MODULATION OF KAINIC ACID NEUROTOXICITY IN RATS.

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SUMMARY.

1. Systemically administered kainic acid causes a dose dependent increase in the amount of peripheral benzodiazepine receptor in the rat hippocampus, as assessed by [^{3}H]PK11195 binding.

2. This increase in binding is due to an increase in B_{max} and not K_D. The increase in PK11195 binding indicates that reactive gliosis has occurred and, by inference, neuronal loss.

3. The kainic acid induced elevation in binding is blocked by the non-NMDA antagonist GYKI 52466 and the NMDA antagonists MK801 and CPP.

4. The adenosine A_1 receptor agonist R-phenylisopropyladenosine (R-PIA) is able to attenuate the kainic acid induced neuronal loss in a dose dependent and time dependent manner.

5. The R-PIA response is blocked by 8-cyclopentyl,-1,3- dipropylxanthine (DPCPX) in a dose dependent manner, although DPCPX is unable to potentiate kainate induced neurotoxicity.

6. 8-*p*-sulfophenyltheophylline (8-SPT), is unable to cross the blood-brain barrier, and is unable to block the R-PIA induced neuroprotection indicating that the R-PIA effect is centrally mediated.

7. Kynurenine, but not kynurenic acid or tryptophan is able to attenuate kainic acid induced neurotoxicity in a dose dependent manner.

8. Kainic acid and potassium chloride (KCl) are able to release [^{3}H] glutamate from hippocampal slices in a dose dependent manner.

9. The kainic acid induced elevation induces a period of heightened release after the first, but not second or third stimulations, and this is not seen after either KCl or kainic acid/KCl stimulations.

10. The adenosine A1 agonist 2-chloroadenosine (5μM) is unable to block the kainic acid induced release of [^{3}H] glutamate.

11. DPCPX (5nM) is able to induce a significant decrease in KCl stimulated release of [^{3}H] glutamate but not the kainic acid induced release.

12. In conclusion, kainic acid is causing neurotoxicity in a dose dependent manner that is mediated through both non-NMDA and NMDA receptors, and can be attenuated by R-PIA, and ascorbate.

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ABBREVIATIONS

ACSF	artificial cerebrospinal fluid.
AD	Alzheimer's disease.
ADC	AIDS dementia complex.
AICAR	5-aminoimidazole-4-carboxamide riboside.
ALS	amyotrophic lateral sclerosis.
ALS-PDC	Guam amyotrophic lateral sclerosis-parkinsonism-dementia complex.
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.
AOAA	aminooxyacetic acid.
AP4	2-Amino-4-phosphonobutyric acid.
AP5	D(-)-2-amino-5-phosphonopentanoic acid.
AP7	D(-)-2-aminophosphonoheptanoic acid.
APNEA	N^6-2-(4-amino-3-iodophenyl)ethyladenosine.
APP	amyloid precursor protein.
βA_4	ß amyloid protein.
BBB	blood brain barrier.
BMAA	ß-methylamino-L-alanine.
BOAA	ß-N-oxalyl-L-alanine.
2CA	2-chloroadenosine.
CGP 37849	DL-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid.
CGS 19755	cis-4-(phosphonomethyl)piperidine-2-carboxylic acid.
CGS 15943A	9-chloro-2-(2-furanyl)-5,6-dihydro-1,2,4-triazole-(1,5-*c*)-quinazoline-5-imine.
CGS 21680	2-*p*-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine.
CHA	cyclohexyladenosine.
ChAT	choline acetyl transferase.
CMZ	chlormethiazole.
CNS	central nervous system.
CPP	4-3-((r)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid.
CPPene	(E)-4-(3-phosphonoprop-2-enyl)piperazine-2carboxlic acid.
CPT	cyclopentyl-theophylline.
CSF	cerebrospinal fluid.
DAG	diacylglycerol.
DMSO	dimethylsulphoxide.
L-DOPA	L-3,4-dihydroxyphenylalanine.
DPCPX	1,3-dipropyl-8-cyclopentylxanthine.
EAA	excitatory amino acids.
EPSP	excitatory postsynaptic potentials.
ER	endoplasmic reticulum.
FALS	familial amyotrophic lateral sclerosis.
FI	focal ischaemia.
FR	free radical.
FRS	free radical scavengers.
GABA	γ-aminobutyric acid.
GAD	glutamic acid decarboxylase.
GI	global ischaemia.
gp_{120}	gene product 120 kiloDaltons.
grp78	glucose regulated protein.
GSH	glutathione (γ-glutamyl-cysteine-glycine).
GYKI 52466	1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine.
HAGU	high affinity glutamate uptake system.
HD	Huntington's disease.
HPLC	high pressure liquid chromatography.
hsp70	70kDa heat shock/stress protein.
hsc73	73kDa heat shock protein.
IDO	indoleamine-2,3-dioxgenase.

γIF	γ-interferon.
IgG	immunoglobulin G levels.
IL1	interleukin-1.
ip	intraperitoneal.
IPSP	inhibitory postsynaptic potentails.
IP_3	inositol trisphosphate.
KA	kainic acid.
LGIC	ligand gated ion channel.
LTP	long term potentiation.
MCAO	middle cerebral artery occlusion.
mGluR	metabotropic receptor.
MK 801	Dizocilipine.
MPP^+	1-methyl-4-phenylpyridine.
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.
NAD^+	nicotinamide adenine dinucleotide.
NADH	nicotinamide adenine dinucleotide hydrogen.
$NADP^+$	nicotinamide adenine dinucleotide phosphate.
L-NAME	L-nitroarginine methyl ester.
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline.
NMDA	N-methyl-D-aspartic acid.
L-NMMA	L-N-monomethyl-arginine.
NOS	nitric oxide synthase.
OPA	O-*p*hthaldialdehyde.
PBR	peripheral benzodiazepine receptors.
PD	Parkinson's disease.
PK 11195	1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline-carboxamide.
R-PIA	R-phenylisopropyladenoisne.
8-PT	8-phenytheophylline.
PS	population spikes.
Quin	quinolinic acid.
Ro 54864	7-chloro-5-(4-chlorophenyl)-1-3-dihydro-2H-1,4-benzodiazepin-2-one.
S1,S2,S3,S4	Stimulations (1^{st},2^{nd},3^{rd},4^{th})
SAA	sulphur containing amino acids.
SALS	sporadic amyotrophic lateral sclerosis.
SHR	spontaneous hypertensive rats.
SOD	superoxide dismutase.
SOD1	cytosolic copper/zinc superoxide dismutase
8-SPT	8-*p*-sulphonylphenyltheophylline.
TBI	traumatic brain injury .
TCP	N-[1-(2-Thienyl)cyclohexyl]-piperidine.
VGCCs	voltage gated Ca^{2+} channels.
4VO	4 vessel occlusion.
WDS	wet dog shakes.
XDH	Xanthine dehydrogenase.
XO	xanthine oxidase.

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"Before I heard him talk, I was just like everybody else. You know what I mean? I was confused and uncertain about all the little details of life. But now, while I'm still confused and uncertain it's on a much higher plane, d'you see, and at least I know I'm bewildered about the really fundamental and important facts of the universe."

Terry Pratchet. **EQUAL RITES.** 1987.

1.0. INTRODUCTION:

1.1 Neurotoxicity.

There are numerous ways to damage neurones in the CNS, but in general they can be divided into either acute or chronic insults depending on the time scale of the insult.

Acute insults result from rapid changes in the brain's microenvironment, be it either reduced blood flow (global or focal ischaemia), decreased blood oxygen or increased carboxyhaemoglobin (hypoxia), traumatic head injury (fractured skull, concussion or missile impact) or chemical poisoning (e.g. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), kainate, domoate, cyanide). In almost all cases of acute insult the biochemical and pharmacological mechanisms appear to be similar, and this means that they are easier to model.

Chronic neurotoxicity, however, varies in time course from days up to decades. Some forms have genetic origins (e.g. Huntington's disease (HD)), others are viral (e.g. AIDS dementia complex (ADC)) and others are of unknown origin (e.g. Alzheimer's disease (AD) or Parkinson's disease (PD)), and also exhibit species differences. One of the main complications in modelling chronic insults is that in human cases there is usually a marked and progressive impairment of cognitive function.

Unlike the acute models, which usually involve large scale neuronal lesioning, the chronic diseases may only affect a small population of neurones in a single region but result in specific behavioural or neurological disturbances that would not be predicted from the same degree of damage in the acute models. This cellular or region specificity adds a further level of complication to chronic neurotoxicity modelling. Unlike the acute models there appears to be no common pathway, but there does appear to be a common final step, that of metabolic failure.

1.1.1 Acute Insults.

As mentioned in the earlier section there are several different types of acute insult and these include; traumatic brain injury, ischaemia, myocardial infarction, poisoning, hypoxia or tonic-clonic seizures. Each of these insults has different pathological and anatomical outcomes but virtually all have a similar biochemical/pharmacological pathway. The exception to this is the acute poisoning: Some neurotoxins are thought to mediate their actions by a mechanism that is analogous to acute insults (e.g. kainate) , whilst others act by a mechanism that is similar to chronic degeneration (e.g. MPTP). In both cases the time course of the 'actual' insult is acute rather than chronic and hence will be dealt with in this section.

1.1.1.1 Traumatic Brain Injury.

Unlike most of the cases of neurotoxicity in human, traumatic brain injury (TBI) occurs predominantly in young adults (late teens to mid 30's). TBI is usually caused by transport accidents, and involves either a fracturing of the skull or severe concussion and brain bruising. In the cases that do not involve missile impacts (ie bullets) there is bruising to the cerebral cortex and damage in the hippocampus. Kotapka *et al.* (1992) reported that in 84% of fatal non-missile cases there was damage in the hippocampus (CA1 most pronounced). As well as this damage, 74% of the cases displayed hypoxic damage in other regions of the brain, in a pattern that is similar to those damaged by experimental head injury models. The model which is mainly used for TBI is that of either solid or fluid percussion.

1.1.1.2 Ischaemia

There are two forms of ischaemia, global or focal, and the type depends on the causative events. Global ischaemia is usually caused by perturbations in either the peripheral blood flow, i.e. by myocardial infarction, or by a perturbation in the blood flow in the main carotid artery. Focal ischaemia occurs if there is a reduction in blood flow along arteries within the brain, be it by haemorrhage or occlusion. Both types of insult result in reduced blood volume, reduced cranial perfusion pressure, and reduced oxygen and glucose availability. Global ischaemia will result in damage to large areas of the brain, and the duration of the perturbation will determine the degree of damage within the affected areas. In general global ischaemia is more likely to prove fatal than focal due to the regions affected, although a focal insult in the hind brain will usually be fatal.

There are several animal models used for both types of ischaemia, and are usually based around either permanent occlusion of a blood vessel or occlusion followed by reperfusion. The middle cerebral artery is the main artery used in the focal ischaemic models, in that the majority of focal ischaemic insults in man are associated with this artery.

Regan & Choi (1994) recently reported that in an in vitro traumatic insult model, antagonists of NMDA but not non-NMDA receptors were able to attenuate the neuronal loss, and this suggests that mechanisms of damage caused by TBI differ from those of ischaemic damage.

1.1.1.3 Seizures.

Seizures occur when specific regions of the brain are undergoing continuous, unregulated neuronal depolarisation, which are epileptiform activities. Epileptiform activity is usually of short duration, and can be combatted by γ- aminobutyric acid (GABA) agonists (specifically $GABA_A$), GABA uptake blockers and inhibitors of the GABA metabolism (GABA transaminase). However if the duration of the epileptiform activity is prolonged, or other brain regions are invaded then there is the possibility of brain damage occurring. Again it is possible to treat these types of seizures with $GABA_A$ agonists. Tonic-clonic seizures are also potentially fatal, and are usually the cause of seizure induced fatalities that do not involve suffocation.

1.1.1.4 Poisoning

There are numerous artificial and naturally occurring agents that are able to cause brain damage in humans. However the brain is protected against most of these agents by the properties of the blood brain barrier (BBB). Five agents are of specific interest and are useful tools in studying neurotoxicity *in vivo*. They are kainic acid (found in Japanese seaweed *Digenea simplex*), domoic acid (*Chondria armata*, seaweed and *Nitschia pungens*, diatom), ß-N-oxalyl-L-alanine (BOAA, *Lathyrus sativus*, chick pea), ß-methylamino-L-alanine (BMAA, false sago) and MPTP (a by-product of synthetic heroin production). These will be dealt with in a later section.

1.1.2 Chronic Insults

As with acute insults there are several different types of chronic insults and these include Huntington's disease, Alzheimer's disease, Parkinson's disease, motor neurone disease, Guam disease and AIDS dementia complex. There are reports in the literature that indicate that some of the chronic diseases have similar biochemical/pharmacological pathways to those of acute insults, although the main difference is time scale (see later sections). For the other chronic diseases the only area of similarity is the final outcome, that of metabolic failure.

1.1.2.1 Huntington's and Parkinson's Diseases.

Although these diseases have different outcomes, ie lack of motor activity in Parkinson's disease (PD) and uncontrolled motor activity in Huntington's disease (HD), they are both diseases of the nigrostriatal system, and are examples of discrete lesions. The causes of these two diseases differ, with HD involving a genetic linkage (although other mechanisms are also implicated), whilst PD is caused by unknown (but not genetic) mechanisms. As with other motor related diseases (Guam disease and Motor neurone disease) Parkinsonism is thought to be caused by factors encountered during life, with aluminium or naturally occuring neurotoxins being implicated as possible causative factors.

Parkinson's disease is caused by a loss of dopaminergic neurones in the substantia nigra, and thus degeneration of the pathway from the substantia nigra pars compacta to the neostriatum. A recent communication by Strange (1994) indicated that the neostriatum is crucial for the control of voluntary movement, with a direct and indirect pathway controlling movement: activation of the direct pathway facilitates movement and activation

of the indirect pathway inhibits movement. He postulated that the direct pathway is regulated by the mesostriatal neurones that are affected by Parkinson's disease. Loss of the direct pathway would result in an inability to facilitate movement, with the indirect pathway dominant, and that movement could be initiated by dopamine.

Until a chance finding in the early 80's there was no animal model for PD, but this changed with the inadvertant formation of MPTP by an American chemist who was preparing pethidine ('new heroin') for use by drug addicts. MPTP is able to cross the blood brain barrier where it is converted to 1-methyl-4-phenylpyridine (MPP^+) by monoamine oxidase B, this charged compound being unable to recross the blood brain barrier. MPP^+ interacts with neuromelanin, found predominatly in dopaminergic neurones. The MPP^+ /neuromelanin complex is relatively short lasting and acts as a sink for future leakage of MPP^+ into the surrounding space (D'Amato *et al.*, 1986, Herrero *et al.*, 1993). MPP^+ also interacts with, and inhibits, the mitochondrial respiratory enzyme NADH dehydrogenase causing the neurones to die due to metabolic failure (Ramsay *et al.*, 1986a, 1986b, 1987). The addicts who were contaminated with MPTP displayed classical parkinsonian paralysis which could only be treated by L-DOPA, the normal treatment for PD. With the finding of MPTP, study into the cause and treatment of PD intensified, but with the drawback that only primates are sensitive to this agent. To date this is the only effective model for PD. There are several reports that N-methyl-D-aspartic acid (NMDA) receptor antagonists (AP7, CPP, or MK801), can prevent MPP^+ neuro-toxicity in the substantia nigra and the striatum (Carboni *et al.*, 1990; Turski *et al.*, 1991; Santiago *et al.*, 1992; Lange *et al.*, 1993), indicating that although there is metabolic failure, cell death also involves NMDA receptor activation. This means that cell death can be elicited by both metabolic failure and excitotoxicity in the MPTP model of

Parkinsonism, although whether this occurs in the disease state itself has not yet been determined.

Huntington's disease results in lesions in the spiny neurones of the neostriatum, and this would result in the abnormal involuntary movements (chorea) displayed by HD sufferers. Although HD is thought to involve a genetic component, it is possible to lesion the spiny neurones with excitotoxins like kainate, indicating that there may be a possible nongenetic mechanism as well, (evidence supporting the excitotoxic component theory is presented in section 1.4.1. NMDA receptor blockade). It is possible, that a genetic defect could result in an increase in glutamate release, an increase in glutamate receptor numbers or a reduction in glutamate transporters numbers/defect reuptake systems, such that the genetic and excitotoxic theories of HD are not exclusive.

1.1.2.2 Alzheimer's Disease.

AD patients suffer from a progressive loss of memory, both the ability to learn new facts and the ability to recall memories. AD is characterised by 2 distinct structures (plaques and tangles) which are found in large numbers in the limbic system, as well as in the cerebral cortex. Normal aged brain only displays small numbers of plaques and tangles within the cerebral cortex, so the presence of these abnormal structures in the limbic system is an indication of AD.

Pharmacological analysis of AD brains indicates that there is a loss of cholinergic neurones and muscarinic acetylcholine receptors. This finding has prompted research into the role that the cholinergic pathways have in the control of memory, and the possible use of cholinergic agents as memory enhancing agents. The cause of AD is unknown although a rare inherited form has recently been linked to mutations of the gene coding

for amyloid precursor protein (APP). A peptide formed from APP, ß amyloid protein (ßAP or $ßA_4$) is a major component of senile plaques. The apolipoprotein, Apo E, is able to bind to the $ßA_4$ and precipitates the protein complex enhancing $ßA_4$ deposits. Normally Apo E is involved in membrane stability, but the ϵ4 allele of Apo E has been reported to have a higher affinity than other alleles of Apo E for $ßA_4$, and there appears to be linkage of Apo E ϵ4 and AD (Poirier *et al.*, 1993; Saunders *et al.*, 1993). The mechanism of $ßA_4$ induced cell death is unknown, but does not appear to be through the excititoxic cascade seen in ischaemia (Busciglio *et al.*, 1993). To date there is no accepted animal model.

1.1.2.3 Motor Neurone Disease and Guam Disease.

Motor neurone disease (or amyotrophic lateral sclerosis (ALS)) exists both as a familial form (about 5% of the total ALS patients, Lou Gehrig's disease) and a sporadic form. In the western Pacific there are several areas of high incidence of an ALS related disease termed Guam amyotrophic lateral sclerosis-parkinsonism-dementia complex (ALS-PDC), which differs from ALS in both the incidence (50-1300 times that of sporadic ALS (SALS)) and the presence of neurofibrillary tangles (a feature implicated in AD). Indeed some ALS-PDC suffers also display AD symptoms (see Garruto & Yase, 1986).

The cause of sporadic ALS is unknown although there are reports of elevated glutamate levels in the spinal fluid of these patients (Plaitakis, 1990; Rothstein *et al.*, 1990). The elevated glutamate level is thought to be due to a dysfunctional glutamate uptake system (Rothstein *et al.*, 1992; Battaglioli *et al.*, 1993; Rothstein *et al.*, 1993) in that glutamic acid decarboxylase (GAD) activity is normal. The mechanism of this impairement of glutamate uptake is unknown, but may involve either an environmental

toxin, a loss of postsynaptic motor neurones and hence a loss of presynaptic glutamate transporters, or a genetic defect. Courathier *et al.*, (1993) recently reported that cerebrospinal fluid from ALS patients is neurotoxic to cortical neurone cultures, and that non-NMDA antagonists can attenuate the neuronal loss. It is unlikely that there is a single cause of both familial ALS (FALS) and SALS.

Recently there have been reports, from one group, of elevated immuno-globulin G levels (IgG) in ALS patients, and this IgG has been reported to interact with voltage gated Ca^{2+} channels (VGCCs) (Appel *et al.*, 1991; Delbono *et al.*, 1991, Kimura *et al.*, 1994; Smith *et al.*, 1994; see also Appel, 1993). Again the cause of this elevated IgG is unknown. If these findings are supported, it would suggest that a possible cause of motor neurone loss is due to elevated calcium influx, as the probability of the VGCCs being open is greater than in control patients and hence a larger calcium influx would be expected to occur following neuronal stimulation. Therefore in these patients, if there is also an impaired glutamate uptake system, the findings suggest that glutamate is more toxic to these patients' motor neurones than those of unaffected patients, due to both a prolonged glutamatergic stimulation and an increased calcium influx.

The cause of the familial form of ALS appears to involve free radicals, with an impairment in the scavenging system. There have been several recent reports that link the cytosolic copper/zinc superoxide dismutase (Cu/Zn SOD or SOD1) with familial ALS (FALS). There are several reports of a mutation in the SOD1 gene, (Rosen *et al.*, 1993; Rouleau, 1993; Deng *et al.*, 1993; Elshafey *et al.*, 1994; Jones *et al.*, 1994) and Robbererct *et al.*, (1994) reported decreased activity of SOD1 in FALS but not sporadic ALS patients. Why the motor neurones are damaged whereas other neurones in the spinal cord are spared in FALS patients is unclear, but probably depends on non-NMDA

receptors and the levels of free radical scavengers within the cell. Motor neurones possess non-NMDA receptors and there is now evidence that links activation of kainate receptors with increased lipid peroxidation in cerebral cultures (Puttfarcken *et al,* 1993), and by implication free radicals. Although this finding was made in the brain there is evidence to suggest that a similar event may be occurring in the motor neurones (Curtis & Malik, 1985; Hugon *et al.*,1989), and Michikawa *et al.* (1994) recently reported that oxygen scavenging enzymes are able to protect spinal cord neuronal cultures *in vitro*.

This link with the glutamate receptors is strengthened by reports of the cause of ALS-PDC. However, ALS-PDC does not seem to be caused by mutations in SOD1, (Figlewicz *et al.*, 1994) i.e. the aetiology is similar to sporadic and not FALS. ALS-PDC affects both motor and brain neurones and the cause appears to be an amino acid, ß-methylamino-L-alanine (BMAA) produced by false sago (Nunn *et al.*, 1968; Spencer *et al.*, 1987; Seawright *et al.*, 1990; Rakonczay *et al.*, 1991; Duncan, 1992; Labella *et al.*, 1993). BMAA and the structurally related compound ß-oxalylamino-L-alanine (BOAA) have been shown to be agonists for the NMDA (Ross *et al.*, 1987; Spencer *et al.*, 1987; Rakonczay *et al.*, 1991; Labella *et al.*, 1993) and non-NMDA glutamate receptor subtypes (Ross *et al.*, 1987; Bridges *et al.*, 1989; Rakonczay *et al.*, 1991) respectively and are able to induce neurotoxicity in cell cultures (Nunn *et al.*, 1987; Ross *et al.*, 1987; Spencer *et al.*, 1987; Weiss & Choi, 1988, Weiss *et al.*, 1989). *In vivo* work was hampered due to the inability of either of these compounds to cross rodent BBB, although they can cross the primate BBB (Spencer *et al.*, 1987). If these agents are able to cross the BBB and activate glutamate receptors *in vivo*, the reports that glutamate receptor activation results in free radical production in the brain (Dykens *et al.*, 1987; Bondy & Lee, 1993; Putfarcken *et al.*, 1993) may also suggest that similar events are occuring at motor

neurones. However, the importance of these agents in ALS-PDC has recently been questioned by some groups (Garruto *et al.*, 1988; Perry *et al.*, 1989; Duncan *et al.*, 1991) who are unable to reproduce the findings of Spencer's group (see Stone, 1993a). A further complication to the understanding of ALS-PDC is that the disease is naturally dying out in the Western Pacific, and the cases that are occuring are not displaying any ALS symptoms, but only AD-like symptoms with PD-like behavioural changes (see Garruto & Yase, 1986; Stone, 1993a).

1.1.2.4 AIDS Dementia Complex

AIDS dementia complex (ADC) is a new disease that has only been observed in the last few years. Unlike PD, HD and AD, ADC appears to be the result of excitotoxicity. ADC is caused by lesions in the limbic system, like AD, but these lesions are not restricted to the cholinergic pathyways. A coat protein of the AIDS virus (gp^{120}) has been implicated in the formation of these lesions. In neuronal/glia cultures gp^{120} has been reported to increase nitric oxide synthesis (NOS), and induce neurotoxicity in a manner that involves NMDA receptors and calcium channels (Lipton, 1992; Ciardo & Meldolesi 1993; Dawson *et al.*, 1993; Diop *et al.*, 1994; Savio & Levi, 1993; Stefano *et al.*, 1993; Sweetnam *et al.*, 1993; Benos *et al.*, 1994; Pietraforte *et al.*, 1994), although Giulian *et al.* (1993) were not able to identify the neurotoxic component released by gp^{120}. Gp^{120} is also a very powerful interleukin-1 (IL1) releasing agent in the CNS (N. Rothwell personal communication). IL1 is found in astrocytes and is released upon stimulation (Guilian *et al.*, 1993; McMillian *et al.*, 1994). IL1 and γ-interferon (γIF) are able to induce indoleamine-2,3-dioxgenase (IDO) (Yoshida *et al.*, 1986; Saito *et al.*, 1991), which in normal brain tissue has a very low activity (Heyes, 1993), and this enzymes converts

tryptophan to formylkynurenine (i.e. the start of kynurenic acid and quinolinic acid pathway, see later). In ADC patients, there is an elevated level of quinolinic acid (Quin) (20-1000 times that of the normal population (Brouwers *et al.*, 1993; Heyes, 1993)), with no change in the kynurenate levels. However, unlike ischaemic insults, the level of Quin, remains elevated for months, rather than the time of the insult itself, although there is a significant increase in IDO activity in the hippocampus in ischaemic brain (Heyes, 1993). This time course suggests that although Quin is elevated it may not be the neurotoxic agent in this disorder, although based on neurological scores there is a good correlation between the Quin concentrations and the severity of the dementia. Its presence may sensitise the neurones to non toxic levels of excitatory amino acids, such that the incidence rate of minor non fatal excitotoxic insults increases, producing widespread minor lesioning throughout the brain.

If IL1 or other cytokines play an important role in ADC, this may explain neuronal damage observed in some viral diseases, such as measles (Heyes *et al.*, 1992c; Saito *et al.*, 1992, 1993, Heyes *et al.*, 1993), Simian immunodeficiency virus (Heyes *et al.*, 1992a) and poliovirus (Heyes *et al.*, 1992b; Heyes, 1993) where there also appears to be a change in the functional state of IDO. This raises possibilities in the treatment of these diseases in adults.

1.2 Biochemical Causes of Neurotoxicity.

As mentioned in the preceding sections, there are several different mechanisms of causing neuronal lesioning, which have different initial pathways, but all appear to possess the same outcome, that of metabolic failure. The biochemical causes and effects of metabolic failure will be dealt with in this section and the pharmacological causes in

succeeding sections.

1.2.1 Energy Production

Under normal circumstances the metabolism of a cell is tightly regulated and is maintained by oxidative phosphorylation. Unlike cells in other parts of the body, neurones are only able to use glucose as the substrate for oxidative phosphorylation, and are unable to convert other carbohydrates or phospholipids to energy producing substrates. The astrocytic population on the other hand is able to utilise other energy rich compounds to maintain cellular function, be it oxidative phosphorylation or anaerobic glycolysis. Astrocytes are able to convert glycogen to glucose intracellularly and release it into the extracellular space for uptake by neurones. Whilst this mechanism is advantageous during normal cellular activity, it does mean that any insult/agent that either specifically or non specifically damages astrocytes will be detrimental to the survival of the surrounding neurones.

Oxidative phosphorylation results in the production of 38 moles of ATP for every mole of glucose converted to water and carbon dioxide. The conversion of glucose to pyruvate (aerobic respiration) or lactic acid (anaerobic respiration) occurs in the cytoplasm and both result in the formation of 2moles ATP/mole glucose (equations 1.1 and 1.2 respectively). Pyruvate is oxidised in the mitochondria to yield a theoretical net production of 38 moles ATP. mole glucose^{-1} (equation 1.1). However, lactate is not a substrate for either the citric acid cyle or oxidative phosphorylation and this results in a decrease in intracellular pH.

$$\text{Glucose} + 2P_i + 2ADP + 2NAD^+ \rightarrow 2\ \text{pyruvate} + 2ATP + 2NADH \quad (1.1)$$

$$\text{Glucose} + 2P_i + 2ADP \rightarrow 2\ \text{lactate} + 2ATP \quad (1.2)$$

$$\text{Glucose} + 10NAD^+ + 2FAD + 36ADP + 38P_i + 2GDP + 12(1/2O_2) \rightarrow 6CO_2 + 6H_2O + 36ATP + 2GTP + 10NAD^+ + 2FAD \quad (1.3)$$

Whilst glucose is the preferred substrate for oxidative phosphorylation, other substrates are available for use including phospholipids, phosphogluconates and amino acids. Neurones are deficient in enzymes that allow for the full utilisation of some of these pathways and hence are heavily dependent on glucose, eg the hexose monophosphate shunt (Bohinski, 1983). Neuronal excitation results in a net loss of glutamate, when the neuronal uptake of glutamate and glutamine are taken into account, and this indicates that for glutamatergic neurones to maintain a viable transmitter pool other precusors must be used, and these include alanine, citrate, α-ketoglutarate, malate and pyruvate (Yu *et al.*, 1983; Shank *et al.*, 1985; Kihara & Kubo, 1989; Sommerwald *et al.*, 1991; Schousboe *et al.*, 1993).

1.2.2 Causes and Effects of Metabolic Failure.

There are several ways of causing metabolic failure including; hypoxia, hypoglycaemia, acidosis, hypercalcaemia and poisoning. Hypoxia and hypo-glycaemia both result in substrate deficiencies, and a termination of oxidative phosphorylation and, if the hypoglycaemia is long lasting, glycolysis. Acidosis alters the tertiary structure of proteins and will affect the enzymes involved in ATP production. Hypercalcaemia will be dealt with in a later section.

Poisons that affect energy production are those that inhibit the oxidative enzymes themselves. Examples of metabolic poisons include cyanide and carbon monoxide (inhibitors of the cytochrome oxidase complex), MPTP (noncompetitive inhibitor of the

NADH dehydrogenase complex, Ramsay *et al.*,1986a,1986b,1987) and heavy metals, although in this case these are nonspecific enzyme inhibitors.

Metabolic failure is a progressive feedforward mechanism, with each failure causing/potentiating the next failure. ATP is generated by the metabolism of glucose (and other energy rich complexes), but is utilised by widely different mechanisms. Maintenance of the intracellular ionic composition is essential for the cell's survival and uses a large percentage of ATP produced. Protein synthesis, cytoskeletal organisation, signal transduction and, in the case of neurones, synthesis, storage and release of neurotransmitters also use large amounts of ATP. In the case of neurones, the astrocytes release substrates for neurotransmitter synthesis and oxidative phosphorylation (Brookes, 1993;and see section 1.2.1). Again this highlights the importance of astrocytes to neuronal survival.

During the initial period of metabolic failure there is a reduction in *de novo* synthesis of 'normal' proteins, which cells are able to tolerate for short periods, as the catabolism of important proteins approaches and then passes the reserve capacity of these proteins. Whilst this is occuring there is also increased production of stress proteins, the presence of which are detrimental to cell survival as illustrated by the neuroprotective action of cycloheximide (a protein synthesis inhibitor), (Schreiber *et al.*, 1993). However, if the metabolic failure continues, then there is a loss of cellular ionic homeostasis. The cause of this loss of ionic homeostasis is the lack of available ATP to drive the ion channel linked ATPases, which are required for the maintenance of the membrane potential. This in turn is required for the energy-dependent ion pumps, which are required for ionic homeostasis. The net result of the loss of ionic homeostasis is the loss of membrane potential, and the permeabilisation of the plasma membrane. A complication

to the above loss of ionic homeostasis is the generation of free radicals which cause lipid peroxidation, and results in actual holes in the plasma membrane (Thaw *et al.*, 1983; Dietrich *et al.*, 1987; Watson, 1993). This means that even if glucose and/or oxygen become re-available to the cell when it has lost ionic homeostasis, there may be no improvement in the chance of the cell's survival (Thaw *et al.*, 1983). The generation of free radicals will be dealt with in a separate section.

1.2.3 Calcium Homeostasis

Another cause of metabolic failure is hypercalcaemia. Under physiological conditions the cellular concentration of free calcium is maintained at between 0.1-1μM. This intracellular concentration is maintained by three membranes; the endoplasmic reticulum, the mitochondria and the plasma membrane, as well as by intracellular calcium binding proteins (figure 1.1). The mitochondria are able to act as a calcium sink with *in vitro* experiments indicating the ability to store 100μM Ca^{2+}.mg protein^{-1} (as Ca_3PO_4) with no apparent deleterious effects on the mitochondrial function (Hunter *et al.*, 1976; Lotscher *et al.*, 1980; Nicholls & Akerman, 1982), although *in vivo* excessive calcium uptake by the mitochondria is linked with metabolic failure (Sciamanna *et al.*, 1992). Calcium is stored in the mitochondria in a calcium phosphate complex, and this means that the more calcium that the mitochondria sequester the less organic phosphate there is available for ATP production (Nicholls & Akerman, 1982). The mitochondria possess 2 separate systems; a calcium ATPase and a calcium pump, driven by the inner mitochondrial membrane potential, and a calcium/sodium/proton exchanger. The direction of calcium influx into the mitochondria via the pump reverses at $[Ca^{2+}]_i$ greater than 10μM (McCormack & Denton, 1986). This means that any large perturbation in the

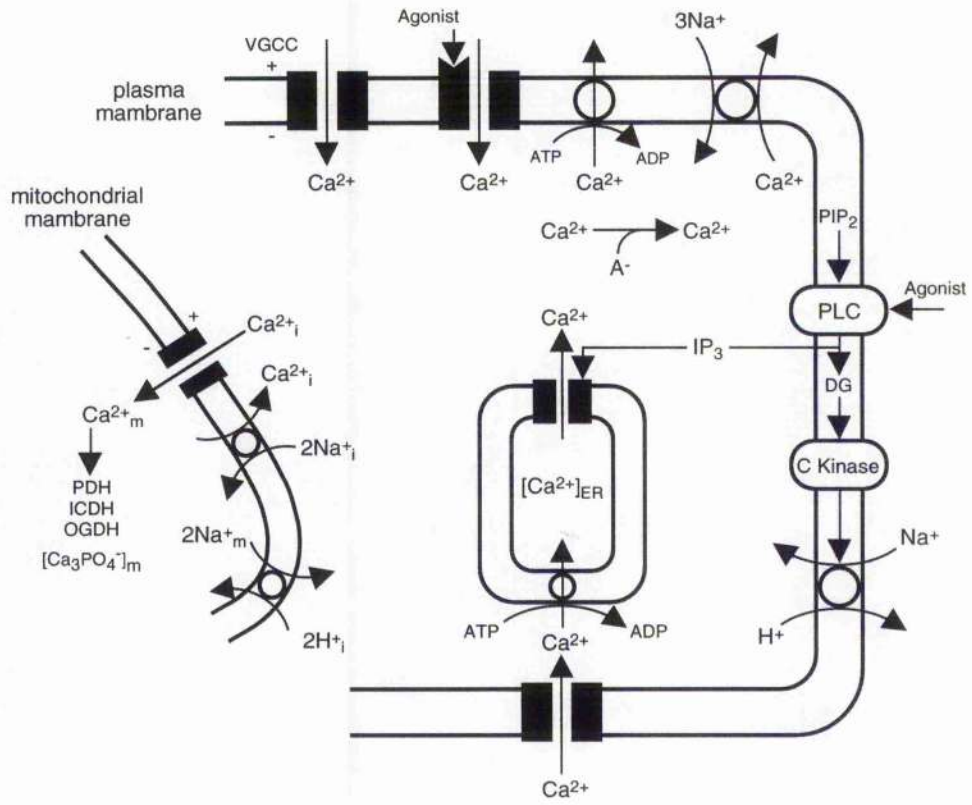


Figure 1.1 Calcium homeostasis systems in mammalian cells.

Key-

A:	Intracellular anions/binding sites/proteins,
DAG:	Diacylglycerol
ER:	Endoplasmic reticular
ICDH:	Isocitrate dehydrogenase
IP3:	Inositol triphosphate
OGDH:	Oxygluterate dehydrogenase
PDH:	Pyruvate dehydrogenase
PIP2:	Phospho-inositol bisphosphate
PKC:	Protein kinase C
PLC:	Phospho-lipase C
VGCC:	Voltage-gated calcium channel

Subscript-

e:	Extracellularly
ER:	Endoplasmic reticularly
i:	Intracellularly
m:	mitochondrially

calcium concentration would be potentiated by calcium leakage from the mitochondria.

The endoplasmic reticulum (ER) possesses a calcium ATPase, and a calcium channel linked to the inositol trisphosphate (IP_3) receptor. IP_3 is generated by cleavage of phosphoinositol bisphosphate by phospholipase C, which also yields diacylglycerol (DAG). The activation of phospholipase C is receptor mediated, and various neurotransmitters, as well as other extracellular messengers, use the generation of IP_3 as a mechanism of intracellular signalling that raises the intracellular calcium concentration. Activation of the IP_3 receptor opens the associated calcium channel, and calcium leaves the ER and locally raises the cytoplasmic calcium concentration (Berridge & Irvine, 1984; Streb *et al.*, 1984). As the amount of calcium present in the ER is finite (Taylor & Putney, 1986) there is no large widespread increase in calcium: the calcium binding proteins involved in signal transduction are concentrated near the ER.

The plasma membrane possesses several different mechanisms for regulating the cytoplasmic calcium levels: voltage-gated calcium channels (dependent on membrane potential: activation results in calcium entry into the cell), a 3sodium/ 1calcium exchanger (dependent on ion homeostasis, and normally an efflux system) and calcium ATPases (dependent on the metabolic state of the cell, and also an efflux system). It is the activation of the plasma membrane calcium channels, rather than the ER or mitochondrial calcium transporters, that results in cell wide elevation in calcium. For a summary of the calcium systems see figure 1.1 and Siesjö & Bengtsson, (1989).

Loss of calcium homeostasis will result in elevated cytoplasmic calcium concentrations and these can have several deleterious effects on the metabolic state of the cell. One of these actions is the collapse of the inner mitochondrial membrane potential with the resulting loss of the proton motive force (the proton gradient driving proton entry

into the mitochondria) and the ensuing loss of oxidative phosphorylation due to decreased NAD^+/NADH ratio. This would result in the reduction in ATP generation, such that the system is now functioning anaerobically. The other serious effect is the activation of calcium proteases. These calcium proteases will enzymically cleave target protein and, in the case of xanthine dehydrogenase, this may result in new enzymic action that is detrimental to the cell's survival.

Xanthine dehydrogenase (XDH) converts uric acid to xanthine and then to hypoxanthine, the reaction using NAD^+ as the electron acceptor. However, if the enzyme is cleaved by a serine calcium protease (Parks & Granger, 1986; Engerson *et al.*, 1987; Parks *et al.*, 1988) the new form is termed xanthine oxidase (XO) and converts xanthine to uric acid, using metabolic oxygen as the electron acceptor:

XDH reaction: $XO + NAD^+ \longrightarrow$ Uric acid + NADH (1.4)
XO reaction: $XO + O_2 \longrightarrow$ Uric acid + O_2^- (1.5)

Under physiological conditions the oxygen radical will be converted to superoxide by reacting with water. Superoxide is normally neutralised by either its conversion to hydrogen peroxide, and hence oxygen and water, or by reduction by intra-cellular reducing agents. If the concentration of superoxide and/or other free radicals reach levels that saturate the neutralising systems then there is the possibility of lipid peroxidation, and the resulting perturbations in the plasma membrane may lead to loss of ionic homeostasis, and hence further reduce the survivability of the cell (see later).

However, not all neurones have the same sensitivity to their micro-environment and some are very sensitive to specific insults whilst others are reasonably resilient to the same insult. This sensitivity appears to be dependent on both the vasculature of the region and the presence of certain proteins (Mattson *et al.*, 1991; Waldvogd *et al.*, 1991). Freund

and co-workers (Freund *et al.*, 1990; Schmidtkastner & Freund, 1991) reported that there is a correlation between the amount of certain calcium binding proteins (Calbindin and Parvalbumin, Leranth & Ribak, 1991; Iacopino *et al.*, 1992) in the cell bodies and cell survival in the hippocampus, the higher the level of these proteins, e.g. in the CA2 cell layer, the greater the cells' chances of survival after an ischaemic insult; conversely in the CA1 and CA3 areas, and the dentate gyrus, all are very sensitive to ischaemia, and here there are low levels of these proteins.

1.3 Neurochemistry of Neurotoxicity.

1.3.1 Glutamatergic Systems.

Glutamate is the main excitatory neurotransmitter in the CNS, but it is also present in millimolar concentrations in the cytoplasm of all cells ($\approx$ 10mM, Greenbaum *et al.*, 1971; Nicholls & Atwell, 1990). Depolarisation of glutamatergic neurones results in the release of glutamate from specific secretory vesicles present near the active zone of the synaptic membrane. Work by Nicholls and co-workers (Nicholls & Sihra, 1986; Sanchez-Prieto *et al.*, 1987; Barrie & Nicholls, 1988; McMahon *et al.*, 1988; Wilkinson & Nicholls, 1988, 1989; Nicholls, 1993) indicates that whilst [^{3}H] glutamate and [^{14}C] aspartate can enter synaptosomes and are found in the cytoplasm only the [^{3}H] glutamate is found in the vesicles. Stimulation of these preloaded synaptosomes results in a calcium dependent release of [^{3}H] glutamate, but if the synaptosomes are in calcium free media then both [^{3}H] glutamate and [^{14}C] aspartate are released. The interpretation of these experiments is that the calcium dependent release is of vesicular contents, whilst the calcium independent release is by reversal of the glutamate uptake systems. This has supported earlier work and it now seems likely that the vesicles possess a glutamate/ATP

transporter (DeBelleroche & Bradford, 1973; Kanner & Sharon, 1978; Kvamme & Lenda, 1981; Disbrow *et al.*, 1982; Kanner & Marva, 1982; Naito & Ueda, 1983, 1985; Maycox *et al.*, 1988; Winter & Ueda, 1993), which is able to maintain an in intravesiclar concentration of ≈60-100mM (Nicholls & Atwell, 1990; Laake *et al.*, 1993).

There are several types of glutamate receptors, and most belong to the ligand gated ion channel (LGIC) superfamily. There are at least three LGIC receptors (the NMDA receptor, and the two non-NMDA receptors (the AMPA receptor and the kainate (KA) receptor)) and they are classified by their sensitivity to agonists (Stone & Burton, 1988; Collingridge & Lester 1989). The other type of glutamate receptor is a member of the G-protein mediated receptor superfamily, and is termed the metabotropic receptor (mGluR), of which there are now at least 6 subtypes (Nakanishi, 1992). A fourth LGIC receptor, the 2-Amino-4-phosphonobutyric acid (AP4) receptor, appears to be presynaptic but is poorly characterised (Conn & Desai, 1991), although AP4 is an antagonist at one group of mGluR's (mGluR1, mGluR5) and agonists at the other group (mGluR2, mGluR3, mGluR4 and mGluR6) (Nakanishi, 1992; Tanabe *et al.*, 1992). It appears that it is the presence of the LGIC glutamate receptors that reduces neuronal survival following an excitotoxic insult. All three of the characterised glutamate LGIC's contain intrinsic sodium channels that are closed at resting membrane potentials. Activation of the AMPA or kainate receptors by an agonist (thought to be either glutamate or aspartate in the brain), will open their intrinsic sodium channels and cause a depolarisation of the membrane.

The NMDA receptor channels are blocked by magnesium, in a voltage sensitive manner, in the open state and so can only be activated when the magnesium has dissociated from its blocking site. This disinhibition of the NMDA receptor occurs near 0mV, i.e. when the cell is already activated, and the channel remains active for longer

periods of time than with other glutamate receptors, (see Collingridge & Lester 1989). The NMDA sodium channel is also larger than most LGIC receptor channels having a conductance of about 50pS (Asher *et al.*, 1988) compared to the ≤20pS for the AMPA and kainate receptor-linked channels (Asher *et al.*, 1986; Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987; Cull-Candy *et al.*,1988; Asher & Nowak, 1988). The NMDA receptor channel is also less ion selective than most LGIC sodium channels, with about 5-10% of its current carried by calcium whereas most of the AMPA and kainate LGIC are relatively impermeable to calcium (Pumain *et al.*, 1987), although there are indications that some subtypes of AMPA/KA receptors are also calcium permeable (Brorson *et al.*, 1992; Rorig & Grantyn, 1993; Brorson *et al.*, 1994). The large conductance of the NMDA receptors means that an over-stimulation would result in a relatively large calcium influx.

The kainate receptor does not possess any allosteric modulatory sites whereas the AMPA receptor is allosterically modulated by zinc. In the case of the NMDA receptor zinc acts as a channel blocker at a site distinct from the magnesium and MK801 sites. The NMDA receptor is allosterically modulated by glycine, at an extracellular site, and occupation of the glycine site potentiates the glutamate signal and also delays desensitisation.

Whilst it is possible to distinguish between NMDA and non-NMDA receptor subtypes by use of agonist/antagonist studies (both binding and electro-physiological studies), the lack of specific antagonists that distinguish between the AMPA and the kainate receptors led to a possibility that the binding differences are due to different states of the same receptor, with the low affinity kainate binding site being considered the AMPA site. The report by Johansen *et al.*, (1993) that there is an antagonist that can

distinguish between the high and low affinity kainate binding sites as well as the AMPA binding site questions this assumption. However, with the advent of molecular biology, it has become clear that specific genes coding for AMPA binding sites and for kainate binding sites, and functional expression of these genes in the Xenopus oocyte model indicate that these genes will produce receptors that possess the characteristics of *in vivo* receptors (Egebjerg *et al.*, 1991; Werner *et al.*, 1991; Blakestone *et al.*, 1992; Petralia & Wenthold, 1992; see also Henley, 1994).

Control of the glutamate system is through receptor desensitisation and glutamate reuptake (Hu *et al.*, 1987), as well as presynaptic control of glutamate release. The glutamate is taken up into neurones and glial cells in an energy-dependent manner through the high affinity glutamate uptake (HAGU) system, although this is also sensitive to changes in the micro-environment, in that the uptake is dependent on the metabolic state of the cell. There are indications that neurones and glia possess different types of HAGU, based on their pharmacological profiles (Griffiths *et al.*, 1992; Albrecht *et al.*, 1993; Clark & Amara, 1993; Kanner, 1993; Kanner *et al.*, 1993; Nakamura *et al.*, 1993; Ohashi *et al.*, 1993, Rao & Murthy, 1993; Robinson *et al.*, 1993; Tanaka, 1993; Voisin *et al.*, 1993; Griffiths *et al.*, 1994; Tanaka, 1994). The fate of transported glutamate is different between the glia and neurones in that in glia it is converted to glutamine by an enzyme that is solely non-neuronal (Norenberg & Martinez-Hernandez, 1979), with the neuronal glutamate being transported into vesicles. There are various reports that in perinatals it is also down regulated during periods of excessive glutamate release (Silverstein *et al.*, 1986; Hu *et al.*, 1991). However, Anderson *et al.*, (1993), reported that after bilateral common carotid occlusion combined with hypotension, there was a long lasting upregulation of HAGU sites. Mahl & Burgesser (1993) reported that under periods

of low ATP uptake was impeded. Astrocytes are able to sustain glutamate uptake during periods of oxygen depletion provided under normo-glycaemia (Swanson, 1992; Ohashi *et al.*, 1993; Swanson & Choi, 1993). However, if glycolysis is inhibited then there is effectively no glutamate uptake. Volterra *et al.*, (1994) have recently found that oxygen free radicals will inhibit glutamate uptake in astrocytes, further indicating that in periods of oxygen/glucose deprivation glutamate homeostasis is compromised. Dysfunction of the HAGU system could result in glutamate being present in the synaptic cleft for prolonged periods, possibly causing over-stimulation of the glutamate receptors following a physiological stimulus. It may be that impairment of the glutamate transporters may play a role in progressive neurological disesase (eg maybe some forms of ALS).

1.3.2 Amino Acids and Neurotoxicity

Early work by Olney (1969) found that glutamate was neurotoxic at high concentrations and, that recording of cells exposed to high concentrations of glutamate indicated that they were being excited to death. This paper, and the report of Rothman (1983) that synaptic activity is required for cell death, has formed the basis for the theory of 'excitotoxicity'. Various reports indicate that during neurotoxic insults there is an elevation of glutamate and aspartate released (Benveniste *et al.*, 1984; Butcher *et al.*, 1987; Globus *et al.*, 1988; Butcher *et al.*, 1990, Mitani & Kataoka 1991) and these reports along with other in vitro experiments (Choi *et al.*, 1988; Koh *et al.*, 1990) indicate that there is a disturbance in the glutamatergic system during periods of insult. Similar disturbances may also be involved in neurodegenerative disorders of different origin (see reviews by Choi, 1988; Choi & Rothman, 1990; Siesjö, 1992).

As well as an increase in glutamate release during ischaemia there are reports that now indicate there is also an increase in extracellular glycine, and that this elevation is also greater and longer lasting than the glutamate elevation (Baker *et al.*, 1991; Cantor *et al.*, 1992).

Compounds that exert their neurotoxicity by metabolic failure (e.g. MPTP), also appear to have a glutamatergic involvement, but in this case it appears to be secondary damage caused by glutamate leakage from dead/dying cells.

1.3.3 Excitotoxic Theory

If there is a period of high glutamate release, and there are NMDA receptors on postsynaptic membranes, then it is probable that the postsynaptic neurones would be exposed to excitotoxic conditions. If the levels of extracellular glutamate remain high then there could be a cascade effect that might be uncontrolled within a small area (see figure 1.2). The current working hypothesis of excitotoxicity is that there is an over-stimulation of the postsynaptic non-NMDA glutamate receptors, either by massive release of glutamate or by excitotoxins, which cause a depolarisation of the neurone. This depolarisation causes the disinhibition of the NMDA receptors and there is a massive influx of calcium through the receptor-associated channel.

In the case of the ischaemic models there is also an increase in glycine release, and extracellular levels recorded by microdialysis, indicate that the glycine sites on the NMDA receptors are fully occupied. Baker *et al.*, (1991) and Cantor *et al.*, (1992) reported that ischaemic levels of glycine and non toxic levels of NMDA would result in neurotoxicity in neuronal cultures. This can be explained by the report of Lerma *et al.*, (1990), that glycine decreases NMDA receptor desensitisation, therefore allowing for the

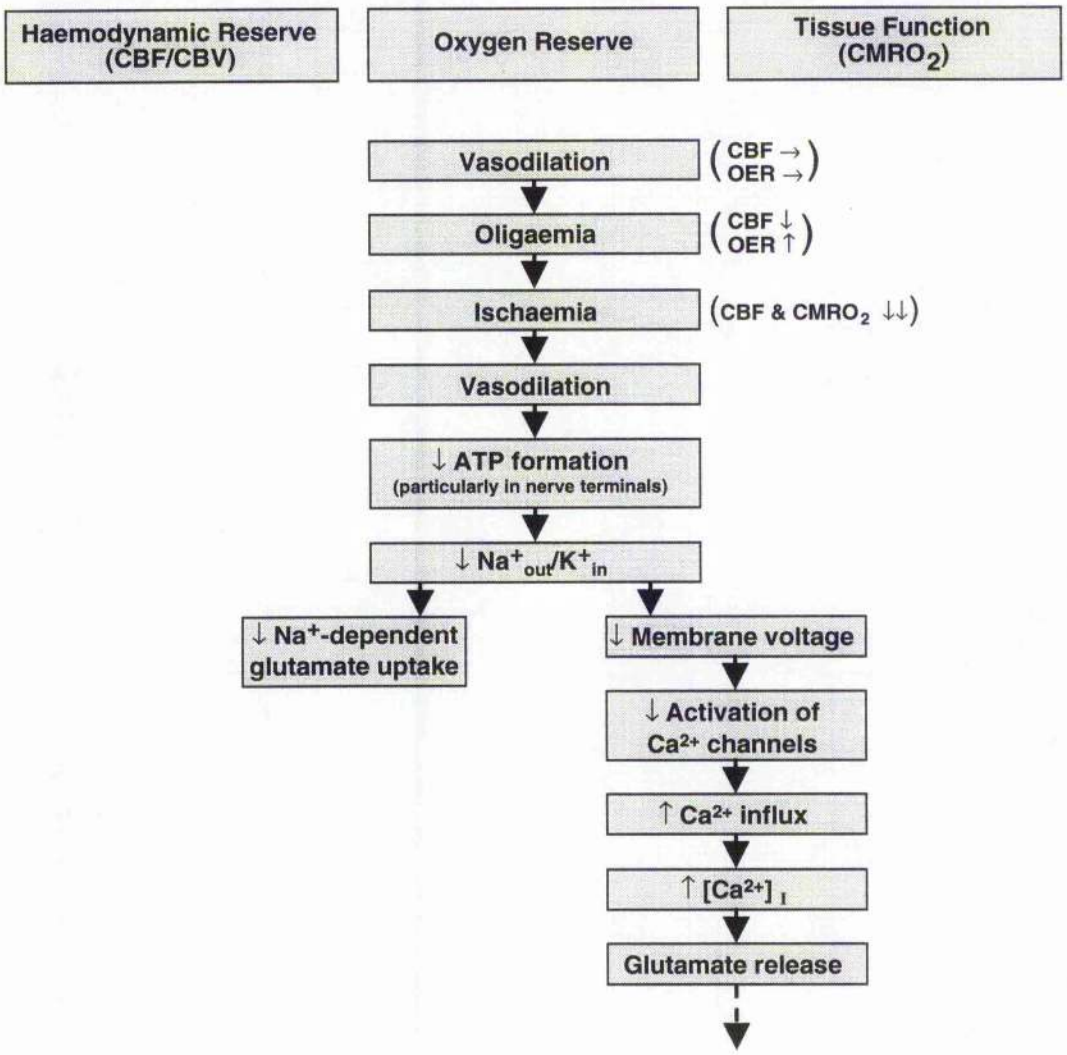


Figure 1.2 Mechanisms of glutamate mediated neurotoxicity

A) Cerebral vascular causes of excessive glutamate release

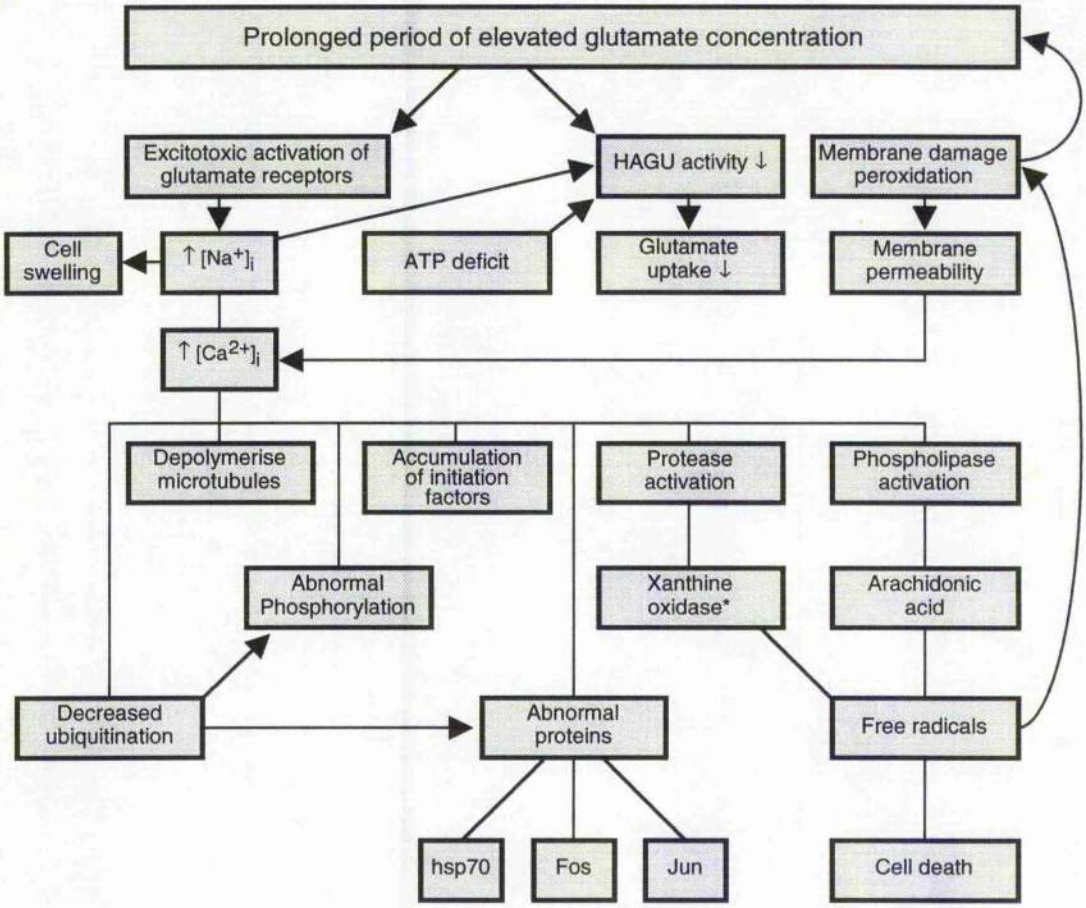


Figure 1.2 Mechanisms of glutamate mediated neurotoxicity

B) Intracellular consequences of excessive glutamate stimulation

receptor to be active longer. Calcium is also entering through the activation of VGCC's (Lin *et al.*, 1990; Ohta *et al.*, 1991; Uematsu *et al.*, 1991; McBurney *et al.*, 1992). This elevation of the intracellular free calcium concentration causes the activation/inhibition of calcium sensitive systems and a decrease in the ATP/ADP ratio (see table 1.1 for a summary of calcium activated events). There would also probably be an initial increase in the metabolic state of the cell to restore this ATP/ADP, by increased oxidative phosphorylation.

