Neuroprotection with the *N*-methyl-D-aspartate antagonist dizocilpine (MK-801) in a model of focal ischaemia

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Abstract

Ischaemic brain damage, due to cerebrovascular disease ("stroke"), head injury, cardiac arrest, surgery (cardiopulmonary bypass and intracranial) and perinatal hypoxia, is a major cause of death and disability in the western world. Stroke alone is the third most common cause of death after cancer and heart disease, accounting for some 10% of all deaths, approximately 60,000 per year in England and Wales.

Recently there has been rapidly growing evidence supporting the "excitotoxic" theory of neuronal damage in ischaemia, in which excitatory amino acid neurotransmitters are implicated as agents of damage to ischaemic brain. Excitatory amino acid antagonists, in particular *N*-methyl-D-aspartate (NMDA) antagonists, have proved to be dramatically effective in the protection of brain parenchyma in experimental models of ischaemia, especially focal ischaemia.

The role of excitatory amino acid neurotransmitters in ischaemia and the experimental evidence for amelioration of ischaemic brain damage by NMDA antagonists, is reviewed. A model of focal ischaemia in the rat is described, which can be used for assessing drugs that might be useful clinically. A method of volumetric analysis of infarct size using conventional histopathology is compared to a much cheaper and faster method using the mitochondrial redox stain, 2,3,5,triphenyltetrazolium chloride (TTC). The later method can be used for the rapid screening of compounds, but it has limitations which are fully explored. The neuroprotective effects of the NMDA antagonist, dizocilpine (MK-801) in acute and chronic ischaemia are compared and the chronic model is used to establish a "dose response" and "therapeutic window" for dizocilpine. Hypertension is a major risk factor in stroke and the neuroprotective effects of dizocilpine are examined in both normotensive and spontaneously hypertensive strains of rat, with pure cortical and combined cortical and striatal lesions. Finally, release of neuron-specific enolase into the CSF is correlated with infarct size in dizocilpine treated and control animals with a view to the possible clinical use of NSE in the quantification of ischaemic damage.

This Thesis is dedicated to

Sue and our son Niall

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All other aspects of the experimental design, execution and data analysis were performed by myself.

Four further publications are in preparation (Hatfield et al., 1991 b, c, d, & e)

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Abbreviations

AP5	D(-)-2-amino-5-phosphonovalerate
BP	Blood pressure
CA	Carotid artery
CBF	Cerebral blood flow
CPP	3-(-)-2-carboxypiperazin-4-ylpropyl-1-phosphonate
CSF	Cerebro-spinal fluid
GLU	Blood glucose
H&E	Haematoxylin and eosin
Hct	Haematocrit
LTP	Long term potentiation
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
NMDA	*N*-methyl-D-aspartate

Abbreviations (continued)

MSDRL	Merck Sharp and Dohme Research Laboratories
NSE	Neuron-specific enolase
PCP	Phencyclidine
SD	Sprague-Dawley
SEM	Standard error of the mean
SHR	Spontaneously hypertensive rat
TCP	Thiennylcyclohexylpiperidine
Temp	Temperature
TTC	2,3,5 triphenyltetrazolium chloride

Chapter 1

Introduction - excitotoxicity and protection in ischaemia via NMDA receptor blockade.

1.1 Cerebral ischaemia

Cerebrovascular disease is the third most common cause of death in Western Europe and North America, after cancer and heart disease (Wolf et al., 1986; Rocket & Smith, 1987). Ischaemic brain damage is a major cause of handicap in both older age groups due to "stroke" and in young adults due to the effects of head injury and perinatal hypoxia. The brain and other structures in the central nervous system, are extremely sensitive to hypoperfusion and ischaemia. If the period of ischaemia persists, cellular injury with attendant neurological dysfunction will occur. The extent of the injury and its potential for reversal depend upon the degree and duration of the ischaemic process. Once ischaemia leads to cellular death (infarction), the injury becomes irreversible and cannot be repaired.

Much time and research effort have been directed at understanding the complex pathophysiological processes underlying cerebral ischaemia in an attempt to form a rational approach to its management.

The protective effects of hypothermia in cerebral ischaemia, particularly global ischaemia, have been known about for many years (Connolly et al., 1962) and hypothermia is still widely used for cerebral and cardiac protection in open heart surgery (Kirklin et al., 1985). Research into hypothermia, however, was almost given up because of management problems such as shivering, arrhythmias, and infection (Dripps, 1956; Steen et al., 1979). More recently there has been a resurgence of interest in the cerebro-protective effects of mild hypothermia (Busto et al 1987; Leonov et al., 1990; Minamisawa et al 1990) but it has not so far proved to be clinically useful in treatment initiated after the onset of ischaemia.

Historically, efforts to develop effective therapy for brain ischaemia have focused on improving either blood flow or oxygen delivery (Grotta 1987). In the last decade, there has been growing enthusiasm for an alternative therapeutic approach directed

at improving the ability of the brain parenchyma to withstand ischaemia (Albers et al 1989). Initial attempts to accomplish this using barbiturates were not encouraging, as massive doses (with consequent side effects) were generally required to produce benefit (Yatsu 1983). There is currently an enormous list of compounds under investigation as potential neuroprotective agents, e.g. calcium channel blockers, aminosteroids, free radical scavengers, kappa opiate agonists, naftidrofuryl gangliosides, 5HT1A agonists, 5HT2 antagonists, alpha2 antagonists, cyclooxygenase and lipooxygenase inhibitors and others (McCulloch et al., 1991). Recently, a further class of drugs, *N*-methyl-D-aspartate (NMDA) antagonists, have stood out in experimental neuroprotective research as having more consistent efficacy in focal ischaemia than any other class of compound. This thesis reviews the role of the NMDA receptor in ischaemia, particularly focal ischaemia, and adds to the experimental evidence that NMDA antagonists can be neuroprotective in a model of focal ischaemia.

1.1.1 Stroke

The major clinical manifestation of focal ischaemia in man is spontaneous "stroke". The human syndrome of stroke consists of the abrupt development of a focal neurological deficit (and/or severe headache/coma) whose origin can be traced to either occlusion of a cerebral vessel (usually an artery), or the spontaneous rupture of an intracranial artery or "aneurysm" with consequent haemorrhage into the brain or subarachnoid space. Approximately 20% of strokes are primarily haemorrhagic. The incidence of new cases is approximately 1.4 per 1000 population per year, but this is age dependent. The approximate annual incidence of first stroke, based on figures from the Oxfordshire community stroke project, is 1/1000/year at 50, rising to 20/1000/year at 70 (Bamford et al., 1990). Mortality one year after a first occlusive stroke is approximately 20%, with 35% of the survivors functionally dependent. Intracranial (intracerebral + subarachnoid) haemorrhage has a worse outcome

with a mortality of approximately 50% at one year. In subarachnoid haemorrhage, delayed ischaemic damage is an important determinant of outcome (Pickard et al., 1989).

1.1.2 Ischaemia in trauma

Ischaemic cerebral damage is an important feature of many conditions encountered in clinical practice other than stroke, in particular, head injury, cardiac arrest, perinatal hypoxia, prolonged seizures and vascular surgery.

Trauma is the leading cause of death under the age of 45 in westernized countries and is the leading cause of male premature mortality, measured by the rate of potential life years lost between 1 and 65 (Rockett & Smith, 1987). Many of these deaths are caused by head injury, in particular, the majority of fatalities in those cases that reach hospital alive. Many of the severely head injured survivors are left permanently disabled. The importance of ischaemic damage in severe head injuries was highlighted by the neuropathological findings of Graham and colleagues (1978 & 1989). They found that in more than 85% of fatal head injuries, ischaemic brain damage was prominent at post mortem, and 40% of these patients had spoken at some time after the injury indicating delayed, secondary damage. The morbidity of survivors is also increased by delayed ischaemic damage (Bullock & Teasdale, 1990).

1.2 The pathology of ischaemia

1.2.1 Thresholds in cerebral ischaemia and the "penumbra".

The events of cerebral ischaemia are extremely complex (Spetzler, 1989). The key element is a reduction of nutrient blood supply to the tissues. Cellular injury from reduced cerebral blood flow (CBF) depends on the depth of the ischaemia and its duration. This concept has been thoroughly explored in primates (Branston et al., 1974, Astrup et al., 1977, Jones et al., 1981) and in cats (Heiss et al., 1976a&b, Carter et al., 1983). Symon and co-workers (Astrup et al., 1977; Branston et al., 1974 & 1977) clearly demonstrated in primates that if CBF was reduced below a threshold of 15 ml/100g/min, somatosensory evoked potentials were abolished; at flows below 10 ml/100g/min ionic pump failure occurred. The levels for electrical failure were similar to those required in man to begin to flatten the electroencephalogram, (EEG)(Sundt et al., 1974; Trojaborg, 1973). Jones et al., (1981) observed absolute thresholds for neurological dysfunction in primates undergoing middle cerebral artery occlusion and noted that the duration of ischaemia at a particular level of flow was important in determining the reversibility of the deficit.

The ischaemic "penumbra" is an important concept in the investigation of focal ischaemia (Astrup et al., 1981). In the clinical setting, following vessel occlusion, there is a range of CBF within the ischaemic area, with a dense ischaemic core and a surrounding zone of oligemia known as the ischaemic penumbra. Within the ischaemic penumbra there is partial energy failure, where blood flow is reduced sufficiently to cause loss of function but remains above the threshold for ionic pump failure. This is the critical zone in which therapeutic intervention can raise the critical thresholds and prolong the time limits of reversible ischaemia.

1.2.2 The role of calcium in neuronal cell death

Intra-cellular ionic calcium serves important functions as a membrane stabilizer, metabolic regulator and secondary messenger. For the normal function of most cells, the concentration of cytosolic calcium requires intricate regulation to maintain levels in the range 50 - 300 nM. Extracellular Ca^{2+} concentrations are approximately four orders of magnitude higher and there are also large intra-cellular stores. This precarious situation is the basis for the long standing hypothesis that Ca^{2+} may be a mediator of anoxic and toxic cell death (Schanne et al., 1979; Farber, 1981; Trump et al., 1981). Sustained elevation of cytosolic Ca^{2+} causes gross disturbances in many biological processes. Toxicity may be mediated by a cascade of events including activation of intracellular proteases and lipases, generation of free radicals, depletion of energy reserves by activation of Ca^{2+}-ATPase, and impairment of mitochondrial oxidative phosphorylation (Cheung et al., 1986).

Ca^{2+} is probably not the final common pathway mediating all ischaemic cell death, as in certain models of hypoxic-ischaemic injury in non-neuronal cell cultures there does not appear to be a correlation between cell viability and Ca^{2+} content (Cheung et al., 1986; Lemasters et al., 1987). However, with regard to the hypoxic-ischaemic injury of central neurones, Choi (1988a) summarised several observations that have supported the supposition that cellular Ca^{2+} overload plays a primary etiological role. Nicholson et al., (1977) demonstrated that hypoxia in the cerebral cortex is associated with a dramatic decline in extracellular Ca^{2+} concentrations and these observations have been confirmed by others (eg Hansen & Zeuthen, 1981; Hansen, 1985); early events in neuronal hypoxia, including transmitter release and liberation of free fatty acids, suggest a rise in intracellular Ca^{2+} (Siesjö, 1988); Ca^{2+} accumulation in hypoxic brain has been correlated with vulnerability to hypoxic injury at both regional levels (Dienel, 1984; Deshpande et al., 1987; Rappaport et

al., 1987) and cellular levels (Simon et al., 1984b) and in fact may occur before neurons appear necrotic under the light microscope (Siesjö, 1988); In cell culture experiments, hypoxic neuronal injury under low Na^+ conditions is highly Ca^{2+} sensitive; and finally, organic blockers of voltage-dependent Ca^{2+} channels can produce a neuroprotective effect in some animal models of ischaemia (Siesjö, 1988; Greenburg, 1987).

This calcium hypothesis of hypoxic-ischaemic neuronal injury has recently gained additional support with the establishment of links between hypoxic-ischaemic neuronal injury and glutamate neurotoxicity, and between glutamate neurotoxicity and Ca^{2+} mediated injury mechanisms (see below).

1.2.3 The Role of excitatory amino-acids

Glutamate and aspartate are excitatory amino acids found throughout the CNS. Fifty years ago Krebs demonstrated that glutamic acid has the unique ability to increase the respiration of the brain and retina (Krebs, 1935). He later demonstrated that glutamate is highly concentrated in the brain with grey matter levels higher than those of white matter (Krebs, 1949). Curtis and Watkins (1959) provided conclusive evidence that glutamate exerts a powerful excitatory action on neurons. It is now widely accepted that acidic amino acids, primarily L-glutamate and L-aspartate, are the major excitatory neurotransmitters of the mammalian central nervous system (Watkins & Evans, 1981; Dingledine et al., 1988; Monaghan et al., 1989; Collingridge & Lester, 1989).

The actions of excitatory amino acids appear to be mediated by as many as five distinct receptor sub-systems (Mayer & Westbrook, 1987; Monaghan et al., 1989; Collingridge & Lester, 1989). These systems include three ion channel-linked receptors mediating neuronal depolarisation, named after the selective agonist

actions of *N*-methyl-D-aspartate (NMDA), quisqualate (Q) and kainate (K) (Watkins & Evans, 1981). A fourth class is defined by the antagonist action of L-2-aminophosphonobutyrate (L-AP4) (Koerner & Cotman, 1981) and a fifth "metabotropic" receptor is linked to phosphoinositide metabolism (Sugiyama et al., 1987).

An understanding of the roles played by acidic amino acids in excitatory neurotransmission has come from studies using specific receptor antagonists. The pharmacology of the quisqualate receptors is similar to the kainate receptors but quite distinct from the NMDA class and it is therefore often useful to refer to "NMDA" and "non-NMDA" receptors. The "non-NMDA" receptors are responsible for fast excitatory post-synaptic potentials throughout the CNS and appear to fulfil a basic signalling function at excitatory synapses (Mayer & Westbrook, 1987). Excitatory transmission along pathways containing NMDA receptors is quite distinct from this "classical" fast-acting synaptic transmission as NMDA receptors are "voltage dependant" (the post synaptic current increases as the cell is depolarised from the resting potential) and the channel is permeable to Ca^{2+} ions (MacDermott & Dale, 1987). NMDA receptors appear to participate in various "plastic" neuronal events including the initiation of long-term potentiation (LPT, Collingridge et al., 1983 & 1987), which is a proposed mechanism of learning and memory (Morris et al., 1986) and the establishment and maintenance of synaptic contacts during neuronal development (Kleinschmidt et al., 1987). NMDA receptors are also involved in certain types of "patterned" neuronal activity (MacDermott & Dale, 1987) and in the processing of sensory information (Salt, 1986). Both NMDA and quisqualate/kainate receptors can be activated by a single type of stimulus (Mayer & Westbrook, 1987) and may coexist at the same synapse, introducing the possibility of complex patterns of activation.

Toxicity of excitatory amino acids

The first evidence that glutamate might have neurotoxic properties was reported by Lucas & Newhouse in 1957. They were screening compounds that they hoped might ameliorate retinal dystrophy and discovered that a systemic injection of glutamate destroys the inner neural layers of the immature mouse retina. Twelve years later glutamate retinotoxicity was confirmed, furthermore, it was shown that systemically administered glutamate causes destruction of neurons in the brains of newborn mice, rats and monkeys (Olney, 1969 & 1978; Olney et al.,1971a). These subsequent studies demonstrated that glutamate and related excitatory amino acids could induce neuronal damage in cerebral structures, such as the arcuate nucleus, where the blood-brain barrier was lacking (Olney, 1969 & 1971). The potency of these compounds as excitatory agonists was correlated with their toxicity and the post synaptic damage they caused involved dendrites and soma while sparing axons and presynaptic terminals (Olney, 1971). High concentrations of glutamate, delivered by chronic infusion, were required to produce neuronal degeneration, which tended to occur in regions where the blood-brain barrier was lacking and glutamate could accumulate, perhaps due to the efficiency of the reuptake systems in other regions (Rothman & Olney, 1986). Exogenous "excitotoxins" such as kainate, quisqualate, and *N*-methyl-D-aspartate are capable of producing axon-sparing lesions but, because these compounds are not substrates for reuptake mechanisms, they are much more toxic (Coyle, 1983).

The term "excitotoxic" was first coined by Olney in 1974 to describe the process of neuronal degeneration following excessive stimulation by the excitatory amino acids glutamate, aspartate and their analogues. The "excitotoxic" concept is that depolarization underlies glutamate neurotoxicity and that the toxic action is mediated through dendrosomal synaptic receptors specialized for glutamatergic (or aspartergic) transmission. Excitotoxic damage has a characteristic cytopathology

(Meldrum, 1990). The damage is entirely postsynaptic, sparing axons and presynaptic terminals. Dendrites show focal swellings; perikarya initially show swollen mitochondria and dilatation of endoplasmic reticulum. Subsequently, nuclear pyknosis and cytoplasmic condensation with multiple vacuolation are seen. Similar acute cytological appearances have been described in the hippocampus and cortex after transient forebrain ischaemia (Arsenio-Nunez et al., 1973; Brown & Brierley, 1972; Simon et al., 1984b; Van Reemps, 1984), after perinatal hypoxia-ischaemia (Ikonomidou et al., 1989), and in the hippocampus after status epilepticus (Evans et al., 1984), suggesting the possibility of an excitotoxic mechanism in these conditions.

Excitatory amino acid release in ischaemia

Benveniste et al. (1984) used the technique of intracerebral microdialysis in the rat hippocampus to provide convincing evidence that there is a dramatic increase in glutamate and aspartate concentrations in extracellular fluid during a period of transient cerebral ischaemia. Hagberg et al. (1985) confirmed the shift of excitatory amino acids from intra- to extra-cellular compartments during ischaemia. Silverstein et al. (1986) demonstrated that forebrain ischaemia causes failure of high-affinity reuptake systems for the transmitters, which are highly energy dependant, suggesting that this causes the increased concentration rather than increased release. Graham and colleagues (1990) demonstrated a marked increase in extracellular glutamate during focal ischaemia, and Shimada et al. (1989), using simultaneous cerebral blood flow measurements and microdialysis, found that there was a threshold for the release of glutamate during global ischaemia in the auditory cortex of cats (at approximately 20 ml/100g/min) which was related to the impairment of auditory evoked potentials. Sandberg et al. (1986) demonstrated an increase in extracellular excitatory amino acids during periods of insulin induced

hypoglycaemia, where again, failure of the reuptake systems due to energy failure was implicated.

1.2.4 The NMDA receptor complex

Glutamate receptors have been described in both vertebrates and invertebrates (Ascher et al., 1986; Meyer & Westbrook, 1987), but NMDA receptors have only been described in vertebrates. Indeed, NMDA receptors appeared to be confined to vertebrate central neurons until Moroni et al. (1986) provided evidence for their presence on enteric neurons. As yet the NMDA receptor protein has not been isolated, purified and sequenced and "images" of the receptor-channel therefore rely almost entirely on electrophysiological data (Ascher & Nowak, 1987). These studies have revealed six main properties of the NMDA receptor complex:

1. NMDA channels are permeable to Ca^{2+} as well as Na^{+} and K^{+} (MacDermott et al., 1986)

2. NMDA channels are blocked by Mg^{2+} in a voltage dependent way (Engberg et al., 1979; MacDonald et al., 1982; Nowak et al., 1984)

3 .NMDA channels may adopt multiple conductance states; some of the minor conductance states (small conductances) resemble the major conductance states opened by non-NMDA agonists. (Ascher et al., 1986)

4. Continued exposure to NMDA agonists produces short-term and long-term decreases in the sensitivity of the NMDA system (Mayer & Westbrook, 1987)

5. Glycine potentiates the response to NMDA (Johnson & Ascher, 1987), and may be a "co-agonist" (Kleckner & Dingledine, 1988)

6. NMDA channels may be blocked by organic molecules such as phencyclidine (PCP) and dizocilpine (Anis et al., 1983; Lodge et al., 1987; Wong et al 1986)

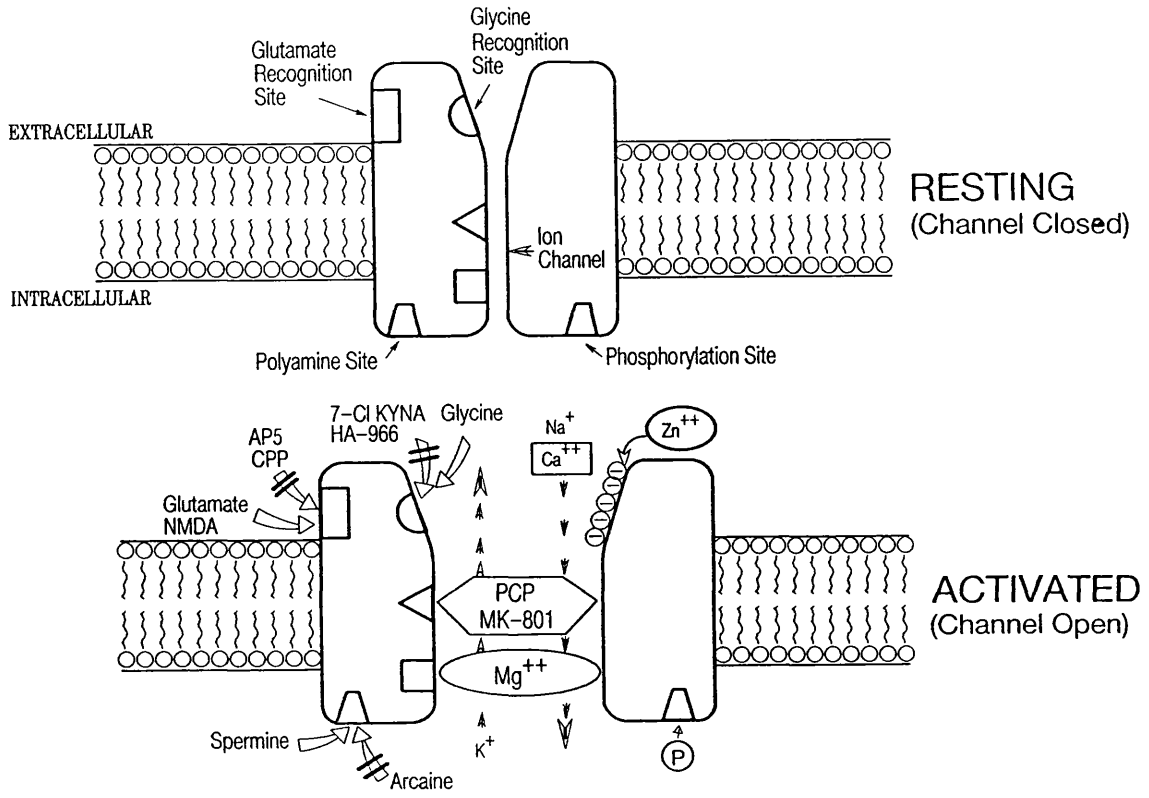

Figure 1.1 A schematic representation of the NMDA receptor-ion channel complex showing potential sites for pharmacological modulation. (reproduced with kind permission from Alan Foster, MSDRL)

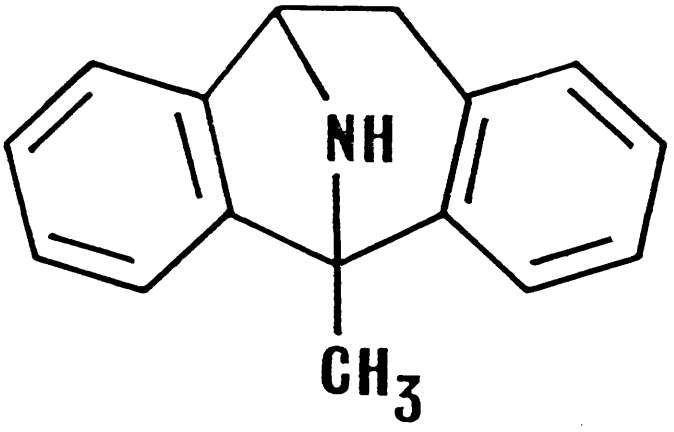

Figure 1.2 Structure of dizocilpine (MK-801), (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine malate.

Schofield et al. (1987) suggested that the structure of NMDA receptors may be similar to that of nicotinic acetylcholine and GABAA receptors, possessing several subunits which associate within the neuronal membrane to form a functional ion channel. Foster & Fagg (1987) have shown that the NMDA receptor consists of several functional domains, namely, the transmitter site, the ion channel and an allosteric site activated by glycine, which may regulate the extent of receptor activation. Figure 1.1 shows a highly schematic model of the NMDA receptor as formulated by Foster et al. (1990; reproduced with their kind permission). The various functional domains can be regarded as potential sites for pharmacological manipulation of receptor function.

1.2.4.1 NMDA receptor antagonists

Several modulatory sites on the receptor-channel complex (figure 1.1) can be considered as possible targets for therapeutic intervention. At the agonist recognition site, the effects of glutamate, aspartate or NMDA are antagonised competitively by structural analogues of glutamate such as CPP (3-(-)-2-carboxypiperazin-4-ylpropyl-l- phosphonate; Davies et al., 1986), AP5 (D(-)-2-amino-5-phosphonovalerate; Watkins & Olverman, 1987) or CGS 19755 (cis-4-phosphonomethyl-2-piperidine carboxylate; Lehmann et al., 1988) and many more compounds have now been shown to have similar action (see Choi, 1990).

There is a site at which glycine acts at low concentration to facilitate the excitatory action of NMDA (Johnson & Ascher, 1987). Glycine is probably an obligatory facilitator and antagonists to this effect such as 7-chlorokynurate (Kemp et al., 1988) and HA-966 (1-hydroxy-3-aminopyrrolidone-2; Fletcher & Lodge, 1988) can block the excitatory effects of NMDA in electrophysiological preparations. Enhanced [^{3}H]MK-801 binding data (Ramsom & Stec, 1988; Williams et al., 1989) suggests the existence of a further facilitatory site at which polyamines such as spermine and

spermidine regulate the excitatory effect of NMDA, and compounds such as Ifenprodil and its derivative SL 82.0715 possibly act at this site (Carter et al., 1989).

There is also evidence for a site at which zinc and tricyclic antidepressants appear to act as NMDA antagonists (Forsythe et al., 1988; Reynolds & Miller, 1988) and intracellular phosphorylation may be required to maintain the receptor-channel complex in its most activatable state (Mody et al., 1988).

Phencyclidine (PCP) ligands, the largest class of noncompetitive NMDA antagonists, do not compete for binding at glutamate recognition sites but act at the so called PCP/MK-801 site within the NMDA receptor-activated membrane channel, and impede the flow of cations through the channel (Kemp et al., 1987; Lodge et al., 1987). The actions of these drugs are considered below .

The Pharmacology of Dizocilpine

Dizocilpine (MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate, fig 1.2) was originally identified as a potent anticonvulsant with anxiolytic and stimulant properties (Clineschmidt et al., 1982 a,b,c), features which are now known to be associated with NMDA receptor blockade. Several chemically different compounds can be classed as non-competitive NMDA antagonists (Kemp et al., 1987). These include the "dissociative anaesthetics", PCP and ketamine; the benzomorphan "sigma opiates", *N*-allylnormetazocine (SKF10047) and cyclazocine; the substituted dioxolanes, etoxadrol and dexoxadrol; the morphinan derivative, dextrorphan; and the benz(f)isoquinoline, LY154045 (Lodge et al., 1987). Wong et al. (1986) found dizocilpine to be the most potent non-competitive NMDA antagonist yet described.

These compounds do not compete with the agonists at the transmitter recognition site of the NMDA receptor but are non-competitive antagonists which are thought

to act on the receptor operated ion channel (Kemp et al., 1987). They are said to be "agonist dependant" ie their blocking effects require agonist activation of the NMDA receptor and their blockade is also voltage dependent suggesting that they bind to a site within the ion channel which senses the transmembrane field (Kemp et al., 1987, Foster et al., 1990).

Dizocilpine, and most other non-competitive NMDA antagonists, are highly lipophilic compounds and they readily penetrate the central nervous system (Kemp et al., 1987). This, together with their "agonist-dependence", gives them some advantage as therapeutic agents. The most obvious disadvantages are their possible psychotomimetic properties, potential lasting changes related to a disturbance of normal NMDA receptor function in excitatory synaptic plasticity (Albers et al., 1989) and possible neurotoxicity (Olney et al., 1989a). A crucial question is whether the adverse effects of such compounds are due to NMDA receptor blockade or some separate action. However, they remain extremely useful tools in the investigation of NMDA receptor blockade in experimental models.

1.2.5 NMDA receptors and hypoxic-ischaemic neuronal injury

In vitro experiments

Rothman (1984) and Goldberg et al. (1986) have demonstrated that when mature cultures of either rodent hippocampus or neocortex are placed in an anaerobic environment, they deteriorate over the next day until only debris remains. Rothman (1983) noted a striking difference between young (2 days in vitro or younger) and mature (at least 2 weeks in vitro) neurons in their sensitivity to anoxia. When young neurons were kept in an environment composed of 95% nitrogen / 5% carbon dioxide, they were not visibly changed after 24 hours. Mature neurons all died after the same exposure. The only difference to explain these findings was that mature

neurones had spontaneous synaptic activity, which was not present early in vitro because all synaptic connections are disrupted before plating and take time to reform (Jackson et al., 1982). Rothman (1984) demonstrated that anoxic destruction of mature neurons could be almost completely prevented if the cultures were pretreated with the non-selective excitatory amino acid antagonist γ-D-glutamylglycine. More recent culture experiments by Goldberg et al. (1987) have demonstrated similar protection from anoxia with much lower concentrations of specific NMDA antagonists.

These experiments provided evidence that accumulation of synaptically released transmitter, probably glutamate, was responsible for anoxic cell death. It is difficult to relate these observations in dispersed tissue culture to the intact central nervous system, but they have been extended to hippocampal slices. Clark & Rothman (1987) demonstrated that NMDA antagonists could prevent anoxia from causing the irreversible loss of normal electrophysiological properties in the CA1 region of rat hippocampal slices.

In vivo studies

Simon et al. (1984a) showed that the excitatory amino acid antagonist aminophosphonohepatanoate, locally injected into the rat hippocampus, dramatically reduced the neuronal damage normally observed after ischaemia. Foster et al. (1987) demonstrated that systemic administration of the NMDA antagonist dizocilpine prevented the neuronal degeneration caused by a direct injection of NMDA into the rat hippocampus. Gill et al. (1987a) showed that systemic administration of dizocilpine protected against ischaemia induced hippocampal neurodegeneration in the gerbil and more recently they demonstrated that dizocilpine was still protective when administered in the post ischaemic period, up to 24 hours after initiation of the ischaemia (Gill et al., 1988). Pulsinelli (1985)

found that removing the glutaminergic excitatory input to the rat hippocampus also reduced the ischaemic changes seen after arterial occlusion. The numerous in-vivo studies of NMDA antagonists in various models of ischaemia are fully discussed in section 1.4.

These studies, and many others, are all consistent with the concept that under anoxic and / or ischaemic conditions there is a build up of a synaptically released neurotransmitter, most likely glutamate, which causes neurodegeneration by acting mainly on the NMDA subgroup of receptors. The early in-vivo work using NMDA antagonists concentrated on only one population of neurons that are particularly sensitive to ischaemia - the CA1 hippocampal pyramids. This region also has the highest concentration of NMDA receptors as shown by autoradiography of binding sites of either NMDA-displaceable [^{3}H]L-glutamate (Monaghan et al., 1983; Monaghan & Cotman, 1985) or [^{3}H]-dizocilpine (Bowery et al., 1988). Evidence that NMDA antagonists can protect these neurons does not necessarily imply that other neurons can be similarly protected. It is unreasonable to expect that blockade of the NMDA receptors will totally protect central neurons from prolonged deprivation of oxygen and all metabolic substrates. Goldberg et al., (1986) demonstrated that when basal ganglia neurons are exposed to an anaerobic environment in the absence of glucose (simulating "ischaemia") they slowly deteriorate in spite of high concentrations of γ-D-glutamylglycine or magnesium (an NMDA channel blocker, see below). Accumulation of glutamate may be a major factor in ischaemic neuronal damage, but it is not the only one.

Hypoglycaemic brain damage

Wieloch (1985a) demonstrated that insulin-induced hypoglycaemic brain damage in the rat can be reduced by NMDA receptor blockade. He also showed that hypoglycaemic striatal damage may be ameliorated by lesions to the glutamatergic cortico-striatal projections in the rat (Wieloch et al., 1985b). Auer et al. (1984) showed that neurons undergoing hypoglycaemia-induced degeneration have a similar appearance, in either light or electron micrographs, to neurons exposed to excitotoxic concentrations of glutamate. It seems likely that hypoglycaemia, like anoxia, interferes with glutamate re-uptake leading to neurotoxic concentrations of the transmitter in the extracellular compartment.

Epileptic brain damage

Many investigators have maintained that prolonged seizures in the absence of anoxia are capable of producing cerebral lesions (Meldrum, 1973). Collins & Olney (1982) demonstrated that an acute seizure focus in the rat sensorimotor cortex produced glutamate-like neurodegenerative lesions in thalamic nuclei that receive a glutamatergic input from the cortex. Prolonged stimulation of the perforant path, a glutamatergic excitatory input to the hippocampus, can produce similar lesions in ventilated anaesthetized rats (Sloviter, 1983). Labruyere et al. (1986) showed that systemic administration of non-competitive NMDA antagonists can prevent seizure mediated brain damage and this has been confirmed by others (eg Clifford et al., 1990). The pathophysiological processes in epileptic brain damage may, therefore, be similar to those in anoxia.

