THE NEUROPROTECTIVE EFFECTS OF A KAPPA-OPIOID AGONIST AND ITS MECHANISTIC BASIS

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SUMMARY

The main aims of this thesis were to investigate the effects of the potent and selective *k*-opioid agonist CI-977 in animal models of focal cerebral ischaemia, and gain insight into a possible mechanism of action underlying the anti-ischaemic efficacy of *k*-agonists. Quantitative neuropathology was used to determine the effect of CI-977 on the volume of ischaemic brain damage after permanent middle cerebral (MCA) occlusion in the rat (outcome assessed at 24h and 4h) and cat (outcome assessed at 6h). Microdialysis techniques were employed *in vivo* to examine the effects of CI-977 on glutamate release after MCA occlusion in the cat and also on neuronal necrosis induced by exogenous glutamate perfused into the rat cerebral cortex by reverse dialysis. The cerebral circulatory effects of CI-977 were examined in the rat using [^{14}C]-iodoantipyrine autoradiography in normal brain and after permanent MCA occlusion, and in the cat after the induction of focal ischaemia using the hydrogen clearance technique. [^{14}C]-2-Deoxyglucose *in vivo* autoradiography was employed to assess the functional consequences as reflected in alterations in local rates of cerebral glucose utilisation in discrete brain regions in conscious rats following the systemic administration of CI-977.

The administration of CI-977 (0.03, 0.3 or 3mg/kg, s.c.) initiated 30 min prior to occlusion of the MCA in the rat (and at multiple times thereafter) produced dose-dependent reductions in the volumes of infarction and of brain swelling when assessed at 24h. The most marked reductions were noted with CI-977 (0.3mg/kg) in both infarction (reduced by 38% from controls) and swelling (reduced by 31%). There was an excellent correlation between the volume of brain swelling and ischaemic damage which was similar for vehicle-treated and CI-977-treated animals (overall correlation coefficient r=0.896). Treatment with CI-977 (0.3mg/kg, s.c.) 30 min before and 30 min after permanent MCA occlusion in halothane-anaesthetised rats significantly reduced the volume of infarction in the cerebral

hemisphere (reduced by 27%) and cerebral cortex (reduced by 32%) despite a marked and sustained hypotension, with only minimal effect on ischaemic damage in the caudate nucleus. Pretreatment with CI-977 (0.3mg/kg i.v. bolus followed by continuous i.v. infusion of 0.15mg/kg/h until death) initiated 15 min prior to MCA occlusion significantly reduced the volume of ischaemic brain damage in the cerebral hemisphere (reduced by 33%) and cerebral cortex (reduced by 42%) in halothane-anaesthetised cats without altering significantly any of the key physiological parameters monitored throughout the post-occlusion survival period.

In a parallel study, the effects of CI-977 (0.3mg/kg, s.c.) on cerebral blood flow were examined 30 min after the induction of ischaemia in halothane-anaesthetised rats. In the hemisphere contralateral to the occluded MCA, pretreatment with CI-977 failed to demonstrate any significant effect on the level of local cerebral blood flow in any of the 25 regions examined. In the hemisphere ipsilateral to MCA occlusion, CI-977 failed to produce statistically significant alterations in either the level of local cerebral blood flow in 23 of the 25 regions or on the volume of hypoperfused tissue determined by frequency distribution analysis. Areas of hyperaemia were observed in CI-977-treated rats with MCA occlusion in both the medial caudate nucleus and lateral thalamus (local cerebral blood flow increased by 65% and 86% respectively compared to vehicle). In an *in vivo* model of glutamate neurotoxicity in anaesthetised rats, pre-and post-treatment with CI-977 (0.3mg/kg, i.v.) had no significant effect on the volume of neuronal necrosis assessed 4h after the onset of glutamate (0.5M) perfusion into the parietal cortex; the NMDA antagonist MK-801 (0.5mg/kg) and the AMPA antagonists (2 x 30mg/kg) significantly reduced the lesion volume by 30% and 23% respectively. Permanent occlusion of the MCA in halothane-anaesthetised cats produced a marked increase in extracellular glutamate levels, determined by microdialysis, when cerebral blood flow fell to a threshold value of approximately 20ml $100g^{-1}$ min^{-1} in vehicle-treated

control animals. The administration of CI-977 (0.3mg/kg i.v. plus an i.v. infusion of 0.15mg/kg/h) 30 min prior to the induction of ischaemia produced no evidence of such a threshold for glutamate release in the ischaemic hemisphere, and markedly attenuated the increase in extracellular glutamate concentration at cerebral blood flow values of less than 20ml $100g^{-1}$ min^{-1}.

The effects of CI-977 upon local cerebral glucose utilisation were examined in the normal brain of conscious, lightly restrained rats. The i.v. administration of CI-977 (0.03, 0.3 or 3mg/kg) induced relatively homogenous patterns of altered cerebral glucose utilisation with moderate statistically significant reductions (approximately 25%) being observed in 29 of the 45 brain regions, and a significant increase (approximately 40%) in one brain region, the lateral habenular nucleus. Glucose use throughout the entire neocortex and inferior colliculus was particularly sensitive to reduction (approximately 35%) following CI-977 administration, although there was only a limited dose-dependency to the response. Minimal alterations in glucose use were observed in 15 brain regions, which were located predominantely in the lower brain stem and forebrain limbic regions.

CI-977 (0.3mg/kg, i.v.) induced minimal cerebral circulatory effects in conscious rats. A significant reduction in local cerebral blood flow was observed in only one brain area, the posterior entorhinal cortex (I). In 44 of the 45 regions examined, local cerebral blood flow was not altered significantly with CI-977 treatment, and no significant increases in cerebral blood flow were noted. Cerebral blood flow remained tightly coupled to oxidative metabolism in the brain regions examined and there was no clear evidence that cerebral blood flow and glucose use could be uncoupled locally, numerically and neuroanatomically after CI-977 administration.

The results of the studies within this thesis suggest that a major component of the mechanism of action which underlies the anti-ischaemic efficacy of CI-977 in animal models of ischaemia is a presynaptic inhibition of glutamate release. Thus

k-opioid agonists such as CI-977 may be of benefit therapeutically for modulating excitotoxic brain damage in cerebral ischaemia in man, and their use is unlikely to be complicated by concerns over CNS safety as with NMDA receptor antagonists.

The investigations in this thesis were conducted in three broadly defined areas:

(1) To examine the neuroprotective effect of the *k*-opioid receptor agonist CI-977 following permanent MCA occlusion in the rat and cat.

(2) To establish a possible mechanism of action underpinning the anti-ischaemic efficacy of CI-977 in animal models of focal cerebral ischaemia.

(3) To assess the functional and cerebral circulatory consequences, as reflected in local cerebral glucose use and local cerebral blood flow respectively of CI-977 in normal rat brain.

This thesis comprises my own original work and has not been presented previously as a thesis in any form. Some of the experiments reported in this thesis were carried out in collaboration with other researchers: Dr. K. Kusumoto performed permanent MCA occlusions in the rat and cat neuropathology studies; the glutamate neurotoxicity *in vivo* study was conducted with Dr. H. Fujisawa; the glutamate release/cerebral blood flow study in the cat was carried out in conjunction with Mr. S. Galbraith and Mr. T.R. Patel.

CHAPTER I

INTRODUCTION

1. CEREBROVASCULAR DISEASE IN MAN

Cerebrovascular disease represents a major cause of disability and mortality in the industrialised world today. Prospective epidemiological studies have identified cerebrovascular disease (cerebral ischaemia) as the third most common cause of death in the Western world, surpassed only by heart disease and cancer, and also the major cause of severe functional incapacity in the adult population (Camarata et al., 1994). It has been estimated that approximately 500,000 people in the U.K. are currently severely disabled by the neurological consequences of cerebral ischaemia (Bamford et al., 1990). The frequency of cerebrovascular disease in modern society and the severity of its sequelae serves to highlight the importance of understanding the pathophysiology of cerebral ischaemia and the necessity to develop effective therapeutic agents for use in the clinic to improve quality of life.

1.1 Stroke and Ischaemic Brain Damage

"Stroke" is the principal clinical manifestation of focal cerebral ischaemic brain damage in man. The worldwide annual incidence of first stroke is estimated at between 150-200 cases per 100,000 population, with mortality in approximately 40% of these patients (Camarata et al., 1994). The underlying trend is age-dependent, with the incidence more than doubling each decade after the age of 55 (Bamford et al., 1990). Those who survive their first stroke are not only often severely disabled, but are at a greatly increased risk of further ischaemic episodes (Haberman, 1984).

Focal ischaemic damage or stroke can be defined as a reduction in cerebral blood flow to a discrete brain area resulting in the abrupt and dramatic development of a focal neurological deficit. Strokes are either

occlusive (due to thrombi or emboli) or haemorrhagic (due to bleeding from a vessel). Haemorrhage may occur at the brain surface (extraparenchymal) e.g. from rupture of vessels damaged by long-standing hypertension, resulting in haematomas within the brain.

Evidence from several large stroke registries indicate that up to 85% of all strokes are thromboembolic in origin and up to 23% of these are cardioembolic (Mohr et al., 1978). About 10-15% of strokes are caused by intracerebral haemorrhage, predominantly in patients 55-75 years old (Foulkes et al., 1988; Mohr et al., 1978). Most of these haemorrhages occur in patients with hypertensive arteriopathy and are located most frequently in the basal ganglia and thalamus, deep hemispheric white matter, cerebellum and the brainstem. Other causes include haemorrhage from arteriovenous malformations and aneurysms, amyloid angiopathy, tumours, vasculitis and coagulopathies. Outcome after intracerebral haemorrhage is poor, with approximately 50% of all patients dead within one year of the initial event (Camarata et al., 1994).

Aneurysmal subarachnoid haemorrhage accounts for about 10% of all first strokes and is more common than primary intracerebral haemorrhage in young adults (Kurtzke, 1985). Of all patients who arrive at the clinic in relatively good condition after subarachnoid haemorrhage, 28% will die within the first 6 months and only 58% will have a favourable outcome (Kassel et al., 1990). In both subarachnoid haemorrhage and intracerebral haemorrhage, secondary, delayed ischaemic brain damage is a crucial determinant of eventual outcome.

The CNS is extremely susceptible to reductions in cerebral blood flow. The final extent of neuronal damage and potential for reversal by pharmacotherapy is related to the severity of reduction of cerebral blood

flow and the duration of the hypoperfusion. A threshold of 17ml/100g/min, or 35% of the basal level of cerebral blood flow, has been found in sustained ischaemia (Jones et al., 1981). Thus, irrespective of whether the primary cause is an occlusive or haemorrhagic stroke, the ultimate consequences of a severe and sustained reduction in cerebral blood flow are the same - irreversible damage to neurones followed by infarction involving all cellular elements, and the production of attendant neurological dysfunctions.

Ischaemic damage is also a feature of head injury and brain trauma. It has been shown that in more than 85% of fatalities resulting from severe head injuries ischaemic brain damage was found at postmortem (Graham et al., 1978). Furthermore, 40% of these patients had spoken at some time after the initial injury on admission to the clinic, which is indicative of delayed secondary brain damage. In addition, clinical studies show that the disability found in many survivors of head injuries are a consequence of delayed ischaemic damage (Bullock & Teasdale, 1990). These findings suggest that these patients may benefit from a neuroprotective agent which reduces or prevents the causes of secondary brain damage. The impact of brain trauma in society is reflected in that it is the leading cause of death under the age of 45 in the Western world, and is the leading cause of premature male mortality, predominantly as a result of ischaemic brain damage incurred after severe head injury (Rockett & Smith, 1987).

1.2 Cerebral Ischaemia in Man and Therapeutic Approaches

A number of premorbid factors presaging stroke have been identified. These include the individually less well established factors such as smoking, alcohol consumption, blood cholesterol level, and obesity, all of which are related to the development of the most important predisposing factors -

atheroma and hypertension (Malmgren et al., 1989). Other major contributing factors are heart disease, diabetes mellitus, and transient ischaemic attacks (TIAs) (Malmgren et al., 1989). Thus, increasing attention is being paid to the identification of patients at risk of stroke, so that appropriate preventative measures, such as the early recognition and treatment of hypertension, can be instituted.

Although there has been an increasing awareness of the need to eliminate factors which predispose an individual to stroke, there is at present no effective neuroprotective agent for use in the clinic to ameliorate the consequences of an ischaemic stroke. Stroke therapy can be directed at a wide range of pathophysiological mechanisms, and there are currently a number of compounds in clinical trials and their results are eagerly awaited (Lipton & Rosenberg, 1994).

The dihydropyridine voltage-sensitive channel antagonist nimodipine, which putatively improves the level of cerebral blood flow to ischaemic tissue (Mohamed et al., 1985), is of clear benefit to subarachnoid patients who are at high risk of delayed ischaemic brain damage due to vasospasm and reduced cerebral blood flow (Pickard et al., 1989). Initial small clinical trials suggested no clear benefit in ischaemic stroke with nimodipine (Gelmers et al., 1988). However, in a multi-centre stroke trial nimodipine showed improvement in a subgroup of patients treated within 12 to 18 hrs after the insult (The American Nimodipine Study Group, 1992).

Several competitive and non-competitive NMDA receptor antagonists, which markedly reduce ischaemic lesion size in animal models of stroke (see McCulloch et al., 1991 for review) are now in clinical trial. Dextromethorphan and memantine are non-competitive NMDA antagonists which can be given orally and have a tolerable side effect profile (Albers et

al., 1991; Chen et al., 1992b). Felbamate, a new anti-convulsant, is a glycine site antagonist with neuroprotective properties (McCabe et al., 1993). The long-term safety of felbamate appears to be acceptable, and the beneficial effects are obtained at well-tolerated doses (White et al., 1992). In addition, CGS-19755 has recently been shown to have tolerable neurological side effects in acute stroke patients (Clarke & Coull, 1994). Thus, these agents, as well as other NMDA antagonists may offer both efficacy and safety for the treatment of stroke.

The ganglioside GM-1 is in clinical trials as an anti-ischaemic agent. In an Italian multi-centre study, treatment with GM-1 within 12h of the onset of stroke produced a significant improvement in the early neurological scores, but by 4 months after the onset of stroke, a non-significant trend in favour of treatment was only observed (Argentino et al., 1989). Studies are currently ongoing to see whether GM-1 might be effective in improving outcome after ischaemic stroke if given early in the course of ischaemia and perhaps for a larger period (Camarata et al., 1994).

The 21-aminosteroids are potent antioxidants which reduce ischaemic damage in animal models of cerebral ischaemia without glucocorticoid or mineralocorticoid side effects (Umemura et al., 1994). Tirilazad is currently undergoing Phase 3 (multi-centre study of efficacy) trial in patients with stroke (Lipton & Rosenberg, 1994). Thus, lipid peroxidase inhibitors and free radical scavengers may offer a potential mechanism for neuroprotection in stroke.

There are enormous financial and research resources being devoted to anti-ischaemic drug development. The importance of cerebral ischaemic brain damage in clinical practice has stimulated the development of many animal models to produce experimentally an ischaemic lesion resembling that

seen in man, and thereby investigate the neuroprotective efficacy of pharmacologic agents. Thus, given the intimate relationship between *k*-opioid systems and excitatory amino acid neurotransmission in the CNS, part of this thesis is devoted to evaluate systematically the anti-ischaemic efficacy of a novel and selective *k*-opioid agonist in experimental models of focal cerebral ischaemia.

2. ANIMAL MODELS OF CEREBRAL ISCHAEMIA

2.1 General Features

In order to systematically investigate the pathophysiology and pharmacotherapy of cerebral ischaemia, reproducible *in vivo* animal models have been developed. There are several advantages in using animal models of cerebral ischaemia. These include: 1) the use of standardised animal strains with no pre-existing disease and uniform cerebrovascular anatomy; 2) control over the severity and duration of the ischaemic episode; 3) direct and instant access to brain tissue for biochemical and neuropathological analyses; 4) the presence of cerebral vasculature for the study of abnormal tissue perfusion, integral in the genesis of ischaemic brain damage, and a feature lacking in *in vitro* models such as brain slices or neuronal/glial cell cultures.

A diversity of animal species, ranging in size from small rodents to large subhuman primates have been used in experimental models of cerebral ischaemia (Ginsberg & Busto, 1989; Hossmann, 1991). However rodent models, particularly the rat, are now used most extensively for a number of pertinent reasons. These include the close resemblance of the cerebrovascular anatomy to that of higher species (Yamori et al., 1976), the detailed information available concerning the anatomical localisation of

neurotransmitter systems, their neurochemistry and neuropharmacology, the development of a range of quantitative autoradiographic techniques to study various dynamic aspects of cerebrovascular function in the rat (e.g. cerebral glucose utilisation and cerebral blood flow), and the greater acceptability in using small, low cost animals when compared to, for example, subhuman primates, from both ecologic and ethical perspectives. However higher species, most notably the cat, will always have an essential contribution to make in the development of anti-ischaemic drugs as large animal models of ischaemia for a number of reasons which will be discussed later (Section 2.3).

Animal models of cerebral ischaemia can be broadly classified into two groups: 1) global or forebrain ischaemia, where there is a transient reduction or cessation of total cerebral blood flow, and 2) focal ischaemia, where blood flow to localised brain areas is compromised.

2.2 Global Cerebral Ischaemia

Models of global ischaemia produce severe reductions in total cerebral blood flow which affect widespread areas of the CNS. The ischaemia is transient (5-30 min) and followed by often prolonged periods of reperfusion (1-7 days). This results in a pattern of diffuse neuronal necrosis within selectively vulnerable areas of the brain, e.g. CA1 region of the hippocampus, neocortex and striatum. The delayed appearance of morphological evidence of irreversible cell death in the hippocampus in models of global ischaemia has raised fundamental questions with regard to its relevance to human stroke. Indeed, delayed cell death has been reported in the human hippocampus after cardiac arrest (Petito et al., 1987), thereby suggesting that models of global ischaemia are most pertinent to cardiac arrest in man.

There are several methods for inducing global cerebral ischaemia in

large animal models (Hossmann, 1991) and almost all are associated with considerable interanimal variability in outcome after a similar ischaemic insult. The species most commonly used in models of transient global ischaemia are the Mongolian gerbil (*Meriones unguiculatus*) and the rat.

Anatomically the gerbil is unique in that it has an incomplete circle of Willis (Levy & Brierley, 1974). Thus global ischaemia can be induced in the gerbil by temporary unilateral or bilateral common carotid artery occlusion (Clifton et al., 1989). This results in selective neuronal necrosis in pyramidal cells of the hippocampal CA1-CA2 subfield. The technical ease of inducing ischaemia in the gerbil is far outweighed by its limitations and on these grounds this model of global ischaemia has been the subject of severe criticism. Hippocampal neurones undergo delayed neurodegeneration (Kirino, 1982) and gerbils are highly susceptible to seizures and hypothermia. As such, it has been proposed that drugs which are neuroprotective in this model may act via hypothermic or anti-convulsant actions, and not by their recognised pharmacological mechanism (Buchan & Pulsinelli, 1990). Moreover, gerbils are so small (50-80g) that physiologic monitoring procedures routinely possible in the rat become technically difficult (e.g. vessel cannulation) or physiologically destabilising (e.g. repeated blood sampling as is required for autoradiographic tracer studies). Therefore crucial physiological variables often go unmonitored in gerbil global ischaemia studies, and consequently this model is of limited value in evaluating anti-ischaemic drug efficacy.

Two models of global cerebral ischaemia predominate in the rat, the two-vessel and four-vessel occlusion models of transient, forebrain ischaemia. In the two-vessel model, originally described by Eklöf and Siesjö in 1972, high-grade forebrain ischaemia is produced by ligation of both common

carotid arteries either permanently, or more usually, transiently for periods of 10-60 min. In addition, this is usually combined with systemic hypotension induced by controlled arterial haemorrhage (Kågström et al., 1983) which is required to reduce cerebral blood flow sufficiently in order to obtain reproducible ischaemic brain damage (Smith et al., 1984). The four-vessel occlusion model involves permanent bilateral occlusion of the vertebral arteries by electrocoagulation (under anaesthesia) followed by, usually 24 hours later, transient bilateral occlusion of the carotid arteries which can be performed in conscious, freely moving rats (Pulsinelli & Brierley, 1979).

The two- and four-vessel models of transient, global ischaemia give rise to ischaemic cell change within selectively vulnerable areas of the brain, including the CA1 pyramidal neurones of the hippocampus, neocortex and striatum (Pulsinelli et al., 1982; Smith et al., 1984). There are reports, however, of inconsistent patterns of ischaemic damage produced between laboratories in both models (see Ginsberg & Busto, 1989 for review). This variability in outcome has been attributed to variations in the collateral blood supply both between and within strains of rat, and a failure to maintain brain temperature following the induction of ischaemia (Ginsberg & Busto, 1989), as moderate reductions in brain temperature (2°C) have been shown to attenuate neuronal necrosis in these animal models of ischaemia (Busto et al., 1987).

2.3 Focal Cerebral Ischaemia

The middle cerebral artery (MCA) is reported to be the vessel most commonly affected in stroke syndromes (Karpiak et al., 1989), and thus animal models of focal ischaemia are believed to be the most pertinent in relation to human stroke. The most widely employed models of focal

cerebral ischaemia employ a permanent or temporary occlusion of a major intracerebral artery, normally the MCA. This produces localised pannecrosis or infarction of the territory supplied by the artery, i.e. the neocortex and striatum, with the final lesion size fully established after approximately 3 hours of ischaemia (Jones et al., 1981; Kaplan et al., 1991). Although models of MCA occlusion were developed originally in large animals such as cats (O'Brien & Waltz, 1973), dogs (Suzuki et al., 1980) and primates (Hudgins & Garcia, 1970), rat models of MCA occlusion have emerged as the most extensively used in studies of the pathophysiology and drug effects in cerebral ischaemia.

The original description of MCA occlusion in the rat described a distal occlusion of the MCA by ligation via a large frontoparietal craniectomy (Robinson et al., 1975). This resulted in a relatively small and highly variable lesion size which was restricted to the cerebral cortex. Subsequent refinements in surgical procedure include a subtemporal approach to the MCA (Tamura et al., 1981a), leaving the zygomatic arch intact (Shigeno et al., 1985a), thereby allowing greater access to the origin of the MCA. Indeed this subtemporal approach with diathermy occlusion has emerged as the standard model of permanent proximal MCA occlusion in the rat, where the MCA is occluded with microbipolar coagulation from its origin to the point where it crosses the inferior cerebral vein, and severed proximal to the lenticulostriate arteries. Subsequent variations in this rat model include: a more distal occlusion to restrict infarction to the cortex (Shigeno et al., 1985a), tandem occlusion of the MCA and ipsilateral common carotid artery (Brint et al., 1988) to improve reproducibility of infarction, and alternative methods of MCA occlusion using microclips, hooks, ligature snares or the abluminal application of endothelin-1 which allow reversal of the occlusion

(Dietrich et al., 1989; Kaplan et al., 1991; Shigeno et al., 1985b; Macrae et al., 1993).

Although rat models of focal cerebral ischaemia are predominantly used in preference to models in higher species (Section 2.1), large animal models, in particular the cat as used in this thesis, are integral to the development of any anti-ischaemic drug. Gyrencephalic species must be examined as the ultimate target is a gyrencephalic species (man) rather than a lissencephalic species (such as the rat). Like man, the cat has a gyrencephalic brain. There has long been concern that the absence of sulci in rodents exaggerates the importance of the wave of spreading depression initiated at the onset of ischaemia. It has been argued that a neuroprotective drug may simply block this electrical depolarisation and thereby reduces ischaemic damage (Gill et al., 1992a; Iijima et al., 1992). Thus, as a result of the general similarity in the cortical architecture of the cat and man, any such indirect mechanism (i.e. attenuating spreading depression) can be discounted as having a disproportionate impact on the conclusions of a study.

The cat also has greater stability than the rat under anaesthesia. In studies with acute neuropathology as an endpoint, at least 3 hours must elapse from the induction of ischaemia for the characteristic histological features of irreversible brain damage to mature (Kaplan et al., 1991). During this period, cardiovascular and respiratory stability must be maintained despite repetitive sampling of blood for blood gas analysis and determination of plasma drug concentration etc., and despite tracheal and bronchial secretions. The degree of cardiovascular and respiratory stability which can be achieved in the anaesthetised cat over several hours is considerably greater than in even the best, well controlled anaesthetised rat. This contributes, therefore, to the certainty placed on cat (and other large animals) studies

with regard to the neuroprotective effects of a drug.

The physical size of the cat brain also underpins the certainty of conclusions derived in investigations of the anti-ischaemic efficacy of a drug. All intracranial interventions produce trauma at the surgical site which in turn produces local tissue damage and more widespread disturbances of vascular reactivity. Such surgical trauma has a quite different pharmacology to ischaemic pathology. In the cat, surgical trauma constitutes a small fraction of the total ischaemic lesion, whereas in the rat, it may comprise up to 15-20% of the total lesion. Furthermore, the crucial ischaemic penumbra and potentially salvageable areas are approximately 25mm from the surgical site in the cat whereas they are only about 4mm distant in the rat. The absolute amount of the ischaemic lesion which is potentially salvageable with neuroprotective drugs is considerably greater in cats than in rats. Not only does this eliminate concern of measurement error or bias from studies in cat, but the use of the cat also facilitates the analysis of drugs which may have small neuroprotective effects, but which are intrinsically safer.

The nature of the focal lesion after occlusion of the MCA is such that it lends itself to volumetric assessment (Osborne et al., 1987) which is much more reliable than assessing the area of ischaemic damage in a few coronal planes (see Mohamed et al., 1985; Park et al., 1988b as examples of how non-volumetric assessment could give rise to false negative drug effects or gross overestimation of efficacy depending on the coronal plane studied.) Models of focal cerebral ischaemia, particularly in the rat and the cat, are becoming increasingly dominant in anti-ischaemic drug development, not just because of their relevance to human stroke but also partly because of the absolute consistency of data which has emerged on the anti-ischaemic efficacy of the NMDA antagonists (see McCulloch, 1992; Meldrum, 1990 for reviews).

2.4 Sources of Variability

The major difficulty encountered when using animal models of cerebral ischaemia in studies of neuroprotective drugs is controlling the level of variability in outcome. A number of different parameters, if not rigorously controlled, can confound the validity of the model and eventual outcome by exacerbating variability. These include:

Temperature

Hypothermia, of even 2°C, has been shown to attenuate ischaemic neuronal necrosis in models of global ischaemia (Busto et al., 1987). In focal ischaemia, mild brain hypothermia (4°C) reduces the volume of infarction (by 48%) following transient but not permanent MCA occlusion (Ridenour et al., 1992). Temperature should therefore be closely monitored and maintained, particularly in brain tissue which has been surgically exposed, and in the whole animal during early recovery from anaesthesia.

Plasma Glucose Concentrations

Hyperglycaemia is known to increase ischaemic brain damage in models of global and focal cerebral ischaemia (Marie & Bralet, 1991). Three-fold increases in plasma glucose increase lesion size by up to 25% after permanent MCA occlusion in the rat (Duverger & MacKenzie, 1988). Thus, monitoring of plasma glucose levels and if necessary overnight fasting of animals should be built into the study design.

Physiological Variables

Blood pressure and respiratory blood gas status (pCO_2, $p0_2$ and pH) outwith the normal limits of autoregulation will influence global cerebral

blood flow, and hence have a major impact on the degree of ischaemic brain injury. For example, transient hypotension (reduced to 60mmHg) in the post-ischaemic period has been shown to increase the volume of infarction by more than 50% in a rat model of focal ischaemia (Osborne et al.,1987). These parameters should therefore be closely monitored, particularly in anaesthetised animals where depth of anaesthesia and artificial respiratory rate represent further sources of variability.

General anaesthesia is a common feature in animal models of cerebral ischaemia, most notably those of focal ischaemia where full monitoring of physiological variables is performed throughout the post-ischaemic period. Anaesthesia can modify cerebrovascular reactivity and the demands for substrate (oxygen and glucose) in discrete regions of the CNS. Therefore, anaesthesia may have an effect on the degree of ischaemic damage, thereby introducing variability to the experimental model. Moreover, the possible interaction between any anaesthetic and the anti-ischaemic drug under investigation (synergistic or antagonistic) in relation to observed neuroprotection is always of concern (Ginsberg & Busto, 1989; Meldrum, 1990).

Surgical reproducibility is another potential source of variability in outcome in animal models of ischaemia. In the permanent MCA occlusion model in the rat, the incidence and extent of focal ischaemic infarction is dependent on both the location and the extent of the diathermy lesion to the MCA (Bederson et al., 1986). In addition the strain of rat used appears to be an important factor. Following proximal, permanent MCA occlusion in 5 different strains of rat, Duverger and MacKenzie (1988) demonstrated considerable variation between strains in the resultant volume of ischaemic damage assessed 48 hours after the induction of ischaemia, despite using

identical surgical techniques and anaesthetic conditions.

Inter-animal variability in outcome, within acceptable limits, is a common feature of many models of cerebral ischaemia. Attempts to reduce the variability by increasing the severity of the insult e.g. lengthening the duration of global ischaemia, combining MCA occlusion with genetic hypertension and/or carotid occlusion must be approached with extreme caution. Whilst this achieves the objective of producing a more reproducible lesion, it may too gross and thus less susceptible to pharmacological intervention.

3. PATHOPHYSIOLOGY OF ISCHAEMIC BRAIN DAMAGE

3.1 Energy Depletion and Acidosis

The energy requirements of cerebral tissue are derived almost exclusively, from the aerobic catabolism of glucose (Sokoloff, 1977). The induction of cerebral ischaemia compromises the supply of oxygen and glucose to the brain, and an imbalance between glycolysis and oxidative phosphorylation results in the accumulation of lactate and H^+, inducing tissue acidosis (Siesjö, 1992a). The accumulation of lactate and H^+ results in severe tissue acidosis after both global and focal ischaemia (Chopp et al., 1988; Wagner et al., 1992b).

The severity of lactic acidosis is dependent upon the availability of glucose and the extent of ATP depletion (Siesjö, 1992a). If glucose is available, as it is via collateral blood supply and residual flow in the ischaemic penumbra after MCA occlusion, the amount of lactate accumulated is determined by the degree of energy failure, and not by plasma glucose concentration (Gardiner et al., 1982). Moreover, as ATP levels are only marginally reduced in perifocal areas after MCA occlusion (Folbergrová et

al., 1992), hyperglycaemia is unlikely to enhance the severity of acidosis (Siesjö, 1992a). This is consistent with observations where hyperglycaemia has no effect on infarct size following permanent MCA occlusion (Nedergaard & Diemer, 1987) although there is controversy (Marie & Bralet, 1991). In contrast, following global and transient severe forebrain ischaemia, where cerebral blood flow and ATP levels are markedly reduced (Pulsinelli & Duffy, 1983), pre-ischaemic hyperglycaemia increases the degree of acidosis during ischaemia (Siesjö, 1992a) and the extent of brain injury (Marie & Bralet, 1991).

Energy failure and acidosis during ischaemia may cause damage to cells by a number of different mechanisms. These include the further inhibition of lactate oxidation, inhibition of H^+ extrusion, denaturation or inactivation of enzymes and other proteins, and the disruption of mitochondrial respiration and the associated promotion of free radical formation through the release of Fe^{2+} (Siesjö, 1988a; 1992b). In addition, the maintenance of the cell membrane potential (approximately -75mV) and of the non-equilibrium distributions of Na^+, K^+. Ca^{2+} and H^+ require energy in the form of ATP. Interruption of the ATP supply during an ischaemic insult has thus two further consequences. First, ionic gradients for Na^+, K^+ and Ca^{2+} are dissipated and the membrane potential is reduced towards zero (Hansen, 1985). Second, the energy deprived cell takes up Na^+ and, when the membrane potential is sufficiently reduced, Cl^-. The intracellular accumulation of Na^+ and Cl^- results in the influx of water into the cell via the osmotic gradient, leading to the formation of cytotoxic oedema (Yang et al., 1992). The reduction in ATP levels will also cause the inhibition (and even reversal) of the energy-dependent membrane-bound uptake carrier mechanisms for glutamate, thereby increasing the concentration of this neurotoxic excitatory amino acid in the

extracellular space (Nicholls & Attwell, 1990). Furthermore, as energy depletion affects both the ATP-dependent extrusion of Ca^{2+} and its intracellular sequestration, the loss of ionic homeostasis will cause a massive elevation in the intracellular concentration of Ca^{2+} (Hansen, 1985; Siesjö & Bengtsson, 1989). Alterations in Ca^{2+} homoeostasis are believed to be central in the genesis of irreversible brain damage after global and focal cerebral ischaemia.

3.2 Calcium

It is well established that Ca^{2+} has an important role in normal cell function. Ca^{2+} has been shown to act as a membrane stabiliser, metabolic regulator, second messenger, to modulate neurite extension and synapse formation, and elicit long-term potentiation (see Siesjö, 1988b; Miller, 1991 for reviews). In the normal resting state, the intracellular concentration of Ca^{2+} in neurones is around 10^{-7}M and the extracellular concentration of Ca^{2+} is about 10^{-3}M (Choi, 1988). It is essential for normal cell function that this ionic gradient is strictly maintained. The regulation of Ca^{2+} homeostasis is maintained by two major membrane-bound pump mechanisms: a $3Na^+/Ca^{2+}$ antiporter driven by the Na^+ gradient created by a Na^+/K^+-ATPase and a Ca^{2+}-dependent ATPase (Siesjö & Bengtsson, 1989). Thus, any mechanism which disturbs the normal Ca^{2+} homeostasis may produce an increase in intracellular Ca^{2+} levels and lead to cell death.

The intracellular levels of Ca^{2+} can be elevated by the influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels (VSCCs) and neurotransmitter receptor ionophores (e.g. the NMDA receptor), as well as by the mobilisation of intracellular stores. At present, 4 types of VSCCs have been identified on neurones, differentiated on the basis of voltage dependency and rates of

inactivation : the L-, N-, T and P-types (See Spedding & Paoletti, 1992 for review). All 4 types of VSCC have been found postsynaptically, although only the N-type have been located on presynaptic nerve terminals, where they putatively control Ca^{2+}-dependent glutamate release (Spedding & Paoletti, 1992).

During ischaemia, there is a loss of normal Ca^{2+} homeostasis. The depletion of ATP leads to the depolarisation of cell membranes and the influx of Ca^{2+} through VSCCs, and the loss of active sequestration of Ca^{2+} into intracellular organelles (Siesjö, 1981). Both elements contribute to an excessive rise in the intracellular concentration of Ca^{2+} which activates a cascade of Ca^{2+}-dependent biochemical process and leads to neuronal death; the first hypothesis to implicate the involvement of Ca^{2+} in the genesis of ischaemic brain damage (Siesjö, 1981). The involvement of Ca^{2+} is supported by recent experimental evidence which describe elevations in the levels of cytosolic free Ca^{2+} after the induction of ischaemia (Benveniste et al., 1988; Uematsu et al., 1989). Although this hypothesis of neuronal necrosis is still viable, it now seems more likely that a pathogenically important part of the Ca^{2+} influx occurs via glutamate-operated calcium channels, most notably the NMDA receptor ionophore (Choi, 1988; Rothman & Olney, 1986; see section 3.3).

Evidence is available, however, to suggest Ca^{2+} is not the principal mediator of all ischaemic cell death. In certain models of hypoxic-ischaemic injury in non-neuronal cells, there does not appear to be a correlation between cell viability and Ca^{2+} content (Cheung et al.,1986); convincingly, a rise in cytosolic free Ca^{2+} does not precede injury in hepatocytes exposed to chemical hypoxia (Lemasters et al., 1987). In addition, in neuronal cell culture, where anoxia was induced by exposure to sodium cyanide, no cell

damage was observed despite massive elevations in intracellular Ca^{2+} concentration (Dubinsky & Rothman, 1991).

There are several observations with regard to hypoxic-ischaemic injury which support a primary etiological role of massive increases in the intracellular concentration of Ca^{2+}. Hypoxia in the cerebral cortex is accompanied by a dramatic decline in extracellular Ca^{2+} concentrations to about 10% of control levels (Nicholson et al., 1977); early events in neuronal hypoxia, including transmitter release and liberation of free fatty acids suggest a rise in intracellular Ca^{2+} (Siesjö, 1988b). Ca^{2+} accumulation in hypoxic brain is correlated at the regional (Dienel, 1984) and cellular (Simon et al., 1984) levels with vulnerability to hypoxic injury, and in fact may occur before neurones appear necrotic under light microscopy (Siesjö, 1988b). In addition, voltage-dependent Ca^{2+}-channel antagonists have demonstrated anti-ischaemic efficacy in some animal models of cerebral ischaemia, although there is controversy (see Hossmann, 1988; Wong & Haley, 1990 for reviews).

There are probably a myriad of reasons why sustained elevations in cytosolic Ca^{2+} after ischaemia are neurotoxic, reflecting summated gross disturbances in the many biological processes regulated by Ca^{2+} availability. Ca^{2+} can impair mitochondrial function by uncoupling oxidative phosphorylation which results in cell dysfunction and death (Siesjö, 1988b). In addition, excess intracellular Ca^{2+} triggers the activation of a cascade of specific enzymatic pathways which can be detrimental to cellular function and neuronal integrity (Siesjö & Bengtsson, 1989; Siesjö, 1992a).

A good example of such a mechanism is Ca^{2+}-activated proteolysis. The continuous stimulation of Ca^{2+}-activated neural proteases (calpains) during ischaemia can result in the abnormal degradation of cytoskeletal proteins, neurofilament proteins, membrane proteins and receptors, and several

enzymes (Melloni & Pontremoli, 1989). The involvement of calpains in ischaemic damage is further demonstrated as a calpain inhibitor has recently been shown to be neuroprotective after MCA occlusion in the rat (Hong et al., 1994). Ca^{2+} can also activate phospholipases - phospholipase A_2 and phospholipase C, which hydrolyse phospholipids giving rise to free fatty acid, lysophospholipid, and diacylglyceride accumulation within the cell. Lysophospholipids and free fatty acids, particularly the unsaturated ones are reported to affect membrane function by acting as membrane detergents and ionophores (Wieloch & Siesjö, 1982). Furthermore, Ca^{2+} can bind to the regulatory protein calmodulin, and thereby directly activate protein kinases and phosphates, resulting in further degradation of the cell structure (Connor et al., 1988; Manev et al., 1989). Thus, irrespective of the enzyme involved, sustained activation by an elevation in intracellular Ca^{2+} clearly compromises neuronal survival as a result of alterations in the function of receptors, ion channels, and membrane structures.

The Ca^{2+} hypothesis of ischaemic neuronal injury has acquired additional support from the recent establishment of relationships both between ischaemic neuronal damage and glutamate neurotoxicity, and between glutamate neurotoxicity and Ca^{2+}-mediated injury mechanisms (see section 3.3).

3.3 Glutamate and Excitotoxicity

It is now generally accepted that the dicarboxylic amino acids L-glutamate and L-aspartate are the major excitatory neurotransmitters ,in the mammalian CNS (Monaghan et al., 1989). Excitatory amino acid (EAA) receptors are the main transmitter receptors mediating synaptic excitation in the CNS, and as such they are involved in many physiological phenomena

ranging from the processing of sensory information and co-ordinated movement patterns to cognitive processes, learning and memory (Collingridge & Lester, 1989; Monaghan et al., 1989; Watkins & Evans, 1981). However, although activation of EAA receptors is integral to the normal functioning of neurones, it is paradoxical that under conditions of excessive EAA receptor stimulation, glutamate can be neurotoxic.

The fundamental observation that EAAs are potentially neurotoxic was made over 30 years ago by Lucas & Newhouse (1957) who found that prolonged exposure to high concentrations of glutamate given to mice systemically, induced degeneration of retinal neurones. This neurotoxicity was confirmed when it was determined that glutamate and related EAAs induce degeneration in areas of the brain not protected by the blood-brain barrier, notably the arcuate nucleus in the hypothalamus (Olney, 1969; Olney et al., 1971; Olney, 1978). The strong correlation between neurotoxic potency and excitatory potency of a series of glutamate analogues suggested a convergence in the mechanisms underlying the two actions. Thus the excitotoxic hypothesis was formulated on the basis that overactivation of EAA receptors was responsible for glutamate-induced neuronal death (Olney, 1978). Moreover, the characteristic "axon-sparing" nature of the excitotoxic lesions was reminiscent of brain damage found in certain human degenerative disorders (e.g. status epilepticus), a similarity which prompted suggestions that excitotoxic mechanisms underlie neurodegenerative disease in man (Olney, 1978; 1985).

The actions of glutamate and related EAAs are mediated by five distinct receptor subtypes, each with characteristic pharmacological and physiological properties (Watkins et al., 1990). The EAA subtypes are : *N*-methyl-D-aspartate (NMDA), 2-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate, metabotropic and L-2-amino-4-phosphonobutyrate (L-AP4)

(Collingridge & Lester, 1989; Monaghan et al., 1989; Watkins et al.,1990) (Figure 1). The NMDA, kainate and AMPA receptor subtypes are all associated with receptor operated ion channels, whilst the metabotropic subtype is thought to be linked to phosphoinositide metabolism as a second messenger system (Schoepp et al., 1990). The L-AP4 receptor subtype signal transduction mechanism is not fully understood, although there is evidence to suggest that it may involve cGMP hydrolysis and function as a presynaptic glutamate autoreceptor decreasing transmitter release (Gasic & Hollmann, 1992).

The non-NMDA, AMPA and kainate receptors, which are permeable to Na^+ and K^+, are responsible for fast excitatory synaptic transmission throughout the CNS and appear to fulfil a basic signalling function at excitatory synapses (Mayer & Westbrook, 1987). Under normal conditions, the NMDA receptor ionophore which is permeable to Na^+, K^+ and Ca^{2+} does not contribute to synaptic transmission because of voltage-dependent blockade at a site within the ion channel by Mg^{2+} (MacDermott & Dale, 1987). However, membrane depolarisation relieves the Mg^{2+} block of the NMDA ion channel and Ca^{2+} is then able to pass freely through the channel into the postsynaptic neurone (MacDermott & Dale, 1987). The activation of NMDA receptors is intimately involved in a number of neurophysiological events such as long-term potentiation (Collingridge & Bliss, 1987), neuronal burst firing (Herrling et al., 1983), the generation of patterned firing in neuronal networks (Dale & Roberts, 1985) and in learning (Morris et al., 1986).

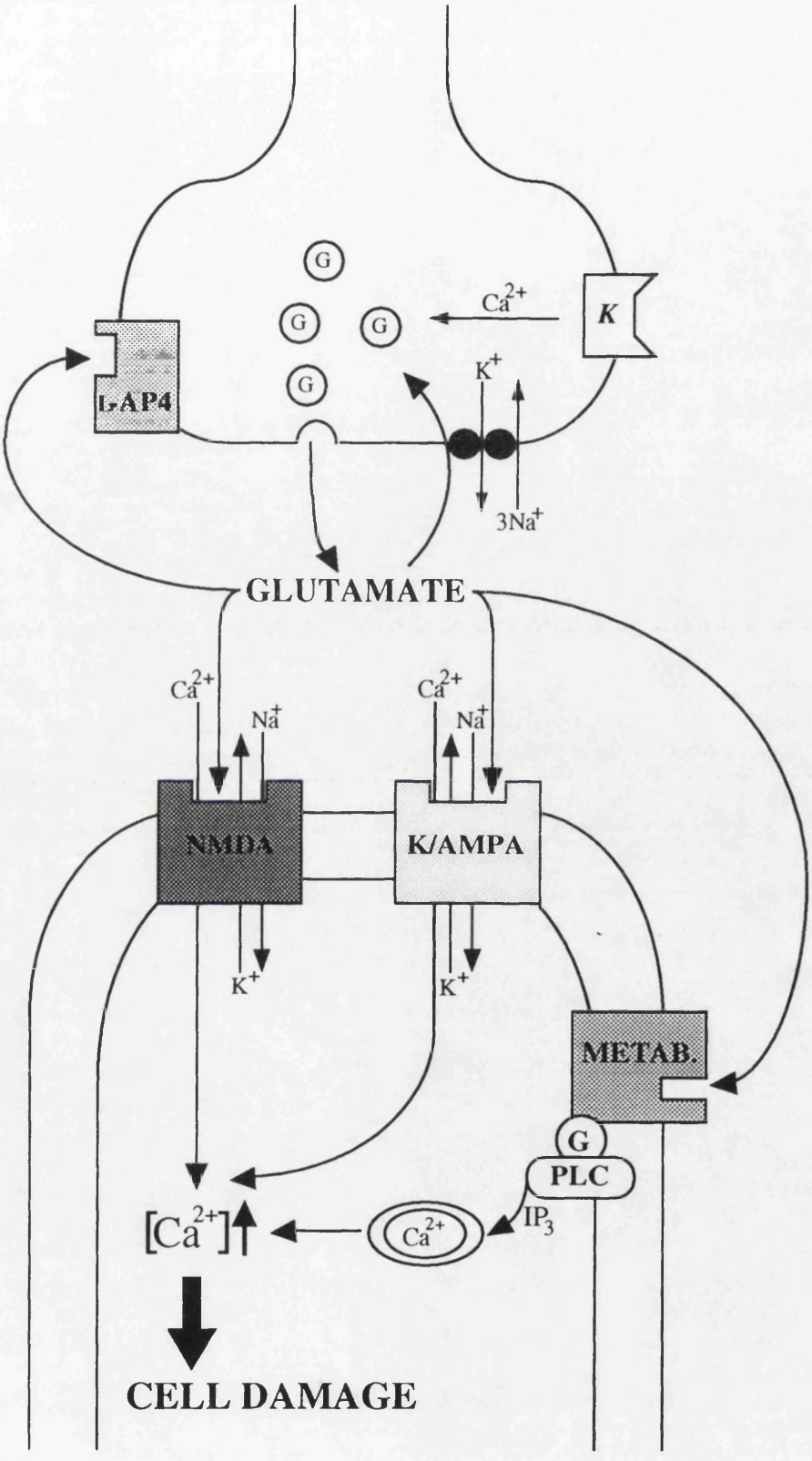
There are a number of distinct sites within the NMDA receptor ion channel complex at which the effects of glutamate can be modulated (Foster & Fagg, 1987). Conceptually, the most simple site at which NMDA receptor activity can be modulated is at the neurotransmitter recognition site for

FIGURE 1

Diagrammatic representation of a glutamatergic synapse. Glutamate released from the presynaptic nerve terminal mediates fast synaptic transmission via the AMPA/Kainate receptor subtypes which are permeable to Na^+ and K^+. Subsequent membrane depolarisation removes the Mg^{2+} blockade of the NMDA receptor ionophore allowing Ca^{2+} influx into the postsynaptic neuron. The intracellular concentration of Ca^{2+} can also be increased by activation of the metabotropic receptor which stimulates the mobilisation of Ca^{2+} from intracellular stores. Overactivation of postsynaptic glutamate receptors can be prevented by a high affinity, Na^+-dependent glutamate uptake mechanism present on neurones and glia which removes glutamate from the extracellular space or by the action of glutamate at the presynaptic L-AP4 autoreceptor to inhibit further release. Agonists at *k*-opioid receptors also putatively attenuate glutamate release presynaptically.

Abbreviations:
NMDA: N-methyl-D-aspartate
AMPA: 2-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
Metab: Metabotropic
L-AP4: L-2-amino-4-phosphonobutyrate.

Presynaptic Terminal



Postsynaptic Terminal

glutamate and NMDA. Competitive inhibition at this site can be produced using the antagonists cis-4-phosphonomethyl-2-piperidine-carboxylic acid (CGS-19755) and D-3(2-carboxypiperazin-4-yl)propenyl-1-phosphonic acid (D-CPPene) (Aebischer et al., 1989; Lehmann et al., 1988). Non-competitive blockade of the actions of glutamate can be produced by agonists such as dizocilpine (MK-801) and the dissociative anaesthetic phencyclidine (PCP), which interact at a site within the ionophore in a use-dependent manner (Kemp et al., 1987). In addition, the divalent cations Mg^{2+} and Zn^{2+} can bind to specific sites within the ion channel to inhibit receptor activity. Glycine acts as a positive allosteric modulator at the strychnine-insensitive glycine site on the NMDA receptor. Antagonists such as 7-chlorokynurenate and L-687,414 attenuate NMDA receptor activity by acting at this site (Kemp et al., 1988; Singh et al., 1990a). Furthermore, ifenprodil and related compounds act as antagonists at another positive allosteric modulatory site - the "polyamine site", to modulate NMDA receptor function (Carter et al., 1989).

There are several lines of evidence from both *in vitro* and *in vivo* studies which support the concept of glutamate excitotoxicity in the genesis of ischaemic brain damage. First, elevated levels of glutamate are neurotoxic to neurones *in vitro* as a result of the lethal influx of Ca^{2+} produced by excessive activation of NMDA receptors (Choi, 1987), although non-NMDA receptors may also contribute (Koh et al., 1990); the accumulation of Ca^{2+} induced by glutamate is closely related to the extent of neuronal death *in vitro* (Hartley et al., 1993). Second, NMDA antagonists (Choi, 1990) and non-NMDA antagonists (Frandsen et al., 1989) are neuroprotective against glutamate toxicity *in vitro*. Third, there is a massive increase in the extracellular levels of glutamate after the induction of global

and focal cerebral ischaemia (Benveniste et al., 1984; Matsumoto et al., 1992), which has been shown to be correlated to the degree of ischaemic brain damage (Butcher et al., 1990; Takagi et al., 1993). Fourth, lesions of the perforant pathway, as well as a number of other chemical and surgical lesions, which reduce excitatory input, reduces ischaemia-induced neuronal necrosis in the hippocampus (Johansen et al., 1986; Kaplan et al., 1989; Onodera et al., 1986; Wieloch et al., 1985).

The most convincing evidence to support the view that glutamate is excitotoxic has been obtained from studies in experimental models of cerebral ischaemia where antagonists at the NMDA and non-NMDA (AMPA) receptor subtypes have been shown to reduce the amount of ischaemic brain damage by up to 50% (see sections 4.1 and 4.2). However, although the blockade of excitatory amino acid receptors, most notably the NMDA receptor subtype, attenuates the transmembrane ionic fluxes which produce neuronal death, their clinical use in man may be limited. This is because in animals, at therapeutic doses, they produce marked effects on behaviour, CNS function and structure (McCulloch & Iversen, 1991; Willetts et al., 1990). An alternative strategy to reduce glutamate excitotoxicity is to inhibit the release of glutamate. Thus, in this thesis the effect of the *k*-opioid agonist CI-977 which putatively reduces glutamate release was determined on glutamate-mediated neuronal damage in animal models of focal ischaemia.

3.4 Ischaemic Penumbra

The induction of focal ischaemia results in a range of moderately and severely reduced blood flow, although the blood flow boundary between normal and damaged tissue can be quite distinct (Tamura et al., 1981b; Tyson et al., 1984). This gradient in cerebral blood flow is representative of a

spectrum of ischaemia from minimal, through moderate to severe ischaemia. Neuronal injury, and the potential for reversal is related to the duration of ischaemia and the degree of reduction in cerebral blood flow. As cerebral blood flow is reduced during ischaemia, a progressive series of morphological, electrophysiological and biochemical alterations occur, thereby indicating thresholds for key events in ischaemia.

The concept of an "ischaemic penumbra" was based on studies in the lightly anaesthetised baboon. The fundamental observation described the loss of evoked electrical activity in the cortex at a threshold blood flow or around 20ml $100g^{-1}$ min^{-1}, whilst the extracellular concentration of K^+ was preserved (Branston et al., 1974). The efflux of K^+ from cells was not observed until the level of cerebral blood flow fell to a threshold value of about 10ml $100g^{-1}$ min^{-1} (Branston et al., 1977). These observations were interpreted to indicate the loss of neuronal function before the loss of ionic homeostasis which was taken as a sign of neuronal membrane failure and hence irreversible neuronal death (Astrup et al., 1977). Absolute functional thresholds were subsequently demonstrated after MCA occlusion in primates where the developing neurological deficit progressed from mild paresis at a blood flow of 22ml $100g^{-1}$ min^{-1} to complete paralysis at 8ml $100g^{-1}$ min^{-1} (Jones et al., 1981). The ischaemic penumbra was thus defined to describe cerebral tissue perfused at a level between the thresholds of functional impairment and of morphological integrity which has the capacity to recover if cerebral blood flow is reverted to values above the 20ml $100g^{-1}$ min^{-1} threshold (Astrup et al., 1981).

Threshold-type relationships have also been reported in the ischaemic penumbra of the lissencephalic brain in the rat. However, it is important to recognise that these threshold values may be different in a gyrencephalic

species for a number of reasons. First, basal cerebral blood flow and cerebral glucose metabolism are approximately 2-3 fold greater in the rat compared to the primate (Jones et al., 1981; Kennedy et al., 1978). Second, the rat brain has a higher cellular packing density relative to the primate brain (Bass et al., 1971; Haug, 1987). Indeed, cell packing density has been invoked to justify a species ranking of cerebral blood flow thresholds causing ischaemic brain damage (Strong et al., 1983; Tamura et al., 1981b).

Mies and co-workers (1991) observed that in the penumbral region 1h after permanent MCA occlusion in the rat, protein synthesis is depressed at a threshold of approximately 55ml $100g^{-1}$ min^{-1} despite the preservation of energy state (threshold of 18ml $100g^{-1}$ min^{-1}). The inhibition of protein synthesis may be associated with progressive tissue injury because with increasing time the threshold of energy depletion gradually rises up to 32ml $100g^{-1}$ min^{-1} at 12h post-ischaemia, suggesting an expansion of the infarct core into the penumbra zone (Mies et al., 1991).

Spreading depression-like depolarisations have been observed in the penumbra of focal ischaemia, although their role remains controversial (Gill et al., 1992a; Iijima et al., 1992). Nedergaard and Hansen (1993) have differentiated between short lasting (spreading depressions) and longer lasting ischaemic depolarisations after permanent MCA occlusion in the rat. They concluded that the penumbral depolarisations are either typical spreading depression waves evoked by an increase in extracellular glutamate and/or K^+ in the core of the infarct, or ischaemic depolarisations elicited by fluctuations of blood flow around the threshold of membrane failure within the perifocal tissue (Nedergaard & Hansen, 1993).

There is evidence to suggest that the penumbra is only gradually recruited into irreversible ischaemic damage. The infarct size has been

shown to gradually increase as the middle cerebral artery is occluded for longer periods, until with an occlusion time of 180 min, the infarct size has reached that found after permanent MCA occlusion (Kaplan et al., 1991; Memezawa et al., 1992). However, although infarct size is believed to be fully established at 3-4h after permanent focal ischaemia in the rat, a 24h post-insult survival period is necessary to allow sufficient development of ischaemic brain swelling. Thus, the ischaemic penumbra is an important concept in anti-ischaemic drug development. First, the penumbra can be defined as ischaemic tissue that is at risk of infarction but is potentially salvageable by pharmacotherapy. Second, the apparent time-dependent recruitment of the penumbral tissue to final infarction suggests a potential window of therapeutic opportunity for intervention with neuroprotective agents.

4. MODULATION OF GLUTAMATERGIC MECHANISMS AND NEUROPROTECTION

4.1 NMDA Receptor Blockade

4.1.1 *Focal Ischaemia*

In experimental models of focal cerebral ischaemia there is compelling evidence that antagonists of the NMDA receptor subtype protect neurones from the effects of cerebral ischaemia (see McCulloch et al., 1991 for review). It is now generally accepted that NMDA receptor blockade reduces the amount of ischaemic damage irrespective of the species (mouse, rat, rabbit, cat, primate), the model of cerebral ischaemia, the time of sacrifice after the induction of ischaemia, whether ischaemia is permanent or temporary followed by a period of reperfusion, and irrespective of the particular site within the NMDA receptor blockade at which blockade is

achieved.

There has been extensive investigation of the efficacy of NMDA antagonists in rat models of focal cerebral ischaemia. There is overwhelming evidence that non-competitive NMDA antagonists (CNS1102, MK-801, PCP) reduce the volume of ischaemic damage after permanent MCA occlusion in the rat (Bielenberg & Beck, 1991; Dirnagl et al., 1990; Gill et al. 1991a, 1992a; Hatfield et. al., 1992; Iijima et al., 1992; Minematsu & Fisher, 1993; Minematsu et al., 1993; Park et al., 1988b; Roussel et al., 1992; Sauer et al., 1993). In addition the weak, non-competitive NMDA antagonist dextrorphan, the active metabolite of dextromethorphan, also affords neuroprotection after permanent focal ischaemia in the rat (Graham et al., 1993b). The anti-ischaemic efficacy of these agents is maintained when administered up to 1h (Graham et al., 1993b; Hatfield et al., 1992; Park et al., 1988b) and even 3h (Bielenberg & Beck, 1991) after the induction of ischaemia. There is an increasing body of evidence demonstrating that competitive NMDA antagonists (AP-7, CGP40116, CGS-19755, D-CPPene) are also beneficial in focal ischaemia in the rat (Park et al., 1992; Roman et al., 1989; Sauer et al., 1993; Simon & Shiraishi, 1990; Takizawa et al., 1991). Moreover, antagonists at the glycine (7-chlorothiokynurenate, L-687,414) and polyamine (SL.82.0715) allosteric sites within the NMDA receptor complex confer neuroprotection after permanent MCA occlusion in the rat (Chen et al., 1993; Gill et al., 1991b; Gotti et al., 1988). The volume of tissue which can be salvaged from irreversible ischaemic damage with NMDA antagonists is approximately 50% of the infarction volume. The maximum anti-ischaemic effects of the drugs are broadly similar irrespective of their precise site of action within the NMDA receptor complex. Interestingly, a recent study has shown that inhibition of NMDA-R1 receptor protein synthesis by

phosphodiester antisense oligodeoxynuycleotides results in the inhibition of NMDA receptor expression and a reduction in volume of ischaemic brain damage by over 40% following focal cerebral ischaemia in the rat (Wahlestedt et al., 1993).

An interesting feature of the neuroprotective efficacy of NMDA antagonists is that the magnitude of the response to MK-801 and kynurenate is markedly attenuated after permanent MCA occlusion in spontaneously hypertensive (SHR) rats, where there is a reduced collateral blood supply (Dirnagl et al., 1990; Roussel et al., 1990, 1992). A possible explanation is that the ischaemic insult is more severe in hypertensive animals when compared to the normotensive strain and thus less amenable to pharmacotherapy (Roussel et al., 1992).

The most convincing demonstrations of the anti-ischaemic efficacy of NMDA antagonists have emerged from investigations of their effects after permanent MCA occlusion in the cat. Pretreatment with a non-competitive antagonist (MK-801) or a competitive antagonist (D-CPPene), and administration of polyamine site antagonists (SL.82.0715, ifenprodil) within 5 min of the induction of ischaemia have been shown to markedly reduce the volume of irreversible ischaemic damage in the cerebral hemisphere (Bullock et al., 1990; Chen et al., 1991b; Gotti et al., 1988; Ozyurt et al., 1988). The administration of MK-801 2h after the onset of ischaemia is as effective as pretreatment in reducing the volume of ischaemic brain damage in the cat MCA occlusion model (Park et al., 1988a) whilst, in contrast, D-CPPene has no significant effect when treatment was initiated 1h after occlusion of the MCA (Chen et al., 1991b). Furthermore, recent evidence suggests that the administration of MK-801 30 min after the induction of ischaemia in a temporary MCA occlusion model in the cat (2h occlusion followed by 4h

reperfusion) (Dezsi et al., 1992) confers a broadly similar magnitude of neuroprotection to that observed with 6h of permanent MCA occlusion (Ozyurt et al., 1988; Park et al., 1988a).

4.1.2 *Global Ischaemia*

The influence of NMDA antagonists on pyramidal neuronal necrosis within the CA1 subfield of the hippocampus in animal models of global ischaemia has been the subject of intense investigation whilst other hippocampal subfields (CA2-CA3, CA4) and other brain regions, such as the neocortex and striatum, have been less studied. These studies have yielded inconsistent effects of NMDA antagonist administration both within the same, and between different, animal models of global ischaemia. Thus, these results have met considerable controversy and been subject to much debate.

In large animal models of global ischaemia MK-801 is the only NMDA antagonist to date which has been investigated. In these studies, MK-801 failed to reduce ischaemic damage to hippocampal CA1 neurones or alter behavioural outcome in cats (Fleischer et al., 1989), dogs (Michenfelder et al., 1989; Sterz et al., 1989) or non-human primates (Lanier et al., 1990) subjected to transient, but severe global ischaemia.

Following transient global ischaemia in the gerbil, MK-801 markedly attenuates CA1 pyramidal neuronal damage in the hippocampus when administered prior to (Gill et al., 1987; Warner et al., 1991) and 24h after (Gill et al., 1988) the induction of ischaemia. Protection against ischaemic injury to hippocampal CA1 neurones in the gerbil has also been observed with the competitive NMDA antagonists CGS-19755 and CPP (Boast et al., 1988) and the non-competitive antagonist ketamine (Marcoux et al., 1988). In addition, there is evidence that MK-801 and CGS-19755 can attenuate

ischaemic damage to the striatum and layer III of neocortex, although no neuroprotection is observed in areas CA2-CA3 and CA4 of the hippocampus (Warner et al., 1991). There are reports however that MK-801 and the polyamine site antagonist ifenprodil confer no neuroprotective effects after transient global ischaemia in the gerbil (Buchan & Pulsinelli, 1990; Sheardown et al., 1993).

There are a number of investigations in rats, using the four-vessel occlusion or two-vessel occlusion plus hypotension models of transient, but severe forebrain ischaemia, which indicate that NMDA receptor blockade with either competitive (AP-7, CGS-19755, CPP) or non-competitive (dextrorphan, ketamine, memantine, MK-801, PCP) antagonists attenuates ischaemic damage to hippocampal CA1 neurones (Church et al., 1988; Rod & Auer, 1989; Sauer et al., 1988; Seif el Nasr et al., 1990; Swan et al., 1988; Swan & Meldrum, 1990). Moreover, there are limited reports that brain regions other than the hippocampus (i.e. the neocortex and striatum) can be protected by blockade of NMDA receptors (Rod & Auer, 1989; Wieloch et al., 1989). Conversely, there is an increasing body of evidence which suggests that the administration of NMDA antagonists (competitive and non-competitive) have no neuroprotective effect on hippocampal neurones in rats subjected to transient forebrain ischaemia (Buchan et al., 1991b; Diemer et al., 1992; Jensen and Auer, 1988; Li & Buchan, 1993; Nellgård & Wieloch, 1992; Nellgård et al., 1991).

The basis for the conflicting data regarding the effects of NMDA antagonism in animal models of global/forebrain ischaemia is believed to be attributable to at least two key variables. First, small differences in brain temperature during and after transient global ischaemia dramatically modify the amount of delayed neuronal damage in gerbils and rats (Busto et al.,

1987; Minanisawa et al., 1990), and it has been suggested that the neuroprotective effects of NMDA antagonists are due principally to an artefactual reduction in brain temperature (Buchan & Pulsinelli, 1990; Corbett et al., 1990). Second, the severity of the ischaemic insult and its impact on energy state may determine the efficacy of NMDA antagonists (Siesjö & Bengtsson, 1989; Wieloch et al., 1989). Following transient, moderate (incomplete) ischaemia, where the energy state is less markedly disturbed, NMDA receptor antagonists may confer neuroprotection, whilst following severe (complete) ischaemia, which is found in many models of global ischaemia (and also in the ischaemic core of a focal insult) NMDA receptor blockade is not efficacious because of a complete breakdown of energy production.

4.2 AMPA Receptor Blockade

The advent of selective antagonists (such as NBQX, GYKI 52466 and LY-293558) for the AMPA receptor subtype has allowed assessment of the role of this receptor in the genesis of ischaemic brain damage in experimental animals.

4.2.1 *Focal Ischaemia*

Although investigations of the anti-ischaemic efficacy of AMPA receptor antagonists in models of focal cerebral ischaemia are numerically limited, a consistent pattern of response is beginning to emerge. In all studies to date, AMPA receptor blockade reduces the volume of infarction produced by permanent or temporary occlusion of the MCA. Administration of the quinoxalinedione NBQX after the induction of ischaemia has been shown to reduce the volume of focal ischaemic damage in the rat after permanent

MCA occlusion (Gill et al., 1992b). In addition, the benzodiazepine non-NMDA receptor antagonist GYKI 52466 markedly attenuates the volume of infarction after permanent focal ischaemia in the rat even when treatment is delayed up to 1 hr after the ischaemic episode (Smith & Meldrum, 1992), although its precise site of interaction within the AMPA receptor ionophore remains to be definitively identified. The delayed administration of NBQX or GYKI 52466 also reduces ischaemic brain damage following temporary MCA occlusion in the rat (2h occlusion followed by 22h reperfusion (Buchan et al., 1991c; Xue et al., 1994). The magnitude of neuroprotection afforded by NBQX in transient focal ischaemia is comparable to that obtained after permanent focal ischaemia in the rat (approximately 30%), although the reductions in lesion size were similar to or smaller than NMDA receptor antagonists in the hands of the same investigators (Buchan et al., 1992; Gill et al., 1991a). A recent study has indicated that pretreatment with a novel AMPA receptor antagonist, decahydroisoquinoline carboxylic acid (LY-293558), modestly reduces the volume of irreversible ischaemic brain damage in the cat after 6h of permanent MCA occlusion (Bullock et al., 1994). No neuroprotection is observed within the basal ganglia after AMPA receptor blockade in MCA occlusion models of focal ischaemia.

4.2.2 *Global Ischaemia*

There have been convincing demonstrations that AMPA receptor antagonists reduce ischaemic neuronal necrosis in rodent models of global ischaemia. The anti-ischaemic efficacy of AMPA receptor antagonists contrasts with the limited and controversial efficacy of NMDA receptor antagonists in the same global models (see McCulloch et al., 1991; Nellgård & Wieloch, 1992). The quinoxalinedione NBQX has been the most extensively

investigated AMPA receptor antagonist in animal models of global cerebral ischaemia. In transient (5 min duration) global ischaemia in the gerbil, NBQX affords almost complete protection of hippocampal CA1 neurones against delayed ischaemic damage, even when administered up to 24h after the onset of ischaemia (Sheardown et al., 1990, 1993). Following transient but severe forebrain ischaemia in rats, a number of investigations indicate that blockade of AMPA receptors with NBQX markedly attenuates ischaemic injury to CA1 neurones (Buchan et al., 1991a; Diemer et al., 1992; Frank et al., 1993; Li & Buchan, 1993; Nellgård & Wieloch, 1992; Xue et al., 1994). Furthermore, this neuroprotective efficacy is maintained when treatment with NBQX is initiated 12h following the ischaemic episode (Li & Buchan, 1993). There is limited evidence available that cortical and striatal neuronal necrosis can be reduced by AMPA receptor blockade with NBQX (Le Peillet et al., 1992; Nellgård & Wieloch, 1992). In contrast to the potent anti-ischaemic effects of NBQX in global ischaemia in rodents, post-ischaemic treatment with NBQX does not improve outcome in a canine model of complete global cerebral ischaemia (Lanier et al., 1993). In addition, it has been shown that GYKI 52466 does not prevent the delayed degeneration of hippocampal CA1 and CA3 neurones in rats (Le Peillet et al., 1992; Xue et al., 1994), although significant neuroprotective against selective cell loss in the cortex and striatum is observed (Le Peillet et al., 1992).

