SEROTONERGIC, PURINERGIC, AND CALCIUM DEPENDENT MECHANISMS IN RAT HIPPOCAMPAL PYRAMIDAL CELLS

BY

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ABSTRACT

This series of investigations using the *in vitro* rat hippocampal slice, examines the role of two important neuromodulatory compounds, serotonin and adenosine. The agonists and antagonists of these compounds were examined for their effects on normal evoked synaptic activity and on epileptiform activity in a low calcium (Ca^{2+}) bursting model.

Perfusion of serotonin (5-HT) in the CA1 region and dentate gyrus (DG) reduced the evoked population spike amplitude. The largest reductions followed the perfusion of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) (a $5-HT_{1A}$ receptor agonist). While application of the $5-HT_2$ receptor agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), did not result any changes in the CA1 region, and only slight reductions in the DG. Application of adenosine elicited a reduction in the evoked population spike, with the specific agonist, 2-chloroadenosine, being much more effective. Perfusion of the degradative enzyme for adenosine, adenosine deaminase potentiated the amplitude of the population spike. Perfusion of the purine antagonist, theophylline, elicited a transient increase in the population spike amplitude, proving that serotonergic and purinergic compounds have the ability to exert inhibitory effects on evoked synaptic transmission in the hippocampus.

Rhythmic synchronous bursting discharges are observed in the CA1 region of hippocampal slices when perfused with artificial cerebrospinal fluid (ACSF) containing low concentrations of calcium. Such bursts are characterized by shifts in the extracellular DC potential upon which population spikes are superimposed: this phenomenon provides a simple model to study cellular hyper-excitability which occurs in the absence of synaptic transmission.

The introduction of 5-HT resulted in a reduction in burst rate only at high doses (e.g. 50uM), while 8-OH-DPAT depressed it significantly at all doses tested. Application of adenosine yielded significant reductions only if high concentrations were used. The catabolic enzyme, adenosine deaminase increased the burst rate 2-10 times above control burst rates. Likewise, addition of two methylxanthines, known adenosine antagonists, 3-isobutyl-1-methylxanthine

(IBMX) and theophylline to the perfusate reversibly increased the burst rate, strongly indicating that large concentrations of purines are being released to control burst behavior. Acting together the serotonergic and purinergic systems modulate hippocampal activity.

Convulsant-induced burst discharges in normal CA1 pyramidal neurons activate the N-methyl-D-aspartate (NMDA) receptor. Surprisingly the NMDA antagonist, D-2-amino-5-phosphonovaleric acid (D-APV), displayed a significant and reversible inhibitory effect on burst rates in the presence of low extracellular Ca^{2+}. This reduction suggests that glutamate or aspartate may released under low Ca^{2+} conditions and that these may be exerting an excitatory effect leading to the bursting activity.

Evidence is presented which demonstrates possible roles for serotonergic, purinergic and possibly excitatory amino acids in modulating the mechanisms underlying the generation of presumed non-synaptic bursting phenomena. Previous studies have suggested that these compounds may exert their effects via modulation of free $[Ca^{2+}]_i$, however, Fura-2 microspectrofluorimetry in cultured hippocampal neurons treated with serotonergic and purinergic agonists and antagonists found no such changes in $[Ca^{2+}]_i$.

Long-term potentiation (LTP) is characterized by a long lasting increase in the efficacy of neurotransmission. An essential function for calcium ions in the induction of LTP has been established and a particular emphasis has been placed on the role of NMDA receptor activation in gating a post-synaptic influx of calcium (Mayer and Westbrook 1987a). The present experiments used dantrolene to blockade intraneuronal calcium release and were able to completely block the induction of both tetanic and Ca^{2+}-induced LTP in the CA1 region of the rat hippocampal slice. This drug inhibits calcium release from the sarcoplasmic reticulum and also diminishes the rise in intraneuronal calcium ion concentrations elicited by NMDA receptor activation in cultured CA1 pyramidal cells. Dantrolene does not block NMDA gated membrane currents or voltage activated Ca^{2+} currents in these cells (Mody et al 1989).

In contrast to reported effects in the DG granule cell layer, NMDA receptors do not seem to be directly involved in Ca^{2+}-induced LTP. In the CA1 pyramidal cell layer the NMDA antagonist D-APV did not block the induction of LTP by transient exposure of hippocampal slices to a high Ca^{2+}-containing medium.

To extend these findings, the role of intraneuronal Ca^{2+} was investigated on cultured hippocampal neurons using Fura-2 microspectrofluorimetry. In neurons perfused with a medium containing normal Ca^{2+}, the resting $[Ca^{2+}]_i$ was approximately 100 nM. Perfusion of high Ca^{2+} for 10 min revealed a rapid and sustained increase in $[Ca^{2+}]_i$ which persisted after the return to normal $[Ca^{2+}]_o$. Application of dantrolene in this experimental paradigm prevented the rise in $[Ca^{2+}]_i$. Interestingly, D-APV applied prior to and during the high $[Ca^{2+}]_o$ perfusion, did not effect the changes in $[Ca^{2+}]_i$.

The combined results from both the hippocampal slice preparation and neuronal cultures suggest that while not excluding pre-synaptic involvement in the maintenance of LTP, the release of calcium from intraneuronal stores, rather than a transmembrane calcium influx may be the critical post-synaptic feature underlying the induction of both tetanic and Ca^{2+}-induced LTP.

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LIST OF ABBREVIATIONS

5-HT - serotonin, 5-hydroxytryptamine
8-OH-DPAT - 8-hydroxy-2-(di-n-propylamino)tetralin
AC - adenylate cyclase
ACh - acetylcholine
ACSF - artificial cerebrospinal fluid
AD - adenosine
ADA - adenosine deaminase
AHP - afterhyperpolarization
ATP - adenosine triphosphate
AV - alveus
CA1,2,3,4 - cornu ammonis regions 1-4
Ca^{2+} - calcium
$[Ca^{2+}]_i$ - intracellular calcium concentrations
$[Ca^{2+}]_o$ - extracellular calcium concentrations
CaBP - calcium binding proteins
cAMP - cyclic adenosine monophosphate
CCK - cholesystokinin
D-APV - D-2-amino-5-phosphonovaleric acid
DG - dentate gyrus
DMSO - dimethylsulfoxide
DOI - 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
DRN - dorsal raphe nucleus
EC - entorhinal cortex
EPSP - excitatory postsynaptic potential
g - channel conductance (e.g. gNa^+ - sodium conductance)
GABA - gamma aminobutyric acid
GAD - glutamic acid decarboxylase
GL - granule cell layer
HF - hippocampal formation
IBMX - isobutyl-methylxanthine
IP - inositol phosphates
IPSP - inhibitory postsynaptic potential
LSD - lysergic acid diethylamide
LTP - long-term potentiation
MF - mossy fiber pathway
MFB - medial forebrain bundle
ML - molecular layer
MRN - medial raphe nucleus
NE - norepinephrine (or noradrenaline)
NMDA - N-methyl-D-aspartate
PAP - papaverine
PI - phosphatidylinositol
PKC - protein kinase C
PL - polymorphic layer
PP - perforant path
PSA - population spike amplitude
PTP - post-tetanic potentiation
PV - parvalbumin
SC - Schaffer Collaterals
SLM - stratum lacunosum/moleculare
SO - stratum oriens
SP - stratum pyramidale

SR - stratum radiatum
THP - theophylline
VIP - vasoactive intestinal polypeptide

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CHAPTER 1 - GENERAL INTRODUCTION

I. THE HIPPOCAMPAL FORMATION

The mammalian hippocampal formation comprises several distinct structures and includes: i) the entorhinal cortex (EC), ii) subicular complex, iii) dentate gyrus (DG), and iv) Ammon's horn or hippocampus proper (Figure 1.1). This composite of brain structures in the temporal lobes is closely associated with other structures of the "limbic" lobe (Broca 1878) which have been the subject of numerous theories. Based on anatomical connectivity, Papez (1937) hypothesized that the limbic lobe, including the hippocampus, was the neural substrate for emotional behavior.

A possible function of the hippocampal formation emerged from the case history of patient HM, who showed severe memory deficits following bilateral removal of his hippocampus (Scoville and Milner 1957). Since that study numerous models of hippocampal function in memory have been published including the works of Thompson, Squire and Cohen, Mishkin, O'Keefe and Nadel, Olton, and Kesner (see Kesner 1986). O'Keefe and Nadel (1978) suggested that space is the critical attribute of specific memories, and that the hippocampus mediates the spatial memory and provides a cognitive map. This cognitive map can be used for place recognition, navigation and coding of context, based on the identification of specific place cells in the hippocampus. However, most learning tasks involve temporal as well as spatial attributes, a factor which their model fails to take into account. Olton (1983) developed a more comprehensive proposal, suggesting that memory can be subdivided into either working or reference memory. Experimental evidence from his and other laboratories has led to the suggestion that the hippocampus and its interconnections mediate working memory while another system, possibly the neocortex, mediates reference memory. However, it should be noted that "memory" is not localized to the hippocampal formation; rather, the hippocampus appears to provide an "index" of memory traces that have been stored elsewhere in the cortex. The hippocampal formation pathways are crucial in the laying down of memory traces for the recall and storage of memory.

More recently, Winson (1985) has proposed that the limbic system and in particular the hippocampus is involved in the gating of information flow, and is dependent on the emotional state of the animal. He contends that while anatomical circuitry of the hippocampus does not change in

response to incoming information, there is a restriction of information flow along certain pathways; i.e.. the neural gates are open or closed. The monamines and in particular norephinephine appear to involved in this gating of hippocampal circuits (Dahl et al 1983).

Although the hippocampus is involved in emotion, gating and memory functions, it has also been found to be very sensitive to various pathological disease states such as epilepsy and ischemic damage. This propensity to pathophysiology in conjunction with its the laminar structure and the ease of its dissection from the brain have led to a proliferation of scientific investigations of its anatomy, electrophysiology, neurochemistry and functional roles. An overview of the work of Seifert (1983), Frotscher et al (1988), Dingledine (1984), Deadwyler et al (1982), Pohorecki and Domino (1987), and Llinas (1988) will serve to outline the current paradigm and establish a platform on which this experimentation of hippocampal modulation by means of putative neurotransmitters and the role of Ca^{2+} in a model for memory may stand.

A. Anatomy

The geometry of the hippocampus is unique in the mammalian central nervous system. It is a bilaterally symmetrical structure and has a distinctive "C" shape. Its two distinct but interrelated cortical fields, the hippocampus proper and the dentate gyrus, interdigitate (Figure 1.1). One interesting characteristic is the high degree of cellular lamination that occurs in the dentate gyrus and the hippocampus. This allows transverse cuts for brain slices, while maintaining functional connectivity (Figure 1.1).

Figure 1.1: The rat hippocampus.

Phantom drawing of the rat brain illustrating the localization of the hippocampus. A schematic of a transverse hippocampal slice is shown, describing the principal lamina and cell types found in the hippocampus proper and dentate gyrus.

Abbreviations:

S. O. - Stratum Oriens

S. P. - Stratum Pyramidale

S. R. - Stratum Radiatum

S. L. - Stratum Lacunosum

S. M. - Stratum Moleculare

G. L. - Granule Cell Layer

M. L. - Molecular Layer

CA1, CA3, CA4 - Cornu Ammonis regions 1,3,4

B.C. - Basket Cell

S.C. - Schaffer Collaterals

4

CA I
(Regio Superior)

B.C.

S.O.
S.P.
S.R.
S.L.

S.C.

Pyramidal Cell

S. M.

CA 4
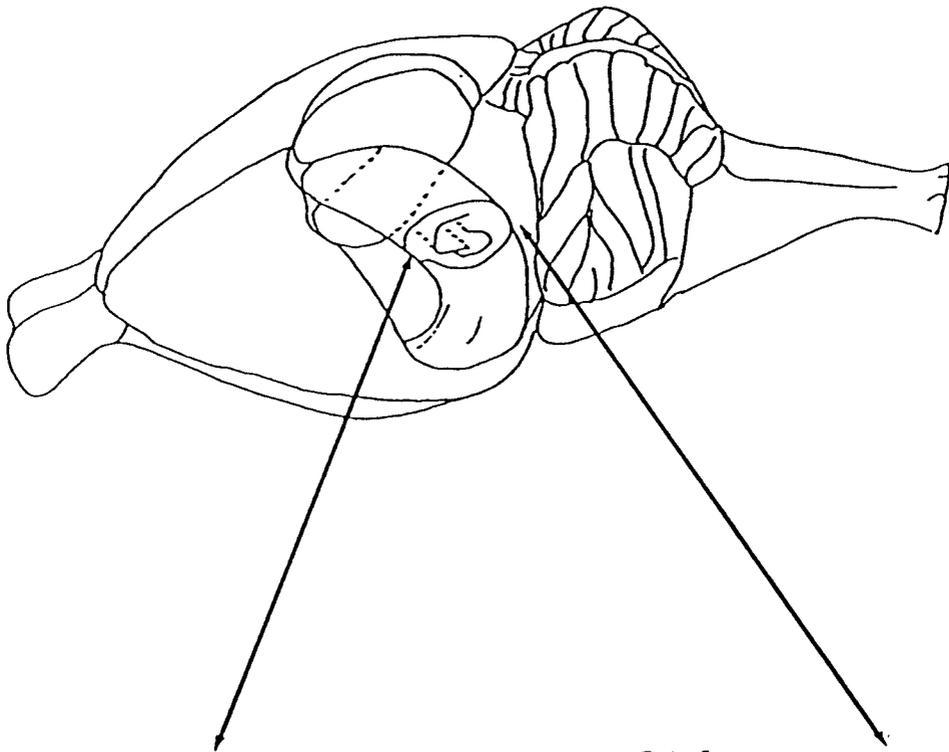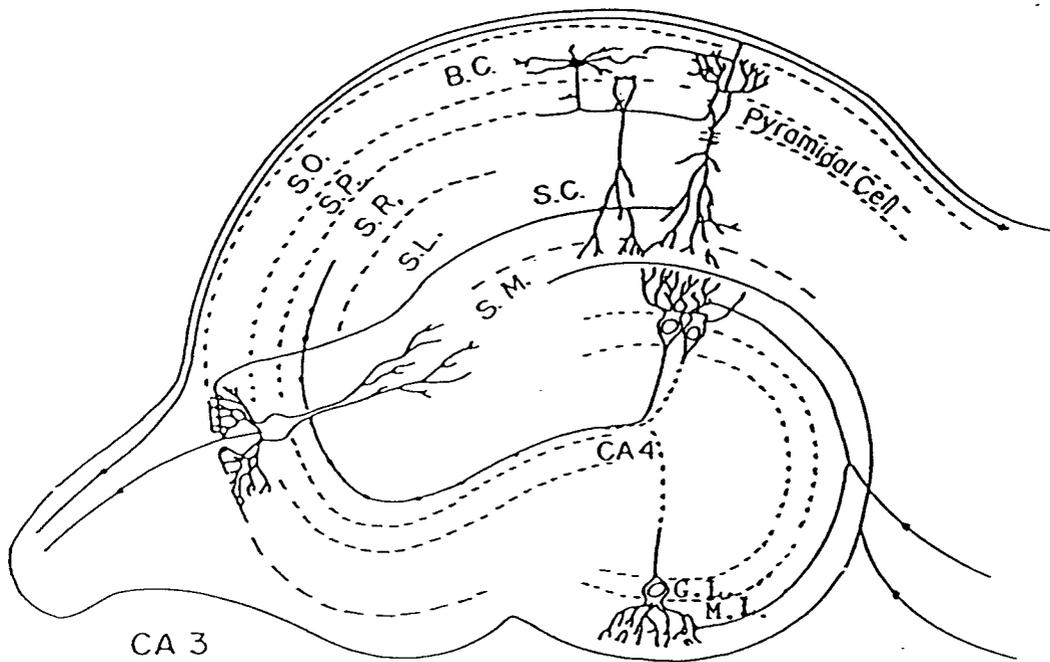
CA 3
(Regio Inferior)

G. L.
M. L.

Area Dentata

1. Cellular Anatomy

Based on early studies of the hippocampal formation by Cajal (1893) and others, the various lamina of Ammon's horn are well described. The CA1 region (Cornu Ammonis 1 or Regio Superior) and the CA3 (or Regio Inferior) have five distinct cytoarchitectural layers. These are (starting from the ventricular cavity; see Figure 1.1) the alveus (AV), stratum oriens (SO), stratum pyramidale (SP), stratum radiatum (SR) and the stratum lacunosum and moleculare (SLM). Each lamina has distinct cell types and characteristics.

The alveolar layer is primarily composed of the axons of the pyramidal neurons. The stratum oriens contains the basilar dendritic tree of the pyramidal neurons and also afferent fibers from other parts of the hippocampal formation. The stratum pyramidale layer is a densely packed region of the CA1-CA4 areas and contains predominantly the cell bodies of the pyramidal neurons. It should be noted that the pyramids of the CA1 region are smaller and more densely packed than the CA3 region. The stratum radiatum is the largest of the lamina in the hippocampus and one of the most complex. This stratum contains the apical dendritic arborizations of the pyramidal neurons, along with numerous afferent fibers that pass through this lamina and play an important role in synaptic integration. In addition, a variety of small neuronal cells (stellate, triangular and fusiform) are present, falling into the category of interneurons which will be discussed later. The stratum lacunosum/moleculare consists of fibers that arise from the regio inferior and extend into the subiculum. This region also contains the tips of the apical dendritic trees.

The principal cell type of the hippocampus is the pyramidal neuron. This neuron type is packed into a dense layer in the stratum pyramidale (SP) and has dendrites that project in both apical and basilar directions. Only one apical dendrite arises from the apical pole of the pyramidal cell and then begins to branch profusely at more distal lamina. From the base of the cell soma an axon and several basal dendrites arise and extend into the SO. The axon makes its way to the alveus, bifurcates, and then travels out of the hippocampus (Frotscher 1988). The principal morphological difference between the CA1 and CA3 pyramids other than size, is that the CA3 pyramidal neurons have large branched spines on the proximal dendritic segments. These

dendritic formations or spines establish synaptic contacts with the mossy fibers, the axonal system of the dentate granule neurons. Another key difference is that, unlike CA1, CA3 neurons send recurrent collaterals to the SR and then to the apical dendrites of the CA1 region.

These collaterals (Schaffer collaterals (SC)) are part of the tri-synaptic excitatory circuit of the hippocampus. A small but significant number of cells in the hippocampus are not pyramidal in shape and are therefore called "non-pyramidal" cells. Although these cells lack dendritic spines, synaptic contact is made by numerous varicose swellings along the length of their dendrites. Most of these neurons can be immunostained for GABA and inhibitory neuropeptides (Dingledine 1984). The fibers of some of these non-pyramidal cells form a basket-like plexus around the cell bodies of the pyramidal and granule cells (Lacaille et al 1987). Although most of these cells are local circuit neurons several studies have demonstrated that they also participate in projections to other brain regions (Totterdell and Hayes 1987, Frotscher et al 1988).

The fascia dentata or dentate gyrus (DG) has three distinct strata; the molecular layer (ML), the granular layer (GL) and the polymorphic cell layer (PL). The ML contains the dendritic arborizations of the granule cells and various other "non-granule" cells. The granular layer contains the tightly packed cell bodies of the granule cells. The PL zone contains a variety of cells including pyramidal like cells. The principal cell of the DG is the small granule cell, which has only an apical dendritic arborization into the ML. From the other side of the cell bodies, axons descend into the PL cell layer and into the dendritic and pyramidal layers of the hippocampus proper. The granule cell axon terminals have a dense mossy appearance and travel in a distinct fiber tract through the regio inferior and then synapse on CA3 pyramidal neurons. Non-pyramidal cells, thought to be inhibitory interneurons, are present in the DG (Seress and Ribak 1985).

2. Chemical Anatomy

The chemical anatomy of the hippocampal formation is slowly being elucidated, although much remains to be studied. The components of the trisynaptic pathways in the hippocampus contain excitatory amino acid neurotransmitters. Release and electrophysiological studies with excitatory amino acids have suggested that glutamate is the primary neurotransmitter of the lateral perforant path as it enters the hippocampus (Dingledine 1984). Storm-Mathisen (1983) provided evidence that suggests that the mossy fiber system also uses glutamate as its transmitter. Aspartate and glutamate are localized in the axons of the CA3 pyramidal neurons which make up the Schaffer collaterals.

The non-pyramidal cells of the hippocampus, in particular the basket cells described by Lorente de No (1934) which surround the pyramidal cells with a dense plexus of synapses, have been shown to contain gamma-aminobutyric acid (GABA) as their principal neurotransmitter (Storm-Mathisen 1977). These basket cells are activated by the pyramidal cells and mediate powerful recurrent feedforward and feedback inhibition. GABA receptors and the GABAergic enzyme glutamic acid decarboxylase (GAD) are found in all layers of the hippocampus.

Extrinsic pathways that modulate activity are presently under scrutiny. One such modulatory system involves the cholinergic (ACh) afferents that arise in the medial septum and the diagonal band, and terminate within the SO of the CA3 region, and in the hilus and the ML of the DG (Geneser 1987a, b). However, in addition to the extrinsic source of ACh, recent work by Frotscher et al (1986), suggests that there are intrinsic ACh-containing fibers located in all layers of the hippocampus, with particularly dense labelling at the CA1/subicular border. Interestingly, Frotscher et al (1986) found all the ACh immunoreactive cells to be non-pyramidal. The locus coeruleus is thought to provide the majority of the noradrenergic innervation to the hippocampus. These noradrenergic neurons form a dense plexus within the hilus of the DG, in the SR of the CA3 region, and SLM of the CA1 region (Storm-Mathisen 1977, Dingledine 1984). Recent work has suggested that NE plays a key role in inhibition and disinhibition (Madison and Nicoll 1986, Leung and Miller 1988). The serotonergic (5-HT) projection to the hippocampal formation arises primarily from the medial and dorsal raphe nuclei and displays a similar distribution to NE (see

Chapter 3). Dense immunoreactive labeling of serotonergic fibers in the SR of the CA1 region is seen with little, if any, distribution in the CA3 (Kohler 1984).

Recent immunohistochemical techniques have localized various neuroactive peptides that may play an important part in modulating synaptic function in the hippocampal formation. Enkephalins have been found in the lateral perforant path (PP) and in the SLM of the CA1 region. The mossy fiber system is heavily immunostained for dynorphin. Basket-like interneurons of the SO of the CA1 region contain cholecystokinin (CCK), along with CCK-positive cells in the SR of the CA1 and the PL zone of the DG. The neuropeptide somatostatin has also been localized in the SO and the hilus of the DG (see Sloviter and Nilaver 1987). Other peptides (see Roberts et al 1984), such as vasoactive intestinal polypeptide (VIP) have the same distribution as CCK labeled neurons. Angiotensin and substance P also appear in the hippocampus and the DG. Of special importance is the finding of various peptides co-localized in the same neuron or interneuron (Sloviter and Nilaver 1987).

B. Neuronal Circuits

The neuronal circuits that modulate hippocampal activity and information flow fall into two types, intrinsic circuitry and extrinsic circuitry. Intrinsic circuits are those that originate only within the hippocampus proper, while extrinsic circuits modulate hippocampal function via inputs from outside the hippocampus or dentate gyrus.

1. Intrinsic Afferents and Efferents

The intrinsic circuitry of the hippocampus and the DG is composed of two primary subsystems: i) the inhibitory circuitry, and ii) the excitatory trisynaptic pathway. The primary inhibitory circuitry within the various lamina is a diffuse system of GABAergic interneurons. In the pyramidal layer, they form distinct synaptic plexuses around individual neurons. Removal of the inhibitory influence often leads to epileptiform activity within the CA3/CA1 regions of the hippocampus (Schwartzkroin and Prince 1977).

The trisynaptic excitatory pathway begins with the perforant path from the entorhinal cortex, and synapse on the dendrites of the granule cells. The mossy fibers (MF) of the DG (the axons of the granule cells) are organized into a distinct band and are restricted to the CA3 pyramidal neurons of the regio inferior. The CA3 pyramidal cells then send axons through the CA1 SR region in a fiber pathway (Schaffer collaterals) which synapses on the CA1 pyramidal dendritic tree in the SR region. In addition, the CA3 pyramids send out axons via the fimbria/fornix pathway. The CA1 pyramidal neurons have only a single axon that bifurcates and enters the alveus sending efferents to the subicular complex where they synapse, although these outputs are not considered part of the trisynaptic pathway. The effects of this powerful excitatory trisynaptic pathway are modulated by the intrinsic inhibitory interneurons noted above. It is this highly laminated trisynaptic pathway that allows one to cut transverse hippocampal slices leaving the intrinsic components intact (Andersen 1975, Frotscher et al 1988).

2. Extrinsic Afferents and Efferents

The major input to the hippocampus and the dentate gyrus is the perforant path (PP) which originates from layer II of the entorhinal cortex (EC), and follows a topographic pattern of termination. The lateral PP projects to the outer part of the dentate ML and the medial PP terminates on the inner part of the ML, closer to the GL. It should be noted that the contralateral EC also sends projections to the dentate in a similar termination pattern. Layer III of the EC sends efferents to the CA1 region which terminate in the distal dendritic zones (SLM) near the hippocampal fissure (Steward and Scoville 1978, Frotscher 1988).

Another afferent input into the dentate region is the hippocampal commissural pathway (Blackstad 1956, Laurberg 1979) which terminates in the ML (Swanson et al 1981). Other inputs to the dentate arise from the septum and terminate in the ML and GL. The ipsilateral associational fibers arise from the same neurons as the commissural sources and project to the same regions (Frotscher et al 1988). Non-pyramidal neurons (GABA immunoreactive (Ribak et al 1986)) as well as pyramidal neurons project via the commissure to the contralateral hippocampus and DG (Schwerdtfeger and Buhl 1986).

The cholinergic input from the medial septum and diagonal band to the CA3 does not have a topographic arrangement and is evident in all layers of the hippocampus and the DG. In addition to the proximal dendritic tree receiving MF input, the CA3 pyramids also have septal and commissural projections (as discussed above (Voneida et al 1981)) synapsing in the SO and SR regions of regio inferior. The CA3 region has an efferent projection to the fornix via the fimbrial pathway.

The CA1 neuron's major afferent input is from the intrinsic Schaffer collaterals and the commissural inputs from the contralateral hippocampus (Voneida et al 1981). In addition axons derived from septal neurons terminate in the SR and SO. The major output from the CA1 region is through CA1 axons projecting via the alveus to the subicular complex. Recent evidence also suggests that there are direct projections to the medial prefrontal cortex from the CA1 (Ferino et al 1987). The CA1 and CA3 pyramids send direct connections to the lateral septal complex, and the subicular connections also show widespread efferent connections to various subcortical and cortical areas (Swanson and Cowan 1977).

Both intrinsic and extrinsic pathways modulate hippocampal and dentate gyrus excitability. It has been suggested that the hippocampus is an association cortex which receives and integrates information from each of the sensory modalities and subsequently projects it back to complex association areas. Thus, "it seems to be in the unique position of influencing, at the cortical level, somatomotor, visceral, motivational and affective, and cognitive mechanisms" (Swanson 1983).

C. Electrophysiology of Principal Cells

The electrophysiological properties of the hippocampal cells have an important function in the integrative properties of the hippocampal formation.

1. Pyramidal Cells

Although the CA1 and CA3 pyramids of the regio superior and regio inferior differ in size and dendritic arborizations, they have very similar electrophysiological properties. Placement of an extracellular electrode in the appropriate cell body layer and stimulation of the principal excitatory pathway to each cell region (MF for CA3; SC for CA1) will result in an evoked population response, termed the "population spike" (Andersen et al 1971). It has been hypothesized that this population spike represents the summation of individual unit discharges in response to synaptic activation; that is, a large number of neurons are simultaneously activated. Similarly if one records extracellularly in the dendritic fields (SR or SO) of the hippocampus one can record a population excitatory post-synaptic potential (EPSP). Analysis of the population spike or the population EPSP is a promising approach since it can reveal much about the synaptic functioning of a population of neurons in the hippocampus.

However, analysis of the population responses does not determine conclusively what synaptic and membrane properties of hippocampal neurons exist at the cellular level. Intracellular recording techniques allow examination of the various ionic conductances that take part in integration of hippocampal activity. In general, the membrane potential at rest is more negative in neurons of slices compared to those recorded *in situ*. The small pyramids of the hippocampus exhibit a high input resistance (approx. 78 Megohms) and a long time constant (15-20 ms), which allow for temporal summation of synaptic potentials. This combination enhances the probability of information transmission from the dendrites to the soma (Biscoe and Duchen 1985).

Using intracellular recordings in hippocampal tissue coupled with pharmacological manipulations, Llinas (1988) describes the following voltage-dependent ionic conductances: 1) a slow background gNa^+ (CA1, CA3) in the soma, 2) a fast activating and inactivating gNa^+, 3) high threshold gCa^{2+} (CA1, CA3) in the dendrites, although there has been one report of a somatic conductance (Johnston et al 1980), 4) low threshold gCa^{2+} (CA1) in the dendrites (Kay and Wong 1987), 5) transient gK^+ (CA1) (Storm 1987), 6) an M-current (possibly a resting gK^+), 7) various anomalous rectifying conductances (CA1) in the soma, 8) Ca^{2+}-activated gK^+

(CA1) at the soma (Alger 1984, Segal and Barker 1986, Lancaster and Adams 1986), and 9) a Ca^{2+}-dependent gCl^- (CA1, CA3, and DG) in both somal and dendritic regions (Segal et al 1987).

Functionally, it is likely that dendritic Ca^{2+} currents are responsible for dendritic amplification, while the slow Ca^{2+} and Na^+ currents are able to transform the post-synaptic potentials which can be integrated at the soma. Slow inward currents also sustain the neuronal bursting activity of pyramidal neurons (Konnerth et al 1986). The various potassium conductances (gK^+)(e.g.. Ca^{2+}-dependent gK^+) control both voltage and transmitter mediated actions.

Although neuronal membrane properties (e.g.. resistance and conductances) regulate hippocampal function, other mechanisms exist which can alter neuronal responsiveness. One such factor is the degree of electrotonic coupling (gap junctions) between neurons, which has been suggested to be about 5% of all the neurons in the CA3 region and up to 10% of the neuronal population in the CA1 region (Dudek et al 1986). The significance of these findings may be important in pathophysiological states such as epilepsy (see Chapter 4).

GABA mediated inhibition in the CA3 region of the hippocampus is similar to that of the CA1 region although some striking differences are also present. Application of selective GABA blockers (picrotoxin, etc) elicit spontaneous synchronous burst activity in the CA3 region. Although similar activity can be seen in the CA1 it appears that the CA3 "drives" the CA1 region (Schwartzkroin and Prince 1977). Hippocampal inhibition due to GABA has very different effects in different regions. The CA3 region has a hyperpolarizing inhibitory post-synaptic potential (IPSP), while the CA1 and the DG have a depolarizing IPSP (but hyperpolarizing at the soma) that may be due to differences in the Cl^- transport systems for the two regions (Misgeld et al 1986). Such a differential could further modulate pyramidal cell responses.

Cholinergic excitation of hippocampal neurons results in a slow EPSP and a overall reduction of the afterhyperpolarization (AHP) that can last for several minutes and is found in all regions (CA1,CA3 and DG). Differences between regions include local excitatory circuits (CA3 has a more dense excitatory circuit than CA1) that are sensitive to glutamate and may play an important role in modulation of epileptiform activity (Christian and Dudek 1988a, b).

2. Granule Cells

Stimulation of the perforant path to the granule cells at a sufficient intensity leads to an extracellular evoked population spike similar to that seen in the hippocampus proper. Intracellular recordings are more difficult on the smaller granule cells but results suggest that similar conductance changes also exist in these neurons (see Durand et al 1983).

Granule cells in response to GABA blockers do not display epileptiform activity, suggesting that these neurons, unlike CA3 and CA1 pyramidal neurons, do not actively participate in epileptiform activity (Misgeld et al 1982). Another significant variation is the large degree of electrotonic coupling seen in DG granule cells (MacVicar and Dudek 1982). Differences in Cl^- conductances have also been noted in GABA mediated inhibition between the CA3 and the DG (Misgeld et al 1986).

3. Interneurons

The non-pyramidal neurons or interneurons represent, in general, GABAergic neurons. These cells are difficult to characterize on the basis of their locale, because unlike pyramidal cells they are not isolated in distinct lamina. Recent studies suggest that these non-pyramidal neurons have sharp short-lasting action potentials, and that the fast repolarizations of these neurons by outward currents enable them to fire at high frequencies (Kawaguchi and Hama 1987). Another distinct feature is their outward rectification (a decrease in input resistance with depolarization) which contrasts with the inward rectification seen in pyramidal and granule cells.

II. NEURONAL CALCIUM HOMEOSTASIS

The extensive use of the hippocampus in brain research has demonstrated that neuronal elements regulate ionic changes very closely. Calcium (Ca^{2+}) is implicated in numerous neuronal functions in both normal and pathophysiological states.

A. Role of Calcium in Biological Functions

Sidney Ringer first reported Ca^{2+} effects on biological tissue in 1883, when he accidentally discovered the importance of Ca^{2+} ions in contractions of cardiac tissue. This pioneering work was furthered by Locke (1894) who demonstrated the importance of extracellular Ca^{2+} in nerve-muscle and nerve-nerve neurotransmission. Others such as Loeb (1906), Mines (1910, 1913), and Loewi (1917, 1918) were able to demonstrate the integral involvement of calcium in the morphology and physiology of cells. Further work by Heilbrun and co-workers (1937) confirmed the importance of intracellular Ca^{2+}, and were able to show that the Ca^{2+} following various stimuli could come from both external and internal pools of bound Ca^{2+}. In 1947, Heilbrun demonstrated contractile responsiveness to Ca^{2+} when he injected Ca^{2+} intracellularly into cardiac cells.

Since these early investigations, evidence has accumulated that Ca^{2+} ions are involved in a variety of cellular functions. Four major roles for Ca^{2+} ions in biological function are: a) structural (skeletal structures, structural maintenance of eukaryotic cells), b) electrical (important charge carrier), c) cofactor for extracellular enzymes and proteins (proteases for blood clotting, DNAase I, etc), and, d) intracellular regulator (cell movement, secretion, cell division, membrane permeability, etc).

Numerous other functions of Ca^{2+} in biological function have been demonstrated which are beyond the scope of this thesis and the reader is referred to Campbell (1983) and Rubin et al (1985) for excellent treatises (see also Rasmussen and Goodman 1977, Rasmussen and Barrett 1984).

B. Calcium as a Second Messenger

The direct actions of Ca^{2+} itself can regulate some neuronal functions, however numerous regulatory processes are Ca^{2+}-activated (i.e.. second messengers). An example of such a second messenger is calmodulin.

In its role as a second messenger, calmodulin, interacts with a large number of cellular enzymes and modulates their activities (see Stoclet et al 1987 for review). Examples include adenylate cyclase, cAMP activation, and a Ca^{2+}-pumping ATPase at the plasma membrane (Klee and Vanaman 1982). It has been estimated that the cytoplasmic concentration of calmodulin in neurons is in the order of $10u$M, the equivalent of approximately $40u$M of Ca^{2+} binding sites (Vincenzi and Hinds 1980). It's buffering capacity is considered to be low when compared to calcium buffering proteins, such as calbindin (Baimbridge and Miller 1982). Calmodulin has also been reported to be localized selectively in the axons of neurons (Carafoli 1987). Various pharmacological manipulations (e.g.. pimozide) that act by interfering with calmodulin reduce the degree of long term potentiation (LTP) seen (Mody et al 1984, Bliss and Lynch 1988), suggesting that Ca^{2+} is an important intracellular modulator.

Another interesting calcium binding protein (CaBP) is calcineurin, which has been found to be involved in the inactivation of Ca^{2+} channels following an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Standen 1981, Eckert and Chad 1986). This system possibly limits the amount of Ca^{2+} that enters the cell during periods of high activity, and thus plays a crucial role in regulation of $[Ca^{2+}]_i$.

C. Neuronal Calcium Homeostasis

Calcium ions have been implicated in nerve membrane excitability, regulation of specific membrane conductances, excitation-secretion coupling, axonal transport and interactions with cyclic nucleotides (Erulkar and Fine, 1979). Considering its many roles in neuronal function, it is important to understand the mechanisms responsible for regulation of intracellular Ca^{2+} levels.

Cellular Ca^{2+} homeostasis is complicated by the large transmembrane gradient of Ca^{2+} concentration. The internal free Ca^{2+} concentration is 0.1-$1.0u$M while extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) ranges from 1.5-2.0mM, a difference of 3-4 orders of magnitude. It is this broad range of concentrations that allows Ca^{2+} to act as a sensitive activator of cellular function. Only a small increase in Ca^{2+}-conductance would lead to a relatively large increase in free $[Ca^{2+}]_i$, thereby activating a host of second messenger dependent reactions.

Estimates of the distribution of Ca^{2+} within a cell vary dramatically and the following values are meant to be representative of a "typical" neuron: nucleus (50%), endoplasmic reticulum (14%), mitochondria (30%), cell membranes (5%), and free intracellular Ca^{2+} (0.005%-0.5%) (based on a total intracellular Ca^{2+} of 2mM). As these values indicate, intraneuronal compartments have the potential to store and release significant quantities of Ca^{2+}.

1. Calcium Entry

There are two major pathways by which Ca^{2+} can gain access to a neuron's intracellular milieu from the extracellular environment. These are calcium channels which open in response to a specific stimulus (ligand, voltage) and Ca^{2+} exchangers that replace Ca^{2+} with another ion (e.g.; Na^+). In addition to extracellular Ca^{2+} entry some intracellular organelles are endowed with Ca^{2+} buffering capacities and can also release Ca^{2+} into the intracellular space thereby increasing its concentrations.

a) Types of Calcium Channels

(1) Ligand Activated Calcium Channels

One entry route for Ca^{2+} is through ligand or neurotransmitter activated channels. The involvement of calcium in neurotransmitter release has been well established (Augustine et al 1987, Smith and Augustine 1988); however, the mechanism by which neuromodulators and hormones act is less clear.

It is uncertain at this time whether these compounds act on a separate class of Ca^{2+} channels or whether they act on specific receptor coupled Ca^{2+} channels. Some types of receptor coupled events may release second messengers and induce release of Ca^{2+} from intracellular stores (see below)(e.g.. inositol phosphate pathway, Taylor 1987, Nahorski 1988).

More recently, N-methyl-D-aspartate (NMDA) receptors found on central nervous system (CNS) neurons have been shown to modulate Ca^{2+} influx. Mayer and Westbrook (1987a) found that activation of the NMDA receptor subtype of excitatory amino acids leads to a transmembrane

Ca^{2+} flux, allowing relatively large amounts of Ca^{2+} to enter the intracellular space (MacDermott et al 1986, Murphy et al 1987). This receptor coupled channel is not specific for Ca^{2+} and also allows other ions (e.g.. Na^+) to flow into the cell. Application of NMDA results in changes in the extracellular $[Ca^{2+}]$ (Lambert and Heinemann 1986). Interestingly, this ionophore is gated by Mg^{2+} ions in a potential dependent manner (Mayer and Westbrook 1987a,b). The involvement of the NMDA receptor has been implicated in long-term potentiation (Collingridge et al 1983; see below), excitotoxicity (Choi et al 1988) and the generation of epileptiform activity (Peet et al 1986, 1987; see below).

(2) Voltage Activated Calcium Channels

The existence of a voltage activated channel involved in action potential generation other than the classical Na^+ channel, was first reported by Fatt and Katz (1953). They were able to demonstrate the existence of a voltage-dependent Ca^{2+} channel in crab muscle. In 1958, Fatt and Ginsborg described the "calcium spike", which based an action potential on the influx of Ca^{2+} rather than Na^+ ions.

The importance of this type of channel in Ca^{2+} influx into the cytoplasm became apparent rather quickly and has been found in almost every type of excitable cell (for review see Reuter 1983, Tsien 1983a).

In recent years much work on various Ca^{2+} channels in neuronal systems has resulted in the classification of three major types of Ca^{2+} channels; designated as T, N, and L types (McCleskey et al 1986, Miller 1987).

The T or transient type channel has a small single-channel conductance (~ 8 pS) and is characterized by a late opening burst followed by rapid inactivation (tau=20-50ms). Both Ba^{2+} and Ca^{2+} pass equally well through it. Omega-conotoxin (w-CGTX) is a weak and reversible blocker of this channel (Fox et al 1987a,b). Transient channels appear to play a role in the production and modulation of rhythmic bursting behavior in neurons (Llinas and Yarom 1981).

The N type of Ca^{2+} channel has an intermediate sized single-channel conductance (~ 13pS) and its kinetics involve a long burst opening followed by moderate inactivation rates

(tau=50-80ms) although rates vary for different cell types (tau=500ms in rat sympathetic neurons) (Hirning et al 1988). Ba^{2+} passes much more easily through this channel than Ca^{2+}. The Ca^{2+} channel blocker, w-CGTX, elicits a persistent blockade. Miller (1987) postulates that the N channel is responsible for neurotransmitter release from pre-synaptic terminals.

The L type of Ca^{2+} channel is characterized by a large single channel conductance ($\sim$25pS) and has very little, if any, inactivation (tau>500ms). Like the N type channel, Ba^{2+} is a better charge carrier, but its most important distinguishing feature is high sensitivity to the dihydropyridine class of Ca^{2+} channel blockers (e.g.. nifedipine). The role of this channel in neuronal signalling is speculative: it may assist in replenishing intracellular stores such as in the sarcoplasmic reticulum (Chapman and Niedergerke 1970) or play a role in neurosecretory functions (McCleskey et al 1986). The L channel displays plasticity and may function to regulate nerve excitability in response to these changes (Miller 1987).

(3) Second Messenger Activated Calcium Channels

Various ligands can bind to a receptor and initiate second messenger actions, which in turn can cause a release of Ca^{2+} from intracellular stores. These ligand initiated actions can result in activation of phosphatidylinositol and enzymes such as adenylate cyclase (see Rasmussen et al 1986, Berridge and Irvine 1984, Nahorski 1988).

The inositol phosphates, a newly discovered second messenger system, appears to be important in controlling cellular functions, including Ca^{2+} release from intracellular organelles (see Nahorski 1988 for a complete review). Upon ligand binding with the receptor, a G protein is activated which leads to the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) by a phosphodiesterase. Breakdown of PIP_2 results in two products: 1) diacylglycerol (DAG) and 2) inositol trisphosphate (IP_3). Stimulation of the DAG pathway activates the enzyme protein kinase C (PKC) at the intracellular surface of the membrane in a process linked with ion channel phosphorylation. Inositol trisphosphate (IP_3) can diffuse within the cytoplasm where it can activate Ca^{2+} channels located on the intracellular storage organelles such as the endoplasmic

reticulum. Upon activation, Ca^{2+} is released into the intracellular space which in turn activates Ca^{2+} dependent events.

Intracellular Ca^{2+} can be released from various sources, including mitochondria, endoplasmic reticulum (Blaustein 1988), and calcium-binding proteins (Michiel and Wang 1986). Together these two separate systems (DAG and IP_3), interact to produce an integrated cellular response (Rasmussen et al 1987).

b) Other Mechanisms of Calcium Entry

There are several other mechanisms by which Ca^{2+} can enter the cell. Most of these have been poorly characterized to date, but include a Na^+-Ca^{2+} exchanger (Baker and Allen 1986), and intracellular Ca^{2+} changes brought about by gap junction modulation (Campbell 1983). The exact significance of these mechanisms of entry and their effects on intracellular Ca^{2+} levels has yet to be evaluated.

2. Calcium Buffering Mechanisms

There are five primary mechanisms of buffering intracellular Ca^{2+}. They are: a) mitochondrial uptake, b) uptake by the endoplasmic reticulum (ER), c) specific calcium binding proteins, d) Ca^{2+}-ATPase transport across the plasma membrane, and e) Na^+/Ca^{2+} exchange. Although all these buffering components have been shown to be active in neurons, their relative contributions to the maintenance of $[Ca2^+]_i$ levels is unclear.

a) Mitochondria

The ubiquitous presence of mitochondria in all tissues of the body has led investigators to determine if these energy-producing organelles have a role in intracellular Ca^{2+} regulation. Using squid axoplasm, Brinley et al (1978) demonstrated the ability of mitochondria in this preparation to buffer and stabilize extramitochondrial Ca^{2+}. The setpoint of this buffering system was regulated by pH, $[Mg^{2+}]$, and the total intracellular Ca^{2+} content.

Evidence has since accumulated suggesting that mitochondria may play an active role in regulating cytosolic Ca^{2+}, presumably via uptake and efflux mechanisms. Uptake of Ca^{2+} into the mitochondria is predominantly via a Ca^{2+} uniporter (Carafoli 1987) driven by a large mitochondrial membrane potential gradient, although evidence for other systems exists (Na^+-Ca^{2+} carrier, Compton et al 1986). Mitochondria also possess the capacity to release Ca^{2+} into the intracellular space, and Compton et al (1986) have presented evidence for the existence of Na^+-dependent and Na^+-independent Ca^{2+} exchange. There is considerable disagreement on the relative importance of these influx/efflux pathways in the regulation of $[Ca^{2+}]_i$ in neurons (see for example Rasmussen and Barrett 1984), but a mitochondrial role in regulation of $[Ca^{2+}]_i$ is "important in the long term regulation of internal Ca^{2+} and in protecting the intracellular environment from exposure to high concentrations of ionized calcium" (Erulkar and Fine 1979, Griffiths et al 1983, 1984).

b) Endoplasmic Reticulum

Since the role of the mitochondria in regulation of $[Ca^{2+}]_i$ has been questioned, the only other intracellular organelle capable of storing and regulating $[Ca^{2+}]_i$ in significant amounts is the endoplasmic reticulum. The importance of intracellular reticula in Ca^{2+} uptake and release has been known for some time: the sarcoplasmic reticulum of both skeletal and cardiac muscle has been thoroughly characterized and it is now apparent that this organelle plays an important role in physiological functions of muscle (Endo 1977, Martonosi 1984). The role of the endoplasmic reticulum in neurons is less well documented, although comparisons between the sarcoplasmic reticulum and the endoplasmic reticulum are inevitable.

The endoplasmic reticulum regulates $[Ca^{2+}]_i$ actively by both uptake and release of Ca^{2+}. The primary regulating mechanism for Ca^{2+} is a Ca^{2+}-dependent ATPase in the endoplasmic reticulum membrane, whose function is to sequester Ca^{2+} into the endoplasmic reticulum in an ATP dependent manner. This pump can be reversed, however, resulting in the synthesis of ATP (Endo 1977). The stoichiometry of the pump is 2 Ca^{2+} for every ATP.

Reversal can occur only if $[Ca^{2+}]_i$ outside of the endoplasmic reticulum falls in the presence of Mg^{2+}.

The endoplasmic reticulum in cerebellar cortical neurons, in response to a prolonged depolarization showed a five fold increase in Ca^{2+} content (Andrews et al 1988). Other neuronal cells such as the *Limulus* photoreceptor cell possess the ability to regulate $[Ca^{2+}]_i$ levels by changes in endoplasmic reticulum Ca^{2+} content (Meldolesi et al 1988). Further evidence for regulation of Ca^{2+} by the endoplasmic reticulum derives from the discovery of a high capacity, moderate affinity Ca^{2+} binding protein named calsequestrin (Meldolesi et al 1988) located within the cisterna of the ER. Other Ca^{2+} binding proteins, such as calmodulin potentially play an indirect role in regulating the Ca^{2+}-ATPase pump of the endoplasmic reticulum (Gupta et al 1988).

The endoplasmic reticulum, in addition to its Ca^{2+} uptake mechanisms, also allows the release of Ca^{2+}. This capacity could have important regulatory effects on second messenger mediated events, and could allow for amplification and integration of various Ca^{2+} signals (Rasmussen and Barrett 1984, McBurney and Neering 1987). One reason for the upsurge of interest in Ca^{2+} regulation by endoplasmic reticulum is the discovery of IP_3 sensitive sites located on the endoplasmic reticulum membrane (McBurney and Neering 1987).

A number of mechanisms allow for release of Ca^{2+} in neuronal endoplasmic reticulum and muscle SR. These are 1) reversal of the Ca^{2+}-ATPase pump (Endo 1977), 2) Ca^{2+} induced Ca^{2+} release (Endo 1977, Martonosi 1984, Fabiato and Fabiato 1975, 1977) and 3) release of $[Ca^{2+}]_i$ by the intracellular messenger IP_3 (McBurney and Neering 1987, Nahorski 1988, Taylor 1987). The above systems are very different; for example, increased $[Ca^{2+}]_i$ is thought to release Ca^{2+} from the endoplasmic reticulum by Ca^{2+} induced Ca^{2+} release and reduced Ca^{2+} pumping, while high $[Ca^{2+}]_i$ concentrations inhibit IP_3 induced release.

c) Calcium Binding Proteins (CaBP)

A large number of CaBPs has been discovered in various tissues the most studied of which are calbindin, parvalbumin, and calmodulin (McBurney and Neering 1987, Heizmann and Berchtold 1987).

The role of calbindin in Ca^{2+} regulation of CNS neurons is interesting because it appears to have a very selective distribution. Some CA1 hippocampal pyramidal neurons and the dentate granule cells have dense immunostaining for calbindin. Cerebellar Purkinje cells also contain this protein and the total calcium binding capacity of calbindin in these cells is estimated to be in the order of $600uM$ (Baimbridge and Miller 1982).

Parvalbumin (PV), first discovered in muscle tissue, has now been localized in the CNS. Interestingly, PV-immunoreactivity in the CNS is almost exclusively localized to interneurons. PV has been found to be co-localized with the inhibitory neurotransmitter GABA, but in the hippocampus and the dentate gyrus only 20-30% of GABAergic neurons contain PV (Heizmann and Berchtold 1987).

The importance of CaBPs is underscored by the finding that changes in calbindin levels occur in cells in pathological epilepsy states (Baimbridge and Miller 1984, Baimbridge et al 1985). Changes in these binding proteins have also been noted in connection with aging (Gibson and Peterson 1987).

d) Calcium ATPase Mediated Extrusion

Calcium also can be removed from the cytosol by an ATP fueled pump located in the plasma membrane. The existence of such a pump was first demonstrated by Schatzmann (1966) in red blood cells. This Ca^{2+} dependent pump has now been described in every type of cell (plant, bacteria, etc), however the relative contributions to Ca^{2+} extrusion by either the Ca^{2+} pump or the exchanger is still unknown (Schatzmann 1986).

Although the early studies suggested that the pump exchanges 2 Ca^{2+} for every 1 ATP, the current consensus is that the exchange is one to one. Also in dispute is whether there is an

absolute requirement for the presence of Mg^{2+}, Ca^{2+} or even free ATP to activate the enzymatic site of the pump (Schatzmann 1986).

Gopinath and Vincenzi (1977) demonstrated that calcium bound calmodulin activates the plasma membrane pump and therefore leads to an enhanced rate of extrusion based on cellular needs.

The stimulation by calmodulin increases the Ca^{2+} affinity of the enzyme and its maximal transport rate. There also exists a calmodulin insensitive ATP driven Ca^{2+} pump in the membranes of the endoplasmic reticulum that has properties different from those of the plasma membrane pump (see section above). It is probable that the Ca^{2+}-activated ATPase pump acts together with the Na^{+}-Ca^{2+} exchanger in regulating $[Ca^{2+}]_i$ (Carafoli 1987).

e) Sodium-Calcium Exchange

A major transport system in neurons is an extracellular Na^{+}-dependent Ca^{2+} exchange (Na^{+}-Ca^{2+} exchange) system, which results in a net movement of Ca^{2+} across the plasma membrane against a concentration gradient (3 Na^{+} in, 1 Ca^{2+} out). The ability of the exchanger to move $[Ca^{2+}]_i$ is not dependent upon ATP levels; however, it ultimately requires ATP hydrolysis to restore the normal Na^{+} gradient (Baker and Allen 1986). The exchanger is a low affinity, high capacity system that is ideally suited to regulating $[Ca^{2+}]_i$ (McBurney and Neering 1987). Under physiological conditions, based on its ability to rapidly clear $[Ca^{2+}]_i$, DiPolo and Beauge (1983) suggested that the exchanger may be important in regulation of $[Ca^{2+}]_i$ levels after stimulation by trains of action potentials. Nonetheless, due to the lack of specific inhibitors of the exchanger, its physiological importance has yet to be determined in neurons.

E. Role of Calcium in Pathophysiology

Calcium has been implicated in aging (Gibson and Peterson 1987), and numerous pathophysiological conditions such as ischemia (Siesjo 1986, Choi 1988), hypoxia (Peters 1986), and epileptiform activity (Delgado-Escueta 1986); the last being one of the most pressing and complex pathological situations where $[Ca^{2+}]_i$ accumulation can lead to neuronal death.

Difficulties quantifying Ca^{2+} changes in epilepsy are inherent in the numerous types of human epilepsy and the experimental animal models that have been developed. Whether an imbalance in the regulation of Ca^{2+} is a causative factor or just a correlate further complicates the problem. Briefly outlined below are some animal models of epilepsy in which Ca^{2+} may play an important role either prior to, during, or after epileptiform discharges.

Various focal epilepsy models (alumina cream, cobalt, and penicillin) all show reductions in $[Ca^{2+}]_o$ and increases in $[K^+]_o$ during the epileptiform activity, in the cell body layer of the hippocampus (Heinemann et al 1986, Lux et al 1986, Somjen 1980, Lux 1974). The kindling model of epilepsy has shown altered Ca^{2+} laminar profiles following stimulation or excitatory amino acid application, particularly in the stratum radiatum (SR) and stratum molecular (SM) of the CA1 region of the hippocampus (Heinemann et al 1986). Comparing normal to epileptic tissue, the above authors suggested that changes in $[K^+]_o$ regulation were not altered, however there was an increased capacity for Ca^{2+} uptake in the apical dendrites of hippocampal pyramidal cells.

Changes in the pyramidal cell membrane properties have been demonstrated following the bath application of kainic acid (Heinemann et al 1986). They were able to show that there was a loss of the calcium-induced K^+-mediated afterhyperpolarization (Ca^{2+}-gK^+ AHP) in CA1 pyramidal neurons.

Alterations in calcium binding proteins such as calbindin and calmodulin have been demonstrated in various models of epilepsy (Baimbridge et al 1985, DeLorenzo 1986). Further evidence has shown that several clinically used anticonvulsants such as phenytoin, diazepam and carbamazepine have been shown to act in part by interfering with Ca^{2+}-binding protein mediated interactions (DeLorenzo 1986, 1988). Other calcium dependent enzyme systems (e.g.. protein kinase C) have been implicated in regulation of specific ionic conductances that utilize Ca^{2+} (DeLorenzo 1988).

A review by Siesjo and Wieloch (1986) compares three different pathological states; ischemia, hypoglycemia and epileptic activity. Although these pathological states have different causes and perturbations, they nonetheless share some common properties. Changes in ion fluxes

as noted above also occur (K^+ out and Ca^{2+} in) and it has been suggested that the Ca^{2+} entry is through post-synaptic glutamate receptor activated channels (Siejo and Wieloch 1986). After the initial Ca^{2+} influx, these authors noted a prolonged period during which there is an enhanced permeability to ions following seizure activity (Delgado-Escueta and Horan 1983). These authors suggested that although electron transport and ATP production are maintained, free fatty acid production and Na^+ influx lead to moderate intracellular acidosis which could then lead to impaired $[Ca^{2+}]_i$ regulation (Siesjo and Wieloch 1986).

Studies of intracellular pH changes indicate a drop of 0.36 units after 5-20 min of seizure activity (Siesjo et al 1984). This acidosis has been reported to inhibit Ca^{2+} uptake by mitochondria, suggesting that low intracellular pH could compromise Ca^{2+} regulation by intracellular organelles (Borle 1981).

Other Ca^{2+} dependent events occur and can modulate intracellular and membrane components. Increased $[Ca^{2+}]_i$ can lead to increased lipolysis, increased protein phosphorylation, increased proteolysis of membrane proteins and inhibition of axonal transport. These Ca^{2+} modulated events can all occur during epileptiform activity and can lead to membrane damage (increased Ca^{2+} fluxes), receptor dysfunction and overall cellular catabolism with eventual cytoclasis (Siesjo and Wieloch 1986).

Although somewhat non-physiological, a novel model was described in 1982, in which the removal of extracellular Ca^{2+} could lead to spontaneous rhythmic bursting activity of CA1 pyramidal neurons (Jefferys and Haas 1982, Taylor and Dudek 1982). This interesting phenomenon has been studied extensively and it is now thought that a number of factors such as changes in K^+ and Ca^{2+} fluxes, membrane destabilization and changes in the cellular currents all contribute to this epileptiform model (see Chapter 4 for a more detailed description).

