# MICRODIALYSIS STUDIES OF PURINE AND MONOAMINE RELEASE FROM THE CENTRAL NERVOUS SYSTEM *IN VIVO.*

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Medicine, University of Glasgow by

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February 1996.

## ACKNOWLEDGEMENTS

Especially, I would like to thank Professor Trevor Stone for his supervision during the course of this work and for giving me this opportunity to continue my education.
Special thanks to Professor A.P. Payne for providing funding for both setting up microdialysis in conscious animals and for my final year and also for his sound advice.
Thanks to The Scottish Home and Health Department and The Royal Society.
Special thanks to Tricia for technical assistance with the high performance liquid chromatography and for enduring the task of dialysis probe-making.
Thanks to Dr. Desmond Gilmore for the use of the high performance liquid chromatography.
Thanks to John Thomson for technical assistance in high performance liquid chromatography and to Neil Bennett and John Lawrie for technical assistance in the AH.
Thanks to Dr. Ying Chen, Professor Charles Marsden and colleagues and Dr. Andrew Young for their advice on establishing the technique.
Thanks to my colleagues for making the dark days brighter: Duncan, Mike, Hussein, Shahin, Alex, Fiona, Paul, Jackie and Bayrum.
Special thanks to Douglas for his encouragement and patience and for helping me put my work into perspective.
Thanks to Richard for being a very willing personal computer advisor and for untangling my computer problems.
Thanks to Fiona, David, Jane and Jilly for believing in me and being there.
Most of all, I would like to thank my parents because without their help I would not have been able to continue my education. Special thanks to my mum for giving me the opportunity to do something that she, too, always wanted to do and to my dad for his advice, his guidance and always sticking by me. It is to my parents that I dedicate this thesis.

## CONTENTS

## List of Figures

## List of Tables

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## ABBREVIATIONS

| | |
|---|---|
| AADC | aromatic amino acid decarboxylase |
| ACPu | anterior caudate putamen |
| acsf | artificial cerebral spinal fluid |
| ADP | adenosine diphosphate |
| AMP | adenosine monophosphate |
| AMPA | (R,S)-a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) |
| (±)-AP-5 | (±)-2-amino-5-phosphonopentanoic acid |
| AS | Albino Swiss |
| AS/AGU | Albino Swiss/ Anatomy Glasgow University |
| ATP | adenosine triphosphate |
| BBB | blood brain barrier |
| CNQX | 6-cyano-7-nitroquinoxaline-2,3-dione |
| CNS | central nervous system |
| CPT | 8-cyclopentyl-1,3-dimethylxanthine |
| DCPu | dorsal caudate putamen |
| DHBA | 3,4-dihydroxybenzylamine |
| DMSO | dimethylsulphoxide |
| DOPAC | 3,4-dihydroxyphenylacetic acid |
| DPCPX | 1,3-dipropyl-8-cyclopentylxanthine |
| ECF | extracellular fluid |
| EHNA | erythro-9-(2-hydroxy-2-nonyl)adenine hydrochloride |
| ESR | electron spin resonance |
| FALS | familial amyotrophic lateral sclerosis |
| fig(s) | figure(s) |
| GP | globus pallidus |
| GPe | external segment of the globus pallidus |
| GPi/SNr | internal segment of the globus pallidus and substantia nigra reticulata |
| GYKI 52466 | 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine |
| HPLC | high performance liquid chromatography |
| hr(s) | hour(s) |
| 5-HTP | 5-hydroxytryptophan |
| HVA | 4-hydroxy-3-methoxy-phenylacetic acid (homovanillinic acid) |
| IB-MECA | N6-(3-iodobenzyl) adenosine-5'-N-methylcarboxamide) |
| IMP | inosine monophosphate |
| i.p. | intraperitoneal |
| i.v. | intravenous |
| KA | kainic acid |
| LGIC | ligand-gated ion channel |
| MAO | monoamine oxidase |
| MCE | 2-mercaptoethanol |
| MCPU | middle caudate putamen |
| MK-801 | (+)-MK-801 hydrogen maleate (dizocilpine maleate) |
| min(s) | minute(s) |
| MPP+ | 1-methyl-4-phenylpyridinium |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |

| | |
|---|---|
| MTA | 3-methoxytyramine |
| NBTG | S-(2-hydroxy-5-nitrobenzyl)-6-thioguanosine |
| NBTI | nitrobenzylthioinosine |
| NECA | 5'-N-ethylcarboxamide adenosine |
| NMDA | N-methyl-D-aspartate |
| OPA | *o*-phthaldialdehyde |
| PCPu | posterior caudate putamen |
| POBN | α(4-pyridyl-1-oxide)-N-tert-butylnitrone |
| PRPP | 5-phosphoribosyl-1-pyrophosphate |
| 8-PST | 8-(p-sulphophenyl)theophylline |
| R-PIA | [R]-$N^6$-(2-phenylisopropyl)-adenosine |
| s.c. | sub-cutaneous |
| S-PBN | N-tert-butyl-α-(2-sulfophenyl)-nitrone |
| TTX | tetrodotoxin |
| U50 488H | trans-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneactemide methanesulphonate hydrochloride |
| VCPU | ventral caudate putamen |
| VTA | ventral tegmental area |

## GENERAL INTRODUCTION

This thesis describes studies using microdialysis to investigate the release of neuroactive compounds from the rat brain. In part I the experiments were directed at examining the ability of kainic acid to release adenosine and its metabolites from the hippocampus of anaesthetised animals. Because of the opportunity to collaborate with Professor Tony Payne, this section of work was curtailed and emphasis was switched to a study of monoamine release in a new strain of mutant rat exhibiting motor dysfunction. This work is presented in part II. Some overlap of interest was retained by including an examination of adenosine release from the mutant rat.

## PART I

### SUMMARY

The effects of kainic acid and potassium on the release of endogenous adenosine and its metabolites, inosine, hypoxanthine and xanthine, from the rat hippocampus have been studied by *in vivo* microdialysis. In the hippocampus of rats anaesthetised with urethane the concentration of extracellular adenosine was estimated to be 0.8μM during the first two hrs after insertion of the dialysis probe.

Kainic acid (0.1-25mM) in the perfusate evoked a concentration-dependent release of adenosine with an $EC_{50}$ of 0.94mM. A 5min pulse of 1mM kainic acid in the perfusate, during a sampling period of one hour, increased the 20μl dialysate levels from 3.68 ± 0.21 to 7.66 ± 0.82 pmol (mean ± sem). A second stimulation (S2) 3hrs (hours) after the first stimulation (S1) also induced adenosine release. The S2/S1 ratio was 0.46 ± 0.02. Kainate-evoked release of adenosine was shown to involve the production of action potentials since TTX (tetrodotoxin) significantly reduced the S2/S1 ratio by 53.85%. The release was reduced by incorporation into the perfusate of CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), a non-NMDA (N-methyl-D-aspartate) receptor antagonist, but not by NMDA receptor blockers, (+)-MK-801 (dizocilpine) or (±)-AP-5 ((±)-2-amino-5-phosphonopentanoic acid), indicating a non-NMDA receptor mediated process. The kappa agonist, U50 488H (trans-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneactemide methanesulphonate hydrochloride), significantly reduced the S2/S1 ratio by 55.77%. Release was reduced significantly by 44.23% by ascorbic acid (an antioxidant), 48.08% by glutathione (a scavenger of hydroperoxides) and 71.15% by oxypurinol (a xanthine oxidase inhibitor) indicating the involvement of free radicals in kainate-evoked adenosine release. Neither the adenosine A1 receptor antagonist CPT (8-cyclopentyl-1,3-dimethylxanthine) nor the A1 receptor agonist R-PIA (R(-) $N^6$-(2-phenylisopropyl)adenosine) affected kainate-evoked release of adenosine. This indicates

that activation of A1 receptors, by endogenous adenosine or an agonist, does not inhibit kainate-evoked release of adenosine.

The present results indicate that kainate-evoked release of adenosine may be mediated by non-NMDA receptor activation, possibly requiring the propagation of action potentials and free radical production.

## 1.0 INTRODUCTION

### 1.1 Adenosine

Kainic acid has been shown to cause neuronal cell death in the CNS (central nervous system) (MacGregor *et al*, 1993) in similar areas to those affected by temporal lobe epilepsy (for review see Sperk *et al*, 1994). Purines have been shown to protect neurones from cell death (MacGregor *et al*, 1993). This project is mainly focused on the release of adenosine into the extracellular space in the presence of the neurotoxin, kainic acid.

#### 1.1.1 Adenosine receptors

Adenosine acts on G-protein coupled receptors, collectively called P1 purinoceptors. Previously, P1 purinoceptors were biochemically distinguished by their effects on adenylate cyclase. Adenosine A1 receptor activation decreased cAMP levels and adenosine A2 receptor activation increased cAMP levels (Stone & Simmonds, 1991). Adenosine responses have been observed without the modulation of cAMP (Stone 1984; Fredholm & Dunwiddie, 1988). For instance, A1 mediated inhibition of neurotransmitter release has been shown to be independent of altered cAMP levels (Dunwiddie 1985; Ribeiro & Sebastiao 1986). More recently, P1 purinoceptors have been studied by molecular cloning and their pharmacological characteristics have been determined. Four members of this family have been cloned and pharmacologically distinguished: A1, A2a, A2b and A3.

#### 1.1.2 Pharmacological classification of adenosine receptors

Pharmacological characterisation of A1 receptors has been aided by the large number of A1 ligands. A frequently used ligand is [3H]DPCPX (1,3-dipropyl-8-cyclopentylxanthine) which is a selective antagonist of the adenosine A1 receptor. Adenosine receptor agonists have potencies at A1 receptors in the order R-PIA (R(-) $N^6$-(2-phenylisopropyl)adenosine) > NECA (5'-N-ethylcarboxamide adenosine) > 2-chloroadenosine and at A2 receptors in the order of NECA > 2-chloroadenosine > R-PIA (Stone & Simmonds, 1991). A2a receptors are thought to be high affinity and A2b receptors are thought to be low affinity adenosine receptors. A2 receptor characterisation has been held back by the lack of selective ligands. Jarvis *et al* (1989) have shown high affinity binding of CGS 21680 to a single class of recognition sites, which they equated with the A2 receptors. However

Johansson & Fredholm (1995) show binding of CGS 21680 not only to classical A2a receptors but also to a receptor site that is different from all known adenosine receptors.

The adenosine agonist IB-MECA ($N^6$-(3-iodobenzyl) adenosine-5'-N-methylcarboxamide) is reported to be a selective ligand at the A3 receptor (Jacobson *et al,* 1993) and, unlike the A1 and A2a receptors, most alkylxanthines are ineffective as competitors in binding experiments at the A3 receptors (Zhou *et al,* 1992). This newly cloned A3 receptor is different from the A3 receptor previously postulated by Ribeiro & Sebastiao (1986), who reported that R-PIA and NECA were approximately equipotent in reducing transmitter release at the neuromuscular junction (Ribeiro & Sebastiao, 1986) and postulated that this effect was mediated by an A3 receptor.

Recently, Cornfield *et al* (1992) have reported the pharmacological characterisation of a so-called A4 binding site, by the binding of CV 1808. This putative receptor has not yet been cloned but its pharmacological profile on rat striatal membranes appears to be as follows: CV 1808> CGS 22988>>NECA≥CGS 21680 (Luthin & Linden, 1995).

1.1.3 Location of adenosine receptors

A1 receptors are highly concentrated in the dendritic zones of hippocampal pyramidal neurones (Reddington & Lee, 1991), in the cortex and in the cerebellum.

A2a receptors are located in the caudate nucleus, putamen, nucleus accumbens and tuberculum olfactorium. In the striatum, expression of the A2a receptor appears to occur in the same cells as those that express dopamine D2 receptors, on the so-called medium sized spiny neurones (Fink *et al,* 1992).

Location of A2b receptors and the novel A3 receptor are less well documented. The A2b receptor appears to be the most abundant receptor on astrocytes (Altiok *et al,* 1992) and evidence shows they exist in cerebral cortical slices Daly *et al* (1983).

The adenosine A3 receptor is reported to be present in highest levels in the testes, moderate levels in the heart and kidney and only low levels in the brain (Zhou *et al*, 1992). This is unlike the A1 and A2 receptors which are highly expressed in the brain. Adenosine A3 receptors are present in the mouse (Jacobson *et al*, 1993), rat (Zhou *et al*, 1992), gerbil (Ji *et al,* 1994), sheep (Linden, 1994) and human (Salvatore *et al,* 1993). The highest

densities of the A3 receptors were found to be present in the striatum and cerebellum in the mouse brain (Jacobson *et al*, 1993).

1.1.4 Responses to adenosine receptor activation: biochemical effects

The A1 receptor couples to the pertussis-toxin sensitive G-protein (Gi, Go) (Freissmuth *et al* 1991; Munshi *et al* 1991). A1 receptor activation is thought to cause 1) the inhibition of adenylate cyclase (via both the α- and the β,γ-subunits of Gi-protein); 2) the activation of several types of K+-channels (Trussell & Jackson, 1985) 3) the inactivation of at least some types of the voltage-dependent Ca2+-channels (Scholz & Miller, 1991) 4) the activation of phospholipase C (via the β,γ-subunits of the G-protein), with subsequent activation of protein kinase C and increase in intracellular Ca2+ (Gerwins & Fredholm, 1992; Fredholm *et al* 1994).

A2a receptors associate with Gs-proteins. Stimulation of these receptors causes activation of adenylate cyclase.

*In vitro* stimulation of A3 receptors results in inhibition of adenylate cyclase (Zhou *et al* 1992). In mast cells, Ali *et al* (1990) postulated a new adenosine receptor which coupled, via a G-protein, to phospholipase C, rather than by Gs or Gi to adenylate cyclase and was not blocked by the conventional antagonists of A1/A2-adenosine receptors (e.g. theophylline and 8-phenyltheophylline). The subsequent cloning of the A3 receptor (Zhou *et al*, 1992), allowed the identification of this receptor in mast cells as the A3 receptor (Ramkumar *et al*, 1993).

1.1.4 Responses to adenosine receptor activation: neuronal effects

The inhibitory actions of adenosine on CNS neurones were first shown by Phillis *et al* in 1975. Adenosine is now thought of as an inhibitory neuromodulator in the CNS (Dunwiddie & Hoffer, 1980; Stone, 1989; Stone & Simmonds, 1991). It is known that adenosine causes suppression of neuronal firing and inhibition of synaptic transmission.

Adenosine exists in the extracellular space in sufficient concentrations to exert tonic inhibitory actions. Endogenous extracellular adenosine has been shown to inhibit transient calcium currents in the hippocampal slice (Wu & Saggau, 1994) and normoxic synaptic

transmission is tonically depressed by ongoing release of adenosine (Zhu & Krnjevic, 1994).

These various inhibitory actions of A1 receptor stimulation presumably form the basis of the reported neuroprotective properties of purines. Adenosine analogues, adenosine uptake inhibitors and adenosine deaminase inhibitors can protect against neurotoxic effects of NMDA and non-NMDA receptor agonists (Arvin *et al,* 1989; Connick & Stone, 1989; Finn *et al,* 1991; MacGregor *et al,* 1993) as well as against ischaemia (Evans *et al,* 1987; Phillis & O'Regan, 1989; Von Lubitz *et al,* 1989; Andiné *et al,* 1990). A variety of pre- and postsynaptic effects of adenosine have been shown which may account for these various inhibitory actions.

1.1.5 Presynaptic Properties of Adenosine

A1 receptors located presynaptically appear to inhibit presynaptic transmitter release. Adenosine has been shown to inhibit the presynaptic release of excitatory neurotransmitters such as acetylcholine (Spignoli *et al,* 1984; Cunha *et al,* 1994) and glutamate (Corradetti *et al,* 1984; Fastbom & Fredholm, 1985; Dunwiddie & Fredholm, 1989; Cantor *et al,* 1992) via action at the A1 receptor (Burke & Nadler, 1988). Duner-Engstrom & Fredholm (1988) and Fredholm & Dunwiddie (1988) suggest that the release of excitatory transmitters is more strongly inhibited than the release of inhibitory transmitters. This would be in keeping with the finding that presynaptic A1 receptors are located on the terminals of some excitatory and not on inhibitory neurones as revealed by autoradiography studies in the cerebellum (Goodman & Snyder, 1982; Goodman *et al,* 1983). More recently, electrophysiological studies provide evidence of adenosine's ability to inhibit excitatory postsynaptic potentials but not inhibitory postsynaptic potentials (Fredholm *et al,* 1989; Yoon *et al,* 1991; Thompson *et al,* 1992).

While a large volume of work shows that glutamate release can be inhibited by adenosine, the evidence for the suppression of GABA release is indeed conflicting. Hollins & Stone (1980b) have shown that 1mM adenosine is required to inhibit [3H]GABA release by 35% from cerebral cortex slices and Burke & Nadler (1988) failed to detect any inhibition from hippocampal slices. Though adenosine may decrease GABA release to some extent,

adenosine does appear to be more selective at reducing excitatory transmission. It may be concluded that inhibitory neurones are much less sensitive to the effects of adenosine.

Adenosine A1 receptor stimulation leads to a number of mechanisms that could all reduce evoked transmitter release, including inhibition of adenylate cyclase, opening of potassium channels or suppressing calcium currents. It is possible that all these mechanisms are operative when reducing transmitter release. The importance of one particular mechanism may depend on which transmitter is being released and how its release is triggered. Conclusions drawn by Dunwiddie (1985) do not support the notion that adenosine derivatives inhibit transmitter release by inhibiting adenylate cyclase.

The regulation of calcium in the nerve terminal by adenosine seems to be a popular explanation. If the intracellular concentration of calcium is lowered then it would be less effective in reducing the energy barrier to secretion of neurotransmitters. Such a lowering of intracellular calcium may be due to the reduction in influx of calcium from the extracellular fluid, decreased mobilisation of calcium from intracellular calcium stores or enhanced removal of calcium from the presynaptic terminal.

In cultured mouse sensory neurones, an adenosine analogue reduced the calcium current through the N-type calcium channel (Gross *et al*, 1989). In rat synaptosomes, adenosine modulated $^{45}Ca2+$ uptake by potassium-depolarised nerve terminals (Ribeiro *et al* 1979; Wu *et al*, 1982). In both synaptosomes and hippocampal slices adenosine decreased calcium uptake across voltage sensitive calcium channels (Bartrup, 1989). In cortical synaptosomes, 2-chloroadenosine attenuated the calcium influx induced by potassium (Arvin *et al*, 1989).

After it was shown that some of adenosine's actions, such as suppression of synaptic transmission, were independent of calcium influx (Dunwiddie, 1984; Halliwell & Scholfields, 1984), several authors failed to inhibit potassium- or veratridine-stimulated synaptosomal $^{45}Ca2+$ uptake by adenosine or adenosine analogues (Barr *et al*, 1985; Michaelis *et al*, 1988; Garritsen *et al*, 1989).

The discrepancies between these groups of results are unexplained and the issue arises of whether the concentrations of purines used to inhibit calcium influx were comparable with

the concentrations used to inhibit neurotransmission. In the study by Ribeiro *et al* (1979), millimolar concentrations of adenosine were used to alter calcium influx, a concentration range around 100 fold higher than that required to inhibit transmitter release. Arvin *et al* (1989), showed inhibition of potassium-evoked glutamate release by 2-chloroadenosine at a concentration as low as 0.01μM, a concentration at which inhibition of potassium-evoked calcium influx was no longer apparent.

Another possibility is that the effects of adenosine on calcium fluxes and transmitter release depends on the preparation used. Interpretation of Ca2+ fluxes in synaptosomes may be misleading since adenosine may affect calcium currents which are only found on neuronal cell bodies and are absent from synaptosomes. Secondly, the interpretation of results may be complicated by the use of potassium to induce calcium influx, since increases in potassium conductances by adenosine have been observed. For these two reasons, Michaelis *et al* (1988) examined the effects of adenosine analogues on veratridine-induced calcium influx and used an *in vivo* model to follow the movement of potassium. They found no effect on veratridine-induced calcium influx by adenosine and an enhancement of potassium current has lead them to postulate that there is a presynaptic receptor-mediated increase in potassium permeability. Other investigators have also revealed an increase in potassium conductance from neurones in the presence of adenosine (Michaelis & Michaelis, 1981; Trussell & Jackson, 1987; Gerber & Gähwiler, 1994).

The mechanism by which adenosine may inhibit calcium influx has been investigated. Calcium current may be inhibited by a secondary effect of adenosine on potassium efflux according to Gerber *et al* (1989) and Greene & Haas (1989) but Schubert & Kreutzberg (1981) and Scott & Dolphin (1981) reported that adenosine inhibits calcium influx which is not mediated by potassium channel activation.

Scott & Dolphin (1981) investigated the involvement of guanine nucleotide binding protein (G-protein) in coupling the adenosine receptor to calcium channels. They found that the non-hydrolysable analogue of GTP enhanced the inhibition of voltage-activated calcium current by 2-chloroadenosine, indicating that the inhibition of calcium currents by

adenosine A1 receptor activation involves the mediation of G-protein (Scott & Dolphin, 1981).

Other mechanisms of presynaptic inhibition of transmitter release have been proposed. These include an enhanced removal of calcium from the presynaptic terminal, a decrease in calcium mobilisation from intracellular stores and a decrease in affinity of calcium for the secretary elements involved in transmission. The latter has been postulated by Silinsky *et al* (1981) who studied adenosine actions on the neuromuscular junction. They suggested that adenosine receptors are linked to calcium binding proteins and stimulation of the receptor results in decreased affinity of the calcium binding protein for calcium.

Presynaptic A2 receptor activation is thought to cause an increase in transmitter release including glutamate (Simpson *et al*, 1992). In general, the A1 receptors are sensitive to low concentrations of adenosine derivatives in the nanomolar range, whereas micromolar concentrations are required for A2 receptor activation (Stone & Simmonds, 1991). Therefore, if extracellular adenosine levels increase to the micromolar range, the effects of A2 receptor stimulation may override the effects of A1 receptor stimulation, resulting in a potentially detrimental response.

1.1.6 Postsynaptic Properties of Adenosine

Adenosine causes suppression of neuronal firing (Gerber & Gähwiler, 1994). A1 receptor activation also causes postsynaptic hyperpolarisation (Trussell and Jackson, 1985; Dunwiddie & Fredholm, 1989; Thomson *et al*, 1992). The mechanism(s) of this hyperpolarisation could involve: 1) an increase in potassium conductance (Segal, 1982; Gerber *et al*, 1989) 2) an increase in chloride influx. Hyperpolarisation of neurones leads to enhanced magnesium block of the ion channel associated with NMDA receptors (Phillis & Wu, 1981), thus preventing the calcium influx which is subsequent to NMDA receptor activation.

Neuroprotective actions mediated via the A2 receptor include: 1) increased cerebral blood flow by vasodilatation (Phillis *et al*, 1984) (direct application of adenosine–induced dilatation of the pial artery in a dose-dependent manner in concentrations as low as 1μM (Berne *et al*, 1974)) 2) inhibition of platelet and neutrophil aggregation (Phillis, 1989) 3)

block of sodium uptake and compound action potentials (Ribeiro & Sebastiao, 1987) 4) increased astrocytic glycogenolysis (Magistretti *et al*, 1986).

1.1.7 The formation and release of adenosine

Cerebral adenosine has been estimated by the microdialysis technique to be present in the extracellular fluid at a concentration of around 1 μM (Zetterström *et al*, 1982, Chen *et al*, 1992). In the CNS the level of extracellular adenosine is influenced by neuronal activity (Richardson & Brown, 1987; Pedata *et al*, 1989; Pazzagli *et al*, 1993). The rate of adenosine formation increases during increased neuronal activity (Hollins & Stone, 1980a; Winn *et al*, 1980; Jonzon & Fredholm, 1985) and neuronal energy metabolism (Hagberg *et al*, 1986).

The only *de novo* pathway which leads to the production of adenosine is via the formation of IMP (inosine monophosphate) (Stone & Simmonds, 1991) (fig (figure) 1.1). The pathway starts with 5-phosphoribosyl-1-pyrophosphate (PP-ribose-P) and uses 5 molecules of ATP (adenosine triphosphate), leading to the formation of IMP. IMP can then be converted to its adenine nucleotide derivative, AMP (adenosine monophosphate), which can be dephosphorylated by 5'-nucleotidase leading to the formation of adenosine. Alternatively AMP can be converted by a reversible reaction to its polyphosphate derivatives, ADP (adenosine diphosphate) and then ATP.

A metabolic source of adenosine is the conversion of hypoxanthine to IMP by the enzyme, hypoxanthine-guanine phosphoribosyltransferase (fig 1.1). This is the so called 'salvage' reaction (Stone & Simmonds, 1991). If hypoxanthine is not converted to IMP, it is metabolised by uric acid via xanthine by xanthine oxidase. Uric acid is the end product and is readily excreted from the body. A second metabolic source of adenosine is the hydrolysis of S-adenosylhomocysteine (SAH) to adenosine and homocysteine (fig 1.1), though the favoured reaction is normally in the reverse direction.

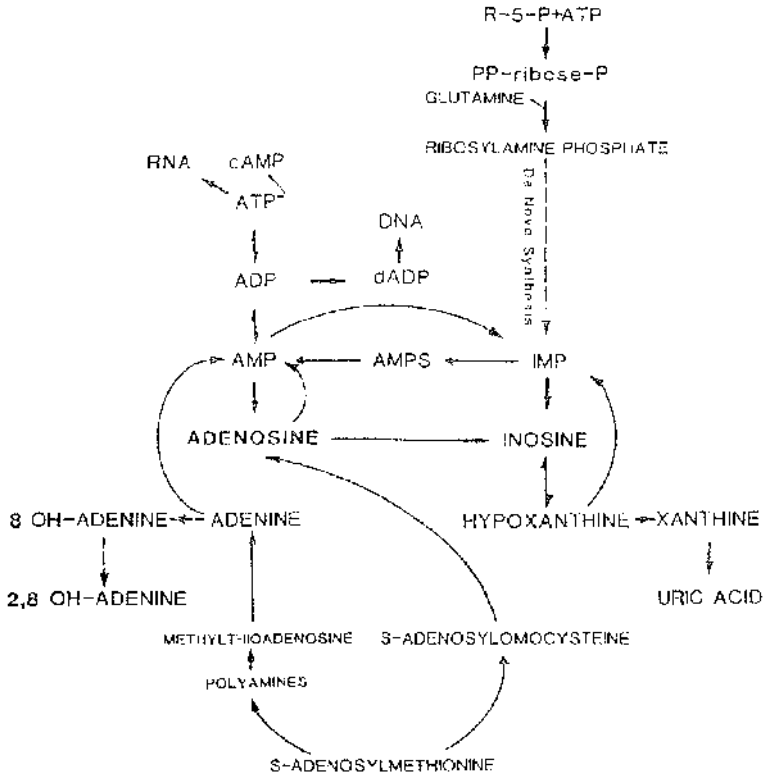



Fig 1.1. Pathways of adenosine production (taken from Stone & Simmonds, 1991).

Dephosphorylation of AMP by 5'nucleotidase (fig 1.1) is generally regarded as being quantitatively the most important reaction for the production of adenosine. Nucleotidases exist intracellularly and extracellularly and respond readily to increases in AMP. Extracellular adenosine may arise from the production of adenosine intracellularly and its subsequent efflux outside the cell by a high affinity nucleoside transport process. An increase in extracellular adenosine may alternatively arise from the prior release of ATP and subsequent extracellular degradation via AMP. ATP can be released on its own from peripheral or central neurones as a transmitter or co-transmitter (Burnstock, 1972, 1986). Using synaptosomes, depolarisation-evoked release of ATP was revealed by White (1978) using potassium or veratridine. Potassium-evoked release of ATP was consistent with the release seen within the CNS of more classically recognised neurotransmitters in that it was calcium-dependent (White, 1978).

In many areas, especially synaptic regions, high ecto-5'-nucleotidase activities have been demonstrated where there is a high adenosine A1 receptor distribution (Goodman & Snyder, 1982; Schubert *et al*, 1983). These similarities in location suggest that ecto-5'-nucleotidase generate synaptically active adenosine to interact with A1 receptors. In agreement with Goodman & Snyder (1982), Fastbom *et al* (1987) showed that there was high receptor binding and enzyme location in the molecular layer of the hippocampus. However, Fastbom *et al* (1987) showed the highest enzyme activity also to be in the globus pallidus (GP), olfactory tubercle and caudate putamen, areas where they found only intermediate or low binding. Enzyme activities and receptor binding were measured and compared using the same species (rats) by Fastbom *et al* (1987) but Goodman & Snyder (1982) compared enzyme activities measured using mice with receptor binding measured using rats. The comparison made by the latter authors may be less accurate than the comparisons made by Fastbom *et al* (1987) since there is large regional variation of adenosine receptor location between the rat and mouse brain (Fastbom *et al*, 1987). Schubert *et al* (1983) found that high adenosine binding can occur without the presence of enzyme activity indicating that there may indeed be a poor correlation between the distribution of binding sites and enzyme activity.

Despite a possible poor correlation between enzyme and receptor sites, evidence that ecto-5'-nucleotidase does play a role in extracellular adenosine production is strong. Using whole rat brain synaptosomes (MacDonald & White, 1985) or rat cortical slices (Hoehn & White, 1990a) basal extracellular adenosine levels were found to be mostly derived from extracellular ATP, since inhibition of ecto-5'-nucleotidase profoundly reduced adenosine release. Also inhibition of ecto-5'-nucleotidase inhibited the release of adenosine in the guinea-pig heart (Imai *et al*, 1989) and prevented the production of extracellular adenosine from ATP which was released by depolarisation of purified rat striatal cholinergic synaptosomes (Richardson *et al*, 1987). The latter group report that the synaptic functioning of adenosine is controlled by the presence and activity of ecto-5'-nucleotidase since the inhibitory effects of adenosine on acetylcholine release were no longer apparent after inhibition of ecto-5'-nucleotidase (Richardson *et al*, 1987).

However, inconsistent results from using ecto-5'-nucleotidase inhibitors on the release of adenosine evoked by potassium or glutamate have been obtained. Potassium-evoked adenosine release from rat brain synaptosomes was decreased slightly by ecto-5'-nucleotidase inhibitors (MacDonald & White, 1985) but was not affected by ecto-5'-nucleotidase inhibitors when adenosine release was evoked from rat cortical slices (Hoehn & White, 1990a). This may indicate that adenosine can be released in the form of ATP only from the nerve terminals (consistent with the release seen within the CNS of more classically recognised neurotransmitters) and this release only contributes slightly to the amount of adenosine released *per se* from the intact neurones and glia found in slices. However, the contribution of nucleotides to potassium-evoked adenosine release in guinea pig synaptosomes (Daval & Barbaris, 1981) was less than that in the rat synaptosomes (MacDonald & White, 1985). Adenosine was released from hippocampal slices without detection of ATP release (Jonzon & Fredholm, 1985) and, despite the rapid dephosphorylation of ATP, the authors state that any released ATP would be measured in their system. The inhibition of ecto-5'-nucleotidase did not reduce adenosine production or release from either amphibian ganglia (Rubio *et al*, 1988) or embryonic chick neuronal and glial culture (Meghji *et al*, 1989). Pons *et al* (1980) have shown that ecto-nucleotidase

activity was not required for glutamate-evoked accumulations of cAMP, indicating that a breakdown of extracellular nucleotides was not required for this effect by adenosine.

Considering this variation of opinion and experimental evidence, it is possible that both the release of adenosine *per se* and the release of adenosine via the prior release of ATP may occur. The importance of one over the other may depend on the species used, the preparation used (e.g. release from isolated nerve terminals (synaptosomes) or from intact neurones and/or glia (slices or *in vivo*)) or the stimuli used (e.g. electrical stimulation or pharmacological agents).

In summary, a poor correlation between location of adenosine receptors and ecto-5'-nucleotidase activities may exist (Fastbom *et al*, 1987) yet a role of ecto-5'-nucleotidase in extracellular adenosine production and functioning has been shown in some circumstances. It may therefore be hypothesised that several pools of nucleotidase exist, only one of which synthesises the "neuromodulator pool" of adenosine. This may lead to a high enzyme activity in areas where there is only intermediate or low adenosine binding sites as reported by Fastbom *et al* (1987).

For adenosine to be released *per se* then it must often be formed intracellularly. It has been suggested that an increase in neuronal activity increases metabolic activity and intracellular ATP breakdown to AMP and thus adenosine. Electrical excitation has been shown to increase dephosphorylation of ATP mainly because it increases intracellular Na+ concentration, thus activating the Na+-requiring ATPase (Pull & McIwain, 1973). The increase in neuronal activity, which occurs during neuronal excitation, may also result in activation of the Na+K+ pump which is driven by ATPase. As three sodium ions and two potassium ions are transported across the membrane, ATP is broken down to ADP inside the cell by endo-nucleotidases. Subsequent dephosphorylation may lead to the accumulation of adenosine which may then be transported out of the cell. However, the breakdown of ATP associated with the activity of the Na+K+-dependent ATPase as a source of extracellular adenosine has been eliminated by a study performed by Hollins & Stone (1980a). In that study, the release of tritium after preloading with [3H]adenosine occurs in the presence of ouabain, an ATPase inhibitor, from slices of rat cerebral cortex.

If the activity of the enzyme Na+-K+-ATPase was responsible for adenosine production, then the ouabain would inhibit, not evoke, adenosine release (Hollins & Stone, 1980a).

The formation and release of adenosine is increased during acute pathological conditions such as hypoxia/ischaemia (Berne *et al*, 1974; Winn *et al*, 1981; Hagberg *et al*, 1987), hypoglycaemia (Butcher *et al*, 1987; White & Hoehn, 1991) and stroke and seizures. Ischaemic insults result in decreased oxidative phosphorylation and anaerobic glycolysis causing intracellular accumulation of adenosine (White & Hoehn, 1991). Depolarisation of cells and the resulting influx of sodium may result in the intracellular accumulation of adenosine and its subsequent efflux by the reversal of the sodium-dependent bidirectional nucleoside transporter which is reported to be present on neurones and glia (Bender & Hertz, 1986; Meghji *et al*, 1989). In support of non-neuronal adenosine release, pools of purines exist in glia, endothelial cells and platelets and adenosine has been shown to be derived from glia by electrical stimulation (Caciagli *et al*, 1988).

1.1.8 Receptor Mediated Release Of Adenosine

Adenosine is released from neurones other than purinergic neurones. Adenosine has been reported to be released from noradrenergic and cholinergic neurones (Pedata *et al*, 1989). Whether release is presynaptic or postsynaptic is another issue waiting to be answered. Work done by Rubio *et al* (1988) on the frog sympathetic ganglion has shown that release of adenosine during synaptic transmission is the result of activation of postsynaptic structures. This conclusion was based on the observation that muscarinic agonists could induce adenosine release, while the release produced by preganglionic stimulation was prevented by muscarinic antagonists.

These experiments indicate that adenosine release can occur in response to activation of neurotransmitter receptors, as well as to direct depolarisation. Release can also be induced by activation of glutamate receptors in the CNS. Both NMDA and non-NMDA receptors have been shown to be involved in glutamate-evoked release of adenosine from rat cortical slices (Hoehn & White, 1990a) since the evoked release was diminished by 50% in the presence of NMDA antagonists and by 66% in the presence of non-NMDA receptor antagonists. Indeed, the local application of NMDA to the hippocampus *in vivo* also

increases the release of adenosine (Chen *et al*, 1992). It has been shown that the NMDA receptor is involved in basal outflow of adenosine in ageing (22 months old) rats but not in adult (3 month old) rats (Pazzagli *et al*, 1995), indicating that the role of excitatory amino acids in regulating adenosine levels is more important in ageing than in adult rats. Work on cortical slices has shown that, with the application of NMDA, only a small fraction of the available NMDA receptors must be activated for adenosine release to be maximal (Hoehn *et al*, 1990). The finding that adenosine release is maximal when the majority of NMDA receptors are still available for activation, indicates that adenosine's neuroprotective properties may not be enough to overcome the consequences of maximal NMDA receptor activation during an excitotoxic insult.

1.1.9 Adenosine removal and degradation

Adenosine can be removed from the extracellular space (the site where adenosine has its receptor-mediated actions) by uptake through nucleoside transporters. The importance of adenosine uptake as an inactivation mechanism is shown by the ability of adenosine uptake blockers to depress neuronal activity (Motley and Collins, 1983), potentiate the ability of adenosine to decrease locomotor activity (Crawley *et al*, 1983, Sanderson & Scholfield, 1986), increase nociceptive threshold (Yarbrough and McGuffin-Clineschmidt, 1981) and exert anti-convulsive effect (Dragunow and Goddard, 1984).

Nucleoside transporters are sub-classified (Geiger & Fyda, 1991) in terms of:

1) high and low affinity for adenosine

2) selectivity for a range of purines and pyrimidines

3) sensitivity and resistance to uptake blockers e.g. NBTI (nitrobenzylthioinosine), dipyridamole

4) ability to translocate nucleosides across cell membrane by passive or facilitated diffusion or by $Na^+$-dependent active transport systems

Once inside the cell, adenosine is converted to AMP by phosphorylation by cytosolic adenosine kinase or, alternatively, to inosine by cytosolic adenosine deaminase and subsequently hypoxanthine and xanthine. The condensation reaction between adenosine

and l-homocysteine produces SAH (Sciotti & Van Wylen, 1993a) and there is also conversion to adenine by adenosine nucleosidase.

Adenosine kinase has a higher affinity for adenosine than does adenosine deaminase (Sciotti & Van Wylen, 1993b). Adenosine is phosphorylated at low micromolar concentrations (Kuroda & McIlwain, 1974; Barbaris *et al*, 1981; Reddington & Pusch, 1983; Wolinsky & Paterson, 1985), the Km value for adenosine of adenosine kinase being 2μM (Phillips & Newsholme, 1979). The Km value for adenosine of adenosine deaminase is 17μM (Phillips & Newsholme, 1979). Thus adenosine is converted to AMP by adenosine kinase under normal physiological conditions.

The rate of phosphorylation of adenosine is maximal at low concentrations of adenosine and decreases with increasing adenosine concentration (Fisher & Newsholme, 1984). During hypoxia or ischaemia, the elevated adenosine levels result in substrate inhibition of adenosine kinase, allowing promotion of deamination of the accumulated adenosine (Nagy *et al*, 1990).

Adenosine deaminase is reported to be intracellular, restricted to oligodendrocytes and endothelial cells (Schrader *et al*, 1987). Cell surface localisation of adenosine deaminase has been reported in human skin fibroblasts (Andy & Kornfeld, 1982), in piglet isolated perfused lung (Hellewell & Pearson, 1983) and in the rat heart (Meghji *et al*, 1988). These reports indicate that adenosine deaminase may contribute to the extracellular metabolism of adenosine. Such existence of exo-adenosine deaminase in cerebral tissue has yet to be been shown.

Adenosine deaminase inhibitors elevate adenosine levels in the brain and have some adenosine-like depressant effects on CNS function (Radulovacki *et al*, 1983). Curiously, they have no effect on hippocampal population spikes (Zhu & Krnjevic, 1994) or on ischaemic-induced increases in cortical glutamate release (Phillis *et al*, 1991). This may imply that adenosine deaminase does not play a critical role in aborting endogenous adenosine's modulatory effects on synaptic transmission.

## 1.2 Excitatory amino acids

1.2.1 Excitatory amino acid receptors

The synaptic responses elicited by excitatory amino acids are mediated by metabotropic and ionotropic receptors. Metabotropic receptors activate phospholipase C via G-proteins leading to increased levels of inositol polyphosphates and the subsequent mobilisation of intracellular stores of calcium and increased production of diacylglycerol with protein kinase C activation and cell protein phosphorylation. There are three subtypes of glutamate ligand-gated ion channels (LGIC): NMDA, AMPA ((R,S)-a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) and kainate receptors, classified by their sensitivity to glutamate agonists (Stone & Burton, 1988; Collingridge & Lester, 1989). The use of antagonists can distinguish between NMDA and non-NMDA receptors but, until recently, such tools were not available to distinguish absolutely between AMPA and kainate receptors. It has been postulated that the kainate receptor exists in high and low affinity states, with Ki values of 1.5 and 19nM for kainic acid, respectively (London & Coyle, 1979). It was revealed that quisqualic acid binds to the high affinity site with 10 fold higher potency than at the low affinity site and that quisqualic acid is 3 fold more potent in binding to either state of kainate receptor than L-glutamate (London & Coyle, 1979). Johansen *et al* (1993) reported that the competitive glutamate antagonist, 5-nitro-6,7,8,9-tetrahydrobenzo[G]indole-2,3-dione-3-oxime (NS-102), was more selective at the low-affinity than the high-affinity [$^3$H]kainate binding site and was a weak inhibitor of AMPA binding. Paternain *et al* (1995) reported that the non-competitive non-NMDA antagonist, GYKI 52466, was more specific for AMPA/ kainate receptors and has little effect at the high affinity kainate.

There are specific genes which code for AMPA and kainate receptors and the functional expression of these genes in the *Xenopus* Oocyte model indicates that these genes will produce receptors that possess the characteristics of the *in vivo* receptors (Egeberg *et al*, 1991; Petralia & Wenthold, 1992; Henley, 1994). Based on the rat glutamate receptor complementary DNA sequences, antipeptide antibodies detected the GluR1, GluR2, GluR3 AMPA receptor subunits in human CNS tissue (Blackstone *et al*, 1992). In addition, the expression of the KA-1 gene which codes for a high-affinity kainate binding site reveals

that this site has similar binding properties to that seen for [$^3$H] kainate using *in vivo* autoradiography in rats (Werner *et al,* 1991). GluR1-4 can be activated by AMPA and kainate but have higher affinity for AMPA than kainate (Bettler & Mulle, 1995). GluR5-7 are selectively activated by kainate and apparent affinity for kainate is higher than GluR1-4 (Egebjerg *et al,* 1991). The cation channel expressed by this receptor has a conductance of about 20pS (Ascher *et al,* 1986; Cull-Candy & Usowiz, 1987; Jahr & Stevens, 1987; Cull-Candy *et al,* 1988).

The NMDA receptor channel has a larger conductance (around 50pS) (Ascher *et al,* 1988) and is less ion selective than most sodium LGIC. About 5-10% of its current is carried by Ca2+, whereas AMPA and kainate receptors are relatively impermeable to calcium (Mayer *et al,* 1987; Pumain *et al,* 1987). However, more recently, evidence indicates that some sub-types of AMPA/ kainate receptors are directly calcium permeable (Brorson *et al,* 1992; Rorig & Grabtyn, 1993; Brorson *et al,* 1994). Homomeric and heteromeric receptors assembled from GluR1/-3/-4 subunits are significantly permeable to calcium (Bettler & Mulle, 1995). All recombinant AMPA receptor subunit combinations containing the edited GluR2(R) subunit are rather impermeable to calcium. Recombinant receptors lacking the edited GluR2(R) are permeable to calcium (this permeability is 5-10 times less than that of NMDA receptors). For kainate receptors, GluR6(R) and GluR6(Q) subunits are permeable to calcium with GluR6(R) having the higher calcium permeability.

1.2.2 Location of excitatory amino acid receptors

NMDA receptors have been shown to have a localisation consistent with a synaptic function, and are present in high density in the cerebral cortex, hippocampus, striatum, septum and amygdala (Greenamyre *et al,* 1985; Choi, 1988(a)). AMPA receptors are located primarily in telencephalic regions, with high levels in hippocampus, cortex, lateral septum, striatum and molecular layer of cerebellum (Nielson *et al,* 1988; Monaghan *et al,* 1989). Kainate receptors are located predominantly on the presynaptic terminals in the CA3 region of the hippocampus (Ferkany *et al,* 1982; Bettler & Mulle, 1995, Malva *et al,* 1995).

Excitatory amino acid receptors are located on the presynaptic terminals or on the cell bodies and activation can produce a depolarisation which initiates an action potential, evoking release from the nerve terminal by exocytosis. NMDA receptor activation evokes release of dopamine and acetylcholine by acting directly on nerve terminals (Krebs *et al*, 1991). Non-NMDA receptor activation has been shown to increase noradrenaline release by a presynaptic action on the terminals of locus coeruleus noradrenergic neurones (Wang *et al*, 1992) and to increase glutamate and aspartate release from rat synaptosomes (Ferkany *et al*, 1982; Connick & Stone, 1986; Poli *et al*, 1991). Non-NMDA receptors have been shown to exist presynaptically on dopaminergic neurones in the striatum (Desce *et al*, 1991).

1.2.3 Excitatory amino acids and excitotoxicity

L-glutamate and L-aspartate are the main endogenous excitatory amino acids in CNS. Paradoxically, the brain is very vulnerable to its own excitatory amino acids. Glutamate is present intravesiclarly, at a concentration of around 10mM (Nicholls & Atwell, 1990). For glutamate to exert its neurotoxic action it must first be released from cells into the extracellular fluid. It is well documented that the release of glutamate and aspartate from neuronal cells into the extracellular space occurs during hypoxia (Benveniste *et al*, 1984; Globus *et al*, 1988; Phillis *et al*, 1991), ischaemia, hypoglycaemia and seizures. Under normal conditions, the extracellular concentration of glutamate is estimated to be about 2μM but during ischaemia this can rise to 1mM (Hagberg *et al*, 1985). Mitani & Kataoke (1991) report that approximately a ten-fold increase in extracellular glutamate for beyond ten minutes may provoke ischaemic-induced neuronal death.

There are thought to be two types of release of excitatory amino acids which result in accumulation of excitotoxic concentrations in the extracellular fluid: $Ca2^{+}$-dependent vesicular release (Nicholls, 1989) or Ca2+-independent depolarisation-induced reversal of the glutamate transporter. Both of these probably occur mainly from synaptic terminals but release from glial cells may also occur by $Ca2^{+}$-independent reversal of the $Na^{+}$-coupled plasma membrane amino acid transporter (Nicholls, 1989)

Once in the extracellular space, neuronal responses to glutamate are terminated by desensitisation of receptors or the uptake of glutamate into nerve terminals or glia. As the concentration of transmitters in nerve terminals is higher than in the synaptic cleft, energy input is required for uptake. It appears that for L-glutamate to interact with the transporter, sodium ions must be on the same side of the cell membrane as L-glutamate and potassium must be present simultaneously on the opposite side (Kanner & Marva, 1982). At the same time as sodium influx is driving the glutamate "uphill", there is potassium efflux (Kanner, 1993). An increase in potassium in the extracellular fluid causes an efflux of L-glutamate (Kanner & Marva, 1982). During loss of ionic homeostasis, for example anoxia and ischaemia, an increase in extracellular potassium may lead to efflux of glutamate. This may significantly add to the vesicular release of glutamate reported to occur under these conditions, thus having further detrimental effects.

In addition to the requirement for sodium and potassium ions, the efflux of L-glutamate via the transporter is faster in the presence of internal or external chloride ions (Kanner & Marva, 1982).

After its uptake into glial cells, glutamate is converted into glutamine by glutamine synthetase, an enzyme located solely non-neuronally (Norenberg & Martinez-Hernandez, 1979). Glutamate taken up into neurones is transported into vesicles. Inhibition of glutamate uptake has been seen in astrocytic cultures during swelling of astrocytes induced by exposure of cultures to a hypotonic or high potassium medium (Kimelberg *et al*, 1995) or during acidosis (Swanson *et al*, 1995). It is postulated that the lack of uptake during conditions of neuronal and astrocytic swelling or acidosis (e.g. during ischaemia) may lead to the neurotoxic accumulation of glutamate in the extracellular space. The high affinity L-glutamate transporter is stereospecific with regard to glutamate, D-glutamate is a poor substrate. D- & L-aspartate have affinities for the transporter in the low micromolar range (Kanner, 1993).

Excitotoxicity is the term used to describe the neuronal degeneration produced by compounds that cause excitation and toxicity in neurones (excitotoxins) (Whetsell & Shapira, 1993). Activation of kainate and AMPA receptors by glutamate opens the

intrinsic sodium channel, that is closed at resting potential, and causes depolarisation of the cell membrane. Depolarisation results in removal of magnesium block from the postsynaptic NMDA receptor, leading to its activation (the NMDA receptor has a voltage-dependent conductance mechanism whereas the kainate and AMPA receptors have voltage-independent conductances). Activation of NMDA receptors and non-NMDA receptors therefore leads to an influx of calcium through receptor-operated calcium channels and voltage-operated calcium channels (Lin *et al,* 1990; Ohta *et al,* 1991; Uematsu *et al,* 1991; McBurney *et al,* 1992). The increase in intracellular calcium causes activation of calcium-sensitive systems. Activation of phospholipase A2 by calcium leads to the production of arachidonic acid. Arachidonic acid causes potentiation of NMDA-evoked currents and inhibition of reuptake of glutamate into astrocytes and neurones. Cytotoxic free radicals are produced during the breakdown of arachidonic acid by cyclo-oxygenase to eicosanoids and by lipoxygenase to leukotrienes. The activation of nitric oxide synthase by calcium produces nitric oxide which interacts with superoxide ions to produce peryoxynitrite ions which lead to production of the toxic hydroxyl ions. Calcium has been reported to cause the conversion of xanthine dehydrogenase to xanthine oxidase, though the existence of the latter in the rat brain is still controversial. The conversion of hypoxanthine to xanthine by xanthine oxidase produces free radicals. Other calcium-sensitive systems include protein kinase C, proteases and endonucleases. Overstimulation of these may be detrimental to cells.

If the supply of oxygen is decreased, then the cells' metabolism would decrease, as would oxidative phosphorylation. Oxidative phosphorylation uses oxygen and produces 36moles of ATP/ mole of glucose. Anaerobic glycolysis would occur instead which produces 2moles of ATP and 2moles of lactate/ mole of glucose as well as hydrogen ions. Therefore there would be a reduction in ATP production and a reduction in pH. A decrease in ATP would cause failure of the Na+/K+-ATPase transport process which would lead to cell depolarisation, a reversal of glutamate uptake and activation of voltage-operated calcium channels. The overall result would be calcium influx, leading to an increase in intracellular

calcium and glutamate release, thus perpetuating a vicious cycle causing further neuronal death.

Glycine may have an importance in neurotoxicity since the ischaemic-induced level of glycine is reported to remain high for longer than that of glutamate (Baker *et al*, 1991; Globus *et al*, 1991) and Wood *et al* (1992) reported that antagonists at the strychnine-resistant glycine site attenuate ischaemic-induced neuronal loss. This would be consistent with the finding that glycine binding to an allosteric extracellular site on the NMDA receptor potentiates glutamatergic signal and also delays desensitisation (Collingridge & Lester, 1989).

1.3. Free Radicals and Excitotoxicity

The acceptance of a single electron by molecular oxygen ($0_2$) forms the superoxide anion radical ($0_2^{\cdot}$). Superoxide is subsequently dismutated by superoxide dismutase producing hydrogen peroxide ($H_2O_2$) (fig 1.2). Hydrogen peroxide has the ability to cross membranes, unlike the superoxide ion. The reaction of superoxide and hydrogen peroxide produces the highly reactive and toxic hydroxyl radical. This oxidation is brought about by the readily available Fe*+ or Cu+ in the CNS, a process called the Fenton Reaction (fig 1.2). In addition, the reaction of superoxide ions and nitric oxide produces the peryoxynitrite ion. The peryoxynitrite ion is cytotoxic through its ability to oxidise thiol groups or through its decomposition to the toxic hydroxyl ion.

Overproduction of these free radicals leads to lipid peroxidation, cell damage and the further leakage of free radicals and cellular components through the cell membrane into the extracellular space. Combination of molecular oxygen with methylene groups of saturated fatty acids in biomembranes makes this part of the membrane become hydrophilic and chemically reactive so that the membrane functions abnormally or is destroyed. Lipid peroxidation involves the removal by free radicals of a hydrogen atom from a fatty acid. This will result in the production of a peroxyl radical which can itself initiate damage to another polyunsaturated fatty acid by removing a hydrogen atom, thus propagating the process of molecular damage across hundreds or thousands of molecules. In addition to

lipids, hydroxyl ions react with virtually any molecule in their path including enzymes which are vital to the cells' function.

The importance of free radicals in neurotoxicity is being realised. A mutant gene that encodes a cytosolic superoxide dismutase has been identified in patients with FALS (familial amyotrophic lateral sclerosis), a degenerative disorder of motor neurones (Rosen *et al*, 1993). An abnormal function of superoxide dismutase may lead to the accumulation of toxic superoxide ions. Free radicals have been proposed to be produced in other diseases involving neurodegeneration or neurotoxicity such as Parkinson's Disease (Olanow, 1993) and ischaemia (Siesjö, 1992). In Parkinson's Disease, dopamine, which has increased turnover in surviving neurones, may be oxidised and produce free radicals. Iron is thought to be present in regions which are known to be involved in Parkinson's Disease (Olanow, 1993) and catalyses free radical generation by the Fenton reaction (Figure 1.2). During ischaemia, the elevated intracellular calcium may activate calcium-sensitive systems which result in free radical production. Phospholipase A2 is activated by calcium and during its breakdown, free radicals are produced. Calcium-activated nitric oxide synthase may lead to the production of the peryoxynitrite ion and thus hydroxyl ions through the synthesis of nitric oxide. Calcium has been reported to cause the conversion of xanthine dehydrogenase to xanthine oxidase, a reaction which produces free radicals.

Given the evidence for production of free radicals under such conditions and the potential role of free radicals in cytotoxicity, free radicals may be involved in neurodegenerative diseases in which there is substantial oxidative stress.

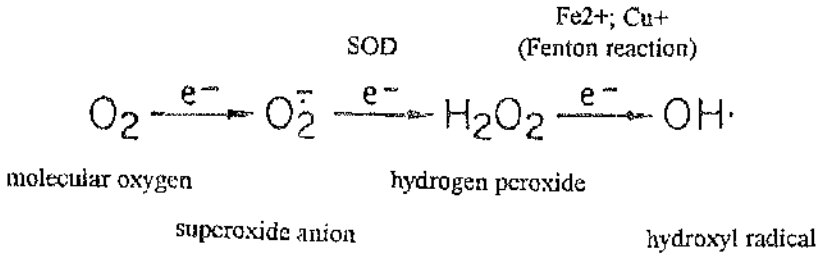



Fig 1.2

The conversion of molecular oxygen to the hydroxyl radical. Molecular oxygen can be reduced to the superoxide anion radical ($O_2^-$). Superoxide dismutase (SOD) catalytically reduces the superoxide anion to hydrogen peroxide ($H_2O_2$). $H_2O_2$ can be reduced in the presence of iron ($Fe^{2+}$) or copper (Cu+) to the highly toxic reactive hydroxyl radical ($OH^-$) by the Fenton reaction.

It has been suggested that activation of kainate receptors leads to the production of free radicals which may contribute to neuronal damage (McNamara & Fridovich, 1993). Stimulation of the non-NMDA receptor has been proposed to activate a calcium-dependent protease that enzymically converts xanthine dehydrogenase to xanthine oxidase which subsequently produces superoxide radicals (McNamara & Fridovich, 1993).

The present project used microdialysis in the hippocampus of anaesthetised rats to:

1) investigate the ability of the non-NMDA receptor agonist and excitotoxin, kainate (Coyle & Schwarcz, 1976), a pyrrolidine isolated from the seaweed *Digenia simplex*, to induce the release of endogenous adenosine and its metabolites, inosine, hypoxanthine and xanthine

2) compare the profile of release induced by kainate with that induced by potassium

3) elucidate the mechanisms involved in the evoked release of adenosine

## 2.0 METHODS

### 2.1 Microdialysis

#### 2.1.1. Summary of technique and development

The analysis of chemical events which occur between cells has been difficult. The dissection of tissue represents a static reflection of synaptic events, mixing cells, organelles and extracellular fluid. To obtain a more dynamic picture of the chemical interplay between cells in living tissue, microdialysis has been introduced. Microdialysis involves perfusing a thin dialysis tube inserted in the tissue. The concentration of compounds in the perfusate reflects the composition of the compounds in the extracellular fluid as a result of the diffusion of substances across the dialysis membrane.

Microdialysis has advantages over older techniques such as the push-pull cannula and cortical cup techniques. The dialysis membrane over the tip of the probe is the main feature of difference between microdialysis and push-pull cannula. This difference means that in microdialysis there is no need to balance the push of the flow of liquid with the pull of the flow. In push-pull cannulae, liquid has to be infused through the tissue and be pulled out through the cannula. As a result, there may be damage to tissue which is minimised in microdialysis. The dialysis membrane also acts as a barrier which improves the sterility of the fluid diffusing into the brain and excludes proteins and other macromolecules from coming out of the brain which would have to be removed before HPLC (high performance liquid chromatography) analysis. These are major advantages over the cortical-cup technique in addition to the ability to perform microdialysis on most organs and tissue of the body.

A powerful feature of microdialysis is the ability to stimulate the tissue locally by including substances in the perfusate. Neurotransmission and release processes may be examined at the same time as the stimulation of receptors by the local administration of a drug via a microdialysis probe (Westerink *et al*, 1987).

However, microdialysis is far from being a simple technique. The complexity of the technique comes from the interactions of the dialysis membrane with the living tissue. The

chemical events taking place in this interaction must be understood, in order to interpret the results.

2.1.2. Complications of interpretation

Insertion of a foreign body, such as a microdialysis probe causes damage to the surrounding tissue. Much work has been carried out to assess the limitations of microdialysis with respect to damage. To review these limitations, the reported effects of insertion of a foreign body into tissue must first be examined. The possible effects are glial reaction, haemorrhage, limited neuronal death (Benveniste & Diemer, 1987), localised haematoma (Yaksh & Yamamura, 1974), alterations in glucose metabolism and blood brain barrier (BBB) function (Benveniste, 1989), reduction in regional blood flow (Tomida *et al*, 1989) and local biochemical disturbances. These changes may be delayed after implantation. For instance, glucose metabolism decreased 24 hrs after probe implantation (Benveniste, 1989), and glial reactions began 2-3 days after implantation (Benveniste & Diemer, 1987). Interruptions of fibres of passage were seen using silver degeneration staining methods both adjacent to the site of implantation and in remote sites e.g. in corpus callosum and contralateral hippocampus when implantation was in the dorsal hippocampus (Shuaib *et al*, 1990). This axonal damage was seen 24 hrs after probe implantation and became increasingly more prominent with longer survival times (Shuaib *et al*, 1990). The following questions must therefore be asked. Are the compounds detected by microdialysis the result of chemical events within the extracellular fluid which occur secondary to the insertion of the probe? Do glial cells, monocytes and endothelial cells which may accumulate around the dialysis probe affect the distribution or metabolism of the detected compounds? Is the BBB disrupted? Plasma constituents and drugs which do not normally pass through the BBB may have increased access to the brain from the surroundings of the dialysis probe.

Work carried out by Westergren *et al* (1995) attempted to answer the final question. They found that the BBB was disturbed 3 hrs and 24 hrs after probe insertion. There was also enhanced passage of CSF and albumin around the probe and increased BBB permeability 24 hrs compared to 3 hrs after insertion. They explain this delay in enhancement by 1)

decreased blood flow due to vasospasm and damaged blood vessels (as blood flow increases after repair of vessels more tracer can enter the brain and dialysate) 2) cell damage around the probe leading to a delayed accumulation of leukocytes and macrophages with the production of free radicals known to increase BBB permeability. However, work carried out earlier by Benveniste *et al* (1984) and Tossman & Ungerstedt (1986) indicate that damage to the BBB is negligible 30min to 2hr after probe insertion. The trauma of implantation may therefore vary with the type of probe used and it is possible that the extent of damage to the BBB is related to the length of time the probe is inserted in the tissue.

2.2 The surgical procedure

All animal procedures were in accordance with the Home Office Guidelines and were specifically licensed under the Animal (Scientific Procedures) Act 1986.

Male Wistar rats (body weight 270-310g) were anaesthetised using urethane 1.25g/kg i.p. (intraperitoneal) and microdialysis probes were stereotaxically inserted bilaterally into the hippocampus. The microdialysis system used for anaesthetised animals is shown in fig 2.1. The coordinates were 5.6mm posterior, 5.0mm lateral and 8.0mm ventral relative to the bregma (fig 2.2) (Paxinos & Watson, 1986). A homeothermic blanket maintained rectal temperature at 36-37$^{0}$C. At the end of each experiment the location of the probes was confirmed by staining with 10% pontamine sky blue. The dye solution was perfused through the probes for 2 minutes after which the brains were removed and placed into 10% formalin pH 7 to fix overnight. The brains were subsequently sectioned manually using a razor blade in order to locate the dye.

The microdialysis probes were continuously perfused with artificial cerebrospinal fluid (acsf), composed of 125mM NaCl, 2.5mM KCl, 0.5mM $KH_2PO_4$, 27mM $NaHCO_3$, 1.2mM $MgSO_4$, 1.2mM $CaCl_2$ and 10mM glucose (pH 7.2) at a flow rate of 2µl/min. Dialysate samples were collected every 20min in tips of eppendorf tubes and either analysed immediately or snap frozen in liquid nitrogen and stored at -20$^{0}$C for later analysis. Two methods of drug administration were used in the present study. Either compounds were dissolved in acsf, pH7.2 and administered through the probe by reverse

dialysis, by means of a liquid switch which caused little disturbance to the flow. Alternatively, drugs were dissolved in saline or vehicle as stated otherwise and injected subcutaneously or intraperitoneally in a volume of 1ml/kg. In the present study, to differentiate between the two methods of administration, administration through the probe will be referred to as intrahippocampal and administration by s.c. (subcutaneous) or i.p. injection will be referred to as systemic.

