# STUDIES ON PRECONDITIONING WITH ADENOSINE, GLUTAMATE AND OUABAIN IN RAT HIPPOCAMPAL SLICES

by

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A thesis submitted in fulfilment of the requirement for the degree of

Doctor of Philosophy to the University of Glasgow

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August 2008

# ABSTRACT

Preconditioning is the phenomenon whereby tolerance to lethal insults is induced by exposing the tissue to a prior sublethal stimulus. This exists in several forms, such as ischaemic preconditioning, adenosine preconditioning and excitotoxic preconditioning. Adenosine preconditioning is known to be mediated by activation of A1 receptors and ATP-sensitive potassium channels whilst excitotoxic preconditioning mainly involves stimulation of NMDA receptors, nitric oxide and most likely ATP-sensitive potassium channel activation. ATP-sensitive potassium channel openers such as pinacidil and diazoxide are also known to exert preconditioning against various types of insults. There have been several models of ischaemia used to study preconditioning in vivo and in vitro leading to some confusion over the effects of preconditioning agents. High concentrations of glutamate or NMDA have been used as models of excitotoxicity in many experimental paradigms. Some molecular changes are associated with preconditioning phenomena, the most prominent being an increased expression of heat shock protein 72 (HSP72). The aims of the current study were to: 1) investigate the effects of exogenous glutamate and other depolarizing agents in the slice preparation and their validity for use as toxic agents 2) examine any potential preconditioning neuroprotection induced by adenosine against various depolarizing agents and elucidate the underlying mechanisms where relevant 3) examine the excitotoxic preconditioning phenomenon and possible underlying mechanisms 4) look at the effectiveness of other known preconditioning agents e.g. ATP-sensitive potassium channel openers against depolarizing agents and identify the underlying mechanisms of protection 5) identify any molecular changes that may occur during acute models of chemical ischemia or acute preconditioning. The rat hippocampal slice preparation was used to investigate the effects of depolarizing agents and preconditioning paradigms upon the extracellularly evoked field epsps, orthodromic and antidromic population spikes. Western blotting was used to detect any changes in the levels of HSP72 in the slices that may have occurred as a result of the depolarizing agents or the preconditioning treatments. It was first established that 5mM and 10mM glutamate induced depressions in the amplitudes of orthodromic population spikes which recovered to a stable plateau. The degree of recovery of the spikes depended partially upon the initial size of the response. As adenosine is known to be released in response to glutamate receptor stimulation, the effects of 5mM glutamate upon the orthodromic spikes were studied in the presence of the A1 receptor antagonist, DPCPX. It was observed that DPCPX did not attenuate the depression of the response during glutamate perfusion but there was a significant elevation in the post-glutamate recovery of the response. This effect was not

observed when the protocol was applied to antidromic population spikes and field epsps, both of which showed a depression in response during 5mM glutamate perfusion but recovered fully when glutamate was removed. The field epsps showed a trend whereby smaller epsps recovered to a far greater degree than population spikes. Although this effect was not significant, the NMDA receptor blocker, MK-801, was co-perfused with glutamate during epsp recordings to examine this further. The degree to which MK-801 alone affected the response correlated with the post-glutamate recovery. To study this effect, isolated NMDA-receptor mediated epsps were recorded and the effects of 5mM glutamate upon them were studied. There was a similar tendency for small NMDA-receptor mediated epsps to recover to a higher level following glutamate treatment compared with larger potentials. In the presence of DPCPX, the larger potentials showed a significant elevation in recovery following treatment with glutamate. It was also shown that the post-5mM glutamate recovery of the orthodromic population spikes was elevated by the presence of the A2a receptor antagonist, SCH 58261. Further experiments using the ATP-sensitive potassium channel blocker, glibenclamide, indicated that this effect may be due to increasing the opening of these channels. Adenosine preconditioning was attempted using 10mM glutamate as an insult. It was shown that adenosine could not precondition against this effect in antidromic or orthodromic population spikes. The effects of the sodium-potassium ATPase inhibitor, ouabain, upon the evoked responses were studied as an alternative insult. It was shown that ouabain induced depressions in field epsps, orthodromic and antidromic population spikes. The antidromic population spikes showed significantly smaller depressions than the orthodromic responses. Further experiments using the glutamate receptor antagonist, kynurenic acid, showed that glutamate receptors mediated the effects of ouabain upon the orthodromic population spikes but not the antidromic spikes. Adenosine preconditioning was attempted against ouabain. It was shown that adenosine preconditioned against the effects of ouabain upon orthodromic and antidromic population spikes but not field epsps. Further experiments were conducted using antidromic population spikes. It was shown using various antagonists, that adenosine protection against ouabain was mediated by A1 receptors, ATP-sensitive potassium channels, NMDA receptors and nitric oxide. To extend these results further, preconditioning using the ATP-sensitive potassium channel opener, pinacidil, was attempted against 10mM glutamate and ouabain. It was shown that pinacidil was able to precondition the antidromic population spike against either insult. Using the NMDA receptor antagonist, DL-AP5, showed that the preconditioning effect of pinacidil against ouabain was mediated by NMDA receptors. Another preconditioning paradigm was attempted to see if glutamate could precondition against ouabain. It was shown that pre-

treatment with glutamate resulted in enhancing the depressant effect of ouabain upon field epsps and antidromic population spikes. To further examine the effects of ouabain upon antidromic population spikes, ouabain was co-perfused in the presence of the intracellular calcium chelator, BAPTA-AM. This resulted in enhancing the depressant effect of ouabain upon the response. A similar result was observed when the calcium concentration in the perfusion medium was lowered to 0.5mM from 2.5mM whereas increasing the concentration to 5mM attenuated the depressant effect. Ouabain was also co-perfused in the presence of charybdotoxin, a blocker of large-conductance calcium activated potassium channels. It was observed that charybdotoxin enhanced the effect of ouabain upon the antidromic spikes. No changes were detected in HSP72 expression in the slices in response to ouabain treatment, 10mM glutamate treatment, pinacidil preconditioning treatment or glutamate preconditioning. The present results show that glutamate and ouabain can induce depressions in the evoked responses from the rat hippocampal slice and that the effects of 5mM glutamate can be attenuated by adenosine receptor antagonists. In addition, adenosine can precondition against ouabain but not glutamate and this effect involves A1 receptors, NMDA receptors, nitric oxide and ATP-sensitive potassium channels. It has also been observed that pinacidil can precondition against ouabain or glutamate and NMDA receptors may be involved in this effect. The inability of glutamate to precondition against ouabain in evoked responses was also demonstrated. The study highlights the effectiveness of preconditioning agents against different depolarizing agents and the interactions between adenosine and glutamate receptors which play a role in preconditioning.

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# DECLARATION

I, Alexandra Laura Ferguson, declare that this thesis was composed by myself, and also that the experiments described therein were performed by myself, except where referenced.

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Alexandra L Ferguson

# ABBREVIATIONS

| | |
|---|---|
| aCSF | Artificial cerebrospinal fluid |
| ADP | Adenosine diphosphate |
| 2-AG | 2-arachidonylglycerol |
| AIF | Apoptosis inducing factor |
| ANCOVA | Analysis of covariance |
| AP -5 | (R)-2-amino-5-phosphonopentanoic acid |
| apaf-1 | Apoptotic protease activating factor-1 |
| ATP | Adenosine 5'-triphosphate |
| AMP | Adenosine monophosphate |
| 5'-AMP | 5'-adenosine monophosphate |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid |
| BAPTA-AM | 1,2-bis(o-aminophenoxy)ethane- n,n,n',n'-tetraacetic acid-am |
| $BK^+$ | Large-conductance calcium activated potassium channels |
| CADO | 2-chloroadenosine |
| cAMP | Cyclic adenosine monophosphate |
| CA1 | *Cornu ammonis* 1 |
| CA3 | *Cornu ammonis* 3 |
| CGP 35348 | P-(3-aminopropyl)-p-diethoxymethyl-phosphonic acid |
| ChTX | Charybdotoxin |
| CNQX | 6-cyano-7-nitroquinoxaline-2,3-dione |
| CPA | $N^6$-cyclopentyladenosine |
| D-AP5 | D-2-amino-5-phosphonopentanoic acid |
| DAG | Diacylglycerol |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DNQX | 6,7-dinitroquinoxaline-2,3-dione |
| DPCPX | 8-cyclopentyl-1,3-dipropylxanthine |

| | |
|---|---|
| E-64 | (2*S*, 3*S*)-3-(*N*-{(*S*)-1-[*N*-(4-guanidinobutyl) carbamoyl]3-methylbutyl}carbamoyl)oxirane-2-carboxylic acid |
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylenediamine tetraacetic acid |
| EGTA | Ethylene glycol tetraacetic acid |
| EPSP | Excitatory post-synaptic potential |
| E-S | Epsp-spike |
| GABA | Gamma-amino butyric acid |
| 5-HD | 5-hydroxydecanoic acid |
| HPLC | High performance liquid chromatography |
| HSP70 | Heat shock protein 70 |
| HSP72 | Heat shock protein 72 |
| i.p. | Intraperitoneal |
| $IP_3$ | Inositol-1, 4, 5-triphosphate |
| IPSP | Inhibitory post-synaptic potential |
| i.v. | Intravenous |
| JNK | C-jun N-terminal kinase |
| L-NAME | $N_\omega$-nitro-l-arginine methyl ester hydrochloride |
| LTD | Long term depression |
| LTD-I | Long term depression of inhibition |
| LTP | Long term potentiation |
| MgATP | Magnesium-bound adenosine 5'-triphosphate |
| mGluRs | Metabotropic glutamate receptors |
| mIPSC | Miniature inhibitory post-synaptic current |
| (+)-MK-801 | Dizocilpine / (5s, 10r)-(+)-5-methyl-10,11-dihydro-5*h*-dibenzo[a,d]cyclohepten-5,10-imine maleate |
| MOPS | 3-morpholinopropane-1-sulfonic acid |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| NBQX | 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione |

| | |
|---|---|
| NMDA | *N*-methyl-d-aspartate |
| nNOS | Neuronal nitric oxide synthase |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NPPB | 5-nitro-2-(3-phenylpropylamino) benzoic acid |
| 3-NPA | 3-nitropropionic acid |
| OGD | Oxygen-glucose deprivation |
| PCP | Phencyclidine |
| $PIP_2$ | Phosphatidylinositol-4,5-biphosphate |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PKG | Protein kinase G |
| $PLA_2$ | Phospholipase $A_2$ |
| PLC | Phospholipase C |
| PMSF | Phenylmethane sulfonylfluoride |
| PVDF | Polyvinylidene fluoride |
| RIPA | Radioimmuno precipitation assay |
| ROS | Reactive oxygen species |
| R-PIA | R-phenyl-isopropyladenosine |
| SAH | S-adenosylhomocysteine |
| SCH 58261 | 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2, 4]triazolo[1,5 c]pyrimidin-5-amine |
| SDS | Sodium dodecyl sulphate |
| SUR | Sulfonylurea receptor |
| TLCK-HCl | Nα-Tosyl-Lys-chloromethylketone·HCl |
| TM | Transmembrane |
| TMD | Transmembrane domain |
| TTBS | Tween Tris-buffered saline |
| ZM 241385 | 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5]triazo-5-yl-amino]ethyl)phenol |

# ACKNOWLEDGEMENTS

I would like to extend my deepest and warmest thanks to my supervisor, Professor Trevor W. Stone, for his invaluable expertise and enthusiasm throughout the completion of this project. The production of this thesis was made possible by his constant availability and support upon overseeing and advising me on the work, all of which have been very much appreciated.

My colleagues in the lab have been very helpful and supportive in their advice throughout my doctoral studies. In particular I would like to thank Dr Clare Guilding and Dr Duncan MacGregor for their advice with the intial setup of the electrophysiology work and Dr Kara McNair and Dr Clare Guilding for demonstrating the Western blot method and their advice during the completion of those experiments. In addition, I wish to thank Professor Brian Morris for allowing me the use of his laboratory to perform the Western blotting. My thanks are also extended to Mr Majed Al-Gonaiah, Dr Amos Fatokun, Dr Caroline Forrest, Dr Gillian Mackay, Dr Andrew Smith, Dr Sarah Mackay and Professor Rob Smith for their support and useful discussions and comments upon the work.

I would also like to thank my brother, Mr John Urquhart Ferguson, who extensively advised me on the type-setting of the thesis and for his IT expertise and knowledge which was always readily to hand on numerous occasions. His friendship and moral support to me has been of immeasurable value for which no amount of thanks can do justice.

Finally, I would like to extend my warmest appreciation to my parents, John and Laura, for their emotional support to me and their generous financial support of this work throughout the past few years. They have endured, with great patience and understanding, my lowest moments throughout my studies and have ceaselessly provided advice and care for which I am forever grateful.

# PUBLICATIONS

Ferguson A.L. & Stone T.W. (2006) A role for adenosine in glutamate preconditioning. FENS Forum Abst., vol. 3, A010.3.

Ferguson A.L. & Stone T.W. (2007) Adenosine preconditions against ouabain but not glutamate in CA1 neurons. Br. Neurosci. Assoc. Abst., 19, 141 [65.05].

Ferguson A.L. & Stone T.W. (2007) Preconditioning interactions in the rat hippocampal slice. Abst. Life Sci., Glasgow 25-26P [PC53].

Ferguson A.L. & Stone T.W. (2008) Role of glutamate in ouabain-induced depression of hippocampal potentials. FENS Forum Abstr., vol.4, 010.13.

Ferguson A.L. & Stone T.W. (2008) Adenosine preconditions against ouabain but not against glutamate on CA1 evoked potentials in rat hippocampal slices. European Journal of Neuroscience DOI: 10.1111/j.1460-9568.2008.06490.x

# 1 - INTRODUCTION

Preconditioning is a phenomenon whereby tolerance to lethal insults is induced by prior sublethal insults. It was first discovered in the canine myocardium (Murry et al., 1986) where the tissue became tolerant to a hypoxic state when pretreated with transient, repeated episodes of hypoxia. This effect has since been studied in a variety of tissues with many investigations focussing on the induction of ischaemic tolerance in neuronal tissues. The first of these exhibited hypoxic preconditioning in rat hippocampal slices (Schurr et al., 1986). Since then, a variety of paradigms have been used to study the mechanisms in neuronal tissue employing a number of different experimental systems. This has revealed two distinct time windows of tolerance: acute and delayed.

Acute preconditioning occurs within a time scale of minutes to hours in the brain and involves several components which include NMDA receptors (Kato et al., 1992; Bandyopadhyay et al., 2002), A1 receptors (Heurteaux et al., 1995; Hiraide et al., 2001; Pugliese et al., 2003) and ATP-sensitive K+ channels (Heurteaux et al., 1995; Pérez-Pinzón & Born, 1999; Garcia de Arriba et al., 1999; Domoki et al., 1999; Blondeau et al., 2000, Nakagawa et al ,2002; Rajapakse et al., 2002) amongst many others. These features also apply to the delayed time window of preconditioning and have been studied to a much greater extent than rapid preconditioning. Although they share similar mechanisms, the induction of delayed preconditioning involves changes in expression of various proteins including inducible heat shock protein 72 (Kirino et al., 1991; Liu et al., 1993) and anti-apoptotic protein Bcl-2 (Wu et al., 2004) whilst molecular changes during rapid preconditioning are unlikely to occur within the time window. Although rapid and delayed preconditioning share some common features, the way in which these mechanisms actually induce protection may vary between the two time windows. A greater understanding of these mechanisms, particularly those of the less studied rapid time window, may lead to the discovery of new drug targets which may ultimately mimic the protective effect of preconditioning.

Various experimental systems have been used to outline the events of preconditioning. Given the two windows of tolerance, acute preparations such as hippocampal slices have been used to study the preconditioning mechanisms within the rapid time window whilst neuronal cultures and in vivo models (global and focal ischaemia) are used more frequently in studies of delayed protection.

There are several pathophysiological and pharmacological stimuli that result in preconditioning in neuronal tissue. Among these are ischaemia (Kitagawa et al., 1990; Kato et al., 1991; Liu et al., 1992; Miyashita et al., 1994), anoxia (Schurr et al., 1986), hypoxia (Schurr et al., 2001), oxygen-glucose deprivation (OGD) (Pugliese et al., 2003),, adenosine (Pérez-Pinzón et al., 1996), glutamate (Schurr et al., 2001), NMDA (Schurr et al., 2001; Bandyopadhyay et al., 2002), nitric oxide (Centeno et al., 1999), ATP-sensitive K+ channel openers (Pérez-Pinzón & Born, 1999; Blondeau et al., 2000, Heurteaux et al., 1995; Nakagawa et al., 2002; Garcia de Arriba et al., 1999; Domoki et al., 1999; Rajapakse et al., 2002) and several others. There are also several models of ischaemia that have been used to observe these effects of preconditioning. These can be physiological (blood vessel occlusion models, OGD, hypoxia or anoxia) or chemical (glutamate, ouabain, etc). As a result, there is a convoluted literature on paradigms and mechanisms of preconditioning.

## 1.1 - Ischaemia

The pathological processes resulting in reduced brain function arise from a decrease in blood supply in vivo or ischaemia (Lipton 1999). When neuronal tissue is deprived of oxygen and glucose, ATP production in the mitochondria for which these are required will slow down and eventually stop. The extent of ATP depletion determines the pathway of damage either by necrosis or apoptosis (Hou & MacManus, 2002).

ATP-dependent processes fail to function when ATP is depleted during ischaemia. Amongst these are the sodium-potassium ATPase pumps which maintain ion homeostasis under normal conditions. In excitable tissues, the electrogenic pumps acts to restore ion gradients following current generation across cellular membranes. They function by exchanging 3 sodium ions from the cytoplasm with 2 potassium ions in a sequential manner. As this actively drives the movement of ions against their concentration gradients, the process requires hydrolysis of ATP. The pump is composed of an α subunit and a β subunit. The α subunit is the site at which the ion transport occurs. The potassium ions bind to an extracellular site upon the α subunit. Magnesium-bound ATP (MgATP) also binds with low affinity to this subunit and accelerates the transport of potassium into the cell. MgATP remains bound with high affinity once the potassium ions have been transported across the membrane. This is because ATP and potassium exert antagonism against each other as they stabilise the pump in two different conformations. The transition between the two conformations is associated with increasing binding energy of ATP to the

subunit. It is the increase in binding energy which constitutes the driving force of the pump.

After the potassium ions have been released in the cytoplasm, 3 sodium ions then bind to the subunit inducing a release of ADP from ATP hydrolysis. This leaves a magnesium ion bound to the phosphorylated subunit with 3 bound sodium ions. The next step involves the conformational change in the pump to transport the 3 sodium ions to the extracellular surface. Potassium binding to the subunit then displaces and releases magnesium and inorganic phosphate leaving the subunit in a conformation available to transport potassium ions again (for review, see Jorgensen and Pedersen, 2001).

The breakdown of the pump during ischaemia is thought to cause the anoxic depolarization (propagating electrical silence across neurons); the depolarization itself has been shown to mediate damage in neocortical brain slices (Jarvis et al., 2001) by cell swelling of the neurons. The resulting membrane depolarisation causes an increase in extracellular glutamate concentration. This increase has been largely attributed to a massive release of neurotransmitter from pre-synaptic neurons; there is growing evidence that this could be mainly due to reversed uptake of glutamate (Madl & Burgesser, 1993; Rossi et al., 2000). The glutamate transporters operate by exchanging $Na^+$ and $K^+$ ions down their gradients to power the transport of glutamate into the neurons and glia. Upon ATP depletion, the sodium-potassium ATPase pump stops functioning and the alteration in $Na^+$ and $K^+$ homeostasis can cause reversal of the transporter; this has been shown in rat hippocampal slices (Madl & Burgesser, 1993; Rossi et al., 2000). Excessive extracellular glutamate can induce excitotoxicity; this is explained later on in section 1.3.2 Excitotoxicity.

In vivo, cerebral ischaemia usually occurs in one of two forms; focal or global. Whilst global ischaemia is non-selective in terms of oxygen and glucose deprivation to the brain tissue, focal ischaemia is confined to a region of the brain and consists of an infarct core which shows severe energy depletion and a delayed cell death form such as necrosis and a surrounding penumbra region which has low ATP levels and is subject to mainly apoptotic delayed cell death mechanisms. The deleterious effects in the larger penumbral region may be reversed by therapeutic intervention so it is therefore of interest to examine the mechanisms of acute damage and delayed cell death in response to ischaemia (for review, see Hou & MacManus, 2002).

## 1.2 - Ischaemic preconditioning – a historical perspective

### 1.2.1 - Evidence and paradigms

Preconditioning was first identified in the canine heart (Murry et al., 1986) when occlusion of the left circumflex coronary artery for a protocol of 5min ischaemia applied four times and separated by 5min intervals produced tolerance against a 40min occlusion ($p<0.001$). The infarct size was 25% of that seen in controls. It was soon discovered that this phenomenon was not limited to the heart but existed in neural tissue also. Using rat hippocampal slices, application of 5min anoxia (by bubbling the perfusing medium with 95% $N_2$/5% $CO_2$ gas mixture) followed by 13 ± 2 min anoxia 30min later resulted in improved recovery of the evoked response of the slice compared to control ($p<0.05$) (Schurr et al., 1986). Other studies have since confirmed these observations in vivo and in vitro.

### 1.2.2 - In vivo preconditioning

Similar to Murry et al. (1986), ischaemic preconditioning of the brain was observed in vivo using bilateral occlusion of the carotid arteries in Mongolian gerbils (Kitagawa et al., 1990). The interval between preconditioning treatment (2min) and ischaemic insult (5min) was at least one day, with several hours at least separating each sublethal preconditioning ischaemia. The time scale of protection also showed a delayed phase of preconditioning. There was also a cumulative effect with multiple sublethal episodes generating greater neuroprotection than single ischaemic episodes.

Another study which implemented bilateral carotid artery occlusion in gerbils showed that 2min occlusion effectively induced tolerance to 3min occlusion but only if the second insult was applied at least 24hrs later (Kato et al., 1991). A 3min occlusion induced an observable decrease in surviving hippocampal CA1 neurons whereas 2min showed no such difference. Focal ischaemia can also induce preconditioning (Liu et al., 1992). Three minutes of focal ischaemia in Wistar rats increased the levels of heat shock protein 72, a protein implicated in the preconditioning phenomenon, in CA1 neurons. After 3 days, protection against 6 and 8min focal ischaemia was observed. A further study showed that focal ischaemia could precondition against global ischaemia (Miyashita et al., 1994). Transient focal ischaemia was induced by unilateral middle cerebral artery occlusion as a preconditioning stimulus; this was able to protect the tissue against damage induced by a further 5min bilateral carotid artery occlusion. There was approximately 40% survival of

CA1 neurons in the left hippocampus with no surviving neurons in the right. Protection was not as robust in this protocol.

The preconditioning effect as observed in vivo suggests that it is physiologically relevant and therefore study of the underlying mechanisms a worthwhile contribution to the scientific literature.

### 1.2.3 - In vitro preconditioning

There are several experimental paradigms used to illustrate in vitro preconditioning such as acute hippocampal slices and cell culture systems. The acute slice system is a good model for illustrating rapid preconditioning owing to the time scale over which they work. Following on from Schurr et al. (1986), it was shown that brief, 2min oxygen-glucose deprivation (OGD) applied to rat hippocampal slices four times preconditioned against a 7min OGD applied 30min later ($p<0.05$) (Pugliese et al., 2003). A similar protocol was adopted by Centeno et al. (1999) using 1min anoxic insults 3 times separated by 10min reperfusion to precondition against anoxia; this was applied until 2min of anoxic depolarization occurred. This resulted in over 90% recovery of the evoked responses from the CA1 pyramidal cells compared with less than 10% recovery of response in controls ($p<0.01$).

Studies using cell cultures have also illustrated the effects of preconditioning. Cortical cultures were exposed to 90min OGD (Bruer et al., 1997). This sublethal insult did not induce changes in lactate dehydrogenase (LDH) release, a measure of the number of damaged neurons. When exposed to 180min OGD after 3 days, there was also no release of LDH compared to a 70% increase in controls. It was noted that tolerance was not observed before 24hrs reperfusion. There are similar observations in hippocampal cell cultures (Khaspekov et al., 1998). 60min OGD preconditioned against a 90min insult applied at 24 or 48 hrs later ($p<0.01$). There was approximately 40-60% protection at the 24 hr time point and complete protection at 48hrs. The time course of protection in cultures matches that of in vivo studies.

## 1.3 - Glutamate

The amino acid, glutamate, is an excitatory neurotransmitter which is ubiquitous throughout the central nervous system (Meldrum, 2000). It is synthesized in the nerve terminals from glucose as part of the Krebs cycle and by transamination of α-oxoglutarate. Glutamate may also be formed via glutamine, another amino acid synthesized in glial cells. Glutamine is transported to the nerve terminals where it is converted into glutamate by the enzymatic action of glutaminase. Glutamate is then taken up into the vesicles within the pre-synaptic neurons. When the propagation of action potentials along the axons of the pre-synaptic neurons reaches the axon terminal, the resulting depolarization of the terminal membrane will open the voltage-gated calcium channels. Upon entry of calcium into the pre-synaptic neuron, the calcium ions will interact with vesicle-associated proteins which lead to the trafficking of vesicles to the synaptic membrane. The vesicles then fuse with the membrane which results in releasing the contents of the vesicles into the synaptic cleft, a process termed exocytosis. Once released into the synaptic space, glutamate diffuses throughout the cleft, mediating several actions at glial, pre- and post-synaptic sites. One of the primary actions is the stimulation of glutamate receptors which are classed into two different families, ionotropic and metabotropic.

### 1.3.1 - Ionotropic glutamate receptors

The ionotropic receptors are ligand-gated ion channels which are dependent upon the binding of glutamate to activate them. Upon activation, the conformation of the receptor is altered so as to allow the channel to become permeable to ions. In the nervous system, the glutamate ionotropic receptors are located on post-synaptic neurons and consist of 3 different types: NMDA receptors; AMPA receptors; kainate receptors (for review, see Ozawa et al., 1998).

#### 1.3.1.1 - NMDA receptors

The NMDA receptors are so named because they are selectively activated by the ligand, N-methyl D-aspartate (NMDA). They are slowly-activating and require glutamate and glycine binding for the channel to open as well as membrane depolarization to relieve the voltage-dependent magnesium blockade of the open pore (Mayer et al., 1984). Once activated, they become permeable to sodium and calcium ions (Ascher & Nowak, 1988). NMDA receptors have a complex pharmacology and function within the brain and mediate various forms of synaptic plasticity within the hippocampus. In addition, they contribute to the excitotoxic effects which occur when the receptors are overstimulated during an

ischaemic event. They have also been shown to mediate some forms of preconditioning in the brain.

#### 1.3.1.1.1 - Molecular biology

There are several forms of the NMDA receptor which exist on account of the various subunits from which a single receptor may be composed. The subunits each contain a large extracellular N-terminal domain, 3 transmembrane segments (TM1, 3 and 4), a re-entrant loop between TM3 and TM4 (M2) and a cytoplasmic c-terminal domain. There are 3 different subtypes of NMDA receptor subunits called NR1, NR2 and NR3. Each of these subunits has various isoforms which affect the functional properties and pharmacology of the NMDA receptor in which it is assembled (for review, see Paoletti & Neyton, 2007).

Each NMDA receptor is made up of 4 subunits with at least one being of the NR1 subtype and one of the NR2 subtype in order for the receptor-channel to function. This is because the NMDA receptor requires activation by two agonists, glutamate and glycine (or D-serine (Mothet et al., 2000)), the binding sites of which are upon the NR2 subunits and the NR1 subunits respectively. The NR3 subunit may also bind glycine but its presence in a receptor assembly negatively affects the channel conductance, calcium ion permeability and voltage-dependent magnesium block.

The NR1 subunit has 8 different isoforms which result from different combinations of alternately spliced cassettes, one in the N-terminal (N1) and two in the C-terminal domain (C1,C2). The properties of the NR1 subunit varies according to which cassettes are present. The presence of N1 reduces agonist affinity 5 fold and increases current amplitude 3 fold. It also reduces polyamine potentiation. C1 enhances the localisation of the NR1 subunit to the synapses and C2 reduces protein kinase C (PKC) potentiation 3 fold and tethers the receptor to the post-synaptic density. The NR1 subunit has a string of glutamic acid residues with negative charges just before M2. This property is considered important for the development of the voltage-dependent magnesium block as the positively charged ion may be pulled into the pore (Dingledine et al., 1999).

The NR2 subunit has 4 isoforms (NR2A, NR2B, NR2C, NR2D). Each of these isoforms exerts their own properties upon the NMDA receptor. For example, the presence of the NR2B subunit increases polyamine potentiation compared with other NR2 subunits. The presence of a subunit within the NMDA receptor assembly may be identified by the subunit pharmacology which is exerted upon the receptor.

#### 1.3.1.1.2 - Pharmacology

There are several sites on the NMDA receptor upon which drugs and endogenous compounds may act (for review, see Stone & Addae, 2002). Glutamate binds to a site within the agonist binding domain upon the NR2 subunit whilst its co-agonist, glycine, binds to a site upon the NR1 subunit. It is at these sites that competitive agonists and antagonists are able to bind, for example, NMDA (glutamate site agonist), AP5 (glutamate site antagonist), D-serine (glycine site agonist), kynurenic acid (glycine site antagonist).

In addition there are binding sites within the channel pore that modulate the activity of the receptor in the open state. When the pore opens, the ion current is initially blocked by the binding of magnesium ion within the pore (Nowak et al., 1984); this occurs as magnesium is attracted to the negative resting membrane potential upon the inside of the cell with respect to the outside. The binding site within the channel is also lined with negatively charged residues to which the magnesium ion is drawn. Usually, the AMPA and kainate receptors will also be stimulated by glutamate and allow the influx of sodium ions which depolarizes the membrane. As the ion influx continues, the membrane depolarization eventually relieves the magnesium blockade upon the NMDA receptors (Mayer et al., 1984). There is also a site which binds phencyclidine (PCP) when the channel is open. Other channel blockers such as MK-801, ketamine and memantine bind to this site.

The NMDA receptor also contains binding sites for allosteric modulators. Some of these agents show some selectivity for the different subunits, for instance, at the relevant site, zinc binds preferentially to NR2A over NR2B subunits to inhibit the receptor activity. Ifenprodil is a modulator which binds specifically to NR2B subunit containing receptors. There is also a polyamine binding site which promotes the receptor activity when bound by endogenous spermine or spermidine.

### 1.3.1.2 - AMPA receptors

Another form of ionotropic receptor is the AMPA receptor. This receptor mediates fast excitatory transmission and once activated by glutamate, becomes rapidly desensitized. Like NMDA receptors, they are involved in synaptic plasticity within the hippocampus and are permeable to sodium ions.

Each AMPA receptor contains 4 subunits. There are 4 different types of subunit, each containing an extracellular N-terminal domain, 4 TM segments and a cytoplasmic C-terminal domain. The subunits are named GluR1, GluR2, GluR3 and GluR4. There is a single residue upon TM2, the subunit segment which lines the channel pore, which

influences the ion selectivity of the channel. In GluR2 subunits, the residue is the positively charged arginine. Its presence in the lining of the pore prevents the permeability of divalent cations such as calcium but allows monvalent sodium ions through the receptor channel. In the other subtypes of subunit, a glutamine residue is present instead of arginine. In AMPA receptors lacking GluR2 subunits, the channel becomes permeable to divalent cations. However, the GluR2 subunit appears to be ubiquitously expressed throughout the nervous system and its presence in the AMPA receptor assembly prevents permeability to calcium, even if GluR1, 3 and 4 form the other three subunits. AMPA receptors are therefore predominantly permeable to sodium ions (Ozawa et al., 1998).

In terms of binding sites, the glutamate binding site is composed of two segments of the subunits; a region of residues between the N-terminal domain and TM1 and another group of residues located in the extracellular loop between TM3 and TM4. Together these regions form a binding pocket for the neurotransmitter. It is at this site that competitive agonists (eg AMPA) and antagonists (eg DNQX) also bind. Within the AMPA receptor, there also exists a site which affects the rapid desensitization of the receptor. This is affected by compounds such as diazoxide, a mitochondrial ATP-sensitive potassium channel opener, which prevents the receptors from desensitizing (Yamada & Rothman, 1992).

#### 1.3.1.3 - Kainate receptors

Kainate receptors are similar in structure, function and pharmacology to AMPA receptors. The receptors are composed of subunits of which there are 5 different subtypes, GluR5, GluR6, GluR7, KA1 and KA2. These all have the same structure as AMPA receptors and may contain either arginine or glutamine at the Q/R site. These subunits have around 40% homology to AMPA receptor subunits. The receptor is selectively bound by the agonist, kainic acid, but there are few selective drugs which can pharmacologically distinguish between AMPA and kainate receptors. They are associated with excitotoxicity as kainic acid is able to cause injury to regions which show a high expression of kainate receptors, eg CA3 of the hippocampus (Sperk, 1994).

### 1.3.2 - Excitotoxicity

Glutamate normally functions to relay chemical messages between neurons at the synaptic clefts. It is crucial in mediating the normal processes of transmission that occur within the brain as well as changes in synaptic plasticity. The excitatory transmitter is normally present in the extracellular space at less than 1μM. If the concentration is elevated to

between 2-3μM such as during ischaemia, it becomes neurotoxic or excitotoxic due to overactivation of glutamate receptors (Obrenovitch et al., 2000). In particular, it appears that excessive calcium influx resulting from overactivation of NMDA receptors induces cell-death processes by activating degrading enzymes and generating harmful free radicals by excessive calcium sequestration within the mitochondria. In addition, the excessive ion influx through all the ionotropic glutamate receptors results in oedematous swelling of the neuron and ultimately, damage to the cell. Excitotoxicity can induce delayed cell death mechanisms via either necrosis or apoptosis. The hippocampal CA1 area seems to be particularly vulnerable to excitotoxcity due to a high quantity of NMDA receptors (Simon et al., 1984).

### 1.3.3 - Glutamate preconditioning

One of the first studies to demonstrate that glutamate can precondition against further glutamate insults was conducted in rat hippocampal slices (Schurr et al., 2001). The protocol consisted of applying 20mM glutamate for 5min to precondition. After a washout of 30min, the same concentration of glutamate was applied for 20min. Preconditioning was observed simply as a return of the evoked responses in the slice as 20mM glutamate is toxic to the point of irreversible damage. They observed that approximately 55% of the slices showed a return in evoked responses after a preconditioning protocol compared with approximately 28% in controls ($p<2.56e^{-7}$). This study drew from previous experiments in cell cultures showing NMDA exposure to neurons could attenuate the damage mediated by glutamate (Chuang et al., 1992; Pringle et al., 1999). Chuang et al. (1992) used cerebellar granule cell cultures to show that a 3hr NMDA exposure blocked the damage mediated by glutamate. This is in agreement with the study by Schurr et al. (2001) using 5min of 20μM NMDA to prevent glutamate toxicity. Similar observations were made by Pringle et al. (1999) regarding NMDA induction of tolerance to glutamate toxicity although it was also observed that sublethal NMDA exposure did not precondition against a subsequent NMDA insult. These studies led onto the supposition that NMDA receptors may mediate excitotoxic preconditioning.

#### 1.3.3.1 - NMDA receptors mediate excitotoxic preconditioning

It has been shown that NMDA receptor stimulation is required for neuronal preconditioning (Kato et al., 1992) as blockade by MK-801, an NMDA antagonist, attenuated in vivo ischaemic preconditioning in Mongolian gerbils. This evidence has been

contradicted in a study using a similar protocol in Wistar rats (Wrang & Diemer, 2004). MK-801 did not decrease the neuroprotection offered by preconditioning. There was no clear indication why opposing results were obtained although there were slight differences in ischaemic insults applied to account for the differential sensitivity of rat and gerbil brains to bilateral carotid artery occlusion. NMDA antagonism has been shown to block ischaemic tolerance in Mongolian gerbils in vivo in another study (Bond et al., 1999). The protocol was almost identical to Kato et al. (1992) using 2min bilateral carotid artery occlusion to precondition, 3min occlusion for a test insult and MK-801 to inhibit NMDA receptors. It was also shown that AMPA receptor competitive and non-competitive antagonism had no significant effect on preconditioning.

A study in hippocampal slices showed that a protocol of 100μM NMDA for 15sec applied every 10min for three applications induced protection against NMDA application for 10min after 30min washout (Bandyopadhyay et al., 2002). This study showed particularly that this protocol decreased cellular swelling in the slices thus attenuating damage caused simply by elevated ion permeability and not necessarily that induced by excessive $Ca^{2+}$ influx. The protocol for NMDA application was adapted from the study of hypoxic preconditioning in slices by Centeno et al. (1999).

### 1.3.3.2 - Mechanism for ischaemic tolerance induced by NMDA receptors

There is little evidence to explain how NMDA receptor activation may mediate preconditioning. Low-level activation is a requirement so as not to induce cell death. A study, which elucidated some part of this protection, used 3-nitropropionic acid (3-NPA), an inhibitor of succinic dehydrogenase in the mitochondria, to precondition against 15min hypoxic insults applied to hippocampal slices (Kasischke et al., 1996). Intraperitoneal injection of 3-NPA (20mg/kg) in rats 1hr prior to slice preparation was performed. Tolerance was observed as population spikes in the preconditioned slices recovered to 90 ± 7% compared with 31 ± 9% in controls ($p<0.05$). This effect was attenuated by 15min perfusion of 100μM AP-5 (NMDA antagonist). As the preconditioning was mediated by either generation of reactive oxygen species produced by the inhibition of the electron transport chain or mild excitotoxicity due to ATP depletion, subsequent membrane depolarization and removal of the $Mg^{2+}$ block at NMDA receptors, this suggests that NMDA receptor activation mediates its own neuroprotection by one or both of these mechanisms. NMDA receptors are known to activate nitric oxide synthase (NOS) thus generating the gaseous particle, nitric oxide (NO) (Yamada & Nabeshima, 1997a + b;

Bredt & Synder, 1989), which has been identified as a mediator of anoxic preconditioning in hippocampal slices (Centeno et al., 1999). In that study, anoxia was generated by bubbling the perfusion medium with 95%N2/5%CO2 in place of oxygenated gas. 1 min of anoxia was applied three times, each separated by 10min, as the preconditioning stimulus. After 30min of reoxygenation following the last 1min anoxia applied, 95%N2/5%CO2 was bubbled into the medium until anoxic depolarization of the evoked response was generated for 2min. This significantly increased the recovery of the evoked response after the anoxic depolarization compared with control responses in which anoxic depolarizations alone were generated. The authors found that repeating the preconditioning protocol in the presence of 7-nitroindazole, a NOS inhibitor, prevented the increase in recovery of the evoked response following 2min of anoxic depolarization. They also found that similar protection could be induced by perfusing diethylamine/nitric oxide complex for 10min onto the slice 30min prior to the generation of anoxic depolarization again. The role of nitric oxide has also been shown in delayed preconditioning protocols in vivo (Gidday et al., 1999) and in vitro (Gonzalez-Zulueta et al., 2000). As Centeno et al. (1999) have observed that NO is involved in anoxic preconditioning, it is very likely that this extends to excitotoxic preconditioning as a result of NMDA receptor stimulation.This has been demonstrated by Youssef et al. (2006) in rat hippocampal slices where a 10μM NMDA application (5min) resulted in preconditioning the population spike against a further 15μM NMDA application applied 30min later. The authors showed that the preconditioning stimulus also impaired the ability of the slice to produce a spike potentiation in response to tetanic stimulation and that this effect was prevented by the NOS inhibitor, L-NAME, further suggesting that nitric oxide may also mediate the preconditioning effect of NMDA.

## 1.4 - Adenosine

Adenosine is an inhibitory neuromodulator which is normally present at 25-150nM in the extracellular medium (for review, see Dunwiddie & Masino, 2001). Its main action in the brain is to suppress excitatory transmission by inhibition of transmitter release from the pre-synaptic terminals. It does have several interactions in neural tissue during ischaemia and preconditioning as well as during synaptic plasticity.

Adenosine is synthesized in the extracellular medium by the dephosphorylation of adenine nucleotides by ecto-nucelotidases, ecto-phosphodiesterases and apyrases. This forms the precursor, 5'-AMP which is then converted to adenosine by 5'-nucleotidase. Intracellularly, adenosine can also be synthesized from 5'-AMP but it may also be formed from S-adenosylhomocysteine (SAH) by the enzyme, SAH hydrolase.

### 1.4.1 - Metabotropic adenosine receptors

Adenosine exerts its effects by its actions upon four G-protein-coupled receptors: A1; A2a; A2b; A3. As metabotropic receptors they have seven transmembrane domains and an N-terminal and C-terminal domain. They are all coupled to G proteins which act as signalling messengers for specific targets to exert their actions upon the cell.

#### 1.4.1.1 - Adenosine A1 receptors

Of these receptors, adenosine shows the highest affinity for A1 receptors which is the most abundant subtype of adenosine receptor in the hippocampus (for review, see Ralevic & Burnstock, 1998). A1 receptors are coupled to inhibitory $G_{i/o}$ proteins which then act to inhibit adenylyl cyclase, hyperpolarize neurons by increasing $K^+$ conductance and suppress neurotransmitter release by inhibiting mainly N-type $Ca^{2+}$ channels at the pre-synaptic neuron of the CA3-CA1 synapse (Manita et al., 2004). Post-synaptically they appear to interact with NMDA receptors and suppress their activity (Canhão et al., 1994; Klishin et al., 1995; de Mendonça & Ribeiro, 1993; de Mendonça et al., 1995).

The A1 receptors activate phospholipase C (PLC) which in turn leads to the production of the second messengers, inositol tris-phosphate ($IP_3$) and diacylglycerol (DAG). $IP_3$ can act upon receptors of the endoplasmic reticulum to mobilise the calcium stores which can affect many calcium dependent processes such as the formation of nitric oxide and phospholipase $A_2$ ($PLA_2$) as well as the activity of calcium-dependent potassium channels. Increasing intracellular calcium availability can also positively modulate PKC, a signalling messenger which is also activated by DAG. PKC can induce the opening of the ATP-sensitive potassium channels which may affect the resting membrane potential and the function of the mitochondria (see section 1.5 - ATP-sensitive potassium channels).

#### 1.4.1.2 - Adenosine A2a receptors

Unlike the A1 receptors, A2a receptors are coupled to $G_s$ protein which increases cAMP production and overall produces an excitatory effect (for review, see Sebastião & Ribeiro, 1996). These receptors are mainly present in the striatum although they are also present in the hippocampus, co-localized with A1 receptors. They disinhibit the A1 receptors in the hippocampus (Cunha et al., 1994) and attenuate the effects of the A1 receptor upon the relationship between presynaptic transmitter release and post-synaptic responses in the rat hippocampal slice (O'Kane & Stone, 1998).

#### 1.4.1.3 - Adenosine A2b receptors

A2b receptors are also coupled to $G_s$ proteins to increase cAMP production. They have been cloned from the human hippocampus (Pierce et al., 1992) and have a much lower affinity for adenosine than A2a receptors. They have not been well characterised on account of the few pharmacological agents available which selectively bind.

#### 1.4.1.4 - Adenosine A3 receptors

A3 receptors are inhibitory in nature. They are coupled to $G_i$ proteins to decrease cAMP production and also increase PLC and $IP_3$ intracellularly which in turn increases intracellular calcium. In the hippocampus, the adenosine A1 receptor rapidly desensitizes in response to agonist exposure within minutes. This desensitization can continue for several hours after the initial exposure and is thought to occur as a result of the action of A3 receptors upon A1 receptors (Dunwiddie et al., 1997).

### 1.4.2 - Adenosine metabolism

Adenosine is catabolised by adenosine kinase intracellularly. The reaction involves dephosphorylation of ATP to produce ADP and AMP. In the extracellular space, adenosine is broken down by adenosine deaminase to form inosine.

### 1.4.3 - Adenosine during ischaemia

There is a huge elevation in adenosine concentration in the extracellular space during ischaemia resulting from ATP breakdown. Adenosine is formed from dephosphorylation of 5-AMP, a metabolite of ATP. In rat hippocampal slices, a 5min exposure of 95% $N_2$/5% $CO_2$ bubbled perfusion medium completely depressed the evoked field epsps from CA1 neurons. The presence of an A1 receptor antagonist, 8-phenyltheophylline, prevented the depression of the evoked response. Measurements of adenosine concentrations indicated that it had greatly increased within 10min of the onset of ischaemia (Pedata et al., 1993). A 5min ischaemic insult in hippocampal slices increased adenosine concentration to 30μM from a basal concentration of 240nM (Latini et al., 1999b). It has also been shown that adenosine mediates the initial depression of electrophysiological responses in the hippocampus during common carotid artery occlusion of rats in vivo (Gervitz et al., 2001). Adenosine normally suppresses glutamate release from neurons and thus the concentration increase would appear to ameliorate the damaging effects of glutamate. It has been shown, however, that at elevated concentrations which occur during ischaemia, adenosine can activate A3 receptors which in turn desensitize A1 receptors, most likely through a G-

protein coupled mechanism (Dunwiddie et al., 1997). As the suppression of glutamate release is mediated via A1 receptors, it is possible that adenosine contributes to neuronal death by blocking protective mechanisms via A3 receptors.

### 1.4.4 - Adenosine preconditioning

Several paradigms in the scientific literature have shown the ability of adenosine to precondition against anoxic and hypoxic insults (Heurteaux et al., 1995; Pérez-Pinzón et al., 1996; Kitagawa et al., 2002). This was first demonstrated in vivo using bilateral common carotid artery occlusion in rats for both preconditioning stimuli and lethal insult (Heurteaux et al., 1995). A 3min sublethal occlusion induced tolerance to a 6min occlusion 3 days later ($p<0.05$). It was noted that if the second occlusion was performed 1hr later, the entire population of CA1 pyramidal cells in the hippocampus were destroyed. The beneficial effects observed were cancelled when DPCPX, an adenosine A1 receptor antagonist was administered intraperitoneally (1 mg/kg) 15min prior to the sublethal ischaemia. Administration of N6-cyclopentyladenosine (CPA), an adenosine A1 agonist, when used in the same protocol, also improved recovery after lethal ischaemia. Adenosine appears to mediate its preconditioning effects in neuronal tissue through stimulation of A1 receptors. This was also observed in hippocampal brain slices (Pérez-Pinzón et al., 1996) using a perfusion medium bubbled in 95% $N_2$/5% $CO_2$ to generate anoxia. 100μM adenosine perfused for 10min prior to anoxia maintained until 2min of anoxic depolarization onset induced tolerance ($p<0.05$). The studies described above show that adenosine is involved in acute and delayed preconditioning.

Further studies have confirmed these observations (Hiraide et al., 2001). In vivo bilateral occlusion of common carotid arteries for 2min followed by 5min ischaemia 24hrs later showed that damage in the CA1 pyramidal cells was attenuated in preconditioned gerbils. This was measured by neuronal density one week later. DPCPX injected intraperitoneally 3hrs following the preconditioning 2min ischaemia enhanced damage to CA1 neurons. Pugliese et al. (2003) also showed similar effects in hippocampal slices with a protocol of 2min OGD with a reperfusion period of 13min. This was repeated four times before 7min OGD was applied 30min later. The preconditioning observed ($p<0.05$) was abolished by perfusion of 100nM DPCPX applied before and during the preconditioning insults ($p<0.01$). It was also noted that an adenosine A3 antagonist (MRS 1523 [100nM]) improved recovery when applied as before. This could be related to the desensitization of A1 receptors via A3 receptors observed by Dunwiddie et al. (1997).

It has been noted that adenosine A1 receptor agonists and antagonists do not always modulate preconditioning (Sorimachi et al., 2004). Using bilateral occlusion of the common carotid arteries in Mongolian gerbils, it was shown that DPCPX did not attenuate preconditioning or alter the ischaemic depolarisation when administered intraperitoneally (1mg/kg) 15min prior to occlusion. There was also no alteration in neuron damage assessed histologically 5 days later. Similar results were observed for CPA except that severe damage was observed in CA1 neurons after 5 days.

### 1.4.5 - Mechanism of adenosine preconditioning

The mechanism by which adenosine induces neuronal tolerance is mediated through a signal transduction pathway consisting of A1 receptor activation leading to PKC activation followed by ATP-sensitive potassium channel activation (Heurteaux et al., 1995; Reshef et al., 2000). In addition to these observations in vivo, the role of ATP-sensitive potassium channels and PKC in anoxic preconditioning was observed in rat hippocampal slices (Pérez-Pinzón & Born, 1999).

It is possible that adenosine may modulate the conductance of NMDA receptors in preconditioning. In a study using tight seal whole-cell recording from rat neostriatal neurons, A2a receptor agonists inhibited the current produced from NMDA receptor stimulation when perfused 5min prior to and during a second application of 10μM NMDA to the neuron (Nörenberg et al., 1997). There was no impact on NMDA conductance using A1 receptor agonists, however, indicating that this may not be a mechanism associated with preconditioning. The abundance of A1 receptors may have been a factor; A2a receptors are most abundant in the striatum whereas A1 receptors predominate in the hippocampus. Adenosine was shown to mediate NMDA preconditioning against glutamate via A1 receptors in a cerebellar granule cell culture (Boeck et al., 2005). The preconditioning effects of adenosine are also present in the heart (Liu et al., 1991) where glutamate presence may be much lower, however, indicating that the mechanism of induced tolerance may be common to all cells.

It has been demonstrated that adenosine delays the onset of anoxic depolarization (Lee & Lowenkopf, 1993). Using brain slices, inhibition of endogenous adenosine using A1 antagonists quickened the onset of depolarization when 99% $N_2$ was bubbled in the perfusion medium. The antagonists were applied prior to and during the anoxic insult. Jarvis et al. (2001) have observed that anoxic depolarization induced by OGD in neocortical slices results in cellular swelling as measured by the intrinsic optical signal (changes in light transmittance associated with ischaemia). This occurred in the presence

of a non-specific glutamate receptor antagonist, kynurenate, thus excluding a role for glutamate in the effect. It has been shown that blockade of the anoxic depolarization prevents cellular swelling, damage and loss of evoked responses in the CA1 region of slices (Anderson et al., 2005). It is possible that by delaying onset of anoxic depolarization, adenosine may reduce the exposure of neuronal tissue to damaging stimuli. Pérez-Pinzón et al. (1996) indicated that whilst adenosine preconditioning protects against anoxic insults, they observed no significant delay in onset of anoxic depolarization. This suggests that preconditioning protection induced by adenosine does not work by delaying the onset of damage in tissue.

## 1.5 - ATP-sensitive potassium channels

ATP-sensitive potassium channels are present in all cells. They are normally closed when ATP is present and open during ATP depletion, an action which decreases the metabolic demand to the cell by increasing potassium conductance. These channels are located on the mitochondrial inner membrane and at the plasmalemmal membrane. Stimulation of the ATP-sensitive potassium channels induces preconditioning protection prompting the examination of mechanisms which may mediate these phenomena.

### 1.5.1 - Molecular biology

The ATP-sensitive potassium channels are composed of two different types of subunit; these are the Kir6 subunits and the sulfonylurea receptor (SUR) subunits. The Kir6 subunits are of two types; Kir6.1 and Kir6.2. The SUR subunits are also of two types; SUR1 and SUR2. The ATP-sensitive potassium channels in neuronal tissue are composed of four Kir6.2 subunits forming the channel pore (Allen & Brown, 2004; Avshalumov & Rice, 2003). Each of the Kir6.2 subunits is associated with a corresponding SUR subunit (which in neurons is probably SUR1 (Chen et al., 2003)) resulting in four surrounding SUR1 subunits around the Kir6.2 pore. The Kir6.2 subunits contain 2 transmembrane helices, M1 and M2 which are bridged by an extracellular loop that is responsible for ion selectivity of the channel. The SUR subunits contain two transmembrane 6 helix domains called TMD1 and TMD2. There is also an N-terminal TMD0 domain in the SUR subunits consisting of 5 transmembrane helices; it has a role in trafficking the Kir6.2 subunits to the cell membrane and may also control the gating of these subunits. SUR subunits contain two nucleotide-binding folds, one of which is located between TMD1 and TMD2 and the other which is found after TMD2.

### 1.5.2 - Pharmacology

ATP interacts with the binding sites on the cytoplasmic domains of all four Kir6.2 subunits to maintain the closed state of the channel. There are also binding sites for phosphatidylinositol 4-5-bisphosphate ($PIP_2$) which acts to stabilize the channel in an open state. Either ATP or $PIP_2$ are bound to a subunit at any one time therefore for the channel to be activated by $PIP_2$, all the ATP-binding sites must be unoccupied as the blockade results from a hinged motion of the M2 portion of any Kir6.2 subunit which is sufficient to maintain the closed state of the channel.

The SUR subunits provide the binding sites for potassium channel openers (eg SCH 58261) and sulfonylurea drugs (eg tolbutamide). They also contain binding sites for Mg-bound ATP or Mg-bound ADP ie the nucleotide binding folds which activate the channel when Mg-bound nucleotides are bound, particularly Mg-ADP. When this is the case, it seems that this overrides the inhibition induced by ATP binding to the Kir6.2 subunits (for review, see Nichols, 2006).

### 1.5.3 - Subcellular localization and function

The ATP-sensitive potassium channels are located upon the mitochondrial inner membrane and the plasmalemmal membrane. The function of the plasmalemmal channels under normal physiological conditions is unclear as there is no apparent effect upon resting membrane potential. Under ischaemic conditions or conditions of ATP depletion, the plasmalemmal channels may act to reduce the excitability of the neuron by allowing potassium efflux to hyperpolarize the membrane. In nerve terminals, this may lead to the inhibition of transmitter release such as glutamate (Soundarapandian et al., 2007a) and GABA (Matsumoto et al., 2002) within the hippocampus. The plasmalemmal channels are also present on post-synaptic sites (Matsumoto et al., 2002, Hosseinzadeh & Stone, 1998) where they hyperpolarize the neuronal membrane although in CA1 neurons, the distribution of functional ATP-sensitive potassium channels may be limited to the somata given that tolbutamide prevents the inhibitory effects of adenosine upon population spikes but not upon epsps (Hosseinzadeh & Stone, 1998). It has also been suggested that the selective vulnerability of the CA1 pyramidal neurons under conditions of cell stress may be due to lower levels of these channels compared with interneurons (Zawar & Neumcke, 2000).

The mitochondrial ATP-sensitive potassium channels are present on the inner membrane and allow potassium influx into the mitochondrial matrix under conditions when ATP

production is decreased. This is thought to protect the mitochondria by preserving the mitochondrial membrane potential and inner mitochondrial volume (Busija et al., 2004).

### 1.5.4 - Role of ATP-sensitive potassium channels in preconditioning

The ATP-sensitive potassium channels have been well documented as a crucial mediator of the preconditioning phenomenon in most tissues. The mitochondrial subtype has been studied in particular detail in neuronal tissues and the roles of both subtypes have been examined extensively in cardiovascular preconditioning studies. Whilst the protective effect of ATP-sensitive potassium channel opening has been studied in neurons mainly with the use of diazoxide, a specific opener of mitochondrial ATP-sensitive potassium channels, the role of the plasmalemmal ATP-sensitive potassium channels has remained unclear. There are conflicting reports regarding the role of the cell surface channels, some studies showing the requirement for such channels (Blondeau et al., 2000, Heurteaux et al., 1995; Pérez-Pinzón & Born, 1999). One study which used knockout mice lacking the plasmalemmal channels, however, showed that a preconditioning stimulus (20min of a bilateral carotid artery occlusion) protected against 40min ischaemia in the hippocampus (Muñoz et al., 2003) indicating that only mitochondrial channels may be required for in vivo preconditioning.

There have been few slice studies in neurons with regard to the effects of ATP-sensitive potassium channels and the acute preconditioning phenomenon. The first of these used pinacidil, a non-selective ATP-sensitive potassium channel opener as the preconditioning stimulus (Pérez-Pinzón & Born, 1999). Hippocampal slices were exposed to three 1min periods of anoxia induced by bubbling the perfusion medium with 95% $N_2$/5% $CO_2$. The lethal insult was applied 30min later consisting of a longer anoxic period. This preconditioning paradigm showed an increase in the recovery of evoked potentials following the lethal ischaemic insult compared with slices exposed to lethal ischaemia alone. This protection was abolished by the presence of tolbutamide in the perfusion medium. It was also shown that 10μM pinacidil, applied for 20min prior to the anoxic episode, induced a higher recovery of the evoked potentials also. Other studies have shown the effects of diazoxide upon slices. Nakagawa et al. (2002) showed that diazoxide delayed the onset of depolarization induced by hypoxia in gerbil hippocampal slices, an effect which was blocked by glibenclamide. This may have similar protective effects like the delay in anoxic depolarization induced by adenosine (Lee & Lowenkopf, 1993). Garcia de Arriba et al., (1999) studied the morphological changes induced by 30min 95% $N_2$/5% $CO_2$

in rat neocortical brain slices and found that prior exposure to 300μM diazoxide attenuated these effects. They were also partly reversed by tolbutamide.

The protective effects of diazoxide have been studied in vivo also. Domoki et al. (1999) reported that neuronal dysfunction resulting from 10min of global ischaemia induced in piglet cortex pial arterioles was attenuated by the prior application of 5-10μM diazoxide. The protection was blocked by the presence of 5-hydroxydecanoate (5-HD). It has also been shown that i.p. injection of diazoxide into 7 day old rat pups decreased the infarct volume in the brain induced by ligation of the common carotid artery for 2.5 hours (Rajapakse et al., 2002). This effect was also blocked by 5-HD confirming the specificity of diazoxide. The authors also confirmed that diazoxide induced its effects upon the mitochondria by using fluoresence studies to show the depolarization of the mitochondria, an effect of diazoxide which has also been reported in rat hippocampal homogenates (Dębska et al., 2001). Reductions in infarct volumes have also been reported in mice where i.v. injection of diazoxide attenuated the effects of a subsequent middle cerebral artery occlusion. The effects of diazoxide in vivo have been attributed to an increase in cerebral blood flow which appears to be a protective mechanism against ischaemia. However, the effects of acute preconditioning in vitro may also be due to the maintenance of the mitochondrial matrix volume and the limitation of calcium influx which preserves respiration within the tissue and subsequently prevents delayed cell death (for review, see Busija et al., 2004).

In addition to ischaemia, the protective effects of ATP-sensitive potassium channel opening have been shown to be effective against chemical insults. This has been studied in delayed preconditioning paradigms moreso than acute. These studies have favoured the use of exogenous glutamate (Nagy et al., 2004; Kis et al., 2004) or rotenone (Tai et al., 2003) as a chemical insult. However, caution must be taken with the interpretation of these effects as diazoxide is reported to affect the conductance of glutamate receptors (Crépel et al., 1993; Lu & Mattson, 2001). It has been particularly noted that diazoxide blocks the rapid desensitization of the AMPA receptors in hippocampal cell cultures (Yamada & Rothman, 1992).

## 1.6 - Heat shock protein 72

The delayed time window of preconditioning protection (24-48hrs) is associated with molecular changes, the most prominent of these being heat shock protein 72 (HSP72) (Kirino et al., 1991; Liu et al., 1993) and the anti-apoptotic mitochondrial protein, Bcl-2

(Wu et al., 2004). HSP72 belongs to the family of heat shock proteins that function as molecular chaperones, maintaining protein structure, synthesis and trafficking. The constitutive form of heat shock protein, HSP70, acts in this housekeeping capacity whilst the stress-induced form, HSP72, is upregulated to prevent protein denaturation and maintain mitochondrial stability in response to cellular stresses such as ischaemia. Although HSP72 is found in higher quantities during cell stress, its presence is indicative of a repair response in tissues in which the damage may be reversed. It is found in high quantities in the ischaemic penumbra of focal ischaemia (Heiss, 2000), the zone of lesser damage surrounding the severely impaired tissues at the core. HSP72 is induced in response to the denaturation of proteins during ischaemia. Protein denaturation activates heat shock factors which phosphorylate and trimerise. This complex then binds to heat shock elements upon the heat shock genes which in turn activate HSP72 transcription. HSP72 then acts to prevent some forms of cell death (for review, see Sharp et al., 1999).

HSP72 has been shown to be upregulated in preconditioning protocols and prevent the onset of apoptosis and necrosis (for review, see Obrenovitch 2008). In vivo, it has been shown that HSP72 expression is elevated in CA1 neurons of male Mongolian gerbils following 2min of induced global ischaemia which in turn increases the neuronal survival following a subsequent 5min global ischaemia induced at one, two or four days post-preconditioning (Kirino et al., 1991). Similar in vivo studies have shown the role of HSP72 in mediating ischaemic tolerance (Liu et al., 1992; Liu et al., 1993; Nishi, 1993; Nishi et al., 1993; Chen et al., 1996).

HSP72 normally stabilises proteins by binding to the hydrophobic regions which become exposed when the protein denatures. During preconditioning however, the stress-induced protein appears to prevent cell death from the intrinsic apoptotic pathway and caspase-independent apoptosis in tissues. The extrinsic apoptotic pathway may also be affected by HSP72 although to a much lesser extent (for review, see Beere, 2005). The HSP72 protection of the first two forms of apoptosis mentioned may occur through several mechanisms. During ischaemia, the pro-apoptotic protein, Bax, is translocated to the mitochondrial membrane where it acts to increase the permeability of the organelle which in turn releases reactive oxygen species, and the apoptotic factors, cytochrome c and Apoptosis Inducing Factor (AIF). HSP72 acts to suppress the activation of Bax by inhibiting c-Jun N-terminal kinases (JNK). It may also enhance the expression of the anti-apoptotic protein, Bcl-2, which opposes the actions of Bax at the mitochondrial membrane. In addition, HSP72 may prevent the effects of cytochrome c and AIF. Cytochrome c normally combines with pro-caspase-9 and apoptosis protein activating factor-1 (apaf-1) to

form the apoptosome, a complex which activates caspase-9 and subsequently the caspase cascade leading to cell degradation. HSP72 binds to apaf-1 and prevents the formation of the apoptosome thus preventing caspase-dependent apoptosis. AIF induces a caspase-independent apoptosis by translocation to the nucleus following its release from the mitochondria. It then associates with endonucleases which results in DNA degradation and cell death. HSP72 acts to neutralise AIF by sequestering it in the cytosol. HSP72 appears to be a highly effective neuroprotective agent on account of its numerous actions in preventing apoptotic cell death (for review, see Obrenovitch, 2008).

The induction of heat shock protein expression has been shown to occur in response to adenosine preconditioning or tolerance induced by an ATP-sensitive potassium channel openers in the hippocampus (Blondeau et al., 2000). It was observed that preconditioning against intraperitoneal injection of kainic acid by a smaller dose of kainic acid administered 3 days earlier prevented apoptosis and necrosis in CA1 and CA3 neurons. This protection was mimicked when the adenosine agonist, R-phenyl-isopropyladenosine (R-PIA), or the ATP-sensitive potassium channel opener, cromakalim, was used as the preconditioning stimulus. This proved effective against global ischaemia induced by four-vessel occlusion as well as kainic acid. It was shown that heat shock protein expression increased over the 3 days in response to kainic acid, R-PIA or cromakalim and was indicative of protection in the tissue. Whilst this study, like many others, has examined the molecular changes in delayed preconditioning, none have assessed the role of HSP72 in acute preconditioning. Given that common factors such as A1 receptor stimulation and ATP-sensitive potassium channel opening can induce acute and delayed preconditioning, it is interesting to see if HSP72 is also common to both. Blondeau et al. (2000) reported that after the second kainic acid administration, changes in heat shock protein expression were observed within 3 hours. This might form part of the mechanism observed in acute preconditioning therefore it may be useful to investigate its role in rapidly induced neuroprotection.

## 1.7 - The hippocampal slice preparation

The hippocampus is a structure within the mesolimbic system which is involved in the processes of memory formation and learning. It has a lamellar structure and receives its major input from the entorhinal cortex. It is divided into approximately 4 regions, CA1, CA2, CA3 and the dentate gyrus (Figure 1). The neurons in each region project onto the next sequentially which forms the trisynaptic pathway. Hence the afferent inputs from the entorhinal cortex form the perforant path fibres which terminate onto the dentate gyrus

granule cells. These granule cells in turn project their mossy fibres onto the CA3 pyramidal cells. The axons of the CA3 neurons, called the Schaffer-collaterals, project onto the CA1 pyramidal neurons. The axons of the CA1 cells then project through the alveus towards the subiculum (Anderson et al., 1971a).

The CA3-CA1 projection has been widely studied. This is because the terminal projections of the Schaffer-collateral axons all synapse onto the CA1 dendrites allowing for easier study of events occurring at one synapse. Also, the lamellar structure of the hippocampus allows it to be used in vitro in slices whilst keeping the major projections intact. The hippocampal slice preparation allows electrophysiological recordings to be made with relative ease, particularly extracellular recordings from the CA1 pyramidal cells. Hence it is possible to record field epsps from the CA1 dendrites and population spikes from the somata. It is also possible to evoke the population spikes which represent the summation of action potentials in either an orthodromic direction by stimulating the CA3 Schaffer-collaterals or in an antidromic direction by stimulating the CA1 axons in the alveus (Anderson et al., 1971b).

## 1.7.1 - Formation of evoked potentials

### 1.7.1.1 - Field epsps

Field epsps are generated by the action of glutamate upon the post-synaptic ionotropic receptors. AMPA and kainate receptors are faster to activate and open and thus allow the initial influx of sodium ions into the post-synaptic neurons. The slower-activating NMDA receptors do not allow any ion influx until the membrane potential has been sufficiently depolarized by ion flux through the AMPA and kainate receptors to relieve the voltage-dependent magnesium block. The AMPA receptors are quick to depolarize so that the NMDA receptors are active for much longer. The epsp parameters that are usually quantified for analysis are the negative going slope of the waveform and its amplitude. The gradient of the slope can serve as an indicator of the strength of the synapse given that it represents the rate at which AMPA receptors are opening in response to glutamate to produce voltage changes. The amplitude represents the overall change in voltage change in the post-synaptic membrane which is indicative of the activity of AMPA and NMDA receptors. The recording of epsps can therefore be a useful indicator of events at the synapse.

