Mechanism and Consequences of Extracellular Adenosine Accumulation in the Hypoxic Hippocampal Slice

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ABBREVIATIONS AND CHEMICAL NAMES

2-CA ~ 2-chloroadenosine
3-IB-MECA ~ N^6-(3-iodobenzyl)-5'-N-methycarboxamidoadenosine
4-AP ~ 4-aminopyridine
8-CPT ~ 8-cyclopentyl-1,3-dimethylxanthine (8-cyclopentyltheophylline)
8-PT ~ 8-phenyltheophylline
8FB-PTP ~ 5-amino-8-(4-flourobenzyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine
ACSF ~ artificial cerebrospinal fluid
ADP ~ adenosine-5'-diphosphate
AHP ~ after-hyperpolarisation
AMP ~ adenosine-5'-monophosphate
AMPA ~ RS-amino-3-hydroxy-5-methyl-4-isoxazolopropionate
AP5 ~ 2-amino-5-phosphonopentanoate
Ap5A ~ p1,p5-di(adenosine-5') pentaphosphate
AP7 ~ 2-amino-7-heptanoate
APNEA ~ N^6-2-(4-amino-3-iodophenyl)ethyladenosine
ATP ~ adenosine-5'-triphosphate
BAPTA ~ 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
cAMP ~ cyclic adenosine-3',5'-monophosphate
CCPA ~ 2-chloro-N^6-cyclopentyladenosine
CGS 15943 ~ 5-imino-9-chloro-2-(2-furyl 1,2,4-triazolo [1,5-c] quinazoline
CGS 21680 ~ 2-[*p*-(carboxyethyl)-phenylethylamino]-NECA
CHA ~ N^6-cyclohexyladenosine
CNQX ~ 6-cyano-7-nitro-quinoxaline-2,3-dione
CNQX ~ 6-cyano-7-nitro-quinoxaline-2,3-dione
CO ~ carbon monoxide
CPA ~ N^6-cyclopentyladenosine
CPP ~ 3-((±)-2-carboxypiperazin-4-yl-propyl-1-phosphinic acid
CTX ~ ω-conotoxin
CV 1801 ~ 2-(phenylamino)adenosine
DPMA ~ N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl]adenosine
DMPX ~ 3,7-dimethy-1-propylargylxanthine
DNP ~ 2,4 dinitrophenol
DNQX ~ 6,7 dinitro-quinoxaline-2,3-dione
DPCPX ~ 8-cyclopentyl-1,3-dipropylxanthine
DPSPX ~ 1,3-dipropyl-8-*p*-sulfophenylxanthine
DTX ~ dendrotoxin
EGTA ~ ethylene glycol-bis(β-aminoethyl ether)
EHNA ~ erythro-9-(-2-hydroxy-3-nonyl)adenosine
EPSC ~ excitatory postsynaptic current
EPSP ~ excitatory postsynaptic potential
fast IPSP/C ~ $GABA_A$-mediated IPSP/C
FCCP ~ carbonyl cyanide p-triflouromethoxyphenylhydrazone
fEPSP ~ field excitatory postsynaptic potential
G protein ~ guanine nucleotide binding protein
GABA ~ γ-amino-butyric acid
Gpp(NH)p ~ guanylyl-5'-imidodiphosphate
GR 79236 ~ N-[(1S,trans)-2-hydroxycyclopentyl]adenosine
H-7 ~ 1-(5-isoquinolinesulfonyl)-2-methylpiperazine
H-8 ~ N-[(2-(methylamino)ethyl]-5-isoquinolinesulfonamide
H-89 ~ N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide
HA-1004 ~ N-(2-guanidinoethyl)-5-isoquinolinesulfonamide
IMP ~ inosine-5'-monophasphate
iodotubercidin ~ 4-amino-5-iodo-7-(β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine
i.p. ~ intraperitoneal
IP_3 ~ inositol 1,4,5-trisphosphate
IPSC ~ inhibitory postsynaptic current
IPSP ~ inhibitory postsynaptic potential
KF17837 ~ (E)-1,3-dipropyl-7-methyl-8-(3,4-dimethoxystyryl)xanthine

metrifudil ~ N-[(2-methylphenyl)methyl]adenosine
MK-801 ~ dibenzocyclohepteneimine
NBTI ~ S-(p-nitrobenzyl)-6-thioinosine, nitrobenzylthioinosine
NECA ~ 5'-N-ethylcarboxyamidoadenosine
NMDA ~ N-methyl-D-aspartate
PCr ~ phosphocreatine
PTX ~ Pertussis toxin
R-PIA ~ R-phenylisopropyladenosine
S-ENBA ~ N^6-endonorbornyladenosine
slow IPSP/C ~ $GABA_B$-mediated IPSP/C
TEA ~ tetraethylammonium
TTX ~ tetrodotoxin
XAC ~ xanthine amino cogener
$\Delta\psi_m$ ~ inner mitochondrial membrane potential

ABSTRACT

This thesis examines the alterations in electrophysiological function during hypoxia in the rat hippocampal slice, in particular those alterations induced by extracellular accumulation of adenosine. Evaluation of electrophysiological responses in the *in vitro* rat hippocampal slice is a standard model for neurophysiological investigations, and has been used extensively in the study of hypoxia. Rat hippocampal CA_1 pyramidal neurons are considered to be selectively vulnerable to hypoxic damage; however, adenosine A_1 receptor-mediated depression of excitatory synaptic transmission during hypoxia may protect neurons. Enhanced excitability of neurons is reported following hypoxia, and is proposed to contribute to neuronal damage.

In the present studies, the post-hypoxic recovery of synaptically evoked field potentials was used to measure the survival of CA_1 pyramidal neurons, and to assess any functional alterations precipitated by hypoxia. Excitatory synaptic transmission in the hippocampal CA_1 area was depressed during 30 minutes of hypoxia due to activation of adenosine A_1 receptors, this depression being sensitive to A_1 receptor antagonism. Synaptic transmission always recovered upon reoxygenation, indicating that CA_1 neurons survived prolonged inhibition of respiration, and that adenosine A_1 receptor activation during hypoxia was not necessary for this neuronal recovery. Following prolonged hypoxia, recovered postsynaptic potentials manifested a permanent hyperexcitability, obvious as multiple population spikes. A method for quantifying this hyperexcitability was developed, which established that it results from a postsynaptic alteration in CA_1 neuronal excitability. Similar hyperexcitability also followed normoxic adenosine exposure, but was not produced by agonists for the A_1 or A_2 adenosine receptor subtypes. Apparently, adenosine activation of an unidentified receptor subtype produces postsynaptic hyperexcitability, and this may be the mechanism of hyperexcitability following hypoxic adenosine accumulation.

This work also examined the mechanism of adenosine accumulation during hypoxia, by assessing A_1 receptor-mediated depression of synaptically evoked field potentials. Hypoxia, respiratory inhibitors and mitochondrial uncoupling agents all caused an adenosine induced depression of synaptic transmission, whereas inhibitors of oxidative phosphorylation did not. Furthermore, alleviation of ATP depletion failed to increase the latency of synaptic depression during mitochondrial uncoupling. It seems likely, therefore, that the massive adenosine accumulation during energy deprivation is not a direct consequence of ATP depletion, but is most likely a result of stimulation of cytosolic 5'-nucleotidase and inhibition of adenosine kinase, possibly by changes in free AMP, pH or cytosolic calcium.

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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signed: .... date: 4/12/95

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1. INTRODUCTION AND OVERVIEW

1.1. GENERAL OVERVIEW OF CEREBRAL HYPOXIA

Cerebral hypoxia results in rapid loss of higher brain functions such as consciousness and purposive behaviour, but these can return with timely reoxygenation (Rossen *et al.* 1943). Despite this apparent recovery, serious consequences may develop later, including epilepsy (Bergamasco *et al.* 1984; Oppenheimer and Hachinski, 1992) and delayed neuropsychological deterioration with associated neuronal damage (Meyer, 1956; Plum *et al.* 1962; Meyer, 1936). Neurons may initially survive hypoxia, but undergo delayed neuronal degeneration in the subsequent hours or days, progressing from microvacuolisation to ischaemic cell change, characterised by shrunken neuronal soma and disorganisation of organelles, and finally disintegration (Brown and Brierley, 1968). Although hypoxic neuronal damage can be widespread, some brain areas are selectively vulnerable and can be damaged by even brief hypoxia, whilst others are not affected; prominent amongst these selectively vulnerable areas is the hippocampus, in particular it's CA_1 region (Sommer's field) (Meyer, 1936; Meyer, 1956; Plum *et al.* 1962; Scholz, 1953; Sommer, 1880; Speilmeyer, 1925). Hypoxia and ischaemia may have different consequences, for instance, hypoxia, but not ischaemia, may produce neuronal necrosis in the globus pallidus, and widespread axonal demyelination (Hirano *et al.* 1967; Plum *et al.* 1962; Meyer, 1936; LaPresle and Fardeau, 1967; Brown and Brierley, 1968). However, while both hypoxia and ischaemia otherwise result in essentially the same pattern of neuronal degeneration, the two are rarely distinct *in vivo* since hypoxia may often be accompanied by hypotension and reduced brain perfusion (Winn *et al.* 1981; Zetterström *et al.* 1982).

There is a long history of neuropathological investigation of the consequences of hypoxia and ischaemia for hippocampal neurons *in vivo* (Meyer, 1936; Speilmeyer, 1925; Brown and Brierley, 1968, and references therein). More recently, *in vitro* models of cerebral hypoxia have allowed investigations uncomplicated by ischaemia. Most significantly, use of the *in vitro* hippocampal slice has contributed to understanding of cerebral hypoxia, so that some aspects are now well defined. This is particularly so in CA_1 pyramidal neurons, where the hypoxic response has some unique features, but is largely characteristic of other principal cortical neurons. Hypoxia causes a rapid depression of synaptic activity in the CA_1 area of the hippocampal slice (Fujiwara *et al.* 1987; Hansen *et al.* 1982), as a result of extracellular accumulation of adenosine and activation of adenosine A_1 receptors (Gribkoff *et al.* 1990; Fowler, 1989). If hypoxia is prolonged, or combined with aglycaemia, neuronal ionic homeostasis and membrane polarisation is lost, after which neuronal recovery is unlikely (Fairchild *et al.* 1988; Reid, 1987; Hansen *et al.*

1982; Fujiwara *et al.* 1987; Taylor and Weber, 1993), due to extracellular accumulation of glutamate (Pellegrini-Giampietro *et al.* 1990) and massive activation of glutamate receptor cation conductances. Such excitotoxic neuronal damage can result from acute swelling due to osmotic imbalance (Rothman *et al.* 1987), or calcium influx can initiate delayed neuronal degeneration (Choi, 1988; Choi, 1990; Rothman and Olney, 1986). However, if oxygen is re-introduced in time, neurons will recover, but may display a form of hyperexcitability characterised by firing of multiple action potentials (Schiff and Somjen, 1985).

Adenosine, aside from it's role in intermediary energy metabolism, has become established as an important neuromodulator (Sattin and Rall, 1970), and is now recognised to participate in hypoxic pathophysiology. Adenosine release is the earliest cellular response to hypoxia, and adenosine-mediated synaptic depression a prominent consequence of hypoxia (Gribkoff *et al.* 1990). Furthermore, adenosine can provide protection of neurons against hypoxic excitotoxicity (Boissard *et al.* 1992). Adenosine is active at a growing list of G protein-linked receptors which modulate ion channels, adenylate cyclase and phospholipase C (Greene and Haas, 1991; Sebastião and Ribeiro, 1992; van Calker *et al.* 1979; Fredholm *et al.* 1985; Ramkumar *et al.* 1993; White *et al.* 1992). Adenosine receptors can both inhibit and stimulate adenylate cyclase activity, and were originally classified as A_1 and A_2 receptor respectively on this basis (Londos *et al.* 1980; van Calker *et al.* 1979; van Calker *et al.* 1978). Adenosine receptors also mediate electrophysiological actions that are not necessarily related to their modulation of adenylate cyclase. Presynaptic adenosine A_1 receptors depress excitatory synaptic transmission (Lupica *et al.* 1992) by inhibiting glutamate release (Corradetti *et al.* 1984) whilst postsynaptic A_1 receptors reduce neuronal excitability by hyperpolarising the neuronal membrane and enhancing the slow after-hyperpolarising potential (Greene and Haas, 1991). In contrast to this, there are high affinity adenosine A_{2a} receptors and low affinity A_{2b} receptors that increase both synaptic transmission and pyramidal neuron excitability (Sebastião and Ribeiro, 1992; Ameri and Jurna, 1991; Mogul *et al.* 1993). A_3 receptors are linked to phospholipase C (Ramkumar *et al.* 1993) with an as yet unidentified action in neurons.

Some aspects of hypoxic pathophysiology have been intensively studied, such as hypoxic excitotoxicity and the membrane currents initiated by hypoxia in hippocampal pyramidal cells, while other aspects have been overlooked. For instance, adenosine has been generally presumed to accumulate during hypoxia as a result of ATP catabolism, despite evidence of relative preservation of ATP during hypoxia (Fredholm *et al.* 1984). It has otherwise been suggested that hypoxia activates 5'-nucleotidase (Lloyd and Schrader, 1987), but the evidence is ambiguous. For instance, the mechanism of adenosine release at the onset of hypoxia has

received little attention, as has the extent and consequences of adenosine receptor activation during hypoxia. Curiously, early post-hypoxic hyperexcitability in the CA_1 area of the hippocampus has been noted several times in the literature but never pursued in depth. Originally, Anderson, commented on the appearance of multiple population spikes in hippocampal field potentials *in vivo* following hypoxia in rabbits, cats and rats (Andersen, 1960). Two decades later, Dunwiddie noted multiple population spikes in the CA_1 in hippocampal slices following prolonged *in vitro* hypoxia (Dunwiddie, 1981) and then Schiff and Somjen devoted a brief paper to describing this phenomenon (Schiff and Somjen, 1985). There is, so far, only a single description of post-hypoxic hyperexcitability using intracellular recording in the rat hippocampal slice (Fujiwara *et al.* 1987). None of these few, brief reports provide any clues as to the cause or underlying mechanism of post-hypoxic hyperexcitability. Post-hypoxic hyperexcitability seems to be the earliest evidence of consequential functional disturbance in neurons following hypoxia. Whether post-hypoxic hyperexcitability is required for hypoxic excitotoxicity is still debated (Imon *et al.* 1991), but delayed neuronal death is dependent on intact excitatory synaptic pathways (Onodera *et al.* 1986; Benveniste *et al.* 1988; Rothman, 1983), and thus may be the result of enhanced sensitivity of pyramidal neurons to synaptic glutamate release.

1.2. AIMS

The role of adenosine in hypoxia in the hippocampus is still only partially understood. Indeed, knowledge of the pharmacology of adenosine receptors is still developing. This thesis examines the earliest responses to hypoxia in the CA_1 area of the hippocampal slice, and in particular, those mediated by adenosine. The mechanism and extent of extracellular adenosine accumulation in the hippocampus still requires clarification, and this thesis seeks to explore the relationship between neuronal oxidative energy metabolism and extracellular adenosine accumulation. Here, the remarkable survival of these selectively vulnerable CA_1 neurons during prolonged hypoxia or mitochondrial poisoning in the presence of high glucose has been investigated. In particular, the role of glycolysis, oxidative phosphorylation and cellular energy state in both adenosine accumulation and neuronal survival during hypoxia have been assessed. The contribution of adenosine A_1 receptor activation to hypoxic depression of synaptic transmission has been confirmed here and used to estimate the concentration of accumulated extracellular adenosine. The role of adenosine A_1 receptor activation in the survival of neurons during prolonged hypoxia *in vitro* has also been addressed. The consequences of activation of adenosine receptors other than the A_1 subtype is not well understood. Of particular significance to this project is that hyperexcitability, somewhat similar to that following hypoxia, accompanies washout after prolonged exposure to exogenous adenosine in the

normoxic hippocampal slice (Okada *et al.* 1992). It is this similarity that lead me to investigate whether hypoxic adenosine release is the sole, or merely a contributing factor, in the production and maintenance of post-hypoxic hyperexcitability that develops upon reoxygenation.

2. INVOLVEMENT OF ADENOSINE IN HYPOXIA IN THE HIPPOCAMPAL SLICE: BACKGROUND LITERATURE REVIEW

2.1. INTRODUCTION

The hippocampus has a long history of anatomical investigation, culminating in the work of S. Ramon y Cajal at the turn of this century which has largely stood the test of time. With nearly as long a history, is the investigation of the hippocampus as a brain area that is selectively vulnerable to hypoxia (Speilmeyer, 1925). Theories of hippocampal function have changed from an earlier presumed role in olfaction or emotion, to its current putative role in memory formation. Despite such changes, the hippocampus has maintained a role as a primary model of cortical organisation and function throughout modern neurophysiology. Surely the pyramidal cells in the hippocampus and their connections are the best studied vertebrate neurons and synapses. Along with basic brain function, the hippocampus has been the model of choice for investigation of cerebral hypoxia. The neuromodulator adenosine has recently received much attention, particularly its actions in the hippocampus which is richly endowed with adenosine receptors. During hypoxia, adenosine accumulates extracellularly in many tissues, notably in the brain, and the consequences of this in the hippocampus have received considerable attention. The present work continues these studies, examining the sources and some consequences of adenosine accumulation during hypoxia in the CA_1 area of the hippocampal slice. This chapter initially provides an overview of hippocampal anatomy, both to illustrate the development of the hippocampal slice technique, and to provide an understanding of the synaptic connections studied in this model. The electrophysiology of the hippocampus and pharmacology of the major transmitters in the CA_1 area are reviewed in brief, to provide a background for the subsequent examination of the actions of the neuromodulator adenosine. The current state of knowledge regarding the consequences of hypoxia on the synaptic transmission and neuronal excitability in the hippocampus and the survival of hippocampal neurons is reviewed. The particular emphasis of these latter is neuronal function and survival during hypoxia, and the possible role of adenosine in these phenomena.

2.2. HIPPOCAMPAL FORMATION

2.2.1. ANATOMY AND CONNECTIONS OF THE HIPPOCAMPAL FORMATION

2.2.1.1. General cortical organisation

The mammalian cerebral cortex can be divided into two morphological types, isocortex and allocortex, this latter including the hippocampus. Isocortex or neocortex is an phylogenetically new tissue and is characterised by six histological layers (Ramon y Cajal, 1952). The most superficial is the molecular or plexiform

layer consisting of fibres running parallel to the surface, including pyramidal cell axons and dendrites, the Horizontal cells of Cajal as well as axons from the Martinotti cells running towards the surface. Next is the outer granular layer, a layer of granule cells and predominantly small pyramidal cells. Below is the pyramidal layer containing typical pyramidal cells of medium size. The deepest part of this layer is crossed by transverse fibres of the outer portion of Baillarger's band. The fourth layer is identified by stellate cells called granule cells. The portion of these previous two layers crossed by the outer band of Baillarger are also called collectively the layer of polymorphic cells. The fifth layer is the ganglionic layer or layer of large pyramidal cells and also contains the inner aspect of Baillarger's band. The deepest layer is the polymorphous layer containing fusiform cells and Martinotti cells and is extensively penetrated by radial fibres to and from the superficial layers.

Allocortex or paleocortex, including the hippocampus, is a phylogenetically old area of cortex. Allocortex is also highly organised, but characteristically only in three distinct layers, with a superficial molecular layer, a cell body layer and a deep layer of fibres and polymorphous cells. Many authors also describe transitional tissues between isocortex and allocortex called variously peri-allocortex or juxta-allocortex.

2.2.1.2. Architectonics of the hippocampal formation

The classic histological work on the hippocampus was done at the turn of the century by Santiago Ramon y Cajal. The hippocampal formation is a convolution of allocortical and transitional tissue occupying an area medial to the temporal lobe adjacent to the lateral ventricle and below the corpus callosum. Its major connections are with the septum, and only a small part reaches thalamic mamillary bodies rostrally and the prorhinal cortex caudally. In humans, the hippocampus is a comparatively small convolution on the ventral, inner aspect of the cerebral hemisphere, limited in size dorsally by the splenium of the corpus callosum. In rodents, however, the hippocampus occupies a relatively large area of the inner side of the hemispheres, stretching from the temporal lobe towards the septum. The following discussion on the morphology and organisation of this structure in the rat is drawn largely from a 1968 translation of Ramon y Cajal and a number of excellent reviews (Bayer, 1985; Angevine, 1975; Chronister and White, 1975)

Figure 1.1 shows a horizontal or transverse section through the hippocampal formation, which shows all the architectonic areas. Such transverse sections are a typical representation of the hippocampus, which is considered to be a lamellar structure, whereby this organisation is repeated along it's septal/temporal (ventral/dorsal) axis. The term hippocampus is often used to denote both the hippocampus and the closely associated structures described below, but this convention will be avoided in favour of the term hippocampal formation in the

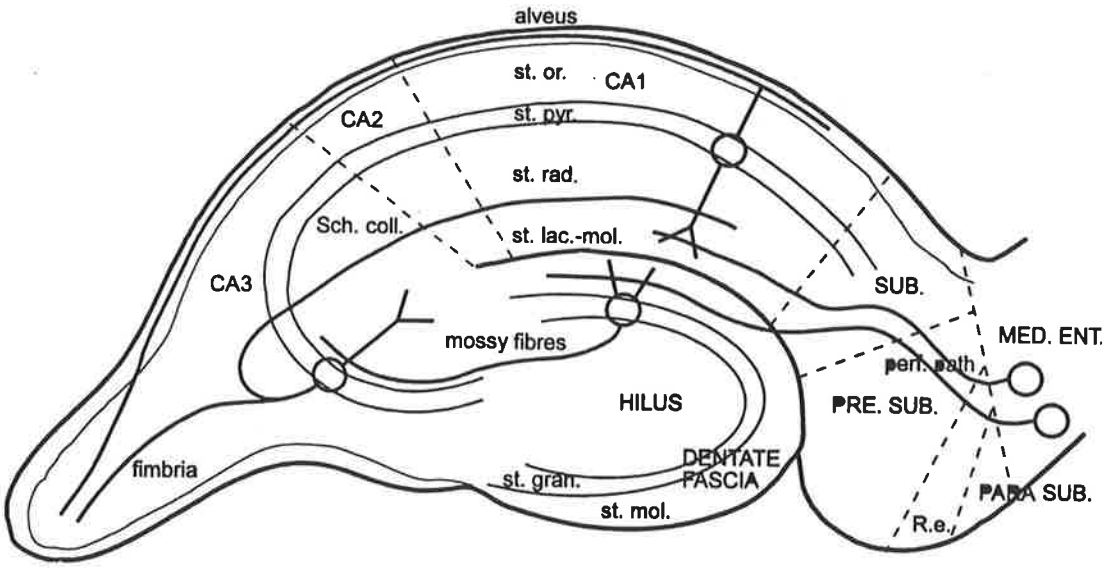

Figure 2.1 Architectonics and major connections of the hippocampal formation
Diagram showing a transverse section of the hippocampus, illustrating the major architectonic areas, cell types and major excitatory pathways. Abbreviations: med. ent. ~ medial entorhinal area; para sub. ~ parasubiculum; perf. path ~ perforant path; pre sub. ~ presubiculum; R.e. ~ retrosplenalis e; Sch. coll. ~ Schaffer collaterals; st. gran. ~ stratum granulosum; st. lac.-mol. ~ stratum lacunosum-moleculare; st. mol. ~ stratum moleculare; st. or. ~ stratum oriens; st. pyr. ~ stratum pyramidale; st. rad. ~ stratum radiatum; sub. ~ subiculum. The alveus is the deepest layers and the molecular layers are the most superficial.

present work. Hippocampus will be used to denote only hippocampal areas CA_1, CA_2 and CA_3, see section 2.2.1.2.4. In normal histological sections the boundaries between the architectonic areas are not always distinct, but rather are gradual changes in cell types and arrangements; however, fibre degeneration studies can reveal sharper boundaries. There are physiological differences, important in the present context, that distinguish not only the hippocampus from adjacent areas, but also subdivisions in the hippocampus. Furthermore, as important hippocampal afferents arise in the adjacent closely associated structures, these will be briefly described.

2.2.1.2.1. Entorhinal area

Adjacent to the isocortical tissue of the piriform lobe is the first peri-allocortical structure, the entorhinal area, divided into lateral intermediate and medial areas. Layer I is the lamina zonalis, a thick plexiform layer containing fibres of the superficial neurons. Layer II and III comprise the laminar principalis externa. Layer II is a mixed population of large and small stellate cells grouped in islands in the lateral area and becoming continuous in the intermediate and medial areas. Pyramidal cells occupy layer III. In the lateral area these two layers are separated by a cell sparse zone. The next layer is the lamina dessicans, a cell poor layer. The lamina principalis interna, layer IV is composed of medium and small pyramidal cells.

2.2.1.2.2. Parasubiculum and presubiculum

The next major divisions are the parasubiculum and presubiculum. Some investigators separate these areas with the area retrosplenalis e. The small pyramidal cells of the lamina principalis externa become increasingly more densely packed. Anterolaterally is the border with the subiculum which also marks the border between peri-allocortex and allocortex proper.

2.2.1.2.3. Subiculum

The subiculum has a wide molecular layer and a pyramidal cell layer continuous with the lamina principalis interna that progressively narrows as it approaches the hippocampus. A deep plexiform layer completes this typical tri-laminar allocortex.

2.2.1.2.4. Hippocampus

Although the hippocampus has the general allocortical structure, detailed examinations shows that the three layers are further subdivided. The hippocampus proper, or Ammon's Horn, is identified by the beginning of a crowded narrow layer of pyramidal cell bodies, four to five cells deep, the stratum pyramidale. The hippocampus is divided into three areas most commonly labelled CA_1-CA_3 (*Cornu Ammonis*) after Lorente de Nó, 1934. Histologically, the pyramidal cells are more densely packed in CA_1 and the proximal dendrites lack the thorny projections of CA_2 and CA_3 cells. The hilus of the dentate gyrus was originally considered part of the hippocampus and called area CA_4 (Ramon y Cajal, 1968), but this nomenclature has been discontinued. Deep to the pyramidal layer is the stratum oriens, a dense plexus of fibres composed of the basal dendrites of the pyramidal cells, pyramidal cell axon collaterals that arise in this layer, ascending branches from alveus fibres and the processes of the dispersed polymorphic cells of this layer. Underlying the stratum oriens is the alveus, containing afferent fibres and efferent axons of the pyramidal cells. The alveus is continuous rostrally and laterally with the fimbria. The superficial molecular layer is further divided into the stratum radiatum and the stratum lacunosum-moleculare. The stratum radiatum is a dense fibre plexus which fades at the end of the CA_3 region, merging with the dentate hilus. The stratum radiatum contains the unbranched portion of the pyramidal cell apical dendrites and horizontal fibres, including many arising from ipsilateral CA_3 pyramidal neurons as well as commissural fibres. The stratum lacunosum-moleculare contains the pyramidal apical dendrites branches, numerous polymorphic cells and horizontal fibre pathways. These include in the lacunosum, collaterals and terminal fibres which have ascended from the alveus forming a dense plexus around lacunosum cells, axon collaterals from the CA_3, and terminal fibres from oriens and radiatum interneurons (Ramon y Cajal, 1968). The molecular layer is composed of some polymorphic cells and a pathway from the subiculum (Ramon y Cajal, 1968).

Several systems of nomenclature for the hippocampal areas co-exist. The classification of Rose, 1926 uses h1-h5 to denote hippocampal and dentate subdivisions and will not be used in this work. As CA_3 and CA_2 do not appear distinct in normally stained sections these are often combined into the regio inferior as opposed to the regio superior comprising the CA_1 (Ramon y Cajal, 1968). The CA areas are further subdivided into areas a, b, and c, based on minor differences in cell morphology and fibrearchitecture, this will not be discussed here.

2.2.1.2.5. Pyramidal neurons

Pyramidal neurons are the primary cell type in the hippocampus. Pyramidal neuron soma are densely packed and surrounded by the axon plexus of the interneurons of the stratum oriens, radiatum, and lacunosum-moleculare (Ramon y Cajal, 1968). Pyramidal dendrites are varicose and spiny; a tuft of basal dendrites descends into stratum oriens and a single pyramidal apical dendrite ascends unbranched through the pyramidal layer and gives of collaterals and processes in the stratum radiatum and lacunosum-moleculare and finally branches into a tuft in the molecular layer (Ramon y Cajal, 1968). The pyramidal axon arises from the soma or a basal dendrite, and descends obliquely to travel in, the alveus (Ramon y Cajal, 1968). The axon gives off collaterals in the stratum oriens which arborise in this layer, and in a few pyramids the axon may also bifurcate in alveus, giving rise to a thinner ascending fibre (Ramon y Cajal, 1968). Pyramidal neuron axon terminals form excitatory, glutamergic synapses, identified both within the hippocampus and externally in the septum (Stevens and Cotman, 1986).

Ramon y Cajal noted the differences between the inferior (CA_2, CA_3) pyramidal neurons and the superior (CA_1) neurons. The apical dendrites of regio inferio pyramidal neurons have wart-like protrusions at which the Mossy fibres synapse. The axons of these neurons also give rise to the Schaffer collaterals, a thick axon collateral more like an axon bifurcation (Ramon y Cajal, 1968). Superior pyramids have delicate local collaterals, but no true ascending axon collaterals (Ramon y Cajal, 1968).

2.2.1.2.6. Dentate gyrus

The dentate gyrus surrounds the CA_3 region of the hippocampus. The stratum granulosum of densely packed granule cell somas 4-10 deep is c-shaped in cross section, thinning towards each end. This layer is divided into a superpyramidale or ectal limb nearest the hippocampal fissure and an infrapyramidale or endal limb opposite the thalamus. The superficial stratum moleculare contains the dendrites of the granule cells and appears continuous with the stratum lacunosum-moleculare of the hippocampus. The deep hilus is transitional to hippocampus. The hilus contains

a variety of neurons, most commonly the mossy cell, but also including scattered large modified pyramidal cells, polymorphs, fusiform and basket cells.

2.2.1.3. Major pathways of the hippocampal formation

The major excitatory pathways in the hippocampus project strongly within the same septal/temporal level, allowing these pathways to be illustrated in a transverse section as in figure 2.1. Excitatory synaptic transmission in the pathways arising within the hippocampal formation is mediated by glutamic acid (Davies and Collingridge, 1989; Colbert and Levy, 1992; Turner and Wheal, 1991; Alzheimer *et al.* 1993).

2.2.1.3.1. Alveus

The alveus is a major afferent and efferent pathway for the hippocampus. The pyramidal neuron axon travels in the alveus to the fimbria. Axons of CA_1 pyramidal neurons may also bifurcate and project via the alveus to the subiculum. The alveus also contains the major hippocampal afferents which arise in the septum.

2.2.1.3.2. Schaffer collaterals

In the CA_3 region recurrent branches of the axons called Schaffer collaterals penetrate to the stratum radiatum and travel to the CA_1 region, where they synapse with the apical dendrites of pyramidal cells (Bayer, 1985). Schaffer collaterals are myelinated for some of their length in the rabbit and cat (Ramon y Cajal, 1968), but only weakly so in the rat (Andersen *et al.* 1978). Schaffer collaterals have a large number of side branches and may synapse *en passant* with hundreds or thousands of CA_1 and CA_2 cells (Andersen *et al.* 1978). CA_3 neurons closest to the dentate gyrus project to near the subicular border and terminate in the superficial stratum radiatum and CA_3 neurons closer to the CA_1 border project to closer CA_1 and terminate deeper in the stratum radiatum and into the stratum oriens (Amaral and Witter, 1989). CA_3 neurons near the dentate gyrus project more septally and CA_3 neurons closer to the CA_1 project more temporally within the hippocampus (Amaral and Witter, 1989). Regardless of their origin within the CA_3, projections originating more temporally tend to terminate superficially in the radiatum near the subicular border, whereas those arising from the septal end tend to terminate nearer the CA_3 border in the deep radiatum and stratum oriens (Amaral and Witter, 1989). CA_3 collaterals also project to other septo-temporal CA_3 regions (Amaral and Witter, 1989).

2.2.1.3.3. Mossy fibres

Dentate granule cell axons, called mossy fibres, project into the hilus where they ramify and synapses with mossy cells, but a long mossy fibre branch projects into the CA_3 region (Bayer, 1985). The mossy fibre is a thin unmyelinated fibre with giant boutons which makes *en passant* synapses with CA_3 pyramidal neurons (Yamamoto, 1972). Close to the hilus, mossy fibres run within the stratum pyramidale, but run for

most of their length in the deep stratum radiatum in a cell poor layer called the lucidum where they synapse with thorny projections of the proximal apical dendrite of CA_3 pyramidal neurons (Blackstad *et al.* 1970). Mossy fibres do not extend into the CA_1 (Blackstad *et al.* 1970). Fibres from cells in the infrapyramidale limb of the stratum granulosum travel in the deepest paths, grading to the fibres running most superficially, which arise from the superpyramidale limb (Blackstad *et al.* 1970; Gaarskjaer, 1978a,b,c). Mossy fibres project entirely within the same septal/temporal layer (Blackstad *et al.* 1970).

2.2.1.3.4. Perforant path

The perforant paths, arising in the entorhinal area, are important hippocampal afferents, one path enters the hippocampus directly, whereas another first crosses the hippocampal fissure to the dentate gyrus. The entorhinal stellate cells from layer II sends fibres which perforate the subiculum and project to the stratum moleculare in the dentate fascia and hippocampal CA_3 (Steward and Scoville, 1976). The lateral perforant path, from the lateral entorhinal area terminates superficially in the dentate fascia and in the CA_3 region. The medial perforant path terminates in the deep part of the CA_3 stratum lacunosum-moleculare and middle third of the dentate fascia stratum moleculare. The intermediate path terminates in intermediate zones in the dentate stratum moleculare (Blackstad, 1958; Steward, 1976; Hjorth-Simonsen, 1972).

The entorhinal pyramidale cells of layer III project directly to all fields of the hippocampus. This perforant path travels entirely within the stratum lacunosum-moleculare (Blackstad, 1958; Steward and Scoville, 1976); indeed, lesions of the entorhinal and parasubiculum demonstrate a sharp boundary between the lacunosum-moleculare and stratum radiatum that is indistinct in normal histological sections (Blackstad, 1958). This projection is arranged in a longitudinal pattern with medial entorhinal afferents terminating closest to the CA_1/CA_2 border and lateral entorhinal afferents terminating near the CA_1/subiculum transition (Steward, 1976). The perforant path synapses *en passant* in dentate stratum moleculare, CA_1 and CA_3 stratum lacunosum-moleculare, and projects within the same septal/temporal layer (Hjorth-Simonsen and Jeune, 1972).

2.2.1.3.5. Commissural connections

As well as the ipsilateral projections each hippocampus also projects to the contralateral hippocampus. In rodents, these projections are through the corpus callosum, the ventral psalterium (hippocampal commissure) and the psalterium dorsalis (dorsal commissure). The psalterium dorsalis does not exist in species with greater callosal development. Contralateral projections are similarly distributed to the ipsilateral projections according to the architectonic divisions and septal/temporal

arrangement (Blackstad, 1956). Additionally, symmetric contralateral fields are connected, and this homotopic symmetry exists for points within architectonic fields as well as between fields (Andersen, 1959).

Connections between the contralateral stratum lacunosum-moleculare of the hippocampus pass through the psalterium dorsalis, and are strongest in the CA_1 and fade towards the CA_3 (Blackstad, 1956; Andersen, 1959). The majority of contralateral projections within the hippocampus, however, cross via the ventral (hippocampal) commissure (psalterium ventralis) and travel in the fimbria. After reaching the hippocampus some of these fibres travel to all hippocampal regions in the stratum oriens, synapsing with basal dendrites of pyramidal neurons (Blackstad, 1956; Andersen, 1959). The majority of fibres originating from the ventral commissure cross the pyramidal layer unbranched near the mid area of the CA_3 and the travel exclusively in the stratum radiatum in the direction of the CA_1 and CA_3 synapsing with the apical dendrites of pyramidal neurons (Blackstad, 1956; Andersen, 1959) and with inhibitory interneurons (Schwartzkroin and Kunkel, 1985). The majority of these fibres travel to the CA_1, but not beyond, and are numerous, as revealed by massive degeneration after contralateral ablation (Blackstad, 1956). Degeneration is less in the CA_3 radiatum but becomes dense in the hilus, and these contralateral fibres do not extend into the dentate gyrus (Blackstad, 1956).

2.2.1.3.6. Associative pathways

Associative pathways in the hippocampus include a longitudinal or axial associative path of CA_2 fibres in the stratum radiatum and stratum oriens associative pathways (Chronister and White, 1975).

2.2.1.3.7. Lamellar organisation

Much of the early histological evidence indicated that the pathways in the hippocampus are organised in a lamellar pattern, with the majority of fibre projecting largely with the transverse axis of the hippocampus within the same septal/temporal layer. This, and the electrophysiological studies of Andersen *et al.* 1971, see below, lead to the proposal of a lamellar organisation of the hippocampus. Whereas this is strictly true for the Mossy fibres and largely correct for other pathways, more recent neuroanatomical studies have shown that there exist significant projections oriented with the septal/temporal axis (Amaral and Witter, 1989). Indeed, as described in section 2.2.1.3.2, the Schaffer collaterals, rather than exclusively connecting particular septal/temporal CA_3 and CA_1 areas, project strongly to different septal/temporal regions (Amaral and Witter, 1989).

The perforant path, mossy fibres, fimbrial axons and Schaffer collaterals and the CA_1 axons in the alveus can be regarded as a four member 'loop' as in figure 2.1. *In vivo*

field potential recordings in response to orthodromic and antidromic stimulation of these pathways at different depths reveal that the hippocampus is arranged in lamellae transverse to its septal/temporal axis which contain all members of a neuronal loop (Andersen *et al.* 1971a). Stimulation of the entorhinal area in anaesthetised rabbits causes firing of the dentate granule cells followed by activity in the CA_1 cell dendrites. The latency of this activity is consistent with a polysynaptic pathway, and is abolished by sectioning of the Schaffer collaterals. This suggests the existence of a tri-synaptic pathway of entorhinal area to granule cells to CA_3 to CA_1 as well as the direct perforant path projections to CA_1 (Andersen *et al.* 1966). This lamellar arrangement has had important experimental ramifications as it was directly responsible for the development of the *in vitro* transverse hippocampal slice.

2.2.1.4. External connections of the hippocampal formation

2.2.1.4.1. Afferents

The lateral input to the entorhinal area is the major cortical input to the hippocampal formation (Segal and Landis, 1974), with input from olfactory cortex, piriform cortex, orbito-frontal cortex and temporal cortex (Chronister and White, 1975; Bayer, 1985; Pribram and MacLean, 1953; MacLean and Pribram, 1953). Supracallosal input is from the cingulate gyrus which projects to the entorhinal area, the presubiculum, the parasubiculum, and to the retrosplenalis e (White, 1959; Pribram and MacLean, 1953). The anterior thalamic nuclei also project to the entorhinal area, via the cingulum, the hypothalamus, amygdala, periamygdala and brainstem (Bayer, 1985; Pribram *et al.* 1950). The pathways within the hippocampal formation described above in section 2.2.1.3 make it clear that the perforant path from the entorhinal area provides strong input to the hippocampus and the dentate gyrus, and the dentate gyrus in turn also projects to the hippocampus. However, there is no direct cortical innervation of either the hippocampus proper or the dentate gyrus (White, 1959; Pribram and MacLean, 1953).

The hippocampus and the dentate gyrus do receive monosynaptic input from outside the hippocampal formation, from the septum via the fornix-fimbrial fibres (Andersen *et al.* 1961a,b) Septo-hippocampal fibres travel to the alveus and terminate in all hippocampal areas and dentate gyrus, and some fornix fibres continue to the subiculum and entorhinal area (Chronister and White, 1975; Meibach and Siegal, 1977; Segal and Landis, 1974). Septal neurons from the midline of the vertical limb of the diagonal band travel via the dorsal fornix to the dorsal hippocampus, cells immediately lateral to the vertical limb of diagonal band project via the medial fimbria to the ventral hippocampus and the intermediolateral septum projects via the lateral fimbria to all levels (Meibach and Siegal, 1977; Segal and Landis, 1974).

The septo-hippocampal projection is unlike the other major connections of the hippocampal formation in that it does not form glutamergic synapses with the apical dendrites of primary neurons. A large portion of septo-hippocampal fibres are cholinergic (Lewis *et al.* 1967), and these terminate in the stratum pyramidale and in the adjacent zones of the stratum oriens and radiatum in the hippocampus, and similarly project predominantly to the granule cell layer and adjacent stratum moleculare and hilus in the dentate gyrus (Frotscher and Léránth, 1985). Dentate granule cells and CA_1 pyramidal cells are activated monosynaptically by septal stimulation (Andersen *et al.* 1961a,b), the current source is on or near the soma (Andersen *et al.* 1961b), which corresponds with the location of cholinergic synapses (Frotscher and Léránth, 1985). Approximately 30% of septo-hippocampal fibres arise from a separate population of septal neurons from the cholinergic neurons and are GABAergic (γ-amino-butyric acid), staining for glutamate decarboxylase (Köhler *et al.* 1984). Interneurons also receive septal cholinergic and GABAergic projections from the septum (Frotscher and Léránth, 1985; Fraser and MacVicar, 1991).

2.2.1.4.2. Efferents

The major target of hippocampal efferents is the septum (Chronister and White, 1975; Powell and Hines, 1975). Stimulation of hippocampal efferents in the fimbria produces glutamate receptor-mediated excitation in the septum, (Stevens and Cotman, 1986). Septal projections from the hippocampus travel via the precommisural fornix. The dorsal hippocampus projects to the medial septal nucleus and dorsomedial lateral nucleus and the ventral hippocampus to the lateral nucleus (Bayer, 1985; Chronister and White, 1975; Powell and Hines, 1975). Other projections include the amygdaloid nuclei, accumbens nucleus, bed nucleus, nucleus of the diagonal band and stria terminalis (Bayer, 1985; Powell and Hines, 1975). Only the fibres of the subicular area terminate outside the telencephalon in the diencephalon, these efferents travel via the postcommisural fornix and the major target is the mamillary bodies of the hypothalamus (Pearson and Pearson, 1976; Powell and Hines, 1975; Bayer, 1985).

2.2.1.5. Local circuits neurons in the CA_1 area

In addition to the primary neurons, the hippocampus has a diversity of non-pyramidal neuronal types forming local connections. These polymorphic interneurons are immunoreactive for GABA, form symmetric, inhibitory synapses with pyramidal neurons and produce inhibitory postsynaptic potentials (IPSP) in pyramids and occasionally other interneurons (Lacaille *et al.* 1987; Buhl *et al.* 1994; Schwartzkroin and Kunkel, 1985; Knowles and Schwartzkroin, 1981; Lacaille and Schwartzkroin, 1988a,b). The intrinsic excitability of pyramidal neurons is dampened by rich supply of inhibitory synapses from these interneurons.

The pyramidal cell bodies are surrounded by a dense plexus of inhibitory fibres and synapses (Ramon y Cajal, 1968), which is derived from interneurons in adjacent layers. Interneurons in the alveus and stratum oriens are fusiform or triangular, with most dendrites parallel to the alveus, although some penetrate into the stratum radiatum, the axon branches profusely in the stratum oriens and stratum pyramidale, forming symmetric synapses with pyramidal neuron dendrites, somata and initial segments (Lacaille *et al.* 1987; Ramon y Cajal, 1968; McBain *et al.* 1994). Similar fusiform or triangular interneuron somata are found in the superficial stratum oriens near the stratum pyramidale, these also have horizontal or descending dendrites with short spines, but the axon ascends and either ramifies in lacunosum-moleculare, or arches in the stratum radiatum and re-enters stratum pyramidale, before branching horizontally among pyramidal neurons in a rich plexus (Ramon y Cajal, 1968; McBain *et al.* 1994). Axon collaterals arising in the stratum radiatum either ramify in this layer and the stratum lacunosum-moleculare, or travel horizontally then enter stratum pyramidale and contribute to the plexus (Ramon y Cajal, 1968; McBain *et al.* 1994). Also in the stratum oriens are large stellate somata with mostly horizontal aspinous dendrites, although some larger dendrites ascend into the stratum lacunosum-moleculare and terminate either without ramification or as roseate branches. The thick, varicose axon courses horizontally or obliquely and covers a large distance, with branches mostly ascending into the stratum pyramidale and contributing to the plexus (Ramon y Cajal, 1968). Similar interneurons, although with fine axons, are described in the stratum oriens and also in the stratum pyramidale layer and form synapses on the soma of many CA_1 pyramidal neurons, and have dendrites that ramify extensively and receive asymmetric (excitatory) synapses in the stratum oriens but also reach into the stratum pyramidale and stratum lacunosum-moleculare (Schwartzkroin and Kunkel, 1985).

Neurons are found in the stratum pyramidale which, although morphologically similar to a small pyramidal cell, have electrophysiological characteristics of interneurons, see section 2.2.2.4.5. These have varicose, smooth or occasionally spiny dendrites, the apical dendrites branches progressively and reaches the hippocampal fissure and the basal dendrite tuft reach the alveus (Schwartzkroin and Mathers, 1978; Buhl *et al.* 1994; Kawaguchi and Hama, 1987). These neurons are distinguished morphologically from pyramidal neurons by the origin and course of their axon, in some cases arising from the apical dendrites, with extensive vertical ramifications in the oriens, pyramidal and radiatum layers (Schwartzkroin and Mathers, 1978). In other cells the axon emerges from the cell body and traverses the lower stratum radiatum with myelinated main branches ramifying horizontally and giving rise to a dense network of terminal branches in the deep stratum pyramidale and shallow stratum oriens (Buhl *et al.* 1994; Kawaguchi and Hama, 1987). The

terminal branches are arranged radially but somewhat oblique in CA_1 in rows following axon initial segments and forming symmetrical synapses (Buhl *et al.* 1994).

Several polymorphic cell types are described in the superficial hippocampal layers, and although these contribute to some extent to the stratum pyramidale plexus, their targets are predominantly the pyramidal neuron apical dendrites. In the stratum radiatum there are stellate or triangular cells with horizontally oriented processes including three or four branching, varicose dendrites with some branches descending into the stratum oriens, and an axon that arborises as fine varicose branches in the stratum radiatum or stratum lacunosum-moleculare. There are also vertically oriented triangular or fusiform cells in the stratum radiatum, sending dendrites to the stratum oriens and axon into the lacunosum which continues horizontally, forming varicose branches (Ramon y Cajal, 1968). Although some of stratum radiatum interneurons have axons which descend and contribute to the stratum pyramidal plexus, these may be displaced lacunosum interneurons (Ramon y Cajal, 1968). Triangular or stellate interneurons in the lacunosum can be identical to displaced neurons just described or else may have a horizontal axon in lacunosum with extensive arborisation in stratum lacunosum-moleculare (Ramon y Cajal, 1968). Fusiform or horizontal multipolar stellate neurons in the molecular layer have varicose dendrites and a delicate, horizontal axon with fine arborisation which ramify over long distances in stratum lacunosum-moleculare, radiatum and sometimes oriens, extending outside the CA_1 region, and some cross hippocampal fissure to dentate molecular layer (Ramon y Cajal, 1968; Lacaille and Schwartzkroin, 1988b; Kawaguchi and Hama, 1987; Williams *et al.* 1994).

2.2.1.5.1. Recurrent inhibition

There is a rich reciprocal innervation between the pyramidal neurons and the interneurons of the deeper hippocampal layers. Recurrent inhibition of CA_1 pyramidal neurons follows sufficiently strong excitation of CA_1 pyramidal neurons by all afferents tested (Andersen *et al.* 1964a). Axon collaterals from the CA_1 pyramidal neurons arise in the stratum oriens and branch among polymorphic cells in this layer (Ramon y Cajal, 1968) forming excitatory synapses with interneurons that make recurrent, inhibitory synapses with the CA_1 pyramidal neurons (Andersen *et al.* 1964a,b; Kandel *et al.* 1961; Dingledine and Langmoen, 1980; Alger and Nicoll, 1982; Knowles and Schwartzkroin, 1981; Lacaille *et al.* 1987; Schwartzkroin and Kunkel, 1985; Schwartzkroin and Mathers, 1978; Lorente de Nó, 1934). Orthodromic excitatory postsynaptic potentials (EPSP) are produced in these interneurons by stimulation of pyramidal neuron afferents in the alveus or by spike trains produced by depolarising steps in CA_1 pyramidal neurons (Knowles and Schwartzkroin, 1981; Lacaille *et al.* 1987; Schwartzkroin and Mathers, 1978).

Similarly, in paired intracellular recordings, spike trains in interneurons produces exclusively IPSPs in pyramidal neurons (Knowles and Schwartzkroin, 1981; Lacaille *et al.* 1987). The horizontally ramifying dendrites of these interneurons must allow integration of signals from many pyramidal neurons. The predominantly horizontal orientation of interneuronal axons allows each cell to synapse with many pyramidal neurons, a single interneuron forming symmetrical, inhibitory synapses with more than 600 pyramidal neurons in a slice (Buhl *et al.* 1994). It is presumably these interneurons which mediate the strong recurrent IPSPs in CA_1 pyramidal neurons produced by stimulation of the alveus in anaesthetised or decerebrate cats (Andersen *et al.* 1964a,b; Kandel *et al.* 1961) or in hippocampal slices (Dingledine and Langmoen, 1980; Alger and Nicoll, 1982; Newberry and Nicoll, 1984).

2.2.1.5.2. Feed-forward inhibition

Whereas hippocampal inhibition was originally considered to be largely recurrent, it was subsequently found that orthodromic stimulation in the stratum radiatum could produce strong, complex feed-forward IPSPs (Alger and Nicoll, 1982; Newberry and Nicoll, 1984). In these studies, antidromic, recurrent IPSPs were completely abolished by bicuculline methiodide, but the feed-forward IPSP contained a bicuculline insensitive component (Alger and Nicoll, 1982; Newberry and Nicoll, 1984), latter shown to be $GABA_B$ receptor-mediated. Antagonism of glutamergic fast excitatory synaptic transmission in the hippocampus abolishes the polysynaptic recurrent inhibition, but monosynaptic IPSPs can be evoked by electrical stimulation sufficiently close to the recording site (Davies *et al.* 1990). Using this technique, one-dimensional current source density analysis of fast ($GABA_A$-mediated) inhibitory field potentials reveals that orthodromic stimulation in the stratum radiatum produces a current source in this layer and current sinks in other dendrite layers (Lambert *et al.* 1991), thus these IPSPs are due to chloride influx at receptors in this dendritic region, rather than the classical somatic synapses of recurrent inhibition. Stimulation in other layers produced current sources at horizontal locations, with stratum oriens stimulation producing a source centred at the stratum oriens/stratum pyramidale border, stratum lacunosum-moleculare stimulation producing a source in this layer and the strongest current source produced in the stratum pyramidale by stimulation in this layer (Lambert *et al.* 1991). However, this alone does not prove feed-forward inhibition as monosynaptic IPSPs could result from direct stimulation of interneurons whose only afferents arise from local pyramidal neurons.

Many interneurons do receive afferents from other than CA_1 pyramidal neurons (Schwartzkroin and Kunkel, 1985; Lacaille *et al.* 1987; Lacaille and Schwartzkroin, 1988a; Lacaille and Schwartzkroin, 1988b; Schwartzkroin and Mathers, 1978). Therefore, the classes of interneurons which mediate recurrent inhibition may also

mediate feed-forward inhibition, indeed all have dendrites ramifying in the apical dendrite fields, away from the site of recurrent synapses in the stratum oriens (Lacaille *et al.* 1987; Schwartzkroin and Mathers, 1978; Schwartzkroin and Kunkel, 1985) and the stratum oriens also receives axons from the white matter which originate remotely, in addition to pyramidal recurrent collaterals (Ramon y Cajal, 1968). The basket cells, for instance, in addition to mediating recurrent inhibition, have asymmetric, presumably excitatory, synapses that degenerate following lesions to CA_1 afferents and commissural connections, suggesting they may also provide feed-forward inhibition (Schwartzkroin and Kunkel, 1985). Stratum radiatum stimulation monosynaptically activates the pyramidal interneuron (Schwartzkroin and Mathers, 1978) and the alveus/oriens interneurons (Lacaille *et al.* 1987). The axo-axonic interneuron in the stratum pyramidale is activated monosynaptically by stimulation in the stratum radiatum or stratum lacunosum-moleculare (Buhl *et al.* 1994). Of all the interneurons in the CA_1 the stratum lacunosum-moleculare interneurons alone do not receive any excitatory input from CA_1 pyramidal neurons (Lacaille and Schwartzkroin, 1988a), however stimulation of the alveus, stratum radiatum, stratum lacunosum-moleculare or the dentate moleculare layer activates EPSPs in these interneurons (Lacaille and Schwartzkroin, 1988b).

2.2.2. ELECTROPHYSIOLOGY

2.2.2.1. Hippocampal slices and whole animal preparations

Whereas many electrophysiological studies of the hippocampus are conducted in anaesthetised, decorticated animals or in animals with chronically implanted electrodes, the *in vitro* hippocampal slice preparation has become popular due to its simplicity. As early as 1951 sections of mammalian cortex maintained *in vivo* demonstrated metabolic responses, such as increased respiration in response to electrical stimulation (McIlwain, 1951), and in the 1960s evoked synaptic potentials were recorded in preparations of guinea pig prepiriform and piriform cortex (Yamamoto and McIlwain, 1966b; Yamamoto and McIlwain, 1966a) and later in dentate gyrus (Yamamoto and Kawai, 1967a; Yamamoto and Kawai, 1968; Yamamoto and Kawai, 1967b). Skrede and Westgaard first took advantage of the previously proposed lamellar structure of the hippocampus (Andersen *et al.* 1971a), and stimulated specific pathways and recorded mono-, di-, and tri-synaptic field potentials from the largely intact excitatory pathways of the *in vitro* transverse hippocampal slice (Skrede and Westgaard, 1971). Intracellular recordings soon followed these first extracellular records (Yamamoto, 1970).

Many studies have shown that the hippocampal slice is a good model for cortical function as it retains activity that is similar to intact animal preparations (Schwartzkroin, 1975; Schwartzkroin, 1977; Skrede and Westgaard, 1971). The

hippocampal slice preparation produces stable field potentials for 9-16 hours, although the amplitude of responses begins to diminish after six or seven hours, and this duration is inversely related to the frequency of stimulation (Schurr *et al.* 1986a; Schurr *et al.* 1984). The hippocampal slice is an important tool for studying cortical synaptic transmission and organisation. Being an *in vitro* preparation obviates the need for anaesthesia and its complicating effects on neural transmission, and better recording is possible because of the mechanical stability of a slice in an incubation chamber. The neural pathways are easily accessible for stimulation and recording and the layered arrangement of pathways within the slice allows stimulation of discrete areas of the dendritic tree.

2.2.2.2. Anatomy of the recording site in the CA_1

The pyramidal neuron is primary cell type in the CA_1 area. A strong mixed pathway travels in the stratum radiatum and makes *en passant* excitatory glutamergic synapses with the proximal apical dendrites of the CA_1 pyramidal neurons. This pathway is composed of the Schaffer collaterals which arise from ipsilateral CA_3 pyramidal neurons and the commissural connections that travel via the ventral hippocampal commissure from the contralateral CA_1 area. The direct perforant path projection travels in the stratum lacunosum-moleculare and synapses *en passant* with the distal portions of the pyramidal CA_1 neuron apical dendrites. Travelling in the alveus and stratum oriens, and synapsing with the basal dendrites of the CA_1 pyramidal cells, are associational and commissural hippocampal pathways and external afferents. The pyramidal neuron axon travels through the stratum oriens and leaves the CA_1 area in the alveus, but axon collaterals arise locally and innervate interneurons that form inhibitory synapses, largely on the pyramidal cell soma, but also on dendrites and initial segments, and mediate recurrent inhibition. Excitatory pathways in all layers innervate local interneurons which form inhibitory synapses with CA_1 pyramidal neurons, mediating feed-forward inhibition. Whereas interneurons may mediate both feed-forward and recurrent inhibition, those in the apical dendritic field may be largely feed-forward and synapse largely with pyramidal dendrites. The organisation of these pathways is shown schematically in figures 2.1 and 2.2.

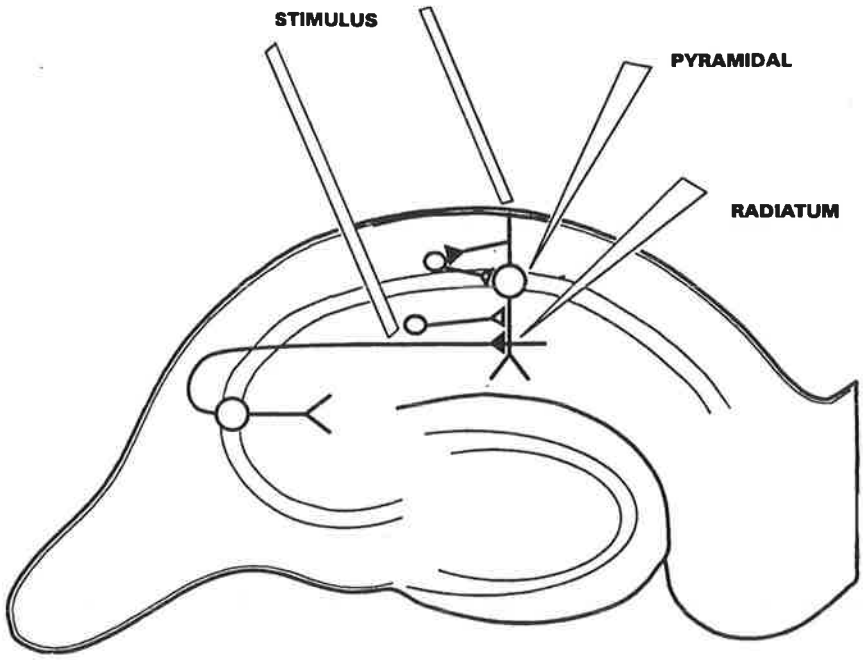

Figure 2.2 Pathways and electrode placements in the CA_1 area.
Stimulating electrodes are shown inserted from the left and stratum pyramidale and stratum radiatum recording electrodes are shown inserted from the right. Neuronal populations are represented by the single CA_3 pyramidal neuron, the CA_1 pyramidal neuron, a CA_1 recurrent interneuron and a CA_1 feed-forward interneuron. Filled triangles represent excitatory synapses and open triangles represent inhibitory synapses.

2.2.2.3. Extracellular recordings

Field potentials, recorded with low resistance extracellular electrodes, represent the summation of synchronous activity in many cells. A negative field potential is recorded in response to current flow into cells at the level of the extracellular recording electrode. Negative fEPSPs can be recorded in stratum oriens, stratum radiatum and stratum lacunosum-moleculare evoked by stimulation at a horizontal site (Ault and Nadler, 1982; Colbert and Levy, 1992) suggesting that there are excitatory synapses in each layer. Such active current sinks produce passive current sources in other layers, recorded extracellularly in other layers as positive potentials as current flows out of the pyramidal neuron and to the active sink. On the other hand, a positive field potential may represent an active current flow out of cells at the level of the recording electrode, such positive field potentials can represent field IPSPs, and although usually masked by the excitatory field potentials, can be recorded when excitatory synaptic transmission is blocked (Lambert *et al.* 1991).

The most common extracellular electrode configuration in the hippocampal slice CA_1 is shown in figure 2.2, and has recording electrodes in the stratum pyramidale and stratum radiatum recording the field potentials at the pyramidal neurons somata and proximal apical dendrites respectively and electrodes in the stratum radiatum to stimulate the Schaffer collateral/commissural fibres for orthodromic activation of synapses horizontal to this electrode. An electrode in the alveus near the pyramidal

layer recording electrode can be used to stimulate pyramidal neuron axons for antidromic activation of spikes. In response to orthodromic stimulation in this setup, the stratum radiatum electrode records a prevolley, a compound action potential which represents the summation of spikes in afferent fibres and is a characteristic of unmyelinated fibres (Andersen *et al.* 1978). The dendritic electrode records a negative field EPSP (fEPSP) as synaptically activated current flows into the dendritic tree and corresponds to the intracellularly recorded EPSP (Andersen *et al.* 1978). Population spikes measured at the stratum pyramidale in slices are a negative wave caused by the flow of cations into the cells (Andersen *et al.* 1971b). The population spike is the record of many individual spikes, the onset and duration coincide with intracellular spikes, and there is a linear relationship between population spike amplitude and the number of spikes from individual cells (Andersen *et al.* 1971b). These active current sinks produce positive waves in other layers, whereby the population spike rides on a positive fEPSP in the stratum pyramidale and a positive population spike is superimposed on the negative wave of the fEPSP in the stratum radiatum (Andersen *et al.* 1971b; Andersen *et al.* 1978).

Masked by these excitatory field potentials are inhibitory potentials. Stratum radiatum stimulation of excitatory afferents that synapse with interneurons can mediate polysynaptic feed-forward inhibition, and stimulation sufficiently close to the recording site can directly stimulate interneurons and activate monosynaptic feed-forward inhibition onto pyramidal neurons. Stratum radiatum and, more effectively, alveus stimulation activates recurrent inhibition onto pyramidal neurons. Inhibitory field potentials cannot be identified directly without pharmacological isolation, but evidence of their modulation of excitatory potentials can be easily demonstrated with paired pulse stimulation. The population spike amplitude is depressed by an appropriately timed preceding stimulus of recurrent or feed-forward inhibition. Longer interstimulus intervals can result in disinhibition via GABA autoreceptors on interneuronal terminals.

To avoid repetition, within the present work, unless specifically stated otherwise, orthodromic and antidromic field potentials in the CA_1 of the hippocampal slice are electrically evoked and recorded from the electrode configuration just described, and are in the rat. Similarly, single cell recording of evoked potentials in CA_1 pyramidal neurons use a stratum pyramidale intracellular or patch electrode and identical stimulating electrode placement.

2.2.2.4. Intracellular recording

Intracellular recording allows identification of single electrical phenomena in single cells. The first intracellular recordings from hippocampal pyramidal cells where made in 1961 in decorticate cats (Kandel *et al.* 1961). These deflections of

membrane potential include action potentials, hereafter called spikes, and single excitatory and inhibitory postsynaptic potentials, EPSPs and IPSPs.

2.2.2.4.1. Spikes

Intracellular recording of spikes in pyramidal neurons from decorticate cat CA_3 and CA_2 areas, in response to orthodromic and antidromic stimuli, show a fast rising positive spike with a mean amplitude of 70 mV above the resting membrane potential of about -52mV and duration of 1.84 ± 0.09 msec (Kandel *et al.* 1961). Orthodromic stimulation gives rise to a fast pre-potential, which is an abrupt depolarisation at the beginning of a spike, about one third the amplitude of the spike, (Spencer and Kandel, 1961). Spikes often are followed by a depolarising after-potential of 10 mV amplitude and 30 msec duration (Spencer and Kandel, 1961), that is due to passive recharging of the soma from the dendrites (Warman *et al.* 1994).

Intracellular CA_1 pyramidal neuron recordings in response to stimulation of the alveus (antidromic) and Schaffer collateral (orthodromic) in guinea pig hippocampal slices display the same characteristics as whole animals. Pyramidal cells have a resting membrane potential near -55mV, an input resistance of about 16MΩ, a spike amplitude of about 65mV and duration of 1.6-1.9 msec. These spikes display a decay half time of 6-14 msec and depolarising after-potentials of 3-8 mV. Summation of depolarising after-potentials during bursts of spikes can trigger further spikes (Schwartzkroin, 1975). Low amplitude fast pre-potentials can be recorded with orthodromic or antidromic stimulation (Schwartzkroin, 1975; Schwartzkroin, 1977), these are likely associated with spike generation at a site distant from the recording electrode (Turner *et al.* 1993).

2.2.2.4.2. IPSP

The IPSP reduces pyramidal excitability by shunting the EPSP current, 'clamping' the cell at the IPSP reversal potential, and by hyperpolarisation of the membrane away from spike threshold (Dingledine and Gjerstad, 1979; Dingledine and Langmoen, 1980). An intracellular record of an IPSP shows a membrane hyperpolarisation, and an increase in conductance associated with opening of chloride channels (Dingledine and Gjerstad, 1979; Dingledine and Langmoen, 1980; Dingledine and Gjerstad, 1980). The IPSP produced by somatic synapses can be reversed by injection of hyperpolarising current or increased in amplitude with depolarising current (Schwartzkroin, 1975; Schwartzkroin, 1977). Whereas it was discovered that the hyperpolarisation and conductance change of the fast IPSP or exogenous GABA could be reduced with penicillin, it was also noted that this did not reduce the duration of the IPSP (Dingledine and Langmoen, 1980; Dingledine and Gjerstad, 1980). It became obvious that there were two pharmacologically distinct GABA receptors in the CA_1 mediating fast, bicuculline sensitive, and slow components of

the IPSP (Alger and Nicoll, 1982), and that the slow component was not mediated by chloride, but by potassium (Newberry and Nicoll, 1984); interestingly this had been foreshadowed in earlier *in vivo* experiments (Andersen *et al.* 1964a). The development of the $GABA_B$ receptor antagonists, phaclofen and 2-OH-saclofen, established that this receptor-mediated the slow IPSP (Lambert *et al.* 1989) and was distinct from the bicuculline sensitive $GABA_A$-mediated fast IPSP, see section 2.2.2.5.1.

2.2.2.4.3. EPSP

The excitatory postsynaptic potential recorded intracellularly in pyramidal cells is a membrane depolarisation (Schwartzkroin, 1975; Schwartzkroin, 1977) that is graded with stimulus intensity and results from synaptic activation of glutamate receptors (Davies and Collingridge, 1989). EPSPs of sufficient amplitude give rise to spikes (Schwartzkroin, 1975; Schwartzkroin, 1977; Davies and Collingridge, 1989). EPSPs have a slow time course, 3-12 msec from onset to peak and 8-15 msec decay half time (Schwartzkroin, 1975). The early part of the EPSP is voltage insensitive whereas the later part of the EPSP has anomalous voltage sensitivity, conferred by a voltage-dependent magnesium block (Davies and Collingridge, 1989). The fast part of the EPSP/C is mediated by the AMPA (RS-amino-3-hydroxy-5-methyl-4-isoxazolopropionate) subtype of glutamate receptor and is blocked by the AMPA selective antagonist CNQX (6-cyano-7-nitro-quinoxaline-2,3-dione), whilst the slow EPSP/C is mediated by NMDA (N-methyl-D-aspartate) subtype and is blocked by the selective antagonist AP5 (2-amino-5-phosphonopentanoate) (Davies and Collingridge, 1989; Hestrin *et al.* 1990), see section 2.2.2.6.

2.2.2.4.4. Pyramidal neuron voltage activated calcium and potassium currents

A thorough review of voltage activated ion channels is beyond the scope of the present account, and is available elsewhere (Dolphin, 1991; Storm, 1990; Storm, 1993). Here it is simply mentioned that hippocampal pyramidal neurons express the low-voltage-activated T-type calcium currents as well as the high-voltage-activated L-type, N-type and P-type calcium currents (Westenbroeck *et al.* 1990; Mogul and Fox, 1991; Mogul *et al.* 1993), as these appear in the later discussions of adenosine actions and are also of importance in hypoxia-induced neuronal calcium overload.

Hippocampal pyramidal neuron potassium currents are presented in brief, since these are important in the context of the present work because some are modulated by adenosine, some are activated by hypoxia, whilst others modulate neuronal excitability and may be important in pyramidal hyperexcitability. Table 2.1 presents a summary of pyramidal neuron potassium currents. I_A and I_D both delay the onset of spikes, I_D causing a long delay and depressing initial firing rate. Both I_A and I_D along with I_K and I_C contribute to spike repolarisation. I_K is the classical delayed outward

rectifying potassium current. I_C, I_M and I_{AHP} underlie the fast, medium and slow after-hyperpolarisations respectively, and contribute to spike train frequency adaptation. I_M also serves to limit persistent depolarisation of the pyramidal neuron membrane, and introduces the sag in depolarising pulses that is typical of pyramidal neurons.

current	blocked by	activated by	activ^n voltage	inactiv^n voltage	activ^n kinetics	inactiv^n kinetics
I_A	4-AP 1-4mM DTX 50-300nM	depol.	-60mV	-60 to -40mV	10ms	100ms
I_D	4-AP 30-40μM DTX?	depol.	-70mV	-120 to -60mV	20ms	seconds
I_K	TEA 5-30mM	depol. increased by phorbol esters muscarinics	-40mV	<-100mV	20-80ms	seconds
I_M	TEA 5-10mM barium 1mM IP_3 activators eg. M2 muscarinics	depol. somatostatin leucotrieines	-60mV	does not inactivate	50ms	does not inactivate
I_C	TEA <1mM CTX 30nM BAPTA Ca^{2+} channel blockers	calcium influx and depol.	-40mV		slow	slow
I_{AHP}	cAMP	calcium influx increased by adenosine	N/A	N/A	400ms	seconds

Table 2.1 Some hippocampal pyramidal neuron potassium currents.
This table summarises the reviews by J.F. Storm (Storm, 1990; Storm, 1993) concerning voltage-activated and calcium-dependent potassium currents. Abbreviations: CTX ~ ω-conotoxin; DTX ~ dendrotoxin; BAPTA ~ 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; 4-AP ~ 4-aminopyridine; TEA ~ tetraethylammonium; N/A ~ not applicable.