1.3 Experimental Models of ischaemia

1.3.1 Classification

Animal models of ischaemia can be broadly classified into two groups, those that produce "global" and those that produce "focal" ischaemia. Global models attempt to simulate the situation following "cardiac arrest" or other clinical situations in which there is profound generalised hypoperfusion of the brain. These models usually involve the temporary occlusion of major extracranial vessels or temporary haemorrhagic / drug-induced hypotension or cardiac asystole. Models of Focal ischaemia attempt to mimic stroke. In the majority of these models, an intracranial vessel is either permanently or temporally occluded or emboli are introduced into the circulation.

Overall there are a plethora of different models, with a wide variety of surgical induction methods, species variations, monitoring and end points, currently in use in the effort to establish a physiological approach to the management of cerebral ischaemia. The significance and clinical relevance of these models is open to continued debate (Molinari, 1988) but a number of factors must be considered.

1.3.2 Species variables

In experimental strokes there are often wide variations in responses to ischaemic challenge among species and among individuals of the same species. These variations are usually attributed to the premorbid vascular anatomy (Molinari, 1986). Traditionally, credibility of a model seems relative to the degree of anatomical, biochemical and physiological similarity to man, making subhuman primates the most relevant species for stroke models. However, in large animals there is greater individual variation in patterns of vascularization (Laurent et al., 1975; Morawetz et al., 1978), making standardization of stroke models more difficult

and necessitating larger numbers for statistically significant outcomes. Ethical issues abound in the use of large animals in experiments and they are much more expensive to obtain and maintain. Conversely, smaller animals are more stereotyped in vascular anatomy, and in post ischaemic behaviours, they are more acceptable from both ecological and ethical perspectives and they are much cheaper. It is quite impractical and morally untenable to test the majority of scientific hypotheses in ischaemia research using primates. This is particularly true in neuroprotection experiments where hard, reliable data, using neurohistopathology as an end point, can be obtained from controlled experiments in lower species, which, in view of the numbers involved, could not be obtained using primates.

1.3.3 Gerbil models

Some experimental models rely on specific anatomical variants from human anatomy, for instance the Mongolian gerbil has been widely used as a model of "hemispheric" ischaemia because the species has an incomplete circle of Willis and ischaemia can be conveniently induced by temporary occlusion of a carotid artery in the neck (Levine & Sohn, 1969). A widely used and more reliable model of "forebrain" ischaemia involves temporary bilateral carotid artery occlusion. The hippocampal neurons (especially the CA1) are particularly sensitive to ischaemia and undergo delayed degeneration. This model has been criticised because many drugs appear to prevent ischaemia induced hippocampal degeneration, including some (eg barbiturates) which are ineffective in other models. It has been proposed that mechanisms such as the hypothermic or anticonvulsant actions of drugs may be responsible for the protection seen (Buchan & Pulsinelli, 1990; see also below).

It is illogical to assume that such species which are anatomically diverse from man should function in physiological and biochemical patterns similar to man. Although

delayed degeneration of neurones in the hippocampus has been observed in man following cardiac arrest (Petito et al., 1987), the exact mechanisms underlying the delayed degeneration of hippocampal neurons in various models of ischaemia and the clinical relevance of the phenomena are still undetermined.

1.3.4 Rat models

During the last two decades, rat models have been used extensively in ischaemia research (Ginsberg & Busto, 1989). The rat has an intact circle of Willis and to produce a consistent degree of "Hemispheric" ischaemia, both carotid and vertebral arteries must be occluded (Pulsinelli & Brierley, 1979) or some other insult must be added as in the model of Smith and colleagues (1984) which involves bilateral carotid occlusion combined with haemorrhagic hypotension.

Rat models have a number of advantages over those using other species, apart from their cost and size. There are many similarities between the anatomy of the cranial circulation of the rat and man (Yamori et al., 1976) especially when contrasted with other species such as the gerbil, cat and dog. More detailed information about the neurotransmitter systems, neurochemistry and neuropharmacology has been obtained in the rat than in any other species and a number of quantitative autoradiographic methods have been developed that measure in a precise anatomical manner various aspects of cerebrovascular function.

The "Tamura" model of focal ischaemia

In 1981 Tamura and colleagues described a method of permanently occluding the middle cerebral artery in the rat. The model has gained wide acceptance over the last decade as a reproducible model of focal ischaemia. The model used in this thesis is based on the Tamura model and is fully described and discussed in chapter 2.

Acute vs chronic models of ischaemia

The overwhelming advantages of acute models of ischaemia in neuroprotection experiments is the degree to which the key physiological variables can be measured and controlled and the lack of any discomfort on the part of the animal if kept anaesthetized. Thus the blood pressure, blood gases, body temperature, and various blood parameters such as haematocrit and glucose concentration, all of which can influence outcome from ischaemia, can be closely monitored throughout the duration of the experiment. The disadvantages of acute models include the lack of "maturity" of the area of ischaemia, the interaction of potential anti-ischaemic agents with the anaesthetics used and the lack of behaviourial measures of outcome. In this thesis a direct comparison was made of the neuroprotective effects of dizocilpine in an acute and a chronic rat model of focal ischaemia. (For these results and further discussion see chapter 4).

1.4 Neuroprotection with NMDA antagonists in Animal Models of ischaemia

1.4.1 Models of "forebrain" ischaemia

Competitive NMDA antagonists

In the first in vivo experiments which demonstrated that NMDA antagonists could be neuroprotective, Simon and co-workers (1984) injected the competitive NMDA antagonist D-AP7 (2-amino-7-phosphonoheptanonic acid) directly into the hippocampus and found that it ameliorated the acute signs of hippocampal neuronal degeneration in a 2-vessel occlusion model of forebrain ischaemia in the rat. The majority of competitive antagonists are polar compounds and therefore systemic

administration results in poor brain penetration. However, Boast and colleagues (1988 a & b) demonstrated reduced hippocampal neurodegeneration in the gerbil model of forebrain ischaemia using repeated systemic (intra peritoneal) injections of the competitive antagonists D-AP7, CPP (3-((±)-2-carboxypiperazin-4-yl)-propyl-l-phosphonic acid) and CGS 19755. The latter compound was effective even when given one or four hours after the onset of ischaemia (Boast et al 1988a). Pre-treatment with CGS 19755 also ameliorated histological damage in a 4 vessel occlusion model of global ischaemia in the rat and post ischaemic treatment, although not improving histological outcome, significantly improved learning ability one month after ischaemia (Grotta et al 1990). In a 7-day recovery model of forebrain ischaemia in the rat, Swan and colleagues (1988) demonstrated protection of hippocampal neurones when D-AP7 was administered intrahippocampally or by high dose intravenous injection.

Two major potential problems in the use of competitive NMDA antagonists are their poor brain penetration and their effects may be diminished in the face of the high extracellular glutamate and aspartate levels which occur during ischaemia.

Non-competitive NMDA antagonists

These compounds are generally lipophilic and have good brain penetration on systemic administration and have therefore received most attention so far as potential neuroprotective agents. Dizocilpine (MK-801) is the most potent of this class of compounds and has been used in the majority of cases (Foster et al., 1990).

Results of experiments using non-competitive NMDA antagonists in models of "forebrain" or "global" ischaemia have varied. Gill and colleagues have consistently demonstrated that dizocilpine can prevent the selective destruction of hippocampal CA1 and CA2 pyramidal neurones which occurs in models of forebrain ischaemia

(Gill et al., 1987a & b, 1988, 1990). Following a 5 min bilateral carotid artery occlusion in the gerbil they found the ED_{50} for dizocilpine was 0.3 mg/kg (i.p.) with 1h pretreatment and with doses of 1-10 mg/kg the majority of animals showed complete protection (Gill et al., 1987a). They also noted that dizocilpine gave full protection when administered 30 min after the ischaemic period and even partial protection when given 24h post occlusion (Gill et al., 1988).

The mechanism of protection of dizocilpine in the gerbil is queried by some authors. Buchan and Pulsinelli (1990) and Corbett and colleagues (1990) furnished evidence suggesting that the protective effect was due to hypothermia rather than NMDA blockade. Small reductions in brain temperature during ischaemia can be neuroprotective (Busto et al., 1987 & 1989) but the "hypothermia" hypothesis for the mode of action of dizocilpine in gerbils has been refuted by Gill and colleagues (1990), who carefully controlled body temperature in a further series of experiments and still showed efficacy for dizocilpine. The gerbil is particularly prone to spontaneous seizures (Thiessen et al., 1968; Cox & Lomax, 1976) and CA1 pyramidal neurones are known to degenerate as a result of prolonged seizure activity (Brierley, 1987). It has therefore been argued that the anticonvulsant action of dizocilpine and various other drugs may underlie their protective effect in this model, but there is little substantial evidence for this (Gill et al., 1987a).

In the rat, results have been even more varied. In a rat model of "forebrain" ischaemia with bilateral carotid occlusion combined with haemorrhagic hypotension (Smith et al., 1984), several groups have reported hippocampal neuronal protection when dizocilpine is given preocclusion (Gill et al 1987b; Church et al., 1988; Rod & Auer, 1988) but Wielock and colleagues (1988) failed to demonstrate a neuroprotective effect. Rod and Auer also found dizocilpine to be ineffective in the model when administered 20 min after the ischaemic period. Jensen and Auer (1988) found that ketamine was ineffective in the same model, but

Church and colleagues (1988) found ketamine to be effective in a similar model. The reasons for these discrepancies are unclear but they presumably relate to methodological differences.

1.4.2 Models of "focal ischaemia"

It is in models of focal ischaemia that NMDA antagonists have been shown to be most effective and this evidence has given us the clearest view of the clinical potential of these drugs. The majority of these models employ permanent or temporary occlusion of a major cerebral artery, usually the middle cerebral artery, as this is widely regarded as being the most relevant to "stroke" in humans (MacKenzie et al., 1986). Virtually all the neuroprotection experiments reported have shown that NMDA antagonists, both competitive and non-competitive, are dramatically effective in a variety of models of focal ischaemia (Table 1.1). This protective effect was apparent in mice, rats, cats and rabbits in both permanent occlusion and reflow experiments in acute and chronic models. When administered in the pre ischaemic period, dizocilpine reduced the signs of acute neuronal damage in the cerebral cortex of the cat by approximately 50% following permanent middle cerebral artery occlusion (Ozyurt et al., 1988). Park and colleagues (1988b) demonstrated similar efficacy for dizocilpine, in the same model, when administered up to two hours after the onset of ischaemia. Reivich and colleagues are reported to have demonstrated a similar protective effect when dizocilpine was administered following temporary middle cerebral artery occlusion in the cat (2 hours occlusion followed by 4 hours reperfusion, see Iversen et al., 1989). Similarly, Dizocilpine was effective when administered 30 minutes pre- and 30 minutes post middle cerebral artery occlusion in the rat (Park et al 1988c), and in a 48 hour recovery model when administered prior to the occlusion (Park et al., 1988d).

Dizocilpine, SL82-715, TCP, ifenprodil and kynurenate have all shown efficacy in a mouse, 4 day recovery, middle cerebral artery occlusion model (Benavides et al., 1990, Gotti et al., 1990). The model uses increases in peripheral-type benzodiazepine binding to reactive astroglia and macrophages as a measure of ischaemic damage, which avoids assessing histopathological damage, a highly time consuming and expensive process. This model may prove extremely useful in examining the dose response and therapeutic window for NMDA antagonists, but the measure of damage is indirect and the experiments un-monitored, necessitating further evaluation in other models (McCulloch et al., 1990).

Several NMDA antagonists have been shown to improve outcome in various models of focal ischaemia in rabbits. Dizocilpine improved survival in a microsphere-induced multi-infarct model in the rabbit when administered during or shortly after the microsphere injection (Kochhar et al., 1988). There are few reports of experiments involving temporary focal ischaemia followed by reperfusion. Kochhar and colleagues (1988) demonstrated than dizocilpine improved outcome in a rabbit model of temporary spinal cord ischaemia. Dextromethorphan and its active metabolite, dextrorphan have been extensively examined in a reperfusion model in the rabbit. Both these agents, with pretreatment and with treatment started with the reperfusion after one hour of ischaemia, reduced the amount of histological damage, reduced the amount of oedema on magnetic resonance imaging and improved somatosensory evoked responses (George et al., 1988, Steinberg et al., 1988a & b, 1989a & b). The minimum anti-ischaemic doses and whether this effect is definitely due to NMDA receptor blockade have yet to be established (Steinberg et al., 1989b; Tortella et al., 1989). The anti-ischaemic dose of dextromethorphan in the rabbit given in the first hour of treatment is 30 mg/kg (iv); the antitussive dose in man is 0.2 mg/kg (po, 3-4 times daily).

Table 1.1 The effects of NMDA antagonists in Models of Focal Ischaemia.

Species	Model	Agent	Treatment	Protection	Reference
Rat	MCA	Dizocilpine	Pre & Post	41%	Park et al.,1988 c
	MCA	Dizocilpine	Pre	40%	Tamura et al., 1988
(SHR)	MCA	Dizocilpine	Pre	15%	Coyle, 1989
	MCA	Dizocilpine	Pre	41%	Bielenberg, 1989
(SHR)	MCA (temp)	Dizocilpine	Pre	19%(ns)	Panetta et al., 1989
	MCA + CA	Dizocilpine	Pre	73%	Buchan et al., 1990
(SHR)	MCA + CA	Dizocilpine	Pre & Post	23%	Dirnagl et al., 1990
	MCA	Dizocilpine	Pre	45%	Lythgoe et al., 1990
	MCA	TCP	Pre	27%	Gotti et al., 1988
	MCA	TCP	Pre	27%	Duverger et al., 1987
	MCA	PCP	Post	57%	Bielenberg, 1989
	MCA	SL82-715	Post	48%	Gotti et al., 1988
	MCA	Kynurenate	Pre & Post	56%	Germano et al., 1987
(SHR)	MCA	Kynurenate	Pre	2% (ns)	Roussel et al., 1989
	MCA	AP7	Post	53%	Roman et al., 1989
	MCA + CA	Dextrorphan	Post	53%	Kent et al., 1989
	MCA	CGS19755	Pre / Post	64 / 50%	Simon et al., 1990
Mouse	MCA	Dizocilpine	Post	86% *	Benavides et al., 1989
	MCA	SL82-715	Post	75% *	"

Table 1.1 (continued)

Species	Model	Agent	Treatment	Protection	Reference
Mouse	MCA	TCP	Post	62% *	Benavides et al., 1989
	MCA	Ifenprodil	Post	60% *	"
	MCA	Kynurenate	Post	60% *	"
Cat	MCA	Dizocilpine	Pre	50%	Ozyurt et al., 1988
	MCA	Dizocilpine	Post	55%	Park et al., 1988 b
	MCA (temp)	Dizocilpine	Post	50%	Reivich (see Iversen et al.,1989)
	MCA	Ifenprodil	Post	42%	Gotti et al., 1988
	MCA	SL82-715	Post	36%	"
	MCA	D-CPP-ene	Pre & Post	65%	Bullock et al., 1990 b
Rabbit	ACA + CA	Dextrorphan	Pre & Post	80%	Steinberg et al., 1988, 1989 a & b
	ACA + CA	Dextromethorphan	"	79%	"

Neuroprotection was assessed by histopathology or with magnetic resonance imaging, except (*) where decrease in peripheral type benzodiazepine binding (ω_3) site density was used. The experimental models were permanent middle cerebral artery occlusion (MCA), temporary middle cerebral artery occlusion (MCA, temp), tamdem occlusion of the middle cerebral and carotid arteries (MCA + CA), temporary occlusion of anterior cerebral and carotid arteries (ACA + CA). Spontaneously hypertensive rats donated (SHR). (ns = not significant). (Table modified after McCulloch et al., 1990) .

1.4.3 Other models

Models of "cardiac arrest"

In several large animal models of cardiac arrest, dizocilpine has failed to improve outcome. Dizocilpine did not improve neurological outcome in cats (Fleischer et al., 1988) or dogs (Michenfelder et al., 1989; Sterz et al., 1989) following cardiac arrest nor did it improve histological outcome in the cat model (Michenfelder et al., 1989). In a study in non-human primates, with 17 minutes of ischaemia (neck tourniquet), dizocilpine failed to provide any evidence of amelioration of the ischaemic damage to the CNS (Lanier et al., 1990). These results may reflect the severity of these models which tend to have a high mortality rate and a variable outcome in the controls. However the same models have been used to demonstrate significant benefit of amino steroids (Perkins et al., 1989) and hypothermia (Leonov et al., 1990). Ketamine has been reported to show a marginal beneficial effect in a dog cardiac arrest model (Natale et al., 1988).

Models of "neonatal hypoxia/ischaemia"

Perinatal hypoxia, like focal ischaemia, is another area where there is convincing evidence for neural protection from ischaemic brain damage. Hypobaric/ischaemic conditions produce cytopathology similar to glutamate toxicity in the infant rat brain (Ikonomidou et al., 1989). Dizocilpine prevents neuronal degeneration in the hippocampus, cortex and basal ganglia produced by combined hypoxia/ischaemia in the neonatal rat, and pretreatment is equally as effective as administration up to 1.25h after the onset of anoxia (McDonald et al., 1987; Olney et al., 1989b; Hattori et al., 1989). Prince and Feeser (1988) also found that Dextromethorphan was effective in the amelioration of ischaemic damage in the neonatal rat.

Models of trauma and intracranial haemorrhage

Although CNS trauma represents one of the potential clinical targets for NMDA antagonist therapy, very little experimental work has been reported in this area. In a rat model of spinal cord trauma, dizocilpine gave a modest improvement of histological and neurological outcome when administered 15 minutes post trauma (Faden et al., 1988 & 1989). In fluid-percussion models of brain damage, dizocilpine (McIntosh et al., 1988), PCP (Hayes et al., 1988) and dextromorphan (Faden et al., 1989) are all claimed to have beneficial effects, but such models poorly replicate the pattern of damage seen after head injury in man (McCulloch et al., 1990). In a rat model of subdural haematoma (a common complication of human head injury), D-CPP-ene pretreatment reduces the amount of ischaemic brain damage (Bullock et al., 1990c).

Although there is little experimental evidence for the value of NMDA antagonist therapy in head injury, ischaemic damage is an almost invariable finding at postmortem following human head injury (Graham et al., 1978 & 1989). Thus the anti-ischaemic efficacy of NMDA antagonists in models of focal ischaemia may be as pertinent to head injury as they are to stroke (McCulloch et al., 1990).

1.5 Scope of present thesis

The aim of this thesis was to define the conditions, in a model of focal ischaemia, for neuroprotection with the non-competitive NMDA antagonist dizocilpine (MK-801), in terms of dose and delay in administration.

In order to accomplish this, a model of focal ischaemia, based on the "Tamura" model (see chapter 2), involving permanent middle cerebral artery occlusion in the rat, was set up and modified to enable recovery experiments to be performed. Initially, various methodological aspects of the model were studied. A method for volumetric analysis of lesion size is described and direct and indirect volume estimations compared (chapter 3). Neuroprotection with dizocilpine in acute and chronic ischaemia was compared. In order to facilitate the large number of studies required for dose response investigations, the mitochondrial redox stain triphenyltetrazolium chloride (TTC) was compared to histopathology as an endpoint in the acute and chronic experiments (chapters 4 & 5). The influence of strain of rat and position of MCA occlusion are also considered (chapter 7). The experiments to determine the dose response and therapeutic window for dizocilpine are described in chapter 6.

Finally, a method for chronic CSF sampling in rats was set up and CSF neuron-specific enolase levels were compared to infarct volumes in control and dizocilpine treated animals to assess the usefulness of this enzyme as a marker of neuronal damage in focal ischaemia (chapter 8).

Chapter 2

The rat middle cerebral artery occlusion model of focal ischaemia

2.1 Introduction

The most widely used reproducible model of focal ischaemia in the rat was described by Tamura et al. (1981a) and this involved permanent, proximal middle cerebral artery occlusion. Prior to this the production of a reproducible, regional ischaemic lesion in the rodent proved elusive. In most previous models an extracranial artery was occluded but owing to the efficiency of the intracranial collateral circulation, the development of brain damage required an additional stress, such as hypoxia (Levine, 1960; Brown & Brierley 1966, 1968 & 1972; Salford et al., 1973a & b) or hypotension (Eklof & Siesjö 1972; Nordstrom & Rehncrona, 1977) to be added. These stresses introduced complicating factors and the results were commonly variable.

Robinson (1981) described a method of occluding the distal middle cerebral artery in the rat but it involved a rather large craniectomy and produces a rather small, inconsistent, frontoparietal infarct (see chapter 7). Distal middle cerebral artery occlusion can only produce consistent infarction in normotensive rats if some other insult is added such as ligation of the ipsilateral carotid (Chen et al 1986, Brint et al 1988). Coyle (1982a) also described a technique for distal middle cerebral artery occlusions and this can produce consistent cortical infarcts in spontaneously hypertensive rats (see chapter 7).

The occlusion of an intracranial artery, usually the proximal middle cerebral artery, is widely used to produce focal ischaemia in larger animals such as the cat (Sundt & Waltz, 1966; Bose et al., 1984), dog (Suzuki et al., 1980), and primate (Hudgins & Garcia, 1970). A reproducible lesion results, provided that the artery is occluded at a proximal site and the cardiovascular and respiratory status is rigorously controlled.

The most common route of occlusion in larger animals is via the trans-orbital approach, but when compared with the cat, dog and primate, the origin of the middle

cerebral artery in the rat lies relatively further from the optic foramen, making a sub-temporal approach more appropriate.

Tamura et al. described the occlusion of the middle cerebral artery in the rat via a sub-temporal approach, using microsurgical techniques. The model was devised as an acute model with continuous monitoring of blood pressure and controlled ventilation throughout the experiment. The model described in this chapter is based on the "Tamura" model with some important modifications, which were primarily aimed at producing a "recovery" model to examine the chronic effects of focal ischaemia.

2.1.2 Anaesthesia

In the Tamura model, anaesthesia was induced with 2% halothane and maintained by controlled ventilation with nitrous oxide / oxygen mixture (70% : 30%) containing 0.5% halothane, via a tracheostomy. This method provides excellent control over the anaesthesia for maintenance of blood pressure and arterial blood gases, but a tracheostomy cannot be used for recovery experiments. For recovery experiments authors mainly use injectable anaesthetics (Germano et al., 1987; Persson et al., 1988), or gaseous anaesthesia via a face mask (Duverger et al., 1988) . In order to maintain the advantages of controlled ventilation in a recovery model, a technique of endo-tracheal intubation in rats was developed.

To avoid repeated exposure to halothane isoflurane was used in all the experiments.

Anaesthesia was induced with 4% isoflurane in oxygen and the animals were deeply anaesthetized to facilitate intubation. The endo-tracheal tubes were made from polythene tubing (external diameter 2.42 mm) connected to a Y connector. The ends of the tubes were bevelled and the animals were intubated "blind" using gentle

tongue retraction and cricoid pressure. Anaesthesia was maintained by ventilation with 1 - 2% isoflurane in nitrous oxide / oxygen mixture (70% : 30%) (Ventilator: Palmer Bioscience Miniature Ideal Pump).

Muscle relaxants were not used in any of the experiments.

2.1.3 Monitoring

Rectal temperature was continuously monitored and maintained at 37 ± 0.5°C by a warming blanket connected to a rectal thermistor probe (Harvard Apparatus). Tamura and colleagues used cannulation of a femoral artery for monitoring blood pressure and for arterial sampling for blood gas analysis. Femoral artery monitoring can be used in recovery models but tail artery monitoring is less disturbing to the animals and this was therefore used in preference.

The tail arteries were cannulated with 15 cm polythene tubes (external diameter 0.96 mm) connected via a three way tap to a blood pressure transducer (Elcomatic EM750). The output from the transducer was fed to a chart recorder (Kipp & Zonen BD41) and to an integrator for digital display of mean arterial pressure. The system was calibrated prior to every experiment using a clinical sphygmomanometer at 0 and 100 mm Hg.

For blood gas analysis 100 μl samples of arterial blood were taken into heparinized tubes. The arterial catheters were flushed with saline containing 20 U heparin/ml after every sample. The samples were analyzed on a Radiometer ABL 30 blood gas analyzer.

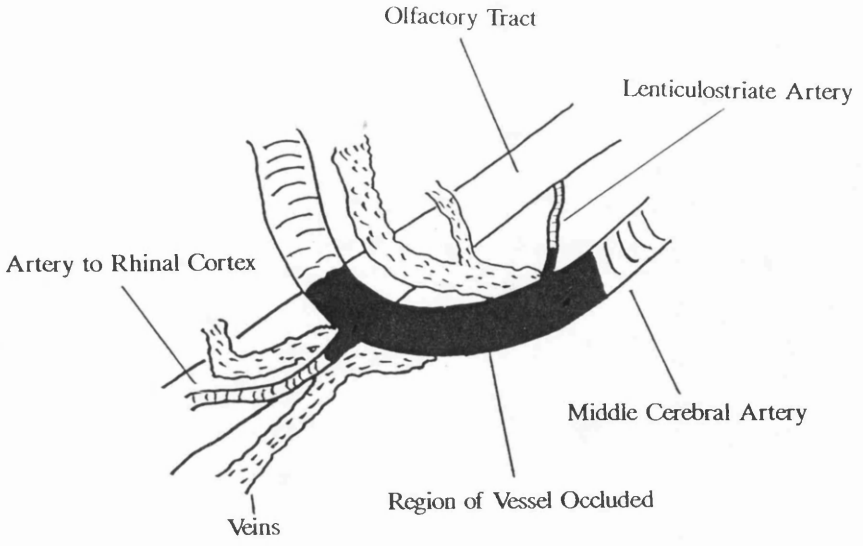

Figure 2.1 Diagram illustrating the anatomy of the middle cerebral artery of the rat, showing the region of vessel occluded in a "proximal occlusion". (Diagram after Shigeno et al., 1985)

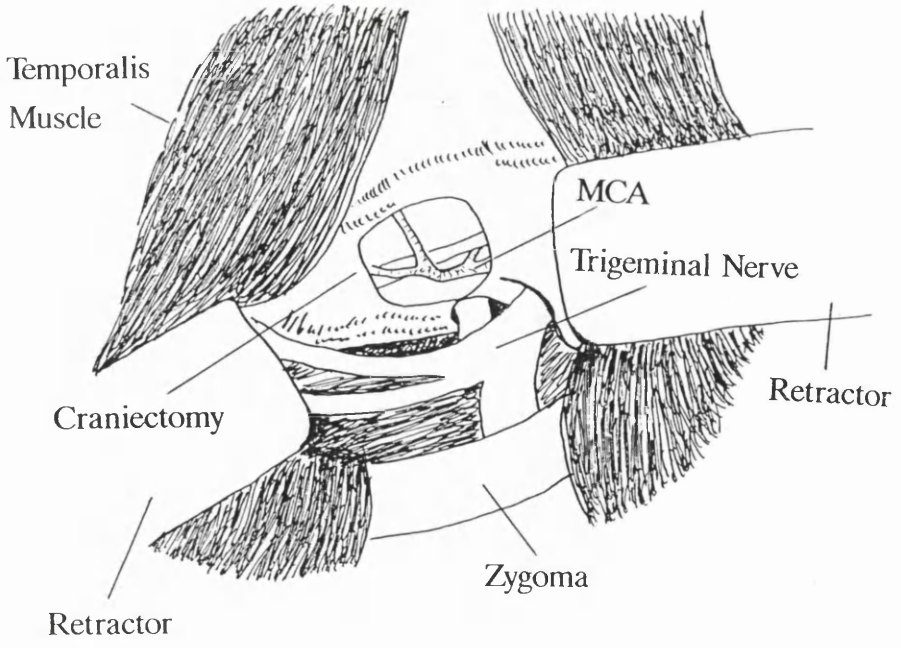

Figure 2.2 Exposure of the middle cerebral artery of the rat via the sub-temporal approach. (After Shigeno et al., 1985).

2.1.4 Surgery

After shaving the region of the incision, the anaesthetized and ventilated rat was placed in the lateral position (left side up) and secured with masking tape. A tarsorrhaphy stitch (4/0 silk) was inserted to protect the left eye. The skin was sprayed with povidone iodine and a skin incision was made half way between the external auditory meatus and the orbit. The skin was retracted with two copper retractors, the temporalis muscle was divided and two further retractors were inserted to expose the infero-temporal fossa. The main difference between Tamura's procedure and the one described here is the preservation of the zygoma and the temporalis muscle. The rest of the procedure was carried out using an operating microscope. The key landmarks were the mandibular nerve and the foramen ovale. A small craniectomy was made using a dental drill, at the junction between the medial wall and the roof of the infero-temporal fossa, as described by Tamura. The dura was opened using a fine needle to expose the middle cerebral artery proximally as far as the major lenticulostriate branch and distally for a short distance after it had crossed the olfactory tract (fig2.2). To occlude the artery it was diathermied using a bipolar coagulator (Malis CMC-2) and divided using micro-scissors. For proximal occlusions, care was taken to include the origin of the lenticulostriate artery. (see fig 2.1)

After occlusion, the retractors were removed and the soft tissues were allowed to fall back into place. The wound was sprayed with povidone iodine and the temporalis muscle was sutured with 4/0 vicryl. The skin was closed with a continuous 3/0 silk stitch.

2.1.5 Recovery

The isoflurane was turned off and the animals were ventilated with nitrous oxide / oxygen (70% : 30%) until they showed signs of recovery. They were then taken off the ventilator and allowed to breath spontaneously via the endo-tracheal tube. When breathing well they were extubated and kept in warmed cages for two hours.

2.2 End Points

Many end points have been used as parameters of neuroprotection including neurological deficit, cerebral blood flow, glycolytic metabolism, water content, or electrical cortical activity (MacKenzie et al., 1986) but more recently there has been emphasis on the importance of quantitative histological studies as a measure of the putative anti-ischaemic effects of therapeutic agents (Graham, 1988). Bederson and colleagues (1986b) described a technique for measuring neurological outcome following middle cerebral artery occlusion in the rat and they demonstrated a consistent correlation between degree of histological damage and severity of neurological deficit at 24 hours. However, neurological examination in the rat is an extremely imprecise measurement of outcome, whereas if volumetric analysis is used, the degree of histological damage can be quantified very accurately (Osborne et al., 1987). It is now generally accepted that volumetric assessment of histological damage is a reliable and sensitive end point for the evaluation of drugs as neuroprotective agents in this model (Graham 1988). Brown and Brierley (1972) demonstrated that perfusion fixation was required for the detection of early ischaemic damage. The general methods used for perfusion fixation in this thesis are described below.

2.2.1 Triphenyltetrazolium Chloride

2,3,5,Triphenyltetrazolium chloride (TTC) is a water soluble salt which is oxidised to a lipid soluble, bright red formazan by mitochondrial enzyme systems. Normal tissues stain a dark red colour when perfused with or immersed in TTC and areas in which the mitochondrial enzyme systems have been incapacitated due to infarction can be clearly demarcated. TTC has been used for more than thirty years to detect ischaemic damage in a variety of mammalian tissues (Sandritter & Jestadt, 1958). It has been used extensively to stain myocardial tissue from humans and experimental animals and is gaining wider acceptance as a stain in experimental models of cerebral infarction because it is quicker and cheaper to use than conventional histopathology (Bederson et al., 1986a; Lundy et al., 1986; Liszczak et al., 1984; Lye et al., 1987). It has been suggested that TTC may be perfused to delineate areas of cerebral ischaemia as early as 4 h after permanent middle cerebral artery occlusion (Park et al., 1988a). Bederson et al. (1986a), however, found that immersion of brain slices in TTC was an unreliable method of determining focal lesions in rat brain when compared to histopathology at 6 h or less after the onset of ischaemia. Taylor et al. (1987) also reported that TTC perfusion at post-ischaemia intervals of less than 6 h was unreliable.

Park and colleagues (1988a) demonstrated that brains stained by TTC perfusion could also be perfusion fixed with formal saline such that a comparison could be made between the two techniques in the same brains. A modified version of their technique of TTC perfusion staining followed by formalin fixation has been used in the majority of the experiments, and is described below.

In this thesis the use of TTC as a method of delineating ischaemic damage following MCAO in the rat has been thoroughly examined and related to histopathological changes with special reference to acute and chronic "neuroprotection" experiments (see chapters 4 & 5).

2.2.2 TTC perfusion staining

TTC perfusion staining can be simple and give reliable results but initial pilot studies revealed that great care must be taken with the perfusion technique to obtain uniform staining. Fig 2.4 is an example of the sort of "patchy" result that can be obtained with inadequate perfusion. This animal had no ischaemic insult and the non-staining white areas do not correspond to any underlying histological abnormality. This problem was also noted by Park and colleagues (1988a). In the pilot studies, four factors stood out as being important in obtaining uniform staining, the quantity of TTC perfused, prior heparinization, the perfusion pressure and the temperature. Both under- and over-perfusion with TTC can produce poor results but the actual volume required depends on the perfusion pressure and technique. More uniform results were obtained at higher perfusion pressures ($\approx$ 200 mm Hg) using continuous perfusion, but the same, if not better, results were obtained using "pulsatile" perfusion. In the technique finally adopted, 20 mls of 2% TTC at 37°C were perfused in a pulsatile manner by hand, at a mean pressure of 100 - 130 mm Hg. Fig 2.5 shows a more uniform perfusion in an unoccluded animal obtained using this technique.