In summary, although variations exist between human and experimental epilepsies, an overwhelming amount of evidence suggests that Ca^{2+} enters the neuron during epileptogenic activity. At the present time it is uncertain whether changes in Ca^{2+} entry and intracellular regulation are causative factors for the onset of epileptiform activity or whether they are simply the result of abnormal discharges. Some studies suggest that aberrant changes in inhibition are

the key factor for the initiation of epilepsy and that changes in Ca^{2+} are only secondary and are involved in reinforcing epileptiform discharges (Dichter and Ayala 1987). Regardless of the initial source of $[Ca^{2+}]_i$, once it has entered the neuron it has wide ranging effects from modulation of ionic conductances to the phosphorylation of proteins important to cell function.

III. THE PRESENT STUDY

Regulation of intracellular Ca^{2+} levels is of crucial importance to neuronal function and homeostasis. Perturbations in these mechanisms can result in numerous pathophysiological conditions. The importance of intracellular calcium in normal synaptic transmission has been known for some time, but increasing emphasis is now placed on the role of intracellular organelles, such as the endoplasmic reticulum, in modulating intracellular events. A number of neurotransmitter/neuromodulatory substances such as serotonin and adenosine may modulate intracellular Ca^{2+} levels (directly or indirectly). The experiments recorded here describe the changes seen during evoked synaptic activity, spontaneous epileptiform bursting, and in the LTP model of memory.

The present study investigates: i) the role of serotonergic and purinergic systems in normal evoked activity and in modulating low Ca^{2+}-induced epileptiform activity, ii) the changes in neuronal $[Ca^{2+}]_i$ in normal, high and low Ca^{2+} states, and iii) the importance and source of Ca^{2+}_i stores in long-term potentiation.

The rat *in vitro* hippocampal slice was used in these experiments due to its: i) relative ease of dissection, ii) laminar structure, iii) well defined pathways, iv) importance in learning and memory, and v) propensity to epileptiform activity.

The experiments presented in Chapter 3, were undertaken in an attempt to define the actions of two classes of neurotransmitter/neuromodulatory compounds, serotonergic and purinergic actions in the hippocampus. These studies were required prior to investigation of these compounds in a model of epilepsy.

In Chapter 4, serotonergic and purinergic modulation of the low Ca^{2+}-induced bursting model of epilepsy was assessed using various agonists and antagonists. This model is of

considerable interest as it underscores the likelihood that non-synaptic mechanisms in modulate epileptiform activity.

A model for memory, LTP, is investigated in Chapter 5. The known involvement of Ca^{2+} with the NMDA receptor in this model led to the investigation of the role of intracellular Ca^{2+} in the induction and maintenance of LTP. A revised model emerges placing a major emphasis on the role of intraneuronal release of Ca^{2+} in the induction of LTP.

Chapter 6 extends the findings described by the electrophysiological investigations by directly examining changes in Ca^{2+}_i concentrations. These experiments used the Fura-2 microspectrofluorimetric technique of intracellular Ca^{2+} determination in cultured hippocampal neurons, a procedure which has uncovered provided evidence of the importance of $[Ca^{2+}]_i$ in the regulation of learning and memory.

CHAPTER 2 - GENERAL METHODS

I. THE HIPPOCAMPAL SLICE PREPARATION

The *in vitro* slice chamber used in the following was designed to allow control of the environment including control of the perfusate. This chamber allows slices to remain viable for up to 8-10 hours (Schurr et al 1984) in an atmosphere of humidified air, and superfused in a solution of artificial cerebrospinal-spinal fluid (ACSF) at a temperature of $34^{\circ}C$..

The chamber is plexiglass and has two components, an outer circular water jacket and an inner circular recording well. The two are connected via six ports allowing for atmospheric exchange. The outer water jacket is partially filled with distilled water and is warmed by a heating jacket. In addition, a gas dispersion ring is located inside the circular water jacket where a mixture of 95% O_2 and 5% CO_2 is bubbled through the water. This combination of warmed water, saturated with oxygen, provides a warm moist atmosphere over the slices in the recording well.

The inner chamber consists of either a single well or double well arrangement (approx. volume 5ml). These wells are fitted with a nylon netting on which the slices are placed. Thermistors in the inner well and outer chamber maintain the temperature to the selected range ($34^{\circ}C$ +/- $1^{\circ}C$), activating a DC temperature control unit. The inner chamber also contains, under the netting, an inlet channel and an outlet channel allowing flow of the ACSF. The level of the ACSF superfusing the slices can then be controlled by adjusting the suction level at the outlet channel.

The ACSF used in these experiments is a modified Ringers solution that duplicates the ionic components of the mammalian CSF. It contains the following (in millimolar): 124 NaCl, 2 KCl, 1.25 $KHPO_4$, 1.5 $CaCl_2$, 1.5 $MgSO_4$, 24 $NaHCO_3$, and 10 D-glucose (Sigma and BDH). When equilibrated with a gas mixture of 95% oxygen-5% carbon dioxide, the ACSF has a pH of approximately 7.4. The ACSF is gassed and warmed on a hot plate elevated above the chamber and is gravity fed through plastic tubing to a flow rate controller (Dial-A-Flo, Sorensen Research

Co.). After adjusting the flow rate to 2-3 ml/min, the ACSF passes through the outer water chamber to facilitate warming and into to the inner chamber. The level of perfusate is controlled by a suction device located at the end of the outlet channel. Medium containing pharmacological agents or of altered ionic constituency can be introduced through a three-way valve without interrupting the flow.

Once slices are placed on the nylon netting within the recording chamber, they can be illuminated from below via reflected light. Visual inspection of the slice is made with the aid of a low magnification dissecting microscope mounted over the recording chamber.

A. Slice Dissection

The experiments were performed on male Wistar rats weighing between 175-275 grams (Charles River, Montreal). Rats were anesthetized in a glass chamber containing diethyl ether. After loss of consciousness the rats were decapitated and the skin and overlying connective tissue were removed from the skull with a scalpel. The cranial bones were removed with small rongeurs and the dura mater was cut with fine scissors and then removed with forceps. Cooled, (0-4 °C) oxygenated ACSF, was then poured over the exposed brain to prevent anoxic damage. A transverse scalpel cut was made across the anterior forebrain and, with the aid of the scalpel handle, the brain was removed and placed in a small beaker of cold ACSF. The brain was then placed on a petri dish covered with an ACSF soaked filter paper and the cerebellum was removed. The right and left hemispheres were separated by a mid-sagittal cut and the right hemisphere discarded. The left hemisphere was then tipped up onto the coronal surface of the frontal cortex and the midbrain was gently separated from the cortical tissue with a blunt spatula. This procedure left the ventro-medial surface of the hippocampus exposed so the spatula could be placed beneath the curvature of the fimbria and fornix to carefully roll the hippocampus away from the subicular cortex (see Chapter 1, Figure 1.1). The remaining cortex was then gently cut away.

During the entire dissection procedure, the brain and the hippocampus were occasionally "washed" with the cold ACSF to remove any debris and to keep the tissue cold.

The isolated hippocampus was then placed on the stage of a McIlwain tissue chopper. The axis of the hippocampus was adjusted so that the longitudinal axis was perpendicular to the angle of the cutting blade. This particular arrangement results in transverse hippocampal slices with their intrinsic circuitry intact (Skrede and Westgaard 1971). The slices (6-10) were cut to a thickness of 400uM. Each slice was lifted from the blade with a fine sable brush and placed in a small petri dish containing cold ACSF. The slices were then transferred to the nylon netting in the recording chamber or placed in an incubation system for use at a later time.

The incubation system consisted of a large beaker, containing oxygenated ACSF sitting in a large heated water bath. The temperature was adjusted to that of the recording chamber. The slices were then placed inside the beaker and were allowed to rest, submerged, on a fine nylon sieve. This arrangement allowed use of slices from the same animal for different ionic and pharmacological manipulations.

In either case, the slices were allowed to equilibrate and recover for a minimum of 45 minutes before the placement of the recording and stimulating electrodes. Reid (1987) showed that 45 min to 1 hour after dissection, stable electrophysiological responses return in the hippocampal slice .

The average dissection time of the hippocampus was 7 minutes. However, more critical than the speed of dissection to the physiological integrity of the slice is care taken during the procedure.

B. Stimulating and Recording Techniques

The bipolar stimulating electrodes were constructed from twisted 62um nichrome wire, the end of each wire cut so as to expose an insulation-free tip. The electrodes were positioned with a micro-manipulator to stimulate afferent fibers (see Chapter 1, Figure 1.1) (Figure 2.1). Square wave pulses of 0.1 ms duration and 0.1-80 volts intensity were delivered by stimulator isolation units (Medical Systems Corp., Model DS2). The delivery of the stimulation pulses was controlled by a four channel pulse generator (Digitimer, Model D4030, Medical Systems Corp.). The

stimulation frequencies during electrophysiological characterization of the slice were 1 every 10 sec for the CA1 region and 1 every 20 sec in the DG, but during the experimental period the pulses were delivered at either 1/min or 1/5min (see each chapter for exact details of stimulation and recording parameters). The rationale for the use of these long interpulse intervals was the prevention of any frequency dependent alterations in the characteristics of evoked potentials.

Extracellular recording electrodes were constructed using a Narishige (Model PE-2) microelectrode puller. The puller was adjusted to produce glass electrodes (Omega Dot tubing, 1.5mm OD, 0.75mm ID, Frederick Haer) with a resistance of 2-8 megohms. The electrodes were filled with 2M NaCl and were positioned under visual guidance either manually or with a Burleigh Inchworm (Model PZ-550) to an approximate depth of 80-150 um below the surface of the slice in the region of interest. The recording electrodes were connected to the headstage preamplifier and lead to an amplifier (WPI Model KS-700 or M707). The amplifier was referenced to the recording chamber by a silver-silver chloride wire in the bath. The evoked responses were filtered at 0.1-10kHz bandwidth and displayed on a dual beam storage oscilloscope (Tektronix), where further filtering and gain amplification could be performed if necessary. The extracellular electrical activity could be photographed on the oscilloscope or stored in a PDP 11/23 computer for later analysis.

Details of the placement of the extracellular recording and stimulating electrodes are provided at the beginning of each experimental chapter. In brief, in the CA1 region, the stimulating electrodes were placed in the dendritic stratum radiatum (SR) while the recording electrodes were placed either in the SR for extracellular population EPSP responses or in the stratum pyramidale (SP) for somatic population responses (Figure 2.1). In the dentate gyrus the stimulating electrodes were placed in either the medial perforant path or in the lateral perforant path. The recording electrodes were either placed in the molecular layer (ML) for dendritic EPSP's or in the granule cell layer (GL) for cell body population responses.

Figure 2.1: Location of stimulating and recording electrodes with representative evoked responses.

A. Schematic of a transverse rat hippocampal slice with the principal cell layers (also see Figure 1.1). Recording and stimulating electrode placement is shown with representative extracellular evoked population responses.

B. Typical evoked population responses seen in the CA1 region of the hippocampus. The uppermost tracing is a population spike, whose amplitude was quantified as the difference in potential between the first peak positivity (P1) and the first peak negativity (N). The reversed field EPSP slopes in the CA1 pyramidal layer, a reflection of the population EPSP, was quantified using the maximal positive slope of the first peak positivity . The lower tracing is a field EPSP recorded from the stratum radiatum. Amplitude was measured from the baseline potential to the first maximum negativity, and the slope was defined as the maximal negative slope.
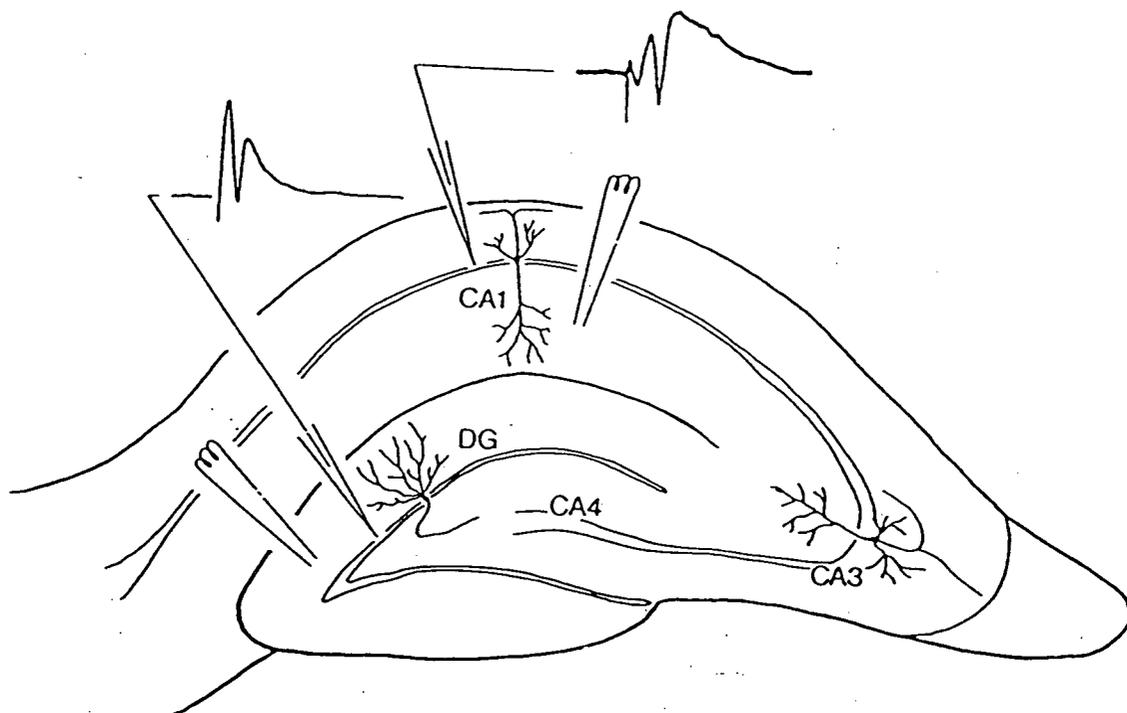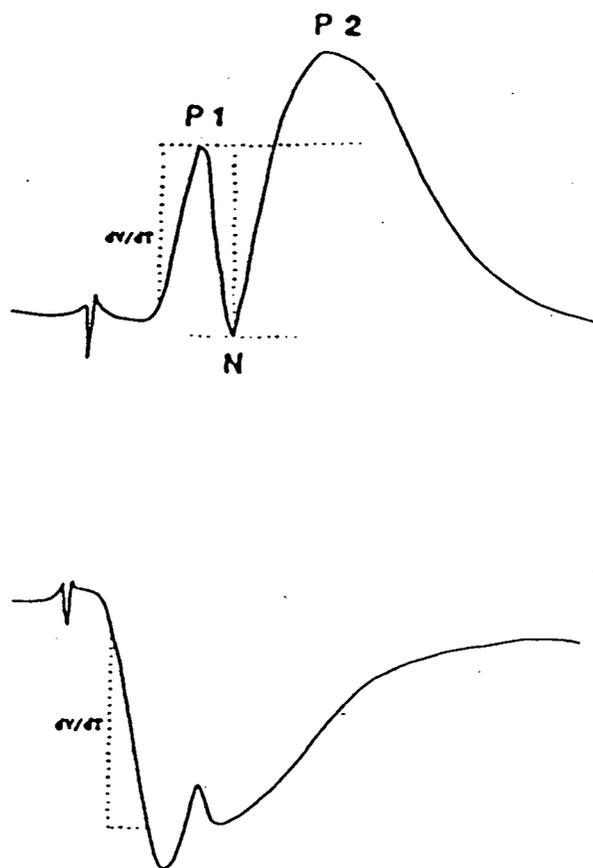
The amplitude of the population spike was measured as the difference in voltage between the peak of the first positive wave (P1) and the peak of the first negative deflection (N) (Figure 2.1). The reversed field EPSP slope was measured as the maximum slope (after differentiation) between baseline potential and the first peak positivity, as the presence of a population spike precluded measurement of the reversed field EPSP amplitude. The field EPSP amplitude of the stratum radiatum (SR) was measured from the baseline to the maximum negativity of the EPSP response. The slope of the SR EPSP was measured as the maximum slope between the baseline potential and the first peak negativity.

The evoked population spike response is the sum of the activity of neurons that are synchronously activated by the stimulation of afferent fiber pathways (Figure 2.1) (Anderson et al 1971). The first positive wave reflects the source current for dendritic excitatory post-synaptic potentials (EPSP), and is unchanged if the action potential is blocked. It is on this positive EPSP wave that the population spike is superimposed. The best measure of the number of action potentials evoked would be calculation of the area of the population spike which is superimposed on the reversed field EPSP. However, as the P2 wave is influenced by ionic fluxes secondary to action potential generation, such as repolarizing K^+ currents, the reversed field EPSP cannot be represented by the summation of the P1 and P2 waves. Therefore, measures of population spike amplitudes involving the P2 component may be less accurate.

C. Computer Facilities

The PDP 11/23 computer used to analyze changes in extracellular waveforms utilized a data collection and graphical analysis system developed by Dr. T. Richardson (Simon Fraser University). This program allows wave forms either to be displayed on a Tektronix 4010 high resolution graphics terminal for on line viewing or to be sent to a Tektronix 4052 plotter for the generation of permanent records. The program allows a

variety of manipulations including averaging, digital filtering, baseline correction, summation, subtraction, differentiation and integration. When set to the users' specifications the program automatically detects multiple maximum and minimum points within a waveform.

Numerical results are displayed on a standard terminal or sent to an IBM PC clone via a terminal emulator (ZSTEM, KEA Systems) in the form of ASCII files. Once stored in the PC, the files could are accessible for analysis in a spreadsheet program (1-2-3, Lotus Corp.). In all cases normal evoked, long-term potentiation and epileptiform data was loaded into 1-2-3 for ease of analysis. Graphical output from the spreadsheet was via Printgraph (Lotus Corp.) or Sigmaplot (Jandel Scientific, Inc.). Statistical analysis was done with either 1-2-3 or using Number Cruncher (Simon and Schuster).

II. LOW CALCIUM-INDUCED BURSTING

Slices were cut and placed in the recording chamber and allowed to equilibrate; then recording electrodes were placed as discussed above. The recordings were primarily made in the CA1 region as the DG did not exhibit regular bursting activity. Once the electrodes were placed and the evoked population responses were elicited from the region of interest, Ca^{2+}-free ACSF was perfused over the slice. In order to prevent the occasional appearance of very large, long lasting depolarizations (spreading depression) the $MgSO_4$ content was increased from 1.5mM to 1.7mM. Within 30-90 min low Ca^{2+} bursts appeared and could be maintained in most slices for the duration of the experiment, usually for 3 hours.

III. LONG-TERM POTENTIATION

Long term potentiation (LTP) can be induced by a variety of different stimuli and paradigms. The reader is referred to a recent review for more detail (Bliss and Lynch 1988).

A. Tetanus Induced LTP

Slices were cut, placed in the recording chamber and allowed to equilibrate. Stimulating and recording electrodes were placed and recordings were made from the CA1 region.

In each experiment, the maximum evoked response was measured and the stimulus intensity reduced until the population spike amplitude was 25% of the maximum response. Test pulses were delivered at a rate of one per 10-30 sec. Baseline stimulation was then delivered at one per 1 or 5 min, and values were taken for 5-10 min. If stable, a tetanic stimulation of 100 Hz for 1 sec was then given. This frequency of tetanic stimulation has been shown to yield reliable LTP in the *in vitro* hippocampal slice. The potentiated response was then followed for 30 min post-tetany. Recording and stimulating electrodes were then moved to another slice and attempts were made to induce LTP at various time intervals during pharmacological manipulations.

B. Calcium-Induced LTP

Another method for inducing LTP in the *in vitro* hippocampal slice is to perfuse a pulse of high Ca^{2+} for a short period (Ca^{2+}-induced LTP). Slices were prepared as discussed above and the electrodes placed in the CA1 region. Again the maximum response was elicited (see section A) and then the stimulation voltage reduced until the population spike was 25% of its maximum value. Several stimulation paradigms, designed to minimize post-synaptic Ca^{2+} entry were used (see Chapter 5 for details).

Once a stable baseline had been reached, a 10 min pulse of ACSF containing 4mM Ca^{2+}, 1mM Mg^+ and 6.25mM K^+ was perfused through the recording chamber containing the slices, producing a potentiation of the population spike responses for the duration of the experiment (60 min post high Ca^{2+}). For details of the various pharmacological manipulations see Chapter 5.

Statistical analyses were performed as indicated in each of the individual chapters. In general, two types of tests were run: i) analysis of variance (ANOVA), and ii) two-sample two-tailed T-tests. Statistical significance was ascribed to P values less than 0.05 ($p < 0.05$).

CHAPTER 3 - IN VITRO ELECTROPHYSIOLOGY OF SEROTONERGIC AND PURINERGIC SYSTEMS

I. INTRODUCTION

Several important neurotransmitter/neuromodulator substances, such as the serotonergic and purinergic compounds have the ability to exert powerful regulatory effects on brain activity. The rat hippocampus is extensively innervated by serotonin containing fibers of the raphe nuclei. Likewise the hippocampus contains large amounts of adenosine. The inhibitory actions of these compounds, albeit by different mechanisms, can have important effects on normal and pathophysiological brain activity.

The aim of this series of studies was to investigate the effects of serotonergic and purinergic agonists and antagonists on normal hippocampal electrophysiology. At the present time, although considerable information is known, neither of these classes of compounds is fully characterized. Using the *in vitro* rat hippocampal slice, experiments were conducted to confirm and amplify the findings in the literature. Extracellular evoked population responses (population spike amplitude, reversed field EPSP slopes) were used as indices of changes in hippocampal excitability.

II. SEROTONERGIC SYSTEMS

In recent years there has been a resurgence of interest in the role of serotonin (5-hydroxytryptamine, 5-HT) in central nervous system (CNS) function. The initial discovery of 5-HT in brain tissue was made in 1953 (Twarog and Page 1953) using a bioassay based on detection of its vasoconstrictive effects. Anatomical and biochemical studies were accelerated with the discovery by Falck et al in 1962 of a histochemical method for the visualization of biogenic amines (see Carlsson 1987 for a review). This method made possible visual detection by light microscopy of 5-HT containing neurons and axonal processes. At about the same time, further evidence concerning the role of 5-HT in the brain was put forth when it was noted that a number of ergot derivatives were found to act as antagonists of 5-HT, including the potent hallucinogen lysergic acid diethylamide (LSD) which is a structural analog of 5-HT (Wooley and Shaw 1954, for

a review see Jacobs and Trulson 1981). By the mid 1960's there was anatomical, biochemical and behavioral evidence that 5-HT played an important role in CNS function.

A. Serotonergic Projections

Using the histochemical method of Falck and Hillarp (1964), Dahlstrom and Fuxe (1964) described the existence of a group of 5-HT containing neurons in the raphe nuclei (for an excellent review of raphe nuclei cytoarchitecture see Petrovicky 1980, 1981). This study demonstrated that serotonergic neurons closely (but not exactly) matched the boundaries of existing raphe nuclei. Although they specifically designated nine groups of serotonergic cell bodies (B1-B9), this nomenclature can be simplified for the purpose of our discussion. The largest group of 5-HT containing neurons is the dorsal raphe nucleus (includes groups B6,7), while B8 and B9 groups correspond to the median raphe nucleus (also known as nucleus central superior). These two raphe nuclei, dorsal and median, are of particular significance as they provide the majority of ascending serotonergic projections to the forebrain.

The primary projection to the hippocampal formation is from the median raphe nucleus (MRN), via the median forebrain bundle where it branches off and the serotonergic fibers course in the fimbria-fornix (Parent et al 1981). This projection innervates the stratum radiatum areas of CA2, CA3, and CA4, with a slight innervation of the stratum oriens of the CA2 to CA4 regions (Azmitia and Segal 1978). Serotonergic fibers also course to the CA1 region of the hippocampus, entorhinal cortex and subiculum, via the cingulum bundle. This innervation of the CA1 region appears to terminate in the stratum radiatum region (Azmitia and Segal 1978), although Lidov et al (1980) suggests that the termination of these serotonergic fibers is the stratum molecular/lacunosum (SLM).

The fimbria fornix pathway also carries fibers from the median raphe nucleus to the dentate gyrus granule cells, the most heavily 5-HT innervated region of the hippocampal formation (Lidov et al 1980). The projection from the dorsal raphe nucleus (DRN) to the dentate gyrus courses via the cingulum bundle which enters the hippocampal formation via the perforant path and appears to terminate primarily in the molecular layer of the DG.

Recent studies have found that there are morphological differences between the axon terminals that arise from the DRN and the MRN (Molliver 1987, Kosofsky and Molliver 1987).

In summary, the serotonergic innervation of the hippocampus comes from two serotonin containing raphe nuclei. The primary fiber pathway is the median forebrain bundle which bifurcates and sends fibers through the fornix and the cingulum bundle. The DRN primarily innervates the DG, and the MRN innervates the CA1 to CA4 regions. The predominant location of synapsing fibers is the dendritic regions of the hippocampal formation (stratum radiatum, molecular layer).

B. Serotonergic Receptors

With the finding that 5-HT fibers project to virtually every part of the cortex, and in view of its possible involvement in mood disorders, it became important to identify the receptor(s) involved in 5-HT function. It had already been demonstrated in the periphery that two binding sites existed, designated M (antagonized by morphine) and D (antagonized by dibenzylline) (Gaddum and Paicarelli 1957). Peroutka and Synder (1979, 1981) on the basis of binding characteristics, were able differentiate two distinct 5-HT membrane recognition sites. Those sites that were labeled by $[^3H]$5-HT were designated 5-HT$_1$ receptors and those labeled by $[^3H]$spiperone were designated 5-HT$_2$ receptors.

It soon became apparent however that the binding of $[^3H]$5-HT was heterogeneous. Displacement of $[^3H]$5-HT by spiperone led to the suggestion that high affinity sites (130nM) for spiperone be designated as 5-HT$_{1A}$ sites, whereas low affinity (47uM) sites for spiperone be designated 5-HT$_{1B}$ (Pedigo et al 1981, Peroutka 1987, also see Leysen 1985 for a comprehensive review). The 5-HT$_2$ binding site is further differentiated by the potent binding of $[^3H]$spiperone in the 0.5nM range. These two receptors have different regional localizations and have been identified in numerous species (Schnellman et al 1984). A number of other types of receptors have been proposed, including a 5-HT$_{1C}$, a 5-HT$_{1D}$, and a 5-HT$_3$ receptor subtype. At the present time these receptor subtypes have not been found to play a role in hippocampal electrophysiology, and will not be discussed further (for more information see Peroutka 1987).

The discussion below will focus on those receptor types thought to be important in the rat hippocampal formation.

1. 5-HT$_1$

As discussed above, the 5-HT$_1$ receptors have been subdivided into two (5-HT$_{1A}$, 5-HT$_{1B}$) subtypes (Pedigo et al 1981). For an excellent discussion of these receptor subtypes and their functional correlates see Hoyer (1988). For a summary of the currently accepted classification systems for 5-HT receptors see Peroutka (1987), Bradley et al (1986) and Glennon (1987).

a) 5-HT$_{1A}$

This particular receptor subtype has been well studied due to the availability of selective agonists for its binding site. At the present time the agonist of choice is 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) (see Hoyer 1988, Gozlan et al 1983, Hamon et al 1984, Middlemiss and Fozard 1983). More recently, several other agonist compounds have shown selective binding to this receptor subtype and include; MDL 72832 (8-[4-(1,4-benzodioxan-2-ylmethylamino)butyl]-8-azaspiro[4,5]decane 7,9-dione (Mir and Fozard 1988), spiroxatrine (Nelson et al 1987), and a buspirone analog BMY7378 (Yocca et al 1987). Whether these agonists will be useful in elucidating the function and distribution of the 5-HT$_{1A}$ recognition site remains to be determined. One of the problems in investigating this receptor type is the lack of potent and selective antagonists.

The distribution of this receptor subtype has been well documented in rodent (Pazos and Palacios 1985) and human (Pazos et al 1987a,b) brains. The highest concentrations of the 5-HT$_{1A}$ receptor in the rodent is found in the dentate gyrus and septal nucleus, and high concentrations were also found in the CA1-4 regions of the hippocampus, amygdala, hypothalamic nuclei and the dorsal raphe (Kohler 1984, Marcinkiewicz et al 1984, Verge et al 1986). These distributions correlate well with human studies (Hoyer et al 1986, Pazos et al 1987a,b). Within the hippocampus, high densities have been found in the stratum radiatum region of the CA1

subfield, and in the stratum moleculare of the dentate gyrus. The majority of these binding sites correspond to post-synaptic receptors (Verge et al 1985, 1986, Hall et al 1985).

Activation of this receptor subtype is thought to mediate a number of distinct physiological processes (see Hoyer et al 1988). Peroutka et al (1981) suggested that the 5-HT_1 receptor was involved in the activation of adenylate cyclase by 5-HT agonists. Evidence from rat and guinea pig hippocampal membranes suggests that the 5-HT_{1A} receptor subtype plays a role in adenylate cyclase activation (Barbaccia et al 1983, Markstein et al 1986) based on studies with agonists and antagonists. Conflicting reports have been published which suggest that inhibition by 5-HT of forskolin stimulated adenylate cyclase is also mediated via 5-HT_{1A} receptors (De Vivo and Maayani 1986, Bockaert et al 1987). In addition, biphasic activation of adenylate cyclase activity has also been reported and has led to the suggestion that the 5-HT_{1A} receptor may mediate both stimulation and inhibition of cyclase activity (Shenker et al 1985, 1987). Additional work is need before definitive conclusions can be made about the 5-HT-sensitive adenylate cyclase system in the hippocampus.

It is suggested that the electrophysiological actions of 5-HT, in particular 5-HT mediated neuronal inhibition are due to activation of the 5-HT_{1A} receptor subtype. Bath application of 5-HT_{1A} selective agents, such as 8-OH-DPAT and buspirone, decreases the amplitude of the evoked population responses (Beck et al 1985, Peroutka et al 1987) in the CA1 region of the hippocampal slice preparation. Intracellular studies also suggest that a 5-HT mediated hyperpolarization is produced by activation of the 5-HT_{1A} receptor (Wu et al 1988, Andrade and Nicoll 1987a). Two separate reports have demonstrated that the 5-HT_{1A} mediated hyperpolarization is due to an increase in gK^+ (Andrade and Nicoll 1987a, Ropert 1988).

In summary, serotonin via the 5-HT_{1A} receptor is able to inhibit hippocampal excitability. This receptor subtype is found in high concentrations in the dendritic regions of the CA1 region and the DG, where it appears to be directly linked to a GABA receptor. In addition, activation of the adenylate cyclase second messenger system could allow for the mediation of 5-HT induced inhibition of adenylate cyclase by activation of the 5-HT_{1A} receptor subtype.

2. 5-HT$_2$

The distribution of this receptor type has been well characterized in the rodent, with the highest labeling in the claustrum, olfactory tubercle and layer IV of the neocortex. Intermediate densities were noted in the dentate gyrus, mammillary bodies, caudate putamen, and layer V of neocortex. Low concentrations of the 5-HT$_2$ receptor were found in the thalamus, hippocampus, brainstem, medulla, cerebellum and spinal cord (Pazos and Palacious 1985). These findings in the rodent have been correlated to the human brain, which shows a similar localization of 5-HT receptors (Pazos et al 1987a). A recent study demonstrates a close anatomical relationship between the regional distribution of the fine 5-HT axon type (from the dorsal raphe) and the 5-HT$_2$ receptor subtype (Blue et al 1988).

The advent of selective antagonists such as ketanserin (Leysen 1985) and agonists like 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) (Glennon et al 1986, 1988, Johnson et al 1987) and 4-bromo-2,5-dimethoxyphenyliospropylamine (DOB) (Titeler et al 1987) have done much to enable the functional characterization of this receptor. The electrophysiological effects produced by alterations in the activity of this class of receptor suggest that the 5-HT mediated depolarization seen in facial nucleus neurons, and the anti-depressant effects of 5-HT on neurons in the prefrontal cortex is thought to be mediated by the 5-HT$_2$ receptor (Aghajanian et al 1987).

In the brain, the 5-HT$_2$ receptor has been linked to the phosphoinositide (PI) second messenger system. Conn and Sanders-Bush in a series of studies (1985, 1986a,b) found a correlation between binding activity and inhibition of PI metabolism, although this response was not as potent as those of other neurotransmitters (see Roth and Chuang 1987). It is interesting to note that in smooth muscle the activation of a Ca^{2+} channel may be under 5-HT$_2$ control, although it is unclear whether the receptor is linked directly or due to activation of the PI system (Cohen et al 1986). In the rat hippocampus, the 5-HT$_2$ receptor appears to be involved in the regulation of cholinergic release of ACh from the terminals of medial septal neurons (Muramatsu et al 1988). An increase in 5-HT$_2$ binding sites in the brains of patients afflicted with major affective disorders has been reported although this finding awaits clarification (McKeith et al 1987).

C. Serotonergic Effects in the Hippocampal Formation

1. Extracellular Recordings

Perfusion of the hippocampal slice with 5-HT produces a dose-dependent decrease in the evoked population spike (Beck and Goldfarb 1985, Rowan and Anwyl 1985). This decrease was sometimes but not always preceded by an increase in the population spike. Some authors have reported changes in the slope of the field EPSP (Rowan and Anwyl 1985, Peroutka et al 1987, Ropert 1988), while others saw no such changes (Beck and Goldfarb 1985). The decrease in population spike amplitude following application of 5-HT is mimicked by application of the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT (see results below, Beck et al 1985, Peroutka et al 1987), although one report has suggested that 8-OH-DPAT may be an antagonist of 5-HT (Colino and Halliwell 1986).

Application of a non-specific 5-HT antagonist, spiperone, blocked the inhibitory effects of 5-HT and 8-OH-DPAT (Beck et al 1985), but application of the selective 5-HT$_2$ receptor antagonist, ketanserin (see Leysen 1985), did not affect the decreases in population spike amplitude produced by application of 5-HT (Rowan and Anwyl 1985). The above data would suggest that the 5-HT$_{1A}$ receptor subtype is responsible for mediating the inhibitory effects of 5-HT. However, confirmation of this proposal awaits the development of specific 5-HT$_{1A}$ antagonists.

At the present time it is unknown what receptor subtype mediates the increase in population spike amplitude seen after application of 5-HT. Several reports have described an increase in either population spike or EPSP amplitude following application of either 5-HT or 8-OH-DPAT (Jahnsen 1980, Peroutka et al 1987, Mauk et al 1988).

Recently, Clarke et al (1987) reported that the decrease in population spike amplitude following 5-HT perfusion could be antagonized by pretreatment with pertussis toxin. Both electrophysiological and biochemical responses could be correlated, and the authors suggest that a guanine regulatory protein, presumably G$_i$, mediates the inhibition seen with 5-HT. This in agreement with the previous findings of Andrade et al (1986).

An important report by Andrade et al (1986) demonstrated the activation of the same type of K^+ channel via both 5-HT and $GABA_B$ receptors. Using a variety of pharmacological tools they were able to show that a G protein couples both of these receptors to a K^+ channel. Direct activation of adenylate cyclase, protein kinase C, or phospholipase C was excluded. A G protein is activated by the 5-HT receptor, and initiates the effects of 5-HT stimulated adenylate cyclase (Berry-Kravis and Dawson 1985). A mechanism involving G proteins would provide a means whereby activation of a variety of receptors results in modulation of the same ion channel.

2. Intracellular Recordings

In CA1 pyramidal cells three distinct effects of 5-HT have been noted: i) membrane hyperpolarization, ii) membrane depolarization, and iii) changes in the afterhyperpolarization (AHP). Numerous studies have reported that the majority of CA1 pyramidal cells exhibit predominantly a hyperpolarizing response, although some neurons reveal a depolarizing response or no change at all to 5-HT application (Jahnsen 1980, Segal 1980, Andrade and Nicoll 1987b, Colino and Halliwell 1986, 1987).

The hyperpolarization seen with 5-HT application is due to activation of the $5\text{-}HT_{1A}$ receptor (Andrade and Nicoll 1987a,b, Wu et al 1988) and is most commonly associated with an increase in K^+ conductance (Jahnsen 1980, Colino and Halliwell 1986, 1987), and a decrease in the calcium activated afterhyperpolarization ($Ca^{2+}\text{-}gK^+$ AHP) (Andrade and Nicoll 1987a,b).

Iontophoretic application of 5-HT was most effective when applied in the stratum radiatum of the CA1 region (Andrade and Nicoll 1987a). This however, differs from a previous report that found the greatest changes in intracellular recording measures when 5-HT was applied to the cell body (Segal 1980). The findings of Andrade and Nicoll (1987a) correlate with binding studies which demonstrate that the $5\text{-}HT_{1A}$ receptor is localized to the dendritic region of the CA1 region in the rat hippocampus.

The increase in gK^+ seen during hyperpolarization appears to be a direct post-synaptic response mediated by 5-HT since blockade of synaptic transmission by high external Mg^{2+} and low Ca^{2+} does not inhibit the increase in gK^+ (Segal 1980). Confirming this study, Segal and

Gutnick (1980) using K^+-ion sensitive electrodes demonstrated an increase in extracellular K^+ following application of 5-HT, which could be blocked by pretreatment with a 5-HT antagonist, methysergide. These findings and others are consistent with the concept that 5-HT mediated hyperpolarization is via a 5-HT$_{1A}$ receptor coupled to a potassium channel, thereby initiating an outward K^+ current. In a later section the effects of 5-HT and its agonists and antagonists will be investigated on $[Ca^{2+}]_i$ changes.

In the dentate gyrus, intracellular responses to 5-HT are similar to those of the CA1 pyramidal cells; hyperpolarization resulting in a decreased Ca^{2+}-dependent K^+ AHP and decreased accommodation to depolarizing stimuli (Segal 1980, Baskys et al 1987). This evidence strongly suggests that similar 5-HT inhibitory mechanisms are present in the DG.

The CA3 region of the hippocampus which shows little or no 5-HT binding is generally unresponsive or not as responsive to applications of 5-HT (Segal 1981).

A great deal more is known about the effects of 5-HT and its agonists in the CA1 region of the hippocampal slice than in the DG region. The purpose of the experiments described below is to compare electrophysiological responses of the CA1 and DG to application of serotonergic agents, using the evoked population response as measure of change in the excitability of the neurons.

D. Methods

Hippocampal slices were prepared as outlined in Chapter 2. The recording electrodes were placed in the cell body layers of the CA1 region and the DG. In the CA1 region, the stimulating electrode was placed in the stratum radiatum to activate the Schaffer collaterals while in the DG they were placed in the outer third of the molecular layer to activate the lateral perforant path. Stimulus parameters have been outlined in the methods chapter. Stimulation and recordings during the experiment were made at 1/min for the duration of the experiment, typically 3-4 hours.

Only one set of recording and stimulating electrodes was used per slice, although recordings were normally taken simultaneously from two individual slices in the recording chamber. After equilibration, the stimulus intensity was adjusted to elicit a population spike of approximately 50% of maximal amplitude.

Drugs were perfused for 10 min after stable baseline population spikes (less than a 10% change in the amplitude) were obtained for 30 min. A total of 260 of 350 hippocampal slices demonstrated stable recordings. The drugs were dissolved in ACSF and entered the recording chamber by means of a three way valve. It should be noted that all the compounds in this series of experiments were mixed into the ACSF 10 min prior to, and oxygenated for only 3 min prior to perfusion, as some compounds (i.e.. 8-OH-DPAT) are sensitive to prolonged oxygenation. The drug solutions contained either 1, 5, 10, 20, 50, or 100 uM 5-HT (Sigma); 1, 5, 10, 20, or 50 uM 8-OH-DPAT or DOI (Research Biochemicals Inc.). Each drug or control solution was tested on 6-10 hippocampal slices for each of the two recording sites.

Control experiments were performed in an identical manner, except that drug free ACSF was prepared and perfused in the same way as drug perfused experiments. Population spike amplitudes and reversed field EPSP slopes were calculated for each response, and these values were normalized relative to the mean of their respective baseline values. Data from each preparation was grouped into five minute bins for analysis and plotting. Analysis of variance (ANOVA) was performed to assess dose-dependent changes, and two-sample two-tailed T-tests were conducted to evaluate significance prior to and following drug application.

E. Results

Control hippocampal slices (N=8 for DG and CA1) exhibited stable population spike amplitudes and EPSP slopes recorded from both CA1 or DG over three hours of recording (Figure 3.1A, B). It should be noted that the population spike amplitude recorded from the dentate gyrus increased significantly during the duration of the experiment (see Figure 3.1A). No significant changes were seen in the CA1 population spike amplitude nor in reversed field EPSP slopes of the DG or the CA1 regions.

1. 5-HT

Perfusion of hippocampal slices with 5-HT produced a significant dose-related reduction in population spike amplitude (ANOVA $p<0.001$) (Figure 3.2, 3.3A, 3.4A) and EPSP slope (ANOVA $p<0.05$) (Figure 3.3B, 3.4B). As expected there was a more pronounced reduction of population spike amplitude in the CA1 region.

The decreases in the population spike amplitude and reversed field EPSP slope produced by application of 5-HT were rapidly returned to control values in the CA1 region, with responses recovering to baseline values usually within 10-15 min (Figure 3.4). However, recovery to baseline values took longer in the DG region.

Comparisons between concentrations revealed that population spike amplitude was significantly reduced by $20u$M and greater concentrations of 5-HT in the DG ($p<0.0002$), and by $10u$M and greater concentrations in area CA1 ($p<0.005$) (see Figure 3.3A). In both hippocampal regions, $50u$M 5-HT produced a maximal effect. At this concentration following a 10 min perfusion of 5-HT the population spike amplitude was reduced to 55 $\pm$ 8% of baseline values in the CA1 and to 80 $\pm$ 7% of baseline values in the DG (Figure 3.4A).

Figure 3.1: Control evoked population spike responses.

Control population spike amplitudes and the reversed field EPSP were measured for the duration of the experimental period following perfusion of drug-free ACSF for 10 min starting at T=0. These controls were necessary to evaluate the effects of the long experimental periods. The population responses were evoked at 1/min and then averaged into 5 or 30 min bins. The responses were normalized to 100% at T=0 (the beginning of the drug perfusion period) to allow comparison between slices and drug compounds. The solid horizontal bar indicates perfusion of control medium. This figure demonstrates the stability of the evoked population spike amplitudes (A) and the reversed field EPSP slope (B).

A. Evoked population spike amplitudes (PSA) for the experimental duration following application of drug-free ACSF (N=8). In the DG a significant increase in the amplitude of the evoked responses was seen (compared to T=0 min; * $p < 0.02$, ** $p < 0.004$) which was not seen in the CA1 region. S.E.M.'s not shown for clarity, but did not vary greater than $\pm$ 5%.

B. Evoked reversed field EPSP slopes following application of drug-free ACSF (N=8). As shown the reversed field EPSP slope did not change for the duration of the experiment. S.E.M.'s not shown for clarity, but did not vary greater than $\pm$ 3%.

The data described in this and subsequent figures have been combined into either 5 or 30 min bins, and represents the preceding 5 or 30 min. The control period of all the slices prior to drug application was normalized to 100% to allow comparison between different slices following the application of various pharmacological compounds.

A.

CONTROLS : PSA
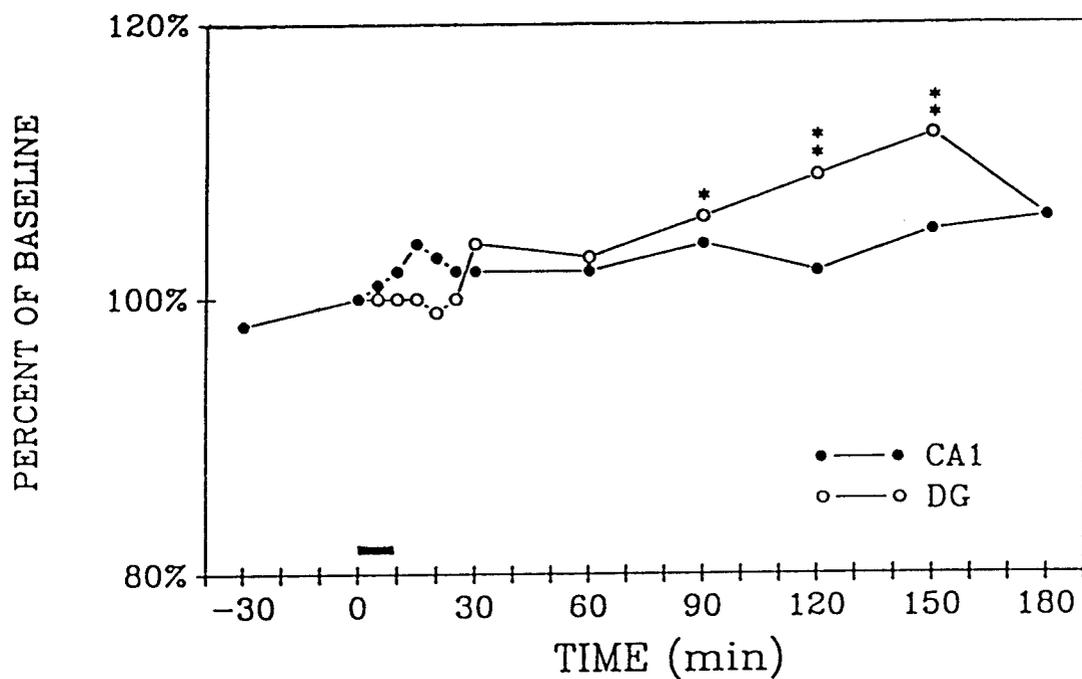
B.

CONTROLS: REVERSED FIELD EPSP SLOPE
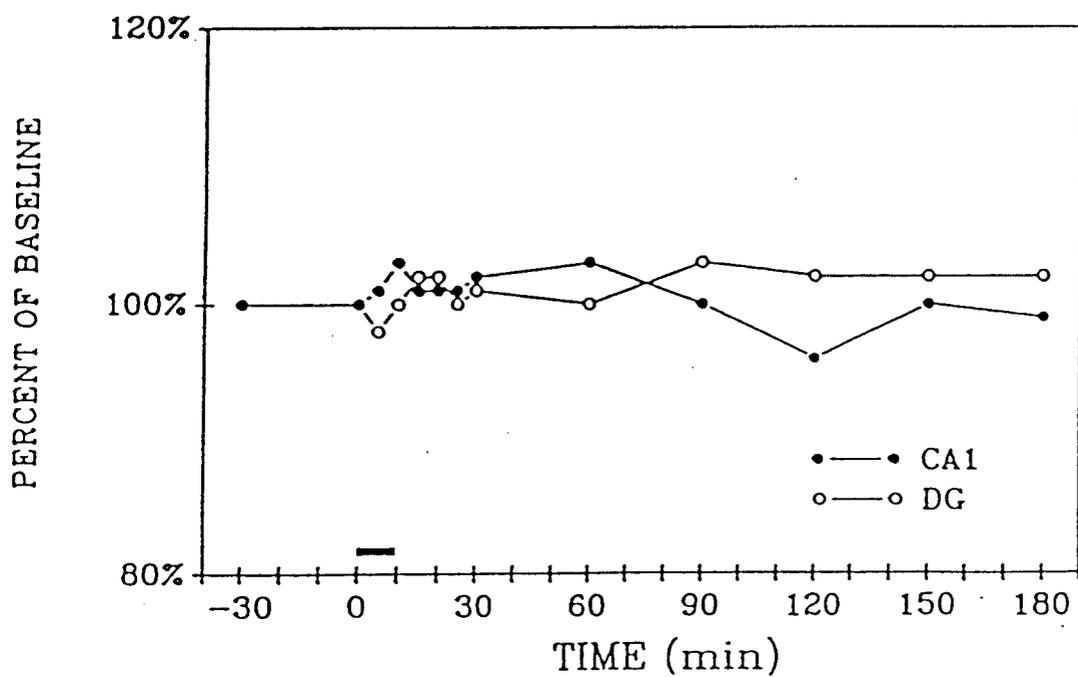
Figure 3.2: Typical evoked population responses in the CA1 and DG regions prior to and following the application of 50μM 5-HT.

Typical evoked responses from the CA1 pyramidal cell layer and DG granule cell layer prior to ("pre") and 10 min following ("post") a 10 min application of 50μM 5-HT. The asterisk denotes the stimulus artifact. Scale is as indicated by the bars.

As illustrated the application of 5-HT is more effective in reducing the population spike amplitude in the CA1 region compared to that in the DG region. In this example the changes seen in the slope of the reversed field EPSP slopes are considerably smaller when compared to the variations in population spike amplitudes.

CA1

POST

←PRE

*

2mV

5msec

DG

PRE

POST

*
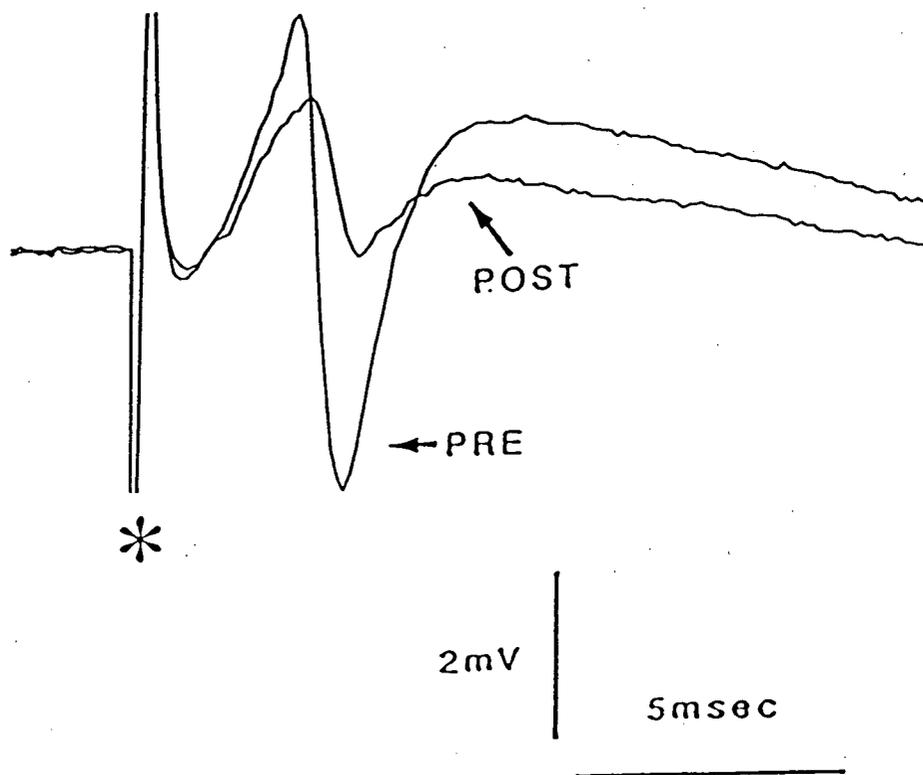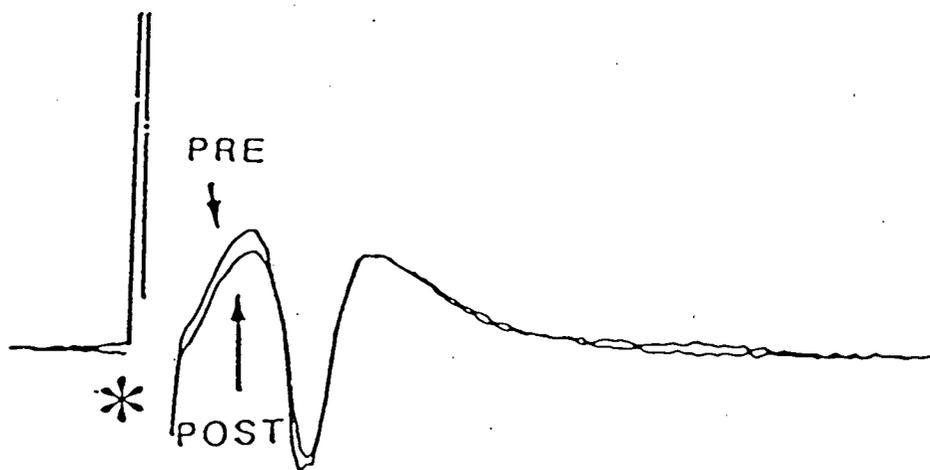
Figure 3.3: Dose response curves of population spike amplitude and reversed field EPSP's following application of serotonin.

Dose response curves following the application of 1, 5, 10, 20, 50, and $100\,u$M 5-HT in the CA1 and DG regions of the rat hippocampus. 5-HT was applied for 10 min, and values were taken at 5 to 10 min following the onset of drug application. Control values are not shown for clarity.

A. Dose response curves of the population spike amplitudes measured in the CA1 and DG (N=6). The application of 5-HT at increasing concentrations is more effective in reducing the population spike amplitude in the CA1 than in the DG region. Maximum reductions in the population spike were seen following the application of $10\,u$M or greater concentrations in the CA1. (* $p < 0.0002$, ** $p < 0.005$)

B. Dose response curves of the reversed field EPSP slope in the CA1 and DG (N=6). The application of 5-HT at increasing concentrations is only slightly more effective in reducing the reversed field EPSP slope in the CA1 than in the DG region. Maximum reductions in the population spike were seen following the application of $50\,u$M or greater concentrations. (* $p < 0.005$, ** $p < 0.01$)

A.

DOSE RESPONSE CURVE: 5—HT PSA

B.
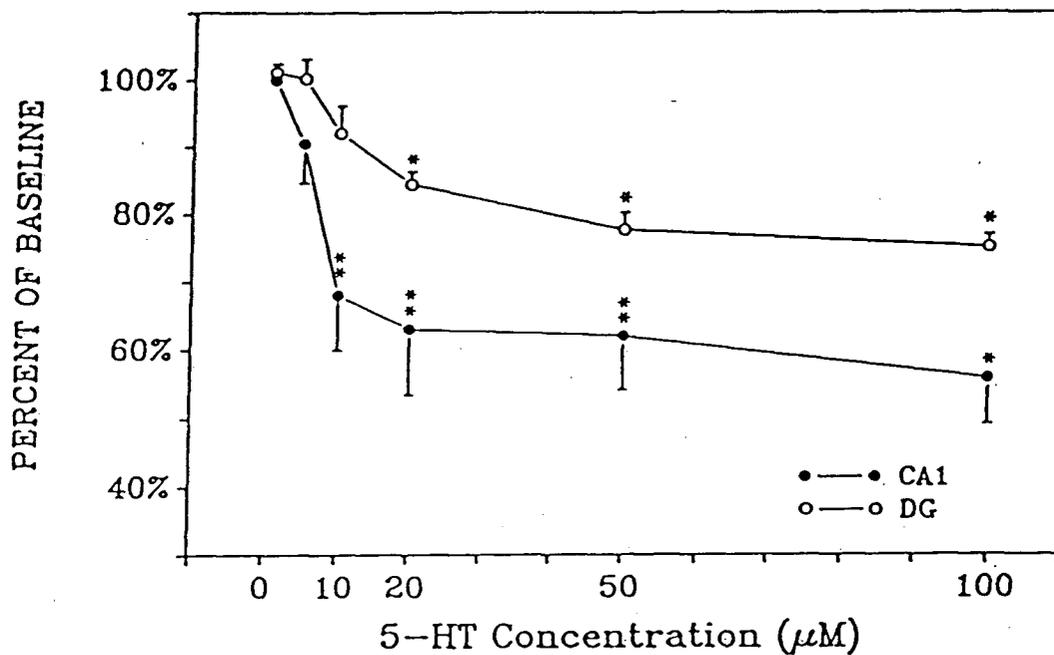
DOSE RESPONSE CURVE
5—HT REVERSED FIELD EPSP SLOPE
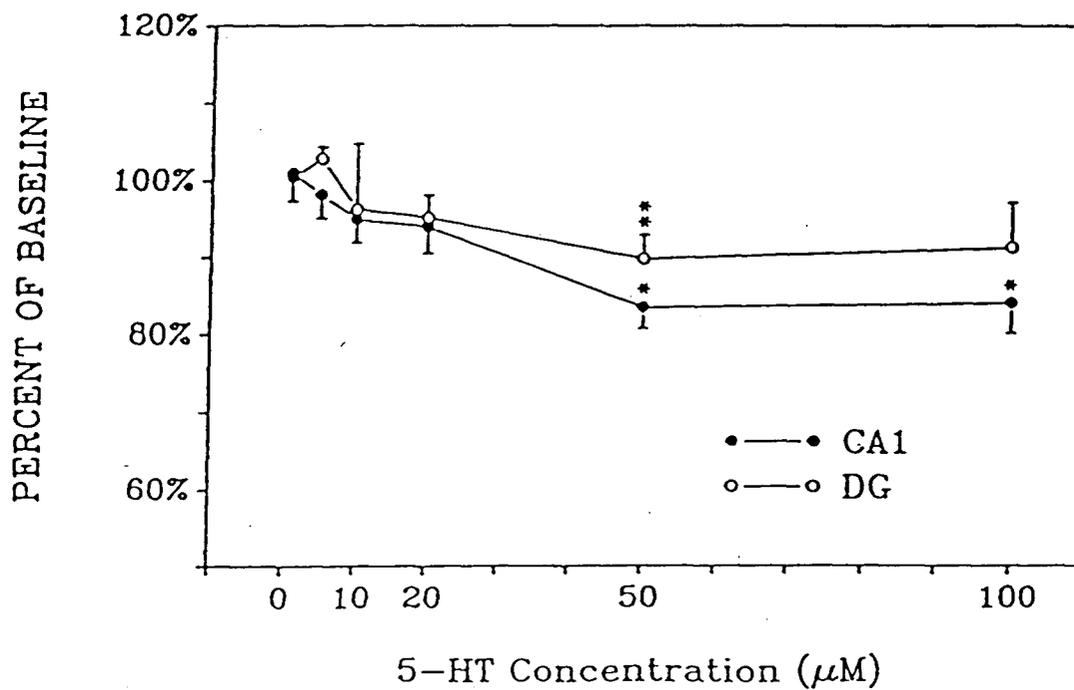
Figure 3.4: Time course of the effects of 5-HT application on population spike amplitudes and reversed field EPSP slope.