Provided there is an ample supply of molecular oxygen and glucose, the cell might be able to survive the insult but possibly in a damaged condition, with an altered metabolic state and the presence of stress proteins. The proteins induced include Fos (c-fos proto-oncogene), 70kDa heat shock/stress protein (hsp70), and Jun B and c (proto-oncogenes c-jun and jun B), and mRNA for zif/268 , 73kDa heat shock protein (hsc73) and glucose regulated protein (grp78) (Nowak, 1990; Nowak *et al.*, 1990; Uemura *et al.*, 1991a; Vendrell *et al.*, 1991; Robertson *et al.*, 1992; Dragunow *et al.*, 1993; Kinouchi *et al.*, 1993; Nowak *et al.*, 1993; Schreiber *et al.*, 1993; Tanno *et al.*, 1993; Walker & Carlock, 1993; Wang *et al.*, 1993; Massamiri *et al.*, 1994). As well as the appearance of these stress proteins there are also abnormal proteins (eg xanthine oxidase) and this might be explained by the observation that after ischaemia there is a reduction in ubiquitin synthesis, resulting in the loss of this indispensible repair protein (Magnusson & Wieloch, 1989; Yamashita *et al.*, 1991). These damaged cells might die at a later date, possibly due to a smaller ischaemic insult, and might explain the phenomenon of delayed neuronal death (Mitani & Kataoka, 1991; Haba *et al.*, 1991). If the supply of oxygen is reduced, then the cells' metabolism would switch from oxidative phosphorylation (38 moles of ATP/ mole glucose) to that of glycolysis (2 moles ATP and 2 moles lactate/ mole

glucose),(see section 1.2.1). This shift would result in decreased ATP production but also a decrease in intracellular pH, both of which are detrimental to the cells' survival. The reduced ATP would mean that the membrane potential would fall and the membrane would become permeable to ions. Whilst this is occurring there would be a failure of the HAGU system, resulting in the prolongation of the excitotoxic stimulus, as well as the production of cytotoxic substances in the cell itself e.g. free radicals, (see figure 1.2).

Table 1.1. Calcium activated events potentially detrimental to neuronal viability.

1) Proteases:
    - Calpains I & II (breakdown of spectrin)
    - Formation of xanthine oxidase
    - Generalised cytoskeletal disassembly
2) Protein kinases
    - Protein kinase C enhancement of glutamate release
    - Calcium-calmodulin-dependent kinase
3) Phosphatases
4) Phospholipases
    - Phospholipase A_2
    - Phospholipase C
5) Ornithine decarboxylase
6) Nitric oxide synthase
7) Endonucleases
8) Induction of stress protein synthesis.

One complication to the theory of glutamate toxicity is the large amount of glutamate ($\approx$500mM) needed to cause neuronal death both *in vivo* and *in vitro* (Choi *et al.*, 1988; Koh *et al.*, 1990). This may be explained by the functioning of the HAGU system: if exogenous glutamate is supplied the HAGU system would be expected to remove large amounts of it as there has been no associated impairment of the metabolic state of the neurones that usually accompanies acute neurotoxicity.

1.3.4 Free Radical Formation and Damage

Free radicals (FR) are short lived reactive atoms or molecules containing an unpaired electron in an outer shell. FRs are produced in the cell by normal reactions and are either intermediates or byproducts. Systems generating free radicals include the respiratory chain in the mitochondria (usually in the vicinity of coenzyme Q) which is where it is terminated by oxygen, prostaglandin synthesis (an intermediate between prostaglandin G_2 and prostaglandin H_2) where it is terminated by either NAD^+ or $NADP^+$, nitric oxide synthesis where it is terminated by oxygen and the monoamine oxidase induced oxidation of catecholamines and dopamine. In these cases the FR are formed in the hydrophillic zone of the cell away from the phospholipids, buried deep in the hydrophobic zone of membranes, that are very susceptible to peroxidation, the ER and mitochondrial membranes being the most susceptible. FRs can also be produced outside of cells in the extracellular fliud by the Fenton iron catalysis, as well as by activation of microglial cells.

Lipid peroxidation would result in perturbations in the membranes resulting in loss of ion homeostasis among the deleterious effects. FR reactions are essentially immortal and can continue to react with any adjacent molecule unless neutralised by reacting with another FR, reacting with an electron donating compound (called antioxidants) or being neutralised enzymatically. FRs can also react with DNA and result in mutations of the bases or cleavage of the DNA strands.

The cellular defences against FR damage involve iron chelators (eg carnosine and hydrocarnosine) to prevent/reduce the Fenton reaction, neutralising enzymes (superoxide dismutase (SOD), catalase, various peroxidases and the glutathione (GSH) oxidase/reductase related enzymes) and FR scavengers (FRS) to 'mop up' the produced

enzymes are found predominantly in astrocytes, and the other enzymes are present in low levels, as is vitamin E. The CSF is also relatively low in iron chelators compared to other extracellular fluids, although there is an active ascorbate carrier system that crosses both the blood brain barrier and the brain CSF barrier (Spector, 1981). The importance of the FRS's to maintaining cell viability was highlighted by Demopoulos *et al.*, (1977) who reported that following middle cerebral artery ligation, there was a reduction in ascorbate concentrations which they postulated was due to its reaction with oxygen free radicals formed in the respiratory chain and that hypoxic tissue damage may be accompanied by FR production. Whilst this postulate has not been proven, the experimental evidence from several hypoxic tissues including gut, heart and brain, that FRS's and/or FR neutralising enzymes reduce the amount of damage, may indicate that FRs are involved in hypoxic damage (see sections 1.4.4 and Discussion).

1.3.5 Excitotoxic Models

1.3.5.1 Ischaemic Models

There are various mechanisms for causing in vivo excitotoxic insults, but they can be separated into two groups; those involving a physical occlusion of cerebral blood vessels and those that cause damage by injections of neurotoxic chemicals. Both methods will cause neuronal damage but result in differing pathological conditions. Middle cerebral artery occlusion models will cause focal ischaemia, four vessel occlusion models will cause global ischaemia and chemical injections will have differing actions depending on the route given.

1.3.5.2 Excitotoxic Models *In Vivo*

Several compounds, structurally related to glutamate and/or aspartate, are also neurotoxic (see figure 1.3). One of the most potent neurotoxins is kainic acid, a dicarboxy containing pyrrolidine (Shinozaki & Konishi, 1970; Olney *et al.*, 1974; Coyle, 1987). In a recent report Winn *et al.* (1991) compared kainate to other neurotoxins, and reported that the excitotoxic potency order in the rodent basal forebrain was:

KA > > Quinolinate > Ibotenate = NMDA.

A report by Stewart *el al.* (1991) reported that domoate, another naturally occurring dicarboxy containing pyrrolidine, is more potent than kainate, and ingestion by humans resulted in behavioural disturbances similar to those reported in the kainate studies (see Heggli *et al.*, 1981; Lothman & Collins, 1981; Sperk *et al.*, 1985). The postmortem pathology is also similar to kainate. This finding now means that the potency order should read as follows:

domoate > kainate > > Quinolinate > Ibotenate = NMDA > glutamate.

The areas sensitive to kainic acid induced damage differ from those affected by other neurotoxins, in that the limbic system is very sensitive to kainic acid but other regions are relatively insensitive. However the behavioural effects of the cerebrovasculature occlusion differ from that of kainic acid injections: after the initial seizures the rat becomes hypersensitive after ip KA injections (Heggli *et al.*, 1981), but they do not exhibit any of the motor dysfunctions associated with an ischaemic insult e.g. loss of limb tone, motor coordination (rope platform climbing), balancing and walking (Hoffman *et al.*, 1991). These kainic acid sensitive areas are similar to those affected by Huntington's disease, temporal lobe epilepsy, and global ischaemia but differ from those sensitive to focal ischaemic insult (Heggli *et al.*, 1981; Kish *et al.*, 1983; see Coyle, 1987; Sperk, 1994 for

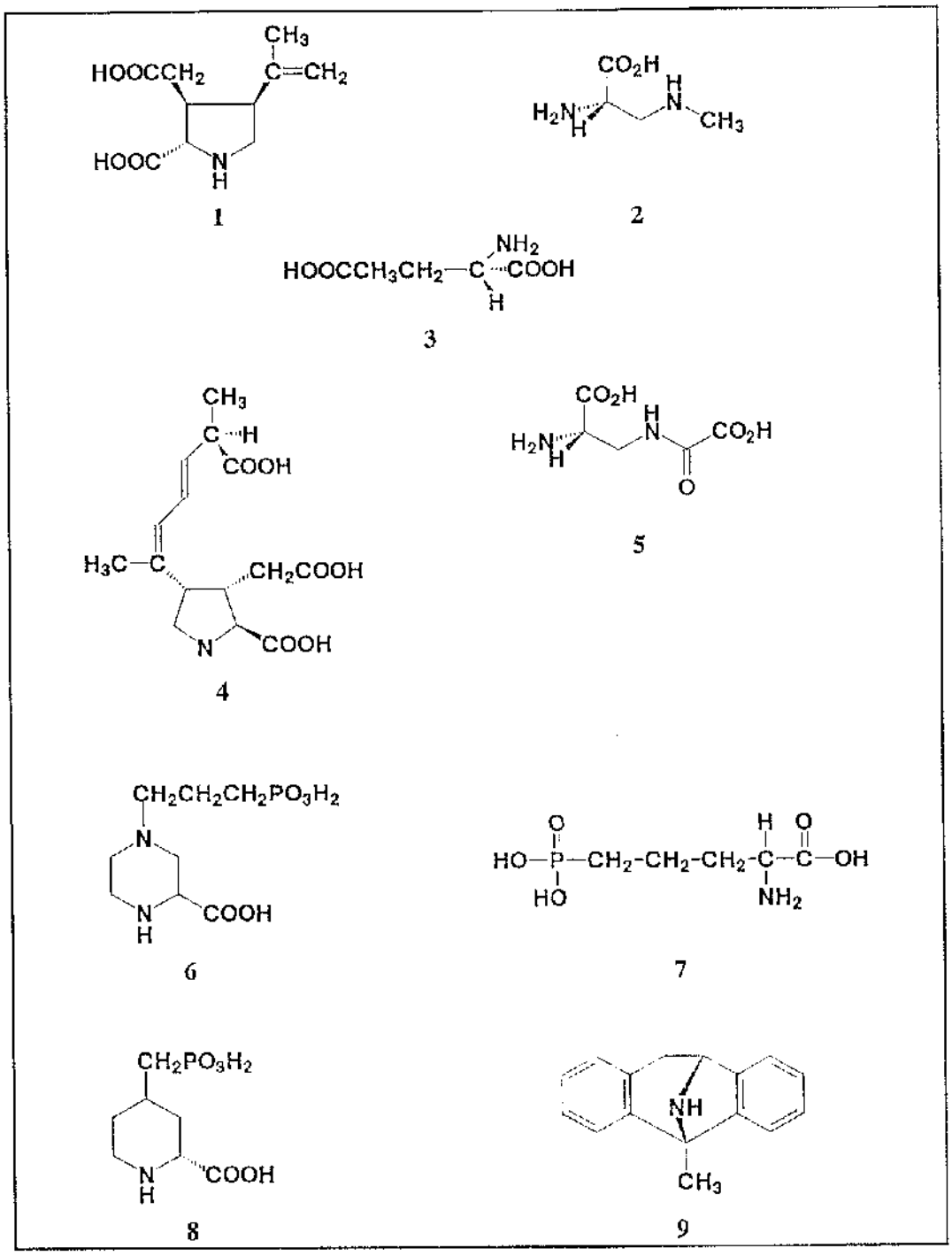


Figure 1.3
Chemical structures of A) glutamate agonists and antagonists
agonists: 1) kainic acid, 2) BMAA, 3) glutamic acid, 4) domoic acid, 5) BOAA,
antagonists: 6) CPP, 7) AP5, 8) CPP 9) MK 801.

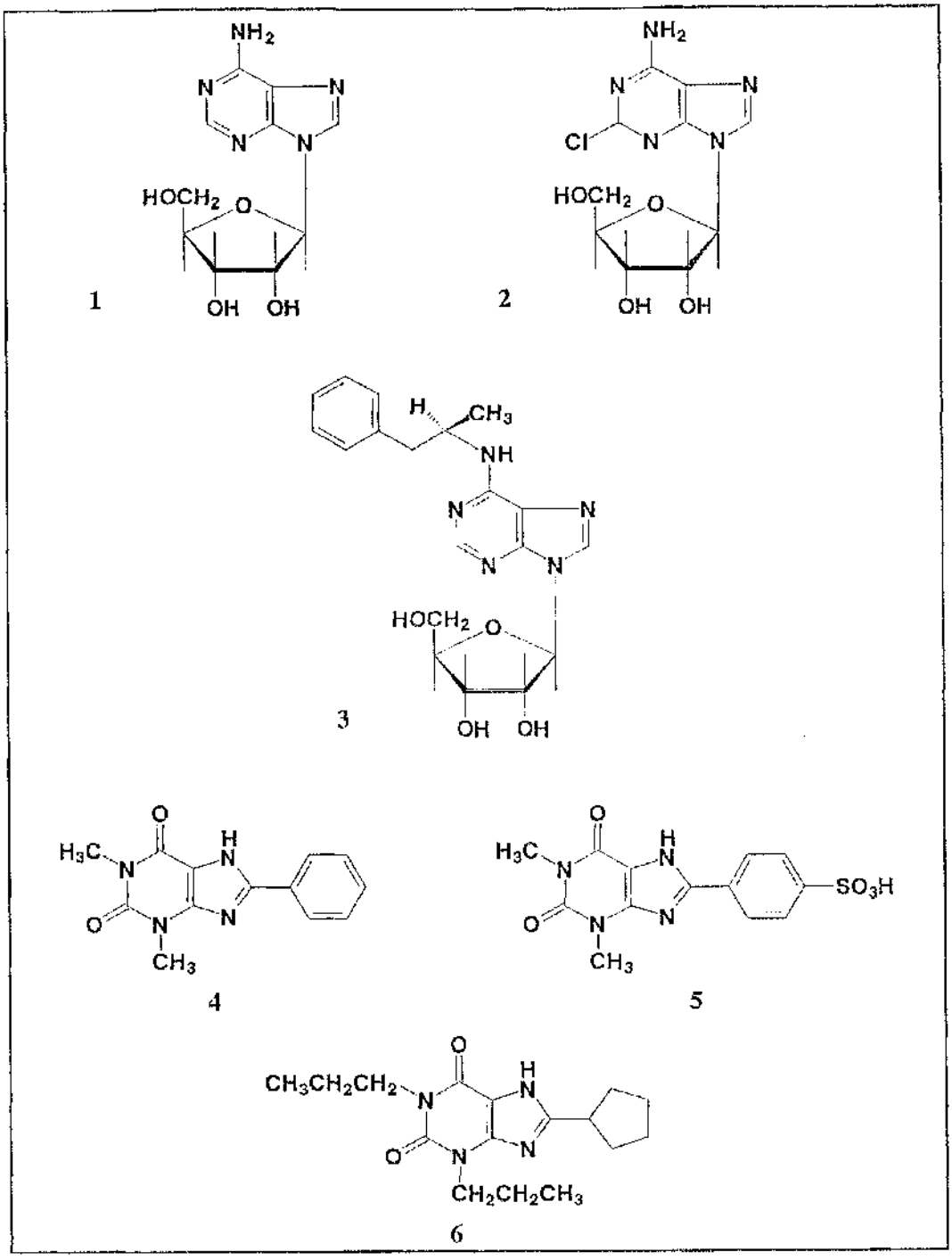


Figure 1.3;
B) purine agonists and antagonists.
Agonists: 1) adenosine, 2) 2 chloroadenosine, 3) R-PIA,
Antagonists: 4) 8-PT, 5) 8-SPT, 6) DPCPX.

reviews). Kainic acid (and domoic acid) also differ from other neurotoxins in that central damage can result from systemic injections, as well as from direct intracerebral administration (see earlier references and, Stewart *et al.*, 1991; Strain & Tasker, 1991; Scallet *et al.*, 1993).

1.3.5.3 Excitotoxin Models *In Vitro*

Two preparations for studying excitotoxicity *in vitro* are those of brain slices and cell cultures. In the brain slice experiments only short term changes can be monitored due to the nature of the tissue preparation. Slices have been used to examine the effects of excitatory amino acid agonists and antagonists, as well as the effect of chemical ischaemia (cynanide and low/zero glucose) or hypoxia (95% N_2/ 5% CO_2), on the functioning of pathways as measured by electrophysiological methods (Reiner *et al.*, 1990).

Cell cultures have the advantage over slice preparations that a longer time scale is available for study, and similar techniques are used to brain slices to induce/reduce neuronal- and nonneuronal-toxicity. Damage in cell cultures can be measured by a variety of methods including lactate dehydrogenase leakage, loss of neuronal markers and induction of genes. However, there are serious drawbacks to the study of neuroprotection in cell cultures, and these are based around:

a) lack of intraregional organisation, and

b) lack of extraregional pathways,

both of which may influence the degree of protection and the areas damaged. However cell cultures do provide an understanding of the biochemical mechanisms of excitotoxicity, that is not possible in other methods of study.

1.4 Sites for Potential Neuroprotective Drugs.

The present theories of EAA induced excitotoxicity indicate that there may be several sites where drugs might be targeted to produce varying degrees of neuroprotection, and these include: blockade of the NMDA receptor, calcium channel blockers, reducing glutamate release and free radical scavengers.

1.4.1 NMDA Receptor Blockade

The main area of interest in neuroprotection against acute neurotoxicity has been blocking the activation of the NMDA receptor or blocking the associated channels, hence preventing the influx of calcium and the neurotoxic cascade that follows over stimulation of this receptor. Several drugs have been tried with varying degrees of success, with the best experimental drug being MK801. Various studies have shown that this compound can reduce the area of damage following Middle Cerebral Artery Occlusion (MCAO), a model that mirrors cerebrovascular occlusion in humans, up to 8 hours after the incident (Foster *et al.*, 1987; Gill *et al.*, 1987; Ozyurt *et al.*, 1988; Park *et al.*, 1988; Michenfelder *et al.*, 1989; Gill & Woodruff,1990; Uematsu *et al.*, 1991; Massieu *et al.*, 1993). However MK801 and other related compounds (e.g. phencyclidine) have several side effects including psychological disturbances (interaction with the sigma receptor), as well as peripheral vasoactive perturbations and respiratory impairment (Mcmanigle *et al.*, 1994; Pechnick & Hiramatsu, 1994). These side effects are thought to occur because these compounds are non-competitive NMDA receptor channel blockers and studies performed in other laboratories indicate that they will block any receptor mediated sodium channel e.g. both the central and peripheral types of nicotinic Acetylcholine channels (S. Wonnacott, personal communication). The main reason that these drugs are still being

used in experimental models is that they are all lipophilic and can cross the blood brain barrier very easily. The more specific competitive NMDA receptor antagonists (e.g. AP5 and AP7) do not produce any major psychological side effects but the use of these drugs is hampered by the fact that they are unable to cross the blood brain barrier. There are now several second generation AP5-like compounds that are able to cross the blood brain barrier, including: CGP 37849 (Schmutz *et al.*, 1989; Fagg *et al.*, 1989; Massieu *et al.*, 1993), CGS 19755 (Boast *et al.*, 1988; Grotta *et al.*, 1990; Aizenman & Hartnett, 1992; Hogan *et al.*, 1992; Massieu *et al.*, 1993) and CPP and CPPene (Boast *et al.*, 1988; Bullock *et al.*, 1990; Massieu *et al.*, 1993).

Another area of interest is in the regulation of tryptophan metabolism. Recent reports have shown that various metabolites of tryptophan metabolism are active in the CNS (Perkins & Stone 1985; Beal *et al.*, 1988; Stone 1990; Jhamandas *et al.*, 1990; Beal *et al.*, 1991; Pearson & Reynolds, 1992; Roberts *et al.*, 1993; see Stone, 1993b). At present there are 2 metabolites that are known to be active (kynurenic acid and quinolinic acid). What is of interest is that kynurenic acid is an NMDA antagonist whilst quinolinic acid is an agonist. As quinolinic acid is produced endogenously (albeit in very small amounts) it has been postulated to be involved in neuro-degenerative diseases e.g. Huntington's chorea, (Connick & Stone 1988; Beal *et al.*, 1990). If this is the case, then any drug that reduces the formation of quinolinic acid will provide some degree of neuroprotection and conversely any drug that increases the formation of quinolinic acid will increase neuronal damage. The opposite seems to be true for kynurenic acid, in that increasing kynurenic acid production should increase neuroprotection whilst decreasing production should lessen its protection. Germano *et al.*, (1988) indicated that treating animals with kynurenic acid produced smaller lesions, in a global ischaemic model.

Various papers have recently indicated that kynurenate only poorly crosses the blood brain barrier (Swartz *et al.*, 1990; Moroni *et al.*, 1991; Wu *et al.*, 1991), although various precursors can (tryptophan and kynurenine) and that treating rats with either of these precursors will elevate brain kynurenic acid levels as assessed by microdialysis. However, after an ischaemic insult there is usually a period of BBB permeabilisation (Dietrich *et al.*, 1993a), and it may be this permeabilisation that is allowing kynurenic acid to exert its neuroprotective action, in that several papers seem to support Germano's report but use different models (Labella *et al.*, 1993 (BOAA); Smith *et al.*, 1993, (TBI); Ghribi *et al.*, 1994, (forebrain ischaemia)). Kynurenic acid is a competitive antagonist of the glycine regulatory site of the NMDA receptor (Kessler *et al.*, 1987; Watson *et al.*, 1988; Williams *et al.*, 1988; Kessler *et al.*, 1989) and there are now several synthetic kynurenines (including 7 chlorokynurenic acid, 5,7-dichloro-kynurenic acid and 7 chlorothiokynurenic acid) that have improved selectivity for the glycine site, and are potentially neuroprotective (Brugger *et al.*, 1990; Pralong *et al.*, 1992; Chen *et al.*, 1993; Lehmann *et al.*, 1993; Wood *et al.*, 1993).

1.4.2 Calcium Channel Blockade

Another main area of interest has been in the blockade of the calcium channels themselves (Sauter & Rudin, 1986; Block *et al.*, 1990; Jacewicz *et al.*, 1990; Lin *et al.*, 1990; Uematsu *et al.*, 1991). The idea behind this is to reduce the influx of calcium through the voltage gated channels and hence reduce the elevation in intracellular calcium (see reviews by Choi & Rothman, 1990; Seisjö, 1993a, 1993b). Although this works well in experimental models it has the drawback of a very short therapeutic time window and is most effective when the drugs are administered prior to the neurotoxic insult.

1.4.3 Regulation of Glutamate Release

Another possible target for neuroprotective drugs is the control of glutamate release. There are various reports in the literature of adenosine reducing the release of glutamate from neurones (Corradetti *et al.*, 1984; Fastbom & Fredholm, 1985; Burke & Nadler, 1988; Arvin *et al.*, 1989; Lekieffre *et al.*, 1991; Heron *et al.*, 1992; Miller & Hsu, 1992; Simpson *et al.*, 1992, Lloyd *et al.*, 1994), or Ca^{2+} uptake (Gonènçalves *et al.*, 1991, Chern *et al.*, 1992; Gonènçalves & Riberio, 1994). In the hippocampus there appear to be three types of adenosine receptors, of which the main two are; the A_1 receptor, which appears to be an inhibitory receptor, linked via G-proteins to potassium channels, and the A_{2a} which appears to be linked to both excitatory and inhibitory G-protein pathways (Cunha *et al.*, 1994). Recently the A_3 receptor has been cloned, and is found to be 80% homologous to the A_1 receptor. Although it is present in the hippocampus, the levels of expression are low (Zhou *et al.*, 1992), and no central function has been found for this receptor. The major problem in the study of this receptor subtype is the lack of selective agonists/antagonists, in that all the agonists reported are have only a 10fold greater specific for the A_3 than the A_1 (Carruthers & Fozard, 1993; Fozard & Carruthers, 1993; Jacobson *et al.*, 1993; Linden *et al.*, 1993, Daly & Jacobson, 1994; Jacobson *et al.*, 1994; Von Lubitz *et al.*, 1994).

Deckert & Jorgensen (1988), reported that after an intraventricular injection of 0.5μg/2μl KA there was a loss in [^{3}H] cyclohexyladenosine (CHA) binding associated with axonal terminals in the CA3 pyramidal cell layer, one of the KA sensitive areas. This location of adenosine A_1 receptors would be able to modulate the amount of glutamate released from the presynaptic terminals and possibly reduce the extracelluar glutamate to a less neurotoxic concentration. It seems that the purinergic system may have a

modulatory action on glutamate excitation and drugs that modulate this system will have an effect on glutamate excitotoxicity. Recently there have been several reports indicating that pretreatment of animals with adenosine agonists or uptake blockers may provide varying degrees of protection against both chemically and physically induced neuronal damage, (Block & Pulsinelli, 1987; DeLeo *et al.*, 1987; Evans *et al.*, 1987; DeLeo *et al.*, 1988; Dragunow & Faull, 1988; Goldberg *et al.*, 1988; von Lubitz *et al.*, 1988; Arvin *et al.*, 1989; Bielenberg, 1989; Connick & Stone, 1989; von Lubitz *et al.*, 1989; Andiné *et al.*, 1990; Finn *et al.*, 1991; Miller & Hsu, 1992; Mori *et al.*, 1992; Simpson *et al.*, 1992; Tominaga *et al.*, 1992; Danielisova *et al.*, 1994; Kano *et al.*, 1994; Stanimirovic *et al.*, 1994; Zhou *et al.*, 1994). Recent reports also indicate that activation of both NMDA and non-NMDA receptors will cause the release of adenosine in the hippocampus (Jhamandas & Dumbrille, 1980; Phillis *et al.*, 1989; Hoehn & White, 1990b; Chen *et al.*, 1992, Sciotti *et al.*, 1993; Ogilvy *et al.*, 1994). In most cases, however, the purines were delivered intracerebrally, whereas any development of purines therapeutically would need to be centred on peripheral administration.

As well as purines, other drugs acting at calcium channels, kappa opioid and $GABA_A$ receptors as well as other, as yet unidentified sites are also able to attenuate glutamate/aspartate release during hypoxic conditions, and hence have therapeutic potential (Brailowsky *et al.*, 1986; Sternau *et al.*, 1989; Gannon & Terrian, 1992; Lyden & Hedges, 1992; Graham *et al.*, 1993a, 1993b; Hayward *et al.*, 1993; Leach *et al.*, 1993; Martin *et al.*, 1993; Lyden & Lonzo, 1994). The sites of adenosine and GABA modulation of KA induced neurotoxicity are shown in figure 1.4.

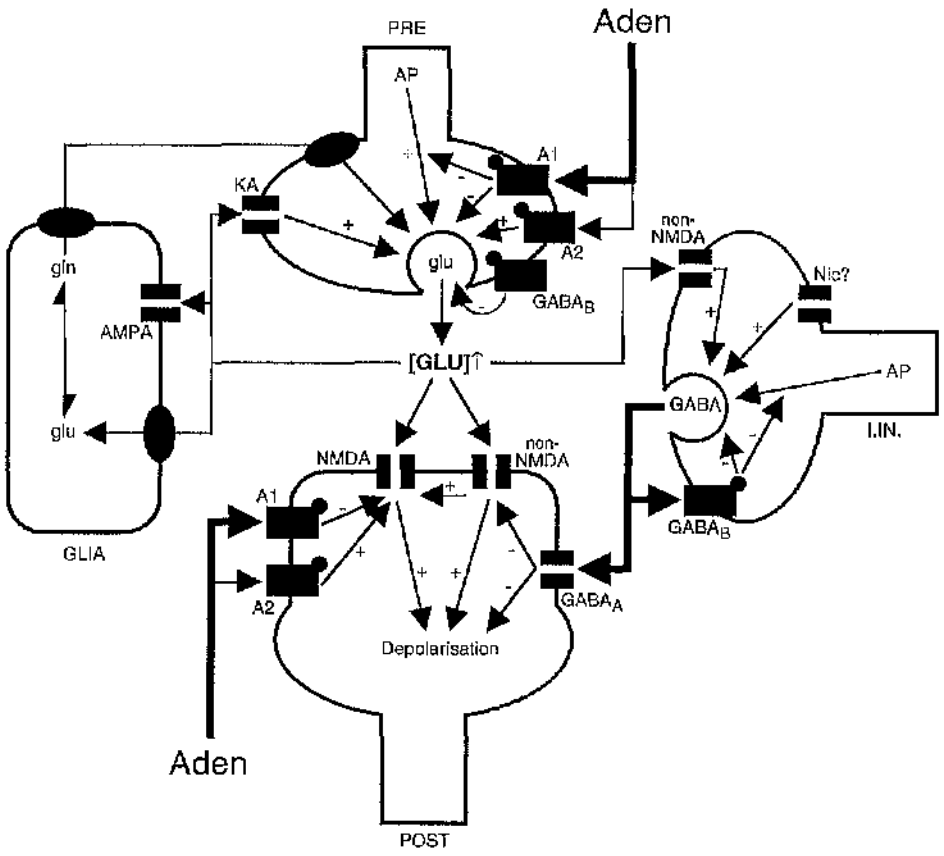


Fig 1.4 Kainic Acid induced glutamate release and modulation by adenosine and GABA.

Key -	Aden:	Adenosine
	AP:	action potential
	I.IN:	inhibitory interneurone
	Pre:	presynaptic terminal
	Post:	post-synaptic terminal
	KA:	Kainic acid
	→ :	inhibitory responses
	(-):	inhibitory effect
	(+):	excitory effect
	⬭ :	Transporter
	▮▮ :	Ligand gated ion channel
	▬● :	G-protein linked receptor

1.4.4 Free Radical Scavengers

Recently there has been interest in the role that free radicals may have in neurotoxicity, with the main focus being that of nitric oxide (Andreeva *et al.*, 1991; Dawson *et al.*, 1991; Lebel & Bondy 1991). There are various reports that activation of NMDA receptors in various parts of the brain will cause the activation of the guanylate cyclase in postsynaptic cells (Garthwaite, 1982; Wood *et al.*, 1982; Garthwaite *et al.*, 1988; East & Garthwaite, 1991). This activation can be inhibited by L-Nitroarginine, which acts by inhibiting the nitric oxide synthase (NOS). Analogues of arginine, e.g. L-nitroarginine can cross the blood brain barrier and inhibit NOS (Haberny *et al.*, 1992; Nagafuji *et al.*, 1992; Nagafuji *et al.*, 1993). However, as further studies are performed, it is becoming apparent that NO has both neuroprotective and neurotoxic actions, dependent on both the model used, time of administration of the NO active agent(s) and the identity of the agents used (Haberney *et al.*, 1992; Lipton *et al.*, 1993; Regan *et al.*, 1993; Snyder, 1993; Tanaka *et al.*, 1993; Maiese *et al.*, 1994; Przegalinski *et al.*, 1994).

The interest in the free radicals is that they will be formed by uncontrolled enzymic activity, anaerobic conditions, depleted levels of anti-oxidants (both intra- and extra-cellularly) and cell death. This may mean that free radicals are not essential in the death of cells expressing excitatory amino acids (EAA) receptors (the cells most at risk), in that they are formed in these compromised cells, but may be involved in spreading the damage to cells that were initially spared from the damage, (delayed spreading neuronal damage) (Haba *et al.*, 1991; Peunova & Enikolopov, 1993; Maiese *et al.*, 1994). If this is the case, then it might mean that drugs that will inhibit the production of free radicals are only effective for a short time period (if at all) (Nagafuji *et al.*, 1993), and it may be that free radical scavengers (e.g. vitamins C and E, and glutathione) are more

effective (Sato *et al.*, 1993 Reiber *et al.*, 1994; Saija *et al.*, 1994). Aspirin (acetylsalicylic acid) has also been reported to be very effective in reducing ischaemic brain damage (Bellavance, 1993), and its protective action may involve both the inhibition of eicosanoid production (and hence free radical formation) and possibly a free radical scavenging action.

1.5 Markers for Neurotoxicity:

1.5.1 Histological

Histological techniques can be employed to study both long and short term changes in the shape and presence of cells within the area of study, and in conjuction with immunocytochemical, ligand binding, and *in situ* mRNA hydridisation techniques can show changes in the biochemical/pharmacological activity of the cells of interest. Although the data obtained from 'normal' histology is important, the technique is not able to identify either the neurones that are still present or the type of neurotransmitter(s) released. If it is used in conjunction with the above techniques then it is a very powerful tool, with the major drawback of the time taken to process each individual animal: histology is therefore usually used to confirm findings used by biochemical/ pharmacological/behavioural studies.

1.5.2 Neuronal

Markers for neurones involve either immunocytochemical, *in situ* hybridisation (see 1.5.1) or pharmacological techniques. The immunocytochemical analysis of neurones can be either specific (eg transmitter synthesis/ uptake/ degradation and receptors) or nonspecific (eg cytoskeletal or synaptic specific proteins). Pharmacological analysis of

neurones usually targets specific populations of neurones, (eg Choline actyltransferase (ChAT) for cholinergic neurones), and hence using specific neuronal markers for neurotoxic studies requires careful interpretation of the results, in that agents that do not alter the presence of these specific markers are not necessarily not neurotoxic, but might just fail to affect the population studied. However the use of several neurone specific markers (eg ChAT and glutamic acid decarboxylase (GAD)) will cover several different neuronal populations. As yet there is no generalised non-specific neuronal marker that is available to neurotoxic studies, although recently there are assays for the widespread voltage dependent sodium channels (Sashihara *et al.*, 1992). The use of this marker will therefore allow neuroscientists to study the actions of neurotoxic agents on the neuronal populations in general, and will provide a model similar to that of the nonneuronal markers (see section 1.5.3.).

1.5.3 Non-neuronal

Following a neurotoxic insult to the brain, by either physical or chemical means, there is a period of neuronal death and 'reactive gliosis'. During gliosis there is a rapid invasion (of the area of damage) by phagocytes, followed by a delayed proliferation of astroglial cells and hence an increase in biochemical markers for glial cells (Andersson *et al.*, 1991, Jørgensen *et al.*, 1993). Benavides *et al.* (1987), have indicated that the increase in peripheral benzodiazepine receptors (PBR), which are associated mainly with glial cells in the CNS, is a more sensitive indirect index for neuronal death than the loss of neuronal markers. The exact location and role of the PBRs are not known except that they purify with the mitochondrial and synaptosomal fractions, (Basile *et al.*, 1986; O'Beirne & Williams, 1988; see Verma & Snyder 1989) and they may to be involved in

the calcium buffering capacity of the mitochondria, and hence energy metabolism (Moreno-Sánchez *et al.*, 1991), although there are also reports that they may be involve in steroidogenesis (Korneyev *et al.*, 1993). There are now numerous reports of studies using ligands for the PBR binding site (PK11195 and Ro5-4864), which have confirmed a significant increase in PBR binding associated with neurodegeneration, (Schoemaker *et al.*, 1982; Benavides *et al.*, 1987; Altar & Baudry, 1990; Bourdiol *et al.*, 1991).

Other nonneuronal markers for neurotoxic study include glial fibrillary acidic protein and related proteins. Recently the findings that kynurenic acid, homocysteic acid and quinolinic acid are synthesised almost exclusively in astrocytes (Roberts *et al.*, 1992; Zhang & Ottersen, 1992; Du *et al.*, 1993 respectively) suggests that these may also be used as indirect markers for damage in later studies.

1.6. Aims

The aims of this project where as follows:

a) to set up a kainate model of neurotoxicity, as quantified by peripheral benzodiazepine binding,

b) to determine the degree of involvement of

   i) the NMDA receptor,

   ii) purine receptors, and

   iii) free radicals,

   in the propagation of neuronal loss caused by systemic kainate administration.

2.0 METHODS

2.1 Kainate Toxicity - Binding

2.1.1 Injection Protocol:

In the *in vivo* experiments 8 week old male Wistar rats, 190 -220g, were used. For the release experiments, section 2.3.1., 210-240g male Wistar rats were used. All animals were either bred in house or purchased from Harlan Olac, and kept under the same conditions, 12hr light/dark regime, with food and water available ad lib. All animals used for each group of experiments came from the same source.

Animals were injected i.p., with drugs in a volume not normally exceeding 1ml. kg^{-1}. The maximal volume advised for i.p. injection was 5ml. for rats of this weight. Kainic acid ($10mg.kg^{-1}$) was dissolved in 0.9% NaCl (saline), R-PIA, normally $25\mu g.ml^{-1}$, in methanol/saline (24/1), APNEA in 10% DMSO in saline, allopurinol in 2% Tween 80/ saline. 8-phenyltheophylline (8PT), ($1mg.ml^{-1}$), was initially dissolved in ethanol and then diluted with saline, and brought to pH 7.4 with NaOH to give final concentrations of 17.5% ethanol and 19.3mM NaOH in 0.9% NaCl. All other drugs were dissolved in saline. Rivotril for injection (clonazepam) was supplied in commercially available ampoules and diluted with the diluent provided. In all cases vehicles were used as control injections. In the experiments using clonazepam the animals were injected 10-15 minutes prior to kainate injection.

2.1.2 Rectal Temperature:

In the 8PT experiments the rectal temperatures were recorded prior to R-PIA/vehicle injection (t_0), and every hour, for 5 hours, after the kainic acid injection, with a digital thermometer probe.

2.1.3 Behavioural Studies

In all experiments the animals were left for 60-90 minutes after the kainate injection before any behavioural observations. The behavioural scores were initially based on those used by Hoffman *et al.* (1991), scoring for consciousness, grip, limb tone, walking and pain reflex. This was repeated 2hr later and on the day of death. This method was discarded as its emphasis is on motor control, which is not affected by kainate lesioning and because scoring caused stress in the handled animals. The replacement method involved simple observation without handling of the animals every hour after kainate injection. Animals were examined for the presence of wet dog shakes, seizures and seizure-like activity, fore-paw elevation, excessive salivation, Straub tail, head weaving and circling. No scoring was used in this test but the presence or absence of these behavioural disturbances was noted.

2.1.4 P_2 Preparation:

The method used was that of Eshleman & Murray (1989) for the preparation of P2 membranes, with slight modifications. On the seventh day of the experiment the animals were killed, within the same 4hr period of the day over the course of the experiments and within 2hrs 30min of each other on the day.

The animals were killed by stunning followed by cervical dislocation and decapitation. The skull was then opened, the dura mater membrane cut and the brain placed in 30ml ice-cold 0.27M sucrose pH7.4. The cerebellum was then dissected away and the brain bisected along the corpus callosum. The hippocampi were then removed and placed in 5ml ice-cold sucrose. This normally took 2.5-4 minutes. In the concentration curve experiments only one animal was killed at any one time; for all other experiments

two animals were used.

The hippocampi were then homogenised with a Braun Homogeniser, 10 X 500 rpm. The crude homogenate was stored on ice, while the homogeniser vessel and pestle were washed twice with 5ml ice-cold sucrose and the washes added to the initial homogenate. This was brought to 100 volumes with sucrose solution and was then stored at -20°C for 2-4hrs.

After completing the preparation of tissue from a group of animals the samples were defrosted at room temperature, centrifuged for 10 min. at 4°C, 1000 x g (IEC DPR 6000 centrifuge), the pellet discarded and the supernatant centrifuged for 20min. at 4°C, 23245x g (Sorval RC 5B Refrigerated Superspeed Centrifuge, SS 34 Rotor). After this step the supernatant was discarded and the pellet was resuspended in ice-cold 50mM Tris HCl buffer pH 7.8 (50 mM Tris salt, brought to pH 7.8 with 11M HCl) 100 volumes and then centrifuged for 20min. at 4°C, 43146 x g (Sorval RC 5B). The new pellet was resuspended in Tris HCl buffer at 100 volumes and stored at -20°C, normally for no more than 24 hrs.

On the day of the assay the samples were defrosted at room temperature and centrifuged for 20min. at 4°C 43146 x g, and the supernatant discarded. The pellet was then homogenised in 5ml Tris HCl buffer 7x1500 rpm, the homogenate kept on ice and the homogeniser chamber and pestle washed twice with 3-5ml Tris HCl buffer. The washes were pooled with the homogenate and the volume brought to 100 volumes, this being the P_2 membrane fraction, and stored on ice until needed.

2.1.5 [^{3}H] PK11195 Assay.

All assays were performed at 4°C (on ice) and samples were incubated for 60min ($\pm$ 1 min). For the concentration experiments the assay was performed in triplicates over the concentration range 0.1 nM - 50 nM [^{3}H]-PK11195, with 10 μM PK11195 as displacing agent, to define non-specific binding, using 250μl P_2 membranes. Both vehicle-treated and kainate-treated animals were examined. The test experiments were performed in duplicates with 500μl P_2 membranes.

The volume of the assay chamber was 2ml, and contained 5μl [^{3}H] PK11195, 5μl cold ligand, and P_2 membranes. The volume was brought to 2ml with Tris HCl buffer. Final assay conditions were 1.75nM [^{3}H] PK11195 in ethanol (0.25% final), 0.25% DMSO $\pm$ 10 μM PK11195 and 100-150μg protein. The assay samples were vortexed at the start of their incubation and approximately every 20 minutes before filtration.

The incubation was terminated by vacuum filtration, with all of the sample being filtered through pre-wetted Whatman GF/C glass filters using a Millipore 12 well 1225 Sampling Manifold. Filters were washed twice with 12ml ice-cold Tris HCl Buffer and vacuum dried, before being placed in scintillation vials. 5ml Ecoscint scintillation fluid was then added, the samples left overnight and then counted using a Packard 2000 Scintillation Counter for DPM, (quenching and efficiency of counting was calculated as being 32% using external standards).

Protein concentrations were measured using the Lowry method (Lowry *et al.* 1951), following solubilisation with 0.25M NaOH and with Bovine Serum Albumin as the standard.

2.1.6 Data Analysis:

Specific binding was calculated by the normal method. Specific binding was also calculated as percent same day control to minimise day to day variations. All values are mean $\pm$ S.E.M. Statistical significance was assessed by unpaired Student's t-test (unequal variance), significance being considered when $p<0.05$. Selected experiments were also analysed by Anova and post-tested with the Student-Newman-Keuls mutlicomparison test. If there was any disparities between the two tests, the Student-Newman-Keuls mutlicomparison test results were used.

2.2 Endogenous Glutamate Release.

Hippocampal slices (450μm thick) were prepared as in section 2.3.1 and were allowed to recover for one hour in an oxygenated ACSF chamber at room temperature. After this hour the slices were transfered to a perfusion chamber and superfused with oxygenated ACSF (1ml.minute^{-1}). The slices were allowed to recover from this transfer for 60 minutes before stimulation with 1ml 50mM KCl. All fractions were collected every 2 minutes and placed on ice before freezing (-20^{0}C). Samples were analysed by an OPA-derivatisation reverse phase ion exchange Gilson HPLC-gradient method.

Solvent A: 90% 0.1M sodium acetate pH 7.2,, 9.5% methanol, 0.5% tetrahydrofuran.

Solvent B: 100% methanol.

40μl samples of perfusate were derivatised with 40μ OPA/2-mecaptoethanol (37mM/5mM respectively) in 100mM borate buffer pH 9.5, and 40μl 100mM borate buffer. The samples were mixed at room temperature and injected into the sample loop 30 seconds after the reaction was initiated. This was performed using a computer

controlled Gilson HPLC 231 Autosampler. The samples were flushed into the HPLC system using a Rheodyne 7010 sample injector. The ratio of solvent A: solvent B was controlled by computer using the Gilson GME_712 programme, each assay run was 25 minutes in duration and the gradient is shown in table 2.1. The amino acids were separated using a Microsorb C_{18}, 3μm, 4.6mm internal diamter, 5cm long column and were detected using a Gilson Spectra/glo fluorimeter, (excitation wave length 390nm: emission cutoff filter 475nm) set at maximum sensitivity.

Table 2.1. HPLC gradient conditions.

Time (minutes)	Flow rate ($ml.min^{-1}$)	% B
0	1.7	0
2	1.7	20
3	1.7	20
9	1.7	45
12	1.7	45
14	1.7	85
16	1.7	85
18	1.7	0

2.3 [^{3}H] Glutamate Release

2.3.1 Hippocampal Slice Preparation.

(The hippocampal slices were prepared by Doctor M.J. Higgins, Department of Pharmacology).

The methods used for these sets of experiments are modifications of Ferkany & Coyle (1983a,b), Connick (1987) and Palmer *et al.* (1992). Male Wistar rats (8 weeks old and 210-240g) were used throughout, either bred in house or bought from Harlan Olac.

In each experiment 5 slices were used from any one animal. The rat was killed by cervical dislocation and decapitation, the skull opened and the dura cut. The brain was then placed in about 30ml ice-cold oxygenated ACSF, the cerebellum removed and the brain bisected along the corpus callosum. The hippocampi were then gently teased away from the surrounding tissue and laid longitudinally upon Whatman filter paper and 300μm slices cut with a McIlwain Tissue Chopper. The hippocampi were then transferred to fresh ice-cold ACSF, the slices teased apart and placed in an oxygen-saturated ACSF environment and left to recover for 90-120 minutes at room temperature.

At the end of this stabilisation period, the slices were transferred to another incubation chamber at 30°C in a temperature controlled water bath (TE-7 Telcom water controller) and left for 15 minutes. After the 15 minutes 10 slices were transferred using a paintbrush from the normal ACSF to 3ml ACSF containing 200nM L-[2,3-^{3}H]-glutamic acid (17-20 Ci/mmol). The slices were then incubated at 30°C for a further 30 minutes.

2.3.2 [^{3}H] Glutamate Release Protocol.

The incubation period with [^{3}H]-glutamate was terminated by the transfer, by paintbrush, of slices to a series of perfusion chambers, 1 slice/ chamber. The incubation chambers were airtight Nuclepore Filtration chambers, with 13mm Pop-Top™ plastic filtration holder 420100 (volume 250μl), and the slice was placed on a 0.5cm diameter Whatman CF/C glass microfibre disc filter. (figure 2.1). Upon insertion of the slices the chambers were closed and were continuously perfused with oxygenated ACSF at 1ml/min, over the course of the experiment. All solutions were maintained at 30°C and were gassed with 95% O_2/ 5% CO_2 for a minimum of 30 minutes prior to introduction into the system

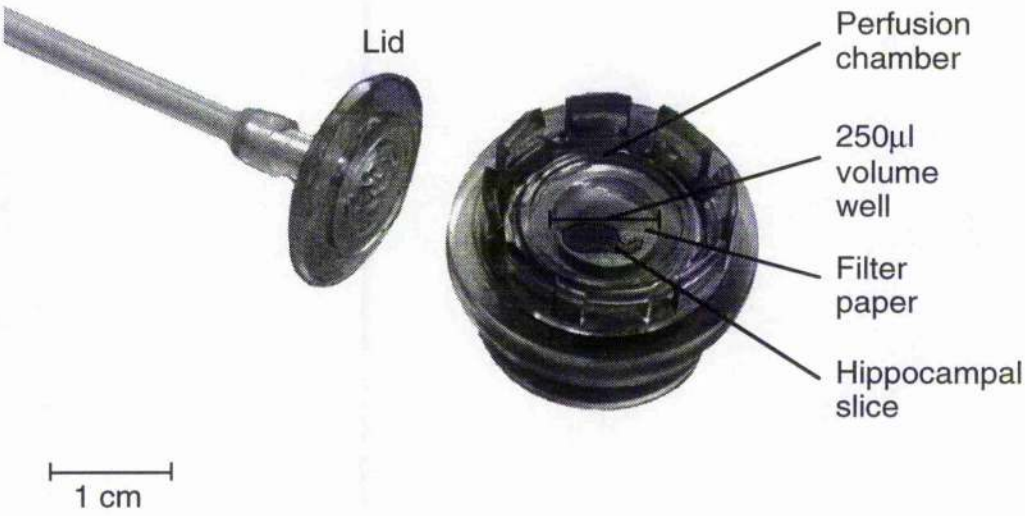


Fig 2.1: Perfusion chamber used in release experiments

(figure 2.2). Samples were collected at various time points (see table 2.2). Solutions were changed 35 seconds prior to their arrival at the perfusion chamber and were preceded and followed by a 3-4 second air bubble. Stimulations were 1 minute duration, and the bracketing with the air bubbles ensured a bolus delivery. During the time when the air bubbles were formed the outside of the tubing was wiped to reduce solution contamination.

Stimulations (S) were timed to arrive every 30 minutes starting at 60 minutes after the start of perfusion. The time taken for a bubble to travel the length of the tubing was timed and solution changes were always calculated to arrive 5-10 seconds after a fraction had started, the time taken was usually 35-40 seconds. The last stimulation was usually 50mM KCl and the slices were left for a further 30 minutes before termination of the experiment. The antagonist(s) were perfused in ACSF over 1 or 2 stimulations (S2 or S3), starting 12 minutes after S1 or S2 and finishing 12 minutes before S3 or S4. In all the stimulations during the antagonist perfusion the stimulating solutions also contained the antagonist.

For detection 4ml Ecoscint scintillation fluid was added to 2ml of perfusate, vortexed and counted using an Kontron 3000 scintillation counter (5 minutes for CPM). Due to the large number of vials produced per experiment (200+) the Kontron counter was used in favour of the Packard 200CA counter used in the binding studies.

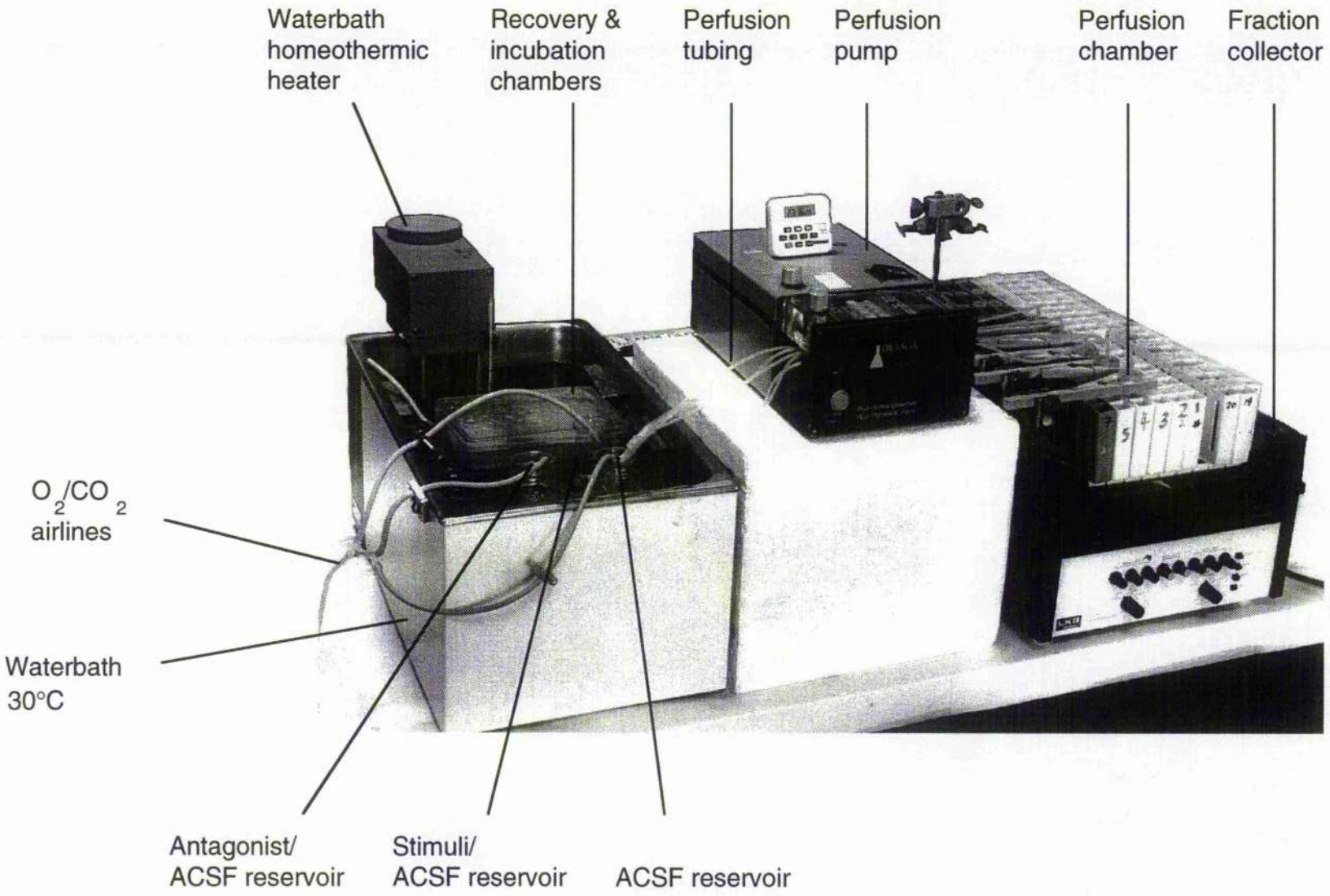


Fig 2.2: Apparatus used in release experiments

Table 2.2. Release experiment protocol.