2.2.2.4.5. Interneuron electrophysiology

In addition to morphology and function (inhibition), electrophysiological characteristics distinguish interneurons from pyramidal neurons. This distinctive physiology is apparently due to differences in neuronal potassium currents between interneurons and primary cell types. All interneurons produce brief (0.4 msec duration at half spike amplitude), small amplitude (43-64mV) spikes compared to pyramidal neurons (Schwartzkroin and Mathers, 1978; Lacaille *et al.* 1987; Kawaguchi and Hama, 1987; Buhl *et al.* 1994; Williams *et al.* 1994), these spikes are followed by a prominent fast AHP (Schwartzkroin and Mathers, 1978; Lacaille *et al.* 1987; Kawaguchi and Hama, 1987; Buhl *et al.* 1994; Williams *et al.* 1994) which is apparently a continuation of spike repolarisation. These characteristics suggest that the potassium currents involved in spike repolarisation, I_A, I_K, and I_C (Storm, 1990; Storm, 1993) are strong. Rapid onset of firing in interneurons (Lacaille and Schwartzkroin, 1988a; Lacaille and Schwartzkroin, 1988b; Lacaille *et al.* 1987; Schwartzkroin and Mathers, 1978) suggests the absence of a potassium current, possibly I_D, which delays spike onset in pyramidal neurons (Storm, 1990; Storm,

1993). In most cases the interneurons lack the slow calcium activated potassium current, the I_{AHP} and spike accommodation is weak or absent, but see below. As a result of this composition of currents, excitatory input to interneurons causes rapid activation of spikes which continue without frequency adaptation, but firing ceases immediately excitatory input is terminated. Interneurons display a near linear current-voltage relationship (Lacaille and Schwartzkroin, 1988b; Buhl *et al.* 1994) and, additionally, have a relatively depolarised resting membrane potential of -51 to -65mV (Schwartzkroin and Mathers, 1978; Lacaille *et al.* 1987; Kawaguchi and Hama, 1987; Buhl *et al.* 1994).

Basket cells, pyramidale interneurons and alveus/oriens interneurons are physiologically similar. They have a high rate of spontaneous spike activity with basket cells firing at 4Hz and others at 20-80Hz (Schwartzkroin and Mathers, 1978; Lacaille *et al.* 1987). Orthodromic stimulation produces bursts of spikes and intracellular injection of depolarising current produces trains of spikes with no accommodation or adaptation (Schwartzkroin and Mathers, 1978; Lacaille *et al.* 1987; Kawaguchi and Hama, 1987). Whereas the slow AHP is usually absent (Schwartzkroin and Mathers, 1978; Lacaille *et al.* 1987; Kawaguchi and Hama, 1987), oriens/alveus and pyramidale interneurons, but not basket cells, produce early inactivation between first and subsequent spikes in a burst (Schwartzkroin and Mathers, 1978; Lacaille *et al.* 1987). Alveus/oriens interneurons and oriens interneurons respond differently to metabotropic glutamate receptor activation, the former displaying a small inward current whereas the latter produce a large inward current with oscillations (McBain *et al.* 1994).

Although still characteristic of interneurons, the stratum lacunosum-moleculare and the axo-axonic stratum pyramidale interneurons form a physiologically distinct group. From the range of interneuron values, they have the more depolarised resting membrane potential (-60 to -65mV), the higher input resistance (63-74MΩ) and the larger action potential amplitude (60 to 64mV) (Buhl *et al.* 1994; Lacaille and Schwartzkroin, 1988b). This group of interneurons rarely fires spontaneous spikes, and orthodromic activation produces usually only one or two spikes (Buhl *et al.* 1994; Lacaille and Schwartzkroin, 1988b; Williams *et al.* 1994). Spontaneous spikes, when present are not blocked by glutamate or GABA receptor antagonism and are apparently intrinsically generated (Williams *et al.* 1994). Depolarising current injection results in a slow AHP in most cells, and variable, generally weak, spike frequency adaptation (Buhl *et al.* 1994; Lacaille and Schwartzkroin, 1988b; Williams *et al.* 1994). Such spike frequency adaptation is different from pyramidal cells, being preceded with an initial acceleration of spikes on a depolarising hump (Buhl *et al.* 1994). Many lacunosum-moleculare interneurons have a delayed onset of firing during depolarisation (Williams *et al.* 1994), presumably mediated by I_A.

2.2.2.5. Inhibitory synapses

2.2.2.5.1. Postsynaptic inhibition

Interneurons form GABAergic synapses (Buhl *et al.* 1994) that produce IPSPs in hippocampal pyramidal cells (Dingledine and Gjerstad, 1980; Langmoen and Andersen, 1981; Lacaille and Schwartzkroin, 1988a; Lacaille and Schwartzkroin, 1988b; Lacaille *et al.* 1987; Andersen *et al.* 1964b; Andersen *et al.* 1964a). There are two main subtypes of GABA receptors, the $GABA_A$ receptor and the $GABA_B$ receptor which mediate the fast and slow IPSPs respectively (Thompson, 1994). Typically, fast neurotransmitter receptors form an integral ion channel which, in the case of the $GABA_A$ receptor is permeable to chloride (Thompson, 1994). The $GABA_B$ receptor is coupled via a G protein to a potassium channel (Thompson, 1994). Although the early studies of recurrent inhibition were made well before the identification of the $GABA_B$ receptor subtype, with hindsight, it is clear that antidromic stimulation produces a primarily fast $GABA_A$-mediated IPSP, blocked by the $GABA_A$ antagonist bicuculline methiodide (Alger and Nicoll, 1982; Newberry and Nicoll, 1984); however, in some studies, the IPSP duration with antidromic stimulation suggests a possible slow $GABA_B$-mediated IPSP of hundreds of milliseconds duration (Dingledine and Langmoen, 1980; Andersen *et al.* 1964a). Whilst orthodromic activation produces bursts of spikes in interneurons, lasting up to 40 msec (Schwartzkroin and Mathers, 1978; Lacaille *et al.* 1987), this is of insufficient duration to produce the long lasting IPSP of pyramidal neurons. The discrepancy between the pure $GABA_A$ recurrent IPSPs in slices and the long recurrent IPSPs *in vivo* may be due to a loss of inhibitory connection in the slice, since inhibition is reduced with transverse as compared to more longitudinal slices of hippocampus. Feed-forward IPSPs produced by stratum radiatum stimulation have a fast component blocked by the $GABA_A$ antagonists bicuculline methiodide or picrotoxin (Alger and Nicoll, 1982; Lambert *et al.* 1991; Davies *et al.* 1990; Newberry and Nicoll, 1984; Williams and Lacaille, 1992), and a slow component blocked by the $GABA_B$ antagonists CGS 35348 or 2-OH-saclofen (Lambert *et al.* 1991; Lambert *et al.* 1989; Williams and Lacaille, 1992).

Whether $GABA_A$ and $GABA_B$ receptors are located at the same or different synapses, or whether fast and slow IPSPs are mediated by different interneurons is not clear. There is evidence that the interneuronal terminals in the stratum pyramidale and in the stratum radiatum are different, being subject to presynaptic modulation by distinct neuromodulator substances (Segal, 1990; Lambert and Wilson, 1993), and may thus arise from different populations of interneurons. There is some evidence that recurrent inhibition is due to $GABA_A$ receptors only (Alger and Nicoll, 1982), and there is also evidence for an interneuron subset that, when stimulated by application of 4-aminopyridine (4-AP), produces only $GABA_B$ receptor-mediated

hyperpolarisation in pyramidal neurons (Segal, 1990). Feed-forward inhibition of pyramidal neurons by the stratum-lacunosum moleculare interneurons gives a slowly decaying IPSP that could be $GABA_B$ receptor-mediated (Lacaille and Schwartzkroin, 1988a). More convincingly, micro-iontophoresis of glutamate onto the stratum lacunosum-moleculare produces tetrodotoxin (TTX) sensitive IPSPs in CA_1 pyramidal neurons, presumably due to activation of lacunosum-moleculare interneurons, which are blocked by the $GABA_B$ antagonist 2-OH-saclofen but not the $GABA_A$ antagonist bicuculline (Williams and Lacaille, 1992). Interestingly, these glutamate evoked IPSPs are not sensitive to phaclofen, unlike electrically stimulated late IPSPs (Williams and Lacaille, 1992).

2.2.2.5.2. Presynaptic inhibition

Excitatory transmission at the Schaffer collateral/commissural synapses in the CA_1 is inhibited by the $GABA_B$ receptor agonist baclofen (Lanthorn and Cotman, 1981; Ault and Nadler, 1982; Blaxter and Carlen, 1985; Colbert and Levy, 1992). $GABA_B$ presynaptic receptors are on the excitatory terminals of CA_3 pyramidal neuron axons, and baclofen inhibits excitatory transmission at the Schaffer collaterals, in the stratum radiatum and associational/commissural fibres in the stratum oriens but not the perforant path in the stratum lacunosum-moleculare (Lanthorn and Cotman, 1981; Colbert and Levy, 1992; Ault and Nadler, 1982). Similarly, local baclofen application on the proximal apical CA_1 pyramidal neuron dendrites, but not distal apical or basal dendrites, in rat hippocampal slices inhibits the Schaffer collateral EPSP (Blaxter and Carlen, 1985). Native presynaptic inhibition is presumably mediated by axo-axonic synapse arising from the interneuron axons which ramify in the stratum radiatum and oriens.

Bath applied baclofen (Peet and McLennan, 1986) or local iontophoresis of baclofen on the proximal apical CA_1 pyramidal neuron dendrites, but not distal apical or basal dendrites (Blaxter and Carlen, 1985), in rat hippocampal slices, inhibits the stratum radiatum feed-forward fast and slow IPSPs. This suggests the presence of $GABA_B$ autoreceptors on interneuronal terminals, which may be activated by GABA released from these terminals.

2.2.2.6. Excitatory synapses

The amino acids glutamate and aspartate are found in high concentrations in the brain where they account for most excitatory neurotransmission (Storm-Mathisen, 1978). Autoradiographic studies of high affinity uptake of these amino acids shows they are localised in the excitatory terminals of the perforant path, mossy fibres and pyramidal cell axons, as well as in hippocampal efferents to the septum and mamillary bodies and contralateral hippocampal projections (Storm-Mathisen, 1978). The highest levels of these transmitters are in the stratum radiatum and oriens as well

as the inner stratum moleculare of the dentate facia, whilst lower levels are in the stratum lacunosum moleculare and outer dentate stratum moleculare (Storm-Mathisen, 1978). Excitatory neurotransmission in the hippocampus is mediated by two groups of excitatory amino acid receptors, originally classified as glutamate preferring and aspartate preferring, but later classified as the receptors activated by the glutamate agonist NMDA and the non-NMDA or AMPA receptors, activated by the agonists, kainate, quisqualate and AMPA (Monaghan *et al.* 1989). These receptor subtypes occur at the same synapses in the hippocampus (Bekker and Stevens, 1989).

2.2.2.6.1. AMPA receptors

Fast excitatory neurotransmission at hippocampal synapses is mediated by non-NMDA receptors activated by the agonists kainate and quisqualate, and most selectively by AMPA (Monaghan *et al.* 1989; Krogsgaard-Larsen *et al.* 1980). This component of synaptic transmission is blocked specifically by the quinoxaline derivatives CNQX and 6,7 dinitro-quinoxaline-2,3-dione (DNQX) (Andreasen *et al.* 1988; Honoré *et al.* 1988; Davies and Collingridge, 1989). The cloned and expressed AMPA receptor binds AMPA > quisqualate > glutamate > kainate, and is an intrinsic cation channel gated by these agonists and blocked by CNQX (Keinänen *et al.* 1990).

2.2.2.6.2. NMDA receptor-ionophore

The NMDA receptor has been well characterised due to the early identification of a specific agonist and a range of antagonists. The transmitter binding site binds the specific agonist NMDA and a range of antagonists such as, α-aminoapidate and the more potent and selective antagonists such as AP5 (aminophosphonovalerate, APV), 2-amino-7-heptanoate (AP7), and the highly potent heterocyclic derivatives of these compounds such as 3-((±)-2-carboxypiperazin-4-yl-propyl-1-phosphonic acid (CPP) (Monaghan *et al.* 1989).

Block of AMPA receptors by CNQX reveals a small persistent synaptic component of synaptic transmission corresponding to NMDA receptor activation (Andreasen *et al.* 1988; Davies and Collingridge, 1989; Hestrin *et al.* 1990). Under physiological conditions the NMDA receptor is blocked in a voltage dependent fashion by magnesium. NMDA currents are greatest if the membrane is depolarised to -20 or -30 mV, which relieves the magnesium block (Mayer *et al.* 1984; Nowak *et al.* 1984). If this magnesium block is relieved by depolarisation, blockade of GABAergic inhibition, or by omission of magnesium from an *in vitro* medium, an NMDA component of synaptic transmission is evident as a slow EPSP and multiple late spikes (Andreasen *et al.* 1988; Davies and Collingridge, 1989). These secondary spikes are blocked in a concentration-dependent fashion by AP5 (Coan and Collingridge, 1987a). The NMDA ion channel differs from the non-NMDA ion

channel as, in addition to sodium, it allows the flow of calcium ions. This calcium current is independent of the voltage-gated calcium currents (Holopainen *et al.* 1989).

In addition to the glutamate binding site, the NMDA receptor contains other distinct ligand sites: a glycine site, a voltage dependent magnesium binding site and an inhibitory site that binds zinc, and a binding site inside the channel (Monaghan *et al.* 1989). Inhibitors that bind at these sites can be exploited as non-competitive antagonists of the NMDA receptor complex. Glycine is an allosteric potentiator of NMDA currents which increases the opening probability of the ion channel (Kessler *et al.* 1989), with the suggestion of an absolute requirement of glycine for NMDA activation (Huettner, 1989). The glycine potentiation is specifically blocked by 7-chlorokynureate, indole-2-carboxylic acid and HA-966 (Foster and Kemp, 1989). The dissociative anaesthetics ketamine and phencyclidine, the structurally related compounds N-[1-(2-thienyl)cyclohexyl]-piperadine and dibenzocycloheptenеimine (MK-801), and the opiate SKF 10047, all bind at the site within the ion channel and block NMDA initiated currents and abolish the NMDA component of synaptic transmission (Coan and Collingridge, 1987b).

2.2.2.6.3. Glutamate inactivation

Uptake by glial cells are responsible for the termination of glutamate neurotransmission, so that gliotoxin treatment *in vivo* leads to an increased accumulation of both glutamate and GABA over controls (Paulsen and Fonnum, 1989). Glial cells import glutamate from the extracellular cleft by an active transport mechanism, metabolise it to glutamine, which is returned to the extracellular space where it becomes available to neurons for the synthesis of more neurotransmitter (Waniewski and Martin, 1986).

2.2.2.7. Other neurotransmitters and neuromodulators

In addition to the major neurotransmitters glutamate and GABA described in the previous sections 2.2.2.5 and 2.2.2.6, the hippocampus also contains many other neurotransmitters and receptors that mediate synaptic transmission or modulate synaptic transmission by other transmitters. Receptors for acetylcholine, noradrenaline, serotonin, histamine, dopamine, ATP and various neuropeptides contribute to hippocampal function (Nicoll *et al.* 1990). In addition to these transmitters and co-transmitters there are receptors for neuromodulators, which differ from neurotransmitters in that they are not released as a result of exocytosis of neurosecretory vesicles. Adenosine is such a neuromodulator, which is released by facilitated diffusion and whose extracellular concentrations reflect intracellular levels. Nevertheless, extracellular adenosine levels can fluctuate greatly and mediate powerful neurophysiological actions.

2.3. ADENOSINE

Although adenosine was long known to cause vasodilatation and bradycardia (Drury and Szant-Gyorgi, 1929), it was Sattin and Rall (1970) who originally demonstrated that adenosine had neuroactive properties and played a role distinct from that as an intermediary in energy metabolism. They showed in guinea pig cortical slices that adenosine or adenine nucleotides stimulate cAMP accumulation, and that this action is blocked by theophylline (Sattin and Rall, 1970).

2.3.1. ADENOSINE RECEPTOR CLASSIFICATION

Adenosine both stimulates and inhibits adenylate cyclase activity, and these two activities are mediated by two pharmacologically distinct receptors, originally named R_i, for inhibition, and R_a, for activation, of adenylate cyclase (Londos *et al.* 1980). The R_a receptor is activated by 5'-N-ethylcarboxyamidoadenosine (NECA) > adenosine > phenylisopropyladenosine (PIA), whereas the reverse order of potency is observed at R_i (Londos *et al.* 1980). By current nomenclature, these are properly called the A_1 and A_2 receptor. Low concentrations of adenosine agonists inhibit cAMP accumulation by an action at the A_1 receptor, and higher concentrations stimulate cAMP accumulation by an action at the A_2 receptor (van Calker *et al.* 1979; van Calker *et al.* 1978). Adenosine receptors also mediate electrophysiological actions that are not related to their modulation of adenylate cyclase. The A_1 receptor mediates diverse presynaptic and postsynaptic inhibitory actions in many preparations. The A_2 receptor has been further subdivided on the basis of binding data (Bruns *et al.* 1986) into the low affinity A_{2b} subtype coupled to adenylate cyclase and a high affinity A_{2a} subtype that mediates poorly characterised excitatory electrophysiological actions. Recently a novel A_3 receptor has been identified (Zhou *et al.* 1992) that is coupled to phosphatidylinositide hydrolysis (Ramkumar *et al.* 1993) which evidently mediates as yet unknown central actions.

Current classification relies on the pharmacology of ligand binding at these adenosine receptors. The first proposed classification of adenosine receptors was based on the earliest available agonists, the A_1 receptor being characterised by the relative affinity for the agonists R-PIA > NECA > 2-chloroadenosine (2-CA), whereas for the A_2 receptor binding affinity is NECA > 2-CA > R-PIA (Williams and Cusack, 1990; Stone, 1991). The most specific and potent A_1 receptor agonists are N^6 substituted analogues of adenosine, including 2-chloro-N^6-cyclopentyladenosine (CCPA), N^6-endonorbornyladenosine (S-ENBA), N^6-cyclopentyladenosine (CPA), and N^6-cyclohexyladenosine (CHA). Less selective agonists include 2-CA and NECA. The relative affinity of the most common agonists for the A_1 receptor is as follows: CCPA > S-ENBA > CPA ≥ CHA ≥ R-PIA > 2-CA > NECA > S-PIA (Williams and Cusack, 1990).

The most selective A_2 agonist available is 2-[*p*-(carboxyethyl)-phenylethylamino]-NECA (CGS 21680) (Jarvis *et al.* 1989), which may be selective for the A_{2a} subtype. Functional studies in peripheral tissues known to express A_2 receptors show a rank order of efficacy of agonists CGS 21680 = 2-(phenylamino)adenosine (CV 1801) ≥ NECA > R-PIA ≥ N-[(2-methylphenyl)methyl]adenosine (metrifudil) > CPA > N-[(1S,trans)-2-hydroxycyclopentyl]adenosine (GR 79236) = S-PIA, whereas in guinea-pig aorta, also known to expresses A_2 receptors, agonist efficacy shows a rank order of NECA > metrifudil > R-PIA = CPA > CV 1801 = GR 79236 > CGS 21680 = S-PIA (Gurden *et al.* 1993). These may represent A_{2a} and A_{2b} receptors respectively, thus metrifudil may represent a reasonably selective A_{2b} receptor agonist.

Whereas the A_3 receptor shows a general similarity in agonist binding profile to A_1 receptors, the affinity for the typical A_1 receptor agonists is 2-3 orders of magnitude lower, closer to the affinity of these agonists for A_{2a} receptors (Zhou *et al.* 1992; Jacobson *et al.* 1993; Ramkumar *et al.* 1993; van Galen *et al.* 1994). In contrast to A_1 receptors, and A_{2a} receptors, the A_3 receptor binds N^6 and 5' di-substituted adenosine analogues with highest affinity (van Galen *et al.* 1994). The A_3 receptor binds N^6-2-(4-amino-3-iodophenyl)ethyladenosine (APNEA), N^6-(3-iodobenzyl)-5'-N-methycarboxamidoadenosine (3-IB-MECA) and N^6-benzyl-NECA with high affinity (Zhou *et al.* 1992; Jacobson *et al.* 1993; Ramkumar *et al.* 1993; van Galen *et al.* 1994). 3-IB-MECA and N^6-benzyl-NECA in particular are potent and selective agonists, binding A_3 receptors with low nanomolar affinity and 10-50 selectivity compared to A_1 and A_2 receptors (Jacobson *et al.* 1993; van Galen *et al.* 1994).

Whereas caffeine and theophylline are non-selective A_1 and A_2 antagonists, the most useful A_1 antagonists are 8-substituted xanthines. These include the potent and highly selective xanthine amino congener (XAC), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and the slightly less selective 8-cyclopentyl-1,3-dimethylxanthine (8-cyclopentyltheophylline, 8-CPT), as well as 1,3-dipropyl-8-*p*-sulfophenylxanthine (DPSPX), and 8-phenyltheophylline (8-PT) (Williams and Cusack, 1990; Stone, 1991). Recently useful A_2 antagonists have been described, and the A_2 antagonist 3,7-dimethy-1-propylargylxanthine (DMPX) (Sebastiào and Ribeiro, 1992a) is readily available. The non-xanthine adenosine antagonist 5-imino-9-chloro-2-(2-furyl 1,2,4-triazolo [1,5-c] quinazoline (CGS 15943) and 5-amino-8-(4-flourobenzyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (8FB-PTP) bind to rat striatal and bovine membrane A_2 receptor more avidly than do the alkylxanthines (Jarvis *et al.* 1989; Dionsotti *et al.* 1994). Recently, a potent antagonist selective for the A_{2a} subclass of receptors, (E)-1,3-dipropyl-7-methyl-8-(3,4-dimethoxystyryl)xanthine (KF17837), has been described (Kanda *et al.* 1994). By contrast, the A_3 receptor is insensitive to alkylxanthine binding and, as yet, no antagonists exist for this receptor

(Zhou *et al.* 1992; Jacobson *et al.* 1993; Ramkumar *et al.* 1993; van Galen *et al.* 1994).

Adenosine receptors have been purified to homogeneity and cloned from several sources. They are typical G protein binding receptors (Mahan *et al.* 1991; Zhou *et al.* 1992; Rivkees and Reppert, 1992), being glycoproteins with an extracellular amino-terminal, seven putative membrane spanning domains connected with three intracellular and three extracellular loops, and an intracellular carboxy-terminal (Taylor, 1990; Boege *et al.* 1991). They share sequence analogy with most other members of the superfamily of G protein linked receptors, and this has been used as a strategy for identifying their cDNA sequences.

2.3.1.1. A_1 receptors

The A_1 receptor has been purified to homogeneity from a number of species including rat (Nakata, 1989) and human (Nakata, 1992). The brain A_1 receptor is a glycoprotein of about 35kDa and binds [^{3}H]DPCPX with high affinity (Nakata, 1992). A rat striatal cDNA fragment encodes a 326 amino acid protein with seven trans-membrane domains typical of G protein linked receptors (Mahan *et al.* 1991). The A_1 receptor expressed from this striatal cDNA binds [^{3}H]DPCPX with high affinity, binds agonists with the appropriate rank order of potency, CCPA = R-PIA > NECA > S-PIA, and ligand-receptor interaction is modulated by G protein binding of Gpp(NH)p (Mahan *et al.* 1991). An A_1 receptor cloned from human hippocampal cDNA has 95% sequence homology with the rat receptor, and when expressed in Chinese hamster ovary cells, binds DPCPX with high affinity, and inhibits adenylate cyclase (Townsend-Nicholson and Shine, 1992).

The hippocampus expresses the highest density of A_1 receptors in the brain, and A_1 receptor mRNA is abundant in hippocampal neurons as revealed by cDNA *in situ* hybridisation (Mahan *et al.* 1991; Cunha *et al.* 1994a). Autoradiography of [^{3}H]CHA binding in rat brain shows that A_1 receptor density is high in the molecular layer of the dentate gyrus and in the stratum radiatum and stratum oriens of all hippocampal areas, whilst the highest binding is in the stratum radiatum of the hippocampal CA_1 area (Fastbom *et al.* 1987; Goodman and Snyder, 1982). Within the hippocampal fields, A_1 receptor density is heterogenous along the septal/temporal axis, with highest numbers of receptors in the septal aspect (Lee *et al.* 1983). A high A_1 receptor density is also found in the cerebellum, in the subiculum, the superior colliculus, and the geniculate nuclei (Goodman and Snyder, 1982; Fastbom *et al.* 1987). Intermediate high levels of A_1 receptors are found in the remaining thalamic nuclei, the lateral septum, and the cerebral cortex (Fastbom *et al.* 1987; Goodman and Snyder, 1982). Lower levels of A_1 receptors are also found in the basal ganglia, the hypothalamus, amygdala, midbrain areas other than superior colliculus (central

grey, substantia nigra), brainstem and stratum moleculare of the CA_1 area (Fastbom *et al.* 1987; Goodman and Snyder, 1982). With the exception of a relatively high density in the granule cell layer of the cerebellum, the receptors are found in the molecular layers of these structures, suggesting they are associated with excitatory synapses (Goodman and Snyder, 1982).

2.3.1.2. A_3 receptors

The rat striatal cDNA clone of the novel A_3 receptor has 58% homology with previously cloned adenosine receptors (Zhou *et al.* 1992). The cloned receptor, expressed in Chinese hamster ovary cells and the native receptor in mast cells binds A_1 receptor agonists, although with an affinity 2-3 orders of magnitude lower for the most selective A_1 receptor agonists, and it does not bind alkylxanthine antagonists (Zhou *et al.* 1992; Jacobson *et al.* 1993; Ramkumar *et al.* 1993; van Galen *et al.* 1994). The A_3 receptor expressed in Chinese hamster ovary cells is linked to a Pertussis toxin sensitive G protein and will inhibit adenylate cyclase (Zhou *et al.* 1992; van Galen *et al.* 1994), whereas the native receptor in mast cells stimulates phospholipase C (Ramkumar *et al.* 1993). Intraperitoneal 3-IB-MECA causes a DPCPX insensitive locomotor depression in mice (Jacobson *et al.* 1993). Low levels of A_3 receptor, determined by [^{125}I]AB-MECA binding in the presence of DPCPX, are found in various regions of the mouse brain, including the hippocampus, cortex, cerebellum and striatum (Jacobson *et al.* 1993). It is worthwhile noting that binding of A_1 receptor agonists in the absence of antagonists could, in fact, represent A_3 receptors, and some incongruous data may need re-interpretation in this light.

2.3.1.3. A_2 receptors

An A_{2a} receptor clone has been isolated from a hippocampal cDNA library, which when transfected into a human cell line expresses a receptor that does not bind CCPA but binds CGS 21680 with high affinity (K_d 10 ± 1nM), and [^{3}H]CGS 21680 is displaced by adenosine receptor ligands with an appropriate rank order of affinity NECA > XAC > 2-CA > CPA (Furlong *et al.* 1992). The A_{2a} receptor clone isolated from rat striatal cDNA library and transfected in COS cells binds CGS 21680 with K_d 38.6nM (Fink *et al.* 1992), and it is interesting to note that this is a lower affinity than the cloned hippocampal receptor. However, [^{3}H]CGS 21680 binds to striatal membranes with a K_d of 16nM (Jarvis *et al.* 1989). The deduced amino acid sequence of the rat A_{2a} receptor shows the seven α-helical membrane spanning domains (IJzerman *et al.* 1994) typical of G-protein coupled receptors.

A_{2b} receptors have been cloned from rat (Rivkees and Reppert, 1992) and human (Pierce *et al.* 1992) brain cDNA libraries. Native receptors in human fibrobasts and those expressed in Chinese hamster ovary cells bind NECA but not A_1 or A_{2a} selective agonists, and are positively coupled to adenylate cyclase (Pierce *et al.* 1992; Rivkees

and Reppert, 1992) Receptors expressed in Xenopus oocytes are positively coupled to phospholipase C (Yakel *et al.* 1994).

Autoradiography of [^{3}H]CGS 21680 shows high A_{2a} receptor density in the striatum, with lower densities detected in the caudate-putamen, nucleus accumbens, olfactory tubercle and globus pallidus (Jarvis and Williams, 1989). Although earlier work failed to demonstrate [^{3}H]CGS 21680 binding in the hippocampus, an adenosine A_{2a} receptor has been cloned from a human hippocampal cDNA library, and A_{2a} receptor mRNA is identified in the soma of rat CA_1 and CA_2 pyramidal neurons and dentate granule cells by *in situ* hybridisation (Cunha *et al.* 1994a), suggesting that this receptor must be expressed in the hippocampus (Furlong *et al.* 1992). This is further supported by enhanced excitatory synaptic transmission and excitability caused by nanomolar concentrations of CGS 21680 in the hippocampus (Sebastião and Ribeiro, 1992a; Sebastião and Ribeiro, 1992b; Cunha *et al.* 1994a). Only recently has specific [^{3}H]CGS 21680 binding been demonstrated in all synaptic layers of the hippocampus, dentate and subiculum, but at levels much lower than detected in even the basal ganglia (Cunha *et al.* 1994a). A_{2a} receptor density in the hippocampus matches the distribution of A_1 receptors and is highest in the stratum radiatum of the CA_1 (Cunha *et al.* 1994a). [^{3}H]CGS 21680 binding exceeds [^{3}H]CHA binding in the stratum lacunosum-moleculare of the CA_3 (Cunha *et al.* 1994a). A_1 and A_{2a} receptors are co-localised on the presynaptic terminals of the rat neuromuscular junction (Correia de Sá *et al.* 1991), and the electrophysiological interaction between A_{2a} and presynaptic A_1 receptors (Cunha *et al.* 1994a) point to a similar arrangement in the hippocampus. A_{2b} receptors are found throughout the brain (Bruns *et al.* 1986).

2.3.2. ADENOSINE A_1 RECEPTORS MEDIATE DEPRESSION OF SYNAPTIC TRANSMISSION AND OF NEURONAL EXCITABILITY IN THE CA_1

Adenosine was first shown to depress CNS neurons in 1974, micro-iontophoresis of adenosine or adenine nucleotides depressed spontaneous activity of corticospinal cells in methoxyflurane/N_2O anaesthetised rats (Phillis *et al.* 1974). In the same year, adenosine was shown to inhibit synaptic transmission in the guinea-pig olfactory cortex slice *in vitro*, evoked by lateral olfactory tract stimulation (Schofield, 1974). In 1979 adenosine was shown to depress synaptic transmission in the hippocampal slice, where postsynaptic excitatory field potentials and the synaptic current sink in the apical dendrites in the CA_1 area, evoked by Schaffer collateral stimulation, showed a concentration-dependent inhibition by adenosine (Schubert and Mitzdorf, 1979). The demonstration that the adenosine depression of postsynaptic field potentials in the CA_1 of rat hippocampal slices could be antagonised by the methylxanthine theophylline (Dunwiddie and Hoffer, 1980) was a major step, identifying that the synaptic depression is mediated by adenosine receptors. Furthermore, it became obvious that endogenous adenosine causes tonic inhibition,

since adenosine degradation with exogenous adenosine deaminase increases, and adenosine uptake block with hexobendine decreases postsynaptic potentials (Dunwiddie and Hoffer, 1980).

2.3.2.1. Adenosine inhibition in the hippocampus is mediated by A_1 receptors

The potency and specificity of ligands at the A_1 receptor parallels their efficacy at neuronal inhibition. In the CA_1 of the rat hippocampal slice, agonist potency at inhibition of orthodromic postsynaptic field potentials (Reddington *et al.* 1982; Fowler, 1988), inhibition of antidromic after-potentials in low calcium, high magnesium medium (Schubert and Lee, 1986), of hyperpolarisation and reduction of input resistance (Ameri and Jurna, 1991), all follow a rank order of potency characteristic of the A_1 receptor. The same rank order of agonist potency is shown for inhibition of aspartate and glutamate release from hippocampal slices or hippocampal synaptosomes (Poli *et al.* 1991; Corradetti *et al.* 1984). Such exogenous agonist-induced inhibition, as well as inhibition from endogenous adenosine, can be blocked by the selective A_1 antagonists DPCPX and 8-CPT (Alzheimer *et al.* 1991; Dunwiddie and Fredholm, 1989; Ameri and Jurna, 1991).

2.3.2.2. Presynaptic actions of adenosine A_1 receptors

2.3.2.2.1. Adenosine inhibits neurotransmitter release

The primary action of adenosine is the depression of excitatory postsynaptic potentials, and electrophysiological evidence suggests that adenosine acts presynaptically to reduce synaptic transmission. In hippocampal slices, the evoked EPSP in CA_1 pyramidal cells can be significantly reduced, without effect on input resistance and membrane potential, by iontophoresis of adenosine onto synaptic region in stratum radiatum or bath application of low adenosine concentrations (Siggins and Schubert, 1981; Segal, 1982). Similarly, adenosine depresses the electrically evoked excitatory postsynaptic current (EPSC) without effect on postsynaptic membrane properties in dissociated peri-natal rat hippocampal neurons (Yoon and Rothman, 1991; Kamiya, 1991). This effect could result from activation of either presynaptic adenosine receptors, or dendritic spine adenosine receptors electrotonically distant from the soma, which do not effect recordings of soma membrane properties. However, strong evidence suggests a presynaptic mechanism in the hippocampal slice since analysis of unitary EPSP variance in whole-cell patch clamped CA_1 pyramidal cells shows a high correlation between depression of the unitary EPSP and the depression of mean quantal content as estimated by M^2/σ^2 (squared average EPSP/variance) in response to low concentrations of adenosine (5-20 μM) (Lupica *et al.* 1992). Furthermore, in guinea-pig olfactory cortex slices, which are devoid of postsynaptic adenosine receptors (Schofield, 1978), synaptic transmission in response to lateral olfactory tract stimulation is depressed by

exogenous and endogenous adenosine (Schofield, 1974; Schofield and Steel, 1988; Motley and Collins, 1983; Schofield, 1978).

Presynaptic inhibition by adenosine and A_1 receptor agonists involves inhibition of neurotransmitter release. In hippocampal slices the A_1 receptor agonist R-PIA inhibits electrical field stimulated release of acetylcholine, noradrenaline, and glutamate (Dunér-Engström and Fredholm, 1988; Fredholm and Lingren, 1987; Fredholm *et al.* 1986). Adenosine, R-PIA, CHA or 2-CA depress the potassium, kainate or 4-AP evoked release of glutamate and aspartate from rat and guinea pig hippocampal synaptosomes (Poli *et al.* 1991; Arvin *et al.* 1989; Barrie and Nicholls, 1993). In hippocampal slices, R-PIA, CHA and adenosine depress release of glutamate and aspartate by electrical stimulation of Schaffer collaterals (Corradetti *et al.* 1984), and in mini-slices of CA_1, lacking stratum lacunosum-moleculare, exogenous adenosine action at an A_1 receptor inhibits potassium evoked glutamate and aspartate release, presumably from Schaffer collateral/commissural fibre terminals (Nadler *et al.* 1990). 8-PT enhancement of evoked glutamate and aspartate release in hippocampal slices indicates a tonic depression of neurotransmitter release, due to basal activation of adenosine receptors by endogenous adenosine (Nadler *et al.* 1990; Corradetti *et al.* 1984). Adenosine also depresses evoked release of glutamate, noradrenaline and dopamine in other central preparations (Harms *et al.* 1978; Dolphin, 1985; Cass and Zahiniser, 1991).

There are variable findings regarding adenosine depression of inhibitory synaptic transmission. Adenosine does not depress a fast IPSC in dissociated peri-natal hippocampal neurons, evoked by extracellular stimulation or intracellular stimulation in paired recordings (Yoon and Rothman, 1991; Kamiya, 1991). In the CA_1 of rat hippocampal slices in the presence of DNQX and AP5, adenosine depresses the monosynaptically activated late IPSP but not the fast IPSP (Lambert and Teyler, 1991a). Since adenosine A_1 receptors are coupled to the same or a similar population of postsynaptic potassium channels as the $GABA_B$ receptor, see section 2.3.4.3.1, it is possible that the adenosine depression of the late IPSP is due to postsynaptic occlusion of the $GABA_B$ current. It appears that adenosine selectively inhibits excitatory neurotransmitter release, whilst sparing inhibitory neurotransmitter release. Adenosine, R-PIA or 2-CA cause a concentration-dependent decrease in potassium or kainate evoked release glutamate and aspartate but not GABA from hippocampal synaptosomes (Poli *et al.* 1991) and from dentate gyrus slices (Dolphin and Archer, 1983). This selectivity concurs with the location of adenosine A_1 receptors on the terminals of excitatory neurons, as has been demonstrated autoradiographically in the cerebellum (Goodman *et al.* 1983).

Presynaptic A_1 receptor activation presumably depresses neurotransmitter release by reducing calcium influx into neuronal terminals. Adenosine causes a concentration-dependent decrease in electrically stimulated or potassium stimulated $^{45}Ca^{2+}$ uptake into rat brain synaptosomes (Gonçalves *et al.* 1991), and CHA reduces both the potassium or 4-AP evoked plateau of cytoplasmic calcium fluorescence in guinea pig brain synaptosomes (Barrie and Nicholls, 1993). Adenosine reduces the decrease in extracellular calcium, due to presynaptic terminal calcium uptake in response to Schaffer collaterals stimulation with synaptic transmission inhibited, measured by ion-selective microelectrodes in the stratum radiatum of the hippocampal slice (Schubert *et al.* 1986).

2.3.2.2.2. Adenosine A_1 receptor inhibition of N-type calcium channels

In a number of preparations used to model presynaptic events, A_1 receptors depress N-type calcium channels. In whole cell voltage clamp of cultured mouse dorsal root ganglion cells, 2-CA reduces the whole cell calcium current and shortens calcium dependent action potentials recorded in tetraethylammonium (TEA), by selectively inhibiting the N-type calcium current but not L-type or T-type currents (Dolphin *et al.* 1986; Gross *et al.* 1989). Increase in intracellular calcium through ω-conotoxin sensitive, N-type channels is depressed by adenosine A_1 receptors in chick ciliary ganglion giant presynaptic terminals (Yawo and Chuhma, 1993) and post-ganglionic neurons (Bennett *et al.* 1992). Furthermore, in whole cell voltage clamp of acutely isolated guinea pig CA_3 pyramidal neurons, from which the Schaffer collaterals arise, current carried through N-type channels is decreased by adenosine or 2-CA (Mogul *et al.* 1993) via an A_1 receptor. However, adenosine does not alter calcium currents in guinea pig CA_1 pyramidal neurons or olfactory cortex neurons (Halliwell and Schofield, 1984).

2.3.2.2.3. Adenosine modulation of presynaptic potassium currents

There is some evidence that A_1 receptor depression of transmitter release may result from enhanced presynaptic potassium currents, and consequent spike narrowing, and therefore a reduction in depolarisation-induced calcium influx. 2-CA inhibition of evoked fEPSP in the CA_1 area of hippocampal slices, a putative presynaptic action, is inhibited by 30μM 4-AP, and, unlike baclofen- and neuropeptide Y induced inhibition, 2-CA induced inhibition is not regained by reducing extracellular calcium, suggesting involvement of potassium channels but not voltage-activated calcium channels (Klapstein and Colmers, 1992). Indirect support of this notion is given by the enhancement of the 4-AP sensitive, transient potassium current, I_A, and shortening of the action potential by adenosine and 2-CA in locus coeruleus neurons (Pan *et al.* 1994) and I_A may be modulated by A_1 receptors in hippocampal pyramidal neurons (Schubert and Lee, 1986). In one report, the potassium channel blocker

barium reduced A_1 receptor-mediated depression of CA_1 EPSP (Birnstiel *et al.* 1992). Also, adenosine reduces the calcium-dependent potassium current, I_C, and the after-hyperpolarisation in presynaptic terminals of chick ciliary ganglia (Bennett and Ho, 1992), this may be secondary to block of the N-type calcium current, but may also be a non-A_1 receptor action.

2.3.2.3. Postsynaptic actions of adenosine A_1 receptors

Adenosine has two actions on postsynaptic neurons. Firstly, it limits large excitatory input by enhancing the long after-hyperpolarisation (AHP) which follows action potential repolarisation and accommodation, causing the slowing of action potential frequency during prolonged depolarisation (Greene and Haas, 1985; Haas and Greene, 1984). Additionally, high concentrations of adenosine, in the range of 10-100μM, reduce neuronal excitability by membrane hyperpolarisation and decreased input resistance (Gerber *et al.* 1989; Siggins and Schubert, 1981; Greene and Haas, 1985; Haas and Greene, 1984; Proctor and Dunwiddie, 1983; Segal, 1982). These different actions are due to enhancement of distinct potassium currents

2.3.2.3.1. Membrane hyperpolarisation

Membrane hyperpolarisation was the first described electrophysiological action of adenosine, in the hippocampus this was initially described in CA_3 neurons (Okada and Ozawa, 1980) then in CA_1 neurons (Siggins and Schubert, 1981). Bath application of 20μM adenosine hyperpolarises the membrane of CA_1 pyramidal neurons and reduces input resistance during hyperpolarising current injection (Siggins and Schubert, 1981). High concentrations of adenosine hyperpolarise rat CA_1 pyramidal neurons, with average hyperpolarisations of 7.1 or 8mV reported from a range of 1-15mV from bath application of 100μM adenosine and direct micro-drop application of 0.1-10mM adenosine (Greene and Haas, 1985; Segal, 1982). Low concentrations of 10 to 20μM adenosine are less consistent, hyperpolarising most CA_1 pyramidal neurons by an average of 3.14mV to 5mV (Siggins and Schubert, 1981; Haas and Greene, 1984). All concentrations of adenosine cause inconsistent decreases in input resistance with mean values reported from 8 to 30% from a range of 0-50% (Siggins and Schubert, 1981; Haas and Greene, 1984; Segal, 1982).

Membrane hyperpolarisation and reduced input resistance from 10-100μM adenosine application in rat hippocampal CA_1 pyramidal cells is due to the activation of an outward potassium current (Gerber *et al.* 1989). Under single electrode voltage clamp, hyperpolarisation by adenosine reverses at membrane holding potentials of -85mV (Haas and Greene, 1984) or -90mV (Segal, 1982) and is also reversed by raising extracellular potassium from 5mM to 12mM (Gerber *et al.* 1989). Input resistance changes by adenosine also vary with alterations in extracellular potassium, consistent with changes in a potassium current (Gerber *et al.* 1989). The identity of

the potassium channel opened by A_1 receptors in CA_1 pyramidal neurons is obscure, but this ligand-gated potassium channel is directly G protein-linked (Van Dongen *et al.* 1988; Trussell and Jackson, 1987) and may be distinct from the voltage-gated channels.

Adenosine hyperpolarisation and reduced input resistance are insensitive to blockade of calcium-dependent potassium currents, I_C or I_{AHP}, by the extracellular calcium channel blockers, cadmium, cobalt or manganese or the calcium chelator EGTA (Haas and Greene, 1984; Greene and Haas, 1985; Segal, 1982). Adenosine hyperpolarisation or outward current are not blocked by TEA or micromolar 4-AP which block I_D, I_M, and I_C (Greene and Haas, 1985; Segal, 1982; Gerber *et al.* 1989). However, the adenosine activated potassium current can be blocked by millimolar concentrations of 4-AP or barium which will block I_A or I_M (Gerber *et al.* 1989). Furthermore, R-PIA inhibition of CA_1 antidromic after-potentials is blocked by 50μM 4-AP (I_D) but not millimolar TEA (Schubert and Lee, 1986), and adenosine and 2-CA enhances the 4-AP sensitive I_A in locus coeruleus neurons (Pan *et al.* 1994). This latter action is due to depolarisation of the I_A inactivation curve, increasing the amount of I_A available near threshold, rather than an increase in maximal stimulation of I_A (Pan *et al.* 1994). Such an action on I_A would not occur under hyperpolarised conditions (Greene and Haas, 1985; Segal, 1982; Gerber *et al.* 1989) where adenosine does not alter I_A (Greene and Haas, 1985).

The apparent inward rectification of the adenosine activated potassium current, originally observed in striatal neurons (Trussell and Jackson, 1985; Trussell and Jackson, 1987), is not observed in most adult rat hippocampal pyramidal neurons (Gerber *et al.* 1989). In those neurons that do display inward rectification (7 of 21, or 38%), it is abolished by 10mM TEA, leaving a potassium current with a linear current/voltage relationship, suggesting the inward rectification may be due to interaction with the TEA sensitive I_C (Gerber *et al.* 1989) or possibly the I_K, reducing the length constant or space clamp, or causing a current shunt.

2.3.2.3.2. Slow after-hyperpolarisation

20-50μM adenosine greatly increases the amplitude and decreases the rate of decay of the slow AHP which follows single spikes, bursts of spikes or TTX insensitive slow calcium spikes in CA_1 pyramidal cells (Greene and Haas, 1985; Haas and Greene, 1984). Adenosine also slightly enhances the early voltage sensitive AHP following bursts of spikes (Haas and Greene, 1984). Adenosine enhancement of the AHP increases spike frequency accommodation (Greene and Haas, 1985; Haas and Greene, 1984). The AHP is usually enhanced despite hyperpolarisation and decreased input resistance, although a sufficient decrease in input resistance with

high concentrations of adenosine will reduce the AHP amplitude (Greene and Haas, 1985; Haas and Greene, 1984).

The slow AHP is mediated by a small-conductance, voltage and TEA insensitive, calcium-dependent potassium current, I_{AHP}, which is blocked by increased intracellular cAMP (Storm, 1990; Storm, 1993; Lancaster *et al.* 1991; Lancaster and Adams, 1986). The I_{AHP} is blocked by a variety of neurotransmitter substances which stimulate adenylate cyclase (Storm, 1990; Storm, 1993), and this may be the mode of adenosine A_1 receptor action. Accommodation, the slowing of frequency of repetitive action potential discharge during long depolarising pulses, is mediated by I_{AHP} and is the main mechanism for reducing large excitatory inputs with relative sparing of small excitatory input (Madison and Nicoll, 1984). Modulation of the AHP may be the most important ascending influence on hippocampal function.

2.3.2.3.3. Other postsynaptic A_1 receptor actions

Adenosine A_1 receptor activation increases the threshold for, and decreases the amplitude of TTX-insensitive slow calcium spikes in CA_1 pyramids in hippocampal slices (Haas and Greene, 1988; Haas and Greene, 1984; Proctor and Dunwiddie, 1983). However in guinea pig hippocampal CA_1 pyramidal cells, adenosine does not reduce calcium currents although it will reduce calcium spikes, suggesting this is not a direct modulation of calcium channels (Halliwell and Schofield, 1984).

In rat CA_1 cells held near resting membrane potential (-60mV), A_1 receptor agonists induce a steady state inward chloride current sensitive to theophylline, PTX pre-treatment or the chloride channel blocker 4,4'-diisothiocyano-2,2'-stilbenedisulfonate (Mager *et al.* 1990). Theophylline induced increase in electrically stimulated calcium flux in rat CA_1 is blocked by the chloride channel blocker 4,4'-diisothiocyano-2,2'-stilbenedisulfonate or the chloride pump blocker furosemide (Schubert *et al.* 1991). This chloride current could be calcium-activated, as a result of adenosine A_1 receptor-mediated phosphoinositide hydrolysis.

2.3.3. ADENOSINE A_2 RECEPTORS

2.3.3.1. Excitatory actions of adenosine receptor agonists

As early as 1983, paradoxical locomotor stimulation was noted following i.p. injection of low doses of R-PIA in mice (Bruns *et al.* 1983), with this low dose of a usually depressant compound suggesting the action of a non-A_1 adenosine receptor. In contrast to the characteristic depressive action of micromolar concentrations of adenosine, low concentrations (10nM-1μM) increase the amplitude of extracellularly recorded, submaximal postsynaptic field potentials in the guinea pig hippocampal CA_1, CA_3 area, superficial grey of the superior colliculus, and dentate gyrus (Nishimura *et al.* 1990; Okada *et al.* 1990; Okada *et al.* 1992). Although the

relatively more selective A_1 receptor agonists NECA and R-PIA are inhibitory in nanomolar concentrations, intracellular recordings show that high, micromolar, concentrations of R-PIA and NECA have excitatory actions, characterised by membrane depolarisation, increased input resistance, enhanced EPSPs, and increased spontaneous action potential frequency, in 50% of CA_1 pyramidal neurons in rat hippocampal slices (Ameri and Jurna, 1991). This effect is seen at low agonist concentrations, and in all neurons if A_1 receptors are blocked by the selective A_1 antagonist DPCPX, and this excitatory action is blocked by the non-selective adenosine receptor antagonist theophylline (Ameri and Jurna, 1991)

2.3.3.2. Post-inhibitory hyperexcitability

In their intracellular study, Siggins and Schubert note a "rebound depolarisation" of hippocampal CA_1 neurons with washout following seven minutes of 20μM adenosine (Siggins and Schubert, 1981). Although concentrations of adenosine higher than 10μM inhibit postsynaptic potentials in the guinea pig hippocampal CA_1 and CA_3 (Okada *et al.* 1990; Okada *et al.* 1992) and dentate gyrus (Nishimura *et al.* 1990; Nishimura *et al.* 1992), washout, after 10-20 minutes application of adenosine, is accompanied by a transient increase in population spike amplitude. Following 30 minute adenosine superfusion, population spike amplitude is persistently increased in the CA_3, CA_1 (Okada *et al.* 1992) and dentate gyrus (Nishimura *et al.* 1992) of guinea pig hippocampal slices. It is curious that hyperexcitability due to low, excitatory, concentrations of adenosine, will persist after only 15 minutes adenosine exposure in the hippocampus (Okada *et al.* 1992) and only 5 minutes adenosine exposure in the superior colliculus (Ishikawa *et al.* 1994), whereas a longer exposure to micromolar concentrations of adenosine is required for persistent post-inhibitory hyperexcitability. Possibly the opposing depressive actions of adenosine A_1 receptor activation restrain the excitatory actions of non-A_1 receptors

In the superior colliculus, adenosine (0.5-10μM) curiously has no inhibitory actions and causes a persistent excitation (Okada *et al.* 1990). The superior colliculus has only A_1 receptors on the presynaptic terminals and A_1 and A_2 receptors postsynaptically (Wan and Geiger, 1990). Why these A_1 receptors fail to produce inhibitory actions is unclear; however, the A_1 receptor binding was determined with [^{3}H]CHA (Wan and Geiger, 1990), and such binding could represent A_3 receptors. Alternatively, collicular A_1 receptors may be differently coupled to effector systems than are A_1 receptors in other tissues. It may be ill-advised to compare adenosine actions in the superior colliculus with those in other tissues. However, in the superior colliculus, persistent increase in the field postsynaptic potential amplitude in the superficial grey, due to optic layer stimulation, occurs during and after even 5 minute superfusion of adenosine, and a similar increase in field postsynaptic

potential amplitude can be produced by both specific A_1 and A_{2a} receptor agonists (R-PIA, CHA, NECA and CGS 21680) at concentrations as low as 1nM (Ishikawa *et al.* 1994). The xanthines theophylline, 8-CPT and DMPX, and the non-xanthine A_2 receptor antagonist CGS 15943 all produce excitability in the superior colliculus in nanomolar concentrations (Ishikawa *et al.* 1994) where they cannot be antagonising tonic A_1 receptor-mediated depression, as this action of A_1 receptors is absent.

Excitability produced by agonists selective at both A_1 and A_2 adenosine receptor subtypes is intriguing, and suggests the possibility of involvement of another receptor subtype. The agonists used in the superior colliculus (Ishikawa *et al.* 1994) all bind the A_3 receptor subtype, but only with high nanomolar affinity (van Galen *et al.* 1994). The excitatory actions of xanthine and non-xanthine adenosine receptor antagonists (Ishikawa *et al.* 1994), which do not bind the A_3 receptor (van Galen *et al.* 1994), is good evidence against A_3 receptor involvement, as is the inhibition of adenosine agonist excitability in CA_1 pyramidal neurons by theophylline (Ameri and Jurna, 1991). Enhancement of excitability by adenosine receptor agonists in the presence of DPCPX eliminates the A_1 receptor (Ameri and Jurna, 1991), and this leaves only the A_2 receptors. Although the published affinity of the A_{2a} receptors for adenosine analogues does not explain the superior collicular data, these affinities are generally established in the striatum (van Galen *et al.* 1994) and there is reasonable evidence that this receptor differs both in affinity and effector coupling from those in other tissues, see section 2.3.3.3. Enhancement of the field postsynaptic potential in the superior colliculus by adenosine can be blocked by the selective protein kinase A inhibitor H-89 as well as the less selective H-7 and H-8 (Hirai *et al.* 1994) and blockade of adenosine enhanced glutamate release by the protein kinase A inhibitor HA-1004 (Hirai and Okada, 1994), indicate an adenosine receptor positively coupled to adenylate cyclase, as are A_{2b} receptors.

2.3.3.3. Adenosine A_{2a} receptor-mediated excitation

Fifteen to thirty minutes perfusion with the selective adenosine A_{2a} agonist, CGS 21680 (3 and 10nM), increases both the amplitude of CA_1 population spikes and the slope of the fEPSP, effects blocked by the A_2 receptor antagonist DMPX (Sebastião and Ribeiro, 1992a; Cunha *et al.* 1994a). The presynaptic inhibition of synaptic transmission by the A_1 receptor agonist CPA is significantly attenuated in the presence of CGS 21680 (Cunha *et al.* 1994a), and the excitatory actions of A_{2a} receptor activation may in part result from alleviation of tonic A_1 receptor action by endogenous adenosine. Indeed, the increase in fEPSP by CGS 21680 (Cunha *et al.* 1994a) is of the same order as that produced by A_1 receptor antagonists (Dunwiddie and Diao, 1994). However, A_{2a} receptor-mediated excitability cannot be entirely due

to alleviation of A_1 receptor depression, since adenosine receptor agonists have excitatory actions in the presence of A_1 receptor antagonists (Ameri and Jurna, 1991).

There is apparently a high affinity A_2 receptor in the rat hippocampus, since the electrophysiological efficacy of low nanomolar CGS 21680 is well below the reported K_d of 16nM for binding to rat striatal membranes (Jarvis *et al.* 1989). Apparently, the A_2 receptor mediating excitatory electrophysiological effects in the hippocampus is distinct from the low affinity, G_s linked A_{2b} receptor mediating cAMP accumulation (Gilman, 1987), since forskolin-induced cAMP accumulation does not cause membrane depolarisation (Dunwiddie *et al.* 1992), as does adenosine induced excitation (Ameri and Jurna, 1991), and whereas CGS 21680 is a weak and non-efficacious agonist of adenylate cyclase in the striatum (Lupica *et al.* 1990), it fails to stimulate cAMP formation in the hippocampus (Lupica *et al.* 1990). Furthermore, only micromolar concentrations of NECA will stimulate cAMP accumulation in the hippocampus (Fredholm *et al.* 1990), and only nanomolar concentrations are required to produce an excitatory action (Ameri and Jurna, 1991). This suggests the presence of a high affinity A_{2a} receptor in the hippocampus, mediating excitatory electrophysiological actions independent of adenylate cyclase modulation, and possibly different from the striatal A_{2a} receptor.

2.3.3.4. Adenosine A_{2b} receptor enhancement of P-type calcium channels

Adenosine causes a large enhancement of P-type calcium currents in acutely isolated hippocampal CA_3 neurons (Mogul *et al.* 1993). This action is mediated via A_{2b} receptors as it is caused by non-selective A_2 agonists adenosine, 2-CA or N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl]adenosine (DPMA), but not CPA or CGS 21680, and is dependent on protein kinase A activation (Mogul *et al.* 1993). The N-type, as well as the P-type or Q-type voltage activated calcium channels contribute to excitatory synaptic transmission between the Schaffer collaterals and the CA_1 area, but the latter appear to be dominant (Wheeler *et al.* 1994). Enhancement of these channels via A_{2b} receptor activation by adenosine could thus cause the increased glutamate release and excitatory synaptic transmission in the CA_1 described in sections 2.3.3.1, 2.3.3.2 and 2.3.3.5.1.

2.3.3.5. Presynaptic and postsynaptic mechanisms of adenosine hyperexcitability

2.3.3.5.1. Presynaptic mechanisms

Data from the superior colliculus shows that the action of 100nM or 100μM adenosine, which causes an increase in the amplitude of the field postsynaptic potential in the superficial grey due to optic layer stimulation, also causes an increase basal and electrically stimulated glutamate release, blocked by the protein kinase A inhibitor HA-1004 (Hirai and Okada, 1994). In patch clamp recordings of whole-cell currents in CA_1 pyramidal neurons, application of 5μM 8-CPT on a background

concentration of 10μM adenosine causes a large increase in the EPSC compared to controls with no drugs or adenosine alone (Garaschuk *et al.* 1992); this enhancement is likely presynaptic as both the NMDA and non-NMDA components of the EPSC are similarly enhanced. Indeed, 100nM adenosine increases electrically evoked glutamate release from hippocampal slices 1.7 fold (Okada *et al.* 1992). This presynaptic hyperexcitability is different from the altered postsynaptic excitability of interest in the present study.

2.3.3.5.2. Postsynaptic mechanisms

It seems likely that the "rebound depolarisation" of hippocampal CA_1 neurons upon washout following seven minutes of 20μM adenosine (Siggins and Schubert, 1981) results from a postsynaptic change. In TTX treated hippocampal slices, synaptic transmission is blocked, and during prolonged application of 50μM adenosine depolarisation follows the initial hyperpolarisation, in 4 of 10 CA_1 neurons (Proctor and Dunwiddie, 1983). A_2 receptor-mediated excitation in the CA_1 is characterised by membrane depolarisation, increased input resistance, enhanced EPSPs, and increased spontaneous action potential frequency, in rat hippocampal CA_1 neurons (Ameri and Jurna, 1991). These actions suggest closure of a potassium channel. Although not discussed, in the CA_3 low adenosine concentrations apparently prevent the development of a slow AHP following stimulus induced bursts of spikes (Okada *et al.* 1992) figure 2A.

2.3.4. ADENOSINE RECEPTOR SECOND MESSENGER SYSTEMS

2.3.4.1. G proteins

Unlike the classic fast neurotransmitter receptors, where agonist binding and channel response are properties of the same protein complex, many receptors modulate ion channels via a guanine nucleotide-binding (G) protein, in either a membrane delimited fashion or via further intracellular second messengers. G proteins are heterotrimers composed of α, β and γ subunits, in order of decreasing mass (Gilman, 1987), that, when activated by binding of GTP, modulate diverse cellular functions. G protein heterogeneity resides largely in the α subunit, whereas β and γ subunits are similar. cDNAs that encode at least 21 distinct α subunits form four families of G proteins: G_i, G_s, G_q, G_{12} (Hille, 1992; Hepler and Gilman, 1992; Gilman, 1987). G_o, common in the brain, is a member of the G_i family, and cellular concentrations of G_i are higher than other G proteins with G_o alone being 1-2% of membrane protein in the brain (Hepler and Gilman, 1992).

The inactive α-GDP complex and the βγ complex combine forming the inactive G-GDP. Receptor-agonist interaction promotes receptor binding of the inactive G-GDP, and promotes the dissociation of GDP. The receptor has high affinity for the αβγ complex without bound guanine nucleotide, and this conformation is also the high

affinity state of the receptor for agonists. The $\alpha\beta\gamma$-receptor complex is transitory, as the nucleotide site is open and GTP binds, causing dissociation of the receptor from the two possible regulatory units, the $\beta\gamma$ complex and the α-GTP complex. Dissociation of the α-GTP complex initiates the intrinsic GTPase activity of the α subunit, which in time de-activates the G protein. See (Hille, 1992; Gilman, 1987; Taylor, 1990; Sternweis and Iok-Hou, 1990; Hepler and Gilman, 1992; Boege *et al.* 1991) for reviews.

Different α subunits with bound GTP activate a variety of effector systems. α_s stimulates adenylate cyclase, α_i inhibits adenylate cyclase, and these effects gave this nomenclature. α_i, and α_o also inhibit calcium channels and open potassium channels. α_q stimulates phospholipase C. α_i and α_o also stimulate phospholipase C and phospholipase A_2 (Hepler and Gilman, 1992; Hille, 1992). It has been suggested that the $\beta\gamma$ complex can activate phospholipase A_2 (Sternweis and Iok-Hou, 1990; Hille, 1992) and possibly some forms of adenylate cyclase (Hille, 1992), as well as directly inhibiting other forms of adenylate cyclase (Hepler and Gilman, 1992; Hille, 1992). $\beta\gamma$ complexes can also indirectly inhibit adenylate cyclase by promoting formation of the inactive $G_{s\alpha\beta\gamma}$ complex (Gilman, 1987). Receptor, G protein and effector systems are not thought to be continuously associated in the membrane, but diffuse separately, making random contacts and allowing amplification, integration, convergence and crosstalk of signals.

2.3.4.2. G protein linked adenosine receptors modulate adenylate cyclase

Modulation of cAMP levels was the first identified action of adenosine on neurons (Sattin and Rall, 1970), and it was established that adenylate cyclase is both stimulated and inhibited by different adenosine receptors (van Calker *et al.* 1979; Londos *et al.* 1980). Adenylate cyclase is modulated by G_s and G_i G proteins as described above in section 2.3.4.1, and these are linked to adenosine A_{2b} and A_1 receptors respectively (van Calker *et al.* 1979; van Calker *et al.* 1978; Fredholm *et al.* 1985).

2.3.4.3. Adenosine A_1 receptor inhibitory actions are G protein linked

Both presynaptic and postsynaptic adenosine A_1 receptors are linked to G proteins. Pertussis toxin (PTX) inhibits G_o and G_i but not G_s G proteins by irreversible ADP ribosylation of the α subunit, and the sulphydryl alkylating agent N-ethylmaleimide also inactivates G proteins with the same specificity (Fredholm *et al.* 1985). PTX or N-ethylmaleimide treatment shifts adenosine A_1 receptors from the high-affinity, G protein coupled, state to the low-affinity, G protein uncoupled, state (van der Ploeg *et al.* 1992; Allende *et al.* 1991), whereas high-affinity agonist binding to the receptor promotes tight coupling of the receptor/G protein complex, and such a coupled G protein is not a substrate for PTX (van der Ploeg *et al.* 1992). Bovine brain A_1

receptors co-purify with α_{i1}, α_o, and α_{i2} G protein subunits, as well as β36 and β35 G protein subunits, and all of these α subunits restore high affinity agonist binding to reconstituted membrane preparations (Munshi *et al.* 1991).

Inhibition of synaptic transmission by presynaptic adenosine A_1 receptors is coupled to G_i or G_o. In hippocampal slices prepared from animals following intrahippocampal PTX injection, adenosine causes no depression of CA_1 evoked postsynaptic field potentials (Stratton *et al.* 1989), and treatment of rat hippocampal slices with N-ethylmaleimide prevents R-PIA depression of electrically field stimulated release of acetylcholine, noradrenaline and glutamate. (Dunér-Engström and Fredholm, 1988; Fredholm and Lingren, 1987; Fredholm *et al.* 1986). Similarly, PTX treatment of cerebellar cultures abolishes the R-PIA inhibition of K^+ or veratridine stimulated efflux of glutamate from cerebellar neurons (Dolphin, 1985), and incubation of mouse dorsal root ganglion cell cultures with PTX prevents 2-CA inhibition of N-type calcium currents (Gross *et al.* 1989). However, in hippocampal slices prepared following intracerebroventricular PTX injection, adenosine presynaptic inhibition of acetylcholine and noradrenaline release and depression of synaptic transmission remains intact (Fredholm *et al.* 1989). These contradictory results may be the result of the limited diffusion of PTX following intracerebroventricular injection (van der Ploeg *et al.* 1991).

Postsynaptic depression of neuronal excitability by adenosine A_1 receptors is similarly coupled to a PTX sensitive G protein. In hippocampal slices prepared from rats after intraventricular injection of PTX, adenosine does not hyperpolarise or reduce input resistance in CA_1 or CA_3 pyramidal cells, and does not inhibit low calcium spontaneous spike bursts (Fredholm *et al.* 1989; Spuler and Grafe, 1989). The adenosine hyperpolarisation recorded in whole-cell patch clamp of cultured hippocampal neurons runs down unless GTP is included in the patch electrode and is abolished by including non-hydrolysable GTP analogues in the patch electrode or by prior incubation of the cultures with PTX (Trussell and Jackson, 1987). 2-CA and R-PIA elicited inward chloride current, in cultured rat CA_1 neurons, is abolished by pre-incubation of cell cultures with PTX (Mager *et al.* 1990).

2.3.4.3.1. G_o proteins mediate A_1 receptor neuronal inhibition

Adenosine A_1 receptor inhibition of adenylate cyclase via a G_i protein may be responsible for the enhancement of the slow AHP, which is modulated by cAMP accumulation (Storm, 1990; Storm, 1993). However, A_1 receptor depression of synaptic transmission and activation of a postsynaptic potassium current is not dependent on modulation of adenylate cyclase. R-PIA reduction of electrically field stimulated release of acetylcholine, noradrenaline and glutamate from rat hippocampal slices, which is sensitive to inactivation of G_o and G_i proteins with N-

ethylmaleimide, is not affected by stimulation of adenylate cyclase with forskolin or inhibition of phosphodiesterase with rolipram (Dunér-Engström and Fredholm, 1988; Fredholm and Lingren, 1987; Fredholm *et al.* 1986). In patch clamped rat striatal neurons adenosine activates a G protein linked outward potassium current, but bath application of forskolin, or cAMP in the patch electrode, does not inhibit the adenosine response (Trussell and Jackson, 1987).

A_1 and $5HT_{1A}$ receptor linked G protein activation mediates both adenylate cyclase inhibition in rat hippocampal membranes and hyperpolarisation in rat hippocampal pyramidal neurons, co-application of saturating concentrations of agonists at both receptors do not produce additive effects (Zgombick *et al.* 1989). The $5HT_{1A}$ receptor is also similarly coupled to the same potassium channels as the $GABA_B$ receptor in hippocampal neurons (Andrade *et al.* 1986). This potassium channel can be activated by G protein α_o subunits with bound GTPγS applied to the cytosolic surface of excised hippocampal neuron patches (Van Dongen *et al.* 1988). It is likely that the electrophysiological actions of adenosine A_1 receptors are G_o linked, whereas the inhibition of adenylate cyclase is characteristically G_i linked.

2.3.4.4. Adenosine A_{2a} receptor is G protein linked

Whereas A_{2b} receptors are well known to be G_s protein linked, the identity of the G protein that mediates A_{2a} receptor electrophysiological actions is unknown. Evidence suggests a G_i or G_o protein, since hippocampal slices from PTX pre-treated rats fail to show adenosine A_2 receptor-mediated excitation (Ameri, 1992).

2.3.4.5. Phospholipase C

Adenosine A_1 receptors in smooth muscle (White *et al.* 1992; Dickenson and Hill, 1993) and A_3 receptors in mast cells (Ramkumar *et al.* 1993) stimulate phospholipase C. Phospholipase stimulation can be mediated by G_i, G_o or G_q proteins (Hepler and Gilman, 1992; Hille, 1992). This may activate the A_1 receptor-mediated chloride current. Note that stimulation of phospholipase C is an early pathophysiological response to ischaemia (Katsura *et al.* 1993).

2.3.5. ADENOSINE METABOLISM AND RELEASE

2.3.5.1. Adenosine formation by 5'-nucleotidase

Adenosine can be formed from a number of sources, including hydrolysis of the α phosphate of AMP by 5'-nucleotidases or non-specific phosphatases, metabolism of cAMP then dephosphorylation of 5'-nucleotidase, and from S-adenosylhomocysteine by S-adenosylhomocysteine hydrolase. Whereas adenosine may be produced from all these pathways, the pool of adenine nucleotides involved in cellular energy metabolism could provide for the large fluctuations in adenosine that occurs under some conditions.

Extracellular AMP can be dephosphorylated by a membrane bound ecto-5'-nucleotidase, and intracellular AMP can be the substrate for at least two soluble 5'-nucleotidases. The ecto-5'-nucleotidase is inhibited by di-nucleotides, particularly α,β-methylene ADP and to a lesser extent by tri-nucleotides. Furthermore, membrane bound 5'-nucleotidase may exist in an intracellular, membrane bound, pool that recycles with the plasma membrane pool, as well as a soluble pool that has detached from its membrane anchor (Zimmermann, 1992). Whereas hydrolysis of released nucleotides by the ecto-enzyme may be responsible for a portion of the basal adenosine tone, it is apparently less important in hypoxia or ischaemia when nucleosides are released (Lloyd *et al.* 1993). The soluble 5'-nucleotidases include an AMP preferring form and an inosine-5'-monophosphate (IMP) preferring form, both of which are stimulated by nucleotides (Zimmermann, 1992). Whereas the 5'-nucleotidases of the brain are not well characterised, adenosine production by dephosphorylation of AMP has been more thoroughly examined in the rat heart and liver.

2.3.5.1.1. Heart and liver

One form of cytosolic 5'-nucleotidase characterised both in rat heart and pigeon heart (Newby, 1988; Meghji *et al.* 1988), strongly prefers IMP over AMP (Skladanowski *et al.* 1990; Newby, 1988; Truong *et al.* 1988) with an apparent K_m (K_{app}) for AMP of 1.8mM and a maximum velocity (V_{max}) of 57.3nmol/min/mg protein compared to K_{app} 0.2mM and V_{max} 245nmol/min/mg protein for IMP in the presence of 5mM ATP (Truong *et al.* 1988). This enzyme is activated by ATP, the K_{app} for AMP and IMP increasing to 6mM and 0.8mM respectively and the Vmax dropping to 13.6nmol/min/mg protein and 121nmol/min/mg protein respectively, in the absence of ATP (Truong *et al.* 1988). This 5'-nucleotidase is inhibited by 5'-deoxy-5'-isobutylthioadenosine (2-6mM) which can penetrate intact cells (Skladanowski *et al.* 1990; Newby, 1988). It is unlikely to be responsible for ischaemic adenosine production, with cytosolic AMP being too low; for instance, a myocardial free AMP concentrations of 1.25μM is calculated for ischaemia (Headrick *et al.* 1989).

The second, AMP preferring form, of rat heart cytosolic 5'-nucleotidase has a higher V_{max} for AMP of 202 nmol/min/mg protein compared to 47.3 nmol/min/mg protein for IMP, although the K_{app} for AMP is higher at 1.2mM compared to 0.8mM for IMP, measured in the presence of ATP. The rat heart AMP preferring 5'-nucleotidase is activated by ATP and ADP and inhibited by P_i, but it shows a sharp increase in activity *in vitro* as adenylate energy charge decreases from 0.9 to 0.7 with maximal activity at 0.6, and whereas overall activity is diminished, this effect is seen in the presence of physiological concentrations of P_i (Itoh *et al.* 1986; Truong *et al.* 1988).

