The development of a novel model of focal cerebral ischaemia using endothelin isopeptides

By

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ABSTRACT

Several animal models of focal cerebral ischaemia have been developed to gain insight into the pathophysiology and possible therapeutic treatment of stroke. This thesis aimed to optimise & characterise a model of focal cerebral ischaemia that utilises perivascular microinjection of endothelin-1 (ET-1) to occlude the middle cerebral artery (MCA) of the rat, and to develop a novel model of endothelin-induced MCA occlusion with controlled reperfusion.

The MCA of the anaesthetised rat was occluded by the intracerebral microinjection of ET-1 into the outer layers of cortex adjacent to the MCA. Histopathological analysis of ischaemic brain damage three days following injection, determined that ET-1 produced a large volume of ischaemic brain damage confined to the vascular territory of the MCA within the dose range 33-300 pmols, whilst a 10 pmol injection was ineffective. The optimal injection volume was found to be in the range 1-3μl. The isopeptide ET-3 was found to be equipotent with ET-1 in the ischaemic insult it produced, having the same minimal effective dose, and being effective over the same dose range. This is the first demonstration that ET-3 is capable of producing ischaemic damage by constricting a major cerebral artery

The equipotency of ET-1 and ET-3 in terms of histopathological outcome in this experimental model of stroke, was supported by laser Doppler flowmetry (LDF) recordings of perfusion velocity from the parietal cortex, in which the severity and duration of ischaemia was found to be the same between isopeptides. These isopeptides are only equipotent at the ET_B receptor, however, ET_B receptor involvement was excluded by the ineffectiveness of the selective ET_B receptor

agonists BQ3020 and IRL1620 in producing MCA occlusion. Furthermore, whilst intracerebral injection of the specific ET_A receptor antagonist FR139317 (3 nmols in 3μl) 10 minutes before ET-3, blocked MCA occlusion, this dose of FR139317 was ineffective against ET-1-induced MCA occlusion. This suggests the involvement of an atypical receptor in the contractile response of the rat MCA to endothelin isopeptdies.

Intracerebral probes determined that striatal temperature remained unchanged following ET-1-induced MCA occlusion. By contrast, striatal oxygen tension, measured by intracerebral oxygen electrodes, and cortical tissue perfusion velocity, measured by laser Doppler flowmetry (LDF) fell rapidly following ET-1 injection. Oxygen tension did not recover for 3 hours following ET-1 injection, whilst the LDF signal showed some recovery but still remained below 50% of baseline levels after 2 hours. [^{14}C]iodoantipyrine autoradiography determined that local cerebral blood flow (CBF) was profoundly reduced throughout much of the vascular territory of the MCA three hours after injection of ET-1. This pathophysiological profile suggests that ET-1-induced MCA occlusion represents a model of permanent focal cerebral ischaemia in which reperfusion is limited.

A 17mer sequence of the secreted form of β-amyloid precusor protein (βAPP), which has previously been shown to possess trophic and neuroprotective properties, was evaluated using this experimental model of stroke. The 17mer peptide was found to confer neuroprotection to both cortex and striatum, when delivered intracerebroventricularly for three days before inducing ET-1-induced MCA occlusion. However, the 17mer peptide did not improve functional recovery

following ET-1-induced MCA occlusion as assessed by a model of skilled motor control.

Both endothelin isopeptides were investigated in the development of a model of MCA occlusion with controlled reperfusion. Quantitative histopathology determined that ET-3-induced MCA occlusion was effectively reversed by intracerebral injection of FR139317 delivered 10 minutes after ET-3. By contrast, injection of 3 or 30nmols of FR139317 10 minutes after ET-1 had no significant effect on the volume of ischaemic brain damage. The histopathological findings were confirmed with LDF recordings of cortical perfusion velocity, in which FR139317 reversed ET-3-induced ischaemia within 5 minutes of injection, whilst FR139317 had little effect on ET-1-induced MCA occlusion. The effectiveness of FR139317 as an agent with which to interrupt ET-3-induced MCA occlusion was confirmed by local CBF measurement using [^{14}C]iodoantipyrine autoradiography. ET-3-induced MCA occlusion with controlled reperfusion was used to investigate very delayed (apoptotic) cell death. FR139317 was injected 30 minutes after ET-3, and histopathology was performed 3 or 14 days later. There was no significant difference in the volume of ischaemic brain damage between groups, and the component of very delayed (apoptotic) cell death in this model is unlikely to contribute significantly to the volume of the ischaemic lesion.

The ET-1-induced MCA occlusion model has the pathophysiological characteristics of permanent arterial occlusion, and is not readily amenable to controlled reperfusion using the ET_A receptor antagonist FR139317. By contrast, ET-3-induced MCA occlusion can be abruptly reversed by the intracerebral injection of

FR139317, and represents a novel model of MCA occlusion with controlled reperfusion.

TO MY FAMILY

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DECLARATION

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

Signed:

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PUBLICATIONS ARISING FROM THESIS

PAPERS

BUTCHER S.P., **HENSHALL D.C.,** TERAMURA Y., IWASAKI K., SHARKEY J. Neuroprotective actions of FK506 in experimental stroke: *in vivo* evidence against an excitotoxic mechanism. (J. Neurosci. - in press)

HENSHALL D.C., BUTCHER S.P., SHARKEY J

Endothelin-induced middle cerebral artery occlusion in the rat: effects on cerebral oxygen tension, brain temperature and cortical tissue perfusion. (manuscript in preparation)

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Pharmacological characterisation of the receptor mediating endothelin-induced middle cerebral artery occlusion in the rat. (In submission to Br. J. Pharmacol.)

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Characterisation of a novel model of MCA occlusion with controlled reperfusion. (manuscript in preparation)

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ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
AP	Anterio-posterior (coordinate)
ATP	Adenosine triphosphate
BBB	Blood brain barrier
bp	Base pairs
BP	Blood pressure
Ca^{2+}	Calcium ion
CBF	Cerebral blood flow
CBV	Cerebral blood volume
$CMRO_2$	Cerebral metabolic rate for oxygen
CO_2	Carbon dioxide
CT	Computed tomography
DA	Dopamine
DAG	Diacyl glycerol
DPMs	Disintegrations per minute
DND	Delayed neuronal death
EAAs	Excitatory amino acids
ET	Endothelin
ET-1	Endothelin-1
ET-3	Endothelin-3
GABA	Gamma amino butyric acid
GPCR	Guanine-nucleotide binding-coupled receptor
H^+	Hydrogen ion
HSP	Heat shock protein
5-HT	5-hydroxytryptamine
ICE	Interleukin 1β converting enzyme
IEGs	Immediate early genes

IL-1β	Interleukin 1β
i.v.c.	Intracerebroventricular
IP_3	Inositol 1,4,5-trisphosphate
K^+	Potassium ion
KA	Kainic acid
L	Lateral (coordinate)
LDF	Laser Doppler flowmetry
μM	Micromolar
MRI	Magnetic resonance imaging
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
mm	Millimeter
mls	Milliliters
mM	Millimolar
mmHg	Pressure in millimeters of mercury
M.W.	Molecular weight
M(Ab)	Monoclonal (antibody)
NA	Noradrenaline
Na^+	Sodium ion
nM	Nanomolar
NO	Nitric oxide
NMDA	N-methyl-D-aspartate
OEF	Oxygen extraction fraction
O_2	Oxygen
pH	Log-molar concentration of hydrogen ions
PMCAO	Permanent middle cerebral artery occlusion
PBS	Phosphate buffered saline
pM / pmol	Picomolar / picomols
PET	Positron emission tomography
PCD	Programmed cell death
rCBF	Regional cerebral blood flow

Rb	Retinoblastoma tumor suppresor protein
SPECT	Single photon emission computed tomography
SNP	Sodium nitroprusside
SHR	Spontaneously hypertensive rat
SD	Sprague-Dawley
s.e.mean	Standard error of the mean
TMCAO	Temporary middle cerebral artery occlusion
V	Vertical (coordinate)
VDCCs	Voltage-dependent calcium channels
VDSCs	Voltage-dependent sodium channels

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CHAPTER ONE

CHAPTER 1. GENERAL INTRODUCTION

1.1. CEREBROVASCULAR DISEASE

1.1.1. Human Stroke: Definitions, causes & consequences

Cerebrovascular disease encompases a number of pathophysiological conditions and includes intracerebral haemorhage, cerebral aneurysm, and in particular ischaemic stroke. The term "ischaemic stroke" or "focal cerebral ischaemia", describes the consequences of CBF reductions that arise from a number of different pathologies. Causes include cardiac arrest, embolism, & thrombosis, reflecting transient global CBF reductions, gradual or sudden narrowing of a major supply artery to the brain or microembolism of smaller arteries (Hossmann, 1982). The underlying cause of most ischaemic strokes is occlusion of an intracranial artery (Garcia et al. 1996b), of which occlusion of the middle cerebral artery (MCA) is most common in humans (Nedergaard, 1988). By convention, symptoms must last for more than 24 hours (Kalimo *et al.* 1997). Focal neurological deficits that resolve within 24 hours are termed transient ischaemic attacks (TIAs). Although not refered to as a stroke, such ischaemic episodes may nevertheless result in some permanent damage despite lacking symptoms (Dyken *et al.* 1984; Kalimo *et al.* 1997).

Worldwide, approximately 4.6 million people die from stroke each year (Bonita & Beaglehole, 1995), making stroke the third leading cause of death among adults (Gorelick, 1995), and the most common disabling and lethal neurological disease of adult life (Dyken *et al.* 1984). In the USA, approximately 500,000 strokes occur a year, with a cost to the nation of $30 billion. In the U.K figures are proportionally similar, with 100,000 people experiencing first-ever strokes, accounting for fatality

rates of 2 in 1000 (12% of all U.K. deaths) (McAuley, 1995). Of the patients affected, roughly one third will die, one third experience a subsequent stroke, and many of the remaining live with some form of disability (Heros 1994). Indeed, cerebrovascular lesions not only result in physical diability such as paralysis, but many emotional and behavioural disorders occur following stroke including depression and anxiety (Robinson, 1997). Despite high prevelance, burden of illnes, economic cost and safe and extensive measures with which to prevent or minimise its occurance (Gorelick 1995), an accepted treatment to limit brain damage following stroke is not in clinical practice.

1.1.2. Human Stroke: Incidence & risk factors

The prevelance of the various forms of cerebrovascular disease vary according to where the studies were performed and whether the data are from hospital records or population surveys (Dyken et al. 1984). Population studies reveal that the large majority of strokes are caused by ischaemia and infarction following small and medium-artery disease, atherothrombotic brain infarction, whilst cerebral embolisation accounts for ~14-20% (Dyken et al. 1984).

There are a number of clearly identified risk factors for stroke, our knowledge of which has advanced considerably during recent decades and indeed exceeds that of other major neurological diseases (Gorelick 1995). Several of these factors are considered non-modifiable such as advanced age, male gender, race and heredity, are not amenable to preventative approaches (Dyken *et al.* 1984). The key predisposing factors include hypertension, cardiac disease, diabetes mellitus, blood lipid levels,

asymptomatic carotid artery stenosis, transient ischaemic attacks (TIAs), cigarette smoking, alcohol and exercise (Gorelick 1995). Hypertension & cardiac disease constitute risk factors where treatment is both feasible and the value has been established, and up to 70% of strokes may be avoidable by treatment of hypertension (Dunbabin & Sandercock 1990). By contrast, factors such as obesity, cigarette smoking and oral contraceptive use, represent risk factors which are treatable but where the value of this has yet to be established (Dyken *et al.* 1984). While cardiac disease and stroke are highly correlated, the effect of prevention and treatment of cardiac disease on stroke requires further study (Gorelick 1995), although it is believed that the treatment and prevention of cardiac disease might shift the balance of stroke risk. For example, treatment of some cardiac disease with antithrombotic agents reduces ischaemic stroke risk. However, the counterargument is that an increase in the survival of cardiac disease patients could increase the pool of people at risk from stoke (Whisnant 1992).

Diabetes is another key risk factor (Dyken *et al.* 1984), although whether control of diabetes mellitus would reduce stroke risk is still uncertain. Similarly, whilst asymptomatic carotid artery stenosis represents risk factors for stroke, myocardial infarction & death, treatment has yet to confirm overwhelming effectiveness in reducing stroke and death incidence, although the incidence of neurological events such as TIAs may be reduced by carotid endarterectomy (Hobson *et al.* 1993). Cessation or reduction of cigarette smoking and heavy alcohol consumption would reverse the predisposition to stroke in these groups within a few years (Gorelick 1995). Since hypercholesterolaemia could account for a significant number of

strokes, treatment of blood lipid level abnormalities seems prudent although further study is needed (Dunbabin & Sandercock 1990; Atkins *et al.* 1993; Gorelick 1995). Prior stroke is also a key risk factor, with the risk of a recurrent stroke increasing many times that of suffering a first-time stroke (Dyken *et al.* 1984).

1.1.3. Brain function & structure

The function of the cerebral circulation is to provide the necessary substrates for the brain, & remove the products of metabolism, and the cause of brain injury following stroke is invariably consequent on the decreased delivery of oxygen and metabolic substrates to the brain (Sokoloff, 1986). The endogenous stores of brain substrates for metabolism are almost neglible compared to the rates of utilisation, and highly specialised and differentiated tissues such as those found in the brain require large and continuous amounts of oxygen and glucose to support function since the brain utilises oxygen and glucose at very rapid rates (Farber *et al.* 1981; Sokoloff, 1986). Despite comprising only 2-3% body weight, the brain receives 15% of the cardiac output, and consumes 20% of the oxygen and 25% of the glucose within circulating blood (Graham, 1985). The oxygen extraction fraction of the brain is 40-50% whilst that of glucose is ~10% (Heiss & Podreka, 1993), and the oxygen content of the blood decreases from 19.6 to 12.9 ml/dl when passing through the brain, whilst carbon dioxide levels increase by 14% from 48.2 to 54.8 ml/dl. The combination of a respiratory quotient close to unity, an almost stoichiometric relationship between oxygen uptake and glucose consumption, and the absence of significant arteriovenous differences for any other energy-rich substrate suggests glucose is the

sole source of oxidative (aerobic) respiration (Graham 1985; Sokoloff, 1986). Under normal circumstances no other energy-yielding substrate other than glucose is significantly extracted by the brain from the blood (Sokoloff, 1986). Very few substrates can restore normal cerebral function during a hypoglycaemic state, and no substance normally present in the blood can replace glucose as the substrate for cerebral energy metabolism (Sokoloff, 1986). There are a limited number of conditions such as starvation, under which the brain can substitute other substances for glucose (Seisjo, 1984). These include ketone bodies and some monocarboxylic acids. However, there is no evidence that these can completely replace glucose (Seisjo 1984; Sokoloff, 1986).

Glucose (a hexose) enters the glycolytic pathway where it is metabolised into two pyruvate molecules. Pyruvate enters the mitochondrion and is metabolised to carbon dioxide (CO_2) and water via the mitochondrial respiratory chain of enzymes. This process produces a total of 42 ATP molecules (the cell's short-term energy storage molecule) from one glucose molecule (Auer & Benveniste, 1997). In the absence of sufficient oxygen, pyruvate is converted into lactic acid, via the enzyme lactate dehydrogenase, which yields a much smaller amount of energy (Stryer, 1991). Production of lactate instead of ATP rapidly depletes cellular glucose levels and causes intracellular acidification from ATP hydrolysis, and directly from lactic acid production (Auer & Benveniste, 1997). Since the brain lacks substantial energy stores (Auer & Benveniste, 1997), dependency of the brain on oxidative metabolism of glucose makes the brain so susceptible to damage during ischaemic episodes (Siesjo, 1992a).

Brain tissue consists of two types of cells, glia and neurones. There are approximately one order of magnitude greater numbers of glia (10^{11}) than neurones (10^{10}), and these are the supporting cells for the neurones. In addition to these cell types, the brain receives its metabolic substrates via the cerebrovascular network, which consists of endothelial and smooth muscle cells. The number of different neurone types are vast and whilst neurones are defined both morphologically (e.g. medium spiny neurones of the striatum) and neurochemically (e.g. dopaminergic). Glia are divisible into three clasess; oligodendroglia (active in the formation and maintenance of myelin), astrocytes (supporting cells which function in neurotransmitter uptake and local metabolism) and microglia, which perform multiple tasks including cellular proliferation and regulation of cytotoxic reactions (Nakajima & Kohsaka, 1993; Auer & Benveniste, 1997). Originally perceived as relatively inactive in metabolic terms compared to neurones, glia are now understood to possess cell surface ion transport systems and neuroreceptors although lacking the metabolic expenditure of axoplasmic transport, a function exclusive to neurones (Auer & Benveniste, 1997).

A crucial component of the brain's structure and function is the blood brain barrier (BBB). Homeostatic maintenance of the internal milieu of the CNS is essential if the brain is to avoid being affected by sytemic fluctuations in blood levels of hormones and ions induced by factors such as feeding, exercise and stress. It is the BBB which provides the ability of the brain to control such fluctuations (Edvinsson et al. 1993c). Ehrlich's classic experiments in 1885 showed that intravenous injection of a dye failed to penetrate the brain, whilst staining the other tissues of the body.

This was supported by Goldmann's studies in 1913 that established the presence of a barrier to the blood. Subsequently, studies have determined the presence of a physico-chemical lining of the cerebral blood vesses (Dermietzel & Krause, 1991). The physical basis for the BBB resides in continuous tight cell junctions between the endothelial cells lining cerebral vessels (Dermietzel & Krause, 1991). This barrier does not cover the entire brain area and a small number of "breaks" exist in the BBB at sites including the pituitary and pineal glands, which allows the passage of blood-borne molecules essential in regualtion of the neuroendocrine system (Dermeitzel & Krause, 1991).

The BBB also constitutes a "chemical" barrier to substance passage. Tight cell junctions physically prevent the passage of large proteins (>m.w.1000) and many micromolecular substances (Dermietzel & Krause, 1991). Small molecules may pass the barrier according to particular properties of that substance, of which lipid soluability is the most decisive (Edvinsson et al. 1993c). Lipid soluble molecules readily cross the BBB, since the membranes comprising the BBB are composed of lipid molecules. By contrast, hydrophilic molecules (e.g. ions, proteins and saccharides) possess poor BBB penetration (Dermietzel & Krause, 1991). Penetration of energy substrates and amino acids that cannot be synthesised within brain cells is achieved by enzyme transporters specific to the nutrient (Edvinsson et al. 1993c). Regulation of molecule passage is also by cellular metabolism within the BBB, whereby a substance entering an endothelial cell may be converted into a form incapable of traversing the antiluminal membrane (Edvinsson et al. 1993c). Disruption to the integrity of the BBB may contribute directly to neuronal injury

(Edvinsson et al. 1993c). Following stroke, the integrity of the BBB may become disrupted, which may lead to exposure of brain tissue to unregulated levels of blood-borne substrates as well as contributing to oedema formation within ischaemic brain tissue (Betz et al. 1994).

1.2. Cerebral circulation

1.2.1. Anatomy of the cerebral circulation

The blood supply to the brain in humans is from two pairs of large arteries, the internal carotid arteries and the vertebral arteries, which provide anterior and posterior flow respectively (Kalimo *et al.* 1997). The internal carotid arteries give rise to the anterior cerebral artery (ACA), MCA and the anterior choroidal artery. By contrast, the vertebral arteries form the basilar artery, posterior inferior cerebellar and anterior & posterior spinal arteries. The basilar then bifurcates to form the posterior cerebral arteries (PCA) and four other vessels including the superior cerebellar (Edvinsson et al. 1993a). The basilar & internal carotid arteries anastomose at the base of the brain to form the circle of Willis. This structure ensures adequate blood supply to the brain despite occlusion of any major artery supplying the circle of Willis (Kalimo *et al.* 1997). From the circle of Willis, the major cerebral blood vessels supply the brain. The anterior cerebral artery is responsible for supply to midline cortical structures and deep structures such as the basal ganglia. The MCA supplies most of the lateral cortex and deep structures such as the caudate nucleus via the lenticulo-striate branches. The anterior choroid artery supplies structures such as

the choroid plexus and hippocampus, whilst the PCA supplies caudal structures such as the cerebrellum.

In the assessment of various animal species as to their relevance as models to study human stroke, the anatomy of the cerebral circulation is of importance. Major differences between a given species and man should be taken into account in the design of models of focal cerebral ischaemia and in the interpretation of pathophysiological outcome. A number of differences exist between the cerebral circulation in man and that of the main animal species used in stroke research (Scremin, 1995). Such differences prompt caution in the adoption of certain species in cerebrovascular disease research. Prominent amongst these is the presence of the carotid rete mirablis, absent in primates, it is an intricate system of branching arteries, veins and sinuses that exists in cats, sheep, pigs and to a lesser extent, in dogs (Scremin, 1995). By contrast the rat and rabbit do not posses this, and the rat, which is currently the most extensively used species in *in vivo* stroke research, shares major similarities to human cerebrovascular anatomy (Yamori *et al.* 1976). Thus the rat brain receives blood from four arteries, the two common carotids and two vertebrals, the former of which divide to form the external and internal carotid arteries. The internal carotid gives rise to the posterior communicating artery, and then is incorporated into the circle of Willis, whilst the vertebral arteries form the basilar artery which later feeds posterior structures of the brain via the posterior cerebral artery. Like the human circulation, a number of major cerebral arteries arise from the circle of Willis and feed anterior and midline structures (ACA), the midway cortical and striatal territories (MCA) and the posterior brain structures such as the

cerebellum (PCA) (Scremin, 1995). The MCA originates at ~2mm caudal to bregma, and courses laterally and rostrally giving off the corticostriate artery, which feeds parts of the piriform cortex, and the anterior & posterior striate arteries, which feed the striatum and are the equivalent of the lenticulo-striate arteries in the human. The MCA then curves over the lateral surface of the cerebral hemisphere giving rise to a variable number of rostral, caudal and medial vessels (Scremin, 1995).

However, whilst there is a general resemblance to the human, a number of differences exist between the human and rat cerebral circulation (Scremin, 1995). These include a (relatively) larger posterior communicating artery and the general absence of an anterior communicating artery, and relatively differential lengths of the internal carotid and posterior communicating arteries (Scremin, 1995).

The appropriateness of the cerebral anatomy & whole animal physiology, the detailed understanding of neurotransmitter systems in the rat as a consequence of this species widespread use in biological research, and the economic & pratical advantage of small animals in research when compared to primates or other larger mammals such as the dog, has fuelled the extensive adoption of the rat within cerebrovascular disease research (Macrae, 1992).

Even within a single species, there may be considerable variation in the outcome following focal cerebral ischaemia. This may arise not only from experimental conditions, such as physiological monitoring, but is inherent as a consequence of the variability of the cerebral anatomy even within a single strain (Brown, 1966). Extensive studies by Brint *et al.* (1988) and Duverger & Mackenzie, (1988) addressed inter-strain differences in the outcome following MCA occlusion in the rat.

Their conclusions were that spontaneously hypertensive rats produced the largest volume of ischaemic damage following MCA occlusion, whilst Wistar/Kyoto rats exhibited the smallest infarcts. Concensus on the most appropriate rat strain is lacking, although consultation of the literature suggest that the Sprague-Dawley rat is the most commonly applied strain. However, whether this is the ideal strain is far from clear and Duverger & Mackenzie, (1988) recommended the Fischer 344 strain based on studies using the main strain variants.

In the development of the most appropriate animal models of stroke there is undoubtedly a need for a degree of consensus between experimental practice to allow closer comparison between studies, whilst maintaining a sufficiently broad range of practical approaches so as not to exclude necessary variability to reflect clinical heterogeneity. There has been an emerging convergence on the use of only a very narrow range of species in models of MCA occlusion (the rat, mouse and cat), whilst studies on forebrain and global models have been predominantly conducted in gerbil. Particular strain use is currently unregulated and this may need to be reviewed if inter-study variability is to be decreased and greater consensus achieved.

1.2.2. Cerebral blood flow autoregulation

The maintenance of cerebral blood flow is controlled within the cranium by a range of autoregulatory, neurogenic and metabolic mechanisms. The relationship between perfusion pressure and cerebral blood flow is primarily controlled by the phenomenon of autoregulation (Graham, 1985). Autoregulation of blood flow refers to an intrinsic ability of an organ or tissue to maintain constant tissue perfusion

despite blood pressure changes (Paulson *et al.* 1990). This may be described as the physiological regulatory mechanism that maintains constant flow over wide ranges of arterial blood or perfusion pressures (Lassen 1959; Paulson *et al.* 1990). The concept of autoregulation was recognised about 100 years ago (Roy & Sherrington 1890; Bayliss 1902). It was Lassen (1959) that firmly established the concept of blood flow constancy during perfusion pressure changes (Strandgaard & Paulson, 1983), establishing the significance of CBF autoregulation and its lower limit, and introducing and applying the term "autoregulation" to the cerebral circulation field (Paulson *et al.* 1990). However, there are limits outwith the organ cannot operate. In the case of the cerebral circulation, autoregulation fails outwith the blood pressure limits of 60-150mmHg (Paulson *et al.* 1990), and disease states may alter these limits. Below ~65mmHg, vessels are maximally dilated and flow is dependent only on perfusion pressures, decreasing with decreasing blood pressure (Graham, 1985). However, studies have brought into question the actual lower limit of autoregulation, and Tuor & Farrar (1984), suggested that whilst the pressure at which CBF begins to decrease is 65mmHg, the true lower limit of autoregulation at which pressure-passive reductions in CBF occurs, is at 35mmHg.

At the upper autoregulatory limit, there is forced dilation of cerebral vessels with the inability of cerebral vasoconstriction to compensate, with subsequent cerebral oedema and the production of hypertensive encephalopathy (Graham, 1985; Edvinsson et al. 1993b). Whilst it is stated that mean cerebral blood flow to the brain is approximately 50ml/100g/min, this does not reflect the constant re-adjustment of local blood flow to meet the various metabolic demands of brain cells. This point is

of fundamental importance not only to understanding the mechanisms controlling cerebral blood flow, but to the appreciation of the consequences of blood flow interruption to the brain. The mechanisms controlling blood supply are fundamentally geared to the fact that the greater the metabolic demand of a brain region, the greater the blood supply and *vice versa* (Villringer & Dirnagl, 1995).

The mechanism of autoregulation of cerebral blood flow remains unclear (Strandgaard & Paulson, 1983). There are three main hypotheses that might account for autoregulation: the metabolic, neurogenic and myogenic hypotheses (Paulson *et al.* 1990; Edvinsson et al. 1993b). The metabolic hypothesis postulates that products of cerebral respiration control blood pressure. The hypothesis postulates that a fall in pressure would lead to a transient accumulation in metabolites that would cause vasodilatation and consequently the restoration of flow. In general, increasing metabolic demands due to neuronal activation leads to highly localised and restricted changes in flow, which might reflect the effects of metabolic mediators. The proposed mediators include CO_2, H^+, O_2, adenosine/adenine nucleotides, K^+ and Ca^{2+} (Kuschinksy & Wahl 1978; Paulson *et al.* 1990; Edvinsson et al. 1993b). The role of these metabolites in autoregulation has yet to be established. For example, adenosine is a product of energy metabolism and a potent dilator of vessels within the brain (Winn *et al.* 1980), but adenosine antagonists do not affect the pressure-flow relationship with autoregulatory limits (Phillis 1989a). The evidence for H^+/pH involvement is also a matter of debate. Cerebral arterioles dilate in response to a decrease in pH and constrict in response to alkalosis. That extracellular pH exerts a dominating or overiding influence is tenous (Siesjo, 1984), while pH has no effect

during hypotension (Edvinsson et al. 1993b), and extracellular K^+ does not change during hypotension (Kuschinsky 1978; Paulson *et al.* 1990).

The neurogenic hypotheis of cerebral autoregulation proposes that vasodilation and vasoconstriction occurs in response to pressure changes under perivascular nervous control. All vascular segments of cerebral vessels are innervated (both extra- and intraparenchymal) by nerves from both the peripheral and central nervous systems, and a number of neurotransmitters and receptors reside within such innervations (McCulloch & Edvinsson 1984; Lou & Edvinsson 1987; Uddman & Edvinsson 1989; Paulson *et al.* 1990; Branston, 1995). While a review of this area of research is outwith the scope of this section, both parasympathetic and sympathetic nerves innervate vessels, whilst transmitters such as DA, substance P and NO are implicated (Paulson *et al.* 1990; Branston, 1995). The extent to which such neurogenic mediators control autoregulation is yet to be determined. Activity of the autonomic nervous system and extrinsic nerves may shift the limits of autoregulation both up and down, and may attenuate the hypercapnic response (Paulson et al. 1990; Edvinsson et al. 1993b). However, autoregulation is generally preserved following surgical or pharmacological blockade of cervical autonomic nerves (Paulson et al. 1990; Edvinsson et al. 1993b; Branston, 1995), suggesting limits in the contribution to autoregulatatory functions.

The myogenic hypothesis proposes that an intrinsic property within the smooth muscle wall of cerebral vessels makes them responsive to changes in flow pressures, whereby vessels contract in response to a rise in transmural pressure (Paulson *et al.* 1990; Edvinsson et al. 1993b). The rapid autoregulatory response to changes in

transmural pressure of resistance vessles does seem to favour this hypothesis, and would account for the vasoconstriction associated with hypertension (Edvinsson et al. 1993b). Such changes rest not on local changes in the milieu but the ability of actin & myosin elements to sense intravascular pressure changes, a principle known as the Bayliss effect (Bayliss 1902; Paulson *et al.* 1990). However, the mechanism behind this property is not well understood. There is some evidence that stretching muscle fibres may alter membrane permeabilities to ions such as Ca^{2+}, whilst studies of vessels from spontaneously hypertensive animals suggest they have altered ionic permeabilities (Harder 1984; Harder & Lombard 1985; Paulson *et al.* 1990). There may be no unifying explanation for the autoregulatory responses to changes in blood pressure or fluctuations in metabolic demand by brain cells, but that rather a collection of factors such as inherent myogenic properties coupled with the effects of metabolic products and nervous innervation act to control and exercise autoregulation. Paulson *et al.* (1990) comment that the mechanism controlling autoregulation may differ from the processes involved in chemical (CO_2 reactivity) and metabolic responses of the cerebral circulation.

1.2.3. Cerebral blood flow regulation (1): Physiological control

Whilst autoregulation maintains constant blood flow within certain perfusion pressures as a result of as yet poorly defined mechanisms, there are myriad of neurotransmitters, neuropeptides and endothelium-derived factors that elicit effects on cerebral blood vessels and consequently modify cerebrovascular tone and CBF. The main agents affecting cerebral vessels are shown in Table 1.

Main mediators exerting effects on cerebral blood vessels

Substance	**Origin**	**Vessels innervated**	**Response**	**Postulated role**
noradrenaline (NA)	sympathetic ganglia & locus coeruleus	arteries, veins & capillaries	mainly constriction	attenuation of CBF change in severe hypertension
acetylcholine (Ach)	sphenopalatine ganglion & 7th cranial nerve	arteries & veins	vasodilatation	unknown
5-hydroxytryptamine (HT)	Raphe nuclei	arteries & veins	dilatation and constriction	tonic vaso-constriction in response to hypercapnia (migraine?)
neuropeptide Y (NPY)	sympathetic ganglia	arteries & veins	constriction	similar to NA
vasointestinal peptide (VIP)	cortical perikarya & sphenopalatine ganglion	arteries & veins	dilatation	unknown
calcitonin gene-related peptide (CGRP)	5th cranial nerve	arteries & veins	dilatation	attenuation of vessel diameter following excess constriction
Substance P	5th cranial nerve	arteries, veins & capillaries	dilatation	nociception & inflammation
nitric oxide	local & pterygopalantine ganglion	arteries, veins & capillaries	vasodilatation	
endothelins	local	arteries & veins	vasoconstriction	mediation of vasospasm

Table 1. Major neural and local mediators with affects on cerebral blood flow (Scremin, 1995).

Whilst detailed discussion of the ralative contributions of these agents is outwith the scope of this thesis, the above table lists those of significance. Regulation of CBF is mainly subserved by the precapillary resistance vessels (Siesjo, 1984). However, activation of neural networks (e.g. serotonergic projections) may modify CBF, although the functional significance of such changes in CBF are a matter of debate since with the notable exception of the endothelins, these mediators are incapable of overwhelming homeostatic mechanisms and reducing CBF to ischaemic levels. However, activation of certain pathways may be of importance in disease states such as stroke and migraine, when autoregulatory capacity is lost. However, as yet no clear functional framework has been established for the roles of neurochemical manipulation by neurotransmitters.

1.2.4. Cerebral blood flow regulation (2): Pathophysiological aspects

Pathophysiological aspects of CBF regulation can be broadly divided into the loss of autoregulatory capacity and blood flow & metabolic abnormalities. CBF autoregulation can be modified or disturbed in several disease states, such as hypertension, diabetes and particularly following cerebral ischaemia (Paulson *et al.* 1990). The extent to which this occurs depends on the disease state, and whilst chronic hypertension induces a re-setting of limits towards higher pressure, ischaemia profoundly affects autoregulatory capacity (Symon *et al.* 1973; Harris & Symon, 1984; Paulson *et al.* 1990). In uni- or bilateral carotid disease regional vasodilatation may maintain CBF within the supply territory of the occluded vessel. However, occlusion of arteries such as the MCA precludes such compensatory

mechanisms (Heiss & Podreka, 1993). Loss of autoregulation within such fields has two major implications for ischaemic tissue. First, the inability of CBF to match metabolic demand, and second, the potential for damage aggravation on recannilisation. The latter predicament arises from the sustained loss of autoregulatory capacity during the initial phase of reperfusion, whereby any blood pressure increase will aggravate tissue oedema and BBB damage (Hoedt-Rasmussen *et al.* 1967; Paulson, 1990).

Pathophysiological abnormalities in blood flow and metabolism have been investigated using modern neuroimaging techniques, although the limited number of studies have left inconsistencies (Heiss & Podreka, 1993). Changes in the local haemodynamics and energy state offer an insight into the interrelationships of perfusion and brain acticvity (Baron *et al.* 1989). Positron emmision tomography (PET) and single photon emission computed tomography (SPECT) studies performed on patients suffering ischaemic stroke have elucidated uncoupling of regional CBF (rCBF), regional cerebral blood volume (rCBV), cerebral metabolic rate for oxygen ($CMRO_2$) and the cerebral meatbolic rate for glucose (CMR_{glu}). Whilst these variables are coupled under homeostatic conditions when parametrs such as pCO_2 are accounted for, occlusive disease states uncouple these (Heiss & Podreka, 1993). These variables are individually affected and uncoupled during ischaemia, and measurement of haemodynamic and metabolic parameters allows distinction of ischaemia from oligaemia or reperfusion which also affect these physiologic parameters (Baron *et al.* 1989; Heiss & Podreka, 1993). Multiple parameter assessment is necessary because, except under conditions of tissue infarction in

which CBF is <12ml/100g/min, CBF measurement alone is insufficient to assess tissue viablility (Heiss & Podreka, 1993).

In chronic states of arterial (e.g. MCA) occlusion, three or more metabolic and perfusion abnormalities are distinguishable consequent on the severity of occlusion (Baron *et al.* 1989). In the first stage, CBV increases in the presence of normal CBF, consequent on the vasodilatory compensatory mechanisms of autoregulation. However, as obstruction becomes more severe, oxygen extraction fraction (OEF) increases consequent on a fall in CBF, and as this reserve becomes exhausted with further perfusional deficits, oxygen consumption falls (Baron *et al.* 1989). In acute stroke, CMR_{glu} is generally reduced within the affected area, although non-oxidative respiration may cause an increased rate, and measurement of CMR_{glu} and pH shifts are a sensitive measure of metabolic disturbance and tissue infarction (Heiss & Podreka, 1993). PET studies have demonstrated with few exceptions that regions demonstrating increased CMR_{glu} and decreased $CMRO_2$ undergo necrosis although such tissue represents that salvageable by therapeutic intervention (Heiss & Podreka, 1993).

Neuroimaging techniques have also provided insight into the more remote effects of ischaemia whereby metabolic and haemodynamic disturbances exceed the morphologically damaged tissue (Heiss & Podreka, 1993). Studies of CBF following stroke have suggested that neuronal connectivity between brain regions may be responsible (Tamura *et al.* 1981). Regions affected include the substantia nigra, which receives GABAergic inhibitory input from the striatum (damaged following occlusion of the MCA), and the cerebellum (Perani *et al.* 1987). In conclusion,

pathophysiological aspects of CBF regulation are manifest as differential alterations in perfusional and metabolic parameters such as CBF and $CMRO_2$, as cerebrovascular compensatory mechanisms compensate within certain limits to acute and chronic ischaemic conditions. Neuroimaging techniques have significantly contributed to our understanding of pathophysiological aspects of cerebral perfusion and metabolic disturbances during ischaemia, and application of such techniques have led to definable alterations in blood flow and metabolic parameters that allow identification of differential vascular occlusion states and morphological outcome following stroke.

1.3. Ischaemic cell death

1.3.1. Histopathology of ischaemic tissue

Correct identification of pathologically damaged tissue following an ischaemic insult is vital since it allows quantification of ischaemic brain damage in experimental models of stroke and facilitates the model's employment as a screen for putative neuroprotectants. A large number of studies and reviews have addressed the histological identification of pathologically damaged cells in ischaemia (Graham, 1985). As a consequence, some generalisations may be made concerning the nature of such cells at the microscopic level. However, as will be addressed subsequently, ischaemic insults do not cause the same form of cell death throughout brain tissue.

1.3.2. Necrosis & apoptosis

There are two basic pathways by which cells may die: necrosis and apoptosis. Apoptosis may also be the mechanism by which cells die which currently come under the term delayed neuronal death (DND) (Du *et al.* 1996). Necrosis may reflect a "passive" cellular response as a consequence of trauma causing acute loss of regulation and function (Kerr & Harmon 1991; Cotman & Anderson 1995). By contrast the term apoptosis refers to a different pathology, where the cell may actively participate in its death by gene-directed suicide, and such a program may be initiated in response to a signal in the form of a toxin, insult or via a physiological pathway (Bursch *et al.* 1992).

Brain lesions of ischaemic origin may be defined according to whether neurones only (selective) or all cell types (pan-necrosis or infarction) are affected. The former represents changes to selected neurones, whilst in pan-necrosis all cells die (Auer & Benveniste, 1997). Definitions of cells irreversible damaged by ischaemia have been documented previously (Farber *et al.* 1981; Farber, 1982; Trump, 1984). Necrotic neurones can be identified as exhibiting pyknosis, karyolysis and cytoplasmic eosinophilia. Cells may exhibit cellular alterations such as scalloping, swelling and breaks in plasma & nuclear membranes, marginal clumping of chromatin (karyorrhexis), dilatation of the endoplasmic reticulum, & ribosome dispersal (Garcia *et al.* 1995). Subsequently, cells undergo rupture of nuclear, organelle and plasma membranes, dissolution of ribosomes & lysosomes, the nucleus swells and the membrane ruptures. Finally, basophilia (staining) is lost, and a "ghost" remains with cell boundaries becoming indistinct. Further to this is the involvement of surrounding cells and exudative inflammation develops in adjoining tissue with damage to other

cells and an inflammatory response, although debris is eventually phagocytosed by specialised cells (Kerr *et al.* 1972; Wyllie *et al.* 1980).

The second morphological and pathophysiological category of cell death is apoptosis. Kerr *et al.* (1972) first coined the term apoptosis to describe a previously recognised process by which cells died in a controlled manner. There are striking differences between necrotic cell death and apoptosis. Work by Kerr *et al.* (1972) and Wyllie *et al.* (1980) established most of the fundamental and significant aspects of apoptosis, identifying its particular morphology, that it is an active, inherently programmed phenomenon, and that certain environmental stimuli can initiate or inhibit it. Furthermore they recognised it as an important basic phenomenon in the turnover of cells in normal and neoplastic tissues. Furthermore, apoptosis commonly affects single cells rather than populations. In the earliest stages of apoptosis, chromatin is seen to aggregate in masses that touch the nuclear membrane, which becomes convoluted and later grossly indented. Following the early changes in the nucleus, cytoplasmic condensation begins, and cell attachments disappear. Organelles become crowded, vacuoles may appear and nucleus fragmentation occurs. Finally, apoptotic bodies form, with the production of membrane-bound spherical shapes containing the cellular contents such as organelles and chromatin still intact. Apoptotic bodies, which may vary widely in size and number are subsequently dispersed into the intercellular space, and then are commonly phagocytosed. Phagocytosis is a rapid process and no inflammation is detectable (Kerr *et al.* 1972). Once ingested autolysis of phagocytosed material occurs and the phagocytosed apoptotic bodies are eventually reduced to residual lysosome bodies prior to

disappearance. The process is of sudden onset and early stages are quickly effected, with the process of cellular shrinkage and membrane "blebbing" being completed often within minutes (Wyllie *et al.* 1980). Wyllie et al. (1980) suggested that there was no answer to how quickly apoptosis might be initiated after a stimulus, with times varying according to stimuli and cell.

In addition to the morphological characteristics that identify cells as apoptotic is the presence of a DNA ladder resolved by gel electrophoresis. This ladder pattern is representative of the actions of endonucleases cleaving cell DNA into multiples of ~180 base-pairs (bp) (Wyllie *et al.* 1980). This pattern of endonucleolytic damage is often used as an indicator that cells having undergone apoptosis (Li *et al.* 1995a).

Considerable research has now addressed the molecular mechanism of apoptosis. Evidence is accumulating that the path by which a cell undergoes apoptosis may be linked with the cell cycle (Steller, 1995). Furthermore, it has been suggested that apoptosis is a premature, abortive mitosis, although distinct biochemistry distinguishes apoptotic and mitotic processes (Meikrantz & Schlegel, 1995). Suggestions have also been made that apoptosis occurs as a consequence of incompatible signals for proliferation and cell cycle arrest (Yonish-Rouach *et al.* 1993). A connection of the two processes was the observation that apoptosis occurs in vivo during proliferation of tissue (Oppenheim, 1991). Similarly, cells undergoing mitosis, mitotic catastrophe (when the cell cycle is disrupted by overexpression of cell cycle components or sustains mutations; Russel & Nurse, 1987) or apoptosis have been seen to share morphological and biochemical similarities (King & Cidlowski, 1995).

There are a number of genetic changes that have been identified in cells undergoing apoptosis. A number of important findings relating to genetic transcription were shown in the studies by Pandey & Wang (1995). They used density arrested Swiss 3T3 cells which die by apoptosis when deprived of serum. Apoptosis was determined based on identification of DNA laddering and morphology, such as plasma membrane blebbing, formation of apoptotic cell bodies and chromatin condensation. Using incorporation of [^{3}H] thymidine and bromodeoxyuridine (BrdU) following serum deprivation, they found cells synthesise a small amount of DNA. Furthermore, using a cell cycle marker, proliferating cell nuclear antigen, they showed that this occurred during the G1 phase of the cell cycle. They also found evidence that synthesis and fragmentation of DNA could occur in the same cell as seen by BrdU incorporation and $3'$-end labelling of DNA by biotinylated uridine. Thus it seems apoptosis in this model is intimately linked with the cell cycle and cells can undergo DNA synthesis and degradation simultaneously. The results of Pandey & Wang (1995) are suggestive of cells being released from the quiescent state to enter the cell cycle *en route* to apoptotic death as indicated by induction of cell cycle proteins. Furthermore these molecular events characterise a G1 phase nucleus, although the re-entry is brief, incomplete and essentially abortive. Pandey & Wang (1995) also suggest c-fos and c-jun, immediate early genes (IEGs), combine to form the AP-1 complex, a transcription factor essential to the apoptosis machinery. Thus the nucleus of a cell must synthesise its own suicide machinery.

Studies in the nematode worm *Caenorhabditis elegans* (*C. elegans*) provide the most direct evidence that apoptosis occurs via a gene directed suicide programme

(Raff *et al.* 1992). In *C. elegans*, 1090 cells are generated during the development of the worm. Of these, 131 die by an intrinsic mechanism (Kumar, 1995). Studies have revealed that 14 genes function in this programmed cell death. Of these genes, ced-3, ced-4 and ced-9 play the most important roles in the control of cell death. The other genes function in the engulfment and disposal of dead cells (Hengartner & Horvitz, 1994). The genes ced-3 and ced-4 are essential for the death of the cells, while ced-9 functions to protect cells from the death programme in cells not destined to die. Elegant studies have been performed manipulating the expression of these genes in order to determine their role in apoptosis. Mutations removing either ced-3 or ced-4 result in the worm having extra cells, although these extra cells do not cause the early death of the worm (Ellis *et al.* 1991). By contrast mutants lacking ced-9, which seems to act as a brake on the suicide program, undergo extra cell death and die early in development (Hengartner *et al.* 1992). Further mutation experiments reveal that the ced-3 expression is not dependent on ced-4 function since mutation of ced-4 has no effect on ced-3 mRNA (Yuan *et al.* 1993). While ced-3 deletion results in extra cells in *C. elegans*, overexpression of ced-3 has been shown to enhance apoptosis (Miura *et al.* 1993).

Cloning of ced-3 reveals a protein of 503 amino acids of a novel cytoplasmic cysteine protease family. The protein has many phosphorylation sites and expression is greatest during PCD execution (Yuan *et al.* 1993). By contrast, ced-4 encodes a novel protein with two potential Ca^{2+} binding domains (Ellis *et al.* 1991).The cloned ced-3 revealed homology to the mammalian enzyme interleukin 1β-converting enzyme (ICE) (Yuan *et al.* 1993), suggesting evolutionary conservation of PCD,

although a mammalian homologue of ced-4 has not been discovered (Kumar, 1995). Consequently a number of researchers have studied ICE with a view to understanding whether it mediates apoptosis in human cells. ICE cleaves a 31 KDa interleukin-1β precursor (pro-IL-1β) to generate the mature 17.5 KDa IL-1β (Kostura *et al.* 1989), a cytokine and mediator of cell death itself (Yamasaki *et al.* 1995). ICE itself is activated by proteolytic cleavage from a 404 amino acid precursor (p45) to form a dimer of p20 and p10, both of which are required for function (Thornberry *et al.* 1992) as a tetramer of $(p20\text{-}p10)_2$. A number of lines of evidence now suggest ICE and its homologs like prICE regulate apoptosis in mammalian cell lines (Kumar, 1995).

Bcl-2 is structurally and functionally homologous to the *C. elegans* gene ced-9 (Vaux *et al.* 1992). As mentioned ced-9 acts as a brake on the actions of ced-3 and ced-4 in *C. elegans* apoptosis. Abnormal activation of ced-9 abolishes the normal developmental cell death, whilst if ced-9 is inactivated, many cells which normally survive now die. Hengartner *et al.* (1992) showed that if ced-3 and ced-9 are both inactivated neither the normal nor extra cell deaths occur, and the animal survives. Bcl-2 encodes a membrane-associated protein (Martinou *et al.* 1994) from its proto-oncogene sequence first identified in human follicular cell lymphomas. It is thought to be associated with the cytoplasmic surface of the nuclear envelope, mitochondria and endoplasmic reticulum. Bcl-2 protein is expressed in most neurones (Martinou *et al.* 1994), but with age, expression declines in most neurones of the CNS but is retained in peripheral nervous system neurones (Merry *et al.* 1994). The exact function of Bcl-2 is unknown (Hockenberry *et al.* 1990; Raff *et al.* 1993). Bcl-2 is

now recognised as one member of a family of related gene-products including bcl-xl and bax (Raff 1993; Martinou *et al.* 1994). It has since been identified as an apoptosis suppresser in a number of models. Bcl-2 oncoprotein has been shown to support cultured cells such as sympathetic neurones (Garcia *et al.* 1992; Allsopp et al. 1993), cerebellar & nigral cells (Zhong *et al.* 1993) and PC12 cells (Mah *et al.* 1993).

It appears that apoptosis is a gene directed program which, while distinct from the cell cycle, may utilise proteins of the mitotic cycle. Specific enzymes and other proteins essential to the cell cycle may be essential to a cells ability to undergo apoptosis, although unique proteins may also be required. Of these genetic products, a number of key regulators are beginning to emerge as well as some enzymes such as ICE which may prove central to apoptosis. A cell may leave the cell cycle to undergo a program of apoptosis, and studies have shown that most cells will in fact undergo apoptosis if presented with the right environment and stimulus.

1.3.3. The ischaemic core

Studies suggest that a number of factors determine the ischaemic damage that results from focal cerebral ischaemia. These include the severity of the blood flow reduction, the duration of occlusion, the regional location of the insult, and the metabolic state of the tissue prior to the ischaemic episode (Traystman *et al.* 1991). Consequently, multiple mechanisms may contribute to cell death, and a given ischaemic insult produces a gradient of challenges to cerebral tissue as a consequence of the vascular blood supply. This translates into a broadly definable separation of the ischaemic

environment. The core or focus of an ischaemic area represents tissue heavily dependent on perfusion from the occluded vessel, and cell death here is likely to be due to loss of perfusional pressures causing severe tissue oedema within a localised area. Cells within this area may well undergo lysis due to subsequent osmotic-mechanical stresses (Siesjo 1992a). The interuption or cessation of blood flow to this region is intense, and the complete collapse of cell membrane ionic gradients means that cells undergo rapid and profound osmotic pressures whereby water enters cells down a osmotic gradient. It is predicted that cell death within the core of an ischaemic insult may result from such chemo-mechanic stresses.

Cerebrovascular morphology allows some generalisation as to the anatomical distribution of core regions of ischaemia. Identification of major anastomoses as well as end-territory vessels means that occlusion of a specific artery produces regions reliably encompases by the term "core" and "penumbra". Rat MCA occlusion models have documented the anatomical regions that fall within these terms in greatest detail. The core region of ischaemia following MCA occlusion lies within the striatum, which recieves its blood supply by the striate (lenticulostriates in humans) branches of the MCA. Brain regions within the supply of this vessel do not receive collateral perfusion, and occlusion of even short duration leads to infarction, as a consequence of rapid energy depletion and osmolytic pressure. Neuroprotection strategies show poor success in reducing striatal damage following MCA occlusion in most cases (Butcher *et al.* 1990). By contrast much of the cortex within the vascular supply of the MCA may receive some collateral circulation and areas distal to the core may be amenable to neuroprotective intervention. However, certain

cortical structures close to the proximal segment of the MCA also represent core regions, such as piriform cortex.

