MODULATION OF KAINATE - INDUCED EXCITOTOXICITY IN RATS

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ABBREVIATIONS

AA	Arachidonic acid
ABC	Avidin/biotinylated enzyme complex
AEC	Aminoethyl carbazole
AMP	Adenosine monophosphate
AMPA	α-Amino-3-hydroxyl-5-methyl-isoxazole-4-propionic acid
4-AP	4-Aminopyridine
AP-5	DL-2-Amino-5-phosphonovalerate
APEC	2-[(2-Aminoethylamino)-carbonylethylphenylethylamino]-5'-*N*-ethylcarboxoamidoadenosine
APP	β-Amyloid precursor protein
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CA	Cornu ammonis
CaM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CGS 21680	2-p-(2-Carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamidoadenosine hydrochloride
CNS	Central nervous system
COX	Cyclooxygenase
CPPene	(E)-4-(3-Phosponoprop-2-enyl)-piperazine-2-carboxylic acid
CPX	8-Cyclopentyl-1,3-dipropylxanthine
DAB	Dimethylaminobenzidine
DPMA	N^6-[2-(3,5-Dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine
EAA	Excitatory amino acid
EDRF	Endothelial derived releasing factor
FMLP	*N*-Formyl-methionyl-leucyl-phenylalanine
GABA	γ-Aminobutyric acid
GYKI 52466	L-(Amino)phenyl-4-methyl-7,8-methylendioxy-5H-2,3benzodiazepine HCl
H2O2	Hydrogen peroxidase
HPLC	High pressure liquid chromatography
IEG	Immediate early gene
I.h.	Intrahippocampal
I.p.	Intraperitoneal
KA	Kainic acid
LDH	Lactate dehydrogenase
LT	Leukotriene
MAP	Microtubule-associated protein
MCAO	Middle cerebral artery occlusion
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]-cycloheptene-5,10-imine maleate
mtDNA	Mitochondrial DNA
L-NA	N^G-Nitro-L-arginine
NAD	Nicotidamide adenine dinucleotide
NADPH	Nicotidamide adenine dinucleotide phosphate
L-NAME	N^G-Nitro-L-arginine methyl ester
NBMPR	Nitrobenzyl-mercatopurine riboside
NBQX	2,3-Dihydro-6-nitro-7-sulphamoyl-benzo(f)quinoxaline
NECA	5'-*N*-ethyl-corboxamindoadenosine
NGF	Nerve growth factor
7-NI	7-Nitroindazole

NMDA	*N*-Methyl-D-aspartate
L-NMMA	N^G-Monomethyl-L-arginine
NO	Nitric oxide
NOS	Nitric oxide synthase
NOS-I	Nitric oxide synthase inhibitor
NPY	Neuropeptide Y
NT	Neurotrophin
$\bullet O_2^-$	Superoxide radical
OH^-	Hydroxyl radical
$ONOO^-$	Peroxynitrite anion
PB	Phosphate buffer
PBS	Double strength phosphate buffer
PG	Prostaglandin
PK 11195	1-(2-Chlorophenyl-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline-carboxamide
R-PIA	R-Phenylisopropyladenosine
PLA2	Phospholipase A_2
PLC	Phospholipase C
8-PST	8-(p-Sulphophenyltheophylline)
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TdT	Terminal deoxynucleotidyl transferase
TGF	Transforming growth factor
TNF	Tumour necrosis factor
Trk	Tyrosine kinase
TTX	Tetrodotoxin
TUNEL	TdT-mediated dUTP-biotin nick end labelling
TX	Thromboxane
U50,488H	3,4-Dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl] benzeneacetamide
XO	Xanthine oxidase
ZM 241385	4-(2-[7-Amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol

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Jones P.A., Smith R.A. and Stone T.W. (1997) Protection against kainate induced excitotoxicity by adenosine A_{2A} receptor agonists and antagonists. In press.

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ABSTRACT

Kainic acid has been used widely as an effective model of excitotoxic neurodegeneration. Using both the intraperitoneal and intrahippocampal routes of injection, we have shown that kainate selectively damages discrete populations of cells in the hippocampus. Following intraperitoneal administration, the A_{2A} agonists 2-p-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamidoadenosine hydrochloride (CGS 21680) and N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine (DPMA), protected against kainate-induced cell death. Protection by CGS 21680 was slightly moderated by the A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPX), but substantially reduced by the addition of the peripherally acting adenosine antagonist 8-(p-sulphophenyltheophylline) (8-PST). The administration of a selective A_{2A} antagonist 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo {2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM 241385) also protected the hippocampus against kainate toxicity. This protection was reversed when ZM 241385 was co-administered with CPX.

After intrahippocampal injections of kainate, neither CGS 21680, R-phenylisopropyladenosine (R-PIA) nor adenosine were protective in the CA3a region of the hippocmpus. ZM 241385 decreased kainate-induced neuronal death, as did the addition of the NMDA receptor antagonist, (+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]-cycloheptene-5,10-imine maleate (MK-801).

Combined results from both intrahippocampal and intraperitoneal studies, suggested that protection by the A_{2A} agonist was mediated mostly from the periphery and not from receptors localised within the hippocampus. Evidence also suggested that stimulation of A_{2A} receptors resulted in an inhibition of the A_1 subtype. Importantly, this study questioned the previous assumption that adenosine, released endogenously during cerebral insults, mediates protection.

The nitric oxide synthesis inhibitors N^G-nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole (7-NI), also protected against intraperitoneally administered kainate. This suggested that inhibition of neuronally (and not endothelially) produced nitric oxide was beneficial.

Studies using the TdT-mediated dUTP-biotin nick end labelling (TUNEL) method for identifying apoptotic cells showed that following intraperitoneal kainate administration, apoptosis was localised to the CA1 region of the hippocampus. Expression of the brain-derived neurotrophic factor (BDNF) protein was investigated, and a possible link between the mechanism of cell death and the inflammatory response also examined.

INTRODUCTION

EXCITOTOXICITY AND ISCHAEMIA

Brain damage can be observed in patients suffering from head trauma, certain types of epilepsy, cerebral infarcts (such as stroke) and in infants as a consequence of a hypoxic birth. Due to its prevalence in society, it is crucial to understand further the basic mechanisms in order to design more effective therapies to eliminate or reduce the damage associated with these conditions. A confusingly large number of both *in vivo* and *in vitro* models for cerebral ischaemia and excitotoxicity have been devised over the years. The models include the direct injection in live animals of compounds designed to induce excitotoxicity and ischaemia, the occlusion of specific blood vessels using an array of mechanical and chemical procedures and a number of models utilising cultured cells.

While none of the models mentioned above, nor the different assortment of cerebral insults are identical, there is a large proportion of common ground. Excitotoxic and ischaemic insults follow a similar physiological pathway, but have different points of origin. For ischaemia, the event at the peak of the cascade is a decrease in cerebral blood pressure below a critical threshold (Siesjö, 1992a). The reduction in blood-borne adenosine triphosphate (ATP) results in the breakdown of energy-dependent ion transport across neuronal membranes within the compromised brain (Siesjö, 1992a). Due to the number and variety of ATP-dependent ion channels situated within the plasma membrane of neurones, any large fluctuation from the normal homeostatic ATP levels would result in alterations in ion flow and subsequent

electrochemical gradients. The loss of K^+ from the cell and the Ca^{2+} influx (Nicholson *et. al.*, 1977), coupled with distorted Na^+ and Cl^- homeostasis (Hossmann *et. al.*, 1977; Hansen, 1985; Hansen & Zeuthen, 1981), leads to the generalised collapse of the cellular membrane, tissue acidosis (Nördstrom *et. al.*, 1978; Siesjö, 1985; Charlton *et. al.*, 1993) and neuronal depolarisation. The influx of Na^+ and Cl^- induces the cell to take up water via osmosis (Nicholson *et. al.*, 1977), causing cell swelling and osmolytic damage.

In ischaemia, the depolarisation of the presynaptic cell following calcium influx leads to the release of large amounts of the excitatory amino acid (EAA) transmitters glutamate and aspartate (Benveniste *et. al.*, 1984), via the reversal of the sodium-dependent transport system (Roettger & Lipton, 1996). In epilepsy this release of EAA transmitters is triggered by a spontaneous, not insult induced, depolarisation of neurones. Once released into the synaptic cleft, glutamate either binds to and activates specific receptors, or is rapidly removed by neuronal and glial uptake systems. Both the ionotropic kainic acid and α-amino-3-hydroxyl-5-methyl-isoxazole-4-propionic acid (AMPA) subtypes of glutamate receptors are initially activated by glutamate, increasing the influx of sodium (and to a lesser degree calcium) into the cell. This postsynaptic membrane depolarisation alters the direction of ionic flow, allowing the magnesium block of the *N*-methyl-D-aspartate (NMDA) type of glutamate receptor to be reversed (Mayer *et. al.*, 1984; Nowak *et. al.*, 1984). Subsequent NMDA receptor activation results in further calcium entry into the cell which, combined with calcium release from intracellular stores and disrupted extrusion, leads to cell death (Choi, 1988a; Choi, 1988b; Siesjö, 1988).

CALCIUM-ASSOCIATED CELL DEATH

Introduction

Calcium-associated delayed cell death occurs through a number of mechanisms and is most likely a result of a combination of factors rather than a single event. Protease activation by calcium may be of significant importance as actions of the proteases include severing the anchoring connection between the plasma membrane and the cytoskeleton as well as breaking down components of the cytoskeleton itself (Choi, 1988a; Cafè *et. al.*, 1993). This would further disrupt the integrity of the membrane and ion flow across it, hastening cell death. Inhibition of the calcium-activated protease calpain protects against vascular occlusion-associated brain damage (Bartus *et. al.*, 1994).

Energy failure, the rise in free intracellular calcium coupled with receptor stimulation, results in increased activity of phospholipase A_2 (Kontos *et. al.*, 1980), phospholipase C (Lynch & Bliss, 1986) and protein kinase (Conor *et. al.*, 1988). Phospholipase A_2 (PLA_2) hydrolyses phospholipids to lysophospholipids and free fatty acids, including arachidonic acid (AA), while phospholipase C (PLC) initiates the inositol pathway (and further release of calcium from intracellular stores) and also leads to the production of AA and other free fatty acids (Bruno *et. al.*, 1993).

Arachidonic Acid

While AA mediates actions of its own (e.g. leakage of molecules across the blood brain barrier; Unterberg *et. al.*, 1987), its metabolism to prostaglandins (PGs),

leukotrienes (LTs) and thromboxanes (TXs), leads to the formation of highly reactive free radicals. However, when the production of free radicals from this pathway was prevented using aspirin (which inhibits cyclooxygenase, preventing PG and LT formation) or BW 775C (a lipoxygenase inhibitor which blocks LT production), no significant protection was observed in high density cortical neurone cultures from kainate induced cytotoxicity (Cheng & Sun, 1994). This does not rule out the importance of the AA pathway in excitotoxicity. The enzyme prostacyclin synthase is inhibited by free radicals *in vivo*, which in turn results in an overactivation of the alternate enzyme in the AA cascade, thromboxane synthase (Siesjö, 1992b). This could exacerbate brain damage through TX-mediated effects on vasoconstriction and platelet aggregation. This effect on blood vessels would not occur within *in vitro* systems and may explain why no effect was noted with aspirin in culture (Cheng & Sun, 1994). In agreement with this suggestion of a TX-mediated increase in neuronal death, cyclooxygenase inhibitors have been shown to decrease damage in the hippocampus of gerbils *in vivo* (Nakagomi *et. al.*, 1989).

O'Regan *et. al.* (1995), suggested that the cleavage of membrane phospholipids by PLA_2, to form arachidonic acid, could alter membrane integrity allowing for the diffusion of charged molecules (including calcium and the excitotoxic amino acids) along their respective concentration gradients, increasing associated damage. Aside from the free radical-related detrimental effects another action of AA is the inhibition of glutamate uptake by neurones (Yu *et. al.*, 1987). Due to most of the uptake of glutamate being the responsibility of glial cells, this inhibition of the neuronal uptake system may not represent a major route of toxicity. AA also inhibits the production

of ATP from the mitochondrial respiratory chain (Takeuchi *et. al.*, 1991), further exacerbating cellular stress.

Alteration in Mitochondrial Function

The mitochondrial respiratory complex, essential for ATP production by oxidative phosphorylation, consists of proteins transcribed from both nuclear and mitochondrial DNA (mtDNA). The ability of the mitochondria to take up calcium renders the structure vulnerable during occasions of high intracellular calcium concentrations (e.g. during excitotoxicity or ischaemia). Alterations in mitochondrial membrane potential and subsequent disruption to the intramitochondrial electron chain by increased levels of calcium, results in the formation and release of free radicals (Piantadosi *et. al.*, 1996; Park *et. al.*, 1996). These radicals may themselves cause point mutations within the mitochondrial DNA, impairing respiration further and increasing the formation of reactive oxygen species (Attardi, 1981). This process is made more likely by mitochondrial susceptibility to DNA damage coupled with limited repair mechanisms (Brandy & Davidson, 1990). In keeping with this theory, the CA1 region of the hippocampus (an area known to be sensitive to glutamate toxicity), shows a selective decrease in mtDNA expression during certain experimental ischaemia models. Levels of mRNA and activity of cyclooxygenase-I (COX-I; one of the mitochondrial DNA encoded protein subunits forming part of the electron transport system) and motor proteins such as microtubule-associated protein 1c (MAP1c) and kinesin (responsible for converting energy from ATP hydrolysis to mechanical work and moving organelles such as mitochondria along microtubules),

are all shown to be sensitive to ischaemia (Abe *et. al.*, 1993). Indeed, it has been shown that during glutamate toxicity, the ability to restore mitochondrial membrane potential is crucial in determining the final outcome of a cell (necrosis or apoptosis - see later; Ankarcrona, 1995). The time for protein synthesis to show disruptive effects (provided the initial calcium overload does not kill the cell) may, in part, explain the phenomenon of delayed cell death. Another factor contributing to the susceptibility of mitochondria to cerebral insults may be the direct inhibitory action of arachidonic acid on ATP production mentioned earlier (Takeuchi *et. al.*, 1991).

FREE RADICALS AND MECHANISMS OF TOXICITY

Origins of Free Radical Molecules

The case for the involvement of free radicals in both excitotoxic and ischaemic neuronal damage has increased in recent years (Kogure *et. al.*, 1985; Bondy & Lee, 1993; Coyle & Puttfarken, 1993). Free radicals are molecules or atoms containing a single unpaired electron. This electron generally confers an extremely high chemical reactivity onto the molecule, leading to very rapid reactions with neighbouring substances and therefore a short half life. Although free radicals are produced naturally within cells by a number of different mechanisms, a large increase in reactive oxygen species has been noted in the brain shortly after cerebral insult (Bondy & Lee, 1993; Keidrowski *et. al.*, 1992; Sun *et. al.*, 1992). These free radicals most probably arise during cerebral insults from a multitude of sources. Arachidonic acid liberated by calcium-activated PLA_2 leads to the production of lipid hydroperoxidases and oxygen radicals (although as stated above, this pathway may

be only of limited importance). Another possible supply of radicals is xanthine oxidase (XO). Under normal conditions xanthines are oxidised by a dehydrogenase enzyme that transfers electrons to the nicotinamide adenine dinucleotide cation (NAD^+). Tissue disruption and calcium influx can convert xanthine dehydrogenase into the oxidase form. This uses oxygen as an electron acceptor, liberating superoxide radicals upon both the conversion of hypoxanthine to xanthine and xanthine to uric acid (Halliwell & Gutteridge, 1989; Coyle & Puttfarcken, 1993, Atlante *et. al.*, 1997). Superoxide radicals could also be formed by the co-oxidation of xanthine oxidase with arachidonic acid (Fridovich & Porter, 1981). The role of xanthine oxidase is once again controversial with some reports showing protective effects of calpain inhibitor I (inhibits calpain-induced xanthine oxidase production) and allopurinol (Cheng & Sun, 1994), while Betz *et. al.* (1991), showed that a protective effect was only observed at a dose where allopurinol was acting as a free radical scavenger.

As stated above, disruption to mitochondria may also result in the release of a large concentration of free radicals.

Nitric oxide (NO), which is released naturally under normal conditions and in higher concentrations during ischaemia, reacts rapidly with superoxide radicals ($\bullet O_2^-$) to form the peroxynitrite anion ($ONOO^-$) which decomposes to the hydroxyl radical ($\bullet OH$) (Beckman *et. al.*, 1990). This pathway may be a major source of damaging free radicals produced during excitotoxicity and ischaemia as a number of studies have shown that prevention of NO formation is protective in experimental models (see page 16).

Neither the superoxide radical nor hydrogen peroxidase (H_2O_2) are reactive enough to generate any significant degree of damage (and certainly not that amount associated with ischaemia), but can by way of the Haber-Weiss and Fenton reactions be converted to the highly reactive •OH radical (see reactions below).

$$2{\bullet}O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

$$\bullet O_2^- + M^{n+} \longrightarrow M^{(n-1)} + O_2$$

$$\bullet O_2^- + H_2O_2 \longrightarrow O_2 + \bullet OH + OH^-$$

$$H_2O_2 + M^{(n-1)+} \longrightarrow M^{n+} + \bullet OH + OH^-$$

Where M is usually the transition metal iron or copper.

The resultant hydroxyl radical formed by the above reactions results in oxidative stress, which is a shift in the pro-oxidant/anti-oxidant balance towards the pro-oxidant state. This can result in severe metabolic dysfunction including peroxidation of lipid membranes, depletion in nicotidamide nucleotides, rises in intracellular calcium, cytoskeleton disruption and DNA strand breakage (Halliwell & Chirico, 1993).

Free Radical-Associated Damage

Lipid peroxidation

Biological material contains a wide variety of unsaturated lipid material, particularly in the form of membranes. The unpaired electron of the free radicals reacts with and removes a hydrogen atom from an unsaturated carbon within these unsaturated fatty

acid chains (Gutteridge & Halliwell, 1990). The removal of hydrogen leaves the resultant carbon chain as a free radical able to bind molecular oxygen to form a lipid peroxide radical. This peroxy radical will then form a hydroperoxide by abstracting a hydrogen from another unsaturated fatty acid, continuing the chain reaction of events (Gutteridge & Halliwell, 1990).

This process of lipid peroxidation, which has been shown to occur in excitotoxicity (Ueda *et. al.*, 1997) and ischaemia (Hall *et. al.*, 1993), can lead to extensive damage to the finely organised structure of biological membranes that are rich in unsaturated carbon bonds (e.g. the cellular and the mitochondrial membrane; Watson, 1993). This in turn can cause a loss in the specialised function of the membrane.

Destruction of thiols

Thiol groups are found throughout the cell including within enzymes responsible for removing free radicals (free radical scavengers). Many important cellular proteins and enzymes (e.g. the structural protein actin or ATPase enzymes involved in the transport of calcium in mitochondria) also depend on thiol groups for functional activity. The oxidation of these thiol enzymes by free radicals can lead to drastic changes in their activity, resulting in effects such as alteration of cellular membrane structure or perturbation of cellular calcium homeostasis (Comporti *et. al.*, 1991). It is probable that this modification of a large number of diverse molecules within the cell may be the initial event that ultimately leads to free radical-associated cell necrosis.

Relevance of Free Radicals in Cerebral Insults

Coupled with the above evidence that excitotoxicity and ischaemia result in the production of large amounts of reactive molecules, the brain itself appears to be at particular risk from free radical damage. Peroxidizable polyunsaturated fatty acids are a major constituent of brain cell membranes. In addition iron, which promotes cytotoxic radical formation, is found in relatively high concentrations in certain brain regions (Floyd & Carney, 1992), although mostly in bound form (Cafè *et. al.*, 1993). Systemic cells are subjected constantly to free radicals from both extracellular sources as well as from intracellular metabolism, requiring the evolution of numerous defence systems (Coyle & Puttfarcken, 1993). These defences include antioxidant enzymes (such as superoxide dismutase (SOD) and glutathione peroxidase), low molecular mass antioxidants (e.g. α-tocopherol and ascorbic acid) and proteins which bind transition metals in a form that disables them from partaking in free radical reactions (Coyle & Puttfarcken, 1993). Free radical toxicity to the cell only occurs when these mechanisms are overloaded. In the brain, levels of these compounds are relatively low and therefore easily saturated. This deficiency in protective mechanisms and high relative concentration of iron, may make brain cells much more susceptible to damage by free radicals.

Free radicals appear to play a significant part in excitotoxic/ischaemic brain damage, as shown by the large number of studies using free radical scavengers which demonstrate at least a partial protective effect (e.g. Bruno *et. al.* 1994; MacGregor *et. al.*, 1996b; O'Neill *et. al.*, 1994; Saija *et. al.*, 1994; Truelove *et. al.*, 1994; Zhao *et. al.*, 1994). Their exact role and importance (whether free radical formation is the

determinant in dictating the fate of the cell, or just a component of a complex which overloads the cell on a number of different fronts) has still to be resolved.

REACTIVE GLIOSIS

Gliosis occurs under conditions of insult within the central nervous system (CNS), where cells of the immune system (e.g. microglia), as well as astrocytes and fibroblasts, undergo morphological changes and produce molecules such as laminin (Liesi *et. al.*, 1983), microtubule associated protein 2 (MAP2; Geisert *et. al.*, 1990), basic fibroblast growth factor (bFGF; Frautschy *et. al.*, 1991), transforming growth factor-β1 (TGF-β1; Morgan *et. al.*, 1993) and β-amyloid precursor protein (APP; Kawarabayashi *et. al.*, 1991). The gliotic response varies depending on the insult. For example isomorphic gliosis, caused by neurotoxic agents where no damage occurs to the blood brain barrier, is considered to be distinct from the anisomorphic gliotic response where there is disruption. After intraventricular injections of AMPA or kainate a gliotic response was observed in selective regions of the hippocampus shortly after the excitotoxic insult (Araujo & Wandosell, 1996).

Due to the rapid inflammatory response to certain cerebral insults (e.g. Morioka *et. al.*, 1991), it has been suggested that while degeneration of neurones may not be the signal which initially triggers the gliotic response, although neurodegeneration may be involved in shaping the specific response by release of molecules (e.g. products of cellular breakdown) after glial activation (Jorgensen *et. al.*, 1993). In excitotoxicity/ ischaemia the highest concentration of reactive astrocytes was observed in the damage-sensitive CA1 and CA3-4 regions of the hippocampus (Araujo & Wandosell,

1996), while microglia were more diffuse and generalised (Marty *et. al.*, 1991). In the first two weeks after central kainate injection, microglia and macrophages appear predominant but, with their decline in number, astrocytes, which remain for a period of months, become the dominant cell type (Dusart *et. al.*, 1991; Marty *et. al.*, 1991).

The inflammatory response consists of a number of different cell types. Neutrophils, which undergo a complex interaction with the endothelial layer of blood vessels, migrate from the circulation. Macrophage, monocytes and mast cells also form part of the interconnecting cascade. Mast cells, via the release of histamine, encourage the binding of neutrophils to the endothelium (by releasing P-selectin). Activated macrophages and monocytes secrete cytokines which in turn induce the further release of P-selectin, as well as priming neutrophils (see Cronstein, 1994).

As well as this release of cytokines, the inflammatory response produces toxic molecules to assimilate cells. While these compounds include enzymes (such as collagenase), it is the generation of toxic oxygen metabolites (e.g. superoxide radicals and H_2O_2) that may be of more importance in increasing local neuronal toxicity. While these latter molecules are produced within vacuoles, there is a degree of leakage into the extracellular space.

Due to the variety of molecules produced by the inflammatory response the benefits versus detriment of reactive gliosis have yet to be determined. The release of bFGF and TGF-β by activated microglia is believed to be protective and important in promoting tissue repair (Rothwell & Relton, 1993; Mattson & Rychlik, 1990). Overproduction of cytokines (e.g. interleukine) may be more harmful (Relton & Rothwell, 1992), by increasing the production and secretion of molecules such as

arachidonic acid and nitric oxide from microglia (Hartung *et. al.*, 1992). Astroglial transport systems are the main source of glutamate removal from the synaptic cleft (Hertz & Schousboe, 1980) and inhibition of these transporters results in pathology similar to that of excitotoxicity (Rothstein *et. al.*, 1996). Conversely, the removal by astrocytes of γ-aminobutyric acid (GABA) from the synaptic cleft (Yu & Hertz, 1982) could be detrimental by increasing the possibility of epileptic seizures (Ravindran *et. al.*, 1994). Glutamate depolarises glial cells directly, by activation of non-NMDA receptors (Muller *et. al.*, 1992), initiating a calcium wave which passes along an astrocytic network, implicating a more complicated role for astrocytes within the neuronal network (e.g. modulation of neuronal activity; Smith, 1992). More importantly the influx of calcium can itself result in the release of glutamate from astrocytes (Parpura *et. al.*, 1994). The complete role of astrocytes within neurological pathophysiology has yet to be fully elucidated and may be much more significant during cerebral insults than was previously assumed.

APOPTOSIS

There is accumulating evidence that while necrosis plays a large and important part of excitotoxic cell death, apoptosis may also account for a proportion of neuronal death (Choi 1996; Chopp, 1996; Weiss *et. al.*, 1996; Honkaniemi *et. al.*, 1996). Apoptosis is an active process, characterised by cell shrinkage, organelle relocalisation and compaction, chromatin condensation and formation of apoptotic bodies (Kerr, 1971; Majno & Joris, 1995). The co-existence of necrosis with apoptosis (Choi, 1996) can make the determination of the predominant mechanism of

cell death difficult. Further complication arises from the observation that even during apoptosis, secondary necrosis can occur. This would most likely only arise on occasions where a large population of cells undergoes apoptosis, overloading the phagocytic removal system. DNA fragmentation, a diagnostic feature of apoptosis (although controversially not always apparent; Ankarcrona *et. al.*, 1995), has been observed in a number of ischaemic models (Filipkowski *et. al.*, 1994; Hill *et. al.*, 1995; Li *et. al.*, 1995; Pollard *et. al.*, 1994). Glutamate or glutamate analogue exposure to neuronal culture systems has been shown to alter the expression of immediate-early genes such as *c-fos* (Kasof *et. al.*, 1995, Lerea *et. al.*, 1992), believed to be a reliable marker for apoptosis (Smeyne *et. al.*, 1993), and induces both rapid necrosis and delayed-onset apoptosis (Ankarcrona *et. al.*, 1995). Death by necrosis or apoptosis was correlated to mitochondrial function. A large number of cells died early after glutamate application, via necrosis. In contrast, Ankarcrona *et. al.* (1995), showed that cells which underwent and subsequently recovered from the initial mitochondrial dysfunction died from an apoptotic mechanism.

NITRIC OXIDE

Nitric oxide is a simple gas that has recently been shown to have an important role within human physiology and pharmacology in such diverse functions as vasodilatation (as the endothelial derived releasing factor or EDRF, NO increases arterial diameter; (Moncada *et. al.*, 1991a), platelet aggregation, neurotransmission and cytotoxicity (Dawson, 1994; Dawson & Snyder, 1994)). These effects are either mediated directly by NO itself, or indirectly via soluble guanylate cyclase activation

leading to an elevation of cyclic guanosine monophosphate (cGMP) levels (Moncada *et. al.*, 1991a).

The conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) enzymes results in the generation of NO. The conversion is an oxidative-reductive process, requiring nicotidamide adenine dinucleotide phosphate (NADPH), oxygen, calcium and calmodulin (Bredt & Snyder, 1994).

Three discrete forms of NOS have been identified: Type I, II, III (Xie *et. al.*, 1992; Bredt *et. al.*, 1991). Type I (neuronal or nNOS) and type III (epithelial or eNOS) are constitutive enzymes and calcium-calmodulin dependent. Inducible NOS, type II, does not require calcium after it's initial expression. Although hippocampal cells show limited immunostaining to nNOS, there is a larger amount of reactivity to eNOS, especially within the CA1 neurones (Dawson & Dawson, 1995). .

Nitric oxide has been targeted as a possible mediator of glutamate-induced neurotoxicity due to both an increase in NO levels after ischaemia (Balcioglu & Mather, 1993; Mülsch *et. al.*, 1994) and excitotoxicity (Faraci *et. al.*, 1994), and an apparent upregulation of nNOS and nNOS mRNA during ischaemia (Zhang *et. al.*, 1994b). The excitotoxic/ischaemic-associated calcium influx activates both constitutive forms of NOS, elevating the levels of NO.

Nitric oxide has a number of actions relevant to excitotoxic/ischaemic insults which may be beneficial or detrimental to the neuronal population. Nitric oxide can decrease cellular calcium influx by inhibiting the opening of the NMDA-associated calcium channel (Manzoni *et. al.*, 1992), or increase the availability of EAAs by stimulating the release of both glutamate and aspartate and inhibiting glutamate

uptake (Lawrence & Jarrett, 1993; Pogun & Kahur, 1993). Nitric oxide has also been shown to facilitate a feedback loop by interacting with the haem moiety of NOS (Moncada *et. al.*, 1991a). Apart from toxic free radical actions of NO mentioned earlier, Dawson & Dawson (1995) speculated that NO may also inhibit the ribonucleotide reductase enzyme and thereby DNA synthesis, due to it's ability to bind to certain non-haem iron complexes. Nitric oxide has further effects on iron metabolism release (e.g. activation of iron regulatory factor; Drapier *et. al.*, 1994), which may be significant in the formation of free radicals via the Fenton reaction.

Nitric oxide activated cGMP exerts a number of effects on calcium levels, by acting on a variety of receptors. cGMP itself inhibits L-type calcium channels, via cGMP-dependent protein kinases or by cGMP stimulated cyclic adenosine monophosphate-(cAMP-) phosphodiesterases. Alterations in Ca^{2+}/Mg^{2+}-dependent ATPase activity by cGMP also affect calcium flux across the cellular membrane and from intracellular stores (see Milbourne & Bygrave, 1995).

A further possible indirect route by which NO may cause damage is by the depletion of the toxic molecule scavenger glutathione, through the formation of intracellular S-nitrosoglutathione (Clancy *et. al.*, 1994), although Wink *et al.* (1993) reported that NO itself is a free radical scavenger. NO has been shown to result in increased release of dopamine within the global temporary cerebral ischaemia model (Kahn *et. al.*, 1995). Dopamine itself has been shown to be toxic to neuronal populations (Filloux & Townsend, 1993).

The evidence for the role of NO in the glutamate neurotoxicity cascade is strong since it has been shown that NOS inhibitors such as N^G-nitro-L-arginine (L-NA), N^G-

monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME) inhibited and sodium nitroprusside (SNP-a nitric oxide donor) mimicked the damage in cortical hippocampal or striatal cell cultures (Corasaniti *et. al.*, 1992; Tryiletti, 1992; Dawson *et. al.*, 1993; Dawson & Snyder, 1994; Dalkara *et. al.*, 1994; Kolleger *et. al.*, 1993). SNP has also been shown to posses anti-convulsant effects mediated through NO, while L-NA prolonged seizure duration (Dawson *et. al.*, 1991; Marangoz *et. al.*, 1994). Many other experiments that reduce the action of NO by various methods (e.g. by preventing its formation, or inhibiting its activity) have also resulted in attenuation of NMDA neurotoxicity (see Dawson, 1994). However, in contrast, other investigators have found no benefit with the addition of inhibitors (Pauwels & Leysen, 1992), and no detrimental effects of NO donors (Garthwaite & Garthwaite, 1994). Some have found opposite effects (Zhang & Iadecola, 1993; Shapira *et. al.*, 1994), with NO inhibitors increasing neuronal damage. NO itself has been shown to be a pro- or anti-convulsant dependent on the dose of the NOS inhibitor.

The above evidence lead to the speculation that low doses of NOS inhibitor are neuroprotective, whereas high doses are ineffective or detrimental, implying that partial inhibition of NOS is sufficient to achieve the optimal effect. The higher dose could adversely affect the feed-back loop or prevent the beneficial vascular effect of nitric oxide on increasing cerebral blood flow. This leads to further speculation that NO production in the brain may be a defence response to decrease EAA toxicity in ischaemia.

The discrepancy in results could also be explained by the existence of two forms of NO (Lipton *et. al.*, 1993; Dawson, 1994). The nitrosonium ion (NO^+) is

neuroprotective by nitrosylating the NMDA receptor modulatory site, while the free radical form ($NO^{\bullet}$) interacts with superoxide forming the peroxynitrite ion ($ONOO^{-}$), which is neurotoxic (Lipton *et. al.*, 1993; Dawson & Dawson, 1995). Differences in results may therefore arise from altered respective concentrations of each form (e.g. this may occur *in vitro* following the addition of the reducing agent cysteine which promotes $NO^{\bullet}$ formation; Dawson, 1994).

7-nitroindazole (7-NI), which is capable of penetrating the blood brain barrier, has been proposed as a potent and competitive selective inhibitor of the neuronal NOS isoform *in vivo* (Babbedge *et. al.*, 1993). Dalkara *et. al.* (1994) showed a decrease in infarct size in focal cerebral ischaemia after systemic injection of 7-NI. This, together with evidence that 7-NI attenuated seizure activity and decreased NO formation *in vivo* after kainate injection (Mülsch *et. al.*, 1994), indicates a possible role for neuronal NOS in ischaemic injury which is masked when using the non-selective inhibitors. 7-NI could, therefore, inhibit the toxic release of NO by the neuronal isoform of NOS, while still preserving the beneficial vasodilatory effects of the endothelial released NO, and has indeed been shown to be protective in global cerebral ischaemia (O'Neill *et. al.*, 1996).

THE HIPPOCAMPUS

The frequent use of the hippocampus as an area of observation is due not only to the specific damage associated with a number of conditions (e.g. excitotoxicity, ischaemia, epilepsy and schizophrenia), its involvement in normal brain function (e.g. long-term potentiation; Bliss & Collingridge, 1993), but also as the

hippocampus is regarded as a source of a relatively homogeneous neuronal population (pyramidal neurones are prevalent at 80-95 %) which is representative of the CNS in general (Banker and Goslin, 1991). The pyramidal cell layer also has the advantage of being a well defined structure that is clearly visible under a light microscope. The hippocampus is known to contain populations of all of the glutamate receptor types (Monaghan *et. al.*, 1983), as well as other groups of receptors thought to be important during cerebral insults, such as those of the adenosine subfamily.

The hippocampus is an arched structure of the brain which displays a large proportion of structural homology between rats, mice, gerbils and humans. The structure itself consists of the dentate gyrus, cornu ammonis and subiculum, and is divided into a number of layers. From the dorsal position the layers run in the order of the ependyma, alveus, stratum oriens, stratum pyramidale, stratum radiatum, stratum lacunosum-moleculare (Cajal, 1911). Three cell types constitute the majority of the hippocampus. The cornu ammonis (CA) contains bipolar pyramidal cells with 'bushy' dendrites protruding both top and bottom. The cells form a prominent, clearly visible layer of a few cells thick, which is sub-divided into five regions, the CA1, CA2, CA3a, CA3b and CA4. The densely packed unipolar granule cells of the dentate gyrus and interneurones make up the majority of the remaining cells (Isaacson, 1982).

Input to the hippocampus arrives through the perforant pathway innervating dentate granule cells from the entorhinal cortex. Mossy fibres from the dentate gyrus subsequently innervate CA3 pyramidal cells, which in turn innervate the ipsilateral

CA1 pryamidal cells via the Schaffer collaterals and the contralateral CA1 pyramidal cells via the commissural fibres (Isaacson, 1982).

KAINIC ACID AS A TOOL FOR THE STUDY OF ISCHAEMIA

Introduction

Kainic acid, originally isolated from a Japanese seaweed as an ascaricide, has been used extensively in neurobiological research for the past several years. Initially, the drug was found to excite neurones when applied iontophoretically to rat cortical neurones (Shinozaki & Konishi, 1970). Subsequently, it was reported that injections of kainate into the brain destroyed specific populations of neurones while sparing axons and synaptic terminals (Schwarcz *et. al.*, 1978; Schwob *et. al.*, 1980). Data have since shown that kainate preferentially activates seizures in the limbic system, particularly the hippocampus (Lothman & Collins, 1981), resulting in the efflux of excitatory amino acids into the extracellular space (Connick & Stone, 1986). Due to the specific pattern of neuronal damage (see below), kainate administration is now considered a good experimental model for temporal lobe epilepsy and global ischaemia (Heggli *et. al.*, 1981; Lothman & Collins, 1981; Sperk, 1994). More recently the kainate model has been suggested to show characteristics similar to schizophrenia (Bardgett *et. al.*, 1995).

Kainic Acid-Induced Cerebral Damage

The mechanism of kainate toxicity is similar to that of excitotoxic/ischaemic damage described above. Kainate has both a pre- and post-synaptic action. Post-synaptically kainate acts on both kainate and AMPA receptors, depolarising the cells, while pre-synaptically inducing the release of glutamate (Ferkany *et. al.*, 1982; Connick & Stone; 1986), resulting in the activation of the remaining glutamate receptors. The following excitotoxicity is as previously described. Kainate has also been shown to dilate cerebral arterioles via an increased production of nitric oxide (Faraci *et. al.*, 1994).

Kainate produces a slightly different distribution of hippocampal damage depending on its route of introduction. Following intracerebroventricular injection of kainate, preferential damage of pyramidal neurones occurs in the CA3 region of the hippocampus (Nadler *et. al.*, 1978; Magloczky & Freund, 1993). The CA1 and CA4 regions also displayed some damage, while the CA2 area showed little evidence of kainate toxicity. Bardgett *et. al.* (1995), showed that neuronal damage following bilateral intracerebroventricular administration of kainate was dependant on dose. Injection of 1.5 nmol of kainate damaged the CA3 region to nearly 100 %, while leaving the CA1 region untouched. Higher concentrations of 4.5 and 6.6 nmol produced 50 % and 75 % damage within the CA1, and showed damage progressing from the CA3 to the CA4.

Systemic injections of kainic acid produce more widespread damage throughout the hippocampus, including the CA1 and CA3-4 regions (Schwob *et. al.*, 1980; Ben-Ari *et. al.*, 1981) This pyramidal cell death is believed to be a direct result of the binding

of kainate to the high-affinity kainate receptors (the highest percentage of which are found in the CA3 region). Damage to the CA1 region was delayed and occurred up to four days after the initial injection (Liu *et. al.*, 1996). Distal damage to the amygdala, entorhinal cortex and medial thalamic nuclei is more likely to be caused by hyperactivity in afferent excitatory pathways (Schwob *et. al.*, 1980), as might be some of the damage in the CA1 region of the hippocampus which is innervated by the Schaffer collaterals from the CA3 region (Magloczky & Freund, 1993). Differences between routes of administration are even more apparent in the immature rabbit where intraperitoneal injections of kainate damaged the hippocampus with a pattern similar to that described above, but intraventricular administration was found not to be toxic (although progressive susceptibility was observed in animals over two months old; Franck & Schwartzkronin, 1984).

Injections of kainate usually cause a sequence of stereotypical events of staring spells, wet dog shakes, mild and severe limbic convulsions, rearing, falling and salivation (Lothman & Collins, 1981). Some reports suggest that damage to the hippocampus is dependant on seizures and that preventing the seizures themselves would dramatically decrease the extent of neuronal death (Ferkany *et. al.*, 1982; Lehmann *et. al.*, 1983). This theory is disputed by Franck and Schwartzkroin (1984), who showed that the immature rabbit hippocampus was relatively insensitive to intraventricular injections of kainate, even though seizures were observed. Evidence with anticonvulsant compounds suggests that seizures are more likely to be a consequence of, rather than an initiator of, excitotoxicity or ischaemia. The anticonvulsant chlormethiazole was protective at a dose below that required to prevent global ischaemically-induced epileptic activity (Cross *et. al.*, 1995). In

contrast, clonazepam abolished seizures without a respective decrease in hippocampal damage (MacGregor *et. al.*, 1996b).

As well as causing neuronal cell death by necrosis, kainic acid has also been found to lead to distinctive morphological and biochemical features associated with apoptosis (Filpikowski *et. al.*, 1994; Pollard *et. al.*, 1994). As mentioned earlier, DNA fragmentation, a feature of apoptosis, has been noted in rats at 18 and 72 hour following kainate injection in the hippocampus, entorhinal and sensory cortex (Filpkowski *et. al.*, 1994). Pollard *et al.* (1994), examining apoptosis in the amygdala and hippocampus using morphological and silver staining techniques, showed that within the hippocampus, pyramidal cells of the CA3a region were preferentially stained, followed by neurones of the CA3c and CA4 which only showed scattered staining. Kainate has also been shown to induce the expression of immediate-early genes (Kasof *et. al.*, 1995a, Willoughby *et. al.*, 1997), believed by many to be necessary for apoptosis (Smeyne *et. al.*, 1993), as well as the expression of target 'effector genes' (Konopka *et. al.*, 1995). Willoughby *et. al.* (1997) showed two phases of *c-fos* induction, an initial induction within the hippocampus followed by a more widespread general event. The mechanism by which kainate induces this expression is not fully determined. Bading *et. al.* (1995), utilising hippocampal neuronal cultures suggested *c-fos* expression was a result of indirect activation of NMDA receptors, whereas evidence with rat cortical cultures suggests L-type voltage-sensitive calcium channel involvement (Murphy *et. al.*, 1991).

Kainate Receptor Localisation

Ligand binding and recombinant experiments show that five genes encode the kainate (high affinity) receptor subunits (GluR5/-6/-7, KA1/-2), while GluR1/-2/-3/-4 encode those of the AMPA receptor (low affinity kainate receptor). The two receptors can be separated by their order of both binding affinities and agonist potency. For AMPA receptors, AMPA binds with much higher affinity than kainate, but kainate does show a degree of receptor stimulation (albeit much lower than that of AMPA itself). AMPA does not display a high degree of binding or agonist potency against the high affinity kainate receptor (see Bettler & Mulle, 1995). Kainate receptors show a rapid desensitisation in the presence of kainate or glutamate, whereas AMPA subunits display desensitisation only in the presence of glutamate or AMPA itself (Fedele & Raiteri, 1996). mRNA for the KA-1 high affinity kainate receptor subunit is localised to the CA3 region of the hippocampus, dentate gyrus (high density), inner cortical layers, cerebellar Purkinje cells and white matter (low density), while the KA-2 subunit mRNA is more diverse. GluR-5 mRNA is found in cingulate and pyriform cortex, subiculum, septal nuclei and Purkinje cells; GluR-6 in cerebellar granule cells, caudate-putamen and hippocampus; GluR-7 in the inner cortical layers, cingulate cortex, subiculum, caudate-putamen, reticular thalamus and stellate/basket cells of the cerebellum (Wisden & Seeburg, 1993).

Autoradiographic studies have localised the kainate receptors to a number of regions in the rat brain including the neocortex, corpus striatum, the CA3 region of the hippocampus, the supragranular layer of the dentate fascia and the granule layer of

the cerebellum (Unnerstall & Wamsley, 1983; Lunn *et. al.*, 1996). Kainate binding sites have been localised to mossy fibre terminals and the soma of CA3 pyramidal cells of the hippocampus (Foster *et. al.*, 1981), suggesting that kainate stimulates the mossy fibre CA3 synapses both post- and pre-synaptically. A decrease in sensitivity of CA3 cells towards kainate-induced neuronal degeneration after mossy fibre lesions (Okazaki & Nadler, 1988) suggests that this presynaptic action, which results in the release of excitatory transmitters glutamate and aspatate from nerve terminals, is important in the excitotoxic effects of kainate. It remains unclear whether this release is mediated by kainate or AMPA receptors. AMPA receptor localisation almost overlaps that of kainate, although the highest concentration is in the dendritic fields of the CA1 (Olsen *et. al.*, 1987; Rainbow *et. al.*, 1984). NMDA receptors are more abundant in the hippocampus, neocortex, amygdala, septal nuclei and striatum (Monaghan *et. al.*, 1983).

ROLE OF ADENOSINE IN CEREBRAL INSULTS

During periods of excitotoxicity, ischaemia and hypoxia, the concentration of adenosine in the extracellular space increases dramatically from a basal level of 0.05-2 μM suggesting the possibility of adenosine as an endogenous protective agent during cerebral insults (Zetterström *et, al,*, 1982; Hagberg *et, al.*, 1987). The adenosine levels increase at a cerebral blood flow level (25 ml. 100 g^{-1}. min^{-1}) above that required for the rise in glutamate (20 ml. 100 g^{-1}. min^{-1}; Matsumoto *et. al.*, 1992) indicating a possible, but time limited protective action of adenosine against glutamate toxicity. This release is also observed within the kainate model of

excitotoxicity, where extracellular adenosine concentration follows a 'bell' shaped pattern, with the peak at 200 minutes after kainate perfusion through a microdialysis probe (Carswell *et. al.*, 1997). Similar release of adenosine has been noted with the introduction of NMDA (Chen *et. al.*, 1992), although the peak occurs earlier, 2 hours prior to that seen after kainate perfusion.

Adenosine receptors have been separated into three major subclasses, A_1, A_2 and A_3. All are linked to a G-protein coupled receptor, A_1 and A_3 receptors to a Gi protein inhibiting adenylate cyclase, while A_2 activates the enzyme via a Gs-protein (Marala & Mustafá, 1993). A_1 and A_3 receptors also activate phospholipase C. A_1 receptors reportedly stimulate phosphoinositide metabolism as well as affecting both potassium and calcium flux. Binding studies have further classified adenosine A_2 receptors into two distinct subtypes, A_{2A} and A_{2B} (Bruns *et. al.*, 1986). While the low affinity A_{2B} subtype is distributed throughout the brain, the high affinity A_{2A} receptors are more localised. Autoradiographic and *in situ* hybridisation studies identified A_{2A} receptors within the neostriatum and to a lesser extent the globus pallidus (Jarvis & Williams, 1989). More recent developments have characterised A_{2A} receptors within the hippocampus and cerebral cortex (Cunha *et. al.*, 1994), although these receptors appear to differ pharmacologically from the typical striatal population (Cunha *et. al.*, 1994). 2-p-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamidoadenosine hydrochloride (CGS 21680), which binds at namomolar concentrations to striatal A_{2A} receptors, displayed an affinity for the A_{2A} agonist at the hippocampal and cortex receptors that was a third of the striatal receptors, and a slightly lower maximal binding (Cunha *et. al.*, 1996).

Analogues of the natural compound adenosine are able to prevent neuronal damage due to kainate. Connick & Stone (1989) showed that direct administration of the stable adenosine analogue phenylisopropyladenosine (R-PIA) into the brain, could protect against the neurodegeneration produced by the NMDA-receptor agonist quinolinic acid. Even at low doses (10 $\mu g.\ kg^{-1}$) systemically injected R-PIA prevented hippocampal damage produced by kainate (Macgregor & Stone, 1993). R-PIA is an effective protectant when given up to 2 hours after kainic acid. Treatment with other A_1 adenosine agonists has been proven to protect cells in a number of different models (Arvin *et. al.*, 1989; Daval *et. al.*, 1989; Evans *et. al.*, 1987, Von Lubitz *et. al.*, 1988), while antagonism of A_1 receptors results in enhancement of damage (Phillis, 1995). In contrast antagonists of the A_2 adenosine receptor, which has opposing effects on the levels of cAMP to A_1 receptors, have been shown to protect the hippocampus (Phillis, 1995; Von Lubitz *et. al.*, 1995). Increasing the availability of adenosine with the addition of adenosine kinase inhibitors (Jiang *et. al.*, 1997a) or upregulation of A_1 receptors by caffeine (Rudolphi *et. al.*, 1989), also protects against cerebral insults.