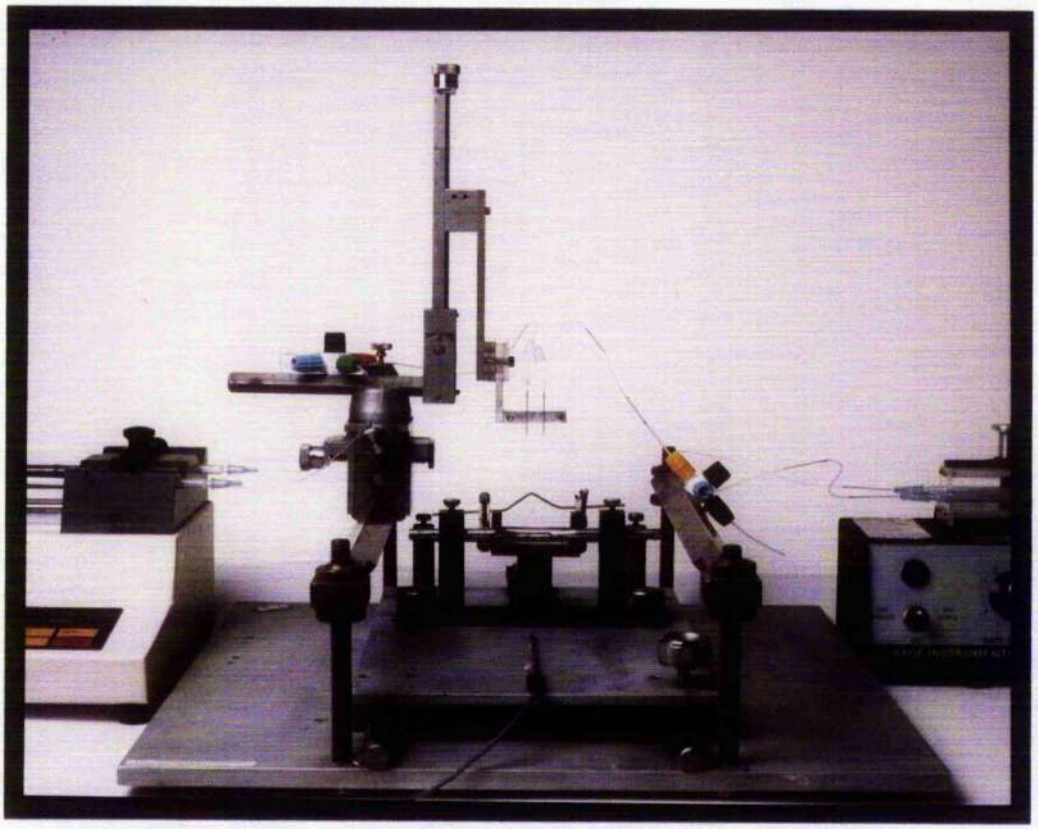


Fig 2.1
Microdialysis system for anaesthetised animals

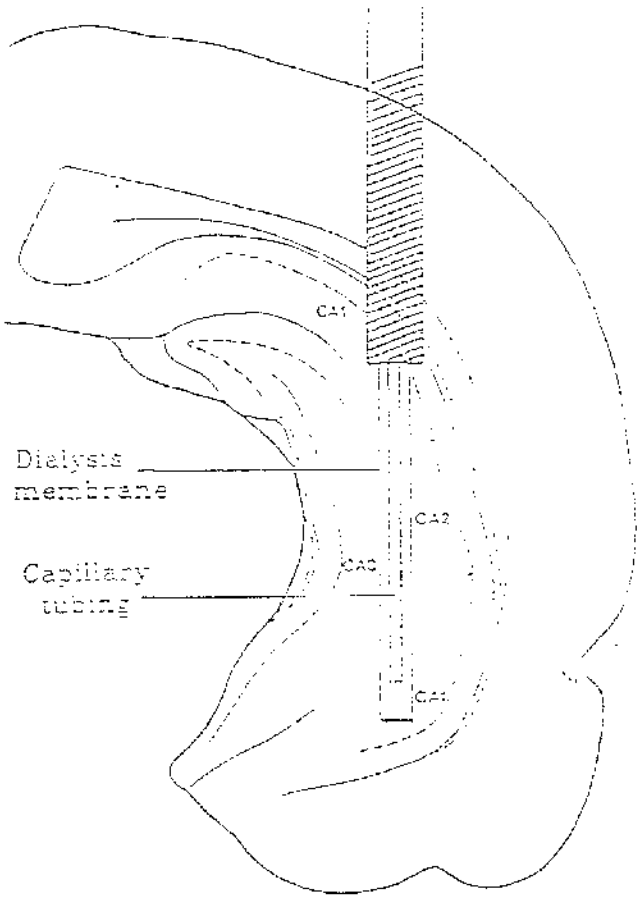



Fig 2.2
Position of probe in the hippocampus

2.3 Materials

The following materials were used:

From Sigma Chemicals: adenosine; L-ascorbic acid; L-aspartic acid; (±)-AP-5; 2-chloroadenosine; dimethylsulphoxide (DMSO); L-glutamic acid; glutathione; hypoxanthine; inosine; kainic acid; α-β-methylene ADP; NBTG (S-(2-hydroxy-5-nitrobenzyl)-6-thioguanosine); NMDA; oxypurinol; R-PIA; TTX (tetrodotoxin); urethane and xanthine.

From Amersham: [$^3$H]kainate

From Anachem: 2-mercaptoethanol (MCE)

From Hoechst: propentofylline

From Ferrosan: CNQX

From Research Biochemicals International: CNQX HBC complex; CPT (8-cyclopentyl-1,3-dimethylxanthine); (+)-MK-801 hydrogen maleate and 8-PST (8-(p-sulphophenyl)theophylline).

From Burroughs Wellcome Co: EHNA (erythro-9-(2-hydroxy-2-nonyl)adenine HCl.

From BDH Biochemicals: formaldehyde; L-glutamic acid; 2-mercaptoethanol; pontamine sky blue.

From Fluka Biochemicals: OPA (*o*-phthaldialdehyde).

From The Upjohn Company: U50, 488H (trans-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneactemide methanesulphonate.

From Astra Neuroscience Research Unit: Chlormethiazole edisylate.

Dr. I. Tarnawa (Budapest, Hungary): GYKI 52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine hydrochloride.

2.4 Microdialysis Probe Construction (concentric probe design)

1) A 23g Microlance needle is filed to a length of 26mm.

2) At least 10mm Hospal polyacrylonitrile membrane (0.3mm, o.d., molecular cut-off 24kDaltons) is fed through the lumen of the filed needle. Forceps are used at all times to avoid plugging pores with grease or dirt

3) 4.5mm of the membrane is allowed to be exposed from inside of the needle and the join between the membrane and the needle is glued, using resin and hardener in a 50: 50 mixture (RS Components), so as not to get any glue on the membrane below the needle join.

4) Portex tubing (0.58mm i.d.) is attached to the opposite end of the needle and the join with the needle is glued.

5) A hole in the Portex tubing is made with a needle and silica tubing (i.d. 40μm) is fed through the lumen of the dialysis membrane towards this hole. The silica tubing is cut and adjusted so that the end is positioned at least 2mm from the tip of the dialysis membrane and with at least 10mm left outside the Portex tubing. The tip of the dialysis membrane is plugged with epoxy and left to dry before the silica tubing is pushed further down so that it is 1.0mm from the tip (0.5mm of which is taken up by the epoxy glue).

6) The hole in the Portex tubing is glued (i.e. the join between the silica tubing and the Portex tubing).

2.5 Microdialysis procedure

Solutions were perfused into the probe from a syringe pump (Harvard Apparatus Syringe Infusion Pump 22 and Sage Instruments Syringe Pump Model 34) at a flow rate of 2μl/min through Portex tubing and samples were collected from the silica tubing.

The relative recovery of each probe was calculated before each experiment by an *in vitro* procedure. In a standard purine solution of 1μM, the microdialysis probes were pumped with acsf at 2μl/min and the dialysates were collected every 20 min and were analysed for purine content. The concentration of adenosine, inosine, hypoxanthine and xanthine in a 20min sample was then measured and divided by the concentration of each purine in the solution to yield the relative recovery of the probe. Three 20 min samples were taken for each probe and the mean value was used to represent that probe.

160 min after probe insertion into the hippocampus, two or three 20 min samples were analysed to assess basal levels. These were followed by a 5min pulse of kainate or $K^+$. To investigate the mechanism of release, two 5 min pulses (S1 and S2) of kainate or $K^+$ were used three hours apart. Drugs which were used to test the mechanisms of kainate-evoked

adenosine release were introduced two hrs before, during and after the second stimulation. The net increase above basal in the first four samples after each stimulation was calculated as a ratio S2/S1. The S2/S1 values obtained for the test experiments were compared to that of control experiments in which no drugs were perfused before and during the second stimulation of kainate or $K^{+}$. Statistical analysis was performed using an unpaired t-test and, where appropriate, a paired t-test.

2.6 Statistical Analysis

Drug-induced release of either purines or excitatory amino acids were analysed by paired t-test against the basal levels which were calculated by averaging two or three levels before the application of the drug.

The S2/S1 ratios were compared in test and control conditions by using ANOVA, followed by Dunnett's t-test. Dunnett's t-test was the recommended t-test using GraphPad Instat version 2.03 package.

Time point release profiles of purines in control and test experiments were compared by ANOVA, followed by Bonferrroni Multiple Comparison's t-test. This t-test was more conservative than the Dunnett's t-test, according to GraphPad Instat version 2.03, and it was used to allow the selection of pairs of columns to be analysed. In the present study, dialysate levels were compared between test and control experiments at the same time point only.

2.7 Purine Analysis

Purines were analysed using an isocratic HPLC (Severn Analytical Solvent Delivery System SA6410B) with ultraviolet detection at 254nm (Severn Analytical uv/vis Absorbance Detector SA6500) and a Rheodyne model 7125 injector (20µl loop). The mobile phase was 0.01M sodium phosphate ($NaH_2$ $PO_4$) with 6% methanol (HPLC Grade) pH 6.1 at a flow rate of 0.8ml/min. A techsphere C18 3µm microsphere column, 10cm by 4.6mm, was used to separate the purines which had the following approximate retention times: hypoxanthine (1.0min), xanthine (1.4min), inosine (2.4min) and adenosine (8.0min) (fig 2.3). The limit of sensitivity for adenosine was approximately 1.5pmol

The identification of compounds was achieved by comparison of retention time with standards. Quantification of the compounds was achieved by parallel chromatography of standards (fig 2.4). At the beginning of the present project, a small study was carried out to compare the quantity of purines obtained by measuring the areas of the peaks in the chromatograms (by multiplying the height of the peaks by the width at half of the height) with that obtained by measuring solely the height of the peaks. The results obtained were very similar and since the latter was a less time consuming process, all compounds (in standards and samples) were subsequently quantified by measuring the purine chromatogram peak height. Table 2.1 shows the correlation coefficient and '$r^2$' values for the calibration graphs seen in fig 2.4.

The following compounds were detected by this HPLC system and for this reason had to be eliminated from the experimental protocol: 2-chloroadenosine and α-β-methylene-ADP. These chromatograms are shown in fig 2.5.

Table 2.1 The correlation coefficients for the purines analysed by HPLC.

| Purine | r | $r^2$ |
|---|---|---|
| Adenosine | 0.9997 | 0.9995 |
| Inosine | 0.9998 | 0.9996 |
| Hypoxanthine | 0.9969 | 0.9937 |
| Xanthine | 0.9980 | 0.9960 |

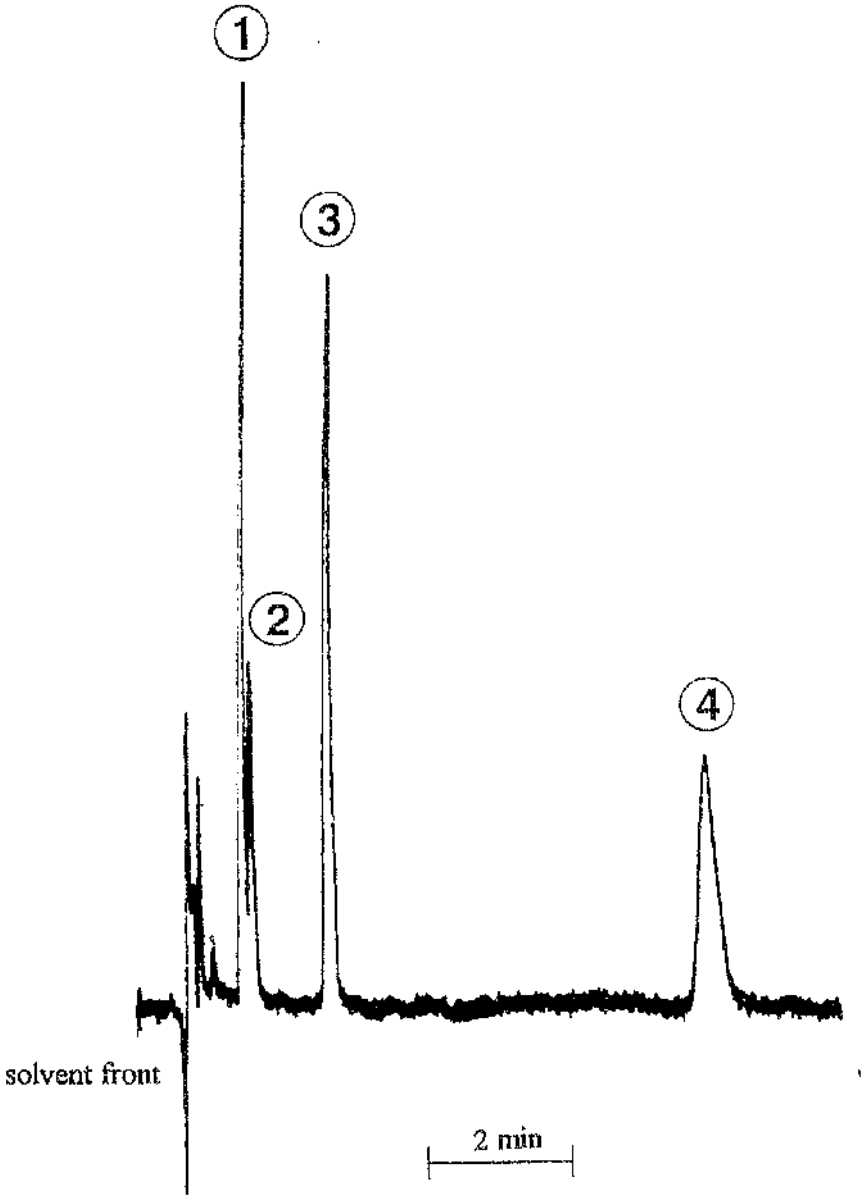



Fig 2.3

Chromatogram of purines 1μM

1 hypoxanthine

2 xanthine

3 inosine

4 adenosine

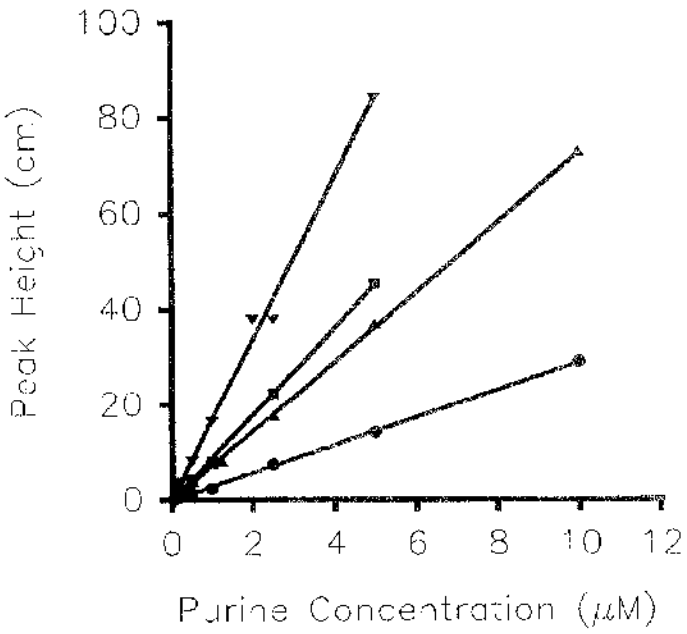



Fig 2.4

Calibration graphs for the determination of purine concentration (μM) by HPLC.

- adenosine
- inosine
- hypoxanthine
- xanthine

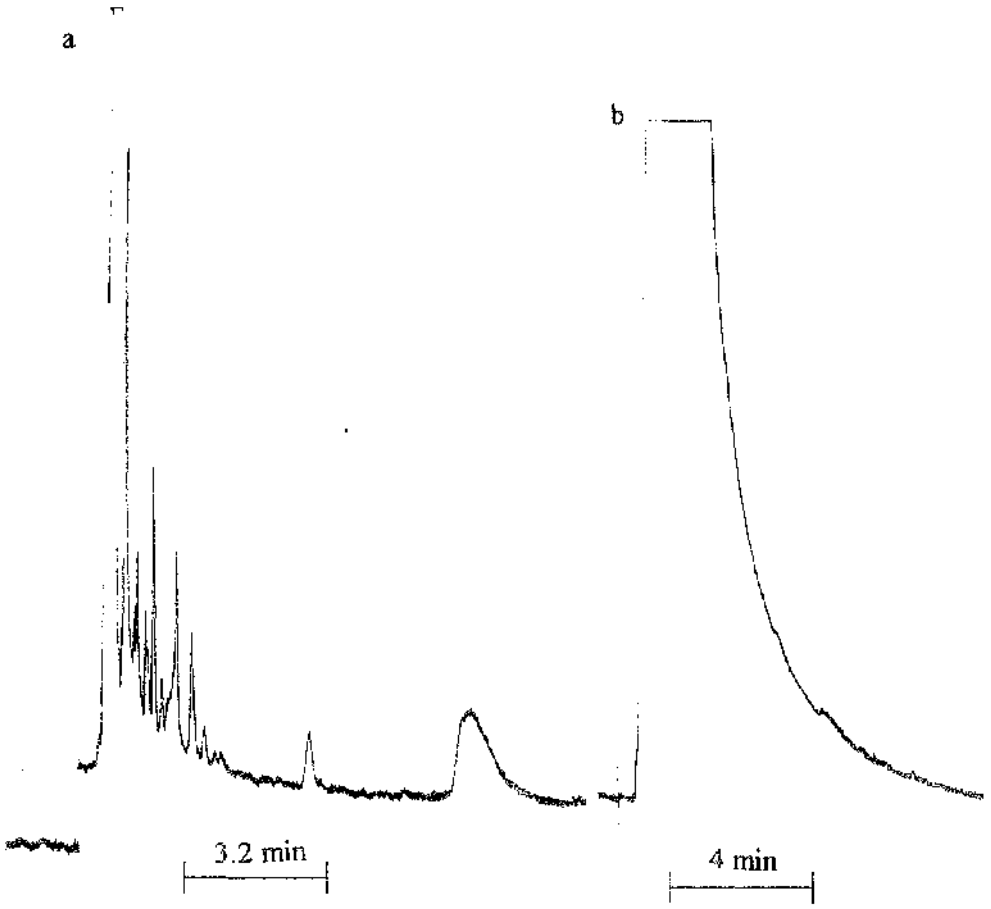



Fig 2.5

Chromatograms of substances in dialysate samples which interfered with the separation of adenosine

a) 2-chloroadenosine 10μM in perfusate (retention time of 8min, same that of adenosine)

b) α-β-methylene-ADP 0.5mM in perfusate

## 2.8 Excitatory Amino Acid Analysis

Amino acids were analysed by a Gilson gradient HPLC consisting of two model 302 pumps, model 712 Gilson Gradient Programmer and a Spectra Glo fluorimeter (Gilson). Aliquots of microdialysate (20µl) were made up to 40µl with acsf, and rapidly mixed with 40µl of OPA/ MCE reagent (0.5% OPA, 0.5% MCE, 10% methanol in borate buffer) and 40µl borate buffer 100mM, pH 9.5. The mixture was allowed to stand for 2 min before injection onto the chromatographic column by a model 231 autosampler and a Rheodyne model 7010 injector (20µl loop). Glutamate and aspartate were separated by a Dynamax microsorb C18 3µm column (5cm by 4.6mm) with retention times of 2min and 4min, respectively (fig 2.6). Solvent A was 0.1M sodium acetate with 9.5% methanol (HPLC Grade) and 0.5% tetrahydrofuran (HPLC Grade), pH 7.2. Solvent B was methanol 100%. Solvent A was mixed in a stepwise gradient with methanol. Flow rate was 1.7µl/min, length of run was 25min and the gradient programme, expressed as time in minutes from injection (% solvent B), was; 0(0), 2(20), 3(30), 9(45), 12(45), 14(85), 16(85), 18(0).

The limits of sensitivity for glutamate and aspartate were both approximately 2pmol. The identification of compounds was achieved by comparison of retention time with standards. Quantification of the compounds was achieved by parallel chromatography of standards (fig 2.7). Peak areas were computer analysed by a model 712 Gilson Gradient Programmer. Table 2.2 shows the correlation coefficients and '$r^2$' values for the calibration graphs seen in fig 2.7.

Table 2.2

The correlation coefficients for the excitatory amino acids analysed by HPLC.

| Amino acid | r | $r^2$ |
|---|---|---|
| Aspartate | 0.9896 | 0.9794 |
| Glutamate | 0.9934 | 0.9868 |

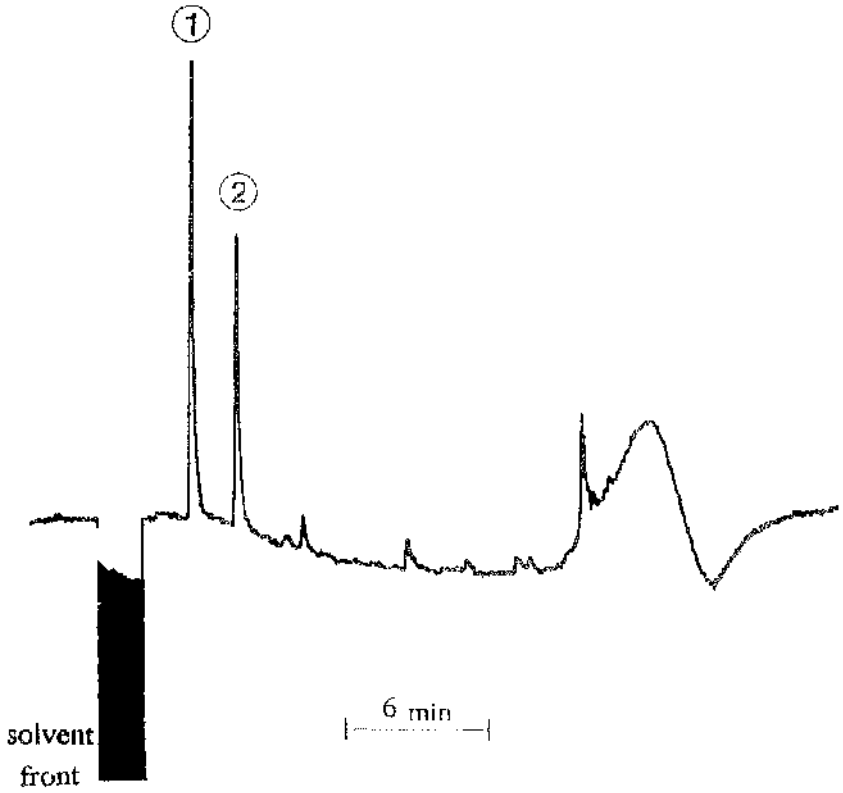



Fig 2.6

Chromatogram of excitatory amino acids 1μM

1 aspartate

2 glutamate

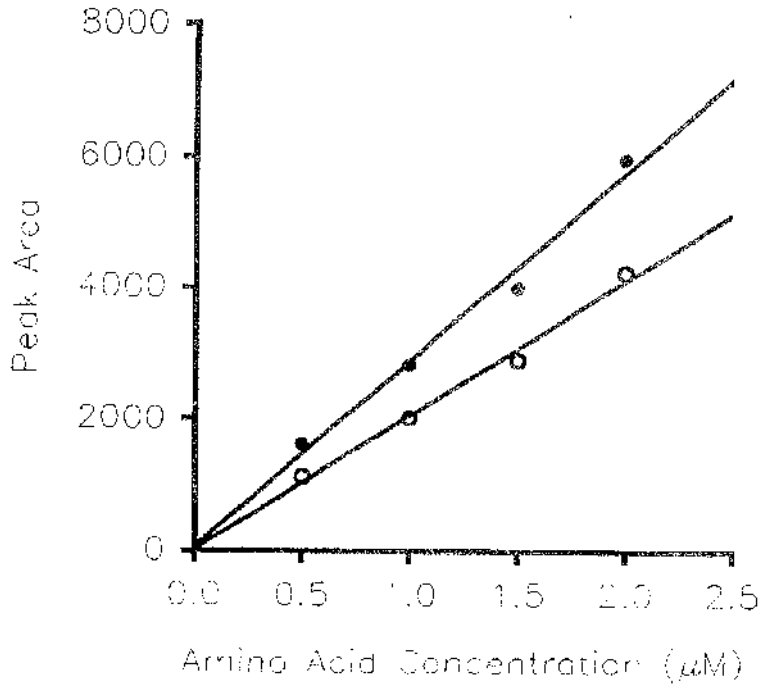



Fig 2.7

Calibration graphs for the determination of amino acid concentration (μM) by HPLC.

- o glutamate
- • aspartate

2.7. Assessment of kainate efflux

To investigate the diffusion of kainate through the rat brain from the microdialysis probe inserted into the hippocampus of urethane-anaesthetised rats, 1mM kainate, spiked with [$^3$H] kainate, was perfused through the probes for 5 min at a flow rate of 2µl/min. 100min after perfusion of the kainate, the rat was killed by an overdose of urethane and the brain removed into dry ice. The brain was kept at -20$^0$C until sectioned into 20µm slices using a cryostat. Every 4th section was placed onto poly-l-lysine-treated slides (slides were treated by dipping into 50ml of poly-l-lysine 5mg/ml (Sigma Chemicals) and then placing in oven for about 2 hrs to dry). The slides were then taken through the following sequence: 1) fix in 4% paraformaldehyde (10g PFA dissolved at 4$^0$C in 25ml 10×PBS (phosphate buffer saline) and 225ml deionised water) for 5 mins 2) rinse for 1 min in 1×PBS 3) rinse for 1 min in each of 70%, 90% and then 100% ethanol) 4) leave the slides to dry and then expose to film (Hyperfilm ([3H]), Amersham) for 3 weeks. The film was developed then soaked in fixer (Kodac Unifix) for twice as long as it took for the water to go cloudy and then clear. Finally, the film was soaked in water for a further 2 min. After drying, the film was placed on a light box and the mean darkness of pixels was measured for each region of the slice by an image analyser and quantified in nCi/mg by comparing with the darkness of known standards. The calibration curve is shown in fig 2.8.

Materials

[3H] Kainate: stock solution 17µM, specific radioactivity 58Ci/mmol. Stock solution was diluted 1 in 10 for the above experiments.

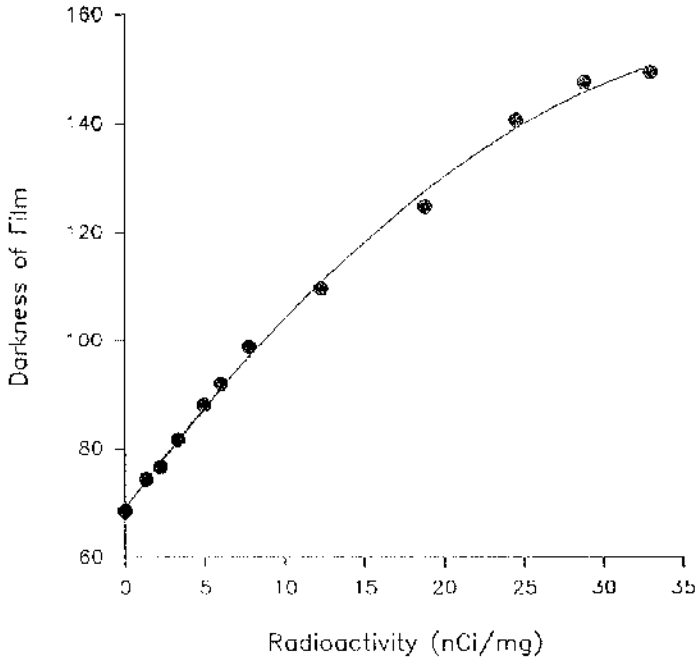



Fig 2.8

Calibration curve for the determination of amount of radioactivity in slices

2.10 Histology of the hippocampus after a 5min exposure to 1mM kainate through the dialysis probe.

Two male Wistar rats (body weight 310g) were anaesthetised using Hypnorm (Janssen Animal Health; fentanyl citrate 0.315mg/ml and fluanisone 10mg/ml) : Hypnovel (Roche; midazolam 10mg/5ml) : water (1:1:2) at a dose of 3.3ml/kg i.p. and microdialysis probes were stereotaxically inserted bilaterally into the hippocampus as before. A homeothermic blanket maintained rectal temperature at 36-37$^{0}$C. The microdialysis probes were continuously perfused with acsf at a flow rate of 2µl/min. A settling period of 1 hour in one of the animals and of 1 hour 40 min in the other was allowed after probe insertion before kainate 1mM was perfused through the right hand probe for 5 minutes by means of a liquid switch. The left hand probe was continuously perfused with acsf to examine the extent of damage by probe insertion.

The interdigital reflex of the rats was continuously tested and anaesthesia was maintained using halothane 0.5-2% in $0_2$ at 1.0 l/min for 1hr 20 min after the kainate pulse. Hypnorm and Hypnovel were chosen to induce anaesthesia rather than halothane since the halothane nozzle did not fit onto the nose bar of the stereotaxic frame. However, the nozzle was close enough to allow maintenance of anaesthesia after the administration of Hypnorm and Hypnovel. Halothane was chosen for maintenance rather than Hypnorm and Hypnovel since anaesthesia by the latter was not so easily reversed.

At the end of the perfusion period the probes were removed, the scalp was sutured and the halothane administration was stopped. The rats were allowed to recover. Seven days later the rats were killed by a lethal dose of urethane and the brain immediately dissected out and immersed into histological fixative (Buffered Formalin Solution, Genta Medical). The brains were allowed to fix for at least 48 hrs and then sliced into 5µm coronal sections. Sectioning and staining were performed by Dr. W. Behan at the Western Infirmary, Dept. of Pathology. Sections from each brain, taken at the level of the hippocampus (levels -4.4 to -6.2mm AP according to the atlas of Paxinos & Watson, 1986), were stained by haematoxylin and eosin mixture and examined by light microscopy for assessment of the extent of cell damage.

## 3.0 RESULTS

### 3.1 The microdialysis probes and their relative recovery

The relative recoveries for adenosine, inosine, hypoxanthine and xanthine were 17.00±0.61%, 13.60±0.52%, 19.91±0.7% and 18.83±0.58% respectively (n=71) (table 3.1). The relative recovery was measured to screen the probes for variations in their properties. The concentration of purines within the ECF (extracellular fluid) may be estimated from the collected dialysate concentration using the relative recovery of the probe. However, these calculated values are not an accurate estimation and are used here primarily to allow comparisons with other studies with different probe construction (see discussion). The basal interstitial concentrations of purines estimated here in the anaesthetised rat hippocampus are summarised in table 3.1.

### 3.2. Adenosine efflux after probe insertion

Immediately upon insertion of the probe into the hippocampus, the efflux of endogenous adenosine, collected in the dialysate, was high but declined to a steady baseline within one hour (fig 3.1). A further 40 minutes was allowed before three successive twenty minute samples were collected to assess basal levels of purines after the attainment of equilibrium. The basal amounts of purines detected in the dialysates are summarised in table 3.1.

**Table 3.1**

The relative recoveries, expressed as percentages, were calibrated in purine solutions (1μM) *in vitro* (n=71). The estimated concentration (μM) in the ECF of adenosine, inosine, hypoxanthine and xanthine (n=10) were calculated from the amount collected in dialysates (pmol/20μl) using the relative recoveries.

| | **Relative Recovery *in vitro* (%) (n=71)** | **Dialysate amounts (n=10, pmol/20μl)** | **Estimated conc. in hippocampus (n=10, μM)** |
|---|---|---|---|
| **ADENOSINE** | 17.00 ± 0.61 | 3.68 ± 021 | 0.80 ± 0.07 |
| **INOSINE** | 13.60 ± 0.52 | 3.12 ± 0.12 | 0.66 ± 0.04 |
| **HYPOXANTHINE** | 19.91 ± 0.7 | 4.96 ± 0.32 | 1.16 ± 0.08 |
| **XANTHINE** | 18.83 ± 0.58 | 13.6 ± 1.14 | 3.60 ± 0.30 |

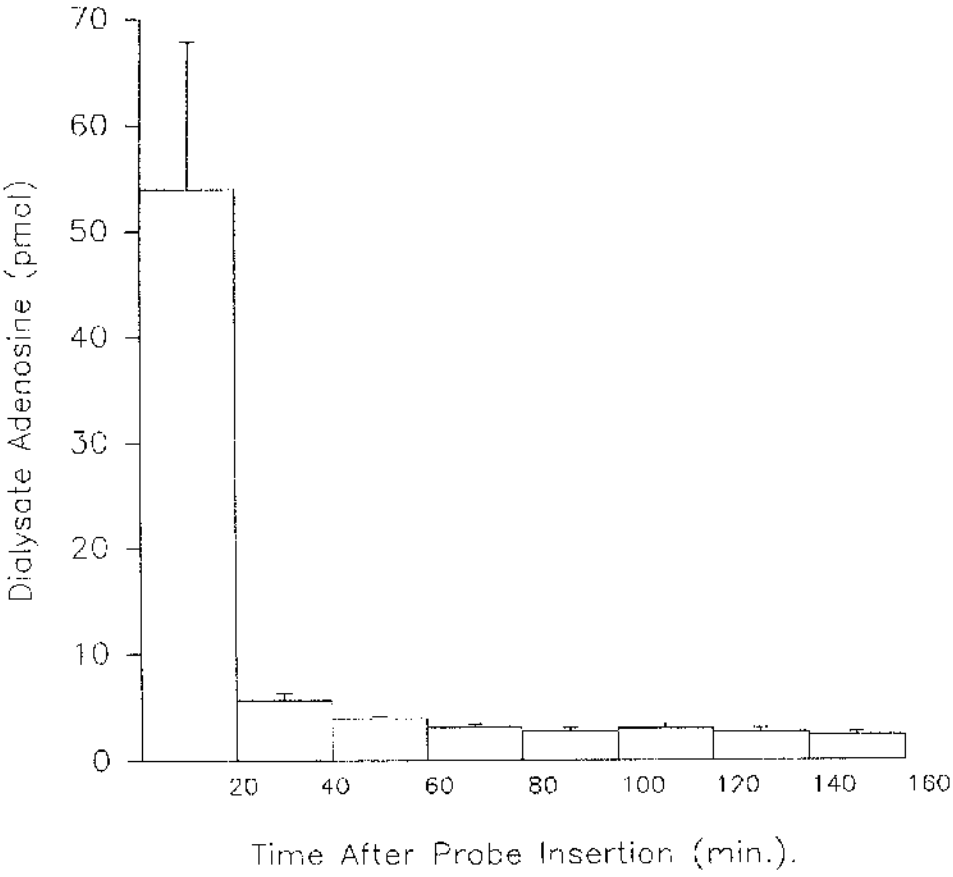



Fig 3.1.

Adenosine dialysate levels in the first 2hrs 40min after probe insertion (pmol, mean ± sem, n=6). There was a high efflux within the first 20min which declined within the first hour. A further 1hr was allowed before two or three 20min samples were taken to assess basal levels. Rate of perfusion was 2µl $min^{-1}$.

### 3.3.1 Potassium-evoked release of adenosine and its metabolites

Adenosine release induced by 100mM potassium reached significance within the first sample of the potassium reaching the tissue (fig 3.2a). Adenosine release peaked within the next sample, the increase being from 3.6 ± 0.2 to 6.26 ± 0.58 pmol/20µl. The delayed peak response was largely due to the lag-time from the liquid switch to the collection vial which was 16 minutes. Potassium was exposed to the tissue during the last 4 minutes of the first sample and the first minute of the next sample. The contact of potassium with the tissue for only 4 min immediately enhanced the release of adenosine significantly, as seen in the first sample, but the bulk of the release occurred in the next sample. Both inosine and hypoxanthine release during the first sample of the potassium reaching the tissue did not reach significance compared to basal (fig 3.2b & c). Significance for these compounds was reached during the second sample of exposure of potassium to the tissue (that in which adenosine release was seen to peak) (fig 3.2b & c). Inosine was increased from 2.71 ± 0.33 to 4.66 ± 0.76 pmol/20µl and hypoxanthine from 6.221 ± 0.93 to 14.21 ± 2.57 pmol/20µl. Xanthine release did not increase after exposure to potassium (fig 3.2d).

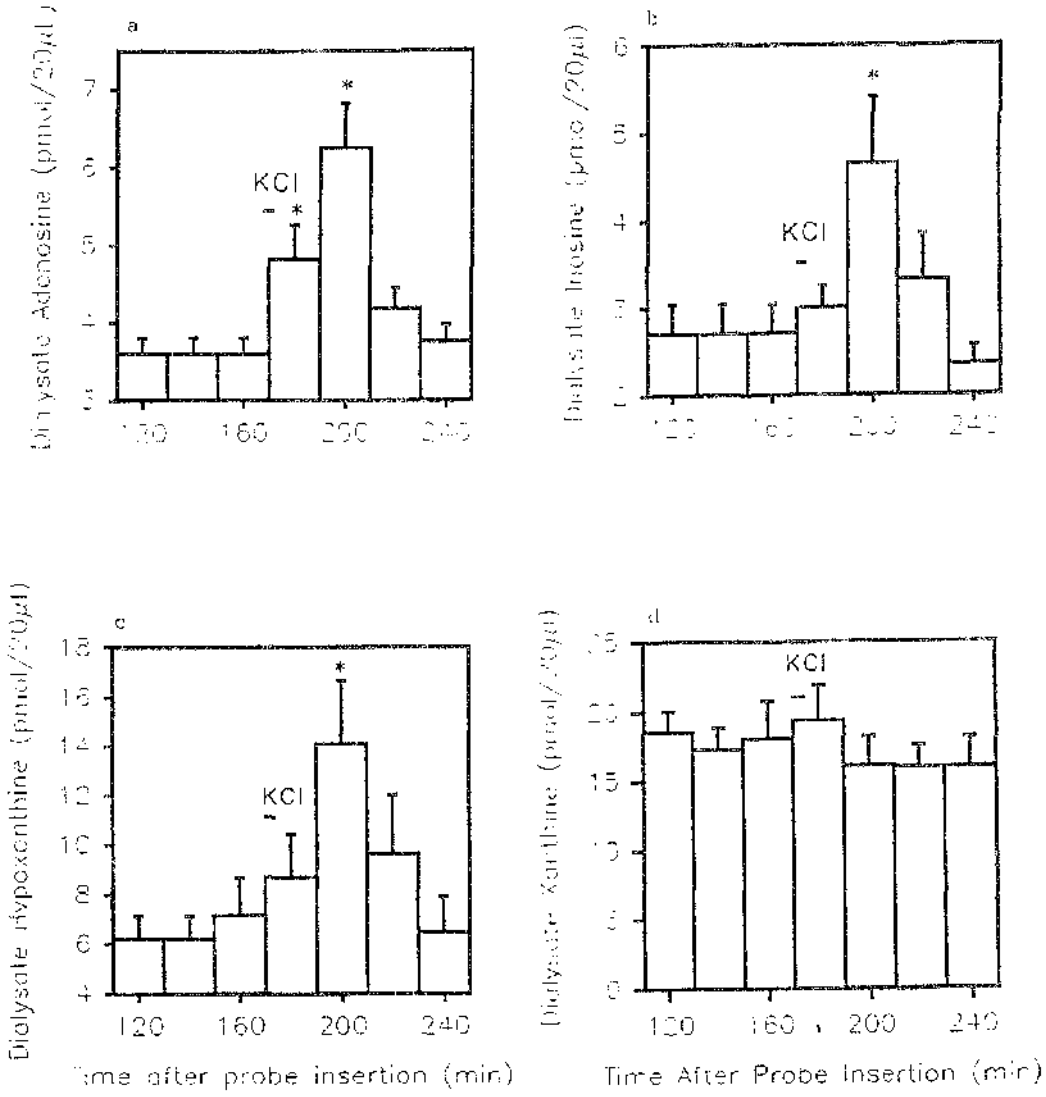



Fig 3.2

The release profiles for adenosine (a), inosine (b), hypoxanthine (c) and xanthine (d) evoked by a 5 min pulse of potassium 100mM, shown by the horizontal bar, (n=7). Levels of adenosine reached significance during the two samples following stimulation, while inosine and hypoxanthine levels were elevated significantly in the second sample following stimulation. The levels of xanthine did not reach significance. Paired t-test was used to show significance against basal (*$p<0.05$).

3.3.2 Release of adenosine evoked by twin pulses of potassium

Three hours after the first 5 minute pulse (S1), a second 5 min pulse (S2) of potassium was perfused through the probe. The first 5 min pulse produced adenosine release which peaked within the second sample of exposure of the tissue (*$p<0.05$, fig 3.3a) and returned to basal levels two hours before the second pulse. The second 5 min pulse produced an adenosine release profile similar to that of the first 5 min pulse in that the release peaked within the second sample of exposure of the tissue (fig 3.3a), however this was not significantly higher than basal release. The amount of release of adenosine above the mean of the two or three basal levels before each stimulation was calculated and termed S1, for the first pulse, and S2, for the second pulse and the S2/S1 ratios were calculated.

To investigate the mechanism(s) of potassium-evoked release of adenosine, a drug was perfused two hours before, during and after the second pulse of potassium and its effect on the S2/S1 ratio was examined. The S2/S1 ratio for the test experiment was compared to that of the control experiment and statistical analysis was carried out using ANOVA, one-way analysis of variance and Dunnett's unpaired t-test.

3.3.3 Release of adenosine and its metabolites evoked by potassium in the presence of CNQX

Potassium-evoked release was not affected by CNQX at a concentration of 4.5μM (fig 3.3b). This is reflected in the unchanged S2/S1 values summarised in fig 3.3d. The S2/S1 value for control was 0.69 ± 0.04 and that of CNQX (4.5μM) was 0.64 ± 0.14. At a ten-fold higher concentration of 45μM, CNQX did produce a reduction of potassium-evoked release of adenosine (fig 3.3c). The S2/S1 ratio was reduced to 0.2 ± 0.08 by 45μM CNQX (*$p<0.05$, $n=4$, unpaired t-test after ANOVA, fig 3.3d).

The profiles of inosine and hypoxanthine release evoked by potassium in the absence and presence of CNQX are shown in figures 3.4 and 3.5. The release of xanthine was not examined in this instance as potassium was not shown to evoke its release (fig 3.2d). The S2/S1 ratios were not calculated for inosine or hypoxanthine as basal levels were not re-established before the termination of some of the experiments.

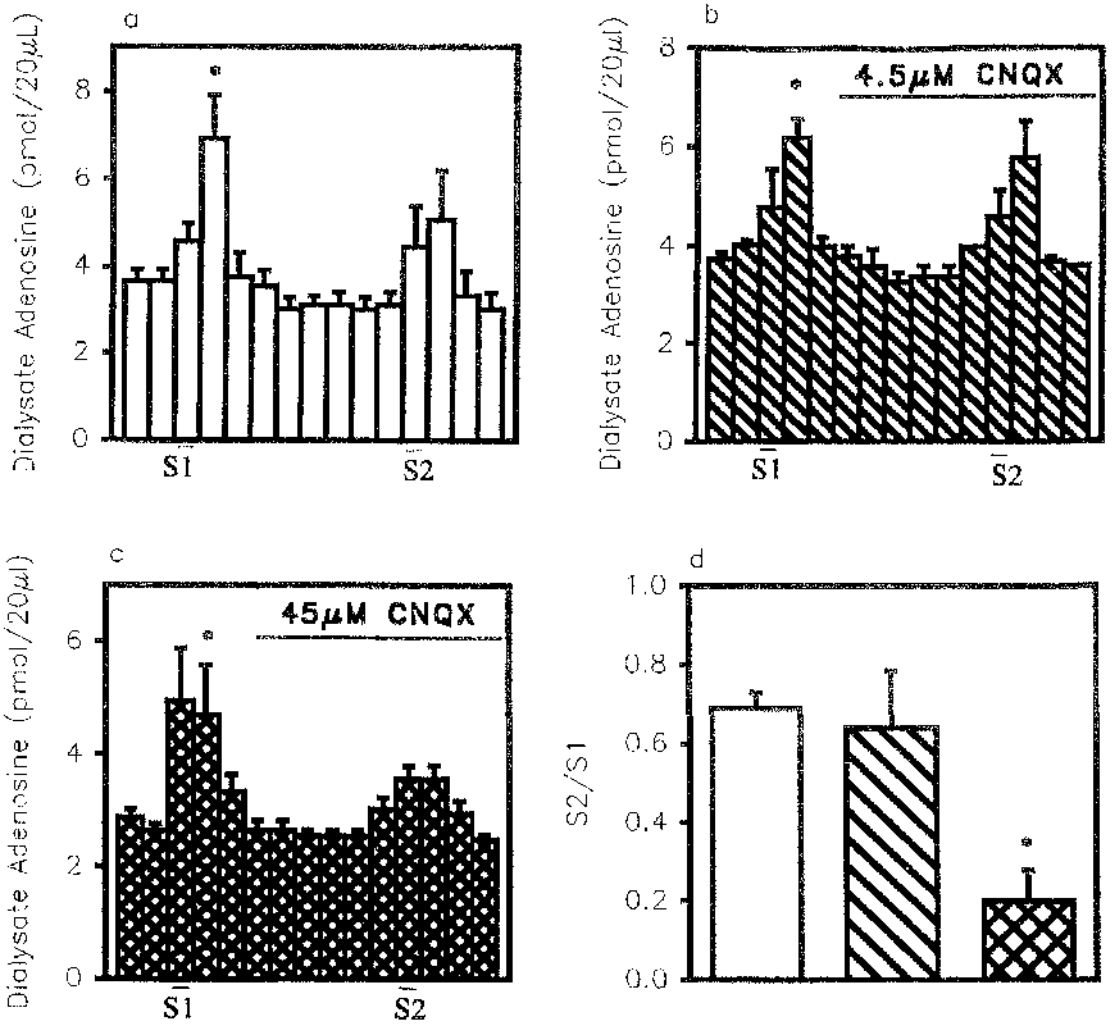



Fig 3.3

Adenosine release evoked by twin pulses of 100mM KCl without the incorporation of any drugs during the second pulse of KCl (n=3) (a) and with the incorporation of CNQX (4.5μM) (n=3) (b) and CNQX (45μM) (n=4) (c), two hours before, during and after the second pulse of KCl, as indicated by the horizontal line (*$p<0.05$ versus basal release, paired t-test). The S2/S1 ratios for (a), (b) and (c) are shown in (d). There was a significant reduction in the S2/S1 ratio in the presence of CNQX 45μM (*$p<0.05$, n=4, using ANOVA followed by t-test).

control (n=3)

CNQX 4.5μM (n=3)

CNQX 45μM (n=4)

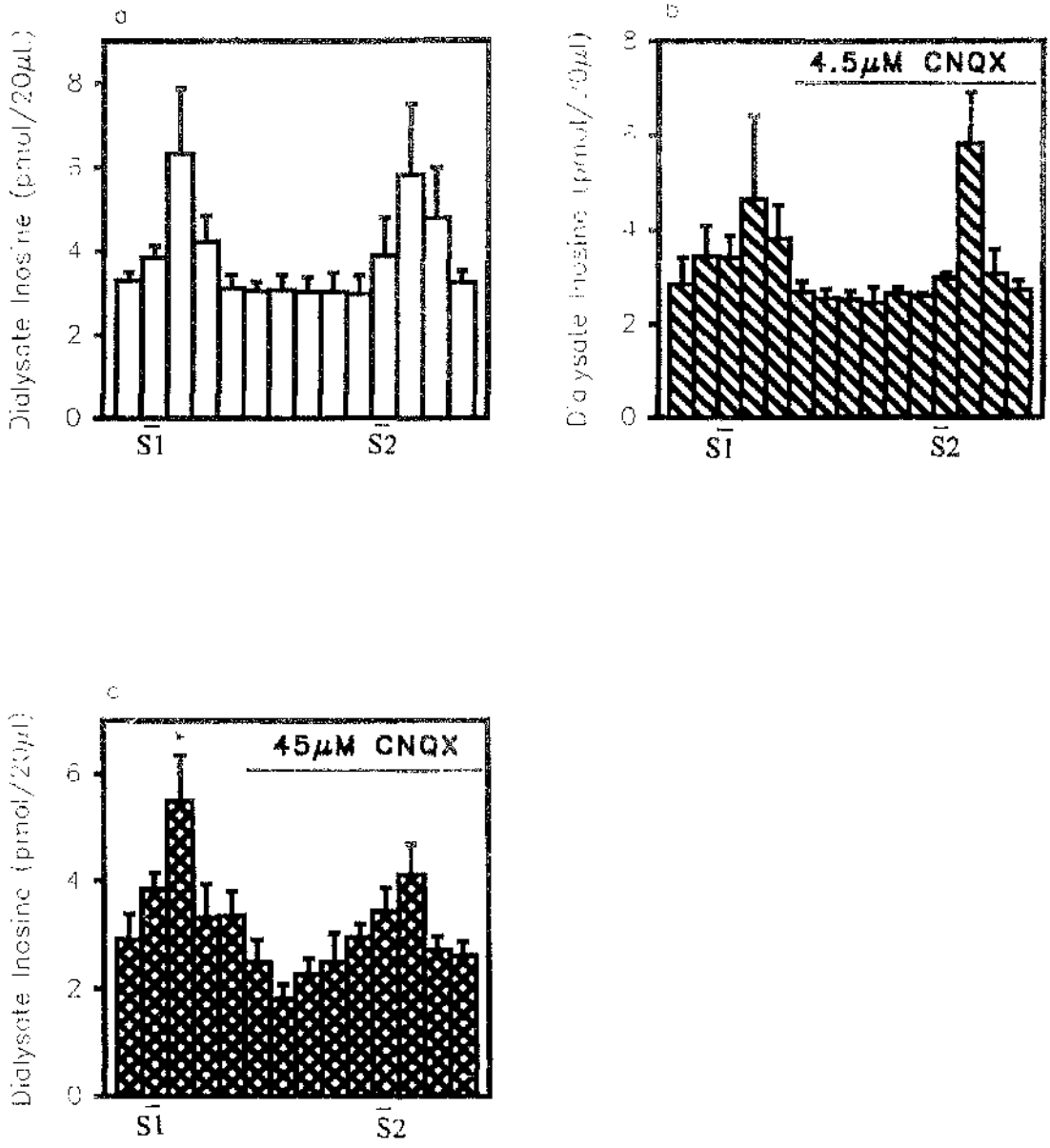



Fig 3.4

Inosine release evoked by twin pulses of 100mM KCl without the incorporation of any drugs during the second pulse of KCl (n=3) (a) and with the incorporation of CNQX (4.5μM) (n=3) (b) and CNQX (45μM) (n=4) (c), two hours before, during and after the second pulse of KCl, as indicated by the horizontal line (*$p<0.05$).

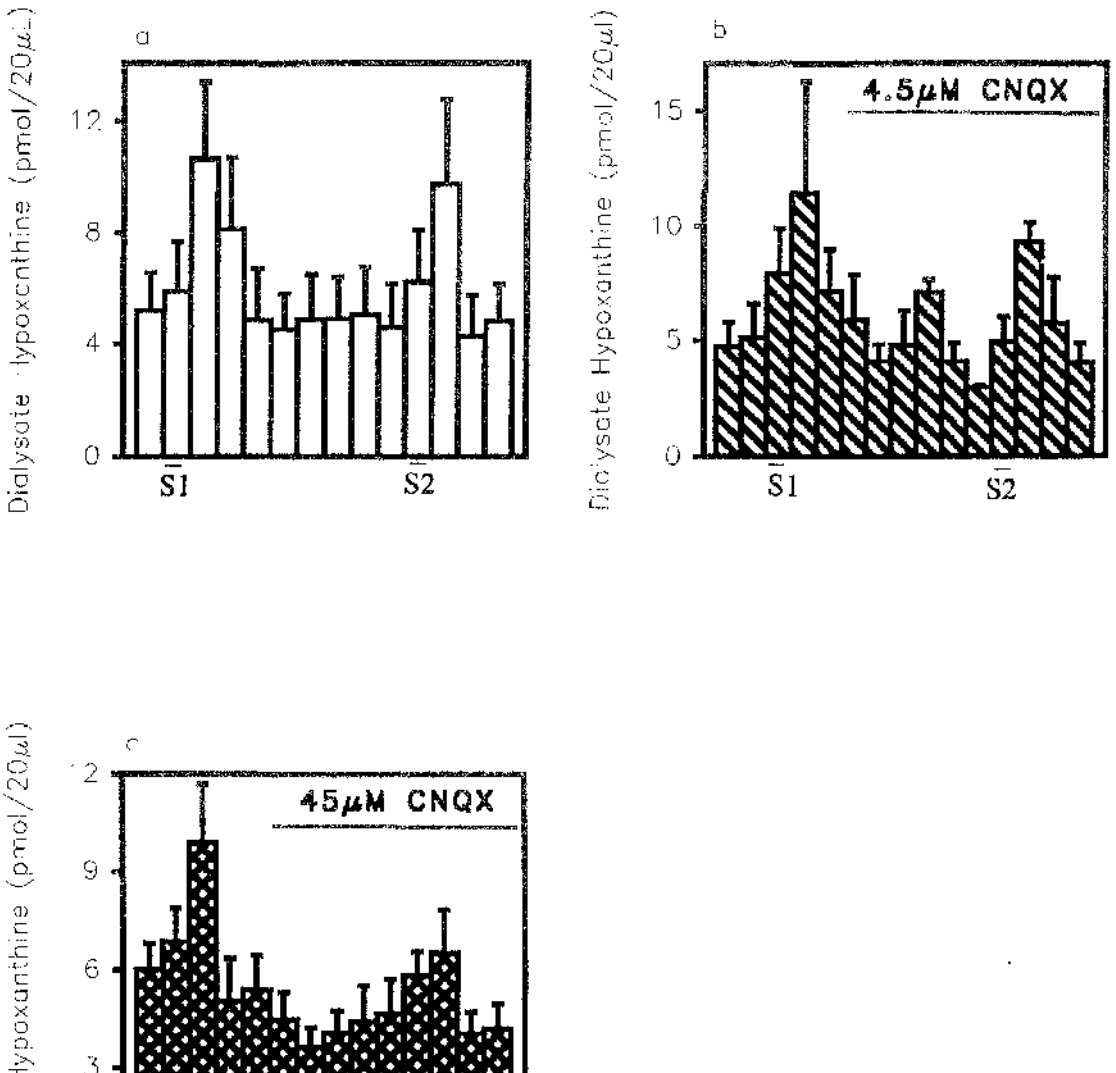



Fig 3.5

Hypoxanthine release evoked by twin pulses of 100mM KCl without the incorporation of any drugs during the second pulse of KCl (n=3) (a) and with the incorporation of CNQX (4.5μM) (n=3) (b) and CNQX (45μM) (n=4) (c), two hours before, during and after the second pulse of KCl, as indicated by the horizontal line (release did not reach significance above basal).

## 3.4 Intrahippocampal kainate-evoked release of adenosine and its metabolites

### 3.4.1 Concentration-dependent release of adenosine by kainate

The perfusion of intrahippocampal kainate for 5 min in a concentration range of 0.1-25mM produced a concentration-dependent release of adenosine (fig 3.6). The $EC_{50}$ concentration was taken as the concentration required to evoke 50% of the maximum response measured. This was estimated to be 0.94mM (Graph Pad version 2.0). The maximum detected concentration of adenosine was 7-8 fold higher than the resting level.

### 3.4.2 Release of adenosine and its metabolites evoked by intrahippocampal kainate at the EC50 of kainate

A 5 min pulse of 1mM kainate produced a release of adenosine, inosine, hypoxanthine and xanthine (fig 3.7). Adenosine release peaked during the sample following that containing kainate. This was largely due to the lag-time from the liquid switch to the collection vial which was 16 minutes. This meant that the tissue was exposed to kainate for the last 4 minutes of one 20 minute period and the first minute of the subsequent 20 min period. Using 1mM kainate, the adenosine content of the dialysate increased from 3.68 ± 0.21 to 7.66 ± 0.83 pmol/20µl (3.7a). The release of both inosine and hypoxanthine reached a peak during the sample after the peak release of adenosine (fig 3.7b & c). Inosine release was increased from 3.12 ± 0.12 to 6.17 ± 0.41 pmol/20µl and hypoxanthine from 4.96 ± 0.32 to 29.48 ± 3.6 (fig 3.7b & c). The release of xanthine rose in the sample after the peak release of inosine and hypoxanthine from 13.48 ± 1.14 to 24.78 ± 1.3 pmol/20µl (fig 3.7d).

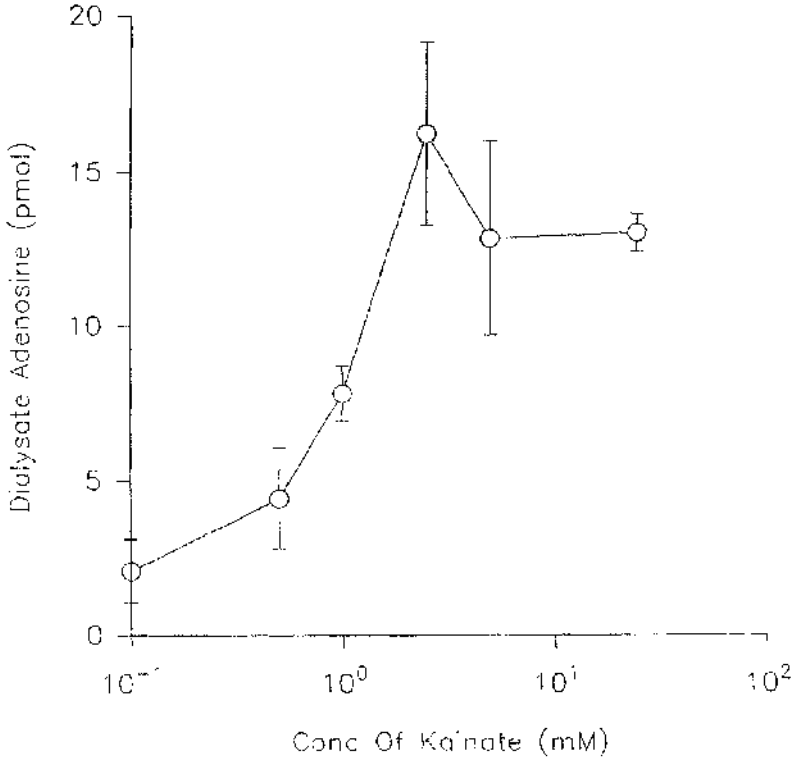



Fig 3.6

Concentration-dependent release of adenosine, expressed as the total amount above basal (pmol), evoked by a 5 min pulse of kainate (0.1-25mM) in the perfusate (mean ± sem, n=3-10). The $EC_{50}$ was 0.94mM. Maximal release was evoked at 2.5mM kainate, total amount released being about 15pmol.

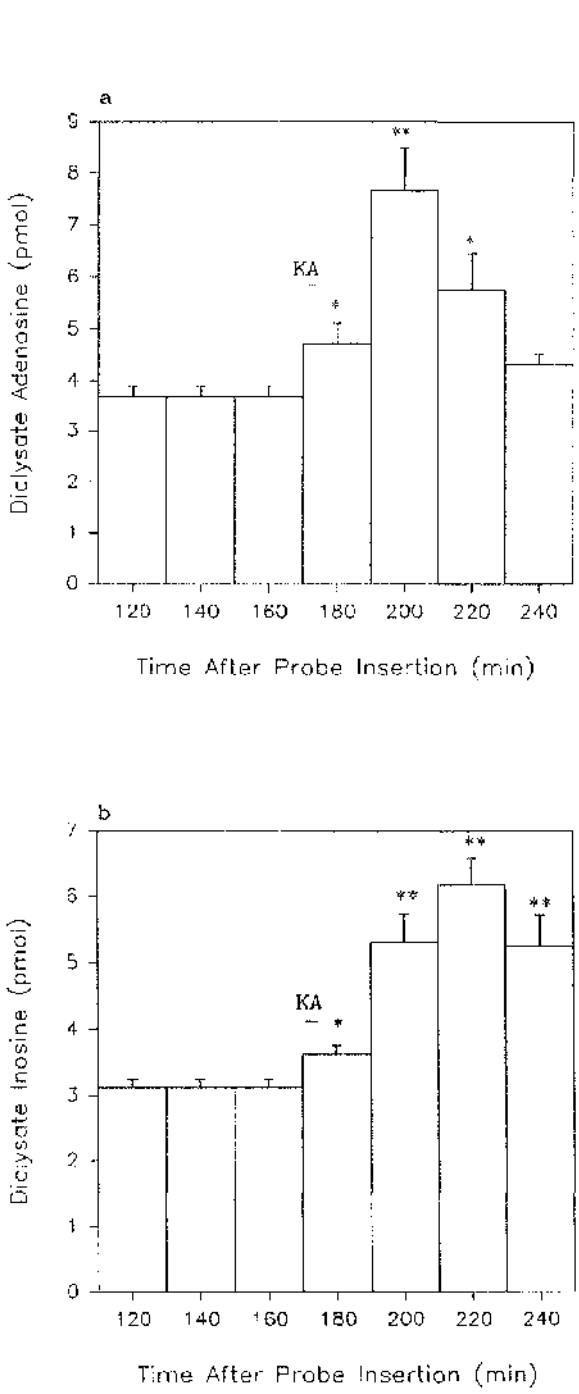
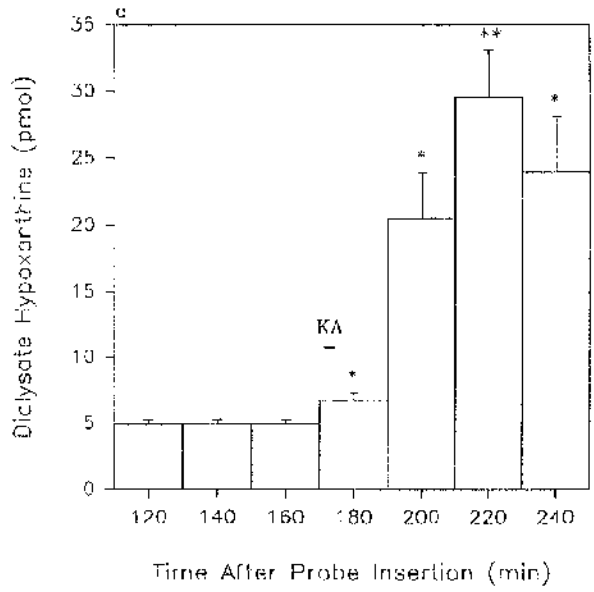



Fig 3.7 a&b

The release profiles for adenosine (a) and inosine (b) evoked by a 5 min pulse of kainate 1mM, shown by the horizontal bar. The paired t-test was used (*$p<0.05$, **$p<0.001$).