The experimental setup for the perfusion fixation is shown in fig 2.3. All three vessels containing perfusion solutions were connected to a regulated air line to enable the perfusion pressure to be set to any desired level. The heparinized saline and TTC solutions were passed through blood warming coils (Travenol no. C2410) in a water bath at 37°C. The 20 ml syringe was used for manual perfusions of TTC and the transducer and digital meter measured the mean perfusion pressure.

TTC (Aldrich cat no. T8,485-9) was dissolved in physiological saline to give a 2% solution. This has a pH of approximately 2.4 but attempts at buffering the solution led to less intense staining and were therefore abandoned.

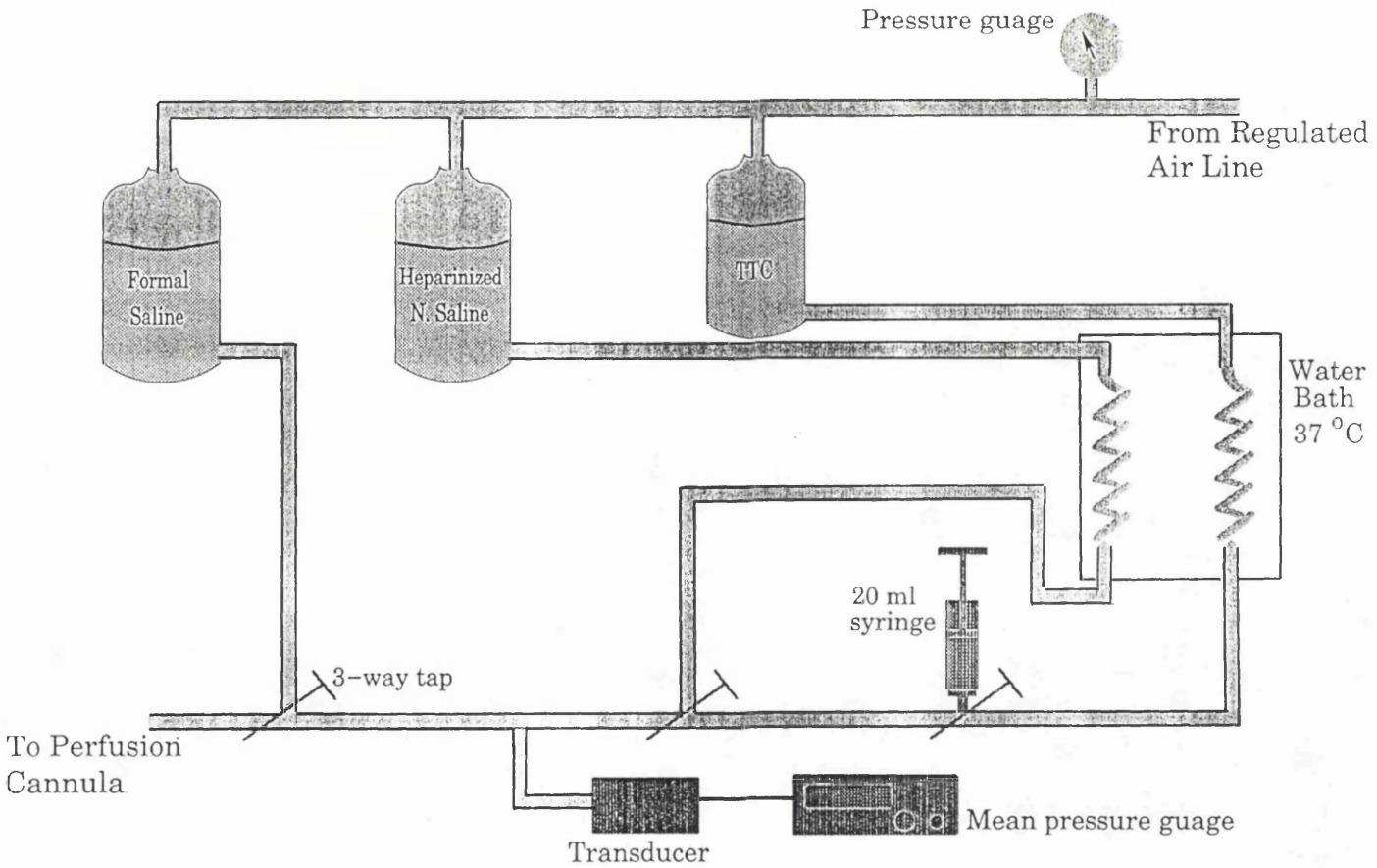

Figure 2.3 Experimental set up for TTC perfusion staining.

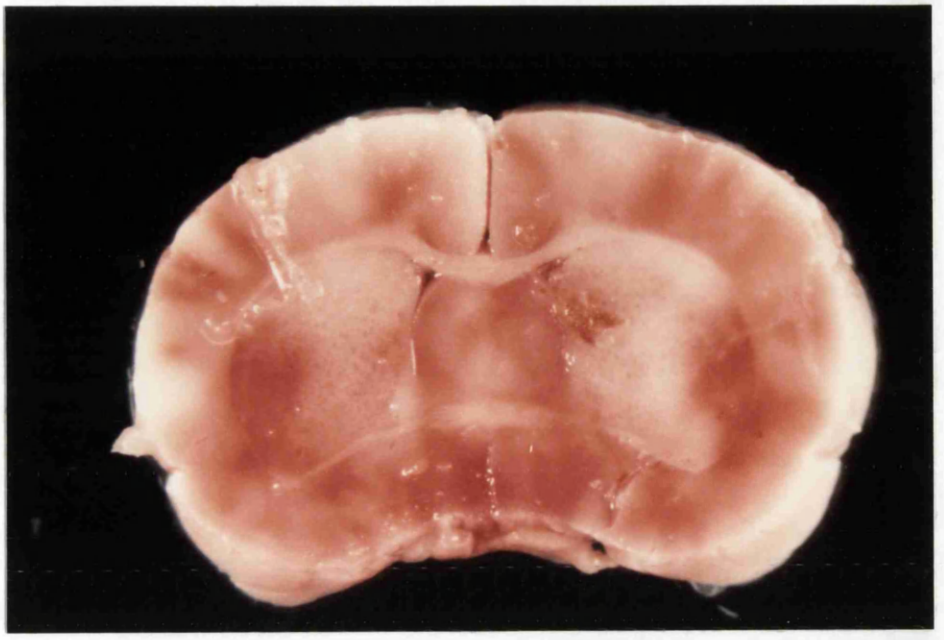

Figure 2.4 "Patchy" TTC staining with inadequate perfusion in an unoccluded animal.

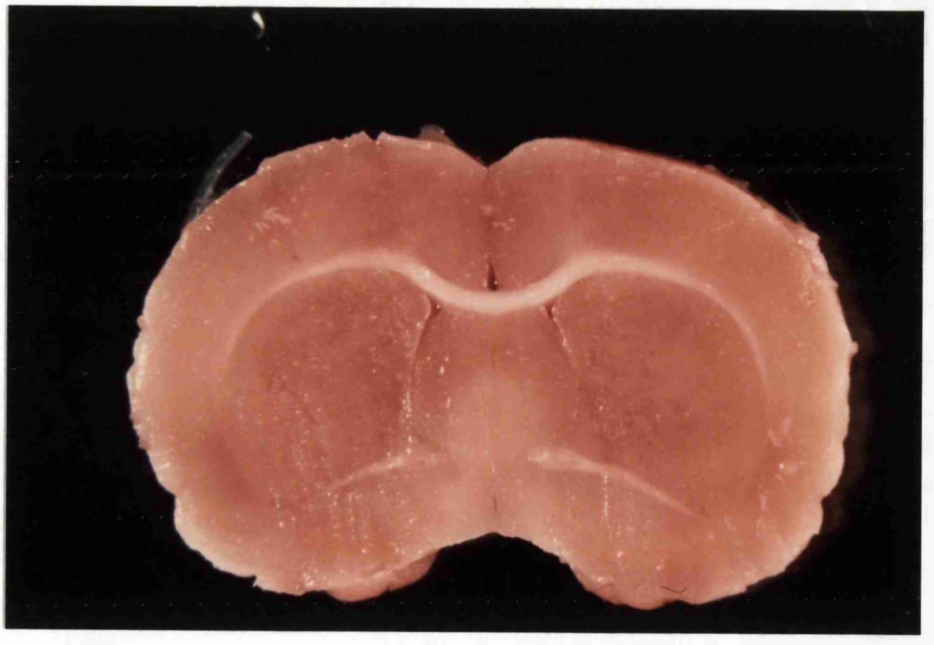

Figure 2.5 Improved TTC staining in a fully heparinized unoccluded animal perfused in a pulsatile manner.

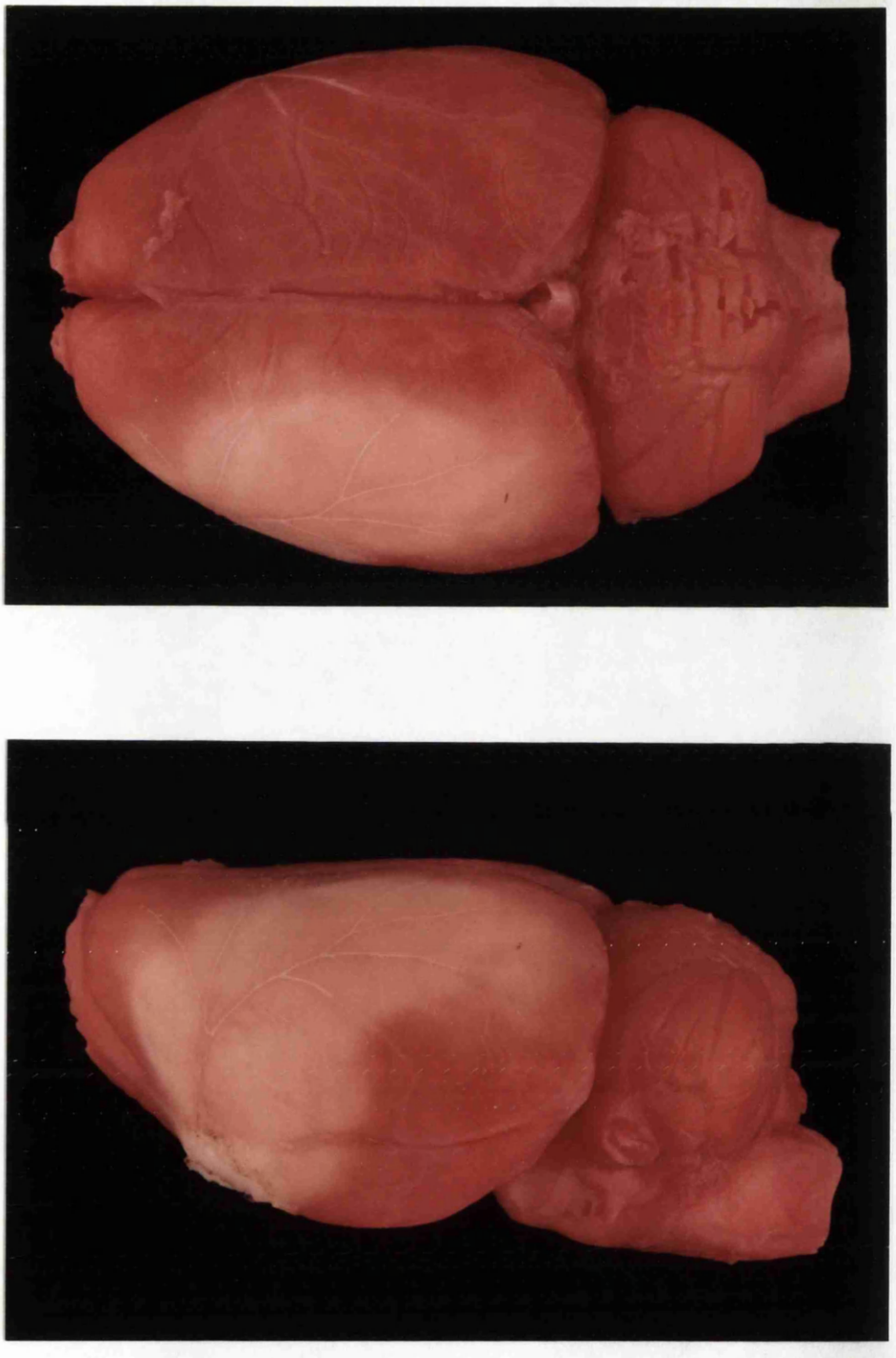

Figure 2.6 Dorsal and lateral views of whole brain following TTC perfusion staining, 24 hours after MCA occlusion.

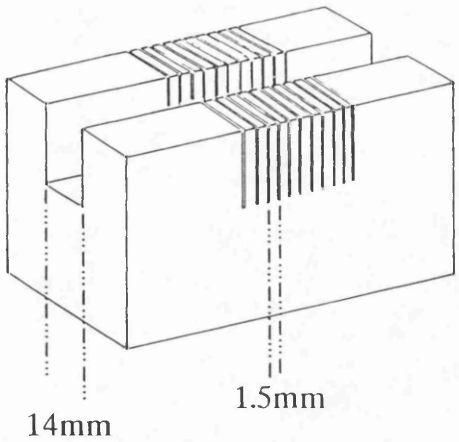

Figure 2.7 Diagram of aluminium block used for sectioning brains into 1.5 mm slices.

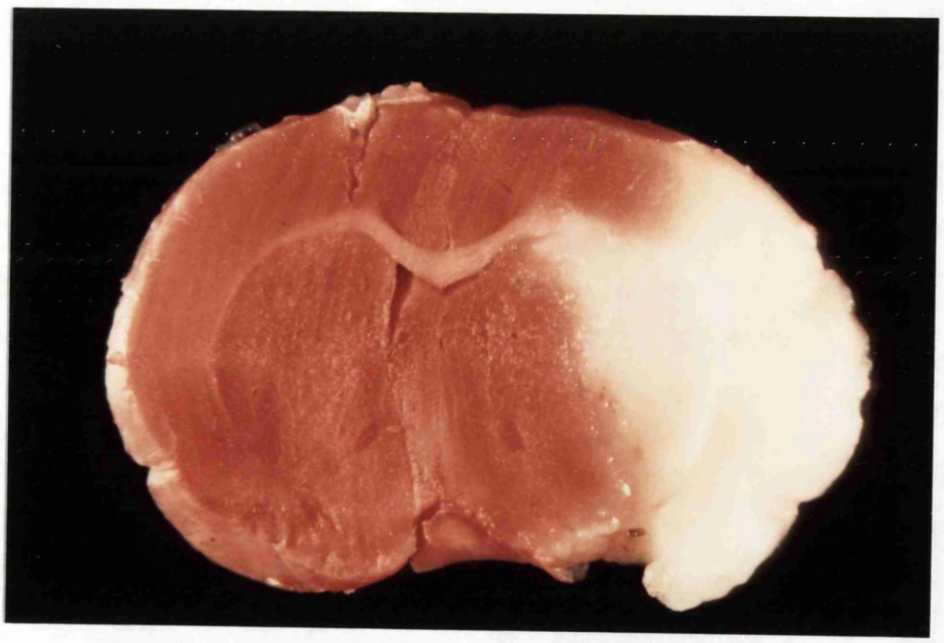

Figure 2.8 Coronal section following TTC perfusion staining, 24 h after MCA occlusion.

2.2.3 Protocol for perfusion fixation

The animals were deeply reanaesthetized with 4% isoflurane in 100% O2. Via a thoracotomy, 1000U heparin was injected into the right atrium, the thoracic aorta was clamped, a catheter was inserted into the ascending aorta via the left ventricle and perfused with warm (37°C), heparinized saline at 100 mm Hg. The right atrium was incised 20 seconds after the perfusion commenced. After approximately 90 sec the right atrium was clamped and 20 ml of 2% TTC in physiological saline at 37°C was perfused in a pulsatile manner at a mean pressure of approximately 120 mm Hg. The TTC was left stagnant for 7 min and 300 ml of 10% formal saline at room temperature was perfused at 100 mm Hg. The heads were left soaking in 10% formal saline for a further 24 hours. They were randomized, the brains removed and the forebrain sliced on a specially made block (see fig 2.7) into 9 x 1.5 mm slices. The rostral surface of each slice was photographed on a low power microscope using ektachrome 160 tungsten slide film (fig 2.8).

2.2.4 Histopathology

The brain slices were embedded in paraffin wax, using a standard neuropathological regime and 8 micron sections were taken from the rostral surface and stained with haematoxylin and eosin and by a method combining cresyl violet and luxol fast blue.

The recognition of ischaemic damage and the volumetric analysis used to quantify it are fully described in chapter 3.

Chapter 3

Quantification of ischaemic damage by volumetric analysis following middle cerebral artery occlusion in the rat.

3.1 Introduction

In order to assess any improvement in histological outcome reliable, accurate, quantification of the ischaemic lesions is essential. In models of global ischaemia ischaemic damage is selective, diffuse and may be delayed, and some type of regional area measurement or cell counting technique must be employed. However, following permanent middle cerebral artery occlusion, the boundaries of the lesion can be delineated as early as 2 hours after MCA occlusion (Tamura et al., 1981), by 24 hours they are very clear and delayed neuronal loss is not a feature of the lesion produced (Nedergaard, 1987). Many methods of quantifying damage following MCA occlusion have been described and these vary from weighing areas of damage cut from scale diagrams (Hayakawa et al., 1979) to computation of infarct size from dorsal and lateral photographs of the brain (Coyle et al., 1984a). Now that image analysis systems are readily available, irregular areas can be measured with excellent reproducibility. Lesion size is often assessed by measuring the area of damage at a particular coronal plane (eg Taylor et al., 1987; Bederson et al., 1986a & b; Persson et al., 1989) but Osborne and colleagues (1987) have clearly demonstrated that volumetric analysis is more sensitive, can be highly reproducible and can be used for the assessment of acute ischaemic damage. The method of volumetric assessment used in this thesis is based on that described by Osborne and coworkers with some important modifications.

3.1.2 Specimen preparation

The technique of perfusion fixation combined with TTC staining is described in chapter 2. The forebrain of a 300-400 g rat perfused as described, after soaking in 10% formal saline for 48 hours, was found to be approximately 15 mm long. An aluminium block was made into which the brains just fitted and this had 11 slots 1.5

mm apart for guiding razor blades (fig 2.7). Using this block the forebrain was conveniently and accurately cut into 10 x 1.5 mm slices. The tips of the frontal poles were discarded (infarction did not occur this far anteriorly) and the rostral surface of the remaining 9 x 1.5 mm slices were photographed under a low power microscope at constant magnification, using tungsten ektachrome 160 colour transparency film. A 1 cm graticule was photographed at the same magnification for later calibration of direct area measurements. For comparison with histological damage the brain slices were then processed for paraffin sections, 8 micron sections were cut from the rostral surface and stained with haematoxylin and eosin (H&E) and by a method combining cresyl violet and luxol fast blue.

3.1.3 Histopathology

In order to clearly delineate acute ischaemic damage in histological sections, perfusion fixation is required to eliminate the cytological artifact of the "dark cell" and "hydropic cell" as these can be confused with ischaemic cell change (Brown & Brierley 1968). As judged by the lack of these artifacts, the absence of blood in the vessels and good neuronal morphology, the method of perfusion described was invariably adequate. 24 hours after the onset of ischaemia the boundary between the infarct and normal brain was very clear and could be seen on H&E sections without magnification in control and dizocilpine treated animals (figs 3.1 & 3.2). After 4 hours of ischaemia, the region of irreversible brain damage had the morphological characteristics as described in perfusion fixed material (Brown & Brierley, 1968)(see fig 3.4). These changes included irregular cell margins, shrunken cell bodies with loss of Nissl substance, and shrunken dark staining nuclei which were often triangular (Compare with normal cortex, fig 3.3). At 4 hours the boundaries were less clear without magnification (fig 3.5) but under the microscope the boundary was still abrupt (fig 3.6).

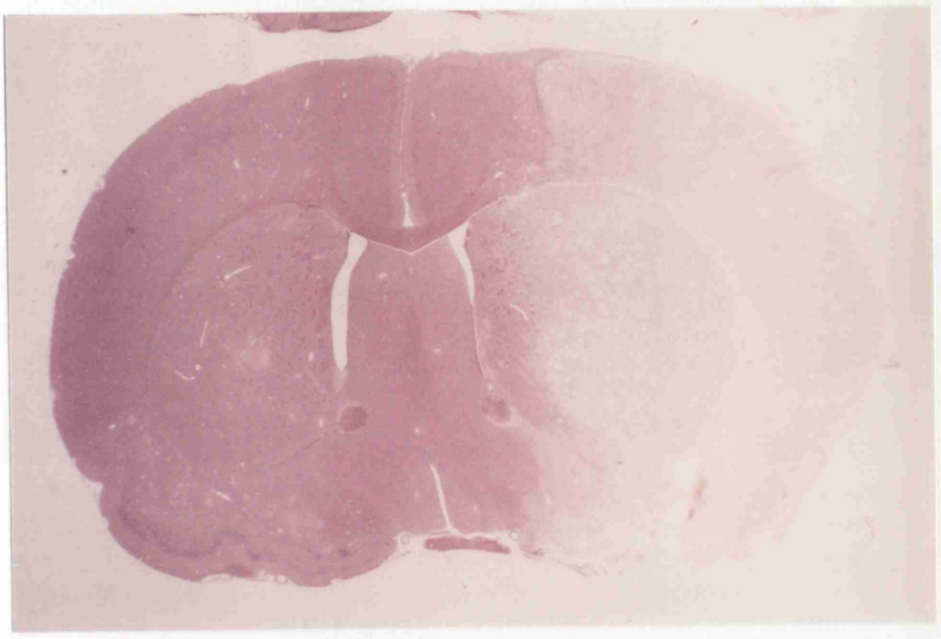

Figure 3.1 Coronal section stained with H & E, perfused 24 h after MCA occlusion (Control animal), with good macroscopic demarkation of ischaemic area (right side of photograph)

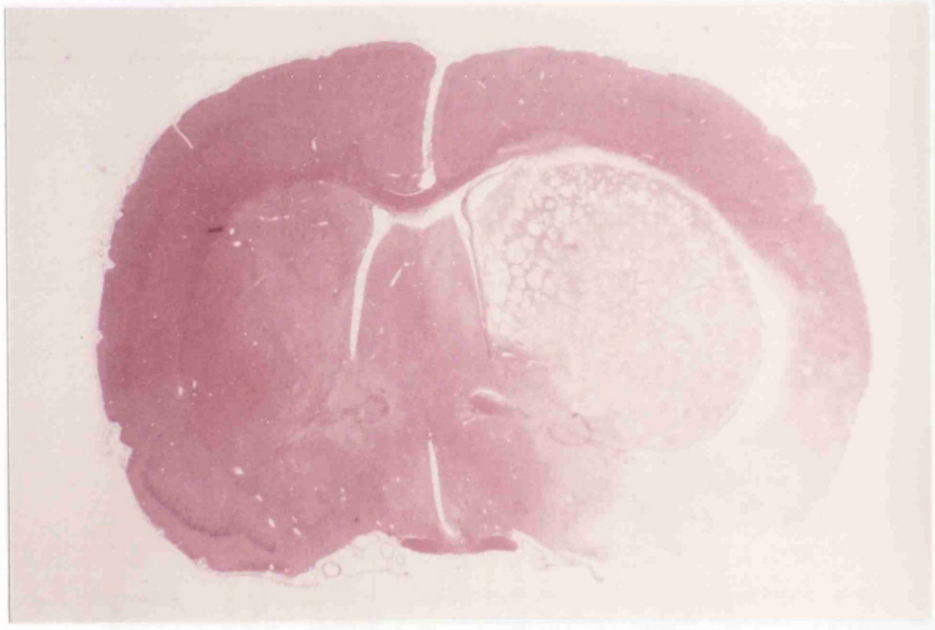

Figure 3.2 Coronal section stained with H & E, perfused 24 h after MCA occlusion (dizocilpine treated animal) showing typical region of cortical protection.

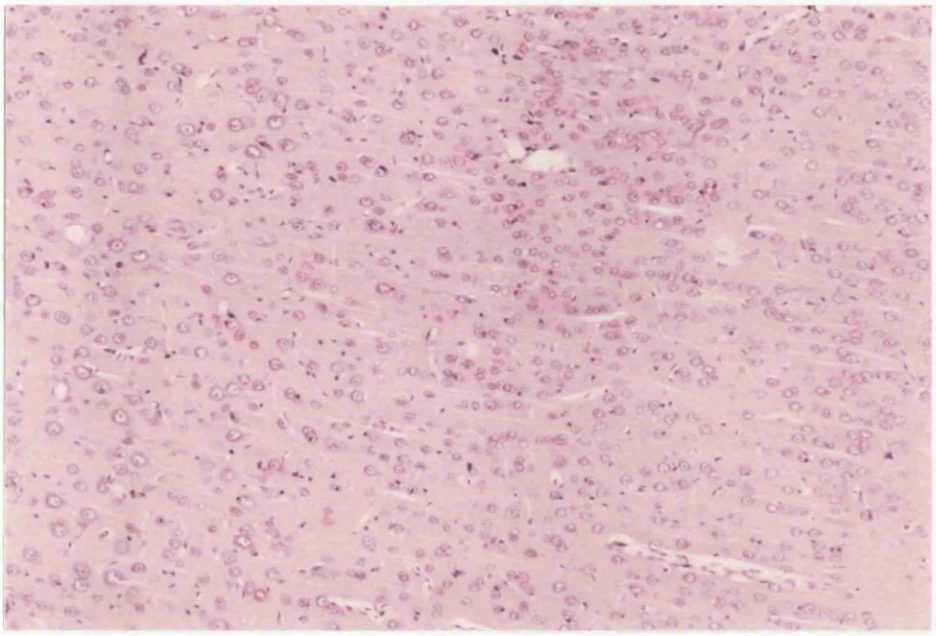

Figure 3.3 High power view of normal cortex, perfusion fixed and stained with H & E.

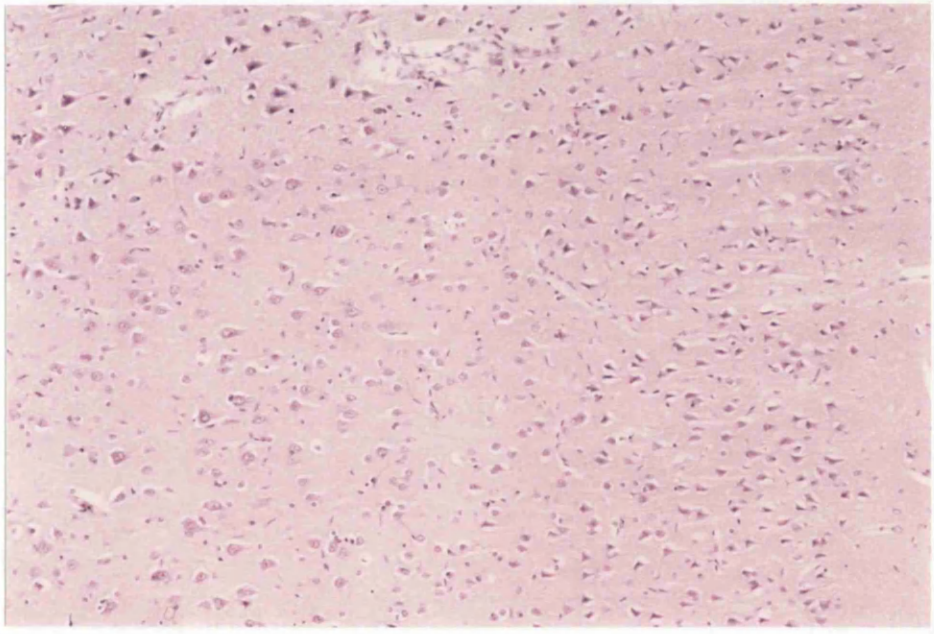

Figure 3.4 High power view of ischaemic cortex, stained with H & E, perfused 4 hours after MCA occlusion showing cortical neurons damaged by ischaemia.

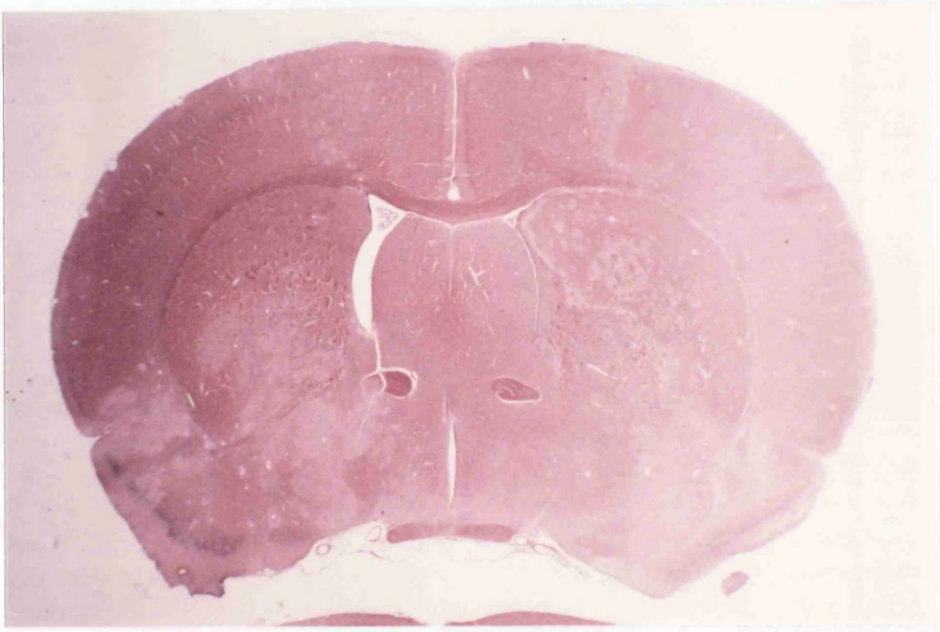

Figure 3.5 Low power view of H & E section perfused 4 h after MCA occlusion showing poor macroscopic delineation of acute ischaemic changes (right side of section).

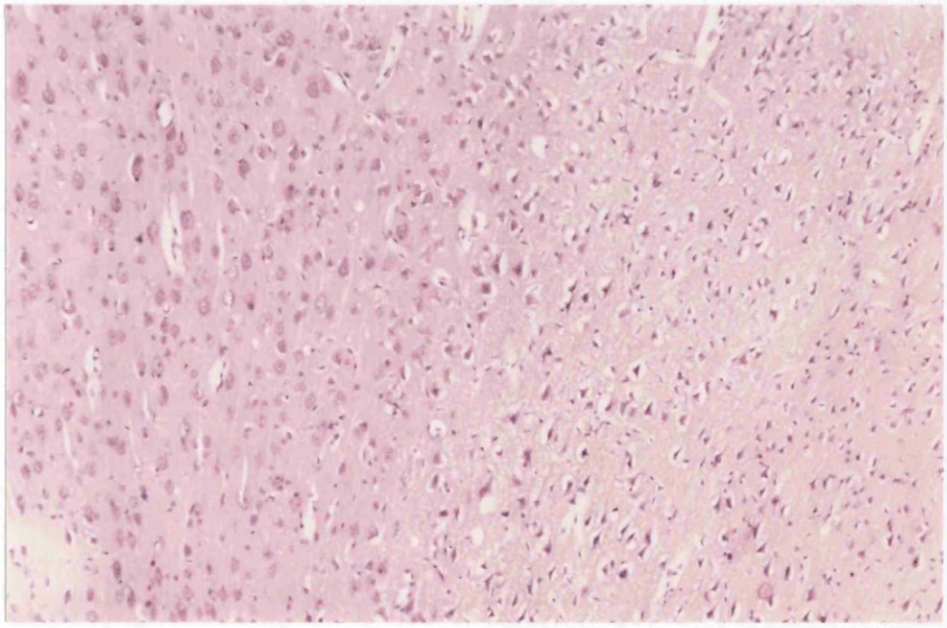

Figure 3.6 High power view of histological section (H & E) showing boundary between ischaemic area and normal brain in the cortex after 4 h ischaemia (ischaemic cortex is on the right side).