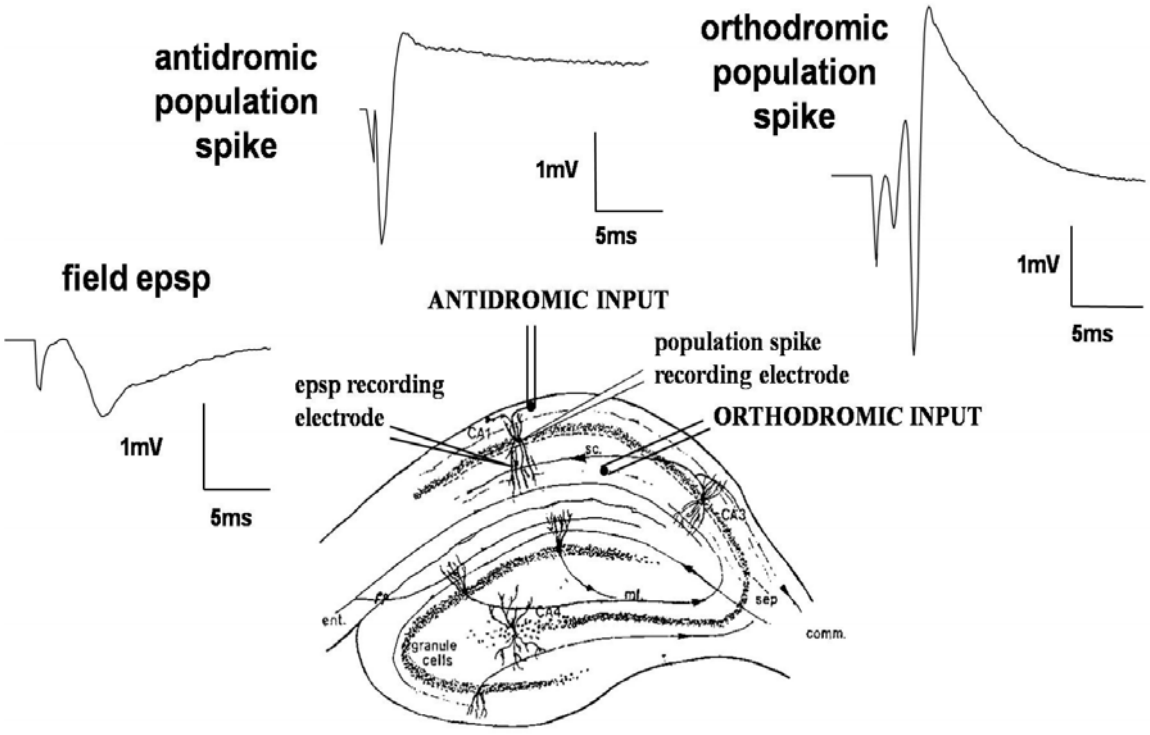


**Figure 1 Diagram of the rat hippocampal slice and electrode positions.** Shown above are the placements for stimulating and recording electrodes upon the preparation to obtain different evoked responses. Examples of the extracellular responses generated by the setup are shown (ent. = entorhinal cortex, sc.= Schaffer-collateral, mf.= mossy fibre, sep.= septum, comm = commissural).

### 1.7.1.2 - Population spikes

The population spikes which can be recorded from the CA1 cell bodies represent the sum of action potentials which propagate into the soma after the threshold for action potential firing has been reached at the axon hillocks. They can be generated by orthodromic current stimulation of the pre-synaptic Schaffer-collateral fibres and are thus generated by synaptic transmission and subsequent epsp formation. The field epsps in turn alter the membrane potential which, if sufficiently depolarized, allows the threshold for action potential firing to be reached at the axon hillock. Alternatively, population spikes can be generated by antidromic current stimulation of the CA1 axons located within the alveus (Anderson et al., 1971b). This allows for the study of population spike generation which is independent of synaptic transmission.

## 1.7.2 - Synaptic plasticity

Synaptic plasticity refers to the changes in synaptic strength between neurons which may occur as part of the formation of memory in vivo or in response to a chemical or electrical stimulus which alters the properties of synaptic transmission in vitro. As changes in synaptic plasticity are thought to underly the processes of memory and learning, the properties of these phenomena have been extensively examined within the hippocampus. The most widely studied forms of synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD).

### 1.7.2.1 - LTP

LTP describes the phenomenon where electrophysiological responses generated from neurons shows a marked increase in size following a brief tetanic stimulation. It was first discovered by Bliss & Lømo (1973) when the authors stimulated the perforant pathways in anesthetized rabbits and recorded the responses from the dentate gyrus granule cells. They noted that epsps and population spikes were both elevated following a 100Hz stimulus. These results were extended by Schwartzkroin & Wester (1975) to show that a similar effect could be obtained from the CA1 neurons of guinea pig hippocampal slices by stimulation of the Shaffer-collaterals. The potentiation of responses appears to be due to an increase in synaptic strength resulting from increased NMDA receptor stimulation (Collingridge et al., 1983) and a potentiation of the epsp-spike relationship where population spikes are disproportionately elevated compared to a more modest potentiation in epsps (Andersen et al., 1980).

#### 1.7.2.1.1 - Synaptic potentiation

The induction of LTP requires NMDA receptor stimulation. At low frequency stimulation, the NMDA receptors retain their voltage-gated magnesium block so that the post-synaptic response remains the same for a given electrical pulse. When the synapse is tetanised by high frequency stimulation, the post-synaptic neurons become more depolarized to the extent that the magnesium blockade is relieved for longer and the NMDA receptors remain in the active open state. This results in a greater calcium influx which is required to induce LTP (Lynch et al., 1983). The greater depolarization of the post-synaptic neurons is observed as a potentiation in the slope and amplitude of the field epsp (Bliss & Collingridge, 1993).

#### 1.7.2.1.2 - E-S potentiation

E-S potentiation was observed by Bliss & Lømo (1973) when the increase in the population spike amplitude in response to tetanisation could not be accounted for by the increase in field epsp slope. This was demonstrated further in hippocampal slices by Andersen et al. (1980) by showing the changes in the input-output curves for epsp slope and population spike amplitude. In dentate granule cells, E-S potentiation has been attributed partially to a decrease in GABAergic feedforward inhibition upon dentate granule cells (Tomasulo et al., 1991).

### 1.7.2.2 - LTD

LTD describes the effect whereby an evoked potential decreases in size in response to a stimulus. The effect was first observed in the hippocampal slice when tetanising one pathway to induce LTP also resulted in depressing the response from a non-tetanised pathway. This was termed heterosynaptic depression (Lynch et al., 1977). It was subsequently shown that homosynaptic depression could be induced by evoking epsps from the CA1 neurons with a stimulation protocol of 1-3Hz frequency for 900 pulses (Dudek & Bear, 1992). It has been found that NMDA receptor stimulation is required to induce LTD along with a post-synaptic change in calcium (Mulkey & Malenka, 1992). LTD may be reversed by applying high frequency stimulation to the pathway (Dudek & Bear, 1992; Dudek & Bear, 1993).

### 1.7.3 - Using hippocampal slices to study preconditioning

The hippocampal slice system may provide useful data regarding the effects of preconditioning. The CA1 neurons are highly sensitive to ischaemia and are therefore useful for studying ischaemia-like changes and the effectiveness of neuroprotective strategies. As the slice system retains the lamellar morphology as it exists in vivo to some degree, it is possible to evoke electrical responses to assess the function of the tissue. In addition to the assessment of electrophysiological changes, it is also possible to examine potential molecular changes which may occur as a result of electrophysiological and chemical experimental protocol. As the preconditioning phenomenon has been clearly demonstrated in hippocampal slices using electrophysiology, it is a suitable choice for pursuing further information on the mechanisms and interactions which may occur during rapid preconditioning.

## 1.8 - Aims of the project

The primary aim of the project was to investigate preconditioning interactions within the hippocampal slice preparation. In particular these included:

- Investigating the effects of exogenous glutamate and other depolarizing agents in the slice preparation and their validity for use as toxic agents
- Examining any potential preconditioning neuroprotection induced by adenosine against various depolarizing agents (glutamate, NMDA, ouabain) and elucidate the underlying mechanisms where relevant
- Examining the excitotoxic preconditioning phenomenon and possible underlying mechanisms
- Looking at the effectiveness of other known preconditioning agents (ATP-sensitive K+ channel openers) against depolarizing agents and identify the underlying mechanisms of protection
- Identifying any molecular changes that may occur during acute models of chemical ischemia or acute preconditioning

# 2 - METHODS

## 2.1 - Electrophysiology

### 2.1.1 - Preparation of rat hippocampal slices

Adult male Wistar rats (100-200g) between 4 and 6 weeks old were used for hippocampal slice preparations. Rats were injected i.p. (1ml/100g) with a solution of 25% urethane in distilled water. Once sufficiently anaesthetised, indicated by the loss of limb and corneal reflexes, the rat was then killed by cervical dislocation. The brain was removed quickly and placed in ice-cold oxygenated artificial cerebrospinal fluid (aCSF) with the following composition (mM): $KH_2PO_4$ (2.2); KCl (2.0); $NaHCO_3$ (25); NaCl (115); $CaCl_2$ (2.5); $MgSO_4$ (1.2); glucose 10; distilled water. Both hippocampi were dissected from the hemispheres, placed on a McIlwain tissue chopper and sliced transversely to a thickness of 450μM. The slices were then immersed in cold aCSF and separated out in a petri dish. The aCSF was then removed until the slices were still sufficiently immersed whilst allowing sufficient oxygen diffusion across the aCSF to reach the tissue. The slices were then placed in a closed incubation chamber continuously gassed with 95% $O_2$/5% $CO_2$.

### 2.1.2 - Extracellular recording

Slices were allowed to incubate for at least 1 hour at room temperature before recording. Slices were placed in a recording chamber that was continuously perfused with oxygenated (95% $O_2$/5% $CO_2$) aCSF at a rate of 3-4ml/min. The slices were held in place within the perfused chamber by placing them on a wire mesh within the chamber and placing small silver bars on top of the slice to hold it in place against the mesh. The temperature was maintained between 28 and $30^0$C. A concentric, bipolar stimulating electrode was placed using micromanipulators under manual control on the stratum radiatum in the CA2 area for orthodromically evoked responses and on the CA1 axons in the stratum oriens for antidromically evoked population spikes. Using a NeuroLog period generator (NL303) and delay-width (NL403), the slice was stimulated via a NeuroLog stimulus isolator (NL800) through the bipolar electrode with 330μs square wave pulses at a frequency of 0.1Hz.

Recording electrodes were made from borosilicate glass capillaries in a Kopf vertical pipette puller. The capillaries were pulled to a fine tip of 1μm. The tip was broken under microscopic control to a diameter of approximately 2μm and the micropipette was then filled with 0.9% NaCl solution with a fine 36 gauge needle. The micropipette was then set

up near the recording chamber for manual manipulation. A Ag/AgCl wire electrode was inserted within the open end of the micropipette. This electrode was connected to the NeuroLog pre-amplifier (NL104) via a headstage. To record a population spike, the recording electrode was placed in the CA1 pyramidal cell layer; for a field excitatory post-synaptic potential (epsp), the electrode was placed in the stratum radiatum to record the apical dendrites of the CA1 pyramidal neurons just above the stratum lacunosum. The evoked response was amplified using the NeuroLog pre-amplifier (NL104) and amplifier (NL106) and filtered before being captured via the Cambridge Electronic Design microplus1401 interface onto the computer-based Signal software. The waveform was captured with a sampling frequency of 10000Hz. The population spike was quantified as the difference between the negative and positive peak potentials. The fepsp negative slope was quantified as the gradient of the line of best fit of all the sampled points taken from between two points determined approximately from after the first quarter and before the last quarter of where the slope formed. The fepsp amplitude was quantified as the difference between the negative peak and the returning baseline. The stimulus amplitude was adjusted by the NeuroLog pulse buffer (NL510) to evoke the maximum response from the slice. In all experiments, a 10min stable baseline of the measured parameter (population spike amplitude; epsp slope; epsp amplitude) was evoked prior to perfusion of any drugs.

As some compounds such as glutamate, NMDA and ouabain, once applied, were shown in initial experiments to induce changes in the maximal response from the slice which lasted beyond one hour, it was determined that at least two slices should be taken from one rat each day to compare the drug treatments on the responses (e.g. slice 1: preconditioning + glutamate; slice 2: glutamate alone). Responses were obtained from the slices at the same time in two different rigs to take account of any time-dependent degradation in the tissue; this was to ensure that results indicating any differences between treatments were not due to a difference in sensitivity or vulnerability of the slice.

Recovery was taken as the mean of the recorded values between 50 and 60 minutes after application of glutamate, NMDA or ouabain for each experiment except where stated otherwise. Percentage values in the text are derived from a 10min stable baseline of the response which is taken as 100%. Time course graphs show the average of the normalized data of several experiments except where a sample time course graph of one experiment is shown. The data from experiments were normalised using the GraphPad Prism software. Tests for linear regression were performed in GraphPad Prism. Where a linear regression test resulted in $p<0.05$, a residual plot showing the deviation of the y values from their

predicted value is shown also. With the exception of ANCOVA, GraphPad Instat software was used for all other statistical analysis. ANCOVA was performed to assess the difference between drug treatments when the size of the response parameter was shown to be a continuous variable affecting the outcome and when all assumptions were met. ANCOVA was performed using the NCSS 97 statistical software. Students t test was used for comparison between 2 groups except where otherwise stated. ANOVA was used for comparisons between multiple groups. Post-tests used are indicated where relevant. All data are presented as mean ± standard error.

Drugs and chemicals were obtained from the Sigma Chemical Company and Tocris Ltd.

## 2.2 - Molecular methods

### 2.2.1 - Protein extraction and Bradford assay

Following electrophysiological experiments, pre-selected slices were placed in an eppendorf tube which was placed in dry ice and kept at -80 degrees Celsius until further use. The protein was extracted from the slices in preparation for Western blotting. 150µl RIPA buffer (composition: 50mM Tris; 150mM NaCl; 1% Igepoal; 0.5% Tritonx100; 0.1% sodium dodecyl sulphate [SDS]; complete Mini EDTA-free protease inhibitor cocktail tablet {composition: aprotinin; bestatin; calpain inhibitor I; calpain inhibitor II; chymostatin; E-64; leupeptin; $\alpha_2$-macroglobulin; petabloc SC; pepstatin; PMSF; TLCK-HCL; trypsin inhibitor (chicken, egg white); trypsin inhibitor (soybean)}) was added to the tissue in the eppendorf tubes and the mixture was ground down with a pestle. The tubes were then placed in a centrifuge and spun at 13,000 rpm at 4 degrees Celsius for 5min. Afterwards, the tubes were carefully removed from the centrifuge so as to not disturb the pellet and the supernatant containing the protein was pipetted out into a new eppendorf tube. The tissue and protein solutions were kept on ice as much as possible during this procedure to minimise degradation of the samples.

The protein concentration was measured for each sample using a Bradford assay. To do this, standard concentrations of Bovine Serum Albumin were prepared in eppendorf tubes at 0, 0.25, 0.5, 1.0, 1.5 and 2.0mg/ml. For the samples, 4µl of protein sample solution was added to 396µl of distilled water. Biorad reageant was diluted with distilled water in a 1:1 ratio and 200µl was added to each tube (standard and protein sample solutions) and mixed thoroughly. 200µl of each solution was then pipetted into a 96 well plate which was then read using fluorometry at 595nm on a plate reader. Once the protein concentration in each

sample was known, all the samples were normalised to the lowest concentrated sample by adding the appropriate amount of RIPA buffer unless the lowest was below 20mg/ml concentration (the minimum concentration recommended by NuPage for use in the mini-gel).

### 2.2.2 - Western blotting

After normalisation, 65µl of each sample was prepared for Western blotting by addition to 25µl of 4 x sample buffer and 10µl of 10 x reducing agent in eppendorf tubes. The solution was mixed thoroughly and heated to 70 degrees Celsius for 10min immediately before loading onto a NuPage 4-12% Bis-tris gel. The gel cassette was cut out of the pack and rinsed with distilled water. The comb was removed from the loading wells and and the wells were then rinsed with NuPage MOPS running buffer mixture with distilled water (buffer 50ml; distilled water 950ml). On the third rinse, the buffer was left in the wells to prevent bubble formation. The gel cassette was set up in the gel tank for electrophoresis. 0.5ml of NuPage antioxidant was added to 200ml of the MOPS running buffer. This was then mixed and added to the central reservoir in the tank. The rest of the buffer was added to the rest of the tank to keep the gel cool while it was running. 10µl of Amersham Full-Range Rainbow Molecular Weight Marker (RPN800V) and 20µl of each heated protein sample were each loaded onto a well. Once loading was complete, gel electrophoresis was run for 45min at 200V. During this time the solutions and equipment for the protein transfer from gel to the Invitrogen PVDF membrane were prepared. 50ml of NuPage transfer buffer was added to 100ml of methanol, 850ml of distilled water and 1ml antioxidant. 200ml of this solution was held back for use in the central reservoir in the gel tank. The rest was used to soak equipment, filter papers, the PVDF membrane and sponges ensuring particularly that the sponges were fully saturated with the solution. Prior to soaking in the transfer buffer mixture, the hydrophobic PVDF membrane (Invitrogen) was soaked for 1min in methanol to enable it to soak in the transfer buffer mixture. At the end of the electrophoresis, the gel cassette was removed from the tank and rinsed in distilled water. The gel cassette was cracked open around the rim with a blade and the top was removed carefully so as not to break up the gel. The equipment for protein transfer was then set up. 3 soaked sponges were placed in a cassette; on top of these was placed a piece of soaked filter paper and then the soaked PVDF membrane. The gel was then released from the rest of the cassette onto the PVDF membrane. Another soaked piece of filter paper was placed on top of the gel. A small roller was used to flatten the stack of sponges, filter paper, gel and membrane in order to remove any remaining bubbles. Another 3

soaked sponges were then placed on top of the stack followed by the cassette cover. This arrangement was carefully placed in the gel tank and secured. The clean transfer buffer was added to the reservoir inside the cassette whilst the rest that was used to soak the equipment, sponges, filter paper and membranes was poured into the rest of the gel tank. Current was applied across the gel and membrane to enable transfer of proteins; this was run for 1 hour at 30V.

At the end of the protein transfer, the PVDF membrane was removed and immersed in Ponceau staining solution for 5min to check if the transfer had worked. This was then rinsed with distilled water and the membrane was checked for protein bands. A digital photographic record was kept of the Ponceau staining to also show the variation in loading of the samples. The membrane was then rapidly immersed in 0.1M NaOH solution for 10-30sec until the bands disappeared. The membrane was then thoroughly rinsed with distilled water.

Prior to blocking, the membrane was cut to separate the protein of interest (HSP72) and the housekeeping protein (actin). This was possible as the two proteins were separated by a distance of more than 2 bands of the rainbow marker. The membranes were then each immersed in 10ml of blocking solution composed of 0.3g dried milk powder and 10ml Tween Tris-buffered saline (0.05% Tween; Tris base 20mM; NaCl 137mM adjusted to pH 7.6 with HCl) (TTBS) in a petri dish and agitated on a shaker for 2.5hours. Following blocking, the membranes were then each placed in 10ml TTBS in 1% milk with a 1:2000 dilution of the primary antibody (mouse anti-HSP72 monoclonal antibody, product no SPA-810 from StressGen) for the membrane containing HSP72 and a 1:200 dilution of the primary antibody (goat anti-actin polyclonal antibody, product no SC1615 from Santa-Cruz) for the membrane containing actin. This was agitated on a shaker overnight at 4 degrees Celsius. Following binding of the primary antibody, the membranes were given 3 separate 10min washes in TTBS on a shaker.

After rinsing with TTBS, the membranes were incubated for 1hour on a shaker with the secondary antibody for the anti-HSP72 antibody (peroxidise-conjugated AffiniPure goat anti-mouse IgG from Jackson ImmunoResearch Laboratories, code 115-035-168) at a dilution of 1:2000 in 1% milk TTBS and the secondary antibody for the anti-actin antibody (donkey anti-goat IgG horse radish peroxidase, product no SC2020 from Santa Cruz) at a dilution of 1:10000 in 5% milk TTBS. Following secondary antibody binding, the membranes were again given 3 separate 10min washes with TTBS. They were then rinsed with distilled water. The membranes were agitated on the shaker with the developing solution from the Amersham enhanced chemiluminescence (ECL) kit. Following agitation,

the excess solution was blotted off of the membranes with filter paper then placed in the developing cassette. The cassette was taken to a dark room where the developing film was then placed into the cassette for an initial exposure time of 30sec under the safelight. This film was developed and based on the outcome, further exposure times were gauged to produce a suitable film for analysis.

The Western blot was analysed for optical density and size of blot using the NIH Image software. The measurements for area and optical density were captured 3 times as well as 3 background measurements of optical density for each band. The corresponding actin band for each HSP72 band was taken as 100% to provide a measurement of HSP72 levels for each sample. The values for HSP72 were used for statistical analysis. One way ANOVA or unpaired t tests were performed to test for differences in HSP72 expression between different groups of slices.

# 3 –STUDIES REGARDING THE EFFECTS OF GLUTAMATE UPON EVOKED RESPONSES AND THEIR MODULATION BY PURINE ANTAGONISTS

## 3.1 – Introduction

As exogenous glutamate has been identified as a chemical mediator of preconditioning, it was of interest to develop a protocol in which glutamate preconditioning could be readily induced during the acute time window of protection. This would enable further study into the mechanisms and interactions with other known mediators of preconditioning e.g. adenosine. To study this in hippocampal slices, exogenous glutamate was included in the perfusion medium and the effects upon the evoked responses were examined. As the results below show, glutamate induced a diverse range of effects upon different responses and potential roles for adenosine receptors in mediating or modulating these effects were observed.

## 3.2 – Results

### 3.2.1 - The effects of glutamate on evoked responses

In order to examine the effect of glutamate as a preconditioning or excitotoxic stimulus, various exposure times and concentrations of glutamate were tested on the slices and the resulting changes in orthodromic population spikes were examined. It was determined that a 10min exposure to 5mM or 10mM glutamate resulted in a depolarization leading to a complete loss of response lasting between 10 and 30min following glutamate perfusion (Fig 3.1a). The response would then return until it reached a stable plateau which was lower than the initial spike size prior to glutamate treatment (Fig 3.1a & b). This effect was concentration dependent with 10mM glutamate producing a more pronounced depression in response size than 5mM. It was also noted that the extent of post-glutamate depression in spike size was to some degree dependent on the initial orthodromic spike amplitude with smaller spikes producing a greater % depression (Fig 3.2). For further experiments, the average size of population spike amplitudes between treatment groups were compared statistically to ensure that the outcomes were not influenced by this covariate.

A preconditioning paradigm was developed using repeated 5min exposures of 5mM glutamate on orthodromic population spikes (Fig 3.3). A 5min exposure allowed a complete recovery of the response before further glutamate treatment. There was no significant difference in the rate of depolarization, the maximal response to glutamate or $\Delta t$ as indicated in Figure 3.3. It was noted however, that by the third treatment the rate of recovery of the response following glutamate perfusion was significantly faster than after the first treatment and remained so for the fourth treatment (Fig 3.4). The rate of recovery was taken as the gradient of the line of best fit; the median value between the maximum amplitude depression and the following recovery and the five values above and below the median were used to calculate the equation of the line. It was therefore determined that 5mM glutamate was an adequate concentration for examining excitotoxic preconditioning interactions.

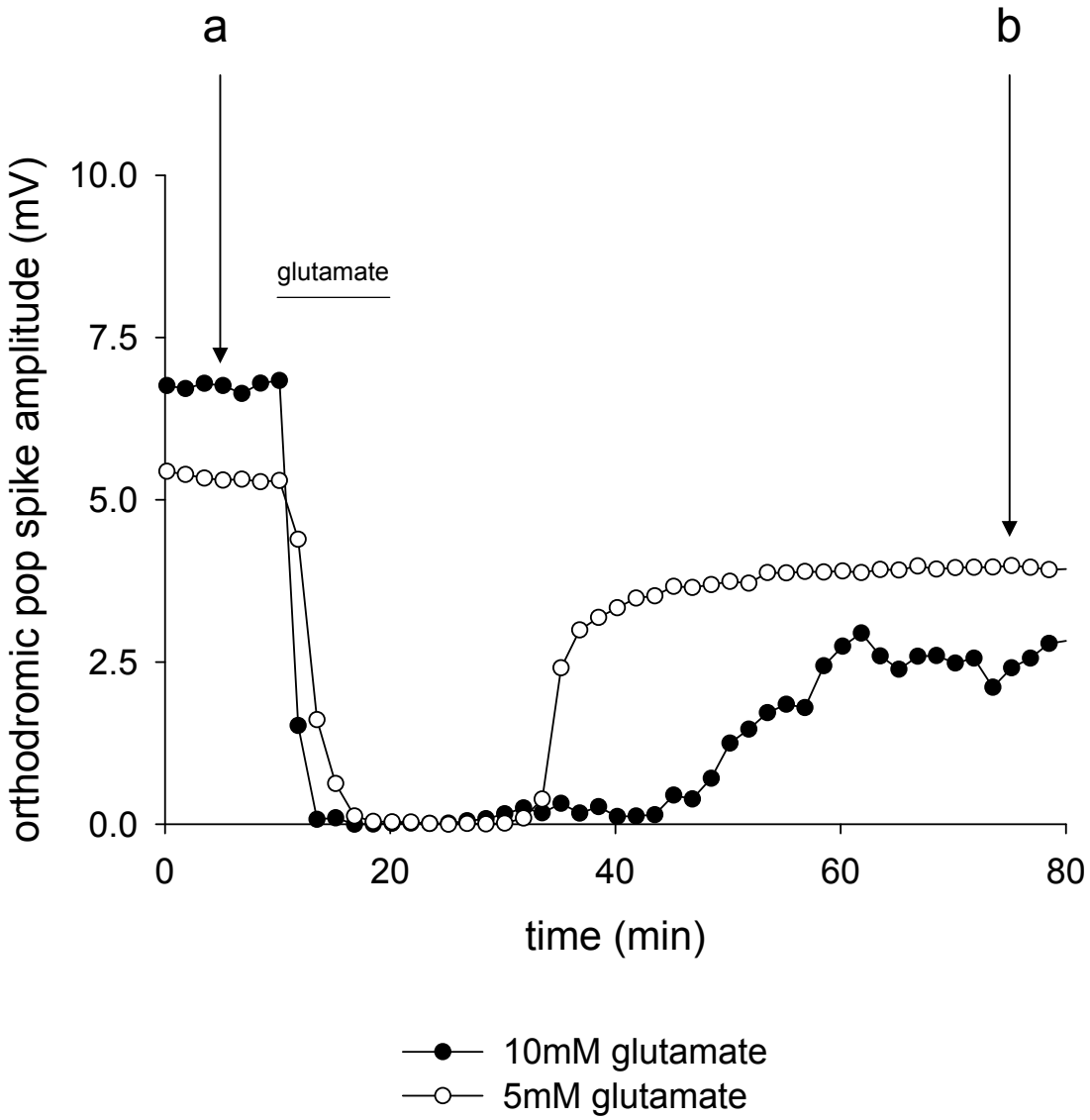


**Figure 3.1a Time course graph showing the effect of glutamate on the amplitude of orthodromic population spikes.** A sample response in the time course changes are shown for each concentration of glutamate. The arrows (a and b) indicate the time points at which the sample waveforms shown in Figure 3.1b are taken.

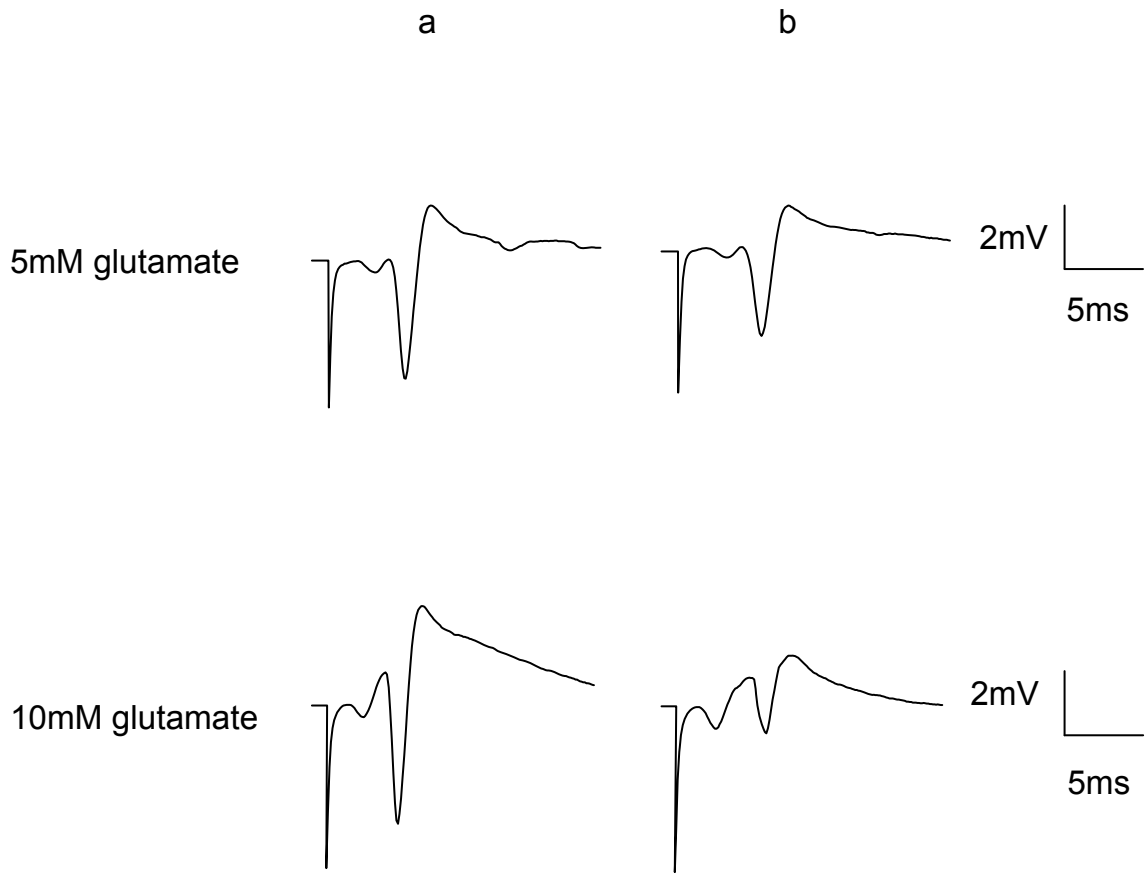


**Figure 3.1b Sample traces of orthodromic population spikes taken from the time points as indicated in figure 3.1a.**

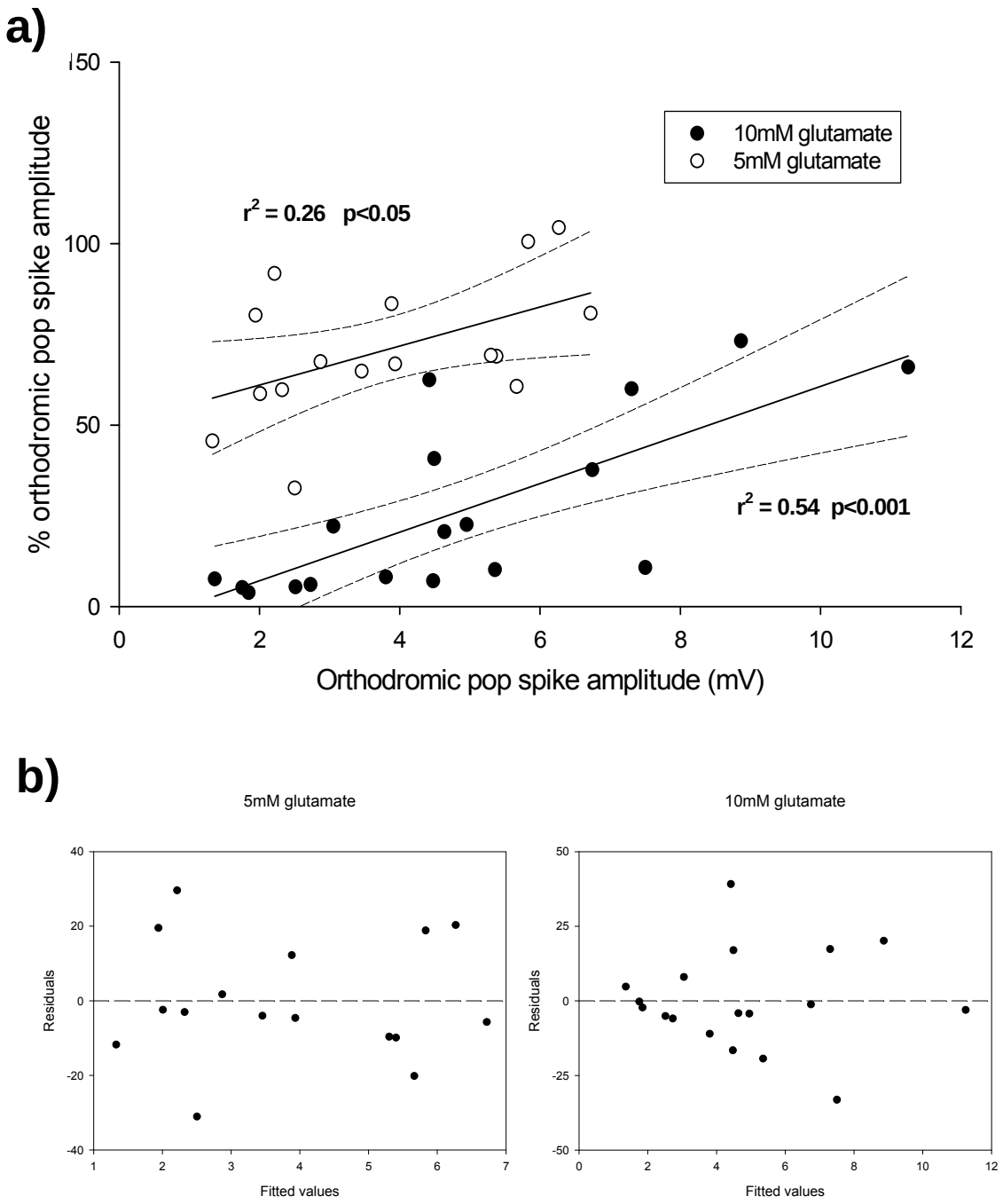


**Figure 3.2 Scatterplot showing the relationship between orthodromic spike size and the % recovery of the response following treatment with 5mM and 10mM glutamate.** Recovery of response is taken as the average % spike size of a stable 10min plateau recovery following glutamate treatment in a). There is a significant difference in recovery between 5mM (74.3 ± 4.2, n = 16) and 10mM (23.2 ± 3.9, n = 18) ($p<0.0001$, ANCOVA) glutamate concentrations. b) shows the residual plots obtained from the regression analy. Note there is a random scatter in both residual plots.

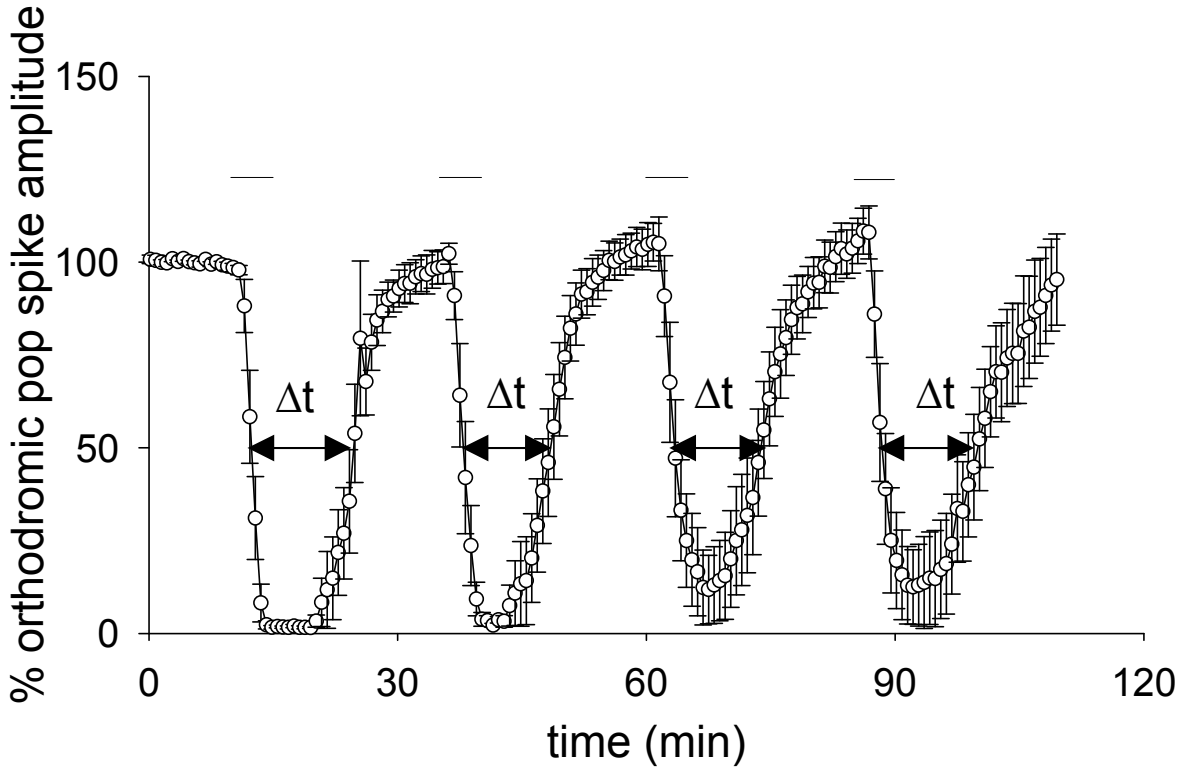


**Figure 3.3 Repeated glutamate application increases the rate of repolarization of the response.** The graph shows the time course of changes in orthodromic spike amplitude. The lines show when 5mM glutamate was perfused onto the slices. Δt is the time taken to return to 50% of the baseline after glutamate application.

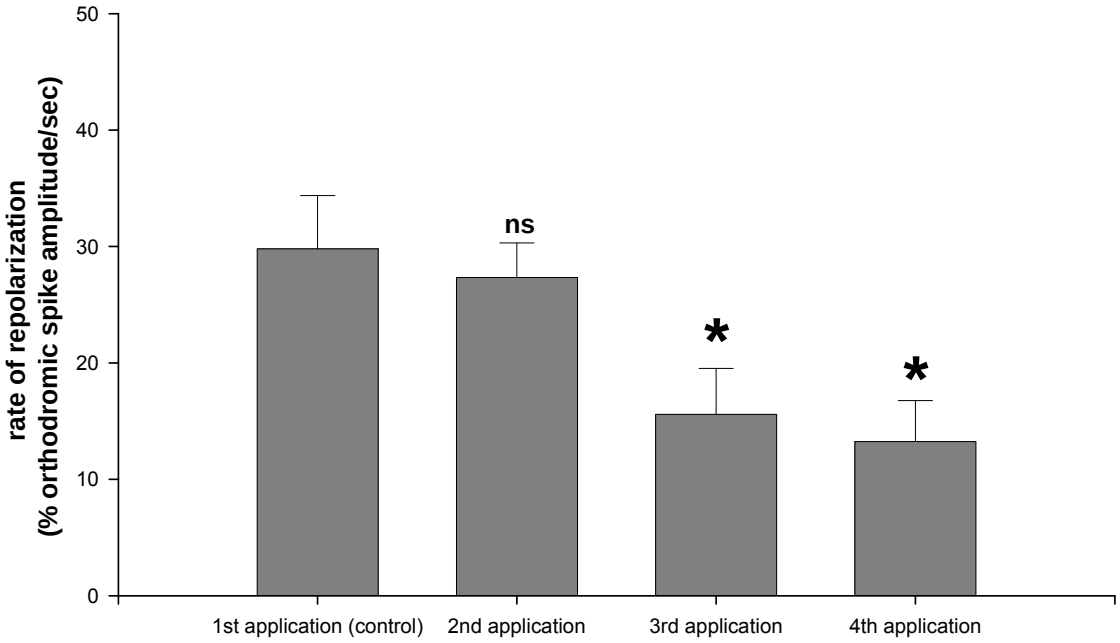


**Figure 3.4 Histogram outlining the changes in the rate of repolarization of the response following glutamate after each application as shown in Figure 3.3.** (ns = not significant *p<0.05 Dunnett multiple comparisons test).

### 3.2.2 - Adenosine antagonists modify recovery from glutamate

Since adenosine is known to be present in the extracellular space of tissue slices and its release can be increased by the activation of glutamate receptors (Manzoni et al., 1994; Hoehn & White, 1990), the effects of adenosine receptor-selective antagonists were examined on responses to glutamate. The A1 receptor antagonist DPCPX, at a concentration of 30nM, was perfused for 10min prior to and during the application of 5mM glutamate. The blockade of A1 receptors did not produce any change in the maximal amplitude of the response to glutamate, but it did result in an improvement in recovery of the population spike amplitude compared with controls, with a larger potential size recorded at the recovery plateau ($p<0.01$; Fig. 3.5a & b).

A similar protocol was adopted using the A2a receptor selective antagonist ZM241385 (50nM) which also produced a significant improvement in recovery of the population spike amplitude after glutamate application ($p<0.01$; Fig.3.5a & b).

Since the effects of DPCPX and ZM241385 were so similar, and it has been reported that the latter compound can block A1 receptors in the hippocampus (Lopes et al., 1999), we tested this possibility by examining the effects of the antagonists on the depression of population spikes induced by adenosine. Adenosine was initially perfused alone for 10min at a concentration of 10μM, after which a recovery period of 30 min was allowed before DPCPX (30nM) or ZM241385 (50nM) was perfused for 10min prior to and then during the application of a further pulse of 10μM adenosine for 10min. Both DPCPX and ZM241385 blocked completely the inhibitory response of adenosine, strongly supporting the suggestion that ZM241385 has an antagonist action at A1 receptors ($p<0.05$ paired t test; Fig. 3.6). To examine any potential role of A2a receptors, we therefore used another A2a receptor antagonist, SCH 58261 (1μM) which has no reported specificity for A1 receptors in the hippocampus. Use of this antagonist resulted again in a significantly greater recovery of spike amplitude following 5mM glutamate compared with controls (Fig 3.7a & b). This result was even more marked by the fact that perfusion of SCH 58261 alone resulted in a significant depression of the response to 85 ± 2.2% ($n = 5$) ($p<0.005$ one sample t test) (Fig 3.7a & b) suggesting that A2a receptors are indeed a component of post-glutamate recovery of spike amplitude.

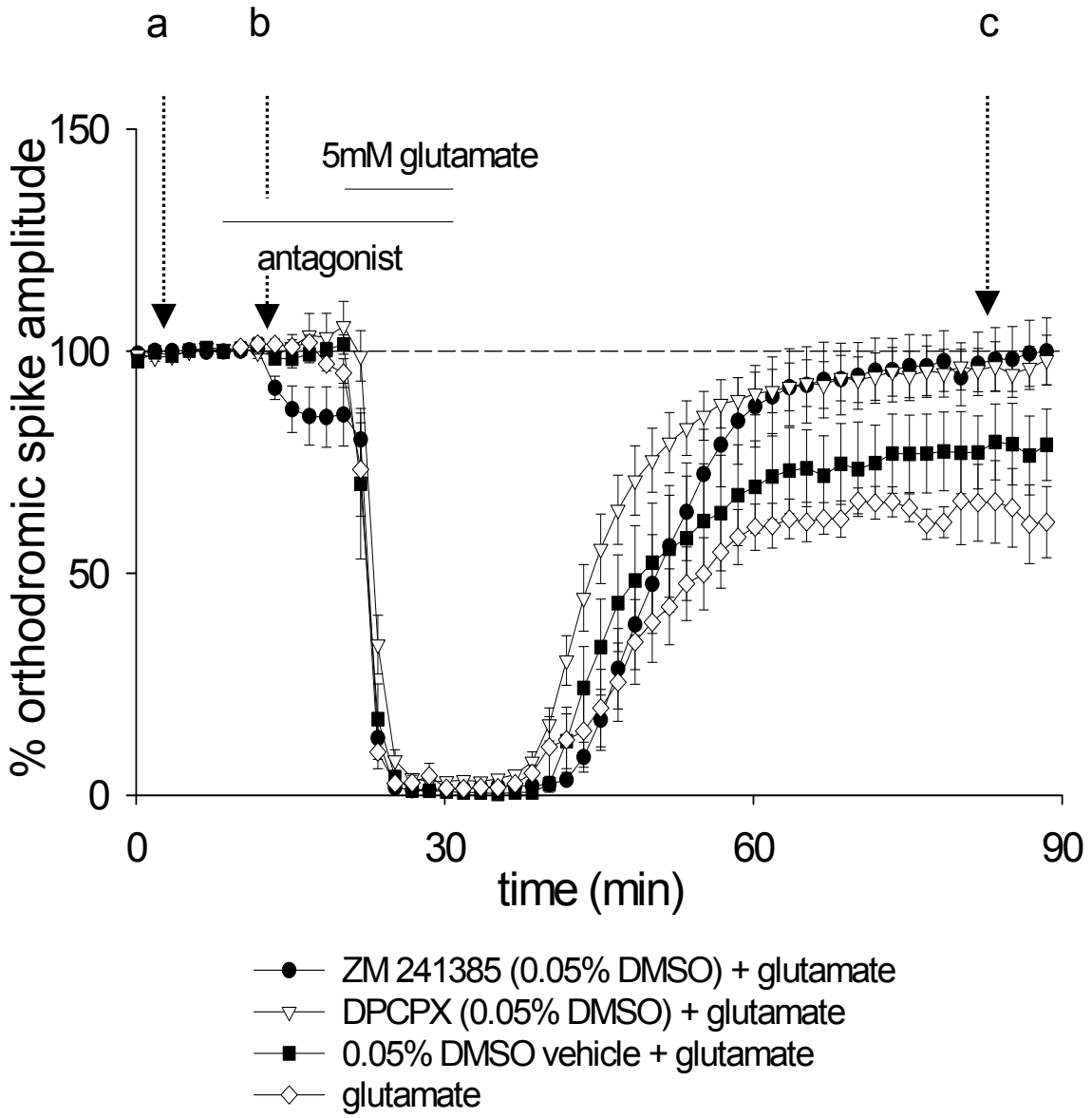


**Figure 3.5a The effects of adenosine antagonists on the recovery of post-glutamate responses.** Blockade of adenosine receptors with 30nM DPCPX (n = 11) or 50nM ZM 241385 (n = 9) significantly increases spike amplitude following 5mM glutamate perfusion compared with 5mM glutamate controls without antagonist (n = 9) (DPCPX, 95.0 ± 5.4%; ZM 241385, 96.0 ± 7.4%; glutamate control, 64.5 ± 3.1%) ($p<0.01$ Bonferroni multiple comparisons test). Perfusion with 0.05% DMSO vehicle alone showed no significant difference compared to glutamate controls (76.0 ± 8.9%, n = 5). Note that the spike amplitude significantly decreases in the presence of 50nM ZM 241385 prior to glutamate perfusion (85.6 ± 7.2%) ($p<0.05$, Wilcoxon rank sum test). The arrows (a, b and c) indicate the time points from which the sample waveforms in Figure 3.5b are taken.

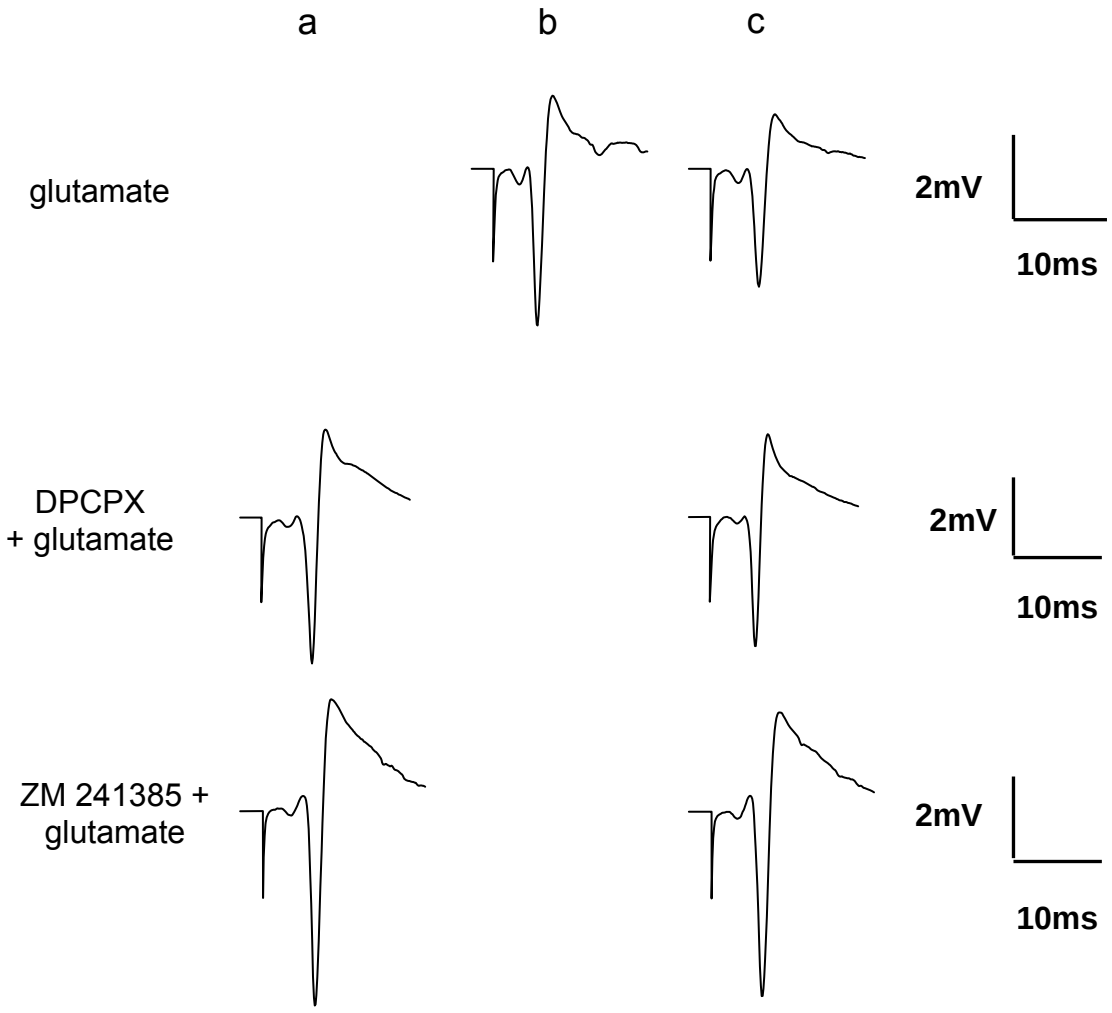


**Figure 3.5b Sample traces taken from time points as indicated in 3.5a.**

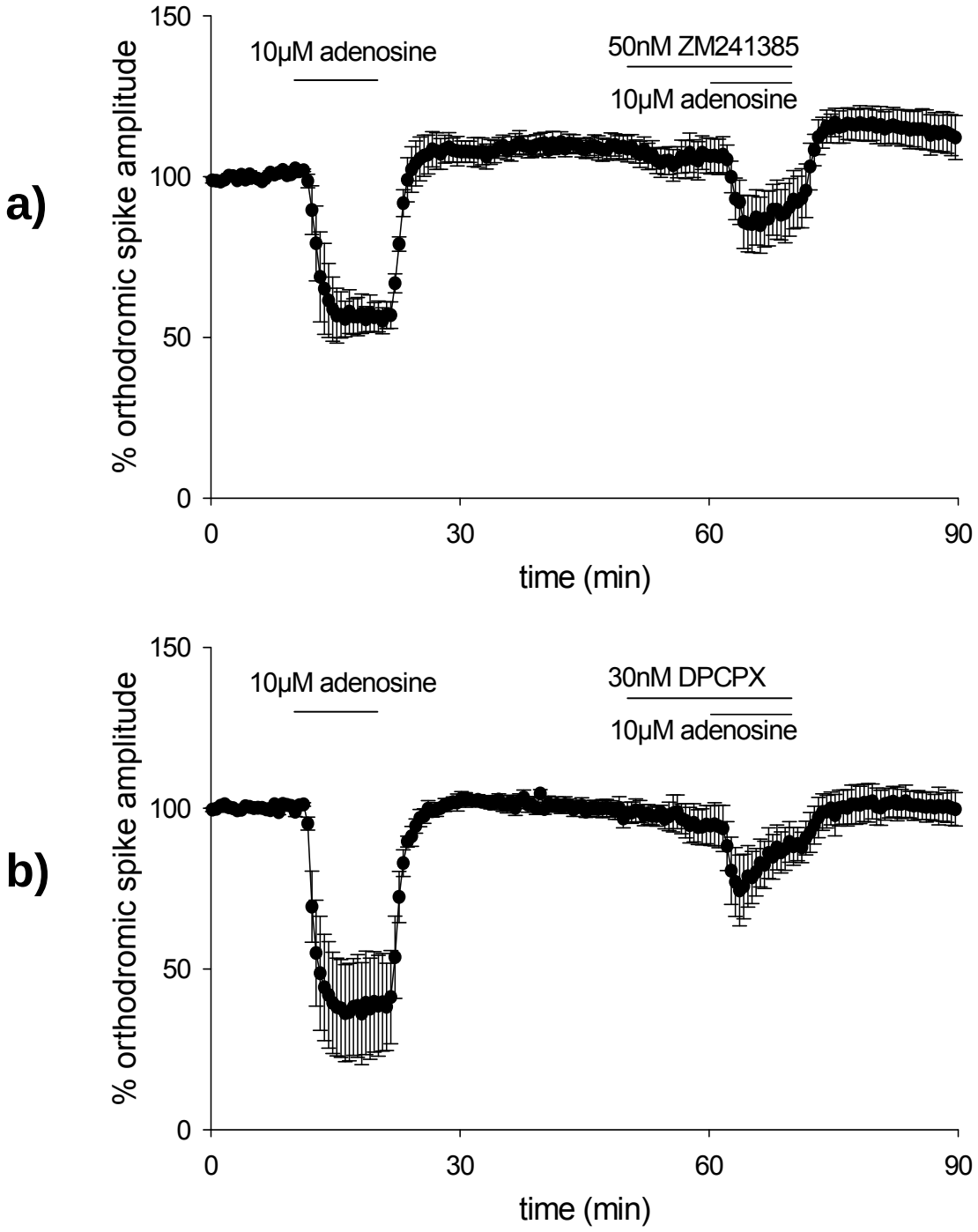


**Figure 3.6 50nM ZM241385 antagonises the inhibitory response of 10μM adenosine.** a) 10μM adenosine caused a 43.6 ± 4.7% depression in orthodromic spike amplitude compared to adenosine perfusion with 50nM ZM241385 which resulted in an 18.0 ± 6.9% depression ($p < 0.05$, $n = 4$). b) 10μM adenosine induced a 47.1 ± 19.1% depression in spike size compared to 3.8 ± 10% with adenosine perfusion with 30nM DPCPX ($p<0.05$, $n =5$). Depression is taken as the mean value of the 2min period preceding adenosine removal from the perfusion medium.

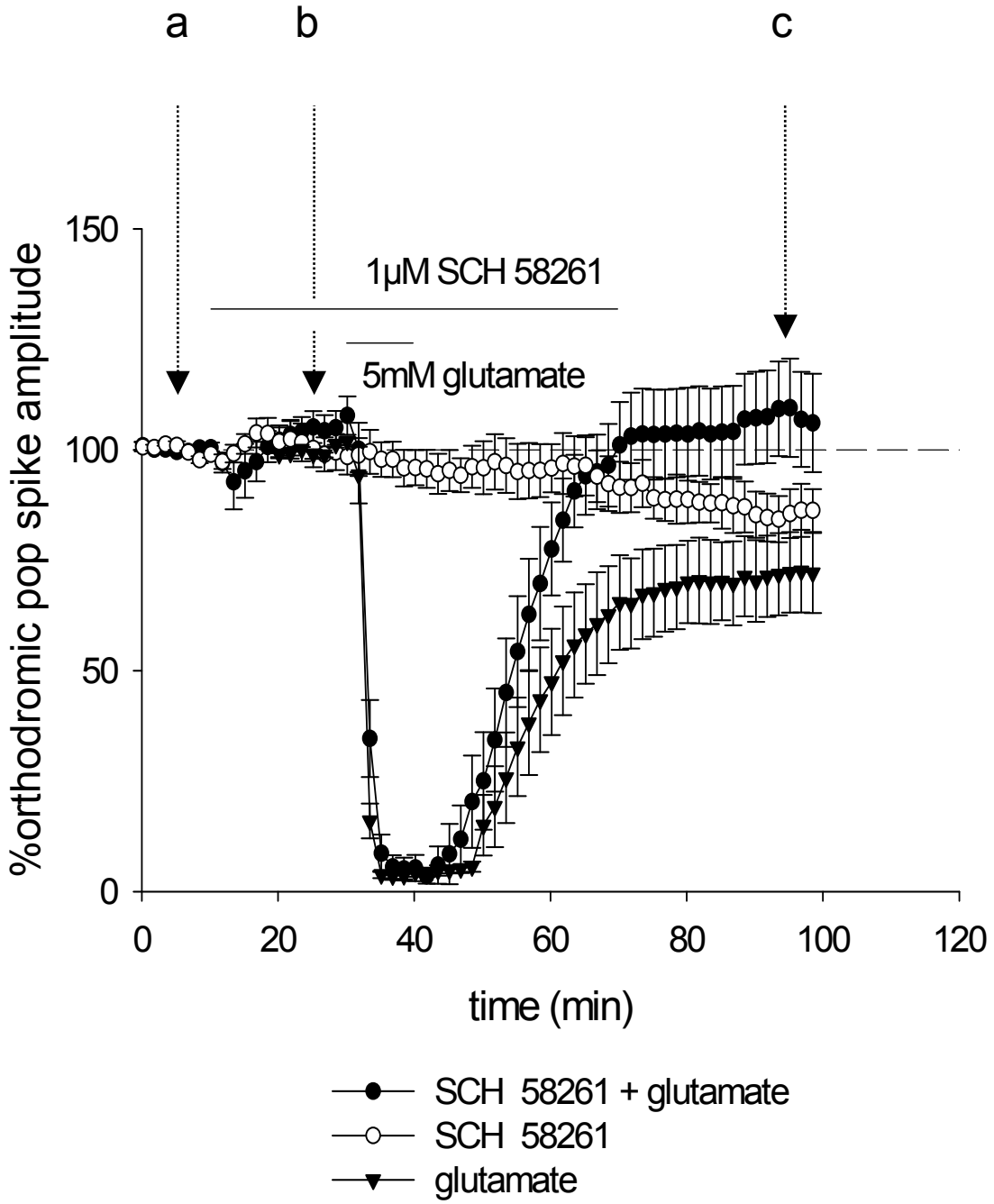


**Figure 3.7a The effect of SCH58261 upon the post-glutamate recovery of the orthodromic population spike amplitude.** 1µM SCH58261 is perfused prior to, during and following 5mM glutamate perfusion (n = 9) or for the same period without glutamate (n = 5). Controls have only glutamate applied (n = 7). There is a significant difference in the recovery of spike amplitude following glutamate with and without SCH 58261 (SCH 58261 + glutamate: 101.7 ± 8.5%; glutamate: 71.7 ± 9.3%) ($p<0.05$). Note that perfusion of SCH 58261 alone resulted in a significant depression of the spike amplitude between 90 and 100min ($p<0.005$ one sample t test). The arrows (a, b and c) indicate the time points from which the sample waveforms shown in Figure 3.7b are taken.

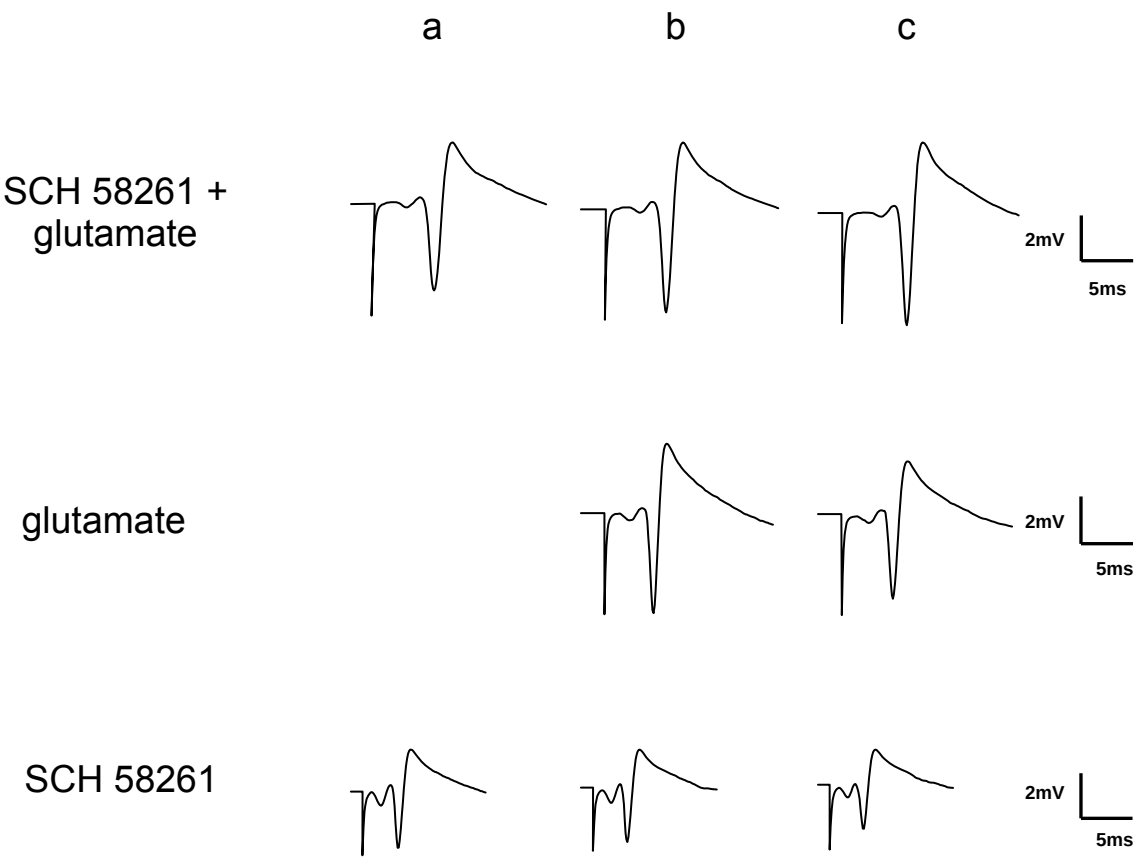


**Figure 3.7b Sample traces taken from time points as indicated in Figure 3.7a**.

### 3.2.3 - ATP-sensitive potassium channels mediate the effect of SCH 58261 on the response to glutamate

The possibility that disinhibition of A1 receptors by an A2a receptor antagonist might result in opening of ATP-sensitive potassium channels and in turn modify the response of orthodromic spikes to glutamate was considered. We repeated the experiment with SCH 58261 described above in the presence of glibenclamide, an ATP-sensitive potassium channel blocker. Glibenclamide suppressed the effect of SCH 58261 on glutamate induced depression (Fig 3.8a & b) suggesting that this effect was mediated by stimulation of ATP-sensitive potassium channels.

### 3.2.4 - Adenosine A1 receptor blockade does not modify the response to NMDA

Having determined that A2a receptor blockade modifies the responses to glutamate and that this is likely due to disinhibition of A1 receptors, the mechanism by which A1 receptor blockade induces a similar effect was still unclear. As A1 receptors are known to interact with NMDA receptors (de Mendonça & Ribeiro, 1993; de Mendonça et al., 1995; Canhao et al., 1994), DPCPX (30nM) was perfused 10min prior to and during applications of NMDA (25μM) for 5min (Fig 3.9a & b). The recovery of potential size was measured as the average of the evoked responses from 50 to 60min after the removal of NMDA, but there was no significant difference observed between responses to NMDA with or without the perfusion of DPCPX (control recovery 12.2 ± 7.2%, treated recovery 13.2 ± 10.6%). It was interesting to note, however, that the resulting recovery of response to NMDA showed a significant linear relationship with spike size (Fig 3.10), suggesting that levels of NMDA receptor stimulation are a factor in determining the % recovery of the response following glutamate perfusion as well as NMDA perfusion.

