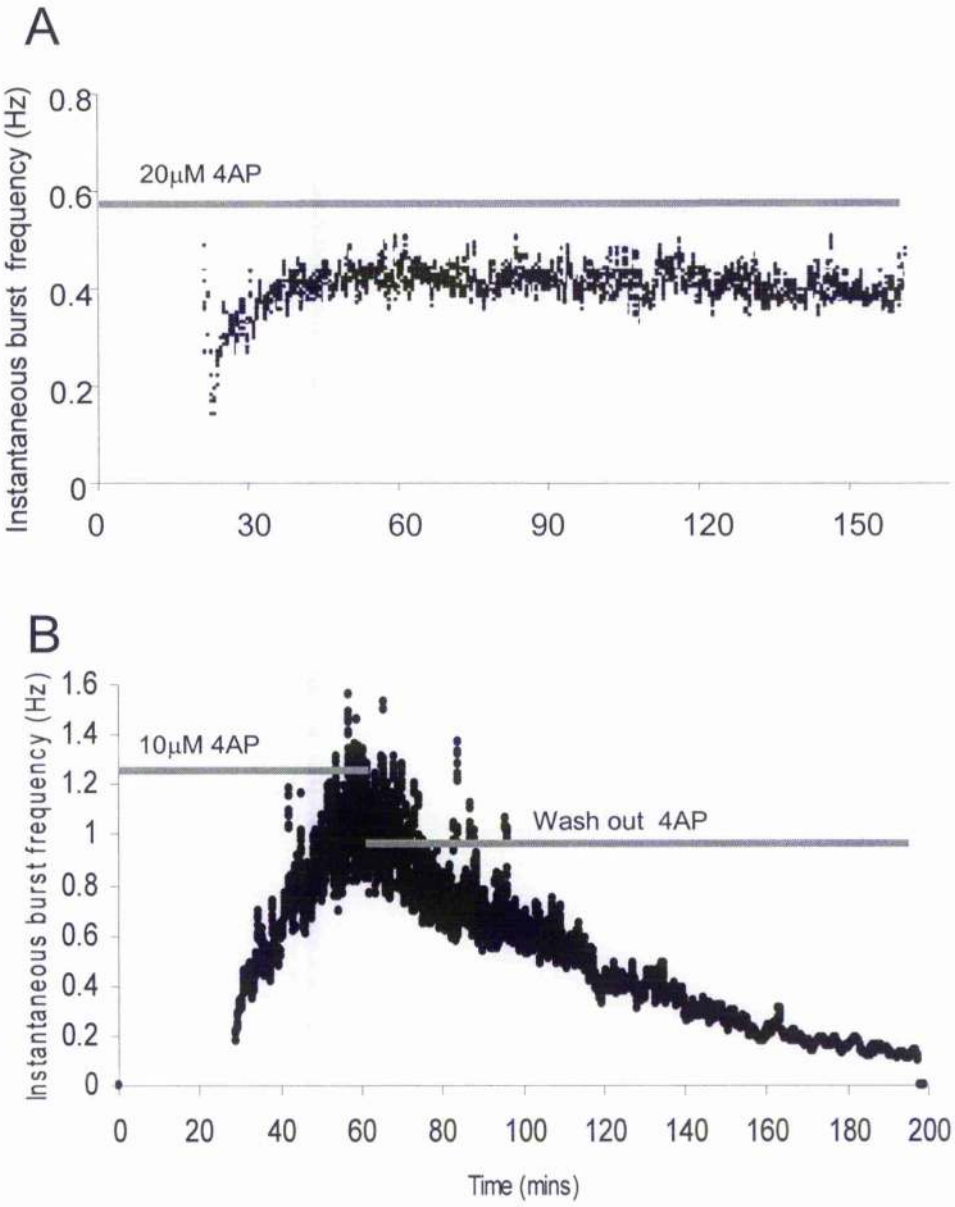

Figure 3.2. *4AP-induced epileptiform discharges remain stable over prolonged periods.*

A. Scatter plot showing the burst frequency for the duration of a typical experiment. Note burst frequency increases as 4AP washes into the bath before settling into a very regular and stereotyped pattern which persists for hours. B. Another experiment in which subsequent washout of 4AP results in a significant reduction in burst frequency but complete abolition is never achieved even after sustained washout.

Interictal activity described above was observed in the overwhelming majority of recordings from hippocampal slices. Less frequent prolonged ictal-like (hereafter referred to as ictal) discharges lasting up to tens of seconds were also observed in small minority of experiments (n=5 of 250, Figure 3.3). In the age group analysed, the occurrence of ictal discharges was inversely related to the age of animal. For instance, all ictal activity was recorded using animals as young as 2-3 weeks old, which was consistent with previously reported studies (Avoli et al., 1993; Fueta and Avoli 1992). In line with this finding, experiments performed under similar experimental conditions in hippocampal slices from older rats (4-5 weeks old) have indicated that 4AP is only capable of inducing interictal discharges (Avoli et al., 1993; Fueta and Avoli 1992).

3.3 Effect of nAChRs agonists on 4AP-induced epileptiform activity

Application of the nAChR agonists nicotine (30μM), 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP, 30μM) and choline (0.3-1mM) to naïve slices in the absence of 4AP did not produce any detectable extracellular field activity in any slices tested (n=6; data not shown). As nAChR activation did not induce any spontaneous network activity, we then went on to assess whether nAChR activation modulates pre-expressed synchronised bursting activity.

Once stable 4AP-induced epileptiform activity was established a range of nAChR agonists were co-applied in order to examine the effect that nAChR activation had upon this form of synchronised bursting. As the basal frequency of 4AP-induced bursting under control conditions varied considerably from slice to slice, all data within a given experiment were normalised to the mean activity recorded in the presence of 4AP alone to enable comparison and pooling of data between individual experiments (control; 100%).

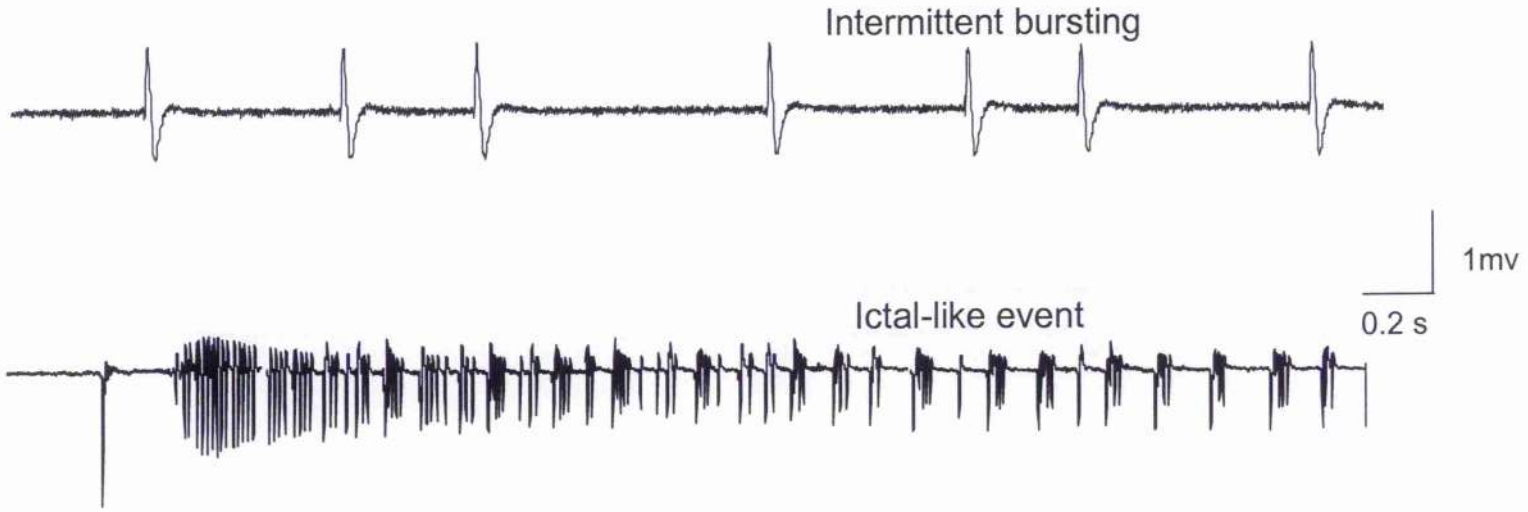

Figure 3.3. *Examples of two types of 4AP-induced epileptiform activities recorded from CA3 area of hippocampal slices .*
Upper trace is an example of short bursts, interictal-like activity, the extracellular correlates of which we shown in figure 1. This type of activity predominated and was seen in 98% of all slices tested. The lower trace shows an example of ictal-like activity. Such after discharges were apparent in only a minority (2%) of slices recorded.

3.3.1 Effect on burst frequency

In a first set of experiments co-application of the selective nAChR agonist DMPP (0.3 to 300 μM) produced a sustained and concentration-dependent increase in the frequency of 4AP- induced epileptiform bursting activity (Figures 3.4 A; *P<0.05*). This robust response was observed in 31 of 37 slices tested and was reversible upon agonist washout (Figure 3.4 A). The concentration response relationship for DMPP induced enhancement of burst frequency was unusual in that it was bell shaped (Stauderman et al., 1998) with a mean maximal frequency potentiation of 37 ± 5% at 30 μM and an EC_{50} calculated from the rising phase of this concentration response relationship of 3.5 μM (Figure 3.6 A). At concentrations beyond 30 μM DMPP still potentiated burst frequency but to a level that was significantly reduced to that induced by 30 μM DMPP.

In a separate series of experiments application of the weak nAChR agonist choline (0.3-1.0 mM) also produced no effect in naïve slices (n=6) but induced a modest increase in the frequency of 4AP-induced epileptiform bursting (Figures 3.4 B and 3.6 A; EC_{50} 150 μM). The magnitude of this effect was more variable than that seen with DMPP and was observed in 23/33 slices tested. Nevertheless the effect of choline was similar to that induced by DMPP as it was reversible upon washout of agonist and produced a maximal frequency enhancement of 27 ± 5%.

In further experiments, application of the selective and potent nAChR agonist lobeline produced a modest yet significant (*P<0.05*) increase (24 ± 11%) in burst frequency at 10-30μM in 8 out of 10 slices tested (Figure 3.5 A). However, further increasing the concentration of lobeline produced a reduction in burst frequency below levels observed in 4AP alone which presumably reflects the non-selective

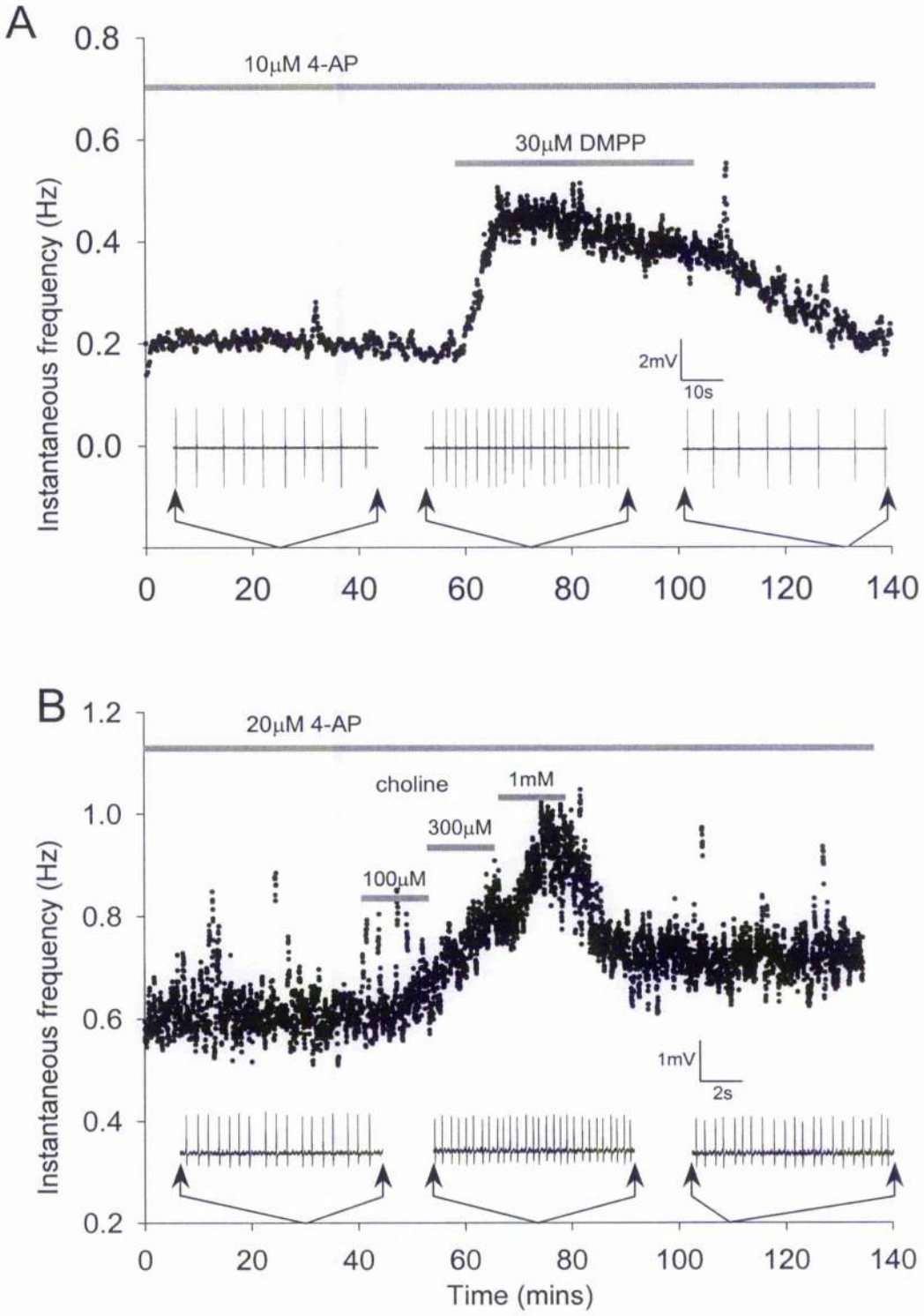

Figure 3.4. *Effect of nAChR agonists on 4AP-induced epileptiform bursting in are CA3 of the hippocampus.*

A. Scatter plot showing instantaneous burst frequency in response to continuous application of 10μM 4AP for the duration of a representative experiment. Co-application of the selective nAChR agonist DMPP (30μM) as indicated by the horizontal bar results in an increase in burst frequency. Inserted voltage traces show rhythmical bursting in the extracellular field potentials before, during and after DMPP application at times indicated by arrows. *B.* Similar scatter plot in which the selective nAChR agonist choline results in a similar dose-dependent and reversible increase in burst frequency. Scales A, 2mV, 10s; B 1mV, 2s.

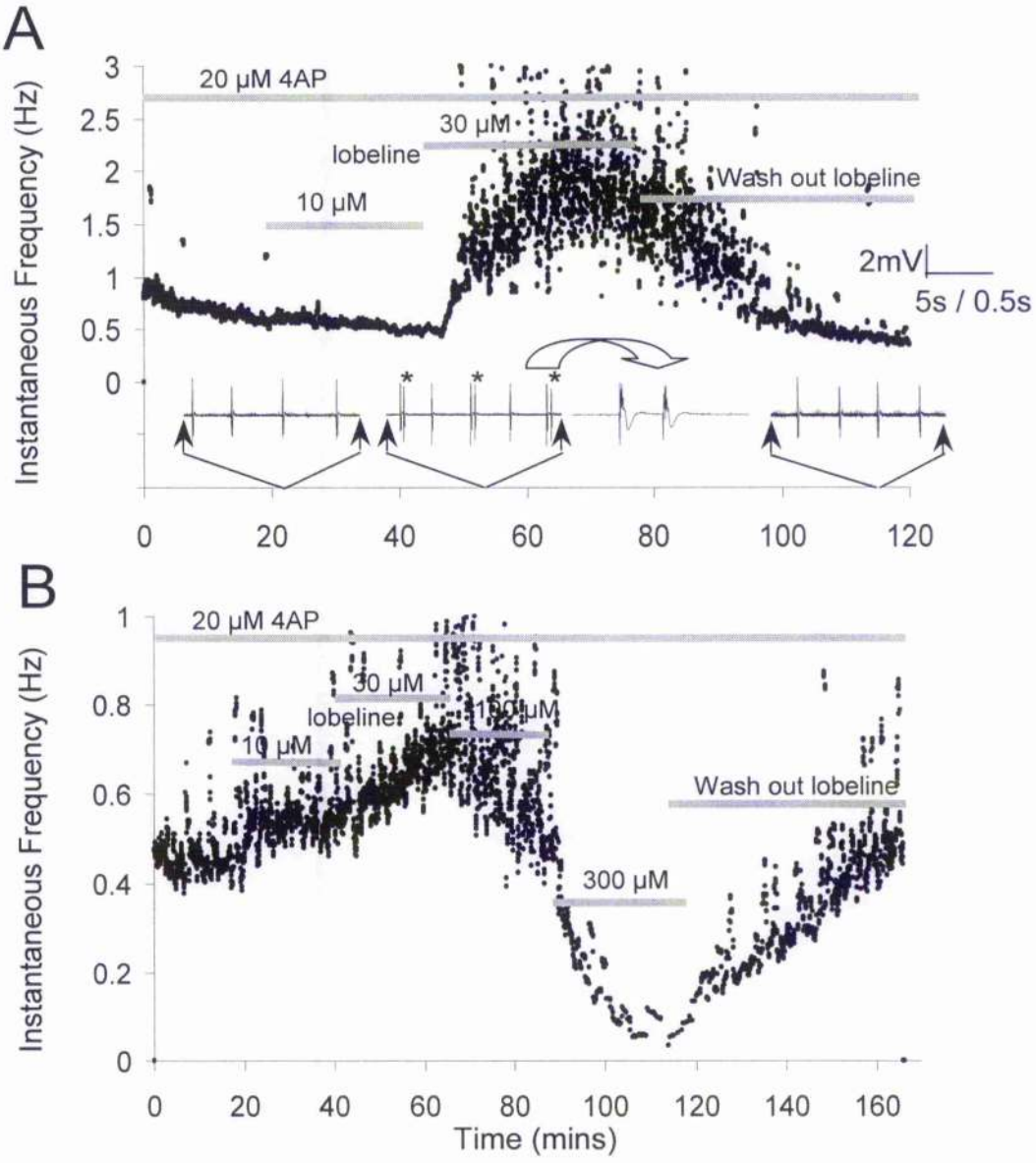

Figure 3.5. *Effect of lobeline on 4AP-induced epileptiform bursting in are CA3 of the hippocampus.*

A. Scatter plot showing frequency of 4AP-induced epileptiform discharges in another hippocampal slice. Sections of voltage trace corresponding to timepoints as indicated by arrows are shown at bottom of graph. Co-application of 30 µM lobeline (horizontal bar) results in a significant increase in burst frequency. Note that the increase in burst frequency in response to lobeline application is accompanied by the appearance of burst doublets (indicated by *). Curved arrows indicate expanded sections of trace illustrating individual field potential events. Subsequent washout of lobeline resulted in the abolition of doublet events and a reduction in burst frequency to baseline levels. B. Similar scatter plot showing an increase in 4AP-induced burst frequency following application of lobeline (10 and 30 µM, horizontal bars). Further increase in lobeline concentration (>10^{-4} M) results in a reduction in burst frequency to levels below control values reflecting a non-selective blockade of calcium channels (Santha, 2000). Note that the decrease in burst frequency in response to high concentration of lobeline is reversed upon washout of lobeline. Scale A, 2 mV, 5 s; 2 mV, 0.5 s for expanded inset

calcium channel blocking activity of this compound (Santha et al., 2000) (Figure 3.5 B and 3.6 A).

In contrast to a constant increase in burst frequency induced by DMPP, choline and lobeline, application of the agonist nicotine (10-100 μM) produced only a transient increase in burst frequency in 7/10 slices tested (Figure 3.6 B) and a sustained increase in the other three slices examined.

Two other nAChRs agonists (±) epibatidine (0.01-10μM, n=10) and cytisine (0.1-30μM, n=10) were examined but found to produce no consistent or significant change in 4AP-induced burst frequency at the range of concentrations tested (Figure 3.7).

Figure 3.8 shows summary of nAChR agonists-induced maximal burst frequency potentiation. A comparison of the effects of various nAChR agonists on 4AP-induced burst frequency exposed that whilst DMPP, choline, lobeline and nicotine could potentiate 4AP-induced bursting activity significantly, two other selective nAChR agonists, epibatidine and cytisine were either poor or ineffective at increasing epileptiform burst frequency.

In addition to fast signalling, acetylcholine is known to mediate a slower modulatory function through both nicotinic and metabotropic (muscarinic) subtypes of acetylcholine receptor (Benardo and Prince, 1982; Cole and Nicoll, 1983; Fraser and Macvicar, 1996; Wonnacott, 1997). In order to rule out any contribution of muscarinic type acetylcholine receptors in the effects described above, further experiments were carried out in the presence of mAChR antagonist atropine at a concentration (10μM) that is known to completely suppress synaptically and pharmacologically evoked mAChR mediated responses in the hippocampal neurones (Cobb et al., 1999; Morton and Davies, 1997). Under these conditions DMPP produced a potentiation of epileptiform burst frequency that was comparable to that

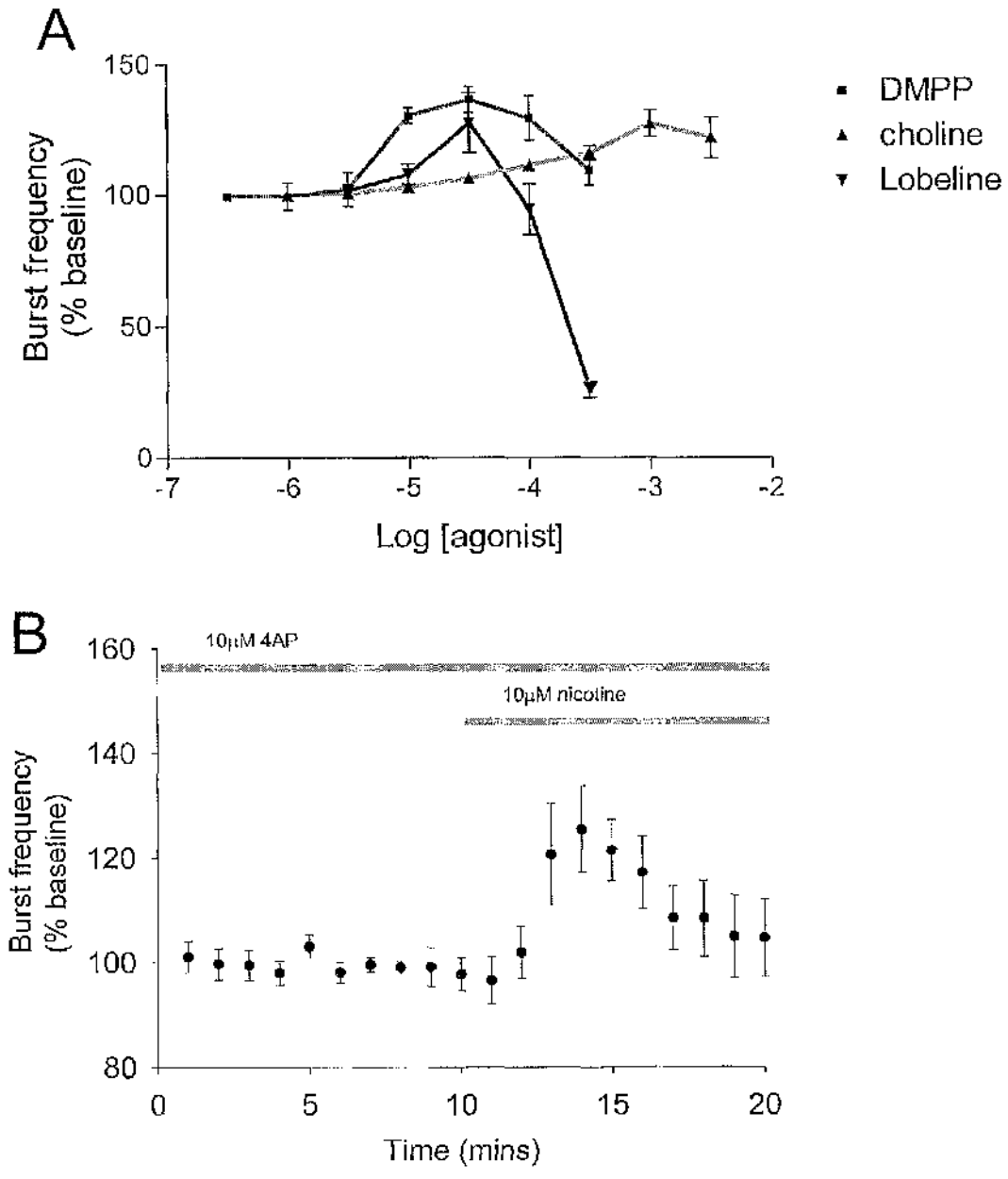

Figure 3.6. *Concentration-response relationship for nAChR agonist-induced potentiation of epileptiform bursting.*
A. Line plot showing the normalised frequency of 4AP-induced bursting events in the presence of DMPP (filled squares), choline (triangles) and lobeline (inverted triangles). Note that all three agonists produce a dose-dependent increase in burst frequency followed by a decrease in burst frequency at higher agonist concentrations (see text). *B.* Scatterplot showing the normalised increase in bursting activity in response to nicotine application as indicated by the solid bar (average of 7 slices). Note the transient nature of the frequency potentiation.

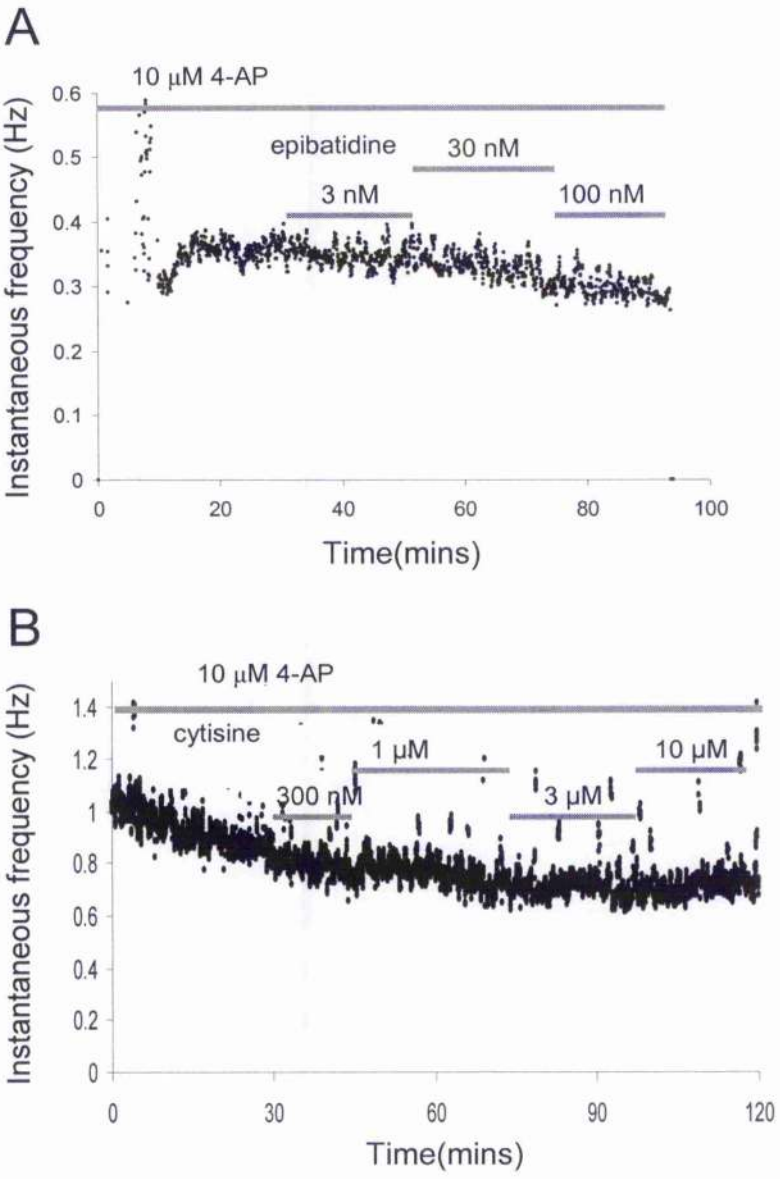

Figure 3.7. *Effect of epibatidine and cytisine on 4AP-induced epileptiform bursting in area CA3 of the hippocampus.*
Scatter plot showing instantaneous burst frequency in response to continuous application of 10μM 4AP throughout two representative experiments. Co-application of the selective nAChR agonists epibatidine (A, 3-100 nM) and cytisine (B, 300 nM-10 μM) for the period indicated by the horizontal bar resulted in no significant change in 4AP-induced burst frequency

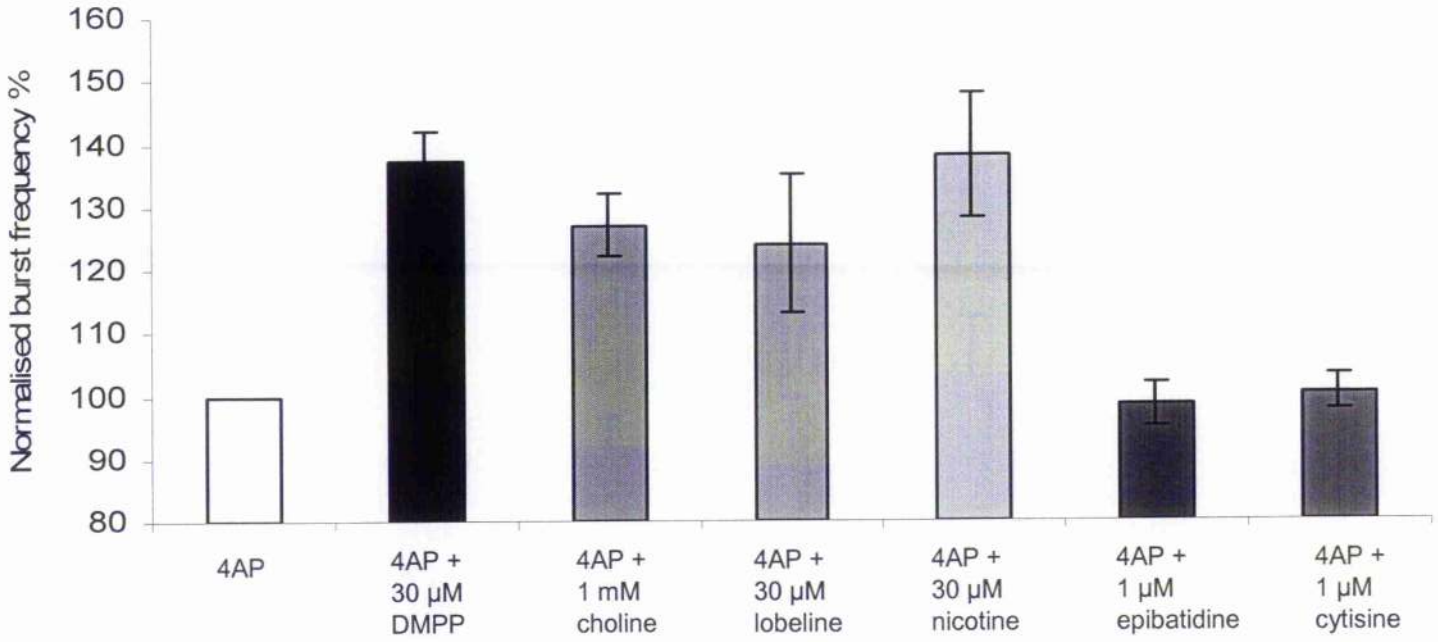

Figure 3.8. *Summary of effects of nAChR agonists on 4AP-induced burst frequency.*
Histogram showing normalised maximal frequency potentiation for each agonist. Note the relative burst frequency enhancement is greatest for DMPP and nicotine. No significant changes were observed following application of epibatidine and cytisine.

in the absence of atropine (n=3; Figure 3.9) suggesting a lack of mAChR involvement in this effect.

In further experiments, application of N-methylcarbamylcholine (MCC), a n-methyl derivative of carbachol that confers enhanced selectivity for nAChRs over muscarinic receptors produced a significant enhancement of 4AP-induced burst frequency when applied at a concentration of 10-30μM (n=3 of 3). However, subsequent experiments (see section 3.5) showed this effect to be principally due to its actions at muscarinic acetylcholine receptors (mAChRs).

DMPP generated the most dramatic alterations in the parameters of the epileptiform discharges, compared to those induced by the other nAChR agonists. For this reason we focused our attention to the effect of DMPP with respect to further and more detailed analysis of the various characteristics of 4AP-induced epileptiform activity (see below).