3.2 Volumetric analysis

Osborne and colleagues (1987) described a technique of volumetric assessment of early ischaemic damage following MCA occlusion in the rat. From histological sections, they drew the region of damage onto corresponding scale diagrams of coronal sections of the rat brain (reduced from the stereotactic atlas of Konig & Klippel, 1963), measured the area of damage on the diagrams using an image analyzer and integrated the areas to give a volume of damage. They clearly showed that the minimum number of sections required for accurate volumetric assessment was eight. The sections chosen from the atlas were not evenly spaced throughout the forebrain but were selected with easily recognisable anatomical landmarks so that corresponding histological sections could be accurately matched. This method was modified so that it could be applied to evenly spaced macro slices to enable direct comparisons to be made between TTC staining and histological sections. As previously stated, the brains of 300-400 g rats, perfusion fixed by the method described in chapter 2, were found to be a constant size, the forebrain being approximately 15 mm long. This is somewhat larger than the forebrains depicted in the atlas of Konig & Klippel, which are approximately 13 mm long. The difference is presumably accounted for because the atlas was made from frozen sections of 150 g rats. The perfused brains were cut into ten 1.5 mm slices on a specially made block as described in chapter 2. This gave nine evenly spaced rostral surfaces and diagrams were chosen from the atlas of Konig & Klippel which approximately represented these sections. The diagrams were reduced from the atlas to 4 x actual size and duplicated on a photocopier (fig 3.7). The boundary between the ischaemic area and normal brain, either from the histological sections or the photographs of the TTC stained slices, was drawn onto these diagrams and area measurements of hemispheric, cortical and striatal damage were made using a Quantimet Image Analyzer system (Cambridge Instruments). A set of TTC stained slices from a typical

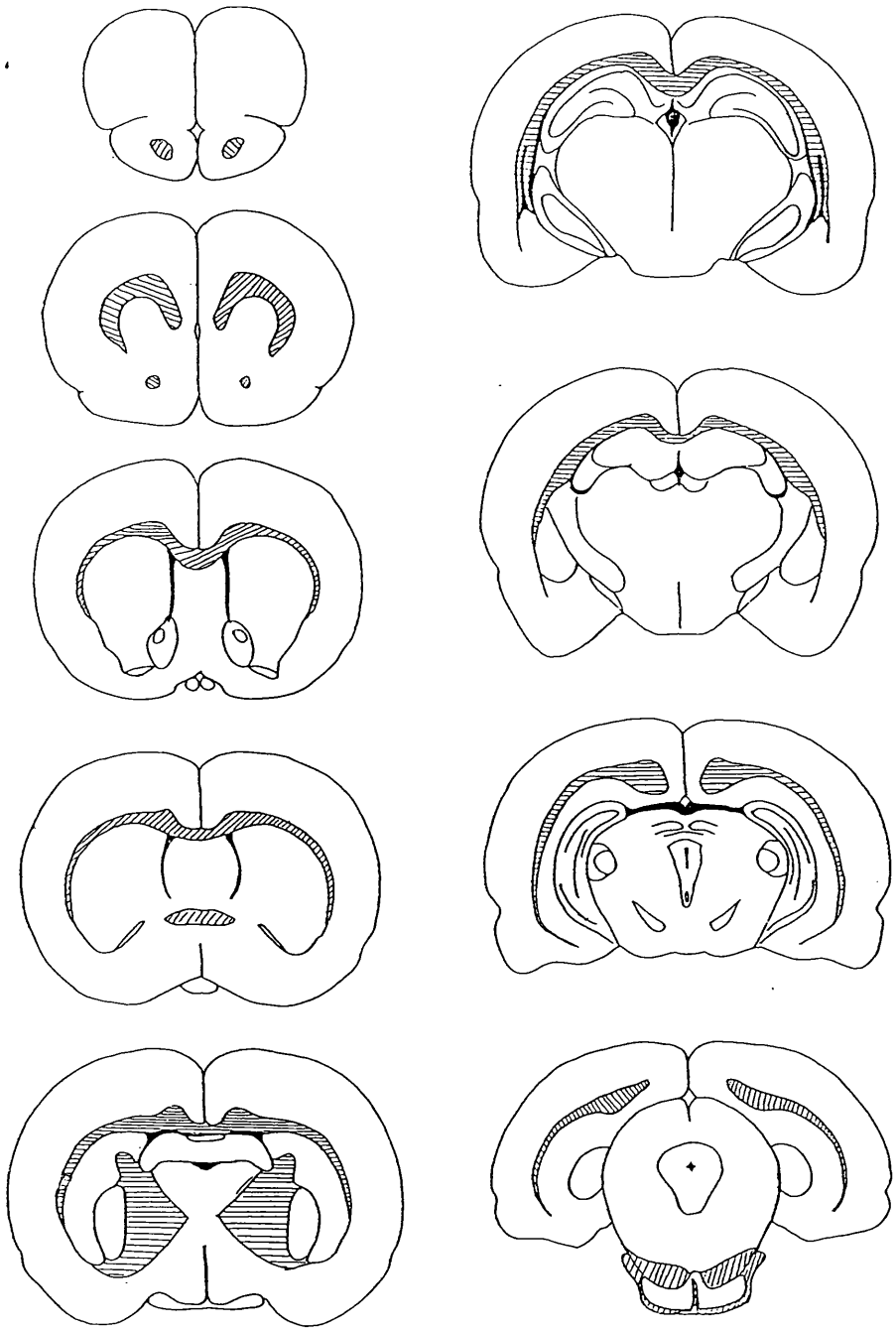

Figure 3.7 Diagrams taken from the atlas of Konig & Klippel used for volumetric analysis of ischaemic damage.

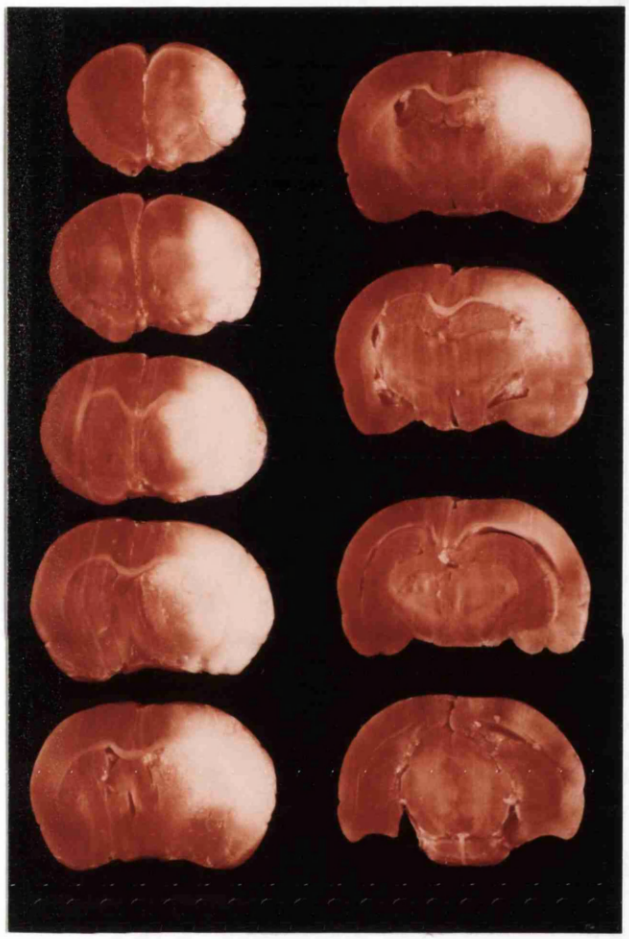

Figure 3.8 Areas of damage found in a control animal at 24 hours after permanent, proximal MCA occlusion as delineated by TTC perfusion staining.

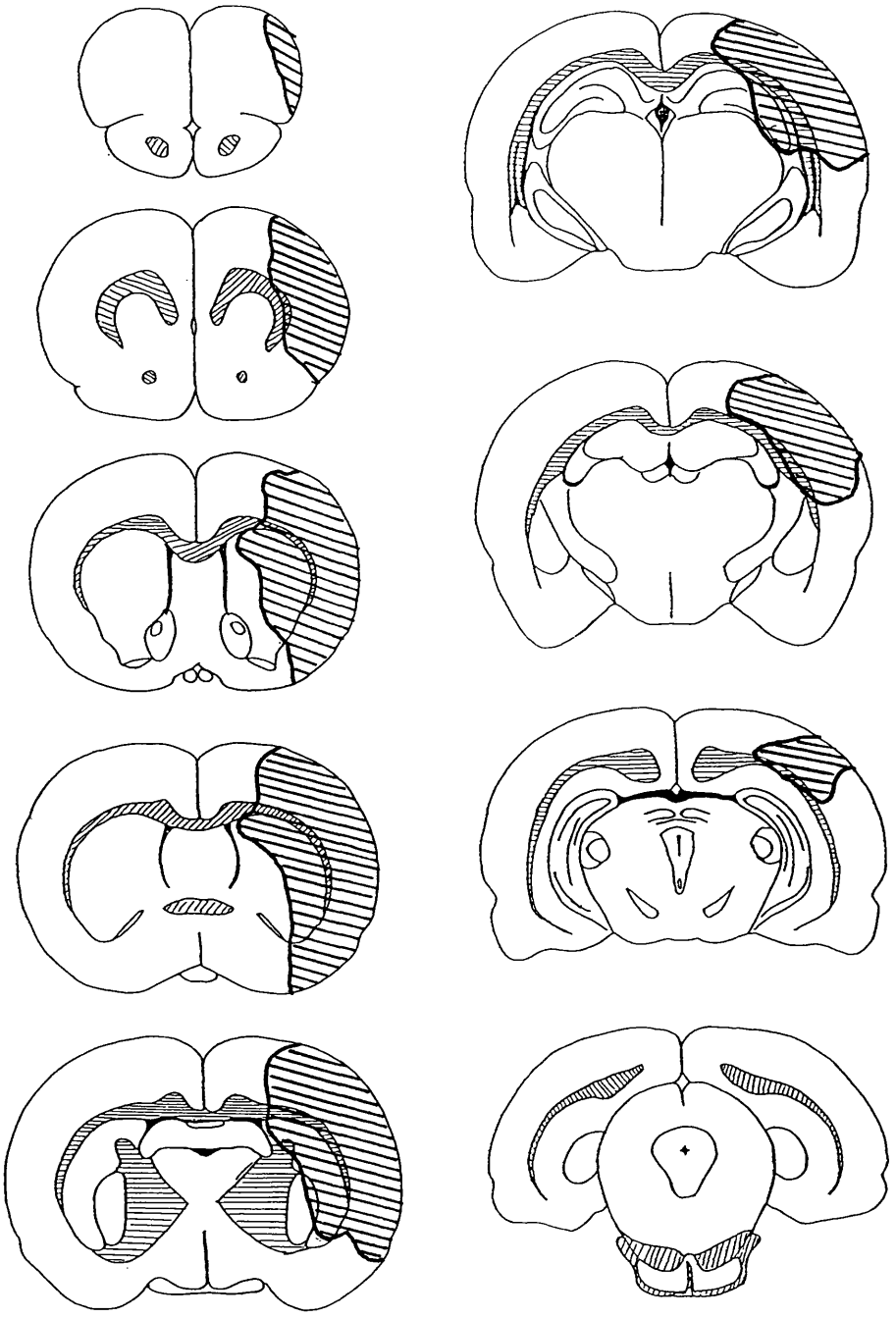

Figure 3.9 Diagrams coresponding to the TTC stained slices (fig 3.8) showing the ischaemic damage seen in a control animal 24 h after proximal MCA occlusion.

control animal at 24 hours is shown in fig 3.8 and the corresponding diagrams are shown in fig 3.9. These areas of ischaemic damage were then integrated on a microcomputer (using the trapezium rule approximation) to give a volume of damage.

3.3 Direct vs Indirect area measurements

There are many advantages in measuring the area of damage indirectly on scale diagrams compared to direct measurements on histological sections. In particular, the latter are prone to distortion and a variable degree of shrinkage during processing (Diemer, 1982) and in acute ischaemia the boundary of the ischaemic area may only be apparent under the microscope (see above). TTC stained, formalin fixed, macro-slices lend themselves more readily to direct measurements as they are less prone to distortion, the boundary is clear and they are not affected by processing. However, there are three main problems associated with direct measurements. Firstly, the formalin fixed brains of 300 - 400 g rats are larger than those depicted in the atlas of Konig & Klippel, the linear ratio being approximately 1.5 : 1.3 (see above), thus the volumes measured will be approximately 1.5 times greater ($[1.5 \div 1.3]^3 \approx 1.5$). Secondly, hemispheric oedema may further increase the volumes measured directly whereas the diagram method automatically compensates for oedema as the area of damage is drawn onto a normal hemisphere using anatomical landmarks. Finally, although the boundaries of the cortex can be clearly defined on TTC stained slices, the boundaries of the caudate and putamen cannot (see fig 2.8), making volume measurements of these nuclei less accurate (see below). The advantages of direct measurements are firstly speed (eliminating the need to produce diagrams for every slice) and secondly, the quantimet image analyzer can be programmed to automatically differentiate between ischaemic and normal brain

by programming its detector with predetermined luminance levels, and if a simplified formula for calculating volumes is used (total area x slice thickness), it can be programmed to work out the volumes directly.

The following experiment was part of a pilot study and is reported to compare and contrast the different methods of estimating lesion size on TTC stained slices.

3.3.1 Materials and Methods

16 Sprague-Dawley rats weighing 290 - 370 g were used in the experiments. Anaesthesia was induced with 4% isoflurane in oxygen, they were intubated and anaesthesia was maintained by ventilation with 1-2% isoflurane in 30% O_2 : 70% N_2O_2. The left middle cerebral artery was occluded as described in chapter 2, with rectal temperature measurement and control but without blood pressure or blood gas monitoring. The animals were recovered immediately after MCA occlusion as previously described. They were divided into two groups, control and dizocilpine (MK-801) treated (1mg/kg given ip 30 min pre- and 30 min post- MCA occlusion, N = 8 + 8). 24 hours after MCA occlusion they were reanaesthetized and perfused with TTC and 10% formal saline, the brains were left soaking in formal saline for 48 hours, randomized and sliced into 1.5 mm sections and photographed (see chapter 2 for details).

The lesions were drawn onto the scale diagrams (fig 3.7) and area measurements were made using a quantimet image analyzer on both the diagrams and on the photographs of the TTC slices directly. The areas measured on the slices included the whole of the left and right hemispheres, as well as lesion size in the hemisphere, cortex and striatum. The volumes from the diagrams were calculated by integrating the slice areas with the distance between the slices on a micro computer, using integration limits for the cortex and hemisphere, anterior 12.5 and posterior 0.05

(see Osborne et al., 1987). The volumes from the direct measurements were calculated by summing the areas and multiplying by the slice thickness (1.5).

3.3.2 Results

There was no mortality associated with this experiment and all animals were included in the analysis. The overall results are summarised in tables 3.1 and 3.2. The direct method demonstrated a significant increase in total volume of the left hemisphere compared to the right, presumably due to cerebral oedema. Interestingly the increase was very similar in treated and control rats (15.8% & 14.4% respectively), inspite of a 30% reduction of infarct volume in the former group. As expected, the direct method measured larger volumes than those based on the diagrams and the average ratio in the estimation of total left hemisphere volumes between the two methods was 1.59 (table 3.1).

Both methods demonstrate a similar difference in lesion size between control and treated animals (table 3.2, fig 3.10). When the lesion volumes measured by the direct method are divided by 1.59 (the average ratio between the two methods in estimating total hemisphere volumes, see table 3.1) there is no significant difference between the two methods in estimating lesions in the hemisphere or cortex, but the direct method significantly over estimates striatal lesion volume. However, this is true for control and treated rats and it does not overestimate the degree of protection in this region (fig 3.11).

The results can be represented graphically as lesion area per section, according to its stereotactic coordinate (fig 3.12) and this demonstrates the levels at which dizocilpine is most effective. However, for comparison of treated animals and controls, total lesion volumes have been used throughout this thesis.

Table 3.1 A. Total hemispheric volumes (mm^3 ± sem) estimated from direct measurements on TTC stained slices.

Group	R hemisphere	L hemisphere	% increase
Control (N=8)	632.3 ± 8.7	731.9 ± 18.0	15.8% *
Dizocilpine (N=8)	618.9 ± 18.0	708.0 ± 14.0	14.4% *

(*$p < 0.01$, 2-tailed t-test)

B. Total hemispheric volume estimated by integrating serial measurements on the scale diagrams.

R = L = 453.2 ±1.9 (mm^3 ± sem, on 4 successive measurements)

Average correction factor between the two methods:
(mean direct L hemisphere volume ÷ 453.2) = **1.59**

Table 3.2 Regional lesion size estimated by direct measurements and using the diagrams.

Group	Hemisphere	Cortex	Striatum
Direct:			
Control (N=8)	262.2 ± 6.3	187.7 ± 4.4	58.4 ± 1.7
Dizocilpine (N=8)	178.1 ± 21.3	111.5 ± 21	53.7 ± 3.5
(% change)	(-32.1%)	(-40.6%)	(-8%)
Diagram:			
Control (N=8)	152.2 ± 3.2	109.5 ± 2.1	28.0 ± 0.7
Dizocilpine (N=8)	105.9 ± 11.7	67.1 ± 11.8	25.5 ± 0.8
(% change)	(-30.4%)	(-38.7%)	(-9%)

(NB. all figures are mm^3 ± SEM)

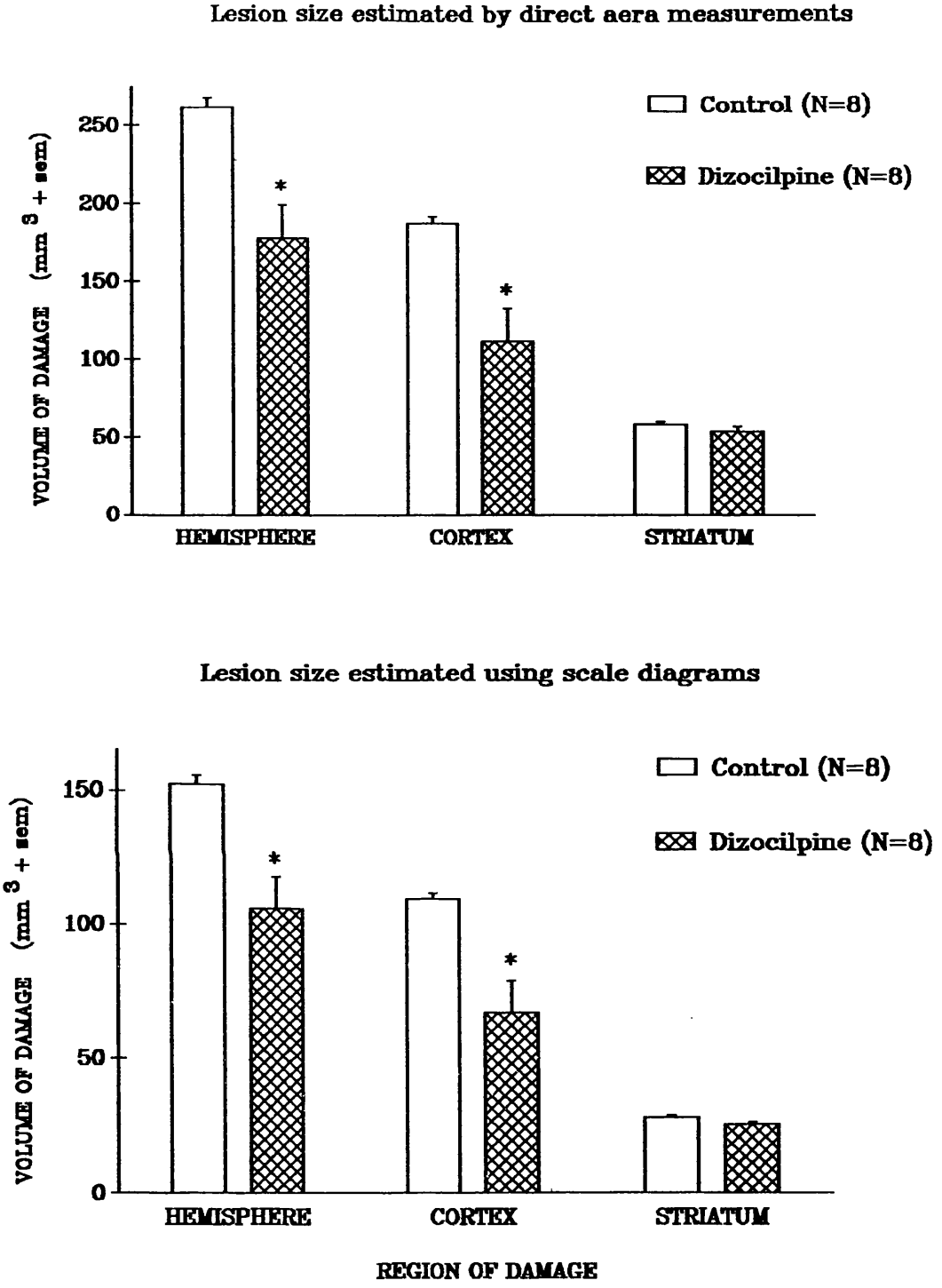

Figure 3.10 Lesion volumes estimated by direct and indirect measurements on TTC stain slices, in control and Dizocilpine treated animals (1mg/kg i.p. 30 min pre & 30 min post MCAO). The direct method (upper graph) leads to larger lesion volumes (note scales on Y axes, *$p < 0.01$, 2-tailed t-test), but the degree of neuroprotection demonstrated in the dizocilpine treated group is similar.

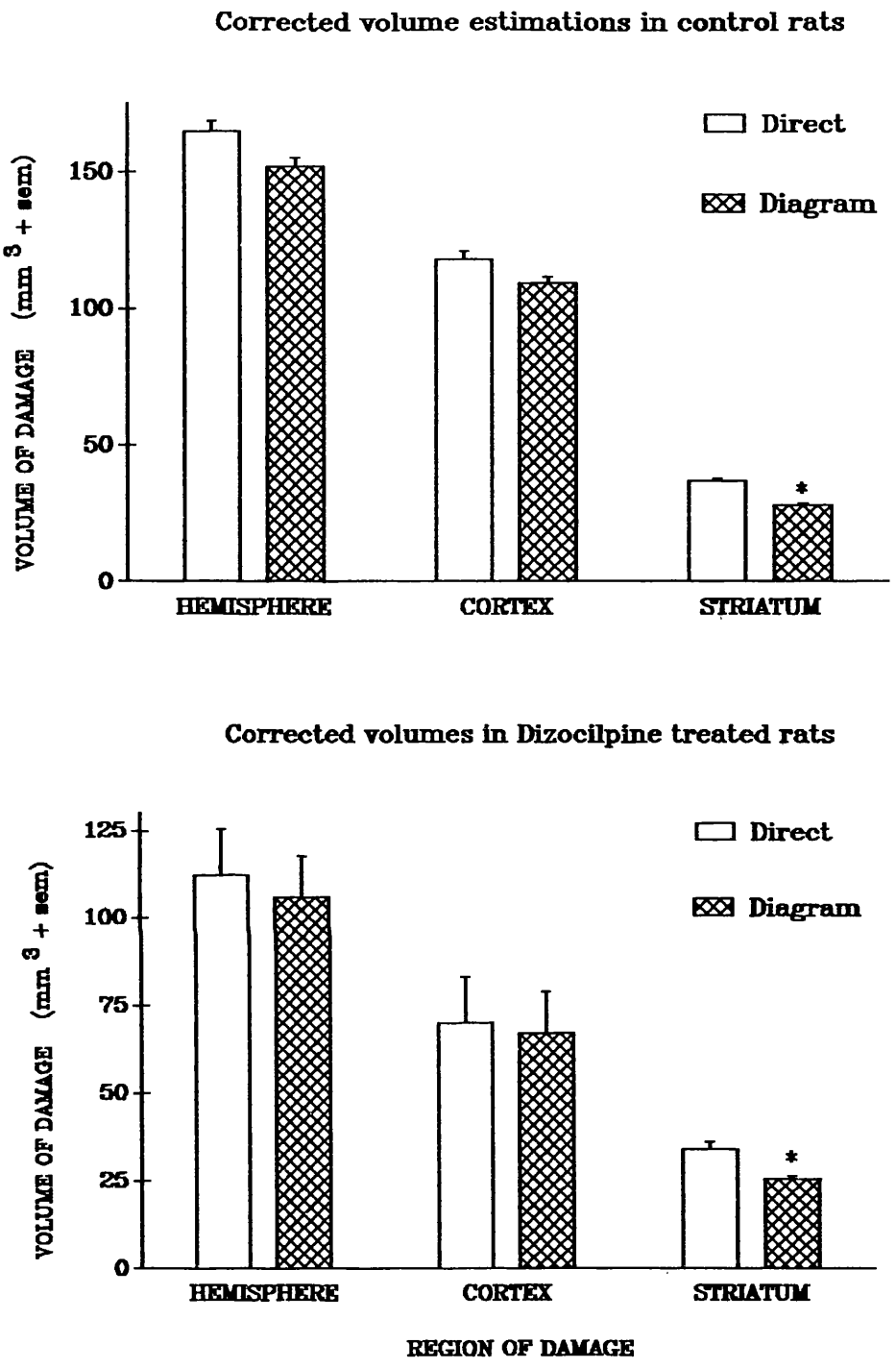

Figure 3.11 Lesion volumes in control (upper graph) and Dizocilpine treated rats (1mg/kg i.p. 30 min pre & 30 min post MCAO, lower graph), comparing direct measurements (corrected for over-estimation of total volume of left hemisphere) to the diagram method, demonstating no difference between the two methods in estimating hemispheric and cortical lesions, but a consistent, significant (*$p < 0.01$, 2-tailed t-test) over-estimation of striatal lesions.

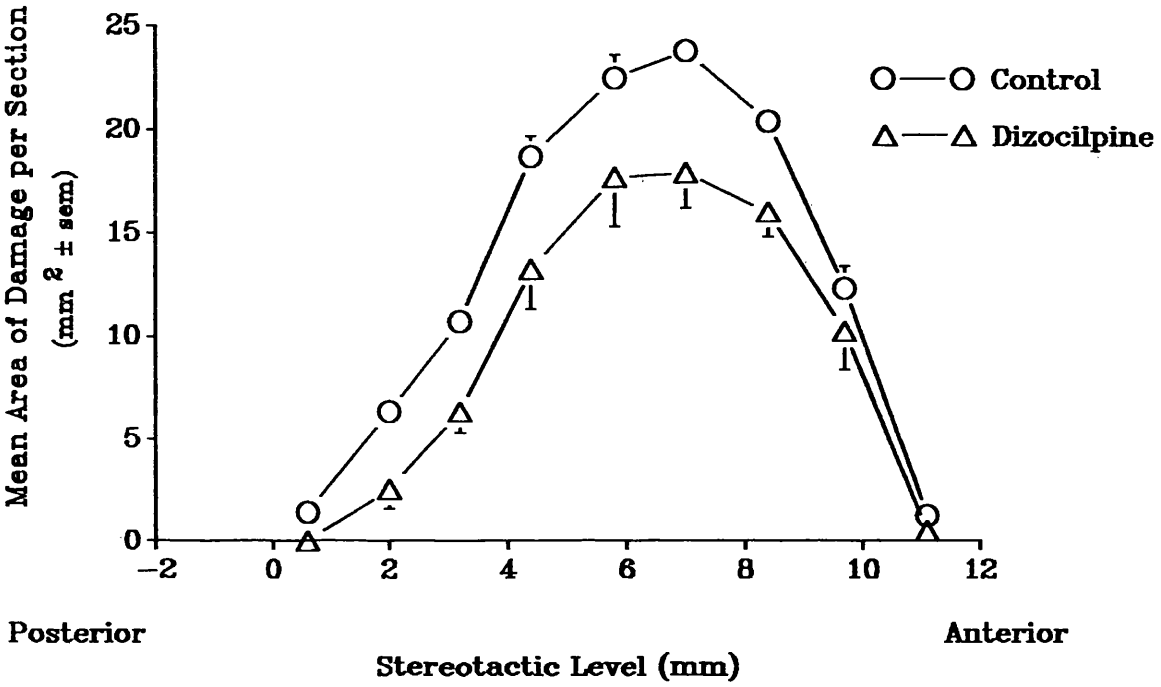

Figure 3.12 Mean area of hemispheric damage per section, using the diagram method, according to the A-P stereotactic coordinate (Bregma = 0), demonstrating the regions in which dizocilpine (1mg/kg i.p 30 min pre & 30 min post MCAO) is neuroprotective.

3.3.3 Discussion

The technique of estimating regional lesion volumes in the rat brain following MCA occlusion using scale diagrams has been thoroughly investigated by Osborne and colleagues (1987). They demonstrated that the method was reproducible and the minimum number of slices required for accurate volume estimations was eight. The diagram method described above uses nine slices and the consistency of the method is demonstrated by the small standard error (2.1%) in the estimated lesion volumes in the control animals (table 3.2). The failure to section the brains at the same levels as the diagrams, transcription of the damaged area to the diagrams and area assessment on the analyzer are all potential sources of error in the method. Even photocopying the diagrams can lead to gradually increasing lesion size over time unless care is taken to use the same master set. Many of these errors are open to operator bias and it is obviously essential to perform the lesion estimations "blind". In all the experiments, the brains were randomized and coded prior to being sectioned and photographed. The method gave consistent results with small standard errors over many experiments.

Provided the volumetric analysis is performed "blind" and concurrently and historical controls are not used, the errors described in the method should not bias the results. In neuroprotection experiments it is essential to use concurrent controls for many other reasons. For example the age, strain, operator and even the batch of animals may be important factors in determining lesion size (Duverger & MacKenzie 1989).

Direct vs Diagram estimations

Measuring lesion areas directly on photographs of the TTC stained slices led to larger volume estimations than the diagram method and the volume ratio between the two methods was approximately 1.59 in the left hemisphere. This difference was due to the fact that brains of 300-400 g rats perfused by the method described in chapter 2 were consistently larger than those depicted in the atlas of Konig and Klippel, which was drawn from frozen sections of 150 g rats. This factor is of little importance in neuroprotection experiments, provided all animals are assessed by the same method. The direct measurements allow a comparison to be made between the total volumes of the right and left hemispheres giving an indication of hemispheric oedema. At 24 hours after MCA occlusion the left hemisphere was approximately 15% larger than the right and this increase was not significantly affected by treatment with dizocilpine (table 3.1). When corrections are made for scale, there is no significant difference between the two methods in estimating hemispheric or cortical lesion volumes in either the control or treated groups (fig 3.10) and the method proved to be quicker and could be semi-automated using a Quantimet image analyzer. In spite of correction for scale the direct method over estimated the striatal damage. This was due to the fact that using the diagrams, striatal damage was measured in the caudate nucleus and putamen but the boundaries of the sub-cortical structures could not be clearly defined on TTC stained slices. This applied equally to treated and control animals and no increased protection was observed using the method (fig 3.11).

3.3.4 Conclusions

The direct method, although over-estimating actual lesion size, gives consistent results, does not over-estimate the degree of neuroprotection seen in the dizocilpine treated animals, and was therefore considered appropriate for estimating lesion volumes in experiments involving large numbers of animals and was used to look at the "dose response" and "therapeutic window" of dizocilpine in this model (see chapter 6). The diagram method offers a high degree of standardization and enables direct comparisons to be made between histological sections and TTC slices (see chapter 4).

Chapter 4

Neuroprotection with dizocilpine in acute and chronic focal ischaemia using TTC staining and Histopathology as end points.

4.1.1 Introduction

Following permanent, proximal, middle cerebral artery occlusion in the rat, there are many factors that affect the volume of the resulting ischaemic damage. Duverger and MacKenzie (1988) have highlighted the importance of the strain of rat. They examined 5 strains (Wistar/Kyoto, Sprague-Dawley, Fisher 344, spontaneously hypertensive rats (SHR) and stroke-prone SHR) and reported that inspite of identical animal house conditions, anaesthesia, and surgical technique, there was considerable variation in the amount of ischaemic damage as assessed by volumetric analysis of histopathology after a survival of 48 hours. Brint and colleagues (1988) have also emphasized the importance of strain and have suggested a number of other factors which may be important such as the supplier of the animals and surgical technique. Coyle has also reported the influence of strain (Coyle, 1986b) and age (Coyle, 1982b) of rats on infarct size.

The importance of physiological variables has been highlighted by many authors. Haemorrhagic hypotension for 30 min following MCA occlusion can have a marked effect on the volume of damage at 4h in this model (Osborne et al.,1987). Small variations in temperature can affect lesion size (Busto et al., 1987) and severe hyperglycaemia can also affect outcome (Duverger and MacKenzie, 1988; Venables et al., 1985; Nedergaard and Diemer, 1987). Blood gases and haematocrit are also important (Graham, 1988).

Given the influence of physiological variables on the determination of the degree of ischaemic damage, much emphasis has recently been placed on the importance of physiological monitoring in experiments evaluating neuroprotective agents (Graham, 1988) and this has led to a debate regarding "acute" vs "chronic" models of focal ischaemia. The great advantage of acute models is that full physiological monitoring may be performed throughout the experiment, but the disadvantages are firstly, that the type of anaesthesia may effect the degree of damage and may

react with the drug under investigation (for dizocilpine interactions see Kurumaji & McCulloch, 1989), and secondly, although with perfusion fixation, early, irreversible ischaemic cell change can be clearly demarcated (Brown and Brieley 1972), infarction per se does not occur until later. With recovery models, the longer the survival, the easier the identification of ischaemic damage, but the greater the possibility of unmonitored post insult complications.

Bederson et al. (1986a) demonstrated that there was no significant difference in the area of ischaemic damage on a single representative coronal section at 6 or 24 hours post MCA occlusion. However, few studies have estimated the volume of ischaemic damage acutely and chronically in the same model.

The following experiment was primarily designed to measure the volume of ischaemic damage at 4 hours and 24 hours following MCA occlusion in control and dizocilpine treated rats.

4.1.2 End points: Histology vs Triphenyltetrazolium chloride (TTC)

The use of 2,3,5,triphenyltetrazolium chloride in the assessment of ischaemic damage has been discussed in chapter 2. TTC is a water soluble salt which is oxidized to a bright red, lipid soluble formazan by various mitochondrial enzyme systems, such as the dehydrogenases. Normal tissues stain a dark red colour when perfused with TTC but ischaemic areas, where mitochondrial enzyme systems have been incapacitated, remain white (see chapter 2, fig 2.8). TTC has been used extensively as a stain in experimental models of ischaemia (Bederson et al., 1986a; Lundy et al., 1986; Liszczac et al., 1984; Lye et al., 1987). Park et al. (1988a) suggested that TTC perfusion may be used as early as 4 hours after MCA occlusion to delineate ischaemic damage but other authors have reported that TTC staining is unreliable up to 6 hours after MCA occlusion (Bederson et al., 1986a; Taylor et al., 1987).