Pigeon heart, which lacks the ecto-5'-nucleotidase (Meghji *et al.* 1988), contains a cytosolic AMP preferring 5'-nucleotidase with a K_m for AMP of 4.6-5.5mM, in the presence of ATP, prefers AMP over IMP by 15 fold, and is not inhibited by α,β-methylene ADP (Newby, 1988; Skladanowski and Newby, 1990). The IMP preferring 5'-nucletidase inhibitor 5'-deoxy-5'-isobutylthioadenosine only weakly inhibits this 5'-nucleotidase, (Newby, 1988; Skladanowski and Newby, 1990). This enzyme, however, is stimulated by ATP and ADP with high level of substrate (Newby, 1988), although at physiological concentrations of AMP (0.1mM) is only stimulated by ADP (Skladanowski and Newby, 1990). This enzyme has a apparent molecular weight of 150 kDa (Skladanowski and Newby, 1990) and may be different from the rat heart enzyme which has a molecular weight of 58 kDa (Itoh *et al.* 1986).

Adenosine may be formed in the rat liver by the action of two soluble 5'-nucleotidases, high and low K_m forms, and a membrane bound ecto-enzyme. The high K_m soluble form is specific for IMP and GMP, has low activity with AMP, and is activated by ATP and inhibited by P_i (Spychala *et al.* 1990). The low K_m soluble form is similar to the membrane bound ecto-enzyme, preferring AMP with a K_m of 12μM and a pH optimum 7.7-8.2 (Spychala *et al.* 1990; Nakamura, 1976). Also similar to the ecto-enzyme it is strongly competitively inhibited by ATP, K_i 13μM, and ADP, K_i 3μM respectively (Spychala *et al.* 1990; Nakamura, 1976) and α,β-methylene ADP, K_i 0.5μM (Spychala *et al.* 1990). This ATP induced inhibition is not substantially increased with ATP in the physiological range of 2-3mM (Spychala *et al.* 1990). With fixed ATP (3mM) and ADP (50μM) 5'-nucleotidase activity increases linearly with increased AMP 1-100μM (Spychala *et al.* 1990).

2.3.5.1.2. Brain

In rat brain several enzymes, including non-specific alkaline or acid phosphatases, can catalyse the removal of the a phosphate from AMP but only the 5'-nucleotidase is active at physiological pH (Nagata *et al.* 1984). Two different cytosolic forms and a membrane bound form of 5'-nucleotidase exist (Meghji and Newby, 1990). In subcellular fractions from whole rat brain, 5'-nucleotidase activity is located mainly in crude mitochondrial fraction, microsomal fraction and nerve end particles, all with high specific activity, and the highest specific activity is in purified synaptic membranes (Nagata *et al.* 1984). High activity of the enzyme in the rat brain is found in the basal ganglia, medulla oblongata, thalamus, and all synaptic layers of the CA_1 area of the hippocampus (Nagata *et al.* 1984; Fastbom *et al.* 1987). The membrane bound form is an ecto-5'-nucleotidase (Meghji and Newby, 1990), shown to catalyse the hydrolysis of extracellular AMP in synaptosomes (Hoehn and White, 1990b; MacDonald and White, 1985) and cortical slices (Hoehn and White, 1990a),

but is not likely to be the 5'-nucleotidase responsible for hypoxic adenosine release which is not dimished by ecto-5'-nucleotidase inhibition (Lloyd *et al.* 1993).

2.3.5.2. Adenosine release

Adenosine may be released through the equilibrative transporter, and thus extracellular adenosine accumulation is essentially a result of facilitated diffusion and reflects intracellular levels. Although adenosine is clearly transported by the nucleoside transporter, adenosine release may be mediated by additional mechanisms, including extracellular metabolism of nucleotides. Nucleotides are not freely transported across the neuronal membrane but may be co-released with true neurotransmitters as part of the vesicle structure. Basal adenosine release (Fowler, 1991; Daval and Barberis, 1981; Craig and White, 1993), glutamate stimulated adenosine release (Craig and White, 1993; Hoehn and White, 1990b), and electrically stimulated adenosine inhibition (Fowler, 1988; Mitchell *et al.* 1993; Lloyd *et al.* 1993) are not entirely blocked by nucleoside transporter inhibition. However potassium evoked adenosine release (Hoehn and White, 1990b; Daval and Barberis, 1981) and possibly hypoxic adenosine release (Meghji *et al.* 1989) are inhibited by nucleoside transporter inhibition.

2.3.5.2.1. Basal adenosine release

Basal extracellular accumulation of adenosine has been demonstrated in cortical slices (Pazzagli *et al.* 1990; Hoehn and White, 1990a), hippocampal slices (Haas and Greene, 1988; Pazzagli *et al.* 1990; Fowler, 1988; Lloyd *et al.* 1993), striatal slices (Pazzagli *et al.* 1990) pontine slices (Regenold and Illes, 1990), cortical synaptosomes (MacDonald and White, 1985), and [^{3}H]adenosine loaded hippocampal synaptosomes (Poli *et al.* 1991). Considerable evidence from the excitatory actions of adenosine receptor antagonists, and from inhibitory actions of adenosine uptake blockers, suggests a basal, inhibitory adenosine tone (Haas and Greene, 1988; Fowler, 1988). Electrophysiological evidence in hippocampal slices suggests that basal adenosine release is independent of presynaptic action potentials and calcium influx, as it continues in low calcium, high magnesium media, and in the presence of TTX (Haas and Greene, 1988; Fowler, 1988). Although the majority of basal adenosine accumulation results from extracellular hydrolysis of a nucleotide, as it is blocked by inhibition of ecto-5'-nucleotidase with α,β-methylene ADP plus GMP, in cortical synaptosomes (MacDonald and White, 1985) and cortical slices (Hoehn and White, 1990a; Craig and White, 1993), this is not the case in hippocampal slices (Lloyd *et al.* 1993). However, indirect evidence supports the release of nucleotides, even in the hippocampus, as adenosine inhibitory tone on electrical responses in hippocampal slices continues in the presence of nucleoside transporter inhibiters (Haas and Greene, 1988; Fowler, 1988), and basal adenosine

release from cortical slices, hippocampal slices, synaptosomes or dissociated brain cells is not blocked by dipyridamole or nitrobenzylthioinosine (NBTI) at concentrations which block both the passive nucleoside transporter and the sodium dependent, concentrative nucleoside transporter (Craig and White, 1993; Johnson and Geiger, 1989; Fowler, 1991; Daval and Barberis, 1981; Dunwiddie and Diao, 1994). This latter could also be due to adenosine release via a mechanism other than the nucleoside transporter.

2.3.5.2.1.1. Basal adenosine levels

Extracellular adenosine measured by microdialysis *in vivo* is 340nM in the rat striatum (Zetterström *et al.* 1982), 130nM in the rat caudate nucleus (Van Wylen *et al.* 1986) and 500nM in the gerbil hippocampus (Dux *et al.* 1990). Basal adenosine in the hippocampal slice estimated from the increase in fEPSP by adenosine antagonists and the EC_{50} for adenosine is in the range 150-200nM (Dunwiddie and Diao, 1994). Although it has been argued that slice preparation or insertion of a microdialysis probe *in vivo* causes cell damage and may release adenosine, the excitatory actions of methylxanthine adenosine antagonists *in vivo* argue strongly in favour of a basal adenosine release and tonic adenosinergic tone. For instance i.p. 8-CPT produces hyperactivity and aggression in rats (Boissard *et al.* 1992), and most readers are probably familiar with the stimulant properties of caffeine.

2.3.5.2.2. Adenosine accumulation secondary to presynaptic nucleotide release

A component of evoked extracellular accumulation of adenosine is dependent on depolarisation of presynaptic terminals and calcium entry, and requires action of the ecto-5'-nucleotidase. Presumably vesicular ATP is released with other neurotransmitters, and is hydrolysed extracellularly to adenosine. Electrically stimulated release of adenosine from cortical, hippocampal and striatal slices and primary striatal cultured neurons, presumably as a result activation of neurons and action potential invasion of axon terminals, is blocked by TTX or calcium free medium (Pazzagli *et al.* 1990; Caciagli *et al.* 1985). Calcium free medium, and to some extent TTX, blocks potassium evoked release of adenosine from cortical and hippocampal synaptosomes (Poli *et al.* 1991; MacDonald and White, 1985). A portion of potassium evoked extrasynaptosomal adenosine accumulation is due to nucleotide release as it is blocked by inhibition of ecto-5'-nucleotidase by α,β-methylene ADP plus GMP (MacDonald and White, 1985). A fraction of total adenosine release from cortical slices by excitatory amino acids or potassium depolarisation is also blocked by TTX or calcium free medium (Hoehn and White, 1990c; Hoehn and White, 1990a; Craig and White, 1993; Hoehn and White, 1989). NMDA receptor evoked extracellular adenosine accumulation in cortical slices is apparently due to presynaptic release of a nucleotide, as it is largely blocked by

inhibition of ecto-5'-nucleotidase with α,β-methylene ADP plus GMP or in calcium free medium (Craig and White, 1993). However, whereas both NMDA and AMPA receptor agonists should produce soma depolarisation, AMPA receptor evoked adenosine release from cortical slices is not inhibited by calcium free medium or inhibition of ecto-5'-nucleotidase with α,β-methylene ADP plus GMP (Hoehn and White, 1990a; Craig and White, 1993).

Some excitatory amino acid-stimulated release of adenosine from synaptosomes is not mediated by action potentials, calcium influx, or ionotropic receptors. Neither NMDA nor non-NMDA receptor antagonists block glutamate evoked release of endogenous adenosine from cortical synaptosomes (Hoehn and White, 1990b) or [^{3}H]adenosine from hippocampal synaptosomes (Poli *et al.* 1991). Similarly, the glutamate agonists NMDA, kainate and quisqualate do not release [^{3}H]adenosine from rat hippocampal synaptosomes (Poli *et al.* 1991). L-glutamate, D-glutamate, D-aspartate, L-aspartate and GABA can cause adenosine release from cortical synaptosomes and [^{3}H]adenosine release from hippocampal synaptosomes (Poli *et al.* 1991; Hoehn and White, 1990b). Potency of glutamate and aspartate stereoisomers at stimulating adenosine release from synaptosomes is paralleled by their affinity for the glutamate uptake site, and is blocked by the glutamate uptake inhibitor dihydrokainate (Hoehn and White, 1990b). Glutamate stimulated extrasynaptosomal accumulation of adenosine apparently involves the extracellular hydrolysis of a nucleotide as it is blocked by α,β-methylene ADP plus GMP, and is not blocked by the nucleoside transporter inhibitor dipyridamole 120μM (Hoehn and White, 1990b).

2.3.5.2.3. Adenosine release with depolarisation

In addition to the calcium dependent extracellular accumulation of adenosine from nucleotides, there is also a calcium independent, TTX insensitive release of adenosine with potassium or veratridine depolarisation (Poli *et al.* 1991; Hoehn and White, 1990b; MacDonald and White, 1985; Hoehn and White, 1990a). Such adenosine release is via the nucleoside transporter, as it is blocked by dipyridamole but not by ecto-5'-nucleotidase inhibition (Hoehn and White, 1990b; Hoehn and White, 1990a). Excitatory amino acid evoked adenosine release from cortical slices and hippocampal slices is mediated by NMDA, AMPA and probably metabotropic receptors (Hoehn and White, 1989; Hoehn and White, 1990a; Hoehn and White, 1990c; Pedata *et al.* 1991). Whereas NMDA evoked extracellular adenosine accumulation is due to presynaptic release of nucleotides (Craig and White, 1993), activation of other glutamate receptors results in nucleoside release, as inhibition of ecto-5'-nucleotidase does not inhibit glutamate, kainate, or AMPA evoked adenosine accumulation (Hoehn and White, 1990a; Craig and White, 1993). Total adenosine released by excitatory amino acids from cortical slices has been shown to be only

partially independent of extracellular calcium, and is only partially blocked by TTX (Hoehn and White, 1990c; Hoehn and White, 1990a; Craig and White, 1993). This suggests that depolarisation from glutamate receptor activation results in adenosine release by mechanism similar to those of potassium or veratridine. This may be due to the increase ATP hydrolysis by ion translocating ATPase during repolarisation, as is seen in synaptosomes with veratridine or potassium depolarisation (Kauppinen and Nicholls, 1986). Such oxidative stress can result in increased production of cytosolic adenosine which is released by facilitated diffusion, see for instance section 2.3.5.2.4.

2.3.5.2.4. Adenosine release during hypoxia

In paralysed, artificially ventilated rats, brain tissue adenosine levels rise from 0.34nmol/g to 1.65nmol/g upon ventilation with 100% nitrogen for 30 seconds, but, although mean arterial blood pressure remains constant at 145mmHg for 15 seconds, it falls to 90mmHg by 30 seconds (Winn *et al.* 1981). With a similar experimental design, 5 minutes P_{a0_2} of 30mmHg with steady blood pressure, also causes a rise of brain adenosine from 0.38nmol/g to 2.73nmol/g (Winn *et al.* 1981). During hypoxia, extracellular adenosine, measured by microdialysis in anaesthetised rats, is elevated three fold from 0.34μM in the striatum and 10 fold from 0.13μM in the caudate nucleus, hypoxia being accompanied by profound hypotension in the study where this was measured (Zetterström *et al.* 1982; Van Wylen *et al.* 1986). Adenosine concentration in the fourth ventricle cerebrospinal fluid of unanaesthetised rats increases from 58.1nM to 95.1nM during 25 minutes of breathing 5% O2 and is still elevated one and three hours following hypoxia, 226.7 and 130.5nM respectively (Barraco *et al.* 1991). In gerbils, whole brain tissue adenosine levels, and interstitial adenosine recovered by microdialysis in the hippocampus and striatum, rise 20-100 fold during cerebral ischaemia (Morimoto *et al.* 1982; Dux *et al.* 1990).

Tissue adenosine levels in hippocampal slices are increased during preparation, which involves brief ischaemia, and also rise in the incubation bath during a hypoxic insult (Fredholm *et al.* 1984). Adenosine collected in the perfusate of hippocampal slices is a minor component of basal purine release but increases dramatically with ischaemia (Lloyd and Fredholm, 1990). ACSF adenosine levels increase in static bath ACSF during hypoxia in hippocampal slices (Fowler, 1991). Ischaemia increases release of adenosine and hypoxanthine from hippocampal slices in the presence of the adenosine deaminase inhibitor EHNA and the ecto-5'-nucleotidase inhibitor α,β-methylene ADP, demonstrating an increase cytosolic production of both nucleosides via hydrolysis of AMP and IMP respectively, and subsequent release (Lloyd *et al.* 1993).

2.3.5.3. Adenosine action is terminated by uptake and catabolism

Inactivation of adenosine requires uptake into cells as the enzymes that promote adenosine catabolism are primarily cytosolic (Meghji and Newby, 1990). Nucleotide uptake in rat brain occurs via an equilibrative transporter and a sodium-dependent active transporter (Plagemann *et al.* 1988; Jarvis, 1987; Johnson and Geiger, 1989). Although NBTI and dipyridamole preferentially block the high affinity, active and low affinity, equilibrative transporters respectively in some systems, the rat brain is comparably insensitive to these inhibitors (Van Belle, 1988; Verma and Marangos, 1985). However, in hippocampal neuron or glial cell cultures, adenosine uptake by either transporter is blocked non-selectively by dipyridamole and NBTI in micromolar concentrations (Ohkubo *et al.* 1991). Such adenosine uptake blockade by NBTI or dipyridamole increases extracellular accumulation of adenosine in rat cortical slices (Craig and White, 1993), hippocampal slices (Fredholm *et al.* 1994; Fowler, 1991), cortical synaptosomes (Daval and Barberis, 1981) and dissociated brain cells (Johnson and Geiger, 1989). Nucleoside transporter inhibitors increase endogenous adenosine induced inhibitory tone on electrical activity in the hippocampal slice (Haas and Greene, 1988; Mitchell *et al.* 1993; Fowler, 1988; Dunwiddie and Diao, 1994).

Most adenosine uptake in the brain is via the equilibrative transporter (Ohkubo *et al.* 1991; Dunwiddie and Diao, 1994), where this low affinity uptake is not saturable and has an apparent K_T of 4μM (Gu and Geiger, 1992). Since the primary nucleoside transporter is equilibrative, net uptake of adenosine is dependent on subsequent intracellular metabolism. In rat cerebral cortical synaptosomes, if adenosine deaminase and adenosine kinase are inhibited, net accumulation of [^{3}H]adenosine does not occur, rather intra- and extrasynaptosomal concentrations equilibrate within 60s and remain stable (Gu and Geiger, 1992). However, with active adenosine deaminase and adenosine kinase, radio-labelled purine nucleotides equilibrate with excess extracellular [^{3}H]adenosine by 15 seconds, and net accumulation of radio-labelled adenosine and purine nucleotides occur with continued incubation (Gu and Geiger, 1992).

Deamination of adenosine produces the inactive nucleoside inosine. Exogenous adenosine deaminase inactivates adenosine and relieves adenosine tone on synaptic transmission and excitability in the hippocampus (Dunwiddie and Hoffer, 1980); however, the hippocampus has the lowest endogenous levels of this enzyme in the brain (Nagy *et al.* 1990). Indeed adenosine deaminase is probably not important in adenosine inactivation, as the inhibitor erythro-9-(-2-hydroxy-3-nonyl)adenosine (EHNA) does not enhance field potential depression due to endogenous adenosine in the hippocampal slice (Dunwiddie and Diao, 1994), and does not prevent post-ischaemic ATP salvage in hepatocytes (Bontemps *et al.* 1993). Inosine and

hypoxanthine production account for less than 5% adenosine catabolism in cortical synaptosomes (Gu and Geiger, 1992). However, the adenosine deaminase inhibitor deoxcoformycin does increase normoxic and hypoxic adenosine accumulation in hepatocytes (Bontemps *et al.* 1993).

Adenosine can be phosphorylated to AMP by adenosine kinase. Blockade of adenosine kinase with iodotubercidin increases extracellular adenosine accumulation in hippocampal slices (Lloyd and Fredholm, 1990). Iodotubercidin prevents adenosine removal upon reoxygenation of hypoxic hepatocytes (Bontemps *et al.* 1993). Adenosine uptake in cortical synaptosomes primarily results in 5'-AMP accumulation (Gu and Geiger, 1992).

2.4. HYPOXIA

2.4.1. HYPOXIA AND ISCHAEMIA

Two insults with similar, but not identical, results are discussed in the literature: hypoxia, reduced oxygen supply to the tissue, and ischaemia, reduced blood flow to the tissue. Ischaemia has received far more attention than hypoxia *in vivo*, where the two insults may not be discrete. Forebrain ischaemia is usually produced *in vivo* by arterial blood vessel occlusion, occasionally accompanied by reduced inspired oxygen concentration or arterial hypotension. The four vessel-occlusion model in the rat, involves permanent occlusion of the vertebral arteries followed, upon recovery from surgery, with transient occlusion of the common carotid arteries; the ischaemia usually being verified by an isoelectric electroencephalogram (Pulsinelli and Brierley, 1979). In the gerbil, forebrain ischaemia can be produced by bilateral carotid occlusion alone, since these vessels do not communicate with the vertebrals, but in the rat, carotid occlusion alone must be accompanied by severe hypotension to produce forebrain ischaemia. Hypoxia can be achieved by reducing the inspired oxygen concentration, but in anaesthetised animals blood pressure may not always be maintained, resulting in some degree of ischaemia.

In vitro the supply of oxygen and glucose to isolated tissue preparations can be easily regulated. For instance, in brain slice preparations, tissue nutrient requirements may be supplied by diffusion from a superfused artificial cerebrospinal fluid. In such a preparation, hypoxia is achieved by dissolving nitrogen rather than oxygen in the superfusate, and 'ischaemia' is modelled by the additional omission of glucose. These *in vitro* insults are not identical with their *in vivo* correlates, in particular, *in vitro* ischaemia is far less complex than *in vivo,* where metabolites build up in the tissues due to the reduced blood flow. *In vitro* simulation of ischaemia by reducing the flow of superfusate or by temporary incubation of the tissue in a small static bath is rarely used, primarily because excised brain tissue nutrient requirements are met

by small static volumes on the order of the tissue volume (McIlwain and Bachelard, 1971).

In the present review, the terms ischaemia or hypoxia will be applied to the common *in vivo* or *in vitro* insults described above, with the following additional proviso. In the hippocampal slice, use of physiological levels of glucose 4mM result in less stable responses both under control and hypoxic conditions, and 10mM glucose is more commonly used. Hypoxic exposure in 4mM glucose or less produces metabolic and physiological responses similar to ischaemia, see section 2.4.5.4, and will thus be considered ischaemic in the present work.

2.4.2. ENERGY METABOLISM DURING HYPOXIA, ISCHAEMIA AND HYPOGLYCAEMIA

2.4.2.1. Mitochondrial respiration

The usual substrate for brain metabolism is glucose. Pyruvate, produced by glycolysis, is metabolised by the enzymes of the Krebs cycle, with free energy conserved in the reduction of NAD^+ and FAD^+. Electrons from NADH and $FADH_2$ are shuttled into the mitochondrial matrix, where they are transported by a number of flavoproteins, cytochromes, non-haem iron and copper proteins, to the final electron acceptor oxygen. Oxidation/reduction of the electron carriers of the inner mitochondrial membrane is accompanied by the release of free energy. At three sites along this electron transport chain, energy is conserved by pumping of protons across the inner mitochondrial membrane, the resulting pH gradient and membrane potential ($\Delta\psi_m$) of 150mV form an electrochemical gradient ($\Delta\mu_{H^+}$). This electrochemical gradient is the common high energy intermediate that drives a number of mitochondrial reactions (Mitchell, 1976), including ATP synthesis via the ATP synthetase, a reversible H^+ ATPase, as well as calcium accumulation into the mitochondrial matrix via a carrier protein, and matrix ATP export via a ADP^{3-}/ATP^{4-} electrotonic exchanger (Hanstein, 1976).

2.4.2.2. Glycolysis

Under normal steady state conditions *in vivo*, glucose is metabolised to pyruvate, most of which is oxidised aerobically in the mitochondrial Kreb's cycle, while approximately 13% of pyruvate produced is converted to lactate and removed in the circulation. The glycolytic rate is controlled by key enzymes, the first, hexokinase, has a higher affinity for glucose, K_m 40μM, than the blood brain barrier glucose transporter (McIlwain and Bachelard, 1971). Glycolytic rate is measured at 0.3 to 0.9 mmoles/kg/min in various species; in the rat, values range from 0.3 mmole/kg/min, in cortical slices, to 0.79±3 mmoles/kg/min in hippocampal slices (Clarke *et al.* 1989; Balázs, 1970; Lowry *et al.* 1964).

In vivo, the brain is an obligate user of glucose. Although glucose uptake by the brain cells themselves is rapid, glucose crosses the blood brain barrier by a specific transporter with a K_m of 7 to 8mM (Clarke *et al.* 1989). Arterial glucose concentration is normally 4 to 5mM, and is lower than the K_m of the glucose transporter. Therefore, brain glucose levels vary directly with the arterial concentration, and glucose level is 1.5mmoles/kg in rodent brain. The brain contains a small supply of glycogen which is continually turned over; in the absence of external glucose, brain glycogen supply could maintain the normal glycolytic flux for some 5 minutes (Clarke *et al.* 1989). During ketosis, the brain can utilise acetoacetate and β-hydroxybutyrate produced by the liver; this ketone body use is passive and reflects blood levels (McIlwain and Bachelard, 1971; Sokoloff, 1989). The brain can also oxidise fats, but these are not abundant in the brain (McIlwain and Bachelard, 1971), and neither fats nor ketone bodies can supply cerebral energy demands in the absence of glucose (McIlwain and Bachelard, 1971; Sokoloff, 1989). Mannose is the only substrate that can successfully replace glucose and maintain brain function *in vivo* (Sokoloff, 1989; McIlwain and Bachelard, 1971; Clarke *et al.* 1989).

Hippocampal slices require at least 2-3mM glucose in the ACSF to support normal electrical function (Fan *et al.* 1988; Schurr *et al.* 1986b), and 2-3mM glucose supports maximal respiration (McIlwain and Bachelard, 1971). In brain slices there is no blood brain barrier to reduce glucose transport into the cells and this process is governed by diffusion of glucose into the slice; it has been calculated that, at the centre of slices 400μm thick, the glucose concentration would be greater than 95% of perfusate glucose concentration (Newman *et al.* 1988a), and indeed uptake of ^{14}C-2-deoxyglucose into the cells is constant throughout slices of this thickness (Newman *et al.* 1988b). Brain slices, devoid of a blood brain barrier, are not obligate users of glucose, and can take up and oxidise lactate, pyruvate, fructose 1,6-bisphosphate, acetate, β-hydroxybutyrate and acetoacetate (Sokoloff, 1989). Ketone bodies and fats will be oxidised, but in the absence of glucose these supply insufficient energy and will not support electrical activity (McIlwain and Bachelard, 1971). Lactate will support electrical activity in brain slices in the absence of glucose, and like glucose must be in excess of 2mM (Schurr *et al.* 1989); however, 10mM lactate is required to support maximal respiration (McIlwain and Bachelard, 1971). Lactate production is initially rapid after brain slice preparation, 50μmoles/g/hr, and lactate levels rise to 3mM after which they stabilise, as above this concentration lactate is used as substrate even in the presence of glucose (McIlwain and Bachelard, 1971).

During hypoxia, oxidative phosphorylation of pyruvate must cease and there is a concomitant increase in the glycolytic flux and lactate production, the Pasteur effect.

Activation of phosphofructokinase the key regulatory enzyme of glycolysis accounts for this effect. Phosphofructokinase is inhibited by ATP, Mg^{2+} and citrate, and is stimulated by NH^{4+}, K^{+}, PO^{4-}, ADP, 5'-AMP, fructose-1,6-bisphosphate and fructose-6-phosphate. During steady state conditions the enzyme is predominantly under the influence of the inhibitors, provided the stimulator concentrations are low. With hypoxia the enzyme is disinhibited by the accumulation of AMP, ADP, and PO_4^{3-} (Clarke *et al.* 1989; Balázs, 1970). Under anaerobic conditions, in brain slices, lactate production increases to 100μmoles/g/hr (McIlwain and Bachelard, 1971). Ischaemia produced by decapitation of adult unanaesthetised mice results in an increase in glycolytic flux of at least 7.4 fold from 0.76mmoles/kg/min, with glucose dropping from 1.5mmol/kg to near zero in 30 seconds, glycogen and ATP dropping to near zero in 60 seconds, and lactate rising steadily over 2 minutes (Lowry *et al.* 1964).

2.4.2.3. Enzymes catalysing nucleotide metabolism

A variety of ATPase enzymes catalyse the removal of the γ phosphate of ATP to drive energy requiring processes. In neurons, maintenance of ionic gradients by membrane Na^{+}/K^{+}-ATPase and Ca^{2+}-ATPase accounts for 50-60% of ATP utilisation (Ereciñska and Silver, 1989; Scott and Nicholls, 1980). A limited amount of ATP can be reformed from ADP, independent of mitochondrial oxidative phosphorylation, through the action of cytosolic creatine kinase which catalyses the transfer of phosphate from phosphocreatine to ADP (Ereciñska and Silver, 1989; Wallimann *et al.* 1992).

$$ADP + PCr + H^{+} \leftrightarrow ATP + Cr$$

The apparent equilibrium constant for this reaction is large, favouring ATP production, thus phosphocreatine must fall considerably before ADP levels rise. Phosphocreatine acts as an energy store, maintains ATP/ADP ratios in a range where they act as regulators of various cellular functions, prevents loss of nucleotides through adenylate kinase and 5'-nucleotidases, and buffers proton production during rapid ATP hydrolysis (Wallimann *et al.* 1992).

Adenylate kinase, which catalyses the conversion of two molecules of ADP to ATP and AMP (Criss and Pradman, 1978), is a salvage pathway that maintains ATP levels when ATP catabolism is high, and is the major pathway for the formation of AMP. Adenylate kinase is unusual in having an equilibrium constant close to unity, allowing the direction of catalysis to be determined by nucleotide concentration. IMP can be formed by the deamination of AMP by AMP deaminase, the rat skeletal muscle form of this enzyme is at half maximal velocity at 0.4-0.5mM AMP (Coffee, 1978).

2.4.2.4. Hypoxic nucleotide metabolism

In the absence of oxygen, mitochondrial oxidative phosphorylation, which provides the majority of ATP during aerobic metabolism of glucose, does not occur. Nevertheless, energy use continues. Hypoxia alone is little studied *in vivo* whereas ischaemia has drawn considerable attention.

In vivo levels of brain labile energy metabolites are similar across mammalian species (Ereciñska and Silver, 1989). The following range of values are reported, in mmol/kg wet weight: phosphocreatine 3.74-6.15; ATP 2.30-3.1; ADP 0.21-0.69; AMP 0.01-0.13 (Ereciñska and Silver, 1989; Katsura *et al.* 1993; Ekholm *et al.* 1992). Whereas total levels of ATP and phosphocreatine approximate free cytosolic levels of these compounds, much of the total ADP and AMP are bound to cellular substituents, and not available for general energy consuming reactions (Ereciñska and Silver, 1989). Free cytosolic AMP and ADP can be estimated from ATP, creatine and phosphocreatine (PCr) from the following equations (Ereciñska and Silver, 1989; Ekholm *et al.* 1992):

$$[\text{fADP}] = \frac{[\text{ATP}] \cdot [\text{Cr}]}{[\text{PCr}] \cdot [\text{H}^+] \cdot K_{CK}} \quad \text{and} \quad [\text{fAMP}] = \frac{[\text{fADP}]^2}{[\text{ATP}] \cdot K_{AK}}$$

Under normoxic conditions, cytosolic fADP is calculated as 0.026 mmol/kg and fAMP as 0.24 μmol/kg (Ekholm *et al.* 1992).

During cerebral ischaemia in the rat (cardiac arrest) and mouse (decapitation), ATP is maintained above 90% of aerobic baseline levels at the expense of phosphocreatine for the first 30 seconds, by which time phosphocreatine has declined to 30% of baseline. ATP declines slowly, being 85% of baseline by 45 seconds and 65% of baseline by 60 seconds, and only drops precipitously to about 25% of aerobic levels at 70-80 seconds, coincident with hypoxic spreading depression (see section 2.4.7.2) (Katsura *et al.* 1993; Ekholm *et al.* 1992; Lowry *et al.* 1964). Total ADP and AMP remain low until 45 seconds of ischaemia, at which time they are increased to 2-3 fold normoxic levels, AMP levels continue to rise, reaching 10-20 fold normoxic levels (Ekholm *et al.* 1992; Lowry *et al.* 1964; Katsura *et al.* 1993). Cytosolic free di- and mono-nucleotides, estimated from ATP and creatine/phosphocreatine levels, rise more quickly. Free ADP doubles by 15 seconds ischaemia and continues to rise to 4 fold pre-ischaemic levels (Ekholm *et al.* 1992). Free AMP increases four fold at 15 seconds ischaemia, 15 fold at 30 seconds, and peaks at 40 fold control levels at 60 seconds ischaemia, before declining to 10 fold control levels after spreading depression (Ekholm *et al.* 1992). Lactate accumulates steadily during ischaemia (Lowry *et al.* 1964; Ekholm *et al.* 1992; Katsura *et al.* 1993). The accompanying accumulation in protons causes an intracellular acidification of about 0.5 pH units (Ekholm *et al.* 1992; Whittingham *et al.* 1984; Kauppinen and Williams, 1990).

In rat and guinea pig hippocampal slices at 37°C, ATP levels are reported over a range of 9 to 29 mmol/kg protein (Okada and Yoneda, 1983; Whittingham *et al.* 1984; Fredholm *et al.* 1984; Yoneda and Okada, 1989; Lipton and Whittingham, 1982) and, similar to that *in vivo*, 4 to 5 mmol/kg wet weight (Kass and Lipton, 1989). Phosphocreatine levels are reported from 27.6 to 56 mmol/kg protein (Lipton and Whittingham, 1982; Whittingham *et al.* 1984; Yoneda and Okada, 1989; Okada and Yoneda, 1983). In the slice, as *in vivo*, ATP is initially preserved at the expense of phosphocreatine during ischaemia or hypoxia. In guinea pig hippocampal slices prepared and incubated under ischaemic conditions following decapitation, the changes in labile metabolites show a similar time course to those *in vivo*, at 2 minutes ATP but has dropped precipitously to 30% of control, phosphocreatine has dropped to 5% and both total ADP and lactate have risen nine fold (Whittingham *et al.* 1984).

With ischaemic superfusion at 37°C *in vitro*, metabolite changes are slower, due to a slow drop in oxygen in a superfusion system compared to the rapid depletion of oxygen with decapitation. ATP is maintained above 75% of control for the first 2 minutes of ischaemic superfusion and drops to about 65% by 5 minutes (Yoneda and Okada, 1989). Phosphocreatine drops to 60% of control by two minutes and 30% by 5 minutes (Yoneda and Okada, 1989; Lipton and Whittingham, 1982). Hypoxic superfusion with 4mM glucose is similar to ischaemia, ATP is maintained near 90% of control for 2 minutes, while phosphocreatine is rapidly depleted to 60%, ATP drops to 60-85% at 5 minutes, and continues to fall (Kass and Lipton, 1989; Wallis *et al.* 1990; Lipton and Whittingham, 1982). Hypoxia in the presence of the more usual 10mM glucose promotes a much slower depletion of ATP, showing an initial drop in the first 5 minutes but is maintained near 85% of control for 30 minutes while phosphocreatine is depleted to near 40% (Fredholm *et al.* 1984; Yoneda and Okada, 1989). Concomitant with the initial drop in ATP is a rise in total ADP and AMP (Fredholm *et al.* 1984). Normoxic adenylate energy charge (ATP + 0.5(ADP))/(ATP + ADP + AMP) in the rat hippocampal slice is reported to range from 0.79 to 0.9 (Cunha *et al.* 1994b; Fredholm *et al.* 1984), this drops as low as 0.34 immediately after slice preparation, but normalises during normoxic incubation due to phosphorylation and loss of nucleotides to nucleosides (Fredholm *et al.* 1984). Hypoxic superfusion drops the adenylate energy charge to 0.8 (Fredholm *et al.* 1984). These energy charges are based on total nucleotides and may not be an accurate representation of the free energy of the cytosolic adenylate pool due to bound mono- and di-nucleotides. During 30 minutes hypoxia in the neocortical slice, nuclear magnetic resonance indicates that ATP is relatively preserved for the first 10 minutes, and declines only slowly thereafter; the earliest

signs are rise in lactate, drop in pH, and a drop in phosphocreatine (Kauppinen and Williams, 1990).

2.4.3. HYPOXIC RELEASE OF AMINO ACID TRANSMITTERS

Hypoxia and ischaemia promote a large extracellular accumulation of amino acid transmitters. Excitatory amino acid induced toxicity (excitotoxicity), appears identical with hypoxic or ischaemic neuronal death, and excitatory amino acid receptor antagonists ameliorate hypoxic/ischaemic neuronal damage. These facts have established the excitotoxic hypothesis of hypoxia, where excessive activation of glutamate receptors and consequent pathological increase in intracellular calcium concentration is considered responsible for hypoxic cell death (Rothman and Olney, 1986; Choi, 1988; Choi, 1990).

Cerebral ischaemia in the rat increases extracellular glutamate (2 to 100 fold) and aspartate (3 to 10 fold), recovered by cortical cup (Simpson *et al.* 1992) or by microdialysis in the hippocampus (Lekieffre *et al.* 1991; Heron *et al.* 1993; Benveniste *et al.* 1984). Ischaemic superfusion increases basal and potassium evoked glutamate and aspartate release from rat and gerbil hippocampal slices (Ikeda *et al.* 1989; Pellegrini-Giampietro *et al.* 1990; Mitani *et al.* 1991), and from cultured hippocampal neurons and astrocytes (Ogata *et al.* 1992).

To what extent *in vivo* ischaemic glutamate release represents exocytotic release of excitatory amino acids is unclear. Microdialysis with calcium free medium has been variously shown to allow ischaemic glutamate release in gerbil brain (Ikeda *et al.* 1989) and prevent ischaemic glutamate release in rat brain (Drejer *et al.* 1985). In hippocampal slices, basal excitatory amino acid release by ischaemia is not calcium dependent, but enhanced potassium evoked glutamate release during ischaemia is entirely calcium dependent (Ikeda *et al.* 1989; Pellegrini-Giampietro *et al.* 1990). Enhanced potassium evoked release of glutamate during ischaemia is calcium dependent in cultured cerebellar granule cells but not in cultured cerebellar astrocytes (Drejer *et al.* 1985); and in the hippocampus, astrocytes account for the majority of glutamate release during ischaemia (Ogata *et al.* 1992). Thus, *in vitro*, ischaemic glutamate and aspartate release appears to be largely from non-transmitter pools, and may be due to reversal of the glutamate uptake mechanism. Glutamate is transported into cells along with 2 sodium ions and the extrusion of a potassium and an hydroxide ion, this ion co-transport may be reversed when ATP is depleted and membrane ion gradients run down (Attwell *et al.* 1993).

It is possible, however, that excitatory amino acids are released from neurons during hypoxia, as such release only occurs with depolarisation during hypoxia. Hypoxia alone does not increase excitatory amino acid release from cultured hippocampal

astrocytes, or from rat hippocampal slices, but does greatly increase the potassium stimulated release of [^{3}H]aspartate and endogenous aspartate (2 to 14 fold) as well as glutamate (15 to 40 fold) from hippocampal slices (Ogata *et al.* 1992; Pellegrini-Giampietro *et al.* 1990).

2.4.4. EXCITOTOXICITY

The primary excitatory neurotransmitter in the brain is the excitatory amino acid glutamate; however, glutamate and its analogues that potently depolarise neuronal membranes are, in excess, potent neurotoxins. The possibility that a pathological increase in synaptic glutamate release can cause neurotoxic effects has fostered the concept of excitotoxicity. Excitatory amino acids can induce neuronal loss by systemic (Olney, 1969) or parenchymal (McBean and Roberts, 1984; Köhler and Schwarcz, 1981) administration *in vivo*, as well as in cortical cell cultures (Frandsen *et al.* 1989; Choi, 1987; Choi *et al.* 1987), in hippocampal cell cultures (Rothman *et al.* 1987; Rothman, 1985) and hippocampal slices (Siman and Card, 1988). Cultures containing astroglial cells are more resistant to glutamate toxicity, presumably due to uptake of the amino acid by the glial cells (Rosenberg and Aizenman, 1989). Similarly, slices, although susceptible to acute damage (Siman and Card, 1988), may be less sensitive to delayed neuronal death. Glutamate neurotoxicity is characterised by an acute phase involving sodium and chloride influx causing rapid but short lived neuronal swelling, and a delayed phase characterised by elevated cytosolic calcium.

2.4.4.1. Acute neuronal damage

Excessive depolarisation causes acute swelling in rat hippocampal cultures (Rothman *et al.* 1987; Rothman, 1985) and mouse cortical cultures (Koh and Choi, 1991; Choi, 1987; Choi, 1985; Koh *et al.* 1990) that results in acute neurological damage. Large amounts of sodium entering neurons, in excess of potassium efflux, must be accompanied by an anion such as chloride to maintain electrical neutrality, and the accumulated sodium and chloride are accompanied osmotically by water, causing cell swelling (Siesjö, 1990). In mouse cortical cultures, five minute exposure to 500μM glutamate, kainate, quisqualate or NMDA but not AMPA produces acute swelling (Koh and Choi, 1991; Koh *et al.* 1990), and a combination of both NMDA and non-NMDA antagonists is required to prevent glutamate-induced neuronal swelling (Koh and Choi, 1991). Massive depolarisation with 100mM potassium can also induce acute neuronal swelling in mouse cortical cultures (Choi, 1987). Cultured mouse cortical neurons can survive acute swelling in calcium free medium and remain stable for days (Choi, 1987; Choi, 1985). However, the hippocampus is particularly sensitive to this acute toxicity, and cell swelling, as a result of sustained glutamate receptor activation, can cause rapid neuronal disintegration in hippocampal cultures (Rothman, 1985) and slices (Siman and Card, 1988). In the

hippocampal slice, cell damage is usually restricted to the soma and dendrites where excitatory amino acid receptor density is highest, whereas axons and glia are spared (Siman and Card, 1988).

2.4.4.2. Delayed neuronal damage

Acute osmotic damage requires massive or sustained receptor activation (Siman and Card, 1988; Koh and Choi, 1991; Rothman, 1985), and may be less important pathologically than the increase of cytosolic calcium that accompanies even a lower level of receptor activation. Excitatory amino acids can elevate intracellular calcium by calcium entry through the NMDA ionophore, by depolarisation and activation of voltage-activated calcium channels, and by release of calcium from intracellular stores. Glutamate-induced elevation of cytosolic calcium can activate calcium-dependent membrane-located proteases with cytoskeleton substrates (Mellgren, 1987), and excess activation may cause membrane damage that may be the mechanism of delayed neuronal death (Choi, 1988).

Both intracellular calcium stores and extracellular calcium contribute to excitotoxic cytosolic calcium overload and delayed neuronal death. Prolonged exposure to low concentrations of glutamate, kainate, quisqualate, or AMPA, will cause delayed neuronal death in murine cortical cultures (Frandsen and Schousboe, 1991a; Koh and Choi, 1991; Koh *et al.* 1990) that is largely a result of release of calcium from intracellular stores. Dantrolene, which prevents release of calcium from calcium-sensitive intracellular stores (Thayer *et al.* 1988), completely blocks hypoxic excitotoxicity in mouse cortical neuron cultures (Frandsen and Schousboe, 1991a; Frandsen and Schousboe, 1991b). In calcium free medium, dantrolene, completely blocks the glutamate-induced rise in cytosolic calcium in murine cortical neuron cultures (Frandsen and Schousboe, 1991a,b). In the presence of extracellular calcium, dantrolene greatly reduces the rise in cytosolic calcium in mouse cortical neuron cultures exposed to glutamate, NMDA and quisqualate (Frandsen and Schousboe, 1991a,b).

Entry of extracellular calcium during brief, large exposures to excitatory amino acids also produces delayed neuronal death. Removal of calcium from the medium of murine cortical cultures, or rat hippocampal cultures, reduces the extent of delayed neuronal death due to excitatory amino acid exposure despite acute swelling (Choi, 1987; Choi, 1985; Rothman *et al.* 1987). If acute swelling of culture mouse cortical neurons due to glutamate exposure is blocked by the replacement of extracellular sodium or chloride, but not calcium, by impermeant ions, delayed neuronal death still occurs (Choi, 1987; Choi, 1985). AMPA and kainate, which do not release calcium from intracellular stores, will produce excitotoxicity that is critically

dependent of extracellular calcium and is not blocked by dantrolene (Frandsen and Schousboe, 1991b).

2.4.4.3. Glutamate receptors mediate excitotoxicity

Both NMDA and non-NMDA receptors are involved in delayed glutamate toxicity. The AMPA receptor antagonists CNQX and DNQX protect cortical cell cultures from kainate, quisqualate or AMPA toxicity, but not from NMDA, and conversely, the NMDA receptor antagonists AP5, AP7, ketamine or HA-966, protect against NMDA but not AMPA or kainate, so that a combination of non-NMDA and NMDA antagonists are required to protect against glutamate-induced delayed death in cortical neuron cultures (Frandsen *et al.* 1989; Koh and Choi, 1991; Koh *et al.* 1990; McDonald *et al.* 1989; Rosenberg and Aizenman, 1989; Lysko *et al.* 1989; Cox *et al.* 1989). However, activation of NMDA receptors seems to be primarily responsible for cell death, since the NMDA antagonists AP5, AP7, ketamine, MK-801 and thienylphencyclidine provide good protection against glutamate-induced, delayed neuronal death in murine and rat cortical cultures (Choi *et al.* 1988; Rosenberg and Aizenman, 1989; Koh and Choi, 1991) and in hippocampal cultures (Michaels and Rothman, 1990; Rondouin *et al.* 1988). AP5 administered even after glutamate exposure still confers added protection against delayed neuronal death in murine cortical cultures (Choi *et al.* 1988). Brief exposure of mouse cortical cultures to glutamate, quisqualate or NMDA but not kainate or AMPA causes delayed neuronal degeneration (Koh and Choi, 1991; Choi, 1987; Choi *et al.* 1987; Choi, 1985; Koh *et al.* 1990). Whereas it is suggested that quisqualate may be taken up by neurons and slowly released in micromolar concentrations (Koh *et al.* 1990), it is also possible that this acute exposure produces long term release of intracellular calcium via metabotropic receptors.

2.4.5. HYPOXIC AND ISCHAEMIC NEURONAL DAMAGE

2.4.5.1. Selective vulnerability of the hippocampus

It has long been known that the hippocampus is among those brain areas that are selectively vulnerable to hypoxic, ischaemic and epileptic damage (Meyer, 1956; Meyer, 1936; Brown and Brierley, 1968; Speilmeyer, 1925; Sommer, 1880). In particular, the hippocampal CA_1 area undergoes delayed neuronal degeneration whilst other brain areas, including the hippocampal CA_3, may be preserved (Pulsinelli and Brierley, 1979; Petito and Pulsinelli, 1984). It is the CA_1 pyramidal neurons that are damaged, whereas interneurons in the hippocampus survive hypoxia, with the exception of the particularly vulnerable somatostatin-containing hilar interneurons which degenerate following the briefest hypoxic insults (Freund and Maglóczky, 1993; Nitsch *et al.* 1989). To what extent the consequences of hypoxia and ischaemia are similar is unclear, since hypoxia may often be

accompanied by hypotension (Winn *et al.* 1981; Zetterström *et al.* 1982). Indeed, insults that may reflect pure hypoxia, such as carbon monoxide or cyanide poisoning more often involve neuronal necrosis in the globus pallidus, and diffuse axonal demyelination, than do hypoxic insults which may involve some degree of hypoperfusion (Hirano *et al.* 1967; Plum *et al.* 1962; Meyer, 1936; LaPresle and Fardeau, 1967; Brown and Brierley, 1968).

Selective vulnerability of hippocampal CA_1 neurons cannot be explained in terms of a disproportionate increase in extracellular glutamate, as glutamate is released equally in the CA_3 area (Mitani *et al.* 1992), but rather must be a characteristic of the CA_1 pyramidal neurons themselves. Possibilities that might explain this selectivity include different glutamate uptake by glia, increased intrinsic excitability of the CA_1 pyramidal neurons due to a high density of glutamate receptors or differences in voltage-activated membrane currents, or susceptibility to intracellular calcium.

2.4.5.2. Synaptic release of amino acids mediates hypoxic neuronal death

Whereas a balance between excitatory and inhibitory transmitters may be required to maintain neural circuits (Mattson and Kater, 1989; Mattson, 1988), excess release of excitatory amino acids can produce neuronal degeneration, and such excitotoxicity may underlie hypoxic and ischaemic cell death. Cultured hippocampal neurons, when exposed to cyanide or hypoxia during the early stages of differentiation, are not visibly affected. In mature cultures, however, neurons which receive synaptic connections die when exposed to cyanide or anoxia (Rothman, 1983). In rats, unilateral intraventricular injection of kainate destroys ipsilateral CA_3 neurons and their projections, the Schaffer collaterals, but preserves the CA_1 pyramidal cells. Subsequent cerebral ischaemia causes delayed neuronal loss in the CA_1 of the hippocampus contralateral to the ablated CA_3 area, but no CA_1 damage occurs ipsilaterally (Onodera *et al.* 1986; Benveniste *et al.* 1988). Evidently, CA_3 projections to the CA_1 area are necessary to produce cell death. Although these synapses are required for glutamate release and excitotoxicity, the actual release of glutamate during hypoxia may not be exocytotic, but due to reversal of the glutamate transporter (Attwell *et al.* 1993).

In hypoxia, delayed neuronal death results from excitatory amino acid accumulation, glutamate receptor activation, and resultant rise in intracellular calcium (Choi, 1988; Choi, 1990; Rothman and Olney, 1986). Hypoxia causes delayed degeneration in hippocampal and cortical neuronal but not glial cultures (Kaku *et al.* 1991). Non-specific glutamate receptor antagonists, or a combination of antagonists for NMDA and AMPA receptors, block hypoxic death in cultured neurons (Rothman, 1984; Goldberg *et al.* 1987; Kaku *et al.* 1991). *In vivo*, NMDA and AMPA receptor antagonists provide some protection against brain damage following hypoxia

(Sheardown *et al.* 1990; Meldrum *et al.* 1987). Delayed neuronal degeneration following hypoxia may, therefore, be a result of hypoxia-induced glutamate release and excitotoxicity due to glutamate receptor-induced increase in cytosolic calcium. In neuronal culture and the hippocampal slice, hypoxia results in an increase in intracellular calcium (Dubinsky and Rothman, 1991; Reid, 1987). *In vivo* calcium uptake evoked by electrical stimulation of the perforant path is enhanced following ischaemia and precedes delayed neuronal death in CA_1 of rat hippocampus (Andiné *et al.* 1988). Intraventricular administration of dantrolene protects CA_1 pyramidal neurons from delayed neuronal death following cerebral ischaemia in gerbils (Zhang *et al.* 1993).

2.4.5.3. Delayed neuronal death

Prolonged ischaemia results in progressive, wide-spread neuronal death over 24 hours, but brief ischaemia may produce delayed neuronal death only in vulnerable areas, where neuronal necrosis does not develop for three to four days following the insult (Schmidt-Kastner and Freund, 1991). Immediately following reperfusion, neurons remain intact, but undergo typical reactive changes evident only by electron microscopy, including transient mitochondrial swelling, disaggregation of polyribosomes, reduced rough endoplasmic reticulum, loss of Golgi apparatus and smooth endoplasmic reticulum proliferation (Petito and Pulsinelli, 1984). Whereas all CA_3 and some CA_1 pyramidal neurons later recover normal ultrastructure, most CA_1 neurons eventually disintegrate.

2.4.5.4. Irreversible loss of synaptic transmission in hippocampal slices

The return of synaptic transmission in the CA_1 area of the rat hippocampal slice following hypoxia is dependent on the degree of hypoxia, the glucose concentration, and the temperature, but these have not been systematically studied. The CA_1 is more sensitive than other hippocampal subareas, and irreversible loss of postsynaptic field potentials occurs in the CA_1 without loss of the prevolley following seven minutes of hypoxia in the presence of 4mM glucose at 37°C (Kass and Lipton, 1986). This loss of synaptic transmission is presumably acute excitotoxicity from massive glutamate receptor activation, and can be prevented by a combination of NMDA and AMPA receptor antagonists (Lobner and Lipton, 1993). Irreversible loss of synaptic transmission is thought to be a consequence of hypoxic spreading depression, secondary to ATP depletion (Kass and Lipton, 1986), see section 2.4.7.2.

The level of glucose appears to be fundamental to this damage, since, in the presence of the more commonly used level of 10mM glucose, synaptic transmission will return following 30 to 60 minutes of hypoxia at 37°C, in rat (Fujiwara *et al.* 1987; Taylor and Weber, 1993) and guinea pig (Hansen *et al.* 1982) hippocampal slices. Indeed, at 34°C, the number of slices in which hypoxic depression of synaptic transmission is

reversible is critically dependent on the glucose concentration between 5 and 20mM (Schurr *et al.* 1987). The extent of recovery of synaptic transmission following 10 minutes hypoxia in low glucose is inversely related to incubation temperatures between 31°C and 37°C, with these extremes showing comlete and no recovery of the population spike respectively (Reid *et al.* 1987). The duration of hypoxia required to produce a large negative shift in DC potential in the CA_1 is dependent on the temperature between 31°C and 36°C (Taylor and Weber, 1993). This large negative shift in DC potential in the CA_1 is likely to be a manifestation of the hypoxia-induced depolarisation that is similar to spreading depression (Czéh *et al.* 1993) after which neuronal recovery is unlikely (Fairchild *et al.* 1988; Fujiwara *et al.* 1987; Taylor and Weber, 1993). Thus, slices at 31°C never display the DC potential shift and always recover synaptic transmission following exposure to 10% oxygen and 2mM glucose, but increasing temperature reduces the duration of hypoxia that can be tolerated (Taylor and Weber, 1993). Temperatures in the vicinity of 32°C are far more commonly used than body temperature (37°C) in hippocampal slice preparations; indeed, lower temperatures are standard in most *in vitro* neurophysiological preparations, as such conditions produce stable and robust electrophysiological recordings.

2.4.6. HYPOXIC INHIBITION OF SYNAPTIC TRANSMISSION

In vivo, cerebral hypoxia or ischaemia produce unconsciousness (Rossen *et al.* 1943), and complete experimental forebrain ischaemia is usually verified by an isoelectric electroencephalogram (Petito and Pulsinelli, 1984). Forebrain ischaemia suppresses hippocampal CA_1 unit activity in gerbil (Suzuki *et al.* 1983b; Mitani *et al.* 1990; Imon *et al.* 1991) and rat (Buzsáki *et al.* 1989; Chang *et al.* 1989) within 20 seconds, the same time course for flattening of the electroencephalogram. Using implanted electrodes, the depression of CA_1 evoked field potentials parallels the drop in hippocampal oxygen tensions during ischaemia (Freund *et al.* 1989), but ATP levels are well preserved (Wallis *et al.* 1990).

Andersen *et al.* originally showed that ventilation of cats, rabbits and rats with nitrogen rapidly inhibits synaptic transmission in the CA_1 area of the hippocampus (Andersen, 1960). In the hippocampal slice preparation, hypoxia was shown to inhibit synaptic transmission at the perforant path-dentate granule cell synapse (Lipton and Whittingham, 1979), and was later shown to inhibit the postsynaptic field potentials as well as intracellularly recorded spontaneous and evoked potentials in the CA_1 (Hansen *et al.* 1982).

Early work suggested that the IPSP is more rapidly suppressed by hypoxia than the EPSP (Leblond and Krnjevic, 1989; Fujiwara *et al.* 1987) and similar findings have been made with patch clamp recording of the IPSC (Hershkowitz *et al.* 1993). More

recently, it has been clarified that polysynaptically evoked IPSP/Cs are more sensitive to hypoxia than EPSP/Cs; evidently, either excitatory synaptic activation of interneurons is more sensitive to hypoxia than is excitation of pyramidal neurons, or transmissin through polysynaptic pathways is more susceptible to depression than is transmission across a a single synapse. It has been suggested that IPSPs recover slowly and sometimes incompletely upon reoxygenation following hypoxia (Krnjevic *et al.* 1991). However, in the presence of glutamate receptor antagonists, monosynaptic $GABA_A$ IPSP/Cs, evoked by stratum radiatum stimulation close to the recording site, are resistant to hypoxia (Zhu and Krnjevic, 1994; Zhazipov *et al.* 1994). On the other hand, the monosynaptically evoked $GABA_B$ IPSC or baclofen induced outward current are reversibly depressed by hypoxia (Zhazipov *et al.* 1993; Zhazipov *et al.* 1994), but this may be a postsynaptic occlusion due to other hypoxia-induced currents, see section 2.4.7.1.

Hypoxia selectively inhibits synaptic transmission but not membrane excitability, as shown by a rapid decrease in fEPSPs amplitude and population spike amplitude, whereas the prevolley persists and the antidromic spike declines only slowly, in parallel with hypoxic depolarisation (Sick *et al.* 1987; Somjen *et al.* 1987). It is now clear that hypoxia blocks synaptic transmission by a presynaptic mechanism, and stimulus evoked presynaptic calcium influx in CA_1 is reduced by hypoxia in the hippocampal slice (Young and Somjen, 1992). In whole cell recording of CA_1 neurons, synaptic transmission can be blocked by hypoxia without alteration in membrane potential or input resistance in the postsynaptic neuron, whilst intracellular GTPγS blocks hypoxic outward current but does not prevent hypoxic inhibition of synaptic transmission (Zhang and Krnjevic, 1993; Krnjevic and Xu, 1990b). Inward current evoked by exogenous glutamate is also not blocked during hypoxic block of synaptic transmission in the CA_1 (Hershkowitz *et al.* 1993).

Synaptic transmission may briefly recover during hypoxia (Fowler, 1992; Sick *et al.* 1987; Fowler, 1993; Fowler, 1990; Fairchild *et al.* 1988; Hershkowitz *et al.* 1993). This recovery occurs just prior to loss of the prevolley and the antidromic population spike if hypoxia or ischaemia is sufficiently prolonged. Synaptic transmission returns when extracellular potassium has risen to 8-10mM, and fails along with the prevolley and antidromic spike during hypoxic spreading depression (Sick *et al.* 1987; Fairchild *et al.* 1988; Fowler, 1990; Fowler, 1992; Fowler, 1993). If hypoxia continues beyond this paradoxical recovery, synaptic transmission rarely returns upon reoxygenation (Fairchild *et al.* 1988). Paradoxical recovery occurs even in the presence of agonists for the G protein-linked adenosine A_1 receptors and $GABA_B$ receptors, that would normally inhibit synaptic transmission, suggesting uncoupling of G proteins during hypoxia (Fowler, 1992).

2.4.7. HYPOXIC ACTIONS ON POSTSYNAPTIC NEURONS

2.4.7.1. Hypoxic hyperpolarisation

Hypoxia causes a hyperpolarisation of hippocampal CA_1 neurons, with a reversal potential and sensitivity to extracellular potassium concentration that indicates it is mediated by an increased potassium conductance (Hansen *et al.* 1982; Fujiwara *et al.* 1987; Leblond and Krnjevic, 1989; Krnjevic and Xu, 1990b). This hypoxic outward current is strongly outward rectifying (Krnjevic and Xu, 1990b), but it's identity remains obscure. Block of synaptic transmission with low calcium and high magnesium, or TTX containing medium does not change the hypoxic hyperpolarisation (Fujiwara *et al.* 1987; Hansen *et al.* 1982; Leblond and Krnjevic, 1989), indicating either a direct effect of hypoxia on the postsynaptic neurons, or activation of a receptor by non-synaptically released agents. Attempts to block this potassium current have yielded mixed results; whereas an early report shows a block of hypoxic hyperpolarisation by 0.4mM 4-AP (Hansen *et al.* 1982), later reports show that the hypoxic hyperpolarisation is not fully blocked by extracellular application of 4-AP, TEA, caesium, apamin or quinine (Fujiwara *et al.* 1987; Leblond and Krnjevic, 1989). Either intracellular caesium or a combination of extracellular caesium and TEA provides a near complete block of the hypoxia evoked outward current in voltage clamped hippocampal CA_1 pyramidal neurons (Krnjevic and Leblond, 1989). Whereas intracellular EGTA does not block the hypoxic hyperpolarisation (Fujiwara *et al.* 1987; Zhang and Krnjevic, 1993; Leblond and Krnjevic, 1989), the possibility remains that it is a calcium-dependent potassium current, as it is blocked by dantrolene (Krnjevic and Xu, 1989).

Carbachol (20μM) action at a muscarinic receptor blocks the hypoxic hyperpolarisation, in an atropine sensitive manner, uncovering a depolarisation and a reduced membrane conductance, coincident with an early transient hypoxic depolarisation that is occasionally seen before hyperpolarisation (Krnjevic and Xu, 1990b); however, carbachol 1μM alone will cause a depolarisation in the CA_1 (Cole and Nicoll, 1984) associated with a sharp inward current (Krnjevic and Xu, 1990b). In comparison to responses recorded with sharp electrodes, whole cell patch clamp of CA_1 neurons produces a smaller hypoxic hyperpolarisation and a smaller reduction in input resistance, and can even be replaced by a depolarisation or inward current, possibly due to dialysis of a diffusible intracellular messenger with patch electrodes contents (Zhang and Krnjevic, 1993; Hershkowitz *et al.* 1993). A similar block of anoxic outward current is achieved by recording with electrodes containing GTPγS which irreversibly activates G proteins (Krnjevic and Xu, 1990b). This indicates that the current is G protein dependent, and carbachol modulation may result from phosphoinositide hydrolysis and subsequent protein kinase-mediated phosphorylation of G protein regulatory domains. Such a block of potassium

currents is produced by muscarinic agonists that increase inositol 1,4,5-trisphosphate (Dutar and Nicoll, 1988a; Madison *et al.* 1987). Carbachol additionally blocks the AHP current by raising cAMP, and produces slow EPSPs in the CA_1, possibly due to block of a voltage insensitive potassium current (Madison *et al.* 1987). However, dibutyl-cAMP superfusion is without effect on input resistance or membrane potential, and does not reduce hypoxic hyperpolarisation (Fujiwara *et al.* 1987).

2.4.7.1.1. ATP-sensitive potassium channels and hypoxic neuronal inhibition

A possible mechanism of hypoxic hyperpolarisation is the activation of ATP-sensitive potassium channels, which are opened by low intracellular ATP concentrations and are closed when ATP concentrations are high. Such an action is evident in the substantia nigra, which is rich in these channels (Mourre *et al.* 1989), where metabolic poisoning causes $^{86}Rb^+$ efflux (Amoroso *et al.* 1990) and an hyperpolarisation that is blocked by the ATP-sensitive potassium channel blocking sulfonylurea, tolbutamide (Murphy and Greenfield, 1991).

Hypoxic hyperpolarisation in CA_3 pyramidal cells in hippocampal slices can be blocked by the sulfonylurea glibenclamide (Mourre *et al.* 1989), suggesting that it is mediated by ATP-sensitive potassium channels. However, [^{3}H]glibenclamide binding in the CA_3 area appears to be mostly on the presynaptic terminals of the Mossy fibres (Tremblay *et al.* 1991). In the CA_1 area of the hippocampus, [^{3}H]glibenclamide binding is lower than in the CA_3 but is prominent in the stratum pyramidale and superficial stratum radiatum, and appears to be confined to the pyramidal cells, with no receptors on the Schaffer collateral terminals (Tremblay *et al.* 1991). In CA_1 pyramidal cells however, the hypoxic hyperpolarisation and fall in input resistance is enhanced by tolbutamide (Leblond and Krnjevic, 1989). Nevertheless, if ATP-sensitive channels contribute to hypoxic hyperpolarisation in the CA_1, hypoxia may override sulfonylurea block of the channel. Indeed, in myocytes, sulfonylurea binding is competitive with nucleotides (Ripoll *et al.* 1993), as are their action against $^{86}Rb^+$ efflux in rat islet cell lines (Schmid-Antomarchi *et al.* 1987). Additionally, sulfonylureas fail to prevent ATP-sensitive potassium channel activation during metabolic poisoning of myocytes with mitochondrial uncouplers (Findlay, 1993). On the other hand, whole-cell recording of CA_1 pyramidal neurons with ATP-free electrodes fails to show hypoxic hyperpolarisation, despite favourable intracellular conditions for opening of ATP-sensitive potassium channels (Zhang and Krnjevic, 1993). ATP-sensitive channels are activated by dinucleotides (Terzic *et al.* 1994), so that the rise in free ADP during hypoxia possibly activates these channels before a drop in ATP occurs. Adenosine receptor activation can relieve the ATP block of these channels by activating G_α G protein subunits in cardiac myocytes (Terzic *et al.* 1994), which is in part responsible for adenosine cardiac protection (Yao and Gross,

1993). Therefore, accumulation of free AMP and adenosine may override sulfonylurea block of ATP-sensitive channels during hypoxia in CA_1 pyramidal neurons, but this has not been verified.

2.4.7.2. hypoxic spreading depression

Hypoxic hyperpolarisation may be followed by a slow depolarisation or inward current. TTX has no effect on hypoxic depolarisation (Fujiwara *et al.* 1987; Hershkowitz *et al.* 1993), suggesting that synaptically released glutamate is not responsible. This slow hypoxic depolarisation results from the increase in extracellular potassium that is due to depression of the Na^+/K^+-ATPase (Fujiwara *et al.* 1987; Hershkowitz *et al.* 1993).

If hypoxia is of sufficient duration it produces a complete loss of neuronal membrane potential in the CA_1 of hippocampal slices that has been likened to spreading depression. Such hypoxic spreading depression can be measured as a rapid depolarisation to zero mV with an undetectable input resistance (Fujiwara *et al.* 1987; Hansen *et al.* 1982), or a rapid inward current that reverses near zero mV (Czéh *et al.* 1993; Hershkowitz *et al.* 1993); these events coincide with an extracellularly recorded negative shift in DC potential (Czéh *et al.* 1993; Taylor and Weber, 1993). Whereas hypoxic depolarisation is associated with a slow increase in extracellular potassium to 10mM, and a slow decrease in extracellular calcium, the onset of hypoxic spreading depression is characterised by a by rapid increase in extracellular potassium to greater than 20mM and a precipitous drop in extracellular calcium from 1.2mM to 0.4mM (Sick *et al.* 1987; Young and Somjen, 1992; Reid, 1987; Hansen *et al.* 1982). With hypoxic spreading depression the antidromic spike and the prevolley are lost (Young and Somjen, 1992; Fairchild *et al.* 1988; Sick *et al.* 1987), stimulus trains no longer produce presynaptic calcium influx (Young and Somjen, 1992), and there is no response to exogenous glutamate analogues (Hershkowitz *et al.* 1993). After hypoxic spreading depression, neuronal recovery is unlikely (Fairchild *et al.* 1988; Fujiwara *et al.* 1987; Taylor and Weber, 1993).

In the hippocampal slice CA_1, hypoxic spreading depression is promoted by physiological temperature and glucose concentration, but is rare at the lower temperatures and higher glucose concentrations more typically used (Taylor and Weber, 1993). For instance, at 36°C, in the presence of 10 mM glucose, a negative shift in DC potential is never seen (Taylor and Weber, 1993) and only a fraction of pyramidal neurons rapidly depolarise (Fujiwara *et al.* 1987). Under similar conditions, CA_3 pyramidal neurons undergo hypoxic spreading depression much earlier than CA_1 neurons (Kawasaki *et al.* 1990).

Hypoxic or ischaemic excitatory amino acid release probably occurs after hypoxic spreading depression (Martin *et al.* 1994). However, hypoxic spreading depression may be partially dependent on glutamate receptor activation as it is delayed, but not prevented, by kynureate or a combination of CNQX and CPP in the hippocampal slice (Young and Somjen, 1992; Hershkowitz *et al.* 1993). Neither TTX, nor intracellular caesium, nor QX-314 inhibit hypoxic spreading depression, suggesting this phenomena is not mediated by potassium or sodium channels (Czéh *et al.* 1993). Instead, it has been proposed that the rapid depolarisation is due to failure of the Na^+/K^+-ATPase (Fujiwara *et al.* 1987; Matsuda *et al.* 1992) and Ca^{2+}-ATPase (Kass and Lipton, 1986), and this notion is strengthened by the observation that rapid ischaemic calcium influx only occurs when ATP levels have dropped (Kass and Lipton, 1986; Katsura *et al.* 1993).

2.4.8. RELATIONSHIP OF NEURONAL DEPRESSION AND ENERGY METABOLISM

Forebrain ischaemia supresses CA_1 unit activity in gerbil (Suzuki *et al.* 1983b; Mitani *et al.* 1990; Imon *et al.* 1991) and rat (Buzsáki *et al.* 1989; Chang *et al.* 1989) hippocampus within 20 seconds, the same time course of flattening of the electroencephalogram. With implanted electrodes it can be determined that the depression of CA_1 evoked field potentials parallels the drop in hippocampal oxygen tensions during ischaemia (Freund *et al.* 1989). This rapid inhibition of synaptic transmission precedes any significant drop in cerebral ATP (Katsura *et al.* 1993; Ekholm *et al.* 1992; Lowry *et al.* 1964). In the hippocampal slice, synaptic transmission between the perforant path and dentate granule cells (Lipton and Whittingham, 1982), and the Schaffer collaterals and CA_1 pyramidal neurons (Wallis *et al.* 1990), fails despite ATP levels being maintained near 90%. CA_1 fEPSPs are completely depressed while adenylate energy charge has only dropped from 0.9 to 0.8 with hypoxia, and the beginning of this synaptic transmission depression precedes any change in adenylate energy charge (Fredholm *et al.* 1984). Potassium conductances in cerebral cortex *in vivo* are activated by 15-30 seconds ischaemia, while ATP exceeds 90% of control (Katsura *et al.* 1993; Folbergrová *et al.* 1990; Ekholm *et al.* 1992). Since in the hippocampal slice with 10mM glucose, ATP is reasonably well maintained throughout prolonged hypoxia (Fredholm *et al.* 1984; Yoneda and Okada, 1989), ATP depletion is not necessary for the CA_1 membrane hyperpolarisation and outward potassium current produced by hypoxia. However, at the time of these depressive actions on synaptic transmission and membrane excitability, calculated free ADP and free AMP have risen enormously, and pH may have dropped by 0.2 units (Ekholm *et al.* 1992), and these cytosolic changes may contribute to neuronal depression.

Hypoxic spreading depression is likely a result of failure of the Na^+/K^+-ATPase secondary to depletion of ATP (Matsuda *et al.* 1992). The rapid depolarisation and rapid extracellular K^+ accumulation by 70-80 seconds ischaemia *in vivo* occurs when ATP is depleted to 40% of control (Katsura *et al.* 1993; Folbergrová *et al.* 1990). Since this phase of hypoxia and ischaemia is associated with irreversible neuronal damage, such damage is thus dependent on failure of energy maintenance. Indeed, the time required to produce irreversible loss of synaptic transmission parallels that required to produce a severe depletion of ATP to 30-40% of control (Kass and Lipton, 1989; Yoneda and Okada, 1989)

Incubation of hippocampal slices with creatine increases their phosphocreatine levels without increasing ATP (Lipton and Whittingham, 1982; Okada and Yoneda, 1983; Yoneda *et al.* 1983). Elevated phosphocreatine slows ATP loss, and increases the time that slices can be ischaemic and still recover synaptic function with reoxygenation in the CA_3 (Okada and Yoneda, 1983). In keeping with this, pre-incubation of hippocampal slices with creatine increases post-ischaemic ATP and reduces the $^{45}Ca^{2+}$ influx associated with hypoxic spreading depression (Kass and Lipton, 1986). Such elevated phosphocreatine increases the latency of inhibition of synaptic transmission and postsynaptic hyperpolarisation due to ischaemia or hypoxia in the dentate gyrus, CA_3 and CA_1 of hippocampal slices (Lipton and Whittingham, 1982; Okada and Yoneda, 1983; Leblond and Krnjevic, 1989). Since synaptic transmission declines before any considerable decrease in ATP, the prolonged latency until synaptic inhibition produced by elevated phosphocreatine may result from reduced accumulation of ADP and AMP via adenylate kinase and nucleoside loss as adenosine.