Time course of 5-HT effects in the CA1 and DG, following a 10 min application of $50\,u$M as indicated by the filled horizontal bar at $T=0$ min.

A. Population spike amplitude reductions following the application of $50\,u$M 5-HT in the CA1 and DG regions (N=6) of the rat hippocampus. Note that within 5 min following the application of 5-HT, the CA1 responses have returned to pre-drug values, while the DG responses took an additional 30 min to return to pre-drug values.

B. Decreases in the reversed field EPSP slopes were similar to the reductions seen in the population spike amplitudes, however the reductions were not as large (N=6).

A.

TIME COURSE: 5-HT PSA
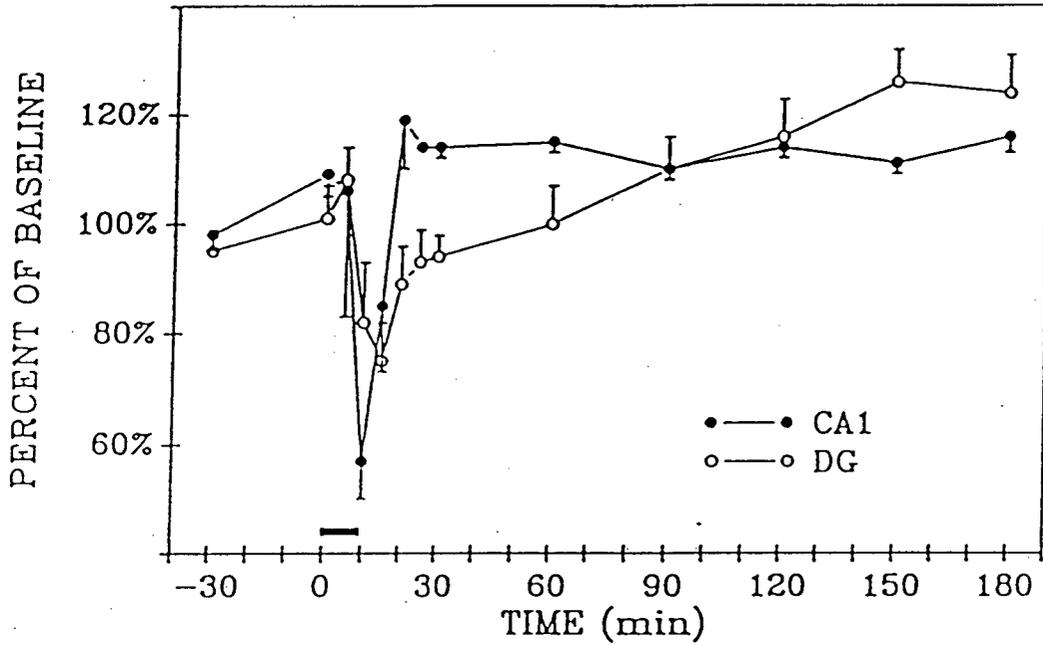
B.

TIME COURSE

5-HT REVERSED FIELD EPSP SLOPE
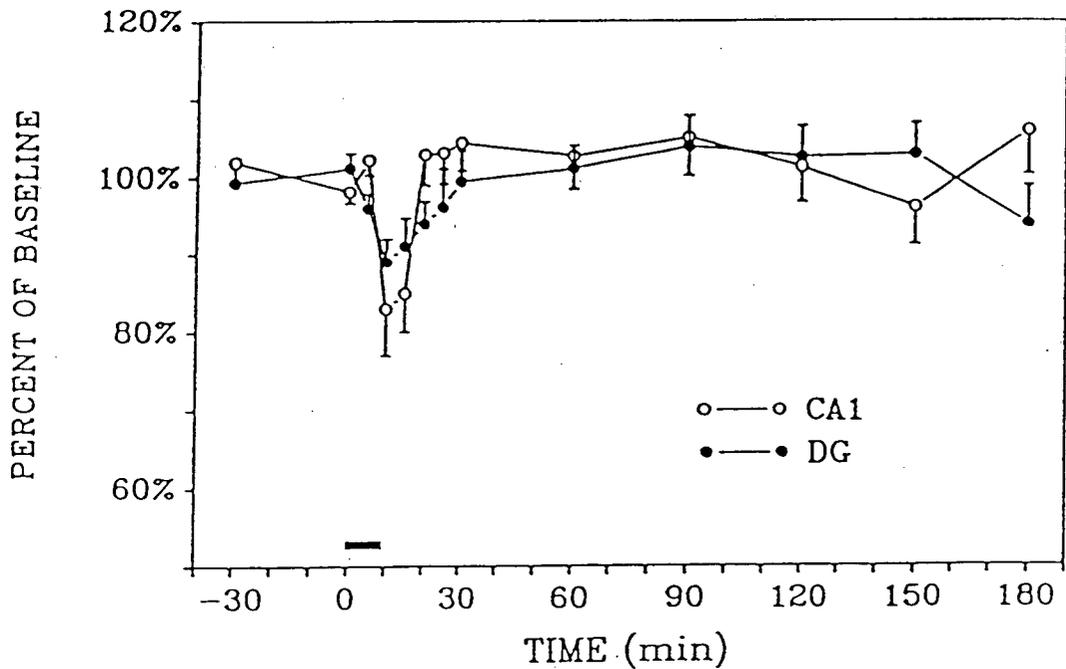
Figure 3.5: Typical evoked population responses in the CA1 and DG regions prior to and following the application of $10u$M 8-OH-DPAT.

Typical evoked responses from the CA1 pyramidal cell layer and DG granule cell layer, prior to ("pre") and 20 min following ("post") the application of $10u$M 8-OH-DPAT. The asterisk denotes the stimulus artifact. Scale is as indicated by the bars.

The application of 8-OH-DPAT is considerably more effective in reducing the population spike amplitude in the CA1 region than it is in the DG region. In this example the changes seen in the slope of the reversed field EPSP slopes are smaller than are the changes seen in the population spike amplitude.
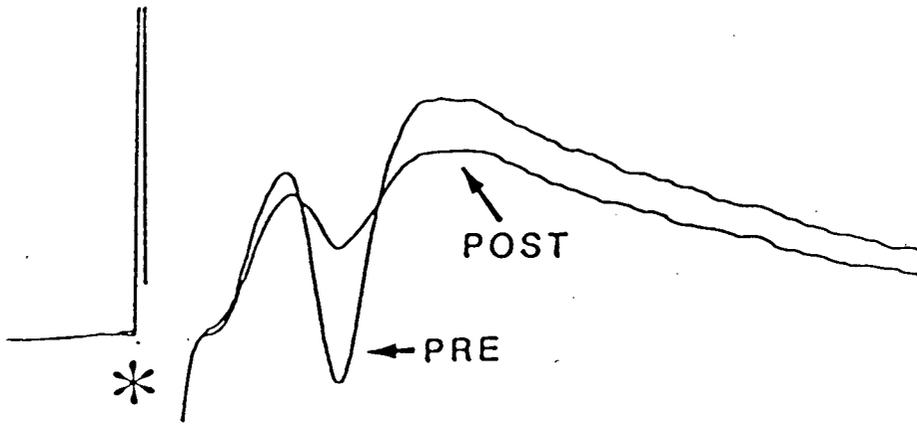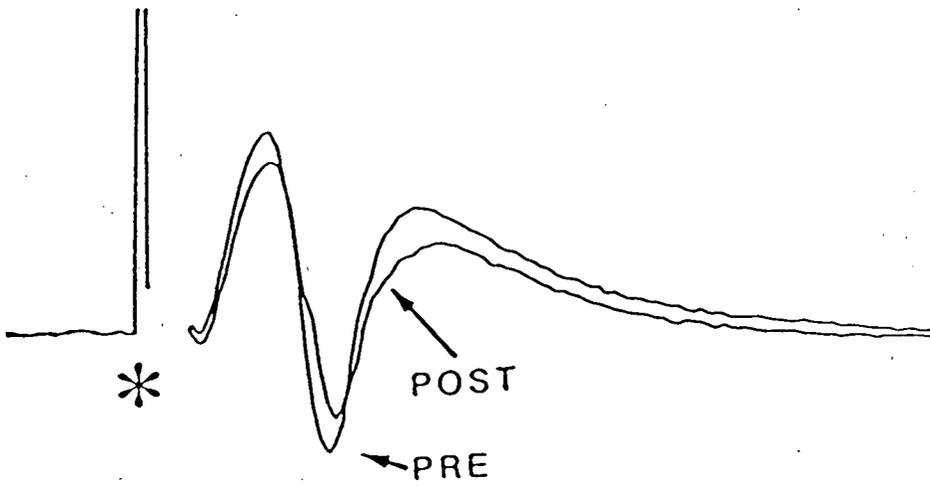
Figure 3.6: Dose response curves of population spike amplitudes and reversed field EPSP slope following application of 8-OH-DPAT.

Dose response curves following the application of 1, 5, 10, 20, and $50\,u$M 8-OH-DPAT in the CA1 and DG regions of the rat hippocampus. 8-OH-DPAT was applied for 10 min, and values were taken 20 to 25 min following drug application. Control values are not shown for clarity.

A. Dose response curves of the population spike amplitudes in the CA1 and DG (N=6). The application of 8-OH-DPAT at increasing concentrations is more effective in reducing the population spike amplitude in the CA1 (with the exception of $50\,u$M) than in the DG region. Maximum reductions in the population spike were seen following the application of $10\,u$M or greater concentrations. (* $p < 0.03$, ** $p < 0.003$)

B. Dose response curves of the reversed field EPSP slope in the CA1 and DG (N=6). Maximum reductions in the slope were seen following the application of $10\,u$M or greater concentrations (except in the CA1). (* $p < 0.03$, ** $p < 0.005$)

A. DOSE RESPONSE CURVE: 8-OH-DPAT PSA
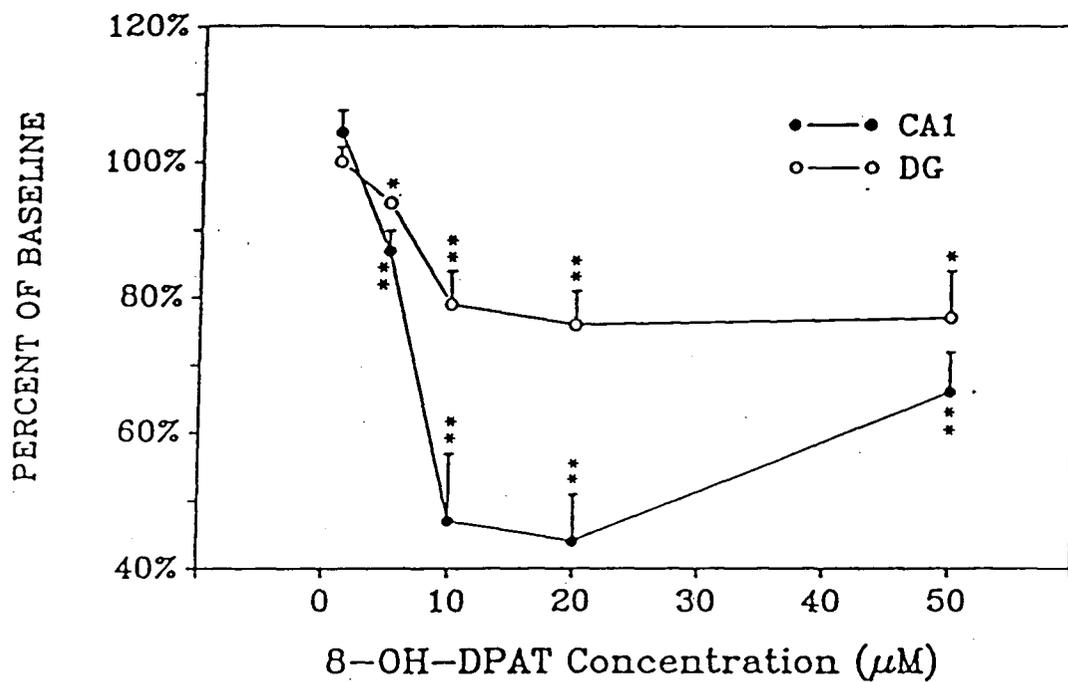
B. DOSE RESPONSE CURVE
8-OH-DPAT REVERSED FIELD EPSP SLOPE
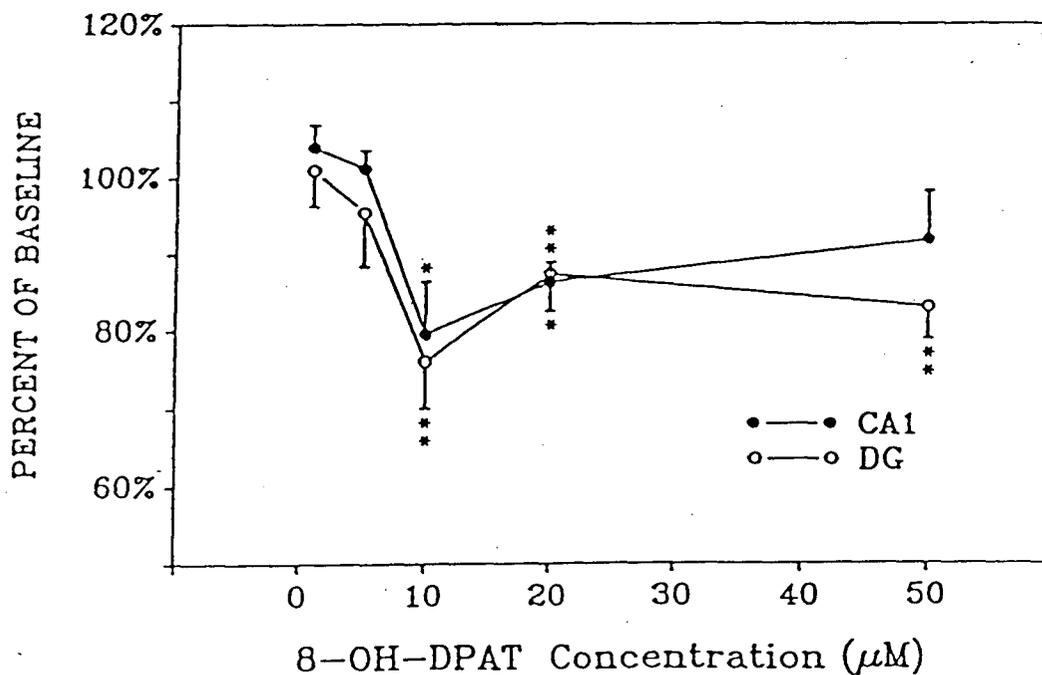
Figure 3.7: Time course of the effects of 8-OH-DPAT application on population spike amplitude and reversed field EPSP slope.

Time course of 8-OH-DPAT effects in the CA1 and DG, following a 10 min application of $10\,u$M as indicated by the filled horizontal bar at T$=0$ min.

A. Population spike amplitude reductions following the application of $10u$M 8-OH-DPAT in the CA1 and DG regions (N$=6$) of the rat hippocampus. Note that within 20 min following the application of 8-OH-DPAT, the CA1 and DG responses were maximally depressed and were maintained for the duration of the experiment. The effectiveness of 8-OH-DPAT in the DG was approximately half of that in the CA1 region.

B. Changes in the reversed field EPSP slopes were similar in both the CA1 and DG regions (N$=6$), reaching a maximal reduction within 30-60 mins.

A. TIME COURSE: 8-OH-DPAT PSA
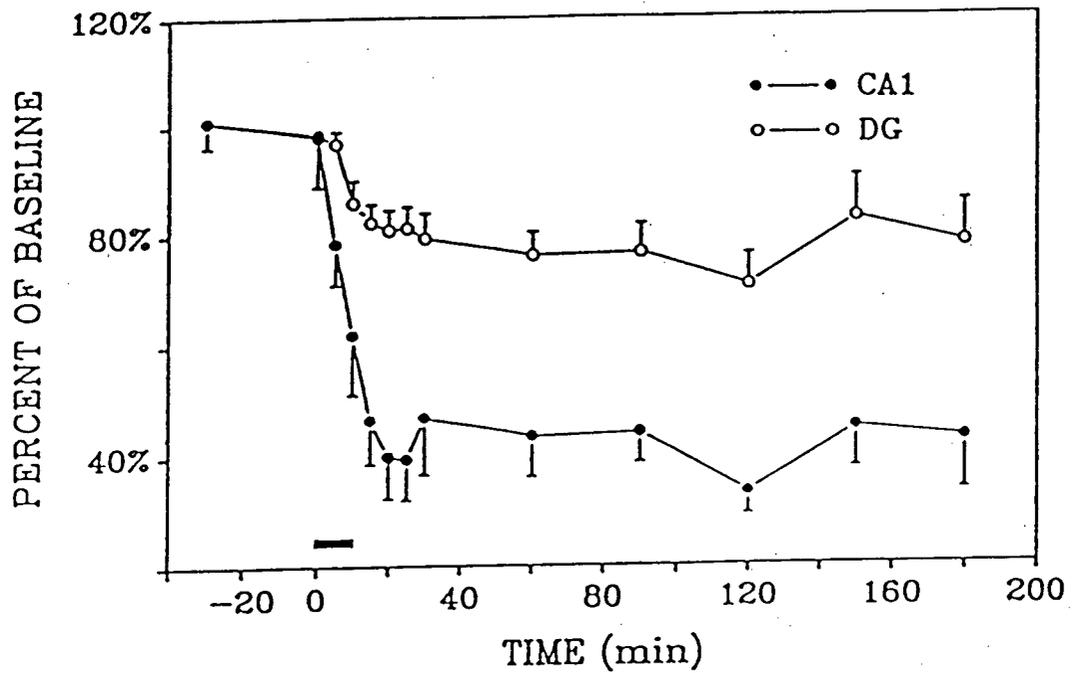
B. TIME COURSE
8-OH-DPAT REVERSED FIELD EPSP SLOPE
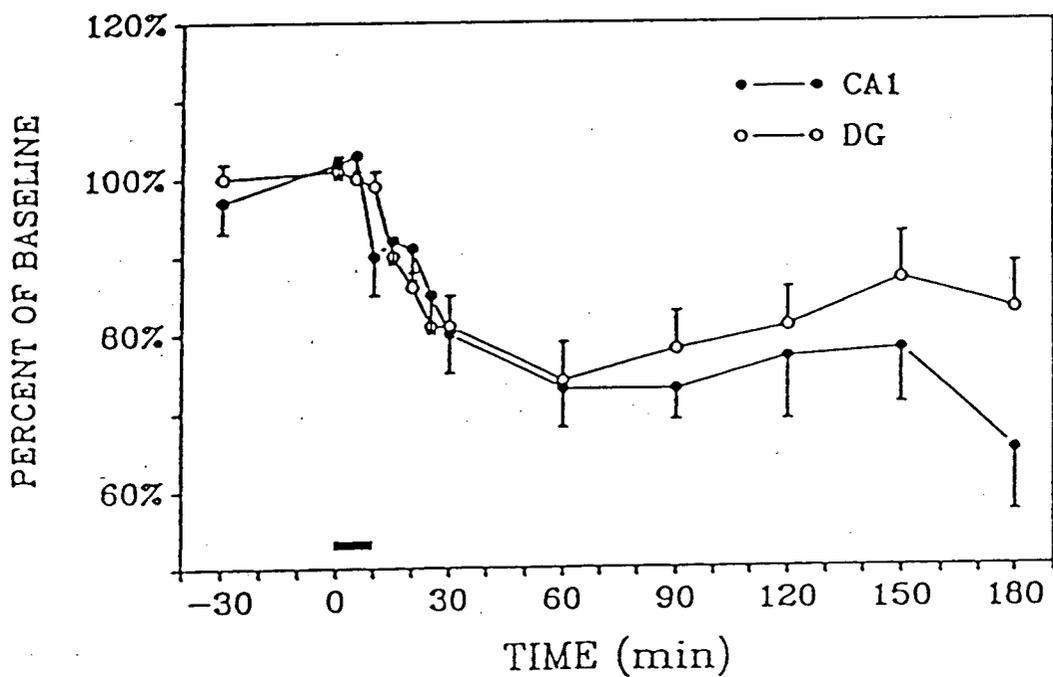
Figure 3.8: Typical evoked population responses in the CA1 and DG regions prior to and following the application of 10uM DOI.

Typical evoked responses from the CA1 pyramidal cell layer and DG granule cell layer, prior ("pre") and 10 min following ("post") the application of 10uM DOI. The asterisk denotes the stimulus artifact. Scale is as indicated by the bars.

The application of DOI is more effective in reducing the population spike amplitude in the DG region compared to the CA1 region. In this example no changes are seen in either the amplitude or the slope of the evoked population responses in the CA1. DOI was only effective in reducing the population spike amplitude and population EPSP slope in the DG region.
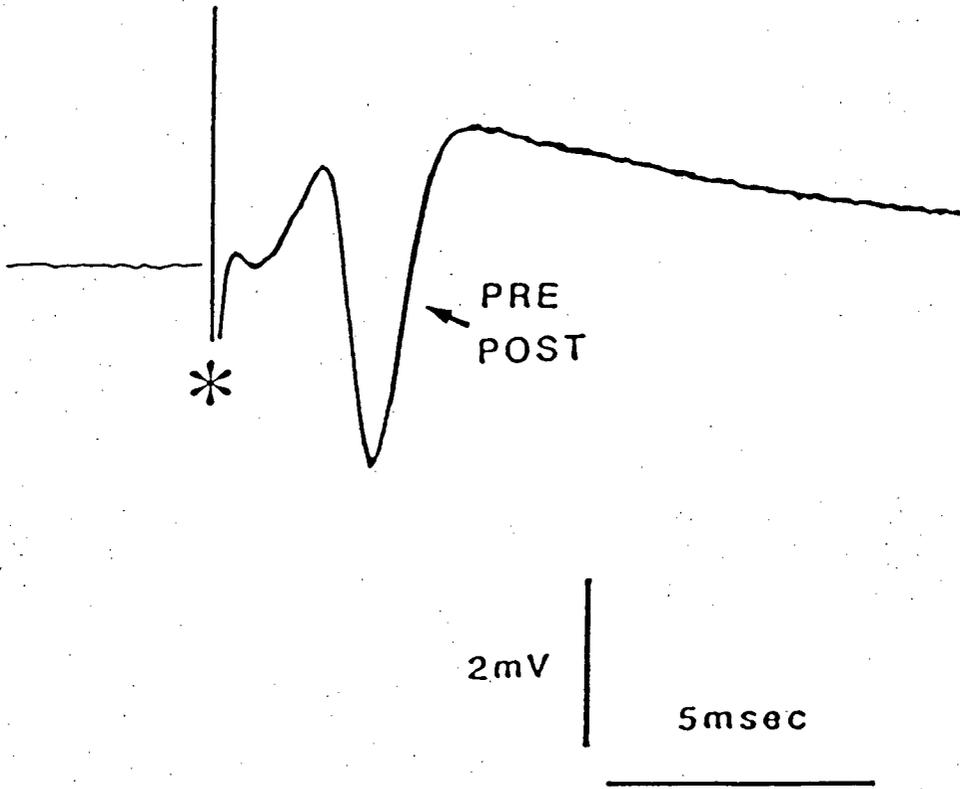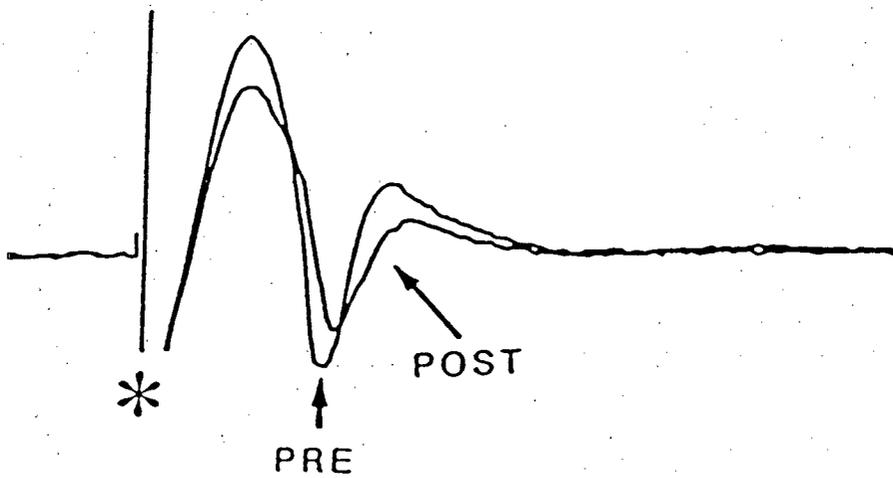
Figure 3.9: Dose response curves of population spike amplitude and reversed field EPSP slope following application of DOI.

DOI dose response curves following the application of 1, 5, 10, 20, and $50\,u$M concentrations for the DG region of the hippocampus. In the CA1 region only 10 and $50\,u$M concentrations were used as DOI had no effect in this region. Values were taken 5 to 10 min following a 10 min DOI application. Control values are not shown for clarity.

A. Dose response curves of the population spike amplitudes in the CA1 and DG (N=6). DOI was applied in the CA1 region at only 10 and $50\,u$M concentrations. Maximum reductions of the DG population spike amplitude were seen following the application of $10\,u$M or $50\,u$M. (* p<0.03)

B. Dose response curves of the reversed field EPSP slope measures in the CA1 and DG (N=6). DOI was applied at only 10 and $50\,u$M concentrations, as it had no effect on the measured slopes in the CA1 region. Reductions of the DG reversed field EPSP slopes were seen following the application of $10\,u$M were not significant. EPSP slopes at 20 and $50\,u$M concentrations returned to pre-drug values.

The lack of effects seen in the CA1 region concurs with the findings that the 5-HT$_2$ receptor is not found in the CA1 region of the rat hippocampus. DOI is a selective ligand for the 5-HT$_2$ receptor subtype.

A.

DOSE RESPONSE CURVE: DOI PSA

B.
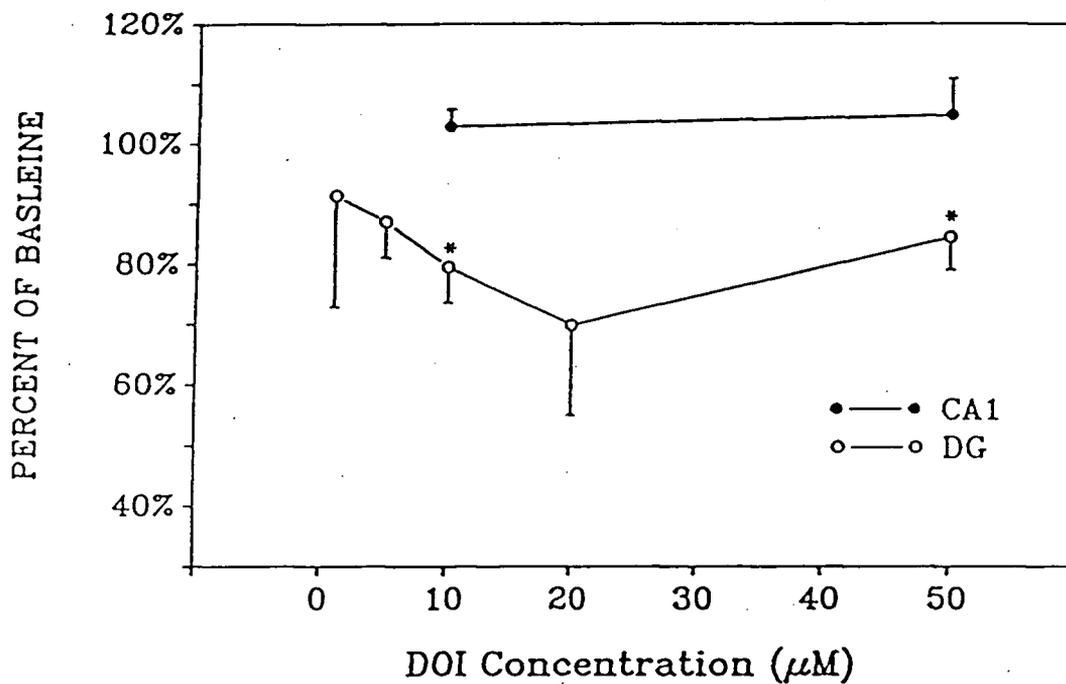
DOSE RESPONSE CURVE
DOI REVERSED FIELD EPSP SLOPE
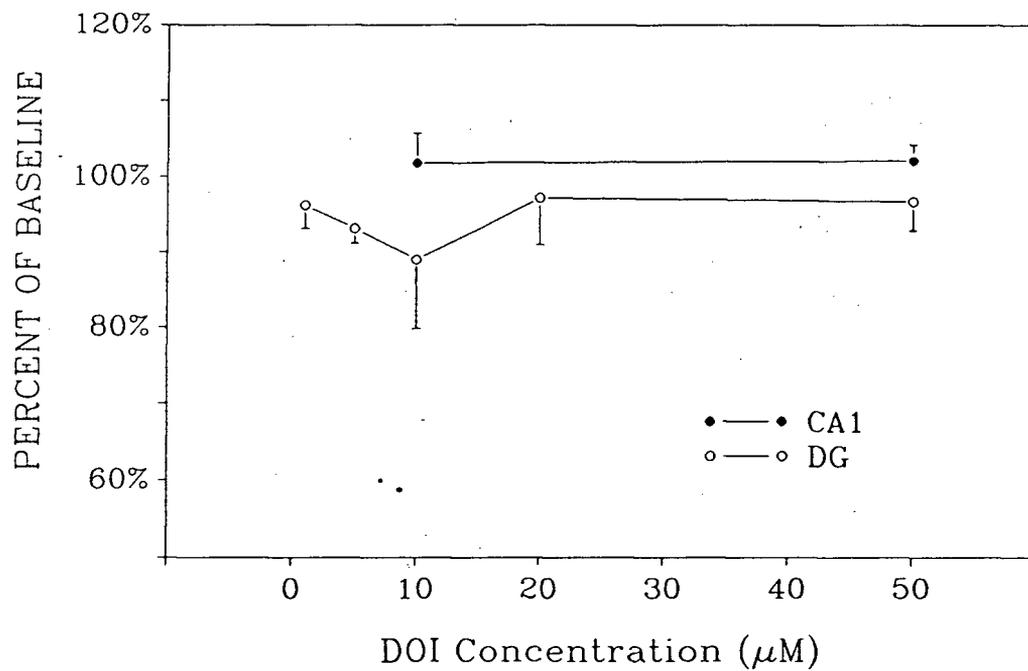
Figure 3.10: Time course of the effects of DOI application on population spike amplitudes and reversed field EPSP slope.

Time course of DOI effects in the CA1 and DG, following a 10 min application of $10\,u$M as indicated by the filled horizontal bar at $T=0$ min. Note the lack of any effect in the CA1 region of the hippocampus.

A. Population spike amplitude reductions were seen only in the DG following application of DOI ($N=6$). A return to pre-drug values was seen within 30 min following the perfusion of DOI in the DG. The lack of any change in the CA1 region confirms the lack of the 5-HT_2 receptor subtype in the CA1 region.

B. Although DOI application elicited a reduction in the reversed field EPSP slope in the DG it was not significant when compared to pre-drug values ($p<0.3$). DOI again had no effect on the CA1 population EPSP slopes ($N=6$).

A.

TIME COURSE: DOI PSA
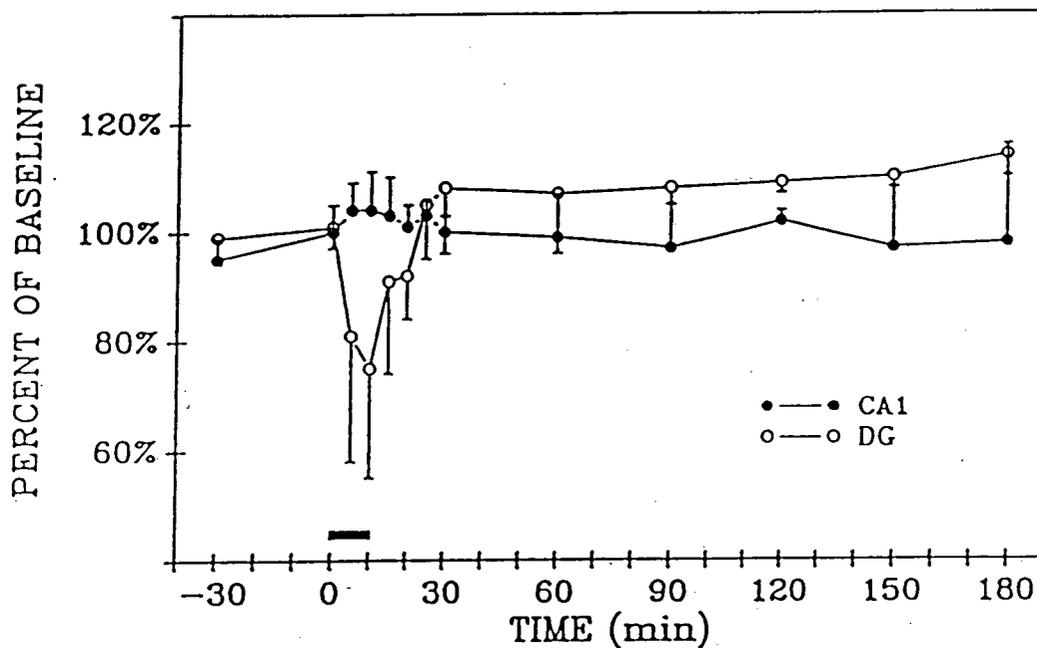
B.

TIME COURSE
DOI REVERSED FIELD EPSP SLOPE
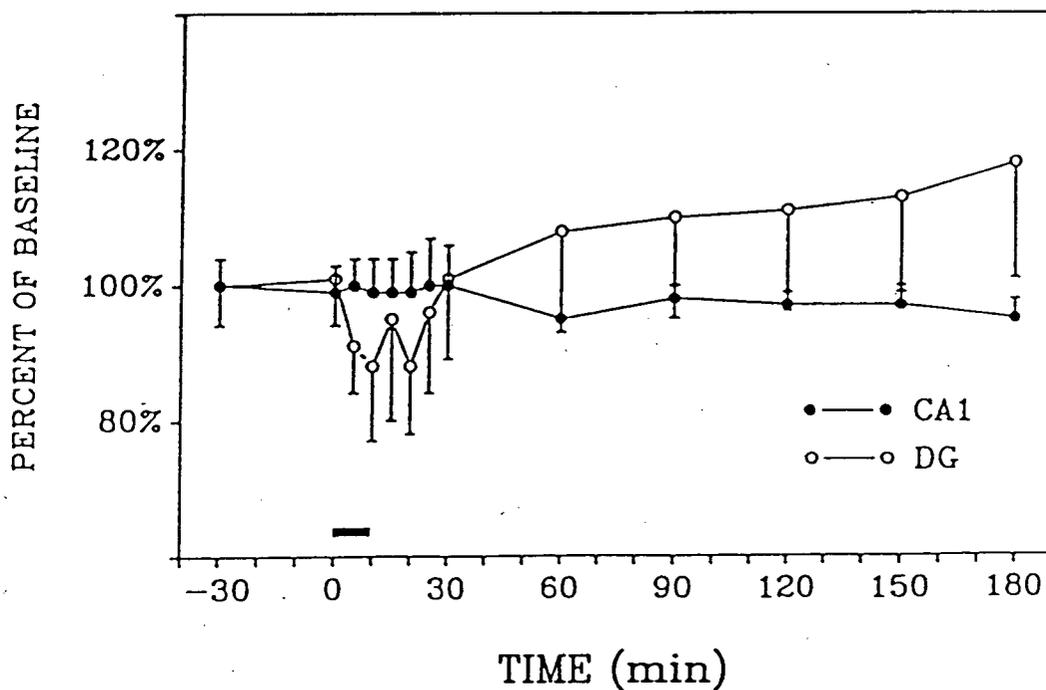
Figure 3.11: Antagonism of the effects of 5-HT, 8-OH-DPAT, and DOI by methysergide and ketanserin.

Application of methysergide or ketanserin alone preceded application of these compounds in combination with 5-HT, 8-OH-DPAT, or DOI by 5 min. The effects of 5-HT and DOI were assessed during the final 5-10 min of application of these compounds; the effects of 8-OH-DPAT were assessed 10-15 min after application. (*p<0.05)

A. CA1 responses.

B. DG responses.

Solid bars: population spike amplitudes; open bars: reversed field EPSP slopes.

5-HT: 20uM 5-HT
5-HT+ME: 20uM 5-HT + 1uM methysergide
5-HT+KE: 20uM 5-HT + 1uM ketanserin
DP: 10uM 8-OH-DPAT
DP+ME: 10uM 8-OH-DPAT + 1uM methysergide
DO: 10uM DOI
DO+KE: 10uM DOI + 1uM ketanserin

A. ANTAGONISTS: CA1 REGION
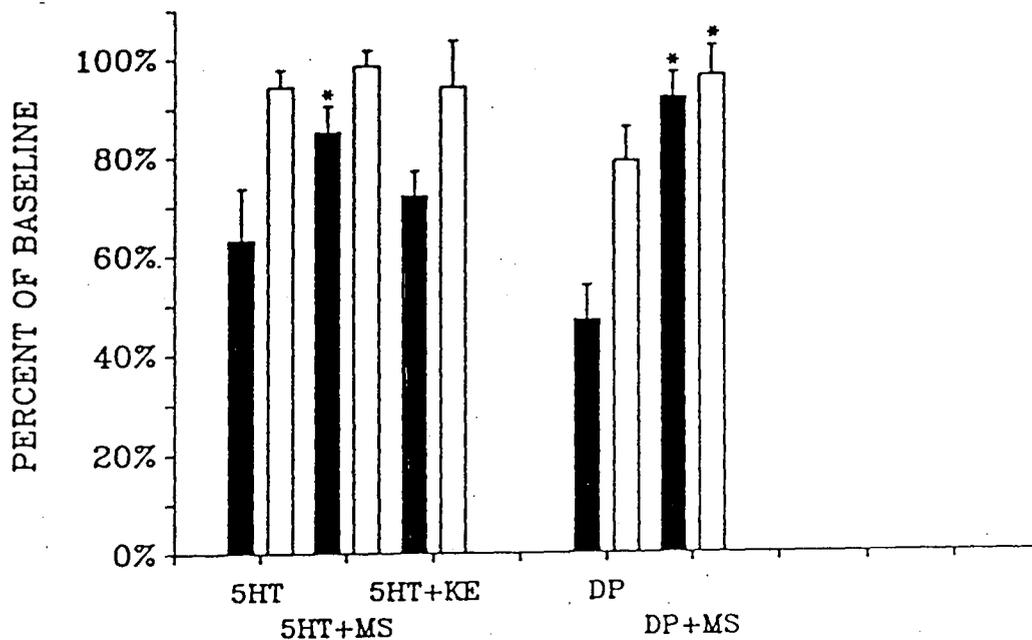
B. ANTAGONISTS: DG REGION
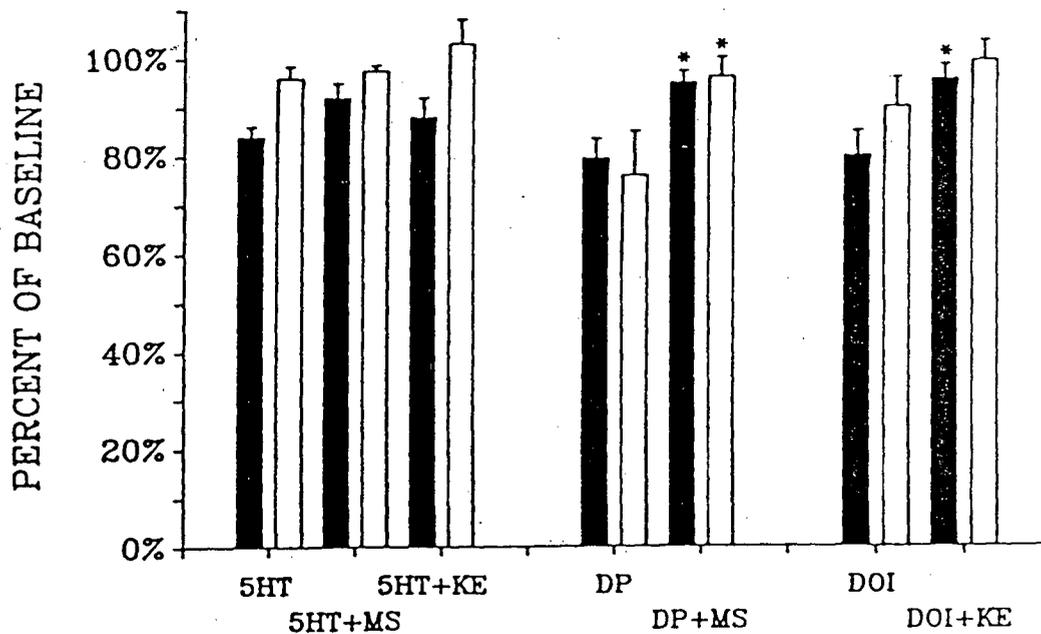
Reversed field EPSP slopes were significantly decreased only at $50 u$M ($p < 0.01$) concentration of 5-HT in the DG, and at $50 u$M and greater in the CA1 ($p < 0.005$). Following perfusion with $50 u$M 5-HT, EPSP slopes reduced to 83% $\pm$ 6% of baseline values in the CA1, and 89% $\pm$ 3% of baseline values in the DG (Figure 3.4B).

In summary, application of 5-HT, resulted in a decrease in population spike amplitude and the reversed field EPSP slope in the CA1 and DG hippocampal regions. The effects of 5-HT were greater in the CA1 than in the DG, and in all cases the CA1 region responses returned to control values more rapidly than did those of the DG (see Figure 3.4A,B). All responses in both hippocampal regions returned to baseline values within 30 min following the perfusion of 5-HT.

2. 8-OH-DPAT

To differentiate between the receptors mediating the inhibitory effects of 5-HT on population spike amplitudes and reversed field EPSP slopes, the 5-HT$_{1A}$ selective agonist 8-OH-DPAT was used. Perfusion of 8-OH-DPAT for 10 min produced a significant dose-related reduction in the amplitude of evoked responses (ANOVA $p < 0.01$) and in the EPSP slope (ANOVA $p < 0.001$) (Figure 3.5). Again a more pronounced effect was observed on the amplitude of population spikes recorded in the CA1 region than in the DG region (Figure 3.6, 3.7).

Comparisons between different doses demonstrated a significant effect of $5 u$M 8-OH-DPAT and higher concentrations on the population spike amplitude in both regions. In both hippocampal regions, CA1 and DG, $20 u$M of 8-OH-DPAT produced a maximal effect on population spike amplitude, and at 20 min following perfusion at this concentration, the population spike amplitude was reduced to 47 $\pm$ 7% of baseline values in the CA1 ($p < 0.003$) and to 79 $\pm$ 5% of baseline values in the DG ($p < 0.003$). An interesting finding was that in the CA1 region, perfusion of $50 u$M 8-OH-DPAT had a smaller effect on population spike amplitude than $10 u$M. This finding was not seen in the DG.

Evaluation of the reversed field EPSP slopes demonstrated a significant decrease in both the CA1 and DG regions after administration of $10 uM$ and greater concentrations 8-OH-DPAT when compared to control values. At 20 min following $10 uM$ drug application values reached 79 $\pm$ 7% in the CA1 ($p < 0.03$) and 76 $\pm$ 6% of baseline values in the DG ($p < 0.005$).

An important finding of these investigations was that the time course of these 8-OH-DPAT-induced changes was very long, in fact at the higher doses the evoked responses never recovered to control values within a four hour observation period. In both the CA1 and DG, the maximum reduction in population spike amplitude was observed 20-30 min after the start of drug perfusion. The duration of these effects varied with drug concentration, as population spike amplitudes returned to pre-drug baseline values within 90 minutes after perfusion with $5 uM$ 8-OH-DPAT, whereas maximum inhibition was observed for the three hour duration of the experiment at concentrations of $10-20 uM$ in CA1 and $10-50 uM$ in the DG. Again interestingly, at $50 uM$, population spike amplitudes in the CA1 returned to control values within two hours after end of the drug application (data not shown).

3. DOI

As noted above, $5\text{-}HT_2$ binding sites appear to be in high concentrations in the DG of the hippocampal formation. Using the selective $5\text{-}HT_2$ agonist DOI, attempts were made to determine the role of this receptor subtype in hippocampal electrophysiology.

Perfusion with DOI produced a significant dose-related reduction in the population spike amplitude of evoked responses in the DG (ANOVA $p < 0.05$), but had no apparent effect on the responses recorded in the CA1 (Figure 3.8). In the DG only $10 uM$ ($p < 0.03$) and $50 uM$ ($p < 0.03$) produced significant reductions in the population spike amplitude (Figure 3.9). In the DG, application of DOI elicited an immediate effect with a maximum decrease within 10 min, and the responses had recovered to pre-treatment values within 30 min after the onset of drug application (Figure 3.10). Preliminary experiments indicated this lack of responsiveness in the CA1 region and experiments were confined to the 10 and 50 uM concentrations of DOI. The significant decreases were seen in the population spike amplitude with DOI application whereas no significant change was observed in the reversed field EPSP slope (Figure 3.9, 3.10).

4. Antagonists

One of the difficulties in characterizing the 5-HT receptor subtypes is the lack of selective antagonists. Antagonists used in the past (e.g.. spiperone) do not differentiate between 5-HT$_1$ and 5-HT$_2$ type receptors. A selective 5-HT$_2$ antagonist, ketanserin, has been found only recently (Leysen 1985). However, antagonists for the 5-HT$_{1A}$ receptor do not yet exist; therefore making it difficult to characterize fully the receptor types mediating the decreases seen in the above experiments. One antagonist that is used in characterizing differences between the receptor subtypes is methysergide (Bradley et al 1987). This antagonist has a higher affinity for the 5-HT$_2$ (2.6nM) than the 5-HT$_1$ receptor (25nM), but nonetheless is useful as an antagonist to identify 5-HT$_1$ binding sites in the brain.

Ketanserin and methysergide were used to examine further the role of 5-HT receptors mediating the decreases described above.

Only slight changes in the drug perfusion techniques were used for this set of experiments. To assess the effects of the antagonists alone, slices were superfused with ACSF containing either 1uM methysergide (N=6) or 1uM ketanserin (N=6) to both DG and CA1 regions for 15 min. The combined effect of agonists and antagonists was assessed when ACSF containing antagonist alone was first superfused for 5 min, followed by ACSF containing a combination of antagonist and an agonist. Recordings from the CA1 and DG were made from 6 slices each at every drug condition, with the exception of the DOI and ketanserin combination, which was assessed only in DG recordings. The following antagonist-agonist combinations and concentrations were used in both the CA1 and DG regions: i) 1uM methysergide and 20uM 5-HT, ii) 1uM ketanserin and 20uM 5-HT, and iii) 1uM methysergide and 10uM 8-OH-DPAT. In the DG region alone the following was used: i) 1uM ketanserin and 10uM DOI.

Data and statistical analysis for the 5-HT and DOI results was performed using the mean normalized responses collected during the final 5-10 minutes of drug application. Comparisons involving the combined 8-OH-DPAT and methysergide condition, due to the long time course of 8-OH-DPAT, were conducted on data collected 10-15 minutes after drug application. Antagonist-

alone perfusions were compared with controls from the previous experiments, while combined agonist-antagonist perfusions were compared with agonist only results.

The results are summarized in Figure 3.11. Neither methysergide alone nor ketanserin alone produced significant effects on population spike amplitude or EPSP slope, in either the DG or CA1 region of the hippocampal slice. The combination of 5-HT and methysergide produced significantly less inhibition of the population spike amplitude in the CA1 region only ($p < 0.05$). However, the inhibition of population spike amplitude produced by 5-HT in either region was not significantly different from that produced by the combination of ketanserin and 5-HT.

The combination of methysergide and 8-OH-DPAT produced significantly less inhibition of population spike amplitude and in the EPSP slope in both the CA1 and DG, than did 8-OH-DPAT alone ($p < 0.05$ for all conditions). The combination of ketanserin and DOI produced significantly less inhibition of population spike amplitude in the DG ($p < 0.05$), than did DOI alone. No change was seen in the EPSP slope.

F. Discussion

The results presented above demonstrate that perfusion of 5-HT or the 5-HT_{1A} agonist 8-OH-DPAT, results in a decrease in both the population spike amplitude and population EPSP slope. This reduction was seen in both the CA1 and DG areas. The reduction of the population spike amplitude following application of 5-HT and 8-OH-DPAT in the CA1 region is in agreement with earlier reports (Beck and Goldfarb 1985, Rowan and Anwyl 1985, Peroutka et al 1987). However, we did not observe the transient increase in population spike amplitude following perfusion of 5-HT, reported by Beck and Goldfarb (1984) and Rowan and Anwyl (1985). Previous studies using intracellular techniques have shown that application of either of these compounds results in membrane hyperpolarization and an increased gK^+, and provide a cellular mechanism for the action of 5-HT and 8-OH-DPAT in the CA1 region (Andrade and Nicoll 1987a,b).

There were notable differences between the hippocampal regions in the degree of inhibition produced by 5-HT and 8-OH-DPAT. Both of these compounds had a greater effect on CA1 pyramidal cells than DG granule cells, and this was particularly the case for the 5-HT_{1A} receptor

agonist, 8-OH-DPAT. A number of factors could account for these differences including variations in the distribution of receptor subtypes between the two regions . The CA1 region has a dense concentration of 5-HT_{1A} binding sites located in the stratum radiatum dendritic zone, with less labelling in the somal regions (Kohler 1984).

Although a dense population of 5-HT_{1A} binding sites has been demonstrated in the DG, this labelling has been seen in the hilar region, just inferior to the granule cells (Kohler 1984, Kohler and Steinbusch 1982). The 5-HT_{1A} binding sites in the DG may be situated on both GABAergic interneurons and dentate granule cells. Serotonin mediated hyperpolarization could serve to reduce inhibitory control of the dentate granule cells by a direct action on the interneuron population. Such an effect may counteract the influence on the direct inhibition of the DG cells while still resulting in an overall decreased inhibition.

Another basic difference between the two regions is their electrophysiological dissimilarity. Segal (1980) reported that the DG granule cells relative to the larger pyramidal cells exhibited a smaller conductance change produced by application of 5-HT_{1A} agonists. In addition, the resting membrane potential is more hyperpolarized in the DG which could account for the smaller hyperpolarization seen after iontophoretic application of 5-HT. Therefore, 5-HT appears to have the same effect on both dentate granule and hippocampal pyramidal neurons.

Application of 5-HT and 8-OH-DPAT produced a decrease in both the population spike amplitude and the reversed field EPSP slope in both hippocampal regions. A decrease in the reversed field EPSP could indicate a reduction in the net post-synaptic current(s) and may underlie the simultaneous decrease in population spike amplitude. A number of physiological processes could account for the decreases seen in the reversed field EPSP slope or the population spike amplitude. These include: i) decreased neurotransmitter release, ii) ephaptic or electrical field effects, iii) increased electrotonic coupling, iv) receptor desensitization (i.e.. 8-OH-DPAT effects), and v) increased membrane conductance. The latter mechanism probably accounts for the effects on the 5-HT_{1A} receptor. This receptor subtype when activated by 5-HT or 8-OH-DPAT mediates an increased gK^+ in pyramidal cells of the CA1 and in the granule cells of the DG.

Application of the general 5-HT antagonist, methysergide, blocked the effects of 5-HT and 8-OH-DPAT in the CA1 region. This finding would suggest that the effects of these compounds are mediated by serotonergic receptors. Application of methysergide has been shown to hyperpolarize hippocampal pyramidal cells in the CA1 region (Andrade and Nicoll 1987b), although the present study found no change in the amplitude or the slope of the population responses following perfusion of methysergide alone.

The 5-HT_2 receptor agonist DOI had no effect on responses recorded in area CA1 (Figure 3.8, 3.9, 3.10). A significant reduction in population spike amplitude was found in the DG, but only at 10 and $50\mu M$. The variable results seen following DOI application could be accounted for by the relatively low affinity of DOI for the 5-HT_2 binding site (10nM, Glennon 1986). Therefore, while DOI may have variable effects, receptor binding studies have demonstrated the localization of the 5-HT_2 receptor binding sites primarily in the DG region of the hippocampus. Agonists with higher affinities and greater selectivity are required to clarify the findings described above.

Application of the selective 5-HT_2 antagonist, ketanserin, did not change the population responses in either the CA1 or the DG regions; however, perfusion of ketanserin prior to and during DOI perfusion reduced the effects of DOI on population spike amplitude in the DG. This finding suggests the involvement of the 5-HT_2 receptor in this region. The implications should be interpreted with caution as the effects of 5-HT were not attenuated by ketanserin, which would suggest that 5-HT and DOI may be active on distinct populations of receptors. In addition, the 5-HT effects mediated by 5-HT_2 receptors, might be masked by other processes, such as those mediated by 5-HT_{1A} receptors.

The long duration of the effects of 8-OH-DPAT on recorded field potentials excited interest, although perfusion of 5-HT and DOI were found to be readily reversible after washout and exhibited no long-term effects. This is consistent with previous studies (Beck and Goldfarb 1985, Olpe et al 1984, Beck et al 1985). Following iontophoretic or pressure application of 8-OH-DPAT, the hyperpolarization seen in the DG and CA1 regions is prolonged relative to that produced by administration of 5-HT (Andrade and Nicoll 1987a,b, Baskys et al 1986). Application of the optimal dose of $10\mu M$ 8-OH-DPAT in the present study resulted in inhibition of population

responses for the duration of the three-hour experiment, and was seen in both the CA1 and DG hippocampal regions. The exact reasons for the long duration of the 8-OH-DPAT effects remain unknown but may be related to the interaction of this agonist with the 5-HT$_{1A}$ receptor. Although very little information is available, changes in lipid solubility, kinetics of drug binding to protein receptor sites and hydrophobic binding may account for the long duration effects seen here. Finally, end product metabolism (compound is sensitive to oxidation; see RBI Summary Sheet) if occurring is also at the present time unexplored and may explain the long time course of the 8-OH-DPAT effects.

In summary, these data suggest an inhibitory role for the 5-HT$_{1A}$ receptor in the CA1 and DG, and an inhibitory role for the 5-HT$_2$ receptor in the DG. These findings are correlated with previous reports describing receptor binding studies, which indicate a high density of 5-HT$_{1A}$ binding sites in both regions, and a large concentration of 5-HT$_2$ sites in the DG.

III. PURINERGIC SYSTEMS

A. Introduction

In recent years purinergic research has moved from the peripheral nervous system to the central nervous system (CNS). Identification of the following purinergic mechanisms are crucial to our understanding of this group of compounds: i) synthesis, ii) storage, iii) uptake systems, iv) receptor subtypes and v) the electrophysiological actions of adenosine.

B. Synthesis of Purines

Purines, such as adenosine, have very well defined pathways for synthesis, uptake and release. Briefly, the two major pathways of producing neuronal adenosine are from the dephosphorylation of 5'-adenosine monophosphate (AMP) by 5'-nucleotidase and the action of S-adenosylhomocysteine hydrolase on S-adenosylhomocysteine. A discussion of the synthetic pathways is beyond the scope of this paper (but see the reviews of Phillis and Wu 1981a).

