

Involvement of interleukin-1 (IL-1) in excitotoxic brain damage

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ABSTRACT

The cytokine, interleukin-1 (IL-1) is synthesised in the brain in response to injury and has been proposed as a mediator of excitotoxic damage. IL-1ra is an endogenous receptor antagonist of IL-1 and has been demonstrated to potently inhibit excitotoxic neurodegeneration due to overactivation of the *N*-methyl-D-aspartate (NMDA) receptor in the rat *in vivo*. However, excitotoxic neuronal damage can also be induced by overactivation of the non-NMDA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), receptor subtype. In the present study the relationship between IL-1 and excitotoxic brain damage (in the striatum and the cortex) induced by pharmacological overactivation of NMDA or AMPA receptors *in vivo* in the rat was further investigated.

Extensive striatal or cortical damage was induced by intrastriatal or intracortical stereotaxic infusion of 7.5-10nmol cis-2,4-methanoglutamate (MGlu) or 10-15nmol S-AMPA activating NMDA or AMPA receptors respectively. Neuronal damage was quantified 24h (for NMDA) or 48h (for AMPA) postinfusions by staining fresh coronal brain sections with tetrazolium chloride.

Results confirm the inhibitory (by 46%) effect of IL-1ra against NMDA receptor-induced lesions in the striatum and also demonstrate that striatal damage induced by selective activation of the AMPA receptor is reduced (43%) by co-infusion of IL-1ra. However, in contrast, co-infusion of IL-1ra failed to inhibit NMDA or AMPA receptor-induced damage in the cortex.

IL-1 β was not toxic to neurones *per se* when infused alone into either the striatum or the cortex. However, a significant fever was observed after infusion of IL-1 β into the striatum, but not after intracortical infusion. The same dose of IL-1 β co-infused into the striatum, did not affect the local striatal neuronal

damage induced by intrastratial infusion of MGlutamate or S-AMPA. However, extensive cortical damage was observed after administration of IL-1 β and S-AMPA into the striatum. This secondary cortical damage, but not the local striatal damage, was reduced (67%) by pretreatment with the NMDA receptor antagonist, MK-801, suggesting that cortical neurodegeneration may be mediated via NMDA receptors. Neuronal damage in the cortex appeared to be complete 24h postinfusions, whilst that in the striatum was submaximal at this time point. In addition to cortical neuronal death, damage in the thalamus was occasionally observed after striatal infusion of S-AMPA and IL-1 β .

IL-1 β administration in the cortex did not affect the local cortical damage induced by intracortical infusion of an NMDA or AMPA receptor agonist. However, infusion of IL-1 β into the ipsilateral or contralateral striatum enhanced this cortical neuronal degeneration.

Studies in mice failed to demonstrate an inhibitory action of IL-1ra against NMDA receptor-induced lesions in the striatum. In addition, genetically-modified mice which had the IL-1 β gene knocked out, or overexpression of IL-1ra also failed to show any changes in the extent of neuronal damage due to striatal infusion of an NMDA receptor agonist.

In conclusion, these studies demonstrate an involvement of IL-1 in the processes leading to neuronal death after NMDA or AMPA receptor activation in the rat *in vivo*. Furthermore, data indicate a novel site of action for IL-1, *the striatum*, and suggest that IL-1 may interact with neuronal pathways (presumably glutamatergic) resulting in neuronal loss within the cortex.

DECLARATION

I, the undersigned, declare that no part of this thesis has been submitted in support of an application for any degree or qualification of the University of Manchester or any other University or Institute of learning.

A handwritten signature in black ink, reading 'C B Lawrence'. The 'C' and 'B' are large and stylized, while 'Lawrence' is written in a more cursive, flowing script.

Catherine B Lawrence.

PREFACE

I obtained my honours degree in Pharmacology (Class Iii) in the School of Biological Sciences at the University of Manchester in 1992. Since then I have remained in Manchester working towards my Ph.D under the supervision of Professor Nancy J Rothwell.

ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisor, Nancy Rothwell, for her support and enthusiasm over the past three (and a bit!!) years. I would also like to thank every one in the lab, both past and present, for putting up with me in my times of happiness(???), distress and most of all stress. Apparently that is what a Ph.D is all about!! I'm afraid there are too many people to mention individually, but I'm thinking of you all (honest). However, I would like to say a special thank-you to good old Giamal, who helped me with the telemetry experiments, even though most of them haven't appeared in this thesis for one reason and another, I still enjoyed the break from *infusions!!!*. A *grand merci* also to Sylvie too for putting up with the few disasters we encountered over infusion of IL-1 into the brain. Thank-you also for the valued reading of this thesis, Dave, Giamal, Sylvie and Paul. I must also thank Ioan Davies (and LEEANNE) for allowing me to use the equipment for the mice experiments, and Andy who was a good companion (and football mate) when stuck in isolation in the quarantine area in our trendy white plastic suits and head gear!! Also so they don't feel left out I must thank the technicians, Louise (for orders, of compounds and equipment that is), Beth (for the tetrazolium) and Anthea (for rats), I couldn't have survived without you! Oh yeh, ta also for being loyal companions during "step classes".

I must say a quick thanks to the Wellcome Trust for their financial support, and to all my buddies in Manchester, Wirral, and further afield for their friendly support! I especially give a big hug to my friends at home, Nikki and Mandie, who have seen me through all my trauma's during the past three years (and more). Without escaping on our nights out, both in Manchester and Liverpool, I wouldn't have survived.

Finally, my biggest thank-you goes to my Mum and Dad who have been great in their support and most of all the love they have given me during my time in Manchester. My sisters Jayne and Anne, and my Nan also deserve a mention. Oh, I have just one more person to express my appreciation to and that is Dave. I can safely say if it wasn't for him I would have packed my bags ages ago and this thesis wouldn't be here now. Thanks Dave for having the patience for coping with my strange behaviour at times, your a star!!

p.s. Now I couldn't forget Norman who has taught that there are people!! that are capable of getting more stressed than myself.

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DEDICATION

I would like to dedicate this thesis to the best parents in the world-
Mum and Dad.

LIST OF ABBREVIATIONS

°	Degree
AA	Arachidonic acid
AC	Adenylate cyclase
ACTH	Adrenocorticotrophic hormone
AIDS	Acquired immunodeficiency syndrome
AMPA	α -amino-3-hydroxy-5-methyl-4-isoazolepropionate
S-AMPA	(S)- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AN	Accumbens nucleus
AON	Anterior olfactory nucleus
L-AP4	L-2-amino-4-phosphonobutanoic acid
AP5	2-amino-5-phosphonopentanoic acid
BBB	Blood-brain barrier
bFGF	Basic fibroblast growth factor
BST	Bed nucleus of the stria terminalis
°C	Degrees centigrade
C	Cortex
CC	Corpus callosum
Ca ²⁺	Calcium
CNS	Central nervous system
CRF	Corticotrophin-releasing factor
CSF	Cerebrospinal fluid
EAA	Excitatory amino acid
EAE	Experimental autoimmune encephalomyelitis
EGF	Epidermal growth factor
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GFAP	Glial fibrillary acidic protein
h	Hours
H ⁺	Hydrogen
HIV-1	Human immunodeficiency virus type 1
HPA	Hypothalamic-pituitary-adrenal axis
ICE	IL-1 β converting enzyme
icv	Intracerebroventricular
IFN	Interferon
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
ILC	Infralimbic cortex
i.p.	Intraperitoneally
IU	International unit
K ⁺	Potassium
LPS	Lipopolysaccharide
LTP	Long-term potentiation
MANOVA	Multivariate analysis of variance
MAP	Mitogen-activated protein
MCAo	Middle cerebral artery occlusion
Mg ²⁺	Magnesium
MGlu	Cis-2,4-methanoglutamate
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]-cyclohepten-

	5,10,iminemaleate
Na ⁺	Sodium
NADPH	Nicotinamide adenosine dinucleotide phosphate
NBQX	6-nitro-7-sulframoylbenzo(f)quinoxaline-2,3-dione
NGF	Nerve growth factor
NMDA	<i>N</i> -methyl-D-aspartate
N ₂ O	Nitrous oxide
NO	Nitric oxide
NOS	Nitric oxide synthase
NS	Not significant
O ₂	Oxygen
OVLT	Organum vasculosum of the lamina terminalis
PAF	Platelet-activating factor
Par	Parietal cortex
PBS	Phosphate-buffered saline
PC	Piriform cortex
PCP	Phencyclidine
PFA	Paraformaldehyde
PKC	Protein kinase C
PG	Prostaglandin
PLA ₂	Phospholipase A ₂
PLC	Phospholipase C
SEM	Standard error of the mean
St	Striatum
TFGβ	Transforming growth factor beta
Th	Thalamus
TNF	Tumour necrosis factors
VSCC	Voltage-sensitive calcium channels
Zn ²⁺	Zinc

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Chapter One

Introduction

Chapter One

Introduction

1.1 Neurodegeneration and excitotoxicity

Neuronal cell death which occurs during development of the nervous system eliminates unwanted neurones. This process is necessary for normal development and function of the brain. However, premature death of brain neurones frequently results from injury, trauma or disease, and can be life threatening. Thus the need for neuroprotective drugs is considerable. As yet no such agents are available that successfully prevent or delay neuronal cell death in humans.

The most common type of neurodegeneration is of the "necrotic" form, where neurones die via the action of external factors. However, there is increasing evidence that apoptosis (or programmed cell death) may occur in the brain, a process by which neuronal cells commit suicide (Rose and Henneberry, 1993; Margolis *et al.*, 1994). The primary cause of neuronal death in most neurological conditions, including stroke, head injury and seizure-induced brain damage and in chronic neurodegenerative states such as Alzheimer's and Parkinson's diseases, is not fully understood, but damage to neurones appears to be predominantly of the classical necrotic form (Ishimaru *et al.*, 1995). Several factors may play important roles in the aetiology of these conditions, including energy deficits, free radicals and environmentally-derived neurotoxins (for review see Lees, 1993a). Studies in recent years have focused on the role of the excitatory amino acid (EAA) neurotransmitters, such as glutamate and aspartate. There is increasing evidence to suggest that these amino acids are important causal factors in the neurodegenerative process. Their excessive release or

impaired uptake can lead to excitotoxic damage and death of neurones.

1.2 Excitatory amino acids (EAA)

The central nervous system (CNS) contains millimolar amounts of glutamate and aspartate and lesser amounts of other amino acids. Glutamate and aspartate are the principal and ubiquitous transmitters mediating fast excitatory synaptic responses at approximately 50% of synapses in the CNS. EAA systems are widely and relatively uniformly, distributed in the CNS. Most of the glutamatergic pathways originate in the cortex and project to a number of different subcortical targets including caudatoputamen (striatum), nucleus accumbens, olfactory tubercle, amygdala, thalamus, substantia nigra, hippocampus and the spinal cord (Fagg and Foster, 1983; Cotman *et al.*, 1987; Storm-Mathisen and Ottersen, 1988). The functionally important glutamatergic pathways are the cortico-striatal path, the perforant path (which originates in the entorhinal cortex and terminates in the hippocampus) and the mossy and climbing fibres (which are the two important afferent pathways of the cerebellum). Most cortico-cortico connections also use glutamate as a neurotransmitter (Fagg and Foster, 1983; Cotman *et al.*, 1987; Storm-Mathisen and Ottersen, 1988; Parent and Hazrati, 1995a, 1995b). The presence of glutamatergic pathways in these regions of the brain strongly indicates an important role of glutamate neurotransmission in cortical and hippocampal cognitive function, pyramidal and extrapyramidal motor function, and cerebellar and sensory function.

Glutamate is stored mainly within presynaptic synaptic vesicles. When the glutamatergic nerve terminals are depolarised, vesicular glutamate is released from its stores into the synaptic cleft by a calcium (Ca^{2+}) dependent process (Nicholls and Attwell, 1990; McMahon and Nicholls, 1991). Termination of the action of glutamate is thought to occur by the uptake of this amino acid from the synaptic cleft by high affinity, sodium (Na^+)-dependent glutamate uptake carriers,

which are present on both nerve terminals and glial cells (Nicholls and Attwell, 1990; Kanai *et al.*, 1993, 1994; Danbolt, 1994). Glutamate is converted to glutamine in the glial cells via the action of the enzyme, glutamine synthetase. Glutamine is then transferred back to the nerve terminals where it acts as the major precursor for newly synthesised glutamate, involving the mitochondrial enzyme phosphate-activated glutaminase (Nicklas, 1986; Schousboe *et al.*, 1992). Glutamate is also synthesised in the nerve terminals from glucose and from transamination of α -ketoglutarate. Newly synthesised glutamate is then actively taken up into synaptic vesicles, and is therefore available for release (Schousboe *et al.*, 1992).

Glutamatergic systems are widely distributed throughout the brain and are involved in numerous brain functions. Glutamate is the predominant fast excitatory neurotransmitter in the CNS and modulates the release of other neurotransmitters, possibly via an action on presynaptic receptors (e.g. Cheramy *et al.*, 1994; Ohta, *et al.*, 1994; Giovannini *et al.*, 1995). However, a unique property of EAA transmitters is their involvement in functions unrelated to synaptic transmission. For example, glutamate is a component of proteins and an intermediate in several metabolic pathways; both carbohydrate and nitrogen metabolism. Glutamate has also been implicated in the development and maintenance of long-term potentiation (LTP), a form of synaptic plasticity which may underlie learning and memory (Bliss and Collingridge, 1993; Collingridge and Bliss, 1995). It has been suggested that EAA's are involved in several processes during development, including regulation of neuronal survival, growth, differentiation, dendritic and axonal structure, synaptogenesis and synaptic plasticity (Collingridge and Singer, 1990; McDonald and Johnston, 1990). EAA's also play an important role in the neuroendocrine regulation of a variety of hormones (Lopez *et al.*, 1992; Brann, 1995). In addition (as discussed later in *Section 1.4*), EAA's may exert neurotoxic actions and contribute in several acute

and chronic neurological disorders, which involve the death of neurones. All these actions of EAA's are mediated via the EAA receptors of which there are several types.

1.3 EAA receptors

The EAA transmitter, glutamate, exerts its effects on CNS neurones and glia by interacting with at least four distinct postsynaptic receptors, although an additional presynaptic L-2-amino-4-phosphonobutanoic acid (L-AP4) receptor has recently been identified (Collingridge and Lester, 1989; Monaghan *et al.*, 1989; Hollmann and Heinemann, 1994). Receptor subtypes have been characterised by electrophysiological, biochemical and anatomical approaches and more recently by molecular studies. They can be distinguished by various properties: for example, the responses they generate, channel kinetics, pharmacological profiles, ion permeability, dependence on endogenous modulators and their detailed anatomical organisation. Glutamate receptors are found throughout the mammalian brain (e.g. Hollmann and Heinemann, 1994) but are specifically distributed in various regions.

There are two main types of glutamate receptors, ionotropic and metabotropic. The ionotropic receptors are ligand gated ion channels which have been classified by actions of the selective agonists, *N*-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoazolepropionate (AMPA) and kainate (Watkins *et al.*, 1990), and are thus divided into NMDA, AMPA and kainate receptors. These receptors are more generally subdivided into NMDA and non-NMDA (AMPA and kainate) receptors and are all directly coupled to cation channels.

Glutamate also activates a metabotropic receptor. This group of receptors are coupled to G proteins, which activate several intracellular enzymes, for example,

adenylate cyclase (AC), and phospholipase C (PLC; Schoepp *et al.*, 1990; Tanabe *et al.*, 1992).

1.3.1 NMDA receptor

The NMDA receptor channel complex is the most well characterised of the EAA receptor subtypes. It derives its name from the observation that the agonist, NMDA, selectively activates this receptor. NMDA is a synthetic analogue of aspartic acid and does not occur naturally within the brain. Much knowledge has been derived about the NMDA receptor due to the discovery of this agonist and selective antagonists, such as 2-amino-5-phosphonopentanoic acid (AP5) and MK-801 ((+)-5-methyl-10, 11-dihydro-5H-dibenzo-[a, d]-cyclohepten-5, 10, iminemaleate or dizocilpine).

The NMDA receptor is complex and has several modulatory sites. This receptor is involved in a wide range of brain functions, most of which are discussed below (for detailed reviews on the NMDA receptor see e.g. Collingridge and Lester, 1989; Scatton, 1993; Stone, 1993; McBain and Mayer, 1994; Mori and Mishina, 1995 and see *Table 1.3.1*).

1.3.1.1 Channel properties/modulation

The NMDA receptor is a ligand-activated, ion channel receptor complex which, following activation by EAA's, allows Na^+ and Ca^{2+} to enter neurones and potassium (K^+) ions to escape from the cytoplasm into the extracellular space. The channel kinetics for the NMDA receptor are relatively slow compared to the AMPA receptor (Jahr and Stevens, 1990; Lester *et al.*, 1990).

NMDA receptors have unusual properties for ligand-gated ion channels. These include a voltage-dependent block by magnesium (Mg^{2+} ; Mayer *et al.*, 1984;

Nowak *et al.*, 1984; Mayer and Westbrook, 1987a), high permeability to Ca^{2+} (Mayer and Westbrook, 1987a; Ascher and Nowak, 1988a), and multiple regulatory/pharmacological domains (e.g. polyamine, glycine, zinc (Zn^{2+})). Occupation of these various sites modulates receptor function in different ways. In addition to the binding of glutamate at a specific site in the receptor, there is also a binding site for glycine. The binding of both glutamate and glycine must occur in order for receptor activation (Johnson and Ascher, 1987; Simpson *et al.*, 1994). Thus, glycine can be considered a "co-agonist" at the NMDA receptor. One of the most important features of the NMDA receptor complex is the voltage-dependent block by Mg^{2+} (Mayer *et al.*, 1984; Nowak *et al.*, 1984; Mayer and Westbrook, 1987a). Mg^{2+} binds to a site within the channel pore. At resting membrane potentials, the NMDA channel is blocked by physiological concentrations Mg^{2+} ions. However, if the membrane is sufficiently depolarised, this block can be relieved, and ions flow through the channel. The NMDA receptor also contains a polyamine site and activation of this site by spermine and spermidine increases the ability of glutamate and glycine to open the ion channel (Williams *et al.*, 1991; Williams, 1994). Extracellular Zn^{2+} ions also modulate NMDA receptor function by interaction with the Zn^{2+} site, which antagonises the NMDA receptor (Westbrook and Mayer, 1987; Christine and Choi, 1990). There is also a domain within the channel pore called the phencyclidine (PCP) site. The action of many drugs which antagonise the NMDA receptor act by binding to this site. Such drugs include the dissociative anaesthetics, ketamine and PCP, and also MK-801 (Rogawski, 1993).

Arachidonic acid (AA) affects the NMDA receptor by potentiating NMDA currents (Miller *et al.*, 1992). The NMDA receptor is also modulated by redox reagents (e.g. Kohr *et al.*, 1994; Omerovic *et al.*, 1994), histamine, neurosteroids and pH (see McBain and Mayer, 1994; Mori and Mishina, 1995) and, maybe regulated by intracellular messengers, such as protein phosphatases and tyrosine kinases (Lieberman and Mody, 1994; Wang *et al.*, 1994b; Wang and Slater, 1994; Sigel,

1995).

1.3.1.2 *Receptor molecular biology and distribution*

The NMDA receptor has been shown, by radioligand binding studies, to be located on neurones throughout the brain, but predominantly within the telencephalon. It is present in high densities in the cerebral cortex, hippocampus, striatum, septum, amygdala and the granule layer of the cerebellum, being most concentrated in the CA1 region of the hippocampus (Cotman *et al.*, 1987; Young and Fagg, 1990). However, NMDA receptors are also present on glial cells (Uchihori and Puro, 1993; Luque and Richards, 1995).

Molecular biological techniques have demonstrated that most NMDA receptors are heteromeric and made up from different receptor subunits (see Monyer *et al.*, 1992; Nakanishi, 1992; Seeburg, 1993; Molinoff *et al.*, 1994; Nakanishi and Masu, 1994). Two families of NMDA receptor subunits have been identified, the NMDA-R1 and NMDA-R2 (NMDA-R2A- NMDAR2D; Nakanishi, 1992). Functionally distinct NMDA receptor channels can be generated by heteromeric assembly of the NMDA-R1 subunit with differing members of the NMDA-R2 subunit family. The NMDA receptor mRNAs display regional differences in their distribution. NMDA-R1 and NMDA-R2A are widely and uniformly distributed throughout the CNS, whereas the NMDA-R2B subunit is expressed only in the forebrain, and the NMDA-R2C subunit is located mainly in the cerebellum (e.g. Monyer *et al.*, 1992; Nakanishi, 1992). These distinct receptor subtypes, distributed in different brain regions probably have different pharmacological and physiological properties.

1.3.1.3 *Function*

NMDA receptors are involved in synaptic transmission at a wide variety of sites

in the CNS. In comparison to AMPA/Kainate receptors, which mediate fast synaptic transmission, the NMDA receptor is involved in slower excitatory synaptic responses. In addition, NMDA receptors seem to be critically involved in synaptic formation and plasticity and exert trophic effects on neurones (Collingridge and Singer, 1990; McDonald and Johnston, 1990). Current evidence shows that NMDA receptors are required for the development and maintenance of LTP (Bliss and Collingridge, 1993; Collingridge and Bliss, 1995). Furthermore, the NMDA receptor has been implicated in the control of normal physiological processes such as respiration and blood pressure (Foutz *et al.*, 1988; Faraci and Breese, 1994) and the regulation of the neuroendocrine system (Brann, 1995). In addition, overstimulation of NMDA receptors has been implicated in various neuronal disorders (e.g. Choi, 1994 and see *Section 1.4*).

1.3.2 The AMPA receptor

In recent years there have been rapid advances in the characterisation of the AMPA receptor. Originally termed the "quisqualate" receptor, this receptor has now been classified into ionotropic (AMPA) and metabotropic receptor subtypes. This has largely followed the discovery of AMPA (Krogsgaard-Larsen *et al.*, 1980), an agonist which shows greater selectivity for the ionotropic (AMPA) receptor, whereas quisqualate activates both the AMPA and metabotropic receptors (for detailed reviews on the AMPA receptor see e.g. Hollmann and Heinemann, 1994; Bettler and Mulle, 1995 and see *Table 1.3.1*).

1.3.2.1 Channel properties/modulation

The AMPA receptor is coupled directly to cation channels and may be associated with a 130kDa modulatory protein (Honoré and Drejer, 1988). The AMPA receptor channel is putatively permeable to monovalent cations, allowing the influx of Na⁺ and efflux of K⁺, but exhibits little permeability to the divalent

ion, Ca^{2+} (Mayer and Westbrook, 1987a, 1987b; Iino *et al.*, 1990). However, under certain circumstances, the AMPA receptor can show Ca^{2+} permeability due to differing subunit compositions of individual AMPA receptors (see Section 1.3.2.2 and e.g. Hollmann *et al.*, 1991; Jonas *et al.*, 1994; and see Bettler and Mülle, 1995). Electrophysiological studies have shown that AMPA receptor-activated channels show little voltage dependence, and display low conductance compared to the NMDA receptor (Mayer and Westbrook, 1987a, 1987b; Ascher and Nowak, 1988b; Bettler and Mülle, 1995).

AMPA receptors have fewer modulatory sites than the NMDA receptor channel complex, although at least one is thought to be present, a 2,3-benzodiazepine site at which some AMPA receptor antagonists bind (Rogawski, 1993; Zorumski *et al.*, 1993), or the desensitisation site at which cyclothiazide and diazoxide can also bind to modify the rate of desensitisation (Bertolino *et al.*, 1993). Unlike the NMDA receptor, rather little is known about the regulation/modulation of responses mediated by AMPA receptors. Barbiturates antagonise (Simmonds and Horne, 1988), Zn^{2+} potentiates (Rassendren *et al.*, 1990), and AA depresses (Kovalchuk *et al.*, 1994) AMPA receptor responses/currents. Additionally, rapid desensitisation appears to be an important regulatory mechanism (see, Bettler and Mülle, 1995; Raman and Trussell, 1995) and, although NMDA receptor activation is thought to promote desensitisation, this is of a slower form (Zorumski *et al.*, 1989). Recent evidence also suggests that the AMPA receptor is regulated by intracellular phosphorylation (Tan *et al.*, 1994).

1.3.2.2 *Receptor molecular biology and distribution*

AMPA receptors are widely and abundantly distributed throughout the CNS (for review on mRNA localisation see Hollmann and Heinemann, 1994), and their distribution generally corresponds to that of the NMDA receptor. High densities of the AMPA receptor are found in the cerebral cortex, hippocampus, striatum,

septum, amygdala, and in contrast to the NMDA receptor, the molecular layer of the cerebellum. Similarly, to the NMDA receptor the highest levels are found in the hippocampus. (Cotman *et al.*, 1987; Young and Fagg, 1990). AMPA receptors are present on both neurones and glial cells (Baude *et al.*, 1994; Lopez *et al.*, 1994; Matute *et al.*, 1994; Patneau *et al.*, 1994).

AMPA receptors (like the NMDA receptor), are multimeric heteromers composed of several distinct subunits (see Barnard and Henley, 1990; Nakanishi, 1992; Seeburg, 1993; Hollmann and Heinemann, 1994; Nakanishi and Masu, 1994). Four families of AMPA receptor subunits have been identified, GluR1-GluR4 (also known as GluRA-GluRD; see Barnard and Henley, 1990; Nakanishi, 1992; Seeburg, 1993; Hollmann and Heinemann, 1994; Nakanishi and Masu, 1994), which can assemble in various combinations to form functional receptors. In most neurones, AMPA receptors exhibit low permeability to Ca^{2+} . However, some AMPA receptors are permeable to Ca^{2+} . These differences in Ca^{2+} permeability have been ascribed to differential receptor-subunit expression (e.g. Hollmann *et al.*, 1991; Jonas *et al.*, 1994 and see Bettler and Mülle, 1995). It appears that the receptor subunits GluR1 and GluR3 form ion channels permeable to Ca^{2+} . However, inclusion of a GluR2 subunit in the receptor, prevents Ca^{2+} permeability (Hollmann *et al.*, 1991; Jonas *et al.*, 1994). Thus, different subunit combinations give rise to receptors with distinct physiological and pharmacological profiles.

1.3.2.3 *Function*

AMPA receptor activation appears to mediate most of the fast synaptic excitatory transmission in the CNS, and like the NMDA receptor, the development and maintenance of LTP (Bliss and Collingridge, 1993). Unlike the trophic effects of NMDA receptors on developing neurones, AMPA receptors appear to exert an opposite effect and do not support the growth of neurones (McDonald and

Table 1.3.1 *Characteristics of NMDA and AMPA receptors*

	NMDA RECEPTOR	AMPA RECEPTOR
Agonists	Glutamate. Aspartate. NMDA. MGlu.	Glutamate. AMPA. Quisqualate.
Molecular weight	209kDa.	52kDa.
Subunits	NMDA-R1- NMDA-R2A-2D	GluR1-GluR4
Distribution	Cortex. Hippocampus. Striatum. Septum. Amygdala. Cerebellum-- (granule layer). Glia.	Cortex. Hippocampus. Striatum. Septum. Amygdala. Cerebellum-- (molecular layer). Glia.
Properties	Voltage-dependent. Na ⁺ /K ⁺ /Ca ²⁺ permeable channels.	Voltage-independent. Na ⁺ /K ⁺ permeable, Ca ²⁺ impermeable channels. Na ⁺ /K ⁺ /Ca ²⁺ permeable channels.
Modulators	Glycine. Polyamines. Hydrogen ion (H ⁺). Zn ²⁺ (inhibits). AA (potentiates)	Barbiturates. Cyclothiazides. Zn ²⁺ (potentiates). AA (inhibits)
Channel Blockers	Mg ²⁺ . MK-801. PCP. Ketamine.	
Function	Slow synaptic transmission. LTP. Synaptic plasticity. Trophic effects.	Fast synaptic transmission. LTP.

Johnston, 1990; Pizzi *et al.*, 1994). There is also evidence for involvement of AMPA receptors in various pathological processes of neuronal death (e.g. Choi, 1994 and see *Section 1.4*).

1.3.3 Kainate receptors

The kainate receptor is similar to the AMPA receptor (see above), and therefore will not be discussed here in detail (see Hollmann and Heinemann, 1994; Bettler and Mulle, 1995 for recent reviews). This receptor is coupled directly to a cation channel, which allows Na⁺ influx and K⁺ efflux from the cell. These fluxes cause depolarisation and, in addition to actions associated with the AMPA receptor, the kainate receptor may mediate fast synaptic depolarisation (see Collingridge and Lester, 1989). However, the pharmacology and function of the kainate receptor, and the physiological importance of its activation remains unclear. This is due to the lack of specific agonists and antagonists for this receptor type. Several studies have suggested that the neurophysiological effects of kainate may be mediated via the AMPA receptor, as kainate can also bind to the AMPA receptor (e.g. Hall *et al.*, 1994; Paternain *et al.*, 1995). In addition, antagonists at the AMPA receptor generally antagonise kainate receptors at higher concentrations. However, kainate has been shown to induce several neuronal responses independently of the AMPA receptor (e.g. Mayer and Westbrook, 1987b; Paternain *et al.*, 1995). Regional distribution studies have revealed that kainate receptors are dissimilar to NMDA and AMPA receptors. The majority of kainate receptors are located in regions of low NMDA receptor site density, for example, the stratum lucidum of the hippocampus (Cotman *et al.*, 1987; Young and Fagg, 1990; Petralia *et al.*, 1994). Kainate sites are also abundant in the hypothalamus and certain areas of the thalamus. As for NMDA and AMPA receptors, kainate receptors are assembled of different subunits, GluR5-GluR7, KA1 and KA2 (see Nakanishi, 1992; Seeburg, 1993; Hollmann and Heinemann, 1994; Nakanishi and Masu, 1994). Again, varying subunit compositions of individual receptors

determine the pharmacological and physiological properties of these putative receptors. Kainate receptor overactivation, in addition to NMDA and AMPA receptors, induces neuronal death (e.g. Choi, 1994).

1.3.4 Metabotropic receptors

Metabotropic receptors are coupled to G proteins and cytoplasmic enzymes. Several different types of pre- and postsynaptic metabotropic receptors have been described and either activate, phosphoinositide hydrolysis (via activation of PLC producing inositol triphosphate and diacylglycerol) or regulate the activity of AC (modulating cAMP formation; Schoepp *et al.*, 1990; Tanbe *et al.*, 1992; Winder and Conn, 1992 and see Hollmann and Heinemann, 1994; Pin and Duvoisin, 1995). At least six genes for the metabotropic receptor (mGluR1-mGluR6) have been cloned. The functional role of the metabotropic receptors has been difficult to define because of the lack of selective agonists and antagonists, although they may regulate neuronal excitability. Their involvement in neuronal death is also unclear (see Choi, 1994).

1.4 Neurodegeneration induced by EAA's

It was discovered over thirty years ago that systemic injection of glutamate destroys the inner neural layers of the immature mouse retina (Lucas and Newhouse, 1957). This observation gave rise to the term "excitotoxicity", describing neuronal injury induced by the neuroexcitatory action of glutamate (Olney *et al.*, 1971). There is now convincing evidence that the same receptors that mediate neuronal depolarisation can also cause neuronal injury (Meldrum and Garthwaite, 1990). Prolonged stimulation of either NMDA or non-NMDA receptors by glutamate eventually results in death of most neurones (Choi, 1994; Rothman and Olney, 1995). The exact mechanism(s) of neuronal injury is complicated, since depolarisation, neuronal swelling, Ca^{2+} influx and possibly

second messengers may contribute. Evidence is accumulating that brain damage associated with anoxia, stroke, head trauma, hypoglycaemia, epilepsy, and perhaps chronic neurodegenerative diseases such as Huntington's, Alzheimer's, and Parkinson's disease, may all be due to the excessive activation of EAA receptors (see Olney, 1990; Whetsell and Shapira, 1993; Zorumski and Olney, 1993; Lipton and Rosenberg, 1994).

High concentrations of glutamate and subsequent EAA receptor overactivation is potentially neurotoxic. However, under normal physiological conditions such toxicity does not arise because of the actions of various control systems. For example, high affinity uptake of glutamate into glial cells and neurones, and voltage-dependent blockade of NMDA channels by physiological concentrations of Mg^{2+} ions (e.g. Mayer and Westbrook, 1987a; Nicholls and Attwell, 1990). However, in pathophysiological conditions, excessive release, or impaired reuptake of glutamate, or receptor abnormalities can allow overstimulation of EAA receptors leading to neuronal death via a complex network of mechanisms.

1.4.1 Mechanisms of excitotoxicity

In vitro studies have shown that there are two main types of neuronal injury induced by exposure of neurones to glutamate (see Choi, 1993, 1994): 1) "Rapidly-triggered excitotoxicity" is the term given to neuronal death observed after a short glutamate exposure (2-3min), and is predominantly dependent on the overactivation of NMDA receptors, and 2) "Slowly-triggered excitotoxicity", after longer exposure times to glutamate, and is dependent on the prolonged overactivation of AMPA/kainate receptors. However, the rapid form of neuronal death is also made up of two components. The first of these is a rapid neuronal swelling, induced by the influx of Na^+ through both NMDA and non-NMDA receptor and followed by passive chloride ion and water influx. The second component is due to the excessive influx of Ca^{2+} through the NMDA receptor-

gated channel. The slow form of excitotoxicity is due predominantly to the overactivation of the non-NMDA receptors, which causes membrane depolarisation via influx of Na^+ . Secondary Ca^{2+} entry then occurs via voltage-dependent Ca^{2+} channels. Additionally, a rise in intracellular Na^+ may activate the reverse operation of the $\text{Na}^+/\text{Ca}^{2+}$ antiport transporter across the mitochondrial and plasma membranes leading to a further increase in intracellular Ca^{2+} (see Choi, 1994; Lipton and Rosenberg, 1994). In addition to the indirect entry of Ca^{2+} after non-NMDA receptor stimulation, Ca^{2+} can also enter directly through channels gated by non-NMDA receptors, some of which have been proposed to be permeable to Ca^{2+} (Hollmann *et al.*, 1991; Jonas *et al.*, 1994). Increases in Ca^{2+} are normally regulated by control mechanisms such as mitochondrial uptake and extrusion via the $\text{Na}^+/\text{Ca}^{2+}$ antiport. However, under excitotoxic conditions these control processes may become impaired.

Thus, excess activation of any of the three ionotropic EAA receptors can lead to neuronal damage which is dependent upon the presence of extracellular ions (Choi, 1987). Ca^{2+} influx via NMDA receptors, as well as that due to activation of non-NMDA receptors, initiates a cascade of metabolic changes which are potentially toxic (Orrenius *et al.*, 1989; Mayer and Miller, 1990). These changes include alterations in mitochondrial function and significant induction of Ca^{2+} -dependent intracellular enzymes (e.g. proteases, phospholipases and protein kinases). These processes may then lead to cytoskeletal and neuronal membrane changes, production of free radicals, increased glutamate release, impaired glutamate uptake as well as interference with normal neuronal maintenance mechanisms (see Meldrum and Garthwaite, 1990; Dugan and Choi, 1994; Farooqui and Horrocks, 1994a, 1994b; Lipton and Rosenberg, 1994 and *Table 1.4.1* and *Figure 1.4.1*).

Thus, it is likely that multiple mechanisms operating in parallel lead to the neuronal damage and degeneration by glutamate, resulting from excessive

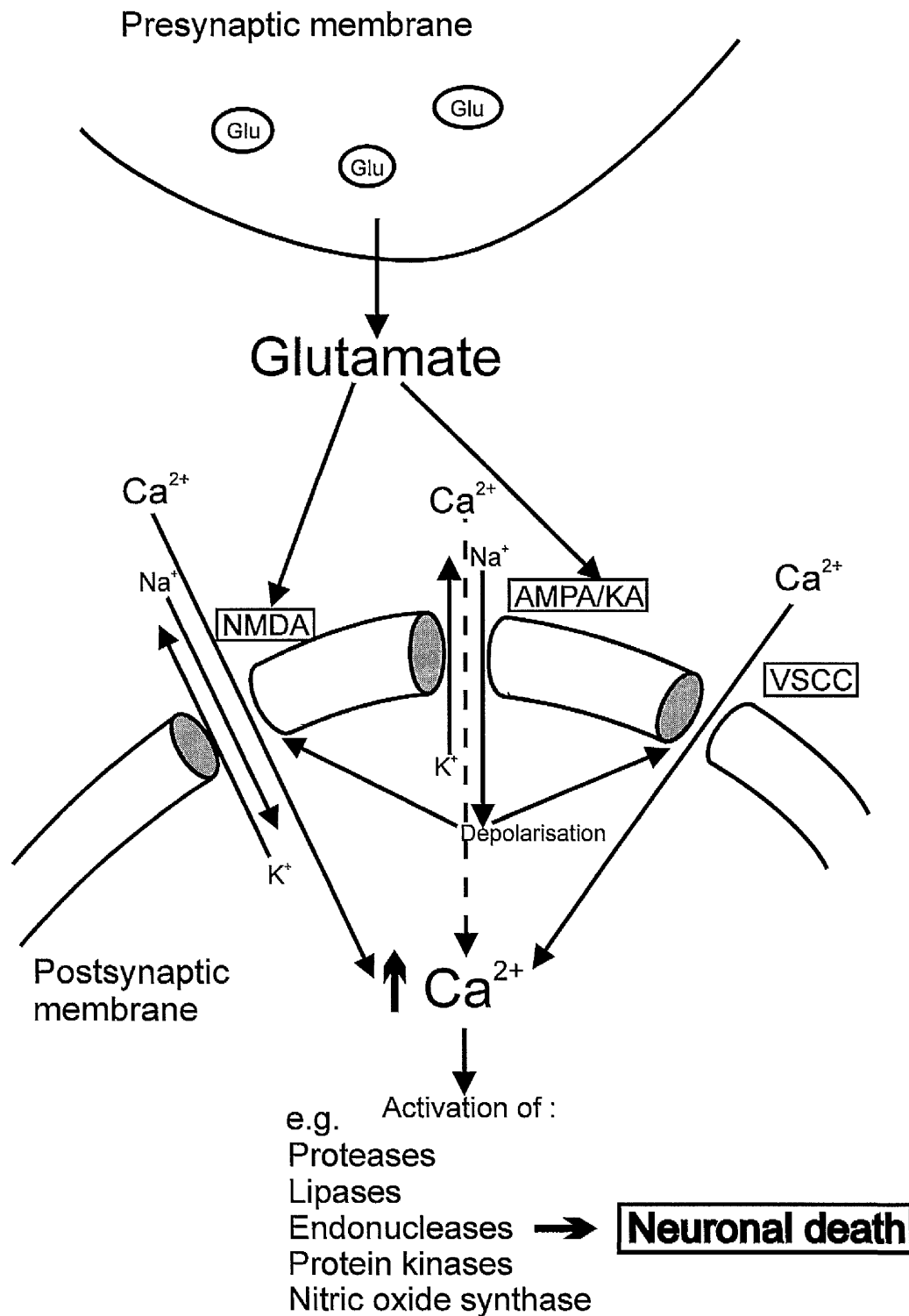


Figure 1.4.1 *Schematic diagram to illustrate a simplified involvement of NMDA and AMPA receptors, and Ca^{2+} in excitotoxic neuronal death*

Glu=glutamate

VSCC=voltage-sensitive calcium channels

Table 1.4.1 *Effects of Ca²⁺-activated enzymes that may be involved in EAA mediated neurotoxicity*

Ca²⁺-activated enzymes.	Product of enzyme activation	Effect/ consequences	Reference
Phospholipase A₂ (PLA₂)	Arachidonic acid (AA)	<ul style="list-style-type: none"> • AA cascade-- synthesis of PG's & PAF → ↑Ca²⁺. • Metabolised to free radicals. • Increase glutamate release. • Inhibit reuptake of glutamate. 	see Farooqui and Horrocks, 1994a, 1994b. Barbour <i>et al.</i> , 1989. Lynch and Voss, 1990. Bito <i>et al.</i> , 1992 Miller <i>et al.</i> , 1992.
Nitric oxide synthase (NOS)	Nitric oxide (NO)	<ul style="list-style-type: none"> • Increase cGMP levels. • Free radical production. • Enhance glutamate release. 	Garthwaite <i>et al.</i> , 1989. Beckman, 1991. Moncada <i>et al.</i> , 1991. Knowles, 1994.
Protein kinase C (PKC)		<ul style="list-style-type: none"> • Phosphorylate enzymes & membrane proteins (e.g. ion channels). • Potentiate glutamate release. • Enhance synaptic (e.g. glutamate) transmission. 	Kaczmarek, 1987.
Proteases (e.g. calpain)		<ul style="list-style-type: none"> • Degradation of cytoskeletal proteins. • Free radical production. 	Melloni and Pontremoli, 1989.
Endonucleases		<ul style="list-style-type: none"> • Condensation of nuclear chromatin. • DNA fragmentation ('apoptosis'). 	Orrenius <i>et al.</i> , 1989.

PG's=prostaglandins, PAF=platelet-activating factor

activation of both NMDA and non-NMDA receptors.

1.4.2 NMDA versus non-NMDA (AMPA) induced neurodegeneration

Glutamate induces neuronal death by activation of both NMDA and AMPA receptors (see *Figure 1.4.1*). The contribution of each receptor in neuronal damage can be assessed using specific agonists and antagonists. Hence, histopathological and pharmacological studies have shown differences in the characteristics of neuronal damage induced by the various receptor subtypes.

Studies *in vitro* suggest that NMDA receptor overactivation induces a rapid cell death termed "oedematous necrosis", whilst in contrast, selective AMPA receptor activation induces a more gradual cell death "dark cell degeneration" (Garthwaite and Garthwaite, 1989; 1991a, 1991b; Garthwaite *et al.*, 1992; Choi, 1993, 1994). In support of this observation, AMPA receptor activation will cause widespread neuronal death *in vitro* only if exposure time is prolonged (~24h), although a small population of neurones is selectively destroyed by shorter exposure to AMPA (~10-60min; see Choi, 1993, 1994). These vulnerable neurones may express Ca^{2+} permeable AMPA channels, which lack the GluR2 subunits (Reid *et al.*, 1993).

Although increases in intracellular Ca^{2+} are probably important in the initiation of neuronal death after overactivation of EAA receptors (e.g. see Frandsen and Schousboe, 1993) the major routes of Ca^{2+} entry still need to be clarified. Different modes of Ca^{2+} entry occur in neurones after NMDA versus AMPA treatment. Exposure of neurones to NMDA *in vitro* results in Ca^{2+} influx as well as release from intracellular stores. In contrast, exposure to AMPA leads to preferential Ca^{2+} influx, with little or no Ca^{2+} release from intracellular stores (Frandsen and Schousboe, 1993; Schousboe *et al.*, 1994; Mody and MacDonald, 1995).

Neurones show marked variations in their vulnerability to the specific EAA receptors agonists (see Meldrum and Garthwaite, 1990). Variations in EAA receptor expression maybe responsible for some of these differences. In the striatum and cortex *in vivo* and in cortical and striatal cell cultures *in vitro*, excitotoxic damage caused by exposure to NMDA or AMPA agonists shows marked variations in the types of neurones affected (e.g. Koh and Choi, 1988a, 1988b; Beal *et al.*, 1989, 1991). Furthermore, in contrast to NMDA receptor agonists, injections of non-NMDA receptor agonists into the rat brain produce damage not only at the site of injection, but also in distant regions (e.g. Köhler and Schwarcz, 1983; Wozniak *et al.*, 1994).

Varied mechanism(s) of neuronal death induced by NMDA and AMPA receptors are implied by differences in the effects of agents on excitotoxic damage caused by either receptor. For example, the divalent cation, Zn^{2+} , decreases neurotoxicity induced by the NMDA receptor, whereas AMPA receptor-mediated excitation is enhanced by this ion (Peters *et al.*, 1987; Koh and Choi, 1988c; Wilson *et al.*, 1992; Weiss *et al.*, 1993). Furthermore, nitric oxide (NO) has been implicated in NMDA receptor-induced toxicity, but its involvement in damage induced by the AMPA receptor remains to be determined (Izumi *et al.*, 1992; Moncada *et al.*, 1992; Kollegger *et al.*, 1993; Hewett *et al.*, 1994). Recent data also suggest that glia differentially modulate excitotoxicity induced by different EAA receptors. Glia appear to exacerbate AMPA receptor-induced neurotoxicity *in vitro*, whereas pure neuronal cultures (with little or no glia present) are more sensitive to the toxic effects of NMDA (Dugan *et al.*, 1995).

In conclusion, several pieces of data suggest that the mechanisms of neuronal damage caused by AMPA receptor agonists differ from those associated with agonists at the NMDA receptor (see *Table 1.4.2*). Furthermore, there are probably differences in the contribution of NMDA and AMPA receptors in various neurological disorders (see *Section 1.5*). However, there are also many common

features between each type of neurodegeneration though the specific mechanisms of excitotoxic neuronal death remain to be fully clarified.

Table 1.4.2 *Some proposed differences between NMDA and AMPA receptor-mediated neuronal death*

NMDA	AMPA	Reference
Rapid cell death "Oedematous necrosis"	Gradual cell death "Dark cell degeneration"	Meldrum and Garthwaite, 1990. Garthwaite and Garthwaite, 1991a, 1991b.
Ca ²⁺ influx and release from intracellular stores important in neurotoxicity.	Ca ²⁺ influx predominantly important in neurotoxicity.	Frandsen and Schousboe, 1993. Schousboe <i>et al.</i> , 1994. Mody and MacDonald, 1995.
NADPH-diaphorase & Somatostatin/ neuropeptide Y containing neurones not vulnerable.	NADPH-diaphorase & Somatostatin/ neuropeptide Y containing neurones highly vulnerable.	Koh and Choi, 1988a, 1988b. Beal <i>et al.</i> , 1989, 1991.
Zn ²⁺ decreases neurotoxicity.	Zn ²⁺ increases neurotoxicity.	Peters <i>et al.</i> , 1987. Koh and Choi, 1988c. Wilson <i>et al.</i> , 1992. Weiss <i>et al.</i> , 1993.
NO synthesis involved in neurotoxicity.	NO synthesis not involved in neurotoxicity.	Izumi <i>et al.</i> , 1992. Moncada <i>et al.</i> , 1992. Kollegger <i>et al.</i> , 1993. Hewett <i>et al.</i> , 1994.
Glia-neuroprotective	Glia-neurotoxic	Dugan <i>et al.</i> , 1995

1.5 Involvement of EAA in neurological disorders

There is now considerable evidence to implicate glutamate in the pathophysiology of acute and chronic neurological diseases, and NMDA and non-NMDA (AMPA) receptors have both been proposed to contribute to neuronal

damage.

1.5.1 EAA neurotoxicity in acute neuronal degeneration

Acute neurodegeneration occurs after a variety of brain insults including cerebral ischaemia, trauma, epilepsy and hypoglycaemia. Strong evidence for a role of endogenous EAA's in acute neurotoxicity includes dramatic increases in the release of EAA's during ischaemia in experimental animals *in vivo* (Benveniste *et al.*, 1984), or traumatic brain damage in humans (Baker *et al.*, 1993). Intervention with excitotoxic processes, for example, by applying EAA antagonists or inhibiting glutamate release, markedly reduces the damage to neurones in experimental models of neuronal damage *in vivo* and *in vitro* (e.g. Choi, 1990; Meldrum, 1990; Lipton and Rosenberg, 1994 and see below).

In vitro and *in vivo* studies have demonstrated impressive neuroprotective effects of NMDA receptor antagonists in experimentally-induced ischaemic neuronal damage, predominantly in rodents (for reviews see Meldrum, 1990; Scatton *et al.*, 1991; McCulloch, 1994). NMDA receptor antagonists are particularly protective in animals subjected to permanent focal ischaemia (e.g. Bullock *et al.*, 1990). However, neuroprotective effects of AMPA antagonists have also been reported in the same type of ischaemic injury (Gill *et al.*, 1992; Smith and Meldrum, 1992). It appears therefore that both NMDA and AMPA receptors are involved in the neuronal damage following focal cerebral ischaemia. Various groups have reported that AMPA antagonists are neuroprotective in transient or global ischaemia and brain damage due to cardiac arrest (e.g. Sheardown *et al.*, 1990; Diemer *et al.*, 1992; Nellgard and Wieloch, 1992). These findings are especially important as NMDA receptor antagonists are ineffective or less efficacious in these situations (Block and Pulsinelli, 1987; Wieloch *et al.*, 1988; Buchan and Pulsinelli, 1991; Diemer *et al.*, 1992; Nellgard and Wieloch, 1992). Neurodegeneration resulting from these different insults may therefore arise from

predominant activation of one receptor subtype (for reviews see Pulsinelli *et al.*, 1993; Gill, 1994).

Excitotoxic processes have also been implicated in neuronal death caused by traumatic brain injury *in vivo* and *in vitro* (see Regan and Choi, 1994). Extracellular concentrations of EAA's rise after traumatic brain damage in humans (e.g. Baker *et al.*, 1993) and NMDA antagonists reduce the extent of neuronal damage in several forms *in vivo* brain injury (e.g. McIntosh *et al.*, 1990; Bullock and Fujisawa, 1992; McIntosh, 1993; Toulmond *et al.*, 1993b), although AMPA antagonists appear to be ineffective (e.g. Qin *et al.*, 1995).

Glutamate also plays an important role in the pathogenesis of epilepsy and subsequent neuronal damage (see Meldrum, 1994). Intracerebral infusion of glutamate or EAA receptor agonists in the rat *in vivo* induces seizure activity and brain damage, which resembles the seizure-related brain damage in patients with epilepsy (e.g. Ben-Ari, 1985). Persistent seizure activity may be due to alterations in EAA physiology and the expression of EAA receptors. Thus, changes in the densities of NMDA, AMPA and kainate receptors have been demonstrated in postmortem tissue taken from patients with epilepsy (e.g. Geddes *et al.*, 1990; Hosford *et al.*, 1991). Furthermore, glutamate antagonists acting selectively at either NMDA or non-NMDA receptors are anticonvulsants in several forms of epilepsy in experimental animals *in vivo* (e.g. Patel *et al.*, 1990; Chapman *et al.*, 1991 and see Meldrum, 1994).

1.5.2 EAA in chronic neuronal degeneration

Although evidence is largely circumstantial, excitotoxic mechanisms have also been implicated in the pathogenesis of various chronic neurodegenerative disorders. These disorders include Alzheimer's disease, Huntington's chorea, motor neurone disease and Parkinson's disease (e.g. Olney, 1990; Whetsell and

Shapira, 1993; Zorumski and Olney, 1993). Abnormalities in EAA physiology and receptor function are found in these diseases, and antagonists at both NMDA and AMPA receptors are partially beneficial in several animal (rat and primate) models of Parkinson's disease (e.g. Brochie *et al.*, 1990; Brochie *et al.*, 1991; Klockgether *et al.*, 1991; Loschmann *et al.*, 1991; Turski *et al.*, 1991).

EAA receptor-mediated neurotoxicity also appears to be involved in the neurodegeneration associated with some viral infections such as acquired immunodeficiency syndrome (AIDS), encephalitis and the Maedi-Visna virus (Hayman *et al.*, 1993; Gendelman *et al.*, 1994; Lipton, 1992, 1994). Studies *in vitro* have demonstrated that production of neurotoxins by human immunodeficiency virus type 1 (HIV-1) infected macrophages, and neurotoxicity induced by the HIV-1 envelope glycoprotein, gp120, are inhibited by NMDA receptor antagonists, but AMPA antagonists are ineffective (e.g. see Lipton, 1992).

In summary, several pieces of evidence implicate EAA's in the pathogenesis of acute and chronic neurological disorders. Modification of EAA processes in these conditions may provide a viable clinical therapy. However, whilst many studies of experimentally-induced neuronal damage in animals have demonstrated the neuroprotective actions of EAA antagonists, their effectiveness in humans remains to be determined. Concern over the clinical use of EAA antagonists has arisen from reports of serious side effects in animals and man, including neurotoxicity and behavioural side effects (see Olney *et al.*, 1989; Albers *et al.*, 1989; Muir and Lees, 1995). Therefore, further clarification of the mechanisms involved in excitotoxic neuronal death is needed in order for novel means of intervention to be targeted.

Complexity in the mechanisms of neurodegeneration has arisen due to the recent discovery of neuroinflammatory mechanisms being involved in the

pathogenesis of several neurodegenerative disorders, and has opened up additional avenues for possible therapeutic intervention.

1.6 Brain inflammation

Under normal circumstances the brain is regarded as immunologically dormant and does not exhibit classical immune responses which are observed in systemic tissues. Acute inflammation in the periphery is defined by a non-specific response to tissue injury which involves the rapid (within minutes) recruitment of neutrophils and monocytes. In contrast, there is no neutrophil recruitment and a delayed increase in the number of microglial/macrophage cells in response to neuronal damage (induced by excitotoxins) in the brain (Andersson *et al.*, 1991a, 1991b; Perry and Andersson, 1992; Akiyama *et al.*, 1994). Additionally, the initiation of an inflammatory response in the brain requires a more potent stimulus when compared to that in systemic tissues (Andersson *et al.*, 1992; Perry and Andersson, 1992).

However, during disease and injury/insult, immune activation and inflammatory responses can occur within the brain (Mucke and Eddleston, 1993; Couraud, 1994; Fabry *et al.*, 1994). For example, neutrophil adherence has been demonstrated on endothelial cells after cerebral ischaemia *in vivo* (e.g. Clark *et al.*, 1993). Activation of resident glial cells, astrocytes and microglia (which can then produce several pro- and anti-inflammatory mediators and, adhesion molecules), and activation of vascular endothelial cells leading to subsequent infiltration of blood leukocytes, occurs after an excitotoxic insult to the brain *in vivo* (e.g. Andersson *et al.*, 1991a, b; Perry and Andersson, 1992; Akiyama *et al.*, 1994). In addition, increases in immune function has been reported in the brain during various chronic neurological diseases (Aisen and Davis, 1994).

It has been proposed that neuroinflammatory mechanisms may contribute to

neuronal damage observed in degenerative diseases such as stroke, head injury, Alzheimer's disease and multiple sclerosis (see 'McGeer and McGeer, 1995; Morganti-Kossmann and Kossmann, 1995; Rothwell *et al.*, 1995b; Shiosaki and Puttfarcken, 1995; Woodroffe, 1995), and immunosuppressives reduce ischaemic neuronal damage in the rat *in vivo* (Sharkey and Butcher, 1994). However, it is not yet proven if the immune reaction occurring in neurodegeneration is completely detrimental, since some aspects may be beneficial, and what are the important mediators of inflammation. A group of soluble polypeptides, *the cytokines*, are the likely mediators of brain inflammatory responses.

1.7 Cytokines

Cytokines are a diverse group of polypeptides including interleukins (IL), interferons (IFN), growth factors and tumour necrosis factors (TNF). Cytokines are classically known as immunomodulatory mediators, affecting most tissues and cells within the periphery (see Hopkins and Rothwell, 1995; Rothwell and Hopkins, 1995), and are potential mediators of peripheral tissue damage and inflammation. They are synthesised predominantly by peripheral immune cells (e.g. macrophages and lymphocytes) and control the growth, differentiation, and function of most cell types. However, a number of cytokines and their receptors are also synthesised in glia (both microglia and astroglia) and neurones within the CNS (see Hopkins and Rothwell, 1995). Furthermore, these molecules exhibit diverse actions in the brain including fever, thermogenesis, cell proliferation, and behavioural changes (see Rothwell, 1990, 1991; Rothwell and Hopkins, 1995). Cytokines form a network of integrated signals and many act in concert to exert their effects, for example IL-1 can act synergistically with other cytokines including IL-6 and TNF α (Dinarello, 1994). However, each cytokine is unique, displays an individual spectrum of actions, and can even exert opposing

effects depending on the cell type affected.

Cytokines coordinate immune responses within the brain in response to neuronal injury or infection. These responses may subsequently affect the outcome of neuronal injury (Shiosaki and Puttfarcken, 1995; Woodroffe, 1995), suggesting an involvement of these proteins in neurological diseases. However, the precise role of cytokines in the response to neuronal injury is unclear, but various reports indicate that these molecules are directly/indirectly involved in neuronal damage and repair (e.g. Relton and Rothwell, 1992; Rothwell and Relton, 1993; Rothwell *et al.*, 1995b; Rothwell and Strijbos, 1995).

1.7.1 *The involvement of cytokines in ischaemic and excitotoxic neuronal injury*

Cytokines are normally present in the brain at low levels. However, during neuronal insults or infection the synthesis of cytokines is upregulated, although the physiological significance of this production is unknown (e.g. see Hopkins and Rothwell, 1995; Woodroffe, 1995). There are many recent reports studying the effects of several cytokines (e.g. IL's and growth factors) on various types of experimentally-induced neurodegeneration *in vitro* and *in vivo*. Historically cytokines are thought to exert predominantly neuroprotective properties. However, several studies have provided conflicting data, for example there are reports of cytokines exhibiting both neuroprotective and neurotoxic responses (e.g. Araujo, 1992; Araujo and Cotman, 1995; Strijbos and Rothwell, 1995). A brief but by no means complete summary on the effect of cytokines on predominantly ischaemic and excitotoxic neuronal injury is given below (and see *Table 1.7.1*).

1.7.1.1 *Neuroprotective effects*

The growth factors including nerve growth factor (NGF), basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF β), and the interleukin, IL-6, protect against cerebral ischaemia (Gross *et al.*, 1993; Nozaki *et al.*, 1993; Prehn *et al.*, 1993; Koketsu *et al.*, 1994; Loddick and Rothwell, personal communication) and excitotoxic damage (Toulmond *et al.*, 1992; Firm *et al.*, 1993; Nozaki *et al.*, 1993; Emerich *et al.*, 1994; Kirschner *et al.*, 1995) in rats *in vivo*, although, Tenjin *et al.* (1995) report no effect of bFGF on cerebral ischaemia in the rat *in vivo*. Glial derived neurotrophic factor has potent inhibitory effects against EAA-induced seizures and also reduces the amount of associated neuronal cell loss (Martin *et al.*, 1995). Many *in vitro* studies in neuronal cultures also reveal neuroprotective effects of the growth factors NGF, bFGF, TGF β , TNF α and β against several ischaemic or excitotoxic neuronal insults (e.g. Mattson *et al.*, 1989; Cheng and Mattson, 1991; Freese *et al.*, 1992; Prehn *et al.*, 1993; Zhang *et al.*, 1993; Chao *et al.*, 1994; Cheng *et al.*, 1994; Yamada and Hatanaka, 1994). The mechanism of these neuroprotective effects is unknown but has been ascribed, in some cases, to be due to a modification of intracellular Ca²⁺ levels (Mattson *et al.*, 1989; Cheng and Mattson, 1991; Mattson and Cheng, 1993; Mattson *et al.*, 1993; Cheng *et al.*, 1994).

1.7.1.2 *Neurotoxic effects*

TGF β_1 has neurotoxic effects against glutamate-induced death in neuronal cultures (Prehn and Kriegstein, 1994) although in the same system TGF β_1 exerts neuroprotective properties. The growth factor, TNF α can kill neurones in culture and potentiate glutamate neurotoxicity *in vitro* (Gelbard *et al.*, 1993; Chao and Hu, 1994), and participates in the destruction of oligodendroglial cells (Selmaj *et al.*, 1991). Furthermore, epidermal growth factor (EGF) enhances

NMDA receptor-mediated increases in intracellular Ca^{2+} concentration in cultured neurones (Abe and Saito, 1992a), suggesting that EGF may play a role in enhancing EAA-mediated toxicity. However, Abe and Saito (1992b) have also proposed that EGF displays protective effects against glutamate neurotoxicity *in vitro*.

In view of these above data, the role of cytokines in neuronal damage is extremely complex and remains to be further clarified. This heterogeneous group of molecules appear to exert opposing actions, displaying neuroprotective or neurotoxic properties. For example, *in vivo* IL-6 protects against excitotoxic damage in the rat (Toulmond *et al.*, 1992), but transgenic mice overexpressing this cytokine exhibit neurotoxicity (Campbell *et al.*, 1993). Some of these discrepancies may arise from comparison of *in vivo* and *in vitro* studies and the use of different animal species or source of cytokine. It is clear that the situation *in vitro* differs from that *in vivo*, for example, in neuronal cultures, glial and endothelial cells are predominantly absent and synaptic connections are not complete. In addition, all of the above reports have focused on the effects of exogenous cytokines on experimentally-induced neuronal death. The concentration of exogenous cytokines employed vary between reports and furthermore, it is questionable that the amount of administered cytokine will relate to that in a physiological/pathophysiological situation.

One of the most relevant pieces of data for the involvement of cytokines in neurodegeneration is the demonstration that inhibition of *endogenous* IL-1 reduces neuronal death induced by cerebral ischaemia and excitotoxin administration *in vivo* in the rat (Relton and Rothwell, 1992). This cytokine has been strongly implicated in neurodegeneration (see e.g Relton and Rothwell, 1992; Rothwell and Relton, 1993; Rothwell and Strijbos, 1995) and there has been an abundance of reports in the past few years concerning the involvement of IL-1 in neuronal cell death. Hence, the following sections will exclusively

concentrate on IL-1 and its role in neurodegenerative processes.

Table 1.7.1 *Summary of the neuroprotective/neurotoxic effects of several cytokines on ischaemic and excitotoxic forms of brain damage in vivo and in vitro*

	Neuroprotective	Neurotoxic
<i>In vivo</i>	NGF bFGF TGF β IL-6 GDNF	IL-1 IL-6
<i>In vitro</i>	NGF bFGF TGF β TNF α TNF β EGF IL-1	TGF β TNF α IL-2 EGF? IL-1

1.8 Interleukin-1 (IL-1)

IL-1, is a 17kDa cytokine classically known to be involved in the peripheral immune system. For example, IL-1 is a potent inducer of T cells, enhances the growth and differentiation of B cells and fibroblasts, and induces the synthesis of adhesion molecules. The term IL-1 actually describes two proteins, IL-1 α and IL-1 β , which are separate gene products and share only 20-30% sequence homology (see Dinarello, 1994), but have almost identical actions on most cells. A third member of the IL-1 family, an endogenous competitive IL-1 receptor antagonist (IL-1ra) also displays significant sequence homology with IL-1 α and IL-1 β (18-26%). This protein binds to the IL-1 receptor, but exerts no known physiological actions other than blockade of receptor function (Dinarello and Thompson, 1991; Dripps *et al.*, 1991a; Arend, 1993). IL-1ra is synthesised within the brain in areas of importance to IL-1 function (e.g. hippocampus,

hypothalamus; Licinio *et al.*, 1991) suggesting an endogenous role in the regulation of IL-1.

All three members of the IL-1 gene family are synthesised initially as a precursor molecules. The IL-1 α and IL-1 β precursors, known as proIL-1 α and proIL-1 β , have a molecular mass of 31kDa and are subsequently cleaved to the 17kDa forms. ProIL-1 α is biologically active, however, proIL-1 β is predominantly inactive and requires cleavage to IL-1 β for optimal activity. This process is performed by an enzyme named IL-1 β converting enzyme (ICE; Thornberry *et al.*, 1992).

1.8.1 IL-1 in the CNS

Constitutive expression of IL-1 in the brain is low, but IL-1 β mRNA and protein have been localised in the hippocampus, olfactory bulb, cerebellum, ventromedial hypothalamus and cortex in the mouse and rat (e.g. Lechan *et al.*, 1990; Ban *et al.*, 1992; Schöbitz *et al.*, 1994). The major cell source of IL-1 in the brain, either in normal conditions or after an injury, is not clear (see Woodroffe, 1995), and may depend on the type of stimulus. It was originally thought that invading monocytes/macrophages, neutrophils and lymphocytes of the peripheral immune system were the only sources of IL-1 in the brain. These cells may enter the brain, especially after injury or infection, possibly at sites which lack a functional blood-brain barrier (BBB; e.g. organum vasculosum of the lamina terminalis, OVLT), or where the BBB is disrupted (e.g. Nieto-Sampedro and Berman, 1987; Blatteis *et al.*, 1989). However, it is now known that native brain cells synthesise and release IL-1, for example, neurones, glia (microglia and astrocytes) and endothelial cells (Giulian *et al.*, 1986; Breder *et al.*, 1988; Hetier *et al.*, 1988; Lechan *et al.*, 1990; Ban *et al.*, 1992; Schöbitz *et al.*, 1994). It has been suggested that microglia are the predominant (or even the only) source of IL-1 within the CNS (Giulian *et al.*, 1986; Hetier *et al.*, 1988).