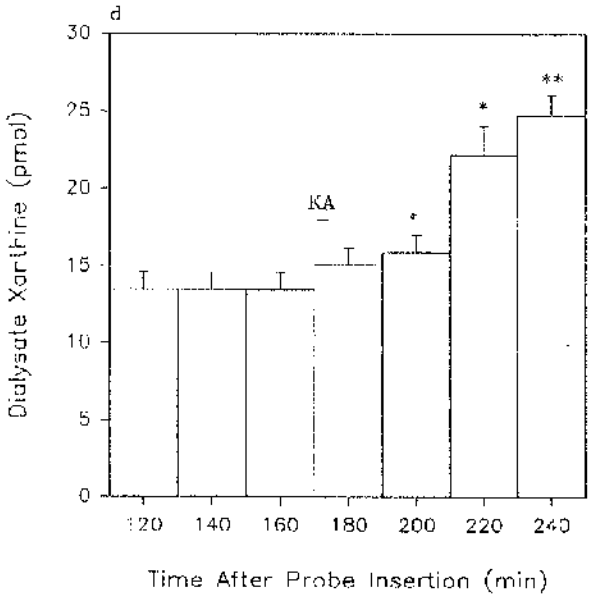




**Fig 3.7 c&d**

**The release profiles for hypoxanthine (c) and xanthine (d) evoked by a 5 min pulse of kainate 1mM, shown by the horizontal bar. The paired t-test was used (*$p<0.05$, **$p<0.001$).**

3.4.3 Release of adenosine evoked by twin pulses of intrahippocampal kainate

Three hours after the first 5 minute pulse of kainate, a second 5 min pulse of kainate was perfused through the probe. Adenosine release returned to basal levels two hours before the second pulse. Adenosine release within the second sample after exposure of the tissue to the second pulse of kainate was not as high as that seen after the first pulse, but was still significantly higher than basal (fig 3.8 a). The S2/S1 ratio for kainate-evoked release of adenosine, 0.465 ± 0.02, was significantly different to that of potassium (0.69±0.04, unpaired t-test, $p<0.05$).

In investigating the mechanism of kainate-evoked release of adenosine, perfusion of a drug two hours before and during the second pulse allowed us to examine the effect it had on the S2/S1 ratio. The S2/S1 ratio for the test experiments was compared to that of the control experiments and statistical analysis was carried out using ANOVA, one-way analysis of variance and Dunnett's unpaired t-test. All S2/S1 ratios are shown graphically after the graphs displaying the effect of all drugs on the profile of release of adenosine. The S2/S1 ratios were not calculated for inosine, hypoxanthine or xanthine as basal levels were not re-established before the termination of some of the experiments.

3.5 Characterisation of receptor mechanisms of kainate-evoked release of adenosine

In the present section CNQX, MK-801, AP-5 and TTX were examined on the profiles of release of adenosine and the S2/S1 ratios are summarised in fig 3.11.

3.5.1 Release of adenosine by intrahippocampal kainate in the presence of CNQX

CNQX, a non-NMDA receptor antagonist was incorporated at a concentration of 4.5μM into the perfusion medium two hours before, during and after the second stimulation. The presence of CNQX at this concentration reduced release evoked by kainate during the second pulse to the extent that release was no longer significantly above basal (fig 3.8b). The resulting S2/S1 ratio of 0.04±0.03 was significantly lower than the control value of 0.465 ± 0.02 ($n=4$, $^{**}p<0.01$ unpaired t-test) (fig 3.11).

3.5.2 Release of adenosine by intrahippocampal kainate in the presence of MK-801

Perfusion with 100μM MK-801 (dizocilpine), a non-competitive NMDA receptor antagonist, resulted in an S2/S1 ratio of 0.36±0.07, not significantly different from control using the unpaired t-test (n=4, fig 3.9a & 3.11).

3.5.3 Release of adenosine by intrahippocampal kainate in the presence of AP-5

The incorporation of 1mM AP-5, a competitive NMDA antagonist, which acts at a different site on the NMDA receptor than MK-801, did not affect the S2/S1 ratio (S2/S1=0.45±0.11, n=5, fig 3.9b & 3.11).

3.5.4 The effect of TTX on intrahippocampal kainate-evoked release of adenosine

TTX at a concentration of 10μM in the perfusate reduced the S2/S1 ratio to 0.184±0.046 (n=4). This was significantly less than the control value of 0.465 ± 0.02 (fig 3.10 & 3.11)

The effects of CNQX, MK-801, AP-5 and TTX on the release profiles for inosine, hypoxanthine and xanthine are shown in figs 3.12-3.14.

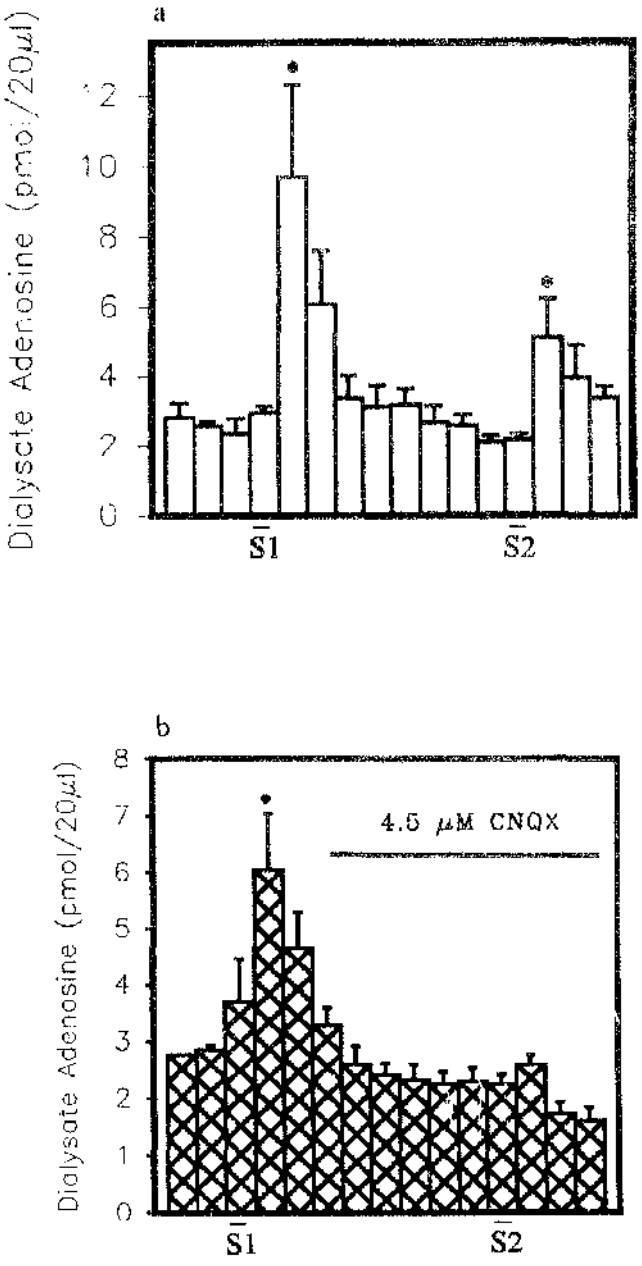



Fig 3.8

Adenosine release evoked by twin pulses of 1mM kainate (a) in the control (without the incorporation of any drugs during the second pulse of kainate) (n=4) and (b) with the incorporation of CNQX (4.5μM), two hours before, during and after the second pulse of kainate, as indicated by the horizontal line (n=4) (*$p<0.05$ versus basal, paired t-test)

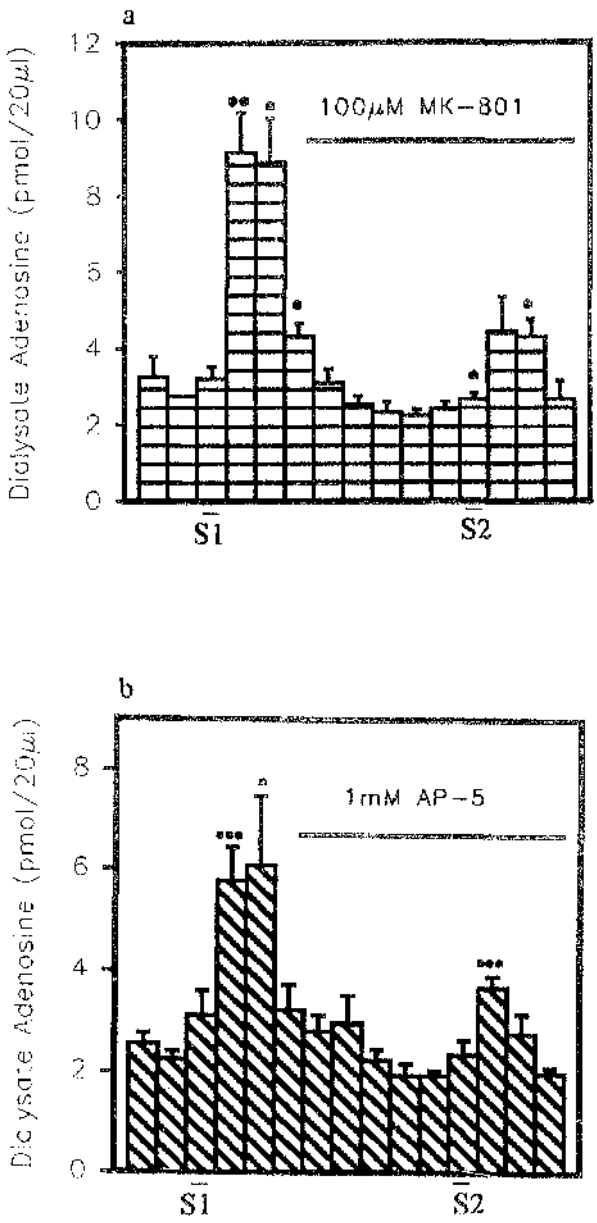



Fig 3.9

Adenosine release evoked by twin pulses of 1mM kainate with the incorporation of (a) MK-801 (100μM) (n=4) and (b) AP-5 (1mM) (n=5), two hours before, during and after the second pulse of kainate, as indicated by the horizontal line (*$p<0.05$, $p<0.01$ versus basal, paired t-test)

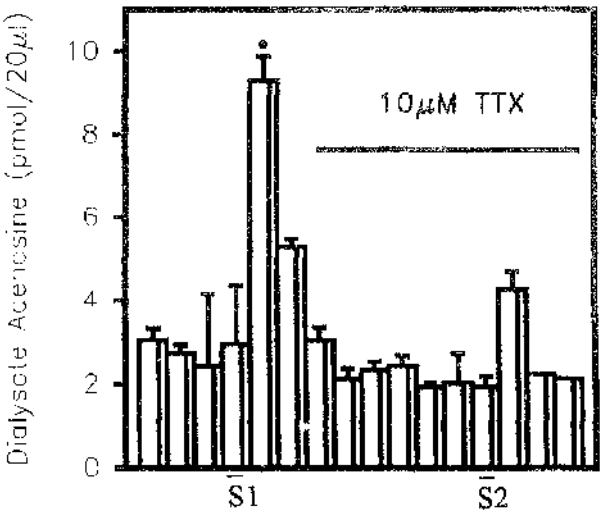



Fig 3.10

Adenosine release evoked by twin pulses of 1mM kainate with the incorporation of TTX (10μM), two hours before, during and after the second pulse of kainate, as indicated by the horizontal line (n=4) (*$p<0.05$ versus basal, paired t-test).

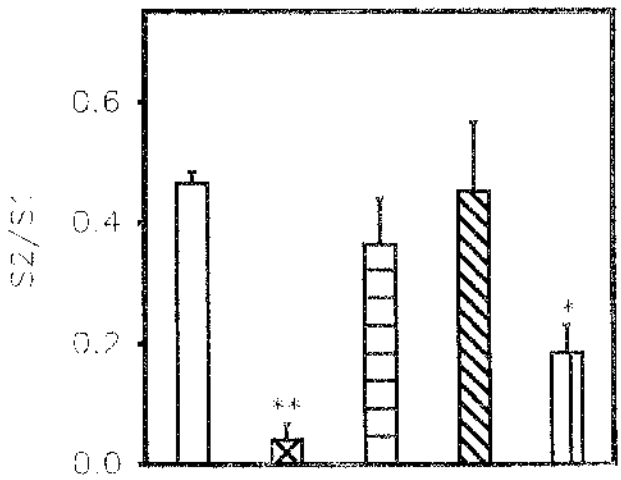



Fig 3.11

The S2/S1 ratios for adenosine release evoked by twin pulses of 1mM kainate for:

- control (n=4)
- CNQX (4.5μM) (n=4)
- MK-801 (100μM) (n=4)
- AP-5 (1mM) (n=5)
- TTX (10μM) (n=4)

There was a significant reduction in the S2/S1 ratio in the presence of CNQX 4.5μM and TTX (10μM) two hours before, during and after the second pulse of kainate (*$p<0.05$, **$p<0.01$, using ANOVA followed by Dunnett's t-test).

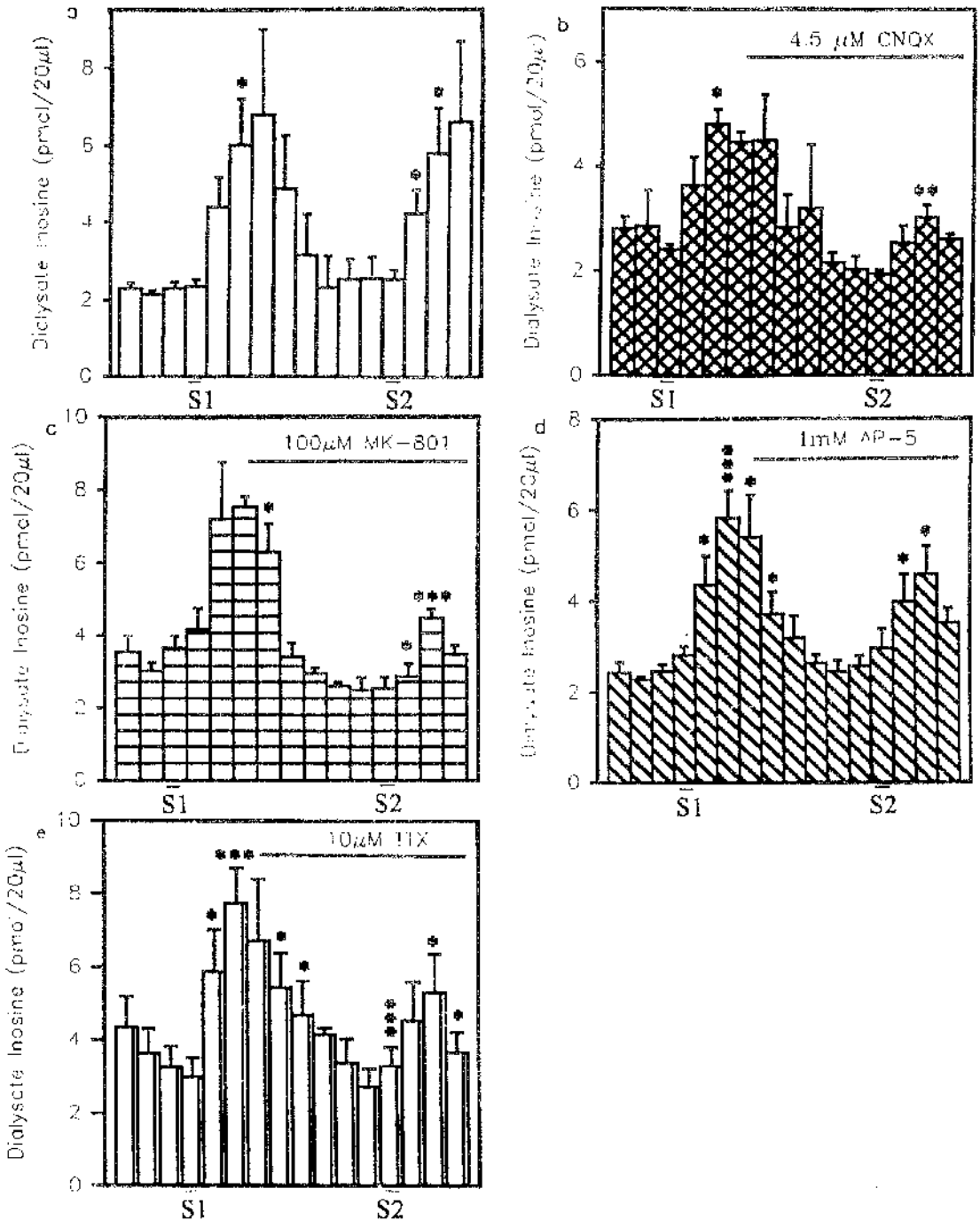



Fig 3.12

Inosine release evoked by twin pulses of 1mM kainate in the control (without the incorporation of any drugs during the second pulse of kainate) (n=4) (a) and with the incorporation of CNQX (4.5μM) (n=4) (b), MK-801(100μM) (n=4) (c), AP-5 (1mM) (n=5) (d) and TTX (10μM) (n=4) (e), as indicated by the horizontal line (*$p<0.05$, **$p<0.01$, ***$p<0.005$ versus basal, paired t-test).

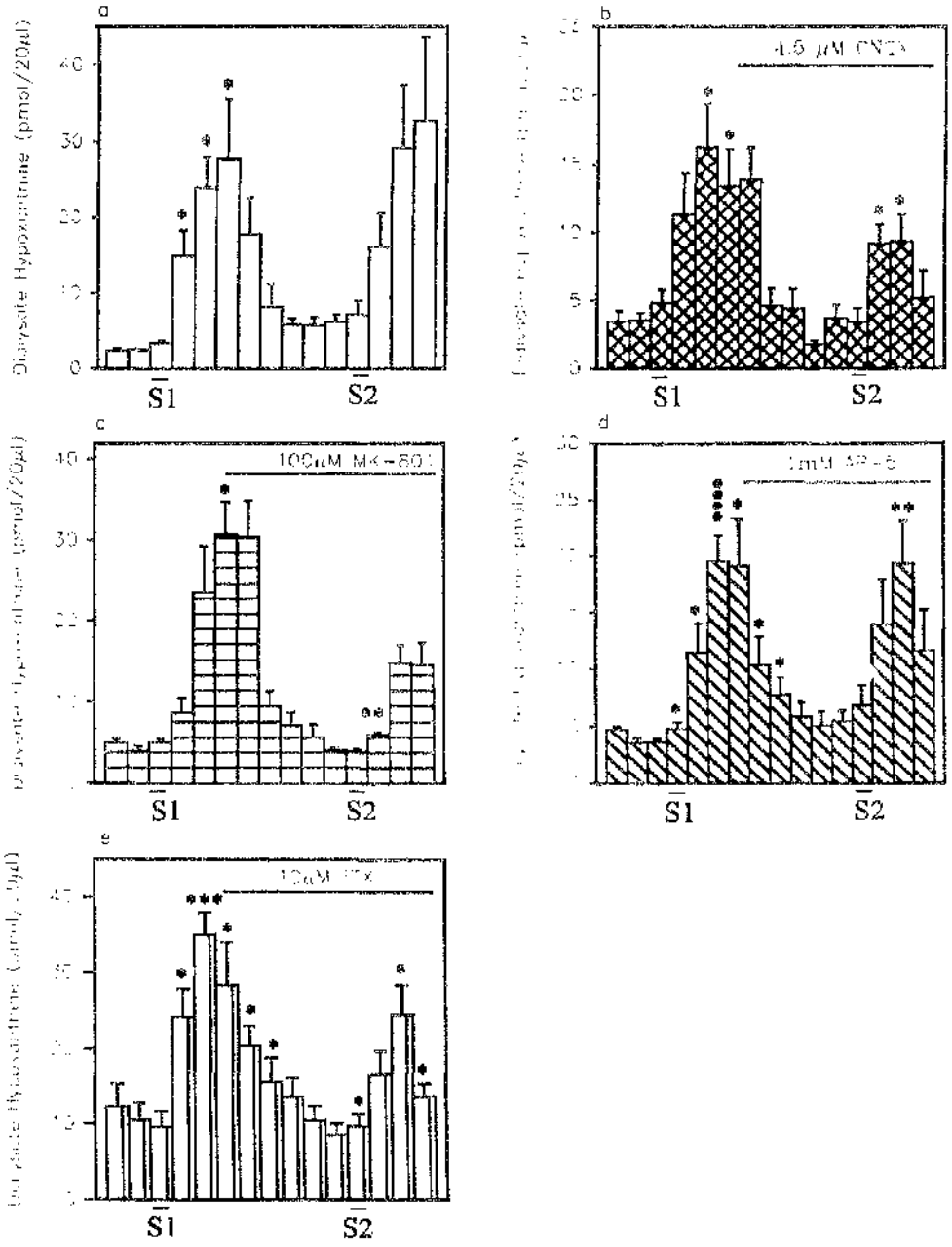



Fig 3.13 Hypoxanthine release evoked by twin pulses of 1mM kainate in the control (n=4) (a) and with CNQX (4.5μM) (n=4) (b), MK-801(100μM) (n=4) (c), AP-5 (1mM) (n=5) (d) and TTX (10μM) (n=4) (e) (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001).

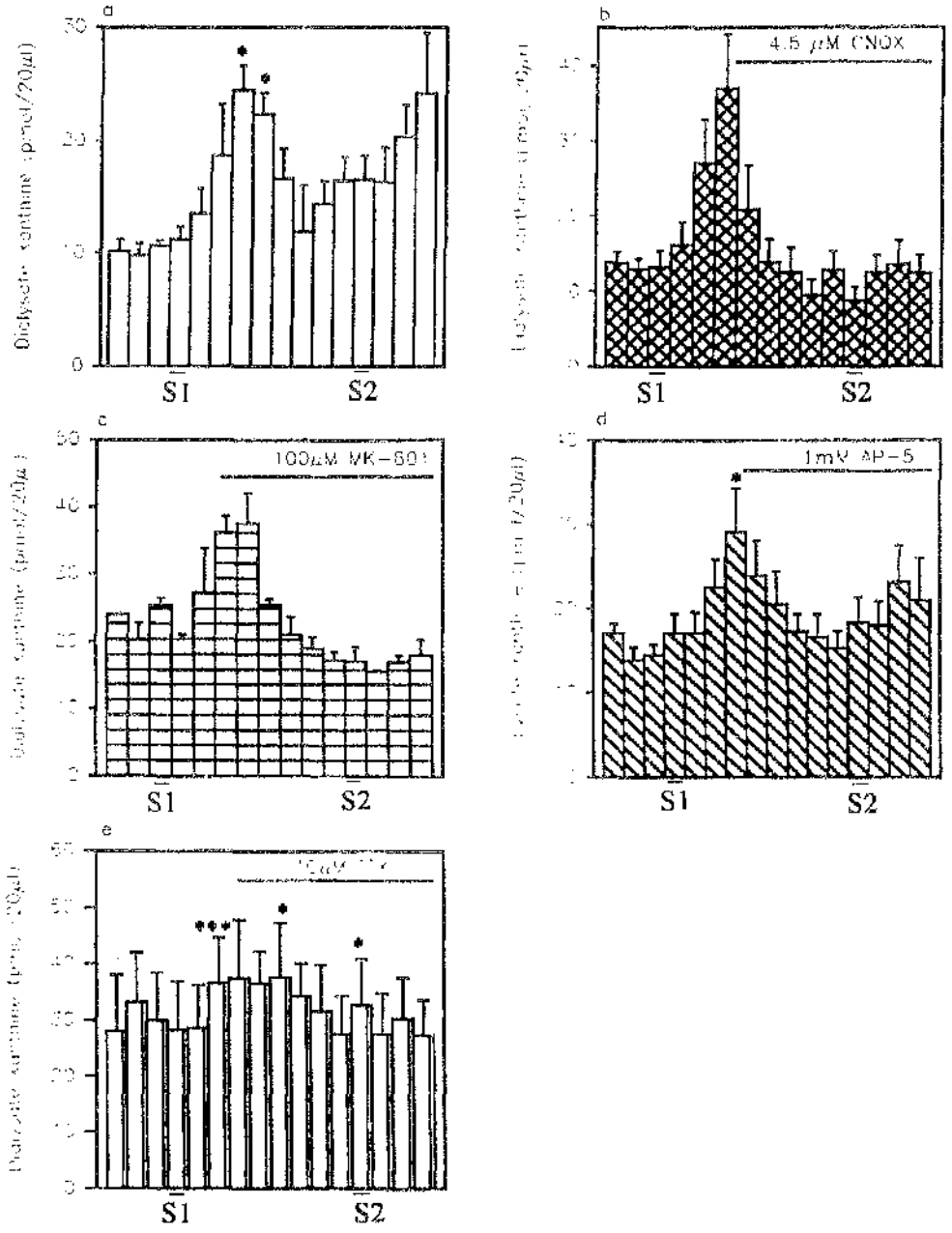



Fig 3.14 Xanthine release evoked by twin pulses of 1mM kainate in the control (n=4) (a) and with CNQX (4.5μM) (n=4) (b), MK-801(100μM) (n=4) (c), AP-5 (1mM) (n=5) (d) and TTX (10μM) (n=4) (e) (*p<0.05, ***p<0.005 versus basal, paired t-test).

3.5.5 Release of adenosine by intrahippocampal kainate in the presence of: a) intrahippocampal GYKI 52466

GYKI 52466 1mM, dissolved in acsf, had a resultant pH of 3.5-4.0. When the pH was brought to 7.2-7.4, GYKI 52466 came out of solution. Intrahippocampal GYKI 52466 (1mM, pH 3.5-4.0) caused a release of adenosine during the two hour perfusion period before the second pulse of kainate (fig 3.15). In the one experiment performed, acsf pH 3.5 caused a release of adenosine during the two hour perfusion period before the second pulse of kainate (fig 3.15).

The effect of intrahippocampal GYKI 52466 1mM dissolved in DMSO 20% in acsf (pH 7.0-7.2) on kainate-evoked release of adenosine was examined and compared to that in the presence of DMSO 20% alone in acsf (fig 3.16a). DMSO alone increased the basal release of adenosine to 8.344 ± 1.182 pmol/20µl (fig 3.16b, $^{**}P<0.01$, $^{***}P<0.001$, ANOVA, Student's t-test, Bonferroni Multiple Comparison Test). DMSO in the presence of GYKI 52466 caused a rise in the basal level of adenosine release ($^{*}p<0.05$, fig 3.16 b). The value before the application of the second pulse of kainate was significantly lower than that in the DMSO alone group ($^{\dagger}p<0.01$, fig 3.16b). Application of the second pulse of kainate caused a further increase to a peak value of 19.17 ± 4.22 pmol/20µl in the presence of GYKI 52466 with DMSO and to 14.829 ± 5.162 pmol/20µl in the presence of DMSO (fig 3.16a). The S2/S1 ratios were not calculated for these experiments since basal levels were not achieved before the second pulse of kainate.

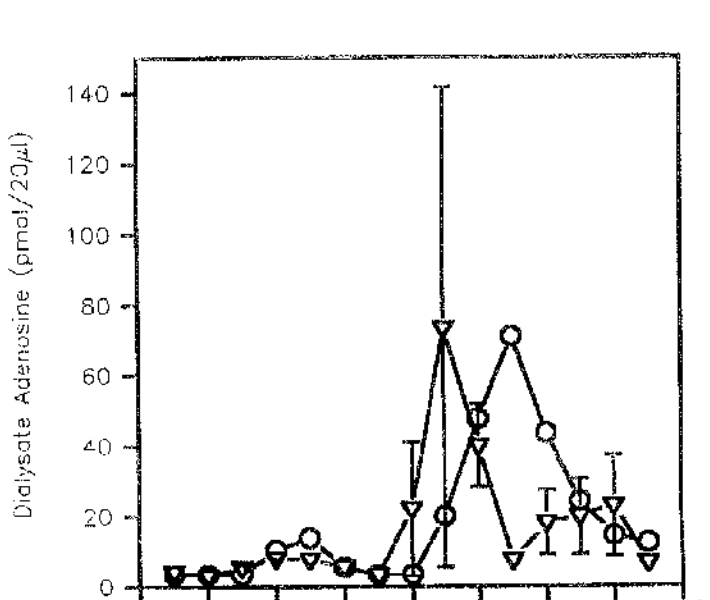



Fig 3.15

Adenosine release evoked by twin pulses of kainate 1mM with the incorporation of

- O acsf (pH 3.4-4)
- ▽ GYKI 52466 1mM (pH 3.5-4)

two hours before, during and after the second pulse of kainate.

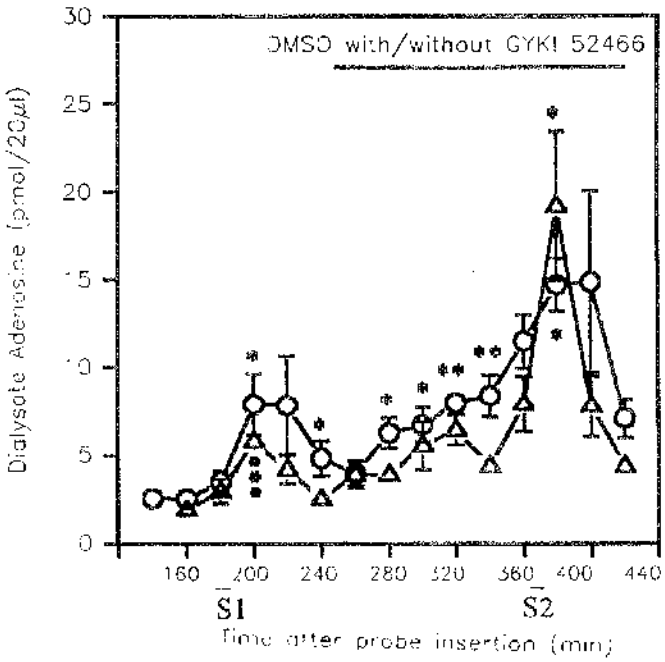



Fig 3.16

(a) Adenosine release evoked by twin pulses of kainate 1mM with the incorporation of

- O DMSO 20% (n=5)(*p<0.05, **p<0.01 vs basal, paired t-test)
- Δ GYKI 52466 in DMSO 20% (n=5) (*p<0.05, ***p<0.005 vs basal, paired t-test)

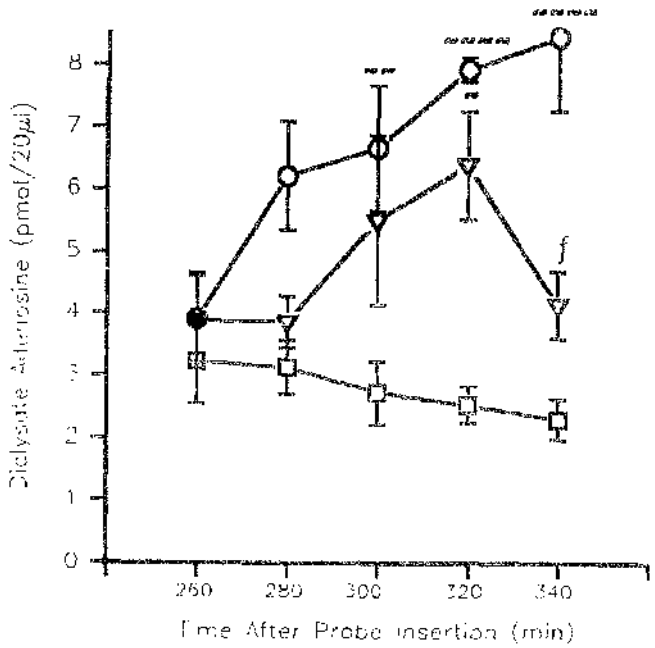



Fig 3.16

(b) adenosine release during the two hours before the 2nd pulse of kainate using ANOVA, followed by Bonferroni Multiple Comparisons Test.

- O DMSO 20% ($^{**}p<0.01$, $^{***}p<0.001$ vs acsf alone)
- ☐ acsf alone (n=4)
- ▽ GYKI 52466 in DMSO 20% ($^{*}p<0.05$ vs acsf alone, $^{f}p<0.01$ vs DMSO 20%)

3.5.5 Release of adenosine by intrahippocampal kainate in the presence of: b) systemic GYKI 52466

The selective non-NMDA receptor antagonist, GYKI 52466 was dissolved in Tween 80 2%, adjusted to pH 6-7 and injected subcutaneously at a dose of 30mg/kg. Three injections were given at times t=-15, t=0 and t=+15 minutes relative to the second pulse of kainate. Systemic GYKI 52466 did not significantly reduce the S2/S1 ratio for kainate-evoked release of adenosine (0.462 ± 0.104) relative to control (s.c. injection of the vehicle, Tween 80 2%) (0.518 ± 0.086) (fig 3.17). Table 3.2 shows the individual values of S2/S1 for each experiment, which reveals that, despite the lack of a significant overall change, there was a substantially smaller S2/S1 ratio in three of the GYKI 52466 experiments.

The effect of systemic injection of GYKI 52466 in Tween 80 on the profiles of intrahippocampal kainate-evoked release of inosine, hypoxanthine and xanthine are shown in figs 3.18-3.20.

Table 3.2

The effect of GYKI 52466 on kainate-evoked release of adenosine

| **Drug inj.** | **S2 : S1 ratio for each experiment** | | | | | | **Mean ±sem** |
|---|---|---|---|---|---|---|---|
| **Tween 80** | 0.639 | 0.576 | 0.56 | 0.333 | 0.214 | 0.789 | 0.518 ±0.09 |
| **Tween 80 + GYKI 52466** | 0.259 | 0.2 | 0.69 | 0.66 | 0.231 | 0.73 | 0.462 ±0.1 |

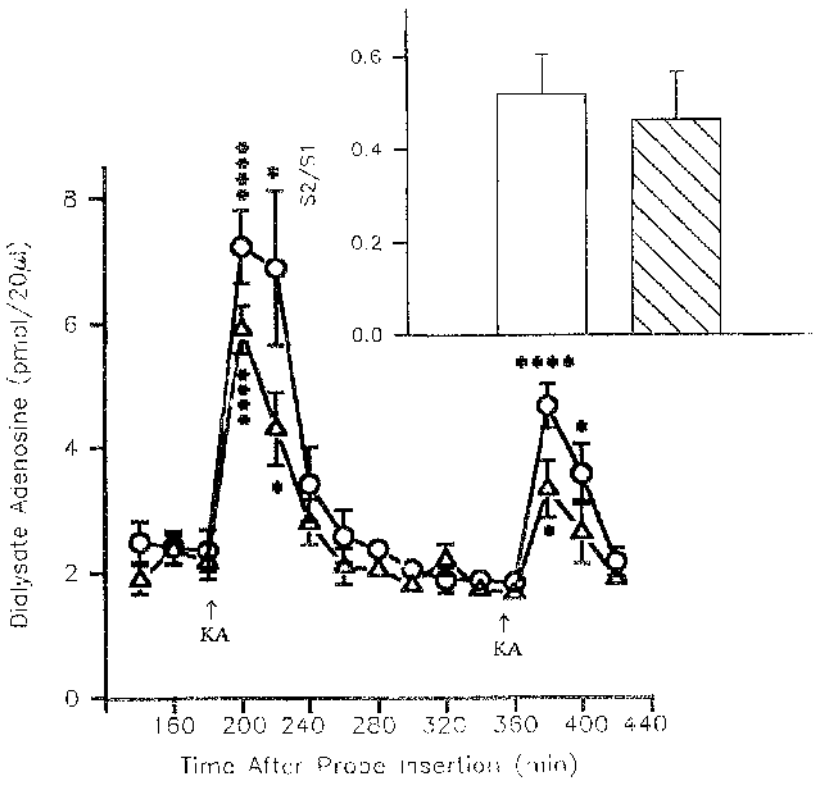



Fig 3.17

Adenosine release evoked by twin pulses of 1mM kainate (arrow) with:

○ Tween 80 2% s.c. (control) (n=6) (*p<0.05, ****p<0.001 versus basal, paired t-test)

△ GYKI 52466 30mg/kg in Tween 80 2% s.c. (n=6) (*p<0.05, ****p<0.001 versus basal, paired t-test)

The injections were t=-15min, t=0min and t=+15min relative to the second pulse of kainate.

Inset: The S2/S1 ratios for adenosine release evoked by twin pulses of 1mM kainate for:

Tween 80 2% (control) (n=6)

GYKI 52466 in Tween 80 2% (n=6) (n.s. to control)

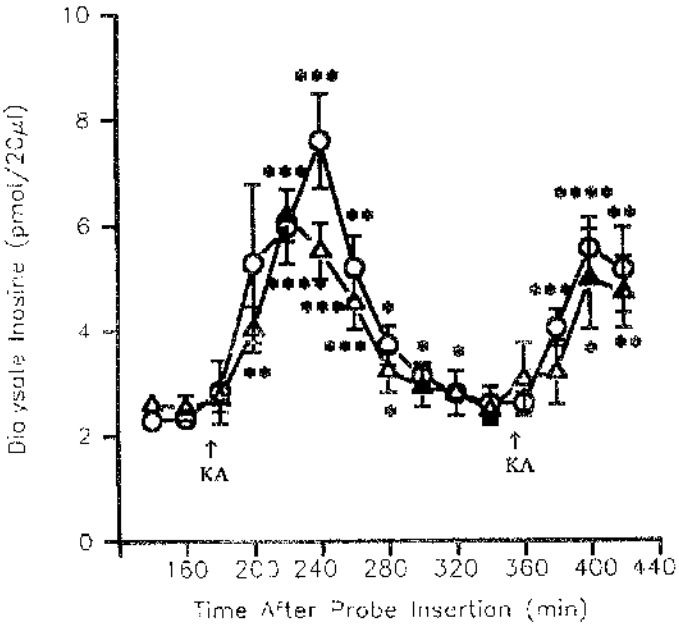



Fig 3.18

Inosine release evoked by twin pulses of 1mM kainate (arrow) with:

○ Tween 80 2% s.c. (control) (n=6) (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001 versus basal, paired t-test)

△ GYKI 52466 30mg/kg in Tween 80 2% s.c. (n=6) (*p<0.05, ***p<0.005, ****p<0.001 versus basal, paired t-test)

The injections were t=-15min, t=0min and t=+15min relative to the second pulse of kainate.

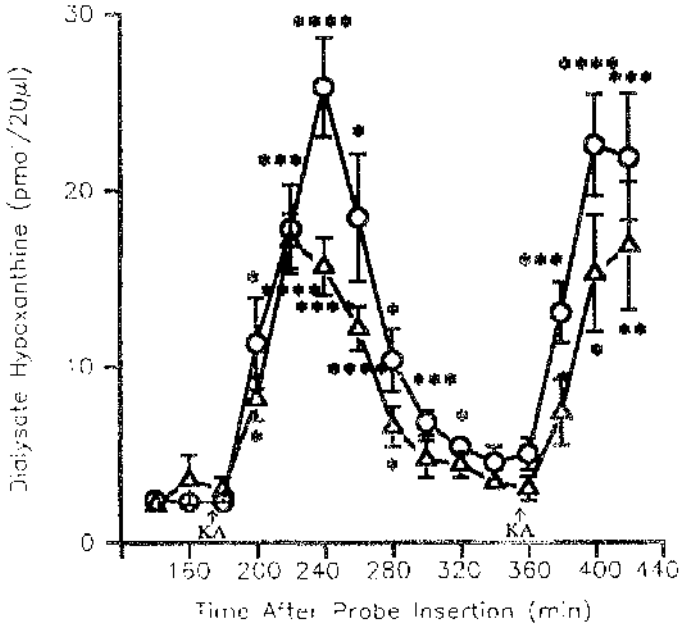



Fig 3.19

Hypoxanthine release evoked by twin pulses of 1mM kainate (arrow) with:

○ Tween 80 2% s.c. (control) (n=6) (*p<0.05, ***p<0.05, ****p<0.001 versus basal, paired t-test)

△ GYKI 52466 30mg/kg in Tween 80 2% s.c. (n=6) (*p<0.05, **p<0.01, ****p<0.001 versus basal, paired t-test)

The injections were t=-15min, t=0min and t=+15min relative to the second pulse of kainate.

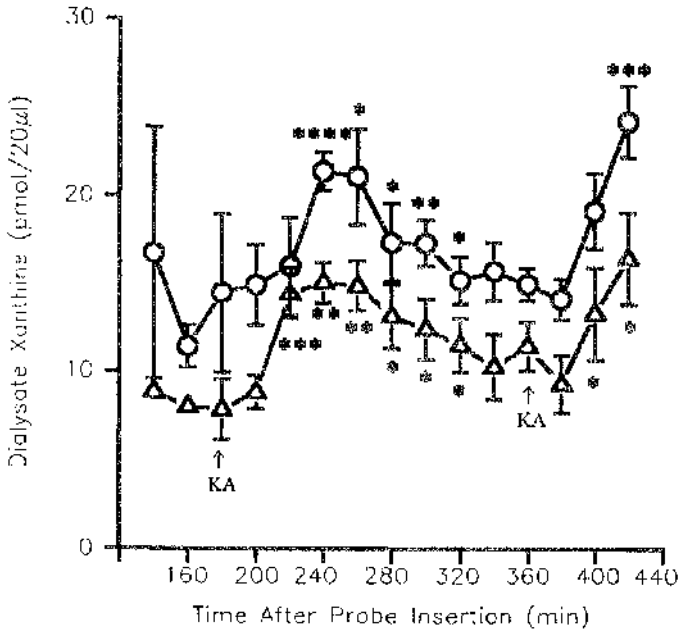



Fig 3.20

Xanthine release evoked by twin pulses of 1mM kainate (arrow) with:

○ Tween 80 2% s.c. (control) (n=6) (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001 versus basal, paired t-test)

△ GYKI 52466 30mg/kg in Tween 80 2% s.c. (n=6) (*p<0.05, ***p<0.005 versus basal, paired t-test)

The injections were t=-15min, t=0min and t=+15min relative to the second pulse of kainate.

3.6 The effect of putative neuroprotective agents on kainate evoked release of adenosine

In the present section R-PIA, CPT, 8-PST, U50 488H and chlormethiazole were examined on the profiles of release of adenosine and their S2/S1 ratios are summarised in fig 3.26.

3.6.1 Release of adenosine intrahippocampal kainate in the presence of R-PIA

The adenosine A1 receptor agonist, R-PIA, at concentrations of 10μM or 100μM did not appear to affect the release of adenosine reflected by the S2/S1 ratios 0.49±0.08 (n=4) and 0.72 ± 0.08 (n=4) (fig 3.21b, fig 3.22a & fig 3.26).

3.6.2 Release of adenosine by intrahippocampal kainate in the presence of CPT

The effect of endogenous adenosine on kainate-evoked release of adenosine was examined by incorporating CPT, an A1 adenosine receptor antagonist, into the perfusate two hours before, during and after the second pulse of kainate. CPT at concentrations of 10μM or 100μM, did not modulate kainate-evoked release of adenosine. The S2/S1 ratios were 0.485 ± 0.143 (n=5) and 0.54 ± 0.09 (n=4) respectively (fig 3.22b, fig 3.23a & 3.26).

3.6.3 Release of adenosine by intrahippocampal kainate in the presence of 8-PST

The non-selective adenosine receptor antagonist, 8-PST, at a concentration of 1mM did not modulate kainate-evoked release of adenosine (fig 3.23b), although the mean S2/S1 for the four experiments (0.84 ± 0.14) was higher than the control (S2/S1 = 0.562 ± 0.05, n.s. fig 3.26).

3.6.4 Release of adenosine by intrahippocampal kainate in the presence of U50 488H

At a concentration of 10μM, the kappa agonist, U50 488H, produced an S2/S1 ratio of 0.308±0.073 (n=5) which was not significantly different from control (fig 3.24a & 3.26). At a concentration 10 fold higher, U50 488H (100μM) significantly reduced the S2/S1 ratio for kainate-evoked release of adenosine by 59% (S2/S1=0.23±0.02, n=5, $p<0.05$, ANOVA, followed by Dunnett's Multiple Comparison's Test, fig 3.26), however the release induced by second pulse of kainate was still significantly above basal (fig 3.24b).

3.6.5 Release of adenosine by intrahippocampal kainate in the presence of chlormethiazole

The incorporation of chlormethiazole (0.5mM) into the perfusate did not modulate kainate-evoked release of adenosine (fig 3.25). The S2/S1 ratio was 0.425±0.073 (n=5)

which is not significantly different from control (S2/S1=0.562±0.05, n=7, ANOVA, followed by Dunnett's Multiple Comparisons Test, fig 3.26).

The effects of R-PIA (10μM), R-PIA (100μM), CPT (10μM), CPT (100μM), 8-PST (1mM), U50 488H (10μM), U50 488H (100μM) and chlormethiazole (500μM) on the release profiles for inosine, hypoxanthine and xanthine are shown in figs 3.27-3.32.

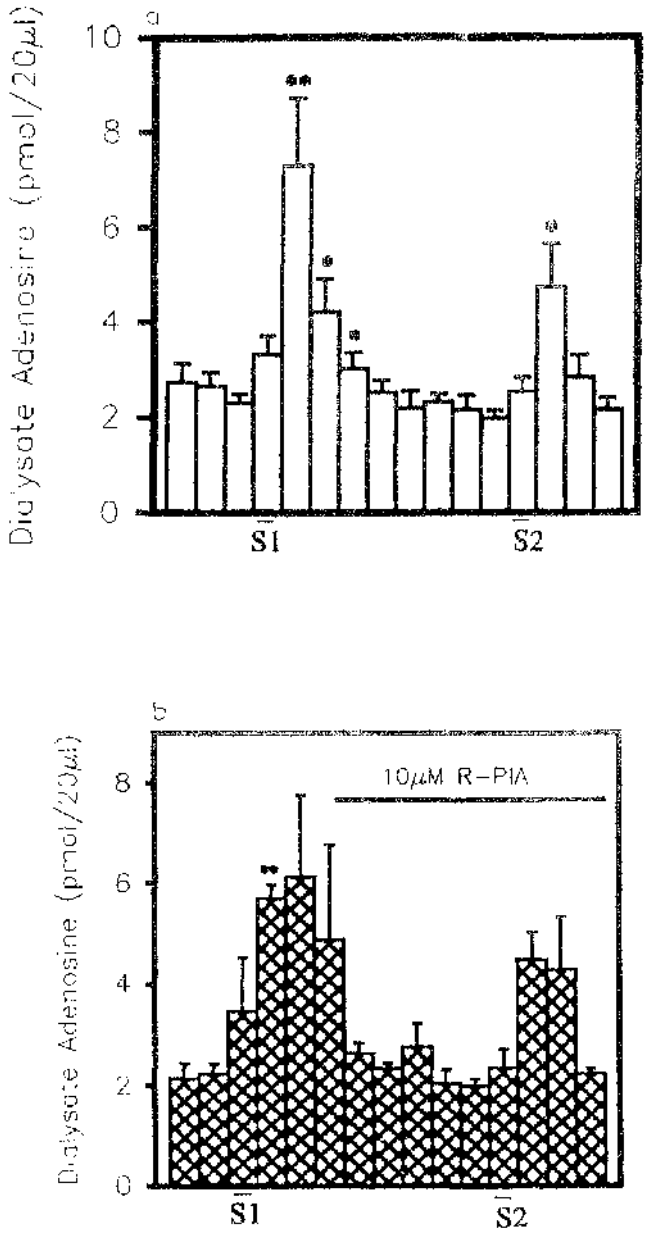



Fig 3.21

Adenosine release evoked by twin pulses of 1mM kainate (a) in the control (without the incorporation of any drugs during the second pulse of kainate) (n=7) (*p<0.05, **p<0.01 versus basal, paired t-test) and (b) with the incorporation of R-PIA (10μM), two hours before, during and after the second pulse of kainate, as indicated by the horizontal line (n=4) (**p<0.01 versus basal, paired t-test).

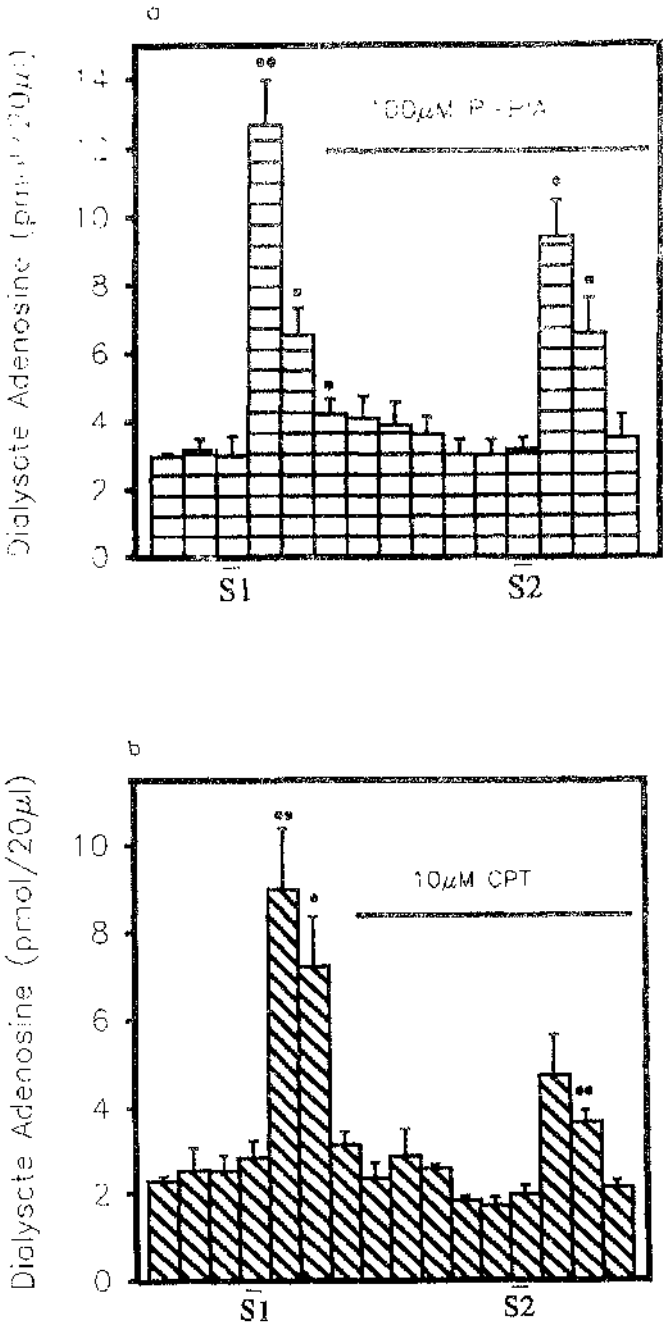



Fig 3.22

Adenosine release evoked by twin pulses of 1mM kainate with the incorporation of (a) R-PIA (100μM) (n=4) and (b) CPT (10μM) (n=5) two hours before, during and after the second pulse of kainate, as indicated by the horizontal line (*$p<0.05$, **$p<0.01$ versus basal, paired t-test)

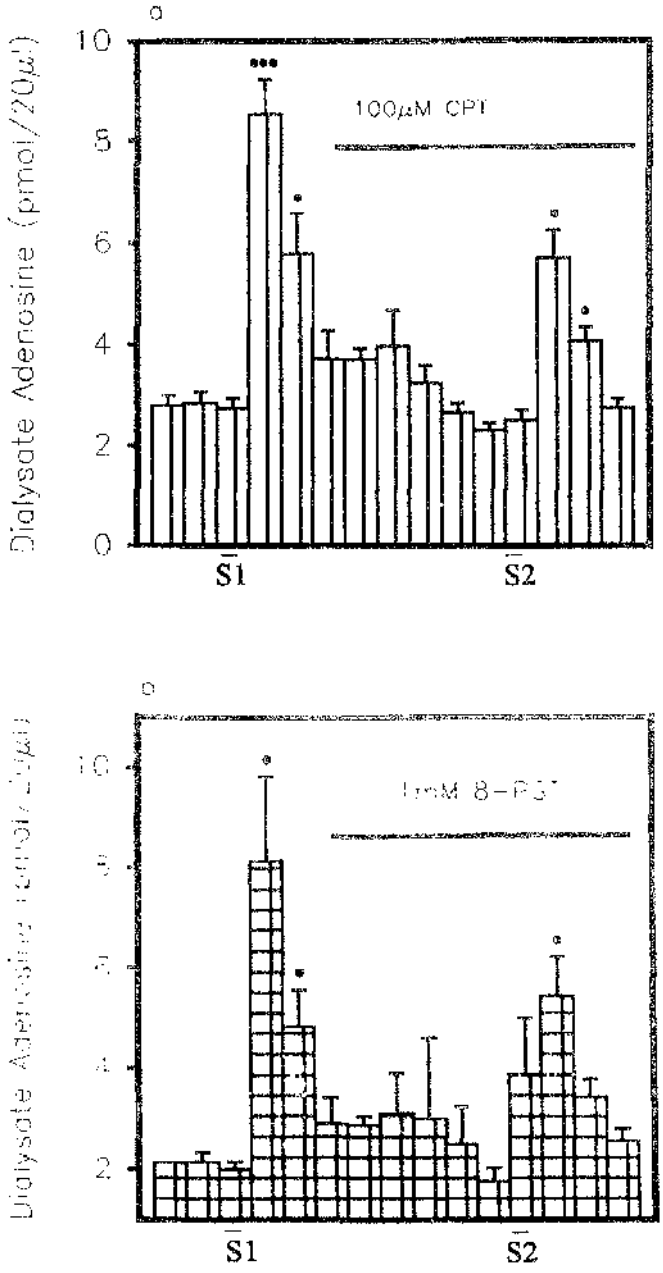



Fig 3.23

Adenosine release evoked by twin pulses of 1mM kainate with the incorporation of (a) CPT (100μM) (n=4) and (b) 8-PST (1mM) (n=4) two hours before, during and after the second pulse of kainate, as indicated by the horizontal line (*$p<0.05$, ***$p<0.005$ versus basal, paired t-test)

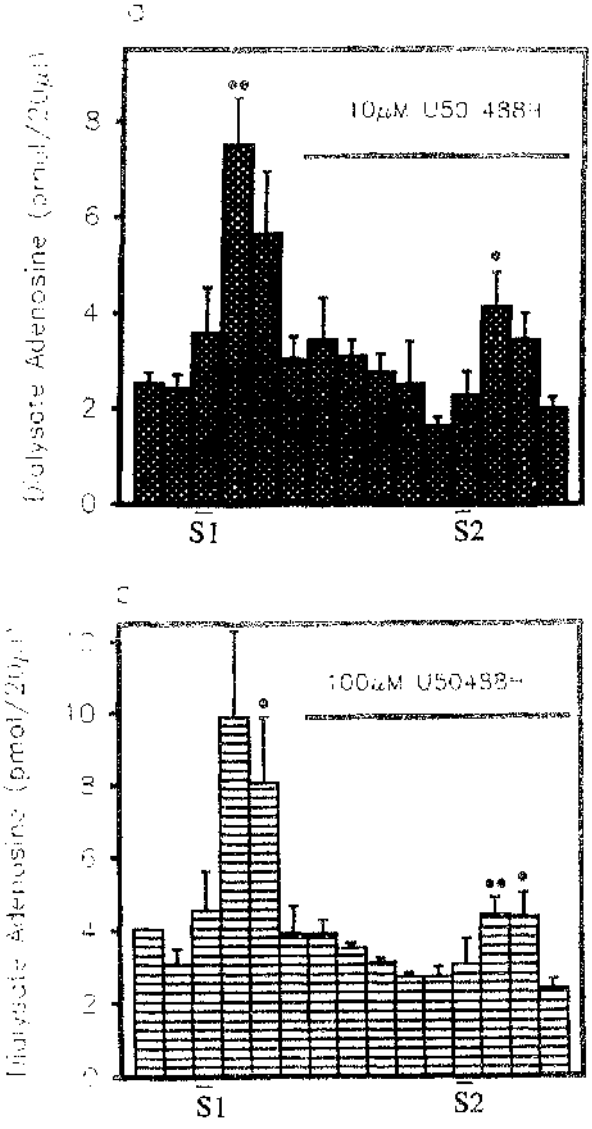



Fig 3.24

Adenosine release evoked by twin pulses of 1mM kainate with the incorporation of (a) U50 488H (10μM) (n=5) and (b) U50 488H (100μM) (n=5) two hours before, during and after the second pulse of kainate, as indicated by the horizontal line (*p<0.05, **p<0.01 versus basal, paired t-test).

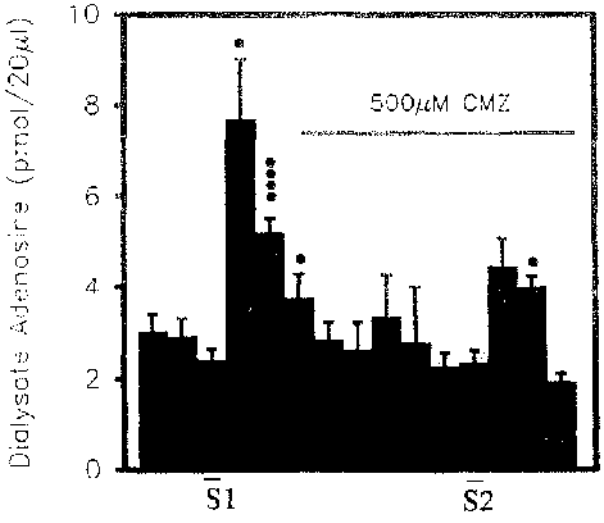



Fig 3.25

Adenosine release evoked by twin pulses of 1mM kainate with the incorporation of chlormethiazole (500μM), two hours before, during and after the second pulse of kainate, as indicated by the horizontal line (n=5) (*p<0.05, ****p<0.001 versus basal, paired t-test).

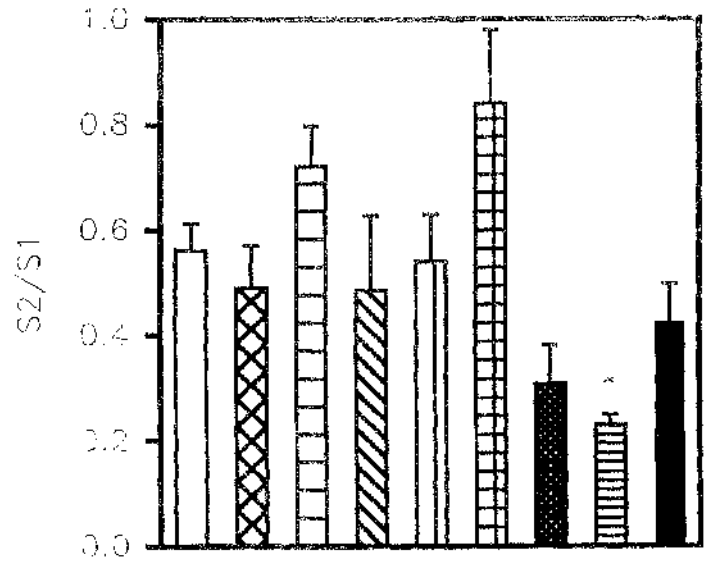



Fig 3.26

The S2/S1 ratios for adenosine release evoked by twin pulses of 1mM kainate for:

control (n=7) R-PIA (10) (n=4) R-PIA (100μM) (n=4)

CPT (10μM) (n=5) CPT (100μM) (n=4) 8-PST (1mM) (n=4)

U50 488H (10μM) (n=5) U50 488H (100μM) (n=5) chlormethiazole (500μM) (n=5)

There was a significant reduction in the S2/S1 ratios in the presence of U50 488H (100μM) two hours before, during and after the second pulse of kainate (*$p<0.05$, using ANOVA followed by Dunnett's t-test)

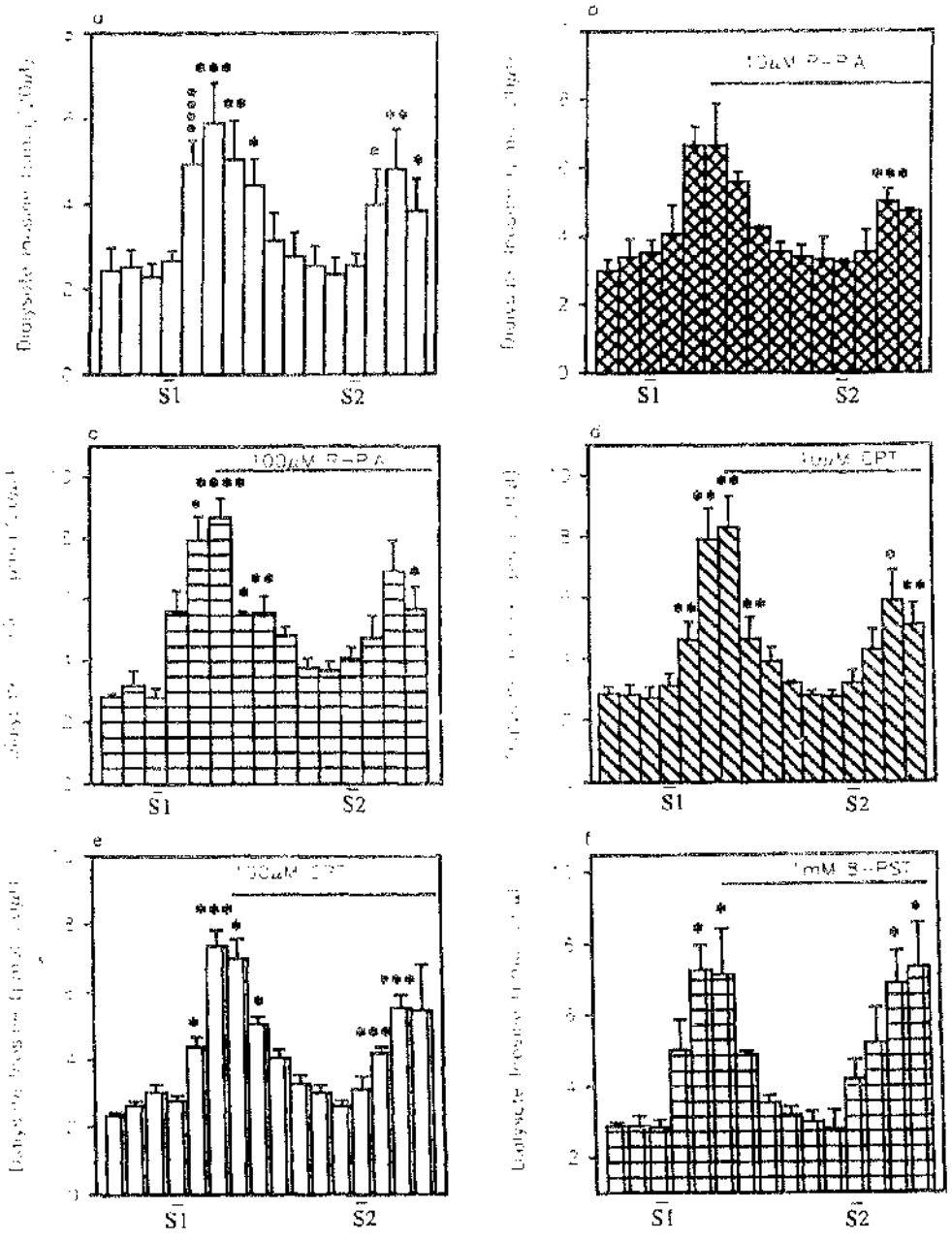



Fig 3.27

Inosine release evoked by twin pulses of 1mM kainate in the control (without the incorporation of any drugs during the second pulse of kainate) (n=7) (a) and with the incorporation of R-PIA (10μM) (n=4) (b), R-PIA (100μM) (n=4) (c), CPT (10μM) (n=5) (d), CPT (100μM) (n=4) (e) and 8-PST (1mM) (n=4) (f), as indicated by the horizontal line (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001 versus basal, paired t-test).