There are several ways in which adenosine may be protective. Adenosine inhibits the release of many neurotransmitters, including glutamate (Poli *et. al.*, 1991). This effect is mediated by presynaptic A_1 receptors linked via G-proteins to both calcium and potassium ion channels. In the postsynaptic neurone, adenosine helps to maintain the intracellular calcium homeostasis, possibly by preventing the depolarisation associated with an influx of ions (Rudolphi *et. al.*, 1992). This would prevent the removal of the Mg^{2+} NMDA blockade and the reversal of the glutamate uptake systems in glial and neuronal cells. This stabilisation of the intracellular

environment is achieved by increasing potassium permeability, hyperpolarising the neurone (Rudolphi *et. al.*, 1992). In addition, there are a number of effects mediated by adenosine acting on A_2 receptors which may be either beneficial or detrimental.

In the striatum A_{2A} receptor stimulation increases the release of the excitotoxic amino acids aspartate and glutamate (O'Regan *et. al.*, 1992, Popoli *et. al.*, 1995) and acetylcholine (Cunha *et. al.*, 1995) but inhibits the release of GABA (Kirk & Richardson, 1994). A_{2A} antagonists, which inhibit the further release of glutamate, have repeatedly been shown to increase neuronal survival within ischaemic models (Phillis, 1995; Von Lubitz *et. al.*, 1995). Activation of the adenosine A_{2A} receptor also increases cerebral blood flow (Stella *et. al.*, 1996; Torregrossa *et. al.*, 1990) via endothelial-derived nitric oxide synthesis (Sobrevia *et. al.*, 1997), and inhibits platelet aggregation (Dionisotti *et. al.*, 1992), although the latter effect may not be via conventional cAMP and subsequent adenylate cyclase activation (Cristalli *et. al.*, 1994). Both effects increase blood and nutrient supply to any ischaemically compromised area of the brain. CGS 21680, an A_{2A} selective agonist (Jarvis *et. al.*, 1989), depresses cerebral glucose utilisation in a large number of brain regions, including the hippocampus (Nehlig *et. al.*, 1994), an effect which may be advantageous to neurones by decreasing the requirement for a depleted nutrient during ischaemia. 2-[(2-aminoethylamino)-carbonylethylphenylethylamino]-5'-*N*-ethylcarboxoamido adenosine (APEC), a centrally acting A_{2A} agonist, increases cerebral blood flow and with chronic exposure and increases neuronal preservation in the hippocampus during forebrain ischaemia (Von Lubitz *et. al.*, 1995). More recently, CGS 21680 has been shown to protect neurones against forebrain ischaemia in gerbils (Sheardown & Knutsen, 1996). A_2 receptors decrease superoxide anion

production in neutrophils (Cronstein *et. al.*, 1985), reducing the possibility of free radical-associated neuronal death which has been implicated in excitotoxic/ischaemic damage (Cheng & Sun, 1994; Kitawaga *et. al.*, 1990; MacGregor *et. al.*, 1996b; O'Neill *et. al.*, 1994). A_{2A} receptor stimulation, therefore, provides a mixture of possible beneficial and detrimental effects, presenting a more confusing picture than that of the A_1 receptor. The present study reports the effects in rats of the A_{2A} agonists CGS 21680 and N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine (DMPA) on excitotoxic neuronal damage induced by kainic acid, and the effects of antagonists selective for A_1 and A_2 receptors.

NEUROTROPHIC FACTORS

Neurotrophins, a family of structurally related polypeptides originally thought to be mainly influential for neuronal survival, differentiation and plasticity during embryonic and early postnatal development, have been shown to possess a significant role within the adult CNS (Hefti *et. al.*, 1993). The neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), NT-4/5 and NT-6 (found in non-mammals only), and which mediate their actions via structurally distinct tyrosine kinase receptors. NGF activates the TrkA receptor (Kaplan *et. al.*, 1991), BDNF and NT-4/5 the TrkB receptor (Klein *et. al.*, 1991; Klein *et. al.*, 1992) and NT-3 the TrkC receptor (Lamballe *et. al.*, 1991). As with all tyrosine kinase receptors their mode of action is a two step process of ligand binding followed by autophosphorylation of their ligand residues (Jing *et. al.*, 1992). These molecules subsequently bind a downstream constituent (e.g. PLCγ).

Within the CNS the TrkA receptor is localised mainly to cholinergic neurones of the basal forebrain and striatum, and less densely in the thalamus and brainstem (Vasquez & Ebendal, 1991; Merlio *et. al.*, 1992; Steininger *et. al.*, 1993; Venero & Hefti, 1993). TrkB is more widespread, but is found in high amounts in the cortical layers of the cerebellum, thalamus and hippocampus (Klein *et. al.*, 1990; Merlio *et. al.*, 1992) . The hippocampus itself contains high levels of BDNF and NGF mRNA, as well as NT-3 and NT-4/5 mRNA (Korsching *et. al.*, 1985, Hofer *et. al.*, 1990, Enfors *et. al.*, 1990; Timmusk *et. al.*, 1993). While protein levels for NGF display a degree of overlapping to areas stained for NGF mRNA, there were areas where protein was found without the presence of mRNA (Connor & Varon, 1992). This was suggested to be due to retrograde transport and accumulation of the protein. Similar accumulation was observed with BDNF protein (Yan *et. al.*, 1997), with discrete areas containing protein, but no mRNA (Yan *et. al.*, 1997; Conner *et. al.*, 1997).

Glutamate and other transmitters are known to influence neurotrophins. After intrahippocampal injection, kainate has been shown to induce BDNF mRNA, in both the ipsilateral and contralateral hippocampus, cortex, amygdala, claustrum and hypothalamus (Ballarín *et. al.*, 1991). This increase was due to both an increase in the number of neurones expressing BDNF and the amount being expressed, and remained high (up to 40-fold) for at least 7 hours. An increase in NGF was also observed, but it displayed shorter term effects with levels decreasing to basal by 7 hours. Evidence from cortical cultures suggests that while this effect is observed by the addition of glutamate analogues (kainate, AMPA, NMDA and quisqualate) it is

not seen with glutamate *per se* and may be regulated by metabotropic glutamate receptors (Bessho *et. al.*, 1993).

Kokaia *et. al.* (1996), has shown similar results after transient forebrain ischaemia. BDNF protein levels were highest in dentate gyrus and CA3 region of the hippocampus and lowest in the CA1 region and neocortex, while mRNA levels were similar (although less comparatively in the CA3 area compared with the dentate gyrus). Using the dentate gyrus-hilar lesion model, Lindvall *et. al.* (1994), showed an increase of NGF and BDNF, both peaking at 8 hours, but with BDNF at a much higher level. It was also suggested that this phenomenon was only observed in mild insults, with the suppression of transcription and/or translation of both BDNF and TrkB genes in severe insults. None of the other neurotrophic factors appear to be upregulated during excitotoxicity/ischaemia.

All the above studies focus on the short-term increases of neurotrophin mRNA or protein. When observed for a longer period a more complex tri-phasic pattern emerges, with a peak after a few hours (~125 % of pre-insult control levels) before a return to basal levels. A larger subsequent peak (~275 %) after seven days was followed by a lower sustained peak sixty days after the original injection of kainic acid (Suzuki *et. al.*, 1995). The levels of BDNF did not return to normal for at least six months.

These alterations in neurotrophin gene expression are probably triggered by calcium influx, and are probably another mechanism by which the brain protects itself against such cerebral insults. The protective ability of neurotrophic factors has been shown in countless studies (e.g. Pringle *et. al.*, 1996; Kubo *et. al.*, 1995, Nakao *et. al.*,

1995), although BDNF can increase glutamate toxicity in pre-treated cerebellar neurones (Fernández-Sánchez, 1995) or cortical cultures exposed to a free radical generator (Gwag *et. al.*, 1995; Koh *et. al.*, 1995)

The protection observed with neurotrophic factors is probably mediated a number of different mechanisms, one of the most important being the stabilisation of calcium homeostasis, which may be via the upregulation of calcium binding proteins. Genes coding for calmodulin show an altered pattern of expression after systemic kainate administration (Sola *et. al.*, 1997). Cell adhesion molecule I (CAM I) mRNA showed an increase five hours after the injection, while CAM II declined over 24 hours. CAM III remained unaltered. Whether these changes were mediated by neurotrophins or other factors was not investigated. BDNF suppresses calcium accumulation by activating Ca^{2+} pumps decreasing cellular calcium levels or a protein kinase which phosphorylates calcium channels, limiting uptake (Jiang *et. al.*, 1997b). BDNF also increases activity of glutathione peroxidase, which scavenges metabolically produced electrophiles. BDNF blocks NOS expression in spinal motor neurones after ventral root avulsion (Novikov *et. al.*, 1995), which as stated earlier may be beneficial to the compromised brain. A further effect of BDNF is the increase of neuropeptide Y (NPY) mRNA within NPY-releasing neurones (Takei *et. al.*, 1997), which may be beneficial due to the observation that NPY-deficient mice are more susceptible to spontaneous and evoked seizures (Erickson *et. al.*, 1996). A detrimental effect of BDNF is that it has been shown to increase the depolarisation-induced (high K^+) release of glutamate from cultured cortical neurones (although no apparent effect was noted on basal release; Takei *et. al.*, 1997). This may help explain observations of a pre-synaptic enhancement of glutamatergic synaptic

transmission (Lessmann *et. al.*, 1994), which may in turn explain the conflicting views of it's ability to attenuate or exacerbate excitotoxic/ischaemic neuronal damage.

OBJECTIVES

The aims of this project were to further explore excitotoxic cell death, as well as the role of adenosine as a possible endogenous neuroprotective agent. Focusing in part on the role of the neurotrophin BDNF and apoptosis, this study examines the mechanisms of both kainate-induced damage to the hippocampus and the protection provided by a number of compounds.

MATERIALS AND METHODS

INTRAPERITONEAL INJECTIONS

Male Wistar rats of weight 190-250 g were used in all intraperitoneal injection experiments. All animals were purchased from Harlan Olac and allowed to settle in the animal house for at least a week under standard conditions (12 hour lighting cycles and both food and water available *ad libitum*) before use.

All injections were administered at a volume no greater than 3 ml. kg^{-1}. Kainate, CGS 21680 and L-NAME were dissolved in saline, DPMA in methanol, 8-(p-sulphophenyltheophylline) (8-PST) in distilled water, 8-cyclopentyl-1,3-dipropylxanthine (CPX) in ethanol, 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM 241385) in polyethylene glycol 300/ 0.1M NaOH (1:1 v/v), R-PIA in methanol and 7-NI in saline/tween 80 (3:1 v/v - a few drops of NaOH were added to lower the pH value to 8), to provide solutions of the correct concentration for injection. The drugs, when added, were all injected 10 minutes prior to the injection of kainate, which was administered at a standard dose of 10 mg. kg^{-1}. The animals were left for seven days under standard conditions.

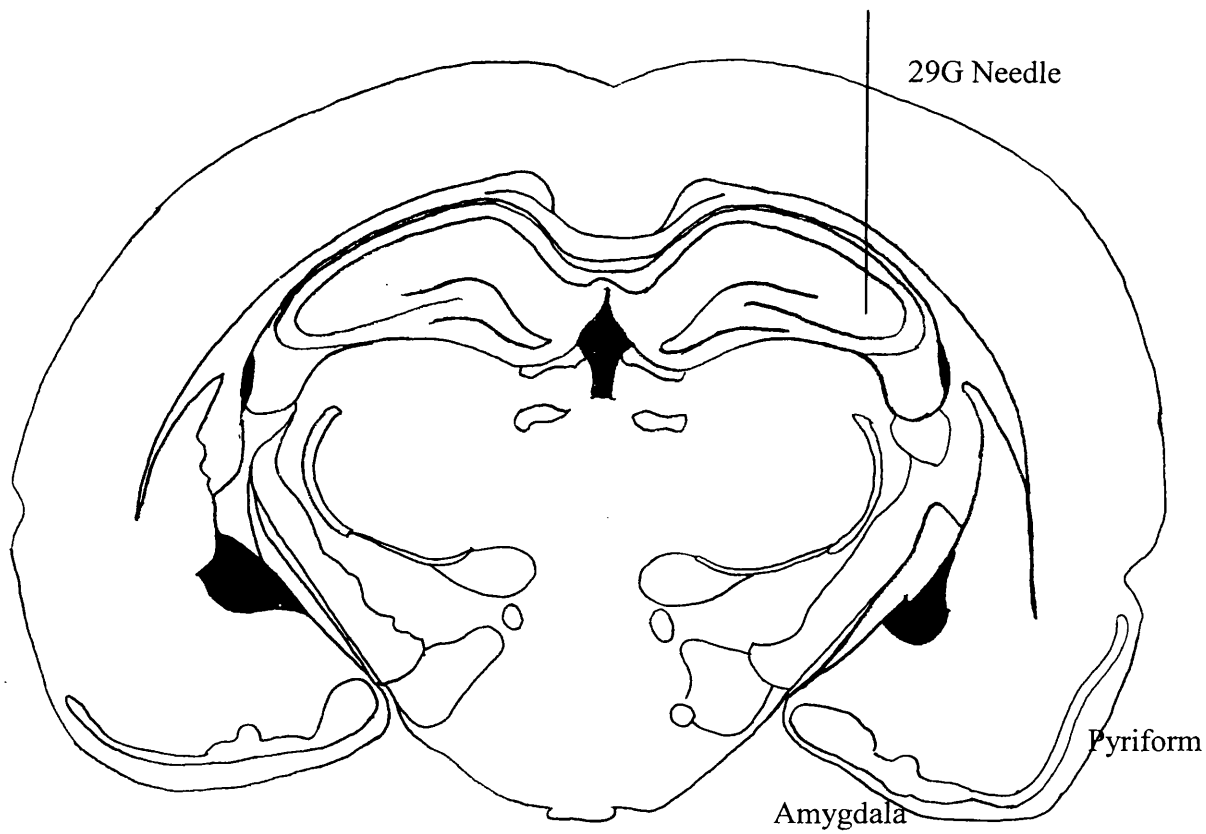
To ensure that the kainate injected was entering the brain (and not having been injected into an organ such as the bladder and rapidly excreted), animals were monitored for epileptic activity. In all animals (except those where only saline or vehicle were administered) some kainate-associated activity was observed. This usually comprised of wet dog shakes, Straub tail, although in some cases seizures were apparent.

INTRAHIPPOCAMPAL INJECTIONS

For this surgical procedure male Wistar rats of weight 270-310 g, were anaesthetised with equithesin (3 mg. kg^{-1}). A small area of the head was shaved before the animal was placed into a stereotaxic frame. A rectal probe and a heating blanket were used to maintain a normothermic temperature throughout the experimental procedure. The co-ordinates for the injections and the position of the tooth bar (-3.5 mm from interaural) were obtained from Paxinos and Watson (1986). The skin over the skull was cut and the exposed area cleared of membranous tissue. The bregma was located and ventral, lateral and anterior co-ordinates were taken. For the injection into the hippocampal structure, the co-ordinates used were -0.36 mm (anteroposterior), -0.32 mm (dorsoventral) and +0.32 mm (lateral) from the bregma (fig. 1). The skull was bored with a drill at the point ventral to the injection site. Bone fragments were removed and the dura gently broken.

The needle was pre-washed with saline and filled with an amount (~ 0.5 ml) of the compound(s) to be injected. The needle (29G) was lowered slowly to the correct ventral position and left for 1 minute, before the drug(s) were infused. A syringe pump (Sage instruments) was used to ensure a constant rate of injection over 3.5 minutes. The injection volume was 1 µl. With the exception of ZM 241385 all co-injected compounds were administered in the same solution. Due to the immiscibility of the A_{2A} antagonist with saline, simultaneous injection of ZM 241385

Fig. 1 Diagram of intrahippocampal injection site. The position of the needle tip below is at a point which is + 3.2 laterally and -3.2 ventrally from bregma. The plane of section is - 3.6 anterior from bregma. Both the pyriform and amygdala, an area noted for extrahippocampal damage in intraperitoneal studies, are also labelled. The diagram has been redrawn and simplified from the original (Paxinos & Watson. The rat brain in stereotaxic co-ordinates. Second edition. Figure 32).



with kainate was unrealisable. ZM 241385 was therefore administered just prior to the kainate injection. Injection volume and time were modified such that the total volume and time remained constant (i.e. each compound was administered in a volume of 0.5 µl over 1.75 minutes).

The needle was left for 2 minutes after drug administration, to allow diffusion away from the injection site, before slow removal. The cut was stitched using a Mersilk thread (Ethicon Ltd.) and needle. To prevent dehydration, two i.p. injections of 2 ml saline were administered post-operatively, 30 minutes apart. Animals were then left to recover under standard conditions for either two or seven days.

In the above experiments, kainate, CGS 21680, adenosine and (+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]-cycloheptene-5,10-imine maleate (MK-801) were dissolved in saline, R-PIA was dissolved in methanol, with subsequent dilutions in saline (such that the final methanol concentration was 0.02 %) and ZM 241385 in polyethylene glycol 300/ 0.1M NaOH (1:1 v/v).

PERFUSION AND TISSUE SLICING

After recovery from either of the above experimental protocols, the rats were anaesthetised with sodium pentobarbitone (60 mg. kg^{-1}). The method of perfusion first described by Brown & Brierley (1972) was used, in which the animal was placed into a supine position and the heart exposed. 50 ml of physiological medium with 12.5 % xylocaine was then perfused through a cannula placed into the left ventricle at mean arterial blood pressure before fixation by 250 ml of FAM (40 % formaldehyde, glacial acetic acid, methanol in the ratio 1:1:8 v/v). The fixative was perfused over a

period of 20 minutes. The animals were decapitated and the heads stored at 4 °C in fixative for up to a week. The brain was subsequently removed and the left hemisphere marked with Indian ink.

The brain was dissected laterally into five 2 mm sections, before placing into a Histokinette (Reichert-Jung) processor for the duration of the 24 hour automated cycle. Processing consisted of dehydration through a series of alcohols (70 %, 90 %, absolute + 2 % celloiden (to soften the tissue), absolute alone and amyl acetate (which removes the alcohol)) and wax (Tissue Tek II; Sakura Fine Technical Ltd.), to impregnate the tissue with wax. To ensure complete infusion of the wax throughout the tissue, the brain slices were left in heated wax in a vacuum for 20 minutes or until air was no longer being drawn from the tissue. The slices were then embedded in wax which is allowed to set on a cold plate. The section of brain was then cut into sections of 6 μm using a microtome (American Optical Company).

Sections were then stretched in warm water and mounted on glass slides pre-coated with an adhesive (albumin). The slides were then allowed to dry at 37 °C.

At two points during the above protocol codes were used to ensure that the observer was blind to the origin of the sections under examination.

HAEMATOXYLIN AND EOSIN STAINING

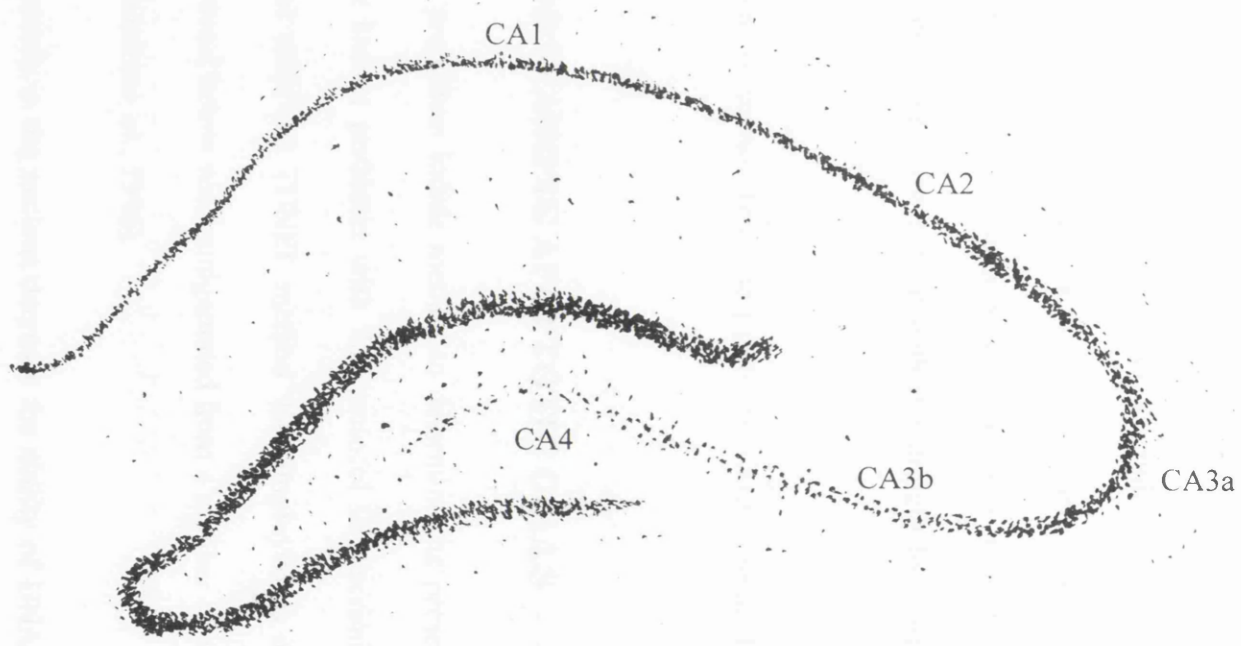
The slides were first dewaxed in xylene (this was later changed to the less toxic alternative, Histoclear; Raymond Lamb Laboratory Supplies) and run through descending ethanol concentrations (absolute (x 2), 90 % and 70 %) before washing in

water. The slides were then immersed in Mayer's haematoxylin for 5 - 6 minutes. After washing in water the slides were placed into Scott's solution (an alkali which changes the acidic haematoxylin pigment red to blue). The section was then examined and restained (if the colour was too light) or dipped into acid alcohol for 5 seconds if the staining was too intense. The sections were subsequently washed, immersed into eosin for 45 seconds - 3 minutes and then washed again. Again re-immersion in eosin would increase the staining if required. The slides were then washed along an alcohol gradient (the time spent in 70 % alcohol was dependent on the strength of the eosin staining as it leaches the stain) to either xylene or Histoclear. After 10 minutes the sections were removed and a coverslip, pre-coated in Histamount (Hughes & Hughes Ltd.), was placed over the brain sections.

QUANTITATIVE ANALYSIS

Sections were examined under a light microscope by observers who as stated above, were unaware of the drug treatment received. The haematoxylin stained cell nuclei blue, while eosin stained the remaining tissues varying shades of pink. With neurones which were damaged, the eosin staining was more intense, staining the cell a deeper shade of pink than the surrounding. Pink damaged neurones were therefore easily distinguished from viable blue stained cells. The left (marked) hippocampus was examined and areas CA1, CA2, CA3a CA3b and CA4 regions scored for damage (fig. 2). The method of determining cell damage was based on a cell count combined with observation and knowledge of the hippocampal structure. As the pyramidal cell layer is a discrete, easily discernible structure, it is possible to observe

Fig. 2 The hippocampus. Diagrammatical representation of the five regions of the hippocampus examined in this study



areas within a cell layer which are devoid of cells 7 days post-treatment. In order to compensate for these gaps, an estimate was made of the percentage area where dead or dying cells had been removed. Cells still present were then counted to obtain a viable cell count of the remaining neurones. These values were then combined to obtain a semi-quantitative value of total damage. For example, if 50 % of the observable hippocampal cells are stained deep pink in a region where a proportion of the cell layer equivalent to 50 % of the total area is devoid of cells, then damage is recorded at 75 %. Figure 3 correlates this method with the straight forward viable cell count ($r = 0.8845$).

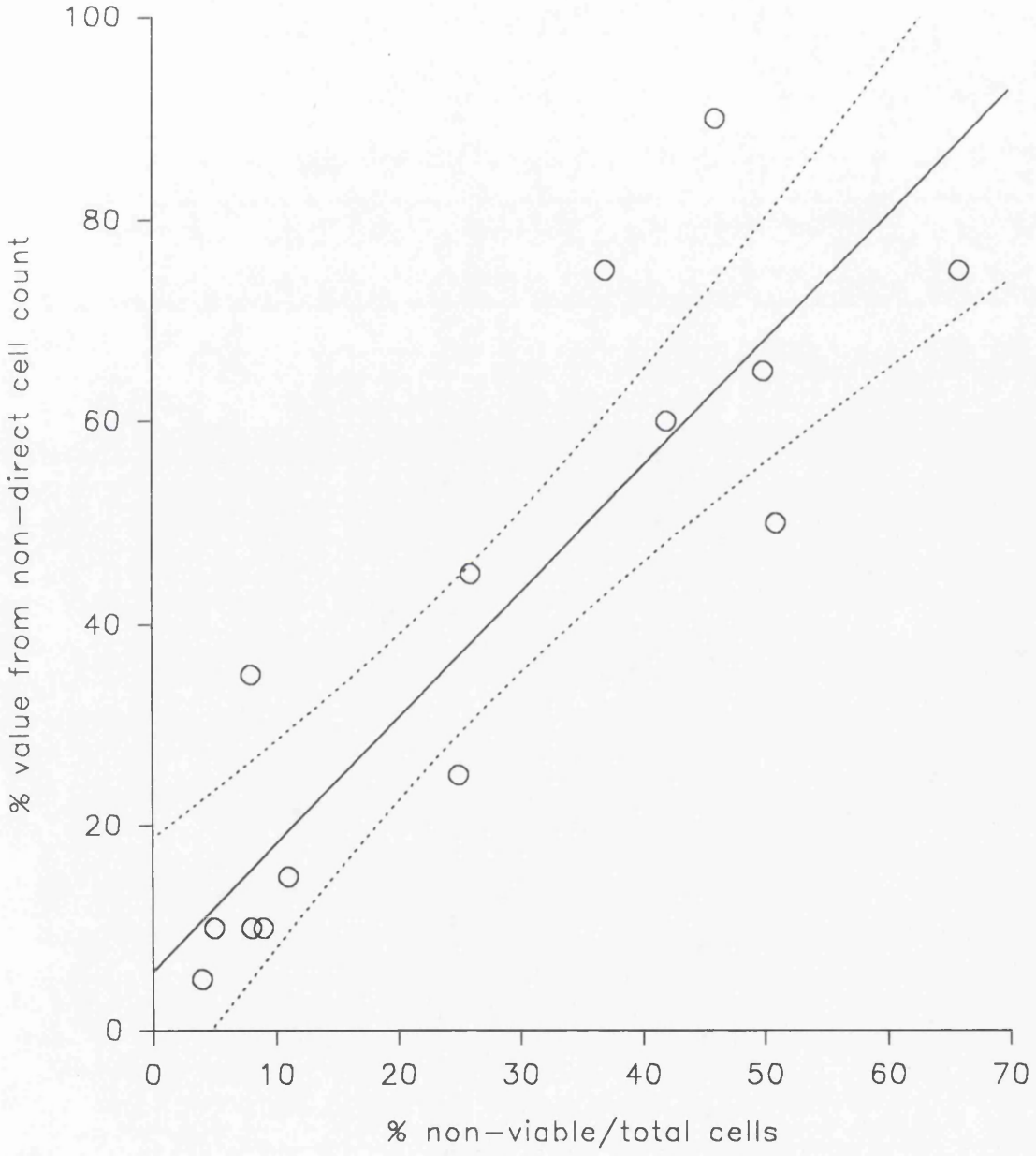
Other areas on the same plane of section (e.g. the pyriform) were examined in the same way as above.

TUNEL METHOD FOR STAINING APOPTOTIC CELLS

Early experiments used the propidium iodide method to determine the presence of apoptotic cells. Due to the known problems with this protocol in discriminating apoptotic from non-apoptotic cells, the TUNEL method was employed as a more selective substitute. The protocol below was amalgamated from a number of sources (e.g. Gavrieli *et. al.*, 1992; Suzuki *et. al.*, 1996).

As the increased levels of protein in the nucleus decrease the ability of DNA break labelling by the TUNEL method (Kerrigan *et. al.*, 1987), the initial step of the protocol was an attempt to lower the protein content. Originally, incubation of the dewaxed brain sections in proteinase K (20 $\mu g.\ ml^{-1}$ for 15 minutes) was used, but following a study discussing the effectiveness of various pre-treatment protocols (Negoescu *et. al.*, 1996), this was altered to microwave irradiation. The sections

Fig. 3 Graph correlating the two methods of cell counting. The values for damage obtained from a simple viable cell count are compared with the respective value when subregions devoid of cells are taken into account. The data below refers to results from the CA3a region of animals injected i.p. with kainate (10 mg. kg $^{-1}$)



were heated in 0.01 M citrate buffer (pH 6) to a temperature of ~85 °C, followed by rapid cooling in citrate buffer and then in double strength phosphate buffer solution (PBS). This method was shown by Negoescu *et. al.* (1996) to double the sensitivity for the TUNEL method without altering the selectivity. The study also showed that the use of formaldehyde as a fixative retained selectivity and sensitivity.

The sections were then washed in both distilled water (four times) and 2 % hydrogen peroxide (to inactivate endogenous peroxidase), before immersion into terminal deoxynucleotidyl transferase (TdT) buffer (5 minutes). Bovine serum albumin (BSA) within the buffer prevented non-specific binding. The slides were subsequently incubated at 37 °C for 1 hour with the TUNEL reagents (0.3 units of TdT (Sigma) and 2 nmol of biotin-16-dUTP (Boehringer Mannheim) per section, dissolved in TdT buffer).

After 1 hour the reaction was halted by soaking the slides for 15 minutes in TB buffer. The slides were subsequently washed in distilled water then PBS twice each. The original staining protocol used aminoethyl carbazole (AEC). Once washed sections were incubated with extravidin peroxidase (1 : 1000 in PBS) for 30 minutes at 37 °C. Washing in distilled water then PBS (both twice) preceded staining the sections with AEC for 30 minutes at 37 °C. Due to the leaching of the stain from sections in alcohol, dehydration and mounting with Histamount was not possible. Sections were instead mounted in aqueous glycine. While this method did highlight apoptotic stained cells, due to the likeness in colour of the stain (brown) and the background glycine (yellow), an alternative was sought. This substitute to the AEC stain was dimethylaminbenzidine (DAB), which was not leached to any significant degree during dehydration. After incubation in TB buffer slides were washed in PBS

three times. Extravidin peroxidase was replaced with incubation of the sections with avidin/biotinylated enzyme complex (ABC; Vectastain, Vector Laboratories) complex for 60 minutes. Subsequent washing was in PBS (twice) and then phosphate buffer (PB), before staining with 0.05 % DAB solution until colour developed (5 minutes). The slides were then dehydrated and mounted in Histamount in the same manner as described above for haematoxylin and eosin staining.

In negative controls incubation with the TUNEL reagents was replaced with TdT buffer (under similar conditions).

A small number of slides were counterstained with eosin (45 seconds - 3 minutes) after DAB staining. These were subsequently dehydrated and mounted as above.

BDNF-PROTEIN STAINING

The procedure used for identifying BDNF protein on brain sections was the standard immunocytochemical protocol. Three washes in PBS preceded incubation in blocking serum (20 % goat serum in 0.3 % Triton/PBS) for 60 minutes. Excess blocking serum was removed before the addition of the primary antibody (rabbit anti-BDNF polyclonal antibody; Chemicon Int. Ltd.). Original experiments using 1 in 1000, 2000 and 3000 dilutions of the BDNF antibody revealed that 1 in 2000 dilution resulted in specific staining without excess background. This concentration, diluted into blocking serum was used in the remaining experiments. The primary antibody was left covering the sections for ~16 hours.

The antibody was removed by washing three times in PBS before the addition of the biotinylated secondary antibody (anti-rabbit IgG; 1:200 in 0.3 % Triton/PBS) for 60 minutes. The remainder of the protocol followed that used in the revised latter portion of the apoptosis experiments. Briefly the sections were washed in PBS, incubated with the ABC complex, washed in PBS and PB and stained with DAB. The slides were then dehydrated and mounted.

STATISTICAL ANALYSIS

All values given are mean ± standard error of the mean. ANOVA was used to compare each value with each and with controls. Any observed statistical significance was investigated by the use Student Newman Keul's t-test, where a value for p less than 0.05 was considered significant.

SOLUTIONS AND MATERIALS

INTRAHIPPOCAMPAL INJECTIONS

EQUITHESIN

16.2 ml	Sodium Pentobarbital
4.25 g	Chloral hydrate
2.12 g	$MgSO_4$
39.6 g	Propelyne glycol
10 ml	Ethanol

HEMATOXYLIN AND EOSIN STAINING

MAYER'S HEMATOXYLIN

1 g	Haematoxylin
50 g	Potassium alum
1 g	Citric acid
50 g	Chloral hydrate
0.2 g	Sodium iodate
1 l	dH_2O

EOSIN

Stock

1 g	Eosin Y (Raymond Lamb Laboratory Supplies)
20 ml	dH_2O
80 ml	95 % Alcohol

Working Solution

100 ml	Stock
300 ml	80 % Alcohol
2 ml	Glacial acetic acid

SCOTTS SOLUTION

80 g	$MgSO_4$
14 g	Sodium bicarbonate
2 l	dH_2O

TUNEL STAINING

TdT BUFFER

3.36 g	Sodium cocodylate
75 ml	Tris-HCl
37.5 mg BSA	
89 mg	Cobalt chloride
75 ml	dH_2O

TRIS-HCl

125 ml	0.2 M Tris
225 ml	0.1 M HCl
150 ml	dH_2O

pH to 7.2 with 0.1 M HCl

TB BUFFER

8.75 g	Sodium chloride
4.4 g	Sodium citrate
500 ml	dH_2O

CITRATE BUFFER (0.01 M)

A: 1.47 g Sodium citrate in 500 ml dH2O

B: 0.21g Citric acid in 100 ml dH2O

For pH 6.0 add solution B to A

PB (0.2 M)

A: 8.72 g NaH_2PO_4 in 600 ml dH_2O

B: 42.45 g Na_2HPO_4 in 1500 dH_2O

560 ml Solution A + 1440 Solution B, pH 7.2

PBS

200 ml	0.2 M PB
36 g	NaCl
1800 ml dH_2O	

AEC

10 g	3-amino-9-ethylcarbazole
2.5 ml	N,N, dimethyl formamide
37.5 ml	0.05 M Acetate buffer (pH 5.0)
7.5 µl	3% H_2O_2

Acetate buffer (pH 5.0)

A: 1.2 ml Glacial acetic acid in 100 ml dH_2O

B: 1.64 g Sodium acetate in 100 ml dH_2O

37 ml A + 88 ml B, made up to 500 ml with dH_2O

RESULTS

NEURONAL DAMAGE

Haematoxylin and eosin:

Saline/vehicle controls

In both intraperitoneal (i.p.) and intrahippocampal (i.h.) models of kainate excitotoxicity, control injections of saline or drug vehicles, did not induce any noticeable damage in any region of the brain examined (including the hippocampus) compared with untreated animal brains (see fig. 5 & 6 and fig. 23 for photomicrographs of i.p. and i.h. experiments respectively). In central experiments, the insertion of the needle did result in a visible tract through the cortex and CA1 region. This tract was surrounded by small dark cells, which corresponded to the description of glial cells. The presence of these cells did not extend beyond 100 μm in any direction. Damage values in the hippocampal regions were not affected outside this sphere and not at the plane of section used in examining hippocampal damage.

Intraperitoneal injections

Kainate

Kainate, injected systemically at a dose of 10 mg. kg^{-1} resulted in significant damage in three of the five regions of the hippocampus when compared with results observed

with saline (which showed no damage in any region; fig. 4). The highest degree of neuronal damage as assessed by haematoxylin and eosin staining was observed in the CA1 region (48.4 % ± 7.4, $p < 0.001$, $n = 16$; fig. 7). The CA3a region displayed slightly less damage, 38.1 % ± 7.0 ($p < 0.001$; fig. 8), whilst the CA2 region exhibited a moderate amount, 22.2 % ± 6.4 ($p < 0.01$). The CA3b and the CA4 regions did not display any degree of damage significantly different from the saline controls.

Adenosine A_{2A} receptor agonists

CGS 21680

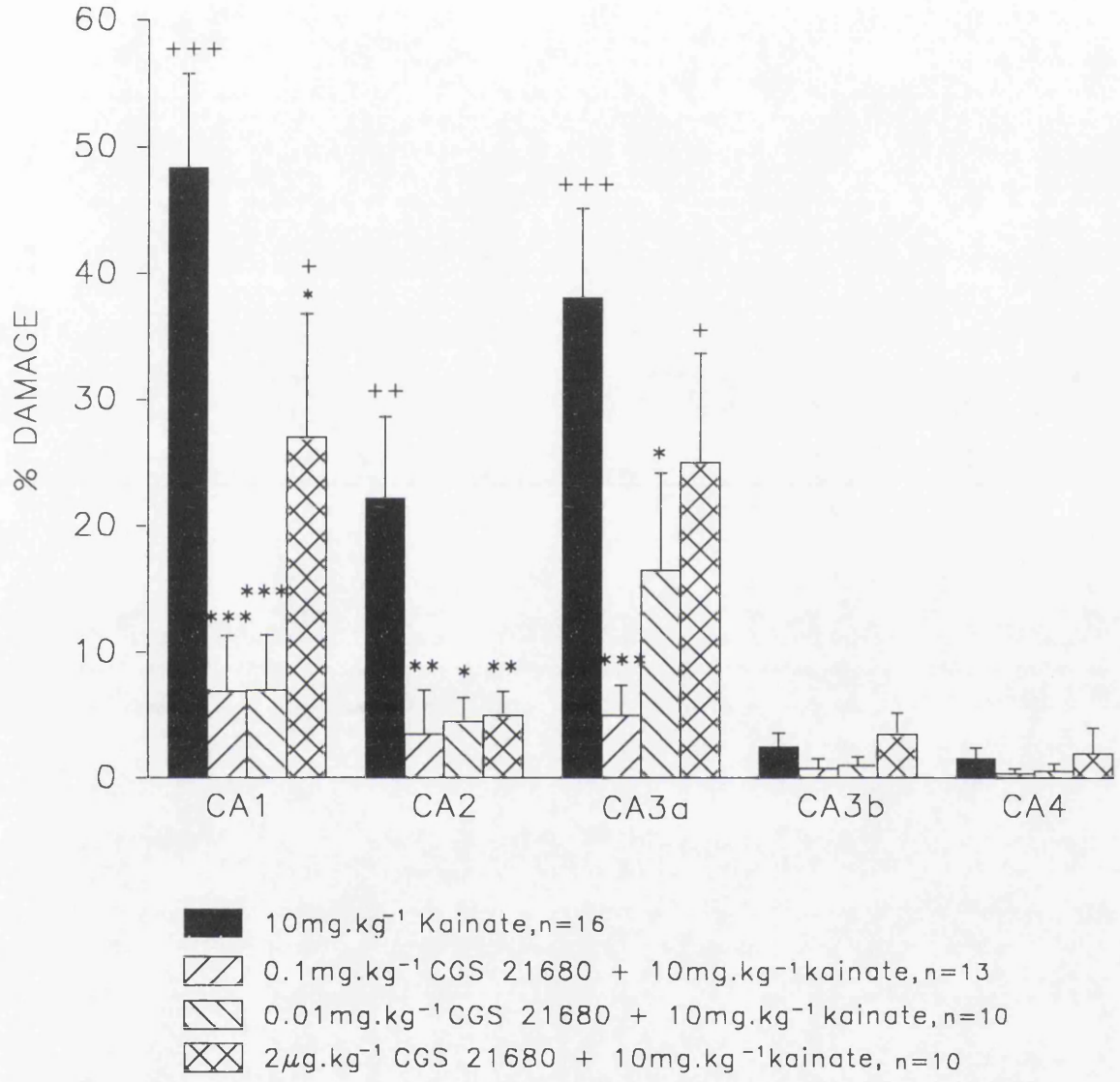
The selective A_{2A} agonist CGS 21680 was administered ten minutes prior to the injection of kainate via the intraperitoneal route at three concentrations, 0.1 mg. kg^{-1}, 0.01 mg. kg^{-1} and 2 μg. kg^{-1}. All three regions significantly damaged by kainate were protected by 0.1 mg. kg^{-1} CGS 21680 ($p < 0.001$ for the CA1 and CA3a regions, < 0.01 for the CA2 region; fig. 4, 9 & 10). Neuronal damage was assessed as 6.9 % ± 4.4 in the CA1, 3.5 % ± 3.5 in the CA2 and 5.0 % ± 2.4 in the CA3a region ($n = 13$), approaching the levels observed with saline controls. No increase in damage was noted in the CA3b (0.76 % ± 0.76) or CA4 regions (0.38 % ± 0.38), which were unaffected by kainate alone. Percentage damage with the introduction of 0.01 mg. kg^{-1} CGS 21680 prior to kainate, was similar to that seen with 0.1 mg. kg^{-1} CGS 21680 in the CA1 and CA2 regions of the hippocampus (7.0 % ± 4.4, $p < 0.001$ and 4.5 % ± 1.9, $p < 0.01$, respectively, $n = 10$). A lower, but still significant, amount of protection was observed in the CA3a region (damage was 16.5 % ± 7.7, $p < 0.05$).

Again there was no significant neuronal death in the CA3b or CA4 regions (1.0 % ±0.7 and 0.56 % ± 0.56, respectively). The lowest concentration of CGS 21680, 2 μg. kg^{-1}, displayed only a very limited degree of protection against kainate. In the CA1 region the value of damage (27.0 % ± 9.8, n = 10) was both significantly different from both saline controls ($p < 0.05$) and kainic acid ($p < 0.05$). Near complete protection was again observed in the CA2 region (damage was 5.0 % ± 1.9, $p < 0.01$), while in the CA3a region the damage observed (25.0 % ± 8.7) was not significant against kainate, but was significantly greater than the saline control ($p < 0.05$). No significant damage was observed in either the CA3b or CA4 regions (3.5 % ±1.7 and 2.0 % ± 2.0, respectively). In view of these results, CGS 21680 in subsequent further studies was injected at an intraperitoneal dose of 0.1 mg. kg^{-1}.

DPMA

Another A_{2A} agonist, DPMA was used at two intraperitoneal injection concentrations, 0.1 mg. kg^{-1} and 1.0 mg. kg^{-1}. Concentrating on only those areas of the hippocampus damaged by kainate (fig. 11), DPMA, when injected at the higher dose, showed protective effects in both the CA1 and CA2 regions of the haematoxylin and eosin stained hippocampal section ($p < 0.01$ and < 0.05 respectively, n = 4). Damage was recorded as 5.0 % ± 3.5 in the CA1 and 1.25 % ± 1.25 in the CA2. No significant neuronal preservation was observed in the CA3a region (27.5 % damage ± 16.4). While no significant change in percentage cell death occurred in either the CA3b or CA4 regions (18.8 % ± 17.1 and 22.5 % ± 22.5, respectively), one animal did show a degree of damage in both areas. Results

Fig. 4 Protection by the A2A receptor agonist CGS 21680, against kainate–induced hippocampal cell death. *** p<0.001, ** p<0.01, * p<0.05 versus kainate alone; +++ p< 0.001, ++ p< 0.01, + p<0.05 against saline controls.



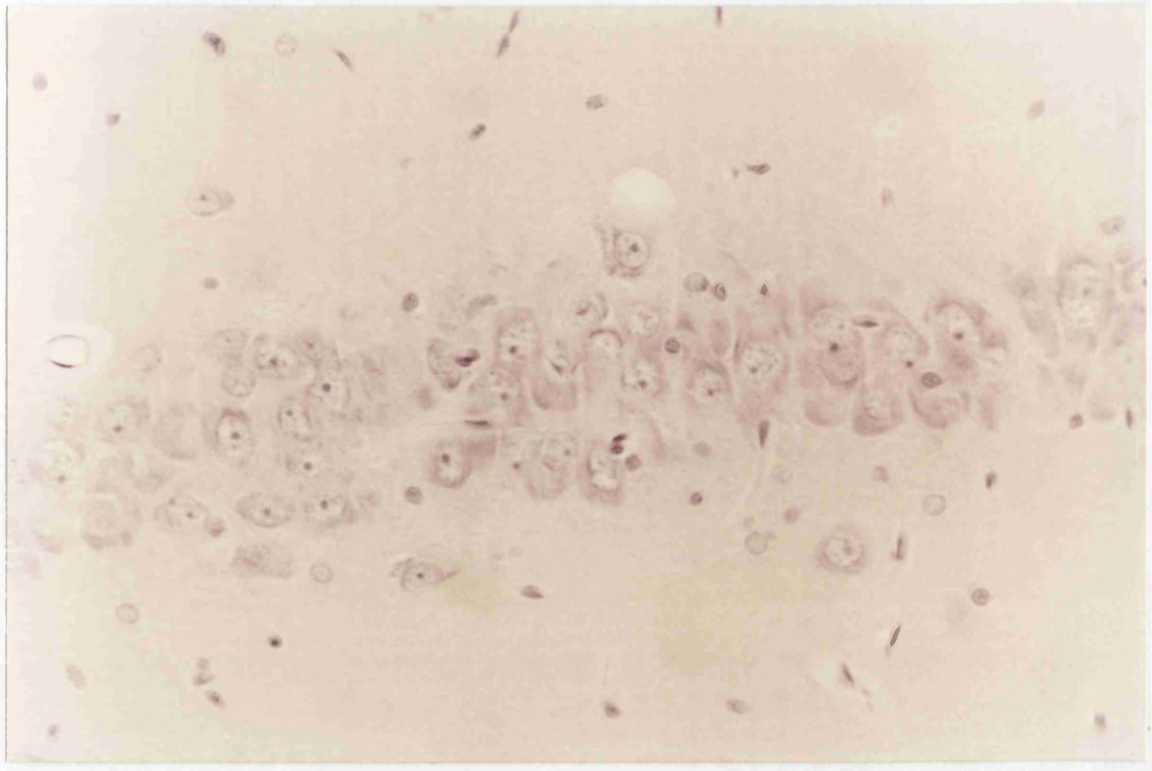


Fig. 5 Haematoxylin and eosin stained CA1 region of the hippocampus after i.p. injection of saline (1 ml). All the cells appear viable and are stained blue with haematoxylin, with no evidence of neurones taking up the deep pink eosin stain. 20 mm : 40 μm.

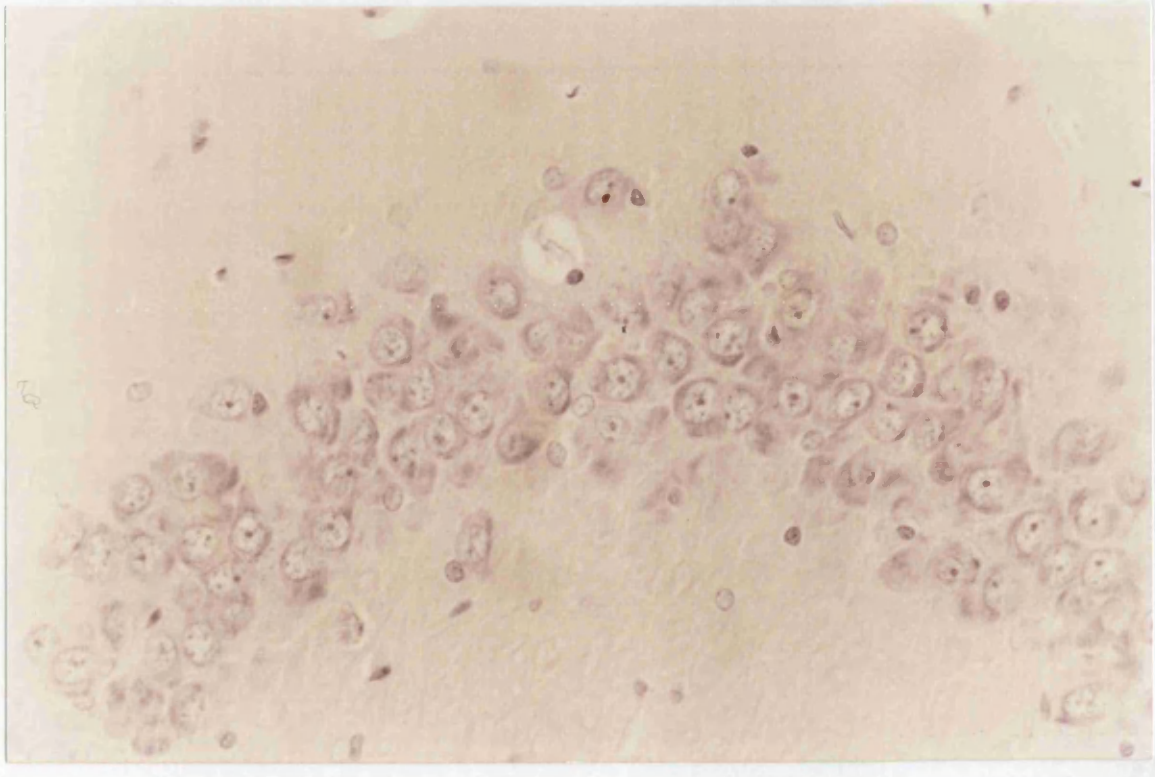


Fig. 6 CA3a region of the hippocampus stained for haematoxylin and eosin following i.p. saline administration. As for the CA1 region, the cells display a healthy morphology seven days after the initial injection. 20 mm : 40 μm.