Various stimuli can increase the expression of IL-1 (see *Section 1.9.1*), including the administration of excitotoxic agents to the rat *in vivo* (Minami *et al.*, 1990, 1991; Yabuuchi *et al.*, 1993).

1.8.2 IL-1 receptors

Two types of IL-1 receptors have been identified in the periphery: the type I and type II receptors. The type I 80kDa receptor is expressed predominantly on T-cells, fibroblasts and epithelial cells, while the type II receptor is 68kDa and is localised on B-cells and macrophages (see Sims and Dower, 1994). IL-1 α and IL-1 β bind with similar affinities to the type I receptor which is thought to mediate the majority of biological actions of IL-1. In contrast, IL-1 β has a higher affinity, than IL-1 α , for the type II receptor (Scapigliati *et al.*, 1989). Recent data suggest that the type II receptor does not activate signal transduction (possibly due to a short cytosolic domain), but acts as a "decoy" receptor which prevents IL-1 binding to the type I receptor (Sims *et al.*, 1994). IL-1ra has a greater affinity for the type I than the type II receptor (Dripps *et al.*, 1991b; Dinarello, 1994).

Whilst these IL-1 receptors have been well characterised on peripheral cells, little is known about their localisation or function in the CNS. IL-1 receptors have been identified on neurones (Ban *et al.*, 1991), and glia (Ban *et al.*, 1993; Ban, 1994; Otero and Merrill, 1994). Autoradiographic and *in situ* hybridisation studies have demonstrated both types of receptors in the mouse brain (Takao *et al.*, 1990, 1992, 1993; Cunningham *et al.*, 1992; Ban, 1994; Parent *et al.*, 1994) in the hippocampus, choroid plexus and cortex, with a predominance in the hippocampus. Several studies have also localised mRNA for the type I receptor in the rat (Yan *et al.*, 1992; Yabuuchi *et al.*, 1994a) and demonstrated IL-1 binding sites in the pituitary (Marquette *et al.*, 1995). However, the presence of high affinity IL-1 receptors (especially the type II receptor) in the rat brain

remains to be fully established. Studies by Luheshi *et al* (1993) using a antibody to the type II receptor, have suggested that this receptor may be functional in the brain and mediate the pyrogenic actions of IL-1 β . Furthermore, it has been proposed that novel or atypical IL-1 receptor(s) may exist in rat brain and that the type II receptor may be functional (Takao *et al.*, 1993; Rothwell and Luheshi, 1994; Marquette *et al.*, 1995). These conflicting data may arise from the different experimental procedures employed (e.g. measurements of message or protein, and binding or functional studies).

Very little is known about the IL-1 signal transduction mechanisms. The pathway to a response induced by IL-1 after binding to its receptor appears to be very complex. Nearly every second messenger pathway that exists to date has been implicated in signal transduction of the IL-1/IL-1 receptor complex. Inositol phosphate hydrolysis, cAMP, G proteins, protein phosphorylation, transcription factors and NO are to name but a few pathways/mediators. Although, major pathways that may be important are a novel mitogen-activated protein (MAP) kinase or the sphingomyelin/ceramide cascade (for reviews see Rossi, 1993; Brooks and Mizel, 1994; O'Neill, 1995).

1.8.3 Biological effects of IL-1 in the brain

IL-1 is primarily associated with immune responses, and acts in the periphery as a mediator of host defence responses to infection, injury and malignant disease. However, this cytokine also acts within the CNS. In addition to several local effects within the brain (e.g. gliosis), IL-1 also mediates various metabolic, endocrine, behavioural and immune responses to systemic disease via actions within the brain.

IL-1 was first identified as an "endogenous pyrogen", and also mediates many other biological functions associated with the acute phase response such as

hypophagia, sleep, "sickness behaviour", anorexia, increased non rapid-eye-movement, and activation of the hypothalamo-pituitary-adrenal (HPA) axis (see Rothwell, 1991; Dinarello, 1994).

Many of the actions of IL-1 have been ascribed to the release of prostaglandins (PG's), since cyclooxygenase inhibitors reduce, for example, IL-1-induced fever, thermogenesis and adrenocorticotrophic hormone (ACTH) release (see Rothwell, 1991; Rothwell and Luheshi, 1994). Corticotrophin-releasing factor (CRF) has also been implicated in many central actions of IL-1 including HPA axis activation, fever and behaviour (e.g. Berkenbosch *et al.*, 1987 and see Rothwell, 1991).

Particular interest has focused on brain IL-1 since it was proposed as a mediator of neurodegeneration (see Relton and Rothwell, 1992; Rothwell and Relton, 1993; Rothwell and Strijbos, 1995; Rothwell *et al.*, 1995b). Many actions of IL-1 may be related to its effect on neurodegenerative processes, and are described below.

1.9 IL-1 and neurodegeneration

Expression of IL-1 is increased after a variety of experimental and clinical neurological insults and in diseased brain (see *Section 1.9.1* and *1.9.2*). Thus, is this IL-1 actively involved in the neurodegenerative processes (either beneficial or detrimental) or is expression just a consequence of injury? As previously discussed (see *Section 1.6*) inflammatory responses, both in the periphery and within the brain, are observed after brain injury (e.g. Giulian *et al.*, 1989; Andersson *et al.*, 1991a, 1991b; Perry and Andersson, 1992; Clark *et al.*, 1993). Since IL-1 is a well known mediator of inflammation, increased expression of this cytokine may be due to the inflammatory reaction. However, it is now emerging that immunological mechanisms may be involved in

pathogenesis of several neurological diseases (e.g. see Sharkey and Butcher, 1994; McGeer and McGeer, 1995; Shiosaki and Puttfarcken, 1995) and IL-1 may be participate in these processes.

Recombinant IL-1 induces glial cell proliferation, neuronal sprouting, neovascularisation and scar formation *in vivo* and *in vitro* (Giulian and Lachman, 1985; Giulian *et al.*, 1988; Lachman *et al.*, 1987). These observations suggest that IL-1 may play a role in neuronal survival and repair after injury. The inflammatory reaction after brain injury may be necessary for the removal of cellular debris and the formation of a glial barrier (via gliosis). Gliosis and scar formation may protect healthy tissue from further injury due to EAA and free radicals which are released from injured tissue. However, an excessive glial reaction may impede the recovery of neuronal function by inhibiting the regeneration of neuronal processes (Reier, 1986; Kimelberg and Norenberg, 1989). This leads to the question of whether IL-1 exerts detrimental or beneficial effects on brain tissue after injury.

Beneficial effects?

IL-1 has been show to act on neurones to augment gamma-aminobutyric acid (GABA)-induced chloride influx (Miller *et al.*, 1991) and inhibit pentylene-tetrazole-induced seizures (Miller *et al.*, 1991), LTP (Katsuki *et al.*, 1990; Bellinger *et al.*, 1993), Ca^{2+} channel currents (Plata-Salamán and Ffrench-Mullen, 1994) and serotonin-induced Ca^{2+} mobilisation in glia (Kugaya *et al.*, 1995). These data suggest an inhibitory action of IL-1 on neurones. Furthermore, IL-1 can induce *in vivo* and *in vitro*, the expression of certain neurotrophic/neuroprotective factors, such as NGF (Spranger *et al.*, 1990; Carman-Krzan *et al.*, 1991). *In vitro* studies on primary neuronal cultures indicate that IL-1 is a neuroprotective/neurotropic factor (Strijbos and Rothwell, 1995). These observations suggest a protective effect of IL-1 on neuronal cell damage.

Detrimental effects?

In contrast to the evidence outlined above, several actions of IL-1 may be detrimental to neurones. IL-1 increases intracellular Ca^{2+} concentrations in cultured neurones (Di Julio *et al.*, 1991) and antagonises the effects of the neuroprotective growth factor, FGF (Logan, 1990). IL-1 can also cause (and may mediate) brain oedema formation (Gordon *et al.*, 1990; Yamasaki *et al.*, 1992, 1994), and induces damage to the BBB (Quagliarello *et al.*, 1991). High concentrations of IL-1 are neurotoxic and reduce the survival of cultured neurones, whereas low concentrations of IL-1 display neurotropic/neuroprotective actions (Araujo, 1992; Araujo and Cotman, 1995; Strijbos and Rothwell, 1995).

Table 1.9.1 *Potentially beneficial and detrimental actions of IL-1 which may influence neurodegeneration*

Potentially beneficial	Potentially detrimental
<ul style="list-style-type: none">• Glial proliferation• Scar formation• Neovascularisation• Enhance GABA receptor responses <i>Inhibit:</i> <ul style="list-style-type: none">• Seizures• LTP• Ca^{2+} channel currents• NGF expression	<ul style="list-style-type: none">• Glial proliferation• Scar formation• Inflammation• Increase intracellular Ca^{2+}• Antagonise FGF• Brain edema formation• BBB damage

The data outlined above (and see *Table 1.9.1*) suggest that IL-1 may have opposing effects in different situations. For example low levels of IL-1 may be neurotropic/neuroprotective whilst abnormally high concentrations may exert a neurotoxic effect. However, more direct evidence for an involvement of IL-1 in neurodegeneration is the effect of inhibition of endogenous IL-1 in several acute forms of neurodegeneration (see *Section 1.9.1*).

1.9.1 IL-1 in acute neurodegeneration

The presence of IL-1 in the brain after several neuronal insults (see *Table 1.9.3*) provides indirect evidence for a role of this cytokine in acute neurodegeneration. In the uninjured brain the constitutive expression of IL-1 is low, but there are numerous reports demonstrating an induction of expression of IL-1 mRNA or protein after various types of brain damage, *in vivo*. For example, IL-1 β mRNA or protein expression is observed in the brain after focal cerebral ischaemia (Iannotti *et al.*, 1993; Liu *et al.*, 1993; Buttini *et al.*, 1994), transient cerebral ischaemia (Minami *et al.*, 1992; Wießner *et al.*, 1993; Wang *et al.*, 1994c; Yabuuchi *et al.*, 1994b), hypoxic-ischaemia (Hagberg *et al.*, 1995; Szaflarski *et al.*, 1995), local brain injury (Woodroffe *et al.*, 1991; Yan *et al.*, 1992), brain trauma (Taupin *et al.*, 1993; Fan *et al.*, 1995), and after administration of excitotoxic agents (Minami *et al.*, 1990, 1991; Yabuuchi *et al.*, 1993; Szaflarski *et al.*, 1995). IL-1 α is also induced after exposure to a neurotoxin in the hippocampus (Maier *et al.*, 1995). In most of these studies maximal increases of IL-1 β mRNA occur between 3-12h. However, significant elevations of IL-1 β mRNA occur within one hour and in some cases as early as 15min after induction of the neuronal insult (e.g. Minami *et al.*, 1992; Liu *et al.*, 1993; Buttini *et al.*, 1994; Wang *et al.*, 1994c; Yabuuchi *et al.*, 1994b). Additionally, IL-1 activity is enhanced in the cerebrospinal fluid (CSF) of patients suffering from a head injury (McClain *et al.*, 1987).

The cell source of IL-1 β produced after acute brain injury is unclear. In the brain astrocytes, microglia, invading immune cells, endothelial cells and neurones have all been suggested to release IL-1 (Giulian *et al.*, 1986; Breder *et al.*, 1988; Hetier *et al.*, 1988; Lechan *et al.*, 1990; Ban *et al.*, 1992; Schöbitz *et al.*, 1994). Some authors have proposed glia to be the main source of IL-1 β after injury (e.g. Yabuuchi *et al.*, 1993, 1994b; Buttini *et al.*, 1994). However, others have

suggested that the maximal increase in IL-1 β occurs prior to activation of microglia or invasion of inflammatory cells (e.g. Liu *et al.*, 1993). Furthermore, the rapid expression (15-30min) of IL-1 β mRNA observed in some situations suggests that neurones may be the predominant source of this cytokine (e.g. Minami *et al.*, 1992; Iannotti *et al.*, 1993). The possibility exists that neurones may contribute to the early release of IL-1 β , while glia and invading immune cells could supply the later production.

These studies provide indirect evidence for a role of IL-1 in neuronal death. However, increased expression of IL-1 may simply be a consequence of neuronal damage or inflammation and does not necessarily prove a causal role of IL-1 in the chain of events leading to neuronal damage. However, there are now extensive data to suggest that IL-1 is indeed a mediator of acute neurodegeneration (for review see Rothwell and Relton, 1993; Rothwell *et al.*, 1994, 1995b; Rothwell and Strijbos, 1995).

Several studies have examined the effect of exogenous administration of IL-1 during cerebral ischaemia *in vivo* in rats. It may be expected that IL-1 would exacerbate neuronal loss in these situations. In accordance, central administration of exogenous IL-1 β enhances the amount of neurodegeneration after permanent focal cerebral ischaemia (Loddick and Rothwell, 1996) and also increases oedema and neutrophil invasion, after transient focal cerebral ischaemia (Yamasaki *et al.*, 1995) in the rat *in vivo*.

However, the first report of a direct involvement of IL-1 in the death of neurones originated from the work of Relton and Rothwell (1992). These authors demonstrated that inhibition of the action of endogenous IL-1 by intracerebroventricular (icv) injection of the IL-1 antagonist, IL-1ra, potently inhibits (by 50-70%) neuronal death resulting from permanent focal cerebral ischaemia (middle cerebral artery occlusion, MCAo) or excitotoxic damage in the

rat. These data suggest that endogenous IL-1 is a mediator of ischaemic and excitotoxic brain damage. Since this study, there have been several further reports (see *Table 1.9.2*) on the effect of IL-1 β /IL-1ra in various types of neuronal injury. Indeed, the inhibitory effect of IL-1ra on the neuronal death induced by permanent focal cerebral ischaemia has been confirmed in the rat (Loddick and Rothwell, 1996) and the mouse (Rothwell *et al.*, 1995a). Systemic administration of IL-1ra also significantly inhibits lesion volume, oedema, the amount of necrotic neurones and leukocyte invasion after cerebral ischaemia in the rat (Relton *et al.*, 1993, 1995; Garcia *et al.*, 1995). Inhibition of IL-1 by central or peripheral administration zinc protoporphyrin (a non-specific inhibitor of IL-1) or an anti-IL-1 β antibody reduces infarct volume, oedema and neutrophil invasion after temporary focal cerebral ischaemia (Yamasaki *et al.*, 1994, 1995; Kadoya *et al.*, 1995). Recent studies have utilised an adenoviral-mediated delivery system to induce chronic overexpression of IL-1ra in rat brain (Betz *et al.*, 1995). This study demonstrates a 64% reduction in the volume of neuronal damage caused by focal cerebral ischaemia in the rat, after central injection of an adenovirus expressing IL-1ra, five days prior to MCAo.

Hence, it appears that both acute and chronic treatment with IL-1ra protects against permanent and transient cerebral ischaemia. Furthermore, IL-1ra treatment inhibits neuronal lesions in experimental studies of hypoxic-ischaemia (Martin *et al.*, 1994), traumatic brain injury (Toulmond and Rothwell, 1995) and heat stroke-induced neuronal damage (Lin *et al.*, 1995) *in vivo* in the rat. Thus, endogenous IL-1 produced after these insults may participate in the pathological processes of neuronal death and IL-1ra treatment may be of significant benefit in acute neurodegenerative disorders.

In all of the studies described above, IL-1 β , or inhibitors of IL-1, have been administered into the cerebral ventricles and hence may have access to the majority of areas within the brain. This experimental protocol therefore, does not

Table 1.9.2 *Effects of modifying levels of IL-1 during several acute neuronal insults*

	Injury	Effect	Reference
Administration of exogenous IL-1β.	• Permanent focal cerebral ischaemia.	Exacerbation of neuronal damage.	Loddick and Rothwell, 1996.
	• Temporary focal cerebral ischaemia.	Exacerbation of neuronal damage, oedema and neutrophil invasion.	Yamasaki <i>et al.</i> , 1995.
Inhibition of endogenous IL-1.	• Permanent focal cerebral ischaemia.	Reduction of neuronal damage.	Relton and Rothwell, 1992. Relton <i>et al.</i> , 1993, 1995. Loddick and Rothwell, 1996 Betz <i>et al.</i> , 1995. Rothwell <i>et al.</i> , 1995a. Garcia <i>et al.</i> , 1995.
	• Temporary focal cerebral ischaemia.	Reduction of neuronal damage, oedema and neutrophil invasion.	Yamasaki <i>et al.</i> , 1994, 1995. Kadoya <i>et al.</i> , 1995.
	• Hypoxic-ischaemia.	Reduction of neuronal damage.	Martin <i>et al.</i> , 1994.
	• Brain trauma.	Reduction of neuronal damage.	Toulmond and Rothwell, 1995.
	• Heat stroke-induced neuronal death.	Reduction of neuronal damage.	Lin <i>et al.</i> , 1995.
	• NMDA receptor-induced excitotoxicity.	Reduction of neuronal damage.	Relton and Rothwell, 1992.

allow for the brain region(s) or site(s) of action of IL-1/IL-1ra to be investigated. IL-1 may not act non-specifically throughout the brain to mediate its effect on neuronal death, but instead act at a defined region.

In summary, there is strong evidence of a role for IL-1 in the neuronal death in several acute neurodegenerative disorders (see *Table 1.9.2*) and possibly even in chronic neurodegeneration (see *Section 1.9.2*).

1.9.2 IL-1 in chronic neurodegeneration

IL-1 may also play a role in several chronic neuronal disorders. However, the evidence for basis of this proposal is largely indirect and circumstantial. For example, increases in the expression of IL-1 have been reported in the brains/CSF of patients with chronic neurodegeneration (see *Table 1.9.3*; Schöbitz *et al.*, 1994). Identification of an involvement of IL-1 in acute neuronal death (see *Section 1.9.1*), may also imply a contribution to the neurodegeneration observed in chronic neurodegeneration. Although, in comparison to the studies on acute neurodegeneration (see *Section 1.9.1*), there are no studies on the effect of inhibition of endogenous IL-1 in patients or animal models of chronic neurodegenerative diseases.

A direct correlation has been proposed between an acute brain injury and subsequent development of Alzheimer's disease (Graves *et al.*, 1990; Mayeux *et al.*, 1993). Expression of IL-1 α and IL-1 β is increased in the brains or CSF of Alzheimer's patients (Griffin *et al.*, 1989; Cacabelos *et al.*, 1991, 1994). Since inhibition of endogenous IL-1 reduces neuronal loss in experimentally-induced brain trauma *in vivo* (Toulmond and Rothwell, 1995), inhibition of this cytokine may be beneficial in preventing the neurodegeneration in Alzheimer's disease. In addition, IL-1 expression correlates with the regions of Alzheimer's-type

pathology and with amyloid plaques (a neuropathological feature of Alzheimer's, e.g. see Griffin *et al.*, 1995; Sheng *et al.*, 1995). Furthermore, IL-1 regulates the synthesis, processing and secretion of the β -amyloid precursor protein (e.g. Goldgaber *et al.*, 1989; Buxbaum *et al.*, 1992; Vasilakos *et al.*, 1994). β -amyloid has been shown to be toxic to neurones and hence, an involvement of IL-1 in Alzheimer's disease may be related to the production of this protein (for reviews see Berkenbosch *et al.*, 1991 and Mrak *et al.*, 1995).

IL-1 has been implicated in multiple sclerosis, a disease thought to be predominantly of an immune origin (see Panitch, 1992). Increased levels of IL-1 are found in the CSF and serum of multiple sclerosis sufferers (Tsukada *et al.*, 1991). The most convincing data for an involvement of IL-1 in multiple sclerosis arises from studies on a relevant animal model of this disease, experimental autoimmune encephalomyelitis (EAE). Jacobs *et al.* (1991) demonstrated that symptoms of EAE are exacerbated by IL-1 and suppressed by inhibition of IL-1.

HIV infection is associated with neuronal death and inflammatory changes, and IL-1 has been proposed as a mediator of this process (see Gendelman *et al.*, 1994; Lipton, 1992, 1994). Elevated levels of IL-1 are evident in the CSF of patients suffering from HIV (Gallo *et al.*, 1989). Neuronal toxicity and release of IL-1 is induced by HIV infection *in vitro* (Merrill and Chen, 1991). NMDA receptor activation appears to be involved in the mechanisms leading to neuronal death in HIV infection *in vitro* (Lipton, 1992, 1994) and IL-1ra inhibits neuronal loss due to stimulation of this receptor *in vivo* (Relton and Rothwell, 1992), suggesting that IL-1 may be involved in the neurodegeneration observed in HIV infection.

Increased levels of IL-1 have also been found in the plasma or brain of patients suffering from schizophrenia, epilepsy and Parkinson's disease (Katila *et al.*, 1994; Mogi *et al.*, 1994; Sheng *et al.*, 1994; Pacifici *et al.*, 1995), although, IL-1 expression could be secondary to the neuronal damage (or infection) which

occurs in these disorders and may not play a role in the pathogenesis. However, Wang *et al* (1994a) have proposed that IL-1 treatment reduces Parkinsonism in rats *in vivo*, suggesting IL-1 may be beneficial in this situation. Thus, the physiological role of IL-1 in these situations remains to be resolved.

In conclusion, therefore, the potential involvement of IL-1 in chronic neurological disorders appears to be complex and probably involves several other inflammatory cytokines.

Table 1.9.3 *Neurodegenerative disorders in which increased expression of IL-1 is observed*

Brain damage	CNS disorders
<ul style="list-style-type: none"> • Cerebral ischaemia <ul style="list-style-type: none"> • Focal • Global • Hypoxic-ischaemia • Local brain injury (e.g. stab wounds) • Excitotoxins • Brain trauma (e.g. fluid percussion) 	<ul style="list-style-type: none"> • Alzheimer's disease • Downs syndrome • Multiple sclerosis • EAE • AIDS • Parkinson's disease • Epilepsy • Schizophrenia

1.9.3 *Relationship between IL-1 and EAA's*

The above data indicate that IL-1 is involved in neuronal death, but the mechanism of this involvement is unknown. However, since EAA's have been implicated in the acute and chronic neuronal disorders discussed in *Section 1.9.1* and *1.9.2* (e.g. Olney, 1990; Whetsell and Shapira, 1993; Zorumski and Olney, 1993; Lipton and Rosenberg, 1994), and in view of inhibition of excitotoxic damage and ischaemic damage (an EAA dependent process) by IL-

1ra, it is likely that endogenous IL-1 may modify or mediate EAA-dependent events.

A relationship between neuronal damage induced by EAA's and IL-1 is indicated by the observation that systemic injection of EAA's (kainic acid) causes induction of IL-1 β mRNA in the rat brain (Minami *et al.*, 1990, 1991; Yabuuchi *et al.*, 1993). Significant increases in IL-1 β mRNA are seen in various brain regions (e.g. olfactory bulb, cerebral cortex, thalamus, amygdala, lateral septum and hippocampus) as early as two hours after excitotoxin injection (Minami, *et al.*, 1991; Yabuuchi *et al.*, 1993). Localisation of IL-1 β expression in the brain correlates with areas which are susceptible to neuronal damage after systemic injection of kainic acid (Schwob *et al.*, 1980; Ben-Ari, 1985). Szaflarski *et al.* (1995) have also reported a marked stimulation of IL-1 β mRNA expression after intrahippocampal infusion of an NMDA receptor agonist, again in areas of the brain where neuronal injury subsequently evolves. These observations suggest that IL-1 β could be induced by the neuronal excitation which is caused by EAA receptor overactivation, and that it may mediate the neuronal death which develops. In addition, since EAA's (and cerebral ischaemia) produce a very rapid (15min-2h) expression of IL-1 β mRNA, neuronal overexcitation probably contributes to this process (Minami *et al.*, 1990, 1992) and the release of IL-1 β is probably neuronal. However, as excitotoxin infusion into the rat brain induces neuronal death and subsequent activation of glia (e.g. Andersson *et al.*, 1991a, b; Perry and Andersson, 1992; Akiyama *et al.*, 1994), it may be these cells which are the predominant source of IL-1 (Giulian *et al.*, 1986; Hetier *et al.*, 1988; Schöbitz *et al.*, 1994). Furthermore, Yabuuchi *et al.* (1993) have proposed that glial cells, possibly microglia, produce IL-1 β after systemic kainic acid treatment. However, the possibility that the process of neuronal death *per se* may cause IL-1 expression can not be ruled out.

It is not yet known if inhibition of endogenous IL-1 can modify the damage induced by overactivation of non-NMDA receptor subtypes *in vivo* (AMPA or kainate; see *Figure 1.9.1*), and if IL-1 plays a role in excitotoxic damage in other brain areas apart from the striatum. Furthermore, it is not yet known if IL-1 can induce neuronal damage directly in the absence of other insults (e.g. EAA's). The aim of this thesis was to answer these questions by studying the effect of IL-1/IL-1ra on an experimental excitotoxic-induced neuronal damage.

1.10 Experimental study of excitotoxic neuronal death

For the experimental study of excitotoxic neuronal death and the putative involvement of IL-1, a variety of *in vivo* and *in vitro* approaches can be employed each with specific advantages and disadvantages.

1.10.1 *In vitro* systems

Primary neuronal cultures and brain slices *in vitro* have been used extensively to study the mechanisms of neurotoxicity induced by EAA's and their specific receptors (e.g. Choi *et al.*, 1987, 1988; Gathwaite *et al.*, 1992), and have provided valuable information regarding mechanisms of neuronal death. The main advantage for *in vitro* systems is the relative simplicity of the techniques employed and the greater degree of experimental control (due to e.g. absence of physiological influences such as changes in blood flow and hypoxia), compared to approaches *in vivo*. However, *in vitro* neuronal systems are clearly different from neurones *in vivo* in many respects and direct comparison of the results may not be always possible (Abdulla and Campbell, 1993; Rothwell and Strijbos, 1995; Strijbos and Rothwell, 1995). For example neuronal cultures are prepared from immature neurones, in which receptors and/or signal transduction mechanisms may not be fully developed. In addition, they lack the complex neuronal connections and organisation present in the intact brain. Neuronal cell

cultures usually lack non-neuronal cell types, for example, they normally have a very low glial content (usually <10%) and lack endothelial cells. When studying the mechanisms involved in neuronal cell death, an interaction between neuronal and non-neuronal cells is probably important. Hence, in neuronal cell cultures this interaction is absent and neurones are normally studied in isolation. However, the use of intact brain slices taken from animals (usually the rat) may avoid some of these problems of neuronal cell cultures (see e.g. Garthwaite and Garthwaite, 1991a, 1991b; Gathwaite *et al.*, 1992). Brain slices maintain a high quality of preservation (e.g. histological) which makes it possible to detect any pathophysiological changes. In contrast to neuronal cell cultures, glia and endothelial cell are present, and since brains are normally taken from adult animals receptors and/or signal transduction mechanisms should be fully developed. Complex synaptic connections should also be preserved, although the fact that slices are usually taken through one plane, and also depending on the thickness of the slice, may mean that not all neuronal connections and pathways are fully complete.

To avoid all these problems accounted with *in vitro* systems the use of valid *in vivo* approaches to study neuronal cell death need to be employed.

1.10.2 *In vivo* systems

There are several animal models of neuronal death *in vivo*. The most clinically relevant are considered to be those of global and focal cerebral ischaemia in several animal species, and some are strongly regarded to highly applicable to stroke observed in humans (e.g. Ginsberg and Busto, 1989; Zivin and Grotta, 1990; Hunter *et al.*, 1995). The latter point is the main advantage when using these approaches to develop neuroprotective drugs, but these procedures are technically demanding and expensive. Furthermore, since the pathogenesis in cerebral ischaemia involves several complex pathophysiological responses (e.g.

changes in blood flow), it is difficult to experimentally manipulate, and not always possible to directly study mechanisms of action of neuronal death at a cellular level. However, the pathogenesis ensued during cerebral ischaemia has been partially ascribed to the overactivation of EAA receptors (see Siesjö, 1992a, 1992b). Neuronal damage induced by direct activation of EAA receptors is a well recognised approach and includes the intracerebral injection of EAA receptor agonists *in vivo* (e.g. McDonald *et al.*, 1989a). The advantages of this technique is that some of the complex responses observed following experimentally-induced ischaemia are omitted, and neuronal death due to specific activation of different types of EAA receptors can be studied individually.

The main objective of this thesis was to study the involvement of IL-1 in neuronal death due to the activation of specific EAA receptors. To address this aim, an *in vitro* system could be employed, or alternatively direct activation of EAA receptors *in vivo* may be used. However, the site(s) of action and cell types participating in IL-1's involvement in neuronal death are not known, and may involve a complex interaction between neurones, glia and possibly endothelial cells. Considering the data described above, an *in vitro* system would not allow these situations to be studied and hence an *in vivo* approach is preferential.

In this project therefore, a simple procedure was used to induce neurodegeneration by infusion of EAA agonists into the striatum or cortex of the rat brain *in vivo*, since these areas have a high density of EAA receptors (Young and Fagg, 1990). These approaches were used to study interactions between endogenous or exogenous IL-1 and EAA's during excitotoxic damage.

1.11 Aims

The overall objective of this thesis was to investigate the involvement of IL-1 in excitotoxic damage and its possible mechanisms of action.

The specific aims were to:

1. Establish and validate a reproducible system of inducing excitotoxic brain damage in the striatum and the cortex in the rat, induced by pharmacological overactivation of EAA receptors by:
 - Determining the optimal dose and time course of selective agonists acting at NMDA and AMPA receptors, which produce quantifiable damage.
 - Testing the effects of selective NMDA or AMPA receptor antagonists on each type of damage.
2. Once reproducible excitotoxic lesions were achieved, the second aim was to:
 - Study the effect of IL-1 α on excitotoxic brain damage induced by different EAA receptor subtypes, and thus to assess the involvement of endogenous IL-1 in these forms of brain damage.
 - Investigate the effect of exogenous IL-1 β on excitotoxic brain damage induced by the different EAA receptor subtypes and to identify possible sites of action of IL-1 .
3. Investigate effects of IL-1 infusion into the striatum or the cortex in the absence of any other insults by:
 - Testing the effects of infusion of IL-1 β on neuronal viability.
 - Assessing effects of infusion of IL-1 β on core body temperature.

4. Establish the effect of chronic endogenous IL-1/IL-1ra manipulation in genetically modified mice by:
 - Identifying the optimum dose and time course of a selective agonist acting at NMDA receptor which produce a quantifiable lesion in the mouse striatum.
 - Study the effect of IL-1ra on excitotoxic brain damage induced by the NMDA receptor subtypes and thus to assess the involvement of endogenous IL-1 in this form of brain damage.
 - Testing the effects of IL-1ra overexpression or absence of IL-1 β on NMDA receptor-induced striatal brain damage in transgenic or knock-out mice respectively.

Chapter Two

Material and Methods

Chapter Two

Material and Methods

2.1 Animals

2.1.1 *Rats*

Male, Sprague-Dawley rats (Charles River, UK), weighing 250-350g were maintained under controlled environmental conditions, at a temperature of 21°C \pm 1°C, a relative humidity of 55 \pm 10% and a 12h light-dark cycle (lights on 0800). Animals were housed in cages of five or six rats and allowed a free access to water and a pelleted diet (Beekay, Bantin and Kingman Ltd, UK).

2.1.2 *Mice*

Male, C57-Black mice (Harlan Olac, UK), or C57-Black/ICRFA^t (BSU, Manchester University, UK), 26-30g body weight were kept under the same conditions as rats (see *Section 2.1.1*), with the exception of being housed in cages of up to 15 animals. Genetically modified mice (transgenic or knock-out) or equivalent wild-type (weighing 28-35g, Merck Research Labs, USA) were kept under MAFF quarantine conditions in an isolated unit and all experiments involving these mice were performed according to quarantine regulations.

All animals were maintained according to British Home Office regulations, and procedures were covered by the relevant home office project and personal licences.

2.2 Materials

2.2.1 *Drugs*

2.2.1.1 *(S)- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (S-AMPA)*

S-AMPA (Tocris Cookson, UK) was dissolved in sterile, 0.1M phosphate-buffered saline (PBS, pH 7.4) to a concentration of 20mM and stored at -80°C. On the day of use this stock solution was diluted with PBS to the required concentration.

2.2.1.2 *Cis-2,4-methanoglutamate (MGlu)*

MGlu (or 1-aminocyclobutane-cis-1,3-dicarboxylic acid $1H_2O$, Tocris Cookson, UK) was dissolved in sterile PBS (pH 7.4) to a concentration of 20mM and stored at -80°C. On the day of use this stock solution was diluted with PBS to the required concentration.

2.2.1.3 *Interleukin-1 β (IL-1 β)*

Human recombinant IL-1 β (E.I Dupont De Nemours and Co, USA; Specific activity (quoted in international units; IU) in a bioassay using the B9 cell line ranged between $1.3-2.5 \times 10^8$ IU/mg compared to the NIBSC standard human recombinant IL-1 β , endotoxin contamination was <2.5EU/mg) was dissolved in 0.9% sterile (non-pyrogenic) saline and stored at -80°C.

2.2.1.4 *Interleukin-1 receptor antagonist (IL-1ra)*

Human recombinant IL-1ra was donated by Dr R Thompson (Amgen, USA). The stock solution of IL-1ra was diluted with sterile PBS (pH 7.4) to a concentration of 5 μ g/ μ l and stored at -80°C until required.

2.2.1.5 *MK-801*

(+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]-cyclohepten-5,10,iminemaleate (MK-

801 or dizocilpine maleate, Merck, Sharp and Dohme, UK) was dissolved in sterile PBS (pH 7.4) to a concentration of 4mg/ml on the day of use.

2.2.1.6 NBQX

6-nitro-7-sulframoylbenzo(f)quinoxaline-2,3-dione (NBQX, Tocris Cookson, UK) was dissolved in 0.2M NaOH, adjusted to pH 7.5-8.5 with HCl and made up to the appropriate volume with sterile PBS (pH 7.4), on the day of use, to give a concentration of 25mM.

2.2.2 Anaesthesia

2.2.2.1 Halothane

Halothane (Fluothane; Zeneca, UK) is an inhalational anaesthetic and was used for all stereotaxic infusions in mice (see *Section 2.3.2*) and abdominal implantation of radiotransmitters in rats (see *Section 2.8.1*). Induction of anaesthesia was performed by placing the animal into a flow chamber filled with 2% halothane, oxygen (O₂; 1.5L/min) and nitrous oxide (N₂O; 1.5L/min) for the mouse or 4% halothane, O₂ (1.5L/min) and N₂O (1.5L/min) for the rat. After loss of consciousness, mice were placed in a stereotaxic frame. Maintenance of anaesthesia was achieved via a nose piece which surrounded the nose bar of the frame, through which 1.5% halothane and the O₂/N₂O mixture flowed. Maintenance of anaesthesia for the rat (in a supine position), was achieved with 2.5% halothane, via a nose piece.

2.2.2.2 Sodium pentobarbitone

Sodium pentobarbitone (Sagatal; Rhône Mérieux, UK) was used for longer periods of anaesthesia in all stereotaxic infusions in rats (see *Section 2.3.1*). The anaesthetic was injected intraperitoneally (i.p.) in hand-held, conscious rats in

a volume of approximately 1ml/kg which resulted in a total dose of approximately 60mg/kg.

2.3 Intracerebral stereotaxic infusions

2.3.1 *Rats*

Male, Sprague-Dawley rats were anaesthetised with sodium pentobarbitone (Sagatal, 60mg/kg, i.p.) as previously described in *Section 2.2.2.2*, and secured in a stereotaxic frame (Narshigue Scientific Instrument Lab, Japan), with the incisor bar lowered -3mm below the intra-aural line, thus ensuring the skull was flat. The ear bars were placed in the external auditory meati. Fur covering the ventral surface of the scalp was removed. A midline incision was made in the skin over the skull, and crocodile clips were used to hold back the skin, to expose the surface of the skull. The membranes (periosteum) overlying the skull were then incised and pushed aside using a clean cotton bud.

The injection needle was positioned over bregma (zero reference point) and lifted vertically clear of the skull. The needle was then moved to the required co-ordinates depending on the area of the brain to be injected. Co-ordinates were determined by reference to the Paxinos and Watson atlas (1986; 0.7mm anterior and 2.7mm lateral for the right striatum or 0.7mm anterior and 5.0mm lateral for the right parietal cortex, see *Figure 2.3.1*). A 1-2mm diameter hole was then made in the skull at the required stereotaxic co-ordinates using a small hand held drill (dental drill, Radio Spares Ltd, UK).

Infusions were made into the right-hand striatum or cortex by means of a 23 gauge stainless steel needle (approximately 3cm; Stainless tube and needle Co. Ltd, UK) fitted to approximately 30cm of polyethylene (PE10) tubing of similar diameter. The system was filled with PBS or saline (depending on the

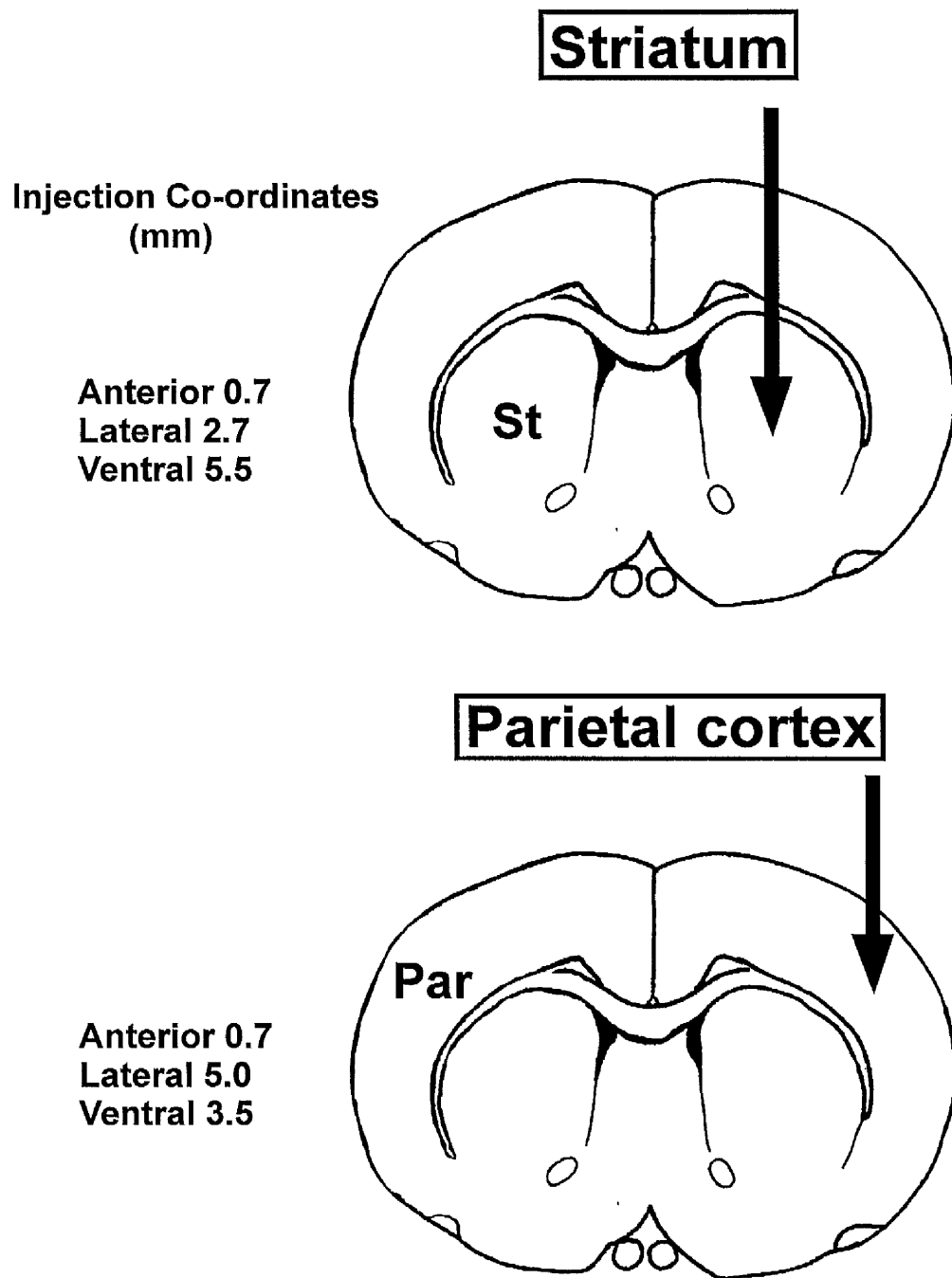


Figure 2.3.1 Rat coronal brain sections at 0.7mm anterior to bregma, illustrating the position of stereotaxic infusions into either the striatum (upper panel) or parietal cortex (lower panel) of the rat. The arrows indicate the site of injection.

St=Striatum.

Par=Parietal cortex.

experiment), and a small bubble introduced into the free end of the tubing which was then connected to a 10 μ l Hamilton syringe. The syringe was placed in an infusion pump (CMA 100, Carnegie Medecine, Sweden), which determined the rate of infusion. Test substances were then introduced, via the Hamilton syringe, into the needle end of the tubing. Approximately 1 μ l of liquid was then dispelled to check that the needle was not blocked and no bubbles were in the tip of the needle. The needle was then stereotaxically located 5.5mm (for the striatum, see *Figure 2.3.1*) or 3.5mm (for the cortex, see *Figure 2.3.1*) below the surface of the dura. Infusions were controlled by the infusion pump at a rate of 0.5 μ l/min. The movement of the bubble was checked to ensure successful injection. If the bubble did not move or the bubble compressed in size the animal was excluded from the study.

After infusion, the injection needle was left in place for 5min to allow diffusion of the injected solution, and subsequently slowly removed. The scalp wound was then sutured and animals placed under a heating lamp to maintain body temperature (for up to two hours). This period ensured recovery from anaesthesia, after which any behavioural observations were noted.

2.3.2 Mouse

Stereotaxic infusions into the striatum of the mouse were performed largely as in the rat with the exceptions described below.

In all experiments male, C57-black or transgenic/knock-out (or wild-type) mice were anaesthetised under halothane (see *Section 2.2.2.1*), and secured in a rat stereotaxic frame (Narshigue Scientific Instrument Lab, Japan). The frame was adapted (in the Department of Geriatric Medicine, University of Manchester, from the design of Jones *et al.*, 1977) for the mouse by means of a small table onto

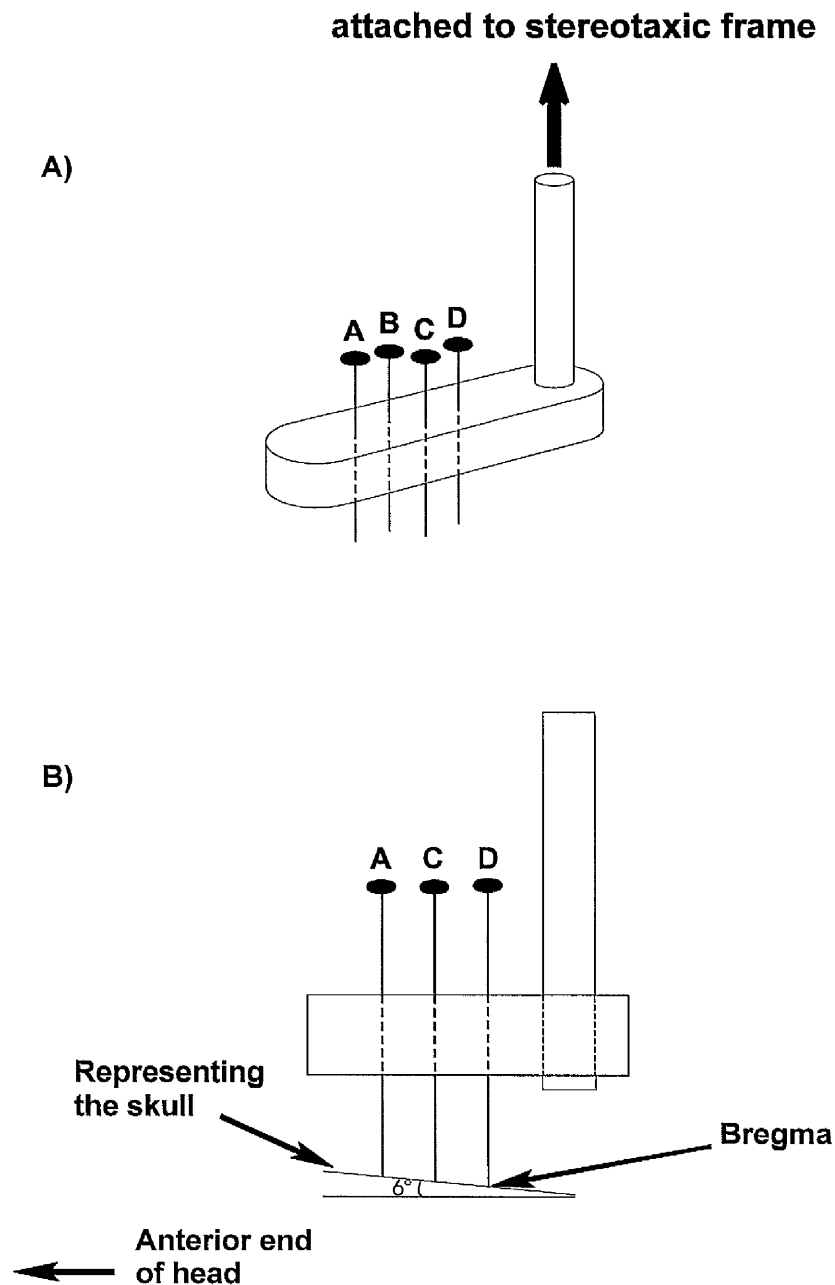


Figure 2.3.2 The alignment device for stereotaxic infusions in the mouse (modified from Fotheringham, 1992).

A) A 3-D diagram of the alignment device which consists of four metal pins (A-D) set into a perspex box. Pin A is anterior and D posterior and are separated by 4mm. Pin B and C are lateral and are separated by 3mm.
NB This diagram is not to scale.

B) A schematic cross section through the device showing the pins simultaneously touching the skull. The posterior pin D is located on bregma. The lateral pin B is not on this diagram as it is superimposed with pin C. The alignment device was removed and the head inclined 6°, anterior end down to bring the head into the true horizontal axis (i.e. the head was flat).

which the animal lay, and a rubber nose piece around the nose bar which allowed the halothane to flow around the face of the animal. Fur covering the ventral surface of the scalp was removed, and a midline incision was made in the skin which was then retracted using a metal clip. In order to ensure the correct orientation of the head (i.e. ensure that the skull was flat), an alignment device (see *Figure 2.3.2*) was used. This consisted of four pins, which were stereotaxically located with the posterior pin on bregma. The head was adjusted in each of the four planes so that all four pins touched the skull simultaneously and the alignment device was removed. The frame was tilted, anterior end down, through 6° thus ensuring that the skull was brought into a horizontal (flat) position. The infusion was then carried out according to the method used for the rat (see *Section 2.3.1*). However, a 31 gauge stainless steel needle with an outer needle coat of 23 gauge was used for the infusion. Co-ordinates for the right striatum were determined by reference to the atlas of Slotnick and Leonard (1975; 0.7mm anterior, 2.1mm lateral and 3.5mm below the surface of the dura).

After infusion, animals were placed on a heated pad, under a lamp and usually recovered consciousness within 30min.

2.4 Dissection of the brain

Animals were sacrificed one to three days after infusions by an overdose of halothane anaesthesia, followed by cervical dislocation. The head was then removed from the body and the skin overlying the skull removed. The skull was carefully cut down the midline and pulled back laterally to exposure the brain. The brain was removed from the surrounding bone by using a spatula, and the forebrain separated from the rest of the brain (the cerebellum or posterior to the hypothalamus) using a razor blade. The brain was then processed accordingly, depending on the experiment (see *Section 2.5*).

2.5 Preparation of tissue for sectioning/staining

2.5.1 *Fresh tissue*

In order to cut fresh sections for tetrazolium analysis, the dissected forebrains (see *Section 2.4*) were fixed to a Teflon chuck using cyanoacrylate Superglue gel (Loctite, UK), and 250 μ m or 500 μ m sections were cut on a vibrotome (Campden Instruments, UK). Tetrazolium staining was then carried out according to *Section 2.6.1*.

2.5.2 *Immersion fixation*

After dissection of the brain (see *Section 2.4*) the cerebellum and approximately 3mm of the forebrain was removed with a razor blade and discarded. The rest of the brain was fully immersed in 4% paraformaldehyde (PFA, pH 7.4; BDH Chemicals, UK, see *Appendix One*), and incubated at 4°C on a rotator for three to seven days, until fully fixed. The brains were rinsed in PBS, and transferred into clean vials containing this buffer. Brains to be sectioned were fixed to a chuck using cyanoacrylate Superglue, and 20-30 μ m sections were cut on a vibrotome (General Scientific Company Ltd, UK). Sections were floated onto clean subbed microscope slides (Chance Propper Ltd, UK, see *Appendix Two*), allowed to dry and were stored, in a clean, dust-free environment before staining (see *Section 2.6.2*).

2.5.3 *Freezing/Cryostat sectioning*

The brains were rapidly removed (as in *Section 2.4*), and frozen in isopentane (BDH Chemicals, UK) chilled with dry-ice to -30°C. The brains were then stored at -80°C until required for sectioning. Frozen brains were placed in a cryostat

(Bright Instrument Co Ltd, UK) set at a chamber temperature of -20°C , and allowed to equilibrate for approximately one hour. The brains were mounted onto a cryostat chuck with embedding medium (O.T.C, Tissue-Tek, Raymond Lamb, UK), and allowed to equilibrate for a further 15min. Coronal sections (at $10\mu\text{m}$) were cut using a blade angle of 12° . Sections which creased or curled were discarded, otherwise the sections were mounted onto subbed microscope slides, at room temperature. Slides were placed in a slide box which contained a small amount of silica gel (BDH Chemicals, UK), sealed and stored at -80°C before staining (see *Section 2.6.2*).

2.6 Histological analysis

2.6.1 *Tetrazolium staining*

$250\mu\text{m}$ (for striatal infusions) or $500\mu\text{m}$ (for cortical infusions and see experiments in *Section 4.3* and *4.4*) coronal sections were cut using a tissue vibrotome (Campden Instruments, UK), as in *Section 2.5.1*. All slices were placed flat in multiwell plates containing tetrazolium chloride dye solution (see *Appendix Three*). This enzyme stains for mitochondrial enzymes (Lojda *et al.*, 1979). Healthy tissue stained a purple/pink colour (formazan dye) due to the reduction of the tetrazolium by the mitochondrial enzymes. Therefore dead tissue, where mitochondria are no longer viable, does not stain and appears white (see *Figures 3.7.1-3.7.3*). The slices were incubated under a heating lamp or in a water bath (37°C) for 20-30min. Stained sections (striatal) were then mounted onto subbed microscope slides and allowed to dry at room temperature and stored until analysis (see *Section 2.7*). Cortical lesions were drawn, by eye, onto scaled stereotaxic brain maps at the appropriate levels. Cortical lesions are sometimes associated with distortion (occasionally accompanied with oedema), and breakage of tissue when the damage is severe, thus direct measurement

of cortical lesions is difficult and may lead to an overestimation of lesion size. However, the method described is independent of oedema and gives greater precision in the measurement of lesion volume.

2.6.2 Detailed histological staining

For more detailed histological analysis various types of staining were carried out on either fixed or frozen sections (prepared as in *Section 2.5.2* or *2.5.3*). Fixed sections were placed in a staining rack and stained with one of the histological stains (e.g. neutral red or haematoxylin and eosin) according to the protocol in *Appendix Four* or *Five*. Frozen brain sections were thawed and allowed to equilibrate at room temperature before staining with cresyl fast violet as in *Appendix Four*.

2.7 Quantification of lesion size

For all striatal lesions the size of damage (area in mm^2) was measured directly by a Seescan image analysis machine (Seescan, UK). Damage was analysed in the striatum of sections corresponding to the co-ordinates ranging from +1.95 to -0.3 (relative to bregma) for the rat and 1.3 to -0.3 (relative to bregma) for the mouse. Total lesion volume (mm^3) was calculated by integration of the individual lesion areas (multiplied by section thickness) for each brain. For cortical neuronal damage, lesion areas were analysed by calculating the area of damage on each brain map section and multiplying by a conversion factor to correct for the actual brain area. Lesion volume was calculated as for striatal lesions.

↳ correction
for oedema?

2.8 Temperature experiments

2.8.1 *Abdominal implantation of radiotransmitters*

Rats were anaesthetised under 2.5% halothane (see *Section 2.2.2.1*) and placed in a supine position. A 3 x 3cm area of the abdomen was shaved, disinfected and wiped clean with ethanol (BDH Chemicals, UK). A small incision was made in the skin and widened using scissors, thus making a hole of approximately 2cm. This procedure allows the underlying muscle layer of the abdominal wall to be exposed, which was opened by a similar technique. Radiotransmitters (Data Sciences, USA) were sterilised with ethanol and inserted into the opened abdominal cavity. The muscle layer was then sutured with 4/0 Ethilon sutures (Ethicon Ltd, UK) and the skin was sutured with 3/0 Mersilk sutures (Ethicon Ltd, UK).

The animals were allowed to recover consciousness (within 30min) and returned to their home cage. This procedure was usually carried out three to five days before experimentation.

2.8.2 *Remote radiotelemetry*

In temperature experiments, remote radiotelemetry (Data Quest III system, Data Sciences, USA) was used to measure core body temperature. At least three days before the start of the experiment, precalibrated transmitters were implanted in the abdominal cavity of the animals (as in *Section 2.8.1*). These transmitters emit temperature-dependent radio-signals. Animals were housed singly the day before the experiment, and the cages placed onto a receiver pad which monitors output frequency emitted from the transmitter. The transmitter was then activated by a magnet held close to the animals abdomen. The emitted radio-signal, via the receiver pad, is converted to a digital signal which is

processed by a PC computer. Temperature recordings (in °C) were recorded on the computer and were taken every 10-30min.

2.9 Statistical analysis

Lesion size is expressed in both area (mm²) per brain section and total lesion volume (mm³). Significant differences of lesion size between two groups of animals were assessed by a Student's t-test for unpaired data. Probabilities are two-tailed, assuming a probability of less than 5% for statistical significance. Results are given as means \pm standard error of the mean (SEM).

Linear regression analysis was performed by the least squares method. Assuming that both variables are normally distributed, the Product-Moment correlation coefficient was calculated to determine correlation.

For temperature data, the effects of treatment on body temperature were studied over the time-course of the experiment. This was performed by using a multivariate analysis of variance with repeated measures (MANOVA). The temperature profiles of the control and treated groups were then compared.

Chapter Three

**Identification of the time course and optimal
doses of EAA-induced neurodegeneration and
modification by EAA receptor antagonists**

Chapter Three

Identification of the time course and optimal doses of EAA-induced neurodegeneration and modification by EAA receptor antagonists

3.1 Introduction

In order to study the role of novel mediators on neuronal damage, an experimental model of neurodegeneration needs to be employed. Most forms of acute neurodegeneration (e.g. cerebral ischaemia, hypoxia and brain trauma) depend on the excessive release of EAA's, and subsequent overactivation of the EAA receptors (see *Chapter One*). Animal models of cerebral ischaemia have been useful in studying the mechanisms of neurodegeneration and anti-ischaemic effect of agents which may modify excitotoxic processes (Ginsberg and Busto, 1989; Zivin and Grotta, 1990; Hunter *et al.*, 1995). However, a more direct method of studying excitotoxic neurodegeneration is derived from the direct overactivation of EAA receptors. This well recognised method of neuronal damage involves the intracerebral injection of EAA receptor agonists (e.g. McDonald *et al.*, 1989a). This experimental approach has advantages over the various clinically relevant animal models, such as cerebral ischaemia. These benefits include the investigation of the direct effect of mediators of excitotoxic damage, without the involvement of complex physiological processes (e.g. neurochemical and vascular disturbances) which occur after cerebral ischaemia.

As discussed in *Chapter One*, the effect of IL-1ra on striatal NMDA receptor-induced damage has already been assessed briefly in a previous study (Relton, 1992; Relton and Rothwell, 1992). Neuronal damage can also occur *in vivo* via overactivation of the AMPA receptor (e.g. McDonald *et al.*, 1992). However, it

is not yet known if IL-1 is involved in damage induced by the AMPA receptor or whether IL-1 mediates excitotoxic damage in other areas of the brain. Such studies require a reproducible model of excitotoxic damage induced by pharmacological overactivation of EAA receptors. A primary objective of this work was therefore to develop an *in vivo* experimental model of neurodegeneration. The general approach was to induce damage by agonists specific at either the NMDA or AMPA receptor in two different areas of the rat brain, the striatum and the parietal cortex. The striatum was chosen because numerous studies have reported the effect of intrastriatal infusion of NMDA receptor agonists (with fewer on AMPA receptor-mediated damage), and this area has a high density of EAA receptors (Young and Fagg, 1990). There are less data on the modulation of excitotoxic damage within the parietal cortex, induced by direct infusion of either NMDA or AMPA receptor agonists. However, this area is also rich in EAA receptors (Young and Fagg, 1990) and is highly susceptible to damage in various forms of brain injury (e.g. ischaemia and head injury).

Thus, optimal doses of EAA agonists for induction of damage, and the time course of both types of excitotoxic lesions, in two different areas of the brain were determined.

3.2 Effect of vehicle

The aim of this study was first, to determine the qualitative effects of infusion of PBS (the vehicle in which all the excitotoxins were dissolved), into either the striatum or the cortex as assessed by tetrazolium staining and second, to validate effects of intrastriatal infusion of PBS on neuronal viability in a qualitative manner, using a more detailed histological method.

3.2.1 Experimental

Striatal or cortical lesions were performed according to the protocol described in *Section 2.3.1*. Sterile PBS (1 μ l, n=5) was infused into the striatum or the cortex. The animals were sacrificed (see *Section 2.4*) 24h or 48h after infusion and neuronal death assessed by the tetrazolium method as in *Section 2.5.1* and *2.6.1*. In separate experiments, animals were infused intrastrially with either 1 μ l of sterile PBS (n=5) or the NMDA receptor agonist, MGlu (10nmol in 1 μ l, n=5) to act as a positive control. Animals were sacrificed 48h later, the brain rapidly removed (see *Section 2.4*), frozen, and 10 μ m sections were cut on a cryostat according to *Section 2.5.3*. Sections were then stained with cresyl fast violet (see *Section 2.6.2* and *Appendix Four*) and examined under a light microscope.

3.2.2 Results

Intrastriatal or intracortical injection of PBS caused no widespread damage as assessed by tetrazolium staining 24h or 48h after infusion (see *Figure 3.7.1* and *3.7.3*). However, discrete neuronal death can be seen around the needle tract. Animals showed no behavioural disturbances after recovery from anaesthesia.

Intrastriatal infusion of MGlu (10nmol) resulted in neuronal death throughout the striatum as assessed by detailed histological staining 48h later. Neurones were virtually completely absent in the immediate site of injection, and appeared unhealthy, shrunken and darkly stained in the surrounding areas (see *Figure 3.2.1*). However, cryostat sections and cresyl violet staining did not allow for more detailed histological alterations assessed. In contrast to tetrazolium staining, detailed histological analysis revealed that intrastriatal infusion of PBS caused discrete and very localised neuronal death at the site of injection. This damage was very small (compared to that seen after MGlu infusion), and seen

Figure 3.2.1

Light micrographs of 10 μ m cresyl violet-stained, coronal sections of rat brain 48h after intrastriatal infusion of 1 μ l PBS (**A-C**), or 10nmol MGlu (in 1 μ l, **D-F**). Panels **G-I** illustrate the striatum of a normal (uninjected) rat brain. Sections are taken approximately at the site of injection (0.7mm anterior to bregma) and are representative of a group of five animals.

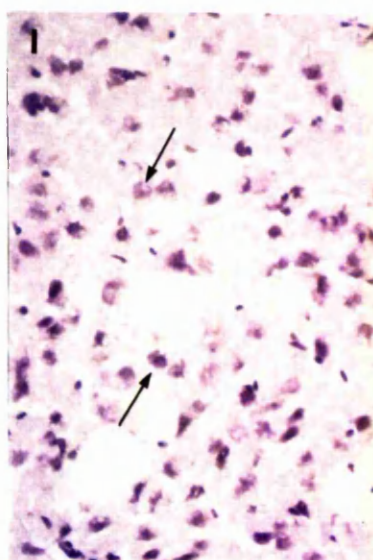
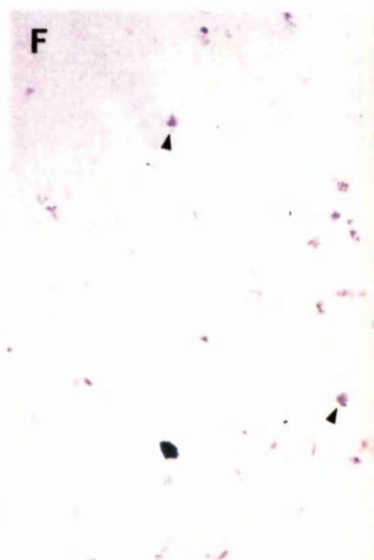
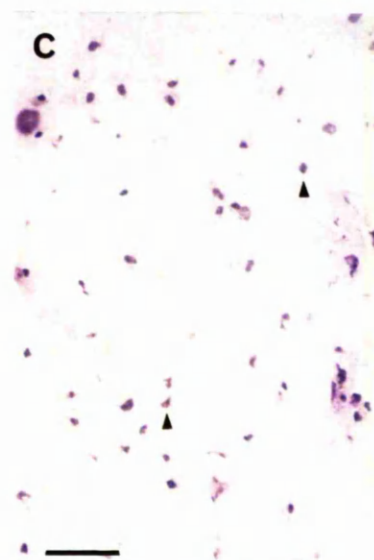
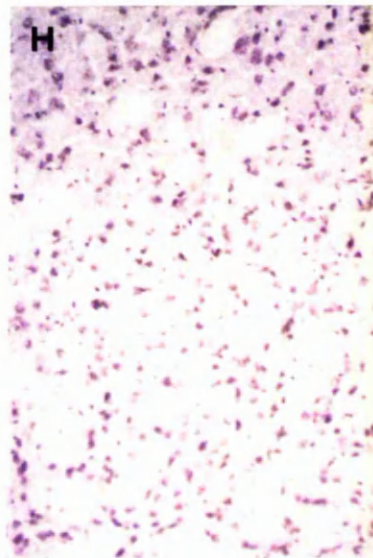
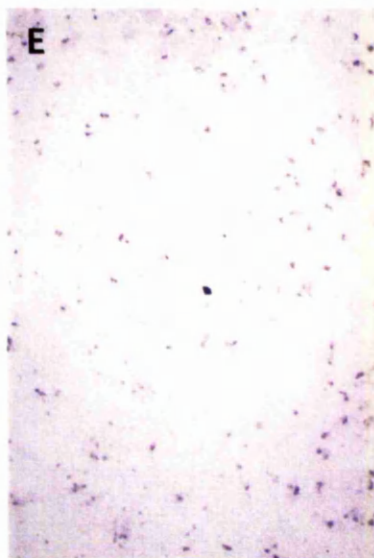
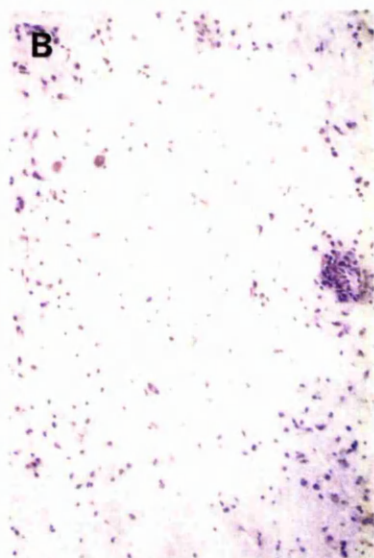
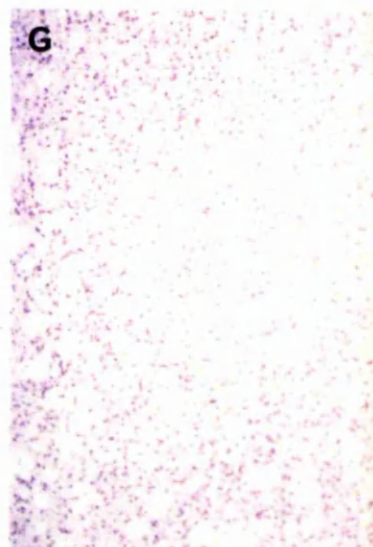
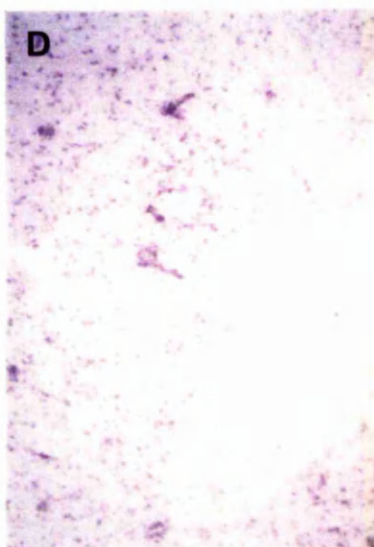
Arrows represent healthy neurones and the arrow heads unhealthy neurones. Note the paler staining of the area surrounding the injection site (in **A, B, D** and **E**), characteristic of damaged tissue and also note the lack of neurones in the MGlu-treated brain (in **F**).