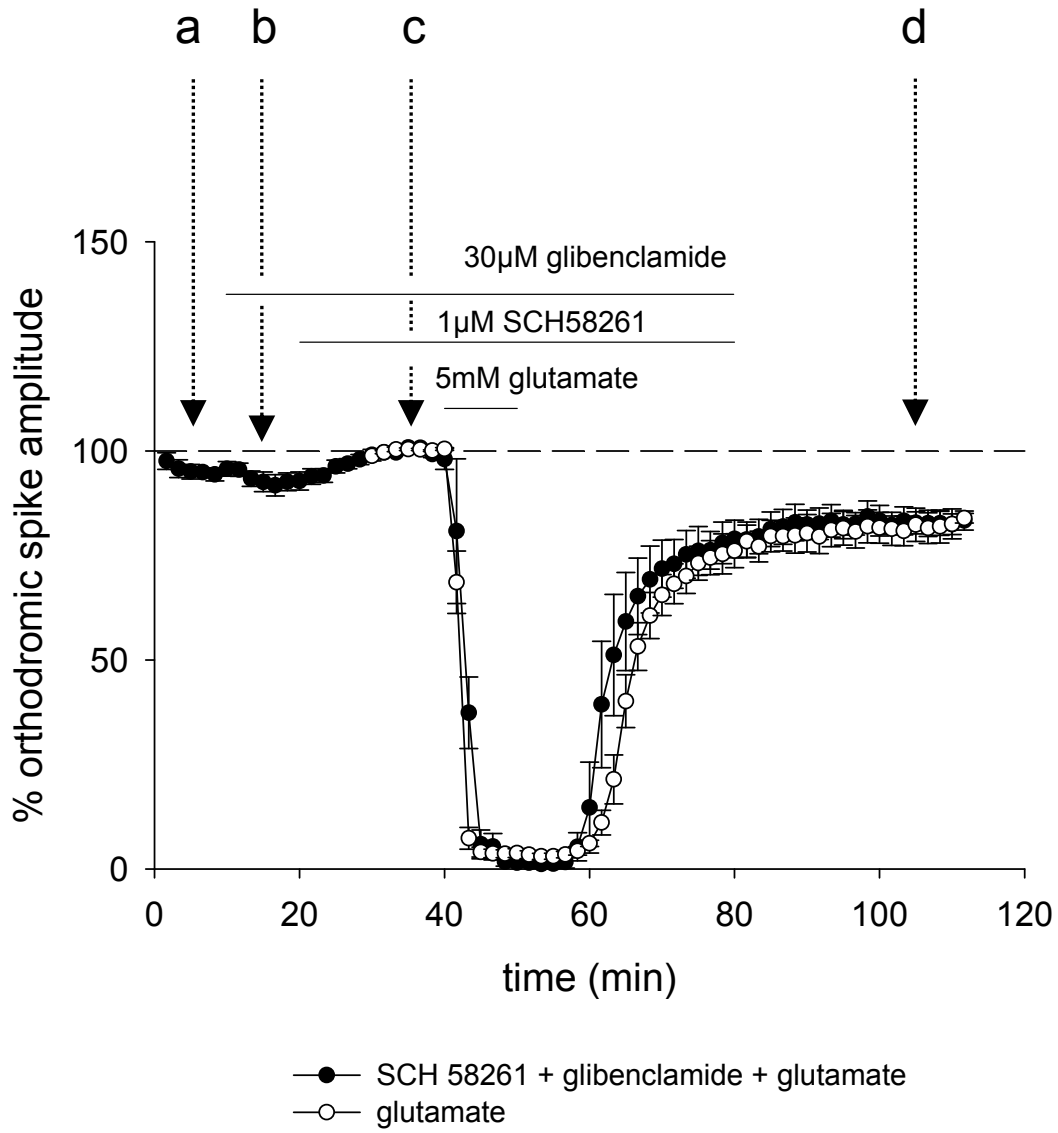


**Figure 3.8a Blockade of ATP-sensitive potassium channels attenuates the modulatory effect of SCH 58261 on glutamate-induced depression of orthodromic population spikes.** 30μM glibenclamide is perfused prior to and during perfusion of 1μM SCH 58261. There was no significant difference in response to glutamate between slices treated with inhibitors (n = 5) and those with glutamate alone (n = 6) (p = 0.83). The arrows (a, b, c and d) indicate the time points from which the sample waveforms shown in Figure 3.8b are taken.

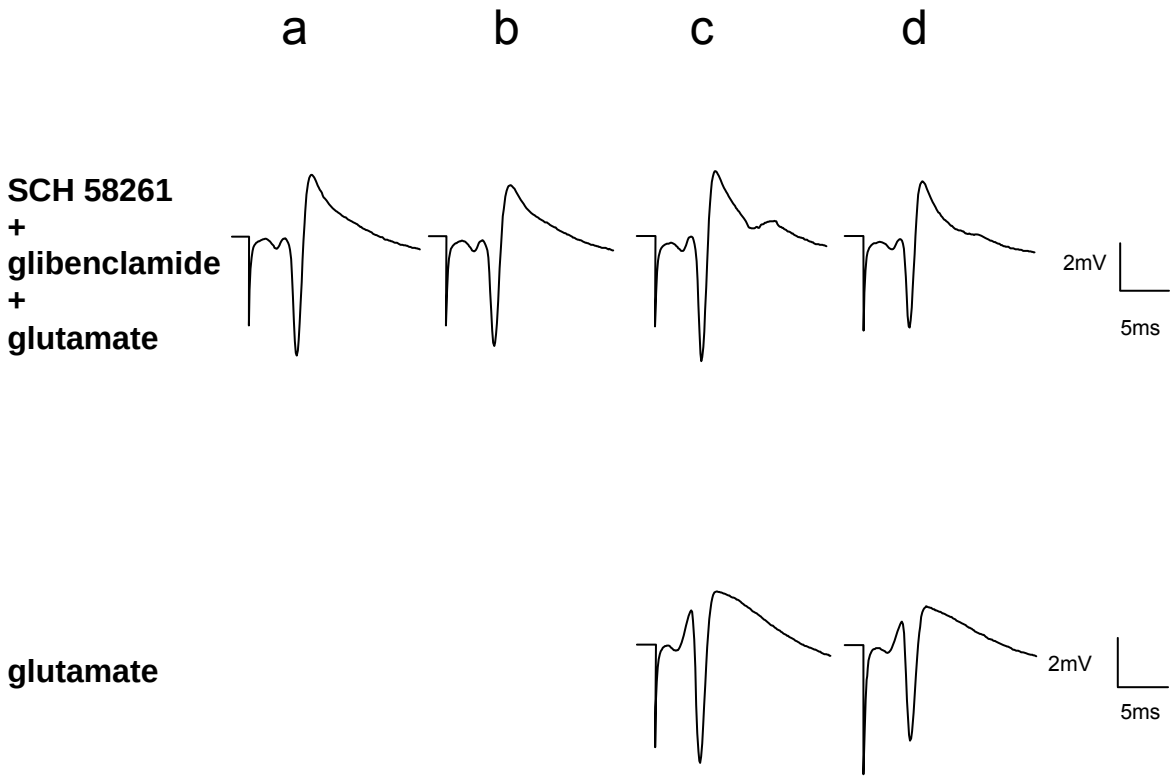


**Figure 3.8b Sample traces taken from the time points as indicated in 3.8a.**

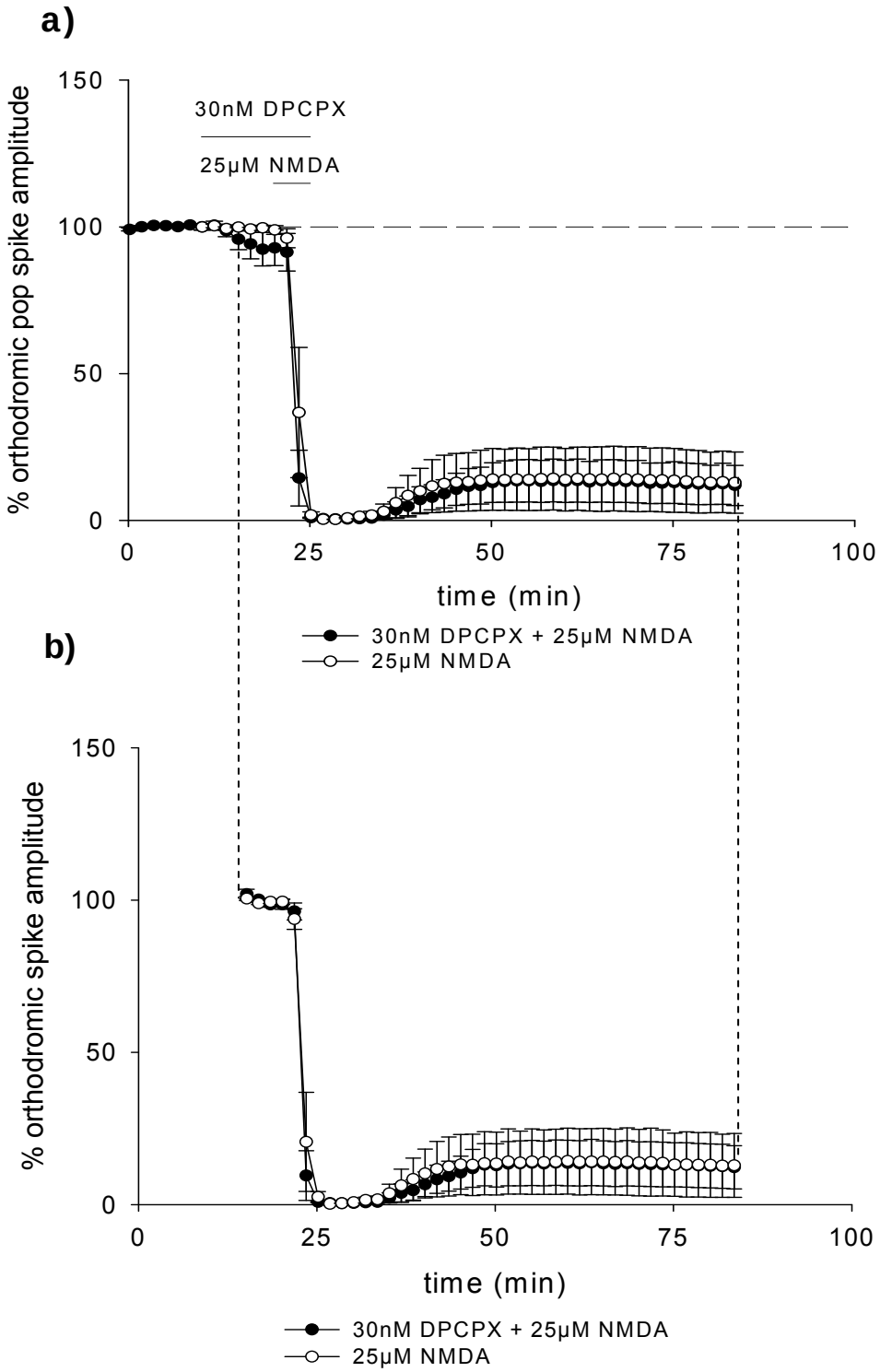


**Figure 3.9 The adenosine A1 receptor antagonist DPCPX does not attenuate the reduced recovery of orthodromic population spikes following depolarization of the response with NMDA.** a) shows the time course of the entire experimental protocol and the changes in orthodromic spike size throughout (DPCPX + NMDA n = 8, NMDA n = 5). b) shows extrapolated data from a) normalised 5min prior to perfusion of 25µM NMDA.

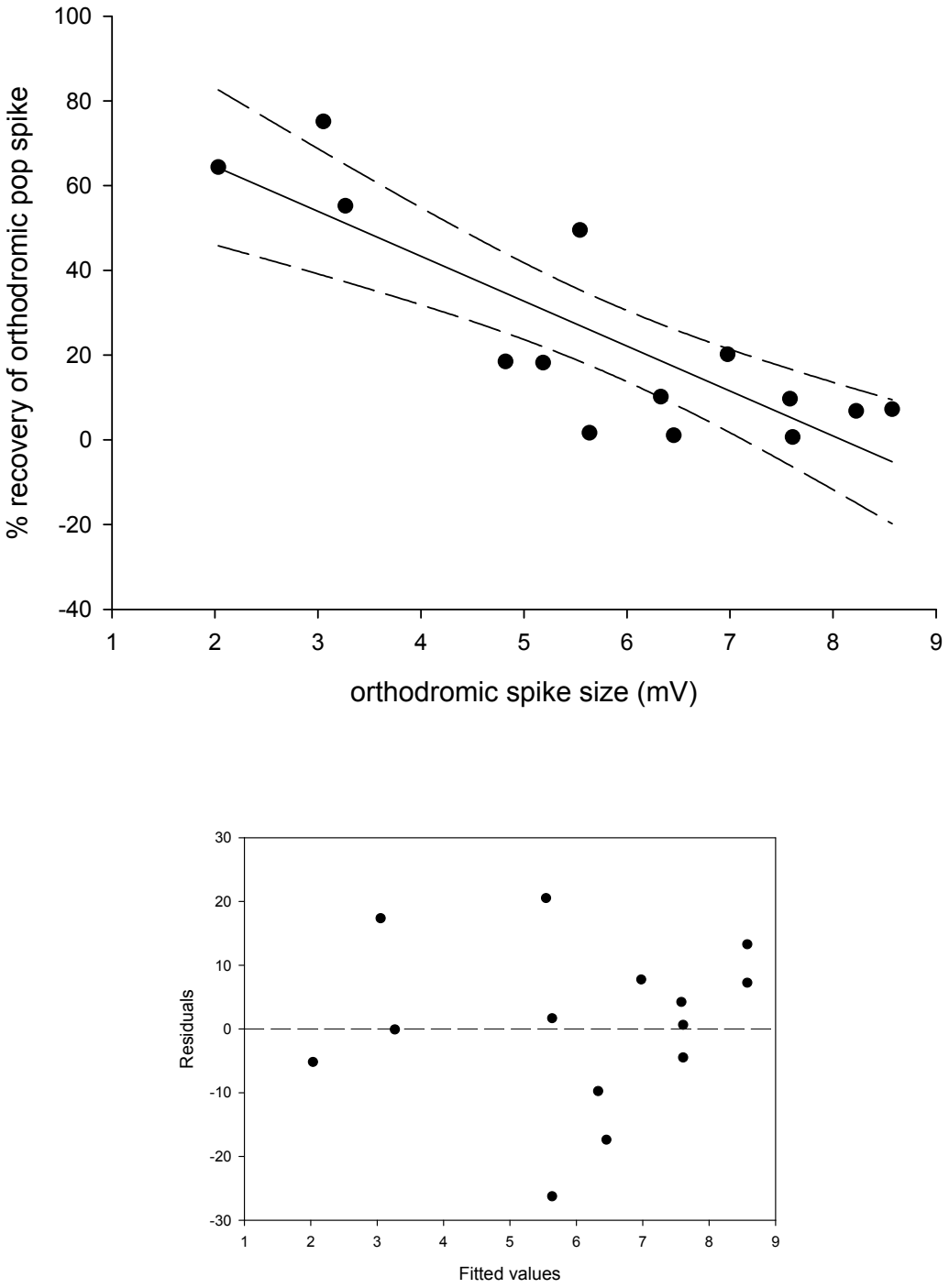


**Figure 3.10 Scatterplot showing the inverse relationship between orthodromic spike size and % recovery of spike size following treatment with 25μM NMDA for 5min.** There is a significant linear relationship ($r^2 = 0.69$, $p<0.0005$). The lower scatterplot shows the residuals obtained from the regression analysis.

### 3.2.5 - Antidromic population spikes and epsps are unaffected in their response to glutamate by A1 receptor blockade

The effect of DPCPX on glutamate was examined further using antidromic population spikes. The antidromic responses depolarized partially in response to 5mM glutamate and recovered fully once glutamate was removed. 10mM glutamate induced a complete loss of antidromic spike amplitude which returned in a similar manner to that observed in orthodromic spikes. There was no correlation observed here between spike size and % recovery following glutamate. When DPCPX was perfused prior to and during 5mM glutamate, there was no observable difference in the observed changes in antidromic spike amplitude (Fig 3.11). It was therefore decided to examine possible changes in epsp slope size and amplitude. 5mM glutamate induced a complete depolarization of the response which lasted between 10 and 20min after glutamate was removed (Fig 3.12). The epsp then recovered but unlike orthodromic spikes, it did not always show a reduced response size compared with pre-glutamate treatment. In some cases, the slope and amplitude of the epsp exceeded that prior to glutamate treatment. A scatterplot of epsp slope or amplitude and % recovery of either parameter showed a trend whereby smaller epsps generally recovered to a greater extent than larger epsps although a statistical correlation for this effect could not be found (Fig 3.13). When glutamate was perfused in the presence of DPCPX, there was no significant difference in either epsp slope or amplitude detected between glutamate only treated responses and glutamate + DPCPX (Fig 3.12). The scatterplots of epsp slope or amplitude versus % recovery of either parameter show that treatment groups were within the same response size range and t-tests showed no significant difference (Fig 3.13) (GLUTAMATE: slope -0.56 ± 0.15 (mV/ms) amplitude 1.3 ± 1.0 (mV)(n = 12); DPCPX + GLUTAMATE: slope -0.92 ± 0.29 (mV/ms) amplitude 1.8 ± 0.5 (mV) (n = 8)).

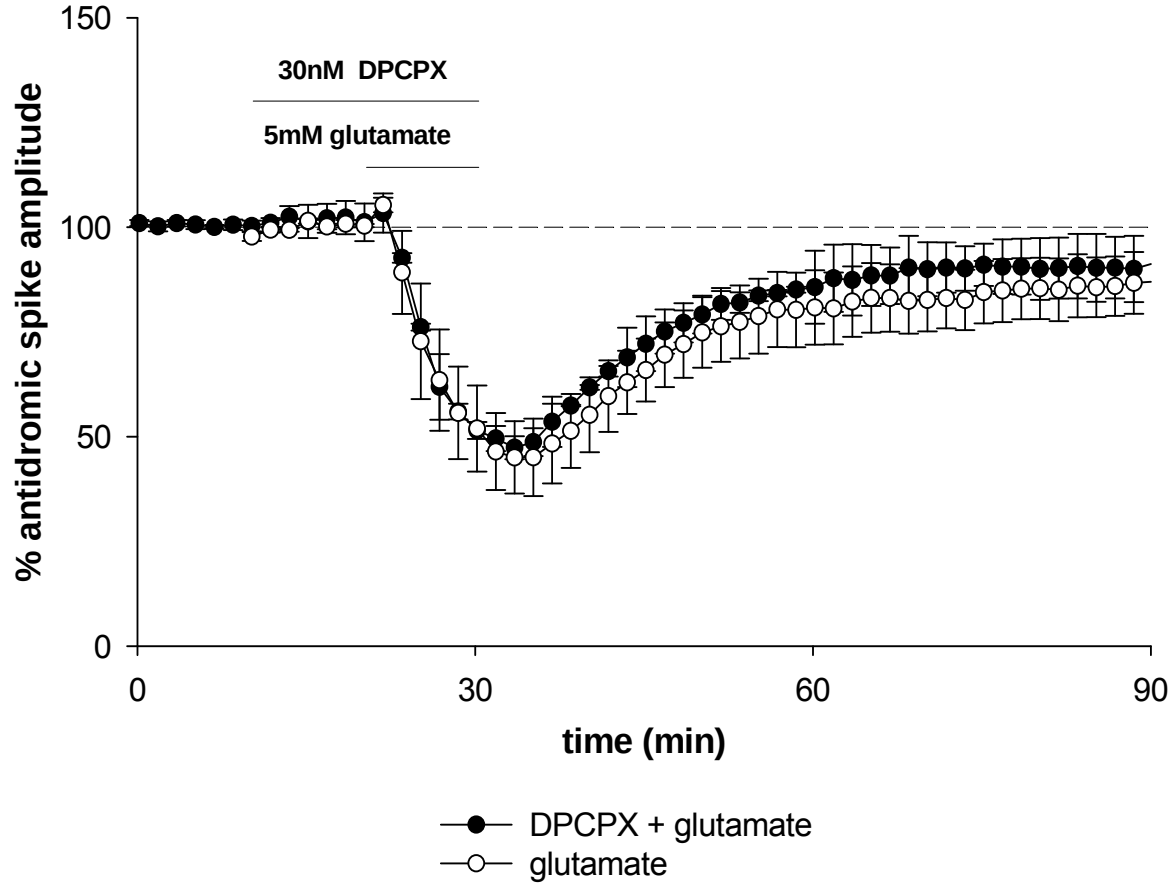


**Figure 3.11 DPCPX does not attenuate the effects of 5mM glutamate on antidromic population spikes.** Antidromic population spikes show a recovery following glutamate-induced depolarization that does not differ significantly from the baseline (50-60min post-glutamate 90.3 ± 8.5%, n = 4). Co-perfusion with DPCPX does not alter the changes in spike size following treatment with glutamate (50-60min post-glutamate 85.7 ± 7.5, n = 4).

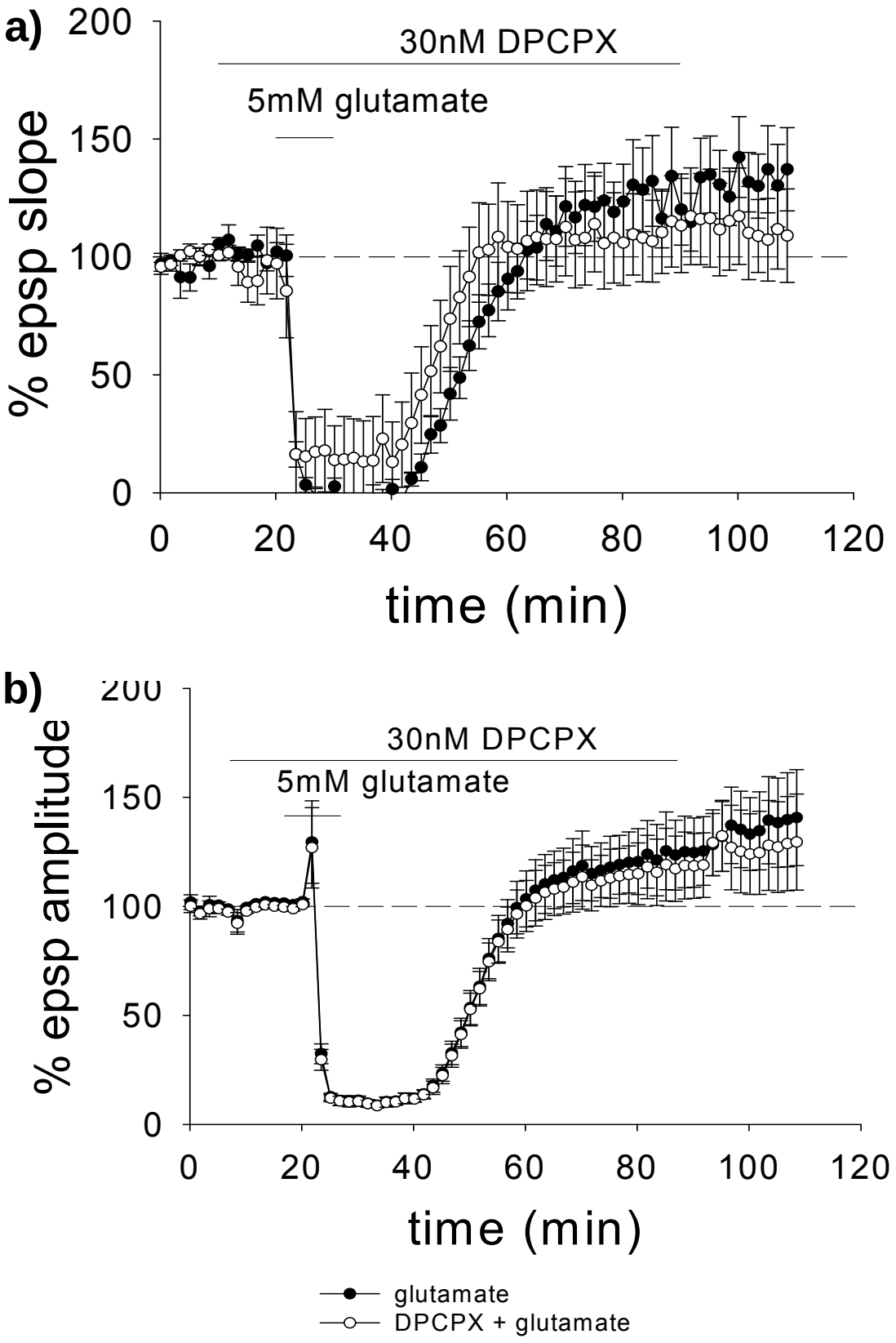


**Figure 3.12 DPCPX does not attenuate the effects of 5mM glutamate on field epsps.** Graphs show the time course changes in a) epsp slope and b) amplitude for slices treated with glutamate alone (n = 12) or in combination with DPCPX (n = 8).

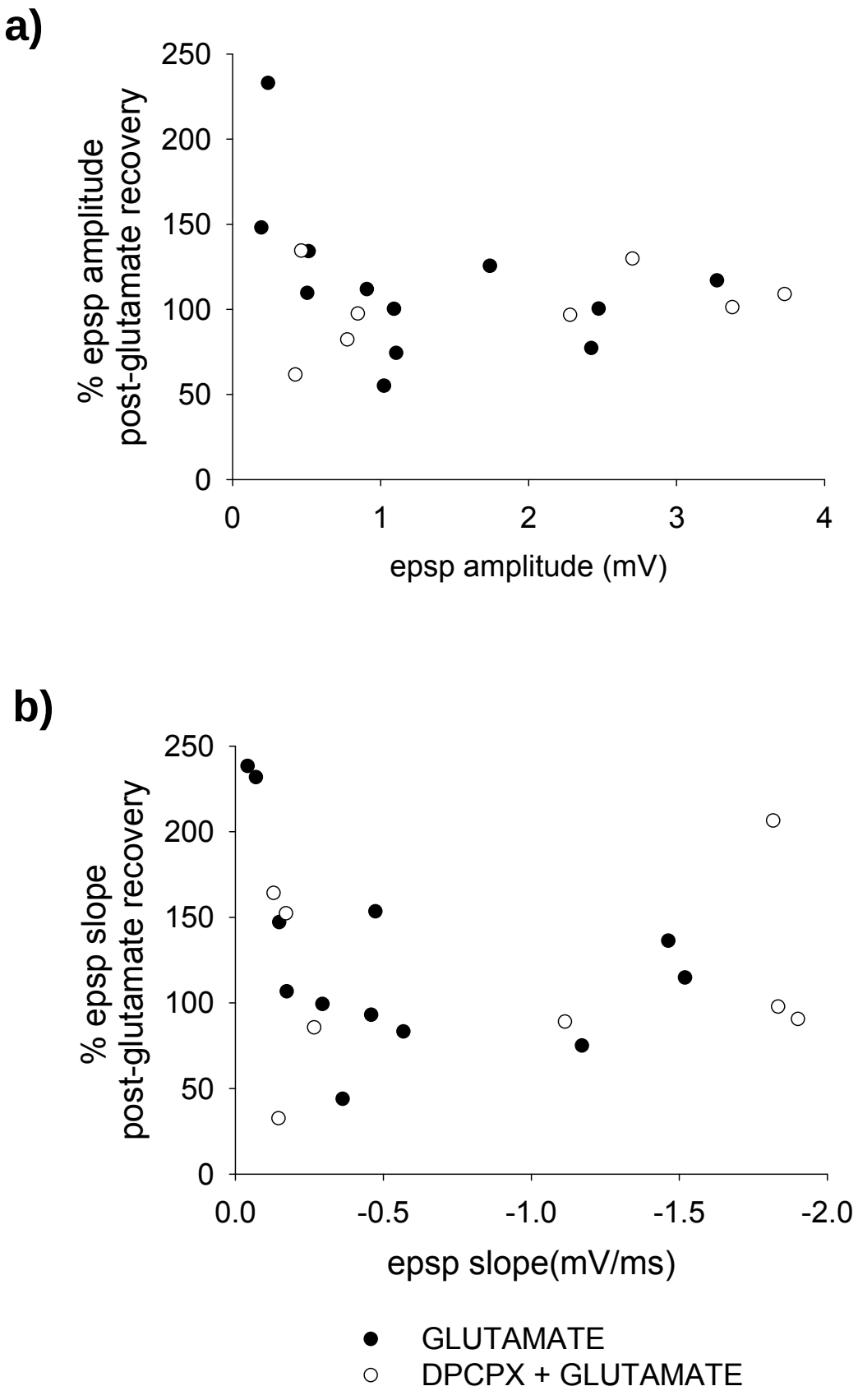


**Figure 3.13 Scatterplots showing the size of epsp parameters against the % recovery of each parameter following treatment with glutamate alone or in the presence of DPCPX.** a) shows the amplitudes and b) shows the slopes.

### 3.2.6 - The effects of NMDA receptor blockade on the response to glutamate in epsps

Given that orthodromic population spikes appear to show a correlation between the outcome following NMDA treatment and the initial spike amplitude and that epsps show a similar trend with glutamate, the effects of NMDA receptor blockade during glutamate perfusion on epsps were examined. The use-dependent NMDA channel blocker, MK-801 (10μM) was perfused prior to, during and after 5mM glutamate exposure (Fig 3.14). The ranges of response sizes versus the % recoveries following glutamate are shown in the scatterplots in Figure 3.15. It was noted that before glutamate perfusion, MK-801 induced either a potentiation or depression in epsp slope and amplitude so the baseline was taken as 5min prior to glutamate perfusion. The majority of responses showed a depolarization upon glutamate perfusion which lasted for approximately 10min post-glutamate. For these responses, the post-glutamate recovery significantly exceeded the initial epsp slope size (137.3 ± 11.1%, $p<0.01$ one-sample t-test) and amplitude (143.7 ± 9.7%, $p< 0.005$ one-sample t-test) and also the post-glutamate recoveries of control responses without MK-801 co-perfusion (SLOPE: 88.2 ± 12.8%, $p<0.05$; AMPLITUDE: 85.1 ± 9.5%, $p<0.0005$). Two of the responses, however, showed a depolarization in response to glutamate followed by an immediate repolarization. This was transient as the response depolarized again in the continued presence of glutamate. The recoveries of these two responses were markedly depressed compared with glutamate treated controls. These responses also showed a marked depression in response size during MK-801 perfusion alone. When the effect of MK-801 on response size was correlated with the outcome following glutamate treatment, it was shown that the % MK-801-induced changes in epsp slope and amplitude were inversely related to the % recovery of epsp parameters post-glutamate (Fig 3.16). This suggested that the extent of NMDA receptor modulation determines the degree of recovery following glutamate.

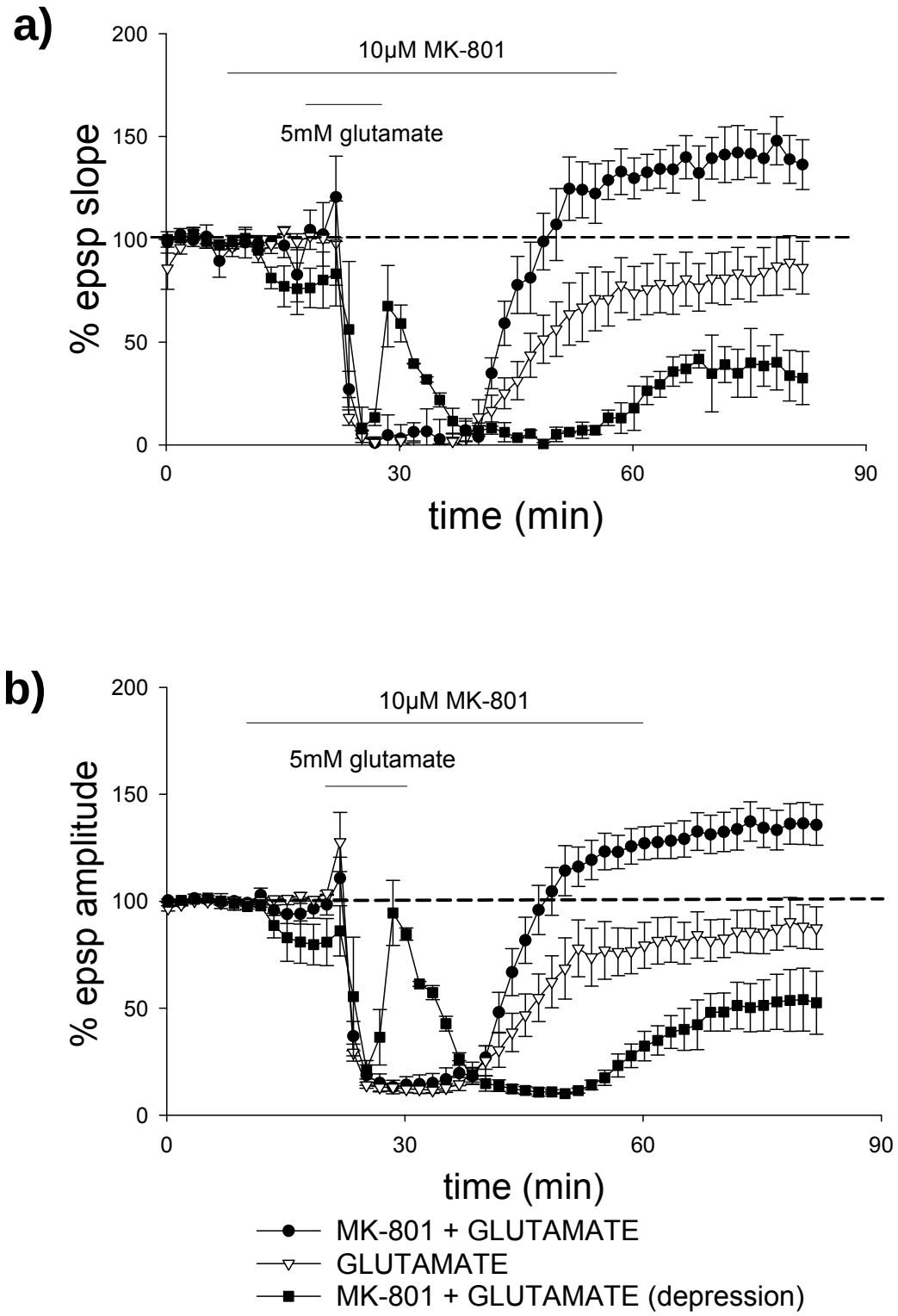


**Figure 3.14 Time course graphs showing the effect of glutamate on epsp a) slope and b) amplitude alone (n = 13) or in the presence of MK-801 (n = 12).** Note that treatment with MK-801 + glutamate is split into two groups based on the response to the protocol (potentiation n = 10, depression n =2).

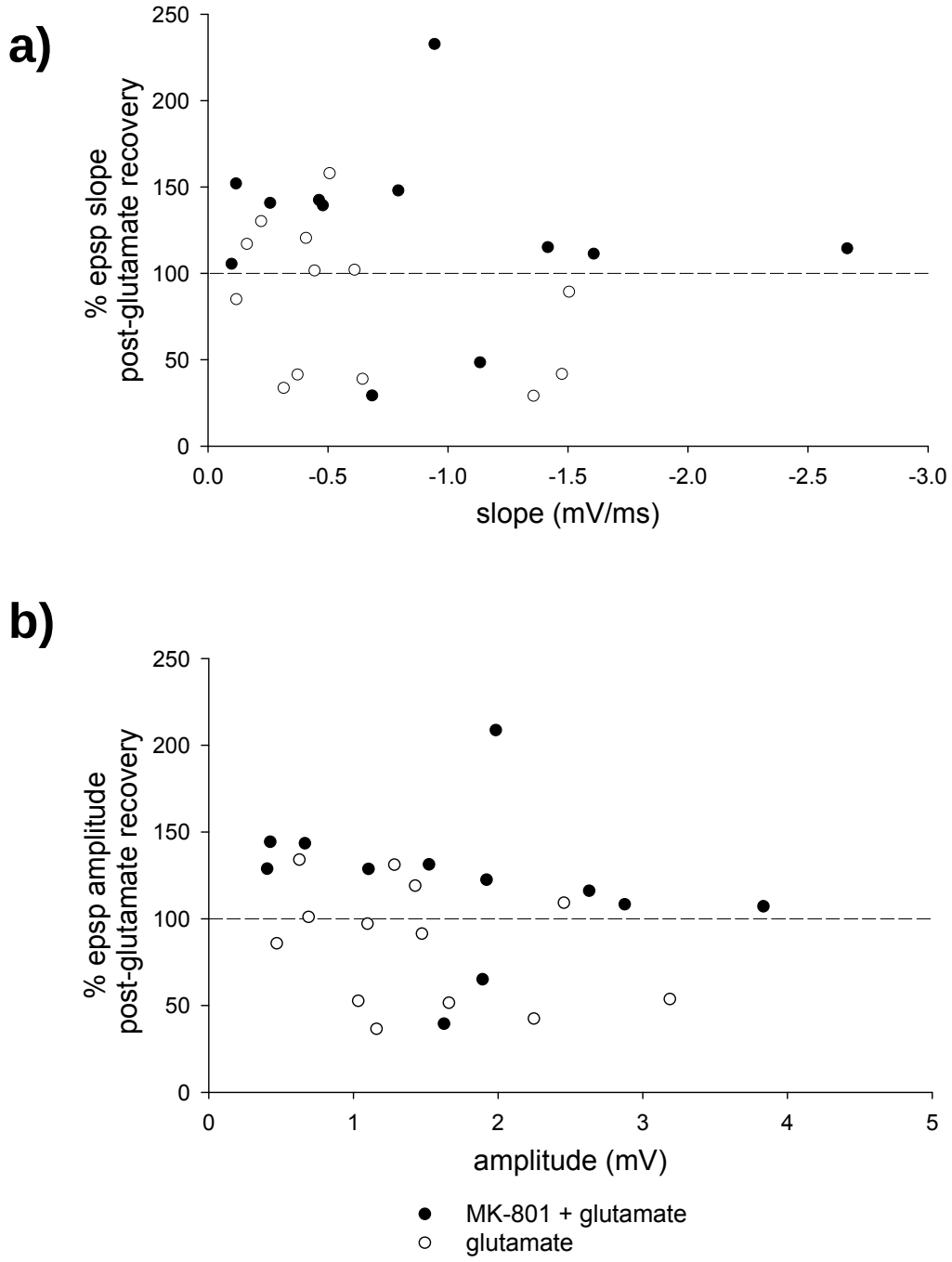


**Figure 3.15 Scatterplots showing the size of epsp slopes or amplitudes against the % recovery of either parameter following treatment with glutamate alone or glutamate in combination with MK-801**. a) shows the gradient of the slope versus the % recovery of the slope after glutamate application. b) shows the size of the epsp amplitude versus the % recovery of the amplitude.

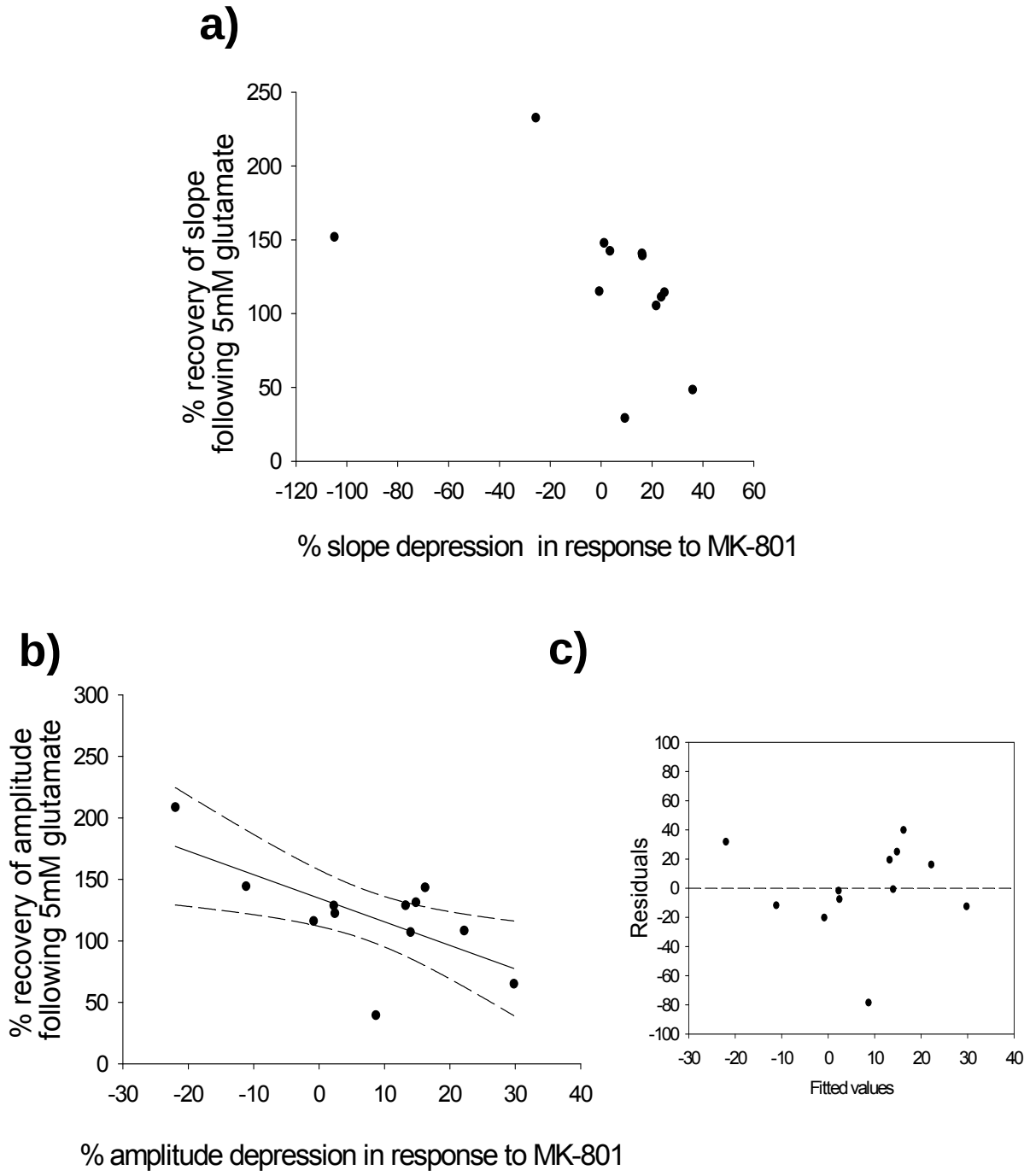


**Figure 3.16 The relationship between the effect of MK-801 on epsps and the resulting post-glutamate recovery.** The scatterplots show the relationship between the effect of MK-801 on a) epsp slope and b) amplitude and the % recovery of each parameter following perfusion with glutamate. Note that there is a significant linear relationship between the effect of MK-801 on epsp amplitude and the % recovery following glutamate ($r^2$ = 0.44, $p<0.05$) and also a significant correlation between the effect on epsp slope and % recovery (Spearman r = -0.7343, $p<0.01$). c) shows the scatterplot of the residuals generated from linear regression analysis for b).

### 3.2.7 - The effects of adenosine A1 receptor blockade on NMDA receptor mediated epsps

To investigate the implications of the above result on the interaction between A1 receptor blockade and the effects of glutamate, the DPCPX-glutamate protocol was repeated in epsps with the NMDA receptor mediated component isolated by perfusing the slice with DNQX and removing Mg2+ from the aCSF. DPCPX was perfused for 10min prior to 5mM glutamate as before (Fig 3.17). NMDA receptor mediated epsps exposed to glutamate alone showed a change in slope and amplitude similar to that observed in composite epsps. The same trend in post-glutamate % recovery compared with epsp slope or amplitude was present although again not significantly correlated (Fig 3.18). Recovery for each experiment was measured as the average of a 10min plateau recovery following glutamate perfusion as soon as such a recovery was obtained. Control responses were selected to match the range of response sizes in the DPCPX + glutamate treated slices (Fig 3.18). It was observed that DPCPX significantly attenuated the depression of NMDA receptor mediated epsp amplitude (Fig 3.19) by glutamate thus confirming that A1 receptor blockade modifies the response to glutamate by interaction with the NMDA receptor.

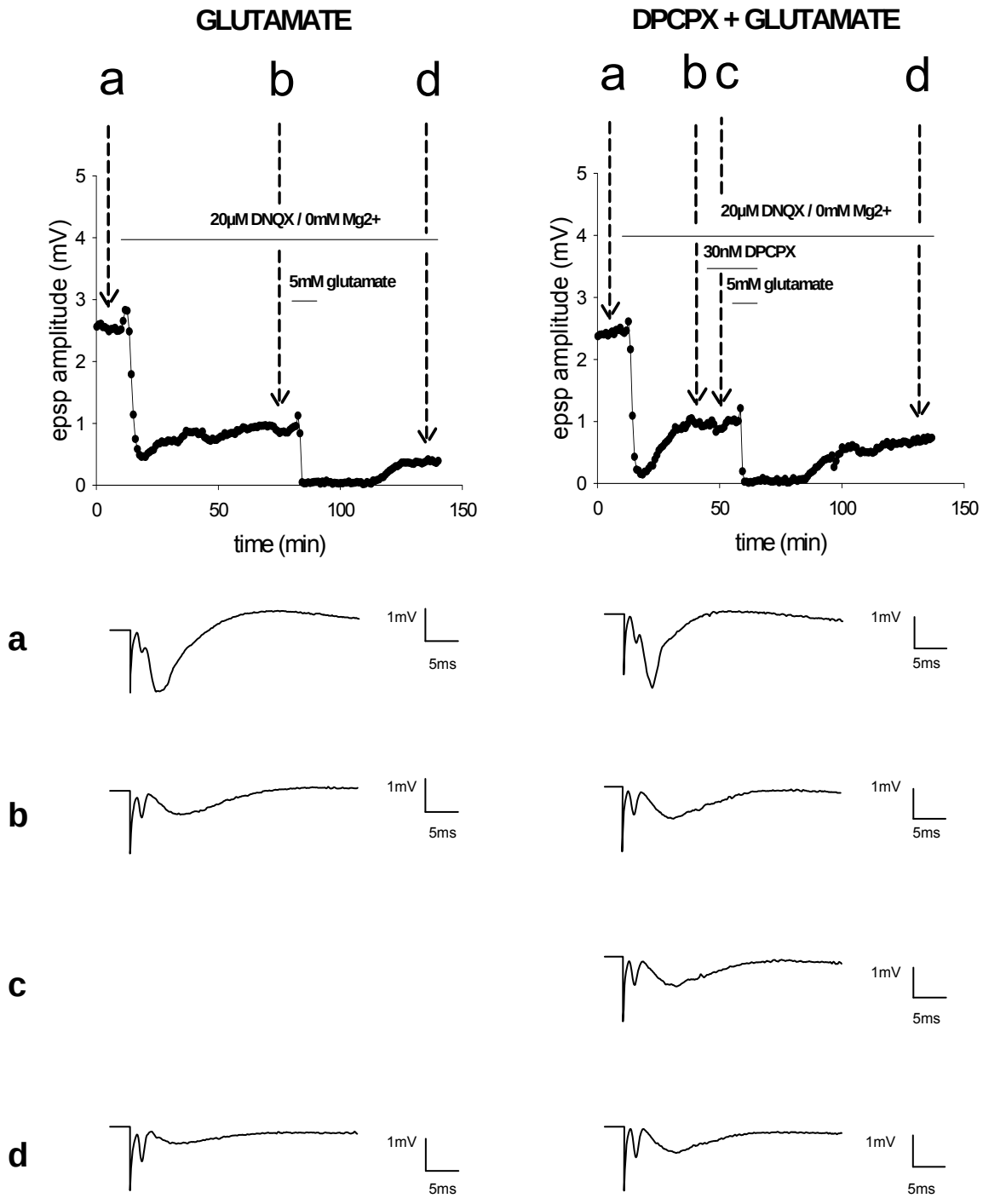


**Figure 3.17 A1 receptor antagonist DPCPX attenuates the depression in NMDA receptor mediated epsp amplitude following depolarization of the response with glutamate.** The graphs show the time course of changes in epsp amplitude for a single experiment for glutamate only and glutamate in combination with DPCPX. The sample traces shown below are taken from the time points as indicated by the letters in the time course graphs. Note that **a** shows a full epsp whilst **b** shows an NMDA-receptor mediated epsp derived from that obtained in **a**.

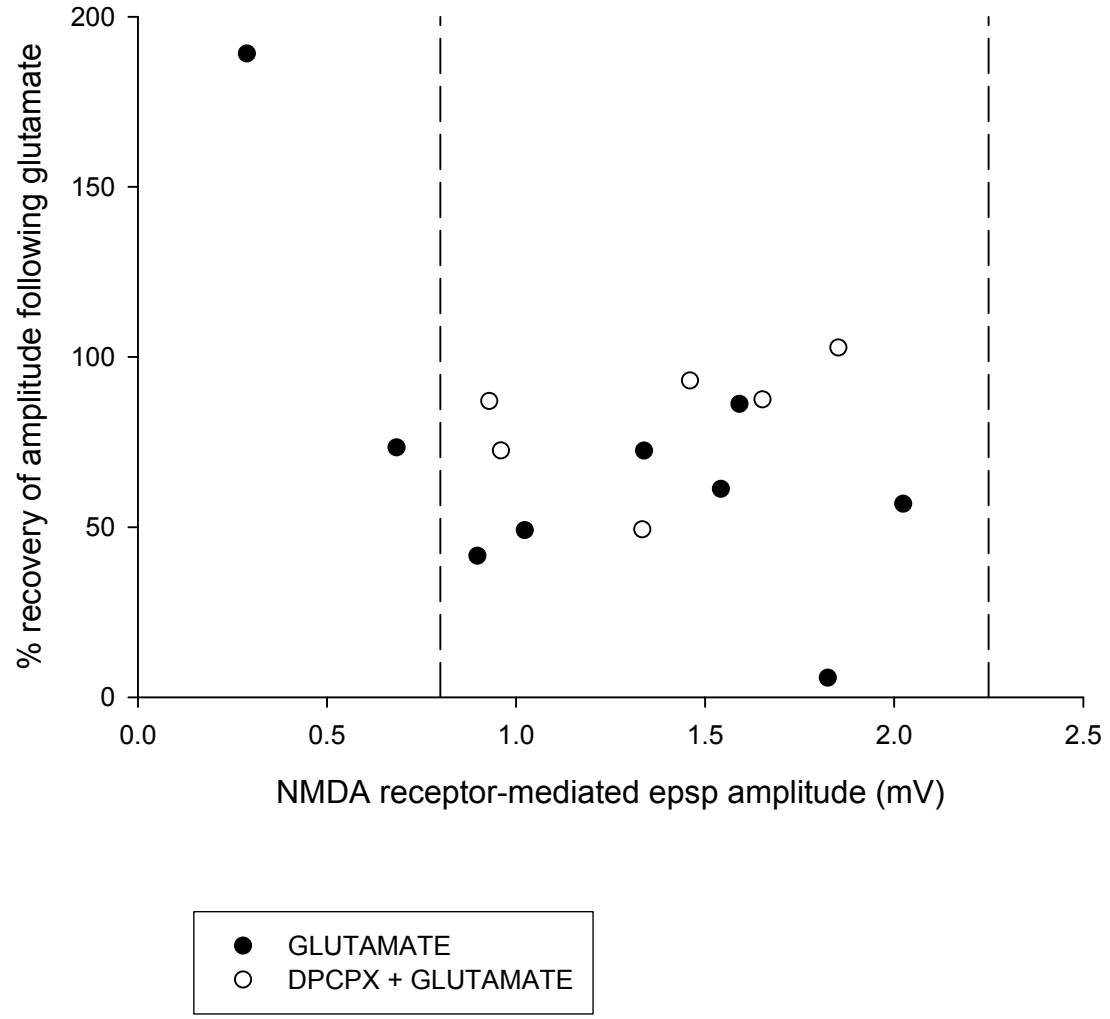


**Figure 3.18 Scatterplot showing the amplitude of NMDA receptor-mediated epsps versus the corresponding percentage recovery following perfusion of glutamate.** Responses were treated with either glutamate alone or in combination with DPCPX as shown in Figure 3.17. The dashed lines enclose the range of values used for comparison between groups as shown in Figure 3.19. Epsps treated with glutamate only and with amplitudes below 0.8mV were excluded.

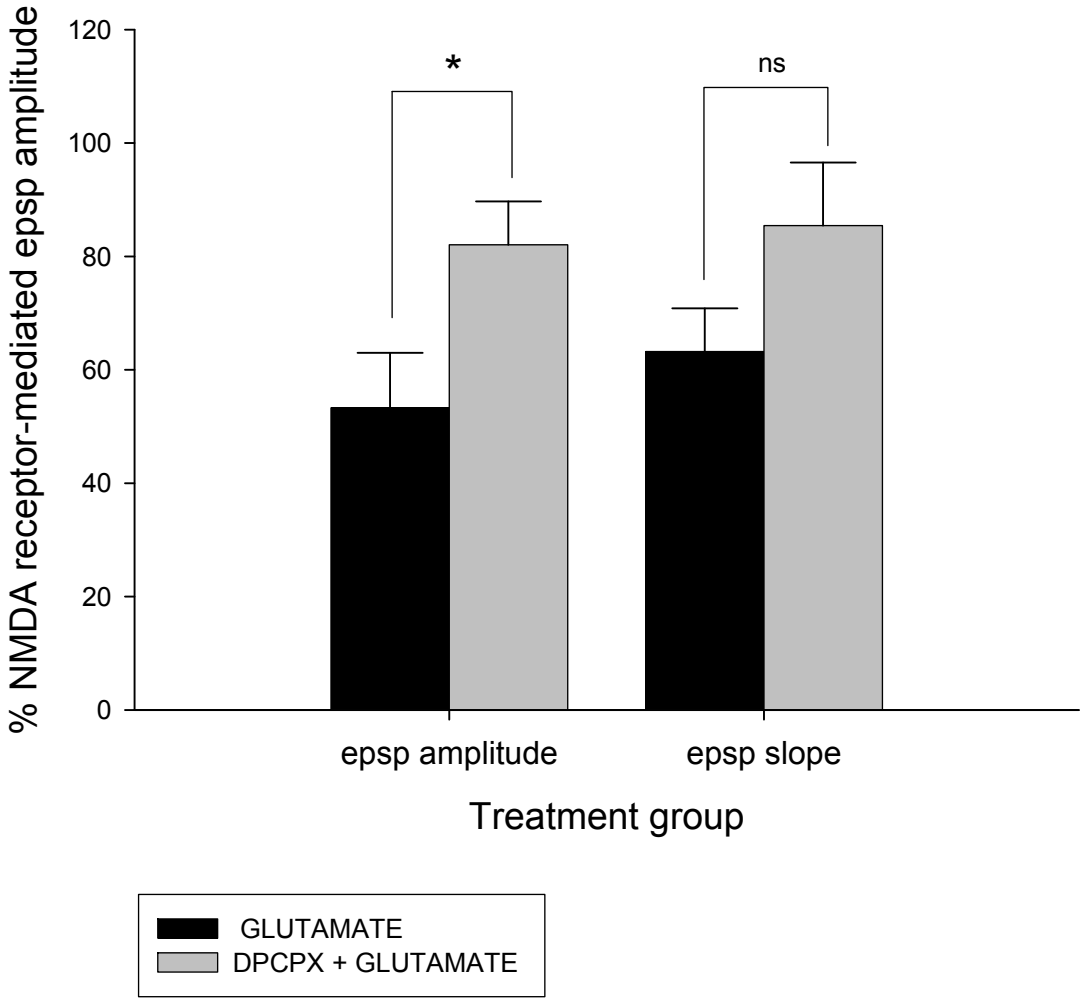


**Figure 3.19 Histogram showing the difference in outcome between glutamate treatment alone (n = 7) and in combination with DPCPX (n = 6) on NMDA receptor-mediated epsp parameters.** A1 receptor blockade significantly attenuates the depressed recovery in epsp amplitude following glutamate ($p<0.05$) but not in the epsp slope. Values for the statistical comparison are those indicated in the scatterplot in Figure 3.18.

### 3.2.8 - Varying the stimulus intensity induces a potentiation of the post-5mM glutamate orthodromic spike

In order to clarify the effects of 5mM glutamate upon the orthodromic population spike, it was decided to vary the stimulus intensity to produce half-maximal and 25% maximal baselines instead of evoking the maximal population spike. After establishing a stable baseline, it was observed that 5mM glutamate applied onto the slices for 10min abolished the response regardless of the stimulus intensity used. During the washout, the response recovered but in slices which used a half-maximal baseline, the recovery of the response was significantly greater than the initial baseline evoked prior to glutamate application (Fig 3.20). It was also observed that the recovery of the responses evoked at the 25% maximal baseline was either increased (n = 3) or significantly decreased (n = 8) ($p = 0.001$, one sample t test) indicating that some kind of threshold existed using this stimulus intensity.

To examine further the threshold phenomenon observed using a 25% maximal baseline, the same protocol was repeated for the 25% baseline applying 5mM glutamate and observing the outcome. The maximal response was evoked three times prior to the application of glutamate to ensure that the response was stable. At one hour post-glutamate, a 100Hz/1sec stimulus was applied to the slice to see if the effects of glutamate were reversed (Fig 3.21). It was observed that the post-glutamate responses, regardless of whether they were higher or lower than the 25% maximally evoked baseline, produced a significant potentiation in response to the tetanising stimulus (Fig3.21a, b & c). This indicated that the effects of 5mM glutamate upon the orthodromic population spike were a form of long-term depression as characterised by its reversibility by a tetanising stimulus.

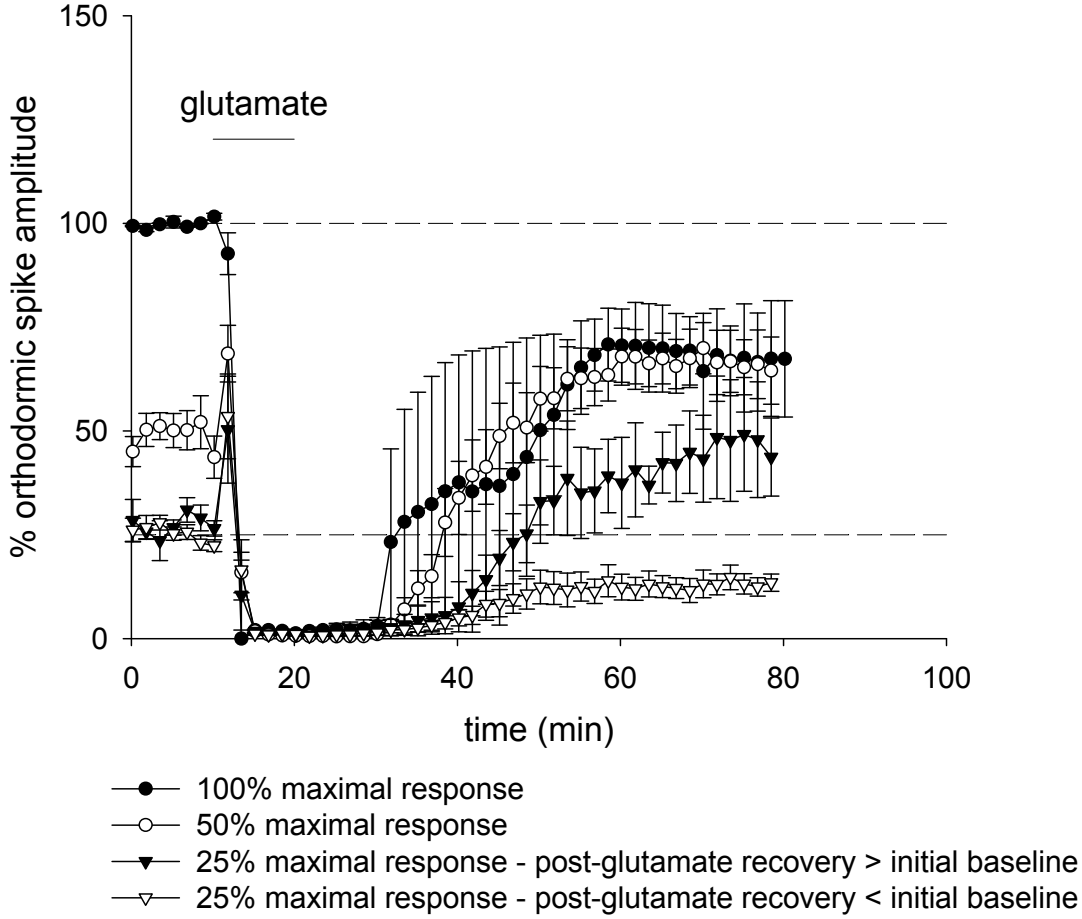


**Figure 3.20 The effects of different stimulus strengths on the response to glutamate in orthodromic population spikes**. The stimulus strength was adjusted to produce population spike amplitudes that were either approximately 50% or 25% of the maximum. At 50% of the maximal response, post-glutamate recovery was significantly greater than the spike amplitude 10min prior to 5mM glutamate perfusion (140.9 ± 16.3%, n = 8, $p<0.05$ one sample t test) unlike the effect of glutamate on the maximally evoked population spike (73.9 ± 7.4%, n = 4, $p<0.05$ one sample t test). At the 25% maximal baseline, post-glutamate recovery showed either an increase from the initial baseline or a depression. This could not be accounted for by differences in absolute maximal baseline (mV), the exact % baseline of the maximum or the absolute baseline when stimulated at 25% of the maximum.

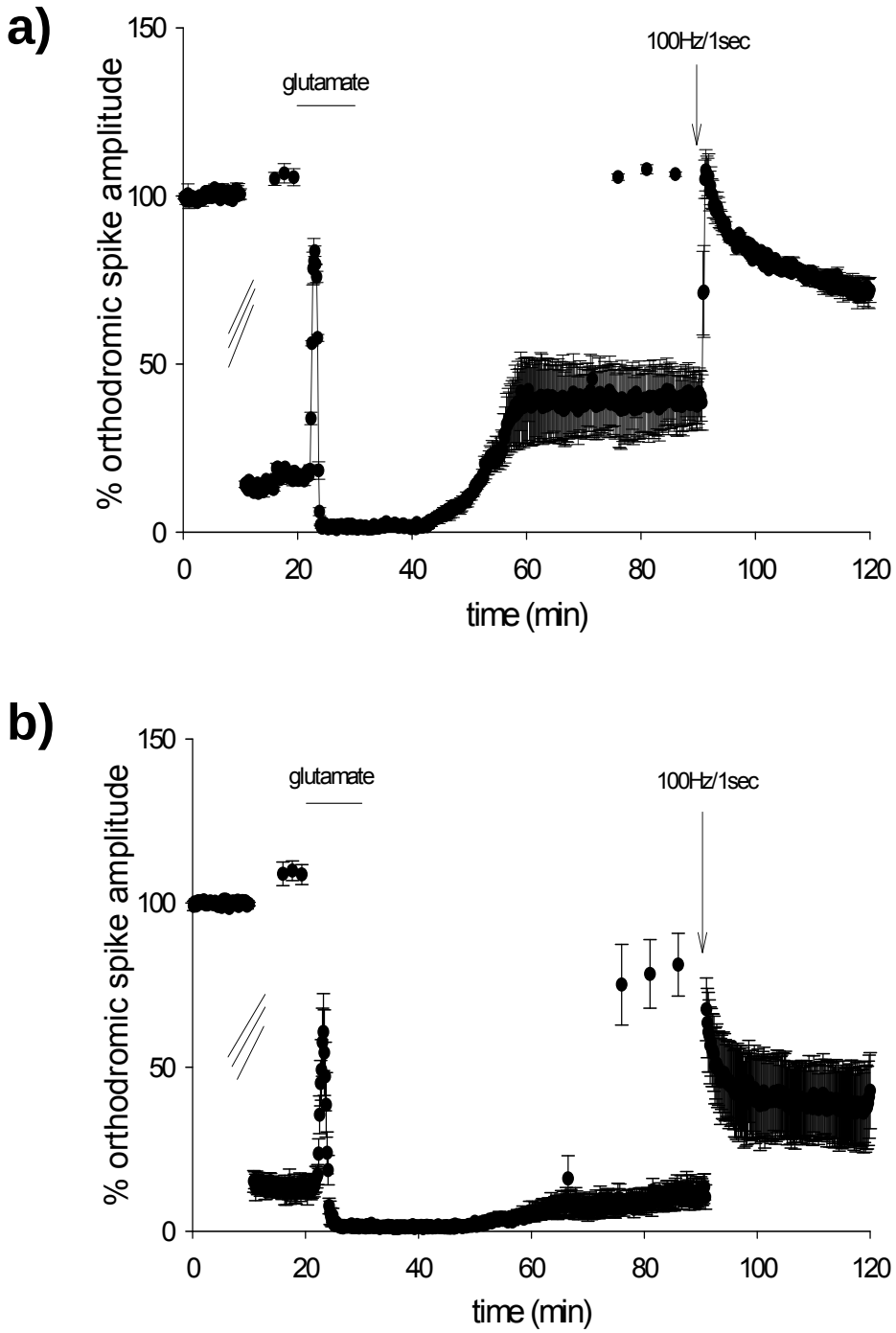


**Figure 3.21a,b (c, overleaf). The effect of glutamate on synaptic plasticity in orthodromic population spikes.** The time course graphs show the changes in orthodromic spike amplitude before during and after 5mM glutamate exposure and after receiving a 100Hz/1sec stimulus. The experiments were separated into two groups based on outcome following glutamate exposure: a) recovery above pre-glutamate baseline (n = 3); b) recovery below pre-glutamate baseline (n = 4)). The responses showing a post-glutamate recovery lower than the pre-glutamate baseline produced a significant LTP in response to 100Hz/1sec stimulation ($p<0.05$ one sample t test). Post-glutamate recoveries were not significantly elevated or depressed unlike the initial experiments above.