2.4.9. ADENOSINE AND HYPOXIC NEURONAL INHIBITION

Adenosine apparently does not contribute to all the postsynaptic effects of hypoxia. Inactivation of G proteins by treatment of rats with PTX, which ADP-ribosylates G_i and G_o proteins, prevents the hyperpolarisation and reduced input resistance to exogenously applied adenosine in hippocampal CA_3 neurons, yet the hypoxic hyperpolarisation and reduced input resistance are preserved (Spuler and Grafe, 1989). Caffeine, theophylline or DPCPX do not prevent hypoxic hyperpolarisation and reduced input resistance in CA_1 neurons (Leblond and Krnjevic, 1989; Croning *et al.* 1994), suggesting that these are not adenosine A_1 receptor-mediated effects. However, hypoxia-induced adenosine release may cause some depression of postsynaptic excitability, as the antidromically evoked after-potentials in calcium free medium are depressed by hypoxia and this depression is blocked by 8-PT (Murphy and Greenfield, 1991). Curiously, A_1 receptor antagonism does depress the rise in extracellular potassium concentration during five minutes hypoxia in the

hippocampal slice (Croning *et al.* 1994), since this rise is likely the result of Na^+/K^+-ATPase depression, adenosine must considerably limit depolarisation of neurons.

A role for adenosine in the inhibition of excitatory synaptic transmission is clear. In the hippocampus, hypoxia rapidly inhibits synaptic transmission at the Schaffer collateral to CA_1 synapse, and the selective adenosine A_1 receptor antagonists DPCPX, 8-CPT or 8-PT, as well as the less selective antagonist theophylline can preserve synaptic transmission during hypoxia at this synapse (Gribkoff *et al.* 1990; Fowler, 1989; Gribkoff and Bauman, 1992; Katchman and Hershkowitz, 1993). Similarly 8-CPT and 8-PT can preserve transmission at this synapse during *in vitro* ischaemia (Fowler, 1990). Perfusion of the isolated rat spinal cord with hypoxic ACSF causes depression of the extracellularly recorded monosynaptic reflex and again, adenosine A_1 receptor antagonist 8-CPT blocks this hypoxic depression (Lloyd *et al.* 1988; Czéh and Somjen, 1990; Moorhouse *et al.* 1991). Also perfusion with low magnesium ACSF, which reduces A_1 receptor agonist activity (Bartrup and Stone, 1988), similarly, reduces the hypoxic depression of the monosynaptic reflex (Czéh and Somjen, 1990). Hypoxic inhibition of EPSCs but not IPSCs in whole-cell patch clamp of CA_1 pyramidal neurons, can be blocked by DPCPX or 8-PT (Katchman and Hershkowitz, 1993).

2.4.10. ADENOSINE PROTECTS AGAINST HYPOXIA, ISCHAEMIA AND EXCITOTOXICITY

Postsynaptic adenosine A_1 receptor actions could provide protection against excitotoxicity during hypoxia and ischaemia. Following adenosine release, adenosine enhancement of I_{AHP} and consequent inhibition of multiple firing could specifically attenuate strong glutamergic activation of neurons. Additionally, the opening of adenosine gated potassium channels should act to shunt glutamate-induced currents, whilst hyperpolarisation of the neuronal membrane by adenosine will help maintain calcium homeostasis, by preventing alleviation of the magnesium block on NMDA receptors, and by reducing activation of voltage-activated potassium channels. Also of possible importance, adenosine hyperpolarisation may prevent inactivation of I_A thus limiting presynaptic action potentials. Furthermore, presynaptic A_1 receptors may limit hypoxic glutamate release, by depressing glutamate release from the neurotransmitter pool.

2.4.10.1. Neuronal survival

Hippocampal injections of 2-CA given immediately prior to or after 10 minutes of forebrain ischaemia, and repeated at 4 and 10 hours, protect against CA_1 cell loss (Evans *et al.* 1987). Intraventricular injection of CHA 15 minutes following 30 minutes of cerebral ischaemia in gerbils, protects hippocampal CA_1 neurons from degeneration (Januszewicz von Lubitz *et al.* 1989; von Lubitz *et al.* 1988).

Endogenous adenosine release provides some protection against ischaemic and hypoxic neuronal degeneration. Increasing extracellular levels of endogenous adenosine with i.p. administration of the nucleoside uptake inhibitor propentofylline or the adenosine deaminase inhibitor deoxycoformycin, significantly reduces the extent of ischaemia-induced CA_1 cell necrosis (Dux *et al.* 1990; Phillis and O'Regan, 1989) and cortical infarct size (Lin and Phillis, 1992). Upregulation of A_1 receptors with chronic caffeine treatment in rats (Sutherland *et al.* 1991) and gerbils (Rudolphi *et al.* 1989) provided protection of CA_1 neurons following ischaemia. Conversely, acute i.p. theophylline or caffeine enhances ischaemic damage in gerbils (Dux *et al.* 1990; Sutherland *et al.* 1991) and i.p. 8-CPT produces hypoxic neuronal damage that is otherwise absent in the CA_1 of rats (Boissard *et al.* 1992).

In foetal mouse cortical cell or rat cortical/hippocampal cell cultures with developed synaptic connections, adenosine or CHA provides concentration-dependent protection against cell loss due to hypoxia, but not against exogenous glutamate (Goldberg *et al.* 1988; Daval and Nicolas, 1994). In hippocampal slices the evoked synaptic population responses survived longer periods of hypoxia in the presence of exogenous adenosine 100μM than control slices (Donaghy and Schofield, 1991).

Adenosine may act postsynapticaly to some extent to reduce excitotoxicity. Co-injection of 2-CA with excitatory amino acids into rat striatum provided partial protection against excitotoxicity (Finn *et al.* 1991; Arvin *et al.* 1989). Hypoxia *in vivo*, largely prevents hippocampal CA_3 damage following i.p. kainate administration. However, CHA fails to protect cortical cell cultures against exogenous glutamate (Goldberg *et al.* 1988). The majority of the neuroprotective action of adenosine in the CA_1 is likely a result of depression of glutamate release.

2.4.10.2. Adenosine modulation of ischaemic excitatory amino acid release

2.4.10.2.1. A_1 receptor inhibition of excitatory amino acid release

Endogenous adenosine, released during cerebral ischaemia in rats, limits the ischaemic glutamate accumulation in the hippocampus, since this is increased 2 to 4 fold by i.p. or local theophylline administration (Lekieffre *et al.* 1991; Heron *et al.* 1993). A_1 receptor activation by local application of agonists, 0.1nM cyclopentyladenosine or 1nM NECA, greatly reduces extracellular aspartate and glutamate recovered by cortical cup during ischaemic and reperfusion (Simpson *et al.* 1992). However, the adenosine deaminase inhibitor deoxycoformycin 500µg/kg i.p. increases extracellular adenosine in the rat brain but does not attenuate ischaemic excitatory amino acid release, measured by the cortical cup technique (Phillis *et al.* 1991), and local application of micromolar R-PIA or CPA does not reduce ischaemic glutamate release in the rat hippocampus (Heron *et al.* 1993; Simpson *et al.* 1992).

This variability may result from confounding A_2 receptor activation by adenosine and high concentrations of R-PIA or CPA.

2.4.10.2.2. A_2 receptor enhancement of excitatory amino acid release

In the four-vessel occlusion model of ischaemia in the rat, micromolar doses of CPA or NECA and A_2 receptor agonist CGS 21680 enhance release of aspartate and glutamate during ischaemic and reperfusion, an action blocked by the A_2 receptor antagonist DMPX (O'Regan *et al.* 1992; Simpson *et al.* 1992). This is probably an A_{2b} effect as it is not produced by nanomolar CGS 21680 (O'Regan *et al.* 1992) and CGS 21680 does not alter acute excitotoxicity assessed by re-appearance of the CA_1 population spike following hypoxia in the hippocampal slice (Zeng *et al.* 1992). However, as the non-selective adenosine receptor antagonist theophylline increases ischaemic glutamate release, it is apparently the A_1 receptor effect that prevails during ischaemic adenosine accumulation.

2.4.10.2.3. A_3 receptors

Acute 3-IB-MECA results in lower post-ischaemic cerebral blood flow, enhanced mortality, and in the 1 of 15 gerbils surviving to 7 days there is increased loss of hippocampal CA_1 neurons (von Lubitz *et al.* 1994). Apart from these vascular actions, it is not known whether adenosine A_3 receptors participate in neuronal modulation during hypoxia.

2.4.11. POST-HYPOXIC HYPEREXCITABILITY

Ischaemia in not well tolerated by hippocampal CA_1 pyramidal neurons. Even brief cerebral ischaemia *in vivo* results in hippocampal damage (Benveniste *et al.* 1988; Evans *et al.* 1987), and brief hypoxia/hypoglycaemia *in vitro* is sufficient to cause acute toxicity in the hippocampal slice (Kass and Lipton, 1986; Okada *et al.* 1988) and delayed neuronal death in neuronal cultures (Kaku *et al.* 1991). However, the extent to which acute or delayed neuronal death due to hypoxia is a significant problem is unclear. Acute cell death in the hippocampal slice requires impractically prolonged hypoxic superfusion (Okada *et al.* 1988; Fujiwara *et al.* 1987; Clarke *et al.* 1989), and delayed neuronal death in neuronal cultures also requires many hours of hypoxia (Goldberg *et al.* 1987; Rothman, 1984), and although hypoxia is little studied *in vivo*, it is probably complicated by hypotension. Hypoxia alone is a poor stimulus for excessive excitatory amino acid release, unless it is accompanied by neuronal depolarisation (Pellegrini-Giampietro *et al.* 1990). However, hypoxia is far from benign, and the earliest, and functionally most obvious, pathology is the persistent increase in neuronal excitability that accompanies the return of synaptic transmission with reoxygenation following hypoxia. Indeed, although controversy exists on this point, such hyperexcitability may be a predisposing factor in hypoxic excitotoxicity.

As with many aspects of hippocampal electrophysiology, the wide ranging work of Andersen *et al.* in 1960 first described post-hypoxic hyperexcitability in the CA_1 area of the hippocampus. Field potentials recorded in the CA_1 area of rabbits, rats and cats in response to contralateral stimulation *in vivo* showed hyperexcitability, evident as multiple population spikes, prior to synaptic depression during brief ventilation with nitrogen, and transient similar hyperexcitability was seen during the early reoxygenation period (Andersen, 1960).

Hippocampal slices exposed to prolonged experimental hypoxic pre-incubation *in vitro*, at and below room temperature, display multiple population spikes in the CA_1 area in response to stratum radiatum stimulation (Dunwiddie, 1981). Hippocampal slices that initially respond with only one population spike, once exposed to 30 minutes hypoxic superfusion at 29°C, display multiple population spikes with recovery of synaptic transmission upon reoxygenation (Schiff and Somjen, 1985). Indeed, such multiple population spikes, presumably evidence of unintentional brief hypoxia at higher temperatures during tissue preparation, are readily evident in much of the literature. In the only study to examine prolonged hypoxia with intracellular recordings of CA_1 pyramidal neurons in the hippocampal slice, 6 of 18 neurons showed a rapid phase of depolarisation at 15-30 minutes hypoxia, that reduced the membrane potential and input resistance to zero, after which reoxygenation was not accompanied by recovery of function (Fujiwara *et al.* 1987). However, 12 of 18 neurons remained 25mV depolarised from resting membrane potential throughout 40 minutes hypoxia; membrane potential returned to normal within 15 minutes of reoxygenation but was accompanied by increase spontaneous EPSP frequency (Fujiwara *et al.* 1987). In neocortical slices, recovery from brief hypoxia is characterised by a slow recovery of the late IPSP, a depolarised membrane potential and an increase in input resistance; apparently this latter persists following prolonged hypoxia (Luhmann and Heinemann, 1992). Brief ischaemia has been shown to cause a long-term potentiation of the NMDA receptor-mediated component of synaptic transmission in hippocampal slices, similar to that seen *in vivo* (Crépel *et al.* 1993). Although post-hypoxic hyperexcitability has been little studied, it has been proposed that post-hypoxic hyperexcitability may result from alteration of adenosine-induced inhibitory tone (Lee and Kreutzberg, 1987), possibly through down-regulation of hippocampal adenosine A_1 receptors after *in vivo* ischaemia (Lee *et al.* 1986). Indeed, down-regulation of adenosine A_1 receptor-mediated inhibition of cAMP production follows prolonged adenosine exposure (Abbracchio *et al.* 1992) as would occur during hypoxia, and this may produce hyperexcitability by preventing adenosine-induced enhancement of the AHP.

There is controversy regarding the existence of post-ischaemic hyperexcitability *in vivo*. In rats, 12-20 minutes ischaemia fails to produce any hyperexcitability of

CA_1 spontaneous or evoked field potentials, whereas both spontaneous and stratum radiatum evoked activity decline in parallel with neuronal necrosis (Buzsáki *et al.* 1989; Miyazaki *et al.* 1993). However, a true hyperexcitability is seen following ischaemia in the rat CA_1, where paired pulse inhibition, in response to contralateral CA_3 stimulation with long inter-stimulus intervals, becomes a facilitation, possibly due to loss of AHP or $GABA_B$ receptor-mediated inhibition (Chang *et al.* 1989). CA_1 pyramidal neurons are poorly resistant to ischaemia, and the inconsistencies in producing hyperexcitability may be due to differences in the duration of ischaemia and possibly the maintenance of brain temperature, both of which may contribute to acute neuronal damage in the population of neurons that are potentially hyperexcitable.

Rather than hyperexcitability, ischaemia may produce an increase in synaptic transmission similar to long-term potentiation, as seen in the hippocampal slice following ischaemic incubation. In gerbil hippocampal slices prepared 5 to 10 hours following *in vivo* forebrain ischaemia, synaptic transmission, as measured by the fEPSP slope, in the CA_1 is increased compared to non-ischaemic slices, whereas the orthodromic and antidromic population spike is reduced (Urban *et al.* 1989) All field potentials are reduced, as a result of neuronal necrosis, in slices prepared in subsequent days (Urban *et al.* 1989). Similarly, with *in vivo* rat hippocampal recordings, the CA_1 fEPSP is potentiated at 6 to 8 hours post-ischaemia without an increase in pyramidal neuron excitability (Miyazaki *et al.* 1993). In gerbils, the spontaneous unit activity recorded in the CA_1 area does not show any increase in frequency or amplitude following 5 or more minutes of forebrain ischaemia (Imon *et al.* 1991; Mitani *et al.* 1990; Suzuki *et al.* 1983b; Katchman *et al.* 1994). However increase in spontaneous unit activity in the CA_1, and a decrease in CA_3, is seen in the days following less than 5 minutes ischaemia in gerbils (Suzuki *et al.* 1983b; Katchman *et al.* 1994), and 10 minutes ischaemia in rats (Chang *et al.* 1989). Unit recordings do not differentiate between pyramidal neurons and interneurons, and the burst-like unit spike discharges (Imon *et al.* 1991) could well be produced by interneurons.

Whereas seizures are common following ischaemia or hypoxia in humans (Rossen *et al.* 1943; Oppenheimer and Hachinski, 1992) and experimental animals, obvious motor seizure are not necessary for neuronal damage, and animals displaying such seizures are usually excluded from study (Petito and Pulsinelli, 1984). Nevertheless, non-motor seizures may occur; indeed, such seizures may account for some of the behavioural changes described during the early recirculation period following ischaemia (Suzuki *et al.* 1983a).

2.5. SUMMARY AND CONCLUSIONS

Electrophysiological recordings in the CA_1 area of the hippocampal slice provide a good model for the study of basic cortical physiology and pharmacology as well as multi-faceted neurochemical interactions, so long as these produce obvious alterations in electrophysiological recordings. The hippocampus slice is a convenient model for a variety of such interactions, notably synaptic plasticity and, more important in the present context, the effects of hypoxia.

Most importantly, the CA_1 area provides a well defined model of cortical synaptic connections between a common primary cortical cell type, the pyramidal neuron, and a diversity of interneurons. The CA_1 pyramidal neurons receive a number of excitatory glutamergic pathways, and a cholinergic pathway, which being anatomically distinct, are easily accessible for selective stimulation. In addition, interneuronal populations mediating a variety of types of GABA induced inhibition can be activated with a moderate degree of selectivity. The hippocampus is also richly endowed with receptors for most neurotransmitters and neuroregulators present in the brain.

Excitatory synaptic transmission, in particular via the Schaffer collaterals, and inhibitory synaptic transmission, along with pyramidal neuron electrophysiology are well characterised in the CA_1, providing a good basis for further studies in this area. In addition, many consequences of hypoxia are well defined in the CA_1 area, indeed, the Schaffer collaterals are of major importance in the outcome of hypoxia. Furthermore, the pharmacology of adenosine A_2 and A_3 receptors in the CA_1 area is developing and adenosine A_1 receptor pharmacology has been well characterised in the CA_1 area, and these receptors are intimately involved in the modulation off excitatory synaptic transmission at the Schaffer collateral synapse. The activation of adenosine receptors during cerebral hypoxia is established, and some consequences of this have so far been investigated.

3. METHODS

3.1. HIPPOCAMPAL SLICE PREPARATION

3.1.1. TISSUE PREPARATION

Hippocampal slices were prepared by standard techniques. Male Sprague-Dawley rats, 250-350g, aged 7-10 weeks, were supplied by the the Animal Resources Centre, Gilles Plain and housed at the Institute of Medical and Veterinary Science Animal House, Adelaide. Rats were decapitated without anaesthesia using a small animal guillotine (Stoelting). The skull was rapidly opened by removing the calvarium, and the brain removed, severing the cranial nerves and olfactory bulb with a spatula. The brain was chilled for 40-60 minutes in ice cold artificial cerebrospinal fluid (ACSF), which serves both to limit cell damage due to the inevitable ischeamia following decapitation and to 'set' the tissue for ease of cutting. On an ice chilled dissecting stage, the hippocampus was dissecting free from the surrounding tissue and an approximately 4mm section cut from the middle of the hippocampus, the section being slightly diagonal from transverse (coronal), judged by alignment with the septal end of the hippocampus. This section was mounted on a teflon block using cyanoacrylate glue, submerged in ice cold ACSF, and 400μm coronal slices were cut with a vibrating blade (Vibroslice, Campden Instruments Ltd.). Slices were stored temporarily at room temperature in a static holding chamber (Medical Systems Corp.) containing approximately 45ml of continually oxygenated and circulated ACSF, before being transferred by pippette to the recording chamber. In most cases this temporary storage of slices was only for the duration of preparation of slices from the two hippocampi, between 5 and 20 minutes. In the recording chamber, slices were held submerged in and superperfused with ACSF at room temperature (18-22°C) for 90 minutes before the ACSF was slowly heated to, and held at 32.0 ± 0.2°C for recording. During heating, electrodes were placed and at least 15 minutes allowed for recovery before experiments began, usually 120 minutes following slice placement in the chamber.

3.1.2. RECORDING CHAMBER

The recording chamber (Medical Systems Corp.) was an acrylic bath of approximately 600μl volume with transverse fluid flow from a bottom fluid inlet at one end to an outlet on top of a dam at the other end. ACSF was delivered via a variable speed peristaltic pump (Ismatec), and removed by vacuum aspiration of the dam overflow. Slices were held at the mid-level of the ACSF between a fixed stiff nylon mesh and a removable weighted nylon net. The slice bath sat on top of an acrylic base unit consisting of a water jacket enclosing a coil of tubing in-line between the pump and bath inlet, and a nichrome wire heating element. The heating element was supplied with proportionally regulated DC current via a model TC-102 temperature controller

(Medical Systems Corp.) which monitored bath temperature via a thermistor probe (Medical Systems Corp.) and maintained the bath temperature. ACSF was superfused at a rate of 2.4ml/min. Further increase in superfusion rate did not improve field potentials, however, if the superfusion rate was reduced, field potential amplitude diminished.

3.1.3. ACSF

ACSF comprised in mM: NaCl 127, KCl 3.5, NaH_2PO_4 1.25, $MgCl_2$ 1.2, $CaCl_2$ 2, $NaHCO_3$ 26, and D-glucose 10. ACSF was oxygenated by bubbling carbogen (95% O_2 / 5% CO_2) through a stainless steel, 10μm pore size, HPLC filter at a rate of 500ml/min for 40 minutes. Hypoxic ACSF was similarly produced by bubbling with 95% N_2 / 5% CO_2. Gases were supplied by Commonwealth Industrial Gas. ACSF was gassed at approximately 32°C to ensure gas saturation at the recording bath temperature. 40 minutes was sufficient time for equilbration, and indeed no increase in oxygenation was found after 20 minutes bubbling. ACSF PO_2 was occasionally verified with a blood gas machine (Corning). Hypoxic ACSF, produced as described had a PO_2 of 24 ± 6mmHg (S.D., n=3). PO_2 of fully oxygenated ACSF, 719 ± 59mmHg (S.D., n=13), varied considerably, being well outside the calibrated range of the blood gas machine. ACSF pH ranged from 7.37-7.40.

For a few studies ACSF was gassed with different gas mixtures containing 5%, 10%, 25%, 50% or 60% oxygen. These were produced with a triple rotameter bank to mix oxygen, carbon dioxide and a diluent gas, either nitrogen or carbon monoxide. The oxygen and carbon dioxide tensions in these mixtures were additionally verified by in-line monitoring. Oxygen was measured by a polarographic electrode at a gel/gas interface (Instrumentation Laboratory), and carbon dioxide by an infrared end tidal carbon dioxide sensor (Nihon Cohden). Results of ACSF PO_2 determinations with the blood gas machine for various oxygen fractions in the bubbling gas are shown in figure 3.1. These show a linear relationship and the slope of the regression line is 0.98, indicating complete equilibration of ACSF oxygen tension with the bubbling gas.

After gassing, ACSF was sealed in an airtight bottle with two outlets, one connected to a flexible resevoir of the same gas used for bubbling and the other connected to the peristaltic pump used to perfuse the recording chamber. Hypoxic and oxygenated ACSF or drug containing ACSF were switched using a two-way tap. All tubing was gas impermeable (Tygon). While it is possible that ACSF gas tensions altered during transit through the recording bath, due to contact with atmospheric gas, alterations were probably minimal as bath ACSF PO_2 and pH, the latter dependent on PCO_2, were not different from those of ACSF in the resevoir.

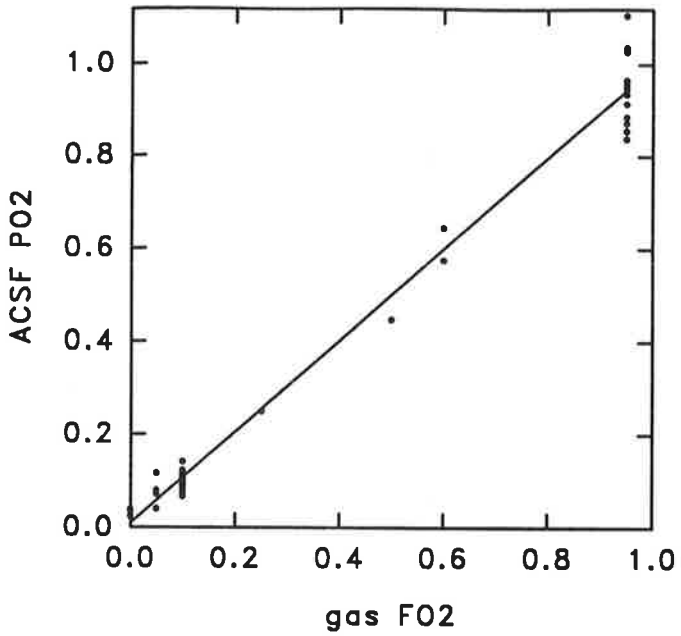

Figure 3.1 ACSF oxygen tension versus bubbling gas oxygen fraction
This figure illustrates the linear relationship between ACSF oxygen tension (PO_2) and oxygen fraction (fO_2) in the bubbling gas. Both PO_2 and bubbling gas fO_2 expressed in bar. The linear regression line was calculated with the least squares method, $r^2 = 0.99$ and slope = 0.98.

Drugs were prepared as stock solutions 100-10,000 times the final concentration in distilled water or 0.05N NaOH and added to ACSF before perfusion. The small quantity of NaOH that was added had no effect on ACSF pH. Occasionally drugs were dissolved in DMSO or ethanol, the maximum final concentrations in ACSF being DMSO 0.1%, and ethanol 0.01% (170mM). At these concentrations, neither DMSO or ethanol alone had any effect on field potentials.

3.1.4. DIFFUSION

Exchange of substances between the capillaries and tissue by diffusion is ensured by short distances and steep concentration gradients. *In vitro* tissue slices are isolated from the vascular system and diffusion distances between the ACSF and the interior of the slice are relatively large, thus permeation of substances, particularly those that are consumed by cells, will be limited. In considering diffusion into a tissue slice, the slice is considered to be a semi-infinite slab with a finite thickness surrounded by a well stirred layer of liquid (Hill, 1966). However, it is likely that the slice is not homogeneous; in particular, the the cells near the surfaces are damaged during cutting (Garthwaite *et al.* 1979), and there is an unstirred layer of liquid of approximately 40μm adjacent to the slice surfaces (Ganfield *et al.* 1970).

3.1.4.1. Oxygen

Oxygen diffusion is governed by the Fick equation with the inclusion of a term for oxygen consumption by cells in the slice (Hill, 1966).

$$\frac{dy}{dt} + a = K\frac{d^2y}{dx^2} \qquad (3.1)$$

Where y is the partial pressure of oxygen, t is time, x is distance, K is the Krough diffusion coefficient (Krough, 1919) and a is the oxygen consumption rate of the tissue. Some models suggest that the diffusion coefficient must be multiplied by α/λ^2, where α is the extracellular volume fraction and λ the tortuosity, values that may vary between viable and non-viable zones of tissue (Lipinsky, 1989). However, as respiratory gases diffuse readily through the cell membrane, α and λ can be safely ignored in the case of oxygen. Indeed, while a smaller value of K is found for animal tissues than water (Krough, 1919), the validity of the equation 3.1 is confirmed experimentally in cat cerebral cortical slices (Ganfield *et al.* 1970).

Of importance in the present context is the supply of oxygen throughout the hippocampal slice under both fully oxygenated and hypoxic conditions. It is well accepted that cells can utilise any available oxygen, and only a minimum oxygen gradient between the cell surface and the mitochondria is required to allow oxidative phosphorylation. For instance, in mitochondrial suspensions or neuroblastoma cell suspensions, a PO_2 of less than 1mmHg is required to reduce oxygen consumption (Wilson, 1990), and oxygen diffusion only departs from that predicted in brain slice areas where the tissue tension is below 2mmHg (Ganfield *et al.* 1970). At steady state, the distance into a slice where PO_2 is zero is defined by an equation derived from equation 3.1 (Hill, 1966).

$$x_i = \sqrt{2Ky_o/a} \qquad (3.2)$$

Where x_i is the distance from the slice surface and y_o is the PO_2 in the bathing medium. The following values can be used to approximate hippocampal slice oxygenation, the Krough oxygen diffusion coefficient for muscle, corrected to 32°C, of 1.57×10^{-5} ml*cm^{-1}*atm^{-1} (Hill, 1966), cortical oxygen consumption of 0.03 ml*g tissue^{-1}*min^{-1} (Leniger-Follert, 1977) and y_o of 0.95 atm for fully oxygenated ACSF, and 0.03 atm for hypoxic ACSF. Substituting these values into equation 3.2 give the distance into the slice from each surface where anoxia occurs as 315μm for oxygenated ACSF and 56μm for hypoxic ACSF, although these are only approximations. The true values may vary considerably, in particular oxygen consumption may be lower at less than physiological temperature. However these calculations suggest that, for the 400μm slices in the present study, control conditions provided adequate oxygenation throughout the slice, and this is supported by measurements in guinea pig olfactory cortex slices which demonstrated 430μm as the

limiting thickness of a slice to prevent an anoxic core at 37°C (Fujii *et al.* 1982). With hypoxic superfusion, the majority of viable cells may be anoxic, as oxygen may not penetrate beyond any unstirred ACSF layer and outer damaged cell layer.

3.1.4.2. Membrane impermeant substances

The diffusion of membrane impermeant substances through the extracellular spaces in the slice are similarly modelled by the Fick equation, with or without the term for consumption as appropriate. Generally for non-gases, the more usual diffusion coefficient k is used, which is the quotient of K and the solubility coefficient, and concentrations rather than partial pressures are used. In this case, however, extracellular volume fraction α and tortuosity λ modify diffusion, such that k is multiplied by α/λ^2. In cerebellar slices, α ranges from 0.21 to 0.28 and λ ranges from 1.55 to 1.84 and are similar to intact brain (Nicholson and Hounsgaard, 1983) but it is possible that these values may be altered in non-viable tissue zones (Lipinsky, 1989). Thus penetration of bath applied substances into the slice is slower than would be calculated for free medium, and several minutes must be allowed for equilibration. Furthermore, substances produced within the slice will accumulate in the vicinity of their source λ^2/α times greater than in free medium.

3.2. ELECTROPHYSIOLOGICAL RECORDINGS

3.2.1. RATIONALE FOR USE OF FIELD POTENTIAL RECORDINGS

For this project, extracellular recording was the preferred methodology. Extracellularly recorded population spikes and fEPSP are described in chapter 2 section 2.2.2.3, and represent the summed action potentials and EPSP respectively in a population of neurons (Andersen *et al.* 1971b; Andersen *et al.* 1978). Thus these field potentials represent graded responses, and can be used to estimate pharmacological responses from a neuronal population. The fEPSP is a graded response, like the underlying EPSP, but also represents the overall strength of synaptic transmission to all neurons contributing to the dendritic plexus at the recording site. The population spike, unlike the underlying action potentials, is also a graded response and can be used to measure alterations in synaptic input or spike threshold where only a portion of the population may be affected. Post-hypoxic and post-inhibitory hyperexcitability, as evidenced by multiple extracellular population spikes, represents multiple action potential firing in some, but perhaps not all, neurons in the recording population. This is clearly seen in the figures of hyperexcitability in slices from kainate lesioned rats (Williams *et al.* 1993) which show paired intracellular and extracellular recordings, the former not always showing multiple spikes evident in the latter. Additionally, the prevolley, for which there is no intracellular counterpart (Andersen *et al.* 1978), provides a more reliable estimate of input strength to the neuronal population than does stimulus strength.

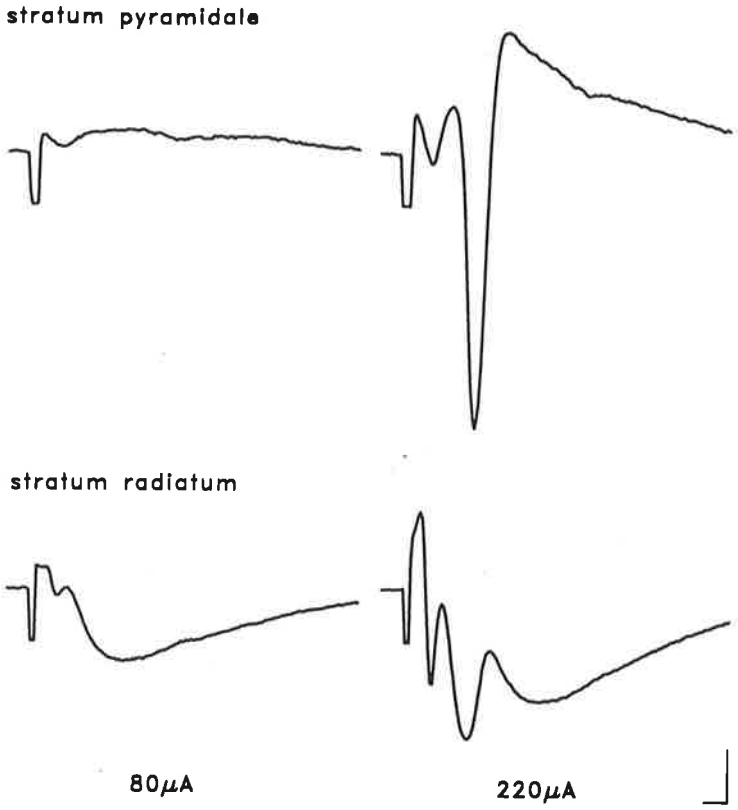

Figure 3.2 Typical averaged orthodromic field potentials
Orthodromic field potentials in the CA_1 area of the hippocampal slice, evoked by stimulation in the stratum radiatum (Schaffer collateral/commissural pathways) and recorded in the stratum pyramidale (cell body layer), top, and stratum radiatum (apical dendrite/synaptic layer), bottom. Stimulus current was set subthreshold for a population spike (left) or to evoke a population spike of 85% maximal amplitude (right). The stratum radiatum electrode records a prevolley, a compound action potential which represents the summation of spikes in the fibres of the Schaffer collateral/commissural afferent pathway and a negative fEPSP as synaptically activated current flows into the dendritic tree (Andersen *et al.* 1978). The population spike, resulting from cation flow into neuronal cell bodies, is the record of many individual spikes, the onset and duration coincide with intracellular spikes, and there is a linear relationship between population spike amplitude and the number of spikes from individual cells (Andersen *et al.* 1971b). These active current sources produce positive waves in other layers, whereby the population spike rides on a positive fEPSP in the stratum pyramidale and a positive population spike is superimposed on the negative wave of the fEPSP in the stratum radiatum. Average of three evoked potentials at 0.05Hz. Calibration bars are 1mV and 1ms, negative down. Stimulus artifacts are truncated for clarity.

Furthermore, assessment of field responses in the present studies reduces bias introduced by variations in concentrations of bath-applied drugs within a slice, this being of particular importance if the substance under study is taken up by cells to any extent (Dunwiddie and Diao, 1994). Of obvious importance, in the present context, are adenosine and oxygen which are taken up and metabolised by cells.

Such field potential recording is a robust technique, recordings are easy to obtain, remain stable over long periods, and experimentally induced hyperexcitability can be reliably assessed. Induction of hyperexcitability requires prolonged hypoxia or drug superperfusion, and the pharmacological assessment of receptor function requires

long periods of recordings during which baseline responses can be safely presumed stable.

3.2.2. INSTRUMENTATION OF THE SLICE

Glass microelectrodes for extracellular recording were pulled from 1.5mm capillary glass with internal filament (World Precision Instruments Inc.) using a Brown-Flaming microelectrode puller (Sutter Instrument Co.). Electrodes were filled with ACSF and the tips broken back to give a resistance of 1-3MΩ. and held with 3M KCl filled Ag/AgCl half-cell holders (World Precision Instruments Inc.). Two unity voltage gain headstages with current gains of x1 (stratum radiatum) and x10 (stratum pyramidale) were used in conjunction with an Axoclamp-2A dual channel microelectrode clamp (Axon Instruments). Axoclamp output from the stratum radiatum electrode was filtered at 30kHz low pass and from the stratum pyramidale at 1kHz low pass. Recording electrodes were referenced to a Ag/AgCl ball electrode at one end of the recording chamber. Monopolar stimulating electrodes were tungsten needles, coated with a two part epoxy resin leaving only the tip exposed. Stimulating electrodes were referenced to a fine Ag/AgCl wire running the length of the recording chamber beneath the slices.

The slice was trans-illuminated with a fibre-optic light source (Volpi) below the recording chamber and viewed at 6-30 times magnification through a conventional stereomicroscope (Wild Heerbrugg). The headstage and electrode assemblies were moved using a combination of 3-dimensional mechanical micro-manipulators and either 1-dimensional or 3-dimensional hydraulic micro-manipulators attached to magnetic pillar stands (Narishige Scientific Instrument Laboratory). The anatomy of the recording area in the CA_1 has been discussed in detail in chapter 2, and the electrode placement illustrated in figure 2.2 is identical to that used in this study. Recording electrodes were placed under visual control in the stratum pyramidale and stratum radiatum of the CA_1 subfield of the hippocampal slice. A tungsten electrode placed in the stratum radiatum, on the CA_2 side of, and approximately 0.5mm from the recording electrode in this layer, was used to stimulate the Schaffer collateral/commisural fiber pathway, the major excitatory input to the CA_1 pyramidal neurons. In addition, this stratum radiatum stimulating electrode would activate the local interneurons evoking monosynaptic feedfoward inhibition to pyramidal neurons. For some experiments, a second stimulating electrode, for antidromic stimulation of the pyramidal neurons, was positioned in the alveus above the stratum pyramidale recording electrode.

Field potentials were evoked in response to constant current stimuli of 0.2ms duration provided by a S-88 dual channel, solid-state square wave stimulator (Grass Instrument Co.) via PSIU6 photoelectric stimulus isolation units (Grass Instrument

Co.). Pathways were stimulated at 0.05Hz to avoid the production of multiple population spikes from each orthodromic stimulus, which occured at stimulus rates of 0.1Hz or higher. Antidromic and orthodromic pathway stimulation were alternated with a 10s interstimulus interval.

3.2.3. DATA ACQUISITION AND ANALYSIS

3.2.3.1. Recording of evoked field potentials

Field potentials were referenced to the recording bath fluid, considered to be 0mV. The Axoclamp-2A output was digitised (DASH-16F analogue/digital I/O expansion board, Metrabyte), recorded to disk for off-line analysis, and displayed on an 80286 microcomputer-based data acquisition and analysis system (Asystant+, Asyst Software Technologies, Inc.). The three waveforms recorded over one minute were averaged for data analysis. Figure 3.2 shows typical averaged submaximal orthodromic field potentials recorded in the stratum pyramidale and stratum radiatum, as described in 2.2.2.3.

3.2.3.2. Field potential measurements

Data and field potential analysis was a modification of the method described by Balestrino *et al.* 1986. Figure 3.3 shows the various components of the post-hypoxic field potentials and how they were measured. These post-hypoxic field potentials differ from pre-hypoxic field potentials in that the former is typified by two populations spikes, while a single population spike is normal for pre-hypoxic recordings. The stratum radiatum field potential in figure 3.3 B. consists of a stimulus artifact, followed by a biphasic prevolley a-b-c, this negative going spike is preceded by a smaller negative going spike which is often masked by the stimulus artefact, as is the case here. Prevolley amplitude is measured from point a to b, as the late upward-component is truncated by the onset of the dendritic field excitatory postsynaptic potential (fEPSP). The fEPSP is the slowly decaying negative wave c-d-e, on which are superimposed, at higher stimulus strenghts, positive waves that result from current flow to the population spikes in the cell soma. fEPSP rate of rise was measured as the maximum slope between c and d, calculated as a first order derivative after digital smoothing through a 6kHz low pass filter. fEPSP rate of rise was contaminated by the prevolley, but since the prevolley was linear with stimulus current yet otherwise unaffected by any treatments in this study, it was ignored for input/output experiments, but the contribution of the prevolley to the fEPSP rate of rise was subtracted for time course experiments, see section 3.2.3.4. The fEPSP amplitude was approximated by subtracting d from the baseline. To measure fEPSP area, a line was fitted manually from point d to exclude any positive wave forms, leaving a pure fEPSP, uncontaminated by the current flow to the population spikes, this is the straight line in figure 3.3 B. This monophasic waveform was inverted, as

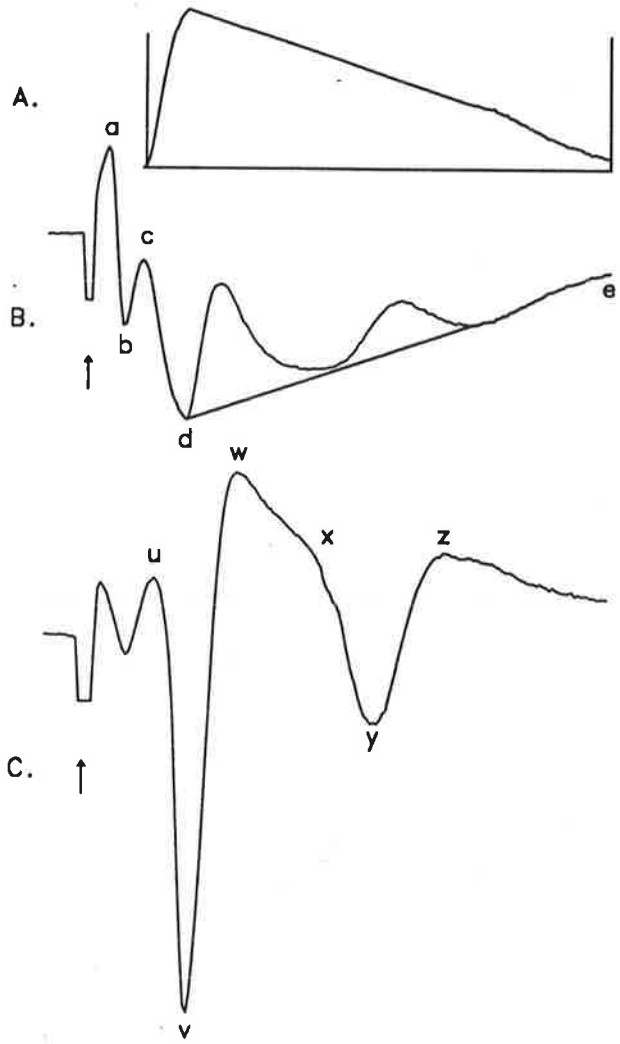

Figure 3.3 Measurement techniques for fEPSP and population spikes
Illustration of the method of field potential analysis, details of these methods are in the text. A. inverted fEPSP section of stratum radiatum field potential used for calculation of fEPSP area. B. Post-hypoxic stratum radiatum field potential showing the prevolley a-b-c and the fEPSP c-d-e. C. Post-hypoxic stratum pyramidale field potential showing two population spikes u-v-w and x-y-z. Small arrows indicate the stimulus artifacts, which have been truncated for clarity.

shown in figure 3.3 A, and the area under the resulting curve integrated to give fEPSP area. Duration of the fEPSP was measured at 20% amplitude, as the wave tends to asymptote and an impractically long recording time was necessary to monitor return to reference voltage.

Figure 3.3 C. shows two orthodromically evoked population spikes, u-v-w and x-y-x, recorded in the pyramidal cell layer. The amplitude of these spikes was measured as the average of the two positive peaks minus the negative peak. The onset of the second population spike at point x was chosen manually as the inflexion point on the descending limb from w where dy'/dx' increased. Later population spikes, if present, were not included in analysis. These population spikes are superimposed on a slow positive wave, the rise from baseline to w and back, that results from current flow to

the fEPSP in the dendrites. The first population spike is preceded by the rising phase of the fEPSP, contaminated by a small prevolley, and the stimulus artifact. Antidromic stimulation evoked a single negative population spike, not pictured, whose onset was often obscured by the stimulus artifact, necessitating that its amplitude measurement taken from the peak negativity to the second positive peak. The waveform events indicated by the letters in figure 3.3 show reliable temporal coincidence between subsequent waveforms evoked in a slice and good coincidence between slices, which aids comparisons before and after treatments and pooling of slice data. Some drugs, notable 8-CPT, cause an earlier onset of the second population spike, requiring adjustment of point x during data analysis.

3.2.3.3. Input/output curves

Input/output curves were constructed by recording orthodromic field potential response to a series of stimulus currents from below threshold for, to supramaximal for population spikes and the relationships shown in figure 3.4 plotted. 1) The prevolley versus stimulus current relationship provides an indication the polarisation of the axonal membrane, the prevolley only being lost in the case of loss of membrane potential (Sick *et al.* 1987). The prevolley varies linearly with the range of stimulus currents used in this study, growing as more axons are recruited. 2) fEPSP rate of rise versus prevolley amplitude measures the strength of synaptic transmission. The fEPSP, reflecting the overall strength of individual EPSPs, directly measures synaptic transmission and is a combined measure ot transmitter release and sensitivity of the postsynaptic receptors, and varies linearly with the prevolley (Andersen *et al.* 1978). 3&4) The amplitude of each population spike versus prevolley amplitude, as well as against fEPSP rate of rise, indicates overall synaptic efficiency and postsynaptic excitability respectively. These are true input/output curves, with input defined here as either synaptic input (prevolley) or synaptic activation (fEPSP). While there is a linear relationship between population spike amplitude and the number of spikes from individual cells (Andersen *et al.* 1971b), the individual neurons only fire spikes above threshold, and these input/output relationships approximate sigmoid curves (Rall, 1955). It is assumed that the excitatory synapses on individual neurons and neuron threshold are both distributed approximately normally, and it follows that the distribution of neurons with respect to both input and threshold will also be normal. Therefore, the fraction of the neuronal population that will fire spikes (as measured by population spike amplitude) plotted against increasing overall input will be the integral of this approximately normal probability density distribution, and thus approximate a sigmoid curve (Rall, 1955).

The first population spike versus the prevolley indicates overall synaptic efficiency. Variations in both the number of activated excitatory synapses on individual neurons

and variations in threshold neuronal threshold will modify this input/output curve. The first population spike versus the fEPSP input/output curve will be modified by alterations in threshold of the underlying neuronal population. Multiple population spikes reflects firing of a multiple action potentials in some of the underlying neuronal population. This is supported by the good temporal coincidence of multiple population spikes and multiple action potentials seen in paired intracellular and extracellular recordings in the hippocampal slice (Meier *et al.* 1992; Williams *et al.* 1993). This multiple firing suggests that neurons are remaining depolarised above threshold for longer duration, either as a result of prolonged excitatory input or reduced inhibition of the postsynaptic neurons. The second population spike versus the prevolley input/output curve could be modified by prolonged activation of AMPA receptor channels or increased activation of NMDA receptor channels. On the other hand, the input/output curves describing the second population spike against either the prevolley or the fEPSP could be modified by changes in the duration of neuronal threshold independent of excitatory input, such as by variation in inhibition or in intrinsic membrane currents.

Input/output curve analysis was essentially as described in the literature (Balestrino *et al.* 1986; Skelton *et al.* 1983), with the modification that second population spike amplitude was also included. For the input/output curves predicted to be linear, the slope of the line of best fit was calculated using the least squares linear regression method. The areas bounded by the approximately sigmoid curves and the abscissa were estimated as previously described (Skelton *et al.* 1983; Balestrino *et al.* 1986), using the following formula.

$$\text{AREA} = \frac{(Y_1+Y_2)(X_2-X_1)}{2} + \frac{(Y_2+Y_3)(X_3-X_2)}{2} + \ldots + \frac{(Y_{n-1}+Y_n)(X_n-X_{n-1})}{2} \qquad (3.3)$$

When comparing curve areas before and after treatment, the same range of abscissa values was used. Input/output curve data for any treatment is shown as a percentage of the pretreatment value, with the exception of the input/output curves for the second population spike which, often being zero pretreatment, were expresses as a percentage of the first population spike pre-treatment input/output curve value.

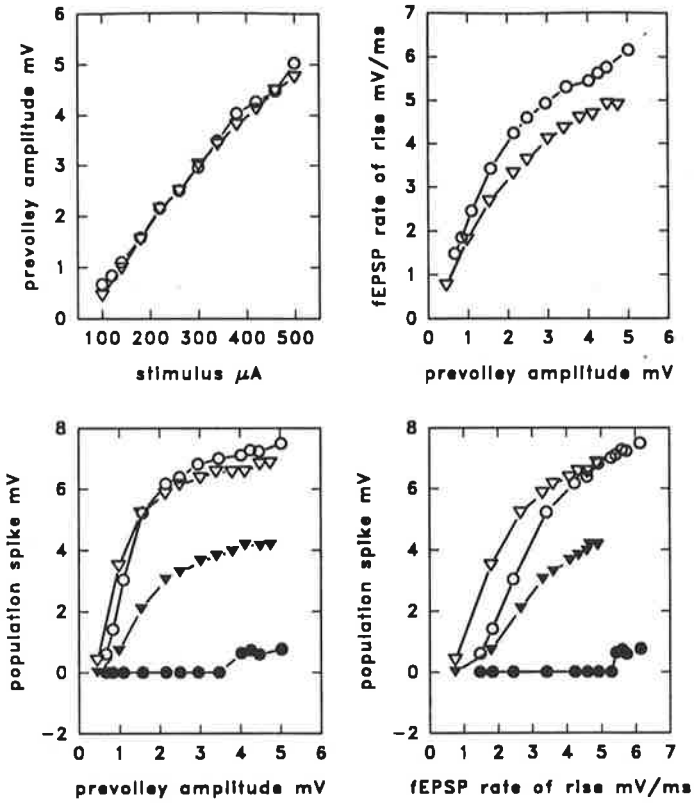

Figure 3.4 Input/output curves of CA_1 field potentials
Input/output curves of field potentials constructed from recordings of prevolley, fEPSP and first orthodromic population spike taken before, open circles, and one hour following 30 minutes of hypoxia, open triangles. The filled symbols are curves constructed from measurements of the second orthodromic population spike.

3.2.3.4. Time course studies

To measure the time course of action of experimental treatments such as hypoxia or drugs during their application to the slice, the stimulus was adjusted to give a first population spike amplitude of 85% of maximum amplitude. This just submaximal response was used to allow identification of changes in excitability without using the more definitive, but time consuming, input/output curve method. Supramaximal responses may mask some minor depressive effects of agents, while just submaximal responses ensures that secondary spikes will be evident if hyperexcitability occurs. Field potentials were recorded for 2 minutes (6 stimuli to each pathway) before introduction of hypoxic or drug containing ACSF. Field potentials were expressed as a percentage of the first minute minute average, with the exception of the second population spike which is expressed as percentage of the initial minute value of the first population spike amplitude. Thus the second minute value is also a control value which, being normally distributed, was used for paired comparisons. Since the prevolley rides on the initial slope of the fEPSP it contaminated the fEPSP rate of rise measurements. The contribution of prevolley to fEPSP rate of rise was subtracted by measuring the fEPSP, at the end of experimentation, under conditions of negligible

synaptic transmission in ACSF containing 4mM magnesium and 0.24mM calcium, and the minimum fEPSP rate of rise obtained was then subtracted from all previous measurements.

3.3. PRESENTATION AND STATISTICAL ANALYSIS OF DATA

Normalised data is presented as mean% ± standard error of the mean (S.E.M.) Raw data is presented as mean ± standard deviation (S.D.). Values of n, generally $n=5$, are stated in text.

Statistics analysis was carried out by paired or unpaired Student's t-test where appropriate, or by 1-way or 2-way analysis of variance (ANOVA) followed by a modified t-test, using the ANOVA within samples mean sum of squares as a pooled estimate of variance and the within sample degrees of freedom to determine the critical t statistic value. Unless stated otherwise in the text, significance was assumed at $P \leq 0.05$ for single comparisons or $P \leq 0.01$ for multiple comparisons, 1-tailed tests. Unless otherwise stated, P values in the text are for 1-tailed tests. When normalised data from different series of slices were compared, raw pre-treatment data was analysed by ANOVA or unpaired t-test to verify that the control populations were the same.

3.4. ETHICAL CONSIDERATIONS

All experimental work involving animals conformed to National Health and Medical Research Council animal usage guidelines, and was approved by the Animal Ethics Committees of the University of Adelaide and the Institute of Medical and Veterinary Science.

4. ACUTE ACTIONS OF HYPOXIA

4.1. INTRODUCTION

For a number of years, the hippocampal slice has been used as a model for investigating the acute effects of hypoxia, including hypoxic depression of synaptic transmission, acute excitotoxicity, and the selective vulnerability of the CA_1 pyramidal neurons. Recently, the use of 10mM glucose has become standard for incubation of hippocampal slices, as it provides more stable electrophysiological responses than lower glucose levels. Nevertheless, some laboratories use a more physiological level of 4mM glucose, but hypoxia in the presence of 4mM glucose, is similar to an ischaemic insult, as discussed in chapter 2 section 2.4.5.4. Consequently, even brief hypoxia in low glucose results in hypoxic spreading depression and irreversible loss of electrophysiological function in the CA_1 (Kass, 1987; Kass and Lipton, 1986). However, considerable work using 10mM glucose has established that even prolonged hypoxia is well tolerated in the *in vitro* hippocampal slice, where synaptic transmission is depressed during hypoxia but recovers with reoxygenation (Fujiwara *et al.* 1987; Schiff and Somjen, 1985). It is also likely that tolerance to hypoxia is further improved with lower incubation temperatures (Taylor and Weber, 1993).

In the hippocampal slice and the spinal cord, the causal relationship between hypoxic release of adenosine and hypoxic depression of synaptic transmission has been well established (Gribkoff *et al.* 1990; Fowler, 1989; Gribkoff and Bauman, 1992; Fowler, 1993; Lloyd *et al.* 1988). Adenosine has also been proposed as a "retaliatory metabolite" (Newby *et al.* 1990), where activation of A_1 receptors *in vivo* is associated with protection of neurons against ischaemic excitotoxic damage. Irreversible loss of synaptic transmission following ischaemia/hypoxia in the hippocampal slice is an indication of acute excitotoxic neuronal damage (Lobner and Lipton, 1993). It is possible that extracellular accumulation of adenosine in the hippocampal slice provides protection of CA_1 pyramidal neurons against excitotoxicity, and contributes to the recovery of synaptic transmission following hypoxia.

In the present model, using 10mM glucose at 32°C, depression of synaptic transmission during prolonged hypoxia, and recovery of synaptic transmission with reoxygenation was examined in detail, to establish the viability of hippocampal slices for further investigation. Adenosine accumulation and adenosine A_1 receptor-mediated depression of synaptic transmission during hypoxia was verified, and the role of this depression in the survival of pyramidal neurons exposed to hypoxia was evaluated. In addition, an estimate was made of the concentration of adenosine that

is achieved during hypoxia in the vicinity of the A_1 receptors on the excitatory synaptic terminals. Thus, it is proposed that superfusion of hippocampal slices with hypoxic ACSF causes a rapid, reversible depression of synaptic transmission; and the CA_1 pyramidal neuron population survives 30 minutes of such hypoxia, as illustrated by full recovery of synaptic transmission. Furthermore, the depression of synaptic transmission during hypoxia, being due to adenosine A_1 receptor activation, is blocked by selective adenosine A_1 receptor antagonists. Such A_1 receptor activation during hypoxia is the result of an enormous extracellular accumulation of adenosine. Finally, antagonism of adenosine A_1 receptor-mediated neuronal depression during hypoxia does not prevent full recovery of synaptic transmission in the CA_1 area.

4.2. METHODS

In the following investigations, hippocampal slices were exposed to only a single drug or hypoxic treatment, thus all comparisons were between different slices, prepared from different animals. In the figures illustrating time course of drug or hypoxia actions, the first two time points are always recorded in drug free, normoxic ACSF, and the first time point is considered baseline. All data is presented as a percentage of the baseline recording. When used, antagonists were superfused onto hippocampal slices for five minutes prior to superfusion with hypoxic ACSF. Input/output curves were typically recorded over 12 minutes. Input/output recordings were made immediately prior to treatment of slices with drugs or hypoxia and then again after one hour of reoxygenation and wash with normoxic ACSF. Headspace gas analysis of ACSF samples for carbon monoxide (CO) was performed by gas chromatographic separation of gases on a molecular sieve column and catalytic conversion of CO to methane for flame ionisation detection. 8-cyclopentyl-1,3-dimethylxanthine (8-cyclopentyltheophylline, 8-CPT), 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX) and cyclopentyladenosine (CPA) were purchased from Research Biochemical Incorporated, 2,4 dinitrophenol (DNP) and glibenclamide were purchased from Sigma, adenosine was obtained from Calbiochem and 2-OH-saclofen was provided by Dr. D. I. B. Kerr. 8-CPT and DNP stock solutions were made in 0.05N NaOH, CPA and glibenclamide stock solutions were made in ethanol, and DPSPX and adenosine stock solutions were made in distilled water. 2-OH-saclofen was dissolved in equimolar NaOH then diluted directly into ACSF.

4.3. RESULTS

4.3.1. HYPOXIA

4.3.1.1. Acute effects of hypoxia

Superfusion of hippocampal slices with hypoxic ACSF caused a rapid, reversible depression of synaptic field potentials, as can be seen in figure 4.1, where the

postsynaptic field potentials are lost while the prevolley is preserved. Upon reoxygenation, synaptic transmission returned to pre-hypoxic levels, but was characterised by appearance of a second population spike; this post-hypoxic hyperexcitability will be addressed in detail in chapter 6. ACSF PO_2 above 450mmHg, produced by gassing with at least 60% O_2, was required to maintain synaptic transmission, any ACSF equilibrated with 50% O_2 or less, caused loss of synaptic transmission. In these studies, hypoxic ACSF was produced by gassing with 95% N_2 / 5% CO_2, unless otherwise indicated. Figure 4.2 shows the time course of the alterations of field potentials induced by superfusion of slices with hypoxic ACSF for 30 minutes (n = 5) compared to a separate set of slices never exposed to hypoxia (n = 5). Evoked field potentials from hippocampal slices not exposed to hypoxic ACSF remained relatively unchanged over a 40 minute time period, figure 4.2. However, in slices exposed to 30 minutes of hypoxic superfusion, the fEPSP and population spike were maximally depressed by 7 and 4 minutes respectively following the switch from normoxic to hypoxic ACSF, and remain depressed throughout hypoxia. Figure 4.3 shows the minimum amplitudes or rate of rise of the field potentials for these same slices. The fEPSP rate of rise in the apical dendrite field was depressed to 5.53 ± 0.68% S.E.M. (n = 5), of its baseline value during hypoxia, this non-zero value results from some contamination of the fEPSP by the prevolley, the latter unaffected by hypoxia; thus, synaptic transmission was probably more completely depressed. The population spike, which measures the summed action potentials in the neuronal population was not evident during hypoxia (n = 5). Both orthodromic field potentials were significantly smaller during hypoxia than the minimum field potentials measured in slices exposed to an identical period of normoxic incubation $P \leq 0.01$. Non-synaptic field potentials were less sensitive to hypoxia, as the antidromic population spike amplitude declined to 79.7 ± 6.2% S.E.M. (n = 5) over 10 minutes, followed by a slower decline to a minimum of 69.7 ± 56.0% S.E.M. (n = 5), just prior to reoxygenation, which was significantly smaller than the minimum amplitude in normoxic slices, $P \leq 0.01$, figures 4.2 and 4.3. The prevolley, a compound action potential which measures afferent input to the CA_1 neurons, was not depressed by hypoxia. Thus, excitatory synaptic transmission and, to a much lesser extent, postsynaptic excitability are depressed by hypoxia, but axonal conduction of action potentials is unaffected. Orthodromic and antidromic field potentials began to recover within two minutes of reintroduction of normoxic ACSF and full recovery took 7 minutes.

4.3.1.2. Input/output curves

Further analysis of synaptic transmission was achieved by producing input/output curves of field potentials immediately before and one hour following hypoxia. Such input/output curves examine alterations in synaptic transmission and neuronal

threshold (Balestrino *et al.* 1986). Figure 4.4 shows sample input/output curves produced before, and one hour following 30 minutes hypoxia, and shows that, in these slices, synaptic transmission was not altered by hypoxia. Figure 4.5 shows the mean slopes or areas of the post-treatment input/output curves of field potential components and additionally the mean maximum first population spike amplitude for slices exposed either to 30 minutes hypoxic superfusion (n = 5) or to continual normoxic incubation (n = 5). These post-treatment values are expressed as a percentage of the pre-treatment values, analysis of raw pre-treatment data shows that these two series of slices form one population, so post-treatment data can be compared. Post-treatment input/output curves did not differ between hypoxia and normoxia, illustrating that axonal conduction, synaptic transmission, and postsynaptic spike threshold are not differently affected by hypoxia or normoxia. Maximum population spike amplitude of post-hypoxic slices was slightly, but significantly, greater than that from normoxic slices ($P \leq 0.05$, n = 5), suggesting that the neuronal population is as well preserved following hypoxia as following normoxic incubation. It is apparent in this figure that the area under the curves relating population spike amplitude to fEPSP rate of rise in both series of slices was greater than 100%, showing this relationship increased with time; however, neither the population spike amplitude or the fEPSP rate of rise increased in comparison to the prevolley. Since neither postsynaptic field potential varied with the prevolley, despite an alteration in their inter-relationship, this suggests that the increase in population spike versus fEPSP is an artefact; indeed, this was typical of most slices. The explanation of this seems to be the slight deterioration of the fEPSP recording with time, insufficient to alter the slope of its relationship with the prevolley, that caused a small left shift in the curve relating population spike to fEPSP, evident in figure 4.4. Since areas were calculated using the same range of abscissa values, this resulted in raised post-treatment area. It is unlikely that this represents a true increase in postsynaptic excitability with time.

4.3.1.3. Miscellaneous hypoxic exposures

To further explore the resilience of the hippocampal slice preparation, some slices were exposed to two 30 minutes superfusions with hypoxic ACSF (n = 3) separated by 90 minutes. Slices survived the second hypoxic exposure with synaptic transmission being preserved. One slice was exposed to hypoxia at 36°C for 15 minutes and synaptic transmission recovered upon reoxygenation. Longer periods of hypoxia near physiological temperature were not examined.

4.3.2. CARBON MONOXIDE

While PO_2 was not measured within the slice during hypoxia or normoxia, it was assumed to be lower than bath PO_2, due to the diffusion limitations imposed by the

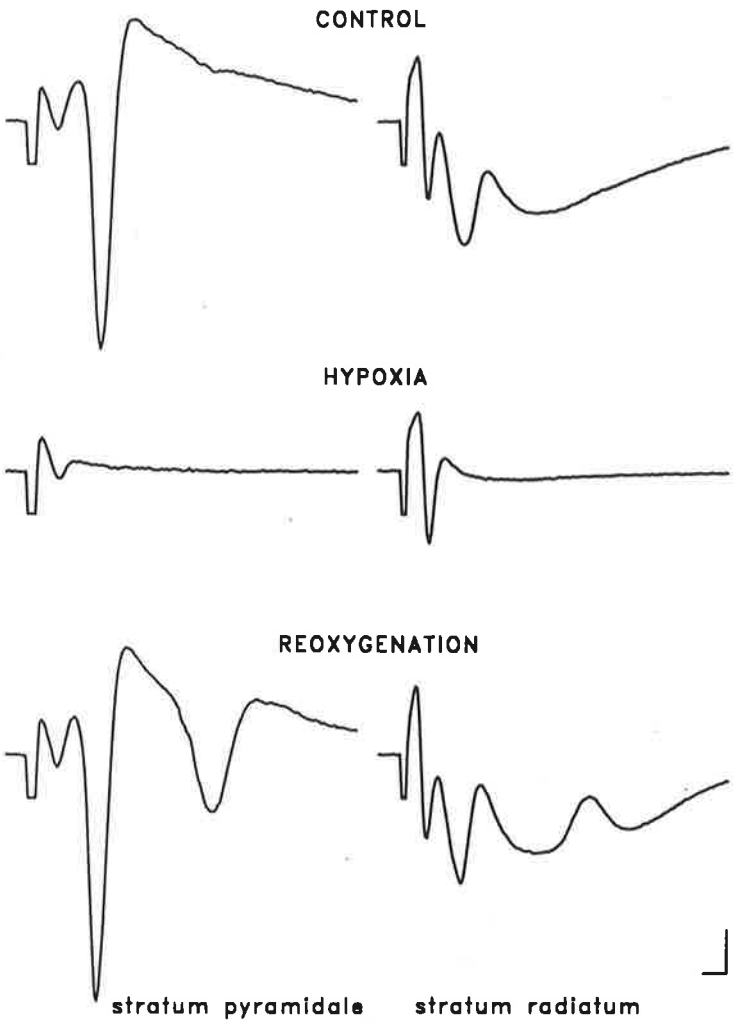

Figure 4.1 Effects of superfusion of hypoxic ACSF on synaptic field potentials in the hippocampal slice
Synaptic field potentials in the CA_1 area evoked by Schaffer collateral/commissural stimulation. The left column shows recordings from the stratum pyramidale (cell body layer) and the right column shows recordings from stratum radiatum (apical dendrite/synaptic layer). From top to bottom are shown respectively response from before, during and 1 hour following 30 minutes of hypoxia. Calibration bars are 1mV and 1ms, negative down. Stimulus artefacts are truncated for clarity.

slice thickness of 400μm and by oxygen consumption, as discussed in chapter 3. It is presumed that the near, or total, anoxia achieved within the slice with hypoxic superfusion inhibits oxidative phosphorylation and alters neuronal energy homeostasis. In order to confirm hippocampal slice survival under severe inhibition of oxidative phosphorylation, investigations of histotoxic hypoxia (Brierley, 1976) were carried out, induced by inhibiting the electron transport chain with CO, and also by uncoupling electron transport from ATP production with DNP.

CO toxicity is associated with delayed neuronal degeneration which resembles ischaemic or hypoxic brain damage (LaPresle and Fardeau, 1967; Plum *et al.* 1962). CO toxicity *in vivo* is generally thought to be a result of tissue hypoxia due to failure of oxygen delivery as a result of the greater affinity of haemoglobin (Hb) for CO than

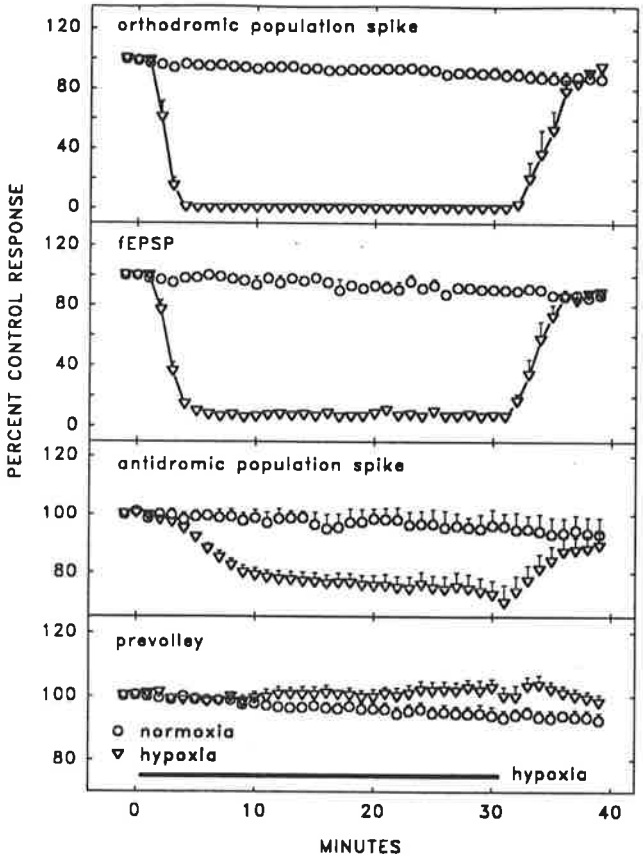

Figure 4.2 Time course of hypoxic alterations in field potentials in the CA_1 area
Field potentials in hippocampal slices before, during and after 30 minutes of hypoxia alone (n = 5) and during a similar period of normoxic incubation in slices never exposed to hypoxia (n = 5). Hypoxia was produced by superfusion of slices with hypoxic ACSF for 30 minutes as indicated by the bar at bottom. Schaffer collateral/commissural fibres were stimulated at 0.05Hz and each three consecutive field potentials averaged to give minute averages. Values are expressed as a percentage of the baseline (first minute average) response. Points represent the mean ± S.E.M. (n = 5) for each condition, where no error bars are evident, they are smaller than the symbol. fEPSP values represent the percentage of the baseline rate of rise of the initial segment and all other values are percentage of baseline amplitude of the respective field potentials. Hypoxia caused significant depression of fEPSP, orthodromic population spike and antidromic population spike compared to normoxia. In slices not exposed to hypoxia, field potential values remained close to 100% of baseline.

for oxygen; however, there is evidence to suggest that CO has direct toxic effects, independent of COHb-induced hypoxia (Haldane, 1927; Piantadosi *et al.* 1988; Orellano *et al.* 1976; Raybourn *et al.* 1978). A possible cellular target for CO in producing direct toxic effects would be cytochrome a_3, part of the terminal electron carrier complex in the mitochondrial respiratory chain. This complex is known to bind CO (Chance, 1953), and the resulting block in respiration would cause histotoxic hypoxia (Brierley, 1976).

The hypoxic ACSF produced here, to compare with nitrogen or CO, was gassed with 10% oxygen and had a mean PO_2 of 79 ± 12 mmHg (n = 7). Headspace analysis indicated that the hypoxic ACSF prepared with 85% CO in the bubbling mixture was

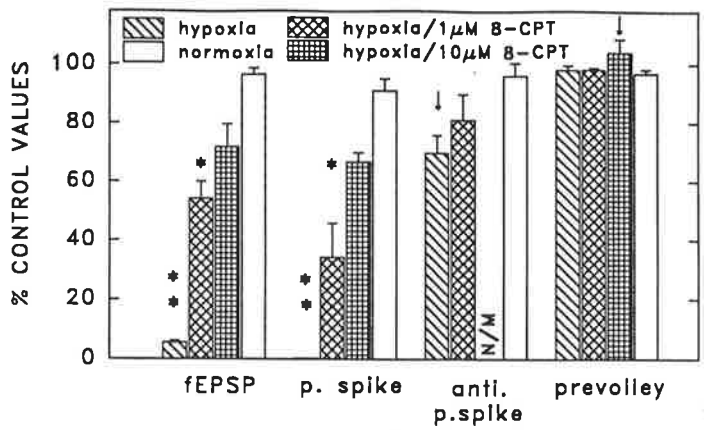

Figure 4.3 Minimum values of field potentials during normoxia and hypoxia with and without adenosine A_1 receptor antagonism
Shows the minimum values, mean + S.E.M., of the field potential components during hypoxia alone (n = 5), hypoxia in the presence of 1μM 8-CPT (n = 5) and hypoxia in the presence of 10μM 8-CPT (n = 3), compared to time matched values during normoxia (n = 5), each condition tested in different slices. Values are expressed as percentage of baseline as in figure 4.2. Minimum values for the fEPSP, population spike, antidromic population spike and prevolley during hypoxia are 5.53 ± 0.68%, 0 ± 0%, 69.73 ± 56.04%, and 98.03 ± 1.56% respectively, for hypoxia in the presence of 1μM 8-CPT are 54.09 ± 5.85%, 34.14 ± 11.47%, 80.98 ± 8.91%, and 98.32 ± 0.64% respectively. Minimum values for the fEPSP, population spike and prevolley during hypoxia in the presence of 10μM 8-CPT are 73.74 ± 7.83%, 66.55 ± 3.08% and 104.10 ± 4.55%, respectively. Double asterisks denotes values that are significantly smaller than all other values, $P \leq 0.01$. Single asterisk denotes values significantly smaller than 10μM 8-CPT and normoxia, $P \leq 0.01$. Arrows denote significantly different from normoxia, $P \leq 0.01$. N/M indicates that antidromic spike was not measured.

saturated with this gas, giving a CO/O_2 ratio of approximately 8 based on the similar solubilities of these gases (Altman and Dittmer, 1971). Assuming similar diffusion coefficients for these gases, tissue CO levels should greatly exceed oxygen and these two gases compete approximately equally for cytochrome a_3 binding (Coburn, 1979; Haab, 1990).