Magnification bar=240 μ m for panel **A, D** and **G**; 95 μ m for panel **B, E** and **H**; and 48 μ m for panel **C, F**, and **I**.

PBS

MGlu

Control



only at the tip of the needle tract. However, in no case was widespread neuronal damage observed after intrastriatal infusion of PBS (see *Figure 3.2.1*).

3.3 Striatal NMDA receptor-induced excitotoxic lesions

The aim of this study was to validate previous work on striatal NMDA receptor-induced lesions, and to examine effects of the non-competitive NMDA receptor antagonist, MK-801 and the AMPA receptor antagonist, NBQX, on striatal damage.

3.3.1 *Experimental*

Striatal lesions were performed according to the protocol described in *Section 2.3.1*. A dose of 5 or 10nmol of the NMDA agonist, MGlu (in 1 μ l, n=10) was infused into the striatum. In separate experiments, animals were injected with either PBS (1ml/kg, i.p., n=11) or MK-801 (4mg/kg, i.p., n=11), 30min prior to intrastriatal infusion of 10nmol (in 1 μ l) of MGlu. In further experiments, NBQX (25nmol in 1 μ l, n=7) or PBS (in 1 μ l, n=7) was infused immediately before 10nmol (in 1 μ l) of MGlu. Animals were sacrificed (as in *Section 2.4*) 24h after infusion and neuronal death assessed by the tetrazolium method as in *Section 2.5.1* and *2.6.1*.

3.3.2 *Results*

Striatal infusion of 10nmol of MGlu caused modest behavioural changes, including episodic "barrel-rolling" activity (ipsilateral rolling), which commenced shortly after recovery from anaesthesia (approximately 90-120min), lasted at least three hours but was not present at 24h. Quantitative assessments of this "barrel-rolling" were not carried out. Previous studies established 24h as the

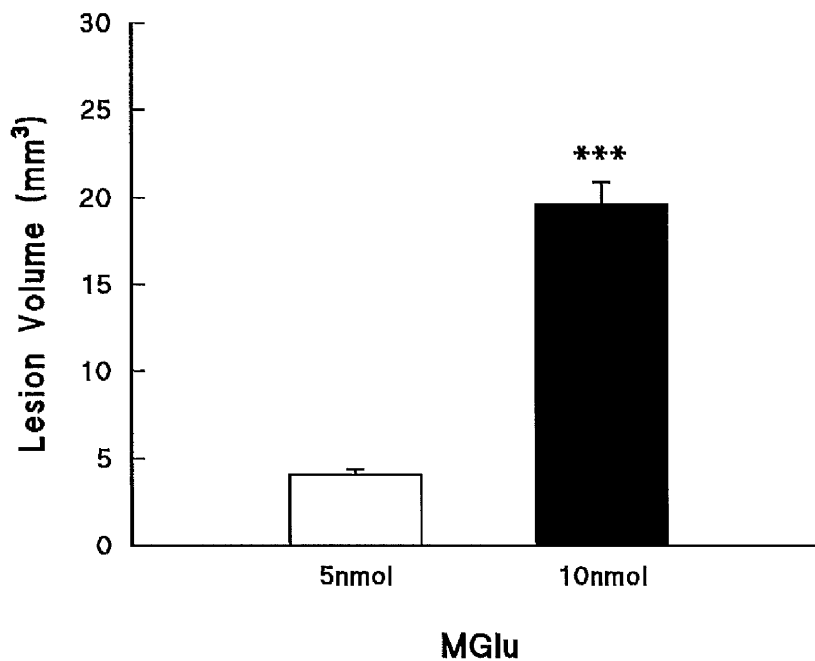
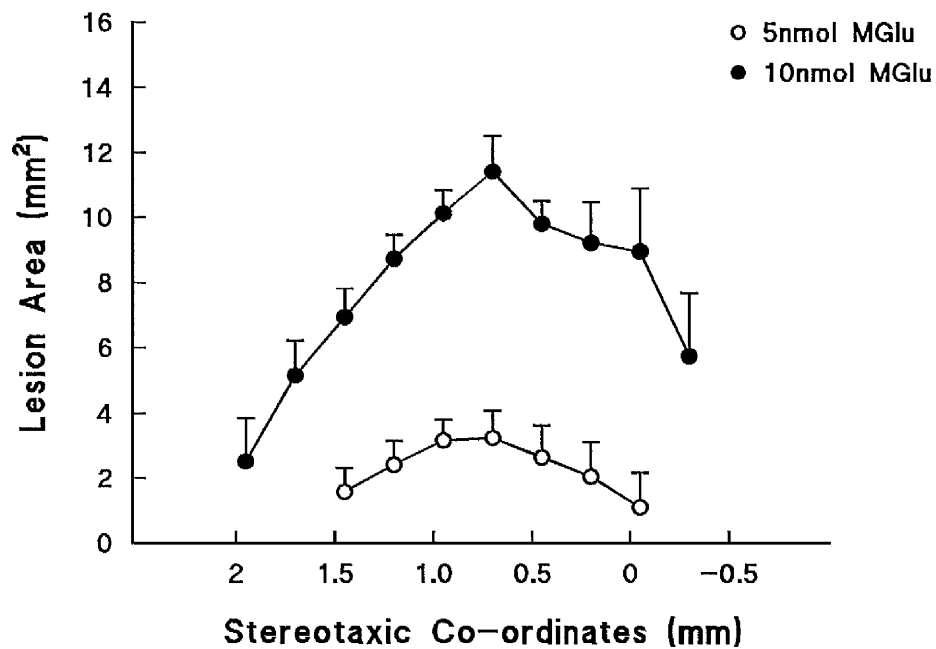


Figure 3.3.1 Lesion size 24h after intrastriatal infusion of MGlU

Damage was induced by intrastriatal infusion of either 5nmol (n=5) or 10nmol (n=5) MGlU (in 1 μ l). Striatal neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 250 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test. *** P<0.001 versus 5nmol-treated animals.

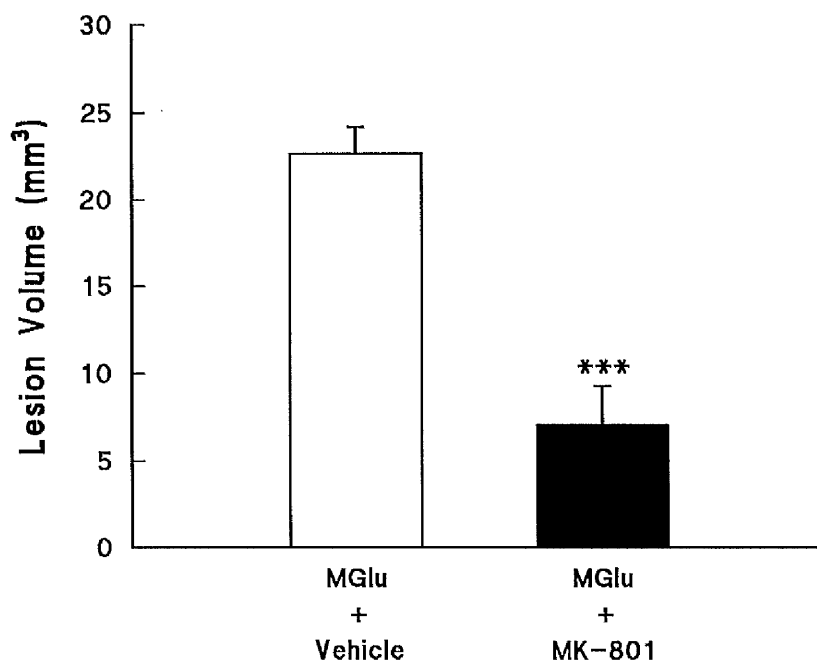
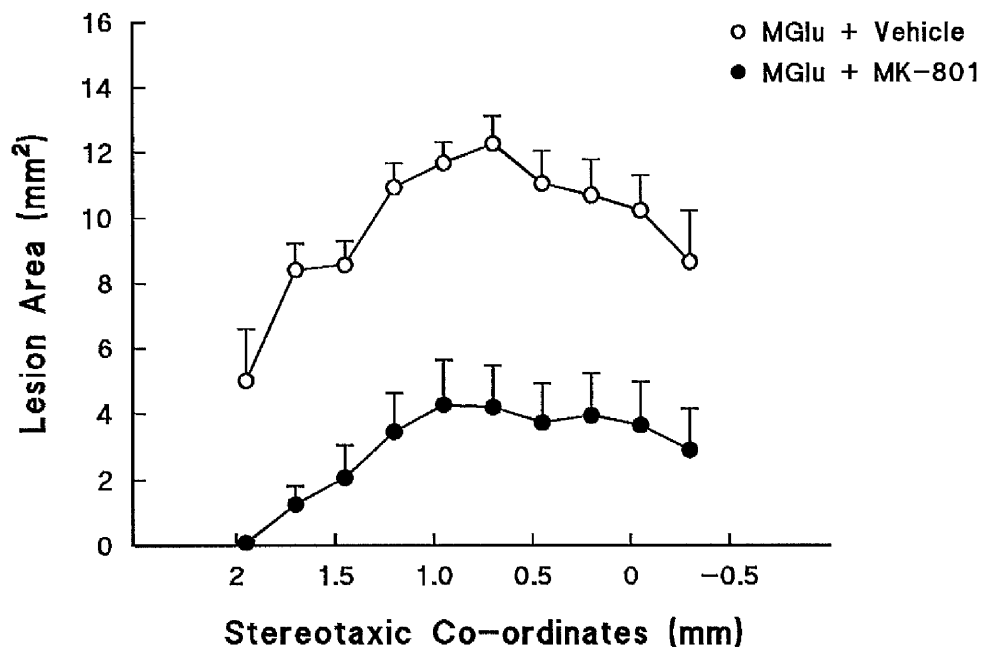


Figure 3.3.2 Effect of MK-801 on striatal NMDA receptor-induced damage

Damage was induced by intrastriatal infusion of 10nmol MGlutamate (in 1 μ l). MK-801 (4mg/kg i.p., n=11) or vehicle (PBS 1ml/kg i.p., n=11) was administered 30min prior to MGlutamate infusion. Striatal neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 250 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test. *** P<0.001 versus vehicle-treated animals.

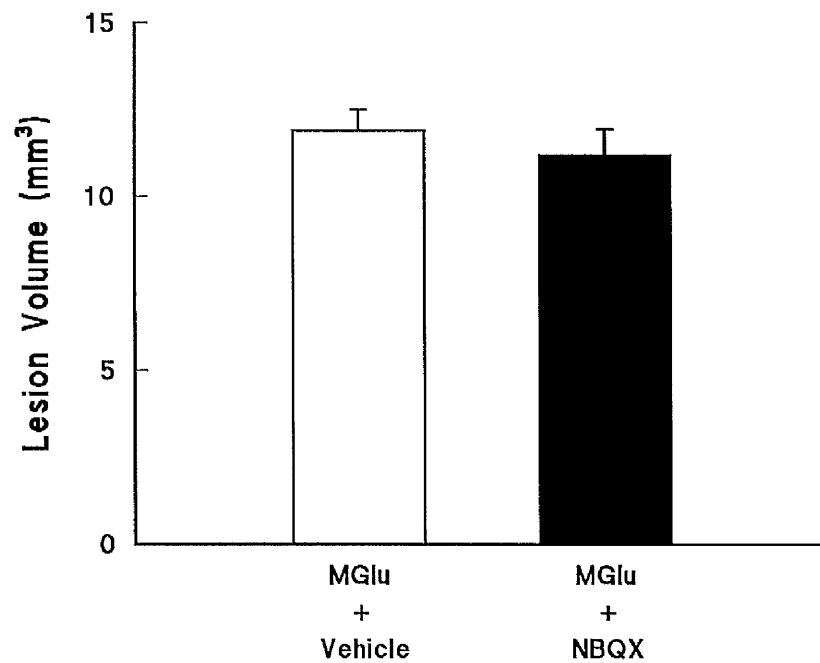
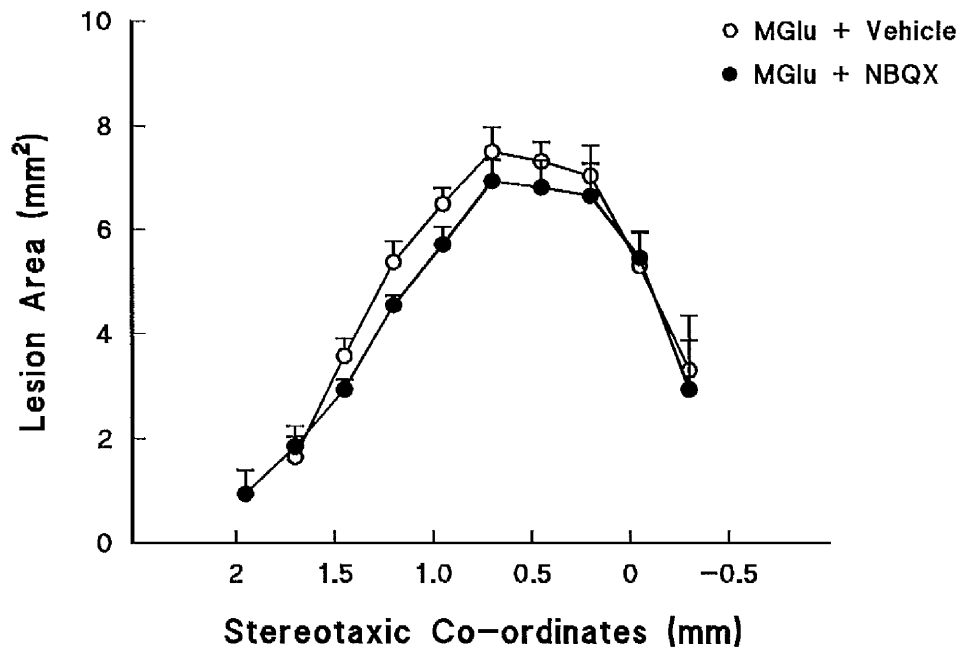


Figure 3.3.3 Effect of NBQX on striatal NMDA receptor-induced damage

Damage was induced by intrastriatal infusion of 10nmol MGlutamate (in 1 μ l). NBQX (25nmol in 1 μ l, n=7) or vehicle (PBS, 1 μ l, n=7) was co-infused with MGlutamate. Striatal neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 250 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test.

optimum time point for measurement of damage, since at earlier time points (two or six hours) the lesion had not fully developed (Black *et al.*, 1992; Relton, 1992), and a dose of 10nmol was chosen according to the data reported by the above authors. Lesions induced by MGlu (5 or 10nmol) were ipsilateral and generally confined to the striatum. However, on some occasions (for 10nmol), damage extended slightly to the overlying ipsilateral frontal cortex, immediately adjacent to the needle tract (see *Figure 3.7.1* and *3.7.4a*). Intrastratial infusion of 5 or 10nmol resulted in reproducible lesions (assessed 24h later) of a volume of $19.64 \pm 1.24 \text{ mm}^3$ (n=5), for 10nmol and $4.05 \pm 0.32 \text{ mm}^3$ (n=5), for 5nmol. *Figure 3.3.1* illustrates the distribution of the striatal neuronal damage after infusion of either 5 or 10nmol MGlu. Pretreatment of rats with MK-801 followed by infusion of 10nmol MGlu significantly reduced lesion volume by 69% (Vehicle $22.63 \pm 1.54 \text{ mm}^3$, n=11 versus MK-801 $7.05 \pm 2.19 \text{ mm}^3$, n=11, $P < 0.001$, *Figure 3.3.2*). In contrast, the AMPA receptor antagonist, NBQX, did not modify MGlu-induced striatal damage (Vehicle $11.89 \pm 0.60 \text{ mm}^3$, n=7 versus NBQX $11.19 \pm 0.74 \text{ mm}^3$, n=7, not significant, (NS), *Figure 3.3.3*).

3.4 Cortical NMDA receptor-induced excitotoxic lesions

The aim of this study was to identify the optimal doses of MGlu which produce large, but submaximal lesions in the parietal cortex (i.e. did not extend throughout the entire cortex), and to study the effect of the NMDA antagonist, MK-801 on the neuronal damage induced by cortical NMDA receptor activation.

3.4.1 *Experimental*

Cortical lesions were performed according to the protocol described in *Section 2.3.1*. A dose ranging between 5-10nmol of the NMDA agonist, MGlu (in $1 \mu\text{l}$, n=17) was infused into the parietal cortex. In separate experiments, animals

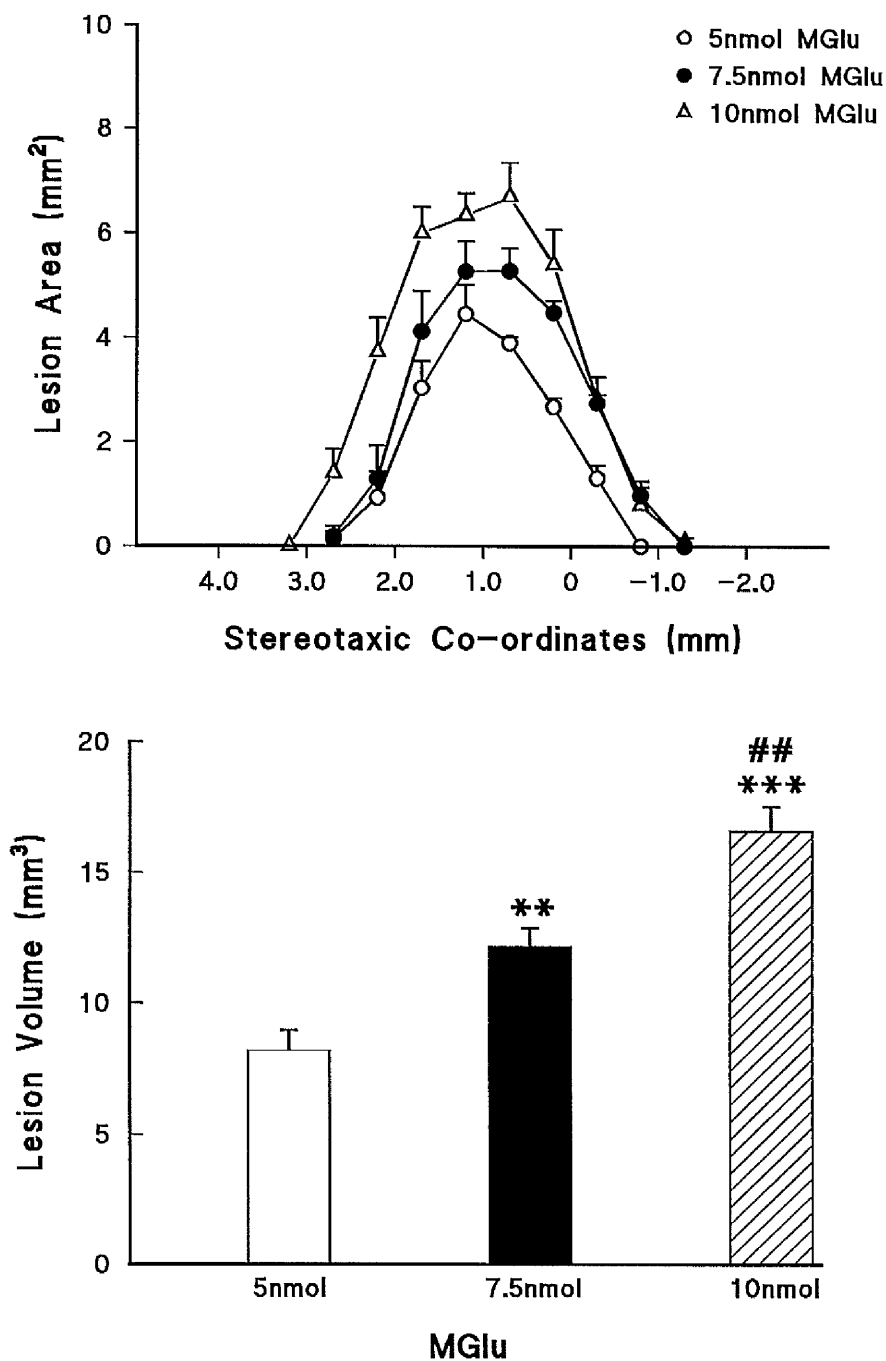


Figure 3.4.1 Lesion size 24h after intracortical infusion of MGLu

Damage was induced by intracortical infusion of either 5nmol (n=6), 7.5nmol (n=5) or 10nmol (n=6) MGLu (in 1 μ l). Cortical neuronal damage was measured 24h later. The upper graph shows lesion area (mm², on 500 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph. Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test. *** P<0.001, ** P<0.01 versus 5nmol-treated animals. ## P<0.01 versus 7.5nmol-treated animals.

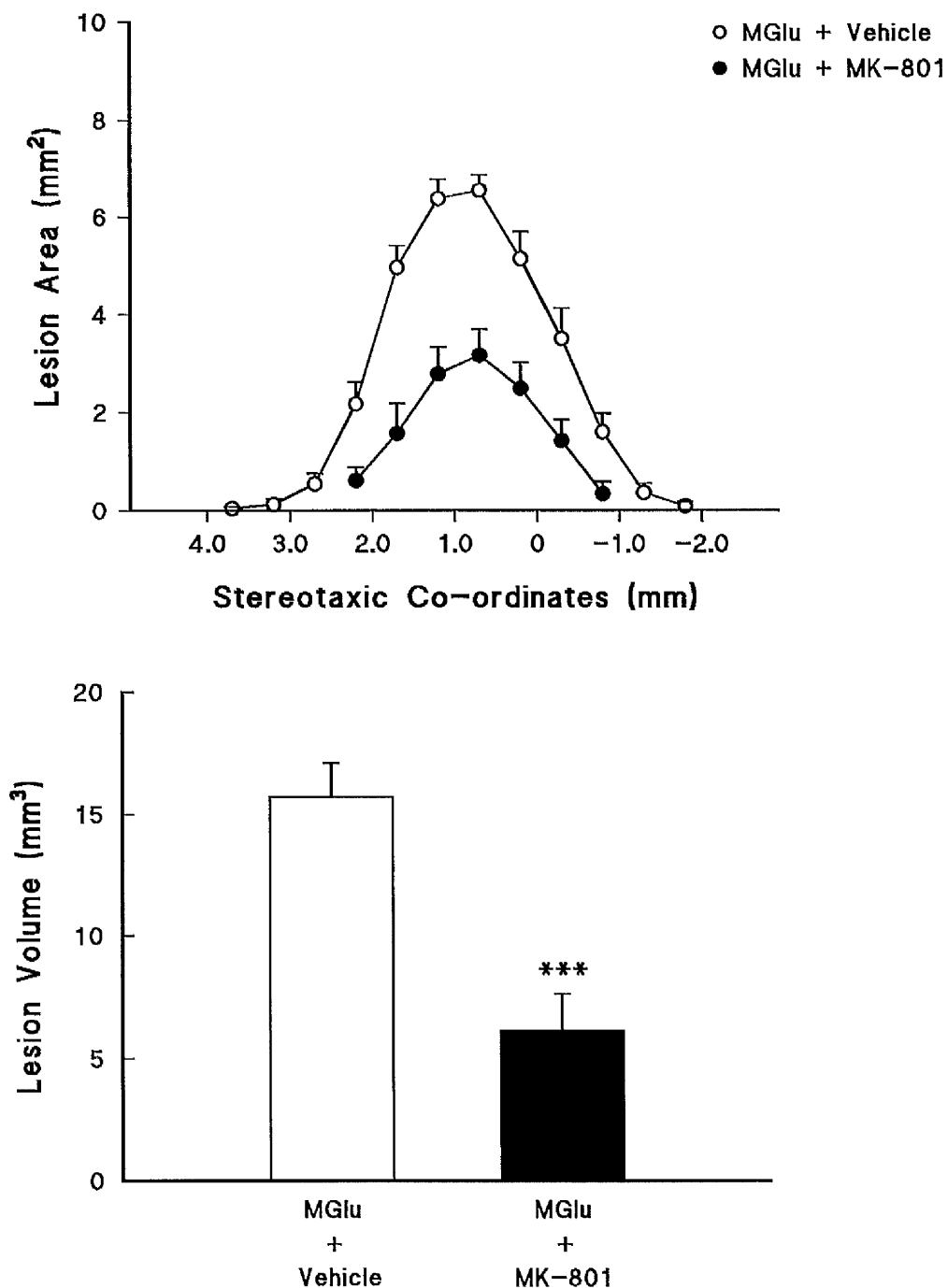


Figure 3.4.2 Effect of MK-801 on cortical NMDA receptor-induced damage

Damage was induced by intracortical infusion of 10nmol MGLu (in 1 μ l). MK-801 (4mg/kg i.p., n=12) or vehicle (PBS 1ml/kg i.p., n=13) was administered 30min prior to MGLu infusion. Cortical neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 500 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test. *** P<0.001 versus vehicle-treated animals.

were injected with either PBS (1ml/kg, i.p., n=13) or MK-801 (4mg/kg, i.p., n=12), 30min prior to intracortical infusion of 10nmol (in 1 μ l) of MGlu. Animals were sacrificed (as in *Section 2.4*) 24h after infusion and neuronal death assessed by the tetrazolium method, as in *Section 2.5.1* and *2.6.1*.

3.4.2 Results

Cortical infusion of 5-10nmol of MGlu resulted in no noticeable changes in behaviour and, in contrast to MGlu-induced striatal lesions, no "barrel-rolling" was observed. Infusion of MGlu produced dose-dependent lesions measured 24h later with 5nmol MGlu resulting in small cortical lesions ($8.20 \pm 0.76 \text{ mm}^3$, n=6) compared to slightly more extensive lesions observed after 7.5nmol MGlu ($12.17 \pm 0.70 \text{ mm}^3$, n=5). The highest dose tested (10nmol) caused the most widespread neuronal death ($16.59 \pm 0.93 \text{ mm}^3$, n=6). *Figure 3.4.1* illustrates the distribution of cortical damage after infusion of either 5, 7.5 or 10nmol MGlu. Pretreatment of rats infused with 10nmol MGlu, with MK-801, significantly reduced lesion volume by 61% (Vehicle $15.72 \pm 1.39 \text{ mm}^3$, n=13 versus MK-801 6.18 ± 1.47 , n=12, $P < 0.001$, *Figure 3.4.2*). See *Figure 3.7.3* for an example of a cortical lesion on a tetrazolium stained section, and *Figure 3.7.4c* for a typical pattern of parietal cortical damage.

3.5 Striatal AMPA receptor-induced excitotoxic lesions

The aim of this study was to identify the optimal doses the AMPA agonist, S-AMPA, which produce large, but submaximal lesions in the striatum (i.e. confined to the striatum), and to establish the optimal end point for assessing neuronal death. The effect of the AMPA receptor antagonist, NBQX and the NMDA receptor antagonist, MK-801 were assessed on striatal damage.

3.5.1 *Experimental*

Striatal lesions were performed according to the protocol described in *Section 2.3.1*. A dose ranging between 10-20nmol of the AMPA agonist, S-AMPA (in 1 μ l, n=11) was infused into the striatum. In separate experiments, animals were injected with either PBS (1ml/kg, i.p., n=8) or MK-801 (4mg/kg, i.p., n=7), 30min prior to intrastriatal infusion of 15nmol (in 1 μ l) S-AMPA. In further experiments, NBQX (25nmol in 1 μ l, n=7) or PBS (1 μ l, n=8) was infused immediately before 15nmol (in 1 μ l) S-AMPA. Animals were sacrificed (as in *Section 2.4*) 24h (for dose response experiments) or 48h (for all experiments) after infusion and neuronal death assessed by the tetrazolium method, as in *Section 2.5.1* and *2.6.1*.

3.5.2 *Results*

Neurotoxicity induced by S-AMPA was assessed 24h and 48h after infusion. Later time points were chosen for S-AMPA-induced lesions as this excitotoxin has been shown to induce excitotoxicity at a slower rate (Garthwaite and Garthwaite, 1989, 1991a, 1991b). Preliminary experiments (data not shown) did indeed suggest that lesions were small and incomplete at 24h, hence all lesions were assessed 48h after infusion.

Striatal infusion of S-AMPA (10-20nmol), caused modest behavioural changes, including "barrel-rolling" which was similar to, but more pronounced than that observed after intrastriatal infusion of MGlu (see *Section 3.3.2*). In some S-AMPA-treated animals intermittent periods of seizure-like motor activity were also observed, which included episodic "barrel-rolling", rearing and movement of the forelimbs and occasional "wet dog shakes". Between these events the animals were hypoactive. In very severe cases some behavioural disturbances

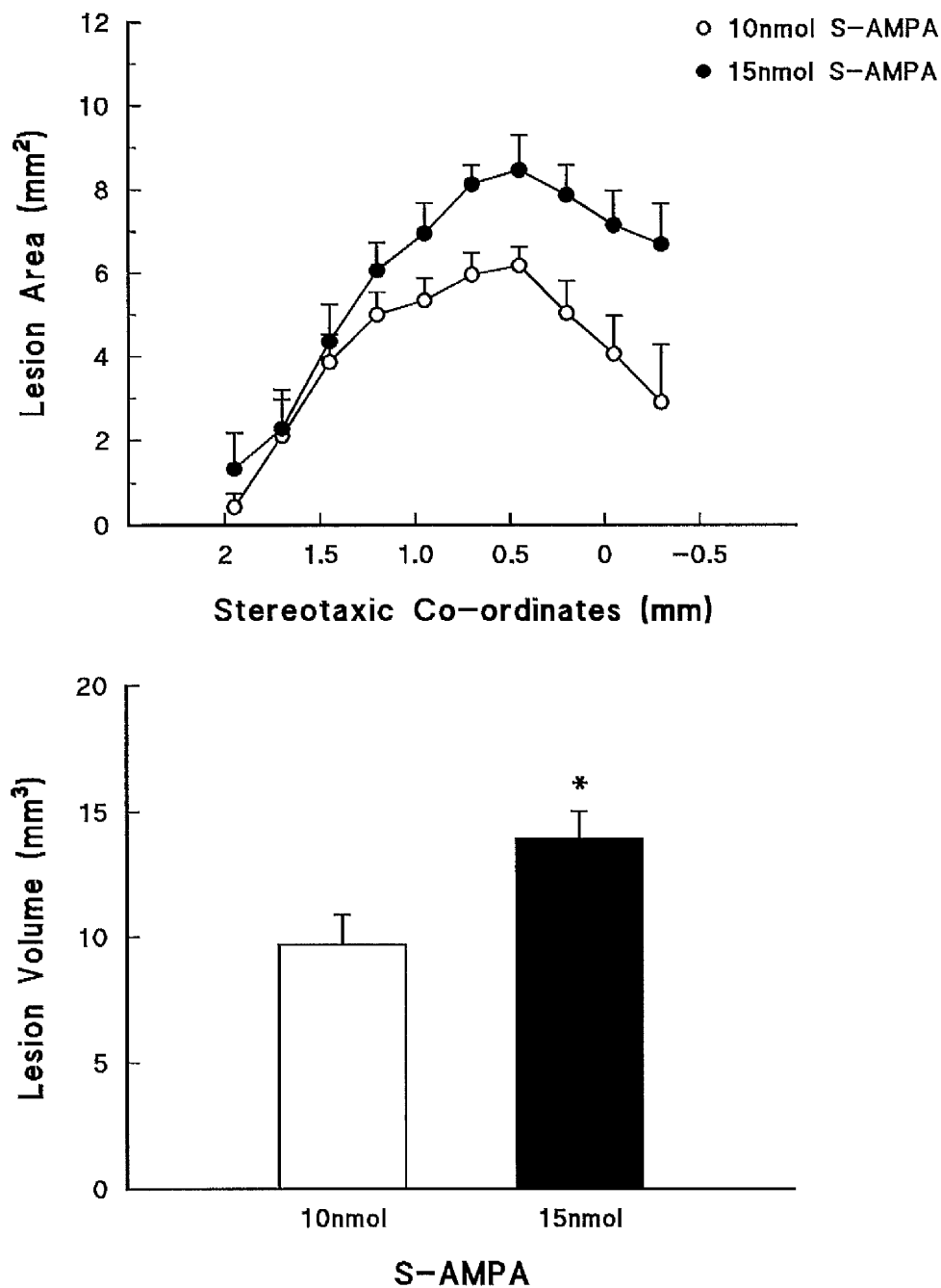


Figure 3.5.1 Lesion size 48h after intrastriatal infusion of S-AMPA

Damage was induced by intrastriatal infusion of either 10nmol (n=5) or 15nmol (n=6) S-AMPA (in 1 μ l). Striatal neuronal damage was measured 48h later. The upper graph shows lesion area (mm², on 250 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph. Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test. * P<0.05 versus 10nmol-treated animals.

were still observed between the 24-48h period. In addition to the striatal damage induced by S-AMPA, modest damage sometimes extended to the overlying frontal cortex and in some cases (approximately 50% of animals) modest ipsilateral extrastriatal damage was observed in the piriform cortex. Damage in the piriform cortex was apparent at all doses of S-AMPA tested, although its occurrence was higher with increasing doses. However, as damage in the piriform cortex was not seen in all cases, it was not quantified, although its presence was noted. See *Figure 3.7.2* and *3.7.4b* for examples of piriform cortex damage in response to striatal S-AMPA infusion.

20nmol S-AMPA produced a lesion throughout the whole of the striatum and usually extended into the ipsilateral cerebral cortex. The damage produced by 20nmol S-AMPA was not quantified because it was so extreme and severely affected extrastriatal regions. In a few cases, infusion of 20nmol S-AMPA produced contralateral as well as ipsilateral piriform cortex damage (not shown). 10nmol S-AMPA caused a smaller striatal lesion ($9.69 \pm 1.21 \text{ mm}^3$, $n=5$) and piriform cortex damage was sometimes observed. Intrastriatal infusion of 15nmol S-AMPA resulted in a more widespread striatal lesion ($13.95 \pm 1.07 \text{ mm}^3$, $n=6$) than the lower dose (10nmol), and the occurrence of piriform cortex neuronal damage was higher. *Figure 3.5.1* illustrates the distribution of striatal neuronal damage after infusion of either 10nmol or 15nmol S-AMPA.

Neuronal damage caused by S-AMPA (15nmol) in the striatum was not significantly affected by peripheral administration of MK-801 (Vehicle $19.70 \pm 1.30 \text{ mm}^3$, $n=8$ versus MK-801 $18.80 \pm 1.11 \text{ mm}^3$, $n=7$, NS, *Figure 3.5.2*). The occurrence of piriform cortex damage was also not markedly altered by MK-801 pretreatment. Sixty three percent of control (S-AMPA and vehicle) animals had piriform cortex damage compared to 83% of MK-801-treated animals, although the volume of these lesions was not quantified. The AMPA receptor antagonist, NBQX, significantly reduced the volume of damage in the striatum

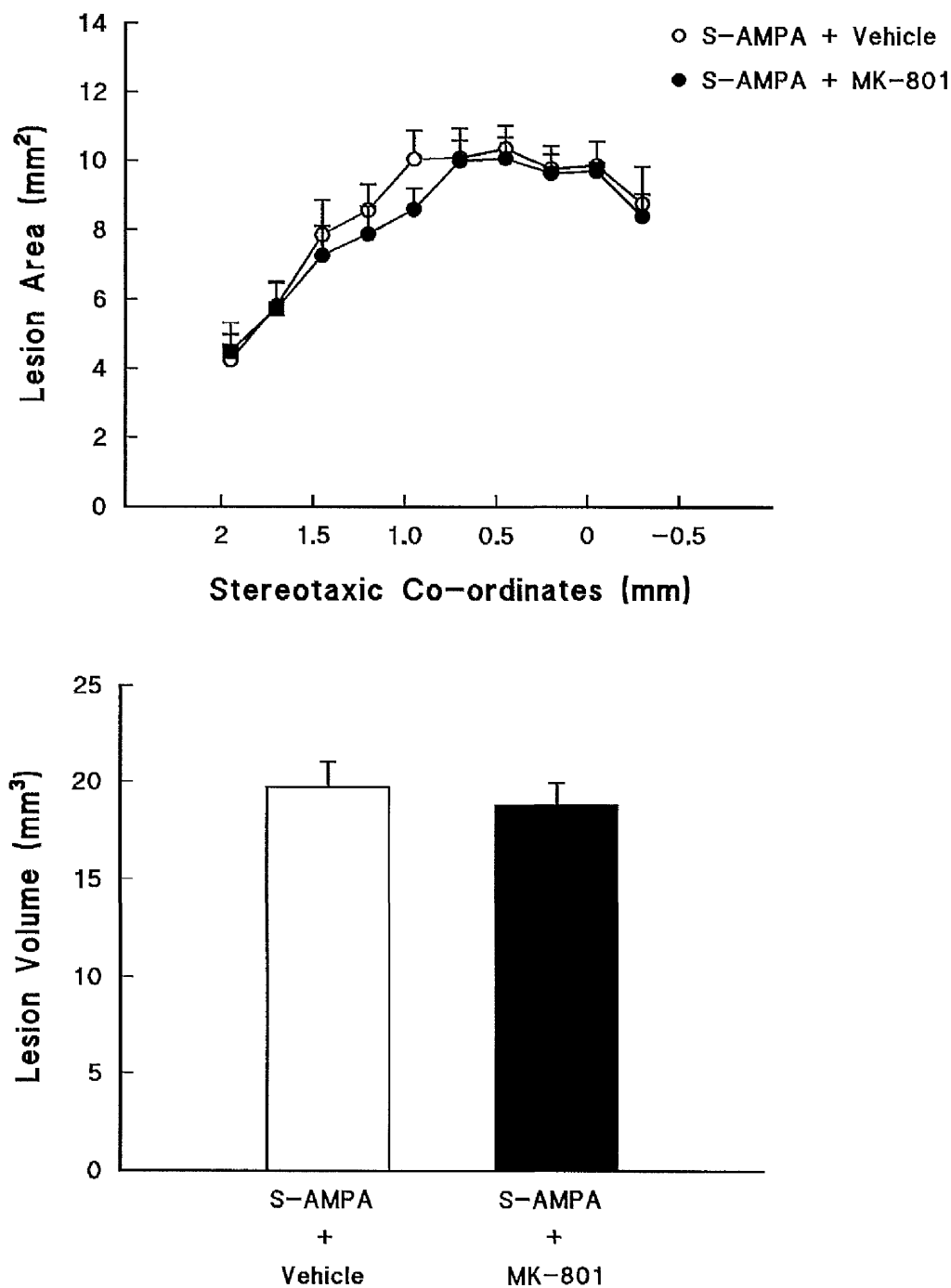


Figure 3.5.2 Effect of MK-801 on striatal AMPA receptor-induced damage

Damage was induced by intrastriatal infusion of 15nmol S-AMPA (in 1 μ l). MK-801 (4mg/kg i.p., n=7) or vehicle (PBS 1ml/kg i.p., n=8) was administered 30min prior to S-AMPA infusion. Striatal neuronal damage was measured 48h later.

The upper graph shows lesion area (mm², on 250 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test.

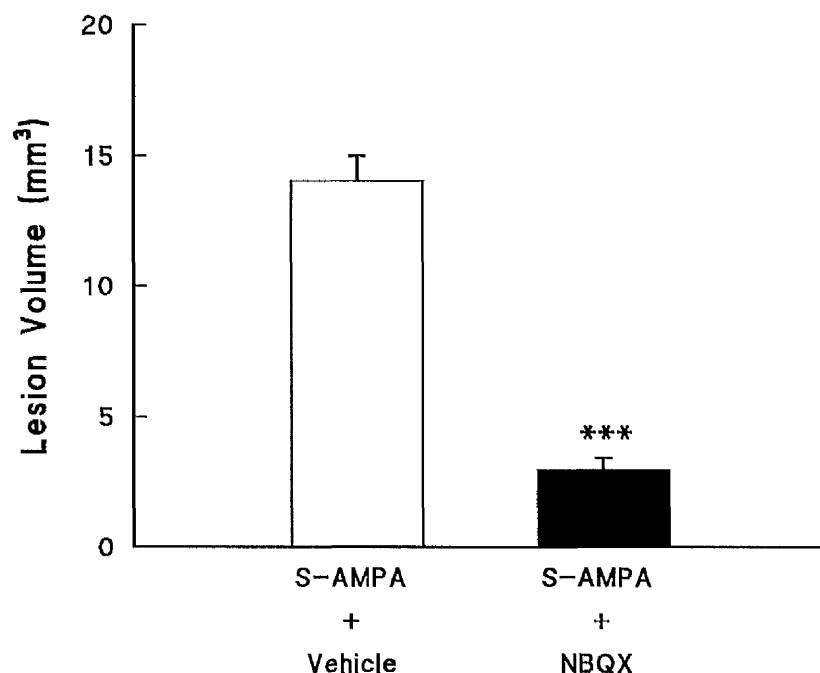
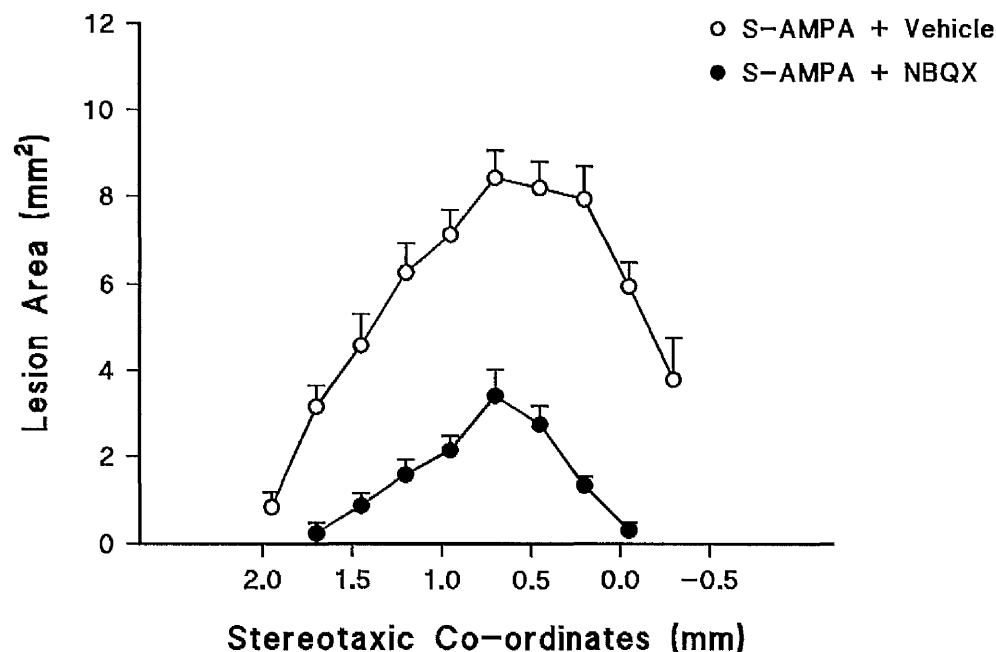


Figure 3.5.3 Effect of NBQX on striatal AMPA receptor-induced damage

Damage was induced by intrastriatal infusion of 15nmol S-AMPA (in 1 μ l). NBQX (25nmol in 1 μ l, n=7) or vehicle (PBS, 1 μ l, n=8) was co-infused with S-AMPA. Striatal neuronal damage was measured 48h later.

The upper graph shows lesion area (mm², on 250 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test. *** P<0.001 versus vehicle-treated animals.

by 79% (Vehicle $14.03 \pm 0.96 \text{ mm}^3$, $n=8$ versus NBQX $3.00 \pm 0.43 \text{ mm}^3$, $n=7$, $P < 0.001$, *Figure 3.5.3*), but did not appear to inhibit the occurrence of S-AMPA-induced piriform cortex damage; 63% versus 86% for control and NBQX-treated animals respectively.

3.6 Cortical AMPA receptor-induced excitotoxic lesions

The aim of this study was to identify the optimal doses of S-AMPA which produce large, submaximal lesions in the parietal cortex (i.e. did not extend throughout the entire cortex), and to establish the optimal time for assessing neuronal death.

3.6.1 *Experimental*

Cortical lesions were performed according to the protocol described in *Section 2.3.1*. A dose ranging between 5-15nmol of the AMPA agonist, S-AMPA (in $1 \mu\text{l}$, $n=34$) was infused into the parietal cortex. Animals were sacrificed (as in *Section 2.4*) 24h ($n=20$) or 48h ($n=14$) after infusion and neuronal death assessed by the tetrazolium method, as in *Section 2.5.1* and *2.6.1*.

3.6.2 *Results*

Cortical infusion of 5-15nmol of S-AMPA resulted in no noticeable changes in behaviour and, in contrast to S-AMPA-induced striatal lesions, no "barrel-rolling" was generally observed. However, on a few occasions, intracortical infusion of 15nmol S-AMPA, resulted in slight "barrel-rolling" activity and mild seizures, and in these animals, damage had spread to the striatum or piriform cortex respectively. Infusion of S-AMPA produced dose-dependent lesions at 24h and 48h (see *Figure 3.6.1* and *3.6.2*). Damage was incomplete at 24h, as all lesions

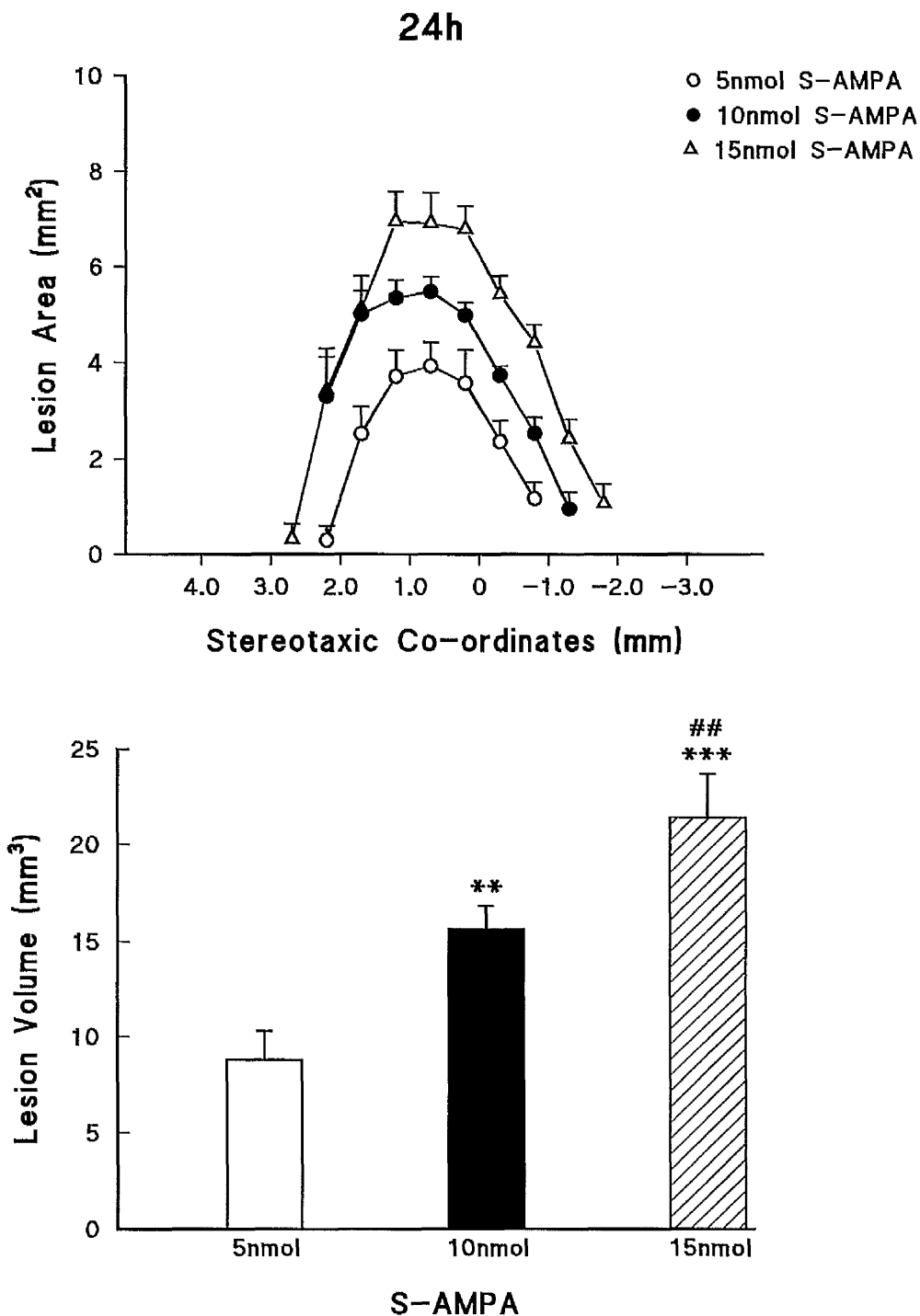


Figure 3.6.1 Lesion size 24h after intracortical infusion of S-AMPA

Damage was induced by intracortical infusion of either 5nmol (n=7), 10nmol (n=7) or 15nmol (n=6) S-AMPA (in 1 μ l). Cortical neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 500 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test. *** P<0.001, ** P<0.01 versus 5nmol-treated animals. ## P<0.01 versus 10nmol-treated animals.

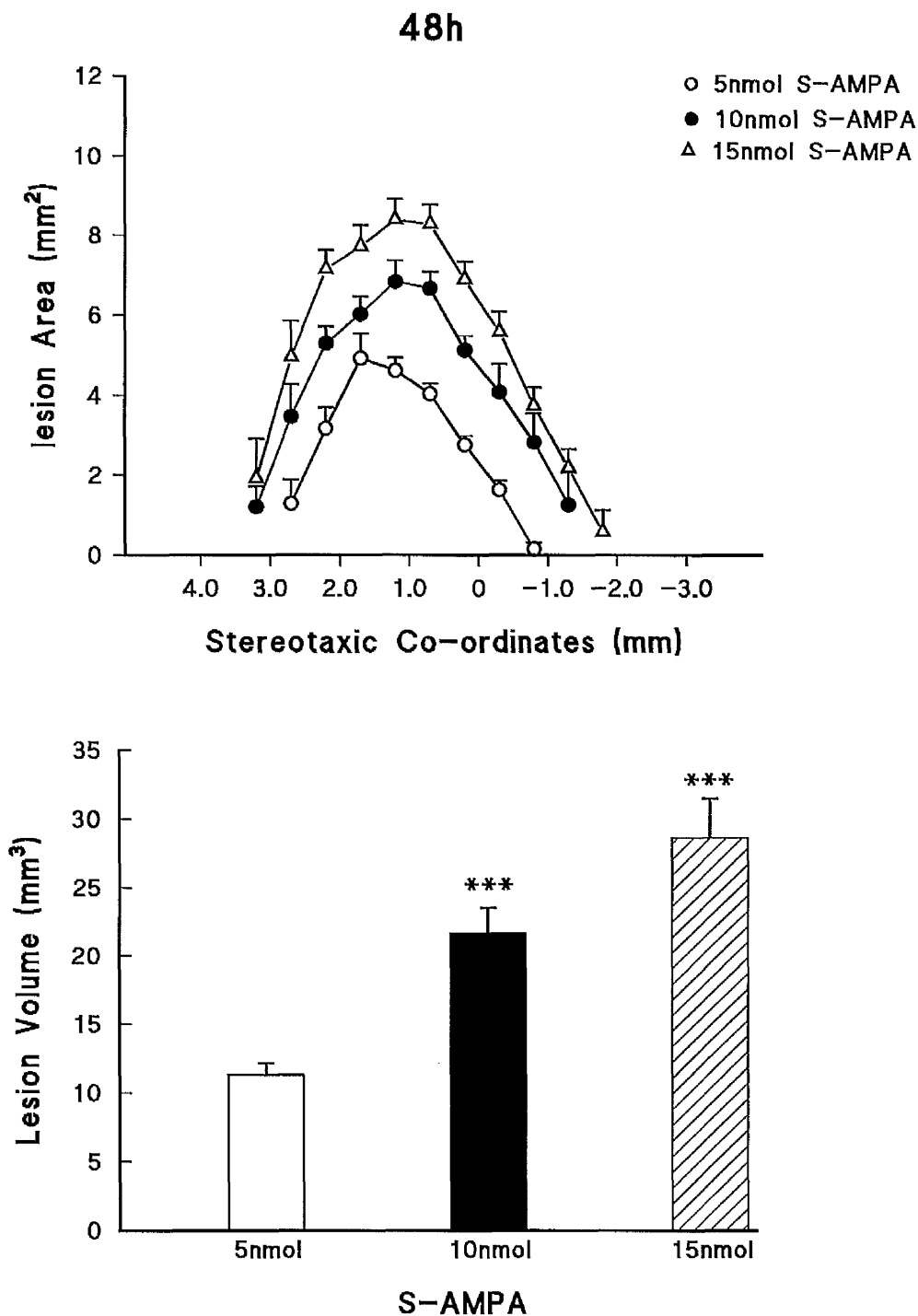


Figure 3.6.2 Lesion size 48h after intracortical infusion of S-AMPA

Damage was induced by intracortical infusion of either 5nmol (n=5), 10nmol (n=5) or 15nmol (n=4) S-AMPA (in 1 μ l). Cortical neuronal damage was measured 48h later.

The upper graph shows lesion area (mm², on 500 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test. *** P<0.001 versus 5nmol-treated animals.

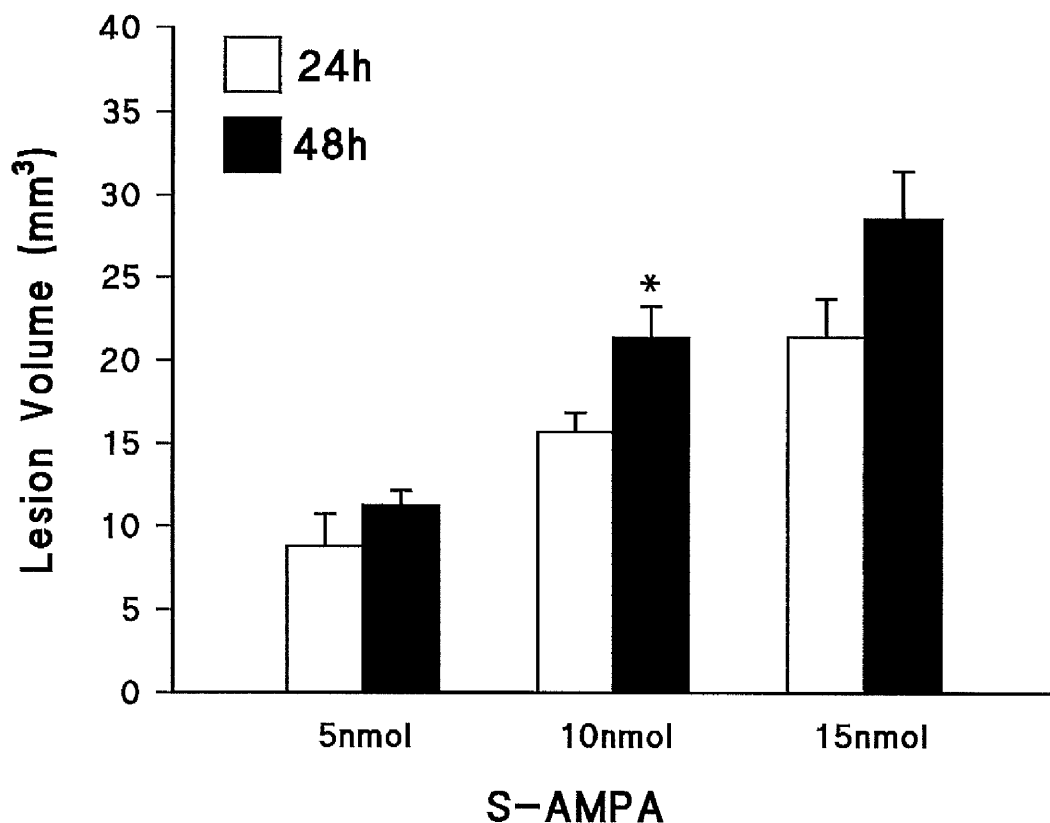


Figure 3.6.3 Lesion volume 24h or 48h after intracortical infusion of S-AMPA

Damage was induced by intracortical infusion of either 5nmol (24h, n=7 and 48h, n=5), 10nmol (24h, n=7 and 48h, n=5) or 15nmol (24h, n=6 and 48h, n=4) S-AMPA (in 1 μ l). The graph expresses cortical lesion volume (mm³, on 500 μ m sections), measured at either 24h (open bars) or 48h (solid bars) later.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test. * P<0.05 versus 24h in 10nmol-treated animals.

assessed at this time point were smaller than at 48h (see later). However, although lesion volumes were different for all doses at the two time points, the volume of damage produced by 10nmol S-AMPA, at 24h compared to 48h, was the only group which reached statistical significance. Thus cortical infusion of 5nmol S-AMPA resulted in fairly small cortical damage (24h $8.78 \pm 1.49 \text{ mm}^3$, $n=7$ versus 48h $11.29 \pm 0.85 \text{ mm}^3$, $n=5$, NS, *Figure 3.6.3*) compared to larger lesions observed after 10nmol S-AMPA (24h $15.69 \pm 1.18 \text{ mm}^3$, $n=7$ versus 48h $21.39 \pm 1.84 \text{ mm}^3$, $n=5$, $P < 0.05$, *Figure 3.6.3*). Infusion of 15nmol S-AMPA into the cortex resulted in the most extensive neuronal damage (24h $21.39 \pm 2.29 \text{ mm}^3$, $n=6$ versus 48h $28.64 \pm 2.82 \text{ mm}^3$, $n=4$, NS, *Figure 3.6.3*), which sometimes spread into the adjacent striatum, however this striatal damage was not measured. See *Figure 3.7.3* for an example of a cortical lesion on a tetrazolium stained section, and *Figure 3.7.4c* for a typical pattern of parietal cortical damage.

3.7 Discussion

The initial aim of this study was to develop a reliable protocol for inducing excitotoxic neuronal damage in two different areas of the rat brain, by two different EAA receptor agonists, acting at either NMDA or AMPA receptors.

3.7.1 *Establishment of excitotoxic damage*

Various studies have employed intracerebral injection of EAA's to study mechanisms of excitotoxicity in experimental animals (e.g. Foster *et al.*, 1988; McDonald *et al.*, 1989a; Fujisawa *et al.*, 1993). The first aim of this study therefore was to establish and optimise a protocol for induction of excitotoxicity which was uncomplicated by factors which are inherent to models of cerebral ischaemia (e.g. reduced blood flow and impaired oxygen delivery). Selective agonists were used in order to study neuronal damage induced by activation of

the two different EAA receptors, NMDA and AMPA. This was attempted by determining the optimum dose of each agonist and the appropriate time point for assessment of damage.

In addition to using different agonists, two different areas of the brain were studied. The areas chosen were the striatum and the cortex (parietal). The aim was therefore to produce large, but submaximal lesions which were confined to either the striatum, or cortex (depending on the area injected), ipsilateral to the site of injection, and did not extend into extra regions. The size of the lesion was crucial in order to observe any enhancement or attenuation of damage which may occur during subsequent experimental manipulation. The results in this chapter clearly demonstrate that the above objectives were accomplished.

Neuronal damage *in vivo* is generally assessed by histological staining methods. For example, haematoxylin and eosin, or cresyl violet staining (Osborne *et al.*, 1987; Fujisawa *et al.*, 1993). However, in this study tetrazolium chloride staining was employed to assess neuronal cell death. It has previously been shown that this staining method clearly delineates areas of neuronal damage at a gross anatomical level (Relton, 1992; Relton and Rothwell, 1992). Tetrazolium staining is dependent on the presence of mitochondrial enzymes (Lojda *et al.*, 1979). Healthy viable tissue with normal levels these enzymes stain pink/purple due to the reduction of tetrazolium chloride to formazan and non-viable tissue remains unstained due to the loss of enzymes in damaged mitochondria. Measurement of damage with tetrazolium has been found to correlate closely with results obtained with haematoxylin and eosin staining (Bederson *et al.*, 1986a; Lin *et al.*, 1993; Bednar *et al.*, 1994). A limitation of this staining method is that tetrazolium also stains intact mitochondria in invading macrophages, although these cells do not appear before two days after excitotoxic damage (Liszczak *et al.*, 1984; Andersson *et al.*, 1991a, 1991b). However, in the present study 48h was the longest time point for assessment of damage, and various other studies on

cerebral ischaemia (stroke), have shown the reliability of this stain in defining regions of neuronal damage from a few hours up to 13 days after the insult (Bederson *et al.*, 1986a; Bederson *et al.*, 1986b; Lye *et al.*, 1987; Isayama *et al.*, 1991; Lin *et al.*, 1993; Saeed *et al.*, 1993; Snape *et al.*, 1993).

Thus, this method allows the rapid and simple assessment of neuronal death, without the requirement of tissue preparation and time-consuming microscopic analysis with other histological methods such as haematoxylin and eosin, or cresyl violet (Osborne *et al.*, 1987; Fujisawa *et al.*, 1993).

3.7.2 Effect of vehicle

Intrastriatal or intracortical infusion of vehicle (PBS, 1 μ l) caused no behavioural changes and no obvious neuronal damage, assessed by tetrazolium 24h or 48h after infusion. Tetrazolium stain measures only gross neuronal damage, therefore when more detailed microscopical histological analysis was carried out, small and very localised neuronal damage was observed, but only in the vicinity of the injection site. This damage can probably be ascribed to mechanical damage produced by the pressure of the needle, and injection of fluid. However, it is assumed therefore, that neither the surgical procedure (injection of needle) or the volume injected produced significant neuronal damage that could be quantified by the tetrazolium staining technique.

3.7.3 NMDA receptor-induced excitotoxicity

It has previously been demonstrated that exogenous administration of 10nmol, MGlu into the rat striatum causes extensive neuronal cell death when assessed 24h after infusion (Black *et al.*, 1992; Relton, 1992; Relton and Rothwell, 1992). Thus, the data obtained in this study, are in agreement with the former studies.

MGlu is a highly selective NMDA receptor agonist which has been well characterized *in vitro* (Lanthorn *et al.*, 1990). Furthermore, MGlu has been used to produce selective striatal NMDA receptor-induced lesion in several studies *in vivo* in the rat (e.g. Black *et al.*, 1992; Schoepp *et al.*, 1994). In the present study, MGlu caused dose-dependent lesions which were maximal after 24h, uniformly stained and clearly delineated. This agonist induced striatal lesions which were largely confined to the vicinity of the injected striatum, apart from slight neuronal damage which was sometimes observed in the cortex immediately overlying the injection site. Cortical damage probably resulted from reflux of infusate up the needle tract as it was withdrawn, rather than remote effects of MGlu. A similar distribution of damage has previously been reported in NMDA receptor-induced brain injury in the rat striatum (e.g. McDonald *et al.*, 1989a; Burtrum and Silverstein, 1993). These authors also report damage in the hippocampus, but in the studies presented here, severe damage was never observed in this region. However, in the former experiments MGlu was infused into the posterior striatum, which is close to the hippocampus, and may explain the damage seen in this structure, whilst injections here are made in the middle of the striatum. Furthermore, MGlu did not appear to induce neuronal damage at sites distant from the injection, i.e. outside the striatum. Lack of damage in extrastriatal brain areas after intrastriatal injection of NMDA receptor agonists in the rat *in vivo* has been well established (e.g. Köhler and Schwarcz, 1983; Schwarcz and Köhler, 1983 and see Section 3.7.6).