1.3.4. The ischaemic penumbra

Whilst the tissue closest to the site of occlusion (eg. piriform & insular cortex and lateral striatum) is the core of the ischaemic area wherein energy failure due to substrate depletion is rapid and often complete, the tissue lying further away from the focus suffers less severe reductions in substrate depletion. Such an area may receive some collateral perfusion, although still subject to oxygen and glucose deprivation, and has been termed the "penumbra". This region displays characteristics specific to its ischaemic predicament and represents the region of ischaemic tissue which may be recoverable with pharmacological agents. The importance of intervening in the penumbral events is illustrated by PET scan findings in human subjects that show that penumbral (or peri-infarct) tissue is recruited to infarct over time (Heiss *et al.* 1992).

Astrup *et al.* (1981) defined the penumbra of an ischaemic region as brain tissue perfused at a level between the thresholds of functional impairment and of morphological integrity, which has the capacity to recover if perfusion is improved. They made the point that the term ischaemic penumbra is descriptive only, constituting tissue experiencing a compromised energy state and existing within the boundaries of upper and lower thresholds but where residual perfusion prevents deterioration towards necrosis. However with subsequent studies it is becoming clear that despite "penumbra" being a term to describe a particular tissue state, within this

delineation some physiochemical factors are constants, some vary and some are still a matter of debate.

Subsequent definitions of the penumbra of ischaemic tissue recognise the complexity of the phenomena and the penumbra must be envisaged as an ischaemic area where perfusional disturbance is tolerated according to the severity and duration of the insult. This relationship and secondary mechanisms that extend this disturbance make the penumbra a dynamic region in which damage progresses with time from the core to the periphery of the ischaemic brain region (Heiss, 1992).

In researching what is a dynamic process of changes in ischaemic tissue (Strong *et al.* 1983; Heiss *et al.* 1994), problems are inherent since the application of techniques often requires established tempero-spatial details of the penumbra itself, which is accepted as poorly definable in absolutes and more reflects a concept than a physically definable entity. (i.e. one needs to know where the penumbra is in order to study it but such a requirement is difficult to address due to the nature of the phenomenon). Hence caution must be exercised in interpreting findings from "the penumbra". Certain events have been used as constants in its definition such as the loss of EEG activity in tissue not in the core of an occluded vessel's vascular territory (Takagi *et al.*1993), and monitoring the region of increased local glucose use (lCMRglc) in combination with moderate acidosis (Peek *et al.*1989). More erroneous methods of definition have also been employed such as assigning a particular region of neocortex (Folbergrova *et al.* 1992), an assumption which may not be altogether valid. The penumbral region of an infarct may also be delineated pharmacologically as that part of the infarct salvageable by drugs (Siesjo, 1992b).

The main metabolic characteristics of the penumbra are increased oxygen extraction, acidosis, high glucose utilisation and residual ATP (Obrenovitch, 1995). This is consequent to anaerobic respiration within the penumbra and increased metabolic demands on the cells due to changes in extracellular EAA levels causing depolarisation. There is not yet a precise consensus as to what level of CBF constitutes "penumbra". Whilst the order of physiological and biochemical variables is consistent between brain regions and tissues, the absolute values for a given region are dependent on the metabolic and functional state of the tissue at the time of the insult (Obrenovitch, 1995).

Many studies have however correlated blood flow reductions to metabolic changes such as increases in lactate, and extracellular potassium. If penumbral tissue represents that which is quiescent but within which the cells retain their structural integrity, quite accurate levels of blood flow can be ascribed to the penumbra. This level must therefore be below the threshold at which protein synthesis is inhibited (~50ml/100g/min), but above that where ion and water homeostasis is lost (6-15ml/100g/min) (Hossmann, 1994), which would represent irreversibly damage tissue.

The penumbra constitutes a relatively narrow rim surrounding the core area of ischaemia (Obrenovitch, 1995). Hossmann (1994) suggests the physical location of the penumbra following MCA occlusion can be visualised by subtracting the region where ATP is reduced from an image of decreased pH, and an emerging consensus on where the penumbra typically exists has been achieved to some extent in models of MCA occlusion. Many studies (Folbergrova *et al.* 1992; Takagi *et al.* 1993;

1994a; Tomlinson *et al.* 1993) identify the area of penumbra following MCA occlusion as a region in the parietal cortex which overlies the core. The basis for this varies between studies but usually derives from CBF reductions in regions not commonly infarcted after MCA occlusion. Neuronal loss does occur within previously penumbral tissue, but not as extensively as in the core, and penumbral tissue will either infarct (if flow is not reestablished), recover (if flow is reestablished) or remain functionally inactive (Tomlinson *et al.* 1993; Heiss *et al.* 1994; Hossmann, 1994). This latter point is poorly understood in a quantitative and temporal context (Siesjo 1992a).

How recruitment occurs and how it can be prevented is much debated (Siesjo, 1992a). Either infarction will proceed, absorbing penumbral tissue until well-perfused tissue is reached, or islands of necrosis develop in penumbral tissue that may coalesce within the penumbra to extend the infarct. The excitotoxicity and anoxic depolarisation theories may well account for the latter process, causing already "at risk" cells to be subjected to conditions they are unable to withstand (Heiss & Graf, 1994). This is supported by the finding that the magnitude of glutamate release during ischaemia is correlated to infarct volume and hence, final outcome (Takagi *et al.* 1993; Taguchi *et al.* 1993; Butcher *et al.* 1990). Evidence exists that spreading depression contributes to recruitment (Obrenovitch, 1995). Nedergaard & Astrup (1986) first described depression-like depolarisations which might account for the expansion of tissue injury. However, SD is rarely observed in humans or cats casting doubt as to its widespread significance in this context (Obrenovitch, 1995).

That tissue within the penumbra can fully recover was demonstrated in the study by Tomlinson et al (1993) in rabbits, using umbelliferone fluorescence to identify changes in pH and CBF. Recordings showed the region comprising much of the penumbra remained acidotic for 45 minutes post occlusion but returned to normal after 3 hours. The pattern of return showed regions closest to collateral vessels recovered first. Acidic foci within this region of mild (pH 6.61) acidosis were seen to coalesce as time proceeded presumably as recruitment to infarct occurred, while other areas recovered, supporting a number of outcomes for the ischaemic penumbra.

Specific protein and nuclear events may occur in the ischaemic penumbra (Heiss & Graf, 1994). Of these, the expression of IEGs and heat shock proteins (HSPs) after focal cerebral ischaemia has stimulated considerable interest (Welsh *et al.* 1992; Kinouchi *et al.* 1993). Welsh *et al.* (1992) and Uemura *et al.* (1991) showed that MCA occlusion in rats induces temporal and spatial patterns of c-fos protein expression although c-fos is not expressed in the core. Furthermore c-fos expression is modulated by MK801, a drug effective in salvaging penumbral tissue. Thus, ischaemia may induce protein synthesis and nuclear transcription not normally occurring in brain tissue. However, complications arise again since the distribution of ischaemia and expression of c-fos, jun-B (another IEG) and HSP may occur in territory not directly affected by MCA occlusion-induced ischaemia (Welsh *et al.* 1992). It is unclear at present as to the implications of IEG and HSP expression, although there is the suggestion that it reflects progression of ischaemic injury as well as the vulnerability of certain cell populations (Heiss & Graf, 1994).

1.3.5. Mechanisms of cell death in stroke (1): Ionic changes

Three factors are of central importance in explaining the response of brain tissue to ischaemia. The first of these is that the energy demands of the intact mammalian brain are met almost exclusively by the oxidation of glucose (Siesjo, 1984; Hossmann, 1994). Second is the high energy demands of the brain, which utilises 20% of circulating oxygen (Graham, 1984), and third is the inability of the brain to store glucose in any significant quantity, making brain cells dependent on the continuous delivery of substrates.

Since oxidative catabolism of glucose is required for adequate ATP production via the mitochondrial respiratory chain, ischaemia rapidly devastates ATP production and inadequate oxygen is available for any remaining glucose to be metabolised efficiently. Neurones are capable of anaerobic respiration although this is an inefficient and inadequate source of cellular ATP. ATP is the cells major source of energy for enzymatic actions. In the resting neurone, an unequal distribution of physiological ions and a membrane potential exist as a consequence of passive and energy driven ion flux across the membrane. This uphill transport depends directly or indirectly on ATP hydrolysis. The membrane potential is maintained by the Na/K-ATPase, with the distribution of the K^+ ion determining to a large extent the membrane potential (Kuffler *et al.* 1984).

The Na^+ gradient, which is distributed across the membrane with a roughly ten-fold difference between extracellular (high) and intracellular (low) concentrations, is also of crucial importance as it is the ion that not only the propagates action potentials but is a source of energy for the movement of other ions and substrates

into and out of the cell and for the reuptake of neurotransmitters (e.g. glutamate). Loss of the Na^+ gradient and hence Na^+ homeostasis will prevent the extrusion of certain ions, prevent cessation of certain neurotransmitter effects, and promote cellular oedema as water is an obligatory molecule in Na^+ movement. The initial event following CBF interuption is thought to be the loss of sufficient ATP production to maintain the crucial energy-dependent homeostatic functions such as the Na/K-ATPase. Pump failure, as well as enhancement of membrane leaks are believed to cause loss of ion homeostasis. Ion fluxes occur in two main phases in cerebral ischaemia. First there is intracellular K^+ loss and H^+ accumulation. The latter is a consequence not only of the switch to anaerobic metabolism which has the consequence of producing H^+, but also accumulation of intra- and extracellular carbon dioxide (CO_2), which is no longer being removed and causes acidification of the milieu as it forms the bicarbonate anion releasing H^+ in water (Siesjo, 1992a; Seisjo, 1984).

During the second phase, K^+ still leaves the cell, and Na^+, Ca^{2+} and Cl^- enter the cell through voltage dependent sodium (VDSCs) and calcium (VDCCs) channels. The Cl^- ion is obligate to Na^+ movement. This causes cellular depolarisation, which is preceded by extensive energy failure (Siesjo, 1992a; Seisjo, 1984).

The deranged pump-leak relationship, and the critically low ATP state of the cell has the consequence that Na^+, Ca^{2+}, K^+ and H^+ ion levels cannot be re-established, and they initiate a series of biochemical events which in the case of Ca^{2+} and H^+ have direct pathophysiological consequences for the cell, while the loss of the Na^+ gradient further promotes Ca^{2+} accumulation. Another pathologically important consequence

of the Na^+ and Cl^- movement into the cell as the Na/K-ATPase fails is that osmotically obligated water enters the cell, causing cell swelling and osmolytic damage (Siesjo, 1992a; Seisjo, 1984).

1.3.6. Mechanisms of cell death (2): Excitatory amino acids and Ca^{2+}

One of the key consequences of ionic homeostasis failure is extracellular accumulation of neurotransmitters and evidence from a range of studies implicate glutamate and other neurotransmitters as key factors mediating ischaemic cell death (Choi, 1990). Indeed, of the neuroactive amino acids, accumulation of glutamate is the most profound (Butcher *et al.* 1990). A number of processess lead to high extracellular levels of glutamate during ischaemia including neuronal vesicle exocytosis, compromised glial and neuronal reuptake (due to the compromised ATP levels which are required for this process) and from the metabolic pool as a consequence of cell lysis.

Glutamate release, and subsequent activation of its receptors during ischaemia, has direct pathophysiological consequences for neurones. Glutamate is the most abundant neurotransmitter in the brain, accounting for neurotransmission at ~95% of all central synapses, and acts at both ionotropic (NMDA and AMPA/KA) and metabotropic (or quisquilate) receptors (Choi, 1990). In the case of the ionotropic receptors, Na^+ entry is gated directly by activation of either of the receptor "groups", leading to cell depolarisation. The NMDA receptor additionally gates Ca^{2+} entry, while the AMPA/KA receptors, although not directly gating Ca^{2+} entry, facilitate Ca^{2+} entry via the opening of voltage dependent calcium channels (VDCCs) of which

the L- (dihydropyridine) N-, P- and T- are best studied. The metabotropic glutamate receptors are G-protein coupled receptors (GPCR) linked to phospholipase C (PLC) and adenylyl cyclase. The former is a membrane bound enzyme which hydrolyses membrane lipid to form diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 is an hydrophilic molecule that triggers release of sequestered Ca^{2+} via the opening of IP_3 sensitive stores in the neuronal sarcoplasmic reticulum. Thus the consequence of glutamate release is the depolarisation of postsynaptic cells, the further propagation of action potential in the postsynaptic cell and a considerable rise in intracellular calcium.

The involvement of glutamate in neurotoxicity can be traced back as far as the demonstration by Lucas & Newhouse (1957) that L-glutamate could destroy neurones in the retina. Excitotoxicity was the term used to describe the effects of glutamate and related compounds, and this led to the postulation that such compounds might contribute to the pathogenesis of CNS cell death in disease states (Olney, 1969; Olney, 1986).

The neurotoxicity of glutamate can be divided into a three stage process (Choi *et al.* 1990) - induction, amplification and expression. This is expressed as the initial ionic intracellular derangements caused by glutamate receptor activation, with resultant accumulations of Na^+, IP_3 and DAG. The derangements are then amplified, most importantly by Ca^{2+} build up, and the pathogenic significance of Na^+ and the products of PIP_2 hydrolysis elevating may be largely due to an enhancing effect on Ca^{2+} accumulation. The final stage of glutamate neurotoxicity as outlined by Choi *et*

al. (1990) is expression via the cascade of events which may include some or all of the aforementioned consequences of raised Ca^{2+}.

In vivo support for this hypothesis has been gained from studies examining changes in excitatory amino acid (EAA) levels during stroke, and studies have correlated increases in amino acids such as glutamate with histopathological ischaemic damage following MCA occlusion in rats. Butcher *et al.* (1990) utilised microdialysis to measure striatal and cortical changes in excitatory amino acids, finding increases of >2000% for glutamate, and other potentially neurotoxic EAAs following MCA occlusion in the rat, correlating these increases to the volume of ischaemic damage. Wahl *et al.* (1994) provided evidence, not only of this raised extracellular glutamate during stroke, but also evidence for the relative contributions of different glutamate "pools" to extracellular accumulation. Using a rat model of focal cerebral ischaemia and microdialysis, they found a biphasic increase in extracellular glutamate after ischaemia was initiated. First phase (~10 min.) saw an increase to ~36μM, the second phase (reaching a maximum after 55min.) reached ~82μM while at resting values levels were ~6μM. The overall changes in extracellular glutamate were similar in zero Ca^{2+} ACSF, although the first phase plateau was absent and extracellular glutamate increased at a slower rate in early ischaemia. They concluded that most glutamate released into the extracellular space in severe ischaemia is not from transmitter pools but metabolic in origin - a consequence of ischaemia-induced neuronal damage, an idea first suggested by Jorgensen & Diemer, (1982). However, this does not rule out the contribution of neurotransmitter glutamate following cell death or consequent on glutamate uptake

reversal. The evidence in this report might be considered cautiously given the complex problems in producing zero calcium in the extracellular milieu and in light of the findings of Obrenovitch *et al.* (1995) who showed that microdialysis can influence pathological conditions during studies by buffering changes in extracellular fluid composition. However, it seems that Ca^{2+}-dependent ischaemia-induced glutamate release (i.e. from neuronal pools) may be minor in comparison to total overflow of extracellular glutamate. This is also supported by the finding that exocytosis would not be sustainable for more than a few minutes as NMRS has shown ATP depletion within 10 minutes of complete ischaemia (Shimizu *et al.* 1993).

Induction of massive glutamate release during ischaemia leads to an unphysiological influx of Ca^{2+} to the cell (Choi, 1990). An increased intracellular Ca^{2+} concentration is one of the mechanisms that contribute to ischaemic cell damage (Heiss & Podreka, 1993), and perhaps the most important consequence of a perturbed cellular energy state (Siesjo & Bengtsson, 1989). Extrusion of Ca^{2+} is decreased due to reduced Ca^{2+}-ATPase or the $3Na^{+}/Ca^{2+}$ exchange activity and decreased binding or sequestration of Ca^{2+} which is compromised by the failure of sarcoplasmic reticulum pumps and displacement of Ca^{2+} from binding proteins due to intracellular acidosis.

Identification of specific downstream effectors of pathological Ca^{2+} levels is incomplete, but activation of one or more Ca^{2+}-dependent enzymes such as PLA_2, PKC or the calpains has been proposed to account for at least some damage that can originate from Ca^{2+}overload and maybe kill neurones.

1.3.7. Free radicals and nitric oxide

There is growing evidence that the formation of free radicals also contributes to ischaemic brain damage (Kiyota *et al.* 1993; Choi *et al.* 1993). The sequential, univalent reduction of molecular oxygen results in the formation of a number of free radicals (molecules with an unpaired electron). These include the superoxide anion ($O_2^{\cdot-}$) and the hydroxyl radical ($OH^{\cdot}$), while another free radical species, nitric oxide (NO), may also be of significance (Werns & Lucchesi, 1990).

Although NO mediates some neurotransmission, it may be neurotoxic under certain conditions (Dawson & Snyder, 1994), and NO has been postulated as having a role in the pathogenesis of brain damage during ischaemia (Dawson *et al.* 1992). Dawson *et al.* (1991) proposed that NO formation by neuronal cNOS (constitutive nitric oxide synthase) mediates NMDA receptor-mediated neurotoxicity. NO has subsequently been implicated in glutamate neurotoxicity from studies in a variety of tissues including striatal slices (Kollegger *et al.* 1993), hippocampal slices (Izumi *et al.* 1992; Moncada *et al.* 1992; Wallis *et al.* 1992) and cell culture experiments (Corasaniti *et al.* 1992, Tamura *et al.* 1992; Dawson & Snyder, 1994). Contradictory evidence of a role for NO inhibition in glutamate neurotoxicity has also been reported (Demerle-Pallardy *et al.* 1991; Pauwels & Leysen 1992; Regan *et al.* 1993).

A role for NO in mediating neurotoxicity are derived from the efficacy of procedures that decrease NO production and effects. These include inhibitors of nitric oxide synthase (NOS), calmodulin antagonists (essential NOS cofactor), flavoprotein inhibitors and reduced haemoglobin (which scavenges NO), which reduced

neurotoxicity in primary cultures of cerebral cortical neurones (Dawson & Snyder, 1994).

Glutamate activates NO production by gating Ca^{2+} entry via NMDA receptor activation and NOS is activated by increases in intracellular Ca^{2+}. It is suggested the neurotoxic effects of NO over-production in cells probably evolves from its interaction with the superoxide anion to form peroxynitrite (Beckman *et al.* 1990; Beckman, 1991). Such species (or NO itself which can exist in free radical form) may then damage intracellular components leading to cell death or lysis. NO may damage cellular DNA (Zhang *et al.* 1994), which may have a secondary neurotoxic effect. Damage to cellular DNA stimulates the nuclear enzyme poly (ADP ribose) synthetase (PARS) to catalyse attachment of 50-100 ADP-ribose units to proteins such as histones as well as PARS itself (de Murcia *et al.* 1991). For each molecule of ADP-ribose added, 1 NAD is used, and 4 ATP is used to regenerate NAD. NO is also known to poison the mitochondrial chain (Dawson & Snyder, 1994), attenuating cellular function on restoration of metabolite levels.

Evidence exists for changes in NO levels during focal cerebral ischaemia. Malinski *et al.* (1993) found increases from <10nM to 2.2μM subsequent to transient MCA occlusion, while Sato *et al.* (1993) reported increases in NO commensurate to the degree of global ischaemia, and Kader *et al.* (1993) reported NO metabolite, cGMP and NOS activity increases following onset of focal cerebral ischaemia. However, it is currently unclear as to the contribution of each potential source of NO (neuronal, glial and/or endothelial.

1.3.8. Intracellular enzymes

Activation of Ca^{2+}-dependent enzymes such as the calpains has been proposed to account for some ischaemic cell damage. Calpain is a Ca^{2+}-activated neutral protease, of which there are two major isoforms. Calpain also appears to be a selective enzyme for a subset of intracellular proteins (Lee *et al.* 1991). Amongst its targets are cytoskeletal proteins (e.g. spectrin), G-proteins, enzymes involved in signal transduction and transcription factors. Cytoskeletal breakdown may therefore be triggered by activated calpain, leading to decreased membrane continuity and as a consequence of compromised energy, loss of the ability of the cell to resynthesize necessary membrane components. This may lead to cell lysis. *In vitro* and *in vivo* evidence for a role for calpain include:

1. The calpain inhibitor MDL 28170 and calpain inhibitor 1 were shown to improve functional recovery of hippocampal and gerbil neocortical slices from hypoxia (Arlinghaus *et al.* 1991; Hiramatsu *et al.* 1993).
2. *In vivo*, MDL 28170 significantly decreased infarct in a rat model of focal cerebral ischaemia (Hong *et al.* 1994), while Rami & Kriegstein (1993) found calpain inhibitor 1 to be protective in the CA1 subfield of the hippocampus in a rat model of global ischaemia.
3. The novel calpain inhibitor AK295 has been shown to be protective in a focal cerebral ischaemia model (Bartus *et al.* 1994).

There is growing evidence for a role for the protein phosphatase calcineurin in mediating cell death. Calcineurin is a Ca^{2+}-activated protein phosphatase of the serine-threonine type that has been identified in a number of tissues including the

brain. Recently the immunosuppresant FK506 has been demonstrated to exhibit potent neuroprotectant actions following focal cerebral ischaemia. Sharkey & Butcher, (1994) demonstrated that FK506 is as efficacious as MK801 in reducing the volume of infarct following MCA occlusion. FK506 (Tacrolimus) is a 23-membered ring macrolide, which has strong immunosuppressive effects (Kino *et al.* 1987; Tamura *et al.* 1994). It has been widely employed for suppressing organ transplant rejection as well as some other applications including treatment of autoimmune diseases (Tamura *et al.* 1994). Its intracellular effects show some mechanistic analogy to other immunosuppressants like cyclosporin A and rapamycin. FK506 binds to an intracellular receptor (immunophilin) now designated FKBP 12 (a 12 kDa molecule). (Harding *et al.* 1989; Siekierka *et al.* 1989a; 1989b). Immunophilins are small soluble receptor proteins that mediate the drugs actions of FK506, rapamycin and cyclosporin (Sabatini *et al.* 1994). Cyclophilin and FKBP differ markedly in amino acid sequence, but both have peptidyl-propyl cis-trans isomerase activity (rotamase activity). This activity is inhibited by cyclosporin and FK506 respectively (Heitman *et al.* 1992). This action alone has been shown not to account for the immunosuppressive effects (Bierer *et al.* 1990; Tropschung *et al.* 1989). In fact the mechanism by which FK506 exerts this effect is by binding of the FK506-FKBP complex to calcineurin (Liu *et al.* 1991). Calcineurin is a Ca^{2+}-activated protein phosphatase of the serine-threonine type, whose activity is required for the Ca^{2+}-dependent step in T-cell activation via the T-cell receptor. Calcineurin inhibition decreases IL-2 mRNA transcription by blocking dephosphorylation of the IL-2 specific nuclear promoter factors (Flanagan *et al.* 1991; Lea *et al.* 1994), and

preventing consequently formation of active nuclear factor of activated T-cells. (Schreiber & Crabtree, 1992).

Rapamycin, is another immunosuppresant which binds the same immunophilin as FK506 and each will compete for this site *in vivo* (Bierer *et al.* 1990; Sharkey & Butcher, 1994). However the intracellular target of the rapamycin-FKBP complex has not yet been identified, but Sabatini *et al.* (1994) identified rapamycin and FKBP targets 1 & 2 (RAFT 1& 2).

An immunosuppressive action could account for FK506's neuroprotection abilities, however there are a number of reasons to suggest this is not its mechanism. The extent to which FK506 can reduce damage is far greater than effects found with whole body irradiation (Kane *et al.* 1992), and rapamycin, which is immunosuppressive and binds FKBP 12, is not neuroprotective (Sharkey *et al.* 1994). Evidence for the presence of components necessary to a neuronal site of action was reported by Dawson *et al.* (1994). They found that FKBP, cyclophilin and calcineurin are heterogeneously distributed, and mainly neuronally located. A very close co-localisation of FKBP & calcineurin, and cyclophilin & calcineurin was reported by Jayaraman *et al.* (1992). Timerman *et al.* (1993) showed that FKBP 12 interacts with the ryanodine receptor which can mediate intracellular Ca^{2+} release and an interaction with the IP3 receptor has also been found (Sabatini *et al.* 1994). An interaction in this process might modify the neurotoxicity of increases in intracellular Ca^{2+} during ischaemia. Furthermore Ca^{2+} has been shown to damage mitochondria, especially under oxidative stress (Connern & Halestrap, 1994), opening pores in mitochondrial membranes which allows potentially irreversible swelling damage. An

immunophilin is believed to mediate the pore formation and cyclosporin A, by binding a specific immunophilin, inhibits this. The process of Ca^{2+}-activated IP3-mediated Ca^{2+} release, which is one of the processes by which the IP_3 Ca^{2+} signal is increased, appears to be Ca/CaM kinase II mediated, and FK506 is found to affect this (Zhang *et al.*1993). FK506 is also neuroprotective against glutamate-induced toxicity in cortical cultures via the prevention of calcineurin mediated dephosphorylation of NOS (Dawson *et al.* 1994).

1.3.9. Thresholds in cerebral ischaemia

Critical threshold CBF values required for maintenance of functional and structural integrity of the brain have been determined through a number of species and models (Kalimo *et al.* 1997). These thresholds for events during ischaemia lie within certain ranges (Hossmann 1994), although differences exist between species. In the rat, these flow thresholds include inhibition of protein synthesis at 55-80ml/100g/min, acidosis at 40-47 ml/100g/min & ATP loss at 13-19 ml/100g/min (Jacewicz et al. 1986; Mies et al. 1991; Hossmann, 1994; Kohno *et al.* 1995). However, it must be recognised that such thresholds are not definitive, and are dependent on the duration of ischaemia (Heiss & Rosner, 1983). Consequently, whilst CBF may need to fall below 15ml/100g/min for infarction, if a level of ischaemia has been maintained for a particular duration, the threshold level will increase such that higher levels of CBF may still lead to infarction despite being above levels that at acute CBF levels are insufficient to cause inraction. Consequently caution must therefore be exercised in interpreting the implications of flow thresholds.

1.4. MODELLING STROKE

1.4.1. Methods of imaging stroke

Techniques which allow the imaging of the brain, both in terms of structure and function can provide experimental research and clinical practice with potentially valuable tools in cerebrovascular disease research. Indeed, there is considerable cross-over between imaging techniques used in the clinic such as magnetic resonance imaging (MRI) and computed tomography (CT), and the experimental situation, although the laboratory research setting enables more invasive procedures to be employed. Neuroimaging techniques are broadly divisible into invasive and non-invasive techniques. Invasive techniques, that include laser Doppler flowmetry (LDF) and [^{14}C] iodoantipyrine autoradiography, will not be addressed in this section since both approaches are described in greater detail in specific sections in which these techniques have been applied. These techniques may be further separated according to whether they measure changes in physical parameters such as water and ions, or are functional, whereby the imaging technique may measure metabolic rates which may (or may not) allow interpretation of cerebral events.

Early imaging of cerebral blood flow was based on ^{133}Xe clearance rates, but modern nuclear medicine techniques allow three dimensional mapping of cerebral imaging and quantification of physiological variables. CT utilises multiple X-ray absorption measurements to construct an anatomical image, whereby X-rays are differentially absorbed according to tissue density (Auer & Benveniste, 1997). Hounsfield & Cormack received the Nobel prize for medicine in 1979 for the development of the first CT system (Auer & Benveniste, 1997). CT has subsequently

been used extensively in clinical practice as it represents a technique with relatively low running cost with good anatomical resolution, although lacks the ability to image CBF or metabolism. Furthermore, whilst CT enables resolution of haemorrhagic stroke better than other imaging techniques, non-haemorrhagic stroke is difficult to resolve early following stroke (Auer & Benveniste, 1997). Its use is still prominant in the U.K. but is declining in the U.S.A. as a consequence of the more widespread introduction of MRI (Bruggen *et al.* 1994).

The technique of transcranial Doppler sonography (TCD) can accurately evaluate cerebral blood flow velocities in intracranial vessels such as the MCA (Kerr et al. 1993). The technique has been claimed to be as useful as a predictor of clinical improvement in acute stroke as cerebral angiography, the technique of x-raying cerebral blood vessels following introduction of a tracer, when performed early (Hasley *et al.* 1988; Kushner *et al.* 1991). TCD provides a simple & cheap (by comparison to techniques such as PET) non invasive method to assess vessel patency and flow levels. As such it provides a method for the rapid assessment of stroke patients when admitted to hospital.

Nuclear magnetic resonance imaging (NMR) or MRI was first applied to medical research 40 years ago. Traditionally used as a non-invasive technique for anatomical imaging with better resolution than CT (Budinger & Lauterbur, 1984), MRI has become the imaging method of choice for CNS diagnostic investigation (Bruggen *et al.* 1994). However, developments have enabled MRI imaging of certain brain metabolites (Auer & Benveniste, 1997), employing MRI-detectable nuclei such as ^{31}P to detect metabolites such as ATP. MRI imaging can be broadly separated into

perfusion and diffusion imaging, which represent blood movement and water diffusion respectively. Blood perfusion may be studied by MR angiography, clearance studies and bolus track imaging with MRI (Hossman & Hoehn-Berlage, 1995). Whilst, MR angiograpy, by which blood perfused vessels can be imaged in a manner similar to conventional angiography, and clearance studies, which determines blood flow according to conventional isotope clearance methods, are somewhat limited in spatial resolution, bolus track imaging provides greater spatial resolution and is the most attractive perfusion technique (Hossman & Hoehn-Berlage, 1995), when combined with fast imaging techniques allowing subsecond temporal resolution (van Bruggen *et al.* 1994). The technique is based on the introduction of a magnetic susceptibitlity contrast agent to the circulating blood, the passage of which is then tracked through the brain. Such agents include metal ions with high magnetic moment which cause perturbations in the normal magnetic field during their passage which can be imaged as a loss of signal intensity (van Bruggen *et al.* 1994; Hossman & Hoehn-Berlage, 1995).

MRI diffusion-wieghted imaging is based on brain water diffusion modulation of signal intensity (Hossmann & Hoehn-Berlage, 1995), and in practice MRI is applied most effectively to the detection of the proton signal from $^{1}H_{2}0$, as the high water content of the brain (80% by weight) means the proton concentration is greater than 80M resulting in high spatial and temporal resolution (van Bruggen *et al.* 1994). Since ischaemia rapidly induces changes in ion and water homeostasis due to membrane potential collapse, diffusion-weighted images (DWI) allow rapid assessment of ischaemia-induced parameters, and diffusion-weighted MRI has been

applied to a large number of focal cerebral ischaemia studies, providing detailed spatio-temporal information both in the clinic and in experimental research (Back *et al.* 1994a; Mintorovitch *et al.* 1991; Zhang *et al.* 1997). The technique has also been correlated and validated against histological end points. Studies suggest diffusion images correlate well with histopathology, although DWI may encompase a larger area than becomes recruited. DWI and brain water changes may not necessarily represent irreversibly damaged tissue (Hossmann & Hoehn-Berlage, 1995). Experimental therapeutic interventions have also been assessed using MRI, to assess the tissue response, and evolving infarct (Seega *et al.* 1993; Zhang et al. 1997).

Whilst, MRI may be applied to resolve water/oedema and metabolite changes in the brain, PET and SPECT allow direct determination of CBF, CBV, $CMRO_2$ and permit assessment of tissue functionality. PET represents the most costly and complex neuroimaging technique, requiring an on-site cyclotron, for the generation of positron-emitting tracers such as $H_2{}^{15}O$, and a team of radiation physicists, chemists, radiologists and specialists in nuclear medicine (Meyer *et al.* 1993). However, since it can use carbon, oxygen and nitrogen tracers, it provides high spatio-temporal resolution of all the main neurological parameters simultaneously, including CBF, CBV, $CMRO_2$, tissue pH and CMR_{glu} (Heiss & Podreka, 1993; Wyper, 1993). By contrast with PET, SPECT utilises commercially available single photon emiting racers such as ^{99m}Tc and employs γ cameras which are available to many nuclear medicine deparatments (Wyper, 1993), but is limited in the parameters that can be studied, and metabolites cannot be measured (Heiss & Podreka, 1993). However, ^{123}I labelled compounds mean that neurotransmitter systems can be

investigated using SPECT for neuroreceptor binding determination (Wyper, 1993). Thus, currently SPECT has found its most widespread application in localising epileptic foci, and *in vivo* neuroreceptor binding (for example in schizophrenia research) (Wyper, 1993), and it is likely to become the functional imaging technique of choice due to its advantages over PET. In conclusion, neuroimaging techniques such as MRI, PET and SPECT offer unprecedented insight into understanding the pathophysiology of stroke, therapeutic intervention and may provide the basis for clinical diagnosis in the future.

1.4.2. Modelling stroke: Medical importance

Understanding of the underlying pathophysiological mechanisms of cerebrovascular disease is still incomplete (Akopov *et al.* 1997). However, most of our understanding of the pathophysiology of cerebral ischaemia has arisen as a consequence of experimental research in animal models of stroke (Overgaard, 1994). Whilst neurological assessment and modern imaging techniques provide the clinician with insight into human stroke, there are many reasons why experimental research in animals is essential. Taking some of the most important examples in turn, animal models provide the researcher with the capacity to control the duration of an ischaemic insult, and include a secondary phase of reperfusion. In human stroke, vascular occlusion may often spontaneously resolve (Overgaard, 1994), and at a time point that cannot readily be controlled without specific intervention. This prevents interpretation of the influence of time over the pathophysiological processes following stroke. Another advantage of experimental modelling of stroke in animals

is the avoidance of co-existent disease states and inter-patient variation which introduce confounding influences in the maturation of a lesion and data interpretation. These include differential vascular anatomy, hypertension and cardiovascular disease which have profound influence on the pathophysiological outcome following stroke (Macrae, 1992; Gorelick, 1995).

Animal models enable the researcher control of physiological parameters such as blood pressure, plasma glucose, temperature and blood gases which may have profound influences on ischaemic events (de Courten Myers *et al.* 1994; Xue *et al.* 1992; Macrae, 1992). For example, high pCO_2 causes a direct increase in CBF (Tuor & Farrar, 1984), whilst temperature may reduce or exacerbate ischaemic damage (Morikawa et al. 1992). Such parameters may vary widely between patients admitted suffering cerebrovascular disease. Further considerations are the relative cost of animal research to that in human subjects, the ability to use invasive methods and ethical considerations.

Animal research also allows the genetic manipulation of certain parameters. This is expressed in two major ways. First is the use of spontaneously hypertensive or stroke prone rats (Coyle *et al.* 1984), which has benefited our understanding of the influence of factors such as hypertension on stroke outcome, as well as providing insight into the genetic basis for these conditions. Second is the breeding of animals lacking (knock-out) or over-expressing particular genes, which has enabled the contributions to ischaemia of very specific variables such as isoforms of nitric oxide (Huang *et al.* 1994; Hara *et al.* 1996) and superoxide dismutase (Yang *et al.* 1994; Kamii *et al.* 1995).

1.4.3. Rodent models of stroke

No one species fulfills the ideal criteria for a practical, convenient, economic and appropriate animal model of stroke. Satisfying criteria such as cost and practicality by the use of rodents such as the rat sacrifices potentially important features of comparativity; for example, unlike the primate and some larger species of laboratory animal like the cat, the rat is not gyrencephalic. However, larger species such as the cat have a different cerebrovascular anatomy to the primate whilst suffering short-falls in terms of practicalities and economic cost. Consequently, a range of species are regularly used to model stroke, each having advantages and drawbacks. However, it is the rat that has been most extensively adopted. An experimental model of stroke in the rat was first described by Robinson *et al.* (1975), whereby the surgically exposed MCA was ligated, which produced a somewhat variable cortical lesion. Prior to this, O'Brien & Waltz (1973) had described MCA in the cat via a transorbital approach.

1.4.4. Models of stroke (1): Focal vs global

Cerebral ischaemic insults to the brain are of two main types: Focal cerebral ischaemia, describes the occlusion or narrowing of the lumen of a cerebral artery that reduces CBF to a level that leads to functional impairment of neuronal function and a "stroke". Global/forebrain cerebral ischaemia describes the situation in which systemic circulation fails and CBF to the whole brain becomes insufficient. The time interval between the onset of ischaemia and the appearance of neuronal damage is

dependent on a number of factors such as the severity and duration of ischaemia, but also on the model type (Overgaard, 1994). Focal cerebral ischaemia usually leads to tissue damage consisting of a central core of densely ischaemic tissue (infarct) surrounded by a rim of selective neuronal loss, as a consequence of differential CBF gradients. Furthermore, studies on a number of species have demonstrated that infarction is uncommon within 3 days when occlusion times are 30 minutes or less (Overgaard, 1995; Du *et al.* 1996). By contrast such gradients are absent following global cerebral ischaemia, irreversible damage usually develops rapidly, and a generalised pattern of tissue damage is found throughout the brain, although some regions are selectively vulnerable (Auer & Benveniste, 1997; Kalimo *et al.* 1997; Overgaard, 1995).

Since the majority of human strokes are caused by the occlusion of an intracranial artery (McAuley, 1995), models of focal cerebral ischaemia have been more extensivley applied to understanding stroke (Kalimo *et al.* 1997). However, application of both have yielded important information regarding the pathophysiology of stroke, and the efficacy of a range of putative neuroprotectants. Studies utilising models of global cerebral ischaemia have also showed that there is a morphological hierachy of cell death following ischaemia, whereby neurones are the most sensitive, and a selective vulnerability when ischaemia is brief (Auer & Benveniste, 1997; Kalimo *et al.* 1997).

1.4.5. Models of stroke (2): Permanent vs reversible

Models of both focal and global cerebral ischaemia may be either permanent or reversible, describing either the permanent interuption of CBF or the re-introduction of CBF respectively (Auer & Benveniste, 1997). The duration of ischaemia seems to be a critical determinant in whether blood flow returns to the intracranial contents (global) or vascular territory of the occluded vessel (focal). Indeed, permanent or non-perfused brain is a clinical phenomoena associated with extended delay before flow return and is believed to be caused by intrinsic resistance of the cerebrovascular bed (Auer & Benveniste, 1997). This no-reflow phenomenon is also seen following focal cerebral ischaemia with reperfusion (del Zoppo, 1994). Thus, pathophysiological outcome in both permanent and reversible focal and global cerebral ischaemia may be dependent on both recirculation as well as events during the ischaemic insyult (Hossmann, 1982).

Models of permanent focal cerebral ischaemia have been of importance in our understanding of the pathophysiology of stroke such as flow thresholds, the ischaemic penumbra, the excitotoxic hypotheis of cell death and the evaluation of putative neuroprotectants (Obrenovitch, 1995; Macrae, 1992; Hossmann, 1994). However, with the understanding that human stroke is predominantly associated with a degree of recannilisation (Overgaard, 1995), incorporation of occlusion with reperfusion is becoming accepted as a prerequiste in models of focal cerebral ischaemia. The addition of reperfusion to models adds a dimension to stroke research that allows the identification of differential contributions of factors such as free radicals and the inflammatory response which have subsequently been found to be of pathophysiological significance following reperfusion. Furthermore, with the advent

of licensing for "clot-buster" such as t-PA (NINDS, 1995), a more complete understanding of the implications of reperfusion, its pathophysiology and temporal importance, will be required.

1.4.6. Subtemporal MCA occlusion

Models of MCA occlusion based on surgical exposure and subsequent occlusion of the MCA artery were developed in the 1930s through work on primates (Peterson & Evans, 1937). More recently the approach has been adapted and applied to small mammals, particularly the rat (Albanese *et al.* 1980; Robinson *et al.* 1975). MCA occlusion following subtemporal craniotomy and proximal occlusion described in the seminal papers by Tamura et al (1981a; 1981b) became the "gold standard" MCA occlusion technique during the 1980s, and is still extensively used. The study described both quantitative histopathology as well as quantitative autoradiographic determination of local CBF by the [^{14}C]iodantipyrine method of Sakurada *et al.* (1978). The model was of permanent occlusion utilising diathermy to occlude the MCA. The approach fulfilled many of the criteria desirable in modelling human stroke, whereby the infarct was extensive, incorporating the cortical & striatal structures within the vascular field of the MCA, reliable, and employed physiological monitoring. Subsequently, the model has been implicit in our understanding of the pathophysiology of stroke establishing flow thresholds for ischaemic injury, the concepts of penumbral and core regions of ischaemic insults and neurological deficits associated with experimental stroke (McAuley, 1995). Subsequent alterations have led to models of cortical-dominant infarction by distal occlusion and early models of

occlusion with reperfusion, that employed clips or ligatures in place of electrocoagulation (Shigeno et al. 1985; Soriano et al. 1997). The model does have a number of constraints. These include the need for craniotomy, which can lead to tissue dessication and thermal injury, and the extensive damage to facial structures incurred during the procedure which can limit application to behavioural analysis and post operative recovery (McAuley, 1995; Sharkey *et al.* 1993).

1.4.7. Intraluminal filament models

Koizumi *et al.* (1986) introduced a new model of focal cerebral ischaemia which utilised an intravascular thread, advanced to the origin of the MCA via the external and internal carotid arteries to occlude the blood supply to the MCA territory. This model, and subsequent variations (ZeaLonga *et al.* 1989), are established now as the model of choice in experimental focal cerebral ischaemia studies on the rat. The model has advantages over the transorbital approach by avoiding need for craniotomy and the extensive surgical intervention incurred in exposing the MCA. Furthermore the model is ideally suited to reperfusion, by simple withdrawal of the suture, which can be performed in the absence of anaethesia. However, many limitations exist with this approach which are not reflected in its extensive adoption. In early studies infarct volume variability was a significant problem, although subsequent modifications by coating the suture, for example in silicon, have improved reliability. A second limitation of the model is damage to structures outwith the vascular territory of the MCA. Damage to midline structures consequent on occlusion of the ACA is common due to the limited spatial separation relative to

the MCA, whilst structures within the thalamus and the hypothalamus commonly undergo ischaemic damage using this technique. This limits interpretation of behavioural studies and may add additional problems such as hyperthermia consequent to damage to such brain regions (Zhao *et al.* 1994). The third limitation of the approach is due to the extensive damage to the endothelial lining of cerebral blood vessels inherent in the occlusion procedure which may introduce an artifactual inflammatory response (McAuley, 1995).

1.4.8. Photothrombotic models

An alternative approach to physical vascular occlusion are photothrombotic models of focal cerebral ischaemia (Watson *et al.* 1985; Dietrich *et al.* 1987). The experimental approach utilises irradiation of a photoreactive dye, systemically introduced, to form a thrombus consequent on the reaction of free radicals that damage the endothelial lining of cerebral vessels & blood elements by peroxidation below the irradiation site. Vascular occlusion is achieved non-invasively, and vessel damage is limited to a relatively small area by comparison to the intraluminal suture approach. The model therefore possess inherent experimental simplicity and versatility of target site. However, the model produces infarction only within cortical structures and variability is high (McAuley, 1995), although the volume of infarction may be as great as that seen in other models (Markgraf *et al.* 1993).

1.4.9. Thrombotic/embolic models

The increasing need for investigation of thrombolytic therapy subsequent to t-PA approval (NINDS, 1995) has driven an interest in the further development of models of embolic and thrombotic stroke that more accurately replicate the clinical setting (Overgaard, 1994). Early models employed the approach of injecting blood clots directly into the carotid arteries (Hill *et al.* 1955), although the variability and mortality rates have led to subsequent modification (McAuley, 1995). Microsphere injection via the CCA allowed more reliable determination of ischaemic duration as occlusion time was not subject to plasmin activation, although again variability in lesion size and location have limited the application of this approach (McAuley, 1995; Overgaard, 1994).

Zhang *et al.* (1997) described a model of thrombotic focal cerebral ischaemia that utilises the intraluminal suture approach to inject a thrombin-blood clot at the origin of the MCA. This model proved reliable, with infarction within both cortex and striatum, and was amenable to thrombolytic therapy (recombinant plasminogen activator). Subsequent studies have demonstrated the efficacy of t-PA in this model (Zhang *et al.* 1997).

1.4.10. Global/forebrain models

Global models of cerebral ischaemia, also termed forebrain ischaemia, have been developed to model the conditions associated with cardiac arrest sensitive (Auer & Benveniste, 1997; Kalimo *et al.* 1997). As with models of focal cerebral ischaemia, a range of laboratory animals have been used including the cat, gerbil and most commonly the rat. There are two basic surgical approaches, although different

species require alterations. In the model described by Pulsinelli & Brierley, (1979), four major arteries in the neck, the two carotids and two vertebrals, are occluded. Bilateral carotid occlusion alone in the rat does not produce cerebral ischaemia due to the vertebral artery supply, and injury is only achieved with 2-vessel occlusion when combined with hypotension (Smith *et al.* 1984). However, in the gerbil, only the carotid arteries need to be occluded to produce global ischaemia (Bode-Greuel *et al.* 1990), due to the absence of the vertebral arteries. Arterial occlusion times vary, but are usually between 5 and 10 minutes, although this has been extended to 30 minutes (Pulsinelli *et al.* 1979). The injury consequent on global ischaemia has been addressed above, and is distinct from focal cerebral injury, with damage observed in selectively vulnerable areas such as the hippocampal CA1 region, which is also seen in humans following cardiac arrest and resusitation (Brierly & Graham, 1984; Petito *et al.* 1987).

1.5. Neuroprotection in stroke

1.5.1. Introduction

Whilst clot-busting drugs such as t-PA, an anti-thrombotic agent, have recently been approved for use in treating stroke victims (NINDS, 1995), this offers a method of reintroducing blood flow to the brain but does not attempt to alter biochemical pathways that may have been activated in cells still salvageable. Despite the unavailability of a "stroke drug", a myriad of approaches have been tried. These range from the early attempts to reestablish blood flow or reduce gross cellular metabolism to current agents which may intervene in the cascade of biochemical

messages which trigger cell death. Extensive research has helped elucidate the mechanisms by which certain drugs or strategies may be neuroprotective which has consequently provided information to facilitate not only our understanding of the mechanisms of action of previously and currently employed research tools, but also a detailed insight into the mechanisms by which cells die in ischaemia and an insight into how we may approach therapy in the future. Historical aspects of both our understanding of the events occurring in ischaemia as well as in drug development means that different approaches have been employed at different times. Thus, initial approaches were to physically reduce molecular events and reestablish blood flow on a gross scale. This was superseded by attempts to interrupt the cascade of ionic changes occurring, while more recently research has begun to centre on specific disruption of the complex molecular cascades which may trigger cell death.

1.5.2. Physiological modulation

Influencing cellular metabolic activity, either to decrease oxygen/substrate demand or improve mitochondrial efficiency, represents an early approach to treatment. Such strategies or treatments include the application of hypothermia or the use of barbiturates.

Interest in the application of hypothermia to reduce ischaemic damage dates back to the 1950s (Lam *et al.*1950; Rosomoff 1956; 1957). Xue *et al.* (1992) conducted extensive animal studies into the effects of a variety of hypothermic conditions in a rat model of permanent MCA occlusion, showing continual mild hypothermia reduced neocortical damage volume, or at least retarded its development, while

transient mild intra-ischaemic hypothermia was seen to dramatically reduce the volume of infarction with a transient MCA occlusion model. Morikawa *et al.* (1992) also addressed the effects of hypothermia in models of both permanent and transient focal cerebral ischaemia. They identified that hypothermia was beneficial in reducing ischaemic damage, and found that hypothermia showed greatest efficacy in models of transient MCA occlusion. Other studies have also demonstrated the efficacy of hypothermia in focal cerebral ischaemi (Busto *et al.* 1987; Buchan & Pulsinelli, 1990; Onesti *et al.* 1991). Whilst hypothermia confers neuroprotection, hyperthermia has been documented as detrimental to outcome following focal cerebral ischaemia (Morikawa *et al.* 1992; Kim *et al.* 1996).

The mechanism of neuroprotection of hypothermia has been addressed. Decreased temperature may inhibit the biosynthesis, release and uptake of various neurotransmitters. Since enzymes perform the majority of cellular reactions, and all these processes are energy dependent, a reduction in the kinetic energy of all cellular molecules will mean all reactions slow down whilst energy containing molecules will be sustained. A number of lines of experimental evidence supports a direct metabolic action, and Hagerdal *et al.* (1975) demonstrated that cerebral metabolic rate (CMR) declines linearly with temperature over the range 37-22°C.

Altering the glucose content of the blood during or after an ischaemic event has been shown to have a number of effects on neuropathological outcome. In 1976, Myers & Yamaguchi showed that hyperglycaemia could aggravate brain damage, and a body of evidence has now accumulated to support a deleterious effect of hyperglycaemia on neuropathological outcome (Myers & de Courten-Myers 1985,

Siesjo, 1988b). Subsequently, effort has gone into determining if hypoglycaemia might confer neuroprotection during ischaemia. The method usually used to produce hypoglycaemia is by insulin infusion, and indeed a number of reports suggest such a change can confer neuroprotection (Robertson & Grossman 1987; LeMay *et al.* 1988; Voll & Auer 1988; Voll *et al.* 1989; Strong *et al.* 1990). However, results from studies where focal cerebral ischaemia models were employed, have not consolidated the work in global models. De Courten-Myers *et al.* (1994) demonstrated that hypoglycaemia, like hyperglycaemia, aggravates brain damage whilst outcome is optimal in normoglycaemic conditions. This is also been supported by work by Yip *et al.* (1991) and Jager *et al.* (1991) who also found that insulin-induced hypoglycaemia aggravated brain injury. Possibilities for these discrepancies may lie in the details of the ischaemic models, with beneficial effects being seen where ischaemia was rapid and complete (<30min), while negative results were received in studies where ischaemia was incomplete or prolonged. The mechanism itself by which hypoglycaemia might work has not been extensively researched, but it is thought that reduced glucose leads to a reduction in lactate production during ischaemia might reduce the acidosis which occurs during ischaemia. However, generation of protons from ATP hydrolysis may contribute to acidosis more than from lactic acid production (Auer & Benveniste, 1997). Current evidence therefore does not suggest therapeutic benefit from modulation of blood glucose.