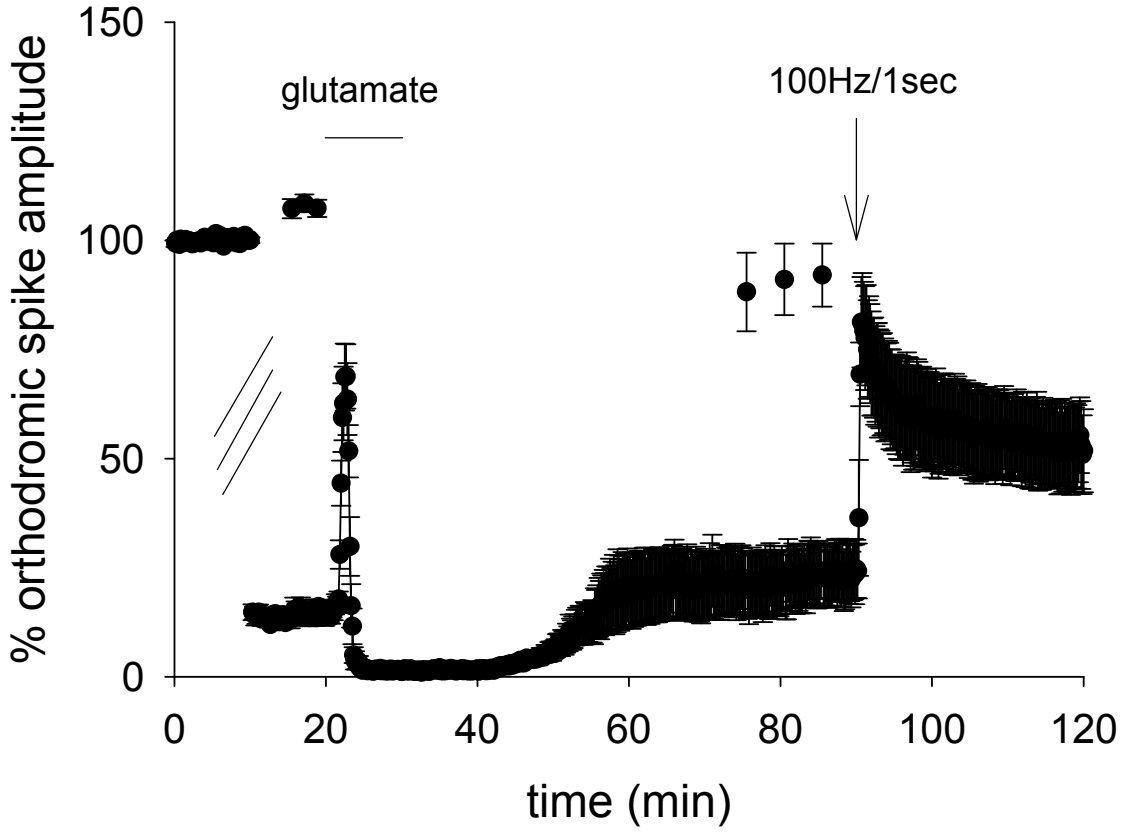


**Figure 3.21c Time course graph showing pooled data from the experiments above.** Overall a significant LTP is induced in response to 100Hz/1sec stimulation following glutamate exposure (239.2 ± 31.2%, n = 7, $p<0.005$ one sample t-test).

# 3.3 - Discussion

## 3.3.1–The effects of exogenous glutamate upon evoked responses from the CA1 neurons

### 3.3.1.1 – The use of exogenous glutamate as an excitotoxin in the hippocampus

Many studies have attempted to induce excitotoxicity in the hippocampal slice preparation with the use of exogenously applied glutamate (Wallis et al., 1994; Alici et al., 1996; Virgili et al., 1997; Schurr et al., 2001; Larsen et al., 2006) or other excitotoxins (NMDA (Bandyopadhyay et al., 2002); kainic acid (Andrew & MacVicar, 1994; Andrew et al., 1996), quinolinic acid (Stone, 1985), domoic acid (Polischuk & Andrew, 1996; Polischuk et al., 1998; Kerr et al., 1999)). In several cases, the changes that are produced in evoked field potentials from slice preparations have been used to quantify the degree of damage induced by application of such agents. The rationale for this can be derived from a prior study in vivo which showed that the extent of evoked field potential changes in the hippocampus induced by glutamate and metabolic inhibitors correlated with histologically assessed cell death four days later (Lees & Sandberg, 1991); thus evoked responses may serve as an indicator of tissue function in response to stressful stimuli. It was noted by Lees & Sandberg (1991), however, that the observed correlation disappeared when potential size was greatly decreased (70% depression or greater). At this level of depression, it is possible that changes in synaptic plasticity dictated the size of the potential rather than toxic effects. Response sizes below 70% may have indicated lower levels of transmission as opposed to reduced cell function; by reducing synaptic transmission excitotoxicity may have been reduced in the hippocampus to a certain extent. This observation was unexplained by Lees & Sandberg (1991) but it does indicate that limitations may exist by assessing tissue injury with the sole use of recorded field potentials. A more recent study has found a correlation between the degree of histologically assessed cell death induced by bilateral common carotid artery occlusion and the degree of depression in the input/output curve of field epsps in the CA1 area of the hippocampus measured at 2 days and 7 days post-ischaemia (Henrich-Noack et al., 2007). Both of these studies suggest that changes in measured field potentials are a reflection of cell damage that occurs in tissue in vivo. These in vivo studies back up many others conducted in vitro, including those examining preconditioning phenomena, which have made assumptions regarding the loss of evoked field potential in relation to toxicity rather than considering changes in transmitter release or synaptic plasticity.

The difficulty in determining tissue toxicity in slice preparations arises from the acute nature of in vitro electrophysiological protocols whereby sufficient time to induce morphological or molecular indicators of cell death is restricted. Some studies have attempted to assess the toxic effects of glutamate in acute hippocampal slices. An early study by Vornov & Coyle (1991) assessed the rate of protein synthesis by autoradiography in hippocampal slices. They noted that, whilst NMDA and glutamate both inhibited protein synthesis, the effects of NMDA were prevented by MK-801 and ketamine whereas the toxic effect of glutamate was not inhibited by glutamate receptor antagonists for any given concentration, exposure duration or by the presence of magnesium in the medium. These results were confirmed and extended in later studies which indicated that 10mM glutamate exposure in hippocampal slices transiently decreased protein synthesis to 50% and ATP levels to 30% (Djuricic et al., 1994a). Unlike a 15min OGD exposure however, the effects of glutamate were reversed within two hours. The same group also showed that the presence of glutamate, quinolinic acid or kynurenic acid did not affect the changes in protein synthesis at 24hours following a 15min exposure to ischaemic conditions in acute slices (Djuricic et al., 1994b). These studies suggest that high glutamate concentrations may have an initial role in decreasing protein synthesis and energy metabolism which is receptor-independent. As the in vivo study by Lees & Sandberg (1991) suggests that cell death is induced a few days following glutamate exposure, exogenous glutamate may be considered a useful tool to inflict acute and delayed damage upon hippocampal tissue.

As a starting point in the present study, exogenous glutamate was applied to hippocampal slices whilst evoking orthodromic population spikes. The results presented here with glutamate showed a complete depolarization of the evoked response that persisted for a while during the washout of glutamate; this was followed by a recovery of the response which showed inconsistencies between epsps, orthodromic population spikes and antidromic population spikes which are discussed below.

#### 3.3.1.2 -The effects of exogenous glutamate on orthodromic population spikes

The initial experiments using orthodromic population spikes showed a complete loss of the response in the presence of glutamate; this may have occurred in a few ways. An overdepolarization of the neurons may have occurred via a massive influx of positive ions through the glutamate ionotropic receptors (Choi, 1987). Depolarization of the membrane potential would effectively prevent the formation of action potentials and thus the orthodromic population spike. It may also be possible that exposure of high levels of

exogenous glutamate to the pre-synaptic neurons may have somehow decreased the release of excitatory transmitters. Alternatively, the exposure of the tissue to glutamate may have induced a release of inhibitory neurotransmitters which may have caused the orthodromic population spike to disappear. The recovery of the responses following glutamate removal could also have a few explanations. When the orthodromic population spikes started to reappear, they recovered to a stable plateau level which was significantly less than the spike amplitude measured prior to the application of glutamate. This reduced amplitude could be the result of a toxic effect, glutamate-mediated long-term depression or increased inhibitory neurotransmission.

As previous studies have shown that short glutamate exposure preconditions against a more prolonged glutamate exposure (Schurr et al., 2001), the use of 5mM glutamate in preconditioning paradigms was determined by repeated exposures on the orthodromic population spike. It was observed that the rate of recovery following later exposures were higher than those previously seen. This established that some degree of preconditioning effects were present with this glutamate concentration and was therefore used initially to probe the mechanisms mediating the effect of exogenous glutamate on the evoked responses.

#### 3.3.1.3 - The effect of exogenous glutamate on antidromic population spikes

The effects of glutamate were examined further in the hippocampal slice by observing the responses of antidromically evoked population spikes. It was observed that exposure to 5mM glutamate caused a reduction in the amplitude of antidromic population spikes but unlike orthodromic population spikes, the response did not completely disappear. This was followed by a return of the spikes to baseline values during washout. 10mM glutamate reduced the spike amplitude until it disappeared and the post-glutamate recovery was significantly lower than pre-glutamate spike size. This indicated that the depressed post-5mM glutamate recovery which occurs in orthodromic population spikes may reflect a change in synaptic transmission but glutamate at 5mM concentration only exerts a transient effect upon the formation of action potentials directly. 10mM glutamate appears to have longer lasting effects upon the cell body as the lack of an orthodromic current pulse did not prevent a long-lasting decrease in action potential formation as observed using antidromic spikes.

Other studies have examined the pathophysiological effects of applying high concentrations of exogenous glutamate. The findings of Larsen et al. (2006) indicated that in isolated CA1 neurons, a 15min exposure of 1mM glutamate in the presence of 10μM glycine and magnesium free aCSF induced an elevation in intracellular calcium that did not return to baseline levels upon washout. In addition, observation of the mitochondrial membrane potential showed an increase of Rhodamine 123 fluorescence (indicating mitochondrial membrane depolarization) in 25% of tested neurons. Whilst the lower glutamate concentration used may not be as potent as that used in the hippocampal slices, the presence of glycine and absence of magnesium would enhance glutamate stimulation of NMDA receptors. Several other studies have shown that mitochondrial depolarization is a consequence of excitotoxicity (Budd & Nicholls, 1996; White & Reynolds, 1997; Ward et al., 2005) although few aside from Larsen et al., have examined this in acute preparations. It is possible that some cellular dysfunction may occur using 5mM glutamate concentrations as Virgili et al. (1997) have reported reduced rates of protein synthesis in rat hippocampal slices although any specific toxic effect is unclear from that study. Any resulting damage induced by such molecular changes may not necessarily be reflected by changes in the extracellular recordings of antidromic spikes. Whilst 5mM glutamate does not exert a long-lasting electrophysiological change upon antidromic population spikes, the reduced recovery in orthodromic responses might have reflected a degree of damage induced at the synapse as opposed to the cell body. To investigate this further, the effects of exogenous glutamate upon the orthodromically evoked field epsps were examined.

#### 3.3.1.4 - The effects of exogenous glutamate upon field epsps

The effects of glutamate on field epsps showed inconsistencies with those observed in orthodromic spikes. Both 5 and 10mM concentrations of glutamate again induced a rapid reduction in the slope and size of the field epsps until the response disappeared. The post-10mM glutamate recoveries were consistently lower than the initial baseline levels. Recovery following 5mM glutamate however showed predominantly a return to baseline levels in most responses with a few small epsps showing a recovery that was markedly higher than the initial baseline levels. As the orthodromic population spikes recovered to a lower level than initial baseline values following 5mM glutamate, the discrepancy in effect between field epsps and orthodromic population spikes might be indicative of a glutamate-induced dissociation in the epsp-spike relationship. The small epsps which exhibited a recovery higher than the initial baseline may have been AMPA receptor-mediated, no NMDA receptor component existing on account of their size (Davies and Collingridge,

1989). To examine the effect of the NMDA receptors upon the post-glutamate recovery of the field epsps, the slices were again exposed to glutamate in the presence of MK-801. By dividing the experiments into groups depending on the effect of NMDA receptor blockade in combination with glutamate exposure, it could be seen that either a potentiated or depressed post-glutamate recovery was induced compared with glutamate alone, although only two slices out of the twelve tested exhibited a depressed recovery which limited a statistical analysis; the results were used for correlation analysis later on (this is discussed further in section 4.5.3). The different effects of NMDA receptor blockade might be accounted for by variations in the numbers and/or subtypes of NMDA receptors at the recording site in each slice. Both factors are varied throughout the hippocampus (Martens et al., 1998; Pandis et al., 2006) and within the slice preparation (Dahl et al., 1990). These results suggest that NMDA receptors determine the post-glutamate outcome in field epsps and mainly prevent the occurrence of a glutamate-induced NMDA receptor-independent form of LTP. The two responses which showed the opposite effect may reflect a role for NMDA receptors in preventing a glutamate-induced NMDA receptor-independent form of LTD. Future experiments into this phenomenon may include quantifying and subtyping receptors from the slice preparation and epsp-spike coupling experiments to further understand these occurring differences in response to glutamate.

### 3.3.2 - Interaction of adenosine antagonists with post-glutamate recovery: orthodromic population spikes

#### 3.3.2.1 - A1 receptor blockade and glutamate

Having observed that 5mM glutamate reduced the size of the orthodromic population spike until it disappeared and that once glutamate was removed, a partial recovery of the response followed, further experiments were conducted to examine the possibility of these effects resulting from the release of inhibitory neurotransmitters. Adenosine was examined as it is known to be released at high concentrations during ischaemia (Latini et al., 1999b) and is a known preconditioning agent (Liu et al., 1991; Pérez-Pinzón et al., 1996). It was found that blockade of adenosine A1 receptors attenuated the depressed post-glutamate recovery. This indicated that endogenous adenosine may be released in the slice during glutamate perfusion which appears consistent with reported observations that NMDA exposure to neuronal tissues increases adenosine levels (Hoehn & White, 1990; Manzoni et al., 1994). As adenosine binding to A1 receptors will decrease in the presence of DPCPX, it is possible that any glutamate-induced increase in extracellular adenosine will increase adenosine binding to the A2a receptor population which in turn may induce a greater

inhibition of A1 receptors (Cunha et al., 1994; O'Kane & Stone, 1999). A2a receptor agonists have been shown to induce protection in the hippocampus in rats against systemic administration of the excitoxin, kainate, (Jones et al., 1998) which seems consistent with the interaction observed between DPCPX and glutamate upon orthodromic spikes here. The possible role of A2a receptors was studied further by observing the effect of glutamate upon the orthodromic population spikes in the presence of an A2a receptor antagonist.

#### 3.3.2.2 - A2a receptor blockade and glutamate

A similar protocol to the experiments examining A1 receptor blockade in the presence of glutamate upon orthodromic spikes was adopted with the A2a receptor antagonist, SCH 58261, used in place of DPCPX before, during and after 5mM glutamate perfusion. As the mechanism underlying the enhanced post-glutamate recovery induced by DPCPX upon orthodromic population spikes was hypothesized to be partly due to an increase in A2a receptor stimulation by endogenous adenosine, it was surprising that a similar result was yielded when the experiment was repeated with A2a receptor blockade instead of A1. Also, SCH 58261 alone caused a significant depression of the orthodromic population spike: this may have been due to a suppression of any disinhibitory effect of A2a receptors upon A1 receptors (Cunha et al., 1994; O'Kane & Stone, 1999) resulting in increasing basal A1 receptor stimulation; it is also consistent with the enhancement of field epsps (Cunha et al., 1994) and population spikes (Sebastião & Ribeiro, 1992) in the hippocampus observed upon exposure to CGS21680, an A2a agonist.

Regarding the effects of A2a receptor antagonism upon the post-glutamate recovery in orthodromic population spikes, it is conceivable that the effects of glutamate upon the orthodromic population spike may represent some form of long-term depression; it is therefore possible that the A2a receptor population acts to mediate such an effect. This is inconsistent, however, with the studies examining the effects of A2a receptor interactions with synaptic plasticity in hippocampal slice preparations. A2a receptor antagonists have been shown to have no effect upon the induction of LTP in population spikes although they can prevent the occurrence of LTP in epsps (Sekino et al., 1991; Fujii et al., 1992; Forghani & Krnjević, 1995; Fujii et al., 1999). A2a receptor antagonists also seem to prevent depotentiation from occurring in population spikes (Fujii et al., 1992; Fujii et al., 1999) although the reasons for this are not understood. It is possible that the 5mM glutamate exposure in this system has a similar physiological mechanism to the

depotentiation phenomenon which could explain why the presence of an A2a receptor antagonist affected the post-glutamate recovery.

It is possible that during glutamate perfusion, sufficient adenosine is released to stimulate A2b or A3 receptors, particularly if higher affinity receptors are blocked by SCH58261 or DPCPX. According to Latini et al. (1999), extracellular adenosine concentrations during ischaemia can reach as high as 23μM in hippocampal slices compared with 240nM under basal conditions. Whilst the exact concentration of adenosine has not been determined during glutamate perfusion in this study, it is possible that adenosine may be increased sufficiently to stimulate A2b or A3 receptors, both of which require an extracellular adenosine concentration over 1μM for binding to occur (Fredholm et al., 1994). As the A3 receptor is known to desensitize the A1 receptor under ischaemic conditions (Dunwiddie et al., 1997), this might explain why A2a receptor blockade produces the same result as A1 receptor blockade.

It is also possible that the effect of SCH 58261 upon post-glutamate recovery is simply mediated by increasing A1 receptor stimulation through suppressing their disinhibition by A2a receptors. As the levels of adenosine were not measured during and after glutamate perfusion, it is possible that they do not change at all; if this were the case, the depression of the post-glutamate recovery is more likely related to glutamate receptor stimulation. This would also mean that A3 receptors are not stimulated as normal levels of adenosine are too low for binding to occur. As this was the simplest interpretation of the results, downstream mechanisms of A1 receptor stimulation were examined as potential mediators of the effects of A2a receptor antagonism upon glutamate-induced changes in orthodromic population spikes.

#### 3.3.3.3 - The role of ATP-sensitive potassium channels in the effect of A2a receptor blockade upon post-glutamate recovery

To further investigate why A2a receptor antagonists were able to prevent the post-glutamate induced depression in spike recovery, the possible role of ATP-sensitive potassium channels was considered as they are known to be activated by A1 receptor stimulation (Heurteaux et al., 1995; Reshef et al., 2000). Using glibenclamide to block the ATP-sensitive potassium channels, it was observed that the post-glutamate recovery of orthodromic population spikes remained significantly depressed below initial baseline levels, despite the presence of SCH 58261. This was consistent with studies showing that

stimulation of ATP-sensitive potassium channels protects against excitotoxicity (Nagy et al., 2004; Kis et al., 2004) although the mechanism for this is unclear. As ATP-sensitive potassium channels are most likely stimulated by A1 receptors, the previously discussed possibility that A3 receptors may act to desensitize A1 receptors during A2a receptor blockade throughout the perfusion of glutamate would now appear unlikely.

### 3.3.3.4 - Selectivity of ZM241385

The experiments initially conducted to examine the effect of A2a receptor blockade upon the glutamate-induced depression in spike recovery used the A2a antagonist, ZM 241385, in place of DPCPX. Using ZM 241385 enhanced the recovery of the orthodromic population spike following glutamate perfusion in a similar manner to DPCPX. This was consistent with the fact that ZM 241385 has been shown to reduce hippocampal cell death induced by kainate in vivo (Jones et al., 1998) suggesting that ZM 241385 can attenuate the effects of excitoxins.

The selectivity of ZM241385 for A2a receptors became a pressing issue, however, as another study had revealed that ZM 241385, at the same concentration used in this study, prevented the inhibitory effects of the A1 receptor agonists, $N^6$-cyclopentyladenosine (CPA) and $R$(-)-$N^6$-phenylisopropyladenosine (R-PIA) upon the population spikes evoked from rat hippocampal slices (Lopes et al., 1999). To investigate this further in the present study, the effect of ZM 241385 on the inhibition of orthodromic population spikes induced by adenosine was examined. It was shown that ZM 241385 attenuated the inhibition of the response induced by 10μM adenosine, which was consistent with a blockade of A1 receptors. This experiment was repeated using DPCPX in place of ZM 241385 to confirm the selectivity of DPCPX as an A1 receptor antagonist. While the results obtained using ZM 241385 were consistent with those using DPCPX and SCH58261, they do highlight problems that can arise regarding the known selectivity of available drugs and subsequent interpretation of data.

### 3.3.3.5 - Interactions between adenosine receptor subtypes

As later experiments using SCH58261 confirmed that blockade of A2a receptors has a similar effect to blockade of A1 upon the post-glutamate recovery of orthodromic population spikes, the possibility of an effect of A1 receptors upon A2a receptors in

mediating these effects seemed unlikely given that ZM 241385 produced a similar result with its non-selective blockade of A1 and A2a receptors. It remains possible that A2a receptors do mediate their effects upon post-glutamate recoveries of orthodromic population spikes by enhancing A1 receptor stimulation in view of the results that show a role for ATP-sensitive potassium channels in mediating their effects. It therefore appears that ZM 241385 may have mediated its effects primarily by A1 receptor inhibition without requiring an interaction with A2a receptors.

This leaves the possibility that endogenous adenosine acts at other receptor subtypes, A2b or A3. It has been recently shown that A3 receptors are indeed present in the hippocampus (Lopes et al., 2003) and may affect synaptic plasticity (Costenla et al., 2001). A2b receptors are present in the hippocampus although their functional characterization is still lacking due to a limited selection of pharmacological agents (Volpini et al., 2003). The role of A2b and A3 receptors cannot be excluded from the data presented here and their role in mediating these effects may warrant future investigation.

### 3.3.4 - The effects of NMDA upon orthodromic population spikes and interactions with A1 receptors

Having established that the effects of A2a receptor antagonists upon the post-glutamate recovery of orthodromic population spikes were most likely mediated by enhancing A1 receptor stimulation, the question still remained regarding the reason for the similar effect induced by A1 receptor antagonism. As the use of a non-selective antagonist of A1 and A2a receptors, ZM 241385, yielded a similar result also, it seemed unlikely that DPCPX mediated this effect through enhanced A2a receptor stimulation as initially thought. The underlying mechanism behind A1 receptor blockade was initially sought by replacing exogenous glutamate with exogenous NMDA. NMDA produced a similar effect to glutamate; initially a complete depolarization which continued for a while after NMDA was removed from the perfusion medium followed by a recovery of the response below its initial size. The presence of DPCPX in the perfusion medium did not affect this response suggesting that it was due to LTD or excitotoxicity, either without release of adenosine. This seems to contradict the findings of Manzoni et al. (1994) and Hoehn & White (1990). Both these studies suggest that NMDA receptor stimulation releases adenosine in cortical and hippocampal slices. Manzoni et al. showed that 10μM NMDA applied for 3min induced a depression which was successfully blocked with 8-cyclopentyltheophylline (8-CPT). Hoehn & White applied 500μM NMDA to cortical slices for 10min and measured

the adenosine content throughout application and washout. Whilst both studies provide clear evidence of adenosine release in response to NMDA, it is critical to note that the effects induced by NMDA in both studies were abolished within minutes of the washout period; there was therefore no post-NMDA recovery which differed from initial baseline values.

## 3.3.5 - Interactions between exogenous glutamate and DPCPX

### 3.3.5.1 - Effects on antidromic population spikes

As replacing glutamate with NMDA had failed to yield any information on the nature of the interaction between DPCPX and glutamate, the protocol previously used for orthodromic population spikes was repeated using antidromic population spikes. It was initially observed that 5mM glutamate lowered the antidromic spike amplitude but it did not disappear; it then continued to recover to initial baseline values after glutamate was removed. This suggested that the effects of 5mM glutamate upon orthodromic population spikes were not mediated solely by the actions of glutamate at the cell body but must be derived from effects at the synapse also. The presence of DPCPX had no impact upon the response of the antidromic population spike to 5mM glutamate therefore the effect of A1 receptor blockade was examined upon field epsps.

### 3.3.5.2 - Effect on composite epsps

The field epsps, as previously discussed in the section 4.1.4, showed a full recovery in both slope and amplitude following treatment with 5mM glutamate. When glutamate perfusion was repeated in the presence of DPCPX, there was no impact of A1 receptor blockade upon the post-glutamate recovery of the response. Given that there were differences in the post-glutamate recovery between orthodromic population spikes and field epsps, it was thought that 5mM glutamate may have induced dissociations between the epsps and the population spikes. This may explain why DPCPX has no effect upon the post-glutamate recovery of the epsps as adenosine receptors are known to affect epsp-spike coupling in hippocampal slice experiments (O'Kane & Stone, 1998). Also, studies examining the effect of adenosine receptors upon synaptic plasticity have reported differential effects between the epsp and the orthodromic population spike in E-S coupling experiments (Fujii et al., 1992: Fujii et al., 1999). These studies have reported that the induction of long-term

potentiation and depotentiation are increased and decreased respectively in epsps when A2 receptors are activated whereas the induction of these forms of plasticity in population spikes are unaffected by the A2 receptors. It should be noted that Fujii et al. (1999) also reported that the presence of the A1 receptor antagonist, 8-CPT, increased the size of both population spikes and epsps in response to a tetanising stimulus (3 bursts each consisting of 5 current pulses at 100Hz, delivered at 20min intervals) without affecting the E-S relationship. These observations seemed to disagree with the apparent lack of interaction between A1 receptor blockade and glutamate observed here with composite field epsps. It was hypothesized that the interaction between DPCPX and exogenous glutamate may be better observed using pharmacologically isolated NMDA receptor-mediated epsps given that there are reported interactions between the NMDA receptors and A1 receptors in the hippocampus (de Mendonça & Ribeiro, 1993; Schubert et al., 1993; Canhão et al., 1994; de Mendonça et al., 1995; Klishin et al., 1995; Nikbakht & Stone 2001). The effects of glutamate and A1 receptor blockade were therefore examined on NMDA receptor-mediated epsps.

#### 3.3.5.3 - Effects on isolated NMDA receptor-mediated epsps

The NMDA receptor-mediated component of the field epsp was isolated by inhibiting AMPA receptors and removing magnesium from the perfusion medium to allow the NMDA receptors to activate in response to glutamate alone without requiring membrane depolarization. The NMDA receptor-mediated epsp was smaller and slower in response compared to the composite epsps. Once the response was stable, the protocol for DPCPX and 5mM glutamate was applied in the same manner as before. The NMDA receptor-mediated epsps decreased in size until they disappeared in response to glutamate alone, as was previously observed for orthodromic population spikes and composite epsps. When glutamate was removed, the responses reappeared and recovered to a stable level that was usually below the initial baseline values for slope and amplitude. The smallest NMDA-receptor mediated epsp recorded, however, showed a recovery that was markedly higher than its initial size. This was similar to the small composite epsps that showed a potentiated recovery following glutamate treatment which strengthened the possibility that the occurrence of higher recoveries may be due to increasing NMDA receptor stimulation.

When DPCPX was applied in the presence of glutamate during the recordings of NMDA receptor mediated epsps, it was observed that the depressed post-glutamate recovery was partially attenuated. The effects of A1 receptor antagonism upon NMDA receptor currents

have been previously studied in the hippocampus and have shown that a consensus that A1 receptor antagonism results in enhancing the effects of NMDA receptors. In particular, it has been observed that DPCPX both facilitates the NMDA receptor-mediated component of epsps in the rat hippocampal slice (Canhão et al., 1994) and suppresses the high-frequency stimulation induced reduction in extracellular calcium, an effect which is dependent on NMDA receptor activation (Schubert et al., 1993). The A1 receptor antagonist, 8-CPT, has also been shown to increase the NMDA receptor component to epsp currents recorded in hippocampal neurons (Klishin et al., 1995). The modulation of NMDA receptors by A1 receptor stimulation is also supported by studies which have observed that the A1 receptor agonist, 2-chloroadenosine (CADO), suppresses the NMDA receptor-mediated epsps in slices (de Mendonça & Ribeiro, 1993) and NMDA-induced currents in isolated hippocampal neurons (de Mendonça et al., 1995). From this evidence it is possible that the increase in post-glutamate recovery observed in orthodromic population spikes and NMDA receptor-mediated epsps when DPCPX is present may be due to a direct effect of A1 receptors upon NMDA receptors. Without glutamate in the perfusion medium, it is possible that the evoked responses would simply increase to higher levels than the post-glutamate recovery observed in the presence of the antagonist. Alternatively, exogenous glutamate may induce adenosine release similarly to NMDA (Hoehn & White, 1990; Manzoni et al., 1994) (see Figure 3.22), the effects of which may be alleviated by DPCPX although the lack of post-glutamate-induced depression in composite epsps suggests that adenosine is not released in response to glutamate. In addition, the results suggest that the NMDA receptor-mediated epsps may modulate the orthodromic population spikes without producing slope or amplitude changes in the composite epsps. The importance of the NMDA receptors involved in epsp formation in the generation of population spikes may be a very interesting avenue of further research.

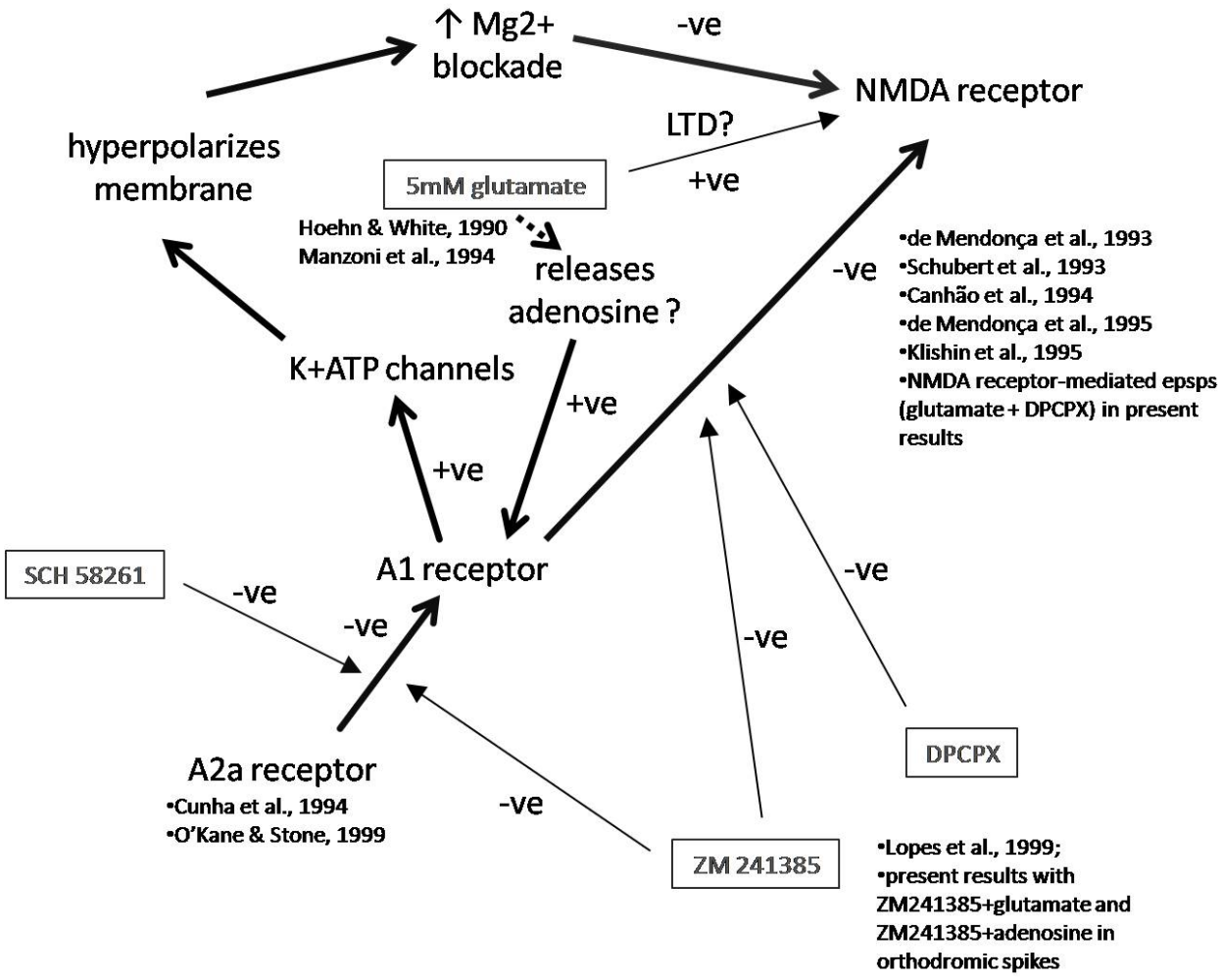


**Figure 3.22 Diagram showing possible interactions between adenosine receptor antagonists and glutamate**. Bulleted points indicate references or results from the present study which support particular interactions.

### 3.3.6 - Relationships between response sizes and their percentage post-glutamate/NMDA recovery

#### 3.3.6.1 - Glutamate and population spikes

Throughout the experiments performed using glutamate or NMDA, correlations emerged regarding the initial size of a given response and the % recovery of the response following glutamate or NMDA perfusion. This was first noted when examining the effects of exogenous glutamate upon orthodromic population spikes where a positive correlation was discovered between the spike size and the degree of recovery of the response. This was initially thought to be due to a probable relationship between the health of the tissue used and the size of the subsequently evoked response. As healthy tissue would have a greater number of functioning mitochondria, a greater capacity to withstand insults or stressful stimuli may exist. Similar correlations between resting membrane potential and total voltage change have been noted for other toxic compounds including cyanide (Englund et al., 2001), a cytochrome oxidase inhibitor that disrupts the electron transport chain within mitochondria. This observation with cyanide was slightly different in that the size of the voltage change depended on how far the initial resting potential was from a fixed potential. The authors observed that cyanide seemed to always return the membrane potential to a fixed point therefore the size of the voltage change would depend on the initial resting potential. It could be interesting to examine the effects of glutamate more thoroughly with intracellular experiments to see if a similar mechanism is responsible for the relationship between initial spike amplitude and percentage recovery following glutamate. However, no correlation was found for antidromic spike size and subsequent % recovery in response to glutamate. If this phenomenon were related to membrane potential, then a similar observation would be expected from antidromic spikes as that found in orthodromic spikes.

#### 3.3.6.2 - NMDA and orthodromic population spikes

It was also noted that a correlation existed between orthodromic population spike amplitude and % post-NMDA recovery. This was unlike glutamate however, in that small spike amplitudes produced larger post-NMDA recoveries whilst larger potentials did not recover well. This might have been due to fewer active NMDA receptors in the smaller responses making NMDA less potent. In light of these data, it became unlikely that the correlation between post-glutamate recovery and spike size was due to fewer functioning mitochondria as initially suggested but instead, it was somehow dependent on NMDA receptor modulation.

### 3.3.6.3 - Glutamate and composite field epsps

As antidromic population spikes showed no correlation between spike amplitude and post-glutamate recovery, this suggested that the relationship observed between orthodromic spike amplitude and post-glutamate % recovery might result from the effects of glutamate upon the epsps. Field epsps showed a negative trend regarding post-glutamate %recovery and initial slope gradient and response amplitude although no correlation could be found for this effect. Nonetheless, it contrasted with the small recovery for small population spikes observed earlier. It was however, consistent with the negative relationship that appeared between orthodromic spike amplitude and post-NMDA % recovery of the response. It seemed possible therefore, that glutamate may act on NMDA receptors to cause dissociations between the epsp and the orthodromic population spike. The effect of the NMDA receptors upon the epsp response to 5mM glutamate was highlighted when co-application of the NMDA receptor blocker, MK-801, induced elevations in the recovery of the majority of epsp responses. It was observed that during the perfusion of MK-801 alone prior to co-perfusion with glutamate, the epsp slope and amplitude became elevated or depressed. The resulting post-glutamate recovery of the response would be correspondingly elevated or depressed compared to the initial baseline value. These data suggested that the degree to which NMDA receptors contributed to the composite epsp formation strongly influenced the size of the recovery of the response during glutamate washout. The overall suppressant effect that NMDA receptors appear to exert upon the recovery of the epsp following 5mM glutamate application is consistent with the idea that the small epsps, which had shown a potentiated recovery to glutamate alone, had little if any NMDA receptor component to them. As the smaller NMDA receptor component of the epsp waveform is much slower to emerge than the AMPA/kainate receptor component of the field epsp (Davies & Collingridge, 1989), the influence of the NMDA receptors upon the recovery of responses during washout from 5mM glutamate application was studied further using pharmacologically isolated NMDA receptor-mediated epsps.

### 3.3.6.4 - Glutamate and NMDA receptor-mediated epsps

Consistent effects were observed when isolated NMDA receptor-mediated epsps, similarly to composite epsps, showed a negative trend between epsp size and post-glutamate % recovery. NMDA receptors are associated with various forms of synaptic plasticity such as LTP (Bliss & Collingridge, 1993) and LTD (Mulkey & Malenka, 1992) and the induction

of LTP and LTD has been observed in intracellular recordings from CA1 neurons with pharmacologically isolated NMDA receptor-mediated synaptic transmission (Xie et al., 1992). As post-glutamate recovery was to some extent dependent on the size of the NMDA receptor mediated epsp, it is likely the correlation between composite epsp size and post-glutamate recovery depends on the degree of NMDA receptor involvement in the evoked response. This is entirely consistent with the effects observed between MK-801 and glutamate upon epsps. It would appear that NMDA receptor stimulation may also induce dissociations in the epsp-spike relationship given that small composite field epsps tended to show a high post-glutamate recovery whilst smaller population spikes had a small recovery. Epsp-spike relationships are known to be affected by NMDA receptors (Daoudal et al., 2002). The apparent epsp-spike dissociation in the present study requires verification with E-S coupling experiments as differences in stimulation strength between separate groups of experiments alone may account for the discrepancy in effect between orthodromic population spikes and epsps. The present experiments were all conducted using the minimum possible stimulation to evoke a maximal response. Epsps and orthodromic population spikes are known to have a non-linear relationship (Andersen et al., 1980; O'Kane & Stone, 1998) therefore a maximally evoked epsp requires a higher degree of stimulation than a maximally evoked population spike.

It is interesting to note that the effects of NMDA on the orthodromic population spikes produced a similar trend in recovery to the NMDA receptor-mediated epsps. It has been shown that a form of inhibitory interaction exists between AMPA receptors and NMDA receptors at CA1 dendrites (Bazhenov & Kleshchevnikov, 1999) whereby antagonism of one receptor-mediated component of the epsp will induce a potentiation of the other. NMDA-induced LTD has also been shown to dephosphorylate GluR1 subunits of AMPA receptors (Lee et al., 1998). These studies suggest that NMDA receptor stimulation depresses the AMPA receptor component of the epsp which is consistent with the effects of MK-801 in potentiating the post-glutamate recovery in epsps. This may relate to the formation of the population spike in one of two ways:-

1. Stimulation of AMPA receptors by glutamate may inhibit NMDA receptor modulation producing a depression in the orthodromic spike

2. Stimulation of NMDA receptors by glutamate inhibits AMPA receptors therefore producing a depression in the orthodromic spike

Given that small population spikes show a low post-glutamate recovery and a high post-NMDA recovery it seems likely that it is the first possibility that is the cause of orthodromic population spike depression of post-glutamate recovery. As the NMDA receptor component increases, AMPA receptors become inhibited therefore the larger population spikes show a higher post-glutamate recovery. The opposite appears to be true for epsps. Small epsps may have no NMDA-receptor component; glutamate stimulation may therefore induce a change in the AMPA/NMDA receptor ratio meaning that the potentiated recovery is NMDA receptor mediated. This is consistent with the behaviour of small NMDA-receptor mediated epsps. NMDA receptor stimulation is required for the induction of LTP (Bliss & Collingridge, 1993). Changes in ionotropic receptor ratios have been previously shown in response to LTP (Isaac et al., 1995; Liao et al., 1995) and LTD (Beattie et al., 2000). As epsps increase in size, the NMDA receptor component may increase until the receptor ratio changes where depression is favoured instead of potentiation. This may also reflect changes in the subunit composition of the NMDA receptors which mediate larger synaptic responses as NR2A subunit containing receptors are associated with LTP induction whereas LTD is associated with the NR2B subunit (Liu et al., 2004). The NMDA receptor is also known to increase the linearity of spatial summation of epsps (Cash & Yuste, 1999). A point to note is that the trends observed with all epsps were in relation to amplitude and not slope. Earlier studies have indicated that NMDA receptor blockade induced a decrease in amplitude but not the negative slope of epsps in the hippocampal slice (Dahl et al., 1990) which indicated that epsp amplitude changes alone are likely to reflect NMDA receptor mediated changes.

### 3.3.7 - Examining the toxicity of glutamate

Experiments were performed to assess the nature of the effects of 5mM glutamate upon the evoked responses from the hippocampal slice. The results of these experiments suggested that the use of 5mM glutamate was non-toxic. The observations that support this conclusion are listed below (1, 2, 3 and 4) and are discussed further in this section.

1. Whilst orthodromic population spikes showed a post-glutamate recovery below initial baseline values, epsps and antidromic spikes showed a complete recovery following glutamate perfusion

2. Lowering the stimulus intensity to obtain 50% maximal orthodromic spikes produced elevations in post-glutamate recovery above the initial 50% baseline

3. Using 25% maximal orthodromic spikes, either elevated or depressed post-glutamate recoveries were observed against the initial 25% baseline

4. Using 25% maximal orthodromic spikes, a tetanic stimulus applied during the post-glutamate recovery produced significant potentiations of the orthodromic population spikes

The first results indicated that 5mM glutamate did not induce membrane dysfunction as antidromic population spikes recorded from the CA1 soma recovered fully. There was no suggestion of damage at the synapse either as epsps showed no significant post-glutamate depression. The effect in maximal orthodromic spikes was therefore interpreted as some form of post-synaptic change in epsp-spike coupling. As post-glutamate depression changed to potentiation using lower stimulation intensity, 5mM glutamate was considered non-toxic. Reversing the depressant effects of glutamate using a 100Hz/1sec stimulus in orthodromic spikes strongly suggests that 5mM glutamate induced changes in synaptic plasticity which was consistent with the elevated post-glutamate recoveries observed in small epsps. The exact nature of the influence of 5mM glutamate on the responses is still unclear as it is impossible to accurately understand the effect on the epsp-spike relationship without E-S coupling experiments which detect the changes in epsps and orthodromic population spikes at the same time in a single slice. Also, the elevated post-glutamate recoveries using small epsps cannot be clearly interpreted as pre-synaptic or post-synaptic effects without paired pulse experiments or measurements of quantal transmission to examine the behaviour of pre-synaptic neurons. It is also important to note that lowering stimulus intensity may be sufficient to change the metabolic demand of the neurons thus better enabling them to withstand stress. It is known that lowering stimulus intensity produces larger potentiations in population spikes in response to theta-frequency patterned primed-bursts (4 trains of 100Hz stimulation applied for 0.1msec and repeated at 200msec intervals) (Leung & Au, 1994). It has also been observed that hippocampal slices show changes in mitochondrial membrane potential which were consistent with theta frequency stimulation in the tissue and which also correlated with the intensity of electrical activity and spatial changes of intracellular calcium induced by the stimulation (Bindokas et al., 1998).

Long-term depression of inhibition (LTD-i) may also explain why lower stimulus intensities produce an elevated post-glutamate recovery. LTD-i is a phenomenon whereby GABA transmission is reduced. The mechanism involves stimulation of post-synaptic group1 mGluRs which generate DAG via phospholipase C (PLC). DAG is then converted to the endocannabinoid, 2-arachidonylglycerol (2-AG) via DAG-lipase. 2-AG is then

thought to stimulate cannabinoid CB1 receptors on GABAergic neurons via retrograde transmission from the post-synaptic neurons which in turn appears to inhibit GABA release (for review, see Freund & Hájos, 2003). The time-course of LTD-i is consistent with the changes observed here (approximately 40min). It is possible that the lower stimulus intensity prevents saturation of the response allowing this effect to emerge. From the mechanism outlined above, this could be shown using a mGluR Group 1 antagonist or a CB1 antagonist and examining the effect on the post-glutamate recovery.

### 3.3.8 – General Discussion

The results presented above have examined the effects of glutamate upon the evoked potentials from the CA1 pyramidal cells and the modulation of these effects by adenosine receptor antagonists. It was observed that 5mM glutamate applied to hippocampal slices induced a complete loss of response size. During the post-glutamate washout, the responses returned to below baseline values in orthodromic populaton spikes and isolated NMDA receptor-mediated epsps. The response recovery using composite epsps and antidromic population spikes was not signficantly different from pre-glutamate baseline values. The A1 receptor antagonist, DPCPX, blocked the effect of glutamate upon the response recovery using orthodromic population spikes and NMDA-receptor mediated epsps whilst no interaction was observed for antidromic population spikes or composite field epsps. From these observations, it would appear that 5mM glutamate applied to hippocampal slices can induce dissociations between the field epsp and the orthodromic population spike. The change in the E-S relationship induced by glutamate can also be modulated by blockade of A1 receptor antagonists, an effect which seems to depend upon interactions with NMDA receptors. Further experiments using E-S coupling are required to verify these results.

It was also observed that blockade of A2a receptors by SCH58261 could enhance the resposne recovery of orthodromic population spikes following application of glutamate. Further experiments revealed that this was due to ATP-sensitive potassium channel stimulation which most likely occurred as a result of removing the inhibitory effect of A2a receptors upon A1 receptors. This may have implications for the use of A2a receptor antagonists as a preconditioning mimetic as ATP-sensitive potassium channels are a central mediator of this phenomenon.

Experiments were conducted to examine the nature of the effects of glutamate upon the orthodromic population spikes. It was revealed that the reduction of response size

following glutamate application could be reversed by a tetanic stimulation. This indicated that the effect of 5mM glutamate upon the slice was induced by a synaptic change and unlikely to be due to a toxic effect. As a consequence of these results, 10mM glutamate was used as toxic insult in further preconditioning experiments.

# 4 – THE EFFECTS OF ADENOSINE PRECONDITIONING AGAINST EXOGENOUS GLUTAMATE AND OUABAIN

## 4.1 – Introduction

Having established that using 5mM glutamate was probably not a concentration sufficient to induce an excitotoxic effect in hippocampal slices, the effects of using a 10mM glutamate pulse were examined in the slice. This could enable the study of any potential protection induced by adenosine preconditioning against excitotoxicity. It was also of interest to examine the effects of a different chemical model of ischaemia, namely the sodium-potassium ATPase inhibitor, ouabain. This allowed for a comparison of the effects of adenosine and other preconditioning stimuli against different mechanisms by which tissue damage may be induced. The results presented below outline the observed effects of glutamate, ouabain and adenosine preconditioning upon the evoked responses in the hippocampal slice.

## 4.2 – Results

### 4.2.1 - The effects of 10mM glutamate upon hippocampal slices

Preliminary experiments established that perfusing slices with 10mM glutamate for 10min yielded the best and most reproducible depressions of electrophysiological activity. When recording orthodromic population spikes, glutamate at 10mM initially induced multiple population spikes, but this period of increased excitability was superceded by a decline of potential size as the pyramidal cells became over-depolarised. Following the removal of glutamate from the superfusion medium evoked potentials gradually returned to a plateau level within 30 to 60 minutes of washout. There was a significant depression in the size of all evoked responses (Fig 4.1a & b).

To investigate if 10mM glutamate induced a form of long-term depression upon the orthodromic responses, the stimulus intensity was reduced to evoke the half-maximal response. Once a stable baseline was established, 10mM glutamate was applied to the slice for 10min. The orthodromic population spike was abolished during glutamate application and throughout the entire washout period. At one hour post-glutamate, a tetanising

stimulus was applied to the slice (100Hz/1sec). There was no reappearance of the spike in response to the stimulus unlike the control LTP responses which were obtained from slices taken from the same hippocampi (Fig 4.2). This indicated that 10mM glutamate exerted an effect other than long-term depression upon the slices.

Western blotting was carried out on slices treated with 10mM glutamate for 10min to detect possible changes in heat shock protein expression (Fig 4.3 & 4.4). There were no significant changes detected between glutamate-treated slices and untreated slices (Fig 4.5).

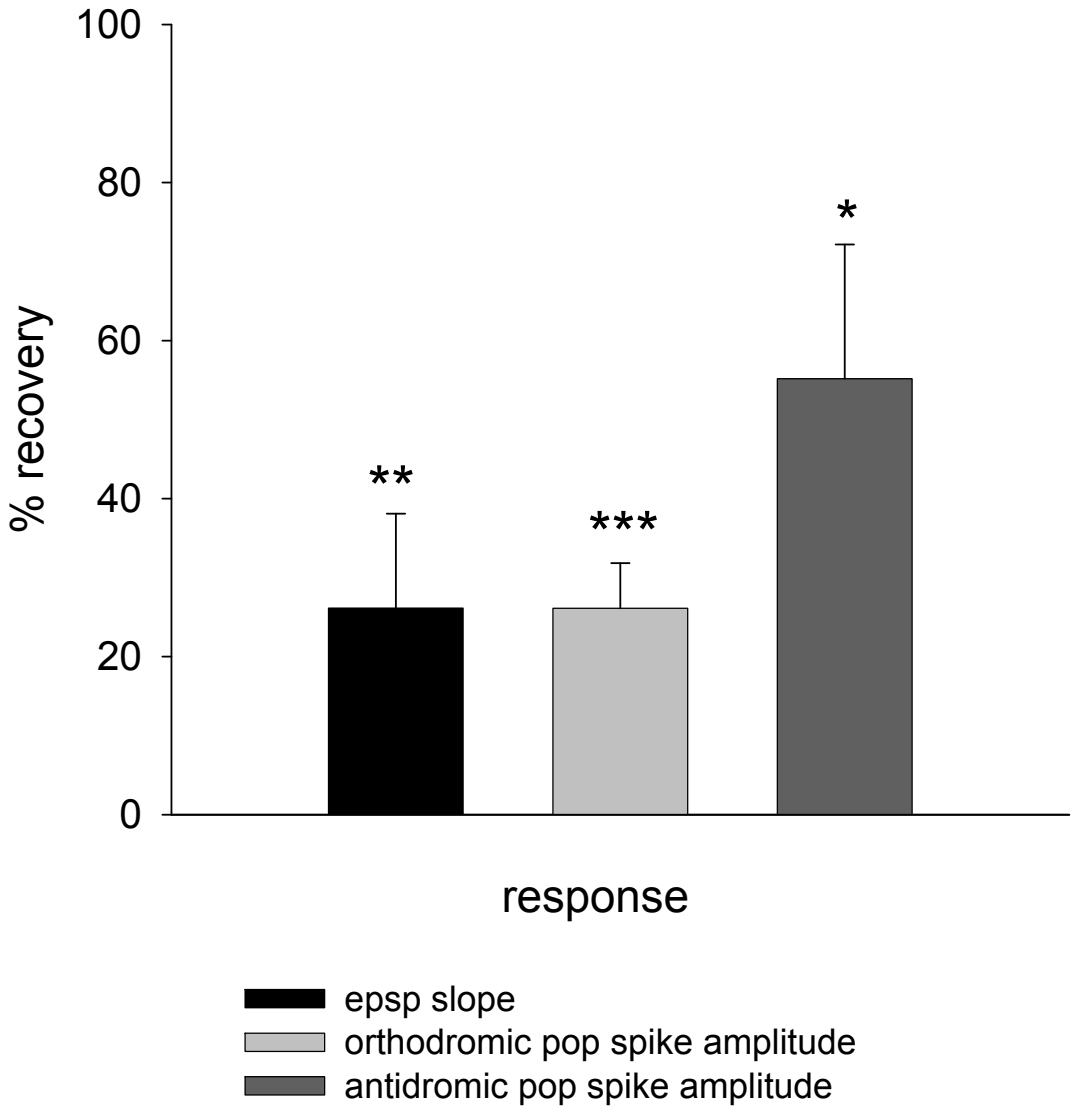


**Figure 4.1a The effects of glutamate on evoked responses from CA1 neurons**

**a)** Epsp slopes (n = 11), orthodromic spikes (n = 18) and antidromic pop spikes (n = 7) all show a significant depression in response size following 10mM glutamate after 50 to 60min (* $p<0.05$ ** $p=0.0001$ ***$p<0.0001$ one sample t- test).

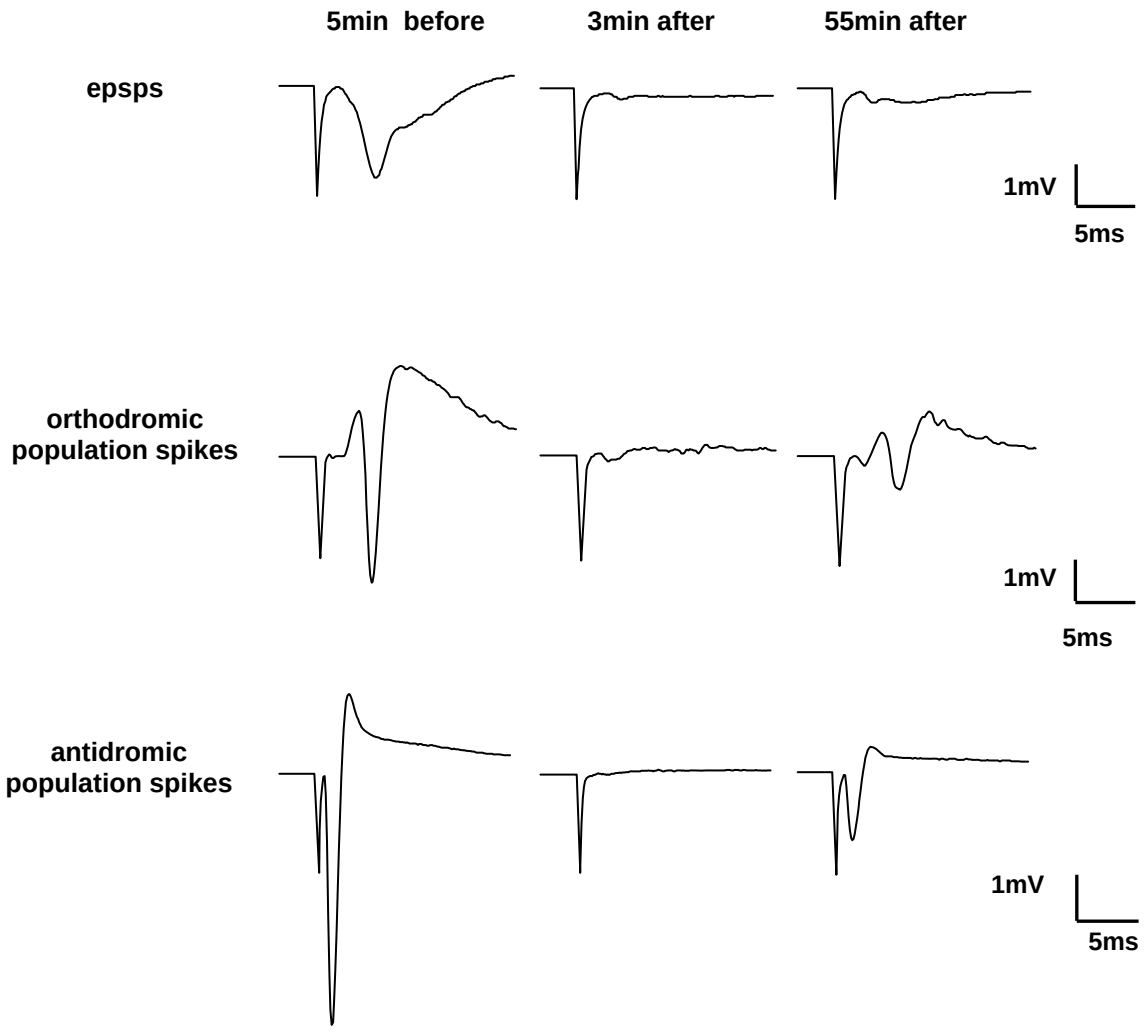


**Figure 4.1b Sample traces showing the effects of 10mM glutamate at various time points on the evoked responses from the rat hippocampal slices.** Time points are in relation to the application of 10mM glutamate.

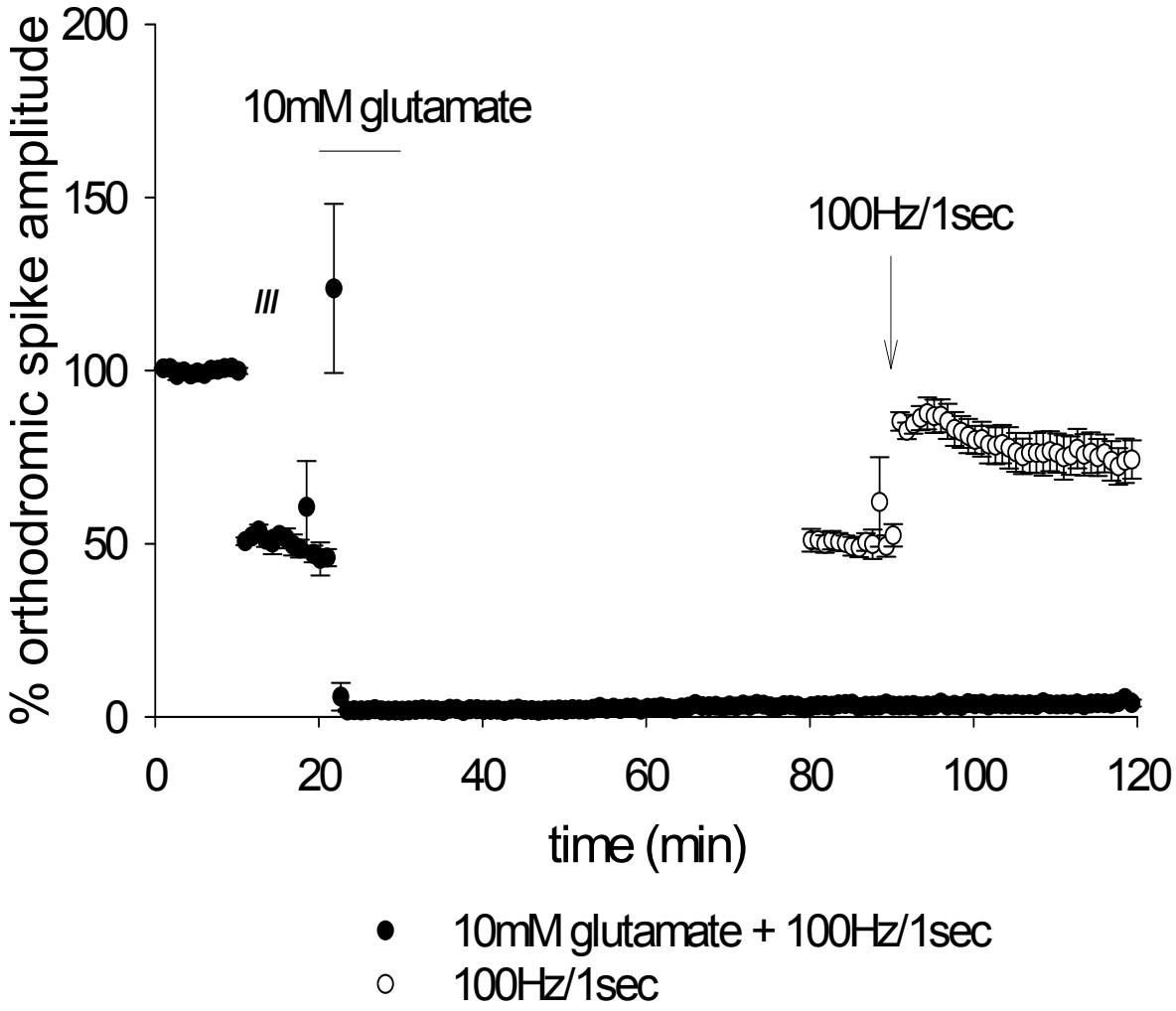


**Figure 4.2 The effect of 10mM glutamate exposure on synaptic plasticity in orthodromic population spikes.** The time course graph above shows the changes in orthodromic population spike amplitude in response to different stimulation intensities and 10mM glutamate perfusion. The responses perfused with 10mM glutamate for 10min show no perceptible recovery after one hour (n = 5). A 100Hz/1sec stimulus at the one hour time-point post-glutamate does not reverse the depression. Control responses show a significant LTP in response to 100Hz/1sec stimulation (140.6 ± 9.4%, n = 5, $p<0.05$ one sample t-test.

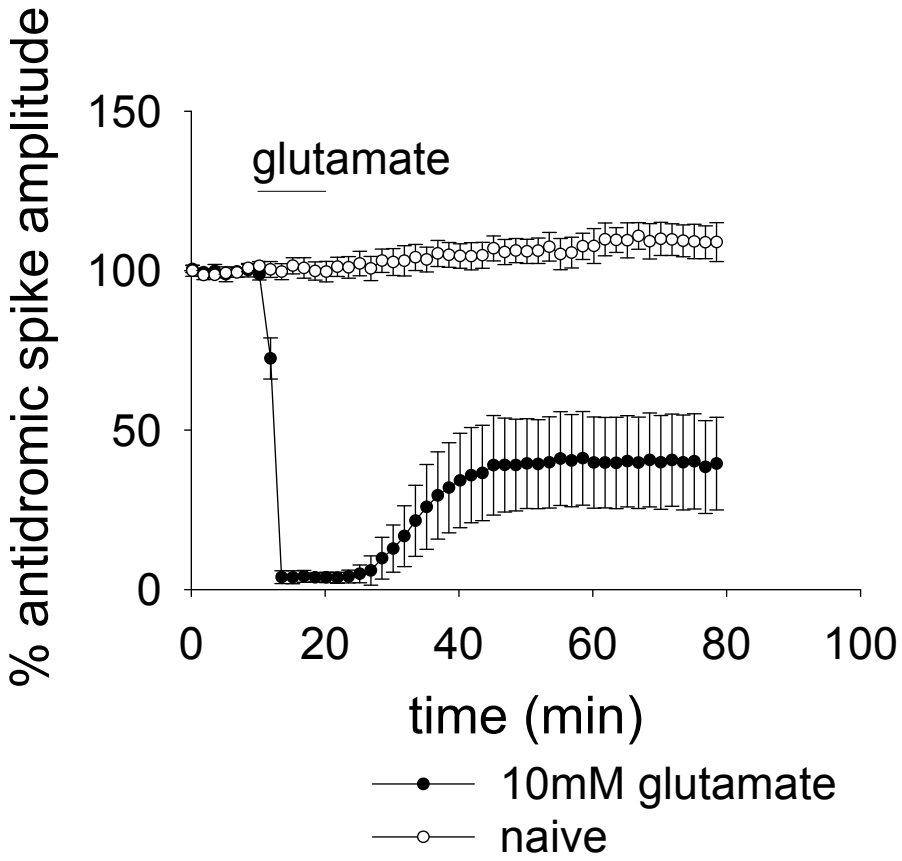


**Figure 4.3 Electrophysiological data from the slices used to generate the Western blots for the detection of HSP72 in Figure 4.4.** A graph of the time course of changes in antidromic population spike amplitude when either treated with 10mM glutamate for 10min or stimulated in the recording chamber for the same length of time is shown.

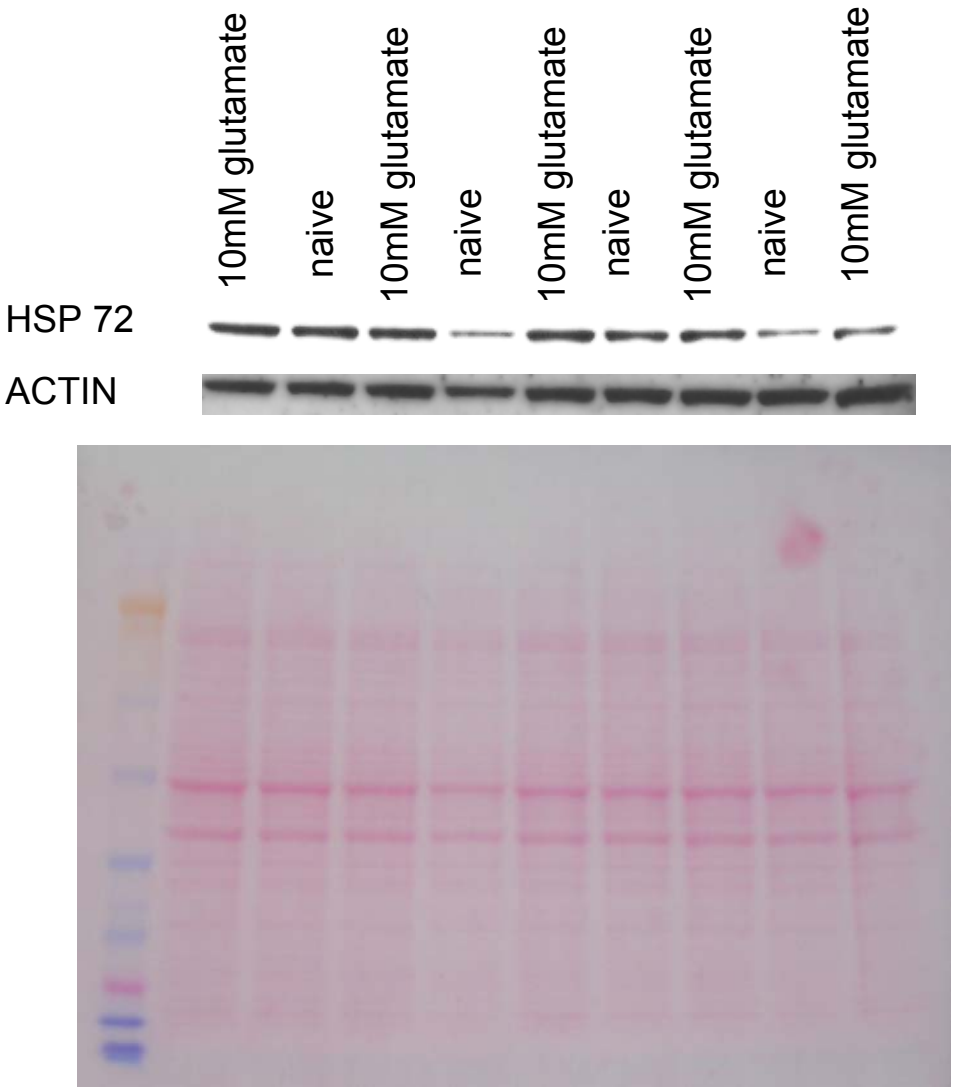


**Figure 4.4 Western blots showing the immumodetection of HSP72 and actin levels in slices receiving glutamate treatments.** The blots for the naive tissue show the detection of HSP72 and actin levels in slices stimulated for the same length of time as the chemically treated slices as shown in Figure 4.3. The corresponding Ponceau stains are shown (in colour).