3.3.2 Effect on burst duration

In addition to its action on burst frequency, DMPP produced a significant reduction in the duration of individual burst events but without causing any overt change in the overall waveform (Figure 3.10 A; n=31). The duration of events decreased as frequency increased and both effects were reversible upon agonist washout (Figure 3.10 B). Detailed analysis revealed a strong correlation between this reduction in burst duration and the increase in burst frequency. Figure 3.10 C shows results from a representative experiment in which the burst frequency could be plotted against the burst duration and the data fitted by linear regression (r2=0.653, *P<0.0001*).

As in the case of DMPP, choline co-application resulted in an increased burst frequency that was accompanied by a reduction in the duration of individual burst events (data not shown).

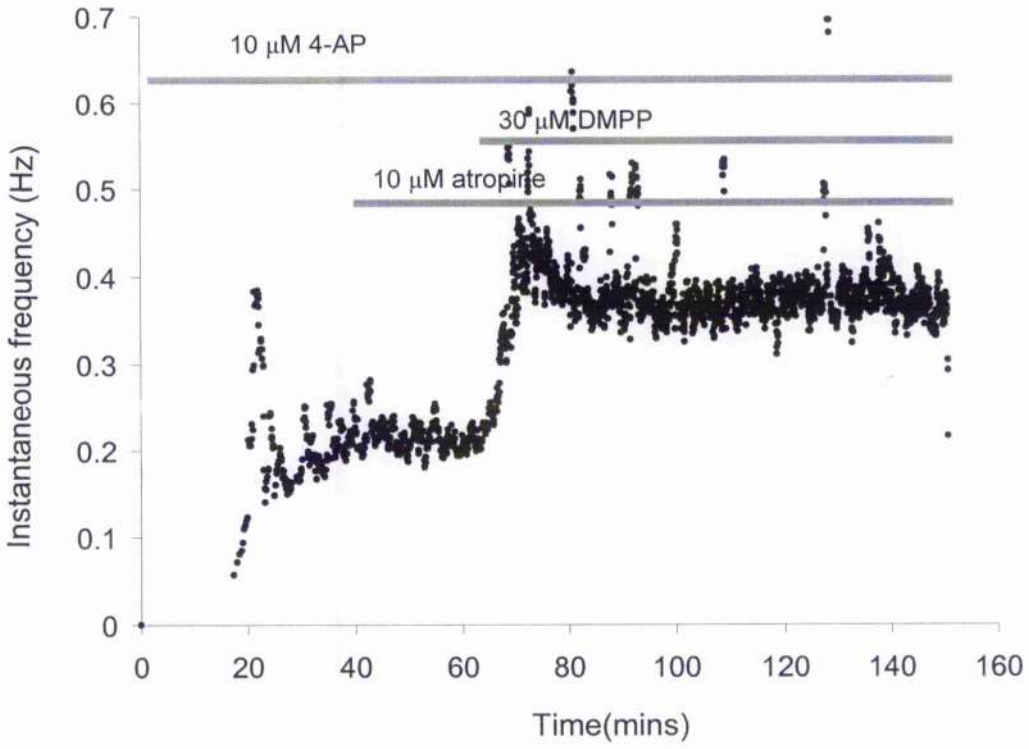

Figure 3.9. *Effect of atropine on DMPP-induced burst frequency potentiation.* Scatter plot shows the action of 10 μM atropine preincubation indicated by horizontal bar on DMPP-induced burst potentiation. Note that the selective mAChR antagonist does not prevent DMPP-induced potentiation of epileptiform burst frequency suggesting that the response to DMPP is due to its action at nAChRs.

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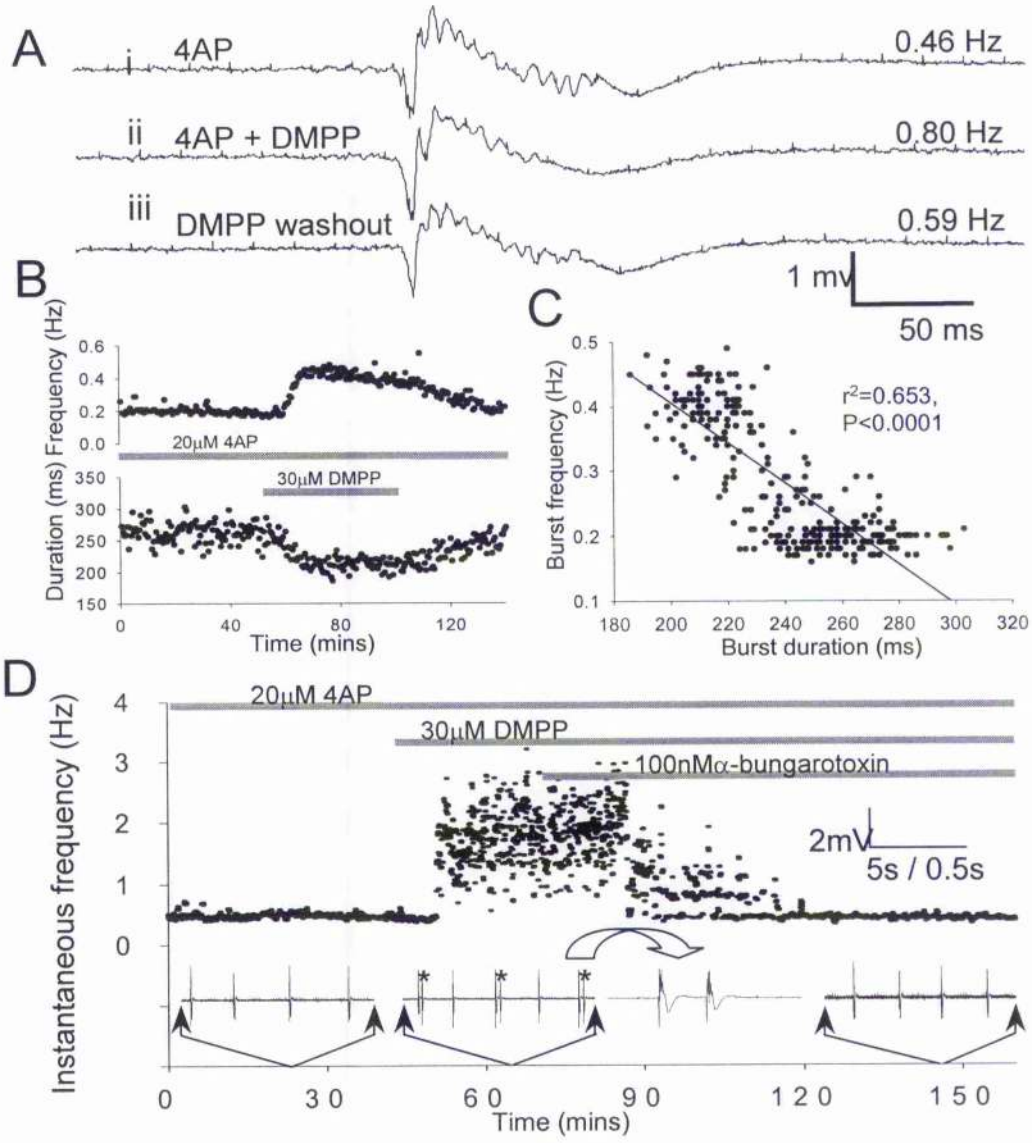

Figure 3.10. *nAChR activation potentiates burst frequency and reduces duration of individual burst events.*

A. Extracellular voltage traces showing individual burst events in response to application of 4AP alone (i, 20μM), co-application of DMPP (ii, 30μM) and following washout of DMPP (iii). Note the fast oscillatory component during each burst event. *B*. Scatter plot showing the action of DMPP on the incidence and duration of individual burst events. Note that the duration of events decreases as frequency increases and that both effects are reversible upon agonist washout. *C* Same data set in which burst frequency is plotted against duration reveals a relationship in which data could be fitted by linear regression (r^2=0.653, P<0.0001). *D*. In a minority of slices (see text), burst frequency increases and displays secondary bursts or afterdischarges. Scatterplot indicates 4AP-induced epileptiform burst frequency for the duration of an experiment. Sections of voltage trace corresponding to timepoints as indicated by arrows are shown at bottom of graph. Note that the increase in burst frequency in response to DMPP application (30μM, horizontal bar) is accompanied by the appearance of burst doublets (indicated by *). Curved arrow indicates expanded section of trace illustrating an individual doublet event. Subsequent co-application of the nAChR antagonist α-bungarotoxin (100nM, horizontal bar) results in the abolition of doublet events and a reduction in burst frequency to baseline levels. Scales A, 1mV, 50ms; D 2mV, 5s; 2mV, 0.5s for expanded inset.

3.3.3 Effect on burst waveform

So far we have shown the action of a range of nAChR agonists including DMPP, choline and lobeline all of which produce a concentration dependent increase in burst frequency and decrease in burst duration without causing any overt change in the waveform of individual bursts. In the majority of slices tested, co-application of DMPP produced a uniform increase in burst frequency (Figures 3.4 A, 3.10 B). However, in a minority of slices (n=6 of 31) besides a general increase in burst frequency, application of DMPP resulted in the appearance of secondary or tertiary bursts resulting in doublets and triplets (Figure 3.10 D, indicated by * in sections of voltage trace at bottom of graph). This effect was reversible upon washout or upon application of the nAChR antagonist (Figure 3.10 D). Similarly, in 2 out of 8 slices tested, lobeline application resulted in doublet and triplets bursts which were reversible upon washout of lobeline (see Figure 3.5 A). However, the emergence of event clusters was not observed following application of choline and nicotine (data not shown).

3.3.4 Effect on burst amplitude

The amplitude of epileptiform bursting is theory provides information regarding the number of neurones firing together with the precise degree of synchrony. However, our recordings revealed considerable variation between as well as within individual experiment. This variation was likely due to factors such as mechanical instability and for this reason further analysis was restricted to burst frequency and pattern rather than overall amplitude.

3.4 Effect of nAChRs antagonists on 4AP-induced epileptiform activity

In the presence of 4AP alone (i.e., no nAChR agonist present), subsequent application of mecamylamine (200μM, n=10) produced no significant change in burst frequency (data not shown) whilst dihydro-β-erythroidine (DHβE, 20μM, Figure 3.11) and α-bungarotoxin (α-Bgt, 100nM, data not shown) produced a modest decrease in burst frequency in 1 of 4 slices tested for each antagonist suggesting that there was little or no basal activation of nAChRs under control conditions in the majority of slices tested.

3.5 Effect of nAChRs antagonists on nAChR-induced burst frequency potentiation

The findings described above using a variety of nAChR agonists suggest that nAChR activation is a key driver for frequency potentiation of 4AP-induced epileptiform activity. To confirm this hypothesis a series of subsequent experiments were performed to establish whether a range of selective nAChR antagonists could reverse the burst frequency potentiation brought about by DMPP and choline. A series of experiments were therefore carried out using a range of selective nAChR antagonists including DHβE, α-Bgt, methyllycaconitine (MLA) as well as the nAChR non-competitive channel blocker mecamylamine. Low concentrations of DHβE (1μM), which are ineffective in antagonising α7 nAChR-mediated currents (Mann and Greenfield, 2003), were found to have no significant effect on the DMPP-induced potentiation of epileptiform bursting. However, the effect of DMPP was completely blocked by the 10-30 fold higher concentrations of DHβE (10-30 μM; n=6 of 8; Figure 3.12 A). At a concentration of 20 μM, such co-application of DHβE caused a

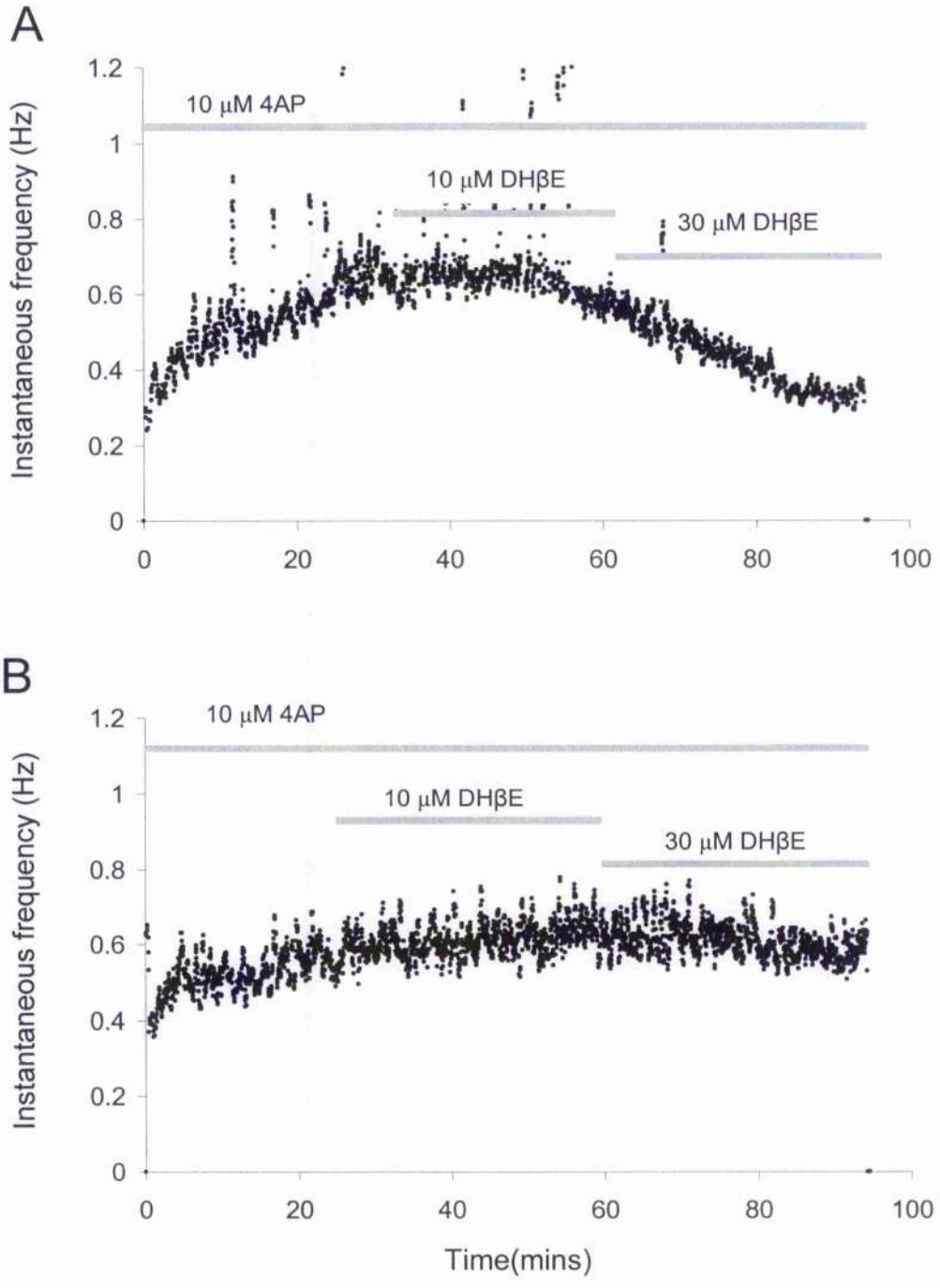

Figure 3.11. *Basal activation of nAChRs under control conditions.*
Application of 10-30 μM DHβE produced a modest decrease in 4AP-induced burst frequency in only one slice (A) but in the majority of slices tested produced no significant change in burst frequency (B) suggesting no or minimal basal activation of nAChRs under control conditions.

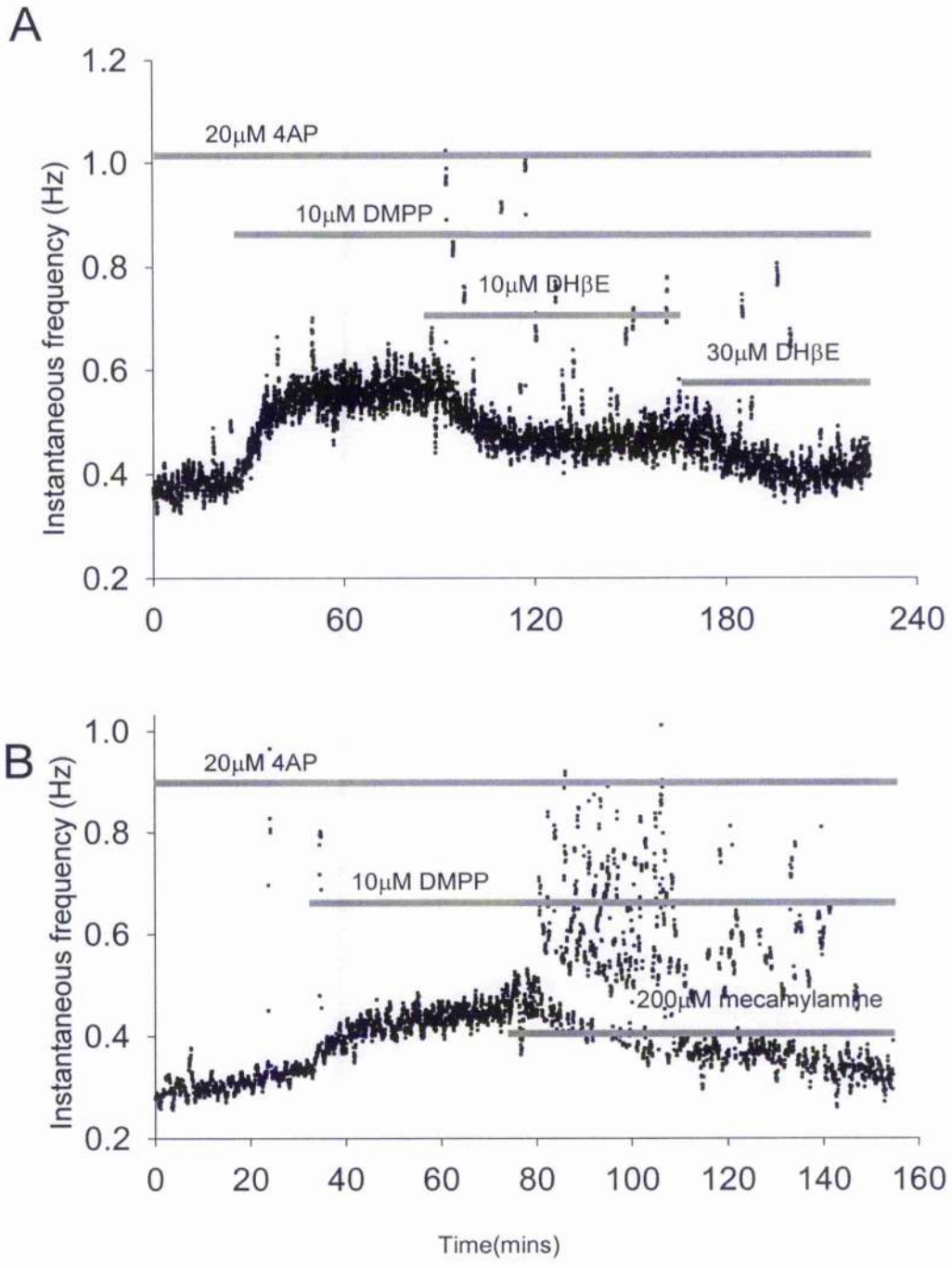

Figure 3.12. *Effect of selective nAChR antagonists on DMPP-induced potentiation of epileptiform bursting in area CA3 of the hippocampus.*
A. Scatter plot showing instantaneous burst frequency in response to continuous application of 20 μM 4AP (horizontal bar) for the duration of a representative experiment. Co-application of the nAChR agonist DMPP (30μM) as indicated by the horizontal bar results in an increase in burst frequency. This is reversed upon subsequent co-appilcation of the selective nAChR antagonist Dihydro-β-erythroidine (10-30μM). *B.* Similar scatter plot in which the potentiating effect of DMPP is reversed upon co-application of non-competitive antagonist and nAChR channel blocker mecamylamine (200μM).

significant reduction in burst frequency by 55 ± 14.6% (*P<0.01*). In further experiments, DMPP-induced (30 μM) burst frequency potentiation was significantly reduced upon co-application of mecamylamine (50-200 μM; n=3 of 4; Figure 3.12 B), α-Bgt (100nM; n=4; Figure 3.13) and MLA (100 nM, n=5) with the three antagonists producing a reduction in burst frequency by 47 ± 14.8%, 86 ± 14% and 47 ± 15.7 respectively (all *P<0.05*).

Likewise, very similar reversals of choline-induced (1 mM) burst potentiation were achieved using mecamylamine (200 μM, n=5) and α-Bgt (100 nM, n=4); these antagonists reducing the potentiation of burst frequency by 67 ± 19% and 65.3 ± 22% respectively (both *P<0.05*). Co-application of MLA (5μM; n=5) also caused a significant reduction in burst frequency by 57.07±9.3%. Figure 3.14 shows frequency plots from a representative experiment showing that co-application of MLA and mecamylamine results in a reduction of burst frequency to near pre-choline levels.

The pooled results for all antagonist studies are summarised in histogram format as shown in figure 3.15 A-D for DMPP-induced burst frequency potentiation and figure 3.15 E-G for choline-induced burst frequency potentiation.

To expend on these experiments and rule out any contribution of muscarinic acetylcholine receptor activation contributes to the burst frequency potentiation, we examined the effect of atropine, a mAChRs antagonist, on DMPP-induced burst frequency potentiation. In the first set of experiments slices preincubated with 10μM atropine, were still able to exhibit burst frequency potentiation upon DMPP application (n=3; Figure 3.10). In other experiments, atropine (10μM) was co-applied following DMPP-induced potentiation but did not reverse or otherwise alter DMPP-induced potentiation (n=3; Figure 3.16 A). Together, these experiments suggest that mAChRs were not involved in the observed burst frequency facilitation.

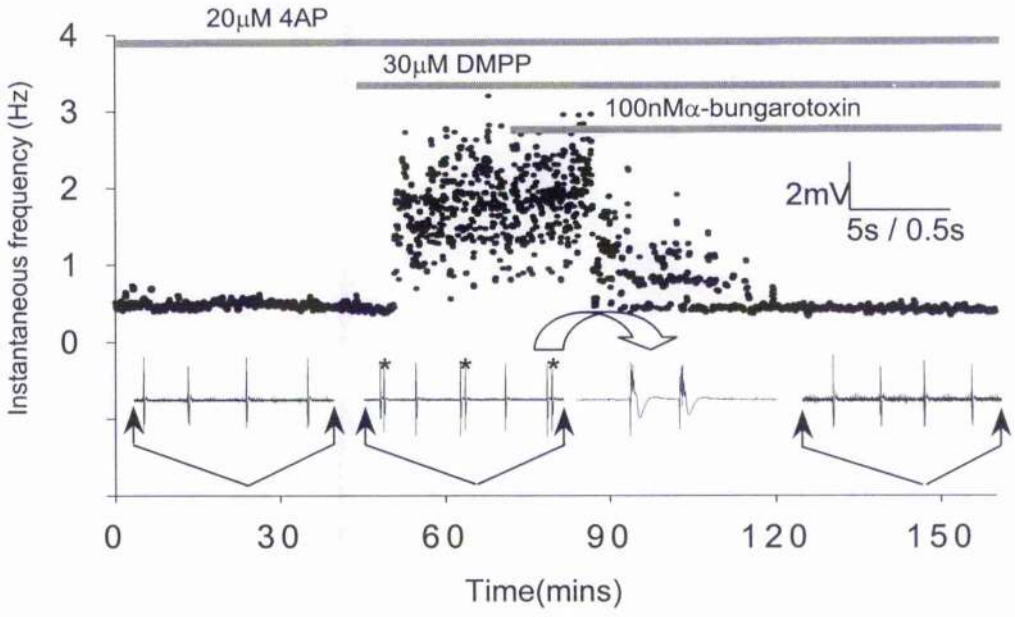

Figure 3.13. *Effect of* α-bungarotoxin *on DMPP-induced potentiation of epileptiform bursting in area CA3 of the hippocampus.*
The scatterplot indicates 4AP induced epileptiform burst frequency for the duration of an experiment. Co-application of the selective nAChR agonist DMPP for the period indicated by the horizontal bar results in an increase in burst frequency. Sections of voltage trace corresponding to the timepoints indicated by the arrows are shown at the bottom of the graph. Note that the increase in burst frequency in response to DMPP application (30 μM, horizontal bar) is accompanied by the appearance of burst doublets (indicated by *) in a minority of slices (see text). Curved arrow points to an expanded section of trace illustrating an individual doublet event. Subsequent co-application of the nAChR antagonist α-bungarotoxin (100 nM, horizontal bar) resulted in the abolition of doublet events and a reduction in burst frequency to baseline levels. Scale *C*, 2 mV, 5 s; 2 mV, 0.5 s for expanded inset.

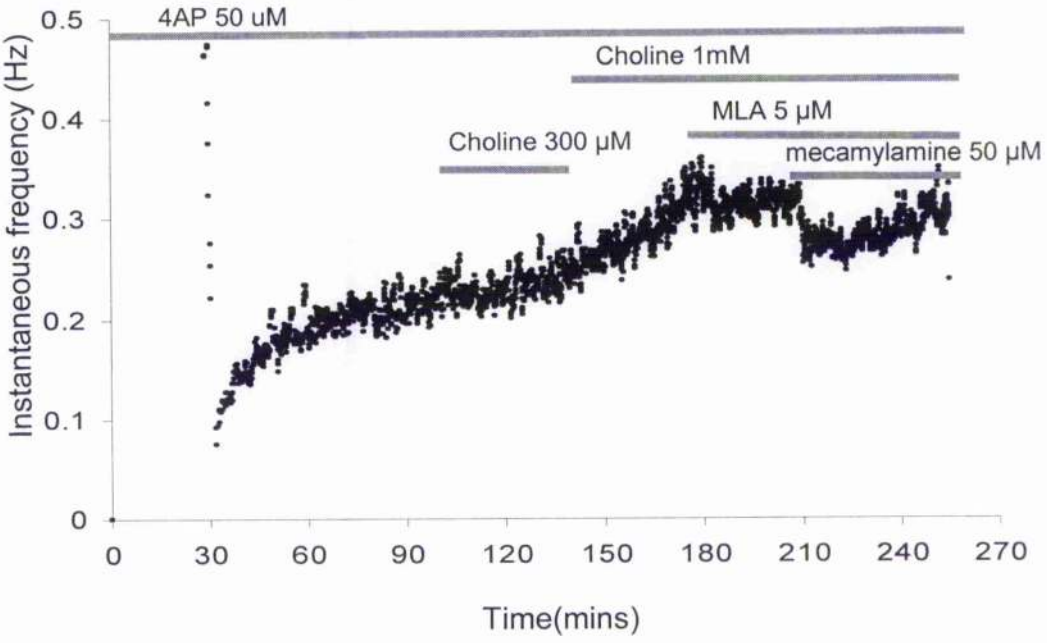

Figure 3.14. *Effect of MLA and mecamylamine on choline-induced potentiation of epileptiform bursting in area CA3 of the hippocampus.*
Scatter plot showing instantaneous burst frequency in response to continuous application of 50 µM 4AP (horizontal bar) for the duration of a representative experiment. Co-application of the selective nAChR agonist choline for the period indicated by the horizontal bar results in an increase in burst frequency. Subsequent co-application of the nAChR antagonist MLA (5 µM, horizontal bar) resulted in a reduction in burst frequency. Further reduction in burst frequency was achieved following application of 50 µM mecamylamine.

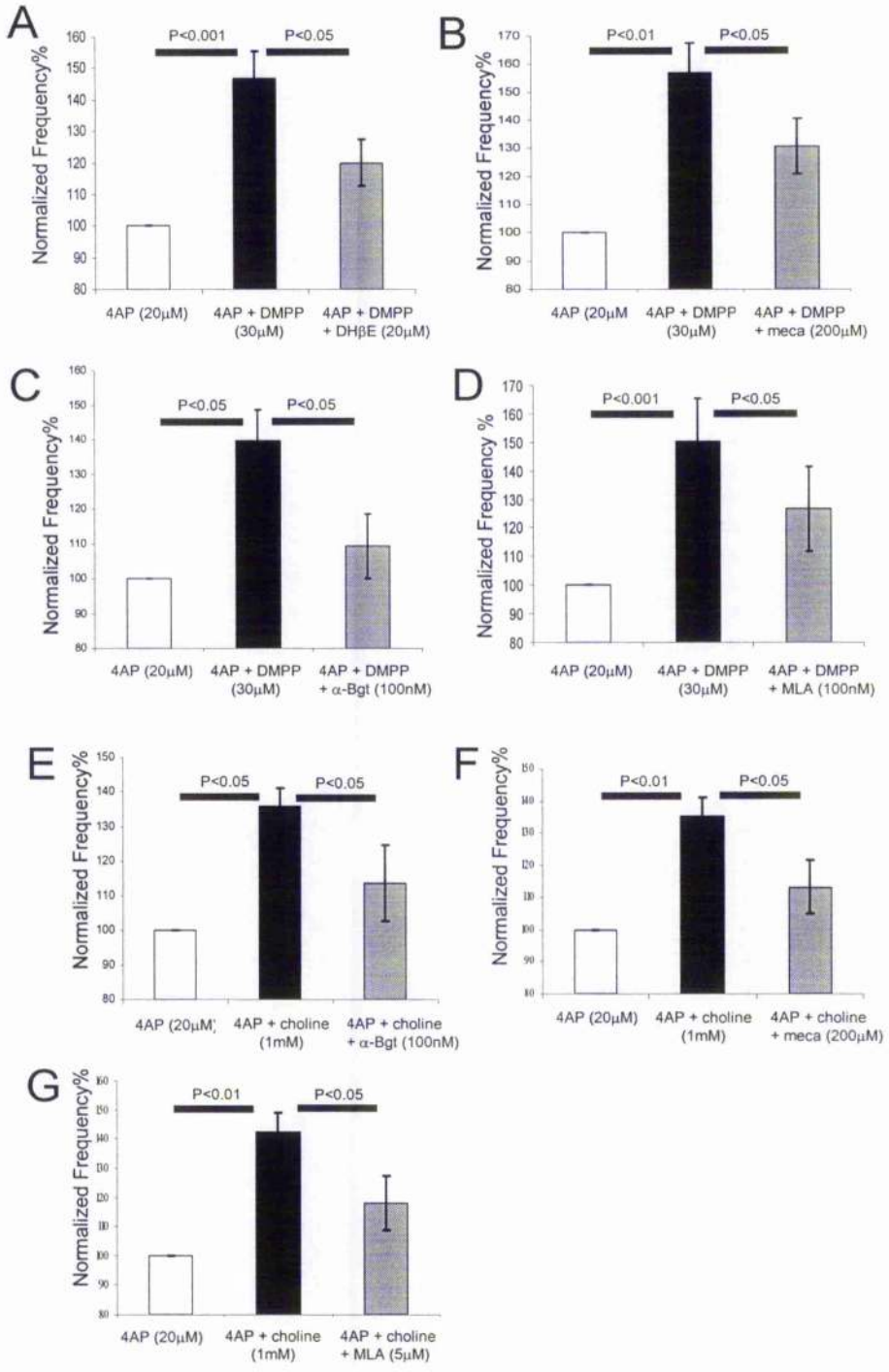

Figure 3.15. *Summary of nAChR antagonist action on DMPP and choline-induced burst frequency potentiation.*

A-D. Histograms showing burst frequency as percentage of baseline (i.e. 20 μM 4AP alone) in response to DMPP (30μM) and subsequent co-application of the selective nAChR antagonists dihydro-β-erythroidine (*A*; 20μM), mecamylamine (*B*; 200μM), α-bungarotoxin (*C*; 100nM) and methyllycaconitine (*D*; 100nM). Note that all antagonists produce a significant reduction in DMPP-induced burst potentiation. E-G. Similar histograms quantifying the effects of the selective nAChR antagonists α-bungarotoxin (*E*; 100nM), mecamylamine (*F*; 200μM), and methyllycaconitine (*G*; 5μM) on choline-induced (1mM) burst potentiation. Note that all three antagonists produce a significant reduction in choline-induced burst potentiation. Horizontal bars indicate P values between respective columns as determined using ANOVA.

The only exception of this finding was in the case of MCC. As mentioned in page 68, application of 10-30 μM MCC produced a significant increase on 4AP-induced burst frequency. However, when 10μM atropine was co-applied following MCC-induced potentiation, a resultant reduction in burst frequency to baseline level was observed (n=3, Figure 3.16 B). Preincubation with 10μM atropine (n=3; data not shown) also prevented the MCC-induced burst frequency potentiation. Together, these data suggest activation of mAChRs to underlie the observed burst frequency potentiation.