Use of drugs to reduce energy demands or substrate requirement for a cell has been investigated previously with the employment of drugs such as the barbiturates and anaesthetics such as isoflurane. Such approaches follow the rationale that where

oxygen supply is limited, depressing cerebral oxygen requirement may improve the balance between supply and demand. However, the mechanism by which hypothermia depresses metabolism (and hence enables the brain to tolerate prolonged periods of circulatory arrest) probably differs from that of drugs such as the baribiturates (Gisvold & Steen, 1985). Hypothermia causes a general depression of cerebral metabolism, while barbiturates and perhaps anaesthetics, depress only that part of metabolism related to active electrical function.

Barbiturate administration before or following vascular occlusion significantly reduces the area of infarction in a number of species studied (Moseley *et al.* 1975; Michenfelder *et al.* 1976; Tamura *et al.* 1979), although prolonged anaesthesia can aggravate ischaemic damage following MCA occlusion (Selman *et al.* 1981; Sharkey & Butcher, 1995). Barbiturates have been shown to have multiple synaptic and nonsynaptic effects in the CNS, including decreasing presynaptic transmitter release & decreasing Ca^{2+} conductances, and potentiating GABAergic responses (Rang & Dale, 1991). Harvey *et al.* (1980) showed that barbiturates produce a dose-dependent reversible depression of neurological function. Associated with this depression is a parallel dose-dependent reduction in CMR and CBF, effects which plateau at the point where the EEG becomes isoelectric (Shapiro, 1985). The suppression of synaptic activity is associated with maximal depression of $CMRO_2$ and CBF of ~50% (Michenfelder, 1974). Furthermore, barbiturates cause non-uniform alterations in local CBF and metabolism in different structures (Shapiro, 1975). Whilst the mechanism by which barbiturates exert a protective effect is often accepted as via an ability to depress brain metabolism during ischaemic periods, since barbiturates do

not depress metabolism past an isoelectric EEG (Steen *et al.* 1983), and the anticonvulsant action, that which decreases neuronal firing, may confer only minimal metabolic depression in the energy-compromised ischaemic zone, the underlying mechanism remains obscure..

1.5.3. Procedures to reintroduce blood flow

Blood flow can be improved by increasing blood pressure, reducing cerebrovascular resistance or decreasing blood viscosity (Hossman, 1982). The reestablishment of flow will theoretically reintroduce substrates to neurones to facilitate their survival. The approach of directly influencing blood flow to an ischaemic region represents one of the mechanisms by which early therapeutic strategies were targeted in stroke. Such an approach may tackle the issue of cellular energy failure restoration, but not the sequelae following energy failure and rather attempts to circumvent such problems before they become irreversible.

Induction of hypertension which prevents or ameliorates the no-reflow phenomenon, may be beneficial in later phases of post-ischaemic recirculation (Hossman, 1982). The no re-flow phenomenon relates to regions of the brain that cannot be reperfused after ischaemia (Gisvold & Steen, 1985). The cause of this hypoperfusion may be due to erythrocyte sludging, platelet or neutrophil aggregation and adhesion, pericapillary oedema or vasospasm (del Zoppo, 1995). Such ischaemia-induced hypoperfusion may contribute further to damage avoidable with adequate perfusion (Cantu *et al.* 1969). However, the benefits of induced hypertension to overcome these events (e.g. with vasopressors) are controversial,

with positive reports of induced hypertension to ameliorate no-reflow (Fischer & Ames, 1972) being contrasted by contradictory results showing no effect (Michenfelder & Milde, 1977) or deleterious effects (Fenske *et al.* 1978). Matsuoka & Hossman (1981) report the lack of beneficial effect of induced hypertension as due to the high resistance of collateral vessels greatly reducing the transmission of a systemic pressure pulse to the ischaemic territory, while aggravation may be caused by subsequent vasogenic brain oedema (Hossman, 1982).

Relief of vascular resistance using drugs such as nitroprusside (which releases NO), Ca^{2+} antagonists, anti-adrenergic agents or by hypercapnia has been attempted to improve reflow. However, with the exception of nimodipine, a Ca^{2+} antagonist (Kazda *et al.* 1979), few of these therapies have been shown to reliably benefit recovery (Hossman, 1982), with some exceptions (Zhang & Iadecola, 1994).

Reduction of blood viscosity has also been attempted, although there is also controversy over its relative benefits for ameliorating ischaemic damage. The use of viscosity reducing measures should improve oxygen delivery to the brain. Such agents include albumin, saline, heparin (Stullken & Sokoll, 1976) and more recently the antiplatelet agents like ticlopidine (Oster *et al.*1994). Use of drugs affecting prostaglandin synthesis (which includes aspirin) have also been implemented, but results have not been successful (Boulu *et al.*1981). However, haemodilution using fluosol-DA, an oxygen carrier, has proved succesful in some focal cerebral ischaemia studies (Peerless *et al.*1981; Sutherland *et al.* 1984).

Despite research in the mid 1970s to early 1980s into ameliorating ischaemia-induced damage by improving blood flow to ischaemic regions, there remains little

compelling evidence that any dramatic effects are consistently seen with the approach of the flow-improvement theory, although application of procedures in combination may still be of therapeutic importance. For example Hallenbeck *et al.* (1982) found the use of a drug combination of heparin with indomethacin and prostacyclin improved neurological function in a canine model of ischaemia.

The most effective approach, and indeed the only therapy currently approved for treatment of ischaemic stroke, is t-PA (NINDS, 1995). This protein activates the plasminogen cascade that dissolves blood clots. This approach circumvents the need to improve collateral perfusion, removing the vascular block directly. In mid-1996, and following the NINDS t-PA Stroke Study, the Peripheral and Central Nervous System Drug Advisory Committee to the US Food and Drug Administration voted unanimously that t-PA was safe and effective therapy for acute ischaemic stroke (Brott, 1996). However, there is a substantial risk of haemorrhage in patients treated later than 3 hours post-stroke and for patients not suffering thrombotic/embolic stroke.

1.5.4. 5-hydroxytryptamine

5-HT has been shown to increase during ischaemia in microdialysis studies (Richards *et al.* 1993; Phebus & Clemens, 1989). However, a role for the serotonergic system in the pathophysiology of ischaemia is still a matter of debate (Prehn *et al.*1993). A role in neurotoxicity was initially suggested by the findings of Berger *et al.* (1982), who showed serotonergic denervation using 5,7 dihydroxytryptamine (5,7 DHT)

partially protected the rat striatum from kainate neurotoxicity, whilst monoamines or their metabolites may can become neurotoxic (Obrenovitch & Richards, 1995).

5-$HT_{2/1c}$ antagonists have been shown to be neuroprotective in models of global ischaemia (Bielenberg *et al.* 1989; Fujikara *et al.* 1989; Globus *et al.* 1992), and protective effects of 5-HT_{1A} agonists have been shown in a number of studies (Bielenberg & Burkhardt, 1990; Bode-Greuel *et al.* 1990; Prehn *et al.* 1993). The site of action of 5-HT during ischaemia has been addressed in a number of studies. Effects on the cerebrovasculature are debatable since Nuglisch *et al.* (1990) showed that the 5-HT_{1A} agonist CM 57493 had no effects on intra- and postischaemic local CBF in a rodent model of ischaemia (Prehn *et al.* 1993). Furthermore, Dietrich *et al.* (1989) found that ketanserin, a 5-HT_2 receptor antagonist increased local CBF in a rat ischaemia model, but did not confer neuroprotection. However, a vascular site of action was supported by the demonstration that 5HT antagonist Ketanserin increased CBF within the area of focal ischaemia in a thrombotic model of stroke (Dietrich et al. 1989). The involvement of the 5-$HT_{2/1c}$ system is confusing since, despite neuroprotective findings of antagonists in models of forebrain and global ischaemia, protective effects in focal cerebral ischaemia models have rarely been observed (Prehn *et al.* 1993). Furthermore, the 5-HT_{1c} antagonist emopamil also blocks the L-type Ca^{2+} channel, complicating interpretation. 5-HT_{1c}-receptors have been reported to mediate excitatory effects (Beck, 1992), while 5-HT_{1A}-receptor stimulation activates a potassium conductance which would reduce neuronal activation by hyperpolarisation (Andrade *et al.* 1986; Colino & Halliwell, 1987). The hippocampus, where 5-HT_{1A} ligands have been shown to be neuroprotective, is

enriched in these receptors (Chalmers *et al.*1991) and, interestingly, the CA2 subfield, which is lowest in 5-HT_{1A}-receptor numbers, has the greater susceptibility to ischaemic damage.

5-HT_{1A} agonists reduce glutamate release (Maura *et al.* 1988) which may offer an explanation for how neuroprotective actions are mediated by certain 5-HT receptors. By contrast, 5-HT_2 receptor activation is detrimental, whilst antagonists of this receptor confer neuroprotection (Zivin & Venditto, 1984), which might be explained by evidence that 5-HT may enhance NMDA receptor responses (Reynolds, 1990). 5-HT, is also released from platelets, which may be involved in ischaemic events by thrombotic occlusion.

1.5.5. Kappa opioids

The kappa opioid receptor (κ receptor) has been clearly distinguished from other opioid receptor subtypes such and selective ligands for the κ receptor have been developed. Tang (1985) and Hall & Pazara (1988) showed that agonists at the κ receptor, U50488 and U62066, reduced hippocampal CA1 cell loss resulting from cerebral ischaemia in the gerbil. Furthermore, U50488 has been shown to reduce mortality and oedema in the rat (Silvia & Tang 1986; Silvia *et al.* 1987) and the cerebral oedema, cortical & striatal neuronal loss, and mortality occurring following focal cerebral ischaemia in the cat (Silvia & Tang 1986; Tang & Silvia 1986). CI-977 (enadoline) has been shown to produce dose-dependent reductions in infarct volume and brain swelling in a rat and cat models of focal cerebral ischaemia (Kusumoto *et al.* 1992; Hayward *et al.* 1993; Mackay *et al.* 1993).

The mechanism by which the effects might be mediated is still unclear, although a number of suggestions have been made. These include a reduction in oedema and a presynaptic mechanism to reduce release of EAAs, although the neuroprotective mechanism is complicated by the number of effects of κ receptor stimulation. k-agonists may modulate the action of glutamate at presynaptic sites by inhibiting its release, or a postsynaptic action by reducing EAA evoked Ca^{2+} entry, which is supported by CI-977 inhibiting seizures in mice caused by NMDA (Singh *et al.* 1990), reducing glutamate release *in vitro* models of ischaemia (Lambert *et al.* 1991) and the demonstration by McFadzean *et al.* (1987) that κ-agonists acting presynaptically inhibit transmitter release and EPSPs. Although κ-agonists may reduce the release of neuronal pools of glutamate, again the evidence that it is glutamate of metabolic origin that comprises the majority of ischaemia-induced glutamate release (Wahl *et al.* 1994), brings into question the significance of this mechanism, although reduction of spreading depression may be a factor.

The other favoured mechanism by which κ-agonists may mediate their effects is by a reduction in cerebral oedema (Silvia *et al.* 1987; Silvia & Tang 1986). Kappa agonists are diuretic and also modulate vasopressin release (Peters *et al.* 1987; Wells & Forsling, 1991). Induction of a profound diuresis would increase plasma osmolarity which may attenuate oedema formation and consequently secondary ischaemic damage. A neuroprotective effect of diuresis has been reported for mannitol, a hyperosmolar agent, which is neuroprotective in some models of ischaemia (Little, 1978; Sutherland *et al.* 1988). However, at the dose where CI-977 produces maximum diuresis, no significant effect was seen on ischaemic damage

(Hayward *et al.* 1993; Mackay *et al.* 1993). Furthermore, CI-977 confers neuroprotection in the gerbil model of global ischaemia despite an absence of a diuretic effect while the diuretic furosemide offers no neuroprotection at doses producing diuresis in the gerbil (Hayward *et al.* 1992). In the study by Mackay *et al.* (1993), plasma hyperosmolarity was not found with CI-977 until 120-180 min after MCA occlusion which is likely to be outwith the therapeutic window of opprotunity, and thus any action of diuresis is occurring at the limits or beyond successful intervention (Mackay et al. 1993).

1.5.6. Calcium antagonists

Calcium channel antagonists have been employed in a number of studies with a view to interrupting the biochemical cascade of events that calcium is believed to cause. Careful consideration of calcium antagonists as a therapeutic approach for blockade of increases in intracellular Ca^{2+} is important since modulation of CBF is a mechanism by which calcium antagonists may mediate neuroprotection. Furthermore, intracellular Ca^{2+} may increase due to stimulation of the sarcoplasmic reticulum stores, via the IP_3 receptor and the ryanodine receptor, so blockade of VDCCs may attenuate only part of the ischaemia-induced Ca^{2+} entry. Conflicting reports exist as to the contributions of VDCCs to events during ischaemia. The spreading wave of depression that is thought to occur during ischaemia is believed to be mediated by glutamate and K^+ (Hansen 1985), the depolarisation consequent to which should open VDCCs (Siesjo & Bengtsson 1989). However, spreading

depression is in fact blocked by NMDA antagonists (Goroleva *et al.* 1987; Hansen *et al.* 1988).

There are a large number of reports in the literature showing beneficial effects of blocking Ca^{2+} entry using ligands selective for L, N & T Ca^{2+} channels in models of stroke (Nakayama *et al.* 1988; Mossakowski & Gadamski 1990; Teasdale *et al.*1990; Kawamura *et al.*1991; Morikawa *et al.* 1991; Shiino *et al.* 1991; Benham *et al.* 1993; Valentino *et al.* 1993; Yamashita *et al.* 1993; Kashiwagi *et al.*1994; Rami & Krieglstein, 1994). However, for both NMDA antagonists and Ca^{2+} antagonists, ameliorative effects are less commonly seen in global ischaemia, although some studies have reported positive effects (Siembowicz & Hansen, 1978; Steen *et al.* 1985; Alps *et al.* 1987; Lin *et al.* 1990). Using Locke-type channel blockers, Mohamed *et al.* (1985), Gotoh *et al.* (1986) and Jacewicz *et al.* (1990) showed that nimodipine reduces infarct size when administered prior to MCA occlusion, while Sauter & Rudin (1989) using isradipine demonstrated infarct reduction by ~50% following MCA occlusion in SHRs. The L-type blockers have been most extensively used, although N-selective antagonists have proved effective (Yenari *et al.* 1996), and Ito *et al.* (1994) reviewed a T-type antagonist, U92032 (which is also an antioxidant confusing interpretation) which, like many of the L-type blockers, was found to reduce neurological deficits in a rodent model of cerebral ischaemia.

Blockade of Ca^{2+} entry to neurones may not be the only mechanism by which VDCC antagonists are effective. Indeed, blockade of vasoconstriction (or vasodilatation) of cerebral vessels may be another mechanism by which VDCC blockers are effective (Gisvold & Steen, 1985). A number of studies have shown L-

type antagonists to improve CBF in peripheral areas (Meyer *et al.* 1990; Sauter & Rudin, 1989). Ohtaki & Tranmer (1993) investigated local CBF changes and neuropathological outcome following administration of the novel calcium antagonist AT877. They found that AT877 significantly improved local CBF during MCA occlusion, as well as improved neurological outcome. They further report that the brain tissue within the ischaemic zone was salvaged by i.v. AT877 primarily through its action to increase CBF. The results of the study by Takayasu & Dacey (1990) with AT877 also propose a vascular effect suggesting the drug can improve cerebral microcirculation since AT877 may produce greater vasodilatation on parenchymal arterioles compared to other Ca^{2+} antagonists.

Consideration of the studies whereby CBF changes have been observed and consequent reductions in infarct volume found, as well as the ability of the drug itself to pass through the BBB must be addressed. Not all studies on CBF increases during ischaemia report beneficial effects and not all Ca^{2+} channel antagonists increase CBF (Siesjo 1992b).

1.5.7. NMDA and excitatory amino acid antagonists

The implication of EAAs, and glutamate in particular, in ischaemic cell death has been discussed above. Significant understanding of the pharmacology of antagonists at glutamate receptors has been achieved in the last two decades (Fagg, 1985; Mayer & Westbrook 1987; Choi 1990; Lodge & Johnson 1990; Watkins et al. 1990). Antagonists for the NMDA receptor and the AMPA receptor are currently the most selective. The NMDA-receptor complex has so far yielded the most sites for

pharmacological modulation. Non-competitive antagonists that block from within the ion channel, and display use-dependence, include MK801 (dizocilpine) and phencyclidine. The NMDA receptor also contains a site for the co-agonist glycine (Johnson & Ascher 1987), and this seems to be where kynurenate acts. Other potential sites for modulation include the zinc and magnesium sites. Competitive antagonists for the NMDA receptor include AP5 (a phosphonate) and CPP, while competitive antagonists for the AMPA receptor include CNQX and NBQX.

Antagonists acting at the NMDA receptor, both competitive (AP5, CPP, CPPene) and noncompetitive (MK801) have been used in both global/forebrain ischaemia and focal cerebral ischaemia models. In global or forebrain ischaemia, neither type of antagonist have proved consistantly successful or efficacious with many studies showing no neuroprotective effects (Buchan *et al.* 1991; Fleischer *et al.* 1989; Lanier *et al.* 1990). However, a number of reports have shown neuroprotection with these drugs in such models (LePeillet *et al.* 1992; Nellgard & Wieloch, 1992). NMDA receptor antagonists have demonstrated greatest efficacy in focal cerebral ischaemia models (Ozyurt *et al.* 1988; Park *et al.* 1988; Buchan *et al.* 1990; Choi, 1990; Gill *et al.* 1991). In the study by Gill *et al.* (1991), using a MCA occlusion model, MK801 was found to reduce ischaemic damage volumes by 60% in the cerebral cortex.

Whilst *in vitro* and *in vivo* work suggest that glutamate antagonists of the NMDA and AMPA receptors are neuroprotective in many models, the mechanism of action remains elusive. Uematsu *et al.* (1991) reported the mechanism to be due to reducing intracellular Ca^{2+} changes and alteration of the NAD/NADH redox state, but

blockade of Ca^{2+} overload may not explain the mechanism by which such drugs exert their effects. Buchan *et al.* (1989) suggests the neuroprotective effect of MK801 in focal cerebral ischaemia may be due to a drug-induced increase in CBF (Gill *et al.* 1991), whilst physiological effects of hypothermia have been demonstrated for MK801. Furthermore studies have demonstrated that the neuroprotective capacity of NMDA receptor antagonists may be facilitated by producing hypothermia in animals, although other reports show contradictory evidence (Kochhar et al. 1991).

1.5.8. Nitric oxide

NO has been postulated as having a role in the pathogenesis of brain damage during ischaemia (Dawson *et al.* 1992). Low doses of nitroarginine compounds, which competitively inhibit NOS, has been shown to reduce infarct volume in models of focal cerebral ischaemia in rats (Buisson *et al.* 1992; Nagafuji *et al.* 1992; Trifiletti, 1992; Dawson *et al.* 1994), cats (Nishikawa *et al.* 1993) and mice (Nowicki *et al.* 1991). However a number of studies have also reported increases in infarct volume with NOS inhibition (Yamamoto *et al.* 1992 Zhang & Iadecola, 1993; Kuluz *et al.* 1993; Morikawa *et al.* 1994), while in some studies no change occurred with NO inhibition (Dawson *et al.* 1992; Morikawa *et al.* 1994). Furthermore, NO donors such as SIN-1 and L-arginine have been shown to be neuroprotective in stroke models (Zhang & Iadecola, 1994; He *et al.* 1995), although again, contradictory findings have been reported (Prado *et al.* 1996).

These discrepancies may be explainable due to the multiplicity and diversity of physiological and pathophysiological actions of NO (Dawson, 1994) and possibly

differences in experimental protocol. Perhaps the most important of these is blockade of NO-mediated vasodilatation which may reduce CBF to cerebral tissue and exacerbate injury (Dawson, 1994). Evidence for a precise role of NO in control of CBF remains to be defined (Iadecola *et al.* 1994). However, NOS containing endothelial cells in the cerebral vasculature and NOS containing adventitial fibres (from the cranial autonomic ganglion) have been demonstrated (Nozaki *et al.* 1993; Irakura *et al.* 1994; Pelligrino *et al.* 1993; Macrae *et al.* 1993). NO is clearly not the only mediator of cerebral vasodilatation since autoregulatory responses and perhaps those to hypoxia are not accounted for by NO effects, and NO may serve different roles according to prevailing conditions (Iadecola *et al.* 1994).

Selective inhibitors, and transgenic animals deficient in a particular form of NOS have been developed to investigate the relative contribution of different NO pools to stroke outcome. These studies have determined that whilst selective neuronal NOS and inducible NOS inhibitors or knockout mice have smaller infarct volumes, endothelial NOS inhibition or knockout aggravates ischaemic damage (Margaill *et al.* 1997; Iadecola *et al.* 1997; Hara *et al.* 1996; Zhang *et al.* 1995). This suggests that with production of more specifc NO inhibitors, targetting may allow therapeutic isolation of the detrimental actions of NO in stroke.

1.6. ENDOTHELIN

1.6.1. Discovery

Endothelins are a family of peptides with potent vascular actions that now occupy a central position within the fields of cerebrovascular, pulmonary, cardiovascular, renal

and hepatic disease research. The rate of discovery and insight has been dramatic and the field is of direct importance to stroke research, not simply because of its possible involvement in aspects of the pathophysiology of the disease, but in its application as a tool to produce focal cerebral ischaemia. The origins of the discovery of endothelin can be traced back to the work by DeMey & Vanhoutte (1983) & Rubanyi & Vanhoutte (1985) examining the role of the endothelium in the facilitation of contractile responses of isolated arteries, and Hickey *et al.* (1985) began work to isolate this contractile substance.

1.6.2. Isolation and sequence cloning

Breakthrough in the endothelin story came as a consequence of the seminal paper by Yanagisawa *et al.* (1988). They isolated a substance from the cultured supernatant of porcine ECs. They discovered a 21-amino acid vasoconstrictor peptide, which they named endothelin (ET), of Mwt. 2,492, which had free carboxy- and amino- termini and had two disulphide bonds. They furthermore cloned the precursor peptide (known as preproendothelin), and identified regional homology to the sarafatoxins (SFXs) venom from the burrowing asp *Atractaspis engaddensis*. They also determined the converting enzyme for the precursor peptide to be a zinc-containing metalloprotease which was inhibited by phosphoramidon. The EC_{50} for ET in a porcine coronary artery assay was $4 \pm 2.2 \times 10^{-10}$M, at least one arder of magnitude lower than any other vasoconstrictor including angiotensin II, and as such has established endothelin as the most potent vasoconstrictor peptide. Subsequently, a family of endothelins has now been discovered within the human genome, and

named endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3) (Inoue *et al.* 1989). All are very similar at the 21 amino acid level, with more significant sequence divergence at stages prior to final cleavage to form ET. Figure 1 illustrates the primary amino acid sequence of the three ET isopeptides. All ET isopeptides are derived from separate pre-proETs, which are processed by endopeptidases to propeptides of 37-41 amino acids (Big ETs) (Rubanyi & Polokoff, 1994). The carboxy terminus is the most highly conserved region between isopeptides, whilst residues 4-7 make up the most variable region with the cysteine disulphide bridges being between Cys^{1} & Cys^{15} and Cys^{3} & Cys^{11} (Rubanyi & Polokoff, 1994).

Potent vasoconstriction was not the only action of ET, with the demonstration by Warner *et al.* (1989) that low doses of proET induced vasodilation, although this was not seen at higher doses. The significance of this finding would prove a key point in the pharmacology of ET. Research into the structure-activity relationship of these vasoconstrictor peptides began by use of substitutions and by truncating peptides. Substitution of the cysteine residues for the pseudoisoteric amino acid alanine were made and the peptides studied in a number of *in vitro*, in situ and *in vivo* models. Work by Hiley *et al.* (1989) & Douglas & Hiley (1991) determined that vasoconstrictor actions of ETs were not dependent on retention of the two sulphur bridges, with $[Ala^{3,11}]$ET-1 and $[Ala^{1,15}]$ET-1, acting as vasoconstrictor agents in the preparations studied, with only some loss of potency. Only the ET-1 peptide with all cysteine residues substituted ($[Ala^{1,3,11,15}]$) lost vasoconstrictor activity, but still potentiated ET-1 responses when administered together. Furthermore, Hiley *et al.* (1989) confirmed observations by Warner *et al.* (1989) of vasodilator actions of ET,

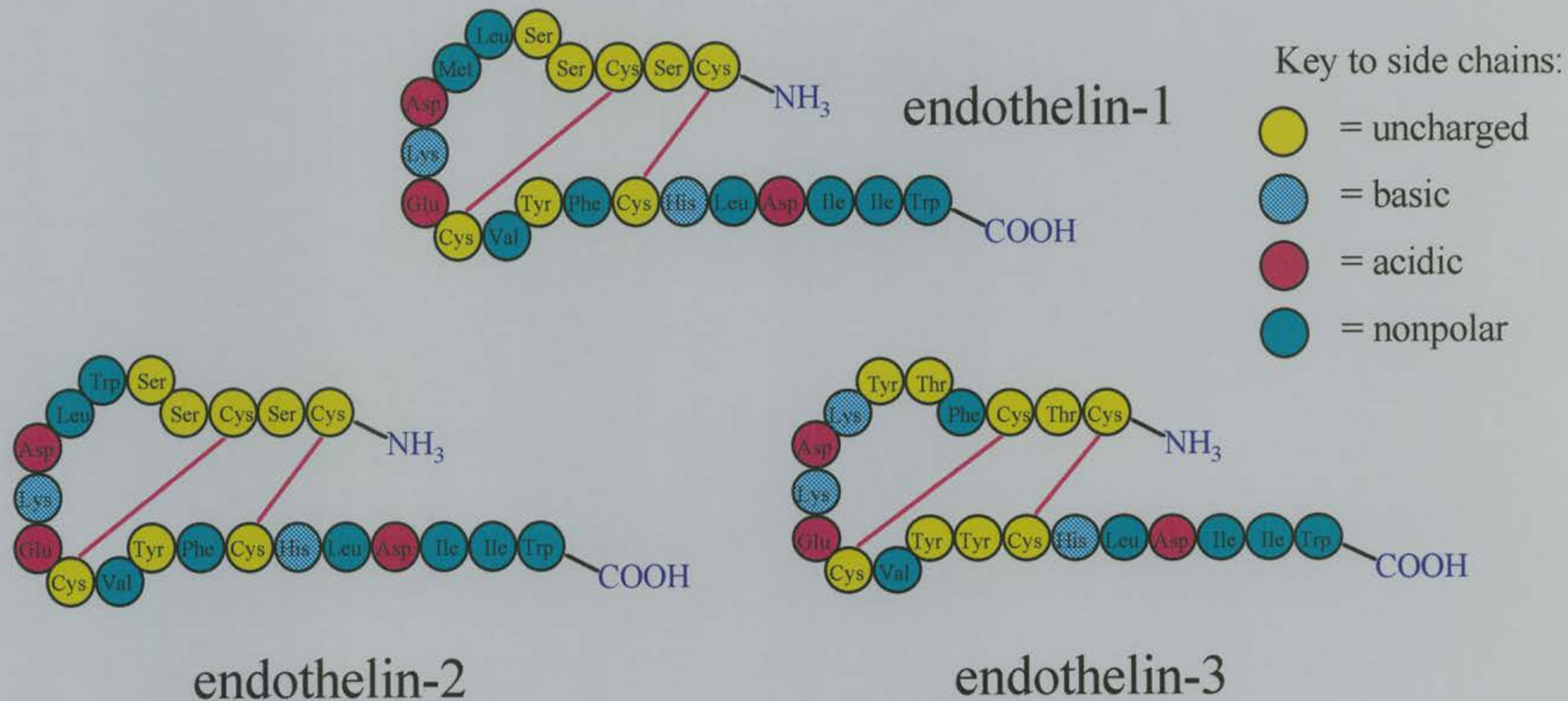


Figure 1. Diagram illustrating the primary amino acid sequences of the three endothelin isopeptides

and that, as opposed to constrictor activity, retention of sulphur bridges was necessary for this action. Evidence that ET-1 released EDRF (either directly or as a consequence of downstream effectors), was supported by the demonstration that removal of the endothelium increased the pressor activity of ET *in vitro*.

1.6.3. Receptor cloning

Hiley *et al.* (1990) furthered initial study of the Ala-substituted ET compounds with work using both the Ala-substituted peptides and truncated peptides in the rat isolated superior mesenteric bed. The work confirmed functional studies suggesting ET receptor heterogeneity, by showing differential orders of potency for vasodepressor action over vasoconstriction with these peptides. While Ala-substituted ET-1 (and a truncated ET_{16-20}) produced pressor responses, both disulphide bonds were required for endothelium-dependent relaxation in the mesenteric vascular bed. This suggests a differing pharmacophore within receptors linked directly to constrictor actions and those linked to relaxation. The order of potency for constriction in most models studied was ET-1=ET-2>ET-3, whilst vasodilator responses showed no isopeptide selectivity.

ET receptor heterogeneity was confirmed conclusively by molecular biology with the cloning of cDNAs encoding an isopeptide selective and non-isopeptide selective receptors. Arai *et al.* (1990) reported the cloning of a cDNA for an ET receptor which exhibited transmembrane topology similar to the G-protein coupled receptors (GPCRs) with an unusually short third intracellular loop, and a long extracellular N-terminal sequence. The cDNA was 3,216 nucleotides long which

encoded 427 residues. Cos cell expression of the cDNA yielded an "ET-1-selective" receptor which bound ET-1 with a Ki of 0.92nM but the Ki for ET-3 was 900nM.

Sakurai *et al.* (1990) cloned a second, non-selective, ET receptor from rat lung and transfected a cDNA library into COS-7 cells producing a single receptor which consisted of 415 residues, Mwt. of 46,901, and the familiar transmembrane topology of GPCRs. The clone was designated as the ET_B receptor and was suggested to be the receptor mediating the vasodilator actions of ETs, at which ETs were equipotent. Studies have subsequently demonstrated that ET_B receptors are activated at lower concentrations than the ET_A receptor subtype (Willette *et al.* 1990).

1.6.4. Mechanism of action

The production of ETs may be regulated by a range of agents, although research has not yet built a complete understanding. In the cerebral circulation, the production of ETs can be regulated by agents such as thrombin, dopamine, bradykinin and angiotensin II (Salom *et al.* 1995). Other factors determined to regulate ET production include cytokines such as TNFs and interleukin-1 (IL-1), and ET-3 may also control the release of ETs (Yokokawa et al. 1991). Receptor expression has been shown to be subject to ligand-induced regulation, whereby ET receptors are internalised following ligand binding, although pathophysiological conditions can alter ET receptor number (Liu *et al.* 1989; Hirata *et al.* 1988)

Cellular localisation of ET receptors is heterogeneous (Salom *et al.* 1995). The vasoconstrictor responses are mediated by ET_A and ET_B receptors are localised to the vascular smooth muscle, whilst vasodilator responses are thought to be mediated by

receptors, predominantly of the ET_B subtype, located on the endothelium of vessels (Salom *et al.* 1995). Association of ET-1 to available membrane receptors is a rapid event, despite contraction developing slowly (Marsault et al. 1993). Binding of ET isopeptides to ET receptors is essentially irreversible (Marsault et al. 1991), and the ability of ET receptor antagonists to inhibit the responses of ETs decreases with incubation time. Indeed, in the study by Wu-Wong *et al.* (1995), incubation of excess ET-1 for 22 hours after $[^{125}I]$ET-1 had been exposed to rat pituitary cells for 2 hours period, failed to displace any bound $[^{125}I]$ET-1.

Research is building a comprehensive explanation of the mechanism by which ETs mediate their actions. Both the vasodilator and vasoconstrictor responses to ETs are mediated in part via an increase in intracellular Ca^{2+}. Intracellular Ca^{2+} stores are mobilised by IP_3 via PLC activation via the GPCRs of ETs. This constitutes the earlier phase of response to ETs. Plasma membrane depolarisation is believed to occur leading to opening of the L-type Ca^{2+} channels facilitating delayed phase Ca^{2+} entry. This late phase entry explains a number of findings that the effects of ETs are either abolished (Saito et al. 1989) or greatly reduced (Jansen et al. 1989; Asano et al. 1990) in the absence of extracellular Ca^{2+} medium, and why L-type blockers often attenuate rather than abolish responses to ET (Rubanyi & Polokoff, 1994). The vasodilator action of ETs are also produced by Ca^{2+} elevations due to ET receptor activation, but these receptors are located on endothelial cells, and the rise in intracellular Ca^{2+} activates the nitric oxide synthase enzyme. The nitric oxide then diffuses to the SMCs overlying these cells to produce the transient relaxations seen with ET. More recently ET receptors have been identified as being linked to release

of prostanoids (TxA_2 and PGI_2) via a GPCR linked to PLA_2 (Rubanyi & Polokoff, 1994). ET-1 has also been demonstrated to be linked to PKC activation, which in turn may regulate a number of downstream effectors such as Na^+-H^+ exchange and nuclear events (Rubanyi & Polokoff, 1994).

The mechanism mediating the extreme duration of action of ET-mediated vasoconstrictions is still poorly understood. However, studies have suggested that the explanation may reside in the rapid process of receptor internalisation. Studies have demonstrated that ET responses undergo quite rapid tachyphylaxis (Sudjarwo *et al.* 1994; Feger *et al.* 1994; Kitazono *et al.* 1995b), and are internalised following binding. However, Marsault *et al.* (1993) proposed that this system of internalisation followed by externalisation may account for the maintained action of ETs (Marsault *et al.* 1993). However, the details of how this sustains ET actions is yet to be fully addressed.

ETs have also been determined to possess long-term mitogenic actions on some cell types. ET-1 has been shown to be a weak mitogen for smooth muscle cells in culture and certain types of glia (Rubanyi & Polokoff, 1994). The signalling pathway for this function is poorly defined but may involve PKC and tyrosine kinases (Simonson & Dunn, 1990; Rubanyi & Polokoff, 1994). Figure 2 outlines a simplified mechanism for typical smooth muscle effects of the ETs, and summarises some of the processing and downstream actions of ET isopeptides.

1.6.5. Pharmacology of ET receptors (1): ET_A receptors & responses

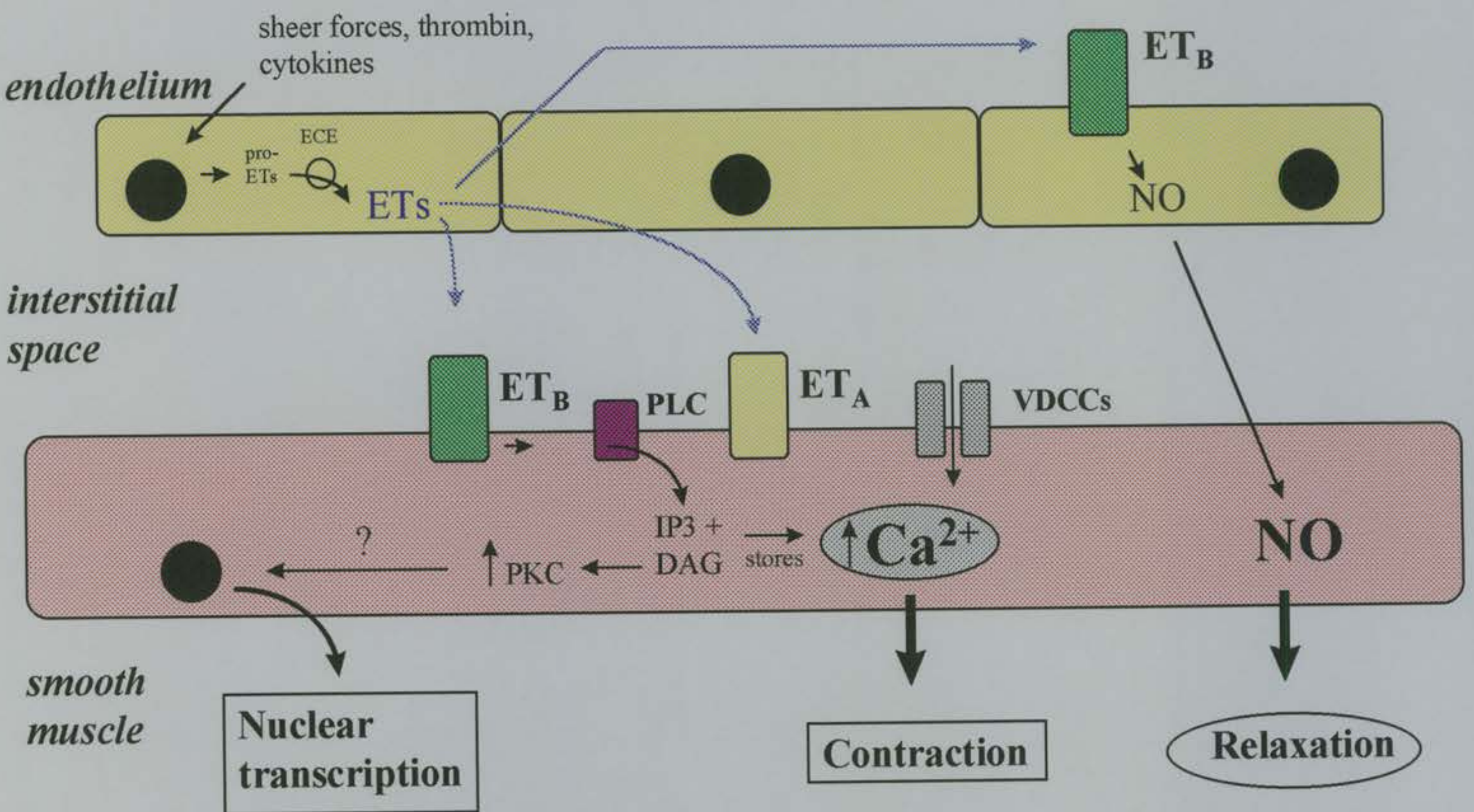


Figure 2. Mechanistic framework for ET receptor-mediated vascular responses. ETs are produced in the precursor form, pro-ET, and subsequently cleaved by ET converting enzyme (ECE) to form mature ETs. Released ETs may act on endothelial ET_B receptors in an autocrine fashion, which mediates smooth muscle relaxation via nitric oxide (NO) release, as well as smooth muscle ET_A & ET_B receptors, which mediate vasoconstriction. Downstream effectors include phospholipase C (PLC)-induced IP_3 & DAG release, and voltage-dependent Ca^{2+} channel activation. Protein kinase C (PKC) is also activated which may mediate downstream nuclear events such as immediate early gene (IEG) expression and further ET production.

Following establishment of receptor heterogeneity, ET_A and ET_B receptors have been documented throughout many tissues studied and in most mammalian species investigated (Rubanyi & Polokoff, 1994). ET-1 is a full agonist on both ET receptors and since an ET_A-selective agonist is still unavilable, determination of receptor pharmacology based on ET-1 effects alone is not possible. However, the development of specific receptor antagonists has facilitated the determination of the role of ET_A receptors. BQ123, modified from the isolated cyclic pentapeptide BE-18257B, has been the most extensively researched ET receptor antagonist (Rubanyi & Polokoff, 1994). Subsequent structure activity studies led to a series of potent and selective ET_A receptor antagonists including FR139317, a linear tripeptide (Sogabe *et al.* 1993).

ET_A receptor antagonists have elucidated a broad range of functions mediated by the ET_A receptor. Most important of these is the mediation of sustained smooth muscle contraction in the majority of systems studied including coronary & cerebral blood vessels, kidney, lung, gastrointestinal tract and liver (Rubanyi & Polokoff, 1994). ET_A receptors have also been identified as mediating the potent neuronal actions of ETs. Buchan *et al.* (1994) characterised the ET receptor in the human isolated pulmonary artery (a potential site for any ET involvement in pulmonary hypertension), finding ET-1 had much greater efficacy than ET-3. This would suggest the involvement of the ET_A receptor, which was confirmed by the finding that the ET_B - selective agonist SFXs6c had no effect, while BQ123 produced sumountable antagonism of ET effects (a shallow schild plot might suggest receptor heterogeneity). Thus an ET receptor antagonist may in the future be a therapeutic

target for treatment of pulmonary hypertension. Bosetan (Ro 47-0203) has been extensively used in functional studies, being a mixed ET_A/ET_B antagonist, and was employed by Lawrence & Brain (1994) with BQ123, an ET_A selective antagonist, to identify the receptor mediating intradermal local microvascular responses to ET-1 and ET-3. Consequently, they determined this to be an ET_A-mediated event since BQ123 was more effective on ET-1 mediated responses, while neither antagonist was as effective at blocking ET-3-induced vasoconstriction. Furthermore, neither antagonist changed basal flow alone (as determined by ^{133}Xe clearance), which represents further evidence against an homeostatic role for endogenous ET. Despite extensive therapeutic indications, the use of ET_A receptor antagonists outside of research is still very limited.

1.6.6. Pharmacology of ET receptors (2): ET_B receptors & responses

Whilst ET_B receptors were originally defined as receptors mediating the vasodilator response to ETs, there has emerged a body of evidence implicating ET_B receptors in a range of actions including vasoconstriction. Unlike the ET_A receptor, selective agonists are available for the ET_B receptor including SFXs6c, BQ3020 and IRL1620 (James *et al.* 1993; Nambi & Pullen, 1995; Widdowson & Kirk, 1996).

As with ET_A selective ligands, ET_B receptor selective ligands have been developed from structure activity studies on ETs. Subsequently, selective antagonists for the ET_B receptor have been described including RES-701-1 and IRL1038 (Sudjarwo *et al.* 1994; Yoneyama *et al.* 1995). The use of selective ligands has facilitated elucidation of the distribution and function of ET_B receptors, which have

been identified in most tissues studied and have confirmed that these receptors mediate constriction in some tissues and may have a role in pathophysiological states (Rubanyi & Polokoff, 1994; Salom *et al.* 1995).

1.6.7. Pharmacology of ET receptors (3): Atypical receptors & responses

A number of studies have now suggested that the classification of ET receptors into ET_A and ET_B is simplistic and does not adequately explain many findings (Bax & Saxena, 1994). Further subtyping of both receptors has now been proposed, as well as the existence of a third (ET_C) receptor.

In the study by Sudjarwo *et al.* (1994), utilising specific receptor antagonists for ET_A and ET_B receptors on a saphenous vein preparation, they identified subtypes of both ET_A and ET_B receptors based on agonist and antagonist selectivity and potency. Subsequent studies have delineated ET_A and ET_B receptors into ET_{AI} and ET_{AII}, with similar classification of ET_B into ET_{BI} and ET_{BII} (Sudjarwo *et al.* 1994; Yoneyama *et al.* 1995). These receptor classifications remain to be widely adopted in the absence of molecular studies and the cloning of receptors.

1.6.8. Physiological implications

Currently, the evidence for a physiological role for ETs is poorly supported by experimental evidence (Rubanyi & Polokoff, 1994), and there is no evidence to date of a role for ETs in the physiological regulation of the cerebral circulation (Patel. *et al.* 1996a; Salom *et al.* 1995), although some evidence suggests that ETs could play a role in blood pressure maintenance (Veniant et al. 1994). However, a number of

findings have begun to shed some light on this, and postulated roles include the maintenance of basal vascular tone, water balance as well as roles in paracrine, autocrine & endocrine signalling (Rubanyi & Polokoff, 1994).

1.6.9. Pathophysiological implications

By contrast to a physiological or homeostatic role, evidence has been rapidly accumulating that ETs have a role to play in pathophysiological events. Subsequently, pathophysiological roles have been proposed for ETs in vasospasm (coronary & cerebral), hypertension (systemic & pulmonary), and ischaemia (myocardial, cerebral & renal) (Rubanyi & Polokoff, 1994).

Work by Hieda & Gomez-Sanchez (1990) showed hypoxic exposure of calf coronary artery ECs produced an increase in ET immunoreactivity in the media. Interestingly, Vanhoutte *et al.* (1989) had argued that ET is not the mediator of the acute hypoxic vasoconstriction (which was the basis for its original discovery) since the response to hypoxia is too rapid.

Hypertension as a pathological sequelae to elevated ET levels has received support. Nakajima et al (1994) demonstrated ET-1 immunoreactivity in the cerebrospinal fluid (CSF) of hypertensive patients, as well as elderly patients with multi-infarction dementia, and have elucidated a correlation between ET-1 levels, hypertension and cerebrovascular injury. However, questions remain as to the physiological relevance of such levels. Indeed, elevated ET-1 concentrations were only around 10pM, levels only just in the active range. However, ET antagonists were effective in attenuating the reduction in the diameter of the basilar artery which

occurs with injection of autologous blood, in dog models of subarachnoid haeorrhage (Nirei *et al.*1993; Itoh et al 1993). Studies have also demonstrated that ETs may contribute to infarct size in a number of organs including the heart and kidney (Watanabe et al. 1991; Kon et al. 1991).

Pathophysiological roles for ETs are also inferred by reduced levels of ETs in depressed patients (Hoffman et al. 1989), and in asthmatic patients, ET levels are increased in the pulmonary lavage fluid (Mattoli et al 1991), whilst a role in the genesis or maintenance of pulmonary disease is being investigated. A role in tumour growth is also suggested by the finding that ET levels are elevated in cancer tissue (Yamashita et al 1991). ET-1 levels have also been shown to be elevated in patients with essential hypertension (Nakajima *et al.* 1994). However, elevated levels of ETs do not allow the determination of cause or effect, or indeed whether such ET levels are detrimental or indeed sufficient to be of pathophysiological significance.

The evidence for a pathophysiological role for ETs in cerebral ischaemia has received considerable support. ET-1 levels have been shown to be much higher in humans who have undergone cerebral ischaemia, SAH and multi-infarct dementia (Hirata *et al.* 1990; Suzuki *et al.* 1990; Ziv *et al.* 1992; Nakajima *et al.* 1994). ET-3 levels have also been shown to be elevated in patients with SAH, vasospasm and head injury (Kraus *et al.* 1991; Salom *et al.* 1995). In experimental ischaemia, ischaemic tissue ET-1 levels are found to be elevated following MCA oclusion (Barone *et al.* 1994), whilst ET_A receptor antagonists have been shown to reduce ischaemic damage following MCA occlusion (Patel *et al.* 1996b).

1.6.10. Cerebral circulation

Early studies showed that ET-1 was capable of producing prolonged vasoconstriction and reducing cerebral blood flow to ischaemic levels when injected into the brain parenchyma (Fuxe *et al.* 1992), intracisternally (Mima *et al.* 1989), or when applied directly onto cerebral blood vessels (Robinson & McCulloch, 1990; Robinson *et al.* 1990). This was supported by findings *in vitro* in which ET-1 produced potent and prolonged constriction of cerebral blood vessels (Jansen *et al.* 1989; Saito *et al.* 1989; Asano *et al.* 1990). The potency and duration of ET-1-induced vasoconstriction has led to its use in models of stroke as an agent capable of producing focal cerebral ischaemia (Robinson *et al.* 1990; Sharkey *et al.* 1993).

Research into the effects of ETs on the haemodynamics of the cerebral circulation began in 1990, with the study by Robinson & McCulloch on the contractions produced by ET-1 on feline cortical vessels. This *in situ* study showed these, vessels like those in the periphery, to be sensitive to ET-1. Resistance vessels responded with vasoconstriction as did the capacitance vessels. Laser-Doppler flowmetry was used in the study by Willette *et al.* (1990) in a subsequent study of the effects of ETs on the cerebral circulation in the rat. The rat cerebrovasculature responded to ET-1, with dilatory as well as constrictory responses of the microvasculature depending on dose and administration (vasoconstriction only prominent at doses >300pM). The BBB seems to limit effects of ETs on cerebrovascular smooth muscle, and circulating ETs are thus unlikely to have pathophysiological implications for the cerebrovasculature, although this may change following focal cerebral ischaemia (Betz *et al.* 1994). The ET receptors mediating

vasoconstrictor actions are solely located on the smooth muscle cells (SMCs), and, at least in the case of the cerebral circulation, responses to ET are likely to be minimal if administered intraluminally due to the effect of the BBB.

1.7. MCA OCLUSION BY INTRACEREBRAL ET-1

1.7.1. The model

The study by Robinson *et al.* (1990) demonstrated the ability of ET-1 to cause sustained constriction of the rat MCA and produce ischaemic brain damage similar to that following permanent MCA occlusion. This model involved the transorbital surgical exposure of the MCA. However, surgical exposure of the MCA has drawbacks in terms of technical requirements and physical damage to facial structure, as discussed previously. In the study described by Sharkey et al. (1993), ET-1 was injected intracerebrally via a previously implanted cannula. This required only a small craniotomy and injection of a small volume of ET-1 into the outer layers of cortex adjacent to the MCA. [^{14}C]iodoantipyrine autoradiography demonstrated that CBF was profoundly reduced within the vascular territory of the MCA. Quantitative histopathology subsequently confirmed extensive infarction within the vascular territory of the MCA (Sharkey & Butcher, 1994; Sharkey & Butcher, 1995). Animals showed good weight recovery and consequently the model has proved useful in behavioural analysis (Marston et al. 1995). A further advantage of this model was the ability to induce MCA occlusion in conscious animals, eliminating confounding influences of anaesthetics (Sharkey et al. 1993). Studies validated the model by demonstrating the neuroprotective efficacy of MK801, a gold standard

neuroprotectant in models of focal cerebral ischaemia (Sharkey & Butcher, 1995). Furthermore, Sharkey & Butcher (1994) and Butcher *et al.* (in press), have demonstrated the neuroprotective profile of FK506.