The reason for the apparent dichotomy between the anti-ischaemic efficacies of NMDA and AMPA receptor antagonists on delayed hippocampal CA1 neuronal necrosis following global ischaemia is not fully understood. A possible explanation as to how blockade of the AMPA receptor attenuates ischaemic neuronal injury at the cellular level is suggested by recent molecular biological studies involving the expression of AMPA receptor

ionophore subunits (GluR1, GluR2, GluR3 and GluR4) in oocytes which have shown that specific heteromeric combinations of subunit molecules are permeable to Ca^{2+} ions (Hollmann et al., 1991). Combinations of AMPA receptor subunits which conduct Ca^{2+} have been found in retinal bipolar cells (Gilbertson et al., 1991), and some hippocampal neurones (Iino et al., 1990). In heteromeric assemblies, the polypeptide GluR2 dictates the conductance and permeability properties of the channel (Hume et al., 1991). When expressed alone or in combination, GluR1 and GluR3 form receptors that have a substantial permeability to Ca^{2+}, but when GluR2 is co-expressed, the receptor channels are virtually impermeable to Ca^{2+} (Hollmann et al., 1991). The elegant observation that following transient severe forebrain ischaemia mRNA transcription in CA1 hippocampal neurones is altered, resulting in reduced production of the GluR2 AMPA receptor subunit (Pellegrini-Giampietro et al., 1992) suggests a switch in glutamate receptor subunit expression and a potential mechanism for a post-ischaemic increase in Ca^{2+} permeability through channels linked to AMPA receptors. This change in mRNA expression occurs in the CA1 subfield of the hippocampus, but not the CA3 zone (Pellegrini-Giampietro et al., 1992). Thus, an increased expression of Ca^{2+}-permeable AMPA-linked channels may account for the selective vulnerability of CA1 neurones to ischaemic injury, and the ability of delayed NBQX treatment to attenuate CA1 neuronal necrosis (Li & Buchan, 1993; Sheardown et al., 1993). In addition, acidosis has been shown to attenuate the influx of Ca^{2+} through NMDA receptor-gated ion channels (Takadera et al., 1992). Thus, the severe acidosis that prevails during global ischaemia and transient forebrain ischaemia (Siesjö, 1988a) may limit the contribution of NMDA receptor-mediated neurotoxicity and increase the relative importance of AMPA receptor-mediated mechanisms. This may also explain

why AMPA antagonists are more efficacious than NMDA antagonists against global ischaemic brain damage.

4.3 Adverse Effects

4.3.1 *Cardiovascular and Respiratory System*

Competitive and non-competitive NMDA receptor antagonists (MK-801 and CPP) depress respiration and induce hypercapnia (Kurumaji et al., 1989). MK-801 increases blood pressure in conscious rats and chloralose-anaesthetised cats (Hargreaves et al., 1993b; Kurumaji et al., 1989; Ozyurt et al., 1988) but markedly reduces blood pressure in halothane-anaesthetised rats (Bielenberg & Beck, 1991; Park et al., 1988b). The non-competitive NMDA receptor antagonist D-CPPene induces hypotension in chloralose-anaesthetised cats at high doses (Bullock et al., 1990; Chen et al., 1991b). Ifenprodil and SL 82.0715, agonists at the polyamine modulatory site on the NMDA receptor complex, reduces blood pressure in halothane-anaesthetised cats (Gotti et al., 1988).

Recent evidence indicates that the AMPA receptor antagonists NBQX and LY-293558 induced marked respiratory depression and evoke dose-dependent increases in pCO_2 and decreases in pO_2 in conscious rats (Browne & McCulloch, 1994). It has also been shown that NBQX induces hypertension in halothane-anaesthetised rats (Gill et al., 1992b). However, the most serious side effect relates to the low solubility of NBQX in aqueous media, because evidence is available which indicates that NBQX crystallises in renal tubules producing an interstitial tubular nephritis and blockage of the glomeruli (Li & Buchan, 1993); Xue et al., 1994).

4.3.2 *Central Nervous System*

The functional consequences of modulation of glutamatergic mechanisms can be mapped using the [^{14}C]-2-deoxyglucose autoradiographic technique developed by Sokoloff and colleagues (1977). The procedure allows an anatomically comprehensive assessment of function-related alterations in cerebral glucose use *in vivo*. Moreover, increases in function-related energy generation have emerged as being particularly important, because they appear to be a prelude to alterations in neuronal morphology within specific regions of the brain.

Non-competitive NMDA receptor antagonists (MK-801, PCP, ketamine) and competitive NMDA receptor antagonists (CPP, CGP 37849) induce dissimilar alterations in function-related glucose use in the CNS at doses broadly comparable in terms of neuroprotective and anti-convulsant efficacy. Marked focal increases in glucose use after MK-801 are observed in regions of the hippocampal formation (subicular complex, molecular layer, dentate gyrus) and in components of the limbic system (posterior cingulate cortex, entorhinal cortex, mamillary body, anteroventral thalamic nucleus), with pronounced reductions in glucose use noted in the neocortex and inferior colliculus (Hargreaves et al., 1993b; Kurumaji et al., 1989; Nehls et al., 1988). In contrast, CPP induces modest changes in glucose use, and only at very high concentrations do a few areas of the limbic system (hippocampus molecular layer, entorhinal cortex) increase their glucose use (Kurumaji et al., 1989; Nehls et al., 1988). It is noteworthy that at high doses, CGP 37849 produces a similar pattern of glucose use response in the limbic system to that observed after MK-801 administration (Hargreaves et al., 1993b). Recent evidence suggests that NMDA receptor antagonists acting at the polyamine (SL 82.0715) and glycine (L-687,414) modulatory sites produce

numerically small and anatomically circumscribed effects on glucose use (Hargreaves et al., 1993a; Scatton et al., 1991). No elevations in glucose use are observed in the limbic system areas with either SL 82.0715 or L-687,414 at doses in excess of those required for neuroprotection in rodent models of focal ischaemia (Hargreaves et al., 1993a; Scatton et al., 1991).

The acute administration of the non-competitive NMDA receptor antagonists MK-801, phencyclidine and ketamine induces transient, reversible cellular swelling and vacuolisation in the multipolar and medium to large sized pyramidal neurones in layers III and IV of the posterior cingulate cortex and retrosplenial cortex in rats (Allen & Iversen, 1990; Auer & Coulter, 1994; Fix et al., 1993; Hargreaves et al., 1993b; Olney et al., 1989, 1991). These vacuolated neurones also go on to later express the heat shock proteins HSP70 and HSP72 (Sharp et al., 1991, 1992). The reversible changes in neuronal structure are noted with MK-801 at doses similar to those at which anti-ischaemic effects and increased glucose use are seen. Similar neuronal swelling and vacuolisation are also observed with the competitive NMDA receptor antagonists (CPP, CGS 19755, CGP 37849) (Hargreaves et al., 1993b; Olney et al., 1991). However, unlike the situation with MK-801, these morphological changes occur at drug doses that are greater than those required to reduce ischaemic brain damage. SL 82.0715 and L-687,414, antagonists of the polyamine and glycine sites respectively on the NMDA receptor complex, do not induce any alterations in cortical neuronal morphology after systemic administration (Duval et al., 1992; Hargreaves et al., 1993a).

The functional consequences of AMPA receptor blockade, as reflected in alterations in cerebral glucose use, have only recently been investigated. The AMPA antagonists NBQX and LY-293558 produce marked, anatomically

widespread, dose-dependent reductions in glucose use throughout the brain (Browne & McCulloch, 1994; Suzdak & Sheardown, 1993). The greatest reductions in glucose use after NBQX or LY-293558 occur in limbic system areas (particularly hippocampal regions and cingulate cortex) and the neocortex. The effects of AMPA antagonists on neuronal morphology remains to be established.

5. THE OPIOID SYSTEM

5.1 Discovery of Opioid Peptides

The importance of opioid drugs is reflected in their extraordinary long use in medical practice (Benedetti & Premuda, 1990). Assyrian tablets dating to the twenty-first century BC describe the extraction of opium from the poppy. Opium is the dried exudate from unripe seed capsules of the opium poppy, *Papaver somniferium.* The analgesic property of opium was recognised by the ancient Greeks, and the therapeutic use of opium was described by Hippocrates during his life from 460-377 BC. There are over 20 alkaloids of opium, which constitute about 25% by weight, although only four - morphine, codeine, papaverine and noscapine (narcotine) have medical uses.

In 1803, a German pharmacist, Sertürner, isolated the chief alkaloid from opium and called it morphia (after Morpheus, the Greek god of dreams). The name morphine is now used in accordance with the convention that the names of alkaloids end in -ine. Noscapine was extracted by Robiguet in 1817 and codeine in 1832; thebaine was extracted by Pelletier in 1835 and papaverine by Merck in 1848. By the middle of the nineteenth century, pure alkaloids were taking the place of opium preparations in medicinal use.

The alkaloids of opium are conventionally divided into two distinct

chemical classes with therapeutic potential, the benzylisoqinolines and the phenanthrenes. The main benzylisoquinoline derivatives in opium, which are non-opioid in nature, are the smooth muscle relaxant, phosphodiesterase inhibitor papaverine (about 1% by weight of opium) and the anti-tussive noscapine (2-8%). More importantly, the main phenanthrene derivatives are principally the opioid 4,5-epoxymethyl morphinans thebaine (0.2%), codeine (0.3-4%) and morphine (9-17%), in ascending order of concentration in opium and potency as opioid agonists. Thebaine is virtually inactive as an opioid agonist but has been an important chemical precursor in the synthesis of 14-hydroxy derivatives of morphine such as the agonist oxycodone and the antagonists naloxone and naltrexone, and of oripavine derivatives such as the potent agonist etorphine, the partial agonist buprenorphine, or the antagonists cyprenorphine or diprenorphine (McKnight & Rees, 1991).

The knowledge that the opium derivative morphine was a potent analgesic led investigators to propose the existence of endogenous opioid-like compounds and postulate the concept of pharmacologically relevant receptors for opioids (Beckett & Casey, 1954). Evidence to support this view emerged with the demonstrations that morphine reduces field-stimulated muscle contraction and acetylcholine release in the guinea-pig ileum (Paton, 1957; Schaumann, 1957), and electrical stimulation of the rat brain stem induces analgesia (Mayer et al., 1971). The actual presence of endogenous opioid compounds was unequivocally established with the extraction of a low molecular weight substance from rat, rabbit, pig and guinea-pig brain (Hughes, 1975). This extract was found to inhibit stimulus-evoked contraction of the mouse vas deferens and guinea-pig myenteric plexus in a manner similar to morphine, and most importantly was antagonised by the opioid antagonists naltreone and naloxone (Hughes, 1975). Purification of the extract revealed

the presence to two endogenous opioid pentapeptides - [Leu^5]-enkephalin (Tyr-Gly-Gly-Phe-Leu) and [Met^5]-enkephalin (Tyr-Gly-Gly-Phe-Met) (Hughes et al., 1975). It was quickly recognised that the [Met^5]-enkephalin sequence was identical to the 61-65 amino acid sequence of β-lipotropin. This discovery led to the characterisation of the opioid properties of the β-lipotropin 61-91 fragment β-endorphin in bovine pituitary (Bradbury et al., 1976; Cox et al., 1975). Furthermore, efforts to identify a non-endorphin opioid substance in porcine pituitary extracts resulted in the isolation of the heptacedapeptide dynorphin A by Goldstein and colleagues (1981).

The discovery that the nervous system contained peptides that function as neurotransmitters or neuromodulators, and have pharmacological effects qualitatively similar to morphine established beyond doubt that the actions of morphine are mediated by physiologically relevant, receptor-mediated mechanisms, to mimic the nature functions of the endogenous opioid neuropeptides. Thus the enkephalins were implicated in important aspects of the operation of the nervous system including the control of sensory function, mood, neuroendocrine function, respiration and gastrointestinal motility (Kosterlitz & McKnight, 1981).

Early evidence suggested that the enkephalins were derived from larger precursor molecules synthesised locally by ribosomal assembly, and not from β-lipotrophin or β-endorphin of pituitary origin (McKnight et al., 1979). However, it was established, ultimately by recombinant techniques, that the nervous system contains three families of endogenous opioid peptides that are formed by proteolytic cleavage of three distinct precursor proteins: the proopiomelanocortin (POMC) precursor (also known as beta-endorphin/ACTH), the proenkephalin A precursor (known as proenkaphalin or enkephalin) and the prodynorphin precursor (also known as dynorphin/new dynorphin or

proenkephalin B) (Akil et al., 1984; Höllt, 1986). The first of these, proopiomelanocortin, contains one copy of the [Met^5]-enkephalin sequence and is the common precursor of α-, β- and γ-MSH, corticotropin, and β-endorphin (Nakanishi et al., 1979, 1981). Proenkephalin A contains the coding for several active peptides all of which are opioid in nature, i.e. six copies of [Met^5]-enkephalin and one of [Leu^5]-enkephalin (Noda et al., 1982a,b). The prodynorphin precursor contains three copies of the [Leu^5]-enkephalin sequence (Kakidani et al., 1982). The precursor molecules contain paired basic amino acids flanking the enkephalin sequences that are believed to act as signals for tryptic-like cleavage. As a consequence, proenkephalin A is a potential source of extended versions of [Met^5]-enkephalin, and prodynorphin is a source of extended versions of [Leu^5]-enkephalin: the "neo-endorphins" and "dynorphins" (Akil et al., 1984).

5.2 Opioid Receptor Classification

The existence of a specific opioid receptor was first suggested by early behavioural and clinical studies, in which a variety of synthetic analgesics based on the structure of morphine were shown to exhibit a high degree of structural and stereochemical specificity (Beckett & Casey, 1954; Portoghese, 1965; Woods, 1956). The complex clinical profiles of morphine-like drugs were subsequently suggested to be attributable to interaction with more than one type of opioid receptor (Martin, 1967). However, the first evidence for multiple opioid receptor types is now generally accepted to have come from the behavioural and neurophysiological observations in dogs with long-term spinal transections (chronic spinal dog preparation) by Martin and co-workers (1976). They proposed the existence of three types of opioid receptors - mu (μ), kappa (*k*), and sigma (σ), to account for the different pharmacological

effects produced by the prototypical agonists morphine (μ:analgesia, miosis, bradycardia, hypothermia, indifference co-external stimuli), ketazocine (k: miosis, general sedation, depression of flexor reflexes, diuresis) and SKF-10,047 (N-allylnormetazocine) σ:mydriasis, increased respiration, tachycardia, delirium).

The opioid nature of the "sigma receptor" has been the subject of much controversy. All the effects produced by SKF-10,047 in the chronic spinal dog were originally reported to be suppressed by the opioid antagonist naltrexone (Martin et al., 1976). A subsequent study, however found that some, but not all, of the effects produced by SKF-10,047 were actually antagonist by naltrexone (Martin et al., 1980). Furthermore, independent studies using the same chronic spinal dog preparation failed to observe antagonism of the effects of SKF-10,047 with the opioid antagonist naltrexone (Vaupel, 1983). It has now been firmly established the putative "sigma effects" of SKF-10,047 (and other benzomorphan opioids) in several behavioural paradigms are not blocked by naloxone, and hence are by definition non-opioid (Mannalack et al., 1986). It is now clear that the affinities of SKF-10,047 and several other benzomorphans for the so-called sigma-binding site labelled in various brain and peripheral preparations, and with several radioligands, reside with the (+)-isomers, and demonstrate a pattern of stereoselectivity that is the reverse of that seen at opioid receptor (Mannalack et al., 1986). Moreover, although it was suggested that the psychtomimetic effects of opioids may be attributable to an effect at the putative sigma receptor compounds such as nalorphine (which are also psychotomimetic in man) have no affinity for the [^{3}H]-(+) SKF10,047 binding sites in guinea-pig brain (Tam, 1985; Pfeiffer et al., 1986). Thus, as naloxone has low affinity and several non-opioid ligands e.g. haloperidol and

phencyclidine have high affinity for this sigma site, the sigma receptor is reported to represent a distinct class of non-opioid binding site possibly associated with the NMDA receptor complex (Quirion et al., 1987).

The discovery of the endogenous enkephalin peptides by Hughes and co-workers provided the "pharmacological tools" with which to further investigate opioid receptor heterogeneity. The activity effects of the enkephalins were subsequently compared with that of morphine in guinea-pig ileum and mouse vas deferens and their relative resistance to antagonism by the non-selective opioid antagonist naloxone compared (Lord et al., 1977). On the basis of the observed pattern of activity with the enkephalins being unlike that of morphine, it was proposed that there was a third receptor type, the delta (δ) opioid receptor for which the enkephalins had highest affinity (Lord et al., 1977).

Through extensive research in *in vitro* and *in vivo* pharmacological models and radioligand binding assays, unequivocal support has been gained for the existence of three types of opioid receptor, μ, δ and *k* in mammalian central and peripheral nervous system, each with a distinct anatomical distribution (Mansour et al., 1988; Martin, 1984). Evidence has been presented, on the basis of complex radioligand binding studies, to suggest the further subdivision of *k*-opioid receptors into at least two separate classes of pharmacologically distinguishable subtypes (see Knapp et al., 1993 for review). These subtypes, termed k_1 and k_2, have been distinguished on the basis of relative sensitivity toward the selective *k*-agonist U-50,488H (Von Voigtlander et al., 1983). The U-50,488H-sensitive site was termed the k_1 receptor and the U-50,488H-insensitive site the k_2 receptor (Zukin et al., 1988). In addition, the existence of a k_3 subtype has recently been proposed, based on the binding profile of a novel ligand naloxone benzoylhydrazone (Cheng et al.,

1992). However, *k*-opioid receptor subtypes have not been fully accepted and will require validation in *in vivo* functional and behavioural tests before this convention is accepted absolutely. Thus, in the absence of unequivocal evidence to support the existence of *k*-opioid receptor subtypes, the fully established *k*-receptor classification will be used within the thesis.

5.3 Opioid Receptor Distribution

Opioid receptors are widely distributed throughout the brain and spinal cord. Quantitative autoradiographic studies have demonstrated that the distribution of opioid receptors vary markedly in both their relative abundance across brain regions and their specific localisation. As comprehensive reviews of this subject are available (see Knapp et al., 1993; Mansour et al., 1987; Tempel & Zukin, 1987 *inter alia*), the following is a brief summary of opioid receptor distribution in rat brain, with particular emphasis on *k*-opioid receptors.

Telencephalon

In the neocortex there is a predominance of μ and δ sites. Although there are regional differences in lamination, μ sites are most prominent in layers I and III/IV of frontal, parietal and temporal cortex, whereas δ-opioid receptors tend to be diffusely localised in layers II, III, V and VI. In contrast, only low levels of *k*-opioid receptors are observed in layers II, III, V and VI of frontal and parietal cortex.

Limbic system structures, like neocortex, have a rich distribution of opioid receptors. For example, in the hippocampal formation there is a relative abundance of μ-opioid receptors in the pyramidal cell layer, stratum lacunosum moleculare, and the molecular and granule cell layers of the

dentate gyrus. In these same areas, comparatively low numbers of *k* and δ sites are observed. Interestingly, the relatively low number of *k*-opioid receptors seen in the rat hippocampus is in contrast to the fairly rich dynorphin innervation observed in this brain region (Chavkin et al., 1985).

In contrast to the situation in the hippocampus, all three opioid receptor subtypes are densely distribution within the caudate-putamen and the nucleus accumbens. μ-Opioid receptors occur predominantly in the sub-callosal region of the caudate-putamen and in receptor "patches" that extend from the striatum to the nucleus accumbens. δ- and *k*-opioid receptors, in contrast, are diffusely distributed within these structures, with *k*-sites particularly dense ventromedially and δ-sites ventrolaterally.

Diencephalon

Compared with the telencephalon, diencephalic structures show a predominance of μ- and *k*-opioid binding. There are high levels of μ-binding in most of the areas of the thalamus, with the exception of the zona incerta, periventricular, central lateral and ventral posteromedial nuclei. Medium to high levels of *k*-opioid receptors are seen in the medial regions of the thalamus including the periventricular, mediodorsal and reuniens nuclei. In the hypothalamus the receptor distribution, is reversed with moderate to high levels of *k*-binding throughout most of the hypthalamic nuclei (ventromedial, dorsomedial, arcuate, paraventricular, supraoptic) with little or no binding to μ-opioid receptors noted. A notable exception is the mamillary body which contains a comparable level of both μ- and *k*-opioid binding.

Mesencephalon and Brainstem

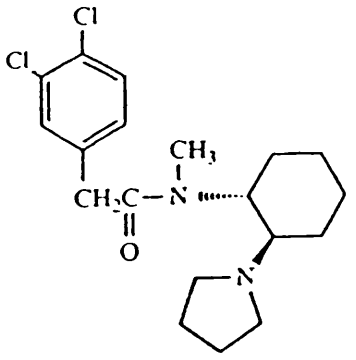
More caudally in the mesencephalon and brainstem, μ- and *k*-opioid receptors show largely parallel distributions with only low levels of υ-sites noted. Both μ- and *k*-opioid receptors are observed in the periaqueductal grey matter, superior and inferior colliculi, interpeduncular nucleus, raphe nuclei, locus coeruleus, parabrachial nucleus, nucleus tractus solitarius, spinal trigeminal nucleus and substantia gelatinosa and ventral horn of the spinal cord. The distributions vary markedly in the substantia nigra where dense μ-opioid binding is observed in the pars compacta, an area devoid of *k*-opioid receptors in the rat. Relatively low levels of δ and *k* binding are observed in the pars reticulata. Negligible opioid receptor binding is detected in the cerebellum of the rat.

An interesting feature regarding the distribution of opioid receptors is the interspecies differences exhibited by *k*-opioid receptors. At the simplistic level, species have been shown to vary dramatically in the abundance of *k*-opioid receptors. In the rat, *k*-opioid receptors compromise approximately 10% of the total number of opioid receptor sites, whereas in most other species such as guinea-pig, rabbit, dog, monkey and man, they represent between 40-50% of the total opioid receptor population (Mansour et al., 1988; Sharif et al., 1990). Anatomically, interspecies differences can be observed within different areas of the brain. In the cerebral cortex high levels of *k*-opioid receptors are found in the deep cortical layers (V-VI) of the guinea-pig, dog, primate and human brain, with only low levels in the rat (Mansour et al., 1988). These differences extend to other areas of the brain including the hippocampus, cerebellum and nigrostriatal system where high levels of *k*-binding are found in the primate in comparison to the low levels of binding in the rat (Mansour et al., 1988).

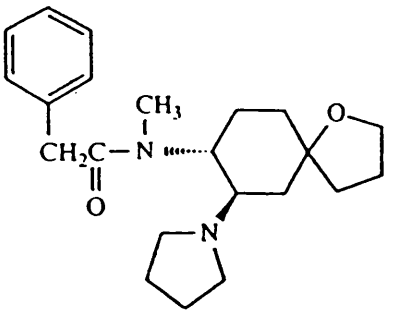
5.4 Kappa Opioid Receptor Ligands and CI-977

The endogenous dynorphin family of opioid peptides, derived from the proteolytic cleavage of prodynorphin, have high affinity for the *k*-opioid receptor (Chavkin & Goldstein, 1981; Corbett et al., 1982). However, it was a non-peptide benzomorphan derivative, ketazocine, which was the compound used to first identify the *k*-opioid receptor following behavioural studies in the chronic spinal dog preparation (Martin et al., 1976). The development of further benzomorphan derivatives (ethylketazocine, bremazocine) and the oripavine ligands, etorphine and diprenorphine, represent the discovery of non-peptide ligands which have high affinity for the *k*-opioid receptor (Chang et al., 1981; Gillan & Kosterlitz, 1982; Kosterlitz et al., 1981; Pfeiffer & Hertz, 1982). However, from *in vitro* functional tests, it has been shown that members of the benzomorphan and oripavine series are intrinsically non-selective and interact with comparable potency at μ- and δ-opioid (Chang et al., 1981; Gillan & Kosterlitz, 1982; Kosterlitz et al., 1981) as well as non-opioid (Su, 1982; Tam, 1983; Zukin & Zukin, 1981) types of receptor. Thus, although the benzomorphan and oripavine ligands have been used in tritium-labelled form to investigate the binding characteristics of the *k*-opioid receptor (Fischel & Medzihradsky, 1986; Gillan & Kosterlitz, 1982; Pfeiffer et al., 1982), selectivity was only achieved by suppressing the binding of these compounds to μ and δ sites by the inclusion of selective unlabelled ligands for these opioid receptors (Chang et al., 1981; Kosterlitz et al., 1981).

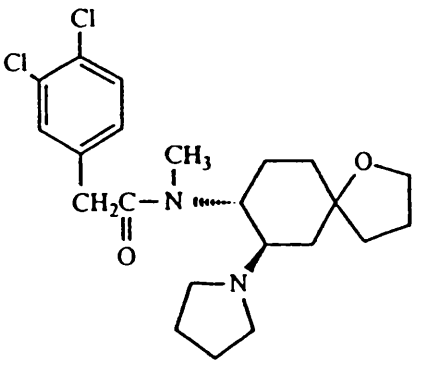
The *k*-opioid receptor is now defined by the selective, non-peptide arylacetamide derivatives U-50,488H (Von Voigtlander et al., 1983), U-69593 (Lahti et al., 1985), U-62066E (Peters et al., 1987), PD117302 (Leighton et al., 1987) and CI-977 (Hunter et al., 1990) (Figure 2) and also by the selective antagonist nor-Binaltorphimine (Portoghese et al., 1987) (Figure 2).



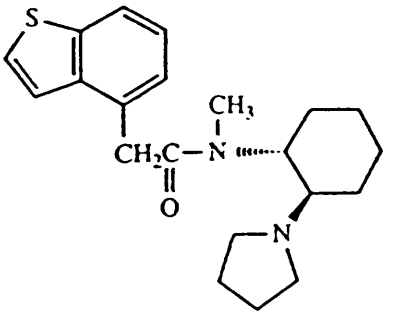
(±) U-50,488H



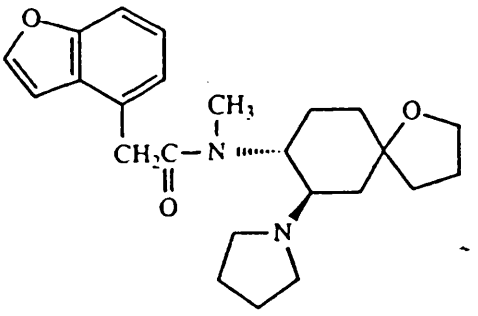
(+) U-69593



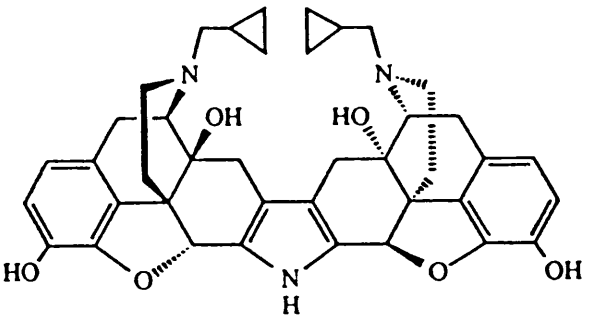
(±) U-62066E



(±) PD 117302



(-) CI-977



NOR-BINALTORPHIMINE

FIGURE 2

Chemical structures of non-peptide agonists and an antagonist at the *k*-opioid receptor.

CI-977 is a highly selective and potent agonist at the *k*-opioid receptor (Hunter et al., 1990). In radioligand binding studies using guinea-pig forebrain homogenates CI-977 binds with high affinity (K_i = 0.11nM) to the *k*-site labelled with [^{3}H]-U-69593 (Hunter et al., 1990). The affinity of CI-977 for radiolabelled μ- and δ-sites is approximately 1000-fold (K_i =99.6nM) and 10,000-fold (K_i = 1036nM) less, respectively, than at the *k*-site. CI-977 also has negligible affinity for other receptor types and most importantly has only 2μM affinity for the putative sigma binding site and greater than 10μM affinity for the phencyclidine site (Hunter et al., 1990). In autoradiographic binding studies, the distribution profile of [^{3}H]-CI-977 labelled sites in the guinea-pig and rat forebrain is consistent with that of a *k*-selective radioligand. In the guinea-pig, the areas most densely labelled were the inner layers (V-VI) of the cerebral cortex, substantia nigra, hippocampus and interpeduncular nucleus. By contrast, in the rat, only low levels of binding were found throughout the cerebral cortex, with the highest densities in the nucleus accumbens, claustrum, dorsal endoperiform nucleus, thalamus and hypothalamus (Boyle et al., 1990).

In antinociceptive tests CI-977 is one of the most potent agents tested. Following the mechanical paw pressure test in the rat, CI-977 was the most potent antinociceptive compound of the arylacetamide series examined (Hunter et al., 1990), and in the monkey tail withdrawal assay, CI-977 was found to be approximately 1000 times more potent than morphine as an analgesic when tested against a moderate (50°C) thermal stimulus (Davis et al., 1992). It has been suggested that agonists selective for the *k*-opioid receptor may be more beneficial as analgesic agents than μ-opioid receptor agonists because at antinociceptive doses they are devoid of the side-effects (emesis, constipation, respiratory depression, physical dependence) associated

with occupation of the μ-opioid receptor (Millan, 1990; Pasternak, 1993). *In vitro* bioassays confirm the very high efficacy of this agent compared to other *k*-opioid agonists (Hunter et al., 1990). Its relative efficacy is at least 10-fold greater than U-62066E and U-50,488H, and may indeed approach that of the endogenous dynorphins.

The prototype opioid antagonist naloxone has been shown to reverse the effects of *k*-opioid agonists, but is not selective for any of the receptor types (Rees et al., 1992). *In vivo*, the analgesic effects produced by CI-977 in animal models of nociception are completely reversed by naloxone (Davis et al., 1992; Hunter et al., 1990). In addition, the potent inhibition of electrically-evoked contractions induced by CI-977 in *in vitro* bioassays (guinea-pig ileum and rabbit vas deferens) is also naloxone reversible (Hunter et al., 1990). These observations therefore demonstrate the opioid nature of these responses to CI-977. The bivalent morphine derivative nor-Binaltorphimine is the most selective antagonist at the *k*-opioid receptor to date (Portoghese et al., 1987). Unfortunately, however, this compound has poor bioavailability and limited penetration to the central nervous system, and thus investigations of the characteristics of *k*-opioid receptors and endogenous *k*-opioid systems are mainly based on the actions of selective *k*-agonists like CI-977 and the endogenous dynorphin opioid peptides.

5.5 The Opioid System and Cerebral Ischaemia

There is good evidence to suggest that the opioid system may be involved in the pathophysiology of cerebral ischaemia. Historically, considerable attention was first directed towards the utility of the non-selective opioid antagonist naloxone as treatment for ischaemic brain damage.

Early studies demonstrated that naloxone reversed hemiplegia and

improved speech in ischaemic stroke patients (Baskin & Hosobuchi, 1981) and reduced neurological deficits in gerbils following permanent unilateral common carotid artery occlusion (Hosobuchi et al., 1982). Subsequent investigations observed a beneficial effect of naloxone in a variety of animal models of global and focal cerebral ischaemia (Baskin et al., 1984b; Levy et al., 1982; Wexler, 1984; Zabramski et al., 1984). On the basis of these findings it was hypothesised that post-ischaemic neuronal degeneration may be a consequence of excessive activation of the endogneous opioid system which could be therapeutically antagonised by opioid receptor antagonists such as naloxone. This theory has not met universal approval and the efficacy of opioid antagonists for the treatment of stroke questioned because the neuroprotective effects of naloxone were not reproduced in many other studies in experimental models of cerebral ischaemia (Gaines et al., 1984; Holaday & D'Amato, 1982; Hubbard & Sundt, 1983; Kastin et al., 1982) or in man (Fallis et al., 1983).

In recent years there has been increasing support for the view that activation of the *k*-opioid receptor can ameliorate rather than exacerbate post-ischaemic neuronal necrosis and promote neurologic recovery and survival. The initial study in this regard demonstrated that treatment with the peptide dynorphin A (1-13), an endogenous *k*-opioid agonist, improves long-term survival in cats following permanent occlusion of the right MCA (Baskin et al. 1984a). In the same study, the non-opioid dynorphin fragment, dynorphin A (3-13), was ineffective. Subsequently it was shown that the non-peptide *k*-opioid agonists U-62066E and U-50,488H reduced hippocampal CA1 neuronal necrosis following transient (5-10 min) bilateral carotid artery occlusion in the gerbil (Contreras et al., 1991; Hall & Pazara, 1988; Tang, 1985). It is notable that U-54,494A, a structural analogue of U-50,488H with

no affinity for the *k*-opioid receptor did not afford neuroprotection (Hall & Pazara, 1988). In addition, there is evidence that U-50,488H reduces the cerebral oedema and mortality after 4 h bilateral carotid occlusion in Fischer 344 rats (Silvia et al., 1987; Silvia & Tang, 1986) and also the cerebral oedema, cortical and striatal neuronal necrosis and mortality resulting from transient (1h) or permanent MCA occlusion in the cat (Silvia & Tang, 1986; Tang & Silvia, 1986). Similarly the *k*-opioid agonist GR89696 has recently been shown to confer neuroprotection following transient, global ischaemia in the gerbil and permanent focal ischaemia in the mouse (Birch et al., 1991).

A precise mechanistic basis for the beneficial effects which *k*-opioid agonists display in some animal models of cerebral ischaemia is presently not known with certainty. Agonists at the *k*-opioid receptor are diuretic (Peters et al., 1987; Slizgi et al., 1984), and thus the prevailing view is that *k*-opioid agonists are neuroprotective by reducing post-ischaemic cerebral oedema (Silvia et al., 1987; Silvia & Tang,1986). However, *k*-agonists are also potent anti-convulsant agents with particular efficacy towards NMDA receptor mediated convulsant activity (Singh et al., 1990b). Indeed a major role of dynorphin peptides in the hippocampus may be to regulate mossy fibre synaptic transmission (Wagner et al., 1991). Prodynorphin is synthesised in the granule cells of the dentate gyrus and dynorphin peptides are densely represented in the mossy fibre pathway (McGinty et al., 1983; Sato et al., 1991). It also appears that the dynorphins are co-localised with aspartate/glutamate in this pathway as well as in other brain areas (Terrian et al., 1988). Electrophysiological studies have shown that *k*-opioid agonists inhibit the excitatory amino acid input to the locus coeruleus (McFadzean et al., 1987), and thus it appears that an intriguing relationship exists between *k*-opioid and excitatory amino acid mechanisms. This is all the more

intriguing given that the marked reduction in the level of the hippocampal dynorphin A immunoreactivity precedes the resultant CA1 neuronal necrosis following transient global ischaemia in the gerbil (Fried & Nowak, 1987) and also that *k*-opioid agonists are neuroprotective in certain animal models of cerebral ischaemia. Thus if a major role of *k*-opioid peptides in the brain is to modulate excitatory amino acid transmission, this would provide a plausible, rational hypothesis to explain the anti-ischaemic efficacy of *k*-agonists.

6. AIMS OF THESIS

The principal objectives in this thesis will focus on investigations into the neuroprotective effect of the selective *k*-opioid receptor agonist CI-977 in animal models of focal cerebral ischaemia and to identify the mechanisms through which *k*-opioid agonists can ameliorate ischaemic brain damage.

The efficacy of CI-977 will be examined after permanent MCA occlusion in a lissencephalic species (rat) and a gyrencephalic species (cat). Outcome will be assessed at 24h after the induction of ischaemia in the rat when infarction and brain swelling are well developed. In addition, the effect of CI-977 on ischaemic damage will be determined at 4h and 6h post-ischaemia in the rat and cat respectively, where key physiological variables which are thought to influence outcome (e.g. MABP, temperature, plasma glucose) can be comprehensively monitored throughout the experimental period.

In order to establish a mechanistic basis for the neuroprotection afforded by CI-977, three different experimental approaches will be employed. First, the effect of CI-977 on cerebral blood flow after permanent MCA occlusion in the rat will be examined using [^{14}C]-iodoantipyrine autoradiography. Second, the effect of CI-977 on the relationship between glutamate release (using the microdialysis technique) and cerebral blood flow (using the hydrogen clearance technique) after focal ischaemia in the cat will be studied. Third, the effect of CI-977 on exogenous glutamate-induced neurotoxicity in the rat cerebral cortex (administered using reverse microdialysis) will be assessed and compared to the effects of NMDA and AMPA receptor blockade using MK-801 and NBQX respectively.

The functional consequences, as reflected in alterations in cerebral glucose use and cerebral blood flow, in the normal brain following systemic administration of *k*-opioid agonists has been minimally investigated hitherto.

In this thesis, the effects of CI-977 in local cerebral glucose utilisation (using [^{14}C]-2-deoxyglucose autoradiography) and local cerebral blood flow (using [^{14}C]-iodoantipyrine autoradiography) will be assessed in the conscious rat to determine whether CI-977, at neuroprotective doses, induces alterations in glucose use or cerebral circulatory effects which may confound its anti-ischaemic action.

CHAPTER II

METHODS

1. ANIMALS

1.1 Rats

All investigations using rats were carried out on adult male Sprague-Dawley rats (Harlan Olac Ltd., Bicester, Oxon, U.K.) weighing 300-450g. The animals were maintained on a 12h light:dark cycle and allowed free access to food and water prior to experimentation. Animals used in cerebral glucose utilisation studies were deprived of food the night preceding the experiment (10-12h) in order to obtain stable plasma glucose levels at the time of study.

1.2 Cats

All investigations using cats were carried out on adult cats of either sex (Hillgrove Family Farm Ltd., Minster Lovell, Oxford, U.K.) weighing 2.2-3.9kg. The animals were maintained on a 12h light:dark cycle and deprived of food the night preceding the experiment (15-18h), but allowed free access to water.

2. THE EFFECT OF CI-977 IN ANIMAL MODELS OF FOCAL CEREBRAL ISCHAEMIA

2.1 24h (Recovery from Anaesthesia) Model in the Rat

2.1.1 *Surgical Preparation*

The rats were anaesthetised initially with a mixture of 4% halothane, 30% oxygen and 70% nitrous oxide, and maintained thereafter with a nitrous oxide/oxygen mixture (70%:30%) containing 0.5-1% halothane delivered by a face mask. Body temperature was monitored throughout the surgical procedure (approximately 1h) by a rectal thermometer, and maintained normothermic (37 ± 0.5°C) via a heating blanket controlled by the thermometer.

2.1.2 *Induction of Focal Ischaemia*

All animals underwent a subtemporal craniectomy and exposure of the main trunk of the left MCA (Tamura et al. 1981a), leaving the zygomatic arch intact (Shigeno et al. 1985a). The animals were placed in the left lateral decubitus position, and an oblique skin incision (2cm) made half way between the left orbit and the external auditory canal. The exposed temporalis muscle was dissected from the cranium and retracted to expose the infero-temporal fossa. Under an operating microscope at high magnification, a small craniectomy was made using a saline-cooled dental drill at the junction between the medial wall and the roof of the infero-temporal fossa, approximately 1mm dorsal to the foramen ovale. The underlying dura was opened with a fine needle to reveal the MCA. The main trunk of the MCA was exposed proximal to the olfactory tract to where it crosses the inferior cerebral vein (Figure 3). The exposed artery was occluded by microbipolar coagulation from its origin to the point where it crosses the inferior cerebral vein. All visible lenticulostriate branches were also occluded. The trunk of the MCA was then severed proximal to the lenticulostriate branches to assure completeness of the vascular occlusion and prevent recanalisation (Figure 3). The temporalis muscle was sutured, the wound closed, and an antiseptic agent (Pevidine) liberally applied.

2.1.3 *Post-Occlusion Recovery*

Following MCA occlusion, anaesthesia was discontinued and the animals allowed to regain consciousness under strict observation in an incubator at 37°C. The rats were then housed singly in the post-operative recovery room, where their overall condition was closely monitored throughout the survival period.

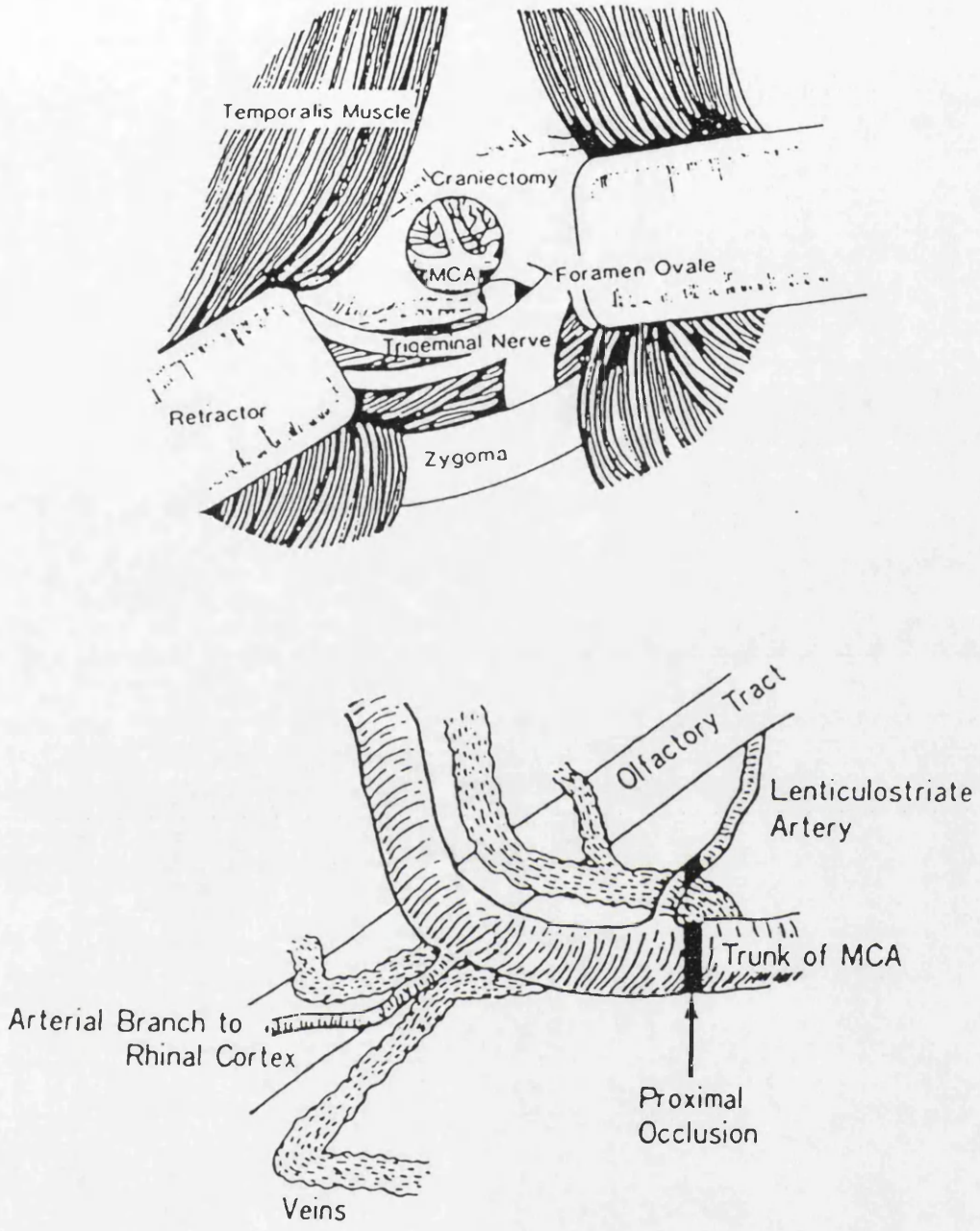


FIGURE 3

Diagrammatic representation of the operative procedure for permanent occlusion of the MCA in the rat. Exposure of the MCA via a subtemporal approach (upper) and proximal occlusion of the MCA by microbipolar coagulation (lower). Black lines indicate the sites of severance. (Adapted from Shigeno et al., 1985a.)

2.1.4 *Neuropathology and Quantitation of Ischaemic Damage and Brain Swelling*

Twenty-four hours after MCA occlusion, the animals were re-anaesthetised with a mixture of 4% halothane, 30% oxygen and 70% nitrous oxide and decapitated. The brains were rapidly removed, frozen in isopentane at -42°C and embedded in a histological medium (Tissutec). The brains were sectioned semi-serially through the cerebrum from the anterior poles to the cerebellum and the sections (20μm) stained with haematoxylin-eosin (H & E).

Areas of ischaemic damage and brain swelling were determined directly from the stained histological sections using a computer-assisted image analyser (Quantimet 970, Cambridge Instruments). This method of quantitative analysis allows the relationship between infarction and brain swelling to be established in the same animals. The areas of ischaemic damage were delineated on those sections which corresponded most closely to 8 preselected coronal levels of rat brain from anterior 10.5mm to anterior 1mm (Table 1) relative to the interaural line from the atlas of König & Klippel (1963) without knowledge of the experimental protocol. The areas of ischaemic damage at each coronal level were integrated with the known distance between each coronal level to derive the total volume of ischaemic damage in each animal (Osborne et al. 1987).

The volume of the cerebral hemispheres ipsilateral and contralateral to the occluded MCA was determined by integration from assessment of the total surface area at the same 8 coronal levels employed in assessing areas of ischaemic damage. The difference between the two hemispheres (ipsilateral-contralateral) provided a measure of brain swelling. End points for integration for cortex and hemisphere were anterior 12.5mm and posterior 0.5mm. The areas and volumes of ischaemic damage and brain swelling were

TABLE 1

CORONAL LEVELS OF RAT FOREBRAIN AND THEIR ANTERIOR CO-ORDINATES

ANATOMICAL LANDMARK	LEVEL	CO-ORDINATE (mm)
Olfactory tract	1	10.50
Nucleus accumbens	2	8.92
Septal nuclei	3	7.19
Globus pallidus	4	6.06
Anterior hypothalamus	5	5.15
Lateral habenula	6	3.75
Medial habenula	7	2.18
Aqueduct	8	1.02

The 8 preselected coronal levels of rat forebrain drawn from the atlas of König & Klippel (1963), with their anatomical landmarks and anterior co-ordinates, used to quantify areas of ischaemic damage after permanent MCA occlusion.

expressed in absolute terms (mm^2 and mm^3).

2.1.5 *Dosing Regimen*

CI-977, dissolved in a vehicle of isotonic saline, was tested at 3 doses; viz. 0.03mg/kg, 0.3mg/kg and 3mg/kg. Drug treatment was initiated with a subcutaneous (s.c.) bolus injection 30 min prior to MCA occlusion. Further s.c. injections were administered at 30 min, 6h, 12h and 18h after MCA occlusion until sacrifice at 24h. Control animals received s.c. injections of the vehicle (1ml/kg) at the same time points.

2.2 4h (Anaesthetised Throughout) Model in the Rat

2.2.1 *Surgical Preparation*

The rats were anaesthetised initially with a mixture of 4% halothane, 30% oxygen and 70% nitrous oxide, a tracheostomy performed and the animals ventilated mechanically thereafter with a nitrous oxide/oxygen mixture (70%:30%) containing 0.5-1% halothane. Inspired halothane concentration was adjusted between 0.5-1% to produce adequate anaesthesia and to maintain MABP at 80mmHg and above prior to drug/vehicle administration. Polyethylene catheters (Portex: external diameter 0.96mm; internal diameter 0.58mm; length 15cm) were inserted into both femoral arteries and one femoral vein to permit the continuous monitoring of MABP (P231D Gould Stratham, Model 2202) and repeated sampling of arterial blood.

2.2.2 *Induction of Focal Ischaemia*

All animals then underwent exposure and proximal occlusion of the left MCA as described in the 24h (recovery from anaesthesia) model (Section 2.1.2). Following MCA occlusion, the temporalis muscle and soft tissues were allowed

to fall back into place and the wound closed.

2.2.3 *Physiological Variables*

Arterial blood samples were taken prior to drug/vehicle administration, at the time of MCA occlusion and at hourly intervals thereafter, until sacrifice at 4h. These samples were used for determination of respiratory status (pCO_2, pO_2 and pH) by a blood gas analyser (238pH/Blood Gas System, Ciba Corning) and plasma glucose concentration by automated enzyme assay (Glucose Analyser 2, Beckman). Respiratory volume was adjusted to maintain pCO_2 between 34-42mmHg (normocapnia), and adequate arterial oxygenation (pO_2 >100mmHg) was maintained throughout. Body temperature was monitored by a rectal thermometer and the animals maintained normothermic via a heating blanket controlled by a thermometer. Extreme caution was taken to avoid any procedure (e.g. direct heating of the head) which could alter artifactually the gradient between deep body temperature and brain temperature.

2.2.4 *Neuropathology and Quantitation of Ischaemic Damage*

Four hours after MCA occlusion, the rats were perfusion fixed with FAM (40% formaldehyde, glacial acetic acid and absolute methanol in a ratio of 1:1:8) for assessment of early ischaemic damage. The halothane concentration was increased to 3% and the animal placed in a supine position. The thorax was opened through a midline incision and a catheter inserted into the ascending aorta via the left ventricle.

The right atrium was incised and heparinised saline infused at a pressure equal to the MABP of the animal until the effusate from the right atrium was bloodless (approximately 75ml). The animals were then perfused with FAM (approximately 200ml) at the same pressure. The animals were then decapitated

and the head stored in FAM for 24h. At this time, the brain was carefully removed and stored in 70% methanol prior to quantitative neuropathology.

The cerebellum and olfactory bulbs were removed and the forebrain cut into 4 equally spaced coronal slices. Each slice was processed, embedded in paraffin wax, and sectioned at multiple levels (approximately 100 sections per brain). The sections (10μm) were stained with H & E and by a method combining cresyl violet and luxol fast blue.

Those sections that corresponded most closely to the 8 preselected coronal levels (Table 1) were examined under light microscopy without prior knowledge of the experimental protocol. Regions showing evidence of ischaemic cell change were delineated and transcribed onto scale drawings of each of the 8 coronal levels (Figure 11). The areas of early infarction in the cerebral hemisphere, cerebral cortex and caudate nucleus were quantified at each level from the scale diagrams using a computer-assisted image analyser (Quantimet 970, Cambridge Instruments). Total volumes of early ischaemic damage were calculated by integration as described previously (Osborne et al., 1987) with the exception that scale drawings were normalised to a hemisphere of 575mm^3 (i.e. for male rats with body weight 350-400g) as distinct to that of 418mm^3 (for female rats with body weight 150g) as employed by Osborne et al. (1987). The ischaemic volumes and areas are expressed in absolute terms (mm^3 and mm^2).

2.2.5 *Dosing Regimen*

CI-977, at the neuroprotective dose of 0.3mg/kg, was given as s.c. injections 30 min before and 30 min after occlusion of the MCA. Control animals received s.c. injections of the vehicle (isotonic saline, 1ml/kg) at the same time points.

2.3 6h (Anaesthetised Throughout) Model in the Cat

2.3.1 *Surgical Preparation*

The cats were anaesthetised initially with saffan (9mg/kg, i.v. total steroids), intubated, and connected to a positive pressure ventilator delivering nitrous oxide (70%) and oxygen (30%) in an open circuit. Polyethylene catheters (Portex: external diameter 1.65mm; internal diameter 1.0mm; length 30cm) were inserted into one femoral vein and one femoral artery for the administration of drugs and the continuous monitoring of MABP and respiratory blood gas status respectively. Anaesthesia was maintained throughout the course of the investigation with a nitrous oxide/oxygen mixture (70%:30%) containing 1-1.5% halothane, in order to maintain sufficient anaesthesia in each animal as determined by loss of the corneal reflex.

2.3.2 *Induction of Focal Ischaemia*

The cat's head was placed in a stereotactic frame (Kopf, Clark Electromedical) and the left MCA occluded via a transorbital approach by an experienced neurosurgeon. With microsurgical techniques, the left orbit was exenterated. The optic foramen was enlarged with a saline-cooled dental drill, and the posterolateral and superior walls of the orbit removed to expose the dura mater overlying the MCA from the origin of the artery to its bifurcation. Under an operating microscope, the dura was incised and the MCA exposed. The trunk of the MCA, all collateral vessels and all visible branches of the lenticulostriate arteries were coagulated with bipolar diathermy, and the main trunk transected with microscissors to assure completeness of the vascular occlusion.

2.3.3 *Physiological Variables*

Throughout the experimental period, MABP was maintained constant between 80-90mmHg by increasing the inclination of the hind limbs and/or intravenous infusion of Hartman's solution (up to approximately 100ml per cat). Regular samples of arterial blood were drawn for determination of respiratory status (238pH/Blood Gas System, Ciba Corning), plasma glucose concentration (Glucose Analyser 2, Beckman), haematocrit and osmolality. Normocapnia (pCO_2 between 28-32mmHg) was maintained by adjusting the stroke volume of the respirator. Metabolic acidosis was corrected by administration of sodium bicarbonate (8.4%). Body temperature was monitored by a rectal thermometer and maintained normothermic by means of a heating blanket controlled by a thermometer. Care was taken to avoid any procedure (such as compromising extracranial flow or direct heating of the head) which could alter the relationship between core temperature and brain temperature.

2.3.4 *Neuropathology and Quantitation of Ischaemic Damage*

Six hours after the induction of ischaemia, the cats were killed by transcardiac perfusion fixation with FAM. The halothane concentration was increased to 3%, and the cat placed in a supine position and heparinised. A thoracotomy was performed and a cannula introduced into the ascending aorta via the left ventricle. The right atrium was cut and physiological saline infused at a pressure equal to the MABP of the animal until the effusate was bloodless (approximately 250ml). The animals were then perfused with approximately 1.5l of FAM at the same pressure. The cats were decapitated, and the head stored in FAM at 4°C for at least 12h. The brain was then removed and stored in 70% methanol prior to neuropathological examination.

The left side of the forebrain was marked with Indian ink. The hindbrain was detached by a cut through the midbrain and the cerebral hemispheres cut into 5 equally spaced coronal slices. The slices were embedded in paraffin wax and sections (7-8μm) cut at 200μm intervals throughout the slices (approximately 150-200 sections per forebrain). The sections were stained by H & E and by a method combining cresyl violet and luxol fast blue.

Those sections that corresponded most closely to 16 preselected coronal levels of cat brain from anterior 22mm to posterior 6mm relative to the interaural line from the atlas of Reinoso-Suarez (1961) were examined by light microscopy without prior knowledge of the animals' history. Areas of early infarction were charted onto scale anatomical diagrams (x 2.21 actual size) at each of the 16 levels (Figure 16). Areas and volumes of ischaemic damage were then derived by integration in a method analogous to that described for the rat 4h (anaesthetised throughout) model (Section 2.2.4). End points for integration for cortex and hemisphere were anterior 28mm and posterior 12mm.

2.3.5 *Dosing Regimen*

CI-977 (0.3mg/kg) dissolved in a vehicle of isotonic saline was administered as a slow (3 min) intravenous (i.v.) injection 15 min before occlusion of the MCA. A constant i.v. infusion of CI-977 (0.15mg/kg/h) was initiated immediately and maintained thereafter until death. Control animals received a bolus injection of the vehicle (1ml/kg, i.v.) 15 min prior to the induction of ischaemia. An i.v. infusion of the vehicle (0.15ml/min) was initiated immediately thereafter and maintained until death.

Samples of arterial blood were withdrawn at hourly intervals throughout the experimental period, centrifuged, and plasma levels of CI-977 determined by radioimmunoassay.

(Determination of CI-977 plasma levels by radioimmunoassay was carried out by Dr. Steven Rose, Parke-Davis, Ann Arbor, U.S.A.).

2.4 Statistical Analysis

All data are presented as mean ± standard errors of the mean (SEM). Volumes of ischaemic damage in the control and drug-treated groups were compared with one-tailed Student's *t*-test because of the *a priori* decision to examine only the ability of the drug to reduce the volume of ischaemic damage. All other statistical comparisons between experimental groups were performed with two-tailed Student's *t*-test. Analysis of variance (ANOVA) with subsequent intergroup comparison by Student's *t*-test with appropriate Bonferroni correction was used when multiple groups were examined. Correlation analysis was carried out by linear regression (Pearson's).

3. INVESTIGATIONS INTO MECHANISMS UNDERLYING THE NEUROPROTECTIVE EFFICACY OF CI-977

3.1 Cerebral Blood Flow after MCA Occlusion in the Rat

3.1.1 *Surgical Preparation*

The animals were anaesthetised, a tracheostomy performed and artificially ventilated under halothane anaesthesia as described elsewhere (Section 2.2.1). Polyethylene catheters (Portex: external diameter 0.96mm; internal diameter 0.58mm; length 15cm) were inserted into both femoral arteries (for continuous monitoring of MABP and repeating sampling of arterial blood) and one femoral vein (for administration of the radioactive tracer).

3.1.2 *Induction of Focal Ischaemia*

All animals underwent exposure and proximal occlusion of the left MCA as described in the 4h (anaesthetised throughout) model in the rat (Section 2.2.2).

3.1.3 *Measurement of Local Cerebral Blood Flow*

Local cerebral blood flow (CBF) was measured 30 min after occlusion of the MCA with the quantitative autoradiographic technique using [^{14}C]-iodoantipyrine as a tracer as described by Sakurada et al. (1978). A ramped infusion of 50μCi of [^{14}C]-iodoantipyrine in 1.5ml of saline was infused intravenously over 30 sec. During the isotope infusion, 15-18 timed samples of arterial blood were collected onto preweighed filter paper discs in a perspex carousel. The rat was killed by decapitation approximately 30 sec after the start of isotope infusion. The brain was rapidly removed and frozen in isopentane (-42°C) for at least 10 min for later processing. The filter paper discs containing the blood samples were placed in preweighed glass vials, sealed, and reweighed to obtain the weight of each blood sample. Hydrogen peroxide (30% wt/vol, 0.4ml) and distilled water (1ml) were added to each vial to bleach the blood sample and extract the radioisotope, and the vials left at room temperature for 30 min. 10ml of scintillant (Ecoscint A, National Diagnostics) was then added. The tracer concentration in each sample was determined 24h later by liquid scintillation counting (Packard Tricarb, 1900CA).

3.1.4 *Physiological Variables*

Arterial blood samples were taken prior to drug/vehicle administration, at the time of MCA occlusion and at the time of cerebral blood flow measurement for determination of respiratory status (238pH/Blood Gas System, Ciba Corning) and plasma glucose concentration (Glucose Analyser 2, Beckman). Body

temperature was maintained in normothermia by means of a heating blanket controlled by the rectal thermometer.

3.1.5 *Processing of Autoradiograms*

The frozen brains were embedded in a histological medium. Coronal sections were cut in a cryostat at -22°C, and 3 in every 13 sections were mounted in glass coverslips and dried on a hotplate at 60°C. Sections were apposed to X-ray film (Kodak GRL) along with a set of precalibrated [^{14}C] standards (concentration range 44-1475nCi/g) in light-tight X-ray cassettes for 14 days.

3.1.6 *Densitometric Analysis of Autoradiograms*

Measurement of optical density was carried out with two different approaches, using a computer based analysis system (Quantimet 970, Cambridge Instruments).

The first approach was to measure the optical density in 25 neuroanatomically predefined brain areas identified according to the atlas of König and Klippel (1963). Optical densities were determined in regions of interest (frame size 0.1-0.3mm^2 depending on region) in the hemispheres ipsilateral and contralateral to the occluded MCA, by taking the average of the results from six adjacent sections at each neuroanatomical locus.

The second approach was to determine the volume of cerebral tissue in both hemispheres perfused with various levels of cerebral blood flow using cumulative frequency distribution analysis. Optical density thresholds were set that corresponded to increments of cerebral blood flow, i.e. 0-10, 10-20, 20-25, 25-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, 100-250ml 100g^{-1} min^{-1}. The blood flow distribution was then assessed in both hemispheres at each of the

8 preselected coronal levels used for volumetric quantification of ischaemic brain damage (Table 1, Figure 11). The area of each hemisphere was delineated with the image analyser over one section at each of the 8 coronal planes, and the total area recorded. By setting limits of optical densities computed to correspond to particular ranges of cerebral blood flow, the size of areas corresponding to the regions within which cerebral blood flow was in this range, was obtained. The total volume of cerebral tissue within each cerebral blood flow range in both hemispheres was then computed by integration in a manner analogous to that used for volumetric assessment of ischaemic damage in the rat (Section 2.2.4). The volumes of cerebral blood flow within each range were then added in sequence to one another, to yield volumes of each hemisphere perfused by increasing ranges of cerebral blood flow.

3.1.7 *Calculation of Cerebral Blood Flow*

With each densitometric approach, local cerebral blood flow was calculated from (a) the time course history of [^{14}C]-iodoantipyrine in arterial blood, (b) [^{14}C] concentration in cerebral tissue and (c) the cerebral tissue:blood partition coefficient for iodoantipyrine (0.79), using the operational equation derived originally by Kety (1951) and as described by Sakurada et al. (1978) (Figure 4).

3.1.8 *Dosing Regimen*

CI-977 (0.3mg/kg), dissolved in a vehicle of isotonic saline, was administered as a s.c. injection 30 min before occlusion of the MCA. Control animals received a s.c. injection of the vehicle (1ml/kg) at the same time point.

$$Ci(T) = \lambda K \int_0^T C_a e^{-K(T-t)}\, dt$$

FIGURE 4

Operational equation for determination of the rate of cerebral blood flow. Ci(T)equals the tissue concentration of tracer at a given time, T, after the i.v. injection of the tracer; λ equals the tissue:blood partition coefficient; C_a is the tracer concentration in arterial blood; t is the variable time. K is a constant defined as m F/Wλ. F/W equals the rate of blood flow per unit mass of tissue; m is a constant equal to 1 that represents a measure of the unrestricted tracer diffusion across the blood-brain barrier.

The rate of blood flow per unit mass (F/W) can be calculated from the final concentration of tracer in cerebral tissue (Ci(T); obtained from autoradiograms), the concentration of tracer in arterial blood from times 0-T, and the tissue:blood partition coefficient of 0.79. (Adapted from Sakurada et al., 1978).

3.2 Glutamate Neurotoxicity *in vivo*

3.2.1 *Surgical Preparation*

Rats were anaesthetised, a tracheostomy performed, and gaseous anaesthesia, delivered via a mechanical ventilator, maintained thereafter as described previously (Section 2.2.1). Polyethylene catheters (Portex: external diameter 0.96mm; internal diameter 0.58mm; length 15cm) were inserted into both femoral arteries and one femoral vein to permit the continuous monitoring of MABP and repeated sampling of arterial blood, and the administration of drugs respectively.

3.2.2 *Microdialysis Probe Implantation*

The animals were turned prone and mounted in a stereotactic frame (Kopf, Clarke Electromedical). The right parietal skull was exposed and, under an operating microscope, a craniectomy was made with a saline-cooled dental drill. The dura was widely opened and the pia incised using a small needle. A microdialysis probe (CMA12: membrane length 3mm, outside diameter 0.5mm; Carnegie Medicine, Sweden) was angled at 15° to the sagittal plane and stereotactically implanted into the parietal cortex at a depth of 3.5mm. Stereotactic co-ordinates for probe placement were anteroposterior 0.0mm, mediolateral 4.0mm relative to Bregma.

3.2.3 *Glutamate Perfusion*

After placement in the cortex, the microdialysis probe was perfused with mock cerebrospinal fluid (CSF) (NaCl 135mM, KCl 1mM, KH_2PO_4 2mM, $CaCl_2$ 1.2mM, $MgCl_2$ 1mM, pH 7.4) at a flow rate of 1.5µl/min using a microinfusion pump (CMA/100; Carnegie Medicine, Sweden) for 30 min. The probe was then perfused with a 0.5M glutamate solution (0.5M monosodium glutamate

dissolved in mock CSF) for 90 min, after which time the probe was removed and the scalp wound closely sutured.

The size and reproducibility of the lesion is related to the glutamate concentration delivered by the microdialysis probe. Thus, on the basis of a prior investigation characterising the dose-dependency of glutamate in this novel *in vivo* model of excitotoxicity (Landolt et al. 1993), 0.5M glutamate was selected.

3.2.4 *Physiological Variables*

Body temperature was maintained in normothermia throughout by means of a heating blanket controlled by a rectal thermometer. Arterial blood gas analysis (238pH/Blood Gas System, Ciba Corning) and determination of plasma glucose concentration (Glucose Analyser 2, Beckman) were performed prior to drug/vehicle administration, at the start of glutamate perfusion and at one hourly intervals thereafter until sacrifice.

3.2.5 *Quantitation of Neuronal Damage*

Four hours after the start of glutamate perfusion (i.e. 2.5h after removal of the microdialysis probe) the animals were killed by decapitation, the brain removed, and frozen in isopentane at -42°C. 20μm coronal sections were cut on a cryostat. Three serial sections were taken at 200μm intervals throughout the brain and stained with H & E. The areas of brain damaged were measured directly from the histological sections on an image analyser (Quantimet 970, Cambridge Instruments). The damaged area at each level was determined by calculating the average of the 3 serial sections. The volume of damage was then derived by summing the damaged areas and multiplying by the interval thickness between sections.

3.2.6 *Dosing Regimen*

CI-977 (0.3mg/kg), in a vehicle of 5.5% glucose solution, was administered as an i.v. bolus injection 30 min before and 30 min after the start of glutamate perfusion. Control animals received the vehicle (1ml/kg, i.v.) at the same time points.

The AMPA receptor antagonist NBQX (30mg/kg i.v.; 30 min pre- and 30 min post-onset of glutamate perfusion) and the non-competitive NMDA receptor antagonist MK-801 (0.5mg/kg i.v.; 30 min prior to the start of glutamate perfusion) were also assessed for their efficacy against glutamate toxicity *in vivo*. The dosing paradigms were selected on the basis of their anti-ischaemic effects in the rat permanent MCA occlusion model (Gill et al., 1992b; Park et al., 1988b). The inclusion of NBQX necessitated the use of 5.5% glucose solution as the vehicle.

3.3 The Relationship between Glutamate Release and Cerebral Blood Flow after Focal Ischaemia in the Cat

3.3.1 *Surgical Preparation*

The cats were anaesthetised, intubated, and maintained thereafter under halothane anaesthesia delivered by a mechanical ventilator as described previously (Section 2.3.1). Polyethylene catheters (Portex: external diameter 1.65mm; internal diameter 1.0mm; length 30cm) were inserted into one femoral vein (for the administration of drugs) and two femoral arteries (for continuous monitoring of MABP, repeated sampling of arterial blood and controlled withdrawal of arterial blood for induction of hypotension). Haemorrhagic hypotension increases the severity of ischaemia (Osborne et al. 1987).

3.3.2 *Microdialysis Probe/Hydrogen Electrode Implantation*

The parietal skull was exposed and, under an operating microscope, craniectomies 7-10mm in diameter were made with a saline-cooled dental drill above the right and left suprasylvian gyri. A microdialysis probe (CMA 10: membrane length 1mm, outside diameter 0.5mm; Carnegie Medicine, Sweden) and an electrode (etched platinum/iridium wire, diameter: 250µm, teflon coated up to 1mm of the tip; Clarke Electromedical) were assembled with a tip-to-tip distance of 1mm. The dura was widely opened, and the pia arachnoid pierced with a small needle. Two probe/electrode assemblies were implanted stereotactically into the left suprasylvian gyrus, and one into the right suprasylvian gyrus, at a depth of 1.5mm. Following placement, craniectomies were sealed with cyanoacrylate glue, and assemblies fixed in place with dental cement.