Using a technique similar to that developed by Park et al. (1988a) which is fully described in chapter 2, TTC perfusion staining was combined with formalin perfusion fixation so that TTC staining could be directly compared with conventional histopathology in the delineation of ischaemic damage at 4 and 24 hours post MCA occlusion, in the control and dizocilpine treated animals.

4.1.3 Materials and Methods

36 male Sprague-Dawley rats, weighing between 280 and 380 g were used in the experiments. They were kept in controlled environmental conditions on a 12 hour light:dark cycle and fed ad libitum prior to starting the experiment.

Anaesthesia was induced with 4% isoflurane in oxygen. The animals were intubated and anaesthesia was maintained by ventilation with 4% isoflurane in a nitrous oxide / oxygen mixture (30% : 70%). A tail artery was catheterised for monitoring blood pressure and for periodic blood sampling. Rectal temperature was monitored and maintained via a warming blanket at 37.0 ± 0.5°C. The left middle cerebral artery (MCA) was occluded as described in chapter 2.

30 min after MCA occlusion, either dizocilpine (3mg/kg) or an equivalent volume of normal saline was given by intraperitoneal (i.p.) injection. One hour after MCA occlusion the tail artery catheter was removed and the wound sealed with cyanoacrylate glue and elastoplast. The isoflurane was turned off and the animals were recovered. Initially they were ventilated with nitrous oxide and oxygen (30% : 70%) until they showed signs of recovery. When breathing well via the endo-tracheal tube, they were extubated. They were kept in warmed cages for the next 2 hours and were allowed free access to water and laboratory chow immediately after recovery.

At either 4 or 24 hours post MCA occlusion the animals were perfusion fixed with TTC and formal saline.

Perfusion fixation

The method of perfusion fixation is fully described in chapter 2. In brief the animals were deeply reanaesthetized with 4% isoflurane in 100% oxygen. Via a thoracotomy, 1000U heparin was injected into the right atrium, the thoracic aorta was clamped, a catheter was inserted into the ascending aorta via the left ventricle and perfused with warm (37°C), heparinized saline at 100 mm Hg. The right atrium was incised 20 seconds after the perfusion commenced. After approximately 90 sec the right atrium was clamped and 20 ml of 2% TTC in physiological saline at 37°C was perfused in a pulsatile manner at a mean pressure of approximately 120 mm Hg. The TTC was left stagnant for 7 min and 300 ml of 10% formal saline at room temperature was perfused at 100 mm Hg. The heads were left soaking in 10% formal saline for a further 24 hours, they were randomized, the brains were removed and the forebrain was sliced on a specially made block into 9 x 1.5 mm slices. The rostral surface of each slice was photographed on a low power microscope using ektachrome 160 tungsten slide film.

Histopathology

The brain slices were embedded in paraffin wax and 8 micron sections were taken from the rostral surface and stained with haematoxylin and eosin.

Quantification of damage

The lesions delineated by TTC and conventional histopathology were drawn onto separate scale diagrams of corresponding sections taken from the atlas of Konig and Klippel (see chapter 3, fig 3.7). The area of damage in the hemisphere, cortex and caudate nucleus for each diagram was measured using a quantimet image analyzer. The areas were integrated to give a volume of damage (see chapter 3).

Statistics

The mean volumes of damage were compared using a 2-tailed t-test and differences were regarded as significant at the $p < 0.05$ level. The data was also analyzed using one-way analysis of variance (ANOVA) with multiple comparisons and Duncan's multiple range test ($p < 0.05$)

The mean values for the physiological parameters were compared using analysis of variance with Duncan's multiple range test ($p < 0.05$). All statistical analysis was performed using SPSS-PC software run on an IBM personal computer.

4.1.4 Results

Following successful MCA occlusion, there were no postoperative deaths and no animals were excluded from the study.

Physiological variables

The mean physiological parameters immediately pre, 30 min post (but immediately prior to treatment) and 1 hour post MCA occlusion (mean ± sem) are shown in table 4.1. There was no significant difference between any of the groups (ANOVA, Duncan's multiple range test, $p = 0.05$) apart from the mean blood pressure in the dizocilpine treated group, which was still significantly lower than that measured in the controls 30 minutes after the i.p. dose (ie 1 hour post occlusion). This hypotensive effect of dizocilpine in anaesthetized rats has been noted by other authors (Park et al., 1988c; Kurumaji & McCulloch 1989) but does not occur in conscious animals (Lewis et al., 1989).

Table 4.1 Mean physiological variables (arterial blood)

Group	Pre MCAO	30 min post	1h post
Blood pressure (mm Hg)			
Control (4h, N = 10)	75.9 ± 1.7	77.2 ± 2.2	74.7 ± 2.4
Dizocilpine (4h, N = 9)	76.0 ± 1.7	81.2 ± 3.5	65.7 ± 2.6 *
Control (24h, N = 9)	82.3 ± 2.3	81.9 ± 1.2	80.1 ± 1.3
Dizocilpine (24h, N = 8)	81.0 ± 2.8	77.6 ± 3.3	63.0 ± 1.7 *
pCO_2 (mm Hg)			
Control (4h)	39.2 ± 1.7	39.9 ± 0.9	38.5 ± 1.0
Dizocilpine (4h)	40.6 ± 1.1	41.3 ± 1.1	39.3 ± 0.6
Control (24h)	38.8 ± 0.9	39.9 ± 0.9	40.2 ± 1.5
Dizocilpine (24h)	37.0 ± 1.2	39.1 ± 0.9	36.2 ± 1.1
pO_2 (mm Hg)			
Control (4h)	138.0 ± 8.2	125.6 ± 7.7	130.5 ± 3.5
Dizocilpine (4h)	131.0 ± 5.4	117.8 ± 3.4	122.5 ± 3.5
Control (24h)	124.3 ± 6.4	122.1 ± 4.4	131.9 ± 4.6
Dizocilpine (24h)	131.2 ± 5.1	129.2 ± 5.7	134.7 ± 3.9
pH			
Control (4h)	7.392 ± 0.013	7.380 ± 0.011	7.393 ± 0.018
Dizocilpine (4h)	7.362 ± 0.011	7.358 ± 0.012	7.381 ± 0.010
Control (24h)	7.398 ± 0.007	7.395 ± 0.016	7.383 ± 0.008
Dizocilpine (24h)	7.403 ± 0.020	7.388 ± 0.013	7.409 ± 0.007

(Readings 30 min post MCA occlusion were taken immediately prior to treatment with either 3 mg/kg dizocilpine i.p. or the equivalent volume of saline)

All values are mean ± sem

* $p < 0.05$ using analysis of variance with Duncan's multiple range test.

Acute vs Chronic ischaemia - Histopathology

Using histopathology as an end point, in the control animals, there was a trend towards increasing hemispheric lesion size at 24 h as compared to 4 h ischaemia (fig 4.1). This was just significant using a 2-tailed t-test ($p = 0.033$) but was not significant using one-way ANOVA with multiple comparisons to all the other groups and Duncan's multiple range test ($p = 0.05$). There was no significant difference in the dizocilpine treated animals using histopathology, between the lesion volumes at 4 h and 24 h of ischaemia in any regions of the brain (fig 4.1). Comparing dizocilpine treated animals with controls, significant protection was seen in the hemisphere and cortex at 4 h and 24 h of ischaemia (fig 4.2).

TTC vs Histopathology

When comparing TTC with histology as an end point, at 4 h TTC staining tended to over estimate lesion volumes in the hemisphere and cortex and under estimate caudate damage (fig 4.3). In the control and dizocilpine treated animals, the difference in the volumes of caudate damage were small but significant ($p < 0.05$, one-way ANOVA, Duncan's multiple range test). The other differences in the control animals were not significant but in the treated group, TTC significantly over-estimated cortical damage (fig 4.3, $p < 0.05$, one-way ANOVA, Duncan's multiple range test).

At 24 h, there was no significant difference between the two methods in either the control or the treated animals in any region of the brain (fig 4.4).

Hemispheric lesion volumes in individual animals, estimated using TTC staining and histopathology, were also directly compared (fig 4.5), the correlation at 24 h was excellent ($r = 0.96$, $p < 0.001$) but at 4 h was very poor ($r = 0.37$, not significant).

Table 4.2 Mean volumes of ischaemic damage (mm^3 ± sem).

Group	Hemisphere	Cortex	Caudate
By Histology			
Control (4h, N = 10)	110.1 ± 7.3	77.2 ± 6.0	25.1 ± 0.7
Dizocilpine (4h, N = 9)	85.1 ± 9.38	54.4 ± 8.6	25.8 ± 0.5
Control (24h, N = 9)	134.4 ± 7.5	92.8 ± 6.1	27.5 ± 0.6
Dizocilpine (24h, N = 8)	89.0 ± 11.0	51.9 ± 9.1	26.7 ± 1.2
By Triphenyltetrazolium chloride			
Control (4h, N = 10)	117.3 ± 5.3	91.6 ± 5.1	20.9 ± 1.1
Dizocilpine (4h, N = 9)	107.3 ± 8.9	81.2 ± 7.7	21.5 ± 1.1
Control (24h, N = 9)	129.2 ± 7.4	95.3 ± 6.0	24.9 ± 0.7
Dizocilpine (24h, N = 8)	84.9 ± 9.0	51.5 ± 8.1	24.6 ± 1.3

(Dizocilpine treated groups were given 3 mg/kg i.p. 30 min post MCA occlusion)
For statistical comparisons between the groups see figures 4.1 to 4.6

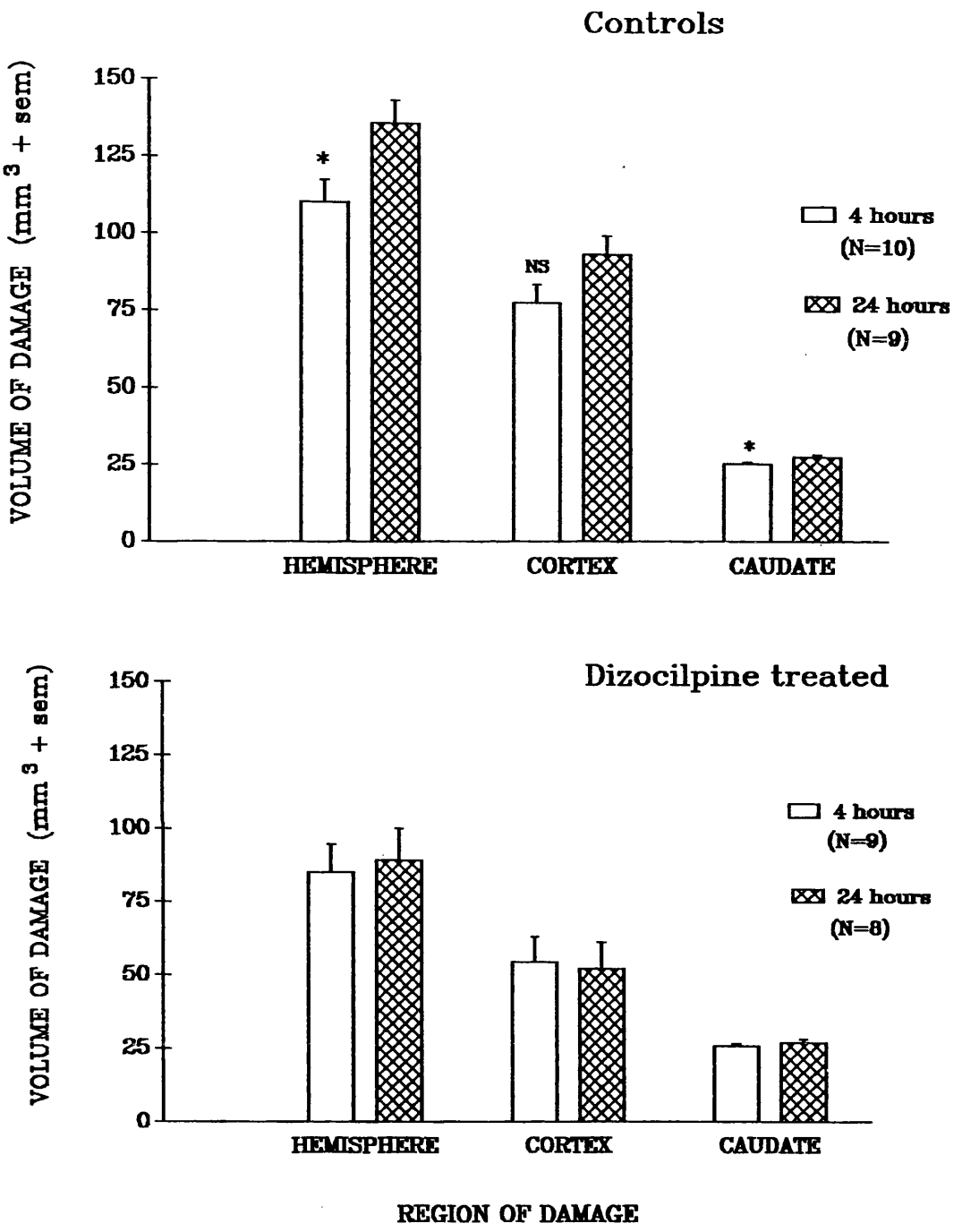

Figure 4.1 Comparison of lesion volumes using **histopathology** in control (upper graph,) and dizocilpine treated (3 mg/kg i.p. 30 min post MCA occlusion) rats (lower graph) at 4 hours and 24 hours post MCA occlusion. In the control animals there was an increase in the lesion volume measured in the hemisphere at 24 hours ($*p < 0.05$, 2-tailed t-test), however, this was not significant using oneway ANOVA with Duncan's multiple range test ($p < 0.05$). In the dizocilpine treated animals there was no significant change in lesion volume at 24 hours.

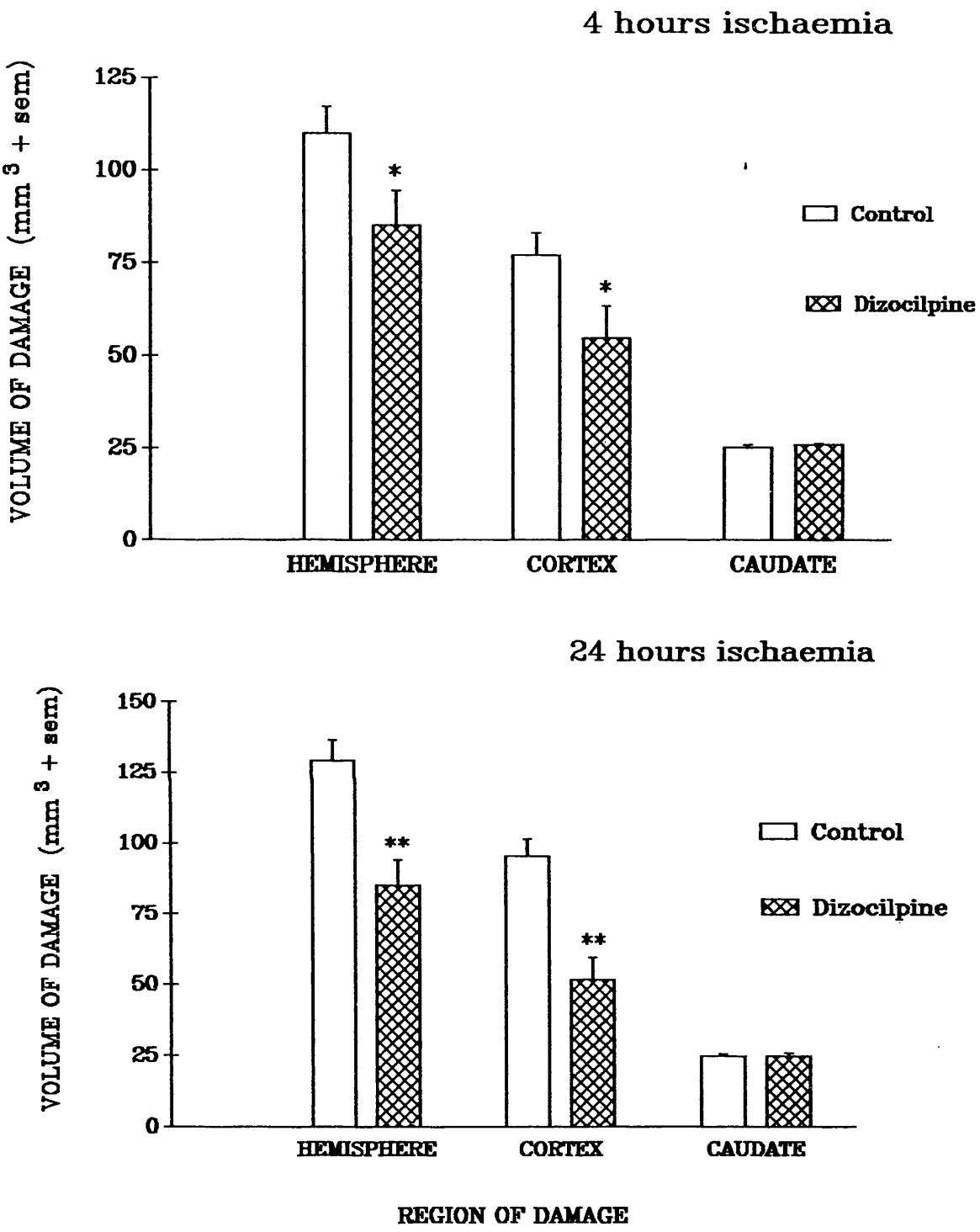

Figure 4.2 Comparison of lesion volumes using **histopathology** at 4 h (upper graph) and 24 h (lower graph) post MCA occlusion, showing the effect of dizocilpine (3 mg/kg i.p.) administered 30 min post occlusion. There was a significant protective effect in the acute and chronic model (*$p < 0.05$, **$p < 0.005$, 2-tailed t-test)

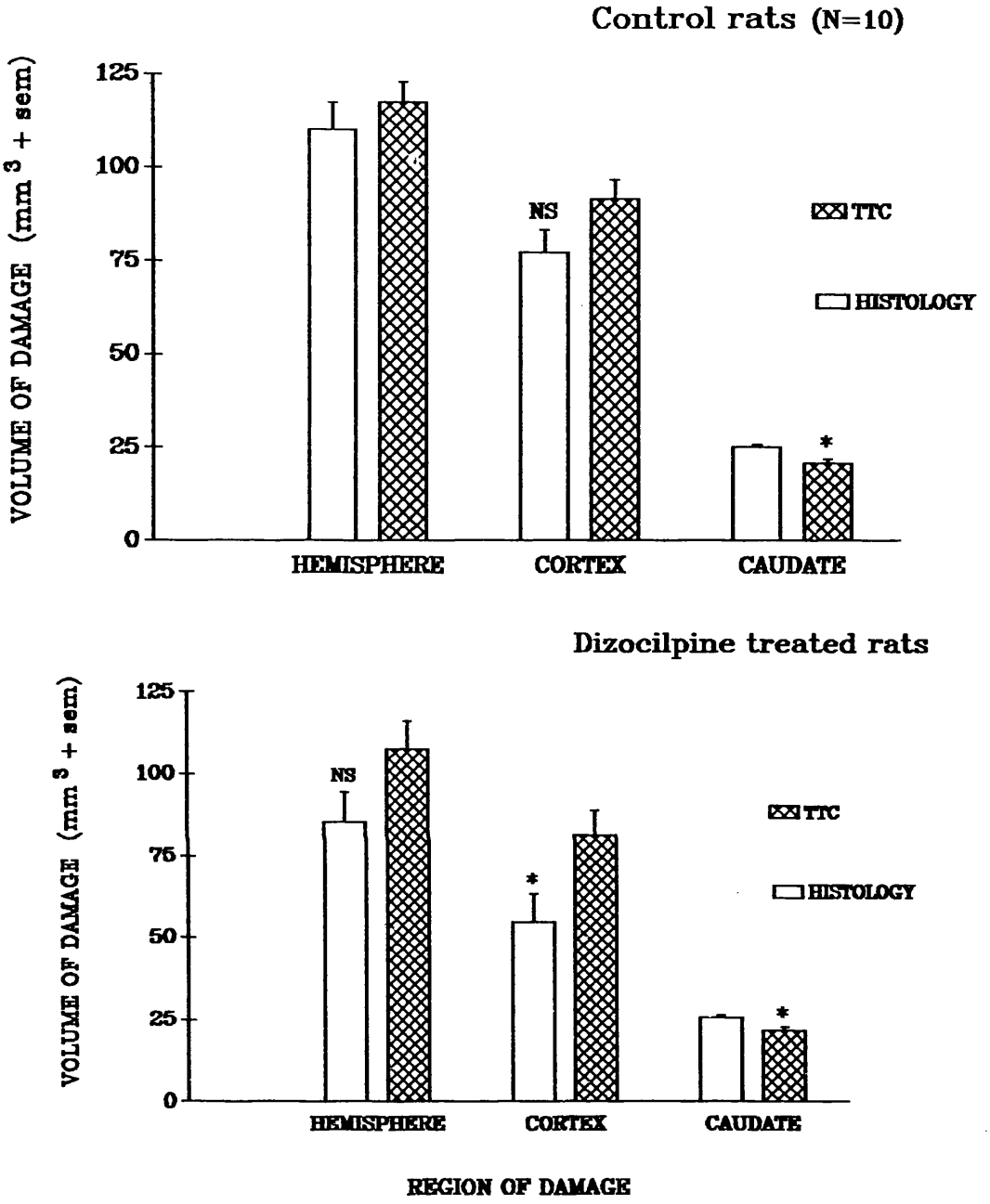

Figure 4.3 Comparison of TTC staining and histopathology in the assessment of the volume of damage at **4 hours** post MCA occlusion in dizocilpine treated animals (3 mg/kg i.p. 30 min post occlusion, N = 9) and saline treated controls (N = 10). In the control animals, TTC staining over-estimated the volume of damage in the hemisphere and cortex as defined by histopathology but this did not reach statistical significance (top graph). In the dizocilpine treated group, TTC staining led to an even greater over-estimate of the volume of damage and this was significant in the cortex when compared to histopathology (*$p < 0.05$, 2-tailed t-test). Caudate damage was significantly under-estimated by TTC (*$p < 0.05$).

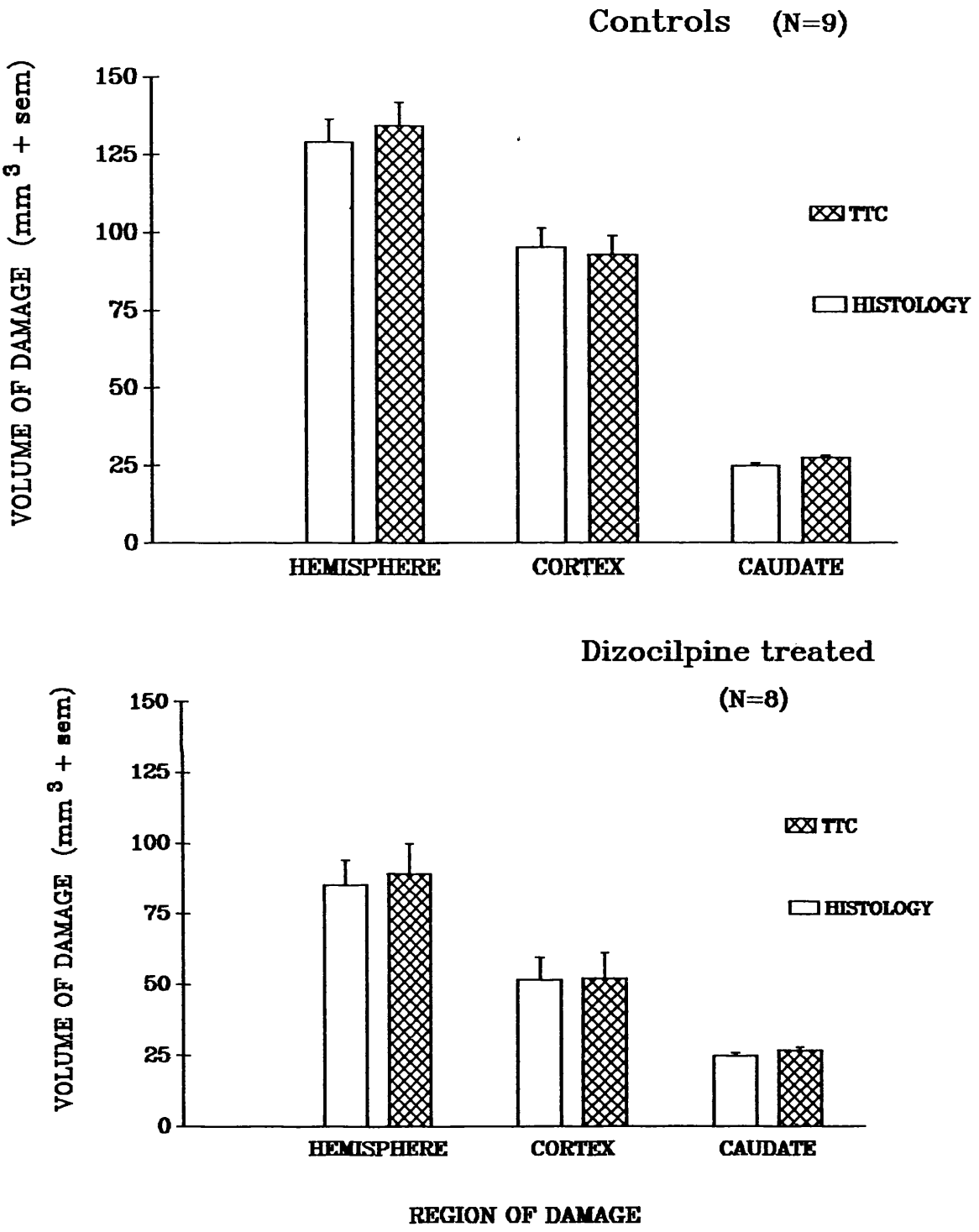

Figure 4.4 Comparison of TTC staining and histopathology in the assessment of the volume of damage **24 hours** post MCA occlusion in dizocilpine treated animals (3 mg/kg i.p. 30 min post occlusion, N = 8) and saline treated controls (N = 9). There was no significant difference between either method in any region of the brain in either the controls or the treated group.

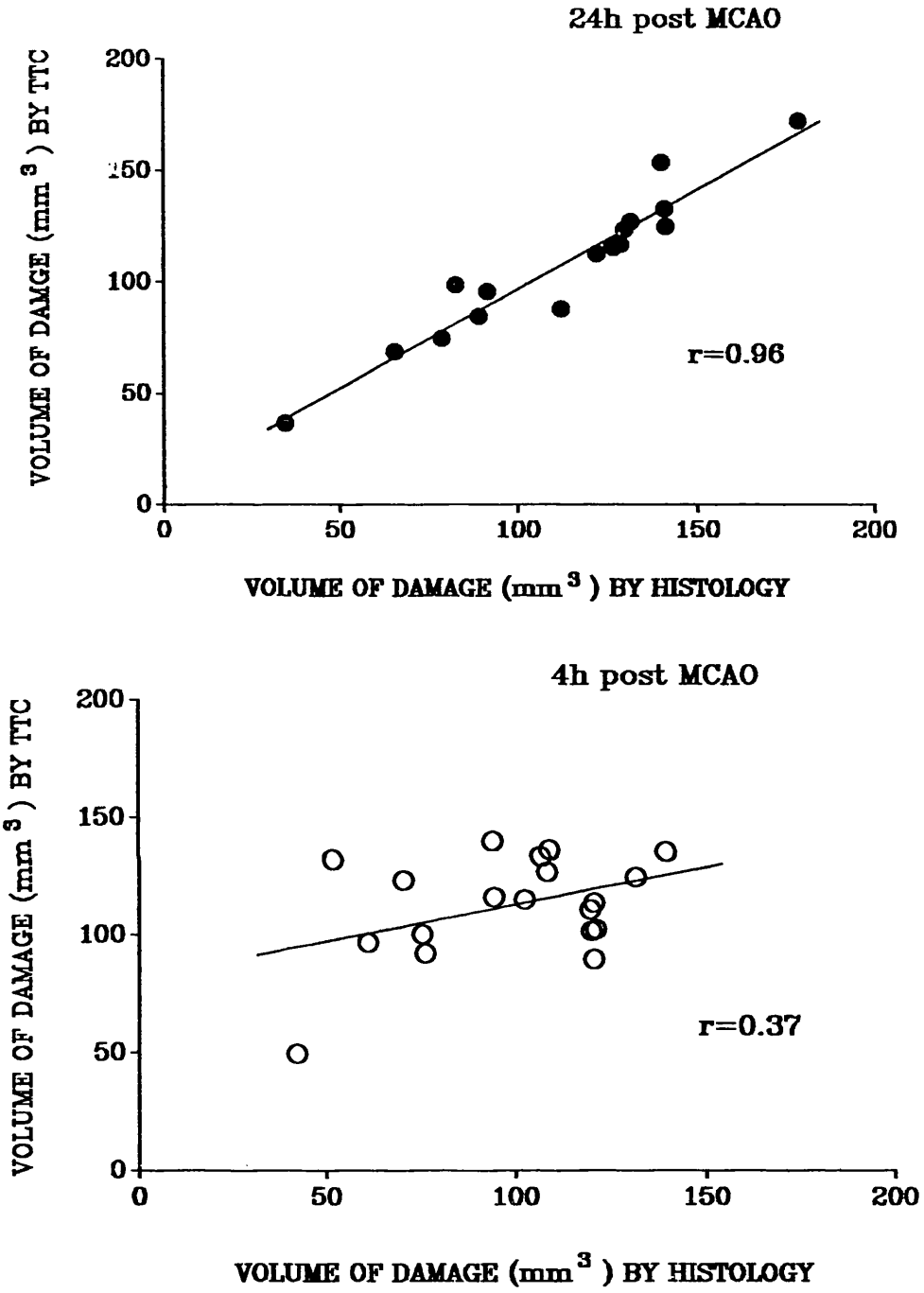

Figure 4.5 Correlation between the estimation of hemispheric lesion volumes by TTC staining and histopathology at 4 h and 24 h post MCA occlusion. After 24h (upper graph) the correlation was excellent ($r = 0.96$, $p < 0.001$) but after 4h (lower graph) the correlation was very poor ($r = 0.37$, not significant).

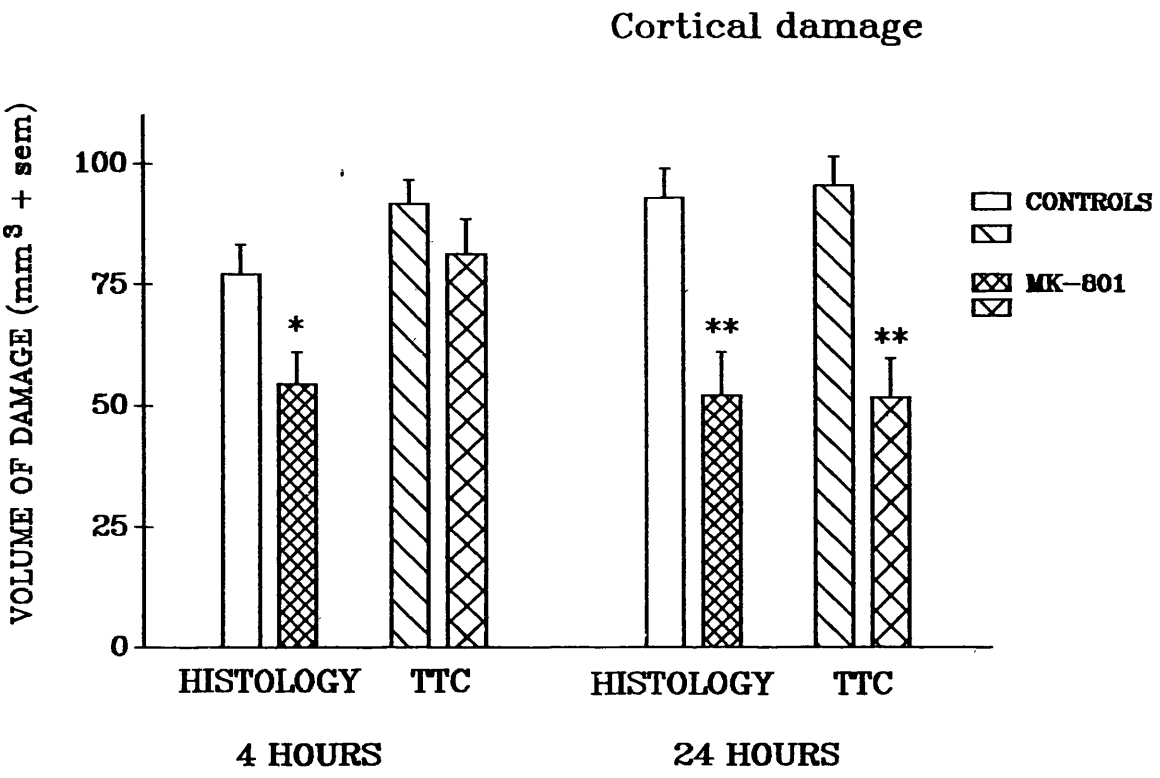

Figure 4.6 Comparison of cortical damage at 4 h and 24 h post MCA occlusion, in control and dizocilpine treated animals (3 mg/kg i.p. 30 min post occlusion) assessed by TTC staining and histopathology (N = 8-10 per group). The 4 bars on the left are the results of the 4 h experiments and the group on the right represent 24 h recovery. The plain and left diagonal bars represent the saline treated animals and the crosshatched bars the treated groups. At 4 hours, significant protection could be seen using histopathology (*$p < 0.05$, 2-tailed t-test) but not using TTC, as the latter failed to demonstrated the reduction in lesion volume in the treated group. At 24 h, highly significant protection was obtained (**$p < 0.001$, 2-tailed t-test) in the dizocilpine treated group and there was no difference between the histopathology and TTC methods. Using ANOVA with Duncan's multiple range test all results marked * or ** are significantly different from all the others ($p < 0.05$).