Hypoxic ACSF equilibrated with CO produced a depression of synaptic transmission identical to that in nitrogen-induced hypoxia. Synaptic transmission, as assessed from input/output curves, one hour following 30 minutes superfusion with hypoxic ACSF, prepared using either CO or nitrogen, is shown in figure 4.6. No significant difference existed between the post-hypoxia values of any input/output curves or maximum population spike height recorded from slices exposed to either nitrogen (n = 5) or CO (n = 5) as the diluent gas ($P \leq 0.05$). Superfusion of slices with ACSF containing 2% CO (n=16), 10% CO (n=8) or 35% CO (n=1), in the presence of greater than 60% oxygen, did not acutely depress, or have any other effects on, synaptic transmission, data not shown. This indicates that, in the hippocampal slice, CO is no more toxic than nitrogen.

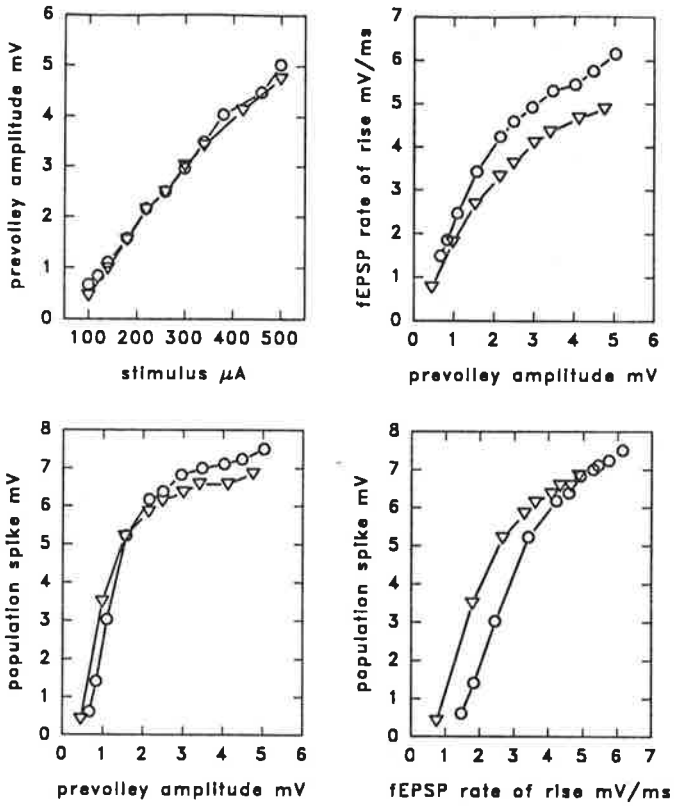

Figure 4.4 Input/output curves
Input/output curves of field potentials from a representative slice constructed before, circles, and one hour following 30 minutes of hypoxia, triangles. Input/output curves were constructed from measurements of prevolley amplitude , fEPSP rate of rise and first orthodromic population spike amplitude recorded over a range of stimulus currents. Each point is measured from an average of three responses evoked using 0.05Hz stimulation.

4.3.3. 2,4-DINITROPHENOL

A further strategy to mimic anoxia was to inhibit mitochondrial oxidative phosphorylation using DNP which dissipates the proton electrochemical gradient across the inner mitochondrial membrane and thus uncouples mitochondrial respiration from ATP production. As with hypoxia, thirty minutes superfusion with normoxic ACSF containing DNP 50 μM (n = 2) produced a rapid, reversible depression of synaptic transmission, as evident in figure 4.7. The onset of synaptic depression was identical with DNP and hypoxia, but the highly lipophilic DNP was slow to washout, and synaptic transmission only returned slowly with reoxygenation, but did recover fully with continued wash. 10μM DNP similarly caused a complete, reversible inhibition of synaptic transmission (n = 1), this slice tolerated two separate 30 minute DNP exposures with complete recovery of synaptic transmission, data not shown. The CA_1 pyramidal neuron population survives prolonged inhibition of energy production by mitochondrial uncoupling.

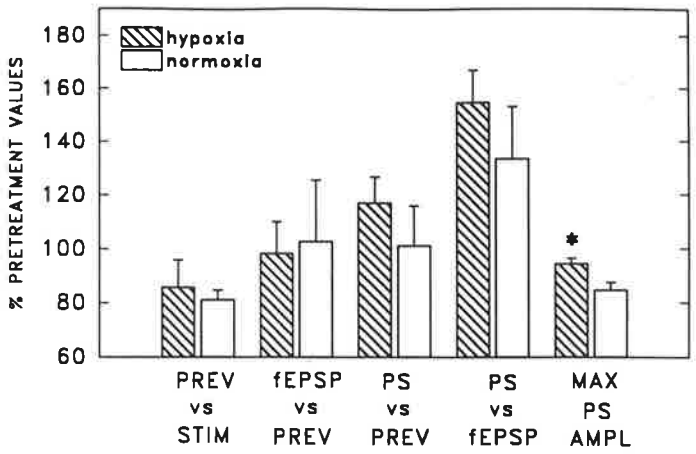

Figure 4.5 Comparison of input/output curves following hypoxia or normoxia
Slopes or areas of the post-treatment input/output curves of prevolley amplitude (PREV), fEPSP rate of rise (fEPSP) and first population spike amplitude (PS) and additionally the maximum first population spike amplitude (MAX PS AMPL) taken one hour following 30 minutes of either hypoxic and normoxic superfusion. Post-treatment values are expressed as a percentage of the pre-treatment input/output or maximum population spike amplitude values for the same slice, mean ± S.E.M. (n = 5). There was no significant difference between the post-treatment input/output curves for hypoxia or normoxia. Asterisk indicates that the maximum population spike amplitude was significantly greater following hypoxia than that from normoxic slices ($P \leq 0.05$).

4.3.4. ADENOSINE A_1 RECEPTOR ANTAGONISM AND HYPOXIA

4.3.4.1. Acute effects of hypoxia during A_1 receptor antagonism

Hypoxic inhibition of synaptic transmission is due largely to activation of A_1 receptors by an increase in extracellular adenosine. Adenosine A_1 receptor blockade during hypoxia therefore resulted in reduced hypoxic depression of field potentials compared to slices exposed to hypoxia alone, figure 4.8. The methylxanthine 8-CPT was used throughout this study as an adenosine A_1 receptor antagonist. 8-CPT has considerable affinity and selectivity for the A_1 receptor in binding assays, A_1 K_i 10.9nM versus A_2 K_i 1400nM (Bruns *et al.* 1986), and has been shown to have an apparent affinity (K_d) of 42nM for the A_1 receptor against agonist induced depression of synaptic transmission in the CA_1 area (Dunwiddie and Fredholm, 1989).

Pre-treatment of hippocampal slices with 1μM 8-CPT for 5 minutes caused a small but significant increase in population spike amplitude to 116.3 ± 2.2% S.E.M. of baseline and in fEPSP rate of rise to 105.2 ± 3.2% S.E.M. of baseline (n = 5, $P \leq 0.01$). During subsequent superfusion of hypoxic ACSF in the presence of 8-CPT, the fEPSP was only reduced to 54.1 ± 5.9% S.E.M of baseline, and the population spike was still evident at 34.1 ± 11.5% S.E.M. (n = 5) of baseline. These values were significantly smaller than time matched normoxic values, but larger than hypoxia alone, $P \leq 0.01$, see figure 4.3. The antidromic population spike declined to 85.4 ± 5.6% S.E.M. of its baseline value by 10 minutes of hypoxia, which was not different from either hypoxic or normoxic time matched values, figure 4.3.

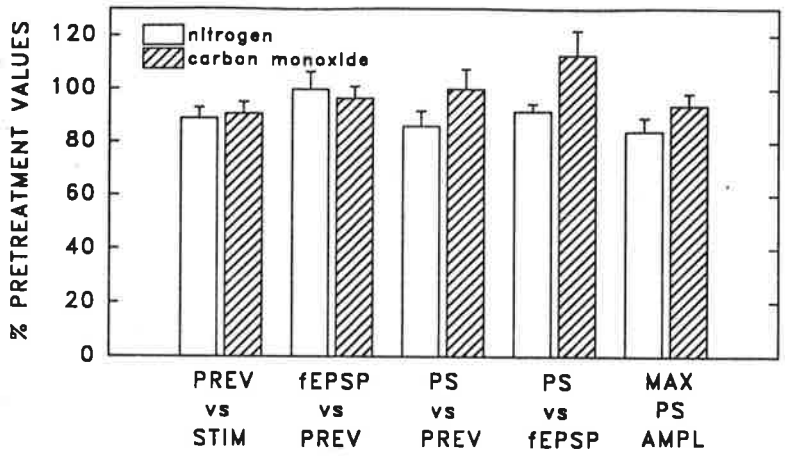

Figure 4.6 Comparison of input/output curves following nitrogen-induced or carbon monoxide-induced hypoxia
Slopes or areas of the post-treatment input/output curves of prevolley amplitude (PREV), fEPSP rate of rise (fEPSP) and first population spike amplitude (PS) and additionally the maximum first population spike amplitude (MAX PS AMPL) taken one hour following 30 minutes of superfusion with ACSF equilibrated with either 85% nitrogen or 85% carbon monoxide. Post-treatment values are expressed as a percentage of the pre-treatment input/output or maximum population spike amplitude values for the same slice, mean + S.E.M. (n = 5). There was no significant difference between the post-treatment input/output curves for nitrogen or carbon monoxide ($P \leq 0.05$).

The block of hypoxic depression of synaptic field potentials by 8-CPT was concentration dependent, figure 4.3. In the presence of 10μM 8-CPT, the minimum hypoxic fEPSP was 73.7 ± 7.8% S.E.M., and the minimum hypoxic population spike was 66.6 ± 3.1% S.E.M. (n = 3). Synaptic field potentials during hypoxia in slices in the presence of 10μM 8-CPT were significantly larger than those from slices made hypoxic either in the presence of 1μM 8-CPT or without antagonist. Although the fEPSP and population spike were clearly depressed during hypoxia in the presence of 10μM 8-CPT, this depression failed to reach significance compared to normoxic field potentials ($P \leq 0.01$).

Normoxic incubation of slices with 1μM 8-CPT for 35 minutes caused a slight significant increase in the fEPSP to 115.0 ± 6.8% S.E.M. and in the population spike to 114.8 ± 6.9% S.E.M. ($P \leq 0.05$, n = 5) of their baseline values, effects which declined with washout of the 8-CPT, data not shown. This increase in field potentials is a result of blockade of the tonic adenosine tone at the A_1 receptors during normoxia. The increase in the fEPSP rate of rise and population spike amplitude prior to hypoxia was similar with 1μM or 10μM 8-CPT; 10μM 8-CPT caused the fEPSP rate or rise to increase to 104.9 ± 4.26% and the population spike amplitude to increase to 110.6 ± 2.6% neither of these were significantly different from 1μM 8-CPT ($P \leq 0.05$), data not shown. This suggests that even 1μM 8-CPT is sufficient to block tonic activation of A_1 receptors by the basal, normoxic levels of adenosine present in the extracellular space of the slice.

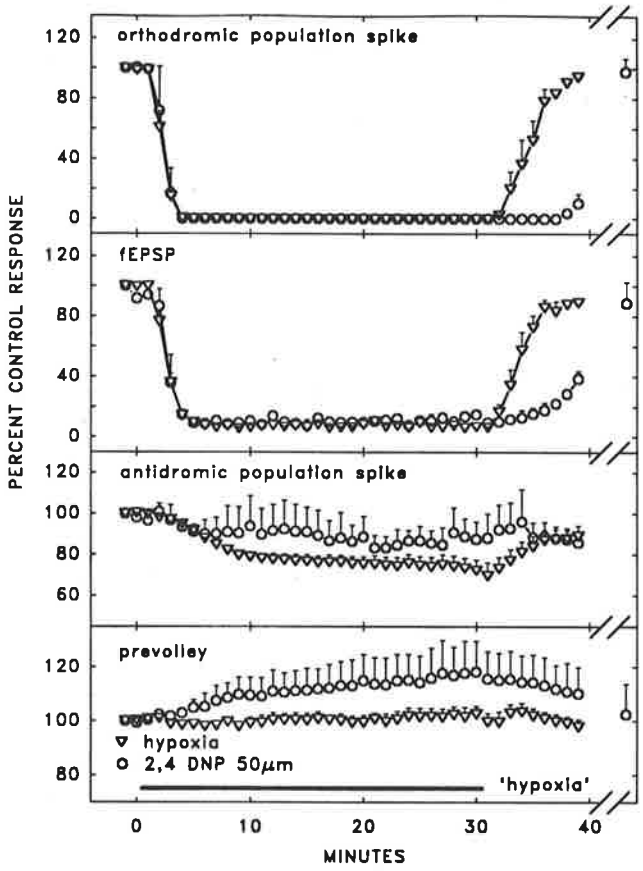

Figure 4.7 Comparison of the time course of alterations in field potentials in the CA_1 area by hypoxia or 2,4 dinitrophenol
Alterations in field potentials in hippocampal slices before, during and after 30 minutes of superfusion with hypoxic ACSF (n = 5) or with normoxic ACSF containing DNP 50μM (n = 5). Hypoxia or DNP superfusion is indicated by the bar at bottom. Data was collected and presented as in figure 4.2. Hypoxia data in this figure is from the same slices as figure 4.2, presented for comparison. DNP, like hypoxia, caused depression of the fEPSP and orthodromic population spike.

In order to verify that the effects of 8-CPT were due to adenosine A_1 receptor antagonism, another adenosine receptor antagonist, DPSPX, was tested against hypoxia. DPSPX has considerably less affinity and selectivity for the adenosine A_1 receptor than 8-CPT: K_i A_1 210nM and A_{2b} 710nM (Daly *et al.* 1985). In addition, this methylxanthine, unlike 8-CPT, is charged and relatively lipid insoluble, and will therefore not penetrate the cell membrane, excluding the possibility of inhibition of intracellular phosphodiesterases, an action typical of methylxanthines (Goodsell *et al.* 1971). DPSPX 75μM (n = 1) reduced hypoxic depression of the population spike and fEPSP, figure 4.9, this concentration being approximately equipotent with 1μM 8-CPT.

4.3.4.2. Input/output curves

Input/output curves were produced immediately before and one hour following treatment with 8-CPT alone, and in combination with hypoxia, to explore any persistent consequences of adenosine A_1 receptor antagonism during hypoxia and

normoxia. Figure 4.10 shows the slopes or areas of the post-treatment input/output curves of early field potential components and additionally the maximum first population spike amplitude, expressed as a percentage of the pre-treatment values. This figure includes the data presented in figure 4.5 for slices not exposed to 8-CPT. 1-way ANOVA indicated that the raw values for the pre-treatment input/output curves for the four treatment groups were from the same population, so post-treatment normalised percentage values between treatment groups can be compared. As an exception were the input/output areas of population spike 1 versus the fEPSP, the pre-treatment raw values of the different groups were significantly different ($P \leq 0.01$), and the post-treatment, normalised values showed significant within sample variation by 2-way ANOVA ($P \leq 0.05$); therefore, no conclusions will be drawn from these data. No post-treatment values of input/output curves of early field potential components showed any differences, demonstrating that early components of synaptic transmission and neuronal excitability were not altered by hypoxia or antagonist treatment. Maximum population spike amplitude of post hypoxic slices was significantly greater than normoxic slices and 8-CPT/normoxic slices ($P \leq 0.05$). Slices exposed to hypoxia in the presence of 8-CPT had maximum population spike amplitudes that were not different from other groups, figure 4.10. Adenosine A_1 receptor antagonism during hypoxia does not compromise survival of the CA_1 neuronal population.

4.3.5. ADENOSINE A_1 RECEPTOR ACTIVATION WITH EXOGENOUS AGONISTS

Superfusion of slices with exogenous adenosine, or with the selective adenosine A_1 receptor agonist CPA, produced a reversible depression of synaptic transmission, without effect on the prevolley or antidromic population spike. Synaptic transmission remained depressed throughout 30 minutes superfusion with these agonists, data not shown. The time course of depression and recovery of synaptic transmission with superfusion of adenosine, the putative endogenous agonist, was similar to that with hypoxia. Synaptic transmission recovered slowly with washout of CPA which, unlike adenosine, is not a substrate for uptake by the nucleoside transporter. Inhibition of synaptic transmission was concentration dependent as shown in the concentration/response curve for inhibition of the fEPSP in figure 4.11. Hill plots, not shown, of these concentration/response curves gave for adenosine ($n = 4$) an EC_{50} of 17μM, Hill slope 1.64 and for CPA ($n = 3$) an EC_{50} of 52nM, Hill slope 1.79.

4.3.6. ADENOSINE A_1 RECEPTORS, BUT NOT ATP-SENSITIVE POTASSIUM CHANNELS, CONTRIBUTE TO 2,4 DINITROPHENOL-INDUCED DEPRESSION OF SYNAPTIC TRANSMISSION

The synaptic depression due to DNP, like hypoxia, is the result of adenosine A_1 receptor activation. In the presence of 1μM 8-CPT, synaptic depression due to 30

minutes superfusion with 10μM DNP was reduced compared to DNP alone (n = 1), data not shown. As will be seen in chapter 5, 1μM 8-CPT reduced the synaptic depression induced by brief application of 20μM or 50μM DNP.

Many studies have demonstrated that hypoxia produces an outward current through potassium channels (Hansen *et al.* 1982; Fujiwara *et al.* 1987; Leblond and Krnjevic, 1989; Krnjevic and Xu, 1990b), that is responsible for hypoxic hyperpolarisation and may be associated with the hypoxic depression of synaptic transmission. DNP produces a similar potassium current mediated hyperpolarisation in cortical neurons (Godfraind *et al.* 1970; Godfraind *et al.* 1971). In addition to depressing synaptic transmission, adenosine, acting at A_1 receptors, is known to increase presynaptic and postsynaptic potassium currents (Pan *et al.* 1994; Greene and Haas, 1985; Haas and Greene, 1984; Segal, 1982). In some brain areas, ATP-sensitive potassium channels open in response to ATP depletion during hypoxia (Mourre *et al.* 1989; Amoroso *et al.* 1990; Murphy and Greenfield, 1991). DNP was used to assess the contribution of ATP-sensitive potassium channels to hypoxic depression of synaptic field potentials. The partial depression of the fEPSP and population spike by DNP in the presence of 1μM 8-CPT was not alleviated by the ATP-sensitive potassium channel blocker glibenclamide 5μM (n = 3). This indicates that in the CA_1 area of the hippocampus, ATP-sensitive potassium channels do not contribute to inhibition of neuronal activity during energy deprivation.

4.3.7. CALCULATION OF ADENOSINE CONCENTRATION DURING HYPOXIA

While adenosine acts at both presynaptic and postsynaptic A_1 receptors, hypoxic block of synaptic transmission at the Schaffer collateral/CA_1 synapse is largely presynaptic (Zhang and Krnjevic, 1993; Krnjevic and Xu, 1990b). Postsynaptic A_1 receptors probably reduce field potentials, particularly the population spike, by hyperpolarisation of the membrane away from threshold, and by current shunting. Adenosine A_1 receptor-mediated presynaptic depression of synaptic transmission is best measured as the reduction in the fEPSP, as this field potential should be less affected by postsynaptic actions of adenosine; indeed, hyperpolarisation and current shunt should have opposing effects on the fEPSP. Conveniently, during hypoxia, antagonism of adenosine A_1 receptors with 1μM 8-CPT reduced depression of the fEPSP to approximately 50%. Comparison of this hypoxic EC_{50} in the presence of antagonist (EC_{50}^B) with the EC_{50} for depression of the fEPSP by exogenous adenosine (EC_{50}), both putative A_1 receptor actions, might provide an estimate of extracellular adenosine concentrations in the vicinity of the excitatory synapses during hypoxia.

Measurement of adenosine EC_{50} and Hill slope is complicated by both the presence of basal adenosine levels and rapid uptake and metabolism of exogenous adenosine. Dunwiddie and Diao carefully examined this problem and arrived at an estimate of

200nM endogenous adenosine present in the hippocampal slice, and calculated the actual EC_{50} for adenosine as 610nM (Dunwiddie and Diao, 1994). These figures are arrived at by measuring exogenous adenosine concentration/response in the presence of large antagonist concentrations and nucleoside transporter blockers to minimise contributions of endogenous adenosine and nucleoside uptake. The EC_{50} is then corrected for the antagonist shift, estimated from the shift of CHA concentration/response curve, and equation 4.1 curve fitted (Dunwiddie and Diao, 1994).

$$R' = \frac{R - R_0}{1 - R_0} \quad \text{where:} \tag{4.1}$$

$$R = \frac{([A] + [A_{end}])^H}{([A] + [A_{end}])^H + EC_{50}{}^H} \tag{4.2}$$

$$R_0 = \frac{[A_{end}]^H}{[A_{end}]^H + EC_{50}{}^H} \tag{4.3}$$

$$[A_{end}] = (FI)^{1/H} \cdot EC_{50} \tag{4.4}$$

Where [A] is exogenous adenosine [A_{end}] is endogenous adenosine, FI is the fractional increase in the baseline fEPSP due to complete block of endogenous adenosine with A_1 receptor antagonists, H is the Hill plot slope for CHA, and EC_{50} is the calculated actual EC_{50} for adenosine. R is the fractional depression of the fEPSP by adenosine, R_0 is the tonic depression due to endogenous adenosine alone and R' is the response redefined with R_0 considered to be baseline, such as is usually done, including in the present investigation.

Dunwiddie and colleagues measured the CA_1 fEPSP amplitude and found an adenosine apparent EC_{50} of 29μM and FI of 0.18-0.20 (Dunwiddie and Fredholm, 1984; Dunwiddie and Diao, 1994). The present work measured fEPSP rate of rise, but similarly found an apparent adenosine EC_{50} of 17μM and FI of 0.15, this latter figure being taken only from slices where the fEPSP rate of rise before 8-CPT was judged to be sub-maximal (114.9 ± 11.7% S.E.M, n = 7). The published K_B for 8-CPT antagonism of A_1 receptor-induced depression of the CA_1 fEPSP amplitude is 42nM, with a Schild slope not different from unity (Dunwiddie and Fredholm, 1989).

The Schild equation relates the dose response, $DR = [EC_{50}]/[EC_{50}{}^B]$ which is the quotient of the EC_{50} for an agonist alone and the EC_{50} in the presence of antagonist, to the concentration of antagonist [B] and the antagonist affinity for the receptor K_B by the equation: $\log(DR-1) = s.\log[B] - \log(K_B)$ (Lazareno and Birdsall, 1993). Extracellular adenosine accumulation during hypoxia provides approximately 50% depression of the fEPSP in presence of 1μM 8-CPT: from figure 4.3, R' = 0.46, using the measured FI of 0.15, R_0 = 0.15/1.15 = 0.13 and rearranging equation 4.1 gives $R = R'(1-R_0) + R_0 = 0.53$. Therefore, the extracellular adenosine concentration

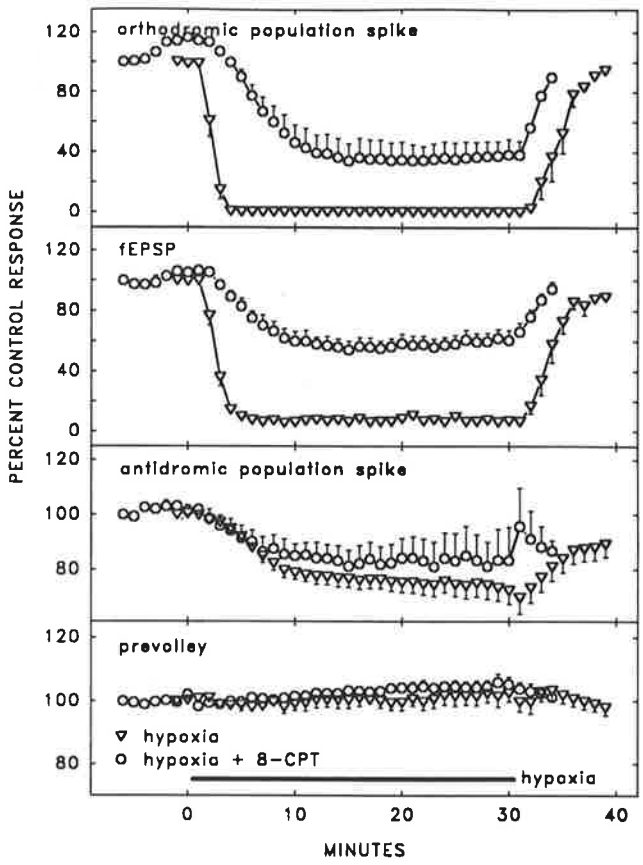

Figure 4.8 Time course of hypoxic changes in field potentials in the CA_1 area in the presence of the adenosine A_1 receptor antagonist 8-CPT
Time course of alterations of the hippocampal slice field potentials before, during and after 30 minutes of hypoxia alone (n = 5) and hypoxia in the presence of 1μM 8-CPT (n = 5). Slices were pre-treated with 8-CPT for 5 minutes before hypoxia. Hypoxia is produced by superfusion of slices with hypoxic ACSF for 30 minutes as indicated by the bar at bottom. Data was collected and presented as in figure 4.2. Hypoxia without antagonist data in this figure is from the same slices as figure 4.2, presented for comparison. Hypoxic depression of fEPSP, orthodromic population spike and antidromic population spike was attenuated by 8-CPT, see figure 4.3.

during hypoxia approximates the $EC_{50}{}^{B}$ in the presence of 1μM 8-CPT and calculating $[EC_{50}{}^{B}]$ = DR• $[EC_{50}]$ should give an estimate of hypoxic adenosine accumulation. Solving the Schild equation for DR using [B] = $1x10^{-6}$M, K_B = $4.2x10^{-8}$M, and s = 1 gives DR = 26. Solving for $EC_{50}{}^{B}$ using the apparent EC_{50} of 17μM gives hypoxic adenosine as 442μM. Solving for $EC_{50}{}^{B}$ using the actual EC_{50} of 610nM gives hypoxic adenosine as 16μM. Using Dunwiddie and Diao's actual EC_{50} and either their Hill slope for CHA of 1.52 or my Hill slope for CPA of 1.79 and my FI = 0.15, solving equation 4.4 gives endogenous normoxic adenosine 175nM or 211nM respectively in the slices in this study.

These two estimates of hypoxic adenosine concentrations differ by an order of magnitude, as the higher estimate is based on an EC_{50} determined with active uptake of adenosine. However, this is not likely the case during hypoxia. The majority of adenosine transport occurs by facilitated diffusion via the low affinity nucleoside

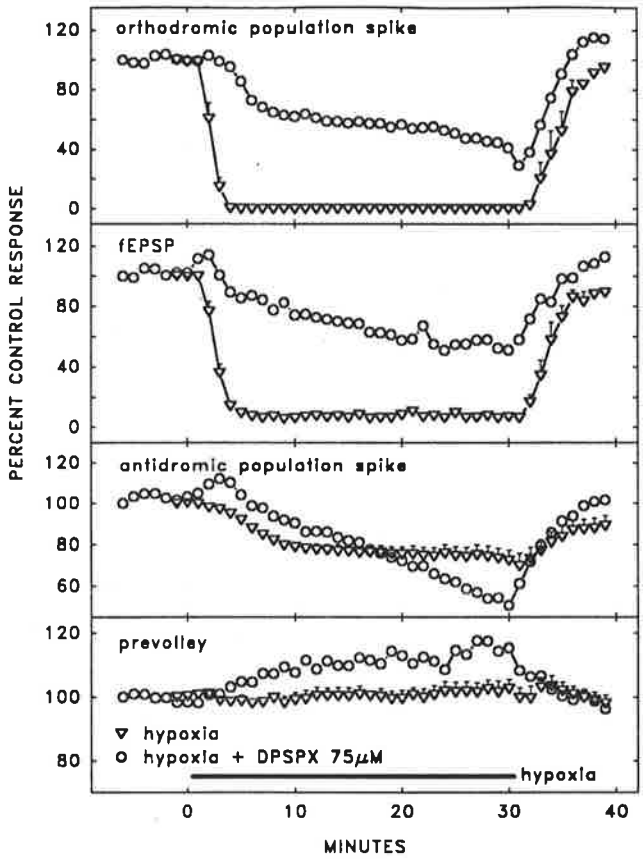

Figure 4.9 Time course of hypoxic changes in field potentials in the CA_1 area in the presence of the adenosine A_1 receptor antagonist DPSPX
Time course of alterations of the hippocampal slice field potentials before, during and after 30 minutes of hypoxia in the presence of 75μM DPSPX (n = 1) and hypoxia alone (n = 5). Slices were pre-treated with DPSPX for 5 minutes before hypoxia. Hypoxia is produced by superfusion of slices with hypoxic ACSF for 30 minutes as indicated by the bar at bottom. Data was collected and presented as in figure 4.2. Hypoxia without antagonist data in this figure is from the same slices as figure 4.2, presented for comparison. Hypoxic depression of fEPSP, orthodromic population spike was attenuated by DPSPX.

transporter, such adenosine uptake is equilibrative so that cytosolic hypoxic adenosine levels probably reflect the elevated extracellular levels (Fredholm *et al.* 1984). In addition, adenosine uptake is limited by subsequent metabolism of adenosine via adenosine kinase (Gu and Geiger, 1992) which is not likely active with the lowered cell energy charge (Bontemps *et al.* 1993). The sodium-dependent, concentrative transporter is probably saturated during hypoxia, and would not contribute significantly to adenosine uptake. Additionally, work in this laboratory, see chapter 5, shows that the nucleoside uptake via the dipyridamole sensitive nucleoside transporter is important for terminating adenosine action during reoxygenation, indicating that uptake may not be active during hypoxia. This suggests that the calculated EC_{50} is the correct choice, and thus it is likely that extracellular adenosine levels rise from the vicinity of 200nM during normoxia to 16μM during hypoxia.

4.4. DISCUSSION

4.4.1. HYPOXIA IS WELL TOLERATED IN THE HIPPOCAMPAL SLICE

Permanent loss of synaptic transmission following hypoxia can be used as an index of acute excitotoxic hypoxic neuronal damage (Kass and Lipton, 1986; Lobner and Lipton, 1993). Orthodromic population spike amplitude correlates well with the number of histologically normal cells in the CA_3 area of hippocampal slice; indeed the population spike amplitude declines proportionally with the increase of swollen versus normal pyramidal cells following hypoxia (Misgeld and Frotscher, 1982). Thus, assessment of synaptic transmission with field potentials allows measurement of partial or complete damage to the underlying neuronal population. At 32°C, synaptic transmission following hypoxic hypoxia or histotoxic hypoxia is not different from that following identical normoxic incubation periods, and the maximum population spike, representing the pool of surviving neurons, is similarly not reduced by hypoxia. The slight increase in the post-hypoxic maximum population spike amplitude may represent an increase number of neurons able to fire action potentials, which is unlikely at maximum stimulation, or perhaps an increase in individual action potential size, which cannot be assessed with field potentials. Alternatively, alterations in the electrical properties of the extracellular space will affect field potentials; possible alterations following hypoxia that would increase the population spike amplitude include an increase in extracellular potassium concentration (Alger and Teyler, 1978), or a decrease in extracellular space. Whatever the reason, this increase in population spike amplitude is only minor. Synaptic transmission also survives multiple 30 minute hypoxic exposures, and additionally, synaptic transmission survives at least 15 minutes of hypoxia at 36°C. This study confirms that in the presence of 10mM glucose, hypoxia is well tolerated in the CA_1 area of the hippocampal slice (Fujiwara *et al.* 1987; Taylor and Weber, 1993; Schurr *et al.* 1987; Hansen *et al.* 1982). No attempt was made to determine the duration of hypoxia required to produce CA_1 neuronal damage in this model, but others have found neuronal survival following 60 minutes hypoxia (Taylor and Weber, 1993).

Irreversible loss of synaptic transmission following ischaemia is presumably acute excitotoxicity from massive glutamate receptor activation (Lobner and Lipton, 1993). Such neuronal death is dependent on hypoxic spreading depression (Fairchild *et al.* 1988; Fujiwara *et al.* 1987; Taylor and Weber, 1993) and these only occur with severe ATP depletion (Kass and Lipton, 1986; Katsura *et al.* 1993; Folbergrová *et al.* 1990; Kass and Lipton, 1989; Yoneda and Okada, 1989). It is apparent from the preservation of the antidromic spike and the prevolley that hypoxic spreading depression did not occur in the present model (Young and Somjen, 1992; Fairchild *et al.* 1988; Sick *et al.* 1987); indeed, it is unlikely to occur at the temperature used here (Taylor and Weber, 1993). It is also likely, therefore, that in the present study, ATP is

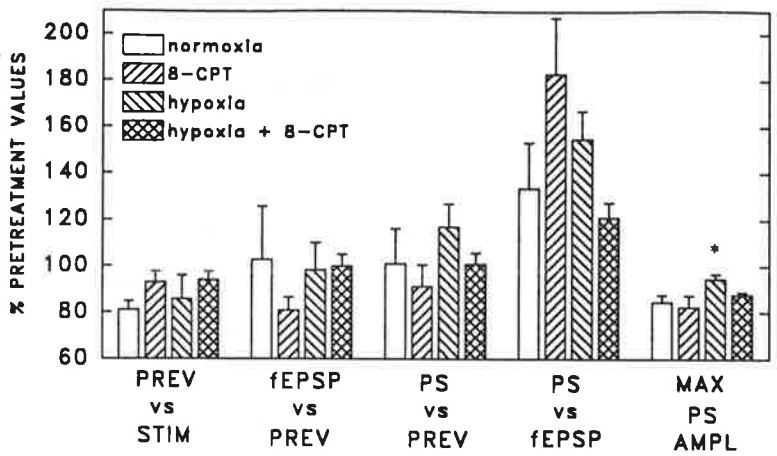

Figure 4.10 Comparison of input/output curves following hypoxia or normoxia alone and in the presence of 8-CPT
Slopes or areas of the post-treatment input/output curves of prevolley, fEPSP and first population spike and additionally the maximum first population spike amplitude for normoxic and hypoxic 8-CPT treatment, combined with the normoxia and hypoxia data from figure 4.5. Data expressed as mean + S.E.M. (n = 5) of percentage of the pre-treatment input/output values for the same slice. There was no significant difference between the post-treatment input/output curves for hypoxia or normoxia. Asterisk denotes significantly different from normoxic control slices and normoxic/8-CPT slices ($P \leq 0.05$).

well preserved in the slice. Indeed, measurements of tissue ATP in the hippocampal slice establish that ATP is reasonably well maintained throughout prolonged hypoxia in the presence of 10mM glucose (Fredholm *et al.* 1984; Yoneda and Okada, 1989; Cox *et al.* 1985).

The present survival times are far in excess of the seven minute survival time of CA_1 neurons *in vitro* in 4mM glucose (Kass and Lipton, 1986). The major factor that determines *in vitro* hypoxic resistance is the level of D-glucose in the ACSF (Schurr *et al.* 1987; Taylor and Weber, 1993). Hypoxia induced in the presence of ACSF glucose concentrations below 4mM can be considered an ischaemic model, and rapidly leads to irreversible loss of synaptic transmission. Apparently anaerobic glycolysis contributes to hypoxic survival of hippocampal slices. While 2-3mM glucose supports maximal respiration in the normoxic slice (McIlwain and Bachelard, 1971), this level may be insufficient to support the glycolytic flux, presumably increased due to the Pasteur effect, during hypoxia. Ischaemia *in vivo* is complicated by the reduced removal of metabolites, in particular, accumulation of lactic acid may have deleterious actions on neuronal survival. However, in the slice lactic acid will not accumulate due to continual superfusion and lack of glucose. Furthermore, *in vitro*, lactic acid is known to be benign during hypoxia (Schurr *et al.* 1988a), and in fact can be used as a substrate in the normoxic hippocampal slice (Schurr *et al.* 1988b).

4.4.2. CARBON MONOXIDE

CO is a commonly encountered toxic gas. Severe poisoning may either be fatal or may lead to neurological dysfunctions identical to those following hypoxia, including coma or seizures, often followed by delayed neuropsychiatric disorders that may occur days or weeks after apparent recovery (Plum *et al.* 1962). This occurs in brain structures vulnerable to hypoxic or ischaemic insults, particularly the hippocampus and striatum, but also typically in the globus pallidus which is less commonly affected by ischaemia (LaPresle and Fardeau, 1967; Meyer, 1936; Meyer, 1963). While direct toxic effects of CO, independent of COHb induced hypoxia (Haldane, 1927; Piantadosi *et al.* 1988; Orellano *et al.* 1976; Raybourn *et al.* 1978) have been proposed, in the present study, CO was no more toxic than nitrogen. One proposed direct toxic mechanism for CO *in vivo* is its ability to bind to reduced cytochrome a_3 in the cytochrome oxidase complex and so inhibit mitochondrial respiration (Piantadosi, 1987). The similarity of neuropathological changes in central white matter with cyanide (Hirano *et al.* 1967) and CO poisoning (LaPresle and Fardeau, 1967) suggests that this occurs, since cyanide also inhibits respiration, but by binding oxidised forms of cytochrome a_3. Exposure of hippocampal slices to cyanide reversibly depresses synaptic transmission in hippocampal slices in fully oxygenated ACSF (Aitken and Braitman, 1989), a finding confirmed in this laboratory, see chapter 5, but low concentrations of CO had no comparable effect. This suggests that, with a PO_2 sufficient to maintain synaptic transmission, CO does not inhibit mitochondrial respiration, although CO might be expected to bind cytochrome a_3. Tissue hypoxia and a high CO/O_2 ratio at the mitochondria are necessary for *in vivo* binding of CO to cytochrome a_3 (Piantadosi *et al.* 1988; Piantadosi, 1987; Brierley, 1976); the hypoxic ACSF in this study had a CO/O_2 ratio of 8, in the range of that previously calculated as required for CO binding to cytochromes (Coburn, 1979; Haab, 1990). It was not possible to determine whether CO binding to cytochrome a_3 did occur during the hypoxic insult; however, the complete recovery of synaptic transmission upon returning to control ACSF shows that there was no persistent inhibition of mitochondrial respiration. Although the cytochrome a_3-CO complex is photo-labile (Chance, 1953), incident light had no observable effect on CO actions on synaptic transmission in the present study.

In addition to providing further evidence for the resilience of the present model, no direct actions of CO poisoning were identified. A previous *in vitro* study used cerebellar Purkinje cell cultures, and demonstrated reduced spontaneous electrical activity during exposure to 3% CO in air (Raybourn *et al.* 1978). It is apparent in the hippocampal slice, that, although evoked potentials are depressed by superfusion with CO-containing ACSF with a PO_2 below the critical level for maintenance of synaptic transmission, no persistent insult from dissolved CO occurs under these

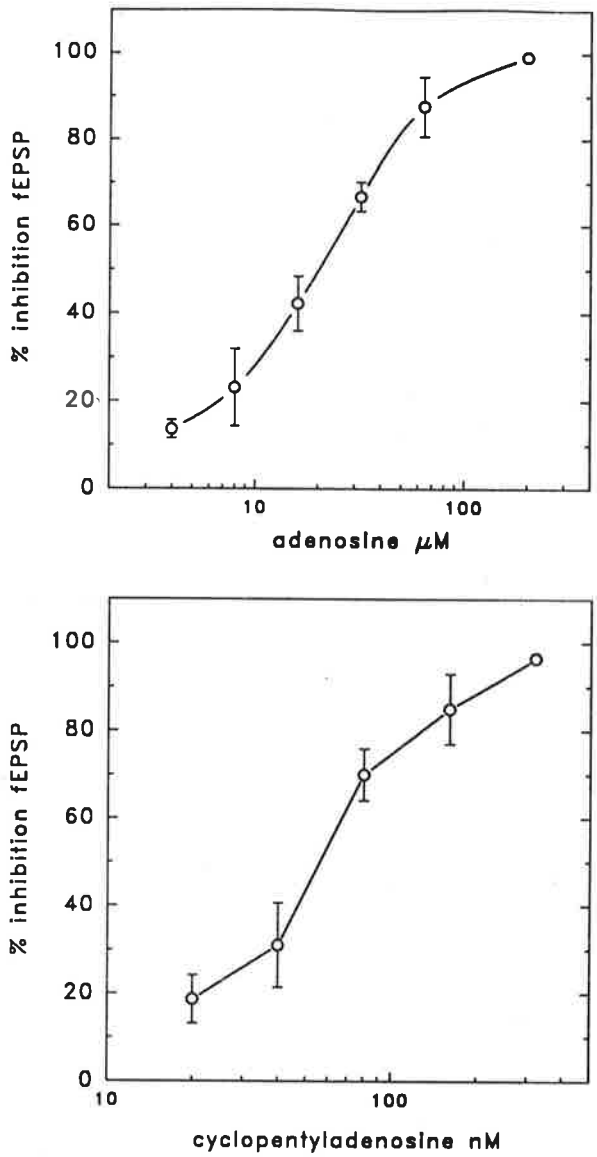

Figure 4.11 Concentration/response curve for adenosine A_1-receptor agonists
Concentration/response curve for adenosine A_1-receptor agonists adenosine (top) and cyclopentyladenosine (below). Points are mean ± S.E.M. percent depression of fEPSP for adenosine (n = 4) and cyclopentlyadenosine (n = 2 or 3).

conditions. The lack of direct effects in the present study could be attributed to the higher PO_2 in the control ACSF required for slice preparations, as compared to mono-layer cell cultures, which can derive sufficient O_2 from media equilibrated with air. This high PO_2 in the control ACSF may displace CO from cellular binding sites, since the relative CO/O_2 affinity of cytochrome a_3 is unity or less (Haab, 1990). Alternatively, cerebellar Purkinje cells may be susceptible to toxic actions dependent on the generation of cyclic GMP (Garthwaite, 1991) that are more prominent in the cerebellum than the hippocampus. Both CO and nitric oxide (NO; endothelial dependent relaxing factor) activate guanylate cyclase (Marks *et al.* 1991), and activation of guanylate cyclase, has also been implicated in glutamate-mediated ischaemic damage (Schousboe *et al.* 1990).

Both endogenous CO, produced by haem oxygenase, and NO, produced from arginine by nitric oxide synthetase, have been implicated as a transmitter substances in the brain (Hirsch *et al.* 1992); and indeed, haem oxygenase and guanylate cyclase are co-expressed in the hippocampal pyramidal cell layer (Verma *et al.* 1993). These gases possibly act as retrograde messengers in hippocampal long-term potentiation, as CO or NO application paired with weak tetanic stimulation can induce long-term potentiation in the CA_1 area and long-term potentiation due to strong tetanic stimulation can be blocked by inhibitors of haem oxygenase or nitric oxide synthetase (Zhou *et al.* 1993). That no potentiation of hippocampal field potentials by CO was observed in the present investigation is of no surprise, since CO exposure was not paired with tetanic stimulation. It remains possible, however, that exogenous CO, acting through guanylate cyclase, may alter neuronal excitability, and possibly lead to delayed neuronal death independent of any hypoxic actions. However, despite the distinct possibility of direct toxic actions of CO in the brain, none were observed in the present study.

4.4.3. DINITROPHENOL

The concentration of DNP used in the current study, 50μM, was sufficiently large to produce mitochondrial uncoupling and promote respiration (Hanstein, 1976) but not so large as to maximally stimulate mitochondrial ATPase activity (Hemker, 1962), a characteristic of uncoupling agents. Thus the concentration used here imitates anoxic energy deprivation, but does not additionally promote excessive ATP hydrolysis. In the present investigation, this chemical inhibition of energy metabolism was no more damaging than hypoxic superfusion. It seems that as long as glycolytic energy production is able to proceed maximally, hippocampal slices will survive prolonged inhibition of aerobic energy production.

4.4.4. HYPOXIC DEPRESSION OF SYNAPTIC TRANSMISSION IS DUE TO A_1 RECEPTOR ACTIVATION

The depression of excitatory synaptic activity during hypoxia is due, at least in part, to adenosine A_1 receptor activation, as is evident here from the alleviation of this depression by the adenosine A_1 receptor antagonist 8-CPT. This finding confirms those of previous studies in the rat hippocampal slice (Gribkoff *et al.* 1990; Gribkoff and Bauman, 1992; Fowler, 1990; Fowler, 1989) and in the hemisected spinal cord (Lloyd *et al.* 1988). An increased adenosine A_1 receptor activation during hypoxia is supported by the rise in total tissue adenosine levels (Fredholm *et al.* 1984) and in the superfusion medium (Fowler, 1991) during *in vitro* hypoxia in hippocampal slice, as well as by the rise in brain adenosine levels with hypoxia in paralysed, ventilated rats (Winn *et al.* 1981). Alternatively, 8-CPT may be acting as a 3',5'-cAMP phosphodiesterase inhibitor, like many other methylxanthines. However, 8-CPT is only a weak inhibitor, with a K_i value of 210μM (Goodsell *et al.* 1971), suggesting

that the observed block of hypoxic depression of synaptic depression by 1μM 8-CPT is unrelated to phosphodiesterase inhibition. This notion is further strengthened by the similar action of DPSPX which is a cell impermeant adenosine receptor antagonist, and would not inhibit intracellular phosphodiesterases.

The K_i of 8-CPT for inhibition of [^{3}H]cyclohexyladenosine binding to A_1 receptors in rat brain membranes is 10.9nM (Bruns *et al.* 1986). Schild analysis of its antagonist actions in the CA_1 area of the hippocampus gives a K_d of 42nM for block of inhibition of the fEPSP by adenosine, a K_d of 45nM for block of adenosine inhibition of first antidromic after-potential, and a K_d of 57nM for block of PIA inhibition of forskolin stimulated cAMP accumulation (Dunwiddie and Fredholm, 1989). The concentrations of 8-CPT used in this study, 1μM and 10μM, are large in comparison to these published values for its affinity at brain adenosine A_1 receptors, yet were insufficient to give complete block of the hypoxic depression of synaptic transmission. This suggests that antagonism of the adenosine A_1 receptor by 8-CPT is surmounted by massive extracellular accumulation of adenosine during hypoxia. Alternatively, some other mechanism, unrelated to A_1 receptor activation, may contribute to the hypoxic depression of synaptic transmission. In the CA_1 of hippocampal slices, hypoxic depression of synaptically evoked field potentials has previously been shown to be antagonised in a concentration-dependent fashion by 0.1 to 2μM 8-CPT (Gribkoff *et al.* 1990; Gribkoff and Bauman, 1992), with a similar potency as found in the present investigation. Furthermore, the depression of orthodromically evoked CA_1 field potentials due to *in vitro* ischaemia is partially blocked by 1μM 8-CPT and nearly completely blocked by 10μM 8-CPT (Fowler, 1990). Similar, near complete, block of synaptic depression in the slice CA_1 is achieved with 10μM 8-phenyltheophylline (8-PT) during ischaemia (Fowler, 1990) and also during hypoxia (Fowler, 1989). Reconciliation of the equal potency of 8-PT and 8-CPT is difficult, as 8-PT has 8 fold less affinity than 8-CPT for the A_1 receptor, with a K_i of 86nM (Bruns *et al.* 1986); however, it is not always possible to equate binding data to antagonist potency (Cheng and Prusoff, 1973). Nevertheless, since the protection of synaptic transmission during hypoxia by 8-CPT was concentration dependent, even at the high concentrations used in this and other studies, and 10μM 8-CPT provides near complete preservation of synaptic transmission during hypoxia, it is most likely that the hypoxic depression of synaptic transmission is due primarily to activation of adenosine A_1 receptors.

Calculations of extracellular adenosine action at the A_1 receptors suggest an 80 fold increase in extracellular adenosine from 200nM during normoxia to 16μM during hypoxia, in the present study. This basal, normoxic level of endogenous adenosine was derived using the method of Dunwiddie and Diao, 1994, and is in good agreement with their findings. Similar basal adenosine levels have been directly

measured in rats and gerbils by extraction from whole brain tissue (Winn *et al.* 1981; Morimoto *et al.* 1982), by extracellular microdialysis (Zetterström *et al.* 1982; Dux *et al.* 1990), as well as with static equilibration of ACSF with hippocampal slices (Fowler, 1991). However, during hypoxia, only 10 fold elevations in adenosine have previously been found (Winn *et al.* 1981; Zetterström *et al.* 1982; Fowler, 1991), but 20 to 100 fold increases have been measured with ischaemia (Dux *et al.* 1990; Morimoto *et al.* 1982). The order of magnitude differences between the actual measurements of hypoxic adenosine release and the present calculations may be explained by compartmentation. The present calculations represent adenosine present at the A_1 receptors, whilst the direct measurements reflect whole tissue levels. A_1 receptors are localised predominantly at synaptic sites (Fastbom *et al.* 1987; Goodman and Snyder, 1982) which are additionally rich in mitochondria. Thus, given the obvious coupling between oxidative stress and adenosine release evident in this work, it is possible that adenosine concentrations rise locally at the synaptic sites during hypoxia, as a consequence of high local mitochondrial density.

4.4.5. POSTSYNAPTIC DEPRESSION OF PYRAMIDAL NEURONS DURING HYPOXIA

The hypoxic depression of the antidromic population spike in this study is of the same maginitude as the slight depression of the first antidromic population spike during hypoxia in low calcium, high magnesium ACSF, previously described (Fowler, 1989). The lack of effect of the A_1 receptor antagonist 8-CPT on this antidromic population spike depression suggests that it is not an adenosine A_1 receptor-mediated action. Consistent with the latter, 50μM adenosine or 50nM CPA cause near complete depression of the fEPSP and population spike but have no effect on the antidromic population spike. A well documented action of adenosine A_1 receptor agonists in the CA_1 is the depression of antidromic *after-potentials* in low calcium, high magnesium ACSF (Schubert and Lee, 1986; Dunwiddie and Fredholm, 1989), but this must represent a different form of postsynaptic depression than the action of hypoxia reported in this study. The depression of the first antidromic spike during hypoxia may reflect the reported 4-15mV hypoxic hyperpolarisation of hippocampal CA_1 neurons (Hansen *et al.* 1982; Fujiwara *et al.* 1987).

Hypoxia causes a hyperpolarisation of hippocampal CA_1 neurons with a reversal potential and sensitivity to extracellular potassium concentration that indicates it is mediated by an increase potassium conductance (Hansen *et al.* 1982; Fujiwara *et al.* 1987; Leblond and Krnjevic, 1989; Krnjevic and Xu, 1990b); however, the identity of this potassium current remains obscure. Attempts to block this current have yielded mixed results (Hansen *et al.* 1982; Fujiwara *et al.* 1987; Leblond and Krnjevic, 1989; Krnjevic and Leblond, 1989), but non-selective blockade with either intracellular caesium, or a combination of extracellular caesium and 10mM TEA gives a near

complete block of the hypoxia-evoked outward current in voltage clamped hippocampal CA_1 pyramidal neurons (Krnjevic and Leblond, 1989). Hypoxic hyperpolarisation is blocked by recording with electrodes containing GTPγS or by extracellular application of 20μM carbachol (Krnjevic and Xu, 1990b), indicating that the current is G protein dependent, and is likely modulated by phosphoinositide hydrolysis. Furthermore, while hypoxic hyperpolarisation is not sensitive to intracellular EGTA (Fujiwara *et al.* 1987; Zhang and Krnjevic, 1993; Leblond and Krnjevic, 1989), the possibility remains that it is a calcium-dependent potassium current, as it is blocked by dantrolene (Krnjevic and Xu, 1989), which prevents calcium release from internal stores. It is most likely that a number of currents are activated during hypoxia.

A further possible mechanism of hypoxic hyperpolarisation is the activation of ATP sensitive potassium channels. Such an action is evident in the substantia nigra, which is rich in these channels (Mourre *et al.* 1989), where metabolic poisoning causes hyperpolarisation (Murphy and Greenfield, 1991) and $^{86}Rb^+$ efflux through potassium channels (Amoroso *et al.* 1990). ATP potassium channels exist on CA_1 neurons (Mourre *et al.* 1989; Tremblay *et al.* 1991); however, while hypoxic hyperpolarisation is blocked by glibenclamide in CA_3 neurons (Mourre *et al.* 1989), hypoxic hyperpolarisation in the CA_1 is not blocked by the ATP potassium channel blocker tolbutamide (Leblond and Krnjevic, 1989), and is therfore not due to an ATP-sensitive potassium current.

It is unlikely that the hypoxic hyperpolarisation in CA_1 neurons is mediated by adenosine A_1 receptors as is not blocked by caffeine, 8-(p-sulfophenyl)theophylline or DPCPX (Leblond and Krnjevic, 1989; Krnjevic and Xu, 1990a; Croning *et al.* 1994). Further evidence from CA_3 neurons from rats pre-treated with Pertussis toxin shows that both baclofen and adenosine are without effect on postsynaptic cell membrane properties, yet hypoxia still evokes a hyperpolarisation and drop in input resistance (Spuler and Grafe, 1989), which therefore can not be mediated by G_i or G_o proteins. Therefore, the block of hypoxic hyperpolarisation in the CA_1 by GTPγS and carbachol (Krnjevic and Xu, 1990b) suggests activation of the Pertussis toxin insensitive G_q. While there is no proven role of adenosine in hypoxic hyperpolarisation, the possibility remains that adenosine activates phospholipase C via A_3 receptors, and that this activates a "metabotropic" potassium current.

4.4.6. ADENOSINE IS NOT NECESSARY FOR POST-HYPOXIC NEURONAL SURVIVAL IN THIS MODEL

Adenosine has been proposed as a "retaliatory metabolite", its depressive actions serving to limit ATP use, and promote cell survival during metabolic stress (Newby *et al.* 1990). While it is attractive to consider that, anaerobic glycolysis is sufficient to

maintain ATP levels only during reduced energy requirements imposed by inhibition of synaptic transmission by endogenous adenosine, this is clearly not the case. 8-CPT, which largely relieves the A_1 receptor mediated block of synaptic transmission, does not promote neuronal damage, nor, apparently, does it allow ATP to be depleted to a point that initiates hypoxic spreading depression. This raises two points, firstly, adenosine is not providing a neuroprotective role in the hypoxic hippocampal slice. Secondly, as synaptic transmission is maintained throughout hypoxia in the presence of 8-CPT, anaerobic glycolysis produces sufficient ATP to maintain neuronal electrical activity for at least 30 minutes. Thus, with the hypoxic challenge presented in these experiments, adenosine does not provide neuro-protection by limiting ATP use.

While endogenous adenosine does not contribute to neuro-protection in the hippocampal slice, endogenous adenosine or exogenous A_1 receptor agonists reduce hypoxic and ischaemic neuronal injury *in vivo* (Dux *et al.* 1990; Evans *et al.* 1987; von Lubitz *et al.* 1988; Phillis and O'Regan, 1989; Januszewicz von Lubitz *et al.* 1989; Lin and Phillis, 1992; Sutherland *et al.* 1991; Boissard *et al.* 1992). Furthermore, exogenous adenosine promotes cell survival following hypoxia in cortical cell cultures (Goldberg *et al.* 1988), and improves recovery of synaptic transmission following ischaemia in the hippocampal slice CA_1 area (Donaghy and Schofield, 1991). Adenosine A_1 receptor activation limits ischaemic excitatory amino acid release (Lekieffre *et al.* 1991; Heron *et al.* 1993; Simpson *et al.* 1992), but does not protect against exogenous excitatory amino acids in cortical neuron cultures (Goldberg *et al.* 1988); therefore, it seems that adenosine neuro-protection results from limiting excitatory amino acid release. Furthermore, in light of the difference between the findings of the present investigation, and those with ischaemic hippocampal slices (Donaghy and Schofield, 1991), it seems likely that pathological excitatory amino acid release does not occur during hypoxia in the current model. Excitatory amino acid release only occurs after ATP depletion and hypoxic spreading depression, as is common during ischaemia; since these are absent during hypoxia, adenosine is not necessary for hypoxic neuro-protection.

4.4.7. SUMMARY AND CONCLUSIONS

Hypoxia causes a massive extracellular accumulation of adenosine, and a consequent adenosine A_1 receptor-mediated depression of synaptic transmission. Although PO_2 was not measured in the slice, calculations of oxygen diffusion suggest complete anoxia within the slice during superfusion of hypoxic ACSF. Such total anoxia for 30 minutes at 32°C in 10mM glucose does not cause acute excitotoxicity and consequent CA_1 neuronal damage, as indicated by the recovery of synaptic transmission following hypoxia, such recovery persisting for at least three hours.

Complete suppression of oxidative phosphorylation was attempted by combining hypoxia with carbon monoxide exposure and also by superfusion with ACSF containing DNP. Neither of these treatments yielded results different from hypoxia, and did not produce acute excitotoxicity. Together these results indicate that as long as glycolysis is allowed to proceed maximally, the CA_1 area of the hippocampal slice survives prolonged hypoxia. Depression of synaptic transmission during hypoxia is largely due to activation of adenosine A_1 receptors, it being blocked by the selective antagonist 8-CPT. During hypoxia, extracellular adenosine concentrations increased 80 fold, from 200nM to 16μM. Neuronal survival during hypoxia was not promoted by the adenosine-mediated depression of synaptic transmission and neuronal energy demands, as antagonism of the adenosine A_1 receptor did not alter neuronal survival, indicated by the post-hypoxic recovery of the population spike.

5. MECHANISM OF HYPOXIC ADENOSINE ACCUMULATION

5.1. INTRODUCTION

Being the terminal electron acceptor in the electron transport chain, oxygen is a fundamental requirement for mitochondrial ATP production. Cyanide, which inhibits electron transfer to oxygen in the mitochondrial respiratory chain, has identical actions to hypoxia in neurons, causing hyperpolarisation (Reiner *et al.* 1990) and inhibiting synaptic transmission (Lees and Sandberg, 1991; Yamamoto and Kurokawa, 1970; Aitken and Braitman, 1989). Presumably hypoxia and cyanide produce neuronal inhibition via the same mechanisms. These studies therefore explore the relationship between the inhibition of mitochondrial energy metabolism and the release of adenosine that causes inhibition of synaptic transmission during hypoxia in the hippocampal slice.

Electrons from NADH and $FADH_2$, produced in the metabolism of substrates, are shuttled into the mitochondrial matrix, where they are transported by a number of flavoproteins, cytochromes, non-haem iron and copper proteins to the final electron acceptor, oxygen. Oxidation/reduction of these electron carriers of the inner mitochondrial membrane is accompanied by pumping of protons out of the mitochondrial matrix. The resulting electrochemical gradient across the inner membrane is the common high energy intermediate that drives a number of mitochondrial reactions (Mitchell, 1976), including ATP synthesis and export, as well as calcium accumulation into the mitochondrial matrix (Hanstein, 1976).

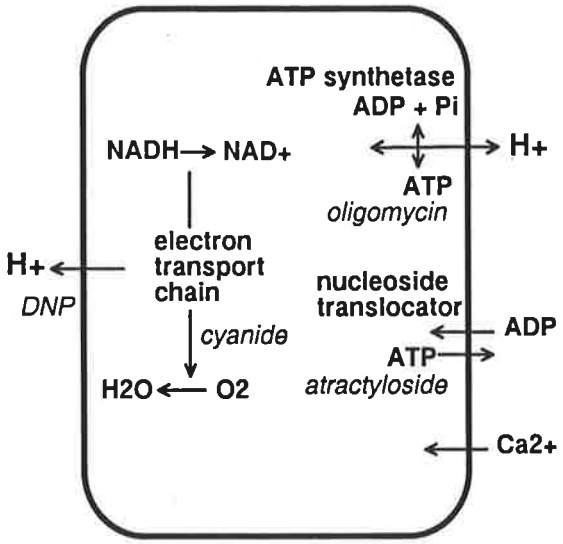

Figure 5.1 mitochondria partial reactions and inhibitors
This diagram illustrates the partial reactions integral to, and associated with, the inner mitochondrial membrane. The principal inhibitors of these reactions used in the present study are indicated in italics.

ATP is hydrolysed to ADP in energy requiring processes. In neurons, the largest energy demand is the maintenance of the membrane potential via the Na^+/K^+-ATPase and the Ca^{2+}-ATPase (Ereciñska and Silver, 1989). Cytosolic salvage pathways for ATP include transfer of the high energy phosphate from phosphocreatine to ADP by creatine kinase, or recycling of two molecules of ADP to ATP and AMP by adenylate kinase. AMP thus produced may either be re-phosphorylated or may be hydrolysed to adenosine by 5'-nucleotidase. Adenosine can also be produced by an alternate route via s-adenosylhomocysteine (SAH) by SAH-hydrolase.

It is generally assumed that hypoxic adenosine accumulation is a result of loss of adenine nucleotides to nucleosides by mass action, secondary to ATP depletion. However, during hypoxia in the cortical slice, ATP may drop initially, but is in fact preserved at a relatively high level (Fredholm *et al.* 1984). Despite this preservation of ATP, adenosine accumulates in brain slices during hypoxia. Adenosine may accumulate as a result of a rise in free AMP, or due to activation of adenosine producing pathways or inactivation of adenosine catabolic pathways. Major metabolite changes during hypoxia include a drop in phosphocreatine and a rise in lactate with a corresponding drop in intracellular pH (Kauppinen and Williams, 1990); possibly these changes may be a signal for adenosine accumulation. Inhibitors are available for many partial reactions involved in energy production and metabolism, and inhibition of some of these reactions mimic hypoxia. Such metabolic inhibitors were used to examine the following hypotheses. Adenosine accumulation during hypoxia is a result of inhibition of mitochondrial energy production but not inhibition of oxidative phosphorylation. Adenosine accumulates despite preservation of total neuronal ATP. Adenosine accumulates during hypoxia as a result of stimulation of 5'-nucleotidase and inhibition of adenosine kinase.

5.2. METHODS

In most instances, the following figures compare various treatments in the same slices. Typically slices were washed for one hour between treatments, and no more than three drug and/or metabolic inhibitor applications were made to any one slice. In the figures illustrating time course of inhibitor actions, all field potential measurements are presented as a percentage of the unique baseline recording for that particular treatment. Where slices were not pre-treated with an antagonist or an enzyme inhibitor, the first two time points are always recorded in drug free ACSF, and the first of these is considered baseline. When used, 8-cyclopentyl-1,3-dimethylxanthine (8-CPT) was superfused onto hippocampal slices for 15 to 20 minutes before application of metabolic inhibitors; however, only the final two minutes of antagonist superfusion are illustrated, and the first of these is used as the

baseline field potential recording. Similarly, with other pre-treatments, baseline field potentials were normalised to 100% at the equivalent time point as tests without pre-treatment. Stimulus current was kept the same between the various treatments in any slice, and in the case of 8-CPT this resulted in a slight increase in the field potentials.

8-CPT and 2,4 dinitrophenol (DNP) stock solutions were made in 0.05N NaOH. Oligomycin B, dipyridamole, rotenone and pentachlorophenol were used from stock solutions in ethanol. Atractyloside, dantrolene, α,β-methylene ADP, (+)catechin, 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX) and p1,p5-di(adenosine-5') pentaphosphate (Ap5A) stock solutions were made in distilled water. S-(p-nitrobenzyl)-6-thioinosine (nitrobenzylthioinosine, NBTI) and 4-amino-5-iodo-7-(β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (iodotubercidin) were dissolved in dimethyl sulfoxide at 1000 times the final required concentration. Sodium cyanide was made up in ACSF immediately before superfusion. Ruthenium red (45% pure) was dissolved directly in ACSF. Creatine monohydrate was dissolved directly in ACSF, which, to compensate for acidification, contained 32mM $NaHCO_3$. 8-CPT, DPSPX and iodotubercidin and were purchased from Research Biochemicals Incorporated, sodium cyanide and creatine monohydrate were analytical reagents, and all other compounds were purchased from Sigma.

5.3. RESULTS

5.3.1. MITOCHONDRIAL UNCOUPLING

Respiration and ATP production are usually tightly coupled in mitochondria; however, in the presence of exogenous uncoupling chemicals, electron transport continues without oxidative phosphorylation, and indeed, a net hydrolysis of ATP occurs (Heytler, 1979; Lehninger, 1964; Lardy and Wellman, 1953). In uncoupled mitochondria, respiration and oxidation of substrate continues at an increased rate, and energy is dissipated as heat (Hanstein, 1976). The classic uncoupling agent is DNP, which, as a lipophilic weak acid, freely exchanges protons across the inner mitochondrial membrane, dissipating the electrochemical gradient (Heytler, 1979). This agent was tested for its action on synaptic transmission in the hippocampal slice.

Superfusion of hippocampal slices with 50μM DNP causes a rapid reversible inhibition of synaptic transmission, as was discussed briefly in the previous chapter, and shown in figure 4.7. Here, figure 5.2 shows that 3.5 minutes superfusion with 50μM DNP caused near complete depression of the population spike amplitude to 0.3 ± 0.3% S.E.M. and fEPSP rate of rise to 6.8 ± 1.0% S.E.M. of baseline, and that these synaptic potentials recovered with washout of the uncoupler (n = 10). DNP had no affect on the prevolley (n = 10) or antidromic population spike (n = 4). As in hypoxia, adenosine release contributes to the DNP depression of synaptic

transmission, which was reduced by the adenosine A_1 receptor antagonist 8-CPT. DNP superfusion in the presence of 1μM 8-CPT (n = 6) depressed the population spike amplitude depressed to 48.4 ± 6.4% S.E.M. and the fEPSP rate of rise depressed to 56.0 ± 4.2% S.E.M. of baseline, both significantly different from DNP alone ($P \leq 0.01$). After one hour washout of 8-CPT, DNP superfusion again completely depressed synaptic transmission (n = 4), and was not significantly different from the pre-antagonist test. Figure 5.3 shows typical orthodromically evoked field potentials before, during and after 3.5 minutes superfusion of 50μM DNP. Note that upon washout of DNP, after this brief inhibition, synaptic potentials recover without the hyperexcitability that followed prolonged inhibition seen in chapter 4. Such hyperexcitability will be discussed in chapter 6.

Superfusion with 20μM DNP for 3.5 minutes caused a reduced depression of synaptic transmission compared to 50μM and was nearly completely blocked by 1μM 8-CPT (n = 3), see figure 5.4. Synaptic depression by DNP 20μM for 3.5 minutes was also completely blocked by the less selective adenosine antagonist DPSPX (n = 1), data not shown.

The uncoupling agent pentachlorophenol, which acts similarly to DNP, depressed synaptic transmission without effect on the prevolley. Unlike DNP, the population spike and fEPSP did not recover during one hour of washout following 3.5 minutes superfusion of 10μM pentachlorophenol. Figure 5.5 shows superfusion of pentachlorophenol and the first 21.5 minutes of wash. In a separate slice, blockade of adenosine A_1 receptors with 1μM 8-CPT delayed the depression of synaptic transmission by pentachlorophenol considerably, but did not prevent loss of the population spike and fEPSP, indicating either that A_1 receptor antagonism was eventually surmounted or that some other action contributed to neuronal depression.

5.3.2. ELECTRON CARRIER INHIBITION

While the uncoupling agent DNP mimics the electrophysiological consequences of hypoxia, its site of action at the mitochondria is distinct from the site of electron transfer to oxygen, and uncoupling stimulates rather than inhibits respiration. Cyanide inhibits electron transfer from cytochrome aa_3 to oxygen, and thus blocks respiration and energy production (Lehninger, 1964). Cyanide has been used extensively in neuropathological (Maduh *et al.* 1990; Oberpichler *et al.* 1990; Goldberg *et al.* 1987; Rothman, 1983) and neurophysiological models (Reiner *et al.* 1990; Aitken and Braitman, 1989; Johnson *et al.* 1987; Yamamoto and Kurokawa, 1970; Murphy and Greenfield, 1991; Dubinsky and Rothman, 1991) to mimic cerebral hypoxia.

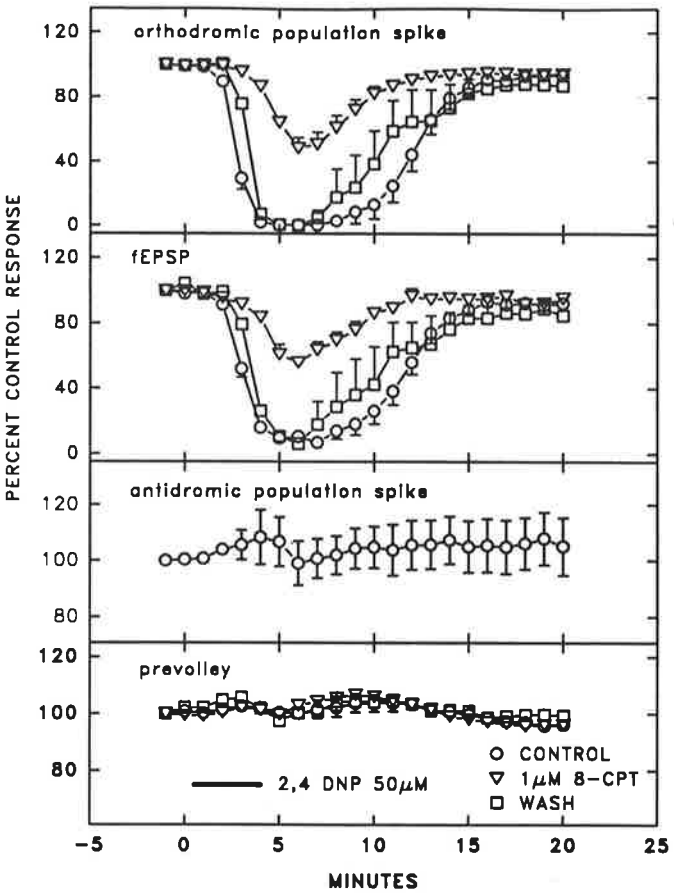

Figure 5.2 Comparison of time course of alterations in field potentials in the CA_1 area produced by 50μM 2,4 dinitrophenol in the presence and absence of the adenosine A_1 receptor antagonist 8-CPT

Field potentials in hippocampal slices before, during and after 3.5 minutes superfusion of 50μM DNP alone (CONTROL, n = 10), DNP in the presence of 1μM 8-CPT (1μM 8-CPT, n = 6) and DNP alone following one hour washout of 8-CPT (WASH, n = 4). DNP superfusion is indicated by the bar at bottom. In the case of adenosine A_1 receptor antagonism (triangles), slices were superfused with 1μM 8-CPT for 20 minutes prior to and during the DNP superfusion, only the last two minutes of 8-CPT pre-treatment are shown. Slices were washed with drug-free ACSF at the end of the DNP superfusion. Schaffer collateral/commissural fibres were stimulated at 0.05Hz and each three consecutive field potentials averaged to give minute averages. Values are expressed as a percentage of the baseline (first minute average illustrated) response for that treatment. Points represent the mean ± S.E.M. for each condition, where no error bars are evident, they are smaller than the symbol. fEPSP values represent the percentage of the baseline rate of rise of the initial segment and all other values are percentage of baseline amplitude of the respective field potentials. Mitochondrial uncoupling caused depression of fEPSP and the orthodromic population spike. Depression of the fEPSP and orthodromic population spike was attenuated by 8-CPT. The antidromic population spike was recorded during four of the CONTROL DNP exposures only.