3.3.3 *Dialysate Collection*

Microdialysis probes were perfused continuously with Krebs-Ringer bicarbonate solution (NaCl 122mM, KCl 3mM, $CaCl_2$ 1.2mM, $MgSO_4$ 1.2mM, KH_2PO_4 0.4mM, $NaHCO_3$ 25mM, pH 7.4) at a constant flow rates of 2µl/min using a microinfusion pump (CMA/100; Carnegie Medicine, Sweden). The probes were then left for a stabilisation period of approximately 2h, during which time any dialysate samples collected were discarded.

After the stabilisation period dialysates were collected in 20 min fractions, total volume 40µl. Two baseline samples were collected, and CI-977 or vehicle was administered 30 min prior to MCA occlusion. A further fraction was collected over the subsequent 20 min and the left MCA permanently occluded. Thereafter, MABP was reduced in 10-15mmHg decrements by withdrawal of arterial blood. Dialysate samples were taken

when MABP had stabilised at each decrement (approximately 10-15 min). This procedure was repeated down to a minimum MABP at 40mmHg. The animals were sacrificed by barbiturate overdose at the end of experiment.

3.3.4 *Measurement of Cerebral Blood Flow*

The platinum electrodes were used to measure cortical cerebral bood flow by means of the hydrogen clearance technique. During the stabilising period after assembly placement, the electrodes were connected to a reference circuit and polarised at a fixed voltage of +700mV. At the time of cerebral blood flow measurement the gaseous anaesthesia delivered was changed to a nitrous oxide/oxygen/hydrogen mixture (30%:30%:30%) containing 1-1.5% halothane. A hydrogen saturation period of 10 min was followed by 10 min for clearance. The cerebral blood flow was calculated using the initial slope index method. Data were collected over a 1 min period, 1 min after the hydrogen gas mixture had been removed. The data were fitted to an experimental curve and the flow calculated from the slope (Young, 1980). The first minute of the clearance curve was discarded to avoid artifacts due to recirculating hydrogen.

Cerebral blood flow values were determined simultaneously with the collection of each sample of dialysate.

3.3.5 *Induction of Focal Ischaemia*

The cat's head was placed in a stereotactic frame. The left MCA was exposed and occluded via a transorbital approach by an experienced neurosurgeon as described previously (Section 2.3.2).

3.3.6 *Physiological Variables*

Regular samples of arterial blood were taken throughout the experiment for determination of respiratory blood gas status (238pH/Blood Gas System, Ciba Corning) and plasma glucose concentration (Glucose Analyser 2, Beckman). Animals were maintained normothermic throughout by a thermostatically controlled heating blanket. A thermistor probe was also inserted periosteally, overlying the parietal cortex, via a scalp incision ipsilateral to the MCA to be occluded.

3.3.7 *Dosing Regimen*

CI-977 (0.3mg/kg) dissolved in isotonic saline was administered as a slow (3 min) intravenous injection 30 min before occlusion of the MCA. A constant intravenous infusion of CI-977 (0.15mg/kg/h) was initiated immediately and maintained thereafter until death. Control animals received a bolus injection of isotonic saline (1ml/kg i.v.) 30 min prior to MCA occlusion. An intravenous infusion of saline (0.15ml/min i.v.) was initiated immediately thereafter and maintained until death. Control animals were treated contemporaneously and randomised with the drug-treated animals. This is the same dosing regimen employed in the neuroprotection study in the cat (Section 2.3.5).

3.3.8 *Determination of Microdialysate Amino Acid Content*

Dialysate amino acids were detected fluorometrically following precolumn derivatisation with orthophthaldialdehyde according to the method of Lindroth and Mopper (1979). Separation of 6 amino acids of interest was achieved under these conditions, in the following order:

Aspartate, Glutamate, Asparagine, Serine, Glutamine, Glycine, Threonine, Arginine, Taurine, Alanine, GABA, Tyrosine.

(Analysis of microdialysate fractions was carried out by Dr. P.K. Hitchcott, Parke-Davis, Cambridge, U.K.).

3.4 Statistical Analysis

All data are presented as mean ± standard errors of the mean (SEM). Comparisons between groups were made using two-tailed Student's *t*-test. Intergroup comparisons were made using a paired *t*-test. One-way analysis of variance (ANOVA) followed by Student's *t*-test with appropriate Bonferroni correction was used for simultanous multiple comparisons.

In the glutamate release study the central hypothesis was primarily to examine the relationship between cerebral blood flow and glutamate release after permanent MCA occlusion in the cat, and whether CI-977 pretreatment had any effect on this relationship. The concept of dimensionality in scientific experiments is commonly ignored. In this study for example, in the control group there are 5 cats, each with 3 microdialysis probes in the cortex, sampling more than once at each cerebral blood flow decrement and thus it is impractical to suggest that each measurement is independent of another. The data in this investigation was therefore statistically analysed using an indicative Student's *t*-test, uncorrected for Bonferroni's adjustment. Parametric statistics were used even although there may have been evidence of an abnormal distribution of data.

4. THE EFFECT OF CI-977 ON CEREBRAL GLUCOSE USE AND CEREBRAL BLOOD FLOW IN THE NORMAL BRAIN

4.1 [^{14}C]-2-Deoxyglucose Autoradiography

4.1.1 *Surgical Preparation*

Polyethylene cannulae (Portex: external diameter 0.96mm; internal diameter 0.58mm; length 15cm) were inserted into both femoral arteries (to allow continuous monitoring of MABP and sampling of arterial blood) and one femoral vein (for administration of drugs and the radioactive tracer) during a brief period (approximately 35 min) of light halothane anaesthesia (1% in 70% nitrous oxide/30% oxygen) delivered via a face mask. The incision sites were infiltrated with a local anaesthetic gel (xylocaine, 2%) and closed. The animals were fitted with a loose fitting plaster cast around the pelvis and lower abdomen, with care taken not to restrict thoracic movements. The anaesthetic gas mixture was discontinued, and the animals allowed to recover for at least 2h before any further manipulations were performed. At this time the conscious, lightly restrained animals displayed normal grooming behaviour and appropriate reactions to slight auditory and sensory stimuli.

4.1.2 *Measurement of Local Cerebral Glucose Utilisation*

Local cerebral glucose use in the fully conscious rat was measured by [^{14}C]-2-deoxyglucose autoradiography as described by Sokoloff et al. (1977). Measurement was initiated by the i.v. administration, over 30 sec, of 50μCi [^{14}C]-2-deoxyglucose in 0.7ml of physiological saline. Fourteen timed arterial blood samples were taken throughout the following 45 min. The blood samples were centrifuged immediately, and the plasma assayed for [^{14}C]-tracer concentration (liquid scintillation analysis) and glucose concentration (Glucose Analyser 2, Beckman). Approximately 35 min after isotope

administration, a sample of arterial blood was taken for analysis of respiratory status (238pH/Blood Gas System, Ciba Corning). At 45 min post-isotope administration the animals were decapitated, and the brain rapidly removed and frozen in isopentane at -42°C prior to processing for autoradiography.

4.1.3 *Physiological Variables*

Samples of arterial blood were taken prior to drug/vehicle and radioisotope administration, and approximately 35 min after [^{14}C]-2-deoxyglucose administration for analysis of respiratory blood gas status (pO_2, pCO_2, pH). Rectal temperature was measured in all animals, and normothermia maintained by external heating lamps. Arterial pressure was monitored continuously.

4.1.4 *Processing of* [^{14}C]-*2-Deoxyglucose Autoradiograms*

20μm coronal sections were cut on a cryostat at -22°C, and 3 in every 13 sections were taken and thaw-mounted onto glass coverslips on a hotplate at 60°C. The brain sections were apposed to X-ray film (Kodak GRL) along with precalibrated plastic standards (concentration range 44-1475nCi/g) in a light-tight X-ray cassette for 14 days.

4.1.5 *Densitometric Analysis*

Local tissue concentrations of [^{14}C] were determined from the autoradiograms by means of a computer-based densitometry system (Quantimet 970, Cambridge Instruments). For each of the 45 brain regions examined in every animal, 12 bilateral density readings were made on a series of 6 consecutive sections in which the structure could be defined anatomically

by reference to the stereotaxic atlas of Paxinos and Watson (1986).

4.1.6 *Calculation of Glucose Use*

Local cerebral glucose utilisation was determined in all 45 regions of interest according to the operational equation described by Sokoloff et al. (1977) (Figure 5) from (a) the arterial plasma [^{14}C] and glucose histories (b) the [^{14}C] concentration in cerebral tissue and (c) the lumped constant and kinetic rate constants for the rat.

4.1.7 *Dosing Regimen*

CI-977 (0.03, 0.3, or 3mg/kg), freshly dissolved in a vehicle of isotonic saline, was injected i.v. over 60 secs, 15 min prior to the measurement of cerebral glucose utilisation. Control animals received a similar volume of the vehicle (1ml/kg) at the same time point.

4.2 [^{14}C]-Iodoantipyrine Autoradiography

4.2.1 *Surgical Preparation*

All rats were surgically prepared for [^{14}C]-iodoantipyrine autoradiography exactly as described previously (Section 4.1.1). The rats were allowed to recover from anaesthesia for at least 2h prior to any further treatment.

4.2.2 *Measurement of Local Cerebral Blood Flow*

Local cerebral blood flow was measured by [^{14}C]-iodoantipyrine autoradiography as described previously in the halothane-anaesthetised rat (Section 3.1.3) but with a minor modification of the method of Sakurada et al. (1978). A ramped infusion of 50μCi of [^{14}C]-iodoantipyrine in 0.7ml of saline was infused over 60 sec. The infusion rate during the first 5 sec was

$$Ri = \frac{Ci^*(T) - k_1^* e^{-(k_2^*+k_3^*)T} \int_0^T Cp^* e^{(k_2^*+k_3^*)t} dt}{K \cdot \left[\int_0^T (Cp^*/Cp)dt - e^{-(k_2^*+k_3^*)T} \int_0^T (Cp^*/Cp) e^{(k_2^*+k_3^*)t} dt\right]}$$

FIGURE 5

Operational equation for determination of the rate of cerebral glucose utilisation. The rate of glucose utilisation, Ri, in any region of cerebral tissue is calculated from: the total tissue concentration of [^{14}C] in that region, Ci^* (obtained from autoradiograms), at a given time T; the concentrations of [^{14}C]-2-deoxyglucose and glucose in the plasma (Cp^* and Cp respectively) at given times, t, throughout the experiment; the rate constants k_1^*, k_2^* and k_3^* for deoxyglucose transport to and from plasma and tissue precursor pools, and for phosphorylation of deoxyglucose by hexokinase; and from the lumped constant, K. K is composed of the relative distribution volumes for deoxyglucose and glucose, the ratio of Michaelis-Menten constants, the maximal velocities of hexokinase for deoxyglucose and glucose, and the fraction of glucose-6-phosphate which continues via the glycolytic pathway for further metabolism. (Adapted from Sokoloff et al., 1977).

13% of the final infusion rate and was increased progressively for each succeeding 5 sec interval. The ramp infusion schedule minimises equilibration in tissues with high cerebral blood flow with arterial blood during the period of measurement. Moreover, even in regions of low cerebral blood flow such as white matter, the intravascular isotope concentration (Ohno et al. 1987) leads to only an underestimation of cerebral blood flow of less than 5% under control conditions (Nehls et al. 1990).

4.2.3 *Physiological Variables*

Samples of arterial blood were analysed prior to administration of the drug/vehicle and radioisotope for determination of respiratory status (238pH/Blood Gas System, Ciba Corning) and arterial plasma glucose concentration (Glucose Analyzer 2, Beckman). Rectal temperature was measured in all animals, and the rats maintained normothermic by external heating lamps.

4.2.4 *Processing of* [^{14}C]-*Iodoantipyrine Autoradiograms*

20μm coronal sections were cut on a cryostat at -22°C, and 3 in every 13 sections were taken and thaw-mounted onto glass coverslips on a hotplate at 60°C. Autoradiograms were generated by exposing the brain sections along with a set of precalibrated standards (44-1475nCi/g) to X-ray film (Kodak SB-5) in light-tight X-ray cassettes for 6 days.

4.2.5 *Densitometric Analysis*

The mean optical density was measured for each region of interest as the average of 12 bilateral measurements on 6 consecutive sections. A computer based densitometer (Quantimet 970, Cambridge Instruments) with

several fixed frame sizes was made to measure the optical density in 45 predetermined loci, defined with reference to the atlas of Paxinos and Watson (1986).

4.2.6 *Calculation of Cerebral Blood Flow*

Local cerebral blood flow was determined in all 45 regions of interest as described previously (Section 3.1.7; Figure 4).

4.2.7 *Dosing Regimen*

CI-977 (0.3mg/kg) was injected i.v. over 60 secs, 15 min prior to the measurement of local cerebral blood flow. Control animals received an i.v. injection of the vehicle (isotonic saline, 1ml/kg) at the same time point.

4.3 Statistical Analysis

All data are presented as mean ± standard errors of the mean (SEM). Comparisons between experimental groups were made using unpaired, two-tailed Student's *t*-test. Analysis of variance (ANOVA) with subsequent intergroup comparison by Student's *t*-test with appropriate Bonferroni correction was used when multiple treatment groups were examined.

The relationship between local cerebral blood flow and local cerebral glucose use in vehicle-treated and drug-treated (CI-977, 0.3mg/kg) animals was assessed using the statistical strategy described previously (McCulloch et al., 1982). This involves a repeated measures analysis of log transformed data to identify the presence of heterogeneities in the relationship between local cerebral blood flow and glucose use. Regions in which the relationship is altered are identified by two sided follow-up tests.

CHAPTER III

RESULTS

1. CI-977 AND ISCHAEMIC BRAIN DAMAGE

1.1 24h (Recovery from Anaesthesia) Model in the Rat

1.1.1 *Post-Operative Recovery*

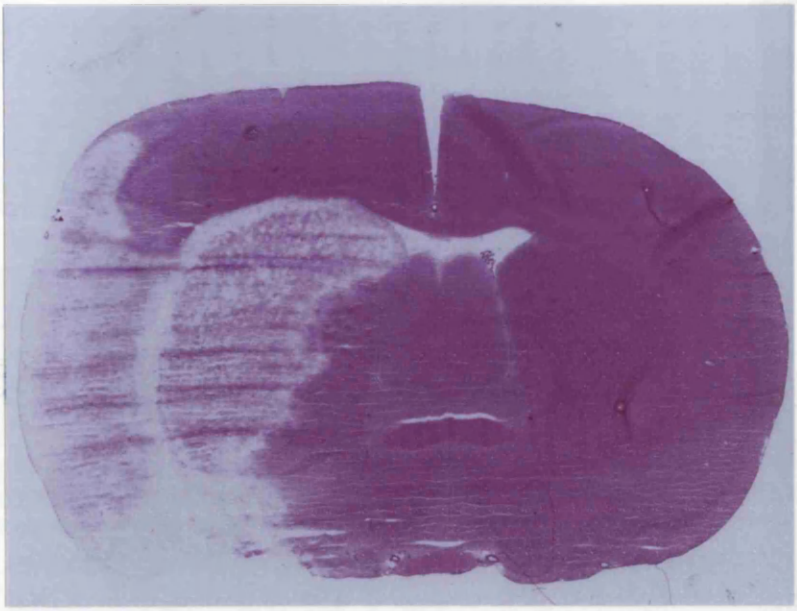
During the 24h post-occlusion survival period, all animals exhibited piloerection and adopted a hunched posture. The animals generally appeared apathetic and reluctant to move either spontaneously or in response to tactile stimulation; normal grooming behaviour and exploratory sniffing movements were absent in all animals.

Following cessation of anaesthesia, moderate to deep sedation (i.e. the animal was arousable with painful stimulation) and delayed awakening from anaesthesia was observed with the highest dose of CI-977 (3mg/kg) examined. Mild sedation (i.e. arousable with gently tactile stimulation and positive spontaneous activity) resulted from treatment with 0.3mg/kg CI-977, whilst no sedation was noted with the lowest dose of CI-977 (0.03mg/kg).

1.1.2 *Neuropathology Examination*

Permanent occlusion of the left MCA produced ischaemic damage predominantly within the frontal, sensory motor and parietal cortices, and the dorsolateral caudate nucleus. When outcome is assessed at 24h after the induction of ischaemia, the boundary between infarcted brain tissue and normal brain tissue is easily seen without magnification in the H & E stained sections (Figure 6). The area of pannecrosis in the cortex and caudate nucleus, and the extent of hemispheric swelling are readily delineated and quantified in the hemisphere ipsilateral to the occluded MCA.

Areas of greater infarction relative to swelling were observed in the rostral stereotactic coronal planes between anterior 10.5mm and anterior 6.06mm (Figure 6). In contrast, areas of greater swelling relative to



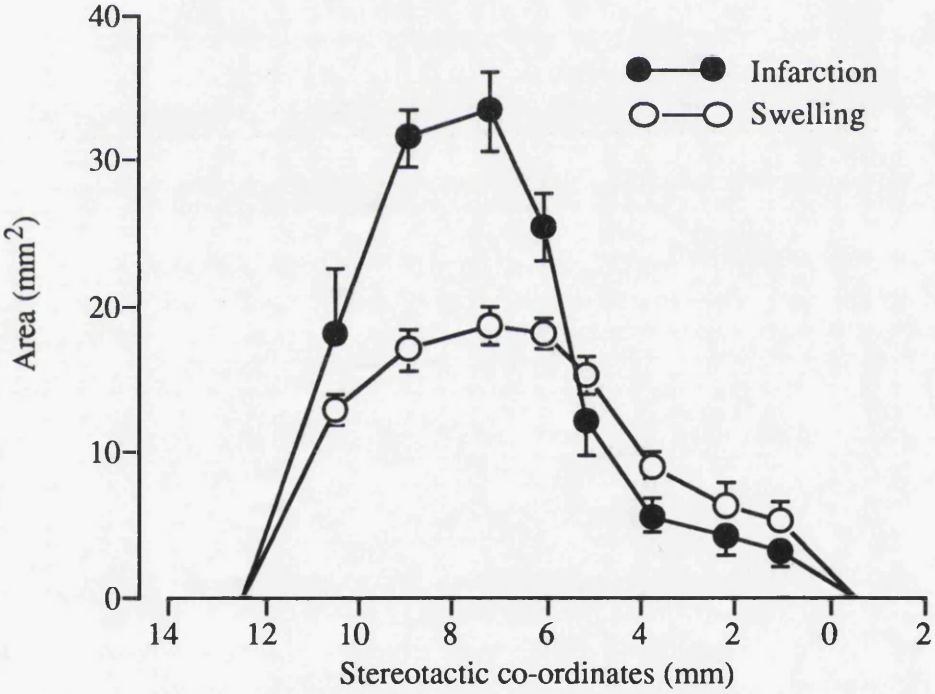


FIGURE 6

A representative photomicrograph (x7) of a coronal rat brain section taken at the level of the caudate nucleus and stained with H & E 24h after permanent MCA occlusion in a vehicle-treated control animal (upper) and the areas of infarction and areas of swelling at each of the 8 pre-determined stereotactic levels in all (n=8) control animals (lower). Data are presented as mean ± SEM. The region of the ischaemic infarct is evident as an area of pallor with a discrete boundary. In addition, note the extent of swelling in the ipsilateral hemisphere when compared to the contralateral hemisphere.

infarction were noted in the caudal coronal planes towards the margins of MCA territory, i.e. stereotactic planes posterior to plane 6.06mm anterior to the interaural line (Figure 6).

1.1.3 *Volumetric Assessment of Ischaemic Damage and Brain Swelling*

The s.c. administration of CI-977 (0.03, 0.3 or 3mg/kg) 30 min before and 30 min after MCA occlusion and at 6 hourly intervals thereafter produced dose-dependent reductions in the volume of infarction in the cerebral hemisphere, the most marked reduction in infarction (reduced by 38% from vehicle-treated controls; $p<0.02$) was observed with CI-977 (0.3mg/kg) (Figure 7).

Treatment with CI-977 initiated 30 min prior to MCA occlusion (and repeated at multiple times thereafter) produced dose-dependent reductions in the volume of brain swelling, with the most marked reduction in swelling (reduced by 31% from vehicle-treated controls; $p<0.01$) being observed with CI-977 (0.3mg/kg) (Figure 7). The volume of the hemisphere contralateral to MCA occlusion was unaltered by CI-977 treatment at any of the doses studied, e.g. control 562 ± 10mm^3; CI-977 (0.3mg/kg) 572 ± 12mm^3.

There was an excellent linear relationship between the volume of ischaemic damage and the volume of swelling (Figure 8) which was similar for vehicle-treated control animals and all CI-977-treated groups with an overall concentration coefficient $r = 0.896$ ($p<0.001$), and the slope of the relationship being 0.489.

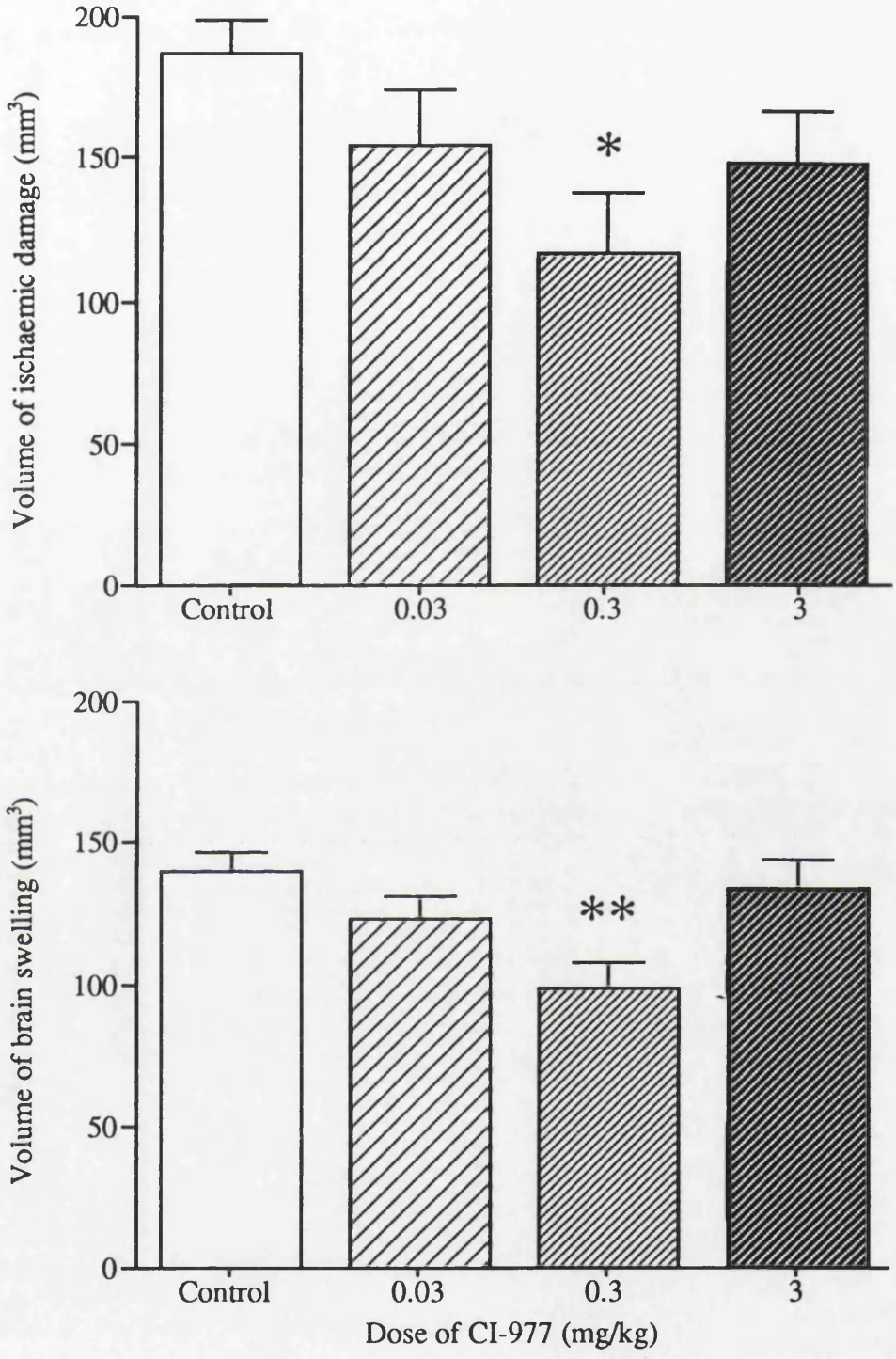


FIGURE 7

The effect of CI-977 upon the volume of infarction (upper) and the volume of brain swelling (lower) assessed 24h after permanent MCA occlusion in the rat. Data are presented as mean ± SEM with controls n=8; CI-977, 0.03mg/kg n=5; 0.3mg/kg n=8; 3mg/kg n=7. *P<0.02, **P<0.01 compared to vehicle-treated control animals (ANOVA followed by one-tailed *t*-test with Bonferroni correction).

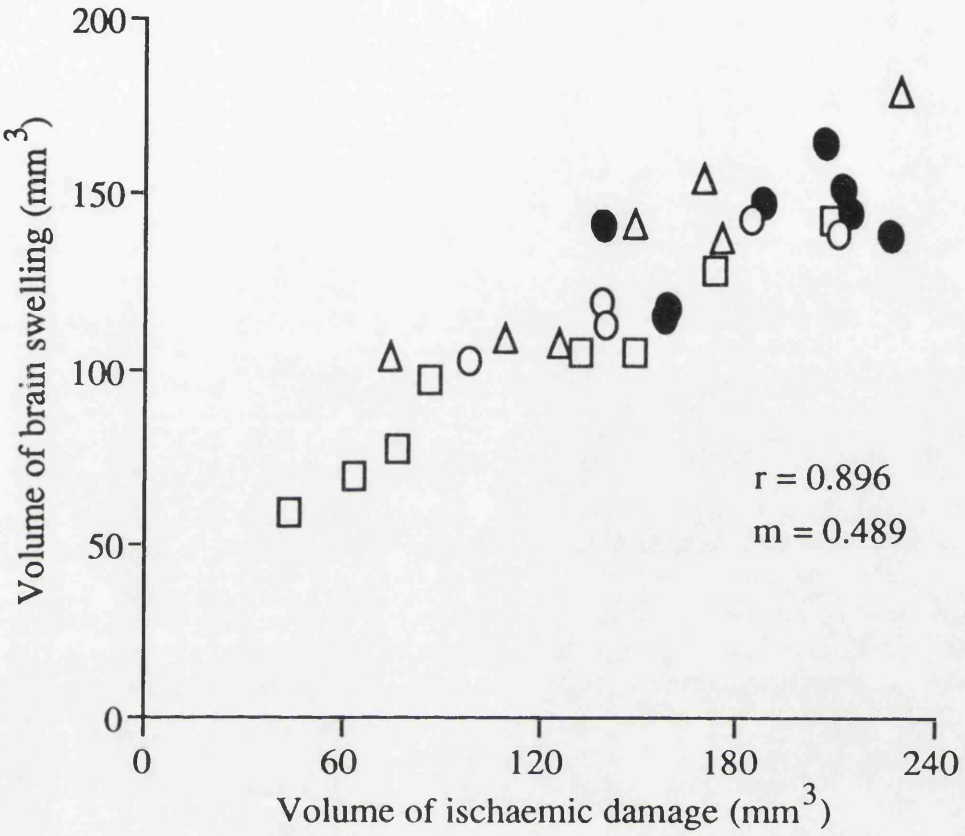


FIGURE 8

The relationship between the volume of infarction and the volume of swelling in individual animals 24h after the induction of ischaemia. There is an excellent ($P<0.001$) linear correlation between the two variables (r = Pearson's correlation coefficient, m = slope) which is similar for all treatments.
●, Vehicle; O, CI-977 (0.03mg/kg);□, CI-977 (0.3mg/kg); Δ, CI-977 (3mg/kg).

1.2 4h (Anaesthetised Throughout) Model in the Rat

1.2.1 *Cardiovascular and Respiratory Status*

The s.c. administration of CI-977 (0.3mg/kg) induced a marked and sustained hypotension. Five minutes after the first injection of CI-977, MABP was reduced by 30% of control levels (control: 93 ± 5mmHg; CI-977: 64 ± 3mmHg, $p<0.001$) (Figure 9). Significant hypotension persisted throughout the post-occlusion period. The second injection of CI-977 (0.3mg/kg) 30 min after MCA occlusion produced no further reduction in MABP (Figure 9). The administration of the vehicle (isotonic saline, 1ml/kg) did not alter MABP. There were no significant differences between the two groups with respect to respiratory blood gas status or rectal temperature, but the administration of CI-977 induced a significant reduction (10%; $p<0.05$) in arterial plasma glucose in the post-occlusion period (Table 2).

1.2.2 *Neuropathology Examination*

All brains were judged to be well perfusion fixed as evidenced by good neuronal morphology, the absence of intravascular blood and the lack of cytological artifacts such as "dark cells" or "hydropic cells" (Brown & Brierly, 1968; Cammermeyer, 1961). Permanent occlusion of the left MCA resulted in ischaemic damage only within the territory of the occluded MCA, i.e. in the dorsolateral cortex and neostriatum. These areas showed the morphological characteristics of early ischaemic cell change, with microvacuolation, shrinkage and triangulation of the nucleus and cytoplasm, increased basophilia of cytoplasm, perineuronal swollen astrocytes, and vacuolation of the neuropil (Figure 10). The border between normal cells and cells showing the morphological characteristics of early infarction was not apparent without magnification (Figure 10), but under light microscopy the

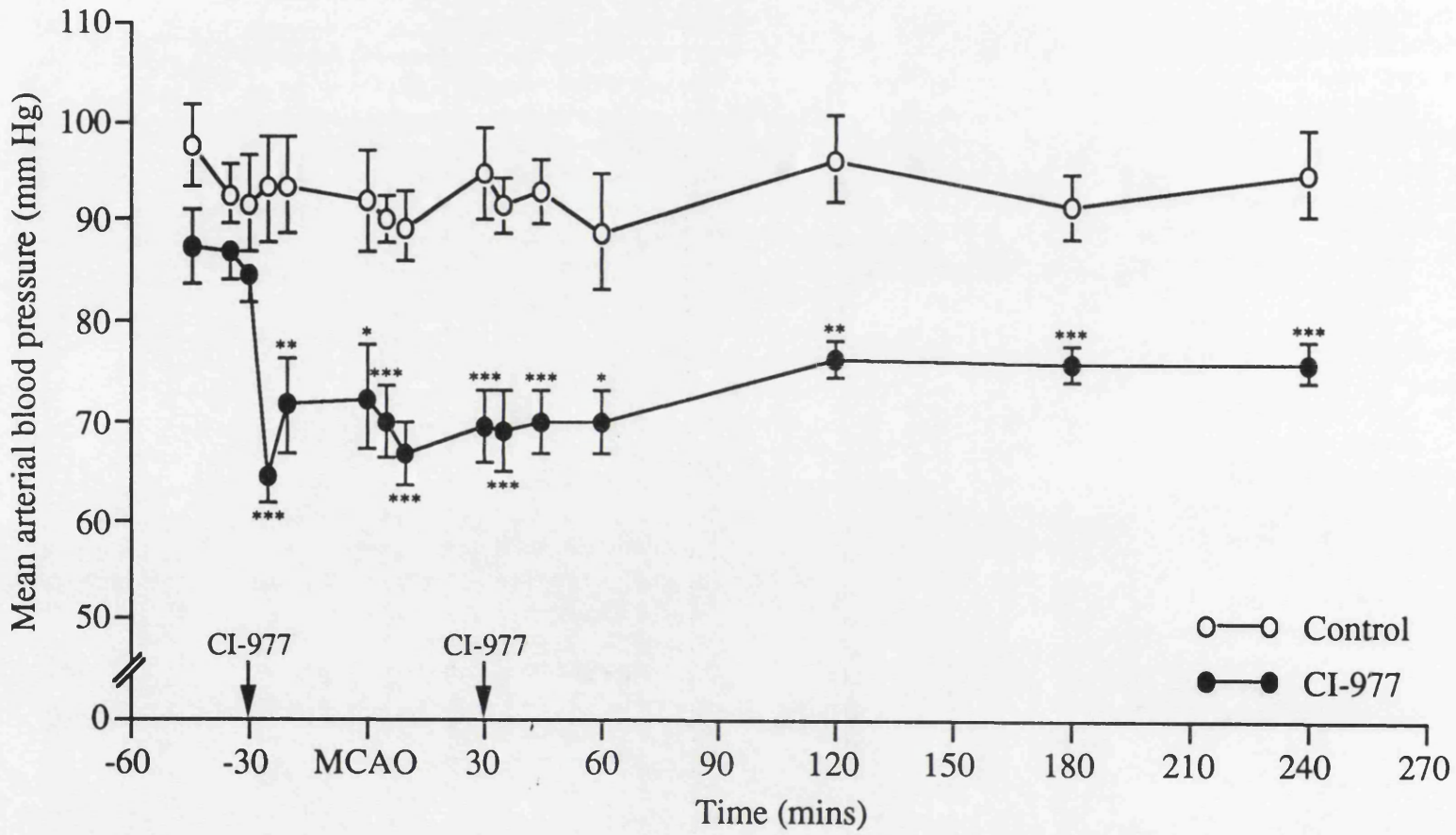


FIGURE 9

Time course of the alterations in mean arterial blood pressure following the administration of CI-977 (0.3mg/kg, s.c.) 30 min before and 30 min after permanent MCA occlusion in halothane-anaesthetised rats. Data are presented as mean ± SEM (n=7 vehicle, n=7 CI-977). *P<0.05, **P<0.01, ***P<0.001 (Student's two-tailed *t*-test).

TABLE 2

CARDIOVASCULAR, RESPIRATORY AND OTHER VARIABLES DURING THE PERIOD OF SURVIVAL AFTER MCA OCCLUSION IN THE RAT: EFFECTS OF CI-977

TIME AFTER MCAO (hrs)	TEMPERATURE (°C)	GLUCOSE (mM)	pO_2 (mmHg)	pCO_2 (mmHg)	pH
			CONTROL GROUP		
0	36.8 ± 0.1	11.0 ± 0.5	134 ± 5	43.5 ± 1.4	7.44 ± 0.01
1	36.9 ± 0.1	10.7 ± 0.5	153 ± 10	39.4 ± 1.6	7.46 ± 0.02
2	36.9 ± 0.1	11.0 ± 0.5	164 ± 10	38.8 ± 1.1	7.43 ± 0.02
3	37.0 ± 0.2	11.0 ± 0.4	168 ± 9	38.5 ± 0.8	7.42 ± 0.02
4	37.0 ± 0.2	10.5 ± 0.3	171 ± 8	40.2 ± 1.0	7.43 ± 0.02
			CI-977 TREATMENT GROUP		
0	36.8 ± 0.2	10.8 ± 0.4	137 ± 8	40.9 ± 1.5	7.50 ± 0.04
1	36.8 ± 0.1	8.9 ± 0.4*	160 ± 5	38.0 ± 1.4	7.47 ± 0.01
2	37.0 ± 0.1	8.9 ± 0.4*	160 ± 8	37.1 ± 0.9	7.46 ± 0.01
3	36.9 ± 0.1	9.4 ± 0.4*	150 ± 9	37.8 ± 0.6	7.44 ± 0.01
4	37.0 ± 0.1	9.6 ± 0.3*	157 ± 7	38.5 ± 0.5	7.44 ± 0.01

Data are presented as mean ± SEM (n=7 in each group). -Data represents values at time of MCA occlusion and at hourly intervals thereafter. CI-977 (0.3mg/kg, s.c.) or vehicle were administered 30 min before and 30 min after the induction of ischaemia. *$P<0.05$ for statistical comparison between vehicle-treated controls and CI-977-treated animals at the same time points (unpaired, two-tailed Student's *t*-test).

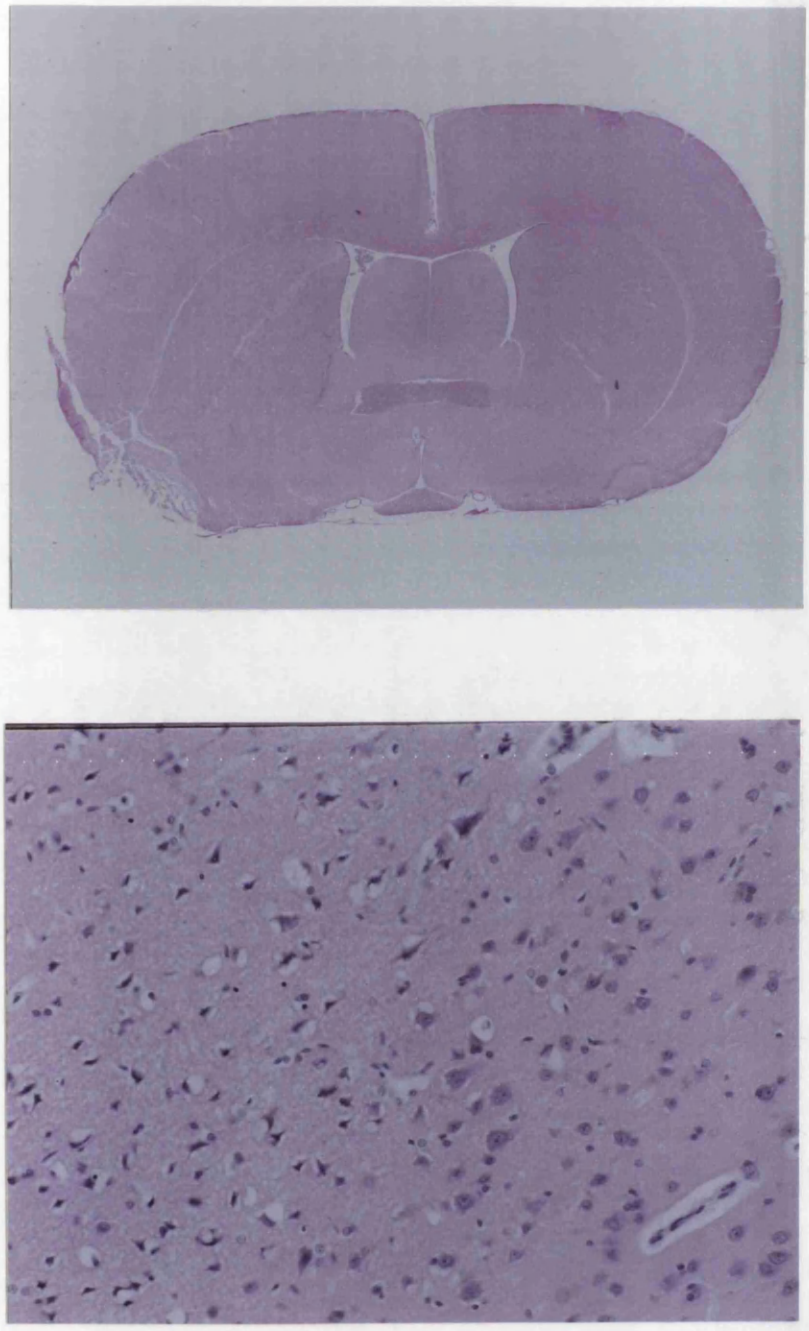


FIGURE 10

Low power (x7) photomicrograph of a coronal section perfusion fixed and stained with H & E 4h after permanent MCA occlusion in the rat showing poor macroscopic delineation of ischaemic cell change (left side of section) (upper) and a high power (x200) view of the same section showing a distinct border between the ischaemic lesion and morphologically normal tissue in the cerebral cortex (lower). Within the lesion, neurones that have undergone ischaemic cell change are triangular in shape with intensely stained nuclei, and the neuropil surrounding these neurones is pale and spongy in appearance. In contrast, neurones within the normal tissue are spherical with less densely stained nuclei and cytoplasm.

border was easily demarcated, thus enabling the delineation of areas of ischaemic damage from these H & E stained sections. The distribution of ischaemic brain damage when assessed at 4h post-occlusion is illustrated in Figure 11. The histological appearance of the ischaemic brain tissue in rats treated with CI-977 (0.3mg/kg) was similar to that in vehicle-treated control animals.

1.2.3 *Volumetric Assessment of Ischaemic Damage*

The administration of CI-977 (0.3mg/kg) 30 min before and again 30 min after MCA occlusion significantly reduced the volume of ischaemic damage in the cerebral hemisphere (reduced by 27% from vehicle-treated controls; $p<0.05$) and cerebral cortex (reduced by 32%; $p<0.05$) (Figure 12). The volume of ischaemic damage in the caudate nucleus was minimally influenced by treatment with CI-977 (Figure 12). The areas of ischaemic damage in the cerebral cortex and hemisphere was reduced by CI-977 to a similar degree at each coronal plane examined with a statistically significant reduction being noted only at plane anterior 3.75mm anterior to the interaural line (Figure 13). The areas of ischaemic damage in the caudate nucleus were not modified by the drug in any plane studied.

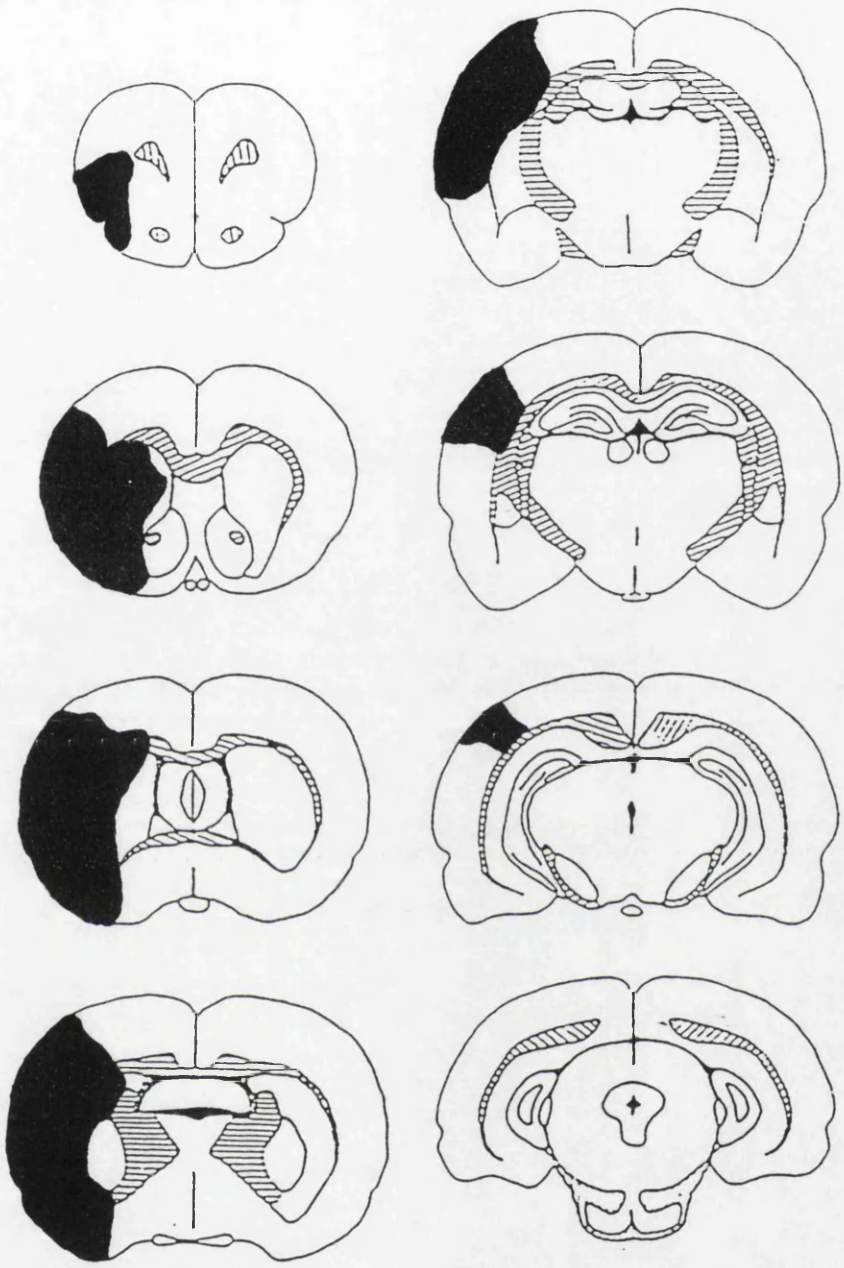


FIGURE 11

Distribution of ischaemic brain damage 4h after permanent MCA occlusion in a vehicle-treated control rat. Regions showing ischaemic cell change (shaded black) were determined at 8 defined coronal planes from plane anterior 10.5mm to anterior 1.02mm relative to the interaural line. The volumes of ischaemic damage in this animal were: cerebral hemisphere 131mm^3, cerebral cortex 109mm^3 and caudate nucleus 34mm^3.

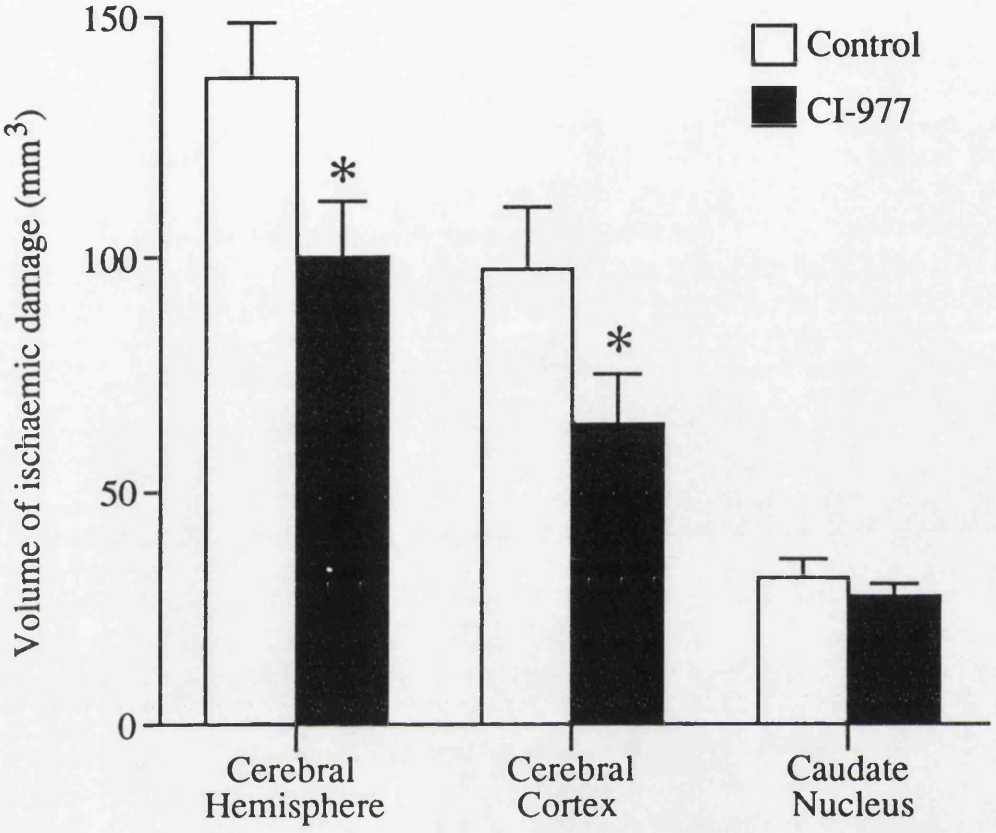


FIGURE 12

The effect of CI-977 upon the volume of ischaemic brain damage in the cerebral hemisphere, cerebral cortex and caudate nucleus assessed 4h after permanent MCA occlusion in halothane-anaesthetised rats. CI-977 (0.3mg/kg, s.c.) was administered 30 min before and 30 min after the onset of ischaemia. Data was presented as mean ± SEM (n=7 vehicle, n=7 CI-977). *P<0.05 (Student's one-tailed *t*-test).

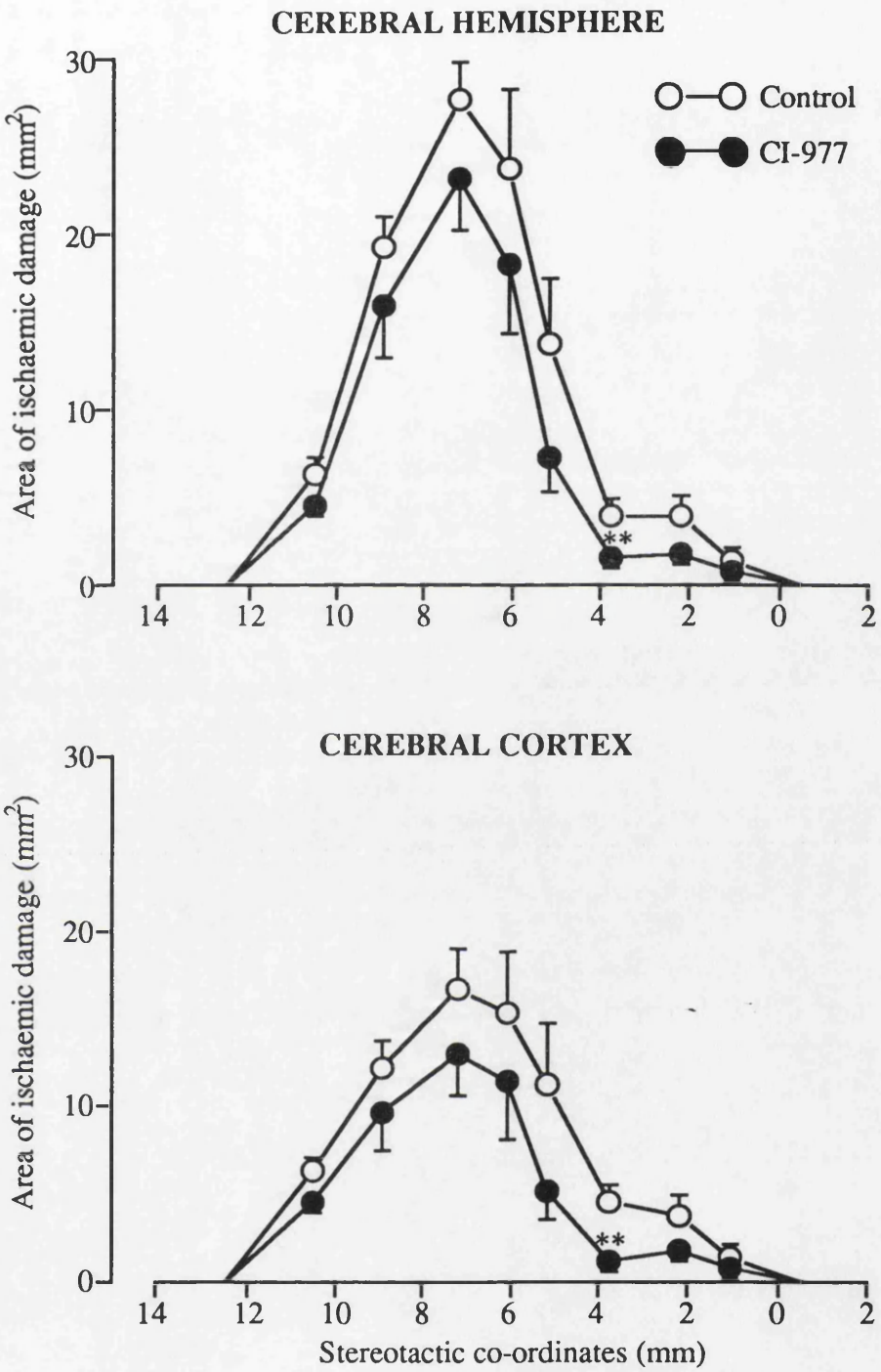


FIGURE 13

The effect of CI-977 upon the areas of ischaemic brain damage in the cerebral hemisphere (upper) and cerebral cortex (lower) at 8 pre-determined coronal planes assessed 4h after permanent MCA occlusion in halothane-anaesthetised rats. Data are presented as mean ± SEM (n=7 vehicle, n=7 CI-977). **P<0.01 (Student's two-tailed *t*-test).

1.3 6h (Anaesthetised Throughout) Model in the Cat

1.3.1 *Physiological Variables*

There were no significant differences between the vehicle-treated control group and CI-977-treated group in key systemic variables that are thought to influence the amount of ischaemic brain damage, e.g. MABP, arterial plasma glucose, core temperature and haematocrit at MCA occlusion and in the post-occlusion survival period (Figure 14, Table 3). There were no significant differences in arterial blood gas status (pO_2, pCO_2, pH) between the two groups at any time point (Table 3). There were also no differences between the control and CI-977-treated groups in either the inspired halothane concentration or the amount of Hartman's solution administered.

The bolus administration of CI-977 elicited a significant reduction in MABP by approximately 25% of control values only within the initial 5 min post-injection period ($p<0.01$, Student's unpaired *t*-test) (Figure 14). The hypotension was relatively transient such that there was no difference between the two groups in the level of MABP at the time of MCA occlusion or during the post-occlusion survival period. The administration of the vehicle (isotonic saline) did not alter MABP (Figure 14).

The plasma level of CI-977 increased slowly but progressively throughout the post-occlusion period (Figure 15). CI-977 induced a progressive increase in plasma osmolality throughout the post-occlusion period, which was significantly different from controls at 240 min ($p<0.01$) and 360 min ($p<0.05$) only (Figure 15).

1.3.2 *Neuropathology Examination*

All brains were judged to be well perfusion fixed as evidenced by good neuronal morphology, the absence of intravascular blood and the lack of

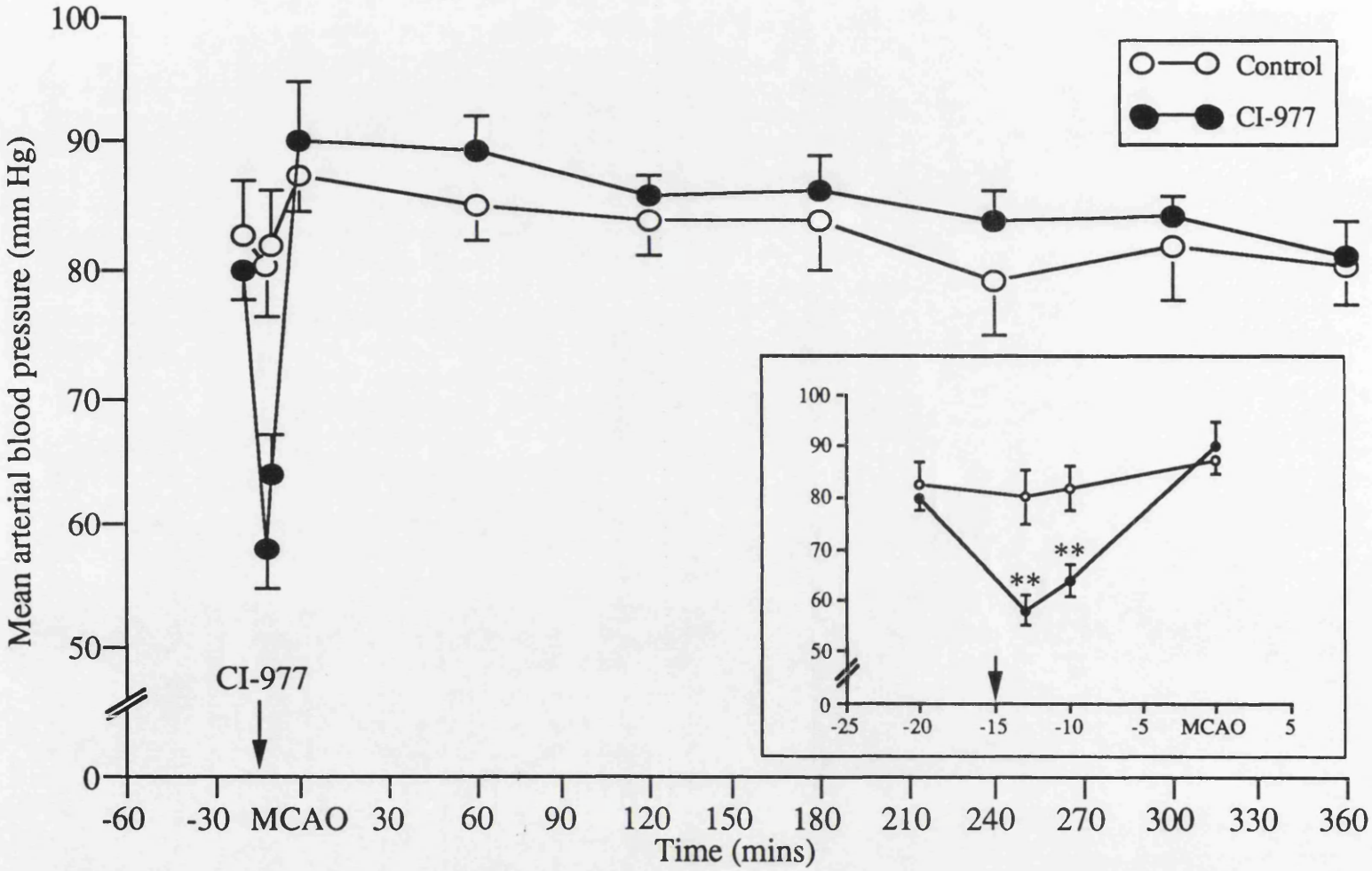


FIGURE 14

Time course of alterations in mean arterial blood pressure following the administration of CI-977 (0.3mg/kg, i.v.) 15 min prior to permanent MCA occlusion (MCAO) in halothane-anaesthetised cats. Insert depicts the alterations in blood pressure in the period immediately after CI-977 administration until the induction of ischaemia. Data are presented as mean ± SEM (n=8 control, n=7 CI-977). **P<0.01 (Student's two-tailed *t*-test).

TABLE 3

CARDIOVASCULAR, RESPIRATORY AND OTHER PHYSIOLOGICAL VARIABLES DURING THE PERIOD OF SURVIVAL AFTER MCA OCCLUSION IN THE CAT: EFFECTS OF CI-977

TIME AFTER MCAO (HRS)	TEMPERATURE (°C)	GLUCOSE (mM)	PO_2 (mmHg)	PCO_2 (mmHg)	pH	HAEMATOCRIT
			CONTROL GROUP			
0	36.7 ± 0.2	7.6 ± 0.4	172 ± 6	35.4 ± 2.1	7.39 ± 0.03	31.9 ± 1.9
1	36.6 ± 0.1	8.4 ± 0.4	164 ± 6	33.4 ± 1.0	7.39 ± 0.02	30.5 ± 1.5
2	36.6 ± 0.1	10.4 ± 0.7	168 ± 7	32.8 ± 1.1	7.39 ± 0.02	29.0 ± 1.8
3	36.6 ± 0.1	12.4 ± 1.0	165 ± 7	32.0 ± 0.7	7.38 ± 0.01	28.9 ± 1.6
4	36.6 ± 0.1	12.8 ± 1.0	168 ± 7	30.0 ± 0.8	7.40 ± 0.01	28.3 ± 2.0
5	36.7 ± 0.1	12.2 ± 0.5	164 ± 4	32.4 ± 1.0	7.37 ± 0.02	27.6 ± 2.2
6	36.7 ± 0.1	12.5 ± 0.6	161 ± 6	31.8 ± 0.9	7.39 ± 0.01	26.6 ± 1.6
			CI-977-PRETREATMENT GROUP			
0	36.4 ± 0.1	8.1 ± 0.6	156 ± 4	33.7 ± 1.6	7.44 ± 0.02	32.7 ± 2.9
1	36.4 ± 0.1	9.2 ± 0.7	161 ± 5	30.9 ± 1.3	7.44 ± 0.02	33.6 ± 2.3
2	36.6 ± 0.1	11.1 ± 0.8	161 ± 5	32.8 ± 1.7	7.40 ± 0.02	29.8 ± 3.4
3	36.7 ± 0.1	11.9 ± 1.0	151 ± 4	33.6 ± 1.4	7.40 ± 0.02	32.4 ± 2.0
4	36.6 ± 0.1	12.3 ± 1.0	162 ± 6	31.5 ± 1.8	7.37 ± 0.02	30.2 ± 2.2
5	36.5 ± 0.1	12.8 ± 1.0	162 ± 3	31.2 ± 1.0	7.39 ± 0.01	31.0 ± 1.9
6	36.6 ± 0.1	13.2 ± 0.9	159 ± 5	30.6 ± 0.6	7.39 ± 0.01	28.8 ± 2.3

Data are presented as mean ± SEM (n=8 control, n=7 CI-977). Data represent values at time of MCA occlusion and at hourly intervals thereafter. There are no significant differences between the vehicle-treated control group and the CI-977 treatment group at any time point for any variable.

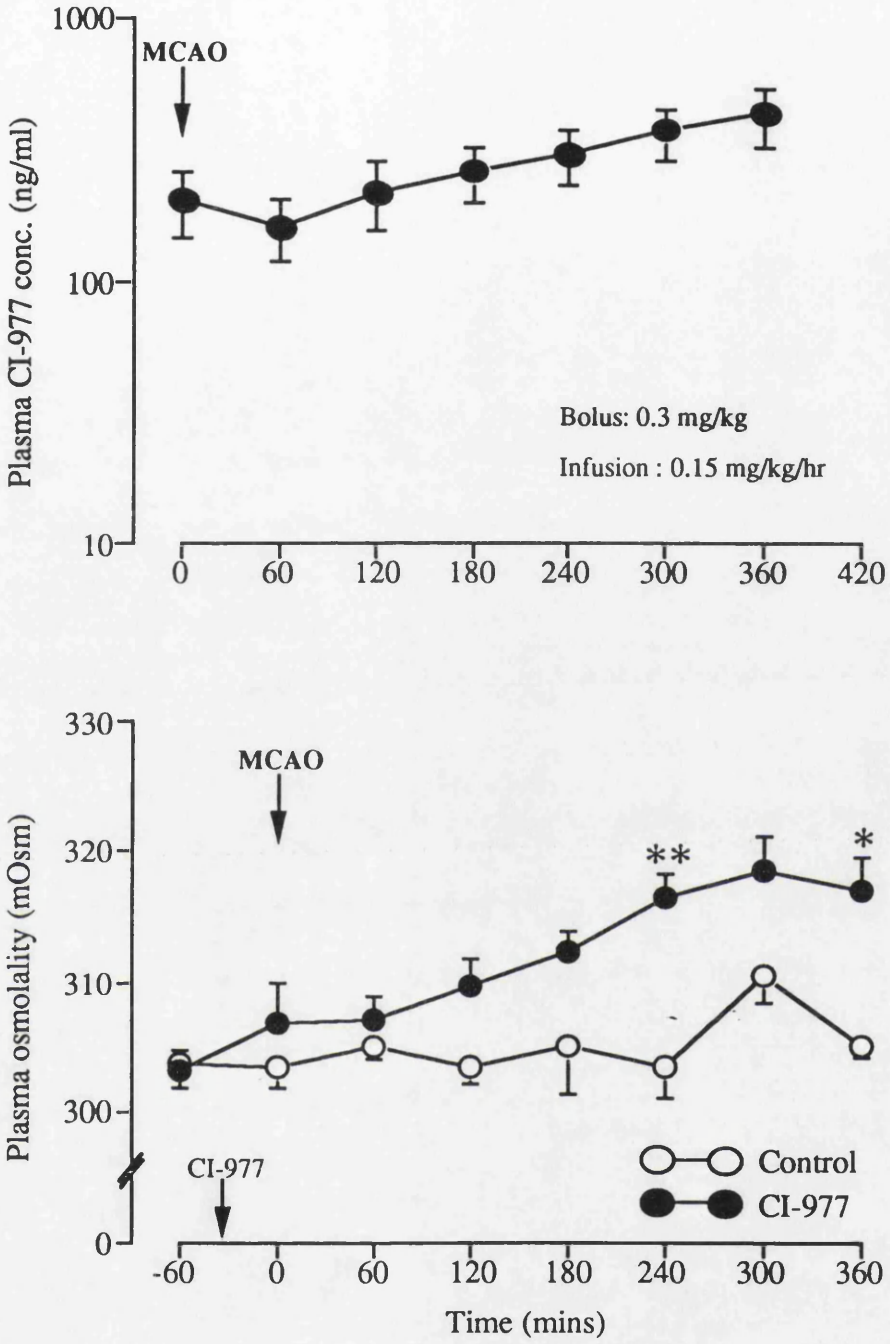


FIGURE 15

Plasma levels of CI-977 presented on a log scale (upper) and the effect of CI-977 upon plasma osmolality (lower). Treatment was initiated 15 min prior to MCA occlusion (MCAO at time zero) in halothane-anaesthetised cats. Data are presented as mean ± SEM (n=3 control, n=7 CI-977). *P<0.05, **P<0.01 (Student's two-tailed *t*-test).

cytological artifacts such as "dark cells" or "hydropic cells" (Brown and Brierly, 1968; Cammermeyer, 1961). Coronal sectioning of the brains revealed the interior of the brains to be well fixed other than in the deeper portions of the left hemisphere where zones of pink discolouration could be seen. Ischaemic damage was observed only within the territory of the occluded MCA, i.e. in the dorsolateral cortex and in the neostriatum. These areas showed the morphological characteristics of early infarction (microvacuolation, shrinkage of the neuropil, triangulation and hyperchromasia of the cell body and nucleus exactly as observed in rat ischaemic territory (Figure 10). The distribution of ischaemic damage in the cat forebrain at 6h after the induction of ischaemia is shown in Figure 16. The histological appearance of the ischaemic tissue was similar in all animals (vehicle-treated controls and CI-977-treated).

1.3.3 *Volumetric Assessment of Ischaemic Damage*

The administration of CI-977 initiated 15 min before MCA occlusion reduced the volume of ischaemic damage due to occlusion of the MCA in the cerebral hemisphere (reduced by 33% from vehicle-treated controls) and cerebral cortex (reduced by 42%) (Figure 17). The volume of ischaemic damage in the caudate nucleus was not altered by treatment with CI-977 (Figure 17). The areas of ischaemic damage in the cerebral cortex and hemisphere were reduced by CI-977 to a similar degree at each coronal plane examined, with statistically significant reductions being noted between planes anterior 14mm and posterior 2mm (Figure 18). The areas of ischaemic damage in the caudate nucleus were not modified by the drug in any plane studied.