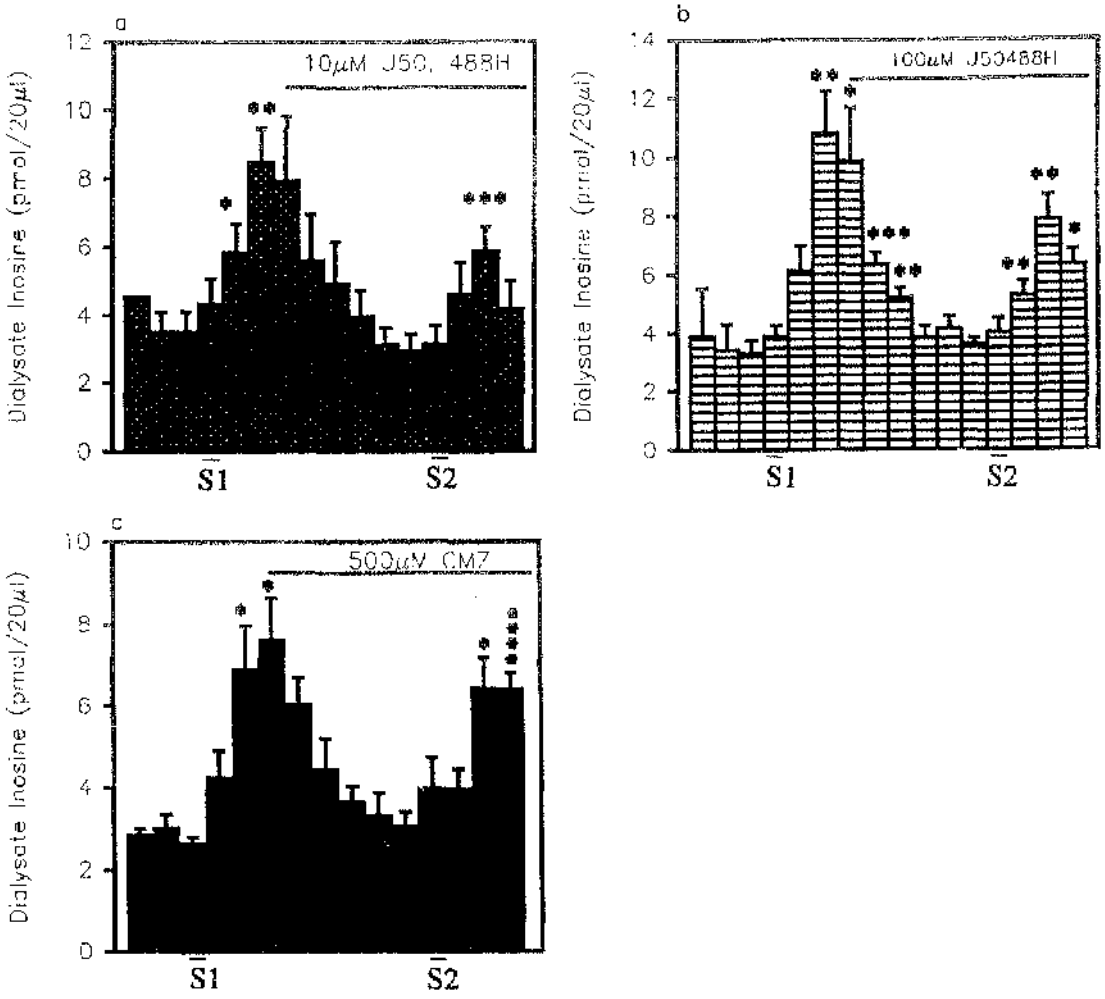



Fig 3.28

Inosine release evoked by twin pulses of 1mM kainate with the incorporation of (a) U50 488H (10μM) (n=5), (b) U50 488H (100μM) (n=5) and (c) chlormethiazole (500μM) (n=5), as indicated by the horizontal line (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001 versus basal, paired t-test).

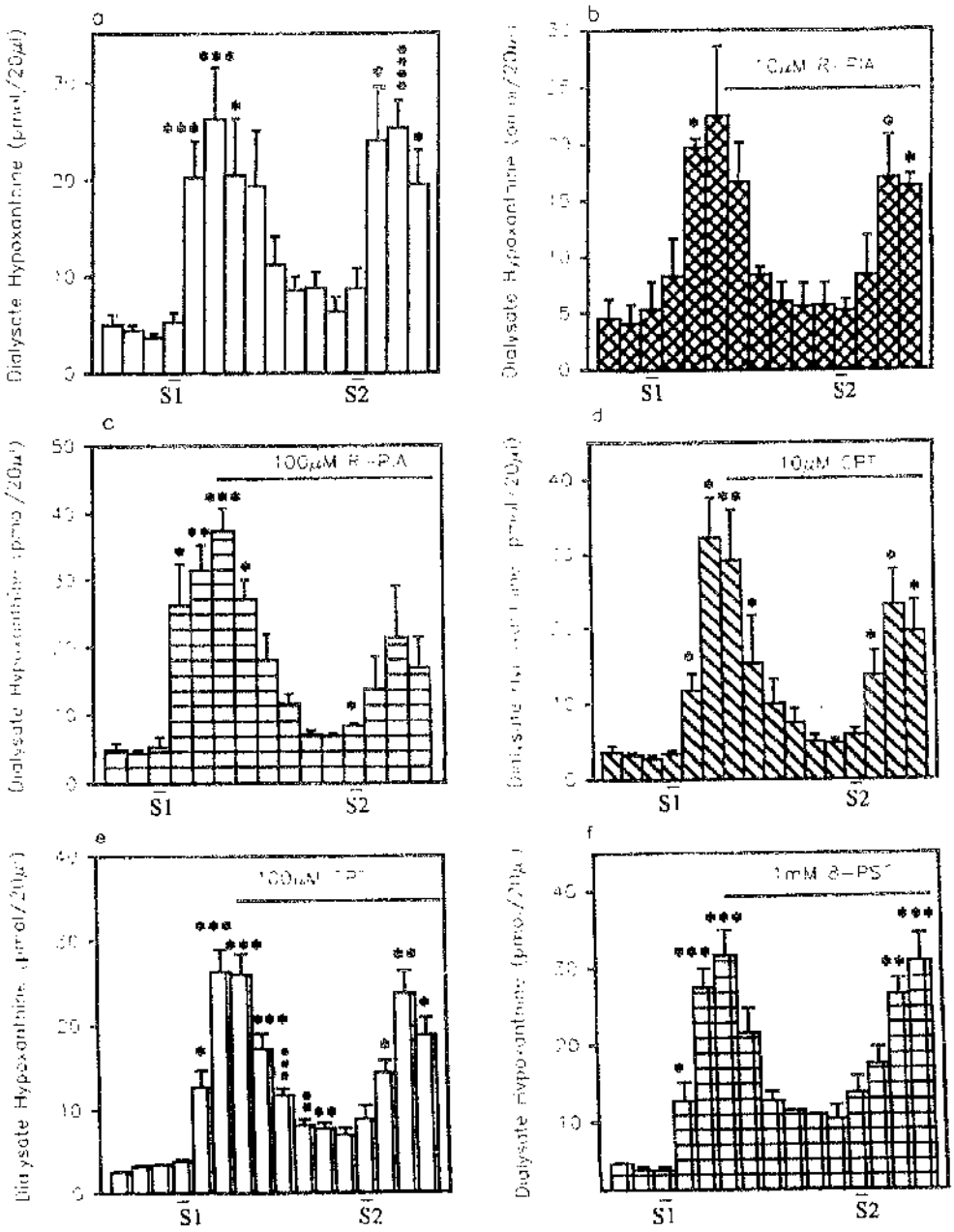



Fig 3.29

Hypoxanthine release evoked by twin pulses of 1mM kainate in the control (without the incorporation of any drugs during the second pulse of kainate) (n=7) (a) and with the incorporation of R-PIA (10μM) (n=4) (b), R-PIA (100μM) (n=4) (c), CPT (10μM) (n=5) (d), CPT (100μM) (n=4) (e) and 8-PST (1mM) (n=4) (f), as indicated by the horizontal line (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001 versus basal, paired t-test).

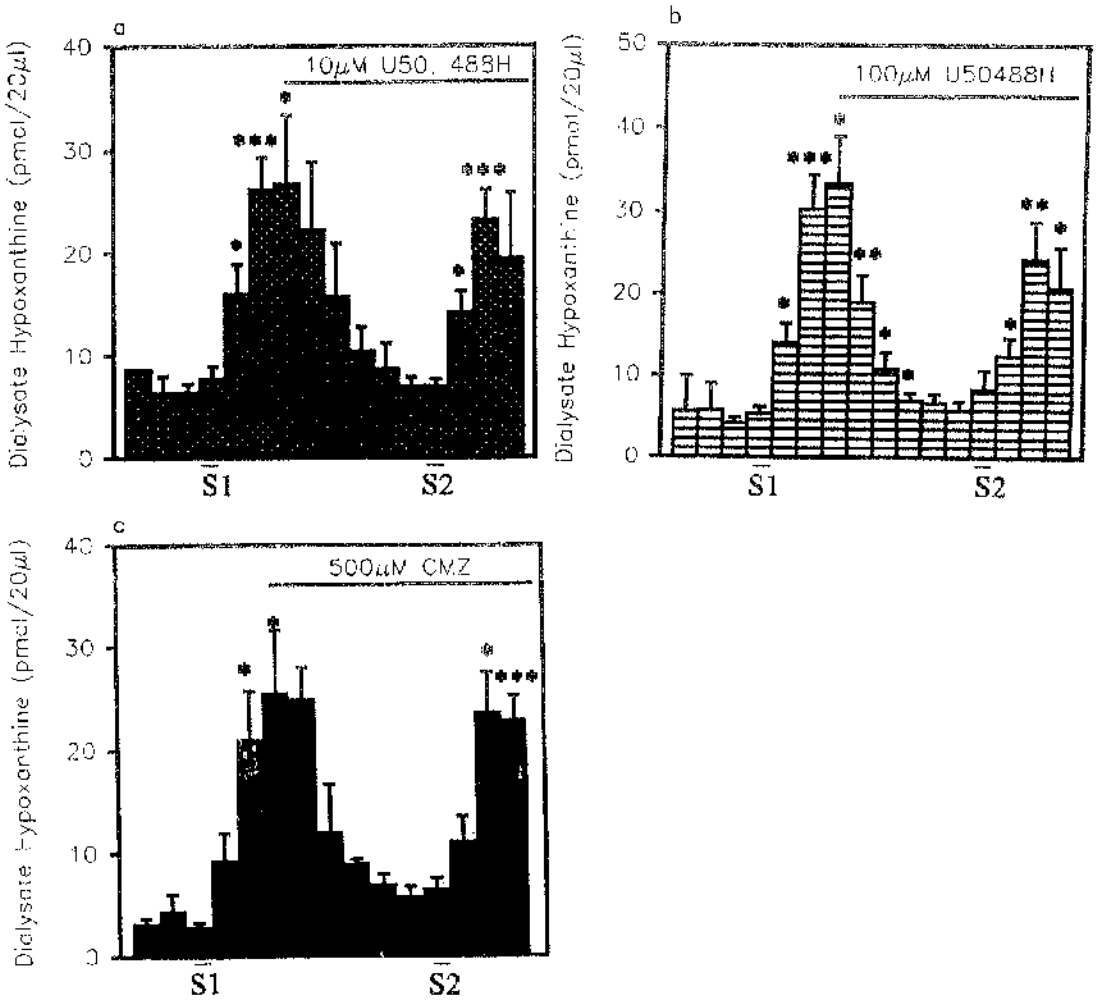



Fig 3.30

Hypoxanthine release evoked by twin pulses of 1mM kainate with the incorporation of U50 488H (10μM) (n=5) (a), U50 488H (100μM) (n=5) (b) and chlormethiazole (500μM) (n=5), as indicated by the horizontal line (*p<0.05, **p<0.01, ***p<0.005 versus basal, paired t-test).

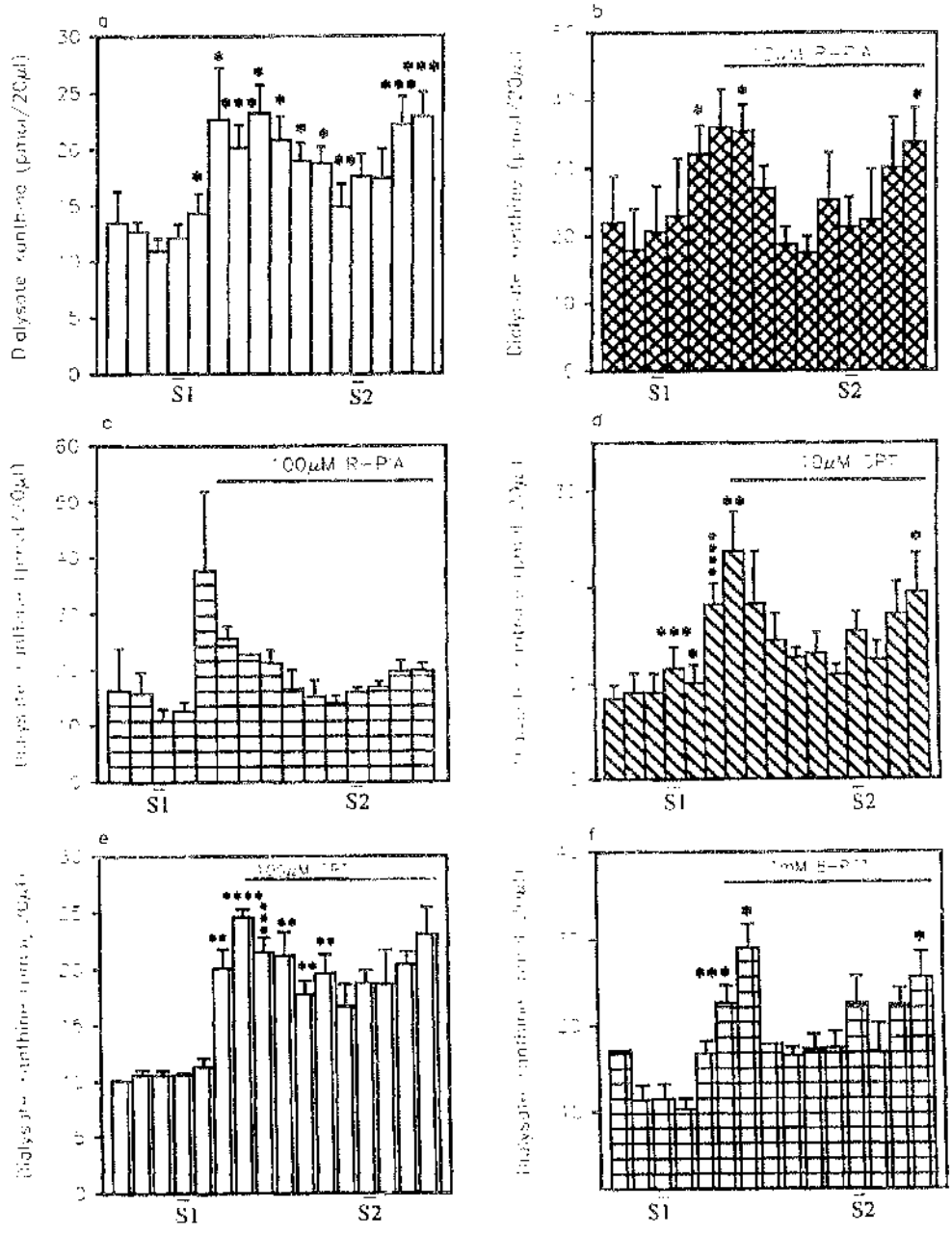



Fig 3.31

Xanthine release evoked by twin pulses of 1mM kainate in the control (without the incorporation of any drugs during the second pulse of kainate) (n=7) (a) and with the incorporation of R-PIA (10μM) (n=4) (b), R-PIA (100μM) (n=4) (c), CPT (10μM) (n=5) (d), CPT (100μM) (n=4) (e) and 8-PST (1mM) (n=4) (f), as indicated by the horizontal line (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001 versus basal, paired t-test).

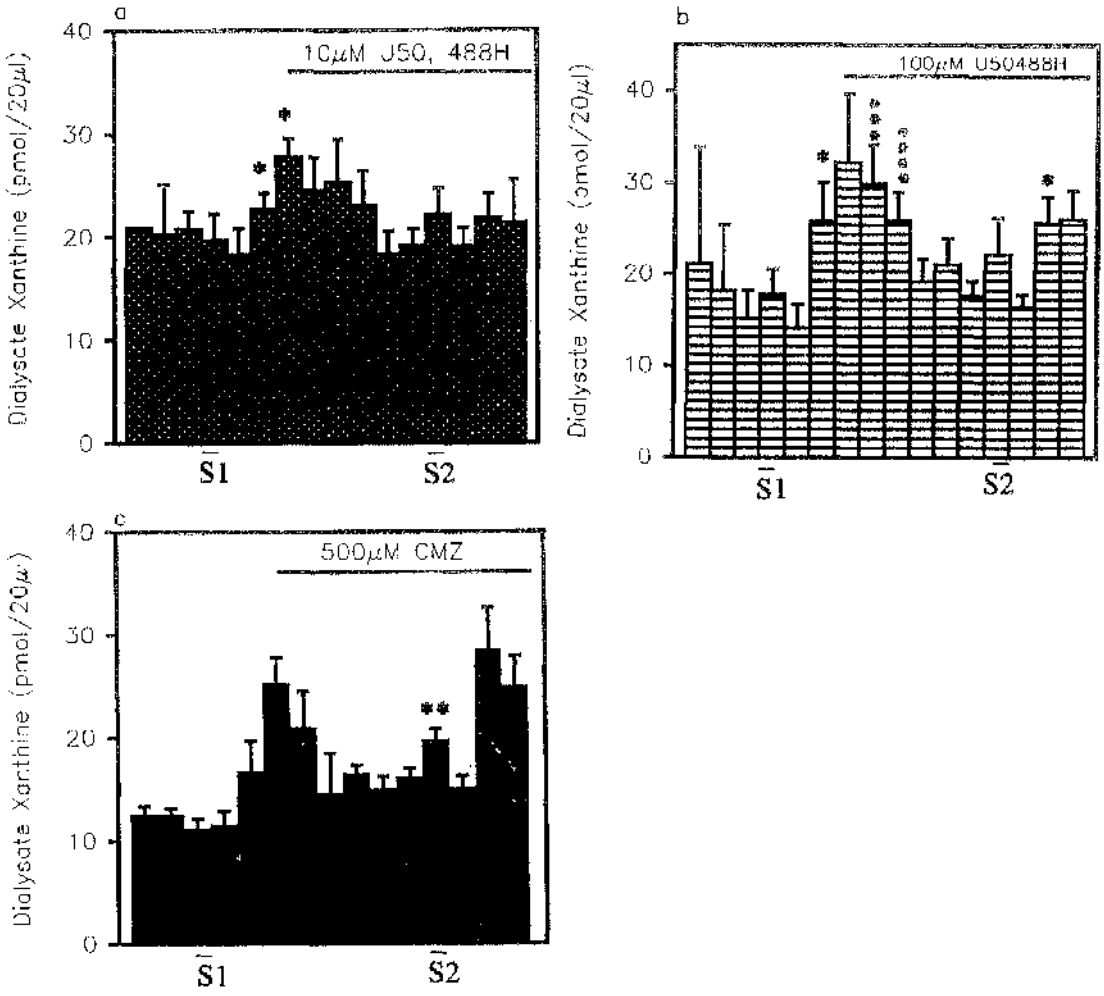



Fig 3.32

Xanthine release evoked by twin pulses of 1mM kainate with the incorporation of U50 488H (10μM) (n=5) (a), U50 488H (100μM) (n=5) (b) and chlormethiazole (500μM) (n=5), as indicated by the horizontal line (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001).

3.6.6 Intrahippocampal kainate-evoked release of adenosine and its metabolites after systemic injection of R-PIA

The s.c. injection of R-PIA (0.25mg/kg) in 43% methanol one hour before the second pulse of kainate did not affect the release of adenosine evoked by 1mM of intrahippocampal kainate (fig. 3.33a & b). The S2/S1 value was 0.41 ± 0.08 (n=4) which was not significantly different when compared to the control: s.c. injection of methanol 43% at t-60 minutes relative to the second pulse which gave an S2/S1 value of 0.412 ± 0.146 (n.s., n=4, fig 3.33c).

The release profiles for inosine, hypoxanthine and xanthine evoked by intrahippocampal kainate after s.c. injection of R-PIA are shown in fig 3.34-3.36.

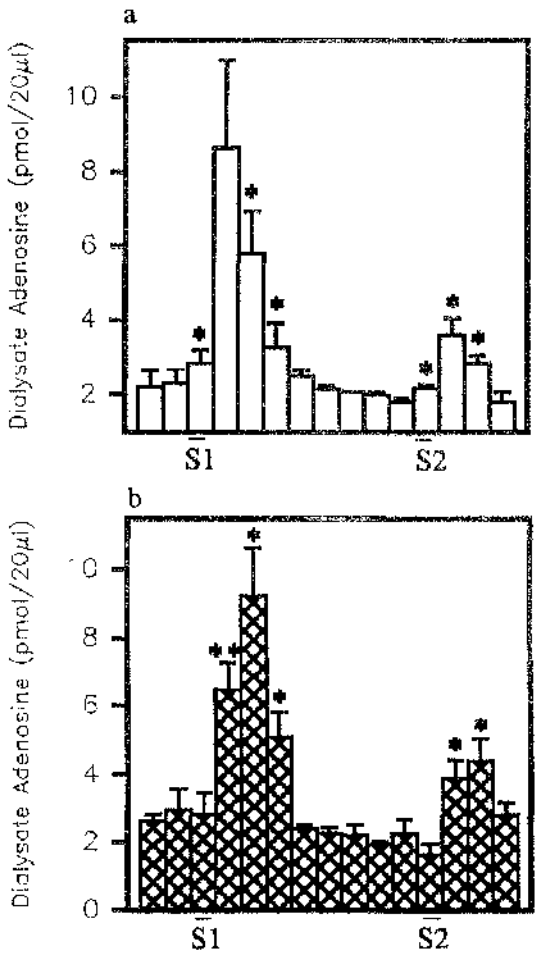



Fig 3.33

Adenosine release evoked by twin pulses of intrahippocampal kainate (1mM) with s.c. inj. of (a) methanol 43% and (b) R-PIA 250µg/kg in methanol 43% one hour before the second pulse of kainate (*$p<0.05$, **$p<0.01$ versus basal, paired t-test)

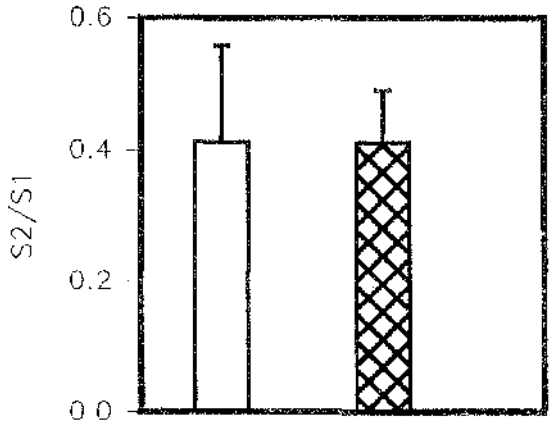



Fig 3.33 (c)

The S2/S1 ratios for adenosine release evoked by twin pulses of 1mM kainate for:

methanol 43%

R-PIA 250μg/kg in methanol 43%

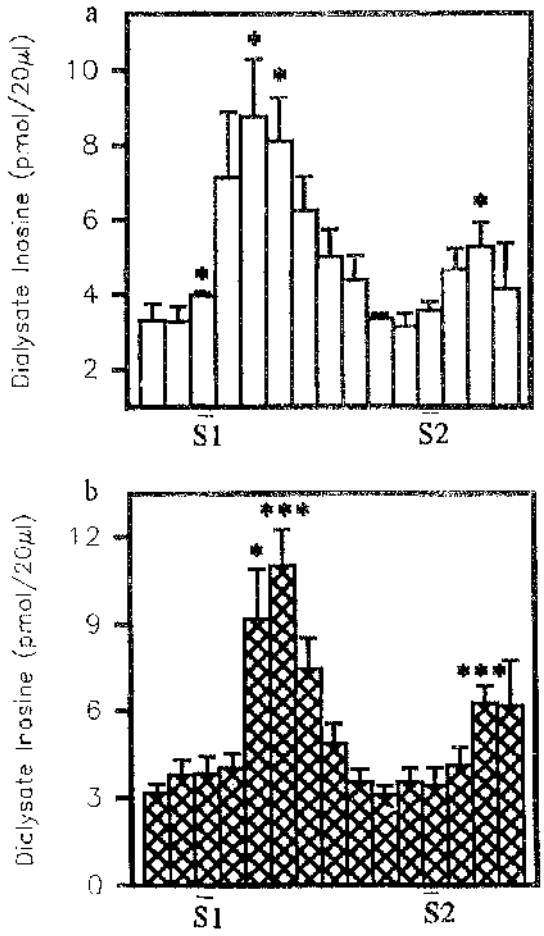



Fig 3.34

Inosine release evoked by twin pulses of intrahippocampal kainate (1mM) with subcutaneous injection of (a) methanol 43% and (b) R-PIA 250µg/kg in methanol 43% one hour before the second pulse of kainate (*$p<0.05$, ***$p<0.005$ versus basal, paired t-test).

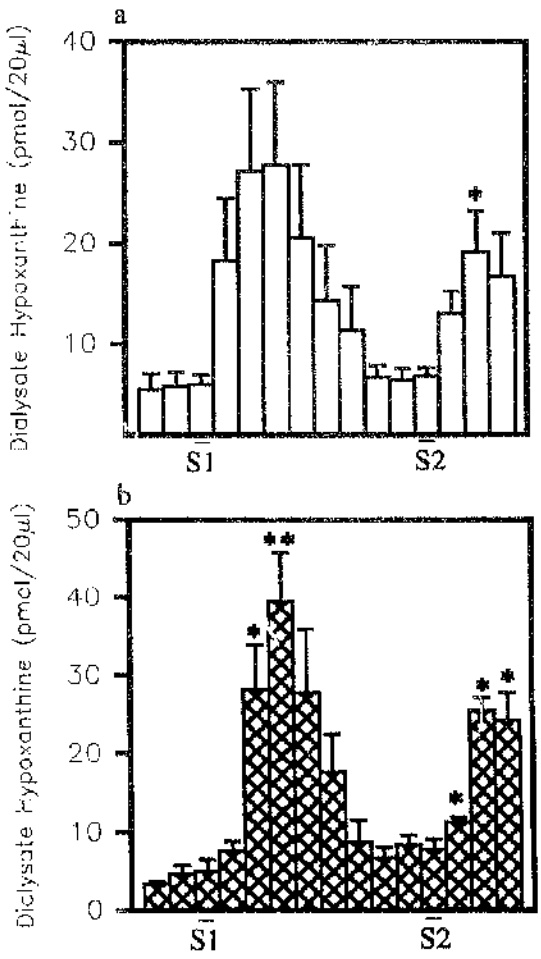



Fig 3.35

Hypoxanthine release evoked by twin pulses of intrahippocampal kainate (1mM) with subcutaneous injection of (a) methanol 43% and (b) R-PIA 250µg/kg in methanol 43% one hour before the second pulse of kainate (*$p<0.05$, **$p<0.01$ versus basal, paired t-test).

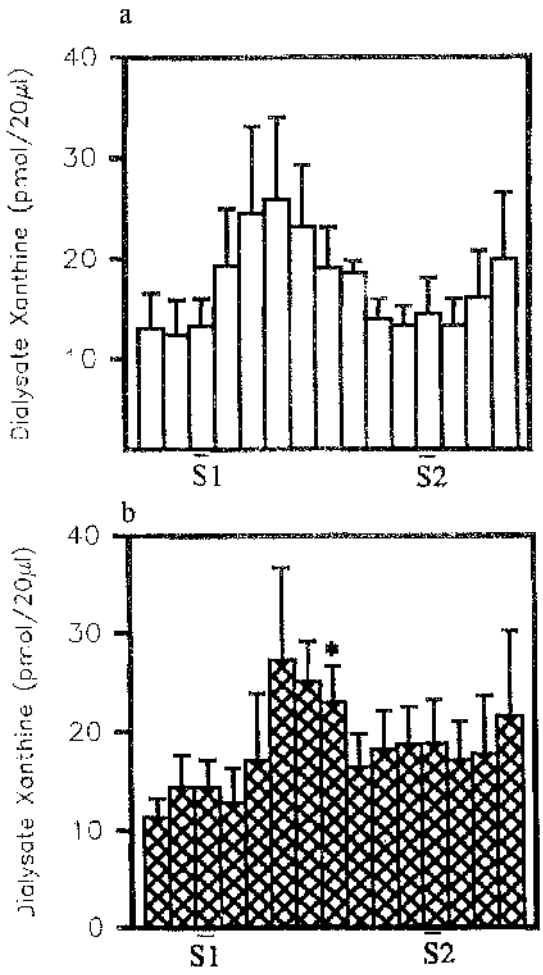



Fig 3.36

Xanthine release evoked by twin pulses of intrahippocampal kainate (1mM) with subcutaneous injection of (a) methanol 43% and (b) R-PIA 250µg/kg in methanol 43% one hour before the second pulse of kainate (*$p<0.05$ versus basal, paired t-test).

3.7 Systemic kainate-evoked release of adenosine in the presence of systemic injection of R-PIA

The release induced by kainate 10mg/kg with methanol 43% injected sub-cutaneously (the control for examining systemic R-PIA with systemic kainate) produced a bell-shaped release of adenosine. The basal level was 2.703±0.226 and the dialysate level reached 3.507±0.421 (*$p<0.05$, paired t-test, n=6, fig 3.37). Adenosine release returned to basal level within 100 min after the injections.

The s.c. injection of kainate 10mg/kg together with R-PIA (0.25mg/kg) in 43% methanol produced a bell-shaped release of adenosine. The release was increased from a basal level of 2.33 ± 0.26 to 4.40 ± 0.55 pmol/20µl (*$p<0.05$, paired t-test, n=4, fig 3.37), and returned to basal level within 140 min after the injections (fig 3.37).

The release of adenosine elicited by systemic kainate and R-PIA is significantly higher 60 min after injections than the release at that time point in the control experiments (kainate and methanol 43% s.c.) ($^{f}p<0.05$, ANOVA, followed by unpaired t-test, Bonferroni Multiple Comparisons Test, fig 3.37). Initially, this may be interpreted as an increase in adenosine release induced by systemic R-PIA. However, the release 60 min after injection of kainate alone is significantly higher than the release at that time point in the control experiments (kainate and methanol 43% s.c.) ($^{\#}p<0.001$, ANOVA, followed by unpaired t-test, Bonferroni Multiple Comparisons Test) but is not significantly different from the release at that time point in the systemic R-PIA and kainate experiments (fig 3.37). The total adenosine release evoked above basal by s.c. kainate 10mg/kg alone was 10.406 ± 1.907 (n=6), by kainate and methanol 43% was 1.494 ± 0.395 (n=6) (****$p=0.001$, unpaired t-test versus kainate alone) and by kainate and R-PIA in methanol 43% was 7.84 ± 2.96 (n=4) (*$p<0.05$, unpaired t-test versus kainate and methanol, fig 3.37 inset). It therefore appears that methanol reduced kainate-evoked release of adenosine and this effect is reversed by R-PIA.

Dialysate inosine levels were elevated significantly above basal after s.c. injection of kainate and R-PIA in methanol (fig 3.38) but not after s.c. injection of kainate and methanol. Dialysate hypoxanthine levels were elevated above basal during both conditions

(*p<0.05, **p<0.01,***p<0.005, ****p<0.001, paired t-test versus basal, fig 3.39), however the elevation after s.c. injection of kainate and R-PIA in methanol was significantly higher than that seen after s.c. injection of kainate and methanol ($^{/}$p<0.05, ANOVA, followed by unpaired t-test, Bonferroni Multiple Comparisons Test, fig 3.39). Dialysate xanthine levels were not significantly higher than basal in either conditions (fig 3.40).

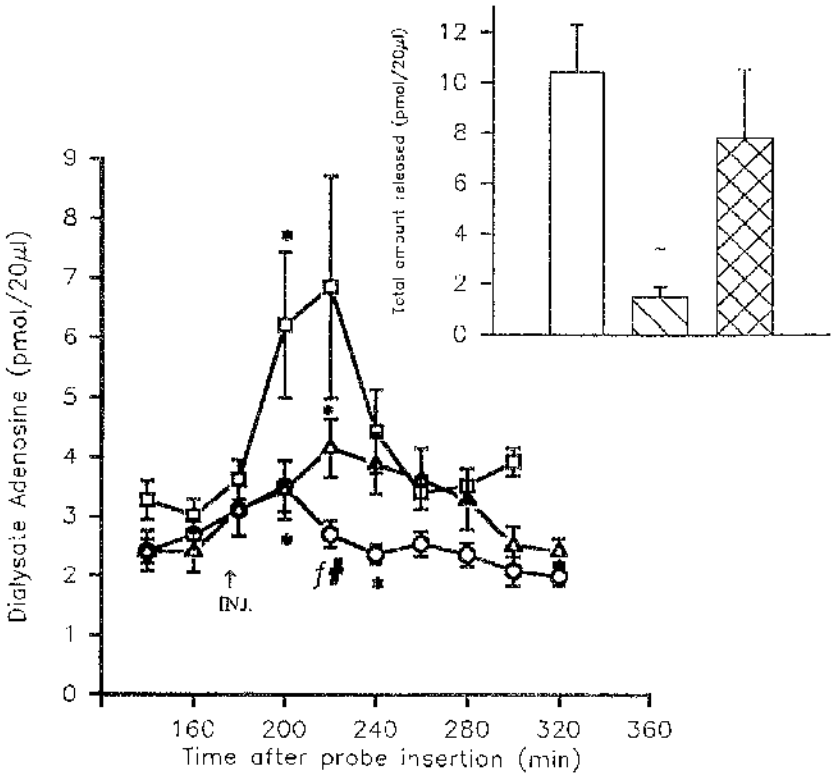



Fig 3.37

The release profiles for adenosine evoked by s.c. injection of:

□ kainic acid 10mg/kg (n=6)

○ kainic acid 10mg/kg & methanol (43%) (n=6). The level of adenosine reached significance 40 min after injection (*$p<0.05$, paired t-test) and returned to basal level 1hr after the injection.

△ kainic acid 10mg/kg & R-PIA 250µg/kg in methanol (43%) (n=4). The level of adenosine reached significance 1hr after the injection (*$p<0.05$, paired t-test) and returned to basal 2hrs 20min after the injections.

The adenosine release evoked by kainate & methanol s.c. was significantly lower 1hr after the injection than kainate & R-PIA in methanol s.c. ($^{f}p<0.05$) and than kainate alone ($^{\#}p<0.001$) (ANOVA, followed by Bonferroni Multiple Comparisons Test).

Inset: The total adenosine release evoked above basal by kainate & methanol s.c. was significantly lower than that of kainate and R-PIA in methanol ($^{\sim}p<0.05$, unpaired t-test).

□ kainic acid 10mg/kg (n=6)

▨ kainic acid 10mg/kg & methanol (43%) (n=6)

▩ kainic acid 10mg/kg & R-PIA 250µg/kg in methanol (43%) (n=4)

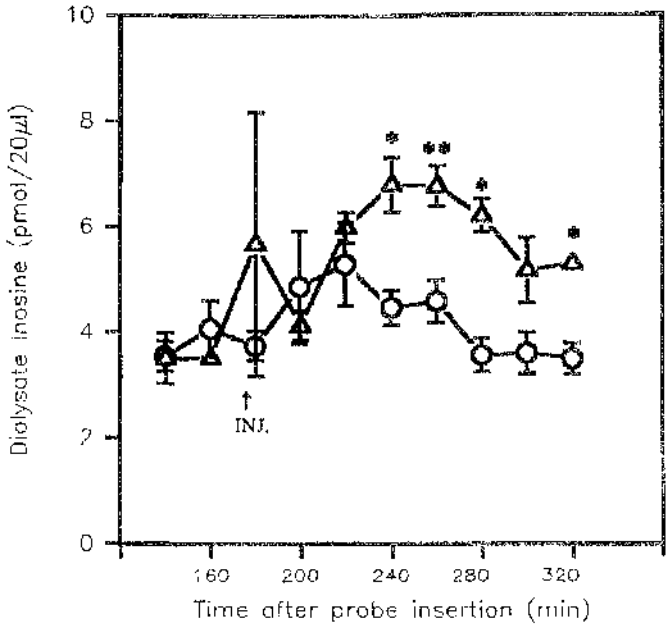



Fig 3.38

The release profiles for inosine evoked by s.c. injection of:

**O** kainic acid 10mg/kg & methanol (43%) (n=6). The level of inosine did not significantly increase above basal

**Δ** kainic acid 10mg/kg & R-PIA 250μg/kg in methanol (43%) (n=4). The level of inosine reached significance 1hr 20min after injection (*$p<0.05$, **$p<0.01$, paired t-test). Inosine release evoked by kainate & methanol s.c. was not significantly lower than that of kainate & R-PIA in methanol s.c. at any time point (ANOVA, followed by Bonferroni Multiple Comparisons Test).

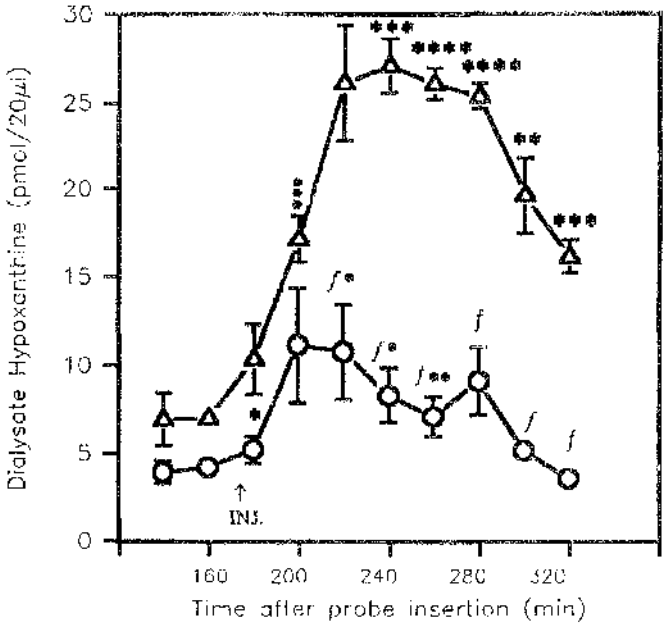



Fig 3.39

The release profiles for hypoxanthine evoked by s.c. injection of:

○ kainic acid 10mg/kg & methanol (43%) (n=6). The level of hypoxanthine reached significance 20 min after injection (*p<0.05, paired t-test versus basal).

▲ kainic acid 10mg/kg & R-PIA 250μg/kg in methanol (43%) (n=4). The level of hypoxanthine reached significance 40min after injection (**p<0.01, ***p<0.005, ****p<0.001, paired t-test versus basal).

Hypoxanthine release evoked by kainate & methanol s.c. was significantly lower 1hr after the injection than kainate & R-PIA in methanol s.c. ($^f$p<0.05, ANOVA, followed by Bonferroni Multiple Comparisons Test).

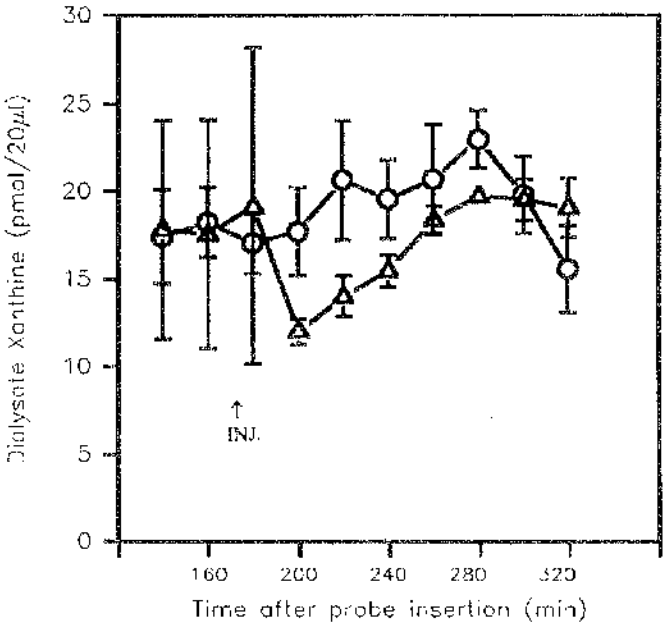



Fig 3.40

The release profiles for xanthine evoked by s.c. injection of:

- O kainic acid 10mg/kg & methanol (43%) (n=6).
- Δ kainic acid 10mg/kg & R-PIA 250µg/kg in methanol (43%) (n=4).

The level of xanthine did not reach significance in either conditions .

3.8 The effect of free radical scavengers/antioxidants on intrahippocampal kainate-evoked release of adenosine

In the present section the effects of ascorbic acid, reduced glutathione and oxypurinol were examined on intrahippocampal kainate-evoked release of adenosine and the S2/S1 ratios are summarised in fig 3.44.

3.8.1 Release of adenosine in the presence of ascorbic acid

At a concentration of 10mM in the dialysis probe (fig 3.41b), ascorbic acid, an endogenous reducing agent, reduced the S2/S1 ratio for kainate-evoked release of adenosine to 0.31±0.05 (fig. 3.44, n=4, significantly lower than control, ANOVA followed by Dunnett's Multiple Comparisons Test, *$p<0.05$), although the release evoked by the second pulse of kainate was still significantly above basal (fig 3.41b).

3.8.2 Release of adenosine in the presence of glutathione

The reducing agent, glutathione (1mM, fig 3.42a) did not reduce kainate-evoked release of adenosine, the S2/S1 value being 0.395±0.05 (n=5, fig 3.44). At a concentration of 10mM, glutathione (fig 3.42b), reduced the S2/S1 value from 0.466±0.035 (control, n=5, fig 3.44) to 0.27±0.06 (n=4, **$p<0.01$, fig 3.44).

3.8.3 Release of adenosine in the presence of oxypurinol

The xanthine oxidase inhibitor, oxypurinol, reduced the S2/S1 ratio to 0.15±0.04 at a concentration of 1mM (fig 3.43b) (n=4, **$p<0.01$, ANOVA followed by Dunnett's Multiple Comparisons Test, fig 3.44) but not at a concentration of 0.1mM (fig 3.43a) (S2/S1=0.485±0.045, n.s. to control, fig 3.44).

The effect of glutathione (1mM) and glutathione (10mM) on the release profiles for inosine, hypoxanthine and xanthine are shown in figs 3.45-3.47.

The effects of ascorbic acid and oxypurinol on inosine, hypoxanthine and xanthine were not examined. During HPLC analysis in the presence of these drugs there was a large solvent front which may have interfered with the peak size and therefore quantification of these metabolites.

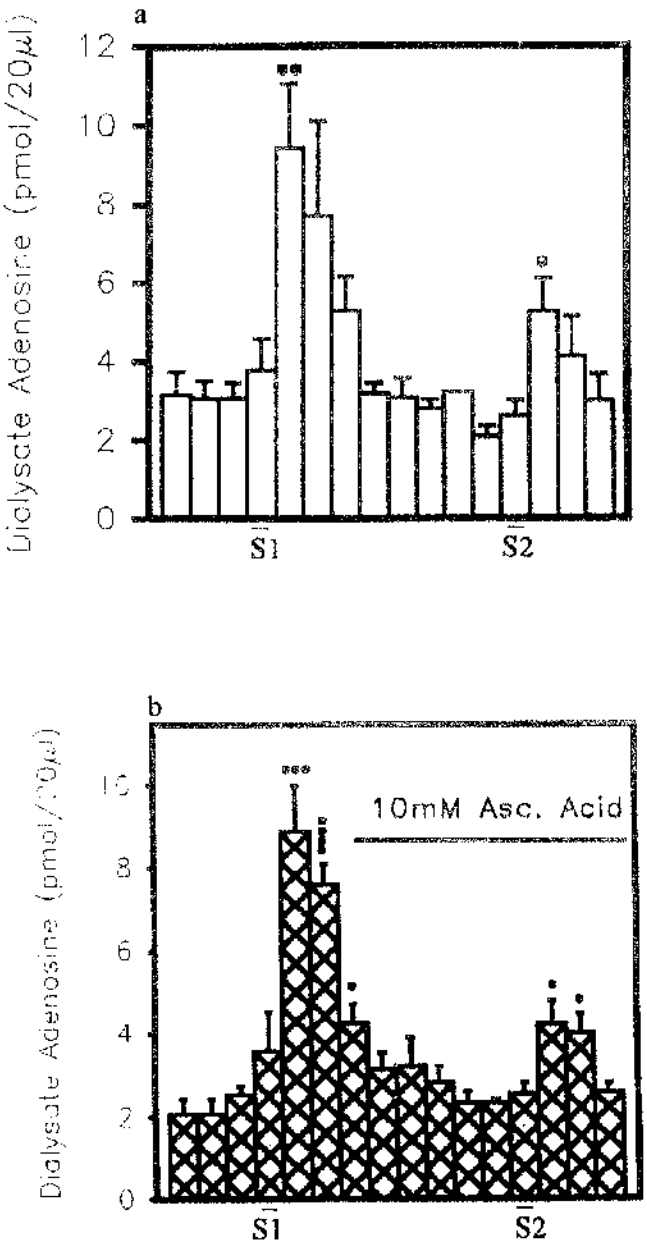



Fig 3.41

Adenosine release evoked by twin pulses of 1mM kainate (a) in the control (without the incorporation of any drugs during the second pulse of kainate) (n=5) and (b) with the incorporation of ascorbic acid (10mM), two hours before, during and after the second pulse of kainate, as indicated by the horizontal line (n=4) (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001 versus basal, paired t-test).

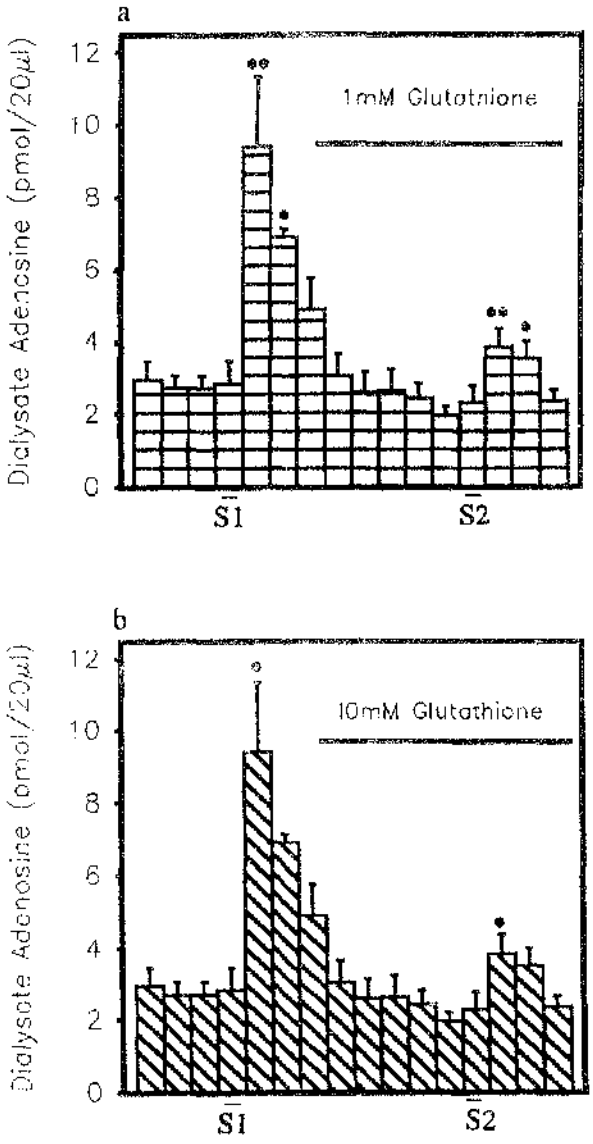



Fig 3.42

Adenosine release evoked by twin pulses of 1mM kainate with the incorporation of (a) glutathione (1mM) (n=5) and (b) glutathione 10mM (n=4) two hours before, during and after the second pulse of kainate, as indicated by the horizontal line (*$p<0.05$, **$p<0.01$ versus basal, paired t-test).

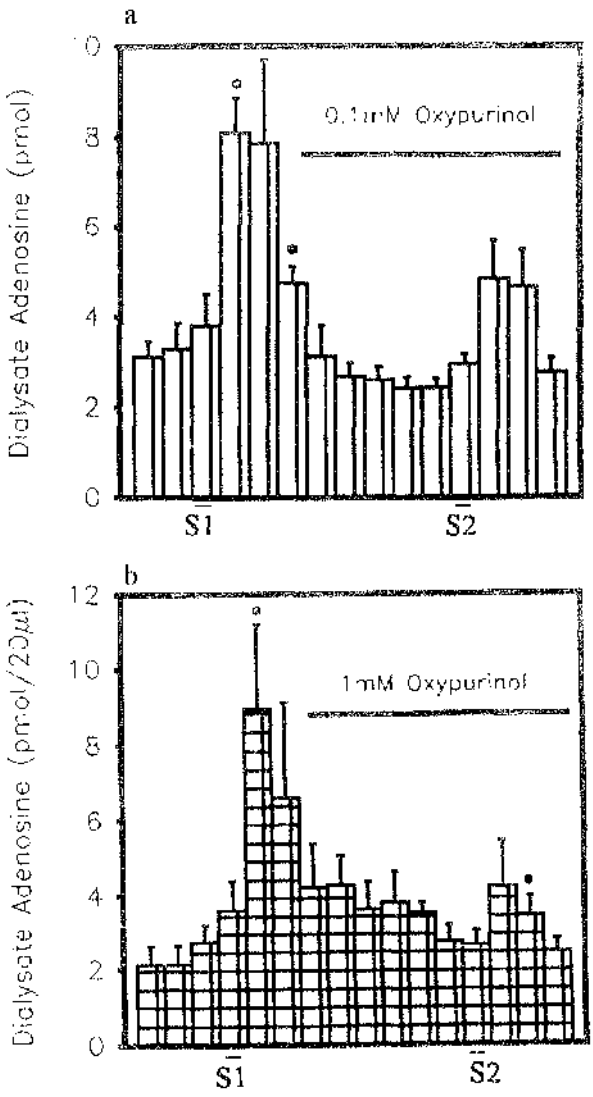



Fig 3.43

Adenosine release evoked by twin pulses of 1mM kainate with the incorporation of (a) oxypurinol (0.1mM) (n=4) and (b) oxypurinol (1mM) (n=4) two hours before, during and after the second pulse of kainate, as indicated by the horizontal line (*$p<0.05$ versus basal, paired t-test).

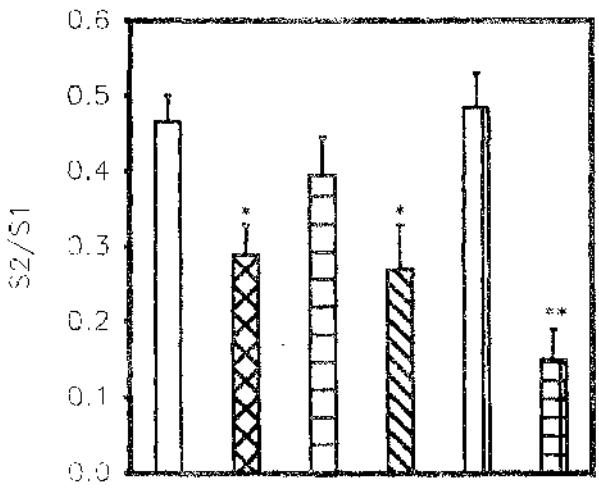



Fig 3.44

The S2/S1 ratios for adenosine release evoked by twin pulses of 1mM kainate for:

control (n=5)

ascorbic acid (10mM) (n=4)

glutathione (1mM) (n=5)

glutathione (10mM) (n=4)

oxypurinol (0.1mM) (n=4)

oxypurinol (1mM) (n=4)

There was a significant reduction in the S2/S1 ratios in the presence of ascorbic acid (10mM), glutathione (10mM) and oxypurinol (1mM) two hours before, during and after the second pulse of kainate (*$p<0.05$, **$p<0.01$, using ANOVA followed by Dunnett's t-test).

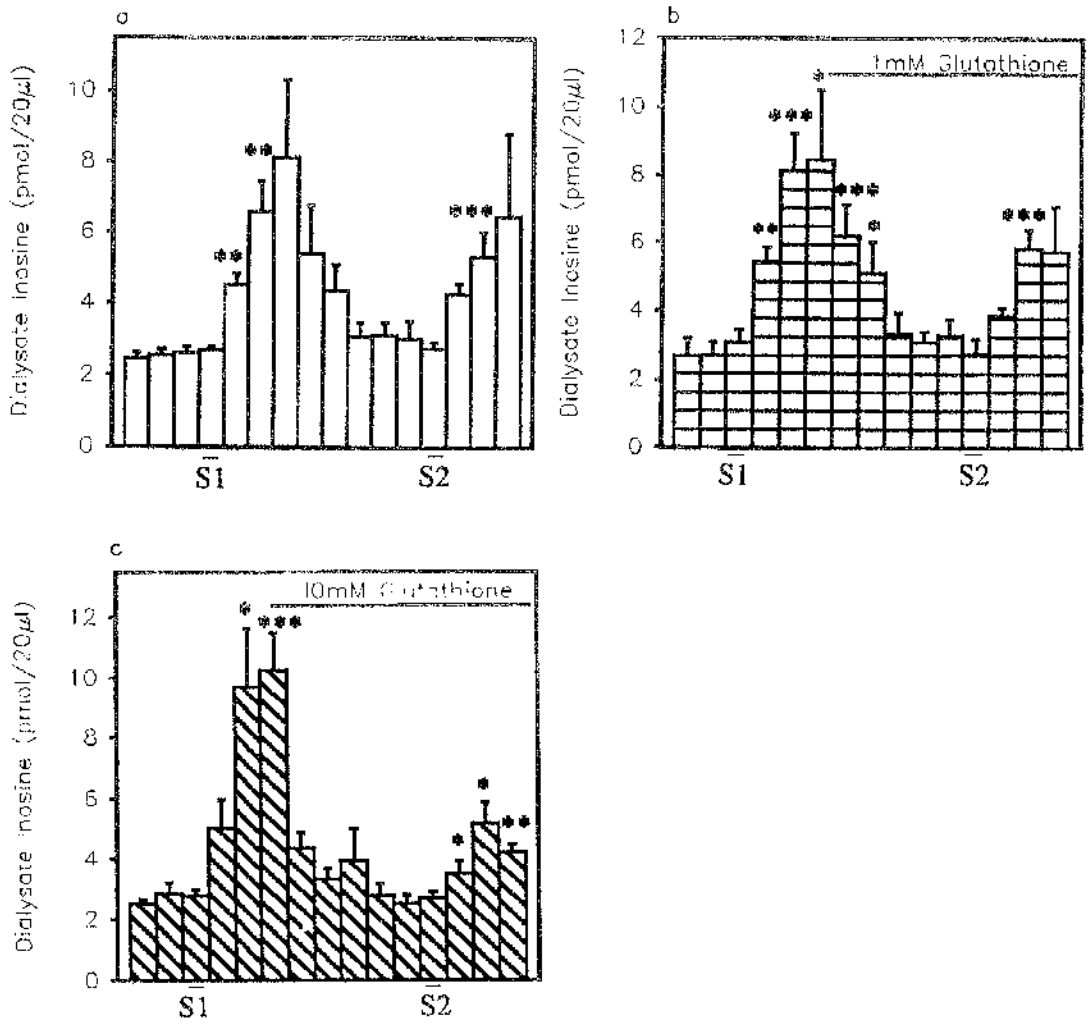



Fig 3.45

Inosine release evoked by twin pulses of 1mM kainate (a) in the control (without the incorporation of any drugs during the second pulse of kainate) (n=5) and with the incorporation of (b) glutathione (1mM) (n=5) and (c) glutathione (10mM) (n=4), as indicated by the horizontal line (*$p<0.05$, **$p<0.01$, ***$p<0.005$ versus basal, paired t-test).

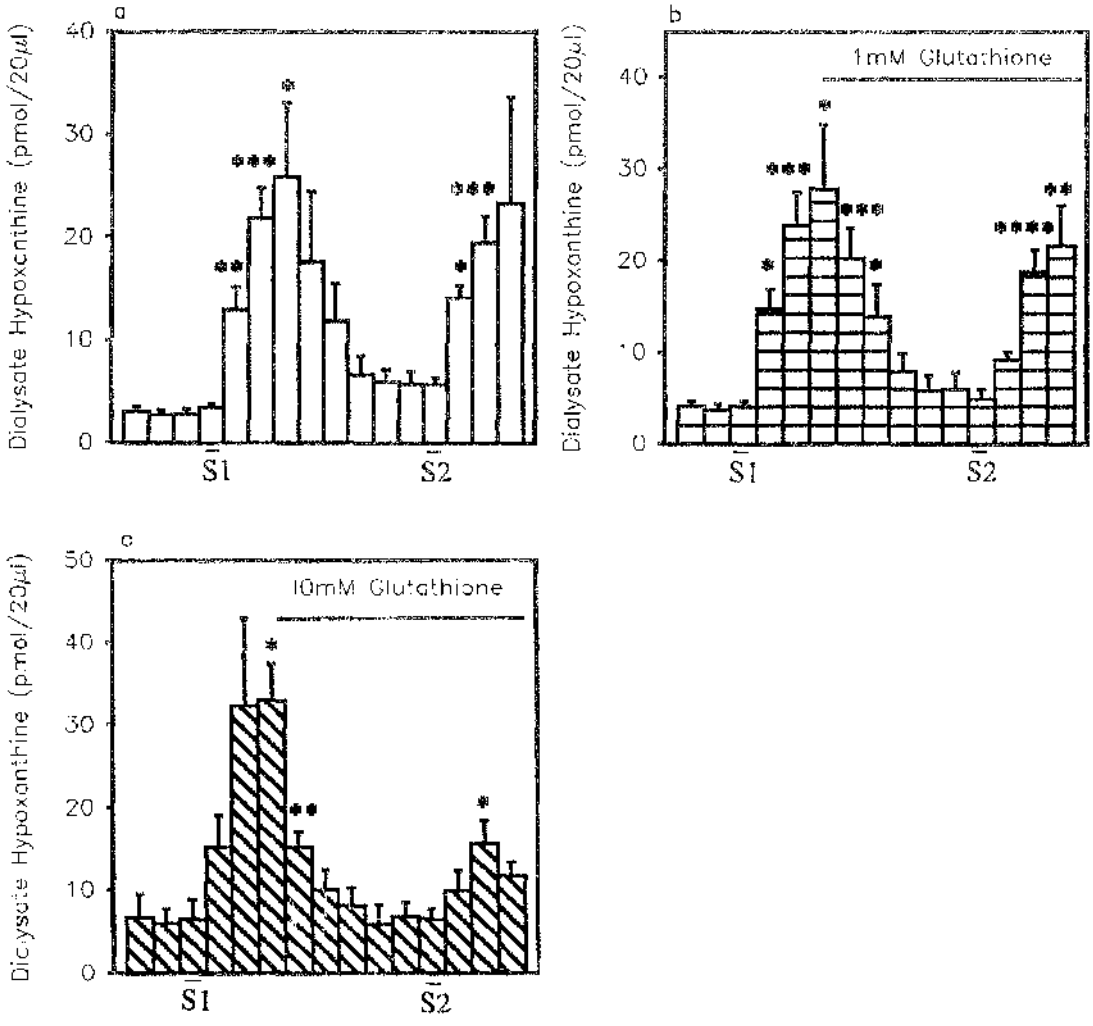



Fig 3.46

Hypoxanthine release evoked by twin pulses of 1mM kainate (a) in the control (without the incorporation of any drugs during the second pulse of kainate) (n=5) and with the incorporation of (b) glutathione (1mM) (n=5) and (c) glutathione (10mM) (n=4), as indicated by the horizontal line (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001 versus basal, paired t-test).

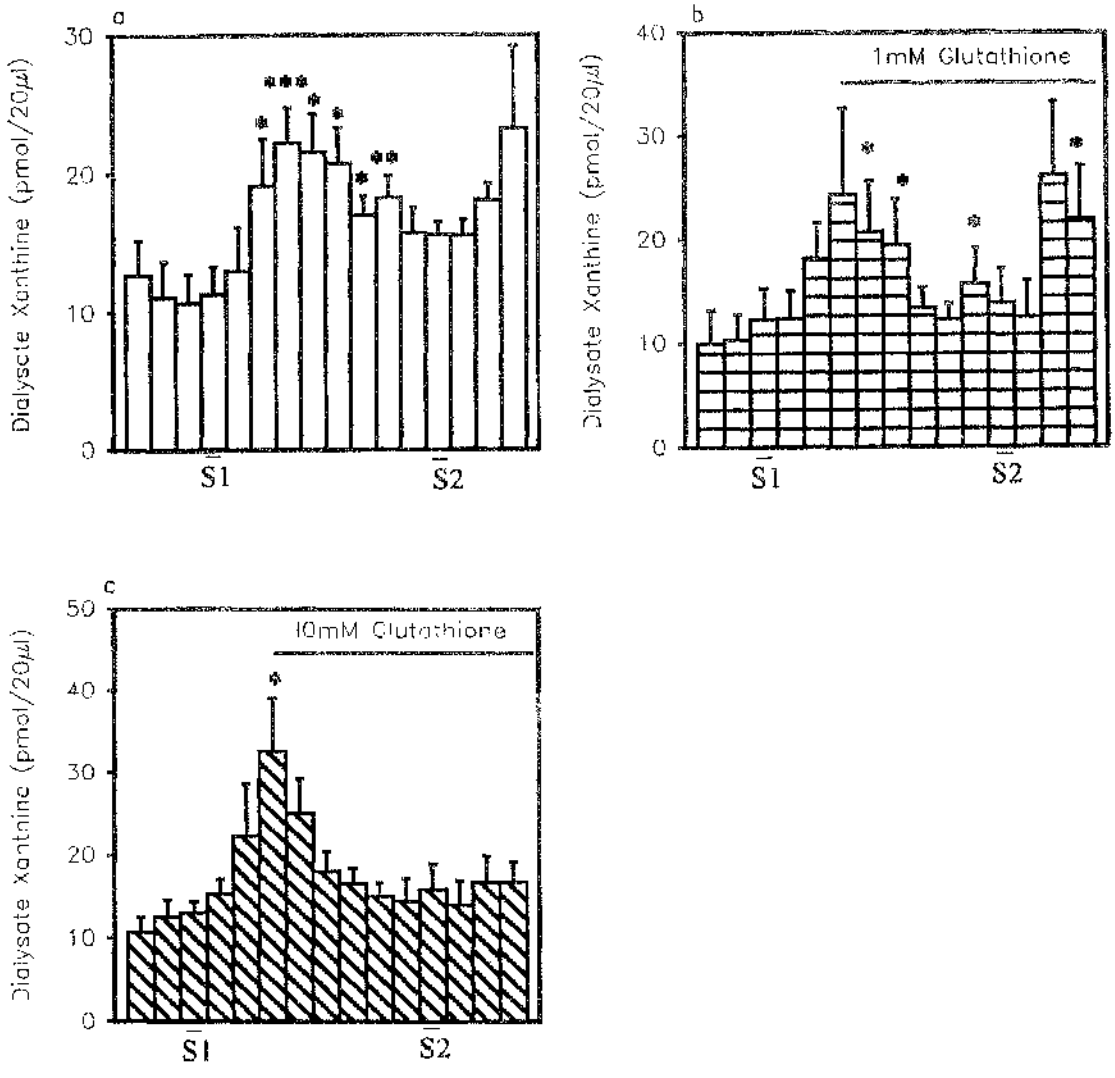



Fig 3.47

Xanthine release evoked by twin pulses of 1mM kainate (a) in the control (without the incorporation of any drugs during the second pulse of kainate) (n=5) and with the incorporation of (b) glutathione (1mM) (n=5)and (c) glutathione (10mM) (n=4), as indicated by the horizontal line (*$p<0.05$, **$p<0.01$, ***$p<0.005$ versus basal, paired t-test).