1.7.2. Aims of thesis

Many characteristics of this model have been studied including the initial ischaemic insult, histopathological outcome and efficacy as a screening tool for putative neuroprotectants. However, key characteristics have yet to be addressed. Amongst these are the optimisation of ET-1 dose and injection volume. Furthermore, the duration of ischaemia has not been documented in this model. Whether this model represents a permanent or transient MCA occlusion, and if reperfusion does occur, is this extensive and does it have any pathophysiological significance, must also be addressed. Finally, and central to this thesis is the question of whether the ischaemic episode can be interupted and controlled reperfusion introduced.

CHAPTER TWO

CHAPTER 2. OPTIMISATION OF ET-1-INDUCED MCA OCCLUSION

2.1. INTRODUCTION

2.1.1. Background

The initial studies described in this thesis address the existing model of MCA occlusion by intracerebral ET-1 injection. Previous studies with this model did not address in detail the optimal dose and injection volume of ET-1. A narrow range of doses (60 and 120 pmols) have been used, and the effect of injection volume has not been reported (Sharkey *et al.* 1993; Sharkey & Butcher 1994; Sharkey & Butcher 1995; Marston *et al.* 1995). Furthermore, changes in animal supplier and equipment since the original studies, necessitated model optimisation.

2.2. METHODS

2.2.1. Surgical techniques

All animals used throughout these studies were supplied by Charles River, and except for section 4.2.3., rats were of the Sprague Dawley (SD) strain. Animals were allowed free access to food and water both before and after surgery, and were maintained in a thermostatically controlled (22±2°C), air conditioned, housing facility on a 14:10 h light/dark cycle (light on 05:00 h) unless otherwise stated. All surgical procedures were performed under asceptic technique. Instruments and non-sterile items (e.g. scalpel blades) were routinely immersed in a sterilising fluid (Novasapa) and/or placed in an instrument steriliser at 200°C prior to use. Animals were returned to clean cages containing bedding materials following surgery after a

recovery period in an incubator which maintained normothermia until recovery from anaesthesia.

Unless otherwise stated, surgical anaesthesia was induced in 5% halothane in a nitrous oxide: oxygen mix (80:20 v/v). Following induction, animals were maintained at 1-1.5% halothane in the same nitrous oxide: oxygen mix. Rectal temperature was maintained at 37.5±0.5°C by a rectal probe connected to a thermostatically controlled heating blanket (CMA) throughout all surgical procedures, unless otherwise stated. Following induction of anaesthesia, the scalp was shaved, and the animal was placed in a stereotaxic frame (Kopf instruments). A midline incision was made by a scalpel blade, the skin and periosteum was cut and retracted. Bregma was located and the desired stereotaxic target co-ordinates marked with a pen on the surface of the skull. A craniotomy (2mm dia.) was then performed over the site using an electric dental drill. The drill site was cooled and irrigated with saline to prevent thermal damage to the underlying brain tissue. Following craniotomy, the dura was incised and reflected using a hooked needle. The cannula was then lowered slowly to the predetermined co-ordinates and injections made (1μl infused per 90 seconds). Following the last injection, the cannula was left *in situ* for a further five minutes before being slowly withdrawn. The scalp wound was then sutured (Ethicon 4/0 silk), the animal was removed from the stereotaxic frame and placed in an incubator. In all studies employing intracerebral injections, separate injection cannulae were made for every drug used.

2.2.2. Histology

In studies where the volume of ischaemic brain damage was determined, animals were allowed to recover for three days unless otherwise stated. Previous studies in this model have determined that the volume of ischaemic brain damage does not increase between three and seven days post-occlusion and therefore three days was considered the most efficient time to allow for the lesion size to be maximal (Sharkey & Butcher 1995). Shorter time periods may curtail maturation of a lesion as ischaemic damage is reported to progress for at least 1 day in studies where ET-1 was applied to the exposed MCA (Dawson *et al.* 1993).

Three days following surgery, animals were deeply anaesthetised by intraperitoneal injection of pentobarbital (50mg/kg), and perfused transcardially. This procedure involved exposure of the thoracic cavity, insertion of a needle connected to a pump into the apex of the left ventricle and incision of the right atrium. Rats were perfused initially with 20mls of heparinised phosphate buffered saline (PBS) (10 IU/ml), followed by 180 mls of 4% paraformaldehyde in PBS (see Appendix B).

On completion of perfusion, the brain was carefully excised and placed into a storage vial containing the paraformaldehyde/PBS solution in 20% sucrose and stored at 2-5°C for at least 48 hours, unless otherwise stated. Brains were then removed from storage, frozen at -42°C in isopentane, and mounted in a plastic embedding matrix (Lipshaw) on a chuck. 20μm sections were cut at -20°C, and collected on gelatine subbed slides and air dried at 20-30°C. All sections were stained with thionin (0.01%). Sections were dehydrated in ethanol, the lipid removed in xylene and then rehydrated prior to immersion in the 0.01% thionin solution for 20-30 minutes. Sections were then differentiated in 0.2% acetic acid, dehydrated again

and then returned to xylene, and subsequently cover slips adhered to the slides with DPX. Sections were examined under a light microscope (Leitz Laborlux) at eight predetermined levels and the volume of ischaemic damage quantified by volumetric analysis according to the principles described by Osbourne et al. (1987).

The mathematical model used to calculate the volume of damage accounts for brain shrinkage and/or distortion, that may occur due to oedema and following perfusion fixation. Ischaemic brain damage was annotated onto enlarged diagrams of the brain at eight stereotaxic levels from frontal cortex (bregma +3.0mm) at its most rostral, to occipital cortex (bregma -7.0mm) at its most caudal. The area of infarct was then cut out and weighed, and quantification of the ischaemic lesion calculated by the equation below:

$$\sum^{T_{1-n}} (A_{n-1} \times T_n) + ((A_n - A_{n-1})\, T_n / T_n) \times 0.763$$

where:

T = distance between sections (thickness)

A = area of ischaemic damage

0.763 = correction factor

The correction factor is determined by:

1) ratio of enlarged section to standard brain size
2) ratio between the weight of tissue to paper for a given area

The equation therefore integrates the cross sectional areas, once corrected for the enlargement factor, with the distance between levels, and has previously shown to correlate well to the same procedure calculated on an image analysis system (M4 MCID). Ischaemic damage is determined as volume, in cubic millimeters (mm^3) for each animal and determined separately for neocortex and striatum, and in some cases the hemispheric volume of damage was calculated.

2.2.3. Experiments (1): Optimisation of previous co-ordinates.

Previous studies with this model had used SD rats from Harlan Nolak within the range 285-315g (Sharkey et al. 1993). However, on beginning this project the routine supplier was Charles River, and a weight range of 280-320g was used. Previous studies have shown that strain and supplier differences can affect the stereotaxic position of brain structures relative to standard atlas references. Furthermore a different sterotaxic frame to that used previously would be used. The first study therefore determined whether the previous co-ordinates were still suitable. Microlitre indian ink injections were employed to determine if co-ordinate modifications were required. Using cadavers of SD rats and following determination of bregma, a craniotomy was performed and a 28 gauge cannula was lowered to the previous co-ordinates and 2μl of indian ink injected. Following injection brains were immediately removed. The brains were sectioned and the ink spot examined under a microscope. Co-ordinates were altered by visual determination and by using the rat brain atlas of Watson & Paxinos (1986).

2.2.4. Experiments (2): Dose response relationship

In order to determine the minimum dose sufficient to reliably cause the MCA occlusion-like pattern of histological brain damage, a range of ET-1 doses were studied. Animals underwent surgery as described in section 2.2.1. Following craniotomy, a 28 gauge steel cannula was then lowered 8.7mm below skull and vehicle or 10,33,100 or 300 pmols of ET-1 were injected in 2μl volume. Animals were allowed to recover for three days before perfusion fixation and histological determination of ischaemic brain damage as described in section 2.2.2..

2.2.5. Experiments (3): Injecton volume

Determination of an optimal volume was assessed by injecting ET-1 (100 pmols) in volumes of 1,2 or 3μl. Animals underwent stereotaxic surgery and histological analysis as outlined in section 2.2.1. and 2.2.2.

2.2.6. Experiments (4): Targeting accuracy

In these studies ET-1 was injected 1mm away from the standard co-ordinates. This was to determine the stereotaxic accuracy required to reliably cause MCA occlusion and provide information as to the surgical skill level required. Second, to confirm that the ischaemic damage following ET-1 injection originates predominantly from an action on MCA located ET receptors and not simply due to intraparenchymal effects of the type previously reported (Fuxe *et al.* 1992) Stereotaxic surgery and histological analysis was performed as outlined in section 2.2.1. and 2.2.2. ET-1 (100 pmols in 2μl) was injected 1mm medial, caudal or dorsal to the standard co-

ordinates. A 1mm rostral injection co-ordinate was not included since the skull narrows rostral to the optimal co-ordinate site and the craniotomy site would miss the skull edge.

2.2.7. Statistical analysis

All data from these studies were analysed by analysis of variance (ANOVA) on ranks with a post hoc Dunn's method test to determine differences between groups. Statistical significance was accepted at the 5% ($p<0.05$) level.

2.3. RESULTS

2.3.1. Optimal co-ordinates

Following microscopic analysis of cerebral cortex from rats injected at a range of stereotaxic co-ordinates, the optimal co-ordinates that delivered the ink to <0.5mm from the MCA were determined. It was also found desirable to lower the tooth bar. This allowed a slightly more caudal craniotomy to be performed, while avoiding the skull edge, which narrows close to the AP co-ordinate. Consequently the vertical depth for the cannula was increased to compensate for the greater tissue penetration depth consequent to this. Results of the study comparing previous co-ordinates for the new variables are shown below:

Optimised stereotaxic co-ordinates for ET-1-induced MCA occlusion:

	previous	**new optimal**
A-P	+0.4mm	+0.9mm
Lateral	-5.2mm	-5.2mm
Vertical	-8.5mm	-8.7mm
Tooth bar	-3.5mm	-3.7mm

2.3.2. Dose-response relationship

Quantitative histopathology determined that intracerebral microapplication of ET-1 in the range 33-300 pmols, yielded a pattern and volume of damage similar to that reported previously (Figure 3). Damage extended from occipital cortex at its most caudal, to frontal cortex at its most rostral, with concommitant damage to medial and lateral striatum. This damage was confined to the vascular territory of the MCA and only 1 animal, in the 300 pmol group, exhibited damage in the hippocampus and thalamus, outwith the MCA-territory. The volume of hemispheric ischaemic brain damage was not significantly different between groups over this dose range, but damage was significantly greater than in the sham-lesioned animals in both cortex and striatum ($p<0.05$). At 10 pmols, brain damage was significantly reduced compared to higher ET-1 doses ($p<0.05$), and consisted predominantly of cannula damage around the injection site. Both cortical and striatal damage in the 10 pmol group were not significantly different from that observed in the sham-lesioned animals ($p<0.05$). Figure 4 shows resolution of ischaemically damaged cerebral cortex following ET-1-induced MCA occlusion, compared to cortex from sham lesioned animals.

2.3.3. Injection volume

Varying the injection volume between 1-3μl had no significant affect on the pattern and volume of ischaemic brain damage in either the cortex or striatum (Figure 5), and

Dose-response relationship for
ET-1-induced MCA occlusion

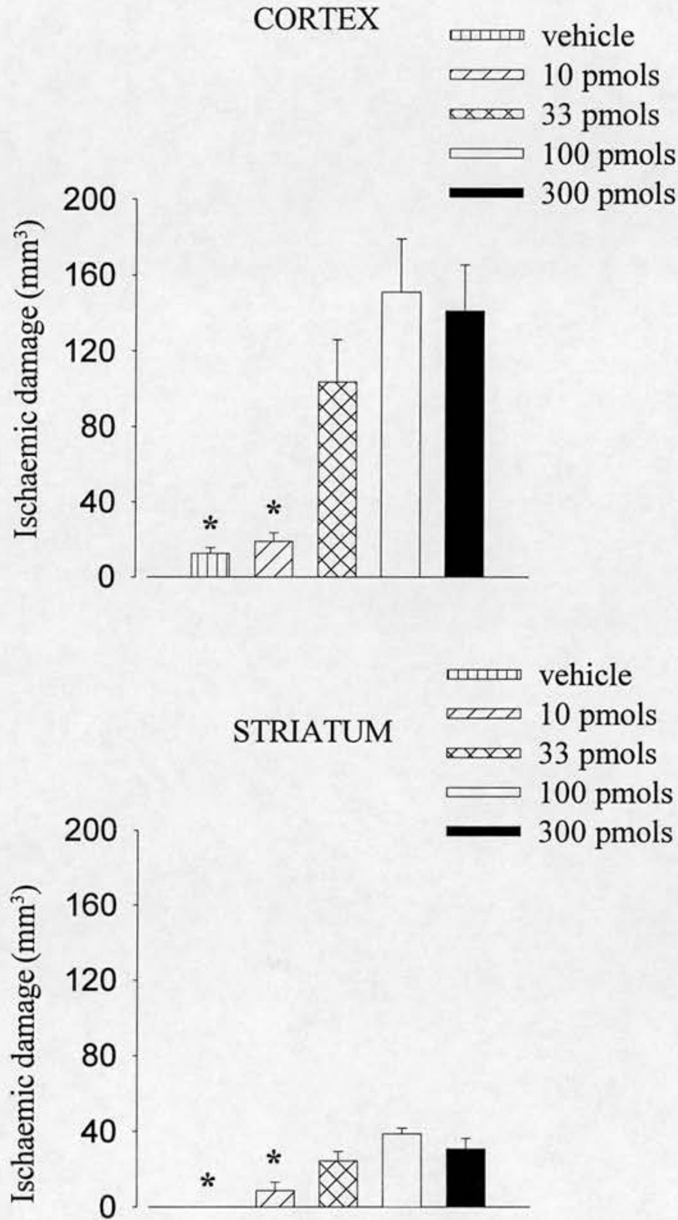


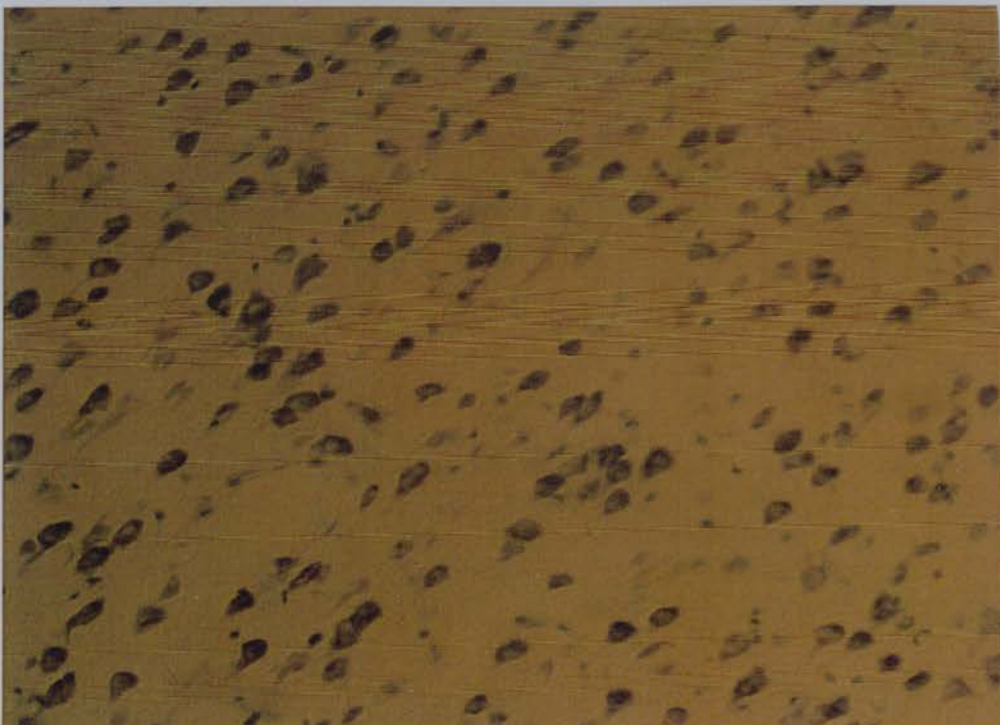
Figure 3. Dose-response relationship for ET-1-induced MCA occlusion. 10 (n=11), 33 (n=12), 100 (n=11), 300 (n=13) pmols or vehicle in 2μl was injected. Ischaemic damage was determined for cortex (top) and striatum (bottom). Data are presented as mean ± s.e.mean. Ischaemic damage was significantly reduced in the 10 pmol group, and not significantly different from vehicle injection. *P=<0.05.

Figure 4.

Light microscope photographs of (Top) thionin-stained ischaemically damaged cerebral cortex three days after MCA occlusion by intracerebral injection of 100 pmols ET-1, and (bottom), cerebral cortex from vehicle injected sham animals. Magnification = x 400

Ischaemic cortex following
ET-1-induced MCA occlusion

Control cortex following sham lesioned animal



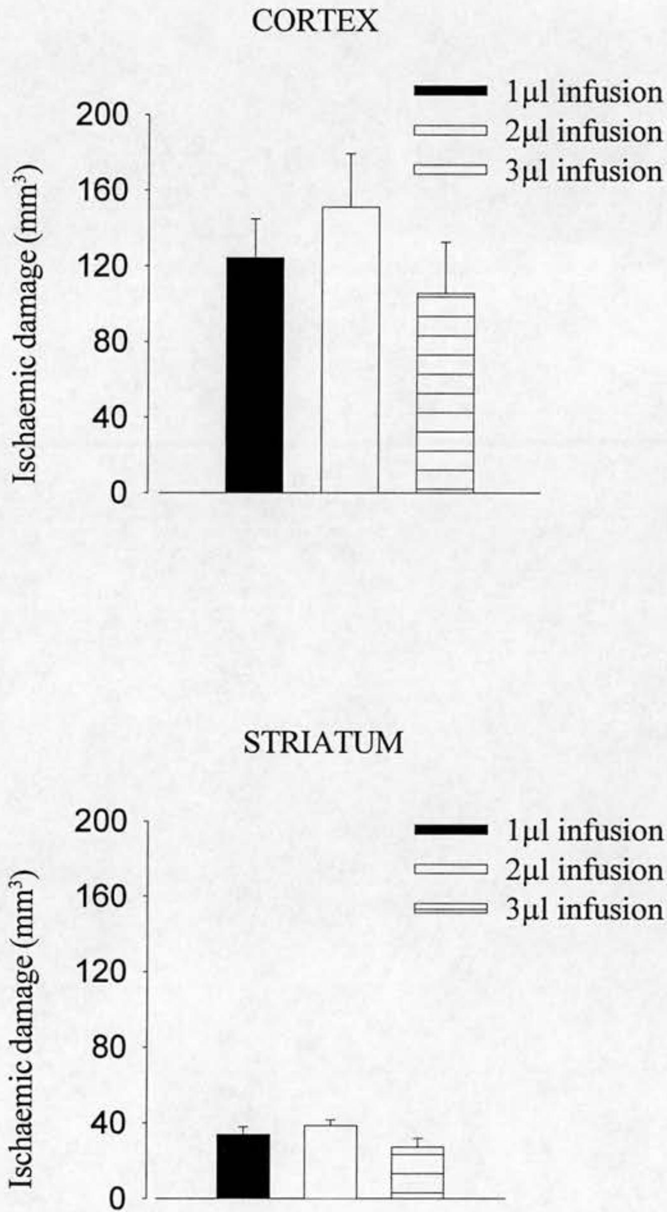


Figure 5. Effects of infusion volume on ET-1induced MCA occlusion. ET-1 (100 pmols) was injected in 1 (n=12), 2 (n=12) or 3 (n=12) microlitre volume. Ischaemic damage was determined for cortex (top) and striatum (bottom). Data are presented as mean ± s.e.mean. No significant differences were found between treatments.

Effects of stereotaxic injection position on ET-1-induced MCA occlsuion

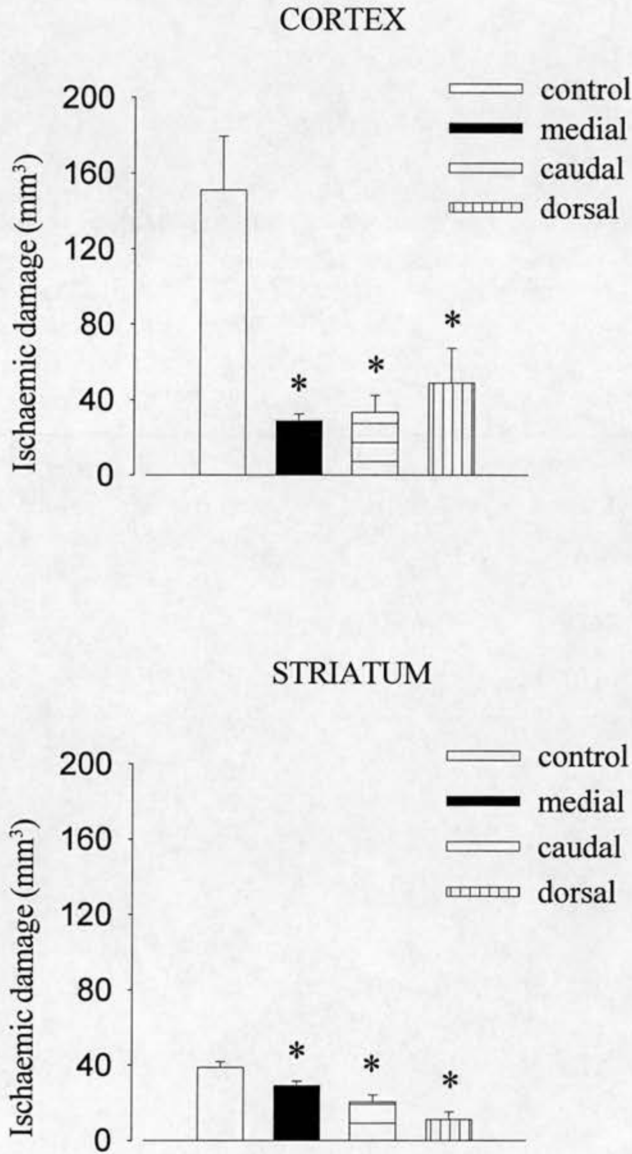


Figure 6. Effects of stereotaxic injection position on ET-1-induced MCA occlusion. ET-1 (100 pmols in 2μl) was injected at the optimal coorodinate (n=11) or 1mm medial (n=10), caudal (n=10) or dorsal (n=10) to the optimal coordinates. Ischaemic damage was determined for cortex (top) and striatum (bottom). Data are presented as mean ± s.e.mean. ET-1 injection 1mm away from optimal coordinates reduced ischaemic damage compared to the standard injection site *P=<0.05 compared to control.

the characteristic pattern of MCA occlusion-induced brain damage was seen in all groups.

2.3.4. Targeting accuracy

Injection of ET-1 into cortical tissue 1mm medial, caudal or dorsal to the optimal co-ordinates, decreased the volume of consequent brain damage. Ischaemic brain damage was significantly reduced in both cortex and striatum at each co-ordinate, when compared to ET-1 (100pmols in 2μl) injection at the standard coordinates ($p<0.05$) (Figure 6).

2.4. DISCUSSION

2.4.1. General

The present studies optimised the model for male SD rats (Charles River) within the weight range 280-320g. Previous studies with this model utilised a different supplier, stereotaxic frame and slightly different weight range. Consequently experiments were performed to determine if a new supplier, frame and weight range might compromise MCA targetting accuracy. Indeed, the previous injection co-ordinates delivered the ink injection slightly dorsally and caudally. This position was changed following the co-ordinate optimising studies to produce the new co-ordinates presented. Following co-ordinate optimisation, dose and injection volume for ET-1-induced MCA occlusion were addressed. The results from these studies outline the optimal dose range and injection volume. We observed that using 33-300 pmols, and with a 1-3μl infusion, ET-1 produced a large ischaemic lesion comparable to

previous studies using this model (Sharkey *et al.* 1993; Sharkey & Butcher 1994; Sharkey & Butcher 1995). Brain damage was restricted to tissue within the vascular territory of the MCA.

Quantification of ischaemic brain damage requires the careful consideration of the criteria for defining irreversibly damaged cells (Nedergaard, 1988). Parameters such as energy depletion or electrical silence are consequently not useable indices (Nedegaard, 1988). An area of ischaemically affected tissue must be examined for cellular signs of irreversible damage that may be termed ischaemic brain damage or "infarct". A cerebral infarct is defined as an area of tissue within which all cells, of neuronal, glial and vascular nature have undergone necrosis (Brierly & Graham, 1984). However, in light of the potential contribution made by apoptosis to ischaemic cell death following focal cerebral ischaemia, such a definition must be adjusted accordingly.

In the hours following MCA occlusion, cells within the MCA territory decrease their ability to take up histological dyes (such as thionin and haemotoxylin & eosin). Cells appear pale and swollen, with evident chromatin dipersal. In areas initially penumbral to the core, neurons appear shrunken with basophilic cytoplasia. These cells later loose their cellular identity in the days following occlusion and the area surrounding the ischaemic tissue becomes more demarcated.

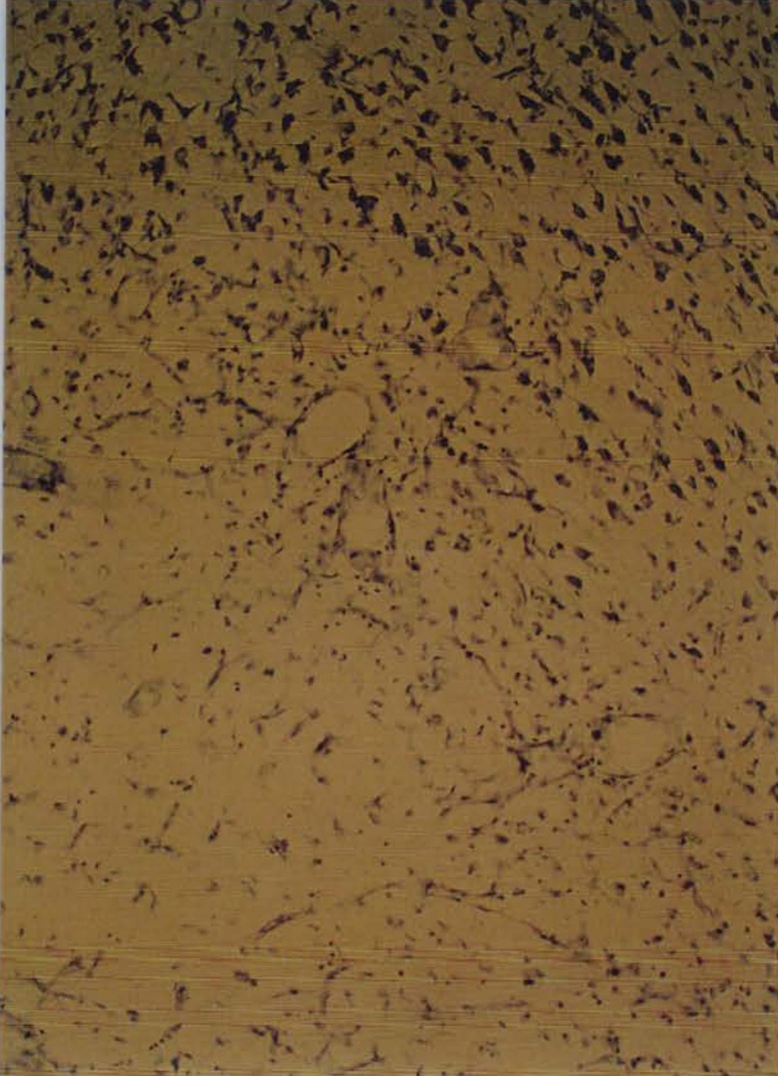
Figure 7 shows the sharp demarcation of ischaemically damages tissue to normal brain tissue, with only a very narrow band of scattered dead neurones, found in the present studies. This pattern of ischaemic damage is similar to that found following surgical exposure via the subtemporal approach whereby the MCA is occluded

Figure 7.

Demarcation of boundary between ischaemically damaged and normal cortex from animals perfusion fixed three days after ET-1-induced MCA occlusion.

Magnification = x 200

Border between ischaemic
and normal cortical tissue



proximal to the circle of Willis. This suggests that brain damage in the ET-1-model results from occlusion of the MCA, proximal to the striate branches, which is supported by the extensive damage noted in the striatum. This pattern contrasts with studies where the MCA was occluded by an intraluminal filament, where damage is often found in structures outwith this vascular field such as the thalamus and hippocampus (Koizumi *et al.* 1986). Furthermore, this model frequently incurs damage to the hypothalamus leading to problems in thermoregulation (Zhao *et al.* 1994), which are avoided by this approach.

The volume of brain damage was not significantly different from sham-lesioned animals when 10 pmols of ET-1 was injected. This suggests that either 10 pmols of ET-1 does not constrict the MCA sufficiently to occlude the vessel, or that the duration of occlusion is insufficient to produce ischaemic damage. These results support an all-or-none response. There was not a statistically significant dose dependent increase in infarct size within the effective range. It might be expected that with increasing dose, the duration of occlusion would increase with subsequently lager infarct volumes. In previous studies in which ET-1 was instilled onto the surgically exposed MCA (Macrae et al. 1993), a dose-response relationship was reported with increasing dose. The explanation for this difference is unclear. However, in the study by Macrae et al. (1993), whilst increasing dose produced a graded ischaemia, severe reductions in CBF (<50% of control) in the lowest effective dose group (10^{-7}M) were of short duration (~30-60 minutes) whilst all other doses (10^{-6} to 10^{-4}M) produced a severe ischaemia of more than 3 hours duration. It is conceivable that this is reflected to an extent in the present data, whereby the lowest

dose (10 pmols) did not effectively produce ischaemia severe enough to produce a lesion, above which the insult was essentially the same irrespective of dose.

The injection volume does not appear to be critical in this model, because similar lesions resulted when 100 pmols of ET-1 was injected in 1-3μl volume, although larger injection volumes would be expected to reduce any errors in ET-1 delivery consequent on inter-animal variations. In other studies, ET-1 was infused one millimetre medial, caudal or dorsal to the optimal site. That this procedure resulted in only a focal lesion confirms two points. First, the volume of ischaemic brain damage produced by infusion of ET-1 at the optimal site must be a direct consequence of occlusion of a major cerebral artery, and second, that careful and precise stereotaxic surgery is required to produce a reliable occlusion of the MCA.

Animal exclusion criteria were established as a consequence of preliminary studies that suggested inter-animal variation in the MCA position leads to occasional "misses" in which the volume of ischaemic damage was $<10mm^3$ in the cortex and absent in the striatum, suggesting failure of ET-1 to reach the MCA. However, exclusion based simply on no striatal damage and $<10mm^3$ of cortical damage was deemed insufficient since the dose and volume were being varied in these studies. Consequently an additional criterion was included as a safeguard. The MCA was marked using indian ink prior to cryostat sectioning. Since the injection cannula tract was found to be visible on a small number of consecutive sections during sectioning, the distance between the MCA and injection site could be determined. If the cannula injection site was >500μm distant to the MCA, this represented an additional exclusion criteria. If an animal fulfilled both lesion volume and cryostat-determined

target inaccuracy criteria, an animal was excluded. These criteria were applied to all studies performed. Approximate rates of exclusion were ~10% or less per treatment for subsequent studies.

In conclusion, these studies demonstrate that there is a relatively broad dose range over which ET-1 effectively causes MCA occlusion. Increasing the dose above 100 pmols does not increase the subsequent volume of ischaemic brain damage. The lesion reliability and volume appears to be independent of injection volume within the range 1-3μl. However, a 2-3μl volume is likely to avoid any "misses" arising from stereotaxic inaccuracy or interanimal variability as the ET-1 diffusion would cover a greater area.

CHAPTER THREE

CHAPTER 3. CHARACTERISATION OF ET-1-INDUCED MCA OCCLUSION MODEL

3.1. INTRODUCTION

3.1.1. Model verification by characterisation

Whilst the ET-1-model has been characterised in terms of histopathological end-point at 3 & 7 days (Sharkey & Butcher, 1995), and local CBF at 10 minutes post occlusion (Sharkey *et al.* 1993), a number of other parameters should be assessed in the process of model characterisation. Of these, perhaps the most important relating to this model is the temporal profile of ischaemia. Since MCA occlusion is not performed by physical means whereby a clip or filament occludes blood flow, the duration of ischaemia cannot be automatically inferred. Whilst the volume of ischaemic damage reported for this model is consistent with that following permanent occlusion of the MCA (Sharkey *et al.* 1993), model characterisation requires an assessment of the duration of ischaemia following ET-1 injection. In the model of ET-1-induced MCA occlusion in which ET-1 was applied to the surgically exposed MCA (Robinson *et al.* 1990), hydrogen clearance measurement of CBF demonstrated recovery of blood flow within a 1-4hr period according to dose (Macrae *et al.* 1993). This finding is significant in the context of this model. One requirement of focal cerebral ischaemia models is the control of the duration of occlusion. Available data suggest that if ischaemia lasts three hours or more, the resulting damage is not different from a permanent occlusion (Kaplan *et al.* 1994). However, if the duration of occlusion is less than three hours, in functional terms the model may represent one of ischaemia with reperfusion. By contrast, if ischaemic

severity remains essentially unchanged for a duration of 3 hours or greater, then the model will constitute one of permanent MCA occlusion in pathophysiological terms. Whilst models of MCA occlusion that employ snare ligatures or the filament approach demonstrate "square wave" patterns of flow reduction and return on release of occlusive procedure (Yang et al. 1994), Macrae *et al.* (1993) showed that at the lower doses of ET-1 gradual reperfusion was seen in this model of MCA oclusion. Consequently, in this section of model characterisation, three methods were employed to determine the duration of ET-1-induced ischaemia. These were the monitoring of cortical tissue perfusion velocity by laser Doppler flowmetry, striatal measurement of oxygen tension and [^{14}C]iodoantipyrine autoradiography of local CBF, three hours after ET-1-induced MCA occlusion. As described above, brain and body temperature can critically affect the outcome of both global and focal cerebral ischaemia, and this parameter was evaluated using an intracranial thermistor. Furthermore, the relationship between body and brain temperature was addressed.

3.1.2. Ottosensor probes

The study utilised miniature multiple thin-film recording probes (Ottosensors Corp.). These microprobes were employed to measure changes in cerebral temperature and oxygen tension within the striatum following ET-1-induced MCA occlusion, and have previously been validated in studies using a model of forebrain ischaemia (Freund *et al.* 1993). The "T.O.P." probe has six sensors, two temperature, two oxygen and two that measure electrical potential, mounted on a glass substrate. The recording area is ~16mm long and 400μm wide x 100μm thick. The probe is

connected via a stereotaxically manipulated holder to an interface unit comprising temperature, oxygen & potential modules, as well as an LCD display module. The probes are rinsed with a solution of acetone and ethanol immediately prior to stereotaxically lowering into the brain parenchyma. The probes gives voltage readings of the concentration of oxygen reacting on their surface, and readings are linear such that voltage can be converted to actual oxygen readings by constructing a straight line between readings from known solutions. The temperature sensors were factory calibrated to read absolute temperature.

3.1.3. Laser Doppler flowmetry

The capability to measure changes in CBF is of central importance in many aspects of stroke research. Whilst the technique of [^{14}C]iodoantipyrine autoradiography gives a quantifiable measure of local CBF in terms of volume per unit time (e.g. ml/100g/min), it allows only single time points to be studied. Methods for continuous measurement of flow changes offers a greater degree of flexibility and versatility in research. Among methods employed for repeated measurements of local CBF are the hydrogen & xenon clearance, the microspheres technique and MRI & PET. However these techniques hold a number of drawbacks such as limitations in rapid flow change monitoring and continuous time point sampling (Skarphedinsson *et al.* 1988).

Nilsson *et al.* (1980a,1980b) introduced a new method which allowed measurement of local changes in CBF in a continuous fashion, and was capable of monitoring rapid changes. The method was based on the principal of Doppler (frequency) shifted light. The term laser Doppler flowmetry (LDF) was coined for

the instrument which uses the frequency broadening of a laser beam back-scattered from moving formed elements of the blood (predominantly red blood cells) (Skarphedinsson *et al.* 1988). The signal is determined on the linear relationship between the relative portion of Doppler-shifted light and the volume fraction of moving red blood cells in the measured tissue, and the relationship between the red blood cell velocity and mean Doppler frequency. Thus, light which is reflected from moving formed elements of the blood produces an altered signal relative to the light received from blood cells and other tissue elements not moving. The output signal of the flowmeter is obtained by real time computation of the number of red blood cells multiplied by their velocity (or red cell flux) (Skarphedisson *et al.* 1988).

The main drawback with the technique is that the Doppler signal values cannot reliably be assumed as CBF in ml/g tissue/min in the way that [^{14}C]iodoantipyrine is quantified (Kirkeby *et al.* 1995). A number of studies have suggested LDF can be calibrated for measurement of absolute CBF (DiResta *et al.* 1987; Eyre *et al.* 1988; Lindsberg *et al.* 1989; Fabricus & Lauritzen 1996). However, these studies determined that such absolutes are only valid when CBF and the brain region is the same in every animal, and baseline values are identical (Fabricus & Lauritzen, 1996). If the probe site is chosen irrespective of baseline values, numerical LDF values will vary accordingly. Consequently, studies employ a strategy for showing relative changes in blood flow. i.e. the LDF values are expressed as % of baseline. This point will be addressed in detail below, and with reference to research findings. Studies also point out that LDF should only be applied to microcirculatory recordings and

not used to record arterial flow for which Doppler sonography is the appropriate technique.

The original technique of LDF was designed for measurement of human skin blood flow and Riva *et al.* (1972) were the first group to employ LDF for blood flow measurements. LDF has since been employed to measure changes in blood flow in tissues as diverse as the spinal cord (Lindsberg *et al.* 1989), intestinal wall & mucosa (Ahn *et al.* 1985; Feld *et al.* 1982), renal cortex (Stern *et al.* 1979) and cerebral cortex (Dinargl *et al.* 1989; Florence & Seylaz 1992; Takagi *et al.* 1994a; Kadoya *et al.* 1995; Zhang *et al.* 1997).

Validation by comparison with established or similar techniques is essential in adopting and deploying a novel technique. LDF has been validated by comparison to known techniques under experimental conditions. Fabricus & Lauritzen (1996) addressed the question of whether changes of CBF measured by LDF correlates with CBF changes measured by [^{14}C]iodoantipyrine in order to determine the valid approach to recording and interpreting LDF values. They found that the numerical values recorded by LDF correlated poorly to [^{14}C]iodoantipyrine values calculated from simultaneous recordings at the same site (i.e. LDF units vs. ml $100g^{-1}$ min^{-1}), concluding that it would be invalid to convert LDF values to absolute CBF. Dinargl *et al.* (1989) also addressed this point. As with the study of Fabricus & Lauritzen (1996), a poor correlation between absolute CBF_{LDF} (CBF measured by LDF) and absolute CBF_{IAP} (CBF measured by IAP), was noted. While concluding that LDF is inaccurate for determination of absolute local CBF in flow units, they found changes in CBF_{LDF} and CBF_{IAP} (with values expressed as % of baseline) correlated well.

Absolute values were highly dependent on probe placement and position and therefore absolute values were not considered a true reflection of CBF. Dinargl *et al.* (1989) offer a number of possibilities for the discrepancy between absolute CBF_{LDF} and CBF_{IAP}. First, avoiding larger vessels is problematic given the density of the microvasculature and the size of the probe, but placement in proximity of larger arteries and veins contributes distortion of the LDF signal. Other contributing factors include microregional variations in perfused capillaries (Kuschinsky *et al.* 1987; Tyson *et al.* 1987; Dinargl *et al.* 1989) and differences in local haematocrit (Mchedlischvili, 1986; Dinargl *et al.* 1989). Both these may influence the LDF signal magnitude. By contrast, local CBF may be recorded consistently by [^{14}C]iodoantipyrine despite differences in haematocrit, flow velocity and capillary recruitment, although CBF_{LDF} might be different in two adjacent regions since all these factors can affect the LDF signal to a different degree (Dinargl *et al.* 1989).

Like Dinargl *et al.* (1989), Fabricus & Lauritzen (1996) found that the ratio of CBF_{LDF} correlated better with the ratio of CBF_{IAP} values between the two hemispheres, and thus under "normal" conditions relative changes of LDF are useful for studies of dynamic changes in CBF. They also found that at increased CBF levels, LDF recorded values greater than CBF_{IAP}. Thus, it seems in non-physiological conditions with high CBF, LDF may tend to overestimate CBF.

A number of studies have also examined CBF as recorded by LDF and compared it to values obtained with the microspheres technique (Eyre *et al.* 1988; Lindsberg *et al.* 1989; Kirkeby *et al.* 1995). In the study by Kirkeby *et al.* (1995), they found that as with [^{14}C]iodoantipyrine, correlation between LDF and

microspheres was not found when LDF values were used as absolute CBF. However, when percent change from baseline was used in the calculation for both LDF and microspheres, a significant correlation was found, although this correlation was weaker than for [^{14}C]iodoantipyrine. This group concluded that LDF, with values expressed as % change, provides a valid method for studying local microcirculatory changes in the brain.

Another common method for repeated measures of local CBF is the hydrogen clearance technique (Haining *et al.* 1968). This technique is quantitative in terms of local CBF in ml/100g/min and has been employed as a technique to determine CBF in a number of models of focal cerebral ischaemia (Heiss & Rosner, 1983; Macrae *et al.* 1993). Skarphedisson *et al.* (1988) compared CBF measurements between LDF and the hydrogen clearance technique, although other studies have been carried out (Diresta *et al.* 1987; Reith *et al.* 1994). While this study does not attempt to correlate actual values, when expressed as a % of control, both LDF and H_2 clearance correlated well over a broad range.

Various studies therefore support the use of LDF for obtaining continuous estimates of CBF changes, although restrictions in interpretation must be addressed. For example, since the volume of tissue sampled cannot be quantified *in vivo*, LDF values cannot be quantified in terms of ml/g tissue/min. Rather LDF values should be expressed and evaluated as % change from control or baseline and consequently results correlate well with other established techniques for measuring CBF (Fabricus & Lauritzen 1996). Furthermore, despite a number of groups referring to LDF recording "CBF", the technique really measures red blood cell flux, and not whole

not whole blood flow/CBF, and should be referred to appropriately. LDF use in research has expanded due to a number of favourable advantages over some of the techniques mentioned in the previous section, including:

1. LDF implies no trauma to the brain, unlike H_2 clearance, with the exception of when used intracerebrally (Skarphedisson *et al.* 1988).
2. LDF allows continuous changes in blood flow to be studied, avoiding the single time point nature of techniques such as [^{14}C]iodoantipyrine autoradiography and microspheres (Kirkeby *et al.* (1995).
3. Simplicity of use. This may not appear to be of importance in some respects, but the ease of use with which it can be applied and the lack of need for i.v. injections, electrolysis and extensive post-study analysis (such as with [^{14}C]iodoantipyrine), is an aid in simplifying the job of the researcher.

As with any technique, the protocol for using LDF is important, and establishing continuity in the use and approach to measurement reduces study-to-study variation and increases comparability between research findings. In table 2, the various procedures and protocols used by groups utilising LDF in cerebrovascular research are listed. This includes factors such as the recording site, condition of the dura and data interpretation and representation.

LDF has been employed in an extensive range of studies into CBF autoregulation & the coupling of blood flow to metabolism (Iadecola & Reis, 1990; Nakai *et al.* 1983; Mraovitch *et al.* 1986; Paulson *et al.* 1990; Florence & Seylaz, 1992; Akgoren *et al.* 1996), the effects of systememic physiological alterations such

Procedural approaches to LDF in focal cerebral ischaemia studies

Author	**Dinargl *et al.* (1989)**	**Tsujimoto *et al.* (1993)**	**Dalkara *et al.* (1994)**	**Reith *et al.* (1994)**	**Takagi *et al.* (1994)**	**Karibe *et al.* (1995)**	**Morimoto *et al.* (1996)**
Species	Rat (Wistar and SHR)	Cat	Rat (SHR)	Rat (Witar)	Rat (SD)	Rat (SD)	Rat (SD)
Stroke model	Subtemporal MCA occlusion	Intraluminal filament MCA occlusion	Subtemporal MCA occlusion	Intraluminal filament MCA occlusion	Subtemporal MCA occlusion	Intraluminal filament MCA occlusion	Subtemporal MCA occlusion
Method to calculate CBF	Absolute LDF units and % change.	% of baseline/control	% of baseline/control	% of baseline/control	% of baseline/control	% of baseline/control	% of baseline/control
Probe site	Parietal CTX	Parietal CTX (Ectosylvian gyrus)	Parietal CTX	Right inferolateral caudate putamen	Parietal CTX	Parietal CTX	parietal CTX
Dura condition	Intact	Intact	Intact.	Went through dura	Intact	Intact	Cut dura
Large vessel avoidance	Yes	not mentioned	Yes	N/A	not mentioned	Yes	Not mentioned
Terminology of what LDF was measuring	"CBF measured by LDF"	"regional CBF measured by LDF"	"regional CBF"	"relative changes in CBF"	"CBF measured by LDF"	"Cortical blood flow monitored by LDF"	"local CBF was monitored by LDF"

Table 2. Examples of procedural approaches to LDF methodology when applied to studies of focal cerebral ischaemia.

as the hypercapnic response of CBF (Haberl *et al.* 1989) and the effects of anaesthetics on CBF (Lee *et al.* 1994; Hudetz *et al.* 1995).

LDF has been applied in cerebrovascular disease research to monitor CBF changes following experimental cerebral ischaemia, and therefore represents a potent tool in such studies, with pre and post occlusion real time measurements of changes in cerebral microcirculation. Furthermore, with the growing emphasis on experimental reperfusion models, LDF can not only confirm successful occlusions, but also monitor CBF recovery during reperfusion. Dinargl *et al.* (1989) were the first group to assess LDF in a model of MCA occlusion. Ischaemic CBF_{LDF} (MCA occluded by transorbital approach) averaged 33% of preocclusion values with some hyperaemia on reperfusion (120-141%). Subsequently, a number of studies have applied LDF in focal cerebral ischaemia studies (Dalkara *et al.* 1994; Reith *et al.* 1994; Morimoto *et al.* 1996; Zhao *et al.* 1997). Studies have examined the effects of glutamate release (Takagi *et al.*1994a), hypothermia and mannitol (Karibe *et al.* 1995), ET receptor antagonists (Patel *et al.* 1996b), ICAM-1 null mice (Connolly *et al.* 1995) and zinc protoporphyrin neuroprotection (Kadoya *et al.* 1995). The parietal cortex is the most commonly studied brain tissue region in studies that employ LDF to monitor CBF following MCA occlusion, and the experimental protocol applied in the present studies has combined many of the common experimental practices.

3.1.5. [^{14}C]iodoantipyrine autoradiography

The quantitative measurement of local CBF within discrete brain regions developed from work by Kety (1951), on the principles of inert gas exchange between blood

and tissues (Sakurada et al. 1978). The approach was based on the Fick Principle, that the rate of change of a chemically inert tracer substance in a given tissue is equal to the difference between the amounts brought to the tissue in the arterial supply and removed by the venous supply (Sokoloff, 1986). A freely diffusible tracer in the blood will be in near instantaneous equilibrium with brain tissue, permitting the measurement of regional blood flow from the rate of accumulation of the tracer in the brain. These basic principles of tissue-tracer equilibration were first applied to the measurement of local CBF using [^{131}I]trifluoroiodomethane in the cat (Landau et al. 1955; Freygang & Sokoloff, 1958; Kety 1960), but the mathematical calculation permitted techniques such as CF_3I, ^{85}Kr, ^{133}Xe and hydrogen clearance to measure blood flow (Landau *et al.* 1958; Glass & Harper 1963; Purves, 1972). The original method involved the intravenous infusion of [^{131}I]trifluoroiodomethane for 1 min during which, timed arterial samples were taken from which the arterial blood concentration could be later calculated. The animal was then decapitated and the brain frozen in liquid nitrogen, sections taken and autoradiographs produced by incubation with X-ray film, whilst maintaining the tissue at -75°C to prevent loss of the volatile tracer. Distribution of the tracer reflects perfusion rates and autoradiograms were pictorial representations of the CBF in various brain structures (Sokoloff, 1986):

The original operational equation was:

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

where Ci(T) was the tissue concentration of the tracer at a given time *T* after tracer introduction, λ was the tissue:blood partition co-efficient, CA the arterial tracer concentration, t was the variable, time. K was defined as equal to $mF/W\lambda$, where F/W is the rate of CBF per unit mass of tissue and m equalled a constant between 0 and 1 that represented the achieved tracer diffusion equilibrium from arterial to venous passage (1 in the absence of diffusion limitation or arteriovenous shunts that are absent in the cerebral circulation). If m=1 then blood flow can be calculated from the time of tracer infusion onset, arterial tracer concentration history and the tissue:blood partition coefficient (Sakurada et al. 1978).

[^{131}I]trifluoroiodomethane was a freely diffusible, chemically inert stable tracer which had a value for m as close to 1 as possible, but suffered disadvantages. These included a short half life, requiring regular synthesis & complex chemical purification, the poor resolution characteristic of [^{125}I] iodine-labelled tracers, alterations in blood soluability with haematocrit, variations in the blood/tissue partition coefficient from region to region & animal to animal and the inconvenience associated with assaying a volatile tracer (Reivich *et al.* 1969; Sakurada et al. 1978; Sokoloff, 1986). Consequently a commercially available, non-volatile [^{14}C] tracer was sought of which [^{14}C]antipyrine seemed to satisfy the requirements (Sokoloff, 1986). However, uptake of this tracer was found to be diffusion limited, leading to CBF values considerably less than those obtained from studies with inert gases (Sakurada *et al.* 1978). Use of 4-iodo-[N-methyl-^{14}C]antipyrine ([^{14}C]iodoantipyrine) circumvented the limitations of [^{14}C]antipyrine possessing 1) isotope stability 2) high oil/water partition coefficient to make it more freely diffusible and 3) a blood/brain

partition coefficient determined as 0.78-0.8 that was uniform throughout the CNS and did not vary with haematocrit (Sakurada *et al.* 1978; Sharkey, 1986; Sokoloff, 1986).