The principal enzyme for production of adenosine in the brain is thought to be membrane bound 5'-nucleotidase. This enzyme is widely distributed but has high concentrations in the

thalamus, temporal lobe, and the medulla oblongata (Nagata et al 1984, Nakamura et al 1983). The 2'-nucleotidase enzyme is located in the cytosolic compartment and is considered unable to play a role in extracellular adenosine production.

Fast and slow uptake systems for adenosine have been found. The slow uptake mechanism has two high affinity components with K_m's of $1 u$M and $5 u$M. Both of these are partially inhibited by 2,4-dinitrophenol which suggests that there are metabolic and passive components present in this process. These uptake systems are sensitive to both Na^+ and Ca^{2+} ions. Evidence has been presented which suggests that the high affinity ($1 u$M) system is a glial uptake system, and the second high affinity system ($5 u$M) represents the neuronal uptake system (Bender et al 1980, Phillis and Wu 1981b).

The fast uptake system (K_m of $0.9 u$M) is thought to be mediated by a nucleoside carrier in the neuronal membrane. This uptake system is inhibited by low temperatures and also by adenosine analogues such as 2'- and 5'-deoxyadenosine, 2'-chloroadenosine and inosine (Bender et al 1981, Stone 1981).

Adenosine and its nucleotides can been taken up into the neuron and stored for reuse or be degraded. The primary degradation enzyme is adenosine deaminase, which converts adenosine to inosine. Two very potent inhibitors of adenosine deaminase, deoxycoformycin and erytho-9-(2-hydroxy-3-nonyl)-adenine (EHNA), when administered *in vivo* increase endogenous levels of adenosine dramatically. It is unknown if this enzyme is responsible for synaptic inactivation, although it is presumed to be the degradative enzyme at this site (Synder 1985).

C. Purine Localization

Early studies have shown little variation in the distribution among different brain regions (Phillis and Wu 1978). Some of the assessment difficulty stems from variations in neuronal size and density, glial distributions, vascular supply and of course metabolic rates (Stone 1981). Other problems have been encountered in the methods used to assess adenosine distribution (Synder 1985, Phillis and Wu 1981a). Concentrations for adenosine and its nucleotides have been

determined in whole brain (0.52μmol/kg) and the cerebral cortex using focal microwave irradiation technique (Phillis and Wu 1981a).

Assessment of possible regional variations of adenosine and its nucleotides has revealed significant variations in the distribution of enzymes responsible for producing adenosine in the CNS (Nagy et al 1984, Nagata et al 1984, Nakamura et al 1983).

High 5'-nucleotidase activity in the rat brain was found in the medulla oblongata, hippocampus and the thalamus. High 2'-nucleotidase activity in the rat brain was found in the cerebellum and the frontal lobe of the cerebral cortex (Phillis and Wu 1981a).

Using microwave irradiation the highest ATP concentrations in the rat brain were found in the sensorimotor cortex, entorhinal cortex, hippocampus, hypothalamus and thalamus (Wojiek and Neff 1982). Another study using the sensitive bioluminescent luciferin-luciferase assay was able to show high regions of ATP concentration in the basal ganglia, hippocampus, cingulate gyrus and the cerebellum (Phillis and Wu 1981a). Other methods of study such as immunohistochemical localization with antibodies sensitive to adenosine, have localized adenosine containing cell groups in the hippocampus (pyramidal cell layer), medial cerebral cortex, caudate and amygdala (Snyder 1985). Immunohistochemical markers for adenosine deaminase, the major degradation enzyme of adenosine, have revealed high adenosine deaminase content in the hypothalamus (Nagy et al 1984).

D. Release of Purines

Purinergic release is thought to be in the form of ATP, which is subsequently converted to adenosine and other purines (Burnstock 1979). Some investigations have demonstrated that adenosine formed approximately 86-99% of the labeled purines released from rat brain synaptosomes during K^+ evoked depolarizations (Di Iorio et al 1988, Phillis and Wu 1981a). Purine release from cortical synaptosomes has been shown to be Ca^{2+} dependent whereas hypothalamic synaptosomes showed a Ca^{2+} independent release.

Further release studies performed in the *in situ* brain demonstrated that upon electrical stimulation of the cortex prompted a five-fold increase in release of adenosine and its derivatives

(Sulakhe and Phillis 1975). This release from the cerebral cortex occurs predominantly as ATP which can then be hydrolyzed to form ADP, AMP, adenosine, inosine and hypoxanthine.

Storage of adenosine as a free nucleotide is very limited since brain concentration levels of adenosine are very low, approximately 2 nmol/g (Stone 1981). Therefore, if increased adenosine concentrations are needed, synthesis and/or degradation could occur, from the "pool" provided by neuronal uptake, catabolism and storage.

In summary, three separate brain regions containing high concentrations of adenosine: the cerebellum, the hippocampus and the thalamus. As is evident, additional work is needed to elucidate the role of adenosine in these adenine nucleotide regions of the brain.

E. Purinergic Receptors

Considerable confusion has existed about the presence of adenosine receptors and their characteristics. This has been complicated further since receptors for ATP, ADP and other nucleotides have also been found (Gehlert et al 1984) not only in the CNS but also throughout the periphery. Variations in nomenclature have also clouded the issue (Stone 1981).

Present studies indicate that adenosine receptors of the cortex are primarily localized at the nerve terminals (Shimizu 1979). Currently, four types of adenosine receptors have been described. These have been designated type A_1, two types of A_2 (A_2 and A_{2H}), and a type P receptor (Snyder 1985). In addition, another receptor type, A_3, has been proposed (Ribeiro and Sebastiao 1986). Only the A_1 and A_3 receptors will be discussed.

1. A1

This high affinity, inhibitory extracellular adenosine receptor is located on neuronal and glial cells. Activation of the A_1 receptor results in the imbibition of adenylate cyclase activity which subsequently leads to decreased levels of cAMP. The A_1 receptor has a K_d for adenosine at 80nM, and several radioligands have been developed. They are: N6-phenylisopropyladenosine (PIA), N6-cyclohexyladenosine (CHA) and 2'-chloroadenosine and have assisted greatly in A_1

receptor characterization (Snyder 1985, Phillis and Wu 1981a). This receptor is competitively antagonized by the methylxanthines (Daly 1983).

Based on binding studies, this receptor is activated by nanomolar concentrations of adenosine and mediates decreases in levels of cAMP (Fredholm et al 1982). The radioligand H^3-CHA is best suited as it binds only to A_1 and not A_2 receptors (Daly 1983).

It should be noted that A_1 receptors are modulated by guanine nucleotides and divalent cations. Binding to this receptor type is enhanced by manganese, magnesium and calcium ions, while sodium ions decrease binding and lithium and potassium are inactive (Goodman et al 1983). This suggests that G proteins couple the receptor complex to adenylate cyclase. This receptor has been found in high concentrations in the cerebellum, hippocampus, medial geniculate body, certain thalamic nuclei and in the substantia gelatinosa (Goodman and Synder 1982). The histochemical localization 5'-nucleotidase corresponds very well with A_1 receptor density for the rat brain.

It appears that electrophysiological responses to adenosine are mediated by this receptor (Dunwiddie and Fredholm 1984). Adenosine induced inhibition of evoked field EPSP's in the rat hippocampus, and inhibition of norepinephrine (NA) release by adenosine are also thought to occur through activation of this receptor (Jonzon and Fredholm 1985).

2. A_3

This receptor subtype has recently been proposed, based on the observations of electrophysiological responses to adenosine (Stone 1985, Phillis and Wu 1981a).

The A_3 receptor is hypothesized to be; i) located externally, ii) sensitive to blockade by the methylxanthines, iii) not coupled to adenylate cyclase, iv) and found in the heart and nerve endings (Ribeiro and Sebastiao 1986).

The physiological effect of adenosine at this receptor site on nervous tissue appears to be a decrease in neurotransmitter release. It has been suggested that the A_3 receptor is linked to a Ca^{2+} channel. A number of mechanisms have been proposed to account for the inhibition of neurotransmitter release including; i) decreased Ca^{2+} entry, ii) adenosine behaving as a Ca^{2+}

channel antagonist, iii) Ca^{2+} antagonism of the effects of adenosine, and iv) a reduction in the efficacy of Ca^{2+} for promoting the physiological response.

In conclusion, the A_1 receptor is thought to be important in mediating electrophysiological responses to adenosine while receptor A_2 may be important clinically in Lesch-Nyhan disease, a disorder of purine metabolism. The A_3 receptor may be important in modulating neurotransmitter release and $[Ca^{2+}]_i$ fluxes. The role, if any , for the P receptor is unknown at this time.

F. Purinergic Effects in the CNS

Behavioral effects of adenosine and its derivatives are manifested as muscular weakness, ataxia, drowsiness, hypothermia epileptiform discharges, and tonic-clonic convulsions (see next chapter, Phillis and Wu 1981, Gehlert et al 1984).

In the brain, adenosine primarily inhibits neuronal activity. The predominant mechanism of inhibition is thought to be a pre-synaptic blockade of excitatory neurotransmitter release but some evidence has accumulated for a mechanism of post-synaptic inhibition directly on the membrane. It should be noted that these processes have a high affinity specifically for adenosine, since other purines and pyrimidines such as inosine, guanosine, xanthine, cytidine, uridine, thymidine and their nucleotides are much weaker or inactive. These adenosine effects are readily blocked by the methylxanthines.

1. Extracellular Recordings

In general, application of adenosine and its agonists in the CNS result in a depression of synaptic transmission (Reddington and Schubert 1979, Schubert and Mitzdorf 1979, Dunwiddie and Hoffer 1980, Okada and Ozawa 1980, Segal 1982). This inhibition has been attributed to both pre-synaptic (Dunwiddie and Haas 1985, Schubert et al 1986) and post-synaptic (Okada and Ozawa 1980, Haas and Greene 1984, Greene and Haas 1985) sites of action.

In the olfactory cortex slice it was found that adenosine depresses the field EPSP in a concentration dependent and reversible manner (Kuroda 1983).

In the hippocampus, application of adenosine depresses the extracellularly recorded field potentials; this effect can be antagonized by the methylxanthines (Dunwiddie and Hoffer 1980). Conversely, the degradation enzyme, adenosine deaminase (ADA), increased the amplitude of the responses (Haas and Greene 1988). Uptake inhibitors of adenosine (i.e.. hexobendine) decreased the amplitude of the recorded potentials. It is interesting to note that the population spike amplitude was much more sensitive to the application of adenosine than the field EPSP amplitude. No differences were noted between the DG and the CA1 region, as adenosine had the same depressant effect.

Similar results in the CA3 region were obtained after perfusion of adenosine (Okada and Ozawa 1980). Current source density analysis has revealed that adenosine reduced synaptic currents, which is in accord with its presumed blockade of neurotransmitter release (Schubert and Mitzdorf 1979).

Using a variety of agonists and antagonists for adenosine Dunwiddie et al (1984) were able to demonstrate that the A_1 receptor is involved in mediating the electrophysiological effects following the application of adenosine.

Adenine nucleotides such as ATP, ADP, AMP, and cAMP have similar inhibitory actions whereas adenine, inosine, guanosine, cytidine and uridine have no depressant effect on the EPSP. Furthermore intracellular recordings (see below) have demonstrated no changes in action potential amplitude, membrane potential and input resistance. This depression of the post-synaptic potential without changing the electrical properties of the post-synaptic membrane may be mediated by a decrease in the efficacy of synaptic transmission possibly via reduced excitatory neurotransmitter release (Phillis and Wu 1981a).

Interactions between various neurotransmitters and adenosine have been reported. One such interaction is that of glutamate and adenosine. Iontophoretic application of glutamate depolarizes neurons by activation of Na^+, K^+ and Ca^{2+} conductances in the membrane (Heinemann and Pumain 1981). A number of studies (see Phillis and Wu 1981a) have suggested that adenosine may decrease Ca^{2+} flux into the cell. It would appear that adenosine can modulate pre-synaptic transmembrane Ca^{2+} fluxes, and thereby decreasing the efficacy of

glutamate application. An alternate explanation, which could account for the decreases described above, is that adenosine may directly inhibit glutamate release. This latter suggestion would be in keeping with the pre-synaptic effects of adenosine in modulating neurotransmitter release (Phillis and Wu 1981a, Snyder 1985).

Adenosine inhibits the release of acetylcholine, dopamine, norepinephrine, serotonin and GABA and these effects are antagonized by the xanthines (see Snyder 1985, Rebeiro and Sebastiao 1986). Both adenosine and norepinephrine increase cAMP levels in brain slices and together exhibit a synergistic effect (Segal 1981).

2. Intracellular Recordings

An early study by Okada and Ozawa (1980), demonstrated that application of adenosine (0.1-1.0 mM) resulted in a 1-5 mV hyperpolarization that could be washed out. In addition, spontaneous and synaptically evoked EPSP's were reduced during adenosine application. A possible explanation for this depression of post-synaptic potentials, without altering the pre-synaptic volley or post-synaptic membrane conductance, could be a reduction in the efficacy of synaptic transmission as a result of reduced neurotransmitter release.

It has been suggested that adenosine may activate a Ca^{2+} conductance however, Greene and Haas (1985) showed that injection of the intracellular Ca^{2+} chelator EGTA did not result in any significant changes in input conductance. Potassium currents were also examined and the delayed outward rectifier was not responsible for the changes seen in the afterhyperpolarization (AHP). Adenosine actions in prolonging the AHP are due to an enhancement of two different gK^{+}'s; one Ca^{2+}-dependent and one independent of Ca^{2+} and voltage (Haas and Greene 1988). This may have significant implications in the modulation of epileptiform activity (Haas et al 1984).

Two recent studies were able to demonstrate that release of adenosine is not Ca^{2+} dependent, as adenosine deaminase (ADA) or 6-(4-nitrobenzyl)thioinosine (NBTI) (an adenosine uptake inhibitor) still had pronounced effects in the absence of Ca^{2+} (Haas and Greene 1988, Scholfield and Steel 1988).

In summary, the primary action of adenosine in the CNS is the inhibition of neuronal activity. This effect appears to be modulated pre-synaptically by a reduction in neurotransmitter release, an increase in a post-synaptic K^+ conductance and an overall decrease in neuronal excitability. Some post-synaptic mechanisms have been postulated and include an increase in the AHP and increases in K+ conductance. In addition, adenosine inhibits the release of certain neurotransmitters and putative neuromodulators. Therefore, this endogenous compound has the ability to modulate a number of key cellular processes that are important in controlling the excitability of the neuron.

The experiments described below examined the effects of purine agonists and antagonists on rat hippocampal neurons.

G. Methods

The methods have been described above and in the previous chapter. The pharmacological compounds were obtained from Sigma Chemical Co. and were applied for a 5 or 10 min as noted in the results section. Some of the experiments described in this section were performed only to confirm the findings in the literature, therefore statistical analysis was not done if the N was less than three.

H. Results

The extracellular responses described below were obtained from a total of 50 rat hippocampal slices. Each slice received only one application of a particular compound. Statistical analysis was performed using two-sample two-tailed T-tests.

1. Adenosine

The hemisulfate salt of adenosine was applied in concentrations of 1, 2, 4, 5, 10, 20, 40 and 80 uM. Application of adenosine for 10 min resulted in no significant depression of the evoked population spike response at the 1 (N=2), 2 (N=2), 4 (N=2), 5 (N=2) and 10 uM (N=6) doses. However, 20 (N=6), 40 (N=6) and 80 uM (N=6) application resulted in a significant dose dependent decrease in the population spike amplitude ($p < 0.008$) (Figure 3.12, 3.13). The reversed field EPSP slope was significantly reduced only at 80 uM concentration ($p < 0.02$) and there was a significant difference between 20 uM and 80 uM ($p < 0.03$) The 80 uM dose reduced the evoked responses with the depression of population spike amplitude reaching a maximum 10-15 min following the onset of adenosine application.

Application of the adenosine agonist, 9-B-RFA (N=6 at all doses), required considerably higher doses, with 100 uM causing a slight decrease in population spike amplitude. This compound was not investigated further.

Figure 3.12: Typical evoked responses of the CA1 region following the application of adenosine, 2-chloroadenosine, adenosine deaminase, or theophylline.

Typical evoked population spike responses from the CA1 region. The left panel illustrates the control population spike response prior to drug perfusion. The center panel is the same evoked response immediately following the application of the drug. The numbers in the parenthesizes indicate the duration of the drug application (i.e.. (10) = 10 min). The right panel shows the same population spike following a 30 min wash with drug-free ASCF. Scale is as indicated by the bars.

Perfusion of AD or the highly selective A_1 receptor ligand, CAD, results in the reduction of the evoked population spike. 30 min following the application of these compounds the population spike had returned to pre-drug values or even potentiated the population spike as seen with CAD.

Perfusion of the degradative enzyme, ADA, caused no immediate effect following application of the compound, but 30 min later the population spike potentiated to almost double pre-drug values. This finding is expected if ADA removes endogenous AD, thereby reducing tonic inhibitory control.

Perfusion of the purinergic antagonist, THP, resulted in potentiation of the evoked population spike and concurs with the results of previous findings. This potentiation is no longer present 30 min after the replacement with drug-free ACSF.

Abbreviations:

AD - Adenosine (Hemisulfate)

CAD - 2-Chloroadenosine

ADA - Adenosine Deaminase

THP - Theophylline

CONTROL   POST DRUG   30MIN WASH

AD (80uM)

CAD (5uM)

ADA (1U/mL)

THP (100uM)

(10)

(5)

(5)

(10)

2mV

5msec

2mV

5msec
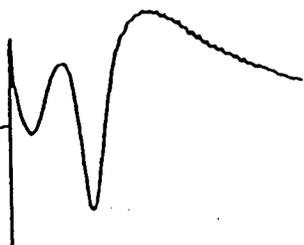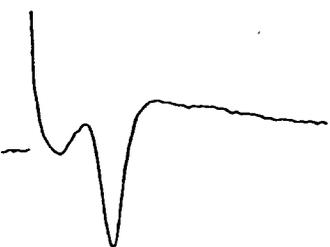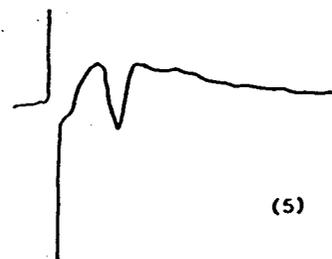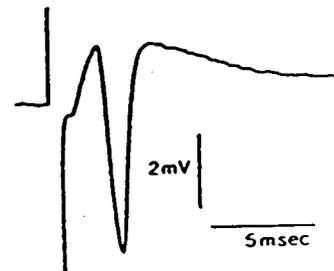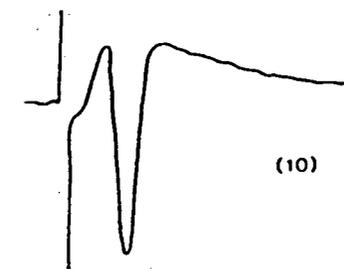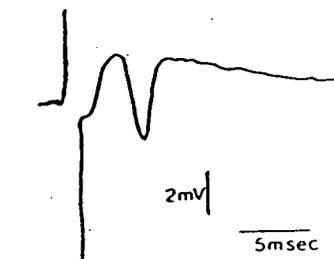
Figure 3.13: The effects of adenosine perfusion on evoked population responses.

Time course of adenosine (hemisulfate) effects in the CA1 region, following a 10 min application of either 20 or $80u$M. Perfusion period is denoted by the filled horizontal bar at T=0 min.

A. Perfusion of $20u$M (N=6) adenosine had a significant effect on the evoked population spike amplitudes, however application of $80u$M (N=6) elicited a massive decrease in the evoked population spike amplitude. It is important to note the long period of time required for $20u$M and $80u$M adenosine to take effect. Error bars are S.E.M's. (* $p < 0.04$)

B. Perfusion of $20u$M (N=6) adenosine had no significant effect on the evoked reversed field EPSP slope. Application of $80u$M (N=6) elicited a significant decrease in the evoked population EPSP slope. Error bars are S.E.M's. (* $p < 0.002$)

A. ADENOSINE (HEMISULFATE) PSA
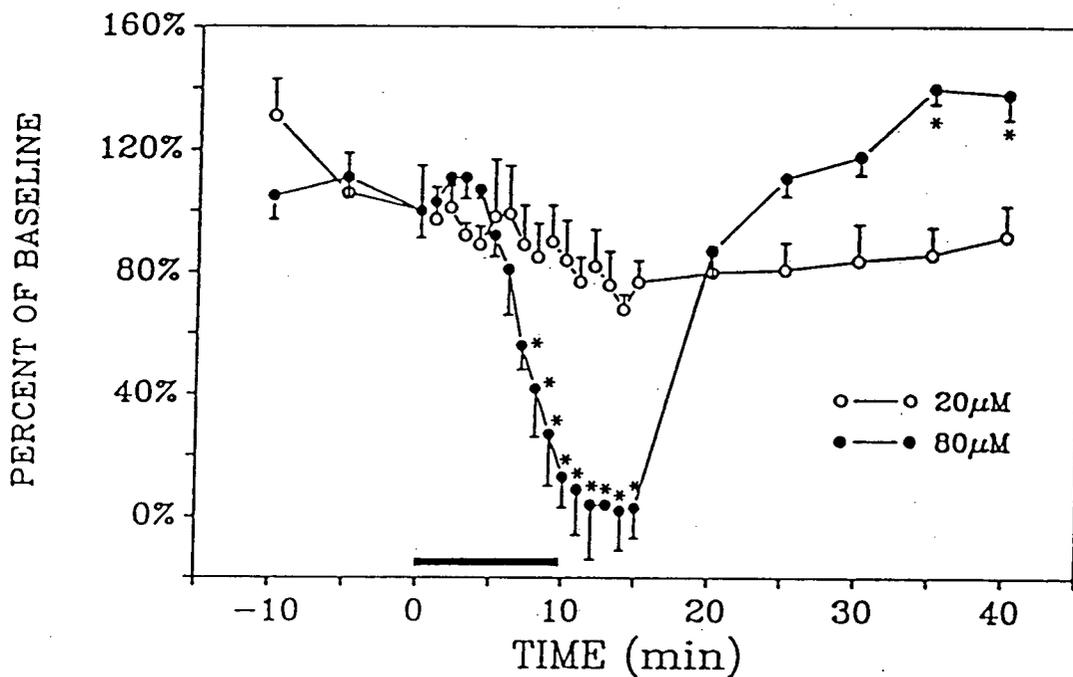
B. ADENOSINE (HEMISULFATE) REVERSED FIELD EPSP SLOPE
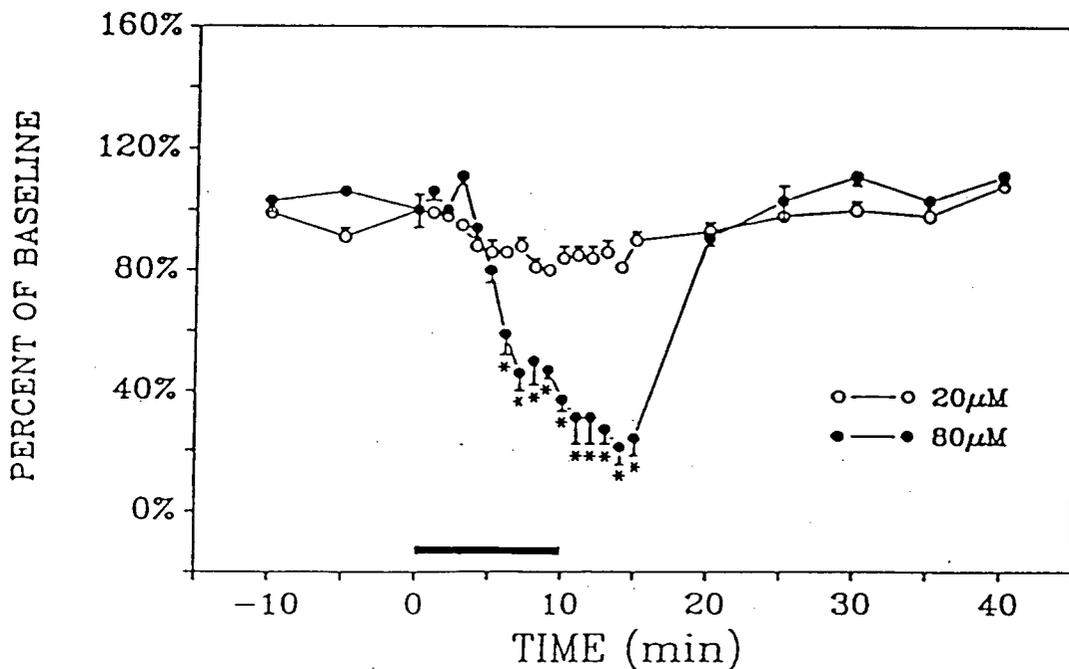
Figure 3.14: Comparison of evoked population spike amplitudes following AD, CAD and ADA perfusion in the CA1 region.

A. Comparison of population spike amplitude changes following perfusion of $5\,u$M solution of adenosine (AD) or 2-chloroadenosine (CAD) in the CA1 region of the hippocampus. In this figure the drug was applied for 5 min as indicated by the filled horizontal bar at $T = 0$ min. While the application of AD elicited no change in the population spike amplitude, application of CAD at the same concentration and duration resulted in a long lasting decrease in the population spike amplitude. This depression by CAD took 35 min to recover to pre-drug values and even exhibited some potentiation ($N = 2$ for both drug concentrations).

B. Perfusion for 5 min of the degradative enzyme, adenosine deaminase (ADA) at a concentration of 0.5 U/ml resulted in no change in the evoked population spike amplitudes. However, application of 1.0 U/ml ADA for 5 min (as indicated by the filled horizontal bar at $T = 0$ min) elicited a transient decrease in the population spike amplitude, but after 30 min following the application of this compound a potentiation of the population spike amplitude was seen ($N = 2$ for both concentrations).

A. ADENOSINE (5μM) COMPARISON
PSA
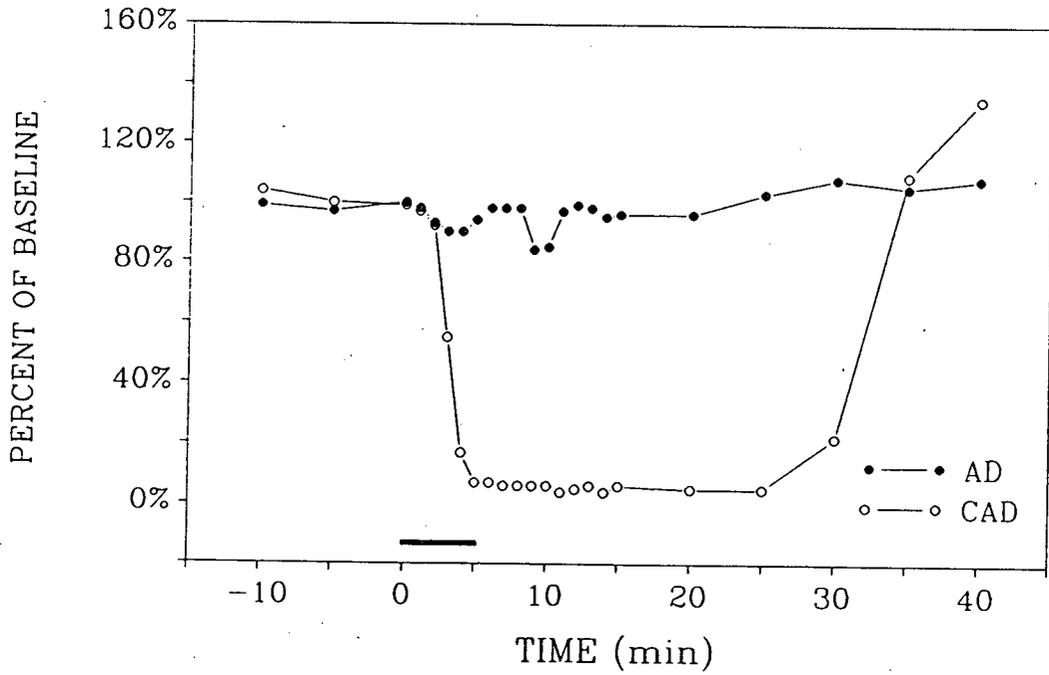
B. ADENOSINE DEAMINASE
PSA SLOPE
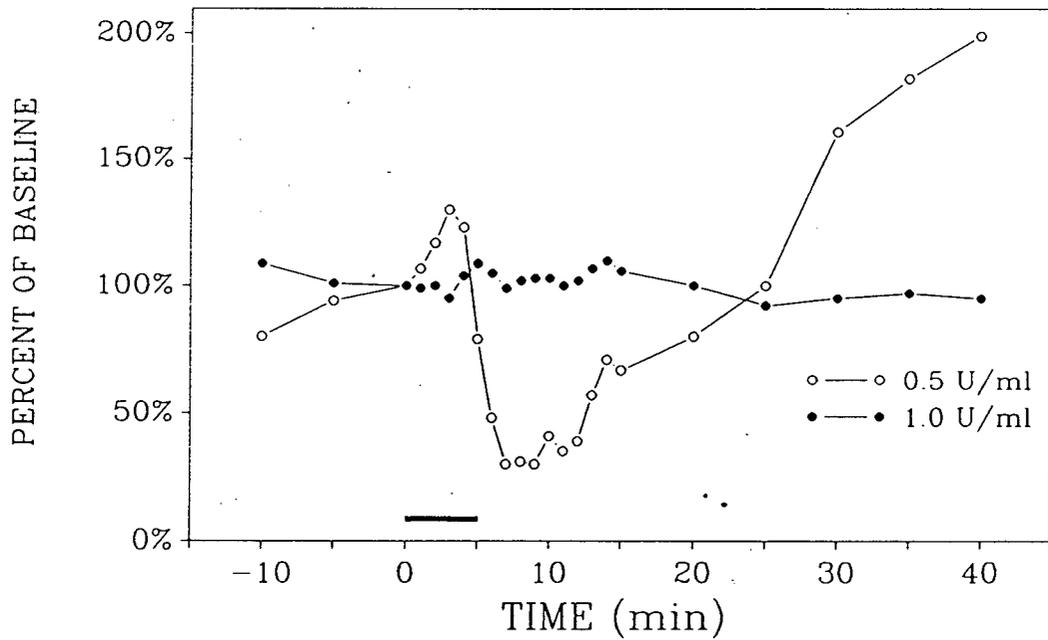
The adenosine agonist, 2-chloroadenosine (CAD), was extremely potent in decreasing both the population spike amplitude and the reversed field EPSP slope. Application of 5 (N=2) and 10uM (N=2) for 5 min abolished the population spike amplitude and significantly reduced the reversed field EPSP slope (Figure 3.12, 3.14A). This abolition was followed by recovery and even slight potentiation of the population spike amplitude. Application of 100uM (N=2) for 10 min abolished the population spike and this blockade was maintained for the duration of the experiment (30 min post drug application). The onset of the effects of CAD was rapid: within 3 min following application of this compound the population spike decreased to 50% of control values. Whenever recovery could be seen there was a rebound increase in the population spike (Figure 3.14A).

In general, the application of adenosine or CAD, elicited an decrease in the evoked parameters measured, and these reductions were dose dependent.

2. Adenosine Deaminase

Application of adenosine deaminase, the degradative enzyme of adenosine, resulted in a delayed potentiation of the population spike. Application of 0.5 U/ml (N=2) for 5 min did not result in changes in population spike or in the slope of the population EPSP (Figure 3.12, 3.14B); however, perfusion of 1U/ml (N=2) elicited a decrease in the population spike (30%) which was followed by a potentiation of the population spike at the end of the 40 min experiment (199%). These results substantiate the hypothesis that extracellular endogenous adenosine modulates hippocampal excitability. Degradation of endogenously released adenosine would thus result in a potentiation of the evoked responses.

3. Theophylline

Application of the adenosine antagonist theophylline, at 100uM (N=2) leads to a reversible increase in the population spike, confirming previous experiments (Figure 3.12).

I. Discussion

The primary electrophysiological action of adenosine is inhibition of neuronal excitability. Using evoked population spike amplitude and reversed field EPSP slope measures, the present results demonstrate that application of adenosine and its agonists results in an inhibition of evoked responses. Although this confirms findings previously reported (Schubert and Mitzdorf 1979, Dunwiddie and Hoffer 1980), the results presented here demonstrate that the effects of purine application are dependent upon the duration of adenosine application, in contrast to several studies which have reported that low concentrations of adenosine elicit reductions of the population spike (i.e.. 10uM, Reddington and Schubert 1979). In those reports, adenosine was added to the perfusing ACSF for an unspecified duration. Although previous studies have reported that maximal inhibition of evoked responses is achieved at concentrations as low as 20uM adenosine (Reddington and Schubert 1979), our own results suggest that larger concentrations are needed when applied for shorter periods of time. These time dependent effects could be due to concentration changes in the amount of endogenous adenosine that is being released during normal synaptic activity, and indicate that perfusion of small doses of adenosine for short periods of time does not significantly alter the purinergic regulatory mechanisms (see Chapter 4). On the basis of the above studies, including our own findings, it is imperative that the duration of application of the compound is held constant for the comparison of the effects of various doses.

Of the purinergic agonists tested, 2-chloroadenosine was found to have the most potent depressant activity. This finding confirms those presented previously (Dolphin 1983, Dunwiddie and Hoffer 1980). Maximal doses required for inhibition of evoked population responses have been reported at 0.5uM (Reddington and Schubert 1979, Dunwiddie and Hoffer 1980).

The results presented in Figure 3.12 and 3.14 illustrate that 80uM AD takes 10 min to have a maximum effect on either the population spike amplitude or the slope of the reversed field EPSP. Conversely, 5uM CAD is maximally effective within 4 min of application and has a long duration effect on the evoked measures. These findings are exactly opposite to those results reported by Dunwiddie and Hoffer (1980), who found that adenosine was effective only during the perfusion of the compound, and CAD was maximally effective only at the end of the drug

application. It should be noted that stratum radiatum field EPSP measures were used in their study. They concluded that adenosine is more effective in the dendritic regions, and comparisons between lamina of the hippocampus have to be made with caution. Evidence also exists that adenosine has its effects predominantly in the dendritic regions of the CA1 hippocampal pyramidal cell (Schubert and Mitzdorf 1979).

Application of adenosine antagonists such as theophylline result in an increase in the electrophysiological measures in the rat hippocampal slice. These findings have been well established (Dunwiddie and Hoffer 1980, Phillis and Wu 1981a).

Adenosine deaminase (ADA), the degradative enzyme for adenosine, should, when applied to the rat hippocampal slice, increase the evoked population responses, if endogenous adenosine is being released. In the experiments described above we report that following ADA application there is in fact a potentiation of the population spike amplitude (Figure 3.12, 3.14). It is interesting to note that application of ADA (1.0 U/ml) elicits a transient decrease in the measured population spike amplitude, not reported previously. Previous studies mention only the potentiation following ADA application (Dunwiddie and Hoffer 1980, Haas and Greene 1988).

In summary, the results presented above demonstrate that adenosine and its agonists inhibit hippocampal excitability. Application of the degradative enzyme or adenosine antagonists leads to potentiation of the evoked responses. These findings are consistent with those previously reported, and further support the hypothesis that adenosine is: i) being released endogenously, ii) exerting a tonic inhibitory influence under normal conditions, iii) modulating pre-synaptic neurotransmitter release, and iv) initiating cellular mechanisms by interacting with adenylate cyclase. Such an endogenous modulation could have significant effects under pathophysiological conditions such as epilepsy.

IV. CHAPTER SUMMARY

Inhibitory mechanisms in the brain are of crucial importance in controlling the excitatory influences in normal and pathophysiological processes. Recent work investigating two such inhibitory classes of compounds has suggested that they may play a crucial role in maintaining a tonic influence. Serotonergic and purinergic systems have been found in the rat hippocampus although key differences exist.

The serotonergic system derives its inhibitory actions from 5-HT containing fibers that arise from the raphe nuclei in the brainstem. Characterization of these fibers and in particular their receptor types has led to the identification of numerous receptor types. The 5-HT_{1A} receptor type is found in high density in the CA1 and DG regions, while the 5-HT_2 receptor type is found primarily in the DG. Based on the experiments presented above, we propose that the 5-HT_{1A} receptor is in a position to powerfully inhibit excitatory influences in this brain region. The proposed mechanism of action for the effects described is most likely an increase in K^+ conductance, which serves to shunt excitatory currents (Haas and Greene 1988).

While the serotonergic system exhibits "classical" neurotransmitter activated inhibition, the purinergic system utilizes a very different mechanism. Differences in the localization of receptors for serotonin and adenosine would suggest by themselves very different functions. Purines in the hippocampus are found in a large number of neurons, unlike the serotonergic system which has a discrete fiber pathway innervating the hippocampus. Due to its ubiquitous presence in hippocampal neurons, and its role in the production of ATP, it seems likely that this compound is released endogenously. This release, as either adenosine or ATP, is independent of Ca^{2+}, another difference from serotonergic systems. Based on the evidence in our results and from other studies, the implication is that the actions of purinergic compounds in the hippocampus are inhibitory. The mechanisms by which adenosine exerts its effects are different from those of serotonin. Although some post-synaptic effects have been postulated, the principal action of adenosine is thought to be via inhibition of pre-synaptic neurotransmitter release (see Phillis and Wu 1981).

CHAPTER 4 - PHARMACOLOGICAL MODULATION OF LOW CALCIUM INDUCED BURSTING

I. INTRODUCTION

There are many types of epileptiform activity and a myriad of models designed to simulate the changes seen in human epilepsies, but no one model addresses all the complexities seen in this prevalent (approximately 1% of the population) disorder. In attempting to discover the underlying causes of epileptiform activity, early experimenters noted that changes in extracellular Ca^{2+} occurred. At that time as today, it was difficult to discern whether or not these Ca^{2+} changes are the inducing factor or the result of the epileptic discharges.

It is of interest then, to ascertain whether or not there is a change in extracellular and intracellular Ca^{2+} levels prior to, during or after an epileptic event. With the advent of Ca^{2+} sensitive electrodes, studies were performed on the cortex of cats during normal synaptic events (Somjen 1980, Nicholson et al 1978). It was shown that in the cell body layer extracellular Ca^{2+} ($[Ca^{2+}]_0$) decreases during these normal synaptic events. These studies were extended by Krnjevic and his colleagues (Krnjevic et al 1980, 1982a,b) who demonstrated that there are decreases in $[Ca^{2+}]_0$ and increases in $[K^+]_0$ following high frequency stimulation of the entrohinal cortex. Heinemann et al (1977, 1986) were able to establish that decreases in $[Ca^{2+}]_0$ in the cell body layer were also prevalent in epileptic tissue. In their model, it was difficult to discern whether these changes were causative or simply a result of changes induced by epileptiform activity. It should be noted that changes in extracellular Ca^{2+} concentrations have not been found for every type of epilepsy.

In 1982 two independent groups reported that the removal of $[Ca^{2+}]_0$ from the perfusion media could induce epileptiform-like bursting in the hippocampal slice (Jefferys and Haas 1982, Taylor and Dudek 1982). This was a surprising and an important finding, as it had until then assumed that synaptic activity was important in the production of epileptiform activity.

Numerous authors have used this model to investigate the anticonvulsant effects of various compounds (Haas et al 1984, Heinemann et al 1985, Snow et al 1985, Agopyan et al 1985, Hood et al 1983).

II. THE LOW CALCIUM INDUCED BURSTING MODEL OF EPILEPSY

In vivo studies by Heinemann et al (1977) showed that $[Ca^{2+}]_0$ levels fall during seizures induced by pentylenetetrazol. Jefferys and Haas (1982) and Taylor and Dudek (1982) simulated these conditions in the *in vitro* hippocampal slice. Following perfusion of ACSF with concentrations of 0.2mM Ca^{2+} and high Mg^{2+} (up to 5mM) for 1-2 hours, slices exhibited bursting behavior which could be reversed by returning them to normal ionic ACSF solutions. These bursts are characterized as negative going shifts upon which population spikes may be superimposed (Figure 4.2B). A characteristic of the bursts is that they occur primarily in the CA1 pyramidal cell layer, spread to the dendritic regions (Figure 4.3), and propagate in either direction from a locus in the CA1 region. Only on rare occasions can such bursts be found in the CA3 region (Haas and Jefferys 1984, Konnerth et al 1986).

Intracellular correlates of these bursts are difficult to obtain but the exchange of normal ACSF with low Ca^{2+} ASCF resulted in a maintained depolarization of approximately 20mV by the time the bursts had started (Haas and Jefferys 1984). While the pyramidal cells were depolarized, the input resistance was slightly but not significantly reduced. However, upon restoration of the resting membrane potential to its original value the input resistance was increased by 25%. In addition, the Ca^{2+}-dependent afterhyperpolarization (AHP) which normally limits the rate of firing in CA1 pyramidal cells (Alger 1984, Lancaster and Adams 1986, Storm 1987) was found to have vanished after incubation in the low Ca^{2+} ACSF. Differential recordings between extracellular and intracellular electrodes verified that the low Ca^{2+}-induced membrane depolarization was sufficient to trigger action potentials (Haas and Jefferys 1984).

Changes in the extracellular ion concentrations were also assessed using Ca^{2+}-sensitive electrodes (Haas and Jefferys 1984, Konnerth et al 1986, Heinemann et al 1982, 1986). Low Ca^{2+} induced bursts have been associated with increases in $[K^+]_0$ of up to 11mM and reductions of $[Na^+]_0$ by 10mM and $[Ca^{2+}]_0$ to 0.1mM, suggesting that there is a significant influx of Ca^{2+} and Na^+ into the neuron (as well as K^+ efflux). It might be argued that this residual Ca^{2+} could be responsible for bursting, however, when the slices are perfused with the Ca^{2+} chelator EGTA (2mM) no significant changes in burst frequency occur. This finding along with other

evidence suggests that changes in Ca^{2+} do not play a role in the maintenance of the bursting response (Haas and Jefferys 1984).

A distinction between generation, synchrony and spread of bursting behavior should be made, but some of the mechanisms responsible for these phases may be interdependent.

A. Burst Generation

While the long period of time required for the onset of low Ca^{2+} bursting is dependent upon a number of factors, two deserve mention One factor contributing to burst generation is the slow depletion of $[Ca^{2+}]_0$ from the hippocampal slice, which can have marked effects on synaptically evoked responses (Jones and Heinemann 1987) (Figure 4.1). Another factor is the slow depletion of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (see Chapter 6).

B. Burst Synchronization

The reduction in extracellular Ca^{2+} could lead to a decrease in the efficacy of GABAergic and other inhibitory synaptic inputs (Heinemann et al 1986). Additional changes that occur when $[Ca^{2+}]_0$ is decreased include; i) changes in certain membrane conductances (e.g.. Ca^{2+}-dependent K^+ conductances) which further affect the ability of the cell to control intrinsic burst capabilities (Schwindt and Crill 1980) and, ii) removal of $[Ca^{2+}]_0$, which leads to a destabilizing influence on the surface of the membrane and further to cellular hyperexcitability (Frankenhauser and Hodgkin 1957, Erulkar and Fine 1979).

Dudek et al (1986) suggest that there are four predominant mechanisms underlying synchronization of epileptiform activity: i) recurrent excitatory chemical circuits, ii) electrotonic coupling via gap junctions, iii) electrical field effects or ephaptic interactions, and iv) changes in extracellular ion concentrations. In the low Ca^{2+} bursting model the recurrent excitatory chemical synapses are inactive due to the blockade of synaptic transmission. New evidence suggests that extracellular ions such as K^+ act on a time scale too slow for the required synchronization of low Ca^{2+}-induced bursts.

At the present time there is evidence from both electrophysiological studies and intracellular dye injections (e.g.. Lucifer yellow) that approximately 5-7% of hippocampal neurons (CA3, CA1) are coupled, presumably by gap junctions (MacVicar and Dudek 1981). An increase in $[Ca^{2+}]_i$ could uncouple cells by the closure of gap junctions between cells (Lowenstein 1981, Rao et al 1987). Although electrotonic coupling (dye coupling, gap junctions) may contribute to seizure synchronization, the degree of involvement remains unclear (Andrew et al 1982; Dudek et al 1983, 1986).

Electrical field effects or ephaptic interactions may contribute significantly to the normal function of the hippocampus (Taylor and Dudek 1984b, Richardson et al 1984, 1987, Yim et al 1986). Furthermore, there is an important role for electrical field interactions as a possible mechanism for synchronization of hyperexcitability (Taylor and Dudek 1984a). This study used current-source density analysis to locate a current sink in the somal region of the CA1 pyramidal cells. This finding corresponds to our findings of burst generation in the somal region of the CA1 region and not the dendritic regions (Figure 4.3). The high cellular density of the CA1 pyramidal layer may also contribute to the maintenance of these seizure-like events for a considerable period of time.

C. Spread of Bursts

Many of the mechanisms suggested to account for the synchrony may also contribute to the spread of the low Ca^{2+} induced bursts. One possible mechanism of spread of bursting activity to neighboring cells might be via changes in extracellular K^+ ($[K^+]_o$). Yaari et al (1986) have shown that an increase in $[K^+]_o$ may precede or be associated with the spread of burst activity from one hippocampal region to another. An increase in $[K^+]_o$ and concomitant decrease in $[Ca^{2+}]_o$ (Konnerth et al 1984; Yaari et al 1986) both enhance neuronal excitability. Lux et al (1986) suggested that the increases in the extracellular space may enhance and be responsible for the spread of $[K^+]_o$ from the focus to other sites of the hippocampus and neocortex. Increased electrotonic coupling may also enhance cell to cell communication and may facilitate electrotonic spread of burst activity (Dudek et al 1986).

Current evidence suggests that non-synaptic mechanisms can play an important role in the generation, spread and synchronization of epileptiform activity. Based on the strong inhibitory influence that serotonergic and purinergic systems have in normal hippocampal electrophysiology, coupled with the findings that these two classes of compounds are important in modulating certain pathophysiological states, the following experiments were performed to assess their ability to inhibit low Ca^{2+} induced bursting in the CA1 region of the *in vitro* hippocampal slice.

III. THE ROLE OF SEROTONERGIC SYSTEMS IN EPILEPSY

The possible involvement of the serotonergic systems in epileptiform activity was first assessed in early studies by Garattini and Valzelli (1957). Using the electroshock model of convulsant activity, they reported a transient increase in brain serotonin concentrations with a return to baseline values by 24 hours after a single convulsive stimulus. Lesions of the 5-HT containing pathways result in facilitation of kindling (Racine et al 1979).

While some models demonstrate an involvement of serotonergic systems in epileptiform activity, others (e.g.. cobalt focus) show no change (Craig and Colsanti 1986). Similiarily, Mongolian gerbils that exhibited generalized tonic-clonic and myoclonic type seizure activity had no change in brain 5-HT content following a seizure (Cox and Lomax 1976), nor did beagle dogs with generalized tonic-clonic convulsions (Edmonds et al 1979).

In contrast, one seizure model that appears to be involved with changes in the serotonergic system is audiogenic mice. Assays of brain 5-HT levels have shown massive reductions accompanied by changes in the rate of monoamine synthesis (Kellogg 1971, Schlesinger et al 1968). Intracerebroventricular administration of 5-hydroxytryptophan (5-HTP) decreased the intensity of audiogenic seizures (Schlesinger et al 1968). Genetically epilepsy-prone rats (GEPR) also showed a decrease in brain 5-HT content (Laird et al 1980), and an increase in brain 5-HT levels (5-HTP injections) in such animals dramatically reduces seizure intensity and susceptibility (Jobe et al 1986).

The involvement of serotonin within the hippocampus and other central nervous system (CNS) regions in epileptiform activity has been recently reviewed by Waterhouse (1986) and Jobe et al (1986). Several studies (Loscher and Czuczwar 1985; Thompson and Neuman 1986) have

shown that the selective high affinity agonist for the 5-HT$_{1A}$ receptor site, 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) (Gozlan et al 1983; Hall et al 1985) is devoid of anticonvulsant activity in several models of epilepsy.

Very few studies have examined the effects of the specific receptor subtypes in epileptiform activity. Only recently have attempts been made to elucidate the role of the 5-HT$_{1A}$ receptor in the kindled (Loscher and Czuczwar 1985) and the noxious stimulation model (Thompson and Newman 1986). Based on this relative paucity of information about the 5-HT$_{1A}$ receptor and its possible role in epilepsy, we have now determined that 8-OH-DPAT has a highly specific mode of action in the low Ca^{2+} burst model of epilepsy.

Similar to the 5-HT$_{1A}$ receptor, the 5-HT$_2$ subtype of serotonin receptors has not been investigated in pathological states such as epilepsy. Using the selective 5-HT$_2$ agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) (Glennon et al 1988) we examined the effects of this compound on the low Ca^{2+} induced bursting model of epilepsy.

In a brief study, Haas et al (1984) demonstrated a reduction in burst rate following application of 5-HT, but no attempts were made to determine the receptor subtype mediating this decrease in burst rate. In addition, this study did not indicate the duration of drug application, and only two doses (10uM and 100uM) were used.

The present study examined the effects of 5-HT, the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT, and the selective agonist for the 5-HT2 receptor site, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) were tested for their ability to inhibit this type of epileptiform activity (Glennon et al 1988). It is important to note that the perfusion of ACSF low in [Ca^{2+}] removes the tonic inhibitory influence of Ca^{2+}-dependent 5-HT release. The removal of 5-HT may be sufficient allow to the initiation or generation of low Ca^{2+} bursts.

A. Methods

Experiments were performed as described in the methods section in Chapter 2. Once the hippocampal slices were prepared, recording electrodes were placed in the stratum radiatum (SR) of the CA1 region and lowered to an approximate depth of 75-150um below the surface of the slice.

Figure 4.1: Changes in the evoked population spike following perfusion with low Ca^{2+} ACSF.

A. Evoked population spike responses illustrating the loss of synaptic efficacy from a typical experiment following the application of low Ca^{2+} ACSF. 1) control response, 2) after 5 min low Ca^{2+}, and 3) after 15 min low Ca^{2+} ACSF. Although not shown similar degradation of evoked responses can be seen in the dendritic stratum radiatum field EPSP's.

B. This figure graphically illustrates the loss of evoked population spike amplitudes following the application of low Ca^{2+} at T=0 min for 30 min (N=2). Note the rapid decline and maintained depression of population spike amplitude in the CA1 region of the hippocampus.

A.

2mV

5msec
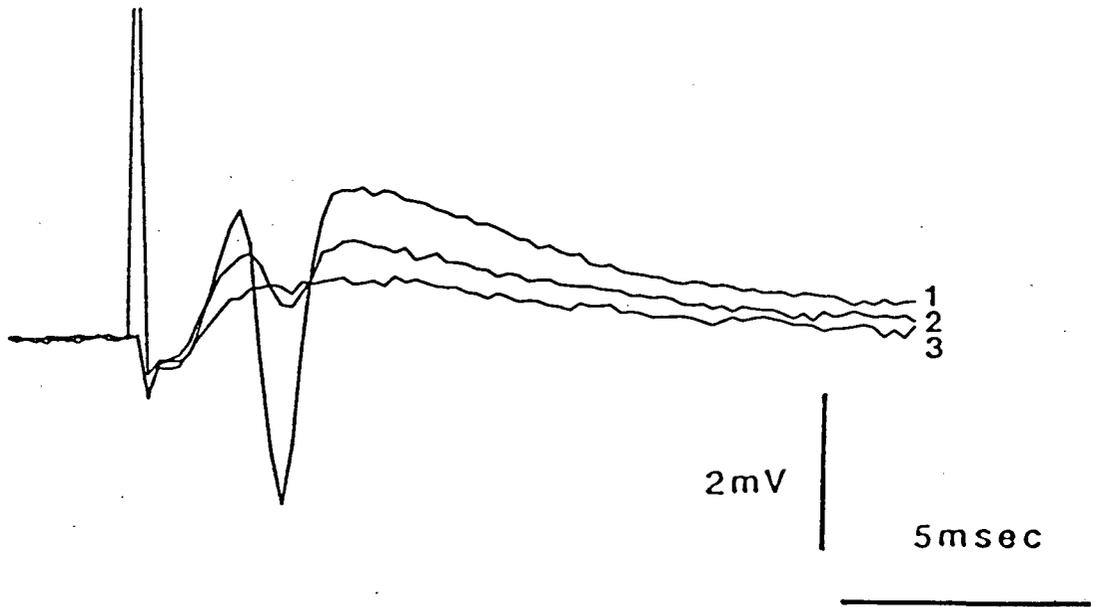
B.
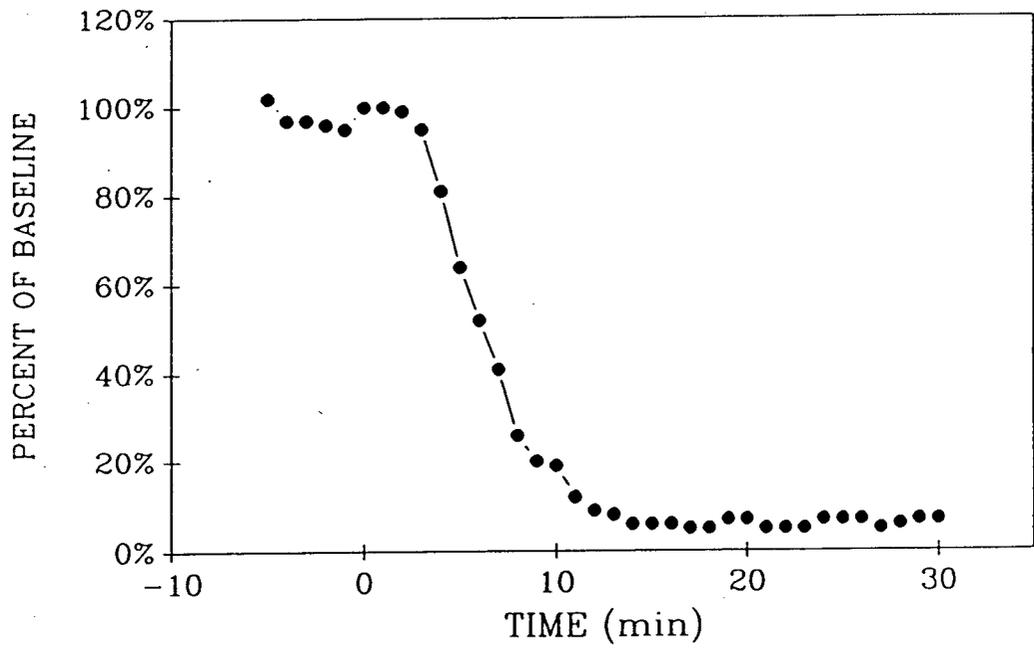
Figure 4.2: Typical low Ca^{2+} induced bursting in the CA1 region.

In hippocampal slices perfused with ACSF low in Ca^{2+}, spontaneous bursting started within 60-90 min and could be recorded for 3-4 hours.

A. This figure schematically illustrates a transverse hippocampal slice indicating the position of the recording electrode with a typical burst recording shown. The bursts are characterized as a rapid depolarization of 2-20mV upon which population spikes may be superimposed (see B below).

B. The majority of bursts demonstrated population spikes superimposed on the depolarizing wave. These are seen more clearly when the time scale is expanded as indicated by the scale bars. The expanded burst is the same burst at all of the time scales shown.

A.

Bursts

4mV

2min

CA1

DG

CA4

CA3

B.