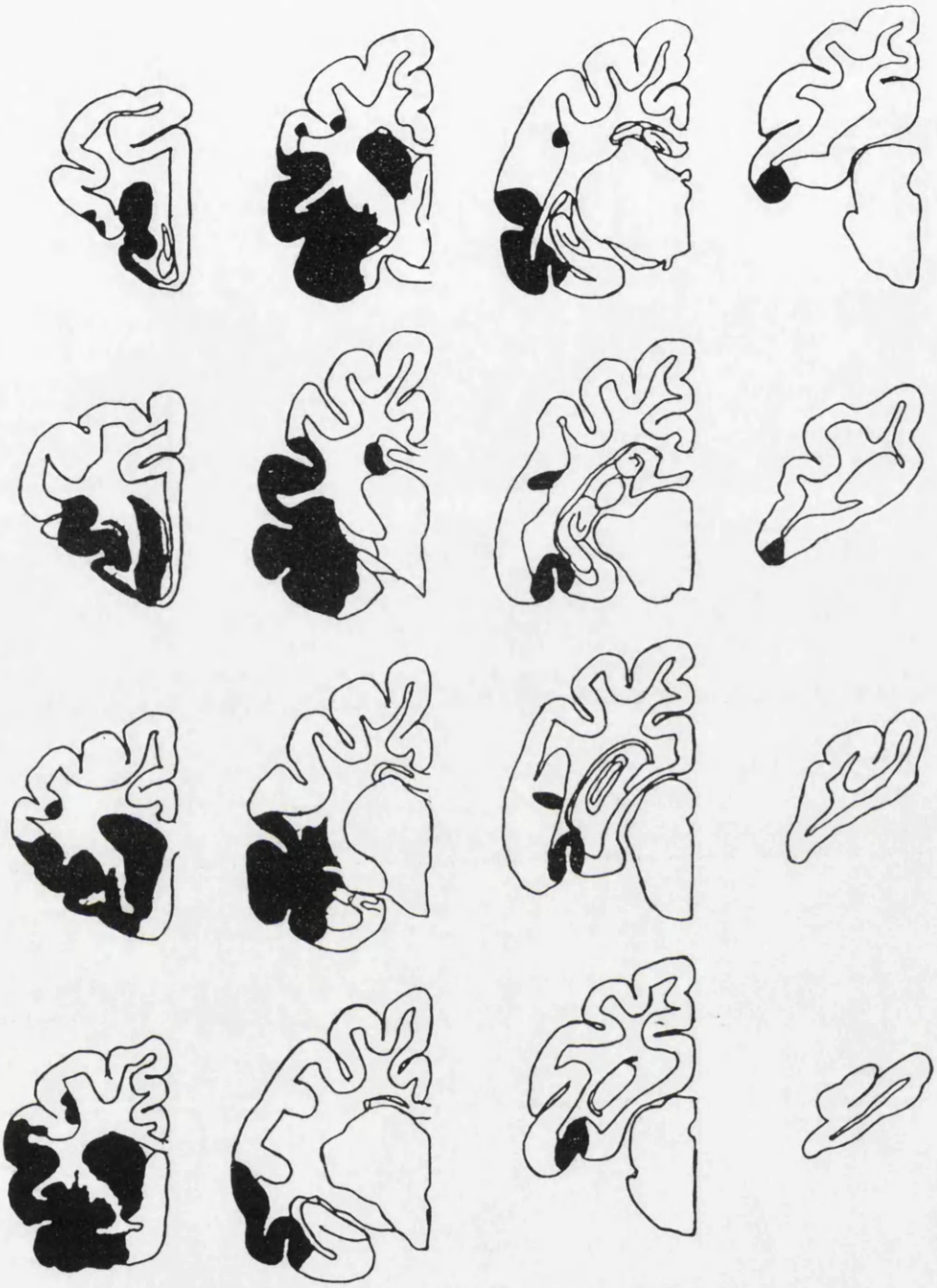


FIGURE 16

Distribution of ischaemic brain damage 6h after permanent MCA occlusion in a vehicle-treated control cat. Regions showing ischaemic cell change (shaded black) were determined at 16 coronal planes from plane anterior 22mm to posterior 8mm relative to the interaural line. The volume of ischaemic damage in this animal were: cerebral hemisphere 2415mm^3, cerebral cortex 1857mm^3 and caudate nucleus 257mm^3.

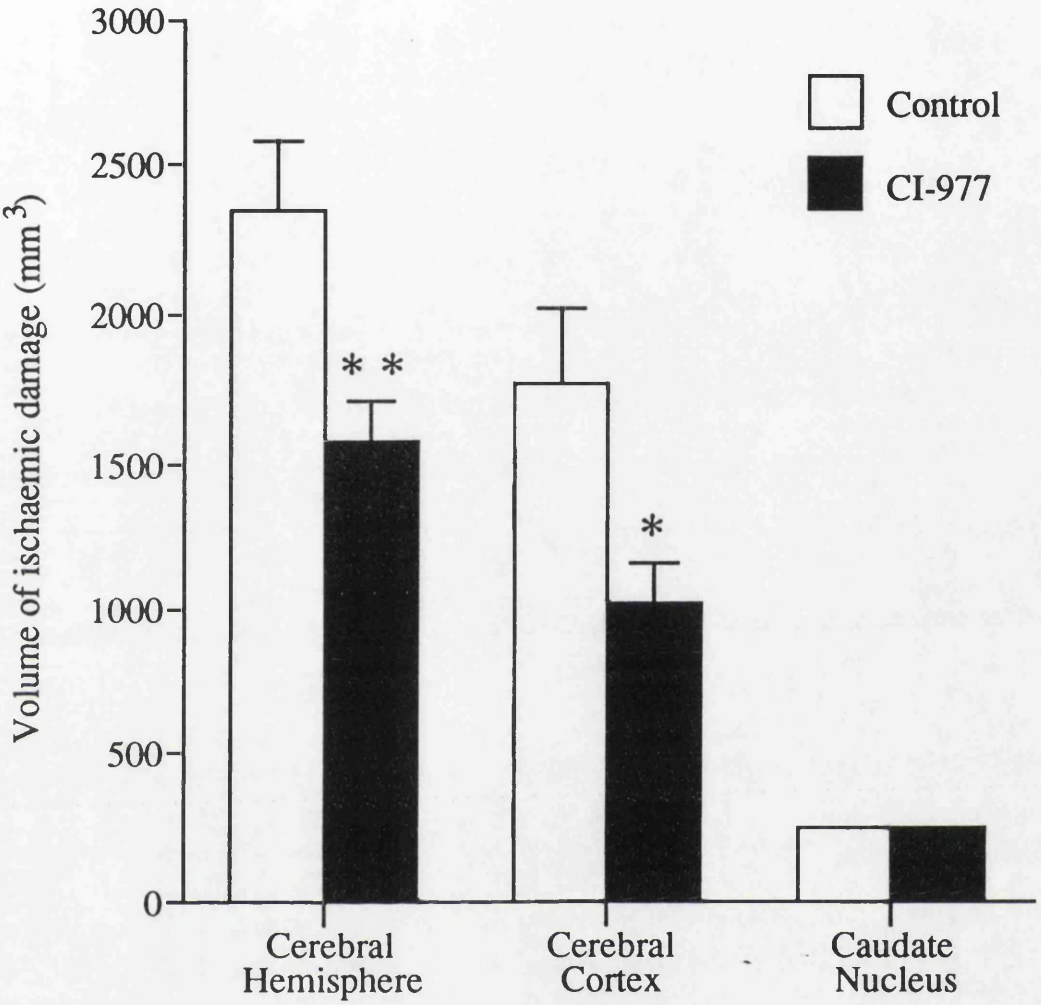


FIGURE 17

The effect of pretreatment with CI-977 (0.3mg/kg, i.v.) upon the volume of ischaemic brain damage in the cerebral hemisphere, cerebral cortex and caudate nucleus 6h after permanent MCA occlusion in halothane-anaesthetised cats. Data are presented as mean ± SEM (n=8 control, n=7 CI-977). *P<0.05, **P<0.01 (Student's one-tailed *t*-test).

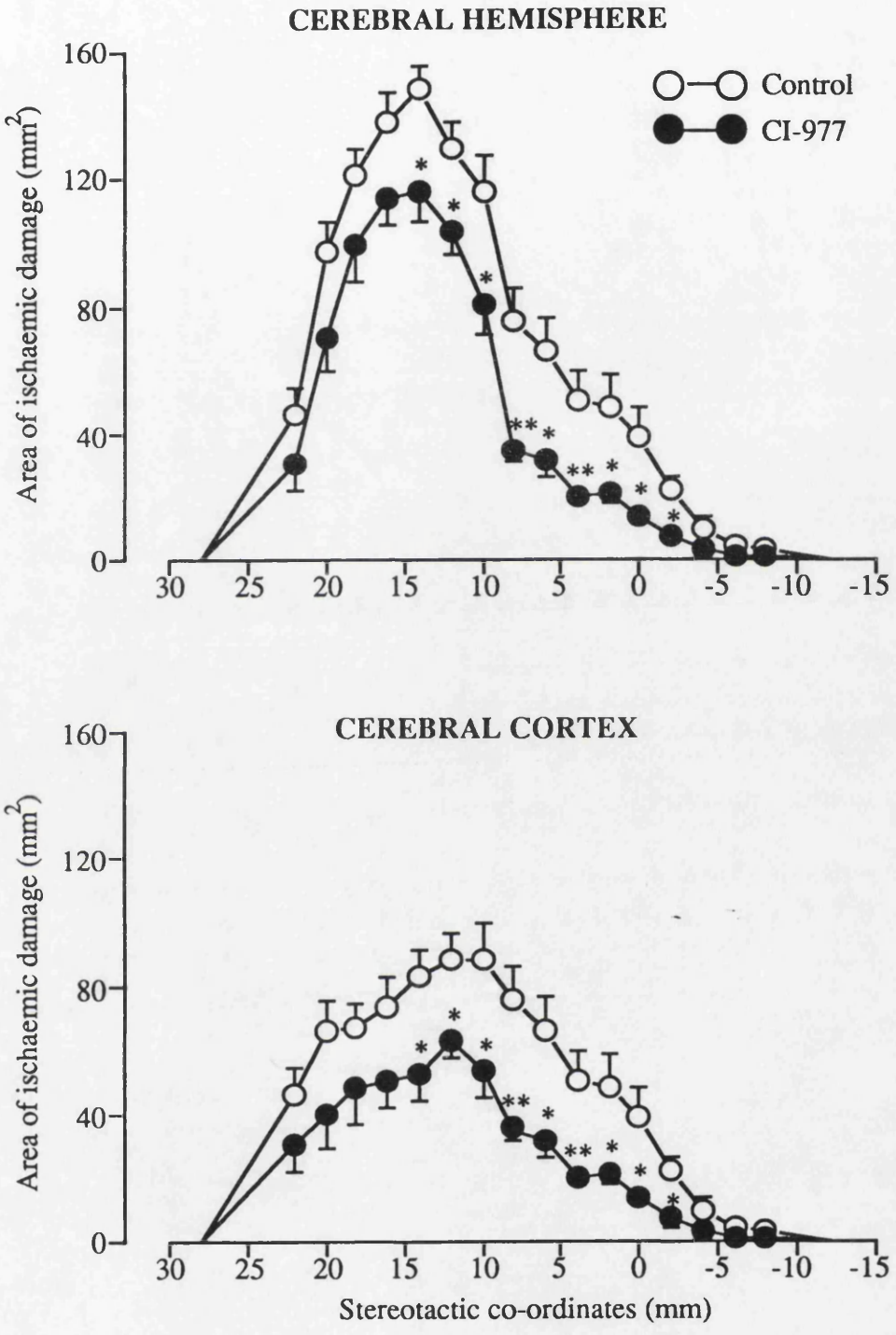


FIGURE 18

The effect of CI-977 (0.3mg/kg, i.v.) administered 15 min prior to MCA occlusion upon the areas of ischaemic brain damage in the cerebral hemisphere (upper) and cerebral cortex (lower) at 16 defined coronal planes 6h after the induction of ischaemia in halothane-anaesthetised cats. Data are presented as mean ± SEM (n=8 control, n=7 CI-977). *P<0.05, **P<0.01 (Student's two-tailed *t*-test).

2. MECHANISMS UNDERLYING THE ANTI-ISCHAEMIC EFFICACY OF CI-977

2.1 CI-977 on Cerebral Blood Flow after MCA Occlusion in the Rat

2.1.1 *Cardiovascular and Respiratory Status*

The administration of CI-977 (0.3mg/kg) effected a marked reduction in MABP (from 92 ± 3mmHg prior to drug administration to 67 ± 3mmHg 5 min after administration; $p<0.01$). Thereafter, MABP returned gradually toward its pre-administration levels. At the time of MCA occlusion, MABP in CI-977-treated animals was still 20% lower than its pre-administration value, and at this stage was significantly different from vehicle-treated controls (Table 4). Significant hypotension persisted throughout the post-occlusion period, such that at the time of cerebral blood flow measurement, MABP was still significantly reduced by 16% of control levels. The administration of the vehicle (isotonic saline, 1ml/kg) did not alter MABP. There were no significant differences between the two groups with respect to respiratory blood gas status or rectal temperature, but the administration of CI-977 induced a significant reduction (15%) in arterial plasma glucose concentration (Table 4). The effects of CI-977 on monitored physiological variables in the present cerebral circulatory study were similar to those observed in a parallel neuropathology study of the neuroprotective effects of CI-977 in the same model (Section 1.2.1).

2.1.2 *Cerebral Blood Flow in Neuroanatomically Defined Regions*

Visual inspection of the autoradiograms from both vehicle-treated and CI-977-treated groups showed marked reductions in local cerebral blood flow in the hemisphere ipsilateral to the MCA occlusion as compared with the contralateral hemisphere. The reductions were most obvious in the neocortex and caudate nucleus.

TABLE 4

CARDIOVASCULAR, RESPIRATORY AND OTHER PHYSIOLOGICAL VARIABLES FOR INVESTIGATION OF CEREBRAL BLOOD FLOW AFTER MCA OCCLUSION IN THE RAT: EFFECTS OF CI-977

	MCAO		CBF	
VARIABLE	CONTROL	CI-977	CONTROL	CI-977
Rectal Temperature (°C)	36.7 ± 0.1	36.9 ± 0.1	36.9 ± 0.1	36.9 ± 0.1
MABP (mmHg)	82 ± 4	71 ± 3*	82 ± 3	69 ± 3*
Arterial Plasma Glucose (mM)	10.2 ± 0.2	8.3 ± 0.2*	10.4 ± 0.2	8.1 ± 0.2*
pCO_2 (mmHg)	38.1 ± 1.1	40.8 ± 1.2	37.6 ± 0.5	39.4 ± 0.7
pO_2 (mmHg)	141 ± 8	132 ± 7	145 ± 8	142 ± 8
pH	7.46 ± 0.02	7.45 ± 0.01	7.46 ± 0.01	7.44 ± 0.01

Data are presented as mean ± SEM (n=7 control; n=8 CI-977). MCAO, data measured at time of MCA occlusion; CBF, data measured at time of CBF measurement. CI-977 (0.3mg/kg, s.c.) administered 30 min prior to the induction of ischaemia. *$P<0.05$ for statistical comparison between vehicle-treated controls and CI-977-treated animals at the same time points (unpaired two-tailed Student's t-test).

In the non-ischaemic hemisphere, the administration of CI-977 (0.3mg/kg) 30 min prior to the induction of ischaemia, failed to produce a significant effect on local cerebral blood flow in any of the 25 areas examined (Table 5).

In the ischaemic hemisphere, CI-977 failed to demonstrate a significant alterations in the level of cerebral blood flow in 23 of the 25 regions examined (Table 5). However, significant increases in cerebral blood flow were observed in the lateral thalamus and medial caudate nucleus at the boundary of the severe hypoperfusion (local cerebral blood flow increased by 86% and 65% respectively when compared to vehicle) (Figure 19, Table 5). In regions in which the blood supply is derived from the occluded MCA (i.e. frontal, sensory motor, parietal and auditory cortices and caudate nucleus (dorsolateral)), no statistically significant differences in blood flow were demonstrated between vehicle-treated and CI-977-treated animals (Table 5).

2.1.3 *Frequency Distribution Analysis of Cerebral Blood Flow*

CI-977 failed to produce a significant effect on the amount of hypoperfused tissue or weighted average cerebral blood flow in either the hemisphere ispilateral to MCA occlusion or in the contralateral hemisphere (Figure 20). The was a negligible amount of tissue (less than 1%) with blood flow less than 30ml $100g^{-1}$ min^{-1} in the contralateral hemisphere in either vehicle-treated or CI-977-treated animals (Figure 20).

Median blood flow in the ipsilateral hemisphere was 53 ± 6ml $100g^{-1}$ min^{-1} in vehicle-treated animals and 72 ± 9ml $100g^{-1}$ min^{-1} in CI-977-treated animals (p=0.119). In the contralateral hemisphere median blood flow was 106 ± 17ml $100g^{-1}$ min^{-1} in vehicle-treated animals and 113 ± 10ml $100g^{-1}$ min^{-1} in CI-977-treated animals (p=0.742).

TABLE 5

EFFECTS OF CI-977 ON CEREBRAL BLOOD FLOW AFTER PERMANENT MCA OCCLUSION IN THE RAT

	IPSILATERAL TO MCAO		CONTRALATERAL TO MCAO	
STRUCTURE	CONTROL	CI-977	CONTROL	CI-977
Frontal Cortex	40 ± 6	64 ± 10	127 ± 13	129 ± 15
Sensory Motor Cortex	30 ± 5	38 ± 11	130 ± 12	116 ± 12
Parietal Cortex	42 ± 6	71 ± 13	120 ± 12	124 ± 8
Auditory Cortex	46 ± 8	68 ± 13	120 ± 14	125 ± 13
Visual Cortex	53 ± 4	69 ± 12	111 ± 14	135 ± 9
Thalamus, Mediodorsal	101 ± 15	122 ± 10	127 ± 16	153 ± 14
Thalamus, Lateral Hyperaemic Zone	105 ± 15	195 ± 21**	105 ± 11	121 ± 10
Lateral Habenula	161 ± 16	195 ± 6	166 ± 18	200 ± 6
Hypothalamus	80 ± 9	108 ± 9	89 ± 11	115 ± 10
Hippocampus, Molecular Layer	100 ± 11	112 ± 9	103 ± 17	97 ± 9
Caudate Nucleus, Lateral	10 ± 2	9 ± 3	126 ± 20	134 ± 16
Caudate Nucleus, Medial	12 ± 1	18 ± 4	121 ± 16	138 ± 16
Caudate Nucleus, Hyperaemic Zone	121 ± 12	200 ± 13***	102 ± 12	111 ± 9
Globus Pallidus	133 ± 18	159 ± 13	95 ± 10	100 ± 8
Red Nucleus	130 ± 17	146 ± 13	137 ± 17	161 ± 15
Subthalamic Nucleus	168 ± 23	190 ± 13	167 ± 20	185 ± 15
Vestibular Nucleus	171 ± 20	178 ± 13	177 ± 20	184 ± 13
Superior Olive	187 ± 25	215 ± 14	188 ± 23	225 ± 22
Inferior Colliculus	151 ± 20	180 ± 15	155 ± 18	189 ± 17
Inferior Olive	166 ± 16	170 ± 15	177 ± 19	179 ± 16
Cerebellar Hemisphere	88 ± 8	96 ± 10	81 ± 8	85 ± 8
Cerebellar Nucleus	193 ± 19	202 ± 18	191 ± 16	191 ± 17
Corpus Callosum	47 ± 6	61 ± 8	47 ± 6	60 ± 8
Genu	37 ± 3	53 ± 9	49 ± 5	58 ± 8
Internal Capsule	64 ± 9	75 ± 5	63 ± 9	77 ± 6

Data are presented as mean ± SEM (n=7 control; n=8 CI-977) and expressed as ml $100g^{-1}$ min^{-1}. MCAO 30 min prior to CBF determination. CI-977 (0.3mg/kg) administered 30 min prior to MCAO. **P<0.01, ***P<0.001 for statistical comparison between vehicle-treated controls and CI-977-treated group in each structure (unpaired, two-tailed Student's *t*-test).

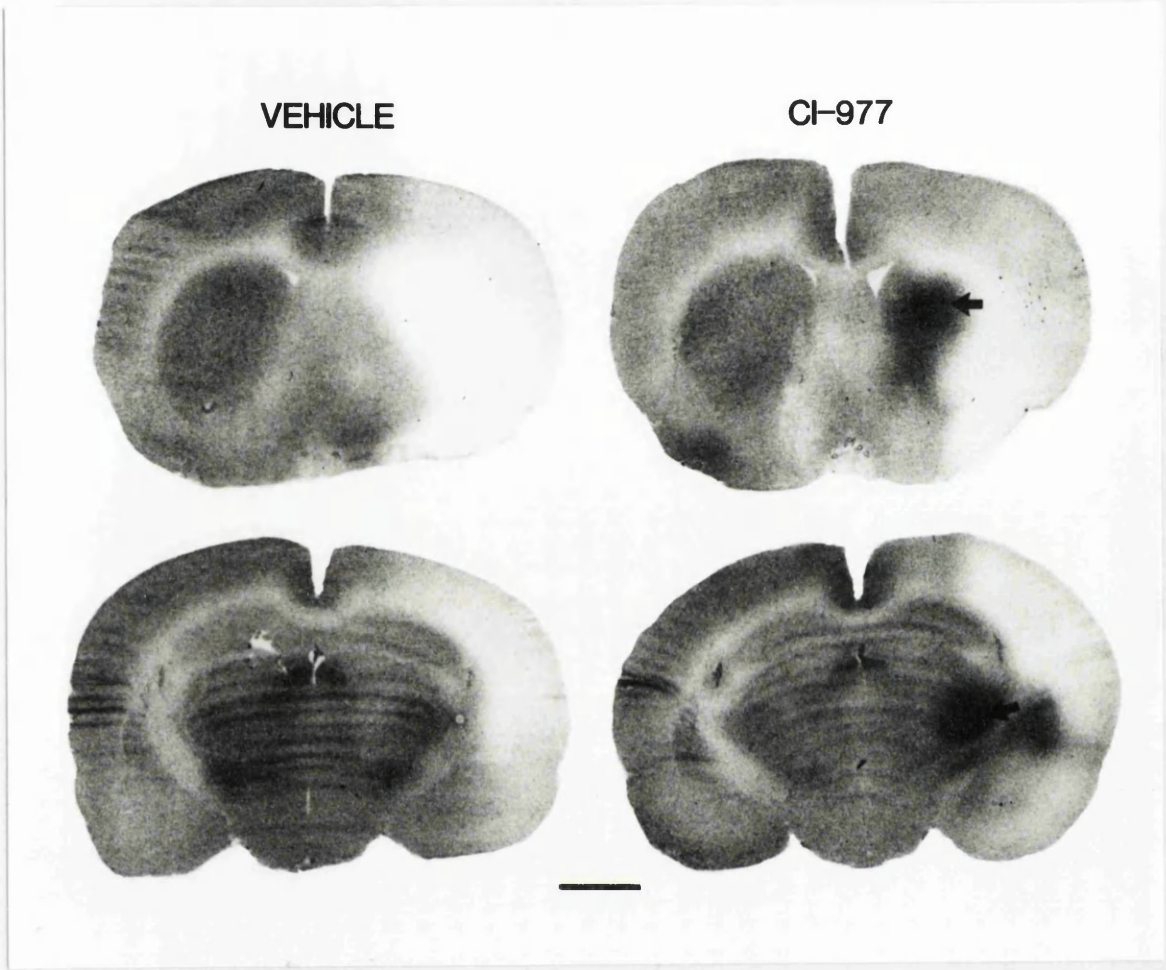


FIGURE 19

[^{14}C]-Iodoantipyrine autoradiograms illustrating the effect of CI-977 upon the pattern of cerebral blood flow at the level of the medial caudate nucleus (upper) and lateral thalamus (lower) 30 min after permanent MCA occlusion in halothane-anaesthetised rats. CI-977 (0.3mg/kg, s.c.) was administered 30 min before MCA occlusion. Relative levels of local cerebral blood flow are directly related to relative optical density. Note the areas of hyperaemia (indicated by arrows) at the boundaries of the zones of severe hypoperfusion. Bar = 2.5mm.

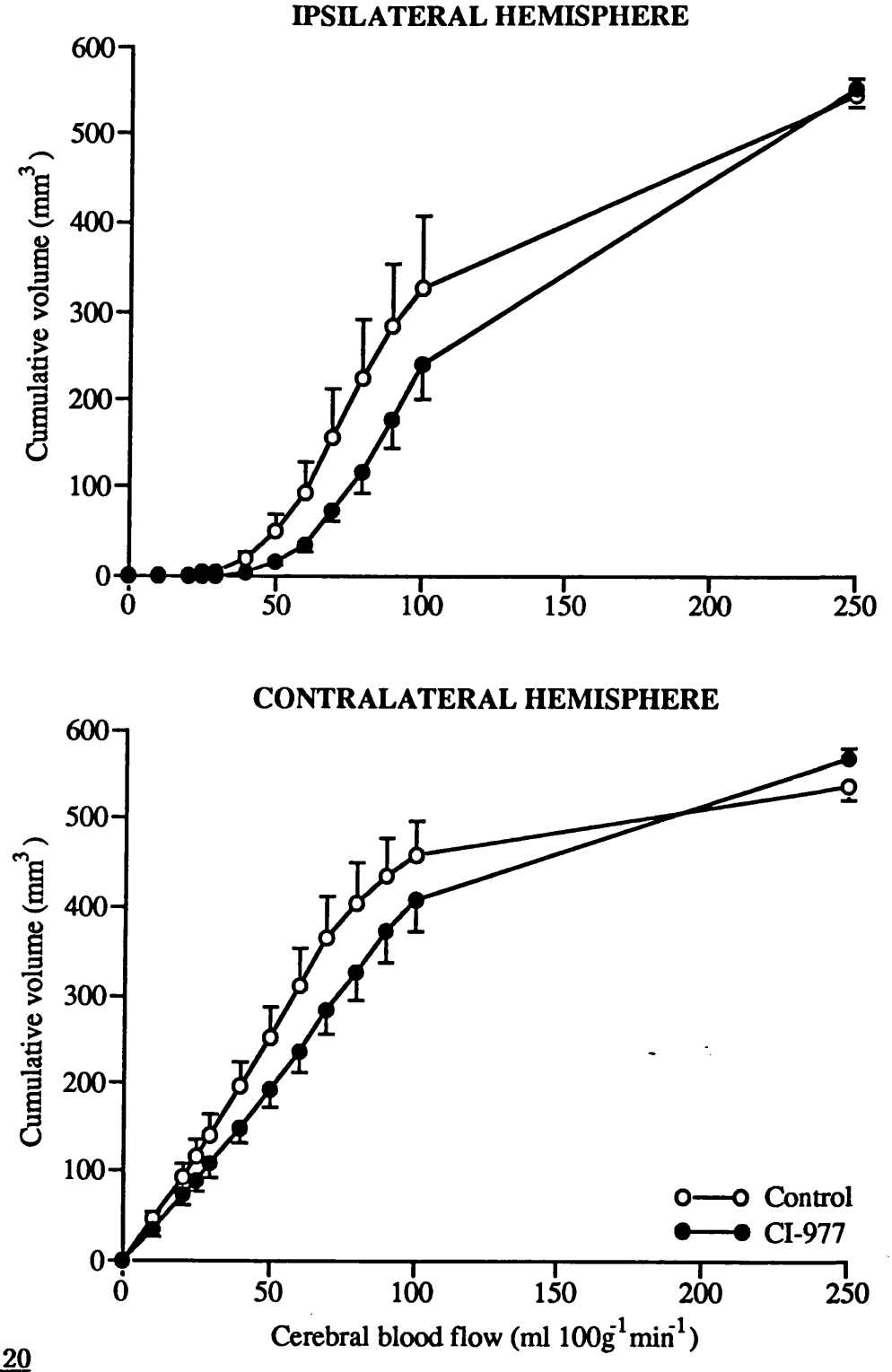


FIGURE 20

The effect of CI-977 (frequency distribution analysis) upon the hemispheric volume, both ipsilateral (upper) and contralateral (lower) to MCA occlusion, perfused by sequentially higher ranges of cerebral blood flow. CI-977 (0.3mg/kg, s.c.) was administered 30 min prior to permanent occlusion of the MCA in halothane-anaesthetised rats, and cerebral blood flow determined 30 min subsequently. Data are presented as mean ± SEM (n-7 vehicle, n=8 CI-977). There was no significant difference between vehicle-treated controls and CI-977-treated animals at any cerebral blood flow range in either hemisphere. Note the larger volume of the ipsilateral hemisphere relative to the contralateral hemisphere perfused with lower levels of cerebral blood flow.

2.2 CI-977 on Glutamate Neurotoxicity *in vivo*

2.2.1 *Cardiovascular and Respiratory Status*

The physiological variables monitored throughout the study are shown in Table 6. There were no significant differences in arterial blood gas studies (pO_2 pCO_2, pH), rectal temperature or plasma glucose concentration between vehicle-treated controls and any of the drug-treated (MK-801, NBQX, CI-977) groups at any time point.

The administration of CI-977 (0.3mg/kg, i.v.) 30 min before and 30 min after the start of glutamate (0.5M) perfusion induced a modest reduction in MABP, which was significantly different from vehicle-treated controls at 120 min ($p<0.05$) and 240 min ($p<0.01$) only (Figure 21). NBQX produced a moderate, sustained increase in MABP throughout the period of glutamate perfusion and in the post perfusion period, and MK-801 treatment induced a modest, non-significant reduction in MABP (Figure 21).

2.2.2 *Histology Examination*

The perfusion of glutamate (0.5M) produced an area of pallor in the cerebral cortex which was visible on the H & E stained sections without magnification (Figure 22). This area of pannecrosis, with a clearly defined border, represents the cortical lesion produced around the microdialysis probe. The lesion extends in anterior and posterior directions relative to the probe (approximately 2mm in each direction (Figure 22). The histopathological features in the area of pallor have been previously described (Landolt et al., 1993), i.e. triangulation of the nucleus, shrinkage of neurones and neuropil, and swelling of perineuronal astrocytes. No histological abnormalities were found in brain regions remote from the dialysis probe.

TABLE 6

GLUTAMATE NEUROTOXICITY IN VIVO: PHYSIOLOGICAL VARIABLES FOLLOWING INTRAVENOUS ADMINISTRATION OF NBQX, MK-801 AND CI-977 TO ANAESTHETISED RATS

TREATMENT	GLUTAMATE PERFUSION (MIN)	RECTAL TEMPERATURE (°C)	PLASMA GLUCOSE (mM)	pCO_2 (mmHg)	pO_2 (mmHg)	pH
VEHICLE	- 30	37.0 ± 0.1	9.8 ± 0.4	39 ± 1	172 ± 6	7.49 ± 0.01
	0	37.2 ± 0.1	9.4 ± 0.3	39 ± 1	181 ± 6	7.48 ± 0.01
	60	37.0 ± 0.1	9.1 ± 0.2	39 ± 1	180 ± 7	7.45 ± 0.02
	120	37.1 ± 0.1	9.5 ± 0.3	36 ± 1	186 ± 6	7.46 ± 0.01
	180	37.1 ± 0.1	10.0 ± 0.2	36 ± 1	172 ± 12	7.47 ± 0.01
	240	37.0 ± 0.1	10.0 ± 0.4	37 ± 1	171 ± 11	7.44 ± 0.01
NBQX	- 30	37.4 ± 0.2	10.9 ± 0.6	39 ± 1	182 ± 5	7.44 ± 0.02
	0	37.1 ± 0.2	11.3 ± 1.1	37 ± 2	189 ± 3	7.45 ± 0.01
	60	37.1 ± 0.2	12.6 ± 2.0	40 ± 1	188 ± 4	7.42 ± 0.01
	120	37.2 ± 0.1	11.8 ± 1.5	40 ± 3	191 ± 7	7.41 ± 0.03
	180	37.4 ± 0.4	11.8 ± 1.2	38 ± 1	192 ± 12	7.40 ± 0.02
	240	36.9 ± 0.1	11.0 ± 0.7	36 ± 1	193 ± 11	7.40 ± 0.02
MK-801	- 30	37.0 ± 0.1	9.8 ± 0.4	39 ± 2	165 ± 13	7.47 ± 0.02
	0	37.2 ± 0.1	8.8 ± 0.4	38 ± 2	174 ± 5	7.47 ± 0.01
	60	37.0 ± 0.1	8.8 ± 0.3	41 ± 1	182 ± 7	7.44 ± 0.02
	120	37.1 ± 0.1	9.4 ± 0.3	38 ± 1	189 ± 10	7.44 ± 0.01
	180	37.1 ± 0.1	9.6 ± 0.3	37 ± 1	181 ± 6	7.45 ± 0.01
	240	37.0 ± 0.1	9.7 ± 0.3	35 ± 3	173 ± 7	7.44 ± 0.02
CI-977	- 30	36.9 ± 0.1	10.3 ± 0.6	40 ± 2	174 ± 9	7.44 ± 0.03
	0	37.1 ± 0.1	8.8 ± 0.4	41 ± 3	176 ± 11	7.42 ± 0.03
	60	37.1 ± 0.1	8.9 ± 0.3	41 ± 1	186 ± 8	7.40 ± 0.02
	120	37.0 ± 0.1	9.7 ± 0.7	37 ± 1	188 ± 8	7.40 ± 0.02
	180	36.9 ± 0.1	9.9 ± 0.7	37 ± 2	192 ± 7	7.39 ± 0.05
	240	37.2 ± 0.2	9.9 ± 0.8	37 ± 1	187 ± 6	7.41 ± 0.02

Data are presented as mean ± SEM (n=10 control; n=5 in each drug-treated group). Data represents values 30 min prior to, at the time of and at 60 min intervals after the start of glutamate perfusion. NBQX (30mg/kg x 2, i.v.), CI-977 (0.3mg/kg x 2, i.v.) and vehicle (5.5% glucose; 1ml/kg, i.v.) were administered 30 min before and 30 min after the start of glutamate perfusion, and MK-801 (0.5mg/kg, i.v.) 30 min before glutamate perfusion only. There are no significant differences between the vehicle-treated control group and the drug-treated groups at any time point for any variable.

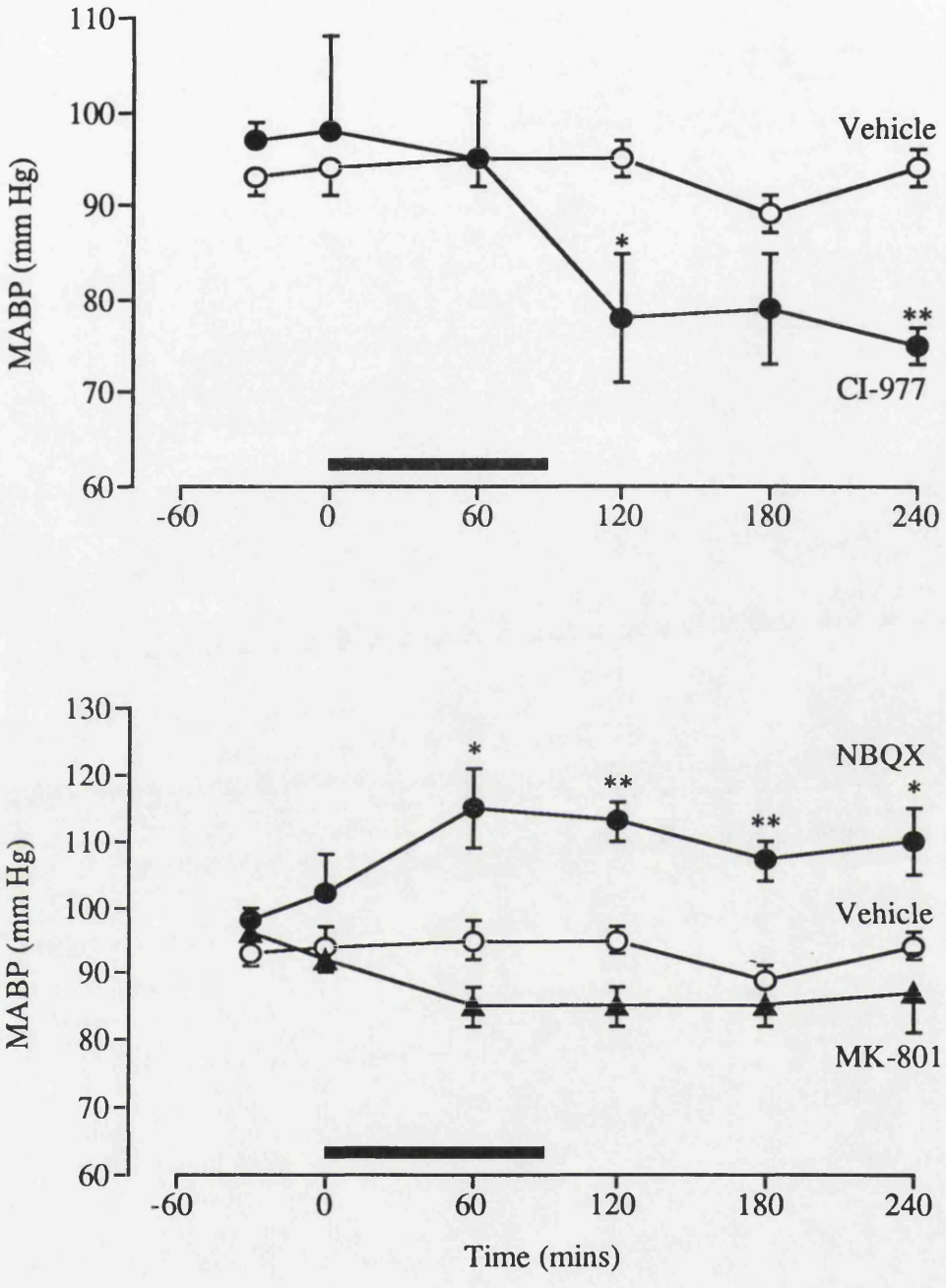
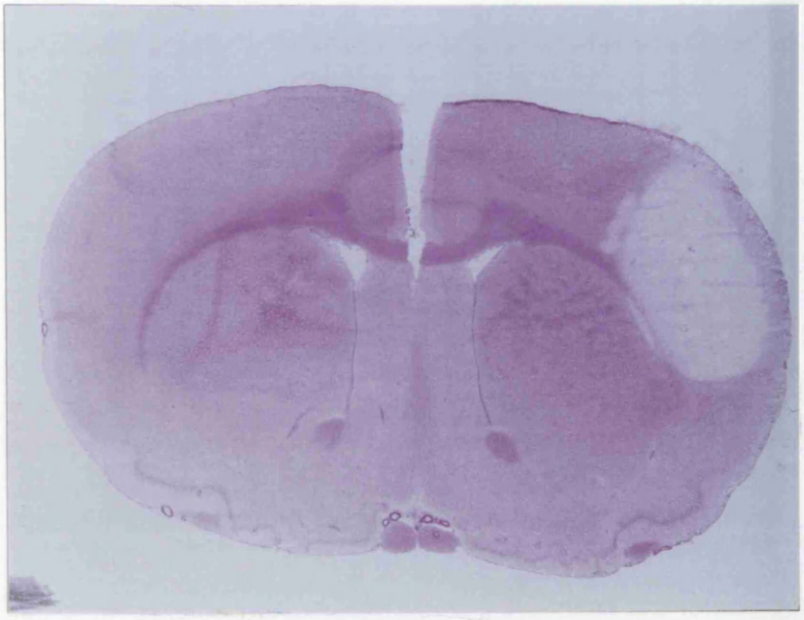


FIGURE 21

The effects of drug treatment on mean arterial blood pressure (MABP) in halothane-anaesthetised rats during the glutamate neurotoxicity *in vivo* study. Data are presented as mean ± SEM (n=10 vehicle; n=5 each drug-treated group). *P<0.05, **P<0.01 relative to contemporaneous vehicle treatment (ANOVA followed by unpaired Student's *t*-test with Bonferroni correction). Solid bar indicates period of glutamate perfusion (0 - 90 min).



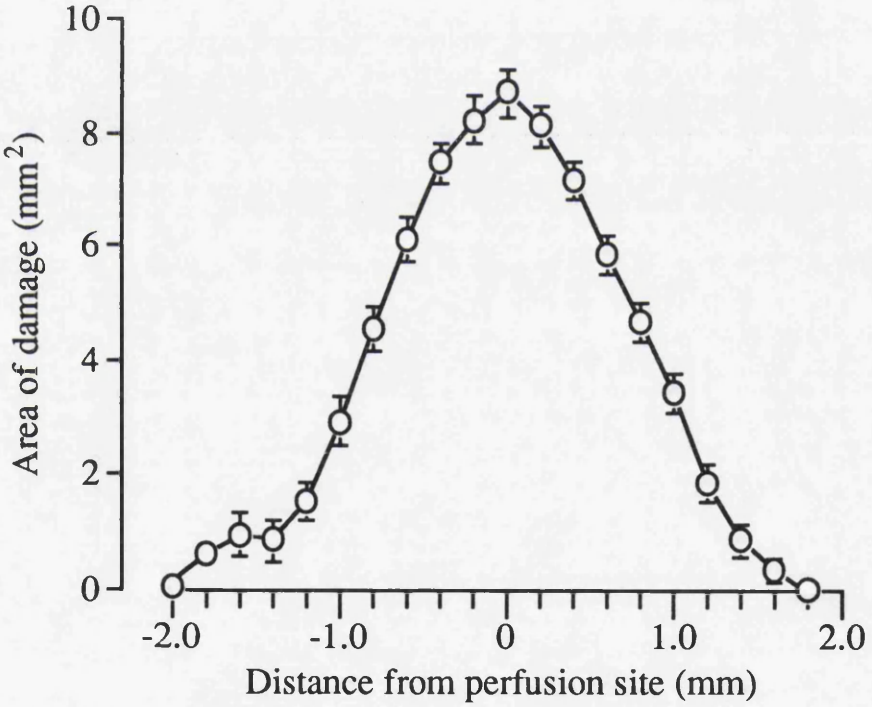


FIGURE 22

Histological assessment of glutamate-induced necrosis. Upper: a representative coronal section (x7) stained with H & E illustrating the area of neuronal damage produced in the same coronal plane as the microdialysis probe in a vehicle-treated control animal. Lower: the areas of damage at 200μm intervals assessed 4h after the onset of glutamate perfusion in vehicle-treated animals. Data are presented as mean ± SEM (n=10).

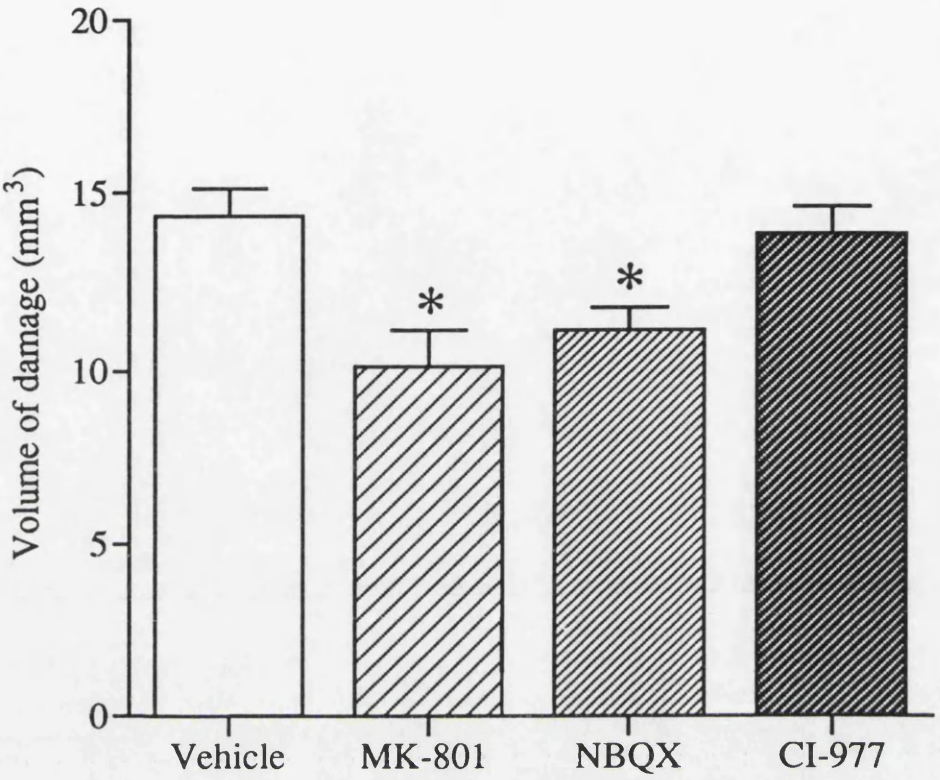


FIGURE 23

The effect of drug treatment on the volume of neuronal damage produced by intracortical glutamate perfusion in halothane-anaesthetised rats. Data are presented as mean ± SEM (n=10 vehicle; n=5 each drug-treated group). *P<0.05 relative to contemporaneous vehicle-treated controls (ANOVA followed by independent two-tailed *t*-test with Bonferroni correction).

2.2.3 *Volumetric Assessment of Histological Damage*

The administration of CI-977 30 min before and 30 min after the initiation of intracortical glutamate perfusion produced no significant reduction in the volume of glutamate-induced damage when compared to vehicle-treated controls (Figure 23). Pre-treatment with NBQX (2 x 30mg/kg) and MK-801 (0.5mg/kg) each significantly reduced the volume of damage produced by intracortical glutamate perfusion. The magnitude of the reductions in lesion volume produced by NBQX and MK-801 were broadly similar, being 23% and 30% respectively (Figure 23).

2.3 Glutamate Release and Cerebral Blood Flow after MCA Occlusion in the Cat: Effect of CI-977

2.3.1 *Physiological Variables*

There were no significant differences between the vehicle-treated control group and CI-977-treated group in respiratory blood gas status (pO_2, pCO_2, pH), temperature (rectal or periosteal), plasma glucose concentration and haematocrit at the time of MCA occlusion and during the subsequent 3h post-occlusion period (Appendix Table).

The bolus administration of CI-977 30 min prior to the induction of ischaemia elicited a significant reduction in MABP by approximately 27% of control values only within the initial 5 min post-injection period (control: 90 ± 6mmHg, CI-977: 66 ± 3mmHg; $p<0.05$ Student's unpaired *t*-test). The hypotension was relatively transient such that there was no difference between the two groups in the level of MABP at the start of dialysate collection and concomitant cerebral blood flow measurement 20 min prior to MCA occlusion (control: 89 ± 4mmHg; CI-977: 81 ± 5mmHg).

2.3.2 *Cerebral Blood Flow*

The administration of CI-977 had no significant effect on the level of cerebral blood flow in the suprasylvian gyrus during the control period prior to occlusion of the MCA in either the ipsilateral or contralateral hemisphere (Figure 24). Following the induction of ischaemia, there was a significant reduction (approximately 60%) in cortical cerebral blood flow in the ischaemic hemisphere in both the vehicle-treated and drug-treated groups ($p<0.05$ Student's paired *t*-test) (Figure 24). A minimal, non-significant reduction (approximately 18%) in cerebral blood flow was observed in the non-ischaemic hemisphere of both groups (Figure 24). CI-977 treatment failed to demonstrate any significant alteration in the level of post-ischaemic cerebral blood flow in either the ipsilateral or contralateral hemisphere (Figure 24).

2.3.3 *Amino Acid Levels in Dialysate*

At normal control cerebral blood flow values, there was no difference in the concentration of glutamate in the ipsilateral hemisphere in either the vehicle-treated control or CI-977-treated groups (Figure 25). After the induction of ischaemia, there was a marked increase in glutamate levels when cerebral blood flow fell to a threshold value of approximately 20ml $100g^{-1}$ min^{-1} in vehicle-treated control animals (Figure 25). In the CI-977-treated group, there was no evidence of such a threshold for glutamate release in the ischaemic hemisphere (Figure 25). In the hemisphere contralateral to MCA occlusion, there was no increase in glutamate levels after the induction of ischaemia in either the vehicle-treated control or CI-977-treated groups (Figure 26). Similarly, there was no elevation in the levels of any of the other 11 amino acids examined in the contralateral hemisphere in either group after the onset of ischaemia.

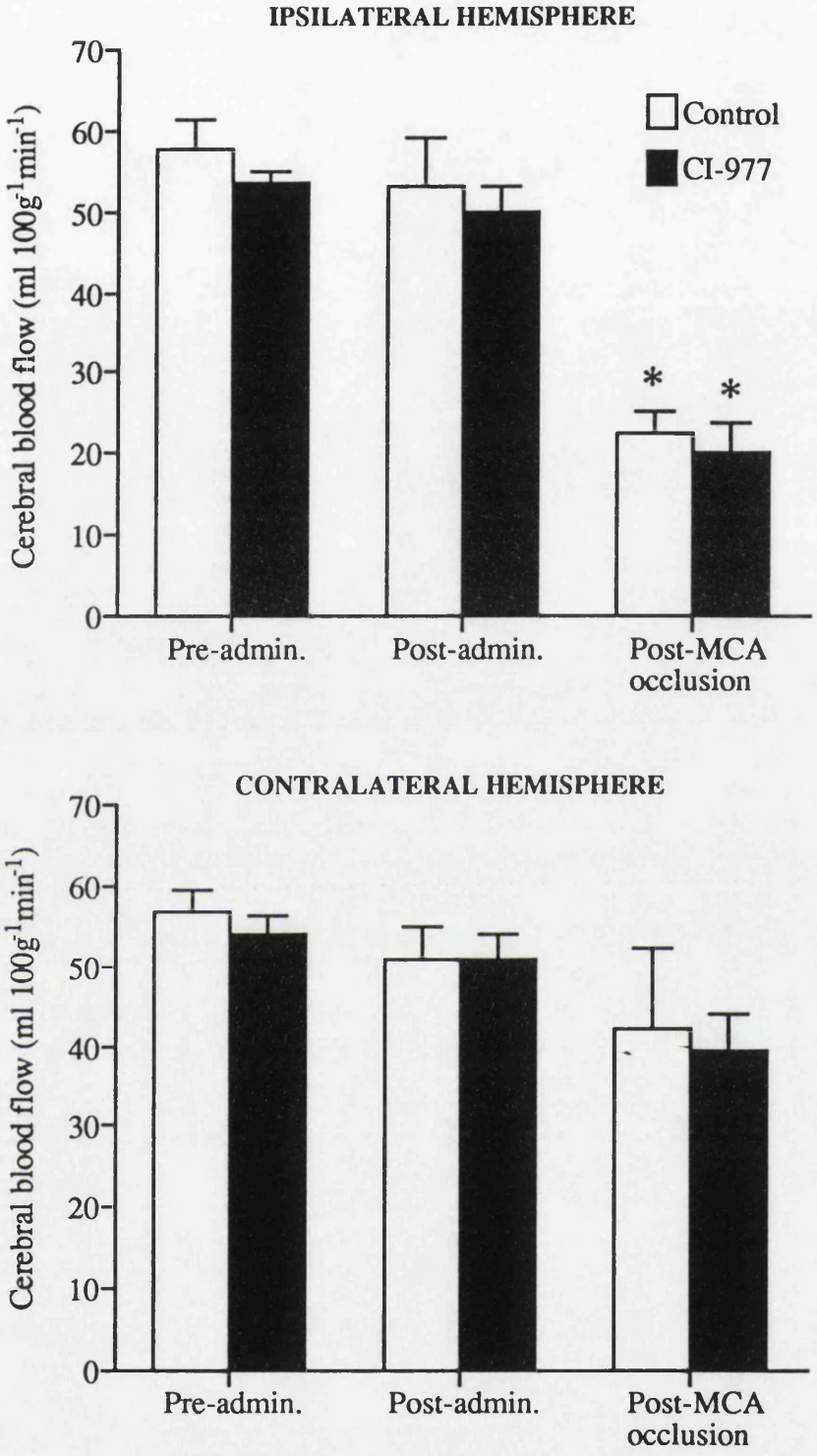


FIGURE 24

The effect of CI-977 administration (admin.) on the level of cerebral blood flow in the ipsilateral (upper) and contralateral (lower) hemispheres immediately before and immediately after permanent MCA occlusion in halothane-anaesthetised cats. Data are presented as mean ± SEM and represent 4-15 measurements in cerebral blood flow in 5 vehicle-treated control and 4 CI-977-treated animals. *P<0.05 (paired *t*-test) relative to pre-ischaemic cerebral blood flow level.

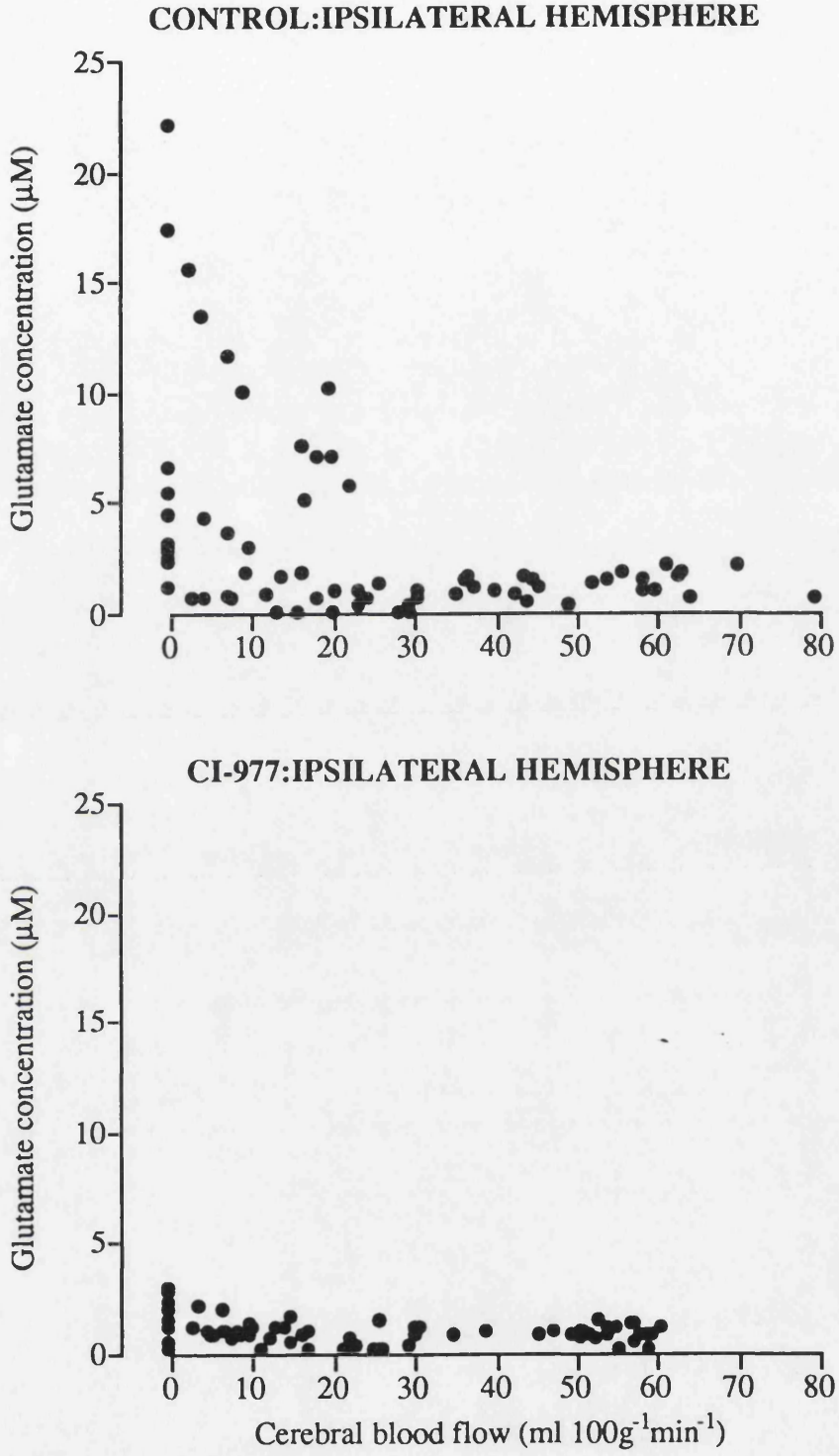


FIGURE 25

Scatterplots of the relationships between cerebral blood flow and the extracellular concentration of glutamate in the ipsilateral hemisphere pre- and post-MCA occlusion in vehicle-treated control (n=5) and CI-977-treated (n=4) animals. Each data point represents all values obtained throughout the course of the investigation.

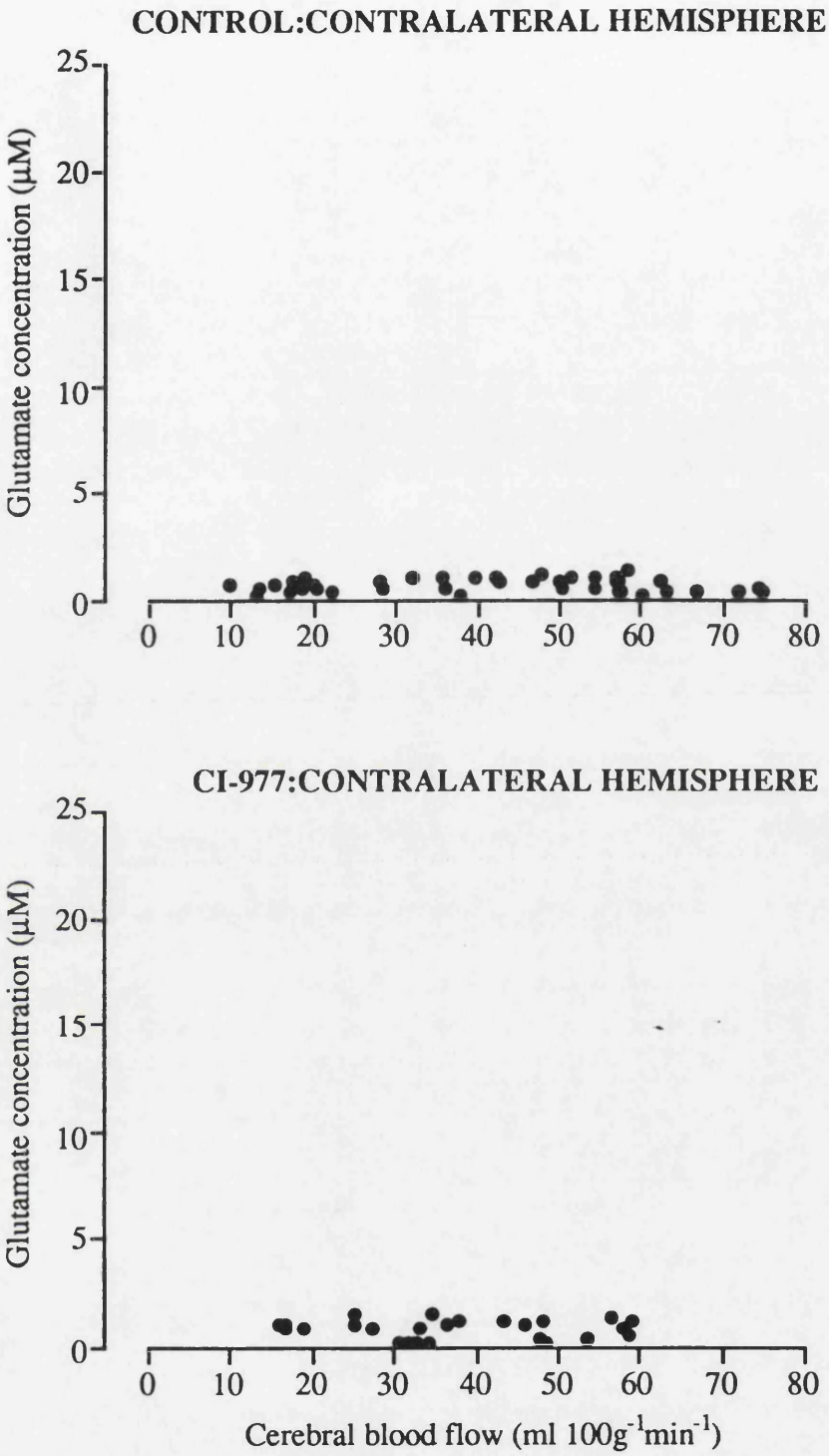


FIGURE 26

Scatterplots of the relationships between cerebral blood flow and the extracellular concentration of glutamate in the contralateral hemisphere pre- and post-MCA occlusion in vehicle-treated control (n=5) and CI-977-treated (n=3; one probe malfunctioned) animals. Each data point represents all values obtained throughout the course of the investigation.

There was evidence of an elevation in the levels of glutamate in the ipsilateral hemisphere when cerebral blood flow fell to values of less than 30ml $100g^{-1}$ min^{-1} in vehicle-treated control animals (Figure 27). This increase in glutamate concentration was attenuated significantly by CI-977 at cerebral blood flow of less than 20ml $100g^{-1}$ min^{-1} (Figure 27). There was no difference between the two groups in the time elapsed after MCA occlusion for cerebral blood flow values to fall to less than 30ml $100g^{-1}$ min^{-1}, where increases in glutamate levels were observed (CBF bin; 0-10ml $100g^{-1}$ min^{-1}: control 123 ± 10 min (n=23), CI-977: 124 ± 11 min (n=35); CBF bin 10-20ml $100g^{-1}$ min^{-1}: control 83 ± 21 min (n=11), CI-977: 78 ± 17 min (n=14); CBF bin 20-30ml $100g^{-1}$ min^{-1}: control: 57 ± 10 min (n=13), CI-977: 51 ± 18 min (n=11)). The extracellular concentration of the excitatory amino acid L-aspartate was also markedly increased at low flow levels (Figure 27). Administration of CI-977 significantly reduced this elevation such that the levels of aspartate did not increase over basal levels in the drug-treated group (Figure 27). There was also an increase in the levels of the inhibitory neurotransmitter GABA at cerebral blood flow values of less than 10ml $100g^{-1}$ min^{-1} which was reduced following treatment with CI-977 (Figure 28). There was no marked elevation in the levels of tyrosine after the induction of ischaemia, although at low flow values there was evidence to suggest an attenuation in CI-977-treated animals (Figure 28).

The effects of CI-977 on the ischaemia-induced release of asparagine, serine, glutamine, glycine, threonine, arginine, taurine and alanine are illustrated in Appendix Figures and summarised approximately in Table 7. The cerebral blood flow bin 0-10ml 100^{-1} min^{-1} was selected for comparison between control and CI-977-treated animals because in this cerebral blood flow bin the response is robust with n numbers typically 20-25. In contrast,

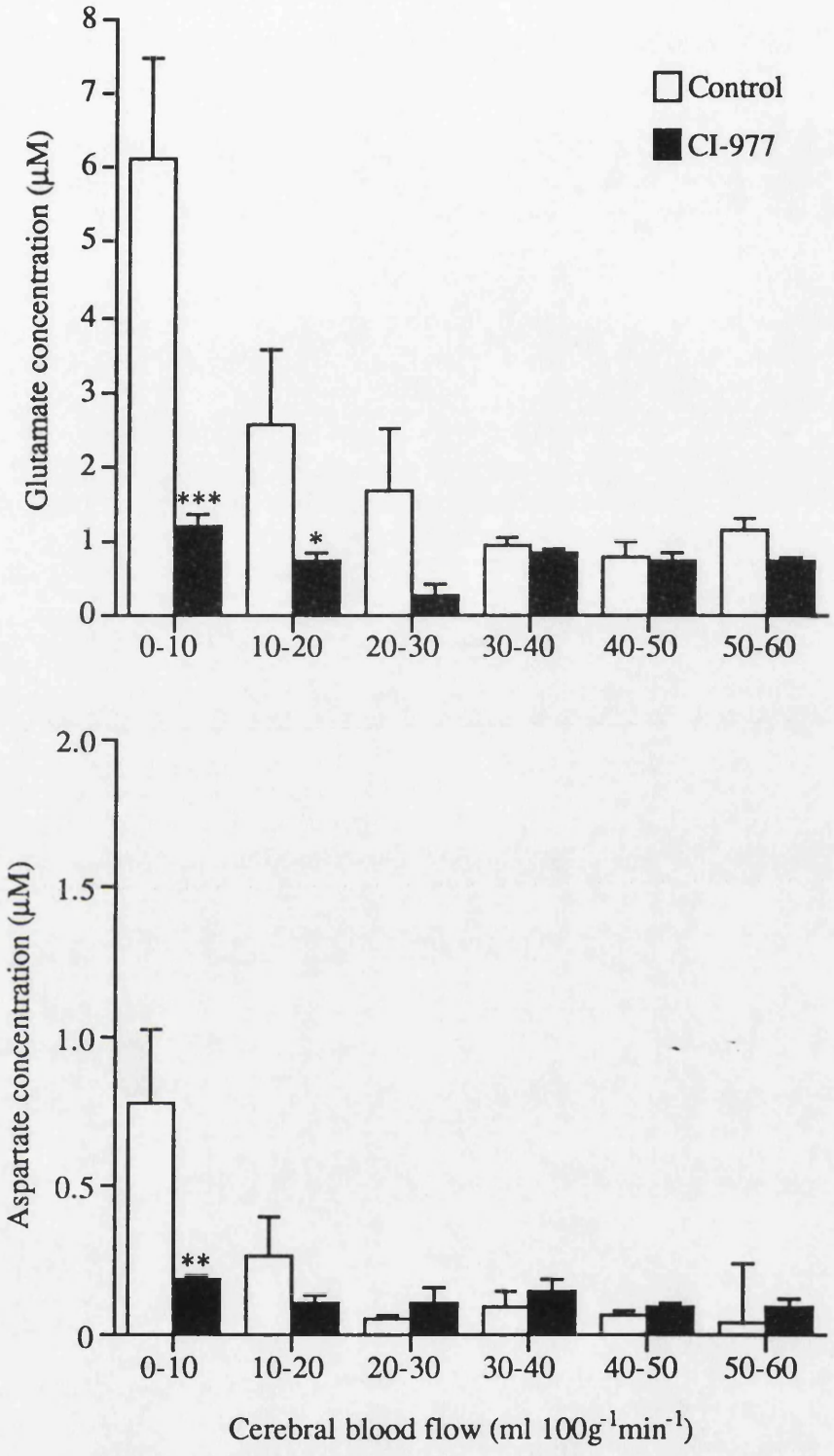


FIGURE 27

The effect of pretreatment with CI-977 on the extracellular concentrations of glutamate (upper) and aspartate (lower) in the ipsilateral hemisphere before and after permanent MCA occlusion in halothane-anaesthetised cats. Data are presented as mean ± SEM and represent 3-26 determinations in 5 vehicle-treated control and 4 CI-977-treated animals. *P<0.05, **P<0.01, ***P<0.001 (Student's two-tailed *t*-test).