3.6 Assess the site of generation of 4AP-induced epileptiform activity in the hippocampus

In our studies we predominantly focused our investigations and recordings within area CA3 of the hippocampus which is known to be the primary generator of epileptiform responses in the hippocampal slices (Lothman et al, 1981). Previous studies have shown that all areas of the hippocampus partake in the synchronous discharged evoked by 4AP (Colom and Saggau, 1994; Perreault and Avoli, 1992). However, a question we wished to address was to assess whether nAChRs can modulate the spread of activity from one area to another. Simultaneous extracellular field recordings were therefore carried out from the cell body layer of area CA3 and at the same time, area CA1 of the hippocampal slice. Concurrent extracellular recording revealed that spontaneous epileptiform activity induced by 4AP was synchronous both within the CA3 and CA1 regions (Figure 3.17 A, n=4). Subsequent bath application of 30 μM DMPP thus resulted in an identical increase in 4AP-induced burst frequency in both CA3 and CA1 regions (Figure 3.17 A,1i-2i). As CA3 and CA1 subfields are mono-synaptically connected via the Schaffer-collateral pathway the question arises: Can synchronous epileptiform discharges in CA1 area be maintained in the absence of this connection? After transection of the Schaffer-collateral afferents (Figure 3.17 B), extracellular field potential activity was abolished in the CA1 subfield but preserved in the CA3 in the majority of slices tested (n=12 of

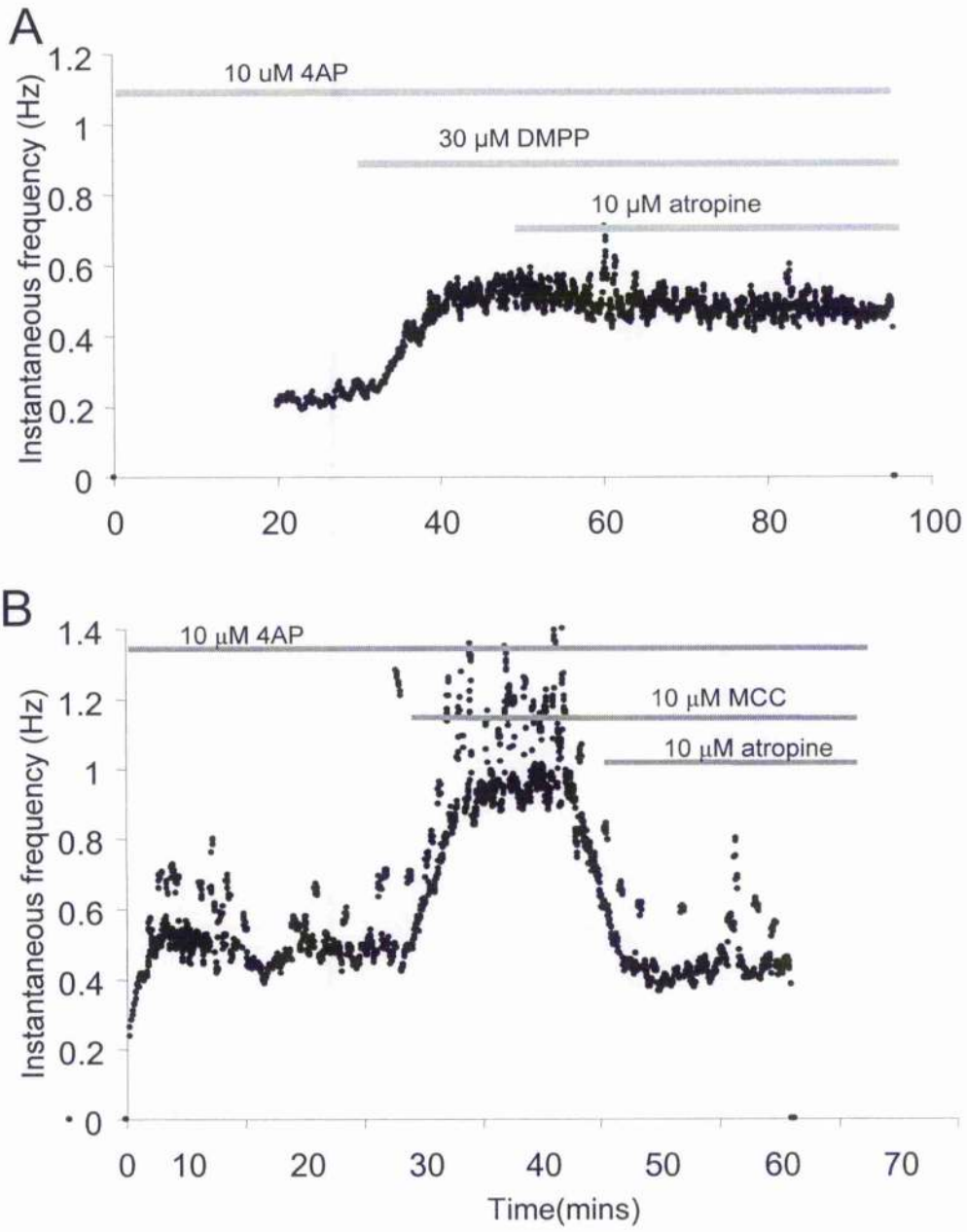

Figure 3.16. *Effect of selective mAChR antagonist atropine on DMPP and MCC-induced potentiation of epileptiform bursting in area CA3 of the hippocampus.* *A*. Scatter plot showing instantaneous burst frequency in response to continuous application of 10 µM 4AP (horizontal bar) for the duration of a representative experiment. Co-application of 30 µM DMPP as indicated by the horizontal bar results in an increase in burst frequency. Subsequent application of 10 µM atropine does not affect DMPP-induced burst frequency potentiation suggesting that this effect is due to activation of nAChRs. *B*. Similar scatter plot in which the potentiating effect of MCC, a n-methyl derivative of carbachol is reversed upon co-application of 10 µM atropine suggesting that mAChRs were involved in MCC-induced burst frequency potentiation.

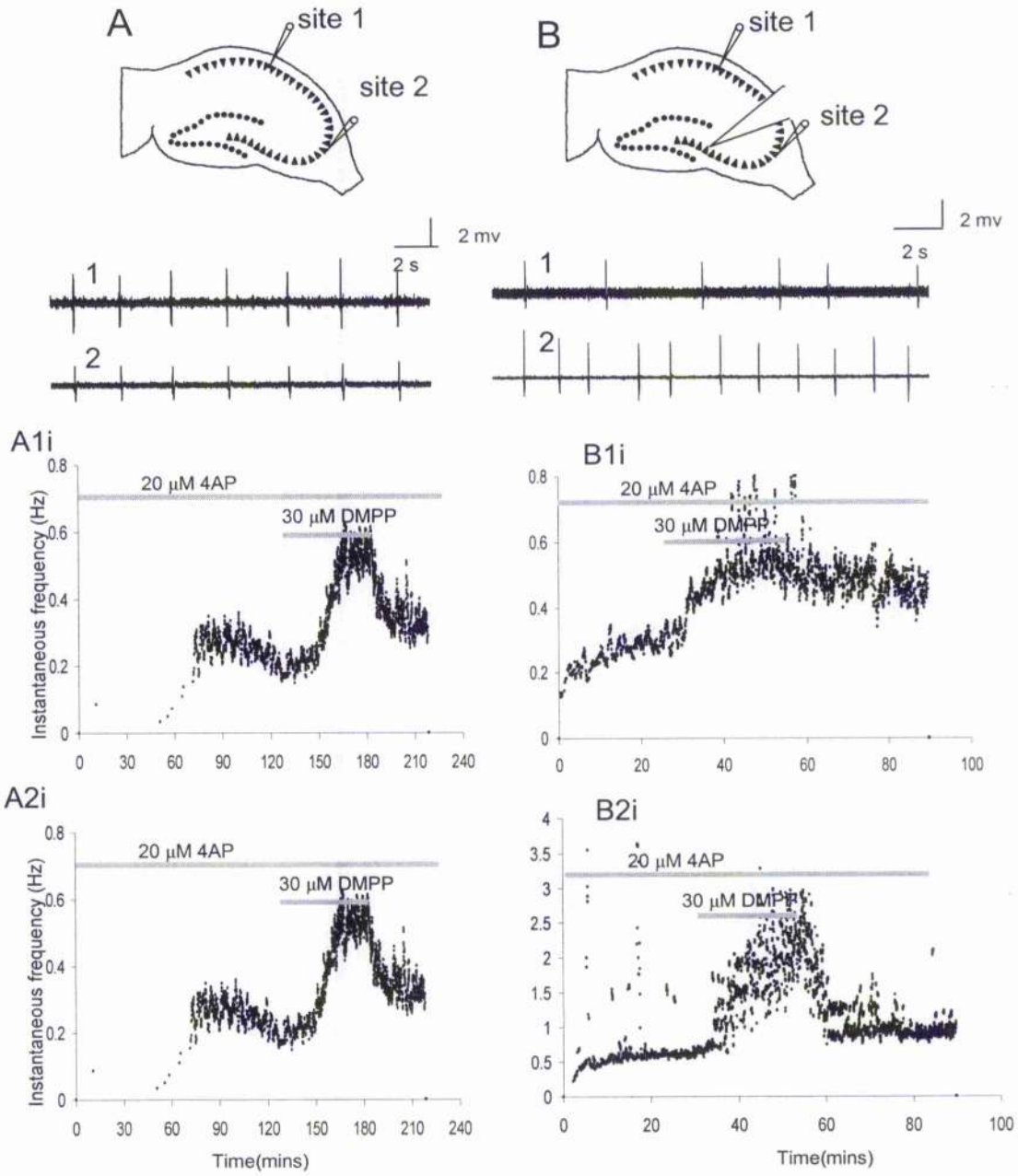

Figure 3.17. *Activation of nAChRs can modulate 4AP-induced epileptiform activity in both CA3 and CA1 regions.*

A. Simultaneous extracellular recording from the CA1 (site 1) and CA3 (site 2) subfields of a hippocampus slice perfused with 20 µM 4AP showing coherent epileptiform activity at both recording sites. Scatter plots showing the effect of application of 30 µM DMPP on 4AP induced bursting activity in CA1 (1i) and CA3 (2i). Note the frequency of 4AP-induced epileptiform activity and also DMPP-induced potentiation are exactly same at both recording sites. B. Transection of the Schaffer-collaterals connections results in a non synchronous and independent epileptiform activity in CA1 (1) and CA3 (2) subfields (in a minority of slices, n= 3 of 15) suggesting that both areas have the capacity to generate epileptiform activity (see text). Application of 30 µM DMPP results in a significant increase in burst frequency in both CA1 (1i) and CA3 (2i) yet their activity remains independent.

15, data not shown). However, in 3 out of 15 slices, a non synchronous and less frequent epileptiform activity was also recorded on CA1 (Figure 3.17 B, a mean instantaneous frequency of 0.6 ± 0.2Hz in CA1 compared to 0.86 ± 0.3Hz in CA3, n=3). This result suggests that epileptiform activity generated in CA3 and propagated to area CA1 is the predominant from of network activity. On the other hand, extracellular activity recorded in CA1 region in the absence of a functional Schaffer-collateral input suggests that the CA1 field can generate epileptiform activity in dependent of the CA3 and under these circumstances, bursting activity recorded in CA1 and CA3 are independent. Subsequent application of 30 μM DMPP also resulted in an asynchronous increase in 4AP-induced burst frequency on CA3 and CA1 regions (Figure 3.17 B,1i-2i; n=3) suggesting that nAChR-induced modulation of epileptiform activity is not unique action with area CA3.

3.7 Discussion

3.7.1 4AP-induced epileptiform activity

The majority of the results described in this chapter were carried out in rat hippocampal slices using the extracellular recording configuration to monitor network activity. Extracellular recording from the *in vitro* hippocampal slice preparation is an ideal method with which to study epileptiform discharges (Jefferys, 1993) in that it allows synchronous activity of populations of neurones to be detected. A limited number of intracellular recordings were also carried out to confirm that the patterns of activity studied at the extracellular (network) level reflected the activity of neurones at the single cell level. Although such cellular recording studies can reveal important mechanistic information regarding the excitability and biophysical properties of individual neurones, they do not truly reflect the pathophysiological aspects of the disease, with regard to excessively co-ordinated firing of groups of nerve cells. This, together with the robust responses and ability to maintain stable recording over long

periods was the justification for utilising the extracellular recording configuration for the majority of experiments described. Similarly, the 4AP model of epileptiform activity was initially chosen for its rather stereotyped nature and ability to give reliable responses that are stable over prolonged periods. The characteristics of 4AP-induced spontaneous bursting activity have been described both in hippocampus (Avoli et al., 1993; Buckle and Haas, 1982; Ives and Jefferys, 1990; Perreault and Avoli, 1989; Perreault and Avoli, 1991; Perreault and Avoli, 1992; Rutecki et al., 1987; Segal, 1987; Traub et al., 1995; Voskuyl and Albus, 1985) as well as other cortical areas (Barkai et al., 1995; Hoffman and Prince, 1995; Mattia et al., 1993). Three types of burst characteristics have been documented to result from the use of 4AP (see section 3.1). In our experiments, negative potentials were never detected and long lasting ictal-activity was only recorded in small minority of slices (2%). The typical activity was a series of interictal epileptiform discharges, which was seen in %98 of all slices tested.

Experimental conditions, such as the cortical area from which the slice originated, species and age of animal used for slice preparations, ionic concentrations, temperature and the exact recording site within the slice all considerably affect the characteristics of spontaneous activity. For example, the neocortex of guinea-pig has a higher propensity to generate 4AP-induced spontaneous epileptiform activity than that of rat (Mattia et al., 1993). Moreover, slices from the somatosensory cortex of adult rats proved to be less prone to epileptic seizures than hippocampal or entorhinal cortex slices or cortical slices prepared from young animals (Courtney and Prince, 1977; Wong and Yamada, 2001). Therefore, we have tried to carry out all of experiments in the same experimental set-up, using same type of preparation and under similar experimental conditions.

The spontaneous extracellular field events described here comprised of a brief initial period of negativity followed by a slower positive and then negative potential which is in agreement with original descriptions (Voskuyl and Albus, 1985). However,

within individual burst events there existed higher frequency oscillatory components in the range of 82 to 175Hz that resembled hippocampal sharp waves described *in vivo* (Buzsaki, 1986) and which have also been described *in vitro* under conditions of elevated extracellular potassium concentrations (Staley et al., 1998). Extracellular field recordings are ideally suited to the study of epileptiform activity and other network responses that are characterised by synchronous activity within neuronal ensembles (Jefferys, 1993). That the fast extracellular potentials were associated with neuronal discharges at the level of single cells as shown in figure 3.1 A confirms that the 4AP-induced field events recorded did indeed represent the coherent activity of populations of neurones within the hippocampus. Furthermore, these data also highlight the precision of firing within individual burst events whereby coherent action potential discharge appears to be regulated on a millisecond time scale (Mainen and Sejnowski, 1995). The precise mechanisms underlying 4AP-induced bursting is not fully characterised but appears to involve multiple synaptic and non-synaptic elements that together contribute to the overall activity (Traub et al., 2001; Traub et al., 1995). An important element of this is likely to be the role of direct electrical coupling through gap junctions. Indeed, recent studies have shown 4AP-induced epileptiform activity to be sensitive to both gap junction blockers as well as connexin 36 gene knockout (Maier et al., 2002; Ross et al., 2000; Traub et al., 2001). Whilst activation of nAChRs resulted in an increase in burst frequency, it is apparent that the mechanisms responsible for the precise firing of action potentials and fast activity within individual bursting events are not compromised upon nAChR activation.

The observation that transection of the Schaffer-collateral connections results in epileptiform activity being restricted to the CA3 region in the majority of slices tested confirms the role of the CA3 region as being the primary generator of 4AP-induced epileptiform bursting (Jefferys, 1993) and thus points to CA3 pyramidal cell recurrent connections as having a critical roll in generation of this activity as has been suggested previously (Christian and Dudek, 1988; MacVicar and Dudek, 1980). On

the other hand, the ability of a minority of isolated CA1 'mini slices' to generate 4AP-induced epileptiform bursting reveals the intrinsically ability of CA1 circuits in generating epileptiform activity. It is likely that this ability is normally masked by the dominant action of the CA3 circuits which are normally propagated via the Schaffer-collateral pathway.

3.7.2 Regulation of burst frequency by nAChR activation

The main finding of this study so far is the demonstration that activation of nAChRs using a range of selective nAChR agonists results in a modest enhancement of 4AP-induced bursting activity in the hippocampus. The pro-epileptogenic action of nAChR agonists was rather subtle however. Indeed, when applied to naïve hippocampal slices on their own, the nAChR ligands tested did not produce any detectable spontaneous extracellular field activity suggesting that they do not produce epileptiform activity *per se* but rather have a modulatory action over pre-expressed synchronised bursting activity.

Irrespective of their differing pharmacological profiles at recombinant nAChRs, most agonists tested produced a concentration dependent increase in burst frequency in the presence of 4AP. Analysis of their potencies for facilitating epileptiform activity revealed that they were consistent with their potencies in functional studies that directly assess nAChR activity (Alkondon et al., 2000; 1997; Chavez-Noriega et al., 2000; Guo and Chiappinelli, 2002; Stauderman et al., 1998). The maximum enhancement induced by lobeline, choline, DMPP and nicotine was relatively agonist independent amounting to a 25-35% potentiation in each case. Barring nicotine which sometimes produced a transient increase in burst frequency, each agonist induced a potentiation that persisted for the period of agonist application. Interestingly, most agonists when applied at high concentrations produced lower levels of potentiation, or even inhibition, thereby generating bell shaped concentration response

relationships; a finding that mirrors the profile of activity of these nAChR agonists in expression systems (Chavez-Noriega et al., 2000; Stauderman et al., 1998). The explanation for this shared yet unusual profile of activity is unclear. Aside from the relative affinity of different agonists for the various nAChR subtypes (Gotti et al., 1997) which will contribute to the differences in potency and duration of effect of each agonist tested it is likely that (a) the degree or state of receptor desensitisation (Fenster et al., 1999), (b) degree of channel block (Marshall et al., 1991; Stauderman et al., 1998) and (c) non specific activity (e.g. in the case of lobeline block of voltage gated calcium channels (Santha et al., 2000) contribute to shaping the concentration response curve. This latter effect may explain the finding that lobeline produced a reduction in burst frequency to levels below control conditions. Another possibility is that there exists a significant degree of tonic activation of nAChRs in some slices causing a basal nAChR-mediated elevation in epileptiform bursting. This is perhaps not surprising given that 4AP is likely to enhance release of neurotransmitters from all terminals within the hippocampus (Rutecki et al., 1987) including cholinergic terminals. The subcortical cholinergic innervation to the hippocampus is very dense and it is possible that there is a constant release of ACh from the degenerating cholinergic terminal field. Moreover, there is also reported to be a sparse population of cholinergic interneurones within the hippocampus (Frotscher et al., 1986; Frotscher et al., 2000) which may contribute to tonic nAChR activation (Cobb et al., 1999). nAChRs are known to exhibit significant receptor desensitisation in the presence of high agonist levels. One explanation therefore is that high concentrations of nAChR agonists may cause overwhelming desensitisation of the hippocampal nAChR population and thus reduce the tonic activation of the receptor population within the slice. We think this is unlikely however, as application of nAChR antagonists in the absence of nAChR agonist failed to produce any detectable change in burst frequency in the majority of slices tested. That said, the lack of effect of nAChR antagonists alone on epileptiform activity in isolated hippocampal slices suggests little or no tonic activation of nAChRs controlling epileptiform activity. However, it should be noted that in such an isolated *in vitro* system the major

cholinergic afferents are severed. Pertinent to this point, additional nAChR-mediated mechanisms to those present in hippocampal slices must operate *in vivo* to explain why nAChR agonists induce epilepsy in their own right in whole animal experiments (Damaj et al., 1999; Miner et al., 1985).

Irrespective of which mechanism(s) is/are operating the finding that choline potentiates epileptiform activity raises the possibility that this chemical entity may provide a physiologically relevant contribution to the patterning of epileptic activity *in vivo* since this natural breakdown product of acetylcholine achieves plasma concentrations close to the levels that we have used to potentiate epileptiform activity *in vitro* (Klein et al., 1992).

A striking feature of the nAChR-induced enhancement of burst frequency described here was that the effect was prolonged, generally lasting for the duration of agonist application. An exception to this finding was the effects produced by nicotine that commonly resulted in only a transient increase in burst frequency. The reason for such differences is unclear but may reflect differences in the degree or state of desensitisation or relative affinity for different nAChR subtypes (Gotti et al., 1997). Whilst nAChRs are classically considered to be for fast signalling and rapidly desensitise, recent reports in the autonomic nervous system suggest that nAChR agonists including nicotine can produce calcium spiking in presynaptic nerve terminals (Brains et al., 2001) which are sustained over prolonged periods of agonist application similar to those used in this study. This supports the proposal nAChRs have a dual role in both mediating fast synaptic excitation in certain hippocampal circuits (Frazier et al., 1998b; Hefft et al., 1999) whilst mediating a slower neuromodulatory action (McGehee and Role, 1996; Role and Berg, 1996; Wonnacott, 1997), in particular the modulation of neurotransmitter release (Albuquerque et al., 1997; Gray et al., 1996; Ji and Dani, 2000) and synaptic plasticity (Ji et al., 2001). Furthermore, it is possible that nAChR activation produces a transient activation of

the receptor, which in turn triggers a more prolonged action perhaps involving calcium-mediated signalling pathways.

A consistent finding in these experiments was the shortening of burst duration as the frequency of bursts increased. Such a relationship between burst duration and frequency has been studied in detail by Staley and colleagues (Bains et al., 1999; Staley et al., 1998) who concluded that both parameters relate to the presynaptic release of glutamate from recurrent circuits. The action of nicotinic acetylcholine receptors with regard to the modulation of glutamatergic recurrent collaterals is addressed specifically in chapter 5.

3.7.3 Pharmacology of nAChR-induced effect

The cholinergic afferents of the septohippocampal pathway is effective in generating limbic seizures (Fraser and Macvicar, 1996) and, thus, injection of cholinergic agents is used as a model for temporal lobe epilepsy (Lothman et al., 1991). Indeed, hippocampal sclerosis after infusion of cholinergic agonists accurately mimics numerous pathological indices of human status epilepticus (Wasterlain et al., 1993). In the hippocampal slice preparation, these same analogs generate prolonged depolarisations (Bianchi and Wong, 1994) and result in a cholinergic-dependent slow afterdepolarisation and long-lasting plateau potential which has properties reminiscent of ictal depolarisations observed during cholinergic-induced seizures (Fraser and Macvicar, 1996). These actions have been attributed to the activation of muscarinic receptors as they are blocked by the selective muscarinic receptor antagonist atropine (Bianchi and Wong, 1994; Fraser and Macvicar, 1996). Activation of muscarinic receptors during blockade of GABA(A)-mediated inhibition also induces synchronous epileptiform activity in immature rat hippocampus (Psarropoulou and Dallaire, 1998) and neocortex (Sutor and Hablitz, 1989).

In addition to the role of muscarinic acetylcholine receptor in modulating epileptiform activity, many reports indicate that nicotinic acetylcholine receptors also regulate neuronal excitability within the CNS (Jones et al., 1999) and may be relevant to several forms of idiopathic epilepsy (Steinlein, 2001). It has recently been found that certain forms of epilepsy may arise from mutations in the genes responsible for encoding of nicotinic acetylcholine receptor subunits (Steinlein et al., 1997; Steinlein et al., 1995). Many reports have also shown that high doses of nicotine induces seizures in animals, an effect shown to be blocked by a variety of nAChR antagonists (Damaj et al., 1999). Therefore, it seems that both nicotinic and muscarinic subtypes of acetylcholine receptors are likely to modulate epileptiform activity. However, the relative contribution of nAChR versus mAChR action in regulating epileptiform activity remains unclear. In our experiments we used very selective nAChR ligands to minimise any probable contribution of mAChRs. Moreover in order to confirm that the observed burst frequency potentiation is through activation of nAChRs and not mAChRs, a series of experiments carried out in the presence of atropine. Such application of atropine did not affect the ability of DMPP to potentiate epileptiform burst frequency suggesting that mAChRs were not involved in the observed burst frequency facilitation. Conversely, the effect of DMPP was significantly blocked by co-application of nAChR selective antagonists confirming the involvement of nAChRs in DMPP-induced burst frequency facilitation. Only in these experiments where the burst frequency potentiation was induced by MCC, was the observed burst frequency potentiation reversed following co-application of atropine. This result indicates that the response to MCC at the concentration tested (10-30 μM) was principally due to its actions at mAChRs. In conclusion these results support a dual influence of muscarinic and nicotinic acetylcholine receptors on epileptiform activity.

Whilst the overall picture that activation of nAChRs can potentiate 4AP-induced epileptiform activity in the hippocampus is evident, the precise pharmacology of such effects were rather complex and difficult to interpret fully. The agonists DMPP, lobeline, choline and nicotine are recognised to be selective nAChRs ligands. Our

results agree with functional studies which show DMPP to have potent agonist actions with EC_{50} values in the micromolar range (Chavez-Noriega et al., 2000; Stauderman et al., 1998). Choline is a weaker agonist yet is nevertheless reported to be an agonist at α7 subunit containing nAChRs (Albuquerque et al., 1997; Alkondon et al., 2000). Moreover, a recent report suggest that choline may also activate a novel form of nAChR that is distinct from the classical α7 subunit containing nAChR (Guo and Chiappinelli, 2002). Again the potency of choline in potentiating 4AP-induced epileptiform responses agrees with published data looking directly at nAChRs (Alkondon et al., 1997; 2000; Guo and Chiappinelli, 2002). Indeed, the finding that choline produced a pro-epileptogenic actions may be significant since, as the natural breakdown product of acetylcholine synthesis, choline may achieve high plasma concentrations approaching those levels used in this study (Klein et al., 1992). Further studies are required however, to establish whether as an endogenous activator of certain nAChRs, choline may serve pathological roles *in vivo*.

The concept that the effects of the agonists used in this study are due to activation of nAChRs is supported by the observations that agonist induced frequency potentiation is inhibited by selective nAChR antagonists. In general terms, all antagonists tested including the non-selective and non-competitive antagonist mecamylamine (Chavez-Noriega et al., 1997) as well as the α4β2 subunit preferring antagonist DHβE (Alkondon and Albuquerque, 1993; Chavez-Noriega et al., 1997) and the α7 subunit preferring nAChR antagonists α-Bgt and MLA (Alkondon et al., 1992) produced a reversal of the burst frequency potentiation induced by DMPP or choline. DMPP, which is known to have affinity for a number of subtypes of nAChR, is predominantly mediating its effects through non-α7 subunit-containing receptors whilst the choline response is primarily mediated through α7 subunit containing receptors and displays specificity for this receptor subunit (Albuquerque et al., 1997; Alkondon et al., 2000). The fact that the effects of both agonist were inhibited by the α7 nAChR-specific antagonist α-Bgt (100 nM), but not by DhβE (1 μM) at

concentrations known to be selective for non-α7 nAChRs perhaps points to these possibility that α7 subunit containing receptors are contributing to the frequency potentiation induced by both DMPP and choline and that the differences may simply reflect the competitive interaction between the ligands. However, in this study it was not possible to define with any certainty the precise subtypes of nAChRs involved in frequency potentiation. Firstly, the pharmacopea of very selective subtype specific nAChRs is as yet rather limited. Few agonists have sufficient nAChR subtype selectivity for the exclusive activation of a particular subtype. Thus, with such a divers range of subunits, which can give rise to a large number of receptor subtypes, determining which nicotinic receptor type is mediating this effect is difficult. Secondly, high concentrations of pharmacological agents were used in this study to overcome tissue penetration problems associated with brain slices maintained in the interface configuration. Most of the drugs lose selectivity or can show some nonspecific effects at high concentrations: DHβE is selective for α4β2 receptors at 10^{-7} to 10^{-6} M. At concentrations as high as 10^{-5} M, it begins to lose selectivity and will block a fraction (30-50%) of α7-mediated response at equilibrium (Alkondon and Albuquerque, 1993; Chavez-Noriega et al., 1997). α-Bgt appears to be specific for α7 receptors in the hippocampus, while MLA is selective for α7 receptors at low nM concentrations, but will begin to block α4β2 receptors at higher concentrations (Alkondon et al., 1992; Alkondon and Albuquerque, 1993). Finally, there was considerable inherent variability in the precise features of epileptiform activity between slices even using the same epileptiform model (Jefferys, 1994). Nevertheless, taken together, the antagonist data suggest that α7 subunit containing receptors are at least in part involved in the regulation of epileptiform busting. Clearly further detailed pharmacological studies using submerged slices and more selective agents are required to determine the important question relating to the relative contribution of different subtypes of nAChR in regulating epileptiform activities. It is interesting to note, however, that recent studies point to specific nAChR subunits being important in certain forms of epilepsy (Elmslie et al., 1997).

Thus, autosomal-dominant nocturnal frontal lobe epilepsy (ADNFLE), which is a form of idiopathic epilepsy, has linkages to genetic mutations encoding a loss of function of the α4 nAChR subunit (Steinlein et al., 1997; Steinlein, 2000; Steinlein et al., 1995) which causes a reduction in seizure threshold (Gotti et al., 1997) and other epilepsies to mutations in the genes encoding the α7 and β3 nAChR subunits (Durner et al., 1999; Elmslie et al., 1997; Neubauer et al., 1998; Phillips et al., 1998); these data supporting our own concept that multiple nAChR subunits are involved in the patterning of epileptiform activity.

CHAPTER 4

REGULATION OF BICUCULLINE AND LOW MAGNESIUM-INDUCED EPILEPTIFORM ACTIVITY BY nAChR ACTIVATION

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REGULATION OF BICUCULLINE AND LOW MAGNESIUM-INDUCED EPILEPTIFORM ACTIVITY BY nAChR ACTIVATION

4.1 Introduction

Epileptiform activity has been reported to be induced following incubation by various GABA receptor antagonists including picrotoxin (Knowles et al., 1987; Kohr and Heinemann, 1990) and bicuculline (Colom and Saggau, 1994; Gulyas-Kovacs et al., 2002; Herron et al., 1985). With respect to the pharmacological action of these compounds epileptiform activity results primarily from reduced $GABA_A$ receptor mediated inhibition. In addition to receptor antagonists, various brain slice preparations generate epileptiform activity when perfused with a medium devoid of added magnesium (Gulyas-Kovacs et al., 2002; Horne et al., 1986; Jones, 1989; Mody et al., 1987; Whittington et al., 1995). The mechanism by which this occurs relates to the ability of the decreased $[Mg^{2+}]_o$ to remove the voltage-dependent Mg^{2+} block from the NMDA-activated channels, thus the excitatory glutamatergic transmission is enormously enhanced (Mody et al., 1987; Traub et al., 1994).

To assess whether the nAChR induced facilitation of epileptiform bursting was unique to 4AP-induced activity or whether nAChR activation had a more general modulatory influence over other forms of epileptiform activity, we examined the effect of the nAChR agonist DMPP on two additional pharmacological paradigms, bicuculline (reduced inhibition) and low magnesium (enhanced excitation) models.

4.2 Bicuculline (BIC)-induced epileptiform model

4.2.1 Characteristics of BIC-induced epileptiform activity

Extracellular recordings were made from the CA3 pyramidal cell layer of rat hippocampal slices, which were disinhibited by bath application of the $GABA_A$ receptor antagonist bicuculline (30 μM). This resulted in a constant, gradual appearance of spontaneous field potentials 125 ± 40 minutes after bath application of bicuculline (30 μM, n= 4). BIC-induced epileptiform discharges occurred every 5 to 20 s with a mean frequency of 0.13 ± 0.04Hz and a mean duration of 392 ± 39ms. An elevated level of K^+ ions in the ACSF to 5mM was used in subsequent bicuculline experiments in order to decrease the latency of appearance of bicuculline-induced epileptiform discharge. Increasing $[K^+]_o$ from 3 to 5mM resulted in an earlier appearance of epileptiform discharges (mean latency=20.8 ± 5.5 minutes after application of 20 μM bicuculline) and brought the bursts slightly closer to the duration and frequency observed in the 4AP induced bursting model (mean burst duration=366.6 ± 64ms, mean burst frequency=0.146 ± 0.02Hz, n=30)(Ives and Jefferys, 1990).