3.8.4 Intrahippocampal kainate-evoked release of adenosine and its metabolites after systemic injection of ascorbic acid

If injected sub-cutaneously, one hour before the second pulse of intrahippocampal kainic acid (1mM), ascorbate (50mg/kg) did not reduce adenosine release when compared to the experiments with injection of saline (control, fig 3.48). The S2/S1 values were 0.48 ± 0.14 and 0.83 ± 0.12, respectively (n.s., unpaired t-test, fig 3.48 inset).

The profiles of release of inosine, hypoxanthine and xanthine are shown in figs 3.49-3.41.

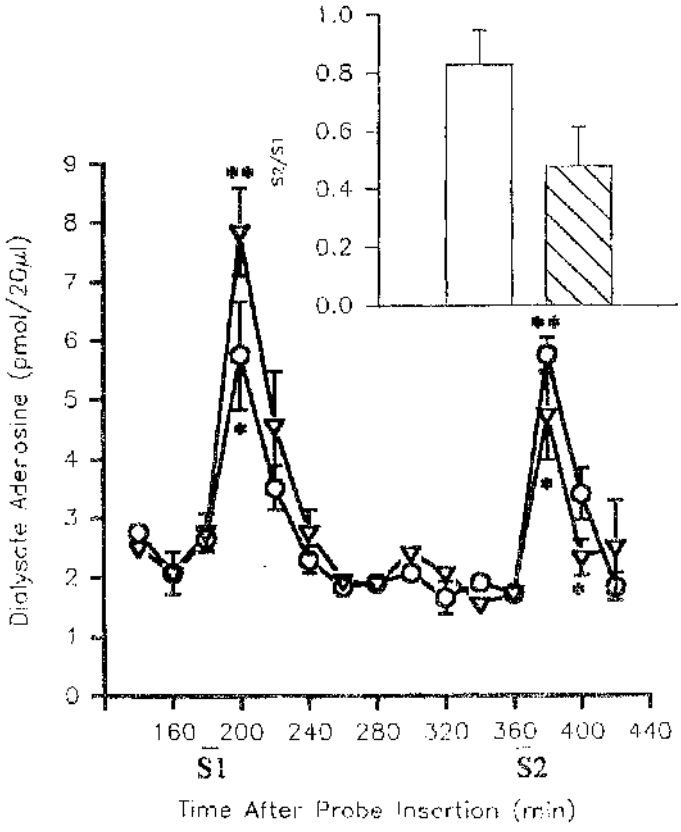



Fig 3.48 Adenosine release evoked by twin pulses of kainate 1mM with subcutaneous injection one hour before the second pulse of kainate of

- saline (*p<0.05, **p<0.01 versus basal, paired t-test)
- ascorbic acid 50mg/kg in saline (*p<0.05, **p<0.01 versus basal, paired t-test)

Inset: The S2/S1 ratios of adenosine release evoked by twin pulses of kainate 1mM with

- saline (control) s.c.
- ascorbic acid 50mg/kg in saline s.c. (n.s. to control)

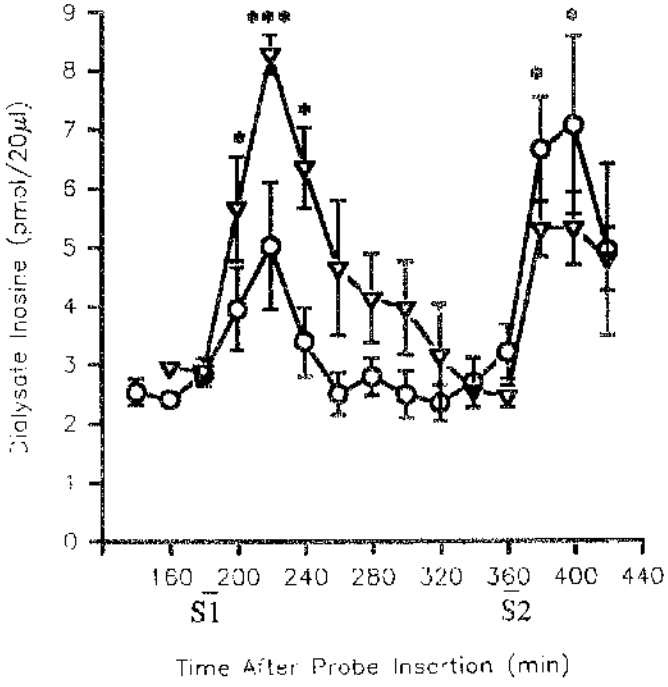



Fig 3.49

Inosine release evoked by twin pulses of kainate 1mM with subcutaneous injection one hour before the second pulse of kainate of

- saline
- ascorbic acid 50mg/kg in saline (*p<0.05, ***p<0.005 versus basal, paired t-test)

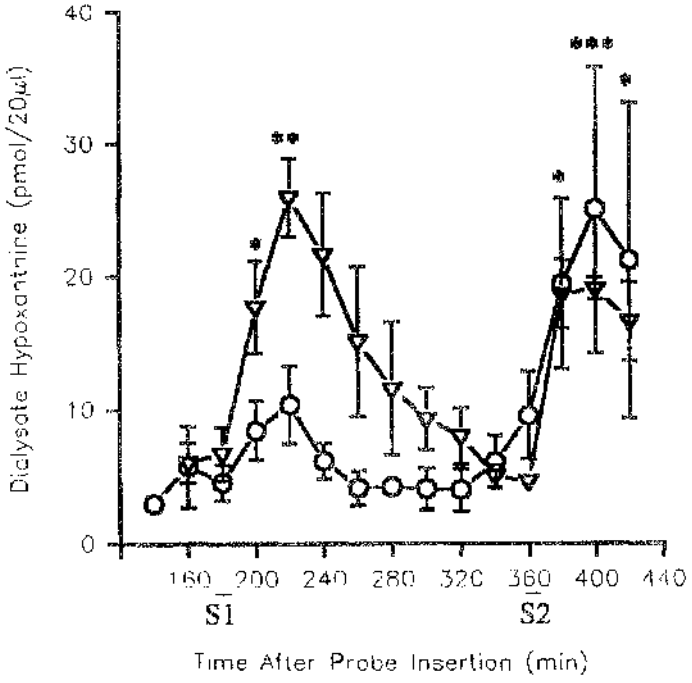



Fig 3.50

Hypoxanthine release evoked by twin pulses of kainate 1mM with subcutaneous injection one hour before the second pulse of kainate of

- saline (*p<0.05, ***p<0.005 versus basal, paired t-test)
- ascorbic acid 50mg/kg in saline (*p<0.05, **p<0.01 versus basal, paired t-test)

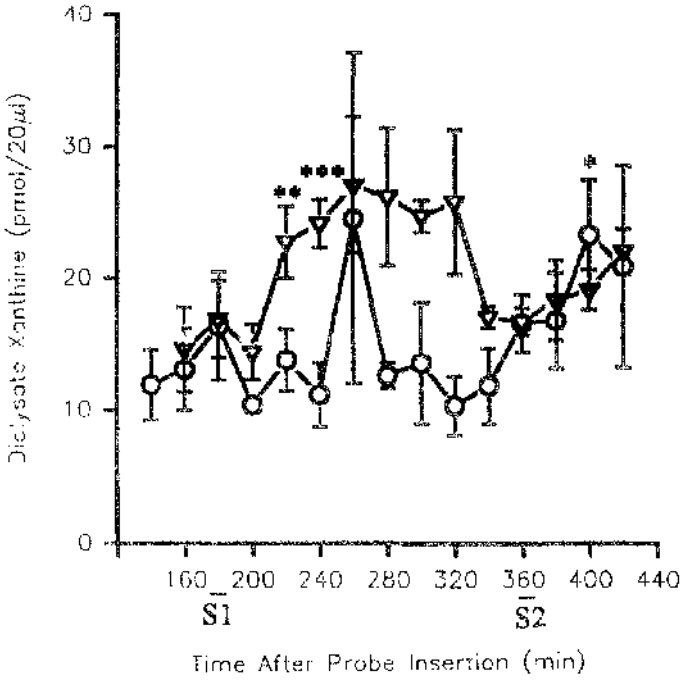



Fig 3.51

Xanthine release evoked by twin pulses of kainate 1mM with subcutaneous injection one hour before the second pulse of kainate of

- saline (*p<0.05 versus basal, paired t-test)
- ascorbic acid 50mg/kg in saline (**p<0.01, ***p<0.005 versus basal, paired t-test)

3.9 Release of adenosine and its metabolites evoked by systemic kainate with and without systemic ascorbic acid

Kainate 10mg/kg i.p., injected at 160 min (following the collection of that sample) after probe insertion, induced adenosine release which reached significance within 40 minutes of injection (fig 3.52). The average of the three basal adenosine levels before kainate injection was 2.157 ± 0.431 pmol/20µl and kainate-evoked release reached 3.364 ± 0.604 pmol/20µl during the second sample after the injection (*$p<0.05$ versus basal before injection, paired t-test). During the next time point, the mean value of adenosine release further increased, although this was not significantly above basal level, using paired t-test. The profile of release was bell-shaped, having reached basal level again 2 hrs after the injection (fig 3.52).

Injection of kainate 10mg/kg i.p. and ascorbic acid 50mg/kg i.p. showed a bell-shaped increase in the release of adenosine, which reached significance above basal within 1hr after injections (increase from 2.56 ± 0.09 pmol/20µl to 4.484 ± 0.796 pmol/20µl, *$p<0.05$, two-tailed paired t-test, fig 3.52). The adenosine release evoked by kainate 10mg/kg i.p. was not reduced when co-administered with ascorbic acid 50mg/kg i.p. (ANOVA, followed by Bonferroni Multiple Comparisons Test fig 3.52).

The total amount of adenosine release above basal is shown in the inset in fig 3.52. Ascorbic acid i.p. did not significantly affect the total amount of adenosine release after kainate i.p.

The release profiles for inosine, hypoxanthine and xanthine after kainate i.p. with and without ascorbic acid i.p. are shown in figs 3.53-3.55. The total amount released above basal was not calculated for these metabolites since their release had not returned to basal level before the termination of some of the experiments.

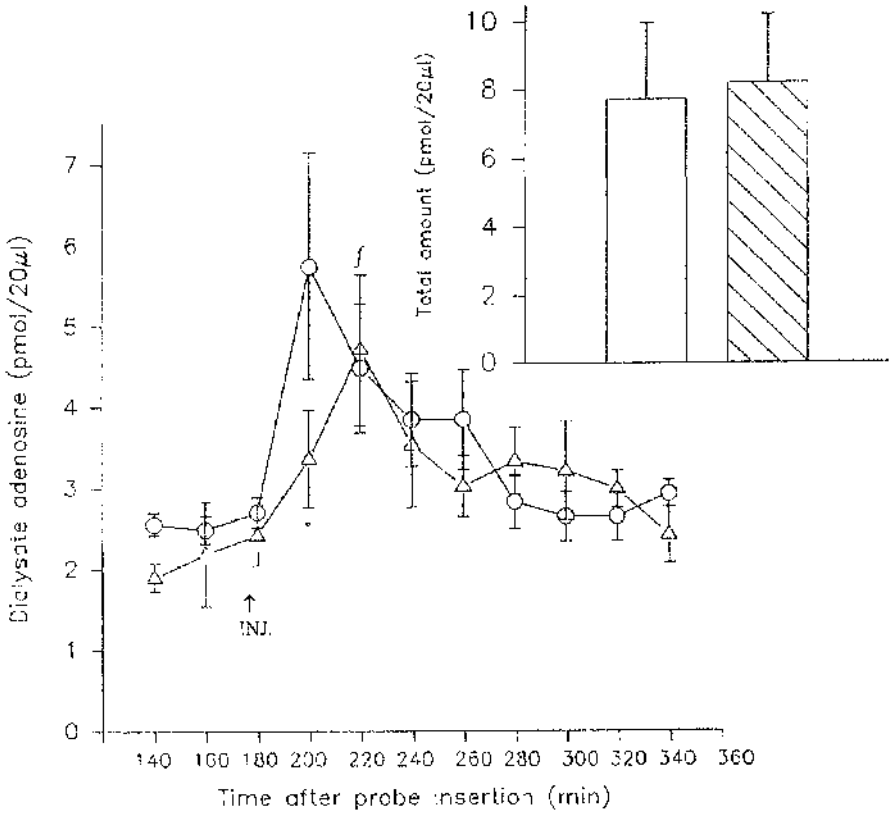



Fig 3.52

The release profiles for adenosine evoked by i.p. injection of:

△ kainic acid 10mg/kg (n=4). The level of adenosine reached significance 40 min after injection (*$p<0.05$, paired t-test) and returned to basal level 3 hrs after the injection.

○ kainic acid 10mg/kg & ascorbic acid 50mg/kg (n=6). The level of adenosine reached significance 1hr after injections ($^{f}p<0.05$, paired t-test) and returned to basal level 2hrs after the injections.

The adenosine release evoked by kainate 10mg/kg i.p. was not reduced by ascorbic acid 50mg/kg i.p. (ANOVA, followed by Bonferroni Multiple Comparisons Test).

Inset: The total amount of adenosine release above basal evoked by:

□ kainic acid 10mg/kg (n=4)

▨ kainic acid 10mg/kg & ascorbic acid 50mg/kg (n=6)

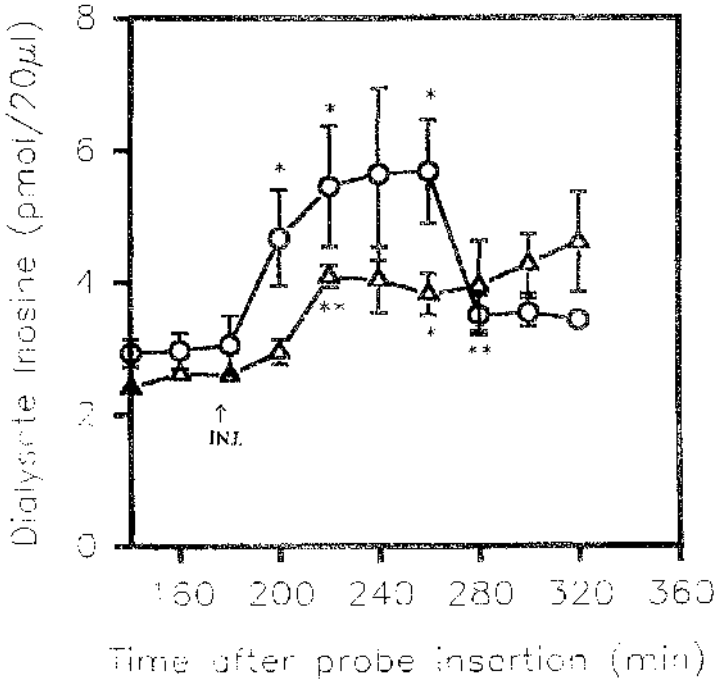



Fig 3.53

The release profiles for inosine evoked by i.p. injection of:

▲ kainic acid 10mg/kg (n=4). The level of inosine reached significance 1hr after injection (*p<0.05, paired t-test) and did not return to basal level within the time-scale of the experiment.

○ kainic acid 10mg/kg & ascorbic acid 50mg/kg (n=6). The level of inosine reached significance 40min after the injection (*p<0.05, paired t-test) and approached basal level 2hrs 40min after the injections.

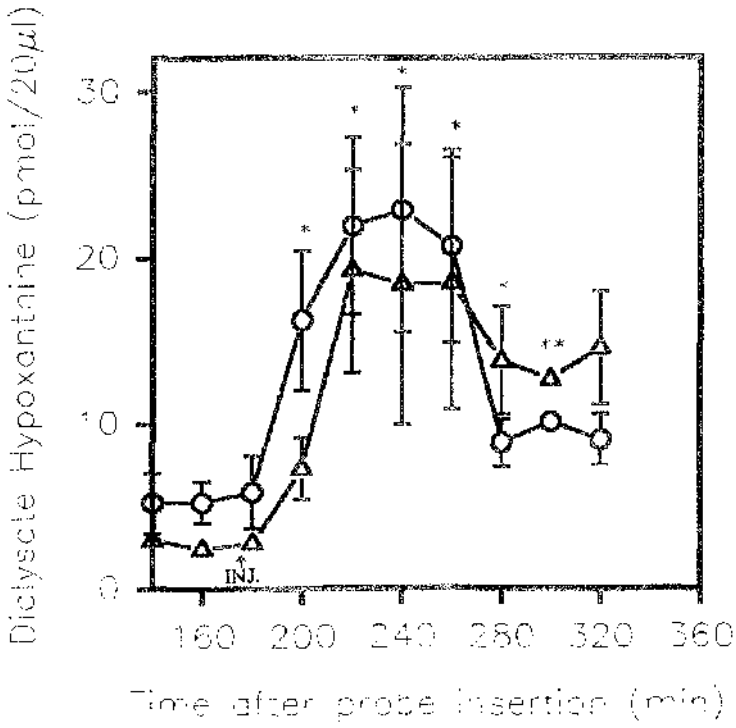



Fig 3.54

The release profiles for hypoxanthine evoked by i.p. injection of:

△ kainic acid 10mg/kg (n=4). The level of hypoxanthine did not reach significance above basal (paired t-test) and the mean values did not return to basal level within the time-scale of the experiment.

○ kainic acid 10mg/kg & ascorbic acid 50mg/kg (n=6). The level of hypoxanthine reached significance 40min after the injection (*$p<0.05$, paired t-test) and approached basal level 2hrs 40min after the injections.

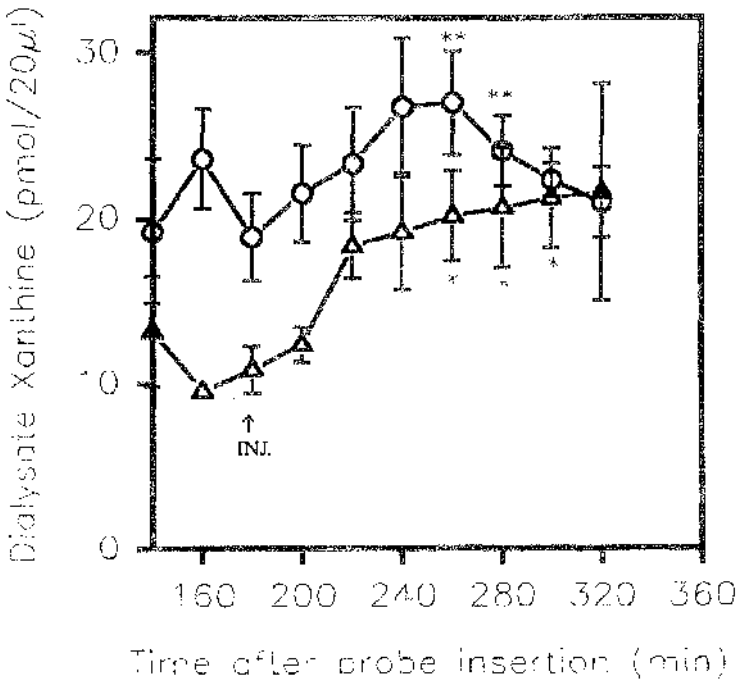



Fig 3.55

The release profiles for xanthine evoked by i.p. injection of:

▲ kainic acid 10mg/kg (n=4). The level of xanthine reached significance 2hrs after injection (*p<0.05, paired t-test) and did not return to basal level within the time-scale of the experiment.

○ kainic acid 10mg/kg & ascorbic acid 50mg/kg (n=6). The level of xanthine reached significance 1hr 40min after the injection (*p<0.05, paired t-test) and returned to basal level 2hrs 40min after the injection.

Subcutaneous injection of kainate 10mg/kg had a similar temporal profile to that of the i.p. injection but the peak mean value was higher (fig 3.56). Adenosine release was increased from 2.973±0.149 to 6.323±1.187 (*$p<0.05$, paired t-test, fig 3.56). Although the mean value of the adenosine release increased further, it was not found to be significantly above the basal level according to the paired t-test.

Injection of kainate 10mg/kg s.c. and ascorbic acid 50mg/kg s.c. also showed a bell-shaped release of adenosine which did not quite reach significance above basal (fig 3.56, paired t-test). The adenosine release evoked by kainate 10mg/kg s.c. was not reduced when co-administered with ascorbic acid 50mg/kg i.p. (ANOVA, followed by Bonferroni Multiple Comparisons Test fig 3.56). The total amount of adenosine release above basal is shown in the inset in fig 3.56. Ascorbic acid s.c. did not significantly affect the total amount of adenosine release after kainate s.c.

The release profiles for inosine, hypoxanthine and xanthine after kainate s.c. with and without ascorbic acid s.c. are shown in figs 3.57-3.59. The total amount released above basal was not calculated for these metabolites since their release had not returned to basal level before the termination of some of the experiments.

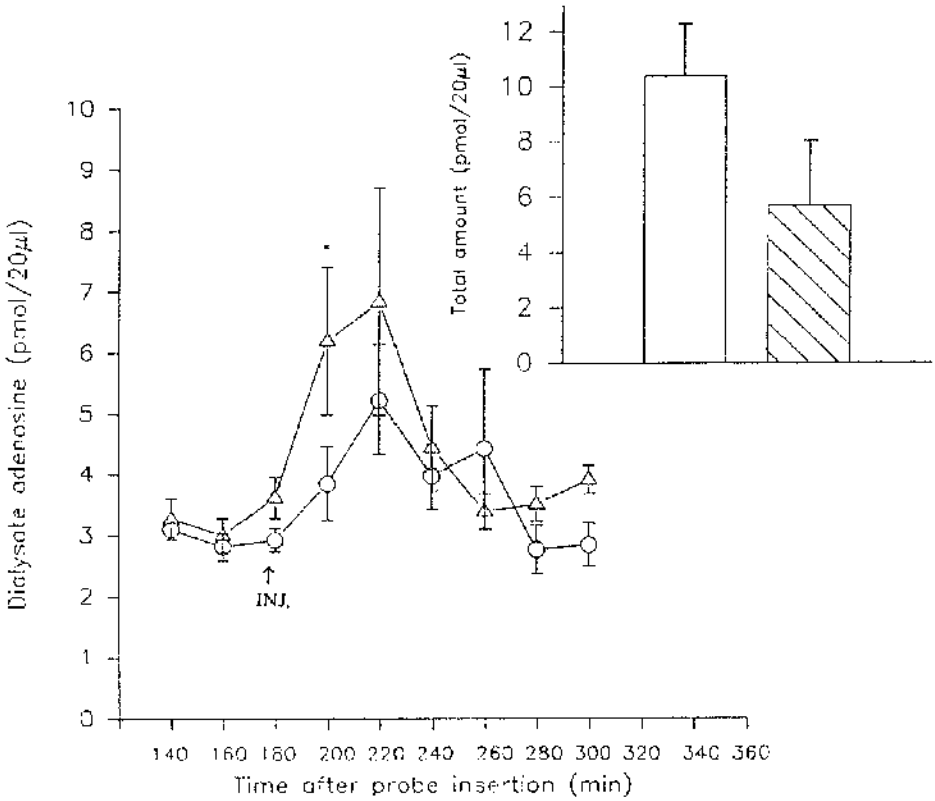



Fig 3.56

The release profiles for adenosine evoked by s.c. injection of:

△ kainic acid 10mg/kg (n=6). The level of adenosine reached significance 40 min after injection (*$p<0.05$, paired t-test) and returned to basal level 1hr 40min after the injection.

○ kainic acid 10mg/kg & ascorbic acid 50mg/kg (n=6). The level of adenosine did not quite reach significance (paired t-test) and the mean values returned to basal level 2hrs after the injections.

The adenosine release evoked by kainate 10mg/kg i.p. was not reduced by ascorbic acid 50mg/kg i.p. (ANOVA, followed by Bonferroni Multiple Comparisons Test).

Inset: The total amount of adenosine release above basal evoked by:

□ kainic acid 10mg/kg (n=6)

▨ kainic acid 10mg/kg & ascorbic acid 50mg/kg (n=6) (n.s., unpaired t-test).

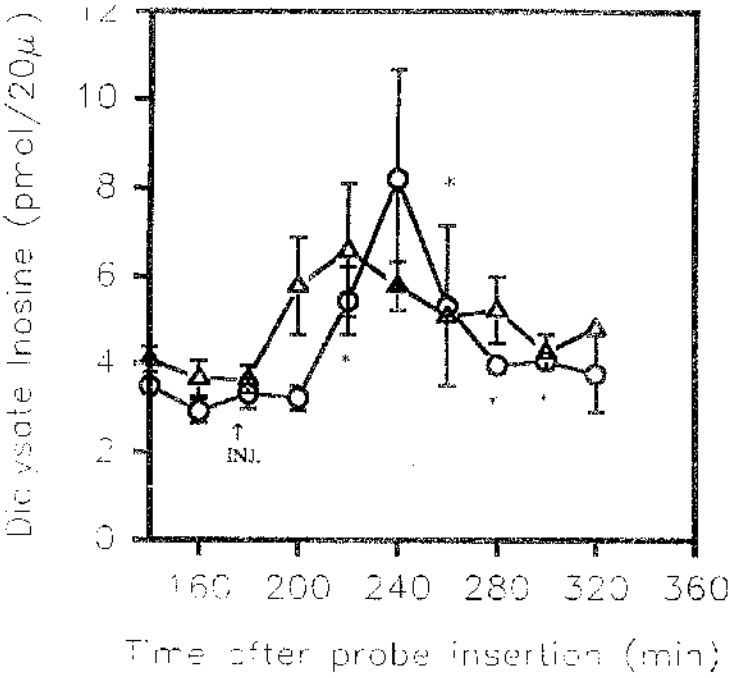



Fig 3.57

The release profiles for inosine evoked by s.c. injection of:

▲ kainic acid 10mg/kg (n=6). The level of inosine reached significance 1hr 40 min after injection (*$p<0.05$, paired t-test) and did not return to basal level within the time-scale of the experiment.

○ kainic acid 10mg/kg & ascorbic acid 50mg/kg (n=6). The level of inosine reached significance 1hr after the injection (*$p<0.05$, paired t-test) and approached basal level 2hrs 40min after the injections.

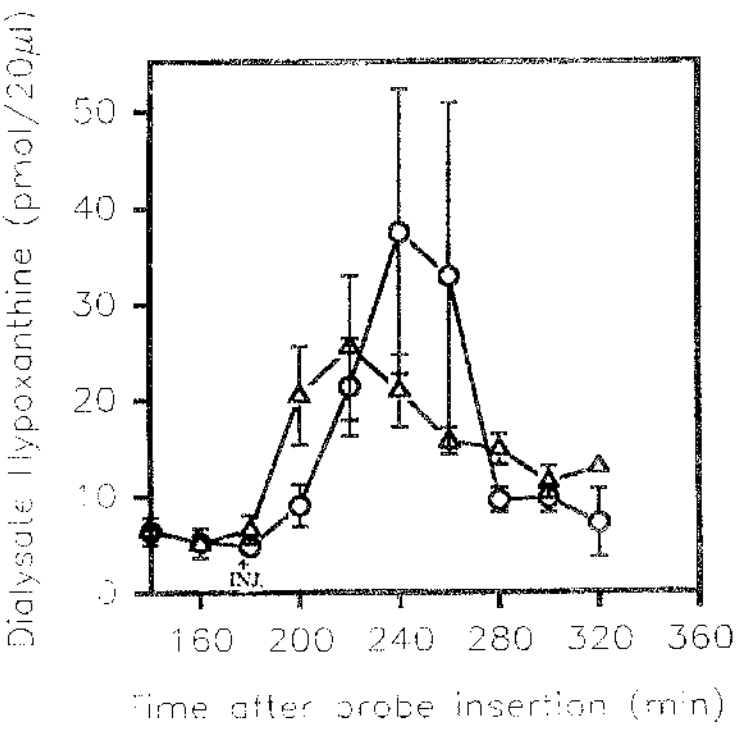



Fig 3.58

The release profiles for hypoxanthine evoked by s.c. injection of:

Δ kainic acid 10mg/kg (n=6). The level of hypoxanthine reached significance 40min after injection (*$p<0.05$, paired t-test) and did not return to basal level within the time-scale of the experiment.

O kainic acid 10mg/kg & ascorbic acid 50mg/kg (n=6). The level of hypoxanthine reached significance 1hr after the injection (*$p<0.05$, paired t-test) and did not return to basal level within the time-scale of the experiment.

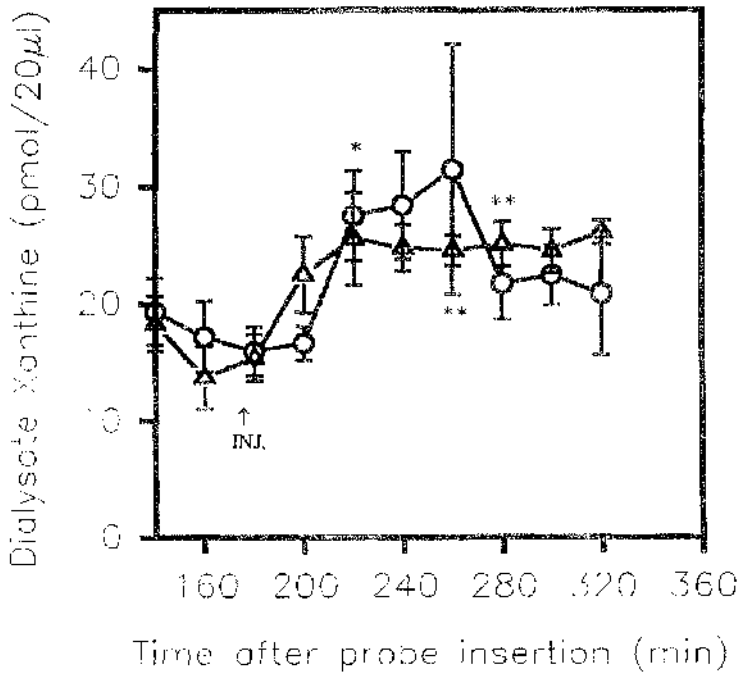



Fig 3.59

The release profiles for xanthine evoked by s.c. injection of:

▲ kainic acid 10mg/kg (n=6). The level of xanthine reached significance 1hr 20 min after injection (*p<0.05, **p<0.01, paired t-test) and did not return to basal level within the time-scale of the experiment.

○ kainic acid 10mg/kg & ascorbic acid 50mg/kg (n=6). The level of xanthine reached significance 1hr after the injection (*p<0.05, paired t-test) and approached basal level 2hrs after the injections.

3.10 Intrahippocampal kainate-evoked release of glutamate and aspartate

Dialysate glutamate and aspartate levels after a 5 min pulse of 1mM kainate were significantly increased above basal and are expressed as a percentage of basal (fig 3.60). The basal levels of glutamate and aspartate were 11.327 ± 2.16 (pmol/20µl) and 4.00 ± 0.89 (pmol/20µl) respectively. Kainic acid caused a bell-shaped release profile of both excitatory amino acids, peaking within one hour of incorporation of kainate into the perfusate. Glutamate and aspartate reached significance within the first collection during which the kainate would have been exposed to the tissue for 4 min (taking into account the 16 min lag-time). The significant elevation was retained for glutamate throughout the profile, even as the level approached basal, 100min after the kainate pulse. The peak glutamate value were 165.61 ± 26.73% of basal (*$p<0.05$) and 161.21 ± 17.55% of basal (***$p<0.005$) during samples collected at 220min and 240min respectively (fig 3.60). The peak aspartate values were 172.75 ± 25.02% of basal (*$p<0.05$) and 185.69 ± 57.25% of basal (n.s.) during samples collected at 200min and 220min respectively (fig 3.60).

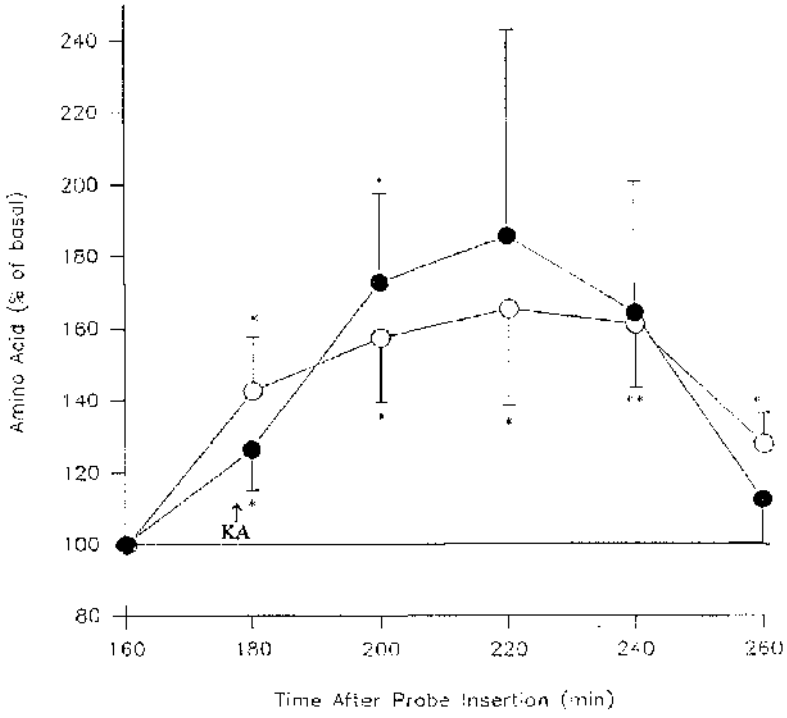



Fig 3.60

The release profiles for glutamate (open circles ) and aspartate (filled circles) evoked by a 5 min pulse of kainate 1mM at the beginning of the sample collected at 180min after probe insertion (shown by the arrow) (n=18). Glutamate and aspartate levels, expressed as % of basal, peaked during the third sample following stimulation. Paired t-test was used (*$p<0.05$, **$p<0.005$). The basal level is indicated by the horizontal line.

3.11 Histology of the hippocampus after microdialysis with and without intrahippocampal kainate exposure

The hippocampi of two rats was examined histologically seven days after exposure to intrahippocampal kainate (1mM). There was gliosis and distinct disruption of the hippocampal cells in the CA3 region of the hippocampus in both rats at the location of the probe through which the kainate was perfused (the result of one of the two rats is shown in fig 3.61, right hand side). Less gliosis and less distinct cell loss was observed as the distance from the location of the probe increased up to 1.25mm anterior. The CA1 layer in the kainate-exposed side showed a slightly thinner cell layer than the contralateral control even in the vicinity of the probe (fig 3.61).

Insertion of the dialysis probe (fig 3.61, left hand-side) alone without drug perfusion caused a small break in the CA1 cell layer and slight gliosis which appeared to be restricted to within 0.35mm of the probe tract.

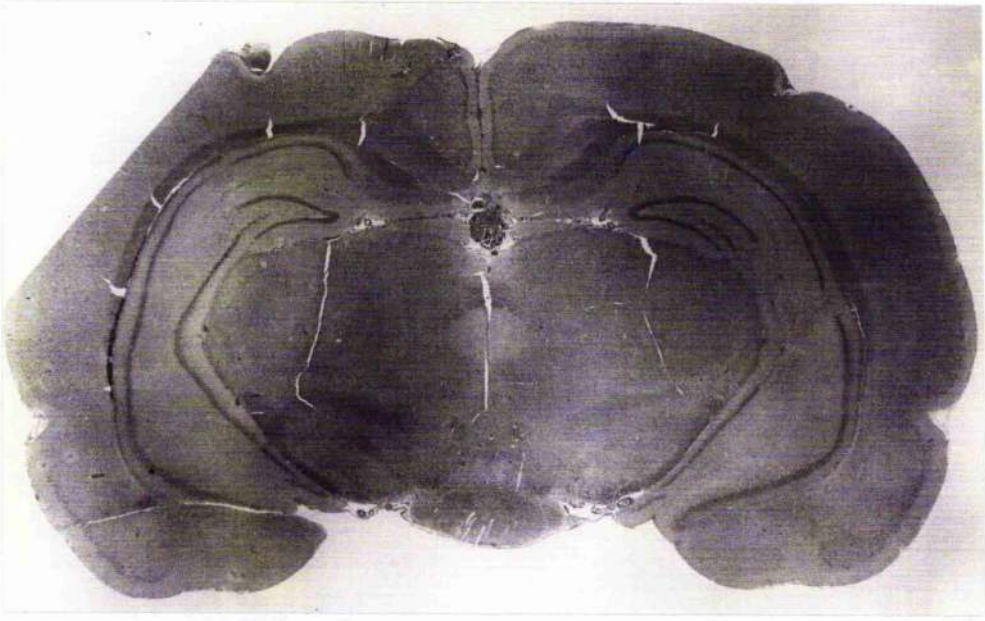


Fig 3.61

Histological examination of the hippocampus seven days after probe insertion and application of a 5 min pulse of kainate 1mM (to right hand side only).

3.12 Assessment of [$^3$H] kainate efflux from the microdialysis probe

The percentage efflux of kainate from the probe to the tissue was examined as well as the extent of its diffusion through the hippocampus. At the plane of the probe position (AP 5.6mm) approximately 65-70% of the kainate was located in the hippocampus (fig 3.62). The rest of the tritium appeared at this plane to diffuse to the cortex. Slices that were more anterior or posterior relative to the probe position, showed approximately 55% of the [$^3$H] kainate in the hippocampus and approximately 45% in the cortical areas.

Using the standard concentration curve (fig 2.8) the amount of radioactivity was calculated in each slice (fig 3.63). From the amount of radioactivity perfused through the probe, the percentage of radioactivity which crossed the probe membrane can be calculated. The amount of radioactivity perfused through the probe was approximately 1μCi. Approximately 598nCi reached the tissue therefore about 60% is crossing the probe membrane.

Estimation of administered amounts of kainate

In order to obtain an estimation of the amount of kainate that had entered the hippocampus during the perfusion of kainic acid, the following calculation was performed. The total amount of kainic acid reaching the via the dialysis membrane at a flow rate of 2μl/min over 5 min, at a concentration of 1000μM, is 6.0nmol ($2 \times 5 \times 1000 \times 0.60$). The last figure 0.60 refers to the assumption that 60% of the kainate present in the probe enters the tissue. The figure is the estimated value of the percentage of radioactive kainate which crossed the probe.

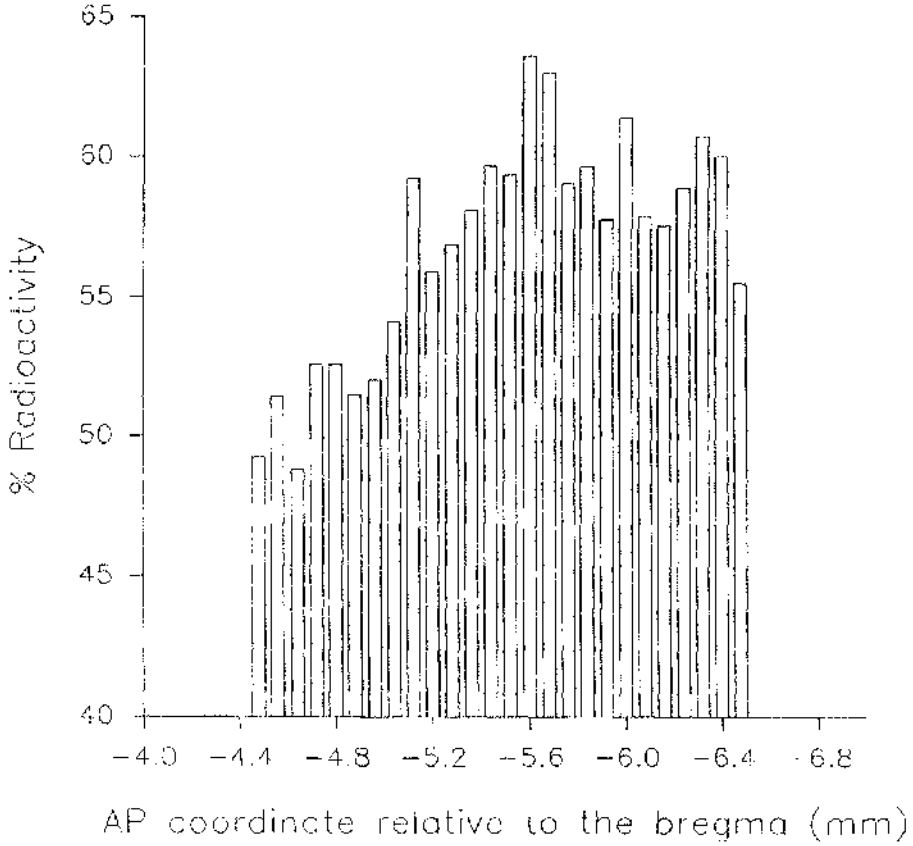



Fig 3.62

The percentage radioactivity ([$^3$H]kainate) found within the hippocampus of each coronal brain section (20μm). The anteriorposterior (AP) coordinates were estimated using Paxinos & Watson (1986).

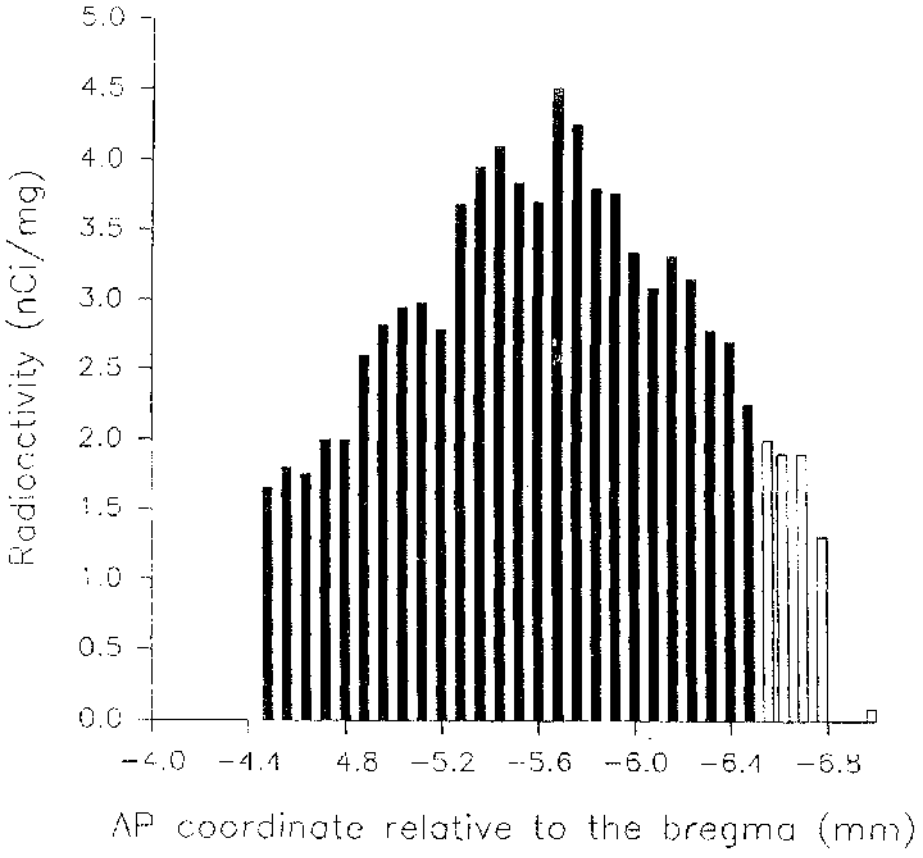



Fig 3.63

The amount of radioactivity ([$^3$H]kainate) found within (solid bar) and outwith (open bar) the hippocampus of each coronal brain section (20μm). The anteriorposterior (AP) coordinates were estimated using Paxinos & Watson (1986).

## 4.0 DISCUSSION

### 4.1 Microdialysis

Microdialysis is a convenient method for monitoring chemical events in the extracellular space within the CNS. The advantages of microdialysis over other *in vivo* techniques such as push-pull cannulae and cortical cups are that all brain regions can be examined and there is minimal tissue trauma (Benveniste, 1989). A semi-permeable membrane allows the diffusion of substances into and out of the microdialysis probe. The molecular cut off of the semi-permeable membrane permits the passage of small molecules including most peptides and drugs but excludes proteins and enzymes. This poses a limitation to the method to the collection of small molecules only, but the advantage is that there is no need for deproteinisation of the dialysate before HPLC analysis and potentially no enzymatic degradation of the compounds in the dialysate (for example, by adenosine deaminase). Microdialysis allows the local application of compounds by incorporating them into the perfusate, a process referred to as reverse dialysis. The main disadvantage is that the percentage of compound which has diffused through the membrane of the probe into the brain, and therefore the amount of compound that reaches the ECF, may not be known with certainty in each case.

Using the relative recovery of each probe, calculated *in vitro*, the interstitial concentration may be estimated from the concentration of a substance in the dialysate. However, the *in vitro* relative recovery does not take into account the mass transport characteristics of a complex medium like the brain. Work carried out by Alexander *et al* (1988) shows that the *in vitro* relative recovery of the probe does not agree with the relative recovery seen in the *in vivo* experiments. They examined the relative recovery *in vivo* by using tritiated water. Tritiated water apparently, at equlibrium, has the same brain extracellular concentration as the plasma concentration (Alexander *et al*, 1988). The *in vivo* relative recovery for tritiated water was calculated by the ratio of the concentration in the brain dialysate to the concentration in the plasma. The authors explain the lack of agreement between the *in vitro* and *in vivo* relative recoveries by portions of the dialysis membrane being blocked by tissue which hinders the diffusion of molecules from the ECF into the probe. In contrast to

the *in vitro* experiments, it is unlikely that the entire surface of the dialysis membrane is in contact with freely diffusible fluid in *in vivo* experiments. As a result, the *in vitro* calibration of the dialysis probe is not a sufficient correction factor for compounds collected *in vivo*. It is for this reason that, in the present study, the compounds are recorded in amounts in pmol in each dialysate. However, the estimated basal interstitial concentration quoted in table 3.1 in the present study is calculated using the *in vitro* relative recovery to allow a comparison with other studies which have done likewise. The basal interstitial concentration of adenosine estimated in the present study in the anaesthetised rat hippocampus is 0.8μM and is consistent with previous findings of around 1μM (Zetterström *et al*, 1982, Chen *et al*, 1992).

Techniques for validating a suitable dialysis probe calibration for *in vivo* use are currently being introduced. The theory that the net increase of a substance in the microdialysate correlates linearly to the concentration of that substance added to the perfusate is called the equilibration calibration (Lönroth *et al*, 1987). The point of no net flux is equal to the interstitial concentration and can be calculated by linear regression.

Immediately after probe insertion into the tissue the efflux of endogenous substances collected in the dialysate was high and declined to a steady level within one hour. This probably represented tissue trauma and is in agreement with Zetterstrom *et al* (1982), Ballerin *et al* (1991), Chen *et al* (1992) and Pazzagli *et al* (1993).

An investigation into the extent of tissue damage caused by probe insertion was carried out in the present study. Two rats were allowed to recover after probes were inserted bilaterally for 3-4hrs under Hypnovel and Hypnorm and halothane, if necessary. Seven days later, the brains were dissected out and examined histologically. The left hand probes were perfused with acsf throughout the experiments and, in the two experiments performed, slight gliosis was seen along the probe tracts. Gliosis around the probe may introduce an extra source of adenosine since pools of adenosine exist in glia. Adenosine released into the ECF from this extra source would cause an overestimation of extracellular adenosine levels which would occur depending on the extent of gliosis. Since

gliosis is evident approximately 24-48hrs after trauma (Jorgensen *et al*, 1993), only experiments over this time-scale would be of concern.

There was a slight break observed in the CA1 cell layer which was presumably where the probe entered the hippocampus.

4.2 Potassium-evoked release of adenosine and its metabolites

The literature strongly suggests that depolarising agents release adenosine. Indeed, potassium, incorporated into the perfusion medium, increased the extracellular concentrations of adenosine, inosine and hypoxanthine in the rat hippocampus *in vivo*, detected by HPLC. The sequential release of purines (adenosine followed by inosine and hypoxanthine) maps the metabolic pathway of adenosine and suggests an important role of adenosine deaminase in the metabolism of adenosine in the CNS. The importance of deamination in regulating extracellular adenosine concentrations has been demonstrated by Pazzagli *et al* (1993) who showed a large increase in adenosine efflux after inhibition of adenosine deaminase in the rat striatum *in vivo*.

The effectiveness of potassium to release adenosine was not altered by CNQX at the same concentration (4.5μM) that abolished 92% of the kainate-evoked adenosine release. However at a 10-fold higher concentration (45μM), CNQX abolished the potassium-evoked release. CNQX was found to inhibit [$^3$H] glycine binding to the glycine binding site of the NMDA receptor (Kessler *et al*, 1989). The concentration required for this response was approximately 7 fold higher ($IC_{50}$ 4.8μM) than that required to inhibit [3H] kainate binding to kainate receptors ($IC_{50}$ 0.71μM). Given that a small percentage of CNQX in the perfusate will reach the tissue from the probe (maybe approximately 10%), in the present study at a concentration of 4.5μM in the perfusate, CNQX is likely to act on kainate/AMPA receptors and, at 45μM, CNQX is likely to act on NMDA receptors. NMDA receptor antagonists have been shown to reduce potassium-evoked release of adenosine by *in vivo* microdialysis by 80% from the rat hippocampus (Chen *et al* (1992), by 52% from the striatum of young rats (Pazzagli *et al*, 1995), by 85% from the striatum of the adult rat (Pazzagli *et al*, 1993) and to a lesser extent (by 30%) *in vitro* from rat cortical slices (Hoehn & White, 1990a). Similar competitive NMDA receptor antagonists

(AP-5 or D-AP7) were used in these studies at similar concentrations to the present study (1mM). The slight variation in the percentage decreased amongst the studies may be due to the variation in preparation. The *in vivo* studies appear to be more affected by the NMDA antagonists than the *in vitro* study by Hoehn & White (1990a) and this may be explained by a higher level of release of excitatory amino acids by potassium *in vivo* than *in vitro*, possibly a result of different extents of calcium-dependent and -independent release of excitatory amino acids.

The surprising, yet substantial, evidence for NMDA receptor-mediated potassium-evoked release of adenosine may be explained by the prior release of excitatory amino acids. This hypothesis is feasible since Burke & Nadler (1988) have shown that potassium depolarisation can induce excitatory amino acid release from hippocampal slices and in turn it has been shown that excitatory amino acids increase adenosine release from the cortex (Hoehn & White, 1990a) and from the hippocampus (Pedata *et al*, 1991; Chen *et al*, 1992).

The lack of the ability of non-NMDA antagonists to affect potassium-evoked adenosine release (shown by 4.5μM CNQX in the present study) is supported by the unaffected potassium-evoked release of adenosine by DNQX in rat cortical slices (Hoehn & White, 1990a). Explanations for this result include the release of excitatory amino acids in closer vicinity of NMDA receptors than non-NMDA receptors. Alternatively, excitatory amino acids released by potassium may be ones which have higher affinity at the NMDA receptor than at the non-NMDA receptors, such as homocysteate (Do *et al*, 1986), N-acetylaspartylglutamate (Zollinger *et al*, 1988) or quinolinate (Stone & Connick, 1985). The final explanation is that activation of non-NMDA receptors does not mediate potassium-evoked adenosine release although the present study has clearly indicated the ability of non-NMDA receptor stimulation to release adenosine.

Twenty-eight percent of the potassium-evoked release of adenosine was not reduced by CNQX 45μM, implying other mechanism(s) may be involved. An increase in intracellular metabolic activity as a result of cell depolarisation may lead to intracellular adenosine accumulation and its subsequent efflux. Alternatively, depolarisation-induced calcium-

dependent vesicular release of ATP may occur and be followed by its extracellular degradation to adenosine.

4.3 Systemic kainate-evoked adenosine release

MacGregor *et al* (1993) showed that systemically administered kainate induced neurotoxicity which was prevented by purines. The present study was therefore undertaken to determine whether systemic kainate, at a dose that causes neuronal damage, induces purine release and whether this purine release is to the extent required to exert neuroprotection.

At the same dose that produced neurotoxicity, systemic kainate (10mg/kg, injected both i.p. and s.c.) produced a significant increase in release of adenosine. The release of adenosine occurred within the first two hours after administration of systemic kainate and reached a maximum of 1.4μM (i.p.) and 2μM (s.c.) (concentration in ECF, estimated by using the relative recovery of the probe for adenosine). The next question to be answered is whether adenosine release evoked by kainate is sufficient to exert neuroprotection against kainate-induced damage. This answer will be approached in section 4.8.

Within the first two hours after systemic administration, kainate has been shown to cause behavioural effects characterised by wet dog shakes and salivation (Worms *et al*, 1981; MacGregor, 1995). The systemically-administered serotonin precursor, 5-HTP (5-hydroxytryptophan), has been shown to cause wet dog shakes and kainate-induced wet dog shakes have been antagonised by serotonin blockers (Worms *et al*, 1981). Worms *et al* (1981) suggested that kainate induces wet dog shakes via an increased serotonergic function. The relevance of this point to the present work is that an increased extracellular adenosine and an increased serotonergic function may occur simultaneously within the first two hours after kainate administration. Whether kainate increases synaptic serotonin release or increases serotonin receptor sensitivity has still to be established. The ability of photochemical-induced ischaemia in the rat to increase cortical serotonin release has been shown by Baldwin *et al* (1993). However these authors have not reported any behavioural effects. The role of the increased serotonergic activity in brain damage is eliminated by

Baldwin *et al* (1993) who showed a lack of alteration of lesion severity after application of serotonergic agonists and antagonists.

Endogenous excitatory amino acids have been implicated in early onset neurotoxicity mediated by kainate. Kainate causes an increase in extracellular glutamate after local administration in the hippocampus. Excitatory amino acids are therefore probably released within the first two hours after administration of systemic kainate. Given the evidence of the ability of glutamate to evoke adenosine release in rat cortical slices (Hoehn & White, 1990a), a prior release of glutamate induced by kainate may cause adenosine release seen within the first two hours of administration of systemic kainate. The role of glutamate release in adenosine release is further investigated in sections 4.8-4.10.

4.4 Release of adenosine by intrahippocampal kainate

The local application of kainate to the hippocampus was used to examine the mechanisms involved in kainate-evoked release of adenosine.

The release of adenosine and its metabolites was evoked by intrahippocampal kainate. There was approximately a 2-fold increase in release of adenosine, inosine and xanthine evoked by kainate. The increase in hypoxanthine release evoked by kainate was approximately 6-fold. A higher increase in hypoxanthine relative to the increase in adenosine caused by hypoglycaemia in the rat striatum has been reported by Butcher *et al* (1987). The reason for this is unclear but may be explained by the conversion of hypoxanthine to xanthine being limited by the availability of xanthine oxidase in the CNS, since the enzyme is mostly in the form of xanthine dehydrogenase *in vivo* (Nishino, 1994), allowing the accumulation of hypoxanthine in the ECF. Elevated levels of hypoxanthine in the presence of xanthine oxidase may be detrimental to surrounding cells since its conversion to xanthine involves the production of superoxide ions (toxic free radicals).

The existence of xanthine oxidase in the rat brain is somewhat controversial. The present study may be considered to provide evidence for the existence (at least to some extent) of cerebral xanthine oxidase or, alternatively, raises the possibility of hypoxanthine and xanthine being released independently of adenosine.

4.5 Twin pulses of intrahippocampal kainate-evoked release of adenosine and comparison with that of potassium

A second period of stimulation by kainate or potassium was able to evoke a significant increase in the release of adenosine although this was substantially smaller than the first. Similar results were obtained for two pulses of endogenous adenosine release evoked by potassium and glutamate in rat cortical slices (Hoehn & White, 1990a) and evoked by potassium and NMDA in the hippocampus of the anaesthetised rat (Chen *et al*, 1992). The diminished response to the second stimulus may be explained by the depletion of releasable adenosine by the first stimulation.

Depletion of adenosine pools may occur by two means. Adenosine, released into the ECF, is taken back up into the cell where it is exposed to enzymatic degradation to hypoxanthine. The conversion of hypoxanthine to IMP by the enzyme, hypoxanthine-guanine phosphoribosyltransferase is the so called 'salvage' reaction (Stone & Simmonds, 1991). The enzyme competing with hypoxanthine-guanine phosphoribosyltransferase for hypoxanthine is xanthine oxidase. Under conditions of elevated intracellular calcium levels, xanthine dehydrogenase is converted to xanthine oxidase. Xanthine oxidase may therefore increase as a result of elevated calcium levels which may occur during a pulse of kainic acid or potassium. If so, hypoxanthine may be converted to xanthine during the first pulse instead of being salvaged to adenosine and therefore pools of adenosine may be depleted for the second pulse. Secondly, depletion of adenosine pools may also come about due to cell death after the first pulse. Cells which are a source of adenosine release may die during the first pulse of kainate or potassium and therefore may not be a source of adenosine release during the second pulse. Evidence of this, in the case of potassium-induced release of adenosine from rat cortical slices, was illustrated by Hoehn & White (1990a) who diminished the effects of the first pulse by using calcium-free medium. They found that a second pulse of potassium in calcium-containing medium was comparable to that elicited by a first pulse in the presence of calcium. The lack of calcium may prevent damage induced by potassium and thus retain releasable pools of adenosine. Another interpretation of this result is that prior calcium-dependent release of ATP or glutamate, which lead to

adenosine release, is prevented by lack of calcium. In either case, the depletion of adenosine is prevented, allowing a larger release during the second pulse (Hoehn & White, 1990a).

In the present study, the S2/S1 ratio for adenosine release produced by potassium is significantly higher than that produced in the presence of kainate. Again a similar pattern was seen in rat cortical slices by Hoehn & White (1990a) when comparing the ratio of adenosine release induced by two pulses of potassium with that of glutamate. This may be explained by several means.

Firstly, there may be a higher intracellular calcium level caused by the first pulse of kainic acid or glutamate compared with the first pulse of potassium. If the levels of xanthine oxidase are higher after kainic acid or glutamate than potassium, less adenosine may be salvaged, as more hypoxanthine is converted to xanthine, and therefore pools of adenosine are more depleted after the first pulse of kainic acid or glutamate than after the first pulse of potassium. Secondly, after the first pulse of kainic acid or glutamate there may be more cell death than after the first pulse of potassium, leading to a more diminished source of adenosine release for the second pulse of kainic acid or glutamate than the second pulse of potassium. Thirdly, there may be desensitisation of kainate or glutamate receptors during the second pulse as a result of stimulation of receptors during the first pulse of kainate or glutamate. Kiskin *et al* (1986), Hori & Carpenter (1988) and Mayer (1989) have shown that kainate receptors do not desensitise although NMDA receptors desensitise rapidly (Krishtal *et al*, 1988). Desensitisation of receptors may therefore be a feasible explanation for the lower efficacy of the second pulse in the case of the application of glutamate (Hoehn & White, 1990a) but only in the case of the application of kainate (the present study) if NMDA receptor activation is involved in kainate-evoked release of adenosine.

The S2/S1 ratios of the metabolites were not calculated since, in most cases, the experiments were terminated before the metabolite levels returned to basal after the second pulse. On examining the graphs displaying the twin pulses of potassium or kainate without the incorporation of any drugs before, during and after the second pulse, it can be seen that the response of inosine, hypoxanthine and xanthine to the second pulse is not diminished to

the same extent as that of adenosine. A similar trend was observed by Chen *et al* (1992) and this may be a reflection of an increase in the deamination of adenosine during the second pulse of kainate or potassium. Adenosine kinase has a higher affinity for adenosine than adenosine deaminase (Sciotti & Van Wylen, 1993b). However, elevated adenosine levels result in substrate inhibition of adenosine kinase, allowing promotion of deamination of the accumulated adenosine (Nagy *et al*, 1990). Thus, the elevated adenosine levels in the first pulse may result in substrate inhibition of adenosine kinase and therefore promote deamination during the second pulse. The proportion of the amount of adenosine being deaminated to the amount of adenosine being phosphorylated may therefore increase in the second pulse compared to the first pulse. Such a scenario would lead to a higher than expected response of the deaminated metabolites of adenosine during the second pulse.

4.6 The effect of TTX on intrahippocampal kainate-evoked release of adenosine

Intrahippocampal kainate-evoked release of adenosine was reduced by 60% by the application of TTX via the dialysis probe. This indicates that part of the release of adenosine depends on neuronal depolarisation and firing brought about by ionic fluxes through voltage-sensitive sodium channels blocked by TTX.

*In vitro* studies show that propagated action potentials contribute only slightly to adenosine release. Endogenous adenosine release from rat cortical slices, where glutamate-evoked release was shown to involve non-NMDA receptors, was reduced by 21% by TTX (Hoehn & White, 1990a) and where NMDA was used to evoke release, was reduced by 35% by TTX (Hoehn *et al*, 1990). TTX-insensitive release of endogenous adenosine has been shown in cortical synaptosomes when induced by glutamate (Hoehn & White, 1990c) and high doses of TTX were required to reduce tritiated adenosine release from cerebral cortex or cerebellum induced by glutamate *in vivo* (Jhamandas & Dumbrille, 1980)

These studies indicate that propagated action potentials are not primarily involved in adenosine release, though in the present study over half of the release showed the involvement of propagated action potentials. The extent of the involvement of propagated action potentials on electrically-evoked and potassium-evoked release of adenosine is also controversial. Electrically-evoked release of tritiated adenosine was blocked by 30-50% by

TTX *in vitro* from guinea-pig taenia coli (Rutherford & Burnstock, 1978) and guinea-pig neocortical tissues (Pull & McIlwain, 1973) and was completely blocked from rat cortical slices (Pedata *et al*, 1988). One reason for the variation in results is the variation in intensity of the electrical stimulation. Pedata *et al* (1988) showed that when the intensity of stimulation was increased, the TTX-sensitive component of adenosine release was reduced. A possible consequence of high intensity stimulation may be the release of adenosine from glial cells as well as from neurones.