Jay et al. (1988) described a correction factor for use of the [^{14}C]iodoantipyrine with small animals such as the mouse. Errors in the original technique arose when applied to small rodents consequent to the relatively low diameter/length ratio of arterial catheters, leading to errors in the time course of [^{14}C]iodoantipyrine concentration in the arterial blood. Two types of arterial sampling distortion occur arising from the delay in the passage of blood from the distal end of the catheter and a washout effect arising from dilution of arterial tracer due to dead space within the catheter (Jay et al. 1988).

[^{14}C]iodoantipyrine autoradiography has successfully been used to measure local CBF, producing accurate blood flow maps of multiple levels of brain tissue at a resolution of ~100μm, which is at least an order of magnitude lower than PET and two orders below current MRI resolution. Furthermore, the technique has been extensively applied to research into cerebral ischaemia.

3.2. METHODS

3.2.1. Indwelling cannula system

In these studies of the characteristics of ischaemia following ET-1-indued MCA occlusion, an indwelling cannula system was used. An indwelling cannula was employed in the original studies with this model when ET-1 was injected in conscious animals. Two factors prevented the use of a stereotaxically lowered

injection cannula in these studies. The holder for the intracerebral probes was too bulky to allow for both a stereotaxically controlled probe placement and a stereotaxically controlled injection needle. Such spatial limitations required an implanted cannula to be used, removing the need for stereotaxic control of the ET-1 injection during intracerebral recordings. The second need for the indwelling cannula system for ET-1 injections arises from the use of LDF. Lowering of a stereotaxically controlled injection needle through the brain parenchyma caused significant deflections of the LDF baseline prior to ET-1 injection in preliminary studies. The use of an indwelling cannula into which the injection cannula could be placed prevented any disturbance of LDF signal prior to ET-1 injection.

Animals were implanted with an indwelling cannula 5-10 days prior to study according to a modification of the method described by Sharkey *et al.* (1993). Animals were anaesthetised, placed in a stereotaxic frame and rectal temperature maintained at 37-38°C. After a midline incision, a craniotomy was performed (co-ordinates from bregma; AP=0.9mm, L=-5.2mm), four surgical screws fixed to the skull and an indwelling cannula (26 gauge with a 31 gauge dummy cannula), cut to 8.5mm, lowered 8.2mm below the skull. The cannula was fixed to the skull by dental cement, the wound sutured (Ethicon 4/0 silk) & sprayed with an antiseptic, and the animal returned to its cage and allowed to recover. Injections were made by withdrawing the dummy cannula and inserting a 31 gauge injection cannula, cut to 9.2mm, which would project below the indwelling cannula to the same co-ordinate site as the stereotaxically lowered cannula reached. The ~0.7mm projection of the injection cannula below the guide ensured injections were made into "undamaged"

tissue. Figure 8 illustrates the in situ recording procedure used in studies with ottosensors probes.

3.2.2. Intracerebral oxygen & temperature

Freund *et al.* (1993) determined that halothane was not a suitable anaesthetic to use with the Ottosensors probes, as this interfered with the oxygen sensor. This was also noted in preliminary studies (data not shown). Consequently, isoflurane, another volatile anesthesic commonly used in *in vivo* studies, was employed.

It was found that a given probe gave inconsistencies in oxygen tension calibration under the approved calibration protocol between studies, so voltage readings were used directly and changes in oxygen expressed as relative (%) changes from pre-MCA occlusion values (taken as 100%-baseline, not 100% oxygen). Temperture probes were calibrated against 2 mercury and 4 electronic thermometers in reference solutions of ACSF within a 32-39°C range, and corrected where necessary.

Following surgery to implant indwelling cannulae, animals were allowed to recover for 5-10 days. On the day of study, rats were reanaesthetised for the duration of the study (this time with isoflurane since the probes do not work with halothane) and placed in the stereotaxic frame. A craniotomy was performed (AP=+1.0mm; L=-2.0 to bregma) and the probe (with its chamber filled) lowered 5-7mm into the striatum to begin recordings.

Oxygen tension and brain temperature readings were recorded from both sets of sensors for 15-30 minutes before ET-1 (450 pmols in 3μl) injection (1μl/90 seconds) via a 31 gauge injection cannula (9.2mm) inserted into the lumen of the indwelling

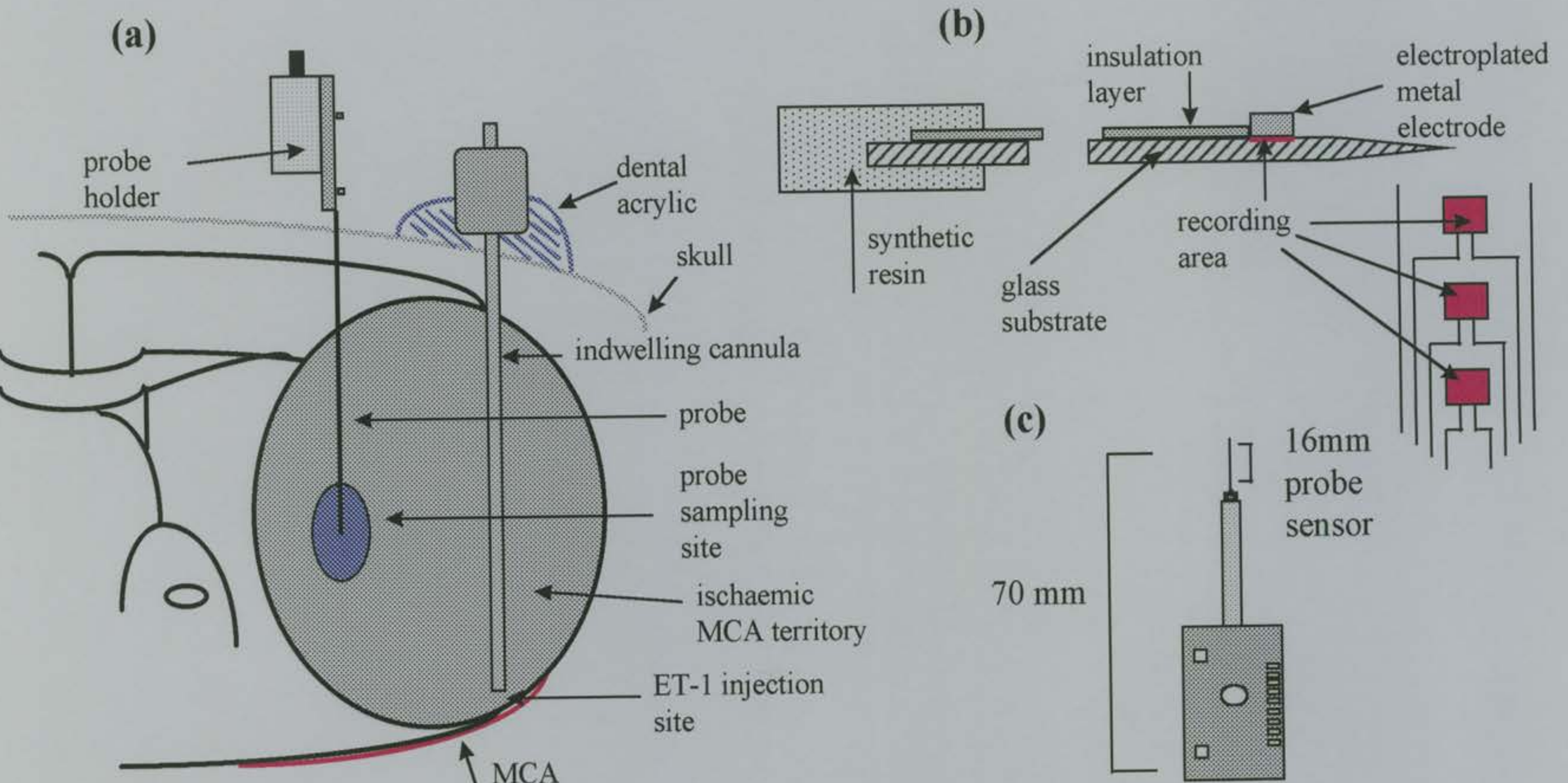


Figure 8. Intracerebral oxygen and temperaure recordings. Details of (a) intracerebral recording site within the rat brain, (b) probe sensor construction & electrode arrangement, and (c) the Ottosensor "T.O.P." probe.

cannula following removal of the dummy cannula. This dose of ET-1 is in fact higher than was determined as necessary during the early characterisation experiments. However preliminary experiments determined the 100 pmol in 2μl injection was unreliable in occluding the MCA using indwelling cannulae. Indeed, in preliminary studies only 1 in 6 studies (17%) showed successful MCA occlusion or signs of ischaemia on inspection of brains. By comparison the higher dose was successful in all experiments. The lower success rate was likely to be a consequence of the use of a guide cannula and the time between implantation and study, with reduced stereotaxic control, small lateral movement of the guide cannula in situ and the re-entering of the rat into the stereotaxic frame. Ischaemic brain damage was therefore quantified in animals three days after injection of 450 pmols in 3μl according to the experimental protocol of section 2.2.1. and 2.2.2.

Following completion of the ET-1 infusion, the injection cannula was removed (5 minutes after last injection) and readings continued for 3 hours. After three hours, the rat was killed while still under anaesthesia and the brain dissected to confirm successful MCA occlusion. This involved microscopic inspection of the MCA vascular territory to determine tissue pallor.

3.2.3. Laser Doppler flowmetry

An indwelling cannula was implanted 5-10 days as described in section 3.2.1.. On the day of study, animals were anaesthetised with halothane and placed in a stereotaxic frame. A craniotomy was performed over the parietal bone following gentle displacement of the top edge of the temporalis muscle. A drop of artificial

cerebrospinal fluid (ACSF) (for composition see Appendix B) at room temperature was added to the craniotomy site. ACSF was found to have no effect on the LDF signal (preliminary studies). A LDF probe (24 gauge) was advanced to just touch the surface of the cortex, and LDF recordings (Transonic systems Inc.) were made for 15-30 minutes prior to ET-1 injection. ET-1 (450 pmols in 3μl) was injected, and recordings were made for 3hours. The animal was then killed and a zero flow value recorded and deducted from the recorded signal. Brains were removed and the MCA territory inspected under a microscope to confirm a successful ischaemic event. LDF data were expressed as a % change from baseline. Figure 9 illustrates the *in situ* recording procedure used in LDF studies.

3.2.4. [^{14}C]iodoantipyrine autoradiography

Animals were anaesthetised and polyethylene catheters (15cm long) were inserted into both femoral arteries and veins to permit the continuous monitoring of mean arterial blood pressure (MABP), the repeated sampling of arterial blood, and the administration of [^{14}C]iodoantipyrine. The wounds were infiltrated with anestheic gel (2% xylocaine) and sutured. The animals were then placed in a sterotaxic frame and the MCA occluded by intracerebral ET-1 injection as described in section 2.2.1. Briefly, following a craniotomy, a 28 gauge cannula lowered 8.7mm below skull and 100 pmols of ET-1 in 2μl, or vehicle (sham animals) in 2μl was injected (1μl/90sec), the cannula left in situ for 5 minutes and then slowly withdrawn. Whilst still under anaesthesia, animals were placed in a plaster-of-Paris cast and secured firmly to a raised support comprised of two house bricks. Animals were connected to a blood

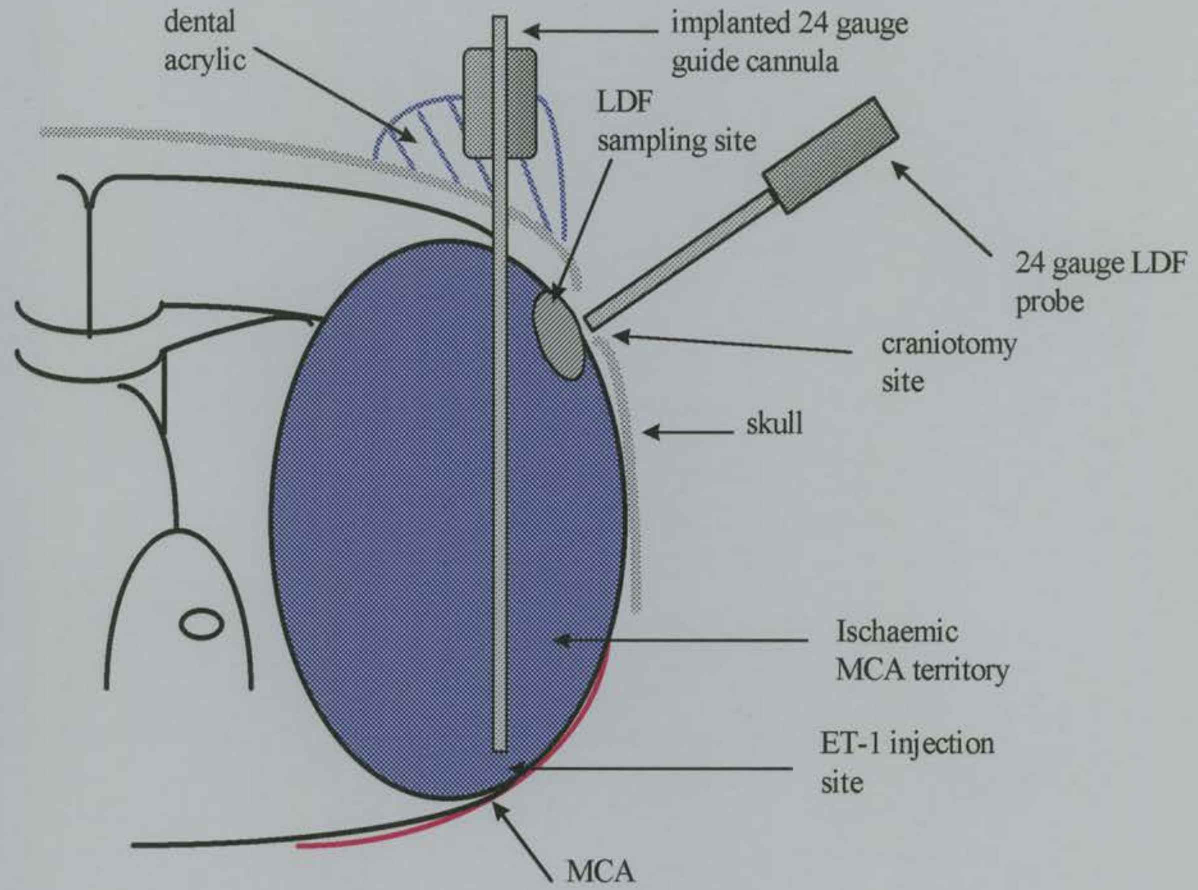


Figure 9. Recording site and surgical preparation for LDF recordings from the parietal cortex.

pressure monitor and allowed to recover from anaesthesia, whilst being maintained normothermic by a heating lamp and rectal probe. The experiment was performed three hours following the first ET-1/saline injection. Blood gases & blood pH (Ciba Corning model 238) and plasma glucose (Beckman glucose analyser 2) were sampled immediately prior to beginning the blood flow measurement. Local CBF was measured according to a modification of the original method of Sakurada *et al.* (1978). Briefly, a ramped infusion of [^{14}C]iodoantipyrine (4.62 MBq/kg (50μCi) in 1ml saline) was administered via the left femoral catheter over a 40 second period. 14-19 timed samples were collected on pre-weighed filter paper discs from the free flowing right arterial catheter. An example of an arterial input function for [^{14}C]-iodoantipyrine loading is shown in Figure 10. Radioactivity in disintegrations per minute (DPMs) were calculated per sample weight and plotted against the time the sample was collected. At the end of the infusion period, the animal was killed by decapitation, the brain excised and frozen in isopentane (-42°C). The frozen brain was transferred to a bed of dry ice where it was afixed to a swivel headed microtome chuck with a plastic embedding matrix (Lipshaw). The brain was then stored at -70°C until sectioning (within 48 hrs). The filter paper discs were re-weighed, 400μl of hydrogen peroxide (40% v/v) added to bleach the colour and 10 mls of scintilation fluid added to each vial. The [^{14}C] concentration was then calculated by scintillation spectrometry.

From each brain, 200 coronal brain sections (20μm) were collected on glass cover slips and rapidly dried on a hot plate (70°C). Autoradiograms were prepared by affixing the coverslips to card, and then sections were exposed to blue-sensitive X-

Arterial input function during [^{14}C]iodoantipyrine autoradiography

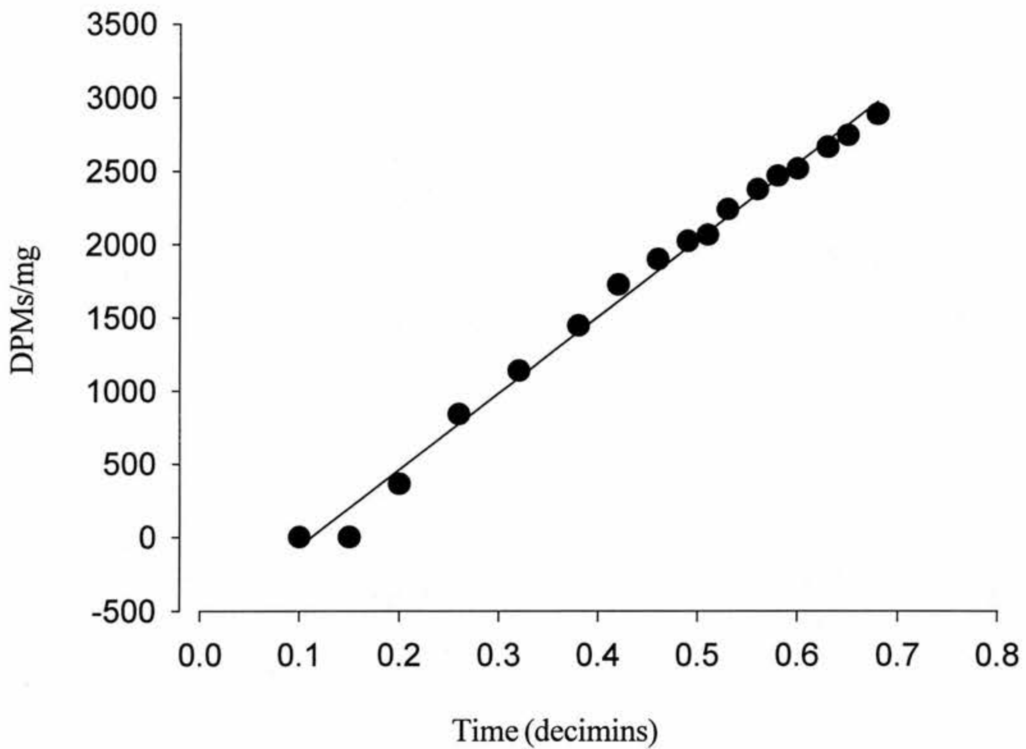


Figure 10. Graph illustrating an arterial input function. A ramped infusion of [^{14}C]iodoantipyrine was delivered intravenously over 35-40 seconds during which timed arterial samples are taken

ray film (Kodak SB-5) along with a series of ^{14}C-labelled standards (concentration range 44-2500 nCi/g tissue equivalents) in light-tight cassettes. These standards include a blank and eight standards with progressively increasing ^{14}C concentrations calibrated to equivalent ^{14}C concentration in brain sections of 20μm thicknes. Films were exposed for 6-7 days and processed according to manufacturer's instructions. Analysis of autoradiograms was performed on a computer-based image analysis system (M4 MCID), following construction of a calibration curve from densitometric measurement of the standards. An accessory file contains the sampling time, sample volume, correction factor and DPM counts from the timed arterial samples, which are integrated into the equation below. Identification of brain areas was made with reference to the atlas of Paxinos & Watson (1986). Bilateral optical density measurements were made from twenty-nine brain regions. Measurements from 4-6 brain sections were analysed per region of interest, and calculation of local CBF made from the optical density value by reference to a calibration curve constructed from the [^{14}C] standards, and from the local [^{14}C] concentration, the history of [^{14}C] in arterial blood and the tissue blood partition coefficient for [^{14}C]iodoantipyrine (0.79) using the equation derived of Sakurada et al. (1978) with the correction factor for distortion of the arterial cathater input function (0.184) of Jay et al. (1988):

$$\mathbf{C_i(\mathit{T}) = \lambda K e^{-K\mathit{T}} \int_0^T C'_a(\mathit{t'} - \Delta \mathit{t}) e^{-K(\mathit{t'} - \Delta \mathit{t})} (1 - K/\mathit{r}) \mathit{dt}} \qquad \mathbf{ml/100g/min}$$

$$\mathbf{+ (K/\mathit{r}) C'_a(\mathit{T})}$$

Where:

Ci (*T*) is the concentration of tracer in tissue at a given time (**T**) following tracer injection

$\mathbf{C'_a}$ is the concentration of tracer in the arterial blood

t is variable time after correction (0.184 x catheter flow rate) for the transit time through the catheter.

K is a constant defined as $\frac{mF}{W\lambda}$ where m=1 (diffusion of tracer across BBB) and F/W is the rate of blood flow per unit mass of tissue

λ is the tissue/blood partition coefficient for the tracer (0.79)

Local CBF within 8 flow thresholds from 0-25 ml/100g/min up to flows >250ml/100g/min was also determined by setting flow thresholds and measuring hemispheric, cortical and caudate nucleus blood flow at the level of the stereotaxic injection site (~1mm rostral to bregma).

[^{14}C]iodoantipyrine studies were performed with the assisstance of Dr. J. Sharkey of the Fujisawa Institue of Neuroscience, University of Edinburgh.

3.2.5. Statistical analysis

All data are shown as mean ± s.e.mean. Temperature and oxygen data were analysed using two-way ANOVA with post-hoc Tukey test, while LDF studies were analysed with one-way ANOVA.

Physiological variables were compared by Students T test. Ipsilateral and contralateral local CBF within 29 specific brain regions following

[^{14}C]iodoantipyrine autoradiography in ET-1-induced MCA occluded animals were compared to sham operated controls by Student's T test. The % of ipsilateral and contralateral hemisphere, cortex and caudate nucleus within 8 specific flow thresholds were compared against flow in sham operated animals by Student's T test.

3.2.6. Experiments performed

Four studies were performed:

1) Body temperature vs brain temperature. Body temperature was varied over the range 30-40°C and corresponding brain temperature recorded in non-MCA occluded animals.
2) Brain temperature and oxygen tension were recorded following ET-1-induced MCA occlusion within lateral striatum.
3) Temporal profile of ischaemia following ET-1-induced MCA oclusion was recorded by LDF
4) [^{14}C]iodoantipyrine autoradiographical determination of local CBF three hours following ET-1 injection. Local CBF for sham and MCA occluded animals was also used to determine the % of hemisphere, neocortex and caudate nucleus within 8 flow thresholds.

3.3. RESULTS

3.3.1. Body & brain temperature

Calibration of temperature sensors in ACSF demonstrated a linear and parallel relationship for the two temperature sensors "a" (proximal) and "b" (distal) in the

range 30-40°C. (r^2(a)=0.98 slope a=1.07±0.02; r^2(b)=0.98 slope b=1.12±0.02). Since changes in temperature for the probe are linear, a correction factor could be simply based on the mean differences between the sensor readings and the rectal probe readings rather than on the differences of slope between sensor and probe readings. This would have required back calculation to absolute zero and a correction factor that took into account slope divergence at different temperatures. Thus the mean differences between sensor readings and the reference electrodes over the range 32-39°C were calculated and then sensor readings corrected where appropriate.

The results for striatal temperature in the range 32-39°C from both probe sensors are plotted in figure 11, against corresponding body temperature. Linear regression gave a r^2 value of 0.96 and slope of 1.03, demonstrating a linear correlation between core body temperature and (non ischaemic) brain temperature in the 32-39°C range.

3.3.2. 450 pmols in 3μl

Injection of 450 pmols ET-1 in 3μl (n=9) produced an hemispheric ischaemic brain lesion of 159 ± 18mm^3. This volume was not significantly different to that seen following injection of 33-300 pmols ET-1 in 2μl.

3.3.3. Cerebral temperature

Striatal temperature and rectal temperature remained between 37-38°C for the duration of recordings with mean values of 37.6±0.03°C and 37.5±0.03°C respectively (Figure 12). Fluctuations in temperature across time were below the limits of thermostatic control of the heated blanket (CMA specification ± 0.2°C) .

Correlation between body and brain temperature

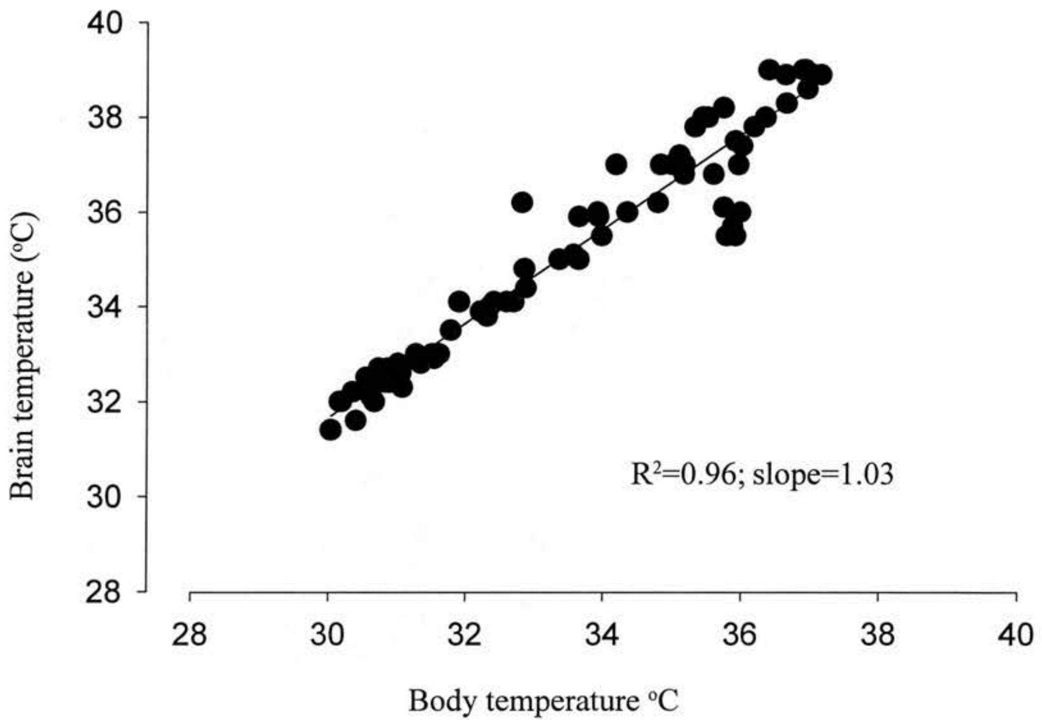


Figure 11. Correlation between body and brain temperature over the range 30-40°C. Body temperature was varied and striatal temperature simultaneously recorded (n=4). A strong positive correlation was found. R^2=0.96; slope=1.03.

Striatal and rectal temperature following
ET-1-induced MCA occlusion

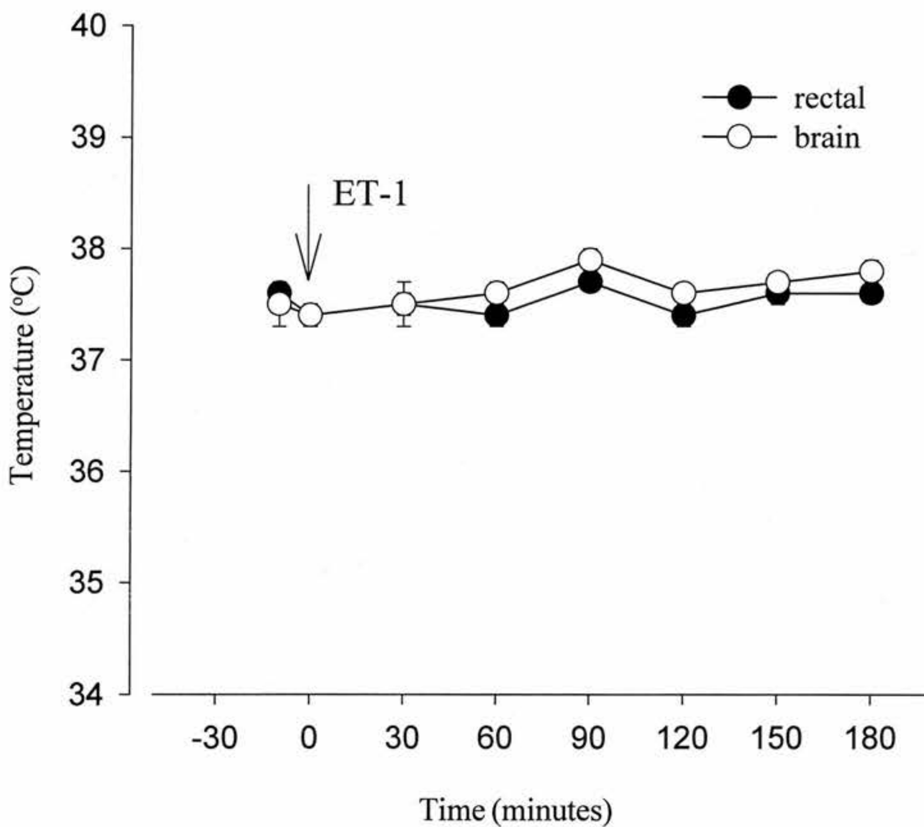


Figure 12. Intracerebral temperature recordings from the striatum following ET-1-induced MCA occlusion (n=3). Data are presented as mean ± s.e.mean. No significant change in brain temperature occured following ET-1-induced MCA occlusion, and no differences were found between striatal and rectal temperature, throughout the 3 hour recording period.

3.3.4. Cerebral oxygen tension during stroke

Striatal oxygen tension remianed unchanged for the duration of recordings in non-MCA occluded (vehicle-injected) animals. By contrast, in animals undergoing ET-1-induced MCA occlusion, oxygen tension fell significantly ($p=<0.05$) within 15 minutes of ET-1 injection to 61% of baseline value (range 50-83%). Oxygen tension was maximally depressed by 85% after 2 hours, remaining below 30% for the duration of recordings and showing no significant recovery (Figure 13).

3.3.5. Cortical tissue perfusion (LDF) - 3hour recordings

LDF recordings from dorsal parietal cortex ipsilateral to the site of Et-1 injection demonstrated that mean flow velocity was reduced by 86% (range 72-99%) within 5 minutes of vessel occlusion (Figure 14). LDF values remained depressed by greater than 50% after 2 hours, and flow was still significantly reduced after 3 hours ($p<0.05$).

3.3.6. Local CBF at 3hours

Physiological variables for sham and MCA occlusion animals are shown in Table 3. Variables were within the normal limits for conscious animals and no significant differences were found between sham and ET-1 treated animals for any of the parameters studied.

Local CBF was found to be generally symmetrical between hemispheres and between sham and MCA occluded animals in brain regions outwith the MCA

Striatal oxygen tension following
ET-1-induced MCA occlusion

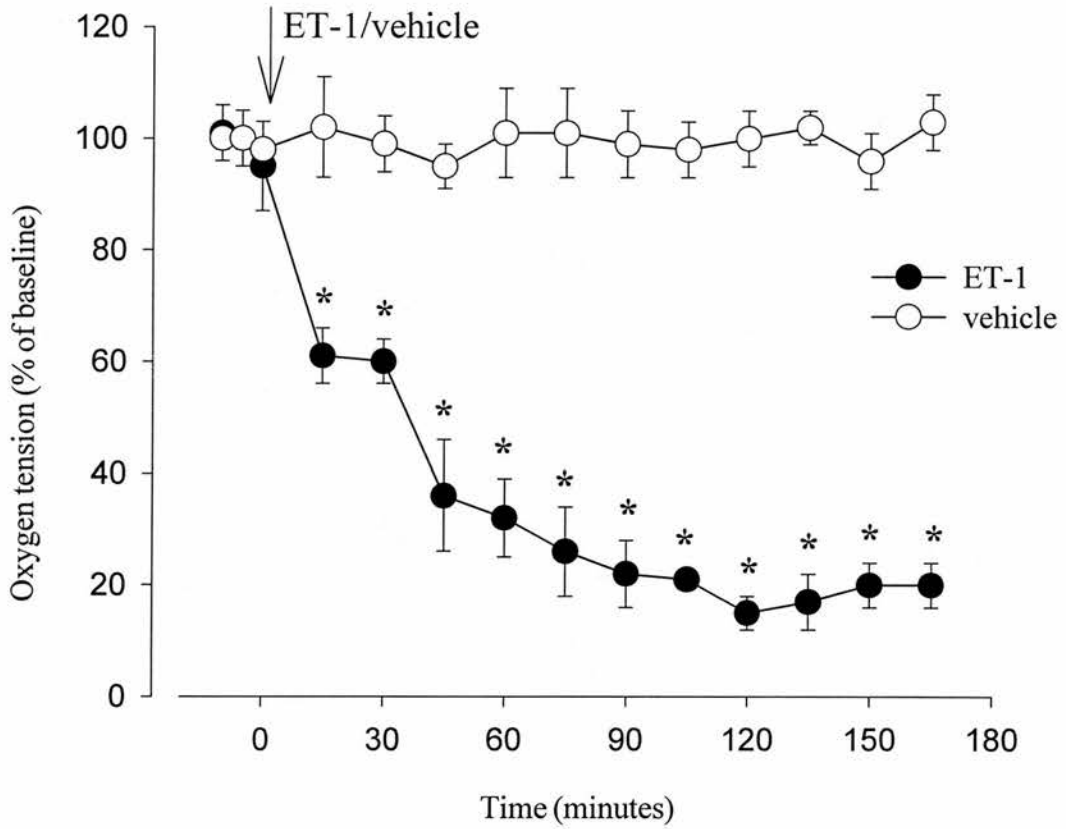


Figure 13. Intracerebral oxygen tension recordings following ET-1-induced MCA occlusion (n=5) or vehicle injection (n=8). Data are presnted as mean ± s.e.mean. Whilst control recordings did not significantly change from baseline, oxygen tension fell significantly within 15 minutes by 39%, and was maximally decreased by 85%, following ET-1 injection. *P=<0.05 compared to vehicle controls.

LDF profile of tissue perfusion velocity change following ET-1-induced MCA occlusion

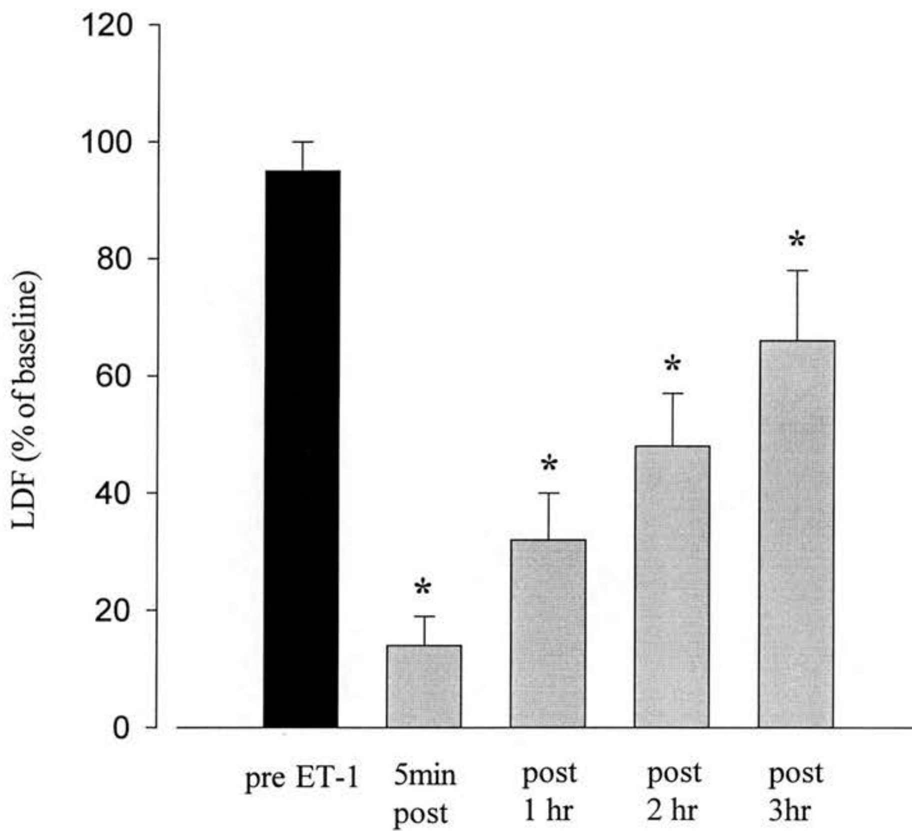


Figure 14. Dorsal parietal cortex LDF recordings of perfusion velocity changes following ET-1-induced MCA occlusion (n=5). Data are presented as mean ± s.e.mean. LDF signal was reduced by 86% within 5 minutes of ET-1 (450 pmols in 3μl), and remained depressed for three hours. **P*=<0.05 compared to baseline.

Physiological variables for [^{14}C]iodoantipyrine determination of local CBF three hours after ET-1-induced MCA occlusion

Variable	**ET-1 injected**	**sham animals**
M.A.B.P. (mmHg)	122 ± 3	117 ± 2
Rectal temperature (°C)	37.1 ± 0.1	37.2 ± 0.1
pH (units)	7.44 ± 0.01	7.42 ± 0.01
pCO_2 (mmHg)	42 ± 1	39 ± 2
pO_2 (mmHg)	93 ± 7	93 ± 7
plasma glucose (mM)	8.8 ± 0.6	8.0 ± 0.5

Table 3. Physiological variables taken immediately prior to [^{14}C]iodoantipyrine determination of local CBF three hours following ET-1 (n=8) or vehicle (n=7) injection. Data are presented as mean ± s.e.mean. No significant differences in physiological parameters were found between groups.

territory. Areas such as the pons, cerebellum and genu of the corpus callosum demonstrated variations in local CBF of <10% between hemispheres and across treatments. Furthermore, there were no significant differences between sham and ET-1 treated animals in any structure contralateral to the side that underwent intracerebral injections. Figure 15 shows pseudocolour transforms of local CBF measured three hours after animals undergoing ET-1-induced MCA occlusion, compared to vehicle injected controls. Note the profound reductions in CBF throughout the MCA vascular territory at both representative stereotaxic levels .

Local CBF determined 3 hours following ET-1 induced MCA occlusion demonstrated significant reductions in blood flow of up to 84% (piriform cortex) in the ipsilateral hemisphere in a number of brain regions compared to sham operated animals (Table 4). These included all cortical structures, except the midline structures of the retrosplenial and cingulate cortices, and the lateral caudate-putamen. A number of structures outwith the MCA vascular territory exhibited small CBF reductions and included the globus pallidus and nucleus accumbens which were reduced by 27% & 43% respectively.

Local CBF at different flow thresholds within hemisphere, cortex and caudate nucleus were calculated and data are presented in figures 16 (hemispheric), 17 (cortex) and 18 (caudate-putamen). Statistical analysis demonstrated a significant shift towards the left througout the three sampled regions. Whilst there were no significant differences in local CBF within any flow threshold for the contralateral hemispheres, the % of ipsilateral hemispheric flow within specific thresholds were significantly increased within the following lCBF bands: 0-25, 25-50 & 50-75

Local cerebral blood flow three hours following
ET-1-induced MCA occlusion

Brain region	Sham MCA occlusion at 3hours		ET-1-induced MCA occlusion at 3 hours	
	Blood flow ipsilateral	Blood flow contralateral	Blood flow ipsilateral	Blood flow contralateral
Superficial cerebral structures				
Medial prefrontal cortex	188±5	188±4	135±23[a]	156±25
Frontal cortex area 8	191±6	191±5	62±19***	188±15
Frontal cortex area 10	184±6	186±5	39±9***	182±16
Piriform cortex	208±8	207±7	33±10***	217±8
Somatosensory cortex	198±2	197±2	42±8***	198±14
Cingulate cortex	226±8	224±7	216±28	224±27
Parietal cortex	202±6	200±6	66±10***	216±17
Temporal cortex	193±6	192±6	64±10***	215±12
Retrosplenial cortex	215±5	213±4	218±27	228±24
entorhinal cortex	140±8	136±9	54±10***	145±10
Primary visual cortex	212±5	213±5	82±15***	221±15
Deep cerebral structures				
Genu of corpus callosum	52±2	51±2	46±9	47±8
Nucleus accumbens	109±6	109±6	63±12**	99±10
Caudate-putamen (dorso-lateral)	131±4	133±4	70±15*	139±11
Caudate-putamen (medio-lateral)	146±8	146±7	80±16**	149±10
Septal nucleus	98±7	98±6	98±13	97±15
Anterior-medial thalamic nucleus	194±7	194±7	173±17[a]	192±14
Ventrothalamic nucleus	149±5	149±6	136±13[a]	178±19
Subthalamic nucleus	147±3	146±5	148±12	167±14
Globus pallidus	101±4	103±4	74±8**	95±6
Hippocampal CA1	84±4	84±4	91±8	98±9
Dentate gyrus	114±4	109±3	122±9	125±10
Internal capsule	84±2	84±2	83±9	89±9
Lateral geniculate nucleus	162±3	158±3	158±12	179±21
Substantia nigra	130±10	131±9	150±16[a]	142±15
Cerebellum & pons				
Pontine nucleus	111±6	112±7	111±7	114±8
Cerebellar white matter	45±2	45±2	44±5	43±4
Cerebellar grey (minimum)	103±1	104±1	108±6	109±6
Cerebellar grey (maximunm)	230±11	229±10	221±13	218±13

Table 4. Local cerebral blood flow (ml/100g/min) in 29 anatomically defined regions of the brain, measured 3 hours after ET-1-induced MCA occlusion or sham (saline) injection. Data are presented as mean ± s.e.mean. Student's T test. *P=<0.05. ***P=<0.05. for comparison to ipsilateral sham animals. [a]no significant difference.

ml/100g/min. By contrast, the % of hemisphere within the bands 100-150, 150-200, 200-250 and >250 ml/100g/min were significnatly reduced in MCA occluded animals.

This pattern was identical to that seen for the cortex and caudate nucleus, whereby the % of the region with the 0-75 ml/100g/min bands were increased, whilst above 100ml/100g/min, the % of the region was significantly reduced compared to sham animals. This separation was qualitatively greatest for the cortex and caudate nucleus.

3.4. DISCUSSION

3.4.1. General

In these studies, intracerebral O_2 & temperature recordings, LDF and [^{14}C]-iodoantipyrine autoradiography were employed to determine the characteristics of the ET-1-induced MCA occlusion model. In establishing a novel model of stroke, a range of parameters should be examined in detail. This allows comparison to existing models and consequently advantageous and disadvantageous aspects to be addressed. These studies suggest that the duration of ischaemia is at least three hours, and that this model is not associated with a reduction in striatal temperature. Intracerebral temperature and oxygen tension were measured within the striatum, since histopathological analysis in chapter 2 demonstrated that the region of the medial and lateral caudate nucleus consistently exhibits ischaemic brain damage. LDF recordings were only made from the parietal cortex, since preliminary studies

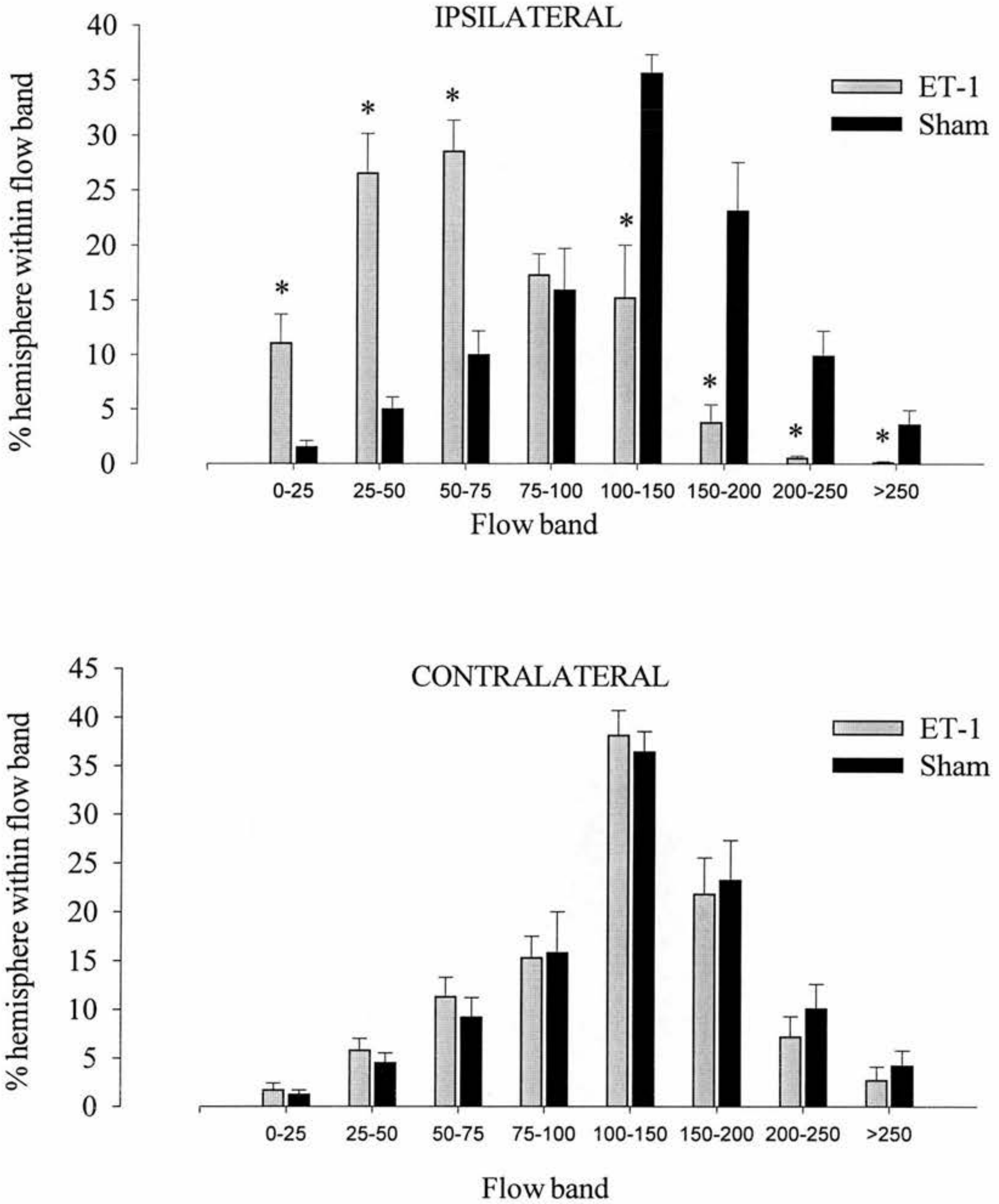


Figure 16. Comparaison of the % of ipsilateral (top) and contralateral (bottom) hemisphere demonstrating local CBF within specific flow bands for ET-1 (n=8) and sham operated (n=7). Data are presented as mean ± s.e.mean. *P=<0.05 compared to sham operated controls.