4.1.5 Discussion

Acute vs Chronic ischaemia

The advantage of using acute models of focal ischaemia in the assessment of neuroprotective agents is the ability to monitor key physiological variables which may influence lesion size, such as blood pressure, blood gases and temperature, throughout the experiment. The major disadvantage is the lack of "maturity" of the ischaemic area and the worry that the agent may delay damage rather than prevent it. This experiment suggests that with dizocilpine in this rat model of focal ischaemia, it is the lesions in the control animals which tends to progress between 4 h and 24 h rather than the lesions in the dizocilpine treated group. This strongly supports the neuroprotective effect of dizocilpine but highlights one of the problems using the "Tamura" model with a histopathological end point at 4 h, which was noted in pilot studies, namely that a degree of anaesthesia induced hypotension is necessary in order to obtain consistent controls at 4 h. It is interesting to note that the coefficient of variation was slightly less at 24 h than at 4 h (19.4% and 24.6% respectively), although this may reflect that it is easier to detect ischaemic areas at the later time point (see chapter 3, figs 3.1 and 3.5).

Given that it is not practical to measure physiological parameters continuously throughout a 24 h period, for how long should such parameters be measured? No single model of focal ischaemia can hope to provide the answers to all the questions concerning a new potential neuroprotective agent. It would seem logical to assess the effects of an agent in a variety of models, including acute, fully monitored experiments and chronic experiments, where the surgical procedure is performed under controlled conditions but recovery is then achieved as quickly as possible, prior to the administration of agents in the post ischaemic period.

Neuroprotection with Dizocilpine

Dizocilpine is a potent, non competitive *N*-methyl-D-aspartate antagonist. It is a powerful neuroprotective agent in models of focal ischaemia and a summary of previous studies, its pharmacology and mode of action are fully discussed in chapter 1. In this study the neuroprotective effects of dizocilpine were examined in acute and chronic focal ischaemia. Following both 4 h and 24 h of ischaemia, dizocilpine (3 mg/kg administered i.p., 30 min after MCA occlusion) was effective in reducing the volume of damage in the hemisphere and cortex (Fig 4.2) but did not influence the degree of damage in the caudate nucleus. The degree of protection was more significant after 24 h ischaemia as compared to 4 h ischaemia, apparently due to an increase in the volume of damage in the control animals. This protection occurred in spite of the hypotensive effect of dizocilpine that occurs in anaesthetized animals which one would expect to increase lesion size (Osborne et al., 1987). The lack of effect of dizocilpine on the degree of damage in the caudate nucleus is consistent with other studies (Park et al., 1988c; Bielenberg et al., 1989) and is presumably due to the fact that the lenticulostriate arteries which supply this region are effectively end arteries (Yamori et al., 1976) and blood flow in this region is reduced to "almost negligible levels" (Tamura et al., 1981b).

These results add to the rapidly increasing number of studies which confirm that dizocilpine and other NMDA antagonists are neuroprotective in both acute and chronic models of focal ischaemia (see chapter 1, table 1.1 for summary) and further supports the "excitotoxic hypothesis" for neuronal damage in focal ischaemia. Furthermore, this protective effect in focal ischaemia is not accompanied by an increase in cerebral blood flow (Park et al., 1989).

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TTC staining vs Histopathology

2,3,5,triphenyltetrazolium chloride (TTC) staining is discussed in chapter 2. TTC has been used to detect experimental myocardial ischaemia in dogs as early as 1 - 2 h after the onset of ischaemia (Lie et al., 1975). The border between normal and ischaemic tissue becomes distinct after 3 - 6 h (Kloner et al., 1981; Fishbein et al., 1981) and occurs before the development of definitive signs of infarction but coincides with the reduction in dehydrogenase activity (Fine et al., 1966), which in ischaemic myocardium of the dog, is an irreversible process (Hamolsky & Kaplan, 1961).

In the ischaemic rat brain, under the light microscope, the earliest change in neuronal morphology following the onset of ischaemia is vacuolation of the cytoplasm and this correlates with the electron microscopy finding of swollen mitochondria (McGee-Russell et al., 1970) and may occur as early as 30 min after the onset of ischaemia. Normal respiratory activity in mitochondria can be correlated with ultrastructural preservation (Ginsberg et al., 1977; Sugano et al., 1970) and since TTC staining reflects intact enzyme systems of the electron transport chain, it might be assumed that it would be a useful agent in early ischaemia. However, irreversible injury to mitochondria in brain cells may not occur until after a long period of ischaemia (Rehncrona et al., 1979).

Park et al., (1988a) demonstrated that there was no significant difference between TTC staining and conventional histopathology in the estimation of lesion volumes, 4 h after the onset of focal ischaemia in the rat, but other authors have found poor correlation between the two methods at less than 6 h of ischaemia (Bederson et al., 1986; Taylor et al., 1987). None of these previous studies involved the assessment of potential neuroprotective agents. The findings of this study closely agree with those of Park et al., (1988) in that there was no significant difference between the two methods in the control animals, with TTC delineating slightly larger lesions in

the hemisphere and cortex and slightly smaller lesions in the caudate nucleus. However, in the dizocilpine treated group there was a significant difference between TTC staining and histopathology in the acute experiments, and hence TTC perfusion staining is unreliable as a method of assessing ischaemic damage in experiments involving potential neuroprotective agents in this acute model of focal ischaemia. In focal ischaemia, dizocilpine does not improve cerebral blood flow in the ischaemic penumbra (Park et al., 1989), thus in the acute model TTC defined lesions may be larger in the treated animals due to lack of stain delivery. The problems associated with TTC staining in acute ischaemia are further explored and discussed in chapter 5.

After 24h of ischaemia there was an excellent correlation between TTC perfusion staining and histopathology in the estimation of lesion volumes and this agrees with the work of Bederson et al. (1986) and Taylor et al. (1987), but importantly, this correlation was maintained equally in control and dizocilpine treated animals, making TTC staining an ideal method for estimating lesion volumes in this model of chronic focal ischaemia.

4.1.5 Conclusions

Dizocilpine significantly reduced the volume of ischaemic damage after 4 h and 24 h of ischaemia in this model, and there was no progression of the lesion in the dizocilpine treated groups between 4 h and 24 h. In the chronic experiments the protective effect was more significant, and this appeared to be due to a slight progression of the lesions in the control animals between 4 h and 24 h.

TTC perfusion staining correlated very well with histopathology as an endpoint in the 24 h study, and the correlation held in both treated and control animals. However, TTC perfusion staining was unreliable in the acute model.

Chapter 5

Triphenyltetrazolium chloride: immersion vs perfusion staining

5.1 Introduction

2,3,5,Triphenyltetrazolium chloride (TTC) staining as a means of delineating experimental brain ischaemia has been discussed in the previous chapter. The experiments so far described have shown that TTC perfusion staining is unreliable when compared to histopathology in the delineation of acute, focal, ischaemic lesions. However it was not clear from the previous study why TTC staining was unreliable and delineated larger lesions. The differences between TTC staining and histopathological changes in acute ischaemia may be due to true differences in mitochondrial dehydrogenase activity, but may also reflect problems with stain delivery. TTC staining may be performed by incubating brain slices in TTC solution, eliminating the problems of stain delivery, but increasing the difficulty of sectioning the brain, and increasing the probability of distortion of the sections making direct area measurements unreliable.

The aim of the following study was to determine whether the pattern of staining obtained with TTC perfusion in the acute phase of ischaemia represents loss of dehydrogenase activity or simply a lack of delivery of the stain to the area of ischaemia. A comparative study of the use of TTC by immersion or perfusion at various times following middle cerebral artery (MCA) occlusion in the rat supported the conclusion that TTC perfusion staining in the acute phase of ischaemia represents a lack of delivery of the stain to the ischaemic area.

5.1.2 Materials and methods

30 male Wistar rats weighing between 260 and 490 g were used in the experiments. 20 animals were studied acutely (5-20 min and 3-4 h) and 10 chronically (24 h) following permanent middle cerebral artery occlusion (MCA occlusion). They were kept under controlled environmental conditions and fed ad libitum prior to the

surgical procedure and upon recovery, in the chronic series.

Surgery was performed as described in chapter 2. The ischaemic lesions were delineated by TTC using the two techniques, and by histopathology, each at three different times.

Acute series

Anaesthesia was induced with 2% halothane and maintained with 0.5 - 2% halothane in a mixture of nitrous oxide and oxygen (70% : 30%). A tracheostomy was performed and mechanical ventilation initiated. A polythene catheter was inserted into a femoral artery for continuous monitoring of blood pressure and periodic sampling for blood gas, haematocrit and plasma glucose analysis. A femoral vein was cannulated for fluid replacement. Rectal temperature was monitored and maintained at 37.0 ± 0.5°C with a warming blanket. Surgery was performed as above and homeostasis was maintained until the end of the experiment.

Chronic series

Anaesthesia was induced with 3% isoflurane and maintained with 1- 1.5% isoflurane in a mixture of nitrous oxide and oxygen (70% : 30%) delivered via a face mask. Rectal temperature was monitored and maintained at 37.0 ± 0.5°C with a warming blanket and surgery was performed as above. Following surgery the animals were allowed to recover from anaesthesia immediately.

At 24 h after surgery the animals were reanaesthetized with 4% isoflurane prior to perfusion or immersion staining.

TTC staining, Group A - Perfusion

The technique used was similar to that described in chapter 2. The animals were heparinized with 1000 U heparin i.v. 5 min prior to perfusion. Via a thoracotomy the thoracic aorta was clamped and a catheter inserted into the ascending aorta via the left ventricle and perfused with heparinized saline at 200 mm Hg. and the right atrium was incised. After approximately 1 min, the right atrium was clamped and 2% TTC in saline at 37°C was infused and left stagnant for 4 - 7 min. The brains were removed, cut into 2 mm coronal sections, placed in formal saline and photographed.

TTC staining, Group B - Immersion

The animals were anaesthetized and the brains removed within 3 min of cardiac arrest. They were cut into 2 mm coronal sections and immersed in a 2% solution of TTC in saline at 37°C, with regular turning. After 15 - 30 min they were removed, placed in formal saline and photographed.

Experimental groups

Acute TTC

Groups 1A and 1B (N = 5 + 5) TTC staining was performed 5 - 20 min after MCA occlusion.

Groups 2A and 2B (N = 5 + 5) TTC staining was performed 180 - 240 min after MCA occlusion.

Chronic TTC

Groups 3A and 3B (N = 5 + 5) TTC staining was performed 24 h after MCA occlusion.

Quantification of ischaemic damage

Measurements were performed "blind" from code numbered photographs. An outline from the drawing in the atlas of Konig and Klippel (1963) of the coronal section at 7.02 mm in front of the AO line was reduced to four times actual size and duplicated. Drawings were made on these diagrams of the lesions at the same level delineated by TTC on the photographs. The area of the lesions on the diagrams was then measured using a Quantimet image analyzer and expressed in mm^2.

Statistics

All values are means ± sem. For the area measurements, comparisons were made using students t-test. Differences were taken as significant when $p < 0.01$. For the physiological variables comparisons were made using Duncan's test ($p = 0.05$).

5.1.3 Results

The mean values for the physiological variables did not differ significantly between any of the groups (Table 5.1). The area of damage determined by TTC was almost identical with perfusion and immersion at 24 h. However, the area of damage determined by immersion was much less at 4 h or less; there was almost no evidence of damage shown with immersion at 5 - 20 min (Table 5.2). By contrast, early perfusion with TTC gave an approximate indication of the ultimate lesion size (fig 5.1).

Table 5.1 Mean physiological variables in the acute experiments (arterial blood).

Group	pH	pO2 (mmHg)	pCO2 (mmHg)	BP (mmHg)	Hct (%)	Glu (mg%)	Temp (°C)
(Group 1A)							
Perfusion	7.40	158.7	33.1	96	40.0	225.4	36.9
(5-20')	± 0.02	± 14.8	± 2.5	± 10	± 0.9	± 17.9	± 0.1
(Group 2A)							
Perfusion	7.39	167.4	38.7	103	37.8	173.6	37.3
(180-240')	± 0.03	± 13.9	± 3.2	± 6	± 1.7	± 19.7	± 0.2
(Group 1B)							
Immersion	7.41	174.6	40.4	108	43.0	219.2	37.0
(5-20')	± 0.04	± 30.6	± 6.2	± 5	± 1.0	± 23.9	± 0.1
(Group 2B)							
Immersion	7.41	150.6	34.1	101	42.0	176.4	37.2
(180-240')	± 0.02	± 6.7	± 1.7	± 6	± 1.8	± 6.9	± 0.1

All readings are expressed as mean ± sem N = 5 for all groups.
There was no significant difference between any of the groups (one-way ANOVA $p < 0.05$).

(Hct = Haematocrit, Glu = Blood Glucose)

Table 5.2 Area of ischaemic damage in mm^2, on sections corresponding to 7.02 mm in front of the AO line from the atlas of Konig and Klippel (1963), determined by TTC staining.

Time post MCAO	Area of damage	
	Perfusion	Immersion
24 h	17.4 ± 1.3	17.6 ± 1.6
3-4 h	14.4 ± 2.3 *	4.4 ± 2.5 †
5-20 min	11.8 ± 2.3 *	0.4 ± 0.5 †

* $P < 0.01$ comparing Perfusion and Immersion (2-tailed t-test)
† $P < 0.05$ comparing the 24 hour study and other times
† (one-way ANOVA, Duncan's multiple range test)
All readings are mean ± sem, N = 5 for each group.

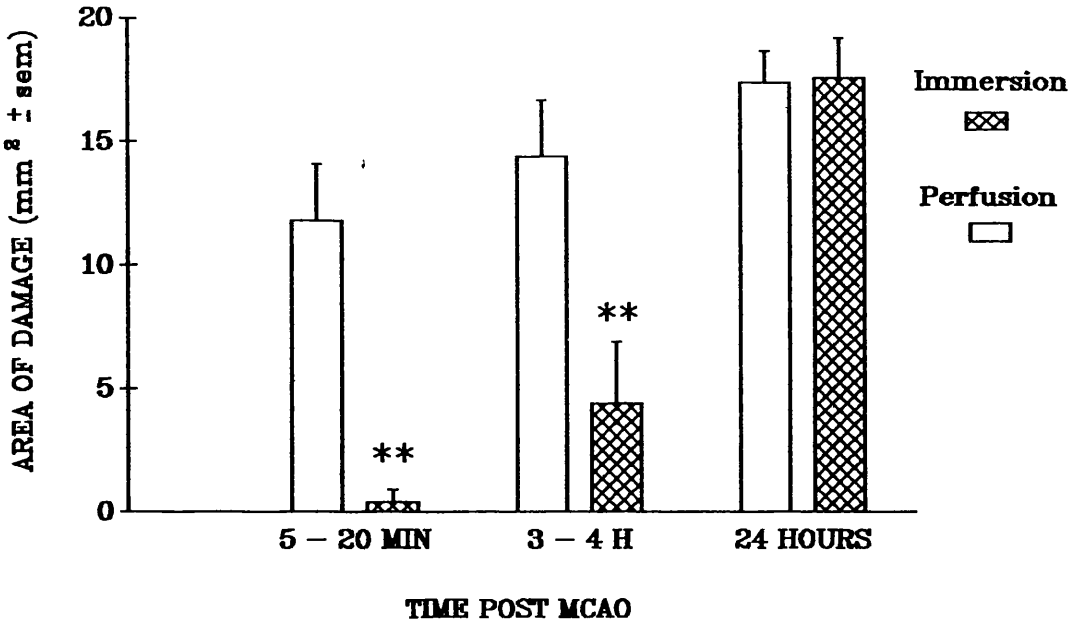

Figure 5.1 Comparison of immersion vs perfusion staining with TTC at various times following MCA occlusion (N = 5 per group, ** $p < 0.01$, 2-tailed t-test).

5.1.4 Discussion

In this study, area measurements of lesions on a single representative slice were used as an end point rather than volumetric estimates for two main reasons. Firstly, pilot studies indicated that there was a dramatic difference between the delineation of lesions by the immersion and perfusion TTC staining techniques in acute focal ischaemia, and secondly, it is difficult to cut fresh brains and perfusion fixed brains at exactly the same levels on multiple sections.

At 24 h following MCA occlusion there was no difference in the area of the cerebral lesion delineated by immersion or by perfusion with TTC. The experiments described in chapter 4 demonstrated that TTC perfusion staining correlated closely

with histopathological damage at 24h. This study suggests that TTC immersion staining also correlates with histopathology, and this agrees with the findings of Bederson et al (1986a). Immersion of brain slices in TTC within 20 min of MCA occlusion revealed virtually no lesion at all, and even at 3 - 4 h post-occlusion there were only small lesions in 2 of the 5 brains examined. However in all brains perfused with TTC within 20 min of MCA occlusion large lesions were evident which were only slightly smaller (not significantly) than the lesions at 3 - 4 h or the lesions at 24 h. The difference in lesion size between perfusion and immersion is most likely to be due to the lack of TTC reaching the ischaemic area rather than the inability of the tissues to oxidise it.

TTC can detect infarcted myocardial tissue in dogs as early as 1- 2 h (Lie et al 1975) but this study suggests that TTC is unreliable in detecting cerebral ischaemic damage of 4 h or less duration and this agrees with the findings of Bederson et al (1986a) and Taylor et al (1987). Early perfusion with TTC defined lesions which were not significantly different from those at 24 h.

It could, therefore, be argued that TTC perfusion immediately after MCA occlusion offers a reasonable predictor of the area which will become infarcted, but this is probably true of any substance which defines lack of perfusion such as Indian ink or carbon black.

5.1.5 Conclusions

Early perfusion studies with TTC therefore give an indication of the final lesion size, but the mechanism by which the ischaemic area is identified differs from that which demarcates it at 24 h. In early perfusion studies TTC is probably acting as a flow marker rather than an indicator of mitochondrial damage. This has major implications in **acute** experiments using neuroprotective agents where neurons may be protected in regions of reduced flow and helps to explain why TTC perfusion staining was unreliable in the acute experiments described in the previous chapter.

Chapter 6

Neuroprotection with dizocilpine: "Dose Response" and "Therapeutic Window"

6.1 Introduction

Dizocilpine (MK-801) has proved to be remarkably effective in reducing infarct size in animal models of focal ischaemia involving permanent middle cerebral artery (MCA) occlusion (see chapter 1, section 1.4, for full discussion). In the rat, dizocilpine has been shown to have beneficial anti-ischaemic properties when administered up to 30 min post MCA occlusion (Park 1988b) and in the cat, up to 2 h post MCA occlusion (Park 1988a). In neither case was a dose-response curve established or the therapeutic window determined and in fact very few studies have attempted to establish these parameters.

The purpose of this study was firstly, to examine the dose effect relationship of dizocilpine when administered by bolus intraperitoneal (i.p.) injection after MCA occlusion, secondly to determine the therapeutic window for a neuroprotective dose of dizocilpine in the same model, and thirdly to measure the plasma concentrations of dizocilpine after a bolus i.p. injection.

6.1.2 Materials and Methods

125 male Sprague-Dawley rats (300-400 g) were used in the experiments; the animals were maintained on a 12 hour light:dark cycle and fed ad libitum prior to the starting the experiments.

Surgical procedure

The animals were anaesthetized with 4% isoflurane in oxygen, they were then intubated and anaesthesia maintained by ventilation with a mixture of 2% isoflurane in 30% oxygen and 70% nitrous oxide. The rectal temperature of the animals was monitored throughout the surgical period and maintained at 37 ± 0.5 °C via a rectal

probe and heating blanket. A tarsorraphy stitch was inserted to protect the left eye and the left middle cerebral artery was exposed via the subtemporal craniectomy and occluded as described in chapter 2. Following surgery the wound was closed and the animals were recovered as previously described.

Immediately after successful MCA occlusion they were allocated to one of the 10 experimental groups.

Experimental groups

A dose response curve for dizocilpine was constructed using doses of 0.3, 0.5, 1.0 and 3.0 mg/kg given intraperitoneally (i.p.) 30 minutes post MCA occlusion. An additional group of animals were given repeated doses of 1 mg/kg i.p at 5 min, 30 min, 1 h and 2 h post MCA occlusion. There were 9-11 animals in each of these groups.

The therapeutic window for the neuroprotective effect of dizocilpine was investigated using a dose of 3 mg/kg which was administered at 30 min, 45 min, 1 h, 2 h and 4 h post MCA occlusion (N = 10-20 per group). The control group of 23 animals received saline i.p..

Perfusion fixation

The animals were allowed to survive for 24 h. Following this they were perfusion fixed with 2,3,5-triphenyltetrazolium chloride (TTC) followed by formol saline as described in chapter 2. Briefly, the animals were deeply reanaesthetized with 4% isoflurane in 100% oxygen. A thoracotomy was performed, 1000U heparin were injected into the right atrium, the thoracic aorta was clamped, a catheter was inserted into the ascending aorta via the left ventricle and perfused with warm

(37°C), heparinized saline at 100 mm Hg. The right atrium was incised 20 seconds after the perfusion commenced. After approximately 90 seconds the right atrium was clamped and 20 ml of 2% TTC in physiological saline at 37°C was perfused in a pulsatile manner at a mean pressure of approximately 120 mm Hg. The TTC was left stagnant for 7 min and 300 ml of 10% formal saline at room temperature was perfused at 100 mm Hg.

The heads were left soaking in 10% formol saline for a further 24 hours, they were randomised, the brains were removed and the forebrain was sliced on a specially made block into 9 x 1.5 mm slices. The rostral surface of each slice was photographed on a low power microscope at constant magnification using ektachrome 160 tungsten slide film.

Quantification of the damage

The area of damage in the hemisphere, cortex and striatum for each slice was measured using a quantimet image analyzer which was calibrated with a 1 cm graticule photographed at the same magnification. For each region the areas on each slice were added together and multiplied by the slice thickness to give the volume of damage. This "direct" method of estimating the volume of damage is fully discussed in chapter 3.

Plasma drug levels

A further experiment was done in unoccluded animals to determine the plasma levels of dizocilpine following a 3 mk/kg (i.p.) dose. Male Sprague-Dawley rats, weighing 244-412g (N = 4) were anaesthetized with a mixture of 1-2% isoflurane in 30% oxygen and 70% nitrous oxide delivered via a close fitting face mask and the tail artery was cannulated to enable blood sampling. The animals were then placed

in small restrainers and allowed to recover for 2 hours. Following this 8 x 200 μl arterial blood samples were taken prior to and at various times up to 4 h after an i.p. dose of dizocilpine. The samples were centrifuged and 100 μl of supernatant was frozen at -70 °C for later analysis by radioimmunoassay (Hichens, Greber and Vyas, in preparation).

Statistics

The dose response data and the repeated administration of dizocilpine group were compared to the controls using a 2-tailed t-test. The same analysis was also performed on the data for the therapeutic window. The same data was also analyzed using analysis of variance with Duncan's multiple range test (significance level $p < 0.05$) using SPSS-PC software. The data is presented as mean ± sem for N animals.

6.1.3 Results

Permanent occlusion of the left MCA resulted in ischaemic damage within the territory of the MCA, that is in the dorsolateral cortex and in the neostriatum. The area of ischaemic damage was delineated in these studies using triphenyltetrazolium chloride. At 24 hours there is no significant difference between the volume of damage delineated by TTC or histopathology (see chapter 4). The direct method of volumetric assessment used here is fully discussed in chapter 3.

The dose response study for dizocilpine revealed that there was a dose related protective effect of dizocilpine against the volume of hemispheric and cortical ischaemic damage when compared to the control animals (Figure 6.1). The 0.3 mg/kg dose did not give significant protection against the volume of hemispheric or cortical damage. However doses of 0.5 ($p < 0.05$), 1 ($p < 0.01$) and 3mg/kg ($p < 0.001$)

of dizocilpine significantly reduced the volume of ischaemic damage in the hemisphere and cortex compared to the saline treated controls (Figure 6.1). The highest dose of dizocilpine of 3 mg/kg produced an approximately 50% reduction in the size of the cortical infarct. There was no significant reduction in the volume of ischaemic damage in the striatum with any of the doses of dizocilpine tested as compared to the control animals. There were 2 post-operative deaths in this study, which were due to respiratory failure, both animals had received a dose of 3mg/kg of dizocilpine.

The group of animals in which 1mg/kg of dizocilpine was given repeatedly at 5 min, 30 min, 1 h and 4 h resulted in a significant ($p < 0.01$) reduction in the volume of ischaemic damage in the hemisphere and cortex (figure 6.1) but not in the striatum when compared to the control group of animals. However it was interesting to note that the degree of protection seen with the repeated dosing was the same as that seen with 1mg/kg of dizocilpine given as a single dose 30 min post-ischaemically (figure 6.1). Using analysis of variance with Duncan's multiple range test, significant protection was obtained in all groups, apart from those given 0.3 mg/kg, in the hemisphere and cortex ($p < 0.05$).

When dizocilpine (3mg/kg, i.p.) was administered at various times post MCA occlusion a significant reduction in the volume of damage in the hemisphere and cortex was seen at 30 min ($p < 0.001$), 45 min ($p < 0.01$) and 1 h ($p < 0.05$) compared to the control animals (Figure 6.2). Although the volume of damage in the hemisphere and cortex was less than that of the controls when administration was delayed up to 2 h and 4 h this reduction did not reach statistical significance (Figure 6.2). Using analysis of variance with multiple comparisons, all treated groups up to 1 h post MCA occlusion showed significant protection in the hemisphere and cortex ($p < 0.05$).

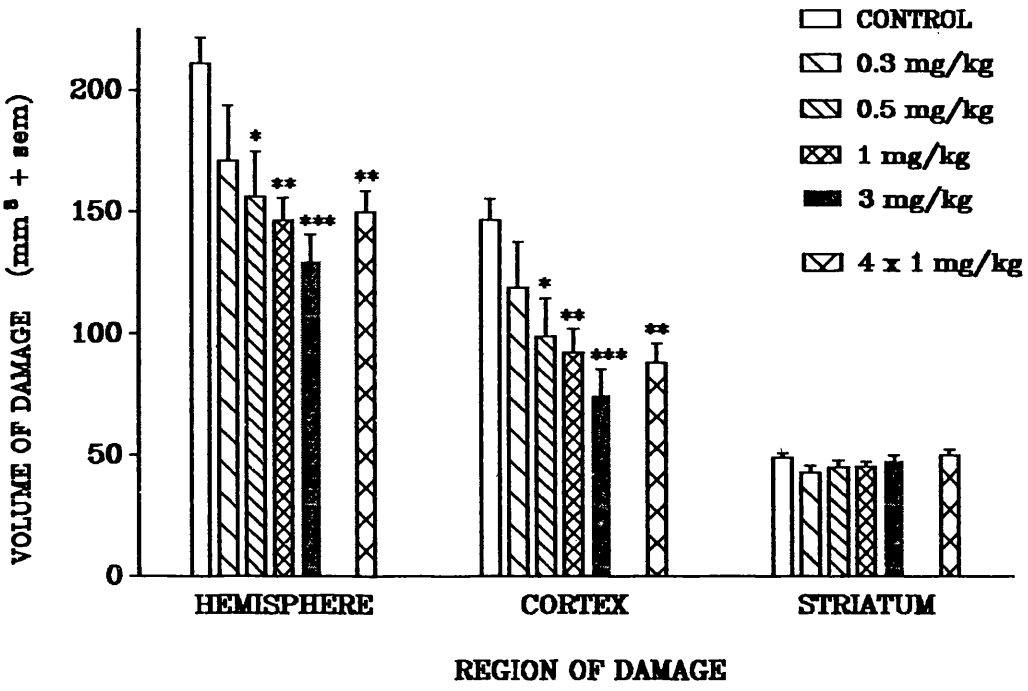

Figure 6.1 Dose response for dizocilpine in the hemisphere cortex and striatum. ED_{50} for cortical protection ≈ 0.3 mg/kg. Doses 0.3 - 3.0 mg/kg were given as a single bolus i.p. 30 min post MCA occlusion. In a further group, 4 doses of 1 mg/kg i.p were given 5 min, 30 min, 1 h and 2 h post occlusion. The control group were given saline i.p. 30 min post occlusion. 0.3 mg/kg was not neuroprotective but all higher doses were, in the hemisphere and cortex ($^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, comparing controls to treated group using 2-tailed t-test). Using analysis of variance with Duncan's correction for multiple comparisons, in the cortex and hemisphere, groups with doses 0.5-3.0 mg/kg were significantly different from the controls but not from each other, 3.0 mg/kg was significantly different from 0.3 mg/kg ($p<0.05$). No significant protection was demonstrated in the striatum at any dose. (Controls N=23, 0.3mg/kg N=9, 0.5mg/kg N=10, 1mg/kg N=10, 3mg/kg N=11, 4x1mg/kg N=10)

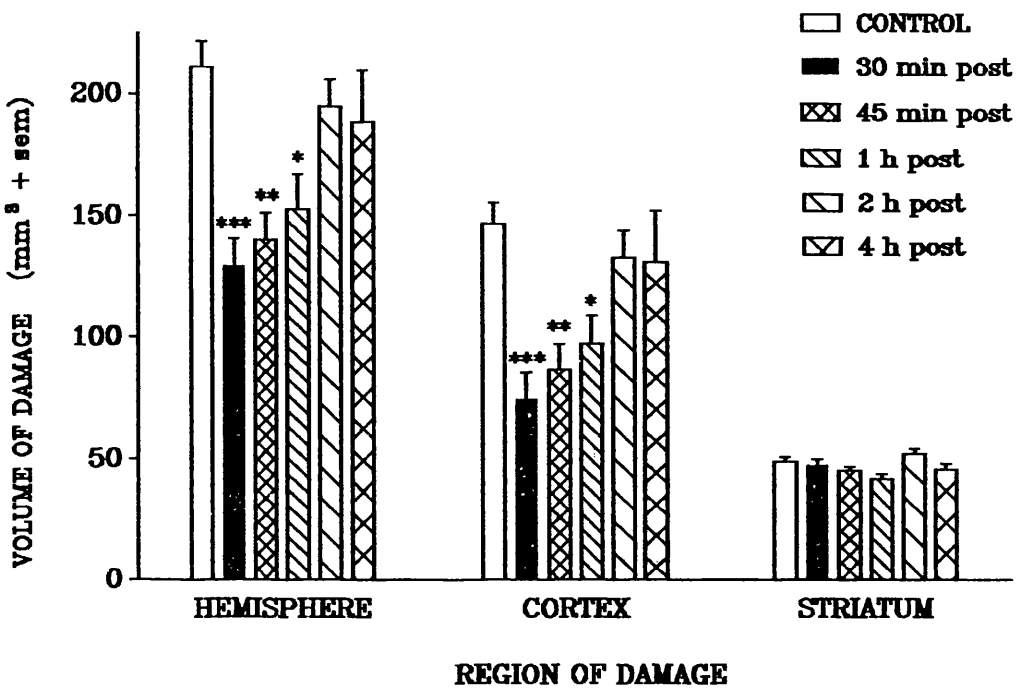

Figure 6.2 Therapeutic window for dizocilpine in the hemisphere, cortex and striatum. 3 mg/kg (i.p.) was administered at various times up to 4 h post MCA occlusion. The control group were given saline. Significant protection in the hemisphere and cortex was obtained up to 1 h post occlusion (*$p < .05$, **$p < .01$, ***$p < .001$, comparing controls to treated groups using 2-tailed t-test). Using analysis of variance with Duncan's correction for multiple comparisons, the 30 min, 45 min and 1 h groups are significantly different from the controls but not from each other, the 30 min and 45 min groups are significantly different from the control, 2h and 4 h groups ($p < .05$). No significant protection was seen in the striatum. (controls N = 23, 30min N = 11, 45min N = 10, 1h N = 20, 2h N = 10, 4h N = 10)

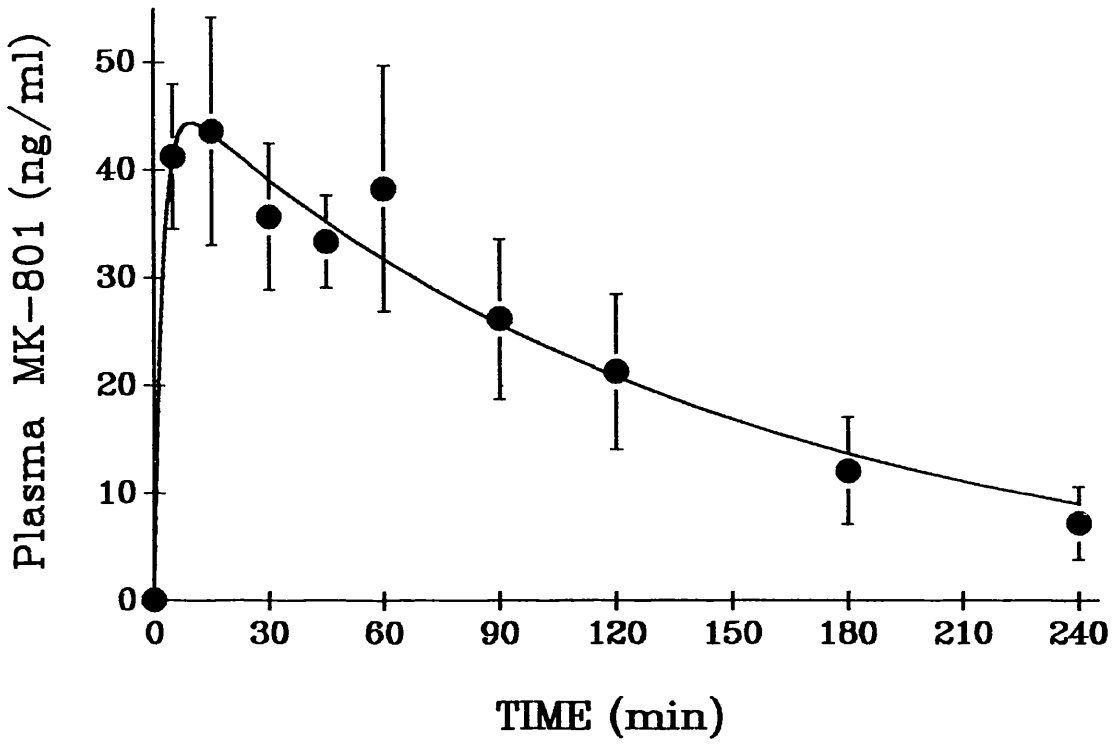

Figure 6.3 Plasma levels of dizocilpine folowing an intraperitoneal injection of 3.0 mg/kg in unoccluded, conscious rats (N = 4). The 2-phase exponential curve was fitted to the data on a computer and has the equation Y = 48.29*exp(-0.007*X) - 46.08*exp(-0.433*X). This gives a maximum plasma concentration of approximately 44 ng/ml and a half life of approximately 1.6 hours.