TIME (minutes)	EVENT.
0	Start Perfusion.
50	Change fraction time 5 to 2 min.
59.30	Start S1 perfusion.
60.05	S1 arrives in chamber.
60.30	Stop S1 perfusion.
61.05	S1 terminates in chamber.
(71.30)	(Start perfusion of drug affecting S2).
89.30	Start S2 perfusion.
90.05	S2 arrives in Chamber.
90.30	Stop S2 perfusion.
91.05	S2 terminates in Chamber.
(101.30)	(stop perfusion of drug affecting S2).
(101.30)	(Start perfusion for drug affecting S3).
119.30	Start S3 perfusion.
120.05	S3 arrives in chamber.
120.30	Stop S3 perfusion.
121.05	S3 terminates in chamber.
(131.30)	(stop perfusion of drug affecting S3).
149.30	Start S4 perfusion.
150.05	S4 arrives in chamber.
150.30	Stop S4 perfusion.
151.05	S4 terminates in chamber.
(163.30)	(Stop perfusion of drug affecting S4).
(181)	(Start perfusing air)
184	Terminate perfusion, end experiment.

2.3.3 Data Analysis.

The data were converted to DPM using an external standard, taking into account efficiency of counting and quenching. The DPM values were then plotted against time using Graph Pad v 2.0. Time points between 54 and 134 (164) were removed and an exponential decay curve ($Y=A^{exp(-BX)}+ C^{exp(-DX)} +E$) was fitted to the remaining values, using the curve fitting by nonlinear regression mode, weighted by 1/Y, least sum of squares (figure 2.3a). The edited-out data points were then returned to the plot and residuals from the curve were then calculated (figure 2.3b & c). The residuals were then loaded into a statistical package (Minitab) and analyzed for change in pattern over time within an experiment and between experimental groups at specific time points. The residuals were also used to determine Sx/Sy ratios. To determine the amount released during a stimulation period, the baseline under the stimulation peak was calculated by averaging 3 time points before and after the peak, and subtracting from the peak values, this value being termed 'stimulated release'. The Sx/Sy ratios were statistically analyzed by U-test, significance being assumed when $p \leq 0.05$ (Mann Whitney U-test).

Slices were discarded if either the S1 was less than 2.27pmol (100 D.P.M. 2 x baseline noise) or if the release showed an unstable baseline.

2.3.4 Solutions and Materials.

The composition of ACSF was as follows (mM): NaCl 125, KCl 2.5, KH_2PO_4 0.5, $NaHCO_3$ 27, $MgSO_4.7H_2O$ 1.2, $CaCl_2$ 1.2 and glucose 10. High KCl solutions were as control ACSF with the exception that KCl replaced NaCl giving the composition KCl 50mM, NaCl 77.5mM or KCl 25mM, NaCl 102.5mM.

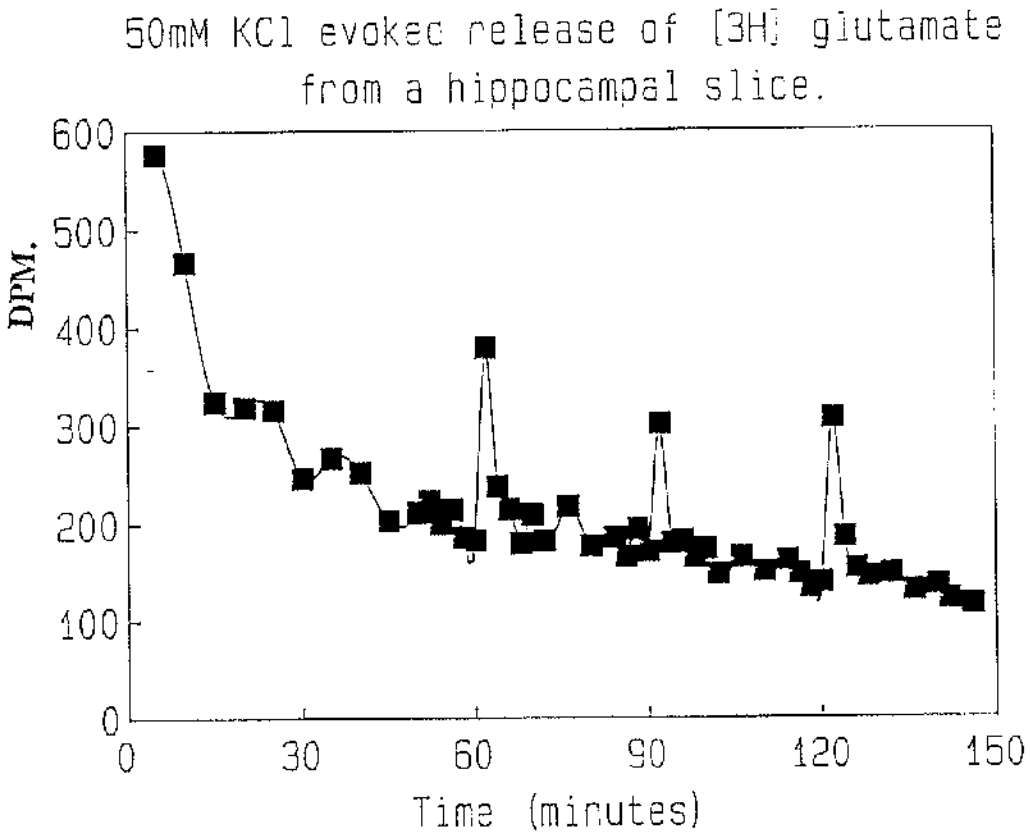
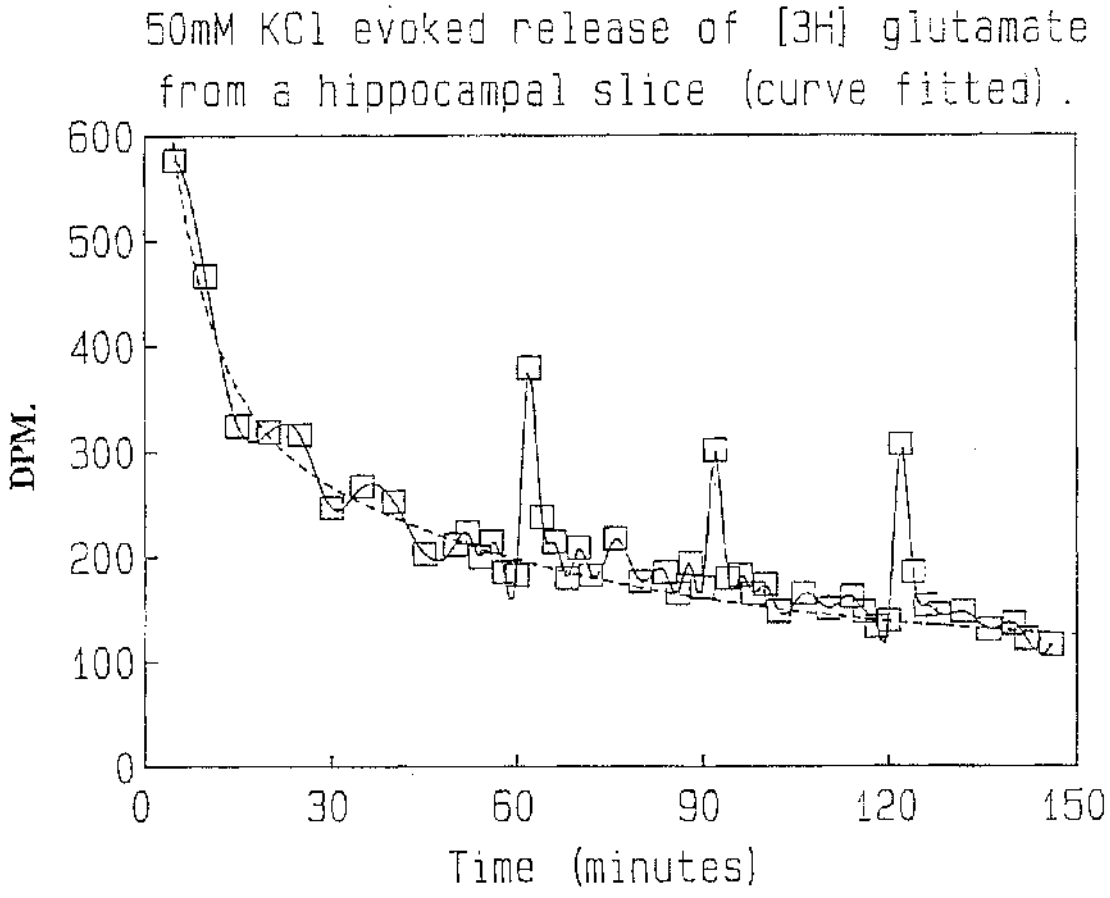
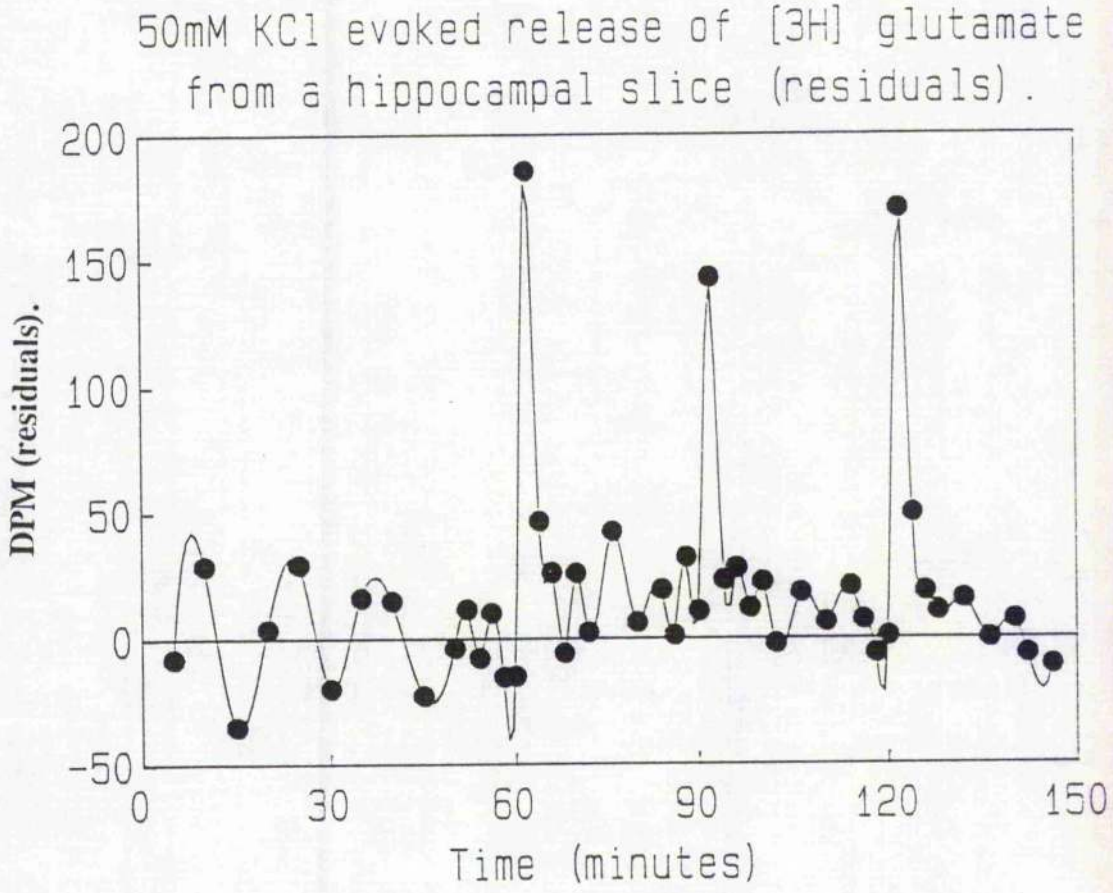


Figure 2.3
Curve fitting of an exponential decay curve to release data.
The data is an example of a single release experiment profile obtained by 50mM KCl stimulation of a hippocampal slice. Time corresponds to time from the start of the perfusion of ACSF through the filtration chambers. The points are joined by a cubic spline calculated curve to connect the fractions collected. The three peaks correspond to S1, S2 and S3 (all 50mM KCl).
A) The initial release profile, obtained after scintillation counting. The data have not been corrected either for efficiency or quenching, this is performed after the residuals have been calculated (100 DPM = 7.75 fmol).



B) The same data expressed overlying the calculated exponential decay curve. The dotted line corresponds to the exponential curve and the solid line to the cubic spline curve.



C) The data are expressed as residual DPM. The method of calculation is described in the methods section, but basically the data values are subtracted from the exponential curve values for that point. The resulting residual values are plotted against time, and the resulting graph represents release above the mathematically determined basal release (0 DPM). The profile illustrates the degree of instability of the release during the initial period of perfusion, (5-50 minutes), after which the fluctuations are smaller and less pronounced.

The Silescol (silica) tubing 1mm inner diameter, 1mm thick wall was used throughout the experiments. The solutions were maintained at 30°C using a Tecam TE-7 Tempette immersion thermostat controlling a Tecam water bath. A Desaga (Heidelberg) PLG-Peristaltic pump was used to pump the solutions from their reservoirs to the release chambers, and the perfusate was collected in 6ml mini-scintillation vials placed in an LKB (Bromma) 2070 Ultrarac° II fraction collector.

2.4 Materials

[^{3}H] PK11195 (specific activity = 80-86 Ci/mmol) was purchased from DuPont/NEN (Stevenage, Herts., U.K.). [^{3}H] glutamate (specific activity = 20-40 Ci/mmol) was purchased from Amersham International plc (Amersham, Bucks., U.K.). 8PT, ascorbic acid, kainic acid, kynreninic acid, L-kynurenine, MK801, R-PIA and tryptophan were from Sigma Chemical Co. (Poole, Dorset, U.K.). 2CA, 8SPT, and DPCPX were from Research Biochemicals Incorporated (Stevenage, Herts., U.K.), Clonazepam and unlabelled PK11195 were gifts from Roche, Switzerland and Pharmuka, France respectively. Chlormethiazole sulphate was a kind gift from Astra Arcus, Sweden. APNEA was a kind gift from Professor R.A. Olsson (University of South Florida, Tampa USA). GYKI 52466 was a kind gift from Egis, Hungary. Rivotril (clonazepam for injection) was purchased commercially.

3.0. RESULTS - NEUROTOXICITY EXPERIMENTS.

3.1 Neurotoxicity

3.1.1 Behavioural Studies;

The behavioural responses observed following 10mg.kg^{-1} kainic acid i.p. correlated well with those in the literature (Ben-Ari *et al.*, 1979; Schwob *et al.*, 1980; Heggli *et al.*, 1981; Lothman & Collins, 1981; Heggli & Malthe-Sorvensen, 1982; Sperk *et al.*, 1983; Ben-Ari 1985; Sperk *et al.*, 1985), with wet dog shakes (wds) appearing before the onset of seizures and salivation, fore paw elevation, Straub tail, circling and penile erection. Each of these different types of behaviour are well characterised in the literature (see above references). A wet dog shake is a whole body shake (not affecting the limbs) of usually a few seconds in duration, seizures being a rapid onset whole body spasm, sometimes involving a vertical translocation. Forepaw elevation involved the animal raising either one or both of its forepaws, until they were level with the rib cage and parallel to the ground, and resting on its haunches to maintain balance. When the animal was displaying forepaw elevation, it rarely moved and any locomotor activity that was observed, usually occurred when only one of the paws was raised. When the animal was displaying Straub tail, its tail was raised off the ground, sometimes vertically but also horizontally, and was rigid, but not necessarily straight. Animals were able to display several behavioural disturbances at the same time, although wet dog shakes were never seen in conjunction with forepaw elevation or seizures. The initial survival rate also agrees well with the previous reports (see above), in that approximately 15% of the kainate injected animals died within the first 6 hour period, with the cause of death probably due to excessive seizure like activity. The onset of wet dog shakes usually occurred within the first hour after the kainate injections and most of the animals had ceased to exhibit

behavioural disturbances six hours after injections. The kainate injected animals showed seizures on the day of injection, but the severity and duration was variable and by the day of killing they showed no signs of motor disturbances as seen in most stroke models. However the animals were hypersensitive to tactile stimuli, in that even a slight pressure resulted in a marked locomotive response, (in some cases the animal leaping 12-18 inches in the air and landing on the ground running).

In general the animals which had been given test drugs, at doses sufficient to afford some degree of neuroprotection, did not display this hypersensitivity and were usually behaviourally similar to the vehicle treated animals. All the animals showed some form of behavioural disturbances following kainate injections, even if they were given drugs that either reduced seizures (clonazepam 1-0.2mg/kg) or gave apparent neuroprotection (i.e. R-PIA). In addition chlormethiazole, clonazepam (1mg/kg), magnesium sulphate or MK801 treated animals were markedly sedated, with little or no WDS activity during this period, but the later onset behavioural disturbances were observed (except for clonic-tonic seizures).

3.2 Binding Studies

3.2.1 Saturation Experiments.

Binding parameters were calculated using Scatchard analysis. The values obtained showed that there was no change in K_d (7.35 $\pm$ 0.27 and 13.44 $\pm$ 3.34 nM vehicle (n=4 animals) and kainate (n=3 animals) respectively), but there was a significant increase (p=0.008) in B_{max} in the kainate treated animals (693.2 $\pm$ 139.2 and 2917.0 $\pm$ 319.2 fmol.mg^{-1} for vehicle and treated animals respectively), (figure 3.1). All subsequent assays were performed using 1.75nM [^{3}H] PK11195 as at this concentration acceptable sensitivity

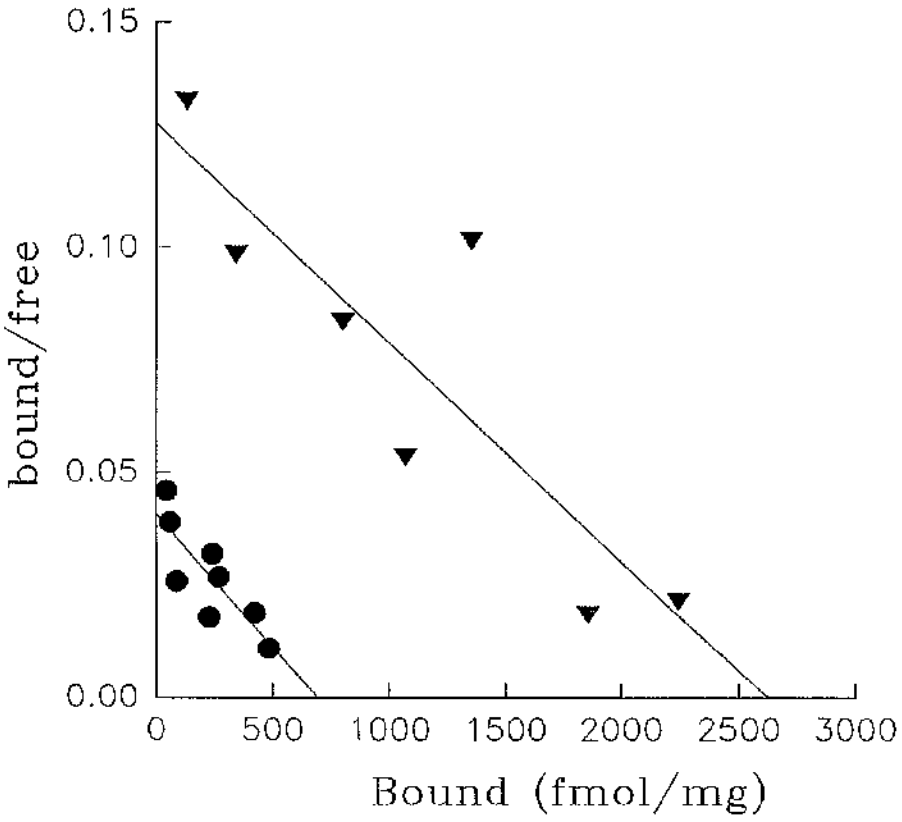


Figure 3.1.

Scatchard analysis of [^{3}H] PK11195 binding to rat hippocampal P2 membranes. The data represents a single experiment prepared as in the methods; ● saline treated rat, ▼ kainate 10 mg kg^{-1} treated rat.

was achieved at a reasonable cost. In the control animals approximately 40% of the total binding was nonspecific, falling to $\leq$10% of total binding with some kainate treated animals.

3.3 Kainate Induced Neurotoxicity.

3.3.1 Kainate Dose Response Curve (Unsedated)

Initial experiments were performed using three groups of animals treated with vehicle, 5mg.kg^{-1} and 10mg.kg^{-1} ip kainic acid respectively. Vehicle (control) and 10mg.kg^{-1} kainic acid groups gave a single tight population of binding, 111.2 $\pm$ 21.1 fmol.mg^{-1} protein (n=12) and 356.8 $\pm$ 49.6 fmol.mg^{-1} protein (n=19) respectively. The 5mg.kg^{-1} kainic acid injected animals showed greater variability and no significant increase in binding was detected, (176.6 $\pm$ 55.8 fmol mg^{-1}, n=9) (see figure 3.2). The 10mgkg^{-1} dose was therefore used for all other experiments as it was most consistent and the increase in binding to 350-500% of control was highly significant, ($p < 0.001$).

When the total amount of protein in the P_2 fraction was examined there was a significant reduction in the 10mg.kg^{-1} but not the 5mg.kg^{-1} treated groups compared to control (4.16 $\pm$ 0.14mg (n=19), 4.79 $\pm$ 0.16mg (n=9) and 4.92 $\pm$ 0.21mg (n=12) respectively) (figure 3.3).

3.3.2 Clonazepam Treatment.

In the initial experiments about 15% of rats injected with kainic acid 10 mg kg^{-1} died during tonic clonic seizures, and a preliminary study was therefore performed to determine whether protection could be afforded against seizures without compromising hippocampal toxicity.

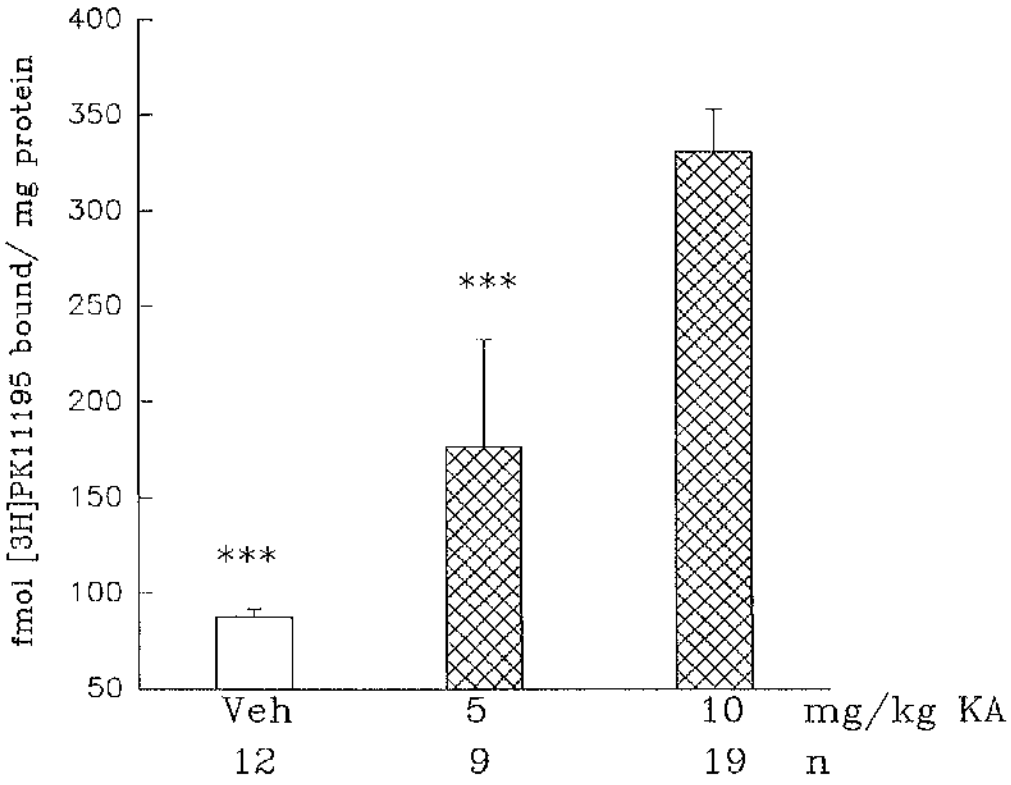


Figure 3.2.

Increase in [^{3}H] PK11195 binding in hippocampal P_2 membranes of kainate treated rats.

Columns indicate mean ± S.E.M. KA = kainic acid, Veh = saline.
*** $p < 0.001$ versus 10mg.kg^{-1} kainate group. Student-Newman-Keuls multiple comparison test.

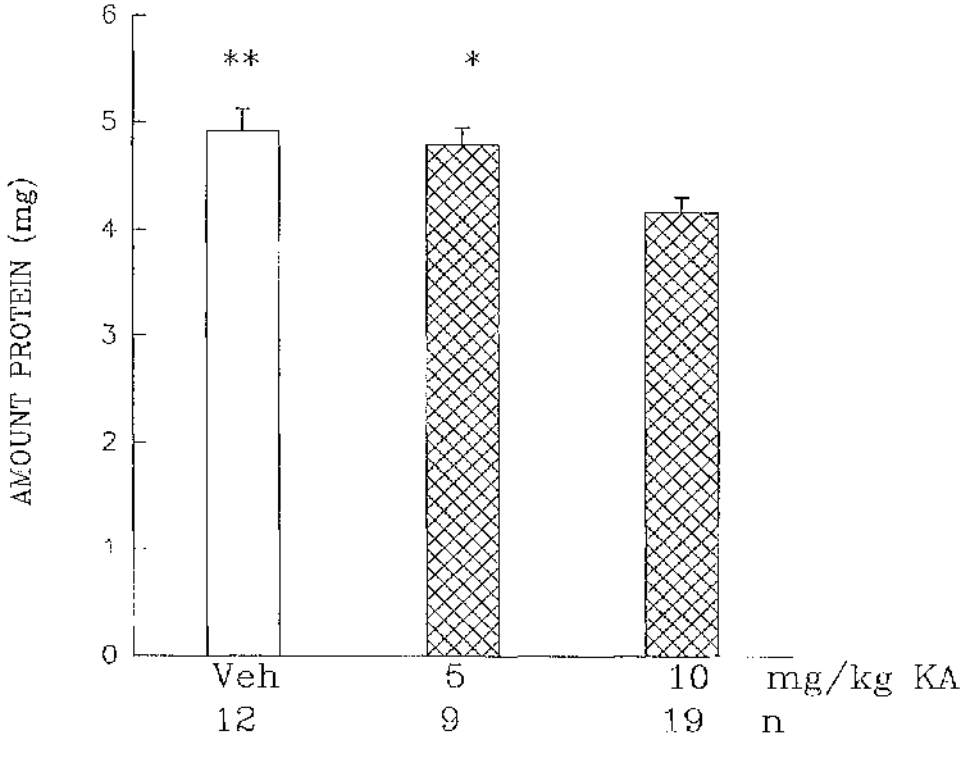


Figure 3.3.

Change in total P_2 protein content in kainate treated rats.

Columns indicate means ± SEM KA = kainate.

The total protein was calculated for each animal by calculating the concentration in the P_2 fraction (obtained by protein Lowry), by the volume of the P_2 sample.

* $p \leq 0.05$, ** $p \leq 0.01$, versus 10mg.kg^{-1} kainate, Student-Newman-Keuls multiple comparison test.

3.3.2.1 Behaviour

Groups of animals were preinjected with 1mg kg^{-1} clonazepam or 1ml kg^{-1} vehicle i.p. alone or followed after 10 minutes by an injection of 10 mg kg^{-1} kainic acid. There was a reduction in both the severity of the seizure activity and the duration of seizure, with a marked sedative effect, but the animals still displayed wet dog shakes, salivation, circling and forepaw elevation as observed in kainate control animals.

3.3.2.2. Binding

The [^{3}H]PK11195 binding was significantly different between the kainate and vehicle treated groups, but no significant difference was obtained between clonazepam and vehicle treated animals in the presence of kainate (figure 3.4). When the dose of clonazepam was reduced to 0.2mg kg^{-1} seizure activity was still reduced but with less apparent sedation and still no effect on [^{3}H]PK11195 binding (240.18 $\pm$ 13.43 (n=12)). The smaller dose was therefore used routinely in subsequent experiments.

3.3.3 Kainate Dose Response Curve (Sedated).

The use of the anticonvulsant, clonazepam, at a dose sufficient to reduce seizure activity but not to affect kainate toxicity, allowed for the re-evaluation of kainate evoked toxicity at higher doses. Even though there was little or no seizure activity the highest dose of kainate used (20mg.kg^{-1}) resulted in 2/8 fatalities. In one of the survivors there was total loss of the hippocampus, striatum and limbic system, although this animal appeared behaviourally normal prior to termination. The dose of 7.5mg.kg^{-1} gave an intermediate level of damage that was significantly different from both the control and the 10mg.kg^{-1} groups but not from the 5mg.kg^{-1} group. The levels of binding obtained after

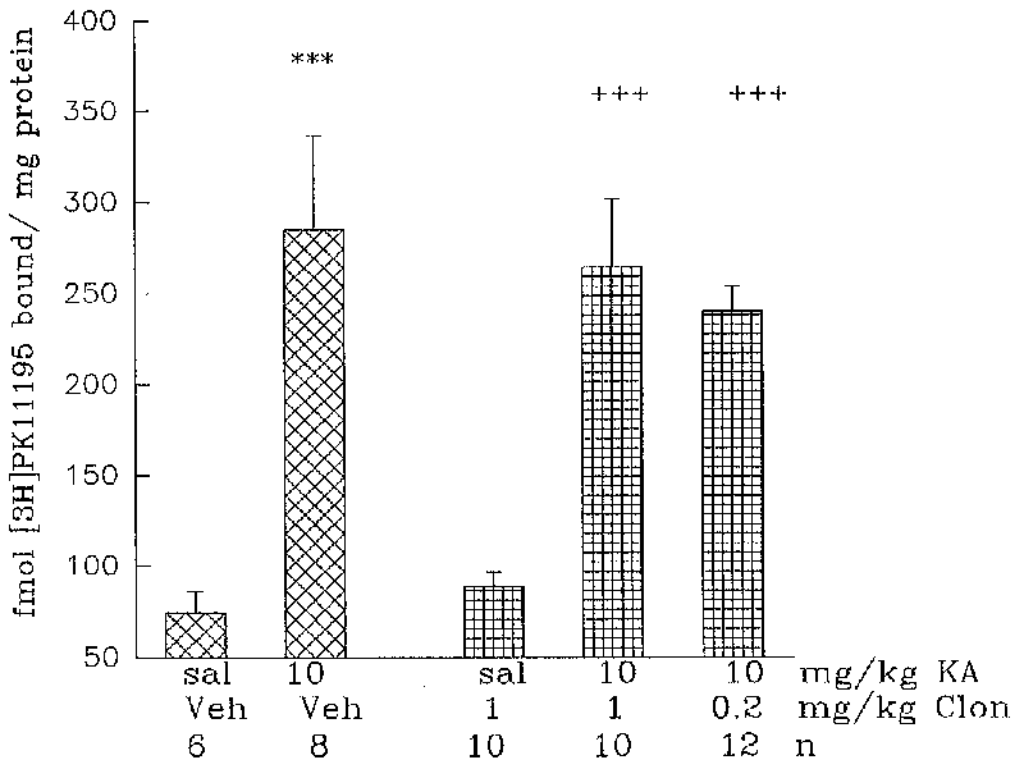


Figure 3.4.

Comparison of clonazepam or vehicle treatment on kainate induced increase in [^{3}H] PK11195 binding to hippocampal P_2 membranes.

Rats were pretreated with clonazepam or vehicle ip 10 minutes prior to kainate 10 mgkg^{-1} or saline ip injection. Animals were left for 7 days and binding performed as in methods. Columns indicate mean $\pm$ s.e.m. sal = saline 1 mlkg^{-1}, KA = kainate 10 mgkg^{-1}, Veh = clonazepam vehicle 1 mlkg^{-1} or
0.2 mlkg^{-1}, Clon = clonazepam.
*** $p \leq 0.001$ versus saline control. +++ $p \leq 0.001$ versus clonazepam control. Student-Newman-Keuls multiple comparison test.

the doses of 12.5, 15 and 20mg.kg^{-1} were not significantly different from the 10mg.kg^{-1} group (figure 3.5).

3.3.4 Non-NMDA Antagonism of Kainate Induced Neurotoxicity.

The non-NMDA antagonist GYKI 52466 is reported to be able to antagonise kainate induced toxicity better than AMPA induced toxicity in *in vivo* studies (Moncada *et al.*, 1991), and was so used in preference to the other non-NMDA antagonists which can cross the blood brain barrier (CNQX, DNQX, NBQX). The same time administration and dosage as of Moncada *et al.*, (1991) was used.

GYKI 52466 was suspended in 2% tween 80 and 30mg.kg^{-1} was administered at times t_{-30}, t_0 and t_{+30}. The control animals received 2% tween 80 solution at the same time. The GYKI 52466 treated animals displayed behavioural disturbances similar to those of the kainate treated rats, but were behaviourally normal by the seventh day. The level of [^{3}H] PK11195 binding in the GYKI 52466 treated animals was significantly less than the kainate/vehicle treated animals (104.61 $\pm$ 23.22 fmol.mg protein^{-1} (n=4) and 234.16 $\pm$ 15.43 fmol.mg protein^{-1} (n=4) respectively, $p \leq 0.05$ Student's unpaired t-test). The binding in the GYKI 52466 group was not significantly different from the control group (figure 3.6).

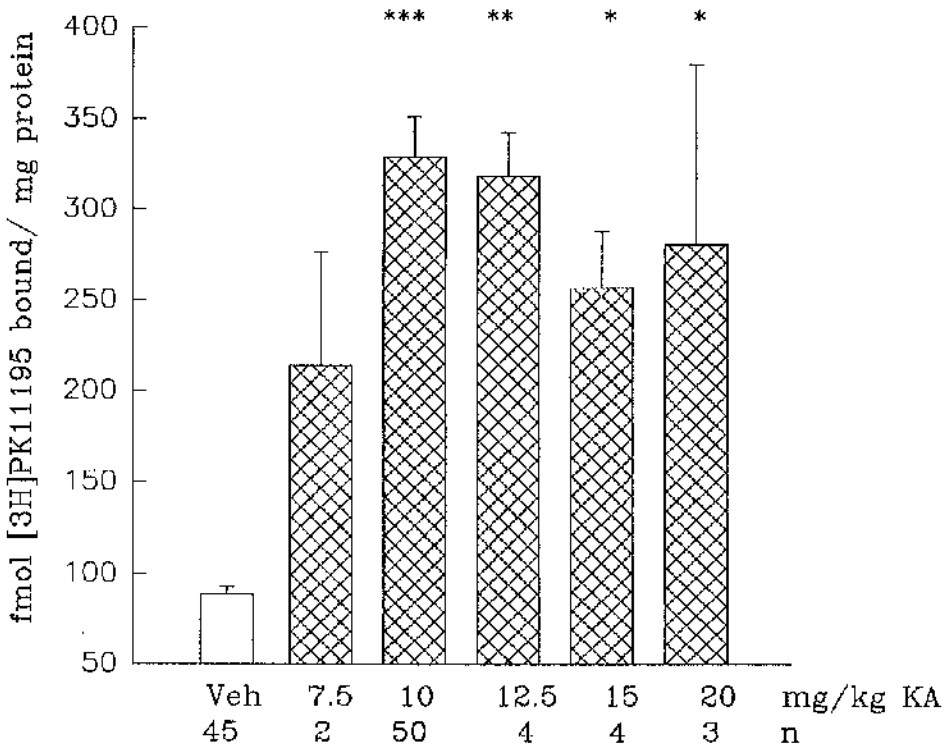


Figure 3.5.

Re-evaluation of kainate dose response curve in clonazepam treated rats.

The animals were pretreated with 0.2mg.kg^{-1} clonazepam 15 minutes prior to kainate/ vehicle injection, and left to recover for 7 days. P_2 fraction prepared as in methods.

Column indicate means $\pm$ SEM, KA = kainate, Veh = vehicle. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ versus control. Student-Newman-Keuls multiple comparison test.

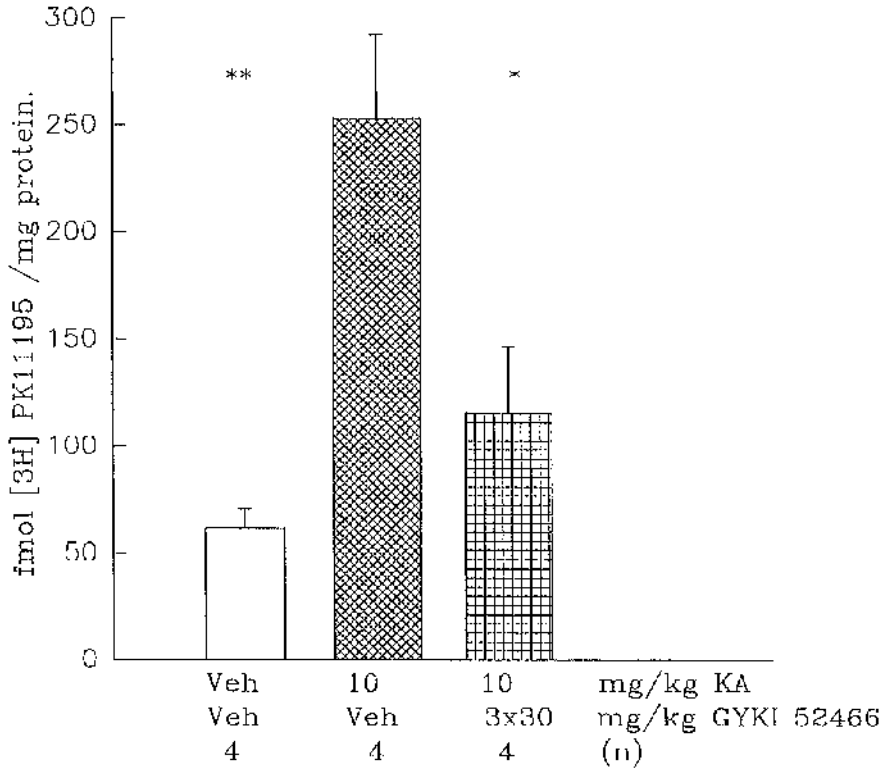


Figure 3.6.

Effect of GYKI 52466 on kainate induced toxicity.

Animals were pretreated with 0.2mg.kg^{-1} clonazepam 10 minutes prior to 10mg.kg^{-1} kainate administration (t_0). GYKI 52466 or vehicle (2% tween 80, 1ml.kg^{-1}) was administered at times

t_{-30min}, t_{0min} and t_{+30min}. The animals were allowed to recover for 7 days before hippocampal P_2 membranes were prepared as in methods.

KA = kainate, KA veh = saline, GYKI veh = 2% tween 80 in saline.

* $p \leq 0.05$ versus kainate group. Student-Newman-Keuls multiple comparison test.

3.4 Purine Neuromodulation.

3.4.1 Adenosine Agonist Injections

3.4.1.1 Adenosine A_1 Agonist Injections.

3.4.1.1.1 Dose Response Curve.

The adenosine analogue R-PIA was injected i.p. at doses of 1mg.kg^{-1}, 100μg.kg^{-1}, 25μg.kg^{-1}, 10μg.kg^{-1} and 2.5 μg.kg^{-1}, but the combination of the 1mg.kg^{-1} with kainic acid was toxic to all the animals treated, with all the animals in this group failing to survive the 1st day and therefore no brains were analyzed from this group (n=4). With the exception of the 2.5μg.kg^{-1} dose of R-PIA (which was not neuroprotective), the amount of binding was not significantly different from the control or any of the groups treated with combinations of R-PIA and kainate. However, all the effective doses of R-PIA significantly reduced the binding caused after treatment with 10mg.kg^{-1} kainic acid, (see figure 3.7). When R-PIA was injected i.p. (25μg.kg^{-1} and 10μg.kg^{-1}) in the absence of kainic acid injections there was no significant difference in the [^{3}H] PK11195 binding compared with the control values (figure 3.7).

3.4.1.1.2 Time Curve (This was performed in conjunction with W.J. Miller, Department of Pharmacology, the University of Glasgow).

As with the other experiments, clonazepam (0.2 mg.kg^{-1}) was co-administered with kainate 10mgkg^{-1} (t_0), and not with the R-PIA administration, which was injected at times up to 3 hours preceding or following the kainate administration. Injection of R-PIA at 1 or 2 hours before or after the kainate induced a significant ($p \leq 0.001$) protection against the neuronal damage (figure 3.8), whereas administration at the 3 hour time points was ineffective. Protection was greater within 1 hour of kainate injection with the maximal

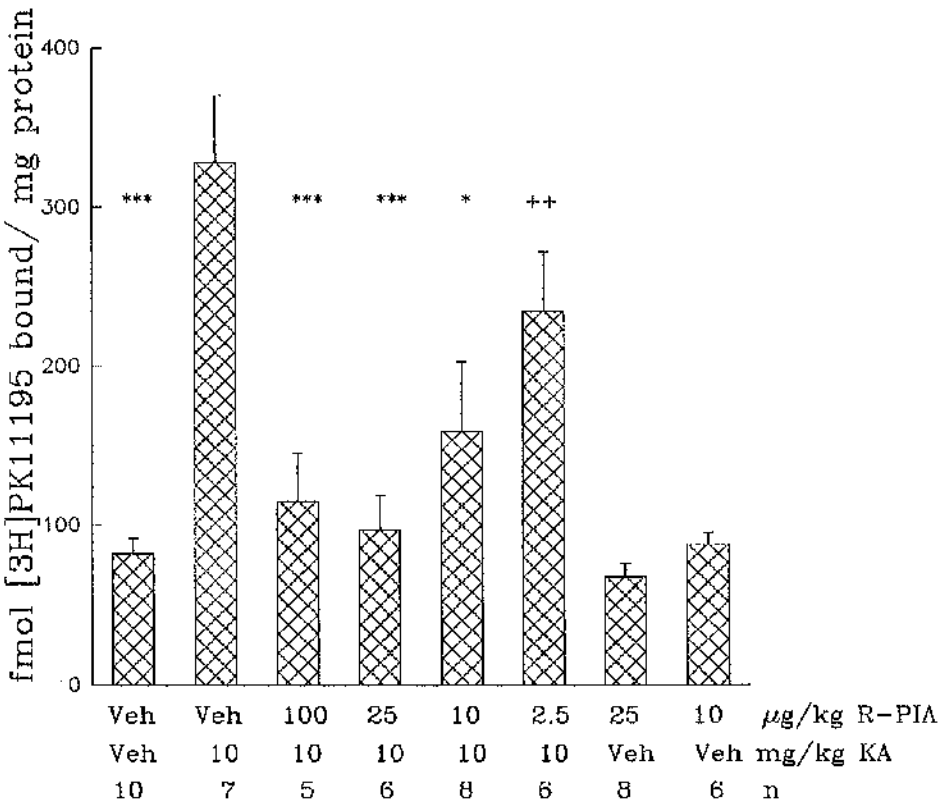


Figure 3.7.

The effect of R-PIA on the kainate evoked increase in [^{3}H] PK11195 binding to hippocampal P_2 membranes.

These experiments were performed without clonazepam

Columns indicate mean ± S.E.M. KA Veh = saline, R-PIA Veh = methanol and saline, KA = kainate.

* $p<0.05$, *** $p<0.001$ significance versus 10 mg kg^{-1} kainic acid. ++ $p\leq0.01$ versus 25$\mu g.kg^{-1}$ R-PIA/10mg.kg^{-1} kainate. Student-Newman-Keuls multiple comparison test.

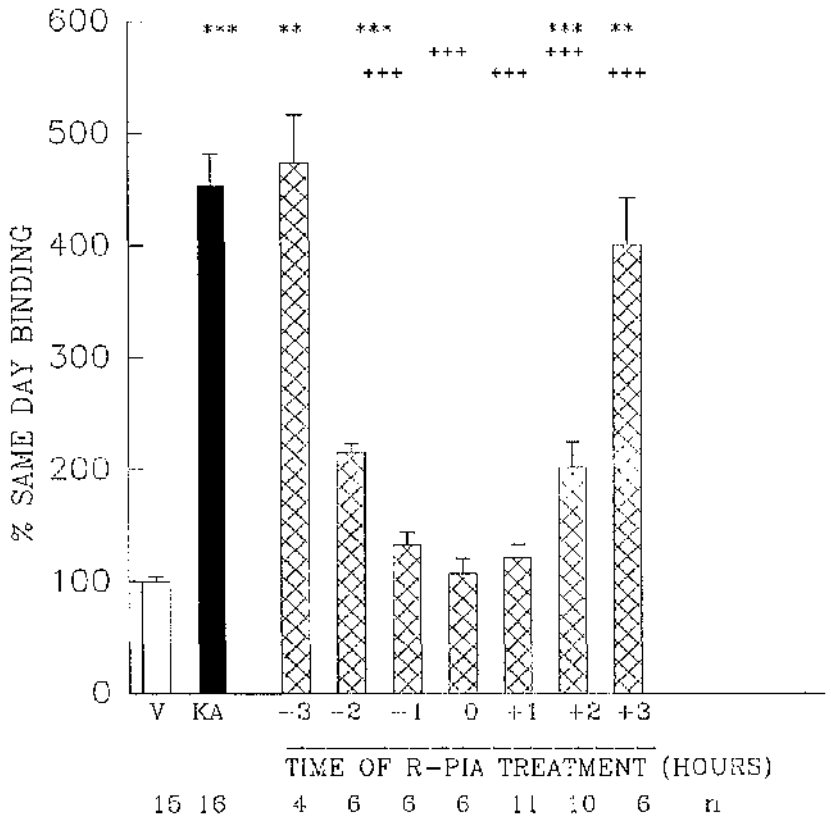


Figure 3.8.

Time course of R-PIA protection against kainate toxicity.

Rats were injected ip with a single dose of 25μg kg^{-1} R-PIA at various times before or after kainate injection. Clonazepam (0.2 mgkg^{-1}) was injected ip 10 minutes prior to ip kainate injection (t_0). Tissue preparation and binding as in methods. Binding as % same day controls.

Columns indicate mean $\pm$ s.e.m.

v = saline and methanol/saline treatment (t_0), KA = kainic acid 10 mgkg^{-1} and methanol/saline treatment (t_0).

** $p \leq 0.01$, *** $p \leq 0.001$ significance versus vehicle.

++ $p \leq 0.01$, +++ $p \leq 0.001$ significance versus 10 mgkg^{-1} kainate.

protection occurring when R-PIA was co-administered with kainate ($p \leq 0.001$), (see figure 3.8).

3.4.1.2 Adenosine A_3 Agonist Injections

The A_1/A_3 agonist APNEA has recently been reported to cause hypotension in anaesthetised rats (Carruthers & Fozard, 1993), although in this study APNEA was administered iv. APNEA was dissolved in 2% DMSO/saline and administered at t_0. Three doses were initially administered; 2,1 and 0.3 $mg.kg^{-1}$, as well as 0.3$mg.kg^{-1}$ APNEA with 50$\mu g.kg^{-1}$ DPCPX. The 2$mg.kg^{-1}$ dose proved to be fatal to all the animals receiving both it and kainate (n=4). The dose of 1$mg.kg^{-1}$ resulted in 6/8 fatalities, whilst the 0.3$mg.kg^{-1}$ dose caused no fatalities. The 0.3$mg.kg^{-1}$ dose resulted in a small but significant reduction in the level of kainate induced binding (200.26 $\pm$ 23.11 fmol.mg $protein^{-1}$ (n=8) compared with the control kainate group of 339.87 $\pm$ 68.34 fmol.mg $protein^{-1}$ (n=5) respectively). This treatment was also significantly different from the vehicle control group, indicating incomplete protection p=0.0179. Treatment with 1$mg.kg^{-1}$ APNEA also resulted in a reduction in the mean value of binding (198.76 and 89.06 (mean 143.91) fmol.mg $protein^{-1}$ (n=2)) compared to the control but this reduction was not significant. Due to the large number of fatalities it was considered unethical to obtain a larger sample number without excessive animal use (figure 3.9). The animals treated with 50$\mu g.kg^{-1}$ DPCPX and 0.3$mg.kg^{-1}$ APNEA, as well as kainate, showed a level of binding that was significantly different from the kainate/APNEA group alone, but not from the kainate group (326.98 $\pm$ 43.29 fmol.mg $protein^{-1}$ (n=5) $p \leq 0.05$ Student's unpaired t-test), figure 3.9).

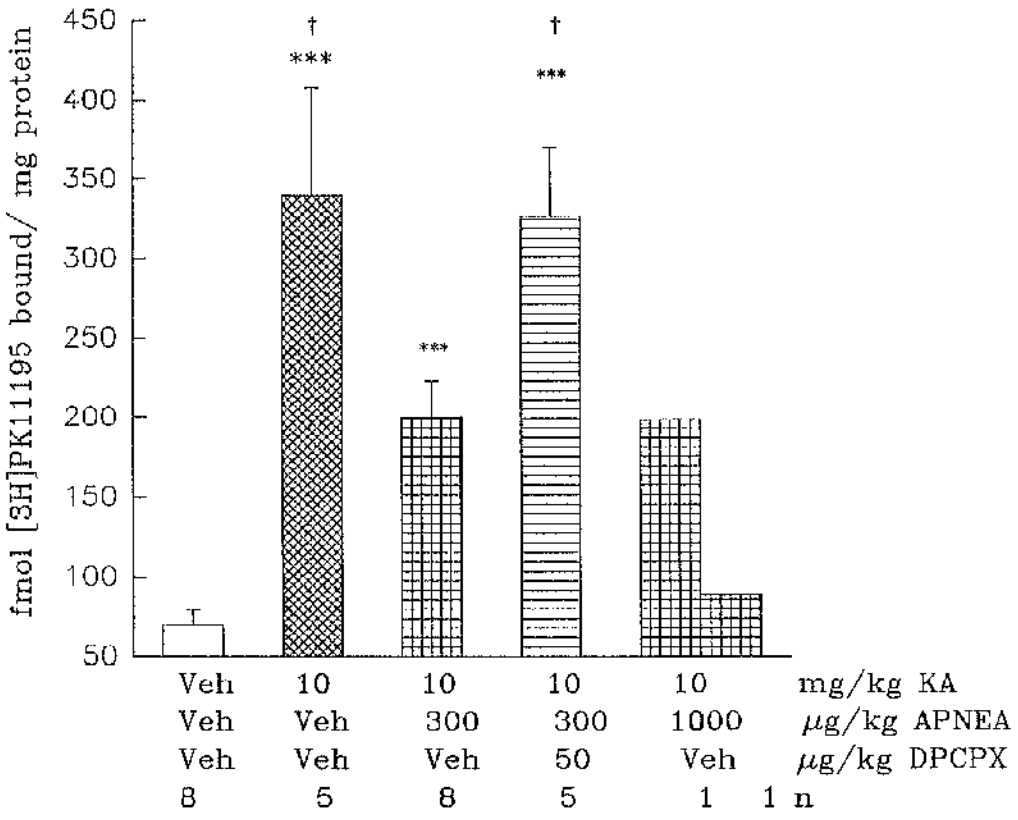


Figure 3.9.

Effect of APNEA on the kainate induced neurotoxicity.

Animals were injected with 0.2mg.kg^{-1} clonazepam 15 minutes before kainate (10mg.kg^{-1}), APNEA or vehicle saline/0.2% DMSO. Animals injected with the vehicle received the same volume as for the 1mg.kg^{-1} APNEA group.

0 = vehicle injected, KA = kainate. Columns = means ± SEM.

*** $p \leq 0.001$ versus control (0 kainate) group,

†† $p \leq 0.01$ versus 0.3mg.kg^{-1} APNEA/kainate group.

3.4.2 Adenosine Antagonist Injections.

3.4.2.1 Non-specific Adenosine Antagonist Injections

The 8-PT vehicle, (ethanol and NaOH) did not significantly alter the level of control or kainic acid binding (figure 3.10). Similarly the vehicle did not significantly reduce the 'protective' action of 25μg/kg R-PIA. The protective action of R-PIA was fully prevented by treatment with 8-PT at the dose of 1mg.kg^{-1} ($p < 0.05$) (see figure 3.10). The kainic acid/8-PT group of rats yielded greater mean levels of binding than that of the kainic acid/vehicles group although the differences were not significant (figure 3.10).

3.4.2.2 Rectal Temperature Experiments

To examine whether any change in body temperature is correlated to final neurotoxicity, a series of experiments was performed using R-PIA $\pm$ 8-PT in kainate treated rats. Contrary to expectations, kainate caused a significant decrease in actual body temperature at the 1 hour time point, but this change was transient and the mean body temperature returned to the starting/normal temperature by the 2 hour time point. In the presence of R-PIA there was no significant change in actual or initial body temperature over the course of the experiment (5 hours). The same was observed for the kainate/R-PIA vehicle/8-PT group, although in this case there was an underlying upward trend over the course of the whole experiment. The kainate/R-PIA/8-PT group displayed a significant decrease in temperature at the 3 hour time point, which returned to normal before being significantly elevated at the 5 hour time point, although the shape of the graph indicates that there may have been an inability to regulate the core temperature, (see figure 3.11).

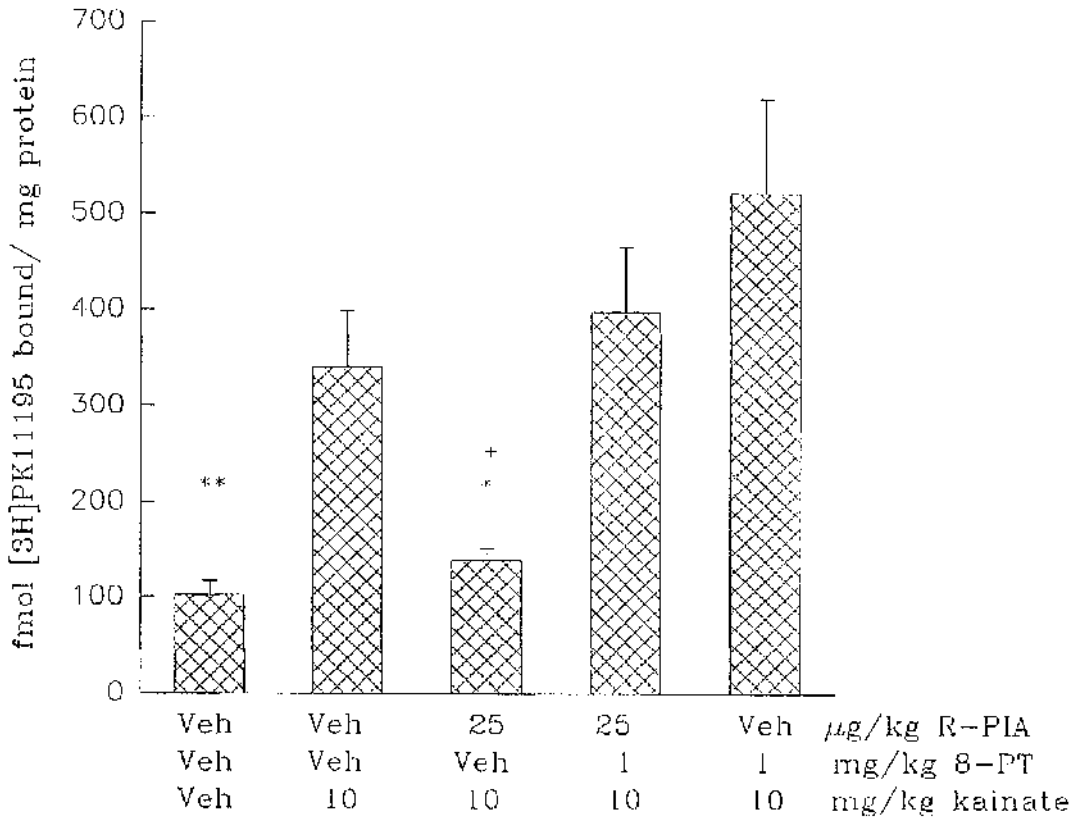


Figure 3.10.