Differences between these results may be explained by the differences in the depolarising stimuli used. For example, the application of glutamate seems to cause the most amount of TTX-insensitive release. This may be because of the reported TTX-insensitive release of adenosine which occurs as glutamate is taken back up into the neurone (Hoehn & White, 1990c). This would only occur in the presence of glutamate as NMDA, kainate and potassium do not use the high affinity glutamate uptake transport.

The preparations used may also contribute to the variation in the results. For example, the use of synaptosomes will produce different results from that of intact neurones if the TTX-sensitive site of neuronal release is postsynaptic, or a large proportion of release occurs from glial cells.

In the present study, the application of TTX has allowed us to define a contribution of adenosine release induced by kainate as partly neuronal. However, this data provides no information about the neuronal source (i.e. postsynaptic or presynaptic) of this contribution. The TTX-insensitive adenosine release may indicate stimulation of glial cells or the release of adenosine from neurones without the requirement of action potentials.

4.7 Characterisation of receptors involved in intrahippocampal kainate-evoked release of adenosine

Excitatory amino acids are released during hypoxia/ischaemia (Benveniste *et al*, 1984; Globus *et al*, 1988; Phillis *et al*, 1991) and epilepsy (Choi, 1988(b)) and hypoglycaemia (Sandberg *et al*, 1986; Choi, 1988(b)). Activation of the three major sub-types of excitatory amino acid receptors is implicated in neuronal death. Activation of NMDA receptors and non-NMDA receptors leads to an influx of calcium through receptor-

operated calcium channels and voltage-operated calcium channels (Lin *et al* 1990; Ohta *et al* 1991; Uematsu *et al* 1991; McBurney *et al* 1992). Intracellular calcium leads to activation of protein kinases, phospholipases, proteases, nitric oxide synthase, impaired mitochrondrial function and the generation of free radicals (Beal, 1992). Uncontrolled activation of one or more of these processes may be potentially lethal to the cell. Calcium-dependent and/ or calcium-independent release of glutamate leads to further activation of NMDA and non-NMDA receptors and the resulting excessive depolarisation of the cell membrane may lead to loss of ionic homeostasis, cell swelling and cell lysis. Indeed, protection against ischaemic-induced cell death has been shown by NMDA receptor antagonists (Bullock *et al*, 1990; Uematsu *et al*, 1991) and non-NMDA receptor antagonists (Le Peillet *et al*, 1992; May & Robison, 1993).

The release of the inhibitory neuromodulator, adenosine, by activation of the three major sub-types of excitatory amino acid receptors is of interest due to its potential to diminish further damage. Adenosine's potential as a neuroprotective agent is revealed by its ability to inhibit presynaptic release of glutamate, cause hyperpolarisation of postsynaptic membranes and prevent loss of ionic homeostasis.

Previous *in vivo* experiments on the hippocampus of the anaesthetised rat have shown that NMDA receptor activation induces adenosine release (Chen *et al*, 1992). In *in vitro* studies on cortical slices, Hoehn & White (1990b) have demonstrated that activation of NMDA and non-NMDA receptors releases endogenous adenosine.

The concentration of CNQX used in the present study (4.5μM) correlates well with the $IC_{50}$ (0.71μM) used to inhibit [3H]kainate binding to the kainate receptor (Kessler *et al*, 1989), given the small percentage which is likely to pass through the semi-permeable membrane of the probe.

In the present study CNQX (a competitive non-NMDA receptor antagonist) abolished kainate-induced adenosine release. This correlates with the inhibition of kainate-evoked release of adenosine from cortical slices by DNQX (Hoehn & White, 1990b) and indicates that kainate-evoked release is mediated by activation of non-NMDA receptors.

Neither MK-801 (dizocilpine), a non-competitive NMDA receptor antagonist, nor ±AP-5, a competitive NMDA receptor antagonist, significantly altered kainate-evoked adenosine release. This is consistent with the lack of inhibition of kainate-evoked release of adenosine from cortical slices by MK-801 (Hoehn & White, 1990b) and indicates that kainate-evoked release is not mediated by activation of NMDA receptors. In contrast, Perkins & Stone (1983) found that the NMDA receptor antagonist, AP-7, blocked 23% of the kainate-evoked release of tritiated purines from rat cortex *in vivo*. The discrepancy between the latter study and the present study or the study performed by Hoehn & White (1990b) may represent differences between the release of endogenous adenosine versus radiolabelled purines. A difference between the release of endogenous amino acids versus radiolabelled amino acids has been shown by Ferkany & Coyle (1983). In hippocampal slices using kainic acid, Ferkany & Coyle (1983) showed calcium-dependent endogenous glutamate and aspartate release, but no [$^{3}$H]-aspartate or [$^{14}$C]-glutamate release. In the radiolabelling technique, the tissue preparation is exposed to the radiolabelled substance and, following a stimulus, the release of the accumulated radioactivity is monitored. The assumption is that the radiolabelled substance is selectively accumulated within the pools of interest and that it mixes with the relevant pool of endogenous substance. An example of a possible flaw in the method of radiolabelled release is that the exogenous pool is different from the endogenous pool.

GYKI 52466 is a 2,3-benzodiazepine which appears to act at an allosteric site on non-NMDA receptors. GYKI 52466 has been shown to act non-competitively to block ion currents mediated through non-NMDA receptors (Zorumski *et al*, 1993). The same authors show that GYKI 52466 has a lower potency for non-NMDA receptors than CNQX. GYKI 52466 and CNQX inhibited neuronal currents in hippocampal cultures gated by 1mM kainic acid with respective EC50 values of 14μM and 1.7μM (Zorumski *et al*, 1993). Electrophysiological evidence indicates that GYKI 52466 is more specific for AMPA/ kainate receptors and has little effect at the high affinity kainate or NMDA receptors (Paternain *et al*, 1995).

GYKI 52466 in solution has a pH of 3.5 and caused a high efflux of adenosine (fig 3.15). To investigate whether this was due to the presence of a low pH, one experiment was performed perfusing acsf, pH 3.5, through the probe two hours before, during and after the second pulse (fig 3.15). As in the experiments where GYKI 52466 was present there was a high efflux with a similar profile. It is therefore possible that a low pH is causing acidosis. An increased hydrogen ion concentration may activate the Na+/H+ transport process, thereby shuttling Na+ ions into the cells causing cell membrane depolarisation and depolarisation-induced adenosine release. Acidosis is also known to retard phosphorylation of ADP to ATP, allowing more ADP available for dephosphorylation to adenosine.

GYKI 52466 came out of aqueous solution when the pH was adjusted to 7.2. GYKI 52466 was therefore dissolved in DMSO 20% and the pH was adjusted to 7.2 for administration intrahippocampally. For systemic injection, GYKI 52466 was suspended in Tween 80 2% and the pH adjusted to 6-7. GYKI 52466 did not reduce the kainate-evoked release of adenosine by either of the two methods of administration when compared to the respective controls.

DMSO was seen to cause adenosine release in its own right (see below) and it is conceivable that under such a compromised condition, the pharmacological effects of GYKI 52466 do not become apparent. Donevan & Rogawski (1993) showed that 100μM GYKI 52466 blocked kainate-activated currents (150μM) in cultured rat hippocampal neurones. In the present study, only a proportion of the GYKI 52466 in the probe will reach the tissue. A concentration of 1mM GYKI 52466 used in the present study, should be sufficient to block non-NMDA receptors and the lack of effect of GYKI 52466 was probably not due to an inadequate concentration.

DMSO has been reported to cause ATP release which is partly calcium-dependent from dorsal spinal cord synaptosomes (Sawynok *et al*, 1993). In an ovarian cancer cell line DMSO acted as a differentiation-inducing agent and caused an increase in the activity of IMP-5'-nucleotidase, although this was 72 hours following exposure (Zoref-Shani *et al*, 1994). Another reported property of DMSO is as a free radical scavenger (Mannion *et al*, 1994). The most likely mechanism of adenosine release by DMSO is the reported increase

in ATP release. By examining the release of adenosine during the two hours perfusion of DMSO and GYKI 52466 in DMSO, it can be seen that there is a significant reduction in release at the last time point before the second pulse of kainate. The rise in extracellular adenosine by DMSO may therefore be partly mediated by the activation of non-NMDA receptors, since GYKI 52466 reduced the release at that time point.

The action of GYKI 52466 on the CNS is very transient, with a maximum effect in the brain reached within 10 min after intravenous administration (Smith *et al*, 1991). The injections of GYKI 52466 in the present study were 15 min before, at the same time and 15 min after the perfusion medium was changed to kainate for the second pulse. Taking the lag-time (16 min) for kainate from the switch to the probe into account, and assuming that the pharmacokinetic profile of GYKI 52466 is the same for s.c. administration as for i.v. (intravenous) injection, the maximum effect of GYKI 52466 should be exerted 21 min and 6 min before and 9 min after the exposure of the tissue to kainate. Thus GYKI 52466 should be exerting its maximum antagonistic effect before and during the peak amount of adenosine is being released. Since the action of GYKI 52466 is so transient, the timing of GYKI 52466 reaching the tissue relative to kainate would therefore be important to its pharmacological effects and slight variations in absorption through the BBB of GYKI 52466 may account for the scatter of data seen in table 3.3. A continuous systemic infusion of GYKI 52466 before, during and after the second kainate pulse would be an appropriate experiment to perform.

Arvin *et al* (1994) have shown that with i.v. infusion of GYKI 52466, the ischaemic-induced release of glutamate is not prevented in the hippocampus but is in the striatum. They postulate that the property of GYKI 52466 to inhibit non-NMDA receptors is likely to be similar in both the hippocampus and striatum and explain the results as a consequence of structural vulnerability to ischaemia. They postulate that the lack of effect of GYKI 52466 is because the release of glutamate in the hippocampus may be related to increased neuronal or metabolic activity and not specifically from presynaptic terminals. Indeed, this may be the case for adenosine in the present study.

4.8 The effect of purines on kainate-evoked adenosine release

Adenosine has been shown to inhibit the presynaptic release of excitatory neurotransmitters such as acetylcholine (Spignoli *et al,* 1984; Cunha *et al,* 1994), glutamate (Corradetti *et al,* 1984; Fastbom & Fredholm, 1985; Cantor *et al,* 1992; Dunwiddie & Fredholm, 1989) and dopamine (Zetterström & Fillenz, 1990).

The adenosine analogue 2-chloroadenosine prevented striatal damage induced by NMDA, kainate and quisqualate (Finn *et al,* 1991) and hippocampal damage induced by ischaemia (Evans *et al,* 1987). The adenosine analogue, R-PIA, prevented the hippocampal damage induced by kainate (MacGregor *et al,* 1993) and quinolinic acid (Connick & Stone, 1989). The adenosine deaminase inhibitor deoxycoformycin, which has been shown to increase extracellular adenosine levels (Phillis *et al,* 1991), prevented *in vivo* hippocampal ischaemic damage (Phillis & O'Regan, 1989).

Kainate has been reported to evoke the release of glutamate *in vivo* from the hippocampus (Lehmann *et al,* 1983) and striatum (Butcher *et al,* 1987) and *in vitro* from the hippocampus and cerebellum (Ferkany *et al,* 1982; Ferkany & Coyle, 1983). Kainate has been reported to evoke the release of aspartate from the striatum *in vivo* (Butcher *et al,* 1987) and *in vitro* (Notman *et al,* 1984) and from the hippocampus and cerebellum *in vitro* (Ferkany *et al,* 1982; Ferkany & Coyle, 1983). The elevated levels of extracellular excitatory amino acids have been implicated in neurotoxicity. Inhibition of synaptic transmission by adenosine, which is reported to be via action at the A1 receptor (Burke & Nadler, 1988), presumably contributes to the reported neuroprotective properties of purines.

In the present study kainate induced elevations in extracellular glutamate and aspartate over 1hr 20min after application of kainate. This is the time span of kainate-evoked adenosine release, thus highlighting the possibility of secondary transmitters such as glutamate and aspartate being involved in kainate-evoked adenosine release. In the present study, an investigation of the contribution of glutamate release to the mechanisms of kainate-evoked release of adenosine was carried out.

R-PIA incorporated into the perfusion medium (intrahippocampal) did not modulate adenosine release evoked by intrahippocampal kainate. The concentration of

intrahippocampal R-PIA correlates well with that used in other studies. Fastbom & Fredholm (1985) showed that 1-10μM PIA blocked glutamate release and Duner-Engstrom & Fredholm (1988) used 1μM to block acetylcholine release. In the present study, only a proportion of the R-PIA in the probe will reach the tissue. A concentration of 10μM or 100μM R-PIA used in the present study, should be sufficient to allow enough R-PIA to reach the tissue and exert its pharmacological action. The lack of effect of R-PIA was probably not due to an inadequate concentration.

Heron *et al* (1992) have shown that R-PIA applied systemically, but not locally, reduced glutamate release during ischaemia. For this reason, R-PIA was administered systemically in the present study, and its effect on adenosine release evoked by intrahippocampal kainate was examined. The systemic dose of R-PIA (250μg/kg) in vehicle (methanol) was the same dose that caused neuroprotection against kainate-induced damage (MacGregor *et al*, 1993). R-PIA administered systemically did not affect intrahippocampal kainate-evoked adenosine release.

MacGregor *et al* (1993) have shown that systemic R-PIA in vehicle (methanol) prevented neurotoxicity caused by systemic kainate in methanol. In the present study, the effect of systemic R-PIA in vehicle (methanol) was examined on adenosine release evoked by systemic kainate in methanol. Methanol reduced kainate-evoked release of adenosine although MacGregor *et al* (1993) showed that neurotoxicity still occurred when kainate was administered with methanol. This may indicate that the mechanisms involved in kainate-induced damage do not bring about kainate-evoked release of adenosine.

Since adenosine is known to be released by increased neuronal activity and since adenosine is known to inhibit neuronal activity, it may be expected that adenosine would inhibit its own evoked release. However, the present study shows that adenosine does not inhibit its own release in the presence of an agent that causes neurotoxicity. Since adenosine prevents neurotoxicity, the finding that adenosine does not inhibit its own release may be advantageous to its neuroprotective properties.

A lack of effect of adenosine has been reported on the Ca2+-independent release of GABA from rat cerebral cortex slices (Hollins & Stone, 1980b). In addition, 2-chloroadenosine

has been shown to block depolarisation-evoked glutamate release from rat hippocampal mossy fibre synaptosomes only after the Ca2+-independent, non-vesicular, cytosolic pools of glutamic acid had been depleted by D-aspartate (Terrian *et al*, 1989). Similarly, in the present study, the Ca2+-independent component of glutamic acid release may be insensitive to R-PIA and may have masked any inhibitory actions of R-PIA on the Ca2+-dependent component. Therefore the possibility that prior release of glutamate by a calcium-independent mechanism contributes to the release of adenosine induced by kainate can not be eliminated.

At postsynaptic sites, A1 receptor activation hyperpolarises the cell membrane leading to magnesium block of the NMDA receptor. The lack of efficacy of R-PIA in reducing kainate-evoked release of adenosine is consistent with the previous conclusion that kainate-evoked release is not mediated by NMDA receptor activation.

R-PIA is a lipophilic compound and the possibility of it either binding to the probe membrane or to cell membranes close to the probe instead of diffusing to the site(s) of adenosine release cannot be excluded. The use of another adenosine agonist, 2-chloroadenosine, which attenuates kainate-induced neurotoxicity in the rat striatum (Arvin *et al*, 1989; Finn *et al*, 1991), was complicated in the present study by its absorbance in u.v. light and no results were therefore obtained (see methods).

Another possibility for the lack of efficacy of R-PIA may be that adenosine was already activating all available A1 receptors, so that the addition of an adenosine agonist had no additional effect. To investigate this, adenosine antagonists were incorporated into the perfusing medium two hours before, during and after the second pulse of kainate.

Kainic acid has been shown to cause glutamate release and glutamate has been shown to cause adenosine release. If kainate-evoked release of adenosine was mediated by the prior release of glutamate and if the endogenous adenosine released by kainate is high enough to inhibit glutamate release then the incorporation of adenosine antagonists, CPT or 8-PST, should increase kainate-evoked release of adenosine. Neither CPT nor 8-PST increased kainate-evoked release of adenosine. The concentrations of CPT used in the present study were 10μM and 100μM. The apparent Ki of CPT for the antagonism of adenosine from

human fibroblasts was found to be 0.71μM (Bruns, 1981). Allowing for the diffusion of CPT through the dialysis membrane, the concentration used in the present study should be sufficient to antagonise adenosine. Likewise, the concentration of 8-PST (1mM) used in the present study should be sufficient to antagonise adenosine since 8-PST was shown to displace [$^3$H]cyclohexyladenosine binding from guinea pig brains with an IC50 of 0.02mM (Bruns *et al*, 1980).

The present results indicate that adenosine does not modulate its own release and does not limit the depolarising activity of kainate. This implies that either that endogenous adenosine is not elevated by kainate high enough to inhibit glutamate release or that kainate-evoked release of adenosine is not mediated by the prior release of glutamate.

Whether 1.4-2 μM endogenous adenosine released by administration of systemic kainate is sufficient to limit kainate-induced neurotoxicity is another question to be answered. Adenosine has been shown to exert inhibitory effects at a concentration of 1μM-20μM on hippocampal slices (Hosseinzadeh, 1994). During hypoxia and ischaemia, the release of adenosine by an *in vivo* microdialysis study of the striatum was shown to be 40μM (Hagberg *et al*, 1987). This is 20-fold higher than the estimated concentration in the present study of kainate exposure in the hippocampus. Evidently the release of adenosine induced by kainate is low relative to the release of adenosine induced by ischaemia.

Blockage of A1 receptors does not potentiate kainate neurotoxicity (MacGregor & Stone, 1994). Thus the adenosine released by kainate at a dose which caused neurotoxicity probably does not exert significant protection against neuronal damage. This may be because adenosine release by kainate is probably not high enough. However, in ischaemia *in vivo*, adenosine A1 agonists failed to suppress the induced release of glutamate (Phillis *et al*, 1993, Héron *et al*, 1993). The explanation for this difference from *in vitro* studies which have clearly shown A1 receptors to inhibit glutamate release (Corradetti *et al*, 1984, Fastbom & Fredholm, 1985, Poli *et al*, 1991) including that induced by kainate (Arvin *et al*, 1989; Finn *et al*, 1991, Poli *et al*, 1991) may lie at least partly in the contribution of calcium-independent glutamate release during ischaemia (Szatkowski *et al*, 1990).

4.9 The effects of kappa agonists on intrahippocampal kainate-evoked release of adenosine

The hippocampus is known to possess the endogenous peptides, dynorphins which are kappa opioid receptor agonists. Moreover, synaptic release of dynorphin has been shown to inhibit presynaptic glutamate release from mossy fibres (Weisskopf *et al*, 1993). Other kappa opiate receptor agonists are able to depress the evoked release of neurotransmitters such as dopamine (Mulder *et al*, 1991; Ronken *et al*, 1993) and glutamate from hippocampal synaptosomes (Gannon & Terrian, 1991), from substantia nigra slices (Maneuf *et al*, 1995) and from rat and marmoset striatal synaptosomes (Hill & Brotchie, 1995).

Inhibition of potassium-evoked release of glutamate by kappa agonists probably results from an inhibition in the potassium-evoked rise in cytosolic Ca2+ levels in presynaptic terminals (Gannon & Terrian, 1991). This effect was elicited by U50, 488H at concentrations greater than 10μM with the EC50 value being estimated at 114μM (Gannon & Terrian, 1991). Kappa opioid receptors have been reported to couple to Ca2+ and K+ channels and modulate ionic conductances through these channels via pertussis toxin sensitive G proteins (North, 1993). Xiang *et al* (1990) and Tallent *et al* (1994) report a decrease in calcium entry through N-type channels. Kappa agonists have also been shown to increase the influx of calcium into astrocytes through L-type calcium channels (Eriksson *et al*, 1993), an effect which would lead to a decrease in available extracellular calcium necessary for presynaptic transmitter release. As a result, kappa agonists are able to protect against neuronal death induced by ischaemia (Tang, 1985; Hall & Pazara, 1988; Contreras *et al*, 1991; Genovese *et al*, 1994). Indeed, U50, 488H reduced the cell damage produced in the hippocampus by kainic acid (Ochoa *et al*, 1992). When incorporated into the perfusion medium in the present study, 100μM (but not 10μM) U50, 488H significantly reduced the release of adenosine induced by kainate. This would suggest that adenosine release is mediated by a calcium-dependent transmitter-like process, or involves the intermediate release of an agent such as glutamate. The present data do not allow a definitive differentiation between these two possibilities. However, although $K^+$-evoked adenosine release from isolated synaptosomes seems to involve N-type calcium channels (Cahill *et al*, 1993), the release induced by non-NMDA receptor stimulation in brain slices

is not calcium-dependent (Craig & White, 1993). Together with the present results, this strongly suggests that kainate triggers the release, of a secondary agent, probably glutamate, by calcium-dependent and calcium-independent mechanisms, which then induces adenosine release. It might be expected that such a release of glutamate would activate NMDA and non-NMDA receptors, inducing a further calcium-dependent and calcium-independent release, respectively, of adenosine (Craig & White, 1993). Such a sequence would compromise the reliability of the earlier conclusion that adenosine release is not mediated by NMDA receptors. However, Pedata *et al* (1991) have shown that glutamate-induced adenosine release is not prevented by NMDA or non-NMDA receptor blockade, and is therefore probably the result of metabolic activation by glutamate (see also Poli *et al,* 1991). To conclude, the results obtained from using U50, 488H indicate that an endogenous secondary agent, released by kainate, may mediate kainate-evoked release of adenosine.

Similar spinal antinociceptive responses to ketamine and kappa agonists, have lead to investigations for a common receptor mechanism. Since ketamine is known to block NMDA receptors, it became relevant to test the hypothesis that kappa agonists are selective antagonists of amino acid responses. Parsons *et al* (1986) reported that U50 488H at concentrations of 1, 10 & 100μM had no consistent effect on ventral root depolarisations produced by quisqualate, kainate or NMDA when superfused over concentration ranges 2.5-169μM *in vitro* in isolated frog hemisected spinal cords (Parsons *et al,* 1986). In addition, these authors have shown, *in vivo*, that intravenous U50 488H does not produce any effect on the neuronal firing in response to any of the three excitatory amino acid agonists on rat spinal neurones in rats anaesthetised by α- chloralose (Parsons *et al,* 1986). This may indicate that U50 488H does not affect the action of glutamate agonists on non-NMDA or NMDA receptors and excludes the possibility that the reduction of adenosine release by U50 488H in our experiments is due to the displacement of kainate from its receptor, a result which would be misinterpreted as an effect of kappa opioid receptor stimulation. It is noteworthy in this context that the concentrations of U50 488H used in the *in vitro* study by Parsons *et al* (1986), cover the

range of concentrations used in the present study. In addition, inhibition of calcium influx, an effect proven to be mediated by kappa opioid receptor activation and not by μ- or δ-opioid receptor activation, has been shown by U50, 488H with an EC50 of 114μM (Gannon & Terrian, 1991). This concentration is higher than that in the present study (100μM) which reduced kainate-evoked release of adenosine, given that only a percentage of this concentration will pass through the membrane.

The above evidence may suggest that U50 488H retains its pharmacological specificity in the present experiments. To confirm a lack of effect of U50 488H on kainate receptors, the displacement of binding of [$^3$H]kainate to kainate receptors by U50 488H was examined. The results can not be clearly interpreted due to experimental problems which are summarised in the Appendix and no conclusion can be drawn from this work.

4.10 The effect of chlormethiazole on intrahippocampal kainate-evoked release of adenosine

Chlormethiazole has anti-convulsant (Greene & Murray, 1989), sedative and hypnotic properties (Ogren, 1986).

Chlormethiazole reduced the antagonism of picrotoxin on muscimol-induced responses in rat caudate nucleus slices at a concentration of 60μM (Harrison & Simmonds, 1983) and increased chloride uptake by a picrotoxin sensitive mechanism into rat cortical synaptosomes with an EC50 of 50μM (Moody & Skolnick, 1989). A direct modulation of chlormethiazole on $GABA_A$ receptor currents has been shown at relatively high concentrations (1-3mM) (Hales & Lambert, 1992). Whilst chlormethiazole (100μM) potentiated strychnine-sensitive glycine inhibition, as measured in voltage-clamped murine spinal neurones, its potentiation of GABA inhibition was greater (Hales & Lambert, 1992). Whether these mechanism(s) of action of chlormethiazole are responsible for the above-mentioned properties of chlormethiazole still remains unclear.

Interestingly, interactions between GABA and adenosine have been reported (Akhondzadeh & Stone, 1994; Fern *et al*, 1994) whereas a lack of interaction between chlormethiazole and adenosine has been shown (Stone, 1988). In CNS white matter baclofen and adenosine synergistically potentiate neuroprotection against anoxia (Fern *et*

*al*, 1994) and in hippocampal neurones muscimol potentiated the ability of adenosine to reduce the amplitude of orthodromically induced population potentials (Akhondzadeh & Stone, 1994). With relevance to this latter report, chlormethiazole neither had the ability itself nor potentiated the ability of adenosine to reduce the amplitude of orthodromically induced population potentials in hippocampal neurones (Stone, 1988). This may indicate that the anticonvulsant action of chlormethiazole is not due to its proposed action at the level of GABA.

Chlormethiazole did not block the electrophysiological effects of NMDLA, kainic acid nor quisqualic acid in the rat cerebral cortex, eliminating the involvement of excitatory amino acid receptors in the anticonvulsant action of chlormethiazole (Addae & Stone, 1988). In addition, chlormethiazole inhibited NMDA-induced convulsions by a mechanism other than binding to the MK-801 site (Cross *et al*, 1993).

Chlormethiazole prevents degeneration of hippocampal neurones induced by transient forebrain ischaemia (Cross *et al*, 1991). Neuroprotection by chlormethiazole against hippocampal damage induced by systemic kainate has also been shown (MacGregor *et al*, 1994). Chlormethiazole has been reported to inhibit glutamate release (Baldwin *et al*, 1994), a possible prerequisite for its neuroprotective properties. .

Chlormethiazole, at a concentration of 500μM (a concentration recommended by A.J. Cross, Astra Neuroscience Research Unit) did not modulate kainate-evoked release of adenosine in the present work. It appears in the present study that increasing the major GABA-mediated inhibitory influence within the hippocampus does not modulate kainate-evoked release of adenosine.

After ischaemia, hippocampal GABAergic neurones have been shown to be preserved (Francis & Pulsinelli, 1982), whereas cortical $GABA_A$ receptors have been reported to be decreased (Baldwin *et al*, 1993b). For chlormethiazole to have GABA-enhancing effects, GABA receptors must be present. By reducing the number of receptors available to chlormethiazole, the proposed GABA-enhancing action of chlormethiazole may be reduced. In keeping with this, MacGregor *et al* (1993) showed that the benzodiazepine, clonazepam, did not reduce kainate-induced hippocampal damage.

In the present study, agents which have the ability to inhibit the release of glutamate have been employed (i.e. R-PIA, U50, 488H and chlormethiazole). To recap, R-PIA and chlormethiazole did not, but U50, 488H did, inhibit kainate-evoked release of adenosine. This indicates that U50 488H reduces kainate-evoked release of adenosine by a mechanism distinct from the mechanisms of action of R-PIA and chlormethiazole. Therefore kainate-evoked release of adenosine is probably not mediated by the prior calcium-dependent release of glutamate.

4.11 The effect of free radical scavengers/ antioxidants on kainate-evoked release of adenosine

Loss of calcium homeostasis, which occurs during brain damage, can lead to elevated levels of intracellular calcium. Elevated intracellular calcium activates phospholipase A2 which releases arachidonic acid for break down to prostaglandins and leukotrienes by cyclo-oxygenase and lipoxygenase, respectively. During these conversions free radicals are produced. The activation of nitric oxide synthase by calcium produces nitric oxide which interacts with superoxide ions to produce peroxynitrite ion which leads to production of the toxic hydroxyl ion. Calcium has been reported to cause the conversion of xanthine dehydrogenase to xanthine oxidase, though the existence of the latter in the rat brain is still controversial. The conversion of hypoxanthine to xanthine by xanthine oxidase produces free radicals.

The toxic hydroxyl radical is not produced directly by any of these mechanisms. The superoxide radical is converted by the enzyme superoxide dismutase to hydrogen peroxide, which together with superoxide ion can form hydroxyl radicals. Oxygen radicals are capable of causing lipid peroxidation, with the destruction of cell membranes.

The production and implications of free radicals in brain damage have become a major focus in excitotoxic brain research. Detection and quantification of free radicals is difficult due to their extreme reactivity. The levels of enzymes which are related to the production and elimination of free radicals and the extent of lipid peroxidation have been used as indices of free radical production and of free radical-induced damage. Bruce & Baudry (1995) reported a hippocampal increase in protein and lipid oxidation within 8 hrs and in

glutathione peroxidase, superoxide dismutase and catalase between two and five days after systemic kainate administration to rats.

The conversion of salicylate to DHBA (3,4-dihydroxybenzylamine) is often used to show the production of hydroxyl free radicals. Schulz *et al* (1995) showed the production of DHBA from salicylate 1hr after the intrastriatal injection of malonate or 3-acetyl-pyridine. The DHBA production was attenuated by pretreatment with S-PBN, showing a free radical scavenging effect (Schulz *et al*, 1995).

The use of electron spin resonance (ESR) has been used to detect more stable free radical adducts of spin-trapping agents such as α(4-pyridyl-1-oxide)-N-tert-butylnitrone (POBN) (Phillis & Sen, 1993).

The neuroprotective action of free radical scavengers is another technique for assessing free radical-induced damage. Schulz *et al* (1995) demonstrated that lesions in rats induced by intrastriatal injections of NMDA, AMPA and kainic acid were attenuated by the free radical scavenger, N-tert-butyl-α-(2-sulfophenyl)-nitrone (S-PBN).

These results suggest the formation of free radicals after kainate administration and it was of interest in the present study to examine the possible role of free radicals in the release of adenosine by kainic acid. Ascorbic acid, reduced glutathione and oxypurinol are the antioxidants and free radical scavengers used in the present study and were chosen due to their spectrum of activity. Ascorbic acid is reported to reduce free radicals, glutathione is reported to scavenge hydrogen peroxide and oxypurinol is a xanthine oxidase inhibitor, preventing the production of superoxide ions.

Ascorbic acid is an endogenous antioxidant, present in the extracellular medium at a concentration of 200-500μM, while estimated whole brain levels are millimolar (Schenk *et al*, 1982). Ascorbic acid reduces free radicals to their neutral state to form ascorbate free radicals. Pairs of ascorbate free radicals are likely to spontaneously form one ascorbate molecule and one dehydro-L-ascorbic acid molecule by disproportionation (one molecule acquires both unpaired electrons to become fully reduced while the other molecule is oxidised).

The free radical scavenging action of ascorbic acid was tested by Maeno *et al* (1989) who reacted hypoxanthine with xanthine oxidase *in vitro* and showed an inhibition of superoxide dependent oxidation of adrenaline (Maeno *et al*, 1989). Majewska & Bell (1990) have demonstrated neuroprotective properties of ascorbic acid in rat cortical neuronal cultures. Preincubation of 1-3mM ascorbic acid for 10 min before adding glutamate 50μM or NMDA 100-500μM protected the cells from dying (Majewska & Bell, 1990). Ascorbic acid (50mg/kg) injected i.p. attenuated kainate-induced hippocampal damage (MacGregor *et al*, 1996).

In the present study, intrahippocampal ascorbic acid reduced intrahippocampal kainate-evoked release of adenosine. However, systemic ascorbic acid had no significant reduction on release of adenosine evoked by either intrahippocampal or systemic kainate. There are several explanations for this discrepancy. Firstly, there may be the lack of absorption of this water soluble vitamin into the brain from the systemic circulation. However MacGregor *et al* (1996) showed a central effect of i.p. ascorbic acid at the same dose and an active ascorbate carrier system that crosses the choroid plexus (the blood-CSF barrier) has been reported (Spector, 1981).

Secondly, the residual urethane present in the peritoneal cavity in the present experiments may interfere with the absorption of ascorbic acid. For the investigation of the effect of systemic ascorbic acid on systemic kainate-induced release of adenosine, urethane 5ml/kg was injected i.p. 2hrs 40min before the i.p. injection of ascorbic acid 1ml/kg. Residual urethane may either chemically interact with ascorbic acid or change the permeability properties of the peritoneal lining. The experiments were therefore repeated by s.c. injection and still no significant effect on kainate-evoked release of adenosine was observed.

Thirdly, another possible explanation for the discrepancy is the chemical interaction of kainate and ascorbic acid in the syringe. Considering a possible interaction between ascorbate and kainate is important since dihydrokainate, the potential product of the reduction of kainate by ascorbic acid, is a compound which may displace kainate from the kainate receptor. However, dihydrokainate binds to the low affinity kainate binding site

with a Ki value of 3150nM compared to kainate which has a Ki value of 19nM (London & Coyle, 1979). Another point of interest is that dihydrokainate is a more effective high affinity glutamate uptake blocker (IC50 ~175μM) than kainate (IC50 ~302μM) (Johnston *et al,* 1979). An elevation in extracellular glutamate by blockage of glutamate uptake by dihydrokainate would possibly enhance adenosine release. However, MacGregor (1995) reported that kainate in the presence of ascorbic acid was stable for up to 5 days in solution. In addition, in the present experiments all drugs were freshly made.

Previous work has shown that chemical reduction of the redox modulatory site potentiated the response to NMDA receptor activation whereas oxidation led to inhibition of the response (Levy *et al,* 1990; Aizenman *et al,* 1992). It therefore becomes relevant to investigate the ability of the reductant, ascorbic acid, to break the disulphide bond on the redox modulatory site of the NMDA receptor which would potentiate the activity of the NMDA receptor. Majewska *et al* (1990) found that ascorbic acid 1mM and 3mM (around the range expected in the ECF in our experiments using ascorbic acid) did not potentiate but actually inhibited NMDA receptor activity. The authors explain that ascorbic acid may alter the electric charge of the NMDA receptor, producing a conformational change in the receptor.

In addition to its antioxidant properties, ascorbic acid has been shown to block calcium channels (Ebersole & Molinoff, 1992). Such a mechanism seems to involve the presence of iron and may be due to the oxidised state of ascorbic acid. The involvement of this effect of ascorbic acid on the reduction of kainate-evoked release of adenosine would be feasible if adenosine release was mediated by the prior release of calcium dependent glutamate or ATP release.

Glutamate uptake is coupled to the efflux of ascorbic acid (O'Neill *et al,* 1984; Cammack *et al,* 1990). This property becomes relevant to the present study since the presence of glutamate in the ECF will be removed from the synaptic cleft by neuronal or glial uptake in exchange for ascorbic acid. Removal of glutamate will reduce release of adenosine mediated by glutamate. A reduction of kainate-evoked release of adenosine may be

occurring by glutamate uptake driven by ascorbic acid efflux in any of the present experiments concerning kainate-evoked release of adenosine.

In order to establish if the reduction of kainate-evoked release of adenosine is mediated by displacement of kainate from the kainate receptor by ascorbic acid, an attempt to examine binding of ascorbic acid in the presence in [$^3$H]kainate was made. However, as explained in the Appendix no conclusion was drawn from these results and it thus remains a possibility. However, Dr M. J. Higgins performed electrophysiological studies using ascorbic acid on the hippocampal slice. The lack of effect on population spikes of ascorbic acid alone (M.J. Higgins, personal communication) confirms a lack of interaction of ascorbic acid with the kainate receptor.

Reduced glutathione contributes to our natural defence against free radical production by scavenging hydrogen peroxide. Oxidised glutathione is present in the brain at higher concentrations (1-2mM) than oxidised glutathione (Slivka *et al*, 1987) and exhibits rapid turnover in the glial compartments (Yudkoff *et al*, 1990). Reduced glutathione (GSH) is oxidised by $H_2O_2$ to produce oxidised glutathione (GSSG). Oxidised glutathione has been reported to be actively transported out of cells (Boobis *et al*, 1989). Oxidised glutathione is recycled to the reduced form by the enzyme glutathione reductase.

The free radical scavenging action of reduced glutathione was tested by Maeno *et al* (1989) who reacted hypoxanthine with xanthine oxidase *in vitro* and showed an inhibition of superoxide dependent oxidation of adrenaline (Maeno *et al*, 1989).

The ability of reduced glutathione to neuroprotect has been shown by Yamamoto *et al* (1993) by a decrease in brain water content after cerebral ischaemia. Kainate-induced brain damage in the rat hippocampus was attenuated by glutathione (Saija *et al*, 1994).

Reduced glutathione was not found to affect the activity of NMDA receptors although oxidised glutathione inhibited NMDA-evoked currents and increases in [Ca 2+]i in retinal and cortical ganglion cell neurones (Sucher & Lipton, 1991). The authors concluded that oxidised glutathione was acting predominantly as an oxidising agent of the redox modulatory site of the NMDA receptor channel complex, while reduced glutathione was not effective at reducing the redox site. Reduced glutathione can be converted to oxidised

glutathione following periods of oxidative stress which may occur after exposure to kainate. It is possible that oxidised glutathione may be responsible for the reduction in kainate-evoked release of adenosine observed after application of reduced glutathione. To clarify this, the experiments could be repeated in the presence of a selective sulphhydryl reducing agent such as dithiothreitol which would break the disulphide bond and reverse any effects that oxidised glutathione may have. However, the present study illustrates that kainate-evoked release of adenosine is not mediated by activation of the NMDA receptor and therefore any influence that oxidised glutathione has on the activity of the NMDA receptor should not affect the release of adenosine evoked by kainate.

Two lines of evidence show that glutathione is not acting at the kainate receptor. Firstly, Sucher & Lipton (1991) found that 10mM reduced glutathione had no effect on calcium responses to kainate 50μM in cultures of rat cortical neurones. Similarly, 0.5mM glutathione did not have any effect on the calcium influx in cultured cerebellar granule cells (Janaky *et al*, 1993). Secondly, Bellis *et al* (1991) examined the chemoreceptor mechanism of glutathione in invertebrates. It was demonstrated that glutamate binding sites exist in two forms in hydras: the glutathione-sensitive and glutathione-insensitive binding sites. The authors discovered a selective association of the kainate binding site with the reduced glutathione-insensitive site.

One report states that glutathione in the reduced form displaced binding of tritiated kainate to kainate receptors in rat brain synaptic plasma membranes with an IC50 of 180μM (Varga *et al,* 1989). Reduced glutathione was more active in the binding to AMPA receptors (IC50 of 8.4 μM). In the present study, glutathione, administered at a dose of 10mM by reverse dialysis, reduced kainate evoked adenosine release. That the observed efficacy of glutathione was due to displacement of kainate from the non-NMDA receptor cannot be excluded in the present study.

Intracellular mechanisms of action by reduced glutathione which would lead to a reduction in adenosine release cannot be eliminated in the present study, however the exogenous reduced glutathione must first be transported into the cells. Since there is normally (under physiological conditions) no, or little, transport of reduced glutathione into intact cells

(Boobis *et al*, 1989; Martensson *et al*, 1989), an intracellular mechanism of action seems unlikely. However, transport of the extracellular degradative products of reduced glutathione may occur into the cell where resynthesis to reduced glutathione may occur.

Xanthine oxidase catalyses the metabolism of hypoxanthine to xanthine and then to uric acid, with a release of superoxide radicals which lead to the production of hydroxyl ions. Oxypurinol is an inhibitor of xanthine oxidase and has been shown to have an inhibitory effect on hydroxyl radical production in the cerebral cortex during ischaemia as measured by ESR and the spin trapping agent POBN (Phillis & Sen, 1993). Dykens *et al* (1987) reported prevention of kainate-induced neuronal death by allopurinol, the precursor of oxypurinol.

The efficacy of oxypurinol in reducing kainate-evoked release of adenosine in the present study may be explained by the high levels of xanthine oxidase reported to be present during ischaemia (Olanow, 1993) and other conditions where there are elevated calcium levels (where xanthine dehydrogenase is converted to xanthine oxidase). Another property of oxypurinol is the attenuation of the metabolism of inosine thus precluding the production of the free radical-generating substances, hypoxanthine and xanthine. It has been shown that oxypurinol pretreatment in cerebral ischaemic rats results in preservation of the high energy phosphates, ATP and ADP, which may lead to one of the mechanisms of cerebral protection caused by oxypurinol (Phillis *et al*, 1995).

Kainate-evoked release of adenosine was reduced significantly by ascorbic acid, oxypurinol and glutathione which may lead to the interpretation that free radicals are involved in the process by which kainate induces adenosine release.

It has been demonstrated that the formation of free radicals, which in turn causes lipid peroxidation, can lead to release of glutamate from neurones (Pellegrini-Giampietro *et al*, 1988). In the present system, the rise in extracellular adenosine after applications of kainate may therefore be the result of free radical action directly on the cells, or could be secondary to a rise in extracellular glutamate which has leaked through cell membranes damaged by free radical induced lipid peroxidation.

Free radicals are reported to cause enzyme inactivation. Through the inhibition of ecto-5'-nucleotidase, free radicals reduced adenosine release during myocardial ischaemia (Takashima *et al*, 1993). The present results indicate that free radicals may mediate the kainate-evoked release of adenosine, implying that a substantial amount of this release is not enzyme-derived.

4.12 Effects of kainate on hippocampal histology

In the present histology experiments, kainate was applied to the microdialysis probe on the right hand side while the microdialysis probe on the left hand side was perfused with acsf only. This was carried out in two animals from which similar results were obtained. There was no cell layer thinning in the CA3 or CA1 regions in the left hand hippocampus (contralateral to the exposure to kainate). Two conclusions can be drawn from this observation. Firstly, there may be limited cell damage due to probe insertion. Secondly, the exposure of the tissue to kainate on one side of the hippocampus does not appear to affect the hippocampal cells on the contralateral side.

The neurotoxic effects of kainic acid have been investigated by Koh *et al* (1990) in murine cortical cultures. A 5 min exposure of 500μM kainic acid produced widespread acute neuronal swelling within one hour but not much late neuronal loss (Koh *et al*, 1990). The acute neuronal swelling was suggested to be caused by influx of $Na^+$ ions through kainate-activated channels, followed by influx of $Cl^-$ ions and water (Koh *et al*, 1990). Intrahippocampal injection of 0.47nmol kainic acid in rats produced an incomplete neuronal loss in most areas of the hippocampus within four days (Lees, 1992). Intrastriatal injection of 2.2nmol kainic acid in rats caused widespread neuronal damage within 2 weeks (Arvin *et al*, 1989).

In the present study, approximately 60% of the kainate delivered in the perfusate is estimated to leave the probe and enter the brain. A 5 min pulse of 1mM kainate at 2μl/min should therefore allow approximately 6.0nmol of kainate to enter the brain. The histological examination of the hippocampus, seven days after the application of kainate through a microdialysis probe in the two rats examined in the present study, revealed hippocampal cell disruption predominantly within the CA3 and to a small extent in the CA1

cell layers. This gives evidence that enough kainate reached the hippocampal tissue to allow depolarisation and damage of neurones. The damage extended 1.25mm anterior to the probe tract. This correlates well with the spread of the radiolabelled kainate, reported in the present study, which was approximately 1.2mm anterior to the probe tract. Approximately 65-70% of the radiolabelled spread throughout the hippocampus in the vicinity of the probe. Approximately 30-35% of the radiolabelled spread into the cortex in the vicinity of the probe, however, there were no signs of cell disruption or gliosis observed in the cortex. This may imply that not enough kainate reached the cortical tissue to allow depolarisation and damage to neurones.

In the present study, gliosis was evident predominantly in the CA3 region of the hippocampus. Jorgensen *et al* (1993) observed signs of gliosis in the CA3 of the hippocampus in kainate-treated rats which occurred with an increased prominence from 2 days to 21 days. In view of this reported delay, gliosis may not occur within the run-time of the test experiments (kainate-evoked release of adenosine was investigated over a time period of 4hrs 20 min after the first pulse of kainate). Activation of kainate receptors, which are reported to be present on glial cells, may contribute to an increase in extracellular potassium ions (Teichberg, 1991). Consequently, there may be a potassium-induced depolarisation of neighbouring neurones leading to initiation of action potentials and transmitter release. A loss of ionic homeostasis may lead to cell swelling which is reported to occur with the early onset of cell death (Koh *et al*, 1990). It is not unlikely that the gliosis observed in the present study indicates the occurrence of early onset neuronal damage after kainate exposure.

Kainic acid has been shown to increase glutamate release *in vivo* from the hippocampus (Lehmann *et al*, 1983) and striatum (Butcher *et al*, 1987) and *in vitro* from the cerebellum (Ferkany *et al*, 1982; Ferkany & Coyle, 1983). It has been shown that both glutamate (Mayer *et al*, 1987) and kainate (Butcher *et al*, 1987) cause the influx of calcium ions. Intracellular calcium has been proposed to accelerate the formation of glial filaments, shown by an increase in glial fibrillary acidic protein, a marker for astrocytes (Aono *et al*, 1990).

Mossy fibres terminals and CA3 pyramidal cells are known to exhibit kainate receptors (Ferkany *et al*, 1982; Bettler & Mulle, 1995, Malva *et al*, 1995) which may explain why the glial cells in the CA3 part of the hippocampus were more susceptible to kainate than glial cells in other parts of the hippocampus (e.g. CA2 and CA1).

The differences in methodology between the present histology experiment and the experiments investigating kainate-evoked release of adenosine (test experiments) must be recognised, especially the anaesthetics used. In the histology experiments the anaesthetics used were halothane and Hypnorm and Hypnovel where as in the test experiments the anaesthetic used was urethane. It has previously been shown that the severity of hippocampal damage following ischaemia varies with the anaesthetic agent (Lees, 1992). Following the intrahippocampal injection of kainate, Lees (1992) has shown that the amount of hippocampal neuronal loss was greater when given under short acting anaesthetics halothane and ketamine, than when given under pentobarbital. This is important in context of the neuronal damage observed in the present histology experiments (under halothane) which may not occur to the same extent in the test experiments (under urethane).

## PART II

### Summary

Recently, in the Laboratory of Human Anatomy, University of Glasgow, a mutant strain of rats has spontaneously arisen with locomotor deficits including general ungainliness, a wide staggering gait and, when fully developed, an inability to initiate movement. The rat is known as the Albino Swiss/ Anatomy Glasgow University (AS/AGU) rat.

So far, previous work has shown that, at 12 months of age, the AS/AGU rats have fewer dopaminergic cell bodies in the substantia nigra pars compacta than control animals (Clarke & Payne, 1994). Based on these preliminary results, their progressive loss of locomotor function and the finding that their post-mortem striatal tissue dopamine levels are lower than in control animals (J.M. Campbell, personal communication), the AS/AGU rat may have the potential to serve as a natural animal model of basal ganglia disorders. The extracellular levels of monoamines, and the effect of L-dopa on these levels, in 5 month old AS/AGU and AS rats have been studied in the present work by *in vivo* microdialysis of the conscious freely-moving rat. The striatal monoamine post-mortem tissue levels have also been studied in 5 month old rats.

There was no significant difference in the basal extracellular striatal DOPAC (3,4-dihydroxyphenylacetic acid) levels between the AS/AGU and AS rats at 5 months of age. L-dopa increased the levels of dopamine and DOPAC in the AS rats to a higher extent than in the AS/AGU rats, indicating that the AS/AGU rats have less ability to convert L-dopa to dopamine and DOPAC than the AS/AGU rat. There was no difference in the striatal tissue dopamine and DOPAC levels between the AS/AGU and AS rats apart from the VCPu which has a drop in DOPAC levels but no difference in dopamine levels. This latter result taken together with the previous finding of a drop in dopamine in the 12 month old AS/AGU rats indicates that the drop in dopamine is probably age related and occurs between the ages of 5 and 12 months.

## 5.0 INTRODUCTION

The search for animal models which accurately mimic the clinical conditions of human movement disorders is ongoing as current paradigms have their drawbacks. Pharmacological manipulations of dopaminergic systems in the CNS (for example, using 6-hydroxydopamine (Ungerstedt, 1968; Voorn *et al*, 1987) or MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Burns *et al*, 1983; Bankiewicz *et al*, 1986) are currently in use for modelling Parkinson's Disease. Mutant strains of mice which have movement disorders exist, some of which have deficits in dopaminergic systems, for example, the weaver mouse (Roffler-Taylov & Graybiel, 1984).

In the Laboratory of Human Anatomy, University of Glasgow, a mutant strain of rats has spontaneously arisen with locomotor deficits including general ungainliness, a wide staggering gait and, when fully developed, an inability to initiate movement. The rat is known as the Albino Swiss/ Anatomy Glasgow University (AS/AGU) rat and has, at 12 months of age, fewer dopaminergic cell bodies in the substantia nigra pars compacta than control animals (Clarke & Payne, 1994). The AS/AGU rats breed well and their life expectancy is around 18 months. The behavioural deficits become apparent approximately ten days post-natal and progressively get worse. So far no detailed work has been carried out on the cerebellum but the animals have been shown to possess both purkinje cells and a granule cell layer.

The advantages of the AS/AGU rat as a natural model of locomotor disorders over other models would be the potential ability to examine an age-related progressive disease and the possibility of being able to isolate the gene.

The aim of the present project was to examine the striatal monoamine content in freely-moving AS/AGU and control rats by microdialysis. In the present study the AS (Albino Swiss) rats were used as controls for the mutant AS/AGU rats.

### 5.1. Basal ganglia

The basal ganglia, as the name suggests, include deep-lying structures of the cerebral hemispheres, notably the corpus striatum (the caudate nucleus, putamen and GP). Compartmentalisation of the caudate-putamen has been detected biochemically by staining

for acetylcholinesterase (AchE) activity. Small AchE-deficient zones have been named the striosomes and make up about 10-20% of the volume of the striatum. These patches of the striatum are also deficient in GABA and neuropeptides. The other 80% of the striatum is the matrix. The neurochemical differences between the striosomes and the matrix are not all-or nothing. One point noteworthy is that the striosomes seem to have the excitatory D1 binding sites whereas the matrix appears to contain the inhibitory D2 binding sites (Graybiel, 1990). The matrix compartment receives the striatal inputs most directly related to sensorimotor processing and projects in turn mainly to the pallidum and the lateral (probably reticular) part of the substantia nigra whose outputs complete the motor circuit via the thalamus (Jiménez-Castellanos & Graybiel, 1989). By contrast, striosomes appear to receive inputs from neural structures affiliated with the limbic system (Donoghue & Herkenham, 1986) and the outputs of the striosomes mainly project to the medial part of the substantia nigra (including apparently the pars compacta) and also to the pars lateralis of the substantia nigra (Jiménez-Castellanos & Graybiel, 1989). It is suggested that dopaminergic drug therapies and cell-replacing grafting therapies could have different functional effects depending on whether they are targeted at the striosomes or the matrix. Modulation of the dopaminergic input to the striatum by striosomes may be related to motivational information from the limbic system whereas the matrix may be tightly linked to specific sensory and motor parameters.

A major breakthrough in the understanding of how basal ganglia dysfunction may lead either to the hyperkinetic state of Huntington's disease or the akinetic state of Parkinson's disease has come from the knowledge that there are two pathways that mediate striatal influences over the activity of thalamocortical neurones. Both pathways go through the GPi/SNr (internal segment of the globus pallidus/substantia nigra reticulata). When animals are at rest, there is little striatal output, while GPi/SNr neurones emit a regular and sustained flow of impulses, producing a tonic suppression of the thalamo-cortical neurones (Chevalier & Deniau, 1990).

*The Motor Circuit:*

The more direct of the two pathways has striatal output mediated by the transmitters GABA and substance P with synapses in the GPi/SNr which inhibit the tonic GPi/SNr suppression of the thalamus.

The more indirect route of the two pathways has striatal output mediated by transmitters GABA and enkephalin. The indirect pathway involve sequential inhibitory synapses in the external segment of the globus pallidus (GPe) and subthalamic nucleus (STN). An excitatory projection from the STN to the GPi/SNr facilitates tonic GPi/SNr suppression of the thalamus.

The direct and indirect pathways have opposing effects on thalamic activity. The direct pathway tends to facilitate ongoing motor activity and the indirect pathway to dampen motor activity (fig 5.1). One of the main points to note is that activation of the direct pathway leads to disinhibition of the GPi/SNr neurones which leads to a hyperkinetic state.

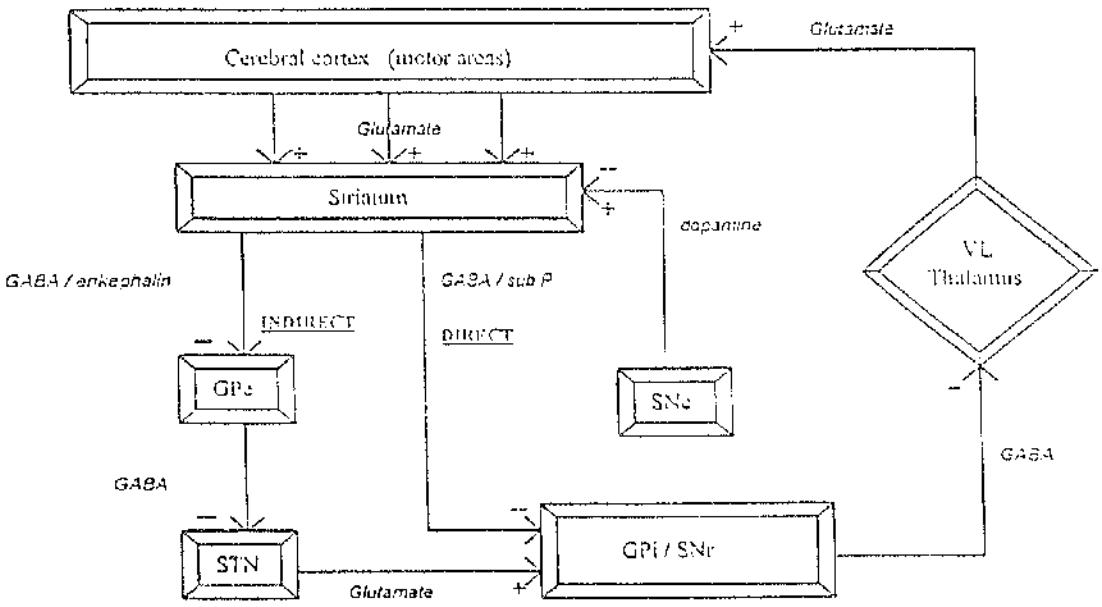



Fig 5.1.

The motor circuit. A simplified diagram of the "direct" and "indirect" pathways of the motor circuit in the Basal Ganglia.

D1 receptors are localised on the cell bodies of striatal neurones that send GABA/substance P projections to the GPi (direct pathway) (Jenner, 1995). Thus dopaminergic inputs to the striatum appear to have a net excitatory effect on this efferent neurone (Alexander & Crutcher, 1990). D2 receptors are localised on the cell bodies of the GABA/enkephalinergic neurones projecting to the GPe (indirect pathway) (Jenner, 1995). Thus dopaminergic inputs to the striatum appear to have a net inhibitory effect on the GABA/enkephalinergic neurone (Alexander & Crutcher, 1990).

Immunocytochemical data from post-mortem human tissue indicate that the loss of the GABA/enkephalinergic striatal projection to the GPe in Huntington's disease is more severe than the loss of the substance P containing projection to the GPi (Reiner *et al*, 1988). A more dominant activation of the GABA substance P neurone may result in the hyperkinetic state observed in Huntington's disease since the direct pathway tends to facilitate motor activity.

6-Hydroxydopamine injections into the substantia nigra, which denervated dopaminergic inputs to the striatum, have been shown to decrease substance P-immunoreactivity and increase enkephalinergic-immunoreactivity (Voorn *et al*, 1987). Thus the innervation of the indirect pathway may be increased when nigrostriatal neurones are denervated. Since the indirect pathway tends to dampen motor activity, this is consistent with the notion of an akinetic state in Parkinson's disease.

Studies on the neuronal activity of MPTP-treated animals have shown a decrease in the mean tonic discharge in the GPe (DeLong, 1990). There was a significant increase in tonic neuronal discharge in GPi/SNr neurones (DeLong, 1990). These observations are consistent with the evidence indicating that the loss of striatal dopamine results in an increase in transmission through the indirect pathway (i.e. from striatum to the GPe, GABA/enkephalin) and a decrease in transmission through the direct pathway (i.e. from striatum to the GPi/SNr, GABA/substance P) (DeLong, 1990). The overall effect would be a tonic GPi/SNr suppression of the thalamus (DeLong, 1990). Thus there is an increased negative feedback to the cortex resulting in decreased instructions to the spinal cord and

suppression of motor activity. This may explain why Parkinsonian patients find it difficult to start moving and why their movements are so slow (Altman, 1990).

5.2 Basal ganglia disorders

1817 was the year that James Parkinson provided the first description of the behavioural symptoms of the disease that is now known as Parkinson's disease. Parkinsonian patients typically show a mild resting tremor, muscular rigidity, postural abnormalities and bradykinesia.

In 1954, a similarity in the behaviour of reserpinised rats (akinesia) and parkinsonian patients, (Plummer *et al*, 1954; Tripod *et al*, 1954) led to the discovery of the striking loss of dopamine in the caudate and putamen of the corpus striatum of post-mortem parkinsonian patients. Mildly affected patients were found to have a deficit of 80% of striatal dopamine and it was discovered that the severity of akinesia and rigidity was correlated with the extent of further dopamine loss (Bernheimer *et al*, 1973; Lloyd *et al*, 1975).

The search for an animal model of Parkinson's disease is ongoing. Pharmacological manipulation of dopaminergic systems in the CNS has been used to model Parkinson's disease, with varying degrees of limitation. Reserpine depletes serotonin as well as dopamine (Shore *et al* 1955) and chlorpromazine, haloperidol and alpha-methyltyrosine induce acute rather than chronic behavioural impairments (Janssen *et al*, 1960; Moore & Rech, 1967).

Permanent depletion of brain catecholamines was found after direct application of 6-hydroxydopamine into the brain (Ungerstedt, 1968). When administered with desmethylimipramine, an inhibitor of high affinity uptake into noradrenergic neurones, 6-hydroxydopamine depletes tissue dopamine but not tissue noradrenaline (Breese and Taylor, 1971). 6-Hydroxydopamine is taken up into the dopamine terminal by the high affinity dopamine uptake system and oxidises to form hydrogen peroxide (Heikkila & Cohen, 1972). The subsequent production of cytotoxic hydroxyl radicals may lead to the destruction of the dopaminergic neurones.

Parkinsonism induced by MPTP was the first model which reproduces the clinical, pathological and biochemical characteristics of the human disorder (Burns *et al*, 1983; Bankiewicz *et al*, 1986). MPTP is converted in the brain to MPP+ (1-methyl-4-phenylpyridinium) by monoamine oxidase (MAO). MPP+ is then selectively taken up into nigrostriatal neurones. MPP+ destroys nigrostriatal neurones by a mechanism which is still unclear but is thought to involve the production of $H_2O_2$ and free radicals. MPTP-treated animals exhibit one of the pathological hallmarks of Parkinson's disease: the loss of melanin-containing neurones of the pars compacta of the substantia nigra, resulting in loss of dopamine in the striatum and the substantia nigra itself. However, drug-induced parkinsonism has the disadvantage that it is not a progressive disease as found in the human disorder.

5.3 The formation and degradation of monoamines

When the neurones are at rest, dopamine metabolism follows leakage of the amine into the cytoplasm. Dopamine is then degraded to DOPAC (3,4-dihydroxyphenylacetic acid) within the dopaminergic neurone (Carlsson & Hillarp, 1962; Roffler-Tarlov *et al*, 1971; Westerink, 1979) although the precise site of metabolism within the neurone is not certain. Efflux of DOPAC from its neuronal site may occur, dependent on its lipophilicity (Trendelenburg *et al*, 1980) or by active transport (Miyamoto *et al*, 1991). Exposure of DOPAC to catechol-O-methyl transferase (COMT), an enzyme thought to be present intraneuronally or extraneuronally (Roffler-Tarlov *et al*, 1971; Westerink, 1979), results in the formation of HVA (4-hydroxy-3-methoxy-phenylacetic acid or homovanillinic acid).

The 0-methylation of dopamine to MTA (3-methoxytyramine) is probably not an important alternative route of metabolism of dopamine. The quantity of HVA formed via MTA has been considered unimportant relative to the quantity of HVA formed via DOPAC Westerink (1979).

When neurones are excited, action potentials invade dopaminergic terminals, and dopamine is released by exocytosis. The bulk of extracellular dopamine is taken back up into the cytoplasm of the neurones. Most of the cytoplasmic dopamine is subsequently transformed

to DOPAC but little seems to be restored into intracellular elements (Zumstein *et al*, 1981).

Thus it appears that DOPAC is formed intraneuronally when neurones are both at rest and excited (Zumstein *et al*, 1981).

It has been proposed that DOPAC is a useful index of dopamine release (Roth *et al*, 1976) since striatal tissue levels of DOPAC have been shown to increase following electrical stimulation of the nigrostriatal pathway but decrease after cessation of impulse flow. Early experiments performed on the superior cervical ganglion of the rat (Karoum *et al*, 1977; Lutold *et al*, 1979) and pig (Pearson & Sharman, 1974) led the authors suggest that the concentration of DOPAC may be a good reflection of the rate of dopamine release due the rapid rate of dopamine metabolism.

Later experiments suggest a lack of relationship between dopamine and metabolite output (Imperato & Di Chiara, 1984). Extracellular dopamine is increased during ischaemia but DOPAC is decreased. This has been explained by the decrease in blood flow during ischaemia causing hypoxia in the tissue which decreases the activity of the enzyme MAO. Imperato & Di Chiara (1984) therefore hypothesise that DOPAC levels are coupled to dopamine metabolism not dopamine release. Another explanation is that depolarisation of neurones causes a failure of monoamine transport. Failure of uptake of dopamine would result in increased extracellular dopamine but decreased DOPAC since less dopamine is being converted to DOPAC intraneuronally.

For the amount of DOPAC in the ECF to reflect the amount of dopamine released, transmitter metabolism must be coupled to transmitter release and the following criteria must be met: Firstly, DOPAC must not be formed from any precursor other than dopamine. To date, no other pathway of DOPAC formation has been realised. Secondly, dopamine must not be metabolised to any product other than DOPAC. There is evidence that dopamine can be 0-methylated to MTA. This metabolic pathway of dopamine appears to be quantitatively unimportant according to Westerink (1979). However, if it does occur then the measurement of DOPAC levels will give an underestimated reflection of dopamine levels.