8 mV

1min

1sec

1sec
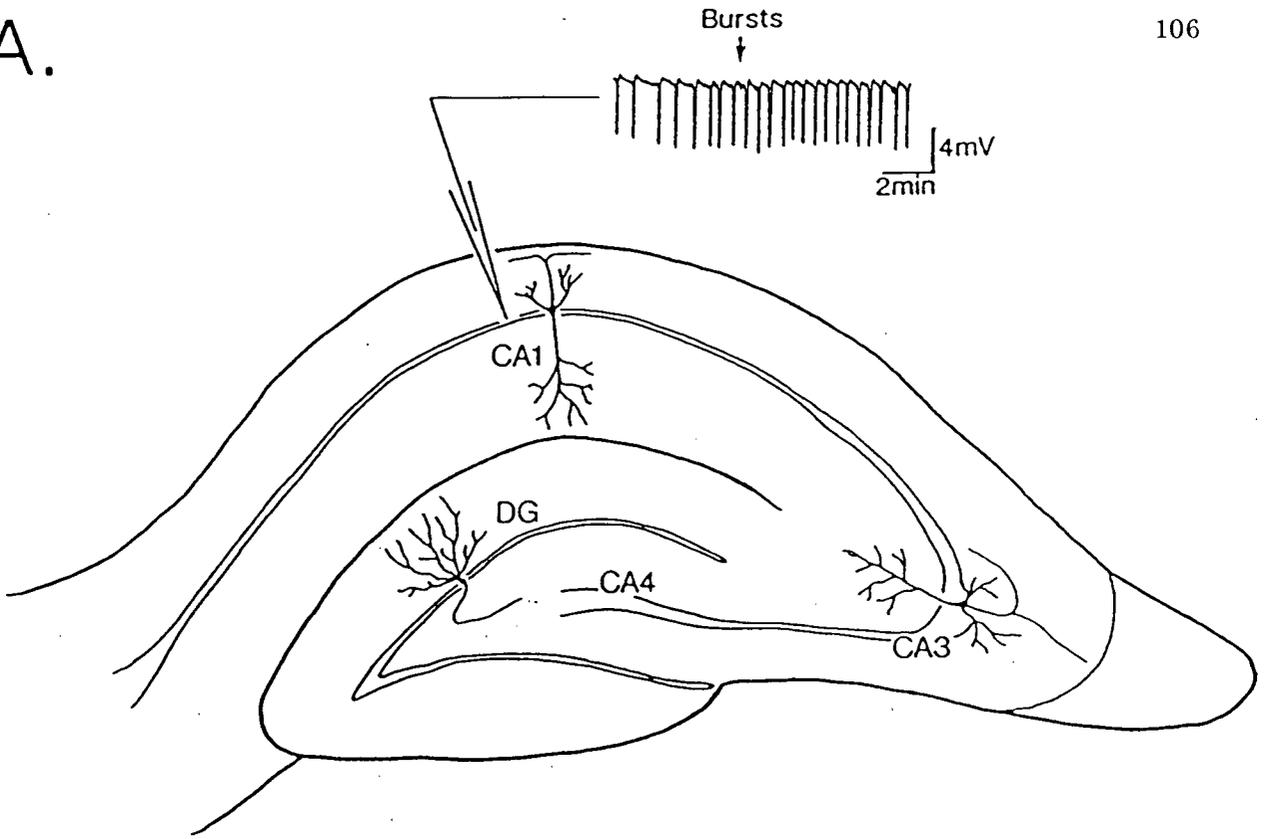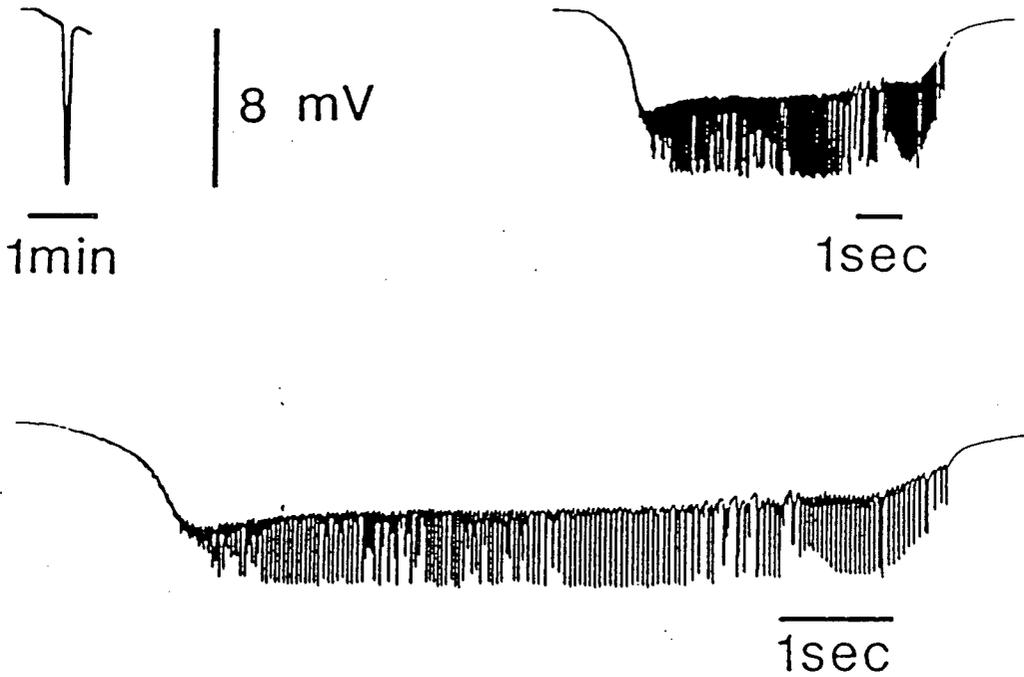
Figure 4.3: Comparison of burst activity between different hippocampal regions.

A. Simultaneous recordings from the CA1 pyramidal cell layer (SP) and the stratum radiatum (SR) in the same slice. Careful analysis demonstrates that the burst activity originates in the SP region, while the smaller inverted bursts in the SR represent the spread of the bursts seen in the SP region.

B. Simultaneous recordings from the CA1 pyramidal cell layer (SP) and the dentate gyrus (DG) granule cell layer (GL) in the same slice. Analysis demonstrates that the burst activity originates in the SP region, while the DG GL bursts represent the spread of the bursts seen in the SP region. This figure also illustrates the occurrence of the small and irregular depolarizations (*) that develop in the granule cell layer of the DG.

A.

CA1 SP

CA1 SR

4 mV

5 min

B.

CA1 SP

DG GL
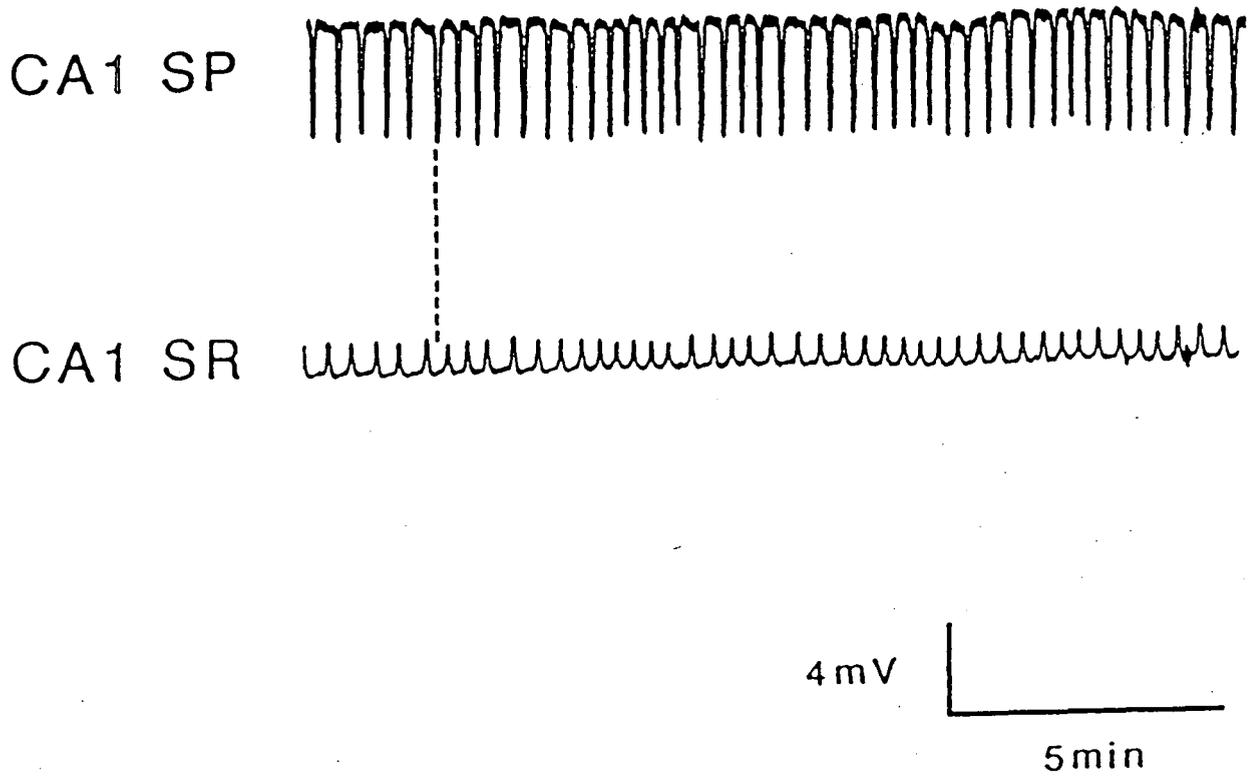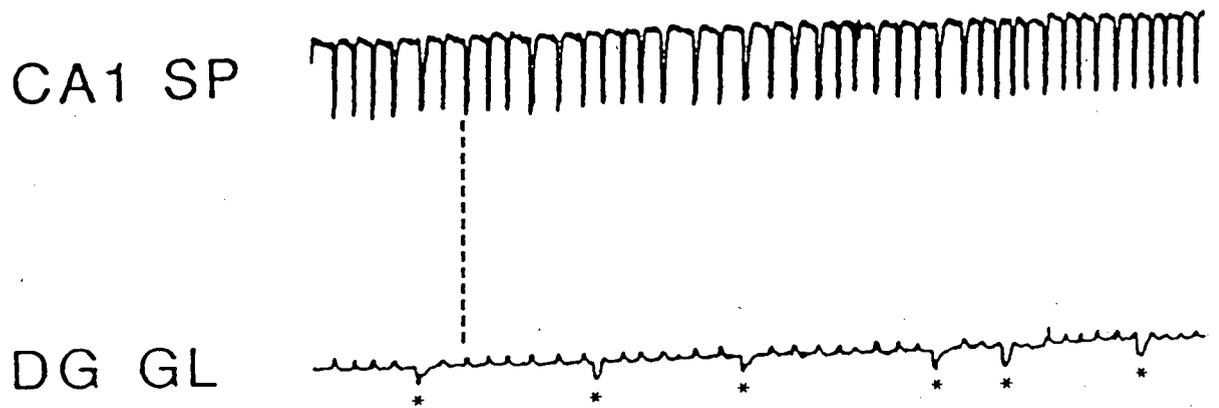
In these experiments, the $CaCl_2$ was not present in the ACSF and the Mg^{2+} concentration was increased to 1.7mM. Introduction of the low Ca^{2+} ACSF resulted in the complete disappearance of the evoked population responses within 10-15 min (Figure 4.1), and subsequent appearance of low Ca^{2+} bursts within 30-95 min (Figure 4.2). An attribute of these low Ca^{2+} bursts is that multiple population spikes are superimposed on each depolarization (Figure 4.2B). These bursts could be followed for 3 or more hours.

After a 30 min baseline period of low calcium induced bursting was obtained, a 10 min pulse of either 1, 5, 10, 20, or 50uM 5-HT (Sigma Chemical Co.), 8-OH-DPAT or DOI (Research Biochemicals, Inc), was administered in low Ca^{2+} media. The 5-HT$_2$ antagonist, ketanserin (Janssen Pharmaceuticals), was applied at 50uM for 15 min, either by itself in controls or in conjunction with DOI. After drug application, perfusion of low Ca^{2+} media without drug was resumed. Since 8-OH-DPAT is rapidly oxidized (see RBI Summary Sheet) the drugs were mixed into the low Ca^{2+} ACSF prior to use and oxygenation was carried out for only 3 min prior to and during the 10 min drug pulse. Control slices were similarly perfused with low Ca^{2+} media but without 5-HT, 8-OH-DPAT or DOI. The low calcium bursting was recorded for 3 hours post drug perfusion.

Data analysis included measurements of burst rates and burst amplitudes prior to and following exposure to 5-HT, 8-OH-DPAT or DOI. The data are expressed as a percentage of the initial baseline period (100% represents baseline) since the burst rate during this period was highly variable from one slice to another (ranges from 5-28 bursts/5 min interval). The data are presented in 30 min bins (unless otherwise noted) and represent the preceding 30 min of low Ca^{2+} burst data. Statistical analysis included analysis of variance (ANOVA) and two-sample two-tailed T-tests.

B. Results

1. Low Calcium Induced Bursting

Control hippocampal slices exhibited a slow steady increase in burst rate for the first 90 min and then slowly declined for the last 90 min of the 180 min experimental period (Figure 4.4, 4.5). Typically, the burst rate of control slices exhibited a steady increase in burst rate, rising from 100% to 146% $\pm$ 22% after 60 min of 0mM Ca^{2+} perfusion, and then a steady decline to 72% $\pm$ 10% of the baseline period at the end of 180 min (N=6). In our experiments low Ca^{2+} bursting was not seen in the DG (N=5) of the hippocampal slice (Figure 4.3).

Once initiated these bursts occurred at a frequency of 2.96 $\pm$ 2.28 per min (mean $\pm$ SD) and had an average width at mid-amplitude of 3.86 $\pm$ 2.74 sec (values were calculated for N=12). The depolarization of these bursts ranged from 2-20mV.

Data was collected from 103 hippocampal slices. Each slice received only one 10 min application of a drug compound (5-HT, 8-OH-DPAT, DOI or ketanserin).

2. Serotonin

Perfusion of serotonin mediated a significant inhibition of the burst rate which was dose-dependent (ANOVA $p < 0.002$) (Figure 4.4, 4.5). All concentrations except $5 u$M (N=6) resulted in a decrease in the burst rate. Application of $10 u$M 5-HT (N=6) paralleled the increase in burst rate seen in control slices to 145% $\pm$ 23% after one hour but was significantly reduced at two hours (75% $\pm$ 9%; $p < 0.02$) (Figure 4.5) . Increasing the concentration of 5-HT to $20 u$M (N=6) resulted in a significant decrease in burst rate after 60 min post drug application (92 $\pm$ 7%; $p < 0.04$) and maintained a significant decrease until 180 min where it resumed control bursting levels ($p < 0.005$). A maximal decrease was seen at $50 u$M 5-HT (N=6), where after one hour the burst rate fell to 77% $\pm$ 13% ($p < 0.02$) and at three hours post drug to 24% $\pm$ 2% ($p < 0.005$) (Figure 4.5B). Interestingly, $1 u$M (N=6) elicited a significant decrease followed by a return to control values after 2 hours ($p < 0.03$).

No significant changes in burst amplitude were noted at any of the concentrations of 5-HT used (data not shown).

Figure 4.4: Chart records of low Ca^{2+}-induced bursting following the application of 5-HT, 8-OH-DPAT, and DOI.

Chart records of burst activity in the CA1 region after control, 8-OH-DPAT, 5-HT, and DOI drug perfusions (10 min as indicated by the arrows). Following application of drug-free ACSF, no change was seen in the burst rate, although at longer periods of time the rate of bursting declined (see Figure 4.5). A 10 min application of $10uM$ 8-OH-DPAT resulted in a decline in the burst rate which was maintained for the duration of the experimental period of 180 min. Application of $50uM$ 5-HT elicited a transient decrease in the burst rate with a partial recovery to pre-drug values after 60 and 180 min. Perfusion of $50uM$ DOI elicited no change in the burst rate during drug perfusion, and only a small change in the burst rate after 60 min with a partial recovery to pre-drug values after 180 min. In addition this figure illustrates the variations in burst rates often seen during this experimental series. It is this variation in burst rate and amplitude between slices that required the normalization (i.e.. set baseline values to 100%, see Figure 4.5) of the data to allow comparison between various pharmacological compounds and slices.

CONTROL

T=0MIN　　　　　　　　　T=10MIN　　　T=60MIN　　　　T=180MIN

4mV |

5-HT (50uM)

4mV |

8-OH-DPAT (10uM)

4mV |

DOI (50uM)

4mV |

112
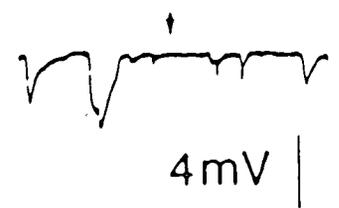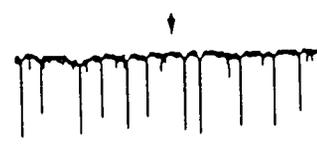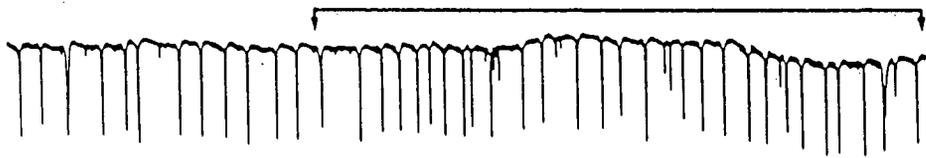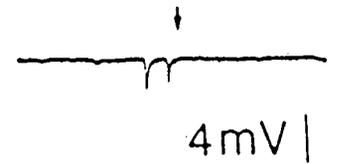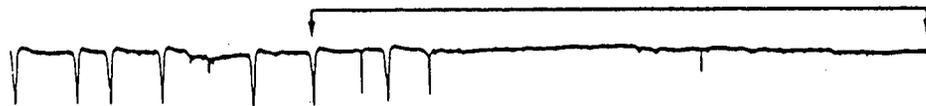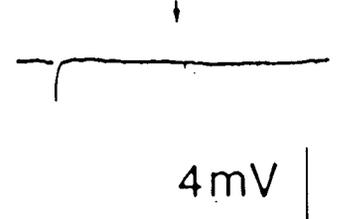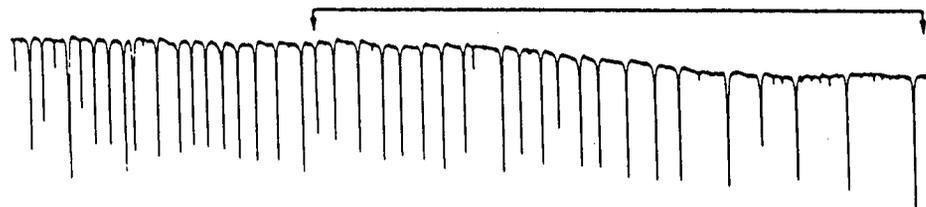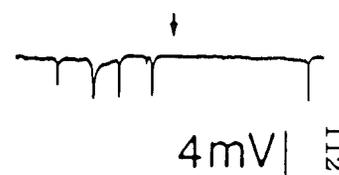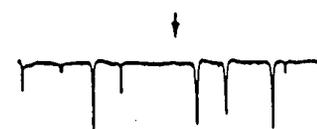
Figure 4.5: The effects of 5-HT on low Ca^{2+}-induced bursts in the CA1 region.

A. Application of 1, 5, 10, 20 and $50\,uM$ 5-HT for 10 min at T=0 (as indicated by the filled horizontal bar). Perfusion of various concentrations of 5-HT (N=6 for all concentrations including controls) resulted in both increases and decreases in burst rate. In general, the higher the concentration the more effective 5-HT was in decreasing burst rates. S.E.M.s are not shown for clarity but were less than 27%.

B. This figure illustrates the effects of 10 and $50\,uM$ 5-HT application on burst rates compared to control bursting. Drug application was for 10 min as shown by the filled horizontal bar at T=0 min. Data is shown with S.E.M.s. (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$)

A. EFFECTS OF 5–HT ON BURST RATE
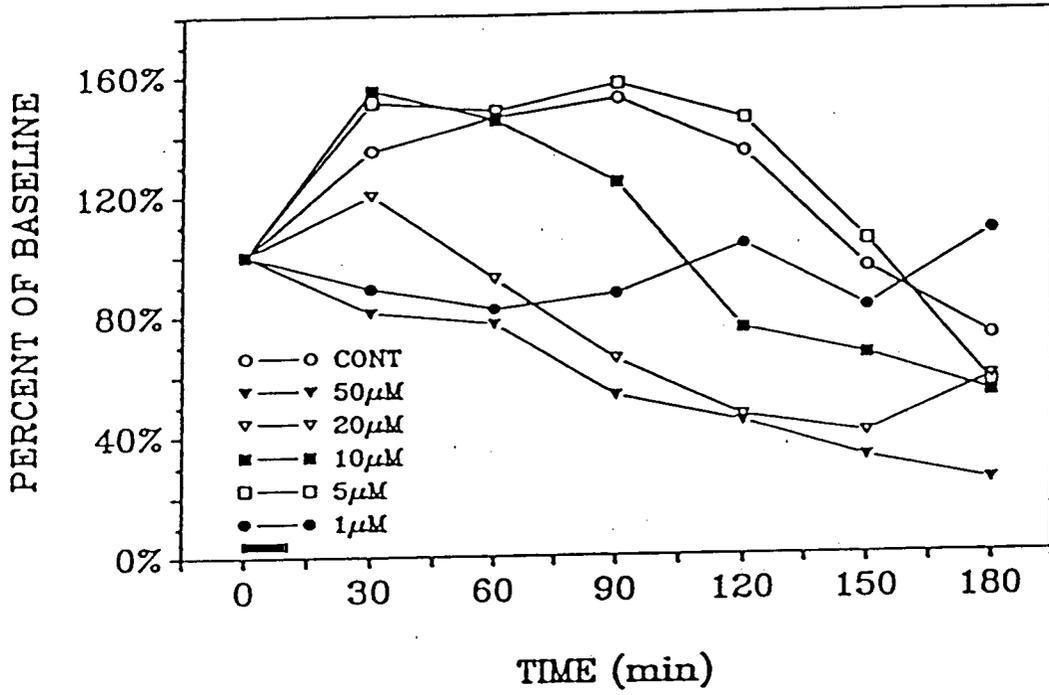
B. EFFECTS OF 5–HT ON BURST RATE
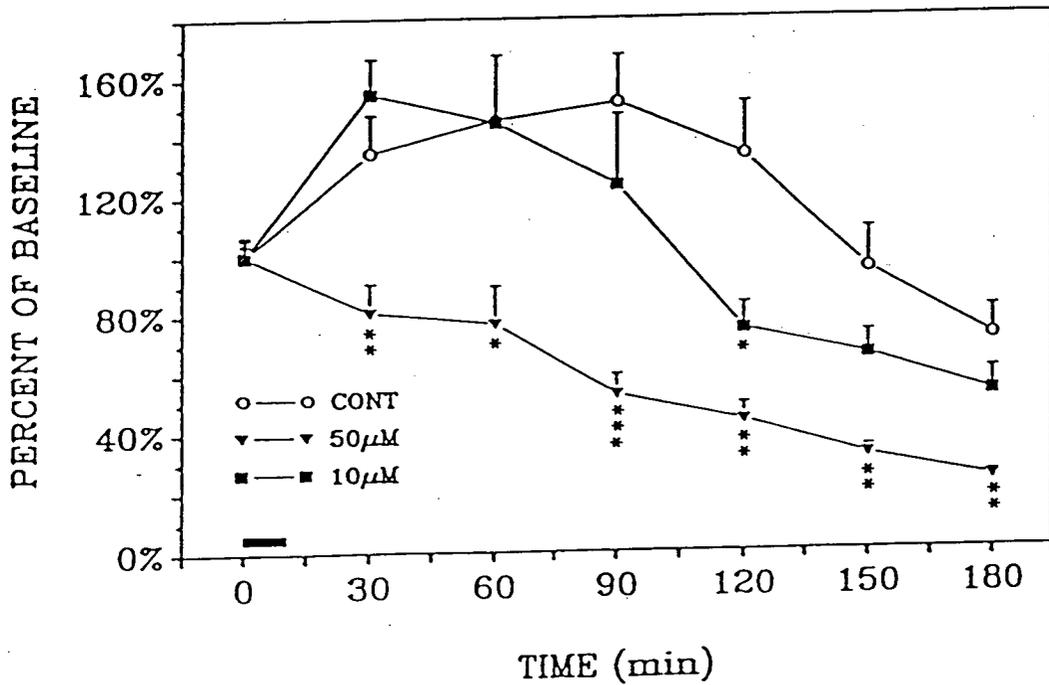
Figure 4.6: The effects of 8-OH-DPAT on low Ca^{2+}-induced bursts in the CA1 region.

A. Application of 1, 5, 10, 20 and $50 u$M 8-OH-DPAT for 10 min at T=0 (as indicated by the filled horizontal bar). This figure illustrates the consistent reductions in burst rate following perfusion of various concentrations of 8-OH-DPAT (N=6 for all concentrations including controls). In general, the higher the concentration the more effective 8-OH-DPAT was in decreasing burst rates. However, there are only slightly significant differences between the $1 u$M and $50 u$M doses ($p < 0.05$). S.E.M.s are not shown for clarity but were less than 22%.

B. This figure illustrates the effects of $50 u$M 8-OH-DPAT application on burst rates compared to control bursting. Drug application was for 10 min as shown by the filled horizontal bar at T=0 min. Note that no recovery to pre-drug values was seen at any of the concentrations tested. Data is shown with S.E.M.s. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

A.

EFFECTS OF 8−OH−DPAT
ON BURST RATE
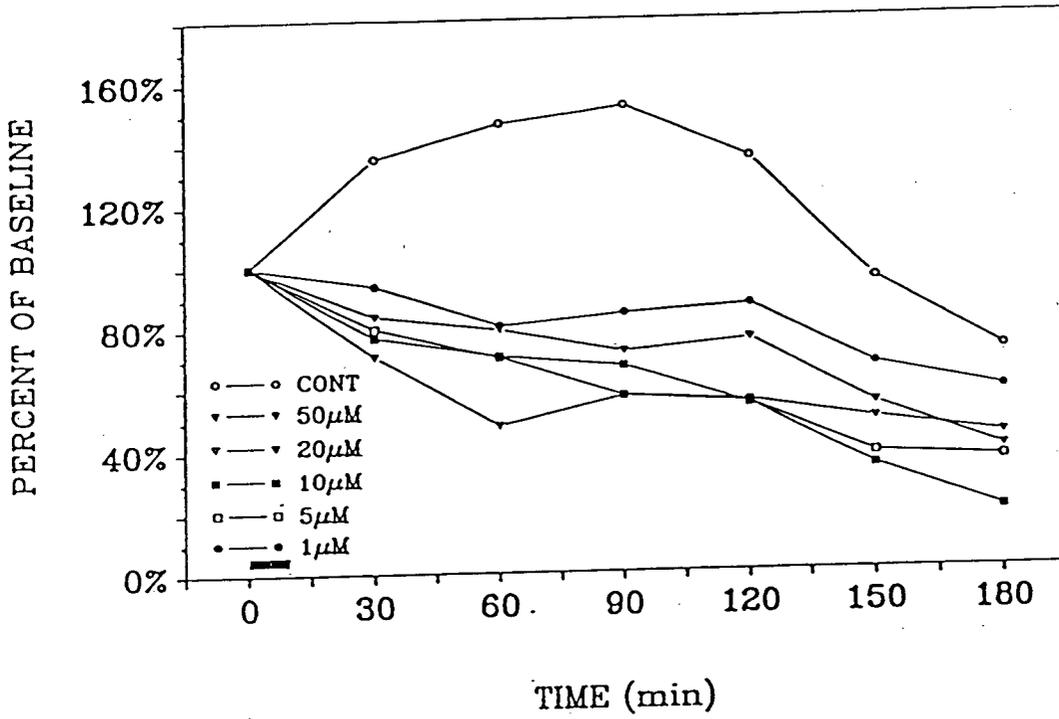
B.

EFFECTS OF 8−OH−DPAT
ON BURST RATE
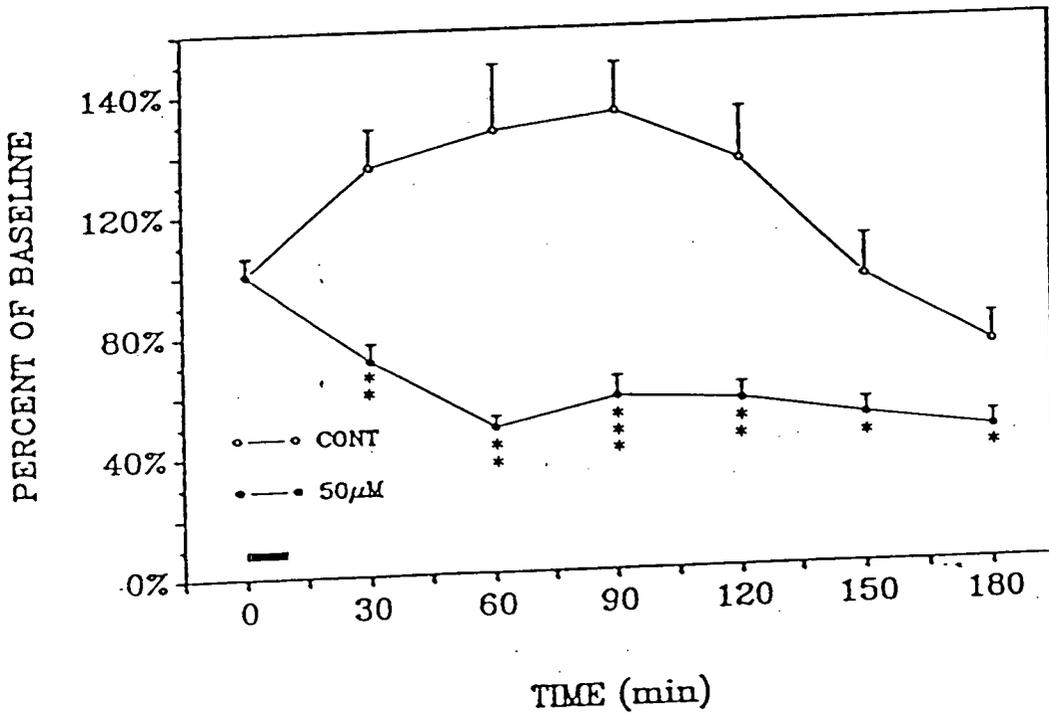
Figure 4.7: The effects of the selective 5-HT$_2$ agonist, DOI on low Ca^{2+}-induced bursts in the CA1 region.

A. Application of 1, 5, 10, 20 and 50uM DOI for 10 min at T=0 (as indicated by the filled horizontal bar). This figure illustrates the various changes in burst rate following perfusion of increasing concentrations of DOI (N=6 for each group including controls). In general, the only significant reductions in burst rate were seen at 10, 20, and 50uM DOI. The results shown are not surprising as the CA1 region has been shown not to contain appreciable binding sites for the 5-HT$_2$ receptor subtype. S.E.M.s are not shown for clarity but are less than 30%.

B. This figure illustrates the effects of 50uM DOI application on burst rates compared to control bursting. Drug application was for 10 min as shown by the filled horizontal bar at T=0 min. Note that recovery to pre-drug values was seen at this concentration. Data is shown with S.E.M.s. (* $p < 0.05$, ** $p < 0.001$)

A. EFFECTS OF DOI ON BURST RATE
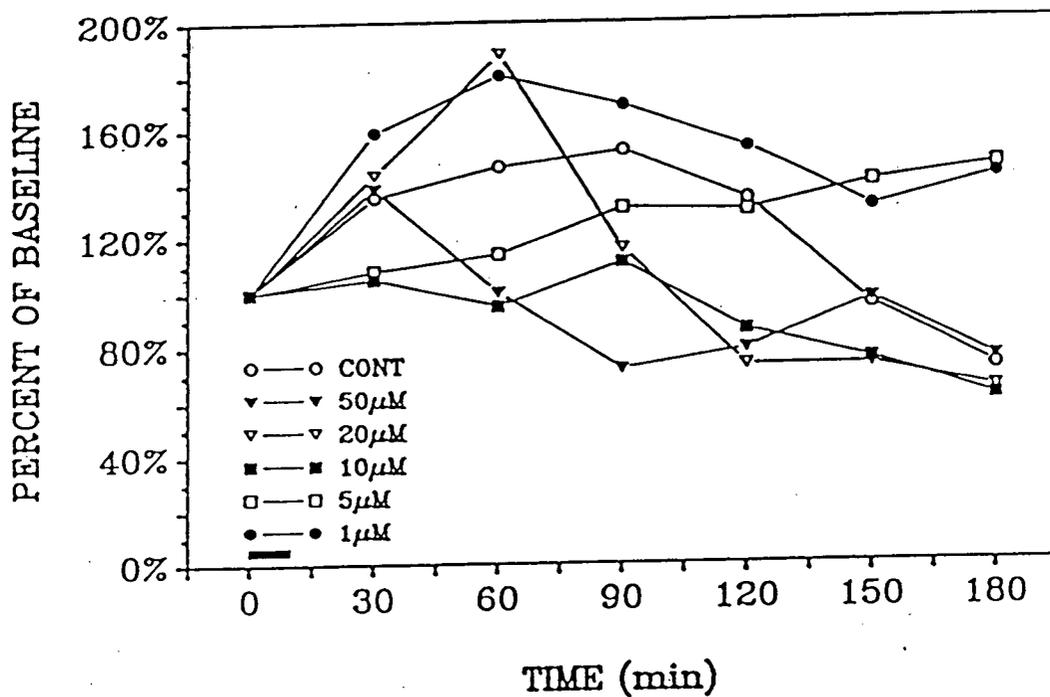
B. EFFECTS OF DOI ON BURST RATE
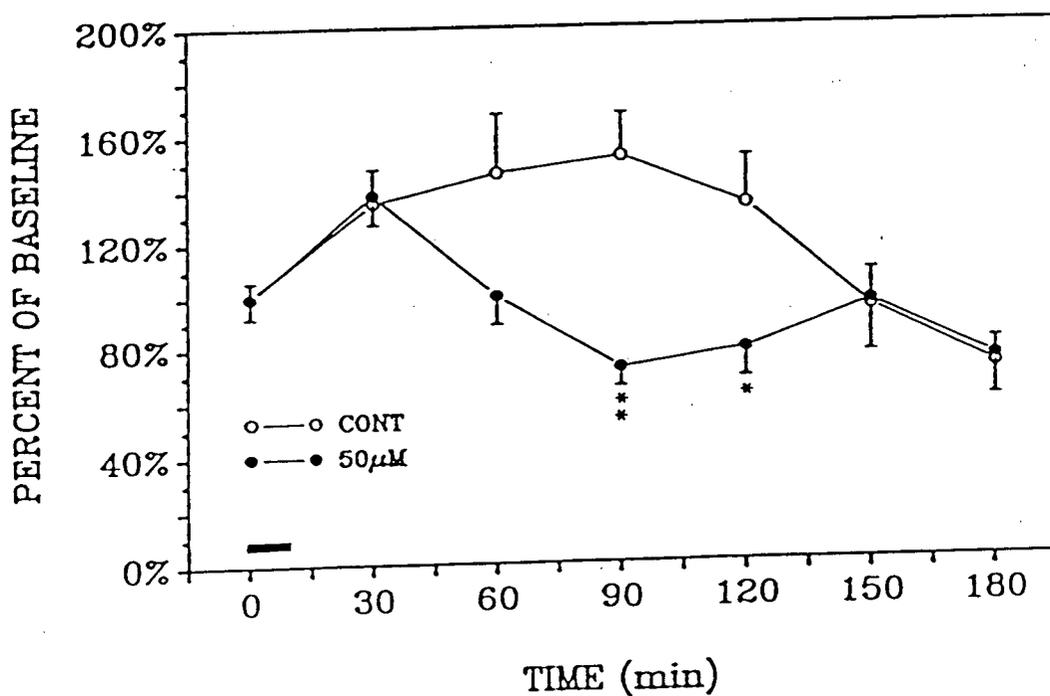
3. 8-OH-DPAT

Application of the selective 5-HT_{1A} agonist 8-OH-DPAT, at all doses resulted in a significant decrease in burst rates that was immediate and long lasting (ANOVA $p < 0.0001$) (Figure 4.6). Significant reductions were observed at all doses when compared to controls ($p < 0.01\text{-}0.005$). However only the $1uM$ concentration was significantly different from $50uM$ ($p < 0.04$). Perfusion of $1uM$ ($N = 6$) resulted in a decrease of the burst rate to $81 \pm 9\%$ at $T = 60$ min ($p < 0.02$) (control, $146 \pm 22\%$) with a return to control values at $T = 180$ ($58 \pm 10\%$; control, $72 \pm 10\%$). Application of $5uM$ ($N = 6$) resulted in a similar decrease in burst rate. However, perfusion of 8-OH-DPAT ($10uM$, $N = 6$) resulted in the greatest overall decrease in burst rate to $70\% \pm 13\%$ after 1 hour and a further reduction to $19\% \pm 2\%$ after 3 hours ($p < 0.002$). At $20uM$ ($N = 6$) 8-OH-DPAT elicited a decrease in burst rate as well. A concentration of $50uM$ ($N = 6$) application elicited a maximum effect on burst rate after 1 hour ($p < 0.007$), with a drop to $48\% \pm 4\%$ and then to $44\% \pm 5\%$ 3 hours post 8-OH-DPAT ($p < 0.03$) (Figure 4.6).

The greatest overall decrease in the burst rate, compared with controls, occurred at both $10uM$ and $50uM$ 8-OH-DPAT. While the $50uM$ concentration exhibited the fastest decrease in burst rate, and $10uM$ brought about the greatest decrease after 3 hours drug application, these changes were not significant (Figure 4.6A).

No significant changes in burst amplitude were noted at any of the concentrations of 8-OH-DPAT used (data not shown).

4. DOI

Statistical analysis of variance of DOI data indicated that there was an overall significant dose-dependent difference between controls and the concentrations used (ANOVA $p < 0.006$). Application of the selective 5-HT_2 agonist DOI had an effect on burst rate only at doses of 10 ($N = 6$), 20 ($N = 6$) and $50uM$ ($N = 6$). Closer comparison between controls and the lower doses of 1 ($N = 6$) and $5uM$ ($N = 6$) did not show any significant effect on burst rate (Figure 4.4, 4.7A). Perfusion of $10uM$ DOI resulted in a decrease in burst rate, with the decrease significant at only $T = 120$ min to $86 \pm 26\%$ compared to control values of $134 \pm 18\%$ ($p < 0.04$). Application of

$20 u$M again showed no significant decreases in the burst rate until T = 120 (DOI 73 $\pm$ 11%, control 134 $\pm$ 18%) (p < 0.005). At $50 u$M the burst rate was significantly reduced only at T = 90 (72 $\pm$ 7%) and T = 120 min (79 $\pm$ 11%) but returned to control values after 3 hours (Figure 4.7).

No significant changes in burst amplitude were noted at any of the concentrations of DOI used (data not shown).

5. Antagonists

Since ketanserin is a highly specific analogue for the 5-HT$_2$ receptor, the effect of this drug was also examined. Application of this compound, $50 u$M ketanserin alone resulted in no significant differences from control values (p < 0.2). Pretreatment with ketanserin ($50 u$M) did however result in the blockade of the DOI induced reduction in burst rate (p < 0.01). Once again no significant changes were seen in burst amplitude.

C. Discussion

Despite the development of numerous compounds that are effective in the treatment of seizure disorders, no unified concept of the pathophysiological or biochemical changes underlying epileptiform activity has emerged. At the present time there is evidence that implicates an involvement of biogenic amines in some seizure disorders (see Waterhouse 1986), however the role of serotonin in controlling or attenuating epileptiform activity is not fully understood. As reviewed above pharmacological agents which decrease 5-HT result in a increase in seizure susceptibility. Glavin et al (1987) has in fact found that there is increased 5-HT turnover (200-400%) from foci of epileptic human hippocampal specimens.

The low Ca^{2+} bursting model has proven useful in assessing the effectiveness and pharmacology of various anticonvulsants (Hood et al 1983; Heinemann et al 1985; Snow et al 1985; Agopyan et al 1985). Using this model of epileptiform activity, we attempted to determine the effectiveness of serotonergic agents, specific for certain receptor subtypes, in attenuating burst activity in the CA1 region of the *in vitro* hippocampal slice preparation. Preliminary work by Haas et al (1984), demonstrated that application of 5-HT to the low Ca^{2+} burst model elicited a

dose-dependent reduction in burst rate, but the duration of application was not apparent from their study and could affect the degree of inhibition of burst activity.

In agreement with Haas et al (1984), 50uM 5-HT rapidly decreased the burst rate, significantly below control values at all times. Lower doses (e.g.. 10uM) may produce a biphasic effect on the burst rate which was not seen at higher doses. The biphasic effects seen here are in agreement with experiments reporting an excitatory and an inhibitory effect of 5-HT (Jahnsen 1980, Andrade and Nicoll 1987a, Ropert 1988).

Recently the 5-HT receptor has been subdivided into a number of pharmacologically distinct receptor subtypes (see Hoyer 1988, Peroutka 1987), and the 5-HT$_{1A}$ receptor is thought to mediate a number of important biochemical events. These include activation/inhibition of adenylate cyclase (Shenker et al 1985, Markstein et al 1986), and activation of a K^+ channel that is coupled to both 5-HT and GABA$_B$ receptors (Andrade et al 1986). Using the 5-HT$_{1A}$ selective agonist, 8-OH-DPAT, we examined its effectiveness as an anticonvulsant on the low Ca^{2+} burst model. We have shown a significant anticonvulsant effect of 8-OH-DPAT in this model at all concentrations. This drug produced a decrease in burst rate but did not alter the burst amplitude. These decreases in burst rate seen after 8-OH-DPAT application are presumably due to activation of the 5-HT$_{1A}$ receptor subtype. The only study to date to examine the effectiveness of 8-OH-DPAT on epileptiform activity found no display of anticonvulsant effects in the electroshock model in rats. 8-OH-DPAT was definitely proconvulsant in the electroshock and pentylenetetrazol induced seizures in mice (Loscher and Czuczwar 1985). The *in vivo* nature of these experiments and the use of large concentrations of 8-OH-DPAT suggest that both pre- and post-synaptic 5-HT$_{1A}$ receptors may be activated thereby reducing endogenous 5-HT (Gozlan et al 1983). The effects of 8-OH-DPAT in the *in vitro* slice (see Chapter 3) with the data presented above, imply that the reduction in burst rate is specific for the 5-HT$_{1A}$ receptor subtype.

Possible mechanisms of action of the 5-HT$_{1A}$ mediated effects, seen with 8-OH-DPAT and 5-HT, include inhibition of adenylate cyclase, alteration of K^+ conductances and activation of PI metabolism (Roth and Chuang 1987). Evidence has accumulated that 5-HT and 8-OH-DPAT, regulate their cellular responses via adenylate cyclase (AC), although whether they increase

(Markstein et al 1986, Shenker et al 1985, 1987) or decrease AC activity (De Vivo and Maayani 1986; Bockaert et al 1987) has yet to be fully determined. Perhaps as suggested by Shenker et al (1987) the 5-HT receptor subtypes, particularly the 5-HT$_{1A}$, may be coupled to both stimulatory and inhibitory proteins for activating the adenylate cyclase system. In this model, a decrease in activation of adenylate cyclase leads to a decrease of intracellular cAMP. Haas et al (1984) demonstrated an increase in the rate of low Ca^{2+} bursting when 8-bromo-cAMP is applied. This would suggest that serotonergic agonists that inhibit adenylate cyclase could decrease the burst rate of the low Ca^{2+} model via a reduction in cAMP levels in the neurons.

Additional experiments revealed that 5-HT (and 8-OH-DPAT) modulate two K^+ conductances which could be responsible for the hyperpolarization typically seen (Andrade and Nicoll 1987a). This finding may account for the initial burst rate increases seen after low concentrations of 5-HT, but the exact mechanisms remain unclear at this time. Increases in $[K^+]_0$ have also been associated with application of 5-HT, although the concentrations used were extremely high (Segal and Gutnick 1980) while no changes were seen in $[Ca^{2+}]_0$. At the present time it is difficult to speculate on the possible involvement of K^+ channel conductances in our model of epileptiform activity.

Electrotonic coupling may play a role in the synchronization of low Ca^{2+} induced bursts, via an increase in coupling or gap junction formation, which could result in an increase and or maintenance of burst rate. In light of this suggestion it is intriguing to note that a recent short report has demonstrated a decrease in neuronal coupling after 5-HT application in *Helisoma* (Mercier and Kater 1987). This decrease would then provide another mechanism by which 5-HT and 8-OH-DPAT could decrease the burst rate, and allow for the implementation of second messenger systems.

The 5-HT$_2$ type has been implicated in some electrophysiological (see Chapter 3) and behavioral effects (Peroutka 1987). Others have offered evidence which suggests that Ca^{2+} antagonists may interact with 5-HT$_2$ type receptors (Defeudis 1987). The 5-HT$_2$ receptor is also involved in mediating inhibition of excitatory amino acid release (Maura et al 1988). Perfusion of DOI at all concentrations did not significantly alter the burst rate during the application period,

and most of the decreases in burst rate were seen only 60 min after drug application. One possibility is that the $5\text{-}HT_2$ receptor is not being activated. Although ketanserin antagonized DOI mediated decreases in burst rate, examination of the time course of these effects is suggestive of secondary effects such as phosphoinositide (PI) turnover (Sanders-Bush and Conn 1986, 1987).

These results form evidence for serotonergic modulation of low Ca^{2+} induced epileptogenesis in the rat hippocampus. Serotonin and the selective $5\text{-}HT_{1A}$ agonist, 8-OH-DPAT produced a dose-dependent decrease in the burst rate but not amplitude, suggesting that the $5\text{-}HT_{1A}$ receptor subtype may modulate this type of epileptiform activity. Speculative mechanisms of action may include; i) a decrease in electrotonic coupling and or electrical field effects, and ii) a decrease in adenylate cyclase activity leading to decreased levels of cAMP.

IV. THE ROLE OF PURINERGIC SYSTEMS IN EPILEPSY

While early work on the peripheral autonomic nervous system led the to "purinergic" nerve hypothesis (see Burnstock 1979), conclusive evidence for such a system in the central nervous system is lacking. At the present time the purines, and in particular adenosine (AD), are considered to be neuromodulators of central nervous system function rather then acting as classical neurotransmitters (Synder 1985).

The first study to examine AD and its metabolites in epilepsy was by Maitre et al (1974), where they presented evidence of blockade of audiogenic seizures following systemic injections of AD. Since then numerous models of epilepsy have been utilized to demonstrate that AD and its agonists are effective anticonvulsants (Dragunow 1988). Models of epilepsy that have been shown to be modulated by purines include; i) kindling (Barraco et al 1984, Rosen et al 1985, Dragunow and Goddard 1984), ii) metrazol induced seizures (Snyder et al 1981, Dunwiddie and Worth 1982, Murray et al 1985), iii) low Ca^{2+}-induced bursting (Haas et al 1984), and iv) electroshock seizures (Lewin and Bleck 1981). Based on the above evidence it is highly suggestive that purine mechanisms may be at work attempting to control or inhibit excessive cell firing.

An association between the effects of AD and calcium has recently become of interest, in light of the proposed A_3 AD receptor that is thought to be linked to a Ca^{2+} channel (Ribeiro and Sebastiao 1986). AD has been shown to modulate the release of excitatory amino acid

neurotransmitters (Corradetti et al 1984). The findings of Crowder and Bradford (1987) demonstrate an interaction between common anticonvulsants (e.g.. phenytoin, carbamazepine) and with neuronal Ca^{2+} uptake and inhibition of excitatory amino acid neurotransmitter release. Other studies have provided verification of an interaction between adenosine and calcium (Dunwiddie 1984, Schubert et al 1986). Both these interactions appear to be pre-synaptic.

Theophylline (THP, 1,3-dimethylxanthine) is an AD antagonist that belongs to the methylxanthine group of compounds. The anticonvulsive effects of AD in the pentylenetetrazol induced seizures can be reversed by application of THP (Murray et al 1985). Using different models of chemoconvulsants, Dunwiddie and Worth (1982) demonstrated that THP could reverse the anticonvulsant effect of AD in pentylenetetrazol induced seizures in rats. THP also has proconvulsant effects on amygdala kindled animals *in vivo* (Dragunow et al 1987). Theophylline has also been shown to antagonize the effects of AD in a low Ca^{2+} model (low Ca^{2+}, high Mg^{2+} model) which utilized stimulus evoked afterdischarges (Lee et al 1984). Although data was not presented, their report suggested the involvement of a post-synaptic site of action for adenosine.

The use of the low Ca^{2+} bursting model of epilepsy circumvents problems of adenosine interactions via synaptic activity, and preliminary investigations has suggested that adenosine does indeed have an inhibitory effect (Haas et al 1984, Lee et al 1984): both of these studies conclude that AD actions were due to post-synaptic activation of AD receptor subtypes.

Thus, the following experiments were conducted to assess the effectiveness of purinergic modulation of low Ca^{2+}-induced bursting.

A. Methods

The methods used here have been described in the methods section of Chapter 2. The purine agonists and antagonists were prepared in ACSF at their appropriate concentrations and oxygenated for at least 10 min prior to bath perfusion. No noticeable pH changes were seen in the drug containing ACSF. After regular burst activity was obtained for 30 min, the various agonists or antagonists were applied for either 5, 10, or 25 min periods. The data is presented as percentages of the control periods, thus allowing data to be normalized across numerous slices. Individual burst amplitudes were measured in mV and calculated into 30 min bins. The data were

then normalized to percentages. The data at the end of the 30 min baseline period were taken to be 100% of burst amplitude. Burst rates were combined into 30 min bins and again data were normalized into percent as described. Statistical analysis utilized the two-sample two-tailed T-test.

Unless otherwise noted all compounds were obtained from Sigma Chemical Co. and stored in a desiccator at -20°C.

B. Results

Control bursting in random slices was obtained, typically increasing to a maximum after approximately 70 min (125 $\pm$ 32%; N=6) and then gradually declining (51 $\pm$ 45%) after 180 min of bursting. In the series of experiments below, data were obtained 20 min prior to and 60 min post drug application, and T=0 indicates the time at which the perfusion of the test compound was begun. Control burst amplitudes gradually declined during the duration of the experimental period.

1. Adenosine (AD)

AD concentrations of 5 (N=5), 10 (N=4), 20 (N=4), 40 (N=9), and 80 uM (N=6) were applied. The only significant difference in burst rate appeared after application of 80 uM AD was seen (Figure 4.8). Application of 80uM AD resulted in a decrease of the burst rate to 55 $\pm$ 9% at T=10 min (p<0.01) (at the end of the AD application) compared to controls (105 $\pm$ 13%). Application for 10 min of 20 or 40uM concentrations did elicit partial reductions in burst frequency in some slices, but no significant effects were seen.

No significant changes in burst amplitude of the low Ca^{2+} induced bursts were seen, except at a concentration of 80uM (data not shown). At this concentration there was an increase in amplitude with a maximum at T=15 min (113 $\pm$ 9% compared to control values of 78 $\pm$ 7%) (p<0.01).

2. Adenosine Deaminase (ADA)

ADA application in our model produced highly significant effects. Dosages of 0.5 (N=4), 1.0 (N=5), 2.5 (N=3), and 5U/ml (N=1) of ADA significantly increased the burst rate in all cases (see Figure 4.9, 4.10). Maximal changes in burst frequency were usually seen within 10 min of

onset of application except for 0.5 U/ml concentration which had its maximum at T=15 min. Comparison of burst frequencies between controls and ADA exposed slices demonstrated significant changes at all concentrations at T=10 min. Control burst rate at T=15 min (i.e. 15 min after onset of drug perfusion) was 91 $\pm$ 7%, while application of 0.5 U/ml increased burst rate to 234 $\pm$ 44% (p<0.05). Application of 1.0 U/ml further increased the burst frequency to 305 $\pm$ 35% (p<0.004) and 2.5 U/ml ADA perfusion to 1348 $\pm$ 181% (p<0.006). Application of 5.0 U/ml was difficult to quantify due to its massive increase in frequency, but an approximate increase of 4900% was seen. An interesting effect of ADA induced increases in burst rate is the initial quiescent period following ADA application which, at higher doses, lasted for up to 10-20 min. Even the lowest dose of 0.5 U/ml resulted in a period of reduced activity for 5 min.

As burst frequency increased, burst amplitude decreased, although these effects were not significant at doses below 2.5 U/ml. As seen in Figure 4.9, significant reductions in burst amplitude occurred as would be expected. Burst rate increases diminish recovery time to control field potentials and in general there is a reduction by 1mV of the field potentials at doses above 2.5 U/ml.

3. Theophylline (THP)

The low Ca^{2+}-induced model of spontaneous bursting THP resulted in a significant increase in the burst rate. Application of $50 u$M for 10 min resulted in no significant increase in burst rate. However, $100 u$M application for the same length of time elicited only a 210 + 21% (N=7) increase in burst rate (p<0.002) (see Figure 4.11, 4.12).

The burst amplitudes were not significantly altered when compared to controls during 10 min applications of THP.

Figure 4.8: Burst rate changes in the CA1 region following the application of adenosine.

A. Chart recordings of burst rate following 20 or $80u$M adenosine (AD) perfusion for 10 min as indicated by the arrows. Note the effect of $80u$M adenosine in reducing burst rates. Scale is as indicated by the bars.

B. This figure represents the resultant transient decrease in burst rate following the application of $80u$M adenosine (N=6 for both control and AD). The filled horizontal bar indicates the period the 10 min of AD perfusion. The burst rate changes returned to pre-drug values within 20 min following the perfusion of AD. (* $p < 0.01$)

A.

Adenosine (AD)

AD 20 uM

2mV
2min

AD 80 uM

B.

BURST RATE CHANGES

PERCENT OF BASELINE

100%

0%

o——o CONT
•——• AD (80 μM)

-20   0   30   60

TIME (min)
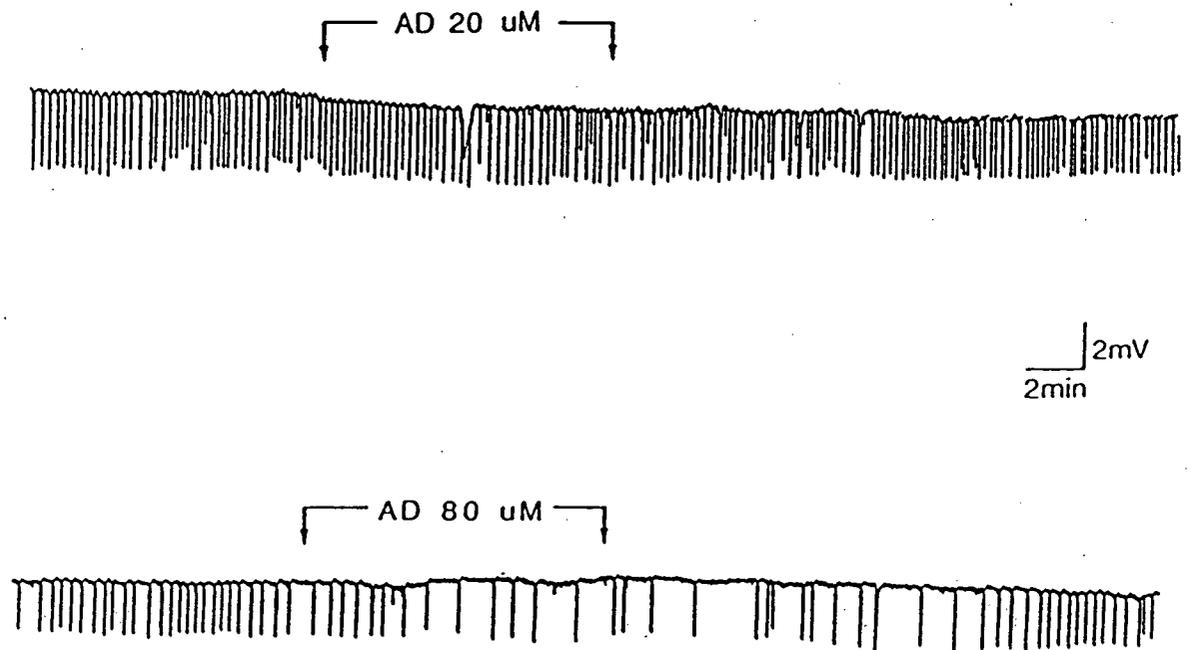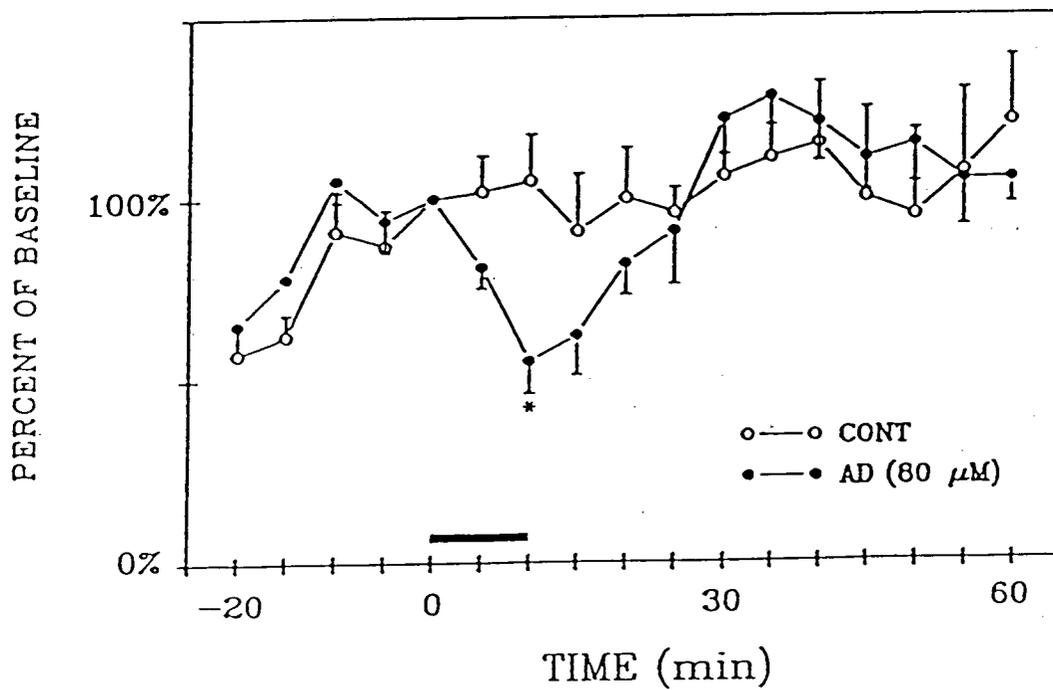
Figure 4.9: Chart records of low Ca^{2+}-induced bursting in the CA1 region following perfusion of the purinergic degradation enzyme, adenosine deaminase (ADA).