The present study demonstrates that infusion of MGlu into the cortex also produces dose-dependent lesions. Limited studies have been published on the neurotoxic effect (especially histological analysis) of NMDA receptor agonist infusion into the parietal cortex *in vivo* (e.g. Beal *et al.*, 1991), although other areas of the cortex, for example the visual and piriform cortex (e.g. Köhler and Schwarcz, 1983; Horsburgh and McCulloch, 1991; Ingvar *et al.*, 1994), and effects cortical infusion of glutamate (Fujisawa *et al.*, 1993) have been examined

and there are much data on the excitotoxicity of NMDA receptors agonists in cortical cultures *in vitro* (e.g. Koh and Choi, 1988b). However, the results presented show that intracortical infusion of MGlu produced dose-dependent lesions, measured 24h later, and resulted in virtually no behavioural changes (see later in *Section 3.7.5*). The damage was largely confined to the parietal cortex, and only in a few cases extended to the adjoining striatum. Striatal damage may have been due to diffusion of excitotoxin from the injection site to the striatum, or to severe or prolonged activation of cortico-striatal glutaminergic neuronal pathways (see Parent and Hazrati, 1995a, 1995b), leading to the excessive release of endogenous glutamate in the striatum, thus resulting in the death of neurones via overactivation of both NMDA and non-NMDA receptors.

In order to establish the specificity of MGlu for the NMDA receptor *in vivo*, in both the striatum and the cortex, rats were pretreated with the non-competitive selective NMDA receptor antagonist, MK-801. The dose used (4mg/kg) has been previously been reported to protect against striatal NMDA receptor-mediated damage in the rat *in vivo* (Beal *et al.*, 1988; Relton, 1992). It should be noted that MK-801 was administered as the total salt and not the free base, therefore the actual dose of the free salt of MK-801 administered was 2.62mg/kg. In this report, MK-801 significantly inhibited neuronal death by 69% and 61% in the striatum and cortex respectively. Due to the severe side effects of this antagonist observed in this study, for example sedation and respiratory depression, higher doses of MK-801 were not tested and may have provided a greater degree of neuroprotection. Several studies concur with the inhibitory effect of MK-801 on striatal NMDA receptor-induced lesions (e.g. Foster *et al.*, 1988; McDonald *et al.*, 1989a, 1989b; McDonald *et al.*, 1990a; Black *et al.*, 1992; Trescher *et al.*, 1994), but there are limited reports of the effect of MK-801 on NMDA receptor-induced damage in the parietal cortex.

In contrast, to the effect of MK-801, co-infusion of the AMPA receptor antagonist,

NBQX, did not affect NMDA receptor-induced striatal lesions. This AMPA antagonist potently inhibits striatal lesions caused by AMPA but not NMDA receptor overactivation (Massieu and Tapia, 1994). However, Massieu and Tapia (1994) did report slight protection against rat striatal lesions *in vivo* induced by the agonist NMDA but not quinolinate, but these authors measured neuronal death indirectly by enzyme assays, which may (although unlikely) pick up subtle changes that could not be detected by the histological method employed in the present study. In addition, McDonald and co-workers (1992) reported a slight neuroprotective effect of the potent AMPA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), on NMDA-induced striatal lesions. However, this antagonist is not selective for the AMPA receptor as it is also a weak antagonist of the NMDA receptor-associated glycine site (Honoré *et al.*, 1988; Watkins *et al.*, 1990).

Overall, the present results suggest that damage induced by MGlu, in the striatum and parietal cortex, is mediated predominantly through selective activation of the NMDA receptor.

3.7.4 AMPA receptor-induced excitotoxicity

AMPA receptor-mediated neuronal injury *in vivo* is less well characterized, and it was therefore necessary to establish an optimal and reproducible protocol for induction of AMPA receptor-mediated neuronal damage. S-AMPA was chosen since it is one of the most selective agonists at the AMPA receptor (Krogsgaard-Larsen *et al.*, 1980; Watkins *et al.*, 1990).

S-AMPA lesions were assessed 24h and 48h after infusion, and it was found that the lesions at 24h were small and submaximal. Therefore, lesions measured after 24h could not be quantified accurately, as they did not reflect the full extent of excitotoxic damage and lesions measured at 48h were consistently larger.

These findings are in accordance with the observation that non-NMDA receptor overactivation induces a slow and delayed type of toxicity *in vitro* (Garthwaite and Garthwaite, 1989, 1991a, 1991b). Furthermore, the limited studies on *in vivo* neuronal damage after intracerebral infusion of AMPA agonists, assess the extent of damage between three and five days, and never before two days (McDonald *et al.*, 1990b, 1992), indirectly suggesting that damage is not complete before 48h.

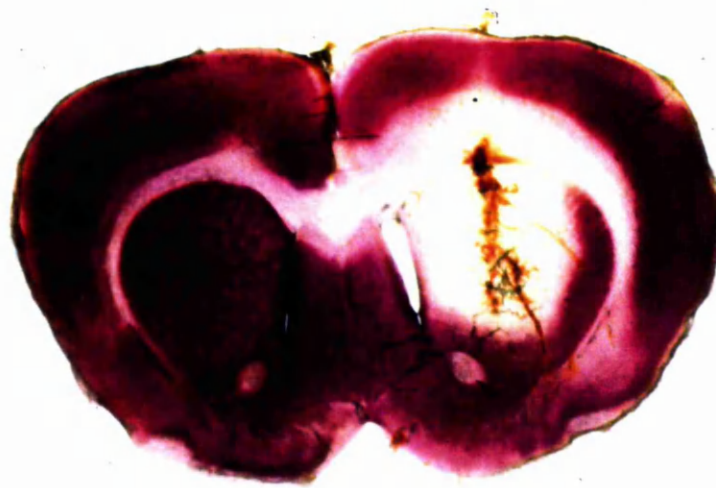
Doses of S-AMPA ranging from 10-15nmol were infused into the striatum and caused dose-dependent and reproducible lesions. McDonald *et al.* (1990b, 1992) have also demonstrated that intrastriatal infusion of S-AMPA (2.5-50nmol) in the rat results in dose-dependent striatal brain injury. In addition to damage within the striatum, neuronal loss was sometimes observed into the overlying cortex, in a similar distribution to that seen for MGlu striatal lesions (see *Section 3.7.3* for discussion). However, in contrast to MGlu-induced striatal lesions, striatal infusion of S-AMPA produced damage not only at the site of injection, but also in structures distant from the striatum. This extrastriatal damage was seen predominantly in the piriform cortex (see *Section 3.7.6* for discussion).

Limited data are available on parietal cortical AMPA lesions *in vivo in the rat* (e.g. see Beal *et al.*, 1991), but the protocol employed here demonstrates that S-AMPA infusion into the parietal cortex resulted in dose-dependent lesions, measured 48h later. The cortical lesions were confined mainly to the parietal cortex, although again (in resemblance to MGlu-induced lesions), damage sometimes spread to the adjacent striatum (see *Section 3.7.3*). In severe cases damage was observed in the piriform cortex and these animals experienced seizures (see *Section 3.7.5*). This damage was probably due to the diffusion of S-AMPA to border of the piriform cortex, which then elicited EAA-induced excitability in this region of the cortex.

In order to determine the specificity of S-AMPA *in vivo* the effects of the AMPA receptor antagonist, NBQX, was tested on striatal damage. This antagonist significantly inhibited, by 79%, striatal neuronal damage, which is consistent with a recent report that NBQX reduces striatal neuronal damage (approximately 71%) induced by overactivation of the AMPA receptor in the rat *in vivo* (Massieu and Tapia, 1994), and that another AMPA receptor antagonist, CNQX, inhibits rat striatal AMPA-induced toxicity *in vivo* by 70% (McDonald *et al.*, 1992). In contrast, S-AMPA-induced striatal damage was not affected by the NMDA receptor antagonist, MK-801, at a dose which significantly inhibited striatal MGLu-induced toxicity. These results concur with previous reports that MK-801 does not prevent AMPA receptor-induced striatal damage in the adult rat *in vivo* (Foster *et al.*, 1988). However, some studies have suggested that in the rat, MK-801 partially inhibits various forms of AMPA receptor-mediated striatal damage *in vivo* (McDonald *et al.*, 1990a, 1992), presumably by limiting the secondary activation of NMDA receptors, which may occur after the release of endogenous glutamate during neuronal excitability. These published studies administered MK-801 after (and also before in McDonald *et al.*, 1990a) the excitotoxin infusion, in comparison to pretreatment only in the present study. Therefore, post-treatment of MK-801 may provide optimal timing to inhibit the action of endogenously released glutamate on NMDA receptors. Furthermore, McDonald *et al* (1990a) used quisqualate to induce AMPA receptor-mediated neuronal injury. However, this agonist is not selective for the AMPA receptor and also activates the metabotropic receptor (see *Section 1.3*)

Overall, the results presented in this chapter indicate that NMDA receptors are probably not involved in the local neuronal damage caused by S-AMPA, and damage induced by this agonist is mediated predominantly by activation of the AMPA receptor.

A)



B)

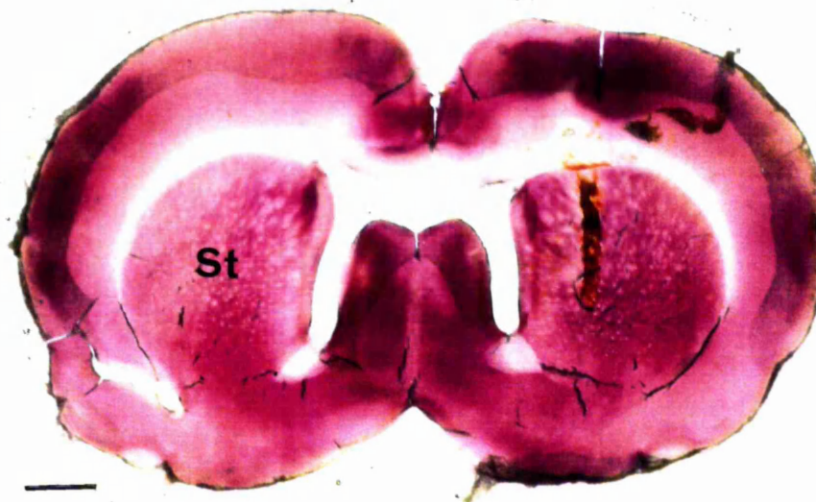


Figure 3.7.1 Striatal lesion caused by intrastriatal infusion of EAA receptor agonists

A representative example of tetrazolium staining of 500 μ m coronal brain sections near the site of injection (1.2mm or 0.7mm anterior to bregma for **A** or **B** respectively) after intrastriatal infusion of:

A). 1 μ l of 15nmol S-AMPA assessed 48h after infusion..

NB. Similar neuronal death is observed 24h after intrastriatal infusion of MGlu (10nmol).

B). 1 μ l of PBS (control vehicle) assessed 48h after infusion.

NB. A similar brain section is obtained 24h after infusion.

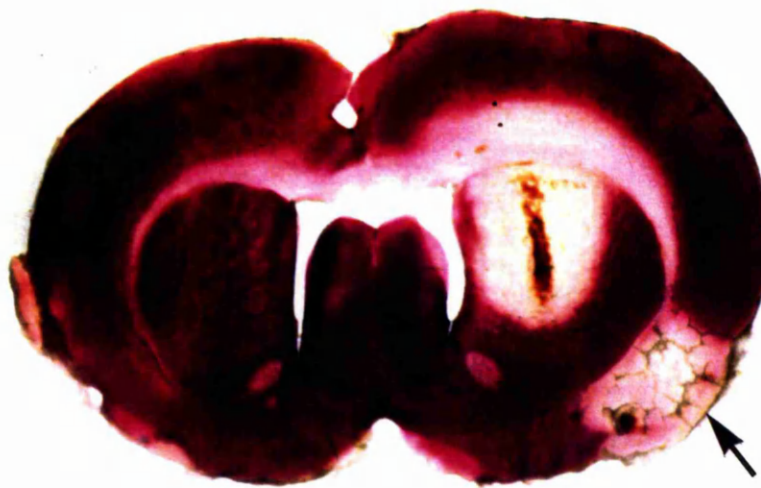
The pale (white) area in the striatum indicates neuronal damage whilst the pink region is healthy tissue.

The dark line in the striatum indicates the site of the needle tract.

St=Striatum

Magnification bar=1.4mm

A)



B)



Figure 3.7.2 Piriform cortex lesions caused by intrastriatal infusion of S-AMPA

Representative examples of tetrazolium staining of 500 μ m coronal brain sections 48h after intrastriatal infusion of 10nmol S-AMPA. NB. Similar piriform cortex damage is observed after intrastriatal infusion of 15nmol S-AMPA.

A). Neuronal damage in the piriform cortex (arrow) in addition to the local striatal damage seen at the site of injection. Section taken at the site of injection (0.7mm anterior to bregma).

B). Neuronal damage in the piriform cortex (arrow) in the absence of striatal damage. Section taken at 1.2mm anterior to bregma

The pale (white) areas indicates neuronal damage whilst the pink region is healthy tissue.

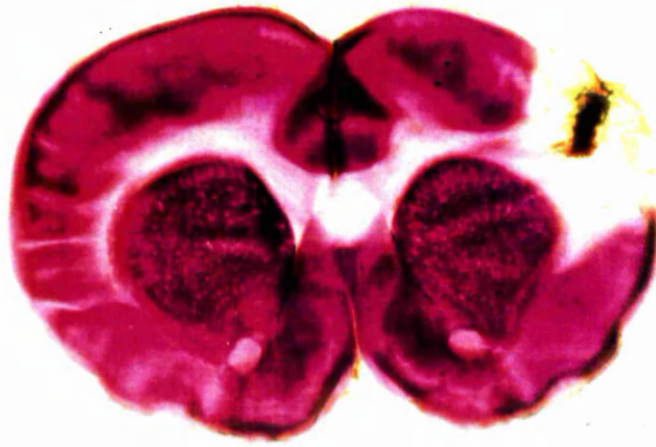
The dark line in the striatum indicates the site of the needle tract.

St=Striatum

PC=Piriform cortex

Magnification bar=1.4mm

A)



B)



Figure 3.7.3 Parietal cortical lesion caused by intracortical infusion of EAA receptor agonists

A representative example of tetrazolium staining of 500 μ m coronal brain sections near the site of injection (1.2mm anterior to bregma) after intracortical infusion of:

A). 1 μ l of 10nmol MGlut assessed 24h after infusion.

NB. Similar neuronal death is observed after intracortical infusion of S-AMPA (15nmol).

B). 1 μ l of PBS (control vehicle) assessed 24h after infusion.

NB. A similar brain section is obtained 48h after infusion.

The pale (white) area in the cortex indicates neuronal damage whilst the pink region is healthy tissue.

The dark line in the cortex indicates the site of the needle tract.

Par=Parietal cortex

Magnification Bar=1.5mm

3.7.5 Behavioural observations

Any behavioural changes observed were predominantly after intrastriatal injection of excitotoxic compounds. There were no noticeable changes in behaviour after injections into the parietal cortex except in a few cases, which will be discussed later. The initial part of this discussion will therefore concentrate on behavioural changes observed after intrastriatal infusions of MGlu or S-AMPA.

Intrastriatal infusion of MGlu or S-AMPA resulted in episodic "barrel-rolling". This behaviour was more pronounced in S-AMPA-treated animals than after MGlu treatment. In addition, S-AMPA-treated animals experienced mild seizure activity (which in some cases included tonic-clonic movements of the forelimbs). This behaviour was rarely observed after treatment with MGlu. It has previously been reported that intrastriatal injection of either NMDA (including MGlu), or non-NMDA receptor agonists result in "barrel-rolling" *in vivo* in the rat (Marrannes and Wauquier, 1988; Vécsei and Beal, 1991; Black *et al.*, 1994). These authors have also observed mild seizure-like activity after intrastriatal infusion of NMDA agonists, either under ether anaesthesia or in conscious rats (Marrannes and Wauquier, 1988; Black *et al.*, 1994). In addition, there are various reports in which striatal NMDA receptor activation results in mild tonic-clonic seizure-like activity in either ether anaesthetised perinatal rats (McDonald *et al.*, 1988, 1989a, 1989b, 1990a) or adult rats (Ciani *et al.*, 1994), or in halothane anaesthetised perinatal or adult rats (Schoepp *et al.*, 1994). However, in the present report, infusions were made in adult rats under the longer-duration anaesthetic, pentobarbitone, and this may have masked any epileptogenic effects of MGlu, which may have occurred. Furthermore, behavioural signs of seizures have found to be much more pronounced in immature rats compared to adult rats (McDonald *et al.*, 1988), and the choice and duration of anaesthetic can effect the toxicity of EAA's (e.g. Zaczek *et al.*, 1978, 1980; Lees, 1992;

Pocock and Richards, 1993; Lees, 1995). It is interesting to note that Ciani and co-workers (1994) reported that intrastriatal injection of an NMDA receptor agonist resulted in considerably shorter and less severe convulsive symptoms when compared to intrastriatal injection of a non-NMDA receptor agonist.

Seizure-like activity in response to intrastriatal injection of excitotoxins has been reported to be due to activation of the limbic circuit (e.g. Ben-Ari, 1985). Some workers have attributed "barrel-rolling" itself to seizures, as this behaviour can be inhibited by anticonvulsants (Marrannes and Wauquier, 1988). However, it has been suggested that "barrel-rolling" is related to increased activity of dopamine-containing neurones in the striatum (Mazzari *et al.*, 1986). Furthermore, "barrel-rolling" can be induced by injection of a variety of substances, such as somatostatin and vasopressin, into different sites of the brain (e.g. Boakes *et al.*, 1985; Diamant and Wied, 1993).

The marked behavioural abnormalities seen after non-NMDA compared with NMDA receptor overactivation may be due to the more widespread lesions (see *Section 3.7.6*) and less specific neurochemical changes induced by non-NMDA receptor activation. For example, somatostatin and neuropeptide Y containing neurones are relatively resistant to damage by NMDA agonists, but are vulnerable to lesions with non-NMDA agonists (Beal *et al.*, 1989; Vécsei and Beal, 1991) and these neuropeptides are suggested to be involved in behaviour. In addition, the lack of seizures due to NMDA, compared to non-NMDA receptor overactivation may be due to the weaker convulsant potential of NMDA agonists in adult rats (e.g. Ciani *et al.*, 1994).

Intracortical infusion of MGlu or S-AMPA resulted in no obvious changes in behaviour. Beal *et al* (1991) also reported no overt behavioural changes after infusion of various excitotoxic compounds into the parietal cortex. However, in a few severe cases, parietal cortical infusion of S-AMPA resulted in seizure-like



activity. Damage was found in the adjoining piriform cortex in these animals. Therefore, the development of seizures could be due to diffusion of the excitotoxin to the piriform cortex, in which epileptogenic activity is initiated (Piredda and Gale, 1986). This behaviour was never seen in MGlu-treated animals, at the doses of MGlu employed, although higher dose may have resulted in seizures.

3.7.6 Extrastriatal damage

In contrast to MGlu-induced striatal lesions, striatal infusion of S-AMPA produced lesions not only at the site of injection, but also in structures distant from the striatum. This extrastriatal damage was observed in the piriform cortex. The occurrence of neuronal loss in areas remote from the site of injection, in response to non-NMDA receptor activation, has been previously reported in the rat (e.g. Ben-Ari *et al.*, 1980; Schwob *et al.*, 1980; Köhler and Schwarcz, 1983; Lerner-Natoli *et al.*, 1991; Wozniak *et al.*, 1994), and the piriform cortex was the most frequently affected region outside the striatum (Schwob *et al.*, 1980; Köhler and Schwarcz, 1983). Other regions, for example the hippocampus and thalamic nuclei, can also be affected by intrastriatal injection of non-NMDA agonists (Ben-Ari *et al.*, 1980; Schwob *et al.*, 1980; Köhler and Schwarcz, 1983). However, due to the staining technique employed in the present study, it is not known if discrete neuronal damage occurred in these regions. It should be noted that all these reported observations (apart from Wozniak *et al.*, 1994) involved the non-NMDA agonist kainate. However, it is quite likely to assume that AMPA and kainate might have similar effects when introduced into the brain *in vivo* (see Bettler and Mülle, 1995), and the results of the present study provide evidence for this proposal. It is surprising, therefore that no mention has been made of piriform cortex damage in various reports involving intrastriatal infusion of AMPA (McDonald *et al.*, 1990b, 1992). However, these authors used immature rats for the majority of their studies, which may be less susceptible to piriform cortex

damage. Subsequently, Wozniak *et al* (1994) have also found piriform cortex damage in response to injection of an AMPA agonist (but not an NMDA agonist), into the nucleus basalis magnocellularis. They also mention that previous workers have not reported this observation.

Distant lesions associated with non-NMDA agonist intracerebral injections are thought to be caused not by the direct toxic action of the excitotoxin, but by accompanying seizure activity (e.g. Ben-Ari *et al.*, 1980; Ben-Ari, 1985). Activation of specific excitatory neuronal pathways, leading to excessive release of endogenous glutamate in target areas, may play an important role as a mediator of such damage (Ben-Ari *et al.*, 1980; Okazaki and Nadler, 1988). In addition, it has been suggested that this seizure-related brain damage is dependent on NMDA receptor activation, because NMDA receptor antagonists prevent neuronal damage in several regions which are remote from the injection site, whilst the local neuronal damage is unaffected (Clifford *et al.*, 1990; Lerner-Natoli *et al.*, 1991; Facchinetti *et al.*, 1992; Ciani *et al.*, 1994; Wozniak *et al.*, 1994). In the present study the occurrence of piriform cortex damage, induced by striatal S-AMPA infusion, was not altered after treatment with MK-801. However, the severity and absolute amount of neuronal death within the piriform cortex may have been affected, as this was not quantified with the method employed here. S-AMPA-induced striatal lesions were significantly inhibited by the AMPA receptor antagonist, NBQX. However, the extrastriatal damage observed in the piriform cortex did not appear to be affected by NBQX. Similarly, recent reports in the rat have reported that NBQX blocks the local excitotoxicity of the non-NMDA agonists, but not the distant neuronal damage *in vivo* (Lees and Leong, 1992, 1994).

In contrast to S-AMPA, no extrastriatal damage in the piriform cortex was observed after MGlut-induced striatal lesions (although mild neuronal damage may have occurred in this structure, which may have gone undetected in

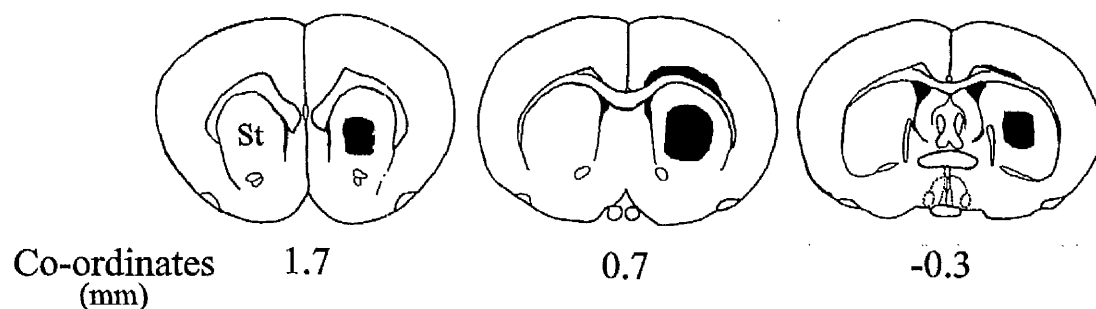


Figure 3.7.4a Pattern of striatal neuronal damage on coronal brain sections typically found 24h or 48h after intrastriatal infusion of 10nmol MGLu or 15nmol S-AMPA respectively. Note the slight damage (in section 0.7) in the ipsilateral cortex overlying the injected striatum.

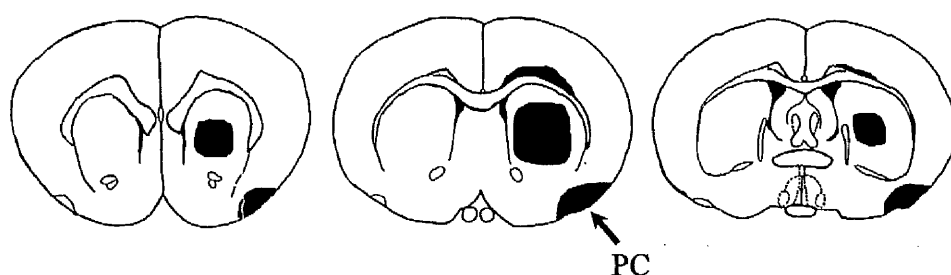


Figure 3.7.4b Coronal brain sections showing a typical pattern of piriform cortex damage, in addition to striatal damage, after intrastriatal infusion of S-AMPA (15nmol). Note the damage in the overlying cortex (see above). NB. Similar piriform cortex damage is observed after intrastriatal infusion of 10nmol S-AMPA.

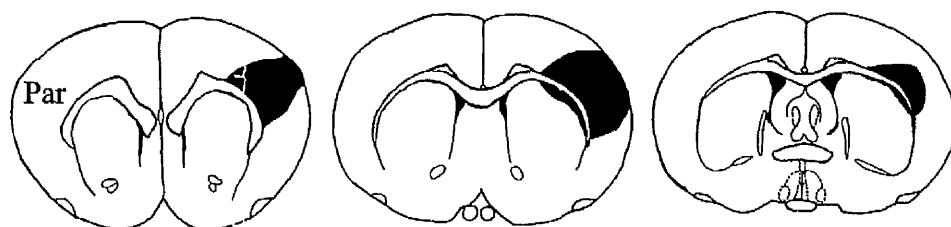


Fig 3.7.4c Pattern of parietal cortical neuronal damage on coronal brain sections typically found 24h or 48h after intracortical infusion of 10nmol MGLu or 15nmol S-AMPA respectively.

N.B Co-ordinate 0.7mm anterior to bregma represents the level of the injection site. Shaded area represents neuronal damage.

St=Striatum

PC=Piriform cortex

Par=Parietal cortex

tetrazolium-stained sections). In support of this observation, it has been reported that NMDA receptor agonists do not cause extrastriatal damage (Köhler and Schwarcz, 1983; Schwarcz and Köhler, 1983). In contrast, Ciani *et al* (1994) have reported slight neuronal damage in the piriform (olfactory) cortex after intrastriatal injection of an NMDA receptor agonist as assessed by detailed histological analysis. However, this damage was only very discrete and markedly restricted compared to the damage seen after intrastriatal injection of a non-NMDA receptor agonist. These authors suggest that the shorter and lower convulsive state elicited by NMDA receptor activation (compared to non-NMDA receptor activation) may explain the lower level of neuronal degeneration observed in distant sites. Furthermore, since in this study, MGlu was found to be only a weak convulsant compared to S-AMPA, the absence of prolonged seizures following striatal injection of MGlu may also account for the lack of extrastriatal neuronal loss.

3.8 Summary

The results of the present chapter demonstrate that MGlu and S-AMPA produced dose-dependent, reproducible striatal or cortical lesions by action on NMDA or AMPA receptors respectively. However, their time courses and effects on distant structures varied significantly. These lesions have been quantified by a histological approach utilising the tetrazolium staining method, which allows a simple, quick and clear way of assessing the neuronal damage in fresh brains.

The protocols used therefore appear to represent a useful *in vivo* model for the investigation of the mechanisms and modifications of excitotoxic damage induced by pharmacological overactivation of the various subtypes of EAA receptors. This approach has certain advantages over other systems involving excitotoxic damage. For example, in experimentally-induced cerebral ischaemia, complex pathophysiological changes occur which are omitted by the direct

pharmacological activation of the EAA receptors. However, there are no direct clinical parallels with acute EAA-induced neuronal damage, although excitotoxic processes are known to occur after various CNS disorders (e.g. stroke and brain trauma). In addition, striatal injection of the NMDA receptor agonist, quinolinic acid, has been reported to reproduce many features of Huntington's disease (Beal *et al.*, 1986).

Chapter Four

Involvement of interleukin-1 in excitotoxic brain damage in the rat

Chapter Four

Involvement of interleukin-1 in excitotoxic brain damage in the rat

4.1 Introduction

The involvement of IL-1 in ischaemic and traumatic brain injury has been previously reviewed (see Rothwell and Relton, 1993; Rothwell *et al.*, 1994, 1995b), and is discussed extensively in *Chapter One*. Both these forms of brain injury are dependent on excessive release of endogenous EAA's (e.g. glutamate) and subsequent overactivation of the EAA receptors (e.g. NMDA and AMPA; for reviews see, Siesjö, 1992a, b; Regan and Choi, 1994). Inhibition of EAA release or action, especially by blocking NMDA or AMPA receptors, reduces the extent of neuronal damage caused by experimentally-induced cerebral ischaemia or brain trauma (e.g. Bullock, 1992; Meldrum, 1990; McIntosh, 1993; Toulmond *et al.*, 1993b; McCulloch, 1994; Regan and Choi, 1994). These observations led us to test the effect of IL-1 on neuronal damage induced by pharmacological activation of NMDA receptors. Thus, it is now established that IL-1 mediates excitotoxic damage due to intrastriatal infusion of an NMDA receptor agonist (Relton and Rothwell, 1992). Neurodegeneration also results from overactivation of AMPA receptors (McDonald *et al.*, 1992), and this form of neuronal death may be mediated by different mechanisms to those underlying NMDA receptor-induced damage (e.g. Garthwaite and Garthwaite, 1991a, 1991b and see *Section 1.4*). Therefore, examination of the effect of IL-1/IL-1ra on AMPA receptor-induced excitotoxicity may provide clues to the mechanism of action of IL-1.

The work of Relton and Rothwell (1992) reports the inhibition of NMDA receptor-

induced lesions by co-infusion IL-1ra in the striatum of the rat. However, the striatum is the only area of the brain these authors studied. The neuronal damage in the experimental models (in the rat) of cerebral ischaemia and traumatic brain injury described above, occurs predominantly in the cerebral cortex, although damage is observed in the striatum after cerebral ischaemia. The cortex and striatum differ, particularly in the type and organisation of their neurones (e.g. Zilles, 1990; Zilles *et al.*, 1990; Nieoullon and Kerkerian-Le Goff, 1992). It is therefore important to compare the effects of IL-1/IL-1ra on damage in both areas.

Thus, the aims of the work presented in this chapter were first, to compare the effects of IL-1ra on excitotoxic damage, induced by overactivation of NMDA or AMPA receptors in both the striatum and the cortex and second, to determine if exogenous IL-1 modifies excitotoxic damage, using the experimental procedures established and verified in *Chapter Three*.

4.2 Effect of IL-1ra on EAA receptor-mediated neurotoxicity

The aim of this study was to validate previous work on the effect of IL-1ra on NMDA receptor-induced striatal lesions, and to test the effect of IL-1ra on AMPA receptor-induced lesions in the striatum, and on both forms of damage (AMPA and NMDA receptor-induced) in the cortex.

4.2.1 *Experimental*

Striatal or cortical lesions were performed according to the protocol described in *Section 2.3.1*. In separate experiments, either vehicle (PBS, 1 μ l) or human IL-1ra (5 μ g/ μ l) was infused into the striatum or cortex of anaesthetised rats (n=8-12), immediately followed by the excitotoxins, MGlu (10nmol in 1 μ l) or S-AMPA

(15nmol in 1 μ l). In addition, in all experiments 1 μ l of IL-1ra alone was infused. Animals were sacrificed (as in *Section 2.4*) 24h after surgery for MGlu infusion and 48h after S-AMPA infusion. Neuronal death was subsequently assessed by the tetrazolium method as in *Section 2.5.1* and *2.6.1*.

4.2.2 Results

Intrastriatal or intracortical infusion of IL-1ra caused no overt lesion as assessed by tetrazolium staining 24h or 48h after infusion, although discrete and localised damage was seen around, and at the tip of the needle tract (not shown). Infusion of IL-1ra alone into either of these areas resulted in no obvious changes in behaviour during the course of the experiment.

Co-infusion of IL-1ra caused a significant reduction in lesion volume (46%) of MGlu-induced striatal damage, compared to vehicle-treated lesions (Vehicle 23.26 \pm 1.19mm³, n=8 versus IL-1ra 12.47 \pm 2.61mm³, n=10, $P < 0.01$, *Figure 4.2.1*). Furthermore, IL-1ra co-infusion also significantly reduced lesion volume (43%) induced by striatal infusion of S-AMPA (Vehicle 12.78 \pm 1.42mm³, n=12 versus IL-1ra 7.26 \pm 1.78mm³, n=12, $P < 0.05$, *Figure 4.2.2*). However, in contrast co-infusion of IL-1ra had no effect on cortical lesion volume induced by cortical infusion of either MGlu (Vehicle 17.68 \pm 1.84mm³, n=9 versus IL-1ra 18.91 \pm 1.41mm³, n=10, NS, *Figure 4.2.3*), or S-AMPA (Vehicle 28.12 \pm 2.80mm³, n=8 versus IL-1ra 25.43 \pm 2.50mm³, n=9, NS, *Figure 4.2.4*).

In summary, IL-1ra reduced NMDA and AMPA receptor-induced lesions within the striatum but not the cortex. In these studies the IL-1ra was co-administered at the site of excitotoxin infusion.

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effective in the striatum
but not in the cortex.
129
What about
extra-striatal lesion with
ACAM

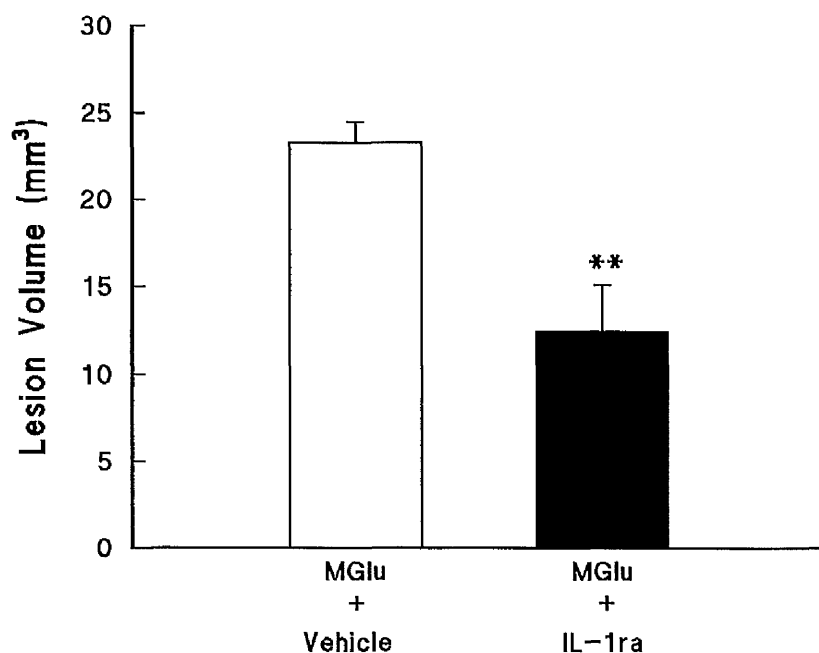
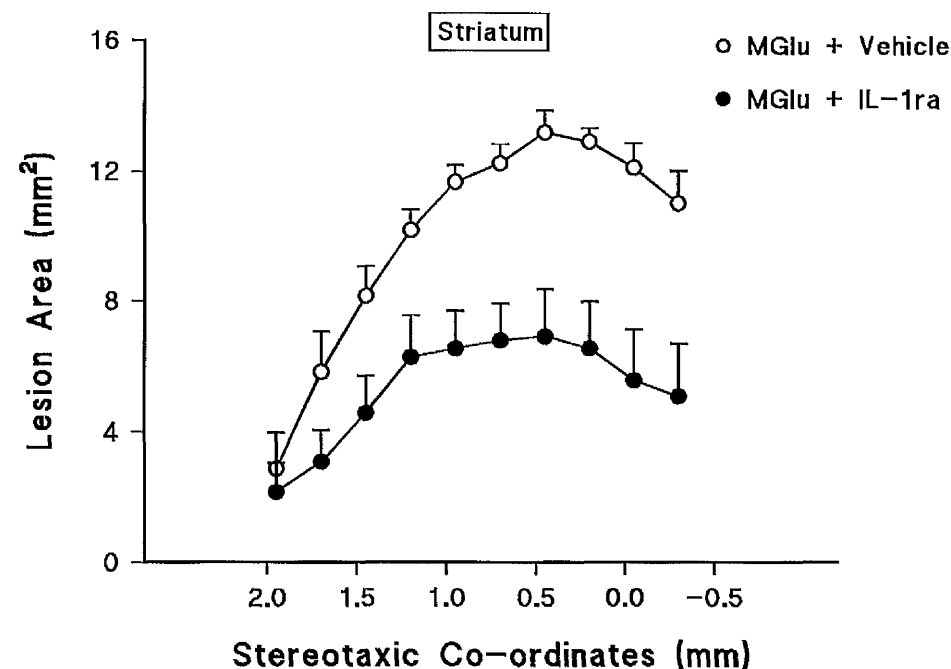


Figure 4.2.1 Effect of striatal infusion of IL-1ra on striatal NMDA receptor-induced damage

Damage was induced by intrastriatal infusion of 10nmol MGlutamate (in 1 μ l). IL-1ra (5 μ g/ μ l, n=10) or vehicle (PBS, 1 μ l, n=8) was co-infused into the striatum with MGlutamate. Striatal neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 250 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test. ** P<0.01 versus vehicle-treated animals.

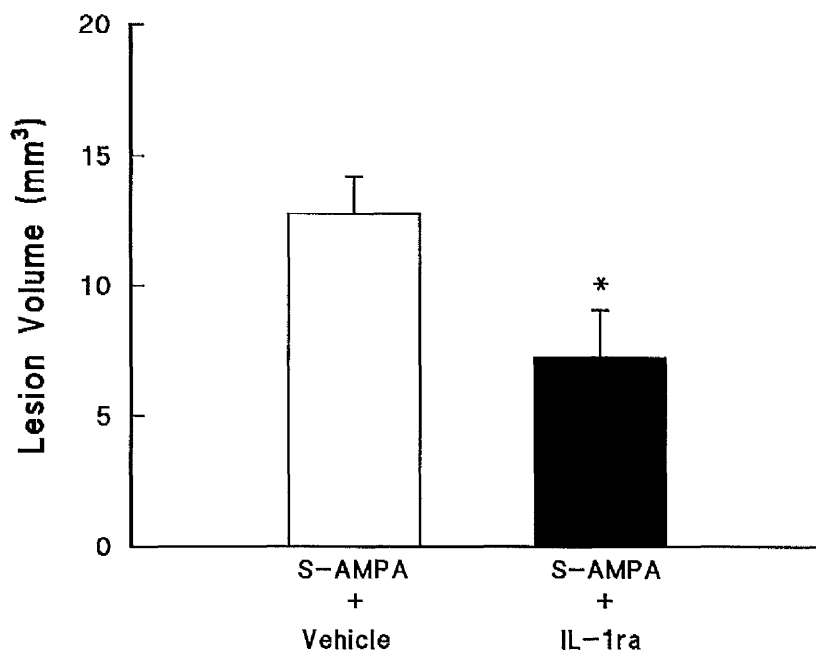
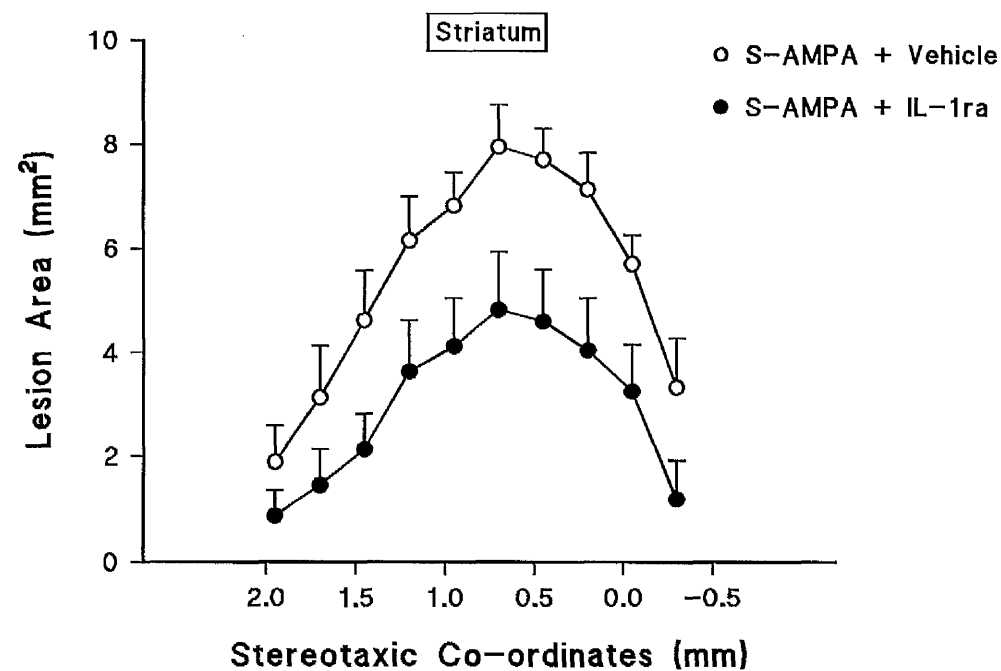


Figure 4.2.2 Effect of striatal infusion of IL-1ra on striatal AMPA receptor-induced damage

Damage was induced by intrastriatal infusion of 15nmol S-AMPA (in 1 μ l). IL-1ra (5 μ g/ μ l, n=12) or vehicle (PBS, 1 μ l, n=12) was co-infused into the striatum with S-AMPA. Striatal neuronal damage was measured 48h later.

The upper graph shows lesion area (mm², on 250 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test. * P<0.05 versus vehicle-treated animals.

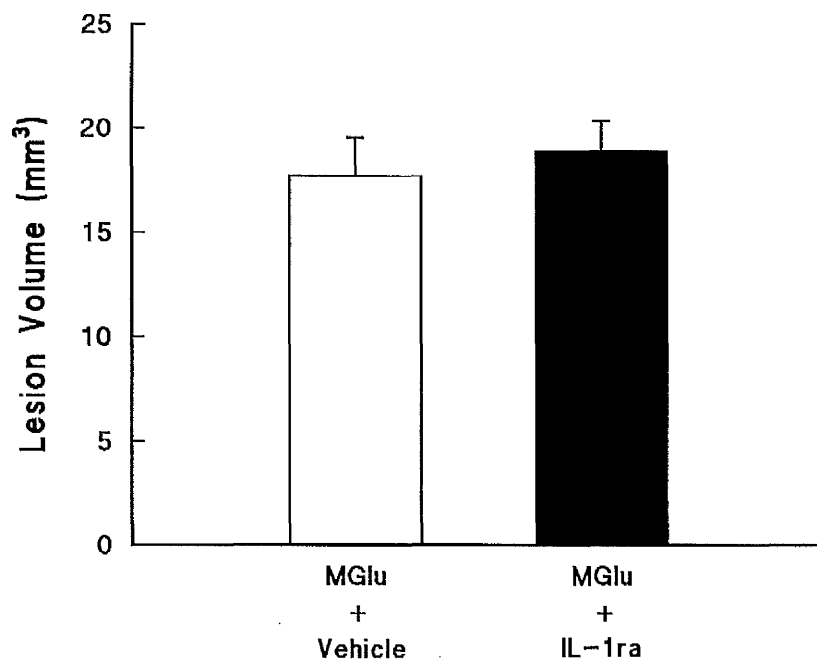
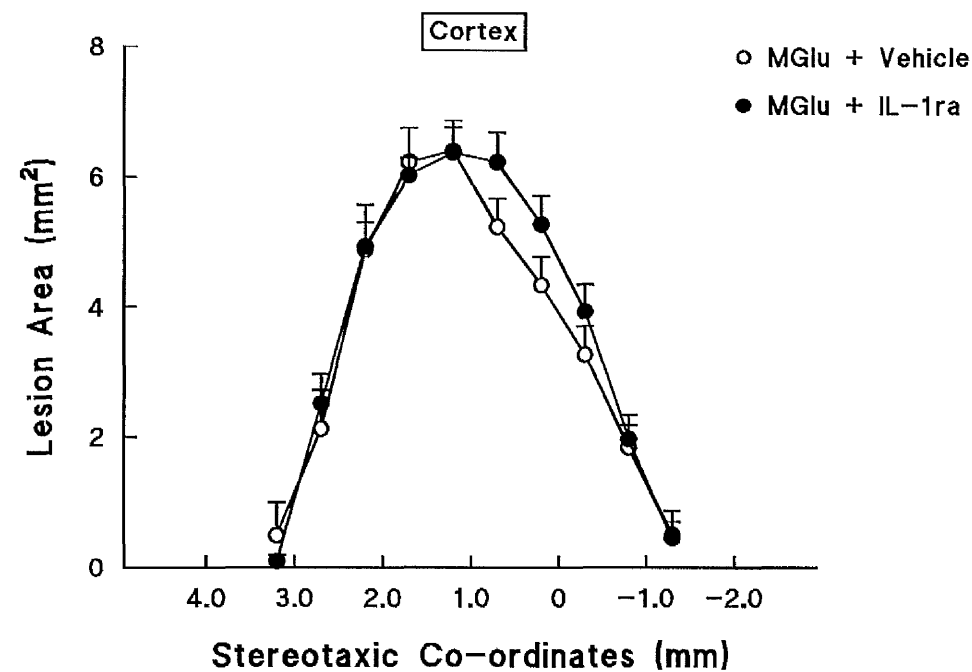


Figure 4.2.3 Effect of cortical infusion of IL-1ra on cortical NMDA receptor-induced damage

Damage was induced by intracortical infusion of 10nmol MGlutamate (in 1 μ l). IL-1ra (5 μ g/ μ l, n=10) or vehicle (PBS, 1 μ l, n=9) was co-infused into the cortex with MGlutamate. Cortical neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 500 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test.

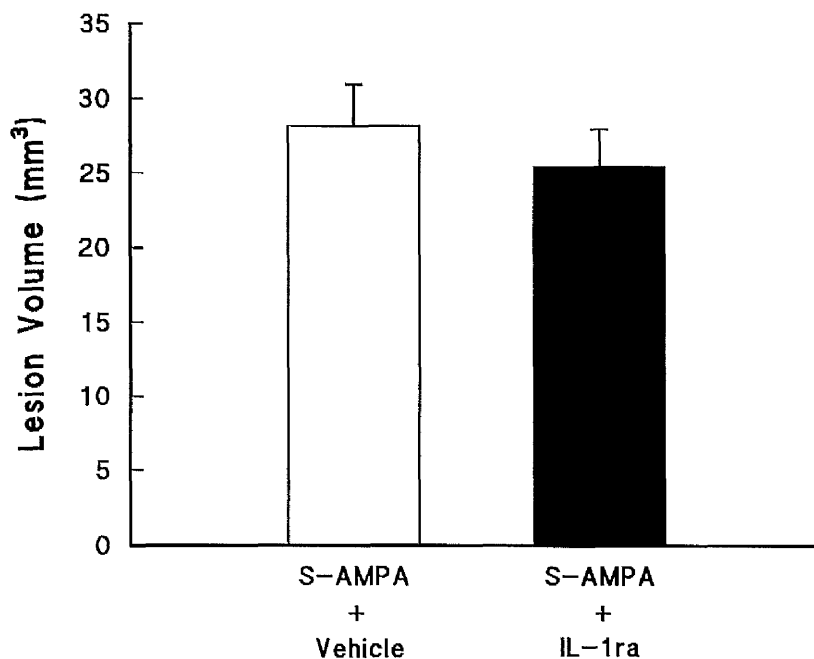
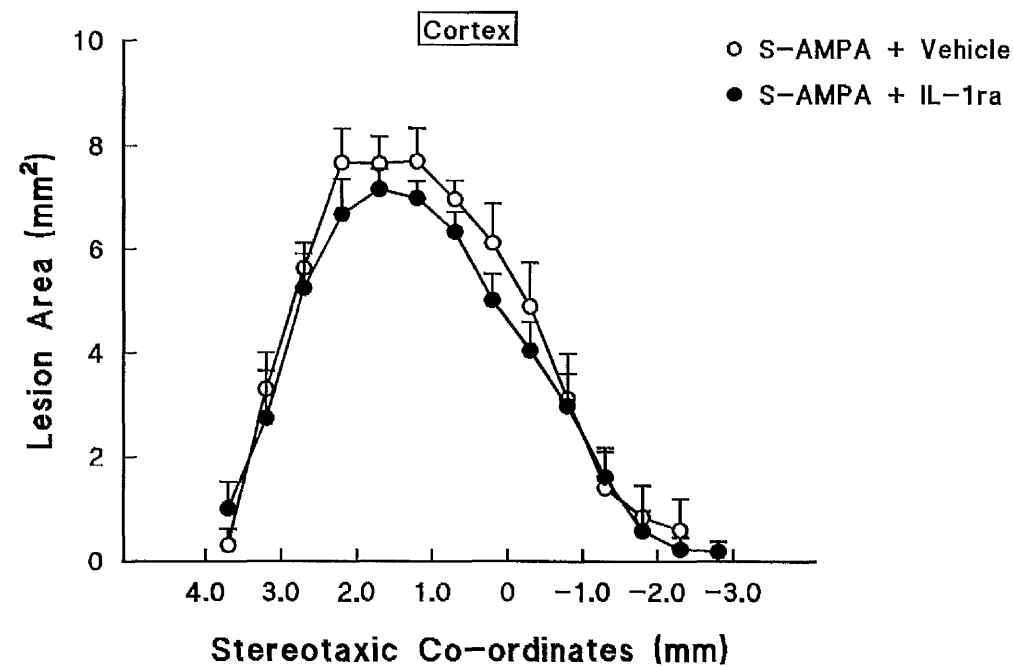


Figure 4.2.4 Effect of cortical infusion of IL-1ra on cortical AMPA receptor-induced damage

Damage was induced by intracortical infusion of 15nmol S-AMPA (in 1 μ l). IL-1ra (5 μ g/ μ l, n=9) or vehicle (PBS, 1 μ l, n=8) was co-infused into the cortex with S-AMPA. Cortical neuronal damage was measured 48h later.

The upper graph shows lesion area (mm², on 500 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test.

4.3 Effect of IL-1 β on EAA receptor-mediated neurotoxicity

The results reported in *Section 4.2.2* on the inhibitory effect of IL-1ra against excitotoxic neuronal injury in the striatum, indicate that IL-1 mediates neuronal death induced by overactivation of EAA receptors. Thus it was proposed that application of exogenous IL-1 may affect, presumably exacerbate, excitotoxic neuronal damage. The aims of this part of the study were first to investigate the effects of IL-1 β on both forms of excitotoxic damage, in the striatum and the cortex and second, to study the possible site(s) of action of IL-1 β .

4.3.1 *Experimental*

4.3.1.1 *Infusion of IL-1 β into the same area as excitotoxin infusion*

Striatal or cortical lesions were performed according to the protocol described in *Section 2.3.1*. Separate experiments were carried out in which human IL-1 β (650IU~5ng in 2 μ l or 1300IU~5-10ng in 2 μ l, n=7-9) or vehicle (saline, 2 μ l, n=6-11) was infused intrastriatally or intracortically immediately after MGlu (7.5nmol) or S-AMPA (10nmol) infusion into the same areas.

4.3.1.2 *Striatal infusion of IL-1 β - Cortical infusion of excitotoxins*

Additional experiments were performed in which 1 μ l of MGlu (7.5nmol) or S-AMPA (10nmol) was infused into the cortex either alone (n=4-6) or immediately followed by infusion of human IL-1 β (1300IU~5-10ng in 2 μ l, n=8-11) or vehicle (saline, 2 μ l, n=9-13) into either the ipsilateral or contralateral striatum.

In both sets of experiments (*Section 4.3.1.1* and *4.3.1.2*), animals were sacrificed (as in *Section 2.4*) 24h or 48h after surgery for MGlu infusion, and 48h

after S-AMPA infusion. Neuronal death was subsequently assessed by the tetrazolium method as in *Section 2.5.1* and *2.6.1*.

4.3.2 Results

Dose of interleukin-1

The dose of IL-1 β is presented as bioactivity, quoted in international units (IU). Due to limited supply, different batches of IL-1 β with different activities were used in this study. Thus, depending on the activity of the IL-1 β batch, a dose of 5-10ng IL-1 β was used to give a total activity of either 650IU or 1300IU.

4.3.2.1 Infusion of IL-1 β into the same area as excitotoxin infusion

In preliminary experiments, co-administration of a lower dose of IL-1 β (650IU~5ng), in the striatum, with MGlu or S-AMPA did not affect striatal damage induced by these agonists (data not shown). Similarly IL-1 β (650IU~5ng) co-infusion in the cortex did not affect cortical lesions induced by either MGlu (Vehicle $14.97 \pm 2.23 \text{ mm}^3$, n=6 versus IL-1 β $17.81 \pm 1.52 \text{ mm}^3$, n=7, NS, *Figure 4.3.1*) or S-AMPA (Vehicle $20.20 \pm 2.48 \text{ mm}^3$, n=6 versus IL-1 β $23.83 \pm 1.95 \text{ mm}^3$, n=9, NS, *Figure 4.3.2*).

A higher dose of IL-1 β (1300IU~5-10ng) co-infused in the striatum also failed to modify the extent of MGlu-induced striatal damage, measured at 24h (Vehicle $5.51 \pm 0.71 \text{ mm}^3$, n=8 versus IL-1 β $6.52 \pm 1.02 \text{ mm}^3$, n=7, NS, *Figure 4.3.3a*) or 48h (Vehicle $6.13 \pm 0.99 \text{ mm}^3$, n=8 versus IL-1 β $4.83 \pm 0.88 \text{ mm}^3$, n=8, NS, *Figure 4.3.3b*) post infusions, and there was no significant difference between lesion volume measured 24h or 48h after insults, in either control- or IL-1 β -treated groups (see *Figure 4.3.3c*). Moreover, MGlu-induced cortical damage was not affected by intracortical co-infusion of 1300IU (~5-10ng) IL-1 β

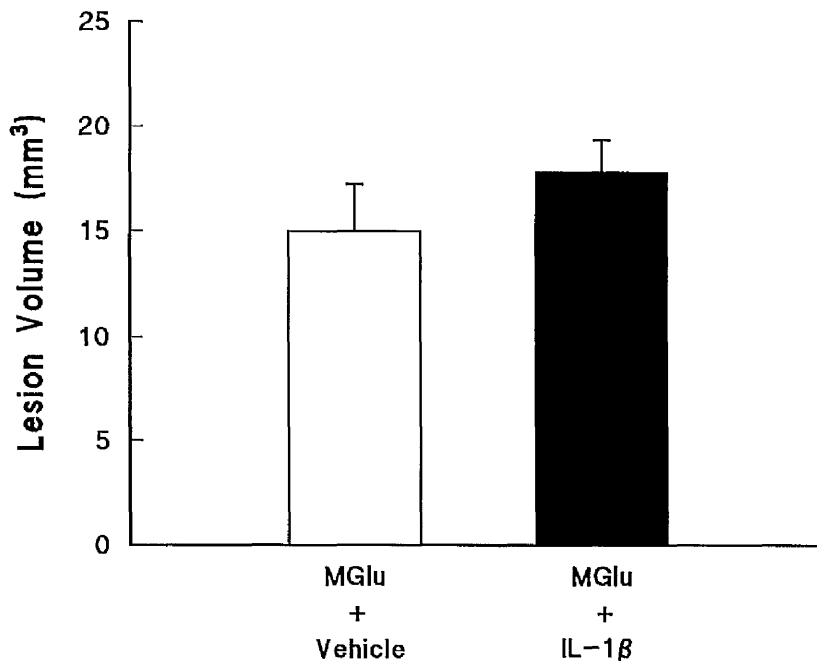
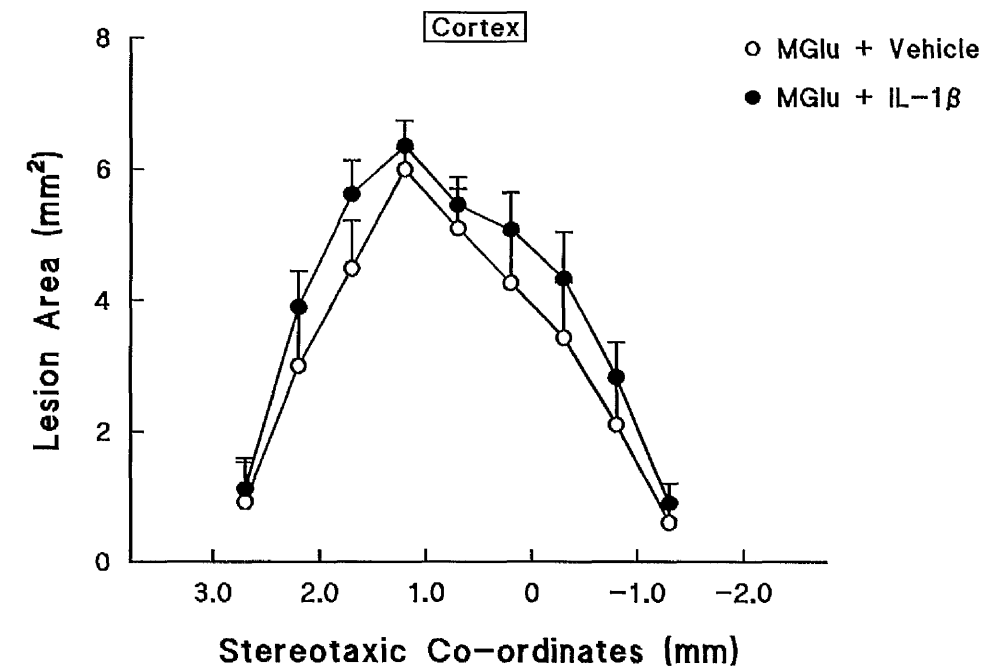


Figure 4.3.1 Effect of cortical infusion of a lower dose of IL-1 β on cortical MGlu receptor-induced damage

Damage was induced by intracortical infusion of 7.5nmol MGlu (in 1 μ l). IL-1 β (650IU~5ng in 2 μ l, n=7) or vehicle (saline, 2 μ l, n=6) was co-infused into the cortex with MGlu. Cortical neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 500 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test.

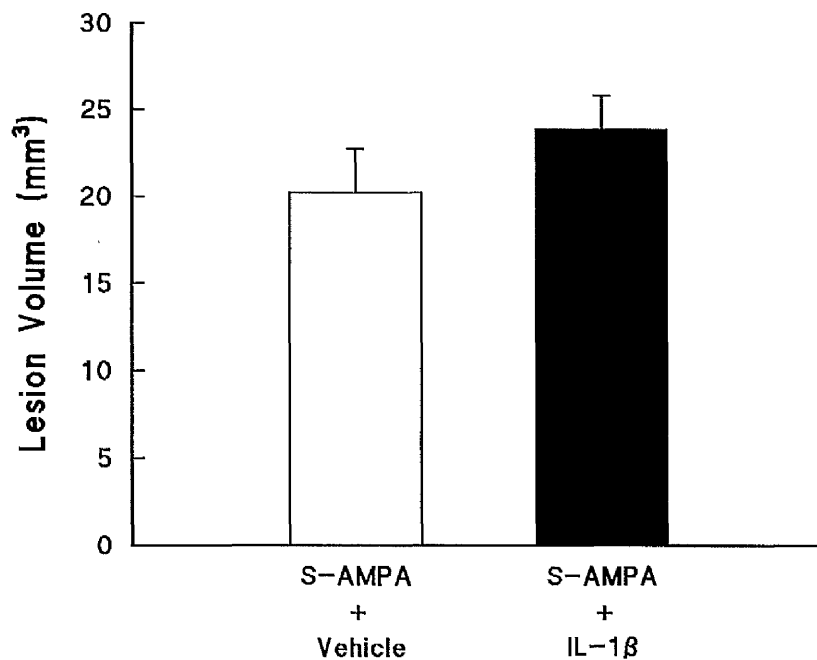
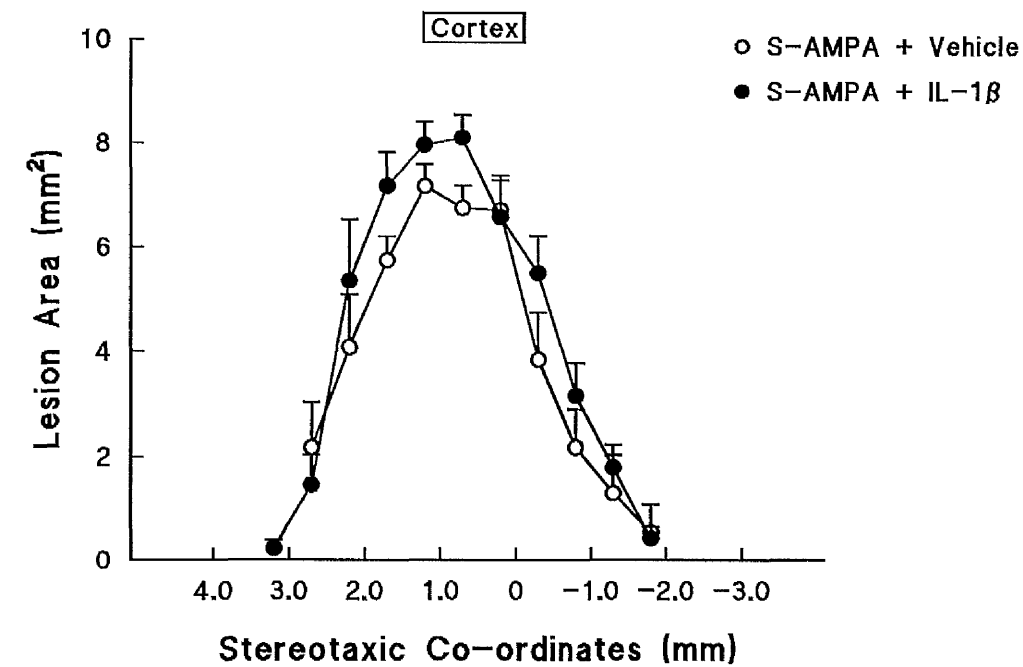


Figure 4.3.2 Effect of cortical infusion of a lower dose of IL-1 β on cortical AMPA receptor-induced damage

Damage was induced by intracortical infusion of 10nmol S-AMPA (in 1 μ l). IL-1 β (650IU~5ng in 2 μ l, n=9) or vehicle (saline, 2 μ l, n=6) was co-infused into the cortex with S-AMPA. Cortical neuronal damage was measured 48h later.

The upper graph shows lesion area (mm², on 500 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test.