Comparison of the binding of [^{3}H] PK11195 to hippocampal P_2 membranes of KA/8PT treated rats and controls.
Columns indicate mean ± S.E.M.
Veh = vehicle, KA = kainate. * $p<0.05$, ** $p<0.01$ versus kainate group.
+ $p\leq 0.05$ versus kainate/R-PIA/8-PT group.

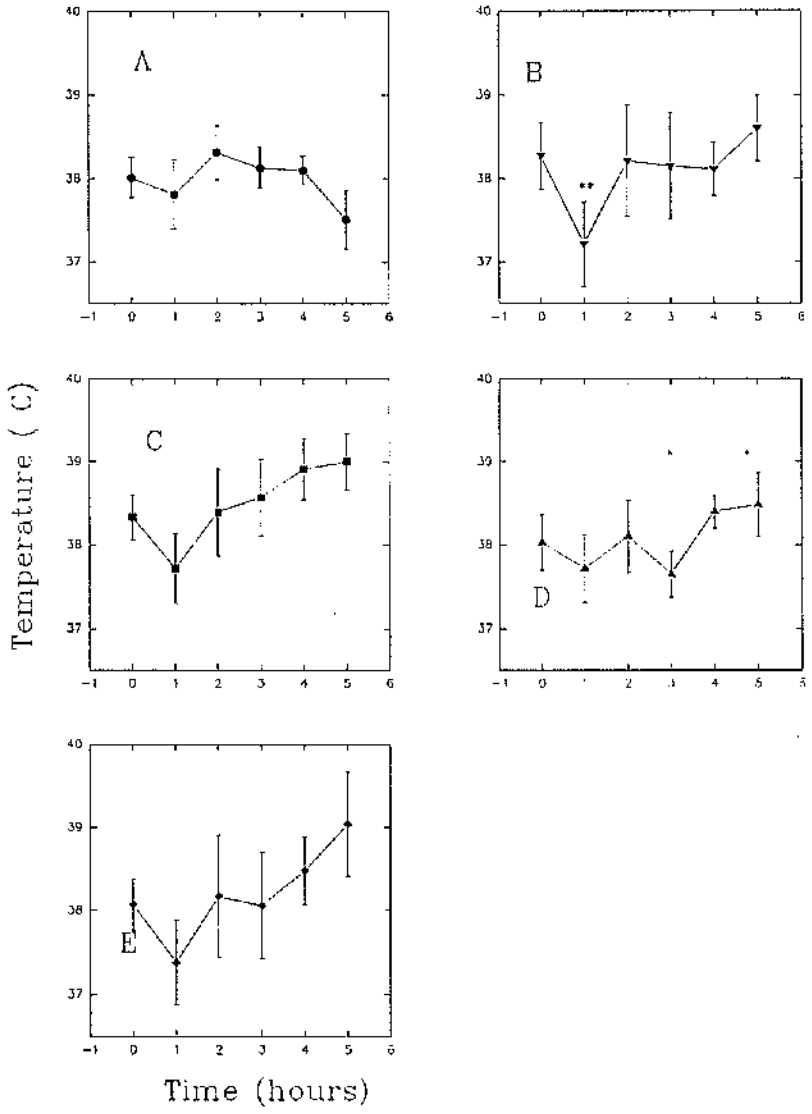


Figure 3.11.

Actual body temperature following I.P. administration of 10 mg.kg^{-1} kainic acid and/or R-PIA/8-PT/vehicles.

a) vehicle treated animals (1mg.kg^{-1} methanol (R-PIA vehicle) and 1 ml.kg^{-1} 8-PT vehicle) (n=7); b) 10 mg.kg^{-1} kainic acid, 1 ml.kg^{-1} methanol and 1 ml.kg^{-1} 8-PT vehicle (n=7); c) kainic acid, 25 μg.kg^{-1} R-PIA,and 8-PT vehicle (n=8); d) kainic acid, R-PIA and 1 mg.kg^{-1} 8-PT (n=10); e) kainic acid, 8-PT and methanol (n=9).

Points indicate mean $\pm$ SEM, students paired t-test.

* $p \leq 0.05$, ** $p \leq 0.01$ versus preceeding time point.

The data were further examined to observe any correlation with the core temperature and binding. There was only significant correlation between temperature and binding at the 5hr time point with increasing core temperature correlating to an increase in [^{3}H] PK11195 binding ($p=0.0191$, $r^2=0.1810$, ($n=41$) Student's t-test), (figure 3.12 a-f). However, there is no correlation between binding and temperature in the groups at 5hrs when examined seperately (data not shown).

If the data are expressed as a change in starting temperature against [^{3}H] PK11195 binding, there is no siginifant correlation, at any of the time points, although the 4hour time point was tending towards significance ($p=0.0738$, $r^2=0.0738$ ($n=41$) figure 3.13).

3.4.2.3 Centrally Active Adenosine A_1 Antagonist Injections.

In the group of animals treated with kainate alone in this series of experiments, the total binding was increased to 300% of control binding (figure 3.14). There was no overall change in the behaviour of the DPCPX treated animals when compared to the kainate treated or kainate/R-PIA treated animals. Animals injected with 250 $\mu g.kg^{-1}$ DPCPX and saline showed no increase in [^{3}H] PK11195 binding when compared to control ($p=0.494$), indicating that there was no induced gliosis or neurodegeneration induced by the antagonist itself.

When DPCPX was co-administered with kainate at doses of 2 to 250$\mu g.kg^{-1}$, in the absence of R-PIA, only the dose of 250$\mu g.kg^{-1}$ resulted in a significant increase in [^{3}H] PK11195 binding (figure 3.14).

However, DPCPX was able to reduce significantly the neuroprotective effects of 25$\mu g.kg^{-1}$ R-PIA at the dose of 10$\mu g.kg^{-1}$, although this level of binding was significantly different from both the vehicle control and the kainate control groups (figure 3.15). At

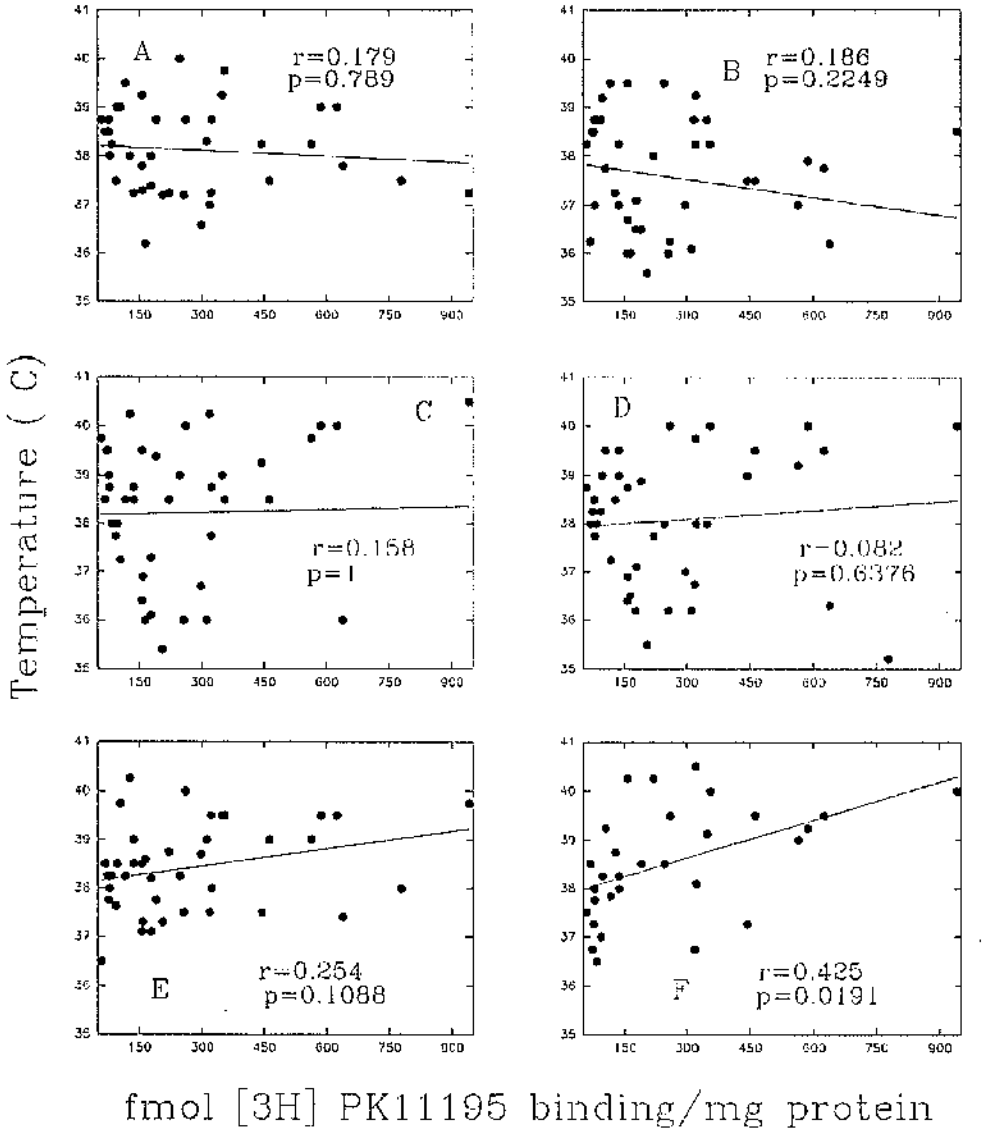


Figure 3.12.

Correlation between actual body temperature and [^{3}H]PK11195 binding in control animals.

The rectal temperature of each animal was plotted against the [^{3}H]PK11195 fmol.mg protein^{-1} binding at each time point. A) starting temperature (t_0 drug injection). B) T_{1hr}, C) T_{2hr}, D) T_{3hr}, E) 4_{hr}, F) 5_{hr}.

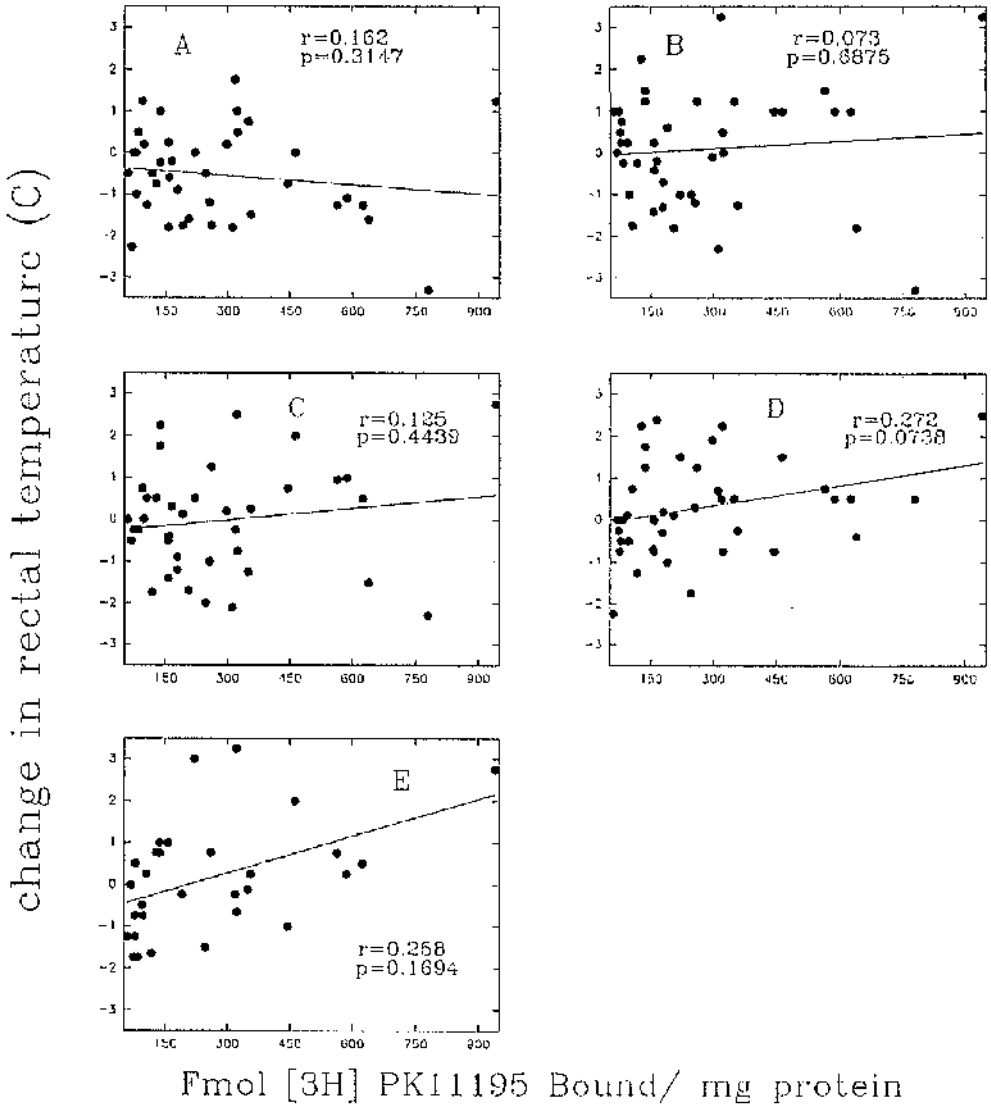


Figure 3.13.

Correlation between change in body temperature and [^{3}H]PK11195 binding in for each injection group (t_1 - $t_5$hours).

The change in rectal temperature of each animal was plotted against the [^{3}H]PK11195 fmol.mg protein^{-1} binding at each time point. the [^{3}H]PK11195 fmol.mg protein^{-1} binding at each time point. (t_0 drug injection). A) T_{1hr}, B) T_{2hr}, C) T_{3hr}, D) 4_{hr}, E) 5_{hr}.

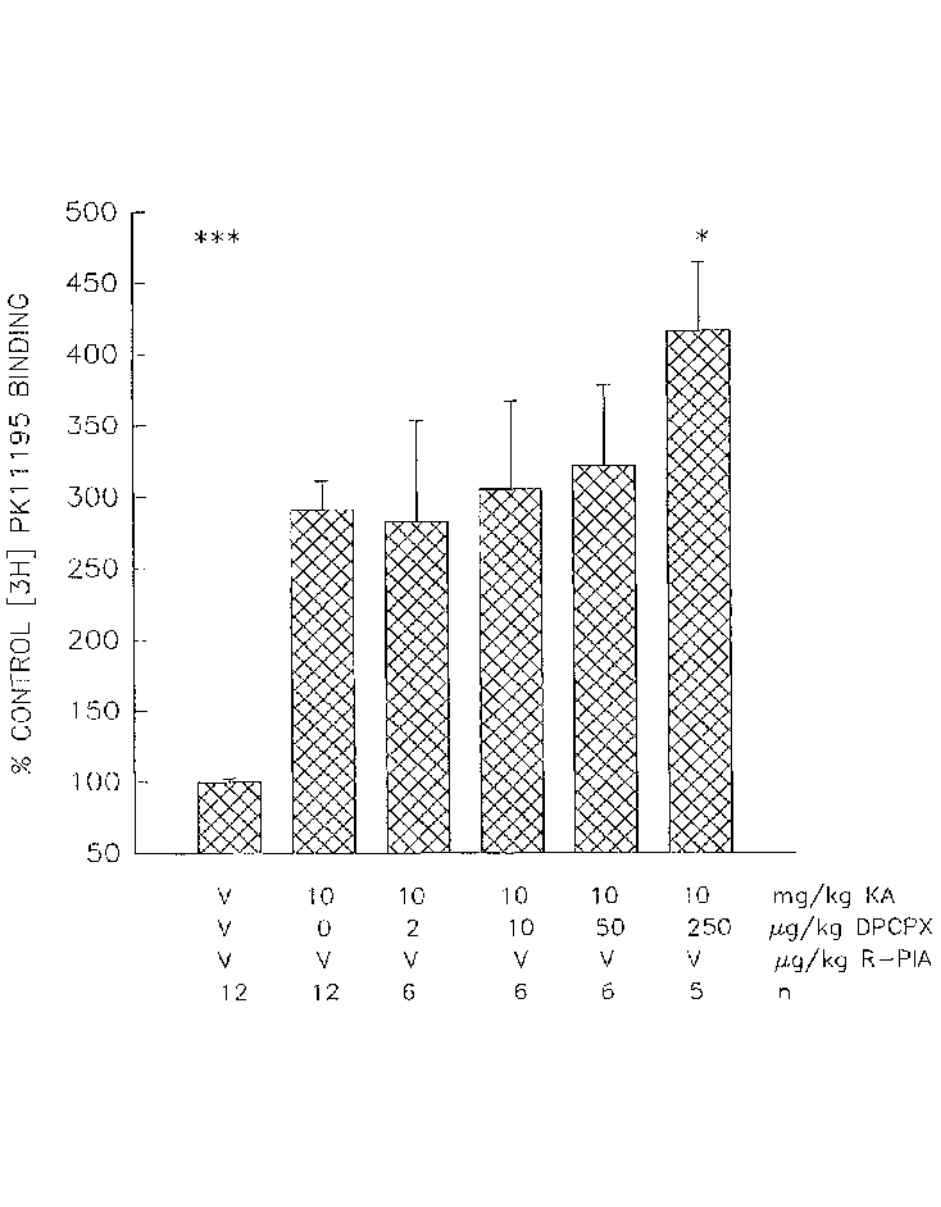


Figure 3.14.

Effect of DPCPX on kainate induced increase of [^{3}H] PK11195 binding.

Rats were pretreated with 0.2 mgkg^{-1} clonazepam 10 minutes prior to kainate (10 mgkg^{-1}) and/or R-PIA/vehicle, DPCPX/saline ip. Binding as in methods. Binding has been calculated as % same day [^{3}H] PK11195 controls. Columns indicate mean ± s.e.m. KA = kainic acid, KA v = saline, R-PIA v = methanol/saline, DPCPX v = saline. * $p \leq 0.05$, *** $p \leq 0.001$ versus vehicles control, Student-Newman-Keuls multiple comparison test.

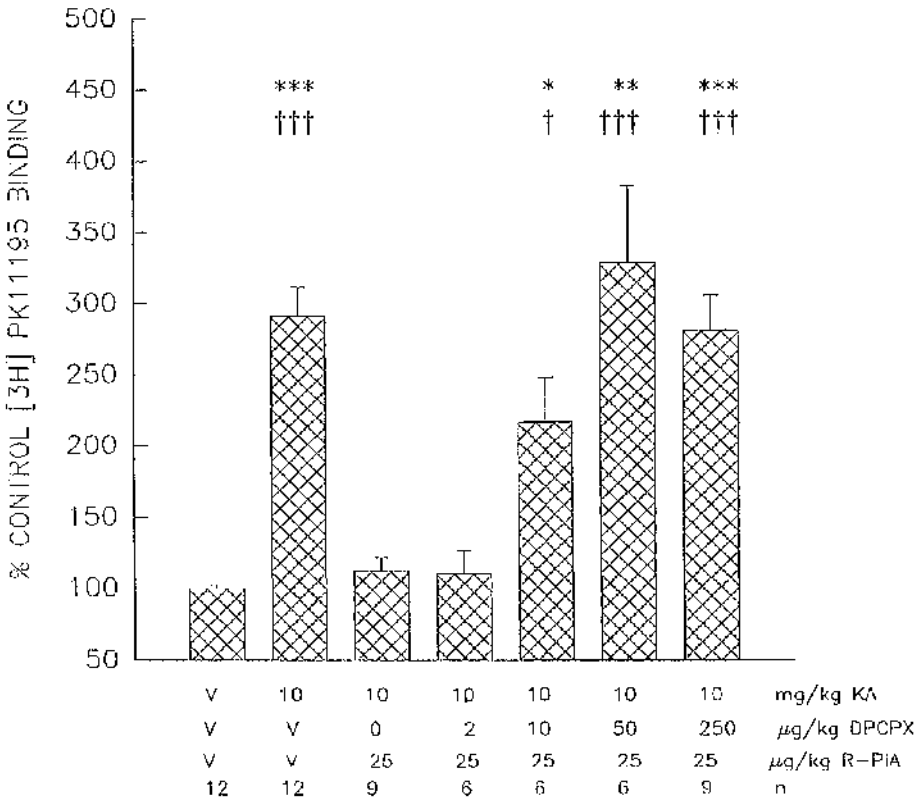


Figure 3.15.

DPCPX dose response curve in kainate/ R-PIA treated rats.

Animals were preinjected with 0.2mg.kg^{-1} clonazepam as per normal, 15 minutes prior to 10mg.kg^{-1} kainate, R-PIA 25μg.kg^{-1} and DPCPX/saline injection. Animals left to recover 7 days before the hippocampi were removed and prepared as P_2 membrane fraction.

Columns indicate means $\pm$ SEM, Student's t-test. Con = vehicles injected, KA= kainate. * $p \leq 0.05$, *** $p \leq 0.001$ versus control. +++ $p \leq 0.001$ versus kainate control. Student-Newman-Keuls multiple comparison test.

higher doses of DPCPX there was complete attenuation of the R-PIA protective action, although there was no difference from the kainate control group, as seen in the experiments without R-PIA (figure 3.14).

3.4.2.4 Peripherally Active Adenosine A_1 Antagonist Injections.

The injection of 20mg.kg^{-1} 8-SPT or 1ml.kg^{-1} saline induced no change in the [^{3}H] PK11195 binding when compared to same day controls ($p=0.638$), though the binding was significantly less than the kainate\saline group ($p<0.001$) (figure 3.16), indicating that there was no gliosis or neuronal damage produced by the xanthine alone. Kainate again produced a 3-fold elevation in [^{3}H] PK11195 binding, which could be prevented by R-PIA at 25μg.kg^{-1}, but the additional presence of 8-SPT at 20mg.kg^{-1} did not prevent that protection. The combined administration of kainate and 8-SPT resulted in a significantly greater degree of binding than obtained with kainate alone (figure 3.16).

3.4.2.5. Comparison of the Potentiation of Kainate Neurotoxicity Induced by Xanthines.

The data from the xanthine experiments were reanalysed, and expressed as percent same day kainate binding, to remove day to day variation. The DPCPX data used were those of 250μg.kg^{-1} (the dose that potentiated kainate toxicity). When the data were expressed in this form, it was found that all the xanthines induced an increase of 140-170% kainate binding (figure 3.17). There was no significant difference between the elevations induced by any of the xanthines.

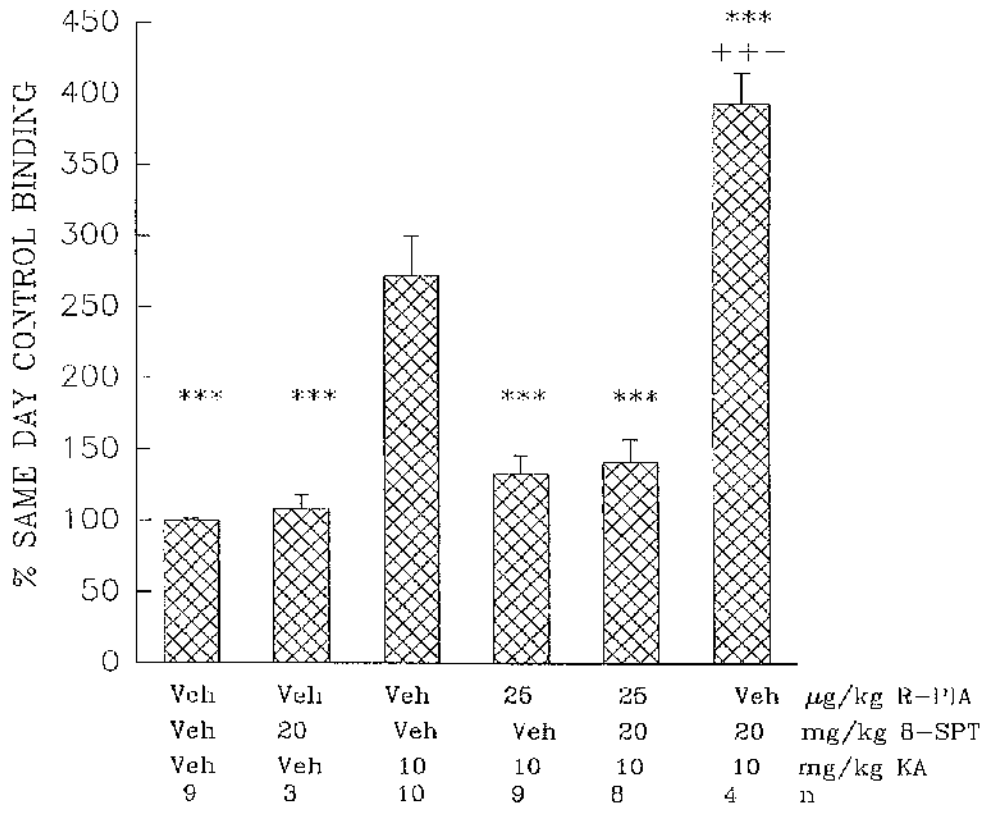


Figure 3.16.
Effect of 8-SPT on kainate evoked increase in [^{3}H] PK11195 binding.

Rats were pretreated with 0.2 mgkg^{-1} clonazepam 10 minutes prior to kainate (10 mgkg^{-1}) and/or R-PIA/vehicle, 8-SPT/saline injection ip. Hippocampal P_2 membranes were prepared as in methods. Binding was calculated as % same day control [^{3}H] PK11195 binding.

Columns indicate mean ± s.e.m. KA veh = saline, KA = kainate, R-PIA veh = methanol/saline, 8-SPT veh = saline.

*** $p < 0.001$ versus kainate control, Student-Newman-Keuls multiple comparison test.

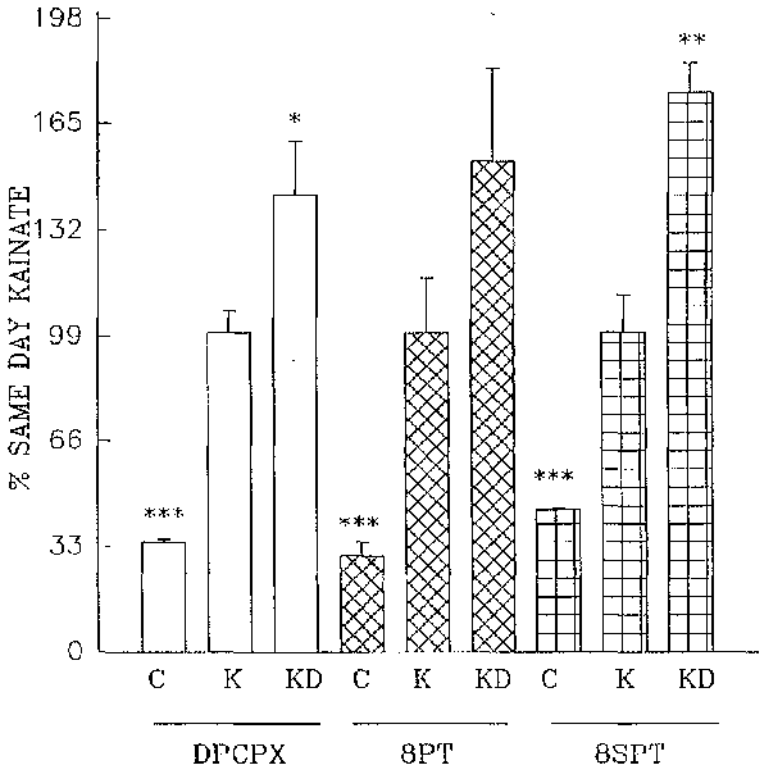


Figure 3.17.

Xanthine attenuation of kainate-induced gliosis.

Data is pooled from the 8-PT, DPCPX and 8-SPT experiments. Data is expressed as same day kainate binding values, and columns represent means ± sem.
C=control, K=kainate control, KD = kainate/adenosine antagonist (drug).
* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ versus same day kainate control group, with experimental groups. Student-Newman-Keuls multiple comparison test.

3.5. NMDA Modulation of Kainate Neurotoxicity.

3.5.1 Exogenous NMDA Antagonists.

3.5.1.1 Non-competitive Antagonists

The non-competitive channel blocking antagonist, MK801 was able to attenuate the kainate evoked increase in [^{3}H] PK11195 binding in a dose dependent manner. At the dose of 3mg.kg^{-1} MK801 was able to protect against the kainate actions significantly, the levels of binding being not significantly different from the control values. The lower dose of 0.3mg.kg^{-1} was also able to reduce the kainate evoked changes, to a lesser extent resulting in a binding level that was significantly different from the kainate but not the vehicle control groups (figure 3.18).

Magnesium sulphate is also a non-competitive channel blocking antagonist of the NMDA receptor complex. At the single dose used here (600mg.kg^{-1}) the animals were markedly sedated and did not display any of the reported behavioural disturbances. After the 7 day recovery period these animals were behaviourally indistinguishable from the vehicle control group. The level of binding in these animals was also similar to that of the vehicle group but was significantly different from the kainate group (figure 3.19).

3.5.1.2 Competitive Antagonist

The competitive antagonist, CPP, was able to reduce the kainate-evoked elevation in [^{3}H]PK11195 binding in a dose dependent manner. Kainate at the dose of 10mg.kg^{-1} was able to elevate the [^{3}H]PK11195 binding from 70.74 $\pm$ 21.37 (control, n=4) to 234.16 $\pm$ 15.43 (n=4) fmol.mg protein^{-1} ($p \leq 0.001$).

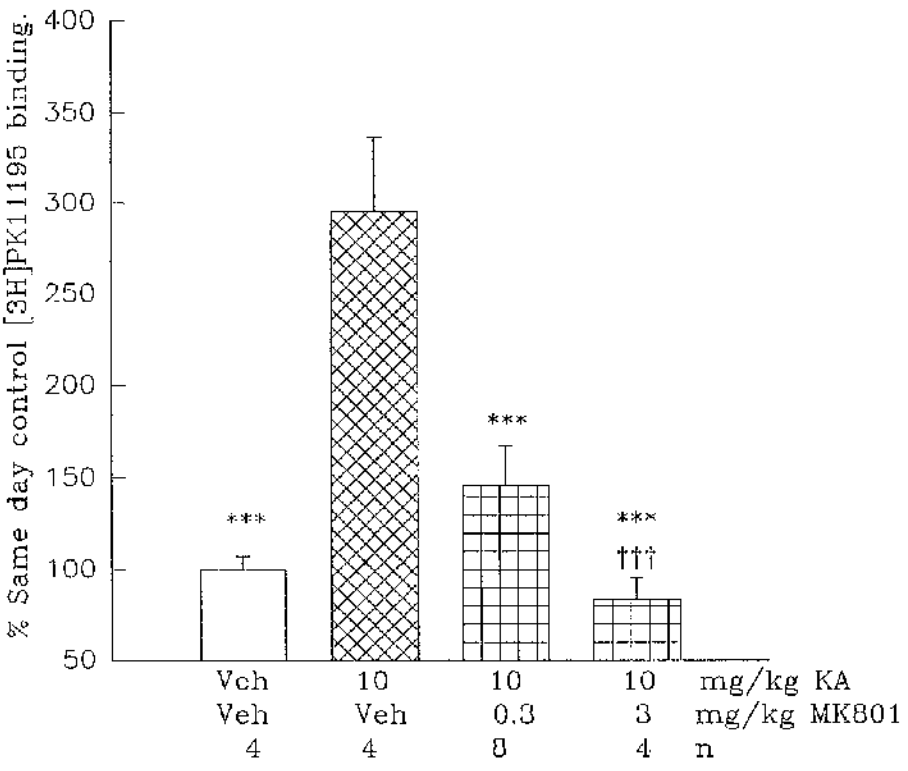


Figure 3.18.

Dose response curve of MK801 attenuation of kainate toxicity.

Animals were injected with 0.2mg.kg^{-1} clonazepam, 15 minutes before kainate and MK801/saline injection. P_2 membranes prepared as in methods.

Columns indicate mean $\pm$ SEM.

Veh = vehicles injected, KA = kainate.

*** $p \leq 0.001$ versus kainate group, ††† $p \leq 0.001$ versus control group. Student-Newman-Keuls multiple comparison test.

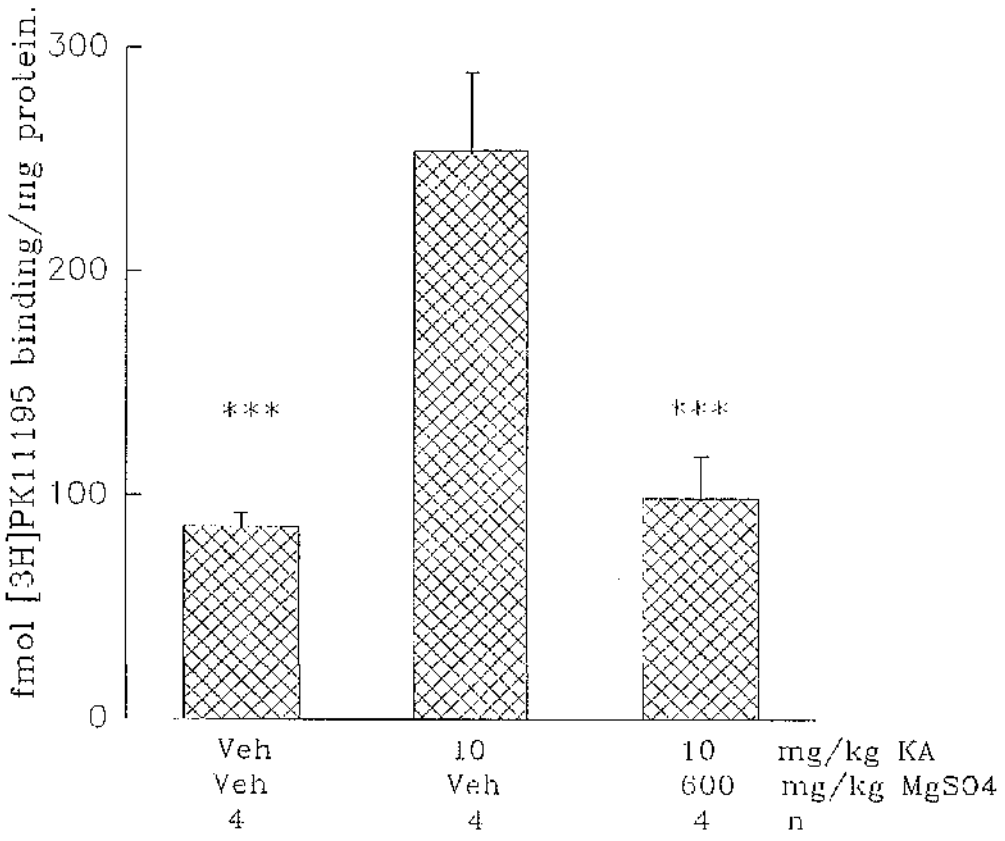


Figure 3.19.

Magnesium attenuation of kainate toxicity.

Animals were injected with 0.2mg.kg^{-1} clonazepam 15 minutes before kainate 10mg.kg^{-1} and magnesium sulphate heptahydrate 600mg.kg^{-1}/saline injection. P_2 membranes prepared as in methods. Columns indicate mean ± SEM.

Veh = saline, KA = kainate.

*** $p \leq 0.001$ versus kainate group. Student's t-test.

When CPP was co-administered, the elevation in binding decreased as the dose of CPP increased, with both 3 and 30mg.kg^{-1} CPP significantly reducing the kainate-induced elevation such that the level of binding was 125.21 $\pm$ 36.49 (n=4) and 95.29 $\pm$ 44.86 fmol.mg protein^{-1} (n=3) (respectively), (figure 3.20).

3.5.2 Endogenous NMDA Antagonists.

Two approaches were used in these experiments, the first was systemic administration of kynurenic acid, and the second was the administration of the precursors tryptophan or kynurenine.

Kynurenic acid 300mg.kg^{-1} administered at t_{-4}, t_0 and t_{+4} failed to alter the kainate induced elevation in binding (311.08 $\pm$ 64.40 (n=4) and 357.73 $\pm$ 40.1 fmol.mg protein^{-1} (n=4) respectively) (figure 3.21) .

One group of animals was treated with a single dose of L-tryptophan 4 hours prior to kainate injection, at the dose of 200mg.kg^{-1}. The mean level of binding of the tryptophan group appeared less than that of the kainate group but, due to the large variation and the small n-number, the two groups were not significantly different (figure 3.22). However, of the four animals injected, 1 died during the course of the recovery, one animal displayed behavioural characteristics similar to kainate treated animals during the recovery period, and the other two were behaviourally normal. Of the 3 surviving animals, the animal which was behaviourally similar to the kainate control gave the highest level of [^{3}H]PK11195 binding whilst the other two gave binding levels in the control range.

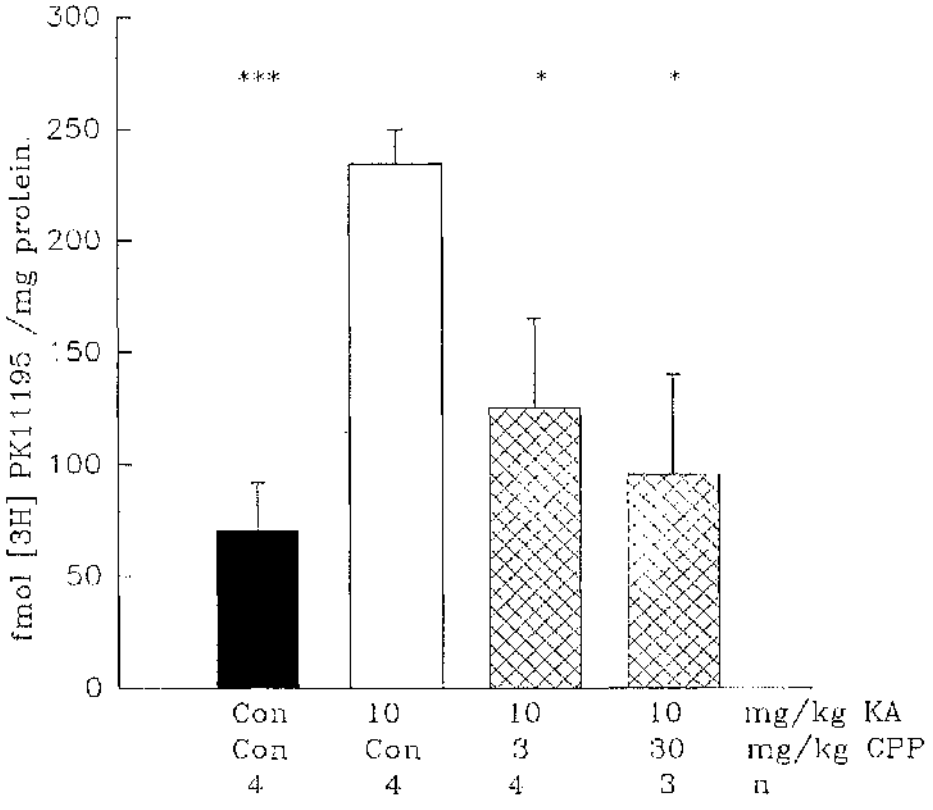


Figure 3.20.

Dose response curve of CPP action on kainate toxicity.

Animals were pretreated with 0.2mg.kg^{-1} clonazepam, 15 minutes prior to 10mg.kg^{-1} kainate administration (t0). CPP or saline was injected at t0. Animals were allowed 7 days to recover before hippocampal P_2 membranes were prepared as in mthods. Columns indicate mean $\pm$ s.e.m.

Con = control (saline injected), KA = kainate.

* $p \leq 0.05$, *** $p \leq 0.001$ versus kainate group, Student's t-test.

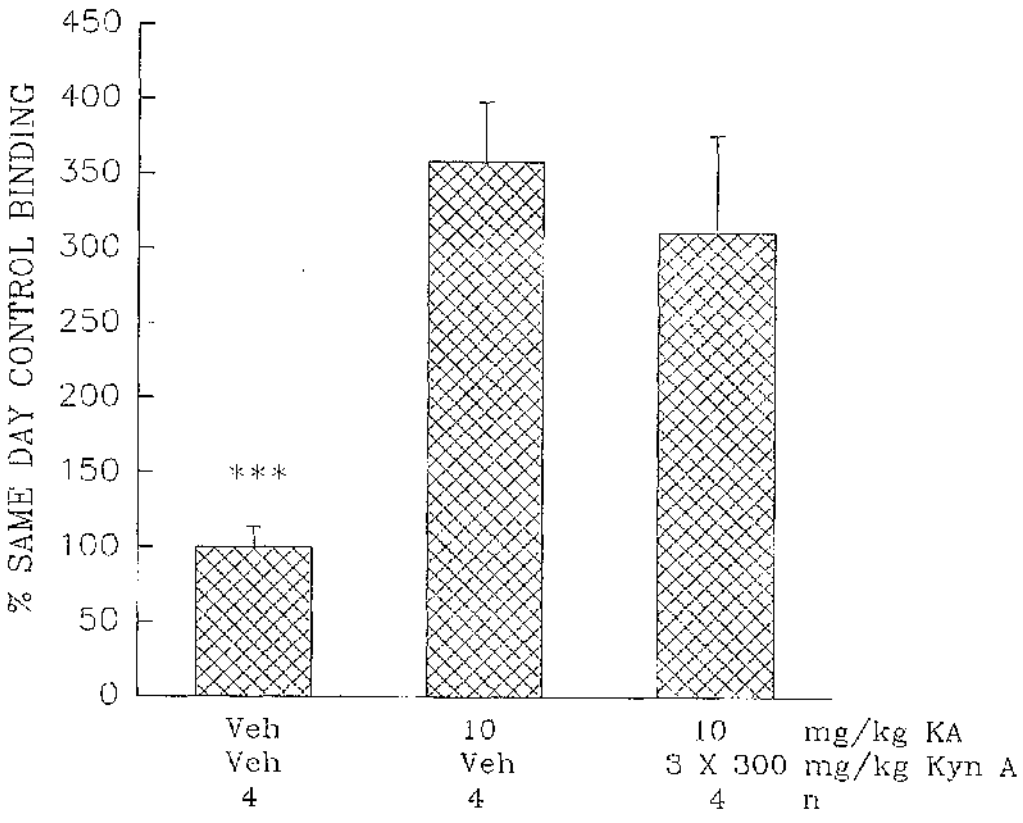


Figure 3.21.

Effect of kynurenic acid treatment on kainate induced toxicity.

Kynurenic acid (300mg.kg^{-1}) was injected at three time points (t_{-4}, t_0 and t_{+4}) in animals injected with kainate 10mg.kg^{-1} (t_0). Columns indicate mean $\pm$ SEM.
KA = kainate, Veh = vehicle injected and KynA = kynurenic acid.

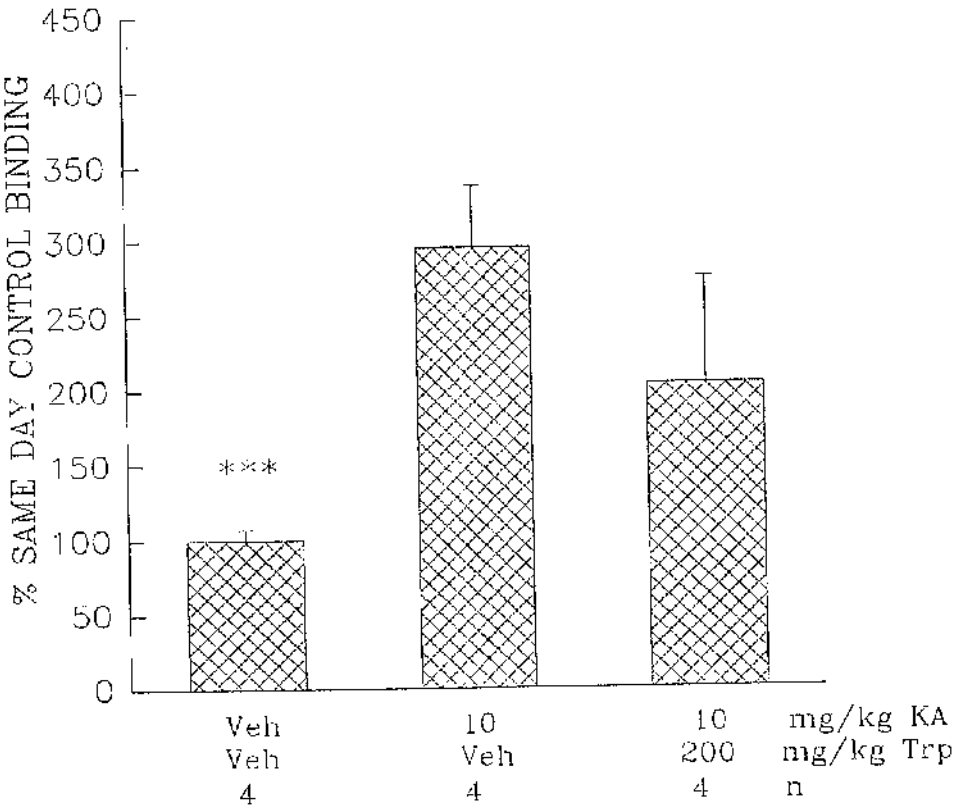


Figure 3.22.

Effect of pretreatment of tryptophan on kainate induced toxicity.

Tryptophan was administered 4 hours prior to 10mg.kg^{-1} kainate injection. Animals were left to recover for 7 days prior to killing and the hippocampi prepared as P_2 membrane fractions as in methods. Columns indicate means $\pm$ SEM.

Veh = vehicles injected, KA = kainate, Trp = tryptophan.

*** $p \leq 0.001$ versus kainate group. Student's t-test.

If the animals were pretreated with L-kynurenine, a slightly different picture was observed. Two time points were used to examine the effect of kynurenine. The initial group of four animals was injected with 150 mg.kg^{-1} L-kynurenine at t_4 hours. As with the L-tryptophan 200mg.kg^{-1} group, one animal died, one was both behaviourally and pharmacologically similar to the kainate group and the other two were similar to the control groups. This resulted in an intermediate level of [^{3}H] PK11195 binding that was not significantly different from either the kainate or the control groups (figure 3.23).

However, if the kynurenine was administered simultaneously with kainate at doses of 30, 75, or 225mg.kg^{-1} a different pattern of binding was observed (figure 3.24). The doses of 75 and 225mg.kg^{-1} produced reasonably consistent binding that was significantly different from the kainate group (figure 3.24). It is interesting to note that at the 225mg.kg^{-1} dose there was no improvement in outcome but there was a smaller variation in the mean than in the 75mg.kg^{-1} group (159.7 $\pm$ 9.75% (n=5) and 150.51 $\pm$ 49.82% (n=5) same day control respectively). The lower dose of 30mg.kg^{-1} (n=5) was not significantly different from the kainate group (231.19 $\pm$ 55.85% same day control (n=4)).

3.6 Modulation of Free Radical Action.

3.6.1 Xanthine Oxidase Inhibition.

The xanthine oxidase inhibitor allopurinol was administered at t_0 in relation to kainate injection. Allopurinol (10mg.ml^{-1}) was prepared in a saline solution containing 2% Tween 80. The control groups received 3.5ml of 2% Tween 80/ saline. All the animals displayed behavioural disturbances similar to those in the kainate control group. Nine animals were injected with a dose of 175mg.kg^{-1} allopurinol, of which six died.

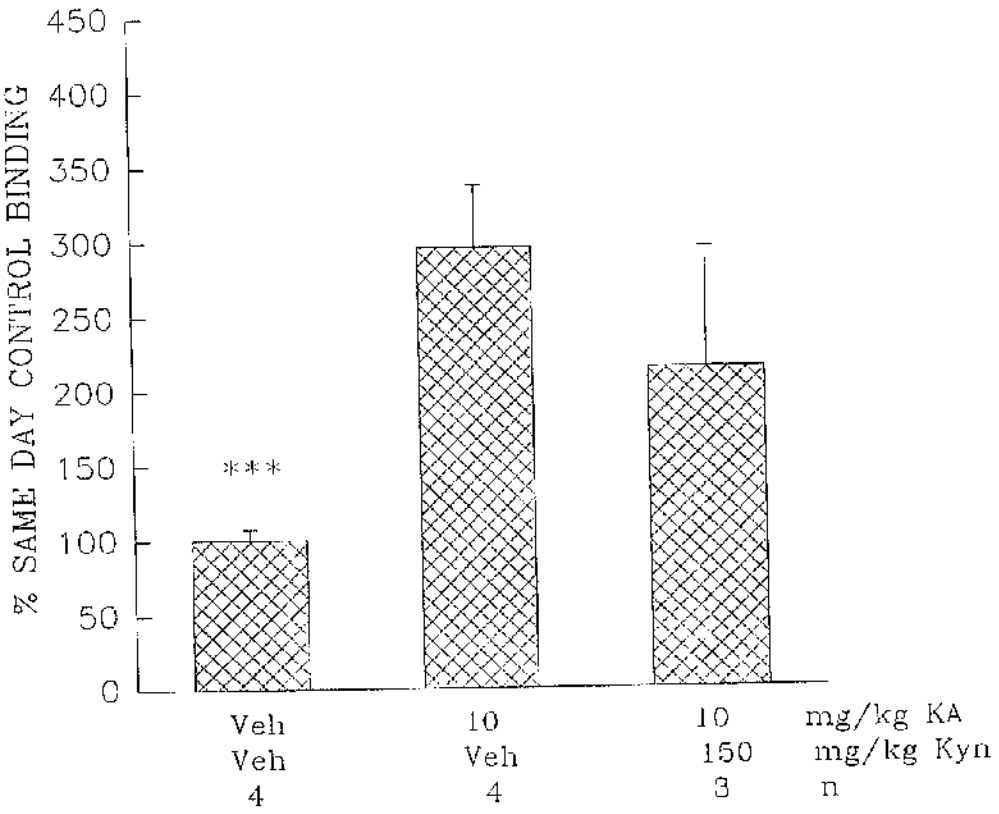


Figure 3.23.

Effect of kynurenine pretreatment on kainate induced damage.

Animals were injected with kynurenine 4 hours prior to kainate 10mg.kg^{-1} injection, and $p_2$membranes prepared as in the methods. Columns indicate means ± SEM. Veh = vehicles injected, KA = kainate, Kyn = kynurenine.

*** $p \leq 0.001$ versus kainate group. Student's t-test.

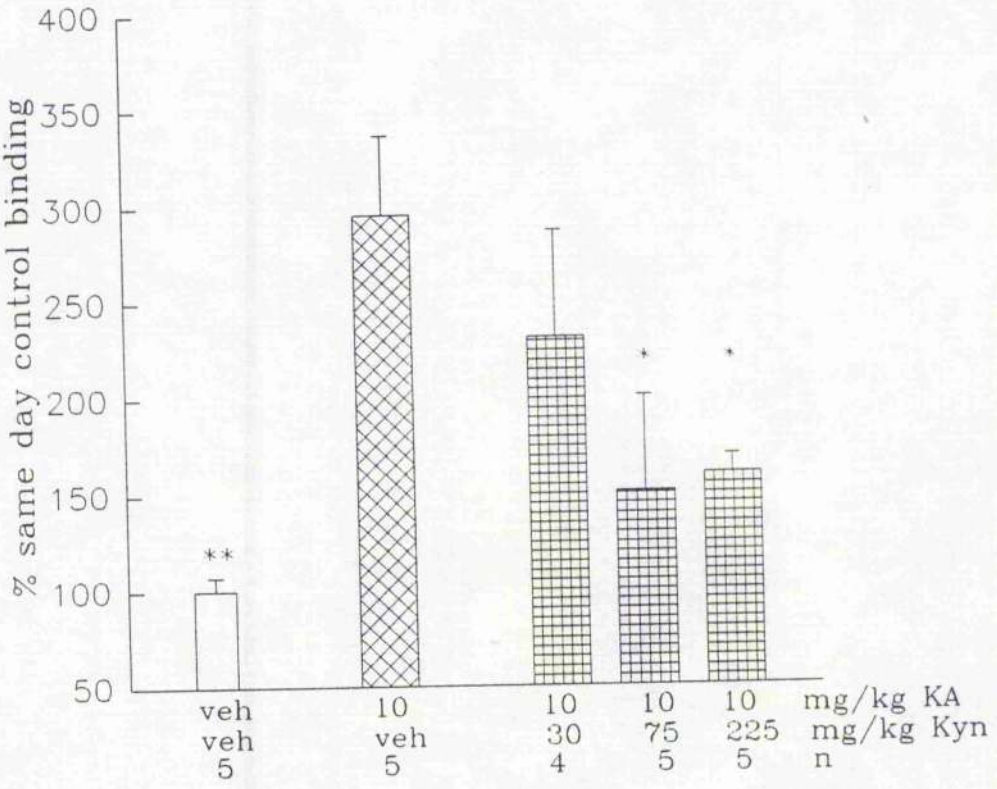


Figure 3.24.

Dose response curve of kynurenine protection against kainate induced neurotoxicity.

Animals were pretreated with 0.2mg.kg^{-1} clonazepam 15 minutes before kainate 10mg.kg^{-1} and kynurenine/vehicle injections. The animals were left to recover for 7 days before the hippocampi were prepared as P_2 membrane fractions as in methods. Columns indicate means ± SEM.

Veh = vehicles injected, KA = kainate, Kyn = kynurenine.

* $p \leq 0.05$, ** $p \leq 0.01$, versus kainate group. Student-Newan-Keuls multiple comparison test.

The time course of the fatalities differed from that of the kainate treatment alone, in that all animals survived the first day, although all the animals were lethargic during the first 4 days of recovery (when the fatalities occurred). The actual cause of death in these animals has not been determined, but is probably due to the intrinsic toxicity of allopurinol itself and not kainate. At a lower dose of 100mg.kg^{-1} there was only one fatality in a group of six rats and the dose of 17.5mg.kg^{-1} produced no fatalities in the five rats treated.

The level of binding mirrored the fatalities in that the 17.5 or 100mg.kg^{-1} groups were not significantly different from the kainate group whilst the 175mg.kg^{-1} group was significantly different (281.03 $\pm$ 23.9 (n=6), 287.06 $\pm$ 33.31 (n=5) and 125.46 $\pm$ 21.69 fmol.mg protein^{-1}, $p \leq 0.001$, (n=3) respectively figure 3.25).

3.6.2 Ascorbate Administration.

3.6.2.1 Ascorbate Dose Response Curve.

The systemic kainate administration caused a significant ($p \leq 0.001$) increase in [^{3}H]PK11195 binding to hippocampal P_2 membranes (250.95 $\pm$ 22.89 fmol.mg protein^{-1} (n=6)) compared to the saline control (81.5 $\pm$ 5.11 fmol.mg^{-1} protein (n=8)). Ascorbate, administered at t_0 produced a dose-dependent reduction in the kainate induced [^{3}H] PK11195 binding with an estimated EC_{50} of 12.9mg.kg^{-1}, and maximal protection at 50mg.kg^{-1} (figure 3.26). The ascorbate gave a significant reduction in [^{3}H]PK11195 binding at 30, 50 and 100 mg.kg^{-1}. As with other drug treatments there was no apparent difference in the behaviour of the ascorbate treated and kainate treated animals during the first 6 hours after kainate administration.