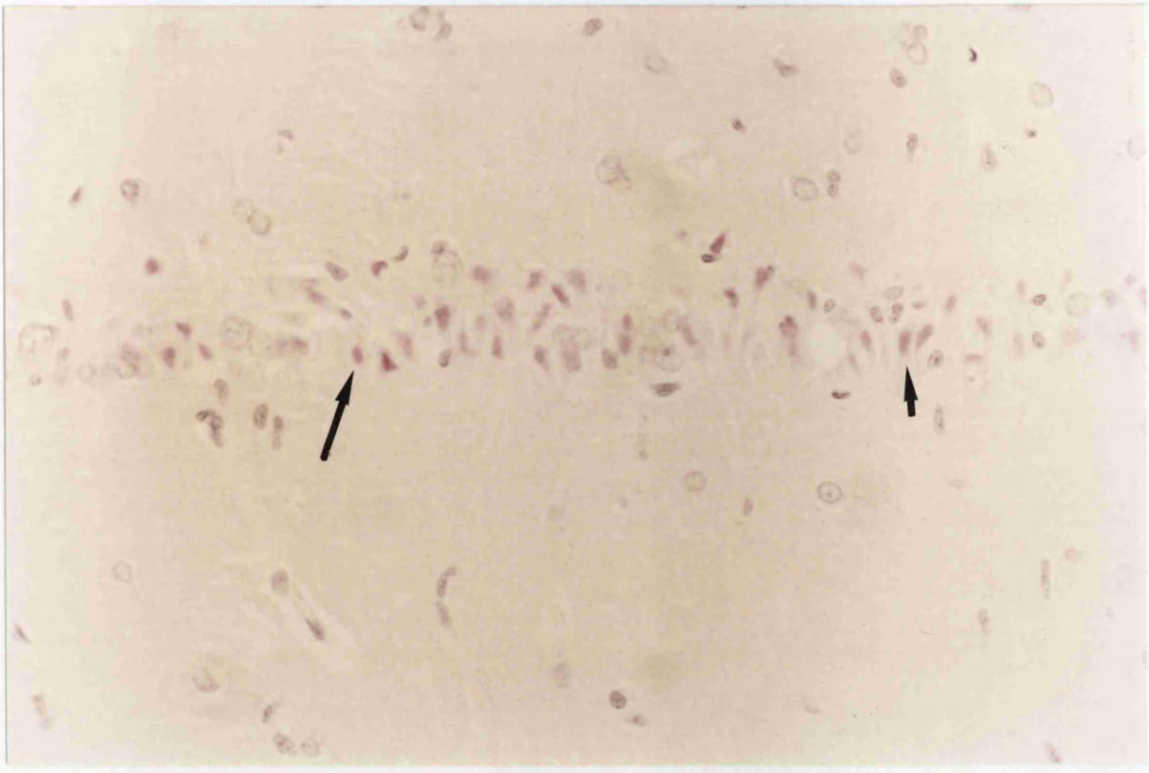


Fig. 7 Haematoxylin and eosin stained CA1 region seven days after i.p. injection of 10 mg. kg^{-1} kainate. Neurones after kainate administration contrast markedly with those after saline control. Note examples of damaged cells (arrows), stained heavily with eosin. 20 mm : 40 μm.

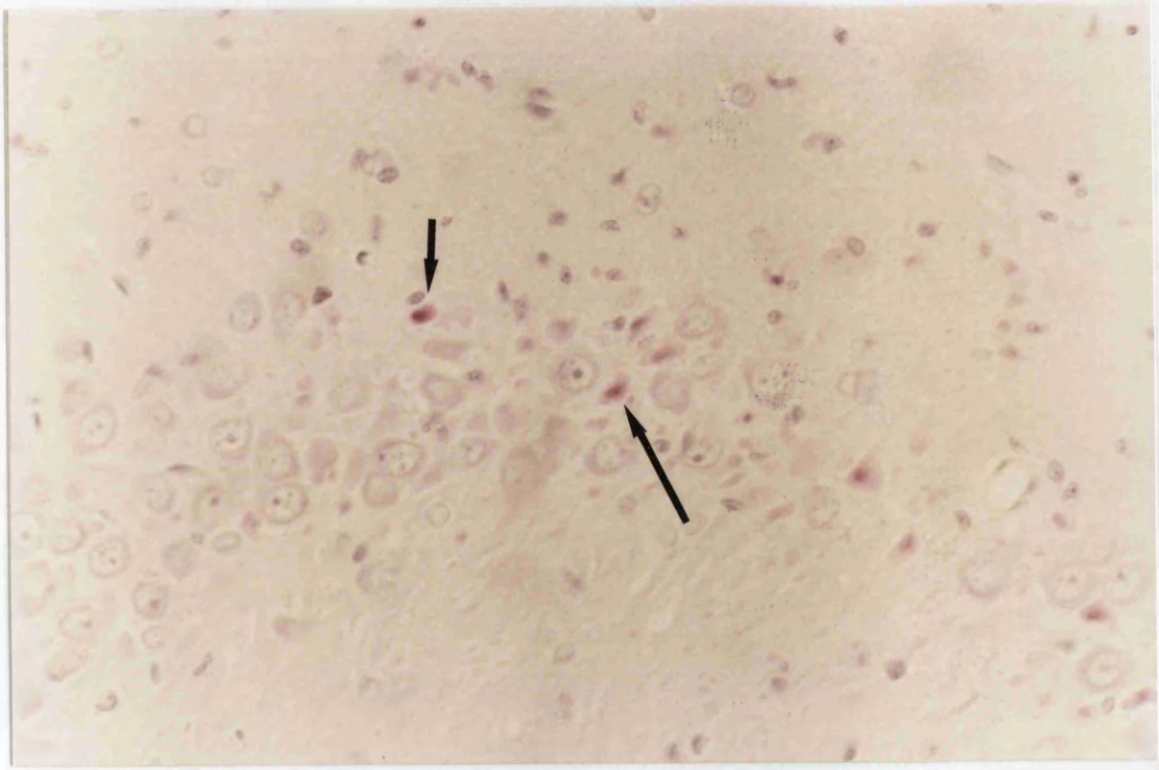


Fig. 8 Damaged CA3a region following i.p. injection of 10 mg. kg^{-1} kainate. Selective neuronal damage is widely apparent throughout this region (arrows indicate non-viable cells). 20 mm : 40 μm.

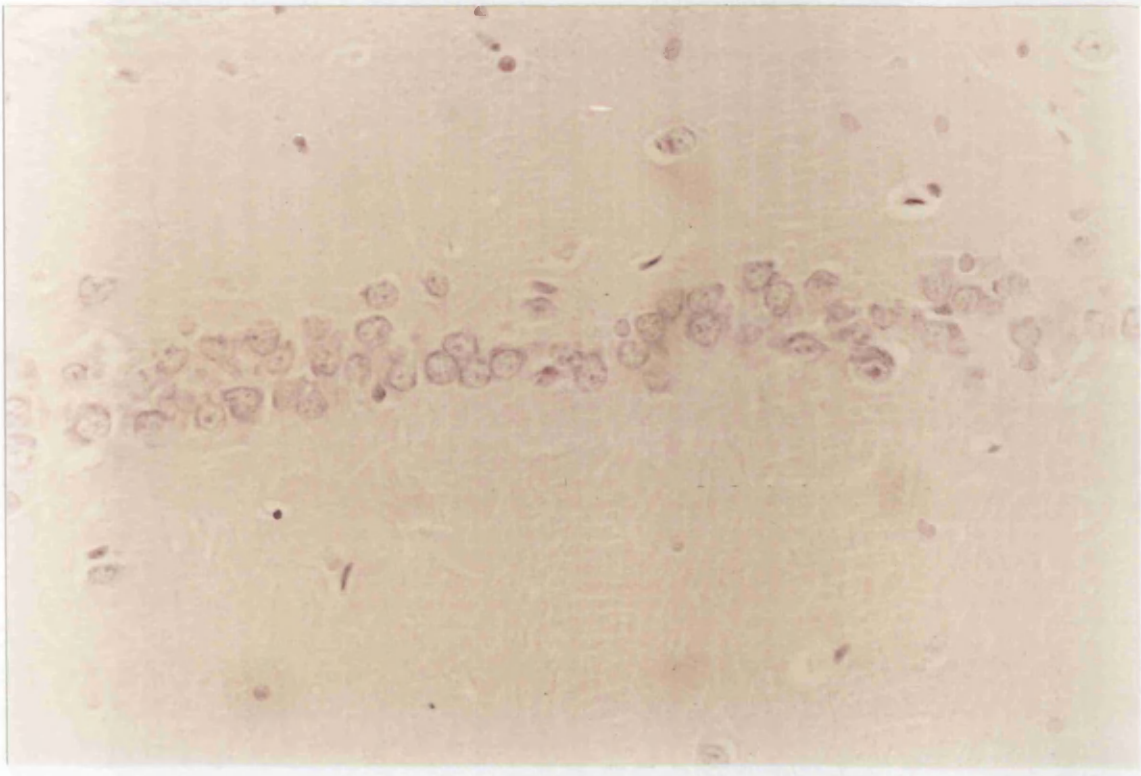


Fig. 9 Protection against kainate toxicity after the i.p. administration of the A_{2A} agonist CGS 21680 in the CA1 region. 0.1 mg. kg^{-1} CGS 21680, injected 10 minutes after 10 mg. kg^{-1} kainate, results in neuronal morphology approaching that of saline controls. 20 mm : 40 μm.

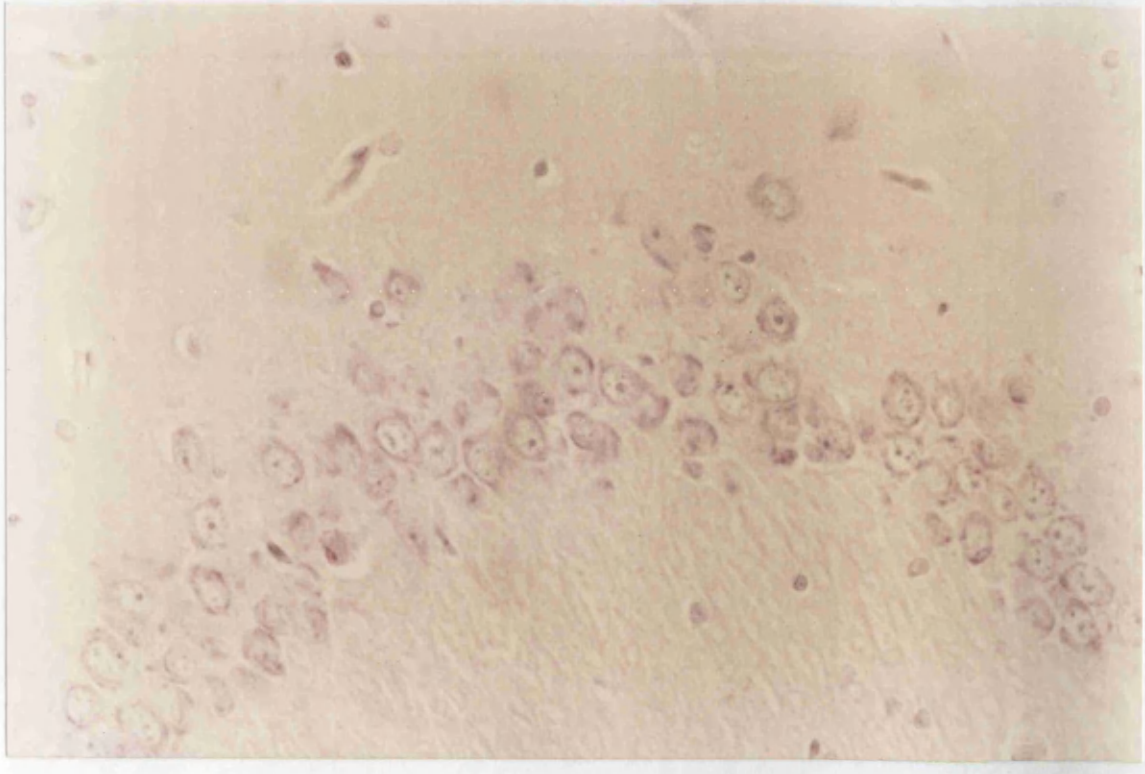


Fig. 10 Kainate toxicity in the CA3a region is prevented by CGS 21680. As for the CA1 region, 0.1 mg. kg^{-1} CGS 21680 prevented the appearance of non-viable cells, when injected 10 minutes after 10 mg. kg^{-1} kainate. 20 mm : 40 μm.

obtained with the lower concentration of DPMA showed no significant reduction in kainate-induced toxicity.

CGS 21680 and adenosine antagonists

To help elucidate the mechanism of protection afforded by CGS 21680, this compound was administered in conjunction with an A_1 receptor antagonist, CPX, or a peripherally acting general adenosine antagonist, 8-PST (fig. 12)

CPX at 50 μg. kg^{-1} did not induce any significant change in protection observed with CGS 21680 alone in the CA1 and CA2 regions (damage was 5.0 % ± 5.0 and 0 % ± 0, respectively, n = 3; fig. 13 for photomicrograph of the CA1 region). Due to the low number of animals treated with this combination, these values were not significantly different from those values obtained for kainate (although they did not represent a significant increase in damage from saline controls levels either). In the CA3a region of the rat hippocampus, CPX did appear to moderate the protective effects of CGS 21680 as damage increased from 5.0 % ± 2.4 (CGS 21680 alone) to 15.0 % ± 12.6 (fig. 14). This value, while not significantly different from CGS 21680 alone, did not represent a statistically significant protection from kainate induced toxicity.

At 20 mg. kg^{-1} the peripheral antagonist 8-PST, negated a proportion of the beneficial effects of the adenosine A_{2A} agonist in the CA1 and the CA3a regions (n = 5; fig. 12). Damage increased in the CA1 region to 28.0 % ± 18.3 (fig. 15) and in the CA3a to 28.0 % ± 13.1 (fig. 16). No such augmentation was noted in the CA2 region (1.0

Fig. 11 Protection observed with the A2A adenosine receptor agonist DPMA. DPMA protected against kainate–induced neuronal damage in the rat hippocampus at 1.0 but not 0.1 mg. kg^{-1} ** $p<0.01$, * $p<0.05$ versus kainate alone.

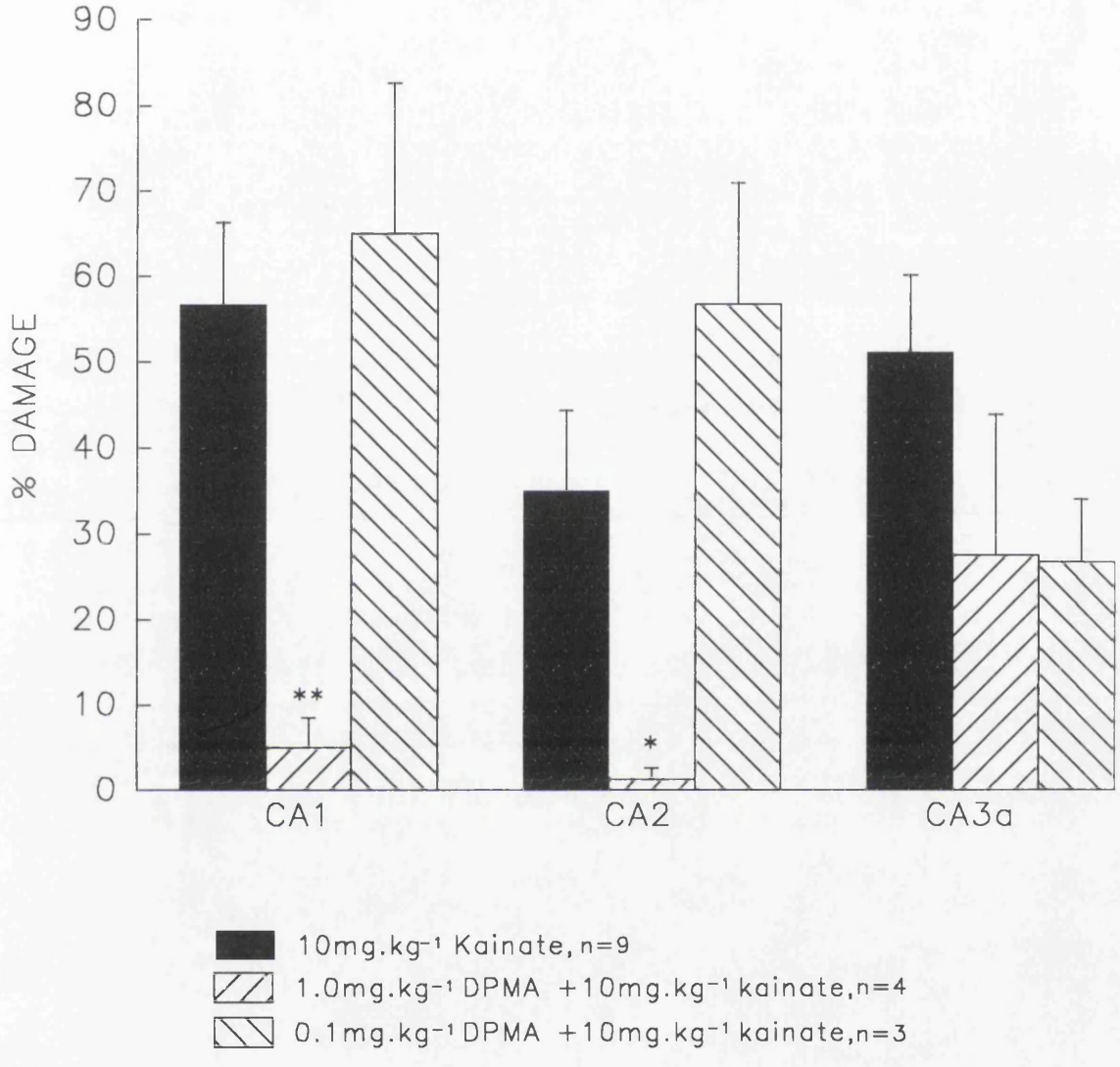
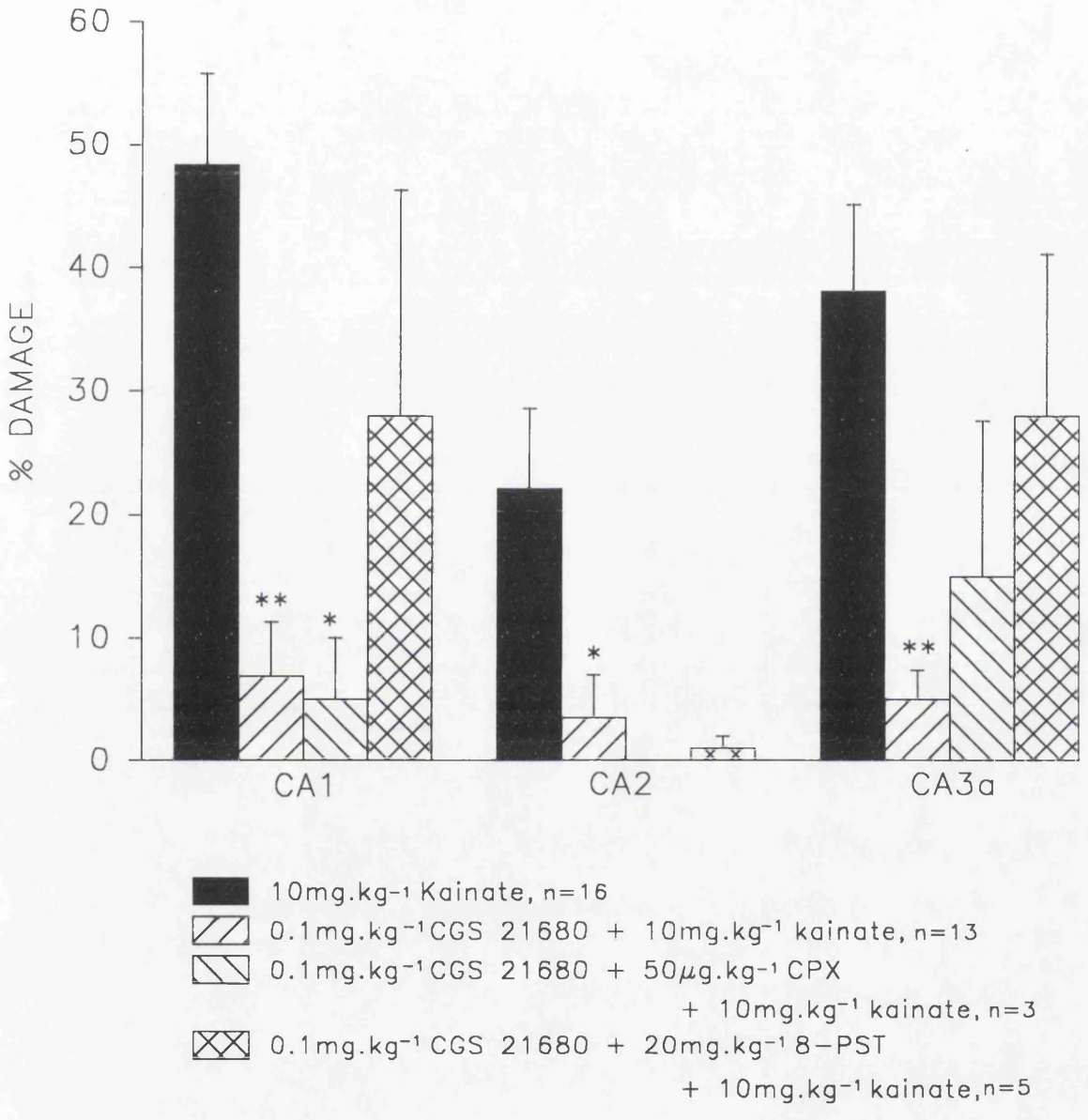


Fig. 12 Effects of the addition of the centrally acting A1 antagonist CPX, and the peripherally acting adenosine antagonist 8–PST, on CGS 21680–mediated protection against kainate toxicity. ** $p<0.01$, * $p<0.05$ versus kainate alone.



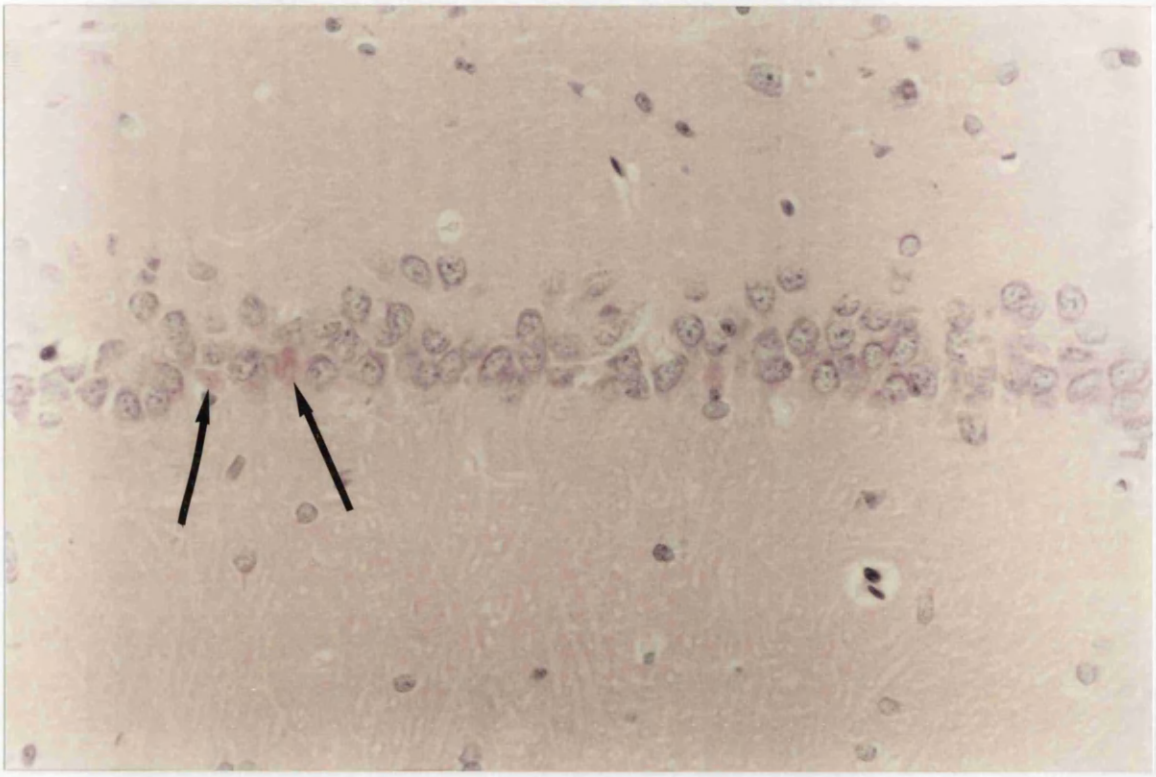


Fig. 13 CA1 region following the i.p. administration of CGS 21680 + CPX + kainate. While a slight increase in the number of non-viable cells is apparent in the above photomicrograph (arrows indicate damaged cells), overall protection by 0.1 mg. kg^{-1} CGS 21680 was not decreased following the addition of 50 µg. kg^{-1} CPX. 20 mm : 40 µm.

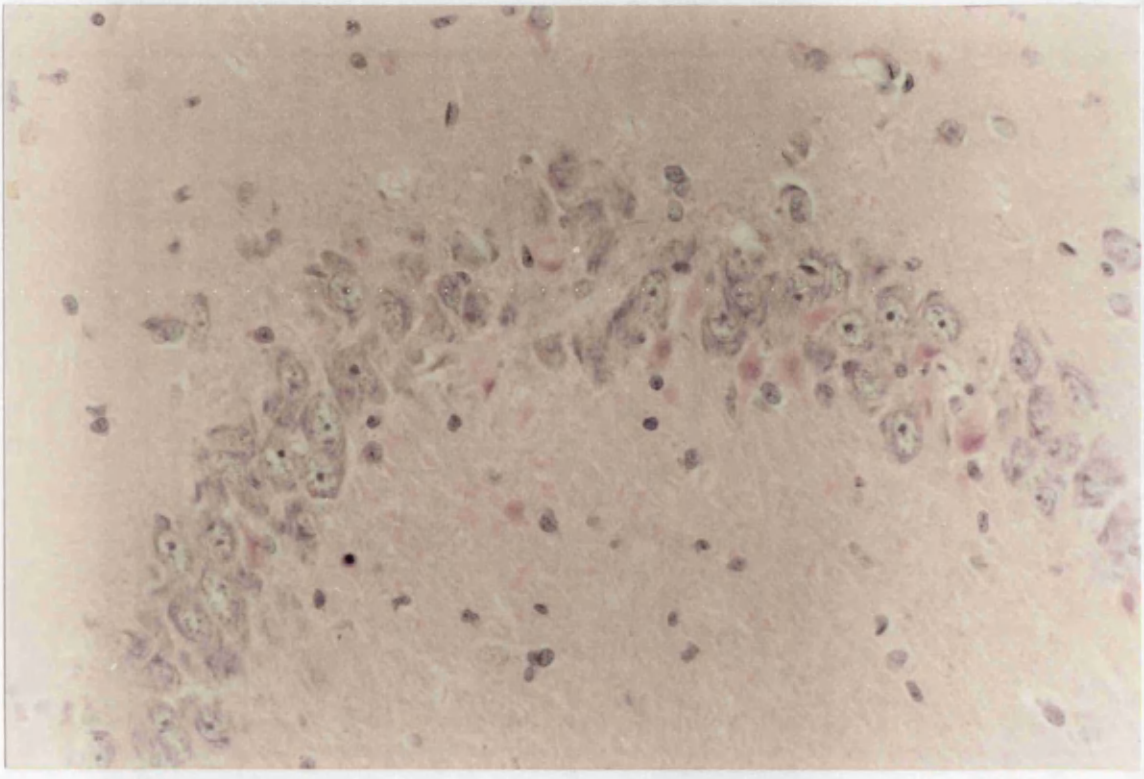


Fig. 14 CA3a region shows a decrease in A_{2A}-mediated protection following the administration of CPX. A larger number of non-viable cells are apparent with the inclusion of 50 µg. kg^{-1} CPX compared to 0.1 mg. kg^{-1} CGS 21680 + 10 mg. kg^{-1} kainate. 20 mm : 40 µm.

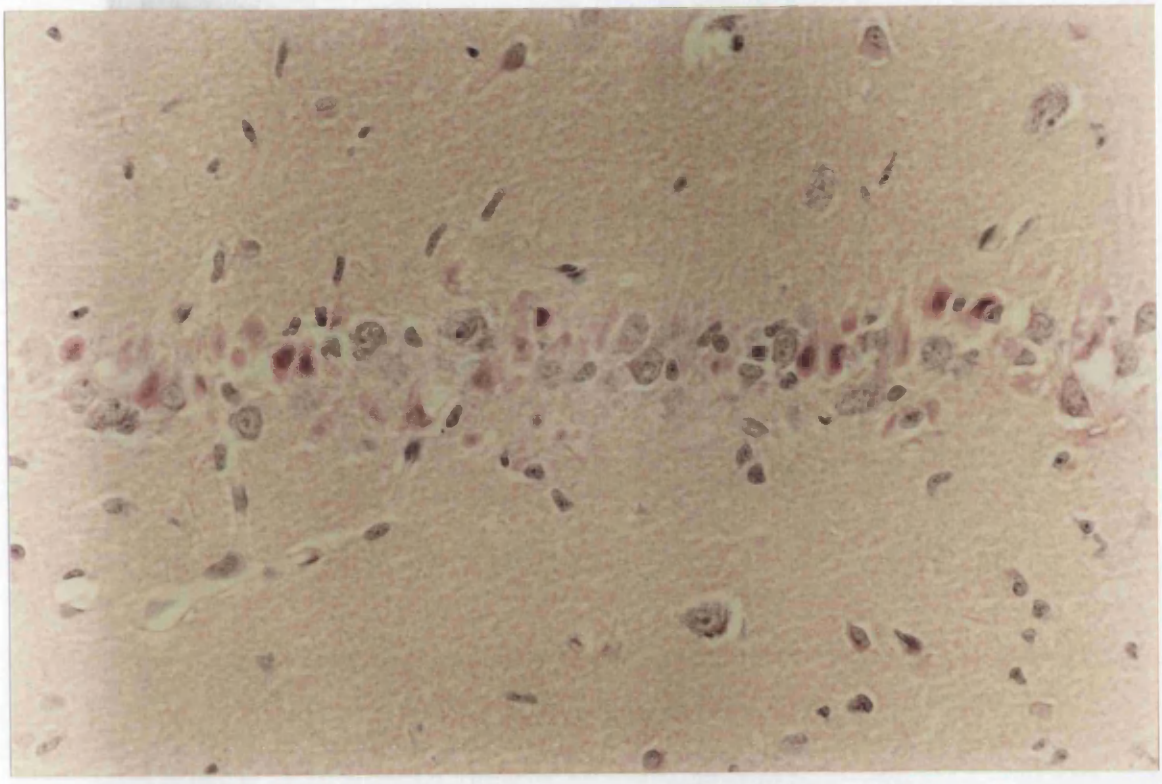


Fig. 15 Substantial damage to the CA1 region after i.p. administration of CGS 21680, kainate and the peripheral antagonist, 8-PST. Levels of cellular damage approach those of kainate alone, following the injection of 0.1 mg. kg^{-1} CGS 21680 with 20 mg. kg^{-1} 8-PST. 20 mm : 40 µm.

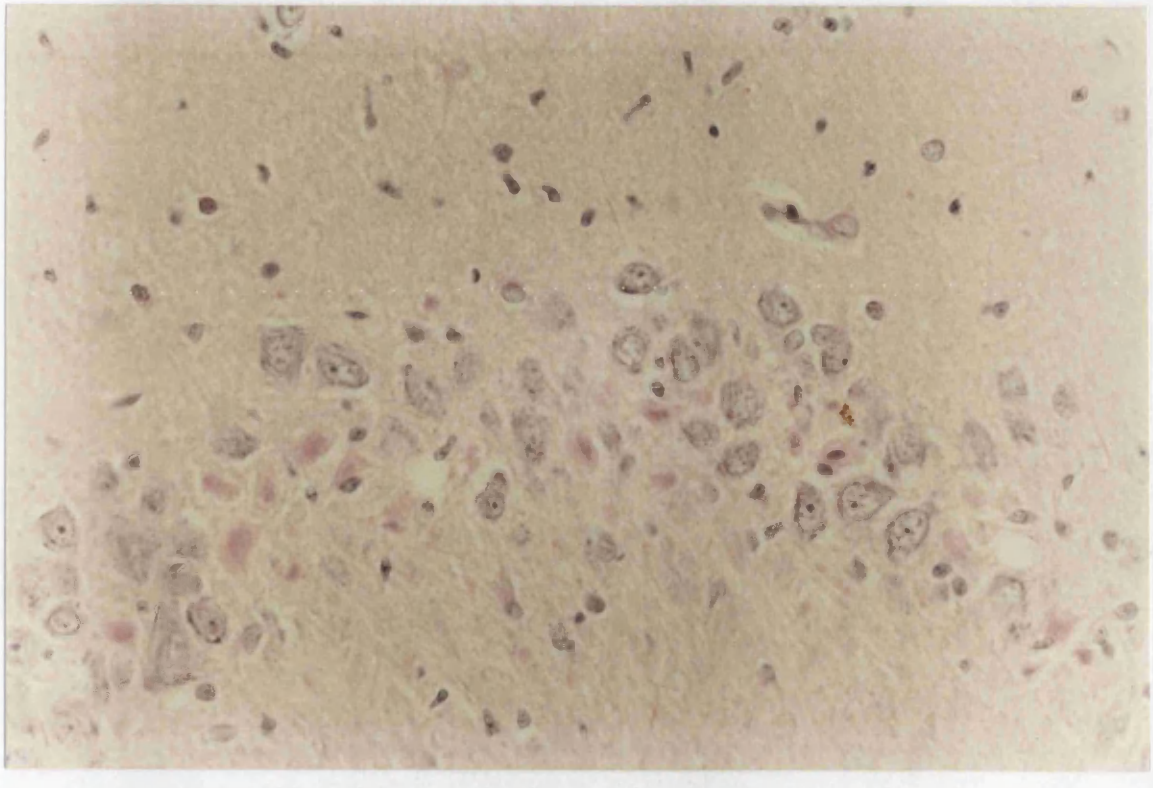


Fig. 16 Damaged CA3a region after i.p. injection of CGS 21680, 8-PST and kainate. The large number of non-viable cells highlights the reversal of protection by 0.1 mg. kg^{-1} CGS 21680 with the administration of 20 mg. kg^{-1} 8-PST. 20 mm : 40 µm.

% ± 1.0). Due to the variability between experiments, these increases were neither significant from kainate nor CGS 21680 (0.1 mg. kg^{-1}) + kainate levels.

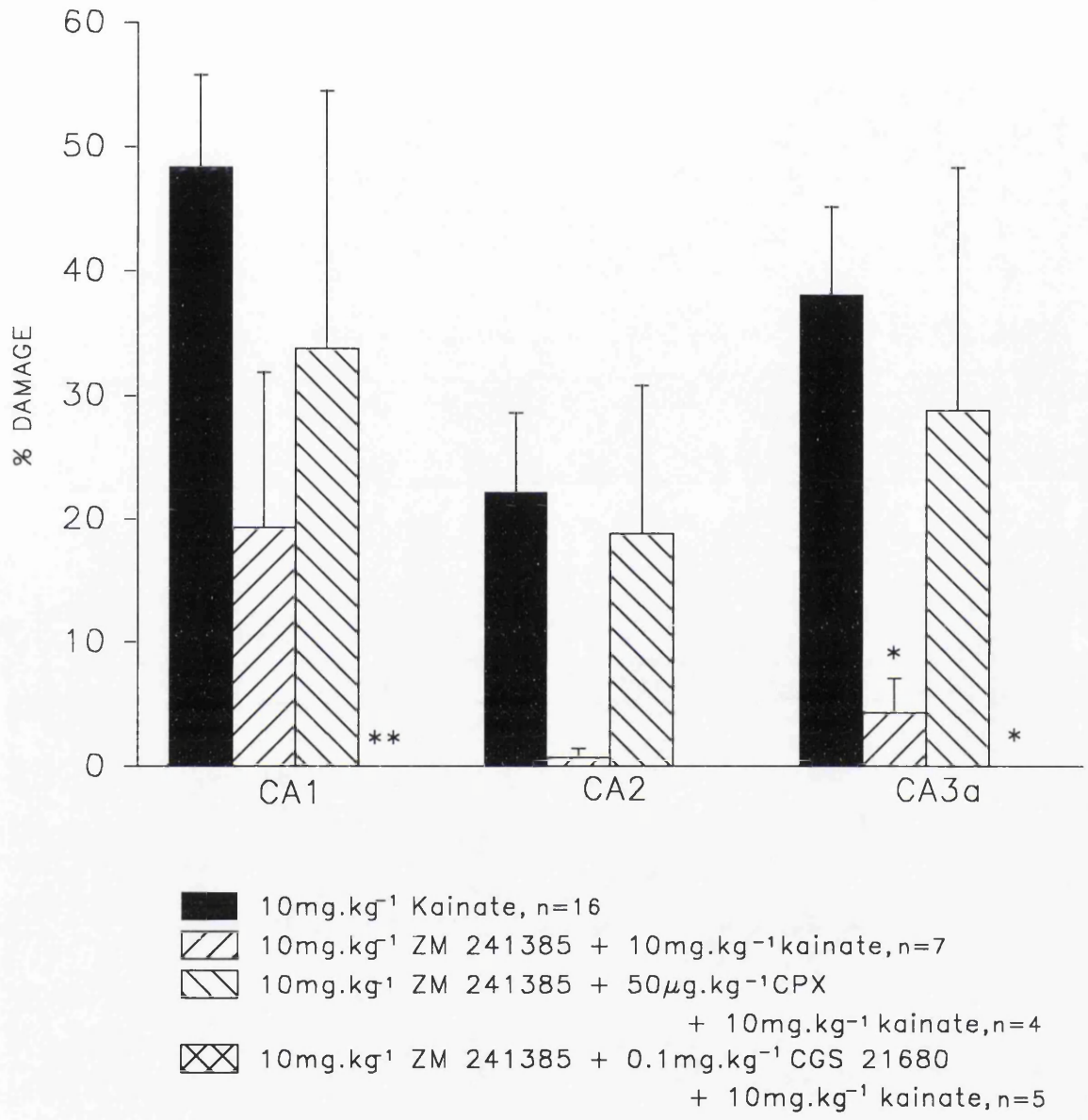
Adenosine A_{2A} antagonist, ZM 241385

The properties of the selective antagonist of the adenosine A_{2A} receptor, ZM 241385, were studied within this model of kainate excitotoxicity (fig. 17). When given alone ten minutes prior to the initial injection of kainate, ZM 241385, at an i.p. dose of 10 mg. kg^{-1}, displayed significant protection against kainate-induced toxicity in the CA3a areas of the hippocampus (4.3 % ± 2.8, $p < 0.05$, $n = 7$; fig. 19). A reduction in damage to 0.71 % ± 0.71 was observed in the CA2 region, although due to the lower amount of kainate-induced damage in this region, this reduction was not significantly different. In the CA1 region damage decreased non-significantly to 19.3 % ± 12.6 (fig. 18).

This protection of the CA3a region by ZM 241385 was not apparent after the further addition of 50 μg. kg^{-1} CPX, where damage at 27.5 % ± 20.1 ($n = 4$; fig. 20), was not significantly different from kainate alone. Damage in the CA1 region was observed at 33.8 % ± 20.8 (fig. 21).

The A_{2A} antagonist compound was also injected in conjunction with the A_{2A} agonist CGS 21680. At 10 mg. kg^{-1} ZM 241385 administered with 0.1 mg. kg^{-1} CGS 21680 displayed total protection against kainate. In none of the animals studied ($n = 5$) was any neuronal damage noted in any region of the hippocampus. While this was significant in the CA1 ($p < 0.01$) and the CA3a ($p < 0.05$), this was not significantly different from kainate in the CA2 region.

Fig. 17 Protection against kainate–induced neurotoxicity by i.p. injection of the A2A receptor antagonist ZM 241385. ** $p<0.01$, * $p<0.05$ versus kainate.



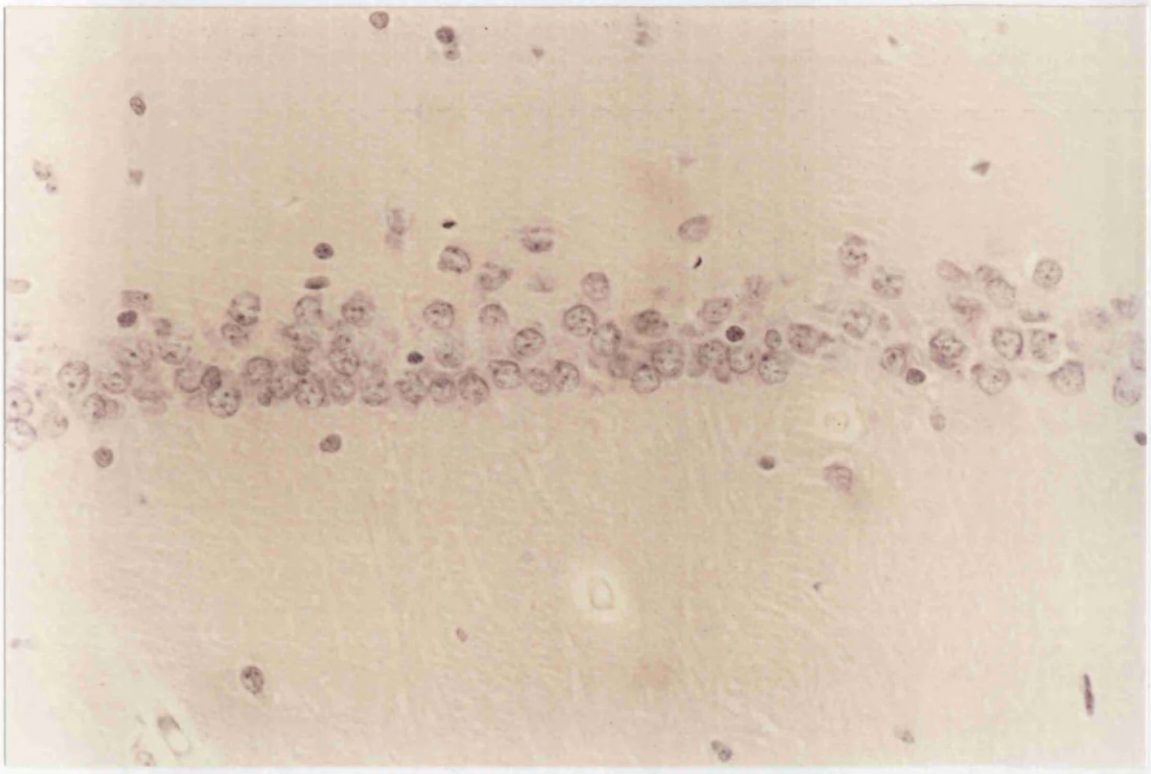


Fig. 18 CA1 region following i.p. administration of ZM 241385 and kainate. While the above photomicrograph does not show the presence of damaged neurones, overall, the level of neuronal death observed with 10 mg. kg^{-1} ZM 241385 + 10 mg. kg^{-1} kainate did not differ from that of kainate alone. 20 mm : 40 μm.

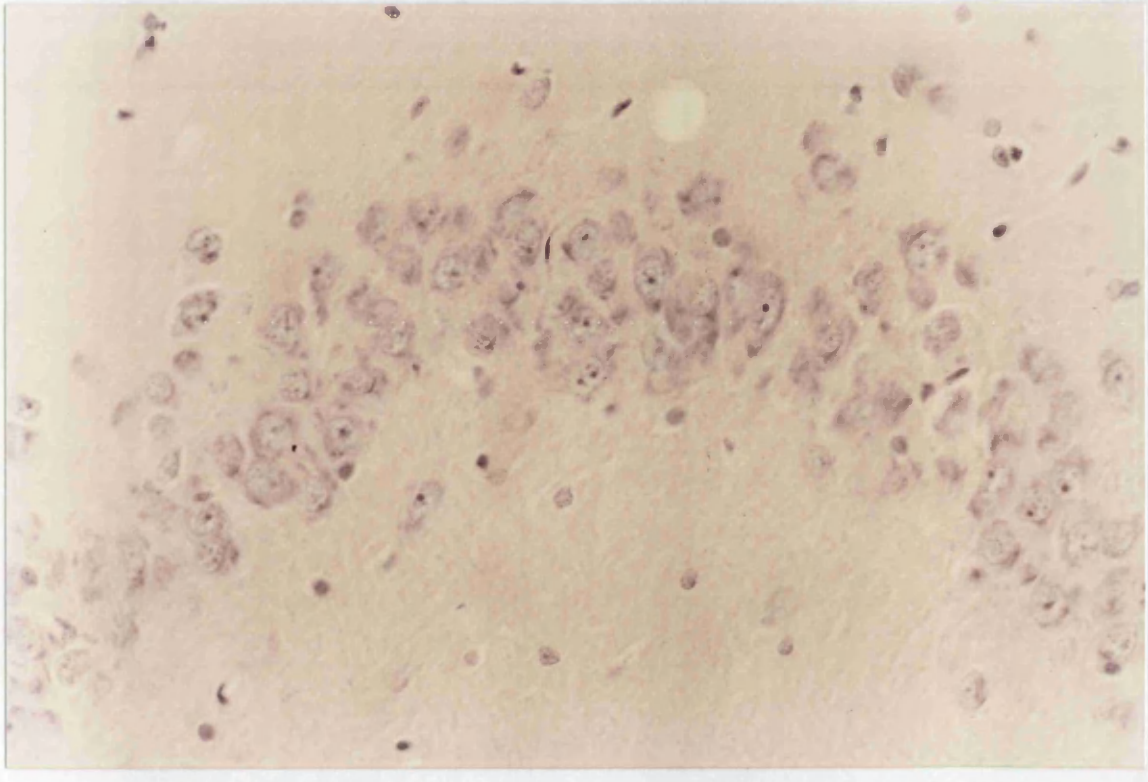


Fig. 19 Protection against kainate-mediated excitotoxicity by ZM 241385 (i.p.) in the CA3a region. The lack of damage above is representative of the group as a whole (n=7), with levels of damage seven days after the injection of 10 mg. kg^{-1} ZM241385 + 10 mg. kg^{-1} kainate approaching those of kainate alone. 20 mm : 40 μm.