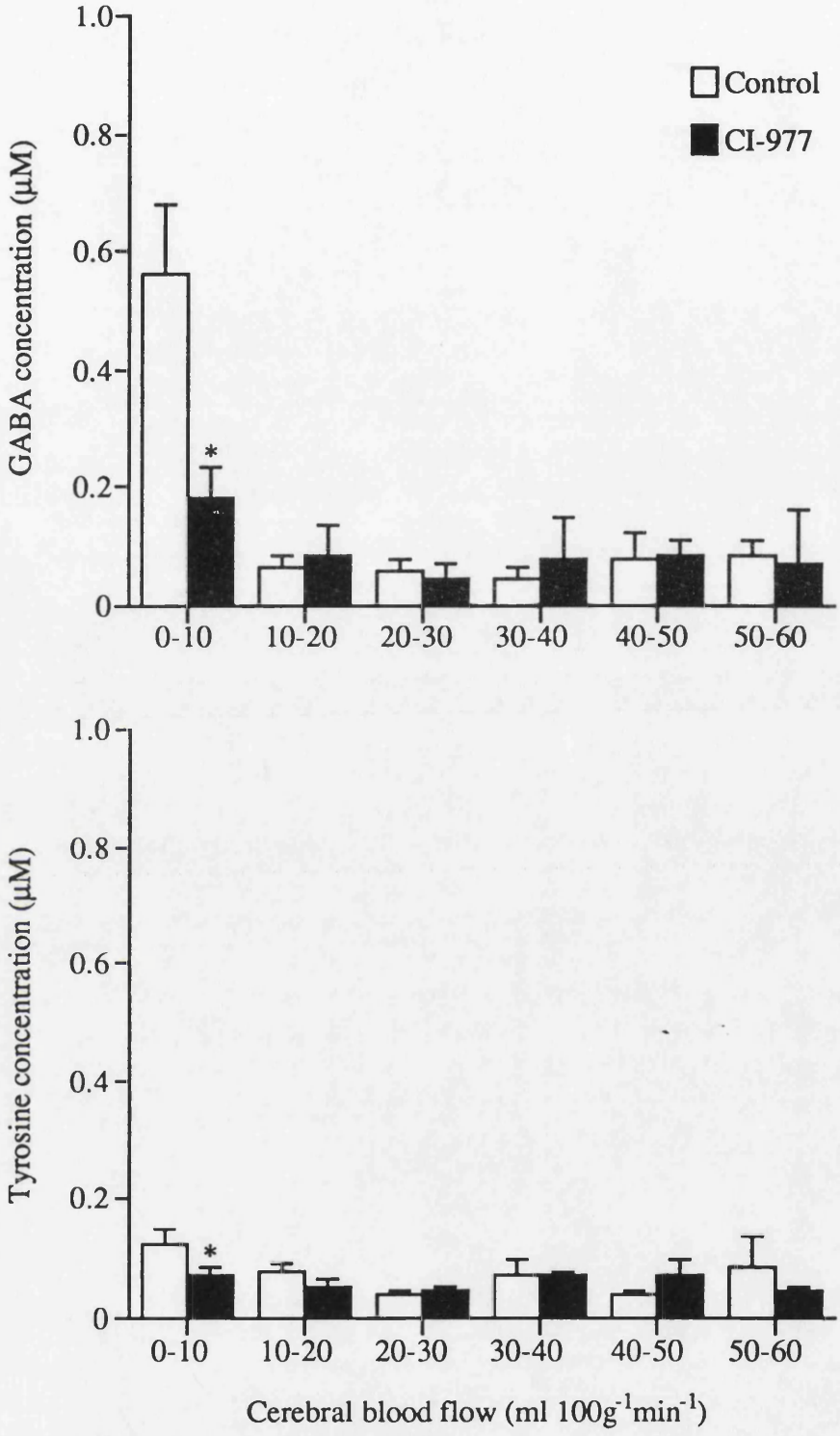


FIGURE 28

The effect of pretreatment with CI-977 on the extracellular concentrations of GABA (upper) and tyrosine (lower) in the ipsilateral hemisphere before and after permanent MCA occlusion in halothane-anaesthetised cats. Data are presented as mean ± SEM and represent 3-23 determinations in 5 vehicle-treated control and 4 CI-977-treated animals. *P<0.05 (Student's two-tailed *t*-test).

TABLE 7

INCREASES IN AMINO ACID LEVELS IN THE CAT SUPRASYLVIAN GYRUS AFTER CEREBRAL ISCHAEMIA

	ALTERATION AFTER ISCHAEMIA	
AMINO ACID	CONTROLS	CI-977
Glutamate	8 fold	0.5 fold
Aspartate	8 fold	0
Alanine	8 fold	3 fold
Taurine	6 fold	3 fold
GABA	5 fold	1.5 fold
Threonine	4 fold	2 fold
Serine	2.5 fold	1.5 fold
Glycine	1.5 fold	0.5 fold
Tyrosine	1 fold	0
Arginine	1 fold	0
Asparagine	0	0
Glutamine	0	0

Approximate increases in amino acid levels relative to control basal values in the CBF range 0-10ml $100g^{-1}$ min^{-1} after permanent MCA occlusion in the cat, and the effect of CI-977 pretreatment. 0 = no change from basal levels. Actual values are illustrated in Figures 27 and 28 and in Appendix Figures.

the cerebral blood flow bin 40-50ml $100g^{-1}$ min^{-1}, where there is an apparent increase after CI-977, is not a robust response, with n numbers approximately 3-4, arising as a consequence of the design of the study. Thus, in such a small group one large value will make a huge impact on the mean of the data in that cerebral blood flow bin.

3. FUNCTIONAL CONSEQUENCES OF CI-977 ADMINISTRATION

The functional consequences of CI-977 as reflected in local cerebral glucose utilisation and local cerebral blood flow were examined in conscious, lightly restrained animals. However, the use of hind limb restraint introduces the possibility that the behavioural responses and CNS effects of CI-977 may be different from those in freely moving animals. Moreover, although indirect indices of acute stress (plasma glucose, pCO_2, MABP) are within normal limits in these lightly restrained rats prior to drug/vehicle administration, chronic, mild stress (and its interaction with CI-977) cannot be discounted entirely. Thus, extreme caution should be exercised in the extrapolation of neuropharmacological data obtained in restrained animals to freely moving animals.

3.1 Cerebral Glucose Use after CI-977

3.1.1 *Behaviour and Physiological Variables*

The intravenous administration of CI-977 (0.03, 0.3, 3mg/kg) resulted in prominent changes in the overt behaviour of the animals. Prior to drug administration, the lightly restrained animals were generally quiescent and displayed normal grooming behaviour and appropriate reactions to slight auditory and sensory stimuli. The behavioural responses following CI-977 were characterised initially by a dose-dependent (although of a minor nature

with 0.03mg/kg) cataleptic state which appeared within 2 min of drug administration, and persisted throughout the measurements of 2-deoxyglucose with the highest dose of CI-977 examined (3mg/kg). The rats stretched their necks, adopted a rigid body posture, gazed in one direction, and exhibited pronounced extension of the forelimbs with splayed-out paws. Moreover, the rats had pinned back white ears, piloerection of the whiskers and a rigid, straightened tail. Grooming, sniffing and exploratory movements of the head and forelimbs (which all control rats displayed) were absent in animals treated with CI-977, characterising signs of CNS depression. In animals treated with the intermediate dose of CI-977 (0.3mg/kg), the behavioural response was replaced progressively (approximately 15-20 min post-injection) by one consisting of abnormal motor activity characterised by intermittent periods of jerky head movements, arching of the neck and upper portion of the body, and frequent vocalisations which persisted until sacrifice. The animals remained conscious throughout, even at the highest concentration studied (3mg/kg).

Treatment with CI-977 produced minor alterations in the monitored physiological variables (Table 8). The injection of CI-977 induced no significant changes in respiratory blood gas status (pCO_2, pO_2, pH) at any dose over the time course of the experiments when compared to vehicle-treated control animals (Table 8). CI-977 had no significant effect on MABP immediately after intravenous administration or at any time point during the experiment (Table 8). In control animals, vehicle injection had no effect on MABP.

CI-977 produced a dose-dependent, moderate reduction in rectal temperature which was sustained throughout the experimental period with CI-977 at the intermediate (0.3mg/kg) and highest (3mg/kg) doses examined

TABLE 8

GLUCOSE UTILISATION STUDY: CARDIOVASCULAR, RESPIRATORY AND OTHER PHYSIOLOGICAL VARIABLES AFTER ADMINISTRATION OF CI-977 TO CONSCIOUS RATS

VARIABLE	TIME AFTER DRUG (min)	CONTROL	CI-977 (mg/kg) 0.03	0.3	3
Rectal Temperature	0	36.9±0.2	37.0±0.2	36.9±0.3	37.0±0.1
(°C)	10	36.9±0.1	36.7±0.1	36.5±0.1*	36.4±0.1**
	50	37.0±0.2	36.8±0.2	36.5±0.3*	36.5±0.1**
Mean Arterial	0	135 ± 3	125 ± 3	134 ± 6	137 ± 3
Blood Pressure	10	131 ± 7	124 ± 4	133 ± 4	141 ± 6
(mmHg)	50	133 ± 4	128 ± 5	130 ± 5	140 ± 7
Arterial Plasma	0	6.6±0.2	6.7±0.3	7.1±0.4	6.9±0.3
Glucose (mM)	10	6.7±0.2	8.3±0.4	10.2±1.4**	8.0±0.3
	50	6.8±0.3	8.7±0.5	12.8±1.3**	10.1±0.6*
pCO_2	0	41.0±1.1	40.6±0.8	37.6±1.4	40.0±1.0
(mmHg)	10	40.0±1.2	40.8±1.0	37.8±1.5	41.7±0.6
	50	38.6±1.3	41.2±0.9	36.9±1.4	40.6±1.6
pO_2	0	93 ± 1	95 ± 2	95 ± 3	94 ± 1
(mmHg)	10	94 ± 3	96 ± 3	100 ± 3	95 ± 2
	50	97 ± 3	99 ± 2	103 ± 3	104 ± 3
pH	0	7.40±0.01	7.41±0.01	7.41±0.01	7.39±0.01
	10	7.40±0.02	7.40±0.01	7.39±0.02	7.38±0.02
	50	7.41±0.01	7.39±0.01	7.40±0.01	7.37±0.02
Number of Animals		6	6	6	6

Data are presented as mean ± SEM. Data represent values at times 0, 10 and 50 min following intravenous administration of CI-977 or vehicle (controls). *P<0.05, **P<0.01 for statistical comparison between each drug-treated group and vehicle-treated controls at each time point (ANOVA, followed by Student's unpaired *t*-test with Bonferroni correction).

(Table 8). CI-977 at the lowest dose of 0.03mg/kg had no effect on rectal temperature. However, the use of external heating lamps throughout the experiment makes accurate interpretation of drug-related temperature effects difficult.

The administration of CI-977 produced significant changes in arterial plasma glucose concentration (Table 8). CI-977 (0.03mg/kg) had no effect on plasma glucose over the time course of the experiments. Plasma glucose levels were significantly increased by CI-977 at 10 min (0.3mg/kg) and 50 min (0.3 and 3mg/kg) post-injection when compared to vehicle-treated control animals.

3.1.2 *Local Cerebral Glucose Utilisation*

The effects of CI-977 (0.03, 0.3 and 3mg/kg) upon local cerebral glucose utilisation were examined in 45 anatomically discrete regions of the CNS (Tables 9-12). In 15 of the 45 regions examined, no significant alterations in glucose use were observed with any dose of CI-977. In one region of the CNS, CI-977 significantly increased glucose with the intermediate and highest doses studied. The administration of CI-977 significantly reduced glucose utilisation, generally with little dose-dependency, in 29 regions of the CNS. Of the 29 regions, 18 displayed significant reductions in glucose utilisation over the entire range of doses studied. The alterations in glucose use following CI-977 administration are considered further below in relation to the functional organisation of the CNS.

Primary Auditory and Visual Areas

The administration of CI-977 reduced glucose utilisation in the visual and auditory cortices (layer IV), superior colliculus, medial geniculate body

and inferior colliculus (Table 9). In these 5 regions the 3 doses of CI-977 were equipotent. Glucose use in the lateral geniculate body, superior olivary nucleus and cochlear nucleus was minimally changed by CI-977 (Table 9).

Extrapyramidal and Sensory Motor Areas

CI-977 treatment resulted in widespread reductions in glucose utilisation in most of the regions examined involved in motor function (Table 10, Figure 29). In the cerebellar nucleus and cerebellar hemisphere, the highest dose of CI-977 failed to induce a significant decrease in glucose use (Table 10). Glucose utilisation in 3 motor areas, the inferior olivary nucleus, red nucleus and vestibular nucleus was minimally altered by CI-977 (Table 10).

Following CI-977 administration, prominent decreases in glucose utilisation (about 30%) were observed in frontal and posterior parietal cortices (layer IV) and ventrolateral thalamus (Table 10, Figure 29). Glucose use was most markedly depressed (by 35%) by CI-977 in the sensory motor cortex (Figure 29), although in all neocortical areas examined, glucose utilisation was decreased by a similar degree with each of the 3 doses studied.

Limbic System and other Brain Areas

Increased glucose utilisation (about 40%) was observed in the lateral habenular nucleus following administration of CI-977 at the intermediate (0.3mg/kg) and highest (3mg/kg) doses examined (Table 11, Figures 29 & 30). In contrast, CI-977 produced significant reductions in glucose use in the hippocampus (dentate gyrus and stratum lacunosum moleculare), amygdala, hypothalamus, interpeduncular nucleus, allocortical regions and dorsal raphe nucleus (Table 11, Figure 29). Of the allocortical regions examined, glucose use was most markedly depressed in the anterior entorhinal cortex (by 45%)

TABLE 9

PRIMARY VISUAL AND AUDITORY AREAS: GLUCOSE UTILISATION FOLLOWING THE ADMINISTRATION OF CI-977

		CI-977 (mg/kg)		
STRUCTURE	CONTROL	0.03	0.3	3
Primary Visual System				
Visual Cortex (IV)	91 ± 4	70 ± 4**	69 ± 2**	68 ± 4**
Lateral Geniculate Body	70 ± 3	59 ± 4	61 ± 1	60 ± 3
Superior Colliculus	82 ± 8	60 ± 4*	68 ± 1	63 ± 4*
Primary Auditory System				
Auditory Cortex (IV)	137 ± 4	95 ± 8**	98 ± 3**	91 ± 5**
Medial Geniculate Body	105 ± 6	79 ± 6**	82 ± 2**	78 ± 3**
Inferior Colliculus	162 ± 6	117 ± 11*	106 ± 9**	105 ± 10**
Superior Olivary Nucleus	124 ± 9	98 ± 8	98 ± 5	112 ± 9
Cochlear Nucleus	137 ± 7	122 ± 8	135 ± 7	130 ± 7

Rates of glucose utilisation (μmol $100g^{-1}min^{-1}$) are derived from 24 animals and presented as mean ± SEM (n=6 per group). Roman numerals indicate the cortical layer examined. *P<0.05, **P<0.01 for statistical comparison between each CI-977-treated group and vehicle-treated controls (ANOVA followed by Student's unpaired *t*-test with Bonferroni correction).

TABLE 10

EXTRAPYRAMIDAL AND SENSORY MOTOR AREAS: GLUCOSE UTILISATION FOLLOWING THE ADMINISTRATION OF CI-977

		CI-977 (mg/kg)		
STRUCTURE	CONTROL	0.03	0.3	3
Caudate Nucleus	101 ± 4	76 ± 5**	74 ± 4**	76 ± 4**
Globus Pallidus	51 ± 2	38 ± 2**	40 ± 3*	37 ± 2**
Substantia Nigra:				
Pars compacta	64 ± 3	49 ± 3**	54 ± 3*	52 ± 2*
Pars reticulata	50 ± 2	38 ± 2**	42 ± 2*	38 ± 1**
Red Nucleus	77 ± 3	62 ± 4	69 ± 4	68 ± 3
Ventrolateral Thalamus	81 ± 4	58 ± 5**	57 ± 4**	59 ± 2*
Subthalamic Nucleus	77 ± 3	62 ± 3*	68 ± 2	63 ± 5*
Inferior Olivary Nucleus	69 ± 2	61 ± 4	66 ± 1	67 ± 4
Vestibular Nucleus	101 ± 4	90 ± 5	92 ± 3	96 ± 6
Cerebellar Nucleus	86 ± 4	69 ± 4*	71 ± 1*	75 ± 3
Cerebellar Hemisphere	49 ± 2	40 ± 3*	41 ± 2	44 ± 2
Frontal Cortex (IV)	101 ± 6	75 ± 6**	82 ± 1	71 ± 5**
Sensory Motor Cortex (IV)	113 ± 6	79 ± 6**	75 ± 2**	70 ± 4**
Posterior Parietal Cortex (IV)	96 ± 5	70 ± 3**	74 ± 2**	68 ± 2**

Rates of glucose utilisation (μmol $100g^{-1}min^{-1}$) are derived from 24 animals and presented as mean ± SEM (n=6 per group). Roman numerals indicate the cortical layer examined. *P<0.05, **P<0.01 for statistical comparison between each CI-977-treated group and vehicle-treated controls (ANOVA followed by Student's unpaired *t*-test with Bonferroni correction).

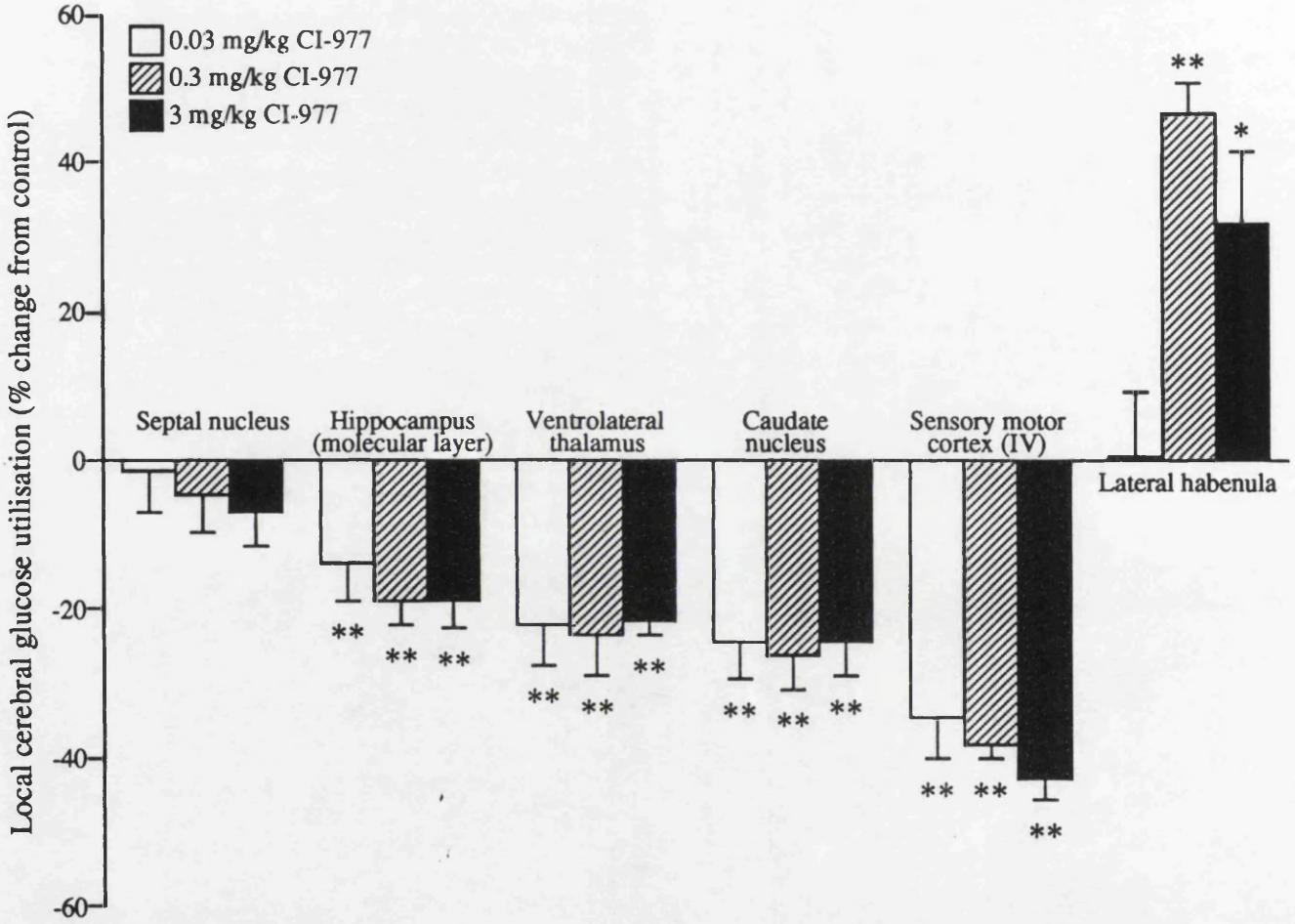


FIGURE 29

Effects of CI-977 (0.03-3mg/kg) upon local cerebral glucose utilisation in the septal nucleus, hippocampus (molecular layer), ventrolateral thalamus, sensory motor cortex (layer IV) and lateral habenula. Data are presented as % change (mean ± SEM, n=6 per group) from the level in each region in saline-treated control animals. *P<0.05, **P<0.01 relative to controls (ANOVA followed by Student's unpaired *t*-test).

TABLE 11

LIMBIC SYSTEM AND OTHER BRAIN AREAS: GLUCOSE UTILISATION FOLLOWING THE ADMINISTRATION OF CI-977

		CI-977 (mg/kg)		
STRUCTURE	CONTROL	0.03	0.3	3
Hippocampus, Molecular Layer	71 ± 3	57 ± 3**	52 ± 2**	52 ± 3**
Dentate Gyrus	62 ± 2	47 ± 3**	46 ± 3**	46 ± 3**
Septal Nucleus	42 ± 3	40 ± 2	37 ± 2	35 ± 2
Nucleus Accumbens	68 ± 4	63 ± 6	65 ± 4	57 ± 5
Amygdala	69 ± 2	54 ± 3*	58 ± 3	50 ± 6**
Lateral Habenular Nucleus	112 ± 10	112 ± 6	159 ± 5**	144 ± 11*
Mediodorsal Thalamus	99 ± 4	83 ± 7	94 ± 3	85 ± 6
Hypothalamus	46 ± 2	38 ± 2	43 ± 3	33 ± 2**
Interpedunclar Nucleus	86 ± 5	70 ± 6	63 ± 6*	62 ± 3*
Anterior Cingulate Cortex	99 ± 5	83 ± 5	80 ± 5*	82 ± 5*
Posterior Cingulate Cortex	83 ± 3	70 ± 5*	67 ± 3*	63 ± 2**
Anterior Entorhinal Cortex (I)	65 ± 2	43 ± 3**	42 ± 3**	36 ± 1**
Posterior Entorhinal Cortex (I)	61 ± 3	46 ± 2**	45 ± 2**	40 ± 2**
Ventral Tegmental Area	57 ± 1	51 ± 3	58 ± 1	53 ± 3
Dorsal Tegmental Nucleus	84 ± 2	71 ± 4	77 ± 3	79 ± 3
Dorsal Raphe Nucleus	78 ± 2	64 ± 4*	69 ± 2	65 ± 4*
Median Raphe Nucleus	90 ± 1	79 ± 5	81 ± 1	78 ± 5
Pontine Recticular Nucleus	60 ± 2	50 ± 3	52 ± 2	53 ± 4
Periaqueductal Grey Matter	52 ± 2	45 ± 2	53 ± 3	45 ± 2

Rates of glucose utilisation (μmol $100g^{-1}min^{-1}$) are derived from 24 animals and presented as mean ± SEM (n=6 per group). Roman numerals indicate cortical layer examined. *P<0.05, **P<0.01 for statistical comparison between each CI-977-treated group and vehicle-treated controls (ANOVA followed by Student's unpaired *t*-test with Bonferroni correction).

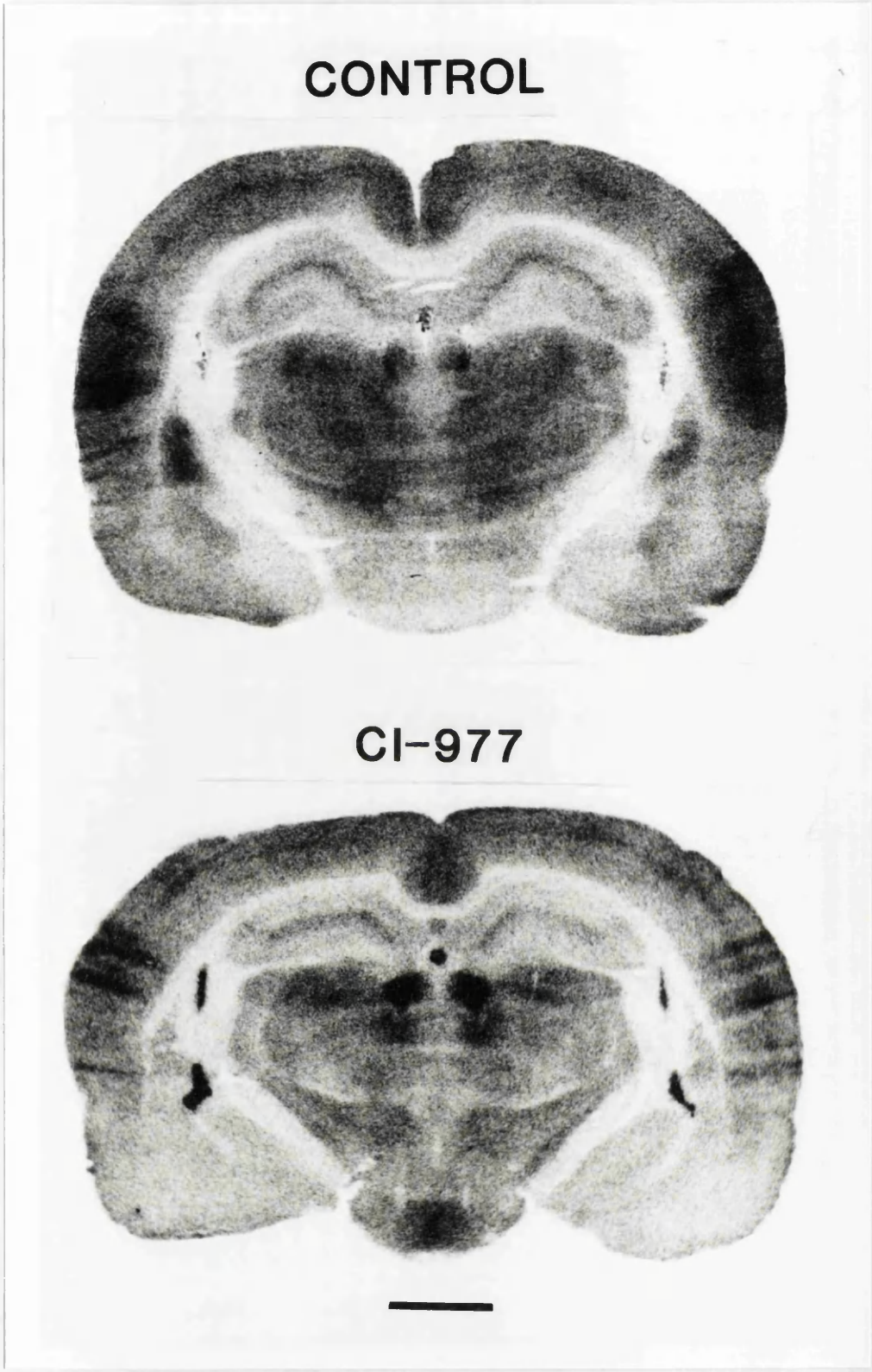


FIGURE 30

[^{14}C]-2-Deoxyglucose autoradiograms prepared from coronal brain sections at the level of the lateral habenular nucleus. Upper: control (saline, 1ml/kg, i.v.). Lower: CI-977 (0.3mg/kg, i.v.). Relative optical density (which is related directly to rate of glucose utilisation) in lateral habenular nucleus is markedly greater than control. Scale bar = 2mm.

TABLE 12

MYELINATED FIBRE TRACTS: GLUCOSE UTILISATION FOLLOWING THE ADMINISTRATION OF CI-977

		CI-977 (mg/kg)		
STRUCTURE	CONTROL	0.03	0.3	3
Corpus Callosum	36 ± 2	28 ± 2*	29 ± 1	28 ± 3*
Genu of Corpus Callosum	25 ± 1	21 ± 2	22 ± 2	20 ± 1
Internal Capsule	35 ± 1	25 ± 1**	24 ± 1**	24 ± 2**
Cerebellar White Matter	34 ± 1	25 ± 2**	24 ± 1**	25 ± 1**

Rates of glucose utilisation (μmol $100g^{-1}min^{-1}$) are derived from 24 animals and presented as mean ± SEM (n=6 per group). *P<0.05, **P<0.01 for statistical comparison between each CI-977-treated group and vehicle-treated controls (ANOVA followed by Student's unpaired *t*-test with Bonferroni correction).

by CI-977 at 3mg/kg (Table 11). No significant alterations in glucose utilisation were displayed in 8 of the 19 limbic areas examined (Table 11, Figure 29).

Myelinated Fibre Tracts

CI-977 produced significant decreases in glucose utilisation in the corpus callosum, internal capsule and cerebellar white matter (Table 12). No significant change in glucose use was observed in the genu of corpus callosum at any dose of CI-977 (Table 12).

3.2 Cerebral Circulatory Effects of CI-977

3.2.1 *Behaviour and Physiological Variables*

The administration of CI-977 (0.3mg/kg, i.v.) produced similar effects on the behaviour of the conscious animals and monitored physiological variables (Table 13) to those described in the glucose utilisation study (Section 3.1.1).

3.2.2 *Local Cerebral Blood Flow*

The effects of CI-977 (0.3mg/kg) on local cerebral blood flow were examined in the same 45 brain regions analysed in the glucose utilisation study (Section 3.1), defined using identical neuroanatomical criteria.

The cerebral circulatory effects of CI-977 in the 45 CNS regions investigated are presented in Table 14. The administration of CI-977 induced a significant reduction in local cerebral blood flow in one brain area, the posterior entorhinal cortex (I). In 44 of the 45 regions studied local cerebral blood flow was not altered significantly with CI-977 treatment, and no significant increases in cerebral blood flow were observed (Table 14).

TABLE 13

CEREBRAL CIRCULATORY STUDY: CARDIOVASCULAR, RESPIRATORY AND OTHER PHYSIOLOGICAL VARIABLES AFTER ADMINISTRATION OF CI-977 TO CONSCIOUS RATS

VARIABLE	TIME AFTER DRUG (min)	CONTROL	CI-977
Rectal Temperature (°C)	0 10	36.9 ± 0.3 37.0 ± 0.1	37.0 ± 0.2 36.5 ± 0.1**
Mean Arterial Blood Pressure (mmHg)	0 10	133 ± 6 135 ± 4	132 ± 4 133 ± 5
Arterial Plasma Glucose (mM)	0 10	8.7 ± 0.3 8.9 ± 0.2	8.4 ± 0.4 11.2 ± 0.5**
pCO_2 (mmHg)	0 10	38.3 ± 0.7 37.1 ± 0.5	39.6 ± 1.1 37.3 ± 1.3
pO_2 (mmHg)	0 10	96 ± 3 97 ± 2	96 ± 4 103 ± 2
pH	0 10	7.41 ± 0.02 7.42 ± 0.01	7.41 ± 0.01 7.41 ± 0.02
Number of Animals		7	8

Data are presented as mean ± SEM. Data represent values at times 0 and 10 min following administration of CI-977 (0.3mg/kg) or vehicle (controls). **P<0.01 for statistical comparison between vehicle-treated controls and CI-977-treated animals (unpaired, two-tailed Student's *t*-test).

TABLE 14

THE EFFECTS OF CI-977 (0.3mg/kg, i.v.) UPON LOCAL CEREBRAL BLOOD FLOW AND LOCAL CEREBRAL GLUCOSE UTILISATION IN CONSCIOUS RATS

	CEREBRAL BLOOD FLOW (ml $100g^{-1}min^{-1}$)		GLUCOSE UTILISATION (µmol $100g^{-1}min^{-1}$)	
BRAIN REGION	CONTROL	CI-977	CONTROL	CI-977
Medulla/pons				
Inferior Olivary Nucleus	130 ± 15	117 ± 7	69 ± 2	66 ± 1
Pontine Reticular Formation	99 ± 10	91 ± 5	60 ± 2	52 ± 2
Vestibular Nucleus	175 ± 13	150 ± 8	101 ± 4	92 ± 3
Cochlear Nucleus	175 ± 13	175 ± 12	137 ± 7	135 ± 7
Superior Olivary Nucleus	163 ± 13	150 ± 9	124 ± 9	98 ± 5
Dorsal Raphe Nucleus	125 ± 12	112 ± 5	78 ± 2	69 ± 2
Median Raphe Nucleus	126 ± 12	119 ± 6	90 ± 1	81 ± 1
Cerebellum				
Cerebellar Cortex	94 ± 11	74 ± 4	49 ± 2	41 ± 2
Cerebellar Nucleus	164 ± 13	136 ± 8	86 ± 4	71 ± 1
Cerebellar White Matter	54 ± 7	44 ± 2	34 ± 1	24 ± 1**
Mesencephalon				
Inferior Colliculus	236 ± 22	189 ± 12	162 ± 6	106 ± 9**
Superior Colliculus	123 ± 13	109 ± 5	82 ± 8	68 ± 1
Substantia Nigra,				
pars reticulata	71 ± 4	67 ± 3	50 ± 2	42 ± 2*
pars compacta	79 ± 3	78 ± 4	64 ± 3	54 ± 3*
Red Nucleus	110 ± 11	99 ± 4	77 ± 3	69 ± 4
Ventral Tegmental Area	75 ± 9	63 ± 3	57 ± 1	58 ± 1
Dorsal Tegmental Nucleus	137 ± 14	120 ± 5	84 ± 2	77 ± 3
Periaqueductal Grey Matter	92 ± 8	74 ± 4	52 ± 2	53 ± 3
Interpeduncular Nucleus	103 ± 7	103 ± 5	86 ± 5	63 ± 6*
Diencephalon				
Medial Geniculate Body	163 ± 18	127 ± 8	105 ± 6	82 ± 2**
Lateral Geniculate Body	120 ± 15	107 ± 8	70 ± 3	61 ± 1
Subthalamic Nucleus	117 ± 13	110 ± 7	77 ± 3	68 ± 2
Hypothalamus	75 ± 7	63 ± 5	46 ± 2	43 ± 3
Lateral Habenular Nucleus	138 ± 11	160 ± 12	112 ± 10	159 ± 5**
Ventrolateral Thalamic Nucleus	125 ± 16	109 ± 8	81 ± 4	57 ± 4**
Mediodorsal Thalamic Nucleus	148 ± 19	135 ± 8	99 ± 4	94 ± 3
Telencephalon				
Hippocampus, molecular layer	95 ± 12	82 ± 7	71 ± 3	52 ± 2**
Dentate Gyrus	99 ± 11	83 ± 7	62 ± 2	45 ± 3**
Amygdala	78 ± 8	65 ± 5	69 ± 2	58 ± 3
Globus Pallidus	74 ± 10	65 ± 4	51 ± 2	40 ± 3*
Septal Nucleus	94 ± 12	88 ± 8	42 ± 3	37 ± 2
Caudate Nucleus	127 ± 16	104 ± 6	101 ± 4	74 ± 4**
Nucleus Accumbens	118 ± 18	117 ± 11	68 ± 4	65 ± 4
Visual Cortex (IV)	120 ± 17	89 ± 7	91 ± 4	69 ± 2**
Auditory Cortex (IV)	228 ± 37	170 ± 14	137 ± 4	98 ± 3**
Posterior Parietal Cortex (IV)	154 ± 22	133 ± 19	96 ± 5	74 ± 2**
Sensory Motor Cortex (IV)	167 ± 25	140 ± 9	113 ± 6	75 ± 2**
Frontal Cortex (IV)	161 ± 21	157 ± 13	101 ± 6	82 ± 1
Anterior Cingulate Cortex	144 ± 27	136 ± 15	99 ± 5	80 ± 5*
Posterior Cingulate Cortex	125 ± 19	98 ± 6	83 ± 3	67 ± 3*
Anterior Entorhinal Cortex (I)	70 ± 7	53 ± 4	65 ± 2	42 ± 3**
Posterior Entorhinal Cortex (I)	92 ± 9	63 ± 5*	61 ± 3	45 ± 2**
Myelinated Fibre Tracts				
Corpus Callosum	43 ± 4	39 ± 3	36 ± 2	29 ± 1
Genu of Corpus Callosum	50 ± 5	48 ± 4	25 ± 1	22 ± 2
Internal Capsule	47 ± 4	40 ± 2	35 ± 1	24 ± 1**

Cerebral blood flow values are derived from 15 animals and presented as mean ± SEM (n=7 control; n=8 CI-977). Roman numerals indicate cortical layer examined. *P<0.05, **P<0.01 for statistical comparison between vehicle-treated controls and CI-977-treated group in each brain region (unpaired, two-tailed Student's *t*-test).

Cerebral glucose utilisation data are extracted from Section 3.1.2 for direct comparison of the relationship between cerebral blood flow and glucose use in all brain regions examined.

Of the 44 brain regions which failed to display statistically significant alterations in cerebral blood flow, modest reductions (about 20%) were observed in 16 areas. These included allocortical and neocortical regions, and the cerebellum (e.g. posterior cingulate cortex: controls = 125 ± 19ml $100g^{-1}$ min^{-1}; CI-977 = 98 ± 6ml $100g^{-1}$ min^{-1}). A modest, non-significant elevation (16%) was observed in 1 brain region, the lateral habenular nucleus (controls = 138 ± 11ml $100g^{-1}$ min^{-1}; CI-977 = 160 ± 12ml $100g^{-1}$ min^{-1}. The autoradiograms from CI-977-treated rats were indistinguishable from those of vehicle-treated control rats, and on detailed visual inspection no regions (not even the posterior entorhinal cortex) could be identified accurately as displaying focal alterations in local cerebral blood flow.

3.3 Relationship between Cerebal Blood Flow and Glucose Utilisation

Data from the studies on glucose utilisation (Section 3.1) and cerebral blood flow (Section 3.2) in conscious rats were combined to investigate the consequences of CI-977 (0.3mg/kg) administration on the coupling of blood flow to oxidative metabolism in normal brain (Table 14).

3.3.1 *Flow-Metabolism Coupling and CI-977*

In vehicle-treated control animals, there was an excellent linear relationship (r=0.93, $p<0.001$) between local cerebral blood flow and local cerebral glucose utilisation (Figure 31). The average ratio of local cerebral blood flow to glucose use in the 45 regions examined was 1.52ml/µmol (range 1.08 - 2.24ml/µmol). Areas in which blood flow is greatest, e.g. inferior colliculus and auditory cortex (IV) displayed also the highest rates of glucose utilisation, whereas areas in which local cerebral blood flow is lowest, such as in white matter tracts, consistently display the lowest rates of glucose use

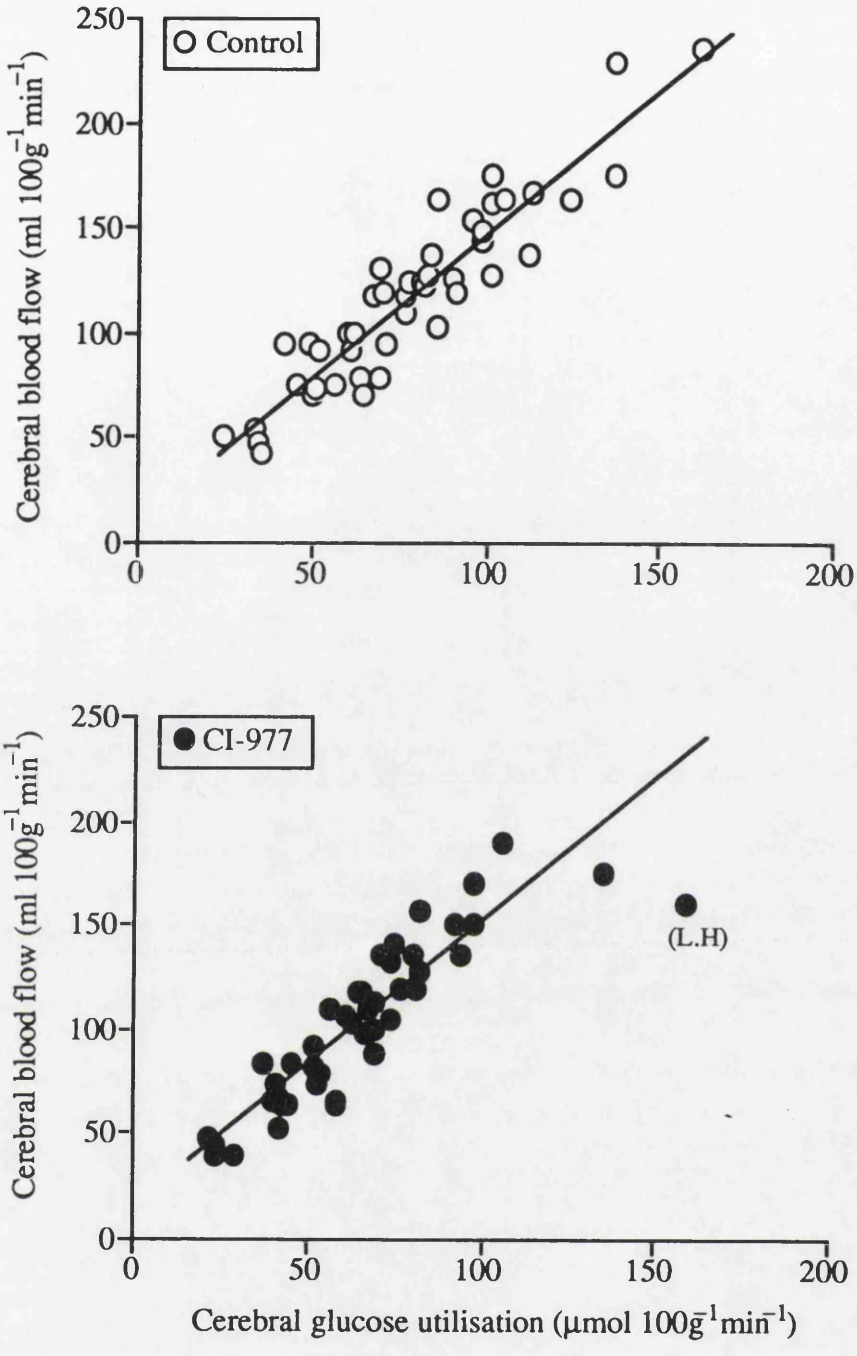


FIGURE 31

Effects of CI-977 (0.3mg/kg, i.v.) on the relationship between local cerebral blood flow and local cerebral glucose use. Upper: vehicle-treated control animals. Lower: CI-977-treated animals. Each point represents the mean cerebral blood flow and mean glucose use in one brain region. Linear regression analysis of this data yielded a slope of 1.37ml/µmol and r = 0.93 for controls and a slope of 1.25ml/µmol and r = 0.88 for CI-977-treated animals. The regression line in both graphs is that for control animals. Note the apparent lack of effect of CI-977 on cerebral blood flow relative to glucose use in the lateral habenula (L.H.) (increased by 16% and 42% from controls respectively).

(Table 14).

In animals which had received CI-977 (0.3mg/kg) there was a strong linear relationship (r=0.88, $p<0.001$) between local cerebral blood flow and glucose utilisation over all regions (Figure 31). The average ratio between local cerebral blood flow and glucose use in the 45 regions examined in animals treated with CI-977 was 1.60ml/μmol (range 1.01 - 2.24ml/μmol). In 44 of the 45 brain regions examined, cerebral blood flow remained tightly coupled to local oxidative metabolism after CI-977 (Figure 31). In only one brain area, the lateral habenula, was there a tendency towards a possible uncoupling of cerebral blood flow and glucose use after drug administration (Figure 31), although this did not reach statistical significance using repeated measures analysis of variance of log transformed data.

The transformation of data to natural logarithms of the mean values for cerebral blood flow and glucose use (McCulloch et al., 1982) reveals a linear relationship between the two parameters, which is characterised by unit slope and intercept α (Figure 32). Statistical comparison of the α values allows changes in the overall relationship to be revealed. Thus, although α changed from 0.80 in vehicle-treated controls to 0.94 in animals treated with CI-977 (Figure 32), this change was not significantly different. Therefore, the association which exists in conscious rats between local cerebral blood flow and local cerebral glucose utilisation was unaltered by CI-977 treatment.

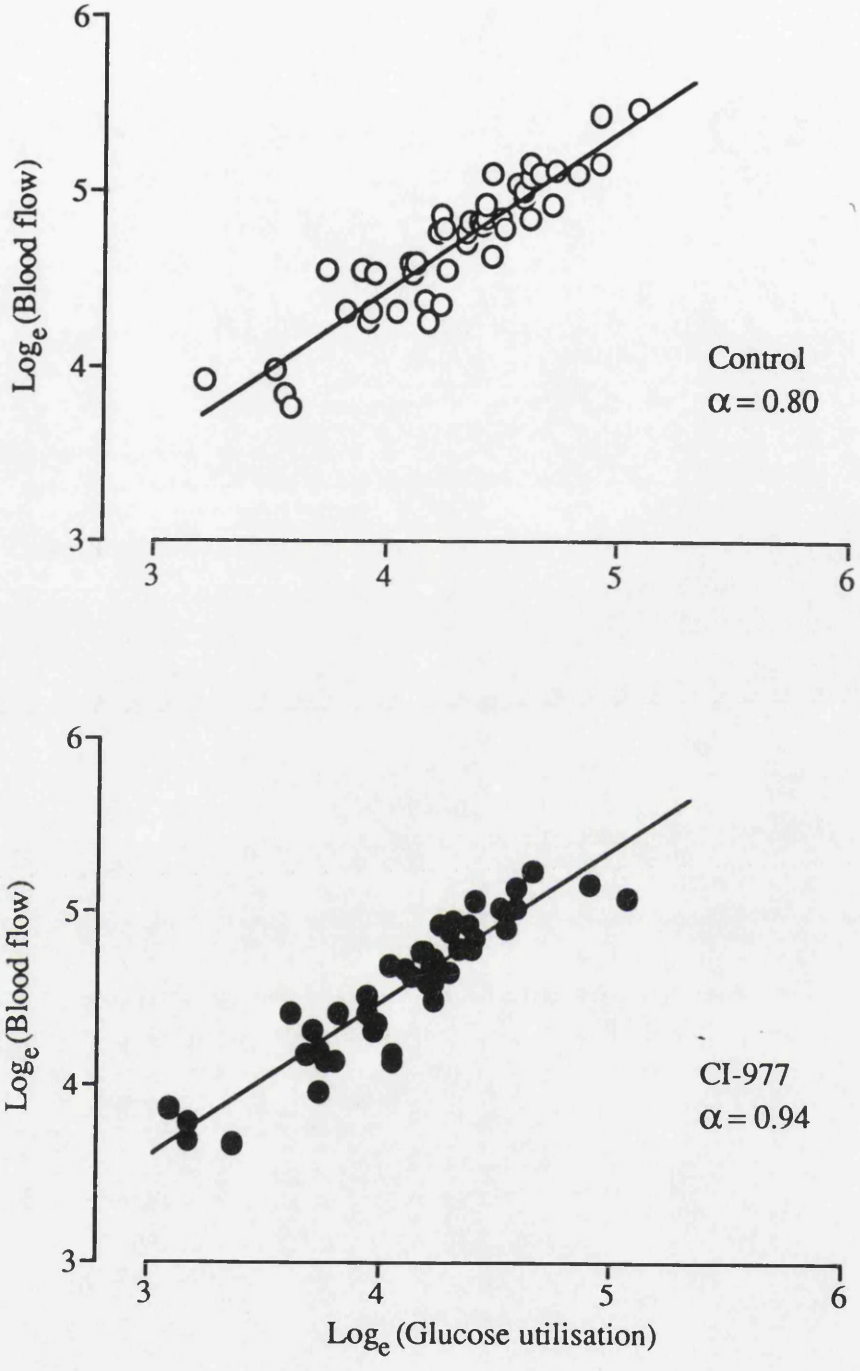


FIGURE 32

Relationship between local cerebral glucose utilisation and local cerebral blood flow presented as a logarithmic transformation of mean values for each of 45 discrete regions of conscious rat brain in vehicle-treated control animals (upper) and animals that received CI-977 (0.3mg/kg, i.v.) (lower). The relationship is described by a line with gradient unity and a point of intercept (α). There is an excellent correlation between local blood flow and glucose use in both the vehicle and CI-977-treated groups (r = 0.92 for both groups).

CHAPTER IV

DISCUSSION

1. ANTI-ISCHAEMIC EFFICACY OF CI-977

There are no pharmacological interventions designed to protect cerebral tissue from ischaemic damage which are in widespread clinical use at the present time. The results presented in this thesis represent fundamental observations that administration of the *k*-opioid receptor agonist CI-977 before and after permanent MCA occlusion in the rat and cat reduces the volume of ischaemic brain damage that develops subsequently. Such data therefore substantiates and extends previous evidence that *k*-opioid agonists can ameliorate the consequences of cerebral ischaemia in certain animal models.

Animal models of focal ischaemia were used in this thesis. Permanent occlusion of the MCA in both the rat and cat have generated the most consistent demonstrations of anti-ischaemia efficacy of NMDA receptor antagonists, and provided the basis that modulation of excitotoxic mechanisms is a sound therapeutic strategy with which to approach the development of neuroprotective agents. However, as crucial as the choice of animal model of ischaemia, is the selection of the experimental endpoint. Hitherto, almost all investigations of the beneficial effects of *k*-agonists have been restricted to rodent models of global ischaemia, with many using experimental endpoints such as brain water content, mortality rate, neurological deficits and duration of survival as measures of cerebral protection (Silvia & Tang, 1986; Silvia et al., 1987; Tang, 1985). These parameters however are not sufficiently unambiguous for use as determinants of anti-ischaemic efficacy. It is now accepted that the demonstration of a reduction in the amount of brain damage (assessed histologically or with MRI) with drug treatment is an absolute requirement in anti-ischaemic drug evaluation. As such, models of focal ischaemia are advantageous in that they lend themselves to volumetric

assessment of lesion size, and therefore the neuroprotective efficacy of CI-977 was assessed using quantitative neuropathology at both the microscopic and macroscopic level.

The present studies provide definitive evidence that *k*-opioid agonists are neuroprotective following focal cerebral ischaemia in both a lissencephalic and gyrencephalic species where key systemic variables which are believed to influence outcome were continuously monitored throughout the post-ischaemic survival period. A previous study describing the efficacy of CI-977 in a rat model of focal ischaemia (permanent MCA occlusion and ipsilateral carotid artery occlusion) assessed outcome at 48h post-ischaemia, which precluded the monitoring of these variables (Boxer et al., 1991), when CI-977 was administered as bolus injections at 30 min and 24h after the ischaemic insult only. The plasma half-life of CI-977 is approximately 1h in the rat (G.N. Woodruff, unpublished observations), and this suggests that the dosing regimen used was insufficient for a 48h survival period (Figure 33). In order to negate similar criticism given such pharmacokinetic considerations, a multiple drug treatment schedule was employed (CI-977 administered 30 min pre- and post-ischaemia and every 6 hrs thereafter) to try and maintain adequate plasma levels of CI-977 throughout the post-ischaemic survival period when outcome was assessed at 24h after permanent MCA occlusion in the rat (Figure 33).

The pivotal observation that CI-977 reduces ischaemic damage in the cat is particularly important for a number of reasons. First, as the ultimate clinical target (humans) for anti-ischaemic drugs is a gyrencephalic species, it would be imprudent to proceed to clinical trials on the basis of solely lissencephalic evidence. Second, the ease with which waves of spreading depression (in which glutamate plays a role) are propagated in the

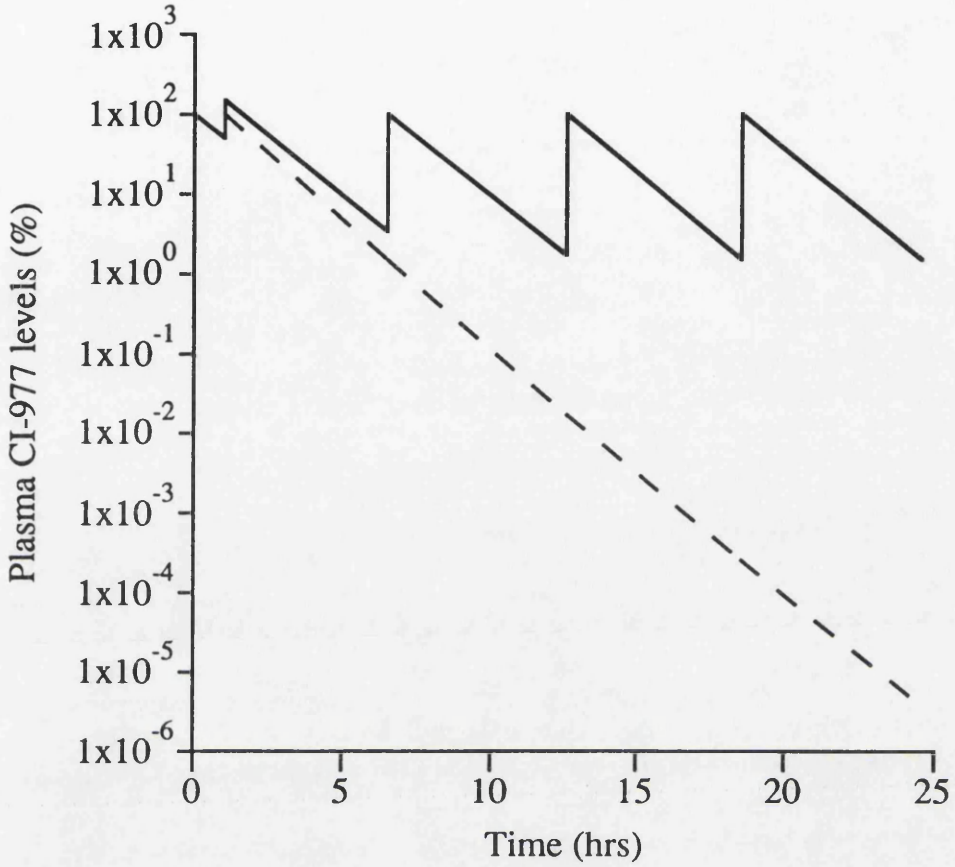


FIGURE 33

Calculated plasma levels of CI-977 (in absolute units) following the administration of CI-977 30 min before and 30 min after MCA occlusion in the rat and at 6 hourly intervals thereafter until sacrifice at 24h. These values are based on a simple one-compartment pharmacokinetic model assuming a linear exponential clearance. The dotted line represents the calculated plasma levels following a single bolus of CI-977 30 min after the induction of ischaemia as used by Boxer et al., 1991. Plasma levels of CI-977 are apparently greater throughout the experimental duration using the multiple dosing paradigm when compared to a single bolus injection. However, recent evidence suggests that there may be a second, slower clearing compartment at approximately 6h after CI-977 administration (Hayward et al., 1993b).

lissencephalic brain and the debate about the contribution of suppression of spreading depression for the anti-ischaemic efficacy of NMDA antagonists (Gill et al., 1992a; Iijima et al., 1992) further emphasise the importance of data from gyrencephalic species. Third, CI-977 was administered as an i.v. bolus followed by a continuous i.v. infusion to maintain constant plasma drug levels for the duration of the study; this dosing regimen is most pertinent to use in humans in the clinical situation.

The relative neuroprotective efficacy of the *k*-opioid agonist CI-977 with postsynaptic NMDA receptor blockade and AMPA receptor blockade remains to be fully established. In the rat, the reduction in the volume of ischaemic damage with CI-977 is of a slightly lesser magnitude to that observed previously with the non-competitive NMDA antagonist MK-801 (Gill et al., 1991a; Hatfield et al., 1992; Park et al., 1988b), but comparable to that obtained with the AMPA antagonist NBQX (Gill et al., 1992b), in similar rat models of focal cerebral ischaemia. In the cat, in the present study, the volume of tissue salvaged with CI-977 (776mm^3) is smaller than that noted with competitive and non-competitive NMDA receptor-antagonists (1300mm^3 and 1600mm^3, respectively) (Bullock et al., 1990; Ozyurt et al., 1988) but similar to that salvaged with polyamine site-receptor antagonists (750-800mm^3) (Gotti et al., 1988) and the AMPA receptor antagonist LY-293558 (600mm^3) (Bullock et al., 1994). One important caveat is that extreme caution must be exercised in comparing data from different research groups and different studies because of subtle but potentially important differences in experimental design (e.g. type of anaesthesia, surgical technical, survival period etc.). It should be recognised that for *k*-opioid receptor agonists, including CI-977 in the present study (as well as for AMPA antagonists elsewhere), much less attention has been focused upon optimising dosing

regimen, rather than establishing anti-ischaemic efficacy, than for NMDA receptor antagonists (see Gotti et al., 1988; Gill et al., 1991a; Chen et al., 1991b for precise definition of dose dependency of the response to NMDA antagonists).

The degree of neuroprotection afforded by CI-977 is similar to that obtained by the *k*-agonist GR89696. The repeated administration of GR89696 produced 35% reduction in cerebrocortical infarct volume in a mouse model of focal ischaemia when treatment was initiated 6h after the induction of ischaemia (Birch et al., 1991). This observation also suggests that a window of therapeutic opportunity may exist for CI-977 following permanent MCA occlusion in the rat and cat.

The administration of CI-977 reduced the volume of ischemic damage only within the cerebral cortex following permanent MCA occlusion in the rat and cat. Minimal protection was observed in the basal ganglia. These effects are consistent with other anti-ischaemic agents, most notably the NMDA receptor antagonists and AMPA receptor antagonists in similar models of focal ischaemia (Bullock et al., 1990, 1994; Gill et al., 1991a, 1992b). The infarct rim of the cerebral cortex where neuroprotection is seen is described as a region which has the characteristics of the ischaemic penumbra (Astrup et al., 1981). Following MCA occlusion, this area has a regional blood flow of approximately 20% normal levels (Tamura et al., 1981b; Tyson et al., 1984), which is the threshold level at which synaptic transmission is abolished, but normal ionic homeostasis is maintained. The penumbra displays an increase in glucose use, putatively due to an increased demand on ionic pumps to maintain ionic homeostasis (Nedergaard & Astrup, 1986). The lack of effect of CI-977 in the caudate putamen is probably due to the fact that the lenticulostriate branches which supply this region are effectively end arteries

(Tamura et al., 1981a), and occlusion of these branches markedly reduces blood flow to less than 10% of normal levels (Tamura et al., 1981b; Tyson et al., 1984). Consequently, there is insufficient energy to enable a return to normal ionic homeostasis (Siesjö & Bengtsson, 1989), even in the presence of CI-977. Thus, as is the case with the NMDA antagonists and presumably AMPA antagonists in models of focal ischaemia, the lack of protection in the caudate nucleus indicates that a minimal level of cerebral blood flow is required for anti-ischaemic efficacy of *k*-opioid agonists like CI-977.

In the present investigations, the effect of CI-977 on histologically defined ischaemic brain damage was assessed at 4h and 6h after the induction of ischaemia in the rat and cat respectively. The principal advantage of assessing early infarction rather than overt infarction which is identifiable 1-7 days after the induction of ischaemia, is that critical physiological variables can be repeatedly assessed (e.g. MABP, plasma glucose) or maintained within defined limits (e.g. core body temperature, respiratory blood gas status). In the absence of such information, the extent to which such systemic factors have contributed or confounded assessment at anti-ischaemic drug action cannot be determined with certainty. However, by assessing drug-induced reductions in early cerebral infarction, it could be argued that CI-977 is delaying the development of, but not actually preventing ischaemic cell death. While such an effect cannot be dismissed absolutely, the demonstrations that permanent MCA occlusion in the rat produces a similar degree of ischaemic brain damage and comparable neuroprotective effect of CI-977 (0.3mg/kg) when outcome is assessed at both 4h and 24h after the induction of ischaemia suggests that CI-977 is not neuroprotective by merely delaying ischaemic cell death in this model. This is concordant with the observations that maximal infarction is seen 3-4h after focal

cerebral ischaemia in the rat (Kaplan et al., 1991; Memezawa et al., 1992) and that, unlike transient models of global ischaemia, there is no delayed neuronal degeneration following permanent MCA occlusion in the rat (Nedergaard, 1988).

The importance of monitoring and controlling physiological variables in studies of neuroprotective drugs in animal models of cerebral ischaemia is increasingly recognised (Ginsberg & Busto, 1989; Graham, 1988). In the majority of investigations, particularly those with outcome assessed 24h or more after the insult, monitoring is effectively restricted to the surgical and immediate post-operative period (Buchan et al., 1991c; Gill et al., 1992b). The results of the present studies provide evidence that the benefit conveyed by the *k*-agonist CI-977 in focal cerebral ischaemia is unlikely to be attributed to artefactual or indeed drug-induced alterations in key physiological variables such as MABP or temperature.

CI-977 induced a marked and sustained hypotension throughout the post-ischaemic period in halothane-anaesthetised rats. Hypotension is an important factor in the evolution of ischaemic brain damage. After the induction of ischaemia, blood flow to perischaemic tissue is linearly related to perfusion pressure, and small reductions in arterial pressure can increase the severity of the insult (Hossmann, 1982). It has been shown in a rat MCA occlusion model that a period of transient haemorrhagic hypotension (MABP of 60mmHg for 30 min) immediately after occlusion of the MCA increased the volume of ischaemic brain damage by more than 50% from that observed in normotensive animals (Osborne et al., 1987) by reducing blood flow to the ischaemic penumbra to even lower levels (Dirnagl & Pulsinelli, 1990). Thus, the hypotension that following the administration of CI-977 might have been expected to exacerbate ischaemic brain damage. The volume of infarction

was, in fact, reduced with CI-977 despite the induced hypotension thereby demonstrating the marked neuroprotective efficacy of this agent. Consequently, it is also possible that the neuroprotection afforded by CI-977 was underestimated. In addition, the hypotensive effect of CI-977 may underpin the U-shaped dose-response curve to CI-977 when outcome was assessed 24h after the induction of ischaemia in the rat. Progressive hypotension with increasing doses of CI-977 would tend to counterbalance the neuroprotective effects of this drug. This would account for the U-shape neuroprotection dose-response curve to CI-977 and the failure of the highest concentration of CI-977 (3mg/kg) to display significant neuroprotection. A U-shaped response of infarction volume versus dose has also been observed with MK-801 in focal ischaemia in the halothane-anaesthetised rat which was attributed to hypotension induced by the highest doses of MK-801 (Gill et al., 1991a).

The administration of CI-977 also induced a decrease in MABP in halothane-anaesthetised cats. In contrast to the hypotensive effect observed in the rat, the reduction in MABP was transient, such that at the time of MCA occlusion, MABP had returned to pre-administration levels, and did not significantly deviate from control levels throughout the entire post-ischaemic period. At present there is no reliable evidence that transient, moderate hypotension prior to the induction of ischaemia by permanent occlusion of the MCA is neuroprotective. Recent evidence suggests that a prior reduction of cerebral blood flow, produced by brief, sublethal periods of global ischaemia reduces the volume of infarction produced by subsequent permanent MCA occlusion in the rat (Simon et al., 1993) and protects hippocampal pyramidal neurones to subsequent, lethal periods of global ischaemia in both rats and gerbils (Kirino et al., 1991; Liu et al., 1992), putatively by inducing

the expression of heat shock proteins (HSP70, HSP72). Thus, if a similar phenomenon of ischaemic tolerance was to occur as a result of the transient, hypotensive effect prior to MCA occlusion in the cat, it may contribute to the neuroprotective effect of CI-977. However, the autoregulatory mechanism will attenuate the reduction in cerebral blood flow in response to the hypotensive episode prior to the induction of ischaemia and the time constant (10-20 sec) will allow episodes of only transient hypoperfusion, and thereby have negligible influence on the neuroprotective efficacy of CI-977.

There is conflicting evidence available regarding the effects of alterations in plasma glucose concentration upon ischaemic brain damage in animal models of cerebral ischaemia (Marie & Bralet, 1991). In models of permanent focal ischaemia, several studies suggest that hyperglycaemia increases the volume of infarction (de Courten-Meyers et al., 1988; Duverger & MacKenzie, 1988; Prado et al., 1988), whereas others suggest that it either decreases (Ginsberg et al., 1987b; Kraft et al., 1990; Zasslow et al., 1989) or has no effect (Nedergaard & Diemer, 1987). At present, there is no reliable evidence describing the effects of reduced plasma glucose on ischaemic brain damage following permanent focal ischaemia. The administration of CI-977 reduced plasma glucose by 10% throughout the post-ischaemic survival period in halothane-anaesthetised rats. However, huge alterations (3-fold) in plasma glucose are required to alter lesion size by 25% after permanent MCA occlusion in the rat (Duverger & MacKenzie, 1988). Thus, it is unlikely that the modest reduction in plasma glucose concentration produced by CI-977 has a major effect, either beneficial or detrimental, on the final volume of infarction.

The issue of brain temperature in the evaluation of anti-ischaemic efficacy of drugs is of major importance (see Ginsberg et al., 1992 for

review). In particular, there is omnipresent concern whether observed anti-ischaemic efficacy can be attributed/ascribed to artefactual alterations in brain temperature. There are three reasons why the neuroprotective effects of CI-977 in the present investigations with permanent occlusion of the MCA (where extracranial perfusion is preserved) are unlikely to be due to such an artefact. First, deep body temperature was similar in both vehicle-treated and CI-977-treated animals. Second, the experimental design and conduct did not involve any procedure (such as compromising extracranial blood flow or direct heating of the head) which could alter artefactually the relationship between deep body temperature and brain temperature in some animals. Third, unlike global ischaemia (Busto et al., 1987) and reperfusion after focal ischaemia (Chen et al., 1992a; Moyer et al., 1992; Zhang et al., 1993), brain temperature has only a minor influence on the amount of ischaemic brain damage after permanent MCA occlusion (Morikawa et al., 1992; Ridenour et al., 1992).

Previous evidence that *k*-opioid agonists are neuroprotective was derived almost exclusively from investigations in rodent models of global ischaemia with outcome assessed at long periods after the ischaemic insult. The observations in this thesis makes three important contributions to support this therapeutic approach. First, unequivocal benefit after treatment with the *k*-opioid agonist has been shown in both a lissencephalic species (rat) and gyrencephalic species (cat). Second, the anti-ischaemic efficacy has been demonstrated in models of permanent MCA occlusion. Third, the reduction in infarction which CI-977 confers is not dependent upon alterations in critical systemic variables (MABP, temperature, plasma glucose) which are thought to influence outcome; for the first time with *k*-opioid agonists, all of these variables have been monitored continuously from the onset of ischaemia until

sacrifice.

2. MECHANISM OF ANTI-ISCHAEMIC ACTION

2.1 Brain Swelling

The pharmacological mechanism to which the neuroprotective effect of *k*-opioid agonists is commonly attributed to a reduction in cerebral oedema (Silvia & Tang, 1986; Silvia et al., 1987). There is a 40-50% mortality rate among severely head-injured patients arising primarily as a result of raised intracranial pressure and subsequent reduced cerebral perfusion due to cerebral oedema and/or non-recovery lesion development (i.e. subdural haematomas, contusions, lacerations) (Lobato et al., 1988; Luersson et al., 1988). This underscores the decision to investigate the efficacy of CI-977 24h after permanent MCA occlusion in the rat. By assessing outcome at 24h, infarction and brain swelling are well developed, and thus it is possible to examine both parameters in the same animal and determine any inter-relationship between the volume of swelling and the volume of infarction after drug treatment.

k-Opioid agonists are known to possess diuretic actions and modulate the release of vasopressin (Peters et al., 1987; Slizgi et al., 1984; Wells & Forsling, 1991). The induction of water diuresis produces an increase in plasma osmolality and the subsequent hyperosmotic state results in the restriction of oedema development and secondary neuronal damage (Silvia & Tang, 1986; Silvia et al., 1987). In addition, preliminary data with MCA occlusion in the rat indicate that CI-977 does not reduce infarct size in Brattleboro rats (in which it does not produce diuresis), although in the Fischer 344 strain CI-977 reduces the volume of infarction to a similar extent as that observed in the present studies (Boxer et al., 1991). On the basis of

such evidence, it was postulated that the primary mechanism to account for the neuroprotection conferred by *k*-opioid agonists is to reduce cerebral oedema through a water diuresis.