The overall shape of an individual epileptiform discharge induced by bicuculline was different from those observed in the presence of 4AP. They usually observed in three types: (1) single, short inter-ictal bursts (Figure 4.1, asterisks in Ai); (2) double bursts (Figure 4.1, double asterisks in Ai) and (3) long polyspike bursts (Figure 4.1, triple asterisks in Bi). The short inter-ictal like bursts (mean burst duration=258 ± 8ms) comprised of an initial short negative potential (<10ms) followed by a biphasic potential, similar to those recorded in 4AP model (Figure 4.1 A, asterisks). A similar high frequency component was also evident throughout the event (Figure 4.1 Aii, arrows). Double bursts consisted of two single bursts each of which had a triphasic waveform (mean burst duration=360 ± 10ms; Figure 4.1 A, double asterisks). The long polyspike bursts consisted of a primary burst, resembling an inter-ictal burst, followed by a train of repetitive short bursts lasting up to 600 ms (mean burst duration=482.7 ± 32ms; Figure 4.1

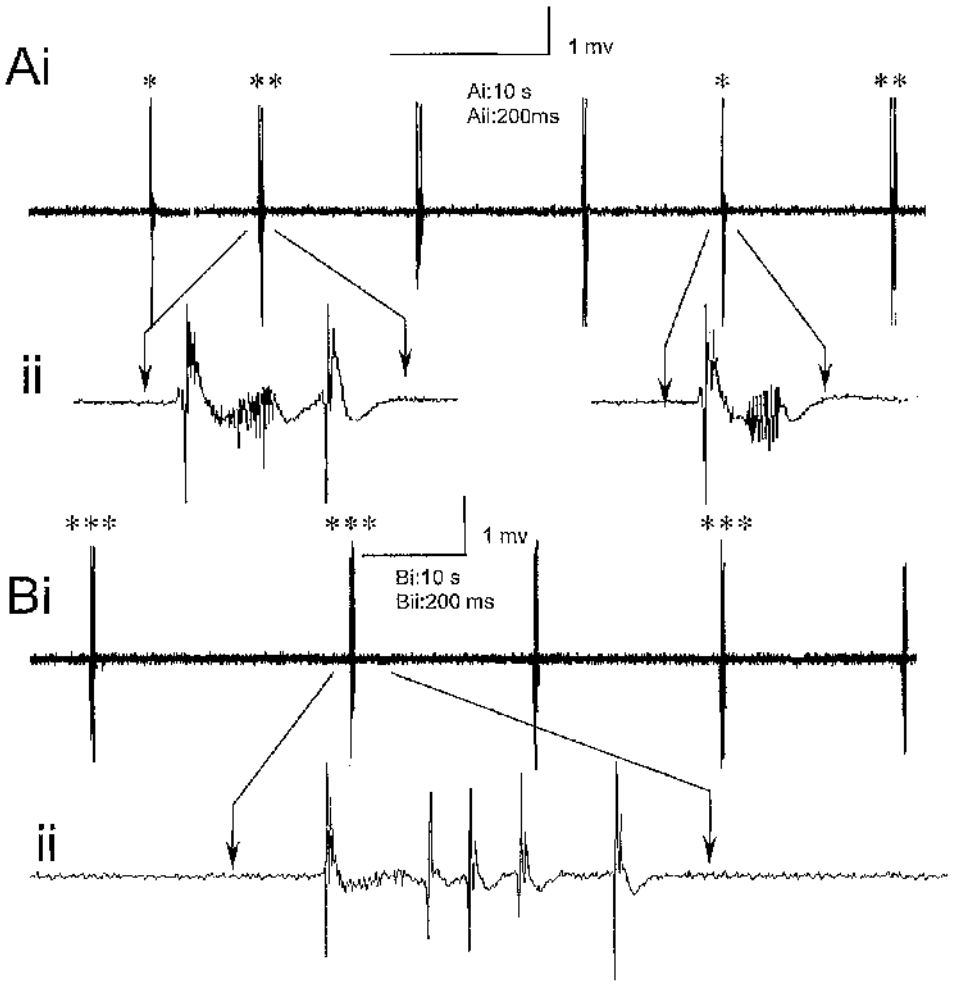

Figure 4.1. *Bicuculline-induced epileptiform bursting activity in area CA3 of the rat hippocampal slice.*
Ai. Extracellular field potential recording from the CA3 stratum pyramidale indicate two types of BIC-induced bursting activity: single short bursts (asterisks) and double longer bursts (double asterisks). A*ii*. Expanded traces showing individual single (right) and double (left) bursting responses at the level of the population response. Bi. Third type of BIC-induced epileptiform bursting indicated by triple asterisks. Bii. Expanded trace showing individual long polyspike bursts consisting of a primary burst, followed by a train of short individual discharges. Scales vertical Ai,Bi: 1mV; horizontal Ai,Bi: 10s; Aii,Bii: 200ms.

B, triple asterisks). However, it is noticeable that the high frequency component is mainly restricted to the initial event in the train. Single and double bursts were observed in most slices and polyspike bursts were present in 7 out of 30 slices.

Scatter plot in figure 4.2 shows a representative experiment in which the slices were incubated in a raised $[K^+]_o$ to 5mM medium prior to disinhibition by application of 20 μM bicuculline. This resulted in a regular synchronised extracellular discharge, which persisted for the duration of experiment (>2hours).

4.2.2 Effect of nAChR agonist on BIC-induced epileptiform activity

Once stable BIC-induced epileptiform discharge was established, the nAChR selective agonist DMPP was co-applied in order to examine the effect of nicotinic receptor activation upon this form of synchronised bursting. In 14 of 14 slices tested, subsequent co-application of DMPP (10-30 μM) produced a significant increase in burst frequency (Figure 4.3; mean maximal frequency potentiation=248 ± 76% of pre-DMPP baseline frequency) that was reversible upon washout of DMPP (n=6, Figure 4.3). In further experiments, co-application of the nAChR antagonists mecamylamine (50-200 μM, n=3; Figure 4.4 A) and DHβE (20-40 μM; n=4 of 5; Figure 4.4 B) were also found to reverse the DMPP-induced burst frequency potentiation.

As in 4AP model, application of DMPP resulted in a significant reduction in the duration of individual BIC-induced burst events from 366.6 ± 64ms (mean of pre-DMPP baseline duration) to 280 ± 25ms after DMPP application.

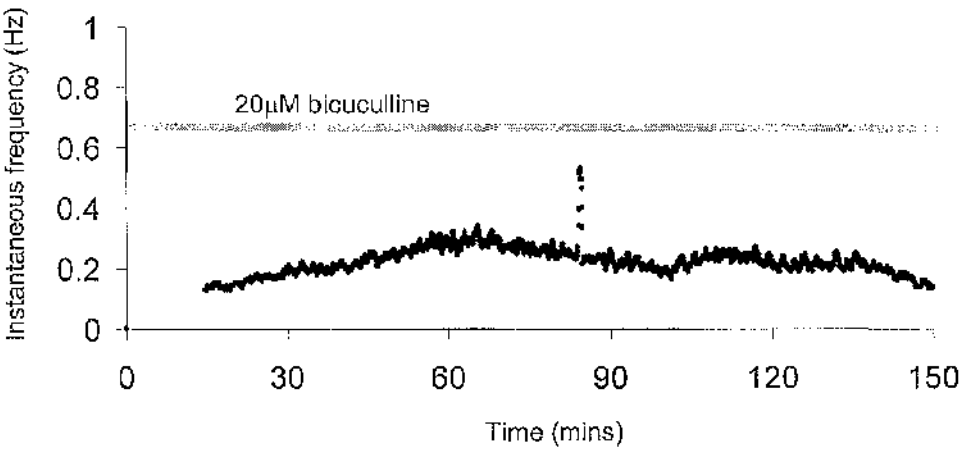

Figure 4.2. *Bicuculline-induced epileptiform discharges remain stable over prolonged recording periods.*
Scatter plot showing a representative experiment in which disinhibition of the slice following application of the $GABA_A$ receptor antagonist bicuculline (20 μM) at time point 0 produced a regular synchronised extracellular discharge. Note that bicuculline-induced burst frequency remained stable over the extended period of experiment.

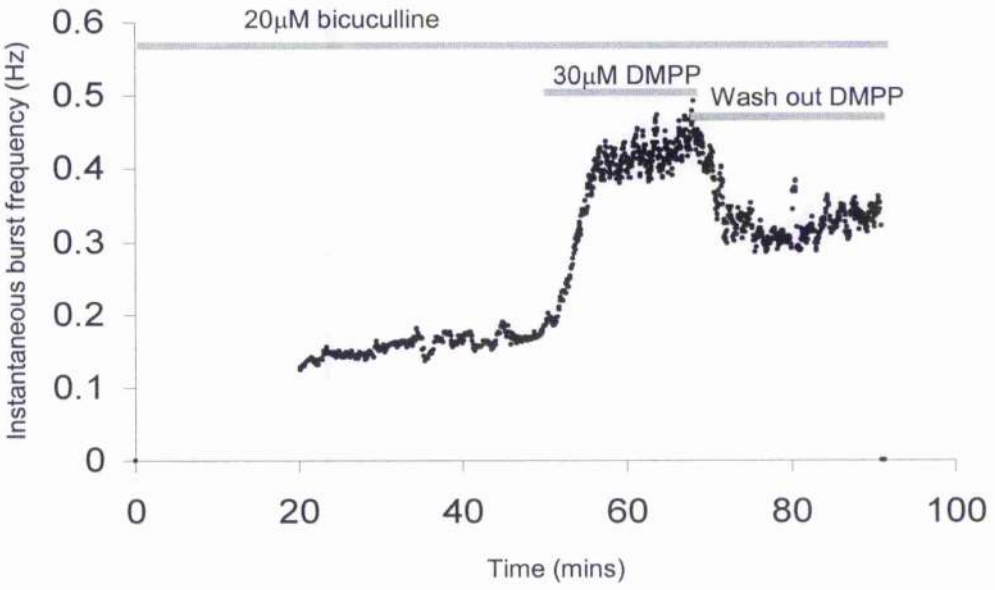

Figure 4.3. *nAChR activation potentiates BIC-induced epileptiform activity in vitro.* Scatter plot showing instantaneous burst frequency in response to continuous application of 20μM bicuculline for the duration of a representative experiment. Co-application of the selective nAChR agonist DMPP (30μM) as indicated by the horizontal bar results in an increase in burst frequency. This was partially reversible upon subsequent washout of DMPP.

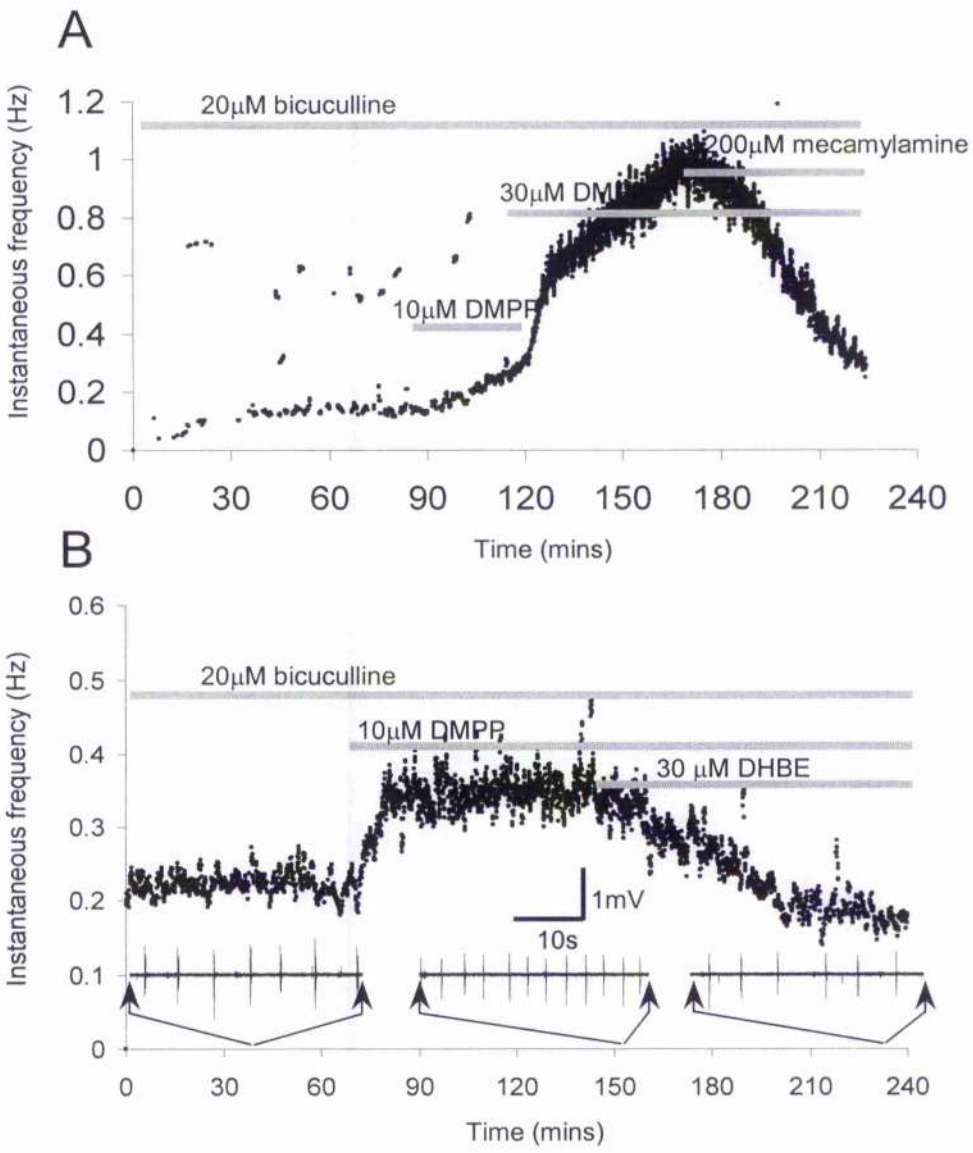

Figure 4.4. *Effect of selective nAChR antagonists on DMPP-induced potentiation of BIC-induced epileptiform bursting in area CA3 of the hippocampus.*
A. Scatter plot showing instantaneous burst frequency in response to continuous application of 20 μM bicuculline (horizontal bar) for the duration of a representative experiment. Co-application of the selective nAChR agonist DMPP (10-30 μM) as indicated by the horizontal bar results in an increase in burst frequency. This is reversed upon subsequent co-appilcation of non-competitive antagonist and nAChR channel blocker mecamylamine (200μM). B. Similar scatter plot in which the potentiating effect of 10 μM DMPP is reversed upon co-application of the selective nAChR antagonist Dihydro-β-erythroidine (30μM). Insets represent spontaneous field potentials before, during and after nAChR activation. Scales vertical:I mv, horizontal: 10 s

4.3 Low magnesium-induced epileptiform model

4.3.1 Characteristics of low magnesium-induced epileptiform activity

Extracellular recordings were made from the CA3 pyramidal cell layer of rat hippocampal slices incubated in ACSF in which Mg^{++} ions had been omitted (n=21). Spontaneous field potentials typically appeared 5-30 minutes following switch over to low magnesium ACSF, with an average latency of 19.8 ± 4 min, similar to that reported previously (Hsu et al., 2000).

Three different types of spontaneous synchronous activity were recorded in the CA3 *stratum pyramidale* of slices exposure to low magnesium ACSF. Brief, single inter-ictal-like discharges (mean duration=204.6 ± 18.9ms; mean frequency = 0.2 ± 0.03Hz; Figure 4.5 A, asterisks) and double bursts (mean duration=383 ± 6ms; mean frequency = 0.16 ± 0.03Hz; Figure 5A, double asterisks) were present in all slices tested (n=21). In addition, more prolonged ictal-like discharges (mean duration=3640 ± 900ms; mean frequency=0.04 ± 0.01Hz) were seen in 4 out of 21 slices (Figure 4.5 B).

4.3.2 Effect of nAChR agonist on low magnesium-induced epileptiform activity

To assess the effect of nAChR activation on ongoing low magnesium-induced epileptiform discharges, the selective nAChR agonist DMPP was bath applied to slices displayed stable epileptiform burst activity. Subsequent co-application of DMPP (30 μM; n=10 of 10 slices) resulted in a significant ($P<0.03$) increase in event frequency (mean maximal frequency potentiation=110 ± 37% of pre-DMPP baseline frequency). DMPP also increased the frequency of pre-existing ictal-like discharges (n=4 of 4 slices). Increase in burst frequency was associated with decrease in the duration of both inter-ictal-like and ictal-like discharges. For

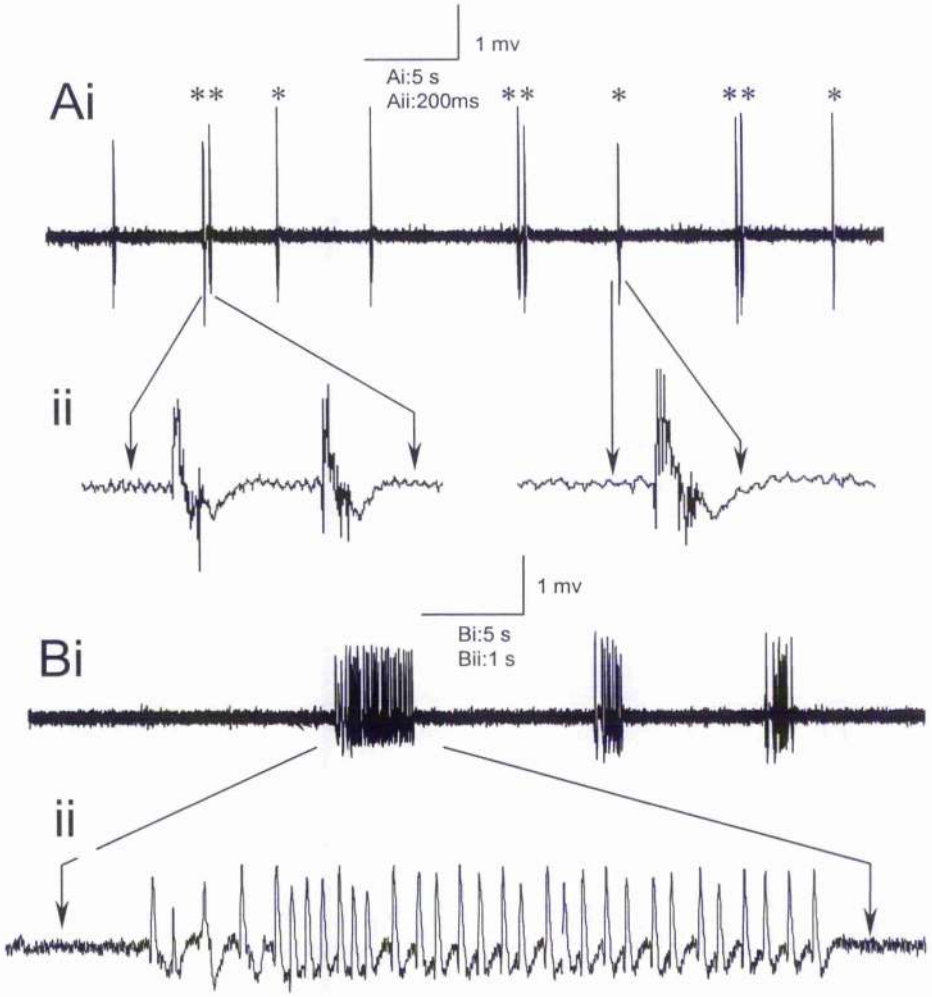

Figure 4.5. *Low magnesium-induced epileptiform bursting activity in area CA3 of the rat hippocampal slice.*

Ai. Representative examples of brief spontaneous synchronous events recorded in the CA3 stratum pyramidal of slices incubated in low magnesium ACSF: Brief, single discharges (asterisks) and double bursts (double asterisks) . Aii. Expanded traces showing individual single (right) and double (left) bursting responses at the level of the population response. Bi. In a minority of slices, low magnesium medium resulted in the appearance of more prolonged ictal-like epileptiform discharges. Bii. Expanded trace showing individual long polyspike bursts consisting of a train of short individual discharges lasting up to several seconds. Scales vertical Ai,Bi: 1mV; horizontal Ai,Bi: 10s; Aii:200 ms; Bii: 1s.

example, there was a reduction in the duration of ictal-like bursts from 3640 ± 900 ms (mean of pre-DMPP baseline duration) to 1100 ± 126.5ms after DMPP application. Furthermore, in 4 of 10 such slices tested, DMPP resulted in the appearance of many more secondary bursts or afterdischarges which had been infrequent or absent prior to nAChR agonist application (Figure 4.6 A).

DMPP-induced effects were reversible upon washout of the agonist (Figure 4.6 A; n=3) or upon co-application of the nAChR antagonists dihydro-β-erythroidine (20-40 μM; n=3 of 5; Figure 4.6 B) and MLA (5 μM; n=2 of 2; data not shown).

4.4 Comparison of spontaneous epileptiform activity in three *in vitro* epilepsy models

Spontaneous, recurring, inter-ictal-like epileptiform bursting developed in all three convulsant solutions. However, the exact characteristics of epileptiform activity were different. Comparing the three models, differences were detected in the time of appearance of the first spontaneous epileptiform discharge and in the frequency of epileptiform discharges after a 30–45-min stabilisation period. The duration of the individual spontaneous epileptiform bursting events was also different. The longest duration of discharges was observed in low magnesium, whilst the highest frequency of spontaneous events was detected in 4AP (Table 4.1). Slices exposed to 20 μM 4AP generated spontaneous epileptiform bursts, which lasted about 200-400ms and recurred regularly at a rate which varied between slices but which were in the range of 18-40 events per minute. Slices exposed to 20 μM BIC and low magnesium medium generated longer, less frequent bursts, lasting about 400ms and recurring irregularly at about 10 events per minute. The shape of spontaneous epileptiform discharges was rather complex. The overall waveform of an epileptiform discharge developed in 4AP was different from those observed in the other two convulsants. In 4AP model, a single triphasic waveform was observed in the majority of slices tested, whilst in BIC and low magnesium models double or polyspike bursts were common.

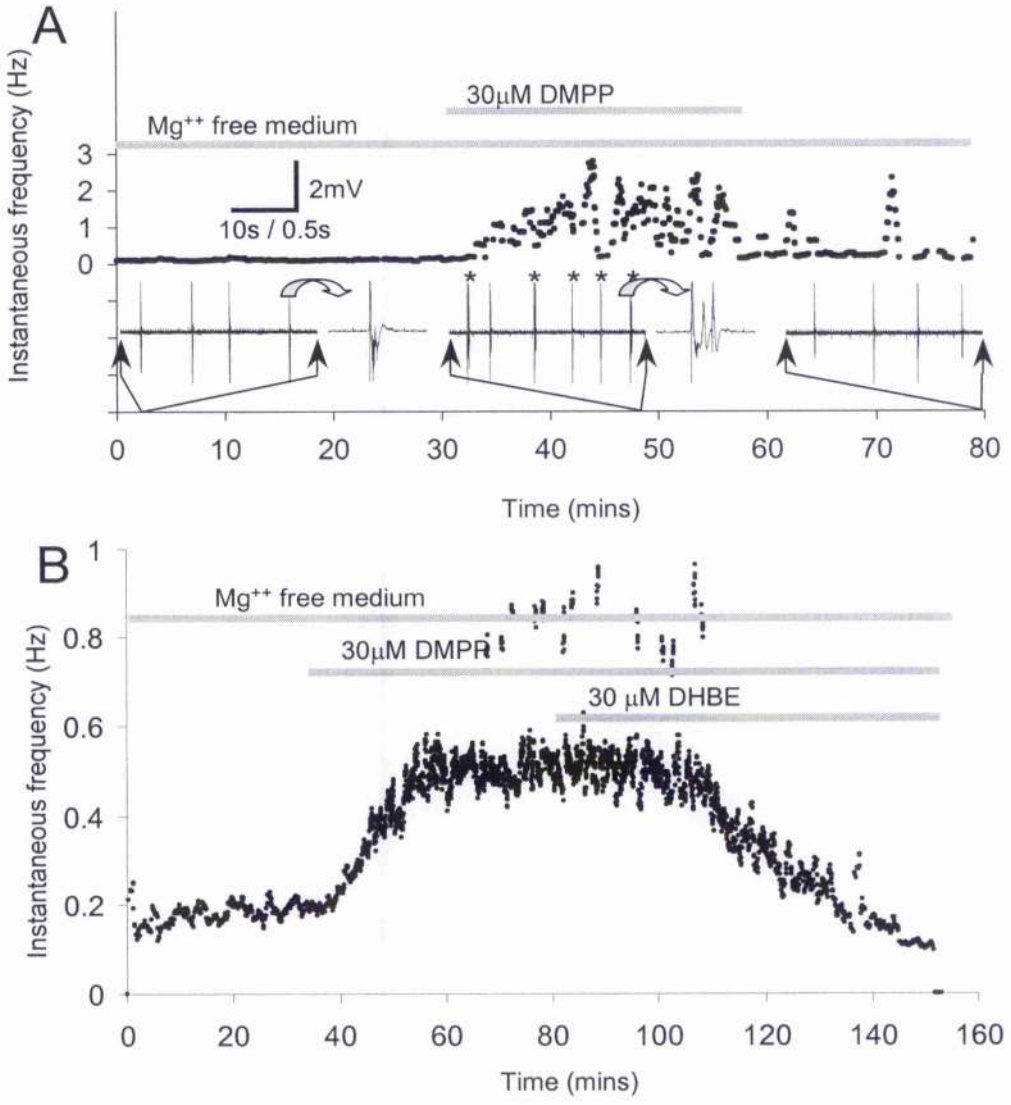

Figure 4.6. *Effect of selective nAChR-antagonists on DMPP-induced potentiation of low magnesium-induced epileptiform bursting in area CA3 of the hippocampus.* A. Scatter plot showing frequency of epileptiform events in another hippocampal slice in which NMDA-mediated excitation was enhanced by removal of Mg^{++} ions from the perfusing medium. Sections of voltage trace corresponding to the timepoints indicated by the arrows are shown at the bottom of the graph. Note that the increase in burst frequency in response to DMPP application (30μM, horizontal bar) was accompanied by the appearance of burst clusters or afterdischarges (indicated by *). Curved arrows point to expanded sections of trace illustrating individual field potential events. Subsequent washout of DMPP resulted in a reduction in burst frequency to baseline levels. Shaded horizontal bars indicate the period over which an individual agent was applied to the bath. B. Similar scatter plot in which the potentiating effect of 30 μM DMPP is reversed upon co-application of the selective nAChR antagonist Dihydro-β-erythroidine (30μM). Scales A, 2mV, 10s, 0.5s for expanded inset.

Indeed ictal-like activity recorded more generally in low magnesium model and observed in 4 out of 21 slices tested.

Table 4.1 *Characteristic parameters of seizure discharges developing in solutions containing different convulsants.*

	Latency (min)	Frequency (Hz)	Duration (ms)
4AP (n=26)	17 ± 2.6	0.4 ± 0.02	257 ± 15.2
BIC (n=30)	20.8 ± 5.5	0.146 ± 0.02	366.6 ± 64
Low magnesium (n=21)	19.8 ± 4	0.165 ± 0.024	711.62 ± 295

A comparison of the effects of DMPP upon the three models of epileptiform bursting revealed that whilst nAChR activation potentiated burst frequency in every model, there were quite marked differences in the magnitude of potentiation induced in each model. Application of DMPP (10-30 μM) resulted in a burst frequency potentiation in 4AP, BIC and low magnesium-induced activity with a mean maximal frequency potentiation of 37 ± 5%, 248 ± 76% and 110 ± 37% for three treatments, respectively ($P<0.01$). Clearly, application of DMPP produced a significant increase in all three models. However, comparing across models, qualitative and quantitative differences emerged: potentiation was most pronounced in the bicuculline model with lesser potentiation in the low magnesium model and lowest potentiation in the 4AP model. These data are summarised in histogram format in figure 4.7. The same data normalised to control (pre-DMPP) frequency show a significant increase in all cases (Figure 4.7 B, $P<0.01$) but with a greater frequency facilitation in bicuculline and low magnesium paradigms.

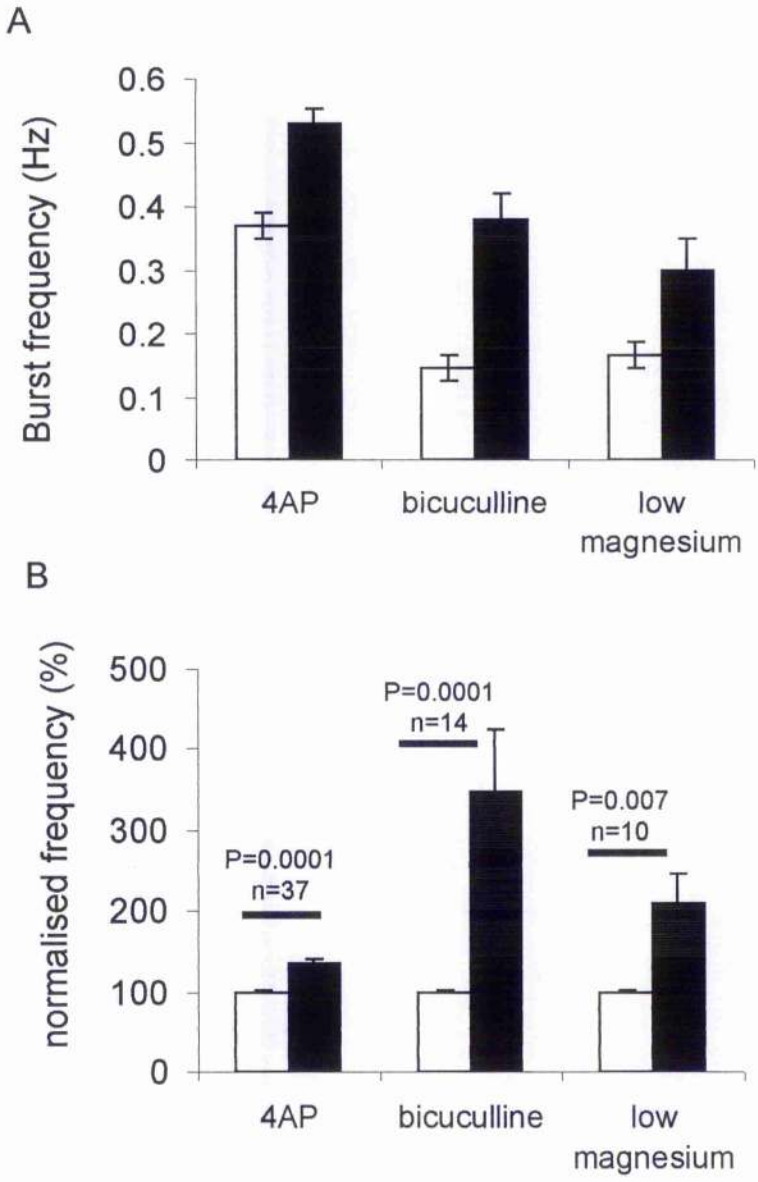

Figure 4.7. *Summary of DMPP-induced burst frequency potentiation in all three models of epileptiform activity.*
A. Histogram summarising 10-30 μM DMPP-induced burst frequency potentiation in 4AP, bicuculline and low magnesium models. B. Same data normalised to control (pre-DMPP) frequency show a significant increase in all cases (Wilcoxon-matched pair test, $P<0.01$). Note the relative burst frequency enhancement is greatest for bicuculline and under low magnesium conditions.

4.5 Discussion

Characteristics of the spontaneous epileptiform activity developed in different convulsants depend on the underlying mechanisms of neuronal synchronisation. 4AP, as a blocker of different K^+ channels (Storm, 1987; Storm, 1988), results in the direct depolarisation of pyramidal cells whilst also directly facilitating synaptic glutamate release (Perreault and Avoli, 1991; Traub et al., 1995). BIC, as a $GABA_A$ receptor antagonist, decreases the amplitude of inhibitory postsynaptic potentials, therefore facilitates propagation of excitatory signals through multisynaptic pathways (Gutnick et al., 1982; Miles and Wong, 1987). Bicuculline will also suppress the basal inhibition due to tonic release of GABA that is reported to occur in the hippocampal formation (Soltesz and Mody, 1994). In low magnesium, voltage-dependent blockade of *N*-methyl-D-aspartate (NMDA) receptors is reduced, thus allowing excitatory glutamatergic transmission to be enormously enhanced (Mody et al., 1987; Traub et al., 1994).

Different types of *in vitro* slice models have been introduced to analyse the development, maintenance and pharmacology of epileptiform activity evoked by the application of these different convulsants (Gulyas-Kovacs et al., 2002; Gutnick et al., 1982; Horne et al., 1986; Jefferys, 1994; Jones, 1989; Mody et al., 1987; Traub et al., 1994). The characteristics of resultant epileptiform activity have varied between studies. This is likely due to differences in the brain region from which the slice originated, in the method of drug application, or in the conditions of the recording epileptiform activity. For this reason, we carried out a series of experiments to compare the characteristics of spontaneous epileptiform activity induced by three different convulsant solutions and the effect of nAChR activation on these three convulsant models in the same type of preparation and under similar experimental conditions. However, characteristic features and pharmacological sensitivity of the discharges differed among the three models despite the fact that recordings were always carried out in the same place: in the cell body layer of area CA3 in hippocampal slices prepared from juvenile Wistar rats. The shortest latency of the first spontaneous epileptiform discharge and the

highest frequency were detected with 4AP. The longest duration of epileptiform discharges and bursts with duration approximating seizures were observed with low magnesium (Jefferys, 1994; Swartzwelder et al., 1987). In deed these results agree well with a recent comparative report, studying the characteristic of seizures developed in somatosensory cortical slices in these three models (Gulyas-Kovacs et al., 2002).

Inter-ictal discharges are characterised by two major abnormal properties: each involved neurone exhibits a transient large amplitude depolarisation (the "depolarisation shift") associated with repetitive spike generation, and this excitation arises with virtual synchrony in the majority of cells in a local population (Prince, 1968; Prince and Connors, 1986). The duration and amplitude of the paroxysmal depolarisation shift (PDS) depend on the actual membrane characteristics and on network properties. These characteristics may vary in different convulsant solutions. In BIC the synchronisation is mainly due to the altered network properties, namely inhibition is reduced in the local circuits. In 4-AP, and especially in low magnesium, changes in the membrane characteristics of neurones play a crucial role in the increased excitability. Thus, the characteristics of epileptiform discharges induced by each of these convulsants vary because of the different basic processes underlying epileptiform activity in each of them. Synchronised PDS activity of all neurones forming an active network determines the shape, duration and amplitude of the epileptiform discharges, which are detected extracellularly as field potentials (Gulyas-Kovacs et al., 2002).