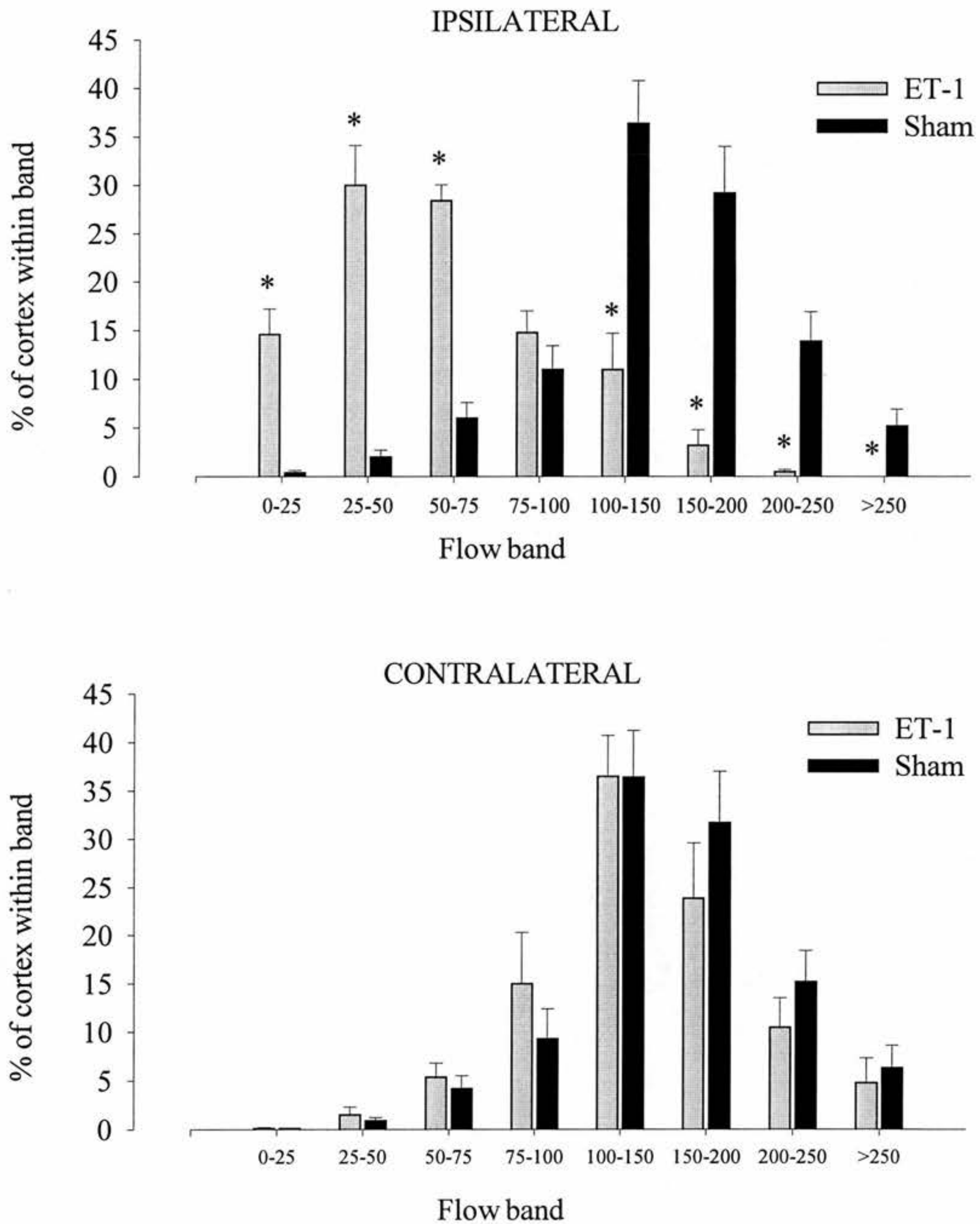


Figure 17. Comparison of the % of ipsilateral (top) and contralateral (bottom) cortex demonstrating local CBF within specific flow bands for ET-1 (n=8) and sham operated (n=7) animals. Data are presented as mean ± s.e.mean. *P=<0.05 compared to sham animals

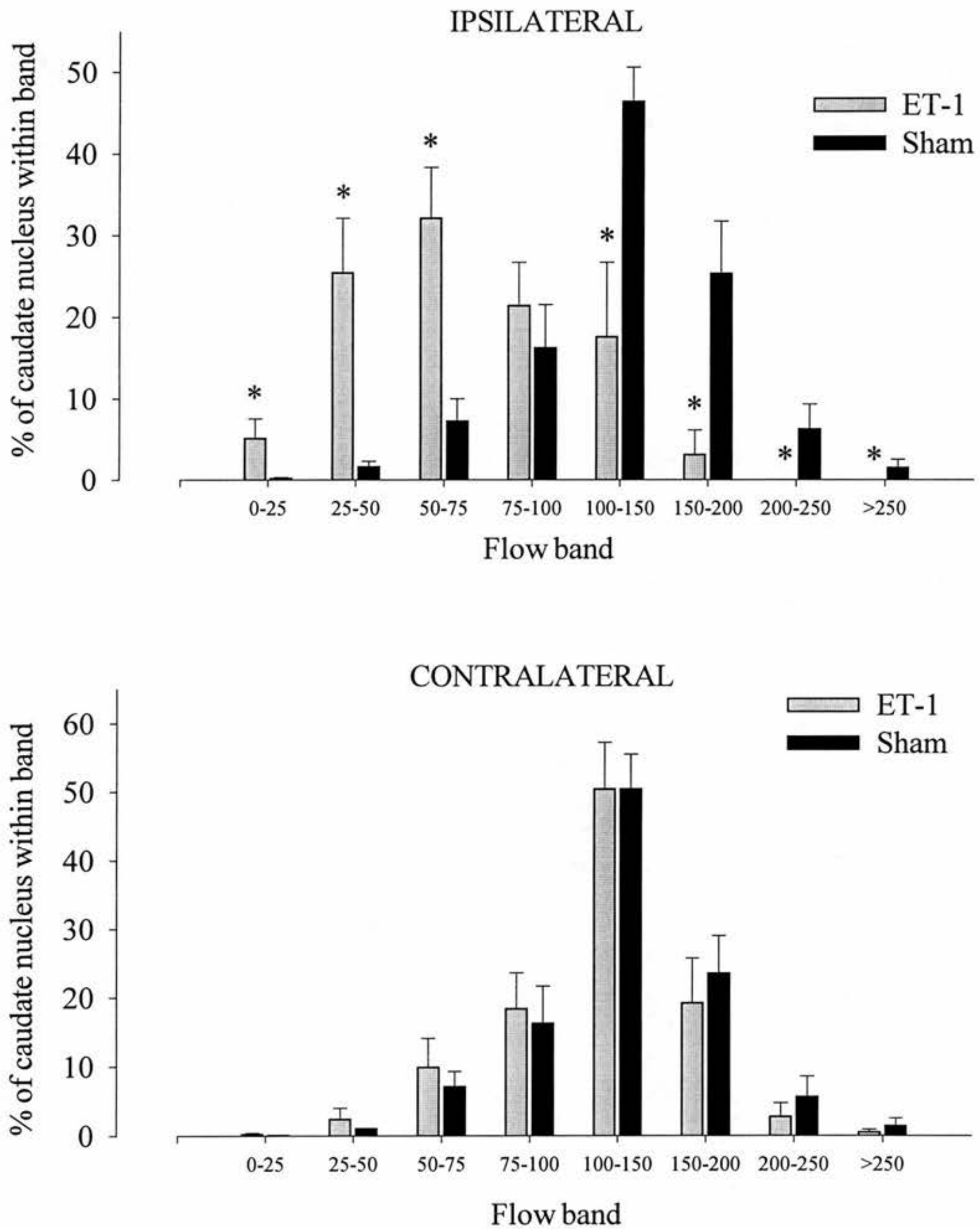
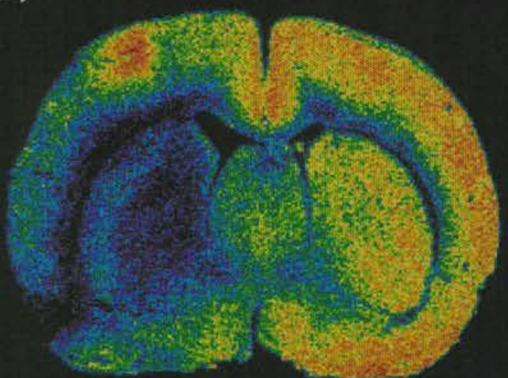
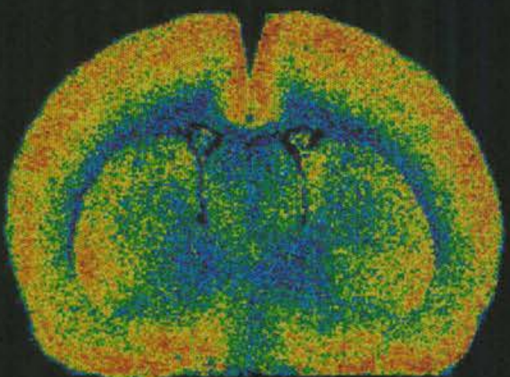


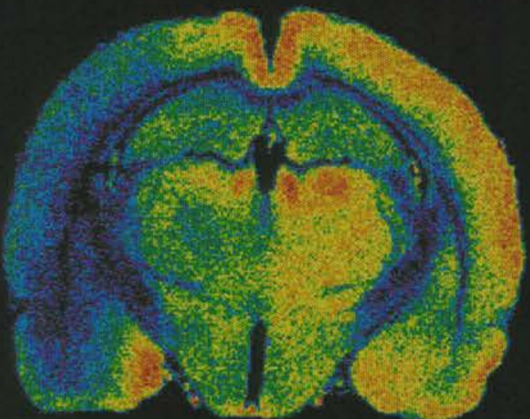
Figure 18. Comparison of the % of ipsilateral (top) and contralateral (bottom) caudater nucleus within specific local CBF bands for ET-1 (n=8) and sham operated (n=7) animals. Data are presented as mean ± s.e.mean. *P<0.05 compared to sham animals.

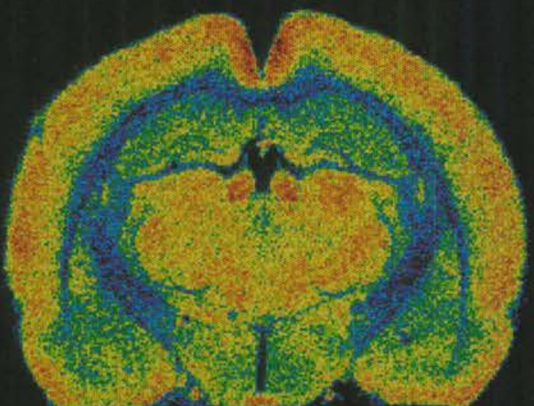
Figure 15.

Local CBF determined by [^{14}C]iodoantipyrine autoradiography three hours after intracerebral injection of 100 pmols ET-1 adjacent to the MCA. Pseudocolour transforms represent local CBF at the level of bregma in (a) ET-1 treated & (b) vehicle injected sham animals at the same level, and local CBF at the level of the hippocampus (Bregma -4.0mm) in (c) ET-1 treated & (d) vehicle injected sham animals (d). Note the profound reductions in local CBF throughout the MCA vascular territory.


(a)


(b)


(c)


(d)
ml/100g/
min
6.3
17.4
30.1
45.0
62.8
84.9
113.1
151.8
210.6
321.5
761.8

determined the LDF signal from intracerebral recordings were unreliable (data not shown).

3.4.2. Body/brain temperature correlation

We found in these studies that there is a linear correlation between core body temperature and (non ischaemic) brain temperature in the 32-39°C range. Previous studies have shown that whilst brain temperature may not be the same as body temperature, there is a direct correlation between them, and the present data are consistent with established findings (Ginsberg et al. 1992; Hasegawa et al. 1994), validating these probes for application to subsequent studies.

3.4.3. Striatal temperature

LDF and intracerebral recordings were made from rats with implanted cannulae. As mentioned, a higher dose and volume was employed in this study using an indwelling cannula, to increase the MCA occlusion success rate. However, histopathological analysis demonstrated that this higher dose and volume had no significant effect on the volume and distribution of ischaemic brain compared to injection of 100 pmol in 2μl using an acutely placed cannula. Intracerebral recordings from the striatum following ET-1-induced MCA occlusion determined that temperature remained unchanged for 3 hours following ET-1 infusion. These results contrast with findings in other models of MCA occlusion, in which the procedures involved have been shown to either elevate (Zhao *et al.* 1994) or decrease (Morikawa *et al.* 1992) brain temperature. However, studies have shown that the

striatal temperature may change by only 0.5°C or less following MCA occlusion (Hasegawa et al. 1994). These differences may be due to a number of factors. First, by contrast to the transorbital approach, occlusion of the MCA in this model circumvents the need for a large craniotomy or displacement of the temporalis muscle. Such surgical procedures may lead to brain cooling consequent on tissue exposure. Second is the possible effect of residual flow within the vascular territory of the MCA, insufficient to prevent ischaemic damage but sufficient to maintain local tissue temperature. This is an important finding because reducing brain temperature ameliorates brain damage from both focal and global cerebral ischaemia (Buchan & Pulsinelli, 1990; Busto *et al.* 1987; Onesti *et al.* 1991; Xue *et al.* 1992), while hyperthermia may exacerbate neuropathological damage (Chen *et al.* 1991; Dietrich *et al.* 1990). Consequently, an advantage of the intracerebral ET-1 model is that neuroprotection studies will not be confounded by changes in brain temperature.

3.4.4. Striatal oxygen tension

The duration of MCA occlusion-induced ischaemia was assessed by measuring oxygen tension within the medial striatum. ET-1-induced MCA occlusion resulted in profound reductions in striatal oxygen tension. This decrease was significant within 15 minutes, and continued to fall reaching a maximum of 85% reduced after 2 hours, thereafter remaining essentially stable. There was no sign of recovery of oxygen tension during the 3 hour recordings. By contrast, vehicle injection had no significant effect on oxygen tension, and there was no significant deviation from baseline recordings. Whilst oxygen tension is not a direct measure of CBF, it is a function of

the perfusion state in ischaemically challenged tissue. That oxygen tension declines following MCA occlusion reflects the reduction in CBF to the tissue and the ischaemic predicament of the brain parenchyma. Measurement of oxygen extraction has been a principle method of assessing cerebral ischaemia using PET methods, and has been addressed above.

Significant falls in brain oxygen levels following experimental cerebral ischaemia have been reported previously (Crockard *et al.* 1975; Martin *et al.* 1982; Nair *et al.* 1987; Freund *et al.* 1989; Liu *et al.* 1995) and the present data are consistent with studies in which the MCA was occluded by intraluminal filament (Back *et al.* 1994b) or transorbital approach (Zauner *et al.* 1995). These results suggest that the duration of ET-1-induced ischaemia is at least three hours.

3.4.5. Cortical perfusion velocity

LDF recordings from the parietal cortex extend the findings of the oxygen tension recordings. The parietal cortex is commonly sampled in LDF studies (Dirnagl *et al.* 1989; Dalkara *et al.* 1994; Kadoya *et al.* 1995; Karibe *et al.* 1995; Takagi *et al.* 1994a) because this region consistently undergoes dense ischaemia following MCA occlusion (Gartshore *et al.* 1996; Sharkey *et al.* 1994; Tamura *et al.* 1981b), and reperfuses on cessation of MCA occlusion (Dirnagl *et al.* 1989; Karibe *et al.* 1995). LDF has also been shown to be an accurate index of histological outcome (Soriano *et al.* 1997). In these studies, LDF values (tissue perfusion units) were expressed as the % change from baseline, which is accepted as the only valid method with which to express LDF data (Fabricus & Lauritzen 1996).

The reduction in parietal cortex flow of -86% within 5 minutes of ET-1 is somewhat greater than that found using [^{14}C]iodoantipyrine autoradiography (~60% reduction) in an earlier study using this model (Sharkey *et al.* 1993), although this may be adequately explained by experimental protocol differences, or the use of anesthetics in the present study, which have significant effects on CBF. Data are also comparable with LDF studies in which the MCA was blocked by an intraluminal filament (Kadoya *et al.* 1995) or by electrocoagulation (Morikawa *et al.* 1992). Flow returned gradually over the three hour recording period but still remained below 50% after two hours, and significantly reduced compared to pre-ET-1 values after three hours. This profile compares well to studies where ET-1 was applied to the exposed MCA (Gartshore *et al.* 1996), in which ^{99m}Tc-exametazime uptake in the parietal cortex was reduced by 84% 5 minutes after ET-1 application and recovered to ~40% 2 hours post ET-1. These findings suggest that CBF may return to structures within the vascular territory of the MCA and some reperfusion may be a characteristic of this model.

These results however do not entirely compliment the findings of striatal oxygen tension, which suggested blood flow has not returned to the striatum after three hours. This difference may be explained by the differential sampling sites. LDF was recorded from dorsal parietal cortex which commonly lies within the ischaemic penumbra (see table 2), and is close to the boundary between normal and ischaemic tissue, such that CBF may return to this area in some cases. By contrast, the medial and lateral striatum represent end-territory structures that do not receive significant collateral perfusion from other vascular fields, and the low efficacy of

neuroprotectants to reduce ischaemic damage in the striatum has been attributed to this (Nagasawa & Kogure, 1989; Sharkey & Butcher, 1994; 1995). These differences are reflected in Figure 33, which is a pseudocolour transform of local CBF measured by [^{14}C]iodoantipyrine 3 hours following ET-1 induced MCA occlusion. A region of hyperperfusion lies within the dorsal LDF sampling site (a), whilst no such hyperperfusion is evident within the striatum. Consequently, the recording position of the LDF probe means that sampling within this region may exhibit a greater degree of reperfusion than core areas of the ischaemic territory.

3.4.6. Local CBF at 3 hours

Previous studies have determined that if ischaemia following MCA occlusion is of three hours duration or greater, the pathophysiological outcome is not significantly different to animals subjected to permanent MCA occlusion (Kaplan *et al.* 1994). Consequently local CBF was determined 3 hours following MCA occlusion using the technique of [^{14}C]iodoantipyrine autoradiography, which is an established method for quantitative determination of local CBF (Sakurada *et al.* 1978).

Twenty-nine brain regions were sampled in these studies which included structures both within and outwith the vascular terrirory of the MCA as well as brain regions with functional connectivity to regions within the MCA vascular field. Generally, ipsilateral local CBF in ET-1 injected rats was not significantly different to the contralateral side and was not significantly different to either ipsilateral or contralateral CBF in sham animals. CBF within the pons, septum and cerebellar white matter was very similar to that reported previously following MCA occlusion

in the rat (Tamura et al. 1981b) and was not significantly different to sham operated animals or the contralateral hemisphere.

CBF was significantly reduced in all ipsilateral cortical structures studied except midline structures such as the cingulate and retrosplenial cortices. These structures receive CBF from the anterior cerebral artery and generally do not undergo significant changes in local CBF following MCA occlusion (Tamura et al. 1981b; Robinson et al. 1990). Local CBF in the frontal and parietal cortices 3 hours after ET-1-induced MCA occlusion was in the range 33-66ml/100g/min, reductions of 63-73%, which compares well to reductions of 82-83% found previously following permanent MCA occlusion of 82-83% (Tamura et al. 1981b), and are almost identical to those found in other models that have employed ET-1, where these cortical structures exhibited reductions of 67-71% (Robinson et al. 1990). Local CBF was reduced by 61% in the more caudal structure of the primary visual cortex, which is in agreement with previous studies (Tamura et al. 1981b; Robinson et al. 1990). This level of CBF reduction has been previously accounted for by this structure receiving some CBF supply from the posterior cerebral artery (Tamura et al. 1981b; Scremin, 1995). The extent of CBF reduction in areas such as the piriform cortex (85%) and somatosensory cortex (79%) suggests that significant reperfusion has not commenced 3 hours following ET-1-induced MCA occlusion. The somewhat higher level of cortical CBF at three hours compared to 30 minutes post-MCA occlusion (Tamura et al. 1981b) may be adequately explained by previous reports that CBF may recover to an extent even following permanent (electrocoagulation) occlusion of the MCA (Hakim et al. 1992).

These results contrast to the study by Gartshore et al. (1996) in which ET-1 was applied to the exposed MCA. In their study, CBF had generally recovered to a greater extent at two hours than was obserevd at three hours in this model. The explanation for this is unclear but is likely to be due to differences arising from injection of ET-1 intraparenchymally compared to application to the exposed MCA, the former of which may limit diffusion and prolong the action of ET-1. Whilst CBF was essentially similar to that following permanent MCA occlusion in this model, the time for half-maximal restoration of pial vessel diameter following ET-1 application is reported to be in the order of 45-60 minutes (Robinson & McCulloch, 1990), which would suggest that the MCA would be more than 50% patent after three hours. Since the MCA is not a resistance vessel such a relaxation would effectively allow full flow through to downstream territories. That structures within the MCA are still densely ischaemic, may therefore be explained in a number of ways. First, the $t_{1/2}$ for ET-1 on the rat MCA may differ. Indeed, the study by Robinson & McCulloch (1990) was performed on the pial vessels of chloralose-anaesthetised cats. Second, post-ischaemic microvascular obstruction may limit reperfusion. This has been proposed to account for the limited reperfusion seen 2 hours following ET-1 application to the exposed MCA (Gartshore et al. 1996). Furthermore, ET-1 itself may diffuse to act on cerebral vessels other than the MCA and limit perfusion from other vessels which has previously been suggested (Gartshore *et al.* 1996).

Local CBF of <25ml/min/100g has been reported as necessary to produce histopathological damage in the rat, although this is somewhat variable (Tamura *et al.* 1981b; Symon *et al.* 1975) and may be higher in primates (Blair & Waltz, 1970;

Tamura *et al.* 1981b). Whilst CBF was still profoundly reduced within the cortex supplied by the MCA three hours after ET-1, the levels reported here (30-60ml/100g/min) are somewhat higher than those reported to produce ischaemic brain damage. However, correlating CBF changes to histopathological outcome is difficult and variable (Hakim et al. 1992). Furthermore, this discrepancy may be explained by the effects of time on the required ischaemic intensity. Heiss & Podreka (1983) determined a relationship between the duration of ischaemia and residual CBF, whereby the severity of ischaemia required to produce irreversible damage decreases with the duration of ischaemia. Whilst very low flow levels below 25ml/100g/min may be required to produce ischaemic damage when measured acutely, this threshold increases with the duration of ischaemia, such that by three hours post MCA occlusion higher flow levels of ~30-60ml/100g/min may be sufficient to produce ischaemic damage. Given difficulties in predicting histological outcome based on CBF and the effects of time on required ischaemic severity following focal cerebral ischaemia, the levels of CBF within the cortex three hours after ET-1-induced MCA occlusion are both compatible with those required to produce histological damage and are essentially consistent with a model of permanent MCA occlusion.

Three hours following ET-1-induced MCA occlusion, CBF in the dorso- and mediolateral caudate nucleus was ~50% of flow in the contralateral hemisphere, which is significantly higher than is found after 10 minutes (Sharkey et al. 1993). The finding of greater CBF recovery in the caudate-putamen in this study is similar to that reported by Gartshore et al. (1996), in which, whilst CBF was still <50ml/100g/min in many cortical structures 2 hours following ET-1-induced MCA

occlusion, CBF in the caudate-putamen was ~80-90ml/100g/min, a similar level to that found in the present studies. The explaination for this is unclear. Indeed, studies suggest that the greater the ischaemic intensity, the more limited subsequent reperfusion is (Hakim et al. 1992; Gartshore et al. 1996). Anatomical differences in the cerebrovasculature may lead to improved CBF to structures within the MCA territory, and the presence of Hubner's arteries, branches of the anterior cerebral artery that feed the caudate-putamen, have been attributed to higher CBF within the striatum following MCA occlusion (Gartshore *et al.* 1996). However, it is generally accepted that the caudate-putamen is an end vessel territory that receives almost no collateral perfusion, which has been proposed to account for the common lack of efficacy of neuroprotectantion in the striatum (Sharkey & Butcher, 1994). Another possibility is that MCA constriction begins to relax after 3 hours leading to a return of CBF. Formation of a thrombus at the site of constriction might mean that whilst flow returns to the proximal region of the MCA branches, such as the lenticulostriate branches that feed the striatum, flow to structures distal to the MCA trunk do not initially receive perfusion. However, this would have to consistently occur, and since these animals were heparinised and no evidence of thrombi within the MCA were visible in any brains, this proposal seems unlikely.

Since ischaemia is densest in the striatum in this model (Sharkey *et al.* 1993), accumulation of vasodilator metabolites such as CO_2 and H^+ will be greatest and may drive any residual CBF within the MCA territory. This may account for the higher flow within the striatum relative to other cortical structures, and has previously been proposed to account for luxury perfusion (Hoedt-Rasmussen *et al.* 1967). However,

since cortical CBF is still profoundly reduced, suggesting the MCA is still constricted, it seems unlikely that physiological function within the cerebrovasculature would be capable of driving this. The exact explanation remains obscure and might be a combination of one or more of these factors.

Comparison of local CBF 3 hours post MCA occlusion compared to 10 minutes also suggests local CBF recovers in a heterogeneous manner across the hemisphere (Table 5), which has been shown previously (Gartshore *et al.* 1996). Local CBF in the pons was included as a control area, and was found to be almost identical between studies and did not change following MCA occlusion which is as reported previously (Tamura *et al.* 1981b). CBF recovery in the present study is correlated to an extent to the initial intensity of ischaemia. The order of cortical ischaemic intensity in the present study was:

piriform > area 10 > area 8 > parietal = temporal

whilst CBF intensity in the cortex in the study by Sharkey *et al.* (1993) was:

frontal area 8 > frontal area 10 > parietal > piriform > temporal

Such a correlation have also been reported previously (Hakim *et al.* 1992; Gartshore *et al.* 1996). CBF in frontal cortex areas 8 and 10 had changed little three hours following MCA occlusion (<10%), however, local CBF had decreased by a further 10-46% in piriform, parietal and temporal cortex, relative to the flow decrease determined 10 minutes following MCA occlusion. By contrast, local CBF had recovered by 13 and 43% in somatosensory cortex and lateral caudate nucleus respectively, by 3 hours post ET-1-induced MCA occlusion.

Comparison of ipsilateral local CBF at 10 minutes (Sharkey et al. 1993) to 3 hours following ET-1-induced MCA occlusion.

	10 min local CBF		3 hour local CBF	
Brain area	**(ml/100g/min) mean ± S.D.**	**% reduction (comparison with contralateral hemisphere**	**(ml/100g/min) mean ± S.D.**	**% reduction (comparison with contralateral hemisphere**
Frontal cortex area 8	30±34	76%	62±53	67%
Frontal cortex area 10	40±64	65%	39±25	79%
Piriform cortex	55±23	51%	33±27	85%
Lateral caudate nucleus	9±5	91%	75±44	48%
Somatosensory cortex	10±3	92%	42±24	79%
Parietal cortex	56±39	59%	66±28	69%
temporal cortex	96±41	24%	64±27	70%
Pons	92±20	no difference	111±20	no difference

Table 5. Comparison of local CBF changes determined by [^{14}C]iodoantipyrine autoradiography 10 minutes (sharkey et al. 1993) and 3 hours following ET-1 induced MCA occlusion. The pons is included as a non-MCA territory brain region as control.

Analysis of local CBF within flow thresholds from 0->250ml/100g/min, demonstrated that whilst only ~1% of the contralteral hemisphere had local CBF below 25ml/100g/min, a significant shift to lower flow levels was found in the ipsilateral hemisphere, cortex and caudate-putamen nucleus. Analysis of the shift determined that at low flow thresholds (CBF 0-75ml/100g/min), the percentage of the region was significantly greater in ET-1 treated animals, whilst at the high flow levels (100->250ml/100g/min) sham operated animals had a significantly greater percentage of the region within that flow band. This supports and compliments the previous findings where lCBF was measured 10 minutes after ET-1 (Sharkey *et al.* 1993). The insight available from comparison may be limited however by the original ET-1-induced MCA occlusion procedure being performed in concious animals using a dose of 120 pmols. However, in those studies, the percentage of the hemisphere with ischaemic levels of CBF <25ml/100g/min was 17%, whilst in these studies ~11% of the hemisphere still had local CBF levels <25ml/100g/min. This demonstrates that the significant and profound reduction of local CBF throughout the MCA territory found 10 minutes post ET-1 are sustained for as long as 3 hours following ET-1-induced MCA occlusion. The finding that CBF had decreased further in some regions 3 hours post MCA occlusion suggests that processes that affect microvascular integrity may be limiting any available CBF. This suggests that the phenomenon of "no reflow" may be an important factor in the profile of blood flow return in this model. "No reflow" (Ames, 1968) refers to limitations in the microvascular patency to flow, which may be caused by a multitude of microvascular changes such as oedema, alterations of endothelial cells, leukocyte-endothelial cell

interaction and coagulation system activation (del Zoppo *et al.* 1991; del Zoppo, 1994; Akopov *et al.* 1996).

A number of brain regions outwith the vascular territory of the MCA undergo CBF changes following MCA occlusion (Tamura *et al.* 1981b), and this includes some of the structures of the basal ganglia. This has been proposed to occur as a consequence of functional connectivity between regions. In the case of the substantia nigra & globus pallidus, this probably occurs due to loss of the inhibitory input from the striatum (Tamura *et al.* 1993; Sharkey *et al.* 1993). In the present study, ipsilateral CBF within the substantia nigra was somewhat higher than in sham lesioned animals (150ml/100g/min compared to 130 ml/100g/min) but this did not reach statistical significance. However, other studies employing ET-1 to occlude the rat MCA have not reported significant increases in local CBF within the substantia nigra (Robinson *et al.* 1990), so the discrepancy noted in this study does is not entirely at odds with the established literature.

CBF within the globus pallidus was found to be significantly decreased in the present study, and has previously been reported to increase (Tamura *et al.* 1981b; Sharkey *et al.* 1993), decrease (Gartshore *et al.* 1996) or remain unchanged (Robinson *et al.* 1990) following MCA occlusion. These discrepancies may be explained by the complex nature of the vascular and neuronal innervation of this structure. The lateral segment of the globus pallidus is perfused from the MCA (Nedergaard, 1988), which may explain the mild reduction in CBF within this territory. However, the globus pallidus receives inhibitory input from the caudate-putamen (Heimer *et al.* 1995), the loss of which has been proposed to account for

hyperperfusion within this region following MCA occlusion (Tamura *et al.* 1981b). Since the globus pallidus receives excitatory cortical input from the neocortex (Heimer et al. 1995), loss of this input subsequent to energy failure within the ischaemic neocortex may lead to reduced metabolic demands on this region with subsequent reductions in CBF. Inter-study discrepancies are also likely to reside on experimental & model differences and the time post-MCA occlusion that CBF was determined. With regards to this latter point, whilst CBF may increase at acute time points when CBF is measured 10-30 minutes post MCA occlusion (Tamura *et al.* 1981b; Sharkey *et al.* 1993), it is conceivable that the hyperperfusion consequent on loss of inhibitory input may be transient. At later time points, hypoperfusion may develop since the globus pallidus is anatomically adjacent to the densely ischaemic caudate-putamen, and mechanisms have been proposed to explain hypoperfusion in territories outwith the MCA vascular field (Slater *et al.* 1977; Tamura *et al.* 1981b). Release of vasoactive agents, of which ET-1 is a likely candidate (Patel *et al.* 1995a), may account for the hypoperfusion in the globus pallidus 3 hours after MCA occlusion.

Ipsilateral local CBF in the nucleus accumbens in the present study was significantly reduced by 36% compared to the contralateral side, which is similar to the reduction of 24% found in the study by Tamura *et al.* (1981b), although this area has been reported not to undergo changes in local CBF following MCA occlusion (Robinson *et al.* 1990; Gartshore *et al.* 1996). The nucleus accumbens is generally considered part of the striatum and is within the structures of the basal ganglia (Heimer *et al.* 1995). It receives at least part of its blood supply from a branch of the

anterior cerebral artery (Scremin, 1995) which explains the relatively minor change in CBF following MCA occlusion, and it is likely that the close proximity of this structure to the ischaemic caudate-nucleus (Heimer *et al.* 1995), as well as loss of cortical input may explain these changes.

Regions within the thalamus, as well as the hippocampal CA1 region have previously been reported to undergo local CBF changes following MCA occlusion. The thalamus and hippocampus receive their vascular supply from branches of the posterior cerebral artery (Scremin, 1995), and the explanation for CBF changes has been based on neuronal connectivity. In the present study, local CBF within the hippocampal CA1 was not affected by MCA occlusion, which has been reported previously (Robinson *et al.* 1990), although mild decreases (Tamura *et al.* 1981b) and increases (Sharkey *et al.* 1993) have been reported.

In this study, CBF in the anterior-medial thalamic, and ventrothalamic nuclei was 9-11% lower ipsilaterally although this was not significant, whilst CBF in the subthalamic nucleus was unaffected which has been reported previously (Tamura *et al.* 1981b). The anterior-medial thalamic nucleus, like the other anterior nuclei, receive the major source of input from the hippocampal formation, and the major cortical projections are to the retrosplenial and cingulate cortices that are outwith the MCA territory (Price, 1995; Scremin, 1995), which would explain why MCA occlusion had only minimal effect on local CBF within these areas.

The ventral thalamic nuclei (lateral and medial) are major relay structures between the cerebellum and motor cortex (Price, 1995), and since the cerebellum is outwith the MCA territory, whilst the motor cortex is at least partially fed by the

posterior cerebral artery (Scremin, 1995), the relatively minor changes in local CBF within this structure would be expected.

The subthalamic nucleus is a strategic relay structure between the globus pallidus and the major output nuclei of the basal ganglia which includes the substantia nigra, whilst there is evidence of some small striatal projections to the subthalamic nucleus (Heimer *et al.* 1995). This structure is not within the vascular territory of the MCA (Scremin, 1995) and given this structure's anatomical connectivity, it is not surprising that this area did not undergo significant changes in CBF following MCA occlusion.

The cause of inter-study discrepancies between CBF changes within thalamic areas and the hippocampus, is unclear. These brain regions receive a diverse and heterogeneous neuronal innervation from both excitatory and inhibitory nerve supplies (Price, 1995), and changes in CBF to these structures after MCA occlusion have been explained based on loss of striatal inhibitory input (Sharkey *et al.* 1993). However, the thalamus is a major relay structure, receiving substantial input from neocortical brain regions such as the somatosensory cortex (Price, 1995), loss of input from which may lead to reductions in metabolic demand, counterbalancing the loss of inhibitory input from the caudate-nucleus. It is conceivable that discrepancies in CBF following MCA occlusion may also be accounted for by the criteria on which the region is selected, model & experimental differences and the time after MCA occlusion at which CBF is measured, and explanations based on assertions of neuronal connectivity are probably superfluous.

3.4.7. Conclusions

The most important conclusions that can be drawn from these studies concern the duration of the ischaemic insult following ET-1-induced MCA occlusion. Two measures of the ischaemic insult, striatal oxygen tennsion and local CBF suggest that there is minimal recovery of blood flow to the vascular territory of the MCA three hours following MCA occlusion in this model, findings complimented by LDF recordings within the parietal cortex. The factors responsible for the lack of reperfusion remain a matter of debate however. Taken with the experimental evidence from previous studies suggesting 3 hour MCA occlusion produces an ischaemic lesion not different from permanent MCA occlusion, it may be concluded that the ET-1 induced MCA occlusion model represents a model of permanent MCA occlusion, in which any reperfusion may be of limited significance.

CHAPTER FOUR

CHAPTER 4: NEUROPROTECTIVE & BEHAVIOURAL EVALUATION OF 17MER SEQUENCE OF β AMYLOID PRECURSOR PROTEIN

4.1. INTRODUCTION

4.1.1. Background

Alzheimer's disease (AD) is the most common cause of progressive intellectual failure in aged humans (Games *et al.* 1995). The accumulation of a truncated form βAPP has been suggested as one of the causes of the pathology of the disease (Checler, 1995; Cotman *et al.* 1995), formation of which may rest on genetic or posttranscriptional processing abnormalities, whilst abnormal phosphorylation of neurofilaments & MAP tau protein have also been implicated (Neve *et al*. 1990; Goldgaber *et al.* 1987). The β-amyloid precursor protein (βAPP) is a large transmembrane protein that is the source of the amyloid β-peptide (βAP), a 40-42 amino acid segment of βAPP, that accumulates in senile plaques in Alzheimer's disease (Smith-Swintosky *et al.* 1994).

βAPP is widely distributed within a number of cell types and is encoded within a single gene on chromosome 21. βAPP's may exist in a number of forms which arise from differential processing (Selkoe *et al.* 1989). Cleavage of βAPP within the βAP region can generate secreted forms of βAPP (APPs) and non-amyloidogenic peptide fragments (Mattson *et al.* 1993). These include forms containing (APP751 & APP770) or lacking (APP695 & APP714) a Kunitz-type protease inhibitor region (Smith-Swintosky *et al.* 1994). APP751 has been identified as nexin-II (Oltersdorf et al. 1989). The most abundant form in the brain is the APP695 (Pluta *et al.* 1994; Smith-Swintosky *et al.* 1994), and both βAPP and βAP are produced and secreted

under physiological conditions (Selkoe 1993). Such plaque accumulation has been investigated in various species of laboratory animals. Whilst βAP accumulates in Alzheimer's brain, Vaughan & Peters (1981) showed that aged rodents do not generally develop βAP plaques, or show them only sporadically (Pluta *et al.* 1994). However, Coria *et al.* (1992) found that rodent brain contains the same forms of βAPP as well as similar immunoreactivity to these proteins, with the greatest expression in neurones, which corresponds with that found in humans. It is possible that sequence differences in the βAPP molecule cause differential processing, making rodents less prone to βAP protein deposits (Pluta *et al.* 1994).

The toxicity of the β-amyloid molecule may reside within an ability to form insoluble sheet-like structures *in vitro* and *in vivo* since injection of aggregated β-amyloid peptides into rat brain has been found to result in some AD-like pathology (Pike *et al.* 1991; Kowall *et al.* 1992). The issue of what causes β-amyloid induced cell death and by what pathway, is poorly understood and controversial (Cotman & Anderson 1995). Evidence for an apoptotic rather than necrotic pathway for β-amyloid-induced cell death has come from the finding that lactate dehydrogenase (LDH) release, a marker of glutamate-induced necrosis (Koh & Choi, 1987), did not correlate to β-amyloid-induced pathology, and β-amyloid-exposed neurones exhibited small, condensed, irregular shaped bodies, a morphology in line with that seen with apoptosis (Loo *et al.* 1993; Cotman & Anderson 1995). Furthermore, post mortem examination of AD brain suggests evidence of apoptosis at the scanning and transmission electron microscope level revealing ultrastructural indicators of apoptosis (Loo *et al.* 1993; Watt *et al.* 1994). Neurones exhibit DNA laddering after

a 24 hour exposure to β-amyloid peptdies (Cotman & Anderson, 1995), although reports of β-amyloid-induced necrotic death are also in the literature (Behl *et al.* 1994). Cotman & Anderson (1995) suggest that some contradictory findings may be consequences of different culture environments, and that secondary necrosis, whereby cells which have proceeded through an apoptotic pathway lyse in a necrosis-like manner due to insufficient phagocytic removal of apoptotic bodies, may be confusing findings in culture. Further evidence for β-amyloid-induced apoptosis, and apoptosis in AD comes from use of histological $3^{/}$ -OH end break labelling, and Cotman & Anderson (1995) demonstrated this marker exists in post-mortem AD brains.

The mechanism of β-amyloid-induced apoptosis has been addressed in a number of studies. Oxidative damage has been suggested as a factor in AD as well as other neurodegenerative disorders such as Parkinson's disease for which there is evidence (Ames *et al.* 1993; Olanow, 1993). Furthermore a number of studies have linked oxidative actions in mediating apoptosis (Hockenberry *et al.* 1993; Kane *et al.* 1993). Furthermore, immunoreactivity for the antioxidants superoxide dismutase and catalase are co-localised in AD plaques (Pappolla *et al.* 1992), and Cotman & Anderson (1995) suggest that β-amyloid and oxidative injury may contribute to AD cell loss.

Calcium has been implicated in βAP-mediated cell death. Orrenius *et al.* (1989) and Mattson *et al.* (1992; 1993) have reported β-amyloids destabilise calcium homeostasis in neurones. Calcium increases have been shown to occur in cells stimulated with apoptosis-inducing agents (McConkey *et al.* 1989) Arispe *et al.*

(1993a; 1993b) have reported that β-amyloid forms calcium channels and multilevel cation channels in bilayers presenting a possible pathway for β-amyloid to induce apoptosis.

The physiological role of βAP proteins in the brain is unclear, although a number of functions have been suggested including non-neuronal cell-proliferation (Palacios *et al.* 1995), a role in blood coagulation, cell adhesion and the potentiation of NGF effects (Smith-Swintosky *et al.* 1994). A role for secreted βAPPs has also been proposed in consolidation or retrieval of memory (Huber *et al.* 1993). Evidence is accumulating for a role for secreted βAPPs in the CNS response to injury (Robinson *et al.* 1993; Tomimoto *et al.* 1994; Pluta *et al.* 1994; Stephenson *et al.* 1992). Nakamura *et al.* (1992) found βAPP deposits after excitotoxic lesions of the rat hippocampus using ibotenic acid, while Wakita *et al.* (1992) showed accumulation of βAPP in rodent models of cerebral ischaemia. Furthermore, Mattson *et al.* (1993) showed protection of cultured hippocampal and cortical neurones with βAPP against excitotoxic lesions.

4.1.2. 17mer in neuroprotection & behavioural studies

Secreted β-amyloid peptides can be both neurotoxic and neuroregenerative (Yankner *et al.* 1990; Takadera *et al.* 1993). These multiple activities are dependent on the form of β-amyloid that was used, with the neurotoxic portion being residues 1-42 containing the hydrophobic rich amino acid end, while the non-toxic region was contained within residues 1-28 (Pike *et al.* 1991; 1993).

A number of studies have demonstrated that a specific 17mer sequence of secreted βAPPs has trophic and neuroprotective actions *in vitro* (Mattson *et al.* 1993; Jin *et al.* 1994; Yamamoto *et al.* 1994), and neuroprotective actions *in vivo* (Bowes *et al.* 1994; Smith-Swintosky *et al.* 1994). The 17 amino acid sequence corresponds to Ala-319 to Met-335 of the APP-695 secreted form of βAPP (Jin *et al.* 1994). This peptide contains a central molecule containing the RERMS sequence which Yamamoto et al. (1994) identified as crucial to the peptide's trophic properties.essential to these properties (Yamamoto *et al.* 1994). The primary amino acid sequence of this 17mer peptide is shown in Figure 19.

Two functional profiles of this βAPP fragment have been demonstrated, that being a neuroprotective ability and a capacity to facilitate functional recovery as a consequence of promoting neurite extension. Jin *et al.* (1994) and Yamamoto *et al.* (1994) demonstrated that this 17mer sequence stimulated neurite extension of CNS neurones in culture, and enhanced neuronal survival in culture. This was mediated via binding to a specifc cell surface receptor, although it is unclear as to whether neurotrophic actions are mediated via a different receptor to that mediating the survival properties (Yamamoto *et al.* 1994). *In vivo* studies in rats demonstrated that intracerebroventricular (i.c.v.) infusion of the 17mer fragment protected hippocampal neurones from ischaemic injury in a four-vessel occlusion model of global ischaemia (Smith-Swintosky *et al.* (1994) and reduced the neurologic consequences of spinal cord ischaemia (Bowes *et al.* 1994). This peptide has not yet been investigated in models of focal cerebral ischaemia. Consequently, two questions have been addressed using the ET-1 induced MCA occlusion model. First, whether the 17mer

AKERLEAKH ***RERMS*** QVM

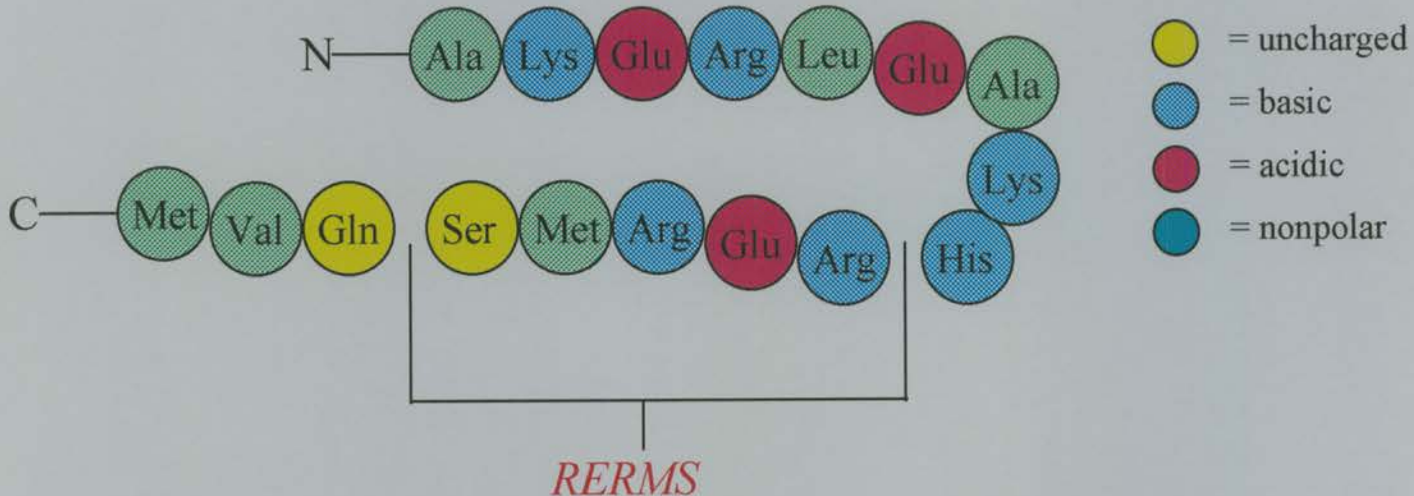


Figure 19. Primary amino acid sequence of the 17mer fragment of the secreted form of the beta-amyloid precursor protein.

peptide is neuroprotective, and second, whether the 17mer peptide may improve recovery as assessed by behavioural outcome, after ET-1-induced MCA occlusion. These questions will consequently address the two properties identified in previous studies, that being a neuroprotective capacity and an ability to mediate functional improvement by promotion of neurite outgrowth.

4.1.3. Experiments (1): Neuroprotection model

The neuroprotection model was a modification of the ET-1-induced MCA occlusion model described in section 2.2.1. The 17mer β-amyloid sequence or a scrambled version of the same amino acids was delivered intracerebrally via mini-pumps for three days prior to ET-1 induced MCA occlusion, with the volume of ischaemic brain damage determined three days later.

4.1.4. Experiments (2): Behavioural model

The ET-1 model has been characterised in terms of the behavioural consequences subsequent to MCA occlusion (Marston *et al.* 1995). In these studies, a skilled paw reaching task, adapted from that described by Montoya *et al.* (1991), was employed to assess behavioural recovery following MCA occlusion. The staircase task is a model of learned paw use, whereby food pellets are retrieved by separate paws across the staircase divide. Following ET-1-induced MCA occlusion, there is a bi-lateral impairment in successful pellet retrieval (Marston *et al.* 1995). This was interpreted as consequent not only on impaired tactile, tacto-motor control and/or control of fine motor movements, but also impairment in some aspects of interhemispheric

communication. Importantly there is a small but significant recovery in pellet retrieval in the days following MCA occlusion. This staircase task was employed in the assessment of behavioural recovery in these studies. The experimental approach was to train the animals on the staircase task until reaching asymtote and then induce MCA occlusion and begin peptide infusion during a single surgical procedure. Beginning infusion immediately after MCA occlusion would circumvent any potentially confusing effects of neuroprotection, and allow only effects of the 17mer peptide on behaviour to be addressed. Then following an initial recovery period, behavioural testing was recomenced for 9 days, and then animals were killed and the volume of ischaemic brain damage quantified.

4.2. METHODS

4.2.1. Mini-pumps

Osmotic mini-pumps were employed in both neuroprotection and behavioural studies in order to deliver the peptide intracerebroventricularly. This circumvents the need to penetrate the BBB and proteolysis consequent on systemic administration of peptides. The pumps (Alzet, model 2002) delivered at a rate of 0.5μl/hr and could run for 14 days. In all studies using the mini pumps, 17mer or scrambled were delivered at a rate of 0.25nmols/hr (0.51μg/hr). This infusion quantity was determined based on previous studies (Roch *et al.* 1994), and a personnel communication from T. Saito. The pumps consist of an L-shaped steel cannula infusion "arm" connected by a short polyethylene catheter (~10cm long) to the pump which contains enough solution to work for 14 days.

4.2.2. Surgery & protocol for neuroprotection study

This study employed SD rats (280-320g) randomly assigned to either the 17mer or scrambled treatment groups. The protocol involved the i.c.v. infusion of 17mer/scrambled sequence for three days prior to MCA occlusion. On the day of mini-pump insertion, animals were anaesthetised as in section 2.2.1., and placed in a stereotaxic frame. Following a midline incision, bregma was located and a craniotomy performed (coordinates from bregma: AP=-1.5mm, L=-2mm). A second craniotomy was drilled a short distance away and a surgical screw affixed to the skull. A minipump, loaded with 17mer or scrambled sequence, was then stereotaxically lowered 3mm below skull and cemented in place. A "pocket" was then opened to accommodate the pump between the scapulae by freeing the skin from the connective tissue behind the shoulders. The scalp wound was then sutured and the animal allowed to recover in an incubator.

Three days later, animals were anaesthetised as in section 2.2.1., and placed in a stereotaxic frame. Following a midline incision, bregma was located and a craniotomy performed (co-ordinates from bregma AP=0.9mm, L=-5.2mm). A 28 gauge cannula was lowered 8.7mm below skull and ET-1 (150pmols in 3μl) injected (1μl/90 sec). The cannula was left *in situ* for five minutes and then slowly withdrawn. The scalp wound was sutured and the animal placed in an incubator to maintain normothermia until the animal had recovered from anesthesia. Three days later, animals were transcardially perfused and the brain removed and stored until

histological processing and volume of ischaemic damage calculation as in section 2.2.2.

4.2.3. Surgery & protocol for behavioural study

Male Lister hooded rats, maintained in the housing conditions previously outlined in section 2.2.1. were maintained on a food restricted diet that maintained animals at 85-90% of their free feeding weight such that animals gained 3-5g per week. The staircase apparatus has been described in detail elsewhere (Marston *et al.* 1995). Briefly, a perspex enclosure (300 x 67 x 95mm, long x wide x high) contained a stainless steel central platform with a staircase either side containing six wells. The apparatus is constructed such that only the ipsilateral paw could reach the pellet and the animal is unable to visually identify pellets. Each well was baited with two pellets (45mg, Noyes formula P). The training regime consisted of two sessions, 4 hours apart during which time the animal was given 5 minutes to recover pellets. Performance was scored as the number of pellets recovered. Training was continued until animals had reached asymtote (9-10 pellets on both sides) which took 9-10 days. Animals were then randomly assigned to ACSF, scrambled or 17 mer sequence, and underwent mini-pump insertion and ET-1-induced MCA occlusion. ET-1-induced MCA occlusion was performed as in section 2.2.1. and a minipump was inserted as described above in section 4.2.2. Animals were perfusion fixed and brains processed for quantitative histopathology as previously described in section 2.2.2.

Paw-reach training was perfromed with the assisstance of Miss T. Higgins of the Fujisawa Institute of Neuroscience, University of Edinburgh.

4.2.4. Analysis of data and statistics

Data are expressed as mean ± s.e.mean. The volume of hemispheric, cortical and striatal ischaemic brain damage was calculated for each treatment, and analysed by Student's T test.

For behavioural data and statistics, paw reach data from left (contralateral) and right (ipsilateral) paws were calated from both a.m. and p.m. testing sessions, after a three day recovery period following MCA occlusion. Data were analysed separately for ipsilateral and contralateral paws, and a.m. and p.m. data combined for 1 day pre-operatively, and in three day blocks (i.e. post operative testing day 1-3, 4-6 and 7-9), postoperatively. The volume of ischaemic damage from animals in the behavioural study as well as the behavioural performance, were analysed by one-way ANOVA.

4.2.5. Drugs/ACSF

ET-1 was dissolved in saline. 17mer and the scrambled sequence of the same peptide were a gift from Tsunao Saitoh, University of California, San Diego. Stock concentration of 17mer and scrambled were made up at 0.5mM, and stored frozen at -70°C. Both the 17mer peptide and the scrambled sequence were dissolved in ACSF (see Appendix B).