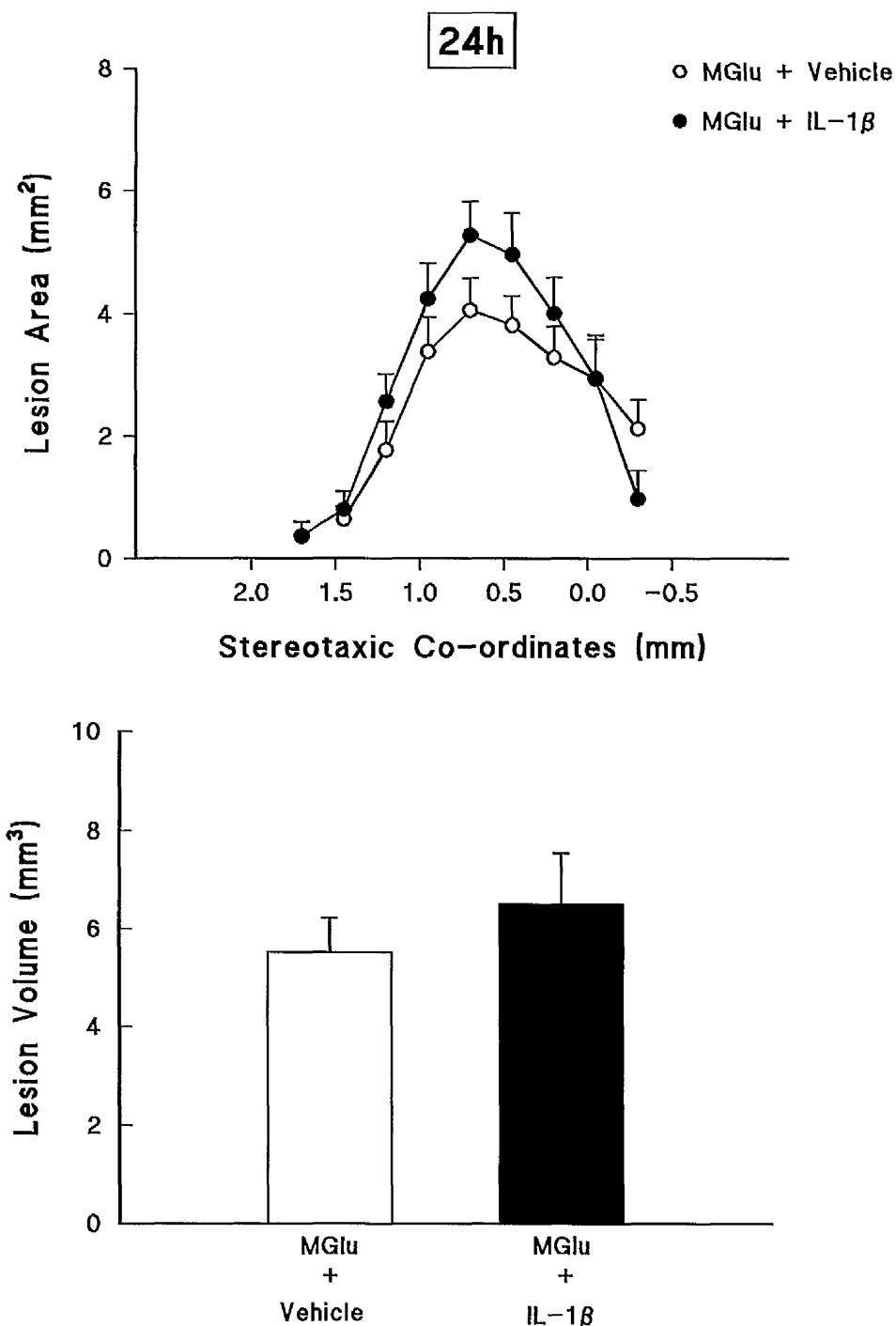


Figure 4.3.3a Effect of striatal infusion of IL-1 β on striatal NMDA receptor-induced damage at 24h

Damage was induced by intrastriatal infusion of 7.5nmol MGlu (in 1 μ l). IL-1 β (1300IU~5-10ng in 2 μ l, n=7) or vehicle (saline, 2 μ l, n=8) was co-infused into the striatum with MGlu. Striatal neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 250 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test.

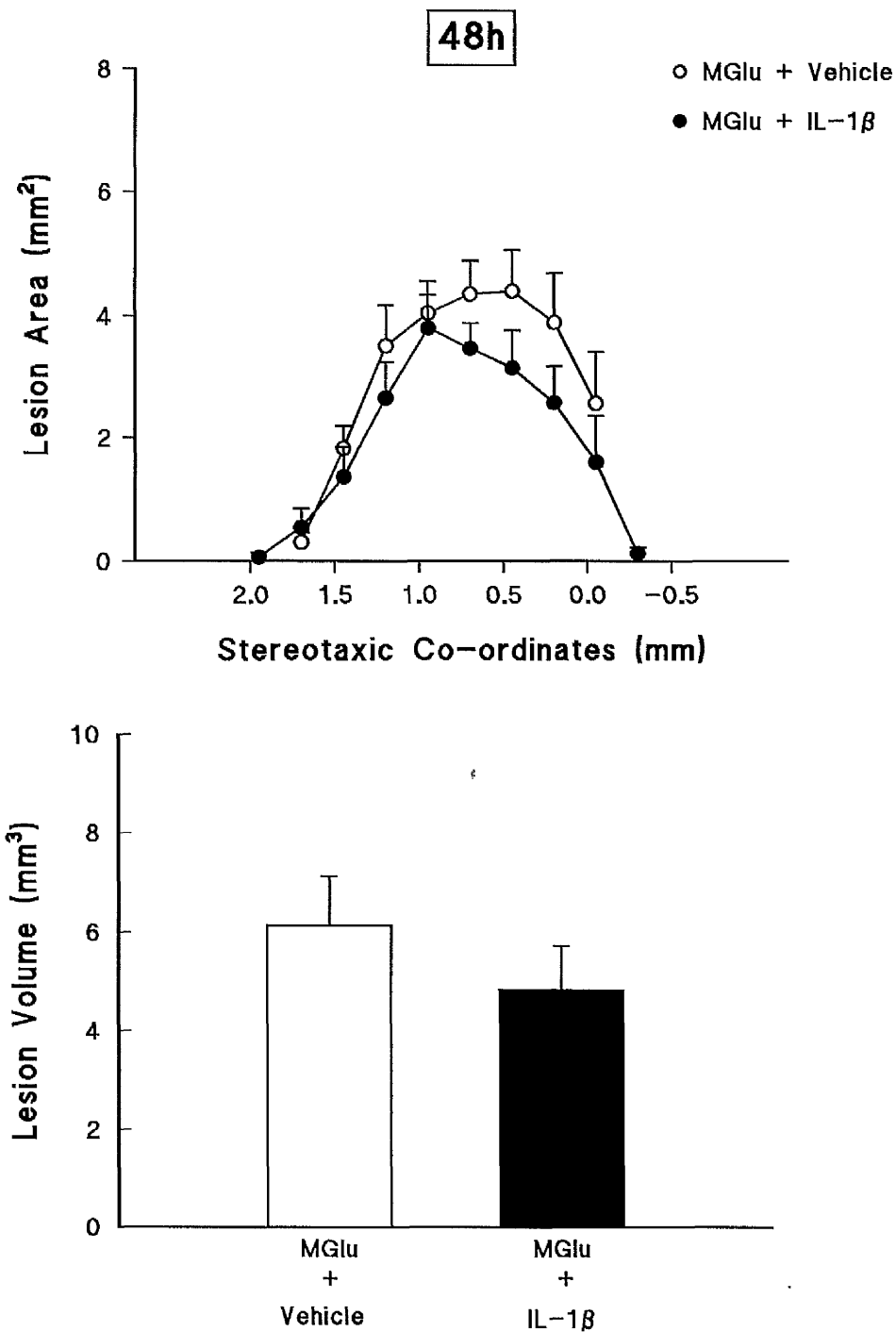


Figure 4.3.3b Effect of striatal infusion of IL-1 β on striatal NMDA receptor-induced damage at 48h

Damage was induced by intrastriatal infusion of 7.5nmol MGlut (in 1 μ l). IL-1 β (1300IU~5-10ng in 2 μ l, n=8) or vehicle (saline, 2 μ l, n=8) was co-infused into the striatum with MGlut. Striatal neuronal damage was measured 48h later.

The upper graph shows lesion area (mm², on 250 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test.

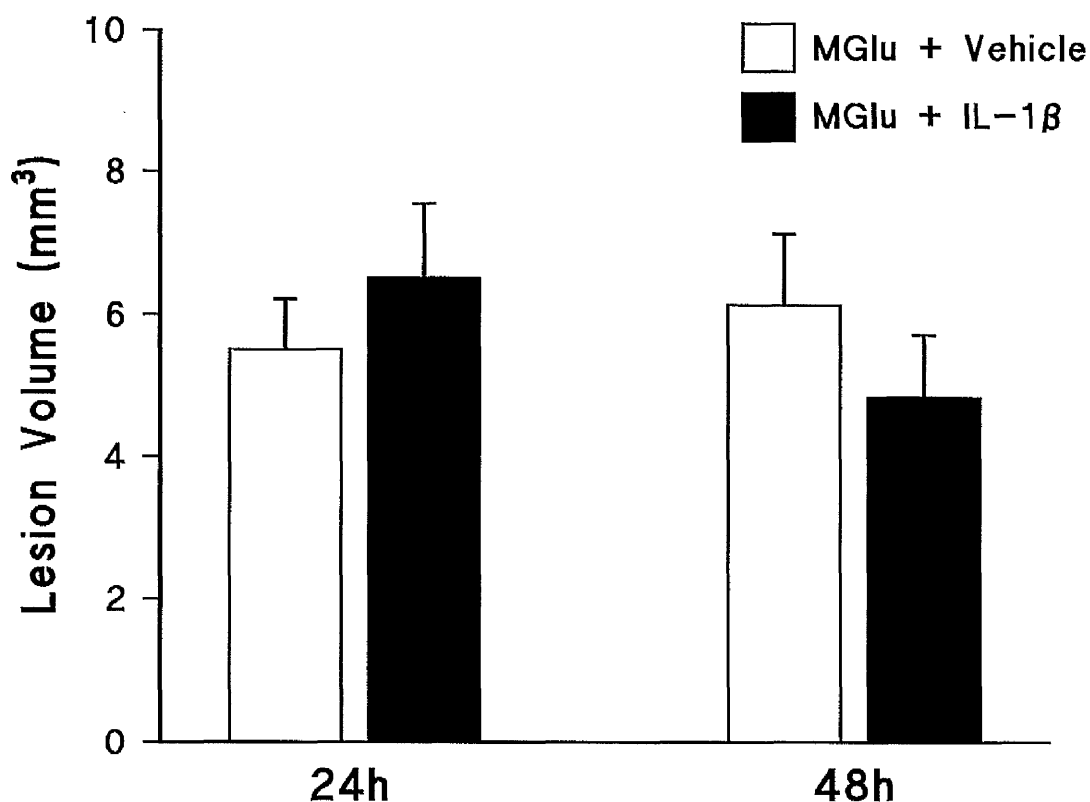


Figure 4.3.3c Effect of striatal infusion of IL-1 β on striatal NMDA receptor-induced damage---Comparison at 24h versus 48h

Damage was induced by intrastratial infusion of 7.5nmol MGlutamate (in 1 μ l). IL-1 β (1300IU~5-10ng in 2 μ l, n=7-8, solid bars) or vehicle (saline, 2 μ l, n=8, open bars) was co-infused into the striatum with MGlutamate. Striatal neuronal damage was measured 24h (two bars on the left) or 48h (two bars on the right) later. Lesion volume is in mm³.

Data are expressed as mean \pm SEM. Statistical analyses were performed using a Student's t-test.

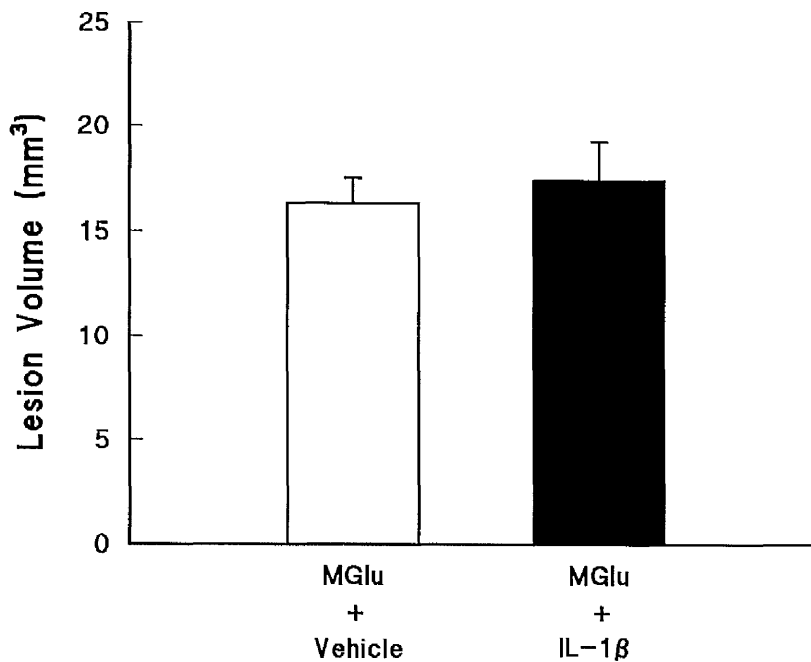
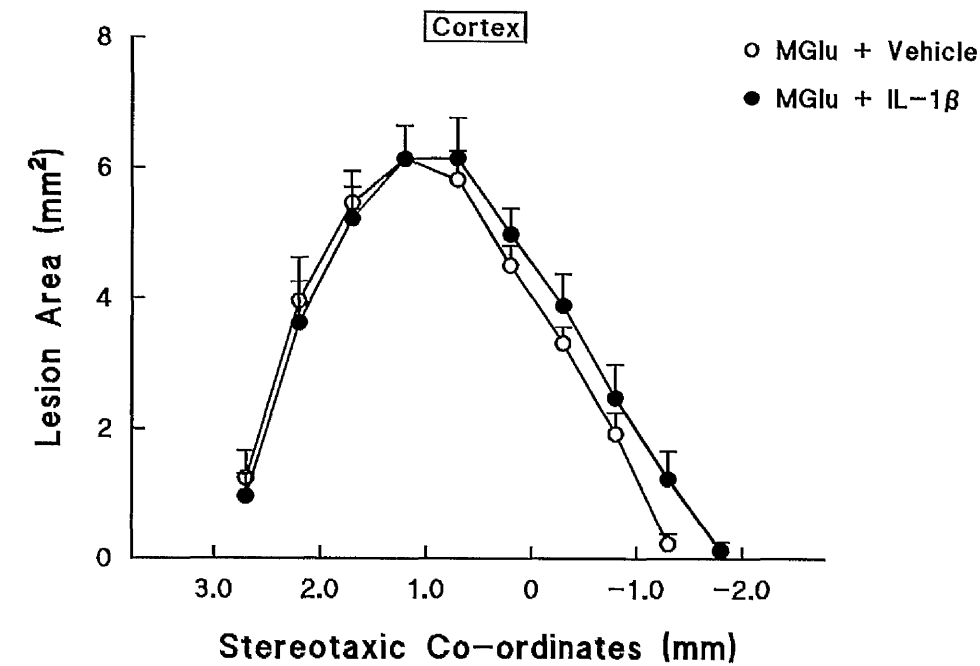


Figure 4.3.4 Effect of cortical infusion of a higher dose of IL-1 β on cortical MGlutamate receptor-induced damage

Damage was induced by intracortical infusion of 7.5nmol MGlutamate (in 1 μ l). IL-1 β (1300IU~5-10ng in 2 μ l, n=9) or vehicle (saline, 2 μ l, n=9) was co-infused into the cortex with MGlutamate. Cortical neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 500 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test.

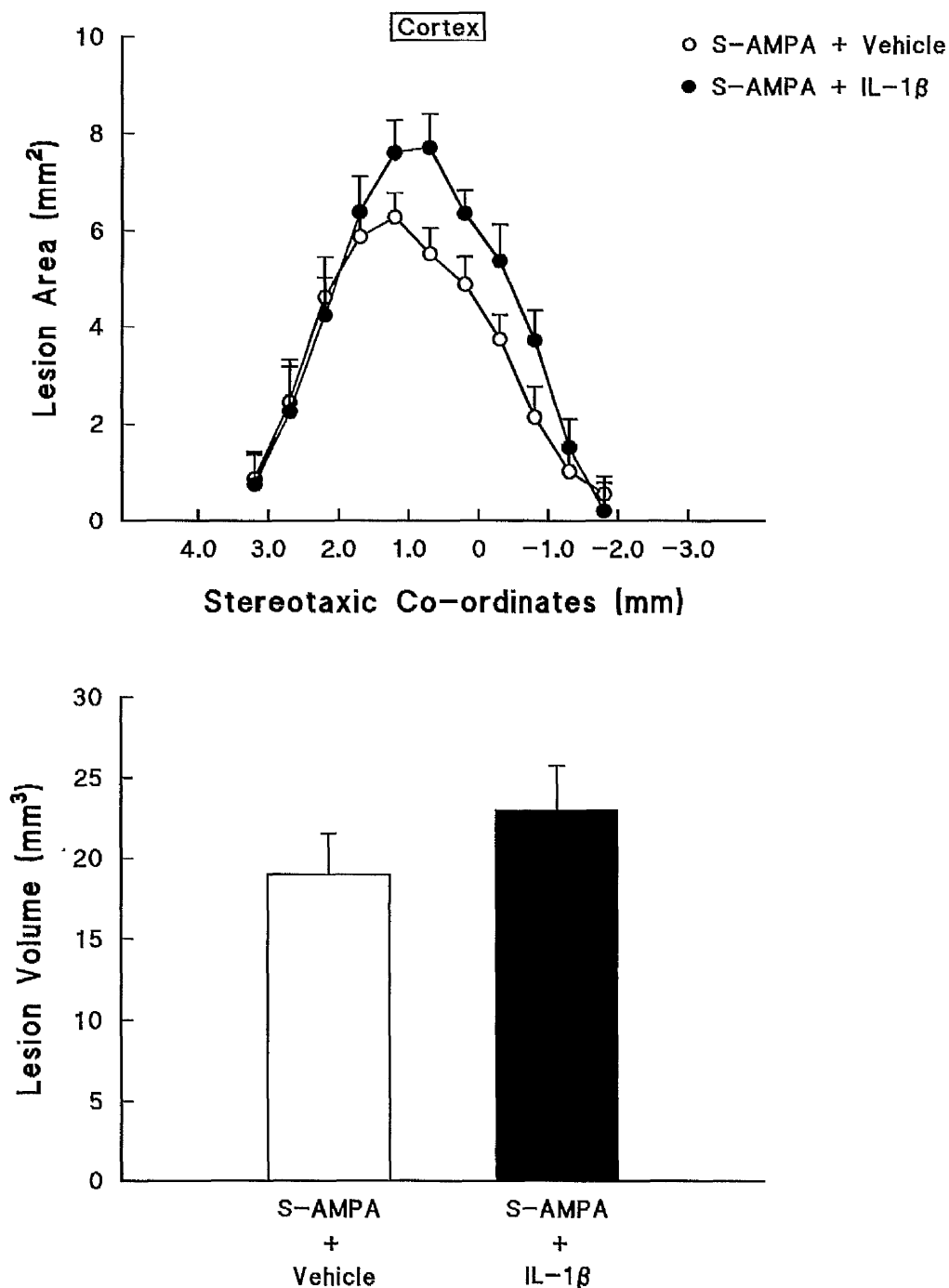


Figure 4.3.5 Effect of cortical infusion of a higher dose of IL-1 β on cortical AMPA receptor-induced damage

Damage was induced by intracortical infusion of 10nmol S-AMPA (in 1 μ l). IL-1 β (1300IU~5-10ng in 2 μ l, n=8) or vehicle (saline, 2 μ l, n=11) was co-infused into the cortex with S-AMPA. Cortical neuronal damage was measured 48h later.

The upper graph shows lesion area (mm², on 500 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test.

(Vehicle $16.30 \pm 1.18 \text{ mm}^3$, $n=9$ versus IL-1 β $17.40 \pm 1.83 \text{ mm}^3$, $n=9$, NS, *Figure 4.3.4*). Local damage was not affected by IL-1 β (1300IU~5-10ng) co-infusion with S-AMPA in either the cortex (Vehicle $18.98 \pm 2.55 \text{ mm}^3$, $n=11$ versus IL-1 β $22.98 \pm 2.77 \text{ mm}^3$, $n=8$, NS, *Figure 4.3.5*) or the striatum (Vehicle $11.15 \pm 0.66 \text{ mm}^3$, $n=10$ versus IL-1 β $11.77 \pm 0.95 \text{ mm}^3$, $n=7$, NS, *Figure 4.3.6a*). However, when IL-1 β (1300IU~5-10ng) was co-infused with S-AMPA into the striatum, an extensive lesion was observed in the ipsilateral cortex (see *Figure 4.3.6a-c*) whilst the striatal damage remained unchanged (*Figure 4.3.6a*). In some cases, this extrastriatal lesion extended over the entire ipsilateral cortex, involving most cortical regions (i.e. frontal, parietal, insular, piriform, and orbital cortices), although the cingulate cortex was consistently unaffected. This cortical damage was significantly larger (1320%) than any cortical neurodegeneration observed after intrastriatal infusion of S-AMPA and vehicle (Vehicle $7.59 \pm 1.17 \text{ mm}^3$, $n=10$ versus IL-1 β $99.30 \pm 17.24 \text{ mm}^3$, $n=7$, $P < 0.01$, *Figure 4.3.6a* and *Table 4.3.6*), but was not present after intrastriatal co-infusion of 650IU (~5ng) IL-1 β and S-AMPA. It should be noted that slices were taken at $500 \mu\text{m}$, as it was impossible to obtain intact thinner sections ($250 \mu\text{m}$, normally taken for striatal infusions) because of the extensive cortical damage.

As discussed previously (in *Section 3.5.2*), minor extrastriatal damage was observed in response to striatal administration of S-AMPA (without IL-1 β) in the immediate overlying ipsilateral cortex and, in approximately 50% of cases, neuronal damage was seen in the ipsilateral piriform cortex. However, these regions were the only cortical areas to be affected, and no widespread cortical damage was observed in the studies presented here in animals treated with 10nmol of S-AMPA and vehicle (see *Figure 4.3.6b* and *4.3.6c*). Damage to the piriform cortex was detected in some animals (4/10=40%) infused intrastriatally with S-AMPA and vehicle, and those treated with IL-1 β showed a similar incidence (4/7=57%) of cortical damage extending into the piriform cortex.

Figure 4.3.6a

Effect of striatal infusion of IL-1 β on striatal and cortical damage induced by striatal AMPA receptor overactivation

Damage was induced by intrastriatal infusion of 10nmol S-AMPA (in 1 μ l). IL-1 β (1300IU~5-10ng in 2 μ l, n=7) or vehicle (saline, 2 μ l, n=10) were co-infused into the striatum with S-AMPA. Striatal (illustrated in the left graphs) and cortical (illustrated in the right graphs) neuronal damage were measured in the same animals 48h later. The upper graphs show lesion areas (mm², on 500 μ m sections), which were integrated to give lesion volumes (mm³), shown in the lower graphs.

Data are expressed as mean \pm SEM. Statistical analyses were performed using a Student's t-test. **P<0.01 versus vehicle-treated animals.

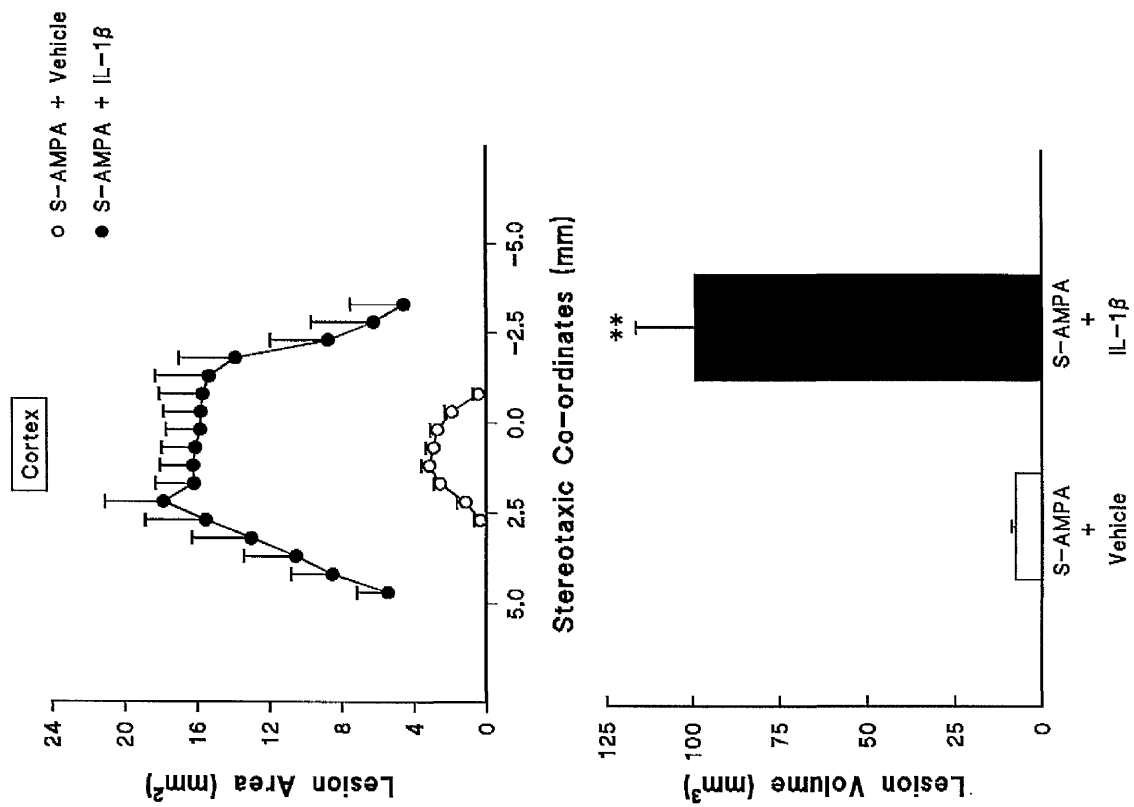
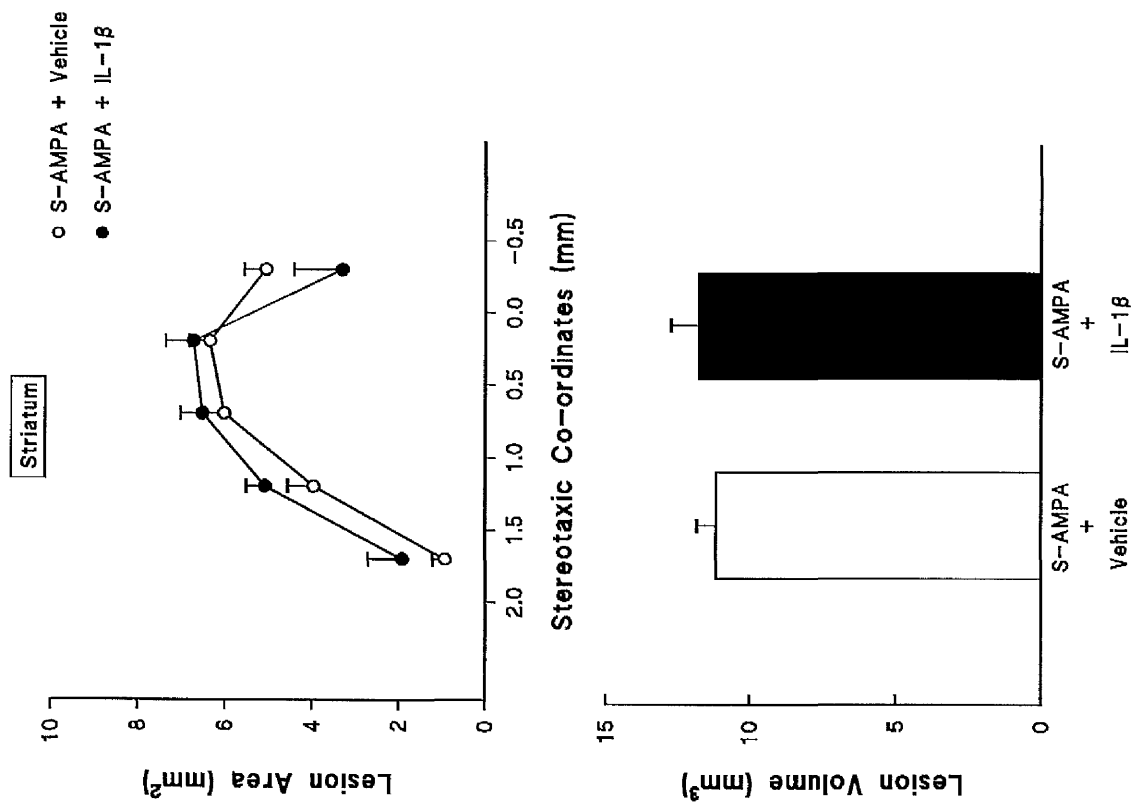


Table 4.3.6

Summary of lesion volumes (mm³) in the striatum and the cortex after striatal infusion of S-AMPA (10nmol) and **A**) vehicle (saline, 2μl) or **B**) IL-1β (1300IU~5-10ng in 2μl).

The presence of piriform cortex damage is noted:

- + Damage present.
- Damage absent.

A)

Striatal S-AMPA (10nmol) + Striatal Saline (2 μ l)		
Striatal lesion volume	Cortical lesion volume	Piriform cortex damage
12.33	6.50	+
10.97	13.64	+
10.02	7.29	+
9.32	2.09	-
9.83	5.31	-
8.21	9.27	-
15.21	13.46	+
13.61	7.25	-
10.75	6.78	-
11.27	4.29	-
Mean \pm SEM 11.15 \pm 0.66	Mean \pm SEM 7.59 \pm 1.17	4/10=40%

B)

Striatal S-AMPA (10nmol) + Striatal IL-1 β (1300IU)		
Striatal lesion volume	Cortical lesion volume	Piriform cortex damage
15.25	60.51	-
7.89	145.86	+
13.20	41.97	-
9.71	81.26	-
10.99	161.60	+
13.65	107.50	+
11.67	156.20	+
Mean \pm SEM 11.77 \pm 0.95	Mean \pm SEM 99.30 \pm 17.24	4/7=57%

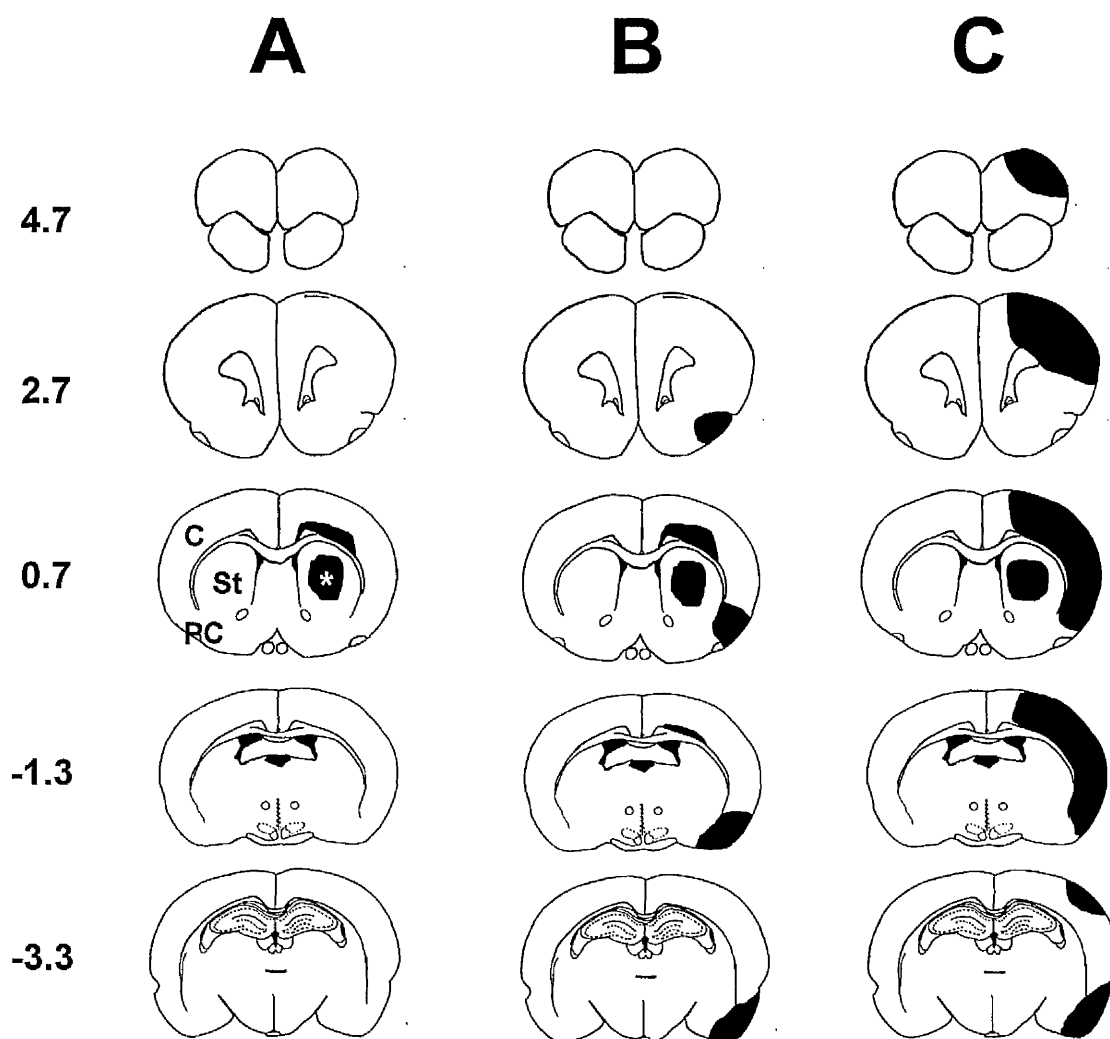


Figure 4.3.6b Local and distant neuronal loss after intrastratial infusion of S-AMPA and IL-1 β

Drawings of coronal brain sections illustrating the characteristic extent of neuronal loss (shaded area) in the striatum (St) and cortex (C) after intrastratial infusion of S-AMPA with or without IL-1 β . Neuronal damage in the striatum and overlying cortex in response to intrastratial infusion of S-AMPA (10nmol) and vehicle (2 μ l saline) is shown in **A**. Panel **B** illustrates additional damage in the piriform cortex (PC) which is sometimes observed after intrastratial infusion of S-AMPA (10nmol) and vehicle (2 μ l saline). Cortical neuronal loss in response to intrastratial infusion of S-AMPA (10nmol) and IL-1 β (1300IU~5-10ng) is shown in **C**. Rostro-caudal co-ordinates (4.7 to -3.3) are indicated in relation to bregma. * indicates the site of injection.

A)



B)

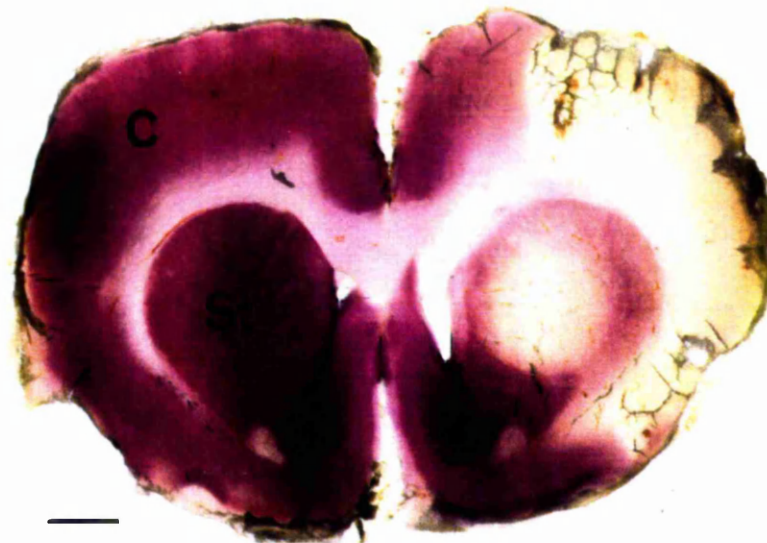


Figure 4.3.6.c Tetrazolium staining to show the effect of striatal infusion of IL-1 β on neuronal damage 48h after striatal infusion of S-AMPA

Coronal brain sections (500 μ m) stained with tetrazolium to illustrate the effect of IL-1 β on neuronal damage after striatal infusion of S-AMPA. The white regions are non-viable neuronal tissue and the pink regions represent viable tissue. The upper panel **A**) shows the striatal damage (white area) produced after striatal injection of S-AMPA (10nmol) and saline (taken just posterior to the site of injection at co-ordinate 0.2, in relation to bregma). The lower panel **B**) shows the striatal and cortical damage (white areas) produced after striatal injection of S-AMPA (10nmol) and IL-1 β (1300IU~5-10ng, taken just anterior to the site of injection at co-ordinate 1.2, in relation to bregma). Note the extensive region of the cortex which is damaged after treatment with IL-1 β . C, Cortex; St, Striatum. Magnification bar=1.4mm

Table 4.3.6 shows the occurrence of piriform cortex damage and illustrates that the extensive cortical lesion formed after IL-1 β co-infusion (with S-AMPA) into the striatum, does not always extend into this structure.

In summary, intracortical co-infusion of IL-1 β did not affect cortical neuronal damage induced by administration of an NMDA or AMPA receptor agonist into the cortex. Similarly, co-infusion of IL-1 β into the striatum did not affect local neuronal damage induced by striatal infusion of an NMDA or AMPA receptor agonist. However, extensive cortical neuronal damage was observed 48h after intrastriatal infusion of S-AMPA and IL-1 β .

4.3.2.2 *Striatal infusion of IL-1 β - Cortical infusion of excitotoxins*

4.3.2.2a Ipsilateral striatal infusion

Infusion of vehicle (2 μ l saline) into the ipsilateral striatum immediately after intracortical infusion of MGlu (7.5nmol) resulted in a significant increase (49%) in cortical lesion volume compared to cortical damage observed after intracortical infusion of MGlu (7.5mol) alone (No striatal infusion 11.44 \pm 1.70mm³, n=6 versus Striatal infusion of vehicle 17.06 \pm 1.12mm³, n=13, P<0.05, *Figure 4.3.7*). Ipsilateral intrastriatal infusion of IL-1 β (1300IU~5-10ng in 2 μ l) immediately after intracortical infusion of MGlu (7.5nmol) significantly increased (261%) cortical neuronal damage compared to that after intracortical infusion of MGlu (7.5nmol) followed by ipsilateral intrastriatal infusion of vehicle (Striatal infusion of vehicle 17.06 \pm 1.12mm³, n=13 versus Striatal infusion of IL-1 β 61.57 \pm 10.43mm³, n=10, P<0.01, *Figure 4.3.7*).

When vehicle (2 μ l saline) was infused into the ipsilateral striatum immediately after intracortical infusion of S-AMPA (10nmol) no change was observed in

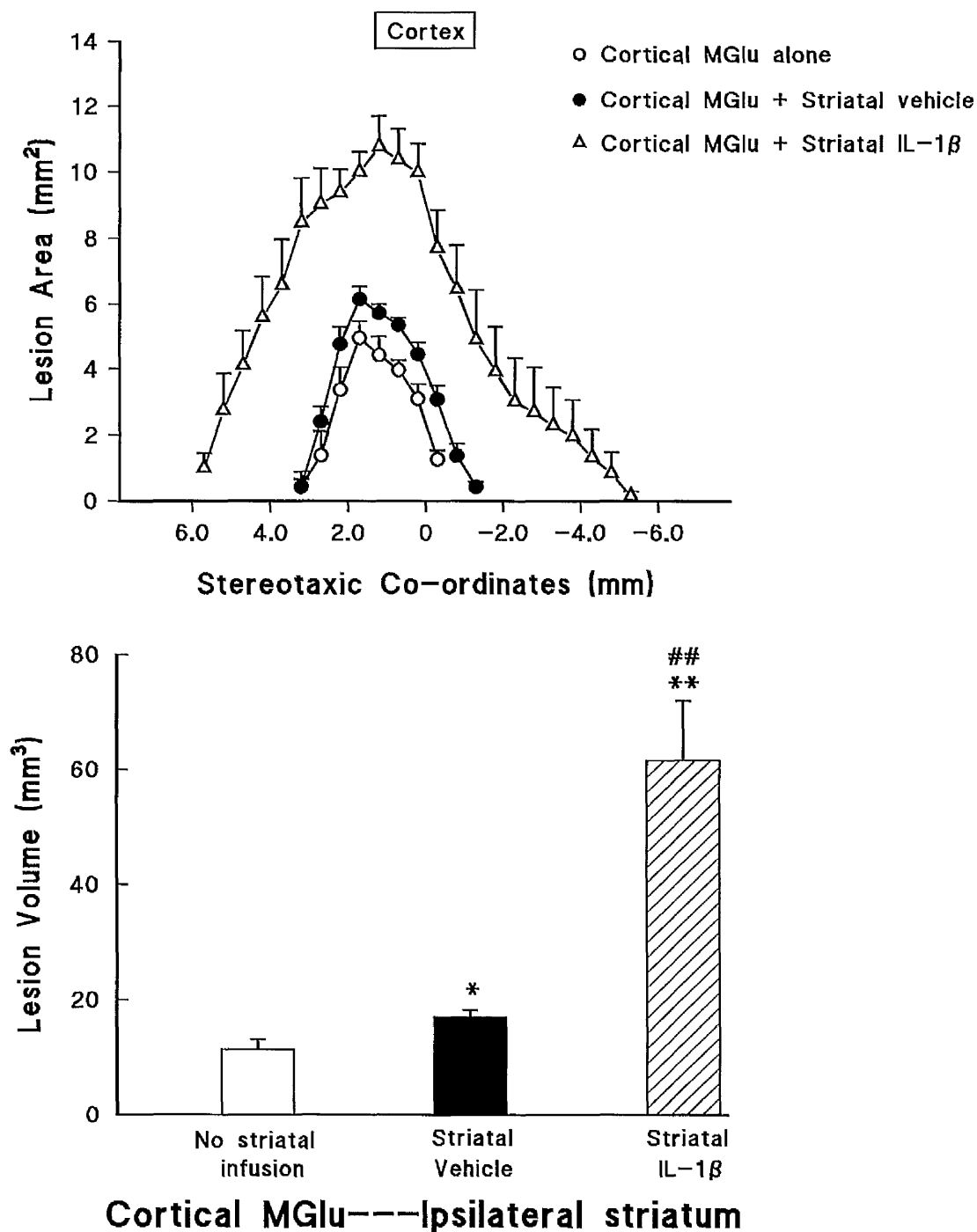


Figure 4.3.7 Effect of ipsilateral striatal infusion of IL-1 β on cortical MGLu receptor-induced damage

Damage was induced by intracortical infusion of 7.5nmol MGLu (in 1 μ l). MGLu (7.5nmol, n=6) was infused alone into the cortex or followed immediately by ipsilateral intrastriatal infusion of IL-1 β (1300IU~5-10ng in 2 μ l, n=10) or vehicle (saline, 2 μ l, n=13). Cortical neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 500 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analyses were performed using a Student's t-test. * P<0.05 versus no striatal infusion, ** P<0.01 versus no striatal infusion, ## P<0.01 versus striatal infusion of vehicle.

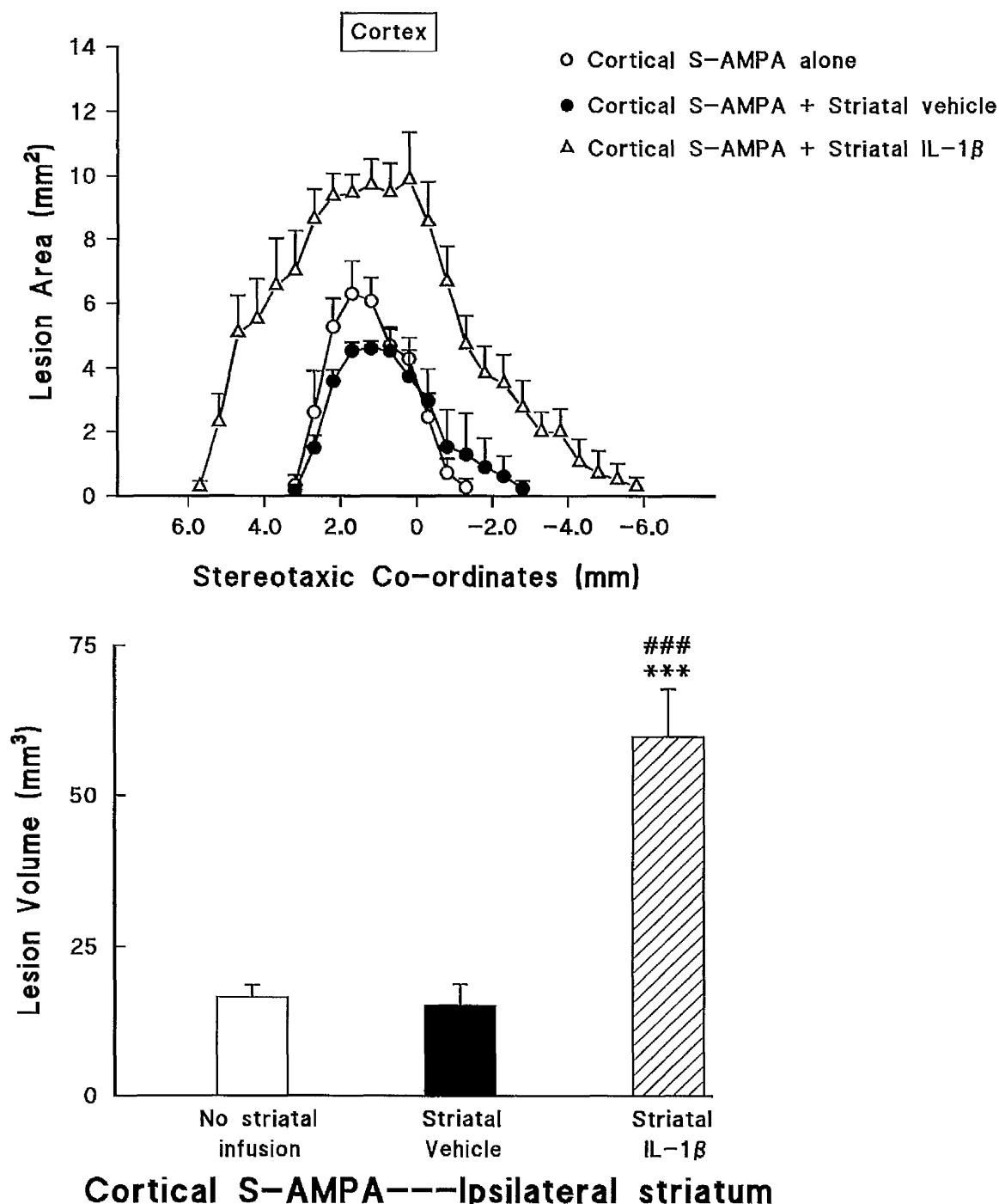


Figure 4.3.8 Effect of ipsilateral striatal infusion of IL-1 β on cortical S-AMPA receptor-induced damage

Damage was induced by intracortical infusion of 10nmol S-AMPA (in 1 μ l). S-AMPA (10nmol, n=4) was infused alone into the cortex or followed immediately by ipsilateral intrastriatal infusion of IL-1 β (1300IU~5-10ng in 2 μ l, n=9) or vehicle (saline, 2 μ l, n=9). Cortical neuronal damage was measured 48h later.

The upper graph shows lesion area (mm², on 500 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analyses were performed using a Student's t-test. *** P<0.001 versus no striatal infusion, ### P<0.001 versus striatal infusion of vehicle.

cortical lesion volume compared that seen after intracortical infusion of S-AMPA (10nmol) alone (No striatal infusion $16.51 \pm 2.01 \text{ mm}^3$, $n=4$ versus Striatal infusion of vehicle $15.12 \pm 3.50 \text{ mm}^3$, $n=9$, NS, *Figure 4.3.8*). In contrast, administration of IL-1 β (1300IU~5-10ng), compared to vehicle, into the ipsilateral striatum markedly enhanced (295%) cortical neuronal damage caused by cortical application of 10nmol S-AMPA (Striatal infusion of vehicle $15.12 \pm 3.50 \text{ mm}^3$, $n=9$ versus Striatal infusion of IL-1 β $59.79 \pm 7.99 \text{ mm}^3$, $n=9$, $P<0.001$, *Figure 4.3.8*).

4.3.2.2b Contralateral striatal infusion

Infusion of vehicle (2 μ l saline) into the contralateral striatum immediately after intracortical infusion of MGlu (7.5nmol) or S-AMPA (10nmol) did not affect the cortical neuronal damage compared to that observed after intracortical infusion of 7.5nmol MGlu (No striatal infusion $13.68 \pm 1.67 \text{ mm}^3$, $n=5$ versus Striatal infusion of vehicle $13.63 \pm 1.01 \text{ mm}^3$, $n=9$, NS, *Figure 4.3.9*) or 10nmol S-AMPA (No striatal infusion $21.60 \pm 1.35 \text{ mm}^3$, $n=6$ versus Striatal infusion of vehicle $22.88 \pm 0.88 \text{ mm}^3$, $n=10$, NS, *Figure 4.3.10*) alone.

In contrast, contralateral intrastriatal infusion of IL-1 β (1300IU~5-10ng in 2 μ l) immediately after intracortical infusion of MGlu (7.5nmol) or S-AMPA (10nmol) significantly increased (100-278%) cortical neuronal damage compared to that after intracortical infusion of MGlu (7.5nmol) followed by contralateral striatal infusion of vehicle (Striatal infusion of vehicle $13.63 \pm 1.01 \text{ mm}^3$, $n=9$ versus Striatal infusion of IL-1 β $51.64 \pm 9.68 \text{ mm}^3$, $n=8$, $P<0.01$, *Figure 4.3.9*), or S-AMPA (10nmol) followed by ipsilateral intrastriatal infusion of vehicle (Striatal infusion of vehicle $22.88 \pm 0.88 \text{ mm}^3$, $n=10$ versus striatal infusion of IL-1 β $45.69 \pm 5.75 \text{ mm}^3$, $n=11$, $P<0.01$, *Figure 4.3.10*).

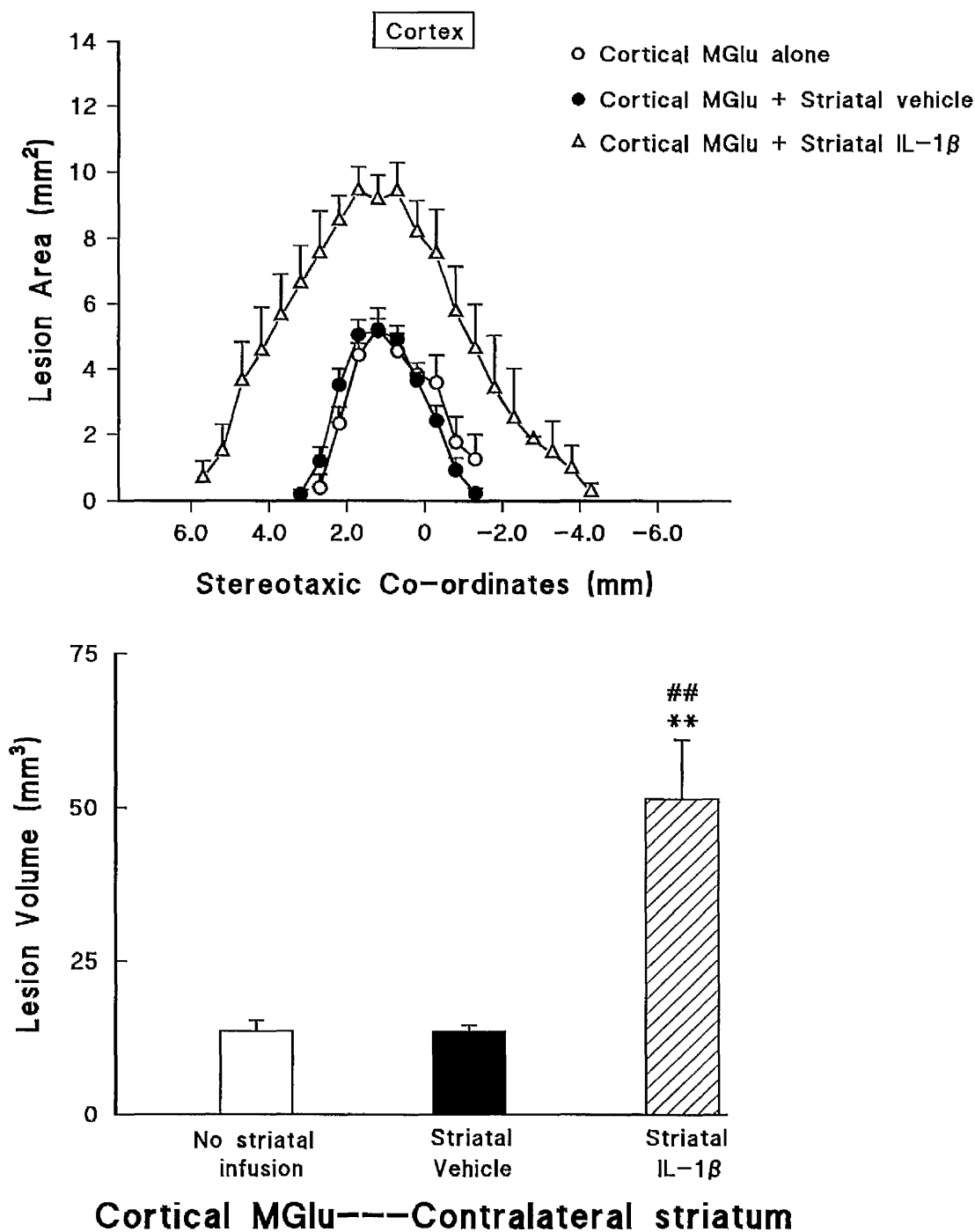
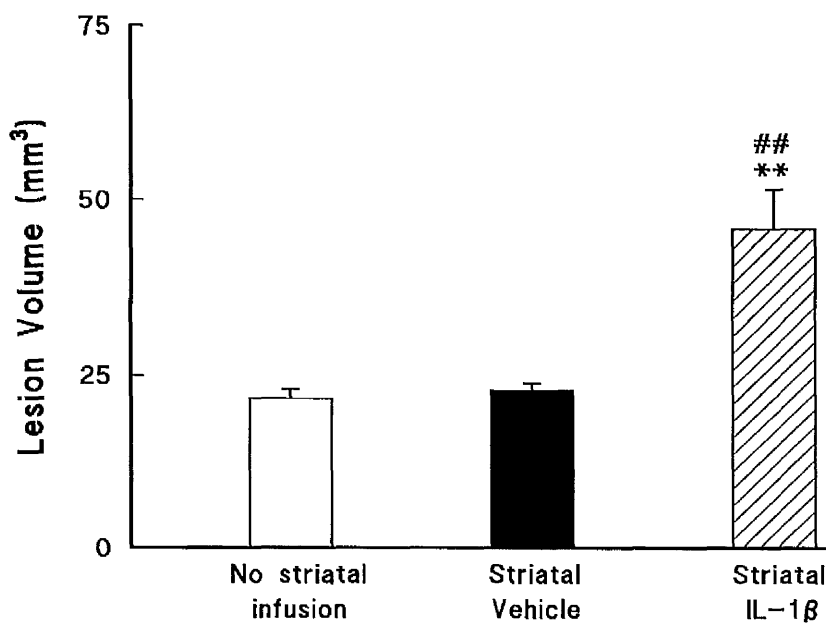
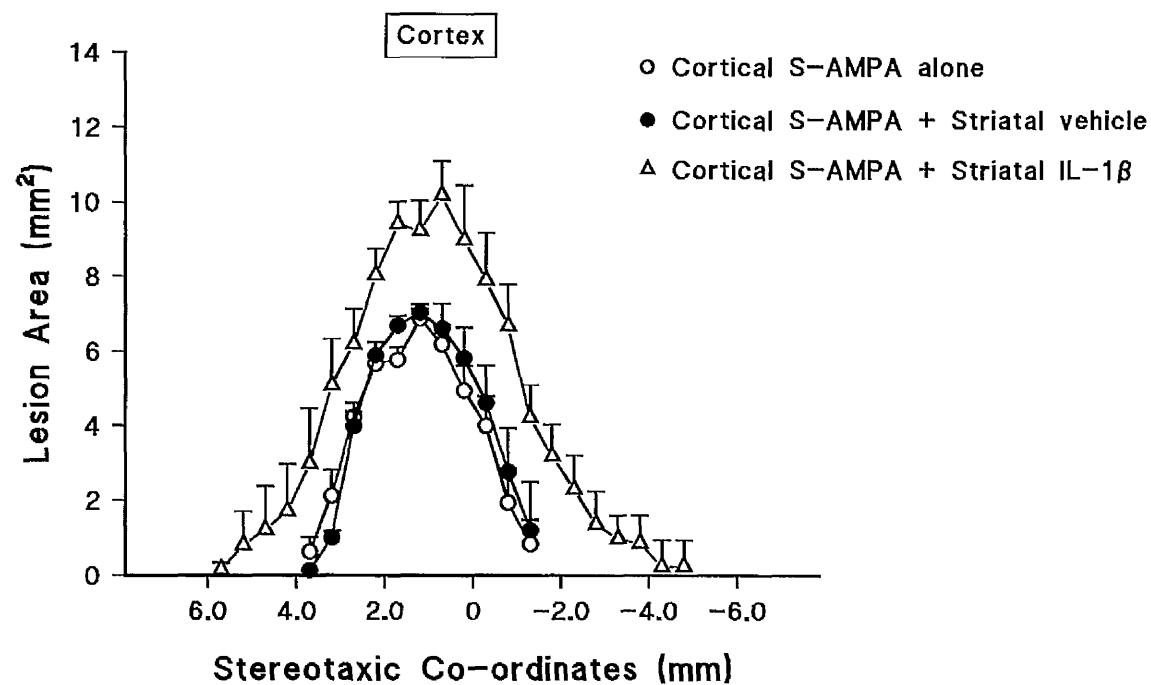


Figure 4.3.9 Effect of contralateral striatal infusion of IL-1 β on cortical MGLu receptor-induced damage

Damage was induced by intracortical infusion of 7.5nmol MGLu (in 1 μ l). MGLu (7.5nmol, n=5) was infused alone into the cortex or followed immediately by contralateral intrastriatal infusion of IL-1 β (1300IU~5-10ng in 2 μ l, n=8) or vehicle (saline, 2 μ l, n=9). Cortical neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 500 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analyses were performed using a Student's t-test. ** P<0.01 versus no striatal infusion, ## P<0.01 versus striatal infusion of vehicle.



Cortical S-AMPA---Contralateral striatum

Figure 4.3.10 Effect of contralateral striatal infusion of IL-1 β on cortical S-AMPA receptor-induced damage

Damage was induced by intracortical infusion of 10nmol S-AMPA (in 1 μ l). S-AMPA (10nmol, n=6) was infused alone into the cortex or followed immediately by contralateral intrastriatal infusion of IL-1 β (1300IU~5-10ng in 2 μ l, n=11) or vehicle (saline, 2 μ l, n=10). Cortical neuronal damage was measured 48h later.

The upper graph shows lesion area (mm², on 500 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analyses were performed using a Student's t-test. ** P<0.01 versus no striatal infusion, ## P<0.01 versus striatal infusion of vehicle.

4.4 Detailed analysis of the cortical response to intrastriatal co-infusion of S-AMPA and IL-1 β

The aim of this study was to investigate, in more detail, the cortical damage caused by co-administration of S-AMPA and IL-1 β into the striatum. Results presented in *Chapter Three* suggest that striatal neuronal damage observed after striatal infusion of S-AMPA was incomplete at 24h post infusions. Therefore, it was determined if the cortical response is present at 24h and, if damage at this time point is significantly different to that at 48h. In addition, to ascertain if secondary cortical neuronal damage resulted from glutamate release and subsequent NMDA receptor overactivation, the effect of the NMDA receptor antagonist, MK-801, was examined. The results of these experiments could provide an indication of whether damage in the cortex is mediated predominantly by the NMDA receptor or if induction of cortical damage occurs via activation of an NMDA receptor-dependent pathway.

4.4.1 *Experimental*

Striatal lesions were induced as described in *Section 2.3.1*. Experiments were carried out in which IL-1 β (1300IU~5-10ng in 2 μ l, n=14) or vehicle (2 μ l saline, n=18) was infused into the striatum immediately after administration of S-AMPA (10nmol) at the same site, and animals were sacrificed (as in *Section 2.4*) 24h (n=15) or 48h (n=17) later. Neuronal death in the striatum and the cortex was assessed on 500 μ m slices by the tetrazolium method as in *Section 2.5.1* and *2.6.1*. The size of cortical damage (after IL-1 β treatment) was determined and related to the size of striatal damage, by calculating the Product-Moment correlation coefficient (according to *Section 2.9*). Finally, damage was noted in other brain regions (e.g. thalamus), and whether piriform cortical damage was present, or if cortical neuronal death extended into this area.

In separate experiments, animals treated with IL-1 β (1300IU~5-10ng in 2 μ l) and S-AMPA (10nmol) in the striatum, were pretreated with either vehicle (PBS, 1ml/kg, i.p., n=7) or the NMDA receptor antagonist, MK-801 (4mg/kg, i.p., n=8), 30min prior to intrastriatal infusions. Animals were sacrificed at 48h later and damage assessed as described above.

4.4.2 Results

4.4.2.1 *Time course of cortical/striatal neuronal damage after intrastriatal infusion of S-AMPA and IL-1 β*

Intrastriatal co-infusion of IL-1 β (1300IU~5-10ng) with S-AMPA (10nmol) did not significantly affect striatal damage measured after either 24h (Vehicle 4.20 \pm 0.60mm³, n=9 versus IL-1 β 5.34 \pm 0.42mm³, n=6, NS, *Figure 4.4.1a*) or 48h (Vehicle 11.52 \pm 0.86mm³, n=9 versus IL-1 β 12.18 \pm 1.44mm³, n=8, NS, *Figure 4.4.1b*) post infusions. However, there was a statistically significant difference in the volume of striatal damage measured at 24h or 48h, in both vehicle- (24h 4.20 \pm 0.60mm³, n=9 versus 48h 11.52 \pm 0.86mm³, n=9, $P < 0.001$, *Figure 4.4.1c*), and IL-1 β -treated animals (24h 5.34 \pm 0.42mm³, n=6 versus 48h 12.18 \pm 1.44mm³, n=8, $P < 0.01$, *Figure 4.4.1c*), suggesting that damage in the striatum was incomplete at 24h in both groups. Furthermore, in agreement with the results obtained in *Section 4.3.2.1*, co-administration of S-AMPA and IL-1 β in the striatum resulted in extensive cortical neuronal death at 48h post infusions, and furthermore, this damage was present at 24h. The extent of damage in the cortex after striatal infusion of S-AMPA and IL-1 β was significantly greater (~980% and 550% for 24h and 48h respectively) than cortical damage in S-AMPA and vehicle-treated animals measured 24h (Vehicle 7.04 \pm 0.82mm³, n=9 versus IL-1 β 76.21 \pm 11.22mm³, n=6, $P < 0.01$, *Figure 4.4.1a*) or 48h (Vehicle 12.31 \pm 2.43mm³, n=9 versus IL-1 β 79.94 \pm 9.71mm³, n=8, $P < 0.001$, *Figure 4.4.1b*)

Figure 4.4.1a

Effect of striatal infusion of IL-1 β on striatal and cortical damage 24h after induction by striatal AMPA receptor overactivation

Damage was induced by intrastriatal infusion of 10nmol S-AMPA (in 1 μ l). IL-1 β (1300IU~5-10ng in 2 μ l, n=6) or vehicle (saline, 2 μ l, n=9) were co-infused into the striatum with S-AMPA. Striatal (illustrated in the left graphs) and cortical (illustrated in the right graphs) neuronal damage were measured in the same animals 24h later. The upper graphs show lesion areas (mm², on 500 μ m sections), which were integrated to give lesion volumes (mm³), shown in the lower graphs.

Data are expressed as mean \pm SEM. Statistical analyses were performed using a Student's t-test. ** P<0.01 versus vehicle-treated animals.