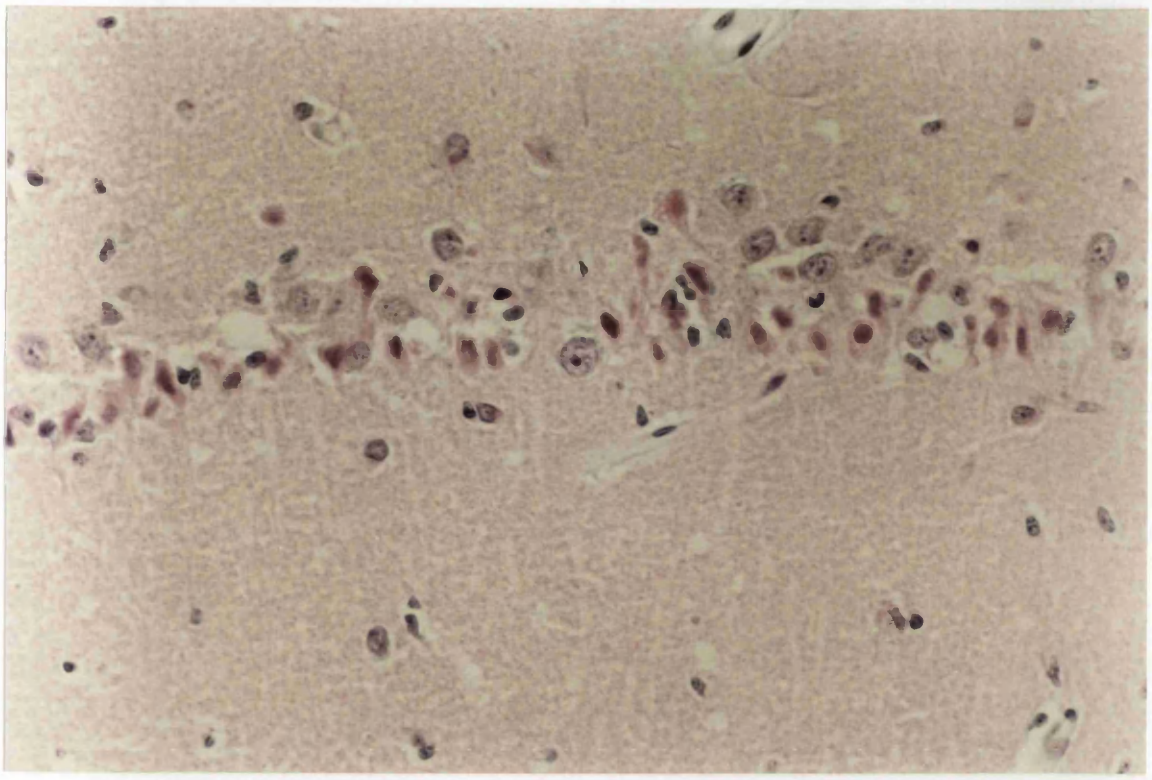


Fig. 20 CA1 region following the i.p. administration of ZM 214385, kainate and the A_1 antagonist CPX. A large number of eosin stained neurones are apparent with the co-administration of 50 µg. mg^{-1} CPX with 0.1 mg. kg^{-1} ZM 241385 and 10 mg. kg^{-1} kainate. The level of excitotoxicity is similar to that observed after kainate alone. 20 mm : 40 µm

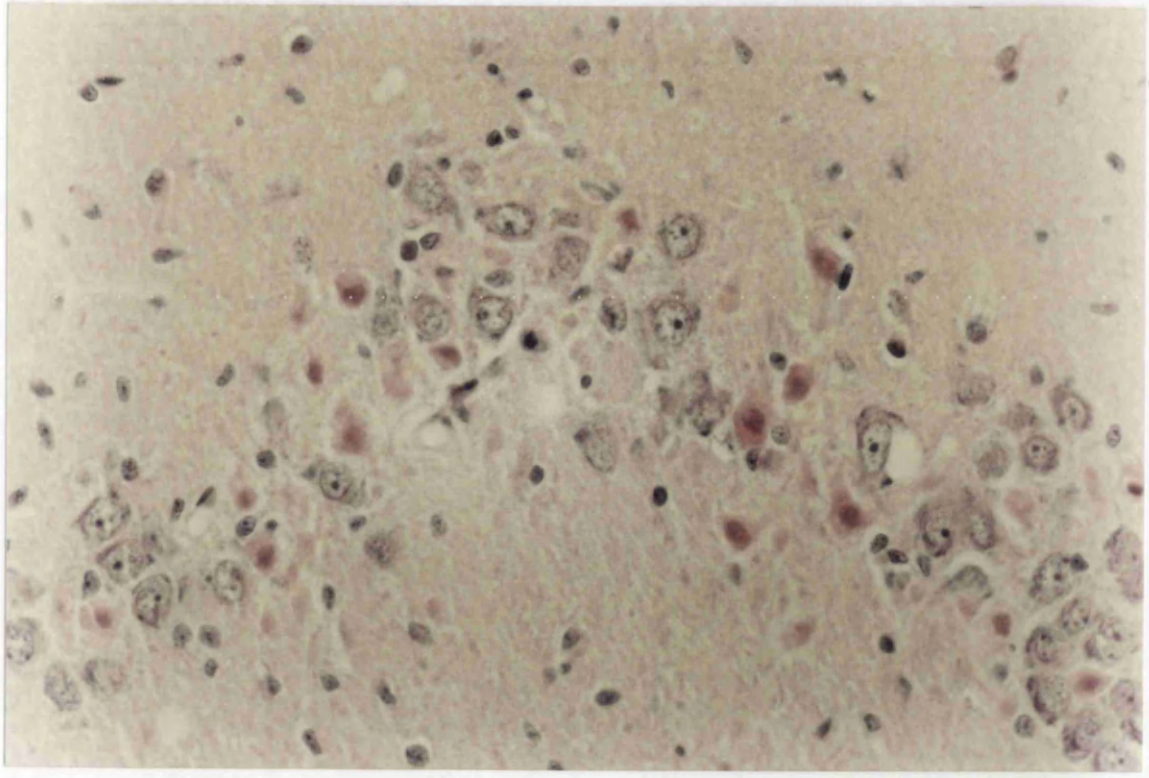


Fig. 21 Lack of protection by ZM241385 in the CA3a region following co-administration of CPX (i.p.). As for the CA1 region above, a large number of non-viable cells are observed following 50 µg. kg^{-1} CPX + 10 mg. kg^{-1} ZM241385 + 10 mg. kg^{-1} kainate. SCALE

Intrahippocampal injections

Kainate

Alongside studies using systemic administration, intrahippocampal injections were also utilised. Two early experiments in which an infusion of 10 nmol kainate in 1μl was made, revealed an ipsilateral pattern of complete neuronal degradation in almost all regions of the hippocampus (only the CA2 region had damage less than 100 % in both experiments, where in one study the damage was recorded as 80 %). Subsequent studies used lower concentrations of 1 and 0.25 nmol. Animals were killed and perfused at either two or seven days after the initial injection (fig. 22).

Experiments with 1 nmol kainate revealed the greatest amount of damage in the CA3a region of the hippocampus. In the CA3a region, the levels of damage were 90.0 % ± 6.7 after two days ($p < 0.01$ compared to saline controls, $n = 3$) and 100 % ± 0 after seven days ($p < 0.01$, $n = 4$). In the CA3b region, damage was assessed as 73.3 % ± 14.5 after two days, but this declined to a level of 25.0 % ± 25.0 after seven days. No significant damage occurred in the CA1, CA2 or CA4 regions.

Studies using the lower, 0.25 nmol, concentration of kainate were also terminated after either two or seven days. Similar results were observed between two and seven days. As for 1 nmol kainate, the highest degree of damage was noted in the CA3a region of the hippocampus after 0.25 nmol injection. After two days damage was 96.7 % ± 3.3 ($p < 0.001$ compared with saline controls, $n = 3$), and 93.4 % ± 2.7 ($p < 0.001$, $n = 10$) after seven days. Values for the CA3b region are also similar between regimens (56.7 % ± 23.3 for two days and 50.0 % ± 8.0 for seven; see fig 24 for photomicrograph of CA3 region of animal injected with 0.25 nmol kainate, killed

after seven days). Again, while small amounts of damage were noted in the remaining regions of the hippocampus, none were significantly different from saline control levels.

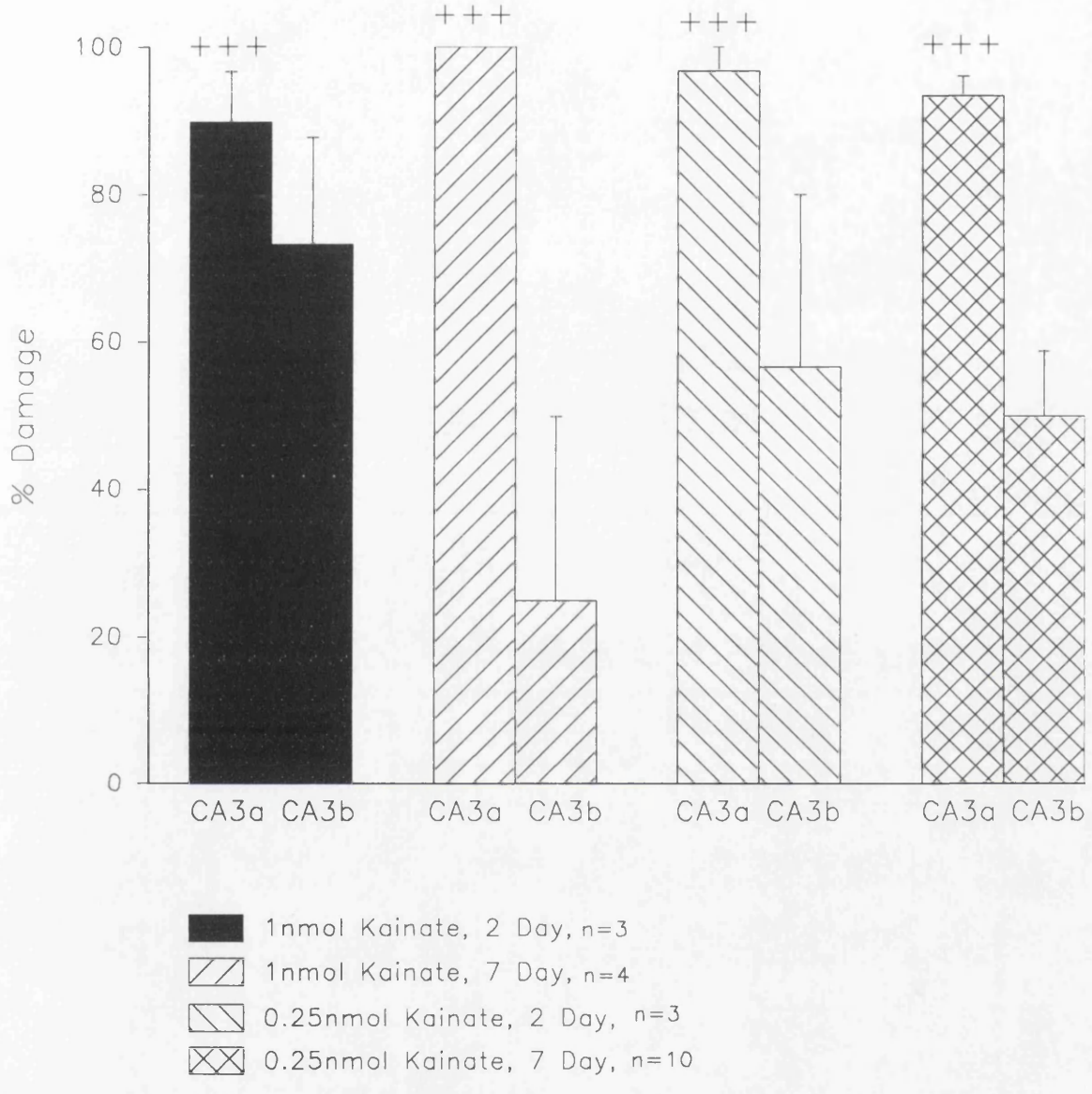
In the remaining experiments kainate was used at the lower concentration of 0.25 nmol to provide a model of near complete neurodegeneration of the CA3a region by excitotoxic mechanisms.

Adenosine A_{2A} agonist, CGS 21680

CGS 21680 was administered with 0.25 nmol kainate at concentrations of 0.01, 0.1, 0.25 and 2.5 nmol (fig. 25). When compared to kainate alone, none of the concentrations alleviated excitotoxic effects. In the CA3a region, CGS 21680 at a concentration of 0.01 nmol produced damage of 75.0 % ± 25.0 (n = 4), 0.1 nmol resulted in 85.0 % ± 10.2 damage (n = 4), 0.25 nmol in 95.0 % ± 2.9 and 2.5 nmol in 100 % ± 0 (fig. 26).

In the CA3b region the highest amount of damage was found with the lowest concentration (71.3 % ± 23.8), closely followed by the largest concentration used (68.3 % ± 8.8). The two intermediate doses (0.1 and 0.25 nmol) induced less damage than the two extreme concentrations, but still offered no significant protection over kainate (28.8 % ± 10.9 and 36.7 % ± 31.8). No significant damage was noted in any of the remaining hippocampal regions.

Fig. 22 Excitotoxic damage to the CA3a and CA3b regions of the hippocampus after central injection of kainate. Animals were killed two or seven days after the initial injection. +++ $p<0.001$ versus saline controls.



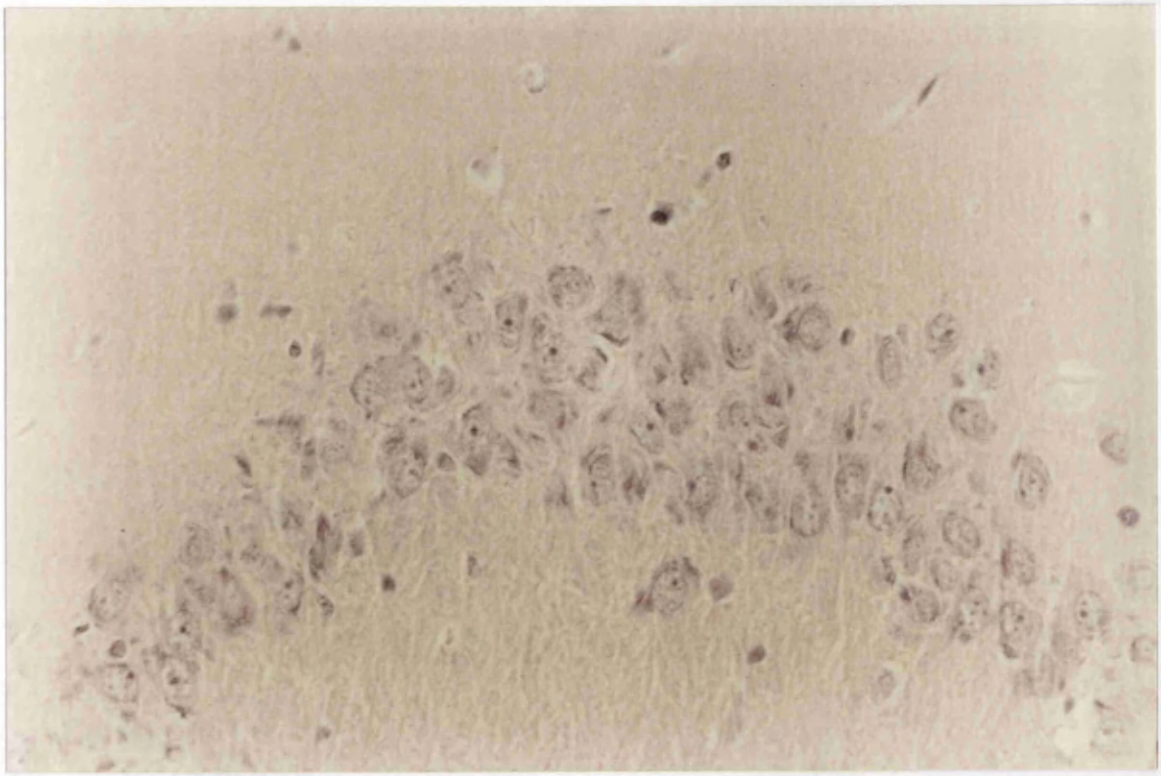


Fig. 23 Photomicrograph of the CA3a region following intrahippocampal injection of saline. The neurones show similar morphology to those after i.p. saline, and do not display any indications of neuronal degradation. 20 mm : 40 μm

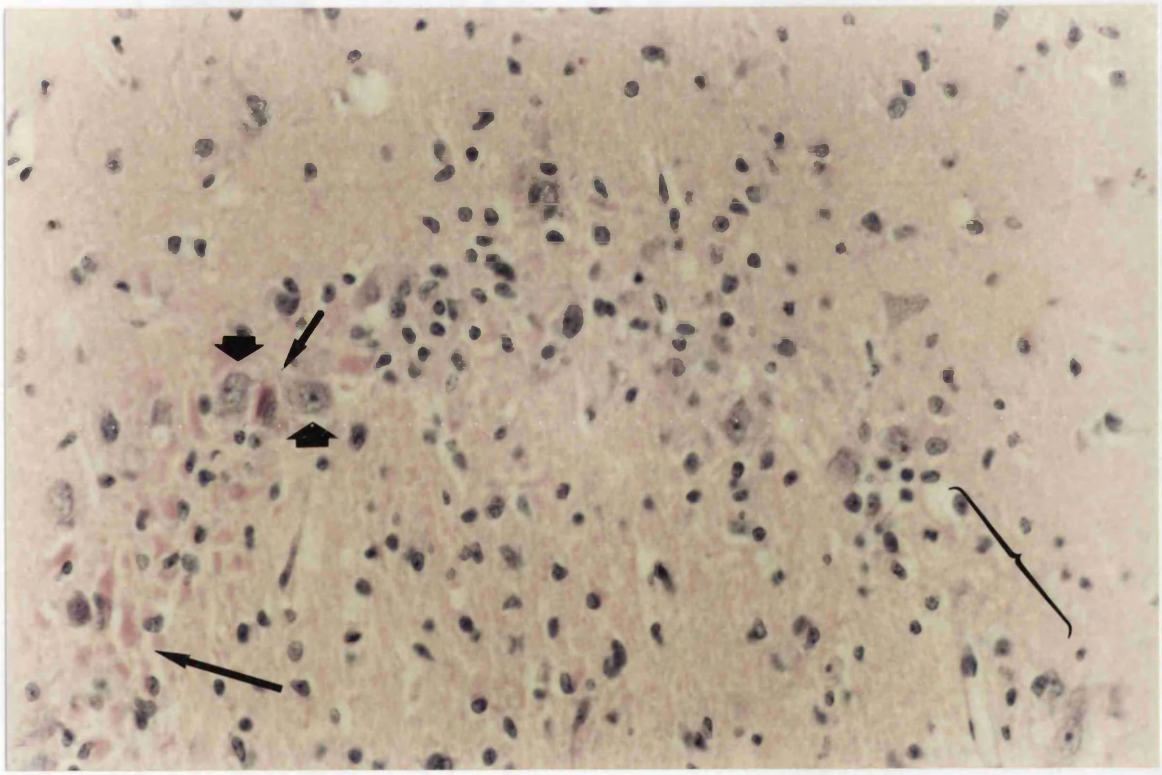
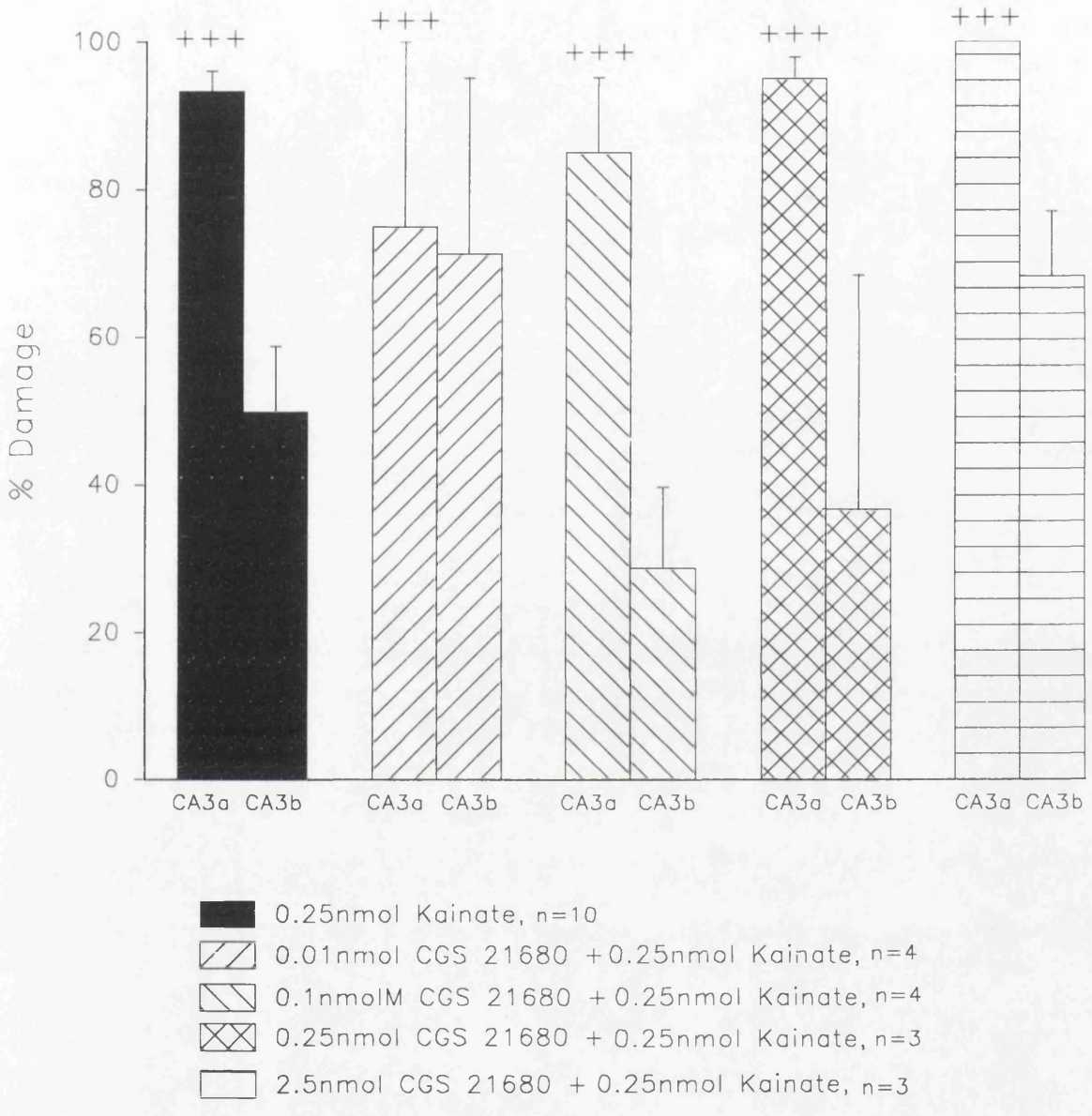


Fig. 24 Damaged neurones of the CA3a region seven days after i.h. injection of 0.25 nmol kainate. The photomicrograph, in contrast to the saline control above, shows very few viable neurones (thick arrows) as well as a number of eosin stained dying cells (thin arrows). The presence of small darkly stained glial is also prominent. Importantly there are also areas of the CA3a region where there is a complete lack of neurones (e.g. see bracketed region). 20 mm : 40 μm

Fig. 25 No protection of the hippocampus from kainate toxicity after intrahippocampal administration of CGS 21680. +++ $p<0.001$ versus saline control levels.



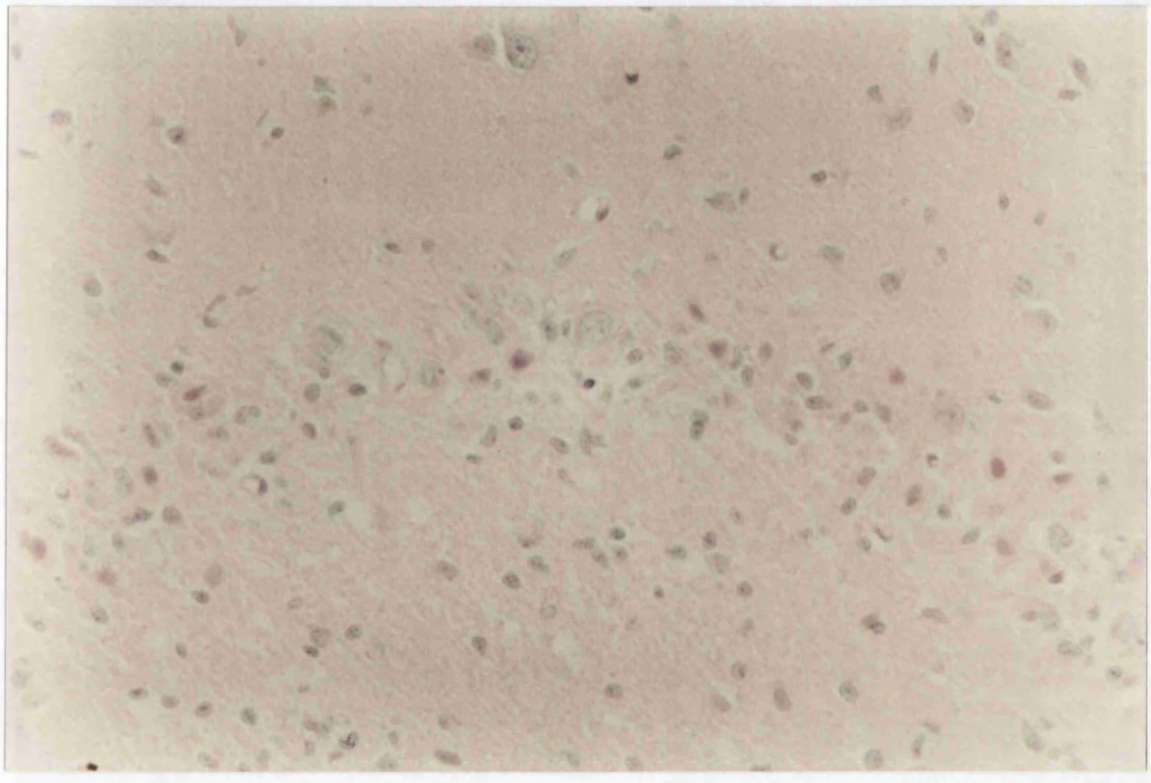


Fig. 26 Lack of protection by the A_{2A} agonist CGS 21680. The photomicrograph of 2.5 nmol CGS 21680 + 0.25 nmol kainate closely resembles that of kainate alone. Almost complete degradation of the region is apparent. As for kainate alone, areas void of neurones can be discerned, as well as an increase in glial cells above that seen in the saline control. 20 mm : 40 μm

Adenosine

The effects of adenosine itself were also examined within the intrahippocampal kainate injection model of excitotoxicity. Adenosine was administered in conjunction with kainate, at three concentrations, 0.01, 0.25 and 2.5 nmol (fig. 27). Although all three reduced the mean level of neuronal damage in the CA3a region, none modified damage to a level which was significantly different from kainate alone. In the CA3a region, adenosine at 2.5 nmol reduced damage to a value of 66.7 % ± 19.6 (n = 6; fig. 31), 0.25 nmol to 75.0 % ± 13.3 (n = 7) and 0.01 nmol to 70.6 % ± 12.6 (n = 8). The kainate-associated damage observed after adenosine administration in the CA3b was 28.3 % ± 16.0 for the highest concentration, 38.6 % ± 14.6 for 0.25 nmol and 41.3 % ± 13.1 for 0.01 nmol. Again, even though the values obtained for damage in the CA3b after kainate administration were lower in the presence of adenosine, none of the concentrations used induced a significantly different amount of damage. No significant increase in damage was observed in the CA1, CA2 or CA4 region.

A_1 adenosine receptor agonist, R-PIA

The adenosine A_1 receptor agonist, R-PIA, was investigated at concentrations of 0.01 and 0.25 nmol (n = 3 for both; fig 28). R-PIA was infused via an intrahippocampal injection with 0.25 nmol kainic acid. The purine agonist did not protect the CA3a region of the hippocampus to any degree. Values of 96.7 % ± 3.3 and 88.3 % ± 6.0 were obtained for damage after R-PIA administration at 0.25 (fig. 32) and 0.01 nmol respectively. Differences in damage appeared in the CA3b region. 0.01 nmol

adenosine significantly reduced damage to 13.3 % ± 8.8 ($p < 0.05$), while 0.25 nmol adenosine tended to increase kainate damage (80.0 % ± 5.8). As with kainate alone, neither R-PIA at 0.1 nor 0.25 nmol resulted in significant damage in the CA1, CA2 or CA4 regions.

Adenosine A_{2A}-selective antagonist, ZM 241385

The selective A_{2A} receptor antagonist compound ZM 214385 was administered as an intrahippocampal injection at concentrations of 0.05, 0.25 and 2.5 nmol (fig. 29). At 2.5 nmol ZM 241385 significantly protected both the CA3a and CA3b regions of the hippocampus (32.9 % ± 15.8 and 9.3 % ± 4.7, respectively, n = 7) against 0.25 nmol kainate ($p < 0.01$; fig. 33). While both 0.25 nmol and 0.05 nmol tended to show a decrease in damage to the CA3a region (65.7 % ± 17.6, n = 6 and 88.3 % ± 1.7, n = 3, respectively), neither was significantly different from kainate alone. No reduction was observed with either of the two lower concentrations in the CA3b region (42.1 % ± 12.4 for 0.25 nmol and 58.3 % ± 8.3 for 0.05 nmol dose). No neuronal damage was noted in any other hippocampal areas, with any concentration.

NMDA-antagonist MK-801

By far the greatest effect of any drug used in alleviating kainate excitotoxic damage was the NMDA receptor antagonist MK-801 (fig. 30). MK-801 was given at both a high (2.5 nmol) and a low (0.01 nmol) dose in conjunction with kainate. Both doses were extremely significant ($p < 0.001$) in reducing CA3a neurodegeneration (3.3 % ±

3.3 for the 2.5 nmol dose (fig. 34) and 10.0 % ± 5.8 for the 0.01 nmol dose). While both doses displayed a reduction in CA3b damage (3.3 % ± 3.3 for the 2.5 nmol dose and 20 % ± 11.5 for 0.01 nmol MK-801), only 2.5 nmol MK-801 was significantly different from damage induced by kainate alone.

Extrahippocampal damage

Other areas of the brain in the same plane of section as the hippocampus (-3.60 from bregma) were also examined for damage using haematoxylin and eosin staining after both i.p. and i.h. injections. Within the hippocampus itself, no other regions of damage were apparent after i.p. or i.h. injections, including the dentate gyrus which appeared to be resistant to all regimens and concentrations of kainate used.

I.p. administration of 10 mg. kg^{-1} kainate proved neurotoxic to discrete populations of cells outwith the hippocampus. Cells within the distinct formation of the pyriform cortex and posterolateral and posteromedial cortical amygdala displayed degrees of damage ranging from 0 to 85 % (fig 35 & 36). Damage within the pyriform cortex displayed a significant linear relationship with that of the CA3a region (fig. 37; slope = 0.71; p = 0.0055) such that damage was only observed in the pyriform when there was a corresponding degree of neurodegeneration in the hippocampus. When examined after administration of kainate in conjunction with other compounds (e.g. CGS 21680) damage to this area was usually only apparent when there was an occurrence of neurotoxicity to the hippocampus. Protection of this region also occurred in parallel to the hippocampus. No compound showed selective preferential

Fig. 27 Lack of protection against kainate following the intra-hippocampal injection of adenosine. +++ $p<0.001$, ++ $p<0.01$ versus saline.

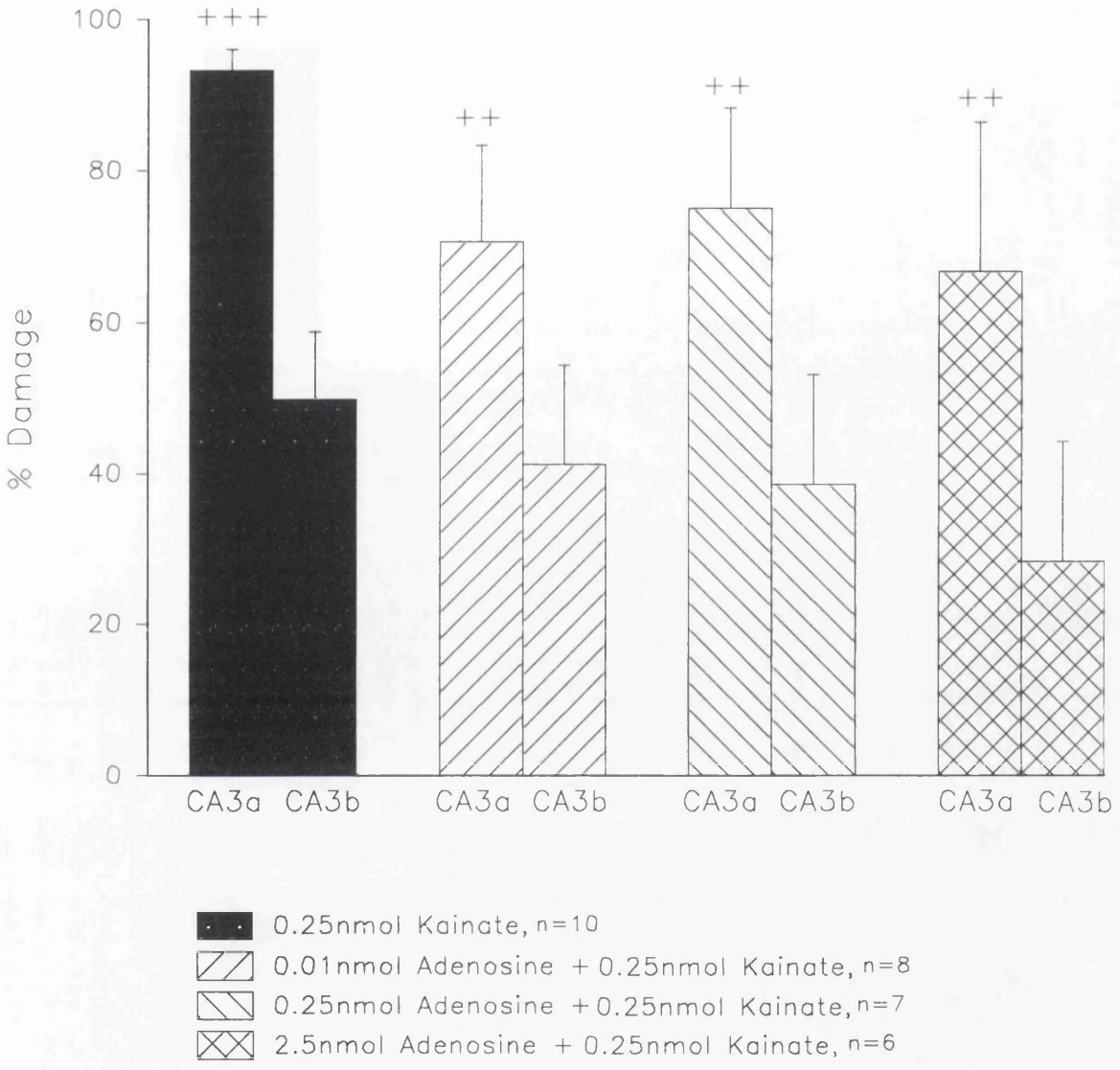


Fig. 28 Protection in the CA3b region only by low intrahippocampal doses of the A1 agonist R–PIA. * $p<0.05$ versus kainate alone. +++ $p<0.001$, ++ $p<0.01$, + $p<0.05$ versus saline control.

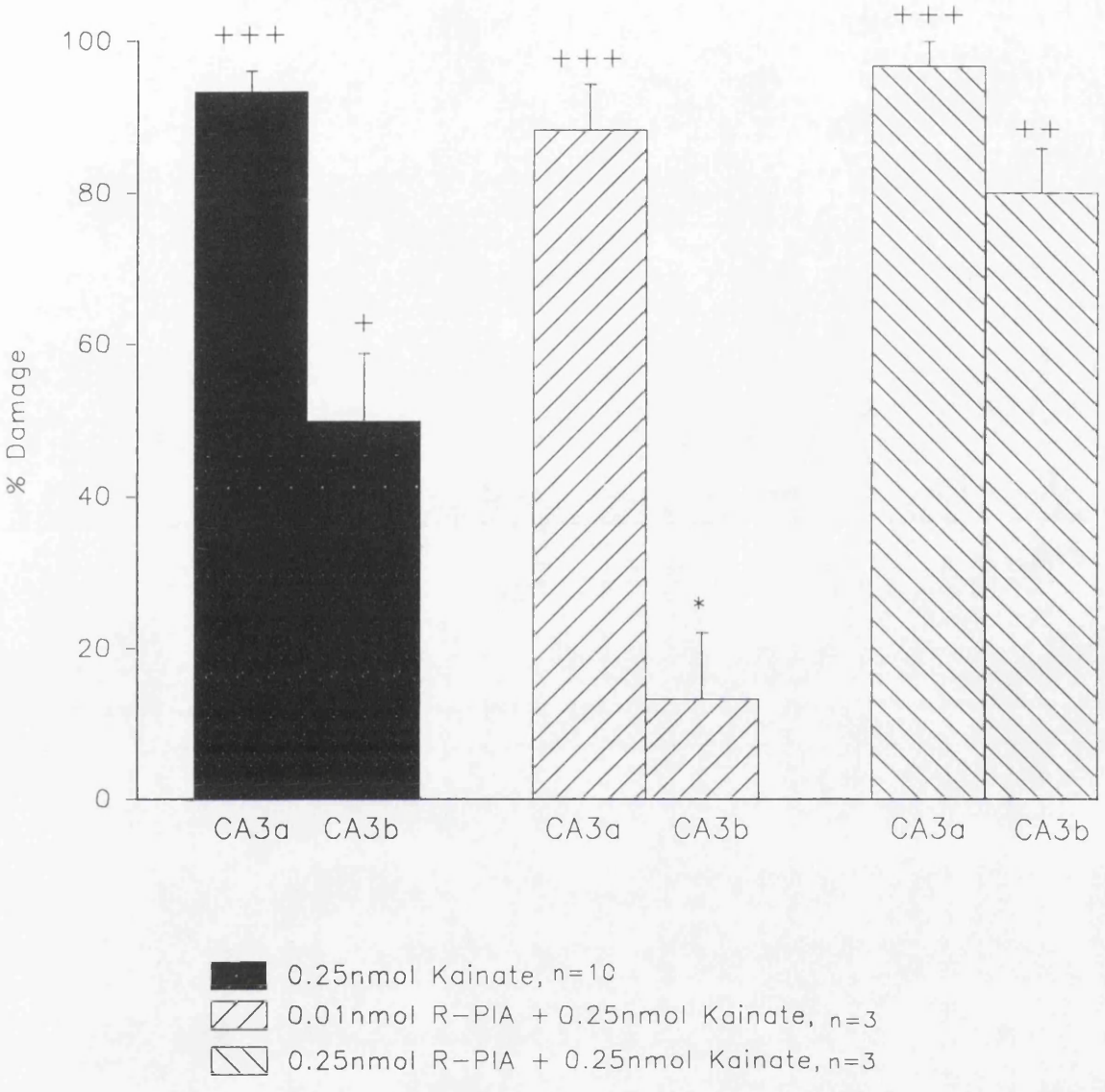


Fig. 29 Protection against intrahippocampal kainate-induced neuronal damage by the selective A2A antagonist ZM 241385. ** $p<0.01$ versus kainate alone; ++ $p<0.01$, + $p<0.05$ versus saline.

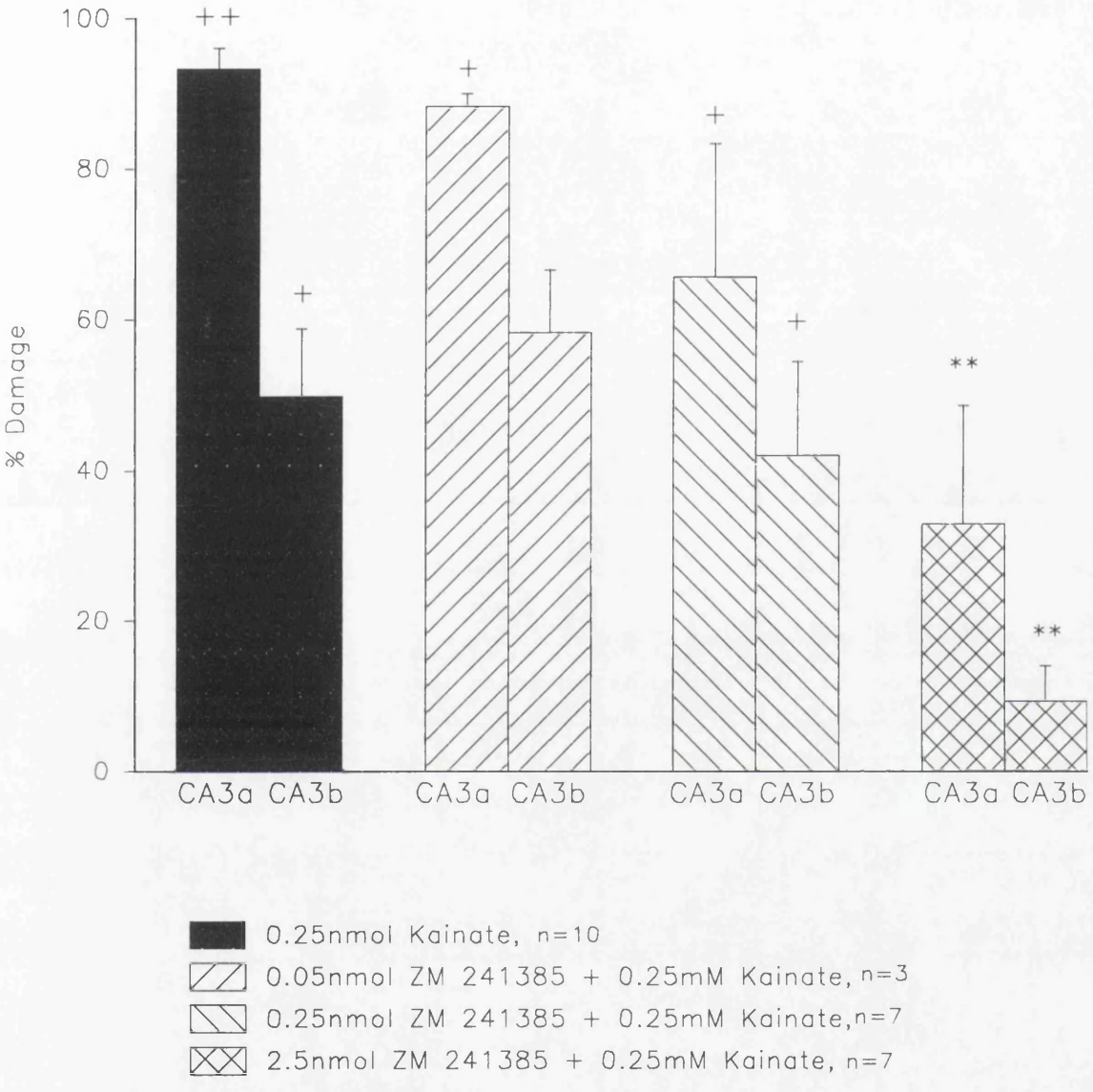
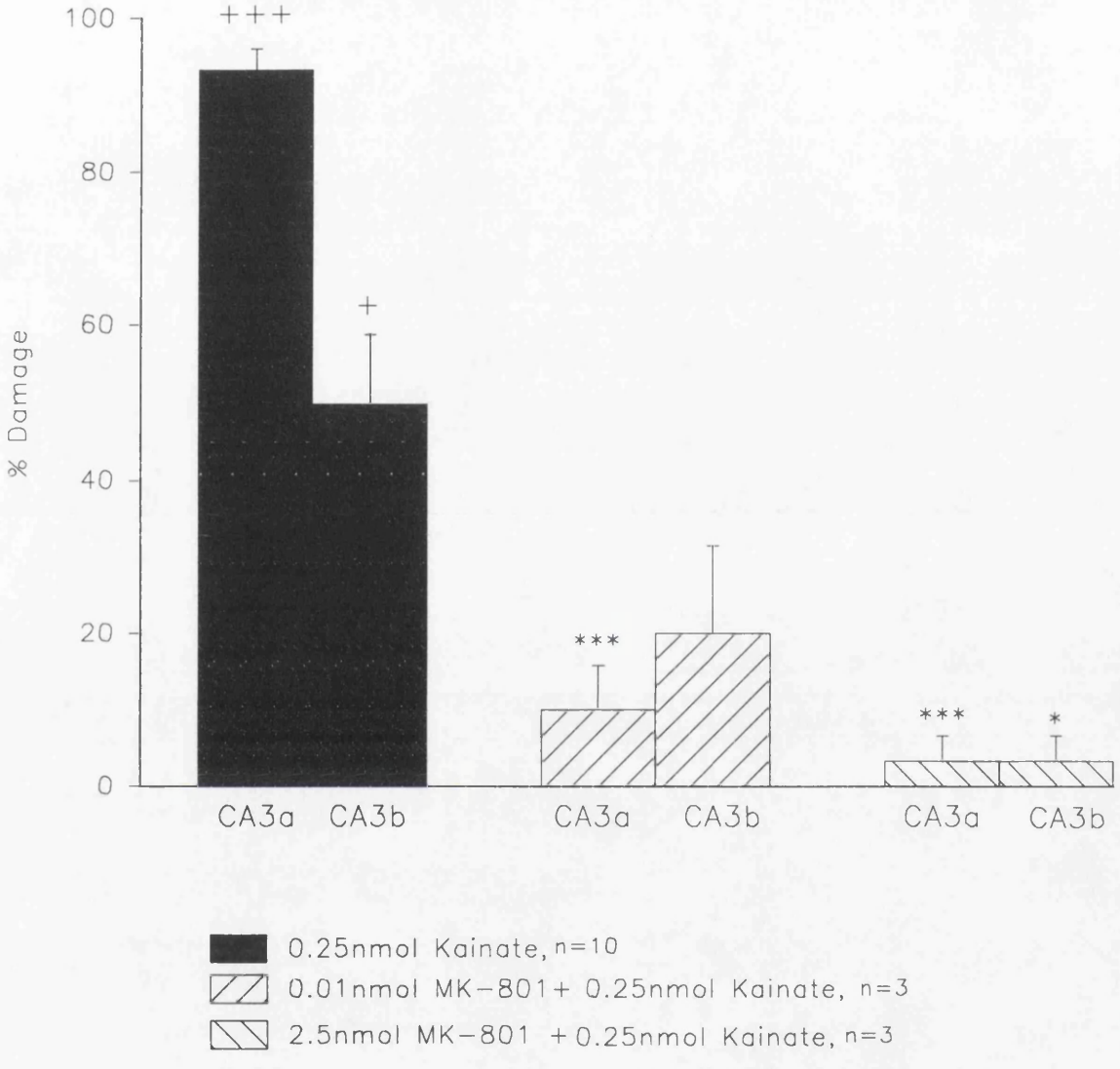


Fig. 30 Intrahippocampal administration of the NMDA antagonist MK−801 protected against kainate−induced excitotoxicity. *** $p<0.001$, * $p<0.05$ as compared with kainate, +++ $p<0.001$ versus saline.