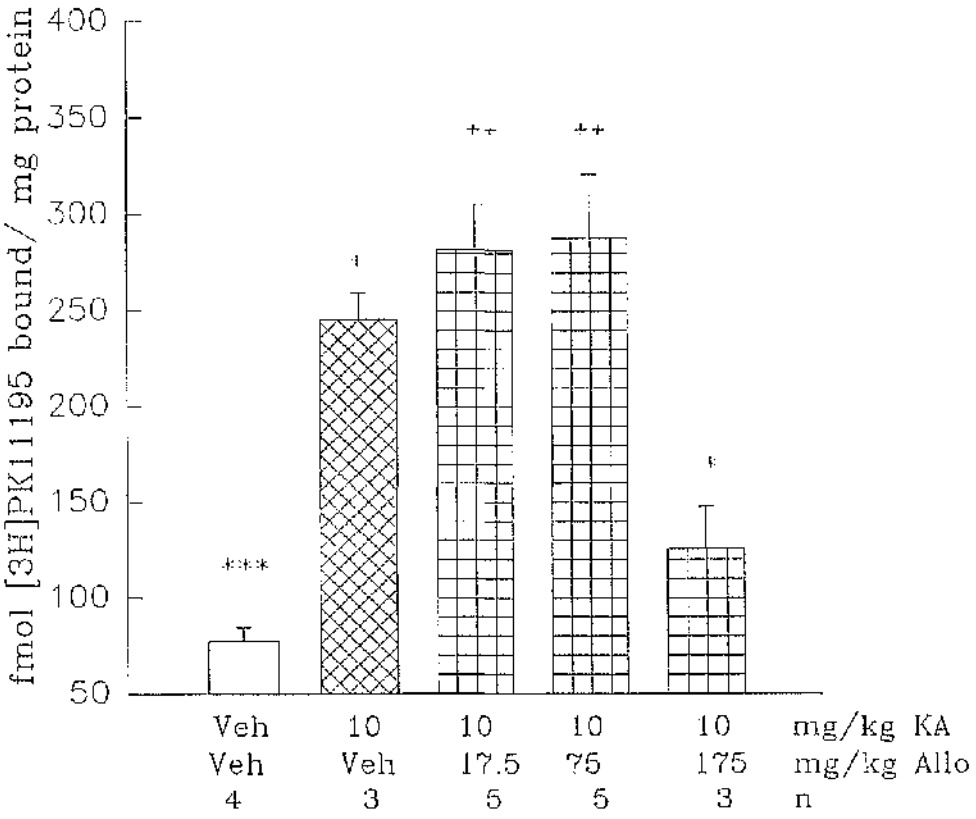


Figure 3.25.

Effect of increasing dose of allopurinol on kainate toxicity.

Animals were injected with clonazepam (0.2mg.kg^{-1}) 15 minutes before kainate 10mg.kg^{-1}. P_2 membranes prepared as in methods. Columns indicate mean ± SEM. Student's t-test.

Veh = vehicles injected, KA = kainate. ALLO = allopurinol.

* $p \leq 0.05$, *** $p \leq 0.001$ versus kainate group. + $p \leq 0.05$, ++ $p \leq 0.01$ versus kainate/allopurinol 175mg.kg^{-1} group. Student's t-test.

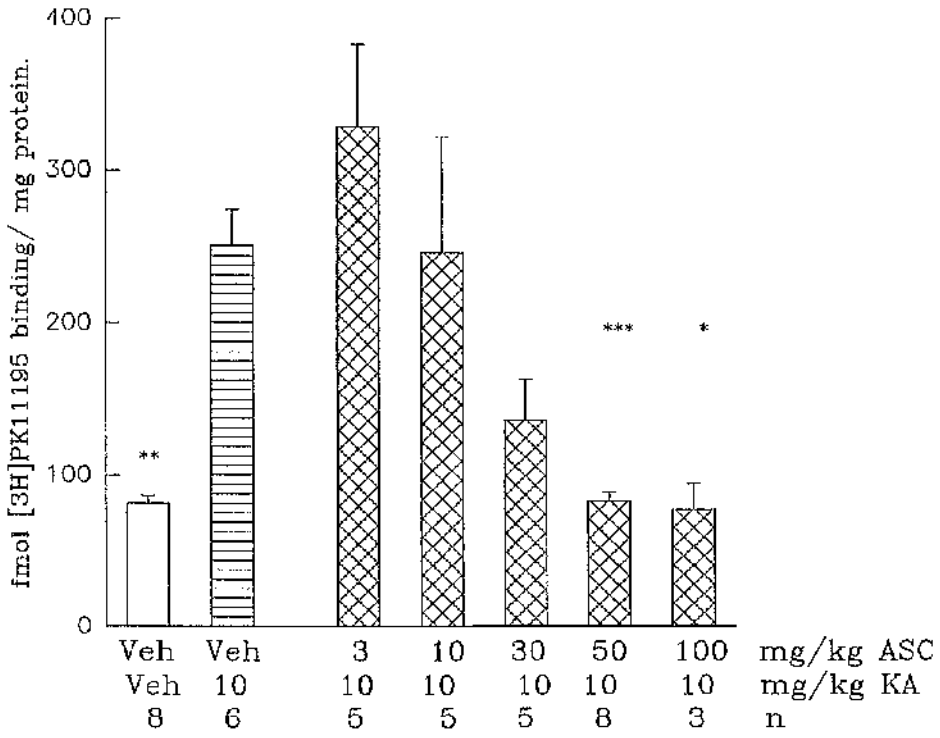


Figure 3.26.

Dose response curve of ascorbate action on kainate toxicity.

Animals were injected with 0.2mg.kg^{-1} clonazepam 15 minutes before kainate 10mg.kg^{-1} and ascorbate/saline injection. P_2 membranes prepared as in methods. Columns indicate mean $\pm$ SEM.

Veh = vehicles injected, KA = kainate. ASC = ascorbate

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ versus kainate group,

Student-Newman-Keuls multiple comparison test.

3.6.2.2 Time Course of Ascorbate Neuroprotection.

In the time course experiments ascorbate 50mg.kg^{-1} was administered as a single injection either before or after kainate injection (t_0). Ascorbate only gave significant protection when administered 1 hour before, or simultaneously with, kainate injection (143.14 ± 22.51 (n=8) p = 0.036, and 85.45 ± 10.64 fmol.mg protein^{-1} (n=8) p=0.002, respectively), but not when given 2 hours before kainate (197.77 ± 45.89 fmol.mg protein^{-1} (n=6) p=0.573) (figure 3.27). Ascorbate treatment 1 hour after kainate administration resulted in an intermediate level of [^{3}H]PK11195 binding but this was not significantly different from kainate treatment (175.20 ± 38.71 (n=6) and 229.51 ± 28.88 fmol.mg protein^{-1} (n=7) respectively p=0.287).

3.7 Modulation of the Gabaergic System.

The anticonvulsant, chlormethiazole (CMZ), has been reported to reduce neurotoxicity in several ischaemic models (Cross *et al.*, 1991), and was tested in the present system. CMZ was dissolved in saline and administered simultaneously with 10mg.kg^{-1} kainate ip. CMZ administration resulted in sedation of the animal, the duration depending on the dose, but once the animals had recovered they displayed classical kainate induced behavioural disturbances, without any observed clonic-tonic seizures.

CMZ was found to attenuate the kainate induced elevation in [^{3}H] PK11195 binding, in a dose dependent manner. Binding after the lowest dose 10mg.kg^{-1} was not significantly different from the kainate group, but was from the control group ($p \leq 0.01$),(173 ± 14.70 (n=3), 260 ± 28.91 (n=4) and 77.51 ± 8.23 (n=4), respectively), (figure 3.28). Binding after treatment with 30mg.kg^{-1} CMZ (118.80 ± 26.78 fmol.mg protein^{-1} (n=4) $p \leq 0.05$) was significantly different from kainate control,

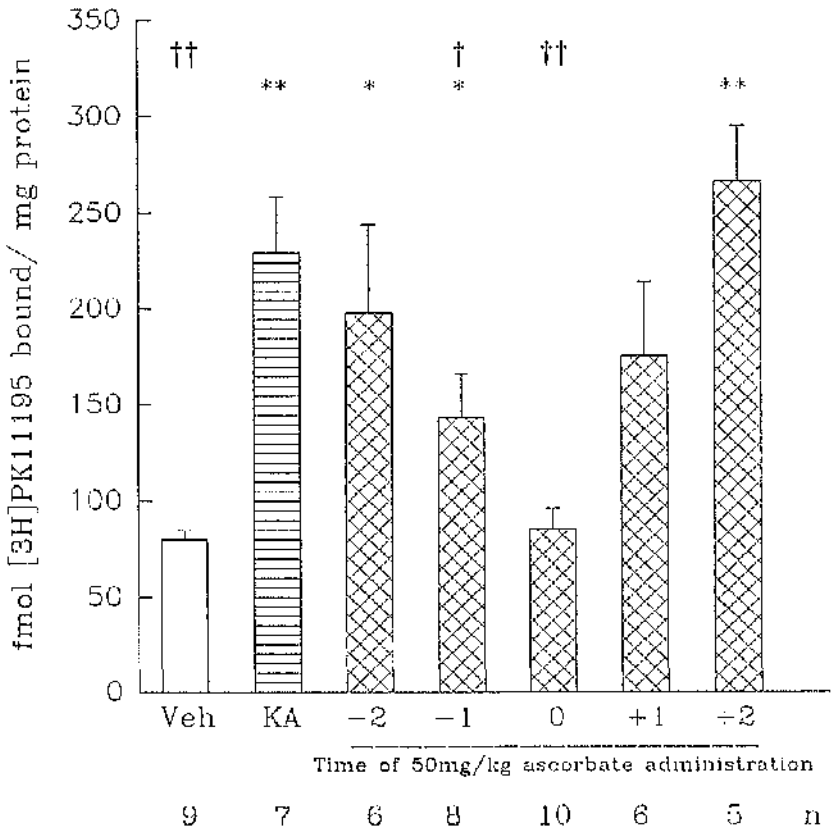


Figure 3.27.

Time course of ascorbate action.

Ascorbate (50mg.kg^{-1}) was injected as a single dose either before or after kainate administration. Clonazepam (0.2mg.kg^{-1}) was administered 15 minutes before kainate 10mg.kg^{-1}. P_2 membranes prepared as in methods. Columns indicate mean ± SEM. Student's t-test. Veh = vehicles injected, KA = kainate. ASC = ascorbate.

+ $p \leq 0.05$, ++ $p \leq 0.01$ versus kainate group.

* $p \leq 0.05$, ** $p \leq 0.01$ versus control group.

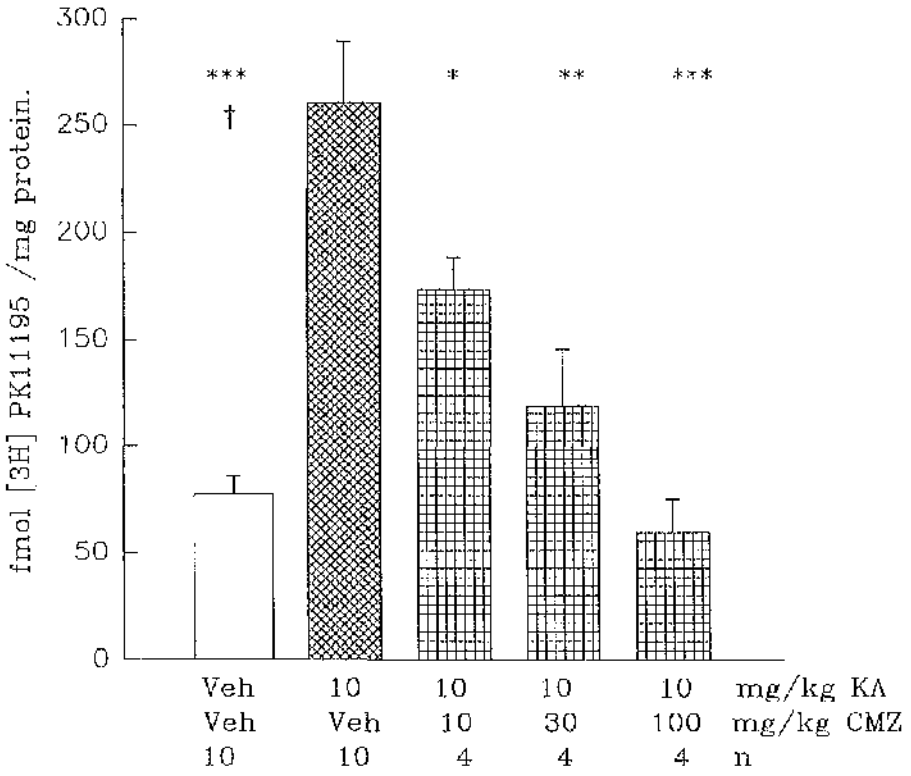


Figure 3.28.

Dose response curve of chlormethiazole induced neuroprotection.

Animals were pretreated with 0.2mg.kg^{-1} clonazepam 10 minutes prior to 10mg.kg^{-1} kainate treatment (t_0). Chlormethiazole or saline (1ml.kg^{-1}) were administered at t_0. Animals were left to recover for 7 days before hippocampal P_2 membranes were prepared as in methods. Columns indicate mean ± s.e.m.

CMZ = chlormethiazole, CON = control (saline) injections, KA= kainate .

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ versus kainate control group.

† $p \leq 0.05$ versus 10mg.kg^{-1} CMZ/KA group, Student-Newman-Keuls multiple comparison test.

whilst after 100mg.kg^{-1} CMZ binding (59.35 $\leq$ 15.69 fmol.mg protein^{-1} (n=4)) was significantly different from both the kainate control and the kainate/10mg.kg^{-1} CMZ groups ($p \leq 0.01$ and $p \leq 0.05$ respectively).

4.0 RESULTS - GLUTAMATE RELEASE EXPERIMENTS

4.1 Endogenous Glutamate Release

Due to unforseen complications with the HPLC fluorimeter, basal levels of aspartate, glutamate or GABA did not register, the peaks being lost in the base line noise. Whilst there were some indications of stimulated release, the peaks were small and non reproducible. To try and overcome the problem of small amounts of amines, samples were concentrated 50 times, by using a vacuum centrifugation step (Gyrovap). Whilst this step gave reproducible standard concentrations, it was unable to concentrate the experimental samples to a concentration such that reproducibility in peaks or a better signal:noise ratio for the basal release was achieved (no results presented).

4.2 Exogenous Glutamate Release.

4.2.1 Potassium Stimulated Release.

High potassium ACSF was able to stimulate [^{3}H] glutamate release from the hippocampal slice to release when containing either 25 or 50mM potassium (figure 4.1 and 4.2, respectively). The stimulations were significantly different from the basal release (40-58 minutes) with a significance level of $p \leq 0.001$. The release was significantly different from basal for 7 fractions after S1 ($p \leq 0.05$), but only 2 after S2 ($p \leq 0.05$) and S3 ($p \leq 0.05$). The extended period of significance following the initial S1 peak was termed the 'Hump' and the release was calculated as the total area under this hump, but this was not significant between 25 and 50mM KCl (table 4.1).

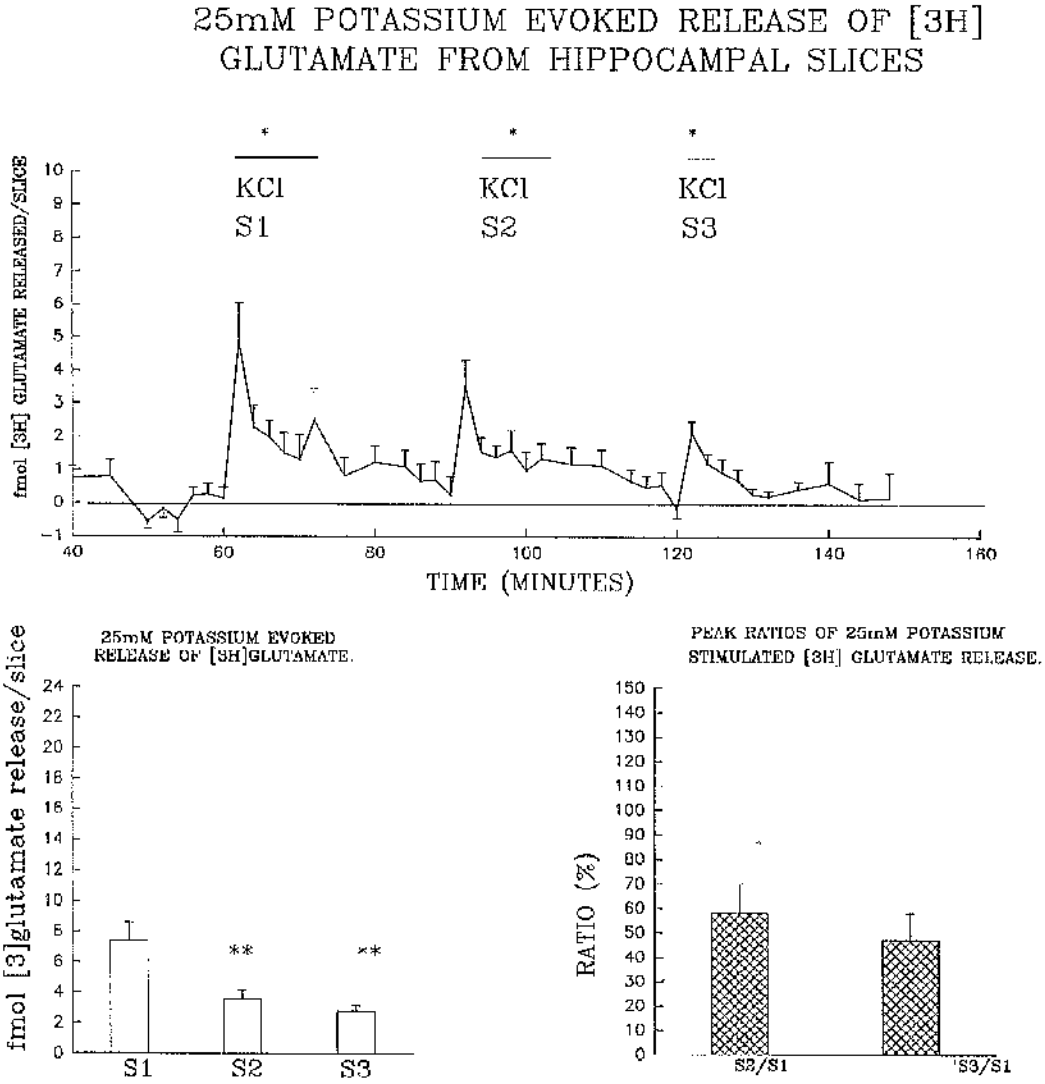


Figure 4.1.

25mM KCl stimulated [^{3}H] glutamate release from hippocampal slices. A) release above predicted basal release (see methods) n=11, * $p \leq 0.05$ versus basal noise (see methods). B) Total release during stimulation, columns indicate mean ± sem. ** $p \leq 0.01$ versus S1 (Mann Whitney U-test). C) Ratio of total stimulated release.

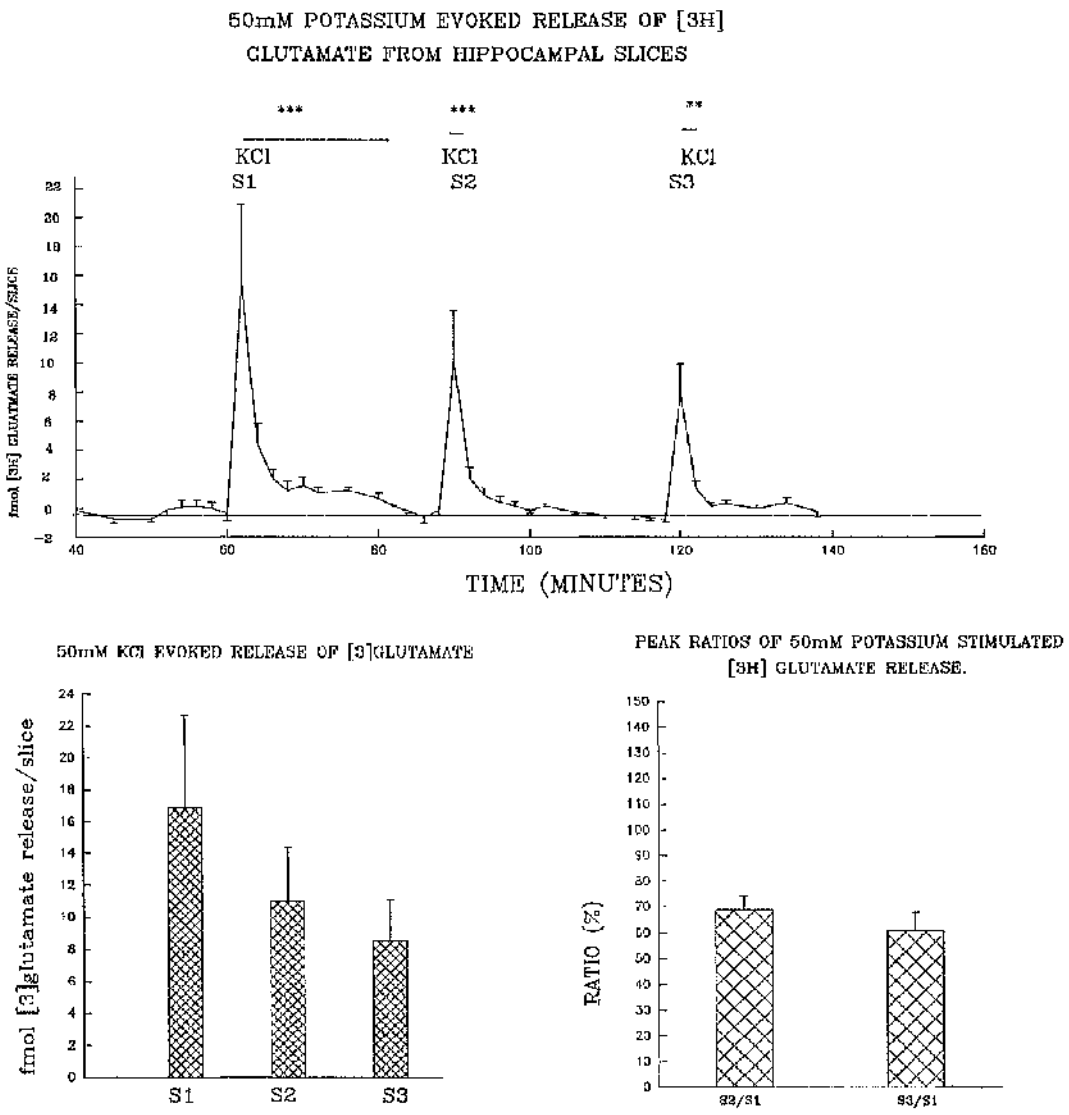


Figure 4.2.

50mM KCl stimulated [^{3}H] glutamate release from hippocampal slices. A) release above predicted basal release (see methods) n=9, ** $p \leq 0.01$, *** $p \leq 0.001$ versus basal noise (see methods) Mann Whitney U-test. Points indicate mean $\pm$ sem. B) Total release during stimulation, columns indicate mean $\pm$ sem. C) Ratio of total stimulated release.

Table 4.1. Comparison of kainate and KCl on stimulated glutamate release.

TYPE / PEAK	S1 (test)	S2 (test)	S3 (KCl)
50mM KCl	16.9 ± 5.8	11.0 ± 3.4	8.6 ± 2.6
25mM KCl	7.4 ± 1.2[a]	3.5 ± 0.6[a]	2.8 ± 0.4[b]
5/25mM KA/KCl	8.6 ± 2.0	4.5 ± 0.8	2.8 ± 0.7[a]
5mM KA	11.5 ± 2.7	15.4 ± 7.2[df]	35.2 ± 10.0[bdf]
1mM KA	3.7 ± 0.4[bdeg]	3.2 ± 0.6[bh]	9.4 ± 2.1[bdfh]

Legend: The figures correspond to total release fmol/slice at each stimulus mean ± sem. The data were obtained as in Methods.
KCl 50mM (n=9), KCl 25mM (n=11), KA/KCl 5/25mM (n=10), KA 5mM (n=6) and KA 1mM (n=10). KA = kainic acid.
[a] $p \leq 0.05$, [b] $p \leq 0.01$, versus same column 50mM KCl
[d] $p \leq 0.01$, versus same column 25mM KCl
[e] $p \leq 0.05$, [f] $p \leq 0.01$ versus same column 5/25mM KA/KCl
[g] $p \leq 0.05$, [h] $p \leq 0.01$ versus same column 5 KA
(Mann Whitney U-test).

There was an apparent dose dependency of the release with 50mM KCl releasing 16.93 ± 5.77 fmol/slice (S1, n=9) and 25mM KCl releasing (7.42 ± 1.22, fmol/slice S1, n=6) although this was not significant (except S3 $p \leq 0.05$, Mann Whitney U-test), due to the variability of the slices (table 4.1). Both concentrations, though, resulted in a decreased release with successive stimulations (S1 > S2 > S3), and the degree of decrease was similar for both doses (table 4.2).

4.2.2. Kainate Evoked Release.

Like KCl, kainate was able to evoke [^{3}H] glutamate release from the hippocampal slice in a dose dependent manner. However, unlike the KCl the area of heightened release after S1 was marked, and prolonged (lasting for 30 minutes in both cases and significantly different from basal, $p \leq 0.05$ for both 1 and 5mM). After the second stimulation (S2) there was no long lasting period of heightened release (lasting only 3 (1mM) and 5 (5mM)

fractions). If the hump (H1) is compared against the KCl humps, the 5mM kainate H1 is significantly different from the humps following both KCl concentrations ($p \leq 0.05$), but the 1mM kainate hump was not significantly different from these or the 5mM kainate H1 (table 4.3). In both experiments there was no period of heightened release after S3 (50mM KCl).

1mM kainate caused a small release (3.71 $\pm$ 0.45 fmol/slice S1, n=6), with a slightly smaller S2 release (3.21 $\pm$ 0.62 fmol/slice) (figure 4.3.). The S1 and S2 values were not significantly different but both were different from the S3 values ($p \leq 0.01$).

Table 4.2. Comparison of the effect of kainate or KCl on the evoked release ratios.

GROUP/PEAK	S2/S1	S3/S1	S3/S2
50mM KCl	69 $\pm$ 5	61 $\pm$ 7	90 $\pm$ 11
25mM KCl	58 $\pm$ 12[a]	47 $\pm$ 11[a]	99 $\pm$ 21
5/25mM KA/KCl	72 $\pm$ 19	51 $\pm$ 14	68 $\pm$ 22
5mM KA	138 $\pm$ 44[c]	341 $\pm$ 71[bdc]	318 $\pm$ 73[dc]
1mM KA	90 $\pm$ 17	318 $\pm$ 41[bdc]	553 $\pm$ 179[dc]

Legend: Values are the percentage of Sx/Sy, as calculated in Methods, and are mean $\pm$ sem. KA =kainic acid. 5omM KCl, n=9; 25mM KCl, n=11; 5/25mM KA/KCl n=10; 5mM KA, n=6 and 1mM KA, n=10.
[a] $p<0.05$, [b] $p<0.01$ versus same column 50mM KCl;
[c] $p<0.01$, [d] $p \leq 0.001$ versus same column 25mM KCl;
[e] $p<0.01$ versus same column 5/25mM KA/KCl (Mann Whitney U-test).

5mM kainate also caused release from the slices (11.52 $\pm$ 2.7 and 15.37 $\pm$ 7.21 fmol/slice (n=6) S1 and S2 respectively, figure 4.4). The S1 value for the 5mM kainate group was significantly different from that of the 1mM group ($p \leq 0.025$, and the S2 or S3 values were also significantly different ($p \leq 0.01$), (table 4.1).

Table 4.3. Total stimulated release and between S1 and S2.

GROUP	Total stimulated	Total 'Hump'
50mM KCl (9)	36.5 ± 11.5	5.1 ± 1.8
25mM KCl (11)	13.7 ± 1.6[a]	8.4 ± 3.5
5/25mM KA/KCl (10)	15.8 ± 2.8[a]	11.5 ± 3.8[a]
5mM KA (6)	62.1 ± 18.3[cd]	29.3 ± 10.0[bd]
1mM KA (10)	18.6 ± 2.8[f]	9.1 ± 2.2[e]

Legend: Total stimulated release was calculated as S1 + S2 + S3, whilst total 'Hump' release was calculated as total residuals between 68 and 84 minutes. KA = kainic acid. Numbers in parenthesis are the number of slices.
[a] $p<0.05$ versus same column 50mM KCl;
[b] $p<0.05$, [c] $p\leq0.01$ versus same column 25mM KCl;
[d] $p<0.01$ versus same column 5/25mM KA/KCl;
[e] $p<0.05$, [f] $p\leq0.01$ versus same column 5mM KA, (Mann Whitney U-test).

When the Sx/Sy ratios were examined it was observed that there was an apparent potentiation in the release of S2 at 5mM kainate (1.38 ± 0.44) compared to either 1mM kainate (0.90 ± 0.17) or the KCl experiments (0.69 ± 0.05, (50mM KCl) and 0.58 ± 0.12 (25mM KCl)) (table 4.2). However, this was only significantly different from the 25mM KCl S2/S1 ratio ($p\leq0.05$).

In both 1mM and 5mM kainate stimulated slices the S3 value was larger than the S2 or S1 (as expected) and was significantly different from both ($p\leq0.05$). An unexpected finding was that the S3 from 5mM kainate treated slices was significantly different from the S3 of 50mM KCl treated slices ($p\leq0.01$), but that in 1mM kainate treated slices was not (table 4.1).

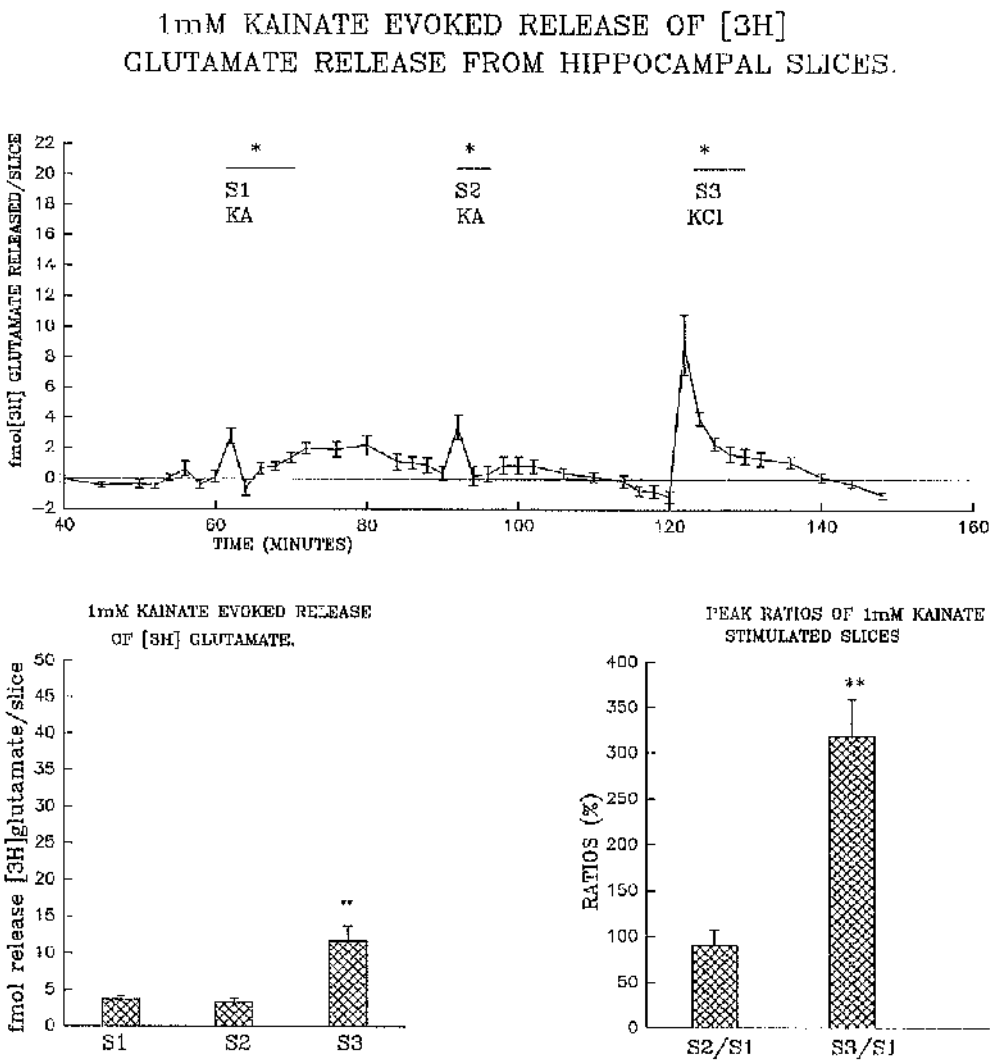


Figure 4.3.

1mM kainate stimulated [^{3}H] glutamate release from hippocampal slices. A) release above predicted basal release (see methods) n=10, points indicate mean ± sem, * $p \leq 0.05$ versus versus basal noise (see methods) Mann Whitney U-test. KA = kainate. B) Total release during stimulation, columns indicate mean ± sem, ** $p \leq 0.01$ versus both S1 and S2. C) Ratio of total stimulated release, ** $p \leq 0.01$ versus S2/S1, columns indicate mean ± sem.

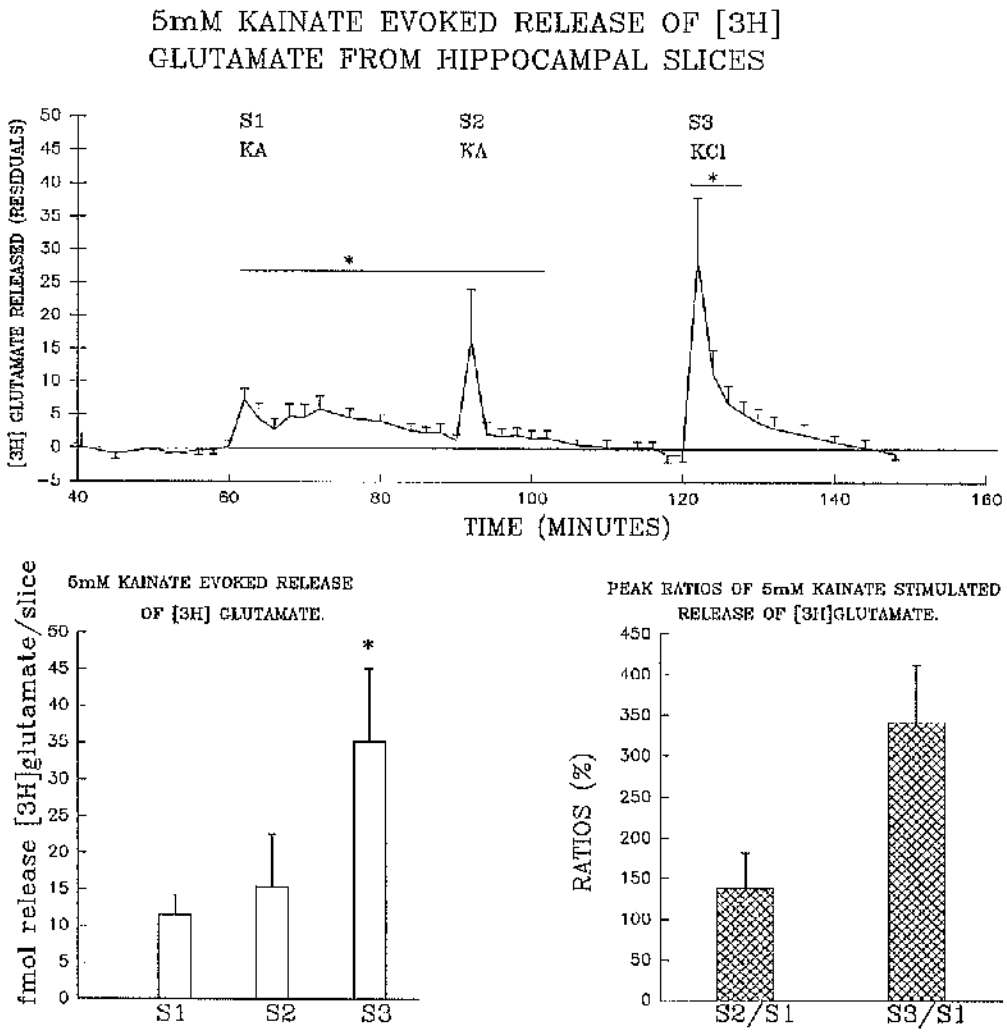


Figure 4.4.

5mM kainate stimulated [^{3}H] glutamate release from hippocampal slices. A) release above predicted basal release (see methods) n=6, * $p \leq 0.05$ versus basal noise (see methods) Mann Whitney U-test points indicate mean ± sem, KA = kainate. B) Total release during stimulation, columns indicate mean ± sem. * $p \leq 0.05$ versus both S1 and S2. C) Ratio of total stimulated release.

To observe what effect, if any, kainate had on KCl stimulated release 2 experiments were performed using 5mM kainate and 25mM KCl as the stimulus for S1 and S2, with S3 being just 25mM KCl. When the actual release was examined (figure 4.5b), the S1 was significantly greater than S2 ($p \leq 0.05$) and S3 ($p \leq 0.01$), but S2 was not different from S3. The release profile obtained from these slices was similar to that of just 25mM KCl and not that from 5mM kainate (figure 4.5a, 4.1a and 4.4a respectively). Even though there was an apparent period of heightened release, this was also of similar shape to 25mM KCl (i,e, tailing off) rather than 5mM kainate (i.e. hump-like).

Indeed, when the actual release is compared for all three groups (table 4.3) this is apparent, but again due to the variability within the 5mM kainate group there is no significance at S1, but both S2 and S3 are different ($p \leq 0.01$). When compared against the 25mM KCl stimulated slices there is no significance. The Sx/Sy ratios also show this tendency with S2/S1 not being significant, but S3/S1 and S3/S2 being significantly different for both groups ($p=0.01$ for S3/S1 and S3/S2 against 5mM kainate), but no significance against the 25mM KCl stimulated slices (table 4.2).

A comparison of the potencies of kainate and KCl is shown in table 4.3. If the total stimulated release is examined (excluding the 'hump'), then the 5mM kainate experiments are significantly different from the 5/25mM kainate/KCl experiments ($p \leq 0.01$,) (table 4.3). If the time period corresponding to the Hump in kainate slices (68-84 minutes) is also examined, then the 5/25mM kainate/KCl is also significant against the 5mM kainate experiments ($p \leq 0.01$), (table 4.3).

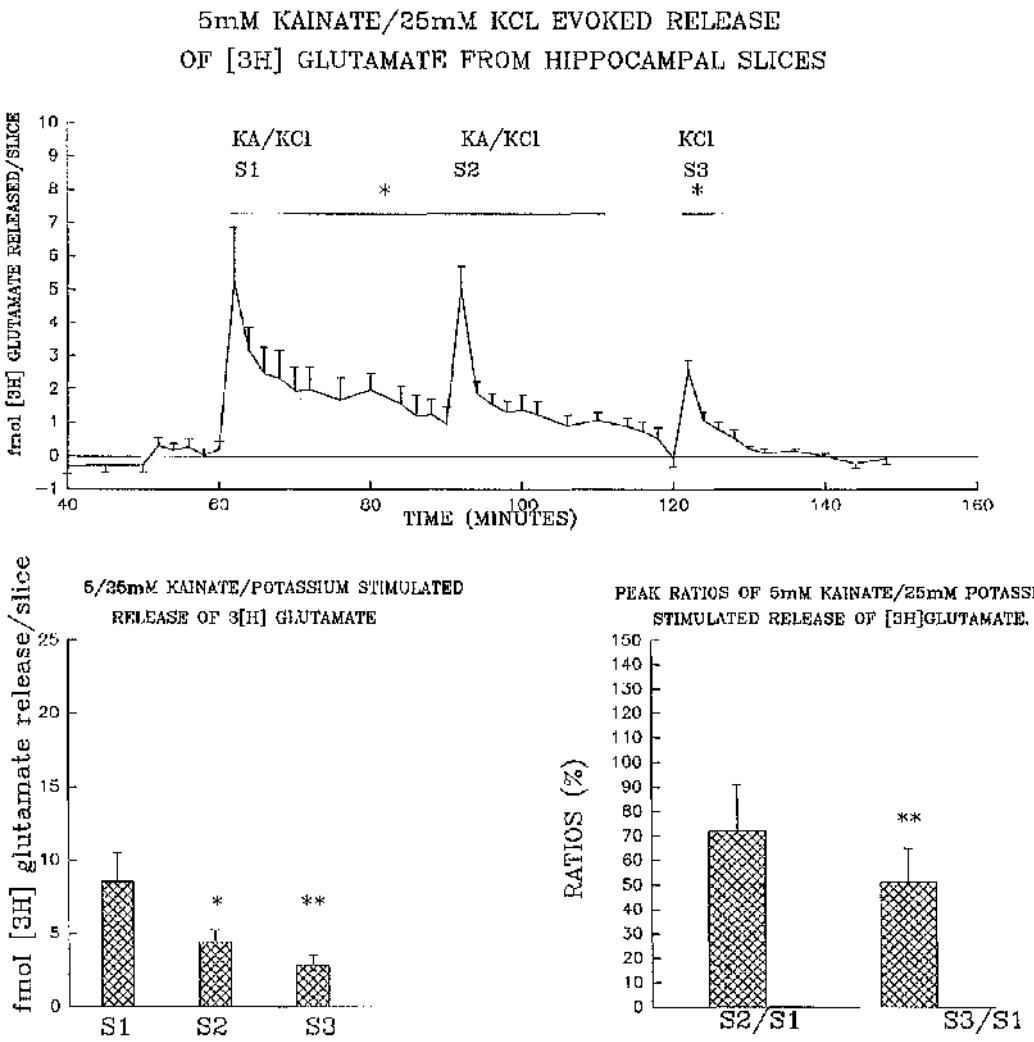


Figure 4.5.

5/25mM kainate/KCl stimulated [^{3}H] glutamate release from hippocampal slices. A) release above predicted basal release (see methods) n=10, * $p \leq 0.05$ versus basal noise (see methods) Mann Whitney U-test, points indicate mean $\pm$ sem. KA/KCl =kainate/KCl stimulation B) Total release during stimulation, columns indicate mean $\pm$ sem,* $p \leq 0.05$, ** $p \leq 0.01$ versus S1 . C) Ratio of total stimulated release, ** $p \leq 0.01$ versus S2/S1.

4.2.3 The Effect of 2-Chloroadenosine on the Kainate Evoked Release.

In the neurotoxicity experiments, R-PIA was used as the adenosine A_1 agonist due to its long lasting action, but this attribute was not desirable in the release experiments and so 2-chloroadenosine was used instead. 2-chloro-adenosine (2CA) was tested against both 1 and 5mM kainate (figures 4.6 and 4.7 respectively), and was perfused during the time period 72 to 102 minutes (stimulus S2, which was in the 92 minute fraction, was delivered in 2CA containing ACSF/kainate).

In the 1mM kainate group the initial S1 was significantly greater than that of the 1mM kainate control experiments ($p \leq 0.025$), as was the S2 ($p \leq 0.01$), but the S3 peaks were not significantly different from the control experiments (table 4.4). The S2/S1 and the S3/S1 ratios were both significantly different from the control ($p \leq 0.01$ and $p \leq 0.05$ respectively), but the S3/S2 was not (table 4.5). Surprisingly, H1 was also significantly greater than the control ($p \leq 0.01$) even though there was a large degree of variability between slices (table 4.6).

Table 4.4 Comparison of the effects of 5μM 2-chloroadenosine on kainate stimulated hippocampal slices.

TYPE /DRUG	S1 (KA)	S2 (KA + 2CA)	S3 (50mM KCl)
1mM KA (10)	3.71 ± 0.45	3.21 ± 0.62	9.37 ± 2.08
1mM KA/CA (6)	6.29 ± 1.46[a]	4.44 ± 1.25[b]	10.42 ± 2.08
5mM KA (6)	11.51 ± 2.70	15.37 ± 7.21	35.18 ± 9.95
5mM KA/CA (6)	4.60 ± 0.88[a]	4.77 ± 1.32[a]	14.49 ± 5.92[a]

Legend: Figures indicate total release, above basal, following stimulation (fmol [^{3}H] glutamate/slice). Numbers in parenthesis indicates number of slices.
KA = kainic acid, CA = 2-chloroadenosine (5μM).
[a] $p \leq 0.05$, [b] $p \leq 0.01$ versus same column controls (Mann Whitney U-test).

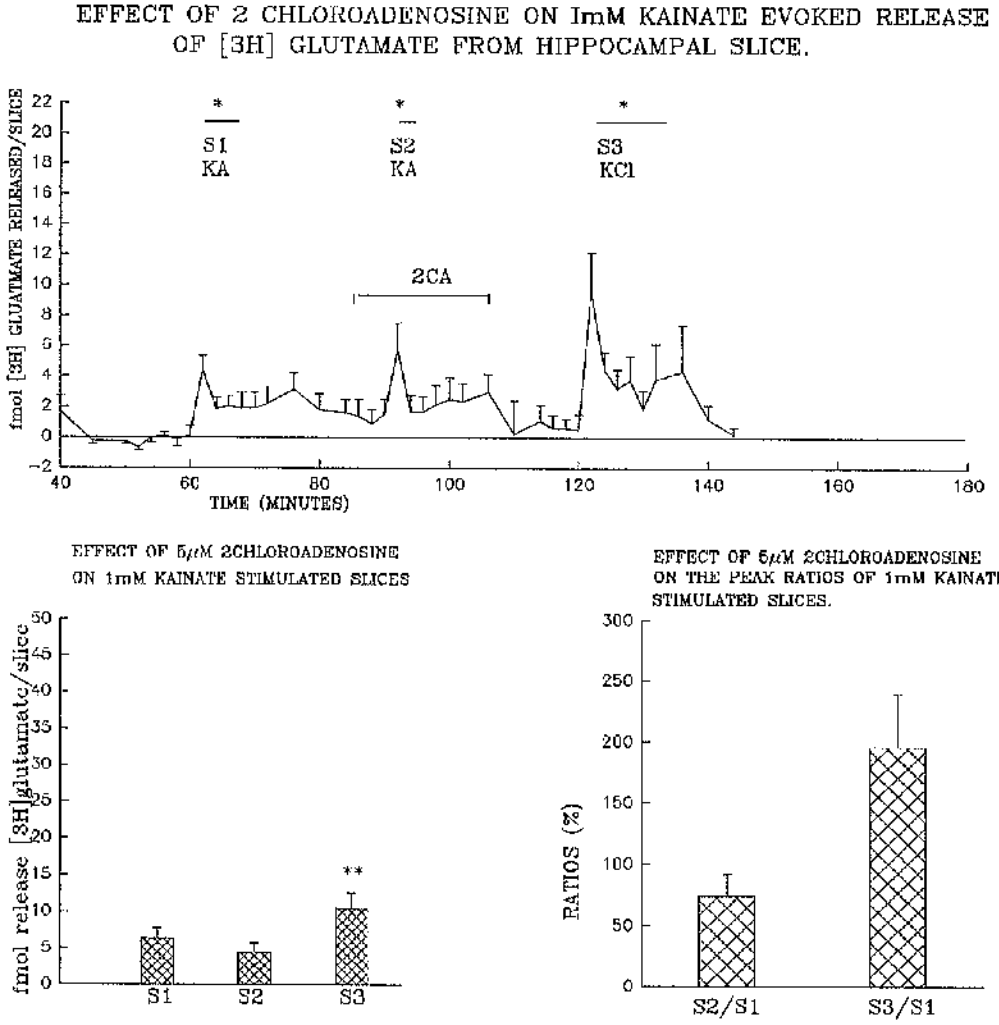


Figure 4.6.

Effect of 2-chloroadenosine on 1mM kainate stimulated hippocampal slices. A) release above predicted basal release (see methods) n=6, * $p \leq 0.05$ versus basal noise (see methods) Mann Whitney U-test. Points indicate mean $\pm$ sem.
KA = kainate stimulation, 2CA = 2-chloradenosine perfusion. B) Total release during stimulation, columns indicate mean $\pm$ sem, ** $p \leq 0.01$ versus both S1 and S2. C) Ratio of total stimulated release.

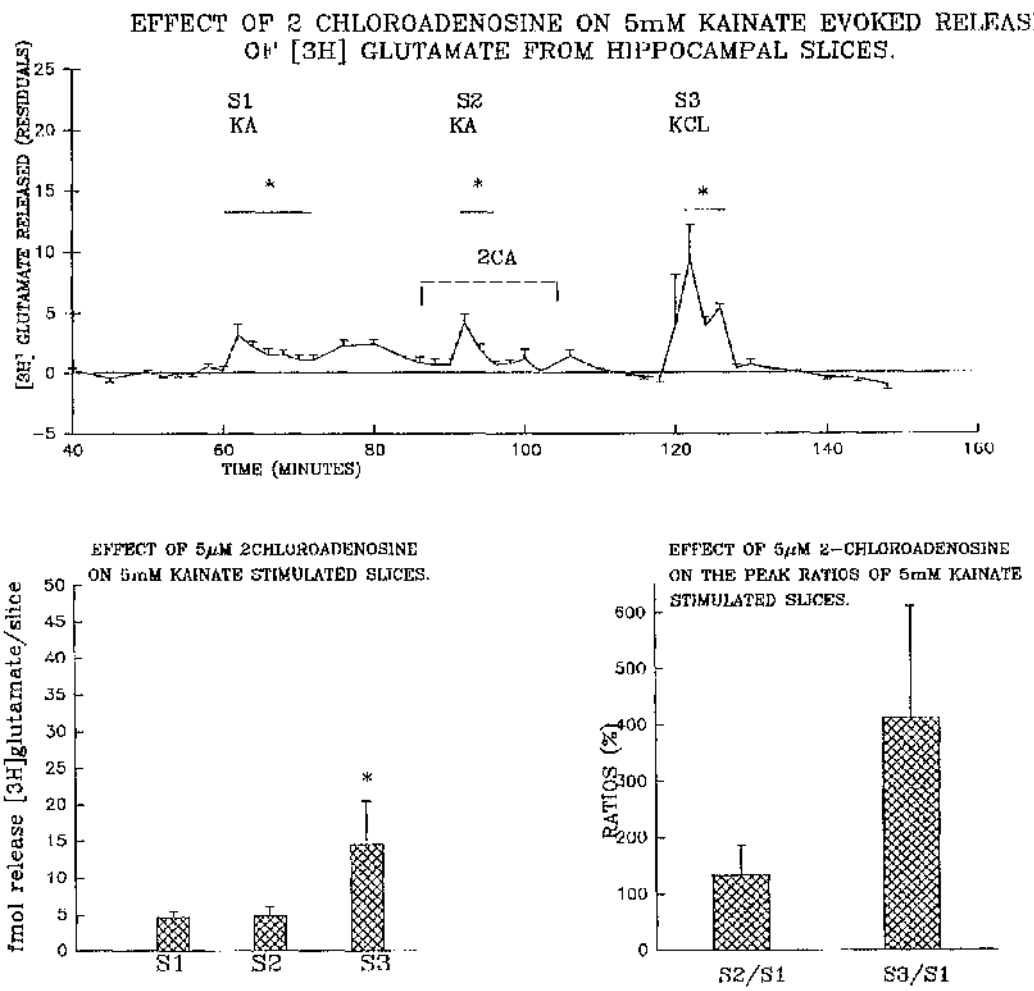


Figure 4.7.

Effect of 2-chloroadenosine on 5mM kainate stimulated hippocampal slices. A) release above predicted basal release (see methods) n=6, * $p \leq 0.05$ versus basal noise (see methods) Mann Whitney U-test points indicate mean $\pm$ sem. KA = kainate stimulation, 2CA = 2-chloradenosine perfusion. B) Total release during stimulation, columns indicate mean $\pm$ sem. * $p \leq 0.05$ versus both S1 and S2. C) Ratio of total stimulated release.

Table 4.5 Comparison of the effects of 2-chloroadenosine on the peak ratios of kainate stimulated hippocampal slices.

GROUP / PEAK	S2/S1	S3/S1	S3/S2
1mM KA (10)	90 ± 17	318 ± 41	553 ± 179
1mM KA/CA (6)	74 ± 18[a]	196 ± 43[a]	367 ± 126
5mM KA (6)	138 ± 44	341 ± 71	318 ± 73
5mM KA/CA (6)	133 ± 52	412 ± 200	388 ± 125

Legend: Figures indicate the ratio of Sx/Sy mean ± sem, as calculated in the methods. KA =kainic acid, CA = 2-chloradenosine (5μM).
[a] $p \leq 0.01$ versus same column 1mM KA (Mann Whitney U-test)

Table 4.6. Total stimulated release and hump release in slices treated with 2 chloroadenosine (5μM).

GROUP	TOTAL	HUMP
1mM KA (10)	18.57 ± 2.81	9.07 ± 2.18
1mM KA/CA (6)	21.15 ± 3.65	12.55 ± 6.00[b]
5mM KA (6)	62.06 ± 18.34	29.29 ± 9.95
5mM KA/CA (6)	23.86 ± 6.04[a]	10.52 ± 1.28

Legend: Columns indicate mean ± sem. Total indicates total stimulated release, whilst Hump indicates release during the time period 68-84 minutes. Numbers in parenthesis indicates number of slices used.
[a] $p \leq 0.05$ and [b] $p \leq 0.01$ versus same column controls (Mann Whitney U-test).

A complication with interpreting the 5mM kainate group arose due to the low amount released compared to the control experiments (S1 4.6 ± 0.88 and 11.52 ± 2.7 fmol/slice respectivcly) and this is shown in tables 4.4 and 4.6. The low levels of release from these slices resulted in all the peaks, the total release and H1, being significantly smaller than those of the control group. This low level of release did not affect the Sx/Sy ratios and there was no significant difference between the kainate and the kainate/2CA groups at any of the levels tested (table 4.5).

4.2.4 The Effect of 8-Cyclopentyl-1,3-dipropylxanthine on Evoked Release.

To determine whether the lack of response to 2CA was due to the adenosine A_1 receptors already being maximally active the selective A_1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was used at 5nM (K_i 0.46nM, Bruns, *et al.*, 1987). Due to the large variability in the S1 peak with 5mM kainate, and also the presence of the 'hump', a third kainate stimulation was added to the experiment, such that S1,S2 and S3 were all kainate and S4 was 50mM KCl (figure 4.8).

Two sets of experiments were performed, the first with DPCPX being washed out 15 minutes after S3, and the second set with the DPCPX present until the end of the perfusion (figure 4.9 and 4.10 respectively).

Unlike the previous 5mM kainate experiments, during the course of these experiments S1 was greater than S2, but again this was not significant (S2/S1 0.87 $\pm$ 0.18 (n=18) and 1.38 $\pm$ 0.44 (n=6) respectively). The amount released during S1 was similar for all three groups (table 4.7). There was a significant difference between the amount released during S3 in the presence of DPCPX (washout experiment) and the kainate control group, but as the S2 amount was also low for the washout group, the importance of this is unclear. However, there was a significant reduction in the amount released by S4 (50mM KCl) compared to the kainate control group (table 4.8, $p \leq 0.01$ for both groups).