Thirdly, after reuptake of released dopamine, dopamine must always be metabolised into DOPAC and not be restored into intracellular elements as proposed by Zumstein *et al* (1981). Wachtel & Abercrombie (1994) reported that dopamine is indeed metabolised to DOPAC since a MAO inhibitor increased extracellular dopamine levels but decreased extracelullar DOPAC levels.

Fourthly, DOPAC must not be formed from dopamine unless the dopamine has first been released into the ECF. Santiago *et al* (1993) report that when impulse flow of the nigrostriatal neurone is suppressed by the application of TTX or baclofen, dopamine is no longer released, though there is an increase in extracellular DOPAC levels. This implies that when dopaminergic neuronal activity is suppressed, dopamine is intraneuronally metabolised instead of being released. Therefore changes in DOPAC levels may reflect changes in intraneuronal dopamine metabolism instead of changes in dopamine release. Therefore an increase in neuronal activity is not a prerequisite for an increase in dopamine metabolism (Santiago *et al*, 1993).

The size of the cytoplasmic pool of dopamine is probably largely dependent on the rate of dopamine synthesis (Zetterstrom *et al*, 1986). AADC (aromatic amino acid decarboxylase) has been shown to exist in non-dopaminergic neurones allowing dopamine synthesis outwith dopaminergic neurones (Lloyd & Hornykiewicz, 1970; Hefti *et al* 1981; Melamed *et al*, 1981). The possibility of dopamine metabolism in such elements where dopamine synthesis occurs cannot be ruled out and indeed an L-dopa-induced increase in DOPAC has been shown after destruction of dopaminergic terminals (Sarre *et al*, 1994).

In any case, for the changes in concentration of dopamine or DOPAC to reflect the changes in dopaminergic neuronal activity, dopamine or DOPAC must not be released from any sites other than from dopaminergic neurones.

For the changes in DOPAC concentration to reflect the changes in dopamine release after pharmacological manipulation, dopamine metabolism must not be stimulated by the drug(s) employed without the release of dopamine. In addition, drug(s) must not impede nor accelerate the removal of DOPAC from its site of production. It has been shown that haloperidol inhibits the transport of DOPAC and HVA away from their sites of production

(Westerink *et al*, 1984) and amphetamine inhibits MAO (Greene & El Hait, 1978). Thus the interpretation of drug-induced DOPAC concentration changes as being changes in either dopamine release or dopaminergic neuronal activity needs caution as the application of haloperidol would give a false positive and that of amphetamine would give a false negative.

The aim of the present experiments was to:

1) compare the basal dialysate and tissue levels of the monoamines in AS/AGU rats with that of the AS control rats

2) compare the effects of L-DOPA on the dialysate monoamine levels of the AS and AS/AGU rats

5.4 Adenosine and dopamine interactions

Recent evidence shows that dopaminergic neurotransmission in striatum can be modulated by adenosine analogues (Zetterstrom & Filenz, 1990). A1 receptor agonists have been shown to decrease dopamine release *in vivo* from the rat striatum (Zetterstrom & Filenz, 1990). A non-selective A1/A2 receptor antagonist has been shown to increase dopamine release in the rat striatum, implying that endogenous extracellular adenosine may tonically suppress dopamine release (Ferre *et al*, 1993). This presynaptic adenosine-dopamine interaction seems to involve A1 receptors since the inhibition of dopamine release by adenosine agonists was blocked by selective A1 receptor antagonists (Zetterstrom & Filenz, 1990). In contrast, most of the evidence regarding adenosine-induced behavioural effects suggests that A2 receptors are involved in the interaction of dopaminergic neurotransmission (Ferre *et al*, 1992). A2 receptor-induced catalepsy has been counteracted by specific D2 receptor agonists (Ferre *et al*, 1991).

An important finding is that A2 receptors have been shown to be co-localised with D2 receptors on the GABA/enkephalinergic neurones which project to the GPe (Fink *et al*, 1992, Ferré *et al*, 1992). Dopamine neurones arising in the substantia nigra, by acting on the D2 receptors inhibit the indirect pathway, causing an inhibition of the GPi neurones and hence a facilitatory effect on motor activity. Activation of A2 receptors has been shown to decrease the affinity of D2 receptors for dopamine (Ferre *et al*, 1994) and A2 receptor

blockade has been shown to enhance postsynaptic D2 receptor transduction (Ferre *et al*, 1993).

Since activation of A1 receptors decreases dopamine release and activation of A2 receptors decreases the affinity of D2 receptors for dopamine, the present experiments were designed to establish the extracellular concentration of adenosine in the striatum in the AS/AGU rats in comparison with that in the AS rats.

## 6.0 METHODS

### 6.1 The surgical procedure

All animal procedures were in accordance with the Home Office Guidelines and were specifically licensed under the Animal (Scientific Procedures) Act 1986.

All animal were bred 'in house', unless stated otherwise, and had free access to food and water at will.

Male Albino Swiss (AS) or Albino Swiss/ Anatomy Glasgow University (AS/AGU) rats (aged 5 months, approximately 330g) were anaesthetised using ketamine (Vetalar 10mg/ml, Parke-Davis Veterinary) and xylazine (Rompun 20mg/ml, Bayer) in a ratio of 2:1, 1.1-1.2ml/kg. A homeothermic blanket maintained rectal temperature at 36-37$^{o}$C during surgery. The rats were mounted in a David Kopf stereotaxic frame with the incisor bar at -3.5mm. The skull was exposed and one drop of lignocaine and adrenaline solution (Norbrook Laboratories (GB) Ltd.) was used to keep the skull free from excess blood. Two skull screws were placed in holes which were drilled on the left hand side of the skull. A 20G guide cannula, 1.6cm long, was stereotaxically inserted into the anterior caudate putamen (ACPu) (+1.0mm AP, 2.5mm L; 3.5mm V) or into the posterior caudate putamen (PCPu) (-1.0mm AP, 4.25mm L, 5mm V) after a hole at the relevant position was drilled and after the dura mater was pierced. All co-ordinates were relative to the bregma suture (Paxinos & Watson, 1986). Before insertion of the guide cannula, dental cement (Redifast pink powder and liquid, Wright Health Group Ltd.) was spread around the screws and the cannula to anchor the cannula in place. The cannula was blocked by a stylet 1.6cm long. The wound was sutured if necessary and the animal removed from the stereotaxic frame and placed in a cage. All animals were watched while recovering from the operation and anaesthetic and occasionally 0.1ml of atipamezole (Antisedan 5mg/ml, Smith Kline Beecham) was administered subcutaneously to allow speedy recovery. A 5ml s.c. injection of sodium chloride (0.18%) and glucose (4.0%) (Baxter) was administered after surgery as a nutrient and for rehydration.

### 6.2 Microdialysis

Two to three days after surgery the animal was placed in a perspex cage and allowed to move freely throughout the experiment. The microdialysis system used for freely moving animals is shown in fig 6.1. Perfusion fluid was perfused through a dual channel liquid swivel and a microdialysis probe. The animal was restrained and the microdialysis probe was then inserted into the striatum through the guide cannula, after removal of the stylet. The resultant depth of the probe tip was 2.5mm lower than the cannula depth i.e. 6mm for the ACPu and 7.5mm for PCPu from the bregma. The position of the probe in the ACPu and PCPu are shown in fig 6.2a & b. Epoxy glue took up 0.5mm of the tip, leaving 2mm of membrane for active diffusion. The probes were continuously perfused with acsf, composed of 125mM NaCl, 3.3mM KCl, 1.25mM $KH_2PO_4$, 2.4mM $MgSO_4$, 1.2mM $CaCl_2$ (pH 7.2) at a flow rate of 2µl/min using a CMA 100 microinjection pump (CMA Medicin, Stockholm, Sweden). Dialysate samples were collected every 20min into vials manually or by CMA 170 Refrigerated Automated Fraction Collector. Sample collection began 120 minutes after the probe was inserted through the cannula, unless otherwise stated. In the case of monoamine analysis, the 40µl dialysates were collected into vials already containing 5µl of DHBA (4.05µM) in HCl (0.9M). DHBA and HCl act as an internal standard for HPLC analysis and a monoamine stabiliser, respectively. The final concentration of DHBA was 0.45µM, and that of HCl was 0.1M. In the case of purine analysis, the 40µl dialysates were collected into empty vials. All samples were either analysed immediately or snap frozen in liquid nitrogen and stored at -80$^0$C for later HPLC analysis.

Drugs administered intrastriatally by reverse dialysis were dissolved in acsf, pH7.2. The lag-time between the syringe selector and the collection chamber was 30 min and this was accounted for in all experiments i.e. the sample was collected 30 min after the syringe with the drug in it was selected. Test and control solutions were changed by means of a CMA 111 Syringe Selector which caused little disturbance to the flow. Three basal levels were achieved before administering drugs, the mean of these basal levels was calculated and compared with evoked release by paired t-test.

At the end of each experiment, one of two procedures were carried out. Either the rats were killed by overdose of urethane. 10% pontamine sky blue dye solution was then perfused through the probes for 2 minutes, after which the brains were removed and placed into 10% formalin pH 7 to fix overnight. The brains were subsequently sectioned manually using a razor blade in order to locate the dye. Alternatively, the rats were anaesthetised by $CO_2$ gas and killed by cervical dislocation. The brains were immediately removed and placed on dry ice for post-mortem analysis to be carried out by micropunch. The probe location and tissue sampling is explained in detail in section 6.4.

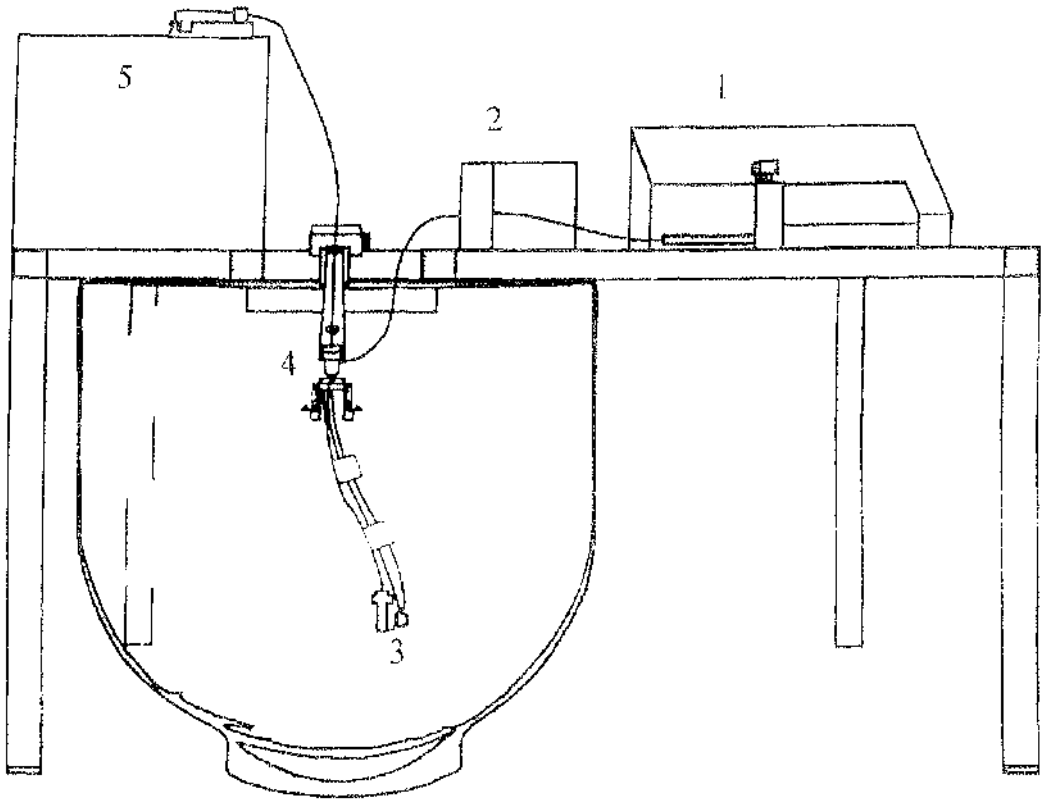



Fig 6.1
Microdialysis system for freely moving animals

1. Microinjection Pump
2. Syringe Selector
3. Microdialysis Probe
4. Liquid Swivel + Counter-Balancing Arm
5. Automatic Fraction Collector

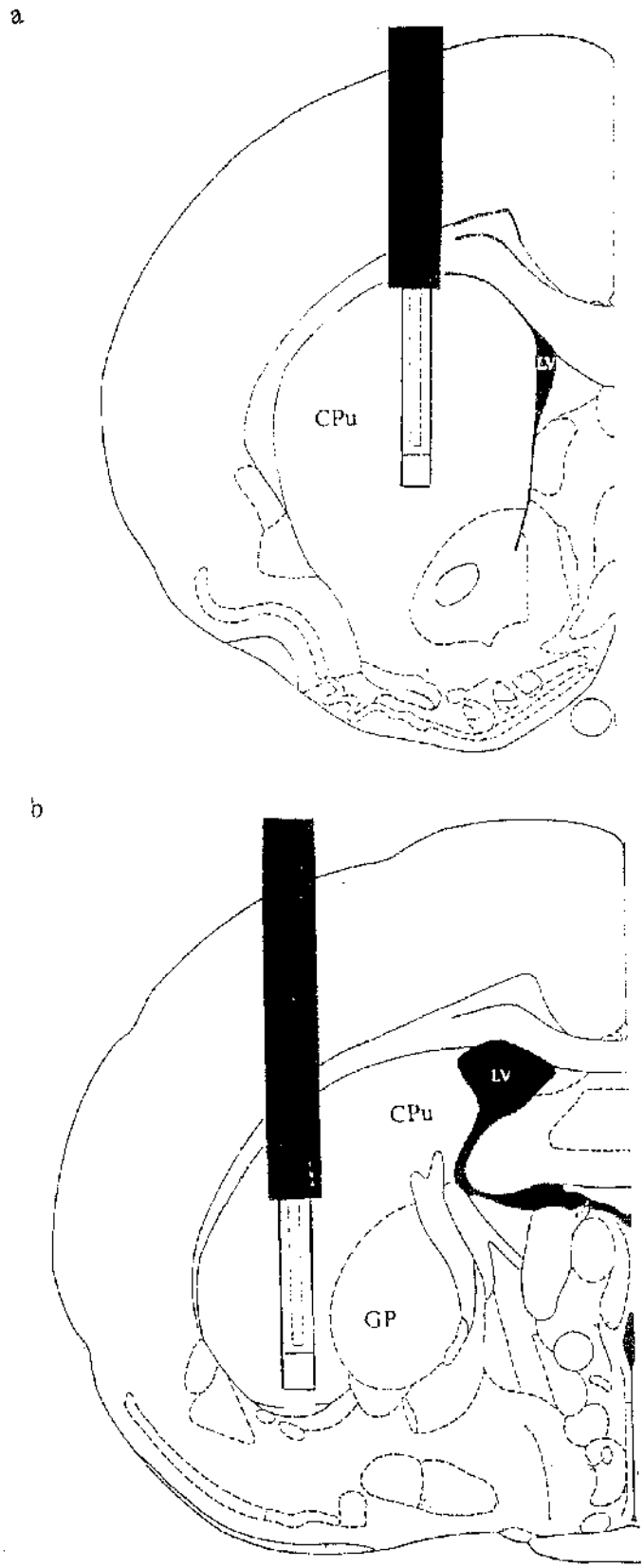



Fig 6.2
Position of the probe within the a) ACPu and b) PCPu

6.3 Protocol for chronic L-dopa injection

L-dopa methyl ester and benserazide were dissolved in sodium metabisulphite 0.1% solution which was filtered and degassed and injected daily into two AS and two AS/AGU rats over 5 days. Microdialysis experiments were carried out on day 5 from the PCPu. L-dopa methyl ester 500mg/kg was injected 30 min after 50mg/kg benserazide once a day for two days. On days three and four of the protocol the doses were halved due to hyperactivity of one of the groups of animals. On day three a guide cannula was surgically implanted into the left hand striatum using the coordinates for the posterior CPu. On day five the animals were injected with the original doses and 1hr 15min after the L-dopa injection, the animals were gently restrained and a microdialysis probe was inserted into the posterior CPu through the guide cannula after removing the stylet. Samples were collected for 3hrs 40min after probe insertion.

6.4 Materials

The following materials were used:

From Sigma Chemicals: benserazide, DHBA, L-DOPA, DOPAC, dopamine and HVA.

6.5 Microdialysis Probe Construction (concentric probe design)

The microdialysis probes have very similar design to those previously described (section 2.6.) with slight modifications for the use in the striatum in conscious animals.

1) A 23G Microlance needle is filed to a length of 20mm. The needle is inserted into the guide cannula to check that it has a smooth fit. The needle is removed from cannula.

2) At least 10mm Hospal polyacrylonitrile membrane (0.3mm, o.d.) is fed through the lumen of the filed needle. Forceps are used at all times to avoid plugging pores with grease or dirt

3) 2.5mm of the membrane is allowed to be exposed from inside of the needle and the join between the membrane and the needle is glued, using resin and hardener in a 50: 50 mixture (RS Components), so as not to get any glue on the membrane below the needle join. It is essential that the minimum amount of glue is on the outside of the needle as glue here would compromise an easy fit into the guide cannula.

4) Portex tubing (0.58mm i.d.) is attached to the opposite end of the needle and the join with the needle is glued.

5) A hole in the Portex tubing is made with a needle and silica tubing (i.d. 40μm) is fed through the lumen of the dialysis membrane towards the hole. The silica tubing is cut and adjusted so that the end is positioned at least 1mm from the tip of the dialysis membrane, with at least 5mm left outside the Portex tubing. The tip of the dialysis membrane is plugged with epoxy and left to dry before the silica tubing is pushed further down so that it is 1.0mm from the tip (0.5mm of which is taken up by the epoxy glue).

6) The hole in the Portex tubing is glued (i.e. the join between the silica tubing and the Portex tubing). The Portex tubing is cut so that it is 2cm long. 2×30cm thinner Portex tubing (0.28mm i.d.) are cut and angled at the ends. One piece of this thinner Portex tubing is slipped into the thicker (0.58mm i.d.) Portex tubing and the join is glued. The second piece of thinner tubing is slipped over the 5mm remaining silica tubing and the join is sealed with epoxy.

6.6 Micropunch

A maximum of seven days after the surgical operation for cannula implantation (3 to 5 days after the microdialysis experiment) the AS and AS/AGU rats were anaesthetised with $CO_2$ gas and killed by cervical dislocation. Only the rats which did not receive any drug treatment were used for micropunch tissue analysis. The brains were immediately dissected out and placed onto dry ice. The brains were sectioned coronally from rostral to caudal into 20μm slices using a cryostat until the corpus callosum was reached. At this point, in the ACPu, a micropunch core (1.0mm id and 1.0mm length) was taken from the right hand side of the brain (the opposite side to that of the microdialysis guide cannula and probe position) (fig 6.3a). If the dialysis experiment was performed in ACPu in that animal, after extracting the tissue core, the brain was further sectioned to the plane of the dialysis probe position in the left hand side and the location was verified by light microscopy. The core was approximately 0.5mm rostral and 0.5mm caudal to the microdialysis probe tract. Sectioning continued until the anterior commissure and fornix joined to make a triangle. At this point micropunch cores were taken from the PCPu in three regions, namely the dorsal

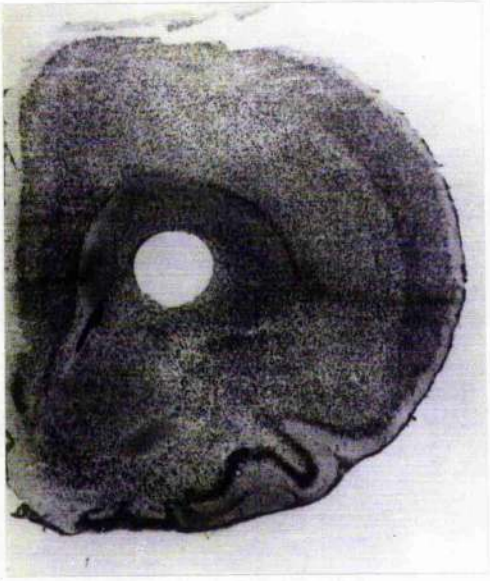
(DCPu), middle (MCPu) and ventral (VCPu) caudate putamen (fig 6.3b). If the dialysis experiment was performed in the PCPu in that animal, after extracting the tissue cores, the brain was further sectioned to the plane of the dialysis probe position in the left hand side and the location was verified by light microscopy. The core was approximately 0.5mm rostral and 0.5mm caudal to the microdialysis probe tract.

All micropunch samples were immediately frozen in liquid nitrogen for deproteinisation and HPLC analysis.

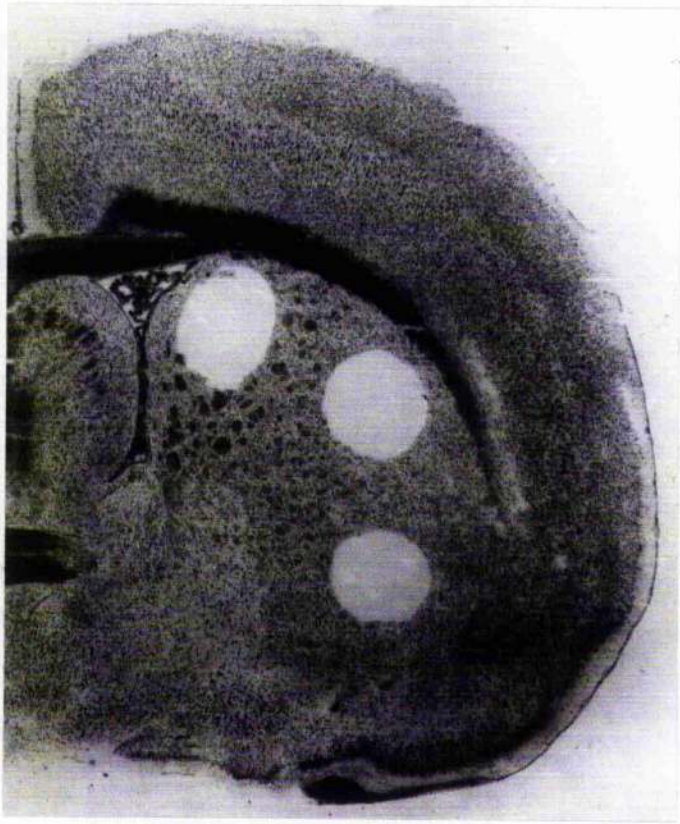
To each micropunch core of tissue 100µl 0.1M HCl and 50µl of 0.45µM DHBA were added. HCl was added to stabilise the amines and break down the protein and DHBA was added as an internal standard. The samples were then centrifuged at 3000g at $4^0$C for 10 min, vortexed for 30 seconds and then centrifuged again at 3000g, $4^0$C for 10 min. Supernatants of the samples were transferred to a fresh eppendorf tube and frozen at -80°C until HPLC analysis. The protein pellets, left-over after removal of the supernatant, were used for protein estimations using the Lowry assay (Lowry *et al*, 1951), the calibration curve of which is shown is fig 6.4.

Statistical analysis was carried out between the AS and AS/AGU groups for the ACPu, DCPu, MCPu and VCPu using an unpaired t-test. DOPAC to dopamine concentration ratios give an indication of dopamine utilisation at the nerve terminal. Ratios were calculated for each tissue extract and comparison between AS and AS/AGU groups were made using unpaired t-test. Statistical analysis was also carried out between each region for both the AS and the AS/AGU groups by unpaired t-test.

a



b



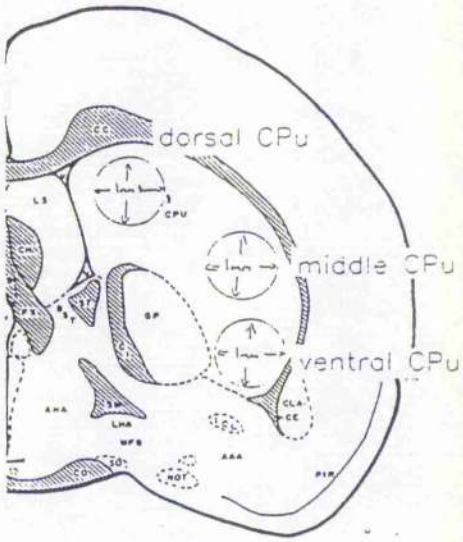



Fig 6.3
Position of the micropunch cores taken from the (a) ACPu (b) PCPu

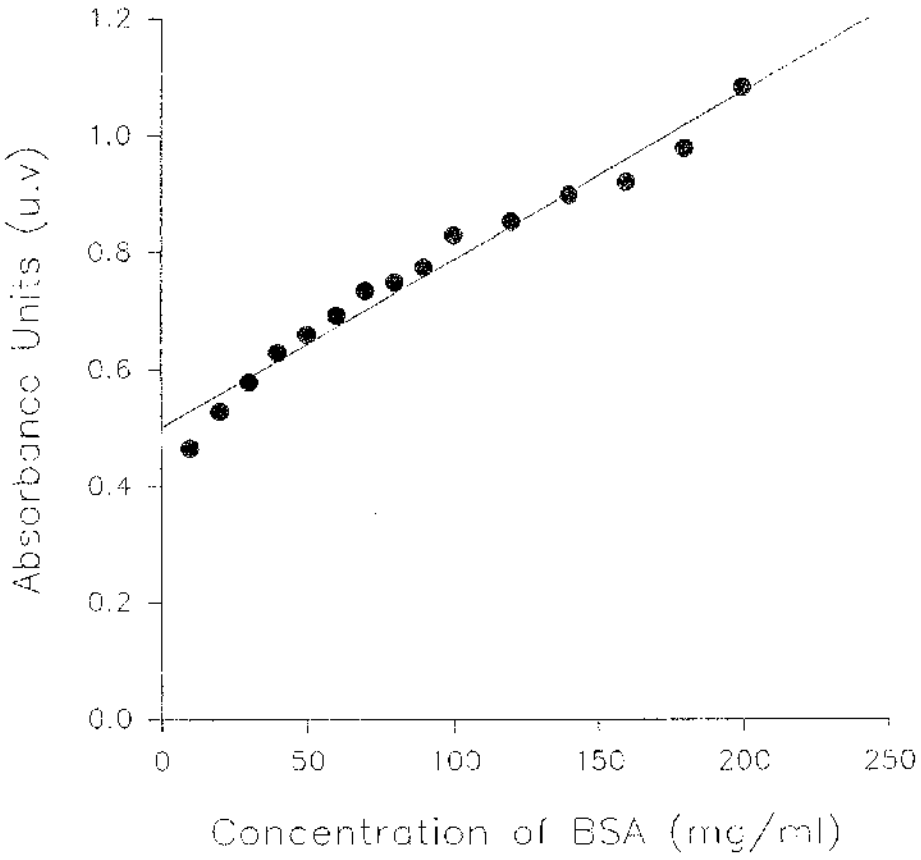



Fig 6.4

Calibration curve for the determination of protein content in micropunch samples using Lowry Assay (BSA; bovine serum albumin).

6.7 Monoamine analysis

Monoamines were analysed by an isocratic ion pair HPLC system using electrochemical detection (Gilson manometric module #802, Gilson electrochemical detection model #141, Gilson pump model #302). Each sample was injected onto a 10µl loop by means of a Rheodyne Switch, model 3175.

For micropunch analysis, an automated system was used (Gilson electrochemical detection model #141, Gilson pump model #307, Gilson sampling injector #231XL, Gilson dilutor #401C and Rheodyne switch 7010).

DOPAC, DHBA, dopamine and HVA in both systems were separated out by a 25 cm × 4.5mm C18 5µm Hypersil column and a mobile phase consisting of 83mM citric acid, 1.0mM ethylenediamine tetra acetic acid (EDTA), 43mM disodium hydrogen phosphate, 0.2mM octane sulphonic acid and 10% methanol at a flow rate of 1ml/min. Each run time was 40 minutes, with retention times of DHBA at 4.2 min, dopamine at 5.4 min, DOPAC at 9.8 min and HVA at 35 min. The detection limit of each sample was 0.053ng/10µl (0.28pmol/10µl) for dopamine, 0.042ng/10µl (0.25pmol/10µl) for DOPAC and 0.063ng/10µl (0.35pmol/10µl) for HVA.

A standard solution was injected onto the HPLC system every seven samples and an example of a typical chromatogram is shown in fig 6.5 a. The identification of compounds was achieved by comparison of retention time with standards. Peak areas were computer analysed by Gilson 712 HPLC software. Quantification of the compounds was achieved by proportion using the internal standard (DHBA). Table 6.1 shows the correlation coefficients and '$r^2$' values for the graphs seen in fig 6.6.

The amount of monoamine in the dialysate samples, as measured by HPLC by manual injection (10µl loop), was corrected for the dilution which occurred from addition of the internal standard and HCl.

A chromatogram from a typical dialysate sample is shown in fig 6.5b. The HPLC did not detect dialysate HVA, presumably because of the sensitivity required for the detection of this compound.

The HPLC system used in the present study was used mainly for teaching and research within the laboratory run by Dr. Des Gilmore. Therefore attempts to increase the sensitivity of the HPLC were restricted.

The injection loop of the HPLC of the automated system used for the micropunch samples was 20µl and the volume of each micropunch sample was 150µl. The amount of monoamine in the micropunch samples, as measured by the automated HPLC, was multiplied by 7.5 to give the total amount of monoamine in each tissue extract. By dividing this by the amount of protein, as estimated by the Lowry Assay, the amount of monoamine per weight of protein was calculated.

Table 6.1

The correlation coefficients for the monoamines analysed by HPLC.

| Monoamine | r | $r^2$ |
|---|---|---|
| DHBA | 0.992 | 0.984 |
| Dopamine | 0.994 | 0.987 |
| DOPAC | 0.994 | 0.989 |
| HVA | 0.994 | 0.988 |

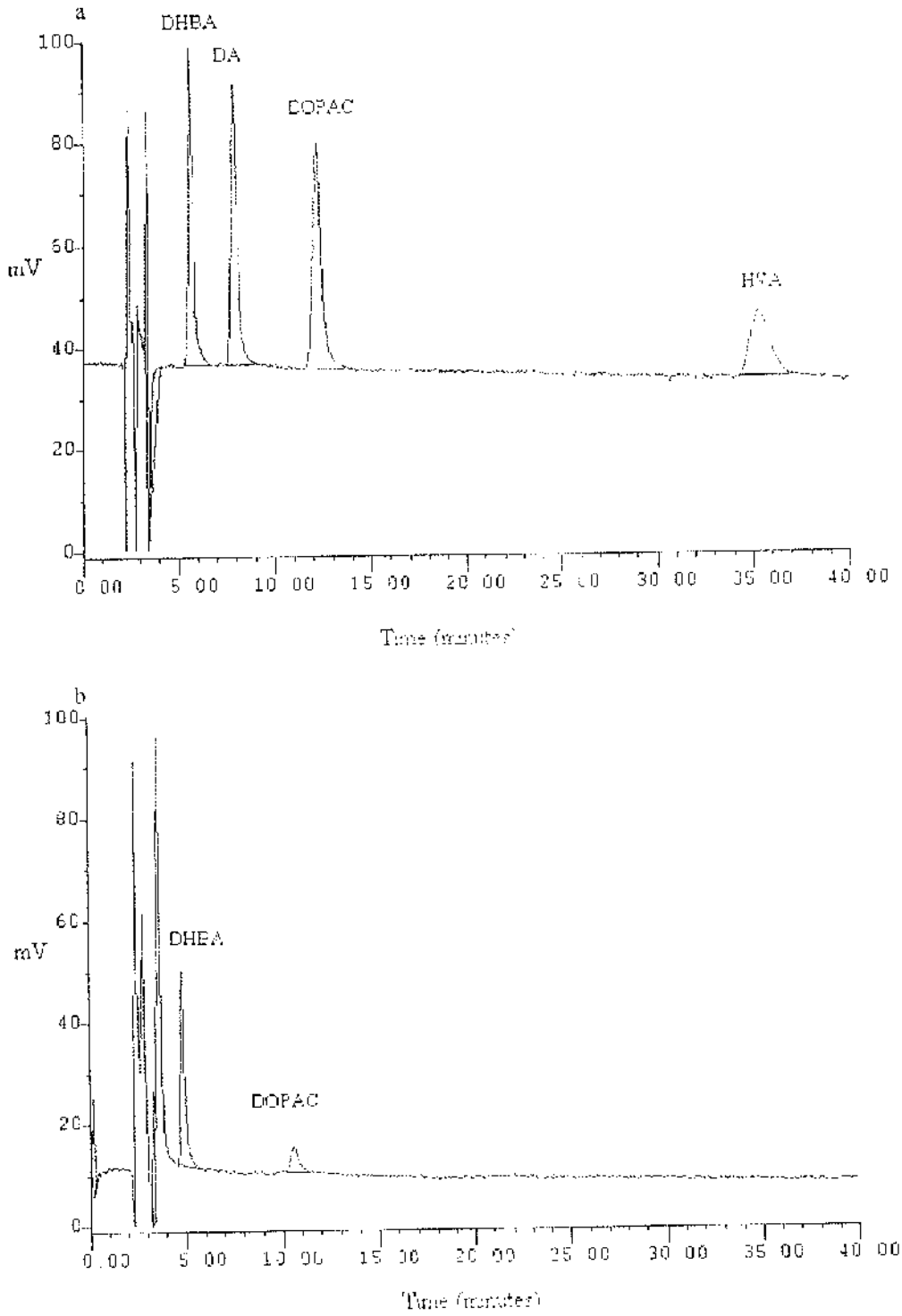



Fig 6.5

**Chromatogram of (a) monoamine standard and (b) an example of a dialysate sample taken from the ACPu 1ng/10µl**

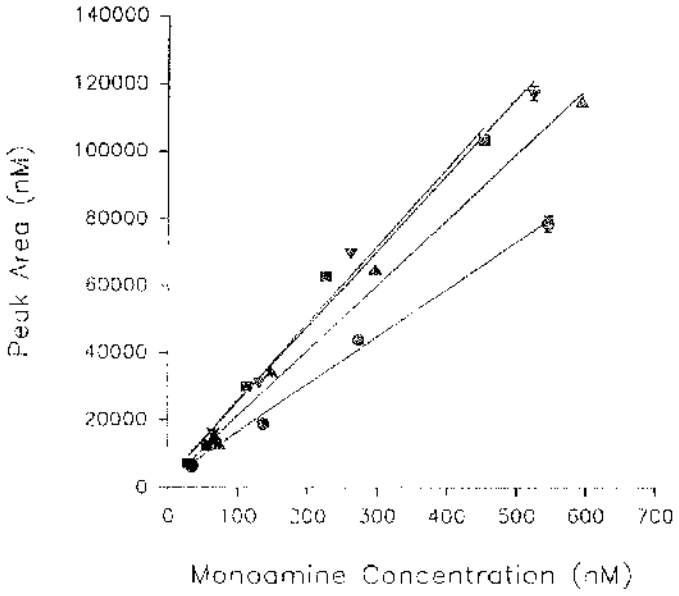



Fig 6.6

Calibration Graph for the determination of DHBA, dopamine, DOPAC and HVA by HPLC.

- DHBA
- DOPAC
- dopamine
- HVA

6.8 Purine Analysis

Purines were analysed using an isocratic HPLC (Severn Analytical Solvent Delivery System SA6410B) and ultraviolet detection at 254nm (Severn Analytical uv/vis Absorbance Detector SA6500) and a Rheodyne model 7125 injector (100µl loop).

Two types of columns and two mobile phases were used. Firstly, the mobile phase used was 0.01M sodium phosphate ($NaH_2$ $PO_4$) with 15% methanol (HPLC Grade) pH 6.1 at a flow rate of 0.8ml/min. The column used with this mobile phase was a techsphere C18 3µm microsphere column, 10cm by 4.6mm, was used to separate out adenosine at 2.4 min retention (fig 6.7). Hypoxanthine, xanthine and inosine were washed off the column with the solvent front using 15% methanol in the mobile phase (instead of 6% methanol which was used with a similar column in Part I of the present thesis) and adenosine was separated out at a retention time of 5.2min. By increasing the methanol component from 6% to 15%, the limit of sensitivity for adenosine was improved from 1.5pmol to 0.14pmol.

Secondly, the mobile phase used was 0.01M sodium phosphate ($NaH_2$ $PO_4$) with 6% methanol (HPLC Grade) pH 6.1 at a flow rate of 0.8ml/min. The column used with this mobile phase was a techsphere C18 3µm microsphere column, 10cm by 2.1mm (a microbore column), and was used to separate out purines at the following retention times: hypoxanthine 2min, xanthine at 2.2min, inosine at 3.6min and adenosine at 10min (fig 6.7). By using the microbore column instead of the normal column, the limit of sensitivity for adenosine was improved from 1.5pmol to 0.03pmol.

Identification of adenosine in the dialysate samples was achieved by comparison of retention time with standards. Chromatogram peak heights were measured manually and quantification of adenosine was achieved by parallel chromatography of standards (fig 6.8). Table 6.2a & b shows the correlation coefficient and 'r' values for the calibration graphs seen in fig 6.8.

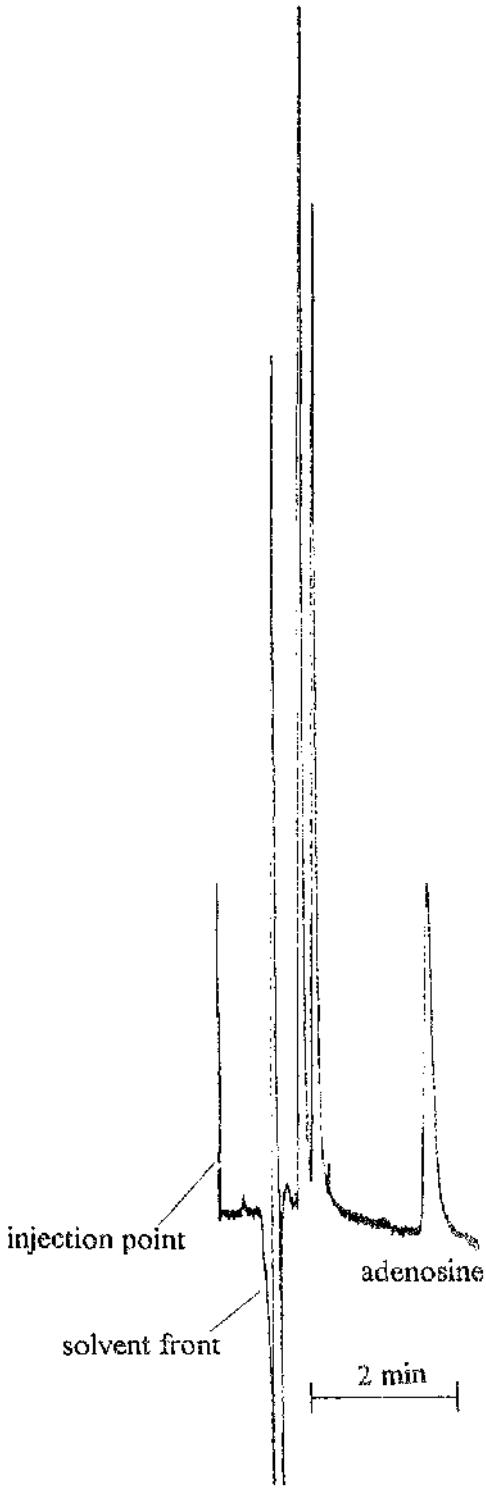



Fig 6.7a

Chromatogram of adenosine using the mobile phase with 15% methanol 1μM

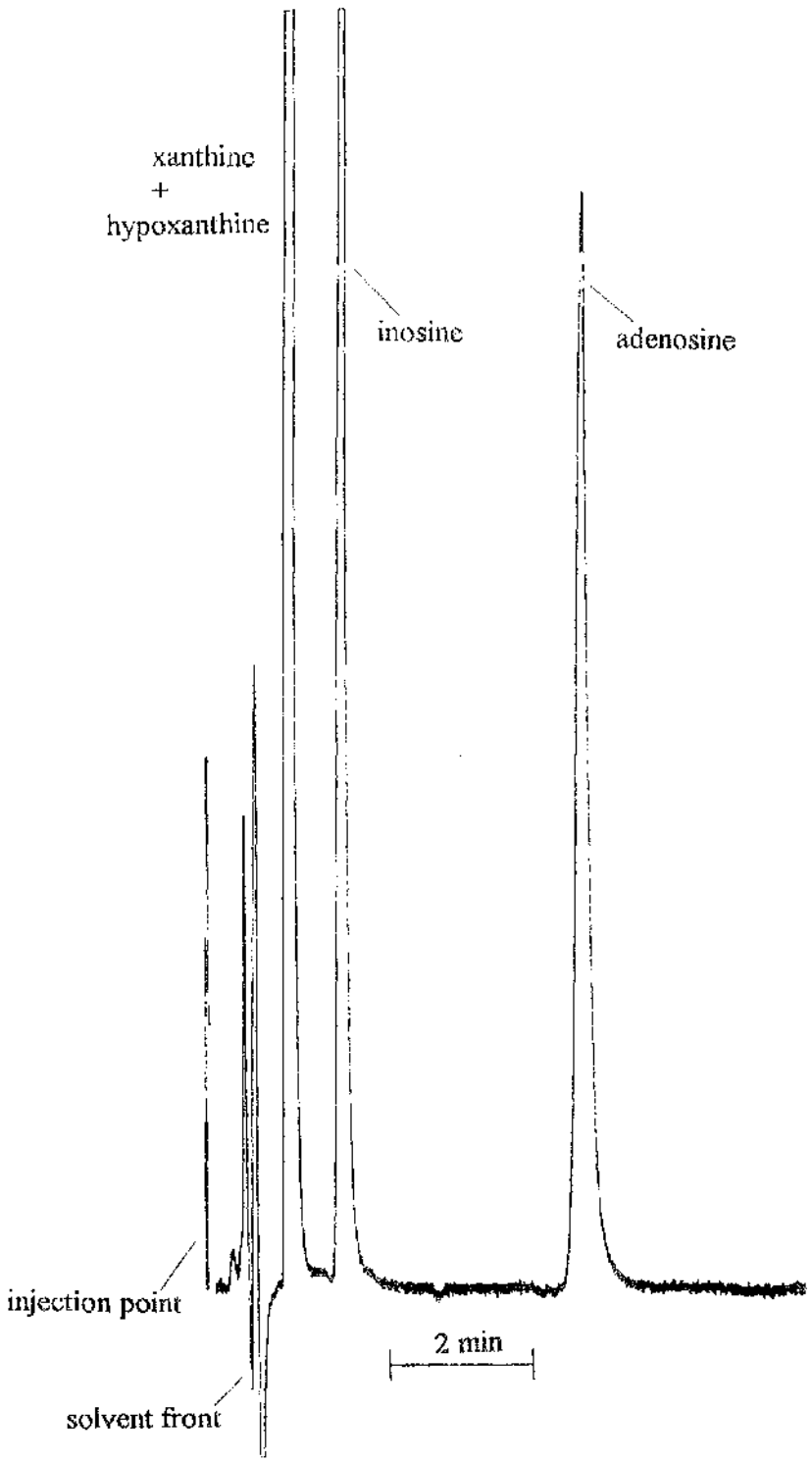



Fig 6.7b

Chromatogram of purines using the mobile phase with 6% methanol and microbore column

1μM

Table 6.2a

The correlation coefficient for adenosine analysed by HPLC using the mobile phase with 15% methanol and the normal column.

| | r | r2 |
|---|---|---|
| Adenosine | 0.9958 | 0.9916 |

Table 6.2b

The correlation coefficient for adenosine analysed by HPLC using the mobile phase with 6% methanol and the microbore column.

| | r | r2 |
|---|---|---|
| Adenosine | 0.9992 | 0.9983 |

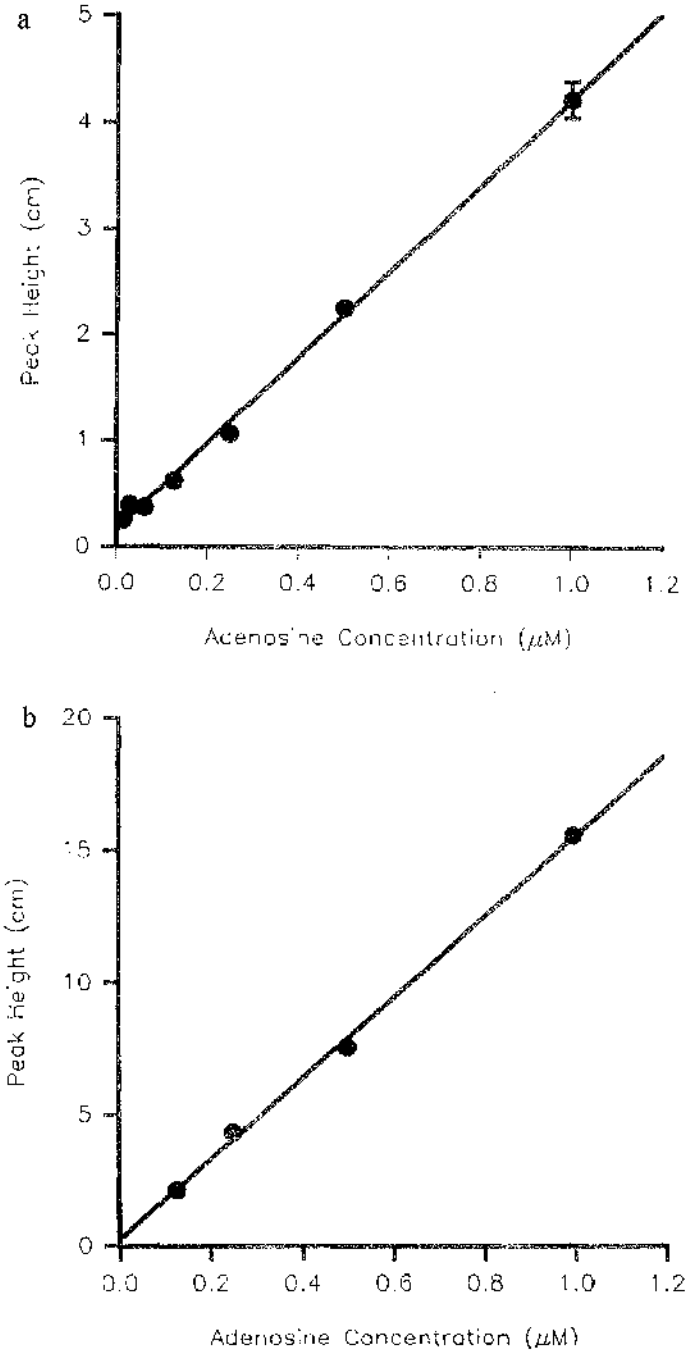



Fig 6.8

Calibration graph for the determination of adenosine by HPLC using (a) the mobile phase with 15% methanol and normal column (b) the mobile phase with 6% methanol and microbore column

## 7.0 RESULTS

### 7.1. Monoamines

#### 7.1.1 Release of monoamines from the ACPu by microdialysis

The basal levels of dopamine, DOPAC and HVA in the AS/AGU rats were compared to that of AS rats. Two hours settling period after probe insertion was allowed before the collection of five basal levels. The lowest mean DOPAC level was 0.223 ± 0.168ng/10µl in the AS/AGU rats and 0.166 ± 0.039ng/10µl in the AS rats and the highest mean DOPAC level was 0.412 ± 0.308 ng/10µl in the AS/AGU rats and 0.248 ± 0.066 ng/10µl in the AS rats (fig 7.1). Statistical analysis using ANOVA followed by an unpaired t-test showed that these differences were not significant.

Dialysate dopamine levels were found to be near the limits of detection of the HPLC system (table 7.1). Dopamine dialysate levels in control AS rats which were detectable were approximately 4 fold lower than those of dialysate DOPAC levels in these rats. Such an assessment could not be made for the AS/AGU rats due to the non-detectable levels of dopamine.

The levels of HVA in the ACPu dialysates were also close to the limits of detection. Table 7.2 illustrates the levels of detectable samples.

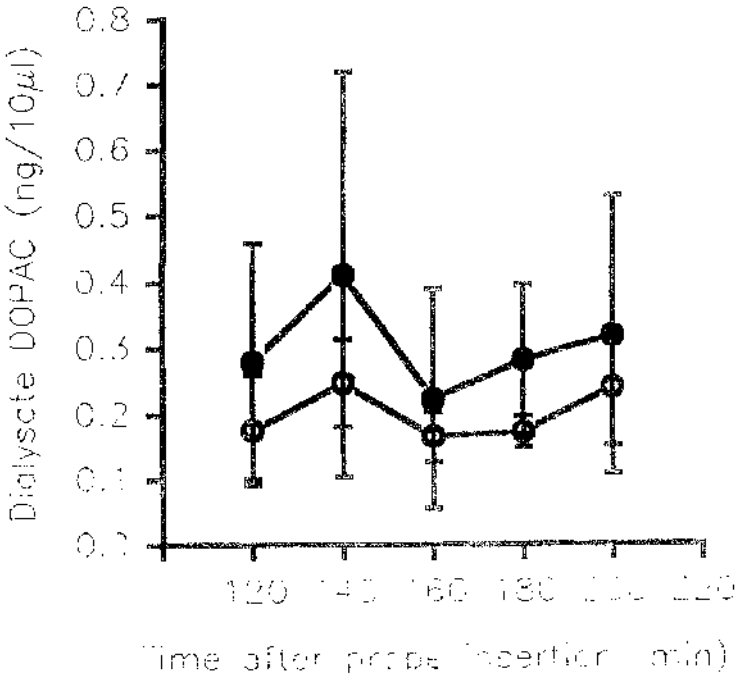



Fig 7.1

DOPAC dialysate levels of 5 month old rats (ng/10µl) in the anterior CPu

- ○ Control n=3
- ● AS/AGU n=4

Table 7.1 Dialysate Dopamine Levels (ng/10μl) in the ACPu in 5 Month Old Rats (N.D.; non-detectable).

| Time (min) | AS | AS | AS | AS/ AGU | AS/ AGU | AS/ AGU |
|---|---|---|---|---|---|---|
| 120 | 0.103 | N.D. | N.D. | 0.07 | N.D. | N.D. |
| 140 | 0.1 | N.D. | 0.176 | N.D. | N.D. | N.D. |
| 160 | 0.09 | N.D. | 0.157 | N.D. | N.D. | N.D. |
| 180 | N.D. | N.D. | N.D. | 0.083 | N.D. | N.D. |
| 200 | N.D. | N.D. | 0.109 | 0.093 | N.D. | N.D. |

Table 7.2 Dialysate HVA levels (ng/10μl) in the ACPu in individual 5 Month Old Rats (N.D.; non-detectable). Time refers to time after probe insertion.

| Time | AS | AS | AS | AS/ AGU | AS/ AGU | AS/ AGU |
|---|---|---|---|---|---|---|
| 120 | N.D. | N.D. | 0.087 | 0.343 | N.D. | 0.29 |
| 140 | 0.25 | N.D. | 0.126 | 0.225 | N.D. | 0.48 |
| 160 | N.D. | N.D. | N.D. | 0.233 | 0.464 | N.D. |
| 180 | N.D. | N.D. | N.D. | 0.211 | N.D. | 0.21 |
| 200 | N.D. | N.D. | N.D. | 0.573 | 0.211 | N.D. |

7.1.2 Monoamine tissue levels from the ACPu by micropunch

The tissue levels determined from ACPu in 5 month old rats for dopamine was 59.0 ± 11.0 pg/μg protein (mean ± sem) for the AS rats and 71.0 ± 33.0 pg/μg protein (mean ± sem) for AS/AGU rats (fig 7.2a, n.s., unpaired t-test). For DOPAC, the tissue levels detected were 4.8 ± 4.0 pg/μg protein (mean ± sd) for AS rats and 17.0 ± 15.0 pg/μg protein (mean ± sd) for AS/AGU rats (fig 7.2b, n.s., unpaired t-test). The DOPAC to dopamine ratio for the tissue levels in AS and AS/AGU rats was 0.156±0.043 and 0.135±0.009, respectively (table 7.3, n.s. unpaired t-test).

Table 7.3

The DOPAC/ dopamine ratios calculated using the tissue of DOPAC and dopamine in the ACPu, DCPu, MCPu and VCPu for AS/AGU and AS rats.

| Region in CPu | AS | AS/AGU | Statistics |
|---|---|---|---|
| ACPu | 0.156±0.043 | 0.135±0.009 | n.s., unpaired t-test |
| DCPu | 0.226±0.041 | 0.23±0.076 | n.s., unpaired t-test |
| MCPu | 0.143±0.031 | 0.197±0.05 | n.s., unpaired t-test |
| VCPu | 0.355±0.091 | 0.413±0.185. | n.s., unpaired t-test |

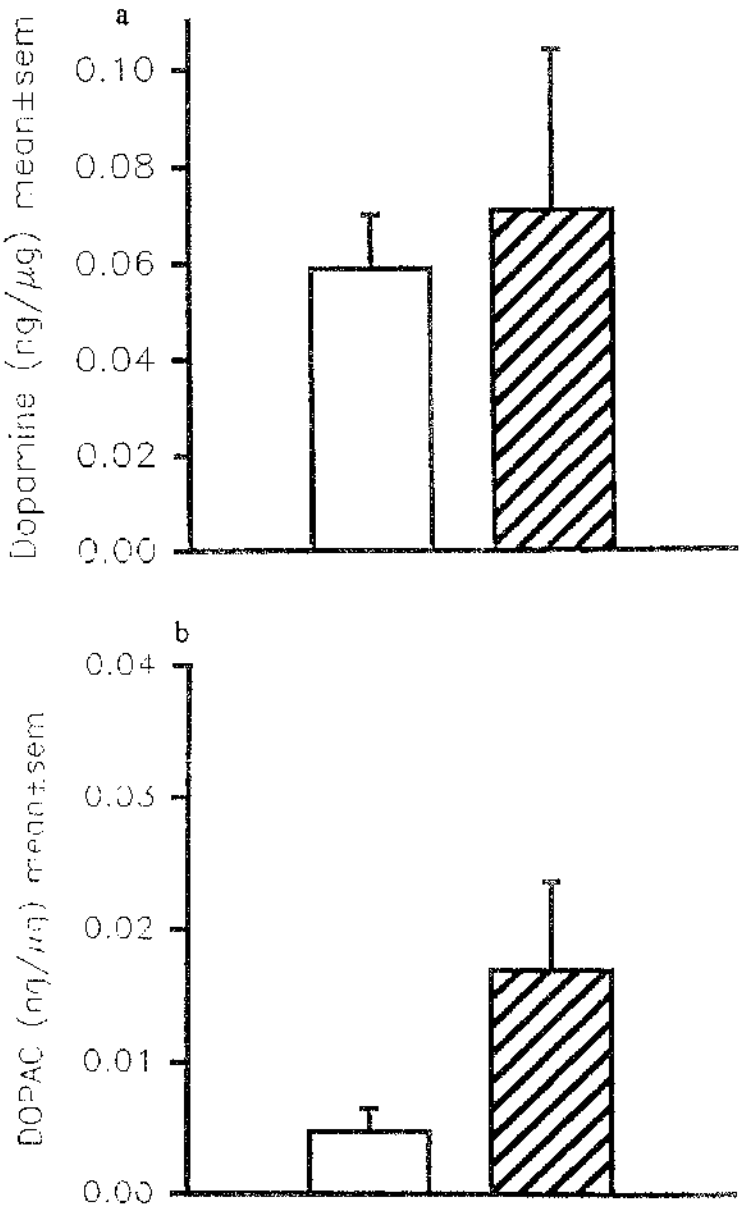



Fig 7.2

Micropunch tissue levels of 5 month old rats (ng/µg) in the anterior CPu for a) dopamine and b) DOPAC.

control n=5 AS/AGU n=5 (n.s. to control).

7.1.3 Release of monoamines from the PCPu by microdialysis

Dialysate levels of dopamine and DOPAC in the PCPu were collected immediately after probe insertion for 3 hrs 40 min. Dialysate dopamine was detected in under half of the experiments carried out on the control AS group of rats and was not detected in any of the AS/AGU group of rats. These values are shown in table 7.4. The DOPAC dialysate levels in the PCPu of 5 month old rats of the AS/AGU rats were not significantly different from the AS values, with levels of approximately 0.1ng/10μl in both groups of rats (fig 7.3).

7.1.4 Monoamine tissue levels from the PCPu by micropunch

The dopamine and DOPAC tissue levels in each of the three PCPu cores were shown not to be significantly different between the AS and AS/AGU rats in any of the regions except for the ventral CPu (fig 7.4-7.6). In the VCPu the DOPAC level for the AS rat was 4.27 ± 0.74 pg/μg protein (mean ± sem) and for the AS/AGU rat was 2.21 ± 0.6 pg/μg protein (mean ± sem) (*$p<0.05$, unpaired t-test, fig 7.6b). Tissue DOPAC/ dopamine ratios for AS and AS/AGU rats are illustrated in table 7.3. There was no significant difference between the AS and AS/AGU rats in any of these regions using unpaired t-test. Comparison of the ratios for each region, DCPu, MCPu, VCPu and ACPu, did not reveal any significant difference within either the AS group of rats or the AS/AGU rats. However the mean of the ratios in VCPu is higher than the ratios in the other three regions for both AS and AS/AGU rats, showing a trend for an increased dopamine utilisation in this area for both AS and AS/AGU rats.

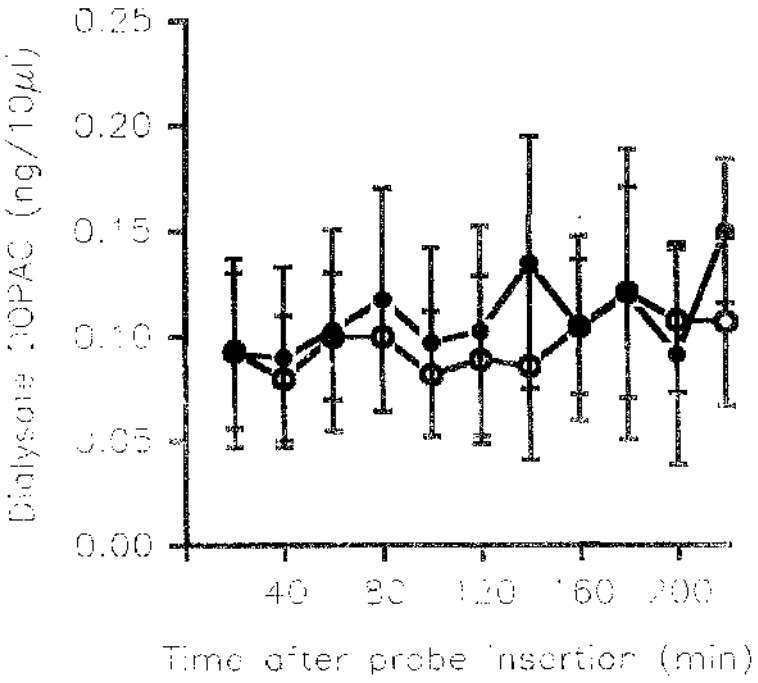



Fig 7.3

DOPAC dialysate levels of 5 month old rats (ng/10µl) in the posterior CPu

- Control n=3
- AS/AGU n=4

Table 7.4

Dialysate Dopamine (ng/10μ) Levels in the Posterior Caudate Putamen in 5 Month Old Rats.

| Sample # | AS | AS | AS | AS/ AGU | AS/ AGU | AS/ AGU |
|---|---|---|---|---|---|---|
| 1 | 0.54 | 0.133 | N.D. | N.D. | N.D. | N.D. |
| 2 | 0.15 | 0.064 | N.D. | N.D. | N.D. | N.D. |
| 3 | 0.104 | N.D. | N.D. | N.D. | N.D. | N.D. |
| 4 | 0.11 | N.D. | N.D. | N.D. | N.D. | N.D. |
| 5 | 0.16 | N.D. | N.D. | N.D. | N.D. | N.D. |
| 6 | 0.11 | N.D. | N.D. | N.D. | N.D. | N.D. |
| 7 | 0.11 | N.D. | N.D. | N.D. | N.D. | N.D. |
| 8 | 0.08 | N.D. | N.D. | N.D. | N.D. | N.D. |
| 9 | 0.06 | N.D. | N.D. | N.D. | N.D. | N.D. |
| 10 | N.D. | 0.176 | N.D. | N.D. | N.D. | N.D. |
| 11 | N.D. | N.D. | 0.062 | N.D. | N.D. | N.D. |

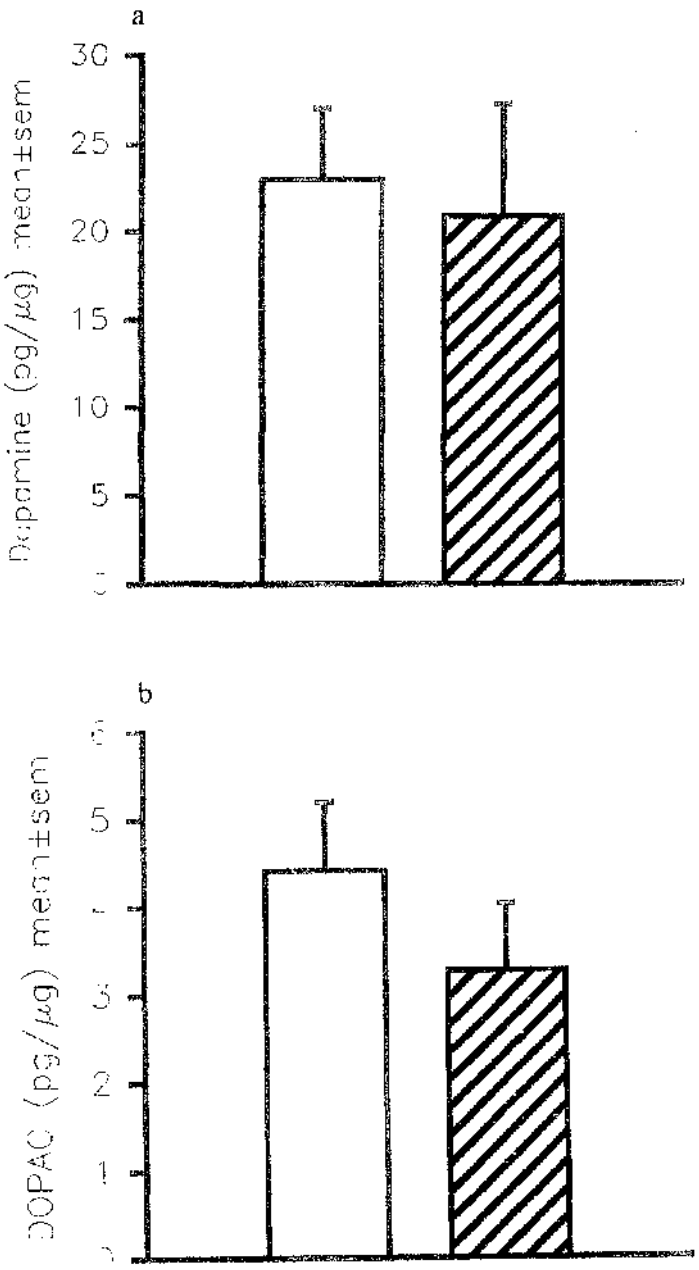



Fig 7.4

Micropunch tissue levels of 5 month old rats (pg/µg) in the DCPu a) dopamine and b) DOPAC

control n=10 AS/AGU n=9 (n.s. to control).