The plasma values obtained at intervals up to 4 h from 4 rats given 3 mg/kg of dizocilpine i.p. are shown in figure 6.3. A 2-phase exponential curve (Y = A*exp(-B*X)-C*exp(-D*X)) was fitted to this data using RS1 software (Figure 6.3) (A = 48.29, B = 0.007, C = 46.08, D = 0.433) and a T1/2 for dizocilpine was evaluated to be 1.65 h and the peak plasma level 44ng/ml.

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6.1.4 Discussion

Although it is well established that *N*-Methyl-D-Aspartate (NMDA) receptor antagonists protect against cerebral ischaemia in a variety of animal models of focal ischaemia, only a few studies have looked at the efficacy of NMDA antagonists at different doses and very few have assessed the maximum time that therapy can be delayed following the onset of ischaemia (Bielenberg 1989).

Dose response

Bielenberg (1989) showed that the non competitive NMDA antagonist, Phencyclidine (PCP), was protective following MCA occlusion in the rat with the volume of infarct at 48 hours as the end point and in his study; PCP efficacy was demonstrated over a dose range of 3 to 30 mg/kg given as a single dose i.p., 30 min pre MCA occlusion. The present study has shown that dizocilpine is neuroprotective from 0.5 mg/kg to 3 mg/kg when given as a single dose i.p. 30 min post MCA occlusion, with increasingly significant protection as the dose is increased. 1 mg/kg given 30 min after MCA occlusion gave the same degree of protection as 4 doses of

1 mg/kg given at 5 min, 30 min, 1 hour and 2 hours after occlusion, suggesting that the early peak plasma concentration may be as important as the mean concentration for this non competitive NMDA antagonist. Doses of dizocilpine greater than 3 mg/kg were not used in the present study as pilot studies indicated that there may be an increased mortality with higher doses, especially if the dose exceeds 10 mg/kg and, the maximum degree of cortical protection reported by any investigators using NMDA antagonists either pre- or post-ischaemically at any dose is approximately 50% (see table 1.1), which is similar to the degree of protection obtained here when dizocilpine (3 mg/kg) was administered as a bolus i.p. 30 min post MCA occlusion. In order to obtain a full dose response relationship, more groups with many more animals per group would be required. However, this study has shown a relatively narrow effective dose range for dizocilpine (0.5-3.0 mg/kg) with a trend towards decreasing protection with decreasing dose (although these groups were not significantly different from each other). If one assumes that 3 mg/kg produced a maximal effect (the degree of protection obtained was similar to pre- and post-ischaemic treatment at a dose of 0.5 mg/kg i.v., Park et al.,1988c) then the ED_{50} of dizocilpine in this model is approximately 0.3 mk/kg. This is similar to data obtained from a model of focal ischaemia in the mouse (Gotti et al., 1990; ED_{50} for dizocilpine = 0.2 mg/kg, i.p.) and also agrees with data from a gerbil model of forebrain ischaemia (Gill et al., 1987a; ED_{50} for dizocilpine = 0.3 mg/kg, i.p.).

Therapeutic window

Park et al., (1988b) demonstrated that dizocilpine was neuroprotective in the cat when 5 mg/kg was administered as a slow intravenous bolus 2 hours post MCA occlusion and the degree of protection was similar when compared to pre-ischaemic administration, their end point being the volume of damage after 6 hours of ischaemia. In this chronic rat model, there was a slight reduction in the volume of

ischaemic damage when dizocilpine was administered at 2 h and 4 h following the onset of ischaemia, but this did not reach significance (fig 6.2). The maximum time that therapy producing significant protection could be delayed was one hour and this is similar to data obtained from the mouse model with another NMDA antagonist SL 82.0715, where significant protection was obtained when therapy was administered 45 min but not 90 min after the onset of ischaemia. However, significant protection has been obtained in a similar rat model of focal ischaemia with the NMDA antagonist PCP with a 3 h delay in administration (Bielenberg, 1989). The reason for this difference is not clear. It may be related to the drug used, but this seems unlikely as PCP has a very similar mode of action to dizocilpine (see chapter 1). It is more likely to be related to either the strain of rat (Fischer 344 as opposed to Sprague-Dawley rats) or some other methodological difference (such as the length of MCA occluded). Further experiments will be required to fully determine the therapeutic window for NMDA antagonists in this model of focal ischaemia. However, this study along with those of Park et al. (1988b, c & d) and Bielenberg (1989), demonstrates the important fact that non competitive NMDA antagonists such as dizocilpine and phencyclidine can have a neuroprotective effect in models of focal ischaemia even when therapy is a significantly delayed after the onset of ischaemia.

Plasma drug levels

There is very little published data on plasma levels of dizocilpine in the rat. Hucker et al. (1982) studied the absorbtion and metabolism of dizocilpine in the rat following oral doses. They found that the drug was almost completely absorbed following an oral dose, the peak plasma concentration after a 1 mg/kg p.o. dose was 46 ± 13.4 ng/ml (mean ± sem for 3 animals) and the plasma half life was approximately 1.4 h, but relatively high concentrations of metabolites (mainly

8-hydroxy and 2-hydroxy dizocilpine) were present in plasma for 24 h or longer, and binding studies have demonstrated that these metabolites are still active (Thompson et al., 1990). The present study is in broad agreement with the findings of Hucker et al. (1982), in that the half life was 1.6 h and this is also similar to the findings of Venzzani et al (1989), who found a terminal T1/2 (elimination) for dizocilpine in the rat to be 1.9 h. The peak plasma level of 44 ng/ml, after an i.p. dose of 3 mg/kg, was somewhat lower than would have been expected according to the study of Hucker et al., (1982) but was considerably higher than that reported by Vezzani and colleagues (12 ng/ml, 1989) after an i.p. dose of 2 mg/ml. The differences in the plasma levels reported in these studies may reflect the different techniques used to detect the drug.

6.1.5 Conclusions

This study has demonstrated that the NMDA antagonist dizocilpine ameliorates ischaemic damage in this model of focal ischaemia in a dose dependant manner. The approximate ED_{50} for dizocilpine, administered by bolus i.p. injection was 0.3 mg/kg. The maximum time that therapy could be delayed was 1 h, although the true therapeutic window for dizocilpine in this model may be a little longer.

Chapter 7

Comparison of neuroprotection with dizocilpine (MK-801) in Sprague-Dawley and spontaneously hypertensive rats following distal and proximal middle cerebral artery occlusion.

7.1 Introduction

A strain of "spontaneously hypertensive" rats (SHRs) was developed in Japan by Okamoto and Aoki (1963) by mating a severely hypertensive male Wistar rat with a moderately hypertensive female from the same colony with continued, selective inbreeding, and since their development they have been widely used in antihypertensive and cerebrovascular research.

The most widely used model of focal ischaemia in the rat for neuroprotection experiments, is the "proximal" (ie proximal to and including the lenticulostriate artery) middle cerebral artery (MCA) occlusion as described by Tamura et al (1981a), a full description of which is given in chapter 2. Duverger and MacKenzie (1988) demonstrated that lesions following MCA occlusion were more consistent in spontaneously hypertensive rats than in normotensive rats and this was also demonstrated by Brint et al. (1988), using a model involving distal MCA occlusion combined with ipsilateral common carotid occlusion. Hypertension is a major risk factor in stroke (Kannel et al., 1970; Dyken & Wolf, 1974; Gautier, 1983) and SHRs demonstrate some of the arteriopathic changes associated with hypertension in humans (Yamori 1976). It has therefore been suggested that SHRs might be more appropriate than normotensive animals for neuroprotection experiments in focal ischaemia, both from the point of view of improved reproducibility of lesions and the possible greater relevance to stroke in man. The vast majority of the reported neuroprotection experiments in focal ischaemia in normotensive animals demonstrate dramatic efficacy of NMDA antagonists (see table 1.1, chapter 1), however, NMDA antagonists do not appear to be as effective in ameliorating focal ischaemic damage in hypertensive animals. Dirnagl and colleagues (1990) demonstrated the failure of dizocilpine to protect in their model of focal ischaemia, which involves a distal MCA occlusion combined with an ipsilateral common carotid occlusion in SHRs, unless the drug was administered both pre and

post-ischaemically. Roussel et al. (1989) demonstrated that the NMDA antagonist kynurenate failed to protect following MCA occlusion in SHRs whereas Germano and colleagues (1987) found that a lower dose of kynurenate improved both neurological and histological outcome following MCA occlusion in normotensive rats. Panetta and colleagues (1989) demonstrated the failure of dizocilpine to improve histological outcome following temporary (2h) MCA occlusion in SHRs whereas Buchan et al. (1990) demonstrated improved histological outcome with dizocilpine following 2h temporary MCA occlusion combined with bilateral carotid occlusion in normotensive animals. It was also noted in pilot studies that the NMDA antagonist dizocilpine is not neuroprotective in SHRs following proximal MCA occlusion (Hatfield, unpublished data) whereas it produces reliable protection with the same lesion in normotensive animals (see chapters 4 & 6). Coyle (1989) has demonstrated that dizocilpine can reduce lesion size in SHRs when a distal MCA occlusion is performed without an ipsilateral carotid occlusion.

The purpose of this experiment was to study the effects of dizocilpine on the lesion size in normotensive and hypertensive rats following distal and proximal MCA occlusions, performed by the same operator in the same laboratory.

7.1.2 Materials and Methods

38 adult, male Sprague-Dawley rats and 32 adult, male spontaneously hypertensive rats (Charles River) weighing between 280 and 420 g were used in the experiments. They were kept under controlled environmental conditions on a 12 hour light:dark cycle and fed ad libitum prior to the experiments.

Surgical procedure

Anaesthesia was induced with 4% isoflurane in oxygen. The animals were intubated and anaesthesia was maintained by ventilation with 2% isoflurane in a mixture of nitrous oxide and oxygen (70% : 30%). Rectal temperature was monitored throughout surgery and maintained at 37 ± 0.5 °C via a warming blanket. The left middle cerebral artery was occluded as described in chapter 2. The artery was occluded either proximal to the lenticulo-striate branch and below the olfactory tract or distal to the lenticulo-striate and above the olfactory tract (fig 7.1) and was divided in all cases. As soon as the MCA was divided the wound was sutured and the animals recovered as previously described. They were kept in warmed cages for the next 2 hours and they were allowed free access to water and laboratory chow immediately after recovery.

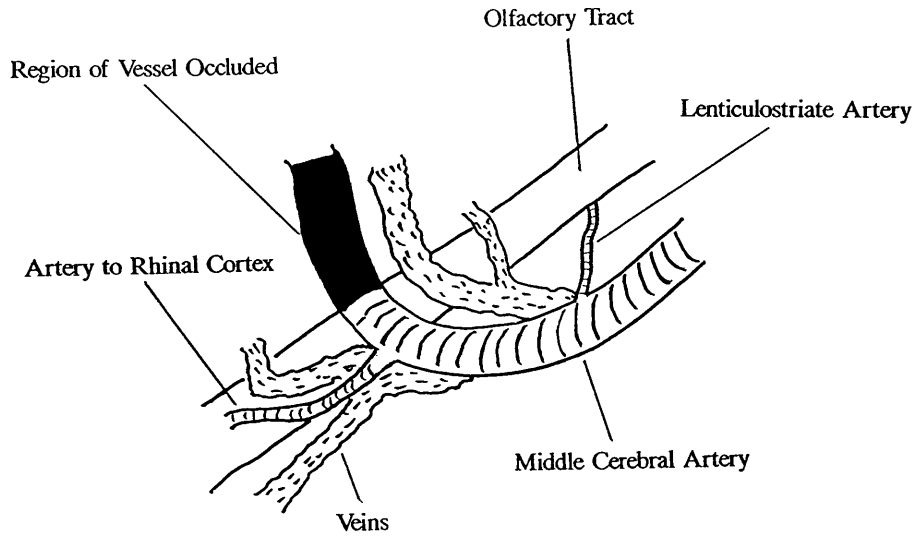
Experimental groups

Immediately after successful MCA occlusion they were either allocated to a control group or were given 3.0 mg/kg dizocilpine i.p., 30 min post MCA occlusion, there were 8-11 animals per group.

Perfusion fixation

24 hours after MCAO the animals were deeply reanaesthetized and they were perfused with TTC followed by formol saline as described in chapter 2. The heads were left soaking in 10% formal saline for a further 24 hours, they were randomised, the brains were removed and the forebrain was sliced on a specially made block into 9 x 1.5 mm slices. The rostral surface of each slice was photographed on a low power microscope at constant magnification using ectachrome 160 tungsten slide film.

Distal MCA occlusions



Proximal MCA occlusions

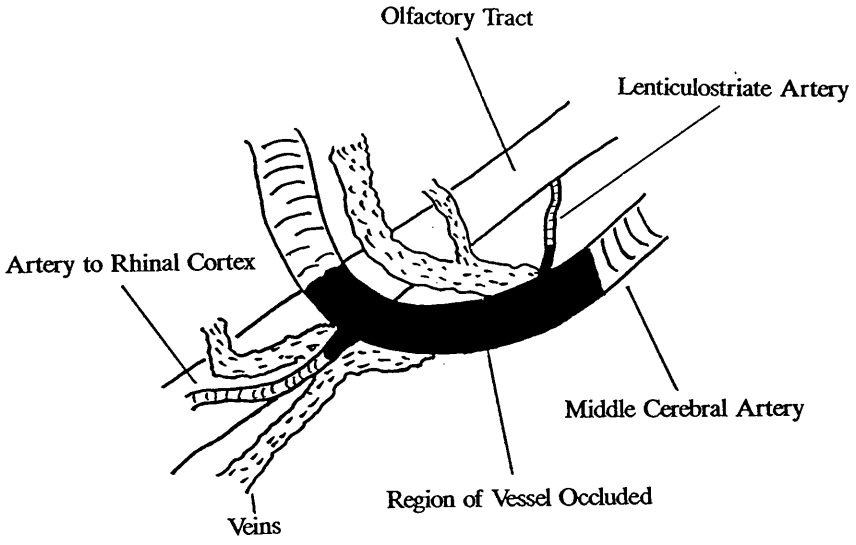


Figure 7.1 Diagrams showing the region of the middle cerebral artery occluded in "distal" and "proximal" occlusions. (Modified after Shigeno et al., 1985)

Quantification of the damage

The lesions delineated by TTC were drawn on to scale diagrams of corresponding sections taken from the atlas of Konig and Klippel. The area of damage in the hemisphere, cortex and striatum for each diagram was measured using a Quantimet image analyzer. The areas on each slice were integrated to give the volume of damage (see chapter 3 for detailed discussion).

Statistics

Each group was compared to the control group using a 2-tailed t-test.

7.1.3 Results

In normotensive, Sprague-Dawley rats, proximal MCA occlusion produced consistent cortical and striatal damage and dizocilpine at a dose of 3mg/kg i.p., 30 min post MCA occlusion, gave significant cortical but not striatal, protection (fig 7.2). Distal MCA occlusion produced almost no striatal damage and the cortical damage was more variable (table 7.1) and on average less than 50% when compared to that following proximal occlusions. The same dose of dizocilpine produced some reduction in cortical lesion volume, but this was not significant (fig 7.3). Proximal MCA occlusions in spontaneously hypertensive rats (SHRs) produced very consistent cortical and striatal damage but dizocilpine failed to protect (fig 7.4). However, distal MCA occlusions in SHRs produced no striatal, but consistent cortical damage and dizocilpine afforded significant cortical protection (fig 7.4).

Table 7.1 Lesion volumes (mm^3) in the hemisphere of the control animals showing the coefficient of variation (C of V)

Group	N	Hemisphere	C of V
SD proximal	9	129.2 ± 22.1	17.1%
SD distal	12	36.6 ± 24.7	68.5%
SHR proximal	8	135.9 ± 12.2	9.0%
SHR distal	7	86.8 ± 9.8	11.3%

Data is expressed as mean ± standard deviation in Sprague-Dawley (SD) and Spontaneously hypertensive rats (SHR) following proximal and distal middle cerebral artery occlusion.

7.1.4 Discussion

Normotensive animals

In normotensive animals, MCA occlusion distal to the lenticulostriate artery does not cause striatal infarction and results in a highly variable degree of cortical infarction (Robinson, 1981; Shigeno et al., 1985; Coyle, 1982a; Bederson et al., 1986b; Brint et al., 1988). These previous studies have reported cortical infarction rates from 0% to 50%, depending on the exact site and the technique used for the occlusion. In the present study, there was virtually no striatal infarction but cortical lesions were demonstrated in 100% of the distally occluded animals. The higher infarction rate in this study may be due to the greater length of MCA occluded (fig 7.1) as compared to these previous studies, but in several cases the lesions were very small and related mainly to the site of the occlusion. The lesions were highly variable with a coefficient of variation for the volume of hemispheric damage of 68.1% (table 7.1). This variability makes the distal occlusion model in normotensive animals quite

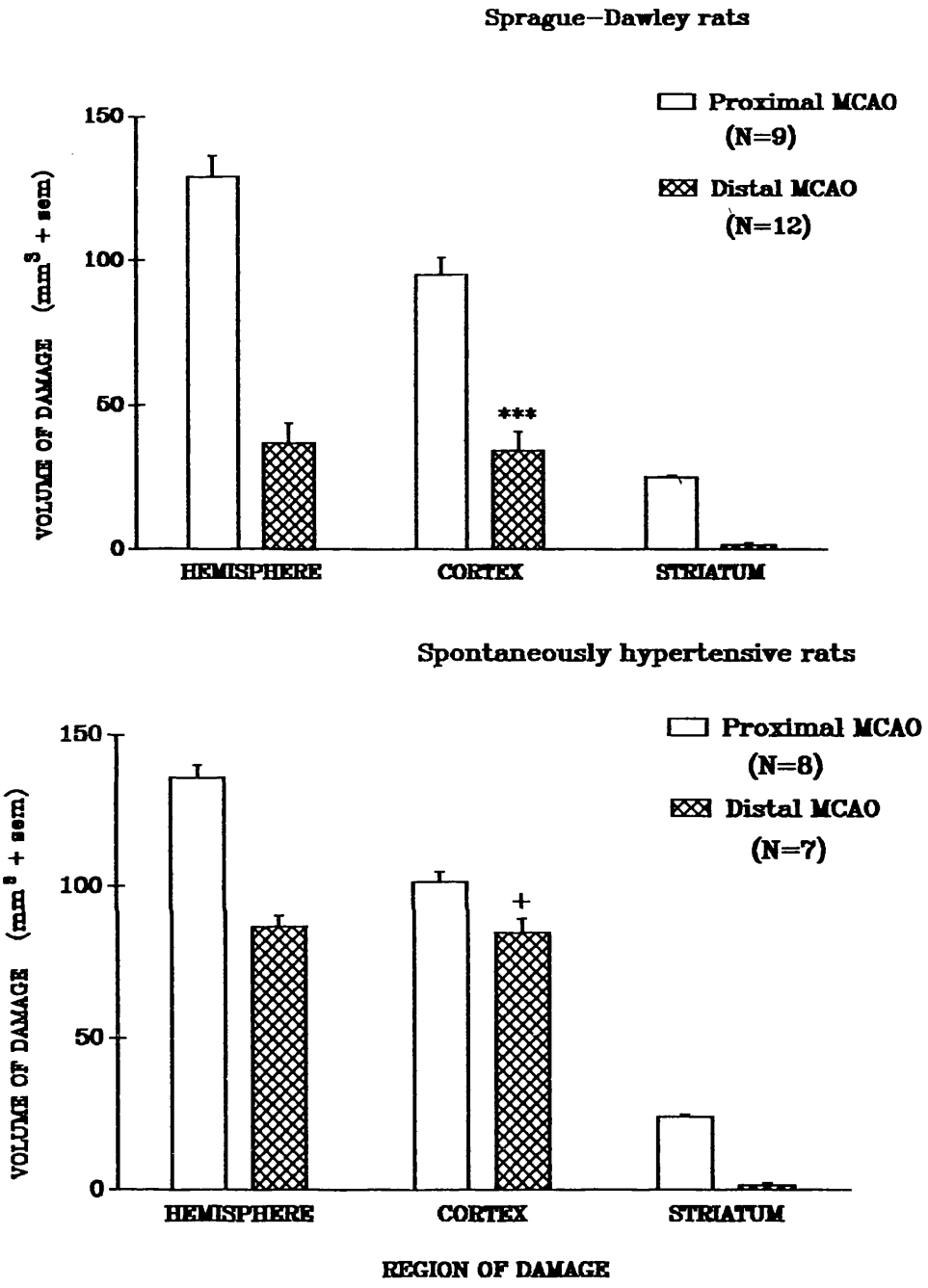


Figure 7.2 Comparison of regional damage in Sprague-Dawley (SD) and spontaneously hypertensive rats (SHR) following proximal and distal MCA occlusions. There was minimal or no striatal damage following distal MCA occlusion in either species. In SHRs, the volume of cortical damage was only slightly reduced following distal MCA occlusions. This reduction is significant using a t-test ($+p = 0.014$) but is not significant using ANOVA with Duncan's test for multiple comparisons ($p < 0.05$). In contrast, the reduction in cortical lesions following distal MCA occlusions in SD rats was dramatic (***$P < 0.001$, t-test) with some animals having only minimal lesions.

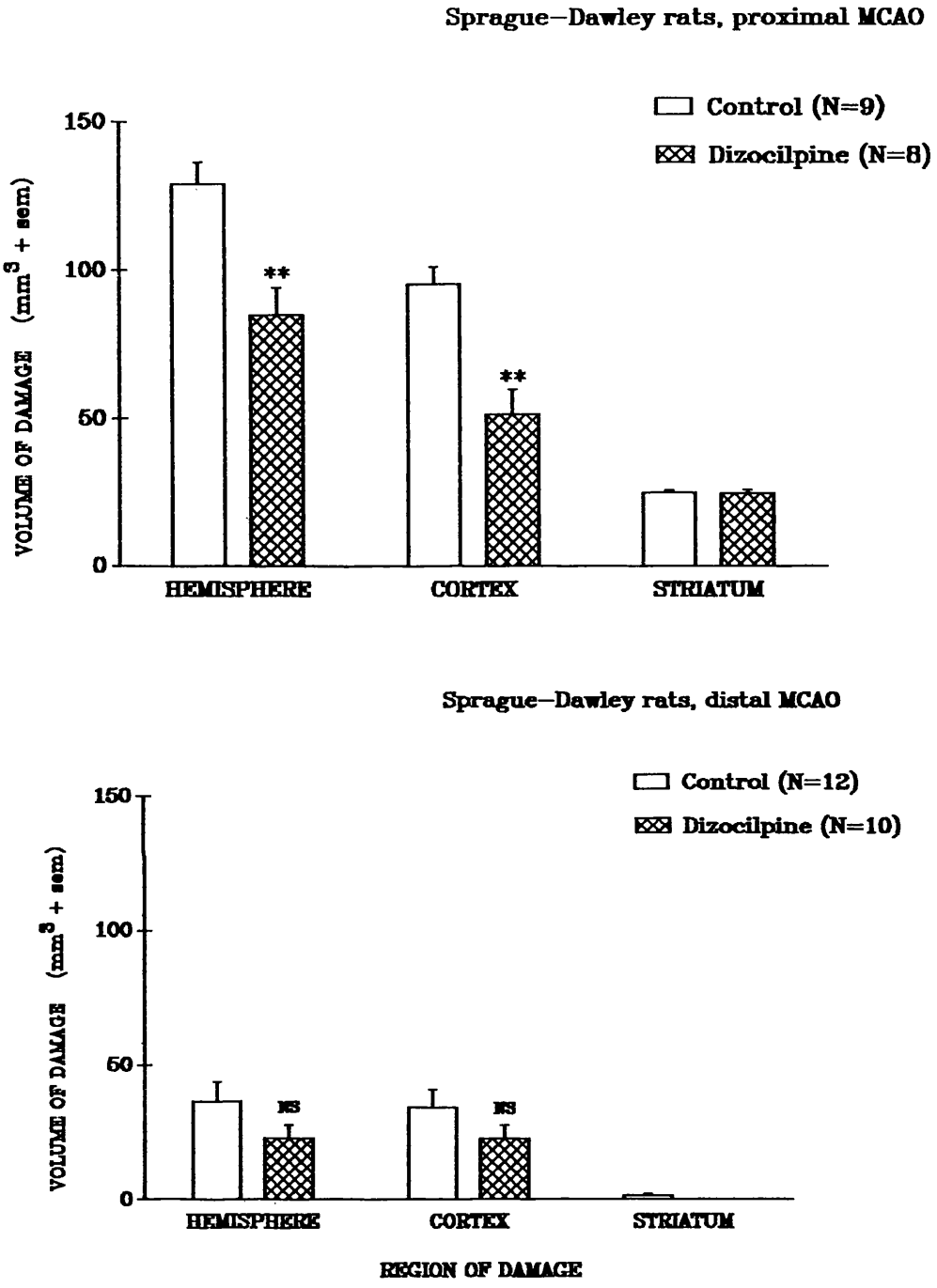


Figure 7.3 Effect of dizocilpine (3mg/kg i.p., 30 min post MCAO) in Sprague-Dawley rats following proximal and distal MCA occlusion. Significant protection was seen following proximal occlusion (**$p < 0.001$, t-test) but not following distal occlusion, which produced small and variable lesions.

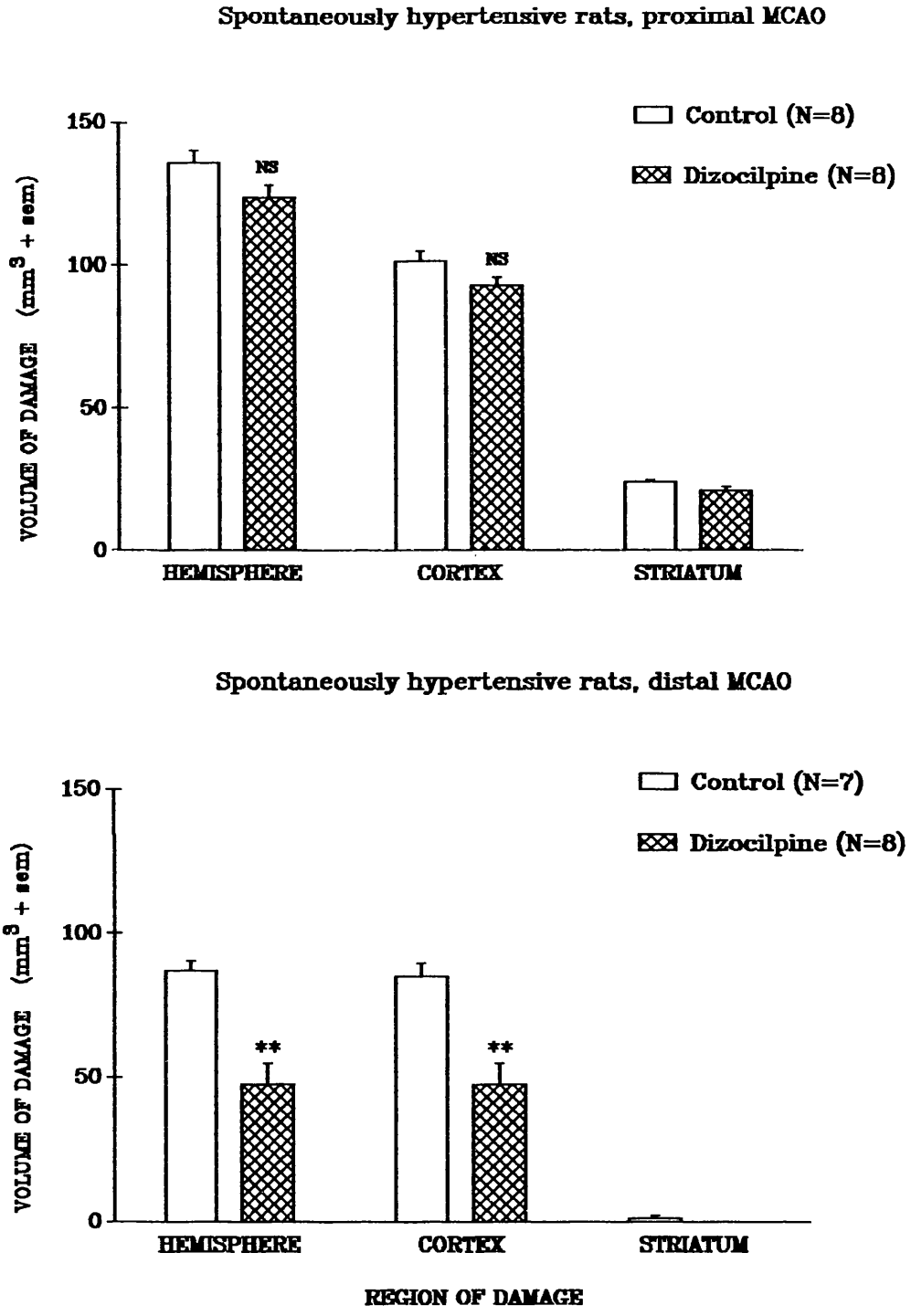


Figure 7.4 Effect of dizocilpine (3mg/kg i.p., 30 min post MCAO) in spontaneously hypertensive rats following proximal and distal MCA occlusion. There was no significant protection following proximal MCA occlusion but following distal MCA occlusion the degree of cortical protection was not only significant (**$p < 0.001$, t-test) but was similar to the cortical protection in Sprague-Dawley rats after proximal occlusion (see fig 7.3).

unsuitable for neuroprotection experiments and although dizocilpine reduced hemispheric lesions by 37%, it is hardly surprising that this was not significant (fig 7.3).

Tamura and colleagues (1981a) demonstrated that following proximal MCA occlusion in normotensive rats, consistent damage is obtained in the cortex and striatum. Many studies have now confirmed this and have shown that dizocilpine improves cortical damage in this model (see table 1.1, chapter 1 and also results chapters 4 & 6). The lack of protection in the striatum is probably due to the fact that the lenticulostriate arteries in the rat are effectively end arteries (Yamori et al., 1976; Coyle, 1982b; Rieke et al., 1981) and blood flow can be reduced to almost negligible levels in the lateral part of the caudate nucleus following their occlusion (Tamura et al., 1981b).

Hypertensive animals

Unlike normotensive animals, spontaneously hypertensive rats (SHRs) undergo consistent extensive cortical infarction following distal MCA occlusion (Grabowski et al., 1988; Coyle et al., 1986b). The increased susceptibility to infarction of hypertensive animals is thought to be due to decreased diameter of pial vessels in SHRs (Johansson et al., 1982 & 1985; Harper et al., 1984) and decreased blood flow through cerebral collaterals following MCA occlusion (Coyle & Heistad 1986; Eke & Halsey, 1985). The present study confirms the increased susceptibility to infarction of SHRs and interestingly, there was only a minimal difference between the volume of cortical damage in SHRs following proximal and distal occlusions (fig 7.2) which also suggests poor collateral circulation.