However, evidence is presented in this thesis to suggest that this pharmacological action is not the principal mechanism to explain the anti-ischaemic efficacy of CI-977 and other *k*-opioid receptor agonists. Comparison of the potency of CI-977 as a diuretic (Hunter et al., 1990) and neuroprotective agent in the rat indicate that, at the dose producing maximum diuresis, CI-977 has no significant effect on the volume of ischaemia following MCA occlusion. In addition, CI-977 is neuroprotective in the gerbil model of global ischaemia in spite of the absence of diuresis, whilst the diuretic furosemide affords no neuroprotection at doses that produce a marked diuresis (Hayward et al., 1992). Furthermore, although brain swelling (an indirect measure of oedema) was clearly reduced by CI-977 treatment when outcome was assessed 24h after the onset of ischaemia, there was no disproportionate reduction in swelling relative to infarction in the rat. A parallel reduction in infarction and swelling has been described with nimodipine, for which no primary anti-oedema action has ever been proposed (rather, the converse) (Harris et al., 1982; Hossmann, 1982; Jacewicz et al., 1990a). In the cat, CI-977 produced a progressive increase in plasma osmolality throughout the post-occlusion period. The magnitude at the increase in plasma osmolality induced by CI-977 is comparable to that obtained clinically in man using the hyperosmolar agent mannitol (Marshall et al., 1978; Wise & Chater, 1962). Mannitol is putatively neuroprotective in some animal models of cerebral ischaemia (Little, 1978; Sutherland et al., 1988). This suggests that the effect of CI-977 is therefore potentially biologically significant. However, clear increases in plasma osmolality were

not observed until 2-3h after MCA occlusion in the cat. The window of therapeutic opportunity in large animal MCA occlusion models is 2-3h (McCulloch et al., 1991), and thus the alterations in plasm osmolality and reductions in size of the ischaemic lesions; cats treated with CI-977 with the numerically greatest alterations in plasma osmolality had larger lesions than those with smallest alterations in plasma osmolality.

Cumulatively, these data suggest that *k*-opioid agonists may not produce their neuroprotective effects principally by reducing cerebral oedema and secondary neuronal death. However, although the reduction in brain swelling may be a consequence of a reduction in infarction, it cannot be discounted absolutely that an increase in plasma osmolality may contribute to the neuroprotection afforded by CI-977 by prolonging the window of therapeutic opportunity.

2.2 Cerebral Blood Flow

The identification of the enkephalins (Hughes et al., 1975) initiated the interest in opioid peptides as possible neurotransmitters or neuromodulators within the CNS. Cerebrovascular interest in opioid peptides originated with the report that the opioid receptor antagonist naloxone reverses ischaemic neurological deficits in man (Baskin & Hosobuchi, 1981). The strategic location of opioid peptides and opioid receptors in central and peripheral structures involved in autonomic function suggests that the opioid system may be involved in cerebrovascular control, either directly or indirectly. Although there have been a number of reports in which opioid-induced contraction or dilatation of cerebral vessels has been demonstrated, there is no clear evidence available at the present time that opioid receptor agonists (or antagonists) have direct vasomotor effects in the cerebral circulation at

concentrations that are pharmacologically selective and clinically relevant.

High concentrations (10^{-4}M) of morphine and other opioid receptor agonists (including U-50,488H) elicit cerebral vasoconstriction, putatively via the activation of opioid receptors (Altura et al., 1984; Recio et al., 1986). The concentrations required are at least 100 times greater than those required in other pharmacological investigations. Relaxation of cerebral arteries has been reported with enkephalin and other opioid agonists, putatively via *k*-opioid receptors (Hanko & Hardebo, 1978; Altura et al., 1984; Harder & Madden, 1984). However, the cerebrovascular relaxations are very small (Hanko & Hardebo, 1978; Harder & Madden, 1984) or are achieved with very high agonist concentrations (Altura et al., 1984). The observations of Wahl (1985) with perivascular microapplication of agents in anaesthetised cats are possibly the most pertinent: leu-enkephalin and morphine are without cerebrovascular effects in the concentration range 10^{-11} to 10^{-5}M, although small increases in pial arteriolar diameter (5-14%) were observed with very high concentrations (10^{-3}M) of opioid agonists. The opioid receptor antagonist naloxone has little direct effect on the cerebral vasculature, except at high concentrations, where a relaxation is observed (Brandt et al., 1983; Wahl, 1985; Sasaki et al., 1984). In addition, the endogenous *k*-opioid peptide dynorphin has been detected in nerve fibres surrounding intracranial blood vessels (Moskowitz, 1987), and the *k*-agonist U-50,488H constricts rat cerebral vessels, an effect which is nalmefene reversible (Chen et al., 1991a).

The mechanistic basis for the effects of opioid agonists and antagonists on cerebral vessels is unknown at the present time. Pial vessels are richly innervated with noradrenergic fibres of sympathetic origin which may manifest themselves through maintenance of vascular tone (Edvinsson & MacKenzie, 1977). Agonists at the μ, δ and *k*-opioid receptors inhibit

noradrenaline release (Werling et al., 1987), and thus opioid agonists may produce their effects on the cerebral vasculature indirectly by modulating sympathetic activity.

The results of the present studies fail to provide any clear evidence to indicate that the *k*-opioid CI-977 has any significant cerebrovascular effects. In conscious rats CI-977 (at the neuroprotective dose of 0.3mg/kg) produced modest, non-significant alterations in cerebral blood flow in the majority of brain regions examined (Figure 34). A significant decrease in local cerebral blood flow was observed only within the entorhinal cortex (posterior) following CI-977. In contrast, the administration of NMDA receptor antagonists produce marked changes in cerbral blood flow in normal, lightly restrained rats using the same techniques as in the present study. Significant increases in local cerebral blood flow have been observed in the limbic system (hippocampus, dentate gyrus, entorhinal cortex, anterior and posterior cingulate cortex), in neocortex (e.g. visual and parietal cortices), in subcortical nuclei (e.g. septal nucleus, caudate nucleus, nucleus accumbens) and even in white matter (corpus callosum), and a significant reduction in local cerebral blood flow in the inferior colliculus following MK-801 (Nehls et al., 1990). A similar pattern of response is seen with ketamine although no increases in cerebral blood flow were observed in neocortical and other forebrain areas (Cavazzuti et al., 1987).

Evidence is also available from the studies in halothane-anaesthetised rats and cats to indicate that CI-977 may have minimal cerebral circulatory effects. In the hemisphere contralateral to the focal ischaemia, an essentially normal, non-ischaemic hemisphere, CI-977 failed to display significant alterations in the level of cerebral blood flow in any of the brain regions examined (Figure 34). Moreover, in the absence of definitive studies,

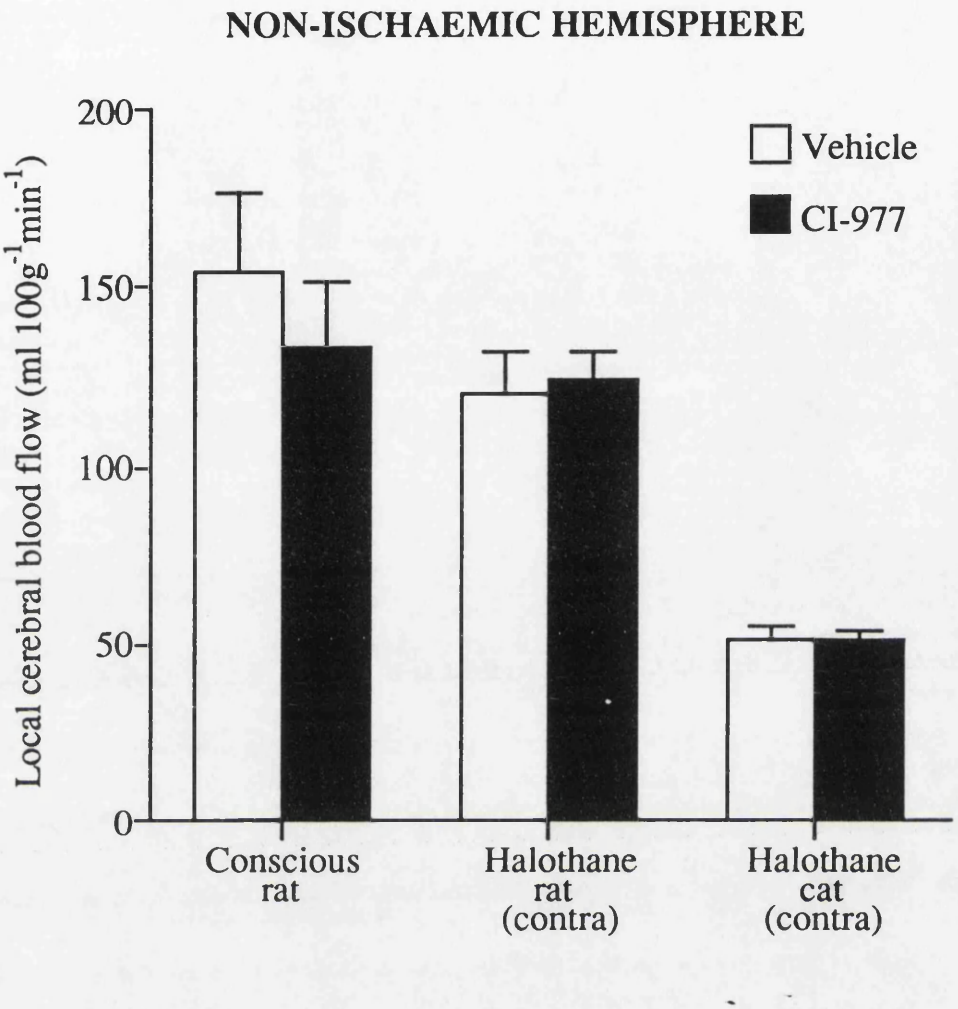


FIGURE 34

The effect of CI-977 upon cerebral blood flow in the parietal cortex in the non-ischaemic hemisphere of conscious, lightly restrained rats and mechanically ventilated halothane-anaesthetised rats and cats. Data are presented as mean ± SEM (n=4-8 per group). There are no significant differences between the vehicle-treated control groups and the CI-977 treatment groups.

these results suggest that halothane-anaesthesia may have a minor influence on the cerebrovascular effects of CI-977 and other *k*-opioid agonists.

The level of cerebral oxidative metabolism is a crucial determinant of the level of cerebral tissue perfusion under normal circumstances (Kuschinsky & Wahl, 1978). In the present studies, there was no clear evidence that cerebral blood flow and glucose use could be uncoupled locally, numerically or neuroanatomically in conscious rats after CI-977. There was, however, a suggestion of a possible uncoupling of cerebral blood flow and glucose use in the lateral habenula following CI-977 administration. This apparent effect may have been a consequence of the lack of spatial resolution in the lateral habenula produced with highly diffusable [^{14}C]-iodoantipyrine when compared to [^{14}C]-2-deoxyglucose, rather than as a response to CI-977. A local dissociation between cerebral blood flow and glucose use has been demonstrated following administration of NMDA receptor antagonists (Cavazzuti et al., 1987; Nehls et al., 1990). The level of cerebral blood flow was increased in excess of metabolic demand in neocortex, cingulate cortex (anterior and posterior), interpeduncular nucleus, and caudate nucleus although the mechanistic basis for such an effect remains to be fully resolved. The principal conclusion which can be drawn from the present study is that the relationship which exists between local cerebral blood flow and glucose in each region of the CNS examined in normal animals is unaltered by CI-977 administration. It would thus appear possible that the alterations in cerebral blood flow observed after CI-977 are a consequence of the reductions in metabolic activity which result from CI-977 treatment, rather than a direct action on cerebral vessels.

The extent to which alterations in cerebral blood flow may contribute to or complicate (e.g. via steal phenomenon) the neuroprotective effect of

CI-977 after permanent MCA occlusion is unknown. Prior to the advent of the excitotoxic hypothesis, anti-ischaemic drug development had been dominated by attempts to convey benefit through improvement in blood flow to ischaemic tissue. The *k*-agonist U-50,488H has been reported to attenuate post-ischaemic hypoperfusion in a mouse head injury model (Hall et al., 1987), suggesting that cerebral circulatory effects may contribute to the anti-ischaemic benefit observed after *k*-opioid agonists. There has long been concern, however, that increasing blood flow to ischaemic brain tissue may have adverse consequences such as increased cerebral oedema and raised intracranial pressure, haemorrhagic transformation or generation of free radicals (Harris et al., 1982; Hossmann, 1982).

There is considerable debate over whether the improved neuropathological outcome after experimental focal ischaemia with NMDA receptor antagonists is attributable to their cerebrovascular effects. Increased cerebral blood flow to the ischaemic areas has been reported with MK-801 and CGS-19755 in the rat MCA occlusion model (Buchan et al., 1992; Takizawa et al., 1991). The non-competitive NMDA antagonists dextromethorphan has been shown to increase blood flow to perifocal tissue after focal ischaemia in rabbits, although global cerebral blood flow values did not differ significantly from control values (Lo & Steinberg, 1991). However, in iodoantipyrine studies conducted in parallel to histopathological studies, Park and associates (1988b, 1989) have described reduced infarct with no alterations in cerebral blood flow within (or adjacent to) the ischaemic territory after MK-801 (Figure 35). Similarly, MK-801 has recently been found to improve the outcome after MCA occlusion in the cat independent of changes in cortical blood flow (Dezsi et al., 1992). These seemingly conflicting results may be due to differences in the animal model,

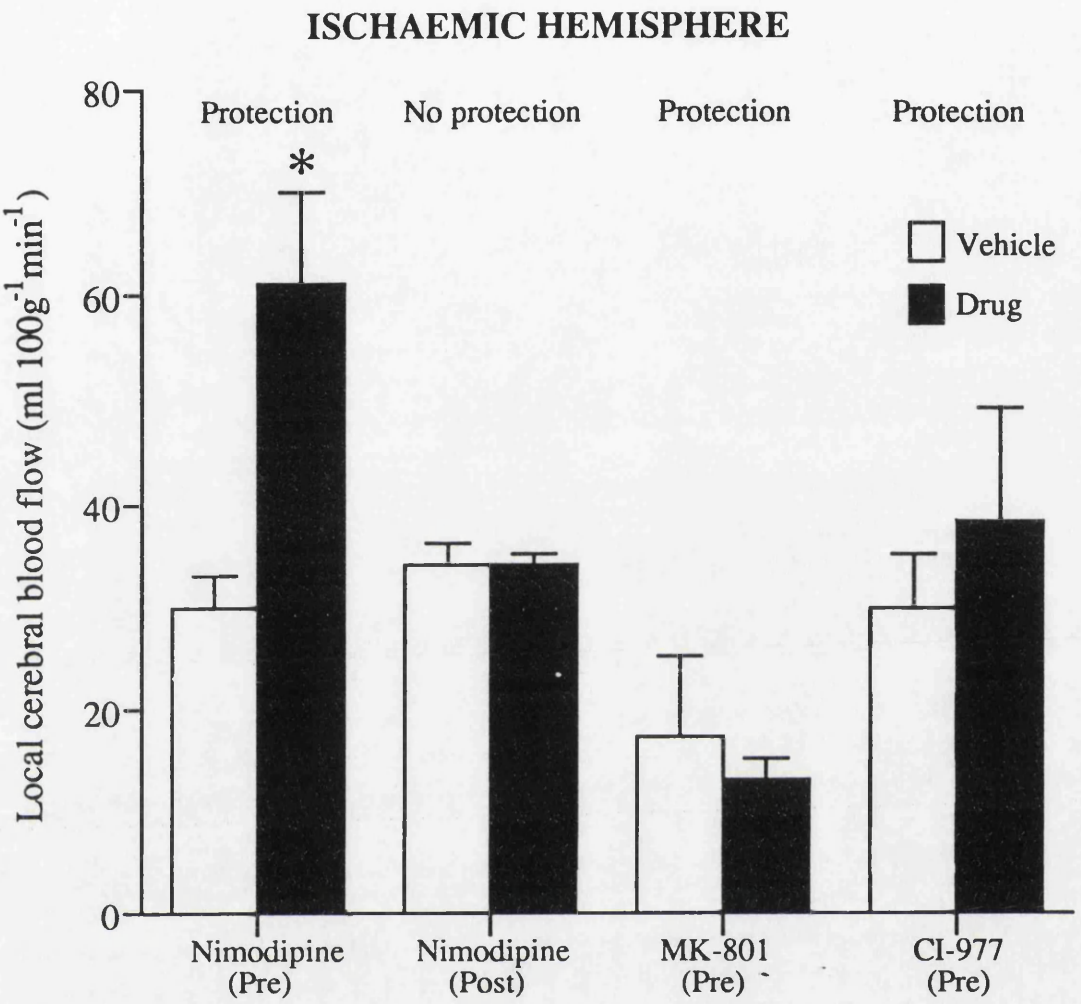


FIGURE 35

Comparison of the effects of pretreatment with MK-801, nimodipine and CI-977 and post-treatment with nimodipine upon cerebral blood flow in the sensory motor cortex ipsilateral to permanent MCA occlusion in halothane-anaesthetised rats. Data are presented as mean ± SEM (n=4-8 per group). *P<0.05 for the difference between vehicle and nimodipine pretreatment. Note that in contrast to nimodipine pretreatment, the neuroprotection afforded by MK-801 and CI-977 pretreatment can be dissociated from their effects on cerebral blood flow. (Redrawn from the data of Gotoh et al., 1986; Mohammed et al., 1985; Park et al., 1989.)

anaesthesia, or method of blood flow determination. In contrast, the reduction in cortical infarction that was observed with nimodipine in the rat MCA model could not be dissociated from an increase in blood flow towards the margins of MCA territory (Gotoh et al., 1986; Jacewicz et al., 1990b; Mohamed et al., 1985) (Figure 35).

In the present study, vehicle-treated animals exhibited an area of profound hypoperfusion that conforms to the pattern of cerebral blood flow previously described in the hemisphere ipsilateral to MCA occlusion in halothane-anaesthetised rats (Tamura et al., 1981b; Tyson et al., 1984). The administration of CI-977 30 min prior to the induction of ischaemia failed to produce marked alterations in cerebral blood flow within the territory of the occluded MCA. In particular, CI-977 failed to demonstrate a significant effect on cortical blood flow in any of the 5 cortical areas comprising the ischaemic cortex (Figure 35), the site at which the neuroprotective effects of CI-977 are observed. Focal increases in cerebral blood flow in circumscribed brain regions in the ipsilateral hemisphere were observed in animals treated with CI-977. The structures involved are the lateral thalamus and medial caudate nucleus ipsilateral to the MCA occlusion. Each of these regions has neuronal connections (putatively glutamatergic) with the ischaemic cortex via corticothalamic and corticostriate pathways (Fonnum, 1984). The delayed degeneration of the lateral thalamus observed several months after MCA occlusion has been attributed to its neuronal connections to the ischaemic cortex (Fujie et al., 1990). The mechanistic basis for the acute increases in blood flow in these regions with CI-977 remains unknown although presynaptic modulation of altered corticothalamic and corticostriate activity would be a possible mechanism (Pinnock, 1992).

CI-977 produced a moderate and sustained hypotension throughout the

cerebral circulatory study, and low arterial blood pressure may be detrimental to local cerebral perfusion. It is generally accepted that cerebrovascular autoregulation to reduced blood pressure is severely impaired by an ischaemic insult (Dirnagl & Pulsinelli, 1990; Symon et al., 1976). Thus, in the present study it is noteworthy that there was no alteration in cerebral blood flow in the ischaemic hemisphere despite the combined effects of impaired autoregulation and a drug-induced reduction in blood pressure. *k*-Opioid receptor agonists may attenuate sympathetic activity (Gulati & Bhargava, 1988), and a reduction in sympathetic activity extends (at least in normal animals) the autoregulatory range (Paulson et al., 1990).

While the same experimental design has been used with success to identify the contribution of cerebrovascular events to neuroprotective agents such as nimodipine (Jacewicz et al., 1990b; Mohamed et al., 1985), it has been unable to demonstrate any contribution of cerebral circulatory effects to the neuroprotection afforded by CI-977 in the present study. However, as with any negative observation, one cannot conclude absolutely that CI-977 is totally devoid of effects on cerebral blood flow, but rather whether the effects are within the statistical power of the design. *Post hoc* power analysis ($\alpha = 0.05$, $\beta = 0.2$) in the present study indicate that the magnitude of changes in cerebral blood flow would have had to be in the order of 35% in the non-ischaemic hemisphere and in excess of 40% in the ischaemic hemisphere to be consistently detected (statistical power 80%). However, the statistical power is similar to, if not better than, previous studies using nimodipine in the same model (Jacewicz et al., 1990b; Mohamed et al., 1985). Thus, whatever changes CI-977 may have on cerebral blood flow in the present study, they are not so readily demonstrable as those seen with nimodipine in this model.

An important general conclusion can be drawn from the results of the present investigation. The improved histological outcome after CI-977 can be dissociated from its effects on cerebral blood flow and suggests that the neuroprotective effects of CI-977 in this model are not principally attributable to cerebral circulatory effects, but via an action on neuronal tissue.

2.3 Glutamate Release

The pivotal observation describing elevations in the extracellular concentrations of glutamate and aspartate measured by microdialysis during ischaemia was reported in the rat hippocampus by Benveniste and colleagues in 1984 (Benveniste et al., 1984). A plethora of studies have since confirmed and characterised this phenomenon, in different brain regions, with various animal models of global (Globus et al., 1988; Hagberg et al., 1985; Shimada et al., 1989, 1990) and focal (Butcher et al., 1990; Hillered et al., 1989; Matsumoto et al., 1992, 1993; Takagi et al., 1993) ischaemia, spinal cord and head injury (Katayama et al., 1990; Panter et al., 1990), and also in man (Baker et al., 1993; Persson & Hillered, 1992) (Table 15). The increase in glutamate release after the induction of ischaemia has been shown to range from 5 to 27 fold in the cerebral cortex (Table 15). It has been estimated that the extracellular glutamate concentration within the synaptic cleft, taking into account shrinkage of the extracellular space of 50% of the normal value during ischaemia, the *in vitro* recovery of the microdialysis probe, and the tortuosity factor (non-linearity of the extracellular diffusion path), can reach up to 1mM (Benveniste et al., 1991).

The precise origin of the elevated levels of glutamate observed during cerebral ischaemia is a contentious issue. The level of glutamate within

TABLE 15

MAGNITUDE OF EXTRACELLULAR GLUTAMATE RELEASE IN ISCHAEMIA/TRAUMA

SPECIES	MODEL	MAGNITUDE OF GLUTAMATE RELEASE	SITE	INVESTIGATORS
Rabbit	Weightdrop spinal cord injury	6 fold	Impact Site	Panter et al., 1990
Rat	Fluid percussion injury	5 fold	Impact Site	Katayama et al., 1990
	Subdural haematoma	3 fold	Hippocampus (non-ischaemic)	Bullock et al. 1991
		7 fold	Ischaemic Cortex	
	Bilateral carotid occlusion and hypotension	8 fold	Hippocampus	Benveniste et al., 1984
	Middle cerebral artery occlusion	17 fold	Ischaemic Cortex	Butcher et al., 1991
		27 fold	Ischaemic Striatum	
Cat	Bilateral carotid occlusion, coagulation of thyrocervical trunk	5-10 fold	Ischaemic Cortex	Shimada et al., 1989
	Middle cerebral artery occlusion	5-12 fold	Ischaemic Cortex	Matsumoto et al., 1992
Man	Subarachnoid haemorrhage	approx. 25 fold	Frontal Cortex	Persson & Hillered, 1992

neurones is around 4 orders of magnitude higher than that in the extracellular space, a gradient maintained across the plasma membrane by Na^+/K^+-dependent carrier-mediated uptake into both neurones and glia (Nicholls & Attwell, 1990). It is generally accepted that glutamate is present in either cytosolic (metabolic) or neurotransmitter (vesicular) pools, which can be discriminated by studying the Ca^{2+}-dependency of glutamate release (Kauppinen et al., 1988; Nicholls & Attwell, 1990). Two distinct mechanisms, alone or combined, may be responsible for the ischaemia induced increase in extracellular glutamate concentration; Ca^{2+}-dependent exocytotic release of vesicular glutamate (Kauppinen et al., 1988) and reversal of the electrogenic uptake transport systems on neurones and glia (Szatkowski et al., 1990).

Under the ionic conditions which prevail during cerebral ischaemia, there is an initial increase in Ca^{2+}-dependent vesicular release of glutamate by ATP-dependent exocytosis (Katayama et al., 1991; Nicholls & Attwell, 1990). Thereafter, as ATP levels decline during the ischaemic episode, the high-affinity energy-dependent uptake systems on neurones and glia, which normally remove extracellular glutamate, fail. There is evidence in severe energy failure that the plasma membrane-bound glutamate uptake carriers can reverse, and pump glutamate out of cells into the extracellular space, a process which is Ca^{2+}-independent (Kauppinen et al., 1988; Nicholls & Attwell, 1990; Szatkowski et al., 1990). The initial increase in glutamate observed after the onset of cerebral ischaemia has been shown to depend on Ca^{2+} (Katayama et al., 1991). In addition phenlysuccinate, which prevents the biosynthesis of vesicular glutamate but leaves the metabolic glutamate pool unaffected, has a minimal effect on glutamate released in the rat hippocampus after transient, severe forebrain ischaemia (Christensen et al., 1991). Such evidence suggests that both Ca^{2+}-dependent and Ca^{2+}-

independent mechanisms are involved in the ischaemia-induced elevation of extracellular glutamate.

The relative contribution of these two distinct mechanisms to the elevation in extracellular glutamate levels will depend upon the severity of the ischaemic insult. During global ischaemia, ATP levels fall to negligible values within a few minutes (Nordström & Siesjö, 1978; Pulsinelli & Duffy, 1983; Welsh et al., 1978, 1982) (Figure 36). However, in animal models of MCA occlusion, although there are marked reductions of ATP levels in the ischaemic focus, there is no complete cessation in cellular energy state (Folbergrová et al. 1992; Nowicki et al., 1988; Welsh et al., 1980) (Figure 36). Furthermore, in the ischaemic penumbra after permanent MCA occlusion where residual blood flow remains between 10-20ml $100g^{-1}$ min^{-1} (Astrup et al., 1981), there are only mild to moderate alterations in ATP levels over several hours (Folbergrová et al., 1992; Nowicki et al., 1988; Welsh et al., 1980) (Figure 36). The residual blood flow in the cerebral cortex would allow replenishment of energy substrates suggesting that glutamate release into the extracellular space by ATP-dependent exocytosis in focal cerebral ischaemia remains possible for long periods.

In the present study, increases in the levels of glutamate and aspartate are observed when cerebral blood flow is reduced to below 20ml $100g^{-1}$ min^{-1} in the cerebral cortex after permanent MCA occlusion in the cat. The magnitude of the elevations in the concentrations of glutamate and aspartate (approximately 3-8 fold and 4-12 fold respectively relative to pre-ischaemic basal levels) are in general agreement with observations reported previously in this model of focal ischaemia (Matsumoto et al., 1992, 1993) (Table 15). The elevations in glutamate release appear to occur at cerebral blood flow values of approximately 15-20ml $100g^{-1}$ min^{-1}, which represents 27-36% of

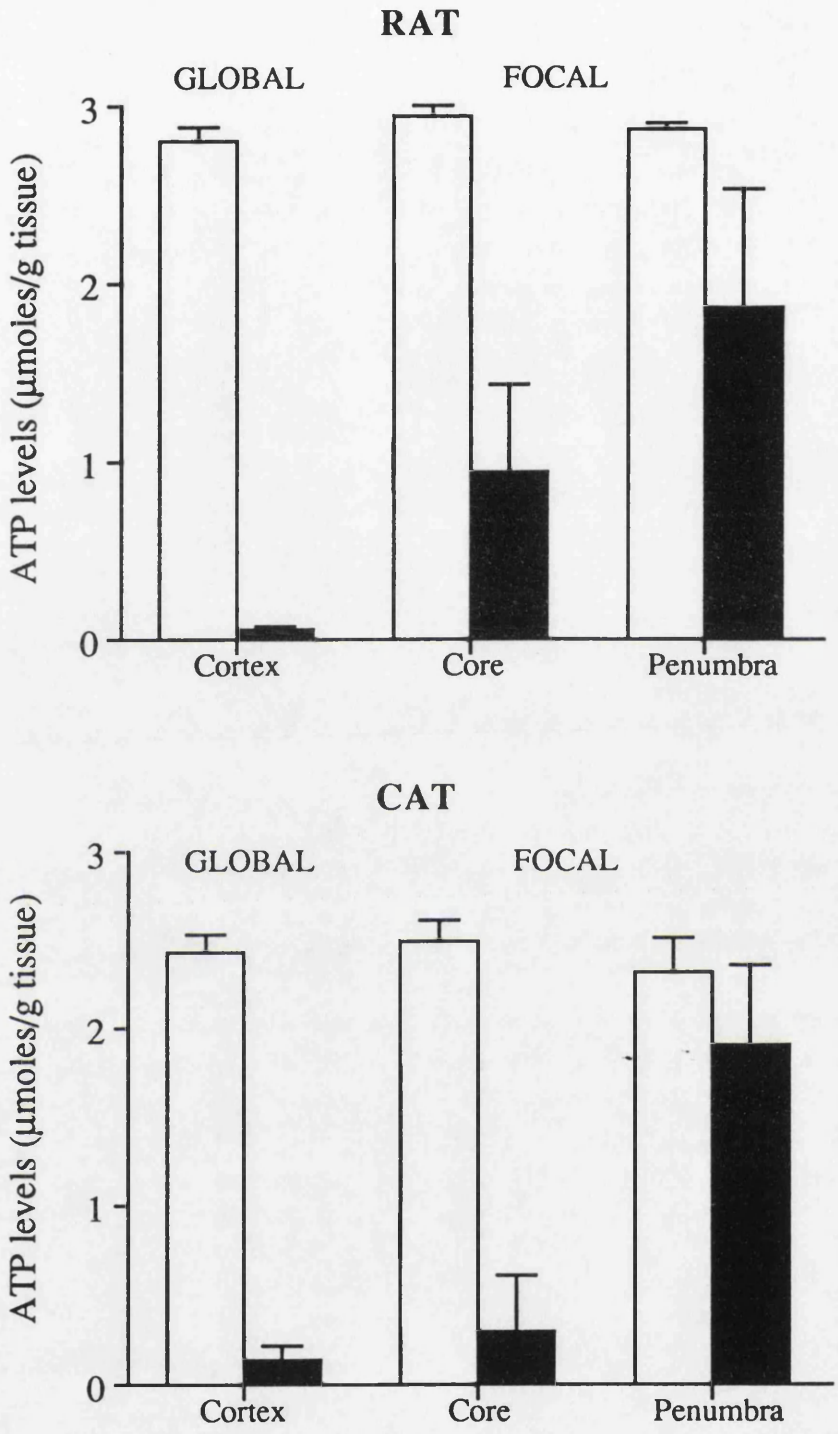


FIGURE 36

Levels of ATP, determined using biochemical enzymatic techniques, following global and focal cerebral ischaemia in the rat (upper) and cat (lower). Note that ATP levels are only minimally reduced in the ischaemic penumbra after the induction of focal ischaemia in both the rat and the cat. Data are presented as mean ± SEM and calculated from mean values given in tables and figures from Pulsinelli & Duffy, 1983; Folbergrová et al., 1992; Welsh et al., 1978, 1980.

pre-ischaemic control levels, and thereby is suggestive of a threshold-like effect for ischaemia-induced glutamate release. There are several reports in which the existence of a threshold-type relationship between cerebral blood flow and extracellular glutamate release has been seen (Matsumoto et al., 1992; Shimada et al., 1989; Takagi et al., 1993). Although these studies differed in the manner in which extracellular glutamate levels were expressed, they demonstrated generally similar ischaemic cerebral blood flow thresholds for glutamate release. Following severe global ischaemia in cats, Shimada and co-workers (1989, 1990) found that the blood flow threshold for glutamate release was 20ml $100g^{-1}$ min^{-1} (36% of pre-ischaemic cerebral blood flow), a value confirmed in the cerebral cortex of cats after permanent MCA occlusion (Matsumoto et al., 1992). Glutamate release in the dorsolateral parietal cortex of rats subjected to transient focal ischaemia was found when cerebral blood flow was less than 48% of control levels (Takagi et al., 1993). Thus the apparent cerebral blood flow threshold value for glutamate release in the present study is comparable to those previously reported.

It has been established that the Ca^{2+}-dependent release of endogenous L-aspartate from isolated terminal preparations is around 10% of that seen for glutamate (McMahon & Nicholls, 1990). This observation is consistent with the specificity of the synaptic vesicle glutamate transporter (Nicholls & Attwell, 1990) and represents a ratio of glutamate to aspartate of 10:1 within the vesicle. The ratio of glutamate to aspartate in the cytosol is approximately 3:2 (Nicholls & Attwell, 1990). In the present study, the ratio of glutamate to aspartate released in the cerebral cortex at blood flow levels of less than 20ml $100g^{-1}$ min^{-1} is about 8:1. This observation suggests that a proportion of the glutamate released is of vesicular origin, and not totally a consequence of glutamate uptake reversal. Moreover, this ratio is

approximately 6:1 in drug-treated animals, which implies that a fraction of ischaemia-induced glutamate release attenuated by CI-977 may be Ca^{2+}-dependent in origin.

A mechanism of action for the neuroprotective effects of *k*-opioid agonists in experimental models of cerebral ischaemia is not known with certainty. In the present studies, CI-977 was found to attenuate the release of glutamate in the cerebral cortex after focal ischaemia in the cat. Furthermore, CI-977 failed to alter the amount of neuronal necrosis produced by exogenous glutamate in the rat cortex; the postsynaptic NMDA and AMPA receptor antagonists MK-801 and NBQX significantly reduced the lesion volume. These results thus provide two lines of evidence which suggest that CI-977 may modulate the neurotoxic action of glutamate in ischaemia via presynaptic inhibition of its release, and such an effect may contribute, at least in part, to the anti-ischaemic efficacy of *k*-agonists.

Excitatory amino acid antagonists have also been reported to reduce the increases in neurotransmitters and other compounds induced by ischaemia or hypoglycaemia. The non-NMDA antagonist GYKI 52466 reduces glutamate, but not aspartate, release from the striatum following transient forebrain ischaemia (Arvin et al., 1994), although neuroprotection is observed in this model in both the cortex and striatum (Le Peillet et al., 1992). MK-801 reduces the release of glutamate and aspartate induced by hypoglycaemia (Westerberg et al., 1988), and NMDA receptor antagonists also attenuate the release of purine catabolites (Hagberg et al., 1987) and lactate (Katayama et al., 1992) following transient ischaemia. These observations are interpreted to result from the neuroprotection afforded by these agents rather than a presynaptic modulation of glutamate release. Similarly it is possible that part of the attenuation in ischaemia-induced glutamate release in CI-977-treated

cats is attributable to the neuroprotective effect of the drug, and reflects a reduction in the number of neurons irreversibly damaged.

The mechanistic hypothesis that CI-977 acts presynaptically to modulate glutamate release is substantiated by evidence obtained both *in vitro* and *in vivo*. Preliminary data indicates that CI-977 inhibits the release of glutamate in the ischaemic cortex after permanent MCA occlusion in the rat (Hayward et al., 1993a). *In vitro*, *k*-agonists inhibit the evoked release of glutamate and aspartate from rat cortical slices (Bradford et al., 1986; Lambert et al., 1991) and guinea pig hippocampal mossy fibre synaptosomes (Gannon & Terrian, 1991a,b, 1992). The inhibition of neurotransmitter release by *k*-agonists is thought to occur by restricting the entry of Ca^{2+} into presynaptic terminals (Gannon & Terrian, 1991a,b, 1992), putatively by closing N-type Ca^{2+} channels (Gross & MacDonald, 1987; Xiang et al., 1990) or by enhancing Ca^{2+} extrusion (Olson & Welch, 1991). Thus *k*-receptor activation by CI-977 may attenuate the rise in presynaptic intracellular Ca^{2+} levels and thereby reduce Ca^{2+}-dependent glutamate release. This is supported by evidence from electrophysiological studies where *k*-agonists, including CI-977, reduce excitatory postsynaptic potentials in the rat locus coeruleus and guinea pig hippocampus, primarily by acting at presynaptic receptors to inhibit calcium influx into the nerve terminal via voltage-sensitive Ca^{2+} channels and subsequent excitatory amino acid release (McFadzean et al., 1987; Pinnock, 1992; Wagner et al., 1992a).

The reduction in the volume of ischaemic damage with CI-977 is less than that observed previously with pretreatment with NMDA receptor antagonists in similar models of focal cerebral ischaemia (see McCulloch et al., 1991 for review), and may reflect a different mechanistic basis of the two classes of drugs. NMDA receptor antagonists will block the neurotoxic

effects of glutamate irrespective of whether the elevated levels are a consequence of Ca^{2+}-dependent or Ca^{2+}-independent release. *k*-Opioid agonists such as CI-977 would be beneficial only against the portion of glutamate released by ATP-dependent exocytosis but in doing so reduce activation, not just of NMDA receptors, but also of AMPA receptors which may also contribute to ischaemic brain damage (Buchan et al., 1993; Bullock et al., 1994; Gill et al., 1992b; Xue et al., 1994).

There is evidence that agents which putatively inhibit glutamate release afford neuroprotection in animal models of cerebral ischaemia and the magnitude of the benefit conveyed is comparable to that obtained with CI-977 in the present study. Most attention has been paid to adenosine analogues which act on presynaptic adenosine A1 receptors, such as 2-chloroadenosine. These compounds have been reported to decrease ischaemic brain injury in models of focal and global cerebral ischaemia by inhibiting receptor activated calcium entry and the concomitant release of glutamate (Rudolphi et al., 1992). Riluzole (2-amino-6-trifluoromethoxy benzothiazole) and BW 1003C87 (5-(2,3,5-trichlorophenyl0-2,4-diaminopyrimidine) are agents which putatively block sodium channels and both drugs have been shown to reduce ischaemic brain damage when administered either before or after the induction of global or focal cerebral ischaemia (Graham et al., 1993a; Lekieffre & Meldrum, 1993; Meldrum et al., 1992; Pratt et al., 1992; Wahl et al., 1993). BW 1003C87 inhibits glutamate release following permanent MCA occlusion in the rat in both the cortex and caudate, although neuroprotection is only afforded in the cortex (Graham et al., 1993a; Meldrum et al., 1992). Interestingly, BW 619C89 (4-amino-2-(4-methyl-1-piperazinyl)-5-(2,3,5-trichlorophenyl)pyrimidine), a structural analogue of BW 1003C87, has recently been reported to reduce both glutamate release and brain damage in the

cortex and caudate after focal ischaemia in the rat (Graham et al., 1994; Leach et al., 1993). Methionine sulfoxamine, an inhibitor of glial glutamine synthetase, which depletes brain glutamine stores and decreases K^+-evoked glutamate release, reduces the volume of infarction after permanent MCA occlusion in the rat (Swanson et al., 1990). In addition, hypothermia has been shown to attenuate glutamate release and the extent of ischaemic damage in various models of cerebral ischaemia (see Ginsberg et al., 1992 for review). These studies support the hypothesis that inhibition of excitatory amino acid release during ischaemia is an effective strategy for reducing ischaemic brain injury.

The neuroprotection afforded by CI-977 is only observed in the cerebral cortex. This may be a consequence of neuropeptide co-existence and neuromodulation. There is evidence that prodynorphin derived peptides co-exist with glutamate in cortical neurones (Gannon & Terrian, 1991b; Terrian et al., 1990) and that glutamate and dynorphin peptides are co-released from hippocampal mossy fibre synaptosomes (Terrian et al., 1988). Moreover, the *k*-agonist U-50,488H inhibits both dynorphin and glutamate release from mossy fibre terminals (Gannon & Terrian, 1991a). Recently, it has been shown that the synaptic release of dynorphin produces a presynaptic inhibition of neighbouring mossy fibres and the induction and expression of long-term potentiation (Weisskopf et al., 1993). Furthermore, the activation of *k*-opioid receptors by the endogenously released peptide dynorphin or the exogenous *k*-agonist U-69593 reduces excitatory transmission in the guinea pig dentate gyrus (Wagner et al., 1993). These observations suggest dynorphin may normally function to protect hippocampal neurones from overstimulation during the intense nervous activity involved in learning by providing a presynaptic feedback control of afferent input to the

hippocampus. Thus, the use of *k*-agonists like CI-977 as neuroprotective agents, and the observations that they may modulate excitatory amino acid release, suggests that they could be viewed as a form of endogenous *k*-opioid replacement or supplement therapy.

The pharmacologic inhibition of glutamate release may have several advantages for neuroprotection over drugs which block postsynaptic excitatory amino acid receptors. The presynaptic inhibition of glutamate release prevents activation of both NMDA and non-NMDA receptors and thus conceptually, with an optimum dosing regimen, could be more effective in reducing ischaemic brain damage than NMDA or AMPA receptor blockade alone. Agents which attenuate ischaemia-induced glutamate release may lack the behavioural and morphological side-effects found with NMDA receptor antagonism. Presynaptic inhibition of glutamate release may also provide a better therapeutic index and offer the possibility of combination therapy with agents acting postsynaptically, including free radical scavengers and drugs modulating calcium toxicity via intracellular mechanisms such as calpain inhibitors.

3. LOCAL CEREBRAL GLUCOSE UTILISATION

The [^{14}C]-2-deoxyglucose technique allows an anatomically comprehensive assessment of function-related alterations in cerebral glucose use *in vivo* (Sokoloff et al., 1977; Sokoloff, 1981). Glucose utilisation constitutes the primary energy-generating metabolic process in cerebral tissue of well-nourished mature animals (Sokoloff et al., 1977). As a consequence of the close coupling between neuronal activity and the energetic requirement for such activity, local alterations in glucose utilisation have been used to demonstrate the involvement of discrete brain areas during various

pharmacological interventions (Kurumaji et al., 1993; McCulloch, 1982). In addition, functional mapping with [^{14}C]-2-deoxyglucose has proven useful as a potential index of structural abnormalities within the brain following drug treatment. Alterations in gene expression and neuronal morphology (cell swelling, vacuolisation) after NMDA antagonists have been shown to be co-incident anatomically with areas of increased cerebral glucose metabolism (Allen & Iversen, 1990; Auer & Coulter, 1994; Fix et al., 1993; Hargreaves et al., 1993b; Olney et al., 1989, 1991; Sharp et al., 1991, 1992).

The use of the [^{14}C]-2-deoxyglucose technique as a measure of CNS function receives its most definitive support from the application of the technique itself in simple sensory stimulation and deprivation experiments. Deprivation of visual stimuli results in reductions of glucose utilisation in neuroanatomical components of the visual system (i.e. dorsal lateral geniculate body, superficial layer of the superior colliculus, and area 17 of the neocortex), whereas stimulation with diffuse light results in intensely-related increments in their glucose use (Kennedy et al., 1975; Miyaoka et al., 1979; Toga & Collins, 1981). Similarly, auditory stimulation or occlusion of the external auditory canals is associated with increases and decreases in glucose utilisation throughout the primary auditory pathway from the cochlear nucleus to the auditory cortex (Sokoloff, 1977). Circumscribed areas of enhanced glucose utilisation can be demonstrated in specific areas of the olfactory bulb in response to stimulation with different odours (Sharp et al., 1975). Furthermore, enhanced glucose utilisation, seen in a discrete column of the somatosensory cortex of the rat brain and resulting from stimulation of a single whisker, can be readily identified (Ginsberg et al., 1987a). Thus, a correlation does appear to exist between the level of glucose utilisation in neuroanatomical components of well-defined functional systems and the

processing of information in those systems.

The present study provides a comprehensive description of the regional changes in functional activity (as reflected in local cerebral glucose use) in the CNS which are involved in the response to the systemic administration of the *k*-opioid agonist CI-977. The results show that CI-977 produces a widespread depression of local cerebral glucose use throughout the CNS. A striking feature of the effects of treatment with CI-977 is how limited in magnitude the alterations in glucose utilisation are in comparison to other neuroprotective agents which modulate excitotoxicity at postsynaptic NMDA receptors (Kurumaji et al., 1989; McCulloch & Iversen, 1991; Nehls et al., 1988; Weissman et al., 1987) and AMPA receptors (Browne & McCulloch, 1994; Suzdak & Sheardown, 1993).

The effect of *k*-agonists on cerebral glucose use has been minimally investigated (Beck & Krieglstein, 1986; Fanelli et al., 1987). Previously, it has been difficult to ascribe with certainty the effects on glucose use to a specific action at the *k*-opioid receptor because of the poor pharmacological selectivity of the agents used. The *in vitro* and *in vivo* pharmacological profile of CI-977 demonstrates that it is a potent and highly selective agonist at the *k*-opioid receptor (Hunter et al., 1990). Thus, it has been possible to attribute the alterations in glucose utilisation in the present study to a *k*-mediated action.

The doses of CI-977 in the present study correspond to those used to investigate the neuroprotective effects of this agent 24h after permanent MCA occlusion in the rat. Maximum neuroprotection with CI-977 was observed at the intermediate dose examined in the present study (0.3mg/kg). The apparent lack of dose-dependent changes in local cerebral glucose utilisation in many of the areas examined may be attributed to a ceiling

effect across the 100-fold dose range used. Thus, although the range of doses used in this study was broad it is possible that significant changes in glucose use might occur at even lower doses. However, the analgesic effects of CI-977 in rats are also observed within the dose range examined (Hunter et al., 1990). This suggests therefore, that the [^{14}C]-2-deoxyglucose technique is as sensitive an end point as is analgesia for CI-977.

The regional hierarchy of changes in glucose utilisation after the administration of CI-977 do not correlate simply with the topography and associated densities of *k*-opioid receptors in the rat brain identified on the basis of radioligand binding (Boyle et al., 1990; Mansour et al., 1987; Tempel & Zukin, 1987). In some regions that exhibit high densities of receptors, such as the nucleus accumbens, CI-977 failed to induce any prominent alterations in glucose utilisation. However, CI-977 reduced the rates of glucose use in the neocortex and molecular layer of the hippocampus, and increased glucose utilisation in the lateral habenula, areas in which the lowest densities of *k*-opioid receptors are observed in the rat CNS. Although all structural elements within a region will contribute to its rate of glucose utilisation, dynamic alterations in glucose use appear to reflect predominantly activity in the axonal terminal of neuronal pathways (Kadekaro et al., 1985). In neuropharmacological studies, alterations in glucose utilisation are not restricted to regions rich in specific receptors for the agent being studied, but are observed also in areas with primary and secondary neuronal connections with the receptor-rich regions (Kurumaji et al., 1993; McCulloch, 1982; Sokoloff, 1981). Alterations in glucose utilisation are thus associated both with the initiation of the pharmacological response and with the polysynaptic neural pathways involved functionally in the expression of the response. However, extreme caution must be exercised as to how such data

can be interpreted. First, the function-related alterations in glucose use are a synthesis of all the pharmacological effects of CI-977 including antinociception, behaviour effects etc., and thus it is impossible to attribute alterations in glucose use to a specific effect. Second, the alterations in glucose utilisation observed after CI-977 in the present study in lightly restrained animals may not accurately reflect the effects of CI-977 in freely moving animals.

The patterns of response to the *k*-agonist CI-977 in the present study bear no obvious similarity to those reported previously with NMDA receptor antagonists acting at either the glutamate recognition site, the glycine modulatory site, or the ion channel site within the receptor complex (Hargreaves et al., 1993a,b; Kurumaji et al., 1989; McCulloch & Iversen, 1991; Nehls et al., 1988; Weissman et al., 1987). The administration of non-competitive NMDA antagonists (such as MK-801 and phencyclidine) or competitive NMDA antagonists (such as CPP and CGP 37849) produces heterogeneous alterations in glucose use, with marked increases noted in anatomical components of the limbic system (posterior cingulate and entorhinal cortices, hippocampus, mamillary body, substantia nigra *inter alia*) (Figures 37 & 38). In contrast, AMPA receptor blockade induces marked reductions in glucose use throughout the brain, with greatest reductions observed in primary auditory areas, limbic structures (particularly hippocampal regions and cingulate cortex), neocortex and some thalamic nuclei (Browne & McCulloch, 1994; Suzdak & Sheardown, 1993) (Figures 37 & 38). However, although the effects of CI-977 on glucose utilisation vaguely resemble those induced by AMPA antagonists, they are quantitatively of a lesser magnitude. Indeed the magnitude of response after AMPA receptor blockade is such that it has been previously observed only with

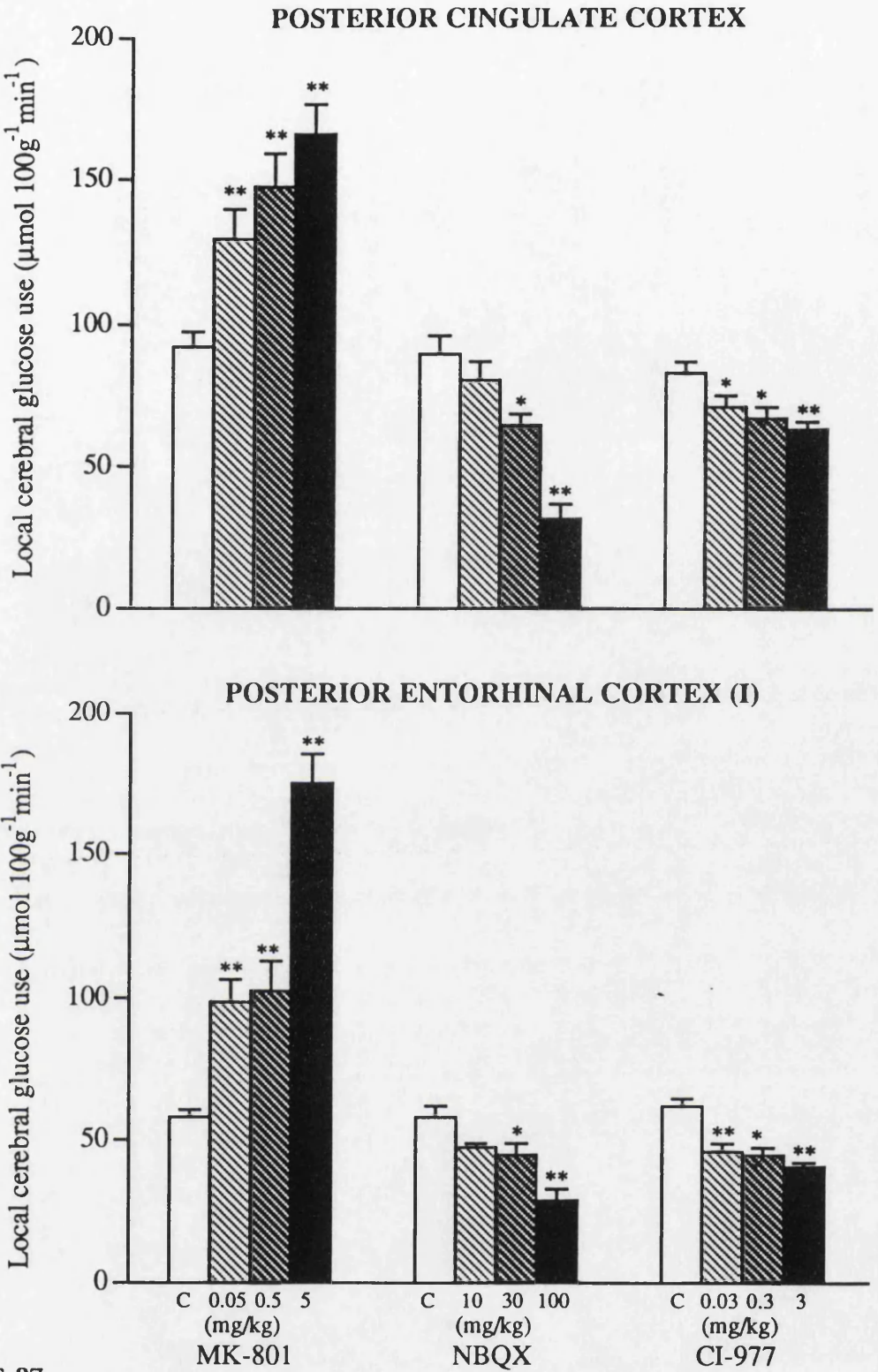


FIGURE 37

Comparison of the effects of the non-competitive NMDA antagonist MK-801 and the AMPA antagonist NBQX with CI-977 on glucose use in the posterior cingulate cortex (upper) and posterior entorhinal cortex (I) (lower) in conscious rats. Data are presented as mean ± SEM (n=4-9 per group). *P<0.05, **P<0.01 relative to vehicle-treated controls. (Redrawn from the data of Browne & McCulloch, 1994; Kurumaji et al., 1989.)

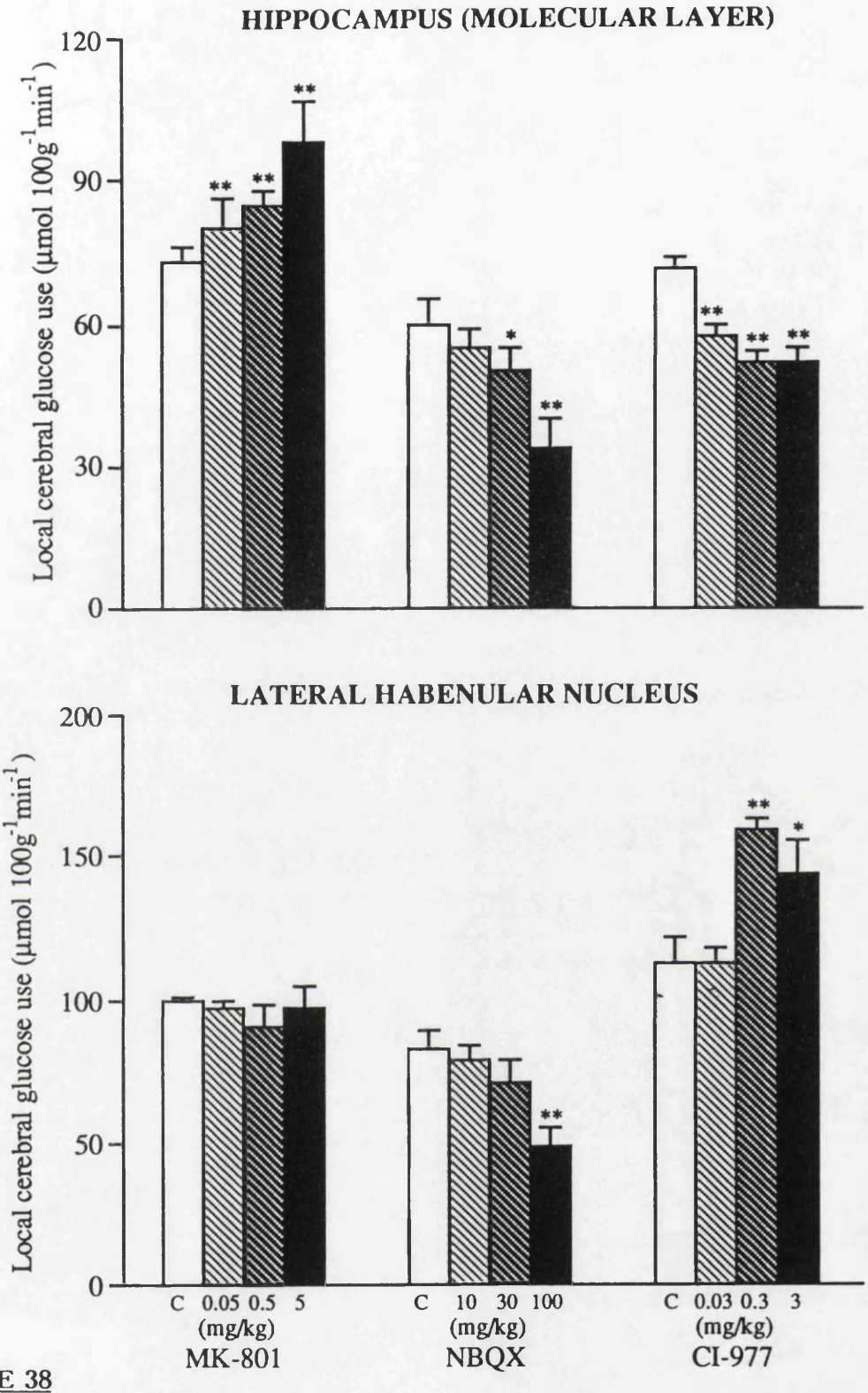


FIGURE 38

Comparison of the effects of the non-competitive NMDA antagonist MK-801 and the AMPA antagonist NBQX with CI-977 on glucose use in the hippocampus (molecular layer) (upper) and the lateral habenular nucleus (lower) in conscious rats. Data are presented as mean ± SEM (n=4-9 per group). *P<0.05, **P<0.01 relative to vehicle-treated controls. (Redrawn from the data of Browne & McCulloch, 1994; Kurumaji et al., 1989.)

barbiturates at doses which produce complete electrocortical silence (Crane et al., 1978).

A prominent finding of the present study is that CI-977 markedly increases functional activity in only one of the brain regions examined, the lateral habenular nucleus (Figure 38). Glucose use in the lateral habenula has previously been found to be enhanced by a number of pharmacological agents including haloperidol (McCulloch et al., 1980), caffeine (Nelig et al., 1984) and oxotremorine (Dow-Edwards et al., 1980). In addition, NBQX reduces glucose use in this nucleus (Browne & McCulloch, 1994) whilst MK-801 has no effect (Kurumaji et al., 1989) (Figure 38). It is noteworthy that previous studies involving non-selective *k*-agonists observed no alteration in glucose utilisation in the lateral habenula (Beck & Krieglstein, 1986; Fanelli et al., 1987). Fibres and cell-containing opioid receptors (Mansour et al., 1987) and opioid peptides (Khachaturian et al., 1983) have been demonstrated in the lateral habenula, thus justifying the sensitivity of this nucleus to CI-977 in the present study. The lateral habenula may serve as a relay station between limbic and striatal forebrain areas and the mensencephalon (Aghajanian & Wang, 1977; Wang & Aghajanian, 1977), and is one of the few forebrain regions which project to nuclei known to be involved in pain modulation such as the dorsal and median raphe, periaqueductal grey matter, and ventral tegmental area (Sutherland, 1982). Stimulation of the habenula is known to induce antinociception which is naloxone reversible (Mahieux & Benabid, 1987; Terenzi & Prado, 1990). Thus, the observation in the present study suggests that the lateral habenular nucleus may be involved in a centrally-mediated mechanism of analgesia of CI-977. Interestingly, CI-977 altered glucose utilisation in only one of the four major habenular efferent projection areas, the dorsal raphe. The reasons for the absence of effects

in the other projection areas are uncertain. The lack of effects may be due to normally low discharge rates in the afferent pathways to those areas which are insensitive to modulation by CI-977.

In most of the neocortical areas examined, marked reductions in glucose utilisation were observed in cortical layer IV after the administration of CI-977. Cortical afferents from the specific thalamic relay nuclei terminate predominantly in cortical layer IV (Herkenham, 1980). GABA agonists have also been shown to reduce glucose utilisation in cortical layer IV, with parallel reductions in glucose use in thalamic relay nuclei (Cuddenec et al., 1987; Kelly & McCulloch, 1982). In the present study CI-977 also reduced glucose use in the medial geniculate body and ventrolateral thalamus, specific thalamic relay nuclei which project to cortical areas with depressed glucose utilisation. This suggests that the observed functional disturbances in cerebral cortex following CI-977 administration are thalamocortical in nature. A previous study using the putative *k*-opioid agonist tifluadom also detected depressed glucose use in a variety of cortical areas, but not in thalamic relay nuclei (Beck & Krieglstein, 1986). The reductions in glucose use were smaller in magnitude, and may be a consequence of an inadequate dose or of poorer selectivity and potency of the agent utilised.

The rates of glucose utilisation in the dentate gyrus and hippocampus (stratum lacunosum moleculare) displayed significant reductions following CI-977 (Figure 38), in spite of the low density of kappa receptors in these regions of the limbic system in the rat (Boyle et al., 1990; Mansour et al., 1987). A marked depression in glucose use has been observed in the hippocampus after NBQX (Browne & McCulloch, 1994; Suzdak & Sheardown, 1993) (Figure 38). In contrast, NMDA antagonists have been shown to markedly enhance glucose use in these limbic regions (Kurumaji et al., 1989;

Nehls et al., 1988; Weissman et al., 1987 (Figure 38). The hippocampus molecular layer and dentate gyrus receive a major glutamateric/aspartergic innervation from the entorhinal cortex via the perforant pathway (White et al., 1977). Prodynorphin-derived opioid peptides have been localised to specific neuronal pathways within the hippocampus, namely the excitatory mossy fibre and perforant pathways (Chavkin et al., 1985; McGinty et al., 1983; McLean et al., 1987). This suggests a potential role of the endogenous dynorphin system in the regulation of normal hippocampal function. In the present study, the reduction in glucose use observed within the hippocampal formation may be a consequence of CI-977 acting on presynaptic *k*-opioid receptors to inhibit excitatory input into both the dentate gyrus and molecular layer of hippocampus. This is substantiated by electrophysiological studies demonstrating that activation of *k*-opioid receptors decrease excitatory synaptic transmission from the perforant path to the dentate gyrus (Wagner et al., 1992a), and that *k*-opioid agonists inhibit glutamate release from hippocampal mossy fibre synaptosomes (Gannon & Terrian, 1991a,b).

The marked focal increases in glucose use after administration of NMDA antagonists (phencyclidine, MK-801, CGP 37849) in components of the limbic system (hippocampus, entorhinal cortex, mamillary body, posterior cingulate cortex) have been of major concern with respect to the clinical use of NMDA receptor antagonists as neuroprotectants (McCulloch et al., 1991; McCulloch & Iversen, 1991). The alterations in glucose use not only provide an anatomical basis for the psychotomimetic properties of agents such as phencyclidine, but in areas of the most intense increases in glucose use, such as the posterior cingulate cortex, these functional alterations have been found to precede the appearance of morphological alterations in neurones (swelling, appearance of cytosolic vacuoles) (Allen & Iversen, 1990; Auer &

Coulter, 1994; Fix et al., 1993; Hargreaves et al., 1993b; Olney et al. 1989, 1991). In contrast, CI-977 induces a marked depression of glucose use in the posterior cingulate cortex. The absence of any focal increases in glucose use following CI-977 administration in the present study suggests that the morphologic changes observed with competitive and non-competitive NMDA antagonists are an unlikely consequence of *k*-opioid agonist administration. Thus, the use of *k*-opioid receptor agonists such as CI-977 in the treatment of cerebral ischaemia in man is unlikely to be complicated by concerns over adverse effects on CNS function and structure.

The cingulate cortex may also be involved in nociception (Sikes & Vogt, 1992). However, the reductions in glucose utilisation observed in the cingulate cortex are not disproportionately different from those in other cortical areas not involved in pain processing. This suggests therefore, that it is inappropriate to ascribe with certainty the alterations in glucose use in the cingulate cortex to a possible mechanism of analgesia for CI-977.

In conclusion, the present investigation provides a comprehensive description of the alterations in local cerebral glucose utilisation associated with systemic administration of the *k*-opioid agonist CI-977. The results highlight the modest nature of the reductions in glucose use, and the lack of dose-dependency with the doses of CI-977 used. Unlike NMDA receptor antagonists, the neuroprotective dose of CI-977 does not produce anatomically widespread elevations in glucose utilisation.

4. CONCEPT TO THERAPY

One of the most pressing issues within the field of cerebrovascular disease research is to discover an effective pharmacological agent for the treatment of ischaemic brain damage in man. The work carried out in this

thesis sought to explore the concept that the selective *k*-opioid receptor agonist CI-977 may offer a potential therapeutic approach to attenuate excitotoxic brain damage in stroke or head injury in humans.

The arylacetamide CI-977 was first synthesised by Parke-Davis, Ann Arbor, U.S.A. in early 1987. Its patent was applied for in July 1987 and was received (U.S. Patent Number: 4,737,493) in 1988. Although CI-977 is currently undergoing clinical trials as an analgesic agent in man, considerable attention has recently focused on the potential of CI-977 as a neuroprotective agent.

The first demonstration of the anti-ischaemic efficacy of CI-977 was reported in abstract form in 1991 by Boxer and co-workers. The intravenous administration of CI-977 once at 30 min and once again at 24h after the induction of ischaemia conferred neuroprotection in a rat model of focal ischaemia when outcome was assessed at 48h. Given that the half life of CI-977 is about 1h in the rat (G.N. Woodruff, unpublished observations), the dosing regimen employed in that study is suggestive of a less than adequate pharmacokinetic design. In the present investigations therefore, a multiple dosing regimen was utilised to circumvent such criticism, and the neuroprotective efficacy of CI-977 was subsequently confirmed at 24h after permanent MCA occlusion the in the rat.

The effects of CI-977 were also determined at 4h after the induction of ischaemia in the rat. This is important, because at this time point, key physiological variables which may influence outcome (e.g. MABP, core temperature, plasma glucose etc.) can be monitored throughout the post-ischaemic survival period. It was shown that CI-977 reduces the volume of infarct assessed at 4h to a similar degree as observed at 24h despite a marked and sustained hypotension. For the first time, the neuroprotective efficacy

of a *k*-opioid agonist has been demonstrated with full monitoring of physiological variables throughout the experimental period. Furthermore, by assessing the extent of histopathological damage at both 4h and 24h after MCA occlusion, the present studies negate the possible criticism that CI-977 merely delays the development of ischaemic cell changes. The observations that CI-977 conveyed benefit in the rat MCA models of focal ischaemia (at 4h and 24h) were integral to the development of this thesis because were it not possible to substantiate and extend the initial observation of the efficacy of CI-977 (Boxer et al., 1991), this thesis may well have followed a different direction, possibly an autoradiographic study investigating the effects of different disease states, e.g. Alzheimer's disease, schizophrenia, head injury, stroke etc. on the distribution and density of *k*-opioid receptors (see Ikeda et al., 1993 and Mackay et al., 1994 for studies in Alzheimer's disease).

The demonstration that CI-977 reduced the volume of ischaemic damage after permanent MCA occlusion in the cat is pivotal to the pre-clinical evaluation of CI-977 as an anti-ischaemic agent. It is necessary to establish efficacy in a gyrencephalic species for a number of reasons. First, the cat has a similar cortical architecture to man, the ultimate clinical target; the rat has a lissencephalic brain. Second, the cat has greater cardiovascular stability under anaesthesia than the rat. Third, the absolute amount of ischaemic tissue which is potentially salvageable in the cat is greater than in the rat, thereby eliminating the concern of measurement bias. All these factors add to the credence that clinicians place in the validity of findings in cat (and other gyrencephalic species) models of ischaemia. It is noteworthy that many of the NMDA antagonists that are in (or have been in) clinical trials have all produced neuroprotective effects in the cat model of MCA occlusion.