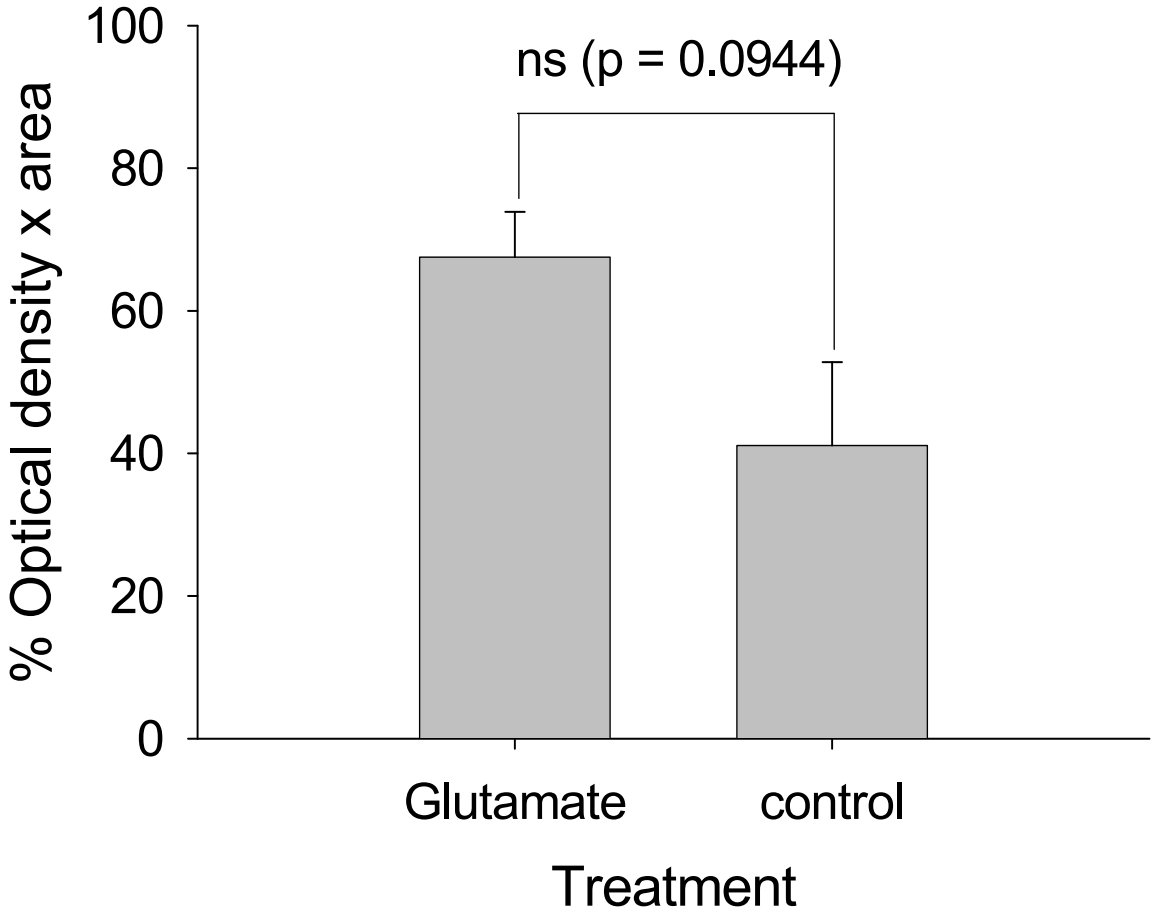


**Figure 4.5 Histogram summarising the changes in HSP72 expression in glutamate treated slices from the Western blot in Figure 4.4.** There are no significant changes in HSP72 expression between glutamate treated groups and naive slices (ns = not significant).

### 4.2.2 - Adenosine does not precondition against glutamate

To investigate preconditioning, a protocol was used similar to that devised by previous groups, in which several mild exposures are given to the preconditioning agent, followed by the application of a stimulus which, when given alone, would induce some degree of irreversible tissue function or damage. To examine the effects of adenosine on glutamate toxicity, the purine was superfused at concentrations of 10μM, 50μM or 100μM for 3 min every 15 mins. Three such applications were made after which a further period of 15min was allowed before a pulse of 10mM glutamate was applied for 10min. A comparison of control responses to glutamate applied alone, with responses obtained after adenosine preconditioning (n = 3 for each concentration) revealed no statistically significant differences between them, either in the amplitude of depression, the extent of recovery measured at the plateau phase, or the rate of recovery measured as the time taken from the end of maximal depression to the attainment of half the plateau recovery (Fig 4.6). A 10min application of 100μM adenosine, shown by Pérez-Pinzón et al., (1996) to protect against anoxia in slices, was employed to examine any protective effect against glutamate. This also failed to protect against 10mM glutamate when treated on orthodromic responses (preconditioned group n = 6; control group n = 9) (Fig 4.7) or antidromic responses (preconditioned group n = 5; control group n = 5) (Fig 4.8).

Using the former protocol it was also found that adenosine did not induce any difference in the depression of potentials produced by 25μM NMDA.

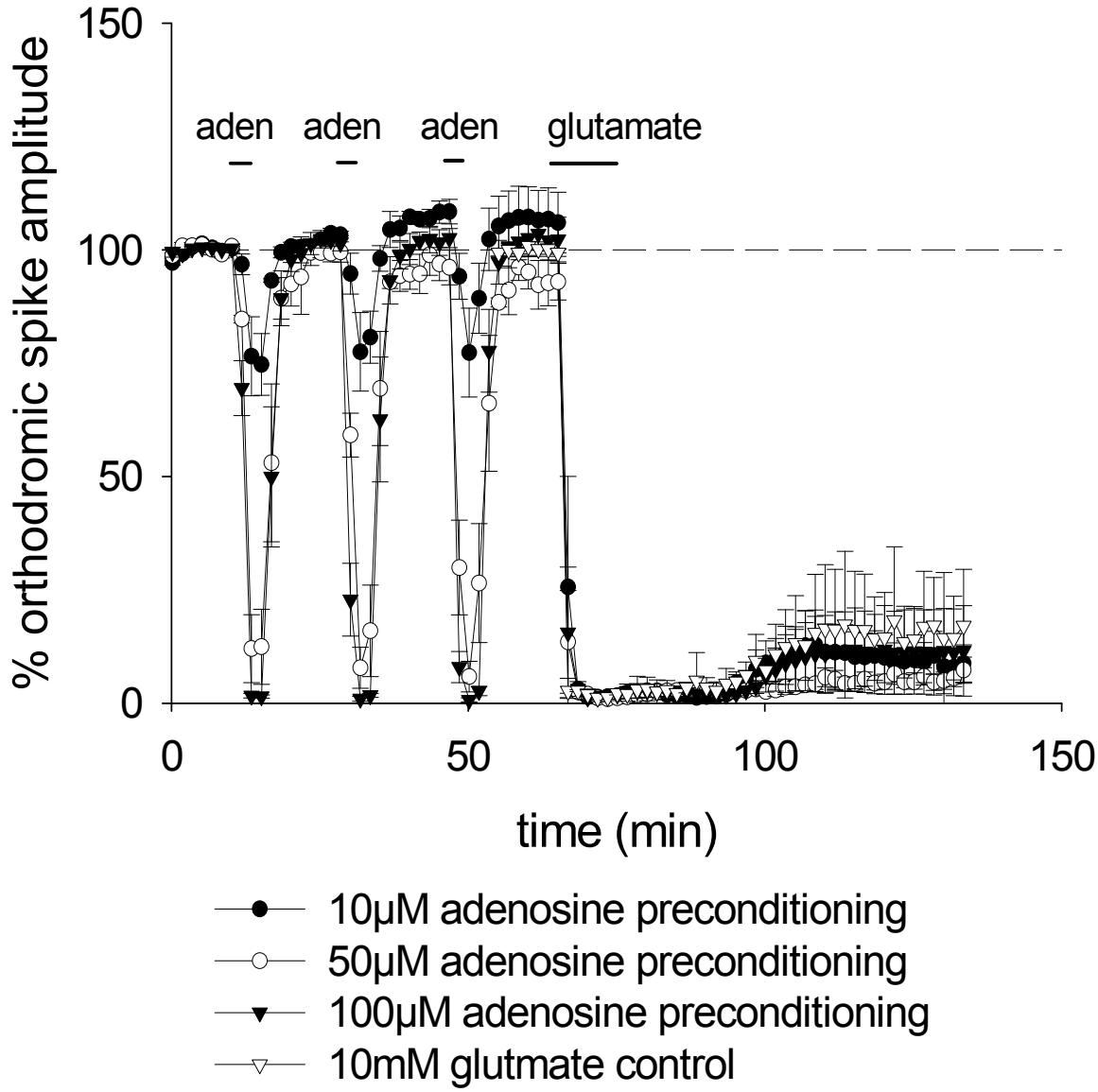


**Figure 4.6 Adenosine does not precondition against glutamate.** Various concentrations of brief adenosine applications failed to precondition against 10mM glutamate (aden = adenosine).

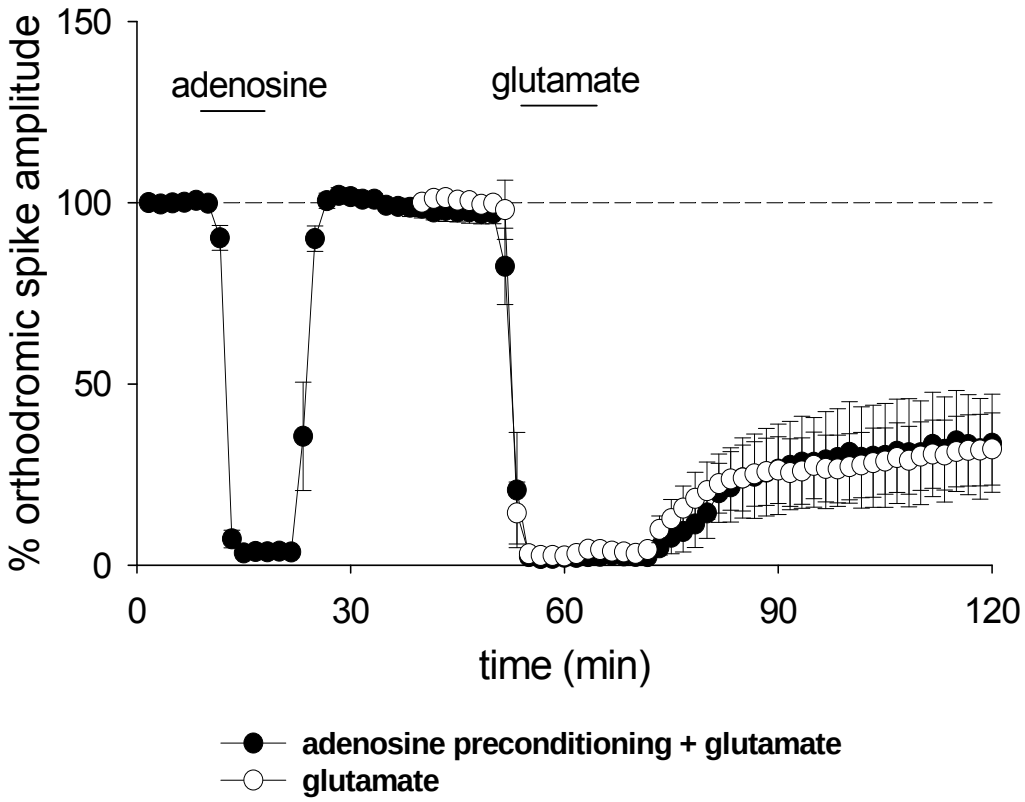


**Figure 4.7 A sustained application of 100μM adenosine failed to precondition against the effects of 10mM glutamate upon orthodromic responses.**

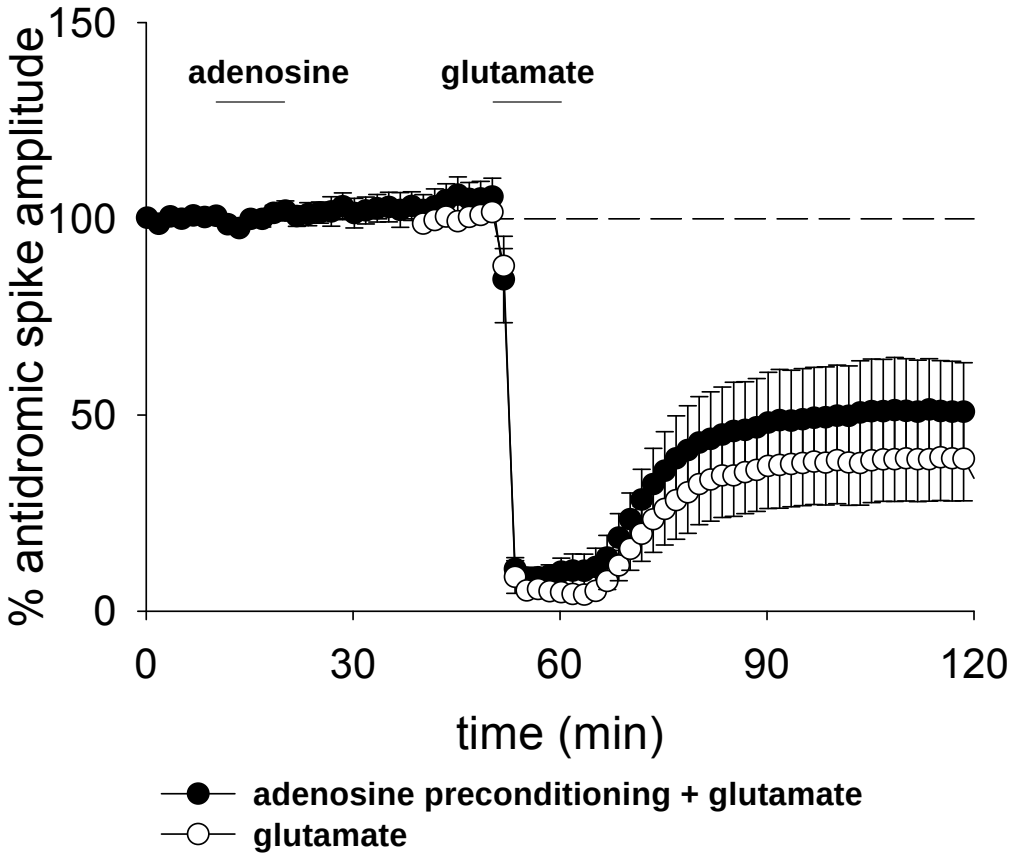


**Figure 4.8 A sustained application of 100μM adenosine failed to precondition against 10mM glutamate in antidromic responses.**

### 4.2.3 - Effects of ouabain, a $Na^+/K^+$ ATPase inhibitor, on evoked responses

In view of the failure of adenosine applications to modify responses to glutamate, we tested an unrelated agent which has, like adenosine, been reported to produce preconditioning against ischaemia (Pierre et al., 2007; Pasdois et al., 2007). In preliminary experiments it was found that a narrow window existed between ouabain applications that produced only a minimal effect on the slices, and applications that generated a complete and irreversible loss of electrical activity. The application of 100μM ouabain for 2 min resulted in an initial increase of neuronal excitability reflected in the recording of multiple population spikes on orthodromic stimulation (Fig 4.10). This was followed by a marked reduction in the size of the evoked potential (Fig 4.9 & 4.10) which did not return to baseline level within 1 hour of washout.

Antidromically evoked population spikes were normally decreased in amplitude by ouabain, though significantly less than orthodromic spikes (see Fig 4.9 & 4.10).

Since ouabain can induce the release of neuroactive compounds (Basarsky et al., 1999; Li & Stys, 2001) and it has been shown to precondition tissues against hypoxic damage, the possibility was considered that the effects of ouabain were due to the release of glutamate or adenosine. When slices were superfused with the non-selective glutamate antagonist, kynurenic acid (Perkins and Stone, 1982; Stone & Darlington, 2002), the potentials that were recorded after orthodromic and antidromic stimulation behaved differently. The depressant effect of ouabain on antidromic evoked potentials was unchanged after perfusion with kynurenic acid at a concentration of 1mM, applied for 10min before, during and for 1h following the inclusion of ouabain (Fig 4.11). Orthodromic potentials, however, exhibited a much greater recovery after the presence of kynurenic acid, compared with control slices in which ouabain was present without any blockade of glutamate receptors (Fig 4.12). These results indicate that glutamate is released by ouabain, but the effects of glutamate are only observed when stimulation involves synapse activation by orthodromic stimulation. It may be that the releasing effect of ouabain is limited to synaptic terminals and not to cell somata or glial cells.

In contrast, adenosine does not seem to contribute to the effects of ouabain. Slices were superfused with DPCPX (30nM) prior to and during the application of ouabain (Fig 4.13). This blockade of A1 adenosine receptors had no effect on the loss of response induced by ouabain (69.4 ± 5.1%, n = 5) compared with control (68.6% ± 5.2%, n = 7). GABA also appears to have no role as blockade of $GABA_A$ and $GABA_B$ receptors did not attenuate the

depression of orthodromic population spikes (Fig 4.14). Indeed blockade of $GABA_A$ receptors enhanced the ouabain-induced decrease in spike size which was significant at the 50min time point of the experimental protocol.

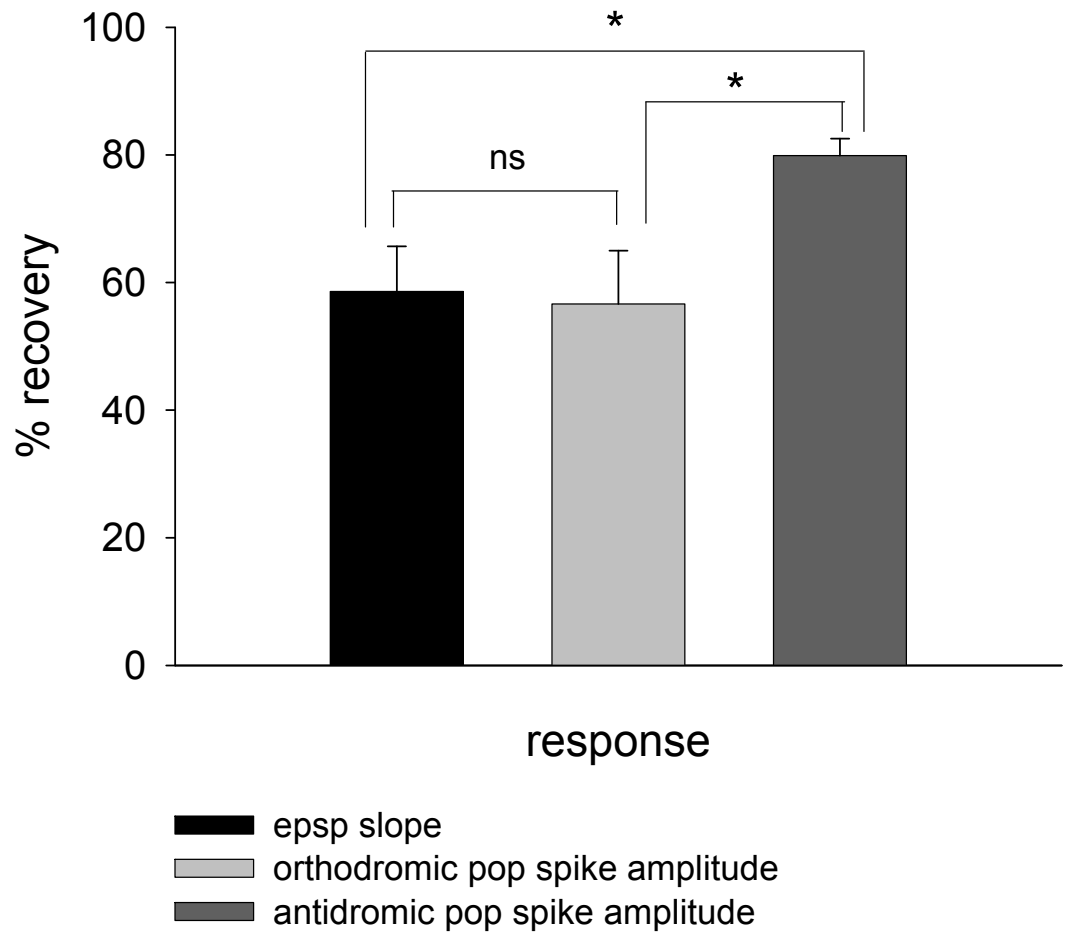


**Figure 4.9 The effects of ouabain on evoked responses from CA1 neurons.** A long lasting decrease in response size after 100μM ouabain perfusion for 2 min is observed for epsps, orthodromically and antidromically evoked population spikes (field epsp 58.59% ± 7.06, n = 7; orthodromic population spike 55.49% ± 7.97, n = 8; antidromic population spike 82.04% ± 1.80, n = 5). The reduction in antidromic spike size is significantly less than that observed for epsp slope and orthodromic spike size (ns = not significant *$p<0.05$ Kruskal-Wallis test).

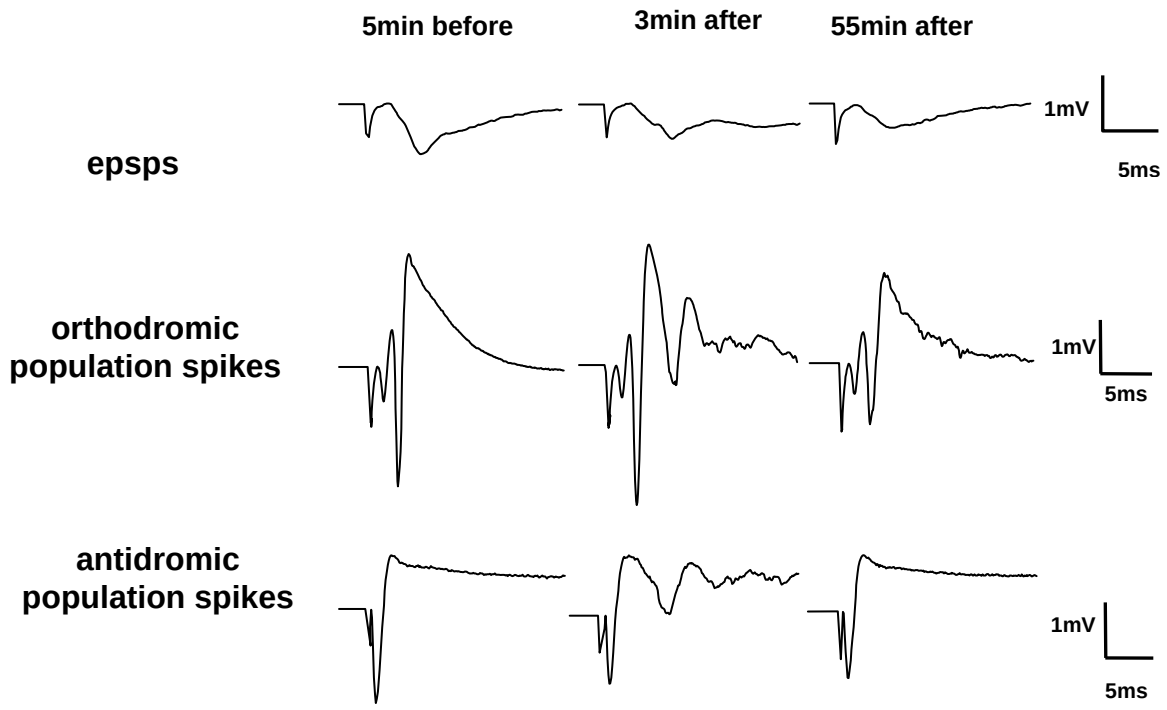


**Figure 4.10 Sample traces showing the effects of ouabain at various time points on the evoked responses from the hippocampal slice**. Times indicated are in relation to application of ouabain.

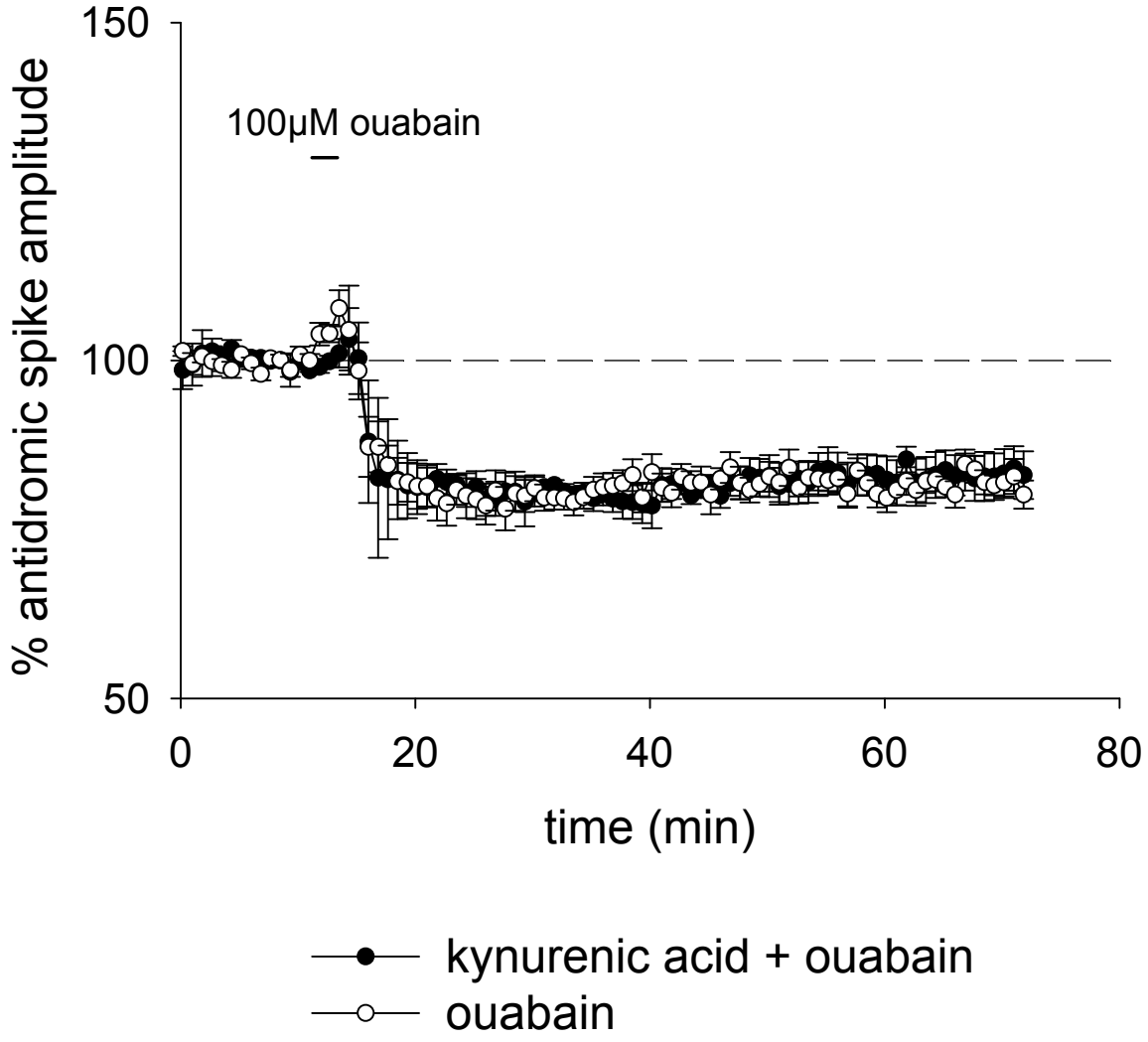


**Figure 4.11 Reduction in antidromic spike amplitude in response to ouabain is independent of glutamate-receptor mediated damage.** The time course graph of antidromic spike amplitude show no difference in response to 100μM ouabain when perfused with (79.8 ± 2.1%, n = 4) or without (79.9 ± 2.7%, n = 6) 1mM kynurenic acid.

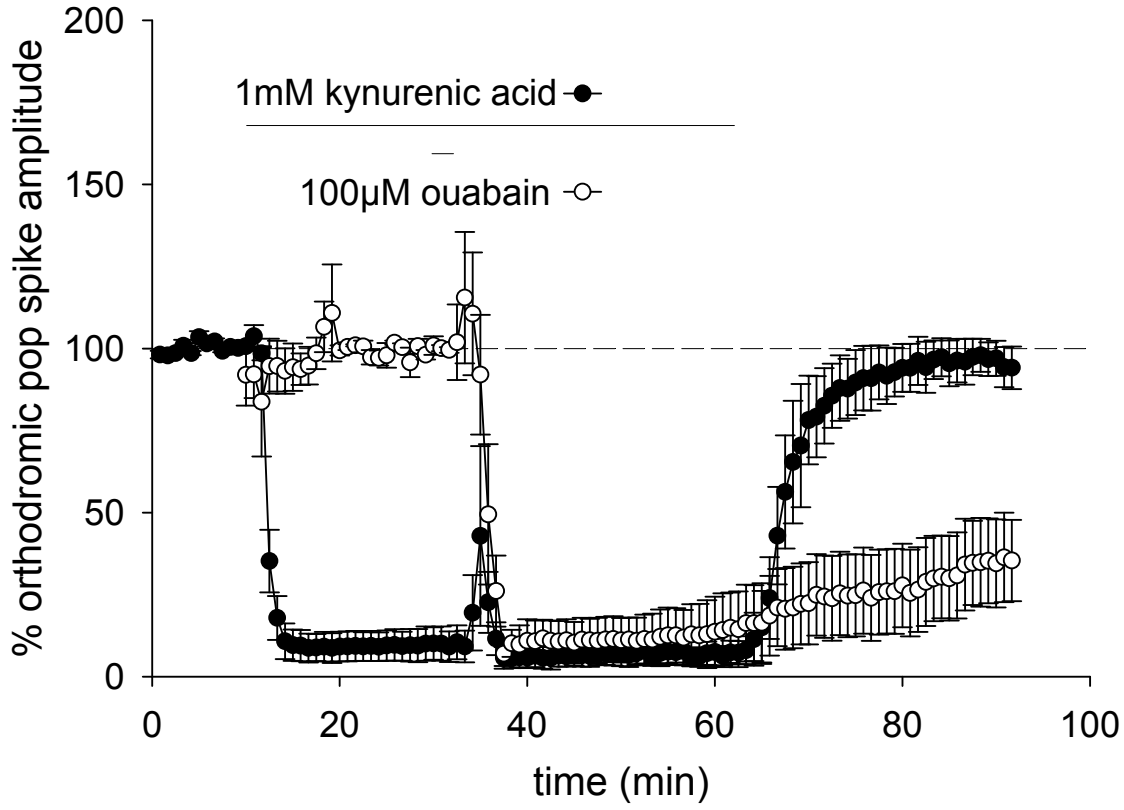


**Figure 4.12 The effect of ouabain upon orthodromic spikes in the presence of kynurenic acid.** Orthodromic spikes show a clear attenuation of the ouabain-induced depression in the presence of kynurenic acid (96.3 ± 5.9%, n = 4) compared with ouabain only controls (39.9 ± 13.1%, n = 4) ($p<0.05$).

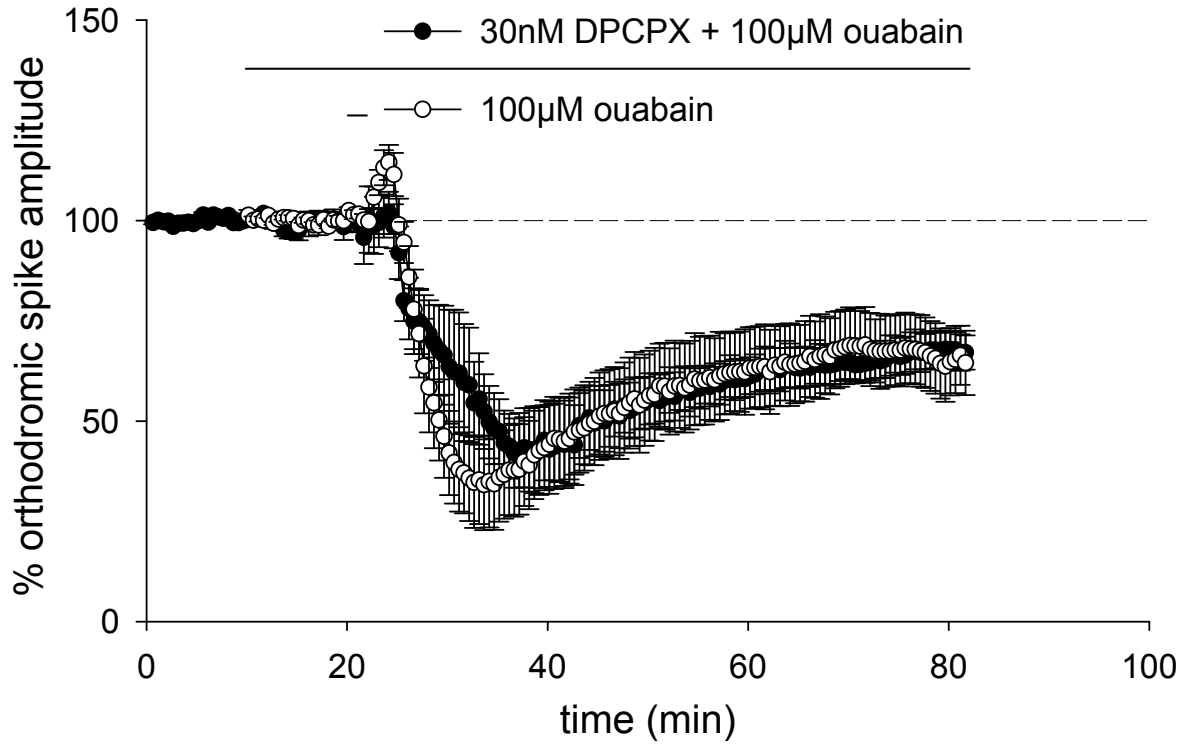


**Figure 4.13 The effect of ouabain upon orthodromic spikes in the presence of DPCPX.** Blockade of A1 receptors does not modulate the response to ouabain in orthodromic spikes (DPCPX + ouabain 69.4 ± 5.1%, n = 5; ouabain only 68.6 ± 5.2%, n = 7).

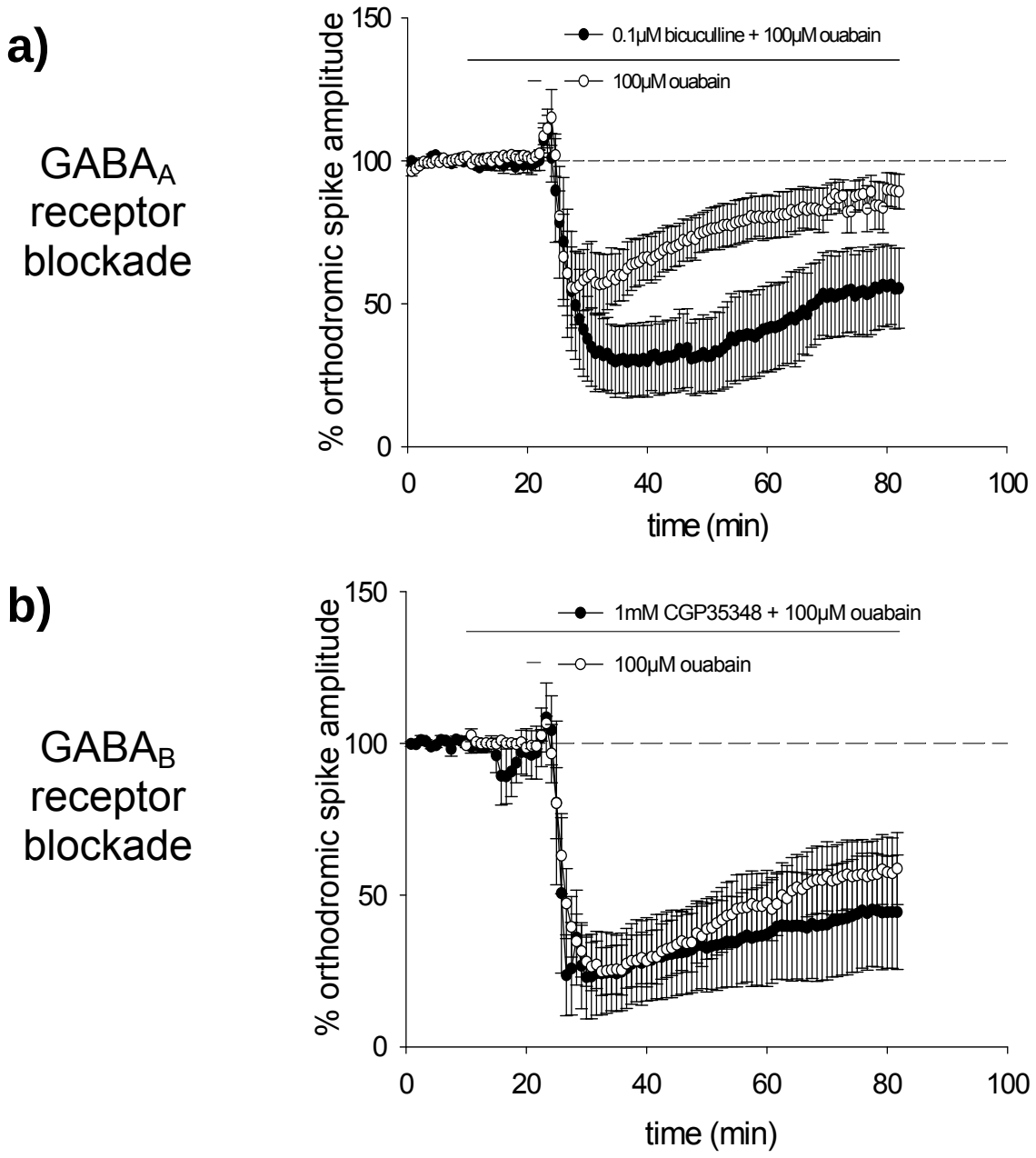


**Figure 4.14 Blockade of GABA receptors does not attenuate the ouabain-induced depression in orthodromic spikes** a) $GABA_A$ receptor blockade during ouabain perfusion with 100nM bicuculline (n = 5) and ouabain only controls (n = 5). Note that $GABA_A$ blockade significantly enhances the effects of ouabain at the 50min time point (31.7 ± 12.9%, n = 5) compared with ouabain-only controls (75.7 ± 7.0%, n = 5) ($p<0.05$). b) $GABA_B$ receptor blockade during ouabain perfusion. (CGP35348 + ouabain 44.0 ± 18.7%, n = 6; ouabain only 56.9 ± 11.4%. n = 10).

### 4.2.4 - The effects of ouabain on synaptic plasticity

Having determined that the post-ouabain induced depression in orthodromic population spikes was due to glutamate receptor stimulation, the nature of this effect on synaptic plasticity was examined. The extent of glutamate receptor stimulation can determine if LTP, LTD or excitotoxicity is induced. Orthodromic population spikes were evoked to obtain the maximum response. When a stable baseline was established, the stimulus was reduced to produce 50% of the maximum response (Fig 4.15a). The maximum stimulus was induced at three time points to determine the exact maximum response during the evocation of the 50% baseline. After establishing a stable baseline at the reduced stimulus, 100μM ouabain was perfused onto the slice for 2min. It was observed that following ouabain treatment, the population spikes showed an initial increase in size followed by a drop. The orthodromic population spikes did not show a significant decrease in size at 50 to 60min post-ouabain (75.2 ± 16.5%, n = 5) compared to the 100% baseline or a significant difference from the half-maximal baseline (50.6 ± 3.2%). The maximum responses were evoked at approximately 45min, 50min and 55min post-ouabain; the average value of the three time points showed no difference in size compared with the average value of those evoked prior to ouabain application (pre-ouabain 114.6 ± 4.8%; post-ouabain 111.1 ± 11.9%) ($p=0.72$, paired t test) (n = 5).

A 100Hz stimulus was applied for 1 second at 1hour post-ouabain. The 100% baseline was taken as the 10min period prior to stimulation for calculating the size of LTP induced. There was a significant potentiation observed which lasted for at least 30min (140.7 ± 11.3%) ($p<0.05$, one sample t test)(n = 5) (Fig 4.15b). This was compared to control LTP responses which were obtained from slices taken from the same hippocampus as those treated with ouabain. The orthodromic population spikes obtained from control slices were stimulated to produce the maximal response followed by establishing a half-maximal baseline. Once a stable baseline was established, a 100Hz stimulus was applied for 1second to these slices. The control responses showed a significant potentiation in response to the tetanizing stimulation (207.6 ± 26.8%, n = 4)($p<0.05$, one sample t test) which were significantly greater than those obtained from ouabain treated responses ($p<0.05$) (Fig 4.15b). It was noted that the half-maximal baselines obtained for ouabain-treated slices and control slices did not vary significantly (ouabain-treated response baseline 50.6 ± 3.2%, n = 5; control response baseline 42.3 ± 3.6%, n = 4) nor did the values of the ouabain treated slices at the 10min period prior to LTP stimulation (75.18 ± 16.5%, n = 5). It can therefore be concluded that ouabain does not prevent the induction of LTP in the hippocampus but it does appear to attenuate the extent of LTP induced.

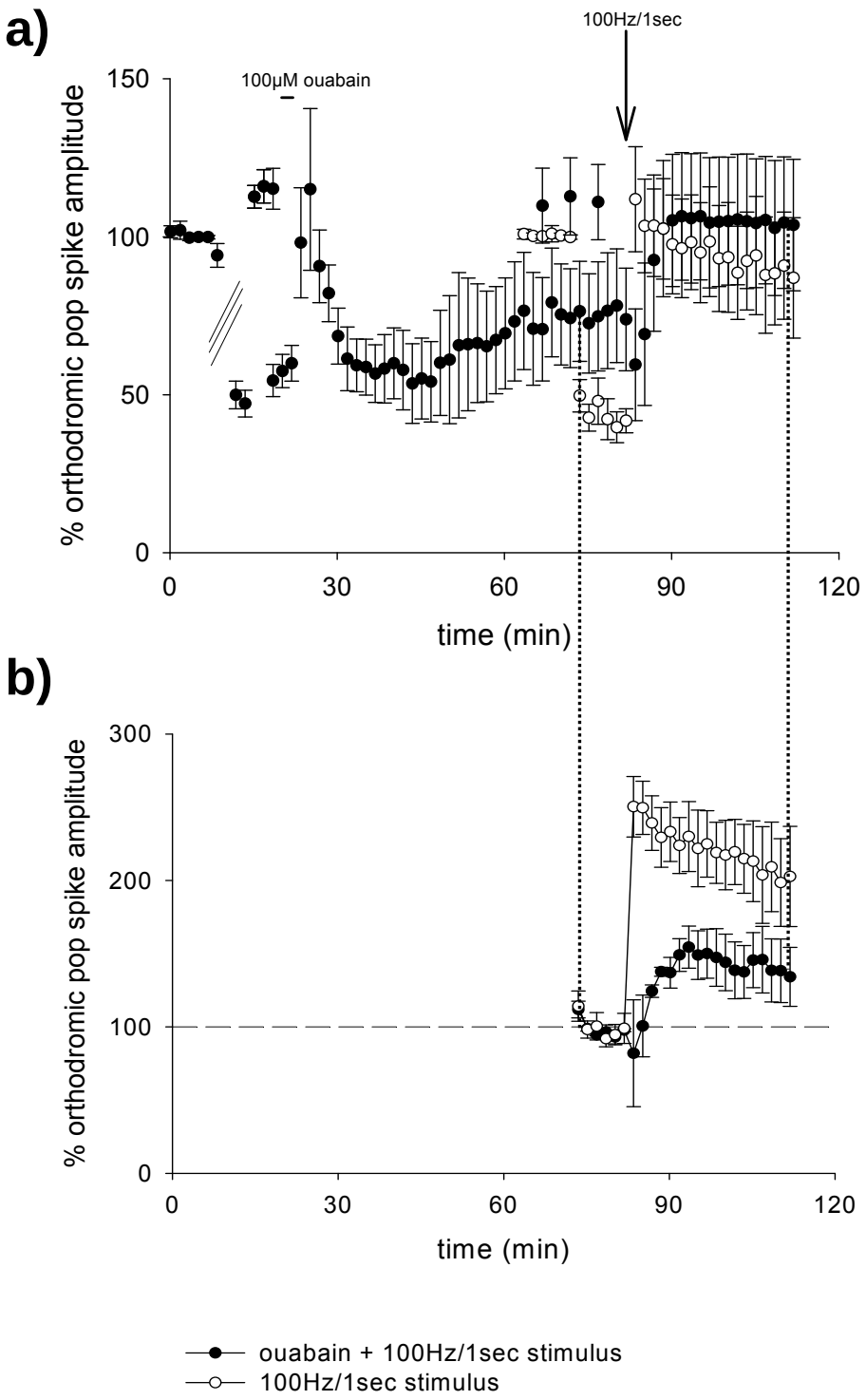


**Figure 4.15 Ouabain impairs but does not prevent the induction of LTP in the rat hippocampal slice.** a) shows the time course of changes in orthodromic population spike amplitude induced in response to altered stimulation and ouabain exposure. The three diagonal lines at approximately the 10min time point indicate the time elapsed before a stable baseline with a half-maximal response was obtained. The times at which ouabain and a 100Hz/1sec stimulus are applied are indicated. b) shows extrapolated data from a) taken 10min prior to the application of a 100Hz stimulus and normalised. The data are normalised from the 10min period prior to LTP.

### 4.2.5 - Adenosine can precondition against ouabain but not glutamate

Since the results presented above indicate that adenosine, although known to produce preconditioning in vivo, did not precondition against exogenously applied glutamate, it was of interest to examine its ability to precondition against the release of glutamate released endogenously by ouabain. A preconditioning stimulus of 100μM adenosine was applied to the slices for 10min, causing a complete loss of the orthodromic population spike which returned within 10min of ending the adenosine perfusion. Indeed, several slices during the return of response showed a recovery of potential size to levels greater than the initial baseline size (Fig 4.16a), an effect of adenosine that has been reported elsewhere and has been attributed to opening of ATP-sensitive potassium channels (Li & Henry, 1992). Recovery from adenosine was followed 30 min later by an application of ouabain (100μM) or glutamate (10mM) applied for 2min and 10min respectively as in the previous experiments (Fig 4.16a & b).

Adenosine applications did not produce any change of the subsequent glutamate response, as reported above, but it did produce a clear modification of the response to ouabain. With a preceding application of adenosine, orthodromic responses recovered after superfusion with ouabain, to a significantly higher level that they did after applications of ouabain alone (Fig 4.16a & b).

The protocol for adenosine preconditioning against ouabain was repeated for recordings of the field epsps (Fig 4.17) using, in separate experimental groups, a stimulus strength to achieve the maximum response (Fig 4.17a) or a reduced stimulus strength to produce a potential size that was 50% of the maximum (Fig 4.17b). At maximum stimulus strength both adenosine and ouabain induced a clear decrease in the amplitude and slope of epsps. The loss of epsp slope size and amplitude induced by ouabain was not attenuated by the preceding application of adenosine, suggesting that the modifications of the ouabain effect on orthodromic somatic spikes was not attributable to changes of synaptic transmission, but probably to changes of postsynaptic excitability. It was noted that post-adenosine treatment, a significant decrease was observed in epsp amplitude (97.8 ± 0.8%, n = 7) ($p < 0.05$ one sample t test), the opposite effect to population spikes. This is consistent to some extent with adenosine's reported effects on epsp-spike coupling (O'Kane & Stone, 1998).

This view was supported when adenosine was applied before ouabain while recording antidromically-induced spikes. After adenosine application, which produced no significant change of antidromic spike size during its perfusion, a significant increase of spike amplitude was observed upon adenosine washout, similar to that seen when using orthodromic spikes ($p < 0.05$) (Fig 4.18a & c). After ouabain was perfused, a similar onset of reduced response size was observed compared with non-preconditioned controls but the response recovered to a level that was significantly greater than control potentials (control recovery to 82.3% ± 2.0, n = 11; preconditioned recovery to 95.2% ± 4.8, n = 8, $p < 0.05$) (Fig 4.18b & c). If slices were perfused with 30nM DPCPX prior to, during and 15min after adenosine applications, the greater recovery from a subsequent application of ouabain was prevented ($p = 0.93$, n = 4) (Fig 4.19).

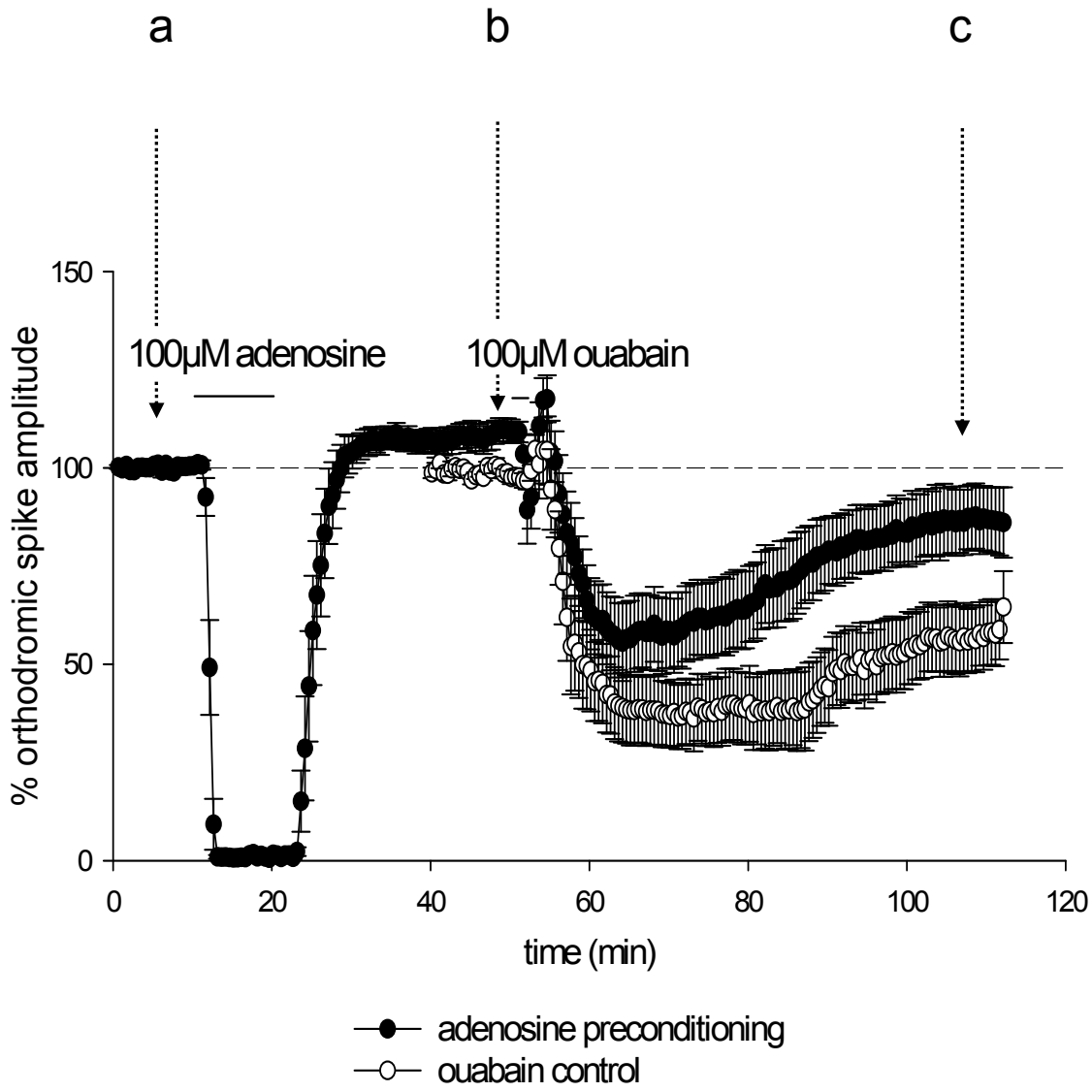


**Figure 4.16a. Adenosine preconditioning attenuates ouabain-induced changes in orthodromic spike size.** a) Preconditioned slices recovered to 94.8 ± 5.5% (n =7) compared to 82.3 ± 2.0% in control slices (n = 11) ($p < 0.05$) when normalised prior to ouabain application. The arrows (a, b and c) indicate the time points from which the sample waveforms shown in Figure 3.37b are taken.

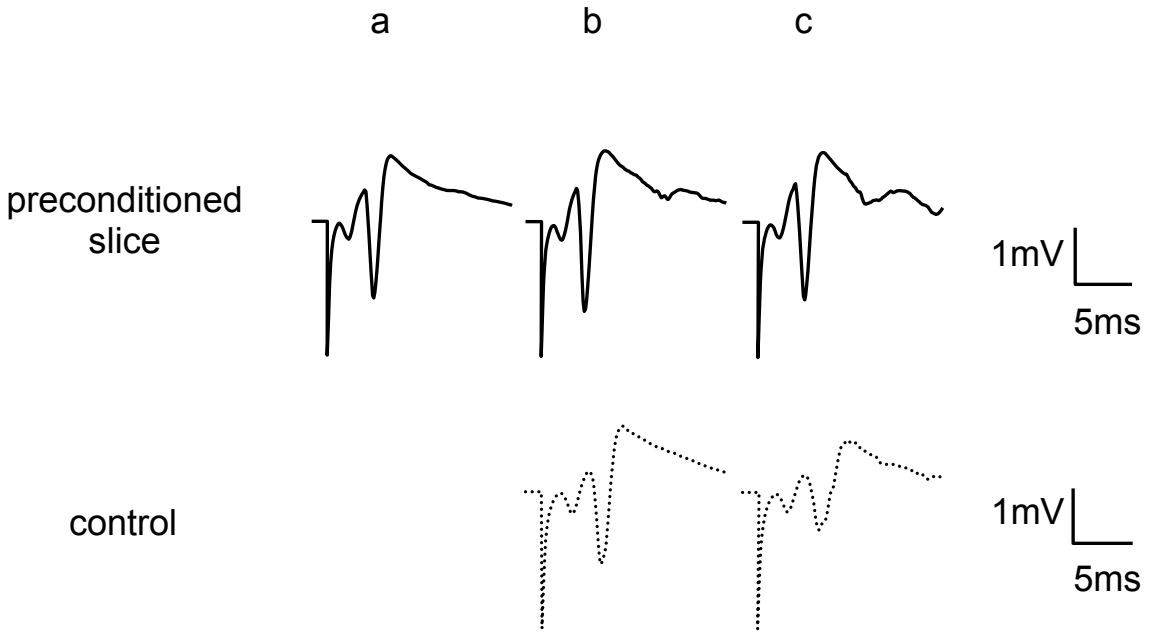


**Figure 4.16b. Sample traces of preconditioning experiments and controls taken from the time points as indicated in Figure 4.16a.**

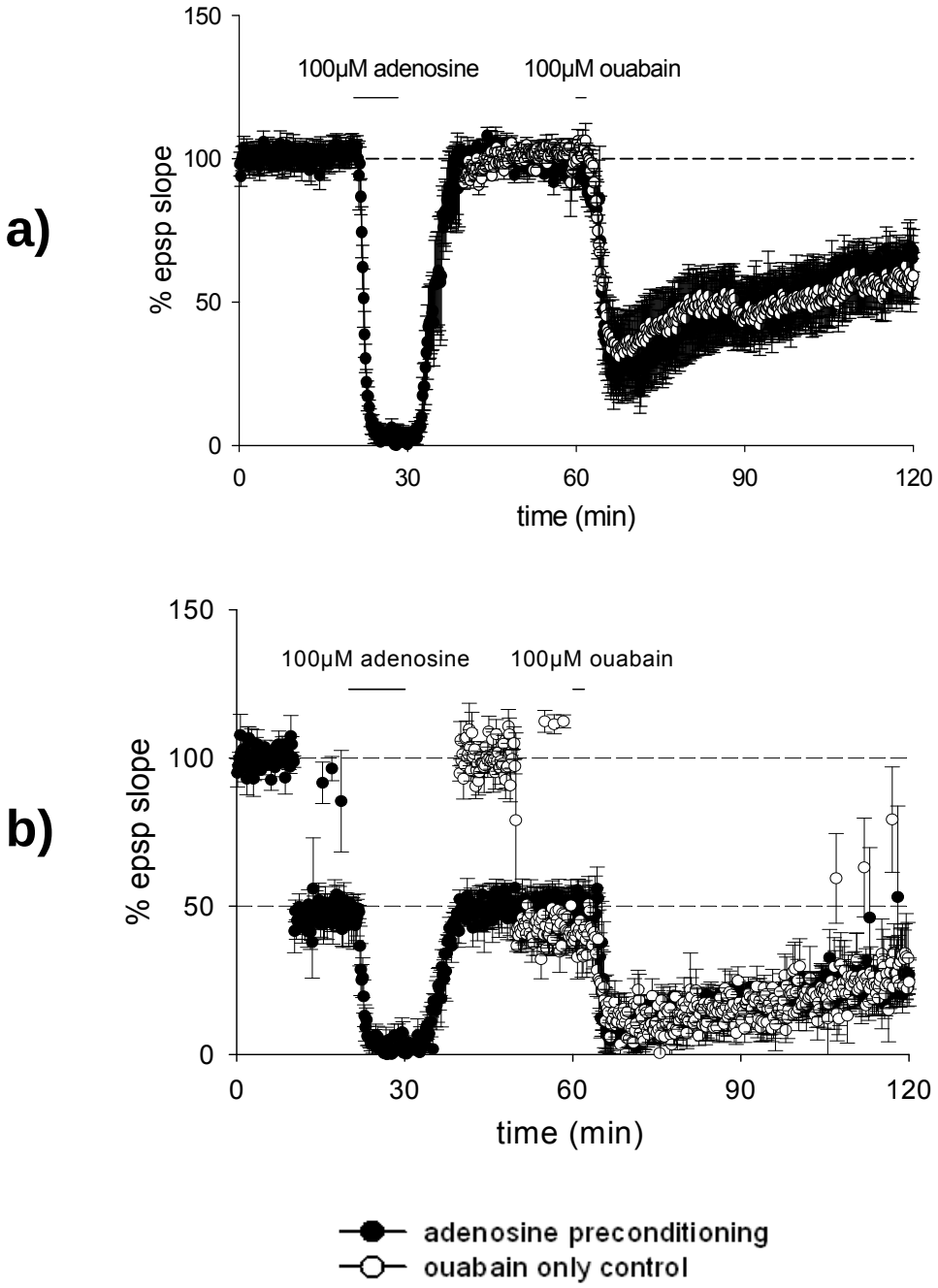


**Figure 4.17 Adenosine preconditioning effects against ouabain are absent in epsp responses.** a) shows experimental protocol using stimulation to evoke maximal epsps. b) shows the same protocol using stimulation to evoke half-maximal epsps.

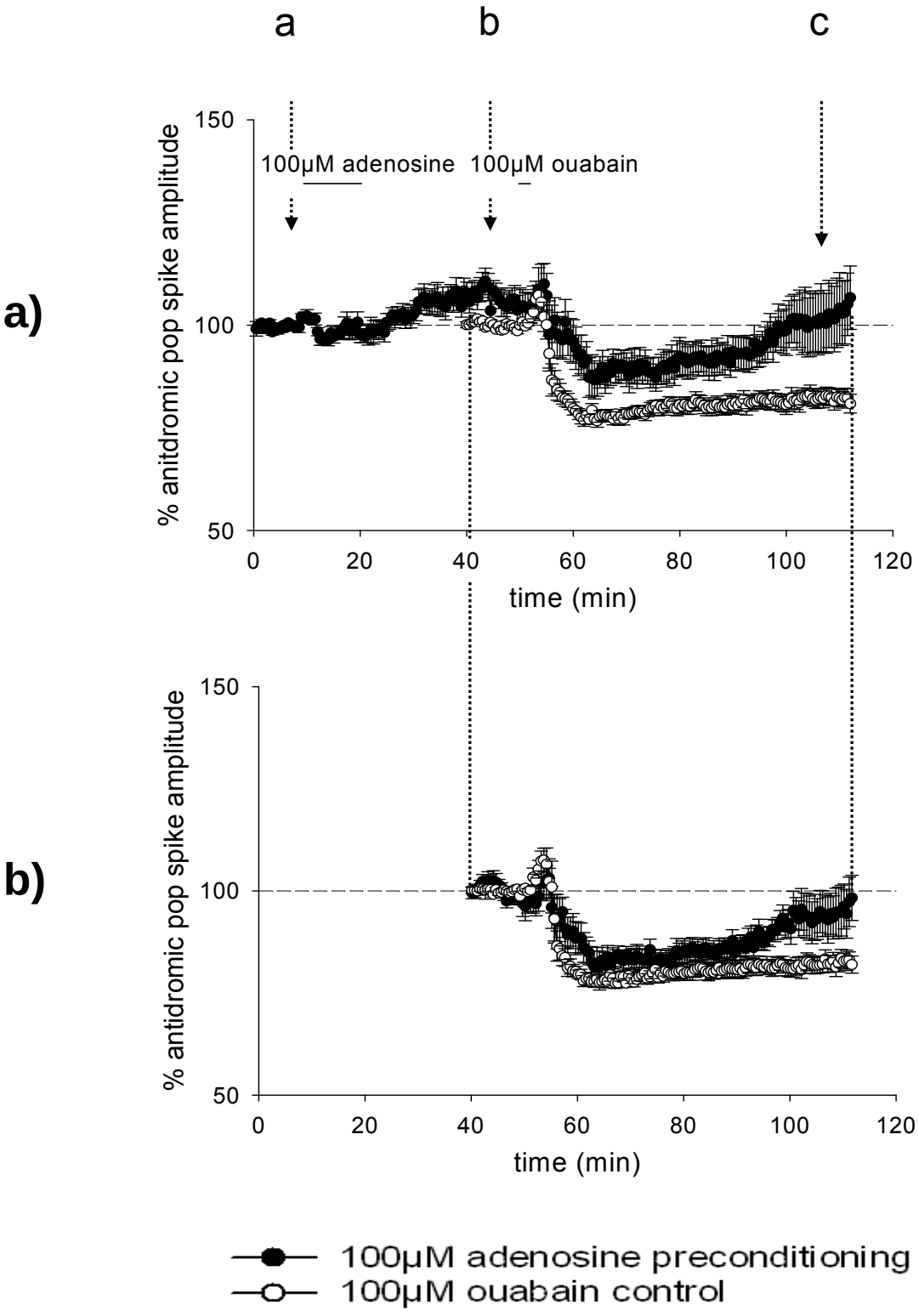


**Figure 4.18a,b/ Protection against ouabain is induced by adenosine preconditioning in antidromic spikes.** a) Time course changes in antidromic spike amplitude in response to adenosine preconditioning and subsequent ouabain application. A significant elevation of response is observable following adenosine. b) Preconditioned responses (n = 8) and controls (n = 11) in antidromics spikes normalised prior to superfusion of ouabain. The arrows (a, b and c) show the time points from which the sample waveform shown in Figure 3.39c are taken.