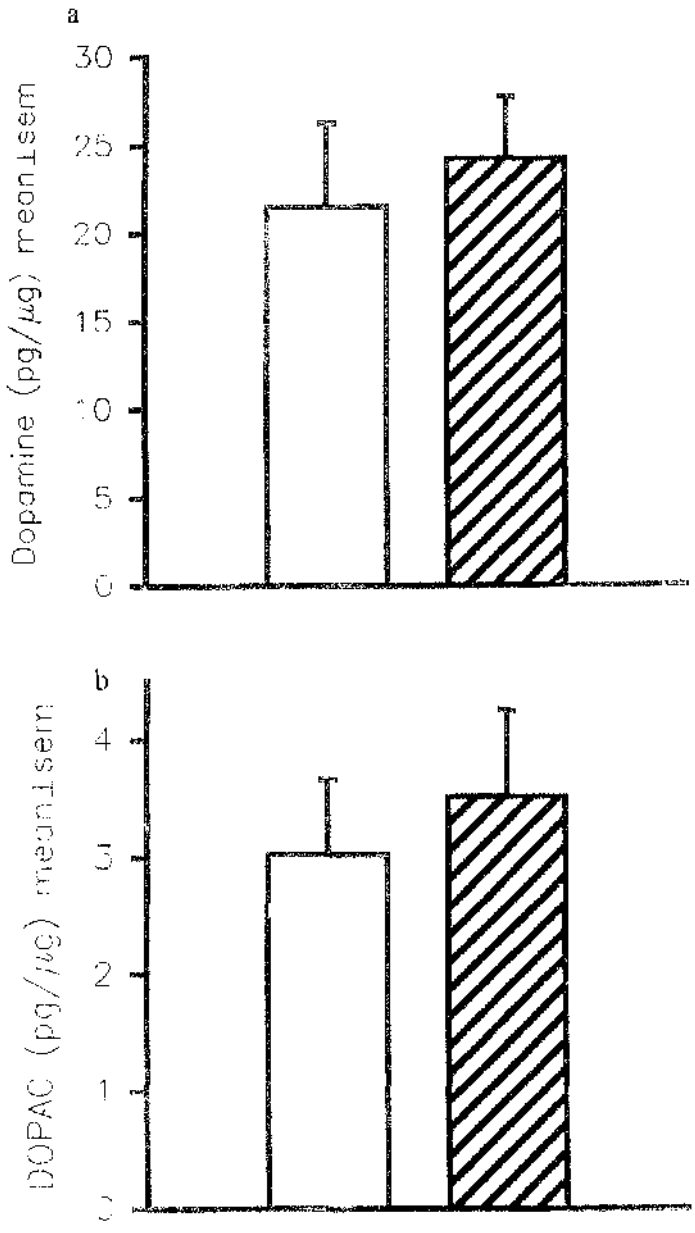



Fig 7.5

Micropunch tissue levels of 5 month old rats (pg/µg) in the MCPu a) dopamine and b) DOPAC

control n=9 AS/AGU n=9 (n.s. to control).

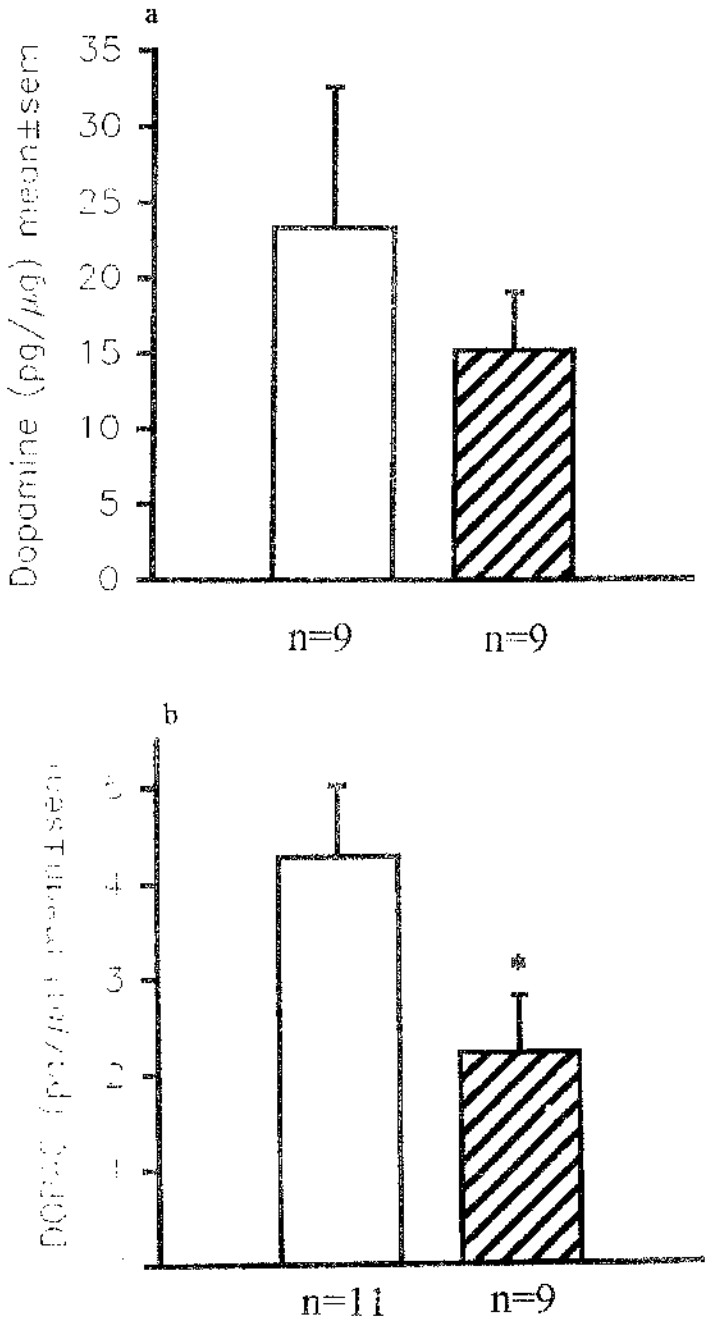



Fig 7.6

Micropunch tissue levels of 5 month old rats (pg/µg) in the VCPu (*p<0.05, t-test) a) dopamine and b) DOPAC

control AS/AGU

7.1.5 The effect of intrastriatal L-dopa on monoamine release from the PCPu by microdialysis

A 30 min pulse of 100μM L-dopa through the probe evoked a release of dopamine and DOPAC in the AS group of rats (fig 7.7 & 7.8). In the AS rats the levels of dopamine increased from non-detectable levels to 1.42 ng/10μl when L-dopa was perfused from the start of the sample collected at time 220 min until 10 min into the following sample (fig 7.7). In the AS rats the DOPAC levels increased from 0.18 ± 0.12 ng/10μl to 2.39 ± 0.49 ng/10μl (*$p<0.05$, paired t-test, n=3, fig 7.8) within the second sample after the start of L-dopa perfusion. The level returned to basal 100 min after the exposure of the tissue to L-dopa.

Dopamine did not increase above non-detectable levels in two of the three AS/AGU rats tested. A slight rise in the third animal occurred during the exposure of the tissue to L-dopa (fig 7.7). In the AS/AGU rats the release of DOPAC, induced by 100μM L-dopa is also bell-shaped (fig 7.8). The dialysate DOPAC levels increased above basal in each of the three animals tested, though the aggregated data does not show a significant increase above basal using the paired t-test. The mean basal level was 0.163 ± 0.094 ng/10μl (average of the four basal levels before L-dopa application) and the peak mean level was 0.605 ± 0.33 ng/μl (fig 7.8).

The total amount of DOPAC above basal released by L-dopa in the AS rats was 4.18 ± 1.12ng/10μl and that for the AS/AGU rats was 1.29 ± 0.55ng/10μl (n.s., unpaired t-test, fig 7.8). The total amount of dopamine above basal was not calculated since basal level was non-detectable and was recorded as zero.

No behavioural effects of L-dopa (when L-dopa was perfused through the probe) were observed in either group of animals.

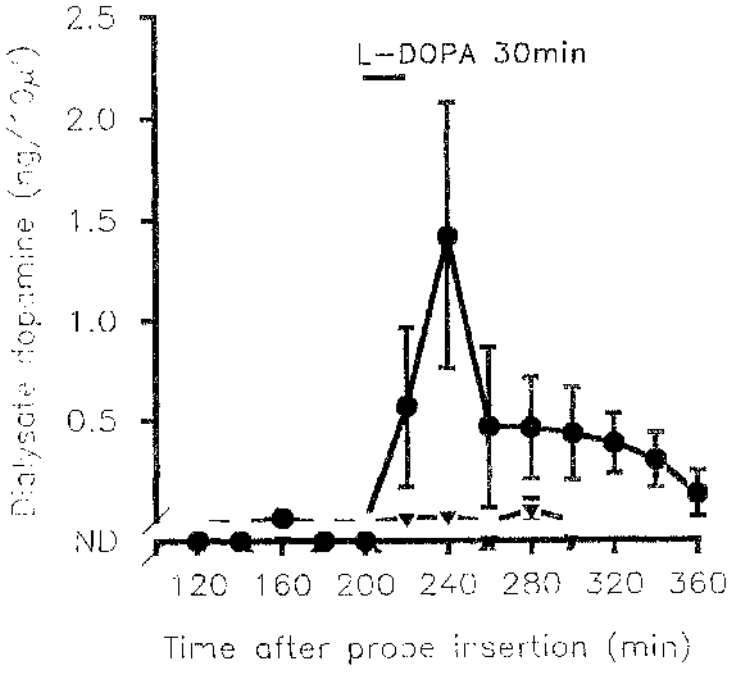



Fig 7.7

The release profiles for dopamine in 10µl dialysates in the PCPu of 5 month old rats evoked by a 30 min pulse of intrastriatal L-dopa 100µM, shown by the horizontal bar.

● AS (n=3)

▼ AS/AGU (n=3)

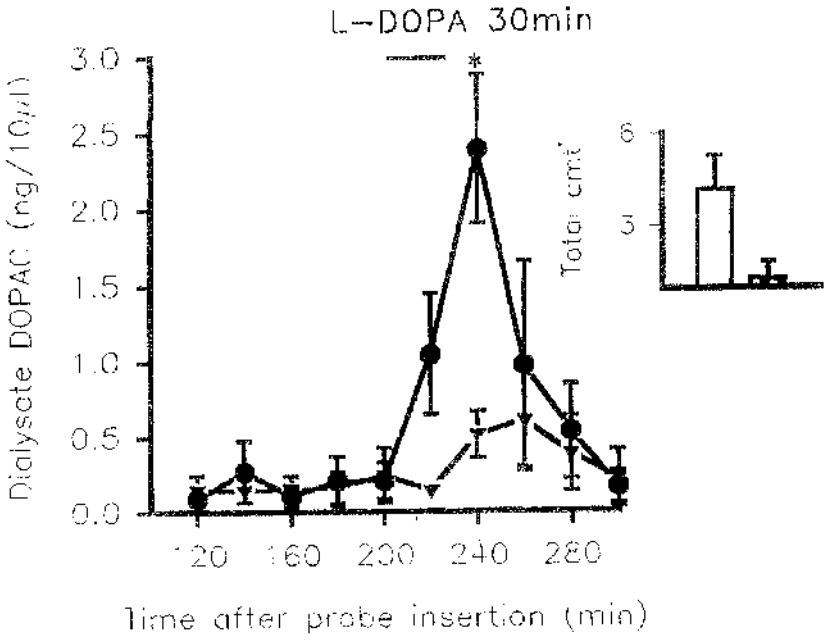



Fig 7.8

The release profiles for DOPAC in 10µl dialysates in the PCPu of 5 month old rats evoked by a 30 min pulse of intrastriatal L-dopa 100µM, shown by the horizontal bar.

- ● AS (n=3) (*$p<0.05$, unpaired t-test)
- ▼ AS/AGU (n=3)

Inset: The total amount of DOPAC released above basal by L-dopa

- ▭ AS (n=3)
- ▩ AS/AGU (n=3)

7.1.6 The effect of chronic systemic injections of L-dopa on monoamine release from the PCPu by microdialysis

Experiments using a chronic injection regimen of L-dopa has been carried out on two AS and two AS/AGU rats. L-dopa injected i.p. over 5 days resulted in a release of clearly detectable levels of DOPAC in all four animals tested (fig 7.9). However, the profiles of release obtained in the two AS rats were very different from the release profiles obtained in the two AS/AGU (fig 7.9). On the fifth day of L-dopa treatment, dialysate DOPAC in the two AS rats increased to a level approximately forty fold and approximately seventy fold higher than the mean basal level of the non-treated AS rats within 3hrs after probe insertion (fig 7.9). The experiment was terminated 3hrs after probe insertion. The dialysate levels within either of the two AS rats may have risen further had samples been taken off beyond the last time point.

The release induced by chronic L-dopa in either of the AS/AGU rats was not to the same extent. Dialysate DOPAC in the two AS/AGU rats increased to a level approximately nine fold and approximately twenty-five fold higher than the mean basal level of the non-treated AS/AGU rats within 1hr after probe insertion (fig 7.9). After the 1hr time point, in one of the AS/AGU rats, the dialysate levels of DOPAC immediately dropped to below the HPLC detection whereas, in the other AS/AGU rat, the dialysate levels of DOPAC slowly dropped over another 1hr to below the detection level of the HPLC (fig 7.9).

On the fifth day of L-dopa treatment, the dialysate dopamine levels of the two AS rats swung in and out of the detection range of the HPLC throughout the experiment (table 7.5). The levels are comparable with the basal levels of non-treated AS rats in the PCPu seen in table 7.4.

Dialysate dopamine release profile in the two AS/AGU rats resembles the profile of DOPAC release in these rats in that release peaked within 1hr in both animals after L-dopa treatment. This is compared with the undetectable levels demonstrated in table 7.4 for the untreated animals. In the L-dopa treated animals, after the 1hr time point, in one of the AS/AGU rats, the dialysate levels of dopamine immediately dropped to below the detection level by the HPLC whereas, in the other AS/AGU rat, the dialysate levels of

dopamine slowly dropped over another 1hr to below the detection level of the HPLC (table 7.5).

Injection of L-DOPA i.p. in the AS/AGU rats resulted in a striking increase in motor activity being observed. For example, injection of L-dopa resulted in AS/AGU rats (normally barely capable of initiating movement) jumping out of cages and fighting which led to their having to be separated. This did not occur in the AS rats. The only time that the behaviour of the AS rats was seen to increase in activity was on the fifth day at the beginning of the microdialysis experiment. In this instance, both animals in this group jumped very high several times consecutively with small periods of rest between each bout, very much like that seen of AS/AGU rats after each injection.

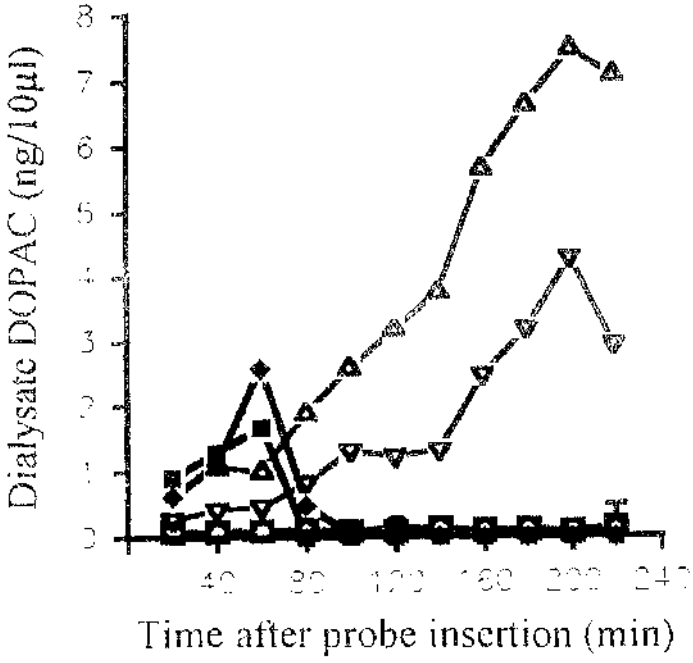



Fig 7.9

The release profiles for DOPAC from the PCPu after no treatment and on the fifth day of a chronic dosage regimen of L-dopa (ng/10µl). The final dose of L-dopa (500mg/kg) was injected 1hr 15min before probe insertion.

- AS no treatment (n=3)
- AS/AGU no treatment (n=3)
- AS chronic L-dopa (n=1)
- AS/AGU chronic L-dopa (n=1)
- AS chronic L-dopa (n=1)
- AS/AGU chronic L-dopa (n=1)

Table 7.5 Dialysate dopamine levels in the PCPu on the fifth day of chronic L-dopa treatment in the AS and AS/AGU groups of rats.

| Time | AS | AS | AS/AGU | AS/AGU |
|---|---|---|---|---|
| 20 | N.D. | 0.079 | 0.506 | 0.052 |
| 40 | N.D. | 0.076 | 0.81 | 0.026 |
| 60 | N.D. | 0.067 | 0.93 | 0.96 |
| 80 | N.D. | N.D. | N.D. | 0.143 |
| 100 | N.D. | 0.041 | N.D. | 0.126 |
| 120 | 0.043 | 0.05 | N.D. | 0.115 |
| 140 | N.D. | N.D. | N.D. | N.D. |
| 160 | N.D. | N.D. | N.D. | N.D. |
| 180 | 0.056 | 0.078 | N.D. | N.D. |
| 200 | 0.065 | 0.089 | N.D. | N.D. |

## 7.2. Adenosine

### 7.2.1 Release of adenosine from the ACPu by microdialysis

Immediately after probe insertion into the ACPu there was a high efflux of adenosine within the first twenty minute sample (fig 7.10). The efflux of adenosine was significantly higher in the AS rats than the AS/AGU (fig 7.10, *$p<0.05$). There was a decline in the release of adenosine in subsequent dialysate samples and basal levels were achieved within 1-2hrs after probe insertion in both AS and AS/AGU rats. Statistical analysis using ANOVA followed by Bonferroni t-test showed that there was no significant difference in the basal levels of AS and AS/AGU rats. However, in most of the experiments in both AS and AS/AGU rats, the basal levels were non-detectable and were depicted as zero in fig 7.10. This introduced an inaccuracy to the analysis and comparison of these levels and clearly a more sensitive system for measuring adenosine was required.

### 7.2.2 Release of adenosine from the PCPu by microdialysis

Experiments measuring potassium-evoked release of adenosine were carried out on two animals in each group. To assist the measurement of dialysate adenosine, an attempt was made to increase the extracellular levels of adenosine. A combination of adenosine deaminase inhibitor (EHNA 100μM) and adenosine uptake blockers (NBTG 100μM and propentofylline 20mM) were incorporated into the perfusion medium throughout the experiment. In addition, to assist the measurement of dialysate adenosine, a microbore column was used in the HPLC system.

The levels of adenosine in the PCPu in the two AS/AGU were compared to that of the two AS rats. Immediately after probe insertion there was a high efflux of adenosine in all four experiments (fig 7.11). The basal level of adenosine, calculated as a mean of the adenosine content in the three or four samples collected 2hrs 20 min after probe insertion, from each of the four rats are illustrated in table 7.6.

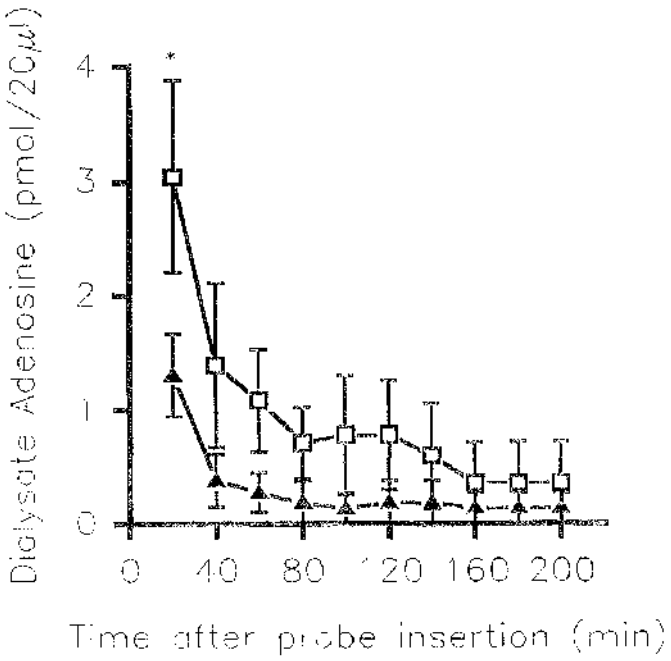



Fig 7.10

Adenosine dialysate levels in the ACPu in the first 2hrs 40min after probe insertion (pmol/20µl, mean ± sem). There was a high efflux within the first 20min which was significantly higher in the AS rats than the AS/AGU (*$p<0.05$, ANOVA, unpaired t-test). There was an immediate decline in release and basal levels were achieved within 1-2hrs after probe insertion in both AS and AS/AGU rats. Rate of perfusion was 2µl $min^{-1}$.

- □ AS rats (n=4)
- ▲ AS/AGU (n=5)

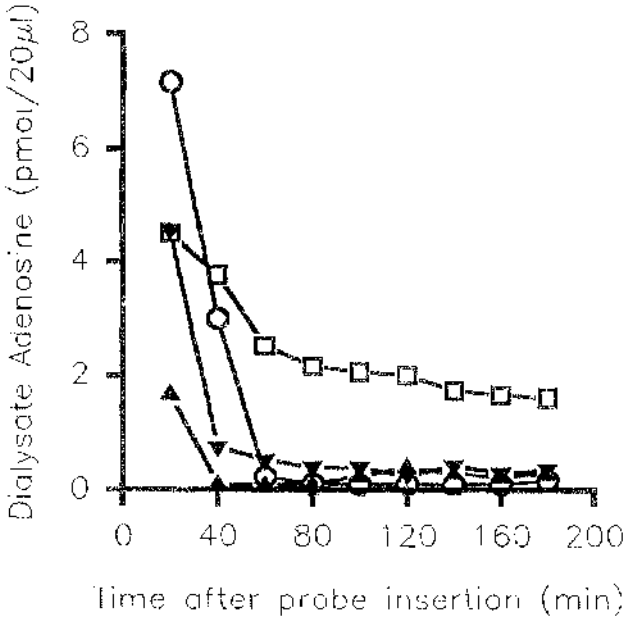



Fig 7.11

Adenosine dialysate levels in the PCPu in the presence of EHNA, NBTG and propentofylline in the first 2hrs 40min after probe insertion (pmol/20µl) in the two AS and the two AS/AGU rats tested. There was a high efflux within the first 20min which immediately declined and basal levels were achieved within 1-2hrs after probe insertion. Rate of perfusion was 2µl/min.

□ AS rats (n=1) ○ AS rats (n=1)

▲ AS/AGU rats (n=1) ▽ AS/AGU rats (n=1)

Table 7.6

Basal levels of AS and AS/AGU rats with the incorporation of EHNA 100μM, NBTG 100μM and propentofylline 20mM in the posterior CPu

| | Mean | | | |
|---|---|---|---|---|
| | AS | AS | AS/AGU | AS/AGU |
| pmol/20μl | 1.79 | 0.99 | 0.27 | 0.34 |

7.2.3 The effect of intrastriatal potassium on adenosine release from the PCPu by microdialysis

A 30 min pulse of potassium, incorporated into the perfusion medium for 3hrs 40 min after probe insertion, elevated adenosine levels in all four rats by approximately 250% of basal (fig 7.12). EHNA, NBTG and propentofylline were perfused through the probes throughout the whole experiment including during the potassium pulse. Due to the scatter of basal levels illustrated in fig 7.11 the release profile was expressed as percentages of basal release was used to compare the effect of potassium on adenosine release in these animals.

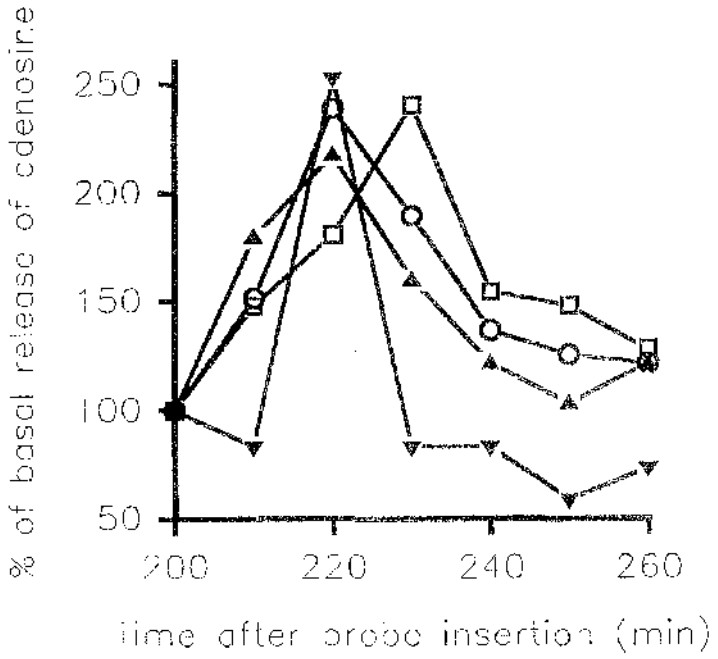



Fig 7.12

The release profiles for adenosine evoked by a 30 min pulse of KCl 100mM, shown by the arrow in the two AS and two AS/AGU rats tested (% of basal). Levels of adenosine peaked at about 250% of basal in all four animals tested.

□ AS rats (n=1) ○ AS rats (n=1)

▲ AS/AGU rats (n=1) ▽ AS/AGU rats (n=1)

## 8.0 DISCUSSION

### 8.1. Microdialysis

In order to validate the technique of microdialysis the following criteria should be met to establish that the compounds detected are a result of neurotransmission. There should be high tissue / ECF ratios because neurotransmitters are stored in vesicles and usually have an efficient system of removal from the synaptic cleft (e.g. high affinity uptake (dopamine, glutamate) or rapid degradation (acetylcholine). There should be a release in response to electrical stimulation of neuronal pathways, depolarising agents and appropriate pharmacological treatment. Release should be blocked by sodium channel blockers and calcium depleting/ calcium-free conditions.

Westerink *et al* (1987) showed that, by using microdialysis in the rat striatum, dopamine, aspartate, glutamate, acetylcholine, and taurine had a tissue / ECF ratio > 1000, whereas their metabolites (e.g. DOPAC, HVA) had tissue / ECF ratios of around 1. The ECF values were calculated by Westerink *et al* (1987) by correcting the dialysate concentrations for the appropriate dialysis efficiency (determined *in vitro*), to the ECF concentration. In the work done by Westerink *et al* (1987) there were reductions in the dialysate levels of dopamine and acetylcholine but not of aspartate, glutamate or taurine after infusion of TTX, indicating a neuronal origin of dopamine and acetylcholine. Further evaluation of neuronal origin of a putative neurotransmitter involves its disappearance from the dialysate when calcium is omitted from the perfusion medium. In the case of dopamine, this was observed by Imperata & Di Chiara (1984). Potassium has been shown to increase dialysate dopamine levels (Westerink *et al*, 1987). These results have lead Westerink *et al* (1987) to conclude that dialysate dopamine levels reflect the changes which occur at the site of release.

Interpretation of measured extracellular dopamine levels by microdialysis requires a review of synaptic homeostasis. To simplify the explanation of this hypothesis the modulation of dopamine release from the nigrostriatal terminal will be examined although these mechanisms of modulation may occur in many efferent neurones. Modulation of dopamine release can occur from the nigrostriatal terminal by several mechanisms. Firstly, recurrent

collaterals may release neurotransmitters onto the nigrostriatal cell body directly. Alternatively, recurrent collaterals may release neurotransmitters onto presynaptic afferents which in turn influence the nigrostriatal cell body. An inhibitory or excitatory neurotransmitter may modulate the activity of the nigrostriatal neurone and may inhibit or enhance synthesis and/or release from the nigrostriatal terminal, respectively. Secondly, dopamine released from the nigrostriatal terminal may act on inhibitory D2 receptors known to exist on presynaptic terminals and inhibit further release of dopamine. Thirdly, levels of dopamine within the ECF may be modulated by the inhibition or enhancement of dopamine reuptake into the presynaptic terminal. Fourthly, release may be modulated by the action of neurotransmitters which are released either via short loop negative feedback onto the presynaptic terminal or via long loop negative feedback onto the cell body.

Recognising the processes of synaptic homeostasis of dopamine may help in the understanding of the compensatory mechanisms which apparently accompany damage to dopaminergic pathways. Nigrostriatal lesioning is accompanied by increased synthesis and apparent increase in release of dopamine from spared terminals and decreased dopamine uptake, which is presumably due to loss of dopaminergic terminals (Zigmond & Stricker 1977; Calne & Zigmond, 1991). Near-normal levels of extracellular dopamine in 6-OHDA-lesioned rats have been reported by *in vivo* microdialysis studies (Robinson & Wishaw, 1988; Abercrombie *et al*, 1990; Castañeda *et al*, 1990; Zigmond *et al*, 1990; Sarre *et al*, 1992). It has been shown that the decrease in dopamine concentration in striatal tissue by 6-OHDA is more than the decrease in dopamine concentration in the overflow from striatal slices ( Snyder & Zigmond, 1990). This may be explained by hyperactivity of the residual neurones, spared by 6-hydroxydopamine lesions. There is an increased synthesis in remaining dopaminergic neurones which may be due at first to activation of existing tyrosine hydroxylase (TH) and later to an increase in the number of enzyme molecules (Zigmond *et al*, 1984). Such an increase in activity of TH may be a result of increased firing rate of the residual neurones. There is an apparent increase in dopamine release from residual terminals *in vivo* (Hefti *et al*, 1980; Zigmond *et al* 1984). The increase in the amount of dopamine release per residual terminal may be a result of an increase in response

of the dopaminergic terminals to depolarisations or reduced D2-mediated presynaptic negative feedback on dopamine release. A decrease in catecholamine uptake has been shown in 6-OHDA-treated rats (Iversen & Uretsky, 1970), probably a result of the smaller number of neurones available to take up dopamine. Due to a reduced number of dopamine uptake sites, the dopamine that is released may diffuse out of the synapses to distant target sites. An increase in dopamine turnover within the residual neurones may also occur. Such compensatory mechanisms have been reported to become apparent 3-4 weeks after the lesion (Snyder *et al*, 1990).

Also, areas deprived of dopaminergic afferents appear to become supersensitive to dopamine agonists, possibly a consequence of an increase in the number of postsynaptic receptors (Creese *et al* 1977; MacKenzie & Zigmond, 1984).

8.2.1 Monoamine levels in the Caudate Putamen

The analysis of monoamine neurotransmitter release was very much hindered in the present study due to the lack of the required sensitivity for dopamine analysis. Basal levels ranged between non-detectable to 0.54pmol/10µl, which is near the limits (0.28pmol/10µl) of our HPLC system. The conditions under which the HPLC was used, were adapted to meet the requirements of all the users of the HPLC within the laboratory and could not be substantially modified for this particular project.

Dopamine levels are lower in the dialysates than DOPAC levels, whereas dopamine is present in larger amounts than DOPAC in tissue extracts. This is consistent with the presence of high affinity uptake within dopaminergic regions and is consistent with results of Sharp *et al* (1986).

The present results indicate that there is no difference between AS and AS/AGU rats in the concentrations of extracellular DOPAC in the ACPu. Basal samples were collected two hours after probe insertion and are consistent with the basal extracellular levels of DOPAC achieved within two hours by Robinson & Camp (1991). There is a slight trend towards an increase in DOPAC in the AS/AGU rats although this was not significant.

The lack of difference in extracellular DOPAC levels can be explained in a number of ways. Firstly, it is possible that there is no difference in striatal dopamine and therefore metabolite content in the ACPu between the AS and AS/AGU rats at 5 months of age.

Secondly, near-normal extracellular levels of DOPAC in the AS/AGU rats may be present owing to compensatory effects which lead to near-normal extracellular levels of dopamine. However, the authors reported a drop in extracellular DOPAC levels after 6-OHDA treatment, despite the lack of a drop in extracellular dopamine levels. Thus, the similar levels of DOPAC in AS and AS/AGU rats is probably not due to compensatory effects which lead to the near-normal extracellular levels of dopamine seen in 6-OHDA-treated rats.

A third reason for the lack of difference in extracellular DOPAC levels can be explained by the microdialysis probe not being located within the appropriate region of the striatum to detect differences of dopaminergic function (i.e. the ACPu may be a location within the striatum which is not associated with motor control and is therefore not where the loss of dopaminergic terminals would result in dysfunction in motor activity) (see below).

The levels of dopamine and DOPAC in tissue extracts were measured. It was found that there was no difference in either the dopamine or DOPAC tissue concentrations between AS and AS/AGU rats in the ACPu.

The lack of difference in the tissue levels of dopamine and DOPAC between the AS and the AS/AGU rats can be explained in a number of ways. Firstly, it is possible that there is no difference in striatal dopamine and DOPAC content in the ACPu between the AS and AS/AGU rats at 5 months of age. Secondly, the micropunch cores and the microdialysis probe may not be located within the appropriate region of the striatum to detect differences of dopaminergic function (see below).

Compensatory mechanisms have been shown in tissue from patients with Parkinson's disease, where the dopamine metabolite concentrations in the post-mortem tissue were less affected by the disease than dopamine (Bernheimer *et al*, 1973). The HVA to dopamine ratio in post-mortem tissue from Parkinson's disease patients has been reported to be 10-fold higher than that from non-affected patients, indicating an increased dopamine

metabolism in the residual dopaminergic terminals of patients with Parkinson's Disease (Bernheimer *et al*, 1973). Higher DOPAC to dopamine ratios have been detected in 6-OHDA-lesioned striatum of rats compared with the unlesioned striatum (Robinson & Wishaw, 1988).

To investigate if DOPAC tissue levels are not reduced in the AS/AGU due to a compensatory increase in dopamine metabolism, the ratios of DOPAC to dopamine tissue concentrations were calculated. The concentration of DOPAC measured in the tissue relative to the concentration of dopamine measured in the tissue reflect the dopamine metabolism in dopaminergic terminals. An increase in the ratio (DOPAC/ dopamine) would reflect an increased metabolism of dopamine to DOPAC within the dopaminergic terminals.

The DOPAC to dopamine ratios, in the present study, for the tissue levels in AS and AS/AGU rats were 0.156±0.043 and 0.135±0.009, respectively. This is in agreement with Sharp *et al* (1986) who found DOPAC to dopamine tissue ratios to be 0.15 in the striatum of normal rats and implies a normal dopamine turnover within the ACPu.

A lack of reduction of tissue DOPAC content in the AS/AGU rats, in the present study, therefore is not due to a compensatory increase in DOPAC in the AS/AGU rats, which may have been caused by the proposed occurrence of a compensatory increase of dopamine metabolism.

Thus, AS/AGU rats at 5 months of age possess similar dopamine and DOPAC levels to control AS rats within the ACPu region.

Work done subsequently on tissue levels in 12 month old AS and AS/AGU rats shows no drop in ACPu tissue dopamine levels but a significant drop in dopamine tissue levels within the DCPu and MCPu (J.M. Campbell, personal communication). The question of probe placement thus becomes of possible importance. For this reason, the regional location of the striatal projections involved in motor control has been investigated.

The caudate putamen is a group of nuclei with a heterogeneous structure and function. In order for the striatum to contribute to sensory and motor functions it must receive afferents from sensory and motor neuronal systems. All major regions of the cerebral

cortex project into the striatum. McGeorge & Faull in 1989 detailed the organisation of the projections from the cerebral cortex to the striatum in the rat. The major subdivisions of the cerebral cortex project into defined but partially overlapping regions of the striatum: neocortex projects to the caudate/putamen, mesocortex mainly to the medial and ventral caudate/putamen but also to the nucleus accumbens and olfactory tubercle. Regions of allocortex project mainly to the nucleus accumbens and olfactory tubercle but also to the medial and ventral regions of the caudate/putamen. The sensory and motor areas of the neocortex project into the dorsolateral caudate/putamen (Cospito & Kultas-Ilinsky, 1981; Domesick, 1988) such that the rostral sensorimotor cortex (head areas) project to the central and medial areas and the more caudal sensorimotor cortex (limb areas) project to the dorsal region of the dorsolateral striatum. Corticostriatal fibres from the primary somatosensory and motor cortices terminate in the dorsolateral region of the striatum (Cospito & Kultas-Ilinsky, 1981). Dopaminergic fibres from the SNr project to the medial-lateral striatum (Beckstead *et al,* 1979). The dorsolateral striatum is therefore well documented to be within the motor circuitry and is therefore a more appropriate location for the dialysis probe than the medial position in the ACPu. Indeed, the dorsolateral striatum is reported to be the area through which motor activity is mainly influenced by dopamine from the SNr (Hirata *et al,* 1984) and behavioural deficits can be produced by injections of 6-hydroxydopamine into the lateral but not the medial portion of the striatum (Snyder *et al,* 1985).

In the present work, the probe was repositioned 4.25mm lateral rather than 2.5mm lateral relative to the bregma. This is in accordance with the co-ordinates used by West *et al,* 1990 (3.5 to 4.0mm lateral, +1.6 to -1.0mm relative to the bregma, Paxinos & Watson, 1986) who show this region of striatum to be within the motor circuit and to be responsible for hind-limb and forelimb movements. Webster (1961) and Richards & Taylor (1982) report that hindlimb movements are located caudally in the lateral caudate/putamen but that responses to forelimb, head and neck stimulation were located rostrally. Since one of the behavioural abnormalities of the AS/AGU rat is slow movement of the hindlimbs,

the probe was moved further posterior relative to the bregma (from +1.0mm to -1.0mm) which is still within the AP co-ordinates stated by West *et al* (1990).

The samples in the PCPu were collected until 3hrs 40 min after probe insertion. For the initial two hours after probe insertion there was a marginal increase in extracellular DOPAC. The mean basal level in the AS for the first two hours was 0.088ng/10µl and for the following 1hr 40min was 0.11ng/10µl. The mean basal level in the AS/AGU for the first two hours after probe insertion was 0.095ng/10µl and for the following 1hr 40min was 0.113ng/10µl. This is consistent with results obtained by Robinson & Camp (1991) who showed that DOPAC levels increase gradually over the first two hours. These authors also show a high efflux followed by a gradual decline in the release of dopamine over the first two hours after probe insertion. The high efflux and decline in release of dopamine reported by these authors is consistent with the high efflux and decline in release of adenosine reported in part 1 of the present thesis. The high initial efflux of dopamine may be due to cell damage by probe insertion and leakage of dopamine from high intracellular storage compartments. This dopamine may subsequently be metabolised to DOPAC resulting in the gradual increase in DOPAC levels. Thus the gradual increase in DOPAC levels may represent the complex series of events leading to the establishment of a new equilibrium.

Dialysis within the PCPu showed no significant differences for DOPAC in AS and AS/AGU rats. Thus, it is possible that there is no difference in striatal dopamine content in the PCPu between the AS and AS/AGU rats at 5 months of age. To strengthen this conclusion, the tissue contents of dopamine and DOPAC were examined.

The tissue concentrations of dopamine and DOPAC in the three regions within the PCPu were measured. There was no difference in the tissue dopamine levels in the three micropunch regions of the PCPu in the present study between 5 month old AS and AS/AGU rats. There was no difference in the tissue DOPAC levels in the DCPu and MCPu in the present study between 5 month old AS and AS/AGU rats, though there was a significant decrease in DOPAC in the VCPu.

The significant drop in DOPAC tissue level in the AS/AGU rats compared with the AS rats in the VCPu (an area where the dialysis probe has not been inserted in the present study) is noteworthy in the context of the observed tendency for an increase in dopamine utilisation in this region (as illustrated by the higher but non-significant DOPAC/ dopamine ratios in the VCPu for both sets of animals than those ratios in ACPu, DCPu or MCPu). The importance of the ventral striatum in the role of motor control must be highlighted. When embryonic substantia nigra cells were transplanted into 6-OHDA-treated rats, there was a reduction in their behavioural impairment only when there was new growth into the ventral and lateral parts of the caudate putamen but not into the dorsal part of the striatum (Dunnett *et al*, 1981). In addition, the VTA (ventral tegmental area) has been shown to project to the ventromedial region of the striatum (Beckstead *et al*, 1979). However, the present results only showed a drop in DOPAC tissue levels, not dopamine. It is therefore concluded that the drop in DOPAC levels may actually be caused by a malfunction of the enzyme MAO within the VCPu in the AS/AGU rats but not to the extent that it is significantly decreases the DOPAC/ dopamine ratio relative to the AS rats.

To investigate whether there is a compensatory increase of dopamine metabolism in the AS/AGU, the DOPAC/ dopamine tissue ratios were calculated for all the regions. Like the ACPu, there was no difference in the DOPAC/ dopamine tissue ratio between the AS and AS/AGU rats for the DCPu, MCPu and VCPu. There was therefore no difference in dopamine turnover between the AS and AS/AGU rats, thus eliminating the issue of compensatory mechanisms resulting in the lack of drop in levels of tissue DOPAC.

To assess whether there was an increased dopamine turnover in one particular region of the striatum, the DOPAC/ dopamine tissue ratios were compared between the ACPu, DCPu, MCPu and VCPu. Though not statistically significantly different, the mean DOPAC/ dopamine tissue ratio was higher, in the VCPu than the DCPu, MCPu and ACPu in both the AS and AS/AGU rats. Thus in the VCPu, there may be a tendency of an increased dopamine turnover.

In the present study, there was no difference in dopamine utilisation in the dopamine terminals between AS and AS/AGU rats shown by the similar tissue DOPAC/ dopamine

ratios in the regions cored within the PCPu. Recently, work carried out by Dr J.M. Campbell (personal communication), has revealed no drop in dopamine tissue levels of the mutant AS/AGU rats aged 10 days or 12 weeks when compared to control AS rats. There was, however, a drop in dopamine DCPu and MCPu tissue levels AS/AGU rats when compared to the control AS rats at 12 months of age and, as shown more recently, at 6 months of age (Dr J.M. Campbell, personal communication). It is finally concluded that dopamine levels in the PCPu drop some time between the ages of 5 and 6 months in the AS/AGU rats.

8.2.2 The effect of intrastriatal L-dopa on monoamine release from the PCPu by microdialysis

L-dopa is currently clinically used to reverse the behavioural effects of Parkinson's disease. L-dopa has been reported to exert little of its effects through D2 receptors but has a more dominant effect on the D1 mediated GABA/substance P pathway (Jenner, 1995).

L-dopa, administered locally into the striatum, induced an increase in release of dopamine and DOPAC in the PCPu of AS rats. This implies the conversion of L-dopa to dopamine and DOPAC is occurring within this region of the striatum in both the AS and AS/AGU rats. However the extent of release of dopamine and DOPAC in the AS/AGU rats was quite different to that in the AS rats. The peak release of DOPAC in the AS/AGU was approximately four fold lower than the peak release in AS rats. This lower response to L-dopa in the AS/AGU rats is reflected by the total amount of DOPAC released above basal by L-dopa. The total amount released in the AS/AGU rats showed a three fold reduction in the mean values compared to AS controls. However, this did not quite reach significance. A similar lower response to L-dopa in AS/AGU rats was indicated by the extent of dopamine release compared with that in the AS rats. Dopamine release in the AS rats rose from non-detectable levels to well within the detection range of the HPLC. However, in the AS/AGU rats the dopamine levels rose from non-detectable levels to just within detection range of the HPLC. Thus it is possible that less dopamine is being converted from the L-dopa in the AS/AGU rats than in the AS rats.

8.2.3 The effect of chronic systemic injections of L-dopa on monoamine release from the PCPu by microdialysis

Chronic injections of L-dopa for 5 days increased the levels of DOPAC in both AS/AGU and AS rats. The means of the first dialysate samples measured after chronic treatment for the two groups of rats are very similar (AS/AGU 0.774ng/10µl and AS 0.744ng/10µl). These values are 7-fold higher than those at the same time point in untreated animals and may be explained by a loading of the tissue due to chronic dosing. The subsequent bell-shaped release may be induced by the dose of L-dopa injected 1hr 15 min prior to probe insertion.

The sudden drop of dopamine and DOPAC release within the two AS/AGU rats might indicate the activation of presynaptic dopamine autoreceptors by an elevated extracellular dopamine release. Activation of dopamine D2 autoreceptors causes a shut-down of release of dopamine. There was no sudden drop in the elevated DOPAC levels in the two AS rats, but the levels actually elevated further. This may indicate an increased number of D2 receptors in the AS/AGU rats, which, interestingly, is postulated to be a compensatory effect of a loss of dopamine. Another explanation for the sudden drop in dopamine and DOPAC in the AS/AGU rats which did not occur in the AS rats may be that AADC in the AS/AGU rats becomes saturated.

8.2.4 Conclusions drawn from the results of induced release of dopamine and DOPAC by perfusions and chronic injections of L-dopa

L-dopa induced release of dopamine does indeed appear to be contradictory. While studies on striatal slices (Snyder & Zigmond, 1990) show L-dopa induced release of dopamine to be markedly reduced, but not abolished, by pre-treatment with 6-hydroxydopamine, studies using *in vivo* microdialysis suggest that the effect of L-dopa on dopamine release is markedly increased after 6-OHDA when compared with that in the intact striatum (Abercrombie *et al*, 1990; Sarre *et al*, 1994). Abercrombie *et al* (1990) and Sarre *et al* (1994) explain this increase by the loss of high affinity uptake sites associated with the decreased number of dopaminergic nerve terminals. The reason for the discrepancy between the results of Abercrombie *et al* (1990) and Sarre *et al* (1994) and that of Snyder

& Zigmond (1990) may be due to the different preparations used. The relatively high superfusate flow rate employed in the *in vitro* studies (i.e. 100µl/min for Snyder & Zigmond (1990)) may have diminished the important contribution of high affinity dopamine uptake to increases in extracellular dopamine following L-dopa. The extent of the lesion induced by 6-OHDA (which may vary between the different studies) may have an important an important effect on the extent of the L-dopa induced dopamine release and this is indicated by Sarre *et al* (1994) who failed to establish a dose-dependent evoked release of dopamine by L-dopa in the lesioned animals.

In the present study, in the two AS rats after the chronic L-dopa it is noted that the dopamine dialysate levels are lower than that of the initial levels measured in AS/AGU rats. Jackson *et al* (1993) report that dopamine terminals play a prominent role in removing extracellular dopamine released by exogenous L-dopa. Dopamine taken up into the terminal is catabolised instead of being used in chemical signalling (Wachtel & Abercrombie, 1994), which may explain our observed elevation of extracellular DOPAC but not dopamine in the two AS rats after chronic L-dopa. An absence of dopamine reuptake sites would therefore result in more dopamine being used in chemical signalling and may explain the increased locomotor activity seen in the AS/AGU rats compared to the AS rats. The postulated increase in dopamine receptors which occur as a compensatory effect may also lead to the increased locomotor activity in the AS/AGU rats.

Langelier *et al* (1973), Lloyd *et al* (1975) and Snyder & Zigmond (1990) have shown that the L-dopa-induced increase in dopamine is lower in striatal tissue after destruction of virtually all of the dopamine terminals than in intact striatum. Sarre *et al*, 1994 showed that the dose-dependent L-dopa-induced release of dopamine in intact rats could not be established in the denervated striatum. These studies are consistent with the lower release of dopamine and DOPAC by L-dopa in the AS/AGU rats found in the present study. In light of the behavioural deficits observed in these rats it is therefore conceivable that the AS/AGU rat possesses less dopaminergic neurones in the striatum than the control strain.

Interestingly, upto 20% of striatal AADC has a non-dopaminergic location (Lloyd & Hornykiewicz, 1970; Hefti *et al* 1981; Melamed *et al*, 1981). The presence of non-

dopaminergic located enzyme may explain the ability of L-dopa to increase the dopamine levels in the denervated striatum, and the saturation of the non-dopaminergic located enzyme may explain why the increase is not to the same extent as in the intact striatum as reported by Langelier *et al*, 1973; Lloyd *et al*, 1975; Snyder & Zigmond, 1990. Non-dopaminergic AADC is possibly found in serotonergic neurones (Ng *et al*, 1972; Hokfelt *et al*, 1973), glial cells (Li *et al*, 1992) or cells in the region of BBB (Bertler *et al* 1966; Langelier *et al*, 1972). It is therefore possible that conversion of L-dopa to dopamine, in the AS/AGU rats, depends upon the AADC which is located in non-dopaminergic sites.

The extensive destruction of dopamine neurones leads to an increase, rather than a decrease, in the behavioural effects of L-dopa (Uretsky & Schoenfeld, 1971; Schoenfeld & Uretsky, 1973; Zigmond & Stricker, 1980). Increased locomotor activity after L-dopa, a dopamine-mediated phenomenon, is generally not readily apparent in intact animals, but it can be seen when high affinity dopamine reuptake has been compromised by drugs possessing dopamine uptake blocking properties or after destruction of dopaminergic terminals (Svensson & Strömberg 1970; Schoenfeld & Uretsky 1973; Zigmond & Stricker 1980). In the present study, L-dopa administered to AS/AGU rats caused hyperactivity during the five day injection regimen, to the extent that the dose of L-dopa had to be halved on the third and fourth days of the five day course and the two AS/AGU rats had to be separated from each other. This hyperactivity was not noted in the AS rats to the same extent as the AS/AGU prior to the fifth day in AS rats. A possible explanation is a prolonged action of dopamine in the ECF after the conversion from L-dopa, consistent with the loss of dopaminergic terminals which possess reuptake sites. These results are consistent with those results obtained from animals with a loss of dopamine function/terminals. The activity noted in both sets of rats was most hyper on the fifth day, the day the microdialysis experiment was carried out. Whether the accumulated L-dopa after the five day regimen or whether a new environment (i.e. the microdialysis cage) provoked the exaggerated hyperactivity, cannot be defined from the present study.

To conclude this section of work, the AS/AGU rats at 5 months of age do not lack striatal dopamine or DOPAC levels. A malfunction in the dopaminergic system may, however,

become apparent when the demand of the function of dopaminergic neurones increases, for example when L-dopa is required to be degraded to dopamine.

8.3.1 Release of adenosine in the caudate putamen by microdialysis

Basal adenosine levels in the ACPu were non-detectable in the majority of AS and AS/AGU, so that an accurate comparison could not be conducted. There was, however, a significant difference in adenosine levels in AS rats compared to AS/AGU rats immediately after probe insertion in the ACPu, a point at which adenosine levels in all experiments were within the range of detection. The lack of sensitivity of the HPLC for adenosine in the striatum of freely-moving rats may obscure any comparison which could be made for the basal levels between AS and AS/AGU rats.

Preliminary studies carried out in the PCPu show a trend for a higher level of adenosine in one of the AS than in either of the AS/AGU rats. These studies were carried out in the presence of adenosine deaminase inhibitors and uptake blockers.

Potassium-evoked release of adenosine appeared to be to the same extent in the two AS as the two AS/AGU.

Activation of A2 receptors is thought to decrease the affinity of D2 receptors for dopamine (Ferre *et al*, 1994). Activation of A2 receptors may therefore lead to an increased activity of the indirect pathway, and thus may pose as a possible explanation for the hypokinesia seen in some basal ganglia disorders. One of the means by which activation of A2 receptors may come about is if there is an elevated concentration of extracellular adenosine. However, the preliminary results in the present work indicate that the extracellular adenosine levels in the AS/AGU rats have a trend for being lower than that in the AS rats. It should be noted that in the PCPu, the uptake blockers or enzyme inhibitors in the perfusate may obscure any difference in basal levels. No definitive conclusion can be drawn from the present results, but the trend indicates that adenosine levels are not responsible for any locomotor deficits in the AS/AGU rats which may be caused by the reported adenosine-dopamine interaction since, if this was the case, a higher (not a lower) basal extracellular level of adenosine would be expected in the AS/AGU rats than in the AS rats.

**Appendix**

Investigation of binding to kainate receptors

Drugs which were effective in modulating kainate-evoked release of adenosine were tested for their ability to displace kainic acid from the kainate receptor. The protocol used was that of Varga *et al* (1989). Four male Wistar rats (270-310g) were killed by stunning and cervical dislocation and the hippocampi were dissected out by Dr. D.G. MacGregor and placed in 5ml ice-cold in 0.32M sucrose containing 0.05M Tris-citrate pH 7.2. The hippocampi were homogenised in a Braun Homogeniser (15×500rpm) in 10 vol of ice cold 0.32M sucrose containing 0.05M Tris-citrate buffer pH7.2 and centrifuged in a Sorval RC 5B Refrigerated Superspeed Centrifuge (SS 34 Rotor) at 3000rpm for 10 min at 4°C. The pellet was discarded and the supernatant was spun at 12000rpm for 20 min at 4°C. The supernatant from this spin was discarded and the pellet was frozen at -80°C for one hour. This P2 pellet was thawed at room temperature and homogenised (10 × 500rpm) in 10 vol of ice-cold distilled water and then centrifuged at 18500rpm for 20 min at 4°C. The supernatant was discarded and the pellet was resuspended in 10ml distilled water and centrifuged at 18500rpm for 20min at 4°C. This washing procedure was repeated twice using 0.05M Tris-citrate buffer instead of distilled water. The supernatant was discarded and the pellet was frozen at -80°C until the day of the binding experiment.

On the day of the binding assay, the pellet was thawed and washed three times as above. The volume of the assay chamber was 500µl. 445µl of membrane were incubated for 10 min on ice in 50µl of either cold kainate (1mM, final concentration) for the non-specific binding, or Tris-citrate for the total binding or test drugs. All membranes were then incubated in 5µl [$^3$H] kainic acid for one hour on ice. All assays were done in triplicate and all vials were vortexed every 20min. Incubation was terminated by vacuum filtration, with all the samples being filtered through pre-wetted Whatman GF/C glass filters using a Millipore 12 well 1225 Sampling Manifold. Filters were washed three times with 5ml of ice-cold 0.05M Tris-citrate buffer and vacuum dried before being immersed into 5ml scintillant (Ecoscint). The amount of tritium was counted by a scintillation counter (Packard 2000CA) for 5 min for CPM (quenching and efficiency of counting was

calculated as being 40% using external standards). The binding assay was repeated (using the same tissue) since inconsistent results were obtained after the first assay.

Data was expressed as percentage of control. The percentage of control was calculated by subtracting the CPM for the non-specific binding from the CPM for the binding in the presence of the displacers and dividing by the CPM for the specific binding. Statistical analysis was performed by a one sample t-test against a hypothetical mean of 100%.

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

In an attempt to establish the best conditions under which to perform the procedure, filters were washed either once, twice or three times with 5ml of 0.05M Tris-citrate buffer, which was either ice-cold (~4$^0$C) or room temperature (~20$^0$C), before vacuum drying as above.

Materials

From Amersham: [$^3$H]kainate from, specific activity 58Ci/mmol, stock concentration 17μM, ethanol: water (2:98).

From Sigma Chemicals: ascorbic acid, glutathione, oxypurinol

From The Upjohn Company: U50 488H

Results

Ascorbic acid (1mM, 10mM, 100mM), glutathione (1mM, 10mM, 100mM), oxypurinol (0.1mM, 1mM, 10mM) and U50 488H (0.01mM, 0.1mM, 1mM) did not significantly affect tritiated ligand binding (fig 1). As illustrated in fig 1 these results have large standard error bars. The number of counts per minute for the vial containing the displacer was often less than that for the vial containing cold kainate (non-specific binding). This may indicate a flaw in the procedure used, possible resulting from inconsistent binding of tritiated kainate throughout the assay (see discussion).

The results obtained from the assay performed to establish the best conditions for the procedure indicate that specific binding was best after washing the filters three times with 5ml of 0.05M Tris-citrate at ~4$^0$C (fig 2). Interestingly, these were the conditions used in

the actual procedure. Due to time constraints no further experiments were carried out to investigate the inconsistency of the presents results.

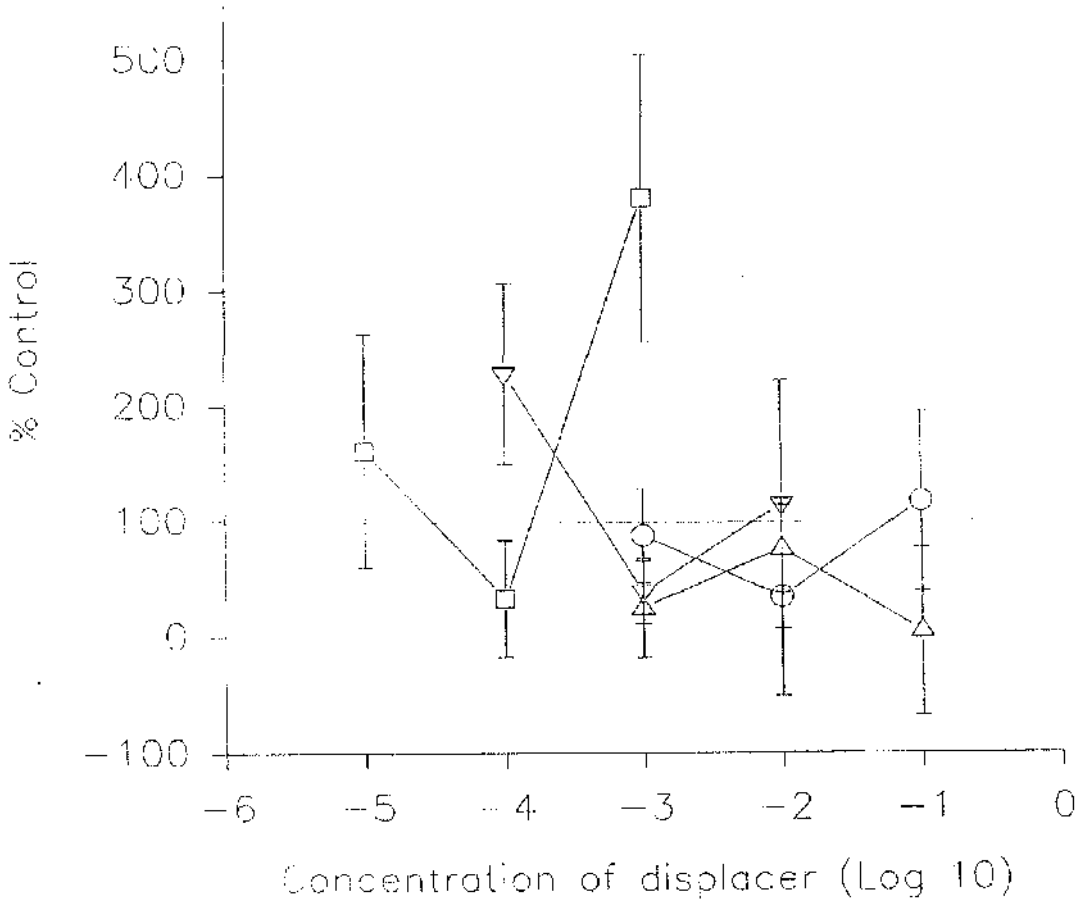



Fig 1

The effect of U50, 488H, ascorbic acid, glutathione and oxypurinol on the binding of tritiated kainate to rat hippocampal membranes.

□ U50, 488H ○ ascorbic acid

△ glutathione ▽ oxypurinol

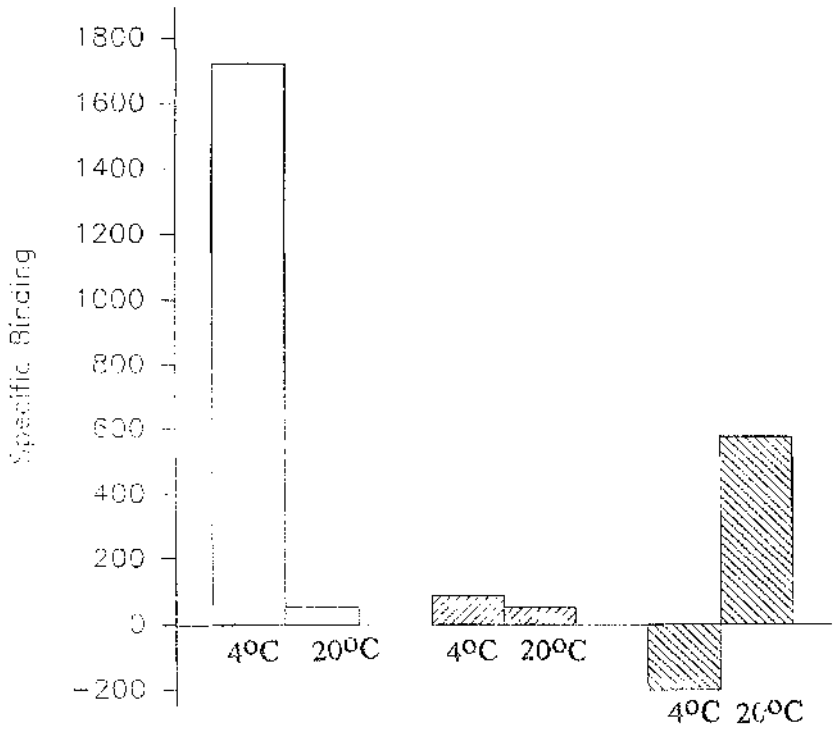



Fig 2

The effect of different conditions in the binding assay procedure on the specific binding of tritiated kainate

3×5ml washes

2×5ml washes

1×5ml washes

Discussion

There was no consistency in the results obtained in the two assays performed indicating that there may be some flaw in the procedure, possibly a lack of consistent binding of the tritiated kainate. A lack of binding of tritiated binding may be a result of several causes. Firstly, a chemical interaction between the displacer and the tritiated kainate may have occurred. For example, ascorbic acid may reduce kainate to dihydrokainate, which may displace kainate from its binding site. This possibility was investigated by MacGregor (1995) who reported that kainate was stable for upto 5 days in solution. Secondly, kainate receptors may either be no longer available on the membranes or have decreased affinity for kainate, possibly a result of being denatured during the preparation of the membranes. The addition of protease inhibitors to the homogenising fluid may prevent such a denaturing. Thirdly, an unspecified contaminant may be present, competing against kainate for the receptors. For example, endogenous glutamate may be present. However this is unlikely since the membranes were washed many times. A fourth explanation is that the tritiated kainate has itself degraded in solution. This could be tested by nuclear magnetic resonance.

The present results do not allow a definitive conclusion as to whether ascorbic acid, glutathione, oxypurinol or U50 488H displace kainate from its binding site.

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