A precise mechanistic basis underlying the neuroprotective effects of CI-977 and other *k*-agonists was unknown at the outset of the investigations in this thesis. The neuroprotective actions of *k*-agonists was generally attributed to a reduction in cerebral oedema via an overt diuresis (Silvia & Tang, 1986; Silvia et al., 1987). However, although CI-977 failed to reduce the volume of infarction after MCA occlusion in the Brattleboro rat (where no diuresis was observed) (Boxer et al., 1991), the NMDA antagonist, MK-801, also failed to demonstrate any neuroprotection in that model (G.N. Woodruff, unpublished observations). This suggests that an anti-oedema action may not be the principal mechanism of neuroprotection. Indeed, the present studies after MCA occlusion in the rat (outcome assessed at 24h) and cat provide no definitive evidence to support an anti-oedema mechanism of action. In addition, the absence of any major effects of CI-977 administration on cerebral blood flow in either conscious or halothane-anaesthetised animals indicates that a cerebral circulatory action is not the principal mechanism underpinning the anti-ischaemic efficacy of CI-977.

There are however, two lines of evidence in the present thesis which suggest that a major mechanism of the neuroprotective effect of CI-977 is via inhibition of glutamate release. First, CI-977 reduces the increase in extracellular glutamate concentration after permanent MCA occlusion in the cat which occurs with cerebral blood flow values of less than 20ml $100g^{-1}$ min after permanent MCA occlusion in the cat. Second, CI-977 has no effect on the volume of damage after the perfusion of exogenous glutamate (0.5M) into the rat cortex, whereas the postsynaptic NMDA and AMPA receptor antagonists MK-801 and NBQX reduce the volume of neuronal necrosis by around 30%. Taken together, these observations indicate that CI-977, and other *k*-agonists, may modulate the excitotoxic action of glutamate at pre-

synaptic sites by attenuating its release, and this would provide a plausible and rational explanation for the anti-ischaemic efficacy of this class of compound.

It is interesting to compare the relative time scales for the *k*-agonist CI-977 and the competitive NMDA antagonist CGS 19755 from compound development to pre-clinical and clinical neuroprotective efficacy trials. CGS 19755 was first reported to be a selective and competitive NMDA antagonist in 1988 (Lehmann et al., 1988), and its neuroprotective efficacy described after MCA occlusion in the rat two years later (Simon & Shiraishi, 1990). However, it has only been recently that reports are emerging from the literature regarding the safety, tolerability and efficacy of CGS 19755 in stroke patients (Clark & Coull, 1994; Grotta, 1994). In comparison, the first demonstration of the anti-ischaemic effects of CI-977 in a model of focal ischaemia to appear in peer-reviewed form was made in 1992 (Kusumoto et al., 1992) and CI-977 is about to enter clinical trials in head injury in humans in late 1994. The shorter duration of taking the concept of *k*-agonists as neuroprotective agents through systematic pre-clinical evaluation of anti-ischaemic efficacy in experimental models of cerebral ischaemia into the clinical setting when compared to CGS 19755, serves to highlight the utmost importance and urgency in acquiring an effective therapeutic agent for use in man.

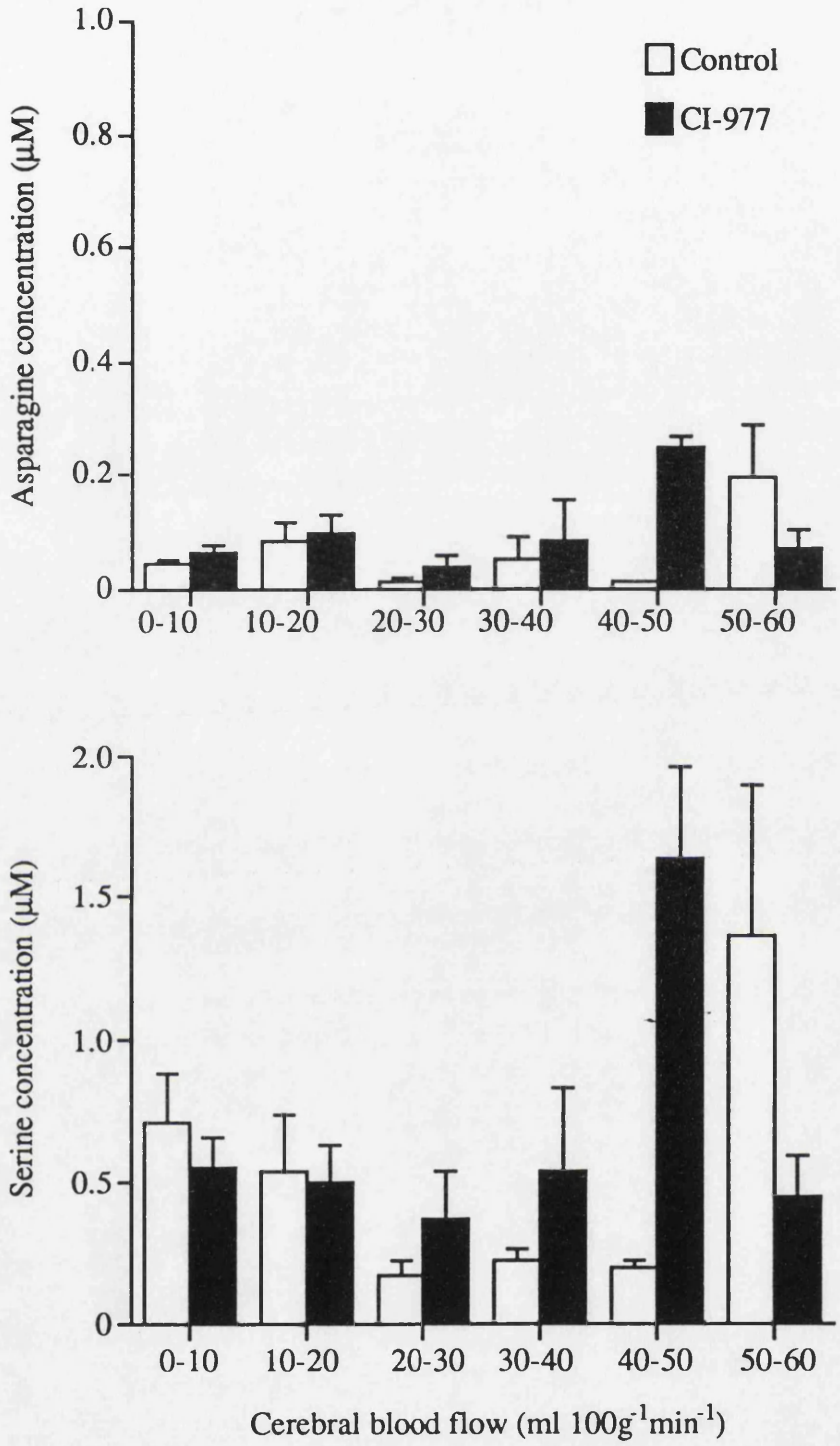
APPENDIX

Glutamate Release and Cerebral Blood Flow after MCA Occlusion in the Cat: Effect of CI-977 on Physiological Variables and Amino Acid Levels in the Ipsilateral Hemisphere before and after the Induction of Ischaemia.

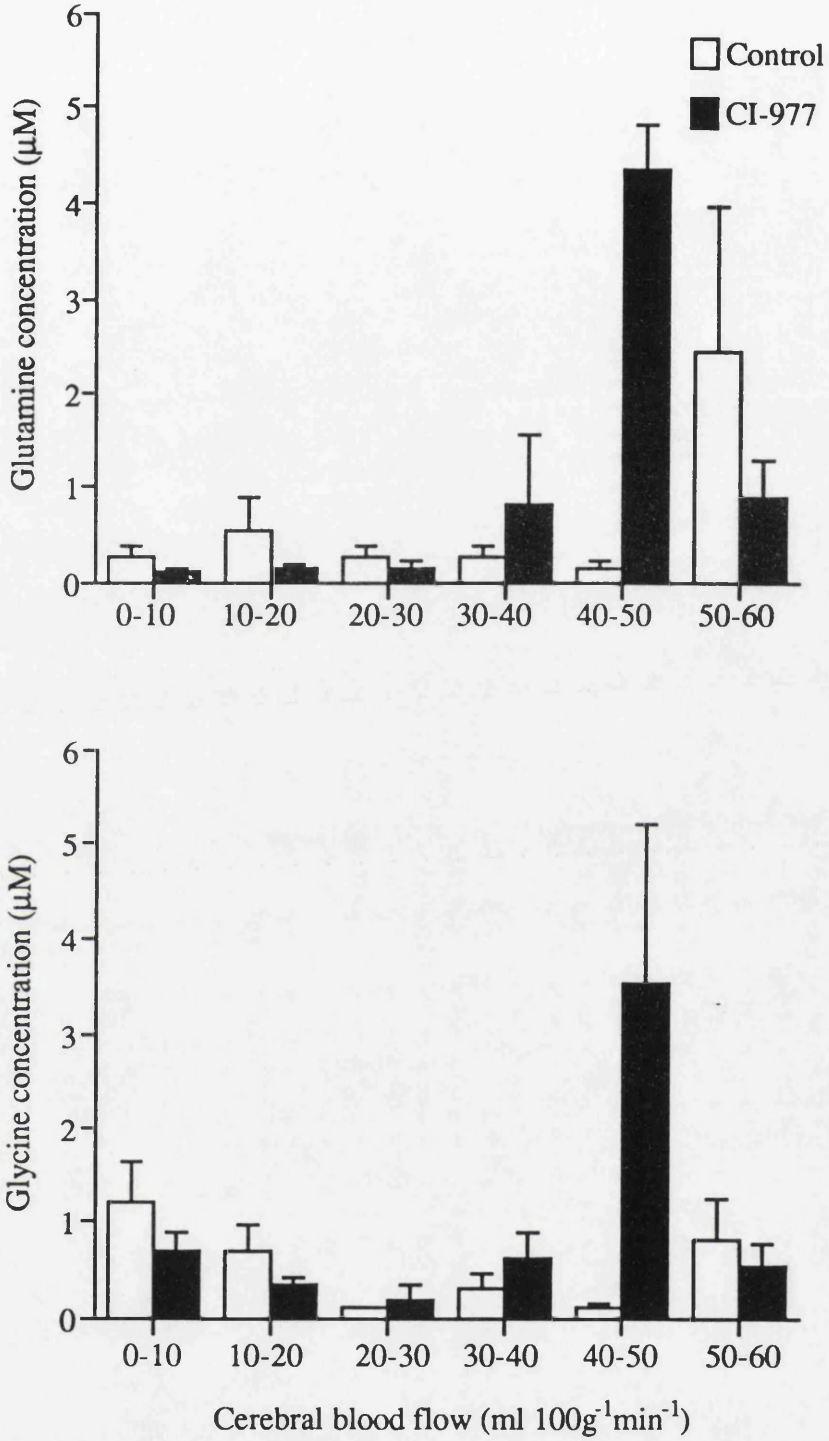
TABLE
CARDIOVASCULAR, RESPIRATORY AND OTHER PHYSIOLOGICAL VARIABLES DURING THE PERIOD OF SURVIVAL AFTER MCA OCCLUSION IN THE CAT IN THE GLUTAMATE RELEASE STUDY: EFFECTS OF CI-977

TIME AFTER MCAO (HRS)	TEMPERATURE °C		GLUCOSE (mM)	pO_2 (mmHg)	pCO_2 (mmHg)	pH	HAEMATOCRIT
	RECTAL	PERIOSTEAL					
			CONTROL GROUP				
0	36.8 ± 0.1	35.1 ± 0.3	11.8 ± 1.7	156 ± 11	30.8 ± 0.8	7.42 ± 0.02	25.4 ± 2.4
1	36.8 ± 0.1	35.1 ± 0.4	11.1 ± 1.8	154 ± 16	29.6 ± 0.9	7.41 ± 0.01	23.4 ± 1.7
2	37.0 ± 0.1	34.6 ± 0.4	11.3 ± 1.1	148 ± 14	28.4 ± 0.8	7.43 ± 0.01	26.6 ± 2.3
3	36.9 ± 0.1	34.5 ± 1.1	11.0 ± 1.7	168 ± 15	31.0 ± 3.0	7.41 ± 0.06	24.0 ± 6.0
			CI-977-PRETREATMENT GROUP				
0	36.6 ± 0.2	34.8 ± 0.6	10.3 ± 1.7	169 ± 8	33.0 ± 1.3	7.39 ± 0.01	26.5 ± 2.5
1	36.8 ± 0.1	34.6 ± 0.2	10.2 ± 1.8	169 ± 7	30.8 ± 1.6	7.41 ± 0.02	25.5 ± 2.8
2	37.0 ± 0.1	34.3 ± 0.4	10.7 ± 1.5	164 ± 16	31.0 ± 1.3	7.43 ± 0.02	24.8 ± 1.9
3	36.8 ± 0.1	34.1 ± 0.5	11.5 ± 1.7	161 ± 17	30.7 ± 1.2	7.38 ± 0.05	22.3 ± 3.9

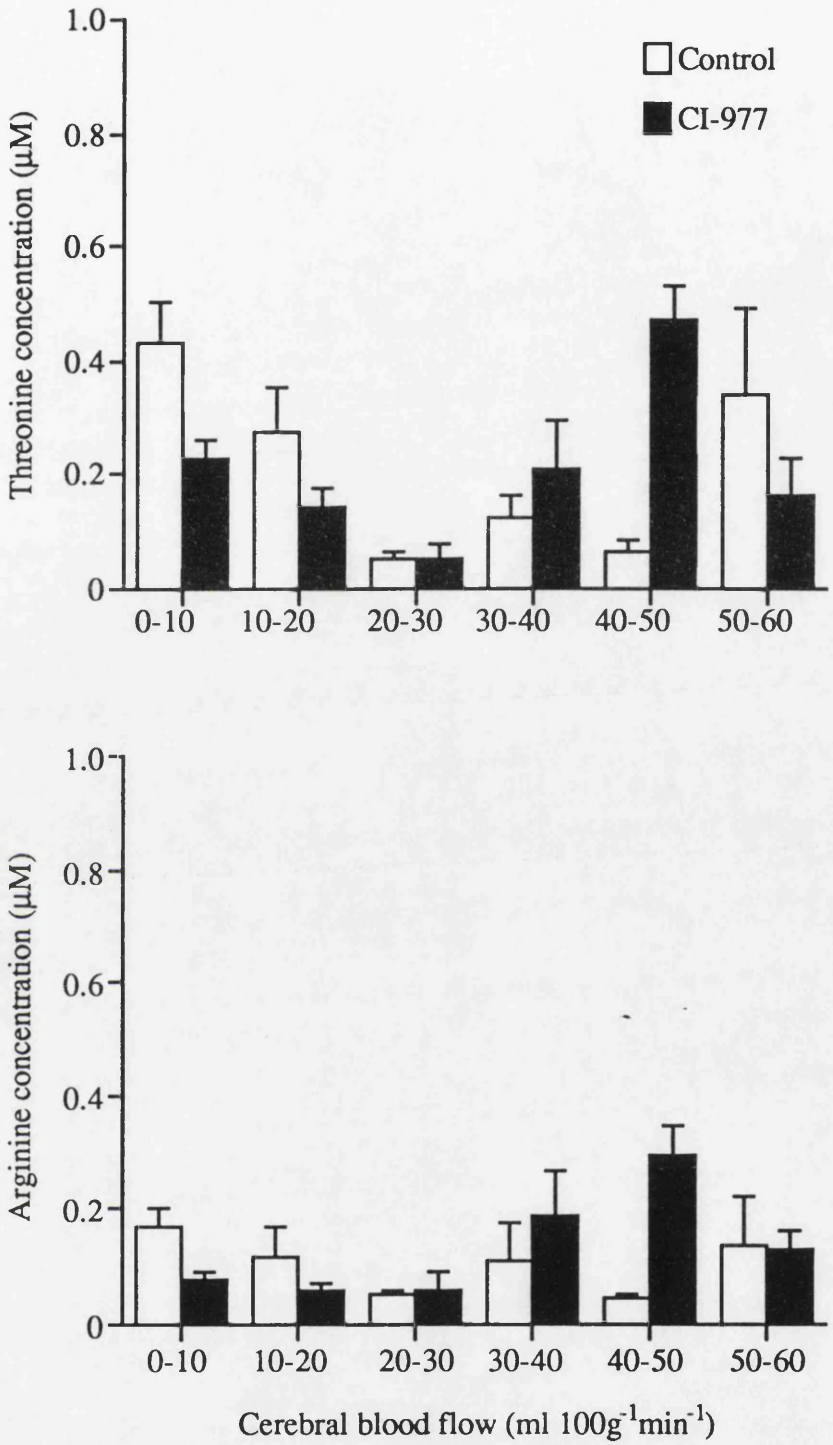
Data are presented as mean ± SEM (n=5 control, n=4 CI-977). Data represent values at time of MCA occlusion and at hourly intervals thereafter. There are no significant differences between the vehicle-treated control group and the CI-977 treatment group at any time point for any variable.



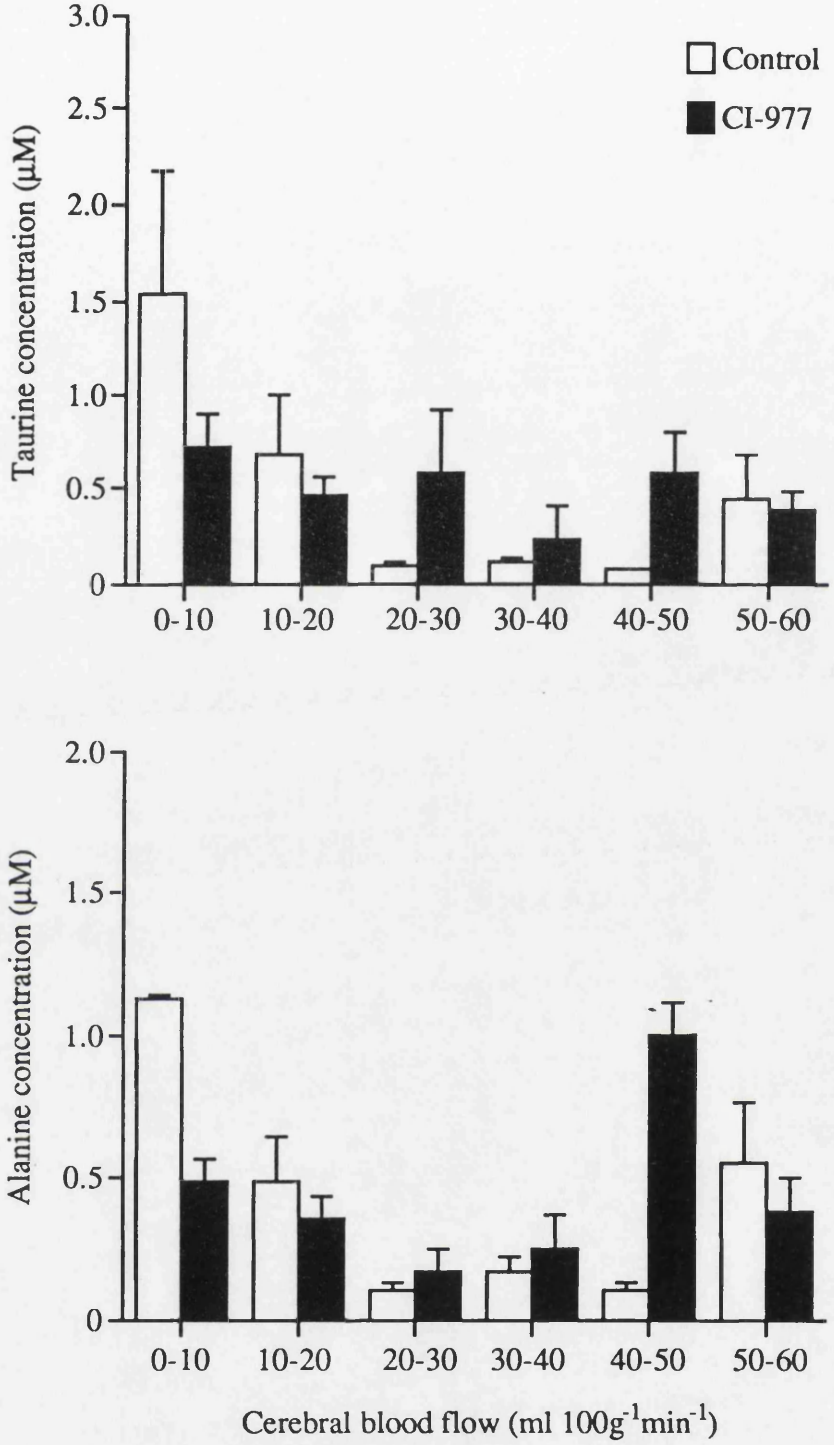
The effect of pretreatment with CI-977 on the extracellular concentrations of asparagine (upper) and serine (lower) in the ipsilateral hemisphere before and after permanent MCA occlusion in halothane-anaesthetised cats. Data are presented as mean ± SEM and represent 3-26 determinations in 5 vehicle-treated control and 4 CI-977-treated animals.



The effect of pretreatment with CI-977 on the extracellular concentrations of glutamine (upper) and glycine (lower) in the ipsilateral hemisphere before and after permanent MCA occlusion in halothane-anaesthetised cats. Data are presented as mean ± SEM and represent 3-24 determinations in 5 vehicle-treated control and 4 CI-977-treated animals.



The effect of pretreatment with CI-977 on the extracellular concentrations of threonine (upper) and arginine (lower) in the ipsilateral hemisphere before and after permanent MCA occlusion in halothane-anaesthetised cats. Data are presented as mean ± SEM and represent 3-23 determinations in 5 vehicle-treated control and 4 CI-977-treated animals.



The effect of pretreatment with CI-977 on the extracellular concentrations of taurine (upper) and alanine (lower) in the ipsilateral hemisphere before and after permanent MCA occlusion in halothane-anaesthetised cats. Data are presented as mean ± SEM and represent 3-26 determinations in 5 vehicle-treated control and 4 CI-977-treated animals.

REFERENCES

Aebischer, B., Frey, P., Haerter, H.-P., Herrling, P.L., Muller, W., Olverman, H.J. & Watkins, J. (1989) Synthesis and NMDA antagonist properties of the enantiomers of 4-(3-phosphonopropyl)-piperazine-2-carboxylic acid (CPP) and of the unsaturated analogue (E)-4-(3-phosphonoprop-2-enyl)piperazine-2-carboxyliuc acid (CPP-ene), *Helvetica Chimica Acta*, **72**, 1043-1051.

Aghajanian, G.K. & Wang, R.Y. (1977) Habenula and other midbrain raphe afferents demonstrated by a modified retrograde tracing technique, *Brain Research* **122**, 229-242.

Akil, H., Watson, S.J., Young, E., Lewis, M.E., Khatchaturian, H. and Walker, J.M. (1984) Endogenous opioids: Biology and function, *Annual Review of Neuroscience*, **7**, 223-255.

Albers, G.W., S'enez, R.E., Moses, J.A. & Choi, D.W. (1991) Safety and tolerance of oral dextromethorphan in patients at risk for brain ischemia, *Stroke*, **22**, 1075-1077.

Allen, H.L. & Iversen, L.L. (1990) Phencyclidine, dizocilpine, and cerebrocortical neurons, *Science*, **247**, 221.

Altura, B.T., Altura, B.M. & Quirion, R. (1984) Identification of benzomorphan-*k*-opiate receptors in cerebral arteries which subserve relaxation, *British Journal of Pharmacology*, **82**, 459-466.

Argentino, C., Sacchetti, M.L., Toni, D. (1989) GM1 ganglioside therapy in acute ischaemic study, *Stroke*, **20**, 1143-1149.

Arvin, B., Lekeiffre, D., Graham, J.L., Moncada, C., Chapman A.G. & Meldrum, B.S. (1994) Effect of the non-NMDA receptor antagonist GYKI 52466 on the microdialysate and tissue concentrations of amino acids following transient forebrain ischaemia, *Journal of Neurochemistry*, **62**, 1458-1467.

Astrup, J., Siesjö, B.K. & Symon, L. (1981) Thresholds in cerebral ischemia - the ischemic penumbra, *Stroke*, **12**, 723-725.

Astrup, J., Symon, L., Brantson, N.M. & Lassen, N.A. (1977) Cortical evoked potential and extracellular K^+ and H^+ at critical levels of brain ischaemia, *Stroke*, **8**, 51-57.

Auer, R.N. & Coulter, K.C. (1994) The nature and time course of neuronal vacuolation induced by the N-methyl-D-aspartate MK-801, *Acta Neuropathologica*, **87**, 1-7.

Baker, A.J., Moulton, F.J., MacMillan, V.H. & Shedden, P.M. (1993) Excitatory amino acids in cerebrospinal fluid following traumatic brain injury in humans, *Journal of Neurosurgery*, **79**, 369-372.

Bamford, J., Sandercock, P. Dennis, M., Burn, J. & Warlow, C. (1990) A prospective study of acute cerebrovascular disease in the community: the Oxfordshire Community Stroke Project, 1981-86, *Journal of Neurology, Neurosurgery and Psychiatry*, **53**, 16-22.

Baskin, D.S. & Hosobuchi, Y. (1981) Naloxone reversal of ischaemic neurological deficits in man, *Lancet*, **iii**, **272-275.**

Baskin, D.S., Hosobuchi, Y., Loh, H.H. & Lee, N.M. (1984a) Dynorphin (1-13) improves survival in cats with focal cerebral ischaemia, *Nature*, **312**, 551-552.

Baskin, D.S., Kieck, D.F. & Hosobuchi, Y. (1984b) Naloxone reversal and morphine exacerbation of neurologic deficits secondary to focal cerebral ischaemia in baboons, *Brain Research*, **290**, 289-296.

Bass, N.H., Hess, H.H., Pope, A. & Thalheimer, C. (1971) Quantitiative cytoarchitectonic distribution of neurons, glia and DNA in rat cerebral cortex, *Journal of Comparative Neurology*, **143**, 481-490.

Beck, T. & Krieglstein, J. (1986) The effects of tifluadom and ketazocine on behaviour, dopamine turnover in the basal ganglia and local cerebral glucose utilization of rats, *Brain Research*, 381, 327-335.

Beckett, A.H. & Casey, A.F. (1954) Synthetic analgesic stereochemical considerations, *Journal of Pharmacy and Pharmacology*, **6**, 986-1001.

Bederson, J.B., Pitts, L.H., Tsuji, M., Nishimura, M.C., Davis, R.L. & Bartkowski, H. (1986) Rat middle cerebral artery occlusion: evaluation of the model and development of a neurological examination, *Stroke*, **17**, 472-476.

Benedetti, C. & Premuda, L. (1990) The history of opium and its derivatives; In *Advances in Pain Research and Therapy*, eds. Benedetti, C., Chapman, C.R. & Giron, G., Raven Press, New York, pp.1-35.

Benveniste, H. (1991) The excitotoxin hypothesis in relation to cerebral ischemia, *Cerebrovascular and Brain Metabolism Reviews*, **3**, 213-245.

Benveniste, H., Drejer, J., Schousboe, A. & Diemer, N.H. (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis, *Journal of Neurochemistry*, **43**, 1369-1374.

Benveniste, H., Jørgensen, M.B., Diemer, N.H. & Hansen, A.J. (1988) Calcium accumulation by glutamate receptor activation is involved in hippocampal cell damage after ischaemia, *Acta Neurological Scandinavica*, **78**, 529-536.

Bielenberg, G.W. & Beck, T. (1991) The effects of dizocilpine (MK-801), phencyclidine, and nimodipine on infarct size 48h after middle cerebral artery occlusion in the rat, *Brain Research*, **552**, 338-342.

Birch, P.J., Rogers, H., Hayes, A.G., Hayward, N.J., Tyers, M.B., Scopes, D.I.C., Naylor, A. & Judd, D.B. (1991) Neuroprotective actions of GR89696, a highly potent and selective *k*-opioid receptor agonist, *British Journal of Pharmacology*, **103**, 1819-1823.

Boast, C.A., Gerhardt, S.C., Pastor, G., Lehman, J., Etienne, P.E. & Liebman, J.M. (1988) The N-methyl-D-aspartate antagonists CGS 19755 and CPP reduce ischemic brain damage in gerbils, *Brain Research*, **442**, 345-348.

Boxer, P.A.B., Cordon, J.J., Rodolosi, L.C. & Marcoux, F.W. (1991) Reduction of infarct volume in a rat model of focal brain ischemia by the kappa opiate agonist CI-977, *Journal of Cerebral Blood Flow and Metabolism*, **11**, S429.

Boyle, S.J., Meecham, K.G., Hunter, J.C. & Hughes, J. (1990) [^{3}H]-CI-977: A highly selective ligand for the k-opioid receptor in both guinea-pig and rat forebrain, *Molecular Neuropharmacology*, **1** 23-29.

Bradbury, A.F.D., Smyth, D.G., Snell, C.R., Birdsall, N.J.M. & Hulme, E.C. (1976) C-fragment of lipotropin has a high affinity for brain opiate receptors, *Nature*, **260**, 793-795.

Bradford, H.F., Crowder, J.M. & White, E.J. (1986) Inhibitory actions of opioid compounds on calcium fluxes and neurotransmitter release from mammalian cerebral cortical slices, *British Journal of Pharmacology*, **88**, 87-93.

Brandt, L., Andersson, K.E., Hindfelt, B., Ljunggren, B. & Pickard, J.D. (1983) Are the vascular effects of naloxone attributable to the preservatives methyl- and propylparaben? *Journal of Cerebral Blood Flow and Metabolism*, **3**, 395-398.

Branston, N.M., Strong, A.J. & Symon, L. (1977) Extracellular potassium activity, evoked potential and tissue blood flow, *Journal of Neurological Sciences*, **32**, 305-321.

Branston, N.M., Symon, L., Crockard, H.A., Pasztor, E. (1974) Relationship between the cortical evoked potential and local blood flow following acute middle cerebral artery occlusion in the baboon, *Experimental Neurology*, **45**, 195-208.

Brint, S., Jacewicz, M., Kiessling, M., Tanabe, J. & Pulsinelli, W. (1988) Focal brain ischaemia in the rat: methods for reproducible neocortical infarction using tandem occlusion of the distal middle cerebral and ipsilateral common carotid arteries, *Journal of Cerebral Blood Flow and Metabolism*, **8**, 474-485.

Brown, A.W. & Brierley, J.B. (1968) The nature, distribution and earliest stages of anoxic-ischaemic nerve cell damage in the rat as defined by the optical microscope, *British Journal of Experimental Pathology*, **49**, 87-106.

Browne, S.E. & McCulloch, J. (1994) AMPA receptor antagonists in local cerebral glucose utilization in the rat, *Brain Research*, **641**, 10-20.

Buchan, A.M., Li, H., Cho, S. & Pulsinelli, W.A. (1991a) Blockade of the AMPA receptor prevents CA1 hippocampal injury following severe but transient forebrain ischemia in adult rats, *Neuroscience Letters*, **132**, 255-258.

Buchan, A., Li, H. & Pulsinelli, W.A. (1991b) The N-methyl-D-aspartate antagonist, MK-801, fails to protect against neuronal damage caused by transient, severe forebrain ischemia in adult rats, *Journal of Neuroscience*, **11**, 1049-1056.

Buchan, A. & Pulsinelli, W.A. (1990) Hypothermia but not the N-methyl-D-aspartate antagonist, MK-801, attenuates neuronal damage in gerbils subjected to transient global ischemia, *Journal of Neuroscience*, **10**, 311-316.

Buchan, A.M., Slivka, A. & Xue, D. (1992) The effect of the NMDA receptor antagonist MK-801 on cerebral blood flow and infarct volume in experimental focal stroke, *Brain Research*, **574**, 171-177.

Buchan, A.M., Xue, D., Huang, Z.-G., Smith, K.H. and Lesiuk, H. (1991c) Delayed AMPA receptor blockade reduces cerebral infarction induced by focal ischemia, *NeuroReport*, **2**, 473-476.

Bullock, R., Graham, D.I., Chen, M.-H., Lowe, D. & McCulloch, J. (1990) Focal cerebral ischemia in the cat: Pretreatment with a competitive NMDA receptor antagonist, D-CPPene, *Journal of Cerebral Blood Flow and Metabolism*, **10**, 668-674.

Bullock, R., Graham, D.I., Swanson, S. & McCulloch, J. (1994) The neuroprotective effect of the AMPA receptor antagonist LY-293558 in focal cerebral ischemia in the cat, *Journal of Cerebral Blood Flow and Metabolism*, **14**, 466-471.

Bullock, R. & Teasdale, G.M. (1990) Surgical management of traumatic intracranial haematomas, in: *Handbook of Clincial Neurology. Head Injury*, Vol.24, Ed. R. Braakman, Elsevier, Amsterdam, pp. 95-118.

Busto, R., Dietrich, W.D., Globus, M. Y-T., Valdés, I., Scheinberg, P. & Ginsberg, M.D. (1987) Small differences in intraischemic brain temperature critically determine the extent of ischemic neuronal injury, *Journal of Cerebral Blood Flow and Metabolism*, **7**, 729-738.

Butcher, S.P., Bullock, R., Graham, D.I. & McCulloch, J. (1990) Correlation between amino acid release and neuropathologic outcome in rat brain following middle cerebral artery occlusion, *Stroke*, **21**, 1727-1733.

Camarata, P.J., Heros, R.C. & Latchaw, R.E. (1994) "Brain Attack": The rationale for treating stroke as a medical emergency, *Neurosurgery*, **34**, 144-158.

Cammermeyer, J. (1961) The importance of avoiding 'dark' neurones in experimental neuropathology, *Acta Neuropathologica* (Berl) **1**, 245-270.

Carter, C., Rivy, J.-P. & Scatton, B. (1989) Ifenprodil and SL 82.0715 are antagonists at the polyamine site of the N-methyl-D-aspartate (NMDA) receptor, *European Journal of Pharmacology*, **164**, 611-612.

Cavazzuti, M., Porro, C.A., Biral, G.P., Benassi, C. & Barbieri, G.C. (1987) Ketamine effects on local cerebral blood flow and metabolism in the rat, *Journal of Cerebral Blood Flow and Metabolism*, **7**, 806-811.

Chang, K.J., Hazum, E. & Cuatrecasas, P. (1981) Novel opiate binding binding sites selective for benzomorphan drugs, *Proceedings of the National Academy of Sciences, U.S.A.*, **78**, 4141-4145.

Chavkin, C. & Goldstein, A. (1981) Specific receptor for the opioid peptide dynorphin:structure-activity relationships, *Proceedings of the National Academy of Sciences, U.S.A.*, **78**, 6543-6547.

Chavkin, C., Shoemaker, W.J., McGinty, J.F. Bayon, A. & Bloom F.E. (1985) Characterization of the prodynorphin and proenkephalin neuropeptide systems in rat hippocampus, *Journal of Neuroscience*, **5**, 808-816.

Chen, H., Chopp, M., Zheng, Z.G. & Garcia, J.H. (1992a) The effect of hypothermia on transient middle cerebral artery occlusion in the rat, *Journal of Cerebral Blood Flow and Metabolism*, **12**, 621-628.

Chen, H.S.V., Pellegrini, J.W., Aggarwal, S.K., Lei, S.Z., Warach, S., Jensen, F.E. & Lipton, S.A. (1992b) Open-channel block of NMDA receptor by memantine: therapeutic advantage against NMDA receptor-mediated neurotoxicity, *Journal of Neuroscience*, **12**, 4427-4436.

Chen, J., Graham, S.H. & Faden, A.I. (1991a) The direct effects of dynorphin A on isolated rat cerebral arteries, *Society for Neuroscience Abstracts*, **17**, 100.11.

Chen, J. Graham, S., Moroni, F. & Simon, R. (1993) A study of the dose dependency of a glycine receptor antagonist in focal ischemia, *Journal of Pharmacology and Experimental Therapeutics*, **267**, 937-941.

Chen, M., Bullock, R., Graham D.I., Frey, P., Lowe, D. & McCulloch, J. (1991b) Evaluation of a competitive NMDA antagonist (D-CPPene) in feline focal cerebral ischemia, *Annals of Neurology*, **30**, 62-70.

Cheng, J., Roques, B.P., Gacel, G.A., Huang, E. & Pasternak, G.W. (1992) K_3 Opiate receptor binding in the mouse and rat, *European Journal of Pharmacology*, **226**, 15-20.

Cheung, J.Y., Bonventre, J.V., Malis, C.D. & Leaf, A. (1986) Calcium and ischaemic injury, *New England Journal of Medicine*, **314**, 1670-1676.

Choi, D.W. (1987) Ionic dependence of glutamate neurotoxicity, *Journal of Neuroscience*, **7**, 369-379.

Choi, D.W. (1988) Glutamate neurotoxicity and diseases of the nervous system, *Neuron*, **1**, 623-634.

Choi, D.W. (1990) Methods for antagonizing glutamate neurotoxicity, *Cerebrovascular Brain Metabolism Reviews*, 2, 105-147.

Chopp, M., Welch, K.M.A., Tidwell, C.D. & Helpern, J.A. (1988) Global cerebral ischemia and intracellular pH during hyperglycemia and hypoglycemia in cats, *Stroke*, **19**, 1383-1387.

Christensen, T., Bruhn, T., Diemer, N.H. & Schousboe, A. (1991) Effect of phenylsuccinate on potassium- and ischemia-induced release of glutamate in rat hippocampus monitored by microdialysis, *Neuroscience Letters*, **134**, 71-74.

Church, J., Zeman, S. & Lodge, D. (1988) The neuroprotective action of ketamine and MK-801 after transient cerebral ischemia in rats, *Anesthesiology*, **69**, 702-709.

Clark, W.M. & Coull, B.M. (1994) Randomized trial of CGS 19755, a glutamate antagonist, in acute ischemic stroke treatment, *Journal of Neurology* (in press).

Clifton, G.L., Taft, W.C., Blair, R.E., Choi, S.C. & De Lorenzo, R.J. (1989) Conditions for pharmacological evaluation in the gerbil model of forebrain ischaemia, *Stroke*, **20**, 1545-1552.

Collingridge, G.L. & Bliss, T.V.P. (1987) NMDA receptors - their role in long-term potentiation, *Trends in Neuroscience*, **10**, 288-293.

Collingridge, G.L. & Lester, R.A.J. (1989) Excitatory amino-acid receptors in the vertebrate central nervous system, *Pharmacological Reviews*, **41**, 143-210.

Connor, J.A., Wadman, W.J., Hochberger, P.E. & Wong, R.K.S. (1988) Sustained dendritic gradients of Ca^{2+} induced by excitatory amino acids in CA1 hippocampal neurons, *Science*, **240**. 649-653.

Contreras, P.C., Raga, D.M., Bremer, M.E., Lanthorn, T.H., Gray, N.M., Iyengar, S., Jacobson, A.E., Rice, K.C. & de Costa, B.R. (1991) Evaluation of U-50,488H analogs for neuroprotective activity in the gerbil, *Brain Research*, **546**, 79-82.

Corbett, A.D., Paterson, S.J., McKnight, A.T., Magnan, J. & Kosterlitz, H.W. (1982) $Dynorphin_{1-8}$ and $dynorphin_{1-9}$ are ligands for the *k*-subtype of opiate receptor, *Nature*, **299**, 79-81.

Corbett, D., Evans, S., Thomas, C., Wang, D. & Jonas, R.A. (1990) MK-801 reduced cerebral ischemic injury by inducing hypothermia, *Brain Research*, **514**, 300-304.

Cox, B.M., Opheim, K.E. Teschemacher, H. & Goldstein, A. (1975) A peptide-like substance from pituitary that acts like morphine. 2. Purification and properties, *Life Sciences*, **16**, 1777-1782.

Crane, P.D., Braun, L.D., Cornford, E.M., Cremer, J.E., Glass, J.M. & Oldendorf, W.H. (1978) Dose dependent reduction of glucose utilization by pentobarbital in rat brain, *Stroke*, **9**, 12-18.

Cuddenec, A., Duverger, D., Lloyd, K.G., MacKenzie, E.T., McCulloch, J., Motohashi, N., Nishikawa, T. & Scatton, B. (1987) Effects of the GABA receptor agonist, progabide, upon local cerebral glucose utilization, *Brain Research*, **423**, 162-172.

Dale, N. & Roberts, A. (1985) Dual component amino acid-mediated synaptic potentials: excitatory drive for swimming in xenopus embryos, *Journal of Physiology*, **363**, 35-39.

Davis, R.E., Callahan, M.J., Dickerson, M. & Downs, D.A. (1992) Pharmacologic activity of CI-977, a selective kappa opioid agonist, in rhesus monkeys, *Journal of Pharmacology and Experimental Therapeutics*, **261**, 1044-1049.

de Courten-Meyers, G., Myers, R.E. & Schoolfield, L. (1988) Hyperglycemia enlarges infarct size in cerebrovascular occlusion in cats, *Stroke*, **19**, 623-630.

Dezsi, L., Greenberg, J.H., Hamar, J., Sladky, J., Karp, A. & Reivich, M. (1992) Acute improvement in histolgical outcome by MK-801 following focal cerebral ischemia and reperfusion in the cat independent of blood flow changes, *Journal of Cerebral Blood Flow and Metabolism*, **12**, 390-399.

Diemer, N.H., Jørgensen, M.B., Johansen, F.F., Sheardown, M. & Honoré, T. (1992) Protection against ischemic hippocampal CA1 damage in the rat with a new non-NMDA antagonist, NBQX, *Acta Neurologica Scandinavica*, **86**, 45-49.

Dienel, G.A. (1984) Regional accumulation of calcium in postischemic rat brain, *Journal of Neurochemistry*, **43**, 913-925.

Dietrich, W.D., Nakayama, H., Watson, B.D. & Kangmitsu, H. (1989) Morphological consequences of early reperfusion following thrombotic or mechanical occlusion of the rat middle cerebral artery, *Acta Neuropathologica*, **78**, 605-614.

Dirnagl, U. & Pulsinelli, W. (1990) Autoregulation of cereral blood flow in experimental focal brain ischemia, *Journal of Cerebral Blood Flow and Metabolism*, **10**, 327-336.

Dirnagl, U., Tanabe, J. & Pulsinelli, W. (1990) Pre- and post-treatment with MK-801 but not pretreatment alone reduces neocortical damage after focal cerebral ischemia in the rat, *Brain Research*, **527**, 62-68.

Dow-Edwards, D., Peterson, J.M., Mahone, P., Rapoport, S.I. & London, E.D. (1980) Effect of oxotremorine, a muscarinic agonist, on local cerebral glucose utilization (LCGU) in the rat, *Society of Neuroscience Abstracts*, **6**, 769.

Dubinsky, J.M. & Rothman, S.M. (1991) Intracellular calcium concentrations during "chemical hypoxia" and excitotoxic neuronal injury, *Journal of Neuroscience*, **11**, 2545-2551.

Duval, D., Roome, N., Gauffeny, C., Nowicki, J.P. & Scatton, B. (1992) SL 82.0715, an NMDA antagonist acting at the polyamine site, does not induce neurotoxic effects on rat cortical neurons, *Neuroscience Letters*, **137**, 193-197.

Duverger, D. & MacKenzie, E.T. (1988) The quantification of cerebral infarction following focal ischemia in the rat: influence of strain, arterial pressure, blood glucose concentration and age, *Journal of Cerebral Blood Flow and Metabolism*, **8**, 449-461.

Edvinsson, L. & MacKenzie, E.T. (1977) Amine mechanisms in the cerebral circulation, *Pharmacology Reviews*, 28, 275-348.

Eklöf, B. & Siesjö, B.K. (1972) The effect of bilateral carotid artery ligation upon the blood flow and energy state of the rat brain, *Acta Physiologica Scandinavica*, **86**, 155-165.

Fallis, R., Fisher, M. & Lobo, R. (1983) A double-blind trial of naloxone in acute stroke, *Stroke*, **14**, 124.

Fanelli, R.J., Szikszay, M., Jasinski, D.R. & London, E.D. (1987) Differential effects of μ and *k* opioid analgesics on cerebral glucose utilization in the rat, *Brain Research*, **422**, 257-266.

Fischel, S.V. & Medzihradsky, F. (1986) Interaction between ^{3}H-ethylketocyclazocine and ^{3}H-etorphine and opioid receptors in membranes from rat brain: a kinetic analysis, *Neuropharmacology*, **25**, 351-359.

Fix, A.S., Horn, J.W., Wightman, K.A., Johnson, C.A., Long, G.G., Storts, R.W., Farber, N., Wozniak, D.F. & Olney, J.W. (1993) Neuronal vacuolization and necrosis induced by the noncompetitive N-methyl-D-aspartate (NMDA) antagonist MK(+)801 (Dizocipline Maleate): a light and electron microsope evaluation of the rat retrosplenial cortex, *Experimental Neurology*, **123**, 204-215.

Fleischer, J.E., Tateish, A., Drummon, J.C., Scheller, M.S., Grafe, M.R., Zornow, M.H., Shearman, G.T. & Shapiro, H.M. (1989) MK-801, an excitatory amino antagonist, does not improve neurologic outcome following cardiac arrest in cats, *Journal of Cerebral Blood Flow and Metabolism*, **9**, 795-804.

Folbergrová, J., Memezawa, H., Smith, M-L. & Siesjö, B.K. (1992) Focal and perifocal changes in tissue energy state during middle cerebral artery occlusion in normo- and hyperglycemic rats, *Journal of Cerebral Blood Flow and Metabolism*, **12**, 25-33.

Fonnum, F. (1984) Glutamate: A neurotransmitter in mammalian brain, *Journal of Neurochemistry*, **142**, 1-11.

Foulkes, M.A., Wolf, P.A., Price, T.R., Mohr, J.P., Hier, D.B. (1988) Design, methods and baseline characteristics, *Stroke*, **19**, 547-554.

Foster, A.C. & Fagg, G.E. (1987) Taking apart NMDA receptors, *Nature*, 329-395-396.

Frandsen, A., Drejer, J. & Schousboe, A. (1989) Direct evidence that neurotoxicity in cultured neurons is mediated via N-methyl-D-aspartate (NMDA) as well as non-NMDA receptors, *Journal of Neurochemistry*, **53**, 297-299.

Frank, L., Bruhn, T. & Diemer, N.H. (1993) The effect of an AMPA antagonists (NBQX) on postischemic neuron loss and protein synethsis in the rat brain, *Experimental Brain Research*, **95**, 70-76.

Fried, R.L. & Nowak Jr., T.S. (1987) Opioid peptide levels in gerbil brain after tranisent ischemia: lasting depletion of hippocampal dynorphin, *Stroke*, **18**, 765-770.

Fujie, W., Kirino, T., Tomukai, N., Iwasawa, T. & Tamura, A. (1990) Progressive shrinkage of the thalamus following middle cerebral artery occlusion in rats, *Stroke*, **21**, 1485-1488.

Gaines, C., Nehls, D.G., Suess, D.M., Waggener, J.D. & Crowell, R.M. (1984) Effect of naloxone on experimental stroke in awake monkeys, *Neurosurgery*, **14**, 308-314.

Gannon, R.L. & Terrian, D.M. (1991a) U-50,488H inhibits dynorphin and glutamate release from guinea pig hippocampal mossy fiber terminals, *Brain Research*, **548**, 242-247.

Gannon, R.L. & Terrian, D.M. (1991b) Presynaptic modulation of glutamate and dynorphin release by excitatory amino acids in the guinea pig hippocampus, *Neuroscience*, **41**, 401-410.

Gannon, R.L. & Terrian, D.M. (1992) Kappa opioid agonists inhibit transmitter release from guinea pig hippocampal mossy fiber synaptosomes, *Neurochemical Research*, **17**, 741-747.

Gardiner, M., Smith, M-L., Kågström E., Shohami, E. & Siesjö, B.K. (1982) Influence of blood glucose concentration on brain lactate accumulation during severe hypoxia and subsequent recovery of brain energy metabolism, *Journal of Cerebral Blood Flow and Metabolism*, **2**, 429-438.

Gasic, G.P. & Hollmann, M. (1992) Molecular neurobiology of glutamate receptors, *Annual Reviews of Physiology*, **54**, 507-536.

Gelmers, H.J., Gorter, K., De Weerdt, C.J. & Wiezer, H.J.A. (1988) A controlled trial of nimodipine in acute ischemic stroke, *New England Journal of Medicine*, **318**, 203-207.

Gilbertson, T.A., Scobey, R. & Wilson, M. (1991) Permeation of calcium ions through non-NMDA glutamate channels in retinal bipolar cells, *Science*, **251**, 1613-1615.

Gill, R., Andiné, P., Hillered, L., Persson, L. & Hagberg, H. (1992a) The effect of MK-801 on cortical spreading depression in the penumbral zone following focal ischaemia in the rat, *Journal of Cerebral Blood Flow and Metabolism*, **12**, 371-379.

Gill, R., Brazell, C., Woodruff, G.N. & Kemp. J.A. (1991a) The neuroprotective action of dizocilpine (MK-801) in the rat middle cerebral artery occlusion model of focal ischaemia, *British Journal of Pharmacology*, **103**, 2030-2036.

Gill, R., Foster, A.C. & Woodruff, G.N. (1987) Systemic administration of MK-801 protects against ischemia-induced hippocampal neurodegeneration in the gerbil, *Journal of Neuroscience*, 7, 3343-3349.

Gill, R., Foster, A.C. & Woodruff, G.N. (1988) MK-801 is neuroprotective in gerbils when administered during the post-ischaemic period, *Neuroscience*, **25**, 847-855.

Gill, R., Hargreaves, R. & Kemp, J.A. (1991b) Neuroprotective effects of the glycine site antagonist (+)cis-4-methyl-HA-966(L-687,414) in a rat focal ischaemia model, *Journal of Cerebral Blood Flow and Metabolism*, **11** (Suppl. 2), S304.

Gill, R., Nordholm, L. & Lodge, D. (1992b) The neuroprotective actions of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) in a rat focal ischaemia model, *Brain Research*, **580**, 35-43.

Gillan, M.G.C. & Kosterlitz, D.H.W. (1982) Spectrum of the μ, σ, and *k*-binding sites in homogenates of rat brain, *British Journal of Pharmacology*, **77**, 461-469.

Ginsberg, M.D. & Busto, R. (1989) Rodent Models of Cerebral Ischemia. *Stroke*, **20**, 1627-1642.

Ginsberg, M.D., Dietrich, D.W. & Busto, R. (1987a) Coupled forebrain increases of local cerebral glucose utilisation and blood flow during physiologic stimulation of a somatosensory pathway in the rat: demonstration by double label autoradiography, *Neurology*, **37**, 11-19.

Ginsberg, M.D., Prado, R., Dietrich, W.D., Busto, R. & Watson, B.D. (1987b) Hyperglycemia reduces the extent of cerebral infarction in rats, *Stroke*, **18**, 570-574.

Ginsberg, M.D., Sternau, L.L., Globus, M. T.-T., Dietrich, W.D. & Busto, R. (1992) Therapeutic modulation of brain temperature: relevance to ischemic brain injury, *Cerebrovascular and Brain Metabolism Reviews*, **4**, 189-225.

Globus, M.Y.-T., Busto, R., Dietrich, W.D., Martinez, E., Valdes, I. & Ginsberg, M.D. (1988) Effect of ischemia on the in vivo release of striatal dopamine, glutamate and γ-aminobutyric acid studied by intracerebral microdialysis, *Journal of Neurochemistry*, **51**, 1455-1464.

Goldstein, A., Fischli, W., Lowney, L.I., Hunkapiller, M. & Hood, L. (1981) Porcine pituitary dynorphin: complete amino acid sequence of the biologically active heptadecapeptide, *Proceedings of the National Academy of Sciences, U.S.A.*, **78**, 7219-7223.

Gotoh, O., Mohamed, A.A., McCulloch, J., Graham, D.I., Harper, A.M. & Teasdale, G.M. (1986) Nimodipine and the haemodynamic and histopathological consequences of middle cerebral artery occlusion in the rat, *Journal of Cerebral Blood Flow and Metabolism*, **6**, 321-331.

Gotti, B., Duverger, D., Bertin, J., Carter, C., Dupont R., Frost, J., Gaudilliere, B., MacKenzie, E.T., Rousseau, J., Scatton, B. & Wick, A. (1988) Ifenprodil and SL82.0715 as cerebral anti-ischemic agents. I. Evidence for efficacy in models of focal cerebral ischemia, *Journal of Pharmacology and Experimental Therapeutics*, **247**, 1211-1221.

Graham, D.I. (1988) Focal cerebral infarction, *Journal of Cerebral Blood Flow and Metabolism*, **8**, 769-733.

Graham, D.I., Adams, J.H. & Doyle, D. (1978) Ischaemic brain damage in fatal non -missile head injury, *Journal of Neurological Science*, **39**, 213-234

Graham, S.H., Chen, J., Lan, J., Leach, M.J. & Simon R.P. (1994) Neuroprotective effects of a use-dependent blocker of voltage-dependent sodium channels, BW619C89, in rat middle cerebral artery occlusion, *Journal of Pharmacology and Experimental Therapeutics*, **269**, 854-859.

Graham, S.H., Chen, J., Sharp, F.R. & Simon, R.P. (1993a) Limiting ischemic injury by inhibition of excitatory amino acid release, *Journal of Cerebral Blood Flow and Metabolism*, **13**, 88-97.

Graham, S.H. Chen, J. & Simon, R.P. (1993b) A dose-response study of dextrorphan in permanent focal ischemia, *Neuroscience Letters*, **160**, 21-23.

Gross, R.A. & Macdonald, R.L. (1987) Dynorphin A selectively reduces a large transient (N-type) calcium current of mouse dorsal root ganglion neurones in cell culture, *Proceedings of the National Academy of Sciences, U.S.A.*, **84**, 5469-5473.

Grotta, J. (1994) Safety and tolerability of the glutamate antagonist CGS 19755 in acute stroke patients, *Stroke*, **25**, 52, 255.

Gulati, A. & Bhargava, H.N. (1988) Cardiovascular responses to kappa-opioid agonists in intact and adrenal demedullated rats, *European Journal of Pharmacology*, **156**, 247-257.

Haberman, S. (1984) Long term prognosis after transient ischemic attacks, *Neuroepidemiology*, **3**, 108-128.

Hagberg, H., Anderson, P., Lacarewicz, J., Jacobsen, I., Butcher, S. & Sandberg, M. (1987) Extracellular adenosine, inosine, hypoxanthine and xanthine in relation to tissue nucleotides and purines in rat striatum during transient ischaemia, *Journal of Neurochemistry*, **49**, 227-231.

Hagberg, H., Lehman, A., Sandberg, M., Nyström, B., Jacobson, I. & Hamberger, A. (1985) Ischemia-induced shift of inhibitory and excitatory amino acids from intra- to extracellular compartments, *Journal of Cerebral Blood Flow and Metabolism*, **5**, 413-419.

Hall, E.D. & Pazara, K.E. (1988) Quantitative analysis of effects of *k*-opioid agonists on post-ischemic hippocampal CA1 neuronal necrosis in gerbils, *Stroke*, **19**, 1008-1012.

Hall, E.D., Wolf, D.L., Althaus, J.S. & VonVoigtlander, P.F. (1987) Beneficial effects of the *k* opioid receptor agonist U-50488H in experimental acute brain and spinal cord injury, *Brain Research*, **435**, 174-180.

Hanko, J.H. & Hardebo, J.E. (1978) Enkephalin-induced dilatation of pial arteries in vitro probably medited by opiate receptors, *European Journal of Pharmacology*, **51**, 295-297.

Hansen, A.J. (1985) Effect of anoxia on ion distribution in the brain, *Physiology Reviews*, **65**, 101-148.

Harder, D.R. & Madden, J.A. (1984) Cellular mechanisms of opiate receptor stimulation in cat middle cerebral artery, *European Journal of Pharmacology*, **102**, 411-416.

Hargreaves, R.J., Rigby, M., Smith, D. & Hill, R.G. (1993a) Lack of effect of L-687,414 ((+)-*cis*-4-methyl-HA-966), an NMDA receptor antagonist acting at the glycine site, on cerebral glucose metabolism and cortical neuronal morphology, *British Journal of Pharmacology*, **110**, 36-42.

Hargreaves, R.J., Rigby, M., Smith, D., Hill, R.G. & Iversen, L.L. (1993b) Competitive as well as uncompetitive N-methyl-D-aspartate receptor antagonists affect cortical neuronal morphology and cerebral glucose metabolism, *Neurochemical Research*, **18**, 1263-1269.

Harris, R.J., Branston, N.M., Symon, L., Bayhan, M. & Watson, A. (1982) The effects of a calcium antagonist, nimodipine, upon physiological responses of the cerebral vasculature and its possible influence upon focal cerebral ischaemia, *Stroke*, **13**, 759-766.

Hartley, D.M., Kurth, M.C., Bjerkness, L., Weiss, J.H. & Choi, D.W. (1993) Glutamate receptor-induced Ca^{2+} accumulation in cortical cell culture correlates with subsequent neuronal degeneration, *Journal of Neuroscience*, **13**, 1993-2000.

Hatfield, R.H., Gill, R. & Brazell, C. (1992) The dose-response relationship and therapeutic window for dizocilpine (MK-801) in a rat focal ischaemia model, *European Journal of Pharmacology*, **216**, 1-7.

Haug, H. (1987) Brain sizes, surfaces, and neuronal sizes of the cortex cerebri: A stereological investigation of man and his variability and a comparison with some mammals (primates, whales, marsupials, insectivores, and one elephant), *American Journal of Anatomy*, **180**, 126-142.

Hayward, N.J., Hitchcott, P.K., McKnight, A.T. & Woodruff, G.N. (1993a) Effect of enadoline in ischaemia-induced glutamate release, *British Journal of Pharmacology*, **110**, 62P.

Hayward, N.J., McKnight, A.T. & Woodruff, G.N. (1992) The neuroprotective action of *k*-opioid agonists in the gerbil, *Molecular Neuropharmacology*, **2**, 221-227.

Hayward, N.J., McKnight, A.T. & Woodruff, G.N. (1993b) Neuroprotective effect of the *k*-agonist enadoline (CI-977) in rat models of focal cerebral ischaemia, *European Journal of Neuroscience*, **5**, 961-967.

Herkenham, M. (1980) Laminar organisation of thalamic projections to the rat neocortex, *Science*, **207**, 532-534.

Herrling, P.L., Morris, R. & Salt, T.E. (1983) Effects of excitatory amino acids and their antagonists on membrane and action potentials of cat caudate neurones, *Journal of Physiology*, **339**, 207-222.

Hillered, L., Hallström, A., Segersvärd, S., Persson, L. & Ungerstedt, U. (1989) Dynamics of extracellular metabolites in the striatum after middle cerebral artery occlusion in the rat monitored by intracerebral microdialysis, *Journal of Cerebral Blood Flow and Metabolism*, **9**, 607-616.

Holaday, J.W. & D'Amato, R.J. (1982) Naloxone or TRH fails to improve neurologic deficits in gerbil models of "stroke", *Life Sciences*, **31**, 385-392.

Hollmann, M., Hartley, M. & Heinemann, S. (1991) Ca^{2+} permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition, *Science*, **252**, 851-853.

Höllt, V. (1986) Opioid peptide processing and receptor selectivity, *Annual Reviews of Pharmacology and Toxicology*, **26**, 59.

Hong, S-C., Goto, Y., Lanzino, G., Soleau, S., Kassell, N.F. & Lee, K.S. (1994) Neuroprotection with a calpain inhibitor in a model of focal cerebral ischaemia, *Stroke*, **25**, 663-669.

Hosobuchi, Y., Baskin, D.S. & Woo, S.K. (1982) Reversal of induced ischaemic neurologic deficit in gerbils by the opiate antagonist naloxone, *Science*, **215**, 69-71.

Hossmann, K.-A. (1982) Treatment of experimental cerebral ischaemia, *Journal of Cerebral Blood Flow Metabolism*, 2, 275-297.

Hossmann, K.-A. (1988) Calcium antagonists for the treatment of brain ischemia: a critical appraisal. In *Pharmacology of cerebral ischemia*, ed. Krieglstein, JD, Wissenschaftliche Verlagsgesellschaft mgH, Stuttgart, pp.53-63.

Hossmann, K.-A. (1991) Animal models of cerebral ischaemia. 1. Review of literature, *Cerebrovascular Disease*, 1, 2-15.

Hubbard, J.L. & Sundt, T.M. Jr. (1983) Failure of naloxone to affect focal incomplete cerebral ischemia and collateral blood flow in cats, *Journal of Neurosurgery*, 59, 237-244.

Hudgins, W.R. & Garcia, J.H. (1970) Transorbital approach to the middle cerebral artery of the squirrel monkey: a technique for experimental cerebral infarction applicable to ultrastructure studies, *Stroke*, 1, 107-111.

Hughes, J. (1975) Isolation of an endogenous compound from the brain with pharmacological properties similar to morphine, *Brain Research*, 88, 295-308.

Hughes, J., Smith, T.W., Kosterlitz, H.W., Fotherghill, L.A., Morgan, B.A. & Morris, H.R. (1975) Identification of two related pentapeptides from the brain with potent opiate agonist activity, *Nature*, 258, 577-579.

Hume, R.I., Dingledine, R. & Heinemann, S.F. (1991) Identification of a site in glutamate receptor subunits that controls calcium permeability, *Science*, **253**, 1028-1031.

Hunter, J.C., Leighton, G.E., Meecham, K.G., Boyle, S.J., Horwell, D.C., Rees, D.C. & Hughes, J. (1990) CI-977, a novel and selective agonist for the *k*-opioid receptor, *British Journal of Pharmacology*, **101**, 183-189.

Iino, M., Ozawa, S. and Tsuzuki, K. (1990) Permeation of calcium through excitatory amino acid receptor channels in cultured rat hippocampal neurones, *Journal of Physiology*, **424**, 151-165.

Iijima, T., Mies, G. & Hossman, K.-A. (1992) Repeated negative DC deflections in rat cortex following middle cerebral artery occlusion are abolished by MK-801: effect on volume of ischemic injury, *Journal of Cerebral Blood Flow and Metabolism*, 12, 727-733.

Ikeda, M., Mackay, K.B., Dewar, D. & McCulloch, J. (1993) Differential alterations in adenosine A_1 and k_1 opioid receptors in the striatum in Alzheimer's disease, *Brain Research*, **616**, 211-217.

Jacewicz, M., Brint, S., Tanabe, J. & Pulsinelli, W.A. (1990a) Continuous nimodipine treatment attenuates cortical infarction in rats subjected to 24 hours of focal cerebral ischemia, *Journal of Cerebral Blood Flow and Metabolism*, **10**, 89-96.

Jacewicz, M., Brint, S., Tanabe, J., Wang, X-J. & Pulsinelli, W.A. (1990b) Nimodipine pretreatment improves cerebral blood flow and reduces brain edema in conscious rats subjected to focal cerebral ischemia, *Journal of Cerebral Blood Flow and Metabolism*, **10**, 903-913.

Jensen, M. & Auer, R. (1988) Ketamine fails to protect against ischemic neuronal necrosis in the rat, *British Journal of Anaesthesia*, **61**, 206-210.

Johansen, F.F., Jørgensen, M.B. & Diemer, N.H. (1986) Ischemic CA1 pyramidal cell loss is prevented by ischemic colchicine destruction of dentate gyrus granule cells, *Brain Research*, **377**, 344-347.

Jones, T.J., Morawetz, R.B., Crowell, R.M., Marcoux, F.W., Fitzgibbon, S.J., Degirolami, U. & Ojemann, R.G. (1981) Thresholds of focal cerebral ischemia in awake monkeys, *Journal of Neurosurgery*, **54**, 773-782.

Kadekaro, K., Crane, A.M., Sokoloff, L. (1985) Differential effects of electrical stimulation of sciatic nerve on metabolic activity in spinal cord and dorsal root ganglion in the rat, *Proceedings of the National Academy of Sciences, U.S.A.*, **82**, 6010-6013.

Kågström, E., Smith, M.-L. & Siesjö, B.K. (1983) Recirculation in the rat brain following incomplete ischemia, *Journal of Cerebral Blood Flow and Metabolism*, **3**, 183-192.

Kakidani, H., Furutani, Y., Takahashi, H., Noda, M., Morimotor, Y., Hirose, T., Asai, M., Inayama, S., Nakanishi, S. & Numa, S. (1982) Cloning and sequence analysis of cDNA for porcine beta-neo-endorphin/dynorphin precursor, *Nature*, **298**, 245-249.

Kaplan, B., Brint, S., Tanabe, J., Jacewicz, M., Wang, X-J. & Pulsinelli, W. (1991) Temporal thresholds for neocortical infarction in rats subjected to reversible focal cerebral ischemia, *Stroke*, **22**, 1032-1039.

Kaplan, T.M., Lasner, T.M., Nadler, J.V. & Crain, B.J. (1989) Lesions of excitatory pathways reduce hippocampal cell deaths after transient forebrain ischemia in the gerbil, *Acta Neuropathologica (Berlin)*, **78**, 283-290.

Karpiak, S.E., Tagliavia, A. & Wakade, C.G. (1989) Animal models for the study of drugs in ischaemic stroke, *Annual Review of Pharmacology and Toxicology*, **29**, 403-414.

Kastin, A.J., Nissen, C., Olson, R.D. (1982) Failure of MIF-1 or naloxone to reverse ischemic-induced neurologic deficits in gerbils, *Pharmacological and Biochemical Behaviour*, **17**, 1083-1085.

Kassell, N.F., Torner, J.C., Haley Jr., E.C., Jane, J.A., Adams, H.P. and Kongable, G.L. (1990) The international cooperative study on the timing of aneurysm surgery. 1. Overall management results, *Journal of Neurosurgery*, **73**, 18-36.

Katayama, Y., Becker, D.P., Tamura, T. & Hovda, D.A. (1990) Massive increases in extracellular potassium and the indiscriminate release of glutamate following concussive brain injury, *Journal of Neurosurgery*, **73**, 889-900.

Katayama, Y., Kawamata, T., Kano, T. & Tsubokawa, T. (1992) Excitatory amino acid antagonist administered via microdialysis attenautes lactate accumulation during cerebral ischaemia and subsequent hippocampal damage, *Brain Research*, **584**, 329-333.

Katayama, Y., Kawamata, R., Tamura, T., Hovda, D.A., Becker, D.P. & Tsubokawa, T. (1991) Calcium-dependent glutamate release concomitant with massive potassium flux during cerebral ischemia in vivo, *Brain Research* **558**, 136-140.

Kauppinen, R.A., McMahon, H.T. & Nicholls, D.G. (1988) Ca^{2+}-dependent and Ca^{2+}-independent glutamate release, energy status and cytosolic free Ca^{2+} concentration in isolated nerve terminals following metabolic inhibition: possible relevance to hypoglycaemia and anoxia, *Neuroscience*, **27**, 175-182.

Kelly, P.A.T. & McCulloch, J. (1982) Effects of the putative GABAergic agonists, muscimol and THIP, upon local cerebral glucose utilisation, *Journal of Neurochemistry*, **39**, 613-624.

Kemp, J.A., Foster, A.C. & Wong, E.H.F. (1987) Non-competitive antagonists of excitatory amino acid receptors, *Trends in Neuroscience*, **10**, 294-298.

Kemp, J.A., Foster, A.C., Leeson, P.D., Priestly, T., Tridgett, R. & Iversen, L.L. (1988) 7-Chlorokynurenic acid is a selective antagonist at the glycine modulatory site of the N-methyl-D-aspartate receptor complex, *Proceedings of the National Academy of Sciences, U.S.A.*, **85**, 6547-6550.