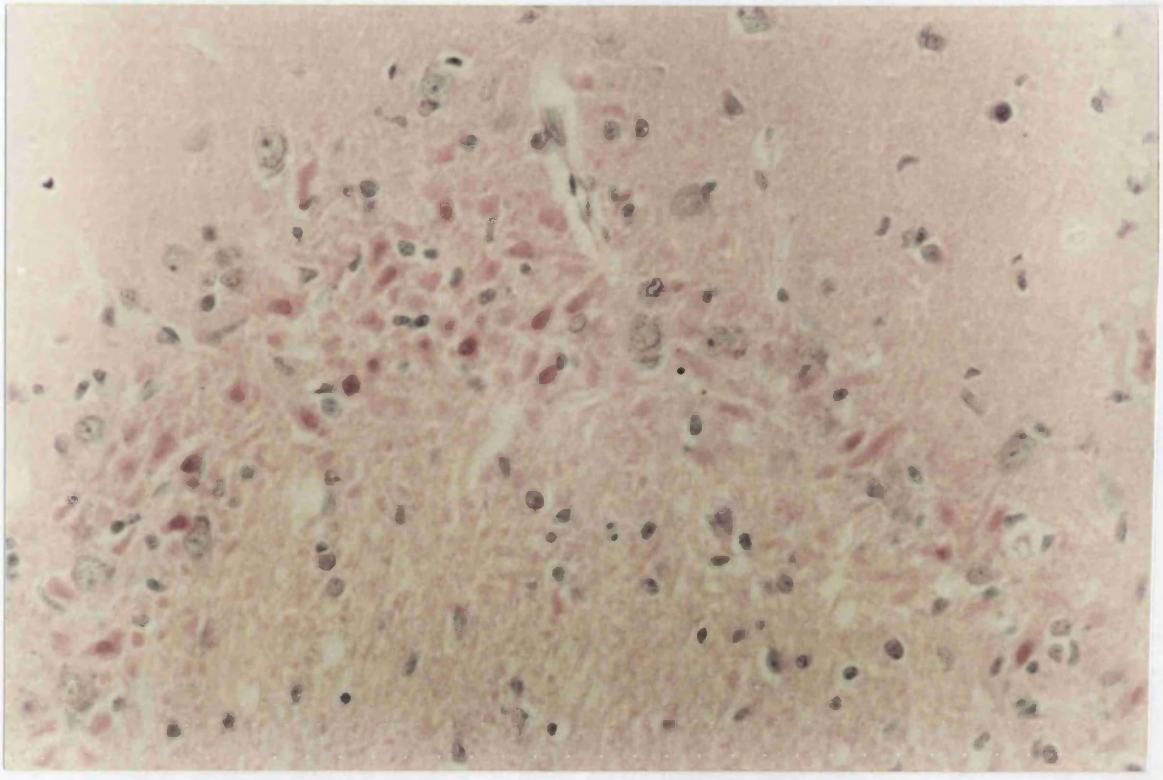


Fig. 31 CA3a region following the i.h. administration of adenosine + kainate. A large number of non-viable cells are apparent throughout this region seven days after an injection of 2.5 nmol adenosine + 0.25 nmol kainate. There is almost a complete absence on blue stained viable cells, showing a lack of protection by adenosine itself. 20 mm : 40 μm.

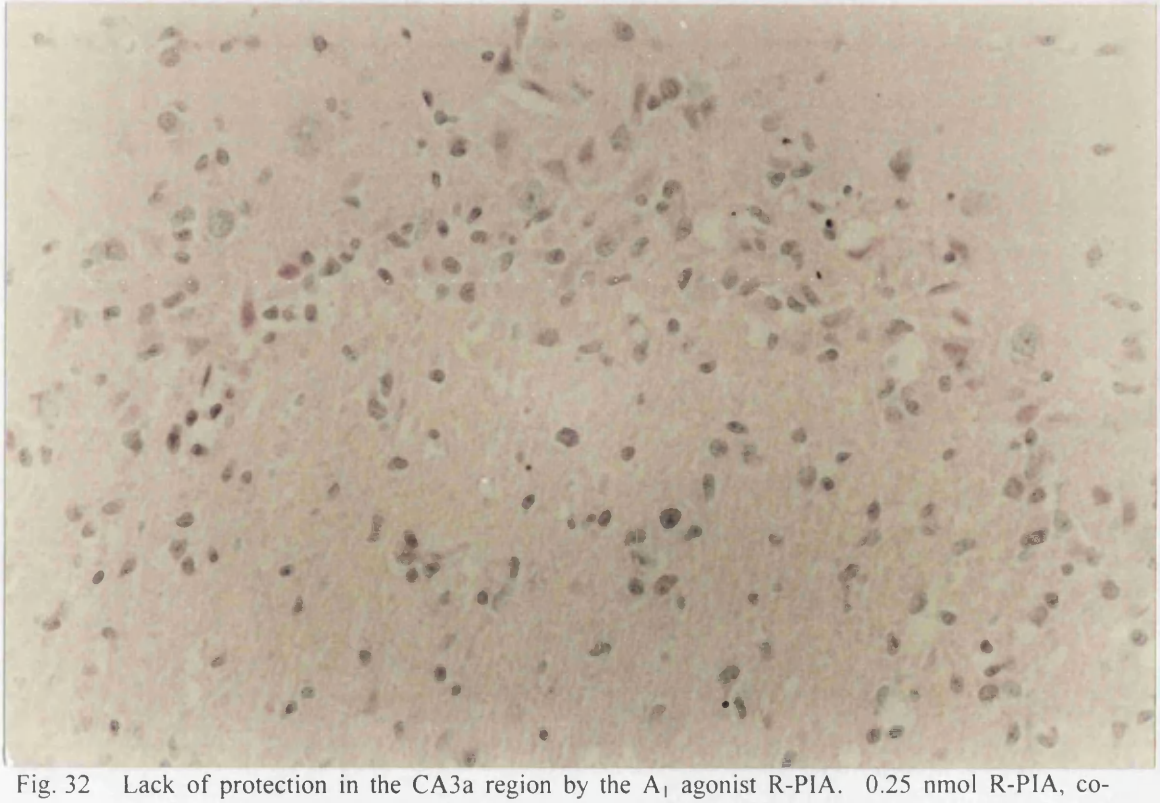


Fig. 32 Lack of protection in the CA3a region by the A_1 agonist R-PIA. 0.25 nmol R-PIA, co-injected with 0.25 nmol kainate did not alter the morphology associated with kainate excitotoxicity, with almost complete degradation of this area. 20 mm : 40 μm.

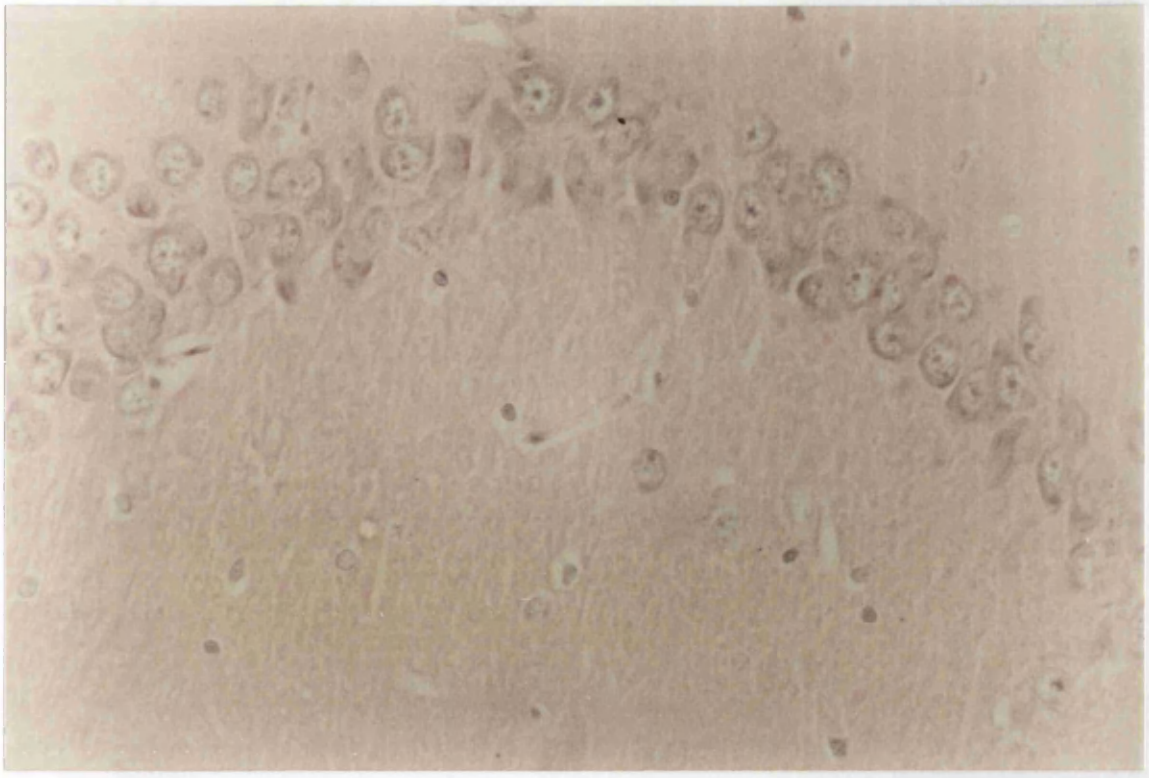


Fig. 33 Protection from kainate toxicity in the CA3a region following the intrahippocampal co-administration of ZM 214385. The above photomicrograph, shows that seven days after the i.h. injection of 2.5 nmol ZM 241385 with 0.25 nmol kainate, there is no damage to the CA3a region. All of the above neurones appear viable and resemble those of the saline control. 20 mm : 40 μm.

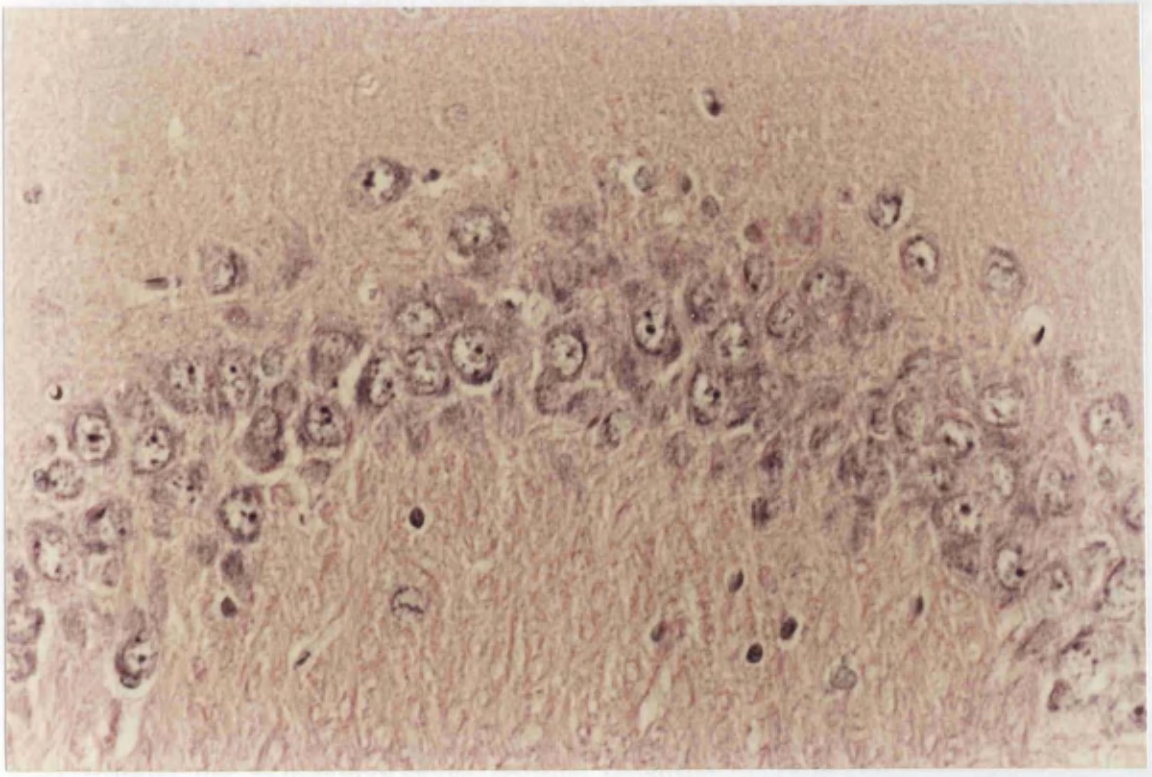


Fig. 34 Photomicrograph of the CA3a region protected by the co-injection of MK-801 with kainate (i.h.). MK-801 at 2.5 nmol prevented the appearance of damaged neurones induced by kainate alone. The region closely resembles that of the saline controls. 20 mm : 40 μm.

protection of either the hippocampus, the pyriform cortex or any other region selectively toxic to kainate.

The excitotoxic effects of kainate to the pyriform cortex or cortical amygdala were not observed after intrahippocampal injections. No other extrahippocampal, nor intrahippocampal areas (apart from those mentioned above) were sensitive to centrally administered kainate. The above figures for damage after intrahippocampal kainate, refer to damage on the ipsilateral hemisphere of the rat brain. Examination of the contralateral hemisphere revealed no damage in either the hippocampus or in any other region on the same plane of section.

Astrocytes in the brain after kainate injection

Due to the suggested importance of astrocytes in cerebral insults, the astrocytic population was observed after haematoxylin and eosin staining. No increase of astrocyte-typical cells (small, dark bodies under light microscopy) was observed after i.p. administration of kainate in the hippocampus compared with saline controls, except occasionally a small increase directly around areas of cell damage (see above photomicrographs). In i.h. studies, a larger number of cells were apparent around the CA3 region in damaged brains (see fig. 24). None were observed in any regimen without corresponding damage. None of the compounds which proved protective increased the number of astrocytes without the presence of damaged neurones. When examining the extrahippocampal regions sensitive to kainate a large number of astrocytic-like cells were apparent when damage was observed. Again, no protective compound increased the number of astrocytic-like cells.

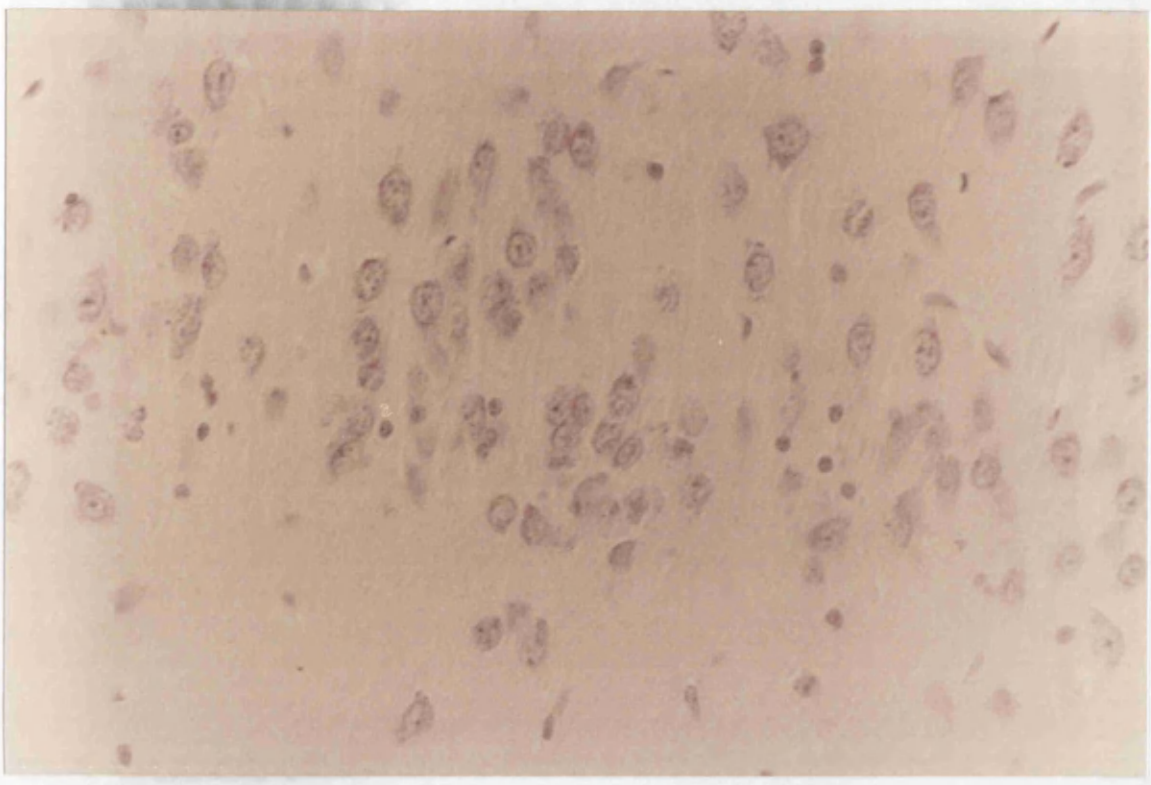


Fig. 35 Photomicrograph of the pyriform cortex from an animal injected with saline (i.p.). The haematoxylin and eosine staining of the control pyriform region shows blue viable cells, without the presence of compromised neurones. The morphology of cells in this region is similar to that of the control hippocampus. 20 mm : 40 μm.

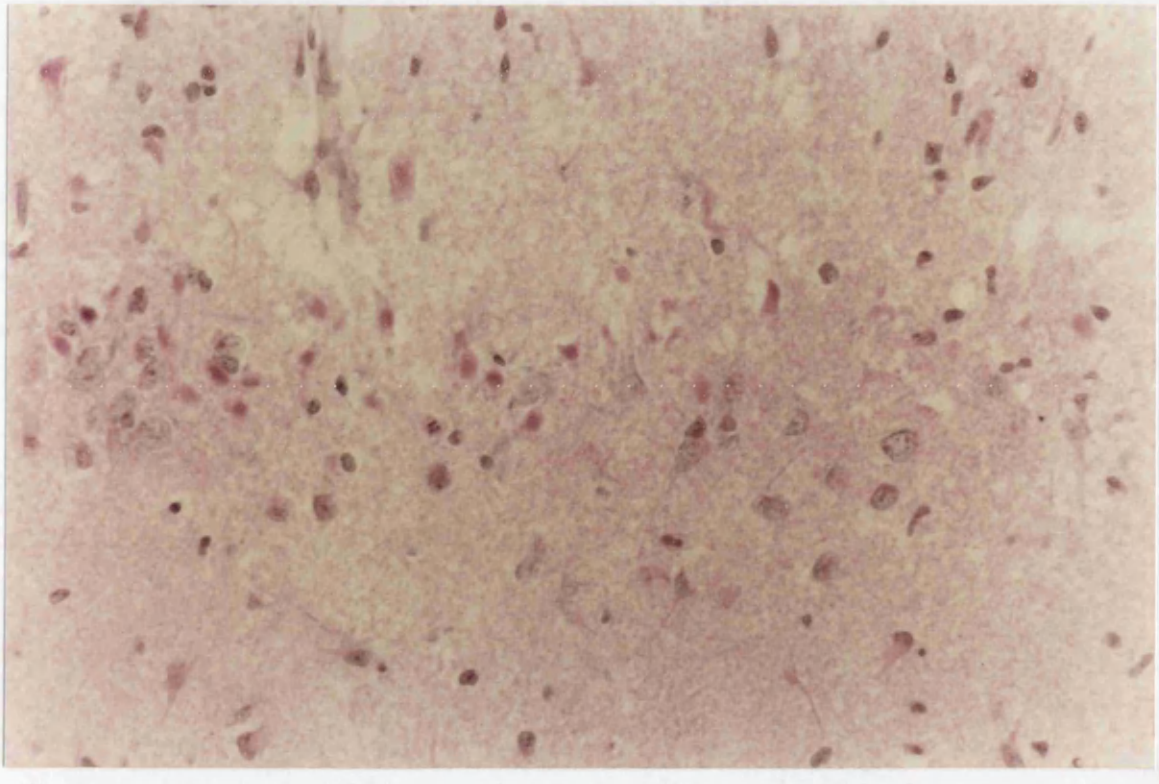
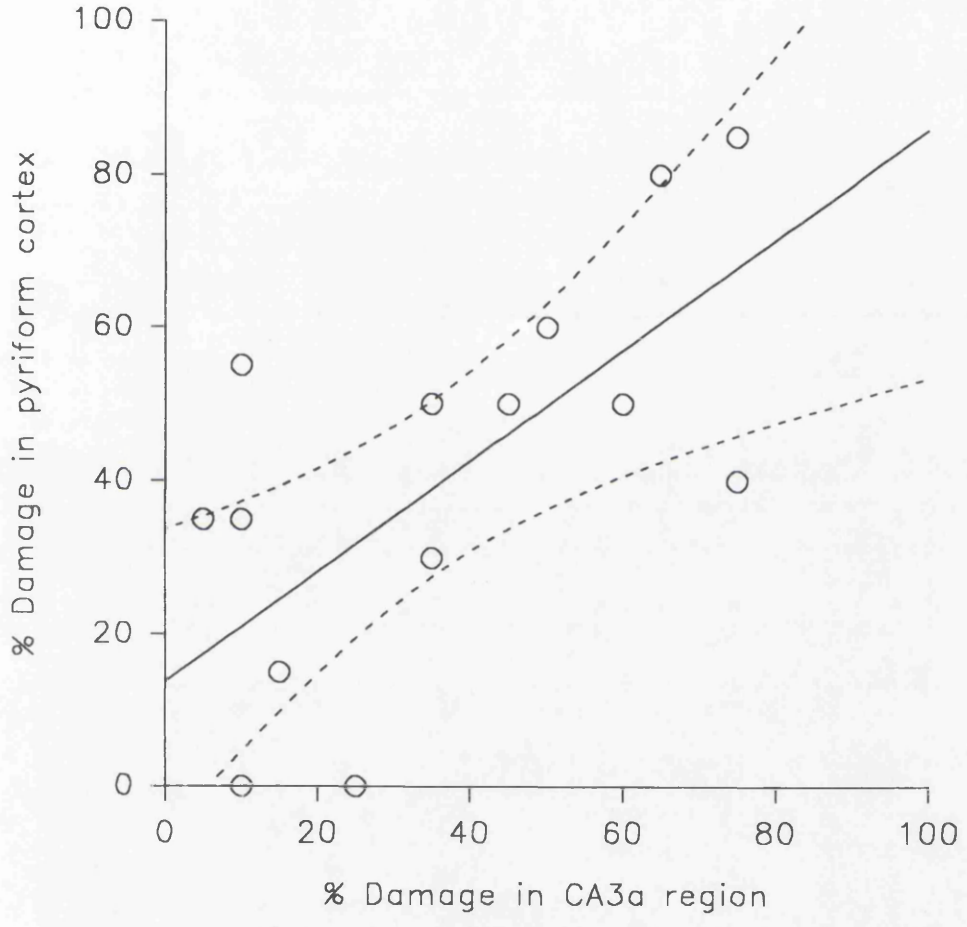


Fig. 36 Pyriform region following peripherally injected kainate. In stark contrast to the control section above, the addition of 10 mg. kg-1 kainate induces widespread neuronal cell death. Very few viable cells are apparent amonst the eosin stained compromised neurones, and large gaps devoid of cells can also be observed. 20 mm : 40 μm.

Fig. 37 Correlation between damage of the pyriform cortex with that of the CA3a region of the hippocampus. Values are percentage damage as assessed by haematoxylin and eosin staining.



TUNEL-POSITIVE CELLS

Intraperitoneal injections

For a number ot sections previously stained with haematoxylin and eosin to determine damage, serial sections were stained with the TUNEL method to identify apoptotic cells (table 1). These serial sections were no more than 20 μm anterior or posterior to the haematoxylin and eosin stained section . For determining whether 10 mg. kg^{-1} kainate induced apoptosis, four sections were used. As determined from haematoxylin and eosin studies, two of these sections were from a hippocampus which was highly damaged (90 % : 75 % and 60 % : 75 % damage for the CA1 : CA3a regions in the two sections respectively) and two represented the hippocampus from an animal with moderate to low damage (50 % : 5 % and 25 % : 10 %). For comparison, fig. 39 & 40 and fig. 41 & 42 are the CA1 and CA3a regions of TUNEL stained sections from a saline treated and a kainate treated animal.

TUNEL staining of brain sections from these animals revealed apoptotic cells only in the CA1 region. This staining was observed only in the three animals where previous haematoxylin and eosin staining of serial sections had observed moderate to high levels of damage in the CA1 region (50-90 %) and not in the animal with damage of 25 %. No apoptotic cells were apparent in the CA3a region (or any other pyramidal region) in any of the kainate treated animals. This increase in TUNEL positive staining in the CA1 region was not observed in animals treated with saline only.

Similarly, TUNEL staining experiments with CGS 21680 (0.1 mg. kg^{-1}) + kainate (10 mg. kg^{-1}) and ZM 241385 (10 mg. kg^{-1}) + kainate (10 mg. kg^{-1}), were performed on a

number of sections covering a range of damage levels. For CGS 21680 (0.1 mg. kg^{-1}) + ZM 241385 (10 mg. kg^{-1}) + kainate (10 mg. kg^{-1}) no animal displayed any hippocampal damage (from haematoxylin and eosin stained sections) and therefore TUNEL stained sections did not reflect this neurodegenerative range.

Only one of these sections stained for TUNEL displayed any apoptosis in the CA1 region, and then the amount was low The amount of damage in this ZM 241385 (10 mg. kg^{-1}) + kainate(10 mg. kg^{-1}) treated animal was 10 %. In the CA3a region the percentage of TUNEL positive cells correlated to damage (from haematoxylin and eosin stained sections).

In all the above experiments, a linear correlation between the percentage of apoptosis and damage was observed (fig. 43; $p < 0.001$, slope = 0.902), such that apoptosis increases proportionally with increased damage. In only one instance was the presence of TUNEL staining apparent without any correlating damage. This may represent an early stage of apoptosis which haematoxylin and eosin staining does not detect.

From the table therefore, it is apparent that none of the above regimens, protective or detrimental, exerts its effect by increasing or decreasing apoptotic cell death within the CA1 above that expected (i.e. low or no damage associated with low or no TUNEL staining, and moderate to high damage with a higher presence of apoptotic neurones).

From sections counterstained with eosin after TUNEL staining, it is apparent that within the CA1, the TUNEL-positive cells were the same cells observed as damaged after haematoxylin and eosin staining (Fig. 44). While the majority of TUNEL-

positive cells were counterstained with the deep pink colour not all of the pink cells in the CA1 stained for apoptosis (10-20 % remained unstained for apoptosis). In agreement with the earlier studies above, there was the presence of pink damaged cells in the CA3a without any indication of TUNEL staining (Fig. 45).

In all the above experiments, TUNEL staining (when present) was localised in the dark nuclear matter within the cell, corresponding with the condensed chromatin feature of apoptosis.

Intrahippocampal injections

A different picture emerged after TUNEL staining of sections for intrahippocampally injected animals (table 2). After kainate injections (0.25 nmol), TUNEL-positive cells were noted only in areas of damage, including a large number along the injection tract. Damage in the CA3a region after kainate injection (0.25 nmol), observed from haematoxylin and eosin stained sections was near maximal in all animals studied (n = 3). Following TUNEL staining a large number of apoptotic cells were also apparent in the CA3a region. In the CA1 region, TUNEL staining was positive only in the one animal which displayed damage (20 %). Similarly, animals injected with CGS 21680 (2.5 nmol) and kainate (0.25 nmol) exhibited 100 % damage in the CA3a (n = 3) and a large number of apoptotic neurones. The only variation in this pattern of high damage, high TUNEL-positive staining, was observed in animals treated with ZM 241385 (2.5 nmol) with kainate (0.25 nmol), where no TUNEL-positive staining was noted in two of the three sections with previously observed CA3a neuronal damage (60-90 %).

Table 1 Table correlating serial sections stained for either damage or apoptosis after i.p. injections Values are percentage damage (determined from haematoxylin and eosin staining) or percentage of positively stained neurones (obtained from TUNEL experiments).

	CA1 Damage	CA1 TUNEL	CA3a Damage	CA3a TUNEL
Kainate 10 mg.kg-1				
	90	90	75	0
	50	25	5	0
	60	40	75	0
	25	0	10	0
Saline				
	0	0	0	0
	0	0	0	0
	0	0	0	0
CGS21680 0.1 mg. kg-1 + Kainate 10 mg. kg-1				
	45	40	10	0
	0	0	0	0
	0	0	0	0
	45	40	25	0
ZM241385 10mg. kg-1 + Kainate 10 mg. kg-1				
	0	0	0	0
	60	60	15	10
	0	0	0	0
	0	0	0	0
	75	70	15	0
CGS21680 0.1 mg. kg-1 + ZM241385 10 mg. kg-1 + Kainate 10 mg. kg-1				
	0	0	0	0
	0	0	0	0
	0	0	0	0
CGS21680 0.1 mg. kg-1 + 8-PST 20 mg. kg-1+ Kainate 10 mg. kg-1				
	50	50	25	0
	90	80	65	0
	0	0	0	0

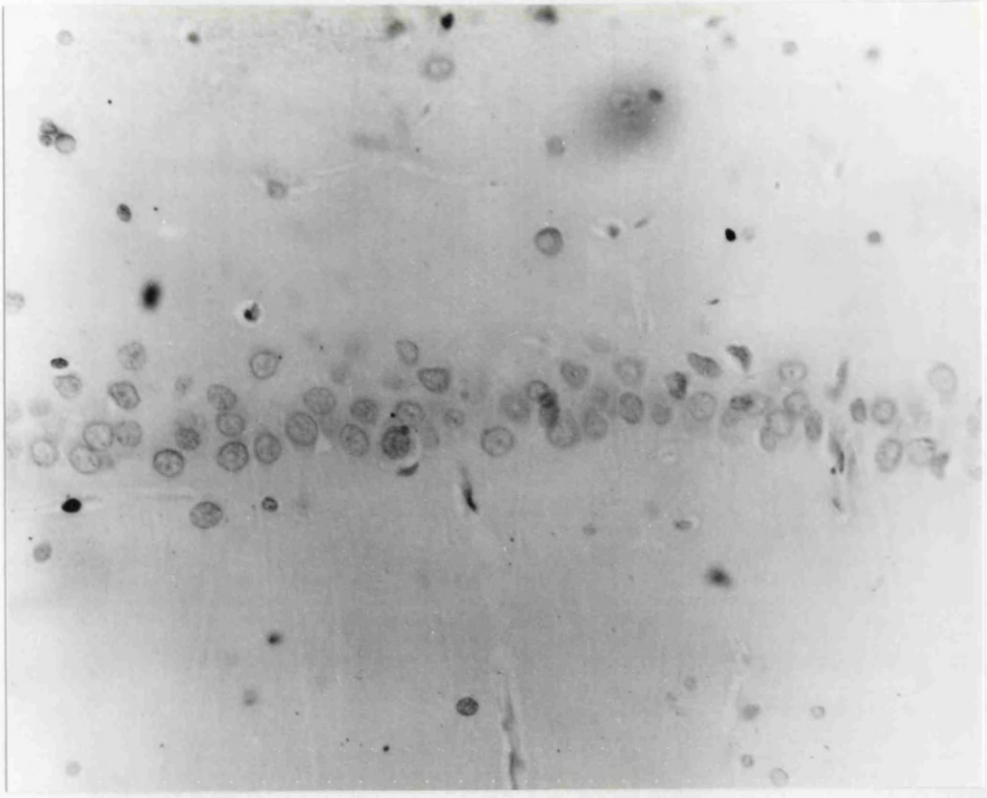


Fig. 39 Lack of TUNEL staining following i.p. injected saline in the CA1 region. The above photomicrograph provides a control for the CA1 region. No staining of neuronal material can be discerned. 20 mm : 50 μm.

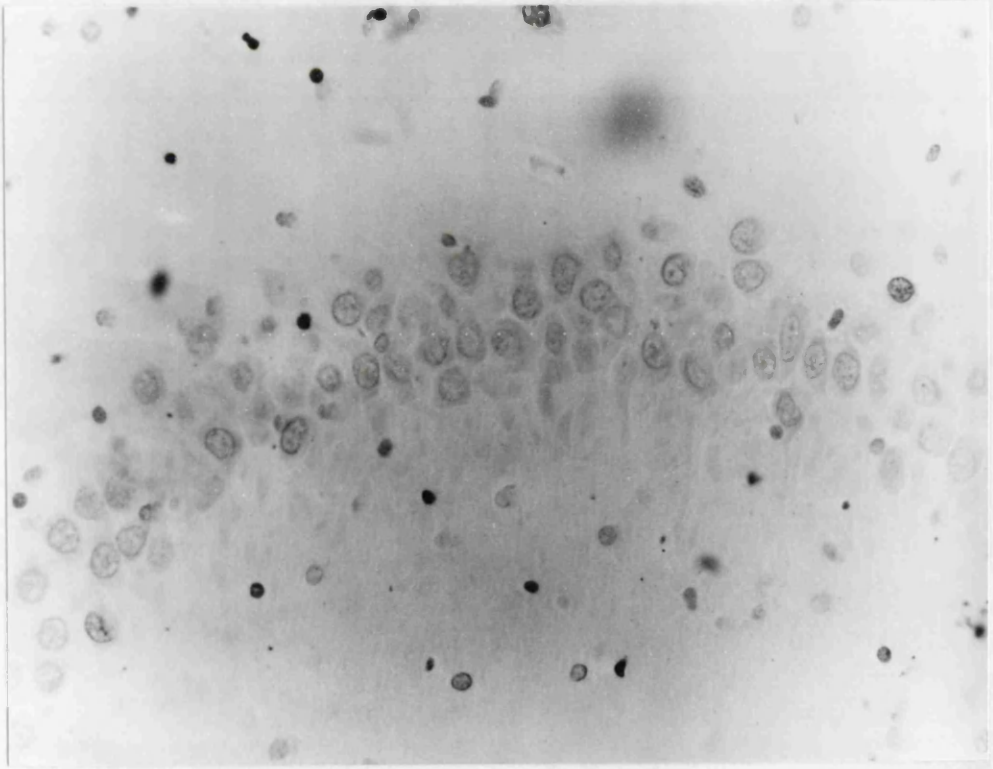


Fig. 40 Control TUNEL staining in the CA3a region after i.p. saline injection. As for the CA1 region, no staining of neurones of this region can be observed in control animals. 20 mm : 50 μm.

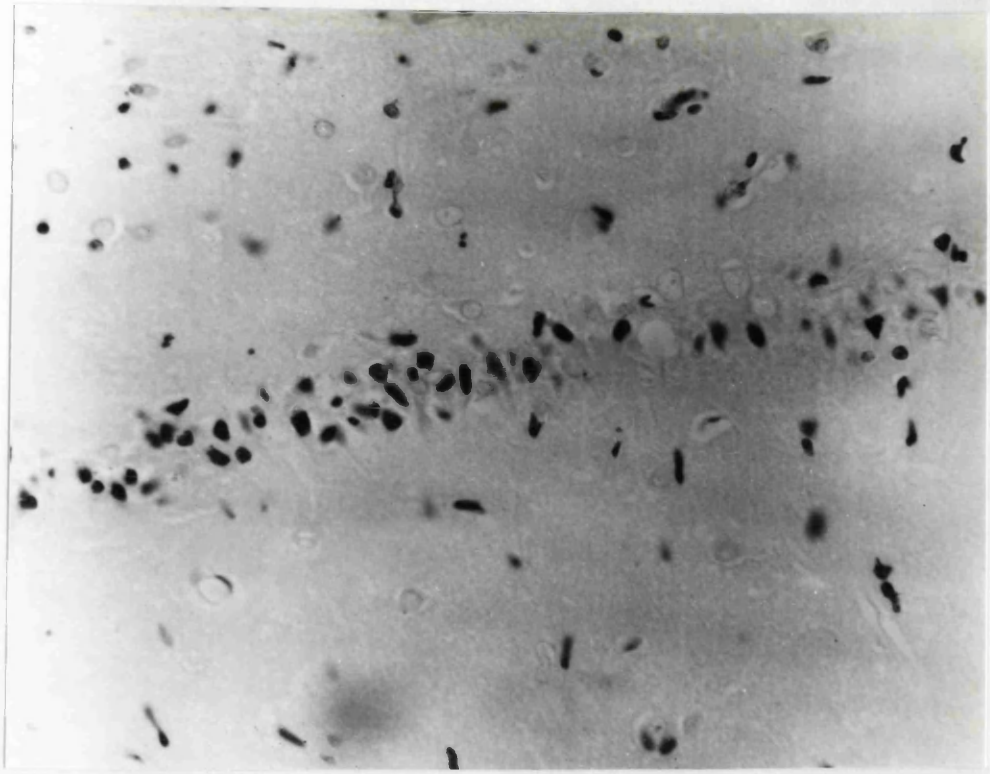


Fig. 41 Kainate-induced TUNEL staining in the CA1 region of the hippocampus. 10 $mg.kg^{-1}$ kainate (i.p.) increasd the TUNEL staining in the CA1 region. As can be seen, the staining is localised to the compact material of the nucleus, with very little non-specific staining. In this photomicrograph most neurones have stained positive for the apoptotic marker. 20 mm : 50 μm

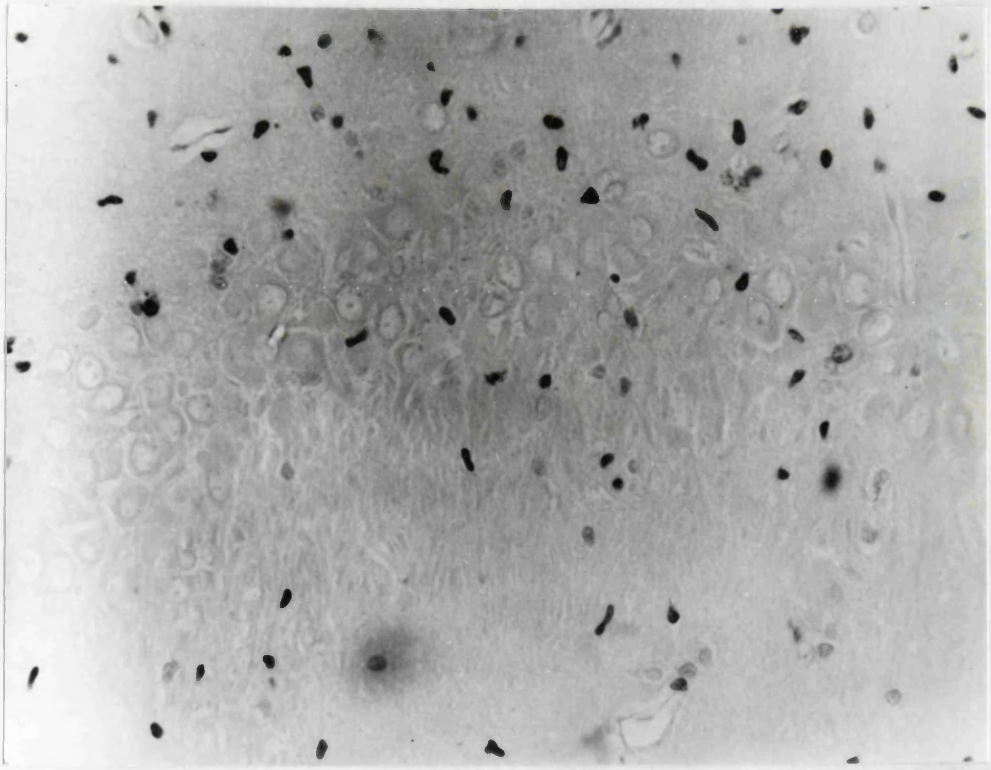
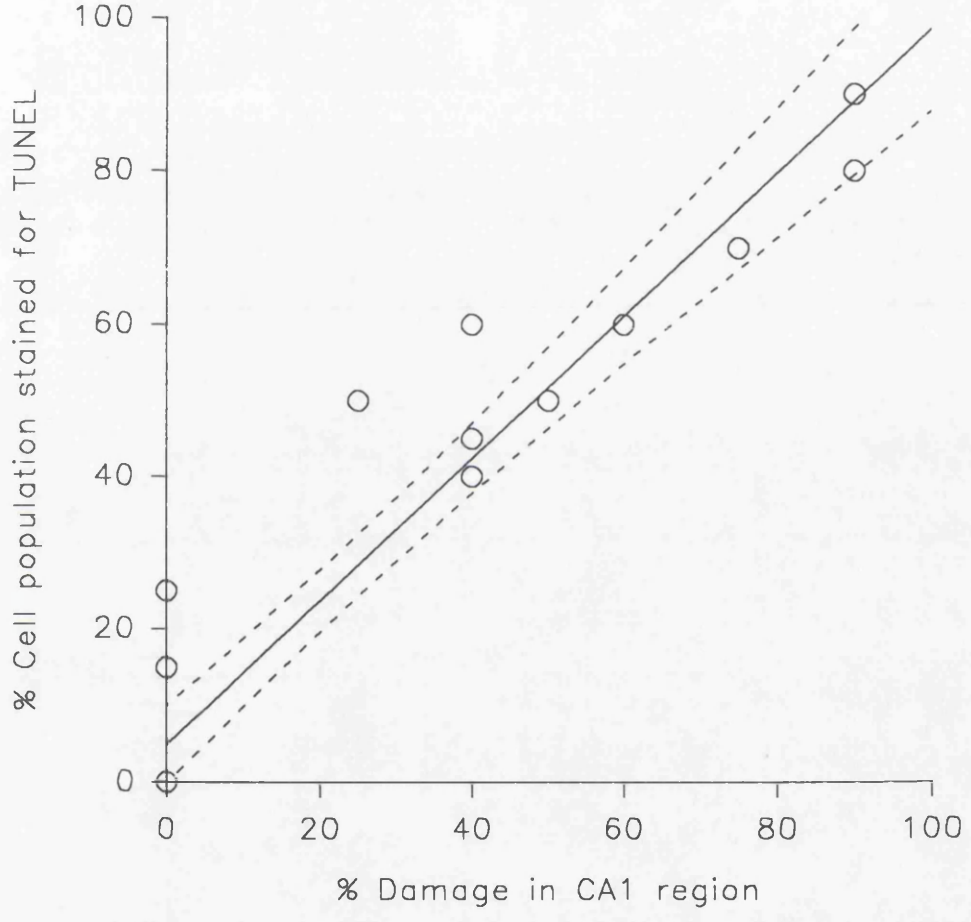


Fig. 42 No TUNEL positive staining of neurones of the CA3a region following kainate administration. The CA3a region of the same animal which produced the above picture of TUNEL staining in the CA1 did not display similar staining for the CA3a region, although non-neuronal cells (e.g. glia) are positively stained. 20 mm : 50 μm

Fig.43 Correlation between damage in the CA1 region and the percentage of cells which stained positive for the TUNEL method.



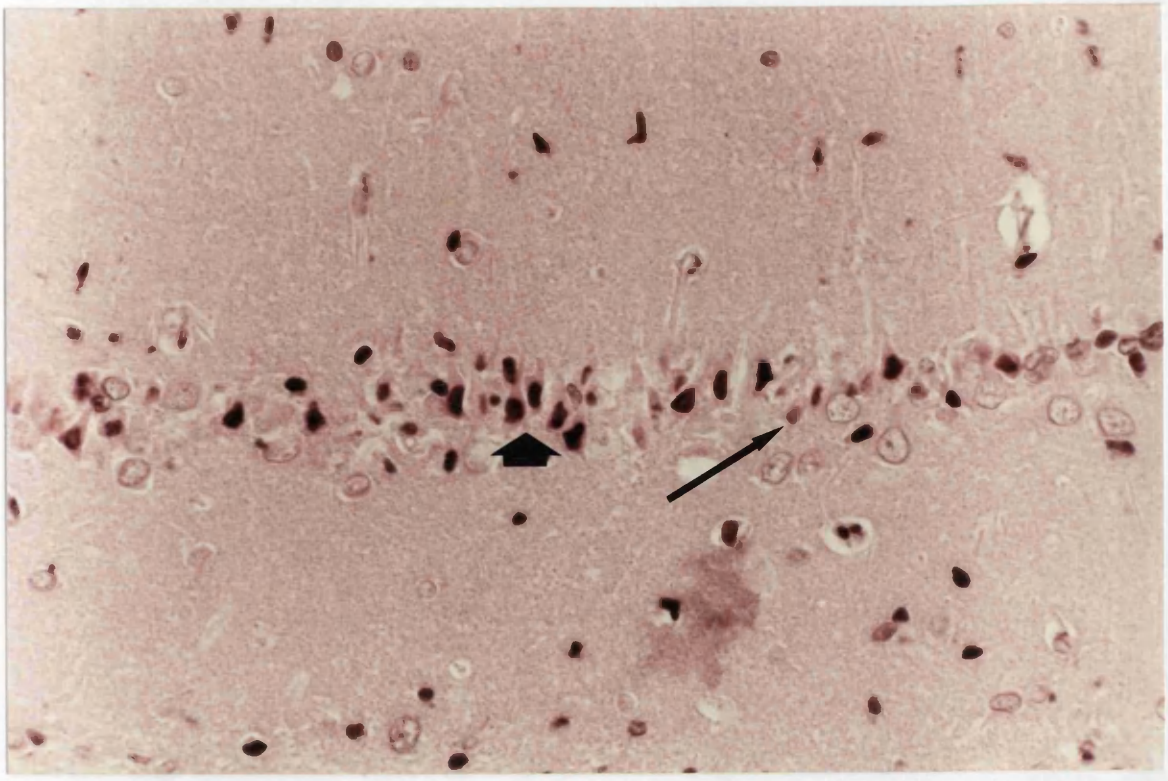


Fig. 44 Eosin counterstaining of the CA1 region following the TUNEL method (i.p.). Cells stained for apoptotsis also take up the deep pink eosin (see thick arrow), although not all of the non-viable cells are positive for apoptosis (thin arrows). While this section is from an animal injected with 2 μg. kg^{-1} CGS 21680 + 10 mg. kg^{-1} kainate, it is typical of the CA1 region following any damaging regimen. 20 mm : 40 μm.

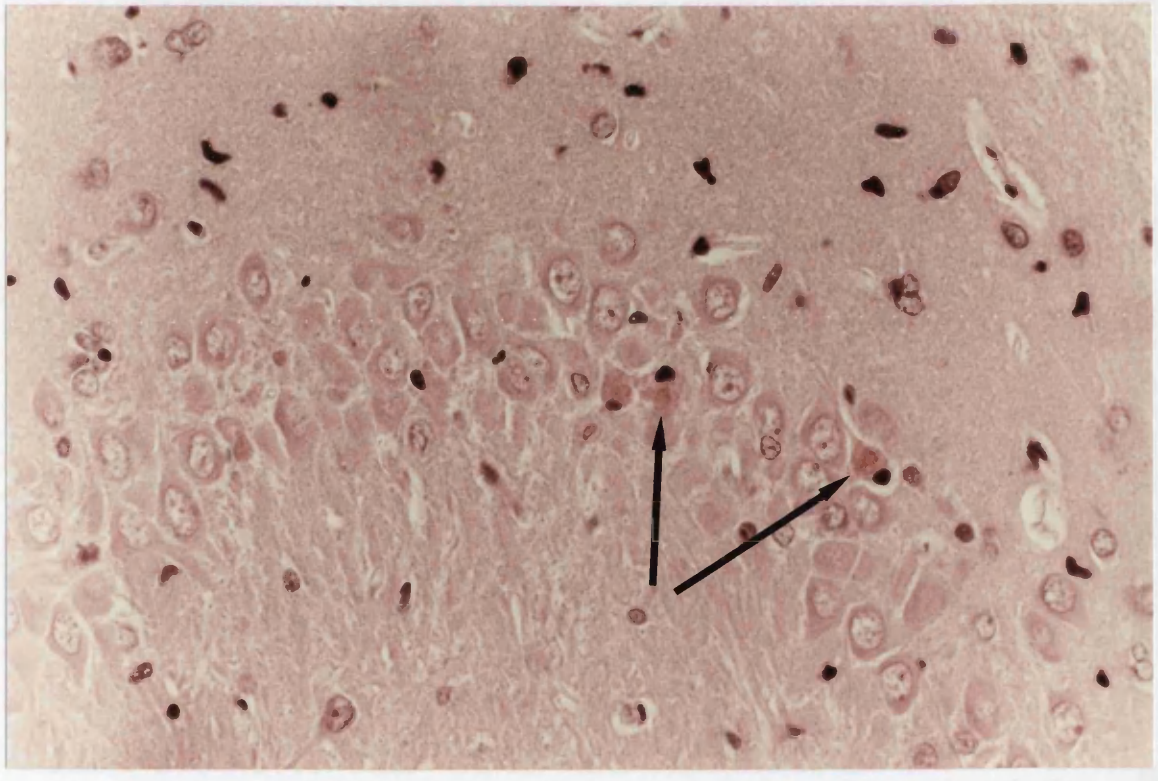


Fig. 45 Damaged CA3a region stained by both the TUNEL method and eosin (i.p.). Although a number of damaged cells are apparent (see arrows), none are positive for apoptosis. Again this was representative of the CA3a region where damage occured after i.p. injections. 20 mm : 40 μm.