The activation of NMDA receptors is accounted in some studies (Siniscalchi et al., 1997; Valenzuela and Benardo, 1995) to be the most important factor in the development of epileptic activity. Moreover, these receptors play a crucial role in the initiation and maintenance of epileptiform activity (Jefferys, 1994), especially in low magnesium (Gulyas-Kovacs et al., 2002). In low magnesium the excitability of these receptors increases extremely, which contributes to the development of long PDSs generating bursts or series of spikes. Both in BIC- and in 4AP-containing solutions, PDSs develop, which contribute to burst generation.

In BIC, however, a regenerative calcium-mediated spike was suggested to initiate the depolarisation (de Curtis et al., 1999). It is also reported that, in disinhibited hippocampal slices non-NMDA type receptors have the primary role in the initiation of epileptiform activity (Traub et al., 1993) and NMDA type receptors are important in the maintenance of epileptiform activity beyond the first 100-200 ms (Jefferys, 1994). While in 4AP enormous excitatory synaptic potentials through both types of ionotropic excitatory amino acid receptors were recorded as contributors to the PDS formation (Perreault and Avoli, 1991). This difference may be an important factor in the alteration of the shape of inter-ictal discharges (Gulyas-Kovacs et al., 2002).

The waveform of the discharges also depends on the relative location of the recording site and the burst initiation site. In different convulsants, seizure initiation may occur in different places. In many cases, including bicuculline and 4AP, the CA3 subfield is the site of initiation of epileptiform discharges (Jefferys, 1993). Where as, the prolonged ictal-like discharges in low magnesium and high $[K^+]_o$ start in CA1, not CA3, thus leading to the proposal that CA1 contains a separate neuronal aggregate necessary for seizures (Jefferys, 1993; Jefferys, 1994; Jensen and Yaari, 1988).

In previous chapter I have shown that nAChRs regulate epileptiform bursting induced by 4AP in rat hippocampal slices. The objective of the experiments described in this chapter was to assess whether nAChR activation has a general excitatory influence over patterned neuronal activity within cortical circuits, we examined the effect of the nAChR agonist DMPP on two additional epileptiform models: BIC and low magnesium models. Our results showed that nAChR activation again potentiates epileptiform activity in both two models. Application of DMPP resulted in an enhanced burst frequency, which was reversed upon washout or co-application of the selective nAChR antagonists. Moreover, DMPP made polyspike bursts or afterdischarges appear when absent in control in all three models or increased the frequency of pre-existing ictal-like discharges in low magnesium model. In all cases increase in burst frequency was associated

with decrease in burst duration and in the case of low magnesium decrease in duration of ictal-like bursting. The finding that nAChR activation also potentiates epileptiform bursting resulting from inhibition of $GABA_A$ receptors by application of BIC and reduction of Mg^{++} ions from the perfusion medium and concomitant elevation in NMDA receptor-mediated excitation (Traub et al., 1994) suggests that nAChRs affect a cellular mechanism that is common to all three epilepsy models investigated. In this respect, a potentially important locus of action is likely to be the recurrent glutamatergic synapses in area CA3 since these recurrent connections are believed to be critical in the generation and regulation of bursting activity within the CA3 network (Bains et al., 1999; Staley et al., 1998; Traub, 1991). Supporting this idea are recent studies that have demonstrated acetylcholine to modulate these synapses directly (Vogt and Regehr, 2001). Thus, it is plausible that frequency potentiation described here reflects the activation of presynaptic nAChRs that facilitate glutamate release which, in turn, forces the network into a higher frequency bursting mode. Consistent with this hypothesis as the frequency of bursting increased so the duration of bursts decreased; a relationship that has been studied in detail by Staley and colleagues during other experimental manipulations that increase burst frequency (Bains et al., 1999; Staley et al., 1998) and who concluded that both parameters relate to the presynaptic release of glutamate from recurrent circuits. An important observation in this study was that nAChR activation appeared to produce a greater enhancement of epileptiform burst frequency in the bicuculline and low magnesium experiments than in the 4AP studies (Figure 4.7). 4AP is known to enhance the release of all transmitters in the hippocampus including glutamate (Rutecki et al., 1987). Should nAChRs be acting through presynaptic facilitation of glutamatergic recurrent collateral connections then it is likely that in the 4AP model these synapses would already be potentiated partially by 4AP itself since this agent greatly promotes glutamate release (Rutecki et al., 1987). Such an explanation would account for the relative greater effectiveness of nAChR agonists in the bicuculline and low magnesium models. A second potential site for the involvement of nAChRs is in modulating GABAergic circuits. Indeed GABAergic hippocampal interneurones are known to express nAChRs (Freedman

et al., 1993; Freund and Buzsaki, 1996) and pharmacological studies have shown that either pharmacological or synaptic stimulation of these receptors produce an postsynaptic depolarisation or inward current (Frazier et al., 1998b; Jones and Yakel, 1997). Clearly however, further studies are required to establish the precise mechanisms by which nAChR activation promotes epileptiform bursting and other pathological network states (see next chapters).

CHAPTER 5

INVESTIGATION OF THE CONTRIBUTION OF GLUTAMATE CIRCUITS IN nAChR-INDUCED BURST FREQUENCY POTENTIATION

CHAPTER 5

INVESTIGATION OF THE CONTRIBUTION OF GLUTAMATE CIRCUITS IN nAChR-INDUCED BURST FREQUENCY POTENTIATION

5.1 Introduction

The numerous subunits that make up nAChRs result in a great diversity of functional receptors, equipping them with different pharmacological and biophysical properties, depending on nAChR subtypes and their location. Located at both pre- post and extra-synaptic sites, nAChRs are ideally placed to regulate neuronal excitability in the CNS (Jones et al., 1999). In the hippocampus, nAChRs, including the calcium permeable α7-type, are present on surface of both GABAergic inhibitory interneurones (Alkondon et al., 1997; Frazier et al., 1998a; Frazier et al., 1998b; Freedman et al., 1993; Freund and Buzsaki, 1996; Jones and Yakel, 1997; McQuiston and Madison, 1999b) and the excitatory glutamatergic pyramidal cells (Albuquerque et al., 1997; Alkondon et al., 1997; Ji et al., 2001), modulating both inhibitory and excitatory circuits. Thus, it is likely that the pro-epileptogenic action of nAChR activation described in previous chapters is mediated through glutamatergic circuits, GABAergic circuits or both.

In 1997 Alkondon reported that pyramidal cells responded to application of nicotinic agonist with a slow depolarisation. However, McQuiston and Madison (1999) reported that only 2 out of 15 pyramidal cells responded to ACh application with a slow depolarisation and inward currents and that such responses were very small and only just at the level of detection. A postsynaptic nicotinic response from pyramidal cells has been the subject of an ongoing dispute with most previous studies reporting that pharmacological activation of nAChRs

do not produce any form of membrane potential response in these cells (Frazier et al., 1998b; Jones and Yakel, 1997). Although pyramidal cells do express nicotinic receptors their role is thought to be presynaptic, important in the modulation of neurotransmitter release (Albuquerque et al., 1997; Gray et al., 1996; Ji and Dani, 2000). It is hypothesised that activation of presynaptic nicotinic receptors enhances transmission directly by elevating presynaptic calcium levels (Gray et al., 1996). However, some other studies were unable to evoke an increase in presynaptic calcium levels with local application of nicotinic receptor agonists suggesting an indirect synaptic modulation could arise from nicotinic excitation (Vogt and Regehr, 2001).

Many reports have shown that high doses of nicotine acting at the neuronal nAChRs can induce seizures in animals (Damaj et al., 1999; Miner and Collins, 1989; Miner et al., 1985). This effect has been studied in detail by Damaj and colleagues (1999) who concluded that nicotine by acting at the presynaptic nAChRs, enhance the release of glutamate, which in turn stimulates NMDA receptors leading to nitric oxide formation and possibly seizure production. However, such studies were all carried out *in vivo* in which there is limited scope for direct investigation of detailed synaptic and cellular mechanisms.

The work presented in this chapter was undertaken in hippocampal slice preparations to establish the mechanism by which nAChRs mediate their pro-epileptogenic actions and to investigate the probable contribution of glutamate circuits in nAChR-induced burst frequency potentiation. The specific aims of this chapter were:

1. To investigate the action of nAChRs on basal glutamatergic transmission
2. To investigate the effect of nAChR activation on epileptiform bursting activity in the presence of glutamate receptor antagonists.

5.2 Investigation of the action of nAChRs on basal glutamatergic transmission

5.2.1 Effect of nAChR activation on evoked glutamatergic synaptic transmission on area CA3

In this set of experiments, field excitatory postsynaptic potentials (fEPSPs) were recorded in the CA3 region of the hippocampal slices, placing recording electrode in the *stratum radiatum* of the CA3 region and stimulatory electrode within the hilus. Typically, fEPSPs evoked by local electrical stimulation consisted of a compound glutamatergic EPSP mediating by the activation of AMPA/Kainate and NMDA receptors. Figure 5.1A shows a representative example of a fEPSP recorded from the CA3 region on which specific glutamatergic antagonists were tested. Bath application of the AMPA/Kainate receptor antagonist, NBQX (2 μM) decreased the amplitude of the EPSP (Figure 5.1 A2) and the further addition of the NMDA receptor antagonist CGP40116 (50 μM) completely abolished the EPSP (Figure 5.1 A3), demonstrating the mixed NMDA and AMPA/Kainate-mediated nature of the evoked synaptic response.

Initial experiments were to investigate the stability of evoked fEPSPs in area CA3. In order to ensure that the amplitude of the evoked EPSPs on area CA3 remains stable over the period of experiments, control fEPSPs were recorded in the absence of DMPP. As illustrated in figure 5.1C, electrical stimulation of afferent fibres within the hilar region resulted in the occurrence of a stable EPSP in area CA3, which was sustained and stable for the extended duration of the experiments (up to 60 minutes, n=4, Figure 5.1 C control).

To investigate the effect of DMPP on evoked glutamatergic synaptic transmission in area CA3, 30 μM DMPP was applied following a stable EPSP baseline. The slices were stimulated once every 20 seconds for a period of 20-30 minutes before, during and after superfusion with DMPP. Bath application of DMPP (30 μM) resulted in a sustained and reversible enhancement of glutamate afferent

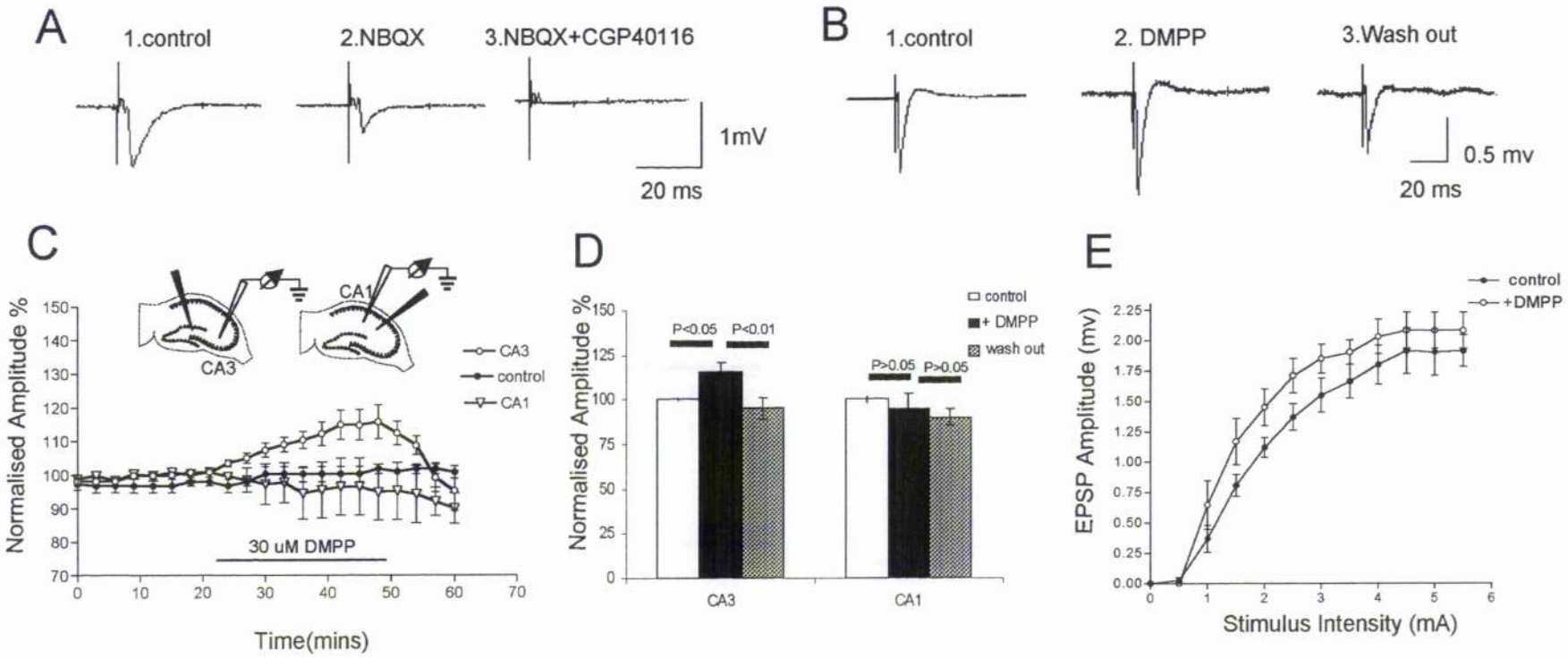

Figure 5.1. *Effect of DMPP on field EPSPs (fEPSPs) recorded in the CA3 and CA1 stratum radiatum of rat hippocampal slice preparation.*
A. Example traces of the response evoked by electrical stimulation in the hilus region (1) before and (2) duing superfusion with NBQX (2μM) alone and (3) combined application of NBQX and CGP40116 (50 μM). B. Similar example traces of the evoked fEPSPs in area CA3 before (1), during (2), and after (3) superfusion with DMPP(30 μM). Note the reversible increase in the amplitude of EPSP in the presence of DMPP. C. Timeplot showing mean evoked EPSP amplitude in areas CA3 and CA1 upon application of DMPP (30μM, horizontal bar, n=12 for CA3, n=4 for CA1) and in area CA3 under control condition (no DMPP added, n=4), all at an intensity producing half of the maximum response. Note the reversible increase in the evoked EPSP amplitude in area CA3 (n=8 of 12) but no consistent change in area CA1 (n=4) following DMPP application. D. Histogram summarising DMPP-induced change in fEPSP amplitude. One-way ANOVA indicated a significant increase in the CA3 region (P=0.007) but not in area CA1 (P=0.4). E. Effect of DMPP on the amplitude of fEPSPs recorded in area CA3 using different intensities. Data are plotted as mean across all intensities ± SEM. Each point represents the average for 4 experiments. Note the clear upward shift in the curve upon DMPP application.

evoked fEPSP amplitude by 15.7 ± 5.1% (mean ± SEM; P=0.007, One-way ANOVA) in the CA3 region of the hippocampus (Figure 5.1 B-D, n=8 of 12). These data suggest that glutamatergic transmission is enhanced by nAChR activation in the CA3 region of the hippocampus.

The Effect of nAChR activation on the amplitude of fEPSPs recorded in area CA3 was assessed at varying stimulus intensities. The result of DMPP action on such stimulus-responses is given in figure 5.1 E. Paired t tests were performed on raw data at each point, revealing that fEPSP amplitudes were significantly increased from baseline following DMPP application across entire range of stimulus intensities ($P<0.05$).

5.2.2 Effect of nAChR activation on evoked glutamatergic synaptic transmission on area CA1

Addition experiments were carried out in area CA1. Field EPSPs were recorded in the CA1 region of the hippocampus by placing recording electrodes within the *stratum radiatum* towards the middle of the CA1 region and subsequently stimulating the Schaffer collaterals with stimulation electrode placed within the *stratum radiatum* close to the CA3/CA1 border. 30 μM DMPP was applied to the bath once a stable EPSP baseline amplitude was achieved. In contrast to findings in area CA3, no significant change (-5.25 ± 8.3%, mean ± SEM; $P=0.4$, One-way ANOVA) was observed on field EPSCs recorded in the CA1 region (Figure 5.1 C-D, n=4) upon application of DMPP suggesting that application of the nicotinic agonist does not alter glutamate transmission at this synaptic connection.

5.3 Effect of glutamate receptor antagonists on 4AP and bicuculline-induced bursting activity

Results obtained from several models of epileptiform activity, both *in vivo* and *in vitro*, have emphasised the important role played by NMDA receptors in the

generation of epileptiform discharges. To test the involvement of NMDA receptors in the 4AP and bicuculline models, we analysed the effects of a competitive NMDA receptor antagonist, D-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid (CGP40116, 50 μM), on spontaneous epileptiform activity induced by either 4AP or by bicuculline. The involvement of other fast excitatory amino acid receptors was also assessed by use of 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo(f)quinoxaline-7-sulfonamide (NBQX, 2 μM) which is an antagonist of AMPA/kainate (non-NMDA) receptors. In the first set of experiments, 50 μM CGP40116 and 2 μM NBQX were co-applied to slices displaying stable epileptiform burst activity to assess the general contribution of glutamate receptors on ongoing 4AP and BIC-induced epileptiform discharges. This combined application of NMDA and AMPA/Kainate receptors antagonists resulted in a gradual reduction and eventually complete abolition of 4AP-induced interictal activity 14.6 ± 6 minutes after bath application of antagonists in all slices tested (n=4). However, this effect was not reversed after (up to 40 minutes) washout (n=2, Figure 5.2 A). A combined application of CGP40116 and NBQX also completely blocked BIC-induced epileptiform activity (n=2, data not shown).

To examine whether NMDA and AMPA/Kainate receptors exerted deferential effects on 4AP and BIC-induced epileptiform activity, we tested the individual effect of either CGP40116 or NBQX on 4AP and BIC-induced epileptiform activities.

For 4AP induced activity, epileptiform discharges persisted following the application of NMDA receptor antagonist, CGP40116 (50 μM), but their frequency significantly decreased from 0.52 ± 0.11 to 0.24 ± 0.03Hz (n=4, *P=0.02*, paired Student's t-tests performed on raw data, Figure 5.2 B).

In a second set of experiments, the involvement of AMPA/Kainate receptors alone on 4AP-induced epileptiform activity was also assessed using the selective AMPA/Kainate receptors antagonist, NBQX. When 2 μM NBQX was added to

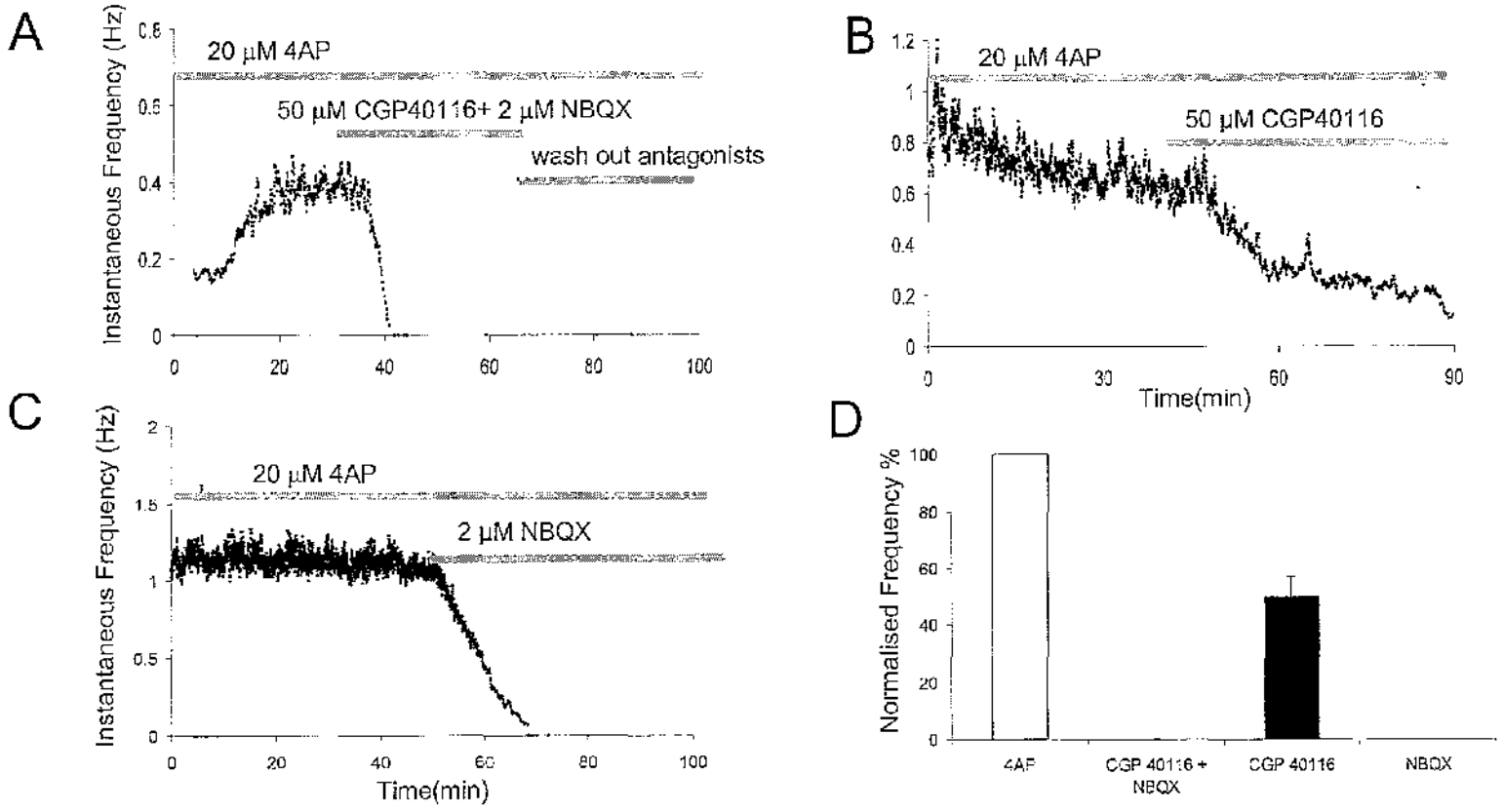

Figure 5.2. *Effects of glutamate receptor antagonists on 4AP-induced epileptiform activity.*
Scatter plots showing representative responses of 4AP-induced burst frequency to (A) the combined application of NMDA and non-NMDA receptor antagonists, (B) application of NMDA receptor antagonist alone and (C) application of non NMDA receptor antagonist alone. As can be seen a complete abolition was achieved following combined application of CGP40116 and NBQX and application of NBQX alone (A,C). A significant decrease in burst frequency was also observed upon application of CGP40116 alone (B). D. Summary of the relative effects of application of CGP40116 and NBQX (n=4), CGP40116 alone (n=4) and NBQX alone (n=6), on 4AP-induced burst frequency.

the bathing medium, 4AP-induced epileptiform discharges gradually reduced in frequency and ultimately abolished completely (n=6, Figure 5.2 C).

In a manner similar to that for 4AP-induced response, co-application of 50 μM CGP40116 also produced a significant decrease in the frequency of BIC-induced epileptiform activity from 0.14 ± 0.01 to 0.06 ± 0.01Hz (n=4 of 6, *P=0.01*, paired-*t*-tests performed on raw data, Figure 5.3 A) but a complete abolishment of epileptiform activity was also observed in 2 out of 6 experiments. On the contrary, application of NBQX (2 μM) produced a complete blockage of BIC-induced epileptiform bursting in all slices tested (n=4, Figure 5.3 B), an effect that was similar to those observed in 4AP model.

The effects of these antagonists on burst frequency are summarised graphically in figure 5.2 D for 4AP and figure 5.3 C for BIC-induced epileptiform activity.

5.4 Experiments to investigate the contribution of ionotropic glutamate receptors in nAChR-induced burst frequency potentiation

5.4.1 Effect of nAChR activation on 4AP and BIC-induced bursting activity in the presence of NMDA receptor antagonist

The previous experiments addressed the action of nAChRs on basal glutamatergic transmission. We next turned our attention to the action of nAChR activation on ongoing epileptiform bursts activity within dynamically active glutamatergic circuits. In order to investigate any possible contribution of NMDA receptors in DMPP-induced burst frequency potentiation, a series of experiments were carried out using the NMDA receptor antagonist CGP40116. Once stable 4AP or BIC-induced epileptiform activity were established, 50 μM CGP40116 was co-applied in order to inhibit any NMDA receptor mediated component to the bursting activity as above. As stated, such application of CGP40116 significantly

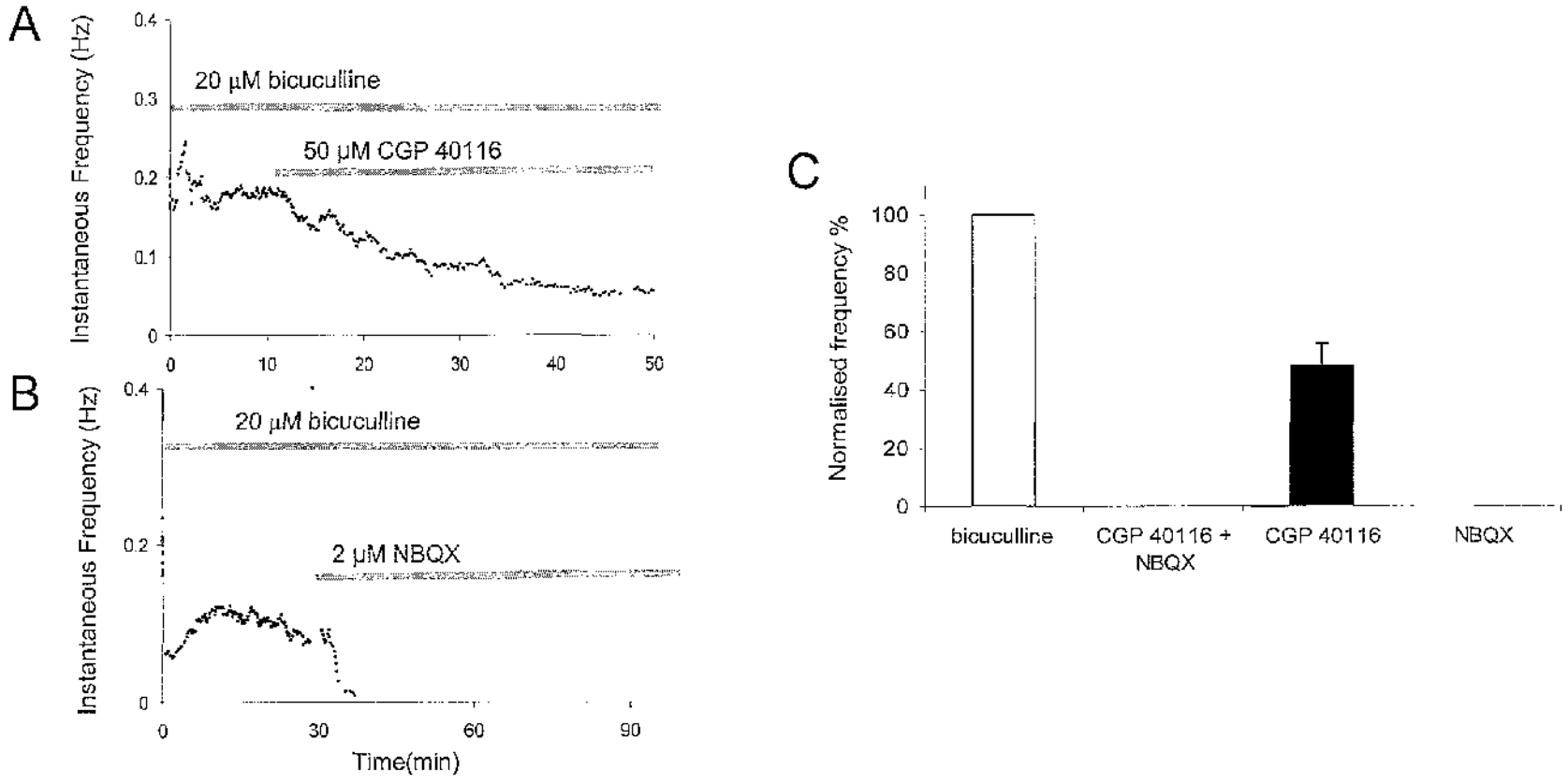

Figure 5.3. *Effects of glutamate receptor antagonists on BIC-induced epileptiform activity.*
Scatter plots showing response of BIC-induced burst frequency to the application of NMDA receptor antagonist (A) and non NMDA receptor antagonist (B) throughout two representative experiments. A significant decrease in burst frequency was observed upon application of CGP40116 and a complete abolition was achieved following application of NBQX (B). C. Summary of the relative effects of application of CGP40116 and NBQX (n=4), CGP40116 alone (n=4) and NBQX alone (n=4) on BIC-induced burst frequency.

decreased the frequency of 4AP and BIC-induced bursting activity. After the stabilisation of the reduced frequency spontaneous epileptiform activity in the presence of CGP40116, 30 μM DMPP was subsequently co-applied to investigate the effect of nAChR activation on ongoing 4AP and BIC-induced epileptiform activity. In such experiments, it was found that the presence of CGP40116 did not compromise the ability of DMPP to potentiate epileptiform burst frequency in the both 4AP (n=4, Figure 5.4 A) and BIC models (n=4, Figure 5.4 B). Indeed, under such conditions, application of 30 μM DMPP produced a reversible increase of burst frequency by 54.5 ± 18.98% and 277 ± 34.7% for 4AP and BIC-induced epileptiform responses, respectively (Figure 5.4 C).

5.4.2 Effect of nAChR activation on 4AP and BIC-induced bursting activity in the presence of AMPA/Kainate receptor antagonist

In contrast to a partial block of epileptiform activity induced by 50 μM CGP40116, application of the AMPA/Kainate receptor antagonist NBQX (2 μM) completely abolished epileptiform activity induced by 4AP and BIC in all slices tested (n=9). We next investigated whether the pro-epileptogenic actions of nAChR activation could reverse the NBQX mediated suppression of 4AP and BIC-induced epileptiform activity.

In the first set of experiments in which 2 μM NBQX abolished 4AP-induced epileptiform responses, subsequent co-application of 30 μM DMPP failed to result in the reappearance of interictal discharges or produce any other form of detectable extracellular field activity, in any slices tested (n=6, Figure 5.5 A). Similarly, 30 μM DMPP was unable to reverse 2 μM NBQX abolition of BIC-induced bursts activity (n=3, Figure 5.5 B).

To investigate further the contribution of AMPA/Kainate receptors during DMPP-induced burst frequency facilitation, we performed experiments using a lower concentration of NBQX (0.2 μM). This concentration of NBQX produces a partial

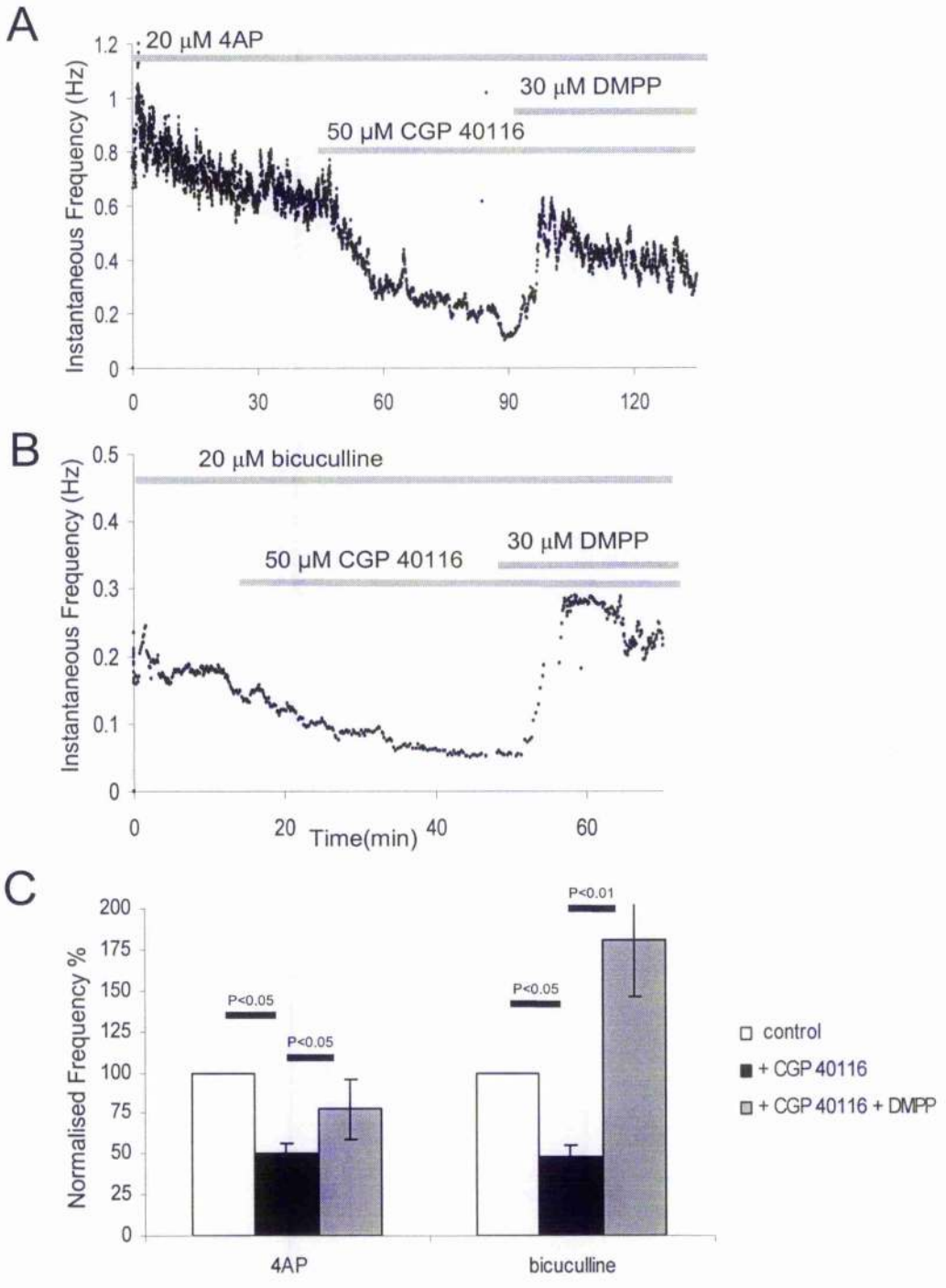

Figure 5.4. *Effect of nAChR activation on epileptiform activity in the presence of NMDA receptor antagonist.*
Scatter plot showing the effect of DMPP on 4AP (A) and BIC (B) -induced burst frequency in the presence of NMDA receptor antagonist, CGP40116. As can be seen, application of CGP 40116 did not affect the ability of DMPP to potentiate epileptiform burst frequency in the both 4AP and BIC models. C. Summary of the effects of application of DMPP on 4AP (n=4) and BIC (n=4)-induced burst frequency, when NMDA receptors are blocked. Horizontal bars indicate *P* values between respective columns as determined using ANOVA performed on raw data.