The chart records of burst activity from the CA1 region illustrate the effects following application of 0.5, 1.0, 2.5 and 5.0 U/ml adenosine deaminase (ADA) in different slices. The period of ADA application (5-10 min) is shown by the arrows. At all doses there was an increase in burst rate. This increase was significantly larger at concentrations above 1.0 U/ml. Recovery occurred at all the doses tested (not shown) but varied from 5-30 min after the application of ADA. The asterisk and star refer to the expansion of oscilloscope traces of the burst records shown in the insets. Inset scale bars (vertical 1mV, horizontal 30 sec) refer to both insets.

4 mV

2 min
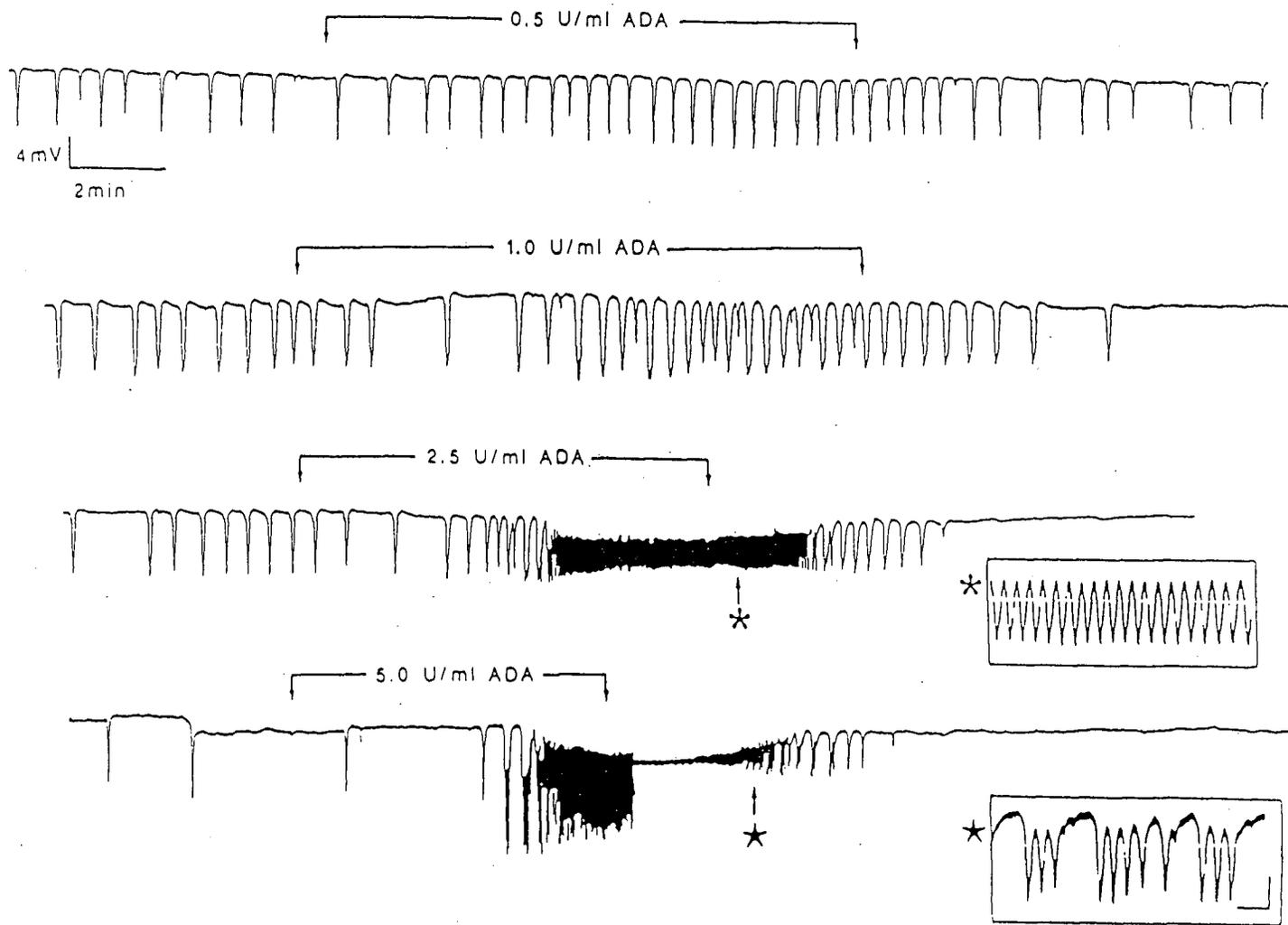
Figure 4.10: Effect of ADA perfusion on burst rate recorded in the CA1 region.

Perfusion of 2.5 U/ml ADA for 10 min as indicated by the filled horizontal bar, elicited a substantial increase in burst rate, which was followed by a return to pre-drug values within 40 min post-drug application. Note that burst activity was reduced for 20 min following the large increase in burst rate. (* $p < 0.005$)

BURST RATE CHANGES
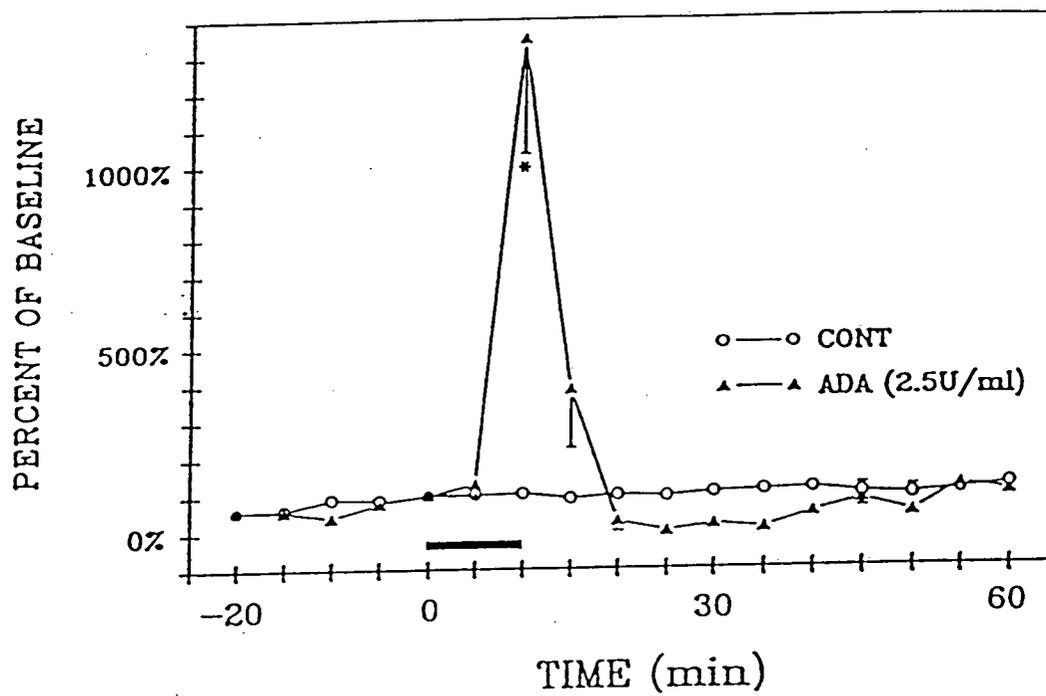
Figure 4.11: Effects of the purinergic antagonists, theophylline (THP) and isobutyl-methylxanthine (IBMX) on low Ca^{2+} induced bursting.

Burst rate changes following a 10 min perfusion of either IBMX or THP. The upper chart records represent pre-drug burst rates, while the center records were taken during the last 5 min of a 10 min drug perfusion. The bottom records illustrate the recovery to pre-drug values of the burst rate after 30 min of re-perfusion with low Ca^{2+} ACSF without drugs.
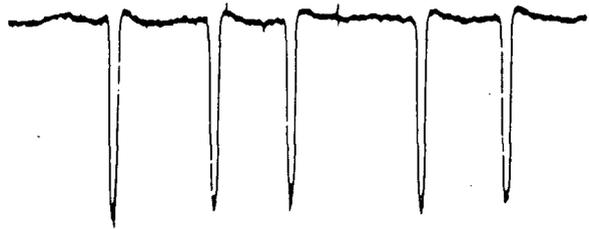
CONTROL
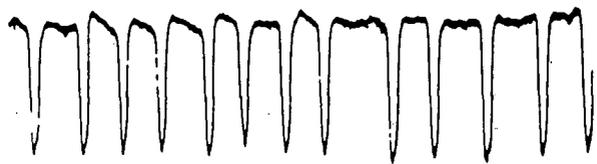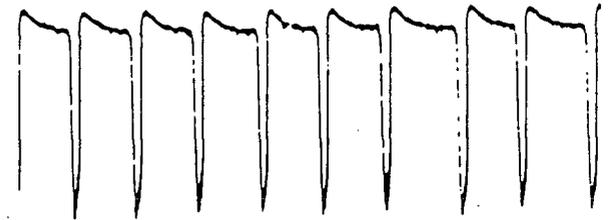
IBMX
(100uM)

THP
(100uM)

WASH

2mV

2mV

1min

1min

134
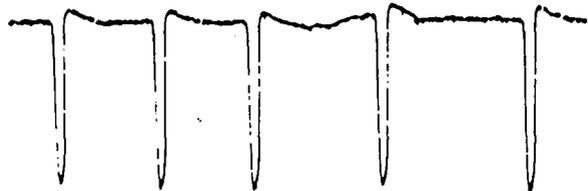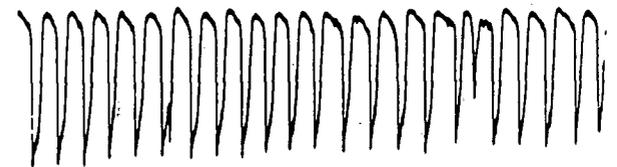
Figure 4.12: Comparison of the time course of the changes seen following perfusion of the purinergic antagonist theophylline (THP) to low Ca^{2+} induced bursting in the CA1 region.

A comparison between burst rate changes following application of control (N=6), 50 (N=4) or $100u$M (N=7) THP. Note that both 50 and $100u$M increased burst rates dramatically. However, only $100u$M concentrations produced significant changes. Application of either dose of this purinergic antagonist was for 10 min as indicated by the filled horizontal bar. This increased burst rate was not accompanied by any change in the burst amplitude. (* $p<0.01$, ** $p<0.001$)

This figure should be compared with figures 4.10 and 4.11. Similar results were obtained from application of the primary degradative enzyme, adenosine deaminase, or the purinergic antagonist THP. The time course of changes in the burst rates were similar.

BURST RATE CHANGES
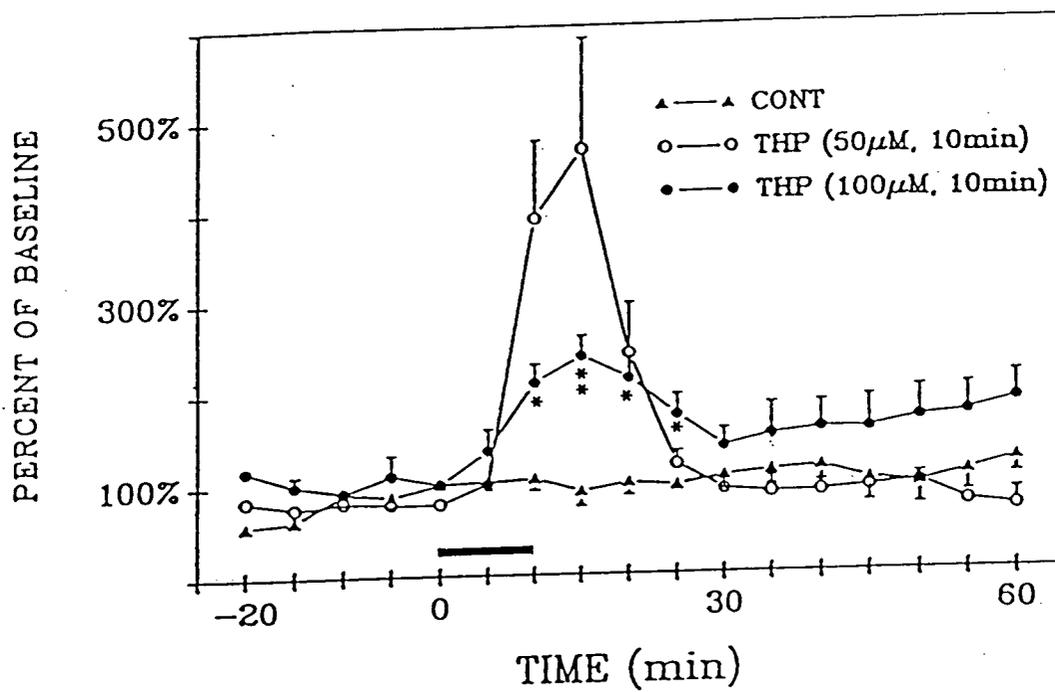
Figure 4.13: Application of the adenosine antagonist, isobutyl methylxanthine (IBMX) to low
Ca^{2+} induced bursting in the CA1 region.

Perfusion of IBMX at $100u$M (N=4) for 10 min as indicated by the filled horizontal bar, elicited a robust increase in the burst rate. This increase in burst rate was maintained for 30 min after the perfusion of this antagonist, and then recovered to pre-drug values. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

BURST RATE CHANGES
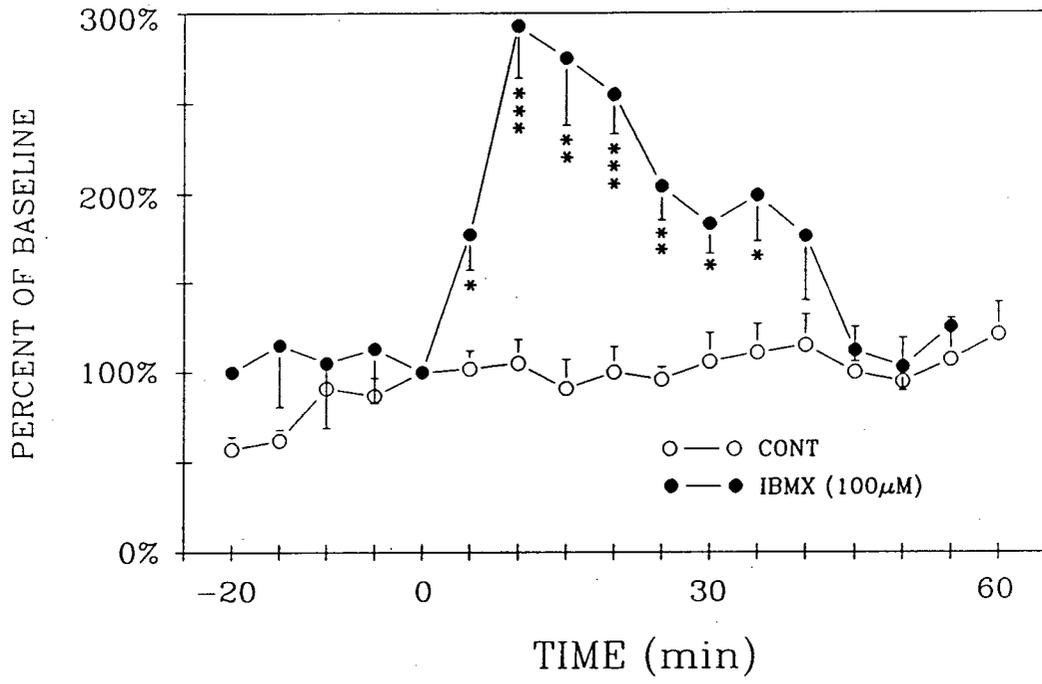
Figure 4.14: Effect of the NMDA receptor antagonist, D-APV on the low Ca^{2+}-induced bursting model.

A. A continuous chart record of burst activity prior to, during and after the application of $50 uM$ D-APV. A slight reduction in burst rate and burst amplitude can be seen. Scale is as indicated by the bars.

B. This figure is a graphical representation of the effects of a 25 min application of D-APV as indicated by the filled horizontal bar. Although there was an almost immediate decrease in the burst rate following the onset of D-APV perfusion this decrease was not maintained for the duration of the 25 min application. Recovery to pre-drug values occurred by the time the drug application was stopped. (* $p < 0.05$, ** $p < 0.001$)

The results seen in this figure would suggest that glutamate might be released during burst activity. This release could come from pre-synaptic sources although it has been previously hypothesized that only post-synaptic mechanisms are active during the low Ca^{2+} induced bursting model of epilepsy.

A.

D-APV 50uM

]2mV

2min

B.

BURST RATE CHANGES

PERCENT OF BASELINE

100%

0%

o——o CONT

▲——▲ D-APV (50μM. 25min)

-20   0   30   60

TIME (min)
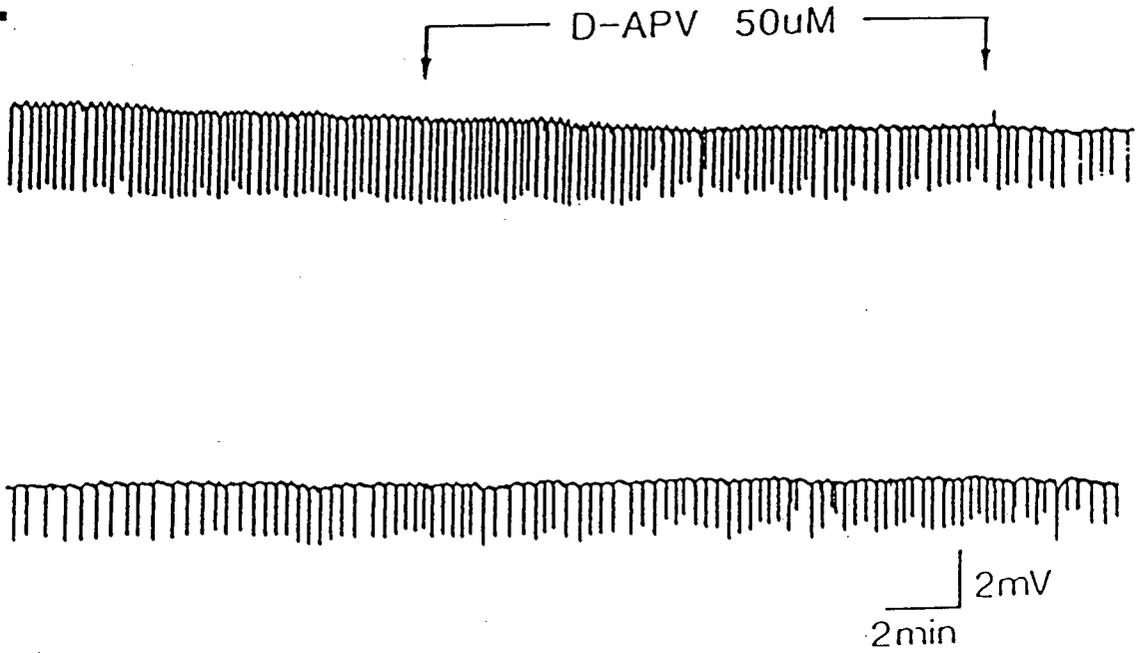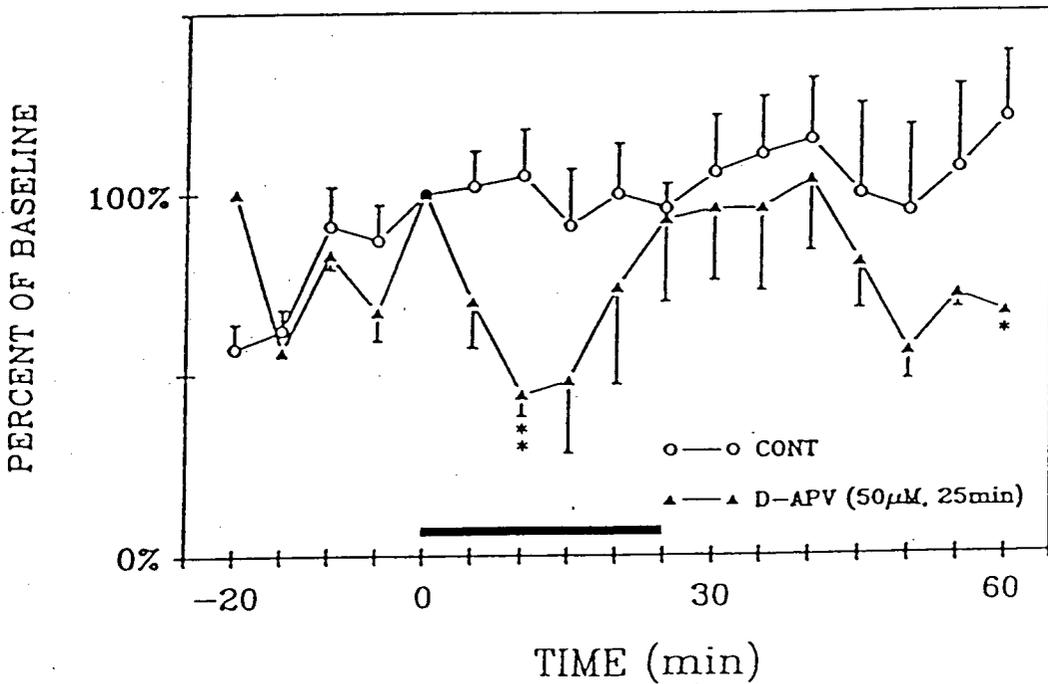
4. Isobutyl-Methylxanthine (IBMX)

Another AD antagonist of the methylxanthine group is IBMX (3-isobutyl-1-methylxanthine). This compound, like THP, above exerts a blocking effect on the anticonvulsant effects of AD (Phillis and Wu 1981a, Dunwiddie and Worth 1982) but has been known along with other methylxanthines to have effects such as intracellular Ca^{2+} mobilization (Endo 1977, Martonosi 1984) and inhibition of the enzyme phosphodiesterase (Amer and Kreighbaum 1975).

In the low Ca^{2+} burst model, this compound displays proconvulsant properties similar to THP. Application of $100 uM$ IBMX for 10 min (N=4) resulted in an increase in burst rate from $105 \pm 13\%$ in controls to $293 \pm 29\%$ (p<0.0006) (Figure 4.11, 4.13). A key difference between IBMX and THP was that the IBMX induced increase in burst rate was maintained for 30 min beyond the period of drug perfusion, whereupon burst rates returned to control levels.

5. Papaverine (PAP)

The isoquinoline derivative and potent phosphodiesterase inhibitor, papaverine (PAP) is a non-methylxanthine compound that is a potent blocker of AD uptake (Phillis and Wu 1981a). This compound has also been found to have anticonvulsant properties (blocking amygdala kindled seizures, Dragunow and Goddard 1984). This AD uptake blocker would then appear to be exerting its effects by prolonging the presence and therefore action of AD.

Application of PAP decreased burst rate as would be expected. PAP at $100 uM$ (N=3) elicited a significant decrease in burst rate, but not burst amplitude. At T=15 min (5 min post drug application) PAP had reduced the burst rate to $17 \pm 17\%$ compared to controls at the same time ($91 \pm 16\%$) (p<0.01) (data not shown). This decrease was maintained for 25 min post drug application, suggesting a potent mechanism of increasing extracellular accumulation of AD, and therefore promoting its anticonvulsive effects.

6. D-amino-5-phosphono valeric acid (D-APV)

It has been suggested that an excessive release of excitatory amino acids, and subsequent activation of voltage dependent Ca^{2+} conductances may play an important role in the initiation and spread of seizure activity (Dingledine 1986); however, in the low Ca^{2+} induced bursting model of epilepsy, extracellular levels of Ca^{2+} have been substantially reduced, and are considered to be too low to sustain the neural Ca^{2+}-dependent mechanisms of neurotransmitter release. Based on this, the assumption that only post-synaptic processes are active has been put forth. The following experiments were designed to determine if all pre-synaptic mechanisms were rendered inactive by the reduction of extracellular Ca^{2+}.

The NMDA receptor antagonist, D-APV was tested on low Ca^{2+} bursting. Various concentrations and drug perfusion times were applied in this model. Three different perfusion times were assessed, 5, 10, and 25 min exposures. Application of the NMDA receptor antagonist D-APV elicited a strong reduction in burst rate. A 5 min application of D-APV ($25 u$M, N = 4) elicited a non-significant decrease in the burst rate ($72 \pm 11\%$) when compared to controls ($105 \pm 13\%$; N = 6) at T = 10 min (5 min post drug exposure) ($p < 0.1$). Perfusion of D-APV for 10 min at $10 u$M (N = 3) elicited a slight decrease in burst rate. Application of $50 u$M for 10 min did not significantly decrease the burst rate. Application of $50 u$M D-APV for a longer period, 25 min resulted in the largest overall decrease in burst rate to $44 \pm 6\%$ ($p < 0.009$) (N = 3) compared to controls ($105 \pm 13\%$; N = 6). This decrease in burst rate returned to control values at all doses following re-perfusion of drug free ACSF (see Figure 4.14).

No changes in burst amplitude were seen at any of the concentrations noted above and no differences were noted when the perfusion times were extended from 5 to 25 min.

C. Discussion

Purinergic neuromodulation of synaptic activity is well established (see Chapter 3, Phillis and Wu 1981a), but appears to have many different functions. Some of these include; i) inhibition of neural activity (Dunwiddie and Hoffer 1980, Schubert and Mitzdorf 1979, Okada and Ozawa 1980), ii) modulation of excitatory amino acid release (Prestwich et al 1983, Corradetti et al 1984), iii) interaction with Ca^{2+} channels (Schubert et al 1986, Morgan et al 1987, Ribeiro and Sebastiao 1986), iv) protection against neuronal injury (Goldberg et al 1988), v) anticonvulsant effects of adenosine (Maitre et al 1974, Dragunow 1988), and vi) interactions with other receptor subtypes (e.g.. benzodiazepines) (Phillis and Wu 1981a).

Numerous studies in recent years have pointed to the possible role of purines in epileptiform activity and its possible clinical usefulness (Coleman et al 1986). It should be noted that the anticonvulsant effects of adenosine (AD) seen in the hippocampal slice (Dunwiddie and Worth 1982, Haas et al 1984), may not necessarily produce the same effects in an *in vivo* model (Bowker and Chapman 1986). The study by Bowker and Chapman (1986) demonstrated that the anticonvulsant effects of AD administration could be attributed to the hypothermic effects of AD, as raising the temperature of the animals antagonized the anticonvulsant effects of AD.

One model that has helped to establish the importance of purines in seizures is the kindling model of epilepsy. Numerous studies of amygdala and hippocampal kindled animals (Dragunow et al 1987, Dragunow and Goddard 1984, Rosen and Berman 1985, Barraco et al 1984) have shown that purinergic mechanisms may be involved. Adenosine and its specific analogues attenuate kindling afterdischarges as well as behavioral manifestations. Antagonists such as theophylline and IBMX, and uptake blockers (i.e.. papaverine) lower seizure thresholds and promote epileptiform activity.

Another type of electrically induced seizure model is that induced by electroshock. In a series of studies, Lewin and Bleck (1981), were able to demonstrate that not only AD but specifically some of its metabolites, such as inosine and hypoxanthine, may be involved

in the termination of epileptic activity. Supportive evidence for AD metabolites as anticonvulsants comes from Skolnick et al (1979), who showed an increase in the latency to seizures induced by pentylenetetrazol. These findings of this model have been extended by Murray et al (1985) who propose that AD and its analogues are also active.

Reduction or elimination of extracellular Ca^{2+} in the ACSF perfusing the hippocampal slice preparation leads to the induction of epileptiform activity (Taylor and Dudek 1982, Jefferys and Haas 1982). Decreases in $[Ca^{2+}]_0$ elicit spontaneous bursting in the CA1 region of the hippocampal slice (Haas and Jefferys 1984). The investigation of adenosine in this model was reported by Haas et al (1984), who demonstrated that AD decreases the spontaneous burst rate. Since this study, several others have examined the effects AD on evoked afterdischarges (Lee et al 1984, Schubert and Heinemann 1988). Lee et al (1984) reported that adenosine appeared to be released endogenously in an effort to tonically reduce repetitive discharges of CA1 pyramidal neurons, and that this effector mechanism was linked to the A_1 receptor subtype located in the apical dendrites. Using Ca^{2+} sensitive electrodes and a modified hippocampal slice, Schubert and Heinemann (1988) reported that physiological levels of AD (1uM, see Zetterstrom et al 1982) may exert a powerful modulatory role on synaptic neurotransmission via a pre-synaptic mechanism. This study attempted to isolate only pre-synaptic portions of the hippocampal slice, and provided evidence that pre-synaptic modulation of neurotransmitter release was not dependent on pre-synaptic Ca^{2+} uptake, but would appear to be due to changes in the intracellular Ca^{2+} levels, or its coupling to the secretory process. Another recent report provided evidence that in the absence of synaptic transmission there was enough endogenous AD available to modulate hippocampal excitability (Fowler 1988).

The low Ca^{2+} induced bursting (low Ca^{2+} and normal Mg^{2+}) model showed evidence that AD possesses anticonvulsant properties. Our model requires very high concentrations of AD to elicit reductions in burst rate, a fact which indicates large

amounts of endogenous AD being released. Some evidence exists for increased release of AD in the presence of low Ca^{2+} (Hollins and Stone 1980).

The most compelling data for evidence of purinergic modulation of low Ca^{2+} induced epileptiform activity is the action of the degradative enzyme for AD, adenosine deaminase (ADA). Application of this enzyme in even small concentrations elicited a significant increase in the rate of bursting: higher concentrations resulted in an extraordinarily high rate. ADA has been examined in other seizure models (Lewin and Bleck 1984, Lee et al 1984, Fowler 1988), and they have confirmed this enzyme's ability to increase epileptiform activity, which is due solely to its ability to degrade endogenous adenosine. Combined results with ADA indicate that considerable amounts of endogenous AD are present.

Supplementary evidence demonstrates that antagonists of AD receptors induce epileptiform activity. Application of theophylline and IBMX to the bursting model resulted in a significant increase in burst rate. Uptake blockers such as papaverine have also been shown to be anticonvulsive in nature, since they reduce uptake of AD in neurons (see Phillis and Wu 1981a).

Our own investigations taken together with other studies suggest a role for high levels of endogenous adenosine in modulating epileptiform activity. The dramatic increases seen with the degradative enzyme for AD, ADA, and the AD antagonists, theophylline and IBMX, all provide further support for the notion that endogenous AD is present.

One of the most novel and exciting faucets of the present investigations is the demonstration that the NMDA receptor antagonist D-APV also reduces the frequency of low Ca^{2+} induced bursts. This finding would suggest that excitatory amino acids such as glutamate or aspartate is being released in large enough quantities to affect the burst rate. Indeed, pre-synaptic release of glutamate could be a factor in the induction of the bursting behavior that is seen in this model. One problem with this suggestion is the lack of extracellular Ca^{2+} to support pre-synaptic glutamate release: nonetheless, direct

measurements of the effect of reduced extracellular Ca^{2+} on $[Ca^{2+}]_i$ levels demonstrated that significant reductions in $[Ca^{2+}]_i$ do not take place following the perfusion of Ca^{2+}-free ACSF (see Chapter 6). Adenosine modulation of glutamate and aspartate release (Prestwich et al 1983, Corradetti et al 1984) has special significance in light of the suggestion that NMDA receptors are involved in epileptiform activity (Dingledine 1986).

V. CHAPTER SUMMARY

Artificial cerebrospinal fluid low in Ca^{2+} will induce spontaneous bursts upon which population spikes may be superimposed. This hyperexcitability occurs in the absence of synaptic transmission and has been used to test the post-synaptic actions of various compounds on epileptogenic activity.

At the present time our understanding of the low Ca^{2+} model suggests that there three primary phases; i) generation, ii) spread, and iii) synchrony of the burst activity. Although they appear to be distinct they may have mechanisms that are interdependent.

The initiation of low Ca^{2+} bursting is likely due to the decreases in the $[Ca^{2+}]_o$. Removal of $[Ca^{2+}]_o$ destabilizes the surface of the neuronal membrane and leads to cellular hyperexcitability (Frankenhauser and Hodgkin 1957, Erulkar and Fine 1979), and a lessening of the efficacy of GABAergic and other inhibitory synaptic inputs (Heinemann et al 1986). Additional changes in membrane conductances (e.g.. Ca^{2+}-dependent K^+ conductances) further affect the ability of the cell to control intrinsic burst capabilities (Schwindt and Crill 1980).

The synchrony of these low Ca^{2+} bursts may be mediated by electrotonic coupling, ephaptic interactions or extracellular K^+ accumulations (Yaari et al 1986, Taylor and Dudek 1984a,b, Richardson et al 1984). The relative density of the CA1 pyramidal layer may also contribute to the duration of these seizure-like events. Although the time course and order of the suggested mechanisms remain unclear, non-synaptic mechanisms can

play an important role in the generation, spread and synchronization of epileptiform activity.

One report suggests a strong role for electrical field interactions (ephaptic interactions) as a possible mechanism of hyperexcitability (Taylor and Dudek 1984a). In the rat hippocampus, electrotonic coupling may contribute to seizure synchronization, although the degree of involvement remains to be elucidated (Andrew et al 1982; Dudek et al 1983). It is interesting to note that serotonin reduces the degree of coupling between neurons of the *Helisoma* (Mercier and Kater 1986), a fact which is of considerable interest since it parallels and could account for the effects seen in the low Ca^{2+} bursting model.

Once the bursts have been generated, a possible mechanism of spread to neighboring cells might be via changes in extracellular K^+ ($[K^+]_0$). Yaari et al (1986) have shown that an increase in $[K^+]_0$ may precede or be associated with the spread of burst activity from one hippocampal region to another, and an increase in $[K^+]_0$ coupled to a concomitant decrease in $[Ca^{2+}]_0$ is known to enhance neuronal excitability (Konnerth et al 1984, Yaari et al 1986). In addition, increased electrotonic coupling would enhance cell to cell communication and may facilitate electrotonic spread of burst activity.

The evidence provided above demonstrates that serotonin is inhibitory on epileptiform activity. The use of selective agonists shows that their inhibitory effects can be attributed to activation of the $5-HT_{1A}$ receptor subtype in the modulation of burst activity. Activation of the $5-HT_{1A}$ receptor leads to a rapid inhibition of adenylate cyclase activity and this in turn could lead to a decrease in intracellular cAMP levels. Such a mechanism is consistent with the effects of the direct application of 8-bromo-cAMP which have been shown to increase burst rate (Haas et al 1984).

Endogenous AD and its metabolites seem to play a critical role in suppression of epileptiform activity. In our hands, using the degradative enzyme ADA, a massive increase in burst rate was observed, which suggests that high extracellular concentrations of AD are present in hippocampal slices where low Ca^{2+} burst activity is established.

Confirmation was obtained when AD antagonists, theophylline and IBMX also produced an increase in burst activity. The long duration of increased burst rate following application to this low Ca^{2+} burst model may be in part due to the other effects of IBMX (i.e. phosphodiesterase inhibitor; see Amer and Kreighbaum 1975). With the onset of epileptiform activity there is a corresponding increase in extracellular levels of AD that may act to increase the threshold for seizure initiation and thus prolong the latency between seizures. Such a conclusion is also consistent with the high concentrations of AD required to elicit a reduction in burst rates. These results would be expected if as suggested above high AD concentrations are already present.

An unexpected finding was that the NMDA receptor antagonist, D-APV, could cause a significant reduction in burst rate. This would suggest that the NMDA receptor may be involved in this model of epilepsy and in fact may play a role in the disease itself (see Dingledine 1986). This finding strongly suggests that pre-synaptic (or post-synaptic) glutamate may be released in sufficient concentrations to assist in the initiation and maintenance of the bursting activity seen in the low Ca^{2+} model. Adequate amounts of extracellular Ca^{2+} may be present to elicit release of this excitatory amino acid. This evidence argues against a purely post-synaptic effect; in fact low Ca^{2+} induced bursting may involve sufficient pre-synaptic release of neurotransmitters and neuromodulatory substances to maintain epileptiform activity.

CHAPTER 5 - LONG-TERM POTENTIATION (LTP) AND NEURONAL CALCIUM CHANGES

I. INTRODUCTION

In 1966, Lømo reported an enhancement of evoked population responses following brief tetanic stimuli to the dentate gyrus perforant path of rabbits. These experiments were continued by Bliss and Gardner-Medwin in 1973, who noted that in chronically implanted rabbits this enhancement lasted for hours and for days to weeks. This enhancement or long-term potentiation (LTP) was seen only in the afferent pathway that received the tetanic stimulation. One reason for the excitement of this discovery is that it may represent a mechanism for learning and memory (Teyler and DiScenna 1987).

Long-term potentiation is defined as a long lasting increase in the synaptic efficacy following a tetanic stimulation of the afferent fibers (Teyler and DiScenna 1987, Bliss and Lynch 1988). Although LTP has been demonstrated in numerous other brain structures, the following discussion of the role of Ca^{2+} in LTP will be limited to the hippocampal formation (see Table 5.1). In the hippocampus, a structure long known for its role in learning and memory, the CA1 and CA3 regions along with the dentate gyrus (DG) all support LTP (Teyler and DiScenna 1987).

Soon after the discovery of LTP, two independent reports demonstrated the importance of Ca^{2+} in the induction of LTP (Dunwiddie and Lynch 1979, Wigström et al 1979). Tetanic stimulation results in an increase in $[K^+]_0$ and a decrease in $[Ca^{2+}]_0$, suggesting that $[Ca^{2+}]_0$ moves into the tetanized cell (Konnerth and Heinemann 1983) and that an increase in $[Ca^{2+}]_i$ is a necessary component for the induction of LTP. Since then numerous other Ca^{2+}-dependent events in LTP have been reported. Turner et al (1982) gave further evidence for extracellular Ca^{2+} involvement when they were able to show that a brief pulse of high Ca^{2+} (4mM) could induce LTP in the CA1 region without tetanic stimuli. This work has been extended to the DG *in vivo* (Bliss et al 1986), and CA3 (Bliss et al 1983, Higashima and Yamamoto 1985); the DG *in vitro* does not exhibit Ca^{2+}-induced potentiation (Melchers et al 1987).

A. Mechanisms for the Induction of LTP

Numerous Ca^{2+} dependent mechanisms have been suggested to account for the induction of LTP in hippocampal afferent fibers. Critical to the induction of LTP in the CA1 region of the hippocampus is the activation of N-methyl-D-aspartate (NMDA) excitatory amino acid receptors. Their importance in LTP is underscored by the now well known fact that pharmacological blockade of the NMDA receptors prevent the induction of LTP in CA1 (Collingridge et al 1983) and DG (Dolphin 1983). Tetanic stimulation which leads to LTP also removes the Mg^{2+} block of the NMDA channel allowing an influx of Ca^{2+} into the neuron (Mayer and Westbrook 1987a).

However, the significance of the NMDA receptor involvement and its Ca^{2+} contribution in the induction of LTP has recently been questioned by several lines of evidence. A recent report by Harris and Cotman (1986) provided evidence that activation of the NMDA receptor did not play a role in the induction of LTP in the mossy fiber afferents to the CA3 region of the hippocampus. Malenka et al (1988) also reported that following current injection during tetanus-induced LTP, they were unable to block potentiation. This finding suggested that, i) post-synaptic Ca^{2+} is essential for the induction of LTP, ii) non-NMDA receptors could contribute to the induction of LTP (i.e. via the quisqualate receptor). However, more recently, the magnitude of the Ca^{2+} influx through the NMDA receptor coupled ion channel has been questioned, causing researchers to examine the notion that intraneuronal Ca^{2+} stores may be crucial to the induction of LTP in the CA1 region (Mody et al 1989, Obenaus et al 1989).

Other mechanisms important to the induction and maintenance of LTP are a variety of pre- and post-synaptic mechanisms. Evidence for pre-synaptic changes has been demonstrated by the extensive work of Bliss and his colleagues. They have shown that there is an increase in release of excitatory amino acids following tetanic stimulation in CA1, CA3 and the DG (Bliss and Lynch 1988) and that this release is Ca^{2+}-dependent (Dolphin et al 1982). More recently the above findings have been questioned, and evidence has been presented which demonstrates that there is in fact no increase in release of either glutamate or aspartate following a tetanic stimulation (Aniksztein et al 1989).

Studies with ion sensitive electrodes have suggested that Ca^{2+} entry is not only post-synaptic but also pre-synaptic and this mechanism might allow for enhanced neurotransmitter

release during LTP (Konnerth and Heinemann 1983). Using the fluorescent dye Indo I, Agaston and Kuhnt (1986) were able to show an increase in $[Ca^{2+}]_i$ in potentiated synaptosomes.

Evidence for post-synaptic mechanisms and the importance of $[Ca^{2+}]_i$ was confirmed by Lynch et al (1983), who were able to block LTP by intracellular injection of the Ca^{2+} chelator EGTA into the cell bodies of pyramidal neurons.

B. Mechanisms for the Maintenance of LTP

Post-synaptic mechanisms that could account for some of the changes important in the maintenance of LTP include: i) maintained increase of $[Ca^{2+}]_i$, ii) increase in the number of glutamate receptors on neuronal membranes, and iii) dendritic morphological changes. Although Ca^{2+} influx into the neuron is an important part of the inductive mechanism of LTP, changes in $[Ca^{2+}]_i$ levels may also play an important role in the processes that underlie its maintenance. It has been well established that cellular incorporation of ^{45}Ca increases following tetanic stimulation, which led the authors to suggest that tetanic stimulation increases $[Ca^{2+}]_i$ (Baimbridge and Miller 1981).

A significant increase in glutamate receptor binding was seen in slices following LTP (Bliss and Lynch 1988, Lynch et al 1982). Lynch and Baudry (1984) proposed that Ca^{2+} entry into the post-synaptic neuron activated various proteases which in turn "uncovered" glutamate receptor sites. At the present time this hypothesis is under question and not thought to play a critical role in the mechanisms underlying induction and maintenance of LTP. Davies et al (1989) reported a temporal change in the sensitivity to non-NMDA receptor ligands (i.e. quisqualate) following the induction of LTP, contending that the sensitivity of non-NMDA receptors increases following the induction of LTP thereby providing a mechanism for functional post-synaptic changes and suggests that pre-synaptic mechanisms may also contribute, but in a temporally distinct manner.

The phosphatidylinositol cascade "triggers" the release of calcium, and recent attempts have been made to determine the importance of this cascade in LTP. Several groups (Bar et al 1984, Lynch et al 1987) have shown an increase in phosphatidylinositol (PI) turnover, suggesting that changes in PI turnover may mediate changes in $[Ca^{2+}]_i$ levels. Ca^{2+}-dependent kinases

such as protein kinase C (PKC) have been shown to play an important part in maintaining the synaptic enhancement seen after LTP. Injection of phorbol esters which activate PKC, induces an LTP-like effect in the CA1 region (Malenka et al 1986), and there is an increase in the amount of PKC that is membrane bound following LTP (Akers et al 1986). Direct injection of PKC into hippocampal pyramidal cells also is able to elicit LTP-like effects (Hu et al 1987) The above evidence would suggest that increases in $[Ca^{2+}]_i$ lead to increased PI turnover and increased PKC activity.

Other Ca^{2+} dependent changes that have been seen following tetanic stimulation include changes in dendritic spine morphology (Fifkova 1985, Brown et al 1988) which may have a role in sustaining the LTP response.

Taken together, the evidence suggests that Ca^{2+} plays an important role in the induction and maintenance of LTP (Table 5.1), and the question becomes one of determining the various intracellular compartments that contribute to the source(s) of Ca^{2+}.

The purpose of the experiments described in this chapter was twofold. First we planned to determine whether Ca^{2+}_i and in particular release from the endoplasmic reticulum pool is important in frequency (tetanic) and Ca^{2+}-induced forms of LTP. This approach was an indirect one using pharmacological agents. Second, we tested in the CA1 region the findings of Bliss et al (1987), who found that the NMDA antagonist D-APV can block Ca^{2+}-induced LTP in the *in vivo* dentate gyrus.

C. Methods

Transverse hippocampal slices ($400um$) were prepared from male Wistar rats as previously described in the general methods. Electrophysiological parameters were followed as outlined in the general methods (Chapter 2). In each experiment, the maximum evoked response was measured and the stimulus intensity was reduced until the population spike amplitude was 25% of the maximal response. Baseline values were taken for 5-10 min and if stable, a tetanic stimulation of 100 Hz for 1 sec was given. The potentiated response was then followed for 20-30 min post-tetanus (see Figure 5.2A).

This set of experiments utilized the pharmacological agent dantrolene-sodium (dantrium (R); Norwich-Eaton) to investigate the possible contribution of the release of Ca^{2+} from the endoplasmic reticulum. This drug inhibits calcium release from the sarcoplasmic reticulum (Desmedt and Hainaut 1977, Fryer et al 1988), and substantially diminishes the rise in $[Ca^{2+}]_i$ elicited by NMDA receptor activation in cultured pyramidal cells without blocking NMDA gated membrane currents (Mody et al 1989).

Dantrolene-Na (MW 324) containing solutions were prepared in ACSF from a 1mM stock solution of dantrolene in dimethylsulfoxide (DMSO) to a $20 uM$ concentration. The actual drug concentration was confirmed spectrophotometrically following each experiment using the extinction coefficient (E $_{1\%/1cm}$ = 608) at an absorption wavelength of 390nm. This method allowed accurate determination dantrolene concentrations. In the tetanic induced LTP experiments, the average concentrations were 21.07 ± 1.70 uM (means $\pm$ S.D.) (N=12). In Ca^{2+}-induced LTP experiments, the concentration of dantrolene averaged 13.88 ± 1.87 uM (means $\pm$ S.D.) (N=18). Care was taken to protect the compound from undue exposure to light as it is susceptible to photo-degradation (Ward et al 1986).

Due to the very low solubility of dantrolene (dantrolene sodium, 1-{[5-(p-nitrophenyl)-furfurylidene]amino}hydantoin sodium hydrate (Figure 5.1)) in aqueous media, it takes a considerable amount of time (one to three hours) for the compound to enter the neurons and affect the intracellular organelles (unpublished observations; Ward et al 1986). Because of this long duration two types of experiments were performed; i) the hippocampal slices were exposed to dantrolene in the recording chamber for the duration of the experiment ("perfusion experiments"), and ii) the slices were allowed to incubate in either control or dantrolene containing solutions in a static incubation chamber, and then removed at the appropriate time intervals and placed in the recording chamber for LTP induction ("incubation experiments").

In the perfusion experiments, once LTP was induced in control slices, the perfusion medium was exchanged with one containing dantrolene. Attempts to induce LTP in naive slices were then made at either 60, 120 or 180 min after the onset of dantrolene perfusion. Following

180 min of dantrolene perfusion the slices were washed with normal medium and further attempts to induce LTP were made 120 min later in naive slices.

In the incubation experiments slices were removed from either control or dantrolene containing media and placed into the recording chamber. Once there, they were allowed to equilibrate further for 30 min. The appropriate ASCF (control or dantrolene) was perfused during this part of the experiment. Recording and stimulating electrodes were placed as described below. Tetanic stimuli (100 Hz for 1 sec) were then delivered to the stratum radiatum region while recording evoked population spike responses in the stratum pyramidal.

In the tetanus induced LTP, the population spikes evoked at 1/min were then averaged together into 5 min bins, and the change in the amplitude of the responses was expressed as a percentage of control (pre-tetanus) values.

In Ca^{2+}-induced LTP experiments, control population spikes and reversed field EPSPs were evoked in the CA1 pyramidal or stratum radiatum region, and adjusted to elicit a 25% maximum amplitude field response. Population responses were evoked once every 5 min, in an effort to reduce any effects of pre- or post-synaptic Ca^{2+} influx. Control responses were evoked for 10 min prior to drug or high Ca^{2+} application. It should be noted that the control ACSF contained 2mM $CaCl_2$, 2mM $MgSO_4$ and 6.25mM K^+ (5mM KCl, 1.25mM KH_2PO_4). The high Ca^{2+} application consisted of ACSF containing 4mM $CaCl_2$, 1mM $MgSO_4$ and 6.25mM K^+ (5mM KCl, 1.25mM KH_2PO_4). This combination of ionic constituents resulted in the induction of stable long lasting LTP (for a least 60 min). The high Ca^{2+} solution was applied for a 10 min duration. The NMDA antagonist, D-APV (50uM) was applied for 5 min prior to and during the high Ca^{2+} perfusion for a total of 15 min. The same controls were used for comparison between dantrolene and D-APV perfused slices.

Statistical analysis was performed using two-sample two-tailed T-tests.

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Figure 5.1: Chemical structure of dantrolene sodium

The chemical structure and chemical name for dantrolene sodium (Dantrium; Norwich Eaton R).
This compound blocks release of Ca^{2+} from intracellular stores such as the sarcoplasmic
reticulum in muscle (Desmedt and Hainaut 1977).
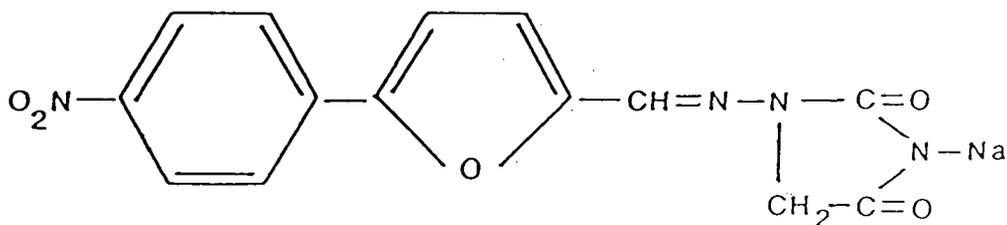
Dantrolene Sodium (Dantrium)

1-{[5-(p-nitrophenyl)-furfurylidene]amino}hydantoin sodium hydrate

Table 5.1

LTP dependent on $[Ca^{2+}]_o$	Dunwiddie and Lynch 1979, Wigstrom et al 1979
increased $[Ca^{2+}]_o$ induces LTP in CA1	Turner et al 1982
increased cell firing, increases Ca^{2+} influx	Konnerth and Heinemann 1983
intracellular injection of EGTA blocks the induction of LTP	Lynch et al 1983
increased Ca^{2+} flux results increased glutamate receptor density	Lynch and Baudry 1984
blockade of calmodulin blocks LTP induction	Mody et al 1984
increased $[Ca^{2+}]_i$ results in increased phosphoinositiol turnover	Bar et al 1984
injection of protein kinase C elicits LTP	Malenka et al 1986 Hu et al 1987
activation of the NMDA receptor results in removal of the Mg^{2+} block and Ca^{2+} influx	Mayer and Westbrook 1987
release of $[Ca^{2+}]_i$ results in LTP	Malenka et al 1988

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Figure 5.2: Typical examples of tetanic and Ca^{2+}-induced LTP.

A. An example of tetanic induced LTP from a single experiment illustrating the enhancement of the evoked population spike amplitude following a tetanic stimulation (100 Hz for 1 sec) at $T=0$ min. The evoked responses shown on the left correspond to the points indicated in the graph on the right, where: A) control, B) 1 min following tetanus, C) 20 min following tetanus.

B. An example of Ca^{2+}-induced potentiation of the amplitude of the evoked population responses in the stratum pyramidal (population spike (PS AMP) $N=5$)) and in the stratum radiatum (field EPSP (EPSP AMP) $N=5$). Recordings were made from both regions of the same slice simultaneously. Solid filled bar indicates perfusion of high Ca^{2+} medium.

In both figures the amplitude of the population spike was normalized to 100% at $T=0$ just prior to the tetanic stimulation. Only slices that exhibited a steady baseline for 5-30 min prior to experimental manipulation were used in subsequent studies. Error bars indicate S.E.M.'s.

A.

TETANIC-INDUCED LTP
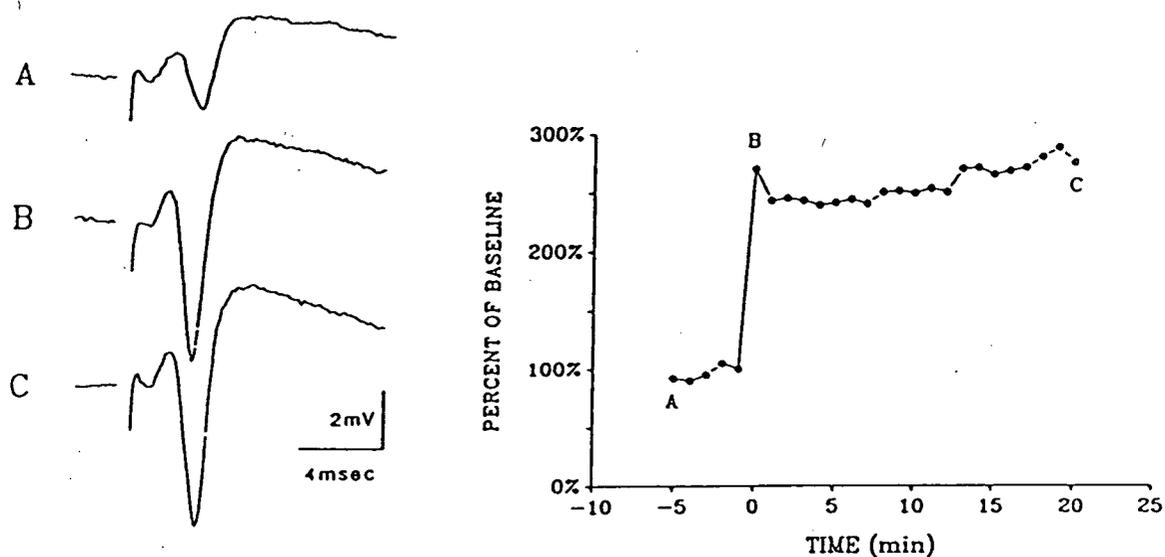
B.

COMPARISON OF Ca^{2+}-INDUCED LTP
IN THE STRATUM PYRAMIDAL AND RADIATUM
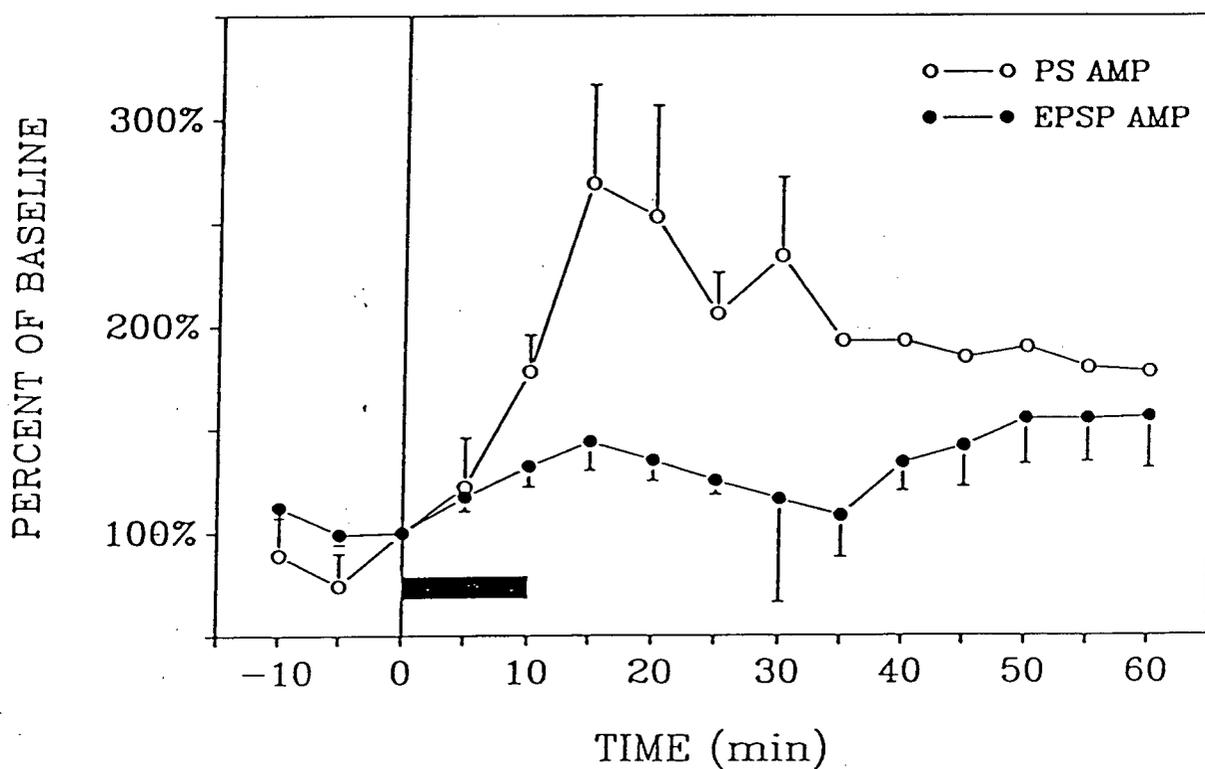
Figure 5.3: Controls required for the dantrium experiments.