Table 2 Table correlating TUNEL positive staining with haematoxylin and eosin assessed damage in the CA3a region of the hippocampus after intrahippocampal injections. Values represent percentages for the respective experiments.

	CA3a Damage	CA3a TUNEL
Kainate 0.25 nmol		
	100	20
	100	100
	95	100
Saline		
	0	0
	0	0
	0	0
CGS21680 2.5 nmol + Kainate 0.25 nmol		
	100	90
	100	60
	100	80
ZM241385 2.5 nmol + Kainate 0.25 nmol		
	0	0
	0	0
	60	0
	100	0
	80	0
Adenosine 2.5 nmol + Kainate 0.25 nmol		
	10	0
	95	80
	95	75

BRAIN-DERIVED NEUROTROPHIC FACTOR

Intraperitoneal injections

Brain sections were examined immunohistochemically for the presence of BDNF protein (table 3). After i.p. injections of 10 mg. kg^{-1} kainate an induction of BDNF protein was observed compared with the saline control treated animals (fig. 48 & 51). The staining for the BDNF protein was observed diffused throughout the cytosol of the cell. Although there was an increase in BDNF induction throughout all regions of the hippocampus after kainate, the percentage of cells stained positive for BDNF did not correlate directly with damage observed from haematoxylin and eosin staining.

With the low amount of damage associated with CGS 21680 (10 mg. kg^{-1}) + kainate (10 mg. kg^{-1}), it was difficult to observe any increase or decrease in BDNF protein binding when compared with kainate alone (i.e. independent of damage). Evidence from the two damaged hippocampi following injection of the A_{2A} agonist with the excitotoxin, suggests that BDNF protein is expressed only in the presence of cellular damage (although as suggested from experiments where kainate was injected alone, the level of induction does not directly correlate to that of damage).

While CGS 21680 0.1 mg. kg^{-1} with kainate 10 mg. kg^{-1} (n = 8) did not show any induction of BDNF staining in any section where damage was not observed, the further addition of ZM 241385 10 mg. kg^{-1}, appeared to alter this pattern of induction as BDNF protein was observed in three of five sections where damage had not occurred. This was also apparent to a lesser extent with ZM 241385 10 mg. kg^{-1} and

kainate 10 mg. kg^{-1}. In complete contrast animals treated with the A_1 agonist R-PIA (when given at 25 μg. kg^{-1}, n = 5), did not display the kainate-associated induction of BDNF staining even though damage occurred in two of the sections.

Intrahippocampal injections

Table 4 shows that effects similar to those seen with i.p. kainate were observed after central injection of 0.25 nmol kainate. BDNF staining was mostly localised to cells within areas of damage (CA3a and CA3b), but displayed no direct correlation between the extent of damage and degree of staining (fig 53 & 54 compares photomicrographs of the CA3a regions of animals treated with saline with kainate). At 2.5 nmol none of the combinations of CGS 21680, adenosine or ZM 241385 with kainate 0.25 nmol displayed this increase in BDNF staining, even though a proportion of animals with each regimen did exhibit damage within the CA3 region of individual experiments. A slight increase was noted at lower concentrations of the above compounds, although still less than the increase seen with kainate alone. In contrast 0.25 nmol R-PIA did not appear to affect the augmentation of BDNF staining by kainate.

NITRIC OXIDE SYNTHASE INHIBITORS

Nitric oxide synthase inhibitors, L-NAME and 7-nitroindazole, were injected 10 minutes before the intraperitoneal injection of 10 mg. kg-1 kainate (Fig. 55). Subsequent haematoxylin and eosin staining revealed significant protection against

Table 3 Correlation of serial section stained for BDNF or damage after intraperitoneal injections. Values represent percentage damage (from haematoxylin and eosin staining) or percentage of cells expressing BDNF protein.

	CA1 Damage	CA1 BDNF-Ab	CA3a Damage	CA3a BDNF-Ab
Kainate 10 mg.kg-1				
	50	80	35	40
	90	50	75	0
	50	30	5	30
	90	5	90	5
	5	0	35	50
	25	30	10	70
Saline				
	0	0	0	0
	0	0	0	0
	0	0	0	0
CGS21680 0.1 mg. kg-1 + Kainate 10 mg. kg-1				
	0	0	0	0
	0	0	0	0
	40	35	25	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	40	10	25	15
	0	0	0	0
ZM241385 10mg. kg-1 + Kainate 10 mg. kg-1				
	0	0	0	0
	0	25	0	0
	0	90	0	70
	75	90	15	50
	0	0	0	0
	0	0	0	0
	60	0	15	5
CGS21680 0.1 mg. kg-1 + ZM241385 10 mg. kg-1 + Kainate 10 mg. kg-1				
	0	40	0	25
	0	0	0	10
	0	30	0	70
	0	0	0	0
	0	50	0	80
R-PIA 25µg. kg-1+ Kainate 10 mg. kg-1				
	5	0	20	0
	0	0	0	0
	0	0	0	0
	50	0	65	0
	0	0	0	0

Fig. 48 Complete absence of BDNF protein staining following i.p. saline injection. While the formation of the CA1 can be discerned, there is an obvious lack of staining to any degree with the saline control. 20 mm : 40 μm.

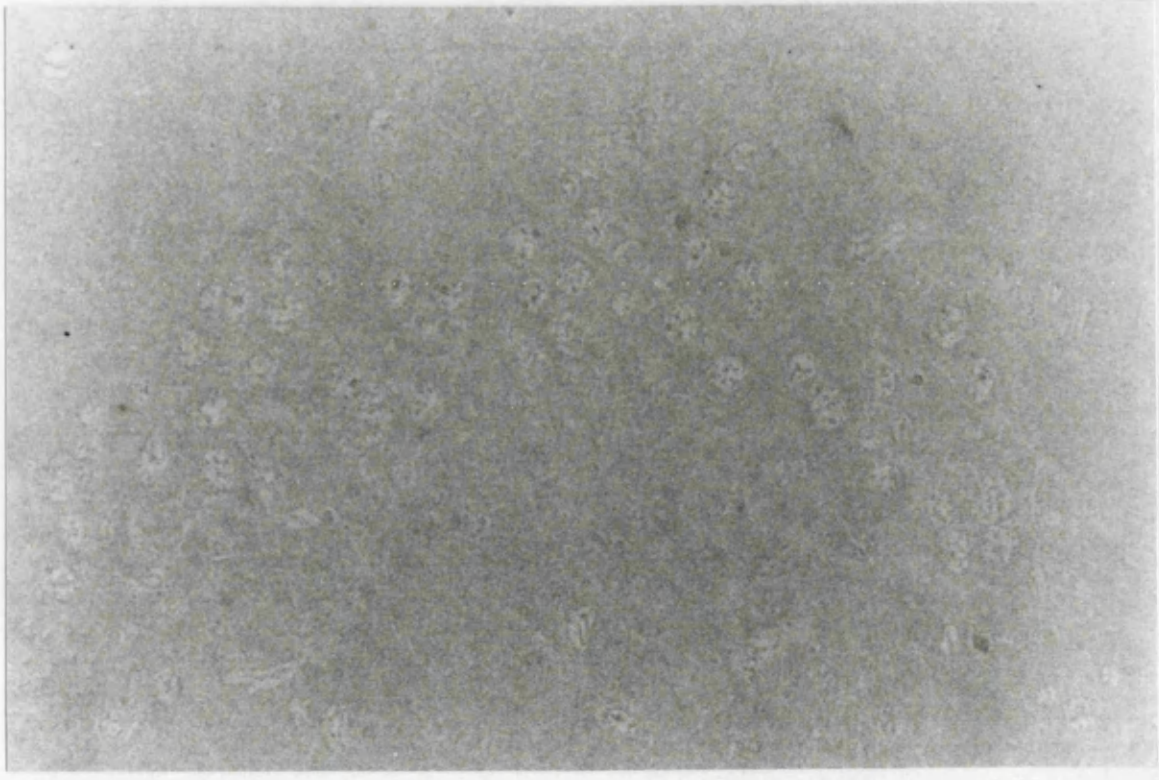


Fig. 49 Lack of BDNF staining in the CA3a region of control animals. As for the CA1 region, the absence of BDNF protein staining makes the CA3a region barely perceptible. 20 mm : 40 μm.

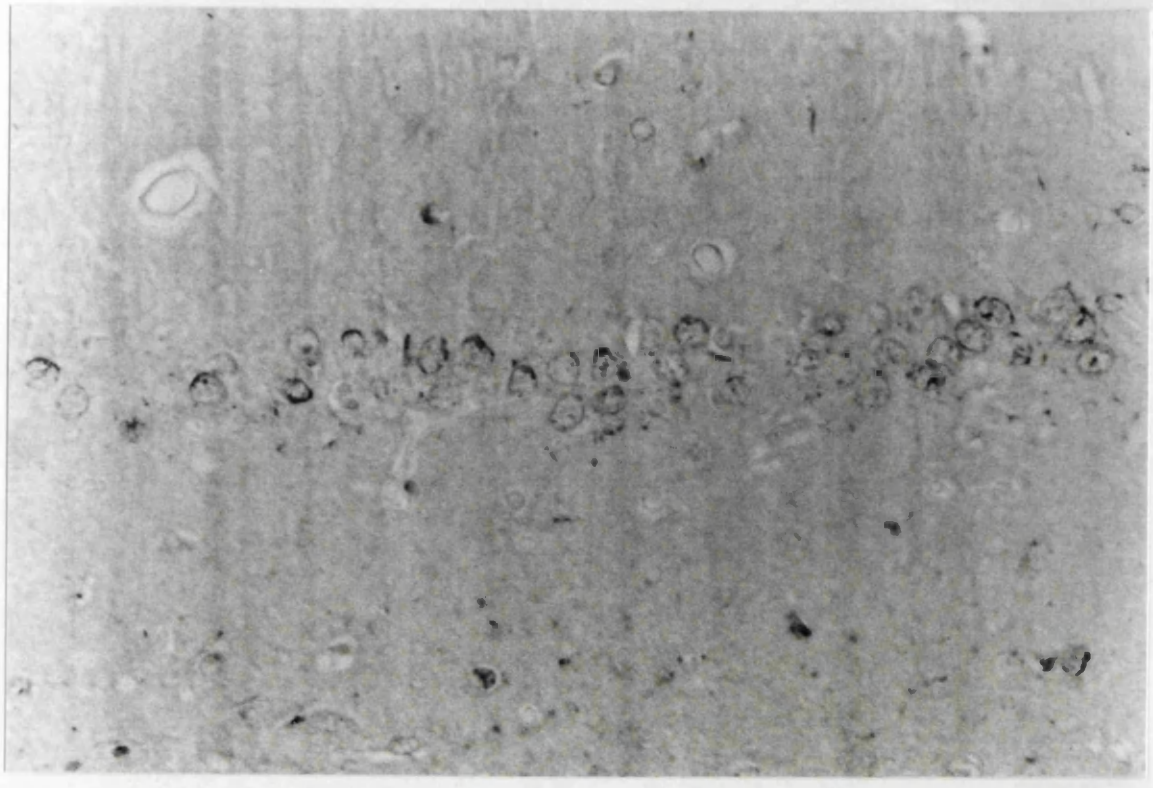


Fig. 50 Kainate-induced BDNF protein staining in the CA1. Compared with the near blank picture obtained with saline controls, 10 mg. kg^{-1} kainate (i.p.) increased BDNF protein throughout the CA1 region. Noticeably, the staining is mostly localised to the neurones. Within the neurones themselves, staining appears to be found in discrete patches on the cellular membrane. 20 mm : 40 μm.

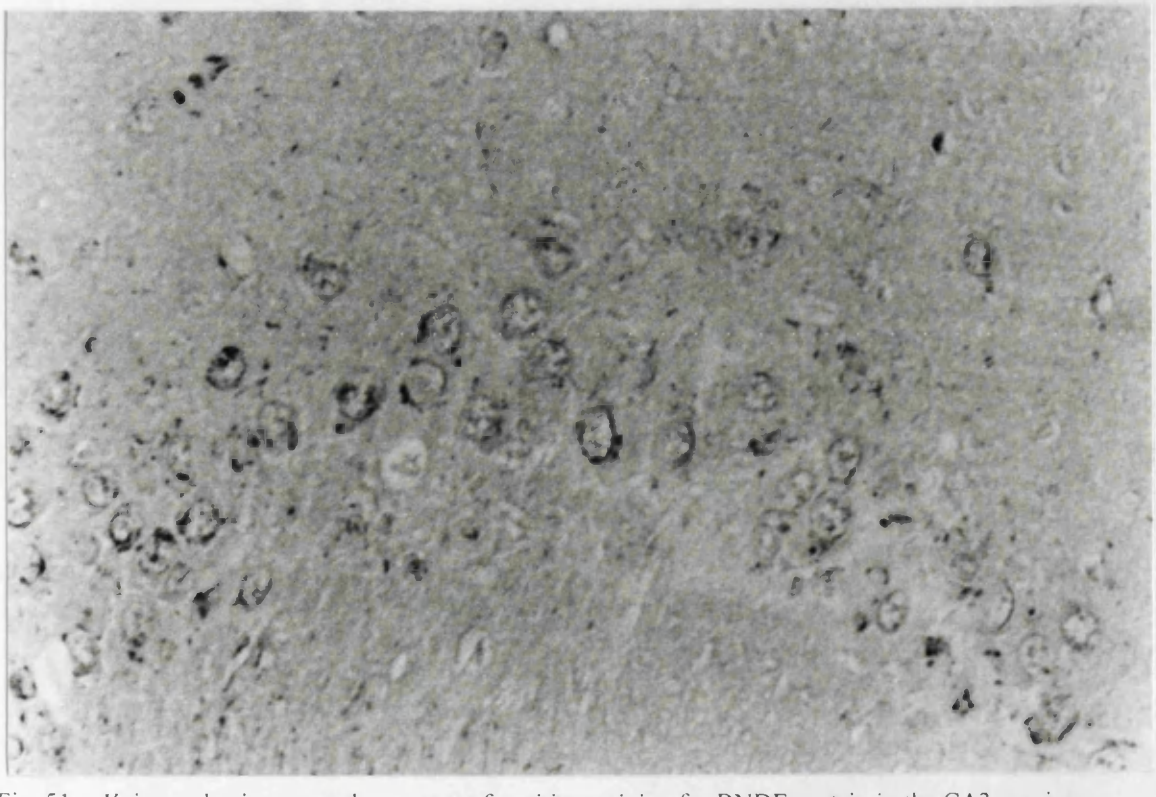


Fig. 51 Kainate also increases the amount of positive staining for BNDF protein in the CA3a region. A very similar picture is observed within the CA3a region after 10 mg. kg^{-1} kainate as for the CA1 region, with most staining localised to patches on nuclear membranes. 20 mm : 40 μm.

Table 4 Table correlating serial sections stained for BDNF protein or damage, after intrahippocampal injections. Values represent percentage of damaged cells or percentage of total cells stained positive for BDNF.

	CA1 Damage	CA1 BDNF-Ab	CA3a Damage	CA3a BDNF-Ab
Kainate 0.25 nmol				
	5	70	100	90
	0	0	100	80
	0	100	95	95
	20	0	100	50
Saline				
	0	0	0	0
	0	0	0	0
	0	0	0	0
CGS21680 2.5 nmol + Kainate 0.25 nmol				
	0	0	100	0
	0	0	100	0
	0	0	100	0
ZM241385 2.5 nmol + Kainate 0.25 nmol				
	0	0	0	0
	0	0	0	0
	0	0	80	0
	0	0	60	0
	0	0	0	0
Adenosine 2.5 nmol + Kainate 0.25 nmol				
	0	0	95	0
	0	0	95	0
	0	0	100	15
	0	0	0	0
	0	0	10	0
CGS21680 0.25 nmol + Kainate 0.25 nmol				
	0	0	90	0
	0	0	95	0
	0	0	100	80
ZM241385 0.25 nmol + Kainate 0.25 nmol				
	0	0	85	0
	0	0	90	10
	0	0	90	20
Adenosine 0.25 nmol + Kainate 0.25 nmol				
	0	0	90	0
	100	0	100	10
	0	0	80	20
R-PIA 0.25 nmol + Kainate 0.25 nmol				
	0	70	100	85
	100	60	90	90
	0	0	30	0

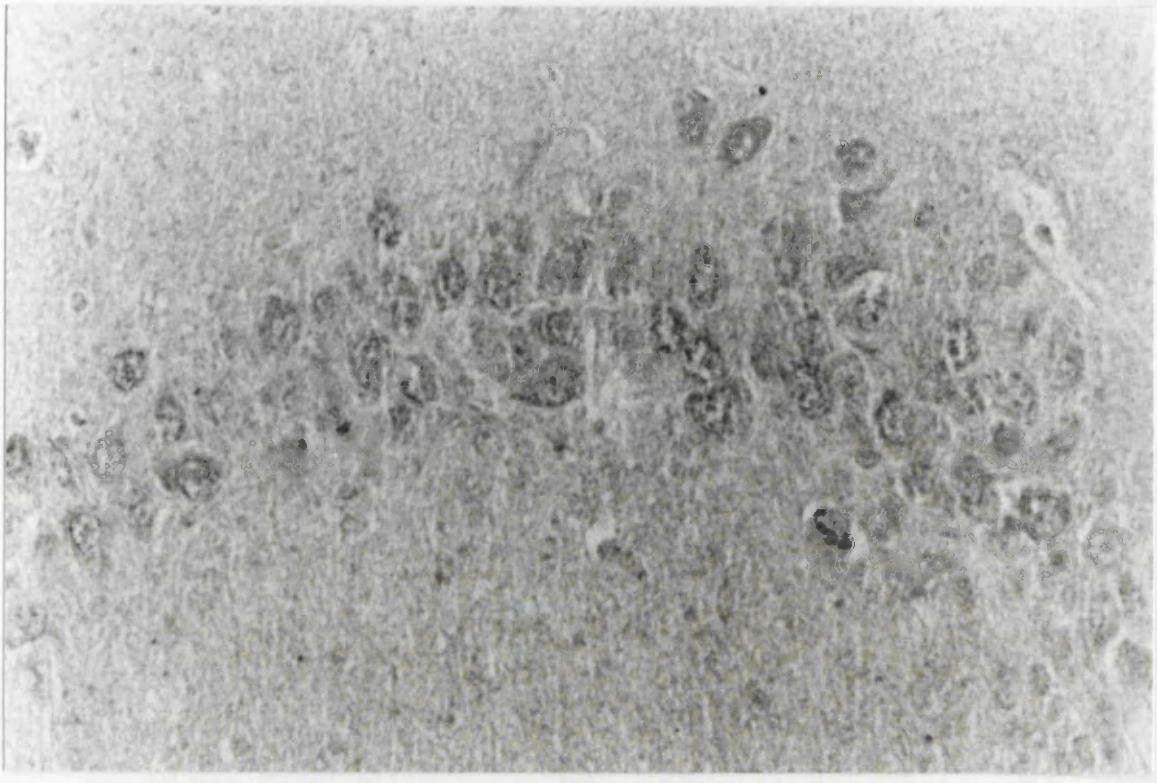


Fig. 53 No BDNF protein-positive staining is apparent following intrahippocampal injection of saline in the CA3a region. Similar to the picture observed after i.p. saline, a complete lack of staining for the protein was observed after the injection into the hippocampus of 1 µl saline. 20 mm : 40 µm.

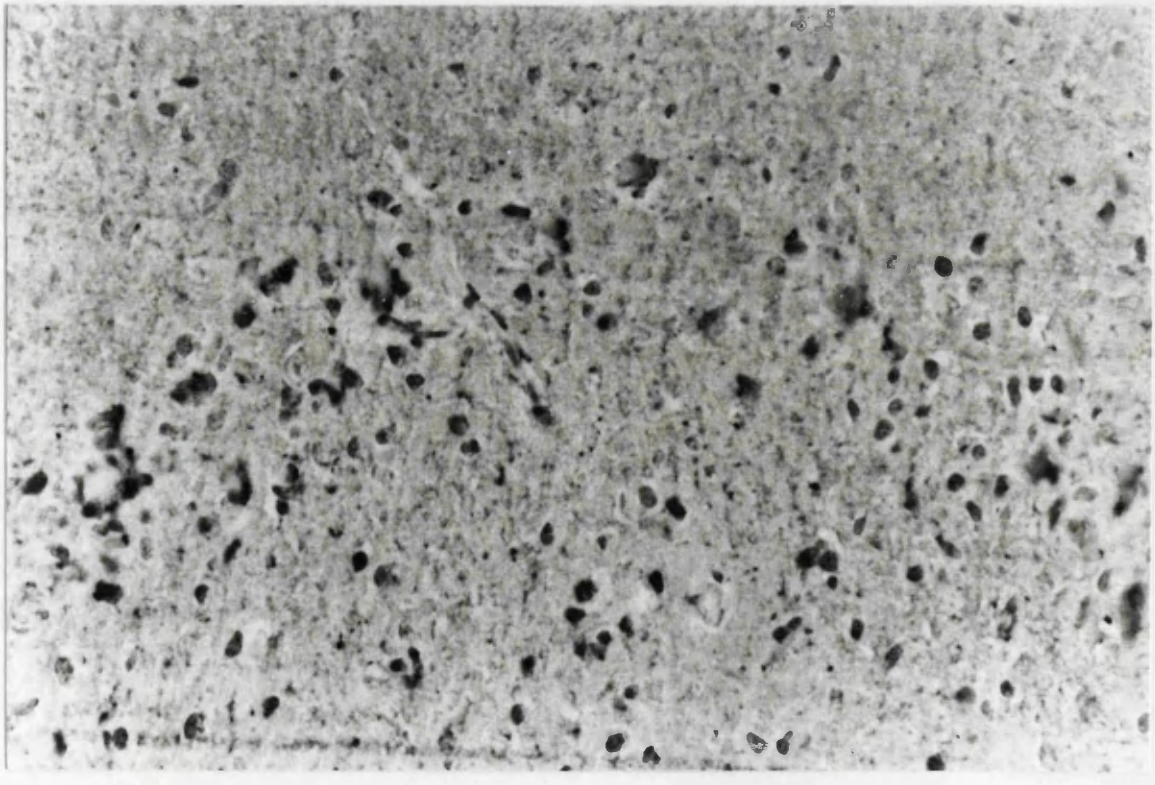


Fig. 54 BDNF staining in the CA3a region following i.h. injection of kainate. A different pattern is observed following i.h. kainate than for systemic administration. Due to the lack of neurones in the CA3a region seven days after the initial injection, the lack of neuronal staining is not surprising. Positive staining of glial cells is apparent. 20 mm : 40 µm.

kainate excitotoxicity in the hippocampus. L-NAME, administered at a dose of 5 mg. kg^{-1}, significantly reduced damage associated with kainate alone in the CA1 (from 56.7 % ± 9.6 to 5.0 % ± 2.9) and CA3a region (51.1 % ± 9.0 to 10 % ± 5.8). Similar significant values were obtained for the i.p. injection of 7-NI in the kainate model. In the CA1 region percentage damage was 2.5 % ± 2.5 and 5.0 % ± 5.0 for the CA3a area. Both regimens tended to reduce kainate-associated damage in the CA2 region of the hippocampus, but in both cases this decrease was not significant.

DISCUSSION

DISCUSSION OF METHODS

Routes of Injection: Advantages and Disadvantages

In this study both intraperitoneal (i.p.) and intrahippocampal (i.h.) routes of kainate administration were used, in order to correlate data between the two and provide further insight into the mechanism of action of purines within the kainate model of excitotoxicity.

After systemic administration of kainate, ≤ 1 % reaches the brain (Nadler *et. al.*, 1980; Berger *et. al.*, 1986). MacGregor (1994) calculated that for a 200 g rat (with an estimated brain weight of 3 g) an intraperitoneal injection of 10 mg. kg^{-1} would result in an intracerebral concentration of ≤ 1.75 nmol. Of course this value is an estimation, with variability between animals in physiology (e.g. pH, blood flow etc.), possibly affecting the concentration.

Another factor which may result in differences of kainate between individual experiments is the ability of kainic acid to cause dysfunction to the blood brain barrier. Saija *et. al.* (1992) noted that kainate increased permeability of the blood brain barrier to [^{14}C]GABA within the cortex, hippocampus and cerebellum, although this was not always constant with either an increase in neuronal injury or an alteration in glucose utilisation. The fact that such a small percentage of kainate reaches the CNS (and is therefore more susceptible to minor changes in

environment), may help explain why kainate, when injected systemically alone, or in conjunction with other compounds, produces variation between experiments in the degree of damage (e.g. Balchen *et. al.*, 1993; MacGregor & Stone, 1994), which is not observed in culture (e.g. Deupree *et. al.*, 1996).

Even though the exact concentration of kainate within the CNS may not be known, systemic injections should result in a widespread (if not necessarily homogeneous) distribution of kainate throughout the brain regions. This is both advantageous, in that it provides an overall picture of effects mediated by kainate, as well as detrimental in studying the effects on specific localities (e.g. the hippocampus), as afferent pathways from one region may distort the picture in another. This may still happen after intrahippocampal administration due to diffusion of kainate, although with only a small volume of drug injected, this effect on afferent pathways is likely to be less significant. The i.h. route may represent a more accurate picture of excitotoxic damage to the hippocampus, since less afferent effects modulate the hippocampal response to kainate. Peripheral effects may also contribute to the overall picture after i.p. injections.

While injections into a specific region of the brain negate a large number of the problems associated with systemic injections (e.g. unknown concentrations, peripheral effects), intrahippocampal administration does possess it's own complications. The process itself is more invasive, requiring the animal to undergo general anaesthesia. Apart from interference to the experiment by the anaesthetic itself (see later), the stress to the animal combined with a recovery period may exert unknown effects on the experiment. Some of the effects were compensated for: (a) Body temperature under anaesthesia was maintained by a thermal blanket coupled to

a rectal probe, (b) The period for which the animals were maintained under anaesthetic was kept to a minimum (~1 hour), and (c) any fluid loss due to inability (or lack of inclination) to drink was corrected by two i.p. injections of 2-3 ml saline post-operatively. Other disadvantages of intrahippocampal injections include the disruption to the blood brain barrier by the insertion of a fine needle into the hippocampus and the possible influx of blood borne products (such as macrophages). The injection also produces a gradient of concentration of kainate away from the needle point. At the tip of the needle the concentration of the administered compounds would be that of the initial solution, but as the compound diffuses outwards, the concentration becomes more dilute.

To help overcome the disadvantages associated with both the i.p. and i.h. excitotoxic models, this study used both in an attempt to fully investigate protection observed with some compounds.

Haematoxylin and Eosin Staining

One of the main problems with any study investigating any form of cellular death is how to determine accurately when a cell is classified as dead (or beyond the point from which it cannot recover). As both the necrotic and apoptotic cell death pathways share a degree of homology in their latter stages (such as ATP depletion, mitochondrial dysfunction and free radical production) the point at which a cell may be classed as dead may be similar. Within either process of cell death, there are known to be stages that are reversible (e.g. blebbing, macromolecular synthesis and chromatin condensation) and those beyond which the cell cannot recover (e.g.

distortion of the cell membrane; Trump & Berezesky, 1995). There are a number of techniques which attempt to focus as close to the 'point of no return' as possible. Most prove ultimately to hold true in the majority of cases, but not universally, resulting in the acceptance that most techniques label markers that suggest and usually correlate with, but do not conclusively prove, cell death. Such techniques include labelling of the gliotic marker [^{3}H]1-(2-chlorophenyl-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline-carboxamide ([^{3}H]-PK 11195) after neuronal insults (MacGregor & Stone, 1993) and measurement of protein synthesis (Karle *et. al.*, 1995) which correlates incompletely with cell viability during apoptosis (Mills *et. al.*, 1995). The result is that most studies resort to the older, simpler methods afforded by histology. Even within histology there is still variation in the stain used and the method of quantification. Stains used for models of cerebral insults are include haematoxylin and eosin (Li *et. al.*, 1992; Aguis *et. al.*, 1994; Dawson *et. al.*, 1994; Fujitani *et. al.*, 1994; Book *et. al.*, 1995; MacGregor *et. al.*, 1996b; Bramlett *et. al.*, 1997), and various methods for staining Nissl substance (Von Lubitz *et. al.*, 1989, Von Lubitz *et. al.*, 1994). While the increase in eosin staining intensity of damaged neurones is apparent in both our studies and those mentioned above, there has been no investigation into this phenomena. The increase in intensity may represent the change in pH of a compromised cell (to which eosin is sensitive) or cellular breakdown (e.g. of the tertiary structure of proteins, providing a larger number of sites for the acid stain to interact with). The cells stained are those at the later stages of cell death.

There is also variation between methods on the quantification of dead cells stained by the specific technique. The most common is a straight forward count of dead cells as

a percentage of total cells (e.g. Fujitani *et. al.*, 1994). This highlights another problem with any histological technique. As the section under examination is a 'snapshot' of an ongoing situation, it takes no account of preceding or subsequent events. After any insult where cells are compromised, mechanisms to remove debris of dead cells are initiated. This results in the possibility that within an area of damage, the cell count of non-viable against total cell population may underestimate actual damage (i.e. discounts any cells that have been removed subsequent to cell death). Other studies overcome this by introducing a semi-quantitative system that covers a wider degree of damage (e.g. Berg *et. al.*, 1993; Magloczky & Freund, 1993). The disadvantage with this method is a loss of accuracy between damage at either end of the bands within the scale. Another method of determining damage by calculating the actual area of damage (e.g. Green & Cross, 1994), brings problems of determining the exact border between areas of compromised and non-compromised cells.

The method for determining cell death in this study is a combination of a direct cell count and quantitative observation. Due to the distinct morphological characteristics of the hippocampus it allows both an accurate calculation of the percentage of dead cells as well as the ability to discern easily areas of neurone depletion. The resultant percentage scale used in this study (in stages of five), is therefore an indication of percentage cell death which takes in to account areas where there is an obvious depletion of visible neurones. Figure 3 shows how the above method correlates with damage calculated using with actual cell counts. While in most experiments there is a high degree of correlation between the two methods, our more versatile protocol gives a more accurate value in instances where a proportion of the hippocampus is

devoid of cells. The method used therefore represents a reliable and reproducible indication of neuronal death within the discrete populations of the hippocampus.

The TUNEL Stain as an Indicator of Apoptosis

As mentioned above staining for cell death (whether apoptotic or necrotic), is not without compromise. Specific for apoptosis are a number of features which can be observed using a variety of techniques. While these markers (such as induction of immediate early genes (IEGs)) are an indication of apoptotic mechanisms, they occur before the onset of terminal cell death (i.e. cells can recover after the induction of IEGs). As both necrosis and apoptosis share a similar final pathway, discriminating between the two after the 'point of no return' may be difficult. Methods identifying apoptotic markers, therefore, do not quantify cell death *per se*, but highlight the mechanism by which cell death is occurring. This definition still has a problem in that a cell may be identified as undergoing apoptosis, before being overcome by necrotic mechanisms. Further complications arise from the evidence that apoptosis may encompass a number of discrete variations. For example, studies of T-cells from p53 knockout mice have found both the dependence and independence of apoptosis on p53 protein (which modulates the expression/induction of genes and proteins believed important in apoptosis; e.g. *c-fos*) in glucocorticoid and γ-radiation induced apoptosis respectively (Clarke *et. al.*, 1993; Lowe *et. al.*, 1993).

Controversy is also apparent in the use of marker methods such as the TUNEL method used in this study, or examination of DNA laddering using gel electrophoresis. Studies have shown that while endonuclease activity is usually

present in models of apoptosis, blockade by DNA-degrading enzyme inhibitors does not affect the final outcome of the cell (Sun *et. al.*, 1994). The TUNEL method for identifying apoptotic cells is also controversial, with a number of studies disputing the specificity of TUNEL to identify only those cells undergoing apoptosis (Van Lookeren Campagne & Gill, 1996; Nishiyama *et. al.*, 1996), suggesting that DNA nick end labelling can also stain positive for necrosis.

DISCUSSION OF RESULTS

Kainate Neurotoxicity, i.p.

In the above study 10 mg. kg^{-1} kainate was enough to result in extensive damage to selected regions in the rat brain. Our experiments showed a large amount of neuronal degradation within the hippocampus, and specifically within the CA1, CA3a and CA2 regions. This correlates well with other studies, where percentage degradation in the CA1 is similar to the CA3a regions (MacGregor *et. al.*, 1996a; Schwob *et. al.*, 1980). Although other reports have shown a similar increase in damage in the CA2 region to the present study (22 %) after systemic injections of kainate (MacGregor *et. al.*, 1996a observed CA2 pyramidal cell damage of ~18 %), only this study found this increase significant. This could be due to a difference in the number of animals tested or in variation of kainate damage between individual experiments distorting any significance in the other reports. In agreement with other studies, no damage due to kainate was observed in the CA3b or CA4 region.

Damage to the CA1 region of the hippocampus is known to be dependant on the dose of kainate since Balchen *et. al.* (1993) observed that while the typical CA1 and CA3a regions were damaged at 10 mg. kg^{-1}, there was no discernible neuronal loss in CA1 after a higher dose of 20 mg. kg^{-1}. At 10 mg. kg^{-1}, in our own study and that by Balchen *et. al.* (1993), it is probable that kainate acts on the CA3a neurones directly (the CA3a region has the highest population of kainate receptors; Wisden & Seeburg, 1993), innervating the CA1 region via the Schaffer collaterals. This may result in indirect distal damage along these fibres to the CA1 region in a manner discussed later.

At 20 mg. kg^{-1}, Balchen *et. al.* (1993) postulated that kainate compromised the CA3a neurones before they were able to stimulate the collateral pathway. Arguing against this view is the fact that after 20 mg. kg^{-1} kainate, over half the neurones still remained (only 15 % less than remained after 10 mg. kg^{-1}). The speculation that kainate damages selective neurones within the CA3a area (possibly those responsible for innervating the CA1 region), cannot be ruled out. Indeed Strain *et. al.* (1991), showed that even at 32 mg. kg^{-1}, kainate only damaged 58.8% of the neurones in the CA3 region. Both DPMA and the low dose of CGS 21680 gave preferential protection of the CA1 over the CA3a region. While this was more obvious in the DPMA injected animals, where there was no significant protection of the CA3a region, 0.01 mg. kg^{-1} CGS 21680 displayed a greater reduction in CA1 neuronal damage than in the CA3 region. This may imply a difference between binding sites within these areas (e.g. in density or affinity), or a difference in the mechanism of toxicity. The latter may be explained by neuronal loss in the CA3a being a direct toxic action of kainate, while CA1 degeneration relies on innervation from the CA3

region. Cells which do not undergo direct toxicity may therefore have a lower threshold for protection.

In our study, extrahippocampal damage was observed in the pyriform cortex and regions of the amygdala. These finding are in agreement with other studies, which have also observed neurodegeneration in the olfactory cortex, the thalamus and neocortex (Schwob *et. al.*, 1980; Sperk *et. al.*, 1983). It is interesting to note that in our study, distal damage to the pyriform cortex significantly correlated with damage to the CA3a region of the hippocampus, but not to the CA1 region, suggesting that pyriform damage is linked to that of the CA3a area.

Protection by A_{2A} Receptor Agonists, i.p.

In this study both A_{2A} adenosine agonists, CGS 21680 and DPMA, proved protective against 10 mg. kg^{-1} kainate, suggesting that the A_2 receptor has a far larger role than was previously assumed. DPMA, which is 13-fold selective for the rat brain A_2 versus the A_1 receptor (Hutchison *et. al.*, 1989), protected pyramidal cells in the CA1 and CA2 region, but not in the CA3a region. This protection was only observed at a high concentration of 1.0 mg. kg^{-1} and not at 0.1 mg. kg^{-1}, suggesting that the protective effects were mediated not by A_2, but by A_1 receptors. On the other hand CGS 21680, showed protection at a concentration of 0.01 mg. kg^{-1}, and even partial protection as low as 2μg. kg^{-1}. CGS 21680 is much more selective than DPMA for the A_{2A} subtype (140-fold versus the A_1 receptor).

While protection has been observed in a number of models with an A_1 receptor agonist (MacGregor *et. al.*, 1993; Arvin *et. al.*, 1989), a similar result with an A_{2A}

agonist was not predicted. As A_1 and A_{2A} receptors exert a number of opposing effects (e.g. on glutamate release (Poli *et. al.*, 1991; O'Regan *et. al.*, 1992) and cAMP production (Lupica *et. al*, 1990)), it was assumed that administration of A_{2A} agonists in a model of excitotoxicity or ischaemia would be detrimental. This belief was further strengthened by evidence that A_{2A} receptor antagonists were neuroprotective (Phillis, 1995; Von Lubitz *et. al.*, 1995). Few reports actually studied the effects of A_{2A} agonists themselves, even though discrete populations of A_{2A} receptors are located in the brain (Jarvis & Williams, 1989), including the hippocampus (Cunha *et. al.*, 1994).

Von Lubitz *et. al.* (1995), showed that chronic pre-operative administration of the weakly selective A_{2A} agonist APEC improved cortical blood flow and survival of hippocampal neurones. More recently, Sheardown and Knutsen (1997) displayed protection by CGS 21680 in a model of temporary forebrain ischaemia in gerbils. The study found protection in the CA1 with CGS 21680 injected post-operatively (30 and 120 minutes) at a concentration of 2×10 mg. kg^{-1}, but not at 2×3.0 mg. kg^{-1}. Effects in other regions of the hippocampus were not reported. Contrary to the belief of Sheardown and Knutsen (1997), it is possible that CGS 21680 may exert some A_1 receptor binding at this high concentration, although due to the lack of investigation using any antagonists, it is difficult to substantiate this proposal for any A_1-mediated protection.

The time of drug administration may also be important, with post-operative injections requiring a higher concentration to be effective. Unlike our own results, DPMA did not protect the hippocampus in the study of Knutsen and Sheardown (1997). This is possibly due to the concentration not reaching levels required to

protect the hippocampus (in our study, DPMA was protective only at a dose 10-fold greater than that of CGS 21680).

Adenosine A_{2A} receptor stimulation has a number of effects, both beneficial and detrimental. As the overall effect of CGS 21680 was to protect the hippocampus, the increase in the excitotoxic amino acids aspartate and glutamate release by A_{2A} receptors (O'Regan *et. al.*, 1992, Popoli *et. al.*, 1995) may not play an important part in kainate-induced excitotoxicity. It seems likely that any further rise in glutamate levels by A_{2A} receptor activation may not have a substantial effect in the excitotoxic brain. Levels of release of the excitotoxin are already elevated by kainic acid, and even increasing the dose of the glutamate analogue does not appear to increase toxicity above a threshold (Strain *et. al.*, 1991).

Effects of the Addition of Adenosine Antagonists, i.p.

The effects observed with 0.1 mg. kg^{-1} CGS 21680 on kainate excitotoxicity were modulated by the addition of either the A_1 receptor antagonist CPX or the peripheral adenosine antagonist 8-PST. CPX, highly selective for the A_1 receptor (700-fold over A_2 receptors), was administered at a dose known to exacerbate kainate-induced neurotoxicity (MacGregor & Stone, 1994). When combined with CGS 21680, no alteration in the previous A_{2A} agonist protection was observed in the CA1 or CA2 region of the hippocampus. In the CA3a there was an increase in pyramidal cell damage to a value of 30 % of that seen with kainate alone. These results show that the beneficial effects of CGS 21680 were mostly due to the A_{2A} receptor, with only a small amount due to A_1 receptor activation. The lack of enhancement of kainate

damage in the CA1 and CA2 regions, suggests the possibility of an inhibitory action of A_{2A} receptors on A_1 receptors. As kainate itself induces the release of adenosine (Carswell *et. al.*, 1997), there should be activation of adenosine A_1 receptors by endogenously released adenosine. With the introduction of CPX, damage should therefore increase due to the inhibition of the protective effects mediated by the A_1 receptor. Alternatively, the protection by the A_{2A} agonists may be such that any decrease in A_1-mediated protection is arbitrary.

In contrast, the addition of 8-PST did exacerbate damage in all three regions. 8-PST, which is highly charged, has been shown not to cross the blood-brain barrier (Baumgold *et. al.*, 1992). It was therefore used as a peripherally acting adenosine antagonist. The decrease in CGS 21680-mediated protection suggests that a large proportion of it's effects on neuronal death are mediated in the periphery. Binding studies have shown that 8-PST given alone increased kainate-mediated damage to 175 % of kainate alone (MacGregor & Stone, 1994). It therefore remains unclear whether our decrease in CGS 21680 protection (to 73 % of kainate) represents the combined effects of CGS 21680 protection and 8-PST toxicity via different pathways or by the direct antagonism of 8-PST on the A_{2A} agonist.

Protection with ZM 241385, i.p.

This modulation of kainate excitotoxicity by a purine analogue was investigated further, with the introduction of a selective A_{2A} antagonist, ZM 241385. ZM 241385 is 80-fold selective for the A_{2A} receptor versus the A_{2B} and 400-1000 fold selective for A_2 versus A_1 (Palmer *et. al.*, 1995; Poucher *et. al.*, 1995), with low affinity for the

A_1 receptor. We observed protection with ZM 241385 against excitotoxic neurodegeneration in all of the hippocampal regions damaged by kainate. While other studies have observed similar effects in different models with A_{2A} antagonists (Phillis, 1995; Von Lubitz *et. al.*, 1995), our study is the first to use a compound which is selective for the A_{2A} receptor with no known activity at the adenosine A_1 receptor. The high degree of selectivity and specificity of ZM 241385 for the A_{2A} receptor suggests that the effects observed with the A_{2A} antagonists are via a direct action on the A_{2A} and not the A_1 receptor. When given in conjunction with CGS 21680, ZM 241385 completely protected all the pyramidal neurones from kainate damage. This increase in protection above that seen with either CGS 21680 or ZM 241385 alone, may suggest an additive effect, although this is hard to substantiate because of the high degree of protection with either compound alone.

The addition of the adenosine A_1 antagonist CPX completely negated the protection observed with ZM 241385. As ZM 241385 itself has no discernible action on the A_1 receptor, this strongly suggests an interaction between the A_{2A} adenosine receptor and the A_1 subtype. Such an interaction has been speculated by Cunha *et. al.* (1994) and O'Kane and Stone (1997), who suggested that activation of A_{2A} receptors inhibits the action of A_1 receptors. Using electrophysiological studies, A_{2A} receptor activation by CGS 21680 was shown to reduce the inhibition of population spikes induced by the A_1 agonist, CPA (Cunha *et. al.*, 1994). Indeed, interaction between the two receptors has since been shown to be a requirement in the potentiation of cAMP in cultured astrocytes stimulated by a metabotropic glutamate receptor agonist (Ogata *et. al.*, 1996).

This theory of A_{2A} activation resulting in A_1 receptor inhibition, would explain why both A_{2A} agonist and antagonists, as well as A_1 agonists are protective and should not be entirely surprising, due to the high degree of co-expression and co-localisation between the two adenosine receptor subtypes in the hippocampus (Cunha *et. al.*, 1994).

In the pathophysiology of excitotoxicity and ischaemia, there is a release of adenosine. This endogenous adenosine activates both A_1 and A_{2A} receptors. If the above theory is correct then a proportion of A_1 receptors will be inhibited. With the further addition of an A_{2A} agonist, a higher degree of protection is observed, mediated by A_{2A} receptors. A selective A_{2A} antagonist, such as ZM 241385, would inhibit A_{2A} receptors, allowing for the activation of A_1 receptors by endogenous adenosine. Subsequent addition of CPX would therefore decrease the protection of ZM 241385. A_1 agonists themselves are protective by overcoming the blockade by A_{2A} receptors.

Further evidence is provided in studies of populations of neurones where both A_1 and A_{2A} receptors co-exist (Correia-De-Sa & Ribeiro, 1994; Cunha *et. al.*, 1996). Corriera-De-Sa and Ribeiro (1994) first showed that at the neuromuscular junction, A_{2A} receptor-facilitated neurotransmitter release predominated over A_1 inhibition. Similarly Cunha *et. al.* (1996), observed that endogenously formed adenosine preferentially activated A_{2A} receptors in the hippocampus, enhancing synaptic transmission.

Microdialysis and high pressure liquid chromatography (HPLC) studies have also revealed an interesting phenomenon relevant to this theory. Carswell et. al. (1997)

showed that kainate induces the release of adenosine by increasing the depolarisation-dependent release of glutamate. When the levels of evoked glutamate release were decreased by 3,4-Dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl] benzeneacetamide (U50,488H), the subsequent endogenous adenosine concentration declined. Subsequent experiments showed no such decrease in extracellular adenosine after the injection of R-PIA, which is also known to decreases the release of glutamate. The possibility that the A_1 receptors were already fully activated before that introduction of R-PIA was dismissed owing to the lack of increase of adenosine following the addition of an A_1 antagonist. One suggestion not examined was the possibility that the lack of effect at the A_1 receptor was due to an inhibition by endogenous adenosine mediated via the A_{2A} receptors.

CPX, which blocks A_1 receptors with high selectivity, has been shown to inhibit the cerebral protection exerted by R-PIA in the kainate treated brain (MacGregor *et. al.*, 1993). Yet when given at the same concentration without the addition of R-PIA, there was no increase in damage (MacGregor & Stone, 1993), even though adenosine released endogenously would be sufficient to activate a proportion of the A_1 receptors. At a concentration at which it has been speculated that CPX would inhibit both A_{2A} receptors and A_1 receptors (MacGregor & Stone, 1993; Simpson *et. al.*, 1992) there is an increase in toxicity. Similar results were obtained by Phillis (1995), who showed that CPX-enhanced neurotoxicity only occurred at 1.0 mg. kg^{-1}, but not at the lower dose of 0.1 mg. kg^{-1}. Although this effect was explained as A_1-mediated, this concentration was greater than that shown previously in the same experimental model to inhibit the depressant action of A_2 receptors (Lin & Phillis, 1991) and much

higher than the 50μg. kg^{-1} required to prevent R-PIA protection (MacGregor & Stone, 1993).

Further evidence for the inhibition of A_2 receptors by CPX at 0.1 mg. kg^{-1} (i.p.) comes from a study examining the effects of the A_1 antagonist on extracellular glutamate detected by microdialysis and HPLC (Simpson *et. al.*, 1992). CPX, which had no effect on glutamate release at an i.p. dose of 0.01 mg. kg^{-1}, decreased the release of glutamate after an injection of 0.1 mg. kg^{-1}. The speculation was that CPX inhibited the A_2-mediated increase in glutamate efflux.