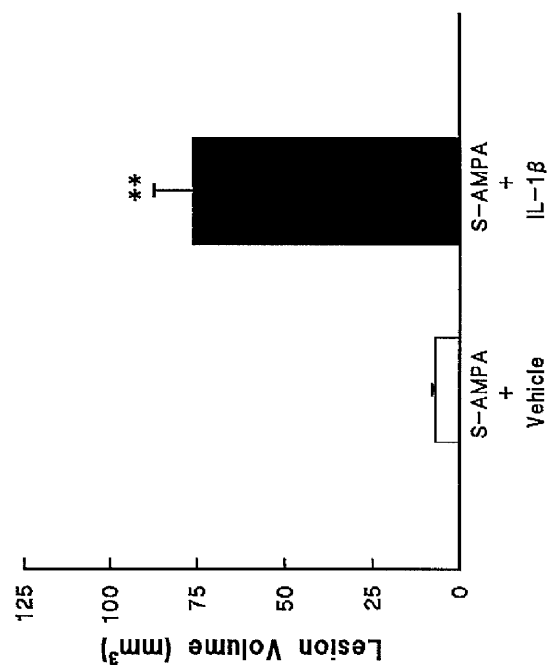
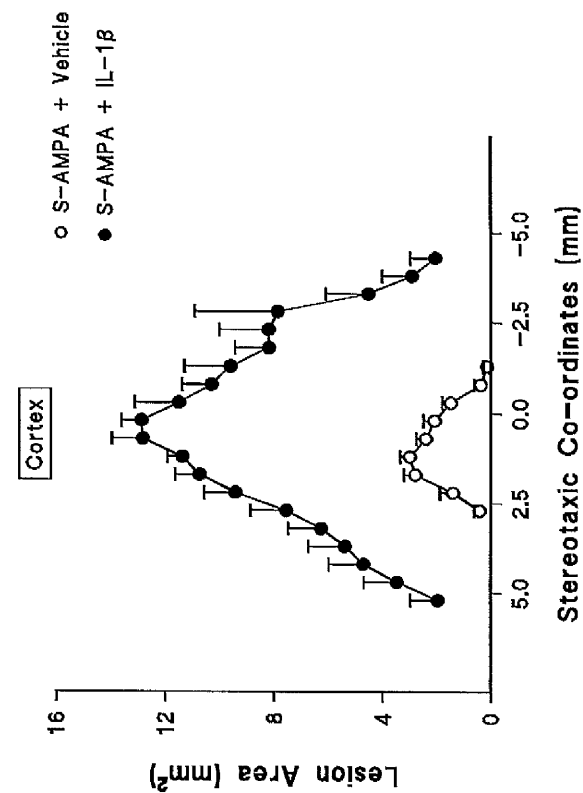
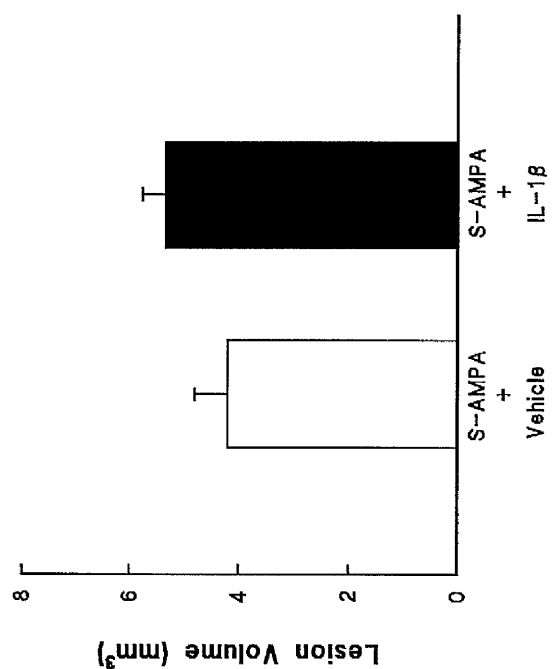
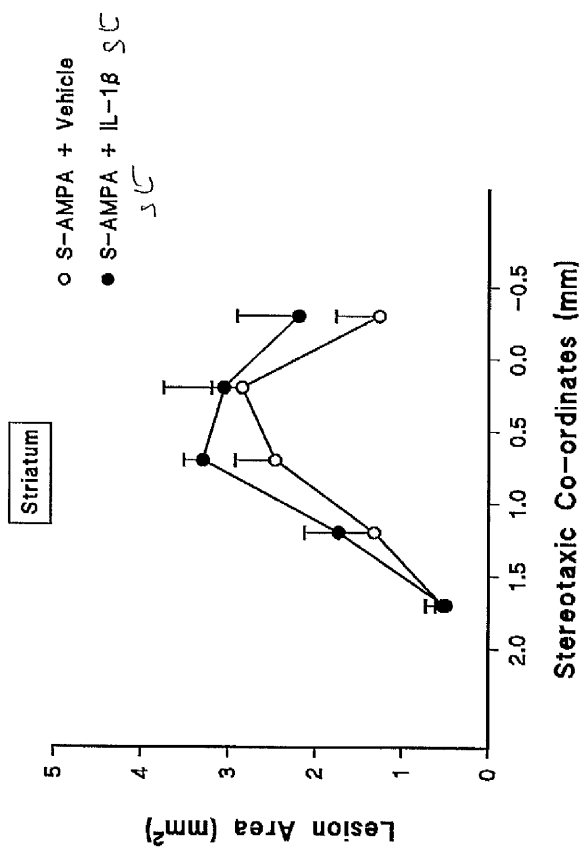
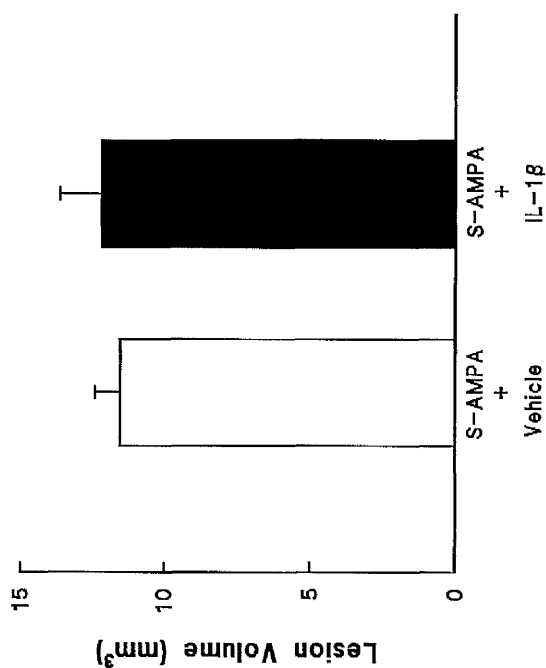
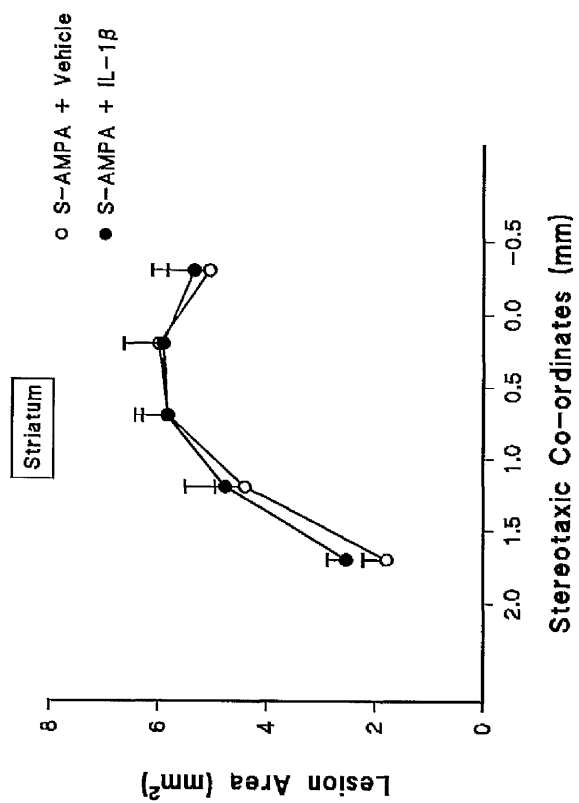
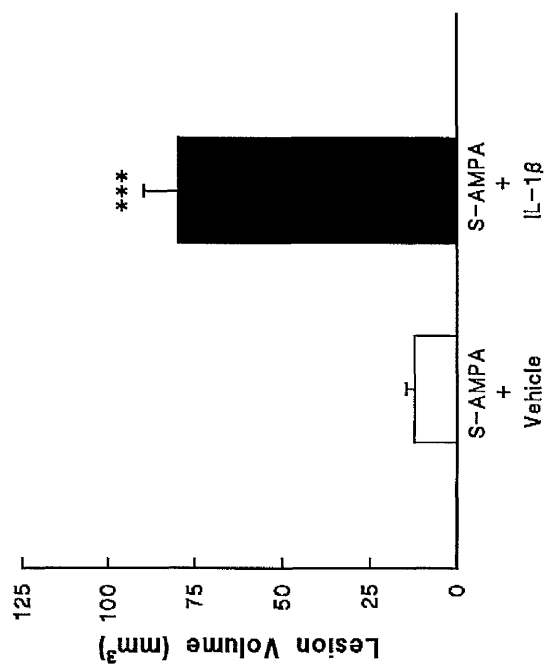
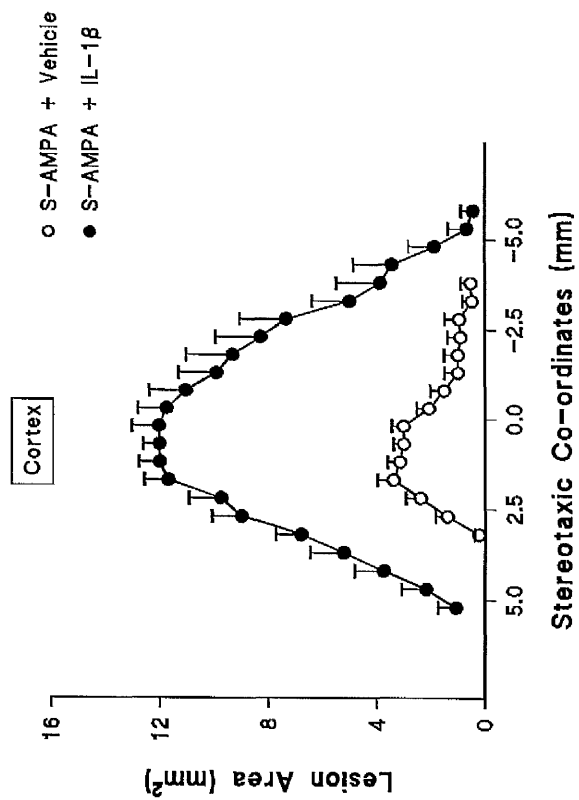


Figure 4.4.1b

Effect of striatal infusion of IL-1 β on striatal and cortical damage 48h after induction by striatal AMPA receptor overactivation

Damage was induced by intrastratial infusion of 10nmol S-AMPA (in 1 μ l). IL-1 β (1300IU~5-10ng in 2 μ l, n=8) or vehicle (saline, 2 μ l, n=9) were co-infused into the striatum with S-AMPA. Striatum (illustrated in the left graphs) and cortical (illustrated in the right graphs) neuronal damage were measured in the same animals 48h later. The upper graphs show lesion areas (mm², on 500 μ m sections), which were integrated to give lesion volumes (mm³), shown in the lower graphs.

Data are expressed as mean \pm SEM. Statistical analyses were performed using a Student's t-test. *** P<0.001 versus vehicle-treated animals.



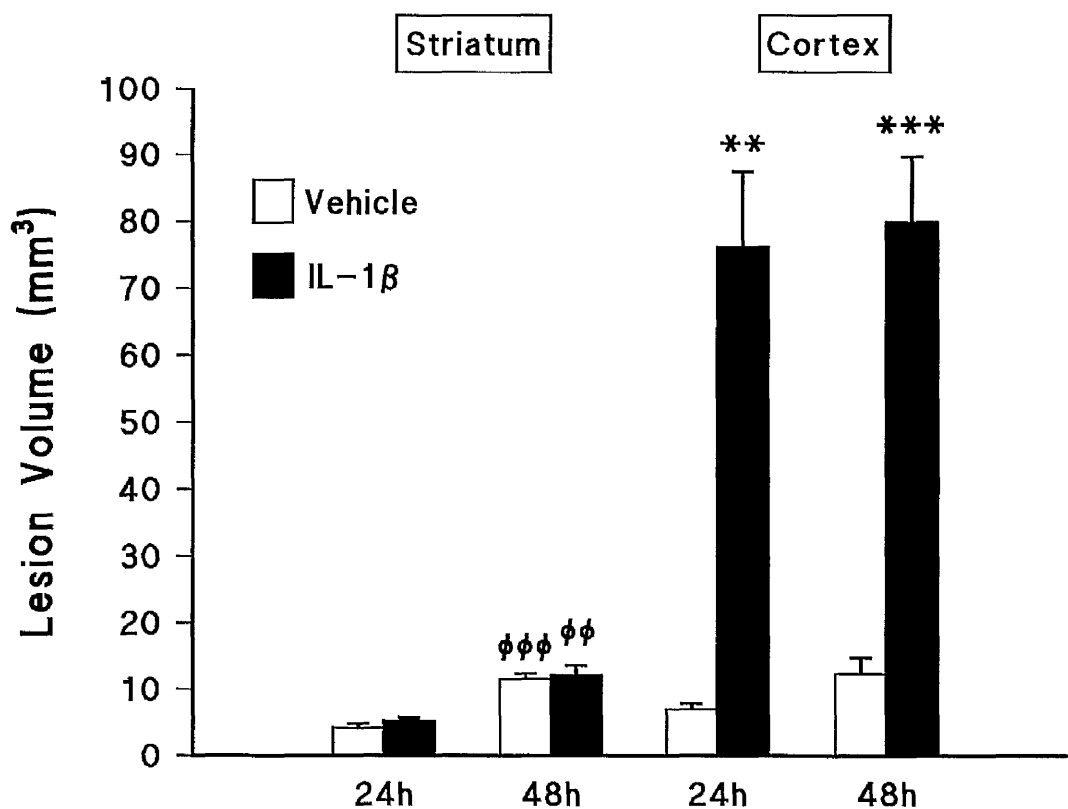


Figure 4.4.1c Effect of striatal infusion of IL-1 β on striatal and cortical damage induced by striatal AMPA receptor overactivation.
 ----Comparison between 24h and 48h

Damage was induced by intrastriatal infusion of 10nmol S-AMPA (in 1 μ l). IL-1 β (1300IU~5-10ng in 2 μ l, n=6-8, solid bars) or vehicle (saline, 2 μ l, n=9, open bars) was co-infused into the striatum with S-AMPA. Striatal (illustrated in the four bars on the left) and cortical (illustrated in the four bars on the right) lesion volumes (mm³) were measured in the same animals at 24h and 48h later.

Data are expressed as mean \pm SEM. Statistical analyses were performed using a Student's t-test. $\phi\phi\phi$ P<0.001 versus vehicle-treated striatal damage at 24h, $\phi\phi$ P<0.01 versus IL-1 β -treated striatal damage at 24h, *** P<0.001 versus vehicle-treated cortical damage at 48h, ** P<0.01 versus vehicle-treated cortical damage at 24h.

after treatments. In comparison to striatal lesions, there was no significant difference between the cortical damage measured at 24h and 48h in either IL-1 β - (24h $76.21 \pm 11.22 \text{ mm}^3$, n=6 versus 48h $79.94 \pm 9.71 \text{ mm}^3$, n=8, NS, *Figure 4.4.1c*) or vehicle-treated animals (24h $7.04 \pm 0.82 \text{ mm}^3$, n=9 versus 48h $12.31 \pm 2.43 \text{ mm}^3$, n=9, NS, *Figure 4.4.1c*). This implies that, in contrast to striatal lesions, the progression of cortical damage produced by striatal infusion of S-AMPA and IL-1 β is developed within 24h.

Figure 4.4.1d illustrates the relationship between cortical and striatal damage after intrastriatal infusion of S-AMPA and IL-1 β . The calculated Product-Moment correlation coefficient indicates that there is no relationship between the extent of damage in these two regions at 24h post infusions ($r = -0.008$). However, at 48h there was a significant positive correlation ($r = 0.79$, $P < 0.05$, $df = 6$) between the lesion volume in the striatum and the cortex. This correlation suggests that the striatal and cortical damage change in parallel, but does not prove a causal relationship.

Damage was observed occasionally in the piriform cortex of animals infused intrastrially with S-AMPA and saline, and neuronal loss was seen in the ventral thalamus in 3 of the 18 animals (17%) measured at 24h and 48h after treatments (see *Table 4.4.1a*). This damage was not quantified, but generally extended throughout a distance of 0.5mm in an anterior/posterior direction. In contrast, animals treated with S-AMPA and IL-1 β exhibited more frequent (7/14=50%) and extensive damage in the thalamus at 24h and 48h after infusions. In IL-1 β -treated animals, neuronal loss often extended throughout an anterior/posterior distance of 1.5-2.0mm and in some severe cases extended over 2.5mm and in animals with most extensive thalamic lesions, both the ventral and some aspects of the medial thalamic nuclei were affected (see *Table 4.4.1b*). As described earlier (see *Section 4.3.2.1*) the cortical lesion caused by S-AMPA and IL-1 β infusion into the striatum did not always extend into the

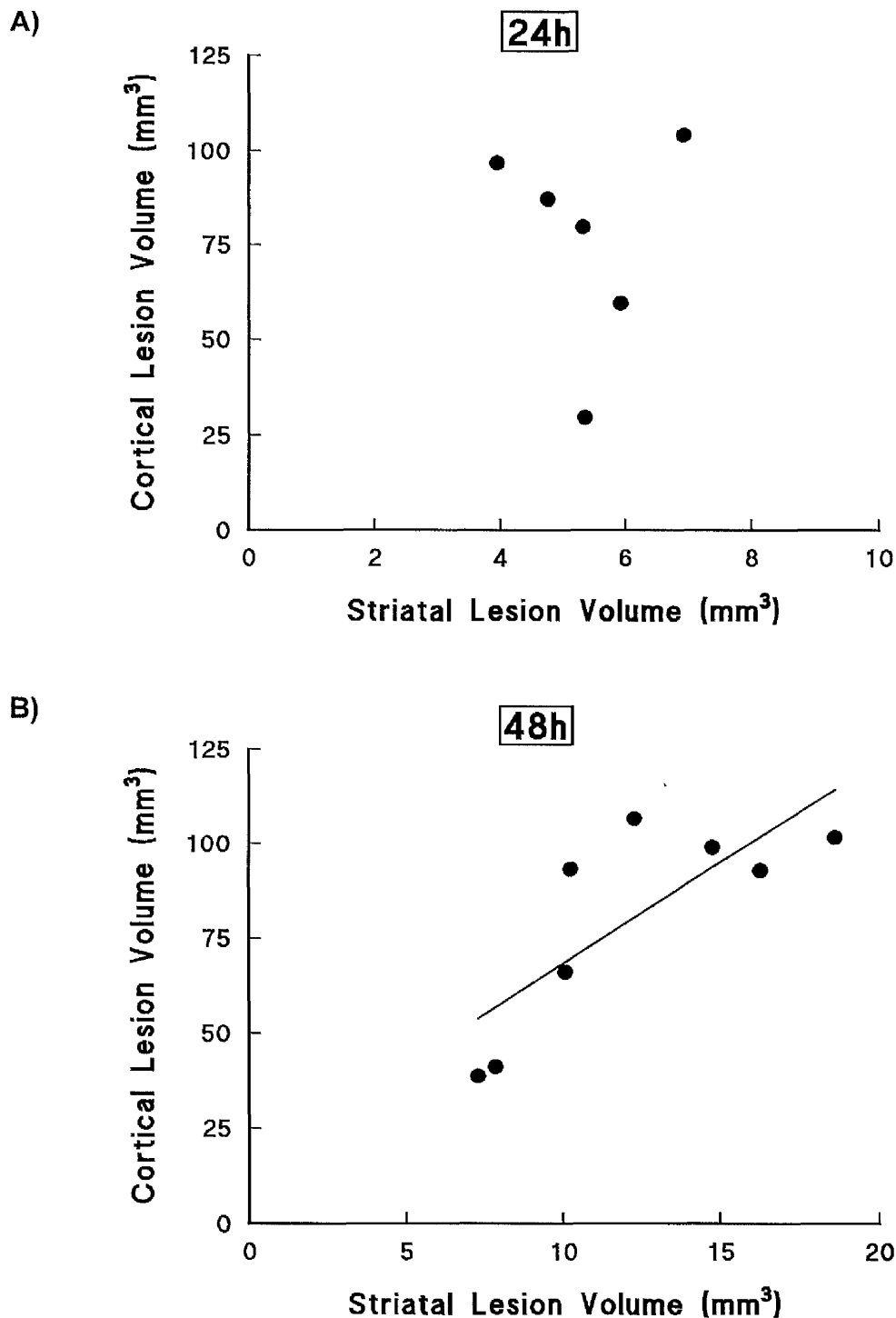


Figure 4.4.1d Correlation between striatal and cortical neuronal damage induced by intrastriatal infusion of IL-1 β and S-AMPA

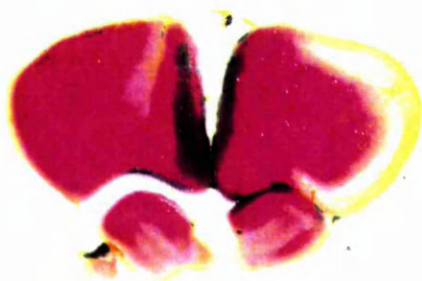
The relationship between striatal and cortical lesion volume at **A)** 24h or **B)** 48h after intrastriatal infusion of S-AMPA (10nmol) and IL-1 β (1300IU~5-10ng). Striatal lesion volume was significantly correlated with the resulting cortical lesion volume at 48h ($r=0.79$, $P<0.05$, $df=6$) but not at 24h ($r=-0.008$).

Figure 4.4.1e Tetrazolium staining to show the distribution of neuronal damage 24h after striatal injection of S-AMPA and IL-1 β

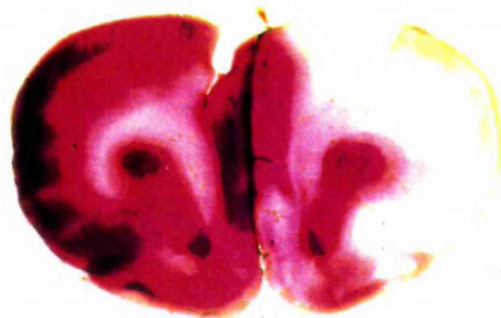
Coronal brain sections (500 μ m) were stained with tetrazolium and illustrate striatal and cortical neuronal damage produced in a representative brain after intrastriatal infusion of S-AMPA (10nmol) and IL-1 β (1300IU~5-10ng). Sections were taken 24h after infusions. The white regions are non-viable neuronal tissue and the pink regions represent viable tissue. Note the extensive region of the cortex which is damaged after treatment with IL-1 β and the smaller amount of damage in the striatum, compared to sections taken at 48h (see *Figure 4.3.6.c*). The dark line represents the site of injection. The numbers represent the co-ordinates of each section in relation to bregma.

C, Cortex; St, Striatum; PC, Piriform Cortex.

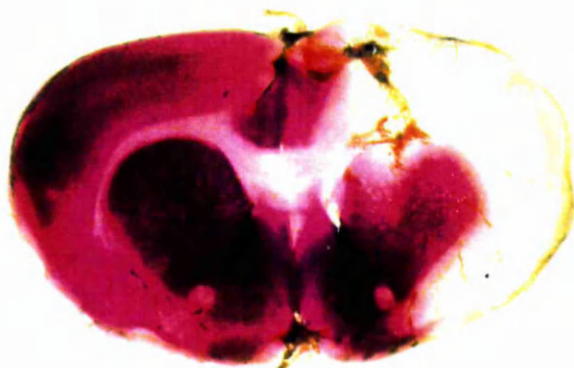
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3.7



2.2



1.2



0.2



-0.3



-1.8

↑
PC

Figure 4.4.1f Tetrazolium staining to show the extrastriatal neuronal damage 48h after striatal injection of S-AMPA and IL-1 β

Coronal brain sections (500 μ m) were stained with tetrazolium and illustrate striatal and cortical neuronal damage produced in a representative brain after intrastratial infusion of S-AMPA (10nmol) and IL-1 β (1300IU~5-10ng). Sections were taken 48h after infusions. The white regions are non-viable neuronal tissue and the pink regions represent viable tissue. Note the extensive region of the cortex which is damaged after treatment with IL-1 β . Note also the extensive damage in the thalamus. The dark line represents the site of injection. The numbers represent the co-ordinates of each section in relation to bregma.

AN, Accumbens nucleus; AON, Anterior olfactory nucleus; C, Cortex; ILC, Infralimbic cortex; St, Striatum; Th, Thalamus.
Magnification bar=1.4mm

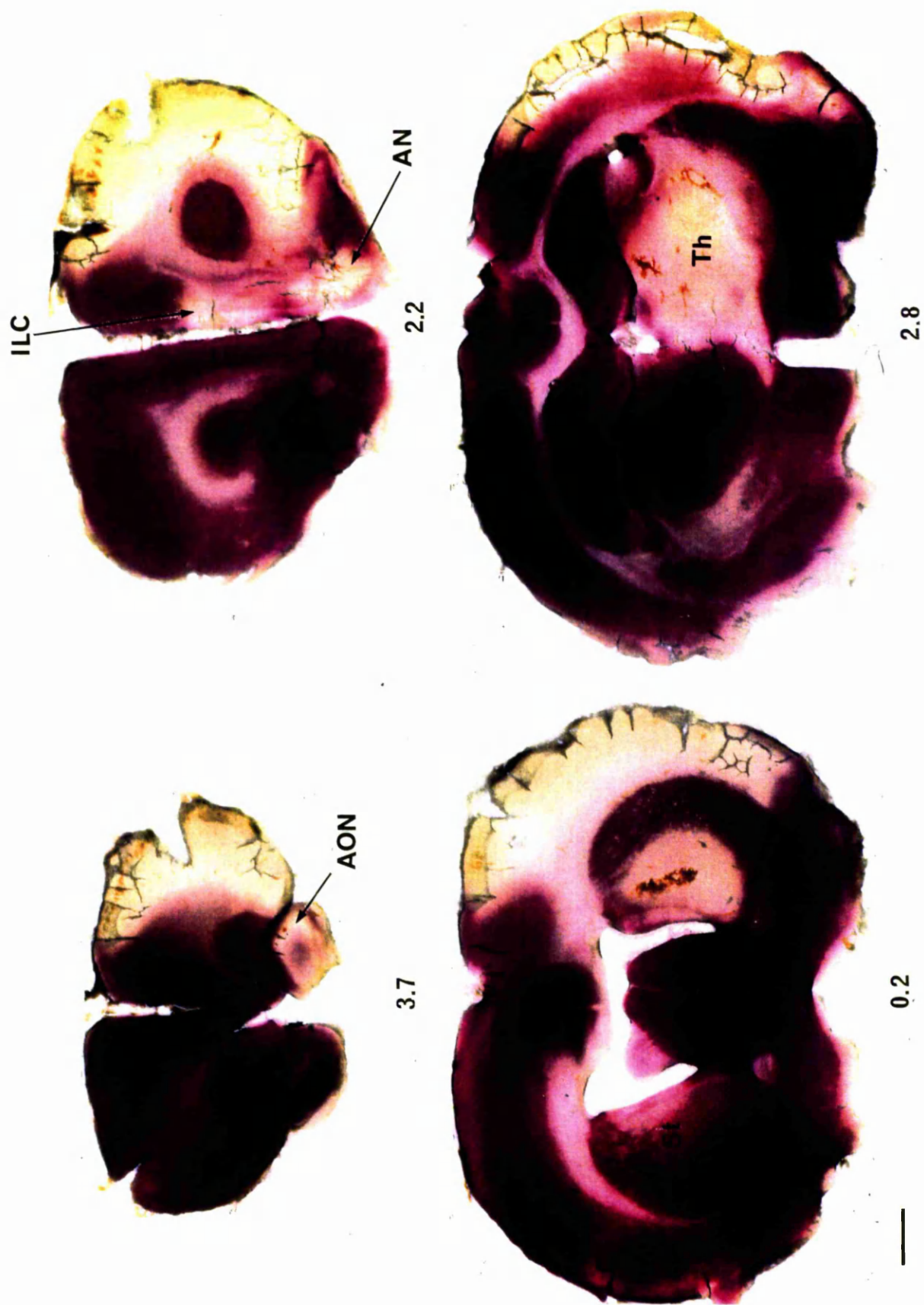


Table 4.4.1a

Summary of lesion volumes (mm^3) in the striatum and the cortex after striatal infusion of S-AMPA (10nmol) and vehicle (saline, $2\mu\text{l}$) **A**) 24h and **B**) 48h later.

The presence of piriform cortex damage is noted and whether damage was observed in the thalamus.

- + Damage present.
- Damage absent.
- + (V) Damage present in the ventral thalamus
extending through a distance of up to 0.5mm.

A)

Striatal S-AMPA (10nmol) + Striatal Saline (2 μ l) 24h			
Striatal lesion volume (mm ³)	Cortical lesion volume (mm ³)	Piriform cortex damage	Thalamus damage
4.65	5.98	-	-
3.31	9.13	-	-
6.32	10.07	-	-
2.17	2.67	-	-
1.76	9.47	-	-
6.02	7.36	+	-
6.61	5.94	-	-
3.28	4.57	-	+ (V)
3.72	8.15	-	-
Mean \pm SEM 4.20 \pm 0.60	Mean \pm SEM 7.04 \pm 0.82	1/9=11%	1/9=11%

B)

Striatal S-AMPA (10nmol) + Striatal Saline (2 μ l) 48h			
Striatal lesion volume (mm ³)	Cortical lesion volume (mm ³)	Piriform cortex damage	Thalamus damage
14.56	5.93	-	-
8.75	5.51	-	-
16.16	15.75	+	-
10.90	15.25	-	+ (V)
8.24	4.10	-	-
10.27	23.95	+	-
12.73	6.35	-	+ (V)
11.54	12.67	-	-
10.51	21.27	+	-
Mean \pm SEM 11.52 \pm 0.86	Mean \pm SEM 12.31 \pm 2.43	3/9=33%	2/9=22%

Table 4.4.1b

Summary of lesion volumes (mm³) in the striatum and the cortex after striatal infusion of S-AMPA (10nmol) and IL-1 β (1300IU~5-10ng in 2 μ l) **A)** 24h and **B)** 48h later.

The presence of damage extending into the piriform cortex is noted and whether damage was observed in the thalamus.

+	Damage present.
-	Damage absent.
+ (V)	Damage present in the ventral thalamus extending through a distance of up to 1.0mm.
++ (V)	Damage present in the ventral thalamus extending through a distance of 1.5-2.0mm.
+++ (V)	Damage present in the ventral thalamus extending through a distance of equal to or greater than 2.5mm.
+++ (V/M)	Damage present in the ventral and medial thalamus extending through a distance of equal to or greater than 2.5mm.

A)

Striatal S-AMPA (10nmol) + Striatal IL-1 β (1300IU) 24h			
Striatal lesion volume (mm ³)	Cortical lesion volume (mm ³)	Piriform cortex damage	Thalamus damage
5.93	59.71	+	+ (V)
6.93	104.2	+	+++ (V)
3.95	96.74	+	-
4.76	87.20	+	++ (V)
5.32	79.75	-	-
5.36	29.67	+	-
Mean \pm SEM 5.34 \pm 0.42	Mean \pm SEM 76.21 \pm 11.22	5/6=83%	3/6=50%

B)

Striatal S-AMPA (10nmol) + Striatal IL-1 β (1300IU) 48h			
Striatal lesion volume (mm ³)	Cortical lesion volume (mm ³)	Piriform cortex damage	Thalamus damage
10.25	93.27	-	+++ (V/M)
10.09	66.14	+	-
14.77	99.05	-	++ (V)
18.62	101.6	+	-
7.85	41.19	-	-
12.29	106.6	-	++ (V)
16.28	92.90	+	+++ (V/M)
7.29	38.75	-	-
Mean \pm SEM 12.18 \pm 1.44	Mean \pm SEM 79.94 \pm 9.71	3/8=38%	4/8=50%

piriform cortex (see *Table 4.4.1b*).

See *Figures 4.4.1e* and *4.4.1f* for examples of tetrazolium stained brain sections illustrating the extent of cortical lesions throughout the brain, and the neuronal damage in other areas (e.g. thalamus), 24h or 48h post infusion of S-AMPA and IL-1 β .

In summary, the local striatal damage produced by intrastriatal infusion of S-AMPA and IL-1 β (or vehicle) was significantly smaller 24h compared to 48h after infusions. However, the volume of cortical neuronal loss observed in response to intrastriatal infusion of S-AMPA and IL-1 β was not significantly different between 24h and 48h post infusions. In addition to neuronal damage in the cortex, neurodegeneration was seen in the thalamus in some animals after intrastriatal treatment with S-AMPA and IL-1 β compared to intrastriatal infusion of S-AMPA and vehicle.

4.4.2.2 *Effect of MK-801 on cortical/striatal neuronal damage after intrastriatal infusion of S-AMPA and IL-1 β*

Co-infusion of S-AMPA (10nmol) and IL-1 β (1300IU~5-10ng) into the striatum resulted in a striatal lesion which was not significantly affected by pretreatment with 4mg/kg MK-801 (Vehicle $11.94 \pm 0.99 \text{mm}^3$, $n=7$ versus MK-801 $12.97 \pm 0.91 \text{mm}^3$, $n=8$, NS, *Figure 4.4.2*). However, the extensive cortical neuronal damage which developed after intrastriatal infusion of S-AMPA and IL-1 β , was significantly reduced (67%), but not completely abolished, by pretreatment with MK-801 (Vehicle $79.17 \pm 15.06 \text{mm}^3$, $n=7$ versus MK-801 $25.91 \pm 3.79 \text{mm}^3$, $n=8$, $P < 0.05$, *Figure 4.4.2*). Degeneration in the thalamus was observed in some animals, in both the vehicle- and MK-801-treated groups. Furthermore, the incidence of this damage was higher after MK-801 (7/8=88%),

Figure 4.4.2

Effect of MK-801 on striatal and cortical damage induced by striatal infusion of S-AMPA and IL-1 β

Damage was induced by intrastriatal infusion of 10nmol S-AMPA (in 1 μ l). IL-1 β (1300IU~5-10ng in 2 μ l) was co-infused into the striatum with S-AMPA. Animals were pretreated with either MK-801 (4mg/kg, i.p., n=8), or vehicle (PBS, 1ml/kg, i.p., n=7), 30min prior to infusions.

Striatal (illustrated in the left graphs) and cortical (illustrated in the right graphs) neuronal damage were measured in the same animals 48h later.

The upper graphs show lesion areas (mm², on 500 μ m sections), which were integrated to give lesion volumes (mm³), shown in the lower graphs.

Data are expressed as mean \pm SEM. Statistical analyses were performed using a Student's t-test. * P<0.05 versus vehicle-treated animals.

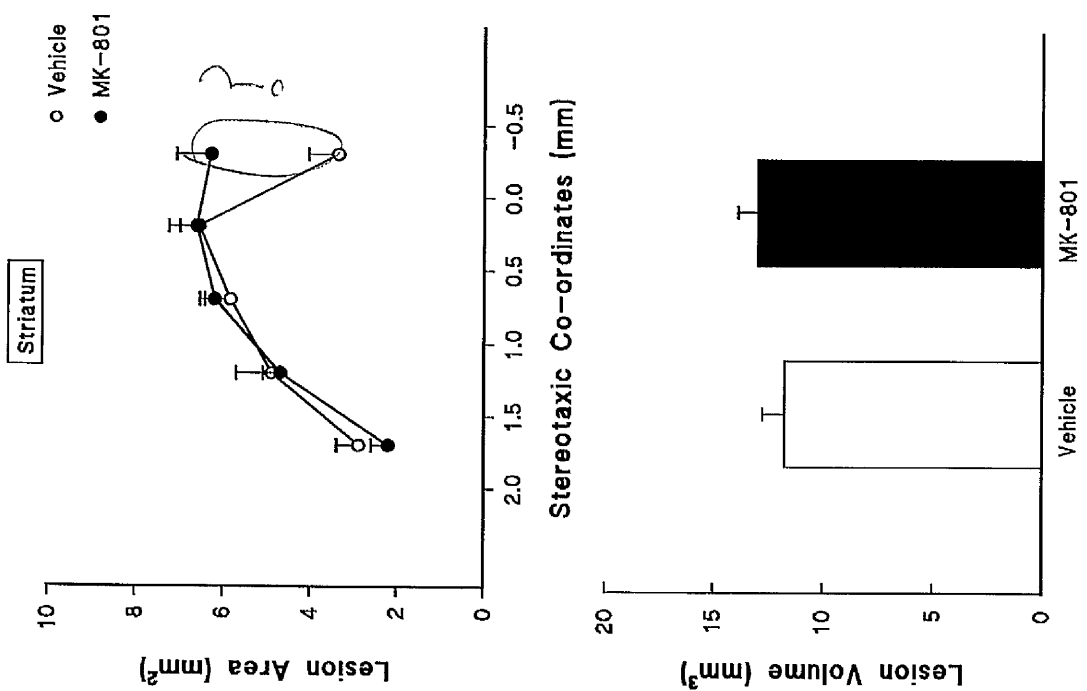
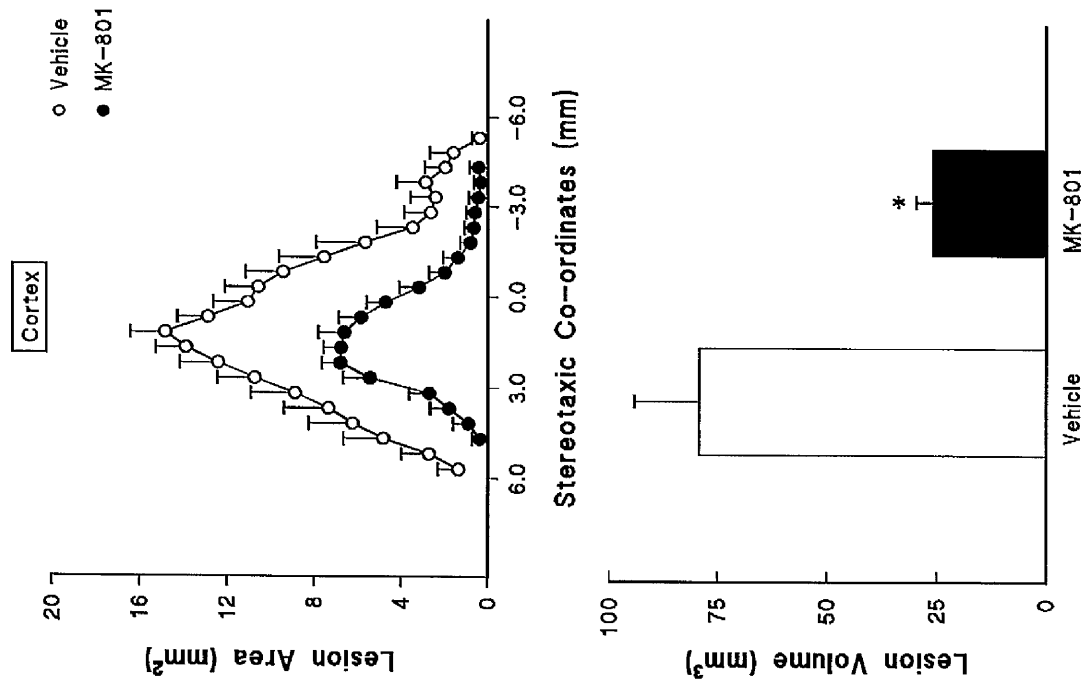


Table 4.4.2

Summary of lesion volumes (mm^3) in the striatum and the cortex after striatal infusion of S-AMPA (10nmol) and IL-1 β (1300IU in 2 μ l) after pretreatment with **A**) vehicle (PBS, 1ml/kg, i.p.) or **B**) MK-801 (4mg/kg, i.p.).

The presence of damage extending into the piriform cortex is noted and whether damage was observed in the thalamus.

+	Damage present.
-	Damage absent.
+ (V)	Damage present in the ventral thalamus extending through a distance of up to 1.0mm.
++ (V)	Damage present in the ventral thalamus extending through a distance of 1.5-2.0mm.
+++ (V)	Damage present in the ventral thalamus extending through a distance of equal to or greater than 2.5mm.
+++ (V/M)	Damage present in the ventral and medial thalamus extending through a distance of equal to or greater than 2.5mm.

A)

Striatal S-AMPA (10nmol) + Striatal IL-1 β (1300IU) + PBS (1ml/kg, i.p.)			
Striatal lesion volume (mm ³)	Cortical lesion volume (mm ³)	Piriform cortex damage	Thalamus damage
16.41	117.4	+	-
10.15	93.33	-	+++ (V/M)
10.33	60.23	-	-
10.78	35.68	-	+ (V)
13.89	78.35	+	+ (V)
11.89	31.91	-	-
8.76	137.27	-	+++ (V)
Mean \pm SEM 11.94 \pm 0.99	Mean \pm SEM 79.17 \pm 15.06	2/7=29%	4/7=57%

B)

Striatal S-AMPA (10nmol) + Striatal IL-1 β (1300IU) + MK801 (4mg/kg, i.p.)			
Striatal lesion volume (mm ³)	Cortical lesion volume (mm ³)	Piriform cortex damage	Thalamus damage
12.12	43.30	-	+ (V)
14.91	9.94	-	+++ (V/M)
14.90	35.95	+	++ (V)
10.15	27.58	-	++ (V)
15.08	30.22	+	++ (V)
11.40	22.57	-	++ (V)
15.99	21.36	+	-
9.21	16.38	-	+ (V)
Mean \pm SEM 12.97 \pm 0.91	Mean \pm SEM 25.91 \pm 3.79	3/8=38%	7/8=88%

compared to vehicle treatment (4/7=57%). MK-801 pretreatment did not appear to affect the occurrence of piriform cortex damage (See *Table 4.4.2*).

In summary, cortical neuronal death in response to intrastriatal infusion of S-AMPA and IL-1 β was significantly reduced by peripheral treatment of the NMDA receptor antagonist, MK-801. In contrast, the incidence of neuronal loss observed in the thalamus, after infusion of S-AMPA and IL-1 β into the striatum, appeared to be enhanced by MK-801.

4.5 Discussion

4.5.1 *Effect of IL-1ra on excitotoxic damage*

Results presented in *Section 4.2.2* show that local infusion of recombinant IL-1ra inhibited NMDA and AMPA receptor-induced lesions in the striatum, but had no effect on lesions induced in the cortex. Intrastriatal or intracortical infusion of IL-1ra alone caused no behavioural effects and no obvious neuronal damage 24h or 48h after infusion, suggesting that this antagonist is not toxic to neurones *per se*.

The volume of NMDA receptor-induced lesions in the striatum was reduced (46%) by co-infusion of IL-1ra, which agrees with the data of Relton and Rothwell (1992), who found 71% inhibition in lesion volume. The reason for the variation in extent of protection is unknown, but may be attributed to batch variability of IL-1ra samples or age of animals used in the experiments. However, in both cases, a significant reduction in lesion volume was found with IL-1ra treatment. Additionally, IL-1ra produced a amount of similar neuroprotection against striatal damage induced by another NMDA receptor agonist, quinolinic acid. Furthermore, the reduction in GAD (a marker for GABAergic neurones), which is seen after intrastriatal infusion of quinolinic acid

is reversed by co-infusion of IL-1ra (Relton, Strijbos and Rothwell, personal communication). These data provide further support the involvement of IL-1 in damage induced by NMDA receptor overactivation.

The effect of IL-1ra on AMPA receptor-induced lesions has not previously been reported. Data reported here demonstrates that IL-1ra reduced damage induced by AMPA receptor overactivation in the striatum (43% inhibition), which is similar in magnitude to the inhibition of NMDA receptor-induced damage. This result is of particular significance since NMDA and AMPA receptors induce neuronal death via differing mechanisms, and are involved in neurological disorders in varying degrees, thus possibly providing clues towards the mechanism of action of IL-1 (see *Chapter One* and *Seven*).

IL-1ra is a highly selective competitive receptor antagonist of IL-1 (Dripps *et al.*, 1991a) and has been reported to have no other agonist or antagonist activities (see Dinarello and Thompson, 1991; Dripps *et al.*, 1991a; Arend, 1993; Dinarello, 1994). Although, partial agonist activity has been reported in human decidual cells (Cole *et al.*, 1993; Mitchell *et al.*, 1993). However, no agonist properties have been reported to date in cells within the CNS. Thus, assuming that IL-1ra acts primarily by blocking the actions of endogenous IL-1 α and IL-1 β , these data suggest that IL-1 mediates, at least in part, neurotoxicity caused by overactivation of either NMDA or non-NMDA (AMPA) receptors within the striatum. It seems therefore that protection by IL-1ra is not specific for neuronal damage induced by overactivation of NMDA receptors. Hence, IL-1ra may have a broader relevance to other forms of neuronal degeneration involving excitotoxic processes.

The dose of IL-1ra (5 μ g/ μ l) was chosen on the basis of previous studies in which this antagonist inhibited striatal NMDA receptor-induced excitotoxicity or central

effects of IL-1 on behaviour (Kent *et al.*, 1992; Relton and Rothwell, 1992). In general IL-1ra needs to be present at a molar excess of 10-1000 fold over IL-1 to reduce responses to IL-1 by 50-100% (see Dinarello and Thompson, 1991; Arend, 1993; Dinarello, 1994). Higher doses of IL-1ra have not been tested and therefore may offer a greater degree of neuroprotection.

In contrast to the effect of IL-1ra on excitotoxic lesions in the striatum, co-administration of this antagonist did not affect damage induced by infusion of either MGlutamate or S-AMPA in the cortex, suggesting site specificity in the neuroprotective actions of IL-1ra. The reason for this difference is largely unknown, however, several suggestions can be proposed:

$\left. \begin{array}{l} \text{IL-1ra} \\ \text{for} \end{array} \right\} \begin{array}{l} \text{it} \\ \text{can} \end{array}$

The cortex is a reported site of IL-1 expression in response to various types of neuronal injury (e.g. Minami *et al.*, 1992; Wang *et al.*, 1994b; Yabuuchi *et al.*, 1994b; Fan *et al.*, 1995). The profile of IL-1 synthesis may vary between the cortex and the striatum after excitotoxic injury. Thus, a higher dose, or a difference in the timing of administration of IL-1ra may be required to affect neuronal damage within the cortex. However, results presented in *Chapter Three* suggest that the mechanism and time course of excitotoxic damage (induced by NMDA or AMPA receptor overactivation) does not differ significantly between the striatum and the cortex. Therefore, it seems unlikely, although unproven, that changing these parameters may result in a significant modification of damage in the cortex.

As discussed in *Chapter One*, IL-1ra binds to two receptor subtypes, type I and type II, with a much higher affinity and selectivity at the type I receptor (Dripps *et al.*, 1991b; Arend, 1993; Dinarello, 1994). Whilst these receptors have been well characterised in the periphery, relatively little is known about their presence in the rat CNS. Several studies have localised mRNA for the type I receptor in the striatum and the cortex in the rat (Yan *et al.*, 1992; Yabuuchi *et al.*, 1994a)

and a recent study has demonstrated IL-1 binding sites in the rat pituitary (Marquette *et al.*, 1995). However, the presence of high affinity IL-1 receptors in the rat brain remains to be fully established and furthermore, it has been proposed that novel IL-1 receptor(s) may exist in rat brain (e.g. Takao *et al.*, 1993; Rothwell and Luheshi, 1994; Marquette *et al.*, 1995). Therefore, there may be differences between the receptors in the striatum and the cortex, with a predominance of high affinity receptors for IL-1ra in the striatum. ^{IL-1}

D. phos. in re. to IL-1 (X)

Another major difference observed between the striatum and the cortex is the fever (rise in core body temperature) produced after intrastriatal, but not intracortical infusion of IL-1 β alone (see *Chapter Five* for experiments and results). Activation of striatal EAA receptors causes rapid local induction of IL-1 β in the rat brain (Van Dam, Strijbos and Rothwell, unpublished data) which could then produce a rise in body temperature and exacerbation of excitotoxic damage (McDonald *et al.*, 1991). Furthermore, IL-1ra inhibits fever induced by icv injection of IL-1 β or IL-1 α (Steffert *et al.*, 1995). ^{Other report of temperature effects or possibly} Therefore, IL-1ra could reduce neuronal damage in the striatum by reduction of the putative rise in body temperature, induced by endogenous release of IL-1 β . Results reported in *Chapter Five* show that there was no change in body temperature after intracortical infusion of IL-1 β alone. Hence, endogenous IL-1 β produced locally after cortical-induced neuronal damage is unlikely to affect body temperature, and cortical infusion of IL-1ra should be ineffective. However, the protective actions of IL-1ra may be independent of changes in body temperature since IL-1ra reduces both cortical and striatal damage caused by cerebral ischaemia ^{what about brain?} without modifying body temperature (Loddick and Rothwell, 1996). Additionally, it has yet to be proven if striatal or cortical infusion of MGlu or S-AMPA causes changes in body temperature and if IL-1 β co-infusion modifies any changes which may occur. Planas *et al* (1995) reported a rise in body temperature after systemic administration of kainic acid (a non-NMDA receptor agonist). However,

this hyperthermia may be due to intensive seizure activity observed in these animals.

The most likely explanation of this difference in effect of IL-1ra between striatal and cortical damage derives from extrapolation of results reported in *Section 4.3.2*. IL-1 β did not affect local cortical damage induced by EAA receptor overactivation when administered within the cortex, but produced a significant increase in cortical neurodegeneration when infused into the ipsilateral or contralateral striatum. Therefore, the site of action of IL-1 may be within the striatum or neuronal structures nearby, for example the hypothalamus; the major site of action of IL-1 within the brain. Thus, if IL-1ra was infused intrastriatally or injected icv, immediately after cortical infusion of the excitotoxins, a significant affect on cortical neuronal damage may have been observed. In support of this proposal, studies on the effect of IL-1ra on neuronal damage after ischaemic or traumatic brain insults have involved lateral ventricle administration of this antagonist (Relton and Rothwell, 1992; Betz *et al.*, 1995; Toulmond and Rothwell, 1995; Loddick and Rothwell, 1996). This method may allow a greater concentration of IL-1ra to be achieved within the striatum or the hypothalamus compared to cortical regions. Furthermore, recent studies performed within our lab have demonstrated neuroprotective effects of IL-1ra against focal cerebral ischaemia when infused into the striatum (ipsilateral or contralateral) but not after cortical infusion (Stroemer and Rothwell, personal communication).

hypothalamic seizure behavior?

Although, the results presented here clearly demonstrate that IL-1ra can modify excitotoxic neuronal damage, there are no direct clinical parallels with this form of acute EAA-induced neurodegeneration, although this type of injury is involved in several neurodegenerative diseases (see *Chapter One* and *Three*). IL-1ra treatment does reduce the neuronal damage which occurs after several clinically relevant animals models of brain damage. For example, IL-1ra reduces neurodegeneration caused by *in vivo* experimentally-induced stroke (Relton and

Rothwell, 1992; Betz *et al.*, 1995; Loddick and Rothwell, 1996; Garcia *et al.*, 1995), or brain trauma (Toulmond and Rothwell, 1995). IL-1ra also inhibits neuronal damage in models of hypoxic- or heat stroke-induced neuronal injury (Martin *et al.*, 1994; Lin *et al.*, 1995) *in vivo*. In contrast, IL-1ra treatment afforded no protection against excitotoxic injury in primary neuronal cultures (Strijbos and Rothwell, 1995; Rothwell and Strijbos, 1995). These conflicting data may provide clues about the mechanism of action of IL-1ra (see *Chapter Seven*).

In conclusion therefore, the fact that IL-1ra reduces neuronal damage induced by NMDA or AMPA receptor overactivation in the striatum, strongly supports a role of IL-1 in excitotoxic neurodegeneration. However, IL-1ra did not protect against cortical-induced neuronal damage when infused into the cortex. This observation (and see below) suggests that the striatum may be the neuronal structure involved in the initiation of neurotoxic effects of IL-1.

downstream

4.5.2 Effect of IL-1 β on excitotoxic damage

4.5.2.1 Striatal infusion of MGlutamate or S-AMPA - Striatal co-infusion of IL-1 β - Effect on striatal neuronal loss

relevant clue?

IL-1ra reduced the amount of neuronal death in the striatum after intrastriatal infusion of an NMDA or AMPA receptor agonist. Thus, it is feasible that IL-1 β infusion may result in an enhancement of damage within the striatum. Results presented in this chapter demonstrate that IL-1 β (at an activity of 650IU or 1300IU) did not increase (or decrease) neuronal loss in the striatum, when co-infused into the striatum with MGlutamate or S-AMPA. Damage in the striatum may be already maximal, although, the doses of excitotoxins chosen (7.5nmol MGlutamate and 10nmol S-AMPA) did not cause damage throughout the striatum, i.e. healthy striatal tissue was present, which could subsequently degenerate.

IL-1 β expression is increased in the striatum after local infusion of MGlu (Van Dam, Strijbos and Rothwell, unpublished data), although IL-1 production has not been quantified. Thus, sufficient IL-1 β may be released during striatal EAA receptor overactivation to maximally activate IL-1 receptors and, if this is the case, addition of exogenous cytokine will have no further effect. However, it should be noted that extensive ipsilateral cortical damage was produced when IL-1 β (1300IU but not 650IU) was co-infused intrastrially with S-AMPA, while the volume of striatal damage remained unchanged (see Section 4.5.2.3 for discussion).

role of
glutamate
receptor

4.5.2.2 Cortical infusion of MGlu or S-AMPA - Cortical or striatal infusion of IL-1 β -Effect on cortical neuronal loss

IL-1 β infusion into the cortex did not affect cortical damage caused by NMDA or AMPA receptor overactivation. However, when the excitotoxins (MGlu or S-AMPA) were infused into the cortex, and IL-1 β was administered into the ipsilateral or contralateral striatum, a dramatic and significant increase in cortical neuronal death was observed in both cases. These results suggest that IL-1 β enhances cortical neuronal damage by acting at a specific site within the striatum on either side of the brain. In other studies reporting exacerbation of neuronal damage by IL-1 β after cerebral ischaemia (Yamasaki *et al.*, 1995; Loddick and Rothwell, 1996), IL-1 β was injected into the cerebral ventricles, so could have acted at numerous sites.

The striatum receives most of its neuronal input from the cortex via glutamatergic cortico-striatal projection neurones. In addition, the striatum receives glutamatergic inputs from parts of the thalamus and most output neurones from the striatum ultimately lead back to the cortex via the thalamus (for reviews see Cotman *et al.*, 1987; Parent, 1990; Nieoullon and Kerkerian-Le Goff, 1992; Parent and Hazrati, 1995a, 1995b). In the striatum, neurotoxicity

induced by striatal infusion of EAA agonists is influenced by glutamatergic innervation from the cortex (e.g. Biziere and Coyle, 1978; McGeer *et al.*, 1978; Jhamandas *et al.*, 1994), suggesting a relationship between cortical and striatal neuronal activation. Furthermore, it has been reported that expression of IL-1 and its receptors are increased rapidly in the cortex in response to brain injury in the striatum (Yan *et al.*, 1992). Thus, IL-1 β may interact anterogradely with specific neuronal pathways between the striatum and the cortex, indirectly resulting in release of glutamate within the cortex and subsequent overactivation of EAA receptors and enhancement of neuronal death. Furthermore, IL-1 β may influence the cortico-striatal projection neurones and could possibly be retrogradely transported along these neurones (Laduron, 1995) to the cortex to interfere with neuronal death. As IL-1 β enhanced cortical damage when infused into the either the ipsilateral or contralateral striatum and 13% of cortical-striatal neurones also project to the contralateral striatum (McGeorge and Faull, 1989), this observation further suggests that IL-1 β may interact with neuronal pathways.

Alternatively, IL-1 β may diffuse from the striatum to another site within the brain to enhance cortical damage. It is unlikely that diffusion to the cortex itself can account for the exacerbation in neuronal death because firstly cortical infusion of IL-1 β did not affect neuronal loss in this region induced by intracortical infusion of MGlu or S-AMPA. Secondly, when IL-1 β was infused into the contralateral striatum, after cortical infusion of either excitotoxin, an enhancement of cortical neuronal loss was observed. In this case it is doubtful whether the IL-1 β would reach the cortex on the other side of the brain. Diffusion of IL-1 β from the ipsilateral or contralateral striatum to the hypothalamus may result in fever (see *Section 5.2.2.1* and Blatteis, 1990; Rothwell, 1991) and the rise in body temperature could increase cortical neurodegeneration (McDonald *et al.*, 1991). However, this can not explain why striatal co-infusion of IL-1 β with MGlu or S-AMPA failed to enhance striatal neuronal death (see *Section 4.5.2.1*), since the studies by McDonald *et al* (1991) demonstrated body temperature

effects striatal neuronal excitotoxic damage. Nevertheless, results discussed in *Section 4.5.2.3* demonstrate that cortical degeneration was observed after intrastriatal infusion of S-AMPA and IL-1 β . Thus, neuronal death in the cortex may be more susceptible to a rise in body temperature. Loddick and Rothwell (1996) reported that injection of IL-1 β (icv) exacerbates ischaemic brain injury in the rat (in the cortex and striatum) and is accompanied by a significant increase in body temperature. However, body temperature was not measured in the studies reported in this chapter, so its influence can not be confirmed. }?

As mentioned above, IL-1 β enhances cortical neuronal damage when infused into the striatum on either side of the brain. In both these situations IL-1 β may cause a fever (see *Section 5.2.2.1*) and thus provides evidence for the role of a rise in body temperature in mediating the increase in cortical neuronal loss. However, it must be stressed that the involvement of IL-1 β interacting with specific neuronal pathways between the striatum and the cortex, to enhance cortical neurodegeneration, must not be ruled out. It is possible that IL-1 β injected in the contralateral striatum may induce responses in the striatum (and then the cortex) on the opposite side of the brain, via neuronal connections (McGeorge and Faull, 1989).

In summary, therefore, when IL-1 β was co-infused with either MGlu or S-AMPA it did not affect the local neuronal death at the site of injection, but co-infusion of IL-1 β and S-AMPA into the striatum caused massive cortical damage (see *Section 4.5.2.3* for discussion). In addition, IL-1 β infused into either the ipsilateral or contralateral striatum, enhanced cortical damage induced by cortical infusion of MGlu or S-AMPA. These results suggest that IL-1 β acts at specific site(s), within the brain, possibly the striatum, to augment excitotoxic damage.

Table 4.5.1 *Summary of effects of IL-1 α /IL-1 β on EAA receptor-mediated neuronal damage in the striatum and cortex*

	STRIATUM		CORTEX	
	NMDA	AMPA	NMDA	AMPA
IL-1α Co-infusion	46% inhibition	43% inhibition	No effect	No effect
IL-1β Co-infusion	No effect	No effect in striatum, but appearance of massive cortical lesion	No effect	No effect
Ipsilateral striatal IL-1β infusion	---	---	261% increase	295% increase
Contralateral striatal IL-1β infusion	---	---	278% increase	100% increase

4.5.2.3 *Striatal infusion of S-AMPA - Striatal infusion of IL-1 β*
-Effect on cortical neuronal loss

Possible explanations for the absence of effect of IL-1 β on striatal neuronal loss, induced by intrastriatal infusion of S-AMPA (or MGlu) and IL-1 β , have been discussed above (see *Section 4.5.2.1*). However, the cortical neurodegeneration observed after S-AMPA and IL-1 β infusion into the striatum has not been discussed in detail. This extensive ipsilateral cortical damage was specific for striatal AMPA receptor overactivation since cortical neuronal loss was not observed when IL-1 β was intrastriatally co-infused with MGlu. The mechanism(s) of action of IL-1 β which result in cortical damage after striatal AMPA receptor overactivation remain to be investigated. The possibility of diffusion of S-AMPA directly to the cortex can be disregarded since extensive cortical damage was never seen in animals in which S-AMPA (10-15nmol) was infused intrastriatally, or when the same doses of S-AMPA were infused directly into the cortex.

It seems unlikely that fever contributed to the cortical neuronal loss after infusion of IL-1 β and S-AMPA into the striatum. Cortical neurodegeneration was absent in animals treated with MGlu and IL-1 β intrastriatally, even though IL-1 β would be expected to cause a rise in body temperature in these animals (see *Section 5.2.2.1*). However, the influence of changes in body temperature in ischaemic brain injury, may depend on the severity of the ischaemic insult (Ginsberg *et al.*, 1993). The data presented in this thesis have shown that, at the doses tested (MGlu=7.5nmol, S-AMPA=10nmol), the average volume of MGlu-induced striatal lesions was approximately half that of S-AMPA-induced striatal death (5.51mm³ versus 11.53mm³ respectively). The effects of IL-1 β on cortical damage in animals with more extensive NMDA receptor-induced lesions in the striatum, remain to be tested. In addition, peripheral administration of MK-801 reduced the amount of cortical neuronal death induced by intrastriatal co-infusion of IL-1 β and S-AMPA. Some authors have ascribed the neuroprotective effects of this

NMDA receptor antagonist, in a variety of insults, to its hypothermic effects (see Ginsberg *et al.*, 1993). Hence the protective effect of MK-801 against the cortical damage in this study, could result from attenuation of IL-1 β -induced hyperthermia.

An other important clue to the question of why IL-1 β induces cortical damage after intrastriatal co-infusion with S-AMPA, but not MGlutamate, may be the different patterns of damage induced by AMPA and NMDA receptor overactivation in the striatum (in the absence of IL-1 β). As previously discussed (in *Chapter Three*), extrastriatal damage is sometimes observed in the ipsilateral piriform cortex following striatal AMPA receptor overactivation, but does not occur after striatal NMDA receptor overactivation. This extrastriatal damage could be due to seizure activity resulting from activation of specific pathways, which may form part of the limbic circuit (Ben-Ari *et al.*, 1980; Ben-Ari, 1985; Okazaki and Nadler, 1988), or possibly neuronal pathways from the striatum, which lead back indirectly to the cortex via the substantia nigra and/or thalamus. Activation of thalamo-cortical fibres is known to produce a potent glutamate-mediated excitatory response in cortical neurones (Parent and Hazrati, 1995a, 1995b). It is possible, therefore, that IL-1 β enhances this initial striatal neuronal activation to a level that produces additional recruitment of thalamo-cortical fibres, and then results in neuronal damage in their target nuclei throughout the cortex. Neuronal loss was also observed in the thalamus in some animals which had extensive cortical damage, supporting the proposal of the involvement of these neuronal pathways. However, it is important to note that not all animals exhibiting cortical damage had neuronal loss in the piriform cortex, or degeneration of the thalamus, although neuronal pathways to the thalamus/piriform cortex may still be activated without resulting in extensive cell loss in these regions. A very small percentage of animals infused with S-AMPA and vehicle in the striatum had limited neuronal loss in the thalamus. Damage in this area was much restricted compared to animals treated with IL-1 β , suggesting that IL-1 β may enhance the mechanisms

leading to thalamic damage.

Striato-cortical connections are mediated mainly by via the substantia nigra and/or thalamus (Parent and Hazrati, 1995a, 1995b). However, it has also been reported that direct striato-cortical connections may exist in the rat, via cholinergic neurones (Sloniewski *et al.*, 1986; Divac *et al.*, 1987; Parent, 1990). IL-1 β could activate these putative pathways during AMPA receptor stimulation, although as these neurones utilise acetylcholine they are unlikely to cause neuronal damage directly. However, acetylcholine has been shown to potentiate glutamate-induced neurodegeneration in cultured hippocampal neurones (Mattson, 1989).

In the present study, striatal damage was measured 48h after S-AMPA infusion, as the striatal damage observed at 24h was incomplete (and see *Chapter Three*), whereas MGlu-induced damage was maximal after 24h. However, no cortical damage was seen 48h after striatal co-infusion of MGlu and IL-1 β . Furthermore, striatal infusion of S-AMPA and IL-1 β resulted in cortical damage within 24h. Hence, the response in the cortex is not simply delayed, and may be due to NMDA receptor activation (see later).

The results of this study suggest that the magnitude of cortical damage induced by striatal infusion of S-AMPA and IL-1 β 48h later is significantly related to the size of the striatal lesion. However, these data must be interpreted with caution. The correlation at 48h post infusion may not be necessarily causal as the size of the cortical lesion could depend on the specific areas/cells of the brain affected, and not on the size of the striatal lesion. There was no correlation between striatal and cortical lesion volume 24h after infusions, but this may reflect the fact that striatal damage was not fully developed at this time.