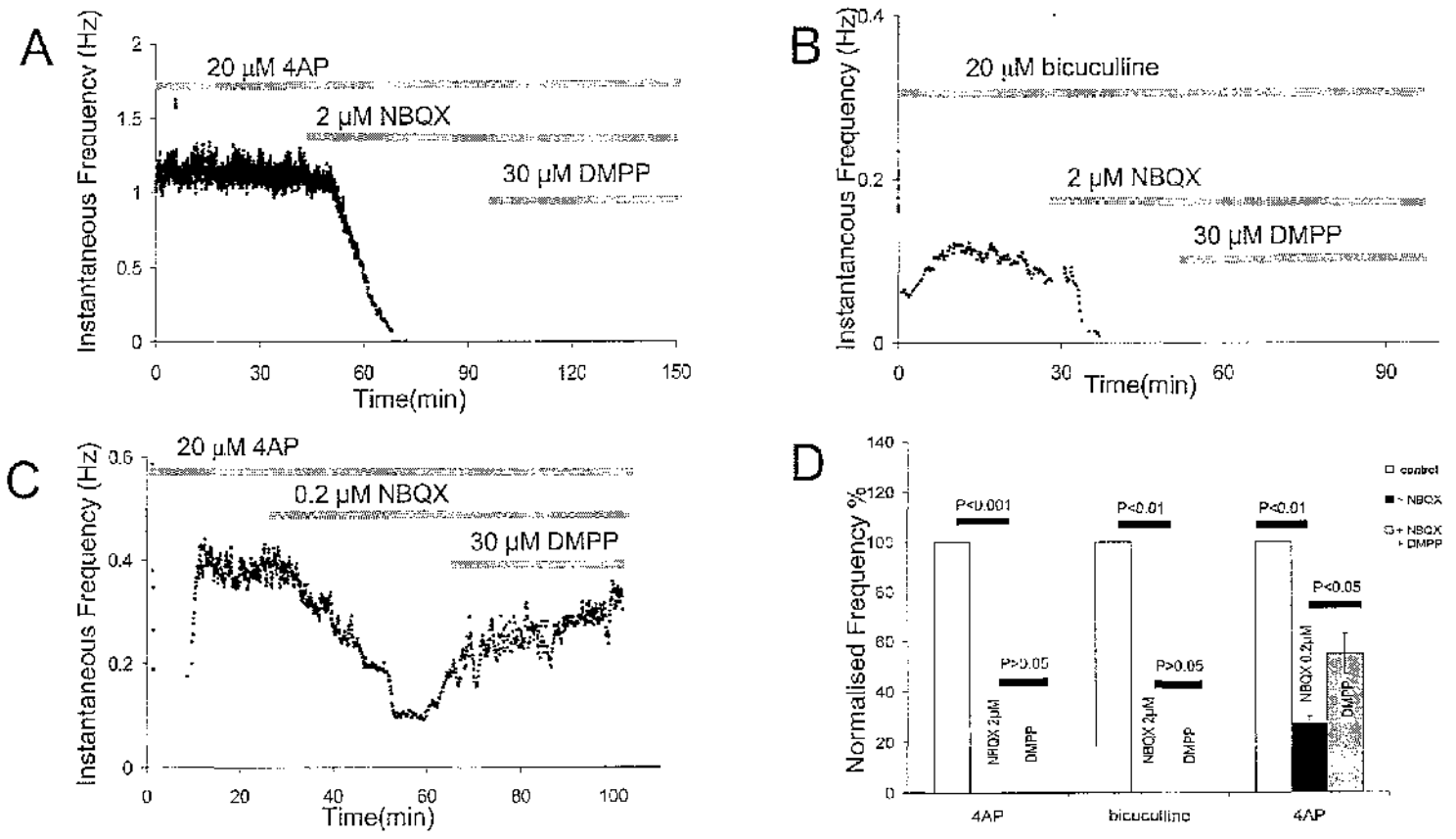

Figure 5.5. *Effect of nAChR activation on epileptiform activity in the presence of non-NMDA receptor antagonist.*
Scatter plot showing the effect of DMPP on 4AP (A) and BIC (B)-induced burst frequency in the presence of 2 µM NBQX in two representative experiments. As can be seen, co-application of 30 µM DMPP did not produce any detectable extracellular field activity in any slices tested in either 4AP or BIC when non-NMDA receptors are blocked in the presence of 2 µM NBQX. C. Slices preincubated with 0.2 µM NBQX, concentration in which produces a partial block of AMPA/Kainate receptor mediated synaptic responses, were still able to exhibit burst frequency potentiation upon DMPP application (n=4). D. Summary of the effects of application of DMPP on 4AP and BIC-induced burst frequency, in the presence of 2 and 0.2 µM NBQX (n=4). Horizontal bars indicate *P* values between respective columns as determined using ANOVA performed on raw data.

block of AMPA/Kainate receptor mediated synaptic responses (Randle et al., 1992). Application of 0.2 μM NBQX resulted in a marked reduction in the frequency of 4AP-induced epileptiform activity (by 72.5 ± 2.5%, n=4, Figure 5.5 C-D) but not a complete abolition of epileptiform activity. Under such conditions, slices preincubated with 0.2 μM NBQX, displayed a pronounced burst frequency potentiation upon DMPP (30 μM) application (96.42 ± 10.5%, n=4, Figure 5.5 C-D). These results suggest an involvement of AMPA/Kainate receptors in DMPP-induced burst frequency potentiation.

5.5 Experiments to investigate the contribution of metabotropic glutamate receptors (mGluRs) in nAChR-induced burst frequency potentiation

Metabotropic glutamate receptors (mGluRs) have been shown to be critically involved in modulating ictal activity during seizures in animal models of epilepsy (Dalby and Thomsen, 1996; Tizzano et al., 1995). Group I mGluRs sustain or promote seizures (Camon et al., 1998; Chapman et al., 2000; Chapman et al., 1999), whereas group II, and group III mGluRs are reported to suppress seizures via presynaptic inhibition of glutamate release (Attwell et al., 1998). Moreover, investigation of the concentration-dependence revealed that sustained low concentration of mGluRs agonist elicited only facilitatory actions, whereas higher concentrations were suppressive (Burke and Hablitz, 1995). These observations suggest the activation of different mGluR subtypes, which may be localised differentially at pre-and postsynaptic sites. Moreover, different neuronal populations, possible expressing different mGluR subtypes or coupling mechanisms, may play integral roles in the induction and generation of epileptiform activities (Burke and Hablitz, 1995). These finding also indicate that a specific mGluR subtype(s) may modulate both excitatory and inhibitory synaptic transmission via a presynaptic reduction of transmitter release (Burke and Hablitz, 1994).

5.5.1 Effect of nAChR activation on BIC-induced bursting activity in the presence of mGluR antagonist

Based on data supporting a critical role of group I mGluRs in promoting seizures (Camon et al., 1998; Chapman et al., 2000; Chapman et al., 1999) and epileptiform activities *in vitro* (Cobb et al., 2000; Merlin et al., 1995; Burke and Hablitz, 1995), a series of experiments were carried out in the presence of mGluR antagonist to investigate the probable contribution of mGluRs in DMPP-induced burst frequency potentiation.

Once stable BIC-induced bursting activity was established, a non-selective group I/II mGluR antagonist (S)-α-methyl-4-carboxyphenyglycine (MCPG, 500 μM) was applied in order to block any contribution of mGluR mediated responses. Subsequent co-application of 500 μM MCPG did not produce any significant change in BIC-induced epileptiform burst frequency, suggesting that activation of such receptor was not a critical factor in the generation of BIC-induced bursting activity (n=4, $P>0.05$, Figure 5.6). Moreover, pretreatment of slices with MCPG did not affect DMPP-induced burst frequency potentiation when DMPP (30 μM) was subsequently applied (n=4, Figure 5.6). This result suggests that mGluRs are not a critical factor in the modulation of BIC-induced epileptiform activity by the activation of nAChRs.

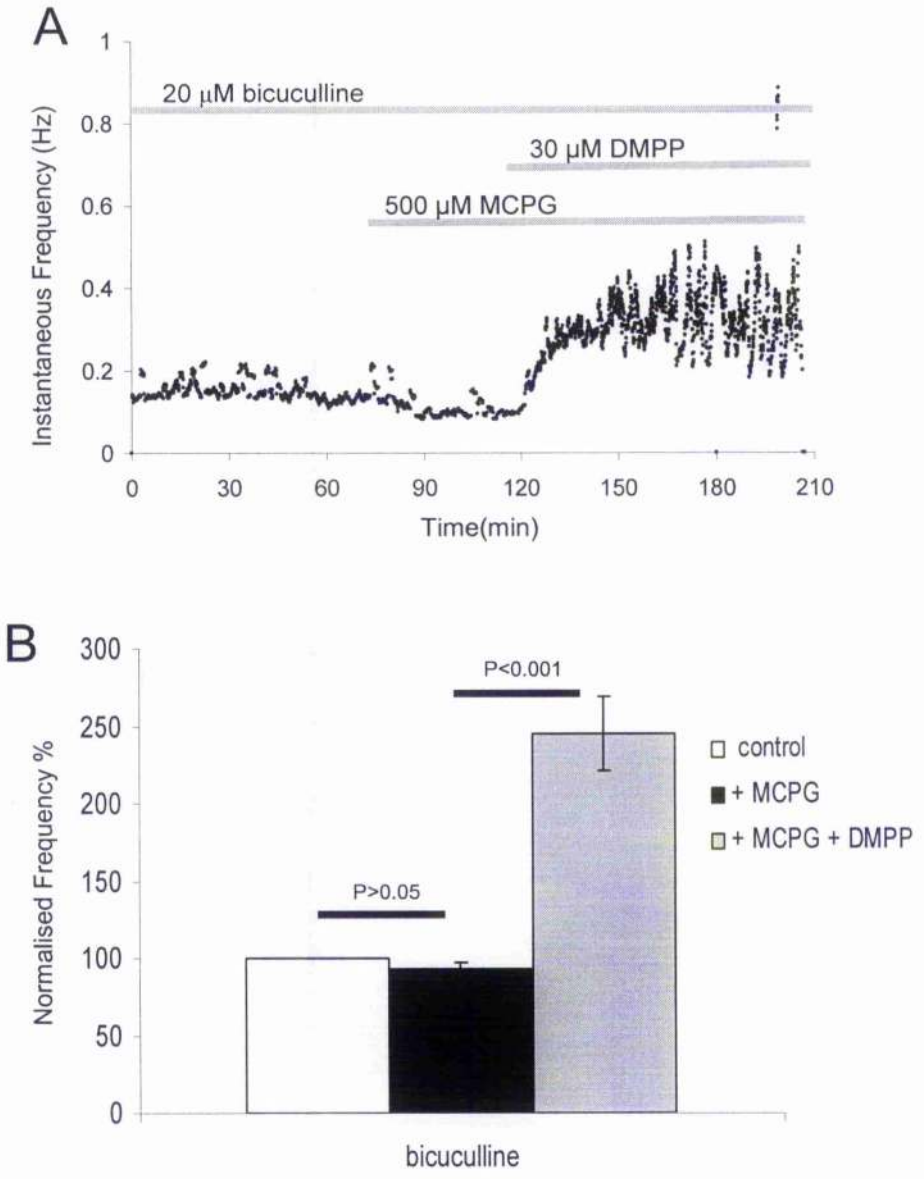

Figure 5.6. *Effect of nAChR activation on epileptiform activity in the presence of metabotropic glutamate receptor antagonist.*
A. Scatter plot showing the effect of DMPP on BIC-induced burst frequency in the presence of the broad spectrum mGLuR antagonist, MCPG. Co-application of 500 μM MCPG did not produce any significant change in BIC-induced epileptiform activity. Subsequent co-application of DMPP produced a significant increase in BIC-induced burst frequency. B. Summary of the effects of application of DMPP on BIC-induced burst frequency in the presence of MCPG (n=4). Horizontal bars indicate *P* values between respective columns as determined using ANOVA.

5.6 Discussion

5.6.1 Effect of DMPP on basal glutamate transmission

The aim of this phase of study was to attempt to assess the precise synaptic mechanisms and identify possible excitatory circuit by which activation of nAChRs produce their pro-epileptogenic effects. In experiments devised to investigate the action of nAChRs on basal glutamatergic transmission, application of selective nAChRs agonist DMPP resulted in a sustained and reversible enhancement of glutamate afferent evoked field EPSP amplitude in the CA3 region of the hippocampus. Previous published results and aspects of the present data indicate that nAChRs may enhance glutamate release from presynaptic terminals. In 1995 McGehee *et al.* showed that nicotine enhanced glutamate transmission in the habenula nucleus of chick (McGehee et al., 1995). This finding is further supported by the observation reported by Gray *et al.* (1996) and Radcliffe and Dani (1998), who showed that nicotine increased the frequency of mini EPSPs in the CA3 region of the hippocampus and hippocampal cell cultures, respectively (Gray et al., 1996; Radcliffe and Dani, 1998). Activation of nAChRs may increase the probability of glutamate release either through direct Ca^{++} entry through the channel itself or indirectly through recruitment of voltage-dependent calcium channels (VDCC) by the local depolarisation. According to Staley et al. (1998), increased probability of release results in a decrease in inter burst interval. This increased burst frequency is associated with a reduction in burst length (Staley et al., 1998). If DMPP acts to open presynaptic nAChR channels, which are permeable to Ca^{2+} (Seguela *et al.*, 1993), resultant changes in calcium dynamics within the presynaptic terminal may thus result in presynaptic modulation of action potential-dependent release. Experiments using the paired-pulse paradigm, which provide indirect evidence of likely presynaptic actions, may be useful to address this point. An increase in the paired-pulse facilitation by DMPP would support a hypothesis that its facilitatory action occurs through activation of pre synaptic nAChRs.

A complementary calcium imaging approach has also been adopted to investigate presynaptic action of nAChRs on calcium transients in glutamatergic terminals. However, such studies are contentious with some groups claiming activation of nAChRs enhances glutamatergic transmission directly by elevating terminal calcium levels (Gray et al., 1996). However, other authors have shown no such actions and suggest nAChRs to effect excitability through other cells types and transmitters notably GABA (Vogt and Regehr, 2001). As our evoked EPSP experiments were not carried out in the presence of GABA receptor antagonists, it is not possible to conclude whether nAChRs modulation is via a direct action on glutamatergic transmission or via a more indirect mechanism, possibly through regulation of GABAergic circuits. However, such an action of nAChRs appears to be independent of fast GABAergic transmission, as detailed in chapter 4 of this thesis, at least with respect to the pro-epileptogenic action. An important finding in this respect was that nAChR activation also resulted in a facilitation of epileptiform burst discharge in the bicuculline model (see chapter 4) in which $GABA_A$ receptors are blocked by high concentrations of the antagonist. However, an action on GABAergic circuits utilising $GABA_B$ receptors can not be excluded since these have recently been shown to exhibit pro-epileptogenic actions (Motalli et al., 1999) (see Chapter 6).

In our experiments increasing the stimulus intensity also increased the amplitude of the evoked EPSP. This presumably reflects the fact that an increase in stimulation intensity would ultimately recruit more fibres and subsequently increases the amount of synaptically released glutamate. Data showing modest but significant increase in the amplitude of evoked EPSPs in the presence of DMPP compared with the control is consistent with a general enhancement of glutamate release across a population of glutamatergic terminals although it is not possible to exclude the possibility of other less direct effect through other systems. Such a finding has been described by Mann and Greenfield (2003) who showed that the inhibition of NMDA receptors revealed a long-lasting excitatory effect of nicotine on hippocampal activity which appeared to be mediated via GABAergic interneurones (Mann and Greenfield, 2003).

In results indicated in this chapter no significant enhancement of EPSP amplitude was seen following DMPP application in the CA1 region. Application of DMPP enhanced glutamate afferent evoked field EPSP amplitude in the CA3 region of the hippocampus but no such effect within area CA1. This observation is consistent with studies reporting that activation of nAChR enhances glutamate release from CA3 pyramidal cells (Gray et al., 1996) but not from CA1 neurones (Alkondon et al., 1997; Frazier et al., 1998b; Jones and Yakel, 1997; McQuiston and Madison, 1999b). Work performed by Alkondon *et al* (1997) showed that CNQX, a competitive glutamate receptor antagonist that inhibits fast glutamatergic transmission mediated by AMPA/Kainate receptors, failed in blocking nAChR-elicited PSCs in CA1 pyramidal cells, which indicated that nAChR activation is not linked to the release of glutamate in CA1 neurones. This finding is further supported by Jones *et al* (1997) and Frazier *et al* (1998) who showed pyramidal cells in the area CA1 to be completely unresponsive to nicotinic receptor activation. McQuiston and Madison (1999) also reported that only 2 out 15 pyramidal cells in area CA1 responded to ACh application and that such responses were barely detectable and significantly smaller than equivalent nAChR mediated responses recorded in hippocampal interneurones.

The conclusion drawn from these data suggests that nAChR activation regulates (potentiates) glutamatergic transmission at a subset of synapses. The apparent selective modulation of glutamatergic transmission by nAChRs mirrors the finding that mAChRs are reported to differentially suppress different excitatory pathways within the hippocampus (Hasselmo and Schnell, 1994). Such an action of nAChRs would be consistent with previous modelling studies which predicted that the susceptibility of the hippocampus to generate epileptiform activity is proportional to the strength of glutamatergic connectivity in recurrent collateral synapses in CA3 region (Bains et al., 1999; Staley et al., 1998) . The terminating of CA3 bursts depends on depletion of the releasable glutamate pool at these recurrent synapses, therefore presynaptic factors controlling glutamate release at recurrent synapses regulate the probability and duration of synchronous discharges of the CA3 network (Staley et al., 1998). According to Vogt and

Regehr (2001) acetylcholine directly modulate these synapses (Vogt and Regehr, 2001). Thus, it is possible that frequency potentiation mediated by nAChRs reflects the activation of presynaptic nAChRs that facilitate glutamate release which, in turn, forces the network into a higher frequency bursting models.

5.6.2 Involvement of glutamate receptors in epileptiform activity

Many factors have been identified as being critical for the generation of epileptiform activity and one of them is activation of excitatory amino acid receptors: NMDA and AMPA/Kainate receptors. The relevance of each of these receptors in focal epileptogenesis, however, remains very controversial because their relative contribution varies greatly depending on the model being studied. For instance, although NMDA receptors have been shown to be involved in the production of epileptiform activity in some experimental models of epilepsy (Meldrum, 1987; Thomson and West, 1986) such as low magnesium model (Horne et al., 1986; Mody et al., 1987; Schneiderman and MacDonald, 1987; Tancredi et al., 1990), NMDA antagonists have little or no effect on the epileptiform discharges seen with other models such as bicuculline and picrotoxin models (Neuman et al., 1988; Thomson and West, 1986) suggesting that NMDA receptors are not necessary for generation of epileptiform activity. Similarly, the importance of AMPA/Kainate in epileptogenesis remains unclear. On the one hand, there are some reports indicating that non–NMDA receptors are involved in the generation of the bursts induced by the convulsants 4AP (Avoli et al., 1993; Perreault and Avoli, 1991) and bicuculline (Gulyas-Kovacs et al., 2002; Traub et al., 1993). On the other hand, it has been shown that AMPA/Kainate receptors have a minor role in the generation of epileptiform discharges in low magnesium model (Gulyas-Kovacs et al., 2002). Finally some studies have provided direct evidence that abnormal excitatory synaptic transmission in the epileptogenic neocortex is primarily mediated by both NMDA and AMPA/Kainate receptors (Lee and Hablitz, 1991; Siniscalchi et al., 1997; Valenzuela and Benardo, 1995). According to these studies there are two distinct phases in the development of epileptic activity, namely induction and maintenance, mediated by either NMDA

or non-NMDA receptors depending on the model being investigated. For example, during exposure to magnesium-free solution, NMDA receptor antagonists prevent the development of the induced epileptiform activity, while the non-NMDA receptor antagonist abolished the epileptiform discharge that persisted after slices were returned to control solution containing magnesium. This data suggests that in this model the enhancement of NMDA receptor activation is responsible for the initiation of spontaneous discharges and that non-NMDA receptors perform a more minor role in the initiation, but contribute to the maintenance of epileptiform discharges (Gulyas-Kovacs et al., 2002; Valenzuela and Benardo, 1995). It has also been shown that, in 4AP model, both types of ionotropic excitatory amino acid receptors are overactivated and contribute to seizure initiation and propagation (Gulyas-Kovacs et al., 2002; Siniscalchi et al., 1997). This finding was further supported by studies investigating the role of excitatory amino acid receptors in generation of picrotoxin-induced epileptiform activity in rat neocortex (Lee and Hablitz, 1991) and in recurrent collaterals of guinea pig hippocampal slice (Miles et al., 1984) that showed a reversible blockade of spontaneous synchronised bursts by the application of several excitatory amino acid antagonists.

The involvement of glutamate receptors in the epileptiform activity was studied in this chapter by the experiments utilising NMDA and AMPA/Kainate receptor antagonists. As detailed above, the majority of previous electrophysiological studies have found that both NMDA and AMPA/Kainate receptors are strongly activated during epileptiform activity (Lee and Hablitz, 1991; Siniscalchi et al., 1997; Valenzuela and Benardo, 1995). According to these studies, we found that 4AP and BIC-induced interictal-like epileptiform activity are completely blocked by NBQX and their frequency significantly reduced by CGP40116. Our findings are different from the data obtained by Avoli and colleagues, who have showed that both interictal- and ictal-like epileptiform discharges induced by 4AP are not affected by NMDA receptor antagonists and who have concluded that 4AP-induced epileptiform activity are mediated through the activation of AMPA/Kainate receptors (Avoli et al., 1993; Perreault and Avoli, 1991).

However, our results are in agreement with those of Gulyas-Kovacs and colleagues (2002), who have shown that NMDA receptor antagonist significantly decrease frequency of 4AP and BIC-induced epileptiform bursting with a similar degree of frequency depression. These authors have also reported that AMPA/Kainate receptor antagonist completely abolished BIC-induced spontaneous activity and significantly decreased the frequency of epileptiform activity in 4AP model. This finding is again in agreement with our results with the only difference being that a complete abolition of 4AP-induced epileptiform activity was observed in our experiments following application of an AMPA/Kainate receptor antagonist. In fact, these authors concluded that there are significant alterations in contribution of NMDA and AMPA/Kainate glutamate receptors to the development and maintenance of epileptiform activity in the different convulsants. However, our results suggest that the relative contributions of NMDA and AMPA/kainate receptors are similar for the 4AP and BIC-induced epileptiform activities described here despite the epileptogenic stimulants acting through distinctly different mechanisms.

Thus, the main conclusion from the work described in this section of the thesis is that, at least in these two models, the epileptic activity requires the activation of both NMDA and AMPA/Kainate glutamate receptors. However, relative contribution of the respective subtypes of glutamate receptor in mediating aspects of epileptogenesis is difficult to assess unequivocally.

In vivo data from animal models of epilepsy have shown modulatory roles of mGluRs on ictal activity during seizures (Dalby and Thomsen, 1996; Tizzano et al., 1995). Studies on *in vitro* brain slices have also suggested that activation of group I mGluRs plays a critical role in the maintenance of the prolonged synchronised discharges (Holmes et al., 1996; Lee et al., 2002; Merlin and Wong, 1997) and in the transition of interictal bursting into ictal activity (Merlin and Wong, 1997). It has been shown in these studies that mGluR antagonists have suppressive effects of prolonged epileptiform activity, suggesting activation of mGluRs during prolonged epileptiform discharges. However, in our experiments

application of MCPG, a non-selective group I/II mGluR antagonist, failed to produce any significant change in BIC-induced epileptiform bursting activity, which was consistent with previously reported studies (Merlin and Wong, 1997; Lee et al, 2002). As only short interictal-like activity was recorded in our experiments, it is likely that this negative finding is attributable to lack of prolonged or ictal like activity in our recordings. This result suggests that group I mGluRs probably does not play a significant role during short interictal activity.

5.6.3 Contribution of glutamate receptors in DMPP-induced burst frequency potentiation

The aims of this set of experiments were to investigate the effect of DMPP on epileptiform activity when glutamate receptors were blocked to uncover possible contribution of glutamate receptors in the pro-epileptogenic action of nAChRs.

In this set of experiments application of NMDA receptor blocker CGP40116 (resulting in a depression of burst frequency) did not affect the ability of subsequent DMPP application to potentiate epileptiform burst frequency. On the other hand, application of the AMPA/Kainate receptor antagonist NBQX (2 μM) completely abolished epileptiform activity suggesting that these receptors are crucial for supporting such activity. Moreover, subsequent co-application of DMPP failed to make any change in extracellular field activity after block with a high concentration of NBQX. However, slices preincubated with a lower concentration of NBQX (0.2 μM), which results in a partial blockade of non-NMDA receptor-mediated synaptic transmission, were still able to exhibit burst frequency potentiation upon DMPP application. These results suggest that NMDA receptors are not necessary in the DMPP-induced burst frequency potentiation and when these receptors are blocked DMPP can potentiate epileptiform activity through activation of AMPA/Kainate receptors. Furthermore, in agreement with earlier chapters, activation of nAChRs may facilitate ongoing epileptiform activity but does not result in the generation of spontaneous activity in otherwise quiescent circuits.

In further studies, application of mGluRs antagonist MCPG again did not affect the ability of DMPP to potentiate epileptiform burst frequency suggesting that the response to DMPP was not due the recruitment of mGluRs.

The main conclusion of these results is:

nAChR–mediated burst frequency potentiation is observed during condition of NMDA receptor block suggesting that under this condition the proepileptogenic action of nAChRs is therefore mediated via enhanced activation of AMPA/kainate receptors. Thus it seems that NMDA receptors are not essential for the proepileptogenic action of nAChRs. However, it is possible that under normal condition part of nAChRs proepileptogenic action is due to an additional component in which NMDA receptor mediated transmission facilitated. Together these data suggest a presynaptic action of nAChRs and increase glutamate release.

CHAPTER 6

INVESTIGATION OF THE CONTRIBUTION OF GABA CIRCUITS IN nAChR-INDUCED BURST FREQUENCY POTENTIATION

CHAPTER 6

INVESTIGATION OF THE CONTRIBUTION OF GABA CIRCUITS IN nAChR-INDUCED BURST FREQUENCY POTENTIATION

6.1 Introduction

Much of the action of acetylcholine within the hippocampus is thought to be mediated through action on the GABAergic system (Gulyas et al., 1999). The main cholinergic input to the hippocampus is through septohippocampal pathway, which originates from the medial septal nucleus and the nucleus of the diagonal band of Broca (Reviewed by Dutar et al., 1995) and contacts both interneurones and principal cells of hippocampus (Heimrich and Frotscher, 1993; Leranth and Frotscher, 1989). ACh may increase or decrease the activity of interneurones via muscarinic receptors and excite interneurones through nicotinic receptors located in the soma-dendritic membrane. ACh may also modulate GABA release through presynaptic muscarinic and nicotinic receptors located on axon terminals. Muscarinic acetylcholine receptor (mAChR) activation increases the frequency of spontaneous IPSCs converging on to pyramidal cells, which is likely the result of a direct postsynaptic excitation of certain interneurones (Behrends and ten Bruggencate, 1993; Pitler and Alger, 1992). On the other hand, mAChR activation decreases the frequency of miniature IPSCs, which is a result of a presynaptic reduction of GABA release (Behrends and ten Bruggencate, 1993; Pitler and Alger, 1992).

The anatomical results, taken together with the electrophysiological data have shown that functional nicotinic receptors are also present on hippocampal interneurones (Alkondon et al., 1998; Frazier et al., 1998a; Frazier et al., 1998b; Jones and Yakel, 1997; McQuiston and Madison, 1999b). It has been reported that

either fast local application of ACh or electrical stimulation of cholinergic afferents depolarises interneurones in the CA1 subfield and the dentate gyrus and induces rapid firing (Frazier et al., 1998a; Frazier et al., 1998b; Jones and Yakel, 1997). Voltage-clamp recordings demonstrated that ACh produces a fast, rapidly desensitising inward current that is blocked by α-bungarotoxin, indicating that the fast excitation is mediated by a Ca^{2+} permeable variant of the nAChR containing the α7 subunit (Frazier et al., 1998a; Frazier et al., 1998b; Jones and Yakel, 1997). In contrast, pharmacological application of nicotinic agonists have been reported not to change the firing or the membrane potential of the majority of hippocampal principal cells (Alkondon et al., 1997; Frazier et al., 1998b; McQuiston and Madison, 1999b).

Located at both pre, post and extra-synaptic sites of interneurones, nAChRs are ideally placed to modulate inhibitory circuits in the hippocampus. ACh increases the activity of interneurones via nicotinic receptors located in the soma-dendritic membrane of interneurones and increases GABA release through presynaptic nicotinic receptors located on axon terminals (Alkondon et al., 1997). Thus, it is likely that the pro-epileptogenic action of nAChR activation described in previous chapters is mediated in part through GABAergic circuits. However, such an action of nAChRs appears to be independent of fast GABAergic transmission since the pro-epileptogenic actions of nAChR activation is resistant to the blockade of $GABA_A$ receptors, as detailed in chapter 4 of this thesis. Thus, nAChR modulation of GABAergic events is unlikely to be important in the pro-epileptogenic action of nicotinic receptor activation, although it is possible that an interaction between nAChRs and $GABA_B$ receptor activation may play a role.

$GABA_B$ receptor-mediated mechanisms are involved in the generation of focal seizures and in epileptogenesis (McLean et al., 1996; Scanziani et al., 1994; Veliskova et al., 1996). Baclofen is a $GABA_B$ receptor agonist which has been shown to activate both pre- and post-synaptic $GABA_B$ receptors (Bowery et al., 1980a; Newberry and Nicoll, 1985) and to inhibit many forms of synaptic

transmission (Ault and Nadler, 1982) including glutamatergic (Lanthorn and Cotman, 1981; Thompson and Gahwiler, 1992), GABAergic (Lambert and Wilson, 1993; Thompson and Gahwiler, 1992) and cholinergic transmission (Morton et al., 2001). However, baclofen may possess a surprising proconvulsant effect as documented in clinical practice (Kofler et al., 1994; Rush and Gibberd, 1990) and in certain models of epileptiform discharge (Motalli et al., 1999; Mott et al., 1989; Watts and Jefferys, 1993). It has also been proposed that the proconvulsant effect of baclofen is caused by a presynaptic, $GABA_B$-mediated inhibition of GABA release from inhibitory interneurones leading to disinhibition (Mott et al., 1989; Watts and Jefferys, 1993). Such an action has also been documented with the epileptiform discharges induced by bath application of 4AP to hippocampal slices (Motalli et al., 1999; Watts and Jefferys, 1993), leading to hypothesise that $GABA_B$ receptor antagonists could exert anticonvulsant actions in this *in vitro* model of epileptiform discharge. Previous studies have indeed shown that the $GABA_B$ receptor antagonist CGP35348 has anticonvulsant actions in rodent models of absence seizures (Bowery and Enna, 2000; Liu et al., 1992). However, an opposite finding has been reported by Motalli *et al.* 2002, who found that the effect of CGP35348 on 4AP-induced epileptiform activity to not be anticonvulsant and to some extent is similar to what was reported in this model during $GABA_B$ receptor activation (Motalli et al., 2002). Given the conflicting results outlined above, the aim of the experiments described in this chapter was to readdress the possible contribution of $GABA_B$ receptors during epileptiform activity and to investigate the involvement of $GABA_B$ receptors in nAChR-induced burst frequency potentiation. A second aim was to further investigate the effect of the nAChR agonist, DMPP on GABA release from hippocampal neurones with a view to uncover possible mechanisms responsible for the pro-epileptogenic action of DMPP.