Duverger and MacKenzie (1988) demonstrated an increased lesion volume in SHRs compared to normotensive rats, following proximal MCA occlusion. In this study

there was a trend towards larger lesions in the SHRs but this was not significant. The reasons for this difference could be due to different sources of SHRs or possibly surgical technique. Pilot studies revealed that more consistent lesion volumes were obtained if a greater length of MCA was occluded (from proximal to the lenticulostriate branch to above the olfactory tract) and it is possible that an increased length of MCA occlusion has led to a decreased difference in lesion volumes between the two strains. It is interesting to note that the coefficient of variation for hemispheric infarct volumes in the Duverger & MacKenzie (1988) series were 33% for Sprague-Dawley (SD) rats and 8% for SHRs, compared to 17% for SD rats and 9% for SHRs in the present series. The improved coefficient of variation in the SD rats may be due to a difference in technique which also accounts for larger lesion volumes in SD rats (129.1 7.4 mm3 compared to 98.8 11.5 mm3) and the decreased difference between the strains.

Coyle (1989) demonstrated that dizocilpine can be neuroprotective in another strain of hypertensive rat, stroke-prone spontaneously hypertensive animals (SPSHRs), following distal MCA occlusion and the present study demonstrates that dizocilpine can be neuroprotective in SHRs following distal occlusion. However, dizocilpine did not ameliorate damage in SHRs following proximal MCA occlusion, presumably due to the less effective collateral circulation in these animals and the decreased "penumbra" resulting from a proximal MCA occlusion.

7.1.5 Conclusions

In normotensive, Sprague-Dawley rats, a proximal MCA occlusion is required to sufficiently reduce the collateral circulation such that consistent infarction is produced. In this model the NMDA antagonist dizocilpine can reduce cortical infarct volumes by up to 50%. With distal occlusions in normotensive animals,

infarct volumes are small and highly variable, and a very large number of animals would be required to show a significant neuroprotective effect. On the other hand, in hypertensive animals, owing to their impoverished collateral circulation and increased susceptibility to infarction, distal MCA occlusion leads to consistent cortical lesions and dizocilpine has a significant anti-ischaemic effect. This protective effect is not seen in hypertensive animals following proximal MCA occlusion, presumably because the collateral circulation is further reduced, beyond the point where there is sufficient "penumbra" in which the NMDA antagonist may operate.

Chapter 8

Neuron-specific enolase (NSE) as a marker of neuronal damage in focal ischaemia.

8.1 Introduction

Neuron-specific enolase (NSE) is the γ-subunit of enolase, a glycolytic enzyme which converts 2-phospho-glycerate to phosphoenolpyruvate, and is found mainly in neurons and cells of neuroendocrine origin (Marangos et al., 1979 & 1987). NSE represents a high percentage (1.5%) of the total soluble brain proteins and is stable in biological fluids (Kato et al., 1981). S-100 protein is a calcium binding protein, which is also regarded as nervous-system specific, and is found mainly in glial cells (Endo et al.,1981). Damage to cells expressing these proteins causes increased concentrations in body fluids and this has led to investigation of their use as biochemical markers of damage to the nervous-system (Royds et al., 1981; Sindic et al., 1982; Scarna et al., 1982; Mokuno et al., 1983; Steinberg et al., 1984; Persson et al., 1987).

Clinical studies have indicated that CSF concentrations of NSE and S-100 proteins may be useful predictors of brain damage from subarachnoid haemorrhage (Persson et al., 1988; Hårdemark et al., 1989b), Stroke (Hay et al., 1984), head injury (Bakay et al., 1986; Steinberg et al., 1984) and cardiac arrest (Roine et al., 1989). In the acute phase, clinical assessment of the extent of brain damage and prediction of potential recovery remain difficult. The treatment of brain injury is geared towards limiting the extent of the injury, in the hope that this will improve outcome. If CSF markers can indicate, quantitatively, the degree of acute damage, then they may be useful in the assessment of different treatment regimes. However, for the meaningful interpretation of CSF marker concentrations, it is imperative to understand their kinetics following the various forms of injury, and this can best be studied under controlled conditions in experimental models.

Hårdemark and colleagues (1988) demonstrated that CSF neuron-specific enolase could be a quantitative marker of focal ischaemic damage, as assessed by the area of damage on a single brain slice, following middle cerebral artery (MCA) occlusion

in rats. The purpose of the present study was to examine the correlation between CSF NSE concentrations and infarct volumes in control and dizocilpine treated animals, with cortical and combined cortical and striatal infarcts.

8.1.2 Materials and Methods

76 male Sprague-Dawley rats (310 - 400 g) were used in the experiments. They were kept in controlled environmental conditions and were allowed food and water ad libitum prior to the experiments.

CSF sampling

Cannulation of the cisterna magna and sampling of the CSF were performed essentially as described by Sarna et al (1983). Sampling catheters were made from approximately 10 cm lengths of fine polythene tubing, internal diameter 0.28 mm (Portex 800/110/100/100). Enamelled copper wire, 0.2 mm diameter (Radio Spares 357-918) was threaded down the centre of the catheters and a small "button" was formed 7 mm from one end, by gently heating the catheter over a soldering iron.

Catheter insertion

For insertion of the CSF sampling catheters the animals were anaesthetized by intraperitoneal injection of 60-100 mg/kg pentobarbital. After shaving the head, they were placed in a stereotactic frame using ear bars only and an incision was made in the midline between the lambda and the external occipital crest. A burr hole was drilled in the mid line, just dorsal to the external occipital crest, taking care not to pierce the dura. A further burr hole was made some 5 mm caudal to the lambda and off the midline to take an 8BA, stainless steel screw. The catheters were inserted

with a piece of copper wire just protruding, to aid entry into the cisterna magna via the extradural route, as described by Hårdemark et al (1988). After insertion of the catheter, it was cemented in place with dental acrylic (fig 8.1), the wound was closed with a continuous 3/0 silk suture and the animals recovered.

CSF sampling was performed by connection of a larger catheter (internal diameter 0.58 mm, Portex 800/110/200/100) and gentle aspiration via a 1 ml syringe. 60 μl samples of CSF were obtained on insertion of the catheters and at daily intervals. Following sampling the CSF remaining in the implanted catheter was carefully returned to the cisterna magna and the catheter was heat sealed.

Experimental groups

Only animals with catheters that remained patent for 2 - 3 days were entered into experimental groups: Sham operation, proximal MCA occlusion dizocilpine (3 mg/kg i.p., 30 min post MCAO), distal MCA occlusion dizocilpine. CSF was sampled for 7 days, after which the rats were perfusion fixed.

MCA occlusion

For middle cerebral artery occlusion (MCAO) the rats were re-anaesthetized, 2-3 days after catheter implantation, with 4% isoflurane in oxygen. Anaesthesia was maintained by ventilation with a mixture of 1-2% isoflurane in 30% oxygen and 70% nitrous oxide. Temperature was monitored via a rectal probe and maintained at 37.0 $\pm$ 0.5 °C by a warming blanket. The middle cerebral artery was occluded, as described in chapter 2, either proximal to the lenticulo-striate branch and below the olfactory tract or distal to the lenticulo-striate and above the olfactory tract (see chapter 7, fig 7.1) and was divided in all cases. As soon as the MCA was divided the concentration of isoflurane was reduced to 0.5%. The wound was closed in layers

and a 4/O silk tarsorraphy stitch was inserted to protect the left eye. The animals were recovered immediately after surgery. Initially they were ventilated with oxygen and nitrous oxide until they showed signs of recovery. They were then allowed to breath spontaneously via the endo-tracheal tube and when breathing well, they were extubated. They were kept in warmed cages for the next 2 hours and they were allowed free access to water and laboratory chow immediately after recovery.

Perfusion fixation

For perfusion fixation the animals were reanaesthetized with 4% isoflurane in oxygen. The right atrium was incised and 100 ml physiological saline followed by 300 ml 10% formal saline was perfused via the left ventricle. The brain was left in situ and immersed in fixative for 24 hours. The brain was then removed and the fore brain sliced into 9 x 1.5mm coronal slices, which were imbedded in paraffin. Sections were cut from the rostral surface of each slice and stained with haematoxylin and eosin. Coded slides were examined "blind" and the region of damage on each section was drawn on to scale diagrams taken from the atlas of Konig and Klippel (1963). The areas were measured on an image analyzer and integrated to give a volume of damage (see chapter 3).

NSE analysis

A total of 420 CSF Samples were analyzed; they were centrifuged at 5000 rpm for 5 min and 40 μl of supernatant was frozen at -70 °C. NSE content was analyzed blind, by radioimmunoassay using a commercially available kit (Pharmacia AB, Uppsala Sweden), using human NSE as a standard.

8.1.3 Results

76 rats had catheters implanted. 13 catheters blocked prior to MCA occlusion and one became infected and was therefore excluded. 62 rats underwent MCA occlusion and there were 6 perioperative deaths, mainly from excessive haemorrhage from the MCA during surgery or post-operative respiratory distress. 29 out of the remaining 56 animals developed blocked catheters in the first 5 days after MCA occlusion, leaving 25 for the final analysis. Most animals lost approximately 10% of body weight in the first 24 hours following MCA occlusion, but showed no other signs of distress.

Histopathological examination showed mature, well defined, infarcts in the regions supplied by the middle cerebral artery. In the animals with proximal MCA occlusions, the infarcts involved cortex and striatum in the same regions as the ones described in chapter 3, 24 hours after MCA occlusion. Following distal MCA occlusion, none of the animals had lesions in the striatum and the cortical lesions were highly variable.

Basal NSE level, estimated by analyzing samples obtained immediately after catheter insertion, was 8.0 ± 0.8 ng/ml. Following sham operation (N = 4), NSE levels rose slightly in the first 24 hours to 12.3 ± 4.4 ng/ml (mean ± sem), but after 48 hours they returned to basal levels (fig 8.2). In all the occluded animals (dizocilpine treated and controls) mean NSE levels peaked on day 3 and by day 7, they had almost returned to basal levels (fig 8.2, day 0 = day of surgery). There was an excellent correlation between the volume of infarction delineated by histopathology at 7 days post MCA occlusion and the integral of the CSF NSE concentration over the first 5 days (fig 8.3, $r = 0.97$, p.001), which did not appear to be dependent on the treatment group or the type of occlusion. There was also a reasonable correlation between the volume of infarction at 7 days and the CSF NSE level 3 days post occlusion (fig 8.3, $r = 0.92$, p.01).

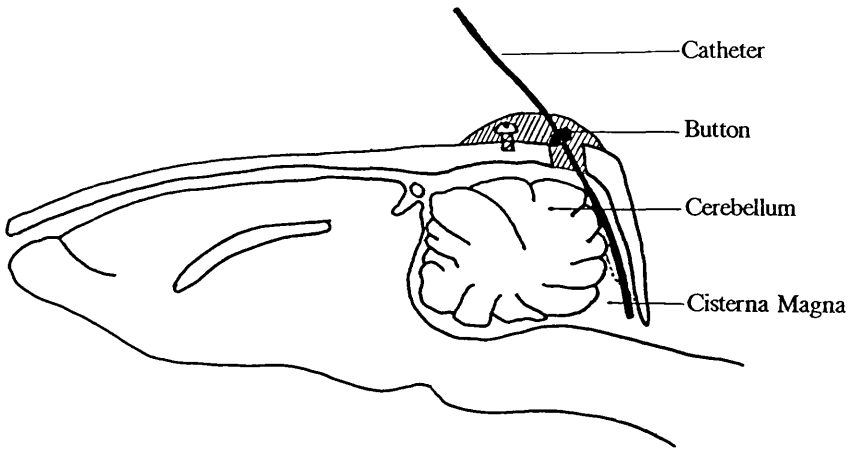


Figure 8.1 Placement of catheter for chronic CSF sampling in rats.

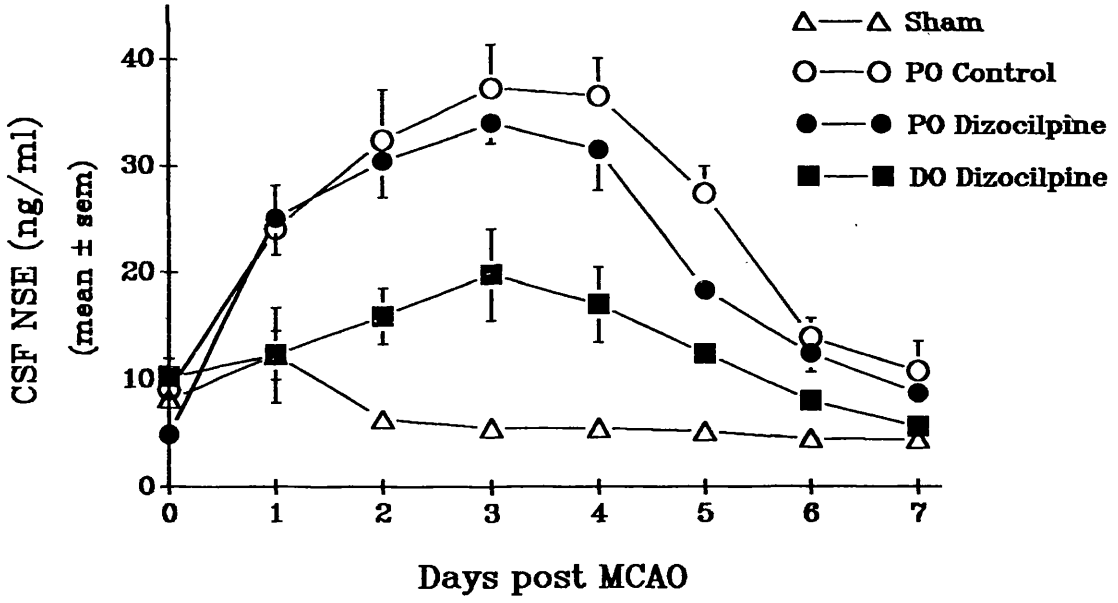


Figure 8.2 CSF Neuron-specific enolase (NSE) concentrations in sham animals and following proximal MCA occlusion (PO) (± dizocilpine) and distal MCA occlusion (DO) treated with dizocilpine (3mg/kg i.p., 30 min post MCAO). Data plotted as mean ± sem.

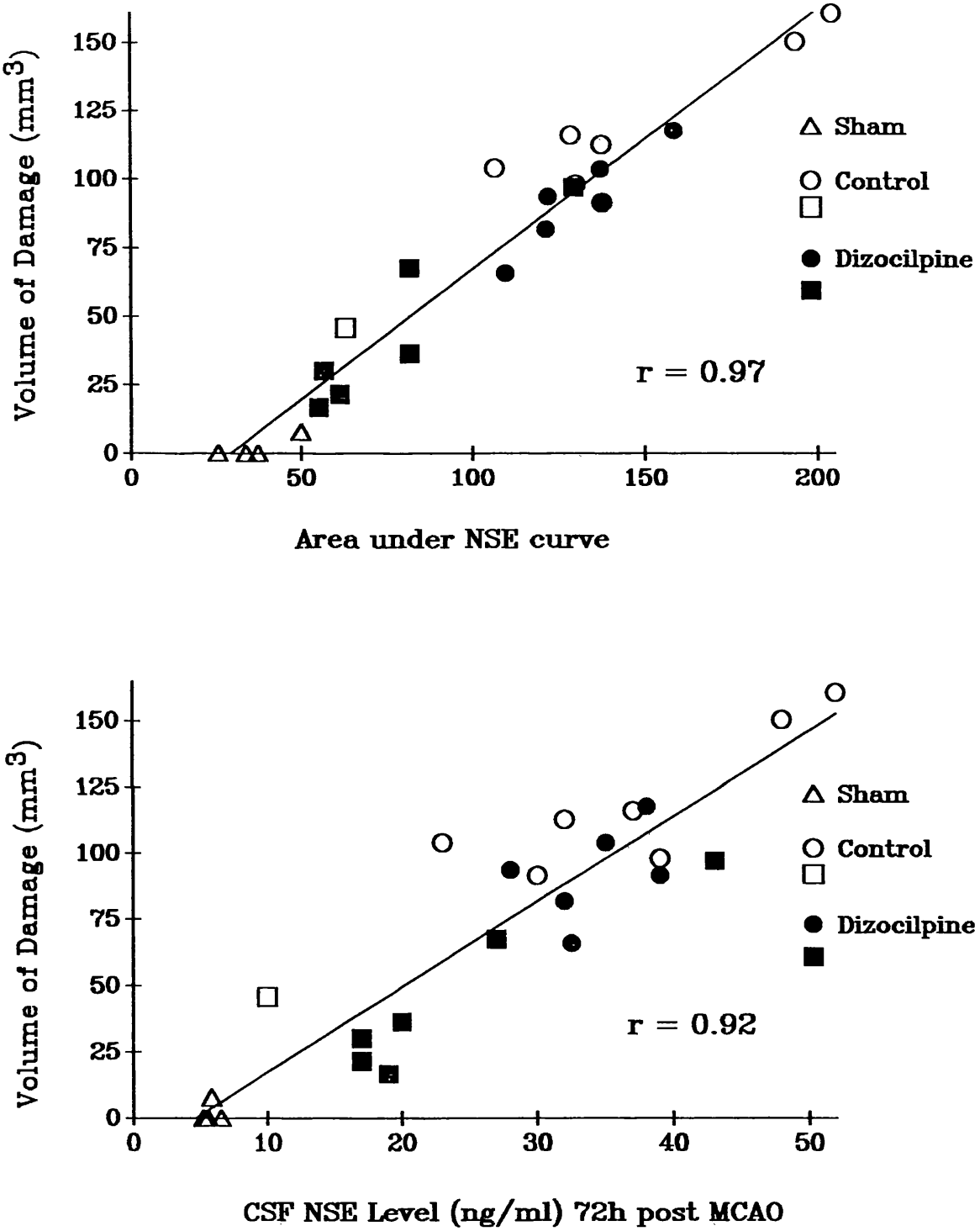


Figure 8.3 The volume of hemispheric damage, defined by histopathology at 7 days correlates well ($r = 0.97$, $p < 0.001$) with the integral of the CSF neuron-specific enolase (NSE) levels over 5 days post MCA occlusion (upper graph). The concentration of CSF NSE at 72h also correlates reasonably well ($r = 0.92$, $p < 0.01$) with lesion size at 7 days (lower graph).

8.1.4 Discussion

This study shows that measurements of NSE in the CSF can reflect the volume of ischaemic brain damage following permanent middle cerebral artery occlusion in the rat. This not only confirms the work of Hårdemark and colleagues (1988), but also demonstrates that the correlation between CSF NSE levels and the degree of brain damage in this model is maintained with pure cortical and mixed cortical/striatal lesions and with animals treated with the NMDA antagonist dizocilpine.

One of the major problems with the technique of CSF sampling used is the high blockage rate of the catheters, especially after MCA occlusion when the problem is likely to be exacerbated by cerebral oedema. Sarna and colleagues (1983), who originally described the technique, reported a blockage rate of 40% at three days after insertion in unoccluded rats, and the pre-occlusion blockage rate (at 2-3 days) in the present study (17%) compares favourably with this. The total blockage rate in this study at 5 days post MCA occlusion (ie 7-8 days post catheter insertion) was 55% which is very similar to the overall rate at 7 days of 53%, reported by Hårdemark et al. (1988). The high dropout rate causes major problems if one tries to compare treated with control animals in neuroprotection experiments, and hence this was not attempted in this small study.

Histopathological examination of infarcts following MCA occlusion in rats allowed to survive, demonstrates a close resemblance to those seen in man (Persson et al., 1989). Modern imaging techniques such as CT, magnetic resonance imaging and positron emission tomography can reveal important information concerning the status of the brain in ischaemic situations but they cannot differentiate between permanently damaged brain tissue and reversible damage or oedema, in the acute phase. It is also impossible to distinguish between permanent and reversible neurological deficit by early clinical assessment. Considerable research has been

done to try and find markers of ischaemic brain damage such as NSE and S-100 which may be clinically useful in the acute assessment of damage (Persson et al., 1987 & 1989; Hårdemark et al., 1989a&b; Hay et al., 1984; Takayasu et al., 1985; Roine et al., 1989). Markers that can quantitatively reflect the degree of acute damage could be of considerable value in the assessment of different treatment regimes, particularly in the evaluation of potential neuroprotective agents. Spontaneous "stroke" in man is a highly variable phenomena and assessment of outcome is difficult (see Adams et al., 1987). Hence large numbers of patients, usually in multicentre, double blind trials, are required to evaluate different therapies. Although ultimately, therapies must be shown to improve clinical outcome if they are to be regarded as effective, quantitative improvement in the degree of acute damage, might be easier to demonstrate and be important as a first step in the evaluation of a new therapy.

8.1.5 Conclusions

The present study provides further experimental evidence that release of NSE into the CSF, quantitatively reflects acute ischaemic brain damage and the size of the final infarct. The samples of CSF taken were approximately 20% of the total CSF volume, but this would have been replaced in less than 30 min, as the production rate of CSF in the adult rat is approximately 2 - 3 μl/min (Sarna et al., 1983). The concentration of NSE rose to peak levels approximately 3 days after the onset of ischaemia and the timing of this peak was not related to infarct size or treatment. A single measurement of NSE on day 3 correlated well with final infarct size, but the integral of NSE levels over 5 days gave a better estimation.

Chapter 9

Conclusions - NMDA antagonists and ischaemic brain damage

9.1 Conclusions

"Within the field of cerebrovascular research, one of the most voluminous and disparate literature concerns the therapy of experimental brain ischaemia" (MacKenzie et al., 1986). The events which follow the onset of cerebral ischaemia are extremely complex and dependent on the exact nature and duration of the ischaemic insult. Given the multiplicity of conditions which may lead to cerebral ischaemia, it is most unlikely that a single agent will be developed that is effective in treating all types of ischaemia and maintenance of tissue perfusion and oxygen delivery will always remain crucial.

In their review of the problems associated with the assessment of anti-ischaemic agents, written before the recent explosion of interest in NMDA antagonists, MacKenzie and colleagues came to three main conclusions. Firstly, focal ischaemia must be differentiated from global ischaemia and focal cerebral ischaemia, induced by middle cerebral artery occlusion, is probably the closest experimental approximation that exists to human stroke: by far the most clinically frequent cerebrovascular disease. Secondly, middle cerebral artery occlusion, especially in the rat using the technique of Tamura et al. (1981), is a useful approach but the only acceptable quantification of the resultant ischaemic damage is histology. Lastly, using their stringent criteria, there was little evidence at that time for pharmacological efficacy in the treatment of focal ischaemia, especially when agents were given after the onset of ischaemia.

This situation has now changed. The excitotoxic hypothesis of brain damage in ischaemia is now firmly established and this opens up new potential avenues for the treatment of ischaemia. Although models of global ischaemia were crucial to the development of the excitotoxic hypothesis, it is models of focal ischaemia which provide the clearest view of the clinical potential of NMDA antagonists. Still using the stringent criteria of MacKenzie et al. (1986), NMDA antagonists have proved

to be remarkably effective in the treatment of experimental focal ischaemia, not only in rats but also in mice, cats and rabbits (see table 1.1).

Although many of the studies showing efficacy for NMDA antagonists in focal ischaemia have used dizocilpine, few have looked at the crucial questions of the dose - effect relationship and the maximum time after the onset of ischaemia that therapy may be delayed. These questions are of fundamental importance in assessing the potential therapeutic usefulness of NMDA antagonists and are central to this thesis. No single model of cerebral ischaemia can hope to provide all the answers concerning a potential therapeutic agent prior to its introduction into clinical trials. In order to obtain dose response and therapeutic window information, the model must be reproducible and designed to facilitate large numbers of experiments. The recovery model of focal ischaemia in the rat described in this thesis fulfils these requirements.

Quantitative histological analysis is now widely accepted as a most important measure of the putative anti-ischaemic effects of therapeutic agents (Duverger & MacKenzie, 1988; Graham, 1988; McCulloch et al., 1990) but conventional histology is both time consuming and expensive. A method of volumetric analysis using triphenyltetrazolium chloride staining has been described, which is very much quicker and cheaper than conventional histology. It has been clearly shown that TTC staining in acute ischaemia is unreliable, particularly in neuroprotection experiments, and this appears to be due to the lack of delivery of the stain if the perfusion method is used and also the continued ability of mitochondria to oxidise TTC even after 3 - 4 h of ischaemia, as shown by the immersion experiments in chapter 5. At 24 h there is excellent correlation between TTC staining and histology in the assessment of focal ischaemic damage and TTC perfusion staining has been used to obtain dose response and therapeutic window information for dizocilpine

in this rat model of focal ischaemia.

Given the assumption that dizocilpine at a dose of 3 mg/kg i.p. produced a maximal effect (see chapter 6) the ED_{50} was approximately 0.3 mg/kg for an i.p. bolus given 30 min post MCA occlusion in this model, and this is in approximate agreement with data obtained from a model of focal ischaemia in the mouse (Gotti et al., 1990; ED_{50} dizocilpine = 0.2 mg/kg) and with data from a gerbil model of forebrain ischaemia (Gill et al., 1987a; dizocilpine ED_{50} = 0.3 mg/kg). The maximum time that therapy could be delayed and still produce a significant response was 1 h and this is also similar to data obtained from the mouse model, where significant protection was obtained with another NMDA antagonist SL 82.0715, after 45 min but not 90 min delay (Gotti et al., 1990). However, significant protection has been obtained with the NMDA antagonist PCP in a similar rat model of focal ischaemia with a delay of three hours following the onset of ischaemia (Bielenberg et al., 1989) and dizocilpine has produced a significant anti-ischaemic effect in an acute cat model after 2 h delay (Park et al., 1988b). The reasons for these differences are unclear at present but the important finding in all these experiments is that therapy can be significantly delayed and still be beneficial, and this is of crucial importance in the treatment of cerebral ischaemia in man.

Hypertension is a major risk factor in human stroke. Spontaneously hypertensive rats (SHRs), have elevated blood pressure, demonstrate some of the arteriopathic changes associated with hypertension in humans (Yamori 1976) and have a more consistent pattern of infarction following MCA occlusion (see chapter 7). Surely this is the ideal strain for neuroprotection experiments using rat models of focal ischaemia, but are NMDA antagonists less effective in this strain? SHRs are more susceptible to infarction than normotensive strains of rat, probably because of reduced collateral circulation in the brain supplied by the middle cerebral artery

(Coyle, 1986a & 1987). Reduced collateral circulation means the ischaemic penumbra will be smaller and this leads to more consistent infarction, but the smaller penumbra means there is less opportunity for drugs to act. In models of ischaemia a balance must be struck between reproducibility and sensitivity. As the penumbra is reduced the model becomes more reproducible but it also becomes less sensitive to drug effects. The experiments described in chapter 6 clearly demonstrate that dizocilpine can be just as effective in SHRs in terms of cortical protection as it is in normotensive animals, provided only a distal MCA occlusion is performed, leaving a sufficiently large penumbra for the drug to act.

Finally, the efficacy of CSF neuron-specific enolase (NSE) as a marker of neuronal damage in neuroprotection experiments has been explored. In this model of focal ischaemia there is an excellent correlation between CSF NSE levels and the volume of ischaemic damage, further supporting the idea that CSF NSE may be a clinically useful measure of ischaemic damage in trials of NMDA antagonists.

9.1.1 NMDA antagonists and the treatment of cerebral ischaemia in man

How can the dramatic neuroprotective efficacy that has been shown for NMDA antagonists in models of experimental ischaemia be translated into the clinical arena? Prior to clinical trials, a number of issues must be addressed.

Safety

The key question is whether doses of NMDA antagonists in man that are high enough to produce neuroprotective levels will be accompanied by an acceptable level of side effects. The non-competitive NMDA antagonist phencyclidine (PCP) has marked psychotomimetic properties and potentially all NMDA channel blocking drugs may suffer from similar problems. Dizocilpine has been shown to cause PCP like behaviourial effects in animals (Koek et al., 1988). However, it may

be possible to modulate the NMDA receptor via other sites, such as the glycine site, without causing such effects (Koek et al., 1990) and in any case, if neuroprotective benefit were proven, many short term, reversible side effects may be acceptable. Of rather more concern are the possible neurotoxic effects of NMDA antagonists (Olney et al., 1989), although the vacuolar changes observed by Olney and colleagues in specific areas of brain appear to be reversible and their exact significance has yet to be established. Another potential problem might be lasting changes relating to normal NMDA receptor function, such as long-term potentiation, causing possible interference with learning and memory function (Collingridge & Bliss, 1987). Again, if these effects were temporary and the anti-ischaemic effects proven, the side effects may be acceptable.

Patient Groups

Clinical trials are time consuming and expensive and results of previous trials of potential anti-ischaemic compounds have been largely disappointing (Yatsu et al., 1987; Millikan, 1985; Barnett, 1987) and have not affected clinical practice. The one major exception so far is the use of the calcium antagonist nimodipine in the treatment of delayed ischaemia following subarachnoid haemorrhage (SAH). Nimodipine is now routinely used in the majority of neurosurgical centres for the prophylaxis of delayed ischaemia in SAH following the unequivocal proof of its efficacy in a well designed, placebo-controlled, double-blind, multi-centre trial (the BRANT study, Pickard et al., 1989). Nimodipine may also be beneficial in occlusive stroke (Gelmers et al., 1988) but trials so far have been rather small and results more controversial. In order to avoid as far as possible further negative or equivocal results, prior to testing NMDA antagonists in man, considerable thought must be given as to which group of patients are most likely to benefit from therapy and in particular, which group would be most appropriate for the first clinical trial.

The results of experiments involving NMDA antagonists in models of global ischaemia remain controversial (Buchan, 1990) and it may be that NMDA antagonists are only beneficial in conditions of partial energy failure, such as the penumbra in focal ischaemia, but not when there is complete energy failure, as in global ischaemia (Wieloch et al., 1989). Undoubtably the most consistent demonstrations of the efficacy of NMDA antagonists as neuroprotective agents have been in models of focal ischaemia (see table 1.1), and it would seem logical to first apply them to clinical situations involving focal ischaemia.

At first sight a trial in occlusive stroke might seem the obvious choice but although stroke must remain the ultimate clinical target for anti-ischaemic therapy, in view of its high incidence, prevalence and morbidity (see chapter 1), there are several problems associated with stroke trials. Many patients are not hospitalised following occlusive stroke in the U.K. at present and very few are admitted within hours of the ictus (Bamford et al., 1988). Early diagnosis may be difficult, even using modern imaging techniques, and transient ischaemic attacks (TIAs) may be diagnosed as stroke. The incidence of stroke is highly age dependant and outcome in the elderly may depend on other cardiovascular factors such as myocardial ischaemia. Finally, in view of the heterogeneity of the condition, large numbers of patients are required to show a beneficial effect.

A first trial of NMDA antagonists in severe head injury?

McCulloch, Bullock and Teasdale (1990) make a strong case for first submitting NMDA antagonists for trial in patients with severe head injuries. Their argument for this is based both on experimental data and practical clinical considerations. Nearly all severely head injured patients have focal cerebral contusions and ischaemic damage plays a crucial role in its pathophysiology (Graham et al., 1978 &

1989). In experimental fluid-percussion injury glutamate induced damage can be ameliorated by NMDA antagonists (Jenkins et al., 1989; McIntosh et al., 1989) and there is further experimental evidence that the ischaemia due to subdural haematoma, one of the commonest complications of severe head injury, can be ameliorated by NMDA antagonists (Bullock et al., 1990c). From the practical point of view, the infrastructure already exists for the intensive management of severely head injured patients. They are routinely admitted within an hour or so of injury to neurosurgical units for assessment and intensive care, which is the ideal environment for trial of a new agent where side effects and progress can be carefully monitored. Finally, head injury remains a major health problem which mainly affects young people and an improvement in outcome would be of considerable benefit to society.

For these reasons it would seem logical to consider a trial of NMDA antagonists in head injured patients, when a suitable agent becomes available.

9.1.2 Claims to originality and contribution to the science of medicine

Triphenyltetrazolium chloride (TTC) has been used as a marker of ischaemic damage in experimental brain ischaemia in several previously reported studies and some of its limitations have been noted. However, the perfusion method has not previously been directly compared to histology in both acute and chronic ischaemia, in particular in experiments involving potential neuroprotective agents, where any degree of flow dependence of a stain may be crucial, as drugs will be exerting their effects in regions of low flow. The immersion and perfusion methods of TTC staining have not previously been compared. These experiments have led to the important conclusions that mitochondria are still capable of oxidizing TTC after several hours

of focal ischaemia and with the perfusion method in acute ischaemia, TTC is probably acting as a flow marker. The effect of dizocilpine in acute and chronic focal ischaemia has not been compared directly previously.

When the experimental work was begun in 1988, there were virtually no published reports of the efficacy of NMDA antagonists in recovery models of focal ischaemia. The dose response and therapeutic window remain the most comprehensive data for dizocilpine in a rat recovery model of focal ischaemia.

There have been no previously published reports of a direct comparison of the efficacy of an NMDA antagonist in normotensive and hypertensive animals with distal and proximal MCA occlusions and these experiments have led to the conclusion that dizocilpine can have beneficial anti-ischaemic effects in hypertensive animals, provided sufficient collateral circulation is left intact.

Neuron-specific enolase levels have previously been measured in rat CSF following MCA occlusion and correlated with the degree of ischaemic damage (Hårdemark et al., 1988). However, the results from very few (4) occluded animals were reported, the lesions were all proximal occlusions and no neuroprotective agents were given. This study adds to the experimental evidence that neuron-specific enolase may be a clinically useful marker of ischaemic damage.

Overall the thesis adds to the rapidly accumulating experimental evidence that NMDA antagonists can ameliorate focal ischaemic brain damage and potentially these may be the most useful agents so far discovered for the treatment of focal cerebral ischaemia in man. However, their efficacy has yet to be proved in clinical trials.

Appendix 1

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