Figure 5.6 shows that 3.5 minutes superfusion of hippocampal slices with 200μM sodium cyanide causes a reversible depression of the fEPSP rate of rise to 11.0 ± 2.7% S.E.M. and the population spike amplitude to 0.2 ± 0.2% S.E.M. of baseline, with no effect on the prevolley (n = 5). As with hypoxia and DNP, the inhibition of synaptic transmission is significantly reduced by adenosine A_1 receptor antagonism, an effect that washes out by one hour. In the presence of 1μM 8-CPT, cyanide

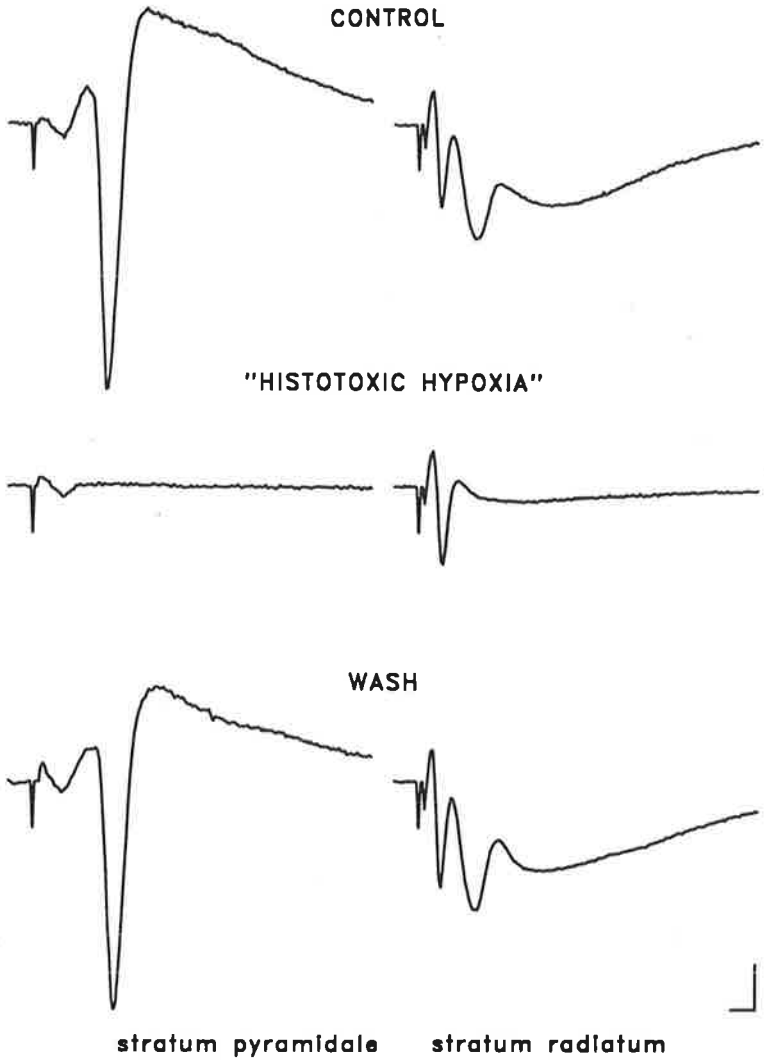

Figure 5.3 Effects of superfusion of 2,4 dinitrophenol on synaptic field potentials in the hippocampal slice
Synaptic field potentials in the CA_1 area evoked by Schaffer collateral/commissural stimulation. The left column shows recordings from the stratum pyramidale (cell body layer) and the right column shows recordings from stratum radiatum (apical dendrite/synaptic layer). From top to bottom are shown respectively response from before, during and 1 hour following 3.5 minutes superfusion of 50μM DNP. Calibration bars are 1mV and 1ms, negative down. Stimulus artefacts are truncated for clarity.

reduced the fEPSP to 77.8 ± 7.8% S.E.M. of baseline and the population spike to 69.5 ± 3.5% S.E.M. of baseline (n = 5), both significantly different from the depression of synaptic transmission by sodium cyanide alone ($P \leq 0.01$). Rotenone, 10μM, which inhibits mitochondrial respiration by blocking electron transfer between NADH dehydrogenase and ubiquinone (Singer, 1979) caused an irreversible depression of synaptic transmission (n = 1), data not shown.

Hydrogen cyanide is volatile and disappears from dilute aqueous solutions (Slater, 1967) and thus sodium cyanide needed to be dissolved in ACSF immediately prior to use. Additionally, the cyanide containing ACSF oxidised the silver/silver chloride bath electrode, and caused rapid baseline drift during superfusion. These technical

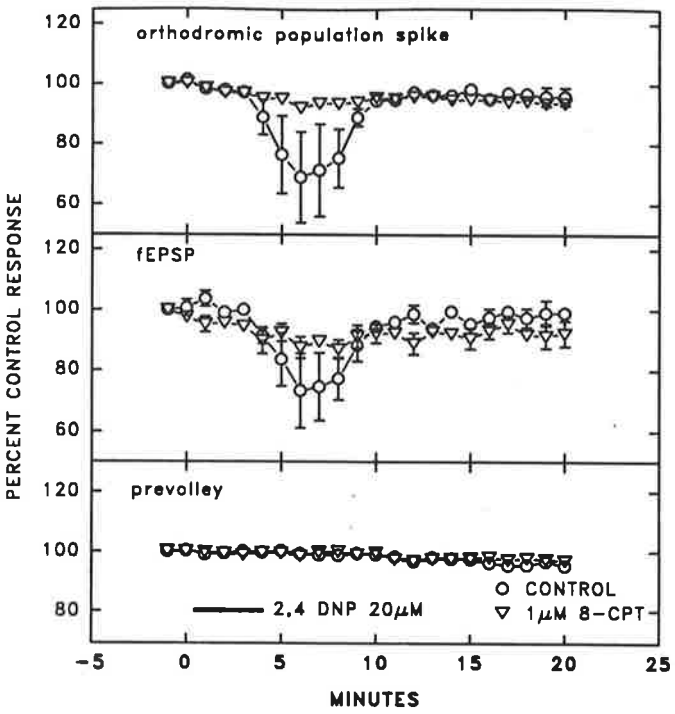

Figure 5.4 Comparison of time course of alterations in field potentials in the CA_1 area produced by 20μM 2,4 dinitrophenol in the presence and absence of the adenosine A_1 receptor antagonist 8-CPT

Field potentials in hippocampal slices (n = 3) before, during and after 3.5 minutes superfusion of 20μM DNP alone (CONTROL) or DNP in the presence of 1μM 8-CPT (1μM 8-CPT). DNP superfusion is indicated by the bar at bottom. In the case of adenosine A_1 receptor antagonism (triangles), slices were superfused with 1μM 8-CPT for 15 minutes prior to and during the DNP superfusion, only the last two minutes of 8-CPT pre-treatment are shown. Slices were washed with drug-free ACSF at the end of the DNP superfusion. Data was collected and presented as in figure 5.2. 20μM DNP caused depression of the fEPSP and the orthodromic population spike that was largely attenuated by 8-CPT.

difficulties, and the similarity of synaptic depression by cyanide or DNP, made DNP a more convenient metabolic inhibitor for further investigation.

5.3.3. INHIBITION OF OXIDATIVE PHOSPHORYLATION

5.3.3.1. ATP synthetase inhibition

True inhibitors of oxidative phosphorylation inhibit ATP synthetase (F_1F_0 ATPase) (Lehninger, 1964), oligomycin B being the classical inhibitor of this enzyme (Linnett and Beechey, 1979). Incubation of substantia nigra slices with a combination of oligomycin B and the glycolytic inhibitor 2-deoxyglucose causes a reduction in ATP content, and activates ATP-sensitive potassium channels (Amoroso *et al.* 1990; Schmid-Antomarchi *et al.* 1987). Incubation of avian neuronal or glial cell cultures with oligomycin B and 2-deoxyglucose likewise reduces ATP content and enhances adenosine release (Meghji *et al.* 1989). While this combination of inhibitors might simulate an ischaemic insult, in order to mimic hypoxia, oligomycin B alone was tested for action on synaptic transmission in hippocampal slices.

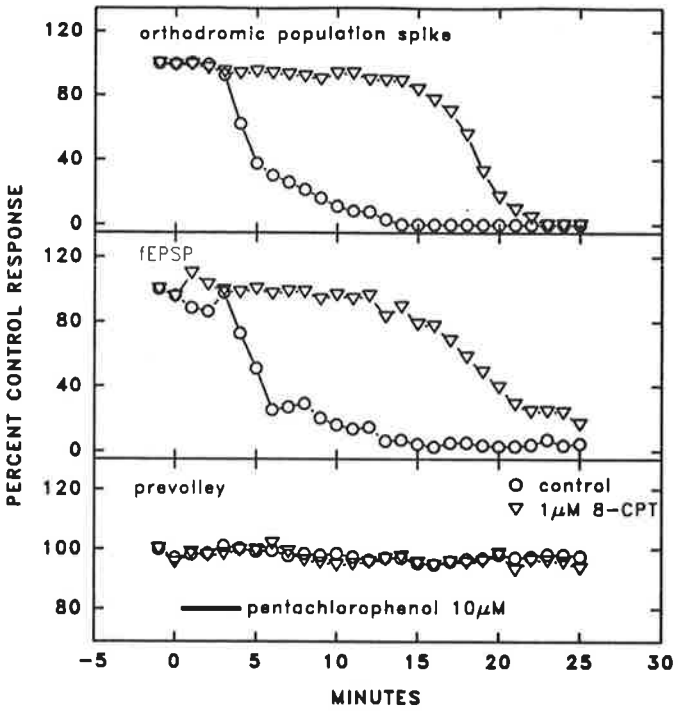

Figure 5.5 Comparison of time course of alterations in field potentials in the CA_1 area produced by 10μM 2,4 pentachlorophenol in the presence and absence of the adenosine A_1 receptor antagonist 8-CPT

Field potentials in a hippocampal slice before, during and after 3.5 minutes superfusion of 10μM pentachlorophenol alone (control, n = 1) and, in a separate slice pentachlorophenol in the presence of 1μM 8-CPT (1μM 8-CPT, n = 1). Pentachlorophenol superfusion is indicated by the bar at bottom, and for adenosine A_1 receptor antagonism (triangles), the slice was superfused with 1μM 8-CPT for 20 minutes prior to and during the pentachlorophenol superfusion, only the last two minutes of 8-CPT pre-treatment are shown. Slices were washed with drug-free ACSF at the end of the DNP superfusion. Data was collected and presented as in figure 5.2. 10μM pentachlorophenol caused depression of the fEPSP and the orthodromic population spike that was delayed by 8-CPT.

Unlike hypoxia or the previously described inhibitors, 3μM oligomycin B did not cause rapid depression of synaptic transmission in the hippocampus. Figure 5.7 shows that 3.5 minutes superfusion of hippocampal slices with oligomycin B 3μM had no significant effect on the population spike, fEPSP or prevolley (n = 4), although both postsynaptic potentials declined slightly. A similar slight decline in field potentials was seen with 3.5 minutes superfusion of 1μM (n = 1) or six minutes of 5μM (n = 1) oligomycin B, data not shown. As can be seen in figure 5.9 the initial 10 minutes superfusion of oligomycin B 3μM also had no affect on synaptic transmission (n = 5). Superfusion of a single hippocampal slice with oligomycin B 3μM for 20 minutes produced a slow decline in synaptic transmission to about 45% of baseline levels, data not shown. In addition, 1mM 2-deoxyglucose was co-administered with 3μM oligomycin B, and this also had no effect on synaptic transmission (n = 1), data not shown.

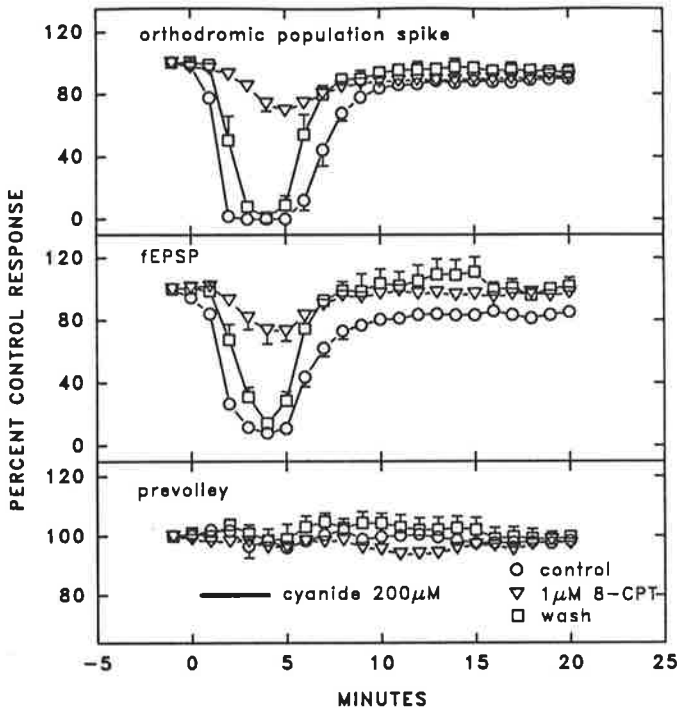

Figure 5.6 Comparison of time course of alterations in field potentials in the CA_1 area produced by 200μM sodium cyanide in the presence and absence of the adenosine A_1 receptor antagonist 8-CPT

Field potentials in hippocampal slices (n = 5) before, during and after 3.5 minutes superfusion of 200μM sodium cyanide alone (control), in the presence of 1μM 8-CPT (1μM 8-CPT) and again alone following one hour washout of 8-CPT (wash). Sodium cyanide was dissolved in ACSF immediately prior to use and superfusion is indicated by the bar at bottom. In the case of adenosine A_1 receptor antagonism (triangles), slices were superfused with 1μM 8-CPT for 15 minutes prior to and during the sodium cyanide superfusion, only the last two minutes of 8-CPT pre-treatment are shown. Slices were washed with drug-free ACSF at the end of the sodium cyanide superfusion. Data was collected and presented as in figure 5.2. Inhibition of electron mitochondrial transport caused depression of fEPSP and the orthodromic population spike. Depression of the fEPSP and orthodromic population spike was attenuated by 8-CPT.

5.3.3.2. Mitochondrial nucleotide transporter inhibition

The adenine nucleotide translocator of the inner mitochondrial membrane electrotonically exchanges cytosolic ADP^{3-} for matrix ATP^{4-}, and may be both physically and functionally associated with the F_1F_0 ATPase in normally energised mitochondria, where nucleotide exchange is essential for ATP synthesis (Lanoue and Schoolwerth, 1984; Vignais, 1976). Atractyloside, by binding to the cytosolic side of the adenine nucleotide translocator, inhibits nucleotide exchange and prevents oxidative phosphorylation (Lanoue and Schoolwerth, 1984; Vignais, 1976).

Superfusion of hippocampal slices with 75μM atractyloside for 8.5 minutes did not alter the population spike, fEPSP or prevolley (n = 4), see figure 5.8. One slice was additionally superfused with 100μM atractyloside for 19 minutes and later with 1mM atractyloside for 4.5 minutes, and in neither case was there any effect on evoked CA_1 field potentials, data not shown. Thus, neither true inhibition of oxidative

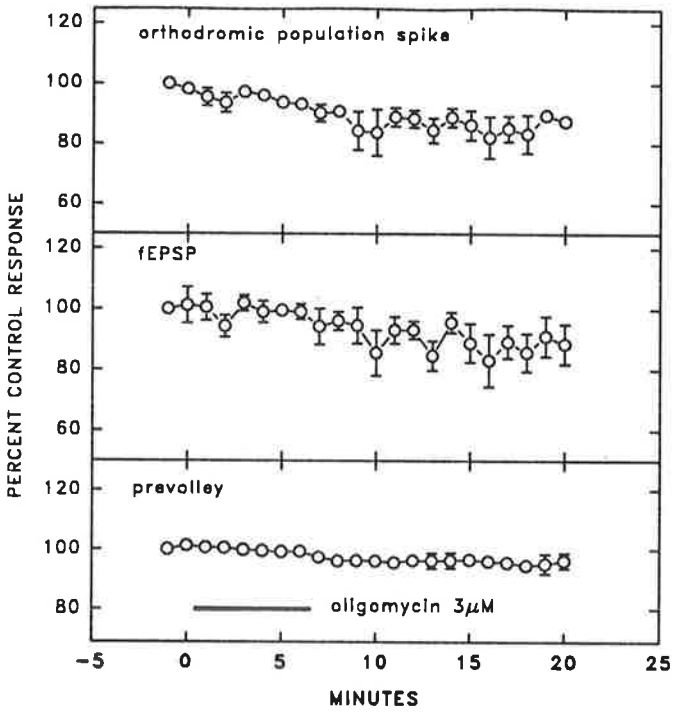

Figure 5.7 Oligomycin B does not cause rapid depression of synaptic field potentials in the CA_1 area
Field potentials in hippocampal slices (n = 4) before, during and after 6 minutes superfusion of 3μM (2.4μg/ml) oligomycin B. Oligomycin B superfusion is indicated by the bar at bottom. Data was collected and presented as in figure 5.2. Inhibition of oxidative phosphorylation had no significant effect on hippocampal field potentials ($p \leq 0.05$).

phosphorylation with oligomycin B nor inhibition of nucleotide exchange produced rapid adenosine release from hippocampal cells.

5.3.4. REDUCING ATP DEPLETION

5.3.4.1. Inhibition of ATPase activity

It is easy to imagine that with inhibition of respiration during hypoxia, cytosolic ATP depletion, and ATP salvage via adenylate kinase, would lead to increased AMP which would drive the equilibrium of the reaction catalysed by 5'-nucleotidases towards adenosine production. Alternatively, reduced free energy of the cytosolic adenine nucleotide pool may reduce the activity of adenosine kinase, an enzyme essential for adenosine salvage. However, this simple explanation for rapid adenosine production during oxidative stress may be insufficient, since inhibition of oxidative phosphorylation does not produce rapid synaptic depression. Therefore, another mechanism may contribute to hypoxic adenosine formation. One possibility explored was that uncoupling, or respiratory inhibition produces a more severe ATP depletion than inhibition of oxidative phosphorylation; indeed, DNP is well known to stimulate ATP hydrolysis by F_1F_0 ATPase-driven proton pumping (Heytler, 1979; Lehninger, 1964; Lardy and Wellman, 1953), and cyanide similarly promotes mitochondrial ATP hydrolysis (Kauppinen and Nicholls, 1986; Scott and Nicholls,

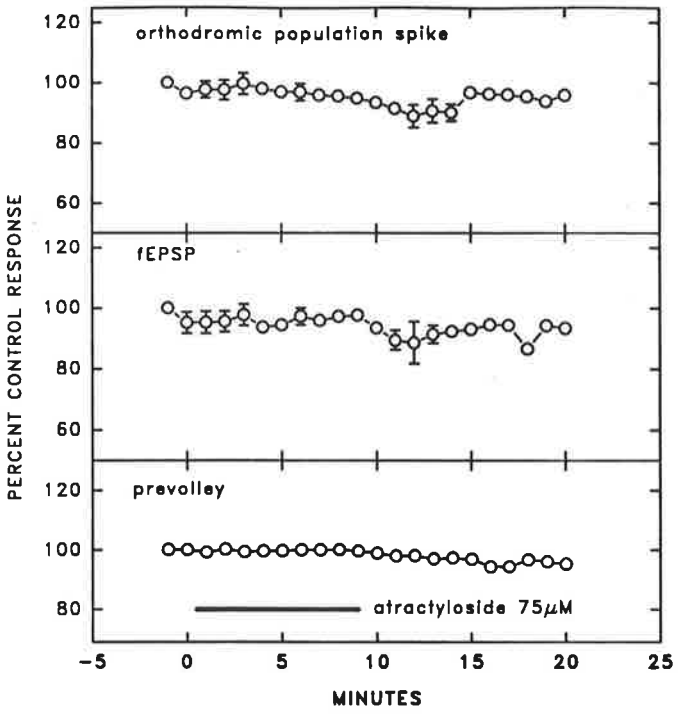

Figure 5.8 Atractyloside does not cause rapid depression of synaptic field potentials in the CA_1 area
Field potentials in hippocampal slices (n = 4) before, during and after 8.5 minutes superfusion of 75μM atractyloside, indicated by the bar at bottom. Data was collected and presented as in figure 5.2. The last six time points are recorded from one slice only. Inhibition of mitochondrial nucleotide exchange had no significant effect on hippocampal field potentials ($p \leq 0.05$).

1980). 50μM DNP provides half maximal stimulation of ATPase activity at physiological pH (Hemker, 1962); therefore, the doses of DNP used in the present study should have given some ATPase stimulation, and this may have contributed to adenosine release.

To investigate the contribution of ATP depletion due to ATPase stimulation in the DNP-mediated depression of synaptic transmission, oligomycin B was co-administered with DNP. Oligomycin B prevents DNP stimulation of F_1F_0 ATPase activity, and additionally inhibits plasma membrane Na^+/K^+-ATPase activity (Jöbsis and Vreman, 1963; Linnett and Beechey, 1979). Five hippocampal slices were superfused with 20μM DNP for 3.5 minutes which depressed the population spike amplitude to 15.7 ± 13.0% S.E.M. and fEPSP rate of rise to 23.4 ± 6.6% S.E.M.. Following one hour of recovery, the same slices were pre-treated for 10 minutes with 3μM oligomycin B, then superfused with 20μM DNP in the presence of 3μM oligomycin B. Oligomycin B failed to prevent the DNP mediated depression of synaptic transmission, see figure 5.9. In the presence of oligomycin B, the maximal depression of the population spike amplitude to 29.2 ± 13.0% S.E.M. and fEPSP rate of rise to 34.3 ± 10.9% S.E.M. was not different ($P \leq 0.01$) from that during the previous DNP exposure.

5.3.4.2. ATP buffering with phosphocreatine

While cytosolic ATP is maintained near 85% of normoxic levels during 30 minutes of hypoxia in the hippocampal slice, phosphocreatine drops precipitously, coincident with the initial small drop in ATP during the first 5 minutes of hypoxia (Fredholm *et al.* 1984; Yoneda and Okada, 1989; Kauppinen and Williams, 1990). Phosphocreatine acts as a cytosolic energy store; during rapid ATP hydrolysis, creatine kinase catalyses the transfer of the high energy phosphate from phosphocreatine to ADP. Thus phosphocreatine can buffer cytosolic ATP, maintain ATP/ADP ratios in a range where they act as regulators of various cellular functions, prevent loss of nucleotides through adenylate kinase and 5'-nucleotidases, and buffer intracellular pH (Ereciñska and Silver, 1989; Wallimann *et al.* 1992). Incubation of hippocampal slices with creatine increases the phosphocreatine levels in the slice, without increasing ATP (Lipton and Whittingham, 1982; Okada and Yoneda, 1983; Yoneda *et al.* 1983), and this elevated phosphocreatine slows subsequent ATP depletion during energy deprivation (Okada and Yoneda, 1983). Elevated phosphocreatine has been observed to increases the latency of depression of synaptic transmission during *in vitro* ischaemia in the dentate gyrus (Lipton and Whittingham, 1982), CA_3 (Okada and Yoneda, 1983), and CA_1 (Leblond and Krnjevic, 1989).

In the present study, hippocampal slices were incubated with 25mM creatine for three hours, which has been shown to produce maximal accumulation of intracellular phosphocreatine (Yoneda *et al.* 1983). During creatine exposure, slices displayed a postsynaptic hyperexcitability with multiple population spikes evoked by a single stimulus, and increased amplitude of the first population spike but a reduced fEPSP, data not shown. Slices were washed with creatine free ACSF for 15 minutes before superfusion with DNP, and washout of the creatine-induced hyperexcitability declined, but not to control levels. Figure 5.10 shows that, 15 minutes following 3 hours creatine exposure, superfusion of slices with 50μM DNP for 3.5 minutes caused a similar rapid complete depression of synaptic transmission as it did prior to creatine incubation (n = 3). One slice was incubated with creatine at 34°C to determine whether improved phosphocreatine formation at a higher temperature would protect synaptic transmission from DNP. There was no difference in DNP action between this and the other two slices, so the data was combined. Adenosine accumulation during uncoupling is not a result of rapid ATP hydrolysis.

5.3.5. INHIBITORS OF NUCLEOTIDE METABOLISM

The enzymes which metabolise nucleotides to adenosine exist as cytosolic forms, and some also as ecto-enzymes. For instance, the existence of an ecto-5'-nucleotidase is well known (Meghji and Newby, 1990) and adenylate kinase may exist as an ecto-enzyme (Nagy *et al.* 1989). Adenosine could be released from cells as a nucleoside, or released nucleotides could be subsequently metabolised. The

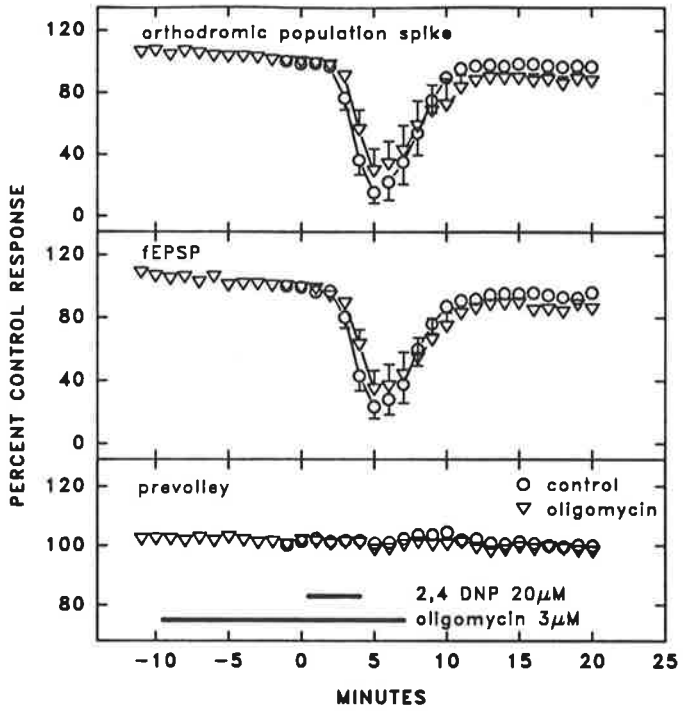

Figure 5.9 Comparison of time course of alterations in field potentials in the CA_1 area produced by 20μM 2,4 dinitrophenol in the presence and absence of the ATPase inhibitor oligomycin B

Field potentials in hippocampal slices (n = 3) before, during and after 3.5 minutes superfusion of 20μM DNP alone (control) or DNP in the presence of 3μM oligomycin B (oligomycin). DNP and oligomycin B superfusions are indicated by the bar at bottom. Data was collected and presented as in figure 5.2. ATPase inhibition had no effect on hippocampal field potentials and did not alter the depression of the fEPSP and the orthodromic population spike by mitochondrial uncoupling

ecto-5'-nucleotidase that metabolises extracellular AMP to adenosine can be inhibited with the non-metabolised nucleotide α,β-methylene ADP, and this compound was used in the hope of elucidating whether adenine nucleotides were released during oxidative stress. However, α,β-methylene ADP alone caused depression of synaptic transmission that was blocked by 8-CPT (n = 1), suggesting that this nucleotide is an agonist at adenosine A_1 receptors. Indeed α,β-methylene ADP has since been shown to depress [^{3}H]-noradrenaline release from rabbit cortex via an action at the A_1 receptor (von Kügelgen *et al.* 1992). Two potential inhibitors of adenylate kinase were examined in the current model. (+)Catechin, which inhibits hepatic adenylate kinase, had no effect on hippocampal field potentials and did not alter the depression of synaptic transmission due to DNP 20μM, (n = 1), data not shown. Also, Ap5A inhibits rat brain adenylate kinase (Nagy *et al.* 1989) and was tested in the hippocampus. Ap5A proved however, to be an adenosine A_1 receptor agonist, causing a depression of synaptic field potentials that was blocked by 8-CPT (n = 1), an action that has recently been confirmed (Klishin *et al.* 1994).

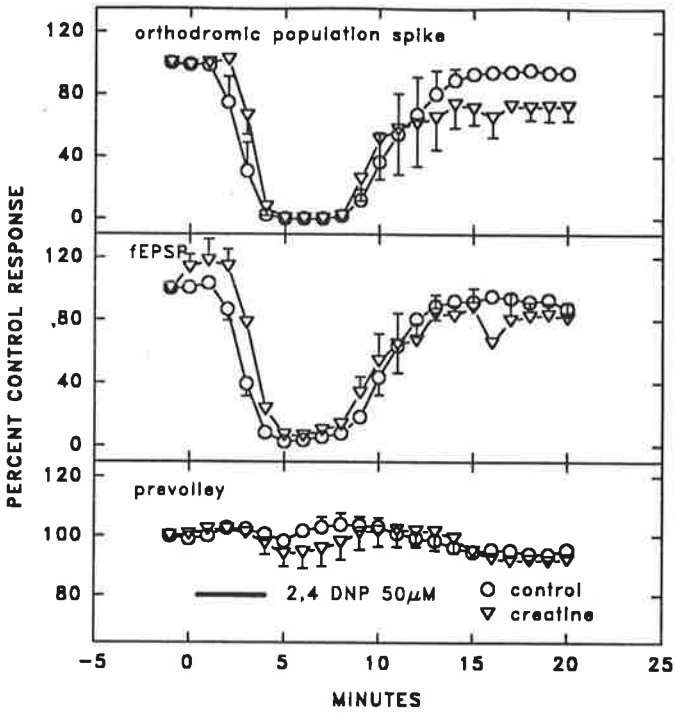

Figure 5.10 Comparison of time course of alterations in field potentials in the CA_1 area produced by 50μM 2,4 dinitrophenol with and without pre-incubation with creatine
Field potentials in hippocampal slices (n = 3) before, during and after 3.5 minutes superfusion of 50μM DNP alone (control) or DNP after pre-incubation in 25mM creatine (creatine). Slices were superfused with ACSF containing 25mM creatine for three hours and then washed in creatine free ACSF for 15 minutes prior to DNP superfusion. DNP superfusion is indicated by the bar at bottom. Data was collected and presented as in figure 5.2. Creatine pre-incubation caused some hyperexcitability (not shown), but did not alter the depression of the fEPSP and the orthodromic population spike by mitochondrial uncoupling.

5.3.6. INTRACELLULAR CALCIUM STORES

In mitochondria, oxidative phosphorylation and calcium uptake are alternative, energy requiring processes, driven by the electrochemical gradient across the inner mitochondrial membrane (Lehninger *et al.* 1967). Uncoupling agents such as DNP prevent calcium uptake and cause calcium efflux from mitochondria, but the inhibitors of oxidative phosphorylation, atractyloside and oligomycin, do not alter mitochondrial calcium homeostasis (Lehninger *et al.* 1967; Carafoli, 1987). The role in intracellular calcium homeostasis performed by mitochondria (Lehninger *et al.* 1967; Carafoli, 1987) may account for the different actions on synaptic transmission of the metabolic inhibitors described above, with mitochondrial calcium release itself initiating adenosine release, or this signal being further amplified via non-mitochondrial, calcium-sensitive intracellular calcium stores. Dantrolene blocks calcium release from calcium-sensitive intracellular calcium stores in the brain (Thayer *et al.* 1988; Frandsen and Schousboe, 1991a), and has been demonstrated to block the rise in intracellular calcium due to cyanide or ischaemia in the hippocampus *in vitro* (Dubinsky and Rothman, 1991; Mitani *et al.* 1993). Indeed dantrolene blocks an hypoxia-activated outward potassium current in CA_1 pyramidal

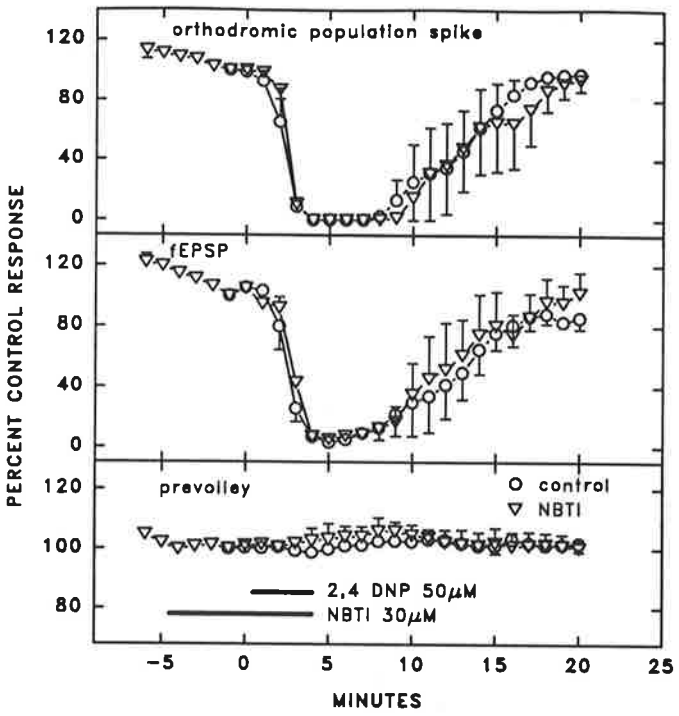

Figure 5.11 Comparison of time course of alterations in field potentials in the CA_1 area produced by 50μM 2,4 dinitrophenol in the presence and absence of the nucleotide transporter inhibitor NBTI

Field potentials in hippocampal slices (n = 3) before, during and after 3.5 minutes superfusion of 50μM DNP alone (control) or DNP in the presence of 30μM nitrobenzylthioinosine (NBTI). DNP and NBTI superfusions are indicated by the bar at bottom. Data was collected and presented as in figure 5.2. Nucleoside transporter inhibition caused a slight depression of synaptic field potentials, but did not alter the depression or recovery of the fEPSP and the orthodromic population spike by mitochondrial uncoupling.

neurons (Krnjevic and Xu, 1989). This prompted investigation of dantrolene in hypoxic depression of synaptic transmission. Superfusion of 30μM dantrolene for 10 minutes had no effect on field potentials. Synaptic depression by superfusion of 50μM DNP for 3.5 minutes was not altered by pre-treatment with dantrolene, (n = 1), data not shown.

Ruthenium red blocks the passive, reversible calcium carrier of the inner mitochondrial membrane (Carafoli, 1987); however, ruthenium red is shown variously to penetrate CA_1 neurons (García-Ugalde and Tapia, 1991), or to be membrane impermeant (Åkerman and Nicholls, 1981), and may also block neuronal membrane calcium channels. Superfusion of 10μM ruthenium red irreversibly depressed hippocampal synaptic transmission, without effect on the prevolley or antidromic population spike, (n = 1), data not shown. During this synaptic depression by ruthenium red, superfusion of 1μM 8-CPT increased the population spike amplitude but not the fEPSP rate of rise, indicating a slight increase in postsynaptic excitability rather than alleviation of synaptic depression. This suggests

that ruthenium red blocked calcium influx into the presynaptic terminals, rather than affecting mitochondrial calcium homeostasis.

5.3.7. NUCLEOSIDE TRANSPORTER INHIBITION BLOCKS ADENOSINE UPTAKE BUT NOT RELEASE

Adenosine crosses the cell membrane via a nucleoside transporter which mediates facilitated diffusion. High and low affinity nucleoside transporters exist that can be blocked by NBTI and dipyridamole respectively, although micromolar concentrations of these inhibitors are reported to block either transporter in the rat (Ohkubo *et al.* 1991). In the hippocampal slice, NBTI or dipyridamole causes an adenosine mediated depression of synaptic transmission (Fowler, 1988; Dunwiddie and Diao, 1994; Haas and Greene, 1988). To determine whether adenosine formed intracellularly is released via the nucleoside transporters during energy deprivation, these inhibitors were investigated in the hippocampal slice.

In the present study 30μM NBTI cause a minor, slow developing depression of synaptic field potentials in two of three slices. Figure 5.11 shows that pre-treatment of slices with 30μM NBTI had no affect on the synaptic depression or recovery due to 3.5 minutes superfusion with 50μM DNP. Dipyramidole is a more effective inhibitor of nucleoside transport in the rat hippocampus than is NBTI, and in two different slices, 8 minute dipyridamole superfusion at 5μM or 50μM caused a slow developing depression of synaptic transmission that did not wash out within one hour, data not shown. In the slice exposed to 5μM dipyridamole, DNP 50μM superfused prior to complete depression of field potentials, still produced rapid depression of synaptic transmission. Thus, while adenosine transport inhibition apparently reduced normoxic adenosine uptake, it did not prevent adenosine release during oxidative stress when transport block may have been surmounted by enormous adenosine production.

5.3.8. ADENOSINE UPTAKE AND CATABOLISM

The action of dipyramidole described above suggests that adenosine uptake is important in termination of adenosine action. It has been suggested that adenosine uptake is limited by subsequent intracellular metabolism, primarily by adenosine kinase (Gu and Geiger, 1992). Co-administration of 100μM iodotubercidin, an adenosine kinase inhibitor (Henderson *et al.* 1972), and 50μM DNP for 3.5 minutes resulted in depression of synaptic transmission that was not reversed by 30 minutes washout (n = 1), indicating that phosphorylation of adenosine to AMP is primarily responsible for termination of adenosine action in the hippocampal slice, data not shown.

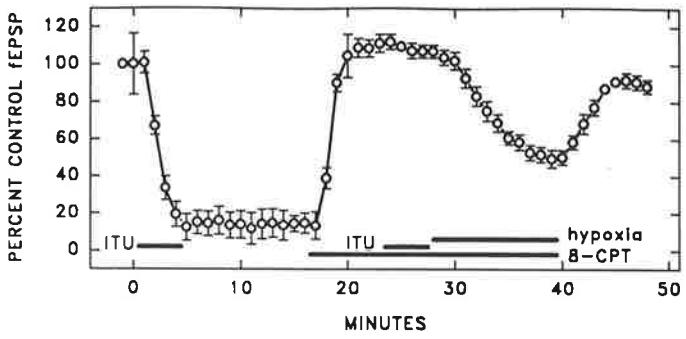

Figure 5.12 Depression of synaptic transmission by adenosine kinase inhibition and by hypoxia
The fEPSP was recorded in hippocampal slices exposed to the adenosine kinase inhibitor iodotubercidin (100μM) alone, and in the presence of 1μM 8-CPT, and subsequently to hypoxia. Iodotubercidin, 8-CPT and hypoxic ACSF superfusion is indicated by the bars at bottom. fEPSP data was collected and presented as in figure 5.2. Iodotubercidin caused a depression of synaptic transmission that was completely reversed by 8-CPT (n = 2). Superfusion of slices with hypoxic ACSF during adenosine kinase inhibition and adenosine A_1 receptor antagonism (n = 4) produced a depression of synaptic transmission.

It is possible that inhibition of adenosine kinase is primarily responsible for adenosine accumulation during hypoxia, and this was investigated. Figure 5.12 shows that superfusion of slices with 100μM iodotubercidin caused an adenosine-mediated depression of the fEPSP, such depression being previously shown to persist for at least 30 minutes. After 12 minutes, slices were superfused with 1μM 8-CPT which completely reversed the depression of synaptic transmission by iodotubercidin. This indicates that adenosine kinase inhibition during normoxia does not produce adenosine accumulation to the same extent as does hypoxia, since 12 minutes hypoxia in the presence of 1μM 8-CPT produced maximal depression the fEPSP to 55.1 ± 5.85% S.E.M. (n = 5) of baseline, see section 4.3.4.1 and 4.3.7. During adenosine A_1 receptor antagonism, slices were again superfused with iodotubercidin to ensure continued inhibition of adenosine kinase, then superfused with hypoxic ACSF for 12 minutes. Figure 5.12 shows that the combination of adenosine kinase inhibition and hypoxia in the presence of 1μM 8-CPT depressed the fEPSP to 49.4 ± 4.73% S.E.M. (n = 4) of baseline, which was not significantly different from hypoxia alone in the presence of 1μM 8-CPT ($P \leq 0.05$). Apparently adenosine kinase is completely inhibited during hypoxia, but this alone cannot account for hypoxic adenosine accumulation, and hydrolysis of AMP to adenosine must also be increased.

5.4. DISCUSSION

In order to examine whether hypoxic inhibition of mitochondrial energy production causes hypoxic production of adenosine, inhibitors of mitochondrial respiration and uncoupling agents were tested, and indeed found to promote adenosine induced

depression of synaptic transmission. However, inhibitors of oxidative phosphorylation did not cause an extracellular accumulation of adenosine, indicating that hypoxic adenosine production is not directly related to block of mitochondrial ATP production.

5.4.1. RESPIRATION

In normally energised brain mitochondria, reducing equivalents from NADH and $FADH_2$, produced in the metabolism of pyruvate, are shuttled into the mitochondrial matrix. NAD^+ is regenerated in the reduction of the flavin mononucleotide prosthetic group of NADH dehydrogenase, a flavoprotein that is the initial electron carrier in the electron transport chain. This chain is a series of electron carrier complexes, comprised of flavoproteins, non-haem iron, coenzymes, cytochromes and copper proteins, either integral to or associated with the inner mitochondrial membrane, see figure 5.13. These carriers, in a series of oxidation/reduction reactions, transfer reducing equivalents from NADH or $FADH_2$ to oxygen, producing water. At the three integral membrane complexes, NADH dehydrogenase, ubiquinone-cytochrome c-reductase and cytochrome oxidase, the transfer of reducing equivalents results in the pumping of protons out of the mitochondrial matrix, with a consequent pH gradient and -150mV membrane potential across the inner mitochondrial membrane ($\Delta\psi_m$) (Scott and Nicholls, 1980; Mitchell, 1976; Lehninger *et al.* 1967). This proton electrochemical gradient ($\Delta\mu_{H^+}$), totalling some 200mV (Åkerman and Nicholls, 1983), conserves the large free energy difference between the reactants and products of the electron transport chain, and drives energy requiring mitochondrial reactions (Mitchell, 1976; Lehninger *et al.* 1967).

The primary function of mitochondria is oxidative phosphorylation, the phosphorylation of ADP to ATP. This is catalysed by ATP synthetase, a member of the class of membrane-associated, reversible ATPases whose catalytic activity is linked to cation translocation (Ragan and Galante, 1985). The ATP synthetase complex consists of two domains, an oligomycin sensitive, membrane domain, F_0, through which protons can flow, driving the phosphorylation of ADP by the water-soluble, F_1ATPase domain (Ragan and Galante, 1985). Respiration and oxidative phosphorylation are usually tightly coupled, transport of two electrons results in phosphorylation of three molecules ADP, and respiration may be blocked if ATP synthesis is inhibited. A further mitochondrial process driven by the electrochemical gradient is the exchange of matrix ATP^{4-} for cytosolic ADP^{3-} by the atractyloside sensitive ADP^{3-}/ATP^{4-} electrotonic exchanger (Lanoue and Schoolwerth, 1984), without which oxidative phosphorylation does not proceed (Vignais, 1976; Lanoue and Schoolwerth, 1984). An alternative energy requiring mitochondrial function is calcium sequestration. Calcium transport across the inner mitochondrial membrane

occurs via a passive, reversible carrier and is driven by the electrical potential of the proton electrochemical gradient (Lehninger, 1970; Carafoli and Sottocasa, 1984).

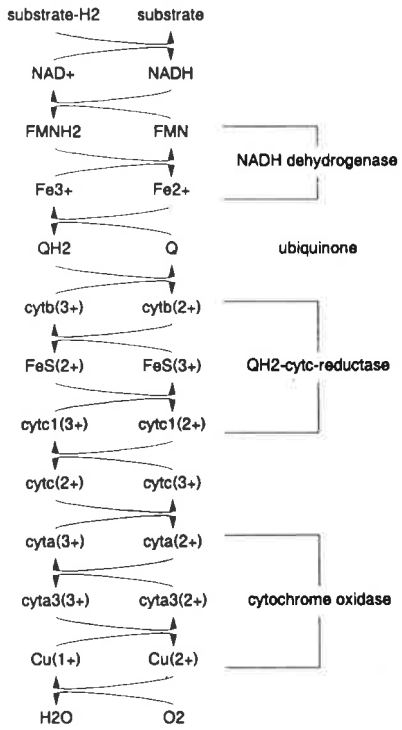

Figure 5.13 Electron transport chain
Diagram of the flow of reducing equivalents from substrate to NAD, and through the electron transport chain to oxygen. Abbreviations: nicotinamide adenine dinucleotide (NAD), flavin mononucleotide (FMN), cytochrome (cyt), and ubiquinone (Q). Protons are pumped at the bracketed, integral inner membrane complexes.

Under normal conditions, where oxygen is plentiful and ADP is low, mitochondria are in state 4, characterised by slow respiration with NADH mostly reduced and subsequent electron transport chain components progressively more oxidised (Jöbsis, 1972). With increased metabolic demand and ATP hydrolysis, the increased availability of ADP allows fast, state 3 respiration, which results in greater oxidation of the all but the terminal electron carriers, which are more reduced by the greater flow of reducing equivalents (Jöbsis, 1972), see table 5.1. When necessary, both the cytosolic glycolytic capacity and mitochondrial respiratory capacity can readily increase to maintain $\Delta\psi_m$ (Kauppinen and Nicholls, 1986), and therefore oxidative phosphorylation.

5.4.2. ENERGY DEPRIVATION

5.4.2.1. Electron transport inhibitors

Inhibiting respiration with cyanide prevents electron transfer from cytochrome oxidase to oxygen (Slater, 1967), this block of the electron transport chain prevents proton pumping and therefore oxidative phosphorylation, and thus closely mimics hypoxia. Consequently, cyanide has been used as an experimental tool for studying

hypoxic mechanisms; for instance, prolonged cyanide mimics hypoxic excitotoxicity in cortical neurons and neuronal cell lines *in vitro* (Rothman, 1983; Oberpichler *et al.* 1990; Maduh *et al.* 1990; Johnson *et al.* 1987; Goldberg *et al.* 1987). In the following discussion, the term respiratory inhibition will be used to indicate both hypoxia and cyanide actions on the mitochondria. It is not surprising, that in the present study, cyanide caused an adenosine A_1 receptor-mediated synaptic depression identical to hypoxia. The present findings confirm that exposure on neurons to cyanide causes rapid, reversible loss of synaptic transmission, previously shown between the perforant path and dentate granule cells *in vivo* (Lees and Sandberg, 1991), at lateral olfactory tract synapses in olfactory cortical slices (Yamamoto and Kurokawa, 1970) and at the Schaffer collateral synapses in the CA_1 of hippocampal slices (Aitken and Braitman, 1989). Intracellular recordings in the CA_1 show that cyanide produces changes typical of hypoxia: a pronounced hyperpolarisation with decreased input resistance, followed by a slow depolarisation and eventual loss of plasma membrane potential (Reiner *et al.* 1990). Rotenone, which also inhibits respiration, but by extremely tight binding to the NADH dehydrogenase complex (Singer, 1979), caused irreversible synaptic depression in the present study.

characteristics						steady-state % reduction of components				
state	$[0_2]$	ADP level	substrate level	resp. rate	rate-limiting substance	a	c	b	flavo-prot.	NAD
1	>0	low	low	slow	ADP	0	7	17	21	90
2	>0	high	0	slow	substrate	0	0	0	0	0
3	>0	high	high	fast	e^- chain	4	6	16	20	53
4	>0	low	high	slow	ADP	0	14	35	40	99
5	0	high	high	0	oxygen	100	100	100	100	100

Table 5.1 Metabolic states of mitochondria
Table compares respiratory rates and substrate availability with steady state reduction of respiratory chain components in mitochondria under different metabolic states. Adapted from Jöbsis,F.F. 1972.

5.4.2.2. Uncoupling

Uncoupling agents typified by DNP are lipophilic, weak acids, and freely exchange protons across the inner mitochondrial membrane, preventing formation of $\Delta\mu_{H^+}$ (Heytler, 1979). Thus, respiration continues at an increased rate, but energy is dissipated as heat (Hanstein, 1976) and no mitochondrial energy requiring process proceed. Like hypoxia, uncoupling produced an adenosine A_1 receptor-mediated depression of synaptic transmission, due to an extracellular accumulation of adenosine.

These studies confirm, in the CA_1, previously described depressive electrophysiological actions of DNP. For instance, lateral olfactory tract synaptic transmission in olfactory cortical slices, and that between the perforant path and dentate gyrus cells in hippocampal slices, is reversibly depressed by DNP (Yamamoto and Kurokawa, 1970; Cox *et al.* 1985). Microiontophoresis of DNP onto cortical and spinal neurons in anaesthetised cats causes a hyperpolarisation of 12mV and reduces input resistance by 6MΩ, and depolarisation or acetylcholine induced firing is inhibited, effects consistent with activation of a potassium conductance (Godfraind *et al.* 1970; Godfraind *et al.* 1971; Krnjevic and Werman, 1978). The inhibition of synaptic transmission and activation of a potassium current by DNP and cyanide are similar to the actions of hypoxia.

5.4.2.3. Mitochondrial poisons selectively inhibit synaptic transmission

Rotenone (Scott and Nicholls, 1980) and cyanide (Kauppinen and Nicholls, 1986) have only minor effect on the plasma membrane potential in synaptosomes, indicating that the depressive actions are not the result of membrane dysfunction. The modest change in membrane potential in cortical and spinal neurons induced by DNP (Godfraind *et al.* 1971; Krnjevic and Werman, 1978) suggests that uncouplers activate a membrane current, but do not collapse the plasma membrane potential as they do to $\Delta\psi_m$. Furthermore, in synaptosomes, the proton translocating uncoupler carbonyl cyanide p-triflouromethoxyphenylhydrazone (FCCP) does not alter the plasma membrane potential measured by ^{86}Rb equilibrium (Scott and Nicholls, 1980). Indeed uncouplers, acting as proton carriers, could not hyperpolarise the plasma membrane, as there is insufficient difference between intracellular and extracellular pH (Martin *et al.* 1994; Ekholm *et al.* 1992; Whittingham *et al.* 1984; Kauppinen and Williams, 1990) to extrude protons against the membrane potential. This indicates a specific action of uncoupling on synaptic transmission and postsynaptic membrane currents, rather than membrane dysfunction.

While the depressive actions of inhibitors of respiration and uncoupling agents on synaptic transmission in the CNS have previously been described, the present studies further demonstrate that, in common with hypoxia, the synaptic depression is a result of activation of adenosine A_1 receptors. Adenosine mediation of synaptic depression by metabolic inhibitors has not previously been described. Indeed there are few demonstration of adenosine release due to these inhibitors in any tissues (Bontemps *et al.* 1993).

5.4.3. SIGNAL FOR ADENOSINE RELEASE

It seems likely that, DNP, cyanide and hypoxia act at a common site resulting in the adenosine mediated inhibition of synaptic transmission, and this site is the mitochondria. The adenosine that accumulates during energy deprivation does not

originate from the mitochondria, as the release of adenosine from isolated mitochondria, via the atractyloside sensitive adenine nucleotide carrier, during state 3 and 4 respiration is not enhanced by rotenone or anoxia (state 5), and is decreased by uncoupling (Bukoski *et al.* 1983). It seems that mitochondrial inhibition must signal cytosolic adenosine accumulation, and cytosolic adenosine will equilibrate with the extracellular space via the nucleoside transporter. Adenosine may accumulate during energy deprivation because of a change in the balance of the cytosolic anabolic and catabolic pathways. Increase adenosine production could result from an increase activity of 5'-nucleotidase as a result of enzyme activation, independent of an increase in substrate. Increase production of adenosine via 5'-nucleotidase could also result from an increase in free AMP, secondary to ATP depletion, or due to local increases in free AMP, with minor overall ATP depletion. Adenosine may accumulate due to reduced salvage due to inactivation of adenosine kinase, which may result from reduced free energy of the ATP pool.

5.4.3.1. ATP depletion is not responsible for initial inhibition of synaptic transmission

Since electron transport inhibition and uncoupling have different actions on mitochondrial energisation, on the surface it would appear that a common action, leading to adenosine release, might be the prevention of oxidative phosphorylation. However, it seems that adenosine release and inhibition of synaptic transmission is not due to ATP depletion or a drop in the free energy of the cytosolic adenylate pool.

5.4.3.1.1. Oxidative phosphorylation

Inhibitors of oxidative phosphorylation prevent mitochondrial production of ATP (Lehninger, 1964). Oligomycin is the classical ATP synthetase inhibitor (Linnett and Beechey, 1979) and, as expected, causes a slight hyperpolarisation of $\Delta\psi_m$ in synaptosomes, but will cause the collapse of $\Delta\psi_m$ if respiration is also inhibited (Scott and Nicholls, 1980). Atractyloside binds the cytosolic side of the adenine nucleotide translocator, inhibiting ATP export from the mitochondrial matrix (Lanoue and Schoolwerth, 1984; Vignais, 1976) and has been used experimentally to produce ischaemic-like changes in canine heart (Bittar *et al.* 1976). However, in the hippocampal slice neither oligomycin B nor atractyloside produced a depression of synaptic transmission; therefore, inhibition of oxidative phosphorylation is an insufficient stimulus for adenosine release. The present findings are at odds with the observation that oligomycin iontophoresis depresses synaptic transmission in cat cortical neurons *in vivo* (Godfraind *et al.* 1971); these different results may be due to the lower glucose *in vivo* further compromising energy production compared to the 10mM glucose used *in vitro*.

Glycolysis can maintain ATP during inhibition of oxidative phosphorylation. In whole cells, oligomycin, in combination with inhibition of glycolysis, will reduce ATP (Schmid-Antomarchi *et al.* 1987; Kristensen, 1989; Meghji *et al.* 1989), but it seems likely that oligomycin B in the presence of glucose will not greatly deplete ATP. While oligomycin in glucose free medium results in ATP depletion in human fibroblast cultures, in the presence of glucose, the ATP drop is only slight, and glucose can regenerate depleted ATP in the presence of oligomycin (Kristensen, 1989). In isolated rat liver cells, atractyloside alone reduces cytoplasmic ATP/ADP ratio and increases AMP (Zuurendonk and Tager, 1974; Kristensen, 1989), but may not result in a large ATP depletion. In the present investigation, prolonged incubation of the slice in oligomycin caused a slowly developing depression of synaptic transmission that may have been adenosine A_1 receptor-mediated, although this was not verified. Such slow adenosine build up may be a result of loss of adenine nucleotides to nucleosides.

5.4.3.1.2. ATP depletion

There is a generally poor correlation between the depression of synaptic transmission by hypoxia and ATP depletion in the gerbil hippocampus *in vivo* (Katsura *et al.* 1993; Ekholm *et al.* 1992; Lowry *et al.* 1964), guinea pig olfactory cortex slice (Yamamoto and Kurokawa, 1970), hippocampal slice CA_1 (Wallis *et al.* 1990) and dentate gyrus (Lipton and Whittingham, 1982). Respiratory inhibitors and uncouplers can inhibit synaptic activity in hippocampal slices without significantly decreasing tissue levels of phosphocreatine or ATP (Cox *et al.* 1985). Hypoxia, while producing an initial small, rapid drop in ATP, and a depletion of phosphocreatine, does not result in a large depletion of total cell ATP in brain slices (Kauppinen and Williams, 1990; Fredholm *et al.* 1984; Yoneda and Okada, 1989). Only prolonged respiratory inhibition reduces cytosolic ATP (Kauppinen and Williams, 1990; Fredholm *et al.* 1984; Yoneda and Okada, 1989). In cortical synaptosomes, maintenance of $\Delta\psi_m$ during inhibition of respiration results from a stimulation of inner mitochondrial membrane F_1ATPase activity to actively pump protons (Kauppinen and Nicholls, 1986; Scott and Nicholls, 1980), indicating that glycolysis can maintain the free energy of the cytosolic adenine nucleotide pool sufficiently for basal energy demands.

5.4.3.1.3. ATP buffering with phosphocreatine

During *in vivo* cerebral ischaemia, while synaptic transmission is inhibited, ATP is maintained at the expense of phosphocreatine (Katsura *et al.* 1993; Ekholm *et al.* 1992; Lowry *et al.* 1964). Similarly, in brain slices during ischaemia or hypoxia, despite an initial drop in ATP and rise in ADP and AMP, ATP is preserved at the expense of phosphocreatine (Kauppinen and Williams, 1990; Fredholm *et al.* 1984;

Yoneda and Okada, 1989). Phosphocreatine acts as an energy store, buffering ATP, maintaining ATP/ADP ratios in a range where they act as regulators of various cellular functions, preventing loss of nucleotides through adenylate kinase and 5'-nucleotidases, and buffering proton production during rapid ATP hydrolysis (Wallimann *et al.* 1992; Ereciñska and Silver, 1989).

Incubation of hippocampal slices with creatine can increase the phosphocreatine levels in the slice without increasing ATP, levels increase linearly with incubation time, doubling from 40 to 80mmol/kg in the first hour (Lipton and Whittingham, 1982; Okada and Yoneda, 1983; Yoneda *et al.* 1983). Elevated phosphocreatine slows ATP loss and increases the time that slices can be ischaemic yet still recover synaptic function with reoxygenation CA_3 (Okada and Yoneda, 1983); it also increases the latency of inhibition of synaptic transmission due to ischaemia and hypoxia in the dentate gyrus (Lipton and Whittingham, 1982), CA_3 (Okada and Yoneda, 1983) and CA_1 (Leblond and Krnjevic, 1989). Creatine pre-incubation did not delay the depression of synaptic field potentials in the present investigation, contrary to previous findings with intracellular recording in some CA_1 neurons (Leblond and Krnjevic, 1989). The failure of creatine pre-treatment to delay synaptic depression provides further evidence against ATP depletion contributing to adenosine release and hypoxic inhibition of synaptic transmission. However, ATP depletion may have a role in hypoxic hyperpolarisation, and this can be delayed with creatine pre-incubation (Leblond and Krnjevic, 1989). Also, ATP depletion may be a source of adenosine during prolonged energy deprivation.

5.4.3.1.4. Hypoglycaemia

Hypoglycaemia provides further evidence that ATP depletion may not contribute to the rapid adenosine accumulation with hypoxia. Hypoglycaemia alone, causes a slow depression of synaptic transmission in the hippocampus, which is, at least in part, mediated by adenosine release (Fowler, 1993). Unlike hypoxia however, hypoglycaemic depression of synaptic transmission in the CA_1 progresses slowly, beginning after 10 minutes of hypoglycaemic superfusion, with full depression of the population spike only after 60 minutes (Fowler, 1993; Schurr *et al.* 1986b). Similarly, *in vivo* recordings from rat dentate granule cell layer show that injection of sodium cyanide causes an immediate depression of field potentials while the glycolysis inhibitor iodoacetate causes synaptic depression developing slowly over 15 minutes (Lees and Sandberg, 1991). [^{3}H]-2-deoxyglucose washes out from brain slices with a half life of 2.6 minutes, suggesting that glucose depletion within the slice following a switch from 10mM glucose to glucose free superfusion should only take 10-15 minutes, (Newman *et al.* 1988a), the time to onset of aglycaemic synaptic

depression. This leaves a 30 minute disparity between the complete depression of the population spike due to hypoxia and that due to hypoglycaemia.

Two possibilities exist to explain this difference. Firstly, the hypoglycaemic slice may derive substrate for oxidation from a source other than glucose, in the slice this substrate would necessarily be endogenous, as no other is provided in the ACSF. Brain glycogen stores can maintain glycolysis for only 5 minutes in the absence of exogenous glucose (Clarke *et al.* 1989). During aerobic glycolysis, 15% of glucose consumed is converted to lactate, which is largely removed in the blood *in vivo*. Brain lactate levels are 1-2mmole/kg wet weight *in vivo* (Balázs, 1970) and lactate levels in rat hippocampal slices are 4 fold higher than *in vivo* (Whittingham *et al.* 1984). Such levels of lactate would be consumed for aerobic ATP production in two to ten minutes (McIlwain and Bachelard, 1971). Although the brain can oxidise fats to acetyl-CoA, and use ketone bodies, acetoacetate and β-hydroxybutyrate , these are not abundant in the brain and cannot supply cerebral energy demands in the absence of glucose (Clarke *et al.* 1989; McIlwain and Bachelard, 1971). It is difficult, therefore, to understand the slow time course of the depression of synaptic transmission in hippocampal slices due to hypoglycaemia, as it seems unlikely that there are sufficient substrate stores. Thus, inhibition of energy production alone may be insufficient stimulus for rapid adenosine accumulation, but may cause a slower loss of adenine nucleotides to adenosine. A different mechanism may be responsible for hypoglycaemic adenosine release compared to hypoxia. If this is the case, mitochondrial status may be the trigger in hypoxia.

5.4.4. ADENOSINE METABOLIC PATHWAYS

The brain does not synthesise nucleosides *de novo* but adenosine can be formed from a number of sources, including hydrolysis of the α phosphate of AMP by 5'-nucleotidases or non-specific phosphatases, metabolism of cAMP then de-phosphorylation by 5'-nucleotidase, and from S-adenosylhomocysteine by S-adenosylhomocysteine hydrolase. Adenosine can be catabolised by phosphorylation or deamination.

5.4.4.1. S-adenosylhomocysteine hydrolase

S-adenosylhomocysteine hydrolase can catalyse the reversible hydrolysis of S-adenosylhomocysteine to adenosine and homocysteine, but unless the products are removed, the equilibrium greatly favours S-adenosylhomocysteine synthesis (Schrader *et al.* 1981; de la Haba and Cantoni, 1959), and the enzyme is subject to product inhibition (de la Haba and Cantoni, 1959). The K_m for hydrolysis of S-adenosylhomocysteine is 2.9μM, and the K_m for adenosine is 0.39μM but is in the millimolar range for homocysteine (Schrader *et al.* 1981). This explains why intracellular trapping of adenosine with homocysteine thiolactone reduces release of

adenosine from ischaemic hippocampal slices (Shirasaki *et al.* 1994). While formation of adenosine from S-adenosylhomocysteine contributes to normoxic basal adenosine levels, adenosine is not produced via this route during hypoxia. In rat hepatocytes, the S-adenosylhomocysteine hydrolase inhibitor, adenosine dialdehyde, causes a decreased adenosine production during normoxia but has no effect during hypoxia (Bontemps *et al.* 1993).

5.4.4.2. 5'-nucleotidase

In rat brain, several enzymes, including non-specific alkaline or acid phosphatases, can catalyse the removal of the a phosphate from AMP, but only the 5'-nucleotidase is active at physiological pH (Nagata *et al.* 1984). In sub-cellular fractions from whole rat brain, 5'-nucleotidase activity is located mainly in the crude mitochondrial fraction, microsomal fraction and nerve end particles, all with high specific activity, and the highest specific activity is in purified synaptic membranes (Nagata *et al.* 1984). High activity of the enzyme in the rat brain is found in the basal ganglia, medulla oblongata, thalamus, and all synaptic layers of the CA_1 area of the hippocampus (Nagata *et al.* 1984; Fastbom *et al.* 1987). There are two different cytosolic forms as well as a membrane bound form of 5'-nucleotidase (Meghji and Newby, 1990). The membrane bound form is an ecto-5'-nucleotidase (Meghji and Newby, 1990), shown to catalyse the hydrolysis of extracellular AMP in synaptosomes (Hoehn and White, 1990b; MacDonald and White, 1985) and cortical slices (Hoehn and White, 1990a), but is not likely to be the 5'-nucleotidase responsible for extracellular adenosine accumulation during hypoxia (Lloyd *et al.* 1993), when nucleosides are released. Cytosolic 5'-nucleotidases have been extensively studied in heart and liver, where many IMP preferring forms have been described (Skladanowski *et al.* 1990; Newby, 1988; Truong *et al.* 1988; Meghji *et al.* 1988). AMP preferring forms of 5'-nucleotidase are described in the rat and pigeon heart and the rat liver (Truong *et al.* 1988; Itoh *et al.* 1986; Skladanowski and Newby, 1990; Newby, 1988; Spychala *et al.* 1990; Nakamura, 1976). This latter enzyme is stimulated by ADP and ATP, although this ATP stimulation may not occur at physiological levels of AMP (Itoh *et al.* 1986; Skladanowski and Newby, 1990). The purified enzyme is activated by falling adenylate energy charge (Itoh *et al.* 1986) and with fixed nucleotide levels, activity increases with AMP concentration (Spychala *et al.* 1990). The AMP preferring 5'-nucleotidase has a pH optimum in the range near pH 7 (Zimmermann, 1992; Newby, 1988). These methods of control of catalytic activity may be important during hypoxia when AMP increases and energy charge and pH fall.

5.4.4.3. Nucleoside transporter

In embryonic chick neuronal or glial cell cultures, where 2μg/ml oligomycin plus 30 mM 2-deoxyglucose increased cytosolic and extracellular adenosine, dilazep, a nucleoside transport inhibitor, decreased release of adenosine by 80-90%, trapping adenosine intracellularly (Meghji *et al.* 1989). Failure of nucleoside transporters to prevent DNP depression of synaptic transmission in the present work is curious. Possibly some adenosine was trapped intracellularly but the transport block was surmounted. Indeed, the nucleoside transporter inhibitors are poorly effective in the rat (Van Belle, 1988; Verma and Marangos, 1985), and the adenosine released in the absence of nucleoside transporter inhibitors is at least an order of magnitude greater than is necessary for complete block of synaptic transmission, see chapter 4.

Extracellular adenosine accumulation during hypoxia or metabolic inhibition is unlikely a result of inhibition of nucleoside uptake, since the majority of transport occurs via a passive carrier (Ohkubo *et al.* 1991; Dunwiddie and Diao, 1994). It is more likely that, during hypoxia, cytosolic adenosine production increases, and adenosine uptake, being mostly equilibrative, is limited by inhibition of subsequent adenosine metabolism via adenosine kinase (Gu and Geiger, 1992; Bontemps *et al.* 1993).

5.4.4.4. Adenosine kinase

Uptake of adenosine and phosphorylation via adenosine kinase is the primary mechanism for removing extracellular adenosine and salvaging nucleotides. This is apparent in the present study from the continual depression of synaptic transmission by the adenosine kinase inhibitor iodotubercidin. In normoxic hepatocytes, iodotubercidin causes an increase in adenosine production (Bontemps *et al.* 1983) to a rate similar to that during hypoxia (Bontemps *et al.* 1993). This indicates a futile cycle of phosphorylation and dephosphorylation of adenosine during normoxia, and the present evidence indicates such a futile cycle occurs in the hippocampus. Evidence suggests that phosphorylation of adenosine is inhibited during hypoxia, and this contributes to adenosine accumulation. Rat hepatocytes in the presence of iodotubercidin and deoxycoformycin produce adenosine at a linear rate of about 18 nmoles/min/g cells, however, this rate only doubles with the introduction of hypoxia, despite a 20 fold increase in total AMP (Bontemps *et al.* 1993). Iodotubercidin alone does not alter hypoxia or cyanide induced accumulation of adenosine, suggesting that adenosine kinase is completely inhibited during hypoxia (Bontemps *et al.* 1993), and, in the present study, iodotubercidin did not increase the adenosine mediated depression of synaptic transmission during hypoxia. However, an 8-CPT concentration that completely reversed the iodotubercidin-induced, A_1 receptor-mediated depression of synaptic transmission, only partially restored the synaptic depression due to hypoxia. It is apparent that, in the hippocampus, hypoxia causes a

considerable increase in adenosine production that far outweighs the adenosine accumulation due to adenosine kinase inhibition.

Nevertheless, enhanced production of adenosine via 5'-nucleotidase during hypoxia cannot account for adenosine accumulation if the adenosine kinase is not saturated or inactivated, since the hypoxic rate of adenosine production in hepatocytes equals the rate of normoxic phosphorylation by adenosine kinase (Bontemps *et al.* 1993; Bontemps *et al.* 1983). In normoxic rat hepatocytes and synaptosomes, adenosine kinase is not saturated by basal adenosine levels, since exogenous adenosine is phosphorylated at about 0.08μmole/min/g cells and V_{max} 0.8pmole/min/mg protein (Bontemps *et al.* 1983; Gu and Geiger, 1992). This suggests adenosine kinase is not saturated by the adenosine produced during hypoxia, and therefore must be inhibited to allow adenosine accumulation.

The rapid recovery of synaptic potentials with reoxygenation demonstrates that adenosine action at the A_1 receptor is terminated rapidly, despite the apparent large concentration of adenosine present extracellularly during hypoxia. If adenosine kinase is inhibited during hypoxia, it must recover immediately and have considerable capacity.