When the Sx/Sy ratios were examined it was noticed that there was a significant reduction in the S3/S1 in the washout experiment compared to the control group ($p \leq 0.01$) but not the continuous DPCPX perfusion group (S3/S1 0.37 $\pm$ 0.14, 0.87 $\pm$ 0.18 and 1.18 $\pm$ 0.57 respectively) (table 4.8).

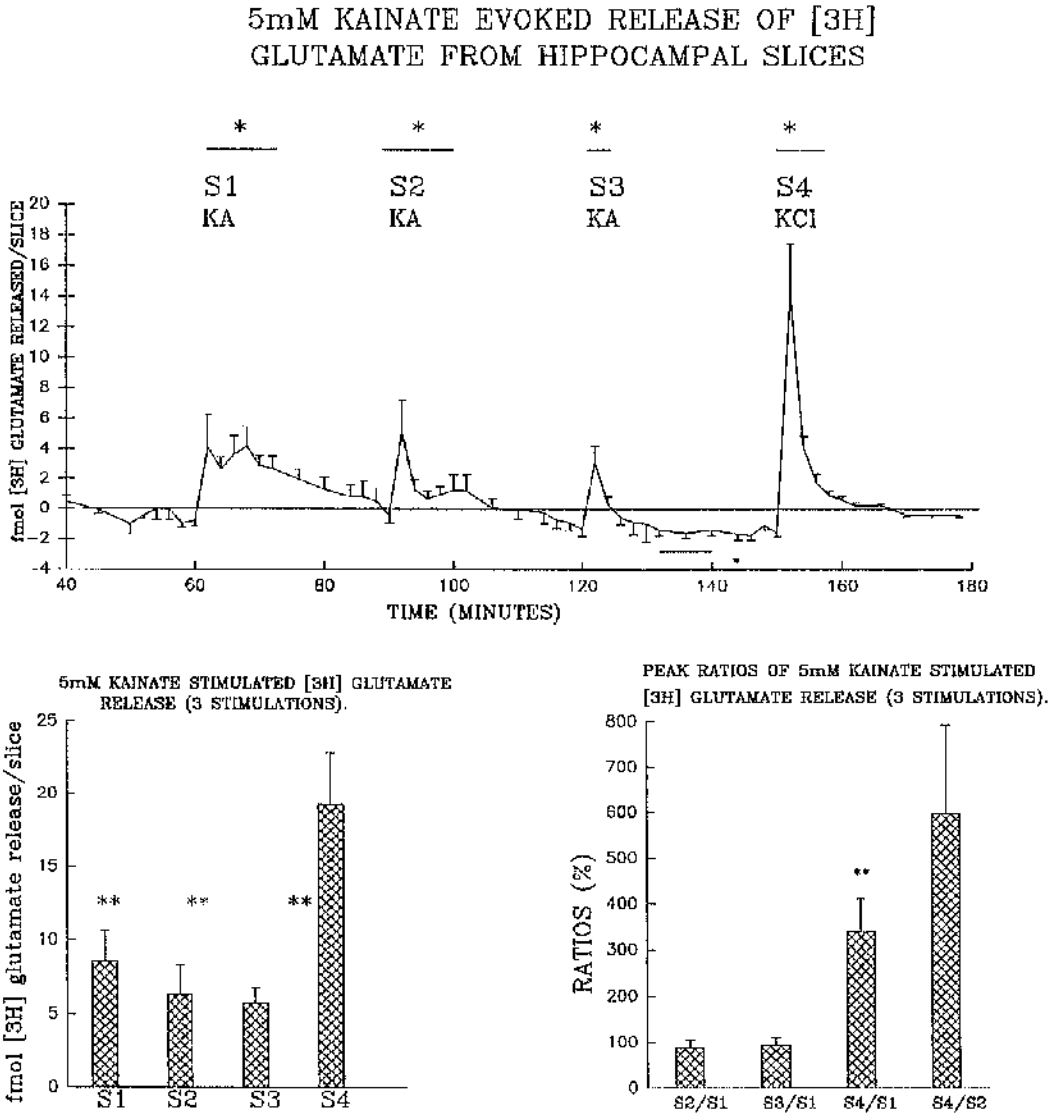


Figure 4.8.

5mM kainate stimulated [^{3}H] glutamate release from hippocampal slices (3 kainate peaks).

A) release above predicted basal release (see methods) n=18, * $p \leq 0.05$ versus basal noise (see methods) Mann Whitney U-test. Points indicate mean $\pm$ sem.

KA = kainate B) Total release during stimulation, columns indicate mean $\pm$ sem. ** $p \leq 0.01$ versus S4. C) Ratio of total stimulated release. ** $p \leq 0.01$ versus S2/S1.

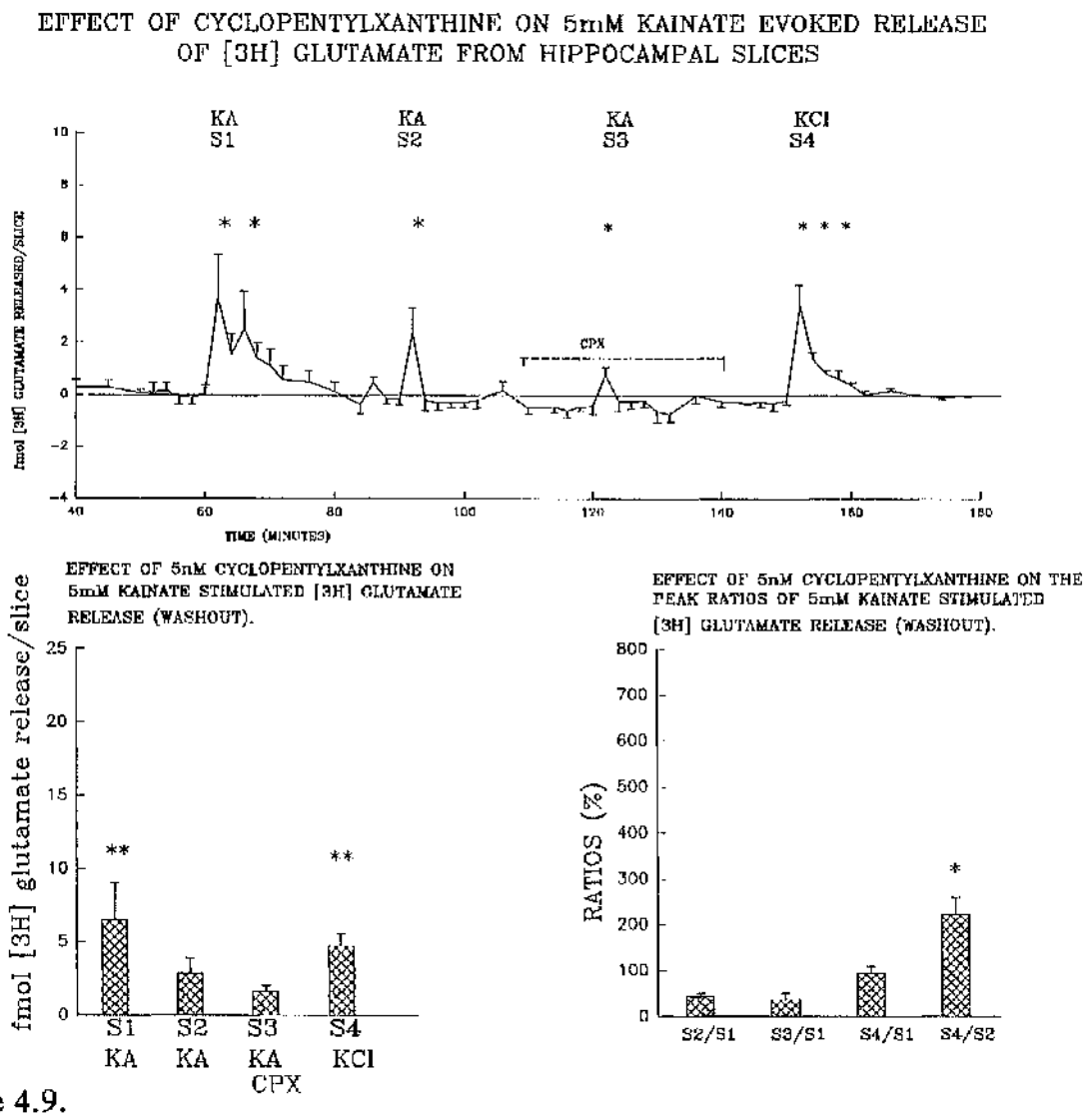
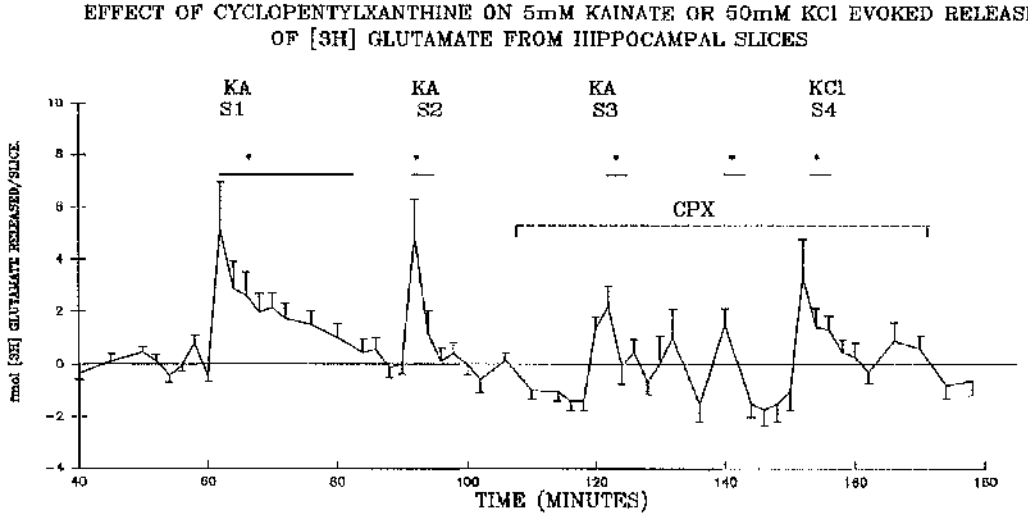
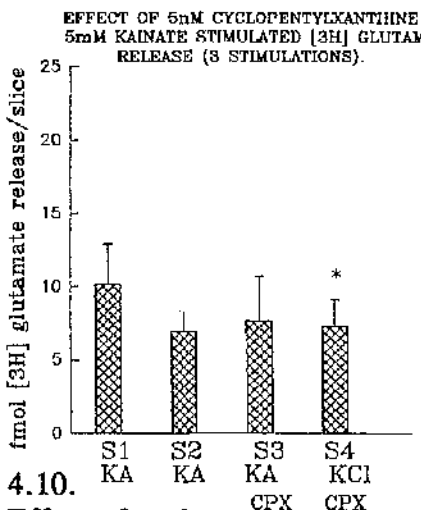


Figure 4.9.

Effect of cyclopentylxanthine on 5mM kainate stimulated [^{3}H] glutamate release from hippocampal slices (washout).
A) release above predicted basal release (see methods) n=6, * $p \leq 0.05$ versus basal noise (see methods) Mann Whitney U-test. Points indicate mean $\pm$ sem. KA = kainate, DPCPX = cyclopentylxanthine perfusion. B) Total release during stimulation, columns indicate mean $\pm$ sem. ** $p \leq 0.01$ versus S3. C) Ratio of total stimulated release. * $p \leq 0.05$ versus S2/S1.





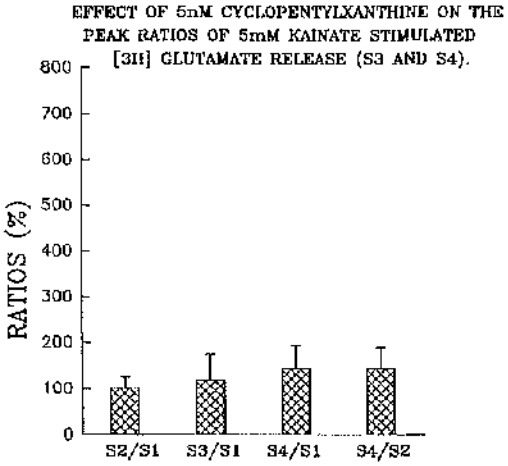


Figure 4.10.

Effect of cyclopentylxanthine on 5mM kainate stimulated [^{3}H] glutamate release from hippocampal slices.

A) release above predicted basal release (see methods) n=11, * $p \leq 0.05$ versus basal noise (see methods) Mann Whitney U-test. Points indicate mean $\pm$ sem. KA = kainate, DPCPX = cyclopentylxanthine perfusion.

B) Total release during stimulation, columns indicate mean $\pm$ sem. ** $p \leq 0.01$ versus S1.

C) Ratio of total stimulated release.

Table 4.7. Comparison of the effect of cyclopentylxanthine on kainate treated slices.

GROUP/DRUG	S1 KA	S2 KA	S3 KA/DPCPX	S4 KCl/DPCPX
5mM KA (control)	8.6 ± 2.1	6.3 ± 1.0	5.7 ± 1.0	19.3 ± 3.5
5mM KA/DPCPX (washout)	6.5 ± 2.5	2.8 ± 1.0	1.6 ± 0.4[b]	4.7 ± 0.9[a]
5mM KA/DPCPX (S3 & S4)	10.2 ± 2.7	7.0 ± 1.4[c]	7.7 ± 3.1[c]	7.3 ± 1.8[a]

Legend:

Figures indicate total release (fmol [^{3}H] glutamate/slice, mean ± sem). KA = kainic acid, DPCPX = cyclopentylxanthine (5nM).
KA control n=18, KA/DPCPX washout n=6, KA/DPCPX (continuous) n=11.
[a] $p \leq 0.05$, [b] $p \leq 0.1$ versus same column control,
[c] $p \leq 0.05$ versus same column DPCPX washout (Mann Whitney U-test).

Table 4.8. Comparison of the effects of cyclopentylxanthine on the peak ratios of kainate stimulated slices.

GROUP/ PEAK	S2/S1	S3/S1	S4/S1	S3/S2	S4/S2	S4/S3
KA (Con)	87 ± 18	94 ± 16	340 ± 71	174 ± 52	598 ± 195	428 ± 94
DPCPX (Wash)	44 ± 6	37 ± 14[b]	94 ± 15[a]	106 ± 55	222 ± 38	423 ± 120
DPCPX (End)	103 ± 23	118 ± 57	144 ± 50[a]	110 ± 33	145 ± 45[bc]	198 ± 57[ac]

Legend

Figures indicate the percentage of Sx/Sy, mean ± SEM.
KA (Con) = control slices, 5mM kainate stimulation, no DPCPX (n=18). DPCPX (wash) = 5mM kainate stimulated slices, DPCPX present between S2 and S3 (S3 in DPCPX/ACSF) (n=6). DPCPX (end) = 5mM kainate stimulated slices, DPCPX present from 102 minutes to end (S3 and S4 in DPCPX/ACSF) (n=11).
KA=kainate, DPCPX = cyclopentylxanthine (5nM).
[a] $p \leq 0.05$, [b] $p \leq 0.01$ versus same column control,
[c] $p \leq 0.05$ versus same column DPCPX washout, (Mann Whitney U-test).

DPCPX did not significantly alter the S3/S2 ratios, although it did affect the S4/Sy ratios. In slices perfused with DPCPX the S4/S1 ratio was significantly different from the control slices, but the two DPCPX groups were not different. Although the S4/S2 ratio for the DPCPX washout was lower than that of the control it was not significantly different, and the S4/S3 ratio was the same as the control. If the DPCPX was continuously perfused the S4/S2 and the S4/S3 values were significantly lower than the control ($p<0.05$ table 4.9).

When the total stimulated release was examined there was no significant difference between the control and the continuous DPCPX perfusion but both were significantly greater than the DPCPX washout experiment (table 4.10). Even though the slices were following a trend similar to the KCl treated slices ($S1 \geq S2 \geq S3$), there was still a period of heightened release after S1, that was significantly greater than the basal release (from S1 to 80 minutes for the control and continuous DPCPX perfusion, none for the washout group, see figures 4.8, 4.9, 4.10) and the H1 values are shown in table 4.9.

Table 4.9. Effect of cyclopentylxanthine on total stimulated release and heightened release ('hump').

GROUP / RELEASE	TOTAL STIMULATED	HUMP RELEASE
KA (control)	39.84 ± 7.37	13.75 ± 4.78
KA/DPCPX (washout)	15.71 ± 4.34	3.37 ± 2.57
KA/DPCPX (continuous)	32.14 ± 4.95	8.84 ± 3.15

Legend: Figures indicate fmol [^{3}H] glutamate release (mean ± sem). Hump release is all the release during time 68-84 minutes.
KA = kainic acid, DPCPX = cyclopentylxanthine (5nM).
[a] $p \leq 0.05$, [b] $p \leq 0.01$ versus same column control,
[c] $p \leq 0.05$, [d] $p \leq 0.01$ versus same column washout (Mann Whitney U-test).

5.0 DISCUSSION

5.1 Discussion of Methods

5.1.1 Neurotoxicity

Previous studies have employed histological or neurochemical analyses to demonstrate that systemic injections of kainic acid to mature rats can induce hippocampal damage (Schwob *et al.*, 1980; Fuller *et al.*, 1981; Lothman and Collins, 1981; Fariello *et al.*, 1989). The present results indicate that the damage induced by kainate is also reflected in an elevation of [^{3}H] PK11195 binding to hippocampal P_2 membranes. Gliosis always accompanies the degeneration of neurones induced by excitotoxins or ischaemia (Schwarcz & Coyle, 1977; Andersson *et al.*, 1991; Haglid *et al.*, 1991; Jørgensen *et al.*, 1993) and the demonstration that binding sites for the peripheral benzodiazepine receptor ligands occur in central glial cells has been proposed as a sensitive (indirect) indicator of neuronal damage (Benavides *et al.*, 1987; Dubois *et al.*, 1988; Altar & Baudry, 1990; Price *et al.*, 1990). Comparative analyses have shown an excellent correlation between the distribution of PK11195 binding studied by autoradiography, and conventional histology (Dubois *et al.*, 1988, Price *et al.*, 1990). Binding techniques, however, are more sensitive, and can reveal changes following concentrations of excitotoxins that are insufficient to cause significant alterations of enzyme markers such as glutamate decarboxylase or choline acetyltransferase (Benavides *et al.*, 1987).

However, although binding is more sensitive than most methods, it should never be used in isolation in neuroprotective studies. Binding is subject to misinterpretation, in that the lack of change in a neuronal marker, for example, does not necessarily indicate that there is no neuronal damage. In this case it may be that the neurones in question are not affected. Another possibility is that there is a change in both the B_{max} and K_D such that

there is no apparent change in saturated binding. Whilst this scenario is unlikely, it should be noted that several receptor subtypes have both high and low affinity binding (eg kainate receptors). To compensate for this possibility, it is usually advisable to perform saturation binding experiments to remove any doubt, by obtaining both K_D and B_{max}, although cost prevented this being performed routinely in the present study, apart from the intial kainate experiments.

Histology is another method of validating results obtained in binding experiments, in that changes in structure are easy to observe, and it is possible to examine areas outwith the areas of study in binding experiments, or to examine if there is any variation in the degree of damage within a region. This is especially true for the hippocampus, where all the regions (CA1 -CA4) and the dentate gyrus are all sensitive to ischaemic-type damage to differing degrees. Histology, though, can only show morphological changes and should be performed in conjunction with other techniques to allow a broader picture to be formed.

5.1.2 Kainate Induced Damage

There are several advantages of using a systemic route for injecting kainate. Firstly, no surgery is required as it is for direct intrahippocampal injections, and the variability inherent in the stereotaxic procedure is avoided. Secondly, the neurotoxin is likely to be more evenly distributed throughout the hippocampus than following localised injections. Thirdly, the concentration of kainate achieved is likely to be lower after intraperitoneal injection, and hence only high affinity kainate binding sites should be affected. The dose of $10mg.kg^{-1}$ would yield a tissue concentration of approximately 38 μM if it were evenly distributed around the body, and probably much less than this in the

brain due to the restrictive permeability of the blood-brain barrier (BBB). Nadler *et al* (1980) and Berger *et al,* (1986) reported that after systemic injection the concentration of kainate in the brain is $\leq 1\%$ of the predicted concentration, and this means that assuming the 200g rat has a brain of 3g then the amount in the brain is ≤ 1.75nmoles. This is of similar dosage to those used when kainate is administered intrahippocampally / intraventricularly (1-20nmoles; see Schwarcz *et al.*, 1978; Franck & Roberts, 1990; Tanaka *et al.*, 1990; Borg, 1991; Moncada *et al.* 1991; Phelps *et al.*, 1991). To achieve neuronal loss in cell culture appoximately 500μM is usually used (Choi *et al.*, 1988; Koh *et al.*, 1990; Koh & Choi, 1991; Puttfarcken *et al.*, 1993). The advantage in the present study is that there is probably an even distribution of kainate in the hippocampus rather than a concentration gradient decreasing from the point of hippocampal administration (600nM throughout the brain compared to 1-20mM at the site of administration, respectively). The doses of kainate used in the present work are comparable with those used by others (e.g. Fariello *et al.*, 1989) but less than the 12mg/kg dose which can induce severe seizure activity (Fuller & Olney, 1981; Altar & Baudry, 1990). Intrahippocampal injections of kainate result in a more severe degree of damage than i.p. injections probably because the dose for a particular area is greater than that achieved by ip injections, and this may mean that some of the damage caused by intrahippocampal injections is mediated by the low affinity kainate receptors/AMPA receptors (Moncada *et al.*, (1991)).

However, this said, the peripheral administration probably has several potential side effects. These include: change in body pH, release of stress hormones, cardiac and respiratory disturbances, temperature changes and, probably most importantly, the behavioural disturbances. All of these have been examined during the initial validation of

kainate as a neurotoxic tool (Schwob *et al.*, 1980; Lothman & Collins, 1981; Sperk *et al.*, 1985; Sperk, 1994). However each of these side effects will be examined in this section.

1) the effect of kainate pH on neurotoxicity:

Although in the present study the kainate was not buffered (pH≈4.0), the damage was similar in magnitude and specific in location to previous reports that used buffered kainate systemically (Schwob *et al.*, 1980; Lothman & Collins, 1981; Heggli & Mathe-Sϕrenssen, 1982; Baran *et al.*, 1994). Several groups have also used unbuffered kainate (Berg *et al.*, 1993, Altagracia *et al.*, 1994) and have also reported the same pattern of damage. As with central administration, *in vitro* studies also indicate that kainate is able to induce neurotoxicity at physiological pH (Schwob *et al.*, 1980; Kato *et al.*, 1991). All these reports indicate that, whilst buffering the kainate when administering centrally is essential, systemically administered unbuffered kainate produces damage that is indistinguishable from buffered kainate, and suggsets that it is not important in the present situation.

2) the effect of kainate on body temperature:

In experiments where kainate is administered centrally the animals are aesthetised and their body temperature is maintained by a homeothermic blanket. This means that the temperature is maintained in the region of 37°C. The findings in the present report and those of Lin *et al.*, 1993, indicate that kainate does not alter the systemic temperature over the course of the experiment, and this argues against the damage being mediated through change in body temperature. However, as mentioned later changes in brain temperature will significantly alter the degree of damage.

3) Behavioural changes:

The importance of the behavioural changes over the whole course of the

experiment is still unclear. The initial disturbances (ie the seizures, the wet dog shakes and the SBS) appear following either systemic or central administration (see Sperk, 1994 for review). This may suggest that the same pathways are activated following either type of administration and that the outcomes are the same but this is still being investigated. The hyperresponsiveness of the kainate damage animals observed after 2-3 days suggests that the animals have elevated levels of the stress hormones (glucocorticoids mineralocorticoids), although there are as yet no reports on the levels of these hormones. Indeed the importance of the stress response is debatable in that adrenalectomony attenuates kainate induced damage (Stein & Sapolsky, 1988; Lee *et al.*, 1989) whilst dexamethasome treatment enhances it (Lenique *et al.*, 1979; Sztriha *et al.*, 1986, Lee *et al.*, 1989). This needs to be further evaluated.

4) the effect of kainate on the cardiac and respiratory systems:

There apppears to be no effect on either of these systems as observed in the present study or reported in previous studies (Schwob *et al.*, 1980; Lothman & Collins, 1981) indicating that damage is not due to respiratory or cardiavascular perturbations.

So, whilst systemic kainate causes behavioural disturbances which are distinct from those observed in the MCAO stroke models, these also occur following central administration. As both the central and systemic administrations result in these behavioural disturbances, and they do not appear to be pH- or body temperature-dependent, these may be important in the mechanism of kainate induced brain damage.

In the present study kainate caused a dose-dependent increase in [^{3}H]PK11195 binding, which was maximal at 10mg.kg^{-1}. This elevated binding was due to an increase in B_{max} but not K_D. Both the K_D and B_{max} are higher than those reported in the literature (in some cases the K_D is 10-100 times greater see table 5.1).

Table 5.1 Experimental conditions used in [^{3}H] PK11195 experiments.

K_D nM	B_{max} fmol. mg^{-1}	SPECIES	TISSUE	SOLUTION	pH	AIC. hr/°C	CL.	REF
NR	NR	mouse	cortex	120mM NaCl 50mM Tris HCl	7.4	1/25	1μM PK11195	1
2.8 - 17.6	NR	human	BCC	PBS + 0.1% BSA	7.4	0.5/25	1μM PK11195	2
2.1	255	human	cortex	50mM Tris HCl	7.4	1/4	10μM PK11195	3
0.96 0.63	2998 131	rat	kidney cortex	50mM Tris HCl	7.4	1/4	10μM PK11195	4
2.3	0.010	rat	heart	PPB	7.4	0.5/25	1μM PK11211	5
1.83	0.012	rat	heart	5mM Tris HCl, 1mM $MgCl_2$, 0.25mM Suc.	7.4	0.5/25	1μM PK11211	6
0.87 2.71	128 0.002	rat	cortex O.Bulb	PBS	7.4	1/4 2/4	1μM Ro5-4864	7
1.88	26300	bovine	pineal gland	50mM PPB	7.4	0.5/ 25	10μM PK11195	8
1.07	0.010	rat	kidney	50mM PPB	7.0	1/4	10μM PK11195	9
1.1	NR	rat	cortex	50mM Tris HCl	7.4	1/4	10μM PK11195	10
1.16	NR	rat	kidney	50mm Tris HCl	7.4	1/4	1μM PK11195	11
5.2	268	rabbit	bladder	10mM Tris HCl	7.5	2/4	10μM PK11195	12
0.74 1.01	20.1 18.3	rat	hippo. cortex	50mM Tris HCl, 120mM NaCl	7.4	1/25	1μM PK11195	13
NR	NR	rat	striatum	50mM Tris HCl, 120mM NaCl	7.4	2/4	1μM PK11195	14
0.58	445	mouse	fore brain	50mM Tris HCl	7.8	1.5/4	10μM PK11195	15
7	14500	human	lympo	50mM Tris HCl	7.4	1/4	10μM PK11195	16
1.71 15.2	6345 13575	rat human	prost.	50mM Tris HCl	7.4	2/4	10μM PK11194	17
NR	NR	rat	hippo. cortex striatum	0.1M PH_2PO_4, 0.1M NaCl.	7.4	1.5/4	100μM Diaz.	18/ 19
7.35	693	rat	hippo.	50mM Tris HCl	7.8	1/4	10μM PK11195	20

Abbreviations: AIC: assay incubation conditions, BCC: blood cell culture, BSA: bovine serum albumin, CL: cold ligand, Diaz.: diazepam, hippo.: hippocampus, lympo.: lymphocytes, NR.: not reported, O.Bulb: olfactory bulb, PBS: phosphate buffered saline, PPB: potassium phosphate buffer, prost.: prostrate, ref: references, suc: sucrose.
References 1 Poignet *et al.*, 1992; 2 Canat *et al.*, 1993; 3 Awad & Gavish, 1991; 4 Awad & Gavaish, 1987; 5 Doble *et al.*, 1985; 6 Doble *et al.*, 1987; 7 Benavides *et al.*, 1983; 8 Basile *et al.*, 1986; 9 Lueddens & Skolnick, 1987; 10 Skowronski *et al.*, 1987; 11 Skowronski *et al.*, 1988; 12 Smyth *et al.*, 1994; 13 Demerlé-Pallardy *et al.*, 1991; 14 Benavides *et al.*, 1987; Eshlemen & Murray, 1989; 16 Ferrarese *et al.*, 1990; 17 Camins *et al.*, 1994; 18 Baldwin *et al.*, 1993a; 19 Baldwin *et al.*, 1993c; 20 MacGregor, this thesis.

Whilst the differences between the B_{max} reported here and in other reports can be due to species/strain variation and possible underlying infections, the difference in K_D is harder to explain away. Possible explanations are differences in the incubation temperature and time, type and concentration of cold ligand, the composition of the buffer itself and the cell type examined (see table 5.1). In the present study the differences were not considered important as the receptor was used as a marker and was not of interest itself.

The elevation in B_{max} indicates that there may be an increase in glia and conversely a loss of neurones but, without any histology it would be impossible to validate that. However the histology experiments also show a loss of neurones in the hippocampus following the kainate injection, and the areas of damage are consistent with previous reports (Schwob *et al.*, 1980; Fuller & Olney, 1981; Lothman & Collins, 1981; Fariello *et al.*, 1989).

In the present study histology was performed in conjuction with Professor D.I. Graham, Department of Neuropathology and Doctor W. Maxwell, Department of Anatomy, The University of Glasgow. In this study, described in appendix 1, the areas of damage caused by the ip administration of $10mg.kg^{-1}$ kainate agreed with reports elsewhere in the literature (e.g. Lothman & Collins, 1981; Heggli *et al.*, 1981; Sperk *et al.*, 1983), with the hippocampal CA1 and CA3 pyramidal cell layers extensively

damaged, widespread damage in the entorhinal cortex, with the entorhinal fissure devoid of cells, as well as damage to the midline thalamus and the amygdala.

5.1.3 Use of Clonazepam.

In the present study clonazepam, at low doses, did not significantly protect against kainate induced damage measured in the hippocampus, but did abolish the tonic-clonic limbic seizures and reduced the frequency of the mild seizure-like activity such as tremor as reported by several other studies (Heggli *et al,* 1981; Lothman & Collins, 1981; Braun & Feed, 1990). There was no apparent effect on wet dog shakes or other behavioural effects of kainate suggesting that these may be initiated in a non-limbic locus by a mechanism independent of seizure activity. At the higher dose used (1 $mg.kg^{-1}$ clonazepam) there was marked sedation but still a significant increase in [^{3}H] PK11195 binding with kainate, a result consistent with the interpretation of Heggli *et al* (1981) that the degree of seizure activity did not correspond to neuronal damage following 12 $mg.kg^{-1}$ kainate. Conversely Lerner-Natoli *et al* (1991) reported that N-[1-(2-Thienyl)cyclohexyl]-piperidine (TCP) was able to block kainate damage, but was unable to block the induced seizures. One possible explanation for the TCP block is that kainate is mediating its neurotoxic but not its convulsive action through glutamate and hence NMDA receptor activation (see later for a full discusion of this).

Several groups have reported that only certain anticonvulsants protect against kainate damage (Zaczek *et al,* 1978; Fuller & Olney, 1981; Voll & Auer, 1991). Benzodiazepines seem unable to prevent kainate damage in the hippocampus (Ben-Ari *et al.,* 1979, Heggli & Malthe-Sorenssen, 1982) and Koh & Choi (1987) found that diazepam or phenobarbitone were not neuroprotective against glutamate neurotoxicity in

cell culture. This separation of convulsions and neuronal damage contradicts the popular view that seizures are needed for the damage (Fuller & Olney, 1981; Lothman & Collins, 1981; O'Shaughnessy & Gerber, 1986). One possible explanation for the difference in effect of anticonvulsants may depend on the experimental technique. Several groups have used high doses of anticonvulsants in the presence of anaesthetics, which themselves have been reported to alter the release of amino acids (Kendall & Minchin, 1982; Richards, 1983; Carla & Moroni, 1992). These alterations may enhance the action of anticonvulsants. Since the present study is based on the change in a glial marker, another possible explanation is that there is an increase either of K_D or B_{max} with no actual neuronal loss. This technique also means that no information could be drawn on the development of damage in different CA areas of the hippocampus. However parallel histological studies support the conclusions presented here and indicate that there is still widespread necrosis in the hippocampus 7 days following 10 $mg.kg^{-1}$ kainate with 0.2 $mg.kg^{-1}$ clonazepam ip (see appendix 1).

As mentioned in the previous section histology was performed in collaboration with several departments and the effect of 0.2$mg.kg^{-1}$ clonazepam was examined by blind analysis. Of the 11 kainate injected animals 2 received 0.2$mg.kg^{-1}$ clonazepam and the others received saline, and there was no observable qualitative difference between these two groups (see Appendix 1).

5.1.4 Behavioural Studies

The behavioural scores were initially based on those used by Hoffman *et al.* (1991), scoring for consciousness, grip, limb tone, walking and pain reflex. This was repeated 2hr later and on the day of death. This score was discarded as its emphasis is on

motor control, which is not affected by kainate lesioning, and because of the stress involved in handling the animals. The replacement method involved simple observation without handling of the animals every hour after kainate injection. Animals were examined for the presence of wet dog shakes, seizures and seizure-like activity, fore-paw abduction, excessive salivation, Straub tail, head weaving and circling. No scoring was used in this test but the presence or absence of these behavioural disturbances was noted.

The behavioural disturbances induced by kainate can be classified into 3 groups; seizures and fits, wet dog shakes, and salivation - fore paw abduction - Straub tail - circling. The use of clonazepam may indicate that the seizure like activity is due to inactivation of the central benzodiazepine binding sites, and hence, by inference the $GABA_A$ receptors. Inhibition of the GABAergic system by kainate has been recently reported (Miligram *et al.* 1992). The wet dog shakes (WDS) behavioural disturbance has been attributed to serotonin receptor activation (specifically the $5HT_2$ receptor), a proposal made by Worms *et al.* (1981). There are reports that ketanserin ($5HT_2$ antagonist) is able to reduce the infarction size, in both the middle cerebral artery occlusion model (Prehn *et al.*, 1993), and transient bilateral carotid occlusion model (Klish & Bode-Greuel, 1992), although Baldwin *et al.* (1993b) failed to get neuroprotection with ritanserin in the photochemical model of focal ischaemia. There is also evidence that $5HT_2$ receptors also inhibit glutamate transmission in the in vitro cerebellar slice preparation (Thellung *et al.*, 1993). The implications for serotonergic modulation in the present model remain to be examined and determined, although Velisek *et al.*, (1994) reported that ritanserin ($5HT_2$ antagonist) failed to suppress kainate induced WDS in developing rats. The third group of behavioural disturbances also indicate a serotonergic involvement since these comprise the serotonergic behaviour syndrome which can be mimicked by $5HT_{1A}$ agonists. As

indicated previously, the involvement of the serotonergic pathway in kainate induced behavioural disturbances and toxicity has not been fully examined and work is underway to evaluate the importance of these behavioural disturbances in kainate induced neurotoxicity.

To determine whether there is any loss or impairment of memory and cognitive function in the kainate model and what, if any, effect neuroprotective agents have on any impairment, complex studies need to be initiated. The most widely used is the Morris water maze, (Morris *et al.*, 1986; Rogers & Tilson, 1990) which examines spatial memory. This model allows for the manipulation of both the learning and retrieval mechanisms, in that NMDA antagonists block learning but not retrieval. This technique was unavailable during the course of the study, but further work should be performed in conjunction with both behavioural and learning models.

5.2 Neurotoxicity.

5.2.1 Glutamate and Kainate Mediated Toxicity.

Intraperitoneally administered kainic acid causes an early increase in local cerebral glucose utilisation, increased neuronal firing, transient permeabilisation of the blood brain barrier and tonic seizures, with associated behavioural disturbances (see Heggli *et al*, 1981; Lothman & Collins, 1981). During this stage there is a transient increase in extracellular glutamate, similar to that seen in ischaemia, probably caused by activation of presynaptic receptors (Ferkany *et al*, 1982; Benveniste *et al*, 1984; Globus *et al*, 1988, 1990, 1991). The released glutamate acts on receptors located both on neurones and glia, (Ferkany *et al*, 1982; Ferkany & Coyle, 1983a,b) to cause the further release of neuroactive substances and depolarisation of the postsynaptic membrane through NMDA

and non-NMDA receptors (Choi, 1988; Choi & Rothman, 1990). Overstimulation of the NMDA receptor is thought to be a critical step in the initiation of excitotoxicity (Butcher *et al,* 1990; Globus *et al,* 1990) and several groups have shown that NMDA antagonists can block kainate evoked neurotoxicity (Fariello *et al.*, 1989; Lerner-Natoli *et al.* 1991; Wolf *et al.*, 1991). The pattern of cell damage which results from this sequence has been detailed in histological work by several groups (Schwob *et al*, 1980; Heggli *et al,* 1981; Lothman & Collins 1981; Altar & Baudry, 1990).

Kainate causes the release of radiolabelled and endogenous excitatory amino acids from intact neurones or synaptosomes (Ferkany *et al.*, 1982; Ferkany & Coyle, 1983a,b; Notman *et al.*, 1984; Gallo & Levi, 1986; Hamberger *et al.*, 1986; Roberts, 1986; Connick, 1987) an effect which may contribute substantially to its excitotoxic properties (McGeer *et al.*, 1978). As well as the release of amino acids, ischaemia causes an increase in extracellular purines (Berne *et al.*, 1974; Winn *et al.*, 1979; Zetterstrom *et al.*, 1982; van Wylen *et al.*, 1986; Hagberg *et al.*, 1987; Phillis *et al.*, 1987, 1988, 1989; Andine *et al.*, 1990; Phillis *et al.*, 1991). Hoehn & White (1990a) reported that elevation in extracelluar adenosine originated from two sources, receptor mediated release of adenosine and a calcium independent release of nucleotides that are broken down to adenosine. The calcium independent release appears to be dependent on glutamate uptake and may be from glial cells. Although this nucleotide component of the total releasable adenosine is small ($\approx 10\%$) it is probably more sensitive to ischaemia than the receptor mediated release, in that the high affinity glutamate uptake systems are down regulated by high extracellular glutamate concentrations (Silverstein *et al.*, 1986; Hu *et al.*, 1991). If, as it seems, there is a reduction in the glutamate uptake during a period of neurotoxicity and there is also a reduction in the adenosine release, then the control of

excitatory amino acid release will decrease and the neuronal damage increase accordingly.

However there is a recent report (Andersen *et al.*, 1993) that raises doubts over the importance of the high affinity glutamate uptake (HAGU) system in attenuating any glutamate induced damage, as it was found that the HAGU system was upregulated in the rat common carotid artery occlusion model used. Again, if this is the case, this highlights the problem of neurotoxicity, in that there are several models for focal ischaemia, several different ones for global ischaemia, and more pharmacological methods of causing damage, and direct comparison between models should be performed with a high degree of caution (see section 5.2.2).

It is possible, however, that any drug, or combination of drugs, that restores control of excitatory amino acid release will possess a neuroprotective component.

5.2.2 Global versus Focal ischaemia.

Although both global ischaemia (GI) and focal ischaemia (FI) result in neuronal death in a calcium dependent manner there is some debate over the differing roles of the NMDA receptor in these two cases. Both scenarios involve a substantial elevation in the extracellular glutamate concentration. The site of action of this glutamate is debated in the GI models. The general consensus is that there is non-NMDA receptor activation, and that non-NMDA antagonists (e.g. GYKI 52466 and NBQX) are neuroprotective (Sheardown *et al.*, 1990; Buchan *et al.*, 1991; Judge *et al.*, 1991; Diemer *et al.*, 1992; Le Peillet *et al.*, 1992; Phillis *et al.*, 1993; Pulsinelli *et al.*, 1993; Sheardown *et al.*, 1993). However it is the NMDA receptor involvement that is uncertain with several reports that find that MK801 is ineffective in severe GI models (4 vessel occlusion (4VO) or bilateral carotid artery occlusion (BCAO)) (Buchan & Pulsinelli, 1990; Diemer *et al.*, 1993; Pulsinelli *et*

al., 1993; Sheardown *et al.*, 1993), but Lin *et al.*, 1993 found some effect in a less severe form of the 4VO GI model.

There are several reports that in the common carotid artery occlusion GI model MK801 is effective at doses compareable to FI models, (Lawerence *et al.*, 1987; Gill *et al.*, 1988; Kato *et al.*, 1990), although Corbett *et al.*, (1990), Buchan *et al.*, (1991b) and Yao *et al.*, (1993) failed to obtain significant neuroprotection. As well as MK801, the GABAergic agent CMZ is also able to distinguish between GI and FI models (Cross *et al.*, 1991).

A possible explaination for the differing actions of the NMDA receptors in the GI and FI models probably involves extracelular acidosis. In the GI model the CSF becomes acidic (ph $\leq$7) and there are reports that the NMDA receptor is desensitised by H+ ions (Morad *et al.*, 1988; Gifford *et al.*, 1990; Tombaugh & Sapolsky, 1990) and this may explain the differences. If this is the case, this would suggest that for GI cases (i.e. cardiac arrest) non-NMDA receptor antagonists are more beneficial than NMDA receptor antagonists (Fleischer *et al.*, 1989; Buchan *et al.*, 1991a; Pulsinelli *et al.*, 1993; Sheardown *et al.*, 1993).

5.3 Kainic Acid Induced Glutamate Release

5.3.1 Endogenous Release Experiments.

In the present system 50mM KCl was unable to release detectable amounts of excitatory amino acids from hippocampal slices, a finding that is at odds with previous reports that endogenous glutamate is releasable from preparations prepared from various brain regions (Ferkany *et al.*, 1982; Collins et al., 1983; Ferkany & Coyle, 1983a, 1983b; Nicholls et al., 1987; Sanchez-Prieto *et al.*, 1987; Nadler et al., 1990; Martin et al.,

1991; Rubio et al., 1991; Palmer *et al.*, 1992; Raiteri *et al.*, 1992; Solyakov *et al.*, 1992). The probable reason for this discrepancy is that the HPLC detector used in the present study was not sensitive enough to measure the small amounts released (fmoles). A further complication with the system was that the signal:noise ratio was such that there was little reproducibility in the peak area of the standards below 10pmoles (amount injected into the HPLC column). These problems prompted the use of [^{3}H] glutamate instead of endogenous glutamate, even though there were reports in the literature that events controlling exogenous transmitter release may be different from those controlling endogenous release (Ferkany & Coyle 1983a,b, Palmer *et al.*, 1992; Solyakov *et al.*, 1992).

5.3.2 [^{3}H] Glutamate Release Experiments

As mentioned in an earlier section (5.2. Kainate toxicity), kainate causes glutamate release (both endogenous and tritiated) from *in vivo* hippocampal preparations (Ferkany & Coyle 1983a,b; Roberts, 1986; Connick; Palmer *et al.* 1992). Using a method that was modified from Ferkany & Coyle, (1983a), Connick (1987) and Palmer *et al.* (1992) it was possible to cause the release of [^{3}H] from hippocampal slices in a dose dependent manner. However, there was no significant attenuation of the release caused by 5μM 2CA. This does not mean though that R-PIA is not acting by attenuating the release of glutamate, as only a single concentration of 2CA was used, and there was a large degree of variability, between slices from the same animal and also between experiments. Even if the S2/S1 or S3/S2 ratios were used (a method that usually reduces the variability between experiments, S. Wonnacott, personal communication) the degree of variability was still too great. This probably suggests that there is either an instrinsic flaw in the method used, or that the

exogenous transmitter pool is different from that of the endogenous pool. Indeed there are reports in the literature that indicate that there are problems measuring exogenous transmitter release (Ferkany & Coyle, 1983a,b) caused by kainate stimulation. It is concluded that for a clearer evaluation of R-PIA action in affecting glutamate release endogenous glutamate (and/or aspartate and glycine) should be measured using an improved HPLC system. During an ischaemic insult the purines do appear to reduce the release of either glutamate or glycine (Baker *et al.*, 1991; Cantor *et al*, 1992), and it is likely that R-PIA also does this in the kainate model.

5.3.3 [^{3}H] Glutamate Release; Kainate and Potassium Stimulation.

Using [^{3}H] glutamate, instead of endogenous glutamate, KCl was able to stimulate release from hippocampal slices at the same flow rate used in the endogenous studies ($1ml.min^{-1}$), in a manner that suggested dose dependency. As expected the S2/S1 ratio was approximately 0.80, with the S3/S1 about 0.60 agreeing with other reports on [^{3}H] transmitter release (e.g. Chen *et al.*, 1992; S. Wonnacott personal communication). The findings that KCl is able to stimulate exogenous glutamate release from the hippocampus agrees with reports in the literature (Ferkany *et al.*, 1982; Ferkany & Coyle, 1983a,b; Connick 1987; Palmer *et al.*, 1992).

Kainate was also able to stimulate [^{3}H] glutamate release from hippocampal slices, at both 1 and 5mM (concentrations similar to those used in other reports (Ferkany & Coyle, 1983a,b; Roberts, 1986; Coyle, 1987; Gannon & Terrian, 1991; Palmer *et al.*, 1992)), and this was dose dependent (5mM > 1mM). The amount released by 5mM kainate was not significantly different from the 50mM KCl stimulation (S1 and S2, see table 4.2.1.1). What was unexpected was that in the 5mM kainate treated slices, the S2

stimulation appeared to release more transmitter than the S1, with the ratio of S2/S1 = 1.38 ± 0.44 compared to the KCl S2/S1 ratio of 0.69 ± 0.55 (50mM) and 0.58 ± 0.12 (25mM), although this was only significant against the 25mM KCl group. The actual release of S2 was not significantly different from that of the S1 in the 5mM kainate treated slices though. The kainate treated slices also displayed a period of heightened release just after S1 that deviated significantly away from the predicted basal release. The amount of glutamate released during this period was dose dependent with the 1mM kainate not being significantly different from the KCl treated slices, but the 5mM kainate releasing significant amounts (greater than 250% S1) compared to either the 1mM kainate or the 50mM KCl treated slices (see sections 4.2.1 and 4.2.2). Kainate did not appear to be delaying the return to basal levels, which would be seen as a tailing off of the peak, but rather as a decrease after the peak and then a humpback shape that slowly returns to basal, in a manner that appears linear. A complication in evaluating this decay of the hump is that S2 usually occurs just after the decay has reached basal. It may have been interesting to determine the time course of this hump, but during the course of the experiments it was not considered important enough to investigate; this may have been an error.

There are several reports that kainate inhibits KCl evoked release of glutamate from synaptosomes (Solyakov *et al.*, 1992, Barnes & Henley, 1994), although the report of Collins *et al.*, (1983) found that kainate enhances K+ evoked glutamate release from rat olfactory cortex. In the present study 5mM kainate did not inhibit 25mM KCl evoked glutamate release, although the release pattern (5mM KA/25mM KCl) was similar to KCl treated slices and not kainate treated slices (i.e. S1 > S2 > S3, with no S1 hump). This was unexpected, in that the slice either behaved as a KCl or a kainate treated slice, with the KCl effect dominant over the kainate effect, and the total release was similar to 25mM

KCl and not 5mM kainate.

Neither this potentiation of kainate evoked glutamate release, nor the period of heightened release following S1, appears to have been reported before, with previous studies usually only performing a single stimulation (Matsui & Yamamoto, 1975; Ferkany *et al.*, 1982; Collins *et al.*, 1983; Ferkany & Coyle, 1983a, 1983b; Sanchez-Prieto *et al.*, 1987; Palmer *et al.*, 1992; Raiteri *et al.*, 1992; Solyakov *et al.*, 1992). Another unexpected finding was that the 50mM KCl S3 stimulation in kainate treated slices was sensitive to the concentration of kainate used to stimulate S1 and S2 (5mM > 1mM), with the 1mM kainate stimulated slices releasing similar amounts to the 50mM KCl stimulated slices. Again, this potentiation of KCl stimulated release has not been reported.

A possible interpretation of these findings is that kainate may sensitise the slice, possibly in a manner analogous to LTP, such that more transmitter is released upon following stimulations. Another possible explanation is that the 5mM kainate is neurotoxic, even at the brief exposure, and that the slice has been damaged (either reversibly or irreversibly). This does not appear to be the case in that the baseline is relative stable and does not deviate away from an exponential decay curve. If the slice has been sensitised, what are the possible mechanisms of this process?

Kainate is a known high affinity glutamate uptake blocker, with activity in the micromolar range (IC_{50} 302μM, Johnston *et al,* 1979) and it is possible that this may be one of the mechanisms by which kainate is sensitising the slice. Evidence in favour of this hypothesis is the period of heightened release after the first kainate stimulation. This hump is dose dependent and the amount released significantly different from the 50mM KCl treated slices, as is the shape of this hump (see figures 4.2 and 4.4). A possible flaw in

this argument, though, is that this hump is not seen after S2 in either the 1mM or the 5mM kainate slices. The shape of the 'interpeak' period is similar to the KCl treated slices, indicating no period of heightened release. This disparity between the release after S1 and S2 also argues against an LTP like process, since the S2 would be expected to be both larger and longer lasting than the S1, whilst the release profiles suggest that the duration of the S2 peak is of the same time period as the S1 peak.

Another possible intrepretation of the hump is that the kainate (S1) stimulates the release of other agents apart from glutamate that initially heighten release, but also affects the synapses in such a way that either there are more kainate receptors available at the plasma membrane, and hence kainate is more effective in releasing glutamate, or that more glutamate containing synaptic vesicles are available for release. Another potential explanation is that kainate caused the release from a slowly replenished pool such that the S2 stimulation is unable to act on this pool as it is depleted. Such a pool could be found in the astrocytes, which are known to contain excitatory neuroactive compounds in discrete releasable pools (ie the sulphur containing amino acids (Griffiths, 1993) and quinolinic acid (Swartz *et al.*, 1989)). These compounds have been shown to be agonists for different types of glutamate receptors and hence release of some or all of these compounds may enhance release. Astrocytes have also been shown to release nonvesicular glutamate via the reversal of the reuptake system (Szatkowski *et al.*, 1990), a process that is thought to be the mechanism whereby neurones release glutamate in a calcium independent manner (Barrie & Nicholls, 1988; McMahon *et al.*, 1988; Wilkinson & Nicholls, 1988, 1989). This release from the astrocytes was by the reversal of the membrane potential in the *in vitro* studies, and this may occur by non-NMDA receptor stimulation of the astrocytes in vivo (Glaum *et al.*, 1990; Stephens *et al.*, 1993;

Holzwarth *et al.*, 1994; see Tiechberg, 1991) as well as by ischaemia, during which there is a change in the extracellular concentrations of several ions including potassium. The importance or degree of astrocytic involvement in the present system remaims to be determined.

Another possibility is that the kainate evoked release of glutamate is activating presynaptic mGluR's. There are several reports that either direct stimulation of mGluR receptors will stimulate release (Tibbs *et al.*, 1989; Herrero *et al.*, 1991a,b) or that arachidonic acid will potentiate mGluR activity, that in turn potentiates AP4 induced release (Nichols *et al.*, 1993, Sanchez-Preisto *et al.*, 1993). There are reports that glutamate causes the release of archidonic acid (Ueno *et al.*, 1988; Lazarewicz *et al.*, 1990; Lynch & Voss, 1990) and this may be occurring in the present system. The mGluR activation may have 2 effects on the slice, firstly to enhance stimulated release (the hump), and secondly to alter the synaptic cytoskeletal organisation such that more glutamate is available for future release. It may be that the mGluR receptors are desensitised/down regulated during the S1:S2 interpeak period and hence unable to enhance the release in successive stimulations.

Elevated extracellular potassium is thought to mediate neurotransmitter release by generalised depolarisation of the plasma membranes (both neuronal and non-neuronal). This may mean that as well as the excitatory transmitters, the potassium evoked release of inhibitory transmitters would antagonise the excitatory agents (causing the hump) released upon kainate stimulation. This might explain why slices stimulated with 5mM kainate and 25mM KCl behave as KCl stimulated slices and not kainate stimulated slices.

However, before the mechanism of enhanced release is determined, it is important to determine that the enhanced [^{3}H] release is glutamate and not glutamine. Glutamate is

converted to glutamine in astrocytes, which is in turn released into the extracellular space to be up taken at the synapses. It is possible, therefore, that the enhanced [^{3}H] release is just glutamine leakage. Whilst it is possible, the evidence to date does not confirm this in that the hump release is greater than S1 evoked release (greater than 250% of S1). At present the cause of this hump is not known, or the mechanism whereby kainate enhances further stimulations.

5.4 Adenosine

5.4.1 Release

Adenosine is present in both the intracellular and extracellular fluid, although it is concentrated intracellularly. Adenosine can be released through calcium dependent and calcium independent mechanisms, and there appear to be several possible sources for the former. Adenosine itself can be released following neuronal activity (Jhamandas & Dumbrille, 1980; Phillis *et al.*, 1989; Hoehn & White, 1990a,b; Chen *et al.*, 1992, Sciotti *et al.*, 1993; Ogilvy *et al.*, 1994), or it could be formed by the breakdown of released ATP, which is present in most types of neurotransmitter vesicle.

Hoehn & White (1990a) reported that *in vitro* adenosine is released in a calcium independent manner during the reuptake of glutamate in hippocampal slice preparations, and Chen *et al.*, (1992) and Ogilvy *et al* (1994) have shown that stimulation of either the NMDA or the kainate receptors *in vivo* will result in an elevation of extracellular adenosine. However in the case of ip kainate Ogilvy (personal communication) reported that the total elevation is only 35pmol in 4 x 20μl samples (175% control) and this is lower than the extracellular elevations of adenosine reported to occur during and after ischaemia (e.g. 2,650% basal, Phillis *et al.*, 1991). This discrepancy between the

ischaemic induced and the kainate induced elevations in adenosine (10-20 fold) may explain the inability of DPCPX to potentiate kainate induced toxicity (reported here) and the findings of Rudolphi *et al.*, (1987), and Simpson *et al.*, (1992) that theophylline potentiated ischaemic damage. Lekieffre *et al.* (1991) reported that inhibition of A_1 receptors by theophylline ($32mg.kg^{-1}$) enhanced glutamate release ($\approx 200\%$), but the enhanced release did not affect the degree of neuronal damage. The role and mechanism of the adenosine A_1 receptor modulating brain damage needs to be further examined.

5.4.2 Temperature

It has been known since the 1950's that patient outcome is improved following neurosurgery if the core body or brain surface temperature is cooled (Lougheed & Kahn, 1955; Little, 1959; Negrin, 1961; Connolly *et al.*, 1962). Indeed, it has now become a standard operating procedure to cool the patient during the operation.

Dietrich *et al.* (1991) reported that during an ischaemic insult in an animal model there is an increase in brain temperature. Prior to this report there had been debate within the literature over whether some agents were neuroprotective, in that there appeared to be disparity when experiments were repeated by other groups. It now appears that protection is affected depending on whether the experiments are performed under homeothermic control.

A number of groups have claimed that changes in body temperature can have a profound effect upon the extent of neuronal damage following ischaemia (Welsh *et al.*, 1990; Buchan & Pulsinelli, 1990; Freund *et al.*, 1992; Dietrich, *et al.*, 1993b; see Dietrich, 1992; Ginsberg *et al.*, 1992,1993; for review). Mitani & Kataoka (1991) have reported that hypothermia can also significantly reduce the neuronal release of glutamate

and aspartate and hyperthermia increases their release. The temperature changes required, however, seem to be substantial (4-6^{0}C), and the relevance of temperature has been disputed by others (Welsh & Harris, 1991; Gill & Woodruff, 1990).