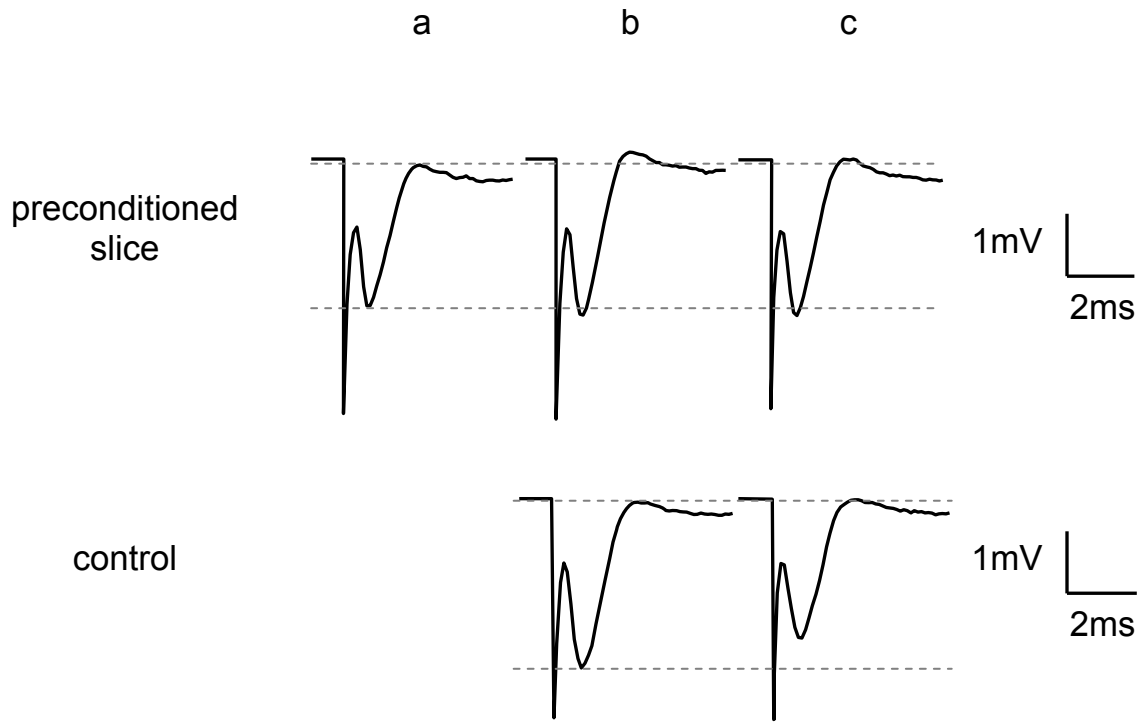


**Figure 4.18c Sample traces taken from time points as indicated in Figure 4.18a.**

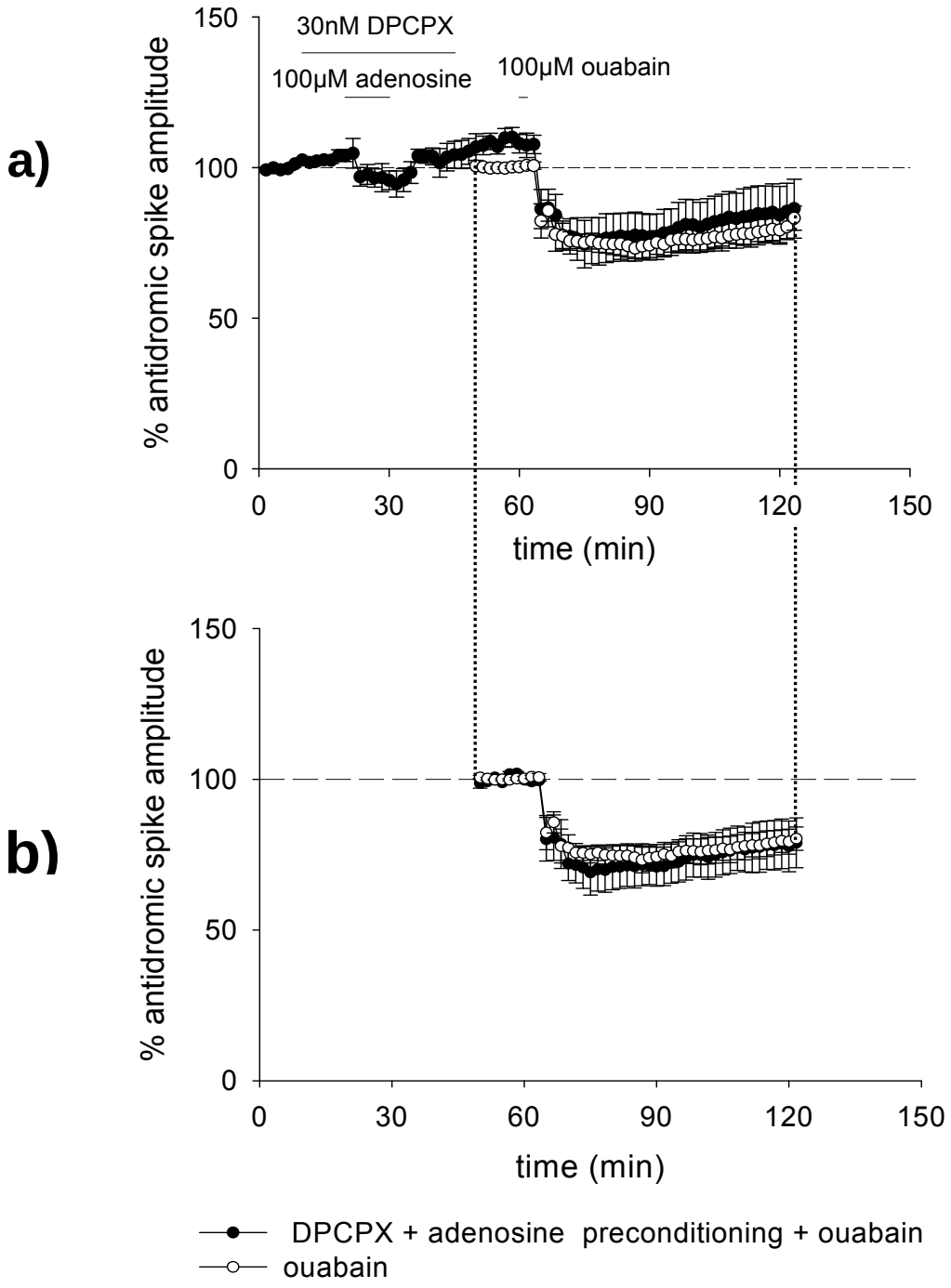


**Figure 4.19 Adenosine preconditioning is suppressed by A1 receptor blockade using 30nM DPCPX.** a) Time course changes in antidromic population spike amplitude during A1 receptor blockade of the adenosine preconditioning protocol. Note that the elevation in spike amplitude following removal of DPCPX (108.1 ± 2.9%) is not statistically significant ($p = 0.07$ one sample t-test). b) The same data from the top graph normalised prior to ouabain application (DPCPX + adenosine preconditioning + ouabain 77.9 ± 8.0%, n = 4; ouabain 78.7 ± 4.1%, n = 4).

### 4.2.6 - Adenosine preconditioning against ouabain requires activation of NMDA receptors

To assess any possible role of glutamate in adenosine's modification of ouabain responses, kynurenic acid (1mM) was perfused throughout the complete experimental protocol, 10 min prior to adenosine and then throughout the adenosine washout and subsequent ouabain perfusion. The sequence was tested using antidromically-evoked spikes. The presence of the glutamate receptor antagonist resulted in a complete suppression of adenosine protection (control recovery to 78.0% ± 3.2, n = 12; preconditioned recovery to 78.3% ± 3.9, n = 9; p = 0.9614) (Fig 4.20). This result suggests that glutamate receptor activation is involved in adenosine's protective effect on ouabain responses. This was further confirmed by blocking adenosine preconditioning using 50μM AP-5 and 10μM DNQX in separate experiments in the same manner as 30nM DPCPX. Adenosine protection against ouabain was still observed during blockade of AMPA receptors with DNQX but was completely suppressed when AP-5 was used to block NMDA receptors (Fig 4.21 & 4.22). It is therefore likely that the role of glutamate in adenosine preconditioning is via NMDA receptor activation.

To probe the mechanism by which NMDA receptor stimulation induces protection against ouabain, we inhibited nitric oxide production with the nitric oxide synthase inhibitor, L-NAME. 100μM L-NAME was perfused 20min prior to, during and 15min after adenosine application (n = 4). This resulted in a complete suppression again of adenosine's preconditioning against ouabain (Fig. 4.23).

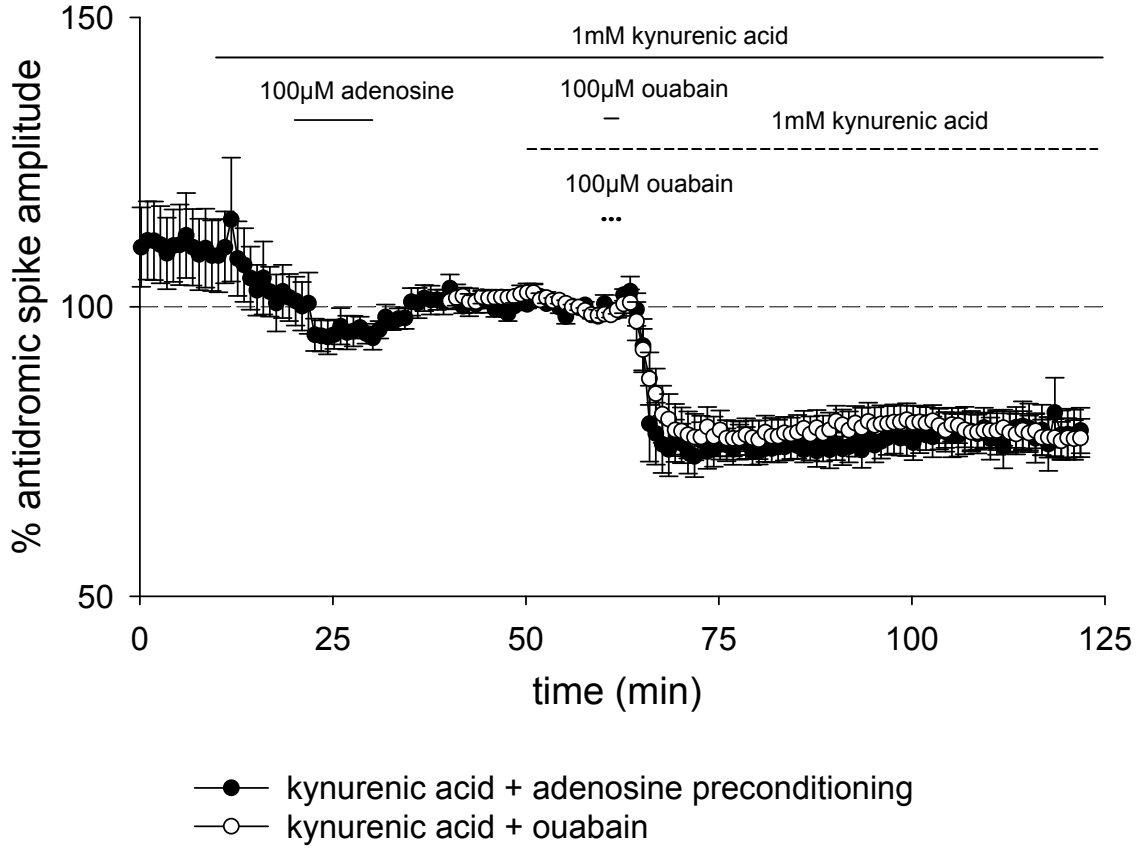


**Figure 4.20 Modulation of adenosine preconditioning by kynurenic acid.** There is no significant difference between preconditioned slices (78.3 ± 3.9%, n = 9) and controls (78.0 ± 3.2%, n = 12) (p = 0.96). All data are normalised 10min prior to ouabain superfusion. Solid lines indicate application of drugs in preconditioning experiments. Dashed lines indicate application of drugs in control experiments.

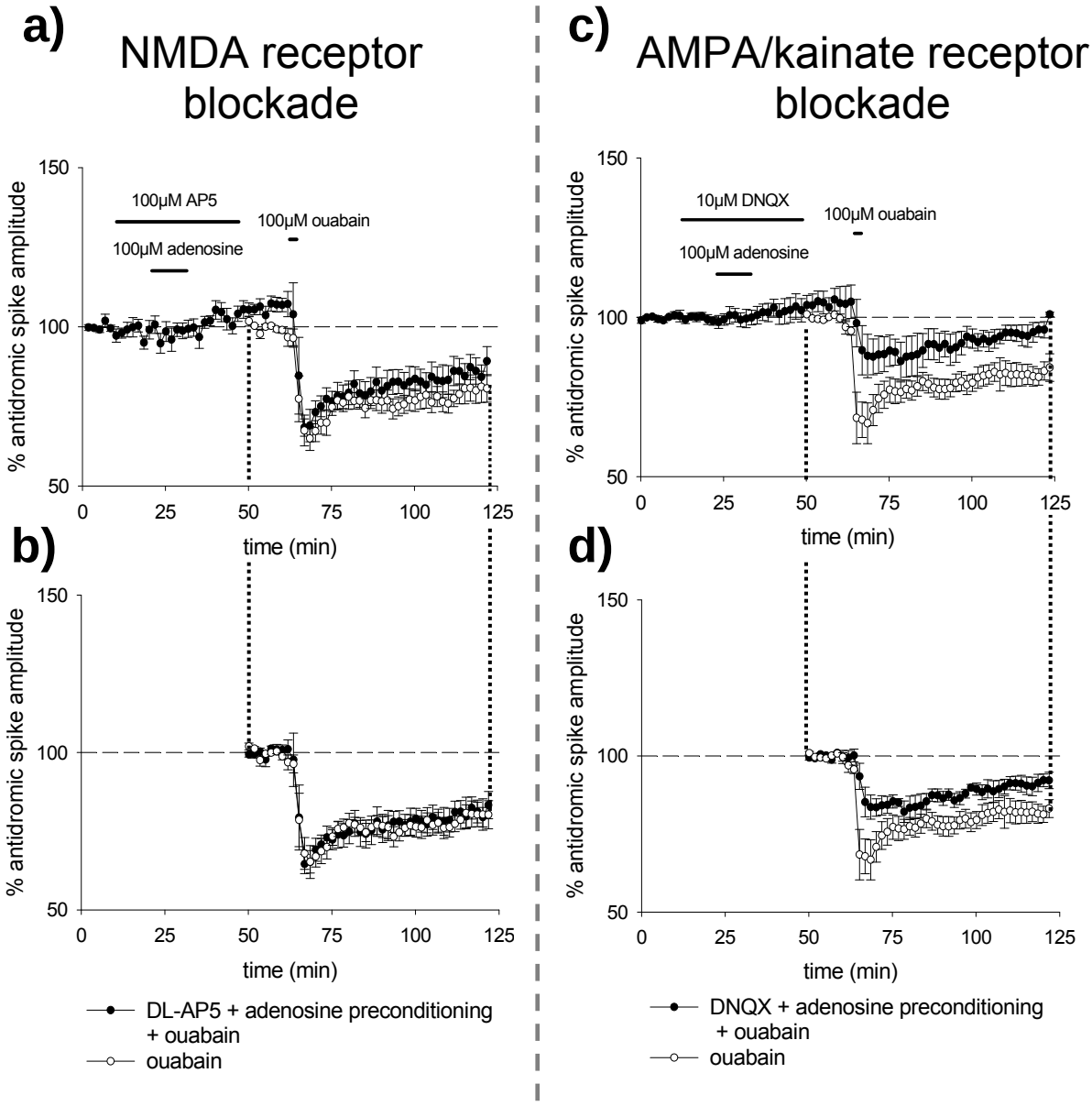


**Figure 4.21 Blockade of NMDA receptors during adenosine preconditioning against ouabain.** a) Perfusion of 100µM DL-AP-5 prior to, during and after superfusion of 100µM adenosine. Note that a significant elevation of antidromic spike amplitude occurs following removal of AP-5 from the perfusion medium ($p<0.05$). b) The same data as above normalised prior to 100µM ouabain perfusion. There is no significant difference between preconditioned responses in the presence of DL-AP5 (n = 5) and control responses (n = 8). c) Perfusion of 10µM DNQX prior to, during and after superfusion of 100µM adenosine. d) The same data as above normalised prior to 100µM ouabain. There is an elevated recovery of preconditioned slices (n = 7) which is significant between 40 and 50min post-ouabain treatment (88.581 ± 1.513%, $p<0.05$ unpaired t-test with Welch correction) compared with controls (81.585 ± 2.614%, n = 10).

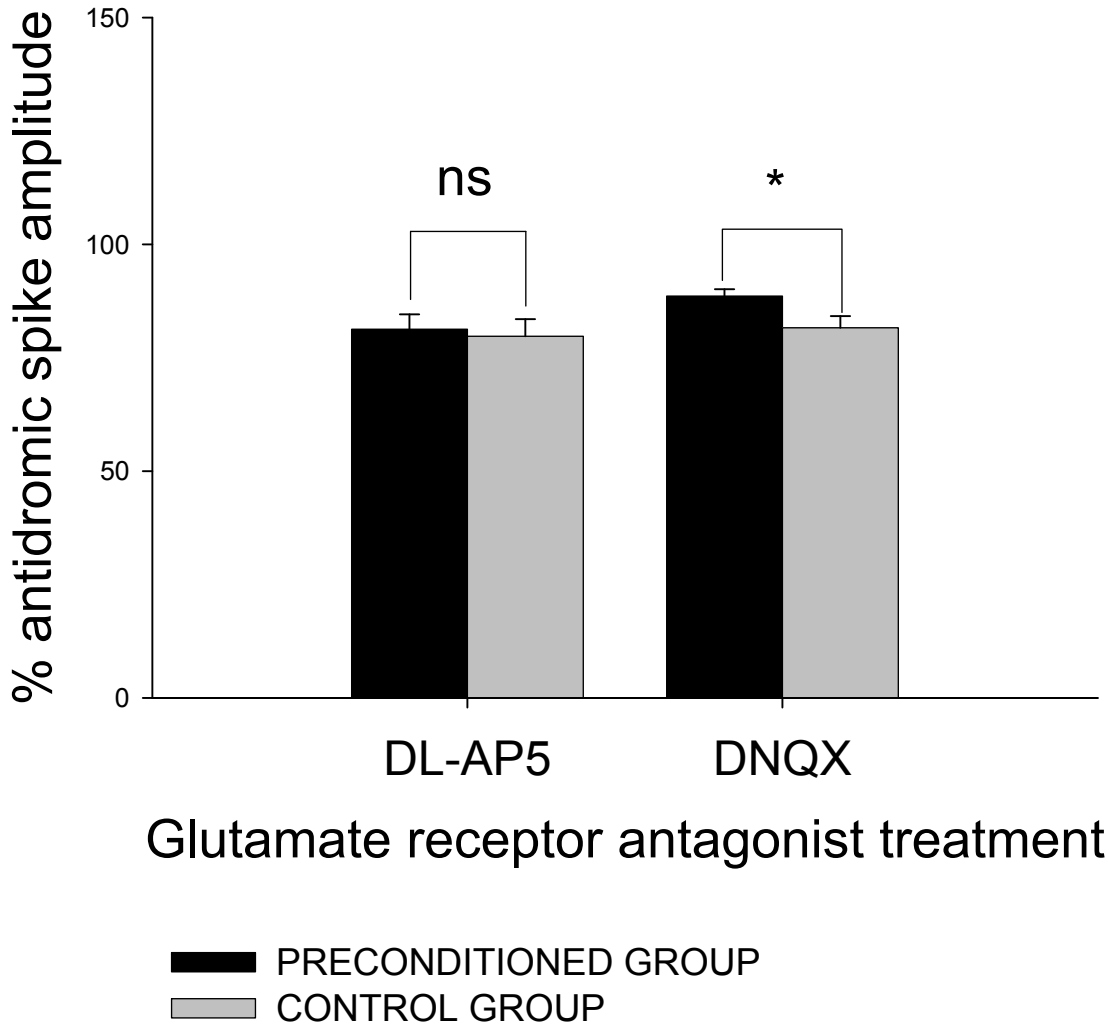


**Figure 4.22 Histogram summarising the results outlined in Figure 4.21.**

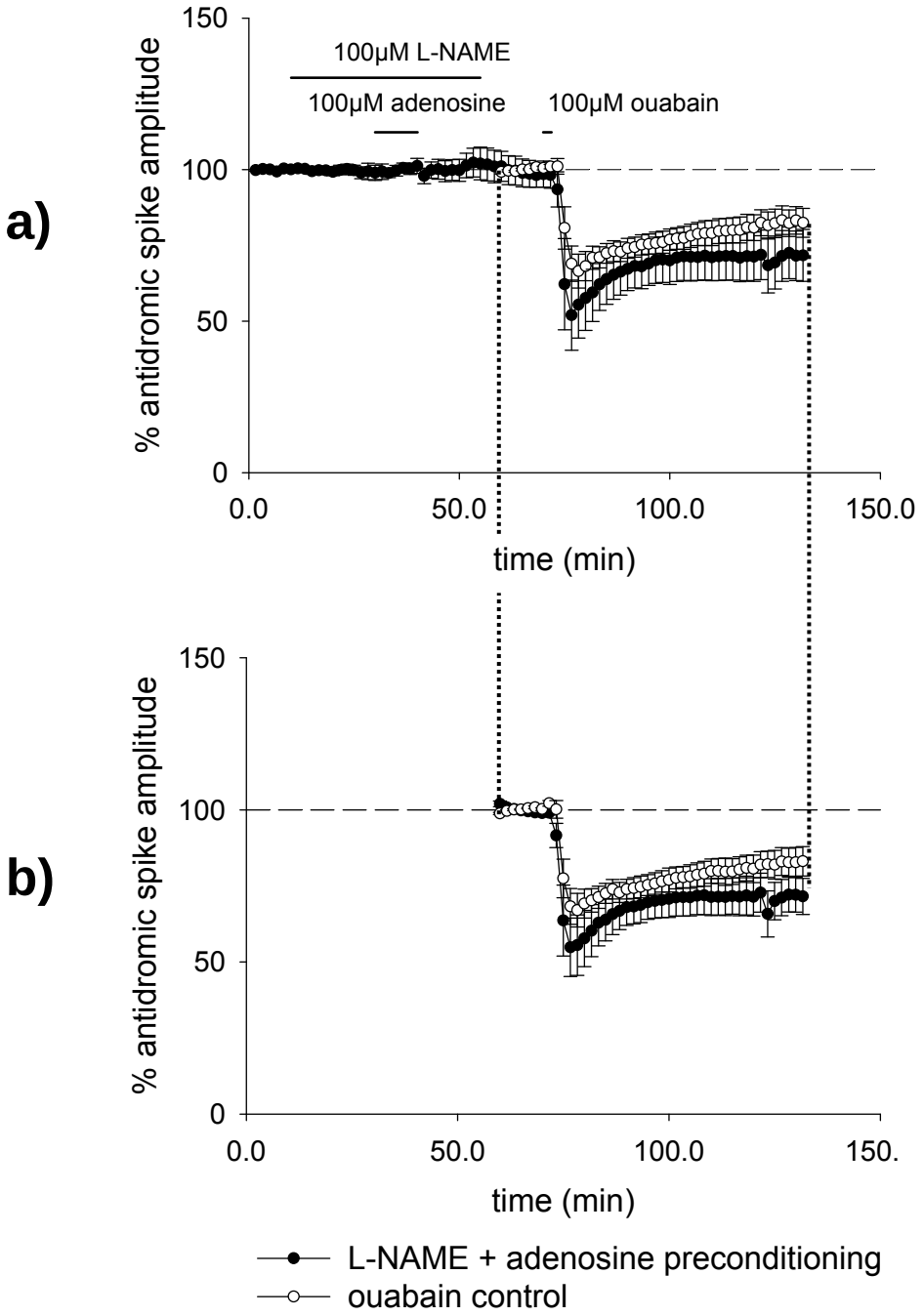


**Figure 4.23 Adenosine preconditioning in the presence of 100µM L-NAME attenuates any modulation of the response to ouabain.** **a)** Perfusion of 100µM L-NAME during adenosine preconditioning. **b)** The same data as above normalised prior to superfusion of 100µM ouabain. There is no significant difference between preconditioned slices (n = 6) and controls (n = 7).

### 4.2.7 - Adenosine preconditioning against ouabain is suppressed by blocking ATP-sensitive K+ channels

A similar protocol to that for A1 receptor blockade was used to examine the role of ATP-sensitive K+ channels in mediating the effects of adenosine. The ATP-sensitive K+ channel blocker glibenclamide was perfused before, during and after adenosine application. This resulted in an almost complete attenuation of adenosine protection against the effects of ouabain (86.6 ± 4.7%, n = 7) compared with controls (82.5 ± 2.5%, n = 10) ($p = 0.4226$) (Fig 4.24) implicating ATP-sensitive K+ channels in mediating this effect. This was examined further by using a selective mitochondrial ATP-sensitive potassium channel blocker, 5-HD (50μM). This attenuated the effects of adenosine confirming the action of glibenclamide (Fig 4.25) and suggesting that adenosine's mechanism involves mitochondrial ATP-sensitive potassium channels.

### 4.2.8 - Pinacidil, an ATP-sensitive K+ channel opener, preconditions against glutamate and ouabain

Having established a role for ATP-sensitive K+ channels which is consistent with reports of adenosine preconditioning (Heurteaux et al., 1995; Reshef et al., 2000) and given that direct modulation of these channels can precondition against hypoxia (Pérez-Pinzón & Born, 1999), we examined the effects of such a modulator, namely pinacidil in this protocol. We perfused 30μM pinacidil for 20min as a preconditioning stimulus followed by either 10min of glutamate (10mM) (Fig 4.27a) or 2min of ouabain (100μM) (Fig 4.26a) at 30min post-pinacidil treatment. Experiments in which there was no perceptible recovery of the antidromic population spike were excluded from analysis. We observed a significant attenuation of the ouabain- and glutamate-induced depressions in antidromic spike amplitude ($p<0.05$) (Fig 4.26b, 4.26c, 4.27b, 4.27c & 4.28) indicating that these channels serve as a common mechanism of protection against Na+/K+ ATPase inhibition and excessive glutamate. The pinacidil preconditioning protocol against glutamate was also repeated using orthodromic spikes. It was observed that the recovery of the responses post-glutamate did not vary between preconditioned and control slices (Fig 4.29).

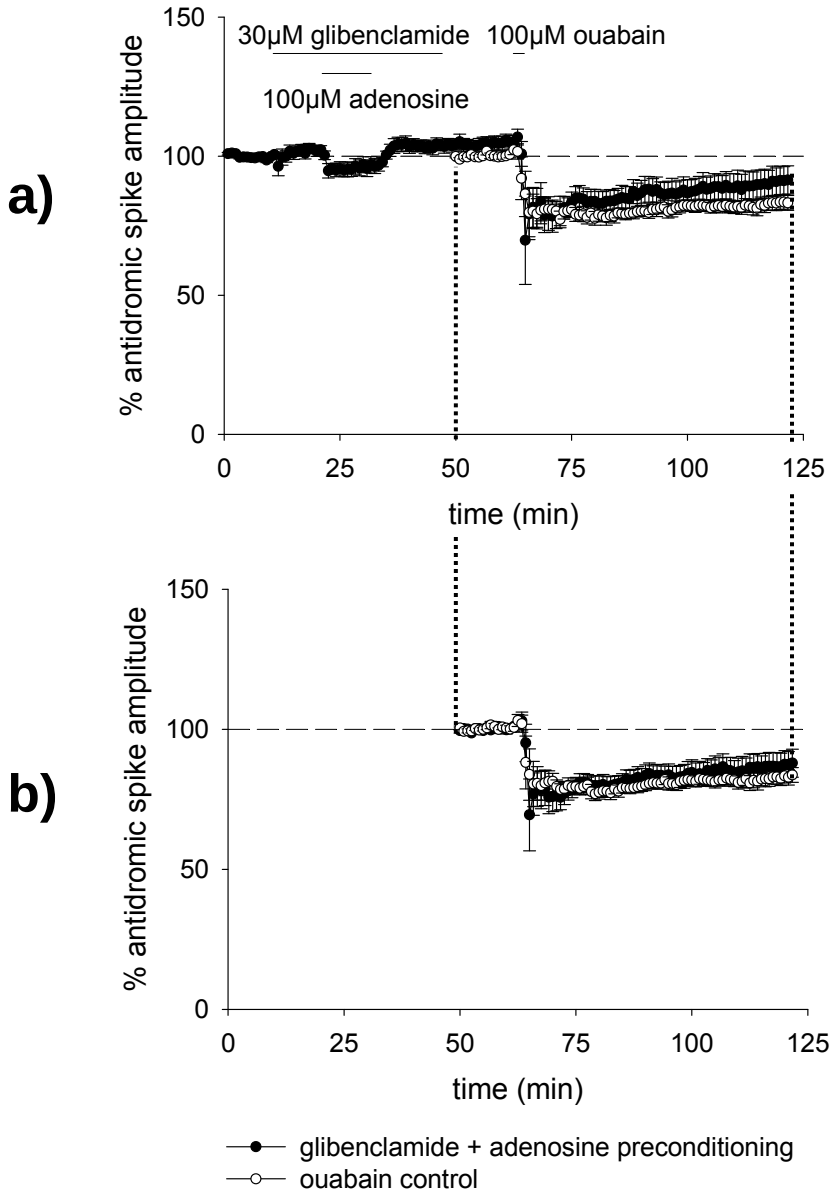


**Figure 4.24 The role of ATP-sensitive potassium channels in mediating the preconditioning effects of adenosine against ouabain.** a) Perfusion of 30μM glibenclamide prior to, during and after superfusion of 100μM adenosine. b) The same data as above taken from and normalised 10min prior to superfusion with ouabain. There is no significant difference between preconditioned responses in the presence of glibenclamide (86.6 ± 4.7%, n = 7) and controls (82.5 ± 2.5%, n = 10).

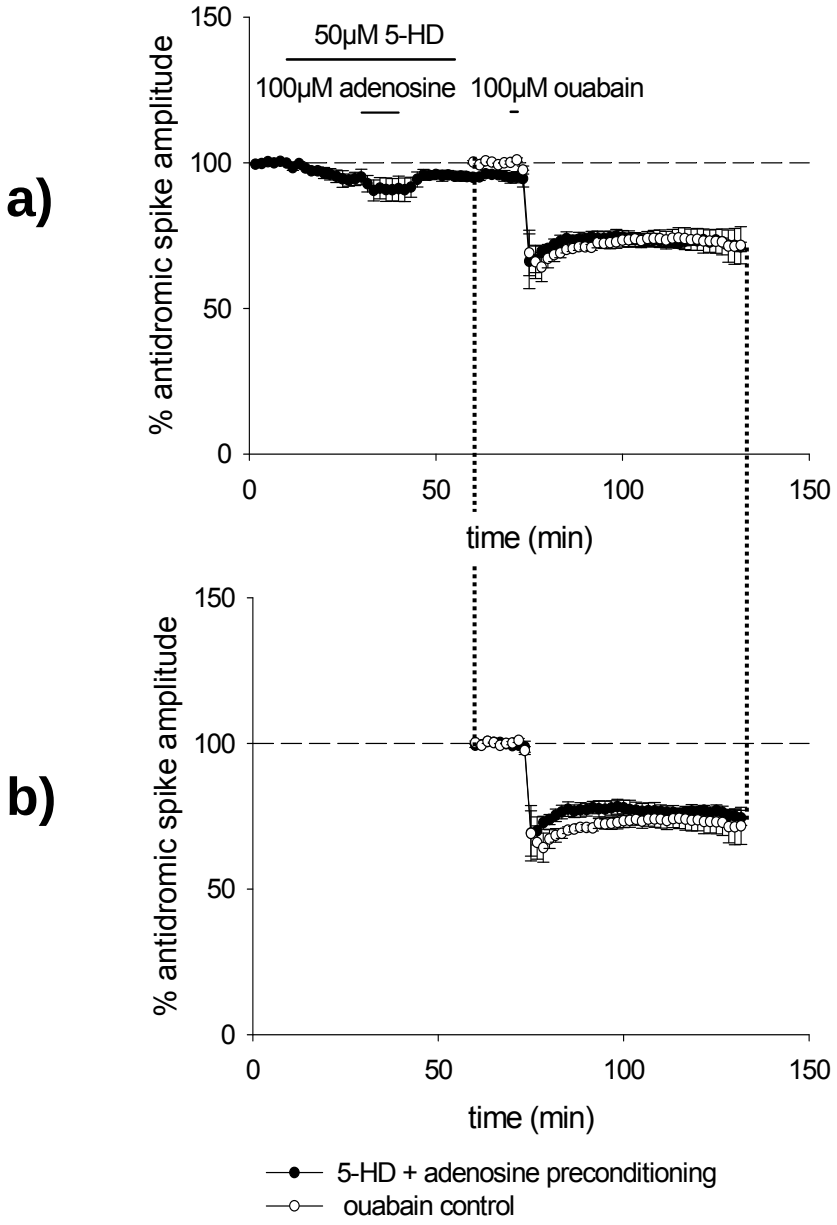


**Figure 4.25 Blockade of mitochondrial ATP-sensitive potassium channels during adenosine preconditioning. a)** Perfusion of 50μM 5-HD prior to, during and after adenosine preconditioning. b) The same data as above taken from and normalised 10min prior to superfusion with ouabain. There is no significant difference between preconditioned responses in the presence of 5-HD (75.6 ± 2.1%, n = 4) and controls (72.4 ± 4.8%, n = 4).

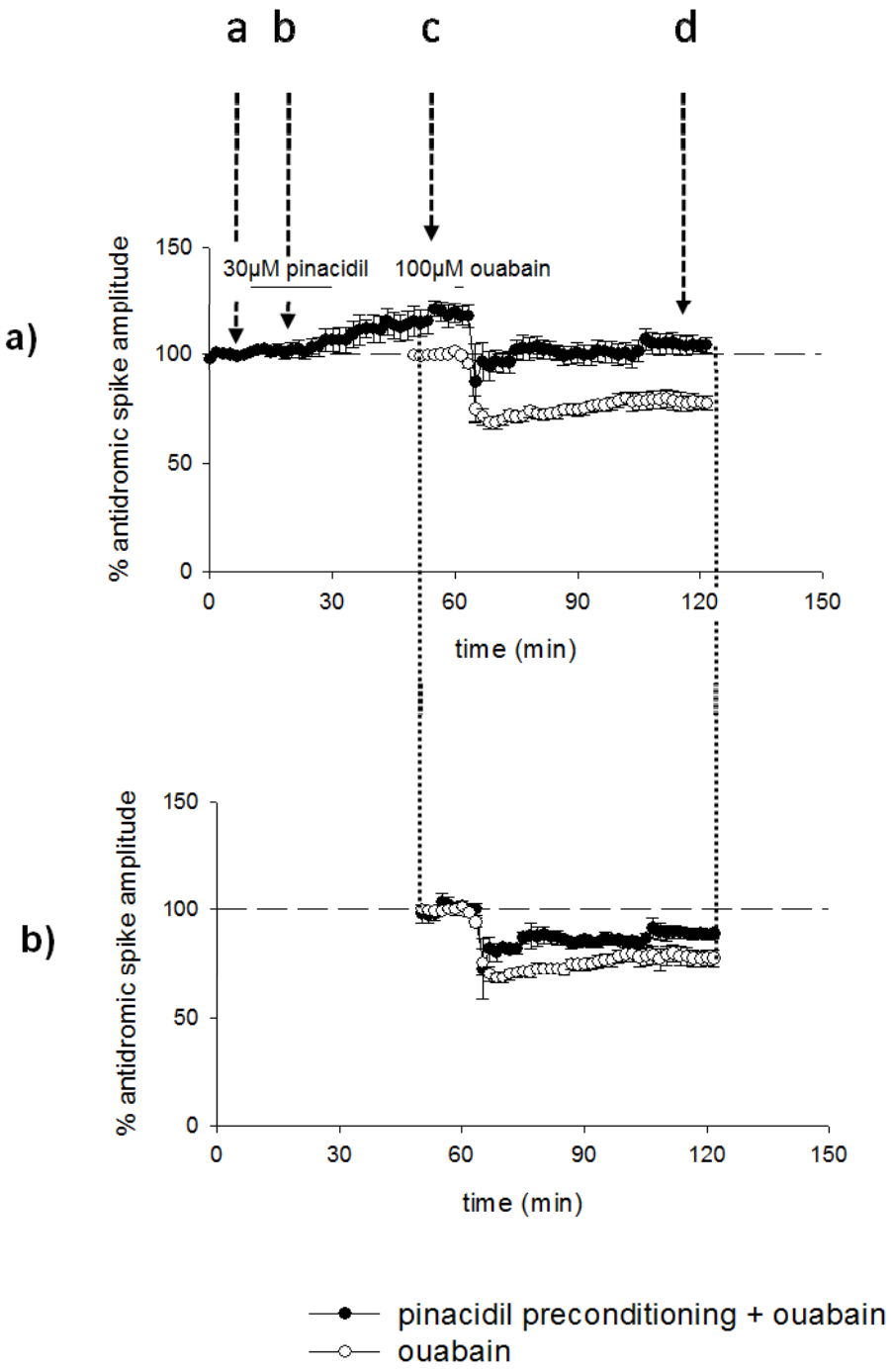


**Figure 4.26a,b. Time course graphs showing the changes in antidromic spike amplitude during the preconditioning protocol with 30μM pinacidil against glutamate.** a) shows the full experimental time course for preconditioned and control slices. b) shows the same data as above normalised prior to addition of ouabain in treated slices (preconditioned group 88.6 ± 2.3% (n = 6); control group 78.3 ± 3.2% (n = 9) ($p<0.05$)). The arrows (a. b, c and d) indicate the time points from which the sample waveforms shown in Figure 4.26c are taken.

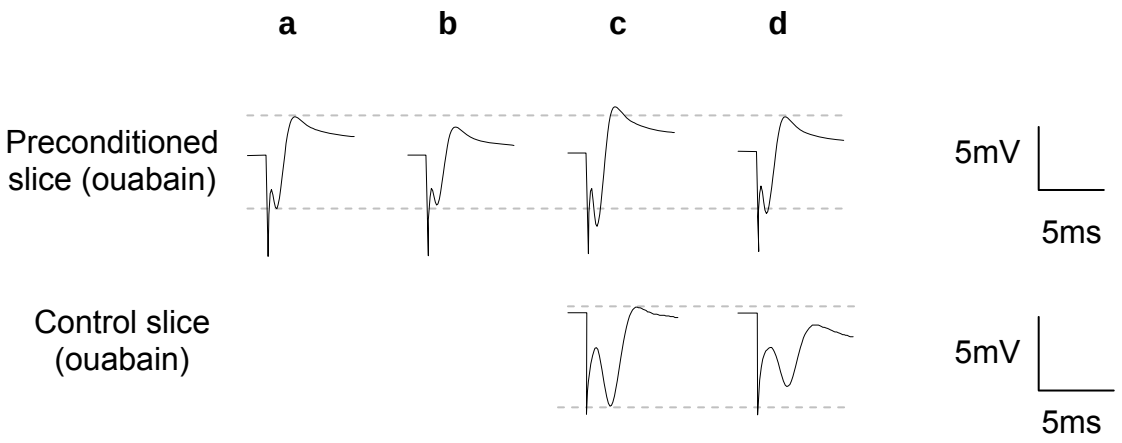


**Figure 4.26c Sample traces of antidromic population spikes taken from the time points as indicated Figure 4.26a.**

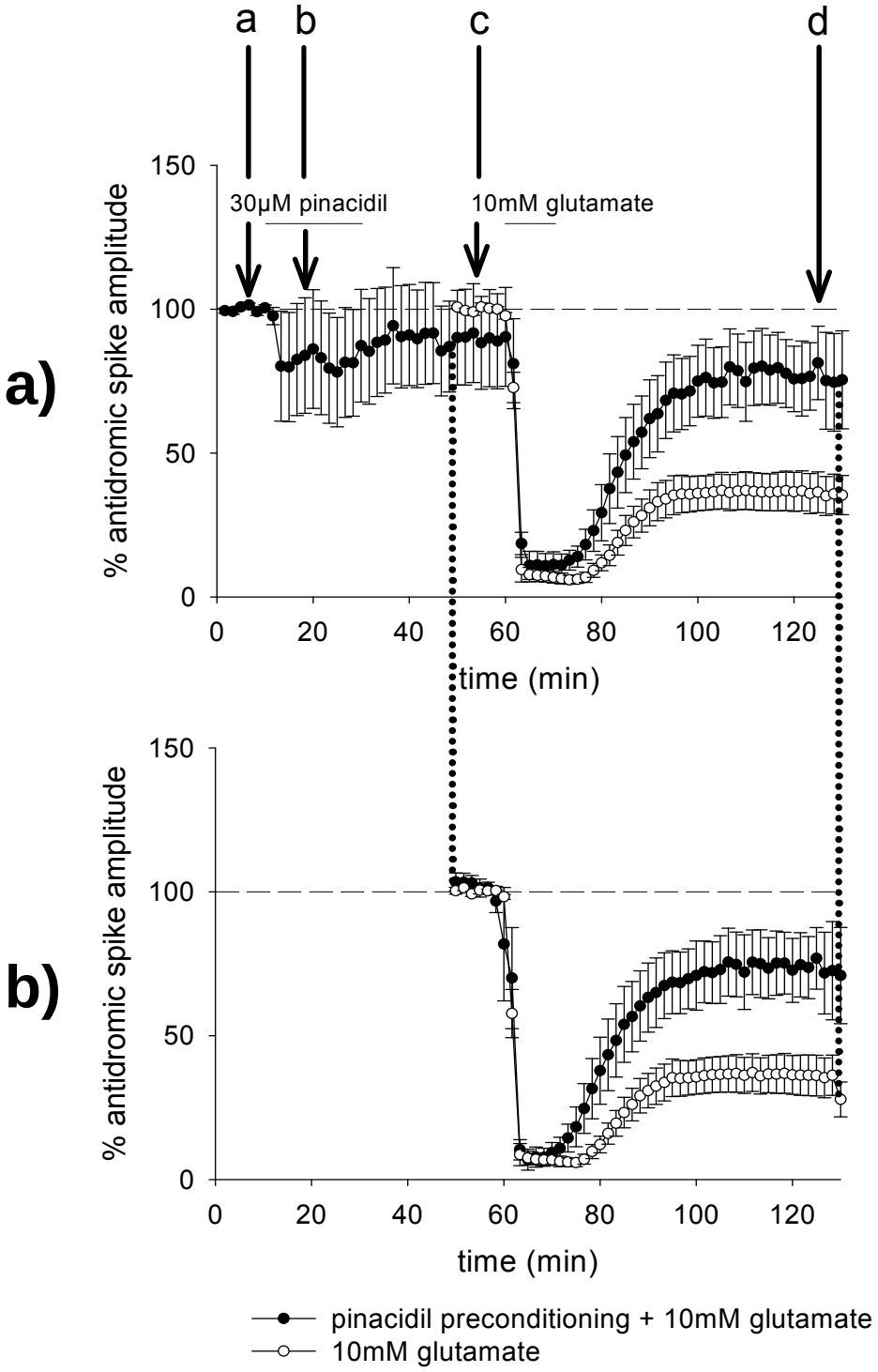


**Figure 4.27a,b. Pinacidil can precondition against the effects of glutamate upon orthodromic population spikes.** a) Time course graph showing the changes in orthodromic spike amplitude during pinacidil preconditioning. b) The same data as a) extrapolated and normalized from 10min prior to the application of 10mM glutamate. Preconditioned slices show a significant elevation in response compared with glutamate only treated controls (preconditioned group 73.3 ± 12.317% (n = 5); control group 36.3 ± 7.1% (n = 6) ($p<0.05$)). The arrows (a, b, c and d) indicate the time points from which the sample waveforms shown in Figure 4.27c are taken.

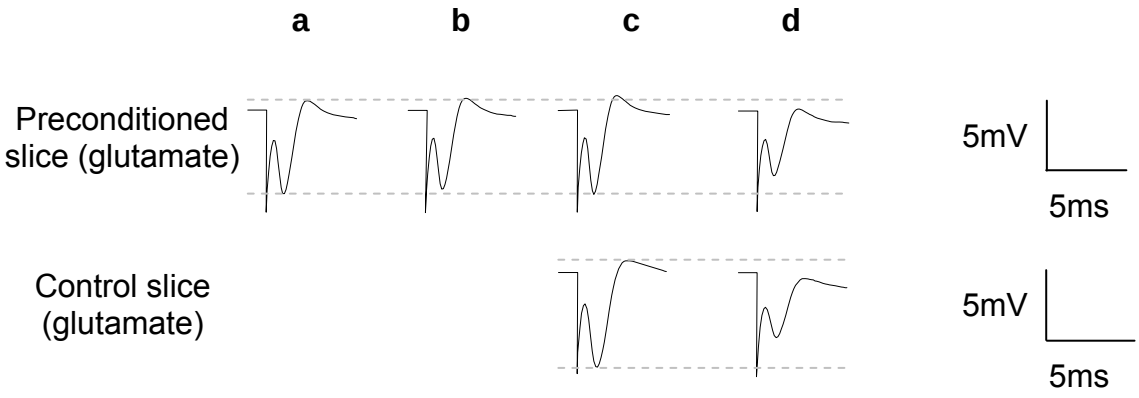


**Figure 4.27c Sample traces of antidromic population spikes taken from the time points inidicated in Figure 4.27a.**

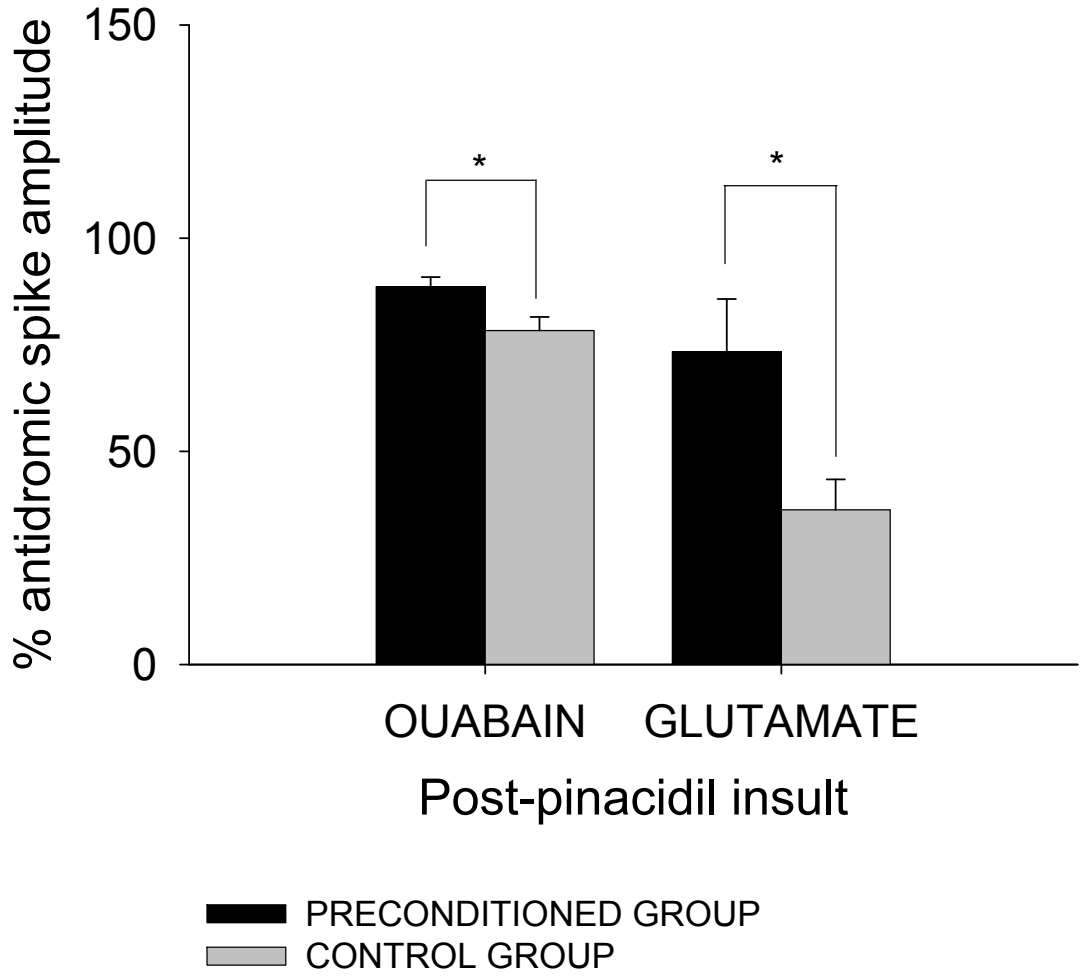


**Figure 4.28 Histogram showing the differences in recovery of antidromic spike amplitude between pinacidil preconditioned groups and controls with either glutamate or ouabain as chemical insults** (GLUTAMATE: preconditioned group 73.3 ± 12.317% (n = 5); control group 36.3 ± 7.1% (n = 6); OUABAIN: preconditioned group 88.6 ± 2.3% (n = 6); control group 78.3 ± 3.2% (n = 9)) (*$p<0.05$).

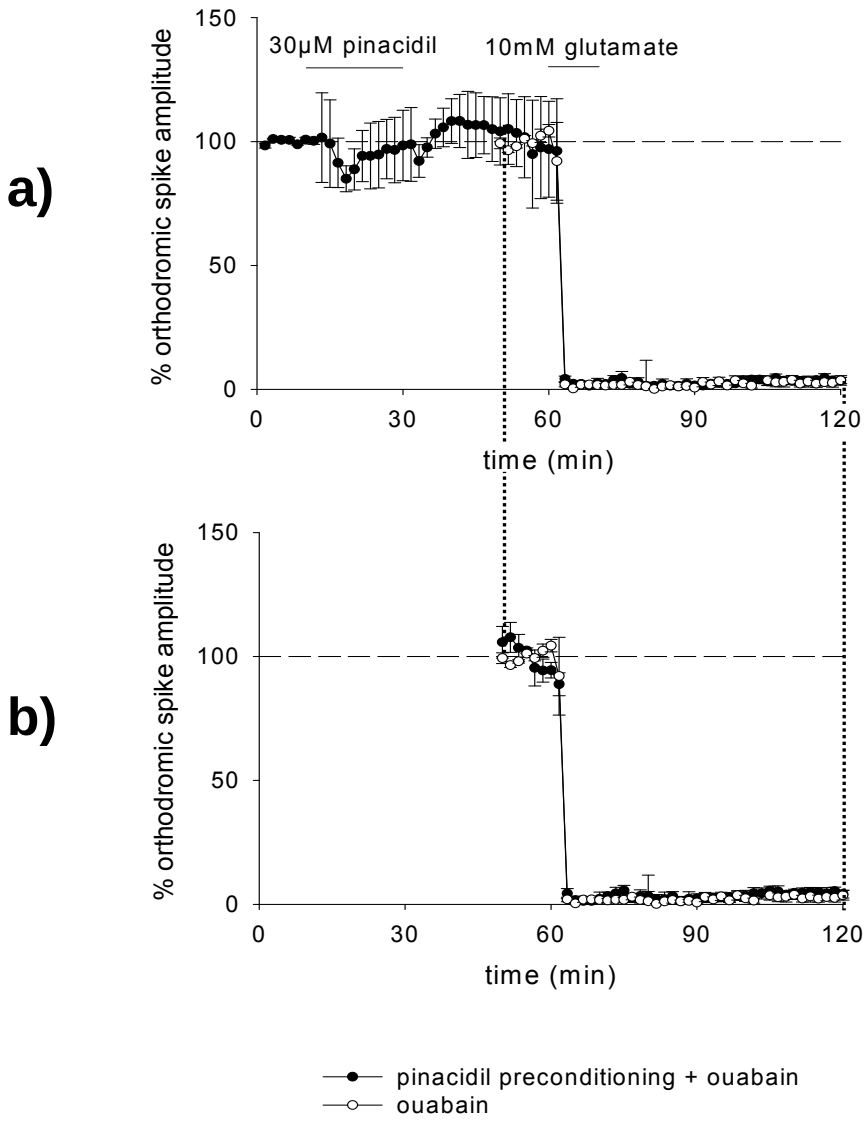


**Figure 4.29 Pinacidil preconditioning against glutamate in orthodromic spikes.** a) Time course changes in orthodromic spike amplitude during 30µMpinacidil preconditioning against 10mM glutamate. b) The same data as the top graph normalised prior to glutamate perfusion. There is no significant difference in post-glutamate recovery between preconditioned (n = 3) and control (n = 3) slices.

### 4.2.9 - Studies on HSP72 changes in response to pinacidil preconditioning against ouabain

Having observed a successful preconditioning outcome with pinacidil against ouabain, some of the slices from these experiments were saved to analyze any molecular changes later on. Naive tissue that was stimulated for the protocol duration for both pinacidil preconditioned and ouabain alone slices were saved for use in Western blotting to account for differences in stimulation times that might induce molecular changes. The electrophysiological data for these slices is shown in Figure 4.30. All these slices were analysed for changes in HSP72 expression. It was shown that there were no differences in expression between preconditioned slices and the corresponding naive slices or control slices and the corresponding naive slices. There were also no changes between ouabain only treated slices compared with pinacidil preconditioned slices (Fig 4.31 & 4.32). HSP72 expression appears to be unaffected by either ouabain treatment alone or preconditioning with pinacidil.

### 4.2.10 - The role of ATP-sensitive potassium channels and NMDA receptors in the pinacidil preconditioning effect against ouabain

To confirm that the preconditioning effects of pinacidil against ouabain observed in antidromic spikes were in fact, due to the opening of ATP-sensitive potassium channels, the protocol was repeated with 30μM glibenclamide, an ATP-sensitive potassium channel blocker, present before, during and after 30μM pinacidil was applied. It was observed the the elevated post-ouabain recovery of the antidromic population spike, previously observed in response to pinacidil preconditioning, was attenuated by the presence of glibenclamide in the perfusion medium (Fig 4.33). This confirmed that the effects were mediated by ATP-sensitive potassium channels.

Having established that NMDA receptors mediated the preconditioning effect of adenosine against ouabain in antidromic population spikes, it was interesting to see NMDA receptors also mediated pinacidil preconditioning. The pinacidil preconditioning protocol was repeated in the presence of the NMDA receptor antagonist, DL-AP5 (Fig 4.34). It was observed that the preconditioning effect was attenuated by the presence of the antagonist indicating that NMDA receptors are involved in the pinacidil preconditioning effect.

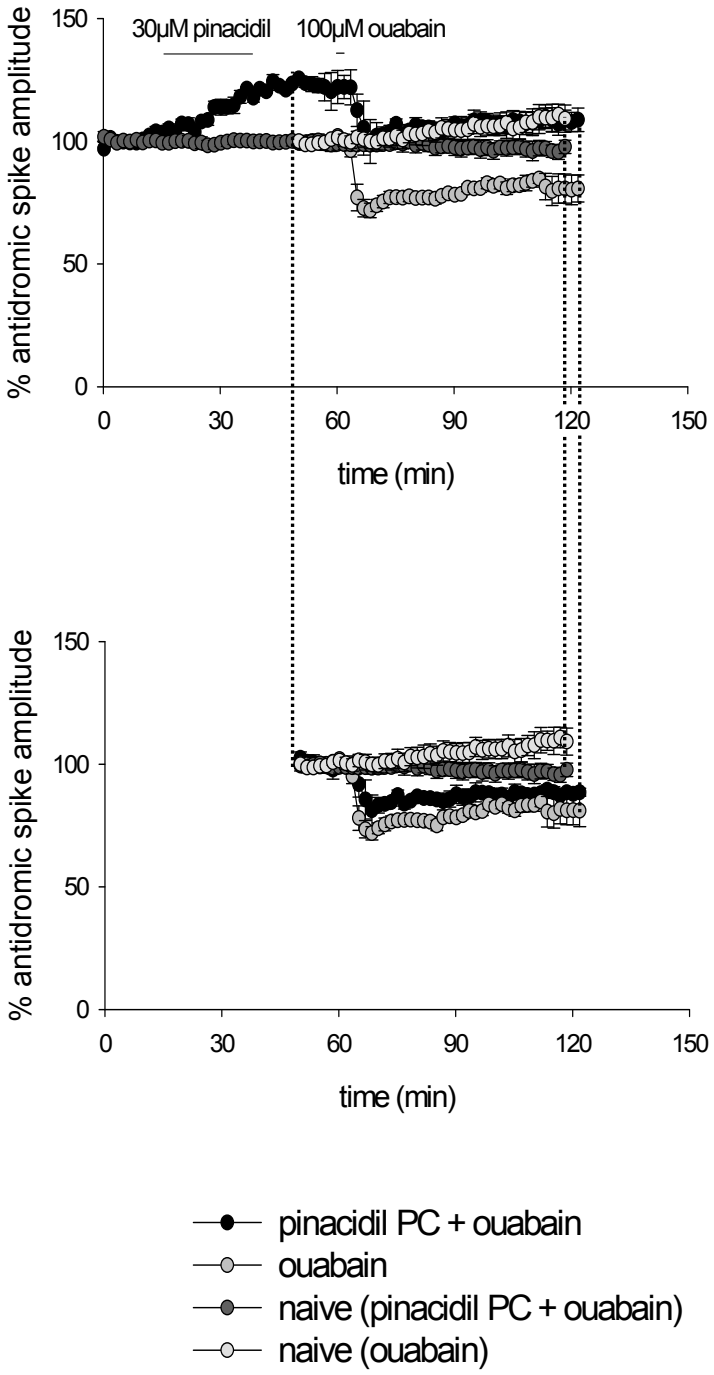


**Figure 4.30 Electrophysiological data from the slices used to generate the Western blots for the detection of HSP72 in Figure 4.31.** Note that these data show no statistical differences between groups; the complete data for this experiment is shown in Figure 4.26. Included also are the electrophysiological time course changes for naïve slices which were used to account for potential molecular changes induced by different stimulation times in the recording chamber. (PC = preconditioning).

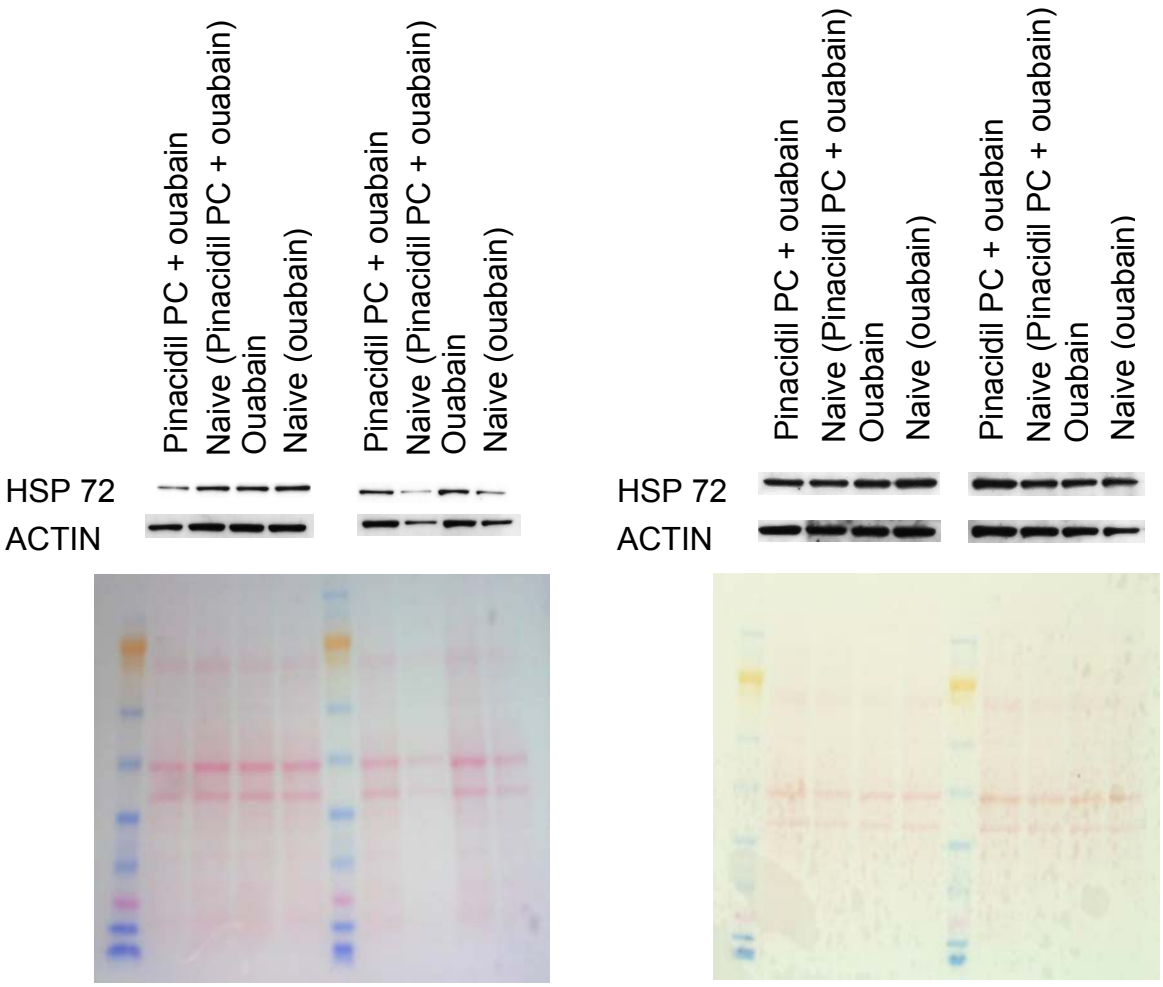


**Figure 4.31 Western blots for the detection of HSP72 from the slices saved from the electrophysiological experiments in Figure 4.30.** The Western blots show detection of HSP72 and actin with different treatments of slices indicated above. Shown below are Ponceau stains for each blot.

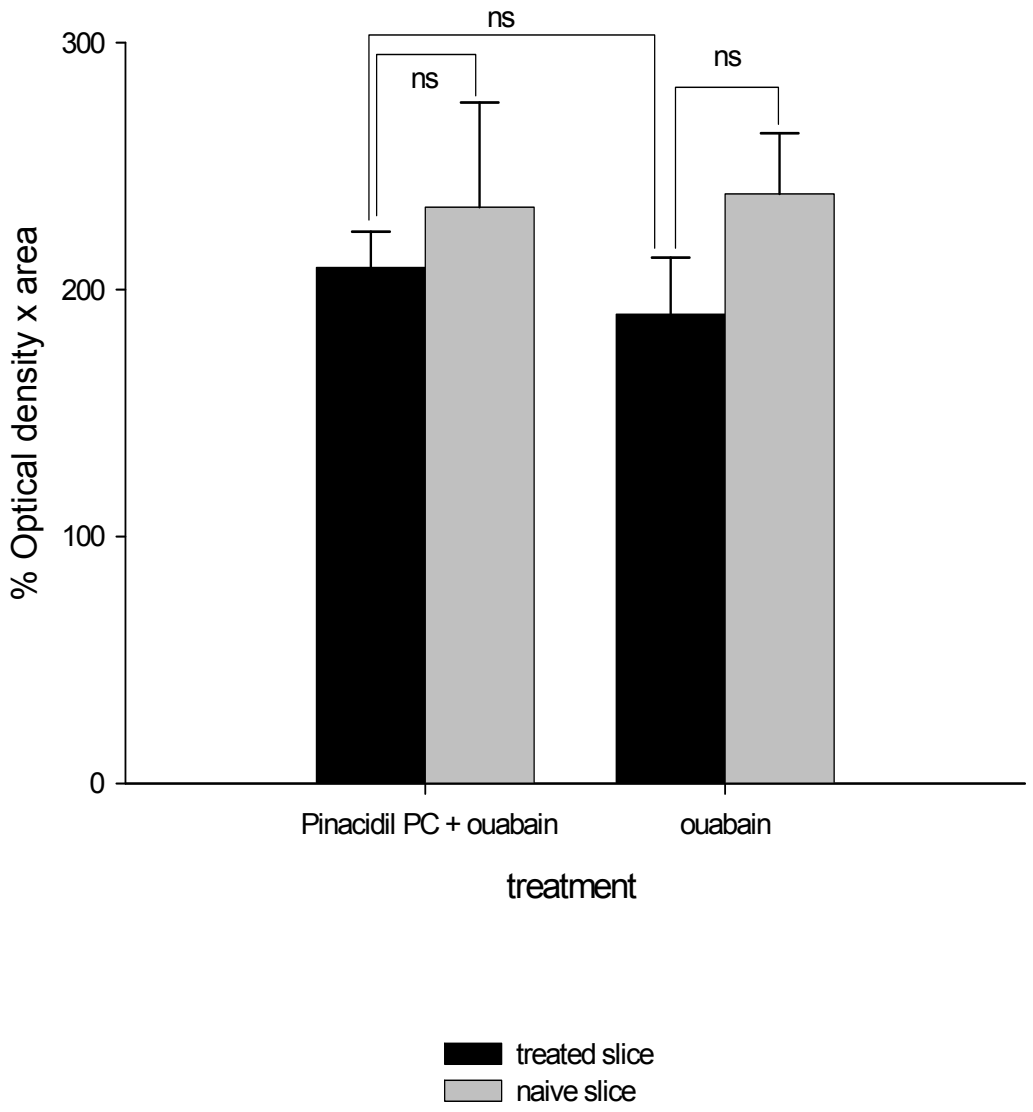


**Figure 4.32 Histogram summarising the results of the Western blots in Figure 4.31.** There are no significant changes in HSP72 between either treated slices and naïve slices or preconditioned slices and ouabain controls.