In the same study (Simpson et. al., 1992), CPA (an A_1 receptor agonist) decreased the level of ischaemia-evoked glucose. 5'-*N*-ethyl-corboxamindoadenosine (NECA) which acts at both A_1 and A_{2A} receptors, inhibited the release of glutamate at a low dose. This was reversed at a higher dose. The authors speculated that NECA was acting only on A_1 receptors at the lower dose and both subtypes at the increased dose. Therefore at the higher dose A_{2A} receptor-mediated effects predominated over those of the A_1 receptor. A similar (albeit less dramatic) effect was observed with CPA, at a high dose which would activate A_{2A} receptors. At this high dose, CPA would still be acting upon A_1 receptors with greater selectivity and potency, and therefore the expected effect on glutamate release would be a net effect of A_1 inhibition and A_{2A} release. As the actual result was a very pronounced reversal of the inhibition which occurred at the lower dose (to control levels), this suggesting that A_1 receptor activation does not assume such a large role as would be expected. This would be explained by A_{2A}-mediated inhibition of A_1 receptors.

Behavioural studies also strengthen this theory. Depression of locomotor activity is believed to be a property of the A_{2A} receptor (Jain *et. al.*, 1995). In contrast neither the stimulation of A_1 receptors by CPA nor inhibition of both A_1 and A_{2A} receptors by caffeine, induced any change in locomotor activity. A subsequent increase in activity was observed after the combination of caffeine and CPX. This suggests that A_1-induced stimulation of locomotor activity requires the inhibition of A_{2A} receptors. This again, would be explained by the inhibition of A_1 receptors by the activation of A_{2A} receptors by endogenous adenosine.

Importantly the above hypothesis questions the role of the A_1 receptor in the neuroprotection afforded by endogenously released adenosine. If A_1 receptor inhibition does not affect the protection mediated by the kainate (or ischaemically) released adenosine, then there may be no A_1 receptor mediated element in the endogenous situation. Alleviation of damage by A_1 agonists occurs only after the exogenously administered analogue overcomes this inhibition of the A_1 receptor by endogenously activated A_{2A} receptors. In contrast, very high doses of A_1 agonists may actually inhibit the actions of the A_1 receptor by stimulating A_{2A} receptors (i.e. after maximal A_1 receptor stimulation, there would be a proportional increase in A_{2A} activation). This is indeed the case in study by Simpson *et. al.* (1992) described above, where a high dose of CPA did not display the inhibition of glutamate release which was observed at a lower dose.

Kainate Toxicity, i.h.

Damage associated with intrahippocampal injections of kainate differed considerably from that of intraperitoneal administration. High concentrations of kainate (10 nmol) resulted in complete destruction of the entire pyramidal cell layer (CA1-4), while lower concentrations (1 and 0.25 nmol) gave near complete damage in the CA3a and a moderate amount in the CA3b region. In contrast to intraperitoneal injections, no significant neuronal degeneration occurred in the CA1 region. The near complete toxicity of the high dose of kainate agrees with Schwob *et. al.* (1980) who showed that almost all neurones within the CNS are sensitive to intracerebral injections of kainate toxicity, provided the local concentration reaches a certain level. The sparing of the dentate gyrus may represent the apparent higher threshold to kainate toxicity of the granule cells (Schwob *et. al.*, 1980).

With the lowest dose (used throughout the remaining experiments), the lack of apparent damage in the CA1 region of the hippocampus is in agreement with a number of studies (Jorgensen, 1993; Magloczky & Freund, 1993; Schwob *et. al.*, 1980). Magloczky & Freund (1993) and Schwob *et. al.* (1980) only found a small proportion of CA1 neurones succumbed to kainate excitotoxicity after intrahippocampal injections, although a degree of cell death was observed in the CA4 region. This slight discrepancy is probably due to the difference in the dose of kainate, as a dose comparable to our own produced a similar lack of CA1 neurodegeneration (Magloczky & Freund, 1993). These results are also consistent with evidence from binding studies, as [^{3}H] kainate is known to have a high degree of binding to the CA3 region, but a lower number of binding sites in the CA1

(Kataoka *et. al.*, 1993). Higher doses of kainic acid produced increasing damage within the CA1 region, although always lower than within the CA3 region (Magloczky & Freund, 1993), as indeed in our own study where 10 nmol kainate destroyed the entire pyramidal cell layer. Comparable results were obtained with kainate displacement of [^{3}H]glutamate from binding sites within these regions (Kataoka *et. al.*, 1993).

Apart from a difference in dose, another reason may be the age of the animals under study. Kesslak *et. al.* (1995) have shown that in young mature animals (3 months old) damage is apparent in both the CA1 and CA3 regions after intrahippocampal injections of kainate, while in older rats (14-20 months) only the CA3 region stained for damage. Again the dose used (0.5 µl of a solution of 0.5 mg kainate in 0.5 ml) was 10 times higher than our own. A difference in age is probably only a very minor factor as, although the ages of the animals were not given, their weights are comparable with (or only slightly lower than) our own (e.g. 190-210 g for Magloczky & Freund, 1993).

A final possible explanation could be the use of different anaesthetics. 0.47 nmol kainate (approximately twice that used in our study) was found to produce less damage in the CA1 region after pentobarbital anaesthesia than after halothane or ketamine (Lees, 1992). Whether similar effects are observed with Equithesin (phenobarbital based) have yet to be determined.

Contralateral damage after kainate injection was not observed in any of the hippocampal subfields. While conflicting with the higher doses used in the Magloczky & Freund (1993) study above, who observed contralateral damage in the

CA1 (but not CA3) region, a similar result was observed with comparable doses. Magloczky & Freund, 1993) also reported that the use of Equithesin as an anaesthetic during surgery prevented contralateral damage. This may be representative of the duration of anaesthetic, which is known to affect kainate (but not NMDA) neurotoxicity (Lees, 1992).

Examination of extrahippocampal areas revealed no other areas of damage on the same plane of section. This is in contrast with both our results obtained after systemic kainate injections, and previous studies. Damage after intrahippocampal kainate has previously been observed in the pyriform, entorhinal and amygdaloid cortices (Sperk, 1994). One reason for the absence of distal damage may be that after kainate injection the pattern is known to be dependant on needle positioning (for example 2 nmol kainate injected into the posterior pyriform cortex results in distal damage to the CA1 and dentate gyrus fields of the hippocampus, whereas the same dose into the anterior pyriform cortex failed to produce any damage to these hippocampal regions (Schwob *et. al.*, 1980). This would not explain the difference between our study and others where intrahippocampal injections are used, as positioning the needle at the point does provide reproducible patterns of damage (Magloczky & Freund, 1993; Schwob *et. al.*, 1980). The lack of distal damage is therefore more likely to be dependent on the differences in dose, as Schwob *et. al.* (1980) showed that distal damage increases with dose. Higher doses have been linked with both a non-specific binding to NMDA receptors and an inhibition of glutamate transport (Deupree *et. al.*, 1996), both of which would increase neurotoxicity. Our study may therefore represent a more accurate model of selective

kainate toxicity, which when combined with i.p. studies may reveal a more complete picture of excitotoxicity.

Mechanism of Kainate Toxicity

The neurotoxic reaction to kainate and glutamate is a complex sequence of events with a number of contradictions that have yet to be elucidated. The pathophysiology is biphasic, involving an initial acutely toxic phase followed by apparent recovery before a further loss of neuronal integrity. The initial stage is believed to be mediated by non-NMDA receptors. Evidence for this is the absence of characteristic cell swelling after glutamate addition to cortical cell cultures in the presence of Na^+-free medium (Choi, 1987), suggesting that the accumulation of intracellular Na^+ (through non-NMDA receptor-associated channels) is responsible. Whether the initial phase is neurotoxic *per se* or requires the later, delayed phase (which may be more dependent on the NMDA glutamate receptors), is unclear. Evidence suggests that the early phase is not excitotoxic as cell death was not observed in the presence of NMDA antagonists, which selectively inhibit the late NMDA-dependent but not the initial non-NMDA-dependent phase (Deupree *et. al.*, 1996). Under culture situations, both the non-NMDA glutamate analogues, kainate and AMPA, require a more sustained exposure than NMDA to produce toxicity (Choi *et. al.*, 1988; Hartley *et. al.*, 1993; Koh *et. al.*, 1990). This further suggests that the delayed phase is NMDA receptor mediated. This simple observation is complicated by the evidence that DL-2-amino-5-phosphonovalerate (AP-5), which both inhibits the NMDA receptor and NMDA toxicity, does not prevent neuronal degeneration by either of the

non-NMDA receptor compounds (Choi *et. al.*, 1988; Koh *et. al.*, 1990). Similar evidence is observed with MK-801, which could not completely block kainate or AMPA toxicity at a dose effective against NMDA (Hartley *et. al.*, 1993; Koh & Choi, 1991). This was not substantiated by our *in vivo* results where MK-801 was found to protect the hippocampus completely from intrahippocampal kainate toxicity, even at a dose as low as 0.01 nmol. Our results were further substantiated by an *in vitro* study where MK-801 did show a complete reduction in the damage marker, lactate dehydrogenase (LDH) release (Deupree *et. al.*, 1996). The discrepancy between studies may therefore reflect a difference in methodology. NMDA toxicity is believed to be dependent on the influx of Ca^{2+} via the NMDA-associated Ca^{2+} channel. The relationship between kainate and AMPA and Ca^{2+} levels is more ambiguous. While the kainate receptor can increase the influx of Ca^{2+} directly, the AMPA receptor has shown no such mechanism of action (Berdichevsky *et. al.*, 1983).

Although glutamate toxicity has been shown to be significantly reduced in neuronal cultures incubated in calcium-free buffer, some incidence of neuronal degeneration still remained (Deupree *et. al.*, 1996). This suggests that a proportion of glutamate toxicity is Ca^{2+}-influx independent. This hypothesis is complicated by the same study (and our own) which found that the addition of the NMDA antagonist MK-801 (which inhibits the NMDA receptor-associated Ca^{2+} channel) is completely protective. As no calcium chelating agent was added to the culture system, the conflict between the results obtained by Deupree *et. al.* (1996) and ourselves may be due to the release of calcium from intracellular stores and across the plasma membrane in the period before the addition of glutamate. This increase in levels of

calcium may therefore be responsible for the limited neurotoxicity. Alternatively, cells compromised in the early calcium-independent phase may also release calcium across disrupted membranes.

While some studies have not observed a relationship between the amount of calcium present within neurones and damage (e.g. Dubinsky, 1993; Dubinsky & Rothman, 1991; Michaels & Rothman, 1990), a correlation between the total amount of calcium influx and neuronal death has been observed in hippocampal cultures (Lu *et. al.*, 1996), which is independent of the route of influx. With the variety of mechanisms within a cell available to maintain calcium homeostasis, the lack of correlation between intracellular calcium levels and toxicity is not surprising. Another argument against presynaptic-dependent excitotoxicity is the apparent contradiction in effective concentrations. *In vitro* studies have observed kainate toxicity at low nanomolar concentrations, yet intracellular calcium increases only in the presence of millimolar kainate (Berdichevsky *et. al.*, 1983; Garthwaite & Garthwaite, 1983; Pastuszko *et. al.*, 1984). The release of glutamate and inhibition of glutamate uptake by kainate also occur only at a millimolar concentration (Poli *et. al.*, 1985). While this implies that the toxicity of kainate is due to postsynaptic effects and not alterations in extracellular glutamate, it may be due to limitations of *in vitro* studies. Kainate may display a direct toxicity at low levels in culture that does not manifest in the more complicated system of the whole brain, possibly due to the existence of other cells (e.g. glia, which take up glutamate) which influence the environment. There is also controversy over the origin of kainate-induced glutamate efflux. A number of studies have found that the release is dependent on the levels of intracellular calcium in the presynaptic cell and that glutamate efflux is exocytotic

from intracellular vesicles. Pocock *et. al.* (1988) challenged these findings by showing in a synaptosomal system that kainate inhibited the excitatory amino acid Na^+ co-transporter, allowing a net calcium-independent leakage of glutamate across the plasma membrane. The glutamate would be from a metabolic rather than vesicular origin. They found no evidence to support the calcium-dependent exocytotic release of glutamate. Due to the large number of studies professing calcium-dependence in glutamate release, it seems unlikely that glutamate release is entirely independent of the dication. The possibility still remains that kainate-induced glutamate release relies on both calcium-dependent and independent mechanisms. The initial calcium- (and ATP-) dependent glutamate exocytotic process being short lasting due to receptor desensitisation and depletion of ATP by kainate (Carver *et. al.*, 1996). The non-receptor-dependent leakage from the cytoplasm may provide a more sustained release over a period of time. A comparable picture of glutamate levels has been observed during cerebral ischaemia (Phillis & O'Regan, 1996), with an initial sharp rise in glutamate concentration, followed by a longer lasting slow decrease over a period of hours. Due to the rapid uptake of glutamate, this slow decline represents a sustained release of the amino acid (although at an ever decreasing rate).

More recently a study has characterised the calcium-dependent release of glutamate and found that high micromolar kainate inhibited 4-aminopyridine (4-AP) stimulated [^{3}H]glutamate release in synaptosomes via an action on the kainate receptor (Chittajallu *et. al.*, 1996). The response was biphasic in that the inhibition was preceded by an initial enhancement of release. This could be explained by an inward depolarising calcium current through the kainate-linked ion channel, exciting the

presynaptic cell, before a long-lasting desensitisation of the channels and subsequent inhibition. AMPA receptor stimulation was shown to enhance [^{3}H]glutamate release. As the net effect of kainate is known to be an increase in the release of glutamate, this release (as shown by Chittajallu *et. al.*, 1996) is likely to be due to the activation of AMPA and not kainate receptors. The release of glutamate by kainate is therefore a combination of enhancement via AMPA receptors and inhibition through interaction of kainate receptors. As the kainate receptor is desensitised by kainate, the less kainate-sensitive AMPA receptor-induced glutamate release is likely to predominate.

Further evidence suggesting that kainate-induced glutamate release is mediated by AMPA receptors on the presynaptic neuronal population, is that AMPA inhibitors and AMPA desensitisation blockers attenuated and potentiated kainate-induced glutamate release respectively (Patel & Croucher, 1996). The lack of complete inhibition with AMPA inhibitors and the presence of glutamate release without the addition of the desensitisation blocker, suggests that activation of the AMPA receptor is not the only mechanism involved. Both these results would be explained by the observation above, that kainate, aside from direct receptor activation, can increase the net leakage of glutamate into the extracellular space. Low concentrations of kainate may inhibit glutamate release by desensitisation of the Ca^{2+} channel, but at a higher (millimolar) concentration induce the leakage of glutamate, thus explaining why kainate is still toxic even though it inhibits calcium influx. Kainate has an action on AMPA receptors, increasing the influx of K^+ and Na^+, (removing the Mg^{2+} block of NMDA receptors), as well as this glutamate leakage. As kainate does not desensitise

AMPA receptors, the influx of ions through the AMPA-linked ion channels is likely to be more sustained.

The complete protection of MK-801 against kainate toxicity implies that calcium influx through the kainate-associated ion channel may not constitute a significant role. Excitotoxicity may therefore be dependent on NMDA-induced calcium influx. Indeed the toxic mechanism of kainate may not depend on its interaction with the kainate receptor at all, but from the evidence above, rely more on the ability of kainate to release glutamate from the cytosol. This glutamate may then act on NMDA receptors (with the Mg^{2+} block removed) increasing intracellular calcium, further release of glutamate and subsequent calcium cell death.

An alternative explanation is that kainate may interact directly with the NMDA receptor. Direct non-specific binding by the non-NMDA agonists on NMDA receptors has been speculated (Deupree *et. al.*, 1996). This speculation was again fuelled by the necessity of high concentrations of kainic acid (1 mM), much more than required to saturate kainate receptors, to produce neurotoxicity. This can now be explained by the toxicity of kainate not being dependent on it's association with the kainate receptor, but with the inhibition of the glutamate co-transporter and direct activation of NMDA receptors, especially at higher doses of kainate.

Oedema, resulting from the diffusion of water into the extracellular space, may also play a role in kainate toxicity (Sperk *et. al.*, 1983). The subsequent constriction of vessels in the vicinity may cause slight alterations in local environment (such as blood flow). Although this is a non-specific effect mediated by kainate it may compound the already poor situation.

Histological Consequences of Kainate Toxicity

The appearance of the brain after kainic acid injection has been well characterised (Jorgensen *et. al.*, 1993; Magloczky & Freund, 1993; Schwob *et. al.*, 1980; Sperk *et. al.*, 1983). Shortly after systemic kainate administration, the hippocampal regions showed a swelling of gliotic cells or postsynaptic dendrites, which was not observed (or greatly reduced) 13 hours later (Schwob *et. al.*, 1980). In the same time period many neurones underwent marked swelling or shrinkage. Interestingly the swelling was associated with glutamate toxicity, while the argyrophilic appearance was associated more with status epilepticus (Schwob *et. al.*, 1980). This stresses that kainate and glutamate do not have identical mechanisms of action. In the hippocampus, damage in the CA3 region is prominent after 8 hours, but damage to the CA1 region does not appear until a later time point (24 hours). Thus either CA1 neurones are more resistant to excitotoxic damage than CA3 neurones (possibly due to a lower density of kainate binding sites) or the CA1 neurones degenerate by a different mechanism from those of the CA3 region. As shown by our results using a low dose of CGS 21680 or DPMA i.p., the latter appears more likely. One of the speculative mechanisms by which the CA1 and other neurones distal to the CA3 region succumb to both i.p. and i.h. administered kainate is by direct innervation along axonal pathways.

Another post-intrahippocampal injection characteristic of kainate is an induction of glial cells in the brain. Within the damaged regions there is an increase in both small rounded macrophages and microglia (identified by a rod-shaped nucleus; Araujo & Wandosell, 1996; Jorgensen *et. al.*, 1993). Both cell types are present by day 2 and

maximal at 7 days. This general gliosis was apparent in the CA3 region, dentate hilus and entorhinal cortex. Consistent with a lack of substantial neurodegeneration to the CA1 subfield, no major influx of gliotic cells was apparent (Jorgensen *et. al.*, 1993). This invasion of blood-borne macrophages was not just dependent on neurodegeneration or blood brain barrier degeneration, as none were observed after lesioning of the dorsal hippocampus, suggesting a more specific response to the kainic acid insult. This is further substantiated by the same report which observed differences between the gliotic response to kainate and AMPA. The gliotic response may be of considerable importance in the overall toxicity of kainate as a mechanism of removing cellular debris and their release into the extracellular environment of a large number of molecules (including free radicals, nitric oxide and neurotrophins; Korsching, 1993). Aside from their role in the short term mediation of excitotoxicity, which is discussed below, these molecules have a long term effect on the environment of the damaged brain. Immediately after the hippocampal insult, astroglial processes extending towards the pyramidal cells of the CA3 region can be observed. A network is formed by day 21. Gliotic cells are still apparent 6 months later (Jorgensen *et. al.*, 1993).

Distal Damage

There is now a large amount of research to suggest that kainate exerts neurotoxic effects to distal regions via afferent fibres. This hypothesis relies on the highly selective pattern of toxicity by kainic acid. Kainate is known to be toxic in areas of high kainate receptor density (e.g. CA3a region), but is also toxic in areas where

there is not such a high degree of kainate binding (e.g. the pyriform; Unnerstall & Wamsley, 1983). Studies of kainate damage have found that areas linked by axonal connections tend to display linked damage. For example, Schwob *et. al.* (1980) observed from differing injection regimens that structures in the thalamus (such as the mediodarsal thalamic nuclei) and cortex (such as the agranular areas), which are linked neuronally, did not show damage independent of each other. Either both regions showed signs of neurodegeneration, or both were undamaged.

A simple experiment by Moncada *et. al.* (1991b), using both NMDA and non-NMDA antagonists after focal injections of kainate, AMPA and NMDA revealed a number of irregularities. Both kainate and AMPA were injected at a dose which caused near complete neuronal death in the subfields CA1-4. The non-NMDA antagonists 2,3-dihydro-6-nitro-7-sulphamoyl-benzo(f)quinoxaline (NBQX) and L-(amino)phenyl-4-methyl-7,8-methylendioxy-5H-2,3benzodiazepine HCl (GYKI 52466) both protected neurones in the CA1 and CA2 from kainate-induced neurotoxicity. There was neither protection of the CA3 region after kainate, nor any hippocampal region after either AMPA or NMDA injection. The NMDA antagonist (E)-4-(3-phosponoprop-2-enyl)-piperazine-2-carboxylic acid (D(-)-CPPene) protected against kainate and NMDA in the CA1 and CA2 regions. The lack of protection observed against AMPA with the non-NMDA antagonists was put down to the dose of the antagonist being such that it inhibited the binding of the less selective ligand (kainate), but was not high enough to compete with AMPA, which has a greater affinity for it's own receptor. This may also explain why no protection was observed in the CA3 against kainate. As both the non-NMDA receptor antagonists used have a greater affinity for AMPA over kainate receptors (Honore,

1991; Ouarduoz & Durand, 1991), it seems unlikely that, if the dose used cannot block AMPA receptors against AMPA, it can inhibit kainate receptors binding kainate.

This study highlighted the concept that the mechanism for kainate toxicity in the CA1 was not the same as for the CA3 region. It suggests that for the CA1 region the toxicity of kainate is dependent on both AMPA and NMDA receptors, while for the CA3 region, kainate excitotoxicity is more dependent on the kainate receptor. This is in stark contrast to our results obtained with MK-801 which showed complete protection in the CA3a region after intrahippocampal kainate injection. Again the deciding factor on the mechanism may be dependent on dose. In the study of Moncada *et. al.* (1991b) the dose used was both higher than our own and sufficient to cause extensive damage throughout the hippocampus (including the less sensitive dentate gyrus). The higher dose of kainate might be enough to activate both AMPA and NMDA receptor populations in the CA1 region to cause direct toxicity, while in our own study, kainate may not be at a concentration high enough to bind indiscriminately to NMDA receptors. In the CA3 region, the high dose of kainate may result in cell death by the activation of a large number of kainate receptors, while our lower dose may require the activation of NMDA receptors (e.g. by kainate-induced glutamate release). A further possibility may be that kainate can activate post-synaptic neurones only over a certain threshold concentration (either directly or by releasing glutamate), resulting in distal CA1 toxicity.

Three speculative mechanisms have been suggested to explain this damage along axonal connections (Schwob *et. al.*, 1980). The first is that either kainate itself or a toxic metabolite is transported the length of the axon. This product then exerts a

toxic effect at the distal population of neurones. As far as is known kainate (or metabolite) is neither taken up by the postsynaptic cell nor incorporated into macromolecules necessary for transport facilitation. This mechanism would also require the presence of binding sites in the distal region for kainate (or the speculative metabolite) to exert the toxic effect. As one of the peculiarities of distal damage is that there is not an abundance of kainate receptors, it seems unlikely that even if kainate were transported it would be unable to exert an effect.

Another possibility is that neuronal degeneration at the primary site results in the production of transneuronal effectors which mediate distal damage. This would suggest that a non-specific mechanism is responsible for the production of distal damage. As physical lesions which damage primary sites do not reproduce distal toxicity in other sites which are linked by neuronal connections, this mechanism is unlikely.

The third and most likely scenario is that kainate acts postsynaptically to initiate neuronal excitability, which is propagated along axons to populations removed from the primary location of kainate action.

Actions of Adenosine Agonists and Antagonists Against Kainate, i.h.

In complete contrast to the effects observed after intraperitoneal injections, CGS 21680 did not appear to protect against kainate excitotoxicity if administered intrahippocampally. This lack of protection was observed using a range of doses, the uppermost of which is believed to activate A_1 receptors (Zhang *et. al.*, 1994a). The lack of protection by the A_{2A} agonist suggests that the beneficial effects are not

mediated locally at the hippocampus. While not measured, it is unlikely that the drug solution would diffuse far from the injection point. It has been observed previously that a 5 μl solution of 10 nmol kainate injected over 10 minutes diffused a radial distance of only 1.5 mm from the tip of the cannula (Schwarcz *et. al.*, 1978). Although the injection volume used in the above experiments was five times greater than our own, the speculated diffusion distance for our study would only be ~0.9 mm (using the assumption that kainate diffuses in a spherical manner). Even considering differences in drug attributes (e.g. lipophillicity), it remains unlikely that any of our compounds injected would diffuse throughout the whole rat brain. The i.h. experiments do not therefore rule out a global centrally-mediated mechanism. Combining these results with the i.p. experiments involving 8-PST, suggests that the effect of CGS 21680 is mediated most within the periphery. The lack of complete reversal of the CGS 21680 protection with the peripheral adenosine antagonist suggests that some of the protection may be due to a central effect, which, as just stated, is likely to be an effect mediated throughout the brain, without any specific localisation to A_{2A} receptors in the hippocampus.

While no protection was observed after central administration of CGS 21680, intrahippocampal injection of the A_{2A} antagonist, ZM 241385 did dramatically reduce kainate-induced toxicity in the CA3a and CA3b regions. This further substantiates the theory of A_{2A}-mediated inhibition of the A_1 receptor, especially when considering that the addition of the A_1 receptor agonist R-PIA, did not protect the CA3a region to any degree. While the inhibition of detrimental effects mediated by the A_{2A} receptor in the hippocampus may account for some of the protection afforded by ZM 241385, combined with i.p. experiments, the evidence suggests the

involvement of the A_1 receptor. Further evidence comes from the lack of protection following the administration of adenosine itself (which would activate both the A_1 and A_{2A} receptor subtypes). While not protecting the CA3a region, R-PIA, at a low dose did protect a different subsection of the hippocampus (the CA3b region). This effect was not observed with a higher dose. Differences between the two cellular sub-populations (CA3a and CA3b), including different A_1/A_{2A} receptor ratio, may account this protection. Alternatively, as peripherally administered kainate selectively damages the CA3a region, while sparing the CA3b area, damage to the CA3b region by intrahippocampally injected kainate may be by a different mechanism to that of the CA3a area.

Mechanism of Adenosine A_{2A} Receptor-Mediated Protection

Adenosine, normally present at 0.05-2 μM in the extracellular fluid, increases dramatically in the presence of kainate. The pattern of adenosine increase is bell shaped, increasing shortly after kainate administration and peaking at 40 minutes (Carswell *et. al.*, 1997).

Adenosine, by A_{2A} receptor stimulation, has been shown to exert effects on blood flow (Phillis, 1989; Torregrossa *et. al.*, 1990), phagocytosis, free radical generation and neutrophil adhesion (Cronstein *et. al.*, 1994).

The vasodilatory effects of adenosine via nitric oxide modulation have also been attributed to the A_{2A} receptor, with little or no implication of any A_1 receptor-mediated interaction. Stimulation of L-arginine transport (system y^+) and a rise in cGMP in endothelial cells, indicative of an increase in nitric oxide synthesis, are

apparent in foetal endothelial cells after A_{2A}, but not A_1 receptor stimulation and are inhibited by ZM 241385 (Sobrevia *et. al.*, 1997).

Adenosine A_{2A} receptors modulate the activity of cells involved in the inflammatory response, by a variety of mechanisms. The adhesion of neutrophils to the endothelium, important for effective invasion of leukocytes, is prevented by A_{2A} agonists, as is the priming of neutrophils by macrophages and monocytes by the release of tumour necrosis factor-α (TNF-α; Cronstein, 1994). While the former is mediated by the A_{2A} receptor (Cronstein *et. al.*, 1985), it is unclear if the latter is due to any receptor-induced changes (Parmely *et. al.*, 1993). Generation of superoxide radicals by neutrophils is enhanced by an action of chemoattractant molecules, e.g. *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), on their receptors (Cronstein *et. al.*, 1990). Coupling of these receptors to the cytoskeleton, increased by the stimulation of A_{2A} receptors, results in receptor desensitisation (Cronstein *et. al.*, 1990). The A_{2A} receptor therefore results in a decrease in neutrophil radical production. The combination of these properties of the A_{2A} receptor suggests the presence of another protective mechanism in the modulation of the inflammatory response, decreasing the overproduction of damaging molecules. To determine how much effect the inhibition of this response has on cells within the compromised areas, it is first necessary to assess any increase in toxicity due to this response. No study has directly measured the leakage of damaging molecules (such as the superoxide radical, hydrogen peroxide and possibly BDNF) into the extracellular space and the effects of released neurotrophic factors have yet to be fully determined.

The above results suggest that A_{2A} agonists work by a mechanism that is not local to the hippocampus. If the inhibition of the local inflammatory response was of major

importance, then it would be expected that intrahippocampal injections of CGS 21680 would retain the protective abilities observed by systemic injections. While the inhibition of adhesion of neutrophils to endothelial cells may not occur due to the limited distance that the adenosine analogue diffuses through the brain, the remaining properties would still be apparent. It therefore appears that the production of neurotoxic effectors by this route may not play an important role in increasing excitotoxic damage. There are a number of explanations to this.

The first is simply that the amount of leakage of molecules such as superoxide radicals, may not be substantial enough to induce damage. As mentioned in the Introduction, cells have an array of different molecules to neutralise toxins (such as glutathione), and it is only after the volume of metabolites overcomes these defences that cellular death occurs. Alternatively, due to the release of large amount of toxic molecules from other sources during excitotoxicity, the leakage of damaging compounds into the extracellular environment by the inflammatory response may be surplus to that required to cause neurotoxicity (such that their removal would make little impact on the survival of neurones). As inflammatory cells are only apparent in areas of damage, the leakage of these molecules will only occur in areas where large amounts of toxic metabolites are already present (e.g. superoxide radicals released by disrupted mitochondria). The reduction or increase in damaging effectors may be offset by an opposite effect of beneficial molecules (such as FGF). As shown in the above experiments, glial cells were only present when accompanied by cellular damage. It is possible, therefore, that the gliotic response occurs after the onset of damage and is concerned with the long-term maintenance of the damaged

environment, with very little involvement in mediating kainate-associated excitotoxicity.

A further explanation for the lack of protection of CGS 21680 after central administration, even though it inhibits potentially detrimental inflammatory effects, involves the route of drug penetration. After i.p. injections the build up of kainate is more gradual than i.h., where the compound is applied directly into the hippocampus. Neuronal degeneration after centrally administered kainate was apparent 1 hour post-lesion (Liu *et. al.*, 1996), while peripheral injections required 3-5 hours to display damage (Schwob *et. al.*, 1980). The speculative reason for a lack of A_{2A} effect may therefore be that the inflammatory response does not contribute to i.h. kainate toxicity, whereas it plays an important role in toxicity after i.p. injections.

With reference to the protection of CGS 21680 (as well as the other compounds) the protective mechanism occurs before the onset of the inflammatory response, preventing it's initiation. This is shown by the absence of any cells involved in the response in the hippocampal environment of undamaged brains.

A_{2A} agonists also have a number of effects on transmitters other than glutamate. A_{2A} receptor stimulation by CGS 21680 enhances the expression of the gene coding for tyrosine hydroxylase in cultured PC12 cells (Chae & Kim, 1997). This in turn modulates the catecholamine biosynthesis pathway, of which tyrosine hydroxylase is the rate limiting enzyme (Nagatsu *et. al.*, 1964). The activation of the A_{2A} receptor can also facilitate dopamine synthesis and release in striatal cell systems (Onali *et. al.*, 1988; Zetterstrom & Fillenz, 1990) and reduce both the affinity of D2 agonists for the D2 dopamine subtype receptors (Ferre *et. al.*, 1991), and the resultant effect of

the receptor (Ferre *et. al.*, 1993). Acetylcholine release from cholinergic neurones can also be increased by CGS 21680 (Cunha *et. al.*, 1995).

Intravenous CGS 21680 has been shown to depress cerebral energy metabolism in a large number of areas in the brain, including the hippocampus (Nehlig *et. al.*, 1994). Regions that displayed this reduction in glucose utilisation included areas of cortex, hypothalamus, thalamus as well as the limbic system. Therefore, there was no correlation between known A_{2A} receptor binding sites and pattern of depressed metabolism. While this lack of conformity between effect and receptor localisation is unusual, it is not unique. Similar mismatched results have been obtained for GABA (Palacios *et. al.*, 1982), dopamine (Brown & Wolfson, 1981) and muscarinic agonists (Dow-Edwards *et. al.*, 1981). The effect is therefore either due to a non-specific interaction or specific action mediated at a location distal to the area of effect. Whether CGS 21680 acts via an adenosine receptor subtype (such as the A_{2B}) receptor or other binding sites dissimilar to adenosine A_1, $A_{2A/B}$ and A_3 receptors (Johansson *et. al.*, 1993), could not be determined by the use of co-injected antagonists. The profile of the location of these sites (the non-adenosine receptor sites were localised mainly to cortical regions) did not correspond to the regions where glucose depression was observed, suggesting that this effect was not mediated by any known binding site of CGS 21680.

Depression of the metabolism of glucose is unlikely to be mediated by the A_{2B} receptor, as at the dose used (0.01 mg. kg^{-1}) CGS 21680 does not appear to significantly bind to the receptor subtype (Jarvis & Williams, 1989; Johansson *et. al.*, 1993; Lupica *et. al.*, 1990). The participation of A_1 adenosine receptors was

dismissed by the lack of duplication of the CGS 21680 depression by an A_1 selective agonist (Nehlig *et. al.*, 1994).

It is conceivable that this non-adenosine receptor-mediated depression of glucose utilisation may be protective against kainate-induced excitotoxicity (where there is a depletion of ATP - Lothman & Collins, 1981; Retz & Coyle 1980) and similarly ischaemia. By decreasing the demand for a limited (and depleted) resource (ATP), CGS 21680 could decrease cellular stress and related damage. Glucose utilisation during ischaemia is decreased in all areas except those areas that are susceptible to damage (Choki *et. al.*, 1983; Jorgensen *et. al.*, 1990; Pulsinelli *et. al.*, 1982). Due to the kainate-mediated action in vasodilatating cerebral blood vessels, any effect CGS 21680 may have is likely to have more importance on peripheral vessels. Unfortunately, while studies have shown the advantage of vasodilatation in ischaemia (where blood flow is restricted;), none have investigated this within the kainate model of excitotoxicity. Therefore whether increasing peripheral blood flow (without the increase of nitric oxide radicals observed with kainate-induced central vasodilatation) is protective is not known. Alongside increasing the nutrient supply to compromised areas, vasodilatation may also increase the washout of kainate (and therefore potentially decrease the toxic effects). This is less likely when considering that Sheardown and Knutsen (1997) showed protection with CGS 21680 when given 30 minutes post-operatively.

In conclusion, the evidence presented suggests that A_{2A} receptor mediated protection is an effect that is not mediated by A_{2A} receptors in the hippocampus. The most likely candidate for this protection is the vasodilatory action on both peripheral and central blood vessels. While this is probably the major beneficial influence, other

factors that may contribute to a lesser degree are those which influence glucose utilisation and the inflammatory response.

In the physiological situation (without external influences) it is unlikely that the adenosine released would have a large effect on peripheral vasodilatation. Therefore the protective mechanisms of endogenously released adenosine would rely on a more locally mediated mechanism. As it seems probable that A_{2A} receptors inhibit the action of A_1 receptors, this mechanism would have to be mediated by A_{2A} receptors. The lack of protection by A_{2A} agonists after i.h. injection (which is more equivalent to the normal physiology of excitotoxicity as there are no peripheral effects), would question the neuroprotective role of endogenously released adenosine. The release of adenosine during kainic acid insults may not, therefore, be an endogenously released neuroprotectant, but simply a part of the excitotoxic pathway. This is more probable when considering evidence that adenosine release is not unique to the brain, but has been shown after stimulation of a number different of preparations, including the stomach, heart, vas deferens, blood vessels, bladder, lung and ileum (see Fredholm & Hedqvist, 1980). Adenosine release may simply be a consequence of the ionic fluxes across the membrane. Further evidence for this comes from our own study where adenosine, given intrahippocampally, did not alleviate kainate-induced excitotoxicity even at a dose of 2.5 nmol. This concentration of adenosine is greater than the 2 μM levels observed after twin pulses of 1 mM kainate (Carswell *et. al.*, 1997).

This does not discount the use of adenosine agonists and antagonists in the prevention of kainate induced excitotoxic damage, but suggests that in the normal physiology of the insult, endogenously released adenosine may not have the protective role previously assumed.

Origin of Adenosine Release

Both *in vivo* and *in vitro* studies have shown an increase in extracellular adenosine after kainate administration and ischaemia (Carswell *et. al.*, 1997; Latini *et. al.*, 1995). Carswell *et. al.* (1997) showed that the 2-fold increase induced by a 5 minute pulse of 1 mM kainate was due to the activation of non-NMDA receptors, as an inhibition of this rise by a non-NMDA antagonist (CNQX), was not observed after NMDA antagonists (MK-801 and (±)-AP-5). *In vivo*, the Na^+ channel blocker tetrodotoxin (TTX) also prevented the kainate-induced change in adenosine levels, implying that neuronal excitation and subsequently generated action potentials are the important mediators. Due to the rapid desensitisation of kainate receptors, excitation (via an influx of Na^+ and/or Ca^{2+}) is likely to be mediated through the kainate-insensitive AMPA receptors. As kainate receptor stimulation may even be inhibitory (Chittajallu *et. al.*, 1996), the net release may represent an aggregation of opposing effects mediated by both non-NMDA receptors. Further release may be a property of subsequently released (or leaked) glutamate, which may involve metabotropic receptors (Carswell *et. al.*, 1997). Inhibition of glutamate release by U50,488H decreased the amount of extracellular adenosine detected by microdialysis after kainate administration (Carswell *et. al.*, 1997).

Exactly how adenosine is formed is not yet entirely clear. Although the purine can be formed by a number of different enzymatic routes, the most common is dephosphorylation by ecto-5'-nucleotidase of 5'adenosine monophosphate (5'AMP). The enzyme is not unique to either the cytosol or the extracellular space and exists within membranes of glial, and the soma and axons of neuronal cells (Newby *et. al.*,

1987). ATP is known to be released from nerve terminals. Release of ATP has been observed from some nerve fibres both in conjunction with (Burnstock, 1976), and independently of (Abood *et. al.*, 1962), neurotransmitter release, implying that most or all of ATP release is not dependent on exocytotic mechanisms. ATP release has been shown to accompany neuronal depolarisation in some nerve systems, implying that such a release during excitotoxicity may increase extracellular adenosine concentrations. In contrast, in other systems (e.g. non-myelinated nerves), activity is coupled with intracellular indications of ATP breakdown (e.g. increases in phosphate levels), before the rise in the extracellular purines, with no release of ATP. While extracellular adenosine concentrations may be dependent on both ATP release followed by extracellular degradation and intracellular breakdown of ATP with subsequent adenosine release, evidence favours the latter as the most important (Fredholm & Hedqvist, 1980). While this appears to be the major route of adenosine formation, a small amount of adenosine may be formed by the S-adenosylhomocysteine hydroxylation of S-adenosylhomocysteine (Schrader *et. al.*, 1981) or from the breakdown of cAMP (Newman & McIlwain, 1977).

Levels of adenosine can be controlled by both intra- and extracellularly located degrading enzymes, as well as adenosine transport systems. Adenosine deaminase (present mostly in the intracellular environment) and adenosine kinase, both breakdown adenosine to inosine or ATP respectively, with the kinase enzyme being most significant (Fredholm & Hedqvist, 1980).

There are also numerous transporter systems for adenosine, increasing adenosine movement to and from the extracellular space. A degree of regional variability in transport. systems is indicated by both a difference between distribution of

transporters inhibited by nitrobenzyl-mercatopurine riboside (NBMPR) and dipyridamole in the brain (Deckert *et. al.*, 1988), and a higher affinity for adenosine of neuronal uptake systems over those of glial cells (Thampy & Barnes, 1983). This, coupled with evidence of variable affinity and selectivity between cell types, suggests there is discrimination in regional uptake.

Unlike adenosine which is rapidly metabolised or taken up (plasma half life of approximately 1 second; Camm *et. al.*, 1991), CGS 21680 is not broken down in the presence of adenosine deaminase, nor does it appear to be taken up (e.g. by blood cells). After an intravenous dose, CGS 21680 shows rapid clearance in an unchanged form (Camm *et. al.*, 1991).

The Role of Apoptosis Within Excitotoxicity

While an earlier chapter opened the discussion on the validity of the research tools used to establish the presence of apoptotic cells, the argument below concentrates on evidence directly linked to excitotoxicity. A large amount of research has linked kainate damage with a mechanism of apoptotic cell death (e.g. Nishiyama *et. al.*, 1996; Pollard *et. al.*, 1994; Simonian *et. al.*, 1996; Weiss *et. al.*, 1996). After an i.p. dose of kainate identical to that used in our study, DNA fragmentation was observed after 18 hours and was apparent after 72 hours in regions that displayed kainate-induced damage (e.g. hippocampus; Filipkowski *et. al.*, 1994). Using a slightly higher dose, Weiss *et. al.* (1996) observed TUNEL detectable fragmentation in the thalamus, amygdala, cortex and the CA1 region of the hippocampus. The staining remained at similar levels for at least 7 days, suggesting a long lasting, dynamic

process. The CA3 and CA4 regions only displayed TUNEL staining if the animal exhibited severe seizures. Nuclear run-on assays have shown that glutamate, by stimulating NMDA-receptor mediated calcium influx, increases the transcription of IEGs (Bading *et. al.*, 1995). Similarly, kainate induced the expression of *c-fos, fos B, c-jun, jun B, zif/268* and *nur/77* in hippocampal culture. While this mechanism was believed to involve the NMDA receptors (Bading *et. al.*, 1995), calcium influx through kainate-linked L-type calcium channels effects similar results in cortical and dentate gyrus cells (Lerea *et. al.*, 1992). The response was biphasic, with the initial expression limited to highly susceptible regions such as the hippocampus and entorhinal cortex, before a delayed, more widespread induction (Kasof *et. al.*, 1995a; Willoughby *et. al.*, 1997).

Although many IEGs are believed necessary for the apoptotic pathway, it has been suggested that *c-fos* induction may be a mechanism of protection. While the majority of pyramidal cells expressed *fos-lacZ*, only limited numbers succumbed to excitotoxic damage (Kasof *et. al.*, 1995a). Further evidence comes from the observation that, during the delayed period of expression, almost the entire forebrain shows an increase in expression, even though not all of the structures undergo apoptosis (Willoughby *et. al.*, 1997). The neurotrophin bFGF, which protects against kainate toxicity *in vivo* (possibly by maintaining calcium homeostasis; Lui *et. al.*, 1993), increased the induction of *c-fos in vitro* (Simpson & Morris, 1994). Evidence suggests that although IEGs may constitute a significant link of the apoptotic pathway, apoptotic cell death is determined by the pattern of IEG expression rather than IEG expression *per se* (Kasof *et. al.*, 1995a). Cells that express *c-Fos*, but do

not proceed to die by apoptosis, may therefore not be exhibiting a pattern of expression necessary for the initiation of apoptosis.

In our own study, i.p. kainate induced damage in both the CA1 and CA3a regions (from haematoxylin and eosin staining), but only induced positive staining for TUNEL in the CA1 region. The CA3a region remained devoid of any positively stained cells. This is in agreement with other studies. Wiess *et. al.* (1996) observed this phenomenon of apoptotic cells localised only to the CA1 region. The disparity mentioned above, where apoptosis occurred in the CA3 and CA4 regions after seizures, may be due to the higher dose used in that study (15 mg. kg^{-1}). Similar results have been obtained in ischaemic models. Honkaniemi *et. al.* (1996) reported a high density of TUNEL-positive cells in the CA1, and a low amount in the CA3a (although none were obviously apparent from the photomicrographs).

From this data it is tempting to suppose that cells which contain a high density of kainate binding sites (such as the CA3a region of the hippocampus) undergo necrosis due to the rapid excitotoxicity of the compound, whereas those sites which are distal (CA1 and pyriform regions) succumb to apoptosis. Ankarcrona *et. al.* (1995) showed that, following glutamate exposure, an initial subset of cells lost mitochondrial function and died by necrosis. Cells which restored mitochondrial homeostasis underwent delayed apoptosis. The ability of cells to recovery shortly after glutamate addition and therefore the route by which cells died, was determined by the concentration of glutamate. Therefore, after i.p. injections, it is probable that cells of the CA3a region do not recover from the original kainic acid insult and die by a necrotic mechanism. Cells distal to the CA3a population die by both necrosis and

apoptosis, as the percentage of TUNEL stained cells in this study was always below the total percentage of damaged neurones.

Brain sections which were stained for both TUNEL-positive cells and eosin, showed that cells of the CA1 region which were stained for TUNEL, also stained bright pink (indicating non-viability), whereas not all of the pink cells were TUNEL-positive. This suggests that the majority of distal cells appeared to follow the apoptotic pathway, and therefore, that in these areas kainate was not exerting a direct toxic effect (such as was observed in the CA3a region).

While some studies which examined neurones at the electron microscopic level after i.p. injection of kainate, suggested that many of the cells stained positive for TUNEL did not share apoptotic morphology (e.g. chromatin condensation), there still remained a proportion of these cells within the population which did display structural apoptotic signatures (Nishiyama *et. al.*, 1996). In contrast, an *in vitro* study did observe chromatin condensation (via the use of a DNA intercalating dye), but showed a lack of either protein or RNA synthesis (Simonian *et. al.*, 1996). The latter observation may help to clarify how apoptosis, generally an energy requiring process, can occur in an ATP depleted environment.

The reliability of chromatin condensation as a marker for apoptosis has been questioned, as it's presence has been observed without other ultrastructural features associated with apoptosis (Sun *et. al.*, 1994). In studies of cortical cells after middle cerebral artery occlusion (MCAO) with NMDA injections in 7 day old rats, DNA fragmentation and nuclear chromatin condensation was observed alongside characteristics not consistent with apoptosis (such as leakage of chromatic material

across the nuclear membrane). 24 hours after NMDA injection, there was a mixture of both TUNEL positive cells which did not display all of the apoptotic criteria with cells which appeared to die by classical apoptosis. This study highlights a major difference between the mechanisms of excitotoxic and ischaemic cell death. As excitotoxic neuronal death appears to require the binding of glutamate analogues to receptors which are located within specific regions, the toxicity is mediated directly within this region or indirectly via neuronal fibres to distal regions. Ischaemic insults achieve a similar mechanism of neuronal death by inducing the release of the excitatory amino acid itself, but also restrict blood flow through a proportion of the brain. It is therefore possible that while both excitotoxic and ischaemic insults initiate a similar sequence of events (inducing apoptosis at distal sites), in ischaemic models, the extra complication of reduced blood flow ensures that the majority of cells do not complete the apoptotic cell death cycle, but are overtaken by necrotic mechanisms. Even within excitotoxic cell death there are three apparent subclasses. The first are those cells which stain bright pink for eosin, indicating cell death, but do not stain for TUNEL; these cells presumably died by necrosis. The second are cells which stain positive for TUNEL, but do not show all the characteristics necessary for apoptotic cell death. These may be neurones where necrosis has overtaken an initiated program of apoptosis. The last are cells where all the criteria of apoptosis are met.

In contrast to our systemic results, central injections of kainate induced TUNEL-positive staining throughout the CA3a region. In agreement with the lack of any distal damage, no other area was found to contain cells that stained by the TUNEL method. The obvious reason for apparent apoptotic damage in a region where only

necrotic damage was observed after i.p. injections, was that the dose was such that cells were not compromised by the initial excitotoxic insult, recovering before undergoing apoptosis. Similar results were observed after kainate was injected at a higher dose (1.2 μg in 0.3 μl) into the amygdala, although in this case the CA3 damage is distal to the injection site (Pollard *et. al.*, 1994). Apoptosis (measured by staining and morphological techniques) also occurred in the amygdala, in the presence or absence (after diazepam treatment) of damage to the CA3. Kainate can therefore initiate local (i.e. non-distal) apoptotic cell death. As this study used higher doses than our own, the difference in mechanism may not, therefore, be related to differences in central concentrations.