Whether or not there is a change in body/brain temperature during excitotoxicity, the above mentioned report by Mitani & Kataoka (1991) had to be considered since the working hypothesis of kainate toxicity is based around the release of glutamate. Synaptic transmission in general is affected by hypothermia in that neurotransmitter synthesis is inhibited and release and reuptake are decreased due to reduced membrane fluidity (Vanhoutte *et al.*,1981; Boels *et al.*, 1985; Haikala *et al.*, 1986; Okuda *et al.*, 1986; Graf *et al.*, 1992). These were not the only considerations, as systemically administered purines can induce significant changes in body temperature to the extent that these may contribute, for example, to the anticonvulsant properties of purines (Bowker & Chapman 1986).

The data in the present study indicate that whilst there is a significant, if transient decrease in body temperature of kainate treated rats at 1 hour, this does not correlate to the final outcome of the level of [^{3}H] PK11195 binding. R-PIA does not alter significantly the body temperature of the animal (although it appears to be reducing the kainate induced drop at 1 hour). 8-PT appears to block the R-PIA action on temperature, but this block is not absolute, and the temperature drop is delayed by two hours. When R-PIA is absent, 8-PT is unable to significantly alter the body temperature in any way, although there is an upward trend, with a non-significant reduction at 1 hour. Whilst there was an underlying trend towards a correlation between increasing temperature and increasing binding, this is only significant at 5 hours (figure 3.12). The importance of these findings is unclear, with no significant changes of temperature observed at doses of R-PIA which provided substantial protection, making it unlikely that R-PIA is mediating its action by

whole body hypothermia in the present study. It should be noted though that there could have been small changes in the brain temperature caused by R-PIA which would not have been observed by measuring the rectal temperature. To examine whether there had been any change in brain temperature an implanted thermometer would have been required, and this would have entailed surgery and a major altering of the experimental protocol. For this reason, brain temperature was not examined.

A report by Liu *et al.* (1993) was published after completion of the temperature experiments which indicated that the kainate model of toxicity was also highly sensitive to changes in body temperature in that hypothermia of 8°C, to 28°C resulted in a 50% reduction in seizure duration and reduced neuronal loss in the hippocampal CA1 and CA3 layers in 12mg.kg^{-1} kainate treated rats. The 6mg.kg^{-1} kainate treatment resulted in a low seizure incidence rate without neurotoxicity. If the rats were treated with 6mg.kg^{-1} kainate and hyperthermia was induced (6°C to 42°C) this dose resulted in elevated seizure activity as measured by EEG and damage comparable to that induced by 12mg.kg^{-1} kainate in the hippocampus. In none of the animals in the study in the present report was there any large change comparable to those reported by Liu *et al.*, (1993) and so the relevance to the present study is still to be determined.

As the current hypothesis concerning ischaemic, or neurotoxin, induced neuronal loss is based on the observation that during a neurotoxic insult there is a period of elevated extracellular glutamate concentration, the report by Mitani & Kataoka (1991) indicates the problems in comparing the effects of neuroprotective agents in temperature regulated and unregulated experiments. Indeed, it is being suggested that attempts to prevent changes in brain temperature will impair the actual neurotoxic mechanisms that are being studied, hence nullifying the observations, and should not be performed.

5.4.3 Mechanism of Purine Action

5.4.3.1 Systemic Application of A_1 Agonists.

Adenosine or its more stable analogues have been shown to reduce neurotoxicity when co-administered into the hippocampus or striatum (Arvin *et al.*, 1989; Connick & Stone, 1989; Finn *et al.*, 1991). When PIA was administered peripherally by Connick & Stone (1989) there was a potentiation of quinolinic acid induced damage, but this was thought to be due to hypotension, as it was possible to mimic this 'toxicity' with ganglion blockers. Roussel *et al.* (1991) reported that R-PIA did not protect against focal cerebral ischaemia induced by main carotid artery occlusion in spontaneous hypertensive rats (SHR). However, there are several differences in the pathological consequences in focal cerebral ischaemia between SHR and normal rats including a larger area of damage in SHR as well as a reduced effect of R-PIA on blood pressure. There are, however, reports that cyclohexyl-adenosine or the adenosine precursor 5-aminoimidazole-4-carboxamide riboside (AICAR) can reduce the degree of neuronal damage following periods of cerebral ischaemia (von Lubowitz *et al.*, 1989; Clough-Helfman & Phillis, 1990; Forlon et al., 1994), or enhance extracellular adenosine levels (White, 1994), findings which prompted the present study to reinvestigate the effects of a systemically administered purine upon neuronal damage. The results reveal that R-PIA was able to protect substantially against the kainate induced damage, at a single administration of $10\mu g.kg^{-1}$, and that $25\mu g.kg^{-1}$ was able to protect when administered at any time 2hour before to 2 hours after kainate administration. At a higher dose of $1mg.kg^{-1}$ in combination with kainate, all treated animals died. An intermediate dose of $100\mu g.kg^{-1}$ produced neuroprotection that was indistinguishable from that of $25\mu g.kg^{-1}$, but with a 33% fatality rate.

The reports by Gustafsson and coworkers (Gustafsson *et al.*, 1989 and Wiklund *et al.*, 1989) indicate that there may be multiple subtypes of the Adenosine A_1 Receptor, differing in their K_D's for R-PIA, with what they term A_{1a} being found centrally (K_D 1.2nM) and A_{1b} being found peripherally (K_D 17.3nM). It is interesting to note that it was the higher dose of R-PIA that increased the mortality rate associated with the $10mg.kg^{-1}$ kainate and that the 'peripheral' A_1 Receptor subtype has a higher K_D than that of the 'central' A_1 Receptor subtype. One of the problems in systemic administration of Adenosine A_1 agonists, to combat neurotoxic insults, is that activation of the peripherally located A_1 receptors produces respiratory and cardiac disturbances that are potentially fatal. If both the existence of two subtypes of adenosine A_1 receptor and the apparent neuro-protective action of adenosine A_1 agonists are confirmed, then centrally acting agonists to the A_1 receptor could be a feasible therapeutic tool for combatting some types of neuronal damage, although the 'effective therapeutic window' might be of shorter duration than is clinically useable (Stanimirovic *et al.*, 1994).

5.4.3.2 Systemic Application of the A_3 Agonist, APNEA

Only the Gustafsson group has been able to provide evidence for this A_{1a}/A_{1b} classification. An alternative explanation for the R-PIA induced toxicity is that it may be activating the recently cloned A_3 receptor (Zhou *et al.* 1992). The presence of an A_3 receptor and the finding that it is highly homologous (80%) with the A_1 receptor (Zhou *et al,* 1992) underlies the importance in synthesising an agonist that is specific for A_1 receptors, that is only centrally active.

This homology between the A_1 and A_3 receptors indicates that there is the likelihood of agonists/antagonists for the A_1 receptor acting on the A_3 receptor and vice

versa. The report by Fozard & Carruthers (1993) that R-PIA is able to activate the A_3 receptor in pithed rats, in conjunction with the observation that R-PIA augments the intrinsic kainate mortality rate at the higher doses used, prompted the study of the A_3/A_1 agonist APNEA. As with R-PIA there was a dose-dependent mortality rate, which appeared to be due to a marked increase in heart rate. The cause of this is unclear although it is postulated that APNEA could release histamine from mast cells (Hannon & Fozard, 1994; Fozard personal communication) which in turn affects heart rate. The intermediate dose ($1mg.kg^{-1}$) gave a level of binding that was not significantly different from the kainate group due to the low number of survivors (2/6 treated). The lower dose of $300\mu g.kg^{-1}$, however, gave both a small but significant protection and an improved survival rate (7/8). As APNEA is able to act on the A_1 receptor, and at the time of the experiment there was no A_3 specific antagonist, the A_1 specific antagonist DPCPX was used. Although DPCPX is able to cross the blood brain barrier, and hence would be unable to distinguish between peripheral or central receptors, it was chosen in preference to the peripherally specific 8-SPT (see later) as a personal communication indicated that 8-SPT was also able to antagonise the A_3 receptor (Professor R.A. Olsson, University of South Florida, Tampa USA).

Since the time of the experiments there have been reports of A_3 agonists and antagonists. Compounds with polar C8 substitutions are the most selective, with DPCPX being a weaker antagonist (Jacobson *et al.*, 1994; Fozard and Olsson personal communications). However, in the present study the dose of DPCPX used was that of $50\mu g.kg^{-1}$, a dose that does not potentiate kainate induced toxicity, but is able to prevent R-PIA protection. In this experiment it was found that DPCPX prevented any APNEA induced protection, suggesting that the protective action is caused by A_1 activation.

Further work with more specific A_3 agonists/antagonists in the presence or absence of A_1 receptor activation is required. It may be, through examination of the literature and with new and selective A_3 agonists and antagonists, that many of the peripheral actions ascribed to the A_1 receptor are mediated by the A_3 receptor.

5.4.3.3 Mechanism of Purine Mediated Protection.

Adenosine is found in nanomolar concentrations extracellularly in the brain (30-300nM, Ballarin *et al.*, 1991; Phillis *et al,* 1991) although the levels are raised substantially by ischaemia and glutamate receptor activation (Phillis *et al,* 1987; Hoehn & White, 1990a,b; Chen *et al,* 1992; Dagani *et al,* 1992; Ogilvy *et al,* 1994). Adenosine receptors (both A_1 and A_2) are found on neuronal and non-neuronal cells (Goodman & Snyder, 1982; Deckert & Jorgensen, 1988), as well as the vasculature and there are numerous reports that activation of A_1 receptors reduces ischaemic or excitotoxic brain damage (Arvin *et al,* 1989; Connick & Stone, 1989; von Lubitz *et al,* 1989; Alzheimer *et al,* 1991; Finn *et al,* 1991; Miller & Hsu, 1992; Rudolphi *et al,* 1992; Simpson *et al,* 1992).

Adenosine may provide protection by several mechanisms, including vasodilatation of the central and peripheral vasculature, hyperpolarisation of neuronal membranes, decreased oxygen consumption by decreased neuronal activity, increased glucose uptake, increased glycolysis in glia, as well as hypothermia (Nehlig *et al,* 1988; Miller & Hsu, 1992; Rudolphi *et al,* 1992; Tominaga *et al,* 1992). As there appears to be no significant change in rectal temperature following systemic kainate/R-PIA treatment in the presence or absence of 8-phenyltheophylline, it seems unlikely that in this model hypothermia has a significant role.

The mechanism of the protection by R-PIA is not clear from this study. Assuming the even distribution of R-PIA around the body, the lowest effective dose of 10 $\mu g.kg^{-1}$ would yield a mean tissue concentration of approximately 24nM. This is close to the low K_D values for the A_1 receptor (Gustafsson *et al.*, 1990; Baumgold *et al.*, 1992) and would be entirely consistent with an action of R-PIA which involved the selective activation of these sites. Baumgold *et al* (1992) reported that *ex vivo* binding of R-PIA in mouse brain gave a K_D of 1.5 $\pm$ 0.2nM and a B_{max} of 398 $\pm$ 29fmol.mg protein^{-1}, indicating that the R-PIA is able to cross the blood brain barrier and that the affinity of the adenosine A_1 receptors is such that, at the doses given during the present report, the receptor would be activated.

A large proportion of A_1 receptors is believed to be located on the terminals of excitatory neurones, including those releasing acetylcholine and glutamate (Goodman & Snyder, 1982; Spignoli *et al.*, 1984; Fastbom & Fredholm, 1985; Dolphin & Prestwich, 1985). Kainate causes the release of radiolabelled and endogenous excitatory amino acids from intact neurones or synaptosomes (Ferkany *et al.*, 1982; Notman *et al.*, 1984; Connick & Stone, 1988), an effect which may contribute substantially to its excitotoxic properties (McGeer *et al.*, 1978).

The most likely explanation for the protection by purines is a suppression of glutamate release. The mechanism of this suppresion is unclear, in that adenosine A_1 receptors have been shown to both reduce calcium current, by inhibiting activation of calcium channels (Schubert *et al.*, 1986) and by increasing potassium current thereby hyperpolaring the synaptic terminals, both of these methods would reduce glutamate efflux, and it is possible they are both activated at the same time.

Heron *et al* (1992) have reported that ip R-PIA (20 $\mu g.kg^{-1}$) given 30 minutes before and 10 $\mu g.kg^{-1}$ 30 minutes after significantly decreased glutamate release following a 20 minute period of ischaemia. Cantor *et al* (1992) reported that A_1 agonists also significantly reduced the ischaemic release of glycine in a dose dependent manner. The importance of glycine in potentiating glutamate toxicity has been examined by several groups (Lerma *et al,* 1990; Patel *et al,* 1990; Wood *et al,* 1992). Glycine decreases the rate of NMDA receptor desensitisation and the reports by Baker *et al,* (1991) and Globus *et al,* (1991) indicate that the elevation of extracellular glycine remains high longer than that of glutamate. The prolonged elevation of glycine may therefore contribute to the delayed neuronal loss reported by other groups (Nakano *et al,* 1990; Haba *et al,* 1991). It is unclear whether kainate itself causes the release of glycine.

Another possibility involves the transport of kainic acid or a derivative across the blood brain barrier. It may be that the action of R-PIA is that it is preventing/reducing the transport of the neurotoxic compound into the brain and this action is reversed by the antagonist, and this needs to be examined further, although based on the behavioural observations this is probably not the case.

Reports by Phillis' group (O'Regan *et al.*, 1992b; Simpson *et al,* 1992) indicates that the A_2 receptor activation may potentiate neurotoxicity following an ischaemic insult, in that the A_2 agonist CGS21680 and the inhibitor CGS-15943A enhanced and inhibited the release of glutamate elicited by 4VO ischaemia, respectively. In the present model it was not possible to examine whether any A_2 active agents affect neurotoxicity due to either lack of specificity (agonists) or their inability to cross the blood brain barrier (antagonists) even when there is a good A_2:A_1 ratio. Again this is an area that needs to be examined in more detail when specific agents become available that can easily traverse

the blood brain barrier.

5.4.3.4 Purinergic Modulation of Stimulated Release.

There are several reports in the literature that adenosine A_1 agonists are able to reduce glutamate release in *in vivo* or *in vitro* studies and in ischaemic preparations (Corradetti *et al.*, 1984; Fastbom & Fredholm, 1985; Burke *et al.*, 1988; Arvin *et al.*, 1989; Lekieffre *et al.*, 1991; Heron *et al.*, 1992; Miller & Hsu, 1992; Simpson *et al.*, 1992, Lloyd *et al.*, 1994). A_1 agonists are also able to inhibit the population peaks (PS) in hippocampal slices (Bartrup & Stone, 1988, 1990; Lupica *et al.*, 1992, 1994; Hosseinzadeh, 1994; Schubert *et al.*, 1994), with antagonists enhancing the PS.

In the present study 5μM 2-chloroadenosine was unable to attenuate either the kainate or the 50mM KCl evoked release, even though this concentration would completely abolish PS (Hosseinzadeh, 1994, & personal communication). It is possible, though, that in the present study the A_1 receptors are already fully occupied and active, even though the slices were prepared in the same way as electrophysiological preparations. The use of either an A_1 antagonist or adenosine deaminase was expected to answer this question, and the adenosine A_1 antagonist DPCPX was chosen in preference to adenosine deaminase. In electrophysiological studies cyclopentyl-theophylline (CPT) was able to potentiate PS size at a dose of 100nM (Hossein, personal communication), but as DPCPX is 10-20 fold more potent than CPT, a dose of 5nM was used in the present study. As shown in the results section DPCPX was able to attenuate kainate evoked release in one group of experiments (DPCPX washed out after S3) but not in the continuous DPCPX perfusion (present in S3 and S4). Given that the S3 of the continuous DPCPX perfusion has a large standard error of the mean (50% of the mean) it may be that the DPCPX

washout experiments are illustrating the action of DPCPX on kainate evoked release, but this is uncertain and no real conclusion can be raised on the action of DPCPX on kainate evoked release.

The findings that DPCPX significantly attenuated the 50mM KCl S4 stimulation in these slices was largely unexpected, in that Simpson *et al.*, (1992) found 0.1mg.kg^{-1} ($\approx$ 140nM, intrahippocampally, based on figures reported by Baumgold *et al.*, (1992)) inhibited ischaemia induced release, and the observations are as yet unexplained. Both the DPCPX washout and the DPCPX continuous perfusion experiments had the same effect on the S4, and this could be explained by the lipophilic properties of DPCPX, that would allow for DPCPX to be affecting the S4 even if the perfusion of DPCPX had stopped. Why is DPCPX inhibiting glutamate release when all the evidence suggested that it should be enhancing the release? Although there is no clear answer, it may be the interneurones that provide clues. Henry finds that (J.L. Henry, personal communication) hippocampal interneurones are inhibited by adenosine A_1 agonists. As most of the interneurones in the hippocampus are inhibitory, then the antagonism of the A_1 inhibition of the inhibitory neurones would result in a net increase in inhibitory transmitter release. Even though there are presynaptic A_1 receptors on excitatory neurones the disinhibition of the inhibitory system may result in a net inhibitory action of DPCPX provided the initial stimulus is submaximal. This is all conjecture, and the DPCPX effect may be an experimental artifact specific to exogenous transmitter release. Adenosine inhibits K^+-evoked potassium release of GABA from cerebral cortex slices (Hollins & Stone, 1980) but not from the CA1 region of the hippocampus (Burke & Nadler, 1988), and during ischemia in the cerebral cortex (O'Regan *et al.*, 1992a). To clarify the effect more experiments are required, studying the action of DPCPX, over a concentration range against different types of

stimulus, on endogenous transmitter release. The study of endogenous transmitter release would answer the question of disinhibition of the inhibitory system since DPCPX would be expected to enhance GABA release. Another possible explanation is suggested by O'Regan *et al.*, (1992) and Simpson *et al.*, (1992), in that there might be an A_2 mediated component to glutamate release and that DPCPX is affecting that. Use of specific A_2 agonists may clarify the situation.

In conclusion high KCl and kainate are able to release [^{3}H] glutamate in a concentration dependent manner. However the pattern of release differs between the two stimuli, with kainate enhancing [^{3}H] glutamate release by an unknown mechanism, and this effect is not seen in slices treated with either a KCl stimulation or a kainate/KCl stimulation. More work is required to clarify the mechanism of enhanced release, and also the paradox obtained when slices were perfused with DPCPX.

5.4.3.5 Time Course of R-PIA Protection.

When adenosine A_1 agonists have been used to protect against ischaemic damage they have been almost invariably administered prior to the onset of ischaemia (von Lubitz *et al,* 1989; Cantor *et al*, 1992; Simpson *et al,* 1992; see Rudolphi *et al,* 1992 for review). In the present work the effective window of protection was found to span 4 hours (-2 to +2h). Protection obtained by R-PIA administration before kainate is probably due to the lipophilic properties of the purine and its persistence in the brain. The protection within the first hour after kainate injection is probably due to the reduction in glutamate and glycine efflux reported by other groups (Cantor *et al*, 1992; Heron *et al*, 1992). The time course of kainate penetration into the CNS and of induced glutamate release has not been reported, but is probably quite rapid in view of the fact that wet dog shakes are seen

in all animals within 40 minutes of systemic administration (Lothman & Collins, 1981). Seizure activity has a longer onset period than wet dog shakes (Heggli *et al,* 1981; Lothman & Collins, 1981) and thus may require higher intracranial kainate concentrations. When kainate 10mg.kg^{-1} is injected ip into anaesthetized rats there was a significant increase in intrahippocampal adenosine release as assessed by microdialysis (H.V. Ogilvy personal communication). This increase in adenosine occurred in the 2nd 20 minute fraction after kainate injection and lasted for a further 3 fractions, although none of the individual fractions were significant. The shape of the adenosine release resembled a bell shaped profile rather than the sharper profiles obtained by intrahippocampal kainate, indicating that the kainate was still exerting its effect for about 80 minutes after the injection. However, as these animals were anaesthetized with urethane, it is difficult to make a direct comparison with conscious animals.

5.4.3.6 Xanthine Blockade.

The protective effect of R-PIA appears to be mediated by a conventional xanthine sensitive P1 purine receptor (Burnstock, 1978; Stone & Simmonds, 1991) since protection was fully prevented by co-administration of 8-phenyltheophylline. Indeed there was an apparent tendency towards an increase in damage in the presence of this xanthine, though the effect was not large and was not statistically significant. This tendency might nevertheless indicate some blockade of an endogenous adenosine mediated neuroprotective tone, and would be consistent with the finding that theophylline itself increases the amount of neuronal damage following cerebral ischaemia (Rudolphi *et al.*, 1987).

5.4.3.7 Central Versus Peripheral Blockade of Adenosine A_1 Receptors.

DPCPX is a highly selective A_1 antagonist (Bruns *et al*, 1987) which has low nanomolar affinity for the receptor (K_i =0.46nM). A recent report by Baumgold *et al*, (1992) indicates that it can cross the blood-brain barrier. A dose of 250 $\mu g.kg^{-1}$ DPCPX would be similar to an intracranial concentration of 0.34μM as calculated by Baumgold *et al*, (1992), and should completely block all A_1 receptors with a degree of A_2 blockade as well. At this dose as well as at the lower dose of 50$\mu g.kg^{-1}$ here, which should block the A_1 sites only, the present results demonstrate a complete inhibition of the neuroprotection produced by R-PIA. This would be entirely consistent with the role of A_1 receptors in mediating inhibition of endogenous amino acid release.

8-SPT was administered to determine the importance of peripheral vasodilatation on the neuroprotective effect of R-PIA, since 8-SPT cannot readily cross the blood-brain barrier: Baumgold *et al.*, (1992) reported that there was less than 5% penetration into the brain after the administration of 50mg.kg^{-1}. In the present work there was no effect of 8-SPT on [^{3}H] PK11195 binding when administered in the absence of kainate and this dose was unable to block the R-PIA protection.

When 8-SPT was administered with kainate and clonazepam the elevation in [^{3}H] PK11195 binding was highly significant when compared to the kainate and clonazepam group. One possible explanation for this could be that 8-SPT is causing vasoconstriction which decreases the blood flow to the brain and increases neuronal metabolic stress and cell damage. An alternative explanation might be that as kainate causes a transient permeabilisation of the BBB (Zucker *et al*, 1983; Saija *et al*, 1992) this permeabilisation would allow the 8-SPT to cross the BBB and enhance the neurotoxicity in a manner similar to that reported by Rudolphi *et al*, (1987) for theophylline and DPCPX reported

here. The main difficulty with such an explanation is that it does not explain why 8-SPT did not prevent the protection by R-PIA. Therefore it is suggested that 8-SPT does not penetrate into the CNS, but that it is a blockade of vascular adenosine receptors, leading to a degree of vasoconstriction, which enhances kainate toxicity. Such a possibility would be consistent with previous indications that any fall in blood pressure, and thus cerebral perfusion can potentiate excitotoxicity (Connick & Stone, 1989). Since neuronal depolarisation by kainate would result in the release of adenosine locally, the greatest influence of 8-SPT would be expected to be in areas most susceptible to the toxin. There may also be some potentiation of damage due to the increased brain reperfusion as the 8-SPT blockade of adenosine receptors decays. Such reperfusion damage may be due to the formation of free radicals (see later).

The idea that peripheral blockade may be potentiating toxicity gains further support when the degree of damage caused by kainate and adenosine antagonists are expressed as a percentage of the same day kainate binding (figure 3.17). In this case 250μg.kg^{-1} DPCPX, 1mg.kg^{-1} 8-PT and 20mg.kg^{-1} 8-SPT all give similar degrees of damage (approximately 150% kainate binding). As the DPCPX response is only seen at the highest dose, it may suggest that this is a non A_1 response and may be due to blockade of either the A_2 or A_3 receptors. 8-PT also has some A_2 action at the dose used in the present study, and the reports in the literature indicate that the A_3 receptor is sensitive to 8-SPT blockade. All this indicates that blockade of peripheral adenosine receptors (probably not the A_1 subtype) potentiate damage caused by kainate. However, the finding that the blockade of the R-PIA response by DPCPX is dose dependent raises the question of whether adenosine is an endogenous neuroprotectant, as doses of DPCPX that block the R-PIA responses should also potentiate toxicity and this dose not appear to be the case.

A possible answer for this is that although kainate does stimulate the release of adenosine in the hippocampus (Ogilvy *et al.*, 1994), the levels achieved are insufficient to provide significant protection. Another point that should be borne in mind is that the half-life of R-PIA is probably far greater than that of adenosine in the synaptic gap, and hence the 'pharmacological window' is far greater than that of adenosine. This may mean that whilst activation of central A_1 receptors provides neuroprotection, blockade of these receptors does not potentiate damage and the converse is true for the peripheral receptors.

5.4.3.8 Conclusions from the Purine Experiments.

In summary the present results indicate that the neuroprotective action of systemically administered R-PIA is via a centrally located A_1 receptor with little or no contribution from the A_2 or systemic A_1 receptors at the microgram doses used in the present study. The finding that single low doses of R-PIA are neuroprotective up to 2 hours prior to or following cerebral insult may suggest the use of selective, lipid-soluble A_1 agonists to be used prophylactically or post-ischaemically as they do not affect the basal release of either the excitatory amino acids or glycine (Cantor *et al,* 1992). Although high doses of APNEA (A_3 agonist) are able to provide a degree of neuroprotection this appears to be acting via the A_1 receptor as well.

5.5 Other Possible Neuroprotective Drugs.

It appears that there are several possible sites of action, whereby drugs can either reduce or abolish the damage caused by overstimulation of the glutamatergic system. Possible pharmacological targets for therapeutic manipulation that have already been investigated include the NMDA receptor antagonists, calcium channel blockers, $GABA_A$

agonists, $GABA_B$ antagonists and free radical scavengers. There is increasing interest in the role of both dopaminergic and serotonergic pathways during periods of ischaemia, and whilst these areas will also be discussed, it will not be to the same degree as the aforementioned targets.

5.5.1 NMDA Antagonists.

The release of glutamate by kainate is considered to be the essential element in the current mechanism of kainate toxicity. Analogy with the ischaemic studies indicates that it is the high extracellular glutamate that causes neurotoxicity, and that this glutamate is mediating its toxicity through the activation of NMDA receptors (see Introduction - Neurotoxicity). Previous studies have shown that the blockade of the NMDA receptor channels will reduce/abolish the ischaemic damage, but that these drugs tend to possess a relatively short ($\leq$5hours) duration therapeutic window (Choi & Rothman, 1990; McDonald & Nowak 1990; McCulloch, 1991). Several reports (Clifford *et al.*, 1990; Wolf *et al.*, 1991) have implied that systemically applied kainate induced toxicity can be blocked by noncompetitive NMDA antagonists (MK801 and $MgSO_4.7H_2O$ respectively), indicating an NMDA receptor involvement, although kainate itself is not able to act directly upon NMDA receptors (Stone *et al.*, 1981; Watkins & Evans, 1981).

Using the [^{3}H] PK11195 assay to evaluate the degree of involvement of the NMDA receptor in the mechanism of kainate induced toxicity, 3 different classes of synthetic/exogenous NMDA antagonists were used (CPP - competitive, MK801 - noncompetitive, and $MgSO_4.7H_2O$ - channel blocker). As predicted all these agents were neuroprotective, and as CPP is highly specific for the NMDA receptor, this supported the earlier findings that there is an NMDA component to kainate toxicity. What was not

expected was that at the highest doses tested they all afforded virtually complete protection.

Moncada *et al.*, (1991) reported that the competitive NMDA antagonist, CPPene, was effective against centrally administered kainate. This report together with those of Fariello *et al.* (1989), Clifford *et al.* (1990) and Wolf *et al.* (1991) would suggest that both routes result in the overstimulation of the NMDA receptor, and, by analogy with the ischaemic models, would mean that the kainate model is an alternate model for ischaemic study.

The reports by Bartrup & Stone (1988, 1990) indicate that adenosine binding to the A_1 receptor requires magnesium, whilst Lloyd *et al.* (1989) found that the effect of 2CA on neonatal rat spinal cord was magnesium sensitive. If this is true *in vivo* as well as *in vitro* then there may be an A_1 component to the $MgSO_4.7H_2O$ protection as well as just the known NMDA receptor blockade. Further work would be required to determine whether this is the case.

5.5.2 Tryptophan Metabolism.

Various groups have reported that products from the metabolism of tryptophan confer varying degrees of protection against excitotoxic insult (Germano *et al*, 1987, Lekieffre *et al*, 1990, Wu *et al*, 1991). Tryptophan is a starting product for several pathways, two of which are the kynurenine and serotonin pathways. In the kynurenine pathway tryptophan is converted to quinolinic acid through several enzymic reactions (see figure 5.1). The products of several of these reactions are reported to be neuronally active, with possible neuromodulatory actions. Kynurenine and kynurenic acid are both reported to be antagonists to the glycine modulatory site of the NMDA receptor (Kessler

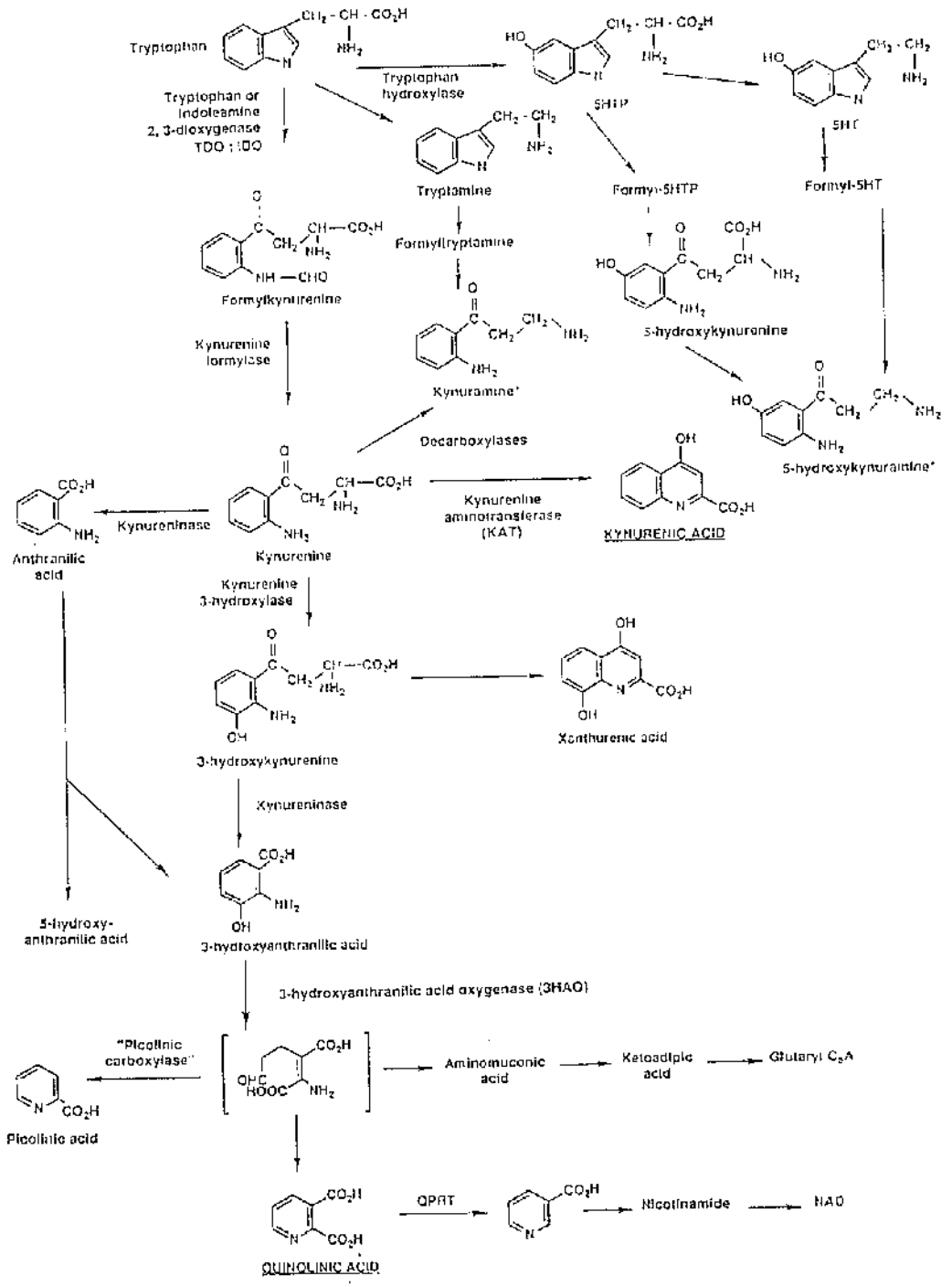


Figure 5.1.

Enzymic pathways of tryptophan metabolism, and kynurenic acid and quinolinic acid synthesis.

et al., 1987; Watson *et al.*, 1988; Kessler *et al.*, 1989), whilst quinolinic acid is an agonist at the NMDA receptor (Perkins & Stone, 1985). If the activity of some of the enzymes were altered then the system might produce an overabundance of either an agonist or antagonist to the NMDA receptor. In the case of Huntington's Chorea there is a reported increase in the amount of kynurenic acid in the primary motor cortex (Connick *et al.*, 1989), although Beal *et al.*, (1990) failed to observe these changes. Reports of quinolinic acid concentrations in the brain indicates that there may be no change in HD patients (Renoylds *et al.*, 1988; Schwarcz *et al.*, 1988).

In a recent study Fukui *et al.* (1991) determined the rate of transport of kynurenines across the blood brain barrier, and found that only tryptophan and kynurenine could cross at useful therapeutic rates. Swartz *et al,* (1990), showed that ip perfusion of either kynurenine or tryptophan produced a significant increase in extracellular kynurenic acid (as assessed by microdialysis and HPLC analysis of the perfusate). In the case of L-kynurenine, there was a 30-40 fold increase in basal levels with 100mg.kg^{-1} L-kynurenine 90-210 minutes after injection. 100mg.kg^{-1} L-tryptophan did not produce such a marked effect, a 10- fold increase being seen 6 hours after injection. In both cases the increase in kynurenic acid could be prevented by aminooxyacetic acid (AOAA), which is known to inhibit kynurenine transaminase (which converts kynurenine to kynurenic acid, Speciale *et al.*, (1990)). AOAA alone can cause lesioning, and this lesioning can be prevented by NMDA antagonists (McMaster *et al,*; 1991, Beal *et al,*; 1991), suggesting that under normal situations the kynurenate/ quinolinate balance is tightly regulated to prevent any excessive NMDA receptor activation. Systemic kainate has been reported to elevate the hippocampal kynurenic acid, not during the period of neurotoxicity but after 1 month, and as there is an invasion of glia following kainate induced toxicity this has

been suggested as the cause of the increase of kynurenic acid (Wu *et at.*, 1991). However several reports from Beal's laboratory (Swartz *et al.*, 1990; Miller *et al.*, 1992) indicate that administration of kynurenine and not kynurenic acid will increase intra-striatal kynurenic acid concentrations 30-40 fold within 2-3 hours. If the organic acid transport inhibitor probenecid is coadministered this increase was almost 600 fold (Miller *et al.*, 1992), indicating that the use of probenecid concentrates kynurenic acid in the extracellular fluid.

All of these findings prompted the investigation into whether systemically administered tryptophan metabolites would enhance the endogenous kynurenine levels and improve neuroprotection. Kynurenic acid was only used in a single pilot experiment, based on the dose regime used by Germano *et al.* (1987). In that report 300mg.kg^{-1} kynurenate was administered at $t_{-3\ hours}$, t_0 and $t_{+3\ hours}$, and provided 50% protection against bilateral carotid artery occlusion. In the present kainate model, though, there was no protection afforded, and the reports by Fukai *et al,* (1990) and Swartz *et al.*, (1990) that kynurenate is unable to cross an intact BBB provides a possible explanation for the differences. In an ischaemic model the BBB is rendered relatively permeable to blood-borne compounds, and although the report by Saija *et al,* (1992) indicates that kainate also causes a permeabilisation of the BBB this is only transient, and so kynurenate is unable to traverse it. This conclusion is supported by the observations reported earlier (section 4.3.3.5) that 8-SPT (another compound that is unable to traverse the BBB) is also unable to prevent R-PIA mediated neuroprotection in the kainate model.

The effectiveness of kynurenine was time dependent, with no protection seen when 150mg.kg^{-1} was administered at $t_{-4\ hours}$, but there was a dose dependent effect when administered at $t_{0\ hours}$. The t_0 administration gave significantly reduced levels of binding

at a dose of 75mg.kg^{-1} ie lower than the dose used in the $t_{-4\ hours}$ experiment. The finding that kynurenine was not neuroprotective when administered at $t_{-4\ hours}$ was unexpected, as reports in the literature indicated that there would be significantly elevated levels in the hippocampus by the time kainate would be expected to be acting. A re-evaluation of the $t_{-4\ hour}$ experiment in conjunction with intrahippocampal microdialysis in conscious animals might provide a possible explanation for this discrepancy.

Tryptophan was only tested at a single dose and time point 200mg.kg^{-1} at $t_{-4\ hours}$, and gave no significant neuroprotection, although the sample size was small. Further work is required to determine whether tryptophan does afford any neuroprotective activity, as the literature indicates, and both time course and dose response experiments need to be performed.

The location of kynurenine transaminase has recently been reported to be vesicular but glial, not neuronal, in origin (Roberts *et al.*, 1992; Du *et al.*, 1993), and also to be activated by neuronal signals (Turski *et al.*, 1989). Kynurenate has been reported to be released from this glial pool by glutamate (Wu *et al.*, 1992), and has led to the term 'gliatransmitters' being used for neuro-active compounds that originate from the non-neuronal pool. If the glia are releasing kynurenate following glutamate release, this would provide an intrinsic antagonist to switch off the NMDA receptors. Quinolinate is also reported to be released from glia (Speciale & Schwarcz, 1993) and would provide another pool of NMDA agonist. It may be that quinolinate is the natural agonist for the NMDA receptor activation, whilst the AMPA and kainate receptors are activated by glutamate, and this method would allow for NMDA receptor activation even during periods of low neuronal stimulation.

Several groups have reported that glia are able to synthesize and release sulphur

containing amino acids (SAA) (Grandes *et al.*, 1991; Tschopp *et al.*, 1992; Zhang & Ottersen, 1992, see Cuénod *et al.*, 1993), although Griffiths and co-workers (Dunlop & Griffiths, 1990; Grieve & Griffith, 1992; Griffith *et al.*, 1992) reported that SAA's are also derived from a neuronal source. There are several different SAA's and there are reports that they are agonists for different glutamate receptors (Dunlop & Griffiths, 1990; Ito *et al.*, 1991; Provoni *et al.*, 1991). Griffith's group (Dunlop & Griffiths, 1990; Griffith, 1993), have shown by HPLC analysis that several SAAs are released during periods of neuronal activation, and this, with Klincnik *et al.*, (1992), supports the earlier reports of Do *et al.* (1986a,b). The presence of several different glutamate agonists and kynurenate in releasable pools may allow for further specific activation of a receptor subtype, beyond that seen with the different affinities glutamate has for its receptors.

5.5.3 GABAergic Modulation.

During excitotoxicity the balance between the excitatory and inhibitory systems is altered, such that inhibitory systems no longer regulate the release of excitatory transmitters (Milgram *et al.*, 1991). This disinhibition of the inhibitory systems during periods of excitotoxicity could also be caused by over-stimulation, but in this case resulting in decreased inhibitory transmitter released. There are reports in the literature (Bliss *et al.*, 1986; Nadler *et al*, 1990) that during periods of high frequency stimulation of the Schaffer Collaterals, axons that run from the CA3 region and synapse on the CA1 dendrites in the hippocampus, there are both enhanced EPSPs and reduced IPSPs. The enhanced EPSPs have been shown to be due to activation of NMDA receptors while the reduced IPSPs are due to presynaptic $GABA_B$ receptor activation that reduce the release of GABA from the inhibitory interneurones (Bliss *et al.*, 1986; Davies *et al.*, 1990, 1991;

see Collingridge, 1992; Bliss & Collingidge, 1993 for reviews). This pattern will result in the formation of long term potentiation (LTP).

If this is the case in pathological circumstances as well, then drugs that enhance the inhibitory system might also prove to be effective neuroprotective agents. One such drug that appears to do this is chlormethiazole (CMZ). Although the actual site of action of this drug is unknown, it does potentiate the action of GABA and prolongs the channel opening of the $GABA_A$ receptor (Harrison & Simmonds, 1983, Moody & Skolnick, 1989; Cross *et al.*, 1989). At present this drug has been shown to provide protection at doses less than are used in present treatment for alcohol dependency (see Green & Cross, 1994 for review), and up to 6 hours post ischemia (both FI and GI models) (Cross *et al.*, 1991; Baldwin *et al.*, 1993a; 1994). When the drug was delivered against intrahippocampally injected NMDA, a focal ischaemic model, it was found to be less effective and have a shorter therapeutic window (Cross *et al.*, 1991). It was to see whether CMZ was effective against a model of global ischaemia that it was used in the present study.

CMZ at 100mg.kg^{-1} would provide virtually complete protection against MCAO induced ischaemia and in the present study also produced the same degree of neuroprotection (see figure 3.28). In the present study, though, there was also a marked sedation when administered with 1mg.kg^{-1} clonazepam. Rats are unable to maintain their body temperature when sedated and hypothermia is reported to offer a degree of neuroprotection (section 5.4.2). This means that in the CMZ treated animals some/all of the neuroprotection could have been through the reported hypothermic neuroprotective mechanism. Further studies need to be performed to see whether this is the case, with dose studies at different doses of clonazepam, as the sedation may have been potentiated

by the higher dose of clonazepam, and the temperature monitored. A further study was performed using 2 lower doses of CMZ (30 or 10 $mg.kg^{-1}$) in animals treated with 0.2$mg.kg^{-1}$ clonazepam. Again these animals showed a marked sedation, but this lasted less than 1 hour, and unlike the higher dose there were also observable behaviour disturbances that were similar to those of the kainate controls. The 30$mg.kg^{-1}$ dose gave approximately 75% protection, whilst the 10$mg.kg^{-1}$ was close to the estimated ED_{50} (10.635$mg.kg^{-1}$). This ED_{50} value of CMZ is lower than that reported by Cross and Green (personal communication) of 35$mg.kg^{-1}$ in an MCAO model.

However, the disinhibition of the GABAergic system is not only due to stimulation of the $GABA_B$ receptor but may also be by stimulation of the adenosine A_1 receptor (Prof. J. Henry, personal communication). Whilst this action of adenosine to inhibit the release of GABA would appear to be contradictory to adenosine's neuroprotective action it might indicate that the GABAergic system has only a transient protective effect (if any) and overstimulation would soon render the GABAergic system inoperable (as is seen in LTP , see earlier). This does not mean that GABAergic drugs are not potentially therapeutically useful against ischaemia, provided long lasting GABAergic agents are used, and indeed there are a few reports in the literature of $GABA_A$ agonists being used to reduce ischaemic brain damage (Brailowsky *et al.*, 1986; Sternau *et al.*, 1989; Hara *et al.*, 1991; Lyden & Hedges, 1992; Lyden & Lonzo, 1994).

5.5.4 Free Radicals.

It is known that during an ischaemic insult the glutamate is initially released from calcium dependent (vesicular) pools, but as the severity of the insult increases the calcium-independent pool (cytoplasmic/astrocytic?) of glutamate becomes the main source of

extracellular glutamate (Sandberg *et al.*, 1986; McMahon *et al.*, 1988; Wilkinson & Nicholls, 1988; Ikeda *et al.*, 1989; Wilkinson & Nicholls, 1989; Rubio *et al.*, 1991, Ogata *et al.*, 1992). In this type of insult NMDA antagonists are effective in reducing the size of infarct but are not able to provide complete protection. Indeed, all neuroprotective agents are only about 95% effective at best. This raises the question of why they are not completely neuroprotective if the damage is mediated through glutamate?

A possible answer is that there is a process that is not dependent on glutamate to cause neurotoxicity. Respiratory failure is a main candidate for this non recoverable component of neurotoxicity, although there has recently been interest in the role of free radical (FR) generation in neurotoxicity and respiratory failure (see Kogura *et al.*, 1985; Kontos & Povlishock, 1986; Schmidley, 1990; LeBel & Bondy 1991; Pazdernik *et al.*, 1992; Coyle & Puttfarcken, 1993; Evans, 1993; Olanow, 1993; Phillis, 1994; for reviews).

The current theories of excitotoxicity indicate that there is an increase in the activity of various enzymes involved in the production of FRs, e.g. phospholipase A_2 (Clemens *et al.*, 1991), superoxide dismutase (Lafon-Cazal *et al.*, 1993), thromboxane synthetase (Pettigrew *et al.*, 1989; Sadoshima *et al.*, 1989), xanthine oxidase (Kinuta *et al.*, 1989). Several vitamins are reported to be FR scavengers, either in the lipophilic or lipophobic enviroments (e.g. vitamin E and vitamin C respectively), and are neuroprotective (Sato *et al.*, 1993). Uemura *et al.*, (1991b) reported that after focal ischaemia there was a significant reduction in glutathione, cysteine and ascorbic acid. This means that during ischaemia, the limited FR scavenger systems are overloaded and the enhanced activities of the FR producing enzymes result in cytotoxic levels of FRs. In the present study only vitamin C was given to the animals, as they can synthesise both

vitamins naturally. Future studies should be performed using guinea pigs, as they lack the enzyme (gulonolactone oxidase) that converts L-gulonolactone to L-ascorbate.

In the present study vitamin C was found to confer neuroprotection in both a time and dose dependent manner, with maximal protection when administered at either $t_{-1\ hour}$ or $t_{0\ hour}$ at a dose of $50mg.kg^{-1}$ ip.

There are several possible mechanisms by which ascorbate is preventing kainate induced neurotoxicity, the first that kainate is being reduced by ascorbate to dihydrokainate, a compound that displays very little affinity for the kainate receptor (Ki = 7.4μM, London & Coyle, 1979), but is a HAGU inhibitor ($\approx$175μM, Bieziere & Coyle, 1978, Johnston et al., 1979). In this scenario, as the dose of ascorbate is increased the ratio of kainate to dihydrokainate decreases. At the low doses this would mean that glutamate is released by kainate and the re-uptake is inhibited by dihydrokainate, and hence increased neurotoxicity. At the higher doses though, when there is more dihydrokainate to kainate, less glutamate is released and so less neurotoxicity occurs, even though the re-uptake is inhibited. However, NMR studies performed by Professor Kirby (Department of Chemistry), indicate that ascorbate is unable to reduce kainate when analysed in saline for upwards of 5 days, and hence this scenario is unlikely, although direct measurement of kainate in the CNS would be required to confirm/refute this conclusion.

The second possibility is that ascorbate is preventing the penetration of kainate into the CNS and hence protecting neurones by preventing their exposure to kainate. Whilst microdialysate studies of CNS kainate levels are required the behavioural observations suggest that kainate is entering the CNS, as the animals display all the 'classical' kainate behavioural disturbances.

A third possibility is that ascorbate has a biochemical action itself. Ascorbate could be acting at several different locations both pre- and post- synaptically, including a) glutamate uptake (Cammack *et al.*, 1991, 1992), b) blockage of dihydropyridine calcium channels (Ebersole & Molinoff, 1992), c) the redox site of the NMDA receptor complex (Levy *et al.*, 1990; Aizenman & Reynolds, 1992) to reduce current (Majewska *et al.*, 1990) and d) a free radical scavenger (Sciamanna & Lee, 1993).

The electrophysiological results, performed by Dr. M.J. Higgins (Department of Pharmacology), suggest that the interaction with the NMDA receptor is not sufficient to prevent the induction and maintenance of LTP, ie the neuroprotective action is probably not through this mechanism, (although Majewskae *et al.* (1990) found that ascorbate inhibited NMDA currents at similar ascorbate concentrations). Beal *et al.* (1988) used intracerebral quinolinic acid to induce neurotoxicity in the cerebral cortex and found that neither allopurinol ($250mg.kg^{-1}$) nor ascorbate $100mg.kg^{-1}$ attenuated the damage significantly. Although we did not examine whether ascorbate is acting through enhancing the uptake system or blockade of calcium channels, the results of Beal *et al.* (1988) would suggest that this is not the case, in that the ascorbate appears to be acting at some point prior to NMDA receptor activation, although Majewska & Bell (1990) found that ascorbate protected cortical neurones from NMDA or glutamate mediated toxicity. Whilst it is not clear how ascorbate is exerting its neuroprotective action, the evidence to date thus suggests that ascorbate is acting as a free radical scavenger in the kainate model system.

There are various methods to generate free radicals, including the oxidation of xanthine to uric acid by xanthine oxidase to produce superoxide and the dismutation to hydrogen peroxide (reactions 1.1 to 1.3), the interaction of superoxide with nitric oxide

to produce hydroxyl radicals (reactions 1.4 and 1.5), and the interaction of superoxide with iron. This is possible if there is a haemorrhagic component to the ischaemic insult in which case the superoxide would be converted to the cytotoxic hydroxyl radical by the following reactions:

$$O^{\cdot}_2 + Fe^{3+} \text{ ---> } O_2 + Fe^{2+} \quad (5.1)$$
$$H_2O_2 + Fe^{2+} \text{ ---> } HO^{\cdot} + OH^{-} + Fe^{3+} \quad (5.2)$$

If the enzymes responsible for dismuting superoxide or hydrolysing hydrogen peroxide are inactive or functioning at reduced efficiency then there would be an increase in the intra- and extracellular concentrations of these toxic free radicals. This would place increasing importance on the free radical scavenging systems, and there is evidence that during a neurotoxic insult, these systems are overwhelmed (Uemura *et al.*, 1991b). There is increasing evidence to suggest that kainate toxicity is also mediated through the generation of free radicals (Dykens *et al.*, 1987; Putfarcksen *et al.*, 1993; Baran *et al.*, 1994). There are several pieces of circumstantial evidence to support this hypothesis. The first is that in familial ALS there is a subpopulation of sufferers who have mutations in the gene encoding the Cu/Zn superoxide dismutase enzyme (SOD1), although the result of the mutation is unclear (Rosen *et al.*, 1993). Non-NMDA agonists have long been implicated in both ALS and Guam Disease, and indeed one of the candidate 'envirotoxins' (BMAA) is an agonist for the AMPA receptor.

The second piece of evidence is the neuroprotective action of the xanthine oxidase inhibitor allopurinol and its metabolite oxypurinol. In the present study, allopurinol was able to prevent kainate induced neurotoxicity at the dose of 175mg.kg^{-1}, a dose that was ineffective in the report by Beal *et al.*, (1988). This result does, however agree with other reports that allopurinol, administered systemically, is able to attenuate the damage caused by a variety of neurotoxic insults including ischemia (Iansek *et al.*, 1986; Martz

et al., 1989; Mink *et al.*, 1991; Williams *et al.*, 1992) and kainate addition to cortical cultures (Dykens *et al.*, 1987).

However, allopurinol is not a specific inhibitor of XO and this may explain the lack of protection reported by some groups (Betz *et al.*, 1991; Hegsted *et al.*, 1991; Lindsay *et al.*, 1991). There are now several reports from Phillis' group that oxypurinol is neuroprotective (Lin & Phillis, 1991,1992; Phillis & Sen, 1993; Sen *et al.*, 1993; and given its greater specificty for XO than allopurinol (Spector, 1987), would indicate that XO generated FRs are probably involved in neurotoxicity.

5.6 Mechanisms of Kainate Toxicity.

5.6.1 Glutamate Mediated Component.

The current theory on the mechanism of kainate toxicity is that it is the release of vesicular glutamate that is neurotoxic. The kainate acts on presynaptic receptors to cause depolarisation and releases vesicular glutamate, which acts postsynaptically to activate the non-NMDA receptors and relieve the voltage block on the NMDA receptors. The hyperstimulation of NMDA receptors causes an influx of Na^+ and Ca^{2+} and through various steps eventual cell death (see section 1.3.3, figure 1.2 and table 1.1).

5.6.1.1 Evidence for Glutamate Component

There are two areas of evidence to support the involvement of NMDA receptors in the kainate toxicity. The first is that kainate releases glutamate in a dose dependent manner (Ferkany *et al.*, 1982; Notman *et al.*, 1984; Connick & Stone, 1988), and that in cell cultures the concentration achieved is sufficient to activate NMDA receptors and induce neurotoxicity. The second is that several reports (Fariello *et al.*, 1989; Clifford *et*

al., 1990; Lerner-Natoli *et al.* 1991; Moncada *et al.*, 1991; Wolf *et al.*, 1991; Lehmann *et al.*, 1993) including the findings in the present report, indicate that NMDA antagonists (MK801, TCP, CGS19755, CPP and $MgSO_4$) all attenuate the kainate neurotoxicity with their effectiveness dependent on the route of delivery of kainate and the target tissue (Rogers & Tilson, 1990).

5.6.1.2 Evidence Against Glutamate Component.

There are however several problems with this hypothesis. The main one is that the concentrations required for kainate to induce depolarisation in hippocampal slices (ED_{50} 1-10μM, (Stone, 1985,1990)) is low micromolar, whilst *in vitro* release of glutamate requires millimolar concentrations in hippocampal slice preparations (Ferkany & Coyle, 1983a,b; Palmer *et al.*, 1992), as well as in vivo kainate-evoked release (Hamberger *et al.*, 1986; Ogilvy *et al.*, 1994)

There are several possible explanations for these discrepancies between the biochemical and pharmacological methods. The first is that they are just variations dependent on the methodology, although this is probably not the case with the hippocampal slice preparation in that it requires micromolar kainate concentrations to depolarise but millimolar kainate to release glutamate (Ferkany & Coyle 1983a,b; Connick, 1987).

A second is that glutamate release is caused by activation of the low affinity kainate receptor (50mM, Henley, 1994), which may be the AMPA receptor, and that the 'true' mechanism of kainate toxicity is mediated through the high affinity kainate receptors (5nM) independent of vesicular glutamate release. This may explain why the striatum is not sensitive to systemic kainate but is to intracerebral or intraventricular injections, in

that higher concentrations would then be achieved at the site of injection, sufficient to initiate neurotoxicity mediated by the low affinity receptors.

A third possible explanation is that the release of glutamate is a secondary, not a primary consequence of kainate receptor activation. If this is the case, then the source of the released glutamate may not be vesicular in origin, but may be from blockade of the high affinity glutamate uptake (HAGU) systems. It is known that kainate blocks HAGU in a dose dependent manner (Biziere & Coyle, 1978; Johnston *et al.*, 1979) and if this is occurring in the in vitro preparations, then the increase in extracellular glutamate may be from a buildup in basal glutamate efflux and not stimulated release, this is unlikely though, given the concentrations used to inhibit HAGU (IC_{50} 302μM, Johnston *et al.*, 1979).

Another possibility is that the release of vesicular glutamate is not cytotoxic. Since a finite amount would be released, the uptake systems would still be functional, (provided the CSF is normal) and the kainate receptors would probably desensitise over a short period. If this is the case, then it argues for the involvement of non-vesicular glutamate for neurotoxicity. The probable source of this glutamate is the cytoplasmic pool ($\approx$ 10mM Nicholls & Atwell, 1990).