It is proposed that the predominant receptor mediating neuronal loss in the

cortex after intrastriatal infusion of IL-1 β and S-AMPA, is the NMDA receptor subtype. Several pieces of data support this proposal. Firstly, neurodegeneration in the cortex appears to be complete 24h after infusions (even though striatal neuronal loss is incomplete) as the amount of cortical damage 48h later is not significantly different. Results reported in this chapter and in *Chapter Three*, indicate that NMDA receptor-induced neuronal death is maximal at 24h after induction, whereas AMPA receptor-induced excitotoxicity is incomplete at this time point and is significantly more extensive 48h later. Secondly, neuronal death in the cortex, but not the striatum, was reduced (67%) by the NMDA receptor antagonist, MK-801, suggesting that NMDA receptor activation mediates cortical damage, and that non-NMDA receptors cause neuronal loss at the site of injection (striatum). This second piece of evidence also supports the involvement of a "seizure-related" pathway in secondary cortical damage caused by IL-1 β (see below).

As previously discussed (in *Section 3.7.6*), several authors have reported that MK-801 (or other NMDA receptor antagonists) can inhibit non-NMDA-induced, seizure-related brain damage at distant sites (but not the damage at the site of injection), and suggested that distant damage is dependent on NMDA receptors (Clifford *et al.*, 1990; Lerner-Natoli *et al.*, 1991; Ciani *et al.*, 1994; Wozniak *et al.*, 1994). However, the results presented here are inconclusive because, although MK-801 inhibited the overall cortical neuronal loss in response to intrastriatal infusion of IL-1 β and S-AMPA, it can not be concluded that this antagonist reduced piriform cortex damage (2/7 animals for control compared to 3/8 for MK-801). In fact MK-801 appeared to increase the occurrence of the distant neuronal loss in the thalamus (4/7 animals for control compared to 7/8 for MK-801).

In conclusion it is proposed that the neuronal death in the cortex after intrastriatal infusion of IL-1 β and S-AMPA is initiated via an NMDA receptor-

dependent pathway, and/or that the damage itself is mediated by cortical NMDA receptors.

4.6 Summary

Taken together, the results in this chapter have demonstrated an involvement of IL-1 in the excitotoxic neuronal death induced by overactivation of both NMDA and AMPA receptors. Furthermore, it appears that IL-1 acts at a specific site, the striatum, to predominantly affect neuronal loss in the cortex.

Chapter Five

Direct effects of interleukin-1 β

Chapter Five

Direct effects of interleukin-1 β

5.1 Introduction

IL-1 has diverse actions within the CNS. One of the primary actions of this cytokine is its ability to produce an increase in body temperature (Rothwell, 1990; Blatteis, 1992), and icv injection of IL-1 β (5-10ng) produces marked fever in rats (see Rothwell, 1991). Hyperthermia has been proposed to affect various types of neuronal injury (Ginsberg *et al.*, 1993; Bowyer *et al.*, 1994) and the severity of excitotoxic brain injury (NMDA and AMPA receptor-mediated) is thought to be dependent, at least in part, on brain temperature after the excitotoxic insult (e.g. McDonald *et al.*, 1991). Since IL-1 causes a rise in body temperature, and assuming this increase is positively related to brain temperature (Opp and Krueger, 1991; Opp *et al.*, 1991), IL-1 may have detrimental effects on the outcome of excitotoxic neuronal injury (seen in *Chapter Four*) through an indirect effect on body temperature. The first aim of this study, therefore, was to ascertain whether IL-1 β alone, caused a rise in body temperature after injection into either the striatum or the cortex (parietal). In addition, as it is not yet known if IL-1 can induce neuronal damage directly in the absence of other insults, the second aim of this study was to examine the effect of IL-1 β alone on neuronal viability after infusion into the striatum or cortex, in the same group of rats.

5.2 Infusion of IL-1 β into the brain

The aim of this study was to examine the effect of IL-1 β on core body temperature, infused into either the striatum or the cortex. In the same group of

rats the viability of striatal or cortical neurones was examined after infusion of IL-1 β into these areas.

5.2.1 Experimental

Temperature-sensitive, radiotransmitters were implanted into the abdominal cavity of anaesthetised rats at least three days before the start of the experiment, following the protocol in *Section 2.8.1*. Core body temperature recordings were carried out (as in *Section 2.8.2*) 24h before the start of all stereotaxic infusions. Animals were anaesthetised and infusions performed as in *Section 2.3.1*. Saline (2 μ l, n=5) or human IL-1 β (5ng in 2 μ l, equivalent to 1300IU, n=5) was infused into the striatum or the cortex. The animals were allowed to recover from anaesthesia for approximately 1.5h before being returned to their individual cages located on the receiver pads. Core body temperature was recorded for a further 12h. Animals were sacrificed (see *Section 2.4*) 24h after infusion and the brains carefully removed, immersed fixed and processed (as in *Section 2.5.2*). Neuronal viability was assessed by staining 20-30 μ m coronal sections with haematoxylin and eosin, or neutral red (see *Appendix Four* or *Five*). The resulting effect of infusions on body temperature was obtained by calculating the change in temperature (in $^{\circ}$ C), compared to the temperature at the same time point, the previous day.

In separate experiments, saline (2 μ l, n=5) or human IL-1 β (5ng in 2 μ l, equivalent to 1300IU, n=5) was infused into the striatum of rats which were sacrificed three days later, and their brains processed as above. Temperature readings were not recorded in this set of experiments.

5.2.2 Results

5.2.2.1 Temperature

Animals which received cortical infusions of IL-1 β or saline, or striatal infusion of saline showed no obvious changes in behaviour. However, animals intrastrially infused with IL-1 β developed a hunched posture and piloerection. Baseline core body temperatures prior to cerebral infusions ranged between 36.6-37.2°C, and were comparable within, and between experimental groups. Striatal injection of IL-1 β (5ng=1300IU) resulted in a significant rise in body temperature (MANOVA, $P < 0.001$, compared to striatal infusion of saline), which started 2h after infusion, peaked at 4h (~2°C maximum), and returned to baseline values 9h after infusion. In contrast, striatal infusion of saline did not result in a significant change in temperature during the course of the experiment. Although during the 3h following striatal infusion of saline there was a slight reduction in body temperature (~-0.5°C maximum), which had returned to baseline values 4h later. Cortical infusion IL-1 β (5ng=1300IU) or saline had no significant effect on body temperature throughout the 12h period. However, a reduction in body temperature was observed in the 1.5-4h after cortical infusion of IL-1 β (~-1.0°C maximum) or saline (~-0.75°C maximum), which returned to baseline after 4.5-6h. See *Figure 5.2.1* for the effect of intrastriatal or intracortical injection of IL-1 β or saline on body temperature.

5.2.2.2 Histology

Striatal or cortical infusion of IL-1 β both produced neuronal death (measured at 24h) in the area immediately surrounding the tip of the injection tract (approximate volume <1mm³). This damage was generally more extensive in the cortex. However, infusion of saline into either the striatum or the cortex also produced localised neuronal damage of similar magnitude. There was no

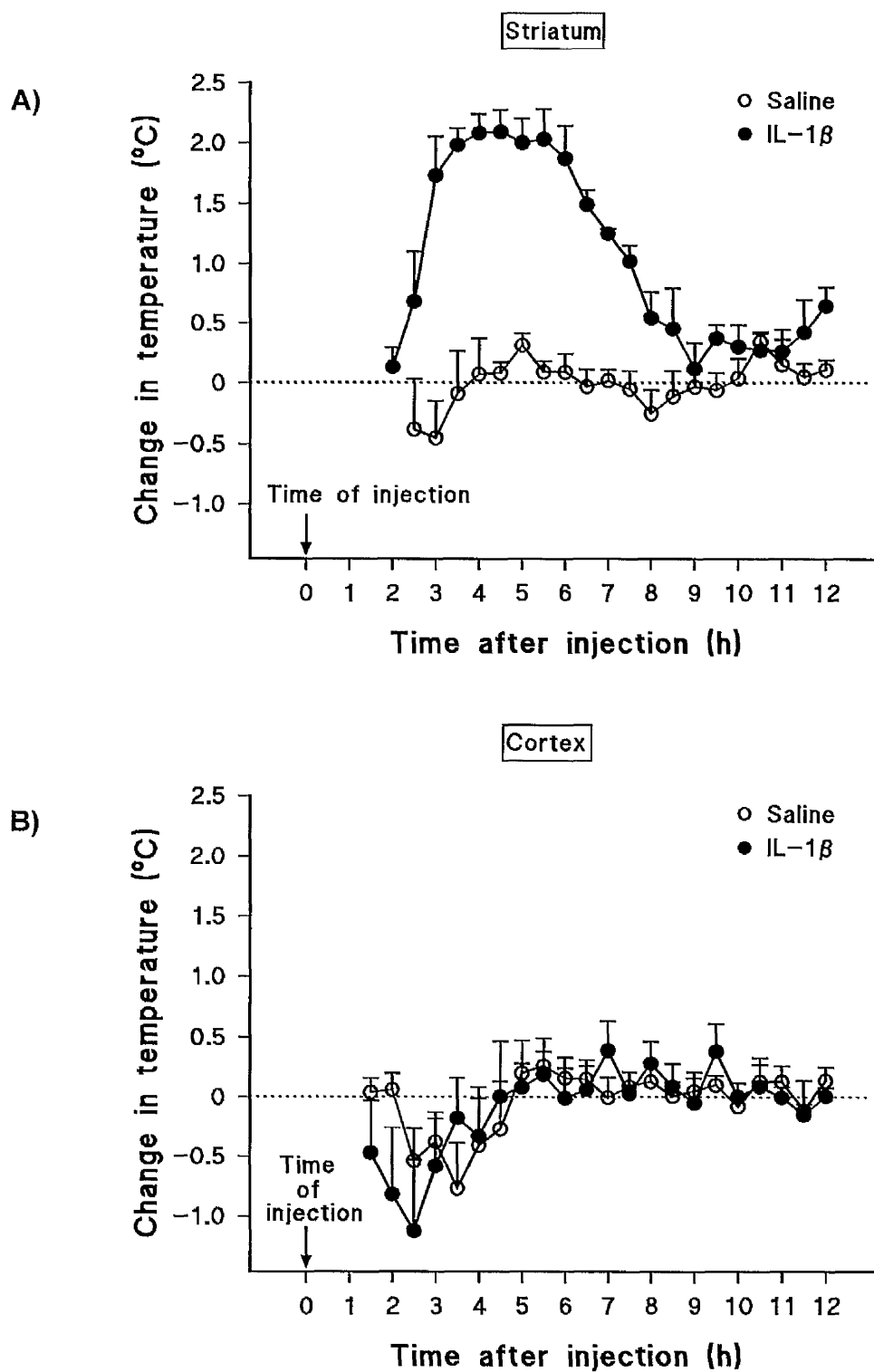


Figure 5.2.1 Effect striatal or cortical infusion of IL-1 β on core body temperature

Change in core body temperature (°C, relative to temperature at the corresponding time points, 24h earlier), 0-12h after intrastriatal (A) or intracortical (B) infusion of either saline (2 μ l, open circles, n=5) or IL-1 β (1300IU, solid circles, n=5). Striatal infusion of IL-1 β produced a significant increase in body temperature compared to striatal infusion of saline ($P < 0.001$, MANOVA).

obvious difference in the extent of neuronal death observed after infusion of IL-1 β , compared to saline, in either area of the brain. Cells resembling glia were sometimes observed in and around the area of neuronal death, and around the needle tract (see Figure 5.2.2a-b and 5.2.3a-b).

Three days after intrastriatal infusion of IL-1 β , neuronal damage was observed at the tip, and around the injection cannula. Again there was no difference in the extent of damage between IL-1 β - and saline-treated groups. However, there was histological evidence of glial scar formation corresponding to the area of neuronal cell death (see Figure 5.2.4a-b), in both saline- and IL-1 β -treated animals, which was not present 24h after infusions (see Figure 5.2.2a-b).

5.3 Discussion

5.3.1 *Pyrogenic effects*

One of the primary actions of IL-1 in the brain is the induction of fever. Both forms of IL-1 (α and β) are involved in the generation of fever (rise in core body temperature) predominantly via an action on the hypothalamus (Moltz, 1993). It has been shown previously that icv injection of 5-10ng IL-1 β elicits maximal increases in body temperature in the rat *in vivo* (Dascombe *et al.*, 1988; Busbridge *et al.*, 1989; Rothwell, 1991; Kent *et al.*, 1992).

The standard method of measuring changes in temperature after experimental manipulation is the change over a period of time from the initial time of infusions, assuming the start of the experiment (time of infusion) is at the same time point for all animals. However, in this study animals did not receive infusions at the same time point, but at different periods throughout the day. Therefore, it would be impossible to pool together the data from all animals in one experimental

Figure 5.2.2a

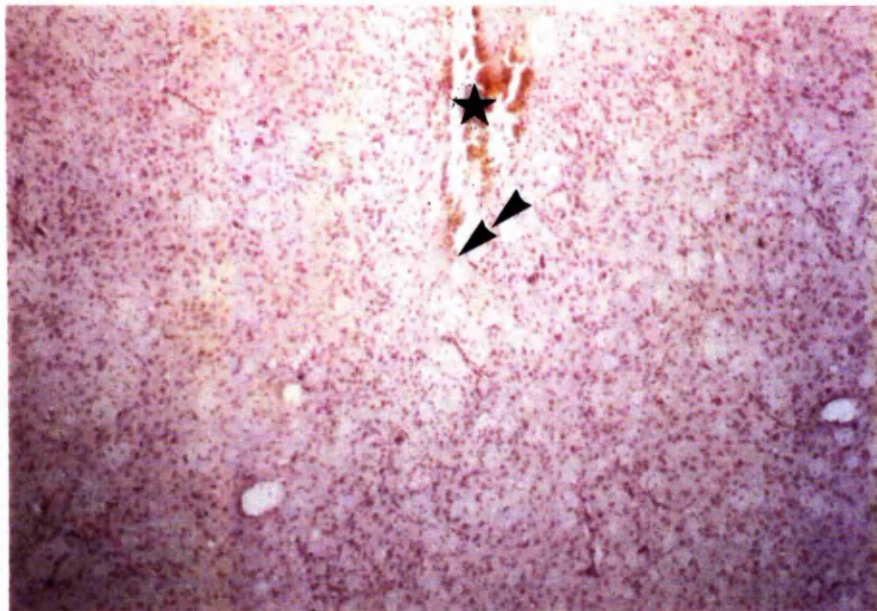
Light micrographs of 30 μ m neutral red-stained, coronal sections of rat brain 24h after intrastriatal infusion of **A)** 2 μ l saline, or **B)** IL-1 β (5ng=1300IU). **C)** Striatum of a normal (uninjected) rat brain.

All sections are representative of a group of five animals.

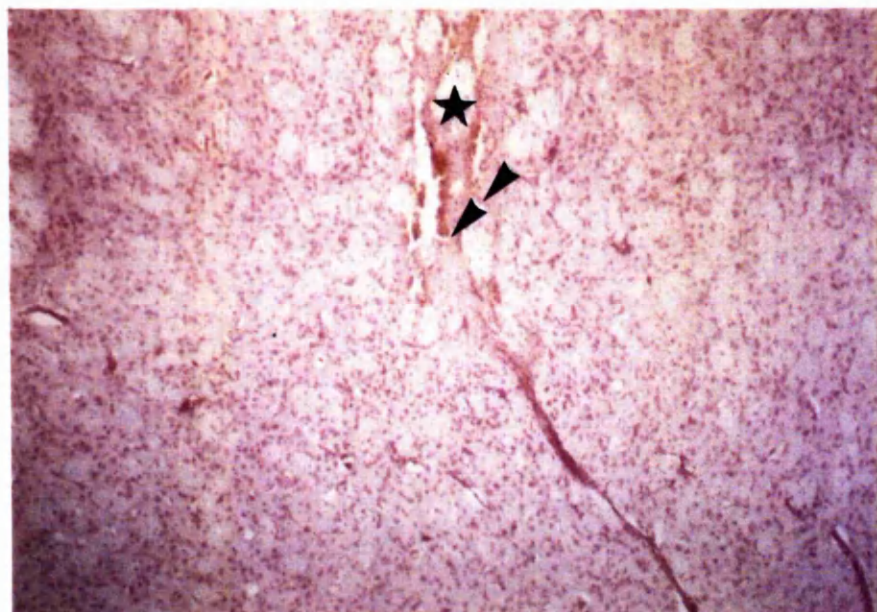
Double arrow heads indicate the sites of injection and the asterisks show the needle tracts.

Magnification bar=170 μ m

A



B



C

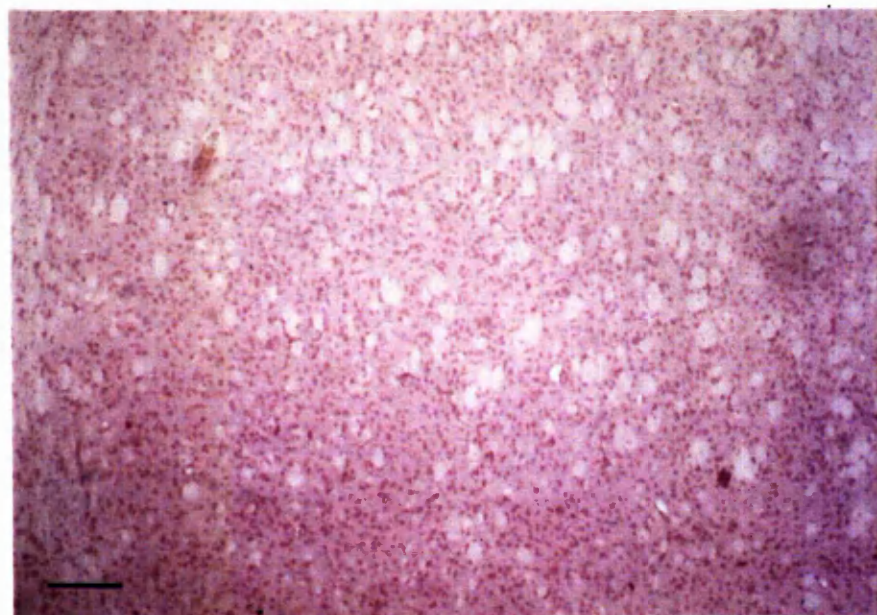


Figure 5.2.2b

Light micrographs of 30 μ m neutral red-stained, coronal sections (the same region as in *Figure 5.2.2a*, but at higher magnification) of rat brain 24h after intrastriatal infusion of **A)** 2 μ l saline, or **B)** IL-1 β (5ng=1300IU).

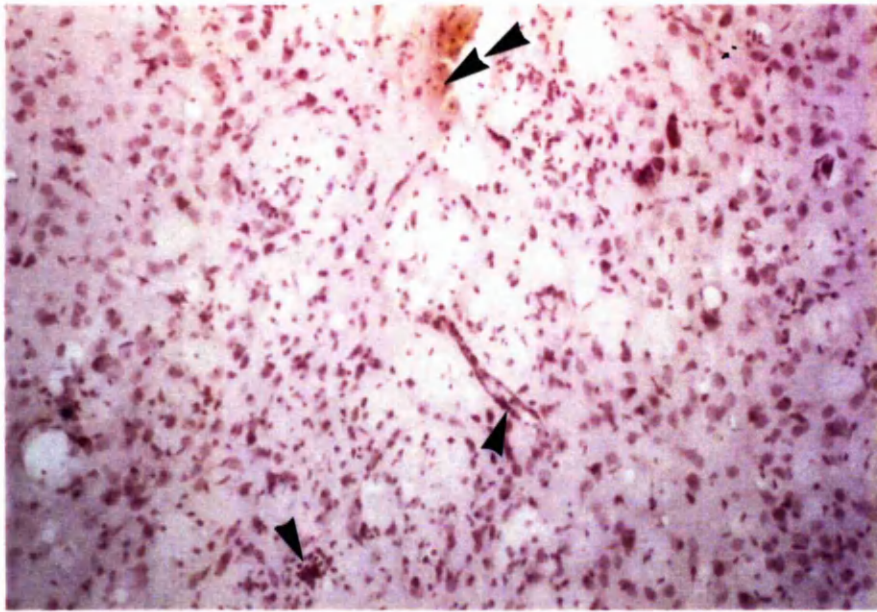
Note the small dot-like cells (arrow heads) in the vicinity of the injection site and along the blood vessels. These cells probably represent glial cells. Note also the lack of healthy neurones in the area surrounding the injection site. **C)** Striatum of a normal (uninjected) rat brain, containing many healthy neurones (arrows). All sections are representative of a group of five animals.

The pale round areas represent fibre bundles (dots).

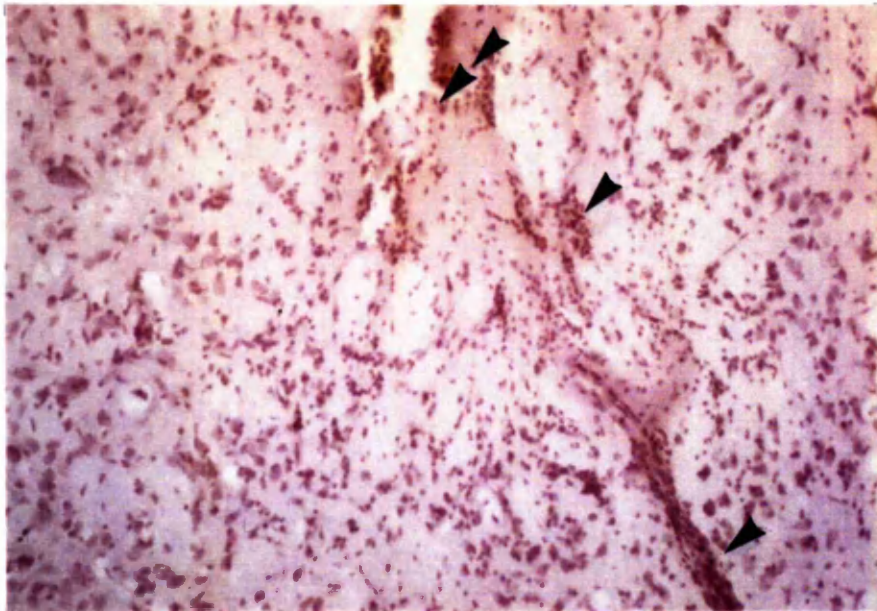
Double arrow heads indicate sites of injection.

Magnification bar=70 μ m

A



B



C

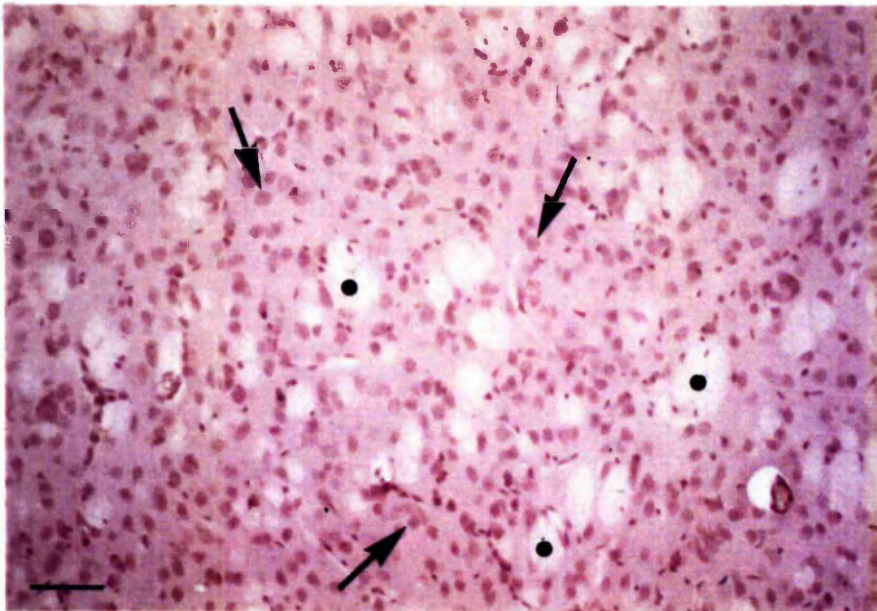
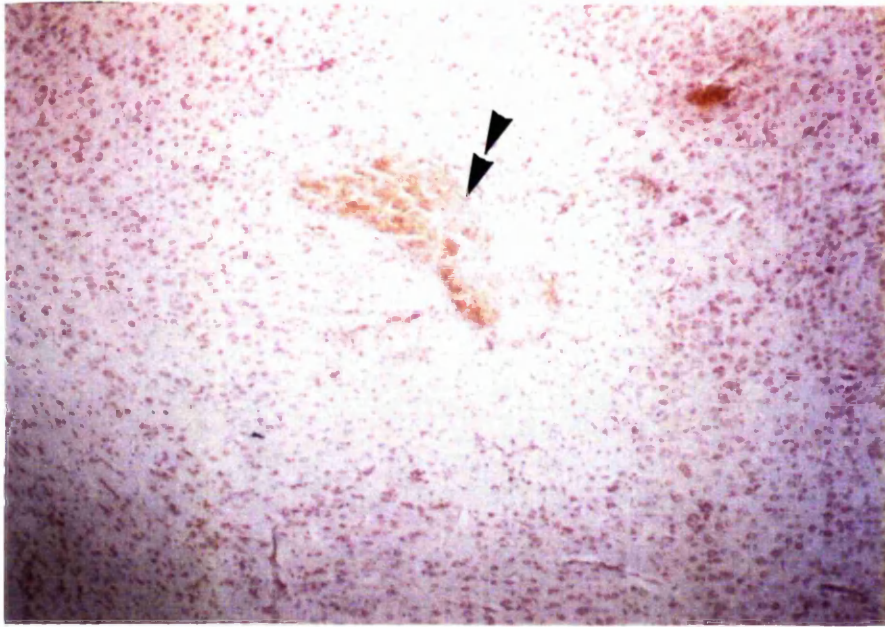


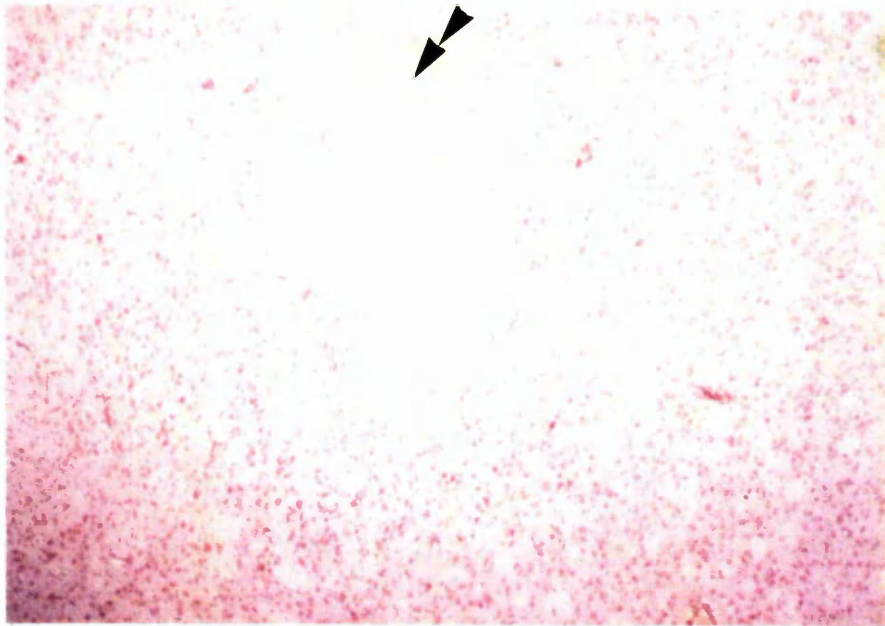
Figure 5.2.3a

Light micrographs of 30 μ m neutral red-stained, coronal sections of rat brain 24h after intracortical infusion of **A)** 2 μ l saline, or **B)** IL-1 β (5ng=1300IU). Note the paler staining of the area surrounding the injection site, characteristic of damaged tissue. **C)** Contralateral cortex of a normal (uninjected) rat brain. All sections are representative of a group of five animals. C=Cortex, CC=corpus callosum, St=striatum. Double arrow heads indicate sites of injection. Magnification bar=170 μ m

A



B



C

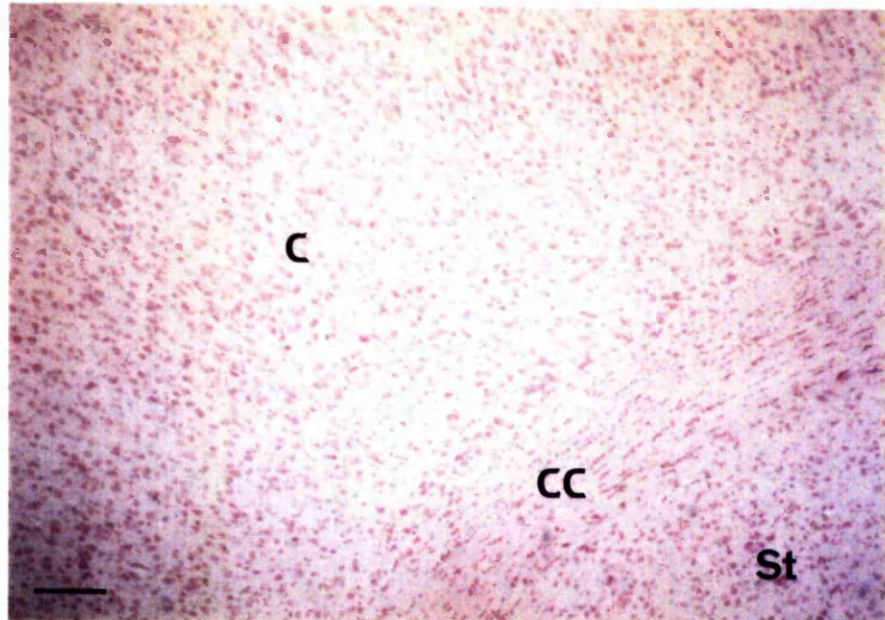


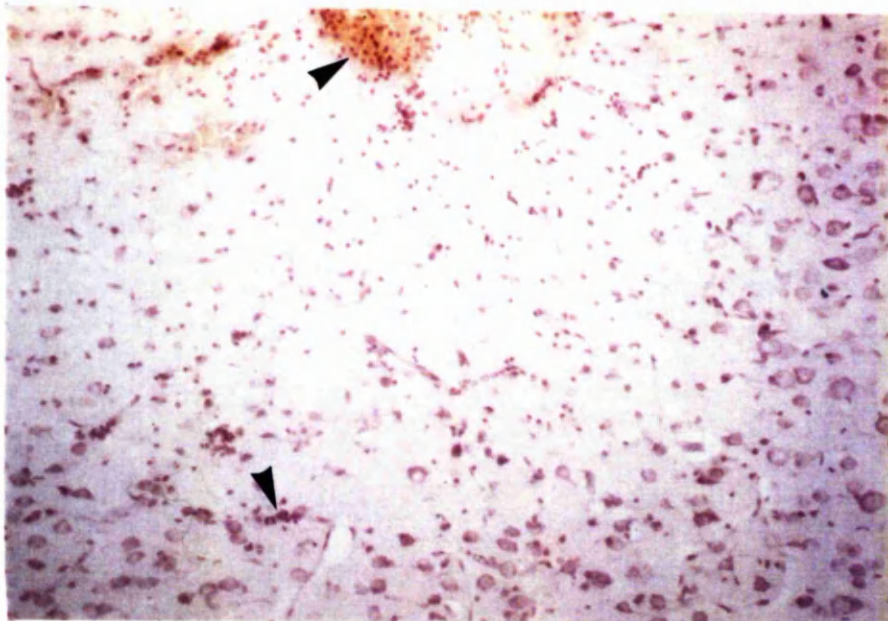
Figure 5.2.3b

Light micrographs of 30 μ m neutral red-stained, coronal sections (the same region as in *Figure 5.2.3a*, but at higher magnification) of rat brain 24h after intracortical infusion of **A)** 2 μ l saline, or **B)** IL-1 β (5ng=1300IU). Note the small dot-like cells (arrow heads) in the vicinity of the injection site and along the blood vessels. These cells probably represent glial cells. Note also the lack of healthy neurones in the area surrounding the injection site. **C)** Contralateral cortex of a normal (uninjected) rat brain, containing many healthy neurones (arrows).

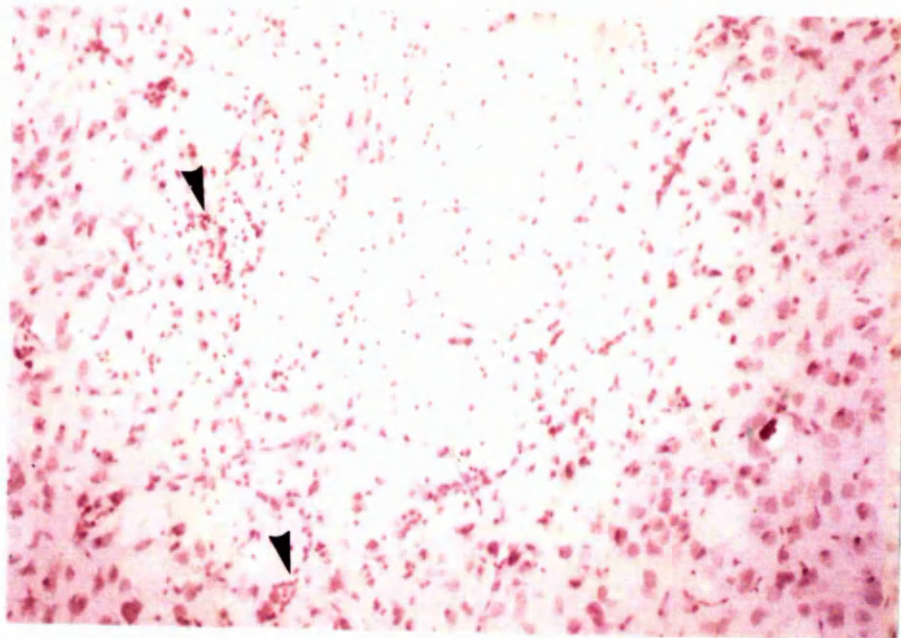
All sections are representative of a group of five animals.

Magnification bar=70 μ m

A



B



C

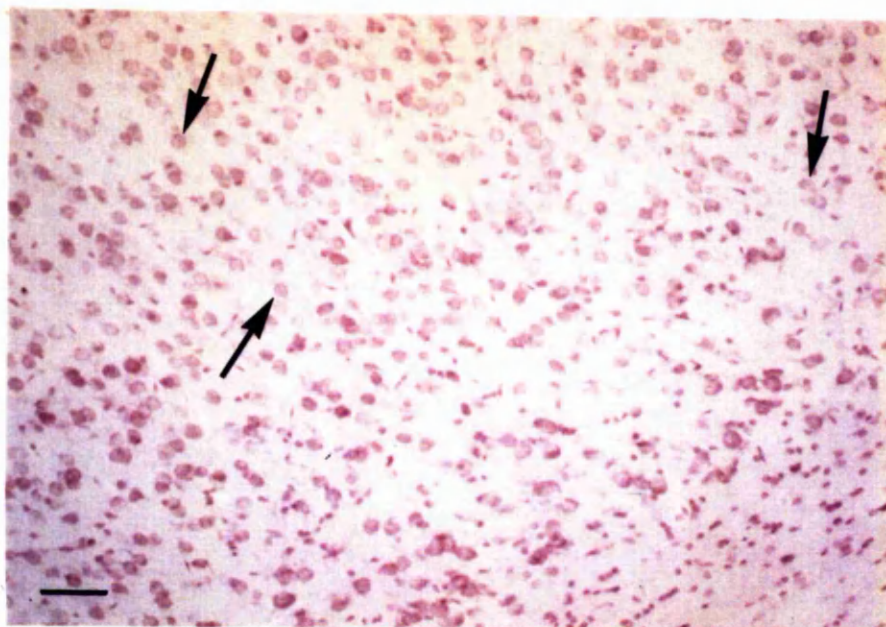


Figure 5.2.4a

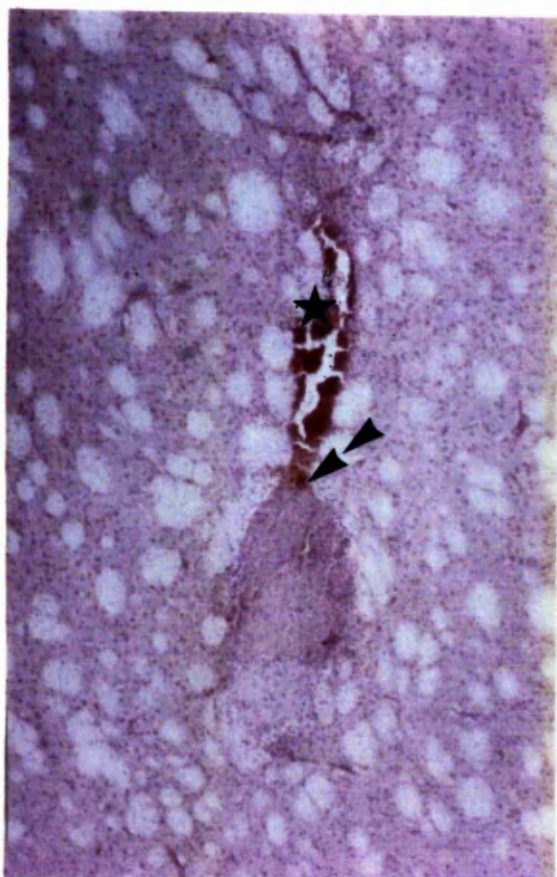
Light micrographs of 20 μ m haematoxylin and eosin-stained coronal, sections of rat brain three days after intrastriatal infusion of **A)** 2 μ l saline, or **B)** IL-1 β (5ng=1300IU). Note that in contrast to 24h post infusion (see *Figure 5.2.2a*), this area of neuronal damage is more clearly defined. This is likely to be due to the formation of a glial scar. **C)** Striatum of normal (uninjected rat brain). All sections are representative of a group of five animals.

The pale, round areas represent fibre bundles (dots).

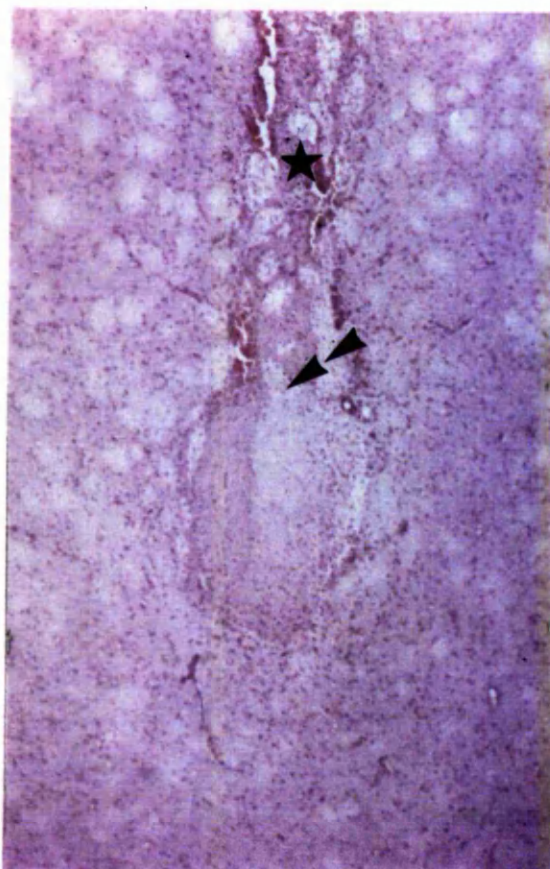
Double arrow heads indicate sites of injection and asterisks show the needle tracts.

Magnification bar=170 μ m

A



B



C

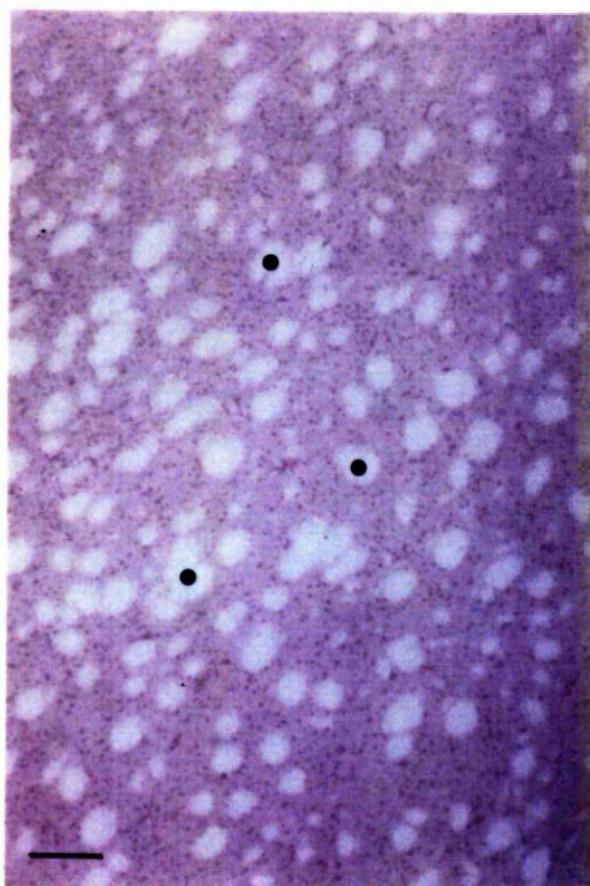


Figure 5.2.4b

Light micrographs of 20 μ m haematoxylin and eosin-stained coronal, sections of rat brain (the same region as in *Figure 5.2.4a*, but at higher magnification) three days after intrastriatal infusion of **A)** 2 μ l saline, or **B)** IL-1 β (5ng=1300IU). Note the small dot-like cells (arrow heads) predominantly surrounding the area of neuronal death. These cells probably represent glia and invading immune cells. Note also the lack of healthy neurones in the area surrounding the injection site and that in contrast to 24h post infusion (see *Figure 5.2.2b*), this area of neuronal damage is more clearly defined. This is likely to be due to the formation of a glial scar. **C)** Striatum of normal (uninjected rat brain). Healthy neurones are indicated by arrows.

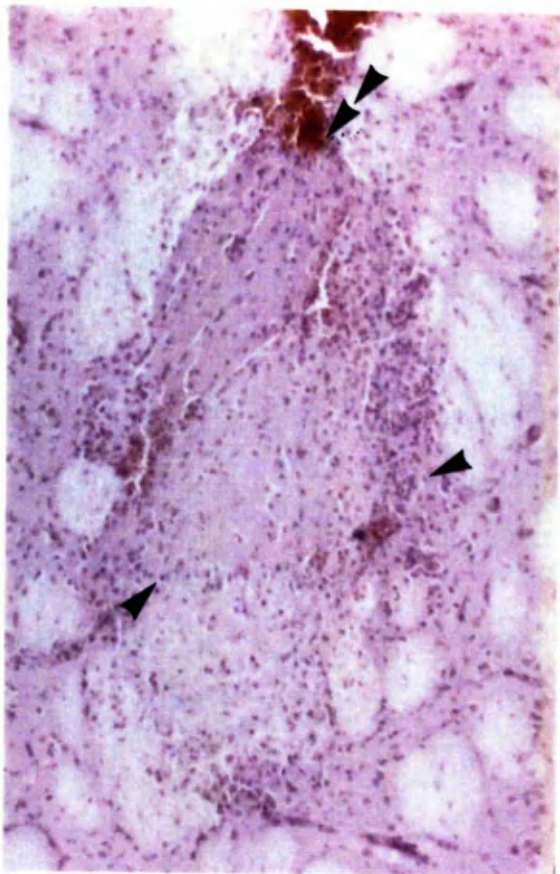
All sections are representative of a group of five animals.

The pale round areas represent fibre bundles (dots).

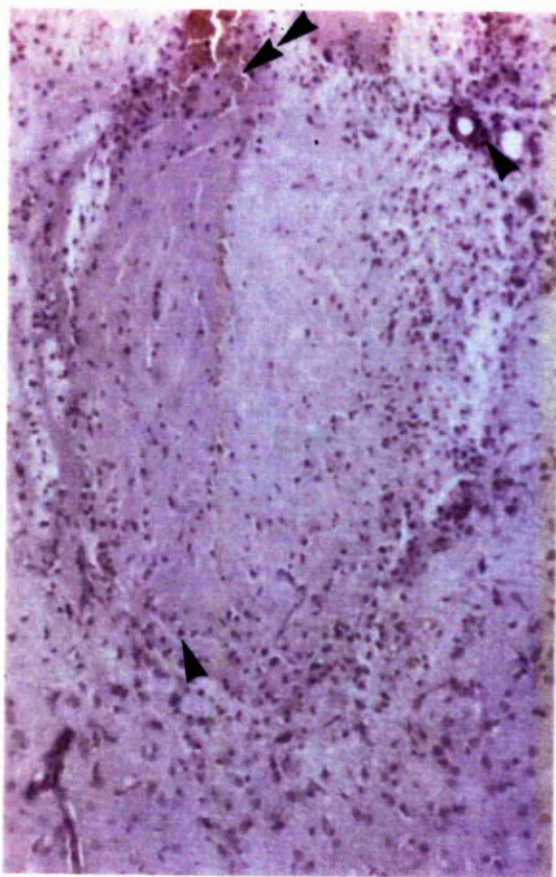
Double arrow heads indicate the site of injection.

Magnification bar=70 μ m

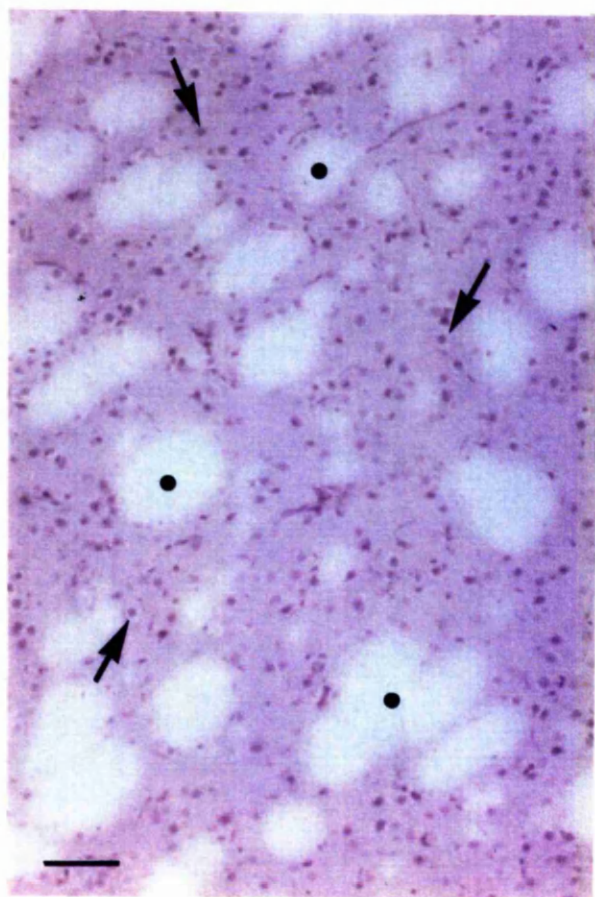
A



B



C



group. In addition, the body temperature of a rat increases towards the evening (~18.00h), as these rodents are nocturnal animals and their activity increases during this time. Hence to avoid these problems and the effect of circadian rhythm-induced fluctuations in temperature, changes in temperatures after infusion of IL-1 β or saline, were calculated by comparison to those, at the same time point, on the previous day.

The severity of excitotoxic brain injury, induced by either NMDA or AMPA receptor overactivation, can be influenced by body temperature (McDonald *et al.*, 1991). Thus, if IL-1 β modifies body temperature after injection into the parietal cortex or the striatum, this may alter the outcome of excitotoxic damage which is induced in these areas. The results in this chapter clearly demonstrate that striatal, but not cortical, injection of IL-1 β resulted in a significant increase in body temperature (~2°C). The initial (1.5-3h) reduction in temperature observed after intrastriatal infusion of saline, or intracortical infusion of saline or IL-1 β , is likely attributed to effects of the anaesthetic. Hypothermia is one of the major hazards associated with pentobarbitone anaesthesia (Green, 1979; Flecknell, 1987) predominantly due to depression of the hypothalamic heat-regulating mechanisms. Hence, during the first few hours after induction of anaesthesia, core body temperature will be reduced until complete recovery from the anaesthetic is achieved. However, animals infused intrastriatally with IL-1 β did not have an initial hypothermic phase. This phase is likely to be offset by the pyrogenic effect of IL-1 β after infusion into the striatum.

The hypothalamus is the main thermoregulatory centre in the brain and direct injection of low concentrations (picogram quantities) of IL-1 β into the hypothalamus induces fever in the rat (see Blatteis, 1990; Rothwell, 1991). However, injection of IL-1 in other brain regions, for example, midbrain reticular formation, pons and medulla oblongata also produces fever in several species (Blatteis, 1980; Blatteis *et al.*, 1988; Blatteis, 1992), suggesting that other sites

may mediate pyrogenic responses. Furthermore, Linthorst *et al* (1994) reported a rise in body temperature after intrahippocampal injection of IL-1 β in the rat, but no effect was observed after injection into the parietal cortex. The results reported in the present chapter, show that intracortical injection of IL-1 β does not modify body temperature, although a rise in temperature was seen after injection IL-1 β into the striatum, indicating that the striatum may also be a site of action in mediating the pyrogenic effects of IL-1 β . }

The mechanism(s) underlying the IL-1 β -induced rise in body temperature after injection into the striatum are unclear. The possibility of diffusion of IL-1 β into the CSF or to the hypothalamus can not be excluded. Sufficient IL-1 β may diffuse, via a direct or indirect route (e.g. via CSF), to the hypothalamus (the principal site of the pyrogenic action of IL-1), thus eliciting a rise in body temperature. This may explain the absence of a pyrogenic effect of IL-1 β after injection into the parietal cortex, due to the greater distance of this structure from the hypothalamus.

Alternatively, IL-1 β may induce a pyrogenic response after infusion into the striatum via activation of specific neuronal pathways between the striatum and the hypothalamus. The exact neuroanatomical connections involved in the pyrogenicity of IL-1 β responses in the striatum are unknown, although certain hypotheses can be proposed. Neuronal pathways have been demonstrated between the hypothalamus and parts of the ventral striatum (Brog *et al.*, 1993). Therefore, it is possible that retrograde axonal transport of IL-1 β may occur along these neurones (Patterson and Nawa, 1993; Laduron, 1995), resulting in a pyrogenic response mediated through the hypothalamus.

The bed nucleus of the stria terminalis (BST) is a forebrain structure which has been implicated in the regulation of body temperature (Kasting, 1989; Moltz, 1993) and is in close proximity to the striatum. IL-1 β has excitatory effects on

neurones of the BST (Wilkinson *et al.*, 1993), and infusion of IL-1 β (picogram quantities) into the BST results in significant increases in body temperature in the rat *in vivo* (Wilkinson *et al.*, 1994). The pyrogenicity of intrastriatal infusion of IL-1 β may, therefore, occur after its diffusion to, and significant activation of, the BST.

5.3.2 Neuronal viability

Infusion of IL-1 β into the striatum or cortex did not result in overt, but very localised, neuronal death at the site of injection. However, there was no obvious difference in the extent of neuronal damage between saline and IL-1 β treatment. Needle insertion or pressure by the infusate, resulting in mechanical injury, are the most likely explanations for the neuronal damage observed after both saline and IL-1 β infusion. Ingvar *et al* (1994) have reported a similar pattern of neurodegeneration after infusion of buffer (PBS) into the cortex.

IL-1 induces astrogliosis and neovascularisation in the rat *in vivo* (Giulian *et al.*, 1988). Because no specific staining for glia was performed, it can not be concluded if intracerebral infusion of IL-1 β resulted in significant gliosis. However, cells resembling glia or invading macrophages were observed around the area of neuronal death and along the needle tract, at 24h after striatal or cortical infusion of saline or IL-1 β and at three days after striatal infusion of saline or IL-1 β . In support of this observation, glial proliferation has been reported after striatal (Bourdiol *et al.*, 1991; Töppler *et al.*, 1993) or cortical (Giulian and Lachmann, 1985; Giulian *et al.*, 1988; Bourdiol *et al.*, 1991; Sievers *et al.*, 1993; Ingvar *et al.*, 1994) infusion of IL-1 β or vehicle in rats *in vivo*. In addition to glial proliferation, a glial scar appeared to be evident at three days post intrastriatal infusion of saline or IL-1 β . Scar formation is normally observed after a penetrating injury to the CNS (e.g. see Maxwell *et al.*, 1990), and has been reported in response to intracerebral infusion of IL-1 β or vehicle (Giulian

5.4 Summary

Striatal, but not cortical, infusion of IL-1 β induced a rise in body temperature. The mechanism of this response is unknown. The fever observed may explain the exacerbation of cortical excitotoxic damage (induced by cortical infusion of EAA receptor agonists) following striatal infusion of IL-1 β (see *Chapter Four*).

However, even though this rise in body temperature may be involved in the actions of IL-1 (observed in *Chapter Four*), it is insufficient to induce overt neuronal death directly after intrastriatal infusion of IL-1 β alone.

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Chapter Six

**Use of transgenic/knock-out mice to study the involvement
of IL-1 in NMDA receptor-induced lesions**

Chapter Six

Use of transgenic/knock-out mice to study the involvement of IL-1 in NMDA receptor-induced lesions

6.1 Introduction

Earlier work reported in this thesis, demonstrated the involvement of IL-1 in excitotoxic neuronal damage induced by overactivation of NMDA or AMPA receptors in the rat (see *Chapter Four*). These data, and most other studies on the effect of IL-1 and other cytokines on neurodegeneration, have focused largely on the use of the neutralising antibodies, recombinant cytokines or antagonists (see Rothwell *et al.*, 1994; Rothwell *et al.*, 1995b). The information obtained from these studies allows us to assess the effects of *acute* cytokine manipulation on neuronal death. However, very little data are available concerning the effect of *chronic* cytokine manipulation on experimentally-induced neurodegeneration *in vivo*. Thus, the development of genetically-modified mice in which the genes encoding for cytokines are overexpressed or deleted, has enhanced our knowledge of the function of these molecules, especially in infection and disease (Taverne, 1993, 1994). In addition, the use of transgenic mice is also advancing the understanding of the pathogenesis of several neurological disorders (for review see Aguzzi *et al.*, 1994). In general, two types of genetically-modified mice are available. Genes encoding cytokines (or their receptors) are mutated to either overexpress its product (termed here "transgenic"), or rendered nonfunctional ("knock-out").

The availability of two types of genetically-modified mice, IL-1 β gene knock-out and IL-1ra overexpressing, enabled the effect of IL-1 β deficiency, or overproduction of endogenous IL-1ra, on neuronal viability after an excitotoxic

lesion, to be studied.

Preliminary studies were performed to identify an appropriate dose of an NMDA receptor agonist for induction of a striatal excitotoxic lesion in the mouse. Subsequently, since exogenous IL-1ra was protective against NMDA receptor-induced striatal lesions in the rat (see *Section 4.2.2*), the effect of acute IL-1ra treatment was established after induction of these lesions in normal mice.

6.2 Striatal NMDA receptor-induced excitotoxic lesions

The aim of this study was to identify the optimal dose of MGlu which produced lesions in the striatum of the C57-black mouse brain.

6.2.1 *Experimental*

Striatal lesions were induced in mice (male C57-black strain, BSU, Manchester University, UK, 26-30g) under halothane anaesthesia, according to the protocol in *Section 2.3.2*. A dose of 1 or 5nmol of the NMDA agonist, MGlu (in 1 μ l, n=14) was infused into the striatum (co-ordinates; anterior 0.7mm, lateral 2.1mm and ventral from dura 3.5mm). The animals were sacrificed (as in *Section 2.4*) 24h after infusion and neuronal death assessed by the tetrazolium method as in *Section 2.5.1* and *2.6.1*.

6.2.2 *Results*

Striatal infusion of 5nmol of MGlu resulted in modest behavioural changes, including "barrel-rolling" activity in some animals. This behaviour was similar to that observed after intrastriatal infusion of MGlu in the rat (see *Section 3.3.2*). However, "barrel-rolling" was not observed after intrastriatal infusion of 1nmol

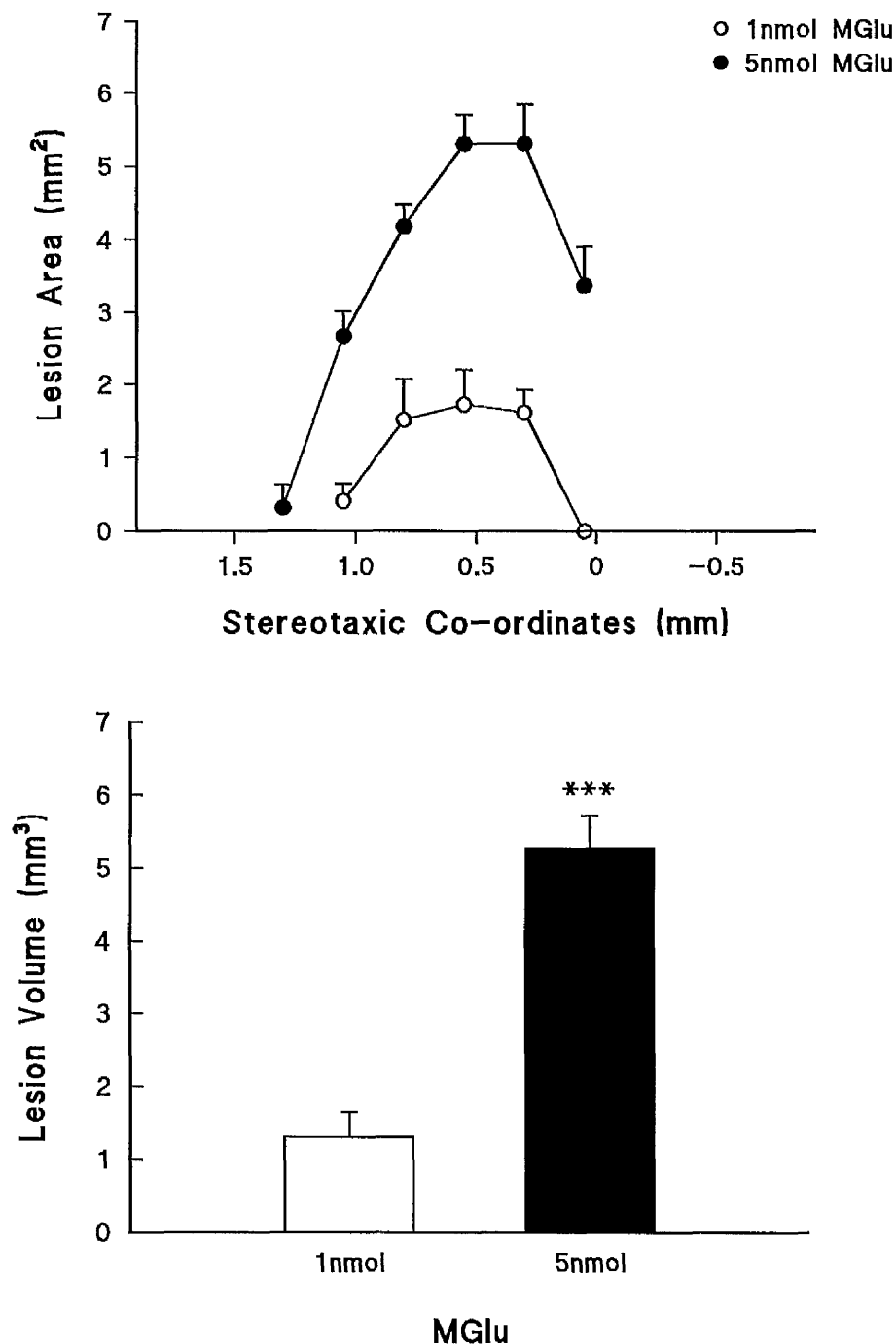


Figure 6.2.1 Lesion size measured 24h after intrastriatal infusion of MGlU

Damage was induced by intrastriatal infusion of either 1nmol (n=4) or 5nmol (n=10) MGlU (in 1 μ l). Striatal neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 250 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test. *** P<0.001 versus 1nmol-treated animals.

MGlu and was not present with either dose 24h after injection. A dose of 1nmol resulted in a small lesion measured 24h later, which was exclusively confined to the striatum (volume of $1.31 \pm 0.33 \text{ mm}^3$, $n=4$). Intrastratial infusion of 5nmol resulted in a significantly larger lesion ($5.28 \pm 0.44 \text{ mm}^3$, $n=10$, $P<0.001$ versus animals treated with 1nmol), which sometimes spread to the overlying ipsilateral frontal cortex. *Figure 6.2.1* illustrates the distribution of striatal damage after striatal infusion of 1 or 5nmol MGlu. A dose of 5nmol was therefore chosen for future studies with both wild-type and genetically-modified mice.

6.3 Effect of IL-1ra on striatal NMDA receptor-induced excitotoxic lesions

The aim of this experiment was to study the effect of IL-1ra on striatal neuronal damage in the mouse, induced by overactivation of the NMDA receptor in order to allow comparison with the results obtained in the rat (see *Section 4.2.2*).

6.3.1 *Experimental*

Striatal lesions were performed in C57-black mice (Harlan Olac, UK) according to *Section 2.3.2*. Animals were infused striatally with either PBS ($1 \mu\text{l}$, $n=9$) or human IL-1ra ($5 \mu\text{g}$ in $1 \mu\text{l}$, $n=9$, the same dose used in rat experiments), immediately followed by $1 \mu\text{l}$ of MGlu (5nmol). Mice were sacrificed (as in *Section 2.4*) 24h after infusion, and neuronal death assessed by the tetrazolium method as in *Section 2.5.1* and *2.6.1*.

6.3.2 *Results*

IL-1ra treatment resulted in no noticeable changes in behaviour in response to striatal infusion of MGlu (e.g. "barrel-rolling"). Co-infusion of IL-1ra ($5 \mu\text{g}$) failed

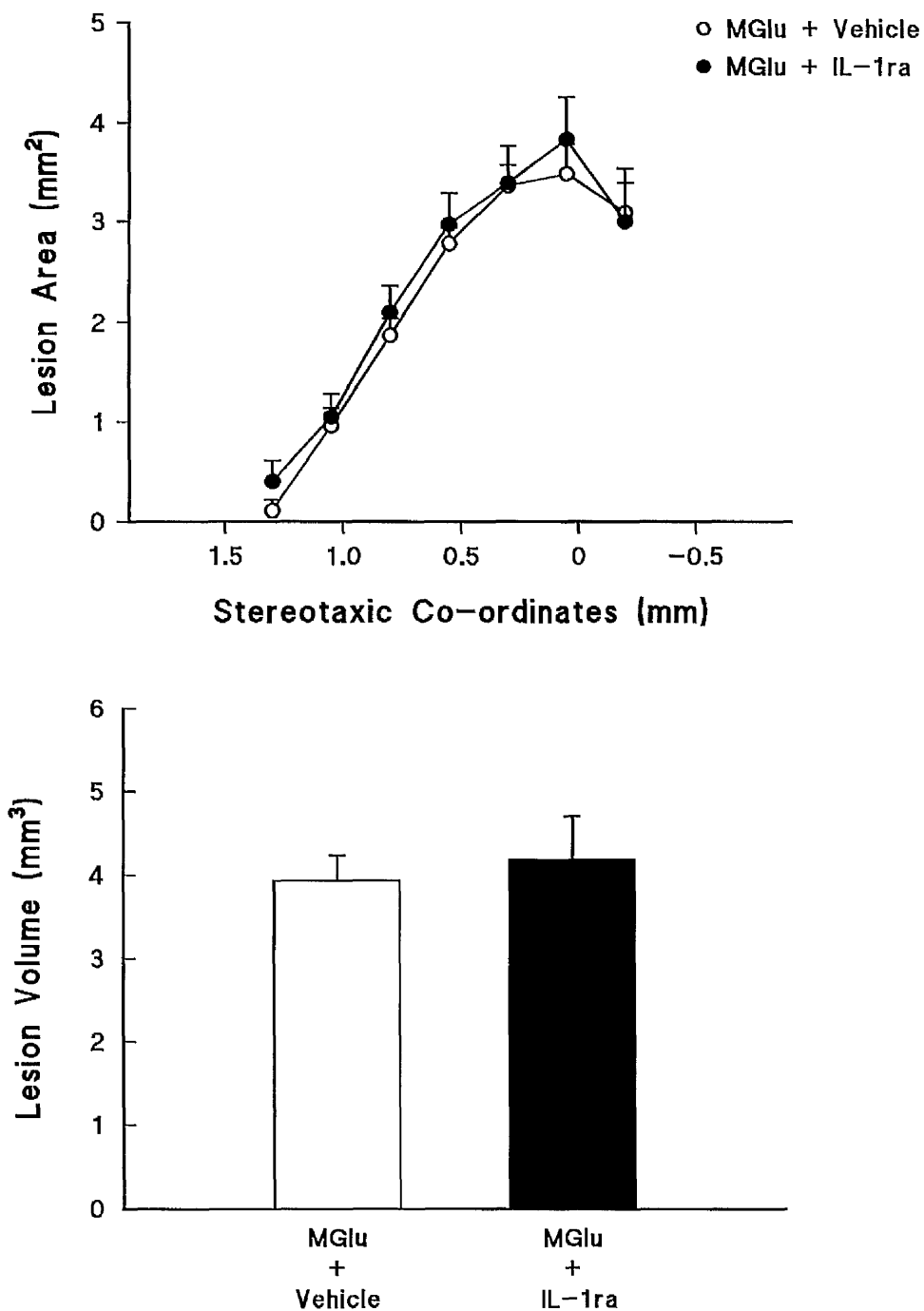


Figure 6.3.1 Effect of IL-1ra on striatal NMDA receptor-induced damage

Damage was induced by intrastriatal infusion of 5nmol MGlutamate (in 1 μ l). IL-1ra (5 μ g/ μ l, n=9) or vehicle (1 μ l of PBS, n=9) was co-infused with MGlutamate. Striatal neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 250 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test.

to affect striatal NMDA receptor-induced damage (Vehicle $3.93 \pm 0.30 \text{ mm}^3$, $n=9$ versus IL-1ra $4.19 \pm 0.51 \text{ mm}^3$, $n=9$, *Figure 6.3.1*), and the distribution of damage was the same in both cases.