Another possibility may be the route of administration. A peripheral injection of kainate, due to the time taken to penetrate the brain, may therefore induction mechanisms that are not initiated by central injections. One of these mechanisms may be the inflammatory response. Due to the more gradual build up of peripherally over centrally administered kainate, cells of the inflammatory response could be stimulated by stressed cells before the extracellular levels of kainate reach toxic levels. The presence of the inflammatory mediators may then induce necrosis in a proportion of cells (e.g. those already stressed), but protect the remainder. After i.h. injections the levels of kainate within the hippocampus are immediately high. Without the presence of any immediate inflammatory response, cells recover from the initial insult, but subsequently undergo apoptosis. This may also explain why even after a high systemic dose of kainate, a population of approximately 40 % of the cells in the CA3a survived (Strain *et. al.*, 1991), while after i.h. administration nearly 100 % succumbed to excitotoxicity.

Our study also showed that the mechanism of protection observed with the i.p. administered adenosine compounds, did not selectively inhibit either necrotic or apoptotic cell death. No unexpected increase or decrease in TUNEL staining was evident. In the CA1 region of animals given an i.p. injection of kainate, the degree of apoptosis correlated with damage such that there was always a high proportion of TUNEL staining in cells where damage was observed. In conjunction with the experiments with kainate alone, no drug administered, induced TUNEL staining within the CA3a region. This is entirely consistent with the proposed mechanism of action for protection by either A_{2A} or A_1 agonists, which suggests that protection is mediated at a point much earlier along the neurodegenerative pathway, before cells have undergone necrosis. Ankarcrona *et. al.* (1995) suggested that apoptosis only occurs after a population of cells, which failed to recover mitochondrial integrity, underwent necrosis. If the mechanisms of protection with any of the above compounds involved mediating against this delayed apoptosis, we would expect to observe a high degree of damage without the presence of apoptotic cells.

The introduction of antagonists revealed a slight discrepancy in this relationship between apoptosis and necrosis. In i.p. experiments, the slight induction of apoptosis in the CA3a after the administration of ZM 241385 or 8-PST, suggests that inhibition of the A_{2A} receptor may increase apoptosis. That both antagonists have a similar effect suggests that it is not mediated by hippocampal A_{2A} receptors. Speculatively, the antagonists may increase apoptosis by preventing the A_{2A}-mediated induction of the inflammatory response. As discussed more fully below, the inflammatory response may increase necrosis while preventing apoptosis. Any inhibition of this

response may therefore increase the proportion of apoptotic cells within the non-viable cell population.

This increase in apoptosis is not observed after the injection of ZM 241385 in the intrahippocampal model. This may be due to the lack of involvement of the inflammatory response in this regimen. That there is a complete absence of apoptosis in the CA3a region after co-injection of ZM 241385 with kainate, is suggestive of a role for the A_{2A} receptor in the promotion of apoptosis.

The A_{2A} receptor may therefore play an important role in determining the pathway by which certain populations die during kainate-induced excitotoxicity, although this role may be dependent on experimental conditions.

The Role of Growth Factors

The role of growth factors is both diverse and not yet fully understood. As the hippocampus itself contains the highest central mRNA levels of NGF, BDNF, NT-3 and NT-4/5, it does not seem much of a presupposition to assume that the family of neurotrophic factors exerts some effects during periods of neuronal insult. Many of the neurotrophins appear to be protective in various models of cerebral damage (Boniece & Wagner, 1995; Cuevas *et. al.*, 1994; Kromer, 1987; Lui *et. al.*, 1993b; Pringle *et. al.*, 1996).

Levels of BDNF mRNA increase after kainate administration (Guilhem *et. al.*, 1996; Rudge *et. al.*, 1995; Suzuki *et. al.*, 1995; Zafra *et. al.*, 1990), with peaks shortly after i.h. kainate administration and after 7 and 60 days. The highest peak was that of 7

days which reached levels of ~270% of control (Suzuki *et. al.*, 1995). The increase in mRNA is apparent in all regions of the hippocampus, with the most dense levels in the dentate gyrus. The possible protective mechanisms of BDNF may explain why the dentate gyrus is less sensitive to the excitotoxicity of kainate, although whether BDNF is protective is still in debate. Amongst the reports of protection by BDNF (Beck *et. al.*, 1994; Lindholm *et. al.*, 1993; Schabitz *et. al.*, 1997) are a few that disagree with these observations (Koh *et. al.*, 1995; Fernandez-Sanchez & Novelli, 1993). Glutamate neurotoxicity was enhanced in the presence of BDNF in cerebellar neuronal cultures (Koh *et. al.*, 1995). This enhancement is believed to be mediated through the NMDA glutamate receptor. Koh *et. al.* (1995), speculated that the detrimental effects of BDNF are constrained to necrotic cell death, with the protective potential reserved for cells following the apoptotic pathway of neuronal death. As many of the effects mediated by BDNF and other neurotrophins are regulated in the embryonic and neonatal developing stages, it would not be surprising if the protective effects were directed mainly against the apoptotic process.

BDNF and other neurotrophic proteins are expressed in both neurones and glia (Rudge *et. al.*, 1995; Korsching, 1993). Under normal conditions in co-cultures of hippocampal neurones and astrocytes, only the neurones appear to be responsible for mediating the levels of BDNF. Upon application of kainate, the protein levels of BDNF dramatically increased, in both neurones and reactive astrocytes peaking at a level 2-3 fold larger at 18 hours (Rudge *et. al.*, 1995). While this increase corresponded with mRNA levels, the experiment did not proceed beyond 3 days. Therefore the possibility of further peaks at 7 or 60 days was not investigated. As BDNF may mediate detrimental or protective effects on cells depending on the

mechanism of cell death (whether necrotic or apoptotic), this increase in BDNF protein levels in astrocytes after activation by kainate, may explain the differences in CA3a damage between i.h. and i.p. administered kainate. Following i.p. administered kainate, the activated inflammatory response and resultant increase in BDNF protein could exacerbate necrotic damage while preventing delayed apoptotic neuronal death. After central administration of kainate, BDNF may not be an important factor in determining the outcome of cells. This speculated absence of interference of BDNF after i.h. kainate may be due to a lack of coincidence in the onset of damage with induction of BDNF. Alternatively, due to the limited sphere of influence of central (as compared with peripheral) injections, the inflammatory response may be more limited than after i.p. injections. Any such decrease in neurotrophic influence on excitotoxic cells after centrally administered kainate, may result in a decrease in the number of neurones recovering from the early excitotoxic shock, but exaggerate the population undergoing apoptosis.

Highlighting the difference in time taken to induce either BDNF protein or mRNA, intrahippocampal injection of kainate does not show an induction of BDNF mRNA until 7 hours after the injection (Ballarin *et. al.*, 1991). After i.p. injections, kainate induced the expression of BDNF mRNA after only 1 hour (3-4 fold), peaking at 3 hours (9-10 fold ; Lee *et. al.*, 1997). From this, it appears that local injections of kainate may not induce the inflammatory response in the same manner as systemic administration. BDNF induction by i.h. kainate injection (mostly from a neuronal, not inflammatory source) does not therefore, appear before the onset of damage. After i.p. kainate, a coincidence of damage and BDNF mRNA increase occurs.

Systemic administration, due to a more extensive diffusion, has the opportunity to induce a much larger inflammatory response.

The time dependence of BDNF protection was highlighted in a hippocampal slice model of ischaemia (Pringle *et. al.*, 1996). Pre-incubation of cultures with BDNF protected the CA1 and CA3 pyramidal cells of the hippocampus, whereas addition of BDNF after the insult did not. In the study by Pringle and co-workers (1996) study, significant protection of CA3 neurones was achieved at a lower dose than that required for neurones in the CA1 region. Even so, at the dose required to significantly protect the CA3 region there was a decrease in percentage damage from ~34 % to ~10 %. In the CA1 region the same dose decreased damage from ~68 % to ~42 %. Therefore in both regions ~25 % of neurones were protected. This may imply that a similar subset of cells were protected in each region. Without examination of the mechanism of cell death (apoptotic or necrotic), it is impossible to tell if BDNF is selectively protecting against one mechanism over the other.

Basal levels of BDNF mRNA *in vivo*, were almost 3 - fold greater in the CA3 than in CA1 region (Kokaia *et. al.*, 1996). After ischaemia an increase in the CA3 region to a 3-fold control levels was observed after 2 hours, coupled with a 131 % increase in protein after 7 days. In contrast, the CA1 region displayed no transient increase in mRNA, but displayed a 25 % reduction in protein levels after 24 hours. The difference in mechanism of cellular death in the CA3 and CA1 region may therefore be due to a difference in level of BDNF protein and/or mRNA expression. In the CA3 region, the higher level may induce necrosis, but restrict apoptotic death. In the CA1 area, the level may not be enough to influence necrosis, nor to prevent apoptosis.

Our own immunohistochemical studies with kainate suggest that after 7 days, the only cells that stained for the presence of BDNF protein were neuronal, with very little, if any cells of the inflammatory response apparent. This is the case after either centrally or systemically administered kainate. This would imply that after 7 days any increase of BDNF is from a neuronal source. In i.h. experiments, this correlates with the study above (Ballarin *et. al.*, 1991), with no role for inflammatory response-mediated increase in BDNF. After i.p. injections, this similarly suggests that BDNF protein is increased after 7 days exclusively in neurones. The initial increase in mRNA after i.p. kainate (Lee *et. al.*, 1997) is unlikely to be due to the hippocampal neurones, as no such increase was observed at a similar time point (1 hour) after i.h. kainate (Ballarin *et. al.*, 1991). The early response to i.p. kainate may therefore be due to inflammatory cell increases. By day 7, any inflammatory mediated effects on BDNF protein levels have disappeared, leaving a neuronal response.

There appeared to be no direct correlation between damage and extent of BDNF protein after 7 days. Due to any sustained levels in BDNF probably being concerned with long-term maintenance, and not a defence against acute toxicity, 7 days after the initial insult may be too late to determine any direct relationship.

Evidence from Rudge *et. al.* (1995) suggests that the degree of upregulation of BDNF protein may not be enough to mediate protection/degradation as there is no evidence of the phosphorylation of the TrkB receptor necessary for signal transduction. Similarly, from *in vivo* experiments, there was no apparent correlation between BDNF mRNA and that encoding the full-length TrkB receptor protein (Falkenberg *et. al.*, 1996) after the central administration of the non-NMDA receptor agonist quisqualate. There was however, a correlation observed between BDNF mRNA and

components of the TrkB receptor (e.g. a 145-kDa glycoprotien, $gp145^{trkB}$). Due to gene splicing and the resultant variation of receptors (two different classes of receptors made from at least eight different transcripts; Barbacid *et. al.*, 1994), a lack of correlation between BDNF and full-length TrkB mRNA expression is not surprising. BDNF itself may not have to act on the TrkB receptor, as while neurotrophins have a receptor to which they most commonly bind, there is evidence suggesting that this relationship is not exclusive (Korsching, 1993). This diversity amongst binding sites may account for the suggested opposing roles of neurotrophic factors under different experimental conditions. Due to the interrelationships between growth factors (e.g. BDNF mRNA stimulation by FGF; Kwon, 1997), it is probable that both the protective and detrimental effects of the neurotrophic factors are mediated through a complex mechanism involving a number of different receptors and receptor subtypes.

From our experiments the adenosine compounds may have an effect on the kainate-induced increase in BDNF protein expression at 7 days. In our i.p. studies, the A_{2A} antagonist ZM 241385 appeared to increase the levels of expression. This is noticeable when given with CGS 21680, but is more startling when given alone, before kainate. In contrast R-PIA did not show any increase in BDNF protein at 7 days, even in damaged hippocampi, while BDNF protein was apparent in CGS 21680 injected animals that were damaged. After i.h. administration, kainate alone showed a high degree of BDNF expression. Co-injection of kainic acid with CGS 21680, ZM 241385 or adenosine resulted in the inhibition of this BDNF induction, even when damage was comparable. Co-administration of kainate with R-PIA did show

BDNF protein levels comparable to those after kainate alone in hippocampi that were severely damaged.

The response of neurones at day 7 may very well depend on a number of other influences in the period after the initial insult. Different effects of the adenosine compounds on effector systems (such as the inflammatory response) early during excitotoxicity may affect how neurones behave subsequently. For instance, differences in levels after i.p. R-PIA and ZM 241385 (both of which should directly or indirectly activate A_1 receptors), may be due to effects on different populations of receptors. For example. the increase in BDNF after ZM 241385 may be due more to inhibition of A_2 effects on the inflammatory response than allowing the activation of A_1 receptors. Without the interference of the inflammatory response (e.g. after i.h. injections), the effects on the neuronal increase in BDNF by the adenosine compounds may alter, with either inhibition or stimulation of the A_{2A} receptor decreasing BDNF levels. While the interactions between adenosine receptors and BDNF levels results in a complex relationship, BDNF levels at 7 days appear to have no effect on the outcome of the neuronal population. This is shown by a lack of correlation between protection/degradation and day 7 protein levels. Therefore these increases may be more relevant to the longer-term repair and maintenance of the hippocampus.

The Dual Role of Nitric Oxide

Nitric oxide has been shown to occupy a double edged role, both to attenuate or exacerbate excitotoxic damage. Kainate, via an interaction with the kainate receptor,

stimulates NO production (Balcioglu & Mather, 1993, Garthwaite *et. al.*, 1989). This release may be further exacerbated by a similar reported action for the NMDA receptor (East & Garthwaite, 1991; Faraci & Brian, 1995). An increase in diameter of cerebral arterioles is also apparent, although only after topical exposure of the blood vessels to 100 μM kainate for 3 minutes (Faraci *et. al.*, 1994). This neuronal NO production was inhibited by the addition of nitric oxide synthase-inhibitors (NOS-I). In our study two NOS-Is were administered systemically 10 minutes prior to kainate. L-NAME is a general NOS inhibitor, while 7-NI is specific for the neuronal subtype (i.e. does not prevent the actions of eNOS which include cerebral vessel dilatation; Faraci & Brian, 1995). In both cases marked protection against kainate-induced excitotoxicity was observed. This suggests that the effects mediated by NO are more harmful than protective in this model, which is in agreement with a large number of other studies (see Introduction). L-NAME at 20 mg. kg^{-1} inhibits almost 50 % of NOS activity (Iadecola *et. al.*, 1993). Our experiments observed protection at a quarter of this lower dose, suggesting that even limiting a small proportion of NO production is highly protective.

As free radical-associated cell death is considered important in excitotoxicity, the prevention of the conversion of NO to toxic metabolites ($ONOO^-$ and $^{\bullet}OH$), may represent the most important mechanism by which NOS-Is protect against cerebral insults. As well as decreasing free radical production, NOS inhibition by L-NAME has also been shown to decrease the extracellular glutamate concentration during seizures after a systemic injection of 10 mg. kg^{-1} kainate (Rigaud-Monet *et. al.*, 1995). This was presumably by the prevention of the presynaptic augmentation of glutamate release by postsynaptically released NO (O'Dell *et. al.*, 1991; Lawrence &

Jarrott, 1993; Sergovia *et. al.*, 1994). The inhibition by L-NAME of the NO-induced inhibition of glutamate uptake may also account for a proportion of the reduction in extracellular glutamate levels (Pogun *et. al.*, 1994).

While the above mechanisms of the NOS inhibitors may confer protection, the role of NO in glutamate release is probably more complex. Other evidence suggests that NO mediates an inhibition of the NMDA receptor, limiting the activity of the glutamate analogue (Manzoni *et. al.*, 1992). While NO may have different actions on the various glutamate receptors, it is more likely that NO has a conflicting role that is dependent on environmental conditions. Both the functional state of the receptor and the local glutamate concentration have been implicated (Rundfelt *et. al.*, 1995). While of possible importance in seizure related activity, the mediation of glutamate release by NO may not be of significance in enhancing neurotoxicity. Overall, the beneficial effects of NOS inhibition on the compromised brain appear to overcome any possible detrimental actions.

The dose of NOS-I appears to be important in determining the outcome of compromised neurones (Rundfelt *et. al.*, 1995). Low doses of NOS-I exert a protection against cerebral insults, which was not apparent (or was even reversed), when administered at higher levels (Carreau *et. al.*, 1994; Shapira *et. al.*, 1994). While the dual role of NO on glutamate release may account for this effect (i.e. at higher doses NOS-Is prevent the NO-mediated inhibition of the NMDA receptor), this is unlikely, as low doses L-NAME enhance NMDA convulsive activity (Buisson *et. al.*, 1993). Low concentrations of the NOS-I have also been shown to increase extracellular glutamate levels *in vivo* (and enhance NMDA-mediated release), but again at a higher concentration, this effect was not observed (Segieth *et. al.*, 1995).

As modulation of glutamate does not appear to correlate with damage, it is unlikely to contribute to excitotoxic insults. The increase in damage by high doses of NOS-I is probably due to the inhibition of blood vessels .

Within an ischaemic model, the time course of nNOS mRNA expression has been correlated with neuronal damage *in vivo*, although NOS containing neurones appeared to be slightly more resistant to degeneration themselves (Zhang *et. al.*, 1994b). This may be accounted for by the ability of nitric oxide to upregulate intracellular antioxidant systems (e.g. glutathione; Nicotera *et. al.*, 1997), whereas conversion in the extracellular space to toxic oxygen radicals initiates the injurious pathway.

CONCLUSIONS

This study has investigated the mechanism of both kainate-induced excitotoxicity and protection mediated by a number of compounds. Evidence from both our experiments and other studies suggest that kainate toxicity is not dependent on the kainate receptor, but relies on activation of AMPA, NMDA receptors and leakage of glutamate across the plasma membrane. We observed an improvement in neuronal survival after kainate-induced excitotoxicity following the administration of A_{2A} agonist. Further investigation suggested that this protection was mediated from the periphery, and not by A_{2A} receptors localised to the hippocampus. Results with the A_{2A} antagonist ZM 241385, provided more evidence for an inhibitory action of the A_{2A} receptor on the A_1 receptor. Importantly, these results suggest that adenosine, released endogenously during the cerebral insult, does not protect the pyramidal cells of the hippocampus.

The role of nitric oxide within this model was examined and although NO has a variety of diverse functions, it was shown that inhibition of a small proportion of NO synthesis prevented a large percentage of neuronal cell death.

The role of both necrotic and apoptotic cell death in excitotoxicity was investigated. Apoptosis was limited to discrete regions of the hippocampus and displayed different patterns of expression dependent on the route of administration. A link between the outcome of cells and the inflammatory response (with particular emphasis on BDNF) has also been postulated.

While the above study focuses on a whole animal model, an attempt was made to correlate this with data obtained from hippocampal studies (see Appendix I). This would have helped to provide further information on the exact mechanism of CGS 21680 protection as well as clarify the relationship between the A_{2A} and A_1 adenosine receptors without the complexity of *in vivo* interactions. Much more work needs to be focused on the role of the inflammatory response and in particular growth factors in excitotoxicity. While it is clear that some neurotrophins mediate protection, our study shows that BDNF may occupy different roles dependent on the situation (and therefore on the model used). A multiple approach (combining both *in vitro and in vivo* techniques) would therefore benefit our understanding. Other *in vitro* studies (such as electrophysiology) would also define the interaction between the two adenosine receptor subtypes, A_1 and A_{2A}. Clinically, if adenosine compounds are to be used to alleviate the cerebral damage associated with excitotoxicity, then the importance of this interaction cannot be overlooked.

APPENDIX I

DISSOCIATED HIPPOCAMPAL CULTURE

Hippocampal cell culture has been used widely to elucidate a number of different areas in various fields of science. This scope extends from examining the effects of free radical scavengers on hypoxic cultures (Rosenbaum *et al.*, 1994), to electrophysiological recordings of ion currents (Wann *et al.*, 1994; Garcia *et al.*, 1994), as well as numerous investigations into neurite growth (Brinton, 1994; Fernaud-Espinosa, *et al.*, 1994; Kobayashi *et al.*, 1994).

This frequent use of the hippocampus is deliberate and due to both specific reasons such as its capacity for activity-dependent changes in synaptic function (e.g. long-term potentiation; Bliss & Collingridge, 1993), its importance in epileptic activity, as well as less-specific influences. These latter incentives include the hippocampus as a source of a relatively homogeneous neuronal population (pyramidal neurones are prevalent at 80-95%), which is representative of the CNS in general (Banker and Goslin, 1991) and its presentation of a clear defined boundary in developed embryos, allowing relatively easy dissection. Due to the homologous cell population, hippocampal cell culture provides an opportunity to examine drug effects/interactions on pyramidal cells without the complications of influence from either afferent input from other cerebral regions or different cell types (e.g. glial cells or cells of the inflammatory response).

Early in this study it was decided to use hippocampal cell culture in conjunction with the *in vivo* experiments to provide a broader interpretation of the events during kainate-induced excitotoxicity and to elucidate mechanisms by which the adenosine compounds may be exerting their effects. Extensive research and experimentation into setting up the technique, which was novel to our laboratory, led to progress in establishing hippocampal neuronal cultures. Unfortunately, the cultures were to prove only partially successful, with inconsistent hippocampal cell survival under apparently identical situations. With this lack of reproducibility after nine months of experiments, we moved away from the *in vitro* approach, concentrating on the histological *in vivo* studies. The chapter below highlights problems with culture systems, not least the huge array of different methods used, and discusses changes made to our original protocol.

To establish hippocampal cultures, tissue from late foetal (E15-18) or early neonatal (D1-3) rat brains (or equivalent times for mice) are most commonly used. This provides the advantages of neurones being much less susceptible to damage due to the absence of a complicated system of axons and dendrites in the immature brain and a lower reliance of the neurones on innervation from other cell populations. In our research we used brains from both late embryonic (E14-15) and postnatal day 1 mice. We found that while hippocampal tissue was easier to remove from neonatal animals (due to a much greater size of the animal), more cultures tended to grow from tissue obtained from foetal animals. Unfortunately in foetal animals the small size of the hippocampal structure made dissection without any surrounding tissue more difficult. This was compounded by the use of mice rather than rats of the same relative age. To overcome this, dissection erred on the side of caution (i.e. removing

all non-hippocampal tissue at the possible expense of some of the hippocampus itself).

In most studies the method of dissection of the hippocampal tissue from embryonic or neonatal brain is similar, utilising previously established methods (Lochter *et al.*, 1991; Banker & Cowan, 1977; Segal, 1983). In our study, pregnant CBA mice were killed by CO_2 asphyxiation followed by cervical dislocation. Embryo sacs were removed and placed in a large petri-dish of Hank balanced salt solution (HBSS, a solution of inorganic salts, glucose and phenol red; Life Technologies Ltd.). The embryos were then separated from the membranous pouch, before the head was removed and placed into a small petri-dish of HBSS. Using sterile needles and forceps the skin covering the head was reflected. Under a microscope the skull was broken open and teased apart exposing the brain, which was subsequently removed and placed into another small petri-dish of HBSS. The brain was then bisected into two cerebral hemispheres. The easily discernible hippocampus was gently teased out using syringe needles. The hippocampi from 6-12 animals were placed into fresh sterile HBSS. The above operation was performed in an open sterile cabinet to allow the use of the microscope. The tissue at this point was transferred to a tissue culture flow hood.

Major differences in methodology appear after the hippocampus has been removed from the surrounding brain. Dissociation and breakdown of the hippocampus is usually achieved by the addition of trypsin to the tissue although some methods require only mechanical breakdown (Garcia *et al.*, 1994; Brinton, 1994). The concentration of trypsin used varies from between 0.08 % (Kieth *et al.*, 1994; Louis

et al., 1993) to 0.25 % (Brinton, 1994; Lochter *et al.*, 1994; Kleiman *et al.*, 1994). DNase (0.01-0.07 %) is often added to hydrolyse any released DNA and prevent its agglutination (Kobayashi *et al.*, 1994; Lochter *et al.*, 1994).

In our own study the hippocampi were diced using sterile needles before transfer, with as little of the surrounding HBSS as possible, to a sterile test tube, where the tissues were allowed to settle in fresh buffer. The buffer was replaced to wash the tissue further, before the tissue was transferred to a sterile pre-warmed test tube of 1 ml 0.25 % trypsin + EDTA (Life Technologies Ltd.) and incubated at 37 °C. Originally the time allowed for the trypsin to remove membrane proteins was 15 minutes. To reduce stress on the cells, this was reduced in later experiments to 10 minutes. The trypsin reaction was inactivated by the addition of 1 ml foetal calf serum (FCS), followed by light centrifugation (500g for 3 minutes). Originally cells were centrifuged at 1000 rpm, but to limit any damage or trauma to the cells this was reduced. The supernatant was removed and cells were washed twice in Dulbecco's modified minimum Eagle's medium (DMEM, a concoction of compounds similar to HBSS, but with a number of additions, including a variety of amino acids and vitamins; Life Technologies). Cells were titrurated through a heat narrowed Pasteur pipette and resuspended in fresh media for plating.

Where many laboratories appear to differ is in the formulation of the culturing medium. Some papers rely on a straight forward medium with only a few supplements while others add numerous compounds to already complex media. There is little correlation between the complexity of culturing media and the type of experiment or length of culturing. The only apparent exception is that short term

cultures (0-2 days) often use Ham's F12 media (similar to DMEM with both essential and non-essential amino-acids, a wide array of vitamins, minerals and metabolites) in conjunction with MEM (which contains only 13 essential amino acids and 8 vitamins) or DMEM (Lochter *et al.*, 1994; Brinton, 1994), whereas long term cultures (> 2 days) tend to utilise MEM or DMEM only (Rosenbaum *et al.*, 1994; Garcia *et al.*, 1994).

Basal medium is not usually sufficient for cell growth and survival, but requires further enrichment. These supplements are often provided by a serum, the most widely used being FCS or horse serum. FCS is generally believed to enhance glial cell growth while horse serum promotes neurone growth (Banker & Goslin, 1991). This belief is not definitive as while some investigators use horse serum alone to culture hippocampal neurones (Garcia *et al.*, 1994; Whitty *et al.*, 1993), others use only FCS (Rosenbaum *et al.*, 1994; Brinton, 1994; Wann *et al.*, 1994), while a further contingent uses both (Louis *et al.*, 1993).

There are disadvantages with serum in that no two batches are identical and the actual concentrations of nutrients and growth factors are unknown. This latter point has numerous consequences in that certain constituents within the serum may interact with the process under investigation. In 1979 Bottenstein and Satto first grew cells in a serum-free supplement medium (N2 supplement). Neuronal cultures have since been maintained in serum-free environments (Lochter *et al.*, 1994; Kleiman *et al.*, 1994; Kobayashi *et al.*, 1994), and in some instances the N2 serum free supplement has been used in conjunction with serum (Louis *et al.*, 1993).

The most common antibiotic added to the medium is a mixture of penicillin (25-100 U. ml^{-1}) and streptomycin (25-100 μg. ml^{-1}; Whitty *et al.*, 1993; Okamoto *et al.*, 1994; Brinton, 1994). Gentamycin (10-100 μg. ml^{-1}) gives a broader protection and is often used against low levels of contamination after neuronal plating (Banker & Goslin, 1991).

The media we used to culture the neurones was DMEM + 10 % FCS, with gentamycin added at a final concentration of 0.1 mg. ml^{-1}. Due to problems with cell growth, the addition of gentamycin was superseded by penicillin/streptomycin (at 100 U. ml^{-1} and 100 μg. ml^{-1} respectively). In an attempt to further remove contamination, some experiments utilised a concentration of penicillin/streptomycin 2.5 times greater in the media used to wash the cells before plating.

Our protocol was also modified in respect of the FCS or penicillin/streptomycin content of the culturing medium. in some experiments FCS or penicillin/streptomycin concentrations were doubled, either independently or within the same protocol. Ultraser G (a serum substitute: Life Technologies Ltd.) was also used in place of FCS at 2 %. None of the changes appeared to increase the reliability of the cultures. In experiments where the antibiotic content was increased, less cultures were successful. The serum substitute seemed no more reliable than FCS.

An area where there appears to be more conformity is in the coating of the culture plates to entice the neurones to adhere. Most protocols use poly-lysine (Brinton, 1994; Kleiman *et al.*, 1994; Tan *et al.*, 1994) in varying concentrations (10 μg. ml^{-1} to 0.1 mg. ml^{-1}). There are, of course, some modifications of this with other

protocols preferring polyethylene (Okamoto *et al.*, 1994) or a mixture of poly-ornithine and laminin (Louis *et al.*, 1993). Although the L-isomers of these amino acids are usually used, the D-isomers are sometimes favoured due to resilience against protease breakdown.

Plating density was altered throughout our experiments to attempt to achieve an optimum density. The density of the cultured cells should be low enough to prevent overcrowding and allow unrestricted growth, but not too low as this appears to be detrimental. 15,000 viable cells. ml^{-1} of medium was the routine plating density used (0.8 ml added to a well of 13 mm diameter), although experiments were also performed with half or double of this. Cell density was calculated by incubating a volume of cell suspension in 0.1 % trypan blue at 37 °C. A hoemocytometer was used to count the viable cell population and density before plating. Banker and Cowan (1977) have found that the plating density was crucial to cell survival and that a narrow range of 15,000 - 20,000 cells. cm^2 was optimal. Our own limited experience tended to agree with this. Cells were left in an incubator under conditions of 95% O_2, 5% CO_2 and high humidity.

One method not discussed here is the use of glial feeder layers in the culture dish over which the neurones are grown. This is because while the neurones may grow better, the glial layer introduces more variation into the experiment. The downside of not using feeder layers according to Banker and Goslin (1991) is that neuronal cells can only survive for a few days without the presence of glial cells. There is conflicting evidence for this throughout the literature, as, although many protocols allow the glial cells to propagate alongside the neurones for up to five days before

halting their proliferation with the addition of cytosine arabinoside for 24 hours (Kleiman *et al.*, 1994; Rosenbaum *et al.*, 1994; Garcia *et al.*, 1994), others add the anti-mitotic agent after only 24 hours (Tan *et al.*, 1994). In both cases, neurones continue to grow after the removal of glial cells. Other studies allow the proliferation of glia alongside neuronal cells by not adding any agent to prevent glial growth (Kobayashi *et al.*, 1994; Keith *et al.*, 1994).

In our own experiments the substrata we used were covered in 5 or 10 $\mu g.\ ml^{-1}$ poly-D-lysine and left for a minimum period of 1 week before use. The solution was removed and allowed to dry. One area within our own experiments where we introduced great variety was in the plating density of the cultured cells.

We used Ara-C at a variety of concentration to determine the optimum which would inhibit glial growth. Ara-C was added after 24 hours to give final concentrations of 0, 0.25 μM, 0.625 μM or 6.25 μM. In agreement with other studies which have found Ara-C toxic at higher doses (Orr & Smith, 1988; Wallace & Johnson, 1989), cultures given the uppermost concentration showed some inhibition of neuronal growth. At the other extreme, cultures where Ara-C was not added did not usually display pyramidal cell growth, although glial cells did proliferate.

Cells were left in an incubator under conditions of 95% O_2, 5% CO_2 and high humidity.

Figures 53-58 show photomicrographs from hippocampal cultures between 6 hours and 48 hours after initial plating. These photomicrographs, taken down a light microscope, show a number of healthy neuronal cells present at all time points.

What is apparent is that as time proceeds the amount of cellular debris increases. This was usually more obvious after the addition of AraC (24 hours). While most of the pyramidal cells appear spherical at the early (6 hour) time point, their more traditional 'pyramid' shape is obtained by 24 hours. Processes appear between 6 and 24 hours, with very few neurite outgrowths before this (see arrow in fig. 2). Prominent growth cones can been seen after 24 hours (see arrows in fig. 3). At 48 hours the origins of a neuronal network can been seen.

While a number of cultures did survive for a period of time, the inconsistency in maintaining hippocampal neurones proved such that obtaining meaningful results would not have been feasible. The study did provide a protocol to reduce this inconsistency, although only with limited success. Due to the seemingly inexhaustible number of alternate techniques, including feeder layers and co-cultures, and it is quite possible that it is this direction which may provide more dependable hippocampal cultures.

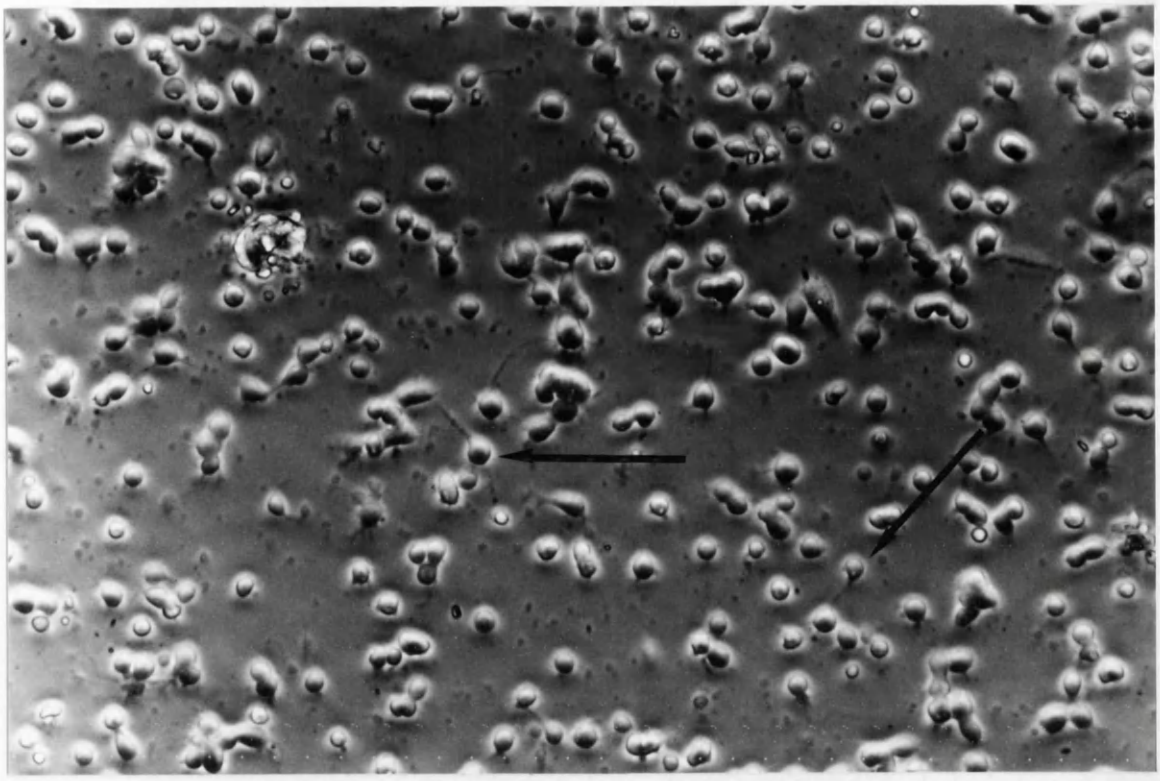


Fig. 56 Hippocampal cell culture 6 hours after plating. The photomicrograph is taken using phase contrast microscopy. While most cells have a sphere-like shape, some (see arrows) are already developing processes. Culture tissue was taken from the optimal experimental conditions. 20 mm : 30 μm.

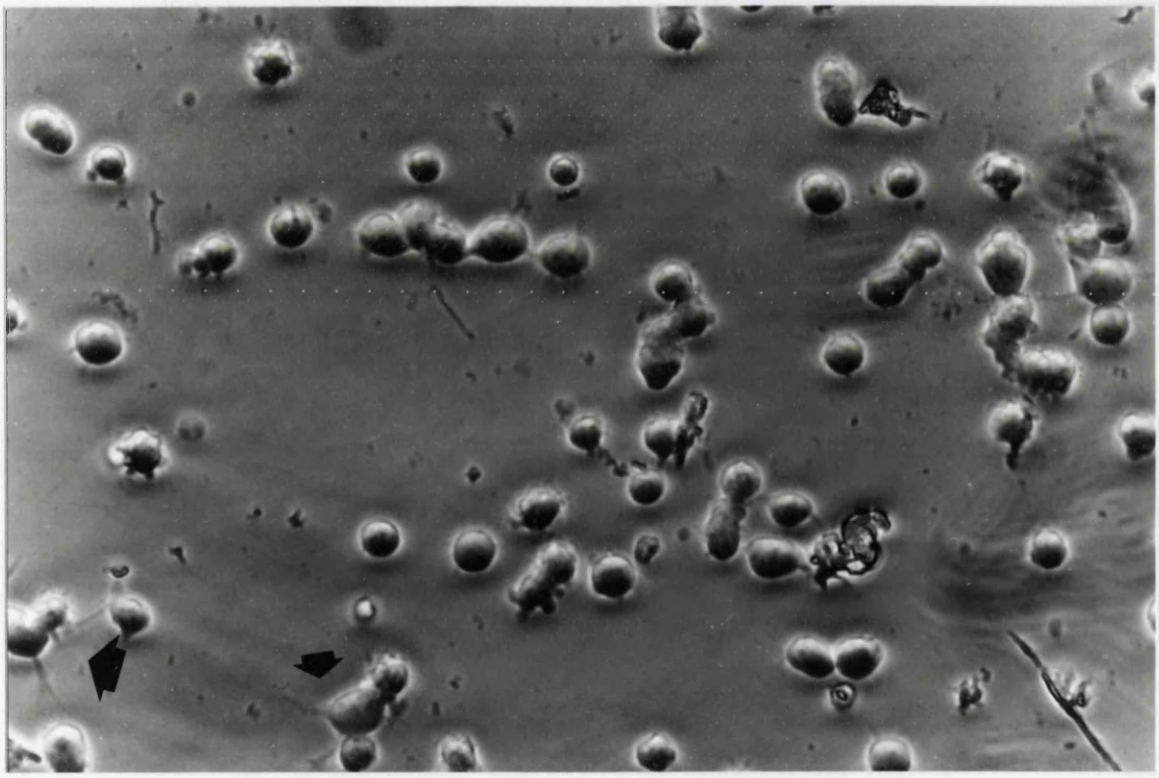


Fig. 57 Hippocampal culture at 6 hours. As above, short processes are visible (arrows). Photomicrograph again taken from an optimal experiment (plating density of 15000 cells. ml^{-1}). 20 mm : 15 μm.

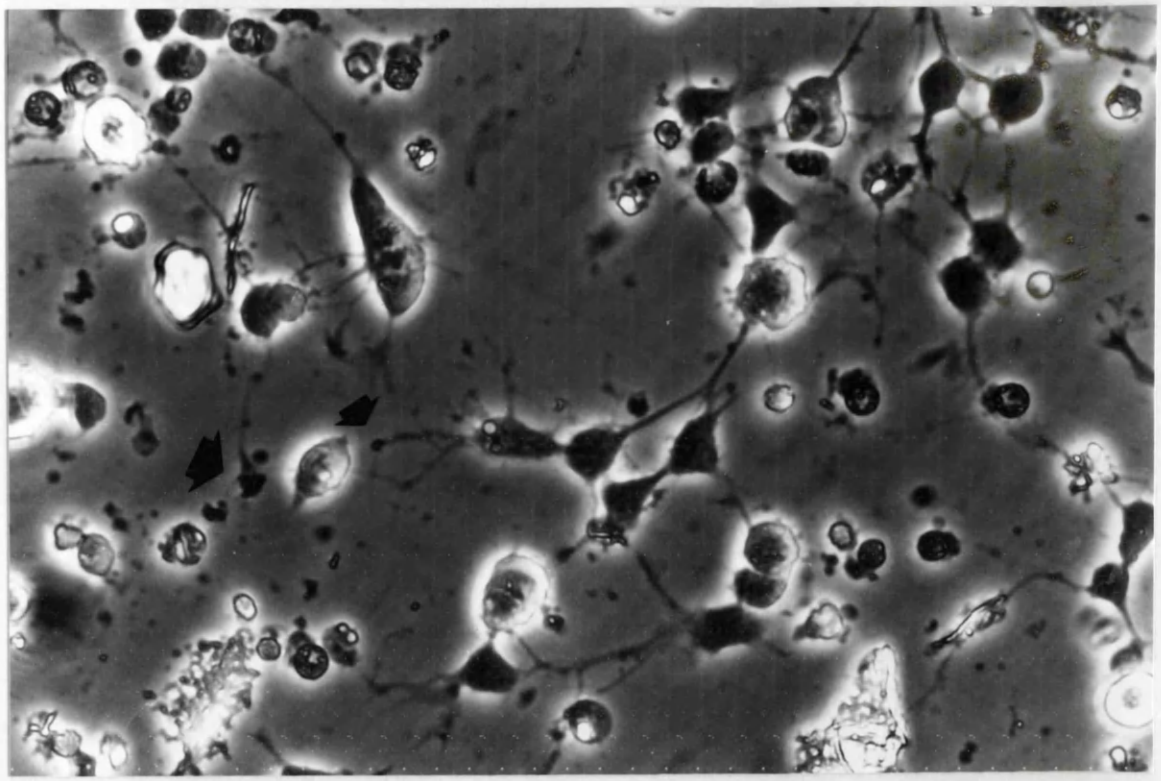


Fig. 5.8 Hippocampal cells, 1 day *in vitro*. 24 hours after initial plating, the more characteristic shape of the pyramidal cell perikaryon is apparent. Numerous processes are also obvious, as are growth cones at the tips of some outgrowths (see arrows). 20 mm : 15 μm.

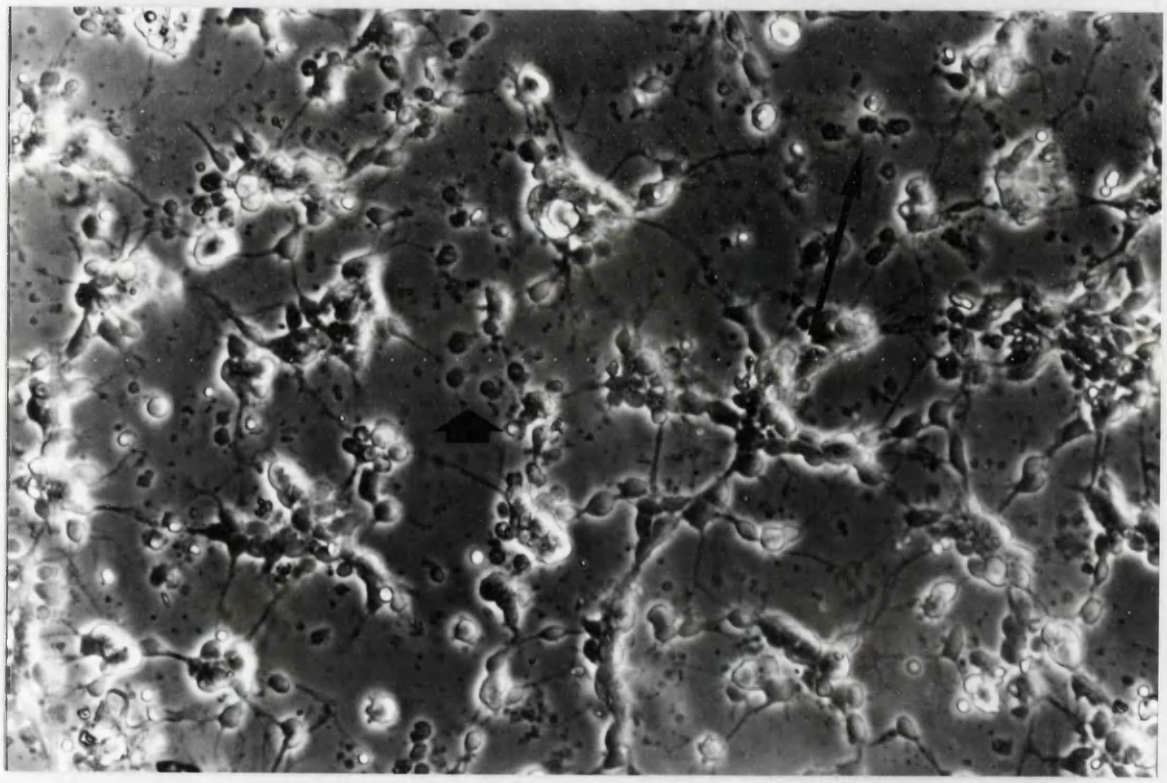


Fig. 5.9 2 day hippocampal culture. A neuronal network is appearing with many more processes than after 24 hours. An increase in cellular debris is also observable. Some cells which have not altered from their original spherical shape, nor emitted processes are still present (arrows). 20 mm : 30 μm.

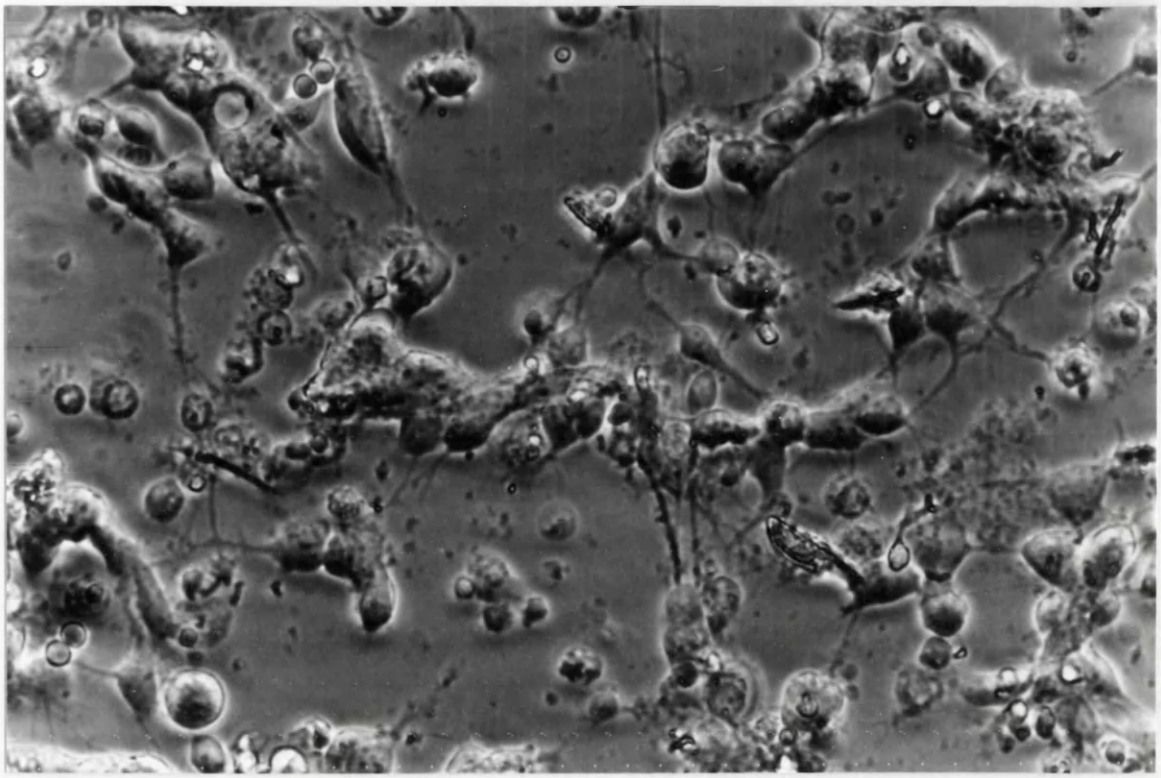


Fig. 60 Hippocampal culture after 48 hours. The intricate network is more apparent at this slightly higher magnification. 20 mm : 15 μm.

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