4.3. RESULTS

4.3.1. Neuroprotection results

In rats treated with the scrambled sequence of the 17mer peptide (n=9) the volume of hemispheric ischaemic brain damage following ET-1-induced MCA occlusion was 231±25mm^3. By contrast, in rats treated with the 17mer sequence (n=7), the volume of hemispheric damage was significantly reduced by 48% (P=<0.05). The volume of cortical ischaemic damage in scrambled treated rats was 191±24mm^3, whilst it was significantly reduced in 17mer treated rats to 100±22mm^3 (P=<0.05). The striatal infarct volume was also significantly smaller in the 17mer group (18±5mm^3) compared to scrambled treatment (40±1mm^3) which was a 55% reduction in ischaemic brain damage (P=0.001) (Figure 20).

4.3.2. Behavioural results

All animals used in these studies reached paw-reach asymtote for at least 1 day before MCA occlusion. Statistical analysis determined that the volume of cortical and striatal ischaemic damage in ACSF, 17mer and scrambled groups was not significantly different between groups when 17mer infusion was initiated after induction of ET-1-induced MCA occlusion (figure 21).

Statistical analysis of paw-reach data in the behavioural model employed in these studies determined that pre-MCA occlusion paw reach data were not significantly different between right and left paws for all three treatment groups (Figure 22). Following MCA occlusion, retrieval significantly fell bilaterally in all three groups. This reduction was profound on the contralateral side, but was

Neuroprotective efficacy of 17mer sequence

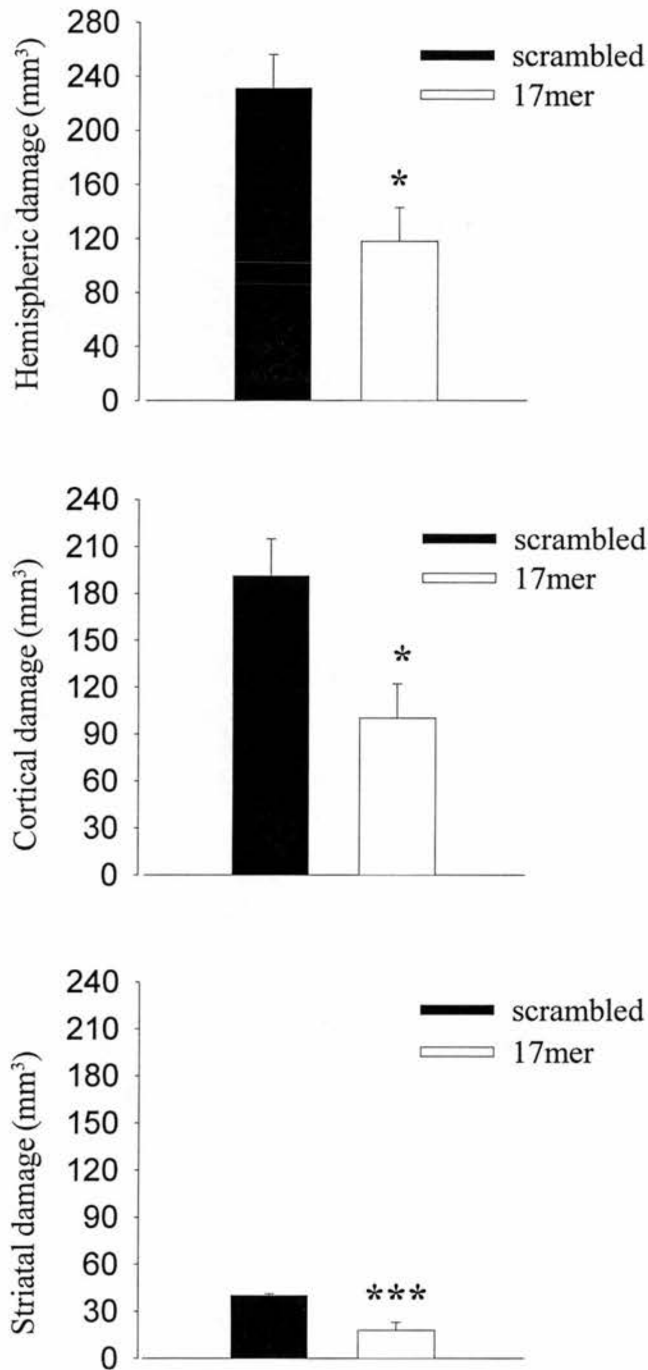


Figure 20. Neuroprotective efficacy of the 17mer peptide, when infused intracerebroventricularly for 3 days prior to ET-1-induced MCA occlusion 17mer (n=7) reduced the volume of hemispheric (top), cortical (middle) and striatal (bottom) ischaemic damage compared to treatment with a scrambled sequence (n=9) of the same peptide. Data are presented as mean ± s.e.mean. *P=<0.05. ***P=<0.001.

Cortical and striatal ischaemic damage for treatment groups in behavioural study with 17mer peptide

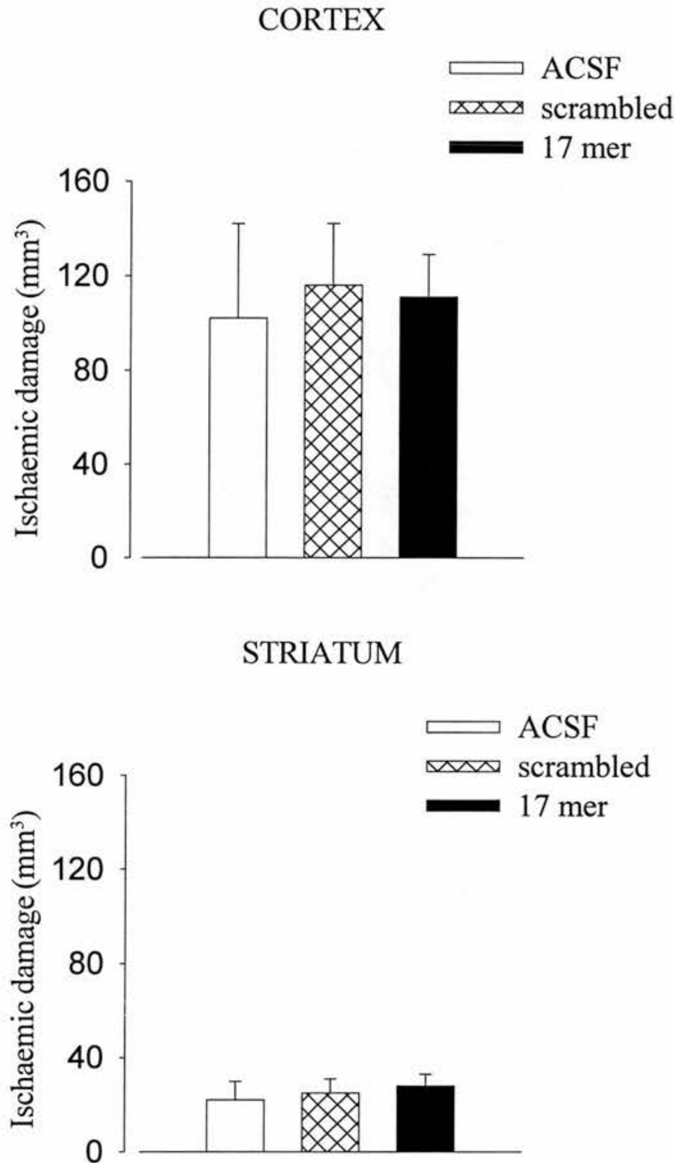


Figure 21. Volume of ischaemic damage following ET-1-induced MCA occlusion for cortex (top) and striatum (bottom) for animals treated with ACSF (n=7), scrambled (n=9) or 17mer (n=9) in behavioural study. Data are presented as mean ± s.e.mean. No significant differences were found between treatments in either cortex or striatum.

Paw-reach paradigm assessment of the effects of 17mer peptide on recovery following ET-1-induced MCA occlusion

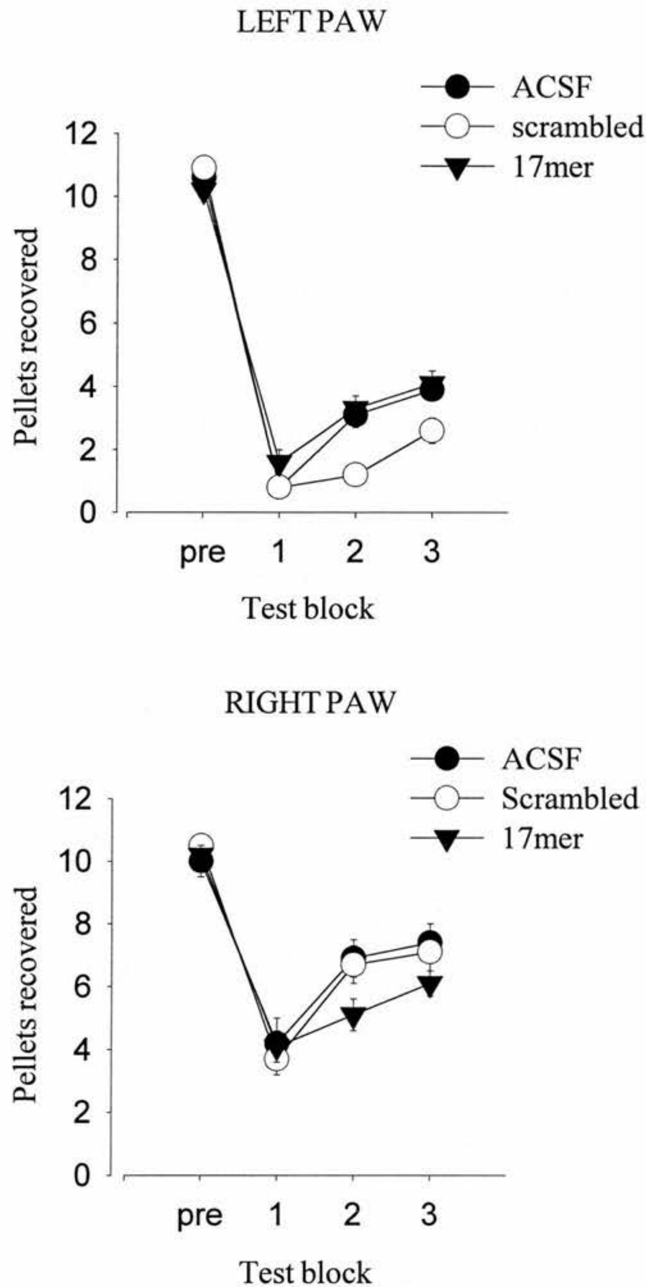


Figure 22. Effects of ACSF (n=7), scrambled (n=9) and 17mer (n=9) on behavioural outcome and recovery for left (top) & right (bottom) paws following ET-1-induced MCA occlusion as assessed in a paw-reach paradigm. Data are expressed as mean ± s.e.mean. No significant differences were found for any treatment for either the ipsilateral or contralateral side.

significant on the ipsilateral side also. Some recovery of function was noted between all three groups was found, but recovery was not significantly different between 17mer, scrambled and ACSF groups at any day post MCA occlusion.

4.4. DISCUSSION

4.4.1. Neuroprotection results

Sequences of the secreted form of the βAPP have previously shown to confer neuroprotection to neurones and promote neuronal sprouting and regrowth (Yamamoto *et al.* 1994; Mattson *et al.* 1993; Smith-Swintosky *et al.* 1994). Other growth factors have also been shown to possess neuroprotective capacity in models of focal cerebral ischaemia (Yamada *et al.* 1991) and in *in vitro* models of ischaemic damage (Finkelstein *et al.* 1993; Mattson & Cheng, 1993; Maiese & Boccone, 1995). The model used in these studies, whereby a peptide was infused intracerebroventricularly prior to MCA occlusion, has also been employed to determine the neuroprotective capacity of IL-1 receptor antagonist (Rothwell *et al.* 1995).

The ET-1 model of MCA occlusion has previously shown to be a useful tool in the evaluation of putative neuroprotectants (Sharkey & Butcher, 1994), whilst the mini-pumps employed have been previously validated in behavioural work (Bannerman et al. 1995). The data from these studies confirm and extend previous studies, demonstrating that intracerbroventricular infusion of 17mer for three days prior to ET-1-induced MCA occlusion, profoundly reduced the volume of hemispheric, cortical and striatal ischaemic brain damage compared to control rats

that were infused with a scrambled sequence of the same peptide. By contrast, when 17mer peptide infusion was begun post MCA occlusion, the peptide was not neuroprotective.

The degree and extent of neuroprotection in the pre-treatment study exerted by the 17mer sequence was unexpected since brain penetration following intracerebroventricular administration of a peptide is often limited. Furthermore neuroprotection was observed in the striatum, an end-vascular territory structure, which is seldom salvaged by neuroprotectants following MCA occlusion (Sharkey & Butcher, 1994).

The mechanism by which secreted forms of βAPPs may exert neuroprotection is unclear although indirect evidence might suggest an involvement in cellular repair (Pluta *et al.* 1994). *In vitro* studies suggest that βAPPs may regulate intracellular Ca^{2+}, which might offer insight into the mechanism by which these proteins confer neuroprotection given the role of Ca^{2+} in ischaemic cell death. In the study by Mattson *et al.* (1993), they found secreted forms of βAPPs reduced intracellular Ca^{2+}, stabilised intracellular Ca^{2+}, and increased neuronal survival in glucose-deprived neuronal cultures. Both APP695 and APP751 peptides caused a rapid and prolonged reduction in $[Ca_{2+}]i$ that accompanied the response to hypoglycaemia. Furthermore, βAPPs protected neurons from glutamate neurotoxicity. Mattson *et al.* (1993), proposed that the secreted forms of βAPP, APP695 and APP751, may have neuromodulatory roles, and their loss in Alzheimer's disease may explain neuronal death. A vascular site of action may explain better the neuroprotection of neocortex by the 17mer, and may represent another mechanism of the 17mer peptide. Further

studies would be required to establish whether 17mer mediated any direct effects on the cerebrovasculature.

It is conceivable that the degree on neuroprotection is artifactually large as a consequence of a toxic effect of the scrambled sequence. However, such a neurotoxic effect has not been reported previously in studies with the scrambled sequence. Furthermore, the volume of ischaemic damage in the scrambled group is not greatly outwith that reported previously in studies with this model (Sharkey & Butcher, 1995), and the volume of ischaemic damage found between 17mer and scrambled sequence in the behavioural study was not significantly different, which would suggest that the scrambled peptide is not exerting a neurotoxic effect. However, it is possible that the scrambled sequence lowers the capacity of neurones to resist focal cerebral ischaemic insults, which would not be detected in the protocol used in the behavioural study. Further studies would be necessary to confirm whether such an action contributed to the large neuroprotective profile of 17mer in these studies.

4.4.2. Behavioural results

In assessing the 17mer for capacity to facilitate behavioural recovery, we infused the 17mer sequence, a scrambled sequence or ACSF following MCA occlusion and then tested the behavioural recovery. An ACSF group was included to determine if either protein alone had a specific effect on behaviour. The intraventricular infusion was begun at the same time as the MCA occlusion was performed to avoid any potential complicating factors that might arise from possible neuroprotective capacity of the 17mer peptide. Consequently, the initial behavioural consequences of MCA

occlusion would be independent of the volume of ischaemic brain damage resulting from ET-1-induced MCA occlusion. Indeed, quantitative analysis of ischaemic brain damage determined that the volume following ET-1-induced MCA occlusion was not significantly different between either 17mer, scrambled or ACSF treated animals in either the cortex or the striatum. That the volume of damage was not different in both the cortex and the striatum was important given the role of both these brain regions in behaviour in this model (Marston *et al.* 1995).

The behavioural model used to assess behavioural impairment of skilled paw reaching in these studies has previously evaluated to assess performance in this stroke model (Marston *et al.* 1995). In these studies, animals from all three groups showed the same extent of impairment in skilled paw-reaching following MCA occlusion.

The similar volume of ischemic damage found between treatment groups was reflected in identical reductions in paw reach performance on postoperative block 1. The impairment was bilateral, as seen by the reduction in right paw performance as well as the contralateral (left) paw. This corresponds to previous findings using this behavioural assessment, whereby the contralateral limb (receiving cortical and striatal input from lesioned side), has the greatest degree of impairment. However, the ipsilateral performance was also impaired, although to a lesser extent.

Behavioural impairment showed some recovery with time over the 9 testing days following MCA occlusion. The rate of functional recovery was not different in either the 17mer, scrambled or ACSF groups. This suggests that 17mer does not promote functional recovery, as assessed in this behavioural model, following ET-1-induced

MCA occlusion. Whether a more sensitive test of behavioural function may have detected an effect of 17mer sequence remains to be determined. Many tests have been employed in the determination of behavioural impairment following MCA occlusion in the rat such as memory and learning tasks. The test used in these studies assess the fine motor control of animals, incorporating both neocortical and striatal components. It is conceivable that behavioural assessment of another parameter might have determined an effect of 17mer.

A different strain of rat was employed in the behavioural model (Lister-hooded) to that used in the neuroprotection study (Sprague-Dawley). The Lister-hooded strain have been demonstrated to possess greater sensory capabilities than the albino SD strain, which are almost blind and do not possess acute hearing (personnel communication - Dr H. M. Marston). The possibility that SD rats might have responded differently to the LH rats in behavioural outcome following 17mer therefore exists.

4.4.3. Conclusions

These studies investigated the effects of a 17mer sequence of the secreted form of βAPP in the ET-1 model. The present data demonstrate for the first time that this peptide is neuroprotective in a model of focal cerebral ischaemia. However, using a behavioural model to assess skilled motor control in the rat, the 17mer sequence was found to have no significant effect on functional recovery following ET-1-induced MCA occlusion.

CHAPTER FIVE

CHAPTER 5. DEVELOPMENT OF A NOVEL REPERFUSION MODEL

5.1. INTRODUCTION

5.1.1. Current reperfusion models

The study of reperfusion in experimental ischaemia has become a central topic in stroke. Reperfusion of occluded vessels occurs in humans following a stroke (Overgaard, 1994), and consequently study of the biochemistry, pharmacology and pathology associated with reperfusion injury is essential in developing our complete understanding of the possible pathophysiological outcomes following stroke (Wardlow *et al.* 1993; Clark *et al.* 1994). With the advent of tissue plasminogen activator (t-PA) and the prospect of future therapies designed as "clot-busters", there exists a need for more accurate experimental modelling of arterial occlusion with subsequent reperfusion. Current models include the intraluminal filament occlusion (Koizumi *et al.* 1986) snare ligature occlusion via the transorbital approach (Shigeno *et al.* 1985), and transient global ischaemia (Pulsinelli & Brierley, 1979). Whilst return of CBF to the previously ischaemic territory effectively provides tissue with the vital metabolic substrates it requires, paradoxically, re-introduction of blood flow to an ischaemic area has been recognised as being potentially detrimental (McAuley, 1995). Thus reperfusion following cerebral ischaemia may provide restoration of substrate, whilst providing a source of tissue damage exacerbation (Hallenbeck & Dutka, 1990).

5.1.2. Biochemical changes related to reperfusion

Studies in models of global and focal cerebral ischaemia have addressed the consequences of reperfusion on the extent of depletion of energy-related metabolites, and the extent to which they return to preischaemic values during the recirculation period. PCr, the substrate which is initially reduced at the expense of maintaining intracellular ATP levels during ischaemia, recovers to preischaemic values, and may overshoot, while the ATP-adenylate energy change (relationship between ATP, ADP and AMP) also fully recovers, requiring up to several hours of reperfusion for full recovery (Lust *et al.* 1985; Nowak *et al.* 1985; Onodera *et al.* 1986a; Crumnne & LaManna 1991; Pulsineli & Duffy 1983; Sims & Zaidan 1995). This time delay is thought to be a consequence of complete adenine nucleotide depletion due to breakdown, and is related to the duration of ischaemia (Lust *et al.* 1985; Onodera *et al.* 1986a; Sims & Zaidan 1995).

Restoration of ATP levels intracellularly is essential to the cell's ability to survive, restoring ionic gradients intracellularly and across the plasma membrane. Restoration of cellular ATP is dependent on the duration of the ischaemic insult (Selam *et al.* 1990). However, within limits, recovery may be essentially complete within the first 20 minutes of recirculation, although this may be duration dependent (Hasen 1985; Silver & Ericinska 1992). In the study by Sun *et al.* (1995), ATP levels decreased to 26% of control following MCA occlusion with bilateral common carotid occlusion, with an increase in AMP levels. Levels recovered to 80% of control following a 60 minute MCA occlusion with 4 hours of reperfusion, whilst ADP and AMP levels fully recovered within the 4 hour recirculation period. Furthermore, restoration of most other metabolites are also generally reversed

quickly (Sims & Zaidan 1995), including extracellular neurotransmitter concentrations (Globus *et al.* 1991; Baker *et al.* 1991). An important point made by Sims & Zaidan (1995) is that whilst energy metabolites may recover on recirculation this does not mean metabolism has returned to a preischaemic state. Almeida *et al.* (1995) examined the activity of the mitochondrial respiratory chain from gerbil brain subjected to ischaemia-reperfusion, using the method of surgical occlusion of the common carotid arteries. Reperfusion was varied between treatment groups while the ischaemic episode was of 30 minute duration. Measurement of respiratory chain components showed complex II-III activity was restored following 5 minutes of reperfusion, activity in complexes I & V required 30 minutes, while complex IV activity was relatively unaffected by ischaemia-reperfusion. Other studies have also shown mitochondrial function to be depressed following ischaemia, while reperfusion reverses those deficits (Rehncrona *et al.* 1979; Hillered *et al.* 1984; Sims & Pulsinelli, 1987).

Further to the issue of delayed full recovery, studies using the deoxyglucose method for assessing glucose utilisation suggest glucose oxidation is depressed following reperfusion and may remain depressed for several days, despite recovery of ATP levels (Pulsinelli *et al.* 1982; Choki *et al.* 1983; Kozuka *et al.* 1989; Jorgensen *et al.* 1990). By contrast, during early reperfusion, glucose utilisation may be exaggerated (Diemer & Siemkowicz 1980; Choki *et al.* 1983), although since calculations with this technique are based on measurements in normal brain, definitive interpretations are precluded (Sims & Zaidan 1995).

Protein synthesis is particularly sensitive not only to ischaemia but to reperfusion, with reductions in synthesis developing following initial reperfusion (Nowak *et al.* 1985; Widmann *et al.* 1991). Restoration of function may not be complete for hours or even days after recirculation (Sims & Zaidan, 1995). The cerebrovasculature may also be affected directly by the effects of reperfusion. Cipolla *et al.* (1997) determined that reperfusion was detrimental to MCA myogenic reactivity, which seems to be dependent on the duration of the reperfusion.

As was mentioned in the introduction to this section, re-introduction of blood flow may bring with it factors detrimental to tissue survival. The involvement of free radicals and oxidative damage in reperfusion has been postulated as a cause of injury and dysfuntion, although the difficulty in measuring such short lived molecules *in vivo* has meant limited understanding of the mechanism of their production (Traystman *et al.* 1991). Oxygen radicals can be generated following ischaemia-reperfusion from sources such as activated leukocytes & endothelial cells (del Zoppo, 1994). However, the most likely source is from the metabolism of free fatty acids and adenine nucleotides, which accumulate during ischaemia (Traystman *et al.* 1991). Targets of free radicals include cell membrane lipid, NADPH, nucleic acids and proteins (Freeman & Chapo, 1982, del Zoppo, 1994). A number of free radical scavengers are endogenous to the brain, which may limit the extent of damage, and include glutathione peroxidase and superoxide dismutase (del Zoppo, 1994). Studies have demonstrated the presence of these highly reactive species, including hydrogen peroxide (Simonson *et al.* 1992) and the products of superoxide and hydroxyl radical interactions (Cao *et al.* 1988; Nelson *et al.* 1992; Zini *et al.* 1992), as well as lipid

peroxidation (Watson *et al.* 1984), in a number of stroke models. These studies indicate that an oxidative burst occurs as a transient peak of free radical production during the first minutes of recirculation following transient cerebral ischaemia (Sims & Zaidan 1995). However, as yet there is insufficient research to determine the differences between global and focal ischaemia, as well as species to species variation, which is another possibility. Indeed, while Oliver *et al.* (1990) determined a protein oxidative loss of glutamate synthetase in gerbil brain following recirculation, Pahlmark *et al.* (1993) did not note a comparable change in the rat, and it is likely the largest contribution of radicals is seen in global rather than focal models of cerebral ischaemia.

The efficacy of radical scavenging agents has helped to establish a significant contribution of oxy-radicals to reperfusion injury. These include the "lazaroids" (Braughler *et al.* 1989), superoxide dismutase (Kitagawa *et al.* 1990; Uyama *et al.* 1992), oxypurinol, a xanthine oxidase inhibitor (Phillis, 1989b) and a lipid peroxidation inhibitor (Hara & Kogure 1990) and many other compounds of this class have been studied and found to be effective in reducing ischaemic brain damage. These studies used global ischaemia models where ischaemia is of short (usually 5 minutes) duration. Whether these compounds are equally effective in longer duration episodes or focal ischaemia is unclear. Indeed, Yue *et al.* (1992) demonstrated reduced efficacy of protection when duration of ischaemia was increased to 10 minutes in their model. While Martz *et al.* (1989) demonstrated a neuroprotective effect of dimethylthiourea (hydroxyl radical scavenger) and allopurinol (xanthine oxidase inhibitor) in a rat model of MCA occlusion, others

have not found protection. Kiyota *et al.* (1993) found that when changes in body temperature were compensated for in control groups (not done by Martz *et al.* 1989) there was no neuroprotective effect of dimethylthiourea in a rat model of MCA occlusion.

Another postulated mediator of reperfusion damage is calcium. It has been suggested that reintroduction of blood flow may kill surviving neurones with a previously intact calcium homeostasis, due to re-exposure to calcium levels (Hallenbeck & Dutka, 1990).

The temporal profile of morphological and inflammatory changes following temporary MCA occlusion with reperfusion (TMCAO) has also been addressed. Reperfusion is associated with inflammatory cell infiltration (del Zoppo, 1994), and this inflammatory cell infiltrate differs from models of permanent MCA occlusion (PMCAO) (Clark *et al.* 1994). Clark *et al.* (1994) found that while there is an inflammatory cell response following PMCA occlusion, it is more rapid and extensive following TMCAO. Clark *et al.* (1994), found a much heavier macrophage and neutrophil infiltrate following TMCAO as well as a pronounced fibroblast and astrocyte infiltrate, absent in the PMCAO model. Whilst re-introduction of CBF in TMCAO models provides an infiltration route, the route of inflammatory cell infiltration in models of PMCAO is currently unclear. Kamijyo & Garcia (1975) attribute the route of infiltration in models of PMCAO as due to the retrograde flow between major arteries. The resolution of the infarct (5-30 days) in terms of macrophages on the matrix, neovascularisation and gliosis, followed by removal of necrotic tissue and glial scar formation progresses at a similar rate in both models

(Clark *et al.* 1994). However, they do report that resolution was more complete at the 15 day time point in the TMCAO model. Thus we can see that while progression of the ischaemic lesion follows some temporal similarities between models, important differences exist in the inflammatory response in both onset and extent.

5.1.3. Endothelium, oedema and structural consequences

The endothelial lining of cerebral blood vessels has a profound role in brain function under homeostatic and pathophysiological situations. Cerebral ischaemia may trigger endothelial reorganisation with cells becoming active in inflammatory processes that may lead to the recruitment of inflammatory cells into the brain parenchyma (Akopov *et al.* 1996). Such blood cell activation may lead to microvascular plugging, tissue oedema and cytotoxic effects which contributes to the development of an ischaemic lesion (Akopov *et al.* 1996). Furthermore, inflammatory activation may reciprocate, and lead to microvascular reorganisation which has implications for ischaemically injured tissue (del Zoppo, 1994).

The endothelial lining of cerebral blood vessels is a cell monolayer that expresses many receptors for interactions with circulating blood elements and the continuous sensing changes to the mechanical and chemical milieu of the vasculature, blood and underlying parenchyma (Akopov *et al.* 1996). There is now extensive evidence that following an ischaemic insult, the endothelium mediates a myriad of responses that alter the usually immunologically priveledged brain parenchyma. Three main factors determine the role of blood-parenchymal signalling carried out by the endothelium. These are the formation of receptors for circulating

blood borne elements, the synthesis and release of chemical mediators and the conversion of circulating signal molecules (Akopov *et al.* 1996). These functions shall be discussed in detail in the subsequent section addressing the role of the immune system in stroke. However, key changes to the endothelium and microvascular environment occur subsequent to ischaemia, which may be detrimental to both brain parenchyma and the endothelium itself (del Zoppo, 1994). Of these perhaps the most important is the development of the no-reflow phenomena identified by Ames *et al.* (1968), in which re-introduction of blood may be impeded by changes within the microvascular environment following ischaemia. This may be caused by mechanical compression of the lumen by tissue oedema, endothelial cell damage, swelling of astrocyte foot-processes, and the attachment of circulating blood elements to the endothelium (del Zoppo, 1994; Garcia *et al.* 1994a; 1994b). Microvascular patency is impeded following ischaemia by the accumulation of leukocytes such as polymorphonuclear leukocytes (PMNLs), which are by far the most abundent leukocytes (del Zoppo *et al.* 1991; Garcia *et al.* 1994b).

The role of inflammatory components is not limited to simple physical impedence that limits blood flow return, but evidence has accumulated to suggest that recruitment of inflammatory mediators contribute directly to ischaemic brain damage. The mechanism by which this occurs will be addressed in the subsequent section.

5.1.3. Inflammation and immune responses

The immune system and inflammatory response have been identified as of key importance as mediators of post-ischaemic pathophysiological changes. The inflammatory response comprises cellular elements, such as the neutrophils & lymphocytes, and a chemical components which includes the cytokines. Interactions between inflammatory cells and chemical signal molecules direct and control inflammatory responses following cerebral ischaemia. Cytokines are low molecular weight glycoproteins, that are released from one cell to be active either on themselves (autocrine) or on other cells (paracrine). They mediate communication between both the immune system cells and noninflammatory cells. Some 40 or more cytokines have been discovered (Bennett & Beeson 1953; Atkins & Wood 1955; Aarden, 1979; Henderson & Poole 1994). The family can be broadly divided into the interleukins (1-12), cytotoxic factors (tumour necrosis factor), interferons (α, β and γ), colony stimulating factors (CSFs), intercrines/chemokines and the growth factors. All cytokines are polypeptides which exert diverse actions on a multitude of cells, and function as important mediators and regulators of inflammation and cell growth & differentiation (Rothwell & Strijbos, 1995). The cytokines exert their effects via cell surface receptors, and binding is of very high affinity, responding in the case of IL-1 to femtomolar concentrations, while expression of receptor numbers may be very low (Rossof *et al.* 1988; Henderson & Poole, 1994).

Under homeostatic conditions circulting leukocytes and inflammatory mediators are largely prevented from entry to the brain by the BBB. Thus in order to enter the

ischaemic territory, leukocytes must move out of the blood stream and pass through this wall in order to contact the brain parenchyma (Sharkey *et al.* 1996).

Membrane glycoproteins, expressed on the surface of cells play a role in cell-cell interactions and cell-matrix functions during haematological and inflammatory responses during tissue injury (McEver, 1991). Cells involved in this process include leukocytes, platelets and endothelial cells, which mediates the migration of cells into injured or infected tissue (Springer 1990; Osborn, 1990). Such molecules enable migration between endothelial cells, through the basement membrane, and in some instances also activate leukocytes (Montefort & Holgate 1991). Expression is transient, and co-ordinated to allow adherence at the appropriate place and time (Kishimoto & Rothlein 1994).

Central to recruitment is the ability of circulating blood cells, which are in a nonadhesive state, to become adhesive and subsequently attach to endothelial cells at an appropriate place and time (Kishimoto & Rothlein, 1994). To date, three families of adhesion molecules have emerged; the integrins, the intracellular adhesion molecules and the selectins. The integrins represent a supergene family of receptor complexes which are present on cell surfaces. They are comprised of a heterodimer of an α and β subunit, and were among the first leukocyte adhesion molecules discovered (Montefort & Holgate, 1991; Kishimoto & Rothlein, 1994). The subfamilies are known after the common β, subunit, $\beta1$, $\beta2$ and $\beta3$, while the basic structure of the α and β units of an integrin comprise an extracellular domain, a membrane spanning region and a cytoplasmic domain (Montefort & Holgate, 1991). The $\beta1$ and $\beta3$ integrins are predominantly extracellular matrix receptors (Kishimoto

& Rothlein, 1994). The β1 integrins include the very late antigens, which may be important in the migration of leukocytes through the endothelial cells and matrix of the extracellular milieu (Montefort & Holgate, 1991). The β3 integrins include the vitronectin receptor and the platelet protein gpIIb/IIIa, a deficiency in the latter of which causes Glanzmann's thrombasthenia, a disease characterised by haemophilia due to failure of platelet-to-platelet aggregation (Ruoslanti & Pierschbader, 1987; Montefort & Holgate, 1991).

The integrin family most relevant to immunological disease are the β2 (CD18) integrins, also known as the leukocyte adhesion protein subfamily (LEUCAMs) (Montefort & Holgate 1991; Kishimoto & Rothlein 1994). The β2 integrins comprise three glycoproteins; lymphocyte-function-associated antigen (LFA-1), Mac-1 and p150.95 (Montefort & Holgate, 1991; Chopp *et al.* 1994). As with the other members of the integrin family, the β2 integrins, while sharing a common β subunit (CD18) have different α subunits (CD11). LFA-1 is composed of CD18 + CD11a, Mac-1 of CD18 + CD11b and p150.95 of CD18 + CD11c (Montefort & Holgate, 1991; Chopp *et al.* 1994; Zhang *et al.* 1995a; 1995b). Each subunit in the β2 integrins has differing molecular weights, with the common CD18 being a 95kDa molecule, while CD11a is 180 kDa, CD11b 165 kDa, and CD11c being 150 kDa. The β2 subfamily are involved in leukocyte-to-leukocyte, leukocyte-to-endothelium and leukocyte-to epithelium interactions (Montefort & Holgate, 1991). Expression levels of the β2 integrins is cell dependent, restricted to leukocytes, and more specifically Mac-1 and p150.95 to macrophages and granulocytes while LFA-1 is expressed on all

leukocytes including T and B lymphocytes, macrophages and granulocytes (Kurzinger *et al.* 1981; Arnaout 1990; Kishimoto & Rothlein, 1994).

The CD18 integrins mediate adhesion through a number of ligands, with LFA-1 binding intercellular adhesion molecules, which are the second family of adhesion molecules (Rothlein *et al.* 1986; Staunton *et al.* 1989; Montefort & Holgate, 1991; deFougerolles & Springer, 1992; Kishimoto & Rothlein, 1994). There are three members of the intercellular adhesion molecule (ICAM) family (ICAM-1,2 & 3) which are members of the immunoglobulin supergene family, and all are related to other Ig-like adhesion molecules such as VCAM-1 and NCAM (Kishimoto & Rothlein, 1994). All are ligands for LFA-1, but ICAM-1 will interact with Mac-1 and CD43 (leukosialin). Regulation and distribution of the three ICAMs are distinct and expression is differential between ICAMs (Kishimoto & Rothlein, 1994). Whilst little is known of the functional roles of ICAM-2 & ICAM-3, ICAM-1 has been implicated in neutrophil recruitment, leukocyte diapedesis, cell mediated cytolysis, antigen presentation, lymphocyte homotypic aggregation, leukocyte-endothelial cell interactions and transendothelial migration (Smith *et al.* 1988; 1989; Buston *et al.* 1989; Montefort & Holgate, 1991; Kishimoto & Rothlein, 1994).

The third family of adhesion molecules are the selectins. This family functions to mediate interactions of leukocytes with the blood vessel wall (McEver, 1991). To date, three selectins have been identified, named LAM-1 (leukocyte adhesion molecule-1 or L-selectin), ELAM-1 (endothelial-leukocyte adhesion molecule-1 or E-selectin) and GMP-140 (granule-membrane protein 140 or P-selectin) (McEver 1991; Montefort & Holgate 1991; Kishimoto & Rothlein, 1994). The selectins all

share a common structure and are all heavily glycosylated (Bevilacqua *et al.* 1987; Johnston *et al.* 1989), and have distinct cellular distributions (Kishimoto & Rothlein, 1994).

Whilst L-selectin is expressed on most leukocytes, E-selectin is confined in expression to stimulated endothelium, and is not present on normal endothelium (McEver, 1991; Kishimoto & Rothlein, 1994). Like E-selectin, P-selectin is expressed on endothelial cells only following stimulation, as well as platelets (McEver *et al.* 1989; 1991), and it is not a receptor for lymphocytes but for neutrophils and monocytes (McEver *et al.* 1991). As yet the precise ligands for selectins (the counter-receptors) are unidentified (McEver, 1991; Kishimoto & Rothlein, 1994), although some of the ambiguity is being cleared up, and some putative ligands (e.g. Lewis X as a P-selectin receptor) have been identified (Kishimoto & Rothlein, 1994).

The process of leukocyte movement (and extravasation) is regulated by the mechanisms of selective leukocyte-endothelial cell recognition (Butcher, 1991), mediated by adhesion receptors. Butcher (1991) in his model of leukocyte-endothelial cell adherence identifies three steps: reversible adhesion, leukocyte activation and activation-dependent binding. Neutrophils begin to loosely interact with the vessel walls, seen as a rolling action at the site affected. It seems that all three selectins are involved in this process (Lawrence & Springer, 1991). Furthermore, certain endothelial stimulators cause translocation to the surface of P-selectin, which is contained in endothelial cell secretory organelles (Weibel-Palade bodies), and once brought to the surface, P-selectin may act to bind surface adhesion

molecules on the neutrophil (Pober & Cotran, 1990a). What determines the specificity of neutrophil or monocyte recruitment is unclear at present although the activating signals which differ between these cell types may be the factor (Butcher, 1991).

Other factors which are involved in the evolution of the inflammatory response in the rapid/acute stages include endothelial secretion of prostacyclin and EDRF as well as endothelial synthesis of PAF (Pober & Cotran 1990b). These events are mediated by factors such as histamine and thrombin. Such factors consequently induce vasodilation and endothelial cell contraction, facilitating the necessary reduction in shear forces. PAF may have a role in activation of bound neutrophils, causing upregulation and activation (Tonnesen 1989; Pober & Cotran 1990b).

The transition from rolling to a resting state requires chemotactic factors (Kishimoto & Rothlein, 1994). The most important change is the shedding of L-selectin from the cell surface and expression of the integrin molecules (Kishimoto *et al.* 1989; Butcher, 1991). The transition marks a change to activation-dependent adhesion receptors and it is Mac-1 (CD18-CD11b), in the case of the neutrophil, that mediates the next stage of leukocyte-endothelial cell interaction (Kishimoto & Rothlein, 1994). While L-selectin was required to localise the neutrophil to the endothelium, it is the CD18 integrins that move the neutrophil across the endothelium (Kishimoto & Rothlein, 1994). As yet the specific factor(s) responsible for activation of rolling neutrophils in situ is not known, and Butcher (1991) suggests that this may vary with the inflammatory state, but include IL-1, IL-8, C5a, fmet-leu-phe and PAF (Pober & Cotran, 1990a; Kishimoto & Rothlein, 1994).

Following activation of neutrophils by the endothelial cell wall and the transition to integrin-dependent adhesion, the third step in the interaction process is initiated, that being stable binding and leukocyte aggregation within the microvascular lumen. The process of aggregation of neutrophils may amplify recruitment and this occurs to such an extent that vessels can become occluded following inflammation (del Zoppo *et al.* 1991; Kishimoto & Rothlein, 1994). Like adhesion, aggregation is a Mac-1-dependent event, although L-selectin may act as a counter receptor in aggregation (Dana *et al.* 1985; Schwartz *et al.* 1985; Anderson *et al.* 1986; Simon *et al.* 1992). Following firm adherence, neutrophils begin the process of transmigration, mediated again by adhesion molecules and promoted and effected by the presence of chemotactants such as cytokines. The neutrophils penetrate the vessel wall by moving by diapedesis between the endothelial cells at the interendothelial cell junction and then proceeding through the underlying basement membrane to the tissue space behind, which does not require disruption of tight cell junctions (Huber *et al.* 1991). Following penetration of the endothelial cell wall, they flatten out before proceeding through the basement membrane (Kishimoto & Rothlein, 1994). However, understanding of this phase, is incomplete (Akopov, 1996). Smith (1992) reports that a chemotactic gradient is crucial for effective transmigration of neutrophils through brain parenchyma, and studies have shown that cytokines such as IL-1 may be involved in production of this chemotactic gradient (Kishimoto & Rothlein, 1994). Many studies have shown transmigration to be a β2 integrin dependent event (Smith *et al.* 1988; 1989b; 1991; Luscinskas *et al.* 1989; 1991; Hakkert *et al.* 1991; Furie *et al.* 1992), whilst the selectins by contrast are found not

to be functional in transendothelial migration of neutrophils (Smith *et al.* 1991; Kishimoto *et al.* 1991).

The expression of the molecules and the temporal profile of leukocyte recruitment during the inflammatory response to ischaemia has been addressed in a number of studies (Garcia *et al.* 1994a; 1994b; Clark *et al.* 1994; Schroeter *et al.* 1994; Jander *et al.* 1995; Wang *et al.* 1994a; 1995b; 1995c; 1995d; Matsuo *et al.* 1994; Okada *et al.* 1994; Zhang *et al.* 1994a; 1995b; 1995c; Kim *et al.* 1995) and is summarised in Figure 23.

Activated, invading leukocytes kill cells by a number of methods including lysozyme, peroxidases, lactoferrin and a range of neurotoxins. The cytokine IL-1β is of key importance as a mediator of brain damage and recent studies have found that endogenous IL-1 can mediate ischaemic cell death (Yamasaki *et al.* (1995), brain oedema (Gordan *et al.* 1990), and breakdown of the blood brain barrier (Quagliarello *et al.* 1991). Studies have demonstrated that Abs and antagonists to IL-1β are neuroprotective in experimental stroke (Yamasaki *et al.* 1995; Yang *et al*. 1995).

The effectiveness of Ab directed against inflammatory cells has also been addressed in a number of neuroprotection studies and is summarised in Table 6. As can be seen, therapies tend to be effective in models of TMCAO, whilst inhibition of the inflammatory process did not alter outcome in models of PMCAO. This compliments the findings of the relative inflammatory components between the two forms of ischaemia. Whilst such therapies have proven successful at limiting ischaemic damage, the most commonly used model for studying stroke with reperfusion is the filament occlusion technique. This has been critised for the pro-

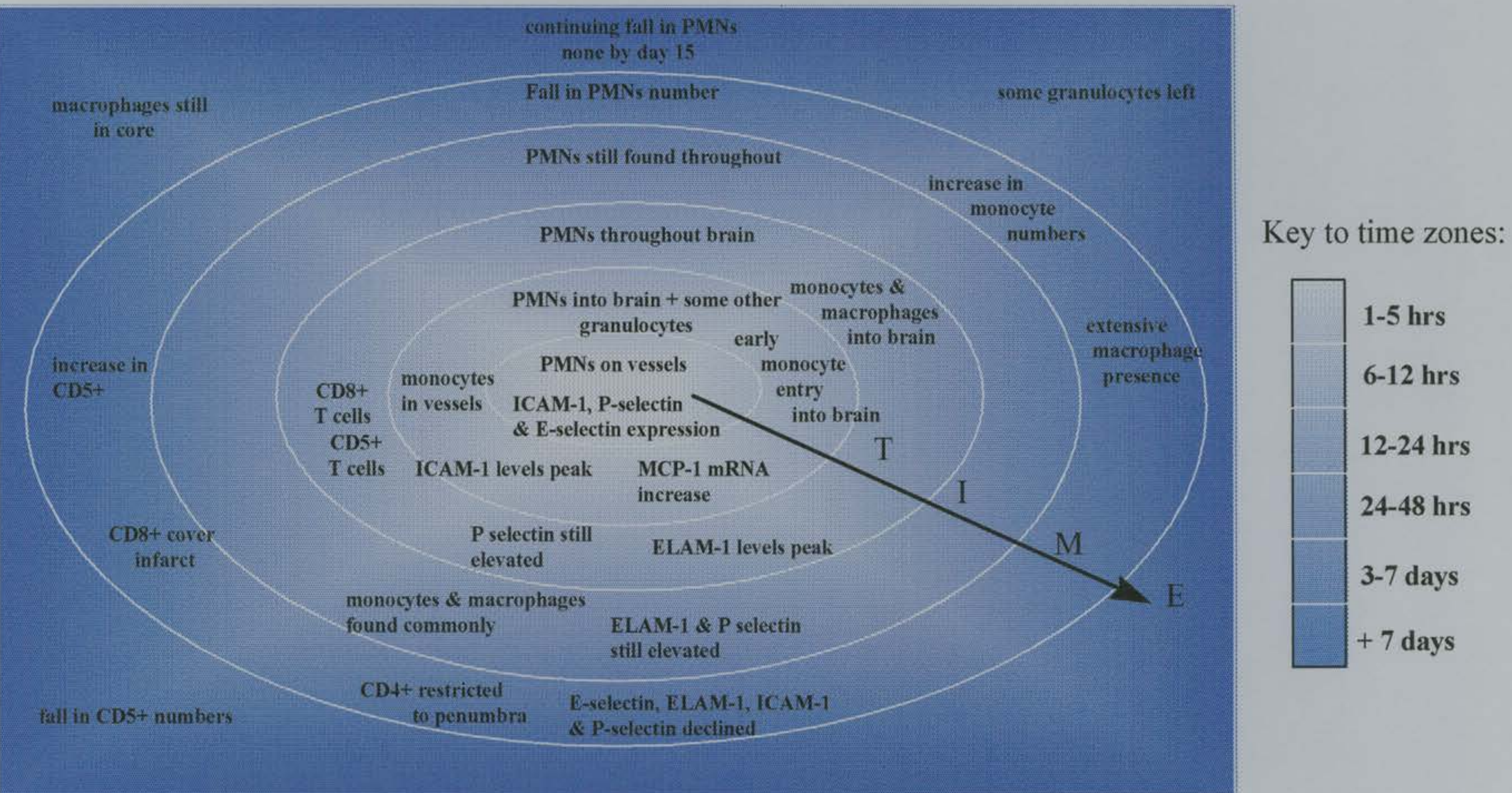


Figure 23. Temporal profile of leukocyte infiltration & inflammatory mediator expression following MCA occlusion by intraluminal filament.

Effectiveness of therapeutic strategies targeted at the inflammatory process

Treatment	Model & species	Results	References
Anti CD18	Transient spinal cord ischaemia and (permanent) multiple cerebral microspheres in the rabbit	Mab reduced neurological deficits in the spinal cord model	Clark *et al.* (1991)
Anti CD18	90 min TMCAO (clip) in the cat	No effect of Mab	Takeshima *et al.* (1992)
Anti CD18	2 hrs TMCAO (filament) in the Wistar rat	↓ infarct ~50%	Zhang *et al.* (1995)
Anti LFA-1	1 hr TMCAO (filament) in the Wistar rat	↓ oedema & infarction	Matsuo *et al.* (1994)
Anti Mac-1	2 hrs TMCAO (filament) in the Wistar rat	↓ infarct by 28%	Chopp *et al.* (1994)
Anti Mac-1	2 hrs TMCAO (filament) in the Wistar rat	↓ infarct by 37-44%	Zhang *et al.* (1995)
Anti Mac-1	2 hrs TMCAO (filament) + PMCAO in the Wistar rat	↓ in damage in TMCAO but not PMCAO	Chopp *et al.* (1995)
Anti ICAM-1	2 hr TMCAO (filament) in the rat	↓ infarct by 41%	Zhang *et al.* (1994)
Anti ICAM-1	2 hrs TMCAO (filament) and PMCAO in the Wistar rat	↓ in TMCAO (44%) but not PMCAO model	Zhang *et al.* (1995)
Anti ICAM-1	Embolic model in the rabbit	Found a neurological improvement	Bowes *et al.* (1993)
Neutrophil inhibitory factor	2 hrs TMCAO (filament) in the Wistar rat	↓ infarct (48%)	Jiang *et al.* (1995)

Table 6. The effectiveness of antibody procedures targeted at components of the immune system in models of cerebral ischaemia. Approaches were predominantly effective in models of transient MCA occlusion that incorporate a reperfusion phase.

inflammatory nature of the procedure, whereby the endothelial lining of cerebral blood vessels is extensively damaged, compromising the BBB integrity, and most likely leading to an artifactually greater inflammatory component which may not be present in the clinical setting. A second critism may be levelled at the time course of the inflammatory process itself. As can be seen from the diagram illustrating the temporal profile of the inflammatory response to cerebral ischaemia (Figure 23), tissue penetration of leukocytes may not peak for up to 12 hours following ischaemia. Since many studies suggest that 3-4 hours of arterial occlusion produces maximal damage, it is questionable as to whether leukocyte recruitment at time points delayed as long as 6-12 hours can significantly affect pathophysiological outcome. Indeed, the release of cytotoxic substances by activated inflammatory cells does not occur until a peak recruitment of inflammatioy cells up to 48 hours following the inital insult (Giulian *et al.* 1993). This consequently brings into question the mechanism by which Ab directed at leukocyte recruitment are effective. It is likely that the mechanism may be due to blocking leukocyte accumulation alone, alleviates microvascular plugging ensuring patency on reperfusion.

5.1.5. Experimental strategy

In these studies the specific ET_A receptor antagonist FR139317, was employed to interupt ET-1-induced MCA occlusion by competing for receptors, to introduce reperfusion. A dual cannula system, whereby a 24 gauge outer cannula contained a 31 gauge needle within its lumen, was employed to deliver ET-1 and FR139317 or vehicle intracerebrally to parenchyma underlying the MCA. The top of the cannulae

were connected to polyethylene catheters to injection syringes. This dual cannula system is illustrated in Figure 24. FR139317 (or vehicle) was injected 10 minutes after ET-1 and histological assessment of ischaemic brain damage performed after three day recovery. In other studies, LDF was employed to monitor the tissue perfusion profile of the effects of FR139317 and SNP on ET-1 induced MCA occlusion.