6.4 Effect of IL-1ra overexpression and IL-1 β deficiency on striatal NMDA receptor-induced excitotoxic lesions

The aim of these experiments were to determine if chronic genetic manipulation of IL-1 β or IL-1ra expression resulted in any modification in the neuronal damage induced by overactivation of the NMDA receptor in the mouse striatum. To address this aim, transgenic mice were used which were genetically modified to either, overexpress IL-1ra, or the IL-1 β gene was knocked out, rendering the animal was incapable of producing IL-1 β (see *Section 6.5.4*). Overexpression of human IL-1ra was achieved under the regulatory control of glial fibrillary acidic protein (GFAP) gene promoter and was therefore produced in the brain by astrocytes. IL-1 β knock-out mice were generated by gene targeting in embryonic stem cells to produce an IL-1 β null allele. The creation of these animals was performed at the Merck Research Laboratories, New Jersey, USA.

6.4.1 *Experimental*

Striatal infusions were performed according to *Section 2.3.2*. A dose of 5nmol of MGlu (in $1 \mu\text{l}$) was infused into the striatum of either IL-1ra overexpressing ($n=9$), IL-1 β knock-out ($n=6$), or the appropriate wild-type control ($n=15$) animals. All mice were sacrificed (as in *Section 2.4*) 24h later, and neuronal death was assessed by the tetrazolium method as in *Section 2.5.1* and *2.6.1*.

6.4.2 Results

Both types of genetically-modified mice did not exhibit any obvious changes in normal behaviour or in their response to surgery, when compared to the wild-type mice. Intrastratial infusion of 5nmol MGlu in all animals resulted in the characteristic behaviour seen after infusion of this agonist into the striatum (see *Section 6.2.2*). There was no obvious difference in this behaviour between the different groups of mice.

Infusion of MGlu (5nmol) into the striatum of IL-1ra overexpressing mice produced striatal lesions which were not significantly different from neuronal damage seen in the wild-type control animals (Wild-type $4.38 \pm 0.58 \text{ mm}^3$, $n=8$ versus IL-1ra overexpressing $4.91 \pm 0.50 \text{ mm}^3$, $n=9$, *Figure 6.4.1*). However, the neuronal damage in IL-1ra overexpressing animals showed a slight, but non-significant increase in the posterior striatum. Neuronal damage due to overactivation of the NMDA receptor in the striatum was also similar for the IL-1 β knock-out group and the equivalent wild-type (Wild-type $4.45 \pm 0.63 \text{ mm}^3$, $n=7$ versus IL-1 β knock-out $4.36 \pm 0.34 \text{ mm}^3$, $n=6$, *Figure 6.4.2*).

6.5 Discussion

Most studies employing intracerebral injection of EAA's to study mechanisms of excitotoxicity utilise the rat (e.g. Foster *et al*, 1988; McDonald *et al*, 1989a; Fujisawa *et al*, 1993). There are limited data on excitotoxic lesions within the mouse brain, although this species is sometimes the chosen for studies on cerebral ischaemia (Gotti *et al*, 1990; Chiamulera *et al*, 1993). However, the mouse is the only genetically-modified animal as yet available in which IL-1/IL-1ra genes have been altered and hence the requirement for the development of a reproducible excitotoxic lesion in the brain of this species. The first aim of this study, therefore, was to establish and optimise a protocol for induction of

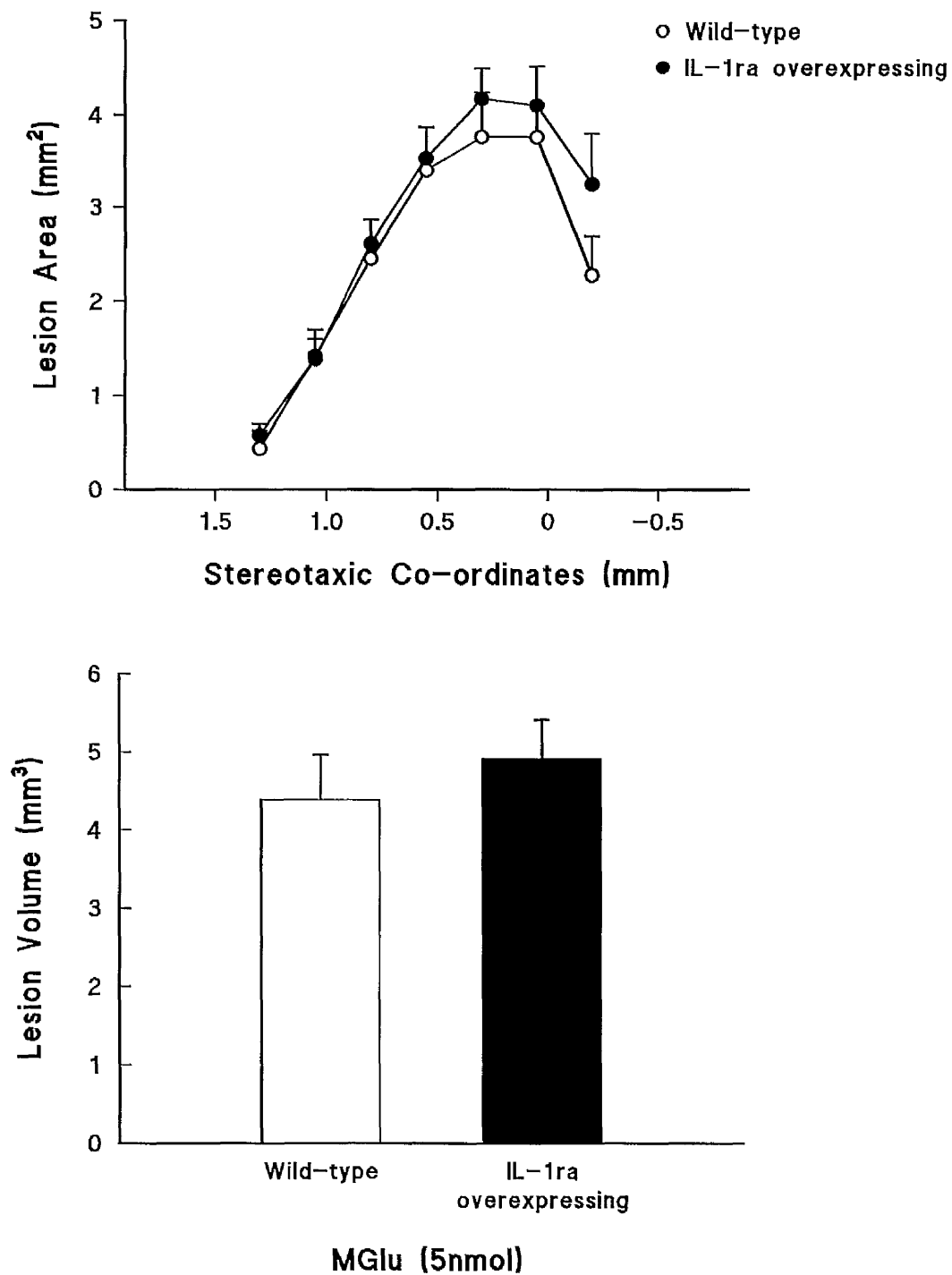


Figure 6.4.1 Striatal NMDA receptor-induced damage in IL-1ra overexpressing transgenic mice

Damage was induced by intrastriatal infusion of 5nmol MGlu (in 1 μ l) in either IL-1ra overexpressing transgenic mice (n=9) or the appropriate wild-type control (n=8). Striatal neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 250 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test.

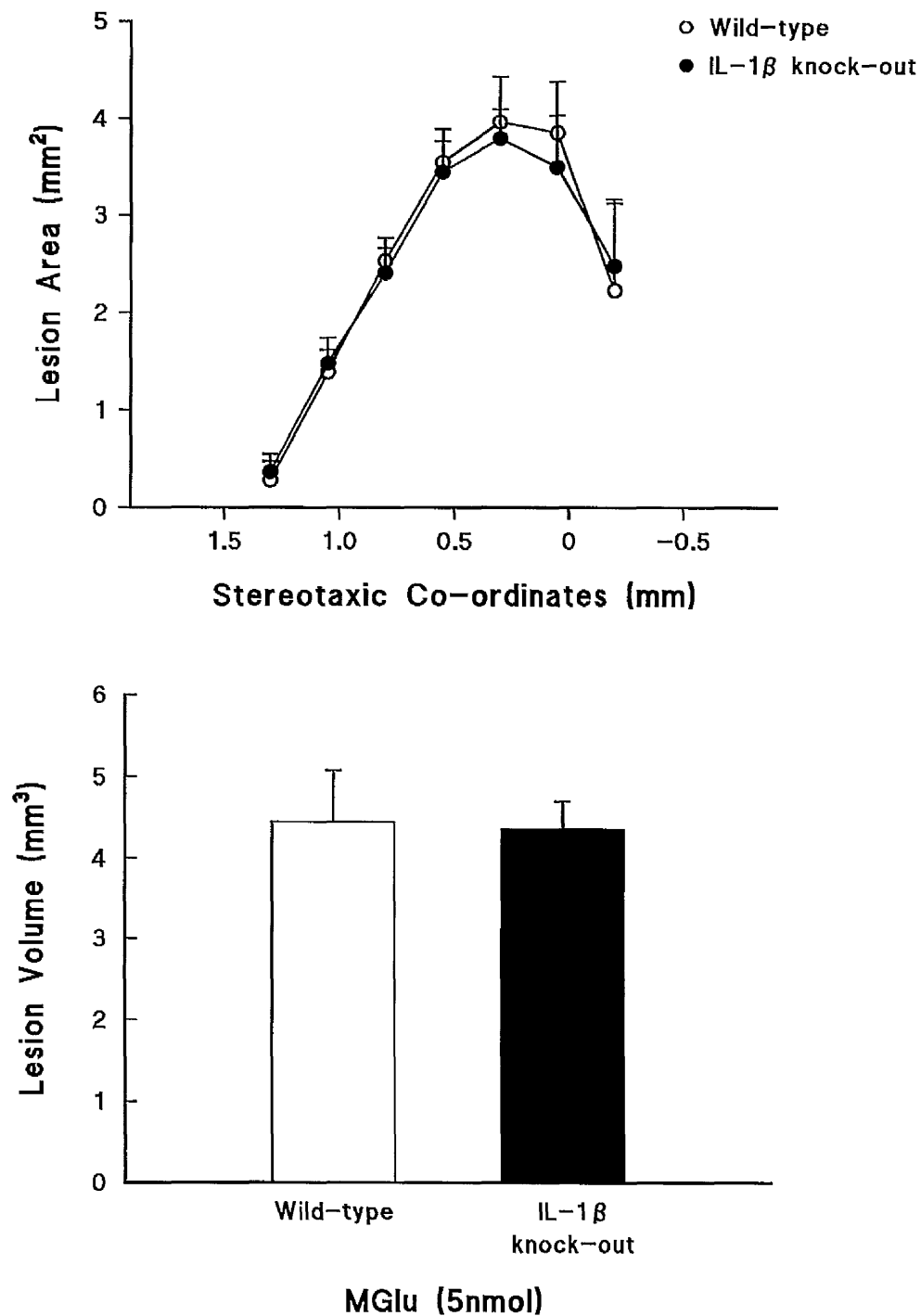


Figure 6.4.2 Striatal NMDA receptor-induced damage in IL-1 β knock-out mice

Damage was induced by intrastratial infusion of 5nmol MGlutamate (in 1 μ l) in either IL-1 β knock-out mice (n=6) or the appropriate wild-type control (n=7). Striatal neuronal damage was measured 24h later.

The upper graph shows lesion area (mm², on 250 μ m sections), which was integrated to give lesion volume (mm³), shown in the lower graph.

Data are expressed as mean \pm SEM. Statistical analysis was performed using a Student's t-test.

excitotoxicity induced by the NMDA receptor in the striatum of the mouse brain.

6.5.1 *Establishment of NMDA receptor-induced excitotoxicity*

Since exogenous administration of 10nmol, MGlu into the striatum of the rat causes extensive neuronal cell death when assessed 24h after infusion (see *Chapter Three*; Relton, 1992; Relton and Rothwell, 1992), a lower dose, 1 or 5nmol, was chosen for infusion into the mouse striatum. MGlu caused dose-dependent lesions of the mouse striatum and since 5nmol produced an excitotoxic lesion encompassing most of the striatum, this dose was chosen for future studies. Behaviour, and the distribution of neuronal death, in the mouse was similar to that observed after intrastriatal infusion of 10nmol in the rat. Thus, for detailed discussion see *Section 3.7.3* and *3.7.5*. However, the work presented here is the first known study on the induction of excitotoxic neuronal death by the NMDA receptor agonist, MGlu, in the striatum of the mouse, and demonstrate that this agonist produced reproducible lesions of the striatum which were clearly delineated 24h after infusion.

6.5.2 *Effect of IL-1ra on NMDA receptor-induced excitotoxicity*

Acute administration of human IL-1ra did not affect the extent of neuronal death induced by striatal infusion of the NMDA receptor agonist, MGlu, in the mouse. This result is in direct contrast to the protective effect of IL-1ra treatment on striatal NMDA receptor-induced damage in the rat (see *Section 4.2.2*), and is surprising, since IL-1ra affords substantial protection against focal cerebral ischaemia in the rat (Relton and Rothwell, 1992; Loddick and Rothwell, 1996) and the mouse (Rothwell, *et al.*, 1995a)

The dose (5 μ g) of IL-1ra employed in this present study was the same as that

used for the rat. However, the mechanism of induction of NMDA receptor-induced toxicity may vary between the mouse and the rat. It is possible, though unlikely, that a higher dose or sustained administration of IL-1ra may be required for protection to be observed in the mouse. IL-1ra (10 μ g) reduced cerebral infarct after cerebral ischaemia in the rat by 60-80% (Relton and Rothwell, 1992; Loddick and Rothwell, 1996). However, the studies on the effect of IL-1ra in cerebral ischaemia in the mouse found a protection of only 40%, even at doses of up to 10 μ g (Rothwell, *et al.*, 1995a). Hence, the mouse may be less susceptible to protection by IL-1ra, explaining the lack of effect of this antagonist in excitotoxic damage, at the dose tested.

IL-1ra binds to type I and type II IL-1 receptors, with a greater affinity for the type I receptor (Dinarello and Thompson, 1991; Dripps *et al.*, 1991b; Arend, 1993; Dinarello, 1994). Both types of receptors are present in the mouse brain (Takao *et al.*, 1990, 1992, 1993; Cunningham *et al.*, 1992; Ban, 1994; Parent *et al.*, 1994) and the type I receptor has been localised in the mouse striatum (Takao *et al.*, 1990). Takao *et al.* (1990, 1993) reported species differences in the binding of IL-1/IL-1ra in mouse and rat. It also seems that a species barrier exists, so that human IL-1ra binds to human type II receptors better than to murine type II receptors (Evans and Robbins, 1994). Therefore, the affinity of human IL-1ra for putative IL-1 receptors in the rat striatum may differ from those receptors in the murine striatum. Hence, a higher concentration of the human IL-1ra used in this study may be required for inhibition of IL-1 receptors in the mouse.

However, in conclusion the results presented here shows that acute IL-1ra treatment fails to inhibit excitotoxic damage in the mouse striatum.

6.5.3 IL-1ra overexpressing transgenic mice-

Effect on NMDA receptor-induced excitotoxicity

Chronic overexpression of human IL-1ra in transgenic mice did not result in any significant change in the extent of neuronal death caused by striatal infusion of an NMDA receptor agonist (MGlut), compared to wild-type controls. Many studies on cytokines involving the use of transgenic mice have resulted in unexpected data (see Taverne, 1993, 1994). For example, various studies using transgenic mice overexpressing IL-6, have shown that these animals exhibit various neurological dysfunctions, including neuronal loss (Campbell *et al.*, 1993; Chiang *et al.*, 1994; Steffensen *et al.*, 1994). However, acute administration of IL-6 is protective against excitotoxic neuronal damage, induced by NMDA receptor overactivation, in the rat brain *in vivo* (Toulmond *et al.*, 1992). Nevertheless, this is the first report to date on the use of IL-1ra overexpressing mice and several explanations can be proposed for the results obtained in this present chapter.

Even though acute administration failed to inhibit neuronal damage in the mouse, it is possible that a prolonged exposure of IL-1ra may be required to observe neuroprotection. Since chronic exposure was also without effect, one of the major questions is whether the expression of IL-1ra is high enough to see a protective effect. Endogenous IL-1ra is usually induced (by several stimuli) 50-100 fold higher compared to the levels of IL-1 and very high concentrations of IL-1ra are required to block the biological effects of IL-1 (see Arend, 1993; Dinarello, 1994). Measurements of the average concentration of human IL-1ra in the brains of the transgenic mice used in this study have shown to be increased (compared to wild-type mice). However, the expression of human IL-1ra was apparently decreased with age; 4126, 125, and 59pg/mg brain tissue at the ages of 2.5, 7, and 24 months respectively (Chen and Zheng, personal communication). The age of the animals used in this study were five to six months. Thus, the expression of IL-1ra in these animals could be significantly

212
do you have an explanation
for that?

reduced and a protective effect may have been observed only if younger animals (e.g. two-months old) were used. *what do happen after a lesion?*

IL-1ra production in the transgenic mice employed in this study was under the regulatory control of the GFAP gene promoter, located in astrocytes. Thus, IL-1ra was produced exclusively in these glia and the expression of IL-1ra described above probably reflects constitutive production in resting astrocytes. However, significant induction of the GFAP in astrocytes may be required to observe a higher level of expression. Injury in the brain is known to transform resting astrocytes to their active form and this correlates with an increase in the expression of GFAP (Mucke *et al.*, 1991; Norton *et al.*, 1992). In addition, an excitotoxic lesion in the CNS activates astrocytes (Dusart *et al.*, 1991). However, the time course of astrocyte induction and subsequent expression of IL-1ra after striatal activation of NMDA receptors may be too delayed to exert a protective effect. Dusart *et al.* (1991) have reported a significant induction of GFAP-positive astrocytes 24h after an excitotoxic insult, although these authors did not study earlier time points. Another study reports a rapid (one hour) induction of GFAP expression after focal mechanical trauma (Mucke *et al.*, 1991). However, the astrocytic response varies depending on the kind of neuronal damage (Norton *et al.*, 1992), and thus may not be significant enough in the 24h period studied in reports presented in this chapter. Furthermore, it needs to be ascertained that, in addition to an increased expression of IL-1ra within astrocytes, this antagonist is also released from these cells. *13*

Since IL-1ra was overexpressed from birth, increased levels of IL-1 or upregulation of IL-1 receptors may result. This could be a compensatory mechanism to overcome the effects of chronic IL-1 inhibition by IL-1ra. Therefore, the animal may be able to respond as if normal levels of IL-1 were present by activating sufficient IL-1 receptors to induce a response. Furthermore, it has been shown that fewer than 5% of IL-1 receptors need to be occupied for

a biological response (see Dinarello, 1994). Hence, even a slight increase in the amount of IL-1 receptors may restore the full biological function of IL-1.

Chronic IL-1ra overexpression also did not affect the neuronal outcome after focal ischaemic brain damage in the mouse *in vivo* (Fotheringham and Davies, personal communication). Hence, it is likely that overexpression of IL-1ra in the mouse does not protect against various forms of brain damage. However, Betz *et al* (1995) have recently reported focal ischaemic damage in the rat is significantly reduced after chronic administration of IL-1ra by adenoviral gene delivery, although overexpression of IL-1ra was for five days compared to five to six months in the animals used in the present study.

However, since acute or chronic administration of IL-1ra did not protect against NMDA receptor-induced lesions in the mouse (see *Section 6.3.2* and *6.5.2*), it can be concluded that in the situations tested here, IL-1ra treatment against excitotoxic lesions was not beneficial in the mouse.

Ischaemic?

6.5.4 IL-1 β knock-out mice-

Effect on NMDA receptor-induced excitotoxicity

The IL-1 β deficient mice exhibited no difference in the extent of neuronal death after NMDA receptor overactivation when compared with wild-type controls. This suggests that IL-1 β itself is not directly involved in the pathogenesis of NMDA receptor-mediated neuronal loss. However, as previously discussed, while some studies on cytokine transgenic/knock-out mice have yielded predictable results, many have provided unexpected data (see Taverne, 1993, 1994).

Zheng *et al* (1995) have reported that the mice used in this present study are completely deficient of IL-1 β and are incapable of IL-1 β expression even after a potent stimulus. Although, IL-1 β -deficient mice are unable to mount a normal

response (acute-phase) to a local inflammatory stimulus (turpentine injection) they do respond similarly to wild-type controls after an systemic inflammatory stimulus (lipopolysaccharide (LPS) injection; Zheng *et al.*, 1995). Since IL-1 β has been implicated in responses due to both turpentine and LPS injections, these reports give conflicting data. IL-1 has many functions which overlap with other cytokines and forms part of the intricate cytokine network (see Akira *et al.*, 1990; Hopkins and Rothwell, 1995; Rothwell and Hopkins, 1995). Because of this, other molecules may act as substitutes when another cytokine is absent (cytokine redundancy). In support of this, IL-6-deficient mice exhibit normal responses to systemic inflammation after injection of LPS (Fattori *et al.*, 1994). This has been attributed to TNF α , since TNF α concentrations in the IL-6-deficient mice are three times greater than in wild-type controls, and may therefore compensate for the absence of IL-6. With regard to the results presented in this chapter, another cytokine may be compensatory to the actions of IL-1 β . Significant activation of IL-1 β -induced responses, which may be involved in excitotoxic neuronal death, could still occur in the brain. For example, IL-1 α is still present in these IL-1 β -deficient mice (Zheng *et al.*, 1995) and, after NMDA receptor activation, expression of IL-1 α may be elevated in the brain of IL-1 β knock-out mice compared to wild-type controls. Enhancement of IL-1 α may therefore compensate for the lack of IL-1 β as many responses induced by latter can elicited by the former cytokine (see Rothwell, 1991). Furthermore, IL-1 α binding has been suggested to be dominant over IL-1 β binding in mice (Takao *et al.*, 1990, 1993). In conclusion therefore, the development of transgenic mice incapable of producing both IL-1 α and IL-1 β , or IL-1 receptor knock-out mice will be required to establish this proposal.

Glial reaction
in these mice?

IL-1 is a potent inducer of IL-6 (see Rothwell, 1991) and the IL-1 β -deficient mice used in this study have a diminished production of IL-6 following turpentine treatment (Zheng *et al.*, 1995). Local infusion of IL-6 attenuates the neurotoxic

effects of NMDA in the rat brain *in vivo* (Toulmond *et al.*, 1992), which suggests that IL-6 is neuroprotective. Hence, if IL-1 β knock-out mice have reduced IL-6 production after MGlut infusion, this could counteract the effect of IL-1 β deletion. Therefore, it could be the balance between the concentrations of IL-1, which may be neurotoxic, and IL-6, which may be neuroprotective, that is important. Thus IL-1 β deficiency could reduce neuronal toxicity directly, but also increase toxicity indirectly by diminishing the amount of IL-6 produced and subsequently halting its neuroprotective effects.

Handwritten note: IL-1 β knock-out mice

In summary therefore, the results in this chapter suggest that in the situations tested here, IL-1 is not involved in excitotoxic neuronal death in the mouse.

Chapter Seven

General Discussion

Chapter Seven

General Discussion

7.1 Introduction

The cytokine, IL-1 has been implicated in excitotoxic damage induced by overactivation of the NMDA receptor in the rat, since IL-1ra inhibits neuronal death due to this receptor in the striatum (Relton and Rothwell, 1992). The main objectives of this thesis were to study the involvement of IL-1 in excitotoxic damage induced by the NMDA receptor and by a second subtype of EAA receptor, the AMPA receptor. Additionally, two areas of the brain were studied to ascertain if IL-1 displayed regional effects on excitotoxic damage.

7.2 Excitotoxin-induced neuronal death

Throughout this thesis, EAA-induced neuronal damage was induced *in vivo* by direct pharmacological overactivation of EAA receptors, induced by intracerebral infusion of specific agonists acting at these receptors. Evaluation and verification of this technique was performed, and the results are presented in *Chapter Three*. Activation of both types of EAA receptors (NMDA and AMPA) produced local damage at the site of injection (striatum or cortex) which was quantified by histological techniques.

The major differences between the neuronal damage induced by either receptor subtype were:

- AMPA receptor-induced damage in either the striatum or cortex was

delayed compared to that caused by NMDA receptor activation.

- Neuronal damage was not only observed at the site of injection after intrastriatal infusion of an AMPA receptor agonist, but also sometimes at a distant site, the piriform cortex.

This latter observation was not seen after induction of both forms of cortical damage or after striatal infusion of an NMDA receptor agonist.

7.3 IL-1 mediates EAA-induced neuronal death

The consequence of IL-1 α or IL-1 β treatment was tested on both types of EAA-induced neuronal damage (NMDA and AMPA receptor) in the striatum and the cortex.

7.3.1 Observations

Effect of IL-1 α on
neuronal death?

- IL-1 α co-infusion inhibited striatal but not cortical neuronal death induced by striatal or cortical infusion of NMDA or AMPA receptor agonists.
- Striatal administration of IL-1 β did not exacerbate local striatal neuronal death induced by striatal infusion of NMDA or AMPA receptor agonists, but caused extensive cortical damage after co-infusion into the striatum with an AMPA receptor agonist.
- Cortical administration of IL-1 β did not exacerbate local cortical neuronal death induced by cortical infusion of NMDA or AMPA receptor agonists, but exacerbated neuronal damage in the cortex (due to cortical infusion of NMDA or AMPA receptor agonists) when infused into either the ipsilateral or contralateral striatum.
- IL-1 β alone was not toxic to neurones *in vivo*.
- Striatal but not cortical infusion of IL-1 β alone caused a rise in core body temperature.

- IL-1ra overexpression or absence of endogenous IL-1 β in transgenic/knock-out mice failed to modify NMDA receptor-induced striatal lesions.

Each of these observations and their implications are briefly reviewed in *Section 7.3.2*.

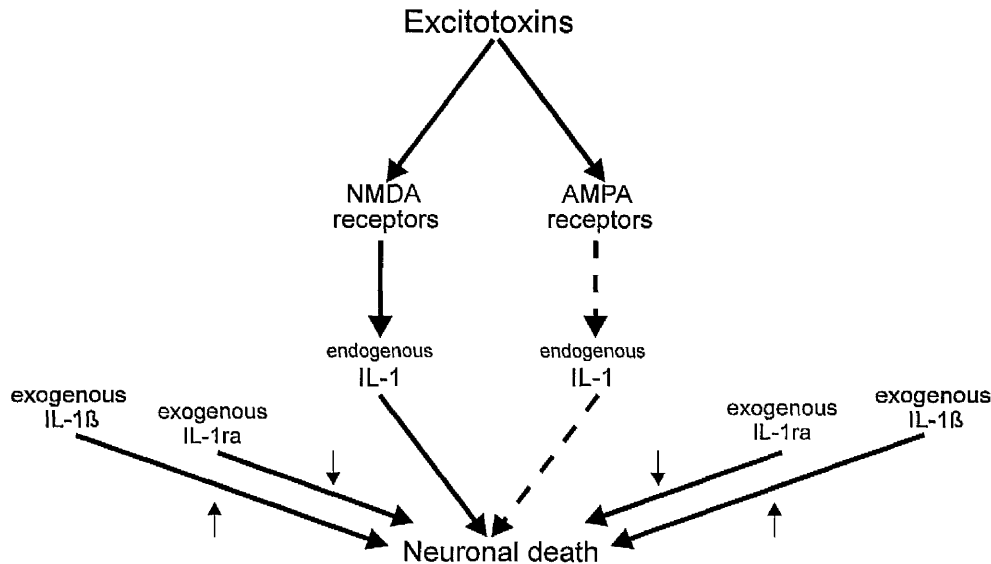


Figure 7.3.1 *Schematic representation of the involvement of IL-1 in excitotoxic neuronal injury. Part II*

↑ exacerbation; ↓ inhibition

7.3.2 Implications

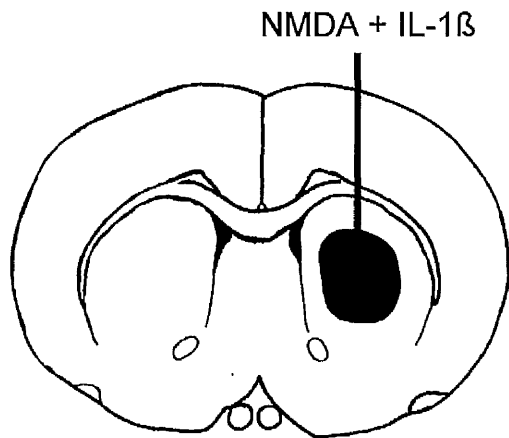
Data presented in *Chapter Four* provided evidence that IL-1ra inhibited both NMDA and AMPA receptor-induced neuronal damage in the striatum. This is the first known study to date showing the effects of IL-1ra on AMPA receptor-mediated excitotoxicity. This is important as the two different receptors induce neuronal damage by varying mechanisms and NMDA and AMPA receptors may

be differentially involved in several types of neurological disorders (see *Chapter One*). For example, AMPA receptors appear to play a greater role in the neuronal death after global ischaemia (e.g. Pullsinelli *et al.*, 1993; Gill, 1994). Therefore, IL-1 may be broadly implicated in a range of neurodegenerative disorders and IL-1ra may have a wider clinical relevance. IL-1ra also inhibits neuronal damage caused by cerebral ischaemia (e.g. Relton and Rothwell, 1992; Loddick and Rothwell, 1996), an EAA-dependent process. Therefore, since this antagonist also reduces EAA-induced neurodegeneration (see Relton and Rothwell, 1992 and *Section 4.2.2*), it can be inferred that the importance of IL-1 probably occurs quite late in the pathway of events leading to neuronal death after cerebral ischaemia and other neuronal disorders where IL-1ra has been demonstrated to be beneficial (see *Section 1.9.1*). In addition, IL-1 may affect excitotoxic damage via a feature which is common to both NMDA and AMPA receptor-induced damage (see *Chapter One* and *Section 7.4*). For example, increases in intracellular Ca^{2+} are fundamental to the process of excitotoxic cell death induced by overactivation of both NMDA and AMPA receptors.

The second major finding in the studies presented here was the fact that IL-1 β was not neurotoxic *in vivo per se*, but the same dose of IL-1 β exacerbated both forms of excitotoxic damage. However, IL-1 β did not exacerbate neuronal damage at the site of injection (the striatum or cortex), but in the case of striatal infusion of IL-1 β with an AMPA receptor agonist, damage developed in the ipsilateral cortex (see *Figure 7.3.2a*), possibly via an NMDA receptor-dependent process. Since cortical damage did not develop after striatal infusion of an NMDA receptor agonist and IL-1 β , it can be inferred that IL-1 β interacts with a process which exclusively occurs after striatal AMPA receptor overactivation. It is proposed therefore, that IL-1 β may interact with the specific neuronal pathways (e.g. cortico-striato-cortical or the limbic circuit) which are thought to be activated during non-NMDA striatal receptor stimulation (e.g. Ben-Ari *et al.*, 1980; Ben-Ari, 1985; Okazaki and Nadler, 1988 and see *Section 4.5.2.3*).

**NMDA receptor-induced
striatal lesions**

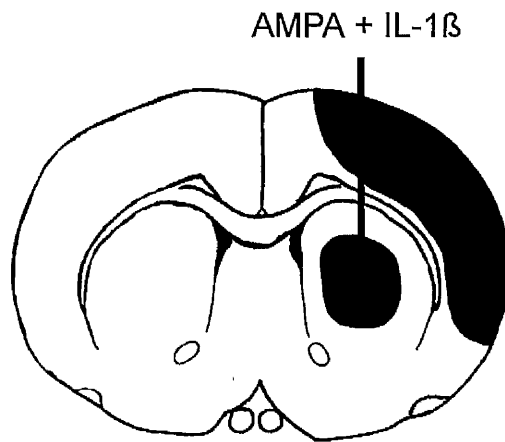
Striatal infusion of IL-1 β



No effect on
striatal damage

**AMPA receptor-induced
striatal lesions**

Striatal infusion of IL-1 β



No effect on
striatal damage

BUT

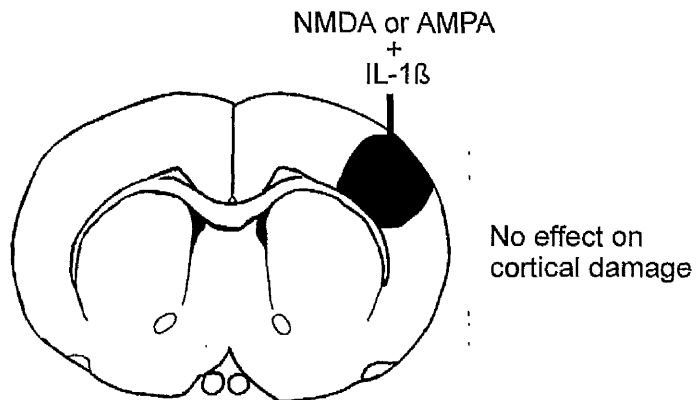
Production of
cortical damage

**Figure 7.3.2a Summary of the effect of IL-1 β on excitotoxic lesions
induced in the striatum**

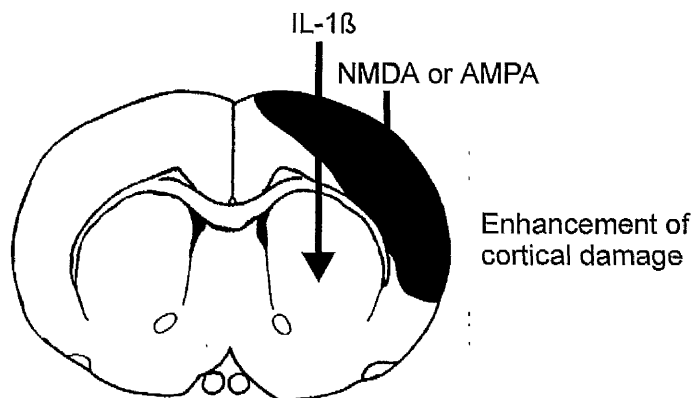
N.B. The agonist MGlutamate was used for NMDA receptor-induced lesions
The agonist S-AMPA was used for AMPA receptor-induced lesions

**NMDA or AMPA receptor-induced
cortical lesions**

Cortical infusion of IL-1 β



Ipsilateral striatal infusion of IL-1 β



Contralateral striatal infusion of IL-1 β

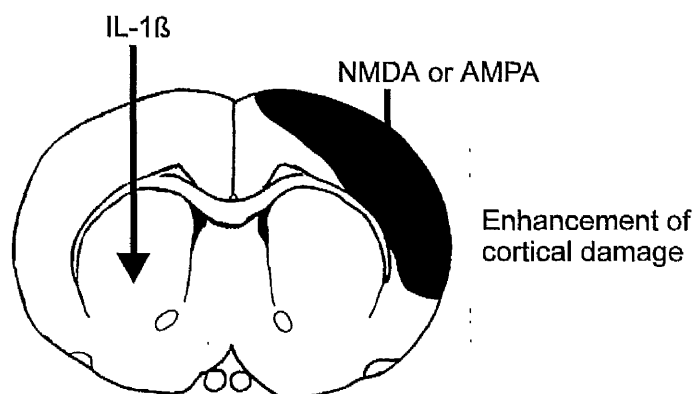


Figure 7.3.2b Summary of the effect of IL-1 β on excitotoxic lesions induced in the cortex

N.B. The agonist MGlu was used for NMDA receptor-induced lesions
The agonist S-AMPA was used for AMPA receptor-induced lesions

Additionally, cortical excitotoxic damage (induced by cortical overactivation of NMDA or AMPA receptors) was not affected by infusion of IL-1 β into the cortex, but was dramatically enhanced when the IL-1 β was infused into the ipsilateral or contralateral striatum (see *Figure 7.3.2b*). These results indicate that a specific site of action of IL-1 may be within the striatum and that IL-1 β may interact with neuronal pathways between the striatum and the cortex in an anterograde or retrograde manner. In support of these data, recent studies performed within our lab have shown that IL-1ra reduces neuronal damage after cerebral ischaemia when infused into the ipsilateral or contralateral striatum (Stroemer and Rothwell, personal communication). Possible mechanisms for these observations have been discussed (see *Section 4.5*) and may be related to the hyperthermic properties of IL-1, although, IL-1ra has been demonstrated to reduce neuronal damage after cerebral ischaemia without any changes in body temperature (Loddick and Rothwell, 1996). Additionally, IL-1ra reduced the hyperthermia after methamphetamine in the rat, but had no affect on neurotoxicity (Bowyer *et al.*, 1994). These two pieces of data suggest that hyperthermia and neuronal death may not be strictly related, and IL-1 β may interact with specific neuronal pathways (presumably glutamatergic) originating within, or structures nearby, the striatum.

How does it
be related?

It appears therefore that IL-1 β *per se*, does not cause rapid neuronal damage, but in the presence of other insults (e.g. during EAA receptor overactivation), IL-1 β (within the striatum) can act synergistically to exacerbate neuronal damage at distant sites (e.g. cortical). This further suggests that IL-1 β interacts specifically with EAA-mediated processes.

Despite the involvement of IL-1 in excitotoxic damage in the rat, studies in this thesis failed to confirm this in the mouse. The use of transgenic/knock-out mice were also employed to determine the contribution of IL-1 in excitotoxic lesions, and negative results were obtained. However, care must be taken in

interpretation of these results, as the use of genetically-modified mice do not always yield predicted data (e.g. Taverne, 1993, 1994 and *Chapter Six*). It can only be concluded that IL-1 may not be involved in the model of excitotoxic brain damage in the mouse employed in this study.

to be observed in the mouse

7.4 Potential cellular mechanisms of IL-1

Potential mechanisms underlying the involvement of IL-1/IL-1ra in excitotoxic neuronal death have been discussed in *Chapter Four* (see *Section 4.5*), in relation to the specific, individual experiments. However, various cellular mechanism(s) by which endogenous/exogenous IL-1 may mediate excitotoxic neuronal death induced by EAA have not been discussed (see *Figure 7.4.1* and *Table 7.4.1*). The precise site or mechanism of action of IL-1 in EAA-mediated damage is unknown but various hypotheses can be proposed, based mainly on indirect evidence. For example, IL-1 could act directly on the EAA receptors, or enhance the release of the endogenous EAA, glutamate, at the presynaptic level or from glial cells. Alternatively, IL-1 may potentiate the actions of glutamate at the postsynaptic level by increasing the release of toxic intracellular mediators (e.g. NO and AA).

what happens with the
proteins when T₂ is
activated!

Studies *in vitro* in neuronal cultures have demonstrated that IL-1ra does not protect against excitotoxin-induced neuronal death, and IL-1 β exerts neuroprotective properties at some doses (Rothwell and Strijbos, 1995; Strijbos and Rothwell, 1995). These data contradict *in vivo* studies and therefore raise the question of whether endothelial or glial cells, which are predominantly absent from neuronal cultures, are involved in the toxic effects of IL-1. In addition, synaptic connections and neuronal pathways are absent in neuronal cell culture and may be essential to the actions of IL-1 (see *Section 7.3.2*).

7.4.1 Glia

Glia (especially astrocytes) are classically thought to regulate the survival of damaged neurones. For example, glia are important in the removal of glutamate from the extracellular space (Nicholls and Attwell, 1990; Schousboe *et al.*, 1992; Kanai *et al.*, 1994) and astrocytes release neurotrophic factors (e.g. Giulian, 1993; Giulian *et al.*, 1993). However, recent evidence suggests that glial cells may also contribute in the destruction of neurones following several neuronal insults (e.g. see Banati *et al.*, 1993; Giulian, 1993; Giulian *et al.*, 1993; Lees, 1993b; Piani *et al.*, 1994). Stimulated microglia or astrocytes have been shown in culture to cause neurotoxicity. The release of several potential neurotoxic factors, for example, glutamate, NO, free radicals and AA have been suggested to contribute to the neurotoxicity of these cells (Piani *et al.*, 1991; Boje and Arora, 1992; Banati *et al.*, 1993; Giulian, 1993; Giulian *et al.*, 1993; Hewett *et al.*, 1994; Skaper *et al.*, 1995). IL-1 causes activation and proliferation of glia (e.g. Giulian *et al.*, 1988) thus, IL-1 may induce the release of neurotoxic factors from these cells. Furthermore, IL-1 β enhances the Ca²⁺ response to EAA receptor stimulation in astrocytes (Holliday and Gruol, 1993) and stimulates astrocytic Na⁺/H⁺ exchange, which may enhance glutamate efflux from these cells (Benos *et al.*, 1994). However, whether effects on glia are relevant to the actions of IL-1 on NMDA and AMPA receptor-induced damage need to be clarified as NMDA receptor antagonists protect against the neurotoxic effects of activated microglia, in contrast to non-NMDA receptor antagonists which are ineffective (Giulian, 1993). Furthermore, glia appear to differentially modulate the response of neurones to the toxic effects of NMDA versus AMPA receptor agonists (Dugan *et al.*, 1995).

↓ IL-1 → glia
activation

7.4.2 Glutamate release/uptake

It is unlikely that IL-1 acts to modulate the release/uptake of glutamate, as IL-1

has no effect on glutamate uptake in astrocytes *in vitro* (Piani *et al.*, 1993) and studies in our lab have failed to demonstrate a change in glutamate release from brain slices after incubation with IL-1 or IL-1ra (Allen *et al.*, 1995). In contrast, Bianchi *et al.* (1995) have recently shown that peripheral injection of IL-1 reduces the levels of several amino acids in the hippocampus (glutamine, glutamic acid and GABA) and suggest that brain amino acid pathways are involved in central modifications induced by IL-1.

7.4.3 Nitric Oxide (NO)

NO has been implicated in excitotoxic damage, and its synthesis and release is increased after NMDA receptor activation (Garthwaite *et al.*, 1989; Moncada *et al.*, 1991, 1992). Inhibition of NOS attenuates neurodegeneration induced by NMDA receptor activation *in vivo* (e.g. Moncada *et al.*, 1992), and induction of astrocytic NOS enhances NMDA receptor-induced neuronal injury in neuronal cultures (Hewett *et al.*, 1994). IL-1 stimulates the production and release of NO from brain endothelial cells (Kilbourn and Belloni, 1990; Murata *et al.*, 1994) and astrocytes (Hewett *et al.*, 1993; Mollace *et al.*, 1993; Hewett *et al.*, 1994; Skaper *et al.*, 1995). Furthermore, IL-1 (in combination with TNF α) stimulation of astrocytes induces NO production and subsequent neuronal degeneration in neuronal cultures *in vitro* (Skaper *et al.*, 1995), and activation of NOS in astrocytes by IL-1 β plus IFN γ potentiated NMDA (but not non-NMDA) neurotoxicity in cortical neurones in culture (Hewett *et al.*, 1994). Hence, IL-1 may exert its effects on neurodegeneration via induction of NO. However, the contribution of NO in the pathogenesis leading to neuronal death after AMPA (compared to NMDA) receptor activation is unclear as NMDA, but not non-NMDA, receptor-induced neurotoxicity appears to involve NO synthesis (Moncada *et al.*, 1992; Hewett *et al.*, 1994).

7.4.4 Arachidonic acid (AA)

EAA's also increase the release of AA from neurones and glia. AA has been implicated directly in neurodegeneration (see Farooqui and Horrocks, 1994a, 1994b) and potentiates the actions of glutamate at the NMDA receptor (Miller *et al.*, 1992). Additionally, AA can act as a retrograde messenger to modify presynaptic and astrocytic glutamate release (Lynch and Voss, 1990; Volterra *et al.*, 1994). IL-1 stimulates AA release from astrocytes (Hartung *et al.*, 1989) and increases the expression and secretion of PLA₂, which is the enzyme involved in AA synthesis (Vadas *et al.*, 1991; Oka and Arita, 1991; Tong *et al.*, 1995). However, only NMDA (but not non-NMDA) agonists have been reported to directly stimulate the release of AA (Dumuis *et al.*, 1988, 1990) and whilst AA potentiates NMDA receptor-induced currents (Miller *et al.*, 1992), a recent report has demonstrated that AA reduces non-NMDA receptor-induced currents (Kovalchuck *et al.*, 1994). Therefore, the involvement of IL-1-induction of AA being involved in both NMDA and AMPA receptor-induced damage seems unlikely.

7.4.5 Summary

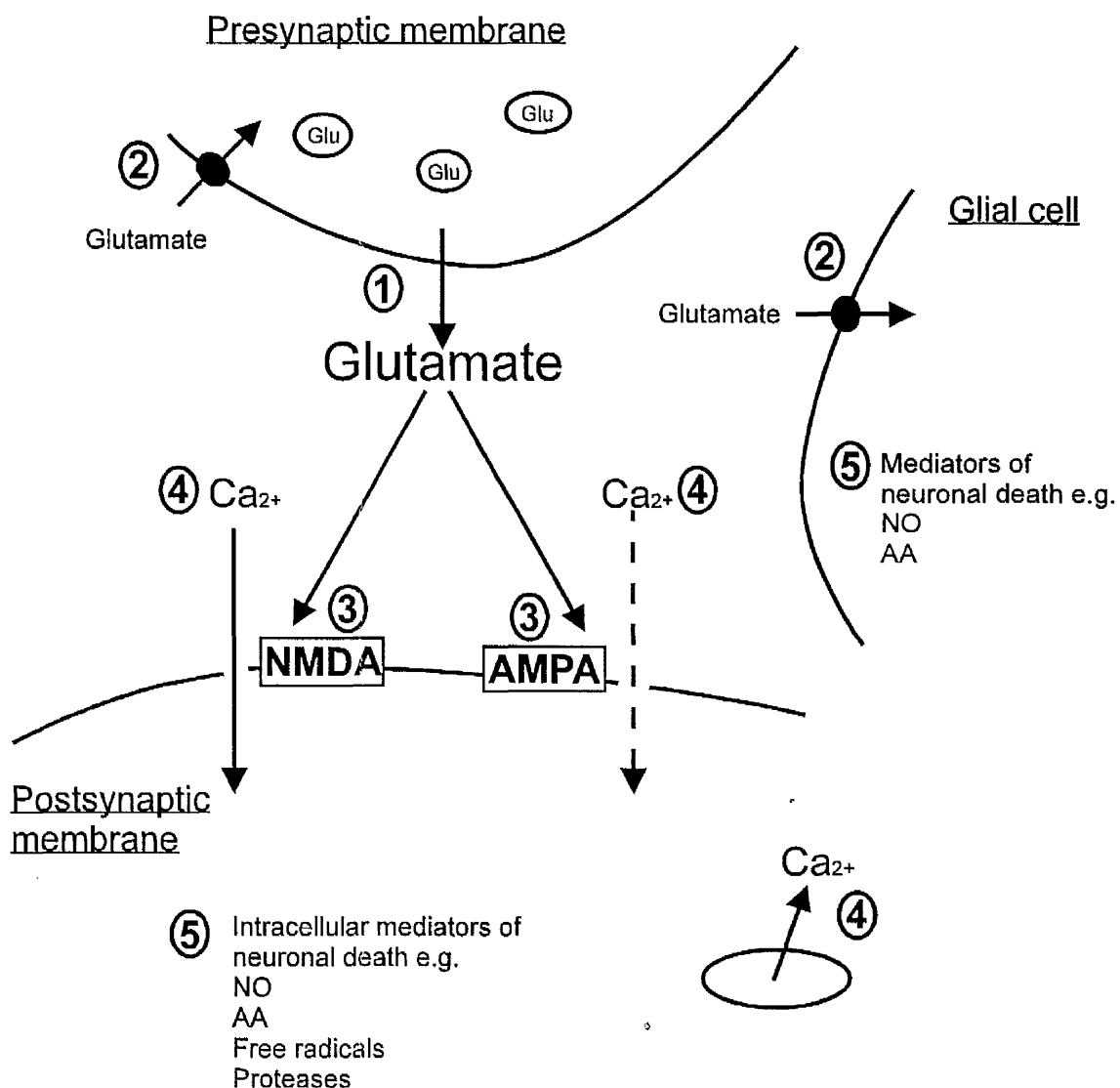
Therefore, the proposed mediators in the mechanism of action of IL-1, in EAA-mediated damage outlined above, appear to involve NMDA, but not AMPA, receptor-induced neurotoxicity. However, in this study, IL-1ra protects against, and IL-1 β exacerbates, both NMDA and AMPA receptor-induced toxicity. Therefore, these proposed mechanisms are unlikely to fully explain the role of IL-1 in both NMDA and AMPA receptor-induced excitotoxicity, although they may contribute, in part, to damage induced by the NMDA receptor. However, several additional possibilities exist (see below).

7.4.6 Calcium

An increased concentration in intracellular Ca^{2+} is the primary mediator in the initiation of events leading to neuronal death and both NMDA and AMPA receptors induce a rise in intracellular Ca^{2+} . IL-1 may affect the levels of this ion, since Di Julio *et al* (1991) have demonstrated an increase in intracellular Ca^{2+} concentrations in cultured neurones in response to IL-1. However, IL-1 inhibits Ca^{2+} channel currents in hippocampal neurones *in vitro* (Plata-Salamán and Ffrench-Mullen, 1994), although the channels involved are the voltage-dependent type of Ca^{2+} channels. Thus, IL-1 could still affect the influx of Ca^{2+} directly through the NMDA and AMPA receptors. It is unlikely that IL-1 affects the release of Ca^{2+} from intracellular stores to exacerbate neuronal death, as the contribution of Ca^{2+} release from these stores, although important during NMDA receptor-induced damage, is probably minimal during excitotoxicity mediated by the AMPA receptor (Frandsen and Schousboe, 1993; Schousboe *et al.*, 1994; Mody and MacDonald, 1995).

7.4.7 Corticotrophin-releasing factor (CRF)

The hormone, CRF may be involved in the actions of IL-1 in neurodegeneration. Injection of a CRF receptor antagonist markedly inhibits transient (Lyons *et al.*, 1991) and focal (Strijbos *et al.*, 1994; Wong *et al.*, 1995a) cerebral ischaemia, and reduces damage caused by striatal infusion of an NMDA receptor agonist (Strijbos *et al.*, 1994) *in vivo* in rats. IL-1 is a potent inducer of CRF expression and release (see Rothwell, 1991), and hence the neurotoxic effects of IL-1 may be due to the release of CRF. CRF is selectively expressed in the ipsilateral amygdala after focal cerebral ischaemia (Wong *et al.*, 1995b) or brain trauma (Roe *et al.*, 1995). The amygdala forms part of the limbic system (other areas include the hippocampus and entorhinal cortex) and is neuronally connected with the striatum (predominantly the ventral striatum; e.g. Carlsen, 1988; Ragsdale



- ① Glutamate release
- ② Neuronal/glial glutamate uptake
- ③ Receptor binding/function
- ④ Ca^{2+} influx/release from intracellular stores
- ⑤ Intracellular mediators of excitotoxic neuronal death

Figure 7.4.1 Possible sites of action of IL-1 in excitotoxic damage

Glu=glutamate

and Graybiel, 1988; Brog *et al.*, 1993). In addition, the amygdala can modulate the activity of the basal ganglia-thalamocortical circuits (e.g. Cortico-striato-thalamocortical) via a "limbic loop" (Alexander *et al.*, 1986). Thus amygdaloid CRF expression after ischaemia or head injury may be involved in the activity of these neuronal loops and could participate in some of the responses induced in the cortex by IL-1 β described in *Chapter Four* and *Section 7.3.2*.

Table 7.4.1 Effects of IL-1 possibly related to excitotoxic neuronal death

Effects of IL-1
<ul style="list-style-type: none"> • Glutamate release • Glutamate uptake • Glia activation • Ca²⁺ influx • NO synthesis/release • AA synthesis/release • Free radical synthesis/release • CRF synthesis/release • Hyperthermia

7.4.8 Hypothalamo-pituitary-adrenal (HPA) axis

Abnormalities of the HPA axis have been demonstrated after ischaemia (e.g. Olsson *et al.*, 1992) and down regulation of the HPA axis reduces brain damage and seizures after hypoxia and ischaemia in the rat (Krugers *et al.*, 1995). Activation of the HPA axis involves an initial release of hypothalamic CRF, release of pituitary hormones which then induce systemic (adrenal) release of glucocorticoids. Since IL-1 activates the HPA axis (see Rothwell, 1991), this may be a mechanism by which IL-1 worsens both ischaemic and excitotoxic neuronal damage, possibly via induction of CRF (see *Section 7.4.7*).

In conclusion, whether the *in vivo* actions of IL-1/IL-1ra on excitotoxic damage are due to alterations in glutamate release from neurones or glia, modifications in the synthesis and release of NO, AA, CRF, or activation of the HPA axis (or a combination), remain to be clarified.

7.5 Clinical relevance

Acute EAA-induced neuronal death in the rat brain *in vivo* has no direct clinical parallels. However, *Chapter One* summarises several acute and chronic neuronal disorders ranging from stroke to Huntington's disease, in which EAA's are implicated in their pathogenesis (see Olney, 1990; Whetsell and Shapira, 1993; Zorumski and Olney, 1993; Lipton and Rosenberg, 1994). Therefore, the studies in this thesis may be relevant to various neurological diseases.

IL-1ra was administered at the same time as the excitotoxic insult. This is a disadvantage when considering the clinical relevance of IL-1ra treatment. In addition, neuronal damage has been assessed only at an acute time point (24-48h) after injury and therefore the possibility that IL-1ra simply delays the development of neuronal death has not been excluded. However, IL-1ra significantly protects against damage caused by cerebral ischaemia and brain trauma when treatment is delayed (up to four hours) and this protection is sustained for at least seven days after the initial insult (Toulmond and Rothwell, 1995; Loddick and Rothwell, 1996). Furthermore, intravenous infusion of high doses of IL-1ra results in no toxicity or serious side effects in volunteers or patients (Dinarello and Thompson, 1991; Granowitz *et al*, 1992; Dinarello *et al.*, 1993) which indicates this molecule may be useful as a neuroprotectant.

IL-1 β exacerbated excitotoxic (and ischaemic) damage and endogenous brain IL-1 β is increased in response to several neurological insults (see *Section 1.9.1-1.9.3* and *Table 1.9.3*). Therefore, the relevance of these results may be

important when considering the neuronal damage in stroke or head injured patients. For example, if a patient contracts an infection, endogenous release of IL-1 both in the periphery and CNS may increase. Hence, neuronal death could then develop in remote areas of the brain, separate from the initial site of infarction.

7.6 Unanswered questions-Future studies

This research has identified a number of important questions (some of which are summarised here) on the mechanisms involved in the interaction between IL-1 and excitotoxin-induced neuronal death.

- What is the contribution of IL-1-induced hyperthermia to the potentiation of excitotoxic neuronal death?

It is possible that IL-1 mediates neuronal damage independent of any changes in body temperature. However, if the involvement of fever is not ruled out, it is still important to determine why the cortex is specifically sensitive to this response after infusion of IL-1 β into the striatum.

- What are the sites (cell type and location) of IL-1 actions?
- What are the neuronal pathways involved?

For example, does IL-1 β mediate its effects in the striatum itself, or a structure (or collection of neurones) nearby? Does IL-1 β interact with a specific neuronal pathway in the striatum? If so, what is the exact neuroanatomical pathway involved? Experiments could be carried out in which *c-fos* expression is determined throughout the brain after striatal infusion of IL-1 β . Results from these studies may indicate which areas of the brain are activated after striatal administration of IL-1 β . Collaborative work has demonstrated that *c-fos*

expression is predominant in the paraventricular nucleus and the supra-optic nucleus (collections of neurones in the hypothalamus) after icv injection of IL-1 β (Lawrence, McGowen, Poat and Woodburn, unpublished data).

Alternatively, IL-1 β may act not in the striatum *per se*, but could be transported anterogradely or retrogradely along neurones originating in the striatum, to a distant site to induce its responses. Tracing studies could be performed to demonstrate if IL-1 β is indeed neuronally transported and, if so, to which sites. The time course of these responses would need to be rapid as the response in the cortex to IL-1 is observed within 24h.

- Which receptors and second or third messengers are involved?

Are the neurotoxic effects of IL-1 induced by activation of the classical type I or II IL-1 receptors, or is a novel receptor involved? Does activation of IL-1 receptors involve induction of a specific second messenger pathway, leading to neuronal death? If these messengers are identified it may provide a novel route for therapeutic intervention.

- Can IL-1 α induce the same responses as IL-1 β ?

Can IL-1 α exacerbate excitotoxic neuronal death, or is this response unique to IL-1 β ? If the latter is true, this may provide important clues towards IL-1's mechanism(s) of action.

Regardless of the mechanism of IL-1's actions, the results reported in this thesis have provided exciting data on the relationship between IL-1 and EAA-mediated neuronal death, and suggests novel sites of action. Further work will certainly need to be performed to determine the physiological relevance of the responses

to IL-1, and whether they relate to neurodegeneration observed in clinical situations.

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APPENDIX ONE

Paraformaldehyde (PFA) solution.

A (1L) 4% solution was made by dissolving 40g PFA powder (BDH Chemicals, UK) in 1L of 0.1M PBS. A magnetic stirrer/hot plate at approximately 60-70°C (no higher) was used to dissolve the PFA as far as possible. To completely dissolve the PFA a few drops of concentrated NaOH was added until the solution went clear. The solution was then allowed to cool to room temperature, filtered and adjusted to pH 7.4 and stored at 4°C.

APPENDIX TWO

Subbing slides.

A 1% gelatin solution (1L) was prepared by adding 10g of gelatin (BDH Chemicals, UK) to 0.5g of chrome alum (Chromium (III) potassium sulphate, BDH Chemicals, UK). A magnetic stirrer/hot plate at approximately 40°C was used to dissolve the gelatin. The solution was filtered through Whatman no.1 filter paper. Microscope slides (Chance Propper Ltd, UK) were placed in slide racks and washed in ethanol (99%) and dried. The clean slides were then placed in the gelatin solution for 5min removed, drained, blotted and allowed to dry before use. Slides were stored at room temperature before use.

APPENDIX THREE

Tetrazolium staining solution

To make 10ml of 0.4% stock tetrazolium blue chloride solution, 40mg 3,3'-[3,3'-Dimethoxy (1,1'-biphenyl)-4,4'-diyl]-bis[2,5-diphenyl-2H-tetrazolium] dichloride (Sigma, UK) was dissolved in 0.5ml dimethyl sulfoxide (DMSO; Sigma, UK) and made up to volume with 9.5ml distilled water. The powder was dissolved by a sonicator for approximately one hour. Stock solution was stored at -20°C until required.

The following solutions were mixed together (on the day of use) in the appropriate quantities to make 40ml of phosphate buffered tetrazolium solution:

- 1) 10ml 0.4% tetrazolium chloride solution.
- 2) 16ml 0.2M Na_2HPO_4 (BDH Chemicals, UK).
- 3) 4ml 0.2M NaH_2PO_4 (BDH Chemicals, UK).
- 4) 10ml 8mM MgCl_2 (BDH Chemicals, UK).

Final concentrations were:

0.1% tetrazolium chloride.

0.1M phosphate buffer, pH 7.4

2mM MgCl_2 .

APPENDIX FOUR

Cresyl fast violet and Neutral red staining.

Brain sections were allowed to reach room temperature (for cryostat sections), placed in a staining rack and stained with 1% cresyl fast violet or 1% neutral red by placing sequentially in the following various solutions:

- | | |
|---|----------|
| 1) Cresyl fast violet (BDH Chemicals, UK) | 5-10min* |
| or Neutral red (Sigma, UK) | 3-5min* |
| 2) Distilled water | 2 washes |
| 3) Dehydrate in methylated spirits (IMS, Genta Medical, UK) | |
| 95% | 5 washes |
| 99% | 5 washes |
| 99% | 5 washes |
| 4) Xylene (Genta Medical, UK) | |
| 1 | 5 washes |
| 2 | 5 washes |
| 3 | 5 washes |

DePeX mounting medium (BDH Chemicals, UK) was then dripped on the slides and a glass coverslip (Chance Propper Ltd, UK) carefully placed on top ensuring no air bubbles developed. Slides were then allowed to dry and examined under the light microscope.

* The length of staining depends on the type (i.e. fixed or frozen) and the thickness of the section.

APPENDIX FIVE

Haematoxylin and Eosin staining

Brain sections were placed in a staining rack and stained with haematoxylin and eosin by placing sequentially in the following various solutions.

1) Haemalum (Mayers, BDH Chemicals, UK)	45sec-2min*
2) Cold water	2 washes
3) Hot water (until blue)	1-2min
4) Eosin [#] (1%, BDH Chemicals, UK)	10-60sec*
5) Cold water	2 washes
6) Dehydrate in methylated spirits	
95%	5 washes
99%	5 washes
99%	5 washes
7) Xylene	
1	5 washes
2	5 washes
3	5 washes

DePeX mounting medium was then dripped on the slides and a glass coverslip carefully placed on top ensuring no air bubbles developed. Slides were then allowed to dry and examined under the light microscope.

* The length of staining depends on the thickness of the section and the age of the stain.

[#] Eosin contained a few drops of glacial acetic acid (BDH Chemicals, UK).

APPENDIX SIX

Publications

A list of publications directly arising from this thesis are listed as follows.

Allen, S.M., Lawrence, C.B. and Rothwell, N.J. (1995) Mechanism of action of interleukin-1 (IL-1) in excitotoxic brain damage. *Soc. Neurosci. Abstr.*, **21**, 37.14.

Lawrence, C.B. and Rothwell, N.J. (1994) Interleukin-1 receptor antagonist inhibits NMDA and AMPA receptor-induced brain damage in the rat. *Br. J. Pharmacol.*, **112**, 484P.

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Lawrence, C.B. and Rothwell, N.J. (1995) Striatal IL-1 actions cause cortical neurodegeneration. *J. Neurosci.*, Submitted. (P)

Rothwell, N.J., Lawrence, C.B., Loddick, S.A., Strijbos, P.J.L.M. and Toulmond, S. (1994) Cytokines and cerebral ischaemia. In: *Pharmacology of Cerebral Ischemia*. (eds, J. Kriegstein. and H. Uberpichler-Schwenk). Medpharm Scientific Publ., Stuttgart, pp419-425.

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