6.2 Investigation of the role of $GABA_B$ receptors in nAChR-induced burst frequency potentiation

6.2.1 Effect of nAChR activation on 4AP-induced epileptiform activity in the presence of $GABA_B$ receptor antagonist

In order to investigate the possible involvement of $GABA_B$ receptors in nAChRs-induced effect, a series of experiments were carried out using the selective $GABA_B$ receptor antagonist CGP55845A. Once stable 10 μM 4AP-induced bursting was established, 1 μM CGP55845A was co-applied in order to block pharmacologically any involvement of $GABA_B$ receptor mediated responses during epileptiform bursting. Such application of CGP55845A did not significantly change the frequency of 4AP-induced bursting activity (mean frequency = 97.8 ± 4% of control 4AP frequency; P>0.05; n=11; Figure 6.1 A-B). After the stabilisation of the 4AP-induced spontaneous epileptiform activity in the presence of CGP55845A, 30 μM DMPP was co-applied to the convulsant containing solution to test the effect of nAChR activation on ongoing 4AP-induced epileptiform activity when $GABA_B$ receptors are blocked. Whereas slices in the absence of CGP55845A would exhibit robust burst frequency potentiation (see chapter 3), slices pre-incubated with CGP55845A (1 μM) failed to exhibit burst frequency potentiation upon DMPP application in 11 of 11 slices tested. Scatter plot in figure 6.1 A shows a representative experiment in which the slices are pre-incubated with $GABA_B$ receptor antagonist CGP55845A prior to application of DMPP. Such application of CGP55845A was found to prevent the ability of subsequent DMPP application to potentiate burst frequency of 4AP-induced epileptiform activity. In the presence of CGP55845A, application of DMPP resulted in no significant enhancement of burst frequency compared to pre-DMPP levels (91.7 ± 7% of pre-DMPP control; P> 0.05; n=11; Figure 6.1 A-B). Overall, these data suggest that $GABA_B$ receptors may play a role in nAChRs-induced frequency potentiation of 4AP-induced bursting.

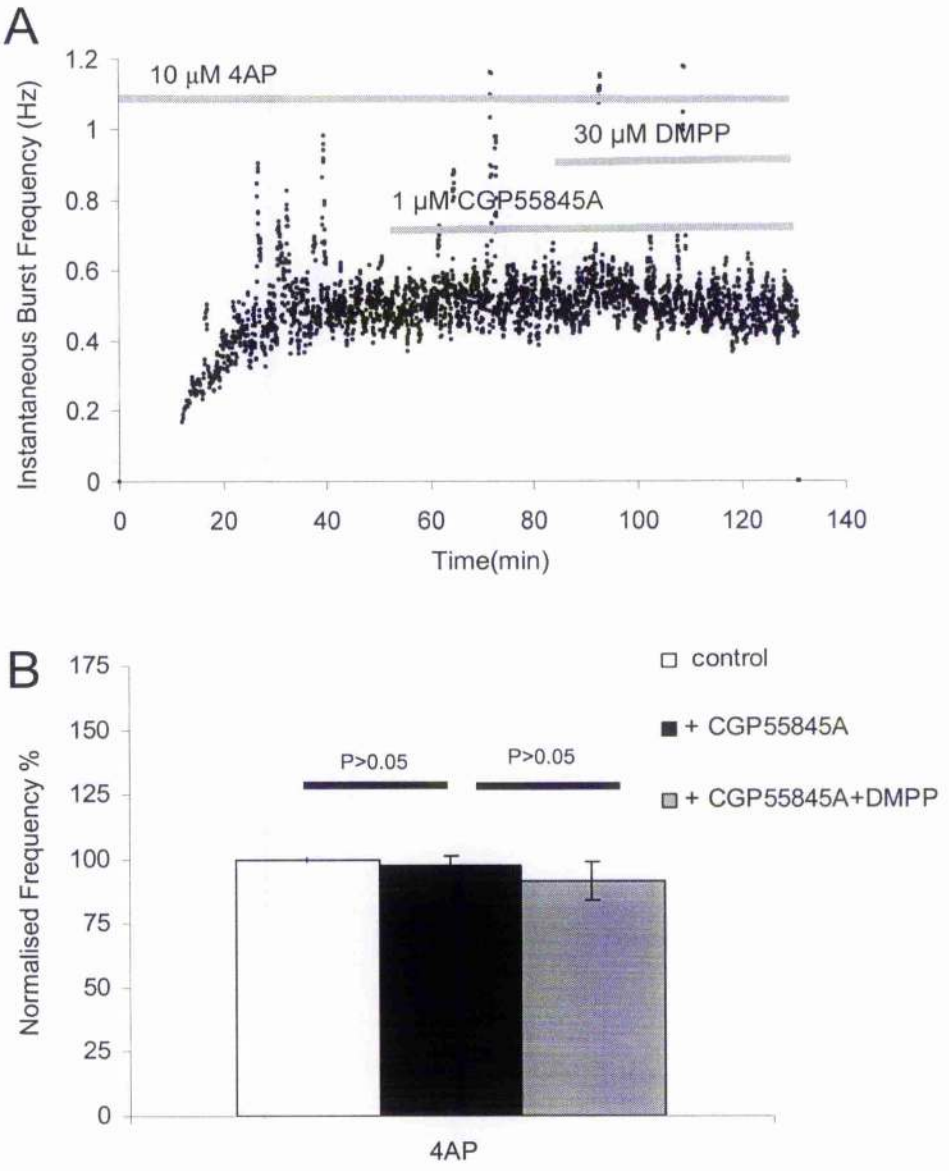

Figure 6.1. *Effect of nAChR activation on 4AP-induced epileptiform activity in the presence of selective $GABA_B$ receptor antagonist.*
A. Scatter plot showing the effect of DMPP on 4AP-induced burst frequency in the presence of $GABA_B$ receptor antagonist, CGP55845A. Co-application of 1 µM CGP55845A did not produce any significant change in 4AP-induced epileptiform burst frequency. Subsequent co-application of 30 µM DMPP again produced no significant change in 4AP-induced burst frequency in the presence of CGP55845A (*P=0.45*, One-Way ANOVA). B. Summary of the effects of application of DMPP on 4AP-induced burst frequency in the presence of CGP55845A (n=11). This $GABA_B$ receptor antagonist prevents the ability of DMPP to potentiate 4AP-induced epileptiform burst frequency. Horizontal bars indicate *P* values between respective columns as determined using ANOVA.

6.2.2 Effect of nAChR activation on BIC-induced epileptiform activity in the presence of $GABA_B$ receptor antagonist

To assess the effect of $GABA_B$ receptor antagonism on ongoing BIC-induced epileptiform discharges and their modulation by nAChRs-induced effect, the selective $GABA_B$ receptor antagonist CGP55845A was bath applied to slices displayed stable bicuculline-induced epileptiform burst activity. Subsequent co-application of CGP55845A (1 or 3 μM in 6 and 3 experiments, respectively) to hippocampal slices generating interictal discharges under control conditions (BIC-containing medium) produced no significant change in burst frequency (mean maximal frequency = 106.8 ± 5% of control BIC frequency, $P>0.05$, Figure 6.2 A-B). The effects induced by these two concentrations of CGP55845A were not different and therefore the data were pooled together for further analysis.

Once stable epileptiform activity was established, 30 μM DMPP was co-applied in order to investigate the effect of nAChRs activation on ongoing BIC-induced epileptiform discharges in the presence of CGP55845A. As detailed in chapter 4 of this thesis, BIC-induced epileptiform activity showed a profound frequency potentiation by subsequent co-application of the selective nAChR agonist DMPP (248 ± 76% of baseline; n=14). In contrast, slices pre-incubated with the $GABA_B$ receptor antagonist CGP55845A (1-3 μM) exhibited negligible burst frequency potentiation upon DMPP application (27.6 ± 17.76% of baseline; $P>0.05$; n=9; Figure 6.2 A-C). Although under this condition, there was no change in the frequency of BIC-induced epileptiform bursting during DMPP application in the majority of slices tested (n=6 out of 9 experiments, Figure 6.2 A), a small increase was observed in the other 3 experiments (Figure 6.2 B). However, these changes did not achieve statistical significance (One-way ANOVA, $P=0.18$, Figure 6.2 C).

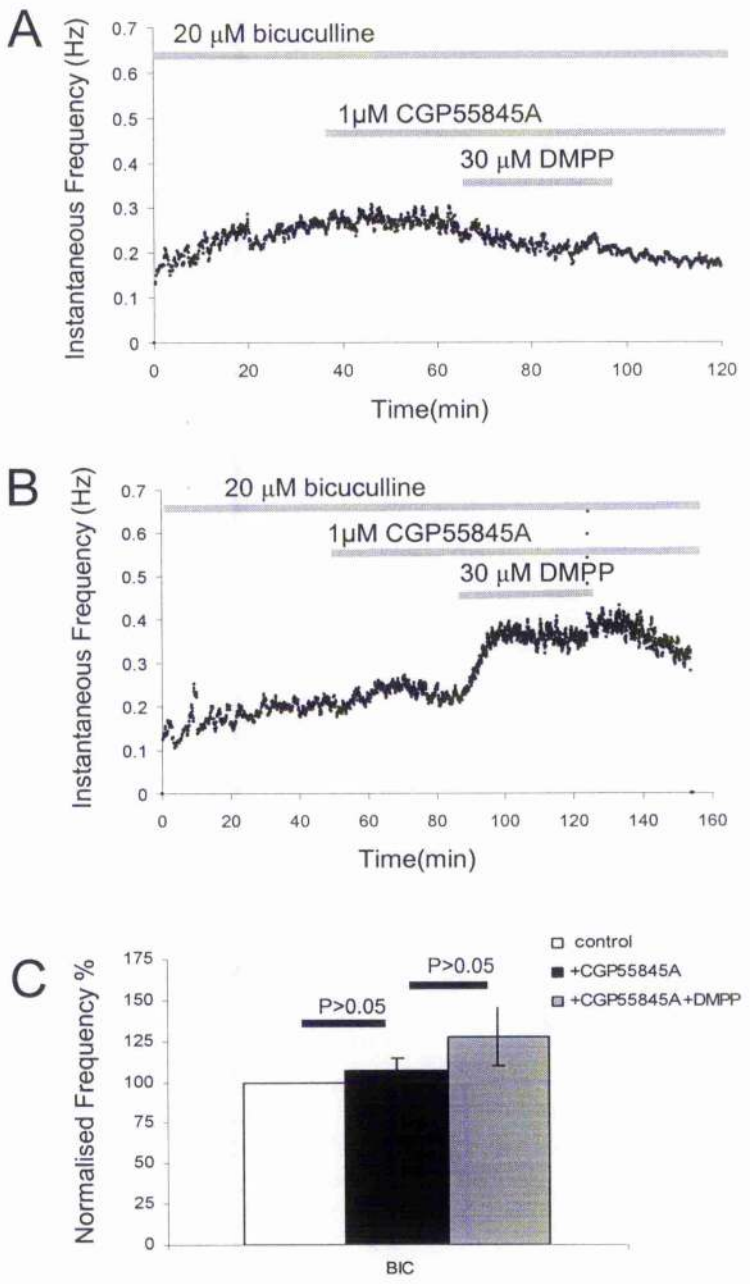

Figure 6.2. *Effect of nAChR activation on BIC-induced epileptiform activity in the presence of $GABA_B$ receptor antagonist.*
A. Scatter plot showing the effect of DMPP on BIC-induced burst frequency in the presence of $GABA_B$ receptor antagonist, CGP55845A. Co-application of 1 μM CGP55845A did not produce any significant change in BIC-induced epileptiform burst frequency. In the majority of the slices, subsequent co-application of DMPP again did not produce any change in BIC-induced epileptiform activity (n=6 of 9). B. In a minority of slices (n=3 of 9) subsequent co-application of DMPP produced a modest burst frequency potentiation of BIC-induced epileptiform activity but which overall did not reach statistical significance (*P=0.18*, One-Way ANOVA). C. Summary of the effects of application of DMPP on BIC-induced burst frequency in the presence of CGP55845A across all slices tested (n=9). Horizontal bars indicate *P* values between respective columns as determined using ANOVA.

6.2.3 Effect of $GABA_B$ receptor activation on 4AP-induced epileptiform activity

Application of the $GABA_B$ receptor agonist baclofen (0.1-30μM) resulted in a concentration dependent reduction and eventual complete blockade of 4AP-induced interictal activity (n=15, Figures 6.3 and 6.4). The effects induced by increasing concentration of baclofen were analysed in 8 slices. With 0.1-10μM baclofen, a decrease in the rate of occurrence of interictal discharges occurred in 5 of 8 slices, whereas abolishment was seen in the remaining 3 experiments. In slices in which interictal activity was not fully abolished by 10μM baclofen, further increasing the concentration to 30μM caused a complete abolishment (n=5 of 5). Complete blockade of interictal discharges was also induced by application of a single concentration of baclofen (30 μM, n=7, Figure 6.4 A).

The suppressant actions of baclofen upon epileptiform bursting were fully reversible upon washout (n=3, Figure 6.3 A) or co-application of $GABA_B$ receptor antagonist CGP55845A (1μM, n=3, Figure 6.3 B). We also analysed the concentration-response relationship of the changes induced by baclofen with respect to the frequency of 4AP-induced epileptiform activity (Figure 6.3 C). Data obtained in 7 slices in which increasing doses of baclofen (0.1-30μM) were sequentially applied indicated an $IC_{50} = 4.7\mu M$, comparable with IC_{50} value of other studies (Avoli et al., 2004; Braun et al., 2004).

Finally, in a minority of slices tested 30 μM baclofen application resulted in abolishment of interictal activity and the appearance of spontaneous ictal activity which was absent in control periods (n=2 of 17, Figure 6.5 B).

6.2.4 Effect of nAChR activation on baclofen-induced suppression of epileptiform activity

In order to assess whether nAChR activation could modulate baclofen-induced suppression of epileptiform activity, a series of experiments were carried out in which DMPP was co-applied following a stable period of baclofen application.

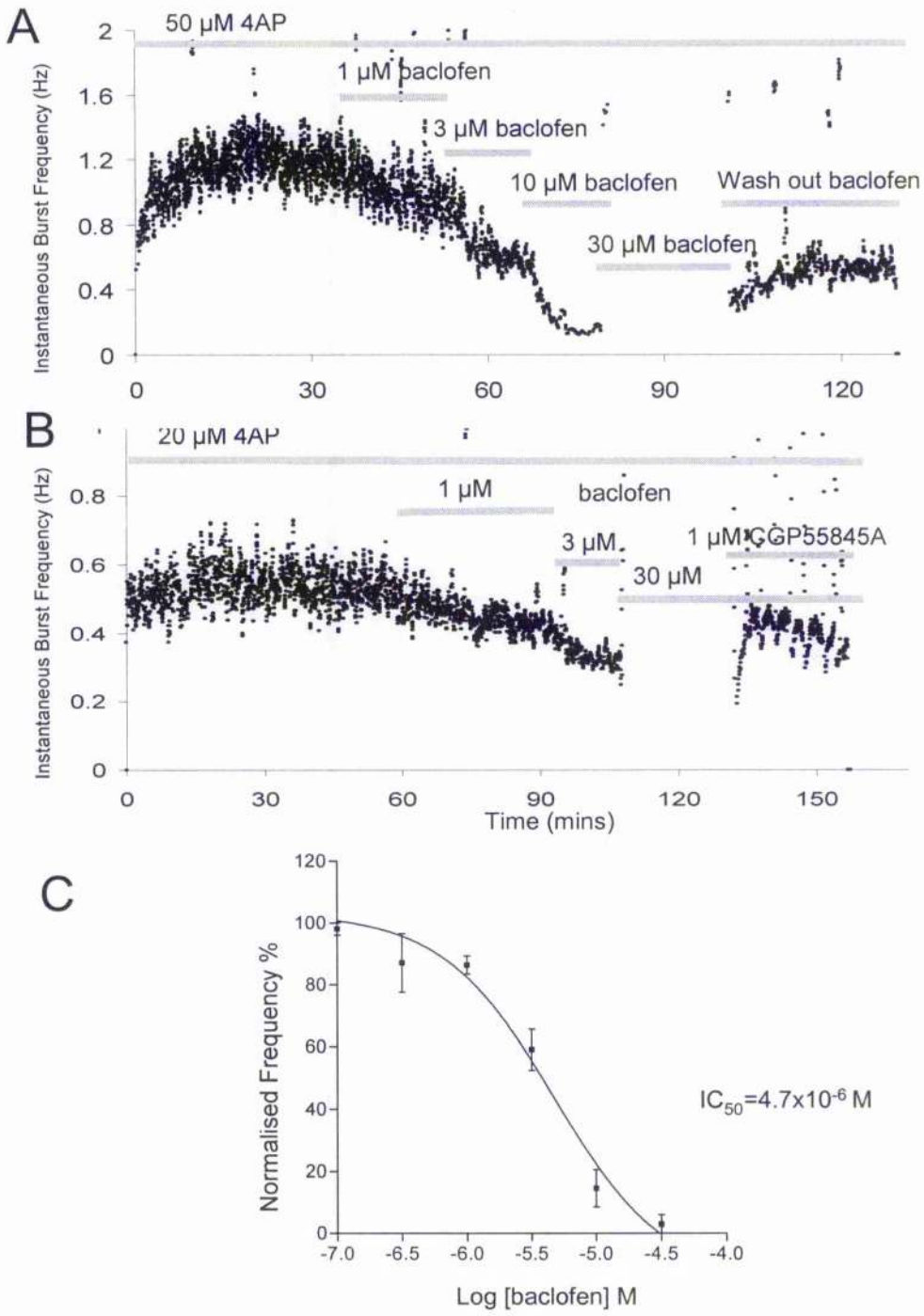

Figure 6.3. *Effect of $GABA_B$ receptor agonist baclofen on 4AP-induced epileptiform activity in area CA3 of the hippocampus.*

A. Scatter plot showing effect of baclofen on frequency of interictal-like events induced by 4AP in the rat hippocampal slice. Application of baclofen (1-30 µM) resulted in a concentration-dependent decrease and eventual complete abolition of the 4AP-induced interictal-like activity. Shaded horizontal bars indicate the period over which an individual concentration was applied to the bath. B. Similar scatter plot in which the effect of 30 µM baclofen is reversed upon co-application of the $GABA_B$ receptor antagonist, CGP55845A (1 µM). C. Concentration-response curve for the decrease in the frequency of 4AP-induced interictal activity, induced by different concentrations of baclofen in 7 experiments. This dose-response curve reveals an IC_{50} of 4.7 µM.

When the nAChR agonist DMPP (30 μM) was applied to slices treated with 4AP + baclofen, interictal activity reappeared upon DMPP application (n=10, Figure 6.4 A-B). Although baclofen-induced suppression of 4AP-induced epileptiform activity was reversed upon application of DMPP, the degree of reversal varied between experiments. In 7 out of 10 experiments, application of 30 μM DMPP partially recovered the epileptiform bursting activity but the frequency recovery did not achieve pre-baclofen levels (39.3 ± 5% of control, Figure 6.4 A-B). In the remaining 3 slices application of DMPP resulted in a complete reverse of baclofen effects to pre-baclofen frequency levels (95 ± 4.5% of control, Figure 6.5 A).

As it is mentioned above, baclofen application elicited ictal-like discharges (which were absent in control) in 2 out of 17 experiments. In all these cases, subsequent application of 30 μM DMPP resulted in the subsequent reappearance of interictal activity whereas the rate of occurrence of the ictal-like events decreased (from 0.17 ± 0.02Hz during 30 μM baclofen to 0.08 ± 0.02Hz after adding DMPP, n=2; Figure 6.5 B). DMPP also reduced the duration of the ictal-like discharges that occurred less frequently than with baclofen only (from 634.8 ± 26 ms during 25 μM baclofen to 321 ± 19ms after adding DMPP, n=2).

6.3 Effect of DMPP on spontaneous IPSCs recorded in hippocampal interneurones

To further investigate the effect of nAChR activation on GABA transmission in the hippocampus and the possible involvement of GABA receptors in nAChR-induced burst frequency potentiation, we used whole cell path clamp configuration to record spontaneous IPSC events in hippocampal interneurones and investigate the action of subsequent nAChR activation.

Interneurones with cell bodies located within *stratum radiatum* were recorded in the whole cell formation under voltage clamp configuration and in the presence of the glutamate antagonists 4 μM NBQX and 50 μM CGP40116. Spontaneous

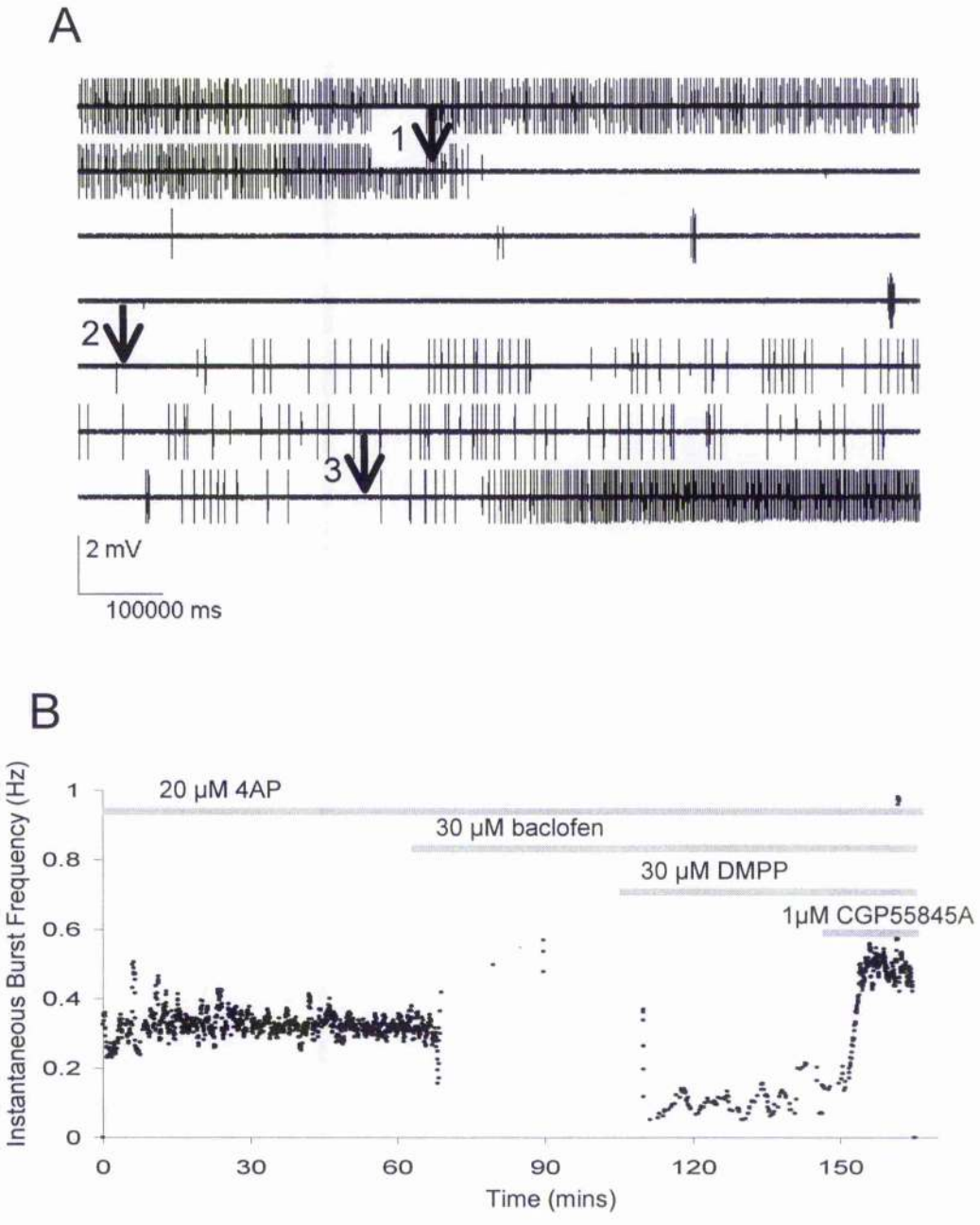

Figure 6.4. *Reverse of baclofen-induced effects on epileptiform activity by DMPP.*

A. Raster plot showing a representative experiment in which interictal bursting activity induced by 4AP is completely abolished following application of baclofen (30 µM, arrow 1 onwards). Subsequent co-application of DMPP (30 µM, arrow 2) and CGP55845A (1 µM, arrow 3) recovered and then potentiated interictal activity, respectively. B. Scatter plot presentation of same experiment showing application of a single concentration of baclofen (30 µM) abolishes 4AP-induced interictal activity which is partially reversible upon co-application of DMPP (30 µM, n=7). Further reverse of baclofen-induced effects achieved following co-application of CGP55845A to medium containing 4AP, baclofen and DMPP.

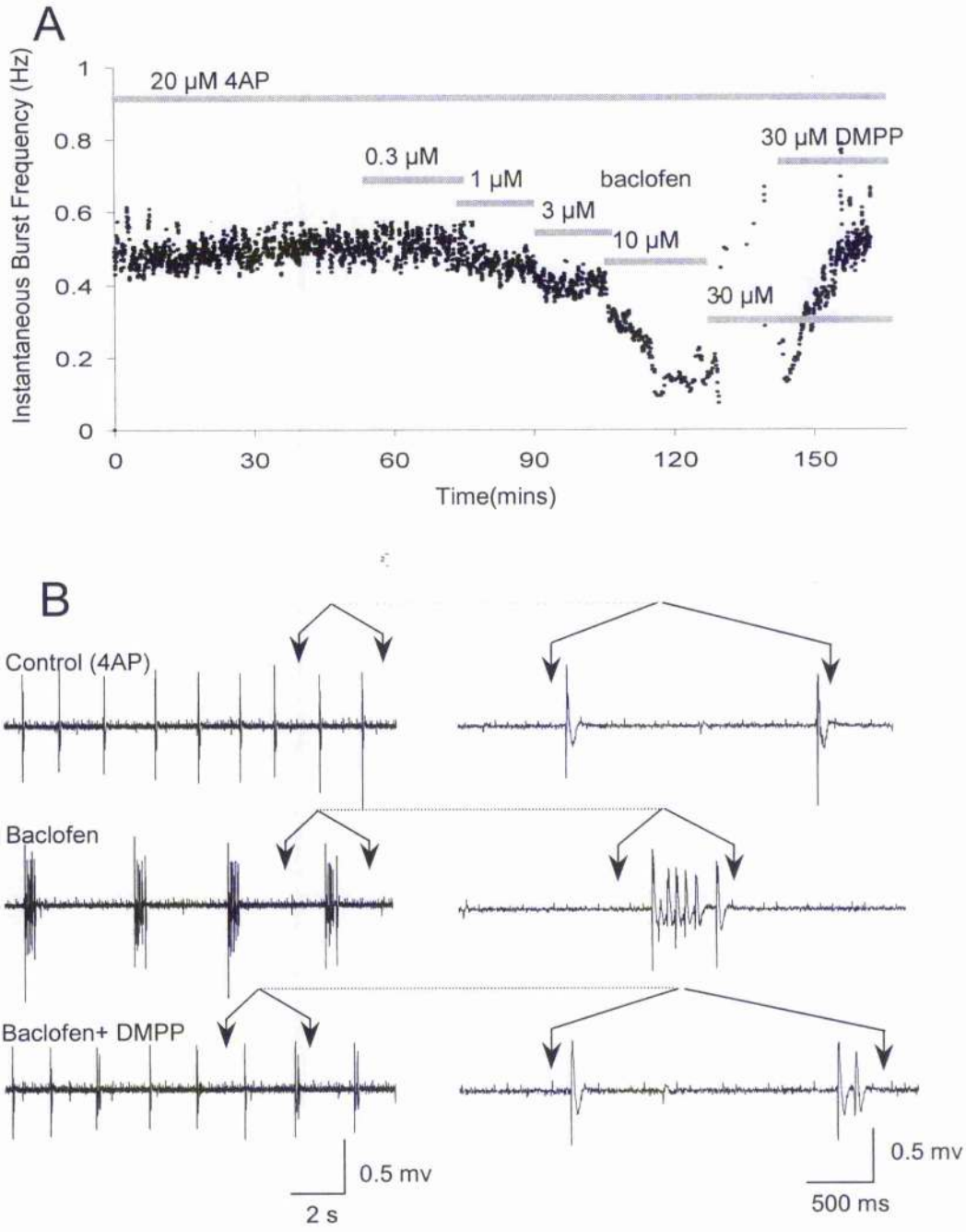

Figure 6.5. *Effect of DMPP on baclofen-induced effect on epileptiform activity.*
A. Scatter plot showing another experiment in which interictal bursting activity induced by 4AP is completely abolished following application of a single concentration of baclofen (30 µM). Subsequent co-application of DMPP (30 µM) and CGP55845A (1 µM) recovered and then potentiated interictal activity, respectively. B. In a minority of experiments baclofen abolished interictal events, while promoting the occurrence of more prolonged ictal-like afterdischarges (n=2 of 17). In these cases, DMPP application resulted in the reappearance of interictal activity and a shortening in the duration and the rate of occurrence of the ictal events. Right column points to expanded sections of each trace illustrating individual field potential events.

IPSCs (sIPSC) were recorded as transient inward currents with fast kinetics and a mean peak amplitude of 20.42 ± 2.47pA (n=6). Perfusion of the hippocampal slices with NBQX and CGP40116 blocked fast AMPA/kainate and NMDA receptors respectively and ensured the spontaneous PSCs recorded were GABAergic in nature (IPSCs). Bath application of 30μM DMPP resulted in a significant increase in frequency of sIPSC from 7.489 ± 1.93Hz to 13.5 ± 2.8Hz (n=6; P=0.0403; Figure 6.6). In contrast, the amplitude of sIPSPs was unaffected by DMPP application (from 20.4 2 ± 2.47pA to 23.41 ± 3.04pA, n=6, P=0.0836).

As well as the increase in IPSCs frequency, DMPP also elicited a pronounced and sustained inward current in 3 of 6 interneurones recorded (Figure 6.6 A). This slow nicotinic current was associated with action potential discharges as indicated by large and uncontrolled current transients (Figure 6.6 A).