Purified adenosine kinase is inhibited by low millimolar concentrations of AMP (Murray, 1968; Hawkins and Bagnara, 1987) but these are not likely to be important physiologically as neither total, nor free AMP achieve these levels, even during ischaemia (Ekholm *et al.* 1992). Drop in the adenylate energy charge below 0.7 causes a steep drop in purified adenosine kinase activity (Murray, 1968). As previously discussed, ATP is not greatly depleted, and free energy of the cytosolic adenine nucleotide pool remains high during respiratory inhibition, and this is therefore unlikely to be the cause of adenosine kinase inhibition. Adenosine kinase from rat brain has a broad pH optimum at 7.5-8.5 but acidification to pH 7 results in a 50% decrease in activity (Yamada *et al.* 1980). Hypoxia results in an intracellular acidification that may be sufficient to deactivate adenylate kinase.

5.4.4.5. Adenosine deaminase

Adenosine deaminase is probably not greatly involved in the deactivation of adenosine, and indeed the hippocampus has only low levels of adenosine deaminase (Nagy *et al.* 1990). While the adenosine deaminase inhibitor deoxycoformycin slightly increases adenosine accumulation and depresses inosine accumulation in rat cortex *in vivo* (Phillis *et al.* 1991) and increases adenosine production in hepatocytes (Bontemps *et al.* 1993) this may be a result of AMP deaminase inhibition. The more selective adenosine deaminase inhibitor erythro-9-(-2-hydroxy-3-nonyl)adenosine (EHNA) does not further increase adenosine mediated inhibition of field potentials in

the hippocampal slice in the presence of nucleoside transport inhibitors (Dunwiddie and Diao, 1994), and does not increase ischaemic release of adenosine from hippocampal slices (Lloyd and Fredholm, 1990).

5.4.5. ACTIVATION OF ENZYMES

5.4.5.1. Mitochondrial signals

It is evident from the preceding discussion that inhibition of oxidative phosphorylation and ATP depletion are not the common mechanism for adenosine accumulation during uncoupling or respiratory inhibition. The best explanation is a hypoxic inhibition of adenosine kinase and an increase activity of 5'-nucleotidase. Yet the common site of action for respiratory inhibition and uncoupling appears to be the mitochondria, and this must signal these changes.

While respiratory inhibition and uncoupling both cause rapid adenosine release, they act differently, and have different consequences on mitochondrial energisation; thus, the common mechanism stimulating adenosine release is unclear. Alterations in the redox potential of the respiratory chain components is not the signal. When the flow of electrons from cytochrome oxidase to oxygen is inhibited, as with hypoxia or cyanide, all components of the respiratory chain will be completely reduced (Jöbsis, 1972); however, with increase electron flow in the presence of an uncoupler, carriers near oxygen will become reduced and carriers near NADH become oxidised to a greater extent than in state 3 (Oshino *et al.* 1974). Furthermore, alterations in the proton electrochemical gradient is not the signal for adenosine release. In cortical synaptosomes, inhibition of respiration causes only a small reduction of $\Delta\psi_m$, from -150mV to -125mV, while uncouplers cause a collapse of $\Delta\psi_m$ (Kauppinen and Nicholls, 1986; Scott and Nicholls, 1980). However the signal may be local adenine nucleotide changes, for instance at the presynaptic sites which are mitochondria rich, rather than whole cell or tissue nucleotide levels, as are usuually measured. It may also be that fluctuations in cytosolic calcium due to release from mitochondrial and non-mitochondrial intracellular stores alters enzyme activation. Intracellular acidification as a result of energy deprivation may alter enzyme function.

5.4.5.2. Increase in AMP

Apparently glycolysis maintains cellular ATP during hypoxia. However, an increase in free ADP or free AMP occurs before ATP depletion during ischaemia; cerebral cortex free AMP raises from 0.24 μmoles/kg wet weight during normoxia to 2-8 μmole/kg wet weight during ischaemia (Ekholm *et al.* 1992), and this rise in AMP might result in a raised adenosine production via 5'-nucleotidases. CA_1 fEPSP is completely depressed while adenylate energy charge has dropped from 0.9 to 0.8 with hypoxia, and the start of synaptic transmission depression precedes any change in adenylate energy charge (Fredholm *et al.* 1984). However, this charge estimate

was based on total nucleotides, not free ADP and AMP. In the isolated perfused rat heart during ischaemia or metabolic stimulation, the drop in phosphorylation potential, [ATP]/[ADP]*[Pi] calculated using free ADP, correlates well with adenosine release (Headrick *et al.* 1989).

While there is certainly an increase in free AMP, this may not be the sole avenue for increased adenosine production. As previously indicated, in hepatocytes, hypoxic AMP increase is disproportionately large compared to the increased adenosine production (Bontemps *et al.* 1993). Elevation of phosphocreatine should delay the rise in free AMP, but creatine pre-incubation of hippocampal slices did not delay the DNP depression of synaptic transmission.

Mitochondrial ATP synthetase can catalyse a close thermodynamic equilibrium between $\Delta\mu_{H^+}$ and the cytosolic ATP pool. Uncoupling with DNP stimulates ATPase activity (Lardy and Wellman, 1953; Lehninger, 1964; Heytler, 1979; Slater, 1967; Hemker, 1962) as ATP is hydrolysed in a futile pumping of protons. Measurement of mitochondrial membrane potential in synaptosomes indicate that inhibition of respiration with cyanide or rotenone also stimulates ATPase activity (Kauppinen and Nicholls, 1986; Scott and Nicholls, 1980). This ATPase activity may promote free ADP and free AMP accumulation and contribute to adenosine release. Inhibition of the ATP synthetase will prevent this ATPase activity. Oligomycin 5μg/ml perfused into *in situ* rat liver for 15 minutes prevents the FCCP drop in ATP content, measured 1 minute after FCCP infusion (Kraus-Friedmann *et al.* 1990). Furthermore, in neurons, maintenance of ionic gradients by membrane Na^+/K^+-ATPase and Ca^{2+}-ATPase accounts for 50-60% of cytosolic ATP utilisation (Ereciñska and Silver, 1989; Scott and Nicholls, 1980) and oligomycin also inhibits Na^+/K^+-ATPase (Jöbsis and Vreman, 1963; Linnett and Beechey, 1979). However oligomycin failed to prevent DNP inhibition of synaptic transmission, suggesting that stimulation of ATPase activity does not promote adenosine release.

5.4.5.3. Enzyme activation

Increase of both adenosine and inosine production in the hippocampus during ischaemia in the presence of EHNA suggests an activation of 5'-nucleotidase acting on both AMP and IMP (Lloyd *et al.* 1993); however, there may also be an increase in IMP production during ischaemia due to deamination of elevated AMP, indeed, AMP deaminase activity is enhanced by a decrease in energy charge (Coffee, 1978). The synthetic nucleoside tricyclic nucleotide (TCN) is taken up by cells and is phosphorylated by adenosine kinase to TCN-P and can be hydrolysed to TCN by 5'-nucleotidase. In hippocampal slices pre-incubated with TCN, ischaemic conditions promote an efflux of TCN as well as adenosine (Lloyd and Schrader, 1987). This suggests that during ischaemia either 5'-nucleotidase is activated or adenosine kinase

is inhibited, as an increase in endogenous mono-nucleotides would not effect TCN-P dephosphorylation.

5.4.5.4. pH

pH changes have been proposed as a mechanism for increasing 5'-nucleotidase activity (Martin *et al.* 1994). Intracellular pH in normoxic cortical slices is 7.2, about 0.29 pH units more acidic than extracellular pH, but anoxia causes an acidification of 0.5 pH units within two minutes (Ekholm *et al.* 1992; Whittingham *et al.* 1984; Kauppinen and Williams, 1990). Intracellular acidification may be a possible hypoxic signal, increasing the activity of 5'-nucleotidase (Newby, 1988) and decreasing the activity of adenosine kinase (Yamada *et al.* 1980). Such hypoxic intracellular acidification is a result of lactic acidosis. Whereas most pyruvate produced by aerobic glycolysis is destined for oxidative metabolism, some pyruvate is reduced to lactate. Lactate accumulates during respiratory inhibition as a result of the block of oxidation of pyruvate, as well as by a stimulation of lactate production concomitant with an increase in glycolysis (McIlwain and Bachelard, 1971). Even though respiration is stimulated during uncoupling, and pyruvate is rapidly utilised, lactate may accumulate due to an increased glycolytic rate; indeed, an increase in aerobic gylcolytic rate produces an accumulation of lactate similar to that produced by hypoxia (McIlwain and Bachelard, 1971). Whereas inhibitors of oxidative phosphorylation can inhibit tightly coupled respiration (Lehninger, 1964), and might be expected to cause lactate accumulation, oligomycin only slightly reduces state 4 respiration in synaptosomes (Scott and Nicholls, 1980), and thus may not produce lactic acidosis. With uncoupling and respiratory inhibition, there could be an initial, lactate independent, drop in pH due to a mismatch between ATP hydrolysis and phosphocreatine hydrolysis (Wallimann *et al.* 1992) when phosphocreatine stores are depleted; however, the failure of creatine pre-incubation to alter the DNP depression of synaptic transmission argues against this being important in adenosine release. Thus, lactic acidosis could be a common mechanism by which respiratory inhibition and uncoupling produce adenosine accumulation.

5.4.5.5. Calcium

5.4.5.5.1. Mitochondrial calcium homeostasis

Although total intra-neuronal calcium is in the range 1.5mM, cytosolic free calcium is approximately 0.1μM (Carafoli, 1987). Calcium is an important second messenger controlling numerable cellular processes, and thus cytosolic calcium levels fluctuate both temporally and spatially. The mitochondria are intimately associated with neuronal calcium homeostasis. Mitochondrial oxidative phosphorylation and calcium uptake are alternative, energy requiring processes, that use the energy of the electrochemical gradient across the inner mitochondrial membrane; as a pair of

electrons traverses the respiratory chain either, six calcium ions can be transported or three molecules of ADP phosphorylated (Lehninger *et al.* 1967; Lehninger, 1970; Carafoli and Sottocasa, 1984; Miller, 1991). Calcium uptake in the mitochondria occurs via a passive, reversible, electrophoretic carrier, and uptake is driven by the electrical potential across the inner mitochondrial membrane (Lehninger, 1970; Carafoli and Sottocasa, 1984). This transport can be specifically blocked by ruthenium red (Moore, 1971). Calcium is normally exported from the mitochondria by an electrically silent Na^+/Ca^{2+} exchanger, Na^+ in turn is exported by the Na^+/H^+ antiporter (Carafoli and Sottocasa, 1984). In the face of high cytosolic calcium levels, the Na^+/H^+ antiporter becomes saturated and net calcium accumulation occurs, to the exclusion of oxidative phosphorylation (Lehninger *et al.* 1967; Lehninger, 1970; Carafoli and Sottocasa, 1984; Miller, 1991). The mitochondrial set point for calcium accumulation is often presumed to be 0.5-1μM, well above the level of cytosolic free calcium (Miller, 1991; Carafoli, 1987). Whether neuronal mitochondria accumulate calcium under physiological conditions is controversial but evidence supports some role in cytosolic calcium buffering in the range of 0.1-1μM (Miller, 1991; Thayer and Miller, 1990). Nerve terminals are rich in mitochondria (Kauppinen and Nicholls, 1986; Scott and Nicholls, 1980) and these terminals are subject to considerable influx of calcium during stimulus/coupling. Indeed, calcium accumulation into mitochondria has been shown to account for most of synaptosomal calcium accumulation (Lust and Robinson, 1969; Åkerman and Nicholls, 1981).

Non-mitochondrial calcium-storage organelles are found within various parts of the neuron including the presynaptic terminals, and are often associated with mitochondria, suggesting the two may cooperate in calcium buffering (Miller, 1991). Calcium-storage organelles are associated with the endoplamic reticulum, and are characterised by Ca^{2+}-ATPase pumps, calcium binding proteins and calcium release channels (Krause, 1991). Some calcium storage organelles can be stimulated to release calcium by calcium itself, or by methylxanthines such as caffeine, and may contain the ryanodine receptor typical of muscle cells, while a second type of calcium-storage organelle has calcium channels opened by the second messenger inositol 1,4,5-trisphosphate (Henzi and MacDermott, 1992; Krause, 1991). Inositol 1,4,5-trisphosphate receptors and ryanodine receptors are found in high levels in the hippocampus but are physically segregated (Henzi and MacDermott, 1992). These intracellular calcium stores have a lower capacity than the mitochondria but a higher affinity for calcium, and can buffer cytosolic calcium in the physiological range (Miller, 1991).

Mitochondrial calcium uptake is dependent on the energisation state, and the ruthenium red sensitive carrier will reverse if the mitochondrial electrochemical gradient is sufficiently low (Carafoli, 1987). Uncoupling agents such as DNP,

dicoumarol or gramicidin prevent calcium uptake and cause calcium efflux from mitochondria; however, atractyloside and oligomycin do not inhibit calcium uptake (Lehninger *et al.* 1967; Lehninger, 1970; Carafoli and Sottocasa, 1984). To what extent respiratory inhibition might affect mitochondrial calcium homeostasis is less clear. While block of electron flow should prevent calcium uptake, in rotenone treated synaptosomes, the maintenance of the inner mitochondrial membrane in an energised state by glycolytic ATP production (Scott and Nicholls, 1980), suggests that hypoxic mitochondria are sufficiently energised to still retain calcium; however, the poisoned mitochondria may be unable to accumulate further calcium if cytosolic calcium rises. In the hippocampal slice in the presence of DNP or cyanide, presynaptic calcium might rise more quickly than under control conditions, since the prevolley is preserved, it is assumed that action potentials are invading the synaptic terminals and causing calcium influx. Such a rise in cytosolic calcium may trigger adenosine accumulation.

Energy deprivation can lead to an increase in cytosolic calcium both from release of calcium and from inhibition of energy dependent uptake of calcium into mitochondrial and non-mitochondrial stores. In isolated fura-2 loaded *Heliosoma trivolis* neurons and in isolated rat hepatocytes, the uncoupler FCCP causes a biphasic release of calcium from mitochondrial stores (Jensen and Rehder, 1991; Fulceri *et al.* 1991). In perfused rat liver and isolated *Heliosoma trivolis* neurons, FCCP causes an increase in intracellular calcium released from non-mitochondrial stores associated with decreased ATP levels (Kraus-Friedmann *et al.* 1990; Jensen and Rehder, 1991). In neonatal rat hippocampal cultures sodium cyanide or azide increases intracellular calcium over five minutes, while oligomycin and DNP cause a slower elevation of cytosolic calcium that is blocked by dantrolene (Dubinsky and Rothman, 1991). In ischaemic hippocampal slices the early rise in intracellular calcium is partially blocked by calcium free media and further reduced by dantrolene (Mitani *et al.* 1993). Respiratory inhibition with antimycin A, but not inhibition of oxidative phosphorylation with oligomycin, blocks substrate driven calcium accumulation into synaptosomes and mitochondria (Lust and Robinson, 1969).

5.4.5.5.2. Calcium evoked hypoxic current

Apparently the hypoxic potassium current in neurons is activated by an increase in cytosolic calcium (Leblond and Krnjevic, 1989; Krnjevic and Xu, 1989) and DNP similarly activates a potassium conductance due to an increase in cytosolic calcium initiated by release of mitochondrial calcium (Godfraind *et al.* 1971). In CA_1 neurons in the hippocampal slice, EGTA-containing electrodes have a variable effect on input resistance (Leblond and Krnjevic, 1989) while the hypoxic hyperpolarisation is unaffected (Leblond and Krnjevic, 1989; Fujiwara *et al.* 1987; Zhang and Krnjevic,

1993). The best evidence that hypoxic potassium current is calcium-dependent is that it is blocked by dantrolene (Krnjevic and Xu, 1989), which prevents calcium release from calcium-sensitive intracellular calcium stores (Miller, 1991; Thayer *et al.* 1988). Whether dantrolene sensitive calcium release activates the hypoxic calcium current, or is an additional requirement for its activity, is unknown. However, in the current work, dantrolene failed to block the DNP depression of synaptic transmission. This is not altogether surprising, as evidence suggests that hypoxic hyperpolarisation and the depression of synaptic transmission are different processes, see chapter 4, section 4.4.5. However, it seems possible that an increase in cytosolic calcium, from mitochondrial stores, during hypoxia, respiratory inhibition or mitochondrial uncoupling may alter the activity of adenosine metabolising enzymes and promote adenosine accumulation.

5.5. SUMMARY AND CONCLUSIONS

The respiratory inhibitor cyanide and the mitochondrial uncoupler DNP produced an adenosine A_1 receptor-mediated depression of synaptic transmission, identical to that produced by hypoxia. However, inhibitors of mitochondrial ATP production and export failed to mimic hypoxia. Buffering of cytosolic ATP with phosphocreatine also failed to alter synaptic depression due to inhibition of mitochondrial energy metabolism. Apparently, neuronal ATP depletion is not responsible for the initial rapid accumulation of adenosine. The best evidence available suggests that adenosine accumulation results from stimulation of 5'-nucleotidase, combined with inhibition of phosphorylation of adenosine by adenosine kinase. Intracellular and extracellular adenosine probably equilibrate by facilitated diffusion via the nucleoside transporter. Both of these effects may be localised in the synaptic terminals which are rich in mitochondria. Possible regulatory mechanisms for 5'-nucleotidase and adenylate kinase that are common to respiratory inhibition and uncoupling include increase in cytosolic calcium secondary to alteration of mitochondrial calcium homeostasis, acidification of the cytosol by lactic acid accumulation, or localised increases in free AMP.

6. PERSISTENT ACTIONS OF HYPOXIA AND ADENOSINE: POST-INHIBITORY AND POST-HYPOXIC HYPEREXCITABILITY

6.1. INTRODUCTION

Lack of oxygen supply to the brain results in a rapid loss of higher brain functions, such as consciousness and purposive behaviour, but these will return with timely reoxygenation (Rossen *et al.* 1943; Sugar and Gerard, 1938). Despite this apparent recovery, the brain may be rendered hyperexcitable, and delayed neuropsychological deterioration with associated neuronal damage can develop (Bergamasco *et al.* 1984; Meyer, 1936). Such post-hypoxic brain damage is found particularly in the pyramidal neurons of the hippocampal CA_1 subfield (Sommer's field) (Sommer, 1880), and is characterised by neuronal degeneration several days after apparent recovery from the hypoxic episode (Speilmeyer, 1925). To date, such delayed neuronal death has attracted most attention, whereas the often persistent hyperexcitability that appears immediately following reoxygenation has been essentially neglected, even though it may foreshadow further brain deterioration.

It is now well established that the loss of neuronal synaptic activity in the CA_1 area of the hippocampus during hypoxia is due to adenosine (Gribkoff *et al.* 1990; Gribkoff and Bauman, 1992; Fowler, 1989), which accumulates extracellularly during hypoxia (Fredholm *et al.* 1984), see chapter 4. This adenosine is a potent neuromodulator that, acting at adenosine A_1 receptors, blocks excitatory synaptic transmission (Siggins and Schubert, 1981), presumably through inhibiting glutamate release (Lupica *et al.* 1992; Nadler *et al.* 1990). These depressive effects of adenosine on glutamate release can protect against hypoxic excitotoxicity. Indeed increasing endogenous adenosine (Dux *et al.* 1990; Phillis and O'Regan, 1989) or administration of exogenous adenosine receptor agonists (Evans *et al.* 1987; Januszewicz von Lubitz *et al.* 1989; von Lubitz *et al.* 1988; Donaghy and Schofield, 1991) promotes the survival of neurons following hypoxia or ischaemia. Given these protective effects of adenosine and the release of adenosine during hypoxia, it has become firmly established in the literature that adenosine release is the brain's own protective mechanism against hypoxia-induced damage (Newby *et al.* 1990).

However, subsequent to an hypoxic or ischaemic episode, synaptic activity may recover, and neuronal hyperexcitability appears. For instance, following brief cerebral ischaemia, spontaneous action potential frequency increases in CA_1 neurons of rats *in vivo* (Chang *et al.* 1989), whilst electrical stimulation of CA_1 afferents evokes epileptiform discharge of pyramidal neurons following *in vitro* hypoxia in hippocampal slices (Schiff and Somjen, 1985). Such hyperexcitability is poorly characterised; for instance, there is evidence both for increased excitatory synaptic

transmission (Miyazaki *et al.* 1993) as well as for an increase in pyramidal neuron excitability (Andersen, 1960; Chang *et al.* 1989), but whether this latter is due to disinhibition or increased intrinsic excitability is unclear.

While it has become lore that the depressive actions of adenosine protect against hypoxic neuronal death, less attention has been focussed on any potential pro-convulsive action of the prolonged, elevated adenosine levels that occur during hypoxia. This aspect of hypoxic pathophysiology is important, since synaptic excitation of CA_1 pyramidal neurons seems a prerequisite of post-hypoxic excitotoxicity (Onodera *et al.* 1986). This chapter presents evidence in hippocampal slices that hypoxia-induced adenosine release itself may contribute to a form of post-hypoxic hyperexcitability that is a result of an increase in excitability of the CA_1 pyramidal neurons. It is hypothesised that examination of input/output relationships for CA_1 synaptic transmission will indicate that post-hypoxic hyperexcitability results from an increase excitability of pyramidal neurons. It is proposed that antagonism of adenosine A_1 receptor activation during hypoxia does not prevent the production hyperexcitability upon reoxygenation. However, application adenosine to slices during normoxia will produce similar hyperexcitability, through an action at another receptor subtype.

6.2. METHODS

In the following investigations, hippocampal slices were exposed to only a single drug and/or hypoxic treatment, thus all comparisons were between different slices, prepared from different animals. In the figures illustrating time course of drug or hypoxia actions, the first two time points are always recorded in drug free, normoxic ACSF, and the first time point is considered baseline. All data is presented as a percentage of the baseline recording. When used, antagonists were superfused onto hippocampal slices for five minutes prior to superfusion with hypoxic ACSF. Input/output curves were typically recorded over 12 minutes. Input/output recordings were made immediately prior to treatment of slices with drugs or hypoxia and then again after one hour of reoxygenation and washout with normoxic, drug-free ACSF. 8-cyclopentyl-1,3-dimethylxanthine (8-cyclopentyltheophylline, 8-CPT), 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX), cyclopentyladenosine (CPA), N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl]adenosine (DMPA), 2-p-(2-carboxyethyl)phenethylamino-5'-ethylcarboxamidoadenosine (CGS 21680), 3,7-dimethyl-1-propylargylxanthine (DMPX), DL-2-amino-phosphono-5-pentanoic acid (AP5), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine hydrochloride (H-7), N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide hydrochloride (H-8) were purchased from Research Biochemical Incorporated; 2,4 dinitrophenol (DNP), staurosporine and 8-bromo-adenosine-3',5'-cyclic-monophosphate (8-Br-cAMP) were purchased from

Sigma; and adenosine was obtained from Calbiochem. 8-CPT, DMPA and DNP stock solutions were made in 0.05N NaOH, CPA stock solution was made in ethanol, and DPSPX, DMPX, AP5, H-7, H-8, 8-Br-cAMP and adenosine stock solutions were made in distilled water.

The data concerning superfusion of hippocampal slices with hypoxic ACSF or with DNP was measured in the same slices from which the data presented in chapter 4 was obtained. Several of the following figures illustrating post-hypoxic hyperexcitability also include, for comparison, data previously presented in chapter 4.

6.3. RESULTS

6.3.1. POST-HYPOXIC HYPEREXCITABILITY

6.3.1.1. Prolonged hypoxia resulted in hyperexcitability upon reoxygenation

As was shown in chapter 4, hypoxia caused a rapid, reversible, adenosine A_1 receptor-mediated depression of synaptic field potentials in the CA_1 area of the hippocampal slice. Coincident with the recovery of field potentials, with the reintroduction of oxygenated ACSF following 30 minutes of hypoxia, was the appearance of a second orthodromic population spike, see figures 6.1 and 6.2. The averaged field potentials in figure 6.1 show that this second population spike of 3.5 ± 0.8mV S.D. maximum amplitude was smaller in than the primary population spike of 7.1 ± 0.6mV S.D. maximum amplitude (n = 5). The second spike was also wider, indicating less synchronous firing of the underlying action potentials in the neuronal population. These second population spikes were never seen to decline over 90-180 minutes of post-hypoxic recordings. Whilst these slices were continually stimulated at 0.05Hz during hypoxia, preliminary studies showed that such stimulation was not necessary for development of multiple population spikes (n = 2), data not shown. Occasionally, slices displayed multiple population spikes from the outset of recording, prior to experimental hypoxia, and it was presumed that this reflected hypoxia during brain slice preparation. Surgical isolation of the CA_1 area with a vertical cut across all layers at the level of the CA_2 did not prevent this hyperexcitability, indicating that, whatever the origin, it is intrinsic to the CA_1 area.

The second population spike arose from the same fEPSP as the first, with an onset delay of a further 4-5ms. Although the post-hypoxic field potentials showed multiple population spikes, the underlying fEPSPs before and after hypoxia were similar. This is evident in figure 6.3A. which shows superimposed stratum radiatum field potentials, arising from the same stimulus strength, before and after hypoxia. Analysis of sub-maximal fEPSPs at a similar prevolley amplitude before and after

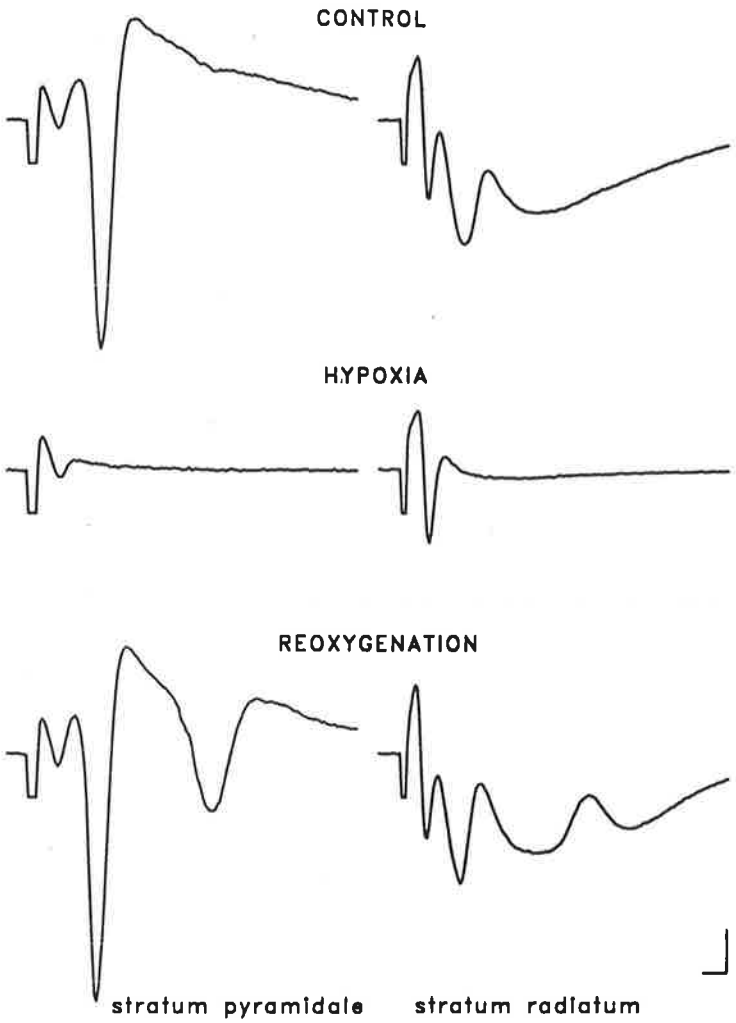

Figure 6.1 Effects of superfusion of hypoxic ACSF on synaptic field potentials in the hippocampal slice
Synaptic field potentials in the CA_1 area evoked by Schaffer collateral/commissural stimulation. The left column shows recordings from the stratum pyramidale (cell body layer) and the right column shows recordings from stratum radiatum (apical dendrite/synaptic layer). From top to bottom are shown respectively field potentials before, during and 1 hour following 30 minutes of hypoxia. Calibration bars are 1mV and 1ms, negative down. Stimulus artefacts are truncated for clarity. Note that this is identical with figure 4.1.

hypoxia showed that fEPSP amplitude, rate of rise, area and duration at 20% of amplitude were not different ($P \leq 0.05$, $n = 5$), figure 6.3B.

6.3.1.2. 2,4-Dinitrophenol induced hyperexcitability

As was illustrated in chapters 4 and 5, the mitochondrial uncoupler DNP and hypoxia have actions similar actions, reversibly depressing synaptic field potentials. In these experiments, washout following 30 minutes superfusion of 50 μM DNP was also accompanied by the appearance of a second orthodromic population spike, figure 6.4. It is only prolonged synaptic depression that produces such hyperexcitability, since 3.5 minutes DNP never caused hyperexcitability ($n = 10$), despite depression of synaptic transmission, see chapter 5. Figure 5.3, chapter 5,

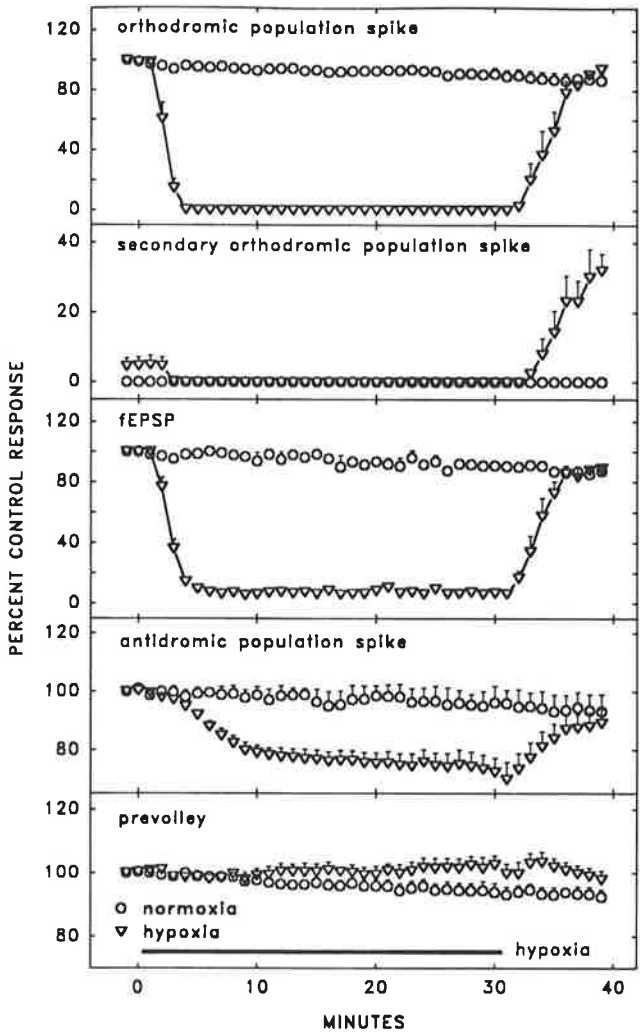

Figure 6.2 Time course of alterations in field potentials in the CA_1 area during hypoxia and reoxygenation

Field potentials in hippocampal slices before, during and after 30 minutes of hypoxia alone (n = 5) and during a similar period of normoxic incubation in slices never exposed to hypoxia (n = 5). Hypoxia is produced by superfusion of slices with hypoxic ACSF for 30 minutes as indicated by the bar at bottom. Schaffer collateral/commissural fibres were stimulated at 0.05Hz and each three consecutive field potentials averaged to give minute averages. Values are expressed as a percentage of the baseline (first minute average) response. Points represent the mean ± S.E.M. (n = 5) for each condition, where no error bars are evident, they are smaller than the symbol. fEPSP values represent the percentage of the baseline rate of rise of the initial segment and prevolley, antidromic spike and first orthodromic population spike values are percentage of baseline amplitude of the respective field potentials. Second orthodromic population spike values are expressed as a percentage of the baseline amplitude of first population spike, to avoid division by zero where the second population spike is absent during baseline recordings. Hypoxia caused significant depression of fEPSP, orthodromic population spike and antidromic population spike compared to normoxia. Recovery of field potentials was accompanied by the appearance of a second orthodromic population spike. In slices not exposed to hypoxia, field potential values remained close to 100% of baseline and a second orthodromic population spike did not develop.

shows typical orthodromically evoked field potentials before, during and after DNP 50μM superfusion for 3.5 minutes, with no second population spike evident upon recovery of synaptic transmission.

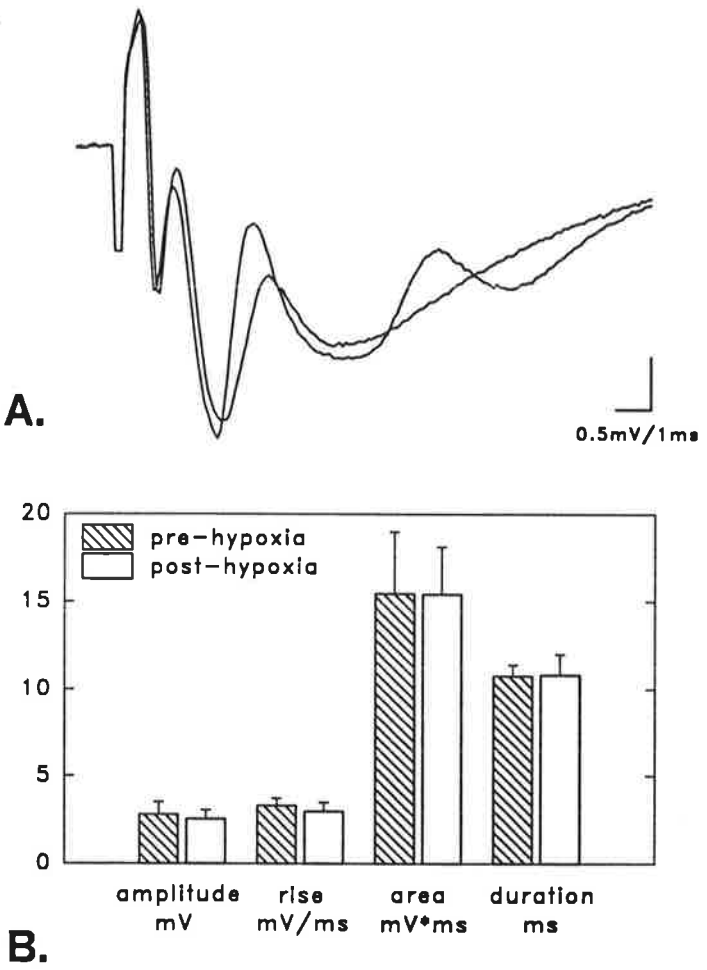

Figure 6.3 Secondary population spikes following hypoxia occurred despite no differences in fEPSP before and after hypoxia
A. Superimposed pre-hypoxic fEPSP and post-hypoxic fEPSP evoked by same stimulus current, note the two population spikes in the post-hypoxic field potential. B. Measurements of fEPSP evoked by the same submaximal stimulus current immediately before and one hour following 30 minutes of hypoxia. Pre-hypoxic values of fEPSP amplitude, rate of rise (slope) of initial segment, area, and duration at 20% of amplitude were 2.8 ± 0.71mV, 3.3 ± 0.42mV/ms, 15.46 ± 3.52mV*ms, and 10.76 ± 0.62ms respectively and post-hypoxic fEPSP values were 2.55 ± 0.52mV, 2.98 ± 0.50mV/ms, 15.4 ± 2.73mV*ms, and 10.82 ± 1.16ms (mean ± S.D., n = 5), pre- and post-hypoxic values were not significantly different ($P \leq 0.05$).

6.3.1.3. Adenosine A_1 receptor antagonism

Adenosine-mediated depression of synaptic transmission is a striking feature of hypoxia in the CA_1 area, and the possible involvement of this adenosine A_1 receptor activation in the production of post-hypoxic hyperexcitability was investigated. Pre-treatment of hippocampal slices with the adenosine A_1 receptor antagonist 8-CPT resulted in the appearance or enhancement of a second population spike in all slices, figure 6.5 and figure 6.6. This disinhibition in the presence of 1μM 8-CPT slowly declined after 8-CPT exposure if the slices were not exposed to hypoxia. However, adenosine A_1 receptor antagonism during hypoxia failed to prevent the appearance of persistent multiple population spikes upon reoxygenation.

Multiple spiking which occurred in the presence of 8-CPT was similar in appearance to that seen following hypoxia but, unlike with hypoxia, was accompanied by small changes in the underlying fEPSP. As previously mentioned, chapter 4, section 4.3.4.1, and can be seen in figure 6.6, the rate of rise of the fEPSP was significantly increased to 105.2 ± 3.2% S.E.M. of baseline ($n = 5$, $P \leq 0.01$). The area of the fEPSP at a similar prevolley amplitude is slightly, but significantly increased from 16.5 ± 2.9mV*ms to 17.5 ± 2.8mV*ms (mean ± S.D., $n = 10$, $P \leq 0.05$) during 8-CPT exposure. However, neither the amplitude 2.9 ± 0.3mV and 2.9 ± 0.3mV (mean ± S.D., $n = 10$), nor the duration at 20% of amplitude 10.3 ± 1.6ms and 10.5 ± 1.4ms (mean ± S.D., $n = 6$), were significantly changed ($P \leq 0.05$) by adenosine A_1 receptor antagonism, data not shown. This slight increase in fEPSP area, without increase in duration, may be due in part to the increase in rate of rise, and in part to an increase in late area of the fEPSP, this latter may be due to alteration of spike after-potentials (Nathan *et al.* 1990), such as the AHP.

Perfusion of slices with 10μM 8-CPT produced a second population spike or increased the amplitude of an existing second population spike, figure 6.7. This effect was only partially reversed by 90 minutes washout of 8-CPT, see figure 6.7 **A.II.** compared to **B.I.** Reoxygenation following subsequent exposed to hypoxia in the presence of 10μM 8-CPT resulted in a further increase in multiple spiking, this is apparent as an increase in amplitude of the second population spike and appearance of a third population spike in figure 6.8 **B.III.**

Disinhibition, manifest as a second population spike also occurred with 75μM DPSPX, and increased during the onset of hypoxia ($n = 1$), figure 6.8. This concentration of DPSPX would be active at both A_1 (K_i 210nM) and A_2 (K_i 710nM) receptors. It seems, therefore that the second orthodromic population spike does not occur as a result of adenosine A_2 receptor activation. Furthermore, the moderately potent adenosine A_2 receptor antagonist DMPX (10μM) promoted the appearance of multiple population spikes, and also failed to depress the second population spike when applied in the presence of 1μM 8-CPT ($n = 1$), data not shown.

During the onset of hypoxia in the presence of 8-CPT, the second orthodromic population spike increased in amplitude and further multiple spiking occurred, prior to depression of these spikes, figure 6.5 and figure 6.6. A second population spike usually occurred transiently during the onset of hypoxia without 8-CPT, but being evident in only one or two responses at 0.05Hz, it was lost in the averaging. This disinhibition seen during the onset of hypoxia may be due to the greater sensitivity of GABAergic inhibitory synaptic transmission than excitatory transmission to hypoxic depression (Hershkowitz *et al.* 1993).

6.3.1.4. Input/output curve changes after hypoxia

Further analysis of field potentials was achieved by producing input/output curves of field potentials immediately before, and one hour following 30 minutes of hypoxia and 1μM 8-CPT, allowing identification of persistent changes in synaptic transmission and excitability. Figure 6.9 and 6.10 show sample input/output curves produced before and after 30 minutes hypoxia and normoxia time control respectively. These figures shows that similar fEPSPs and first population spikes were evoked before and after hypoxia. A second population spike occurs only in response to strong input before hypoxia but this relationship is strongly left-shifted and enhanced following hypoxia. As was shown in chapter 4, figure 4.10 no post-treatment values of input/output curves of early field potential components, the prevolley, fEPSP and first population spike, showed any differences ($P \leq 0.01$), demonstrating that fast components of synaptic transmission and neuronal excitability were not altered by hypoxia or antagonist treatment.

Following recovery from hypoxia, the most striking alteration in neuronal activity was a prolongation of excitation in response to a single orthodromic stimulus, evident in the enhancement of the second population spike, as seen in the representative slices of figures 6.1 and 6.9 and in the mean areas of the input/output curves of the second population spike shown in figure 6.11. Hypoxia produced significant increases in input/output curves describing the second population spike, compared to normoxic controls, normoxia with 8-CPT (1μM) or the pooled pre-treatment values, ($P \leq 0.01$). Hypoxia alone or hypoxia in the presence of 8-CPT (1μM) produced a similar increase in these input/output curves. Normoxia alone or normoxic 8-CPT (1μM) superfusion did not result in persistent alterations in the input/output curves second population spike in the compared to pre-treatment, ($P \leq 0.01$), except where the 8-CPT (1μM) normoxic value for population spike 2 versus prevolley was larger than pre-treatment, which was a consequence of considerable hyperexcitability appearing in only one of the 5 slices in this 8-CPT (1μM) group. Clearly, hypoxia produces an increase in excitation that persists for at least one hour after reoxygenation, and this disinhibition is not altered by adenosine A_1 receptor blockade.

Although 1μM 8-CPT produces a disinhibition during superfusion as already noted, this effect declines with washout of the drug, whereas, disinhibition induced by 10μM 8-CPT did not completely washout within 90 minutes. Input/output curves following hypoxia in the presence of 10μM 8-CPT were not derived in all cases; however, 10μM 8-CPT yielded results qualitatively similar to 1μM 8-CPT, see figure 6.7. The areas of the second population spike curves were increased following 10μM 8-CPT alone, and further increased in the same slice by subsequent hypoxia in the presence of 10μM 8-CPT, data not shown. The relative contributions of hypoxia and 10μM 8-CPT to this hyperexcitability could not be determined. Similar results

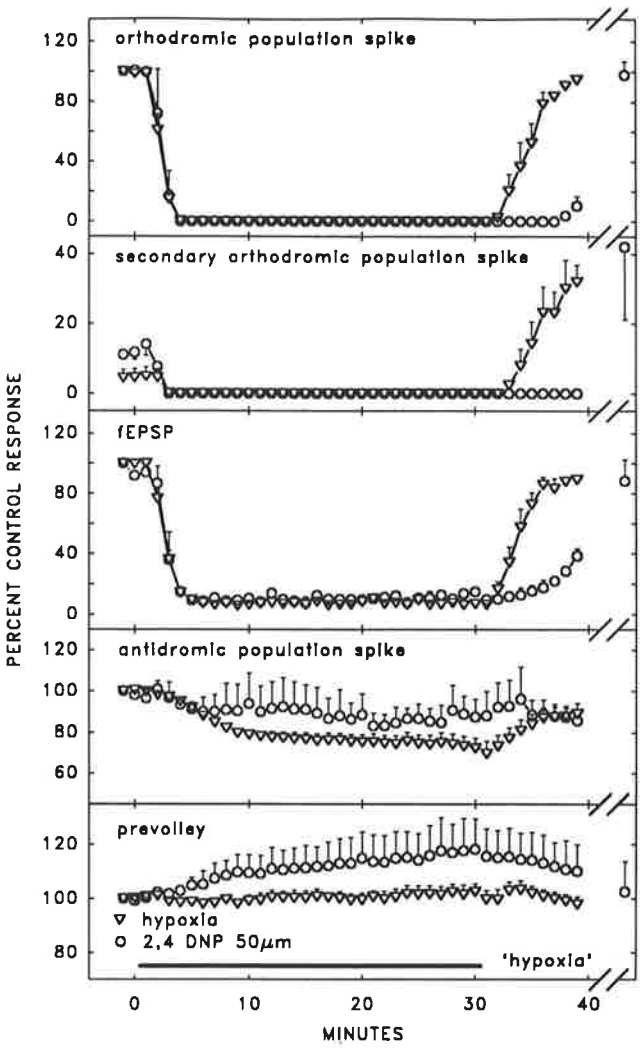

Figure 6.4 Comparison of the time course of alterations in field potentials in the CA_1 area by hypoxia or 2,4 dinitrophenol
Alterations in field potentials in hippocampal slices before, during and after 30 minutes of superfusion with hypoxic ACSF (n = 5) or with normoxic ACSF containing DNP 50μM (n = 5). Hypoxia or DNP superfusion is indicated by the bar at bottom. Data was collected and presented as in figure 6.2. Hypoxia data in this figure is from the same slices as figure 6.2, presented for comparison. DNP, like hypoxia, caused depression of the fEPSP and orthodromic population spike, and recovery synaptic transmission after DNP washout was characterised by the appearance of a second orthodromic population spike.

were obtained in the two slices exposed to hypoxia in the presence of 75μM DPSPX. DPSPX failed to prevent post-hypoxic hyperexcitability (n = 1), but the hyperexcitability induced by normoxic superfusion with DPSPX also failed to washout in 90 minutes (n = 1).

Adenosine A_1 receptor antagonists produce presynaptic disinhibition, characterised by an increase in fEPSP rate of rise and population spike amplitude, which decline immediately with washout of the antagonist, and postsynaptic disinhibition, characterised by multiple population spikes, which persist for some time after drug washout. It has recently been described that postsynaptic disinhibition of CA_1

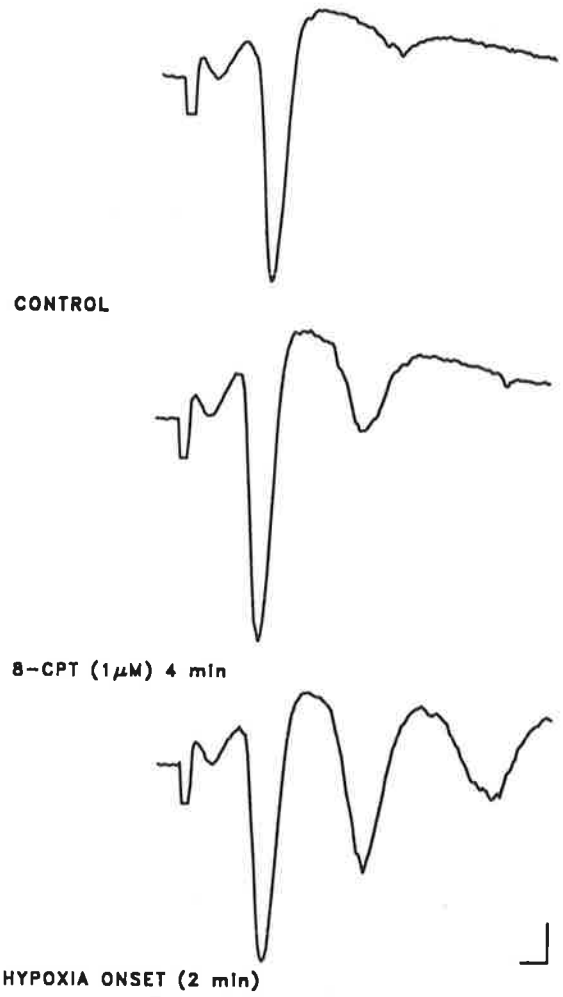

Figure 6.5 8-CPT disinhibition and hypoxic onset disinhibition
Averaged pyramidal layer field potentials taken before, after 4 minutes of 1μM 8-CPT perfusion and during the onset of hypoxia in the presence of 1μM 8-CPT. Note the increase in amplitude of the second population with 8-CPT which further increased in amplitude during the onset of hypoxia. Stimulus artefacts are truncated for clarity. Calibration bars are 1ms and 1mV, negative down.

pyramidal neurons in the hippocampal slice by 8-CPT involves a preferential enhancement of the NMDA component of the EPSC (Klishin *et al.* 1995), and this may be responsible for the multiple population spikes seen in the present study. The persistent postsynaptic disinhibition after normoxic superfusion of adenosine A_1 receptor antagonists represent a form of synaptic plasticity that warrants further investigation, particularly since A_1 receptor-mediated hyperpolarisation is preserved (Alzheimer *et al.* 1989).

Superfusion of 20μM DMPX (n = 1) during hypoxia failed to prevent the development of persistent multiple spiking after hypoxia, data not shown. However, DMPX is a relatively weak and non-selective antagonist at adenosine A_2 receptors, and further trials were not conducted in light of the large extracellular concentration of adenosine obtained during hypoxia, see chapter 4, section 4.3.7.

6.3.2. ADENOSINE A_1 RECEPTOR INHIBITION IS NOT REDUCED FOLLOWING HYPOXIA

It has been proposed that adenosine A_1 receptor desensitisation could account for post-hypoxic hyperexcitability (Lee and Kreutzberg, 1987), and indeed, both post-hypoxic hyperexcitability and adenosine A_1 receptor antagonism are characterised by the appearance of multiple population spikes. This possibility was tested by measuring the depression of the synaptically evoked field potentials using the selective A_1 receptor agonist cyclopentlyadenosine before and after hypoxia. The concentration/response curves for the fEPSP in figure 6.12 show that there was no difference in A_1 receptor-mediated depression of synaptic transmission before or after hypoxia. Similarly, the depression of the population spike by cyclopentlyadenosine was no different before and after hypoxia, data not shown. In addition, antagonism of adenosine A_1 receptors with 8-CPT following a period of hypoxia resulted in an increase in fEPSP rate of rise, and in the amplitude of both population spikes, similar to that found before hypoxia, data not shown. This indicates the continued presence, following hypoxia, of a depressive tone due to basal extracellular levels of endogenous adenosine.

6.3.3. ADENOSINE RECEPTOR AGONISTS DURING NORMOXIA

6.3.3.1. Adenosine caused rebound hyperexcitability

In order to explore whether adenosine action, possibly at some receptor subtype other than A_1 receptors, may be responsible for rebound hyperexcitability, hippocampal slices were exposed to a variety of adenosine receptor agonists for 30 minutes. Hyperexcitability was produced by adenosine, the endogenous ligand, but not by selective agonists for the A_1 and A_2 receptors. Application of exogenous adenosine under normoxic conditions produced effects much like those following an hypoxic episode. Superfusion of hippocampal slices with 50μM adenosine for 30 minutes caused a rapid, reversible depression of synaptic field potentials; the population spike was completely abolished and the fEPSP was reduced to a minimum of 13.5 ± 0.8% of baseline value (n = 4), while the prevolley persisted at 96.3 ± 1.3% (n = 4); the antidromic population spike persisted at 95% in the one slice where it was measured. Moreover, upon washout of the applied adenosine, recovery of synaptic field potentials was accompanied by the appearance of a second population spike, despite the absence of hypoxia, see figure 6.13.

6.3.3.2. Low dose adenosine did not produce multiple population spikes

Superfusion of guinea pig hippocampal slices with low micromolar or nanomolar concentrations of adenosine produces presynaptic enhancement of excitatory synaptic transmission to CA_1, CA_3 and dentate granule cells, by a mechanism that can persist following washout (Okada *et al.* 1990; Nishimura *et al.* 1992; Okada *et al.* 1992). To examine whether the post-inhibitory rebound excitability described in the

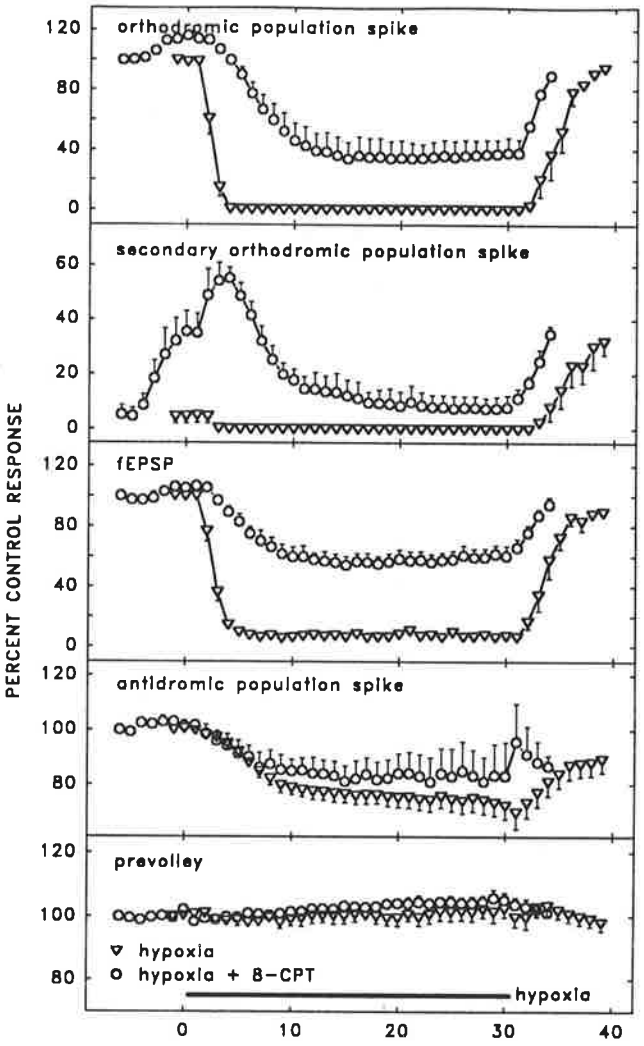

Figure 6.6 Time course of hypoxic changes in field potentials in the CA_1 area in the presence of the adenosine A_1 receptor antagonist 8-CPT
Time course of alterations of the hippocampal slice field potentials before, during and after 30 minutes of hypoxia alone (n = 5) and hypoxia in the presence of 1μM 8-CPT (n = 5). Slices were pre-treated with 8-CPT for 5 minutes before hypoxia. Hypoxia is produced by superfusion of slices with hypoxic ACSF for 30 minutes as indicated by the bar at bottom. Data was collected and presented as in figure 6.2. Hypoxia without antagonist data in this figure is from the same slices as figure 6.2, presented for comparison. Hypoxic depression of fEPSP, orthodromic population spike and antidromic population spike was attenuated by 8-CPT. Prior to hypoxia, 8-CPT caused disinhibition, evident as an increase in fEPSP, first population spike and in particular second population spike. Note the increase in second population spike amplitude during the onset of hypoxia, see figure 6.5.

present study can be similarly produced, hippocampal slices were exposed to low adenosine concentrations. However, 30 minutes superfusion of slices with 100nM adenosine (n = 2), 1μM (n = 1) adenosine or 10μM (n = 1) adenosine did not produce multiple population spikes either during or following drug application.

6.3.3.3. A_1 and A_2 receptor agonists did not produce multiple population spikes

Adenosine, being the endogenous ligand, will activate all adenosine receptor subtypes. Superfusion of hippocampal slices for 30 minutes with selective agonists

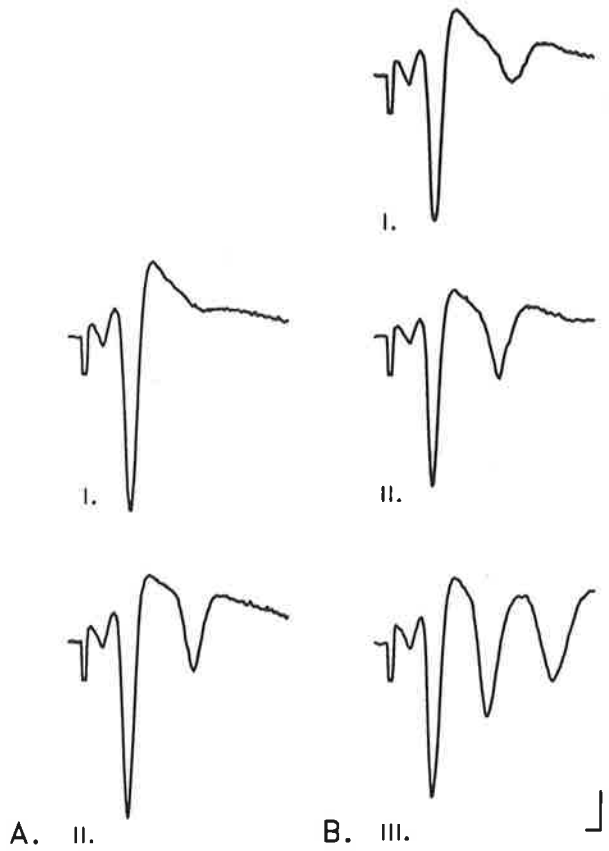

Figure 6.7 Field potentials during 10μM 8-CPT and hypoxia
AI. shows a control field potential, AII. shows field potentials during superfusion with 10μM 8-CPT. BI. shows field potentials following 90 minutes washout of 10μM 8-CPT. BII. shows field potential during a subsequent superfusion with 10μM 8-CPT. BIII. shows field potentials following 30 minutes hypoxia in the presence of 10μM 8-CPT. Note the increase in excitability following hypoxia. Calibration bars are 1mV and 1ms, negative down. Stimulus artefacts are truncated for clarity.

for the A_1 and A_2 adenosine receptor subtypes failed to produce hyperexcitability like that following adenosine. The most obvious action of hypoxia, or exogenous adenosine, is the A_1 receptor-mediated depression of synaptic transmission; however, although 8-CPT (1-10μM) reduced synaptic depression during hypoxia, nevertheless this A_1 receptor antagonism failed to alter the appearance of post-hypoxic hyperexcitability. This suggests that hypoxic activation of A_1 receptors is unlikely to be responsible for the generation of post-hypoxic, rebound, hyperexcitability. Indeed, superfusion of hippocampal slices with the selective adenosine A_1 receptor agonist CPA 50nM for 30 minutes produced a complete depression of the population spike, and depressed the fEPSP to 21.1 ± 1.6% S.E.M (n = 2) of baseline. Nevertheless, recovery of the population spike and fEPSP upon washout of CPA, was not accompanied by appearance of a second population spike (n = 4).

Possible involvement of an adenosine A_2 receptor in rebound hyperexcitability was explored, using two different agonists. The adenosine A_2 receptor agonist DMPA, which does not discriminate between A_{2a} or A_{2b} receptors, at 50nM caused a small,

steady decline in the fEPSP to a minimum of 77.6 ± 6.7% of baseline, and in the population spike to a minimum of 83.37 ± 7.1% (n = 4) of baseline values, over 30 minutes exposure. Again, washout of DMPA was not accompanied by the appearance of a second population spike. On the other hand, CGS 21680 is thought to act at the A_{2a} subclass of adenosine A_2 receptors, which have been proposed to mediate a form of hyperexcitability (Cunha *et al.* 1994). Superfusion of hippocampal slices with CGS 21680 (50nM) for 30 minutes caused a slow, steady decline in the fEPSP to 85.6 ± 1.4% of baseline. The population spike, however was initially increased to 107.8 ± 4.9% S.E.M. (n = 3) of the baseline value at seven minutes superfusion of CGS 21680, whereafter it declined over the remaining period of superfusion to 98.8 ± 3.5% of baseline (n = 3). This initial increase in population spike amplitude was variable, a 10-15% increase occurring in two of the three slices tested and no increase in the remaining slice. CGS 21680 had no effect on the amplitude of a small second population spike which was already present in these slices prior to drug superfusion. In one slice, a combination of 50nM CPA, 50nM CGS 21680 and 50nM DMPA also failed to produce any hyperexcitability. Attempts to replicate adenosine mediated post-inhibitory hyperexcitability by using selective agonists for adenosine A_1 and A_2 receptor subtypes thus failed.

6.3.3.4. Input/output curve changes after adenosine receptor agonists

As with hypoxia, the input/output curves relating to the first population spike, and the maximum population spike amplitude, were not useful indicators of hyperexcitability induced by adenosine, as there were no significant differences between their pre- and post-treatment values, data not shown. Hyperexcitability is evident, however, in figure 6.14 which shows the mean post-treatment values of the area under the input/output curves relating the second population spike to the prevolley and the fEPSP, for slices exposed to the various adenosine receptor agonists and control slices; the figure additionally shows the combined pre-treatment values of all these slices. Adenosine exposure produced significant hyperexcitability compared to the control slices, and to all other agonists treated slices, as well as to the combined pre-treatment values for all slices, ($P \leq 0.01$). No other adenosine receptor agonist produced input/output curve areas for the second population spike greater than control or pre-treatment. This hyperexcitability produced by adenosine, however, was itself significantly less than that produced by hypoxia, ($P \leq 0.05$). However, the production of hyperexcitability by adenosine may be concentration dependent since 50μM, but not lower concentrations, were effective. The concentration of exogenous adenosine tested in the present study was lower than that calculated to be present at the receptors during hypoxia, and future testing of higher adenosine concentrations is warranted.

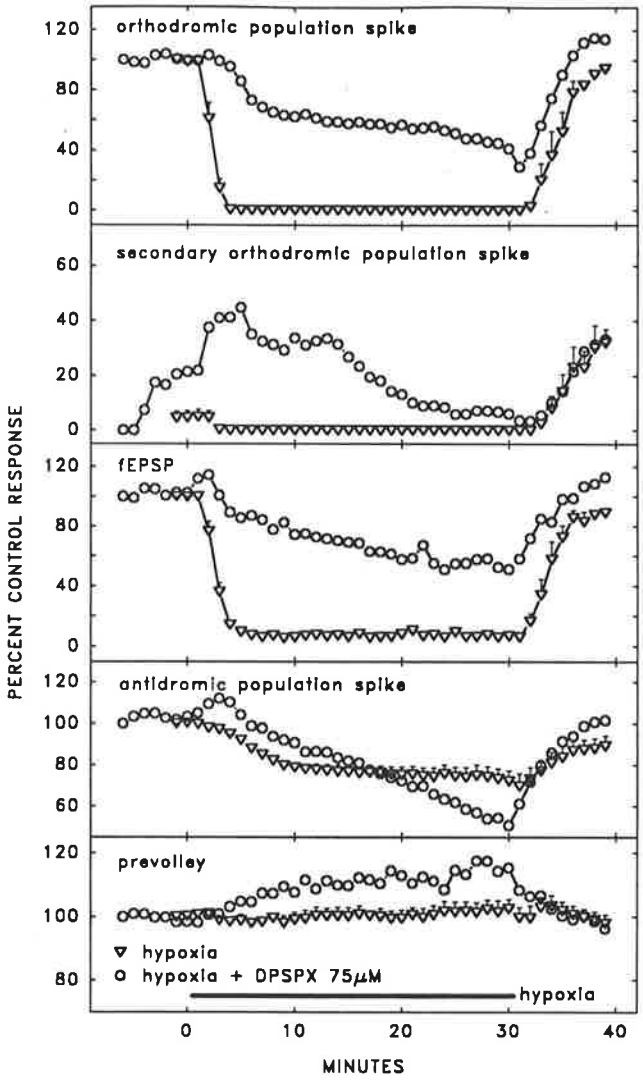

Figure 6.8 Time course of hypoxic changes in field potentials in the CA_1 area in the presence of the adenosine A_1 receptor antagonist DPSPX
Time course of alterations of the hippocampal slice field potentials before, during and after 30 minutes of hypoxia in the presence of 75μM DPSPX (n = 1) and hypoxia alone (n = 5). Slices were pre-treated with DPSPX for 5 minutes before hypoxia. Hypoxia is produced by superfusion of slices with hypoxic ACSF for 30 minutes as indicated by the bar at bottom. Data was collected and presented as in figure 6.2. Hypoxia without antagonist data in this figure is from the same slices as figure 6.2, presented for comparison. Hypoxic depression of fEPSP, orthodromic population spike was attenuated by DPSPX. Note the increase in disinhibition during hypoxia, similar to that with 8-CPT in figure 6.6.

6.3.4. GLUTAMATE RECEPTORS IN POST-HYPOXIC HYPEREXCITABILITY

It is well known that glutamate accumulates extracellularly in the brain during and following ischaemia, and may accumulate with hypoxia if neurons are depolarised (Pellegrini-Giampietro *et al.* 1990; Ogata *et al.* 1992), see chapter 2, section 2.4.3. However, such glutamate release is unlikely in the present model where hypoxic spreading depression does not occur (Martin *et al.* 1994), see chapter 2 section 2.4.7.2 and chapter 4, section 4.4.1. Nevertheless, even in the absence of enhanced

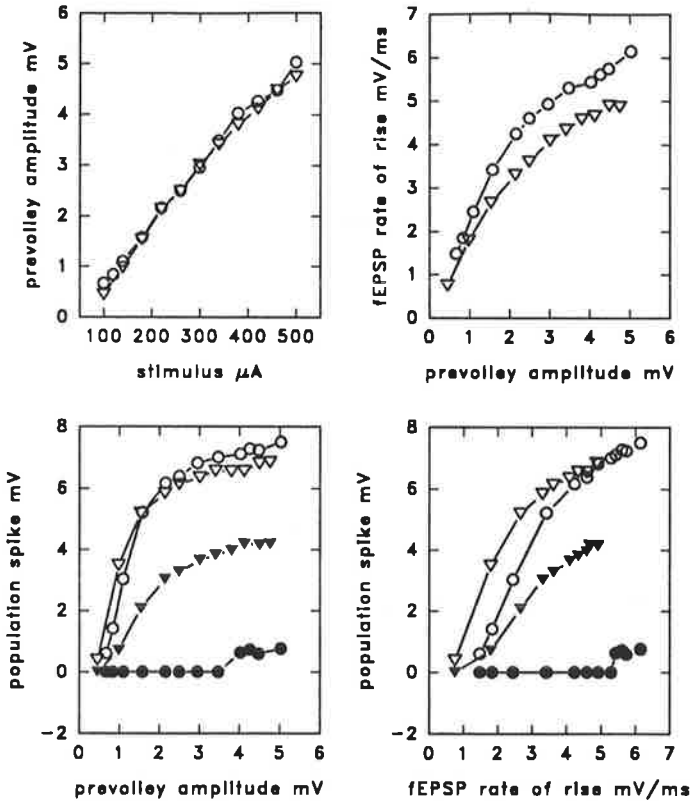

Figure 6.9 Input/output curves of CA_1 field potentials before and after hypoxia
Input/output curves of field potentials from a representative slice constructed before (circles) and one hour following 30 minutes of hypoxia (triangles). Input/output curves were constructed from measurements of prevolley amplitude , fEPSP rate of rise, first orthodromic population spike amplitude (open symbols) and second orthodromic population spike amplitude (filled symbols) recorded over a range of stimulus currents. Each point is measured from an average of three responses evoked using 0.05Hz stimulation. Note the strong left-shift of, and increase of area under, the second population spike curves.

glutamate release, glutamate action at the NMDA or metabotropic receptors may produce multiple population spikes, and this was investigated.

6.3.4.1. NMDA receptor antagonism

Under physiological conditions, the NMDA receptor is blocked in a voltage dependent manner by magnesium (Mayer *et al.* 1984; Nowak *et al.* 1984). If this magnesium block is relieved by depolarisation, blockade of GABAergic inhibition, or by omission of magnesium from an *in vitro* medium, an NMDA component of synaptic transmission is evident as a slow EPSP and multiple late action potentials (Andreasen *et al.* 1988; Davies and Collingridge, 1989). These secondary spikes are blocked in a concentration-dependent fashion by the NMDA receptor antagonist AP5 (Coan and Collingridge, 1987a). In the present investigation, antagonism of NMDA receptors with 50μM racemic AP5 was ineffective in depressing the second population spike following hypoxia in the hippocampal slice (n = 2), see figure 6.15. This indicates that the second population spike is generated from the initial, AMPA receptor-mediated, depolarisation, as seemed apparent from the fEPSP measurements

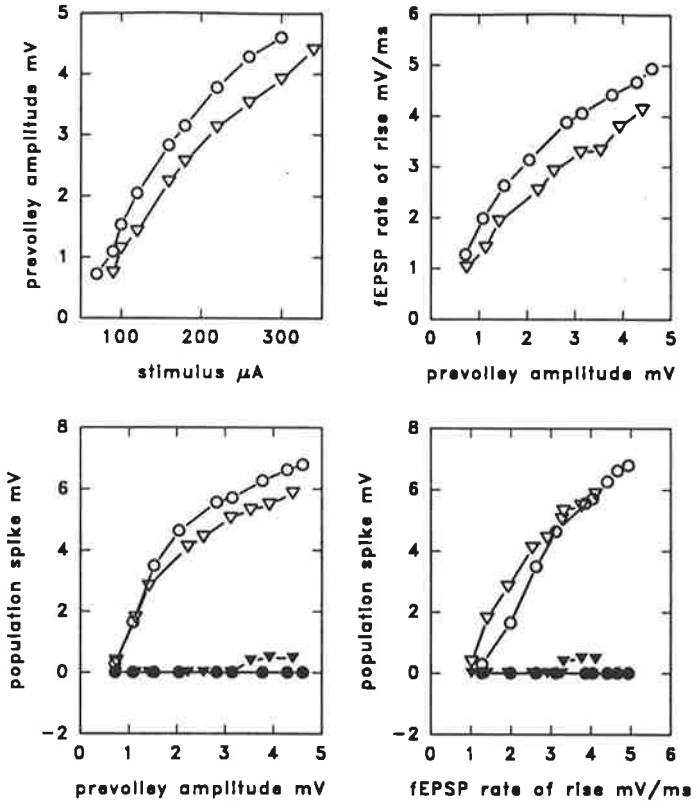

Figure 6.10 Input/output curves of CA_1 field potentials of a slice never exposed to hypoxia

Input/output curves of field potentials from a representative slice constructed before (circles) and one hour following 30 minutes of normoxic control incubation (triangles). Input/output curves were constructed from measurements of prevolley amplitude , fEPSP rate of rise, first orthodromic population spike amplitude (open symbols) and second orthodromic population spike amplitude (filled symbols) recorded over a range of stimulus currents. Each point is measured from an average of three responses evoked using 0.05Hz stimulation.

above. Also, NMDA receptor activation and tetanus of synaptic input can produce long-term potentiation of synaptic transmissions (Bliss and Collingridge, 1993). NMDA receptor activation during hypoxia does not contribute to the development of post-hypoxic hyperexcitability since superfusion of 30μM AP5 throughout hypoxia did not prevent the development of multiple population spikes (n = 1).

6.3.4.2. *trans*-ACDP

Metabotropic glutamate receptor activation combined with tetanus of the Schaffer collaterals can produce long-term potentiation mediated by protein kinase C, characterised by multiple population spikes (Aniksztejn *et al.* 1992). Here, 100μM *trans*-ACDP application caused an initial appearance of a second population spike but eventual depression of synaptic transmission. No post-inhibitory hyperexcitability was evident upon washout, see figure 6.16.