5.6.2 Free Radical Mediated Component.

The findings of Dykens *et al.* 1987, and ourselves that allopurinol is able to attenuate kainate induced neurotoxicity, indicates that superoxide generation by xanthine oxidase plays some role in the kainate neurotoxicity. Puttfarcken *et al.*, (1993) report that kainate is able to cause lipid peroxidation in cerebral granule cell culture, and by inference increased free radical production, although whether the free radical production precedes

or is in parallel to the lipid peroxidation is not known. All these findings suggest that kainate toxicity may be caused by processes that are occurring in addition to the glutamate release, as observed by Kato *et al.*, 1991.

5.6.3 Proposed Mechanism of Kainate Mediated Neurotoxicity.

The proposed mechanism of kainate toxicity in the hippocampus following systemic administration is as follows: kainate activates both pre- and postsynaptic receptors and results in a transient release of glutamate from presynaptic vesicular pools. This released glutamate is rapidly removed from the synaptic cleft before it reaches toxic concentrations by the active HAGU systems. At the same time as the glutamate release, the kainate receptors are causing activation of a calcium dependent protease to enzymically convert xanthine dehydrogenase to xanthine oxidase. The xanthine oxidase converts xanthine to uric acid and an FR anion (reaction 1.5). Due to some mechanism not yet understood, the free radical scavenger enzymes and systems are not able to cope with this increase in superoxide anions and hydroxyl radicals and singlet oxygen radicals start to form intracellularly. As the concentrations increase the phospholipid bilayers start to be permeabilised by uncontrolled peroxidation. This permeabilisation of the plasma membrane would allow the efflux of cytoplasmic glutamate into the synaptic cleft and the surrounding area. At this stage, the damage would be propagated both by kainate induced free radical formation and hyperstimulation of NMDA receptors. This mechanism would allow for the action of various agents that are neuroprotective against kainate neurotoxicity, eg RPIA, allopurinol, $MgSO_4$, MK801 and kynurenines. The adenosine A_1 agonist (RPIA) would be hyperpolarising both the pre- and post-synaptic membranes to prevent kainate induced depolarisation, whilst the A_1 antagonist DPCPX, would prevent

endogenous adenosine activation. Allopurinol would reduce the production of superoxide anions and ascorbate would oxidise any free radicals formed. The NMDA antagonists would be acting after the initial cell loss and would be attenuating the spreading glutamate induced toxicity (and this is summarised in figure 5.2). Whilst there is no direct support for this hypothesis, the time courses reported both here and in Beal's paper (Beal et al., 1988), would suggest that free radical damage occurs prior to glutamate release, and that R-PIA is hyperpolarizing the kainate receptor containing neurones initially, before acting post synaptically to modulate the NMDA receptor containing neurones. MK801 has a relatively long therapeutic window in ischaemic models (4 hours plus), and would be expected to have a similar window in the present study.

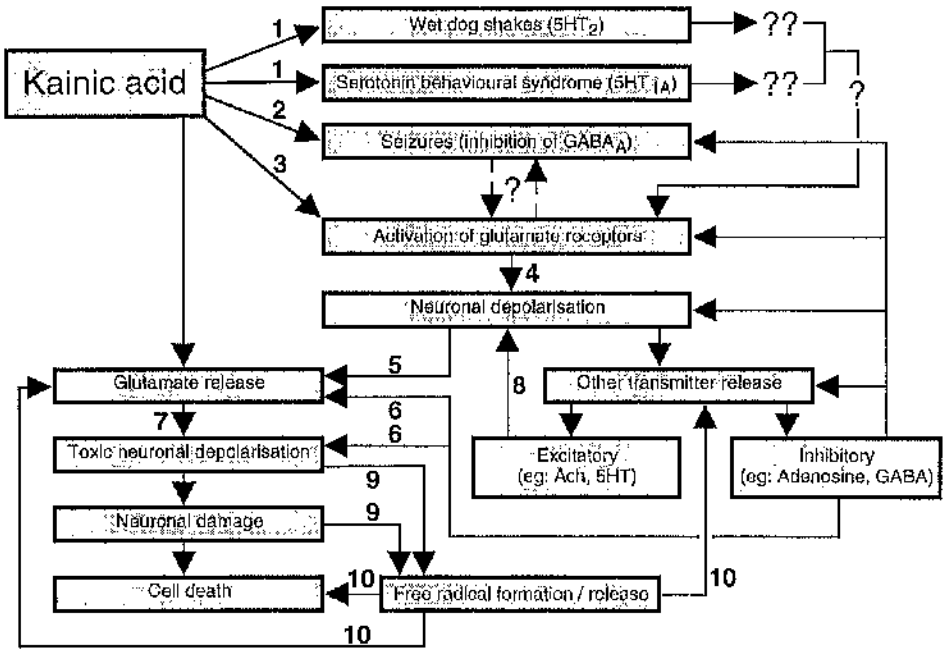


Fig 5.2 Current mechanisms of kainate induced neurotoxicity , and potential sites of intervention.

Key-

1: 5HT antagonists, to block the behavioural side effects.

2: Anticonvulsants to reduce/abolish seizures and possibly secondary damage.

3: Glutamate receptor antagonists, to inhibit this activation. The NMDA site appears to be the main receptor involved in the ensuing toxic cascade.

4: Voltage dependant calcium channels. Blockade of these channels will reduce calcium influx into cells.

5: Inhibition of glutamate release. Targets for this include calcium channel blockers, and agonists for kappa-opioid, $GABA_A$ or adenosine A_1 receptors.

6: Inhibitory transmitter agonists will reduce toxicity, while antagonists appear to enhance it. Receptors appear to be the $GABA_A$ or adenosine A_1 targets here.

7: Inhibition of toxic depolarisation. Targets for this include non-NMDA and NMDA receptor antagonists, inhibitory receptor agonists and calcium and sodium channel blockers.

8: There may be activation of non-glutamatergic neurones, whose depolarization will release other neuroactive agents into the site of injury. Possible targets here have yet to be identified.

9: Inhibition of free radical formation. Targets here include phospholipase A_2 and xanthine oxidase.

10: Free radical scavengers. Targets here include both the O_2/H_2O, NO derived radicals and preoxidised lipids.

?: Possible connections to unknown role in toxicity.

5.7 Areas of Further Research.

The work presented above indicates that the neurotoxic action of kainic acid could be prevented by various endogenous neuromodulators and exogenous drugs. More histology is required though before it is certain that the apparent lack of gliosis is truly neuroprotection. Several other areas also need to be examined including;

1) the treatment of the animals with [^{3}H] kainate to ensure that it is entering the brain, possibly sampled by microdialysis;
2) improved *in vivo* and *in vitro* release studies, using microdialysis and brain slice perfusion, in both cases analysing the perfusate by HPLC for purines and amino acids. The *in vitro* studies could also be used with [^{3}H] and [^{14}C] labelled compounds (possibly glutamate and adenosine), although care should be taken in the exogenous transmitter release experiments;
3) the importance of the serotonergic pathways in the mediation of neurotoxicity;
4) whether free radical production is a cause or an effect of kainate induced toxicity; and
5) determining whether any of the previously used agents can protect against centrally administered excitotoxins (kainate, NMDA, quinolinic acid, the plant neurotoxins BOAA and BMAA), ischaemic insults (both focal and global) or possibly chronic aluminium treatment.

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APPENDIX 1.

PREVENTION BY A PURINE ANALOGUE OF KAINATE-INDUCED NEUROPATHOLOGY

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Abstract.

Systemic injection of the glutamate analogue kainic acic produces a regional and cellular pattern of neuronal loss in the central nervous system which mimics that produced by transient global ischaemia or repeated convulsive seizures. It has previously been found, by measuring the binding of a glial marker ligand, that analogues of adenosine, such as R-N6-phenylisopropyladenosine (R-PIA), can prevent kainate induced damage at doses as low as 10 $\mu g.kg^{-1}$, i.p. The use of gliotic markers, however, is open to misinterpretation, and the present work was designed to re-examine purine protection against kainate using histological methods. The results show that R-PIA, at a dose of 25 $\mu g.kg^{-1}$ i.p. in rats, can protect against the neuronal damage caused by kainate in the entorhinal cortex, septum and amygdala as well as the hippocampus. This protection could be completely prevented by the simultaneous administration of 1,3-dipropyl-8-cyclopentylxanthine, indicating the involvement of A1 receptors in the protection.

INTRODUCTION:

Evidence from a variety of sources indicates the involvement of excitatory amino acids in several CNS disorders. These include seizure disorders, since the use of antagonists at either N-methyl-D-aspartate (NMDA)[26,45,46] or non-NMDA receptors [15,72,83,86] can abolish or reduce the severity of convulsions in animal models of epilepsy, as well as disorders in which the most prominent feature is neuronal degeneration. The neuronal damage resulting from focal cerebral ischaemia, for example, can be prevented by agents which block the neurotoxic effects of amino acid agonists applied into the CNS [37,38,64-66,71].

The glutamate analogue, kainic acid, originally attracted much interest because of its ability to produce axon-sparing lesions in the brain [25], raising the possibility that it might represent a useful tool in the study of neuronal communication. In addition, kainate is able to cross the blood-brain barrier to a limited extent, causing neurotoxicity after parenteral injection [1,43,44,52,70,75].

The regional distribution of the neurotoxicity produced by systemically administered kainate bears marked similarities to some pathological conditions, leading to the use of systemic kainate as a model for the development of appropriate pharmacological therapies. In particular, parts of the limbic system exhibit a high sensitivity to kainate induced damage. The nature and pattern of damage resembles closely that seen to follow global ischaemia or temporal lobe epilepsy [6,24,36,52,69], while being quite different from that seen in focal ischaemia paradigms [24,44,48]. It has also been remarked that the regional pattern of kainate-induced neuronal damage is similar to that seen in Alzheimer's disease [1].

The similarity between kainate and ischaemic neuropathology is consistent with the view that during episodes of cerebral ischaemia, the tissue hypoxia results in a release of endogenous glutamate and aspartate [7,13,41,51,67,68] which can then activate NMDA and non-NMDA receptors to cause further excitation and transmitter release. This cycle of events may develop and persist over many hours since agents able to disrupt the cycle, for example by blocking amino acid receptors, are effective in the prevention of neuronal damage even when applied several hours after a transient cerebral insult [38,42].

An alternative approach to breaking the depolarisation\release cycle is to suppress amino acid release [39] and several groups have now reported on the ability of adenosine, or its metabolically stable analogues, to prevent amino acid induced neuronal damage [2,20,33,53]. Adenosine is an ubiquitous agent which exerts a number of actions on neurones with the common consequence of depressing neuronal function [77,78]. Thus via activation of A1 receptors the nucleoside directly hyperpolarises neurones by increasing potassium conductance [40,81,82], it suppresses the excitatory actions of some neurotransmitters such as acetylcholine [10,23] and it inhibits the release of excitatory, but not inhibitory transmitters [50,81,87]. A combination of these effects probably accounts for the ability of adenosine analogues to block the amino acid release and neurotoxic effects of kainic acid.

The systemic administration of the purine derivative R-N^6-phenylisopropyl-adenosine (R-PIA) can prevent the hippocampal damage produced by systemically injected kainate [53,54]. This occurs at doses as low as 10 $\mu g.kg^{-1}$ i.p. and can be demonstrated with R-PIA injection up to 2 hours after the administration of kainate. However, the neurotoxicity produced by kainate in these latter studies was assessed using the binding of the peripheral benzodiazepine site ligand PK11195 as a reflection of the gliosis

occurring in response to neuronal damage. The measurements were also confined to the hippocampus.

In view of the success of the neuroprotection seen in those studies we have now extended the investigation to report a direct histological examination of the interaction between kainate and R-PIA. Such an examination overcomes the objection made to binding studies that R-PIA may simply prevent gliosis and not modify neuronal damage itself. It also allows the examination of the extent of protection throughout a number of brain regions other than the hippocampus.

METHODS:

All experiments employed 8-10 week old male Wistar rats, 190-280g, which were kept under standard conditions. Animals were injected intraperitoneally with drugs in a volume not exceeding 1 $ml.kg^{-1}$. Kainic acid was dissolved in saline, and R-PIA in methanol. In all cases vehicles were used as control injections and all animals were pretreated with clonazepam ($0.2mg.kg^{-1}$) i.p. 10 minutes prior to kainate injection. The animals were allowed to recover, and were killed 7 days later.

Tissue fixation.

After 7 days all the animals in the four groups were given an overdose of sodium pentobarbitone (5 ml of 60 $mg.ml^{-1}$) and perfusion fixed with 40% formaldehyde, glacial acetic acid, absolute alcohol in the ratio 1:1:8, v\v (FAM), using the method described by Brown & Brierley [11]. Briefly the animals were placed in the supine position and heparinised ($1000\ IU.kg^{-1}$). A thoracotomy was performed, and a cannula introduced into the ascending aorta via the left ventricle. Physiological saline was infused into the animal at mean arterial blood pressure for 5-10 seconds after incising the right atrium. This was followed immediately by 200ml of FAM fixative at the same pressure. After infusion the rats were decapitated and the head was stored and fixed at 4^0C for at least 12 hours. The brain was then removed and the left cerebral hemispheres marked with indelible ink. The hindbrain was detached by a cut through the midbrain and the cerebral hemispheres were cut into five coronal slices, each 2mm thick. The brain stem was cut at right angles to its long axis into slices 2mm thick and the cerebellum into 2 slices perpendicular to the folia on the dorsal surface of each hemisphere. Bilateral blocks of brain were embedded in paraffin wax and sections 7-8 um thick were stained by haematoxylin and eosin and by

a method combining cresyl violet and Luxol fast blue. An 8μm section was examined by conventional light microscopy at $\times$400 magnification, at the level of the mid dorsal hippocampus, by three observors (PAJ, DGM & TWS) semi-blind, and the lefthand hippocampus was scored for percentage damaged/dead neurones in the cell layer. The hippocampus was examined at 5 sites, (as indicated in figure 1) corresponding to the CA1, CA2, CA3a, CA3b and CA4 regions. Zero damage, but disruption of the cell layer was scored as 10% damage, whilst complete loss of the cell layer in the field of vision was scored as 100% damage.

Due to the small n-number used in the control group (n=2), only the kainate and kainate/R-PIA were statistically analysed using Student's t-test.

RESULTS

The injection of kainate induced a series of behaviours similar to those described previously [54,70,73] and which include wet dog shakes, hind-limb abduction, Straub tail and excessive salivation. These were observed in all animals receiving kainate, irrespective of the treatment received. The administration of clonazepam prevented the occurrence of tonic-clonic seizure activity.

Neuropathology

Macroscopic description

Adequate perfusion was achieved in all animals as shown by the lack of blood in the blood vessels and both firmness and pallor of the brain.

Microscopic changes

As judged by the absence of blood in the vessels, good neuronal morphology, and the absence of the cytological artefacts (the "dark cell" and "hydropic cell" [11,14] perfusion fixation appeared to be adequate in all animals.

a). Control-saline group

No histological abnormalities were seen in either of the two animals within this group.

b). Kainate group

Evidence of neuronal damage was present in each of the 11 specimens within this group. Affected neurones were more angular in shape, showed loss of nuclear detail and shrinkage and marked eosinophilic staining of cytoplasm with loss of Nissl substance.

There was marked swelling of associated dendrites, appearances being typically those seen in "dendrosomatotoxic" but "axon sparing" lesions [25,63]. In the least severely affected

areas neuronal damage was restricted to single neurones or small clusters of neurones separated by normal cells; in the most severely affected areas neuronal changes were observed in a larger proportion of the neurones, the associated neuropile of which had a loose texture and stained only lightly with eosin. Apart from slight swelling of astrocytes no changes were present in glia. In those areas where all or most of the neurones were affected the endothelium of some of the small blood vessels was prominent.

No histological abnormalities were seen in one of these animals. In the remaining ten specimens, the distribution of lesions was similar and symmetrical. Principal sites of involvement were the subfrontal cortex including the pyriform cortex, the cortex related to the rhinal fissure and the cortex of the inferomedial quadrant of each frontal lobe immediately in front of the genu of the corpus callosum, the entorhinal cortex, each amygdaloid nucleus, and the nuclei of each septum principally medial but also lateral groups. The hippocampus was involved in all animals but to a variable extent. In the least affected animals there was the occasionally damaged cell in CA1 sector compared with those animals in which the hippocampi were extensively involved when there was involvement of CA1, CA3 and CA4, the greatest damage always being in CA1 followed by CA3 and then CA4. In none of the animals was there more than moderate damage in the CA2.

In none of the animals was there any involvement of the basal ganglia and in 2 of the 11 animals changes were present in the upper brain stem (in one there were foci of neuronal damage in the periaqueductal grey matter and in another animal there was extensive change within each substantia nigra.

In none of the animals was there involvement of the cerebellum.

c). Kainate/PIA group

No abnormalities were seen in the brains of 2 of the 4 animals of this group. The pattern of the damage in the 2 affected animals was similar although they differed in severity, and in both animals it was similar to that already described in the kainate group (group b).

Relative extent of neuronal damage.

The left side-handed hippocampus was scored for percentage damage, as there was symmetry in damage in both hippocampi (within $\approx 10\%$ damage).

CA1 Cell layer damage.

Neither the control group, or the kainate/R-PIA group showed any damage in the CA1 cell layer (0 ± 0 n=2 and 4 respectively), but there was $49.09 \pm 12.82\%$ damage in the kainate treated animals ($p \leq 0.01$ versus the kainate/R-PIA group, Student's t-test see figure 2). The kainate group 4 animals had 90-100% damage in the CA1 cell layer.

CA2 Cell Layer

As with the CA1 cell layer, both the control and the kainate/R-PIA groups displayed no damage, whilst the kainate group showed 50% damage in one animal, with 0-30% in the rest, (including disruption of the cell layer). The damage caused by kainate ($10.91 \pm 4.76\%$) was significantly different from the kainate/R-PIA group ($p \leq 0.05$) see figure 3.

CA3 Cell Layer

The CA3 cell layer displayed a marked regional variation in the degree of damage with the angle being damaged to $\geq 70\%$ in six of the kainate group, but in the cell layer after the angle only $\geq 30\%$ in one of the kainate group (this animal had 90% damage in both the CA1 and CA3a regions). This variation in the degree of damage between cells in the angle and in the cell layer succeeding the angle, resulted the angle being termed the CA3a and the cell layer after the angle the CA3b (see figure 1).

In one of the control animals there was a slight disruption of the CA3a cell layer but no change in the CA3b cell layer (5 ± 5 and $0 \pm 0\%$ respectively, see figures 4A, 4D & 4E).

Kainate caused $54.55 \pm 11.23\%$ damage in the CA3a but only 13.64 ± 3.105 in the CA3b, (see figures 4B, 4D & 4E). Unlike its action in the CA1 region R-PIA was unable to prevent kainate induced damage in both the CA3a and CA3b regions ($27.5 \pm 21.36\%$ and $5 \pm 2.89\%$ respectively, see figures 4C, 4D & 4E). However, in the case of the CA3a region this was probably due to one animal having 90% damage whist the rest had $\leq 20\%$ damage. In the CA3b region the p value was tending towards significance ($p=0.069$). In both cases more animals are required to confirm/refute the assumption that R-PIA is not protecting the CA3 regions.

CA4 Cell Layer.

As with the CA2 region there was $\leq 20\%$ damage in all kainate treated animals bar two (both of which had 90% damage). There was no damage with either control or kainate/R-PIA treatment but one R-PIA animal did display a disrupted cell layer). The

kainate treatment was not significantly different from either of the other groups, (see figure 5).

DISCUSSION

Previous studies of kainic acid neurotoxicity using the peripheral benzodiazepine site ligand PK11195 as a marker for reactive gliosis [53,54] are open to the criticism that apparently protective agents may not actually modify the occurrence or extent of neuronal damage, but may simply prevent the gliotic reaction. The present work, however, reveals that this is not the case with R-PIA, since this agent is able to prevent histologically demonstrated kainate-induced neuronal damage.

The cellular damage seen here following the systemic administration of kainate resembles closely that reported in previous studies [43,44,47,70,73]. Damaged neurones appear dark and shrunken but in less affected areas of the CNS are found intermingled with normal cells. In more severely affected regions most neurones degenerate and vascular endothelium appears prominent. There appears to be some upper limit to the degree of damage, however, since even in the CA3 region only about half the neurones exhibited signs of damage. In a previous study Strain & Tasker [79] reported that with a systemic dose of even 32 $mg.kg^{-1}$ of kainate, only 59% of CA3 neurones were killed. The neuronal changes are similar to those which have also been reported using in vitro studies of kainate induced damage [8].

Regional distribution

The kainate treatment produced signs of damage in a number of subcortical areas, most notably the hippocampus, entorhinal cortex, septum, dorsomedial thalamus and all nuclei of the amygdaloid complex. This regional distribution is again entirely consistent with that described in detail in earlier histological studies [43,44,70,73] and also parallels results obtained when the degree of neuronal activation is assessed directly using the 2-

deoxyglucose method [52]. The similarities using 2-deoxyglucose involve not only the increased activity in entorhinal, septal and amygdaloid areas, but extends to the greater sensitivity of CA3 pyramids compared with CA4 and CA2 cells. The distribution is also consistent with the view that systemic kainate reproduces the pattern of neuronal damage occurring in some human epileptic conditions [4,29,80].

The pattern of hippocampal damage deserves a closer examination. As reported in previous histological studies, the CA4 region was affected by systemic kainate less severely than CA1 or CA3, and CA2 was essentially unaffected. However, the CA1 region tended to be damaged more consistently in the present work, and often to a greater degree than CA3. This represents a difference from earlier findings of Franck,[35] Nadler et al. [58] and Cook & Crutcher [21], but may be explained simply by different sensitivities in the animal strains used or the use of different doses of kainate. Previous studies have often employed doses of 12 to 32 $mg.kg^{-1}$ kainate, and Balchen et al.[3] have pointed out that the size of dose can determine the pattern of hippocampal damage: the higher the dose, the less the damage seen in CA1. This may be the result of the CA3 region being exceptionally sensitive to kainate. The density of kainate binding sites is higher in CA3 than in any other region of the CNS, and this correlates with a higher sensitivity to the excitatory effects of the compound [27,28,76]. A higher dose of systemic kainate may therefore cause such a rapid excitation, inactivation and death of CA3 cells that the Schaffer collateral projection, which is believed to be necessary for the destruction of CA1 neurones [57] becomes inactive[3]. This sequence probably explains the consistent finding that local intrahippocampal or intraventricular injections of kainate destroy the CA3 region selectively and induce almost no damage in CA1. In our study a relatively low systemic dose of 10 $mg.kg^{-1}$ kainate was employed, in combination with clonazepam, in order to

minimise seizure activity and thus confine the effects of kainate to direct activation of neurones. It is possible, therefore, that in this situation a less dramatic activation of CA3 cells may have permitted a greater degree of CA1 neuronal damage.

The use of clonazepam was introduced in previous studies because interest in peripheral benzodiazepine binding was limited to the hippocampus, and it had been reported that diazepam could suppress behavioural seizures and distant neuronal damage while having much less, if any, inhibitory effect on the hippocampal toxicity of kainate [5,16,43]. In the present study, however, clonazepam did not diminish the pattern of distribution or the severity of neuronal damage seen with kainate in any brain region, despite the almost total suppression of overt seizure behaviour. While the explanation of this is unclear, it is possible that the profile of action of diazepam, used in earlier studies, differs from that of clonazepam. Certainly the dose of clonazepam used here is around one tenth to one hundredth the dose of diazepam, yet it fully blocked the seizures. Clonazepam may thus have a higher ratio of antiseizure to neuroprotective activity. Alternatively, the difference may simply reflect our deliberate choice of the minimum dose of clonazepam needed to suppress behavioural seizures, a consideration which was not evident in earlier studies.

Protection by R-PIA.

The ability of R-PIA to prevent kainate induced neurotoxicity supports the previous work involving the use of PK11195 as a marker for reactive gliosis [53,54] and is consistent with studies from other laboratories which have demonstrated protection by 2-chloroadenosine against excitotoxin induced damage [2,20,33] or by cyclohexyladenosine against ischaemic damage [85].

Purines may be able to afford protection by virtue of their ability to hyperpolarise neurones directly to a level at which calcium influx is minimal. However, it seems probable that an inhibitory action on synaptic terminals is importantly involved. It is clear from studies in which the destruction of afferent pathways reduces kainate induced excitation and neurodegeneration that intact terminals are required for excitotoxicity [34,43,49,55,57,61,62,76]. This may result from a presynaptic action of kainate to evoke the release of other compounds such as glutamate and aspartate which are primarily responsible for the subsequent degeneration, or it may indicate the need for such presynaptic factors to exert a permissive action on the effects of kainate.

The former is made more likely by direct demonstrations of the release of glutamate and aspartate by kainic acid [19,32,59,88] and by the reports of kainate receptors on synaptosomal preparations of CNS tissue [9]. Such an involvement of released amino acids would help to account for the frequent observation that kainate induced damage can be at least partially prevented by antagonists at NMDA receptors [18,30,84] which would be activated by glutamate released from terminals. Indeed, since kainate may also release glutamate from glial cells [51], these may provide an additional source of potentially toxic amino acids.

Adenosine and its analogues are known to act at A1 nucleoside receptors to suppress the release of a variety of neurotransmitters including glutamate [22,31], acetylcholine [74], dopamine [12,17,56], and peptides. This activity could therefore lead to the blockade of amino acid release induced by kainate, and the protective effect observed here. It is certainly relevant that other agents which can suppress the release of excitatory amino acids, such as the pyrimidine derivative BW1002C87 (5-(2,3,5-trichlorophenyl)-pyrimidine-2,4-diamine-1,1-ethanesulphonate) and kappa opiate receptor agonists are able

to prevent neuronal damage in studies of ischaemia [39,60].

In summary, this study has confirmed the neuroprotective activity of the purine analogue R-PIA against neurotoxicity induced by systemic injections of kainic acid. In view of the similar pathology of kainate damage and that resulting from transient global ischaemia or repeated seizures in humans, it would appear that purines may represent a promising avenue for the development of clinically useful neuroprotectant agents.

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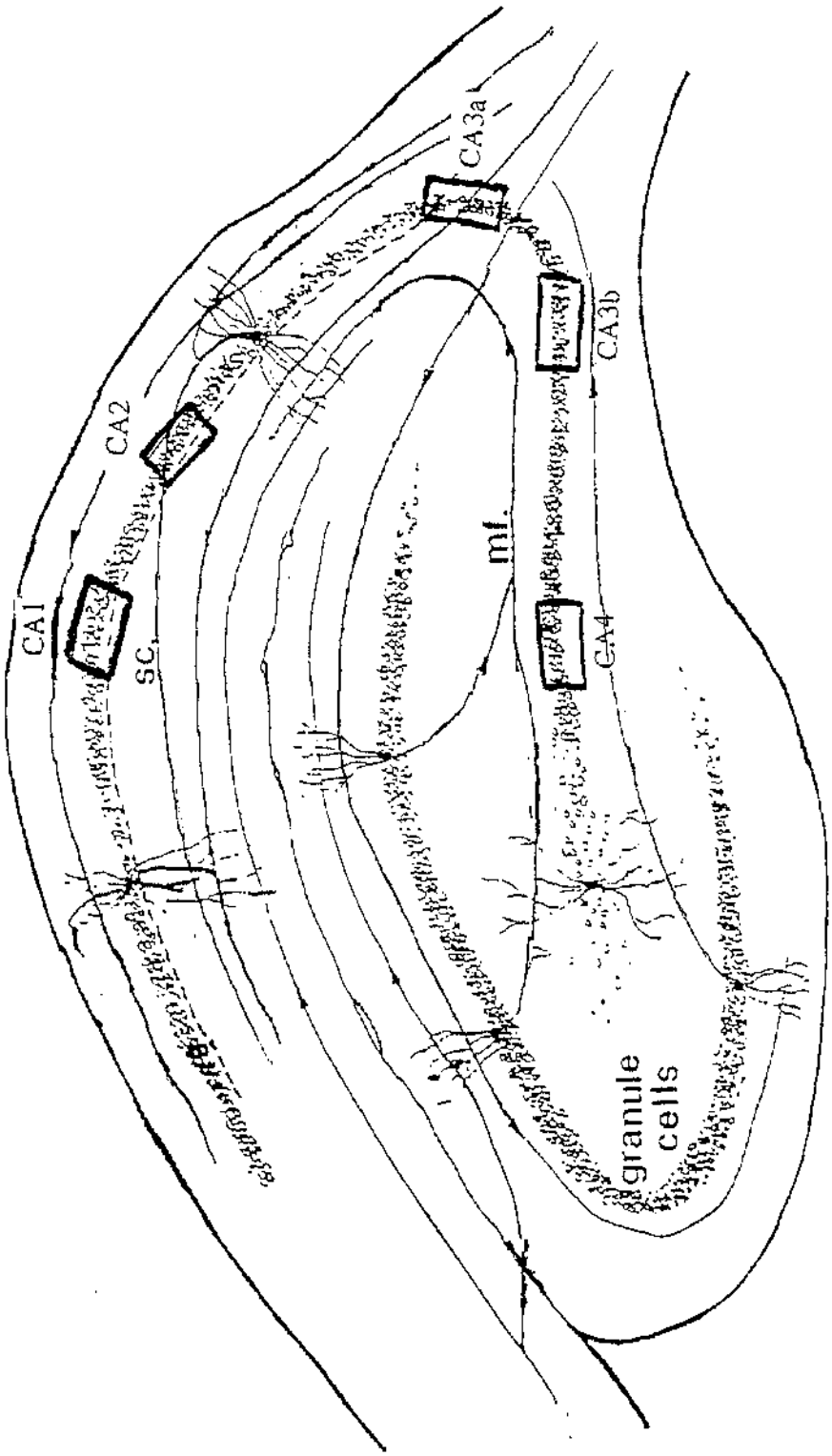

Figure 1:

Areas of the hippocampus analysed for neuropathological.

Legends: CA = Cornu ammonis subfields, mf. = mossy fibres, sc. = Schaffer collaterals.

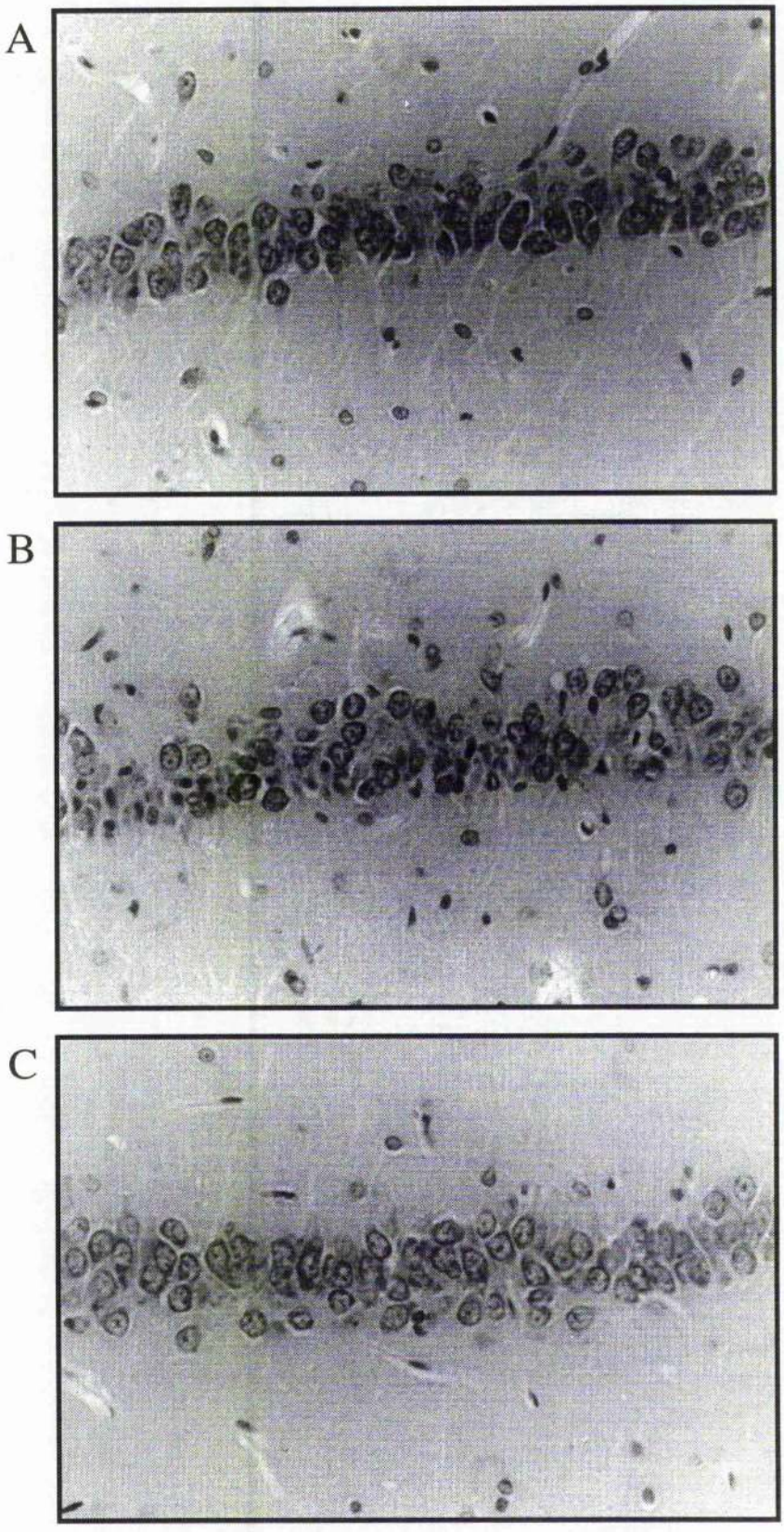

Figure 2. Examination of CA1 cell layer

A) control (saline/methanol injected) animal
B) 10mg.kg^{-1} kainate/methanol injected animal
C) 10mg.kg^{-1} kainate/25mm.kg^{-1} R-PIA

} H & E stained, x330

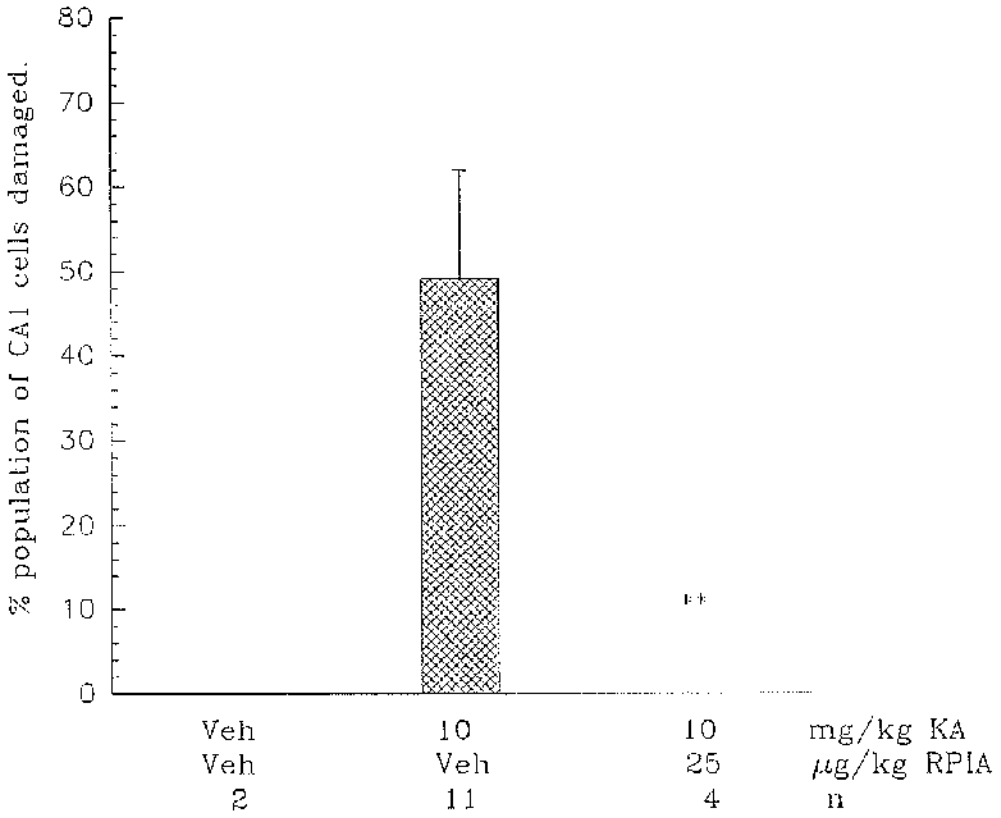

Figure 2D. Analysis of damage in the CA1 cell layer.
Columns indicate mean ± sem. ** $p \leq 0.01$ versus kainate group, Student's t-test.

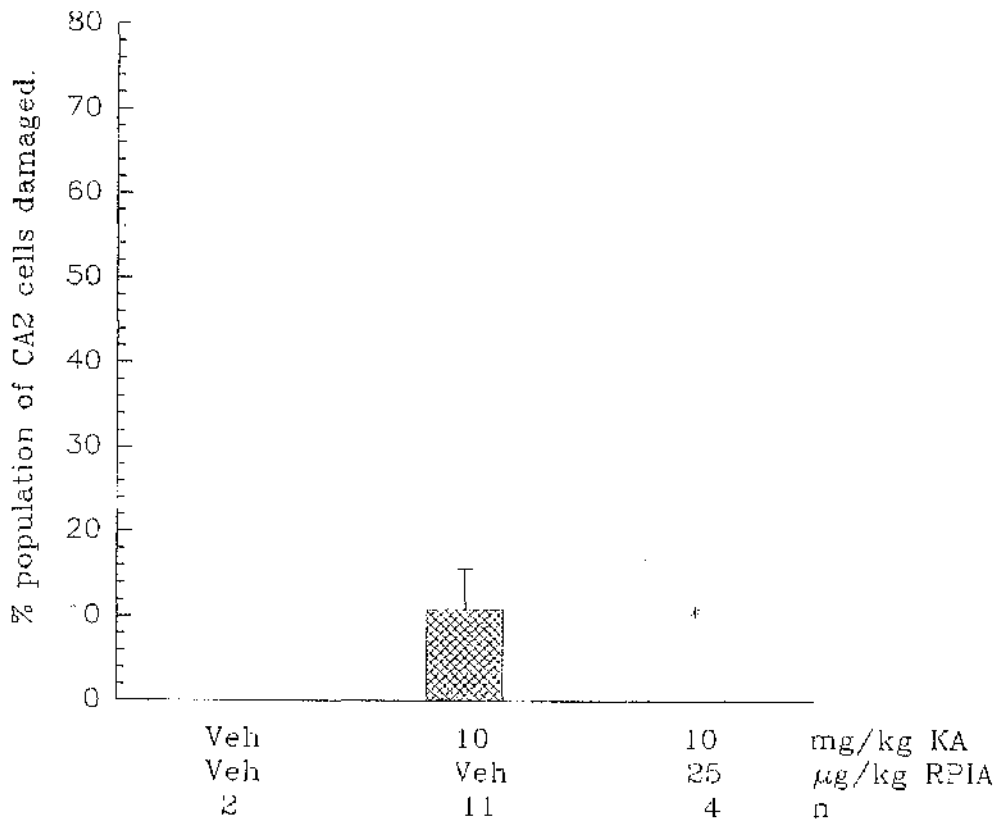

Figure 3.

Analysis of damage in the CA2 cell layer.
Columns indicate mean ± sem. * $p \leq 0.05$ versus kainate group, Student's t-test.

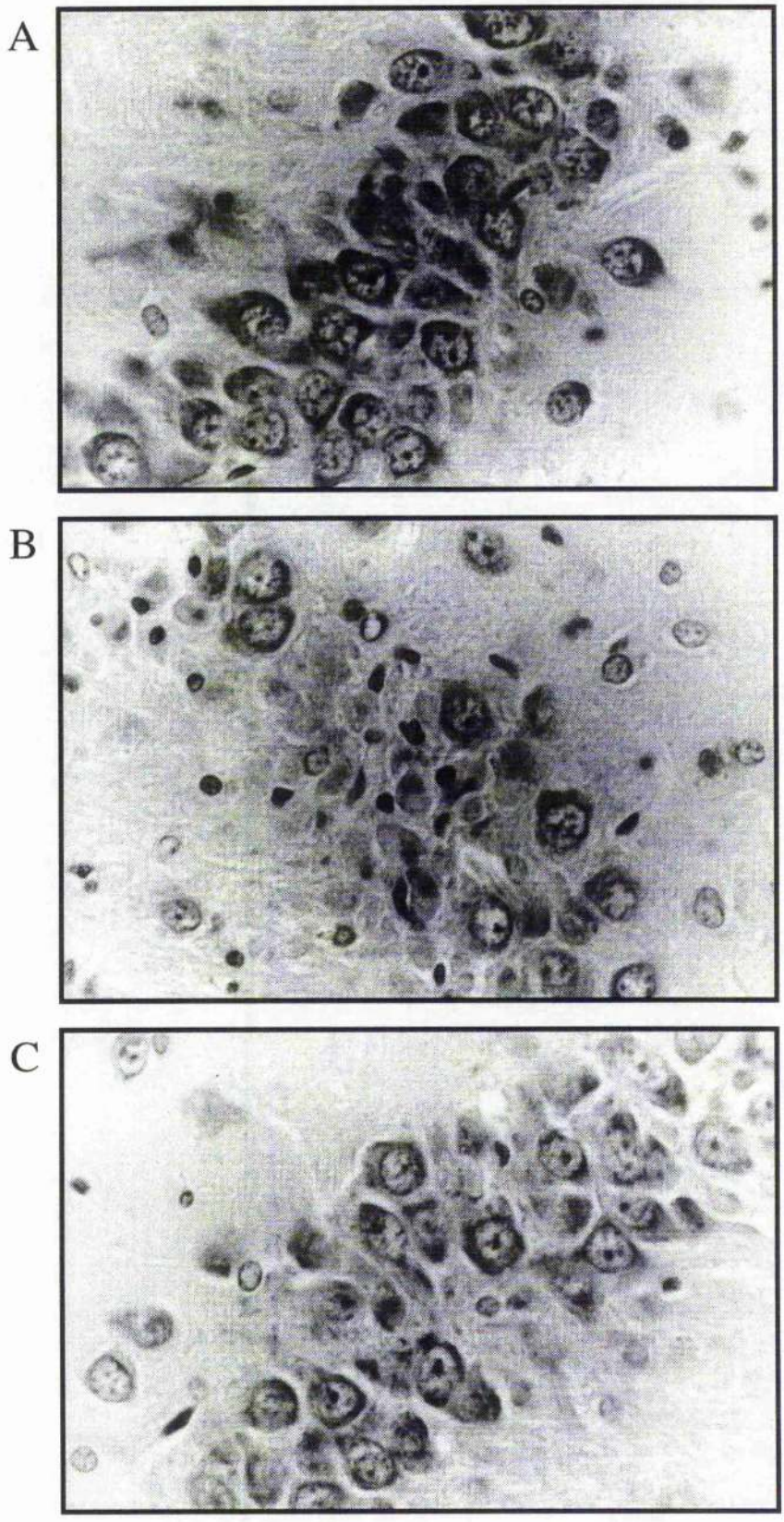

Figure 4. Examination of CA3 cell layer

A) control (saline/methanol injected) animal
B) 10mg.kg^{-1} kainate/methanol injected animal
C) 10mg.kg^{-1} kainate/25mm.kg^{-1} R-PIA

} H & E stained, x530

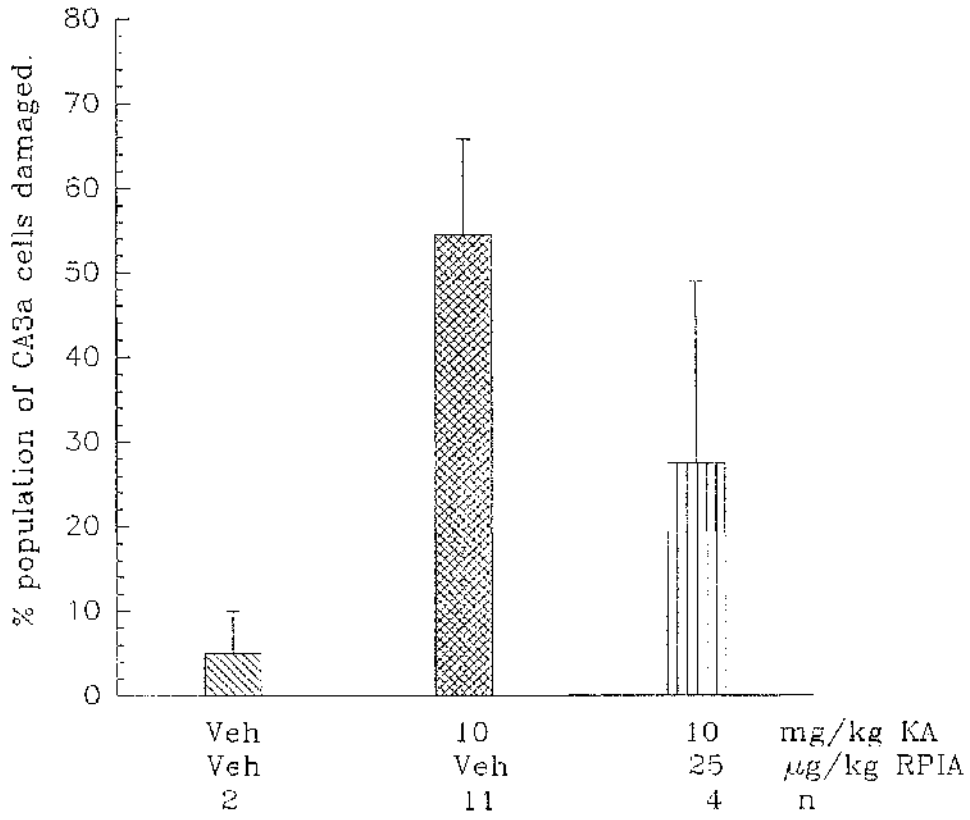

Figure 4D.
Analysis of damage in the CA3a cell layer.
Columns indicate mean ± sem.

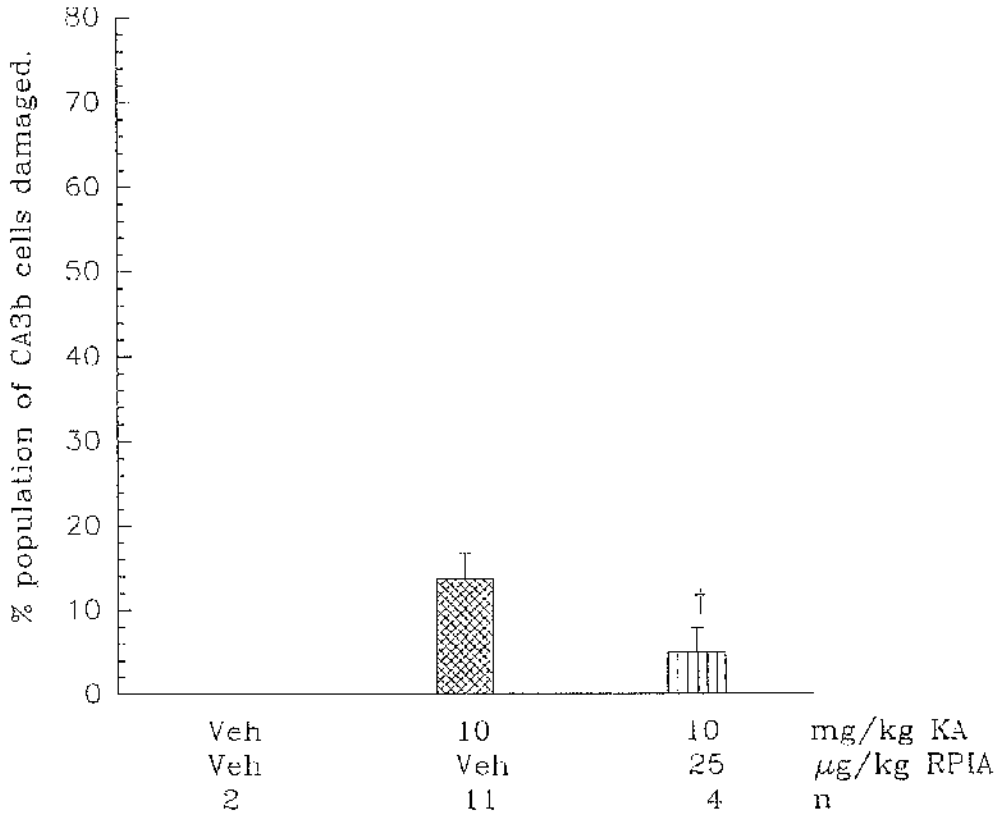

Figure 4E.

Analysis of damage in the CA3b cell layer.

Columns indicate mean ± sem. + $p \leq 0.069$ versus kainate group, Student's t-test.

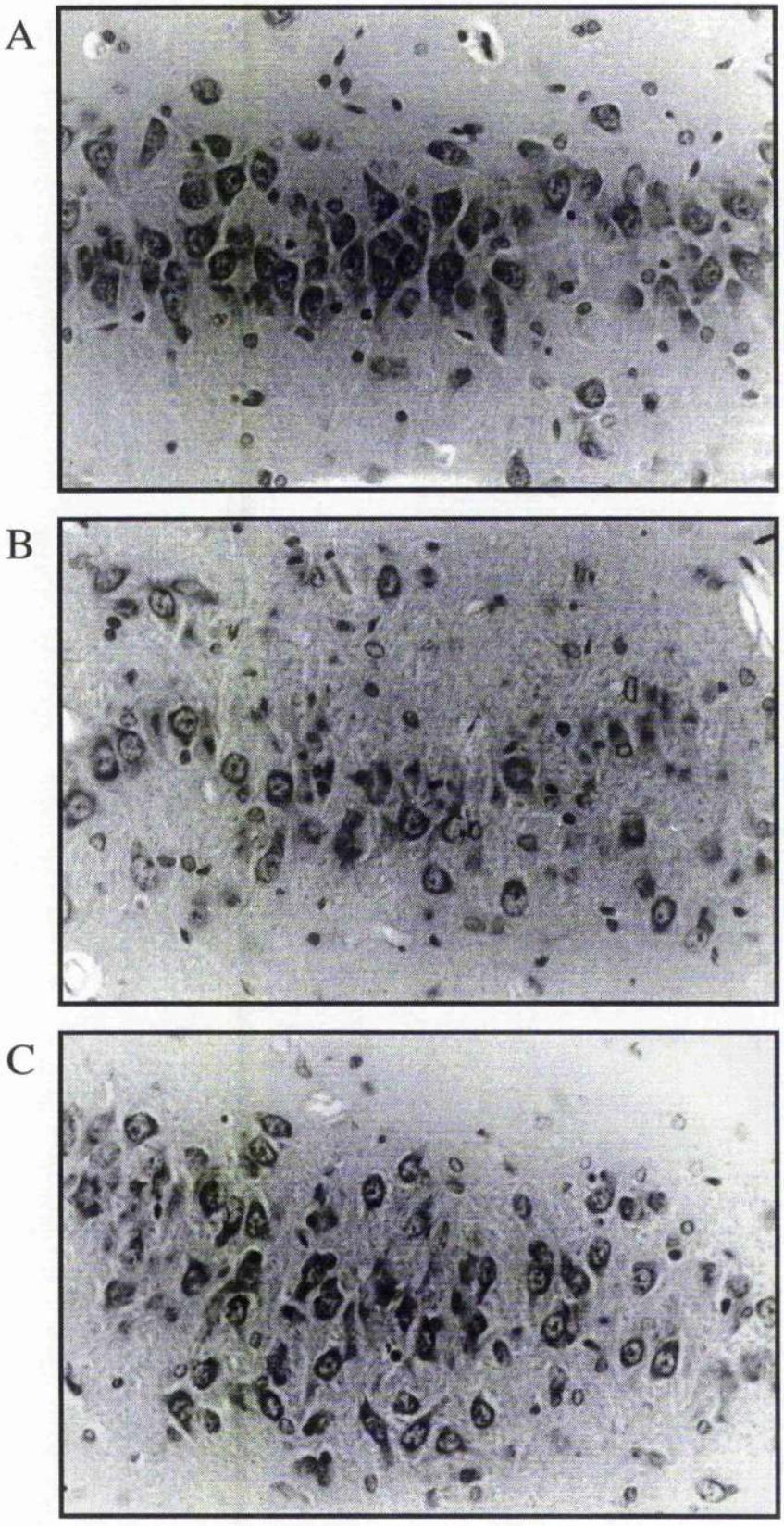

Figure 5. Examination of CA4 cell layer

A) control (saline/methanol injected) animal
B) 10mg.kg^{-1} kainate/methanol injected animal
C) 10mg.kg^{-1} kainate/25mm.kg^{-1} R-PIA

} H & E stained, x330

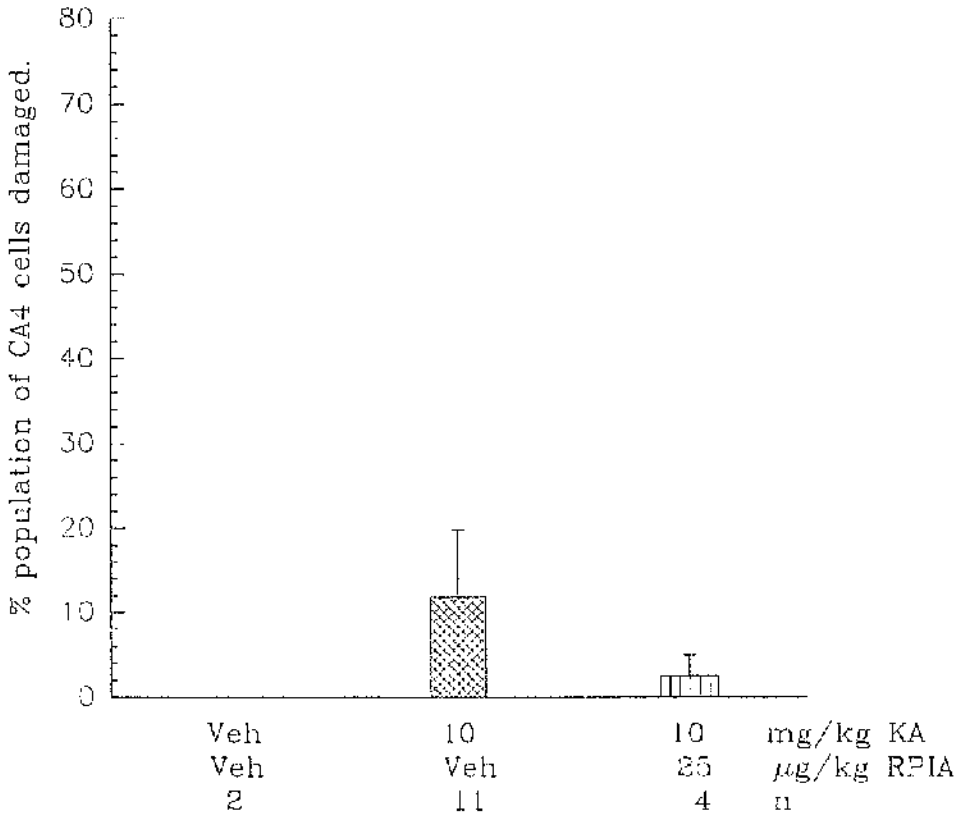

Figure 5D.

Analysis of damage in the CA4 cell layer.

Columns indicate mean ± sem. * $p \leq 0.05$ versus kainate group, Student's t-test.