A. Preliminary experiments indicated that the effects of dantrolene required long application periods and the following experiment was performed to assess the degree of LTP following a tetanic stimulation in hippocampal slices perfused for long periods of time. This figure illustrates the population spike amplitudes of control LTP evoked at T = 30 min (N = 5) and following 180 min (N = 5) after equilibration of the slices in the *in vitro* recording chamber. Tetanic stimulation was delivered at T = 0 min in both experiments. A significant reduction in the degree of LTP was seen only after T = 20 min (* $p < 0.05$)

B. The effect of dantrolene on the amplitude of the evoked population spikes was tested to determine any changes due to the long perfusion times required for this compound. This continuous recording of evoked population spikes amplitudes measured in slices perfused for an additional with normal ACSF and then for an additional 180 min (N = 2) with dantrolene containing ACSF ($\sim 20u$M) (filled horizontal bar). As can be seen from this figure, there is only a slight difference in the population spike amplitude between normal and dantrolene exposed slices. The filled horizontal bar indicates the last 40 min of a 180 min dantrolene perfusion.

A.

CONTROL LTP
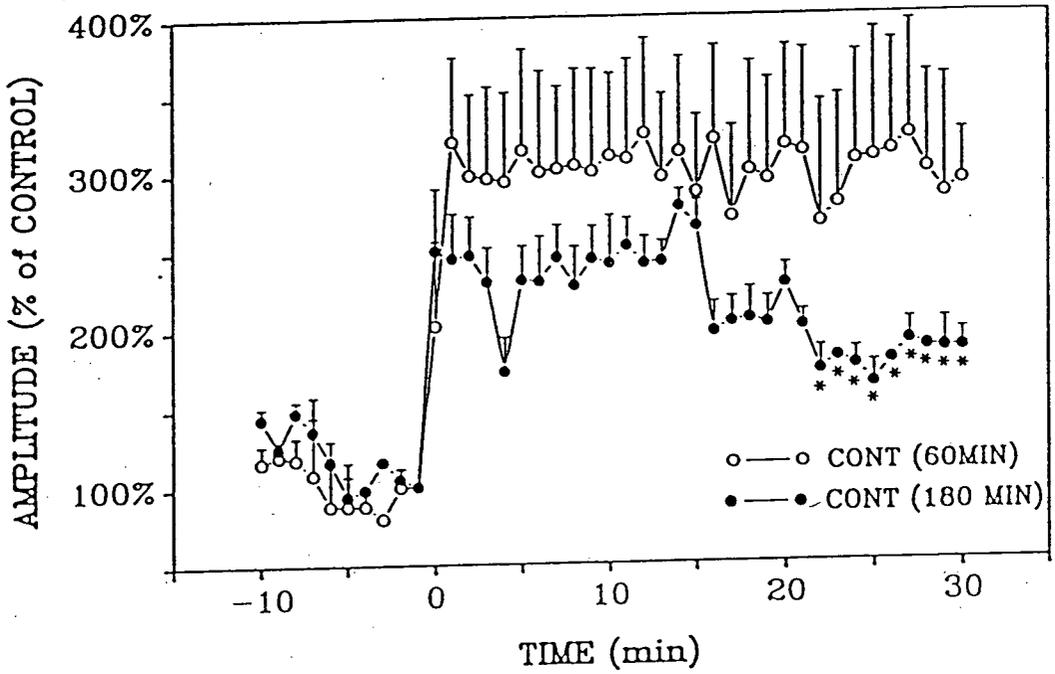
B.

CONTROL AND DANTRIUM PERFUSION
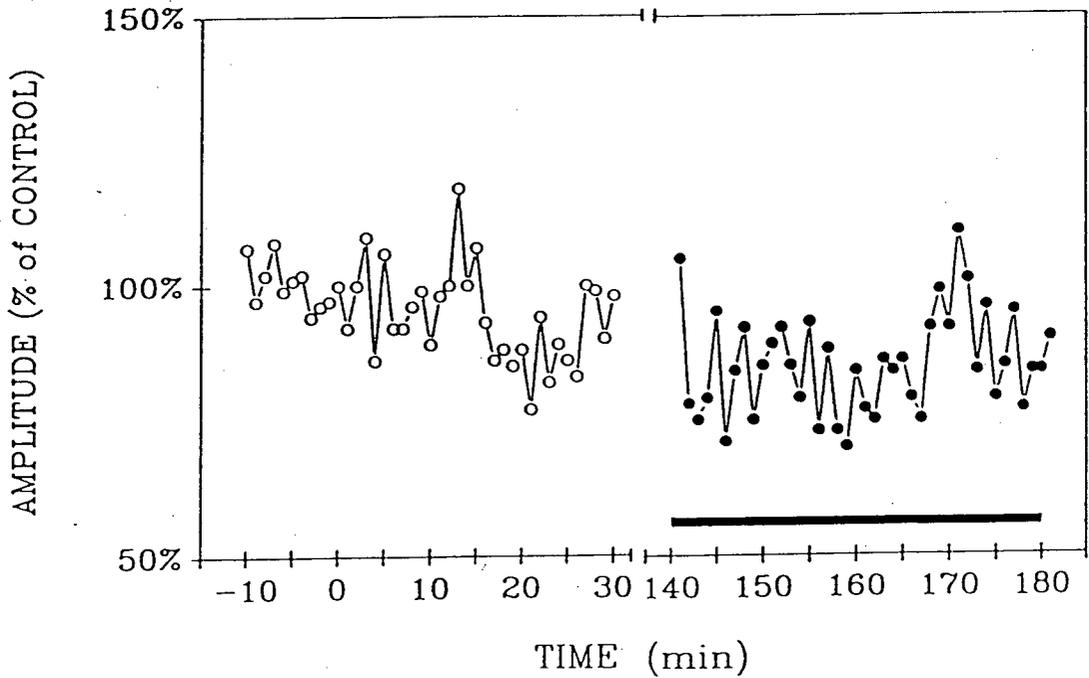
Figure 5.4: The blockade of LTP following dantrolene perfusion.

A. Tetanic stimulation of control slices (filled circles) (N = 4) elicited a robust and maintained LTP following equilibration for 60 min. In slices perfused with 20 uM dantrolene for 180 min, a tetanic stimulation (100 Hz for 1 sec) failed to induce LTP (filled triangles) (N = 4) but exhibited a short post-tetanic potentiation. In slices exposed to 180 min dantrolene and then perfused with drug-free ACSF for 120 min, tetanic stimulation did not result in a significant recovery of the slices ability to induce LTP (filled squares) (N = 4). This failure to recover could be due to the slow washout of dantrolene. In each experimental slice, only a single tetanic stimulus was delivered. The tetanic stimulus was delivered at T = 0 min in the figure.

B. Individual evoked population spikes where in each case the smaller trace is the control response and the larger is the evoked response 10 min after a tetanic stimulation. The evoked responses correspond to the figure above; control slices (filled circles), after 180 min dantrolene (filled triangles), and perfusion of drug-free ACSF for 120 min after 180 min dantrolene perfusion (filled squares). One important feature to note is the gradual broadening of the population spike following the application of dantrolene. At the present time the cause for this change is unknown (see Discussion). Scale is as indicated by the bars.

A.
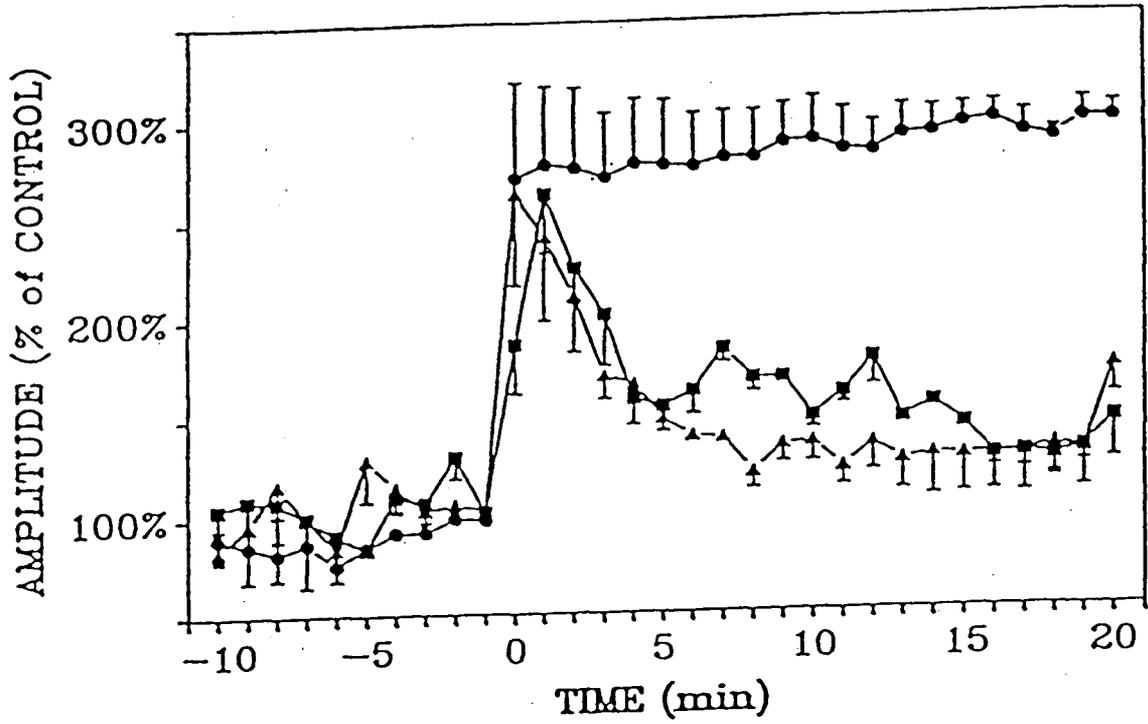
B.
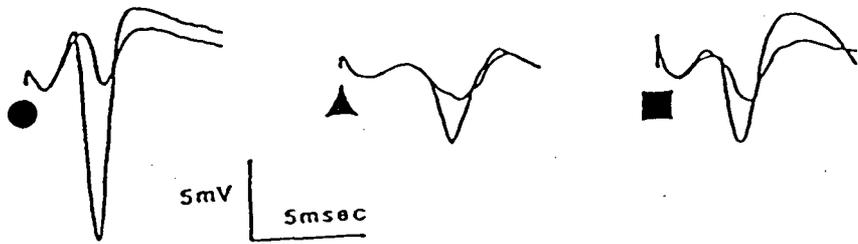
Figure 5.5: The blockade of LTP following dantrolene incubation.

Preliminary experiments demonstrated that dantrolene blockade of tetanic induced LTP required long perfusion periods. In light of these findings a separate series of experiments was conducted in which the hippocampal slices were incubated outside of the recording chamber in normal or dantrolene containing ACSF. This method allowed not only the exposure of both sides of the slice to the dantrolene containing ACSF but also, transfer of the slices to the recording chamber at various time intervals. The results of those experiments are shown here. Control slices (N=12) were still able to sustain a robust LTP, while the ability to induce LTP in dantrolene incubated slices (N=12) was significantly impaired when measured at T=10 min (p<0.005). Tetanic stimulation was delivered at T=0 min.

INCUBATION EXPERIMENTS
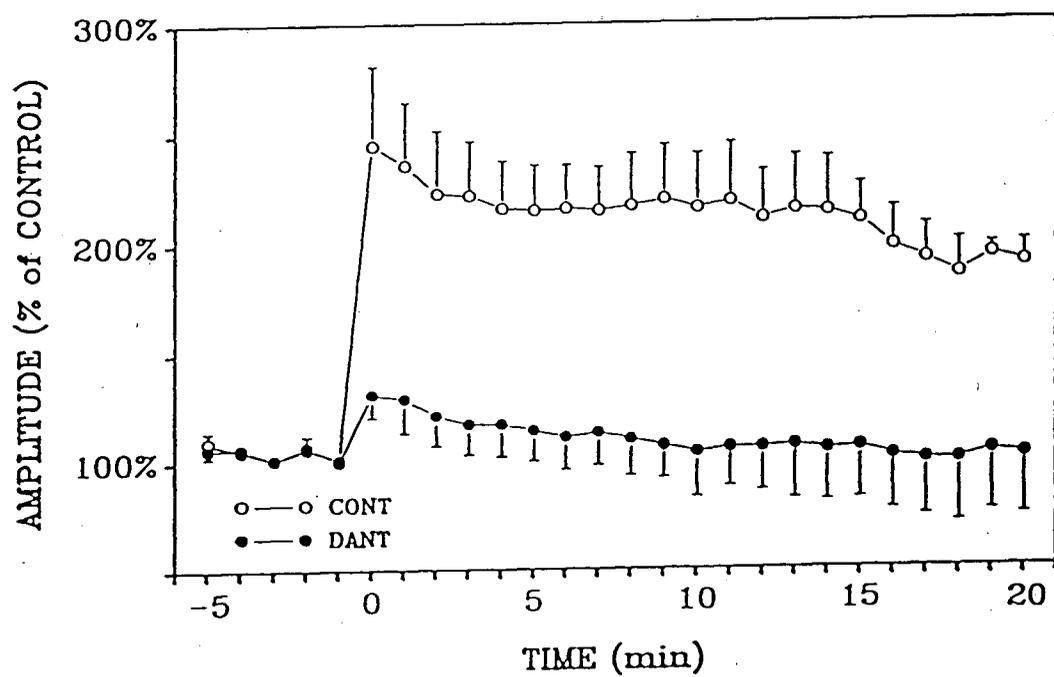
D. Results: Tetanic Induced LTP

1. Perfusion Experiments

In all experiments where control media were perfused a stable LTP could be induced by tetanic stimulation. Figure 5.3A illustrates that there is a slight tendency for LTP to decrease with time. When control ACSF was perfused for either 60 or 180 min prior to tetanic stimulation a robust potentiation was achieved. Measurement of the evoked population spike 10 min after a tetanic stimulation the degree of LTP was $310 \pm 54\%$ and $240 \pm 31\%$ respectively (T=0, where 100% represents baseline). The degree of potentiation was significantly decreased when measured at 30 min following the tetanic stimulation, to $292 \pm 33\%$ and $185 \pm 12\%$ ($p<0.05$) (N=5 slices for each control group). Short duration perfusions with dantrolene (10-20 min) had no effect on the ability to induce LTP.

A second set of controls was initiated to ensure that dantrolene itself had no effect on the amplitudes of the population spikes during the duration of the experiments. Figure 5.3B demonstrates that there was a slight tendency in the population spike amplitude to decrease when compared to slices exposed to control ACSF for 60 min and then dantrolene for 180 min. These slices were not given a tetanic stimulation.

After application of dantrolene ($\sim 20u$M) for 60 min to 180 min, a tetanic stimulation resulted in a brief period of potentiation (less than 5 min), which has been referred to by others (Bliss and Lynch 1988) as post-tetanic potentiation (PTP) (see Figure 5.4A). This PTP was control-like, or in some cases, even enhanced. However, the ability to induce LTP decreased progressively with the duration of dantrolene perfusion. Comparison between control potentiation and potentiation following 180 min dantrolene application demonstrated a significant reduction of the population spike amplitude at 10 min post tetanus ($135 \pm 10\%$; N=4) ($p<0.0005$); however,comparison between pre- and post-tetanic conditions of slices exposed to dantrolene for 180 min demonstrated no significant differences. In slices exposed to dantrolene for 180 min and subsequently washed in normal media for 120 min, no significant recovery of the LTP response ($148 \pm 6\%$; N=6; T=10 min) ($p<0.3$) was obtained (Figure 5.4A, B). The slope of the reversed

field EPSP (see methods) was measured as a index of change in cellular excitability. In these experiments (where 100% indicates baseline just prior to a tetanic stimulation), there was no change in the EPSP slope determined either 5 min prior to the tetanic stimulation ($106 \pm 3\%$) or 10 min post tetanus (103 $\pm$ 6%) when compared to control EPSP values ($90 \pm 6\%$; $99 \pm 6\%$). The concentration of dantrolene used ($\sim 20 u$M) had no significant effect on the amplitude of population spike recorded for up to 180 min prior to tetanic stimulation (Figure 5.3B).

Representative individual evoked population spikes obtained during the experiments are shown in Figure 5.4B. In each group the smaller evoked response is the control response prior to tetanic stimulation and the larger evoked response is 10 min following a tetanic stimulation. It should be noted that there is a gradual widening of the evoked population spike, however at the present time the cause for this change unknown (see Discussion).

2. Incubation Experiments

In general, the results from this series of experiments were the same as the perfusion experiments. The control slices exhibited a similar degree of potentiation with the population spike amplitude increasing to $216 \pm 25\%$ (T $=10$ min; N $=12$) and then declining slightly to $182 \pm 10\%$ (T$=20$ min). The slices incubated in dantrolene ($\sim 20 u$M) for a total of 120 min prior to tetanic stimulation demonstrated no LTP with values of $104 \pm 21\%$ and $102 \pm 28\%$, 5 min prior to and 10 min post tetanus respectively (Figure 5.5). The slope of the reversed field EPSP was measured and again no significant differences could be found following tetanic stimulation in normal ACSF or following dantrolene application.

One advantage of the incubation experiments is that the slices were being saturated with dantrolene from all sides, as compared to the perfusion experiments where only one side of the hippocampal slices was being exposed to the ACSF containing the drug dantrolene. The perfusion experiments do however have the advantage that slice movement and manipulations are kept to a minimum, thereby reducing damage to the afferent pathways and the cells of the slice. Taken together these two separate sets of experiments confirm that dantrolene is able to block the induction of LTP regardless of the mode of application utilized.

Figure 5.6: The blockade of Ca^{2+}-induced LTP by dantrolene.

A. LTP was induced by a transient (10 min) perfusion with high Ca^{2+} ACSF media containing 4mM Ca^{2+} and 6.25mM K^+ (filled horizontal bar at T=0 min), which could reliably induce an enhancement of the amplitude of the evoked population spikes (N=10). This LTP was maintained for the duration of the experiment. If slices incubated in dantrolene ($\sim 14\,u$M) for 120 min were exposed to this high Ca^{2+} ACSF, they were unable to elicit a significant enhancement of the population spike amplitude ($p < 0.005$) (N=5).

B. Examination of the slope changes of reversed field EPSPs demonstrated an enhancement (N=10) after transient application of high Ca^{2+}. However, incubation with dantrolene ($\sim 14\,u$M) for 120 min significantly increased the slope of the reversed field EPSP when compared to control slices. This enhancement was seen only during the first 10 min. (* $p < 0.001$)

A.

HIGH Ca^{2+} LTP: PSA

HIGH Ca^{2+} LTP

REVERSED FIELD EPSP SLOPE

B.
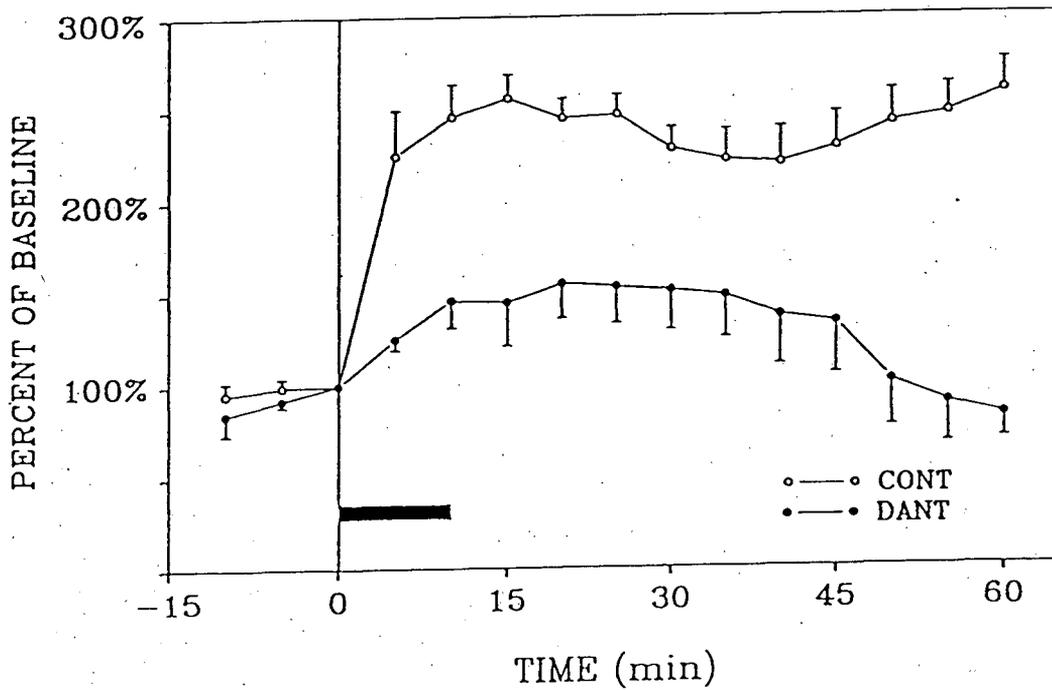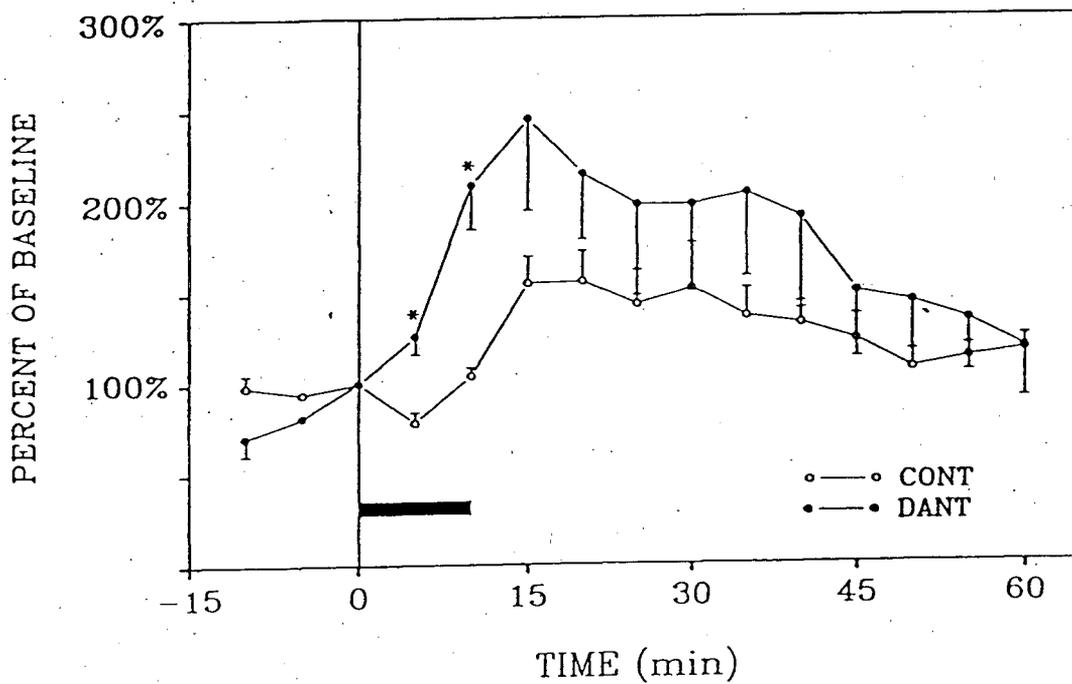
Figure 5.7: Typical evoked responses following perfusion of high Ca^{2+} ACSF.

This figure compares the effects of a transient (10 min) of high Ca^{2+} on the individual evoked population spikes in the CA1 region. Control slices displayed a robust enhancement of the evoked responses that was maintained for the 60 min duration of the experiment. Slices that were incubated in dantrolene for 60 min and then exposed to a pulse of high Ca^{2+} failed to elicit a robust increase in the population spike (also see Figure 5.6). It should be noted that the reversed field EPSP did increase (see Figure 5.6). Scale is as indicated by the bars.

CONT   High Ca^{2+} 1min WASH   60min WASH

CONT

DANT

2mV

2msec

170
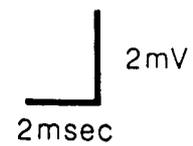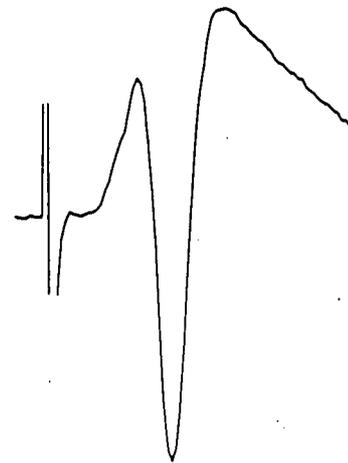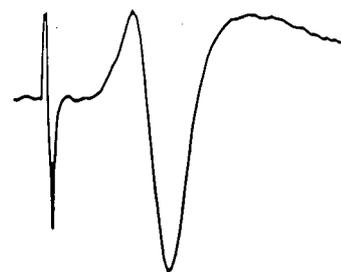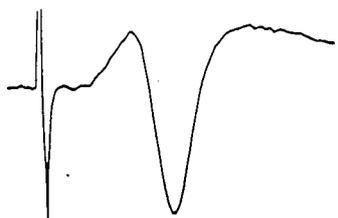
Figure 5.8: No change in Ca2+-induced LTP following pretreatment with the NMDA antagonist, D-APV.

A. As shown in this figure, a transient (10 min) perfusion of high Ca^{2+} (filled horizontal bar) resulted in a robust enhancement of the evoked population spike amplitude (N=10). This enhancement was maintained for the duration of the experiment. Pretreatment for 5 min prior and during the transient high Ca^{2+} by $50uM$ D-APV (cross hatched bar) did not block the induction of LTP (N=8). This novel finding is opposite to that found for the *in vivo* dentate gyrus, where it has been demonstrated that pretreatment with D-APV could block the induction of high Ca2+-induced LTP (Collingridge et al 1983).

B. A similar examination of the effects of high Ca^{2+} on the reversed field EPSP slopes demonstrated an increase in the slope of the EPSP (N=10). Pretreatment with $50\,uM$ D-APV for 5 min prior and during (cross hatched bar) the high Ca^{2+} perfusion resulted in a slight change between control and D-APV perfused slices (N=8) but only during high Ca^{2+} perfusion (* $p<0.05$).

A.

HIGH Ca^{2+} LTP: PSA
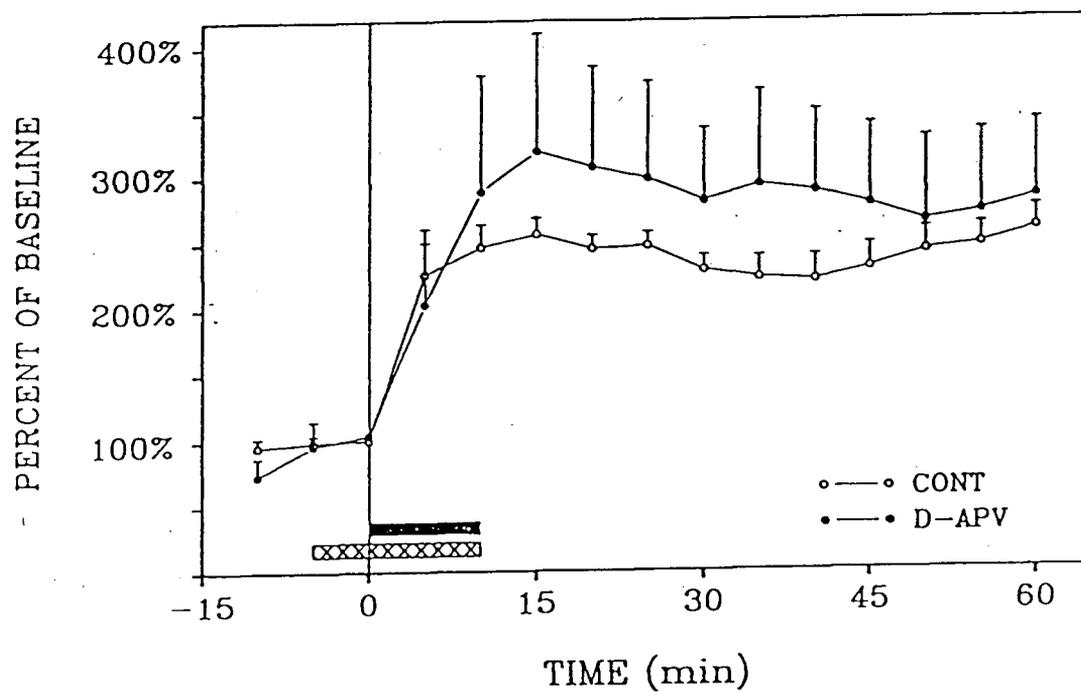
B.

HIGH Ca^{2+} LTP

REVERSED FIELD EPSP SLOPE
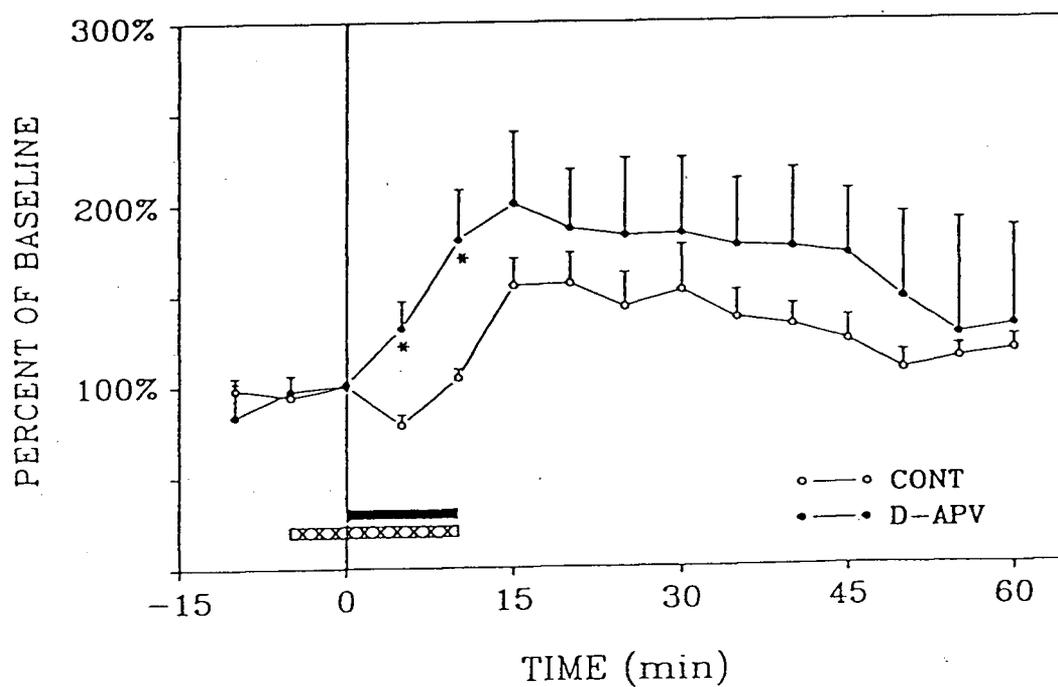
Figure 5.9: Typical evoked responses following perfusion of high Ca^{2+} ACSF.

This figure compares the effects of a transient (10 min) perfusion of high Ca^{2+} on the evoked population spikes. Control slices were able to display a robust enhancement of the evoked responses that was maintained for the 60 min duration of the experiment. Pretreatment of the NMDA antagonist, D-APV (50μM), 5 min prior to and during the transient perfusion of high Ca^{2+} failed to reduce the robust increase in the population spike (see also Figure 5.8). This finding is opposite to that of the findings with tetanic induced LTP (Collingridge et al 1983). Scale is as indicated by the bars.
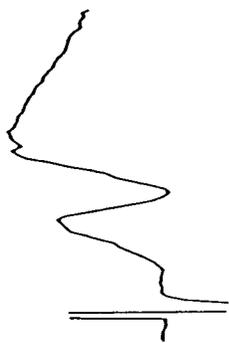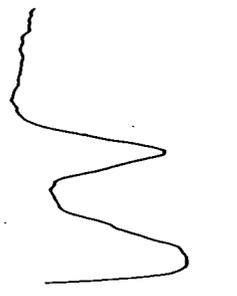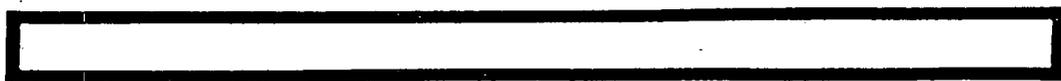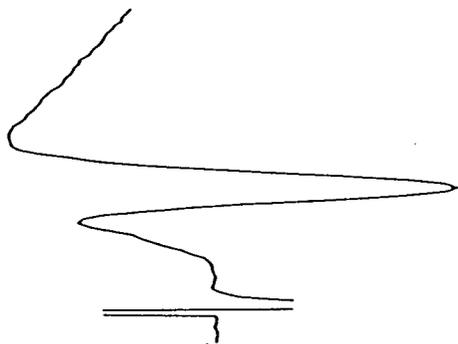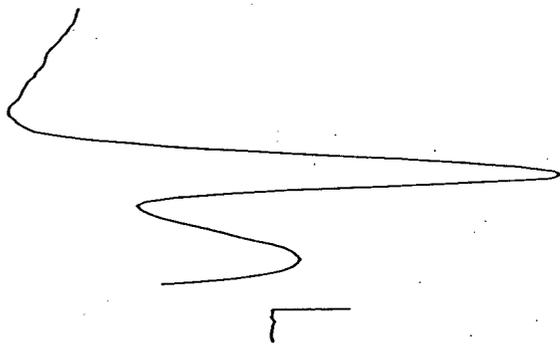
E. Results: Calcium-Induced LTP

1. Dantrolene Experiments

Enhanced synaptic responses in the CA1 region could be reliably induced following a 10 min transient perfusion of 4mM Ca^{2+} to the hippocampal slice while recording in the stratum pyramidal and radiatum region. Measurements of the amplitude of the evoked responses at 5 min prior to and at 15 min after the onset of the high Ca^{2+} pulse (5 min post high Ca^{2+} application) demonstrated a robust LTP in the CA1 stratum pyramidal with a smaller increase in potentiation in the dendritic layers (see Figure 5.2B). This enhancement was maintained for up to 60 min in our experiments.

Additional comparisons of the latency of the evoked response (from the time of the stimulus) showed no change in either region after LTP had been induced (data not shown). The above findings would suggest that the largest changes seen in the high Ca^{2+} experiments were those seen in the somatic CA1 region. It is important to note that the recordings were made in both regions of the slice at the same time, and the stimulus intensity was adjusted so as to evoke a 25% of maximum population spike. This method could in fact modify and modulate the responsiveness of the dendritic EPSP (see below).

A separate series of controls was run in conjunction with the dantrolene experiments and the results are presented in Figure 5.6A and B. The average amplitude at 5 min prior to high Ca^{2+} perfusion (T=-5) was 99 $\pm$ 5% (N=10). Following a 10 min application of 4mM Ca^{2+} the mean amplitude of the population spikes increased to 256 $\pm$ 13% at T=15 min (5 min post high Ca^{2+} pulse) and was maintained at T=60 min (259 $\pm$ 17%). After incubation and perfusion of dantrolene for 120 min, the pre-high Ca^{2+} values were no different at T=-5 (92 $\pm$ 4%; N=5) but in contrast the degree of potentiation was significantly reduced at T=15 (145 $\pm$ 24%) ($p < 0.005$) and at T=30 min (151 $\pm$ 22%), and by T=60 min population spike amplitudes had returned to control levels (82 $\pm$ 13%) (see Figure 5.6A). Representative population spikes evoked in control or dantrolene perfused slices are shown in Figure 5.7.

Measurements of the reversed field EPSP amplitude and the rate of rise (slope) in the above experiments, demonstrated significant differences ($p < 0.001$). The only noticeable difference in the reversed field EPSP was seen within the first 30 min after the application of the high Ca^{2+} (see Figure 5.6B).

Two sets of experiments were performed using different frequencies of stimuli to test whether the changes we observed above were due to stimuli delivered during the transient exposure to high $Ca2+$-induced changes. In one series of experiments a higher stimulation rate of 1 per min (0.0167 Hz) was used. The results were very similar if a slower (1 per 5 min, 0.0033 Hz) stimulation rate was used with the notable exception of a distinct post-tetanic potentiation (PTP). The data for the high Ca^{2+}-induced LTP experiments was obtained using the slower stimulation rate.

2. D-APV Experiments

Prior to using the NMDA antagonist D-APV in the Ca^{2+}-induced model of LTP, the efficacy of this compound was assessed in blocking tetanic-induced LTP (data not shown). Following tetanic stimulation (100 Hz for 1 sec), control slices elicited a robust LTP (greater than 300%). However, when D-APV (50uM) was applied for 15 min prior to tetanic stimulation, this NMDA receptor antagonist could block the induction of LTP.

The effects of D-APV were investigated further in $Ca2+$-induced LTP and were tested under the same conditions as described above, with the exception that the NMDA receptor antagonist D-APV (50uM) was applied for 5 min prior to and during the high Ca^{2+} perfusion for a total duration of 15 min. The results are shown in Figure 5.8 and 5.9.

Pretreatment with the NMDA receptor antagonist, D-APV, (at the same dose which was sufficient to block tetanic induced LTP) failed to block LTP induced by a 10 min high Ca^{2+} perfusion. Application of D-APV did not alter the magnitude of the evoked responses (when compared to controls) at pre-LTP levels (96 $\pm$ 19% (N=8)) (control 99 $\pm$ 5% (N=10)), at 5 min post high Ca^{2+} (T=15 min)(319 $\pm$ 91% (control 256 $\pm$ 13%) p=0.52) and at the end of the experiment at T=60 min (283 $\pm$ 59% (control 259 $\pm$ 17%)). Representative evoked population spikes are shown in Figure 5.9 comparing the effects of control and D-APV exposed slices.

Measurement of the reversed field EPSP slope demonstrated that there were significant changes in the slopes between control slices and slices where D-APV had been applied. However, this significance between slopes was seen only during the 10 min of high Ca^{2+} perfusion ($p < 0.05$) (Figure 5.8B). The reasons for these differences are unknown at this time (see Discussion).

II. DISCUSSION

The present findings support a crucial role for $[Ca^{2+}]_i$ in both tetanic and Ca^{2+}-induced LTP. The application of dantrolene, a compound which inhibits the release of Ca^{2+} from the sarcoplasmic reticulum of muscle (Desmedt and Hainaut 1977, Ward et al 1986), blocked the induction of LTP in both tetanic and Ca^{2+}-induced forms of LTP in our experiments. The time courses of these effects are similar in both forms of potentiation. The differences between the population spike amplitude data and the reversed field EPSP data following tetanic or Ca^{2+}-induced LTP provides evidence that following the induction of LTP there is a dissociation between the amplitude of the population spike and the EPSP slope (Taube and Schwartzkroin 1988a). In the extracellular recordings of the reversed EPSP slope, it is possible that while there may be blockade of the underlying EPSP (i.e. D-APV or dantrolene). This blockade could lead to an increase in the inhibitory post-synaptic potential (IPSP) which would increase the EPSP slope. Because the recordings were performed in the pyramidal cell layer, increases in the IPSP would be manifested as an increase in the slope of the EPSP/IPSP. If there is increased $[Ca^{2+}]_i$ (see Chapter 6) following a high Ca^{2+} application this increase could effectively reduce GABAergic release and action.

Another observation was that there was a gradual broadening of the population spike following long-term exposure to dantrolene. The underlying mechanisms are unknown, although this spread suggests that there may be a decrease in the total number of pyramidal cells that are firing action potentials in synchrony, resulting in a broader evoked population spike.

An additional finding of this chapter is the ineffectiveness of the NMDA receptor antagonist, D-APV, to block the enhancement seen following a transient perfusion of high Ca^{2+}. D-APV has been shown to be effective in blocking tetanic induced LTP (Collingridge et al 1983,

Harris et al 1984). Pretreatment of hippocampal slices with D-APV for 5 min prior to and during the high Ca^{2+} perfusion did not significantly reduce the enhancement of the population spike amplitude or the reversed field EPSP slope seen during high Ca^{2+} perfusion alone.

If both tetanic and Ca^{2+}-induced LTP do indeed share similar mechanisms, why then is the application of this antagonist ineffective in Ca^{2+}-induced potentiation? In the *in vivo* dentate gyrus, Bliss et al (1987) reported that Ca^{2+}-induced LTP could be blocked by NMDA receptor antagonists. Using a low Mg^{2+} model for the induction of potentiation, Hamon et al (1987) was able to demonstrate that $30uM$ D-APV was ineffective in blocking the enhanced responses during the perfusion of 1mM Mg^{2+} ACSF. Previous studies have demonstrated that removal of Mg^{2+} leads to the development of epileptiform activity that can be blocked by D-APV application (Stanton et al 1987). The results of Bliss et al (1987) were obtained from *in vivo* experiments which in themselves have differences. Their high Ca^{2+} pulse contained 10mM Ca^{2+} and was applied to the dentate gyrus. A recent study demonstrated differences in Ca^{2+}-induced LTP between these two regions; the CA1 region showed potentiation and the dentate gyrus actually showed a decrease in the size of the field potentials (Melchers et al 1987). In the present investigation, a high Ca^{2+} pulse elicited a stable, long lasting LTP, and pretreatment by the NMDA antagonist D-APV did not alter the evoked responses. Thus, blockade of the NMDA receptor in the high Ca^{2+} model does not block the induction of LTP. The most likely trigger for the induction of LTP in this model is an increase in post-synaptic $[Ca^{2+}]_i$ (see Chapter 6).

One possible difference between the findings reported in this chapter and other studies could be the D-APV concentration used. However, $50uM$ has been shown to be completely effective in blocking tetanic induced potentiation (Harris et al 1984). In addition, the low Mg^{2+} models also were able to prevent the enhancement of the population spike using only $30uM$ D-APV (Hamon et al 1987). The *in vivo* dentate gyrus study by Bliss and colleagues (1987) used a $100uM$ D-APV concentration. The results presented in this chapter used a $50uM$ D-APV, a concentration which is considered to be maximal for NMDA receptor blockade. An explanation might be that D-APV may act as a partial agonist. The K_d for the NMDA receptor is $1uM$ but

concentrations of 25-50uM are required to block this receptor, suggesting that the D-APV is a partial agonist.

The findings described above would suggest that intrinsic differences in the requirement for NMDA receptor activation exist between the dentate gyrus and the CA1 region of the rat hippocampus. Support for this suggestion comes from the NMDA receptor binding studies which demonstrate that the CA1 region of the hippocampus has one of the highest concentrations of this receptor (Monaghan et al 1984, Geddes et al 1986). In addition there could be different alterations in the sensitivity to excitatory amino acids following Ca^{2+}-induced LTP, as has been described for tetanus induced LTP (Davies et al 1989)

At the present time, there is considerable controversy over the relative contributions from pre-synaptic and post-synaptic elements to either of these two phases (induction versus maintenance), but it is well recognized that the induction of potentiation requires post-synaptic activation. Maintenance of LTP has advocates supporting both pre- (Bliss and Lynch 1988, Sastry 1982, Landfield et al 1988) and post-synaptic (Wigström and Gustafsson 1988, Brown et al 1988) elements as critical factors for long term potentiation.

The following hypothetical mechanism takes into account the results presented above and allows for both pre- and post-synaptic elements to contribute to the enhancement of the evoked responses. Following a tetanic stimulation to afferent fibers of the CA1 region of the hippocampus, the pre-synaptic fibers become depolarized and allow for pre-synaptic Ca^{2+} influx and subsequent neurotransmitter release (probably glutamate, see Storm-Mathisen 1977). An increase in neurotransmitter release follows, presumably from pre-synaptic terminals after tetanic stimulation has been reported, which was maintained for at least 1 hour post-tetanus (Bliss et al 1986). These findings have been question recently by Aniksztejn et al (1989).

Pre-synaptic depolarization and the release of glutamate during tetanic stimulation leads to depolarization and activation of the post-synaptic NMDA receptor complex. Early work by Collingridge et al (1983) demonstrated that blockade of the NMDA receptor by application of the NMDA receptor antagonist, D-APV, could block the induction of LTP (see also Harris et al 1984). NMDA receptor activation of the post-synaptic neuron leads to removal of the voltage dependent

block by Mg^{2+} of the channel (Nowak et al 1984, Mayer et al 1987). This "unblocking" of the NMDA receptors is thought to result in the post-synaptic entry of Ca^{2+} (Jahr and Stevens 1987). The resulting increases in $[Ca^{2+}]_i$ may then interact at various intraneuronal sites such as Ca^{2+} binding proteins including calmodulin (Mody et al 1984, Popov et al 1988), protein kinase C (Malinow et al 1988, Akers and Routtenberg 1987, Akers et al 1986, Alkon and Rasmussen 1981, Linden et al 1986, Lovinger et al 1985, Malenka et al 1986, Nishizuka 1988) and calcium dependent proteases (Lynch and Baudry 1984).

Previous studies have demonstrated the importance of $[Ca^{2+}]_o$ in the induction of LTP (Dunwiddie and Lynch 1979, Wigstrom et al 1979). A recent report by Auyeung et al (1986) described the induction of LTP in area CA1 by combining low-frequency afferent stimulation with K^+-induced depolarization of hippocampal slices in Ca^{2+}-free medium. In addition, conjunction of depolarizing pulse with afferent activity also induced LTP in CA1 neurons in the absence of Ca^{2+}. These results await further elucidation as does the importance of glia in LTP (Sastry et al 1988).

An elevation of $[Ca^{2+}]_i$ following activation of NMDA receptors has been implied both from electrophysiological and ^{45}Ca uptake studies (for a review see Bliss and Lynch 1988). Using Fura-2 microspectrofluorometry (Grynkiewiez et al 1985), an NMDA induced increase in $[Ca^{2+}]_i$ has been measured directly in a number of preparations, including hippocampal pyramidal cells maintained in tissue culture (Connor et al 1988)(see Chapter 6).

Once Ca^{2+} has entered the intraneuronal space of the neuron it can activate numerous Ca^{2+} dependent events that can lead to maintenance of the LTP response. It has been suggested that the activation of the NMDA receptor and the Ca^{2+} flux that occurs is sufficiently large enough and the duration is extensive enough to lead to the permanency of LTP (see Bliss and Lynch 1988).

The results presented above demand some modification of this proposed scheme to account for the role of intracellular stores of Ca^{2+} (see Chapter 6). Dantrolene blocks calcium release from the sarcoplasmic reticulum (Desmedt and Hainaut 1977, Roed 1982) and also reduces substantially or blocks the rise in $[Ca^{2+}]_i$ induced by NMDA receptor activation in cultured

(predominantly CA1) pyramidal cells (Mody et al 1989). The present experiments have shown that this drug also blocks the induction of LTP without significantly affecting the amplitude of the population spike. Whole-cell voltage-clamp recordings have ruled out a direct blockade of NMDA receptors or voltage operated Ca^{2+} channels (Mody et al 1989), suggesting that the action of dantrolene is probably due to antagonism of intracellular calcium release.

The source of this blockade of intracellular Ca^{2+} release is thought to be the endoplasmic reticulum of the neuron. The physiological functions of the muscle sarcoplasmic reticulum have been well documented and play an important role in $[Ca^{2+}]_i$ regulation (see Endo 1977, Martonosi 1984, for excellent reviews). Comparisons have been drawn between the sarcoplasmic and the endoplasmic reticulum. One reason for the interest in Ca^{2+} regulation by the endoplasmic reticulum is the finding that there are inositol phosphate sensitive sites located on the endoplasmic reticulum membrane (McBurney and Neering 1987). Further evidence for regulation of Ca^{2+} by the endoplasmic reticulum rests on the discovery of a high capacity, moderate affinity Ca^{2+} binding protein named calsequestrin (Meldolesi et al 1988, Volpe et al 1988, Pozzan et al 1988). Although the location of these organelles is uncertain, it is presumed that they lie within the cisterna of the endoplasmic reticulum. It appears then that the endoplasmic reticulum is in a unique position to regulate intracellular Ca^{2+} levels effectively. Given the activation of the NMDA receptors, one might envision the following sequence of events. Depolarization and activation of the NMDA receptors leads to a transient increase in $[Ca^{2+}]_i$, but at levels insufficient to result in maintained intracellular Ca^{2+} levels. This primary influx of Ca^{2+} could then be adequate enough to elicit a secondary release response (Ca^{2+}-induced Ca^{2+} release similar to that documented for cardiac muscle; Endo 1977, Martonosi 1984, Fabiato and Fabiato 1975, 1977).

This Ca^{2+}-induced Ca^{2+} release results in enhanced $[Ca^{2+}]_i$, which could activate the biochemical mechanisms underlying the maintenance of LTP, such as protein synthesis (Stanton and Sarvey 1984, Frey et al 1988, see also Charriaut-Marlangue et al 1988), gene transcription and changes in protein phosphorylation (Akers and Routtenberg 1987, Melchers et al 1988). Thus, the maintenance of the potentiated response is dependent on the release of the $[Ca^{2+}]_i$ from

intracellular stores such as the endoplasmic reticulum. This proposed cascade does not preclude a mechanism by which gating of NMDA receptors could initiate second messengers or other "factors" responsible for release of Ca^{2+} from intraneuronal stores.

The NMDA receptor is not activated in tetanic-induced LTP of the CA3 mossy fibers (Harris and Cotman 1986). The NMDA antagonist D-APV is however, effective in blocking LTP in the CA1 region when applied during tetanic stimulation of the Schaffer collateral/commissural fibers (Collingridge et al 1983, Harris et al 1984). Other glutamate receptor subtypes may play an important role in LTP (Izumi et al 1987). Davies et al (1989) reported an increase in the sensitivity of the quisqualate receptor following the induction of LTP. This finding provides evidence for a post-synaptic change: pre-synaptic elements may contribute but in a temporally distinct manner. Therefore, although evidence suggests that the NMDA receptor plays a role for the induction of LTP in the CA1 region of the hippocampus, other brain regions may not require activation of the NMDA receptor for potentiation to occur.

In previous discussions of the role of $[Ca^{2+}]_i$ in LTP, little has been made of the possible spatial requirements. The changes in $[Ca^{2+}]_i$ resulting from activation of voltage-gated calcium channels may be highly restricted to the cytoplasmic volume immediately adjacent to the channel (Chad and Eckert 1984, Simon and Llinas 1985). Other mechanisms of increasing $[Ca^{2+}]_i$, in particular the involvement of phosphatidylinositol turnover (Alkon and Rasmussen 1988) may affect a much larger volume of the cell. A requirement for intraneuronal calcium release would be consistent with many of the treatments which induce LTP such as perfusion of phorbol esters (Malenka et al 1986) and perhaps the transient elevation of calcium itself (data presented above, Bliss et al 1987, Turner et al 1982). The site-specificity of LTP may still require some restriction of Ca^{2+} release post-synaptically or may reside in the pre-synaptic terminal.

In conclusion, the data presented above is consistent with the hypothesis that release of calcium from intraneuronal stores in the post-synaptic neuron is a critical pre-requisite for LTP to occur. As such, NMDA receptor activation, increasing $[Ca^{2+}]_0$ or application of phorbol esters, may all be capable of inducing LTP due to their ability to mobilize intraneuronal calcium stores. This hypothesis predicts that any neurotransmitter or other agents capable of stimulating the

release of post-synaptic calcium stores should also induce LTP. An important role for a pre-synaptic involvement in the specificity and/or maintenance of long-term potentiation is not, however, precluded.

CHAPTER 6 - Determination of Intracellular Calcium Levels

I. INTRODUCTION

In previous chapters it has been suggested that changes in $[Ca^{2+}]_i$ might be responsible for some of the effects seen in the various experimental paradigms, particularly in the low Ca^{2+} bursting and the LTP experiments. With the advent of fluorescent dyes that bind Ca^{2+} it has become possible to accurately and rapidly determine $[Ca^{2+}]_i$ levels in living cells (Grynkiewicz et al 1985). Using this methodology applied to *in vitro* cultures of dispersed hippocampal neurons, the experiments presented in this chapter were designed to investigate directly the changes in resting $[Ca^{2+}]_i$ after: i) application of serotonin and adenosine, ii) perfusion of low Ca^{2+} ACSF, and iii) changes in $[Ca^{2+}]_i$ levels following the perfusion of high Ca^{2+} ACSF.

The use of Fura-2 as an intracellular calcium indicator has several distinct advantages: i) it can be loaded into the cytoplasm without puncturing the neuronal membrane, ii) it has a weaker Ca^{2+} affinity which allows for the measurement of higher Ca^{2+} values (several micromolar), iii) it allows spatial imaging of signals, iv) it has a faster response time than ion sensitive electrodes, and v) it can be re-excited numerous times without photo-bleaching (Tsien 1988).

While there are numerous advantages, there are also some limitations to using fluorescent dyes: i) these dyes cannot be used on photosensitive tissue, ii) fluorescent dyes have low dissociation constants (100-400nM) and can become saturated making it difficult to distinguish between $[Ca^{2+}]_i$ levels above several micromolar, and iii) the possibility exists that dyes may of compartmentalize into intracellular organelles (Tsien 1988). Despite these limitations, experiments using Fura-2 have provided some fascinating insights into neuronal Ca^{2+} functions in both the dendritic and cell soma regions (see Connor et al 1988).

The present experiments carried out in an attempt to: i) quantify $[Ca^{2+}]_i$ changes following application of 5-HT and its agonists and antagonists, ii) assess $[Ca^{2+}]_i$ changes following application of adenosine and its agonists and antagonists, iii) determine the effects of reducing extracellular Ca^{2+} on $[Ca^{2+}]_i$ in cultured hippocampal neurons, and iv) measure $[Ca^{2+}]_i$ following a transient perfusion of high Ca^{2+}.

II. METHODS

Intracellular Ca^{2+} levels can be measured and quantified using one fluorescent metallochromatic indicator. One such hydrophilic indicator is Fura-2. An acetoxymethyl (AM) ester of this fluorescent dye can permeate cells and in the cytosol non-specific esterases cleave the AM side chains to produce the membrane-impermanent free indicator. When the dye chelates Ca^{2+} ions, the absorption peak shifts to a shorter wavelength (from 362 to 335nm) and the ratio of the concentrations of free indicator to the chelated form can be monitored by double wavelength microspectroscopy.

Several Ca^{2+} chelating dyes exist and selection of the appropriate dye depends on the particular use. In our experiments Fura-2 was the dye of choice. It is highly fluorescent in both bound and unbound states, and can be used at low intracellular concentrations due to an approximate 30 fold greater fluorescence per mole of dye when compared to Quin-2. It is resistant to fading, so that measurements can be made over a longer period of time without photobleaching (see Tsien et al 1982, 1984, Grynkiewicz et al 1985 for details).

We have applied this methodology to a cell culture system in which $[Ca^{2+}]_i$ can be determined in individual neurons (see Figure 6.1 for a schematic). Extension of this experimental procedure to the slice preparation has several problems including difficulty in visualizing individual neurons and requires sophisticated computer imaging analysis unavailable to us at the present time.