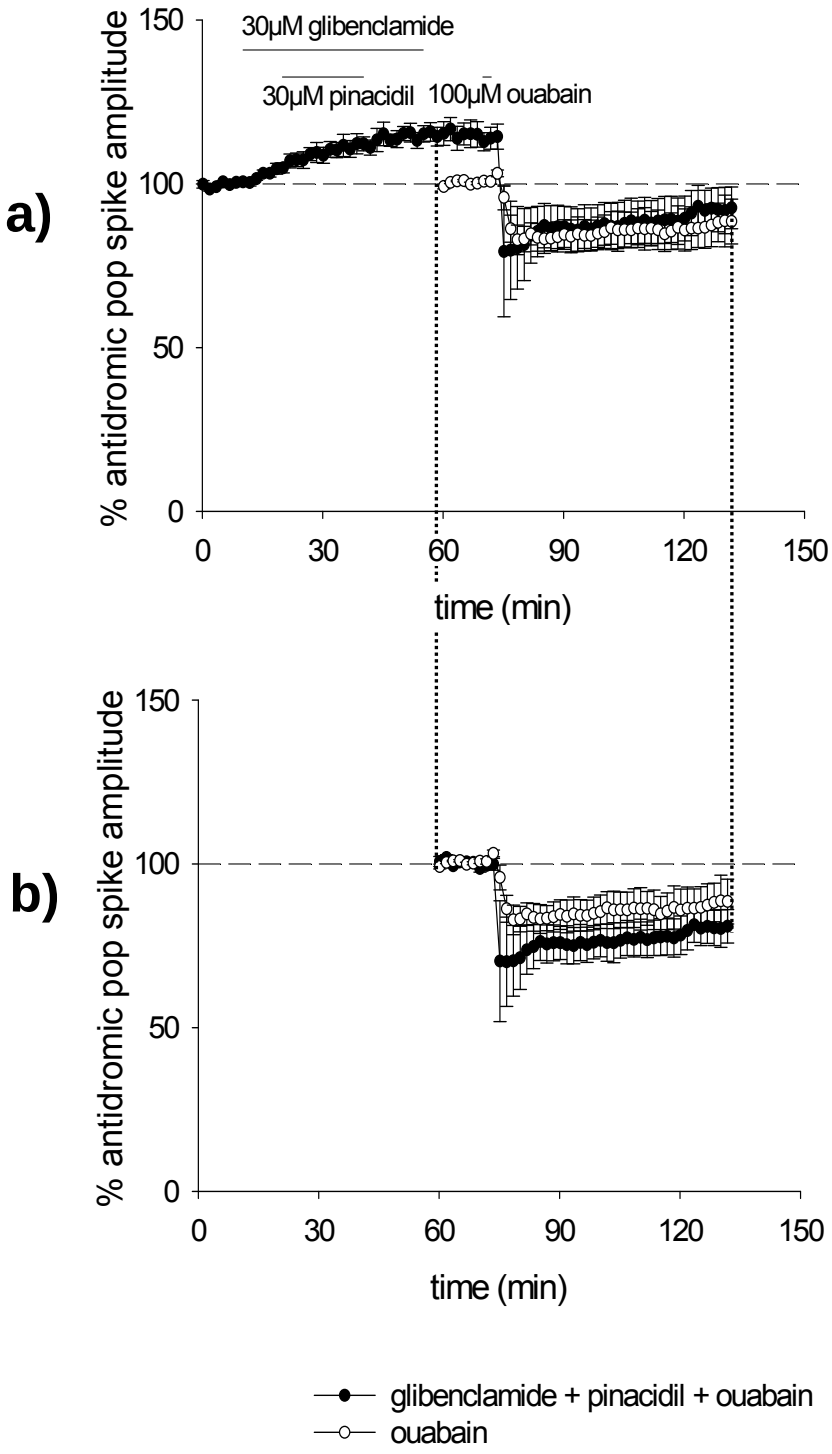


**Figure 4.33 Pinacidil preconditioning is prevented by blockade of ATP-sensitive potassium channels.** a) Perfusion of 30µM glibenclamide during 30µM pinacidil preconditioning against 100µM ouabain. Note that the elevation in antidromic spike size during and after glibenclamide perfusion is significant (114.6 ± 3.6%, $p<0.05$ one sample t test**).** b**)** the same data as in a) normalised prior to ouabain perfusion. There is no significant difference between preconditioned slices (n = 4) and controls (n = 4).

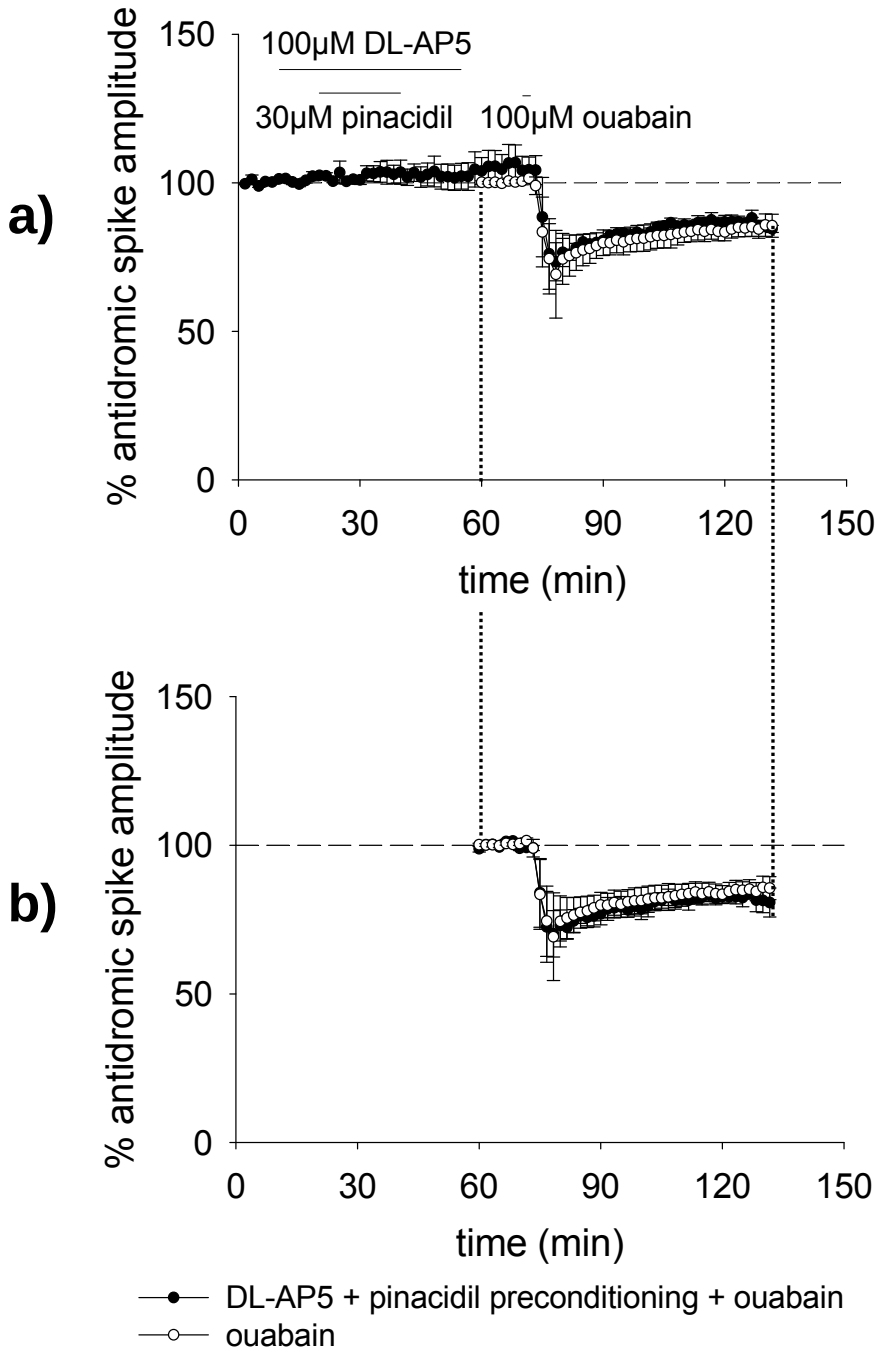


**Figure 4.34 Pinacidil preconditioning against ouabain in the presence of a NMDA receptor antagonist.** a**)** Perfusion of 100µM DL-AP5 during pinacidil preconditioning. b**)** The same data as above normalised prior to superfusion of 100µM ouabain. There is no significant difference between preconditioned slices (n = 3) and controls (n = 5).

### 4.2.11 - Stimulation with brief applications of glutamate causes a reduction in the size of evoked responses following ouabain

Since there is previous evidence that low concentrations of glutamate can precondition against a toxic application of glutamate (Schurr et al., 2000), it was decided to determine whether glutamate could also precondition responses to ouabain. Glutamate was applied 3 times at a concentration of 10mM for 2min. After the third glutamate application, ouabain (100μM) was applied for 2min resulting in a dramatic reduction of antidromic (Fig 4.35) and orthodromic (Fig 4.36) responses. The depression of potential size (epsps or antidromic) was significantly different from control responses to ouabain applied without preceding compounds (antidromic potentials: glutamate-treated n = 3; ouabain only n = 4, $p<0.05$; epsps: glutamate-treated n = 5; ouabain only n = 3; $p<0.05$ unpaired t-test with Welch correction).

As the interaction of glutamate upon the post-ouabain recovery of the responses was so profound, some of the slices were saved for later analysis of molecular changes. Naive tissue which was stimulated in the recording chamber for the corresponding length of time of the preconditioning protocol and the control slice protocol was also obtained to account for any differences that may occur on account of variable stimulation times. The electrophysiology for these slices is shown in Figure 4.37. Western blotting was used to detect any changes in HSP72 expression in the slices (Fig 4.38). The results showed that there were no discernable changes in HSP72 between naive slices and the corresponding treated slices or between preconditioned and control slices (Fig 4.39). This indicated that the glutamate-ouabain protocol did not induce any changes in HSP72.

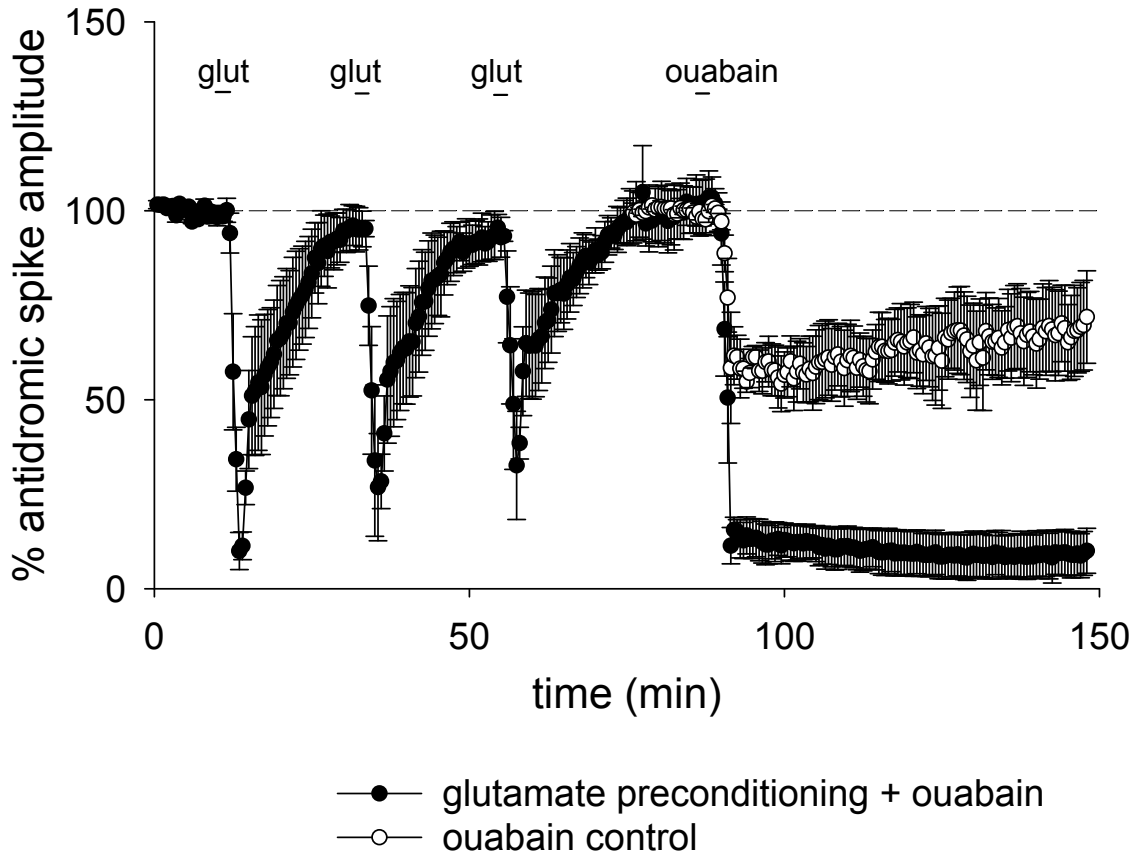


**Figure 4.35 The effects of glutamate preconditioning against ouabain upon antidromic population spikes.** 10mM glutamate preconditioning prior to application of ouabain in antidromic responses results in a significantly greater loss in spike size (26.2 ± 5.4%, n = 3) compared with ouabain only treated responses (68.1 ± 11.0%, n = 4) ($p<0.05$).

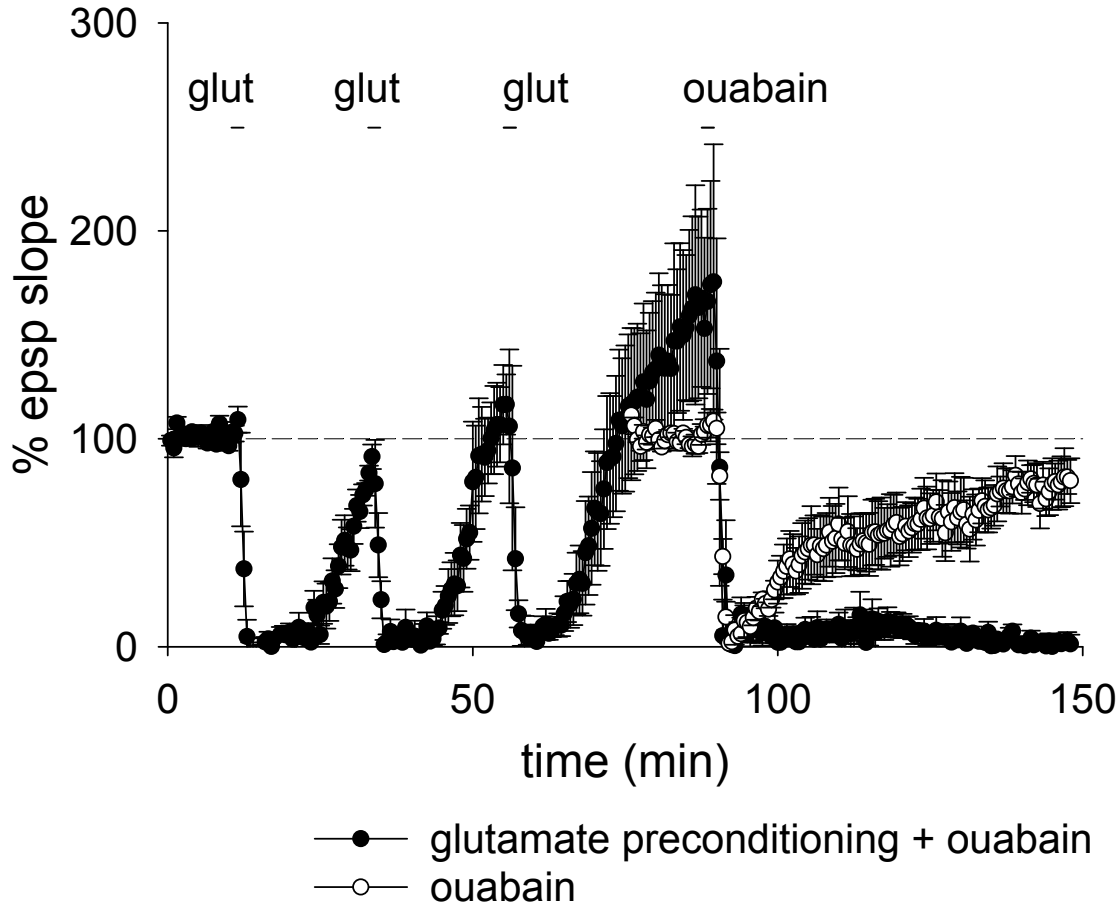


**Figure 4.36 The effects of glutamate preconditioning against ouabain upon field epsps.** 10mM glutamate preconditioning prior to application of ouabain in epsp responses results in a significantly greater loss in slope size (1.4 ± 2.2%, n = 5) compared with ouabain only treated responses (77.3 ± 9.7%, n = 4) ($p<0.005$) unpaired t test with Welch correction *(glut = glutamate). Note that percentage values in the legend are derived by taking the average epsp slope size in the 10min period prior to ouabain application as 100%. The figure shows 100% baseline as the 10min period prior to the first application of glutamate for the preconditioned responses.

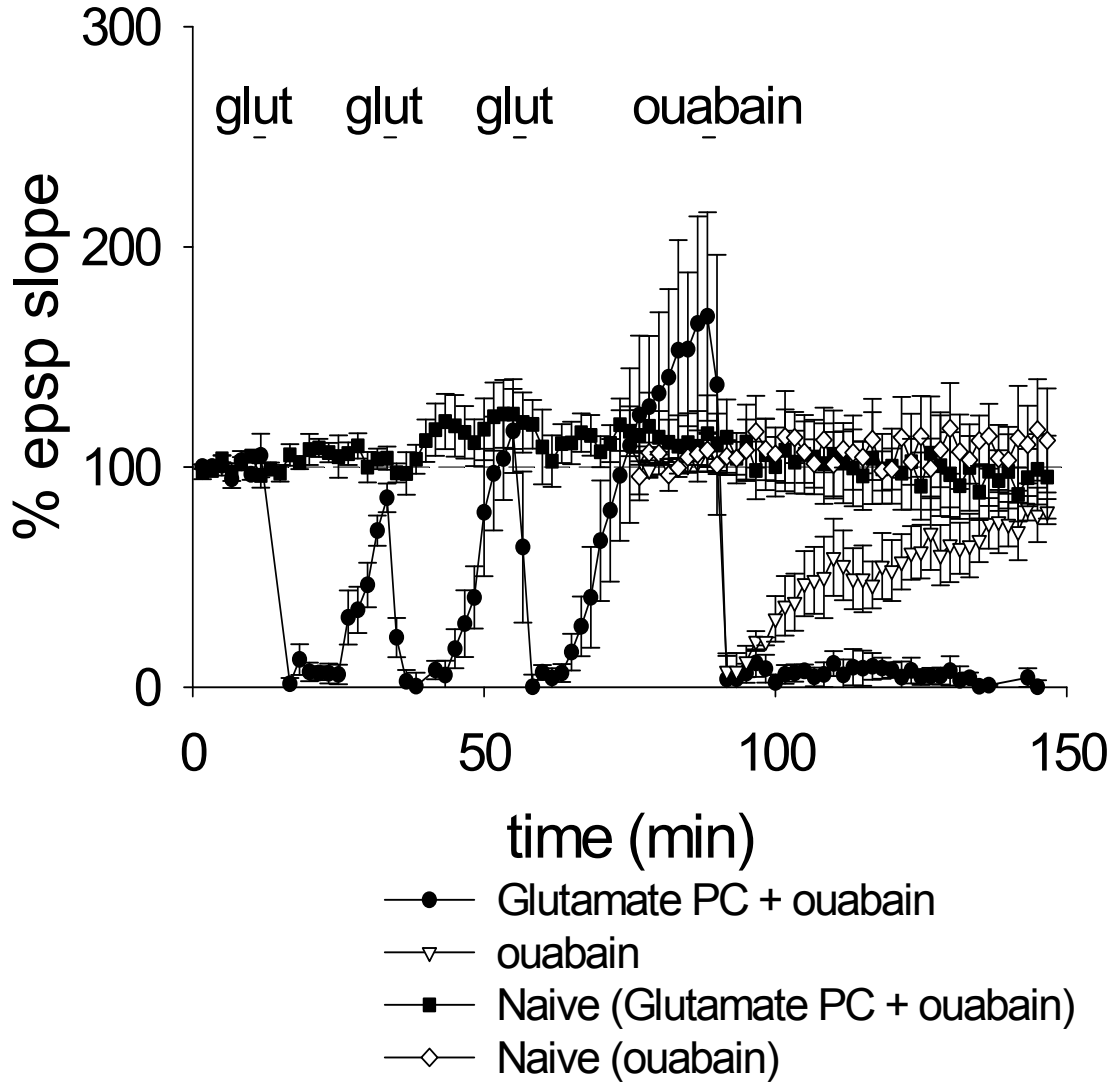


**Figure 4.37 Electrophysiology of the slices used in the Western blots in Figure 4.36.** The time course graph shows in the changes in epsp slope in response to 10mM glutamate preconditioning prior to application of ouabain. Preconditioned epsp responses show a significantly greater loss in slope size in response to ouabain (2.0 ± 5.6%, n = 4) compared with ouabain only treated responses (77.3 ± 9.7%, n = 4) ($p = 0.005$ unpaired t test with Welch correction). Also shown are the time course changes in epsp slope recorded from slices which were stimulated for the same length of time as each treatment protocol, referred to as naive slices. *(glut = glutamate) (PC = preconditioning).

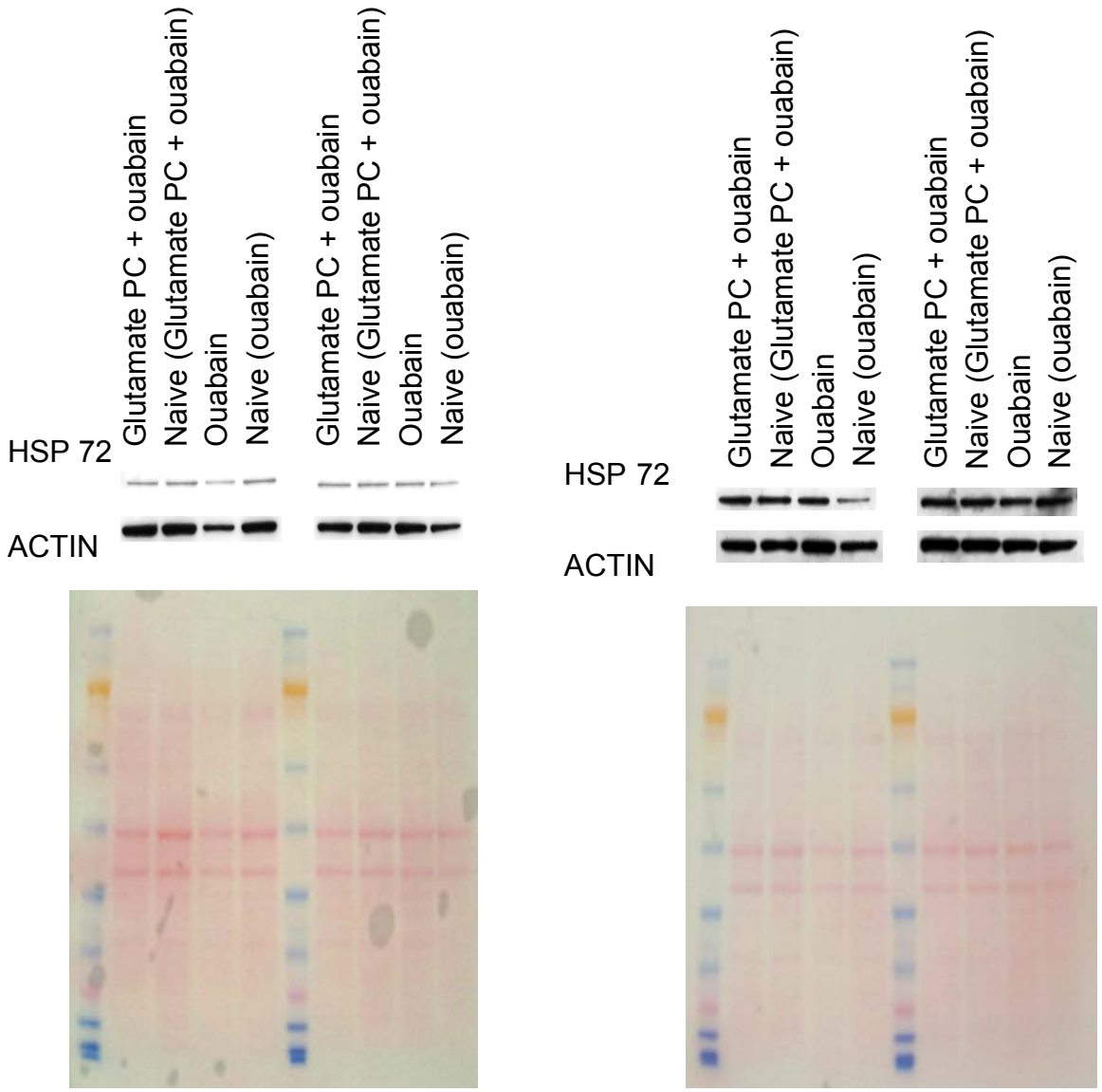


**Figure 4.38 Western blots showing the immumodetection of HSP72 and actin levels in slices receiving glutamate preconditioning treatments followed by ouabain or ouabain only.** The blots for the naive tissue show the detection of HSP72 and actin levels in slices stimulated for the same length of time as the chemically treated slices as shown in Figure 4.37. The corresponding Ponceau stains are shown (in colour). (PC = preconditioning).

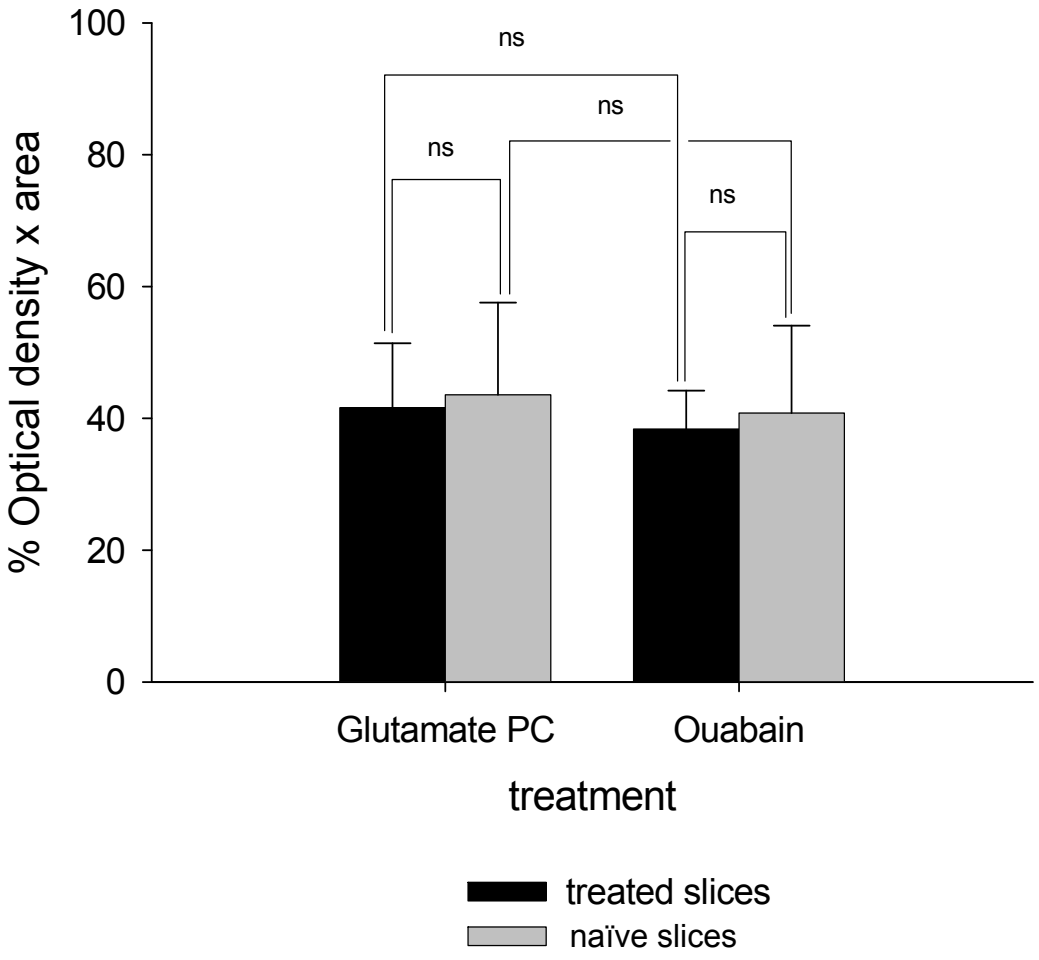


**Figure 4.39 Histogram summarising the changes in HSP72 expression in glutamate preconditioned slices and ouabain treated slices.** There are no significant changes between treatment groups (PC = preconditioning) (ns = not significant Bonferroni multiple comparisons test).

### 4.2.12 - Modulation of the effects of ouabain in antidromic spikes by calcium manipulation

It was decided to examine the role, if any, of calcium in the effects of ouabain upon hippocampal responses as changes in calcium dynamics may alter to preconditioning (Bickler & Fahlman, 2004; Pérez-Pinzón et al., 1999). The intracellular calcium chelator, BAPTA-AM (10μM) was perfused for 20min and had no effect on the antidromic population spike ($p = 0.6885$) (Fig 4.40). 100μM ouabain was then perfused for 2min in the presence of BAPTA-AM resulting in a dramatic loss in antidromic spike amplitude (2.4 ± 1.9%, n = 4) compared to ouabain only (83.5 ± 2.5%, n = 4) ($p<0.001$) (Fig 4.40) indicating that intracellular calcium is needed for recovery. To determine the exact nature of the role of calcium, we altered the concentration of $CaCl_2$ in normal aCSF (2.5mM $CaCl_2$) to 0.5mM or 5mM. Changes in [$CaCl_2$] alone did not significant alter the antidromic spike amplitude after 30min (Fig 4.41) as expected from the absence of synaptic involvement in the antidromic potential. When 100μM ouabain was perfused onto the slice after 30min of altered [$CaCl_2$], O.5mM $CaCl_2$ produced dramatic reductions in response size similar to that seen for BAPTA-AM whilst 5mM $CaCl_2$ prevented any significant change in the size of the response (107.9 ± 12.8%, $p = 0.5568$ one-sample t-test). Given that BAPTA-AM or low extracellular calcium appear to enhance the depressant effect of ouabain and that the effect of ouabain can be attenuated by raising extracellular calcium concentration, it seems that ouabain induces its reduction in antidromic spike amplitude by decreasing intracellular calcium.

To investigate further the possibility that intracellular calcium reduction is an effect of ouabain, potential downstream consequences on calcium-activated K+ channels were examined. The large conductance calcium-activated K+ channels (BK+ channels) were selected in particular as they have a reported role in preconditioning in cardiovascular tissue (Shintani et al., 2004; Feng et al., 2006). Charybdotoxin (ChTX), a selective channel blocker for BK+ channels was used at a concentration (25nM) that is reported not to induce changes in population spike amplitude although it may cause some spike broadening (Shao et al., 1999). We perfused ChTX for 1min prior to perfusion of ouabain and then for 3min after ouabain removal from the medium (Fig 4.42). The ChTX + ouabain group showed an enhanced depression of antidromic spike amplitude which continued until the end of the experiment (58.4 ± 7.0%, n = 4) compared to ouabain alone (74.9 ± 3.5%, n = 6)($p<0.05$). This suggests further that intracellular calcium concentration is decreased upon ouabain application resulting in an inhibition of BK+ channels.

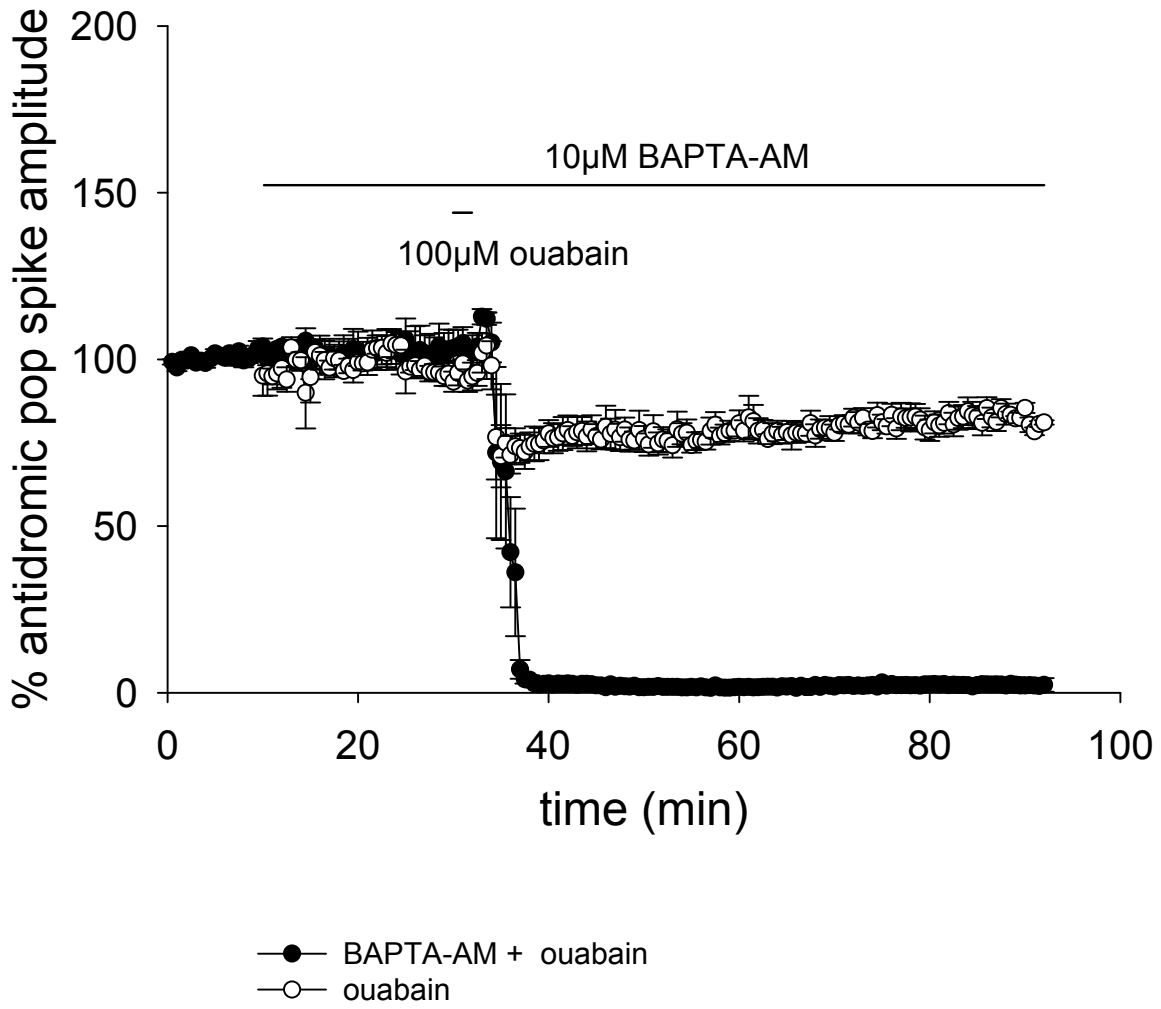


**Figure 4.40 Modulation of the effect of ouabain upon the antidromic spike by BAPTA-AM** a) Chelation of intracellular calcium using BAPTA-AM induces a dramatic loss in spike amplitude in response to ouabain perfusion. Note that BAPTA-AM alone does not affect antidromic spike amplitude.

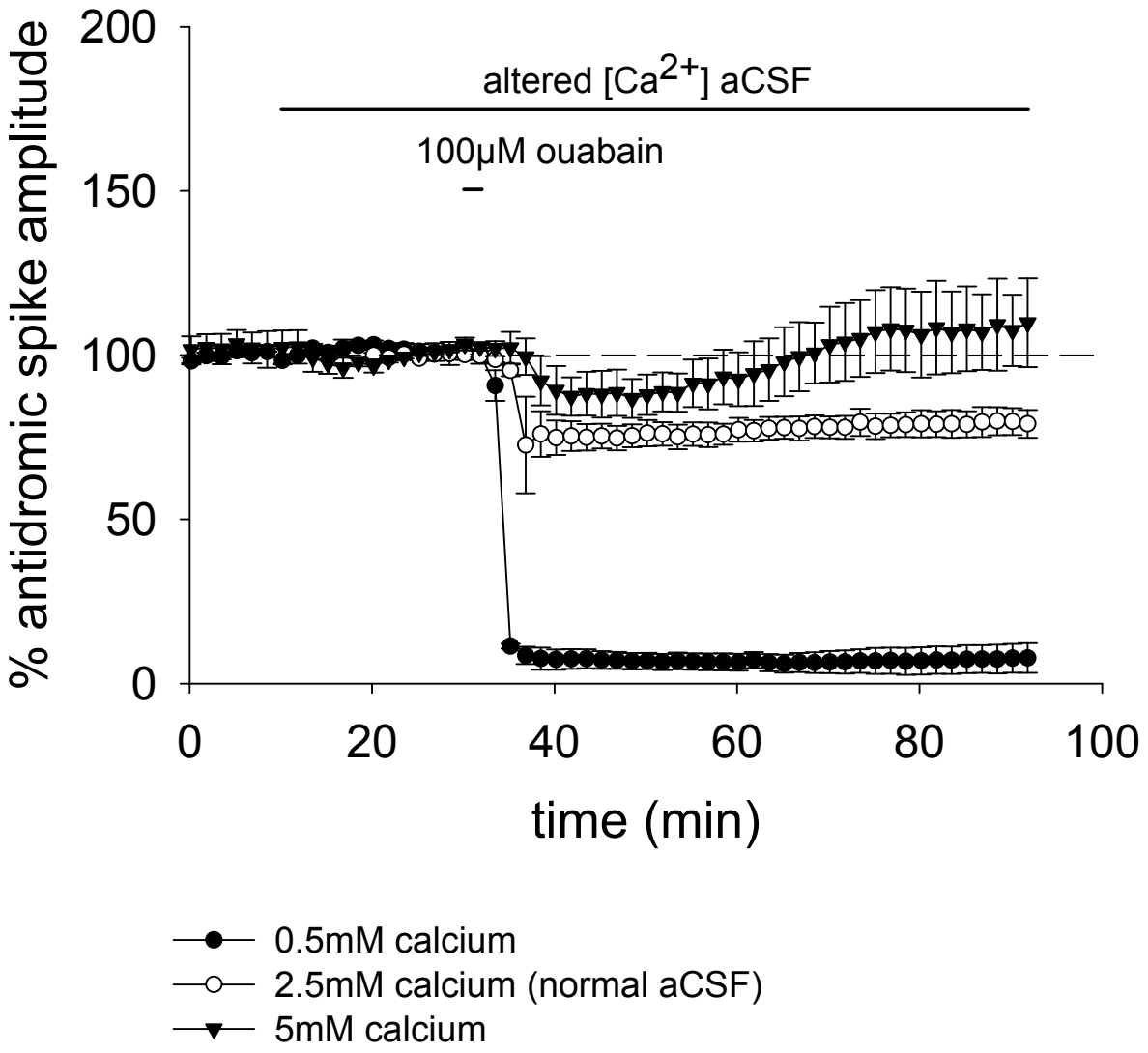


**Figure 4.41 Altered effects of ouabain in the presence of higher and lower concentrations of $CaCl_2$.** 0.5mM $CaCl_2$ enhances the depression of antidromic spikes by ouabain (n = 3) compared to normal aCSF (n = 5) ($p<0.0001$). Ouabain is unable to produce a significant change in spike amplitude in the presence of 5mM $CaCl_2$ (107.9 ± 12.8%, p = 0.5568 one-sample t-test). Note that spike amplitude is unaffected by changes in [$CaCl_2$] alone.

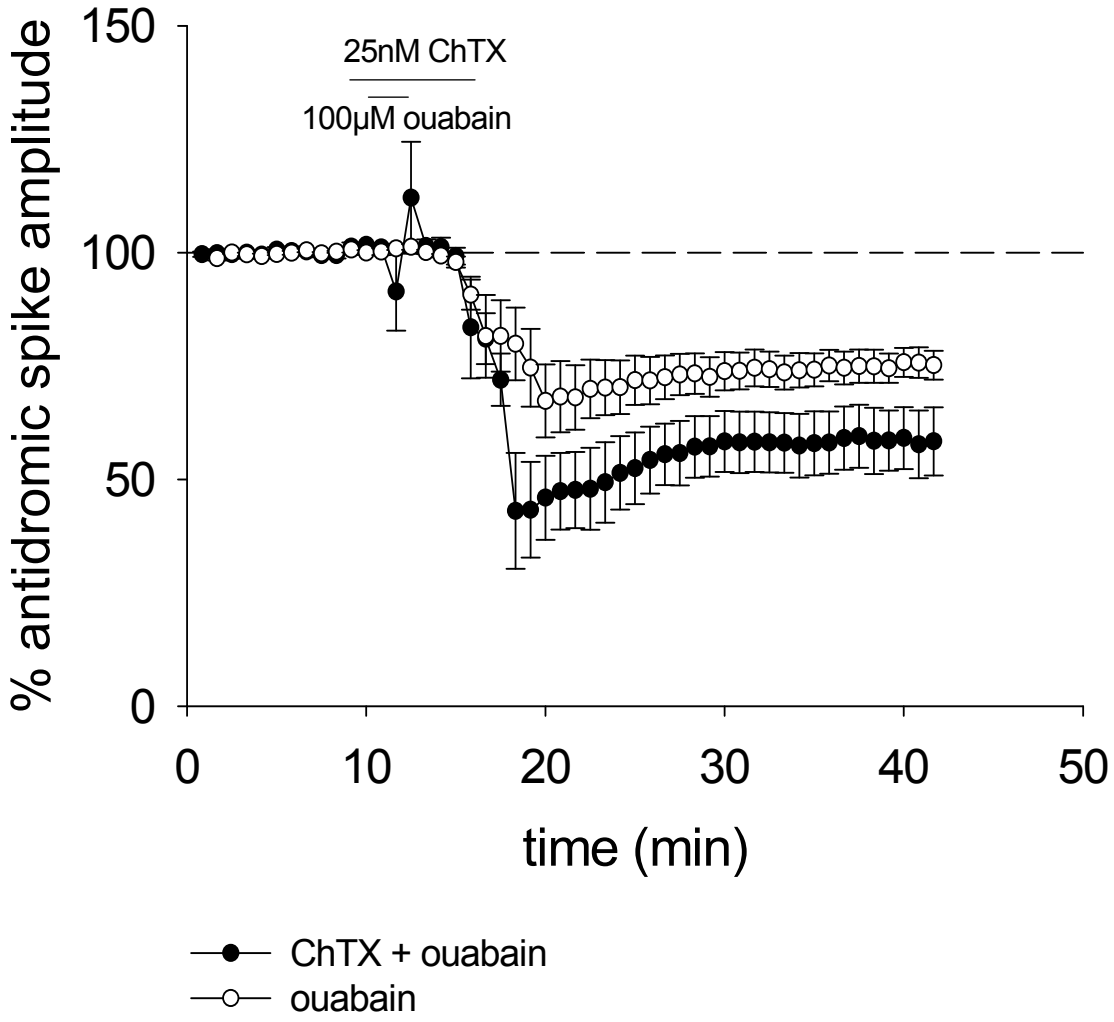


**Figure 4.42 BK+ channel blockade enhances the ouabain-induced depression of antidromic spikes** (ChTX = charybdotoxin).

# 4.3 – Discussion

## 4.3.1 –The effects of 10mM glutamate upon the hippocampal slice

### 4.3.1.1 - 10mM glutamate: effects on evoked responses

Unlike 5mM glutamate, 10mM glutamate induced a significant depression in antidromic responses. The reduced action potential firing cannot be due to a glutamate-induced change in synaptic plasticity but probably some membrane dysfunction resulting from the likely entry of sodium and calcium ions through the glutamate ionotropic receptors. This would result in a change in resting membrane potential and subsequent interference with the opening of voltage-gated ion channels. Although the sodium-potassium pump should restore the ion equilibrium after membrane depolarization, the lasting depression in post-glutamate recovery in the antidromic spikes may indicate that excessive ion influx has resulted in a cell swelling and subsequent damage to the structural integrity of the neuron. Bandyopadhyay et al. (2002) demonstrated cell swelling in slices in response to 100μM NMDA by using optical image scattering. The reduced potential size in the orthodromic responses following glutamate may be indicative of a similar effect or it could be due to a change in synaptic plasticity. This might include glutamate-induced long-term depression or increased inhibitory neurotransmission.

The effects of glutamate itself on the evoked responses from the hippocampal slice are consistent with those reported in the literature (Wallis et al., 1994; Alici et al., 1996; Obrenovitch et al., 1997). Some studies confirm a loss of electrophysiological responsiveness that is highly comparable to that reported here, mainly a complete loss of evoked responses upon application of millimolar concentrations of glutamate for several minutes which returns during washout to a reduced level (Wallis et al., 1994; Alici et al., 1996). Wallis et al. (1994) observed these effects using 9mM glutamate for 8min; this resulted in approximately 25% recoveries for antidromic and orthodromic population spikes at 1hour post-glutamate. They concluded that the reduced recovery for antidromic population spike amplitude was evidence of excitotoxicity. The epsp slope showed no recovery using the same protocol. The authors also noted that 5mM glutamate for 8min exposure did not induce a significant depression in the recovery of antidromic and orthodromic population spikes and concluded that 5mM glutamate was not a toxic concentration. Alici et al. (1996) applied 5mM and 10mM glutamate to hippocampal slices for 20min and measured the extracellular calcium concentration using calcium-sensitive electrodes. They observed that extracellular calcium concentration decreased from 1.2mM to approximately 0.83mM and 0.79mM for 5 and 10mM glutamate respectively. They

noted that the calcium concentration returned to baseline levels within 15-20min of the washout period, an effect which is consistent with the return of the evoked responses in the results presented in this thesis. Alici et al. (1996) also observed that antidromic and orthodromic responses recovered to baseline levels following treatment with 10mM glutamate whilst higher concentrations (20mM and 50mM) induced an irreversible loss of response. The effects of glutamate on evoked responses presented in the results appear to agree with those of Wallis et al. (1994) and disagree with Alici et al. (1996). This might be on account of the calcium concentrations used in each study. Alici et al., (1996) used 1.6mM $CaCl_2$ in the aCSF whilst Wallis et al. (1994) used 2.4mM $CaCl_2$ which is closer to the concentration used in the present study (2.5mM $CaCl_2$). This could explain the difference in sensitivity of evoked responses to glutamate shown in each study as calcium influx has been shown to mediate glutamate-induced extended neuronal depolarization in hippocampal neuronal cultures (Limbrick et al., 2003).

The effects of exogenously applied glutamate have also been examined in other neuronal preparations. Obrenovitch et al. (1997) have observed a concentration-dependent depolarization of the intracellular potential upon intracerebral application of 5 or 20mM glutamate for 2min to striatal tissue. Sombati et al. (1991), Coulter et al. (1992) and Limbrick et al. (2003) have observed similar changes in membrane potential in hippocampal neuronal cultures using 500μM glutamate in the presence of 10μM glycine. In these studies glutamate exposure produced burst firing followed by a depolarization of the membrane potential. During glutamate washout, the membrane potential remained depolarized to approximately -5mV for hours post-treatment; this effect is termed "extended neuronal depolarization" by the authors. Coulter et al. (1992) demonstrated that there was no change in responsiveness to glutamate or cell morphology at 4hours post-glutamate but that cytotoxicity occurred at 24hrs post glutamate. It was also shown that calcium influx is required for the induction (Coulter et al., 1992) and maintenance (Limbrick et al., 2003) of extended neuronal depolarization. Hippocampal neuronal cultures are more sensitive to glutamate than acute slice preparations and die from exposure to micromolar concentrations. This increased sensitivity is presumably due to the lack of glia and their glutamate transporters to reduce extracellular glutamate accumulation. In spite of the difference in glutamate concentration used, the time course and nature of changes in membrane potential in hippocampal cultures are similar to those in the evoked responses presented here. It is possible that extended neuronal depolarization underlies some of the effects observed in hippocampal slices in relation to low post-glutamate recovery of evoked responses.

#### 4.3.1.2 - 10mM glutamate: effects on LTP

The post-glutamate response to a 100Hz/1sec stimulus again differed greatly between 5mM and 10mM glutamate concentrations. The depression in post-10mM glutamate responses was not reversed using a 50% baseline. The effects of stimulus intensity on the degree of LTP induced by theta frequency stimulation as reported by Leung & Au (1994) may explain the discrepancy between 5mM and 10mM glutamate concentrations which used 25% maximal and 50% maximal baselines respectively in the present study. Leung & Au reported that larger potentiations were obtained using smaller baselines therefore the use of a 25% maximal baseline for 5mM glutamate may have induced LTP whereas 50% maximal baselines for 10mM glutamate may not. However, control LTP responses were obtained in slices taken from the same hippocampus as those which received 10mM glutamate stimulation; as a 100Hz/1sec stimulus would be expected to reverse the effects of LTD (Dudek & Bear, 1992; Dudek & Bear, 1993), the failure of such a stimulus to reverse the effects of 10mM glutamate indicates that this concentration does not induce LTD. Taken together with the reduction of antidromic spike amplitudes following treatment with 10mM glutamate, it can be suggested that 10mM glutamate exerts a toxic effect upon the tissue.

#### 4.3.1.3 - 10mM glutamate: effects on heat shock protein expression

Potential changes in heat shock protein expression were also examined in response to 10mM glutamate exposure in hippocampal tissue. No statistically significant difference in heat shock protein 72 expression between naive tissue and glutamate-exposed tissue. Changes in heat shock protein expression have been noted in hippocampal tissue in response to excitotoxicity in vivo (Ayala & Tapia, 2003). In the study by Ayala & Tapia (2003), rats were treated with 4-AP (a potassium channel blocker) via microdialysis in the hippocampus. Electroencephalogram recording showed seizure activity in response to 4-AP which was inhibited by the glutamate antagonists, MK-801 and NBQX. It was also noted that extracellular glutamate concentration was increased in 4-AP exposed tissue. However, they were unable to detect changes in heat shock protein 72 until at least 6 hours post-treatment had elapsed. A different study has shown dectection of changes in heat shock protein expression within 30min within the hippocampus in vivo (Galli et al., 2006) although it has also been reported that HSP72 expression in the CA1 region does not appear until 16hours after 5min ischaemia induced by a four-vessel occlusion (Nishi et al., 1993). The 1hour time point post-glutamate at which the slices were frozen may explain

why no significant changes were detected in protein expression in the results presented here.

#### 4.3.1.4 - Assessing toxicity to acute injury

Direct assessment of glutamate toxicity in hippocampal slices has been difficult in this study and others. Lozovaya et al. (2004) have indicated that exogenously applied glutamate only reaches the cell body in hippocampal slices as the synapses are protected by the uptake mechanisms of the glutamate transporters; that study however used micromolar concentrations of glutamate which are much lower than the millimolar concentrations used in the present study. Virgili et al. (1997) have detected reduced rates of protein synthesis following exposure to 5mM glutamate in hippocampal slices. Studies examining positive indicators of cell death such as morphological changes or molecular markers in response to such exogenous chemical insults are lacking in slice systems, mainly because of the acute nature of electrophysiological responsiveness compared with the time scale taken to induce such changes which may extend from several hours to days. It has been noted in hippocampal neuronal cultures that exposure to 500μM glutamate shows a neuronal survival of 70-80% in the short-term; at 24hours it was observed that more neurons then died highlighting the difficulty in assessing short-term changes with long-term (Coulter et al., 1992). Lees and Sandberg (1991) conducted electrophysiology in vivo to obtain histological changes several days after a glutamate insult although they were able to correlate changes in evoked responses with delayed cell death. For in vitro systems, slice cultures may prove a more suitable tool for assessing electrophysiology with delayed cell death. For acute changes in evoked responses however, visual analysis may be better to confirm tissue injury using agents such as propidium iodide or Rhodamine 123, both of which require confocal microscopy. Optical image scattering may also be used to assess cell swelling rather than molecular markers or changes in morphology of hippocampal slices.

### 4.3.2 - Adenosine preconditioning: interaction with exogenous glutamate

No interaction was found between adenosine preconditioning and exogenous glutamate using various concentrations of adenosine and different preconditioning protocols. This was a surprising result as adenosine has been shown to precondition against hypoxia (Pérez-Pinzón et al. (1996) using 100μM adenosine for 10min, a protocol which was

attempted against glutamate in the present study. Adenosine is released during hypoxia (Pedata et al., 1993) and also during NMDA receptor stimulation (Manzoni et al., 1994; Hoehn & White, 1990) so that a potential interaction seemed likely. In addition adenosine may trigger glutamate release (Okada et al., 1992) in some circumstances leading one to hypothesize that an indirect excitotoxic preconditioning effect might occur. No interaction was observed in orthodromic or antidromic population spikes here suggesting that any potential glutamate release is not sufficient to precondition against 10mM glutamate or that adenosine release during hypoxia or NMDA receptor stimulation is unaffected by adenosine preconditioning.

Adenosine might be expected to precondition against excitotoxicity by inhibiting glutamate release pre-synaptically via A1 receptor stimulation. This may explain the discrepancy between anoxia used by Pérez-Pinzón et al. (1996) and glutamate used in the present study.

### 4.3.3 - Effects of ouabain on evoked responses: excitatory transmission

The sodium-potassium ATPase inhibitor ouabain produced multiple spikes in evoked field potentials followed by a depression in response size. Ouabain-induced multiple spiking of evoked responses has been attributed to calcium influx (McCarren & Alger, 1987). The depression in potential size was the parameter examined in the present study. It was first noted that antidromic population spikes showed a significantly smaller depression in response size than orthodromic spikes and field epsps. The discrepancy in effect seems to be due to differences in glutamate receptor stimulation at the cell body and dendrites in response to ouabain exposure. The broad spectrum glutamate receptor antagonist, kynurenic acid (Perkins & Stone, 1982; Stone & Darlington, 2002), was able to prevent the ouabain-induced depression in orthodromic population spikes which are driven by synaptic transmission whilst the significantly smaller depression in antidromic population spikes was unaffected by glutamate receptor blockade. These results are consistent with those of Jarvis et al. (2001) who showed that neocortical brain slices showed a decrease in light transmittance in response to ouabain which is consistent with water uptake into the cell. The authors also showed that this effect occurred in the presence of kynurenic acid. As the protocol for ouabain application to the tissue was exactly the same as that used in the present study (100μM ouabain applied for 2min), it seems likely that the results presented here using antidromic spikes in the presence of ouabain reflect a degree of cell swelling in the hippocampal slice that occurs independently of glutamate. This is also consistent with a reported observation that exogenously applied glutamate only reaches the cell body in

hippocampal slices as the synapses are protected by the uptake mechanisms of the glutamate transporters (Lozovaya et al., 2004). It can therefore be assumed that synaptically released glutamate would not reach the cell body as a result of the same uptake mechanisms. This may be considered as a useful model of ischaemia in the rat hippocampal slice as 10min OGD exposure in this experimental system has yielded cell swelling and loss of electrophysiological responsiveness in CA1 neurons that was not mediated by glutamate (Obeidat et al., 2000); these effects were attributed to damage that occurred from spreading depression, a phenomenon that induces a wave of electrical silence over neuronal tissue and which occurs independently of glutamate. Ouabain has been considered to be a useful model of spreading depression in other studies which utilize hippocampal slice systems (Barsarksy et al., 1998; Obeidat & Andrew, 1999; Basarsky et al., 1999; Balestrino et al., 1999).

Whilst the antidromic spike depression occurs without glutamate receptor stimulation, it is apparent that the depression in orthodromic responses occurs in response to glutamate accumulation at the synapse leading to enhanced glutamate receptor stimulation. Glutamate may accumulate in the extracellular space in a few ways:

1. Sodium-potassium ATPase inhibition may depolarise the pre-synaptic neuron allowing calcium entry and therefore exocytotic release of glutamate (Drejer et al., 1985)

2. Sodium-potassium ATPase inhibition may result in cell swelling leading to activation of glutamate-permeable volume-sensitive anion channels, therefore increasing efflux and extracellular accumulation of glutamate (Basarsky et al., 1999).

3. Glutamate uptake in the pre-synaptic nerve terminals and glia is driven by the gradient of sodium (for binding glutamate) and potassium (for net transport) ions at the glutamate transporter (for review see Tzingounis & Wadiche, 2007). This process is therefore dependent on the ability of the sodium-potassium ATPase to maintain the ion equilibrium across the membrane. Inhibition of the pump results in accumulation of extracellular glutamate due to reduced uptake at the transporters (Madl & Burgesser, 1993; Roettger & Lipton, 1996; Rossi et al., 2000).

The effects of ouabain on orthodromic responses are consistent with the report of Basarsky et al. (1999) which showed that 100μM ouabain perfused for 90seconds onto hippocampal

slices produced cell swelling detected by intrinsic optical imaging and glutamate efflux measured by high performance liquid chromatography. The glutamate efflux was prevented by blocking glutamate permeable volume-sensitive organic anion channels with 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) in the presence of zero calcium and EGTA. In the present study, adenosine only preconditioned against ouabain-induced depressions in the antidromic and orthodromic population spikes and not the field epsps, therefore the mechanism of glutamate accumulation at the synapse was not investigated further as it seemed unlikely that adenosine exerted its effects by modulating synaptic glutamate levels. The source of glutamate accumulation could be investigated by co-perfusing adenosine with ouabain in a similar manner to the experiments conducted with kynurenic acid and ouabain in orthodromic potentials. Adenosine inhibits exocytotic release of glutamate therefore if the ouabain-induced depression was blocked in the presence of adenosine, this would suggest that glutamate is pre-synaptically released by exocytosis. Alternatively the pre-synaptic release of glutamate could be directly measured by examining potential changes in the frequency of spontaneous mepsps by quantal analysis. If the result suggested that glutamate accumulation resulted from blockade of transporters, one could selectively block glial or neuronal transporters. Madl & Burgesser (1993) used high performance liquid chromatography (HPLC) to directly measure ATP content and the uptake and release of excitatory amino acids in hippocampal slices. Using D, L-threo-β-hydroxyaspartate, a substrate for the transporters, it was determined that ATP depletion decreased uptake into neurons and glia. Decreased glutamate immunoreactivity also occurred primarily at axonal terminals suggesting that extracellular glutamate accumulation was mainly due to pre-synaptic reversal of transporters. Calcium was removed from the medium to exclude an exocytotic mechanism. The authors also showed that 1μM ouabain acted in synergy with ATP depletion to increase extracellular glutamate. Similar observations have been made in rat spinal dorsal white matter showing that reduction in compound action potentials in response to ouabain exposure were attenuated by blockade of glutamate receptors and sodium-dependent glutamate transporters (Li & Stys, 2001). Voltage-sensitive dyes have been used to examine glutamate transporter activity in rat hippocampal slices showing a role for glia in glutamate removal from the extracellular space (Nakamura et al., 2003).

Rossi et al. (2000) showed that current changes generated in CA1 cells during perfusion with 95%$N_2$/5%$CO_2$ bubbled aCSF containing 7mM sucrose in place of 10mM glucose were the result of glutamate release; they further showed that these current changes occurred when the neuronal glutamate transporters were reversed and not by other

proposed mechanisms of glutamate accumulation such as exocytosis, cell swelling-induced release and release from glia.

From the present results it appears that glutamate release in response to ouabain is spatially limited to the synapse and does not affect the changes observed in the antidromic population spike. This appears consistent with the known literature reporting the effects of ouabain such as glutamate-independent cell swelling (Jarvis et al., 2001) and extracellular glutamate accumulation via reversed uptake into neurons (Rossi et al., 2000) and release through volume-activated channels (Basarsky et al., 1999).

### 4.3.4 - Effects of ouabain on evoked responses: inhibitory transmission

#### 4.3.4.1 - Adenosine

Experiments were also conducted to investigate the role of the inhibitory neurotransmitters, adenosine and GABA, in mediating the ouabain-induced orthodromic spike depression. Adenosine A1 receptor blockade during application of ouabain indicated that adenosine was not released in response to sodium-potassium pump inhibition. Other adenosine receptors were not examined on account of the lack of effect of A1 receptor blockade. If adenosine were released, it would first bind to the higher affinity A1 receptors before binding to others. This is consistent with the observations of Vaillend et al. (2002) which show that potentiation of E-S- coupling in response to dihydroouabain, a low affinity analogue of ouabain, was not prevented in the presence of DPCPX. It should be noted that in the study by Vaillend et al. (2002), dihydroouabain induced epileptiform activity without altering resting extracellular potassium ion concentration. They also noted a potentiation in E-S coupling in response to dihydroouabain signifying an increase in population spike amplitude whilst the observations in the present study show that ouabain induced the opposite effect upon population spikes. Despite the differences in electrophysiological effects and the use of different sodium pump inhibitors, Vaillend et al., support the results of the present study which show an absence of adenosine A1 receptors in the effects of ouabain upon orthodromic population spikes.

#### 4.3.4.2 - GABA

The role played by GABA in the ouabain-induced effects in orthodromic potentials is less clear than that of adenosine. The changes in orthodromic population spikes in response to ouabain were examined in the presence of $GABA_A$ and $GABA_B$ receptor antagonists. $GABA_B$ receptors had no clear role in the ouabain-induced effects. In the presence of the $GABA_A$ receptor antagonist, bicuculline, the ouabain-induced depression in orthodromic population spikes was enhanced. Having established that extracellular glutamate probably increases during ouabain perfusion, the most likely explanation is that inhibition of $GABA_A$ receptor blockade increased glutamate release from pre-synaptic neurons. As $GABA_A$ receptor blockade enhanced the effect of ouabain, this suggests that synaptic glutamate accumulation resulting from sodium-pump inhibition occurs primarily as a result of reduced glutamate uptake from the synaptic space as opposed to exocytotic release of excitatory neurotransmitter (see Figure 4.43). It is possible however that ouabain, by depolarizing the pre-synaptic membrane, induces vesicular release of glutamate. The vesicles may not be completely depleted of neurotransmitter after ouabain-induced exocytosis therefore $GABA_A$ receptor blockade might contribute to further exocytosis. The possibility of GABA release during ouabain perfusion is therefore unlikely; it cannot be completely dismissed however as the effect of any blockade of GABA inhibition on the orthodromic responses may have been hidden by the resulting increase in glutamate release which would enhance the glutamate-induced depression in the orthodromic responses (see Figure 4.43). However, given that there was no change in the nature and time course of the orthodromic population spike in response to ouabain in the presence of a $GABA_B$ receptor antagonist, release of GABA in response to sodium-potassium ATPase inhibition seems unlikely. It has been shown by Matsumoto et al. (2002) that mIPSCs recorded from dissociated CA1 neurons with intact pre-synaptic terminals increase in frequency to approximately 160% in response to 100μM ouabain. Vaillend et al. (2002) report a similar observation with dihydroouabain on mIPSC frequency in hippocampal slices. These studies both indicate that GABA is released pre-synaptically in response to ouabain. Earlier studies however, report a decrease in the size of the evoked IPSP in response to strophanthidin, a cardiac glycoside that induced burst firing in orthodromic population spikes (McCarren & Alger, 1987). The authors attributed the strophanthidin-induced epileptiform activity to a decrease in GABAergic inhibition. This study examined hyperexcitability induced by various cardiac glycosides similarly to Vaillend et al. (2002). As there appears to be some disagreement in the literature regarding the effects of sodium-potassium ATPase inhibition upon GABAergic transmission, it may be beneficial to clarify

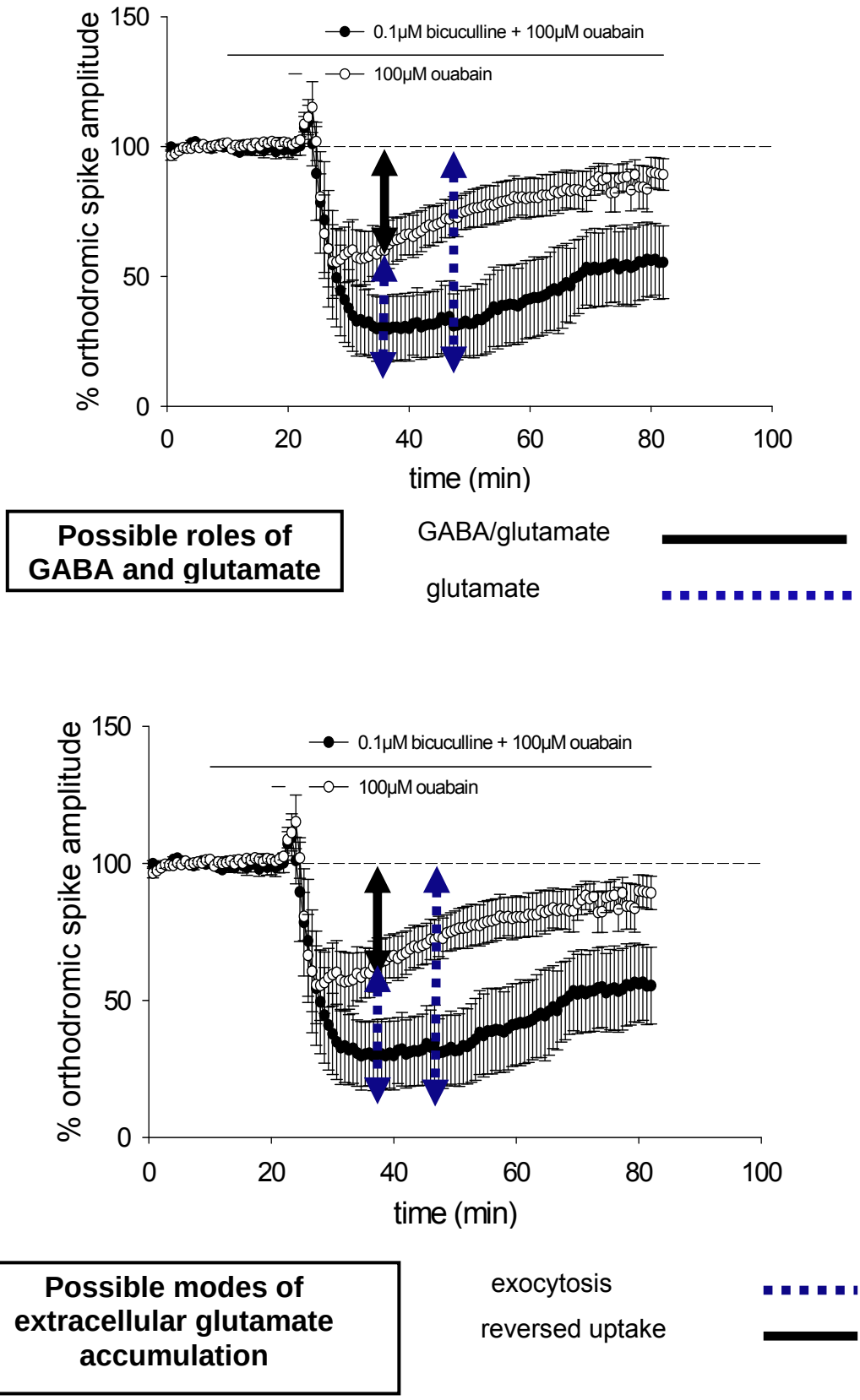


**Figure 4.43 Possible mechanisms underlying the effects of ouabain on orthodromic population spikes during $GABA_A$ receptor blockade.** The upper graph indicates how GABA and glutamate may affect the time course of the experiment. The lower graph indicates the possible ways that glutamate is released into the synaptic space during $GABA_A$ receptor blockade and $Na^+/K^+$ ATPase inhibition.

the effects of ouabain upon GABA in the present study with future experiments that examine the effect of ouabain upon IPSPs.