Kennedy, C., Des Rosiers, M.H., Jehle, J.W., Reivich, M., Sharp, F. & Sokoloff, L. (1975) Mapping of functional neural pathways by autoradiographic survey of local metabolic rate with [^{14}C]deoxyglucose, *Science*, **187**, 850-853.

Kennedy, C., Sakurada, O., Shinohara, M., Jehle, J. & Sokoloff, L. (1978) Local cerebral glucose utilization in the normal conscious macaque monkey, *Annals of Neurology*, **4**, 293-301.

Kety, S.S. (1951) The theory and applications of the exchange of inert gas at the lungs and tissues, *Pharmacology Reviews*, **3**, 1-41.

Khachaturian, H., Lewis, M.G. & Watson, S.J. (1983) Enkephalin systems in diencephalon and brainstem of the rat, *Journal of Comparative Neurology*, **220**, 310-320.

Kirino, T. (1982) Delayed neuronal death in the gerbil hippocampus following ischaemia, *Brain Research*, **239**, 57-69.

Kirino, T., Tsujita, Y. & Tamura, A. (1991) Induced tolerance to ischemia in gerbil hippocampal neurons, *Journal of Cerebral Blood Flow and Metabolism*, **11**, 299-307.

Knapp, R.J., Hunt, M., Wamsley, J.K. and Yamamura, H.I. (1993) CNS receptors for opioids; In *Imaging Drug Action in the Brain*, ed. London, E.D., CRC Press, pp.119-176.

Koh, J.-Y., Goldberg, M.P., Hartley, D.M. & Choi, D.W. (1990) Non-NMDA receptor-mediated neurotoxicity in cortical culture, *Journal of Neuroscience*, **10**, 693-705.

König.F.R. & Klippel, R.A., *The Rat Brain: A Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem*, Krieger, New York, 1963.

Kosterlitz, H.W. & McKnight, A.T. (1981) Opioid peptides and sensory function, *Progress in Sensory Physiology*, **1**, 31-95.

Kosterlitz, H.W., Paterson, S.J. & Robson, L.E. (1981) Characterization of the *k*-subtype of the opiate receptor in the guinea-pig brain, *British Journal of Pharmacology*, **73**, 939-949.

Kraft, S.A., Larson Jr., C.P., Shuer, L.M., Steinberg, G.K., Benson, G.V. & Pearl, R.G. (1990) Effect of hyperglycemia on neuronal changes in a rabbit model of focal cerebral ischemia, *Stroke*, **21**, 447-450.

Kurtzke, J.F. (1985) Epidemiology of cerebrovascular disease. In: McDowell, F.H. & Caplow, L.R. (eds.) *Cerebrovascular Survey Report*, NINCDS, NIH Bethesda, MD, pp.1-34.

Kurumaji, A., Dewar, D. & McCulloch, J. (1993) Metabolic mapping with deoxyglucose autoradiography as an approach for assessing drug action in the central nervous system, in *Imaging Drug Action in the Brain*, Ed. E.D. London, CRC Press, pp.207-246.

Kurumaji, A., Nehls, D.G., Park, C.K. & McCulloch, J. (1989) Effects of NMDA antagonists, MK-801 and CPP, upon local cerebral glucose use, *Brain Research*, **496**, 268-284.

Kuschinsky, W. & Wahl, M. (1978) Local chemical and neurogenic regulation of cerebral vascular resistance, *Physiology Reviews*, **58**, 656-689.

Kusumoto, K., Mackay, K.B. & McCulloch, J. (1992) The effect of the kappa-opioid receptor agonist CI-977 in a rat model of focal cerebral ischaemia, *Brain Research*, **576**, 147-151.

Lahti, R.A., Mickelson, M.M., McCall, J.M. & Von Voigtlander, P.F. (1985) [^{3}H]U-69593 a highly selective ligand for the opioid *k* receptor, *European Journal of Pharmacology*, **109**, 281-284.

Lambert, P.D., Woodruff, G.N., Hughes, J. & Hunter, J.C. (1991) Inhibition of L-glutamate release: A possible mechanism of action for the neuroprotective effects of the *k*-selective agonist CI-977, *Molecular Neuropharmacology*, **1**, 77-82.

Landolt, H., Bullock, R., Fujisawa, H., McCulloch, J. & Miller, S. (1993) Glutamate diffusion characteristics determine neurotoxicity in the rat brain: 14C glutamate autoradiography, *Journal of Cerebral Blood Flow and Metabolism*, **13**, S753.

Lanier, W.L., Perkins, W.J., Karlsson, B.R., Milde, J.H., Scheithauer, B.W., Shearman, G.T. & Michenfelder, J.D. (1990) The effects of dizocilpine maleate (MK-801), an antagonist of the N-methyl-D-aspartate receptor, on neurological recovery and histopathology following complete cerebral ischaemia in primates, *Journal of Cerebral Blood Flow and Metabolism*, **10**, 252-261.

Lanier, W.L., Christopher, T.J., Scheithauer, B.W., Milde, J.H., Hofer, R.E. & Michenfelder, J.D. (1993) Post-ischemic treatment with NBQX does not improve outcome in a canine model of complete cerebral ischemia, *Journal of Cerebral Blood Flow and Metabolism*, **13** (Suppl. 1), S701.

Le Peillet, E., Arvin, B., Moncada. C. & Meldrum, B.S. (1992) The non-NMDA antagonists, NBQX and GYKI 52466, protect against cortical and striatal cell loss following transient global ischaemia in the rat, *Brain Research*, **571**, 115-120.

Leach, M.J., Swan, J.H., Eisenthal, D., Dopson, M. & Nobbs, M. (1993) BW619C89, a glutamate release inhibitor, protects against focal cerebral ischemic damage, *Stroke*, **24**. 1063-1067.

Lehmann, J., Hutchison, A.J., McPherson, S.E., Mondadori, C., Schmutz, M., Sinton, C.M., Tsai, C., Murphy, D.E., Steel, D.J., Williams, M., Cheyney, D.L. & Wood, P.L. (1988) CGS 19755, a selective and competitive N-methyl-D-aspartate-type excitatory amino acid receptor antagonist, *Journal of Pharmacology and Experimental Therapeutics*, **246**, 65-76.

Leighton, G.E., Johnson, M.A., Meechan, K.G., Hill, R.G. & Hughes, J. (1987) Pharmacological profile of PD117302, a selective *k*-opioid agonist, *British Journal of Pharmacology*, **92**, 915-922.

Lekieffre, D. & Meldrum, B.S. (1993) The pyramidine-derivative, BW1003C87, protects CA1 and striatal neurons following transient severe forebrain ischaemia in rats. A microdialysis and histological study, *Neuroscience*, **56**, 93-99.

Lemasters, J.J., Diguiseppi, J., Nieminen, A.L. & Herman, B. (1987) Blebbing, free Ca^{2+} and mitochondrial membrane potential preceding cell death in hepatocytes, *Nature*, **325**, 78-81.

Levy, D.E. & Brierley, J.B. (1974) Communications between vertebrobasilar and carotid arterial circulations in the gerbil, *Experimental Neurology*, **45**, 503-508.

Levy, R., Fenstel, P., Severinghaus, J. & Hosobuchi, Y. (1982) Effect of naloxone on neurologic deficit and cortical blood flow during focal cerebral ischemia in cats, *Life Sciences*, **31**, 2205-2208.

Li, H. & Buchan, A.M. (1993) Treatment with an AMPA antagonist 12 hours following severe normothemic forebrain ischemia prevents CA_1 neuronal injury, *Journal of Cerebral Blood Flow and Metabolism*, **13**, 933-939.

Lindroth, P. & Mopper, K. (1979) High performance liquid chromatographic determination of subpicomole amounts of amino acid by precolumn fluorescence derivatization with δ-pthaldialdehyde, *Analytical Chemistry*, **51**, 1667-1674.

Lipton, S.A. & Rosenberg, P.A. (1994) Excitatory amino acids as a final common pathway for neurologic disorders, *The New England Journal of Medicine*, **330**, 613-622.

Little, J.R. (1978) Modification of acute focal ischemia by treatment with mannitol, *Stroke*, **9**, 4-9.

Liu, Y., Kato, H., Nakata, N. & Kogure, K. (1992) Protection of rat hippocampus against ischemic neuronal damage by pretreatment with sublethal ischemia, *Brain Research*, 586, 121-124.

Lo, E.H. & Steinberg, G.K. (1991) Effects of dextromethorphan on regional cerebral blood flow in focal cerebral ischemia, *Journal of Cerebral Blood Flow and Metabolism*, **11**, 803-809.

Lobato, R., Sarabia, R., Cordobes, F., Rivas, J., Adrados, A., Cargrere, A., Bombez, P., Madera, A. & Lamas, E. (1988) Post-traumatic cerebral hemispheric swelling, *Journal of Neurosurgery*, **68**, 417-423.

Lord, J.A.H., Waterfield, A.A., Hughes, J. & Kosterlitz, H.W. (1977) Endogenous opioid peptides: multiple agonists and receptors, *Nature*, **267**, 495-499.

Lucas, D.R. & Newhouse, J.P. (1957) The toxic effect of sodium L-glutamate on the inner layers of the retina, *Archives of Opthalmology*, **158**, 193-201.

Luersson, T., Klauber, M. & Marshall, L. (1988) Outcome from head injury related to patient's age, *Journal of Neurosurgery*, **68**, 409-416.

MacDermott, A.B. & Dale, N. (1987) Receptors, ion channels and synaptic potentials underlying the integrative actions of excitatory amino acids, *Trends in Neuroscience*, **10**, 280-283.

Mackay, K.B., Dewar, D. & McCulloch, J. (1994) Kappa-1 opioid receptors of the temporal cortex are preserved in Alzheimer's disease, *Journal of Neural Transmission*, **7**, 73-79.

Macrae, I.M., Robinson, M.J., Graham D.I., Reid, J.L. & McCulloch, J. (1993) Endothelin-1-induced reductions in cerebral blood flow: dose dependency, time course and neuropathological consequences, *Journal of Cerebral Blood Flow and Metabolism*, **13**, 276-284.

Mahieux, G. & Benabid, A.L. (1987) Naloxone-reversible analgesia induced by electrical stimulation of the habenula in the rat, *Brain Research*, **406**, 118-129.

Malmgren, R., Bamford, J., Warlow, C., Sandercock, P. & Slattery, J. (1989) Projecting the number of patients with first ever strokes and patients newly handicapped by stroke in England and Wales, *British Medical Journal*, **298**, 656-660.

Manev, H., Favaron, M., Guidotti, A. & Costa, E. (1989) Delayed increase of Ca^{2+} influx elicited by glutamate role in neuronal death, *Molecular Pharmacology*, **36**, 106-112.

Mannalack, D.T., Beart, P.M. & Gundlach, A.L. (1986) Psychomimetic σ-opiates and PCP, *Trends in Pharmacological Sciences*, **7**, 448-451.

Mansour, A., Khachaturian, H., Lewis, M.E., Akil, H. and Watson, S.J. (1987) Autoradiographic differentiation of mu, delta and kappa opioid receptors in the rat forebrain and midbrain, *Journal of Neuroscience*, **7**, 1445-2464.

Mansour, A., Khachaturian, H., Lewis, M.E., Akil, H. & Watson, S.J. (1988) Anatomy of CNS opioid receptors, *Trends in Neurosciences*, **11**, 308-314.

Marie, C. & Bralet, J. (1991) Blood glucose level and morphological brain damage following cerebral ischemia, *Cerebrovascular and Brain Metabolism Reviews*, **3**, 29-38.

Marcoux, F.W., Goodrich, J.E. & Dominick, M.A. (1988) Ketamine prevents ischemic neuronal injury, *Brain Research*, **452**, 329-335.

Marshall, L.F., Smith, R.W., Rauscher, L.A. & Shapiro, H.M. (1978) Mannitol dose requirements in brain-injured patients, *Journal of Neurosurgery*, **48**, 169-172.

Martin, W.R. (1967) Opioid antagonists, *Pharmacological Reviews*, **19**, 463-521.

Martin, W.R. (1984) Pharmacology of opioids, *Pharmacological Reviews*, **35**, 283-323.

Martin, W.R., Eades, C.G., Gilbert, P.E. & Thompson, J.A. (1980) Tolerance to and physical dependence on *N*-allylnormetazocine (NANM) in chronic spinal dogs, *Substance of Alcohol Actions/Misuse*, **1**, 269-273.

Martin, W.R., Eades, C.G., Thompson, J.A., Huppler, R.E. & Gilbert, P.E. (1976) The effects of morphine- and nalorphine-like drugs in non-dependent and morphine-dependent chronic spinal dogs, *Journal of Pharmacology and Experimental Therapeutics*, **197**, 517-532.

Matsumoto, K., Graf, R., Rosner, G., Shimada, N. & Heiss, W.-D. (1992) Flow thresholds for extracellular purine catabolite elevation in cat focal ischemia, *Brain Research*, **579**, 309-314.

Matsumoto, K., Graf, R., Rosner, G., Taguchi, J. & Heiss, W.-D. (1993) Elevation of neuroactive substances in the cortex of cats during prolonged focal ischemia, *Journal of Cerebral Blood Flow and Metabolism*, **13**, 586-594.

Mayer, D.L., Wolfe, T.L., Akil, H., Carder, B. & Liebeskind, J.C. (1971) Analgesia from electrical stimulation in the brainstem of the rat, *Science*, **174**, 1351-1354.

Mayer, M.L. & Westbrook, G.L. (1987) The physiology of excitatory amino acids in the vertebrate central nervous system, *Progress in Neurobiology*, **28**, 197-276.

McCabe, R.T., Wasterlain, C.B. Kucharczyk, N., Sofia, R.D. & Vogel, J.R. (1993) Evidence for anticonvulsant and neuroprotectant actions of felbamate mediated by strychnine-insensitive glycine receptors, *Journal of Pharmacology and Experimental Therapeutics*, **254**, 1248-1252.

McCulloch, J. (1982) Mapping functional alterations in the CNS with [^{14}C]deoxyglucose. In: *Handbook of Psychopharmacology*, Vol. 15, eds. Iversen, S., Iversen, L.L., Snyder, S.H., New York, Plenum, pp.321-410.

McCulloch, J. (1992) Excitatory amino acid antagonists and their potential for the treatment of ischaemic brain damage in man. *British Journal of Clinical Pharmacology*, **34**, 106-114.

McCulloch, J., Bullock, R. & Teasdale, G.M. (1991) Excitatory amino acid antagonists: opportunities for the treatment of ischaemic brain damage in man; In *Excitatory Amino Acid Antagonists*, ed. Meldrum, B.S, Blackwell, pp.287-325.

McCulloch, J. & Iversen, L.L. (1991) Autoradiographic assessment of the effects of N-methyl-D-aspartate (NMDA) receptor antagonists in vivo, *Neurochemical Research*, **16**, 951-963.

McCulloch, J., Kelly, P.A.T. & Ford, I. (1982) Effect of apomorphine on the relationship between local cerebral glucose utilization and local cerebral blood flow (with an appendix on its statistical analysis), *Journal of Cerebral Blood Flow and Metabolism*, 2, 487-499.

McCulloch, J., Savaki, H.E. & Sokoloff, L. (1980) Influence of dopaminergic systems on the lateral habenular nucleus of the rat, *Brain Research*, **194**, 177-124.

McFadzean, I., Lacey, M.G., Hill, R.G. & Henderson, G. (1987) Kappa opioid receptor activation depresses synaptic input to rat locus coeruleus neurons *in vitro*, *Neuroscience*, **20**, 231-239.

McGinty, J.F., Henriksen, S.J., Goldstein, A., Terenius, L. & Bloom, F.E. (1983) Dynorphin is contained within hippocampal mossy fibers: immunochemical alterations after kainic acid administration and colchicine-induced neurotoxicity, *Proceedings of the National Academy of Sciences, U.S.A.*, **80**, 589-593.

McKnight, A.T. & Rees, D.C. (1991) Opioid receptors and their ligands, *Neurotransmissions*, **VII**, 1-6.

McKnight, A.T., Hughes, J. & Kosterlitz, H.W. (1979) Synthesis of enkephalins by guinea-pig striatum *in vitro*, *Proceedings of the Royal Society, London*, **206**, 199-207.

McLean, S., Rothman, R., Jacobsen, A., Rice, K. & Herkenham, M. (1987) Distribution of opiate receptor subtypes and enkephalin and dynorphin immunoreactivity in the hippocampus of squirrel, guinea pig, rat, and hamster, *Journal of Comparative Neurology*, 255, 497-510.

McMahon, H.T. & Nicholls, D.G. (1990) Glutamine and aspartate loading of synaptosomes: A re-evaluation of effects on calcium-dependent excitatory amino acid release, *Journal of Neurochemistry*, **54**, 373-380.

Meldrum, B. (1990) Protection against ischaemic neuronal damage by drugs acting on excitatory neurotransmission, *Cerebrovascular and Brain Metabolism Reviews*, 2, 27-57.

Meldrum, B.S., Swan, J.H., Leach, M.J., Millan, M.H., Gwinn, R., Kadota, K., Graham, S.H., Chen, J. & Simon, R.P. (1992) Reduction of glutamate release and protection against ischemic brain damage by BW 1003C87, *Brain Research*, **593**, 1-6.

Melloni, E. & Pontremoli, S. (1989) The calpains, *Trends in Neuroscience*, **12**, 438-444.

Memezawa, H., Smith, M-L. & Siesjö, B.K. (1992) Penumbral tissues salvaged by reperfusion following middle cerebral artery occlusiaon in rats, *Stroke*, **23**, 552-559.

Michenfelder, J.D., Lanier, W., Scheithauer, B.W., Perkins, W.J., Shearman, G.T. & Milde, J.H. (1989) Evaluation of the glutamate antagonist dizocilpine maleate (MK-801) on neurologic outcome in a canine model of complete cerebral ischaemia: correlation with hippocampal histopathology, *Brain Research*, **481**, 228-234.

Mies, G., Ishimaru, S., Xie, Y., Seo, K. & Hossmann, K.-A. (1991) Ischaemic thresholds of cerebral protein synthesis and energy state following middle cerebral artery occlusion in the rat, *Journal of Cerebral Blood Flow and Metabolism*, **11**, 753-761.

Millan, M.J. (1990) *k*-Opioid receptors and analgesia, *Trends in Pharmacological Sciences*, **11**, 70-76.

Miller, R.J. (1991) The control of neuronal Ca^{2+} homoeostasis, *Progress in Neurobiology*, **37**, 255-285.

Minanisawa, H., Nordström, C.-H., Smith, M.-L. & Siesjö, B.K. (1990) The influence of mild body and brain hypothermia in ischaemic brain damage, *Journal of Cerebral Blood Flow and Metabolism*, **10**, 365-374.

Minematsu, K. & Fisher, M. (1993) MK-801 reduces extensive infarction after suture of middle cerebral artery occlusion in rats, *Cerebrovascular Disease*, **3.** 99-104.

Minematsu, K., Fisher, M., Li, L., Davis, M.A., Knapp, A.G., Cotter, R.E., McBurney, R.N. & Sotak, C.H. (1993) Effects of a novel NMDA antagonist on experimental stroke rapidly and quantitatively assessed by diffusion-weighted MRI, *Neurology*, **43**, 397-403.

Miyaoka, M., Shinohara, M., Batipps, M., Pettigrew, K.D., Kennedy, D. & Sokoloff, L. (1979) The relationship between the intensity of the stimulus and the metabolic response in the visual system of the rat, *Acta Neurologica Scandinavica*, **60, 16-17.**

Mohamed, A.A., Gotoh, O., Graham, D.I., Osborne, K.A., McCulloch, J., Mendelow, A.D., Teasdale, G.M. & Harper, A.M. (1985) Effect of pretreatment with the calcium antagonist nimodipine on local cerebral blood flow and histopathology after middle cerebral artery occlusion, *Annals of Neurology*, **18**, 705-711.

Mohr, J.P., Caplan, L.R., Melski, J.W., Goldstein, R.J., Duncan, G.W., Kistler, J.P., Pessin, M.S. & Bleich, H.L. (1978) The Harvard Cooperative Stroke Registry: A prospective registry, *Neurology*, **28**, 754-762.

Monaghan, D.T., Bridge, R.J. & Cotman, C.W. (1989) The excitatory amino acid receptors: their classes, pharmacology, distinct properties ain the function of the central nervous system, *Annual Review of Pharmacology and Toxicology*, **29**, 365-402.

Morikawa, E., Ginsberg, M.D., Dietrich, W.D., Duncan, R.C., Kraydieh, S., Globus, M.Y-T. & Busto, R. (1992) The significance of brain temperature in focal cerebral ischemia: Histopathological consequences of middle cerebral artery occlusion in the rat, *Journal of Cerebral Blood Flow and Metabolism*, **12**, 380-389.

Morris, R.G.M., Anderson, E., Lynch, G.S. & Baudry, M. (1986) Selective impairment of learning and blockade of long-term potentiation by an N-methy-D-aspartate receptor antagonist, AP5, *Nature*, **319**, 774-776.

Moskowitz, M.A., Saito, K., Brezina, L. & Dickson, J. (1987) Nerve fibres surrounding intracranial and extracranial vessels from human and other species contain dynorphin-like immunoreactivity, *Neuroscience*, **23**, 731-737.

Moyer, D.J., Welsh, F.A. & Zager, E.L. (1992) Spontaneous cerebral hypothermia diminishes focal infarction in rat brain, *Stroke*, **23**, 1812-1816.

Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A.C.Y., Cohen, S.N. & Numa, S. (1979) Nucleotide sequence of cloned cDNA for bovine corticotropin-betalipotropin precursor, *Nature*, **278**, 423-427.

Nakanishi, S., Teranishi, Y., Watanabe, Y., Notake, M., Noda, M., Kakidani, H., Jingami, H. & Numa, S. (1981) Isolation and characterization of the bovine corticotropin/betalipotropin precursor gene, *European Journal of Biochemistry*, **115**, 429-438.

Nedergaard, M. (1988) Mechanisms of brain damage in focal cerebral ischaemia, *Acta Neurologica Scandinavia*, **77**, 81-101.

Nedergaard, M. & Astrup, J. (1986) Infarct rim: Effect of hyperglycemia on direct current potential and [^{14}C]2-deoxyglucose phosphorylation, *Journal of Cerebral Blood Flow and Metabolism*, **6**, 607-615.

Nedergaard, M. & Diemer, N.H. (1987) Focal ischaemia in the rat, with special reference to the influence of plasma glucose concentration, *Acta Neuropathologica (Berl.)*, **73**, 131-137.

Nedergaard, M. & Hansen, A.J. (1993) Characterization of cortical depolarizations evoked in focal cerebral ischaemia, *Journal of Cerebral Blood Flow and Metabolism*, **13**, 568-574.

Nehlig, A., Lucignani, G., Kadekaro, M., Porrino, L.J. and Sokoloff, L. (1984) Effects of acute administration of caffeine on local cerebral glucose utilization in the rat, *European Journal of Pharmacology*, **101**, 91-100.

Nehls, D.G., Kurumaji, A., Park, C.K. & McCulloch, J. (1988) Differential effects of competitive and non-competitive N-methyl-D-aspartate antagonists on glucose use in the limbic system, *Neuroscience Letters*, **91**, 204-210.

Nehls, D.G., Park, C.K., MacCormack A.G. & McCulloch, J. (1990) The effects of *N*-methyl-D-aspartate receptor blockade with MK-801 upon the relationship between cerebral blood flow and glucose utilisation, *Brain Research*, **511**, 271-279.

Nellgård, B., Gustafson, I. & Wieloch, T. (1991) Lack of protection by the N-methyl-D-aspartate receptor blocker dizocilpine (MK-801) following transient severe cerebral ischemia in the rat, *Anesthesiology*, **75**, 279-287.

Nellgård, B. & Wieloch, T. (1992) Postischemic blockade of AMPA but not NMDA receptors mitigates neuronal damage in the rat brain following transient severe cerebral ischemia, *Journal of Cerebral Blood Flow and Metabolism*, **12**, 2-11.

Nicholls, D. & Attwell, D. (1990) The release and uptake of excitatory amino acids, *Trends in Pharmacological Sciences*, **11**, 462-468.

Nicholson, C., Brugggencate, G.T., Steinberg, R. & Stockle, H. (1977) Calcium modulation in brain extracellular microenvironment demonstrated with ion-selective micropipette, *Proceedings of the National Academy of Sciences, U.S.A.*, **74**, 1287-1290.

Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Hirose, T., Inayama, S., Nakanishi, S. & Numa, S. (1982a) Cloning and sequence analysis of cDNA for bovine adrenal preproenkephalin, *Nature*, 295, 202-206.

Noda, M., Teranishi, Y., Takahashi, H., Toyosato, M., Notake, M., Nakanishi, S. & Numa, S. (1982b) Isolation and structural organization of the human preproenkephalin gene, *Nature*, **297**, 431.

Nordström, C.-H. & Siesjö, B.K. (1978) Effects of phenobarbital in cerebral ischemia. Part I: Cerebral energy metabolism during pronounced incomplete ischemia, *Stroke*, **9**, 327-335.

Nowicki, J-P., Assumel-Lurdin, C., Duverger, D. & MacKenzie, E.T. (1988) Temporal evolution of regional energy metabolism following focal cerebral ischemia in the rat, *Journal of Cerebral Blood Flow and Metabolism*, **8**, 462-473.

O'Brien, M.D. & Waltz, A.G. (1973) Transorbital approach for occluding the middle cerebral artery without craniectomy, *Stroke*, **4**, 201-206.

Olney, J.W. (1969) Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamatea, *Science*, **164**, 719-1290.

Olney, J.W. (1978) Neurotoxicity of excitatory amino acids. In *Kainic acid as a tool in neurobiology*, eds. McGeer, E.G., Olney, J.W. & McGeer, P.L. Raven, New York, pp.37-70.

Olney, J.W. (1985) Excitatory transmitters and epilepsy-related brain damage, *International Reviews of Neurobiology*, **27**, 337-362.

Olney, J.W., Ho, O.L. & Rhee, V. (1971) Cytotoxic effects of acidic and sulphur containing amino acids on the infant mouse central nervous system, *Experimental Brain Research*, **14**, 61-76.

Olney, J.W., Labruyere, J. & Price, M.T. (1989) Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs, *Science*, **244**, 1360-1362.

Olney, J.W., Labruyere, J., Wang, G., Wozniak, D.F., Price, M.T. & Sesma, M.A. (1991) NMDA antagonist neurotoxicity: mechanism and prevention, *Science*, **254**, 1515-1518.

Olson, K.G. & Welch, S.P. (1991) The effects of dynorphin (1-13) and U50,488H on free intracellular calcium in guinea pig cerebellar synaptosomes, *Life Sciences*, **481**, 575-581.

Ohno, K., Pettigrew, K.D. & Rapoport, S.I. (1978) Lower limits of cerebrovascular permeability to non electrolytes in the conscious rat, *American Journal of Physiology*, **235**, H299-H307.

Onodera, H., Sato, G. & Kogure, K. (1986) Lesions to Schaffer collaterals prevent ischaemic death of CA1 pyramidal cells, *Neuroscience Letters*, **68**, 169-174.

Osborne, K.A., Shigeno, T., Balarsky, A.-M., Ford, I., McCulloch, J., Teasdale, G.M. & Graham, D.I. (1987) Quantitative assessment of early brain damage in a rat model of focal cerebral ischaemia, *Journal of Neurology, Neurosurgery and Psychiatry*, **50**, 402-410.

Ozyurt, E., Graham, D.I., Woodruff, G.N. & McCulloch, J. (1988) Protective effect of the glutamate antagonist, MK-801 in focal cerebral ischemia in the cat, *Journal of Cerebral Blood Flow and Metabolism*, **8**, 138-143.

Panter, S., Yum, S.W. & Faden, A.I. (1990) Alteration in extracellular amino acids after traumatic spinal cord injury, *Annals of Neurology*, **27**, 96-1900.

Park, C.K., McCulloch, J., Kang, J.K. & Choi, C.R. (1992) Efficacy of D-CPPene, a competitive N-methyl-D-aspartate antagonist in focal cerebral ischemia in the rat, *Neuroscience Letters*, **147**, 41-44.

Park, C.K., Nehls, D.G., Graham, D.I., Teasdale, G.M. & McCulloch, J. (1988a) Focal cerebral ischaemia in the cat: Treatment with the glutamate antagonist MK-801 after induction of ischemia, *Journal of Cerebral Blood Flow and Metabolism*, **8**, 757-762.

Park, C.K., Nehls, D.G., Graham, D.I., Teasdale, G.M. & McCulloch, J. (1988b) The glutamate antagonist MK-801 reduces focal ischemic brain damage in the rat, *Annals of Neurology*, **24**, 543-551.

Park, C.K., Nehls, D.G., Teasdale, G.M. & McCulloch, J. (1989) Effect of the NMDA antagonist MK-801 on local cerebral blood flow in focal cerebral ischaemia in the rat, *Journal of Cerebral Blood Flow*, **9**, 617-622.

Pasternak, G.W. (1993) Pharmacological mechanisms of opioid analgesics, *Clinical Neuropharmacology*, **16**, 1-18.

Paton, W.D.M. (1957) The action of morphine and related substances on contractions and on ACh output of coaxially stimulated guinea-pig ileum, *British Journal of Pharmacology*, **12**, 119-127.

Paulson, O.B., Strandgaard, S. & Edvinsson, L. (1990) Cerebral autoregulation, *Cerebrovascular and Brain Metabolism Reveiws*, **2**, 161-192.

Paxinos, G. & Watson, C. (1986) *The Rat Brain in Stereotaxic Co-ordinates*, 2nd edition, Academic Press, Sydney.

Pellegrini-Giampietro, D., Zukin, R.S., Bennett, M., Cho, S. & Pulsinelli, W. (1992) Switch in glutamate receptor subunit gene expression in CA1 subfield following global ischemia in rats, *Proceedings of the National Academy of Sciences, U.S.A.*, **89**, 10499-10503.

Persson, L. & Hillered, L. (1992) Chemical monitoring of neurosurgical intensive care patients using intracerebral microdialysis, *Journal of Neurosurgery*, **76**, 72-80.

Peters, G.R., Ward, N.J., Antall, E.G., Lai, P.Y. & De Maar, E.W. (1987) Diuretic actions in man of a selective kappa opioid agonist: U-62,066E, *Journal of Pharmacology and Experimental Therapeutics*, **240**, 128-131.

Petito, C.K., Feldmann, E., Pulsinelli, W.A. & Plum, F. (1987) Delayed hippocampal damage in humans following cardiorespiratory arrest, *Neurology*, **37**, 1281-1286.

Pfeiffer, A., Brantl, V., Herz, A. & Emrich, H.M. (1986) Psychotomimesis mediated by *k*-opiate receptors, *Science*, **233**, 774-776.

Pfeiffer, A. & Herz, A. (1982) Discrimination of 3 opiate binding sites with the use of a computerised curve fitting technique, *Analytical Biochemistry*, **21**, 266-271.

Pfeiffer, A., Pasi, A., Mehraein, P. & Herz, A. (1982) Opiate receptor binding sites in human brain, *Brain Research*, **248**, 87-96.

Pickard, J.D., Murray, G.D., Illingworth, R., Shaw, M.D.M., Teasdale, D.M., Foy, P.M., Humphrey, P.R.D., Lang, D.A., Nelson, R., Richards, R., Sinar, J., Bailey, S. & Skene, A. (1989) Effect of oral nimodipine on cerebral infarction and outcome after subarachnoid haemorrhage: British aneurysm nimodipine trial, *British Medical Journal*, **298**, 636-642.

Pinnock, R.D. (1992) A highly selective *k*-opioid receptor agonist, CI-977, reduces excitatory synaptic potentials in the rat locus coeruleus *in vitro*, *Neuroscience*, **47**, 87-94.

Portoghese, P.S. (1965) A new concept in the mode of interaction of narcotic analgesics with receptors, *Journal of Medical Chemistry*, **8**, 609-616.

Portoghese, P.S., Lipkowski, A.W. & Takemori, A.E. (1987) Binaltorphimine and nor-binaltorphimine, potent and selective *k*-opioid receptor antagonists, *Life Sciences*, **40**, 1287-1292.

Prado, R., Ginsberg, M.D., Dietrich, W.D., Watson, B.D. & Busto, R. (1988) Hyperglycemia increases infarct size in collaterally perfused but not end-arterial vascular territories, *Journal of Cerebral Blood Flow and Metabolism*, **8**, 186-192.

Pratt, J., Rataud, J., Bardot, F., Roux, M., Blanchard, J-C., Laduron, P.M. & Stutzmann, J-M. (1992) Neuroprotective actions of riluzole in rodent models of global and focal cerebral ischaemia, *Neuroscience Letters*, **140**, 225-230.

Pulsinelli, W.A. & Brierley, J.B. (1979) A new model of bilateral hemispheric ischemia in the unanaesthetized rat, *Stroke*, **10**, 267-272.

Pulsinelli, W.A., Brierley, J.B. & Plum, F. (1982) Temporal profile of neuronal damage in a model of transient forebrain ischemia, *Annals of Neurology*, **11**, 491-498.

Pulsinelli, W.A. & Duffy, T.E. (1983) Regional energy balance in rat brain after transient forebrain ischemia, *Journal of Neurochemistry*, **40**, 1500-1503.

Quirion, R., Chicheportiche, R., Contreras, P.C., Johnson, K.M., Lodge, D., Tam, S.W., Woods, J.H. & Zukin, S.R. (1987) Classification and nomenclature of phencyclidine and sigma receptor sites, *Trends in Neuroscience*, **11**, 444-446.

Recio, L., Marin, J., Reviriego, J., Salaices, M. & Lopez-Rico, M. (1986) Effect of morphine on the cat middle cerebral artery, *Brain Research*, **376**, 262-267.

Rees, D.C. (1992) Chemical structures and biological activities of non-peptide selective kappa opioid ligands; In *Progress in Medicinal Chemistry*, Vol. 29, eds. Ellis, G.P. & Luscombe, D.K., Elsevier Science Publishers B.V., pp.109-139.

Reinoso-Suarez, F. (1961) *Topograpischer Hirnatlas der Katze, fur experimental-physiologische Untersuchungen.* Darmstadt: E. Merck.

Ridenour, T.R., Warner, D.S., Todd, M.M. & McAllister, A.C. (1992) Mild hypothermia reduces infarct size resulting from temporary but not permanent focal ischemia in rats, *Stroke*, **23**, 733-738.

Robinson, R.G., Shoemaker, W.J., Schlumpf, M., Valk, T. & Bloom, F.E. (1975) Experimental early infarction in rat brain: effect on catecholamines and behaviour, *Nature*, 255, 332-334.

Rockett, I.R. & Smith, G.S. (1987) Injuries in relation to chronic disease: an international view of premature mortality, *American Journal of Public Health*, **77**, 1345-1346.

Rod, M.R. & Auer, R.N. (1989) Pre- and post-ischemic administration of dizocilpine (MK-801) reduces cerebral necrosis in the rat, *Canadian Journal of Neurological Sciences*, **16**, 340-344.

Roman, R., Bartkowski, H. & Simon, R. (1989) The specific NMDA receptor antagonist AP-7 attenuates focal ischaemic brain injury, *Neuroscience Letters*, **104**, 19-24.

Rothman, S.M. & Olney, J.W. (1986) Glutamate and the pathophysiology of hypoxic-ischemic brain damage, *Annals of Neurology*, **19**, 105-111.

Roussel, S., Pinard, E. & Seylaz, J. (1990) Kynurenate does not reduce infarct size after middle cerebral artery occlusion in spontaneously hypertensive rats, *Brain Research*, **518**, 353-355.

Roussel, S., Pinard, E. & Seylaz, J. (1992) Effect of MK-801 on focal brain infarction in normotensive and hypertensive rats, *Journal of Hypertension*, **19**, 40-46.

Rudolphi, K.A., Schubert, P., Parkinson, F.E. & Fredholm, B.B. (1992) Adenosine and brain ischaemia, *Cerebrovascular Brain Metabolism Reviews*, **4**, 346-369.

Sakurada, O., Kennedy, C., Jehle, J., Brown, J.D., Carbin, G.L., Sokoloff, L. (1978) Measurement of local cerebral blood flow with iodo[^{14}C]antipyrine, *American Journal of Physiology*, **234**, H59-H66.

Sasaki, T., Kassell, N.F., Turner, D.M., Maixner, W., Torner, J. & Coester, H.C. (1984) Effects of naloxone on canine vascular smooth muscle, *Journal of Cerebral Blood Flow and Metabolism*, **4**, 166-172.

Sato, M., Mortia, Y., Seika, T., Fujita, M., Ohhata, K. & Tohyama, M. (1991) Localization and ontogeny of cells expressing preprodynorphin mRNA in the rat cerebral cortex, *Brain Research*, **541**, 41-49.

Sauer, D., Nuglisch, J., Rossberg, C., Mennel, H.-D., Beck, T., Bielenberg, G.W. & Krieglstein, J. (1988) Phencyclidine reduces postischaemic neuronal necrosis in rat hippocampus without changing blood flow, *Neuroscience Letters*, **91**, 327-332.

Sauer, D., Allegrini, P.R., Cosenti, A., Pataki, A., Amacker, H. & Fagg, G.E. (1993) Characterization of the cerebroprotective efficacy of the competitive NMDA receptor antagonist CGP40116 in a rat model of focal cerebral ischemia: an in vivo magnetic resonance imaging study, *Journal of Cerebral Blood Flow and Metabolism*, **13**, 595-602.

Scatton, B., Cudennec, A., Duverger, D., MacKenzie, E.T., Nowicki, J.P. & Zivkovic, B. (1991) Effect of SL 82.0715, an NMDA receptor antagonist acting at the polyamine modulatory site, on local cerebral glucose use in the rat brain, *Journal of Cerebral Blood Flow and Metabolism*, **11**, S312.

Schaumann, W. (1957) Inhibition by morphine of the release of ACh from the intestine of the guinea-pig, *British Journal of Pharmacology*, **12**, 115-118.

Schoepp, D., Bockaert, J. & Sladeczek, F. (1990) Pharmacological and functional characteristics of metabotropic excitatory amino acid receptors, *Trends in Pharmacological Sciences*, **11**, 508-515.

Seif el Nasr, M., Peruche, B., Rossberg, C., Mennel, H.-D. & Krieglstein, J. (1990) Neuroprotective effect of memantine demonstrated in vivo and in vitro, *European Journal of Pharmacology*, **185**, 19-24.

Sharif, N.A., Durie, E., Michel, A.D. & Whiting, R.L. (1990) Dog cerebral cortex contains μ, σ- and *k*-opioid receptors at different densities: apparent lack of evidence for subtypes of the *k*-receptor using selective radioligands, *Brain Research*, **510**, 108-114.

Sharp, F.R., Butman, M., Wang, S., Koistinaho, J., Graham, S.M., Sagar, S.M., Noble, L., Berger, P. & Longo, F.M. (1992) Haloperidol prevents induction of the hsp70 heat shock gene in neurons injured by phencyclidine (PCP), MK-801, and ketamine, *Journal of Neuroscience Research*, **33**, 605-616.

Sharp, F.R., Jasper, P., Hall, J., Noble, L. & Sagar, S. (1991) MK-801 and ketamine induce heat shock protein HSP72 in injured neurones in posterior cingulate and retrosplenial cortex, *Annals of Neurology*, **30**, 801-809.

Sharp, F.R., Kaver, J.S. & Shepherd, G.M. (1975) Local sites of activity-related glucose metabolism in rat olfactory bulb during olfactory stimulation, *Brain Research*, **98**, 596-600.

Sheardown, M.J., Nielsen, E.Ø., Hansen, A.J., Jacobsen, P. & Honoré, T. (1990) 2,3-Dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline: A neuroprotectant for cerebral ischemia, *Science*, **247**, 571-574.

Sheardown, M.J., Suzdak, P.D. & Nordholm, L. (1993) AMPA, but not NMDA, receptor antagonism is neuroprotective in gerbil global ischaemia, even when delayed 24h, *European Journal of Pharmacology*, **236**, 347-353.

Shigeno, T., McCulloch, J., Graham, D.I., Mendelow, A.D. & Teasdale, G.M. (1985a) Pure cortical ischaemia versus striatal ischaemia. Circulatory, metabolic, and neuropathologic consequences, *Surgical Neurology*, **24**, 47-51.

Shigeno, T., Teasdale, G.M., McCulloch, J. & Graham, D.I. (1985b) Recirculation model following MCA occlusion in rats, *Journal of Neurosurgery*, **63**, 272-277.

Shimada, N., Graf, R., Rosner, G. & Heiss, W.-D. (1990) Differences in ischemia-induced accumulation of amino acids in the cat cortex, *Stroke*, **21**, 1445-1451.

Shimada, N., Graf, R., Rosner, G., Wakayama, A., George, C.P. & Heiss, W.-D. (1989) Ischemic flow threshold for extracellular glutamate increase in cat cortex, *Journal of Cerebral Blood Flow and Metabolism*, **9**, 603-606.

Siesjö, B.K. (1981) Cell damage in the brain: a speculative synthesis, *Journal of Cerebral Blood Flow and Metabolism*, **1**, 155-185.

Siesjö, B.K. (1988a) Acidosis and ischemic brain damage, *Neurochemical Pathology*, **9**, 31-88.

Siesjö, B.K. (1988b) Historical overview. Calcium, ischemia, and death of brain cells, *Annals of the New York Academy of Sciences*, **522**, 638-661.

Siesjö, B.K. (1992a) Pathophysiology and treatment of focal cerebral ischemia, Part I: Pathophysiology, *Journal of Neurosurgery*, **77**, 169-184.

Siesjö, B.K. (1992b) Pathophysiology and treatment of focal cerebral ischemia, Part II: Mechanisms of damage and treatment, *Journal of Neurosurgery*, **77**, 337-354.

Siesjö, B.K. & Bengtsson, F. (1989) Calcium fluxes, calcium antagonists, and calcium-related pathology in brain ischemia, hypoglycemia, and spreading depression, a unifying hypothesis, *Journal of Cerebral Blood Flow and Metabolism*, **9**, 127-140.

Sikes, R.W. & Vogt, B.A. (1992) Nociceptive neurons in area 24 of rabbit cingulate cortex, *Journal of Neurophysiology*, **68**, 1720-1732.

Silvia, R.C., Slizgi, G.R., Ludens, J.H. & Tang, A.H. (1987) Protection from ischemia-induced cerebral edema in the rat by U-50488H, a kappa opioid receptor agonist, *Brain Research*, **403**, 52-57.

Silvia, R.C. & Tang, A.H. (1986) Protection of ischemic induced cerebral edema by the kappa opioid agonist U-50488; In *Pharmacology of Cerebral Ischemia*, ed. Krieglestein, J., Elsevier, Amsterdam, pp.381-384.

Simon, R.P., Niiro, M. & Gwinn, R. (1993) Prior ischemic stress protects against experimental stroke, *Neuroscience Letters*, **163**, 135-137.

Simon, R., Swan, J., Griffith, T. & Meldrum, B. (1984) Calcium overload in selectively vulnerable neurons of the hippocampus during and after ischaemia: an electron microscopy study in the rat, *Journal of Cerebral Blood Flow and Metabolism*, **4**, 350-361.

Simon, R. & Shiraishi, K. (1990) N-methyl-D-aspartate antagonist reduces stroke size and regional glucose metabolism, *Annals of Neurology*, **27**, 606-611.

Singh, L., Donald, A.E., Foster, A.C., Hutson, P.H., Iversen, L.L., Iversen, S.D., Kemp, J.A., Leeson, P.D., Marshall, G.R., Oles, R.J., Priestly, T., Thorn, L., Tricklebank, M.D., Vass, C.A. & Williams, B.J. (1990a) Enantiomers of HA-966 (3-amino-1-hydropryrrolid-2-one) exhibit distinct central nervous system effects: (+)-HA-966 is a selective glycine; N-methyl-D-aspartate receptor antagonist, but (-)-HA-966 is a potent γ-butyrolactone-like sedative, *Proceedings of the National Academy of Sciences, U.S.A.*, **87**, 347-351.

Singh, L., Vass, C.A., Hunter, J.C., Woodruff, G.N. & Hughes, J. (1990b) The anti-convulsant action of CI-977, a selective *k*-opioid receptor agonist: a possible involvement of the glycine/NMDA receptor complex, *European Journal of Pharmacology*, **191**, 474-480.

Slizgi, G.R., Taylor, C.J. & Ludens, J.H. (1984) Effects of the highly selective *kappa* opioid, U-50,488, on renal function in the anaesthetized dog, *Journal of Pharmacology and Experimental Therapeutics*, **230**, 641-645.

Smith, M.-L., Auer, R.N. & Siesjö, B.K. (1984) The density and distribution of ischemic brain injury in the rat following 2-10 min of forebrain ischemia, *Acta Neuropathologica*, **64**, 319-332.

Smith, S.E. & Meldrum, B.S. (1992) Cerebroprotective effect of a non-N-methyl-D-aspartate antagonist, GYKI 52466, after focal ischemia in the rat, *Stroke*, **23**, 861-864.

Sokoloff, L. (1977) Relation between physiological function and energy metabolism in the central nervous system, *Journal of Neurochemistry*, **29**, 13-26.

Sokoloff, L. (1981) Localization of functional activity in the central nervous system by measurement of glucose utilization with radioactive deoxyglucose, *Journal of Cerebral Blood Flow and Metabolism*, **1**, 7-36.

Sokoloff, L., Reivich, M., Kennedy, C., Des Rosiers, M.H., Patlak, C.S., Pettigrew, K.D., Sakurada, O. & Shinohara, M. (1977) The [^{14}C]deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetised albino rat, *Journal of Neurochemistry*, **28**, 897-916.

Spedding, M. & Paoletti, R. (1992) Classification of calcium channels and the sites of action of drugs modifying channel function, *Pharmacological Reviews*, **44**, 363-376.

Sterz, F., Leonov, Y., Safer, P., Radovsky, A., Stezoski, W., Reich, H., Shearman, G.T. & Greber, T.F. (1989) Effect of excitatory amino acid blocker MK-801 on overall neurologic and morphologic outcome after prolonged cardiac arrest in dogs, *Anesthesiology*, **71**, 907-918.

Strong, A.J., Venables, G.S. & Gibson, G. (1983) The cortical ischaemic penumbra associated with occlusion of the middle cerebral artery in the cat: 1. Topography of changes in blood flow, potassium ion activity and EEG, *Journal of Cerebral Blood Flow and Metabolism*, **3**, 86-96.

Su, T.-P. (1982) Evidence for sigma opioid receptor: binding of [^{3}H]SKF-10047 to etorphine-inaccessible sites in guinea-pig brain, *Journal of Pharmacology and Experimental Therapeutics*, **223**, 284-290.

Sutherland, R.J. (1982) The dorsal diencephalic conduction systems; a review of the anatomy and functions of the habenular complex, *Neuroscience Biobehaviour Reviews*, **6**, 1-13.

Sutherland, G., Lesiuk, H., Bose, R. & Sima, A. (1988) Effect of mannitol, nimodipine and indomethacin singly or in combination on cerebral ischemia in rats, *Stroke*, **19**, 571-578.

Suzdak, P.D. & Sheardown, M.J. (1993) Effect of the non-NMDA receptor antagonist, 2,3-dihydro-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline, on local cerebral glucose uptake in the limbic forebrain, *Journal of Neurochemistry*, **61**, 1577-1580.

Suzuki, J., Yoshimoto, t., Tanaka S. & Sakamoto, T. (1980) Production of various models of cerebral infarction in the dog by means of occlusion of intra-cranial trunk arteries, *Stroke*, **11**, 337-341.

Swan, J.H., Evans, M.C. & Meldrum, B.S. (1988) Long-term development of selective neuronal loss and the mechanism of protection of 2-amino-7-phosphonoheptanoate in a rat model of incomplete forebrain ischaemia, *Journal of Cerebral Blood Flow and Metabolism*, **8**, 64-78.

Swan, J.H. & Meldrum, B.S. (1990) Protection by NMDA antagonists against selective cell loss following transient ischaemia, *Journal of Cerebral Blood Flow and Metabolism*, **10**, 343-351.

Swanson, R.A. & Sharp, F.R. (1994) BW619C89, a glutamate release inhibitor, protects against focal cerebral ischemic damage, *Stroke*, **25**, 225.

Swanson, R.A., Shiraishi, K., Morton, M.T. & Sharp, F.R. (1990) Methionine sulfoximine reduces cortical infarct size in rats after middle cerebral artery occlusion, *Stroke*, **21**, 322-327.

Symon, L., Branston, N.M. & Strong, A.J. (1976) Autoregulation in acute focal ischemia, *Stroke*, **7**, 547-554.

Szatkowski, M., Barbour, B. & Attwell, D. (1990) Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake, *Nature*, **348**, 443-446.

Takadera, T., Shimada, Y. & Mohri, T. (1992) Extracellular pH modulates N-methyl-D-aspartate receptor-mediated neurotoxicity and calcium accumulation in rat cortical cultures, *Brain Research*, **572**, 126-131.

Takagi, K., Ginsberg, M.D., Globus, M. Y.-T., Dietrich, W.D., Martinez, E., Kraydieh, S. & Busto, R. (1993) Changes in amino acid neurotransmitters and cerebral blood flow in the ischemic penumbral region following middle cerebral artery occlusion in the rat: correlation with histopathology, *Journal of Cerebral Blood Flow and Metabolism*, **13**, 575-585.

Takizawa, S., Hogan, M. & Hakim, A.M. (1991) The effects of a competitive NMDA receptor antagonist (CGS-19755) on cerebral blood flow and pH in focal ischemia, *Journal of Cerebral Blood Flow and Metabolism*, **11**, 786-793.

Tam, S.W. (1983) Naloxone-inaccessible receptors in rat central nervous system, *Proceedings of the National Academy of Sciences, U.S.A.*, **80**, 6703-6707.

Tam, S.W. (1985) (+)-[^{3}SKF10,047, (+)-[^{3}H]ethylketocyclazocine, μ, *k*, σ, and phencyclidine binding sites in guinea pig brain membranes, *European Journal of Pharmacology*, **109**, 33-41.

Tamura, A., Graham, D.I., McCulloch, J. & Teasdale, G.M. (1981a) Focal cerebral ischaemia in the rat: I. Description of technique and early neuropathological consequences following middle cerebral artery occlusion, *Journal of Cerebral Blood Flow and Metabolism*, **1**, 53-60.

Tamura, A., Graham, D.I., McCulloch, J. & Teasdale, G.M. (1981b) Focal cerebral ischemia in the rat: 2. Regional cerebral blood flow determined by [^{14}C]iodoantipyrine autoradiography following middle cerebral artery occlusion, *Journal of Cerebral Blood Flow and Metabolism*, **1**, 61-69.

Tang, A.H. (1985) Protection from cerebral ischemia by U-50,448E, a specific kappa opioid analgesic agent, *Life Sciences*, **37**, 1475-1482.

Tang, A.H. & Silvia, R.C. (1986) Protection from cerebral ischaemia by U-50488 in cats subjected to occlusion of the middle cerebral artery, *Society for Neuroscience Abstracts*, **12**, 64.

Tempel, A. & Zukin, R.S. (1987) Neuroanatomical patterns of the μ, δ and *k* opioid receptors of rat brain as determined by quantitative *in vitro* autoradiography, *Proceedings of the National Academy of Sciences, U.S.A.*, **84**, 4308-4312.

Terenzi, M.G. & Prado, W.A. (1990) Antinociception elicited by electrical or chemical stimulation of the rat habenular complex and its sensitivity to systemic antagonists, *Brain Research*, **535**, 18-24.

Terrian, D.M., Gannon, R.L. and Rea, M.A. (1990) Glutamate is the endogenous amino acid selectively released by rat hippocampal mossy fiber synaptosomes concomitantly with prodynorphin-derived peptides, *Neurochemical Research*, **15**, 1-5.

Terrian, D.M., Johnston, D., Claiborne, B.J., Andsah-Yiadom, R., Strittmatter, W.J. & Rea, M.A. (1988) Glutamate and dynorphin release from a subcellular fraction enriched in hippocampal mossy fiber synaptosomes, *Brain Research Bulletin*, **21**, 343-351.

The American Nimodipine Study Group (1992) Clinical trial of nimodipine in acute ischemic stroke, *Stroke*, **23**, 3-8.

Toga, A.W. & Collins, R.C. (1981) Metabolic response of optic centres to visual stimuli in the albino rat: anatomical and physiological considerations, *The Journal of Comparative Neurology*, **199**, 443-446.

Tyson, G., Teasdale, G.M., Graham, D.I. & McCulloch, J. (1984) Focal cerebral ischemia in the rat: topography of hemodynamic and histopathological changes, *Annals of Neurology*, **15**, 559-567.

Uematsu, D., Greenberg, J.H., Hickey, W.F. & Reivich, M. (1989) Nimodipine attenuates both increase in cytosolic free calcium and histologic damage following focal cerebral ischemia and reperfusion in cats, *Stroke*, **20**, 1531-1537.

Umemura, K., Wada, K., Uematsu, T., Mizuno, A. & Nakashima, M. (1994) Effect of 21-aminosteroid lipid peroxidation inhibitor, U74006F, in the rat middle cerebral artery occlusion model, *European Journal of Pharmacology*, **251**, 69-74.

Vaupel, D.B. (1983) Naltrexone fails to antagonize the σ effects of PCP and SKF 10,047 in the dog, *European Journal of Pharmacology*, **92**, 269.

Von Voigtlander, P.F., Lahti, R.A. & Ludens, J.H. (1983) U-50,488: a selective and structurally novel non-mu (kappa) opioid agonist, *Journal of Pharmacology and Experimental Therapeutics*, **224**, 7-12.

Wagner, J.J., Caudle, R.M. & Chavkin, C. (1992a) *k*-Opioids decrease excitatory transmission in the dentate gyrus of the guinea pig hippocampus, *Journal of Neuroscience*, **12**, 132-141.

Wagner, J.J., Evans, C.J. & Chavkin, C. (1991) Focal stimulation of the mossy fibers releases endogneous dynorphins that bind k_1-opioid receptors in guinea pig hippocampus, *Journal of Neurochemistry*, **57**, 333-343.

Wagner, J.J., Terman, G.W. & Chavkin, C. (1993) Endogenous dynorphins inhibit excitatory neurotransmission and block LTP induction in the hippocampus, *Nature*, **363**, 451-454.

Wagner, K.R., Kleinholz, M., de Courten-Myers, G.M. & Myers, R.E. (1992b) Hyperglycemic versus normoglycemic stroke: Topography of brain metabolites, intracellular pH, and infarct size, *Journal of Cerebral Blood Flow and Metabolism*, **12**, 213-222.

Wahl, M. (1985) Effects of enkephalins, morphine, and naloxone on pial arteries during perivascular microapplication, *Journal of Cerebral Blood Flow and Metabolism*, 5, 451-457.

Wahl, F., Allix, M., Plotkine, M. & Boulu, R.G. (1993) Effect of riluzole on focal cerebral ischemia in rats, *European Journal of Pharmacology*, **230**, 209-214.

Wahlestedt, C., Golanov, E., Yamamoto, S., Yee, F., Ericson, H., Yoo, H., Inturrisi, C.E. & Reis, D.J. (1993) Antisence oligodeoxynucleotides to NMDA-R1 receptor channel protect cortical neurons from excitotoxicity and reduce focal ischaemic infarctions, *Nature*, **363**, 260-263.

Wang, R.Y. & Aghajanian, G.K. (1977) Physiological evidence for habenula as major link between forebrain and midbrain raphe, *Science*, **197**, 89-91.

Warner, M.A., Neill, K.H., Nadler, J.V. & Crain, B.J. (1991) Regionally selective effects of NMDA receptor antagonists against ischemic brain damage in the gerbil, *Journal of Cerebral Blood Flow and Metabolism* **11**, 600-610.

Watkins, J.C. & Evans, R.H. (1981) Excitatory amino acid transmitters, *Annual Review of Pharmacology and Toxicology*, **21**, 165-204.

Watkins, J.C., Krogsgaard-Larsen, P. & Honoré, T (1990) Structure-activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists, *Trends in Pharmacological Sciences*, **11**, 25-33.

Weisskopf, M.G., Zalutsky, R.A. & Nicholl, R.A. (1993) The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fibre synapses and modulates long-term potentiation, *Nature*, **362**, 423-427.

Weissman, A.D., Dam, M. & London, E.D. (1987) Alterations in local cerebral glucose utilization induced by phencyclidine, *Brain Research*, **435**, 29-40.

Wells, T. & Forsling, M.L. (1991) *k*-Opioid modulation of vasopressin secretion in conscious rats, *Journal of Endocrinology*, **129**, 411-416.

Welsh, F.A., Ginsberg, M.D., Rieder, W. & Budd, W.W. (1978) Diffuse cerebral ischemia in the cat: II. Regional metabolites during severe ischemia and recirculation, *Annals of Neurology*, **3**, 493-501.

Welsh, F.A., Greenberg, J.H., Jones, S.C., Ginsberg, M.D. & Reivich, M. (1980) Correlation between glucose utilization and metbolite levels during focal ischemia in cat brain, *Stroke*, **11**, 79-84.

Welsh, F.A., O'Connor, M.J., Marcy, V.R., Spatacco, A.J. & Johns, R.L. (1982) Factors limiting regeneration of ATP following temporary ischemia in cat brain, *Stroke,* **13**, 234-242.

Werling, L.L., Brown, S.R. & Cox, B.M. (1987) Opioid receptor regulation of the release of norepinephrine in the brain, *Neuropharmacology*, **26** (7B), 987-996.

Westerberg, E., Kehr, J., Ungerstedt, U. & Wieloch, T. (1988) The non-competitive NMDA antagonist MK-801 reduces extracellular amino acid levels during hypoglycemia and protects against hypoglycemic damage in rat neostriatum, *Neuroscience Research Communications*, **3.**, 151-158.

Wexler, B.C. (1984) Naloxone ameliorates the pathophysiologic changes which lead to and attend an acute stroke in stroke-prone/SHR, *Stroke*, **15**, 630-634.

White, W.F., Nadler, J.V., Hamberger, A. & Cotman, C.W. (1977) Glutamate as transmitter of hippocampal perforant path, *Nature*, **270**, 356-357.

White, H.S., Wolf, H.H., Swinyard, E.A., Skeen, G.A. and Sofia, R.D. (1992) A neuropharmacologic evaluation of felbamate as a novel anticonvulsant, *Epilepsia*, **33**, 564-572.

Wieloch, T., Gustafson, I. & Westerberg, E. (1989) The NMDA antagonist, MK-801, is cerebroprotective in situations where some energy production prevails but not under conditions of complete energy deprivation, *Journal of Cerebral Blood Flow and Metabolism*, **9** (Suppl.1), S6.

Wieloch, T., Lindvall, O., Blomqvist, P. & Gage, F. (1985) Evidence for amelioration of ischemic neuronal damage in the hippocampal formation by lesions of the perforant path, *Neurological Research*, **7**, 24-26.

Wieloch. T. & Siesjö, B.K. (1982) Ischemic brain injury: the importance of calcium, lipolytic activities and free fatty acids, *Pathological Biochemistry*, **30**, 269-277

Willetts, J., Balster, R.L. & Leander, J.D. (1990) The behavioral pharmacology of NMDA receptor antagonists, *Trends in Pharmacological Sciences*, **11**, 423-428.

Wise, B.L. & Chater, N. (1962) Thje value of hypertonic mannitol solution in decreasing brain mass and lowering cerebrospinal fluid pressure, *Journal of Neurosurgery*, **19**, 1038-1043.

Wong, M.C.W. & Haley, E.C. (1990) Calcium antagonists: stroke therapy coming of age, *Stroke*, **21**, 494-501.

Woods, L.A. (1956) The pharmacology of nalorphine (N-allylnormorphine), *Pharmacological Reviews*, **8**, 175-198.

Xiang, J.-Z., Adamson, P., Brammer, M.J. & Campbell, I.C. (1990) The *k*-opiate agonist U50488H decreases the entry of ^{45}Ca into rat cortical synaptosomes by inhibiting N- but not L-type calcium channels, *Neuropharmacology*, **29**, 439-444.

Xue, D., Huang, Z.-G., Barnes, K., Lesiuk, H.J., Smith, K.E. & Buchan, A.M. (1994) Delayed treatment with AMPA, but not NMDA, antagonists reduces neocortical infarction, *Journal of Cerebral Blood Flow and Metabolism*, **14**, 251-261.

Yamori, Y., Hori, R., Handa, H., Sato, M. & Fukase, M. (1976) Pathological similarity of strokes in stroke-prone spontaneously hypertensive rats and humans, *Stroke*, **7**, 46-53.

Yang, G.Y., Chen, S.F., Kinouchi, H., Chan, P.H. & Weinstein, P.R. (1992) Edema, cation content, and ATPase activity after middle cerebral artery occlusion in rats, *Stroke*, **23**, 1331-1336.

Young, W. (1980) H_2 clearance measurement of blood flow: A review of technique and polargraphic principles, *Stroke*, **11**, 552-564.

Zabramski, J.M., Spetzler, R.F., Selman, W.R., Roessmann, U.R., Hershey, L.A., Crumrine, R.C. & Macko, R. (1984) Naloxone therapy during focal cerebral ischemia. Evaluation in a primate model, *Stroke*, **15**, 621-627.

Zasslow, M.A., Pearl, R.G., Shuer, L.M., Steinberg, G.K., Lieberson, R.E. & Larson Jr, C.P. (1989) Hyperglycemia decreases acute neuronal ischemic changes after middle cerebral artery occlusion in cats, *Stroke*, **20**, 519-523.

Zhang, R-L., Chopp, M., Chen, H., Garcia, J.H. & Zhang, Z.G. (1993) Postischemic (1 hour) hypothermia significantly reduces ischemic cell damagë in rats subjected to 2 hours of middle cerebral artery occlusion, *Stroke*, **24**, 1235-1240.

Zukin, R.S., Eghbali, M., Olive, D., Underwald, E.M. & Tempel, A. (1988) Characterization and visualization of rat and guinea-pig brain K opioid receptors: Evidence for K_1 and K_2 opioid receptors, *Proceedings of the National Academy of Sciences*, 85, 4061-4065.

Zukin, R.S. & Zukin, S.R. (1981) Demonstration of [^{3}H]cyclazocine binding to multiple opioid receptor sites, *Molecular Pharmacology*, **20**, 246-254.

Peer Reviewed Journals

Kusumoto, K., Mackay, K.B. & McCulloch, J. (1992) The effect of the kappa-opioid receptor agonist CI-977 in a rat model of focal cerebral ischaemia, *Brain Research*, **576**, 147-151.

Fujisawa, H., Dawson, D., Browne, S.E., Mackay, K.B., Bullock, R. & McCulloch, J. (1993) Pharmacological modification of glutamate neurotoxicity in vivo, *Brain Research*, **629**, 73-78.

Ikeda, M., Mackay, K.B., Dewar, D. & McCulloch, J. (1993) Differential alterations in adenosine A_1 and k_1 opioid receptors in the striatum in Alzheimer's disease, *Brain Research*, **616**, 211-217.

Mackay, K.B., Kusumoto, K., Graham, D.I. & McCulloch, J. (1993) Focal cerebral ischemia in the cat: pretreatment with a kappa-1 opioid receptor agonist, CI-977, *Brain Research*, **618**, 213-219.

Mackay, K.B., Kusumoto, K., Graham, D.I. & McCulloch, J. (1993) Effect of the kappa-1 opioid agonist CI-977 on ischemic brain damage and cerebral blood flow after middle cerebral artery occlusion in the rat, *Brain Research*, **629**, 10-18.

Mackay, K.B., Dewar, D. & McCulloch, J. (1994) Kappa-1 opioid receptors of the temporal cortex are preserved in Alzheimer's disease, *Journal of Neural Transmission*, **7**, 73-79.

Mackay, K.B. & McCulloch, J. (1994) Distribution of effects of the kappa opioid agonist CI-977 on cerebral glucose utilization in rat brain, *Brain Research*, **642**, 160-168.

Mackay, K.B., Patel, T.R., Galbraith, S. & McCulloch, J. (1994) The relationship between glutamate release and cerebral blood flow after focal ischaemia in the cat: Effect of CI-977 pretreatment, (in preparation).

Mackay, K.B., Kusumoto, K. & McCulloch, J. (1991) The neuroprotective effect of the kappa agonist CI-977 in a rat model of focal cerebral ischaemia. *British Journal of Pharmacology*, **104**, 303P.

Mackay, K.B., Ikeda, M., Dewar, D. & McCulloch, J. (1992) Adenosine A_1 binding is reduced in the temporal cortex and caudate in Alzheimer's disease, while kappa-opioid binding is preserved, *Neuroscience Letters*, **42**, S38.

Mackay, K.B., Kusumoto, K., Graham, D.I. & McCulloch, J. (1992) The effects of a kappa opioid agonist in experimental focal cerebral ischemia, In *Pharmacology of Cerebral Ischemia*, eds. Krieglstein, J. & Oberpichler-Schwenk, H., Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, pp.207-215.

Mackay, K.B., Kusumoto, K., Graham, D.I. & McCulloch, J. (1992) Effect of CI-977 on ischemic brain damage and cerebral blood flow after middle cerebral artery (MCA) occlusion in the rat, *Society for Neuroscience Abstract*, **18**, 522.6.

Fujisawa, H., Bullock, R., Dawson, D., Browne, S., Mackay, K.B. & McCulloch, J. (1993) Pharmacological intervention in a new *in vivo* model of glutamate neurotoxicity, *Second International Neurotrauma Symposium*, P57.

Kusumoto, K., Mackay, K.B., Graham, D.I. & McCulloch, J. (1993) The kappa-1 opioid agonist enadoline: ischaemic brain damage and cerebral blood flow after permanent middle cerebral artery occlusion, *Journal of Cerebral Blood Flow and Metabolism*, **13** (Suppl.1), S697.

Kusumoto, K., Mackay, K.B., McCulloch, J., Graham, D.I. & Asakura, T. (1993) Effect of the kappa agonist CI-977 on ischaemic brain damage and cerebral blood flow after middle cerebral artery occlusion in the rat, In *Recent Advances in Neurotraumatology*, eds. Nakamura, N., Hashimoto, T. & Yasue, M, Springer-Verlag Tokyo, pp.240-243.

Mackay, K.B., Browne, S.E. & McCulloch, J. (1993) Modulation of glutamate mechanisms with enadoline and NBQX: local cerebral blood glucose utilisation in the rat, *Journal of Cerebral Blood Flow and Metabolism*, **13** (Suppl.1), S786.

Macrae, I.M., Dawson, D.A., Mackay, K.B., Graham, D.I. & McCulloch, J. (1993) Acute tissue swelling associated with transient but not permanent focal cerebral ischaemia, *Second International Neurotrauma Symposium*, P48.

Macrae, I.M., Dawson, D.A., Mackay, K.B., Graham, D.I. & McCulloch, J. (1993) Comparison of infarct size and degree of swelling in permanent and reversible acute focal cerebral ischaemia, *Journal of Cerebral Blood Flow and Metabolism*, **13** (Suppl.1), S94.