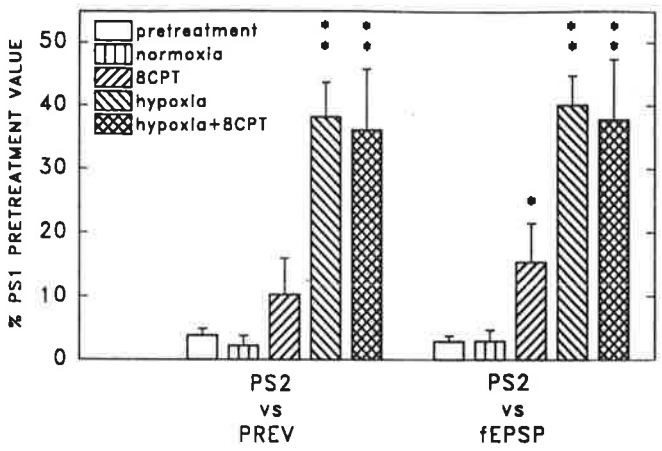

Figure 6.11 Second population spike input/output areas following hypoxia or normoxia
Areas of the input/output curves for hypoxia and 8-CPT relating the second population spike amplitude to prevolley amplitude or fEPSP rate of rise (n = 5) and, for comparison, the combined pre-treatment value for these curves (n = 20). The figure shows the mean ± S.E.M. value for area expressed as a percentage of the corresponding first population spike pre-treatment input/output curve area. Double asterisks denote that the hypoxic curves whether in the presence or not of 8-CPT are significantly larger than either normoxic curve ($P \leq 0.01$), and are also significantly larger than the combined pre-treatment value ($P \leq 0.01$). The hypoxic curves are not different from each other, and the normoxic curves are not different from each other. Single asterisk shows the area of the curve of population spike 2 versus fEPSP for 8-CPT application during normoxia is larger than the pre-treatment control ($P \leq 0.01$), but no other normoxic values are different from pre-treatment.

6.3.5. PROTEIN KINASE

Several lines of evidence suggest a possible protein kinase involvement in the generation of post-hypoxic and adenosine rebound hyperexcitability. Firstly, adenosine A_1 and A_3 receptors activate phospholipase C (White *et al.* 1992; Dickenson and Hill, 1993; Ramkumar *et al.* 1993), and modulation of adenylate cyclase was the first described action of adenosine A_1 and A_2 receptors (Sattin and Rall, 1970; Londos *et al.* 1980). Such modulation may promote excitability, for instance, adenosine A_{2b} receptor-mediated cAMP accumulation and protein kinase A activation in CA_3 neurons enhances P-type calcium currents (Mogul *et al.* 1993). Also, both protein kinase C and protein kinase A have been implicated in the adenosine-mediated increase in excitatory synaptic transmission in the superior colliculus and dentate gyrus (Okada and Hirai, 1993; Hirai *et al.* 1994; Nishimura *et al.* 1992). While these represent presynaptic actions of adenosine, protein kinases may also participate in postsynaptic excitation. For instance, protein kinase C is apparently required for long-term potentiation in the CA_1 area (Malinow and Tsien, 1990). Also, adenylate cyclase activators or cAMP analogues produce a potentiation of sub-maximal population spikes, without any increase in the fEPSP in the CA_1 area, which persists or decays only slowly after washout of the drug (Dunwiddie and Hoffer, 1980; Dunwiddie *et al.* 1992; Pockett *et al.* 1993; Slack and Pockett, 1991).

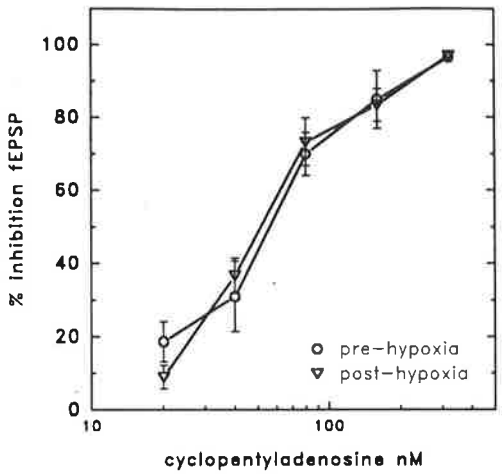

Figure 6.12 CPA concentration/response curves before and after hypoxia
Concentration-response curve for the adenosine A_1-receptor agonist cyclopentyladenosine before (circles) and after (triangles) hypoxia. Points are mean ± S.E.M. percent depression of fEPSP (n = 2 or 3).

H-7 is a relatively non-selective protein kinase inhibitor, active at both protein kinase A and protein kinase C, with micromolar efficacy (Hidaka *et al.* 1984). Superfusion of one hippocampal slice with 100μM H-7 throughout 30 minutes of hypoxia did not prevent the hypoxic depression of synaptic transmission, and also failed to prevent post-hypoxic hyperexcitability. Interpretation of this result is difficult because, as has been previously described (Leahy and Vallano, 1991; Corradetti *et al.* 1989), H-7 alone produced increase in excitability, manifest as multiple population spike and an enormous increase in fEPSP duration, see figure 6.17. However, while the fEPSP returned to baseline duration, multiple population spikes persisted.

H-8 is a moderately selective protein kinase A inhibitor (Hidaka *et al.* 1984). Staurosporine, like H-7, inhibits protein kinase C at the magnesium-ATP site, but is more potent and selective with an IC_{50} in the range 2.7-10nM (Tamaoki *et al.* 1986; Robinson, 1992). Neither 10μM H-8 (n = 1), nor 100nM staurosporine (n = 2) superfused during hypoxia prevented the hypoxic depression of synaptic transmission or the development of multiple population spikes upon reoxygenation, data not shown. Neither of these compounds alone had any effect on CA_1 field potentials, indicating that the H-7-induced excitability is probably unrelated to protein kinase inhibition.

Superfusion of a slice with 500μM (n = 1,) but not 250μM (n = 1), of the cell permeant cAMP analogue 8-Br-cAMP depressed synaptic field potentials in an 8-CPT sensitive manner, see figure 6.18. This indicates that 8-Br-cAMP is an agonist at the A_1 receptor, as are many adenine nucleotides (Phillis *et al.* 1974), see also chapter 5, section 5.3.5. Figure 6.18 shows that In the presence of 1μM 8-CPT, 500μM 8-Br-cAMP, rather than depressing synaptic transmission, instead increased the amplitude

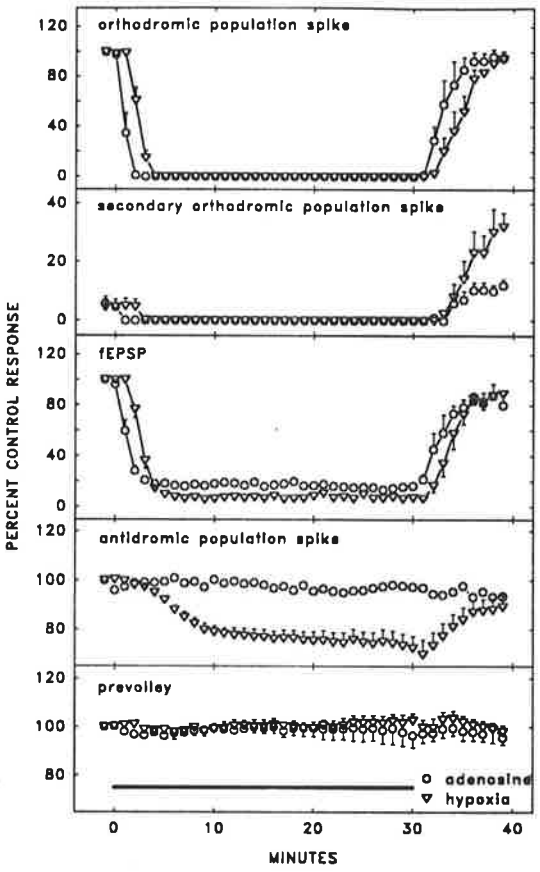

Figure 6.13 Comparison of the time course of changes in field potentials in the CA_1 area by 50μM adenosine and hypoxia

Alterations in field potentials in hippocampal slices before, during and after 30 minutes of superfusion with hypoxic ACSF (n = 5) or with normoxic ACSF containing 50μM adenosine (n = 4), except antidromic spike (n = 1). Hypoxia or DNP superfusion is indicated by the bar at bottom. Data was collected and presented as in figure 6.2. Hypoxia data in this figure is from the same slices as figure 6.2, presented for comparison. Adenosine, like hypoxia, caused depression of the fEPSP and orthodromic population spike, and recovery of synaptic transmission after adenosine washout was characterised by the appearance of a second orthodromic population spike.

of the second population spike without altering the fEPSP. Although this enhancement of the second population spike could represent 8-Br-cAMP action at an undefined adenosine receptor, it may also be a protein kinase A action.

6.4. DISCUSSION

6.4.1. POST-HYPOXIC HYPEREXCITABILITY IS AN INCREASE IN POSTSYNAPTIC NEURONAL EXCITABILITY

Using a new method of analysis of hippocampal evoked field potentials, input/output curves of the second population spike were constructed in this study as an indicator of hyperexcitability induced by hypoxia or adenosine. This striking form of hyperexcitability is rarely discussed in the literature, despite being apparent in figures from some papers (Okada *et al.* 1992). Importantly, the present method provides a means to quantify alterations in neuronal excitability that are not revealed in

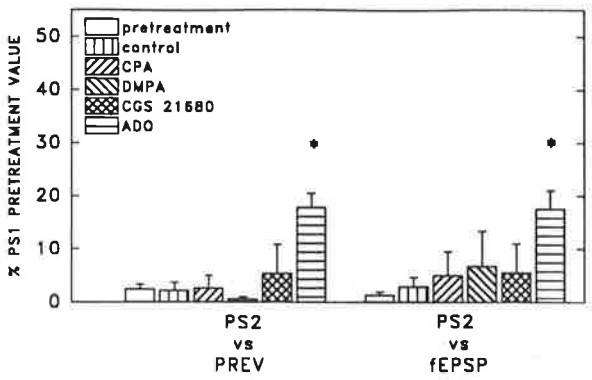

Figure 6.14 Second population spike input/output areas following adenosine receptor agonists

Areas of the input/output curves relating the second population spike amplitude to prevolley amplitude or fEPSP rate of rise (n = 5) and, for comparison, the combined pre-treatment value for these curves (n = 25) for various adenosine receptor agonists. The figure shows the mean ± S.E.M. value for area expressed as a percentage of the corresponding first population spike pre-treatment input/output curve area. Asterisk denotes significantly different from combined pre-treatment value ($P \leq 0.01$) and greater than any other agonist treatment ($P \leq 0.05$).

established methods using field potentials. In conjunction with the input/output curves of the early synaptic components, it can provide further information as to the presynaptic or postsynaptic site of increases in excitatory synaptic transmission.

It is apparent that a 30 minute period of hypoxia does not result in an increase in fast synaptic transmission, relative to afferent input. In chapter 4, comparison of input/output curves relating prevolley amplitude to either fEPSP slope, or first population spike amplitude, revealed no significant difference between normoxic or hypoxic incubation of slices, suggesting that there is no increase in excitatory transmitter release following recovery from hypoxia. It is likely that the component of the stratum radiatum fEPSP following the population spikes is modulated to some extent by spike after-potentials and IPSPs, indeed, increase in this late area of the fEPSP can be produced by paired pulse depression of the fast IPSP (Nathan *et al.* 1990). Also, the amplitude of the fEPSP may be curtailed by the onset of a population spike. Such contamination complicates interpretation of fEPSP amplitude, area and duration. However, measurements of fEPSPs, resulting from inputs of similar strength, showed no differences before and after hypoxia, despite the obvious multiple population spikes following hypoxia. Taken together, these results indicate that there is no increase in the net excitatory input to the CA_1 neurons. Nevertheless, post-hypoxic hyperexcitability is evident in the input/output curves of the second population spike, and is likely, therefore, to result from a prolonged postsynaptic neuronal excitation in response to stimulation. This prolonged excitation is in response to AMPA receptor activation and not NMDA receptors, since NMDA receptor antagonism did not reduce any of the population spikes. This multiple spiking following hypoxia indicates that, in some of the neuronal population,

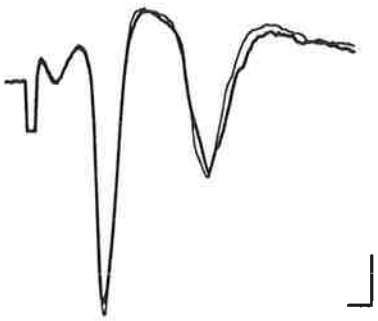

Figure 6.15 Second population spike is not NMDA generated
Stratum pyramidale field potentials following 30 minutes of hypoxia. Superimposed multiple orthodromic population spikes recorded in the presence (thick line) and absence (thin line) of the NMDA antagonist AP5 (50μM). Calibration bars are 1mV and 1ms, negative down. Stimulus artefacts are truncated for clarity.

membrane depolarisation exceeds threshold for a prolonged period in response to excitatory input, as a result of either disinhibition or an alteration in intrinsic neuronal excitability.

CA_1 pyramidal neurons are intrinsically excitable, but are under the influence of polysynaptic feedback and feedforward GABA mediated inhibition, as well as an inhibitory tone from the action of basal levels of endogenous adenosine, which limit their activity. Alleviation of either of these forms of inhibition could be responsible for post-hypoxic hyperexcitability. Indeed, disinhibition leads to multiple population spikes; for instance, in the present study, adenosine A_1 receptor antagonism with 8-CPT produced multiple population spikes, whilst multiple population spikes accompany depression of $GABA_A$ receptor function in hippocampal slice (Garcia-Ugalde *et al.* 1992; Watson and Lanthorn, 1993). However, in both cases this disinhibition is accompanied by an increase in the first population spike amplitude that is not observed in post-hypoxic input/output curves such as figure 6.9, see also chapter 4, section 4.3.1.2. Also, the lack of change in fEPSP area and duration, a putative fast IPSP action (Nathan *et al.* 1990), suggests no change in the fast IPSP. Although hypoxia can depress monosynaptically evoked IPSCs in CA_1 pyramidal neurons in hippocampal slices (Zhazipov *et al.* 1994; Katchman *et al.* 1994), there is no evidence that this persists with reoxygenation. It is well established that GABAergic interneurons in the CA_1 are resistant to ischaemia, and will survive when CA_1 pyramidal cells have degenerated (Schlander *et al.* 1988; Nitsch *et al.* 1989; Schmidt-Kastner and Freund 1991); therefore, post-hypoxic hyperexcitability is unlikely, to result from selective loss of these inhibitory interneurons. Furthermore, [^{3}H]GABA binding, GABA release (Johansen *et al.* 1991), and fast IPSPs (Johansen *et al.* 1991; Jensen *et al.* 1991) are not reduced in rats following ischaemia. Nevertheless, excitation of interneurons is particularly sensitive to hypoxic depression (Leblond and Krnjevic, 1989; Fujiwara *et al.* 1987; Krnjevic *et al.* 1991),

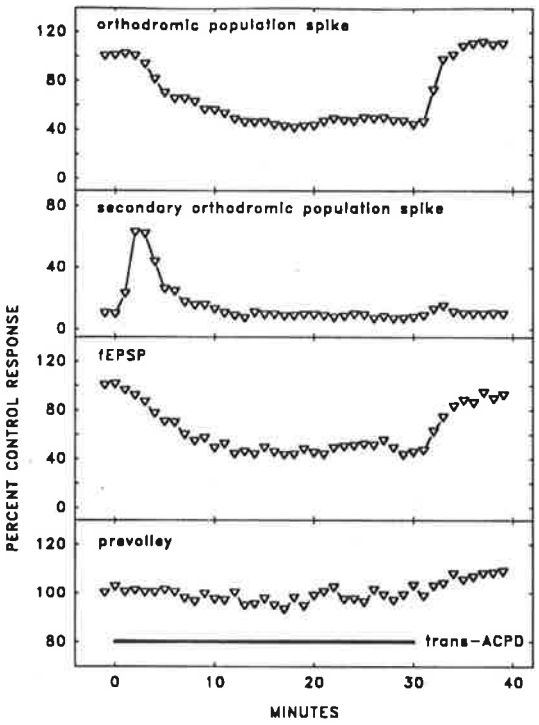

Figure 6.16 Time course of alterations in field potentials in the CA_1 area by *trans*-ACPD
Alterations in field potentials in hippocampal slices before, during and after 30 minutes of superfusion with 100μM *trans*-ACPD (n = 1), indicated by the bar at bottom. Data was collected and presented as in figure 6.2. trans-ACPD initially caused the appearance of a second population spike without enhancement of the first population spike or fEPSP. Continued trans-ACPD superfusion caused depression of the fEPSP and both population spikes. Synaptic transmission returned to normal, with no second population spike, after trans-ACPD washout.

and polysynaptic IPSPs are suggested to recover incompletely from hypoxia (Krnjevic *et al.* 1991). Thus, reduced recurrent inhibition may contribute to post-hypoxic hyperexcitability, and is an avenue for further investigation.

Desensitisation of A_1 receptors as a consequence of exposure to adenosine during hypoxia has been proposed as a mechanism of post-hypoxic hyperexcitability (Lee and Kreutzberg, 1987). Indeed, prolonged exposure of striatal slices to adenosine A_1 receptor agonists does desensitise the A_1 receptor-mediated inhibition of adenylate cyclase (Abbracchio *et al.* 1992). However, in the present study, A_1 receptor-mediated inhibition of synaptic transmission is not altered following hypoxia. This is not to say that A_1 receptor-mediated inhibition of adenylate cyclase is not desensitised following hypoxia in the hippocampus, and indeed this could alter intrinsic neuronal excitability by reducing the I_{AHP} as discussed below. However, failure of A_1-receptor antagonism during hypoxia to alter post-hypoxic hyperexcitability is evidence against this phenomenon being mediated by desensitisation of A_1 receptors. Secondly, a reduced endogenous adenosine level after hypoxia is unlikely, as antagonism of the adenosine A_1 receptor with 8-CPT still caused increase excitability.

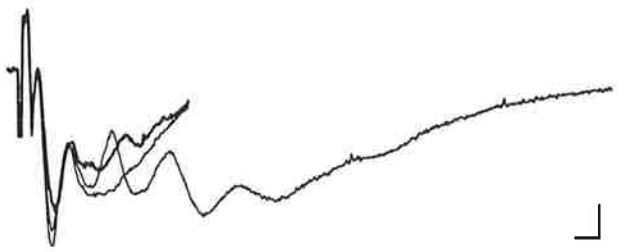

Figure 6.17 H-7 increases synaptic transmission in the CA_1 area
Stratum radiatum field potentials recorded in a slice pre-treated with 100μM H-7 for five minutes then exposed to 30 minutes hypoxia in the continued presence of H-7. Field potentials recorded before drug treatment and during normoxic H-7 pre-treatment are shown as thin lines, and the field potential shown as a thick line was recorded following hypoxia, after one hour reoxygenation and wash in normoxic, drug-free ACSF. Note the increase in fEPSP duration during normoxic H-7 superfusion and evidence of two population spikes after hypoxia. Calibration bars are 0.5mV and 2ms, negative down. Stimulus artefacts are truncated for clarity.

An increase in intrinsic excitability of pyramidal neurons could arise from reduced potassium currents. A diversity of potassium currents exist in the CA_1 pyramidal neurons, where these currents contribute to maintenance of the resting membrane potential, spike repolarisation and frequency accommodation, and the control of neuronal excitability by some receptors (Storm, 1993). In the only study found, that made intracellular recordings from hippocampal slice CA_1 pyramidal layer neurons before, during, and after prolonged hypoxia, neither the resting membrane potential nor the input resistance were any different before and after hypoxia (Fujiwara *et al.* 1987), suggesting that the potassium currents that contribute to the resting membrane potential are not altered by hypoxia. Since multiple population spikes, induced here by hypoxia, represent multiple underlying action potentials, it is possible that the potassium channels that mediate early frequency accommodation and spike repolarisation are involved. The fast, medium and slow afterhyperpolarisations (AHP) that mediate accommodation are caused by the I_C, I_M and I_{AHP} respectively (Storm, 1993), and it is possible that one of these is depressed following hypoxia. For instance, desensitisation of adenosine A_1 receptor-mediated adenylate cyclase inhibition (Abbracchio *et al.* 1992) could promote a cAMP-dependent depression of the I_{AHP}. Whether or not reduction of any of these currents contribute to post-hypoxic hyperexcitability awaits investigation with single cell techniques.

6.4.2. SUBSET OF NEURONS BECOME HYPEREXCITABLE

It is possible that only a subset of neurons develop hyperexcitability following hypoxia, and this would explain why the second spike is always smaller in amplitude than the first population spike (Andersen *et al.* 1971b). This might correspond to the portion of neurons that show post-hypoxic hyperexcitability in intracellular recordings, 38% of spiny pyramidal neurons in layer II/III of neocortical slices

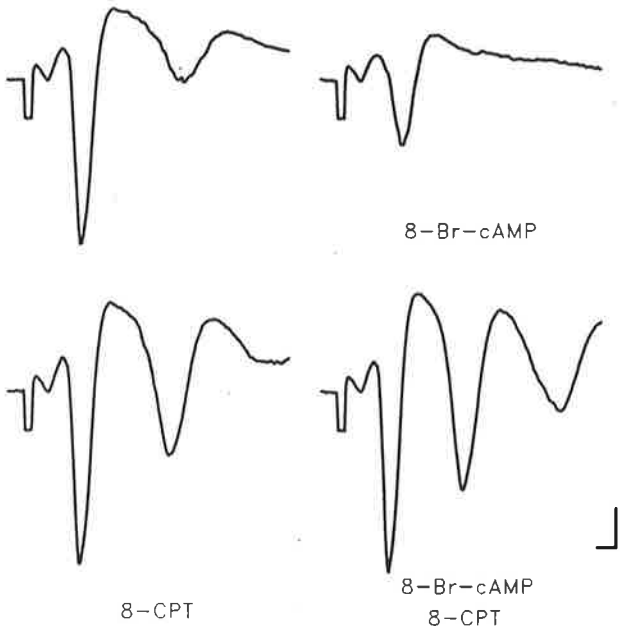

Figure 6.18 8-Br-cAMP is an A_1 receptor agonist and also produces hyperexcitability
Field potentials in the CA_1 area of the hippocampal slice. At top left is a control recording and top right is during superfusion of 500μM 8-br-cAMP. The bottom row shows the same treatment but in the presence of 1μM 8-CPT. Calibration bars are 1mV and 1ms, negative down. Stimulus artefacts are truncated for clarity. Note that the depression of the first population spike by 8-br-cAMP is blocked by 8-CPT and instead 8-br-cAMP increases the amplitude of the second population spike.

(Luhmann and Heinemann, 1992) and 43% of CA_1 pyramidal neurons in hippocampal slices (Fujiwara *et al.* 1987).

6.4.3. GLUTAMATE RECEPTORS AND LONG-TERM POTENTIATION

Ischaemia can produce a form of long-term potentiation in the hippocampus, presumably due to glutamate release and NMDA receptor activation. In the CA_1 of hippocampal slices, NMDA receptor activation during one to three minutes of ischaemia produces a potentiation of the NMDA component of evoked field potentials (Crépel *et al.* 1993). Population spike and fEPSP are potentiated following *in vivo* ischaemia in the rat (Miyazaki *et al.* 1993) and the fEPSP is potentiated in hippocampal slices prepared from gerbils following *in vivo* ischaemia (Urban *et al.* 1989). However, the post-hypoxic hyperexcitability described here is different from typical long-term potentiation. Long-term potentiation is usually characterised by an increase amplitude of the fEPSP and first population spike (Bliss and Lomo, 1978), and not the appearance of multiple population spikes like those evident following hypoxia. Long-term potentiation requires synaptic activation of NMDA receptors (Bliss and Collingridge, 1993) but this is unlikely to occur during hypoxia, since post-hypoxic hyperexcitability can occur without Schaffer collateral stimulation during hypoxia, non-synaptic glutamate release is unlikely in the absence of hypoxic spreading depression (Martin *et al.* 1994) and hypoxic hyperpolarisation of CA_1

pyramidal neurons (Fujiwara *et al.* 1987) would maintain the magnesium block of NMDA receptors (Mayer *et al.* 1984; Nowak *et al.* 1984). Furthermore, here, NMDA receptor antagonism during hypoxia with AP5 did not prevent development of post-hypoxic hyperexcitability. Metabotropic glutamate receptor activation with *trans*-ACPD can produce a form of long-term potentiation independent of NMDA receptor activation, but also requires presynaptic activity (Bortolotto and Collingridge, 1993; Aniksztejn *et al.* 1992). Application of *trans*-ACPD in the absence of tetanic stimulation failed to produce a long-term potentiation of any kind in the present investigation.

6.4.4. HYPEREXCITABILITY DURING ONSET OF HYPOXIA

Synaptic excitation of interneurons is particularly sensitive to hypoxic depression (Leblond and Krnjevic, 1989; Fujiwara *et al.* 1987; Krnjevic *et al.* 1991). If hypoxic onset disinhibition represents depression of recurrent inhibition, it's potentiation during A_1 receptor antagonism suggests either an absence or insensitivity of presynaptic A_1 receptors on the CA_1 recurrent collaterals or reduced adenosine release in the vicinity of the recurrent collateral synapses. However, CA_1 pyramidal neurons express A_1 receptor mRNA (Mahan *et al.* 1991; Cunha *et al.* 1994a) and A_1 receptor binding is high, not only in the stratum radiatum, but also in stratum oriens, and is reasonably high in the stratum pyramidale (Fastbom *et al.* 1987), these latter being the site of many recurrent synapses with interneurons, see chapter 2, section 2.2.1.5.

6.4.5. ADENOSINE MEDIATED HYPEREXCITABILITY

Of particular interest, in this study, is the similarity of persistent multiple population spikes produced by 30 minutes superfusion with 50μM exogenous adenosine and those that appear following a similar period of hypoxia. The adenosine-induced hyperexcitability is not as great as that produced by hypoxia but, due to rapid uptake, the concentration of exogenous adenosine used probably represents a smaller concentration at the receptors than that obtained during hypoxia, see chapter 4, section 4.3.7. While adenosine itself produced hyperexcitability, none of the agonists selective for adenosine receptor subtypes produced persistent multiple population spikes. Of those used here, CGS 21680 is a putative selective A_{2a} adenosine receptor agonist, and CPA is a selective A_1 receptor agonist, whilst DMPA is an less selective A_2 receptor agonist. But although it binds the A_{2a} receptor with high affinity (van Galen *et al.* 1994), the present study and another (Mogul *et al.* 1993) have shown it to have actions different from those of CGS 21680, raising the possibility it may act at the A_{2b} receptor. Possibly, the concentrations of agonists used were not appropriate, or adenosine post-inhibitory hyperexcitability results from activation of an as yet poorly characterised receptor subtype, such as the recently

described A_3 receptor (van Galen *et al.* 1994) or even a novel receptor subtype, or possibly requires simultaneous activation of more than a single receptor type.

Persistent post-inhibitory hyperexcitability, consisting of multiple population spikes following adenosine application, has not been previously reported, although the figures in Okada *et al.* (1992) suggest multiple population spikes recorded in the CA_3 region following washout of adenosine. Most other evidence supports an adenosine receptor mediated hyperexcitability of a different, presumably presynaptic, kind, characterised by an increased amplitude of the first population spike and EPSP following adenosine application, in the dentate gyrus and in the CA_3 area (Nishimura *et al.* 1990; Okada *et al.* 1990; Nishimura *et al.* 1992; Okada *et al.* 1992). In superior collicular slices, a persistent increase in postsynaptic potential amplitude in the superficial grey, with optic layer stimulation, occurs during and after even 5 minute superfusion of adenosine (100nM), and similar increases in postsynaptic potential amplitude can be produced by agonists specific for both A_1 and A_{2a} receptors, at concentrations as low as 1nM (Ishikawa *et al.* 1994). In the superior colliculus, the adenosine-mediated increase in synaptic transmission is mediated by an increase in glutamate release; adenosine produces an increase in basal and electrically stimulated glutamate release, which is dependent on extracellular calcium and is blocked by TTX and by the protein kinase A inhibitor HA-1004 (Hirai and Okada, 1994). It may well be that a similar hyperexcitability can also occur in the CA_1. In the presence of A_1 receptor antagonists, adenosine or phenylisopropyladenosine increase whole-cell EPSCs in CA_1 pyramidal neurons (Garaschuk *et al.* 1992). The A_{2a} receptor agonist CGS 21680 transiently increases the first population spike amplitude in the CA_1 area in a some slices and reduces the depression of synaptic transmission by CPA (Cunha *et al.* 1994a), the former finding being confirmed here. The enhancement of synaptic transmission in the hippocampus by adenosine may be related to adenosine enhancement of P-type calcium currents in CA_3 neurons (Mogul *et al.* 1993). This action is mediated via A_{2b} receptors as it is caused by adenosine, 2-chloroadenosine or DPMA, but not CPA or CGS 21680, and is dependent on protein kinase A activation (Mogul *et al.* 1993). On the other hand, adenosine agonists in the presence of A_1 receptor antagonists produce depolarisation with an increased input resistance (Ameri and Jurna, 1991), and rebound depolarisation occurs after adenosine withdrawal (Siggins and Schubert, 1981). This may represent a postsynaptic form of hyperexcitability, such as that described in the present investigation. It is interesting to note that, similar to the present study, hyperexcitability persisting beyond the drug application in slices is reported only with exogenous adenosine, not with A_1 and A_2 selective agonists.

6.4.6. POST-HYPOXIC HYPEREXCITABILITY IS NOT MEDIATED BY A_1 RECEPTORS

Whether adenosine release by hypoxia is solely responsible for the observed post-hypoxic hyperexcitability has not yet been established conclusively. Nevertheless, it seems unlikely that post-hypoxic hyperexcitability results from the obvious adenosine A_1 receptor activation during hypoxia, since post-hypoxic hyperexcitability still occurred if the adenosine A_1 receptor antagonist 8-CPT was present throughout the period of hypoxia. Preliminary studies using the adenosine A_2 receptor antagonist DMPX, found that this compound will neither block the development of post-hypoxic hyperexcitability nor depress the secondary population spikes after they become established. DMPX, however is a relatively weak antagonist with only moderate selectivity at A_2 receptors , and is likely of limited use in the face of the evident massive adenosine release during hypoxia.

6.4.7. SECOND MESSENGERS

If post-hypoxic hyperexcitability is mediated by adenosine receptor activation, it represents a different electrophysiological action from the well known direct G protein/ion channel interaction typically ascribed to these receptors. Nevertheless, rebound hyperexcitability may be mediated by G protein-coupled second messengers produced by adenosine receptor activation. To date, identified adenosine receptor/G protein effector systems include phospholipase C and adenylate cyclase, the latter being subject to both positive and negative modulation (van Calker *et al.* 1979; Sattin and Rall, 1970; Londos *et al.* 1980; White *et al.* 1992; Dickenson and Hill, 1993; Ramkumar *et al.* 1993). Thus, adenosine receptor activation could modulate cAMP-dependent protein kinase A, calcium/phospholipid-dependent protein kinase C or inositol 1,4,5-trisphosphate calcium stores, and these second messengers can have excitatory effects in pyramidal neurons.

6.4.7.1. Second messenger regulation of potassium channels

Potassium channels are regulated by the second messengers, and inhibition of potassium channels would promote hyperexcitability; in particular, inhibition of I_C, I_M and I_{AHP} may lead to production of multiple population spikes. The I_{AHP} is blocked by cAMP, and this is presumably the mode of action of many of those neurotransmitters positively linked to adenylate cyclase, which depress the slow AHP (Storm, 1990; Storm, 1993; Sim *et al.* 1992; Gerber *et al.* 1992). Calcium/calmodulin-dependent protein kinase II activation, secondary to an inositol 1,4,5-trisphosphate induced raise in intracellular calcium by muscarinic receptors, also blocks the slow AHP (Muller *et al.* 1992). Activation of protein kinase C with its natural activator, diacylglycerol, a synthetic analogue, 1-oleyl-2-acetyl-glycerol, or the membrane permeant tumour-promoting phorbol esters reduces the amplitude of the slow AHP and reduces I_{AHP} elicited by synaptic activation or depolarising current

in CA_1 pyramidal neurons, in hippocampal slices and acutely dissociated hippocampal cultures, but may not be the mode of action of receptor depression of the I_{AHP} (Alger and Williamson, 1988; Dutar and Nicoll, 1988a; Baraban *et al.* 1985; Doerner *et al.* 1988; Gerber *et al.* 1992). I_M can be reduced by intracellular application of inositol 1,4,5-trisphosphate in hippocampal neurons (Dutar and Nicoll, 1989; Dutar and Nicoll, 1988a). Insertion of rat brain membrane calcium-activated maxi-K^+ channels into lipid bilayers results in two calcium activated potassium channels, one of which is characteristic of I_C (Reinhart *et al.* 1991). Most of these I_C channels are up-regulated by protein kinase A action, and down-regulated by phosphatase 2A action, but some are down regulated by phosphorylation (Reinhart *et al.* 1991). In *Xenopus* oocytes expressing cloned mouse brain potassium channels, activation of co-expressed cloned mouse brain serotonin $5HT_{1c}$ receptor, direct activation of G proteins, or intracellular injection of calcium, reversibly suppresses I_K amplitude for 20 minutes. This probably occurs via calcium/calmodulin-dependent phosphatase since calmodulin antagonists, but not protein kinase inhibitors block the I_K suppression (Hoger *et al.* 1991). Intracellular perfusion of calcium blocks I_A in acutely dissociated guinea pig neurons (Chen and Wong, 1991), and purified protein kinase C in the presence of phorbol 12-myristate-13-acetate depresses chick brain I_A expressed in *Xenopus* oocytes (Lotan *et al.* 1990). Thus, any number of potassium channels could be modified by second messengers produced by adenosine. Verification of such an effect will require single cell recording and intracellular application of second messenger inhibitors or activators, to identify any current modified by a preiod of hypoxia or prolonged adenosine appplication.

6.4.7.2. Receptor mediated hyperexcitability

Activation of a variety of receptors G protein linked receptors can produce postsynaptic hyperexcitability, manifest as an increase in either the first population spike or development of a second population spike, without an increase in synaptic transmission, as evidenced by no increase in the fEPSP. A single G protein subtype may stimulate different effectors, whilst multiple subtypes can modulate the same effector system (Taylor, 1990). The fact that agonist binding and channel response are not properties of the same protein complex in G protein linked receptors allows amplification, integration, convergence and crosstalk of different receptor mediated signals. Here two such receptors will be discussed. The extrasynaptic β-adrenergic receptors produce a well characterised form of hyperexcitability similar to that described here following hypoxia, and muscarinic receptors produce an increase in pyramidal neuron excitability via second messengers which are up-regulated following hypoxia (Ninomiya *et al.* 1989).

6.4.7.2.1. β-adrenoceptor and cAMP mediated hyperexcitability

The hippocampus receives adrenergic input from the locus coeruleus, the brain's adrenergic nucleus (Nicoll *et al.* 1990). Axon varicosities from locus coeruleus neurons do not form specialised synapse with target neurons (Seguéla *et al.* 1990) and can exert a tonic control of neuronal excitability by activation of non-synaptic receptors. Locus coeruleus stimulation and local application of norepinephrine in the hippocampus excites pyramidal neurons via β-adrenoceptors (Pang and Rose, 1987; Summers and McMartin, 1993). β-adrenoceptors are characteristic G protein linked receptors which are positively coupled to adenylate cyclase via G_s, and 80-90% of hippocampal β-adrenoceptors are of the β_1 subtype (Summers and McMartin, 1993). Activation of β-adrenoceptors in the CA_1 area of the hippocampal slice produces long-lasting potentiation of evoked population spikes. A ten minute application of the β-adrenoceptor agonist isoproternol (500nM) causes a 200-400% increase in the amplitude of sub-maximal orthodromic CA_1 population spikes, and antidromic population spikes, which persists after washout of the agonist (Mueller *et al.* 1981; Dunwiddie *et al.* 1992; Heginbotham and Dunwiddie, 1991). This potentiation occurs post-synaptically and does not reflect increased excitatory neurotransmitter release, as the evoked fEPSP is not increased (Heginbotham and Dunwiddie, 1991; Dunwiddie *et al.* 1992; Mueller *et al.* 1981). While β-adrenoceptors activation depolarises the postsynaptic membrane, it also depresses the amplitude and duration of the slow AHP, this latter action being evident even at concentrations too low to produce membrane depolarisation (Dunwiddie *et al.* 1992; Haas and Konnerth, 1983; Madison and Nicoll, 1986). Such potentiation is independent of membrane depolarisation, as it is evoked both in 3.5mM potassium (Mueller *et al.* 1981) and 6mM potassium (Dunwiddie *et al.* 1992), and electrical stimulation during isoproterenol perfusion is not necessary (Heginbotham and Dunwiddie, 1991). β-adrenoceptor-induced hyperexcitability is likely mediated by elevation of cAMP.

Application of cAMP analogues or forskolin, an adenylate cyclase activator, to hippocampal slices produces postsynaptic hyperexcitability, evident as a slow decaying, or persistent potentiation of sub-maximal population spikes, without increase in the fEPSP in the CA_1 area after washout of the drug (Dunwiddie and Hoffer, 1980; Dunwiddie *et al.* 1992; Pockett *et al.* 1993; Slack and Pockett, 1991). The longer the application the more persistent is the potentiation, with 20 to 40 minute drug application producing population spike potentiation lasting over 120 minutes (Pockett *et al.* 1993; Slack and Pockett, 1991). Forskolin and cAMP analogues apparently produce a second population spike from a single stimulus, which although evident in the figures, it is not discussed in the text (Pockett *et al.* 1993). Intracellular recording from CA_1 neurons shows that the cAMP analogues and forskolin greatly depress the amplitude of the AHP, but do not cause depolarisation

or effect the EPSP (Dunwiddie *et al.* 1992). This hyperexcitability is distinct from tetanus-evoked long-term potentiation, as such potentiation rarely produces second population spikes, and long-term potentiation can be produced in slices already potentiated with dibutyl-cAMP (Pockett *et al.* 1993). Dibutyl-cAMP (200μM) is a weak agonist at the adenosine A_1 receptor, and causes a depression of synaptic transmission that is blocked by the A_1 receptor antagonist 8-(p-sulfophenyl)theophylline (10μM); such receptor antagonism does not prevent the potentiating action of dibutyl-cAMP (Pockett *et al.* 1993), a similar action was confirmed for 8-Br-cAMP in the present investigation.

6.4.7.2.2. Cholinergic receptor mediated hyperexcitability

The septum provides a major input to the hippocampus via the fornix and alveus, and about half of this projection is cholinergic. These unmyelinated fibres are concentrated in the stratum pyramidale, deep stratum radiatum and superficial stratum oriens, and synapse with pyramidal cell soma and proximal dendrites (Frotscher and Léránth, 1985; Lewis *et al.* 1967). Electrical stimulation of cholinergic afferents can produce an increase in input resistance, slow EPSPs and spikes, and depress the slow AHP in CA_1 pyramidale neurons (Madison *et al.* 1987). Inhibitory cholinergic actions are also present in the hippocampus, including a muscarinic receptor-mediated depression of the fEPSP and EPSC (Sheridan and Sutor, 1990; Kamiya, 1991; Dutar and Nicoll, 1988b) and enhancement of I_K (Zhang *et al.* 1992). Nevertheless, in CA_1 neurons, cholinergic receptors, which are all of the muscarinic subtype, are mostly excitatory, producing depression of potassium currents (Cole and Nicoll, 1984; Madison and Nicoll, 1984). Muscarinic M1 and M3 receptor activation blocks voltage-insensitive potassium leak currents, producing depolarisation and spike discharge (Cole and Nicoll, 1984). It also blocks I_A resulting in increased spike amplitude and duration (Nakajima *et al.* 1986), as well as inhibiting the slow AHP and underlying I_{AHP} (Dutar and Nicoll, 1988b; Madison *et al.* 1987; Cole and Nicoll, 1984; Madison and Nicoll, 1984; Lambert and Teyler, 1991b). M1 and M3 receptors, coupled to G_q, activate phospholipase C, and stimulate hydrolysis of phosphatidylinositide (Offermanns *et al.* 1994; Dutar and Nicoll, 1988b; Fisher *et al.* 1992). This results in the production of the second messengers inositol 1,4,5-trisphosphate and diacylglycerol. While it is well known that activation of protein kinase C will depress I_{AHP}, (Alger and Williamson, 1988; Sim *et al.* 1992), the protein kinase C inhibitor staurosporine does not prevent cholinergic agonist depression of the I_{AHP} (Sim *et al.* 1992). However, inhibition of the calcium/calmodulin-dependent protein kinase II prevents the muscarinic block of the slow AHP (Muller *et al.* 1992), presumably this is due to phosphorylation of the potassium channel and is secondary to an inositol 1,4,5-trisphosphate induced raise in intracellular calcium. Activation of muscarinic M2 receptors, coupled to G_i (Offermanns *et al.* 1994), blocks the non-

inactivating I_M (Madison *et al.* 1987; Dutar and Nicoll, 1988b; Dutar and Nicoll, 1988a). Depression of I_M appears critically dependent on strong agonists of stimulation of phosphatidylinositide hydrolysis (Dutar and Nicoll, 1988b; Dutar and Nicoll, 1988a), but this maybe because of the relative inefficiency of M2 receptors at stimulating phosphatidylinositide hydrolysis (Hosey, 1992). Activation of protein kinase C fails to block I_M, but intracellular application of inositol 1,4,5-trisphosphate reproducibly reduces I_M (Dutar and Nicoll, 1988b; Dutar and Nicoll, 1988a). Stimulation of cholinergic afferents in the stratum oriens blocks the leak currents and can depress slow AHPs, but does not inhibit I_M (Madison *et al.* 1987); this may be due to higher concentrations of acetylcholine needed to activate the I_M response.

6.4.7.3. Second messengers in post-hypoxic hyperexcitability

As previously noted, the presynaptic form of adenosine-mediated hyperexcitability in the dentate gyrus and superior colliculus is apparently mediated by protein kinase A, being inhibited by H-7, H-8, HA-1004 and H-89 (Okada and Hirai, 1993; Nishimura *et al.* 1992; Hirai *et al.* 1994; Hirai and Okada, 1994), and P-type calcium channel enhancement by adenosine A_{2b} receptor activation in the hippocampus is blocked by the protein kinase A inhibiting peptide WIPTIDE (Mogul *et al.* 1993). Neither of these are likely to be the same phenomenon as the postsynaptic excitability described in the present study. However, the involvement of cAMP and protein kinase A in post-hypoxic or post-inhibitory excitability warranted investigation, since these are involved in previously described forms of postsynaptic excitability. Furthermore, the G_i or G_o linked receptors, serotonin or $GABA_B$, which share effector systems with adenosine A_1 receptors, can actually enhance the G_s linked action of β-adrenoceptors on the slow AHP in hippocampal neurons (Andrade, 1993), and thus could enhance tonic adrenergic excitation. However, neither of the protein kinase A inhibitors H-7 or H-8, nor the protein kinase C inhibitor sphingosine prevented post-hypoxic hyperexcitability. Clarification by the use of a more potent and selective protein kinase A inhibitor is necessary, in particular since 8-Br-cAMP produced some hyperexcitability.

An inositol 1,4,5-trisphosphate mediated rise in intracellular calcium may mediate post-hypoxic hyperexcitability, by an action similar to the cholinergic calcium/calmodulin-dependent protein kinase II depression of the slow AHP (Muller *et al.* 1992) or a calcium/calmodulin-dependent protein phosphatase depression I_K (Hoger *et al.* 1991). This may be a direct action of adenosine receptor phospholipase C activation, or may be due to interaction of adenosine receptors with, for instance cholinergic muscarinic receptors, at the level of second messengers. For example, electrical stimulation of cholinergic afferents in the presence of eserine, or application of exogenous cholinergic agonist that are full agonists at stimulating

phosphatidylinositide hydrolysis, such as carbachol, inhibits adenosine A_1 receptor and $GABA_B$ receptor mediated depression of field potentials in the CA_1 area of the hippocampus (Worley *et al.* 1987). Indeed, transient severe hypoxia, or chronic, mild *in vivo* hypoxia, causes up-regulation of muscarinic cholinergic receptor-coupled phosphatidylinositide turnover in the rat brain, lasting for 48 hours (Ninomiya *et al.* 1989). Cholinergic fibre terminals in the hippocampus are a source of normoxic, basal adenosine, since ecto-5'-nucleotidase is located on cholinergic synapses (Sebastiào *et al.* 1993), and adenosine is produced from ATP at cholinergic synapses (James and Richardson, 1993). ATP is involved in the packaging of acetylcholine in vesicles, and these are likely co-released from cholinergic terminals. Hippocampal cholinergic terminals have high affinity A_1 receptors which depress acetylcholine release in the CA_1 (Dunér-Engström and Fredholm, 1988; Fredholm, 1990; Cunha *et al.* 1994b), and these may be altered by hypoxia.

6.5. SUMMARY AND CONCLUSIONS

Depression of synaptic transmission in the CA_1 area of hippocampal slices for 30 minutes by either hypoxia or adenosine superfusion resulted in an increase in postsynaptic excitability upon return of synaptic transmission following reoxygenation or drug washout. This hyperexcitability, manifest as firing of multiple spikes in response to unchanged AMPA receptor-mediated excitatory synaptic transmission, may have resulted from reduced GABAergic inhibition but more likely resulted from increased pyramidal neuron excitability. Post-hypoxic hyperexcitability was not blocked by adenosine A_1 receptor antagonism or by NMDA receptor antagonism during hypoxia, nor by inhibition of protein kinases. While adenosine produced hyperexcitability, agonists selective for adenosine A_1, A_{2a} and A_{2b} receptor subtypes did not, and adenosine may act at A_3 receptors or an unidentified receptor subtype to produce rebound hyperexcitability.

While the cause of post-hypoxic hyperexcitability remains unclear, this study has focussed on the possible involvement of adenosine in its generation. Indeed, it is attractive to consider that the post-inhibitory hyperexcitability following adenosine application, and the post-hypoxic hyperexcitability described here may be the same phenomenon. If this is the case, it represents a novel, and possibly deleterious, action of adenosine that should be considered in future design of therapeutic purinergic agents. Indeed, adenosine enhances ischaemic damage in the superior colliculus (Fujiwara *et al.* 1994), and a similar action may co-exist with "protective" actions of adenosine in the hippocampus and other brain areas. Confirmation of these findings will require potent and specific antagonism of the as yet unidentified adenosine receptor involved in post-hypoxic hyperexcitability.

7. CONCLUDING REMARKS

7.1. GENERAL SUMMARY OF EXPERIMENTAL WORK

The aim of this thesis was to investigate the earliest responses to hypoxia in the CA_1 area of the hippocampal slice, and in particular, those mediated by adenosine. The present study explored the relationships between neuronal oxidative energy metabolism, extracellular adenosine accumulation, and the remarkable survival of these selectively vulnerable CA_1 neurons during prolonged hypoxia. The present work also investigated whether hypoxic adenosine release is a contributing factor in the production of the post-hypoxic hyperexcitability that develops in the hippocampus upon reoxygenation following prolonged hypoxia. A summary of the contributions of this thesis to these areas is now presented.

7.1.1. CHAPTER 4

Procedural differences between laboratories has resulted in varying estimates of the acute damage caused by hypoxia in the hippocampal slice. This chapter examined the depression of synaptic transmission during prolonged hypoxia and the recovery of synaptic transmission upon reoxygenation in 10mM glucose at 32°C, to establish the viability of hippocampal slices in the present model. Also, the reported adenosine A_1 receptor-mediated depression of synaptic transmission during hypoxia was verified. These studies allowed estimation of the extracellular concentration of adenosine that is achieved during hypoxia, and evaluation of the role of adenosine-mediated neuronal depression in recovery of synaptic transmission following hypoxia in the present model. In hippocampal slices, 30 minutes of hypoxia at 32°C did not cause acute excitotoxic CA_1 neuronal damage, as indicated by the preservation of synaptic transmission following re-admission of oxygenated ACSF. More aggressive blocking of oxidative phosphorylation was attempted by combining hypoxia with carbon monoxide exposure, and also by superfusion with oxygenated ACSF containing the mitochondrial uncoupler DNP. Neither of these treatments yielded results different from those with hypoxia, and did not produce acute excitotoxicity. These results show that, the CA_1 area of the hippocampal slice survived prolonged hypoxia, under conditions which allowed glycolysis to proceed at maximum rate. Hypoxia or chemical inhibition of mitochondrial energy metabolism caused massive extracellular accumulation of adenosine, and an adenosine A_1 receptor-mediated depression of synaptic transmission. It was estimated that, during hypoxia, extracellular adenosine concentrations rose 80 fold, from 200nM to 16μM. Neuronal survival during hypoxia was not promoted by the adenosine A_1 receptor-mediated depression of synaptic transmission, as antagonism of the adenosine A_1 receptor did not alter neuronal survival, indicated by the unchanged post-hypoxic recovery of neuronal function in its presence. Future studies might examine the role of

adenosine A_1 receptor activation in protecting slices incubated at physiological temperature and glucose concentration and exposed to a duration of hypoxia following which synaptic transmission only partially recovers.

7.1.2. CHAPTER 5

Since ATP is relatively preserved during hypoxia (Fredholm *et al.* 1984), it has never been clear whether adenosine accumulation during hypoxia is a result of loss of adenine nucleotides to nucleosides by mass action, or whether the activities of the enzymes involved in adenosine metabolism are altered. This chapter employed inhibitors for some of the partial reactions involved in energy production and metabolism to investigate adenosine-mediated synaptic depression during hypoxia. Blockade of the mitochondrial electron transport chain or dissipation of the inner mitochondrial membrane electrochemical gradient produced an adenosine A_1 receptor-mediated depression of synaptic transmission, identical with that produced by hypoxia. However, this initial rapid accumulation of adenosine was evidently not due to neuronal ATP depletion, since inhibition of mitochondrial ATP production or export failed to mimic hypoxia. Also, buffering of cytosolic ATP concentrations with phosphocreatine failed to delay synaptic depression arising from inhibition of mitochondrial energy metabolism. Adenosine accumulation may result from stimulation of 5'-nucleotidase and increase in free AMP, possibly combined with inhibition of adenosine salvage via adenosine kinase. Possible regulatory mechanisms for adenylate kinase include increase in cytosolic calcium secondary to the alteration of mitochondrial calcium homeostasis, acidification of the cytosol due to lactic acidosis, or localised increase in free AMP. A cell permeant and selective adenylate kinase inhibitor, as well as a 5'-nucleotidase inhibitor, would be useful to investigate the relative contributions of loss of adenine nucleotides and 5'-nucleotidase activation in hypoxic adenosine accumulation.

7.1.3. CHAPTER 6

Subsequent to an hypoxic or ischaemic episode, synaptic activity may recover, and neuronal hyperexcitability can appear, both *in vivo* and *in vitro* (Chang *et al.* 1989; Schiff and Somjen, 1985). Such hyperexcitability is poorly characterised; for instance, there is evidence both for increased excitatory synaptic transmission (Miyazaki *et al.* 1993) as well as for an increase in pyramidal neuron excitability (Andersen, 1960; Chang *et al.* 1989). This chapter presented evidence in hippocampal slices that hypoxia-induced adenosine release itself may contribute to a form of post-hypoxic hyperexcitability that is a result of an increase in excitability of the CA_1 pyramidal neurons. Depression of synaptic transmission in the CA_1 area of hippocampal slices for 30 minutes by either hypoxia or adenosine superfusion resulted in a postsynaptic hyperexcitability upon return of synaptic transmission

following reoxygenation or drug washout. This represents a novel form of hyperexcitability, quite distinct from classical long-term potentiation or other forms of adenosine receptor-mediated hyperexcitability. Post-hypoxic hyperexcitability is not blocked by adenosine A_1 receptor antagonism or NMDA receptor antagonism during hypoxia, nor by inhibition of protein kinases. While adenosine produces hyperexcitability, agonists selective for adenosine A_1, A_{2a} and A_{2b} receptor subtypes do not, and adenosine may be acting at A_3 receptors or an unidentified receptor subtype. This hyperexcitability, manifest as firing of multiple spikes in response to otherwise unchanged excitatory synaptic transmission, may have resulted from reduced GABAergic inhibition but more likely resulted from increased pyramidal neuron excitability. Further studies are required to characterise the pharmacology of excitatory actions of adenosine in the hippocampus. In particular the adenosine receptor responsible for the postsynaptic rebound excitation described here needs to be identified and antagonist developed; such antagonists would help investigation of the mechanism of post-hypoxic hyperexcitability.

7.2. FUTURE DIRECTIONS

A number of areas would prove profitable for future research. The mechanism of post-hypoxic and post-inhibitory rebound hyperexcitability can be examined, concentrating on two areas. First would be the second messengers involved in production of hyperexcitability using intracellular application of kinase and phosphatase inhibitors, and modulation of calcium release from intracellular stores. Second would be identifying of receptors other than adenosine that might participate in the production of hyperexcitability via crosstalk. For instance, adenosine receptor activation may up-regulate excitatory actions of β-adrenergic receptors and thus enhance adrenergic tone. The actual underlying membrane currents that are reflected in multiple population spikes should be identified; initially currents responsible for the various spike after-hyperpolarisations would be examined.

The mechanism of hypoxic excitotoxicity should be examined. On one hand, it may be that sufficiently prolonged hypoxia results in ATP depletion and excitotoxicity identical to ischaemia. On the other hand, the role of post-hypoxic hyperexcitability in delayed neuronal death following hypoxia may be amenable to study in hippocampal cultures or slice cultures. This would hinge on the ability to block the induction or development of post-hypoxic hyperexcitability, or the ability to successfully mimic hyperexcitability by normoxic application of adenosine agonists. The role of adenosine A_1 receptor activation by endogenous or exogenous adenosine in the protection of neurons from damage due to hypoxia should be examined, again in culture.

Perhaps the globus pallidus, potentially an area selectively vulnerable to hypoxia rather than ischaemia (Plum *et al.* 1962; Meyer, 1936), may be the place to examine pure hypoxic damage. Also, examination of the effects of hypoxia in the superior colliculus might provide useful information, in light of the curious adenosine receptor pharmacology in this brain area (Ishikawa *et al.* 1994).

7.3. FRAMEWORK OF EARLY HYPOXIC EVENTS INCLUDING THE FINDINGS OF THE PRESENT STUDY

Chapter one presents a framework of events associated with cerebral hypoxia, from the loss of consciousness through to delayed neuropsychiatric deterioration and neuronal degeneration *in vivo*, which can be reflected *in vitro* by the depression of synaptic transmission through to acute and delayed excitotoxicity. The present studies have added to the understanding of these processes, and an expanded framework of the early hypoxic events in the CA_1 area of the hippocampal slice is now presented.

During hypoxia, the absence of oxygen as the terminal electron acceptor in the electron transport chain prevents mitochondrial respiration. This results in local cytosolic changes in the synaptic terminals which are rich in mitochondria. These changes may include increase in cytosolic calcium secondary to alteration of mitochondrial calcium homeostasis, acidification of the cytosol due to lactic acid accumulation, localised drop in free energy, and accumulation of free AMP. Some of these changes could initiate a rapid cytosolic accumulation of adenosine, due to an increase in free AMP, activation of 5'-nucleotidase and depression of adenosine kinase activity. Intracellular and extracellular adenosine equilibrate, through the passive nucleoside transporter, causing a rapid increase in extracellular adenosine to 16μM, from the basal normoxic concentration of 200nM. The resulting increased activation of presynaptic adenosine A_1 receptors depresses synaptic activity in the CA_1 area.

Hypoxic depression of synaptic transmission is accompanied by hyperpolarisation of the pyramidal neurones that results from activation of a potassium conductance (Leblond and Krnjevic, 1989; Hansen *et al.* 1982; Fujiwara *et al.* 1987). This hyperpolarisation is not mediated by activation of ATP-sensitive potassium channels (Leblond and Krnjevic, 1989) or adenosine A_1 receptors (Croning *et al.* 1994), but could be due to activation of another adenosine receptor. If hypoxia is prolonged, neurones may depolarise, and eventually ionic homeostasis and membrane polarisation is lost, after which neuronal recovery is unlikely (Fairchild *et al.* 1988; Reid, 1987; Hansen *et al.* 1982; Fujiwara *et al.* 1987; Taylor and Weber, 1993). Such hypoxic depolarisation, and the associated glutamate release (Attwell *et al.*

1993), only occurs once ATP depletion is sufficiently severe that membrane-associated ion-translocating ATPases cannot function (Kass and Lipton, 1986); however, neuronal energy demands during hypoxia are usually met by glycolytic ATP production (Fredholm *et al.* 1984; Kauppinen and Nicholls, 1986; Scott and Nicholls, 1980). If oxygen is re-introduced before hypoxic depolarisation, synaptic transmission recovers, indicating survival of a majority of the neuronal population. Such neuronal survival during hypoxia is not dependent on adenosine A_1 receptor-mediated depression of neuronal activity and energy consumption.

Following hypoxia, hippocampal slices may display a form of hyperexcitability characterised by firing of multiple population spikes in response to unchanged AMPA receptor-mediated excitatory synaptic transmission. This hyperexcitability may result from reduced GABAergic inhibition, but more likely is due to increased pyramidal neuron excitability. This post-hypoxic hyperexcitability results from prolonged activation of adenosine receptors, probably of a type which activate phospholipase C. Subsequent second messenger activation of a calcium-dependent protein kinase or phosphatase may promote hyperexcitability by persistent closure of a neuronal potassium channel.

7.4. RELEVANCE OF THE PRESENT FINDINGS

At a fundamental level these investigations provide further insight into some modulatory mechanisms governing synaptic transmission and excitability in the hippocampus. In particular, they provide a better knowledge of the actions of adenosine in modulating excitatory synapses and neuronal excitability, and the alterations precipitated by hypoxic adenosine release. Secondly, these studies provide an improved definition of the earliest changes in hippocampus induced by hypoxia. Post-hypoxic hyperexcitability is particularly important since increased neuronal excitability following reoxygenation may well be a prerequisite for some forms of hypoxic or ischaemic excitotoxicity.

7.4.1. NEURONAL DEATH AND HYPOXIA

7.4.1.1. Hypoxia and ischaemia

To address the *in vivo* relevance of the present hypoxia studies, it is necessary to summarise the relationship between ischaemia and hypoxia. While both ischaemia and hypoxia inhibit oxidative phosphorylation and cause adenosine release, only ischaemia inhibits glycolysis. During ischaemia, extracellular accumulation of glutamate may occur due to ATP depletion and loss of membrane ion homeostasis and polarisation, resulting in reversal of the glutamate/sodium/potassium/hydroxide co-transporter (Attwell *et al.* 1993). The resulting massive activation of glutamate receptor cation conductances leads to excitotoxic neuronal damage, including acute

swelling due to osmotic imbalance and calcium influx-initiated delayed neuronal degeneration (Rothman *et al.* 1987; Choi, 1988; Rothman and Olney, 1986; Choi, 1990). Acute hypoxic excitotoxicity occurs in the hippocampal slice with ATP depletion and hypoxic depolarisation, and this is demonstrated with ACSF containing low glucose (Rose, 1926; Kass and Lipton, 1986). However, in the present investigation with 10mM glucose, prolonged hypoxia was well tolerated in the hippocampal slice at 32°C, and in other studies this is also the case at 37°C (Fujiwara *et al.* 1987; Taylor and Weber, 1993). Due to continued glycolytic flux during hypoxia in the presence of 10mM glucose, ATP levels and cytosolic free energy are maintained and neurons do not depolarise; therefore, glutamate is not released by transporter reversal. Additionally, during hypoxia, adenosine accumulation inhibits exocytotic glutamate release. If glutamate receptor activation does not occur during hypoxia, any hypoxic excitotoxicity must develop in response to normal synaptic glutamate release upon reoxygenation. In the present study, glutamate-mediated synaptic transmission is not enhanced following hypoxia; however, post-hypoxic hyperexcitability may promote of form of excitotoxicity that develops subsequent to hypoxia in response to normal synaptic glutamate release. Indeed, intact synaptic pathways are required for delayed neuronal death following *in vitro* hypoxia (Rothman, 1983).

It seems that as long as glycolysis can proceed maximally, CA_1 neurons *in vitro* are not intrinsically susceptible to hypoxia; therefore, any *in vivo* hypoxic damage may be a result of other factors. The lower blood concentration of glucose compared to ACSF may result in insufficient glucose supply to neurons in the face of increased glycolytic flux during hypoxia. However, glucose concentrations are probably optimal as adverse effects of hyperglycaemia during hypoxia are reported *in vivo*. Hypoxia may result in reduced glucose supply due to hypoxic hypotension (Van Wylen *et al.* 1986). A combination of hypo-perfusion and low glucose concentrations may sufficiently decrease ATP production such that glycolysis will eventually be compromised by preventing phosphorylation of glucose to glucose-6-phosphate.

Conversely, during cerebral ischaemia, so called penumbral areas may exist where energy deprivation is of reduced severity due to residual perfusion (Astrup *et al.* 1981). In this ischaemic penumbra, hypoxic mechanism, such as those examined in the present work, may take precedence over ischaemic pathology. It is important to develop an understanding of hypoxia alone, both as an insult in its own right, but also to aid further investigation into ischaemia.

7.4.1.2. Is post-hypoxic hyperexcitability required for delayed neuronal death?

The development of enhanced excitability following ischaemia, and any involvement of this hyperexcitability in neuronal degeneration *in vivo*, is controversial. Seizures are common following ischaemia (Rossen *et al.* 1943; Oppenheimer and Hachinski, 1992) but do not influence morbidity or mortality (Oppenheimer and Hachinski, 1992). In experimental models, ischaemia either results in a postsynaptic hyperexcitability (Chang *et al.* 1989) or a synaptic long-term potentiation (Urban *et al.* 1989), or results in no change in excitability (Buzsáki *et al.* 1989; Thayer and Miller, 1990). It has been shown that hippocampal delayed neuronal degeneration occurs following ischaemia in gerbils despite an absence of hyperexcitability in unit recordings (Imon *et al.* 1991).

While it seems that hyperexcitability is not required for post-ischaemic neuronal degeneration, the situation may be different for hypoxia. Adenosine provides some protection against ischaemic excitotoxicity; however, adenosine does not provide protection against exogenous glutamate-mediated excitotoxicity. It seems likely that adenosine provide protection against ischaemia by acting presynaptically to prevent glutamate release, yet ischaemic glutamate release is not exocytotic. Possibly adenosine is acting in an ischaemic penumbra where excitotoxic damage is a result of enhanced excitability in response to synaptically glutamate release.

7.4.1.3. Therapeutics and drug design

Although delayed neuronal death often follows hypoxia or ischaemia, it is not an obligatory consequence, since the pathological changes may be alleviated by pharmacological intervention in the recovery phase (Meldrum *et al.* 1987; Evans *et al.* 1987). An understanding of the pathophysiology of the initial reoxygenation period following hypoxia or ischaemia is important since it may well permit more effective treatments for the brain damage that occurs during the first 24 hours following hypoxia, a time when neurons otherwise destined for disintegration can be rescued.

Adenosine has been implicated in the survival of neurons during ischaemia (Newby *et al.* 1990). Within the present model, adenosine A_1 receptor activation did not contribute to the survival of CA_1 neurons during 30 minutes of hypoxia at 32°C. Adenosine may improve the outcome following *in vivo* ischaemia, by increasing blood flow via A_2 receptors. However, *in vivo*, activation of adenosine A_1 receptors by endogenous adenosine provides protection of neurons during hypoxia (Boissard *et al.* 1992). Such A_1-mediated protection is presumably due to limiting glutamate release and excitation of neurons. However, adenosine excitatory actions may promote neuronal damage. Indeed, in the superior colliculus, where inhibitory actions of adenosine are absent, exogenous adenosine enhances acute ischaemic

neuronal damage (Fujiwara *et al.* 1994). Such an action may co-exist with inhibitory actions of adenosine in other brain areas. In these other areas, initiation of hyperexcitability, that becomes apparent after uptake of adenosine with reoxygenation, may promote excitotoxicity in response to normal synaptic glutamate release.

While the cause of post-hypoxic hyperexcitability is not completely clear, this study has focussed on the possible involvement of adenosine in its generation. Indeed, it is attractive to consider that the rebound hyperexcitability following adenosine application, and the post-hypoxic hyperexcitability described here may be the same phenomenon. If this is the case, it represents a novel, and possibly deleterious, action of adenosine that should be considered in future design of therapeutic purinergic agents. For instance, while adenosine may be provide some protection against ischaemia, it may be of no value, or even detrimental, for hypoxia. Glutamate antagonists provide a better avenue for anti-hypoxia and anti-ischaemia drugs, as they avoid the excitatory actions of adenosine agonists.

7.4.2. ADENOSINE RELEASE

Investigation into the mechanisms of adenosine accumulation provides an example of non-synaptic neuromodulation, a fundamental form of communication within the brain. Synaptic transmission is traditionally characterised by vesicular release of a neuromodulator substance and resultant activation of an extracellular receptor both occurring within a specialised synapse; however this scheme is being redefined, with the recent identification of gaseous neuromodulators, which diffuse between neurons and act intracellularly. Furthermore, synaptic transmission is not the exclusive means of communication in the brain; for instance, neuromodulators can perform a paracrine role, being released from axon varicosities that do not form specialised synapses with neurons, and activating remote extra-synaptic or synaptic receptors. Also, neuromodulators can be released at synaptic or non-synaptic sites via reversible transporters rather than by exocytosis. Such forms of non-synaptic neuromodulation may mediate generalised and sustained brain activity, such as level of arousal or mood (Bach-Y-Rita, 1993), whereas synaptic transmission mediates discrete neuronal activation. Adenosine release during hypoxia has been investigated in the present study, and has been shown to provide a direct connection between oxidative energy production and modulation of synaptic transmission. Extracellular adenosine accumulation probably causes the unconsciousness which accompanies hypoxia, and thus, provides a simple functional example of non-synaptic neurotransmission. Adenosine also accumulates during repetitive electrical stimulation of neuronal afferents (Mitchell *et al.* 1993). Such use-dependent release of adenosine may occur via a similar mechanism as during hypoxia, for instance, increase membrane ATPase

activity may lead to a rise in free AMP and lactic acid, or calcium influx into the presynaptic terminals may alter the balance of adenosine anabolism and catabolism. Such mechanisms are worthy of further investigation.

At a practical level, regulation of adenosine release mechanisms themselves may have clinical applications for early treatment of hypoxia or ischaemia. Also estimates of extracellular adenosine accumulation during hypoxia provide a reference for further investigation of possible pharmacological consequences of hypoxic adenosine receptor activation, such as rebound hyperexcitability.

7.4.3. APPLICATION OF FIELD POTENTIAL RECORDINGS AND PHARMACOLOGICAL METHODS TO THE STUDY OF HYPOXIA IN THE HIPPOCAMPAL SLICE

7.4.3.1. Success of field potential studies

Pharmacological manipulation of field potentials with receptors antagonists and enzyme inhibitors has proven to be a highly successful strategy for investigating the role of adenosine in hypoxia. Extracellular adenosine accumulation provides a direct link between cellular oxidative energy metabolism and synaptic transmission and neuronal excitability, and such adenosine accumulation can be assessed by adenosine A_1 receptor-mediated synaptic depression. Adenosine release may be a good indicator of brain tissue energy status, since acute measurements of total nucleotides do not accurately reflect neuronal free energy, and certainly not oxidative energy production. The present pharmacological studies provided, for the first time, some insight into the connection between adenosine accumulation during hypoxia and a potentially deleterious hypoxic outcome, namely, post-hypoxic hyperexcitability.

7.4.3.2. Advantages and limitations of field potential recordings

Extracellularly recorded population spikes and field excitatory post-synaptic potentials (fEPSP) represent the summed action potentials and excitatory post-synaptic potentials respectively in a population of neurons. Such field potential recordings allow assessment of the mean response of a population of neurons, rather than that from a single neuron which could be unrepresentative of the whole population. In this context, diffusion limitations in tissue slices may result in gradients of drug concentration from exogenously applied compounds, or from those produced within the tissue, and field potentials will be less biased than single cell techniques by the recording location within such gradients across the depth of the slice. Also, post-hypoxic and rebound hyperexcitability, as evidenced by multiple extracellular population spikes, represents multiple action potential firing in some, but perhaps not all, neurons in the recording population, and thus can be best assessed from population responses. The influence of an extracellular recording electrode on the

population response is minor in comparison to single cell techniques where the intracellular or patch electrodes may dialyse the intracellular milieu, leading to run-down or complete alteration of the response. This is particularly important in the investigation of an integrated response such as hypoxia where adenosine is produced in the cytosol, and cytosolic second messengers may be important in persistent actions of adenosine receptor activation. On the other hand, extracellular recording does not provide access to the cytosol for application of cell impermeant compounds active intracellularly. Field potential recording is a robust technique, recordings are easy to obtain, and remain stable over long periods. Experimentally-induced hyperexcitability requires prolonged hypoxia or drug perfusion, and the pharmacological assessment of receptor function requires long periods of recordings during which baseline responses can be safely presumed stable. On the other hand, in assessing electrophysiology, extracellular recording is limited to a reflection of what is occurring in individual neurons, whilst changes in individual spike amplitude or threshold are masked, and spike after-potentials as well as inhibitory potentials are relatively inaccessible. Nevertheless, field potentials have provided a number of advantages in the study of hypoxia in the hippocampal slice.

APPENDIX A: PUBLICATIONS DURING CANDIDATURE

Chapter 4

Doolette, D.J. and Kerr, D.I.B. (1992) Comparison of carbon monoxide and nitrogen induced effects on synaptic transmission in the rat hippocampal slice. *Neurosci. Lett.* **138**, 9-13.

Doolette, D.J. (1990) Comparison of nitrogen and carbon monoxide hypoxia in the rat hippocampal slice. *Clin. Exp. Pharmacol. Physiol.* **17 Supplement**, 19(Abstract)

Chapter 6

Doolette, D.J. and Kerr, D.I.B. (1995) Hyperexcitability in CA_1 of the rat hippocampal slice following hypoxia or adenosine. *Brain Res.* **677**, 127-137.

Other contributions

Kerr, D.I.B., Ong, J., Doolette, D.J., Abenante, J. and Prager, R.H. (1992) 3-Amino-2-(4-chlorophenyl)-nitropropane is a new $GABA_B$ receptor agonist, more active peripherally. *Eur. J. Pharmacol.* **236**, 239-245.

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