Although pharmacological blockade of GABA receptors did not conclusively show if GABA was or was not released during ouabain perfusion, it is possible that the blockade by kynurenic acid of ouabain-induced depression of orthodromic population spikes was a result of reduced feedback inhibition (Belan & Kostyuk, 2002). Release of GABA to mediate feedforward inhibition is unlikely as glutamate receptor blockade would not have prevented the effects of ouabain in orthodromic spikes (Tomasulo et al., 1991). The GABAergic neurons comprise less than 10% of the overall neuronal population in the CA1 area (Aika et al., 1994) therefore it is unlikely that they release GABA in sufficient quantities to mediate the ouabain-induced depression in orthodromic population spikes.

### 4.3.5 - Effects of ouabain on evoked responses: calcium modulation

Having established that the ouabain-induced depression in antidromic spike amplitude was independent of glutamate, alternative mechanisms were sought to explain this phenomenon. The role of calcium was examined in mediating this effect for various reasons: blockade of the sodium-potassium ATPase results in intracellular calcium accumulation in the heart (Reuter et al., 2002) and neuronal tissue (Saghian et al., 1996; Dietz et al., 2007); the relationship of the sodium-potassium ATPase to the regulation of the sodium-calcium exchange mediates the action of cardiac glycosides in the heart (Reuter et al., 2002); increasing intracellular calcium in hippocampal slice cultures (Bickler & Fahlman, 2004) and acute slices (Pérez-Pinzón et al., 1999) is implicated in preconditioning.

#### 4.3.5.1 - Intracellular calcium

The effect of using an intracellular calcium chelator on the response of the antidromic population spike to ouabain was initially examined. BAPTA-AM alone produced no change in the antidromic population spike amplitude. Upon co-perfusion with ouabain in the presence of BAPTA-AM, there was a dramatic reduction in the size of the antidromic population spike which greatly exceeded the degree produced by ouabain alone. From this it is possible that ouabain in normal aCSF increases or decreases intracellular calcium concentration; a decrease in calcium would explain why ion chelation enhanced the effect of ouabain although it might therefore be expected that a decrease in antidromic spike amplitude would occur in response to BAPTA-AM alone. BAPTA-AM has been used at

much higher concentrations in hippocampal slices without any disruption of the recorded parameter (Niesen et al., 1991; Fredholm & Hu, 1993; Spigelman et al., 1996; Tonkikh et al., 2006) consistent with its action in the present study.

It has been shown that intracellular calcium accumulation from oxygen and glucose deprivation can directly decrease the activity of the sodium-potassium ATPase in rat cortical slices (Matsuda et al., 1992). The authors observed that this effect was blocked by BAPTA-AM but not by removing extracellular calcium. If intracellular calcium accumulation itself decreases the activity of the sodium-potassium ATPase then it will be more vulnerable to inhibition by ouabain. The present results appear to disagree with Matsuda et al. (1992) as BAPTA-AM potentiated the deleterious effect of ouabain on the antidromic population spike. However the observation by Matsuda et al. (1992) that removal of extracellular calcium did not prevent the inhibition of sodium-potassium ATPase activity is inconsistent with their result using BAPTA-AM and the present results. BAPTA-AM is reported to block the large conductance calcium-activated potassium channels (Storm, 1987; Niesen et al., 1991) which contribute to repolarization of the membrane potential (Poolos & Johnston, 1999). It is possible that the Matsuda et al. (1992) results reflect such a change induced by intracellular calcium which is independent of extracellular calcium concentration.

The other explanation of the present results is that ouabain increases intracellular calcium; an accumulation of positive charge intracellularly would result in increasing the membrane potential; this may lead to initial increases in spike discharges as the membrane potential is closer to firing threshold and eventually result in overdepolarization of the neuron thereby reducing action potential firing. This would be consistent with the epileptiform activity induced upon initial exposure to ouabain which has been previously attributed to elevations in intracellular calcium (McCarren & Alger, 1987; Alger & Williamson, 1988). Either increasing or decreasing calcium may result in disrupting action potential firing and therefore decreasing the size of the antidromic population spike. To examine this further, the effects of modulating extracellular calcium on ouabain-induced changes in evoked responses were studied.

#### 4.3.5.2 - Extracellular calcium

It was observed that ouabain produced similar effects in the presence of low extracellular calcium concentration as those in the presence of BAPTA-AM. Like BAPTA-AM, low

calcium concentration alone did not affect the antidromic population spike. Using twice the concentration normally present in aCSF resulted in attenuating the ouabain-induced depression in antidromic spike amplitude; overall the results indicated that ouabain acts to decrease intracellular calcium concentration. This is consistent with the report of Bickler & Fahlman (2004) which showed, using hippocampal slice cultures, that increasing intracellular calcium with calcimycin or ionomycin, small lipid-soluble molecules which bind to calcium ions and carry them across the cell membrane without inducing excitotoxicity, resulted in reducing cell death in the CA1, CA3 and dentate gyrus areas in response to a 45min exposure to OGD. Pérez-Pinzón et al. (1999) also showed that an anoxic preconditioning paradigm in acute hippocampal slices failed to promote recovery of evoked responses when the calcium concentration in the perfusion medium was lowered to 05.mM from 2mM. These studies support the damaging effect of decreasing calcium levels.

It has also been observed however, that 30μM ouabain in acute hippocampal slices results in increased intracellular calcium detected by Fura-2 in CA1 neurons (Dietz et al., 2007), an effect that was reduced by KB-R7942, an inhibitor of the sodium-calcium exchange in its reverse mode. The authors noted that intracellular calcium initially increased at 8min post-ouabain then returned to basal levels. This was followed by a second increase in calcium levels which did not recover. Zhan et al. (1998) observed that 50μM ouabain exposed for 4min onto acute hippocampal slices increased intracellular calcium which resulted in reducing intracellular pH. Removing calcium from the perfusion medium resulted in no elevation of intracellular calcium in response to ouabain. The measurements for calcium were taken no longer than 15min post-ouabain exposure. The studies by Dietz et al. (2007) and Zhan et al. (1998) are consistent with the known mechanism of action of cardiac glycosides in the heart which involves inhibiting the sodium-potassium ATPase resulting in reversal of the sodium-calcium exchange; this in turn causes intracellular calcium accumulation which strengthens the contractility of myocytes (Reuter et al., 2002). The relationship between the sodium-potassium ATPase and the sodium-calcium exchanger has also been characterised in cultured cerebellar granule cells (Czyz et al., 2002a; Czyz et al., 2002b). The mechanism of calcium accumulation by sodium-potassium ATPase inhibition is also consistent with an OGD model of ischaemia which showed increases in intracellular calcium during 5min of OGD in hippocampal slices (Lobner & Lipton, 1993). This study showed that the initial influx of calcium occurred via NMDA receptors which was then superceded by calcium influx via voltage-gated calcium channels and reversal of the sodium-calcium exchange after 2.5min; the last two pathways accounted for approximately 60% of the overall influx. It has also been specifically shown

that inhibition of the reverse mode of the sodium-calcium exchange by KB-R7943 in hippocampal slices improves the recovery of population spikes following low oxygen and low glucose exposure (Schroder et al., 1999).

The smaller concentrations of ouabain used by Dietz et al. (2007) and Zhan et al. (1998) induced increases in intracellular calcium. This is consistent with the use of low affinity analogues of ouabain to induce multiple spiking in responses without changing potential size (McCarren & Alger, 1987; Vaillend et al., 2002). It is also consistent with the epileptiform activity observed upon initial ouabain exposure prior to decreasing potential size in the present study. It is possible that the continued accumulation of calcium in the present results may induce calcium sequestration and/or efflux via the plasmalemmal calcium-ATPase resulting overall in reduced calcium levels in the cytoplasm. As ATP levels were not depleted in this study by removal of oxygen and/or glucose, this pump may have compensated for any resulting calcium accumulation. The intermittent increases in intracellular calcium observed by Dietz et al. (2007) suggest that different calcium influx, efflux, release or sequestration mechanisms occur in a time-dependent manner which may also be true of the present results. As noted above, the studies by Dietz et al. (2007) and Zhan et al. (1998) took intracellular calcium measurements shortly after ouabain exposure which reflects initial changes in calcium dynamics only. The results presented here imply that ouabain initially increases intracellular calcium in CA1 neurons due to the presence of epileptiform activity. Intracellular calcium then appears to diminish if correlating with the reduction of spike size. Further changes in intracellular calcium may occur in response to ouabain similarly to Dietz et al. (2007), but the calcium manipulations prior to, during and after ouabain is applied may prevent their detection. It may be useful to alter calcium concentration after ouabain exposure in future experiments to better understand the complex effects of this ion.

#### 4.3.5.3 - Large-conductance calcium-activated potassium channels

As the antidromic population spike represents the summation of synchronously firing action potentials from a population of neurons (Anderson et al., 1971b) generated in the absence of synaptic input, a decrease in spike size probably reflects a change in the intrinsic conductances that generate action potentials. The large conductance calcium-activated K+ channels (BK+ channels) mediate the fast afterhyperpolarization which contributes to the repolarization of action potentials in the CA1 somata (Poolos & Johnston, 1999). The BK+ channels were selected in particular as they have a reported role

in preconditioning in cardiovascular tissue in vivo (Shintani et al., 2004) and in vitro (Feng et al., 2006) and BAPTA-AM is reported to block the fast afterhyperpolarization mediated by BK+ channel opening in CA1 neurons (Storm, 1987; Niesen et al., 1991). They are also regulated by voltage in addition to calcium; the voltage sensor on the channel is responsible for its activation which can occur in the absence of calcium. It is important to note that the modulatory effect of calcium seems necessary to allow proper functioning to continue (Barrett et al., 1982; Cox et al., 1997). Charybdotoxin (ChTX), a selective channel blocker for BK+ channels was used at a concentration (25nM) that is reported not to induce changes in population spike amplitude although it may cause some spike broadening (Shao et al., 1999). Co-perfusion of ouabain and ChTX resulted in enhancing the ouabain-induced depression in antidromic spike amplitude thus suggesting that sodium-potassium ATPase inhibition decreases the open probability of BK+ channels. Given the results with calcium concentration manipulation and BAPTA-AM, it is likely that a decrease in intracellular calcium in response to ouabain results in blocking the BK+ channels although the change in membrane potential from ouabain-induced depolarization may activate the channels. This has implications for the formation of action potentials as the membrane potential will take longer to repolarise by reducing the fast afterhyperpolarization.

It has been reported by Reich et al. (2004) that a depression in orthodromic population spikes in response to dihydroouabain was prevented by iberiotoxin, another inhibitor of BK+ channels. The discrepancy between this result and those in the present study may be due to the low affinity ouabain analogue used by Reich et al. (2004). They noted that the depression induced in orthodromic population spikes occurred independently of NMDA receptor stimulation and attributed this to decreased pre-synaptic transmitter release on account of BK+ channel blockade at the nerve terminal. This is in contrast to the present results which show that glutamate receptor stimulation is required to induce a depression by ouabain in orthodromic responses. Like the other studies using low-affinity analogues (McCarren & Alger, 1987; Vaillend et al., 2002) or smaller concentrations of ouabain (Dietz et al., 2007; Zhan et al., 1998) there may be a threshold of sodium-pump inhibition that is not reached in these studies to produce a resulting decrease in intracellular calcium following an initial increase. If calcium levels keep changing throughout the post-ouabain recovery, it may be useful to block the BK+ channels after exposure to ouabain to fully elucidate its mechanisms. It may also be useful to observe the effect of ouabain in the presence of a BK+ channel opener such as NS 1619.

It has been shown that ChTX at 15-20nM concentrations was able to inhibit the afterhyperpolarization of stimulus-induced epileptiform activity in the hippocampus (Alger & Williamson, 1988) suggesting that BK+ channels open during such activity. This would seem consistent with the current results on account of the appearance of multiple spiking and the subsequent blockade of BK+ channels by ChTX. The greater reduction in antidromic spike amplitude in response to ouabain in the presence of ChTX is also consistent with a report that opening of BK+ channels reduces the effects of hypoxia-induced spreading depression in rat hippocampal slices (Hepp et al., 2005). Overall it appears that initial activation of BK+ channels is followed by reduced opening in response to ouabain on the antidromic population spike in CA1 neurons. This would be consistent with a biphasic effect on calcium levels following sodium-potassium pump inhibition.

### 4.3.6 - Effects of ouabain on the induction of LTP

As glutamate receptor stimulation mediates the effects on orthodromic population spike amplitude, the subsequent effects on synaptic plasticity were investigated. It was observed that using a 100Hz stimulus for 1second appeared to reverse the effects of ouabain on the evoked potential although it was significantly less than the LTP induced in control slices. This may have been due to variations in the percentage baseline obtained for each individual slice as according to Leung & Au (1994), lower baselines will produce greater potentiations. Statistical analysis of the baselines obtained between groups showed no significant differences however, indicating that this probably does not account for the effect observed.

It was also noted that when the baseline had been set to 50% of the maximum value, ouabain failed to induce a significant depression in the orthodromic population spikes. It is possible that lowering the stimulation strength lowers basal metabolic rate as fewer energy-dependent processes (e.g. transmitter uptake, synthesis, degradation) required for neurotransmission will be active. The neurons could be effectively preconditioned against the subsequent ouabain insult as the slice has adapted to a lower metabolic rate. It is also possible that there will be a longer time to overdepolarization of the neurons using a smaller baseline. This could enhance protection as the damage produced by ouabain-induced depolarization is due to cell swelling (Jarvis et al., 2001). With initially fewer electrically active neurons, the onset of depolarization will take longer. While the active neurons may still suffer some damage due to cellular swelling, the electrically silent neurons may become more excitable and thus are stimulated at a lower threshold. Also, delay in the onset of hypoxia-induced depressions in hippocampal responses have been

shown to be neuroprotective (Balestrino & Somjen, 1986). However, using a 50% baseline for epsps in the adenosine preconditioning experiments against ouabain did not seem to alter the extent of depression induced by ouabain. This may simply be due to the fundamental differences in epsp and population spike generation as only the latter highlighted the preconditioning effect of adenosine against ouabain.

It was therefore concluded that ouabain has a deleterious effect on synaptic plasticity in the hippocampal slice indicating that the glutamate-induced effects may comprise a mixture of long-term depression and toxicity.

### 4.3.7 - Post-adenosine effects on evoked responses

It was observed that following adenosine exposure, antidromic and orthodromic population spikes increased significantly in size by approximately 10% whilst epsp slope showed no change and epsp amplitude decreased significantly; this suggested that dissociations may occur between epsp amplitude and population spike amplitude if they are recorded simultaneously. The effect on population spike amplitude may be attributed to opening of ATP-sensitive potassium channels. ATP-sensitive potassium channels do not affect the response of epsps to adenosine but do mediate the inhibitory response of adenosine on orthodromic population spikes (Hosseinzadeh & Stone, 1998) and exhibit different properties at pre- and post-synaptic sites (Hosseinzadeh & Stone, 1998; Matsumoto et al., 2002). This is consistent with the occurrence of elevations in population spikes only and not epsps in the present results. However, adenosine-induced hyperpolarizations in CA1 neuronal membrane potentials are attributed to the opening of ATP-sensitive potassium channels due to the inhibition of this effect by glibenclamide (Li & Henry, 1992). Also, ATP-sensitive potassium channels are reported to mediate hypoxia-induced hyperpolarizations in CA1 neurons (Fujimura et al., 1997). The authors reported here that post-hypoxic hyperpolarization was always greater than hypoxic hyperpolarization. These observations suggest that potassium efflux through these channels changes the resting membrane potential to a more negative value which moves it further away from the firing threshold for generating action potentials. In the present results, the elevation in population spike amplitude may occur as a result of the increase in electrochemical gradient for sodium and potassium ions which result from hyperpolarization of the resting membrane potential. As the slices were stimulated maximally with current pulses, the membranes should depolarize sufficiently to prevent any decrease in the firing of action potentials resulting from more negative resting membrane potentials. As sodium ion influx will continue until the voltage-gated sodium channels inactivate, this will result in a larger

action potential. Overall, the spike amplitude will increase. The study by Li & Henry (1992) agrees with the role of ATP-sensitive potassium channels in mediating the inhibitory response of adenosine on orthodromic population spikes (Hosseinzadeh & Stone, 1998). In the present study, the increase in response size occurs during adenosine washout, not during application. The previous observations by Li & Henry (1992) and Hosseinzadeh & Stone (1998) examined the role of ATP-sensitive potassium channels during adenosine application. While these channels appear necessary to mediate some of adenosine's inhibitory effects, the other actions of adenosine may result in inhibitory activity during its application which disappears upon washout. The ATP-sensitive potassium channels appear to remain active following withdrawal of adenosine. This is consistent with the effects of pinacidil upon the population spikes which start to elevate during pinacidil perfusion and continue to do so after washout.

It is also possible that the rebound elevation in spike size following adenosine stimulation is due to some direct effect on excitatory transmission. The present results show some similarity to the reports of Okada et al. (1990) who showed an excitatory effect of adenosine at submicromolar concentrations which were later shown to be a result of enhanced glutamate release (Okada et al., 1992). It is possible that in the present results, following adenosine perfusion, the endogenous adenosine content does not re-equilibrate completely back to its original levels, resulting in higher basal adenosine concentrations which could mediate a potential excitatory effect as indicated by Okada et al. (1992). However the studies of Okada et al. (1992) were conducted in CA3 cells from guinea pig hippocampal slices and measured the intracellular membrane potential and the epsp amplitude. In the present study the adenosine-induced reduction of epsp amplitude did not recovery fully to baseline but showed a very small but significant depression. Thus, these results differ from the study by Okada et al. (1992). This may simply be due to differences in species or the hippocampal recording site.

It may be possible that the high level of adenosine exposure may have altered the kinetics of the intracellular ecto-nucleotidases which generate adenosine from ATP or adenosine kinase which converts adenosine to 5'-AMP, a precursor to ATP. Extracellularly, adenosine deaminase may also have increased the breakdown of adenosine to form inosine. The ecto-nucleotidases may have decreased their enzymatic activity whilst adenosine kinase increased in order to favour the production of ATP in response to higher adenosine levels. This could promote greater excitatory effects as a result of increased sodium-potassium pump function and increased transmitter uptake. This could explain the elevation in spike size observed during adenosine washout. However, the ATP-sensitive

potassium channels appear to open in response to adenosine (Li & Henry, 1992) and close in the presence of ATP (Noma, 1983). If higher levels of ATP were generated by exposure to high adenosine concentrations then it would be expected that the ATP-sensitive channels will close. Alternatively, it could be that adenosine is broken down more quickly by adenosine deaminase so that following adenosine removal the basal adenosine concentration is lower than before. This could increase the excitability of the tissue as the inhibitory effects of endogenous adenosine on potassium channels and calcium channels will be reduced. The role of enzyme activity in mediating these effects or their role in the preconditioning phenomenon was not examined, partly because there are few suitable pharmacological modulators of the ectonucleotidases. This could be a very interesting avenue for future study using enzyme assay techniques or Western blotting.

Another explanation of the present results is that the intensity of the adenosine exposure stimulated all adenosine receptor subtypes; A3 receptors have a much lower affinity for adenosine than A1 receptors but at the concentrations used for preconditioning here (100μM adenosine), they are probably stimulated resulting in a desensitization of A1 receptors (Dunwiddie et al., 1997). This could also explain the rebound excitation of the population spikes as there is no existing basal adenosine inhibition via A1 receptors. The report of Pérez-Pinzón et al. (1999) showed that blockade of adenosine A1 receptors with 10μM DPCPX for 10min resulted in a significant potentiation of the orthodromic population spike during drug application and washout. It was also observed that such exposure preconditioned against a following 95% $N_2$/5% $CO_2$ anoxia for 5 minutes. Although this report appears consistent with the explanation of a desenzitization of A1 receptors in the present results, it does, however, seem to be at complete odds with the fact that A1 receptor blockade prevented adenosine preconditioning against ouabain in the present results, similarly to several other preconditioning paradigms (Heurteaux et al., 1995; Pérez-Pinzón et al., 1996; Hiraide et al., 2001; Kitagawa et al., 2002; Pugliese et al., 2003). It is possible that, upon intial stimulation by adenosine, the A1 receptors become subsequently desensitized so that, overall, there is blockade of A1 receptors, in agreement with the observations of Pérez-Pinzón et al (1999). This may be consistent with the opening of ATP-sensitive potassium channels which mediate hyperpolarizations or preconditioning protection; initial stimulation of A1 receptors may induce a sustained open state of the ion channels which continues in the absence of further A1 receptor stimulation.

The decrease in epsp amplitude may be due to a decrease in the NMDA receptor-mediated component of the epsp as it was shown in the present study that blockade of adenosine A1 receptors protected the response from post-glutamate induced depression. A change in epsp

amplitude in the absence of any change in epsp slope is indicative of NMDA receptor modulation (Dahl et al., 1990) as the slope represents the fast AMPA-receptor mediated component of the waveform. Stimulating A1 receptors may therefore be expected to have the converse effect on epsp amplitude. However, blocking A1 receptors did not appear to alter the isolated NMDA-mediated epsp, only its response to glutamate. Also no such interaction between A1 receptor blockade and post-glutamate recovery was observed in composite epsps such as those used in the adenosine preconditioning experiments. The effect of adenosine preconditioning on epsp amplitude was very small (approximately 2-3% depression of epsp amplitude) and while it probably reflects a change in receptor activity, its significance in the context of the aims of the present study argued against further examination. In future investigations, it would be very interesting to study this dissociation between the effects on population spike amplitude and epsp amplitude using E-S coupling experiments.

The post-adenosine effects on evoked responses were taken into account when calculating the response recovery following either ouabain or glutamate. Whether the post-adenosine occurrence of a population spike increase is required to induce a preconditioning effect is unclear, although the increases generally occur in groups of experiments where there was a successful preconditioning outcome (orthodromic population spikes, antidromic population spikes, AMPA/kainate receptor blockade during adenosine preconditioning) with some exceptions (NMDA receptor blockade during adenosine preconditioning).

### 4.3.8 - Adenosine preconditioning: interaction with ouabain

Having observed that adenosine did not precondition against glutamate using orthodromically or antidromically evoked responses, ouabain was used in place of glutamate to investigate adenosine preconditioning as sodium-potassium ATPase inhibition induced glutamate-dependent and independent effects in population spikes. It was initially observed that adenosine was able to effectively precondition against ouabain-induced changes in orthodromic population spike amplitude. Adenosine inhibits pre-synaptic glutamate release via exocytosis but not by reversing glutamate uptake. The results from $GABA_A$ receptor blockade during ouabain exposure using orthodromic population spikes suggested that ouabain releases glutamate into the extracellular space by reversed uptake in neurons and glia so that adenosine preconditioning would probably have no interaction with ouabain-induced glutamate release. This was entirely consistent with the lack of interaction observed in recorded epsps between adenosine preconditioning and ouabain and the absence of protection in the adenosine preconditioning/glutamate insult paradigm. In

the tests of antidromic population spikes, it was shown that glutamate released by ouabain was not the mediator of the depression in potential size. The adenosine used to produce preconditioning against ouabain may therefore affect the glutamate-independent effect of ouabain. This was confirmed when ouabain-induced depression in antidromic population spikes was attenuated by a prior application of adenosine. From these observations, it appears that the preconditioning effect of adenosine against ouabain is post-synaptic and limited to the cell body of CA1 neurons at the CA3-CA1 region.

### 4.3.9 - Mechanism of adenosine preconditioning against ouabain

From the present results, it appears that the mechanism of adenosine preconditioning against ouabain involves the stimulation of A1 receptors, NMDA receptors, generation of nitric oxide and stimulation of ATP-sensitive potassium channels. This section will discuss the role of each of these factors in mediating the effects observed.

#### 4.3.9.1 - Role of A1 receptors

The involvement of A1 receptors is entirely consistent with the reported mechanisms of preconditioning (Heurteaux et al., 1995; Pérez-Pinzón et al., 1996; Hiraide et al., 2001; Kitagawa et al., 2002; Pugliese et al., 2003). As A1 receptor stimulation is the dominant effect of adenosine in the hippocampus, further investigations into the other actions of adenosine which may have potentially mediated this effect were not pursued.

There is increasing evidence that A3 receptors may mediate preconditioning in cardiac tissue (Armstrong & Ganote, 1994; Liu et al., 1994; Wang et al., 1997; De Jonge et al., 2002) and it has been recently shown that they are indeed present in the hippocampus (Lopes et al., 2003) and may affect synaptic plasticity (Costenla et al., 2001); however it is also known that A3 receptors disrupt the protection of preconditioning in hippocampal slices (Pugliese et al., 2003) and even cause cell death if stimulated excessively (Kohno et al., 1996).

A2b receptors are present in the hippocampus although their functional characterization is still lacking due to a limited selection of pharmacological agents (Volpini et al., 2003). They upregulate in myocardial preconditioning and A2b receptor knockout mice cannot be preconditioned against myocardial infarction (Eckle et al., 2007). The role of A2b and A3

receptors cannot be excluded from the data presented here and considering their potential involvement in preconditioning they may warrant investigation in the future.

#### 4.3.9.2 - Role of NMDA receptors

The involvement of NMDA receptor activation in mediating adenosine preconditioning against ouabain was surprising given that glutamate release induced by sodium-potassium ATPase inhibition did not affect the antidromic population spike. The role of NMDA receptors may be related to their calcium permeability (Ascher & Nowak, 1988) when they are in an open state and unblocked by magnesium (Nowak et al., 1984). The experiments examining the role of calcium in mediating the effects of ouabain upon antidromic population spikes revealed that the reduction in potential size was enhanced by the presence of the BK+ channel blocker, ChTX. Extra-synaptic NMDA receptors have been shown to regulate the opening of BK+ channels from outside-out membrane patch recordings taken from rat olfactory bulb granule cells (Isaacson & Murphy, 2001) and this is thought to underlie some observations of NMDA receptor mediated inhibition in the hippocampus (Nicoll & Alger, 1981; Zorumski et al., 1989). It is possible therefore that adenosine induces BK+ channel opening via NMDA receptor stimulation which attenuates the effects of ouabain in hippocampal slices. The BK+ channels have shown a post-synaptic co-localization with PSD95 protein in the hippocampus and do not occur in hippocampal neurons lacking NMDA receptors (Sailer et al., 2006). BK+ channel opening has been reported to induce preconditioning in cardiovascular tissue (Shintani et al., 2004; Feng et al., 2006) therefore it is likely that it is involved in this effect. It is also consistent with the fact that BK+ channels do not contribute to action potential repolarization in the dendrites of CA1 neurons but are localized to the soma (Poolos & Johnston, 1999); this might explain the localization of the preconditioning effect of adenosine against ouabain. The role of BK+ channels could be confirmed by observing the effect of adenosine preconditioning against ouabain in the presence of a BK+ channel blocker.

It is surprising that the activation of NMDA receptors by adenosine required to induce protection against ouabain appears to have no preconditioning effect against a 10mM glutamate pulse. It may be that the threshold of NMDA receptor stimulation required to induce excitotoxic preconditioning in hippocampal slices was not reached in this study. The concentration of ouabain used appears to be a milder insult than 10mM glutamate given that the effects of ouabain are partially reversible by tetanic stimuli; a lower degree of NMDA receptor stimulation may therefore be sufficient to induce protection. A

preconditioning protocol in hippocampal slices showed that cell swelling induced by a lethal NMDA insult was reduced by prior transient NMDA applications (Bandyopadhyay et al., 2002). Cell swelling has also been shown to be a consequence of ouabain application to neocortical slices (Jarvis et al., 2001). It may be that in the present study, adenosine is acting to decrease cell swelling induced by ouabain via NMDA receptor stimulation. This could be confirmed by using optical scatter imaging similarly to Jarvis et al. (2001) and Bandyopadhyay et al. (2002) to view the changes in cell structure during the adenosine preconditioning protocol against ouabain.

#### 4.3.9.3 - Role of nitric oxide

##### 4.3.9.3.1 - Interaction with ATP-sensitive potassium channels

If NMDA receptors are stimulated as a result of adenosine preconditioning then it is likely that nitric oxide is generated also. By blocking the formation of nitric oxide using an inhibitor of nitric oxide synthase, L-NAME, the preconditioning effect of adenosine upon ouabain was abolished in the present results. This indicates that nitric oxide production constitutes part of the mechanism of adenosine preconditioning. It has been documented that nitric oxide mediates the protection induced by anoxic preconditioning in hippocampal slices (Centeno et al., 1999) and studies in cardiovascular tissue have shown that this free radical induces the opening of mitochondrial ATP-sensitive potassium channels (Sasaki et al., 2000) via a cGMP-protein kinase G pathway (Han et al., 2002a; Deka & Brading, 2004; Xu et al., 2004; Kuno et al., 2007). This pathway has been implicated in preconditioning against anoxia in rat hearts (Cuong et al., 2006). The role of nitric oxide in stimulating ATP-sensitive potassium channels is also implicated in delayed cardiovascular preconditioning; diazoxide induced an upregulation of inducible NOS (iNOS) when administered 24hrs earlier in wild type mouse hearts. iNOS knockout mice were not protected after diazoxide treatment 24hrs prior to a 40min global ischaemia (Wang et al., 2001). The authors suggested that increases in nitric oxide following upregulation of iNOS open mitochondrial ATP-sensitive potassium channels.

The peroxynitrite anion, $ONOO^-$, which is the product of a reaction between the free radicals, superoxide anion ($O_2^-$) and nitric oxide, has also been shown to open brain mitochondrial ATP-sensitive potassium channels (Lacza et al., 2003). Another study using a neuroblastoma cell line in culture showed that a preconditioning protocol consisting of 2hours of serum deprivation induced an increase in neuronal NOS (nNOS) which in turn resulted in mitochondrial ATP-sensitive potassium channel opening via a nitric oxide-

cGMP-PKG dependent pathway. This protocol protected against administration of the neurotoxin, MPTP (Chiueh et al., 2005). An acute study showed that the nitric oxide donor, sodium nitroprusside, enhanced the current induced by pinacidil in cell-attached patch recordings from rabbit ventricular myocytes (Han et al., 2002a) indicating that the effects of nitric oxide upon mitochondrial ATP-sensitive potassium channels may occur in the time scale of the preconditioning protocol used in the present study. The role of nitric oxide in the present preconditioning protocol using adenosine may possibly include an effect upon the opening of mitochondrial ATP-sensitive potassium channels. There are few, if any, reports in the literature on the effect of nitric oxide on plasmalemmal ATP-sensitive potassium channels.

Also, it has been shown in rabbit heart tissue that mitochondrial ATP-sensitive potassium channels, when opened, generate free radicals (Han et al., 2002b). It is possible that the source of nitric oxide production required to induce protection against ouabain is via adenosine-induced opening of mitochondrial ATP-sensitive potassium channels. The exact source of nitric oxide generation required in mediating adenosine preconditioning remains unclear from the present results; however the involvement of NMDA receptors in adenosine preconditioning shown here and the known receptor-mediated production of nitric oxide (Yamada & Nabeshima, 1997a + b; Bredt & Synder, 1989) make NMDA receptor-induced nitric oxide generation the most likely mechanism of adenosine preconditioning.

#### 4.3.9.3.2 - Interaction with BK+ channels

Another possibility regarding the role of nitric oxide is in relation to its actions on BK+ channels. There are conflicting reports on the nature of nitric oxide modulation of BK+ channel activity. Studies in non-neuronal (Bolotina et al., 1994) and neuronal (Shin et al., 1997; Ahern et al., 1999) tissue suggest that nitric oxide stimulates the opening of BK+ channels. Tjong et al. (2008) have recently shown that intermittent hypoxia experienced by rats results in a decreased function of BK+ channels in acutely dissociated CA1 neurons, an effect which is reversed by nitric oxide donors. One report however, indicates that nitric oxide attenuates a voltage- and calcium-dependent potassium current in whole-cell recordings from CA1 neurons (Erdemli & Krnjević, 1995b). As the effects of ouabain upon antidromic population spikes might be due to the closing of BK+ channels as implied by the enhancement of ouabain-induced depressions by ChTX, it is possible that the nitric oxide is required during adenosine preconditioning to open BK+ channels in order to

promote recovery of the population spike following sodium-potassium ATPase inhibition. Further studies regarding the nature of nitric oxide modulation of BK+ channel activity would be useful in clarifying this possibility.

#### 4.3.9.3.3 - Interaction with sodium-potassium ATPases

Nitric oxide donors have been reported to reduce the activity of the sodium-potassium ATPases in hippocampal slices (Delwing et al., 2007) and from porcine cerebral cortex (Sato et al., 1995). It is possible that this reduction in sodium-potassium ATPase activity is due to a potential action of nitric oxide upon the ATP-sensitive potassium channels and the BK+ channels. Opening of these channels would result in decreasing cytoplasmic potassium ions; in the case of plasmalemmal BK+ channels and ATP-sensitive potassium channels, this would result in increasing extracellular potassium available for binding to the extracellular site on the ATPase pump. ATP and potassium binding exhibit an antagonism where ATP affinity for the pump is lower when potassium ions are bound. As the conformational state of the pump alters as potassium ions are transported across, the affinity of ATP for the enzyme pumps increases. The difference in energy for ATP binding to the pump in its different conformations drives the ion transport. By raising extracellular potassium through opening of potassium channels, binding of the potassium ions will increase and further displace ATP binding. This will maintain the pump primarily in the conformational state of high potassium binding/ low ATP binding (Jorgensen & Pedersen, 2001). This could explain the effect of nitric oxide upon the sodium-potassium ATPase activity in the studies by Delwing et al. (2007) and Sato et al. (1995). As high potassium will also displace ouabain binding at the potassium binding site of the pump, this might also explain the requirement for nitric oxide production in preventing the effects of ouabain.

### 4.3.9.4 - Role of ATP-sensitive potassium channels

Given the extensive literature regarding the importance of ATP-sensitive potassium channels in preconditioning phenomena, experiments were conducted to examine the potential effects exerted by activation of these channels by adenosine in the present study. Adenosine is reported to activate the ATP-sensitive potassium channels in neurons via PKCepsilon (Pérez-Pinzón et al., 1996; Hearteaux et al., 1995). A role for these channels in the preconditioning effect induced by adenosine against exposure to ouabain was

conclusively shown when the preconditioning effect was abolished by the presence of the ATP-sensitive potassium channel blocker, glibenclamide, during the preconditioning stimulus. It was unlikely that this was due to any non-specific actions of glibenclamide as the specific blocker of mitochondrial ATP-sensitive potassium channels, 5-HD, produced similar results when used in the same manner as glibenclamide. The results therefore suggest that activating the mitochondrial ATP-sensitive potassium channels, which will produce a depolarization of the mitochondrial membrane potential (Ardehali & O'Rourke, 2005) is required to mediate adenosine preconditioning against ouabain. Mitochondrial potassium influx via ATP-sensitive channels prevents excessive calcium influx into the mitochondria and thus maintains the volume of these organelles under conditions where excessive calcium could disrupt mitochondrial oxidative phosphorylation (Busija et al., 2004); ATP production should therefore be maintained under ischaemic conditions.

These reported effects of the mitochondrial ATP-sensitive potassium channels appear to maintain cellular viability following ischaemia but the role of mitochondrial ATP-sensitive potassium channels in attenuating the effects of ouabain upon the sodium-potassium pump remains unclear. As ouabain inhibits the pump by occupying the extracellular potassium binding site, any change in the mitochondrial production of ATP will not alter the potassium-ATP binding activity which drives the electrogenic ion transport. It is possible that the sodium-potassium ATPase activity is modulated by free radicals produced as a result of mitochondrial ATP-sensitive potassium channel activation. Diazoxide, a selective opener of the mitochondrial ATP-sensitive potassium channels is known to generate free radicals within minutes in cultured cortical neurons (Kis et al., 2003) and free radicals are reported to inhibit the sodium-potassium ATPase pump (Huang et al., 1992; Kurella et al., 1999, Boldyrev et al., 2004). The potential mechanism by which nitric oxide and other reactive oxygen species may act to reduce the effects of ouabain is outlined in the previous discussion section dealing with possible interactions between nitric oxide and sodium-potassium ATPases, as are the possibilities regarding the plasmalemmal ATP-sensitive potassium channel subtype in mediating these effects.

## 4.3.10 - Pinacidil preconditioning

### 4.3.10.1 - Rationale for the use of pinacidil over diazoxide

As ATP-sensitive potassium channels appear to have a central role in mediating various preconditioning phenomena, the role of these channels was explored further by developing

another protocol using an ATP-sensitive potassium channel opener, pinacidil, in place of adenosine and testing its effectiveness against ouabain and glutamate insults. Potential interactions with NMDA receptors, having been identified as mediators of adenosine preconditioning against ouabain in the present study were also examined. The use of pinacidil in cardiovascular preparations as a preconditioning stimulus has been vast but conversely, its use in neuronal studies has been extremely limited. Neuronal preconditioning studies have tended to utilise diazoxide in favour of its specificity for mitochondrial ATP-sensitive potassium channels over the plasmalemmal subtype. Its use in the present study was not pursued after initial attempts had revealed that a low solubility (50mM in DMSO) coupled with the need for a high concentration (approximately 500μM has been reported (Erdemli & Krnjevic, 1995a) might have compromised the results on account of a 1% presence of the free radical scavenger, DMSO. This level of DMSO has been previously shown to exert a delay against the loss of response under anoxic and OGD conditions in hippocampal slices when present in aCSF at 0.4% (Hülsmann et al., 1999; Greiner et al., 2000). DMSO is also reported to suppress whole-cell currents induced by glutamate, NMDA and AMPA in hippocampal cell cultures (Lu & Mattson, 2001). In addition to the problems of using DMSO as solvent, 500μM diazoxide is reported to block the rapid desensitization of AMPA receptors in hippocampal cell cultures (Yamada and Rothman, 1992). Supporting this are the observations of Crépel et al. (1993) who have shown that exposure of acute hippocampal slices to 600μM diazoxide enhances the amplitude of field epsps recorded from CA1 and CA3 neurons, an effect not prevented by glibenclamide or tolbutamide. The authors also indicated the occurrence of an enhancement of AMPA-, quisqualate- and NMDA-induced currents and a suppression of the isolated fast and slow IPSPs in the presence of diazoxide. Although the preconditioning effects of diazoxide have been confirmed as the result of opening of mitochondrial ATP-sensitive potassium channels by the use of the specific blocker, 5-HD (Domoki et al., 1999; Rajapakse et al., 2002; Liu et al., 2002), effects of diazoxide upon glutamate transmission, particularly for studies like the present which examine effects upon synaptic transmission, make its use unfavourable in spite of its specificity for mitochondrial ATP-sensitive potassium channels. As the role of plasmalemmal ATP-sensitive potassium channels was not ruled out as a mechanism of adenosine preconditioning in the present study, pinacidil was adopted for further experiments in view of the evidence of its effectiveness as a preconditioning agent in cardiovascular and neuronal (Pérez-Pinzón & Born, 1999) tissue.

#### 4.3.10.2 - Protection against ouabain and the requirement for NMDA receptor stimulation

Similarly to the present studies examining adenosine-induced preconditioning, pinacidil was shown to precondition against the ouabain-induced depression of antidromic spike amplitude. To confirm that this effect was mediated by opening of the ATP-sensitive potassium channels, this experiment was repeated in the presence of the blocker glibenclamide which, by its abolition of the preconditioning effect, confirmed the specificity of pinacidil for the ATP-sensitive potassium channels.

To understand how the activation of the ATP-sensitive potassium channels fits in with the other mechanisms of preconditioning examined in the present study, namely the requirement for NMDA receptor stimulation to mediate adenosine preconditioning against ouabain, the pinacidil preconditioning protocol was repeated with NMDA receptor blockade during pinacidil exposure to the slices using the antagonist, DL-AP-5. The result indicated that NMDA receptor stimulation is also required to mediate pinacidil preconditioning as well as adenosine preconditioning. This was a surprising result given that the adenosine A1 receptor directly interacts with the NMDA receptor in the hippocampus and that nitric oxide, which is generated from NMDA receptor stimulation, opens the mitochondrial ATP-sensitive potassium channels in heart tissue.

These observations suggest that ATP-sensitive potassium channel opening would occur downstream of NMDA receptor activation rather than the other way around; however, the requirement for NMDA receptor stimulation to mediate the actions of pinacidil upon the effects of ouabain as shown here does not necessarily preclude a reciprocal action of NMDA receptors upon ATP-sensitive potassium channels. Indeed, co-dependent relationships between ATP-sensitive potassium channels and preconditioning signalling messengers such as nitric oxide (Centeno et al., 1999) and PKC (Pérez-Pinzón & Born, 1999; Hearteaux et al., 1995) are thought to underlie the continuing action of preconditioning agents after they are no longer exposed to the tissue. This would explain why, for instance, after adenosine or pinacidil removal from the perfusion medium, the ATP-sensitive potassium channels remain open until a harmful insult is applied. If a co-dependent relationship is activated such as those mentioned above, each participant in the relationship will continue to re-generate each other after the initiating stimulus is removed. This is consistent with the reports that nitric oxide both opens mitochondrial ATP-sensitive potassium channels (Sasaki et al., 2000; Wang et al., 2001; Lacza et al., 2003; Han et al., 2002a; Han et al., 2002b) and is also generated when these same channels are opened (Horimoto et al., 2000; Lebuffe et al., 2003; Horinaka et al., 2001). PKC has been shown

in cardiovascular tissue to have a co-dependent relationship with ATP-sensitive potassium channels (Gaudette et al., 2000).

It seems odd, if co-dependent relationships are initiated, that the time window for rapid preconditioning should be limited and not continue into a delayed preconditioning effect. If these co-dependent relationships do form the basis of preconditioning, the most probable reason that they do not continue into perpetuity is that the ATP-sensitive potassium channels do eventually "run-down" as has been shown by Thuringer et al. (1995) thus explaining an apparent gap between acute and delayed preconditioning. Why the protection should restart again after the rapid time window of protection has run-down is another issue but one that is not addressed in these acute experiments.

Understanding the basis for the activation of NMDA receptors by pinacidil is far from clear. Pinacidil acts at plasmalemmal and mitochondrial ATP-sensitive potassium channels so this may occur by either type of channel. Plasmalemmal potassium channels when opened will allow potassium efflux from the intracellular compartment; the loss of intracellular positive charge will result in a more negative resting membrane potential which will enhance the voltage-dependent magnesium block of the NMDA receptors.

With this rationale it seems unlikely that ATP-sensitive potassium channels modulate the currents generated by NMDA receptors to enhance protection. Studies that have examined the neuroprotection afforded by ATP-sensitive potassium channels against excitotoxicity have proposed that such effects occur as a result of the enhanced voltage-dependent blockade of the NMDA receptors (Soundarapandian et al., 2007a) or the inhibition of excitatory transmitter release at pre-synaptic terminals (Soundarapandian et al., 2007b), actions which act to reduce NMDA receptor stimulation.

There are few studies in the literature regarding the stimulation of NMDA receptors by ATP-sensitive potassium channels. NO is generated from activation of mitochondrial ATP-sensitive potassium channels (Sasaki et al., 2000; Wang et al., 2001; Lacza et al., 2003; Han et al., 2002a; Han et al., 2002b); it is possible that excessive production of NO results in *S*-nitrosylation of the NMDA receptor leading to its inactivation (Gow et al., 2000). This is maybe a necessary step to allow continuous blockade of NMDA receptors after pinacidil is removed which is why blockade by AP-5 therefore prevents preconditioning. The possible roles of NMDA receptors in mediating adenosine preconditioning may not apply to pinacidil as it probably stimulates ATP-sensitive potassium channels more potently than adenosine, so that any need for BK+ channels may not be required in this paradigm. The mechanism of pinacidil may rely very much upon hyperpolarization of the

cell membrane to decrease the effect of ouabain at the sodium-potassium pump, moreso than adenosine, so that the depolarizing currents produced by NMDA receptor activation may stifle this protection. Pinacidil may therefore show a divergent mechanism of protection from adenosine by virtue of its specific and potent effects at ATP-sensitive potassium channels. This may also explain the different effect of pinacidil preconditioning against 10mM glutamate as compared to adenosine preconditioning. This is discussed in the next sub-section 4.3.10.3 – Protection against glutamate.

It is possible that pinacidil may have similar non-specific effects to diazoxide which could have a confounding impact on the results shown here.

### 4.3.10.3 - Protection against glutamate

The requirement for NMDA receptor stimulation during pinacidil preconditioning against ouabain is consistent with the observed protection against glutamate in addition to ouabain in the present study. NMDA receptor stimulation has been shown in several studies to precondition against various insults (Chuang et al., 1992; Kato et al., 1992; Pringle et al., 1999; Schurr et al., 2001; Bandyopadhyay et al., 2002). Pinacidil only preconditioned against glutamate in antidromic population spikes and not orthodromic population spikes. Having shown that 10mM glutamate disrupts the induction of LTP in orthodromic responses, this may explain why pinacidil was unable to precondition against pre-synaptically evoked responses. Also, the ATP-sensitive potassium channels show different effects on epsps and population spikes in CA1 neurons (Hosseinzadeh & Stone, 1998) and have different properties at pre- and post-synaptic sites (Matsumoto et al., 2002). From the adenosine preconditioning results, the ATP-sensitive potassium channels appear to mediate their protective effects post-synaptically. This is also consistent with the lack of interaction between adenosine preconditioning and ouabain in epsp responses.

In spite of the opening of ATP-sensitive potassium channels required to mediate adenosine preconditioning against ouabain, adenosine was unable to precondition against glutamate in neither antidromic nor orthodromic responses. This may be due to a difference in the level of potassium channel stimulation induced by pinacidil and adenosine. It is possible that adenosine does not stimulate the ATP-sensitive potassium channels sufficiently to induce protection against glutamate. Adenosine seems to protect against the glutamate-independent effects of ouabain.

The observation that successful pinacidil preconditioning against glutamate was observed only in antidromic spikes and not orthodromic spikes may be related to the actions of glutamate at the synapse and the cell body. Although there were no significant differences between the depressant effects of 10mM glutamate upon epsps, orthodromic and antidromic population spikes, the attenuation of epsp size by glutamate will prevent the formation of orthodromic population spikes in spite of any changes in the activity of potassium channels. This is probably why the effect of pinacidil on glutamate–induced depression in response size was observed only in antidromic population spikes. Changes in E-S coupling as a result of ATP-sensitive potassium channel stimulation could have allowed for observation of changes in population spike amplitude without concurrent alterations of epsps. This is probably why adenosine preconditioning against ouabain was observed in orthodromic population spikes and not epsps. The different results between preconditioning protocols for ouabain and glutamate could be accounted for by the different effects that each compound exerted on synaptic plasticity. Glutamate induced a depression in orthodromic population spikes which was not reversible by a 100Hz/1sec stimulus whilst ouabain did not completely prevent the induction of LTP. The depressant effect of glutamate on orthodromic population spikes was therefore more profound than that of ouabain. As changes in the E-S relationship account partly for the occurence of LTP (Andersen et al., 1980), the effect of glutamate on epsps could have masked any changes in population spikes if they were orthodromically evoked. In the present results, either no change in E-S coupling was induced during pinacidil preconditioning using orthodromic population spikes or such changes were insufficient to observe a significant effect of pinacidil upon glutamate.

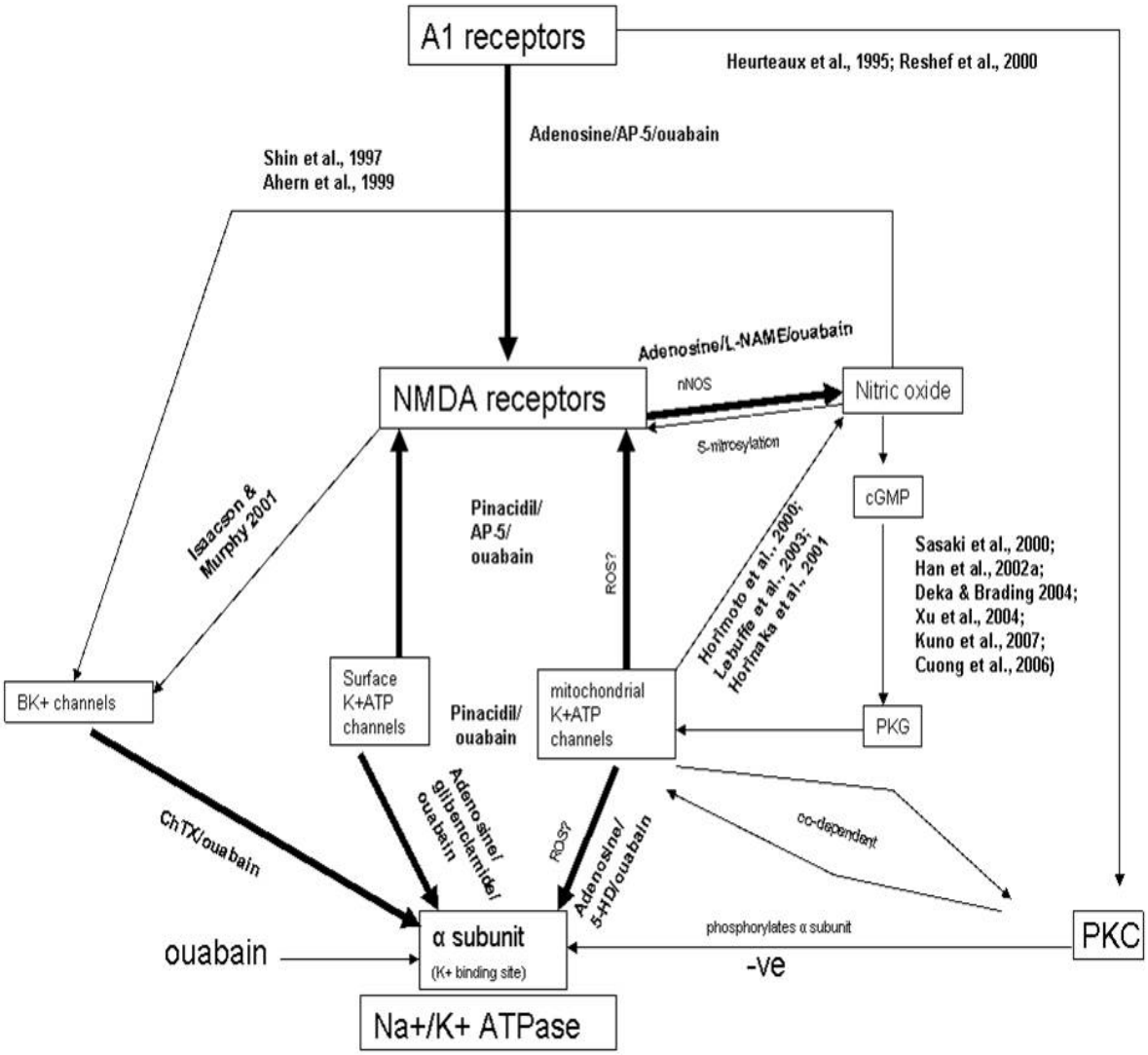


**Figure 4.44 Diagram of possible preconditioning interactions.** The figure shows interactions based on the presented results and observations from the scientific literature. NO = nitric oxide, PKC = protein kinase C, nNOS = neuronal nitric oxide synthase, PKG = protein kinase G, ROS = reactive oxygen species, ChTX = charybdotoxin, 5-HD = 5-hydroxydecanoate. The arrows in bold indicate particular interactions which have been examined in the present results. References and experiments for particular actions are indicated.

### 4.3.11 - The interaction between glutamate preconditioning and ouabain

Having observed that ouabain and glutamate could both be preconditioned by ATP-sensitive potassium channel activation, the interaction between these two agents was investigated as both are reported to induce preconditioning effects (Schurr et al., 2001; Pierre et al., 2006; Pasdois et al., 2007; Li et al., 2006). Pulses of glutamate applied to antidromic population spikes and epsps resulted in brief reductions in potential size following each transient exposure to the agonist which rapidly returned during each washout. When ouabain was applied after glutamate treatment, the potentials rapidly disappeared and did not return after one hour of observation. This effect is remarkably similar to those reported by Stel'mashuk et al. (1996) who reported that cerebellar granule cell cultures, when pre-exposed to 100μM glutamate for 15min, showed significantly higher cell deaths in response to 10μM ouabain exposure for 3 hours compared with no pretreatment. Interestingly, they also reported that pre-exposing the cells to ouabain before glutamate reduced cell death. Boldyrev et al. (2004) have suggested that NMDA receptor stimulation results in suppressing ouabain-sensitive sodium-potassium ATPase activity as a result of NMDA receptor generated reactive oxygen species acting upon the pump based upon their observations using cerebellar granule cell cultures. If generation of free radicals upon glutamate application to the slices results in suppressing sodium-potassium ATPase activity, then the pump will be more sensitive to ouabain causing a greater depolarization of the membrane and resulting in the loss of potential size observed in the present results. Although NMDA receptor stimulation appears to attenuate the effect of ouabain as demonstrated in the adenosine preconditioning experiments, the level of receptor stimulation may not be sufficient to generate free radicals in quantities that will cause suppression of sodium pump activity. Repeating these experiments with lower glutamate concentrations that do not reduce the potential size may therefore yield different results.

Using the antidromic potentials highlighted the similarity between the results using pulses of glutamate to precondition against ouabain and the effects of calcium modulation upon the response of the potentials to ouabain. As low calcium and chelation of intracellular calcium resulted in potentiating the depressant effect of ouabain, this may suggest that glutamate preconditioning as performed here results in decreasing intracellular calcium. A decrease in calcium might result from NMDA receptor desensitization upon repeated stimulation with glutamate therefore inactivating this entry pathway for calcium; the influx of ions into the neurons from glutamate receptor stimulation could also act to increase the activity of ion extrusion or sequestration mechanisms which may result in decreasing intracellular calcium; in the case of the sodium-calcium exchange however, this is not

likely as the activity of this exchange will decrease if the sodium-potassium ATPase activity is suppressed by free radical generation via NMDA receptors.

A novel mechanism which could explain these effects derives from the recent discovery of an endogenous ouabain-like compound which inhibits the sodium-potassium ATPase similarly to ouabain. It is termed endobain E and has been isolated from rat cerebral cortex and has been shown to modulate the binding of zinc (Reinés et al., 2004) and MK-801 (Reinés et al., 2001). The same authors have also shown that endobain modulates the NMDA receptor in the hippocampus during bilateral occlusion of rat common carotid arteries suggesting a role for this compound during ischaemia (Reinés et al., 2005). It is possible that in the present results, glutamate stimulation may increase the presence of endobain E which will, in turn, exert its effects upon both the NMDA receptor and the sodium-potassium ATPase pump. When the slices are then exposed to ouabain, the combination of endobain E and ouabain upon the pump will result in a greater suppression of its activity which may result in a larger depolarization of the membrane and the massive loss of potential size observed in the present results.

### 4.3.12 - Issues regarding the interpretation of Western blots

Western blotting can produce difficulties in the quantification of proteins and the choice of loading controls. Housekeeping proteins used as loading controls can vary on account of the experimental paradigm (Ferguson et al., 2005), a striking example being β-actin in the hippocampus, which is altered in response to plastic changes or an enriched environment in vivo (McNair et al., 2006; McNair et al., 2007). The statistical analysis of the molecular study results in this thesis indicated no significant differences in actin between experimental groups so it may be safe to assume that changes in housekeeping proteins between samples vary as a result of loading. The converse may also be true however: errors in loading may hide any potential changes in housekeeping proteins as a result of the experimental protocol.

The efficiency of the protein transfer from the running gel to the membrane is also impossible to determine accurately. Ponceau staining of the membrane was employed in the present study but this only provides a qualitative confirmation that the transfer was successful in order to proceed with protocol.

If results of a particular experiment are across more than one blot, factors such as blocking the membrane, efficiency of antibody binding and exposure time to chemiluminescence

can affect the final optical density, which is used to quantify the result. As some of the results in the present study were obtained across only two blots, the influence of these factors may have been much greater in determining the outcome. To minimise the effects of these variables, one would require a high n number and as many blots as possible.

For these blots, the constitutive form of HSP (HSP70) may have been a better choice of loading control rather than actin as this would have provided an indication of the level of heat shock protein that might therefore be induced in response to various stimuli. As there is a mere 2kDa difference in size between these proteins, this would prove technically difficult to obtain clear bands for the two proteins. There are available antibodies however, that bind to both forms so as to prevent stripping of the blot (eg mouse monoclonal anti-HSP72/HSP70 product no SPA-822 from Stressgen). With the appropriate choice of gel and running time, it is possible to obtain clear bands (Moon et al., 2001) if only after much trial and error.

A final point regarding the length of time allowed for molecular changes to occur following drug treatment (or untreated slices in the recording chamber): 1hour post-treatment is a very short time in which to see a molecular change with 4 samples. HSP72 was selected for this reason as it has been shown to upregulate in 1 hr (Araki et al., 1994) whereas other molecular indicators of cell damage or death (eg caspases) take several hours to observe a change. A Western blot analysis of hippocampal slices may therefore not be the most suitable indicator for assessing cell damage unless the slices are allowed to incubate for several hours post-treatment. Other methods such as propidium iodide staining may have been more appropriate as this fluorescent molecule is taken up into cells in which the membrane has degraded. Membrane lysis occurs over a shorter time frame than molecular changes and fluorescence is quantified by confocal microscopy.

### 4.3.13 - General Discussion

In the present results, it has been observed that 10mM glutamate induces effects upon field epsps, orthodromic and antidromic population spikes. All responses were initially lost upon application of glutamate; during the washout period, the responses recovered to levels significantly below the pre-glutamate values. It was also observed that the effects of 10mM glutamate were not reversed by a tetanic stimulus suggesting that this concentration of glutamate is sufficient to induce some dysfunction in the slice as opposed to a change in synaptic transmission.

The effects of ouabain upon the responses showed that sodium-potassium ATPase inhibition reduced the response sizes from the slice but that antidromically evoked responses were affected to a lesser degree than the orthodromic responses. By blocking the glutamate receptors, it appeared that the reduction in orthodromic responses was due to an increase in glutamate receptor stimulation whilst the effects in antidromic population spikes were independent of glutamate. It was also observed that, for orthodromic population spikes, the effects of ouabain could be partially reversed by a tetanic stimulus but that the potentiations induced were significantly lower than in control responses. Similarly to 10mM glutamate, this implied that some dysfunction occurred in the slice from sodium-potassium ATPase inhibition although to a lesser extent than that induced by 10mM glutamate.

For preconditioning experiments, it was observed that adenosine was able to induce tolerance against ouabain but not against exogenously applied glutamate. Further study showed that this effect occurred in population spikes but not in field epsps. This suggested that adenosine protected against the glutamate-independent component of the ouabain effect which was observed upon antidromic population spikes. By blockade of some known mediators of preconditioning, it was shown that the mechanism of adenosine preconditioning against ouabain involved adenosine A1 receptors, NMDA receptors, ATP-sensitive potassium channels and nitric oxide.

Pinacidil, an opener of the ATP-sensitive potassium channels, was used as a preconditioning stimulus against glutamate and ouabain. It was shown to protect against both types of chemical insult although with glutamate, the effect was observed in antidromically evoked responses only. It was also shown that NMDA receptors were required to mediate pinacidil preconditioning against ouabain, consistent with the mechanism of adenosine preconditioning.

To examine any potential interaction between glutamate and ouabain, a preconditioning protocol was developed where glutamate pulses were applied at intervals and the effect upon a subsequent application of ouabain was observed. It was shown that glutamate preconditioning had a deleterious effect upon the responses of both field epsps and antidromic population spikes to ouabain suggesting that, instead of cross-tolerance between the two agents, the combination of the two resulted in a greater loss of slice function.

# 5 – CONCLUDING DISCUSSION

## 5.1 Main Findings

1. Glutamate and NMDA induce changes in field epsps, orthodromic and antidromic population spikes. The changes in the orthodromic responses may be due to the degree of NMDA receptor involvement in the response.

2. The changes induced in orthodromic population spike size following 5mM glutamate treatment can be attenuated using adenosine A1 and A2a receptor antagonists.

3. The A1 receptor antagonist, DPCPX, attenuates the post-5mM glutamate effects on potential size by modulating the NMDA receptor-mediated component of the field epsp. This appears to extend to the effect of glutamate upon the orthodromic population spikes.

4. The A2a receptor antagonist, SCH 58261, appears to attenuate the reduced post-5mM glutamate potential by promoting the opening of ATP-sensitive potassium channels.

5. The post-5mM glutamate recovery is depressed using a maximally evoked orthodromic spike but is potentiated when using a half-maximally evoked orthodromic spike. Using a 25% maximal baseline elicits either a potentiated or depressed post-5mM glutamate recovery of the response.

6. A tetanising stimulus is able to reverse the post-5mM glutamate effects upon the orthodromic population spike but not upon the post-10mM glutamate effects.

7. Ouabain is able to induce depressions in field epsps, orthodromic and antidromic population spikes. The depression in antidromic population spikes is significantly less compared to the orthodromic responses.

8. The effects of ouabain upon antidromic population spikes are independent of glutamate receptors whilst the effect upon othodromic population spikes is attenuated by glutamate receptor blockade.

9. The ouabain-induced depression in the antidromic population spike amplitude can be altered by modulating calcium in the perfusion medium and blocking BK+ channels.

10. Ouabain impairs the ability of the orthodromic population spike to produce a potentiation in response to a tetanising stimulus.

11. Adenosine is able to precondition against the effects of ouabain in population spikes but not against the effects of glutamate.

12. Adenosine preconditioning against ouabain requires A1 receptors, NMDA receptors, NO and ATP-sensitive potassium channels.

13. Pinacidil is able to precondition against ouabain and glutamate in antidromic population spikes. The protective effect of pinacidil against ouabain depends on NMDA receptor activation.

14. Glutamate preconditioning enhances the depressant effects of ouabain upon field epsps and antidromic population spikes.

## 5.2 - Future directions

This work has studied the preconditioning phenomenon as it occurs in the rat hippocampal slice preparation and has also examined the interactions of adenosine receptors with high glutamate levels. The latter observations expand upon the work in the current scientific literature, showing that the NMDA receptor/adenosine A1 receptor interaction extends beyond epsp recordings to affect the population spikes in CA1 neurons. Such interactions are useful to study as they may lead to a greater understanding of the cognitive processes at the level of the organism and possibly to future cognitive therapies. These results require greater study with E-S coupling experiments to understand how NMDA receptors and adenosine receptor antagonists appear to affect this relationship in the present study.

This study has also highlighted the use of different chemical models of ischaemia and the various questions that these may raise. It questions the usefulness of such models and whether they do in fact provide an accurate model of ischaemia. The relevance of a compound like ouabain may until recently have seemed dubious as an ischaemic mimetic on account of the way in which it blocks the sodium-potassium ATPase. The discovery of an endogenous ouabain-like compound (Reinés et al., 2001; Reinés et al., 2004, Reinés et al., 2005) however, sheds an interesting light on the present results. The role of endobain E during ischaemia is currently unknown but its presence does give a greater physiological relevance to the use of ouabain. As strokes consist of complex and different mechanisms of cell death, the use of different models to isolate specific cell death events can provide

useful information and greater understanding of the pathology. Further study into the changes induced by glutamate and ouabain as models of chemical ischaemia should be continued with these advantages in mind.

One of the main findings of the thesis is the novel interaction which exists between adenosine and ouabain. Having observed adenosine protection in one ischaemic model only may highlight the limitations of adenosine-based compounds as a preconditioning therapy. The involvement of NMDA receptors in mediating these effects shows a novel interaction which might be exploited with further study.

An interesting future avenue of research may be in extending a possible role of BK+ channels in preconditioning. The BK+ channel activity increases to induce an ethanol tolerant state (Dopico et al., 1998; Cowmeadow et al., 2005). This is potentially of interest as studies have shown that moderate intake of alcohol leads to a decreased incidence of stroke compared with heavy intake or abstinence (Sacco et al., 1999). Whether this is related to some form of preconditioning is unknown but the BK+ channels may represent a viable drug target in which the preconditioning phenomenon is utilised. Leptin increases the activity of BK+ channels in the hippocampus (Shanley et al., 2002); an interesting possibility is that this hormone may itself become the basis of future targets in preconditioning studies also.

The relevance of preconditioning to stroke therapy, while still far from becoming a clinical therapy, may prove to be vital as cardiac preconditioning strategies become more developed. The nature of preconditioning is such that a cardiac or neuronal infarct would not be prevented but that the post-ichaemic tissue will be resilient to damage. Preconditioning agents for the cardiovascular system may therefore not be as effective working in isolation as the non-preconditioned tissues, such as the brain and other organs, may still suffer the effects of reduced blood flow. As the promise of clinical cardiovascular preconditioning agents seems to be drawing nearer, the speedy development of neuronal preconditioning agents may be required to work in conjunction with these therapies.

## 5.3 – Conclusion

The present work has shown the effectiveness of preconditioning agents against different depolarizing agents and the interactions between adenosine and glutamate receptors which may play a role in preconditioning. This data may be of value in the pursuit of preconditioning-based therapies for the treatment of stroke.

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