A. Cell Culture

Dispersed rat hippocampal neuron cultures were prepared from 18-day-old embryos according to Banker and Cowen (1977) with the following modifications. The fetal tissue was treated with 0.1% trypsin for 15 min and the dispersed cells plated at a density of 100,000 cells/cm^2 directly onto 18mm diameter glass coverslips previously coated successively with 10 ug/ml poly-D-lysine in 0.15 M borate buffer, pH 8.4 at room temperature overnight, and then with 16.7 ug/ml laminin (Gibco) in Ca/Mg free Hanks solution for two hours at room temperature just before use. After 30 min the coverslips were drained carefully and placed upside down into 3.5 cm culture dishes containing 2 ml Dulbecco's minimum essential medium supplemented with 10%

horse serum. Two days later 0.2 ml of 2 mg/ml 5-fluoro-2'-deoxyuridine and 5 mg/ml uridine were added to the medium to suppress glial growth and division. Under these conditions we were able to obtain evenly dispersed neuron cultures with very little glial contamination.

B. Fura-2 and Cell Loading

Cells were loaded with Fura-2AM (Molecular Probes, Eugene, Oregon) as described in Poenie et al (1986) (see Figure 6.1). Pluronic F-127 and bovine serum albumin were used to maintain the Fura-2 in suspension and the cells were loaded for 1.5 hours at 37°C. Under this method cells showed no indication of compartmentalization into intracellular organelles and could be used for up to 4 hours after loading with only minimal signs of leakage of Fura-2. Washing out of unconverted Fura-2AM occurred during the equilibration time when the coverslips were mounted on the recording chamber.

C. Calibration of Fura-2 Fluorescence

For calibration, neuronal preparations identical to those used in experimental manipulations were loaded with Fura-2AM and mounted on the chamber. The ratio of fluorescence induced by excitation at 350 and 380 nm was determined in the presence of the non-fluorescent calcium ionophore $5uM$ Br-A23187 first in the presence of zero external Ca^{2+} (with 1mM EGTA) and then in the presence of excess (1mM) Ca^{2+}. The choice of wavelengths was dictated to some extent by the emission peaks of the mercury arc lamp and the limited transmission of glass optics and filters below 350nm. Measurements were taken from at least 20 neurons under each condition and the values of R_{min}, R_{max} and Beta determined to be 0.341, 2.991 and 9.72 respectively (see Grynkiewicz et al. 1985).

D. Microspectrofluoroscopy

For recordings the coverslips were mounted with the cells down onto a laminar flow-through chamber (volume approx. 400 ul). Silicone rubber sealant was used to complete a water-tight seal, the chamber inserted into a stainless steel holder and the entire assembly mounted onto the stage of a Zeiss Jena Jenalumar microscope equipped for epifluorescence. The light source was a 200 Watt mercury arc lamp powered by a DC power supply. The light was passed first

through one of two differential interference filters (350 or 380 nm $\pm$ 5 nm) mounted in a computer controlled turret, and then through a 410 nm dichroic mirror and a 100X apochromat oil immersion lens with a numerical aperture of 1.4 and an adjustable diaphragm to reduce the light intensity. A circular diaphragm in the light path was used to reduce the area of excitation to the size of a single neuron. The fluorescent light was passed back through the dichroic mirror and a bandpass filter (490 nM) and then deflected either to the eyepieces or to the camera port in which was mounted a photomultiplier tube. Continuous measurements of fluorescence ratios were obtained on a 5 sec time base and individual measurements (eg. of resting $[Ca^{2+}]_i$ prior to and between stimulations) were also taken when appropriate. The data were stored on a computer for later determination of free calcium concentrations.

E. Chamber Design

The chamber holder was constructed of stainless steel and was designed such that the incoming perfusate passed first through a narrow bore 15 cm long channel coursing through the holder. The perfusate then entered an open well, connected to the laminar flow chamber via a 1 cm long 2 mm diameter channel. The outflow was collected in a second open well on the opposite side of the chamber and the excess perfusate removed by suction. This design enabled the temperature to be accurately controlled to within +/- 0.5°C in the range of 22-34°C (24°C unless otherwise stated) by the use of two heating elements placed under the chamber holder which were regulated by a thermistor-feedback heating control unit. Drugs were added via a syringe pump connected to a timer such that 50 ul of drug was delivered over a 3 second period. A suitable extension of cannula tubing was attached to the syringe in the pump and the tip placed directly into the chamber entry port via the first open well. The chamber itself was constructed of Perspex and the floor of the holder below the chamber was made of glass in order that the cells could also be viewed with transmitted light.

F. Calculations and Calibration

The intracellular calcium concentrations of the cells are calculated using the following formula:

$$[Ca^{2+}]_i = K_d * B * ((R - R_{min})/(R_{max} - R))$$

where:

K_d = rate constant for the association of Fura-2 with cytosolic free calcium (200nM)

B = ratio of fluorescence intensities (380nm with 0 $[Ca^{2+}]_0$)/(380nm with infinite $[Ca^{2+}]_0$)

R = experimental ratio of fluorescence intensities (350nm/380nm)

R_{min} = R with 0 $[Ca^{2+}]_0$

R_{max} = R with infinite $[Ca^{2+}]_0$

To obtain R_{min} cells were incubated for 20 min with 5mM Br-A23187 in the presence of a medium containing no added Ca^{2+} and 10mM EGTA. R_{max} was then determined on the same population of neurons by substitution of a medium containing ionophore and 1.8mM calcium.

Periodic measurements of $[Ca^{2+}]_i$ were taken from single, randomly selected neurons. The perfusion media used in the following experiments consisted of artificial cerebrospinal fluid (ACSF) as defined in the previous chapters: i) normal ACSF, ii) low Ca^{2+} ACSF, and iii) high Ca^{2+} ACSF.

III. RESULTS

A. Resting Intracellular Calcium Levels

Intracellular calcium levels were determined from cultured rat hippocampal neurons, which had been loaded with Fura-2AM. Figure 6.2 illustrates that resting $[Ca^{2+}]_i$ were consistently less than 150nM and were stable for at least 1.5 hours. The neurons in this experiment were perfused with normal ACSF which was bubbled with 95% O_2/5% CO_2.

Figure 6.1: Procedural steps required for the determination of $[Ca^{2+}]_i$ levels using Fura-2 microspectrofluorimetry in cultured rat hippocampal neurons.

Schematic of the three principal steps involved in preparing rat hippocampal cell cultures for determination of intracellular Ca^{2+} levels using Fura-2 microspectrofluorimetry

Rat hippocampal neurons were cultured on coverslips for two weeks prior to loading with the Ca^{2+} sensitive fluorescent dye, Fura-2AM. The loaded cultures could then be used on an epifluorescent microscope for the determination of $[Ca^{2+}]_i$ levels of individual neurons.

1 Preparation of Neuronal Cultures:

a. 18 day old Wistar rat embryo

b. Hippocampus dissected out

c. Cells dissociated & plated onto 18 mm coverslip. Incubated for 2 weeks face down in 5% CO_2 at 37°C

2 Loading of Fura-2 AM:

a. 50 µL DMSO

Fura-2AM

EBSS media solution

b. Add solution to culture

c. Incubate for 1.5 hours at 37°C with 5% CO_2 with Fura-2AM.

d. Cells were washed with standard EBSS and post-incubated for 0.5 hours in standard EBSS.

e. Cells are now ready to use.
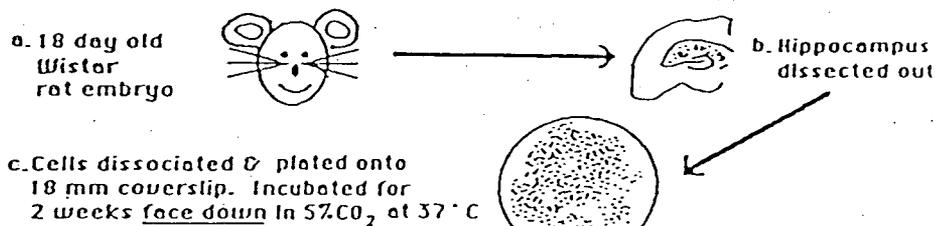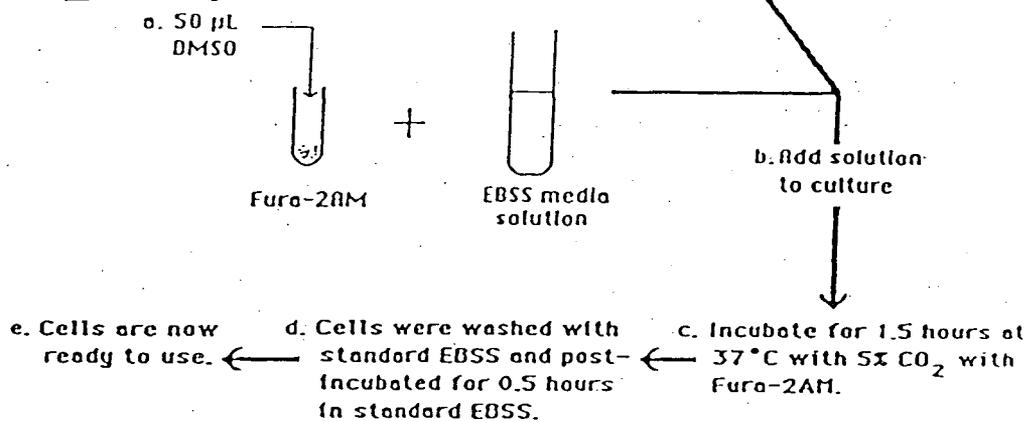
3 Determination of Intraneuronal Ca^{+2}:

After incubation, place culture face-down on well filled with EBSS.

Calculation of intracellular Ca+2 in hippocampal neurons.
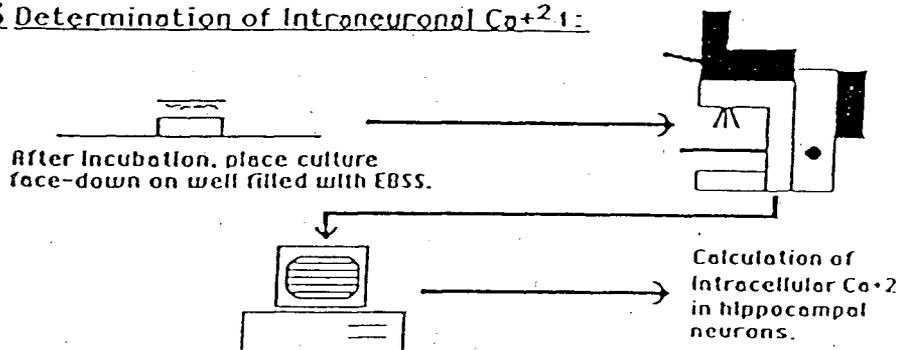
Figure 6.2: Effects of Fura-2 on $[Ca^{2+}]_i$ levels.

Intracellular Ca^{2+} levels were measured with the fluorescent probe Fura-2 in cultured hippocampal neurons prepared from embryonic rats (E18). The ratio of Fura-2 fluorescence at 350 and 380 nm was determined on a 5 sec time base using a Jenalumar microscope equipped for epifluorescence. Individual cells were isolated with an adjustable circular diaphragm and viewed with a 100X oil immersion lens. The fluorescent light was diverted either to the eyepieces or to a photomultiplier tube mounted in the camera port. The signal was converted to digital form and stored on a computer for later calculation of $[Ca^{2+}]_i$ levels (see Figure 6.1). Calibration was achieved using neurons exposed to the Ca^{2+} ionophore Br-A23187 either in the absence of external Ca^{2+} (with 1mM EGTA) or in the presence of 1.8 mM Ca^{2+}.

This figure illustrates that determination of $[Ca^{2+}]_i$ levels in cultured rat hippocampal neurons using Fura-2AM does not overtly affect levels of $[Ca^{2+}]_i$. Each data point represents the determination of $[Ca^{2+}]_i$ levels from 5-10 randomly selected neurons. It is important to note that in this and all subsequent figures, each data point represents the combined values of the randomly selected neurons in the 2-3 min preceding and after the data point. If repeated determinations were made from the same neuron, similar results could be obtained.

Resting $[Ca^{2+}]_i$ – Control
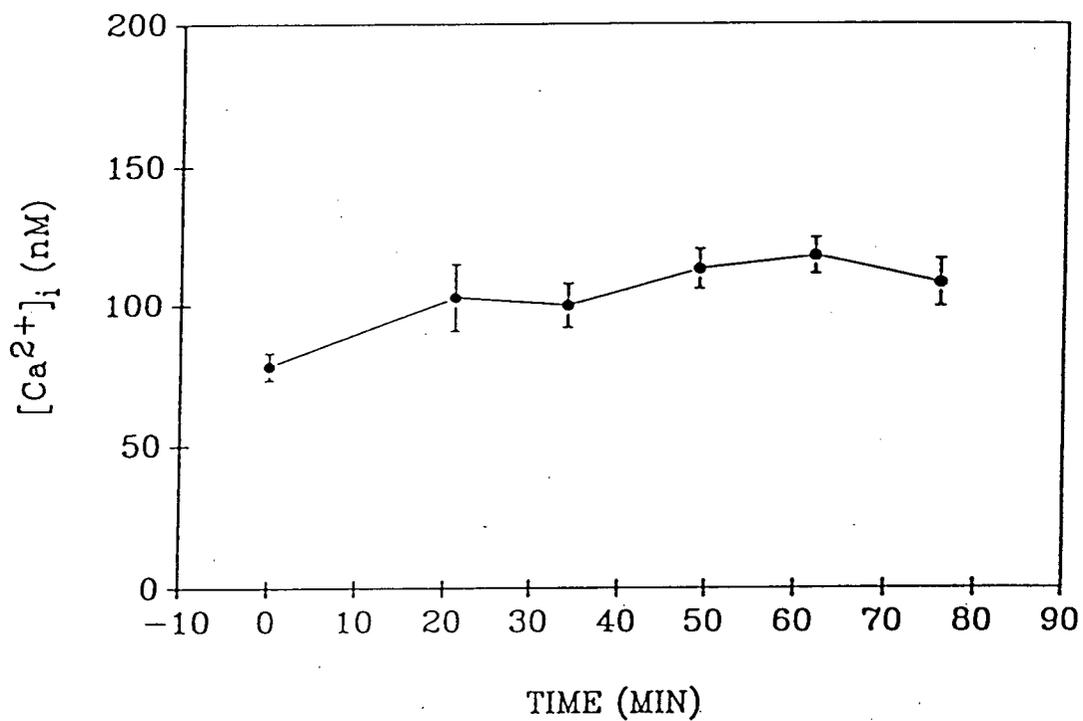
Figure 6.3: The effect of low Ca^{2+} ACSF perfusion on cultured hippocampal cells

Intracellular Ca^{2+} levels were determined prior to and after perfusion of low Ca^{2+} ACSF.

A. This figure illustrates that after perfusion of low Ca^{2+}, each individual neuron had a decrease in $[Ca^{2+}]_i$ levels (N=15). Intracellular Ca^{2+} levels were determined for each neuron prior and following the perfusion of low Ca^{2+} (onset at T=0 min)

B. This figure presents the average $[Ca^{2+}]_i$ values prior to and during the low Ca^{2+} perfusion. Data are from the same neurons shown in part A above. (* p<0.002)

A.

$[Ca^{2+}]_i$ MEASUREMENTS FROM INDIVIDUAL NEURONS
PRIOR AND DURING LOW Ca^{2+} PERFUSION
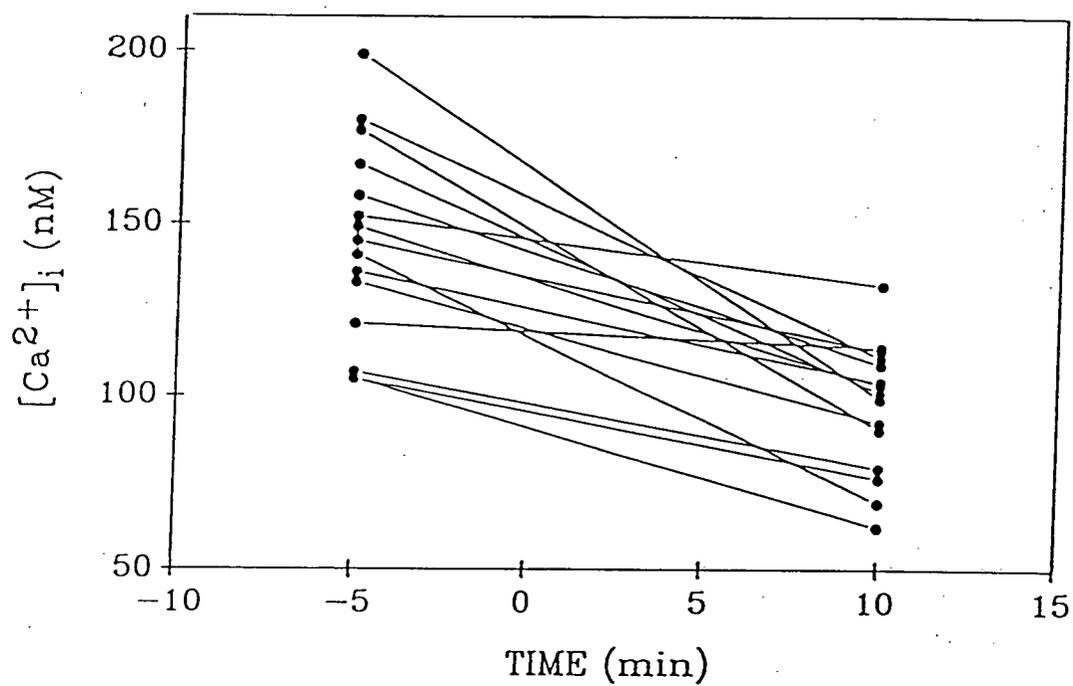
B.

LOW Ca^{2+} PERFUSION WITH FURA-2
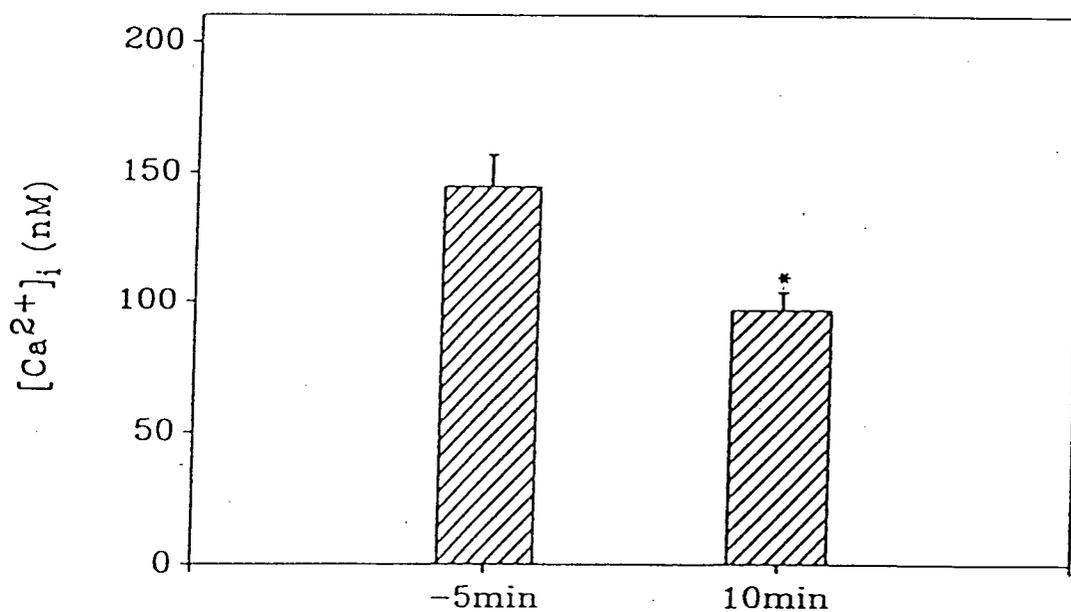
Figure 6.4: Intracellular Ca^{2+} level changes following application of high Ca^{2+} ACSF.

A. Using Fura-2 microspectrofluorimetry, the effects of a transient (10 min) perfusion of 4mM Ca^{2+} on the $[Ca^{2+}]_i$ of randomly selected cultured hippocampal neurons was examined. Application of high Ca^{2+} (filled horizontal bar) resulted in a gradual and maintained increase in $[Ca^{2+}]_i$ levels. These enhanced levels of Ca^{2+} were maintained for the duration of the experiment. Each data point represents $[Ca^{2+}]_i$ determinations from 7-10 hippocampal neurons.

B. Although $[Ca^{2+}]_i$ levels were significantly elevated, application of glutamate ($50\,ul$, $50\,uM$) was able to elicit a typical rise in $[Ca^{2+}]_i$ levels. This experiment was done to assess the responsiveness of neurons with elevated $[Ca^{2+}]_i$ levels. This finding demonstrates that although $[Ca^{2+}]_i$ levels are elevated, the neuron is still capable of responding to neurotransmitter application.

A.

Effect of Transient High Ca^{2+}
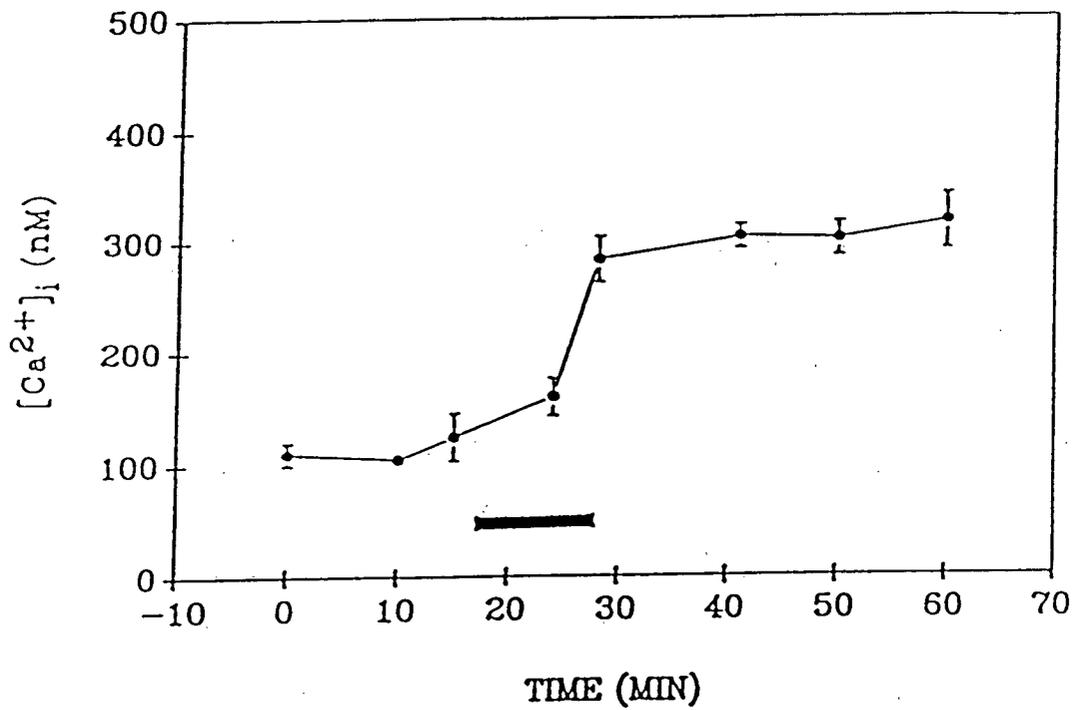
B.

Effect of Transient High Ca^{2+}
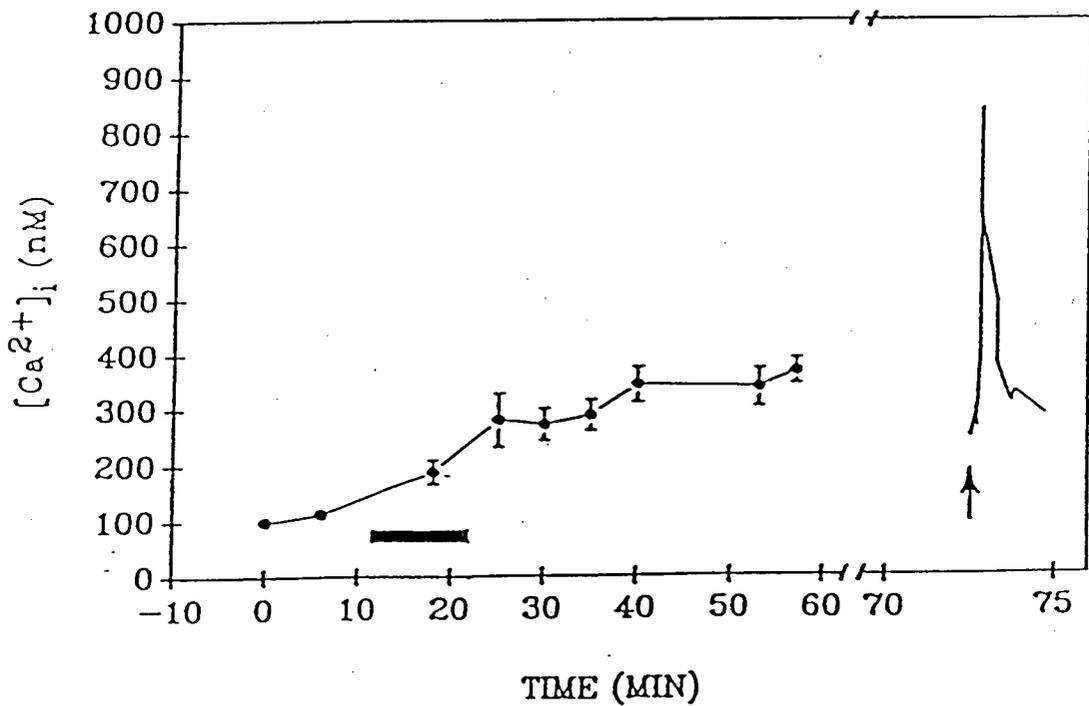
Figure 6.5: The effect of dantrolene on cultured hippocampal neurons.

A. Application of dantrolene from a DMSO stock solution $(20u\text{M})$ (filled horizontal bar) for extended periods of time had no effect on intracellular Ca^{2+} levels when applied to cultured hippocampal neurons. Each data point represents the determination of $[Ca^{2+}]_i$ levels from 7-12 neurons.

B. This experiment demonstrates the ability of dantrolene to block the rise in $[Ca^{2+}]_i$ levels following a transient exposure of high Ca^{2+} ACSF. DMSO $(100ul/100\text{ml})$ was applied first as a control, as dantrolene is very insoluble. Dantrolene application for 15 min prior and during the 10 min high Ca^{2+} pulse did not elicit any appreciable changes in intracellular Ca^{2+} concentrations. Re-perfusion with medium containing dantrolene only elicited no changes in $[Ca^{2+}]_i$ levels. This would suggest that dantrolene is capable of blocking the high-Ca^{2+} induced increases in intracellular Ca^{2+} concentrations.

A. Dantrolene Control
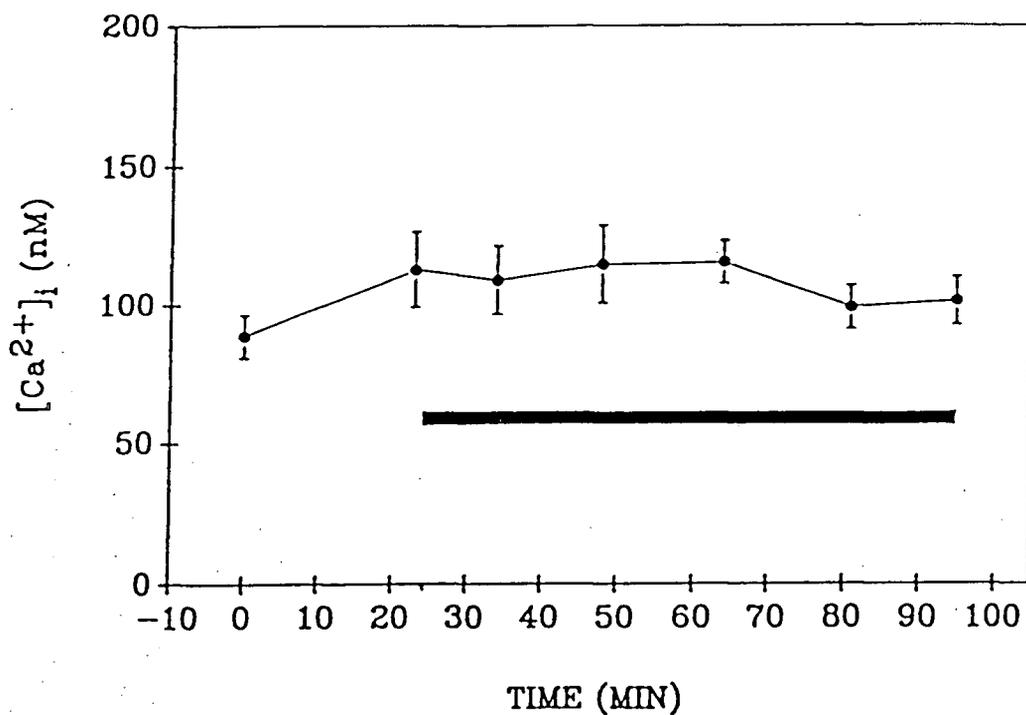
B. Effect of Dantrolene on High Ca^{2+}
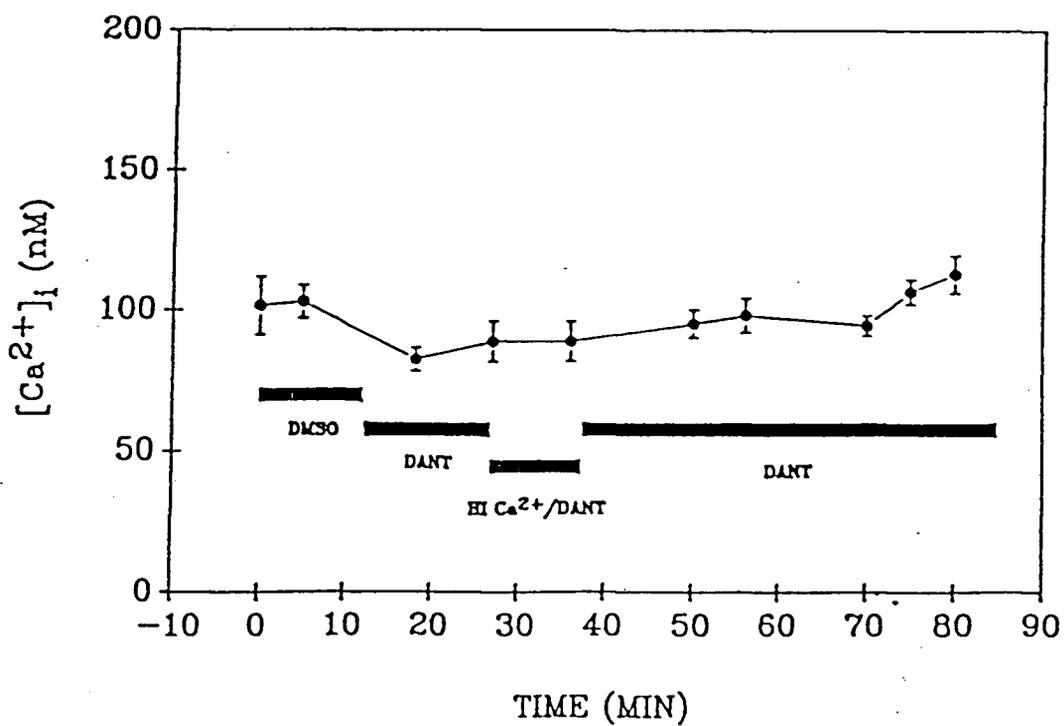
Figure 6.6: The effect of D-APV on a transient pulse of high Ca^{2+} in cultured hippocampal neurons.

Application of D-APV (50uM) for 5 min prior to and during (cross hatched horizontal bar) the transient (10 min) exposure to high Ca^{2+} (4mM) (solid horizontal bar) did not prevent the rise in intracellular Ca^{2+}. These [Ca^{2+}]$_i$ measurements were taken from 6-9 cultured hippocampal neurons. This figure suggests that blockade of the NMDA receptor does not prevent the rise in [Ca^{2+}]$_i$ from intraneuronal stores.

EFFECT OF D–APV ON HIGH Ca^{2+}
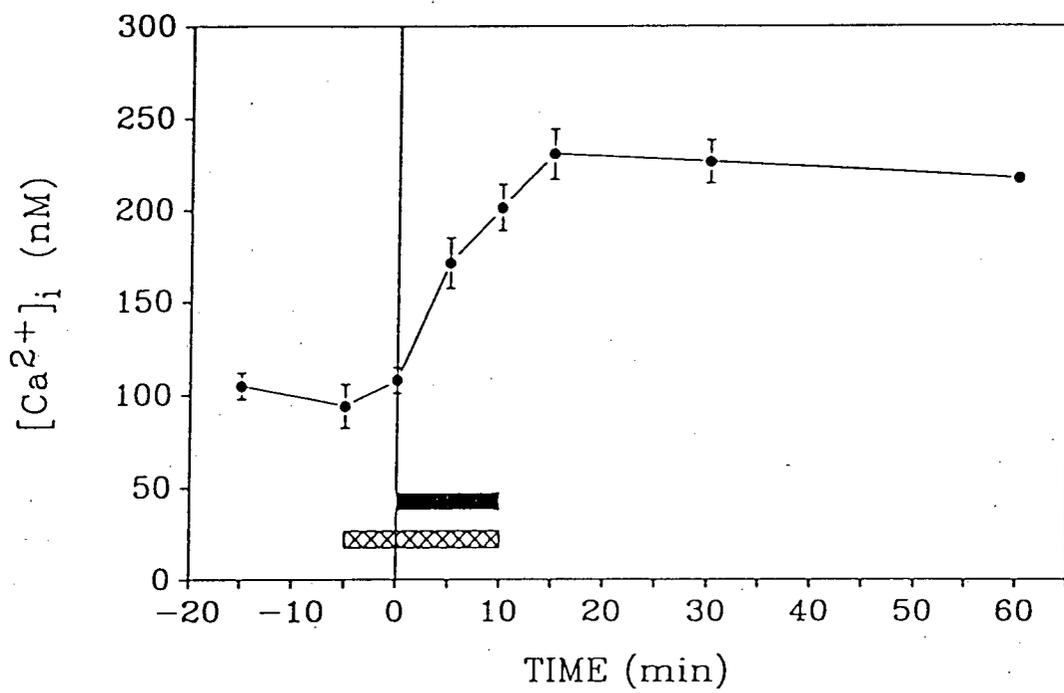
B. Serotonergic and Purinergic Effects on Intracellular Calcium

Based on reports of serotonergic and purinergic modulation of $[Ca^{2+}]_i$, repeated attempts were made to determine the effects of these agonists on resting $[Ca^{2+}]_i$ in hippocampal cultured neurons. No changes in intracellular Ca^{2+} levels were seen following either transient application or continuous perfusion of 5-HT ($50uM$), 8-OH-DPAT ($50uM$), DOI ($50uM$), AD ($25,100uM$) and 2-chloroadenosine (CAD) ($100uM$). Co-application of 5-HT or AD with glutamate ($50uM$) did not result in any consistent differences in $[Ca^{2+}]_i$ levels when compared to responses induced by glutamate alone (data not shown).

C. Perfusion of Low Calcium ACSF: Changes in Intracellular Calcium

Perfusion of hippocampal neuron cultures with low Ca^{2+} ACSF resulted in a marked decline in $[Ca^{2+}]_i$ levels in 15 of 15 neurons examined. The greatest change in $[Ca^{2+}]_i$ occurred within the first 10 min following the perfusion of low Ca^{2+} ACSF and remained stable at this lower level for the duration of the experimental period (greater than 40 min). Figure 6.3A illustrates the individual responses of these 15 neurons which had resting $[Ca^{2+}]_i$ in the range of 105-199nM. Perfusion of low Ca^{2+} ACSF resulted in a decrease in $[Ca^{2+}]_i$ to the range of 62-132nM (Figure 6.3B). Intracellular $[Ca^{2+}]$ levels fell significantly ($p<0.002$) to their new values within 10 min.

The low Ca^{2+} ACSF used in these experiments was identical to that used in Chapter 4. It is important to note that removal of Ca^{2+} from the perfusate does not eliminate residual amounts of Ca^{2+} that may be a contaminate of the other ionic constituents. It is estimated that the low Ca^{2+} ACSF may still have contained $1-5uM$ Ca^{2+}: EGTA was not included in order to be consistent with the medium used for induction of bursting activity in the hippocampal slice preparation.

D. Effects of Elevated Calcium

Using an experimental protocol similar to that used for Ca^{2+}-induced LTP (Chapter 5), application of a 10 min pulse of 4mM Ca^{2+} to cultured hippocampal neurons resulted in an increase in $[Ca^{2+}]_i$ which was maintained for at least 30 min following the return to normal

ACSF (Figure 6.4A). The increase in $[Ca^{2+}]_i$ was approximately 3-fold and highly significant (control 110 $\pm$ 10nM (N=10); high Ca^{2+} 304 $\pm$ 11nm (N=7))(where N= indicates the number of cultured cells examined) (p<0.0001). In a repeat of this experiment, cell viability was tested by a transient pulse of L-glutamate. A rapid and transient increase in $[Ca^{2+}]_i$ was obtained indicating that despite an elevated $[Ca^{2+}]_i$ hippocampal neurons were still responsive to glutamate (Figure 6.4B).

Perfusion of ACSF containing approximately $20uM$ dantrolene for longer than 60 min had no significant effect on resting neuronal $[Ca^{2+}]_i$ (Figure 6.5A). Since dantrolene is prepared from a DMSO stock solution the effect of DMSO (100ul/100ml) was also tested. Treatment of these cultured neurons with a solution containing DMSO also elicited no changes in resting $[Ca^{2+}]_i$ as shown in Figure 6.5B. However, treatment with dantrolene ($\sim 20uM$) for 15 min prior to and during a 10 min perfusion with high Ca^{2+} ACSF effectively blocked the sustained rise in $[Ca^{2+}]_i$ seen with high Ca^{2+} alone (compare Figures 6.4 and 6.5B). Values of $[Ca^{2+}]_i$ did not change significantly from pre-high Ca^{2+} (82 $\pm$ 4nM; N=10) to post high Ca^{2+} (95 $\pm$ 5nM; N=11) (p<0.06).

In a similar experimental design, perfusion of high Ca^{2+} ACSF containing $50uM$ D-APV, prior to and during the transient pulse of high Ca^{2+} (4mM) did not block the rise in $[Ca^{2+}]_i$ (Figure 6.6). This rise remained elevated for the duration of the experiment (compare Figures 6.4A and 6.6). Intracellular Ca^{2+} rose significantly from resting levels of 105 $\pm$ 7% (N=8) to 226 $\pm$ 12% (N=6) (p<0.0001).

IV. DISCUSSION

While the hippocampal slice technique allows examination of the electrophysiological changes which occur for example following a high frequency stimulation or a transient perfusion of high Ca^{2+}, measurements of resting $[Ca^{2+}]_i$ of single neurons in this preparation require sophisticated imaging techniques. Using cultured hippocampal neurons, assessment of $[Ca^{2+}]_i$ is relatively simple with Fura-2AM microspectrofluorimetry. The results from such experiments may provide an estimate of those changes in $[Ca^{2+}]_i$ which may occur in the slice preparation

under similar conditions. The purpose of this chapter was therefore to simulate as much as possible the experimental protocols in slice experiments.

A number of methods have determined indirectly that neuronal resting $[Ca^{2+}]_i$ levels are in the order of $10^{-7}M$ free Ca^{2+}. In the cultured hippocampal neuronal preparation used in our experiments the average free Ca^{2+}_i concentration is between 100-150nM, which concurs with other findings in hippocampal (Connor et al 1988), dorsal root ganglion (Thayer et al 1988), cerebellar neurons (Tank et al 1988).

Despite reports that have suggested that serotonin or adenosine may modulate changes in intracellular Ca^{2+}, no evidence was found that either of these compounds consistently affected $[Ca^{2+}]_i$ (Kendall and Nahorski 1984, Ribeiro and Sebastiao 1986). The suggested mechanism of action for 5-HT or 8-OH-DPAT via increasing K^+ conductances may explain the lack of change in $[Ca^{2+}]_i$ (Andrade and Nicoll 1987a). Modulation of Ca^{2+} by adenosine has been suggested; however the site of action (i.e. channel vs intracellular stores) is unknown (Schubert et al 1986, Haas and Greene 1984). Additional evidence has been provided that revealed that the post-synaptic effects of adenosine are to reduce gK^+ (Haas and Greene 1988). Maura et al (1988) provided some evidence that 5-HT and AD (Fastbom and Fredholm 1985) can modulate glutamate effects, however our findings suggest that these changes are not via $[Ca^{2+}]_i$ alterations.

Removal of extracellular Ca^{2+} had a significant effect on $[Ca^{2+}]_i$ levels, although these changes were not extraordinarily large. These small reductions in $[Ca^{2+}]_i$ may be due to low micromolar concentrations of residual Ca^{2+} present in the low Ca^{2+} media. It should be noted that the calcium chelator EGTA was not included in the perfusion media in these Fura-2 since it was also not included in the hippocampal slice, low Ca^{2+}-induced bursting experiments. While it is conceivable that this modest fall in $[Ca^{2+}]_i$ could account for the induction of low Ca^{2+}-induced bursting (eg. perhaps by a reduction in Ca^{2+}-dependent membrane conductances such as Ca^{2+}-gK^+) it is more likely that the cause of burst activity in the low Ca^{2+} model of epilepsy may be generated by: i) removal of charge screening influences of Ca^{2+} leading to destabilization of the membrane (Frankenhaeuser and Hodgkin 1957), and ii) a reduction in the efficacy of GABAergic interneurons (Heinemann et al 1986).

Investigation of the effects of elevated $[Ca^{2+}]_0$ on $[Ca^{2+}]_i$ has led to some unexpected results. A transient perfusion (10 min) of high Ca^{2+} (4mM) induced long-lasting increases in $[Ca^{2+}]_i$ which were maintained for the duration of the experiment. Dantrolene has been shown to block the release of Ca^{2+} from the sarcoplasmic reticulum in muscle cells (Desmedt and Hainaut 1977). Based on proposed similarity of function between the sarcoplasmic reticulum and the endoplasmic reticulum, it was thought that this compound may also act at this neuronal Ca^{2+} store. Application of dantrolene to these hippocampal cultured cells completely blocked the increase in $[Ca^{2+}]_i$ seen during the high Ca^{2+} perfusion. In hippocampal slices using a similar experimental design, perfusion of dantrolene was able to block the induction of both tetanic and Ca^{2+}-induced LTP. Comparisons between the hippocampal slice and cultured neurons strongly argues in favor of a role for intraneuronal organelles in the release of Ca^{2+} into the intracellular space of the neuron. Further evidence comes from the findings of Mody et al (1989) which question the importance of Ca^{2+} entry via the NMDA receptor. They were able to demonstrate that dantrolene did not affect Ca^{2+} channels nor Ca^{2+} fluxes through the NMDA receptor-coupled ionophore. Therefore, this suggests that the source of increased $[Ca^{2+}]_i$ is from intracellular organelles such as the endoplasmic reticulum and that this release and maintenance of $[Ca^{2+}]_i$ could play a critical role in the enhancement of electrophysiological responses.

While in tetanic induced LTP the NMDA receptor activation is necessary, Ca^{2+}-induced LTP is not blocked by application of the NMDA receptor antagonist D-APV to the hippocampal slice. Interestingly, in cultured hippocampal neurons application of D-APV fails to block the rise in $[Ca^{2+}]_i$ following a transient elevation of $[Ca^{2+}]_0$. This, and the effects of dantrolene would suggest that the increase in $[Ca^{2+}]_i$ seen following elevated Ca^{2+} is not via Ca^{2+} entry through the NMDA receptor-coupled ionophore but is rather release of Ca^{2+} from intracellular stores. By monitoring the release of intracellular Ca^{2+} from a "caged" fluorescent Ca^{2+} chelator by light, Malenka et al (1988) were able to demonstrate that increasing Ca^{2+}_i levels was sufficient for the induction of LTP (see Chapter 5 discussion for alternate interpretations). One possible mechanism for inducing these changes in $[Ca^{2+}]_i$ may be that of Ca^{2+}-induced Ca^{2+} release (Fabiato and

Fabiato 1975, 1977), where a small amount of Ca^{2+} entry into the neuron could trigger the release of Ca^{2+} from intraneuronal stores.

In summary, while caution is needed in extrapolation of the cultured hippocampal neuron data to the hippocampal slice, the results described here provide crucial evidence that release of calcium from intraneuronal stores is a critical prerequisite for LTP to occur.

CHAPTER 7

General Discussion

The hippocampal formation influences and is influenced by a wide of variety of other brain regions. Discussions of the possible function(s) of the hippocampus have included its role in emotional behavior (Papez 1937) and involvement in memory (Scoville and Milner 1957). The use of the hippocampal slice technique has greatly aided research on both the physiology and pathophysiology of the hippocampus. The investigations described in this thesis used the hippocampal slice technique for measurement of electrophysiological parameters (e.g. population spike amplitude), and then extended those findings using cultured hippocampal neurons together with Fura-2 microspectrofluorimetric determinations of $[Ca^{2+}]_i$.

There are numerous neurotransmitter/neuromodulatory substances in the brain that can regulate hippocampal excitability. One of these compounds is the neurotransmitter serotonin (5-HT), which has shown dense radioligand binding in the hippocampus (Kohler and Steinbusch 1982). The effects of 5-HT and its agonists were examined under three different conditions: i) normal evoked synaptic activity, ii) the low Ca^{2+}-induced bursting model of epilepsy, and iii) changes in $[Ca^{2+}]_i$ following application of this monoamine.

Application of either 5-HT or the $5-HT_{1A}$ receptor ligand, 8-OH-DPAT, elicited a decrease in both the evoked population spike amplitude and in the reversed field EPSP. Comparisons between hippocampal regions demonstrated that these effects were greater in the CA1 region than in the DG. This correlates well with binding studies that demonstrate a high degree of radiolabeling in the CA1 region of $5-HT_{1A}$ receptors (Pazos and Palacios 1985). The inhibitory effects of both 5-HT and 8-OH-DPAT have been attributed to an increase in gK^+, and studies have suggested that both GABA and 5-HT may share a common potassium channel (Andrade and Nicoll 1986, 1987a). The $5-HT_2$ receptor agonist, DOI did not have any effect on evoked responses in the CA1 region, however, application to the DG resulted in a significant inhibition of evoked responses. Again this finding correlates well with binding studies (Pazos et al 1987a).

Investigations of these serotonergic compounds in the low Ca^{2+} model of epileptogenic activity further outline a role for the 5-HT$_{1A}$ receptor. Perfusion of either 5-HT or 8-OH-DPAT resulted in a decrease in the burst rate recorded from the CA1 region, while DOI had a delayed effect. The selective agonist, 8-OH-DPAT, was much more potent in reducing the burst rates at all doses. The possible mechanisms of action for this reduction include: i) a reduction in adenylate cyclase activity, thereby reducing cAMP levels, ii) activation of gK^{+} as in the normal evoked studies, and iii) non-synaptic mechanisms such as a decrease in electrotonic coupling which has been demonstrated by Mercier and Kater (1986). Evidence from previous studies suggests that 5-HT may act via changes in $[Ca^{2+}]_i$. In an attempt to confirm these suggestions, changes in $[Ca^{2+}]_i$ were measured in cultured hippocampal neurons following application of 5-HT or its agonists. Short term perfusion or transient pulses of 5-HT, 8-OH-DPAT, or DOI failed to alter changes in $[Ca^{2+}]_i$. In addition, co-application of these compounds with glutamate failed to alter the increases in $[Ca^{2+}]_i$ seen by application of glutamate alone.

Overall, these findings suggest that 5-HT, and in particular activation of the 5-HT$_{1A}$ receptor subtype, is important for the inhibition of neuronal activity during both normal synaptic activation and the type of epileptiform discharges seen under low extracellular Ca^{2+} conditions. The most likely mechanisms by which these effects of 5-HT$_{1A}$ activation occur include activation of a hyperpolarizing gK^{+}, changes in adenylate cyclase activity, and/or a reduction in electrotonic coupling between neuronal populations.

The purines have been shown to dramatically modulate hippocampal activity, in that adenosine (AD) perfusion results in an inhibition of neuronal evoked responses (Greene and Haas 1985). The degree of inhibition is dependent upon the duration of purine application and the selective agonist 2-chloroadenosine (CAD) is much more potent than AD itself. The results presented in this thesis confirm previous reports that application of AD results in an inhibition of evoked responses, while antagonists such as theophylline (THP) cause an increase in the evoked electrophysiological potentials. Similarly, application of the degradative enzyme, adenosine deaminase (ADA) results in potentiation of the evoked responses suggesting that large amounts of

endogenous adenosine are present in the extracellular milieu. Some authors have suggested that AD effects evoked responses by pre-synaptic inhibition of neurotransmitter release via a regulation of the flow of Ca^{2+} ions into pre-synaptic terminals (Ribeiro and Sebastiao 1986, see also Phillis and Wu 1981a).

In the low Ca^{2+}-induced bursting model substantial doses ($>80u$M) were required to reduce significantly the rate of bursting activity. This is larger than those required for inhibition of synaptically evoked responses (Reddington and Schubert 1979). In a dramatic result however, perfusion of ADA, resulted in massive increases in burst rate. Furthermore, application of AD antagonists, such as THP, or IBMX resulted in an increased rate of bursting activity. Taken together these results provide very strong evidence that AD is being released under these low Ca^{2+} conditions. This release could be an attempt to control this aberrant electrophysiological behavior.

Application of AD failed to change the resting $[Ca^{2+}]_i$ in our preparations of cultured hippocampal neurons. In addition, AD co-application with glutamate did not alter the increases in $[Ca^{2+}]_i$ observed with glutamate alone. It must be made clear that in our experiments $[Ca^{2+}]_i$ was measured in the cell soma, and not in pre-synaptic terminals. Therefore, a highly localized effect of AD on Ca^{2+} fluxes in the terminals, as suggested by Ribeiro and Sebastiao (1986), has not been excluded. The present results strongly suggest that AD is being released endogenously and the hypothesis that AD exerts most of its effects by pre-synaptic modulation of neurotransmitter release remains tenable.

The serotonergic and purinergic compounds are in a unique position to modulate hippocampal activity, albeit by different mechanisms. The serotonergic system is a discrete fiber system that can control hippocampal excitability by activation of specific receptor types. In contrast, the purines form a diffuse system that can be found within the entire hippocampus and primarily regulates the release of neurotransmitters. The importance of these two groups of compounds in both normal synaptic activity and in epileptiform discharges has been demonstrated.

The role of Ca^{2+} in central nervous system function is crucial to many processes within the neuron. The neuron has developed very rigorous systems for controlling intracellular Ca^{2+} levels, and these include; i) Ca^{2+} binding proteins, ii) ATP dependent mechanisms for efflux, and iii) intracellular organelles, such as the endoplasmic reticulum. In recent years particular emphasis has been placed on the endoplasmic reticulum as a structure not only involved in intraneuronal Ca^{2+} buffering but as a source of Ca^{2+} for the induction of various Ca^{2+} dependent biochemical events. Although $[Ca^{2+}]_i$ levels are normally regulated very precisely, it has been proposed that an excessive Ca^{2+} entry may be the cause of neuronal death in disease states such as ischemia, hypoxia and epileptiform activity (Siesjo 1986, Choi 1988).

Removal of extracellular Ca^{2+} will in itself elicit a type of epileptiform activity referred to as low Ca^{2+} induced bursting. The exact mechanism(s) underlying this aberrant activity are unknown, however it has been suggested that a reduction in $[Ca^{2+}]_i$ after $[Ca^{2+}]_o$ may be responsible. Such a reduction in $[Ca^{2+}]_i$ could compromise activation of the inhibitory Ca^{2+}-dependent gK^+ (Heinemann et al 1986). It should be noted that residual Ca^{2+} from other ionic constituents is present, and may be sufficient to maintain burst activity. Similar experiments were performed using Fura-2 microspectrofluorimetry. These experiments were performed on cultured hippocampal neurons and demonstrated that when neurons are bathed in a medium low in Ca^{2+} there is a significant (but not large) decrease in $[Ca^{2+}]_i$ that reaches a new plateau which is maintained for up to an hour. The findings that $[Ca^{2+}]_i$ is well maintained during low Ca^{2+} induced bursting supports the proposed mechanism(s) for the generation and synchronization of burst activity which include: i) destabilization of the membrane leading to hyperexcitablity, ii) a reduction in the efficacy of GABAergic interneurons, and iii) a reduction in Ca^{2+}-dependent conductances (e.g. Ca^{2+}-dependent gK^+).

In the last several years a prominent role has been suggested for the NMDA receptor subtype in epilepsy and long-term potentiation. Blockade of this receptor with the specific NMDA antagonist, D-APV, surprisingly elicited a reversible decrease in burst rate induced by low Ca^{2+} ACSF. This bursting activity is generally considered to occur in the absence of synaptic

transmission, however the findings presented above suggest that sufficient concentrations of glutamate (or some other excitatory amino acid neurotransmitter) is interacting with the NMDA receptor either to initiate or maintain burst activity. The present experiments cannot exclude spontaneous release of neurotransmitter, however, removal of $[Ca^{2+}]_0$ does result in a loss of evoked responses, indicating that insufficient $[Ca^{2+}]_0$ is present in this model to support neurotransmitter release. It would seem therefore unlikely that the residual $[Ca^{2+}]_0$ is the source for glutamate release but it is possible that the release from intracellular stores may support neurotransmitter release under some circumstances. The present findings do not exclude a role for glia in modulating glutamate release or uptake in this model of epileptiform activity.

The importance of excitatory neurotransmitter release in low Ca^{2+} induced bursting is unknown but may include a role in the initiation and maintenance of burst activity. Thus, although the low Ca^{2+} bursting model has been suggested to be purely post-synaptic, evidence exists which suggests a pre-synaptic component exists (see Dichter and Ayala 1987).

The NMDA receptor has been implicated in frequency dependent events such as the model for learning and memory, long-term potentiation (LTP). The importance of Ca^{2+} in LTP has been well established. One possible source of Ca^{2+} required for the induction of enhanced synaptic responses, is an influx of Ca^{2+} through an NMDA gated channel (Mayer and Westbrook 1987a), but this has been questioned (Mody et al 1989). In examining alternate sources of Ca^{2+}, experiments were conducted using dantrolene, a compound that has been shown to block the release of Ca^{2+} from sarcoplasmic reticulum. This compound effectively blocked the induction of both tetanic and Ca^{2+}-induced LTP, and it has no effect on NMDA activated currents and voltage dependent Ca^{2+} currents (Mody et al 1989). These results suggested that a common mechanism, such as an increase in intracellular Ca^{2+}, was responsible for the induction of LTP in these two methods of enhancing synaptic responses.

Previous reports have demonstrated that the NMDA receptor activation is important in tetanic induced LTP (Collingridge et al 1983) and Ca^{2+}-induced LTP (Bliss et al 1987). A recent finding that the increase in $[Ca^{2+}]_i$ seen following NMDA receptor activation comes from

intraneuronal stores (Mody et al 1989) suggested that $[Ca^{2+}]_i$ may be important in LTP. Application of the NMDA antagonist, D-APV did not block the induction of Ca^{2+}-induced LTP. This indicates that while NMDA receptor activation is important in tetanic-induced LTP, it does not play a critical role in Ca^{2+}-induced LTP. These results in conjunction with the dantrolene experiments demonstrate that an essential component for the tetanic or high Ca^{2+} induction of LTP in the CA1 region of the hippocampus is release of $[Ca^{2+}]_i$ from intraneuronal stores.

Experiments using Fura-2 microspectrofluorimetry on cultured hippocampal neurons replicated the same conditions as the slice experiments, in an attempt to determine the role of $[Ca^{2+}]_i$ stores following a transient elevation of Ca^{2+}. Application of a transient pulse of high Ca^{2+} elicits a sustained increase (3-fold) in $[Ca^{2+}]_i$. Perfusion of dantrolene, was able to block this rise in $[Ca^{2+}]_i$. Similar experiments were performed to assess if the NMDA receptor was activated during the high Ca^{2+} pulse, but perfusion of D-APV did not block the rise in $[Ca^{2+}]_i$.

Cautious extrapolation from cultured neurons to hippocampal slice data would suggest that intraneuronal stores of Ca^{2+} are critical for the induction of long term potentiation. While the NMDA receptor may be important in some forms of LTP, it is not the unifying factor between tetanic and Ca^{2+}-induced LTP. In addition, these results suggest that the neuron has the ability to regulate and maintain $[Ca^{2+}]_i$ changes for extended periods of time. Intracellular organelles such as the endoplasmic reticulum are thought to play an important part in neuronal homeostasis and we propose that this organelle is the source of the intracellular Ca^{2+} required for LTP.

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