6.4 Discussion

The main finding of this chapter was the demonstration that preincubation of slices with a $GABA_B$ receptor antagonist can prevent the ability of the nAChR agonist DMPP to induce epileptiform burst frequency potentiation. This is consistent with the proposal that nAChRs might affect epileptiform activity through modulating GABAergic circuits. GABAergic interneurones in the hippocampus are known to exhibit functional nAChR-mediated responses (Alkondon et al., 1998; Frazier et al., 1998b; Jones and Yakel, 1997; McQuiston and Madison, 1999b). nAChRs are thus ideally placed to regulate neuronal excitability within hippocampal circuits. Strong neurochemical evidence indicates that presynaptic nAChRs are involved in the enhanced release of a number of transmitters, including GABA (Alkondon et al., 1997; McMahon et al., 1994; Wonnacott et al., 1989). Comparable to those reported by these authors, our group have found that nAChR selective agonist DMPP increases the frequency of spontaneous IPSPs recorded in the hippocampal CA1 interneurones (L.Ferrigan; personal communication). This result indicates that nAChR agonists can

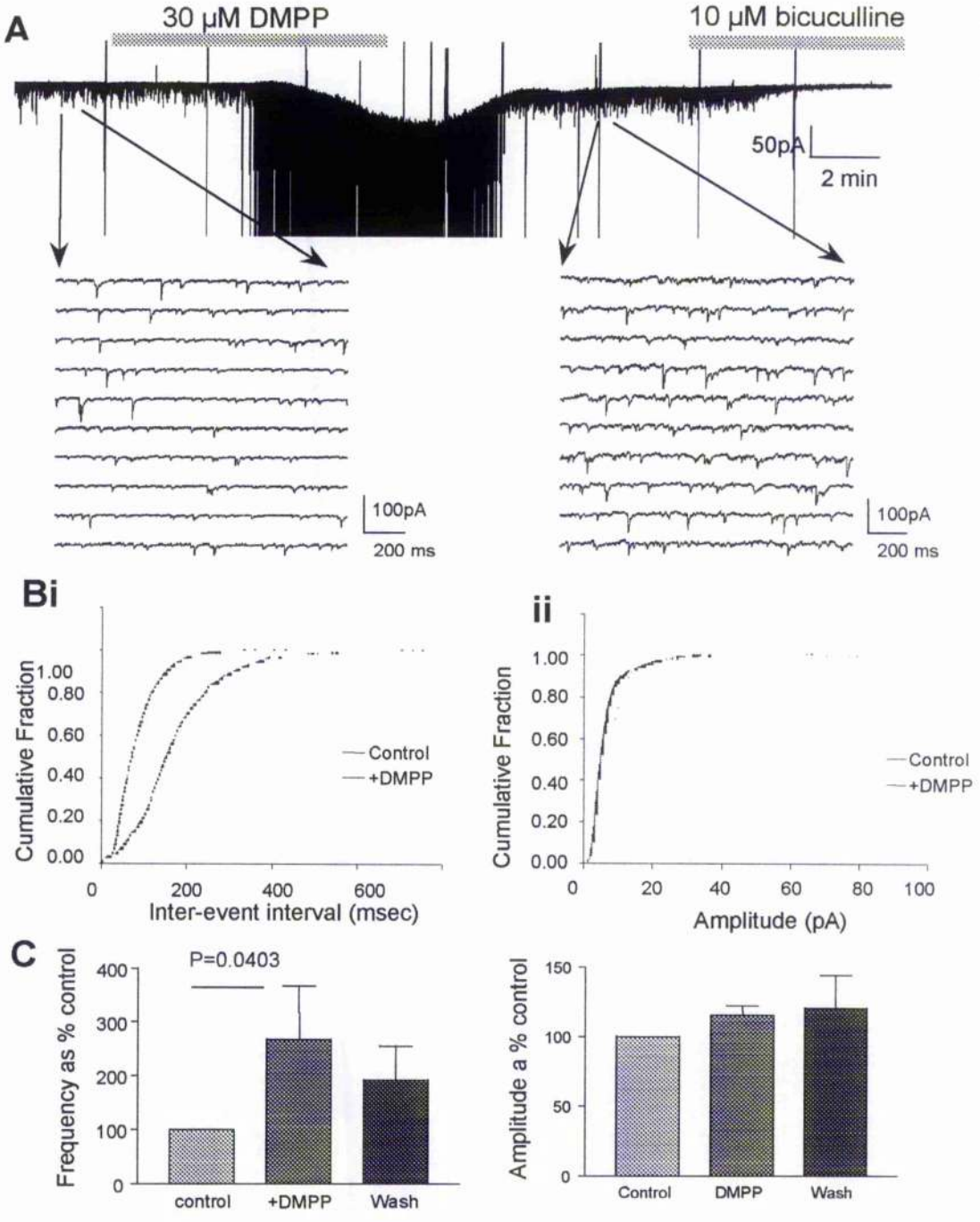

Figure 6.6. *Effect of DMPP on spontaneous IPSCs (sIPSCs) in CA1 interneurones*
A. Representative current trace and raster plots illustrating sIPSCs recorded from a CA1 interneurone. Note the inward current and increase in sIPSC frequency in response to DMPP application. Subsequent co-application of the selective $GABA_A$ receptor antagonist completely blocks spontaneous inward currents confirming the GABAergic nature of the events. B. Representative cumulative probability plots showing (i) sIPSC inter-event interval and (ii) sIPSC amplitude before and following DMPP application. C. Summary histograms showing the effect of DMPP on mean sIPSC frequency and amplitude (n=6). A one-way analysis of variants and Tukeys post test performed on the raw data indicated a significant increase in sIPSC frequency (P=0.0403) and no change in IPSC amplitude (P=0.0836).

depolarise CA1 interneurones and increase GABA release within the CA1 region of the hippocampus. Whilst nAChRs in the CNS are traditionally considered to exert a modulatory influence (Role and Berg, 1996; Wonnacott, 1997), recent studies have demonstrated nAChRs to also mediate fast acetylcholine-mediated neurotransmission in hippocampus and neocortex (Alkondon et al., 1998; Frazier et al., 1998b; Jones and Yakel, 1997; McQuiston and Madison, 1999b). Given these evidences, it is likely that the pro-epileptogenic action of nAChRs is mediated in part through GABAergic circuits. However, such an action of nAChRs appears to be independent of fast GABAergic transmission. An important finding in this respect was that nAChR activation also resulted in a facilitation of epileptiform burst discharge in the bicuculline model (see chapter 4) in which $GABA_A$ receptors are blocked by high concentrations of the specific $GABA_A$ receptor antagonist. This contrasts with a previous report that cholinergic modulation of bicuculline-induced epileptiform activity is exclusively mediated through mAChRs (Sutor and Hablitz, 1989). However, this study was carried out in neonatal tissue and developmental differences may therefore account for such discrepancies. We believe that this is unlikely however, and indeed have shown that nAChRs potentiate bicuculline-induced epileptiform bursts in both neonatal and juvenile rats (2-5 weeks old). Furthermore, nACh receptors show complex developmental expression profiles but important subunits such as the α7 subunit are generally expressed in hippocampus throughout postnatal development and into adulthood (Adams et al., 2002). The present results suggest that the primary means by which nAChR activation are facilitating epileptiform activity is through mechanisms other than affecting fast GABAergic transmission. Indeed, this agrees well with a recent report showing the ability of nicotine to potentiate paroxysmal depolarising shifts following GABA withdrawal syndrome (Silva-Barrat et al., 2001).

Previous *in vitro* works have shown that antagonising $GABA_B$ receptor is not sufficient per se to cause epileptiform synchronisation (McCormick, 1989; Sutor and Luhmann, 1998), but it can potentiate epileptiform responses induced by $GABA_A$ receptor antagonists (Karlsson et al., 1992; McCormick, 1989; Scanziani

et al., 1994; Sutor and Luhmann, 1998). These data suggested that weakening or abolishing $GABA_A$ receptor-mediated inhibition should be a sine qua non condition for expressing the proepileptogenic effects of $GABA_B$ receptor. On the other hand, Motalli et al, 2002, have demonstrated that similar effects can be obtained by blocking $GABA_B$ receptors in the 4AP model in which $GABA_A$ receptor inhibition is potentiated as the result of an increased release of GABA from interneurone terminals (Perreault and Avoli, 1991; Perreault and Avoli, 1992; Rutecki et al., 1987). Overall, these data suggest that the capacity of $GABA_B$ receptor-mediated mechanisms to modulate epileptiform synchronisation in cortical networks maintained *in vitro* does not depend on $GABA_A$ receptor antagonism but rather on the ability of ambient GABA to activate type B receptors. This condition can be achieved by increasing neuronal excitability, and thus GABA release, either by blocking the $GABA_A$ receptor function (Karlsson et al., 1992; McCormick, 1989; Scanziani et al., 1994; Sutor and Luhmann, 1998) or by applying drugs capable of augmenting transmitter release (e.g., 4AP) (Motalli et al., 2002). It has been hypothesised that the facilitation of ictal activity by $GABA_B$ receptor antagonist is mainly caused by the blockade of presynaptic $GABA_B$ receptor, leading to an increase in GABA release and subsequent larger $[K^+]_o$ elevations (Motalli et al., 2002). However, in our experiments, application of a specific $GABA_B$ receptor antagonist, CGP55845A produced a very modest burst frequency potentiation in the BIC model and also did not change frequency of epileptiform activity in 4AP model. It is reported that 5-10 fold higher concentration of $GABA_B$ receptor antagonists are required to block presynaptic as opposed to postsynaptic receptors when these are activated by synaptically release GABA (Pozza et al., 1999). It is likely therefore that this negative finding is attributable to the concentration of CGP55845A (1 μM) added to perfusing medium in our experiments being not high enough to substantially block presynaptic $GABA_B$ receptors.

Moreover, it has been reported that $GABA_B$ receptors exist as subtypes having distinct neuronal locations, functions and pharmacological properties (For review see Bonanno and Raiteri, 1993; Mott and Lewis, 1994). Thus, the different

$GABA_B$ receptor antagonists may reflect different competitive interaction between different receptor subtypes and exhibit different pharmacology at pre and post-synaptic $GABA_B$ receptors (Pozza et al., 1999). Indeed, different subtypes of $GABA_B$ receptors have been found at pre-and postsynaptic sites within the rat dorsolateral septal nucleus (Yamada et al., 1999). If this distinct pharmacology and $GABA_B$ receptor distribution also extends to other CNS structures, such differences may therefore account for the findings described here.

As detailed in chapters 3 and 4 of this thesis, the frequency of 4AP and BIC-induced epileptiform activity were potentiated by subsequent co-application of the selective nAChR agonist DMPP. In 4AP model, application of DMPP (10-30 μM) resulted in a mean burst frequency potentiation of 37 ± 5%. In contrast, slices pre-incubated with the $GABA_B$ receptor antagonist CGP55845A (1μM) were not able to exhibit burst frequency potentiation upon DMPP application. Similarly, in the presence of 1μM CGP55845A, slices exhibited negligible burst frequency potentiation of bicuculline-induced (20μM) epileptiform activity upon DMPP application (27.6 ± 18%), in comparison to those observed in the absence of CGP55845A (248 ± 76 %). A comparison of the effects of DMPP upon these two models of epileptiform bursting in the absence and presence of $GABA_B$ receptor antagonist is summarised in histogram format in figure 6.7. As illustrated in figure 6.7 C, the maximal burst frequency potentiation to DMPP application in 4AP and BIC models in the absence of CGP55845A is 45 and 220% higher in comparison to those in the presence of this antagonist.

These data suggest that $GABA_B$ receptor activation is necessary or at least an important element in the nAChR-induced burst frequency potentiation effect and nAChRs may regulate the excitability of hippocampal networks through $GABA_B$ receptor-mediated mechanisms.

Overall the data presented in this chapter are robust and reproducible. However, the data at the same time appears paradoxical and defy any simple mechanistic explanation. One possible explanation for this result is that increased nAChR

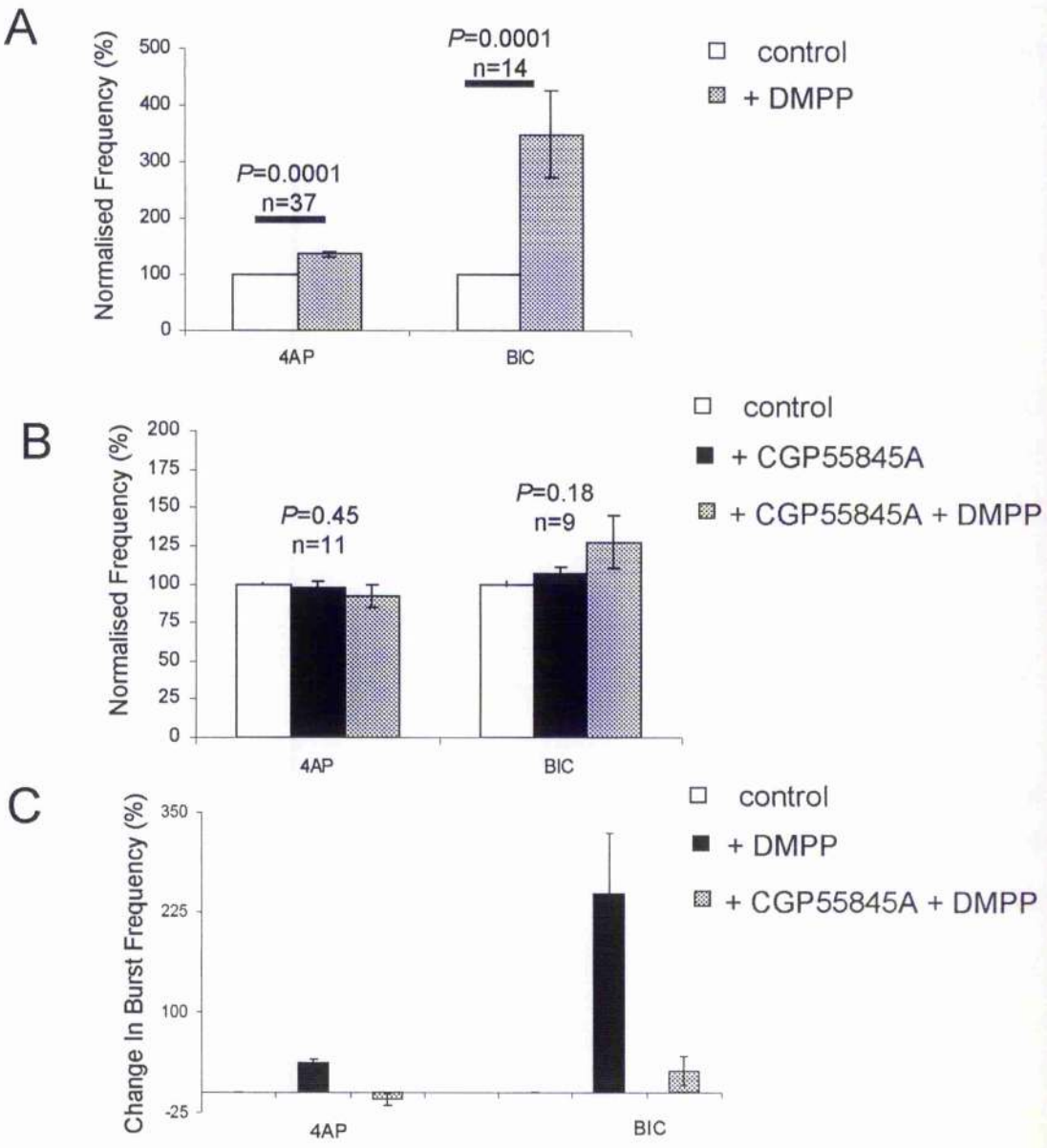

Figure 6.7. *Comparison of DMPP-induced burst frequency potentiation in 4AP and BIC models of epileptiform activity in the absence and presence of $GABA_B$ receptor antagonist.* A. Histogram summarising 10-30 μM DMPP-induced burst frequency potentiation in 4AP and BIC models normalised to control (pre-DMPP) frequency. In the absence of $GABA_B$ receptor antagonist, activation of nAChRs by DMPP results in a significant increase in burst frequency in both models (Wilcoxon-matched pair test, $P<0.01$). B. Quantitative summary of the effect of DMPP on 4AP and BIC-induced epileptiform activity in the presence of $GABA_B$ receptor antagonist, CGP55845A (1-3 μM). Application of CGP55845A blocked the ability of DMPP to potentiate 4AP-induced bursting (n=11) and also substantially decreased DMPP-induced maximal burst frequency potentiation in BIC model (n=9). One-Way ANOVA indicated no significant change in burst frequency upon application of DMPP in the presence of CGP 55845A in 4AP ($P=0.45$) and BIC models ($P=0.15$). C. Histogram summarising DMPP-induced change in burst frequency in the absence and presence of CGP55845A. Note the maximal burst frequency potentiation in BIC model in the absence of CGP55845A is 200 fold higher in comparison to those in the presence of this antagonist.

function leads to an increase in GABA release from GABAergic interneurones. This leads to a subsequent recruitment of $GABA_B$ receptors, which in turn can modulate neuronal excitability via several mechanisms including:

- Activation of $GABA_B$ receptors induces an increase in K^+ conductance (Gahwiler and Brown, 1985; Newberry and Nicoll, 1984; 1985) which modulates seizure activity in both *in vivo* and *in vitro* preparations (Traynelis and Dingledine, 1988; Zuckermann and Glaser, 1968). Hence the proconvulsant action of $GABA_B$ receptors activation may have resulted from changes in $[K^+]_o$ homeostasis. Indeed, GABA-mediated $[K^+]_o$ elevations have been shown to initiate ictal discharges in the 4-AP model (Avoli et al., 1996). However, $[K^+]_o$ recordings reported by Motalli et al (1999) indicated that the proconvulsant action of $GABA_B$ receptors is not accompanied by any measurable change in either $[K^+]_o$ baseline or $[K^+]_o$ transient elevations (Motalli et al., 1999).

- Activation of $GABA_B$ receptors induces presynaptic inhibition of GABA release in turn leading to a depression of IPSPs and subsequent increases in network excitability [e.g., $GABA_B$ mediated inhibition of GABA release from inhibitory interneurones leading to a disinhibition (Mott et al., 1989; Watts and Jefferys, 1993)].

- Another possibility is that increased GABA release by nAChRs activates postsynaptic $GABA_B$ receptors. Activation of postsynaptic $GABA_B$ receptors can induce a membrane hyperpolarisation of inhibitory interneurones again leading to disinhibition of pyramidal cells and increase in excitability.

- However, such a disinhibitory action must be associated with the ability of excitatory terminals to release transmitter, this being essential for proconvulsant activity. An important mechanism underlying the proconvulsant action of $GABA_B$ receptors has been suggested to be related to activity-

dependent changes in CA3 area network excitability that occur during $GABA_B$ receptor activation (Motalli et al., 1999). It is possible that an accumulation of glutamate-containing vesicles docking at presynaptic terminals results from the block of interictal activity and asynchronous excitatory synaptic potentials caused by $GABA_B$ receptors activation (Motalli et al., 1999). This should lead to an increased availability of excitatory transmitter. Presynaptic factors controlling glutamate release have been proposed to regulate the probability and duration of synchronous discharges generated by the CA3 network (Staley et al., 1998).

Application of the $GABA_B$ receptor agonist baclofen decreased and eventually blocked the 4AP-induced interictal activity in all experiments. In our study most baclofen effects were antagonised by CGP55845A, thus indicating that they were mainly caused by the activation of $GABA_B$ receptors. In particular, we propose that baclofen abolishes 4AP-induced interictal activity by decreasing the release of transmitter from excitatory and inhibitory terminals. This may be caused by activation of presynaptic $GABA_B$ receptors inhibiting both GABA and excitatory transmitter release (Lambert and Wilson, 1993; Lanthorn and Cotman, 1981) and by a postsynaptic $GABA_B$-mediated hyperpolarisation that decreases excitability of principal cells (Newberry and Nicoll, 1984; 1985) and interneurones (Misgeld et al., 1989; Williams and Lacaille, 1992). Reappearance of interictal activity by DMPP following application to medium containing 4AP and baclofen, could be as a result of effect of nAChR activation in enhancing neurotransmitter release from presynaptic terminals (Gray et al., 1996; Role and Berg, 1996; Wonnacott et al., 1989). However, this effect of DMPP can not completely overcome the inhibition of transmitter release exerted by baclofen since the effect of baclofen was only partially reversible upon application of DMPP in most experiments. Subsequent application of CGP55845A blocked baclofen-induced inhibition of neurotransmitter release and produced additional enhancement of neurotransmitter release.

In summary, whilst we have found robust evidence that $GABA_B$ receptor activation is an important element in the nAChR-mediated burst frequency potentiation effect, the precise mechanistic detail is unclear and requires further investigation.

CHAPTER 7

GENERAL DISCUSSION

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The major outcome of this study was the demonstration that activation of nAChRs has pro-epileptogenic actions within hippocampal circuits. This was observed across a range of different forms of pharmacologically-induced epileptiform activities including 4AP, bicuculline and low magnesium models suggesting that nAChRs may have a general action in exacerbating epilepsy-like discharges. The mechanisms by which this exacerbation of epileptiform activity occurs does not significantly compromise the mechanisms responsible for the precise firing of action potentials and fast activity within individual bursting events as represented by the relatively small effect of nAChR agonists on high frequency aspects of burst waveform recorded in the presence of 4AP. Our findings of this study suggest that activation of nAChRs potentiates pre-established epileptiform bursting activity in the hippocampus without demonstrating any overt epileptogenic activity *per se*. This has to be viewed with caution since it is likely that the actions of nAChR activation may be more pronounced in the intact hippocampal structure *in vivo*. Whilst the hippocampal slice retains many of the transverse pathways including the trisynaptic circuit, longitudinal pathways are lost and recurrent connections reduced. The exact subtypes of nAChRs mediating the pro-epileptogenic actions can not be fully established in this study. It was our experience that relatively high concentrations of nAChR ligands were necessary to obtain reliable effects within the interface-type recording chamber. Nevertheless, it is apparent that at least $\alpha 7$ and probably $\alpha 4\beta 2$ subunit containing receptors are involved.

It has been shown that pharmacological activation of nAChRs can potently modulate the induction of synaptic plasticity including hippocampal long-term

potentiation (Ji et al., 2001; Mann and Greenfield, 2003). Indeed several aspects of the data presented in this thesis support potential plasticity that may occur at synapses in the network as a result of the activation of nAChRs. For example, the inability of nAChR antagonists to fully reverse the effects of DMPP (Figure 3.15) and the lack of full reversibility of DMPP effects upon wash out (Figure 4.3) support the possibility that plasticity events may be an important factor in the action of nAChRs in epileptogenesis. Depending on the distributions of various nAChR subtypes and the timing of nAChR activity, cellular and synaptic events can be modified in many different ways (Ji et al., 2001). Presynaptic nAChRs can increase the probability of neurotransmitter release this increasing the fidelity of synaptic transmission at any given excitatory synapse. Postsynaptic nAChRs can further increase the depolarization and calcium signal associated with successful transmission, helping to initiate intracellular cascades. On the other hand, nAChRs can have a potent impact upon the activity of interneurone populations which themselves are important regulators of network excitability and synaptic plasticity (Davies et al., 1991).

That said, the question still remains as to the precise mechanistic detail by which nAChRs enhance epileptiform activity? It is not possible from this study to identify the precise means by which nAChRs facilitate epileptiform activity although it is likely that a number of factors may contribute to their pro-epileptogenic actions. An important locus of action is likely to be that of the calcium-dependent signalling pathways. Classically nAChRs are considered to participate in fast signalling (Frazier et al., 1998a; Hefft et al., 1999). However, recent reports in the autonomic nervous system suggest that nAChR agonists, including nicotine, can produce sustained calcium spiking in presynaptic nerve terminals (Brain et al., 2001) during prolonged periods of agonist application. It is possible that this type of neuromodulatory effect at recurrent glutamatergic terminals may be responsible for generating the potentiation of epileptiform activity reported here. Relevant to the hippocampus there is a precedent for nAChRs exhibiting a slow neuromodulatory action (McGehee and Role, 1996; Role and Berg, 1996; Wonnacott, 1997) with the strongest evidence for

modulation of neurotransmitter release (Albuquerque et al., 1997; Gray et al., 1996; Ji and Dani, 2000) and/or regulation of synaptic plasticity (Ji et al., 2001). In both cases transient activation of nAChRs can potentially trigger either a transient or persistent effect that, by analogy to the autonomic nervous system, could possibly involve calcium-mediated pathways within nerve terminals.

The data presented in this thesis provide some additional insight into the potential mechanisms underlying nAChR-induced frequency facilitation. Although the mechanisms underlying the pro-epileptogenic action of nAChRs are complex and not simply due to activation of nAChRs, clearly involve the modulation of both GABAergic and glutamatergic circuits (see Figure 7.1). In experiments devised to investigate the action of nAChRs on basal glutamatergic transmission, application of DMPP resulted in a sustained yet reversible enhancement of glutamate afferent evoked field EPSP amplitude in the CA3 region of the hippocampus suggesting that nAChRs enhance glutamate release from presynaptic terminals. Whilst we can not rule out a possible postsynaptic sensitisation-type action on glutamatergic transmission we feel that this is unlikely. Instead, we hypothesise that activation of nAChRs by DMPP enhances glutamate release, which in turn can strengthen recurrent excitatory connections in area CA3 and thus facilitate polysynaptic reverberations throughout the network. The strength of such recurrent connections have been proposed to be critical in the generation and regulation of bursting activity within the CA3 network (Bains et al., 1999; Staley et al., 1998; Traub, 1991) and recent studies have shown acetylcholine to modulate these synapses directly (Vogt and Regehr, 2001). The experiments using glutamate receptor antagonists (Chapter 5) further confirm the involvement of glutamate receptors in pro-epileptogenic effect of nAChRs. In agreement with previous studies, our results have shown that nAChRs unlikely to have a direct postsynaptic effect in principal cells.

On the other hand, in experiments devised to investigate whether nAChRs regulate GABAergic cells and circuits, application of DMPP revealed an inward current and increased the frequency of spontaneous IPSCs in a subpopulation of

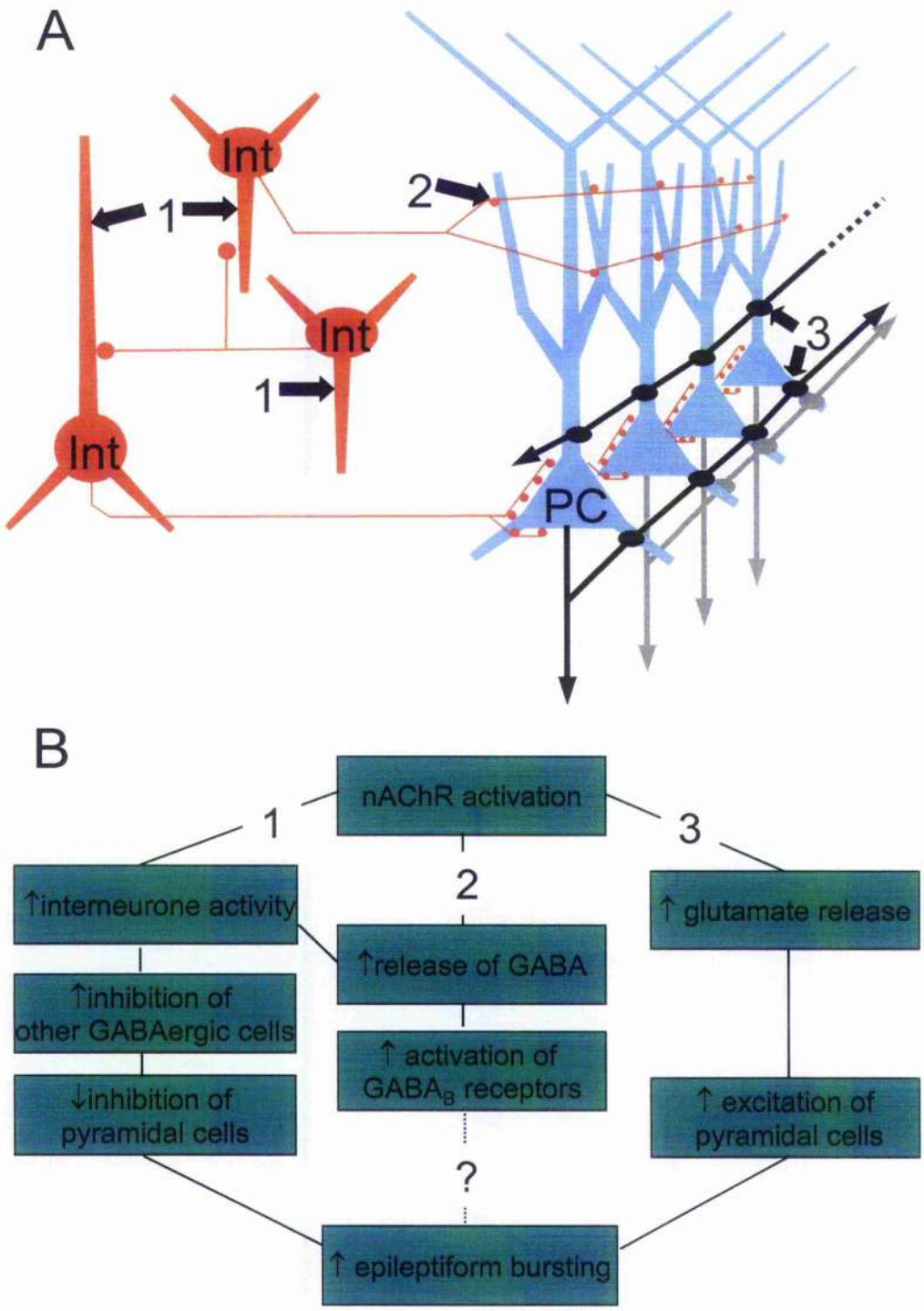

Figure 7.1. *Summary of possible loci and mechanisms for nAChR-mediated pro-epileptogenic action.*
A. Schematic drawing showing the possible sites at which nAChRs may influence hippocampal microcircuits including (1) postsynaptic sites on GABAergic interneurones (including interneurone-selective cells), (2) pre-synaptic GABAergic terminals and (3) glutamatergic terminals. B. Flow chart indicating possible routes by nAChR modulation may ultimately result in pro-epileptogenesis (see text for details). Abbreviations: int - interneurone; PC - pyramidal cell.

CA1 interneurones suggesting that nAChR agonist can directly depolarise CA1 interneurones and increase GABA release within the CA1 region of the hippocampus.

Together, these results suggest that activation of nAChRs may produce pro-epileptogenic actions through regulating both glutamatergic and GABAergic circuits, resulting in both direct excitatory and modulation of GABAergic (probably via $GABA_B$ receptors) inhibition respectively. This is consistent with studies suggesting the involvement of either glutamatergic or GABAergic transmission in the mechanisms underlying nicotine-induced seizures *in vivo* (Damaj et al., 1999). However, despite the functional expression of nAChRs on hippocampal GABAergic interneurones (Alkondon et al., 1997; Frazier et al., 1998a; Frazier et al., 1998b; Jones and Yakel, 1997; McQuiston and Madison, 1999b) it is unlikely that nAChRs are significantly affecting GABAergic circuits to promote epileptiform activity since frequency facilitation is observed in the presence of the $GABA_A$ receptor blocker bicuculline. Indeed, a somewhat surprising finding was that nAChR-mediated burst frequency potentiation was particularly pronounced in the bicuculline model. However, an action on $GABA_B$ receptor mediated synaptic inputs cannot be excluded since $GABA_B$ receptor-mediated mechanisms are involved in the generation of focal seizures and in epileptogenesis (McLean et al., 1996; Scanziani et al., 1994; Veliskova et al., 1996) and also in facilitating epileptiform activity (Motalli et al., 1999; 2002). In experiments to identify possible contribution of the $GABA_B$ receptors in the nAChR-induced effect, application of $GABA_B$ receptor antagonist prevented DMPP-induced burst frequency potentiation of 4AP and bicuculline-induced epileptiform activity suggesting that nAChRs may regulate the excitability of hippocampal networks through $GABA_B$ receptor-mediated mechanisms. This has important consequences for investigating network dynamics in that suggests the basal neuromodulatory tone of transmitters and their interacting may be a significant consideration.

Whilst we hypothesise that activation of nicotinic acetylcholine receptors has pro-epileptogenic actions within hippocampal circuits through regulating either glutamatergic, GABAergic or both circuits further effort is required to establish (a) how different nAChR subtypes impact on this cellular process and (b) the precise nature of their interaction. Furthermore, it is also necessary to establish whether nAChR activation may indirectly modulate epileptiform activity through other transmitter systems. The hippocampus is reach in a variety of neurotransmitters (Reviewed by Vizi, 1998) and ACh is known to modulate the release of many of these.

A key requirement is to establish whether discrete subtypes of nAChRs are implicated in epileptogenesis. The limited pharmacopea has hampered progress in this area to date. However, introducing novel significantly more selective ligands would be useful to address this issue.

The hypothesis that choline as a selective agonist at certain nAChRs has a role in modulating epileptiform activity is significant. Despite acetylcholine being broken down and inactivated by acetylcholinesterase rapidly, it is possible that the sustained presence of choline may have a longer lasting neuromodulatory influence. An interesting study would be to examine plasma choline levels across a large bank of epilepsy patient volunteers to assess the possible potential contribution of brain choline levels to epileptogenesis.

Another avenue in attempting to elucidate the mechanisms underlying pro-epileptogenic actions of nAChR activation would be to take a calcium imaging approach. Recent studies using confocal imaging have shown nAChRs to be an important regulator of spontaneous neurotransmitter release in the peripheral nervous system (Brain et al., 2001). Similar studies using a cell imaging approach whereby discrete cells and circuits are labelled with a fluorescent calcium indicator could be useful to assess the action of the cholinergic system on cellular processes and importantly, calcium transients within nerve terminals at discrete circuits.

Studies are on going with respect to identifying the precise inhibitory circuits under modulation by nAChR activation. These findings may reveal potentially more selective pharmacological means by which to regulate specific GABAergic circuits and this avoid the side effects associated with current anticonvulsant drugs which potentiate the entire GABAergic system without discrimination.

Despite these unresolved questions, the observation that brain nAChRs regulate synchronised neuronal activity suggests that nAChRs may represent a potential target in developing novel treatments for the control of epilepsy. Since many epilepsies are resistant to current drug therapies which influence neuronal activity by affecting ion channels responsible for cell excitability or acting to potent GABAergic synaptic transmission, there is a push to identify new therapeutic agents based upon novel mechanisms of action. As a result, nAChRs are currently the subject of intense research interest with respect to their therapeutic potential in a series of neurological and psychiatric conditions including epilepsy. It has been reported in animal seizure models that preferential nicotinic acetylcholine receptor antagonists are potent anticonvulsants in the maximal electroshock seizure test (Loscher et al., 2003) and against nicotine-induced seizures (Damaj et al., 1999). The anticonvulsant potency in the maximal electroshock seizure test was decreased by injection of a subconvulsant dose of nicotine, suggesting the contribution of nAChR mechanisms (Loscher et al., 2003). In conclusion, it may be suggested that nicotinic acetylcholine receptor antagonism might be a valuable future therapeutic approach to treat epileptic seizures. The work in this thesis has gone some way to providing a basis upon which future studies to this aim can be based.

CHAPTER 8: REFERENCES

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