5.2. METHODS

5.2.1. Surgery for FR139317 post treatment

Surgery was performed as previously described in section 2.2.1. Following a craniotomy, a 24 gauge dual cannula was stereotaxically lowered into piriform cortex approximately 0.5mm dorsal to the MCA (co-ordinates from bregma; AP=0.9mm; L=-5.2mm, and -8.7mm below skull; tooth bar at -3.7mm). FR139317 (3 or 30nmols in 3μl) or vehicle (3μl) was injected 10 minutes after ET-1 (100 pmols in 2μl). The cannula was left in situ for 5 minutes, and then slowly withdrawn. The wound was sutured with Ethicon 4/0 silk, and animals were returned to an incubator where normothermia was maintained until the rat had fully recovered from anaesthesia. Three days later animals were reanaesthetised, transcardially perfused with paraformaldehyde in PBS as previously described in section 2.2.2. and the brains processed for quantitative histopathology.

LDF was employed to record cortical CBF changes following ET-1 induced MCA occlusion, with FR139317 injection 10 minutes afterwards. Sodium nitroprusside (SNP) post injection was included for comparison. The protocol was as

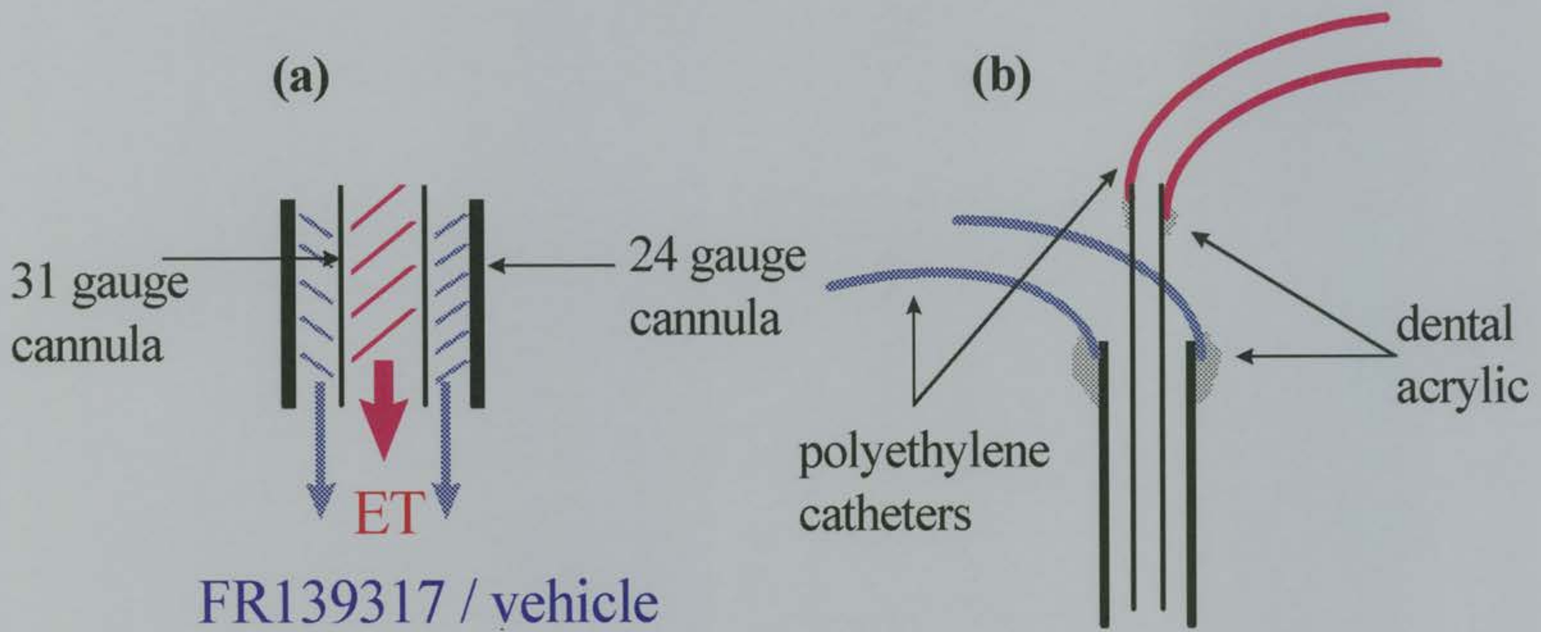


Figure 24. Diagram illustrating bottom (a) and top (b) of the dual cannula constructed for co-injection of FR139317 / vehicle before or after endothelin isopeptides.

described for previous LDF experiments performed in section 3.2.3., with minor modifications. Animals were implanted with an indwelling cannula for intracerebral injection of ET-1 and other drugs. Following 5-10 day recovery, animals were reanaesthetised and a polyethylene catheter inserted into the right femoral artery for blood pressure monitoring and blood gas analysis. Animals were then ventilated artificially (Harvard Rodent ventilator) and placed in a stereotaxic frame. A craniotomy was performed over the parietal cortex, and a LDF probe advanced to touch the intact surface of the dura. The site was irrigated with ACSF and recordings made for 15-30 minutes prior to ET-1 injection (100 pmols in 2μl). 10 minutes following ET-1 injection, FR139317 (3 nmols in 3μl), SNP (3 nmols in 3μl) or vehicle (3μl) was injected and recordings were made for a further 90 minutes. Blood analysis was performed at regular intervals for determination of pH, pCO_2, pO_2 and plasma glucose concentration.

At the end of the experiment, a zero flow reading was taken and the value subtracted from LDF recordings. At the termination of the experiment, brains were excised and inspected under an operating microscope for evidence of reperfusion and/or ischaemia (tissue pallor).

5.2.2. Drugs

ET-1 and the lower dose of FR139317 were dissolved in saline to the appropriate concentration that would deliver 50 pmols/μl and 1 nmol/μl respectively. Stock FR139317 for 30 nmol injection, was made up in Tween (<1%) in saline vehicle.

5.2.3. Statistical analysis

All data are presented as mean ± s.e.mean. Histological data were analysed by Student's T test. Physiological variables for LDF studies were compared by ANOVA on ranks. LDF data were expressed as % change from baseline and data from LDF recordings following FR139317/SNP injection were compared by Two-way ANOVA with post-hoc Tukey test to vehicle post-injected controls.

5.3. RESULTS

5.3.1. Post treatment with FR139317/saline

In control, vehicle post-injected controls (n=9), the volume of damage following ET-1-induced MCA occlusion was 115 ± 24 mm^3. The volume of ischaemic damage when FR139317 (3nmols; n=9) was delivered 10 minutes following ET-1 injection was 80 ± 35mm^3, which was not significantly different. Furthermore, the 10-fold higher dose of FR139317 (30 nmols; n=6) did not significantly reduce ischaemic damage compared to vehicle treatment (n=4) (108 ± 62mm^3 vs 170 ± 67mm^3 respectively) (Figure 25).

5.3.2. LDF assessment of reperfusion

Physiological variables were kept within normal limits for both the FR139317 and SNP post-injection experiments compared to controls, and no significant differences were found between groups at any time point (Tables 7 & 8 respectively). ET-1 injection reduced the LDF signal to 84 ± 5% in FR139317 (n=4) post-treated animals, and 75 ± 6% in vehicle post-treated controls (n=5), which was not

Effects of intracerebral FR139317 10 minutes after ET-1-induced MCA occlusion

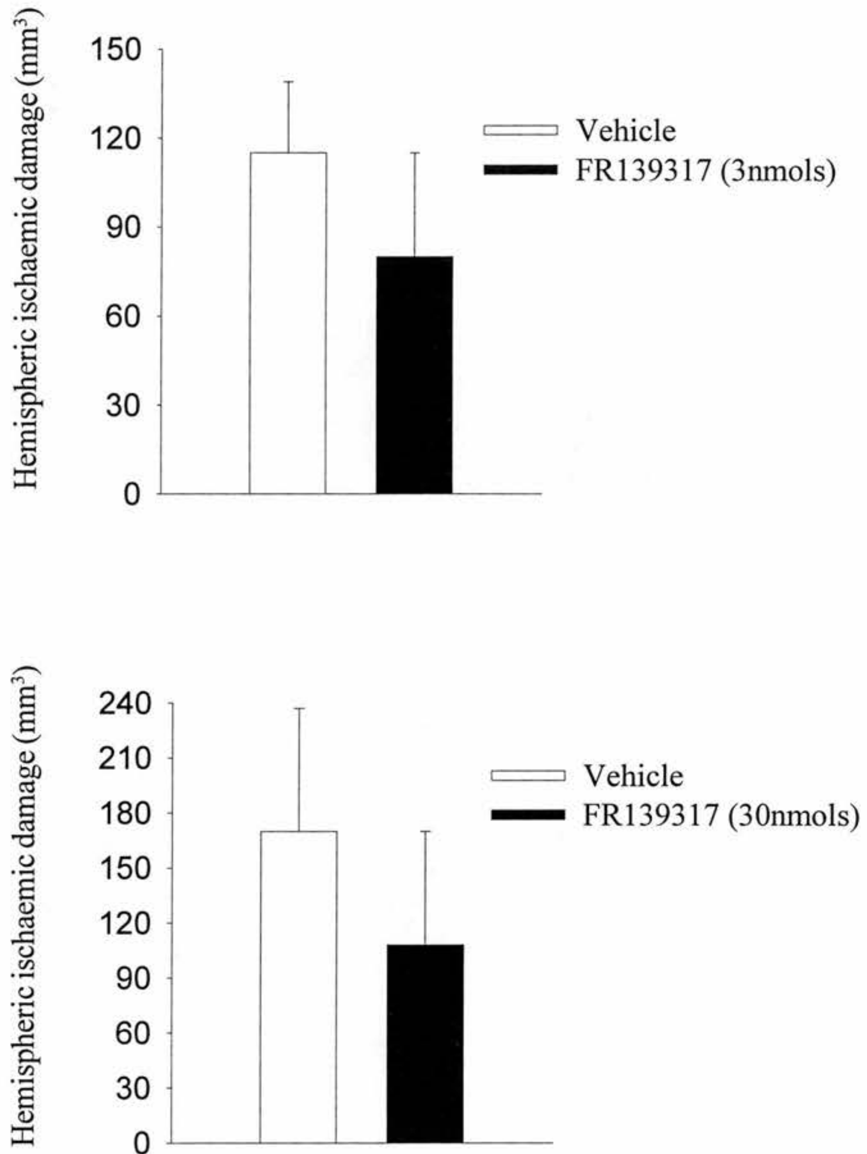


Figure 25. Effects of intracerebral injection of FR139317 10 minutes after ET-1-induced MCA occlusion. 3nmols of FR139317 (n=9) or vehicle (n=9) (top), or 30 nmols of FR139317 (n=6) or vehicle (n=4) (bottom) were injected 10 minutes following ET-1 (100 pmols). Data are presented as mean ± s.e.mean. No significant differences were found between vehicle and FR139317 post-treatment on ET-1-induced MCA occlusion at either dose.

Physiological variables for LDF determination of effects of FR139317 on ET-1-induced MCA occlusion

Variable	**ET-1 + vehicle**			**ET-1 + FR139317**		
	Pre ET-1	**Post ET-1**	**Post 1 hr**	**Pre ET-1**	**Post ET-1**	**Post 1 hr**
M.A.B.P. (mmHg)	82±1	82±1	82±1	83±1	82±1	83±1
Rectal temperature (°C)	37.4±0.05	37.4±0.05	37.5±0.05	37.4±0.1	37.3±0.1	37.3±0.05
pH (units)	7.47±0.01	7.47±0.01	7.45±0.02	7.46±0.01	7.46±0.03	7.42±0.05
pCO_2 (mmHg)	38±1	38±1	38±1	39±1	36±2	37±1
pO_2 (mmHg)	103±2	99±4	104±6	102±3	108±8	105±6
plasma glucose (mM)	9.2±0.4	9.3±0.5	9.8±0.6	8.2±0.5	8.1±0.7	8.6±0.4

Table 7. Physiological parameters for LDF profile of the effects of FR139317 (3 nmols; n=4) or vehicle (n=5) injection 10 minutes after ET-1 (100 pmols). Data are shown as mean ± s.e.mean. No significant differences in physiological parameters were found between groups at any time point.

Physiological variables for LDF determination of effects of SNP on ET-1-induced MCA occlusion

Variable	ET-1 + vehicle			ET-1 + SNP		
	Pre ET-1	**Post ET-1**	**Post 1 hr**	**Pre ET-1**	**Post ET-1**	**Post 1 hr**
M.A.B.P. (mmHg)	81±1	82±1	81±0.5	83±1	82±1	82±1
Rectal temperature (°C)	37.4±0.1	37.4±0.05	37.5±0.05	37.4±0.05	37.4±0.1	37.4±0.05
pH (units)	7.47±0.01	7.47±0.02	7.45±0.02	7.47±0.01	7.46±0.01	7.46±0.01
pCO_2 (mmHg)	39±1	39±1	39±1	39±1	39±0.5	37±0.5
pO_2 (mmHg)	102±2	97±5	99±5	109±3	106±8	100±2
plasma glucose (mM)	9.0±0.5	9.2±0.6	9.5±0.6	9.0±0.3	10.4±1.3	9.6±0.5

Table 8. Physiological parameters for LDF profile of the effects of SNP (3 nmols; n=4) or vehicle (n=4) injection 10 minutes after ET-1 (100 pmols). Data are shown as mean ± s.e.mean. No significant differences in physiological parameters were found between groups at any time point.

significantly different between treatments. Injection of FR139317 (3 nmols) 10 minutes following ET-1 did not effectively reverse the reduction in perfusion compared to vehicle injection, and LDF was not significantly different between treatments at any time point. (Figure 26). Microscopic inspection of the vascular territory of the MCA after termination of recording revealed profound tissue pallor throughout the MCA territory.

LDF was reduced to 73 ± 4% in SNP post-treated rats, and 79 ± 7% in vehicle controls, 5 minutes after ET-1-injection, a reduction not significnatly different between treatments. By contrast to FR139317, the same dose of SNP effectively reversed the ET-1-induced reduction in the LDF signal within 15 minutes, compared to the vehicle injection group, in which the LDF signal did not recover. The LDF signal then remained stable for the duration of recordings thereafter (Figure 27).

5.4. Discussion

5.4.1. General

The studies described in this section addressed whether ET-1-induced MCA occlusion could be interupted by the intracerebral co-injection of an ET receptor antagonist. Previously, studies described in chapter 3 suggested ET-1-induced MCA occlusion has the pathophysiological profile of a model of permanent MCA occlusion. The extent of reperfusion was limited, and not controlled. Models of focal cerebral ischaemia that incorporate a phase of reperfusion are however essential in improving our understanding of the pathophysiology of stroke. Consequently, the

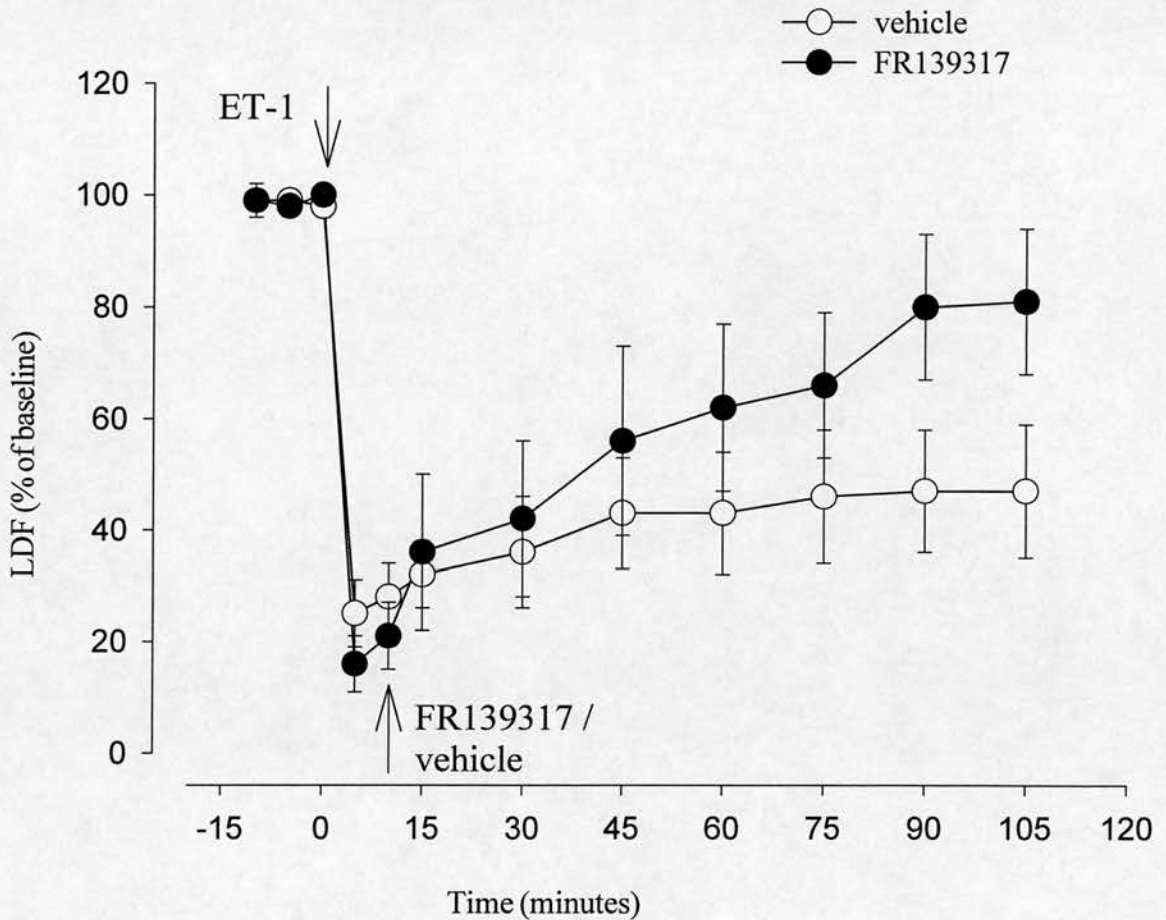


Figure 26. LDF recording of the effects of FR139317 (3nmols; n=4) or vehicle (n=5) injection 10 minutes after ET-1-induced MCA oclusion. Data are presented as mean ± s.e.mean. LDF signal fell to ~30% of baseline following ET-1 (100 pmols). Two-way ANOVA determined LDF in FR139317 injected animals was not show significantly different from vehicle controls at any time.

Effect of sodium nitroprusside on the LDF profile of ET-1-induced MCA occlusion

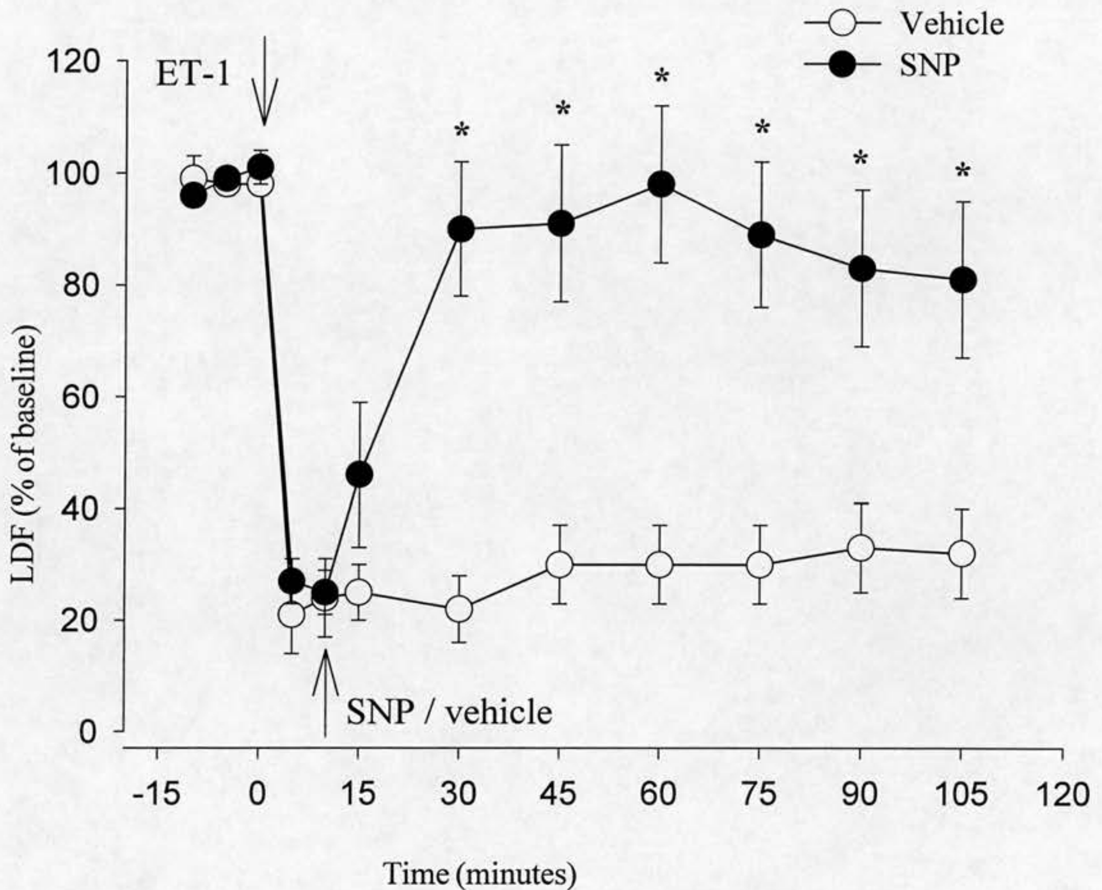


Figure 27. LDF recording of the effects of SNP (3nmols; n=4) and vehicle (n=4) on ET-1-induced MCA oclusion. Data are presented as mean ± s.e.mean. LDF signal fell to ~25% of baseline following ET-1 (100 pmols) in both treatment groups, 5 minutes after injection. LDF had recovered to ~50% 5 minutes after SNP injection, and had recovered to ~95% of baseline after 20 minutes, remaining stable for the duration of remining recordings. By contrast, LDF in vehicle controls showed no significant signal recovery during the recordings. *P=<0.05 compared to vehicle controls.

manipulation of the current model was investigated with a view to producing a model of MCA occlusion in which reperfusion could be introduced in a controlled manner.

A number of models of focal cerebral ischaemia have been developed that encompass a reperfusion phase, including compression occlusion of the surgically exposed MCA by reversible snare ligature (Shigeno et al. 1985), intraluminal occlusion by filament (Koizumi *et al.* 1986), and abluminal endothelin-1 (Macrae *et al.* 1993). However, the intraluminal filament model has been the most extensively adopted for MCA occlusion with reperfusion. Models which employ physical approaches to arterial occlusion inherently cause damage to the artery, and the intraluminal filament approach causes extensive damage to the endothelial lining of the cerebral arteries. The consequences of these may be two-fold. Mechanical damage to the cerebral artery and endothelial denudation will impair vessel responsivity and cerebrovascular tone (McAuley, 1995). Second, denudation of the endothelium will compromise BBB integrity, promoting oedema, whilst potentially exacerbating the inflammatory signal (McAuley, 1995). Both these events are likely to affect the pathophysiological response to ischaemia, due to the role of the microvasculature in stroke (del Zoppo, 1994). Consequently there exists a need to investigate focal cerebral ischaemia with reperfusion using a model that does not incur such extensive physical damage to the cerebrovasculature. The use of ET-1 to produce MCA occlusion circumvents the physical damage associated with the other models of stroke (Macrae, 1992; Sharkey *et al.* 1993). Whilst studies in which ET-1 was applied to the exposed MCA document reperfusion to the MCA territory in a

dose-dependent manner, the control of reperfusion is limited when compared to the rapid reflow possible with ligature and filament approaches.

The approach employed in the present studies, was to intracerebrally inject microlitre volumes of an ET receptor antagonist after ET-1 to reverse MCA occlusion. The rationale for this was based on a number of decisions. First, use of a specific ET receptor antagonist may interupt ET-induced MCA occlusion by blocking the site at which the agonist acted. Consequently this would inhibit contraction and lead to reperfusion. Second, the intracerebral injection minimises potentially confounding effects of a systemic injection of antagonist, and systemic administration of an ET receptor antagonist has been shown to decrease ischaemic brain damage after MCA occlusion (Patel et al. 1996b). The alternative approach to pharmacological interuption of ET-induced MCA occlusion is physiological antagonism by agents that indirectly block or reverse the effects of the ETs. Early research on ET demonstrated that agents such as isoprenaline, a non selective β adrenoceptor agonist, and sodium nitroprusside (SNP) or SIN-1, which releases nitric oxide, was capable of reversing the effects of ET-1 (Yanagisawa *et al.* 1988; Gologorsky *et al.* 1994). Consequently the duration of ET-1-induced MCA occlusion could be interupted by injection of one of these agents. However, the disadvantage with such approaches are the complications in data interpretation. Specifically, in the case of SNP, the problem is the role of nitric oxide in the brain (Dawson, 1994). It could be argued that a small intracerebral infusion of SNP would be unlikely to have profound effects on CBF outside of a localised site, given the short half-life of NO. However, a factor of perhaps greater importance is the role of NO in the

pathophysiological process itself. NO has been suggested as a mediator of ischaemic brain damage (Boje & Arora, 1992; Dawson et al. 1993; 1994) which would complicate interpretation. However, since the site of NO action is within the core region of ischaemia, the tissue would enevitably become infarcted at the injection site. NO is also implicated in the inflammatory response, inhibiting a number of platelet activation-related processes, potentiating the actions of prostaglandin I_2 (PGI_2), blocking PAF production, and preventing blood cell activation & adhesion (Akopov *et al.* 1996). Consequently, additional introduction of NO into the microvascular environment central to the site of potential inflammatory response would complicate interpretation of the pathophysiological response to ischaemia, introducing artifactual components and limiting the application of the model.

In these studies FR139317, a specific ET_A receptor antagonist was used. The rationale was based on autoradiographic data (De Oliveira *et al.* 1995), suggestting that the ET_A receptor predominates on the cerebrovasculature of the rat. Furthermore, the specific ET_A receptor antagonists BQ123 and FR139317 prevent ET-1 induced contraction of cerebral arteries *in vitro* (Salom *et al.* 1993; Feger *et al.* 1994; Kitazono *et al.* 1995a; Goadsby *et al.* 1996). This is supported by the order of ET isopeptide potency (Feger et al. 1994), whilst ET_B selective agonists such as IRL 1620 do not produce vasoconstriction when applied to cerebral arteries *in vivo* (Kitazono *et al.* 1995a).

FR139317 is a potent and selective ET_A receptor antagonist both *in vitro* and *in vivo* (Sogabe *et al.* 1993; Gross et al. 1994; Goadsby et al. 1996), displaying 20-200,000 fold selectivity for the ET_A receptor, and subnanomolar affinity for the ET_A

receptor with a $K_D = 0.4nM$ (Bacon & Davenport, 1996; Peter & Davenport, 1996). Another possible advantage of using FR139317 is that it is a peptide-based antagonist (Sogabe et al. 1993) which is unable to cross the BBB. This would have the effect of minimising tissue distribution of FR139317.

A number of potential cannula systems were considered for experiments in which an ET antagonist was delivered intracerebrally to the same site as an ET isopeptide. Two main approaches could be considered. Antagonist and agonist could be delivered via the same cannula, with each drug loaded separately, with an air bubble or mineral oil separation. Second, the two drugs could be physically separated by employing a dual cannula system, whereby a large gauge cannula contained a narrower gauge needle within the lumen. Such a system prevents the possibility of drug mixing which might interfere with drug action. The advantage of the former would be less needle tract damage and the simplicity of the system. However, the latter, dual cannula system, approach was employed.

5.4.2. Histological and LDF assessment

The data from these studies have shown that ET-1 does not represent a suitable candidate for MCA occlusion with pharmacological reversal of ischaemia. Whilst intracerebral injections prevent determination of drug concentration, the 30 and 300-fold higher dose of antagonist used in these studies compares well to the dose ratio of FR139317 capable of blocking the effects of endothelin *in vitro* and *in vivo* (Sogabe *et al.* 1993; Gross *et al.* 1994). The antagonist was injected 10 minutes after ET-1 rather than at a later time point since MCA occlusion of sort (30 minutes) duration

does not produce an ischaemic brain lesion when assessed after 3 days (Du *et al.* 1996). If FR139317 effectively blocked the action of ET-1, then very little brain damage would result. In these studies, injection of 3 or 30 nmols FR139317 failed to block ischaemic damage consequent on ET-1-induced MCA occlusion, as assessed by a histological endpoint 3 days after drug injection. These findings were complimented by LDF, which determined that injection of FR139317, 10 minutes following ET-1 injection, did not effectively reverse the ET-1-induced reduction in the LDF signal, and the vascular territory remained ischaemic afterwards. By contrast to FR139317, LDF determined that the physiological antagonist SNP, effectively reversed the effects of ET-1, which confirms previous studies that have shown NO donors reverse the effects of ET-1 (Goligorsky *et al.* 1994).

The ineffectiveness of FR139317 against ET-1 may be explained by the nature of ET-1 to ET_A receptor interactions. ET-1 receptor binding is pseudoirreversible, and with increasing incubation time, the effectiveness of antagonists decreases further (Sagher *et al.* 1994; Hay & Luttmann, 1997; Marsault *et al.* 1991; 1993; Waggoner *et al.* 1992; Ohlstein *et al.* 1995; Wu-Wong *et al.* 1995), although some studies have suggested that ET receptor antagonists may be effective after ET-1 has been exposed to tissue (Patel *et al.* 1995a; 1996b). This suggests that ET-1 binding to the ET_A receptor displays non-equilibrium (irreversible) binding characteristics that will not obey typical dose-shift pharmacological responses to competitive antagonists. *In vitro* studies report that ET_A receptor antagonism is only effective when the antagonist is introduced to the test system in conjunction with ET-1 or prior to ET-1, and antagonism was ineffective when the antagonist was delivered after ET-

1 had been exposed to the tissue (Sagher et al. 1994; Yoneyama *et al.* 1995; Hay & Luttmann, 1997; Awane-Igata *et al.* 1997; Warner *et al.* 1993). It is conceivable that even if FR139317 was effective at an early time point, since incubation time increases the binding affinity of ET-1 (Wu-Wong *et al.* 1995), reversal at later (practical time point of 90 / 120 min occlusion) would still be ineffective. In conclusion, ET-1 is unlikely to be a suitable candidate substrate with which to modify the ET-1 model to produce controlled reperfusion.

CHAPTER SIX

CHAPTER 6. ENDOTHELIN-3-INDUCED MCA OCCLUSION

6.1. INTRODUCTION

6.1.1. Background

This chapter addresses the pharmacological characteristics of the receptor mediating ET-induced MCA occlusion, and whether ET-3 represents a more suitable substrate for MCA occlusion with controlled reperfusion. The previous chapter demonstrated that ET-1 is not a suitable substrate for controlled reperfusion using a specific ET_A receptor antagonist (FR139317). Consequently, a novel approach was taken to develop a model of controlled reperfusion that utilised ET-3, an isopeptide of ET-1, to produce MCA occlusion.

6.1.2. ET-3 pharmacology

Identification of the gene for the ET isopeptide, ET-3 was described by Inoue *et al.* (1989) and its location found to be on chromosome 20. ET-3, and ET-2, were shown to possess a high degree of amino acid sequence homology to ET-1 (see Figure 1). These peptides were also 21 amino acid peptides containing two disulphide bonds and an identical hydrophobic C-terminal hexapeptide sequence (Goto *et al.* 1996). The structure of ET-3 has been analysed by proton NMR (Bortman *et al.* 1991; Mills *et al.* 1992).

ET-3 has a lower affinity to ET-1 at the ET_A receptor (Miller *et al.* 1993), and ET-3 has a lower potency than ET-1 at the ET_A receptor (Feger *et al.* 1994). By contrast ET-3 is equipotent with ET-1 at the ET_B receptor. A third, ET-3-selective (ET_C)

receptor has been proposed at which ET-3 is more potent than ET-1 (Emori *et al.* 1990).

A number of studies have addressed the effects of ET-3 on the cerebrovasculature. ET-3 has been demonstrated to mediate both vasoconstriction and vasodilatation of cerebral arteries *in vitro* (Schilling *et al.* 1995), with a lower affinity and efficacy than ET-1 & ET-2. *In vitro* studies on cerebrovascular cells have demonstrated that ET-3 has lower potency at stimulating an intracellular increase in [Ca^{2+}] (Salom *et al.* 1995).

6.1.3. Rationale for studies

Both ET_A and ET_B receptor subtypes have been identified in the cerebrovasculature (Salom *et al.* 1995), although radioligand binding studies suggest that the ET_A subtype predominates (Davenport *et al.* 1995; De Oliveira *et al.* 1995; Pierre & Davenport, 1995). This proposal is supported by *in vitro* functional studies that employed specific ET receptor antagonists (Kitazono *et al.* 1995a).

Central to the rationale behind using ET-3 to produce MCA occlusion is based on the pharmacological evidence that ET-1 has a higher affinity than ET-3 at the ET_A receptor. By contrast, the ET_B receptor is an isopeptide non-selective receptor (Masaki *et al.* 1994; Rubanyi & Polokoff, 1994; Salom *et al.* 1995; Goto *et al.* 1996). According to receptor binding theory, a lower affinity ligand exhibit a faster dissociation rate. This would suggest that whilst ET-1 binding is too permanent to facilitate reversal by an antagonist within a suitable operating regime, the lower affinity of ET-3 for the ET_A receptor suggests that it may be more amenable to

reversal by an ET receptor antagonist. In these initial studies, the ability of ET-3 to produce MCA occlusion and ischaemic brain damage has been addressed. Furthermore, the characteristics of the receptor mediating ET-induced MCA occlusion were addressed.

6.2. METHODS

6.2.1. Surgery & histology

Surgery was performed as described in section 2.2.1. Following a craniotomy, a 28 gauge steel cannula was stereotaxically lowered into piriform cortex approximately 0.5mm dorsal to the MCA (co-ordinates from bregma; AP=0.9mm; L=-5.2mm, and -8.7mm below skull; tooth bar at -3.7mm), and 2μl of ET-3, ET-1, BQ3020, IRL1620 or vehicle was injected (1μl/90 seconds). The cannula was left in situ for a further 5 minutes and then slowly withdrawn. The wound was sutured, and animals were returned to an incubator where normothermia was maintained until the rat had fully recovered from anaesthesia. Three days later animals were reanaesthetised, perfusion fixed and the volume of infarction was calculated as previously described in section 2.2.2..

For studies using co-intracerebral application of FR139317 with ET isopeptide, a 24 gauge dual cannula was employed as previously described in section 5.2.1. ET-1 or ET-3 (100 pmols in 2μl) was administered via the internal cannula 10 minutes after FR139317 (3nmols in 3μl) or vehicle which was delivered via the outer cannula.

6.2.2. LDF experiments

Laser Doppler flowmetry (LDF) was utilised to assess tissue perfusion velocity in the cerebral cortex following ET-1/ET-3 injection. Animals were implanted with an indwelling cannula prior 5-10 days prior to study as described previously in section 3.2.1.

On the day of study, animals were anaesthetised, and a catheter inserted into the right femoral artery for blood pressure monitoring (Transonic Systems Inc.), blood gas analysis (Ciba Corning model 238) and plasma glucose determination (Beckman Glucose Analyzer 2). A craniotomy was performed in the parietal bone at a site more ventral than was employed in section 3.2.3. to ensure the doppler probe was located in a region closer to the core of the ischaemic area (co-ordinates from bregma: AP=0mm; L=-5-6mm, and 5-6mm below skull level). LDF probes (24 gauge) were advanced by a micromanipulator to rest on the surface of the parietal cortex. Recordings were made for 15-30 minutes prior to the insertion of a 33 gauge needle into the lumen of the indwelling cannula to deliver ET-1 or ET-3 (100 pmols in 2μl). Recordings were then continued for 3hrs. At the end of the recording period animals were killed by anaesthetic overdose (5% Halothane) and a zero flow recording taken which was later subtracted from LDF signal values.

Arterial blood gases (pCO_2 and pO_2), blood pH and plasma glucose concentration were determined for animals in the LDF study. Variables were measured before and 15-30 minutes after ET-1/ET-3 injection, and then subsequently 1,2 and 3 hours post ET-1/ET-3.

6.2.3. Experiments performed

The dose response relationship of ET-3 (10-300 pmols in 2μl) was examined, as were the effects of two ET_B receptor agonists, BQ3020 (100 pmols in 2μl) and IRL1620 (100 and 1000 mols in 2μl). The temporal profile of ischaemia following ET-1 (100 pmols in 2μl) and ET-3 (100 pmols in 2μl) induced MCA occlusion was addressed by LDF.

Finally, FR139317 (3nmols in 3μl) was injected 10 minutes before ET-1 or ET-3 to determine the pharmacological profile of the receptor mediating MCA occlusion.

6.2.4. Drugs

BQ3020 was purchased from Research Biochemicals Inc. IRL1620 was from NovaBiochem. Both drugs were dissolved in an alkaline saline vehicle, pH adjusted with sodium hydoxide to pH ~9. ET-1 and FR139317 were made up as previously described. ET-3 was dissolved in saline, and aliquots frozen at -70°C until use, as previously described for ET-1.

6.2.5. Statistical analysis

All data are presented as mean ± s.e.mean. Data from the dose response study and the effects of ET_B receptor agonists were analysed by ANOVA on Ranks with post hoc Dunn's method. LDF data were analysed by two-way ANOVA. Data from the comparison of 100 pmols of ET-1 with ET-3-induced MCA occlusion, and the effect of FR139317 pretreatment on ET-1 & ET-3-induced MCA occlusion, were analysed by Student's T test or Mann-Whitney Rank Sum test.

6.3. RESULTS

6.3.1. Dose-response relationship for ET-3

Intracerebral injection of ET-3 into the piriform cortex adjacent to the MCA produced a large ischaemic brain lesion of 78 ± 22mm^3 (33 pmols; n=11), 127 ± 21mm^3 (100 pmols; n=11) and 96 ± 20mm^3 (300 pmols; n=11), which was confined to the vascular territory of the MCA. Damage extended from the frontal cortex through to occipital cortex with concomitant damage to medial and lateral striatum. There were no significant differences in the volume of hemispheric ischaemic damage produced by these doses . At the lowest dose studied (10 pmols), volume of hemispheric ischaemic damage was 5 ± 2mm^3 (n=10), which was significantly reduced compared to the other doses (P<0.05), and was not significantly different from vehicle (sham lesioned) treatment (Figure 28).

Comparison of the effects of 100 pmols ET-3 to the same dose of ET-1 on the volume of hemispheric ischaemic damage revealed no significant differences between the isopeptides. Comparison of the area of ischaemic damage at 8 stereotxic levels also determined no significant differences between isopeptides at the 100 pmol dose (Figure 29).

6.3.2. Temporal profile of ET-3-induced MCA occlusion (comparison to ET-1)

During LDF studies, physiological parameters were kept within normal limits, and no significant differences were found between groups (Table 9). LDF determined that ET-1 and ET-3-induced MCA occlusion produced maximal reductions in parietal cortex perfusion velocity within 5 minutes, of 78±6% and 68±6% respectively. The

Dose-relationship for ET-3-induced MCA occlusion

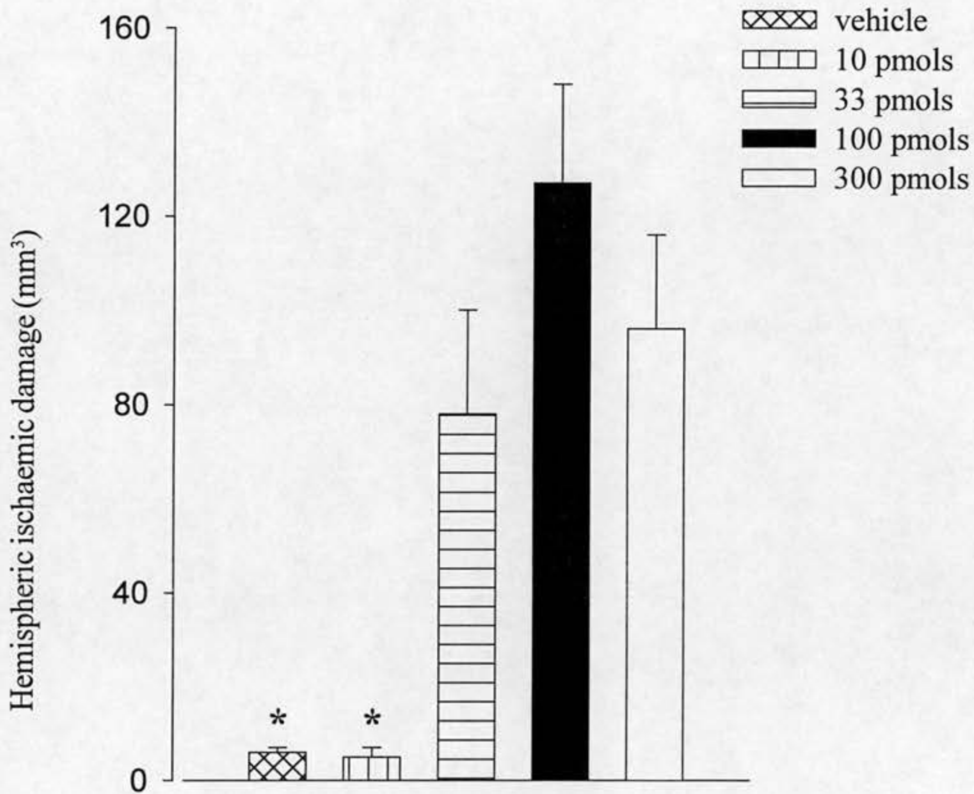


Figure 28. Dose-response relationship for ET-3-induced MCA occlusion. Vehicle (n=10), or 10 (n=10), 33 (n=11), 100 (n=11) or 300 (n=11) pmols ET-3 was injected, and hemispheric ischaemic damage determined. Data are presented as mean ± s.e.mean. *P=<0.05 compared to other doses.

ET-1 vs ET-3-induced MCA occlusion

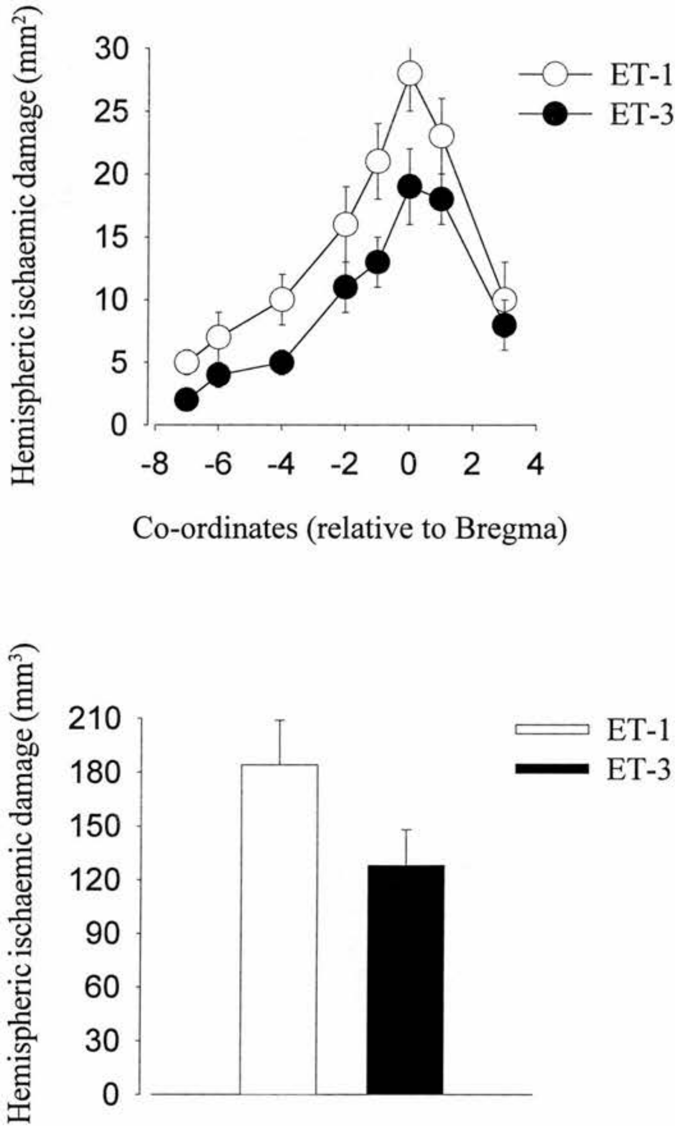


Figure 29. Comparison of the area at eight stereotaxic levels (Top) and hemispheric volume (Bottom) of ischaemic damage following injection of 100 pmols of ET-1 (n=12) and ET-3 (n=13). Data are presented as mean ± s.e.mean. No significant differences were found between ET-1 and ET-3 in either the area or hemispheric volume of damage.

Physiological parameters for LDF profile of ET-1 and ET-3-induced MCA occlusion.

Time	M.A.B.P. (mmHg)		Rectal temperature (°C)		pH (units)		pCO_2 (mmHg)		pO_2 (mmHg)		Plasma glucose (mM)	
	ET-1	ET-3	ET-1	ET-3	ET-1	ET-3	ET-1	ET-3	ET-1	ET-3	ET-1	ET-3
Pre	82±1	83±3	37.4±0.1	37.5±0.1	7.47±0.01	7.49±0.01	38±1	38±2	104±3	112±18	9.2±0.6	8.5±0.5
Post	83±1	82±1	37.5±0.1	37.5±0.1	7.48±0.02	7.46±0.01	38±1	39±1	103±1	98±11	9.5±0.6	8.4±0.4
Post 1hr	82±1	81±1	37.5±0.1	37.5±0.1	7.45±0.02	7.46±0.01	38±1	38±1	107±7	98±7	9.8±0.8	8.6±0.2
Post 2hrs	82±1	81±1	37.5±0.1	37.3±0.1	7.44±0.02	7.53±0.05	39±1	32±5	109±5	100±12	9.4±0.8	7.9±0.2
Post 3hrs	82±1	80±0.5	37.4±0.1	37.4±0.1	7.43±0.01	7.46±0.01	38±1	38±1	101±6	96±6	9.0±0.6	7.5±0.2

Table 9. Physiological variables for LDF study comparing ET-1 (n=4) and ET-3 (n=4) induced MCA occlusion. Data are shown as mean ± s.e.mean. No significant differences in physiological parameters were found between groups at any time point.

LDF signal remained depressed by >50% following both ET-3 and ET-1 for three hours. Statistical analysis determined that the maximal reduction, and temporal profile of the reduced tissue perfusion following ET-1 and ET-3, was not significantly different at any time points (figure 30).

6.3.3. Effects of ET_B agonists

Intracerebral injection of the putatively selective ET_B receptor agonists BQ3020 (100 pmols; n=5) and IRL 1620 (100 pmols; n=8) (1nmol; n=8) did not produce an ischaemic brain lesion, and damage consisted only of a small needle tract injury within the cerebral cortex, which was not significantly different from that found in sham operated animals (hemispheric ischaemic damage was <8mm^3 for all treatments) although it was significantly different (P<0.05) to that produced by 100 pmols ET-3 (120 ± 15mm^3; n=13) (Figure 31).

6.3.4. Effects of FR139317 on ET- induced MCA occlusion

Intracerebral injection of FR139317 (3nmols) 10 min before ET-1 (100 pmols; n=12) produced an hemispheric ischaemic brain lesion of 147±25mm^3, which was not significantly different to the volume of ischaemic brain damage in ET-1 + vehicle (n=12) treated animals (140±27mm^3). By contrast, intracerebral injection of FR139317 10 minutes prior to ET-3 significantly reduced the volume of ischaemic brain damage to 7±1mm^3 (n=10), compared to 116±23mm^3 in the vehicle (n=10) pretreatment group (P<0.001) (Figure 32).

LDF profile for ET-1 and ET-3-induced MCA occlusion

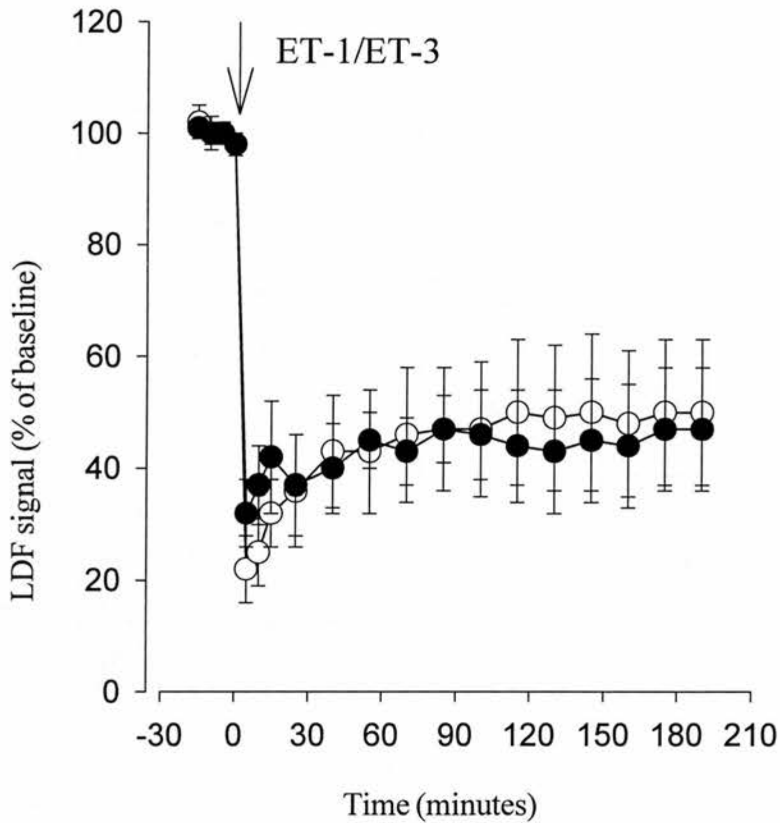


Figure 30. LDF profile of ET-1 (n=4) and ET-3 (n=4) induced MCA occusion. Data are presented as mean ± s.e.mean. No significant differences were found between the isopeptides for either the maximal reduction or the temporal profile of ischaemia.

Effects of intracerebral ET_B receptor agonists

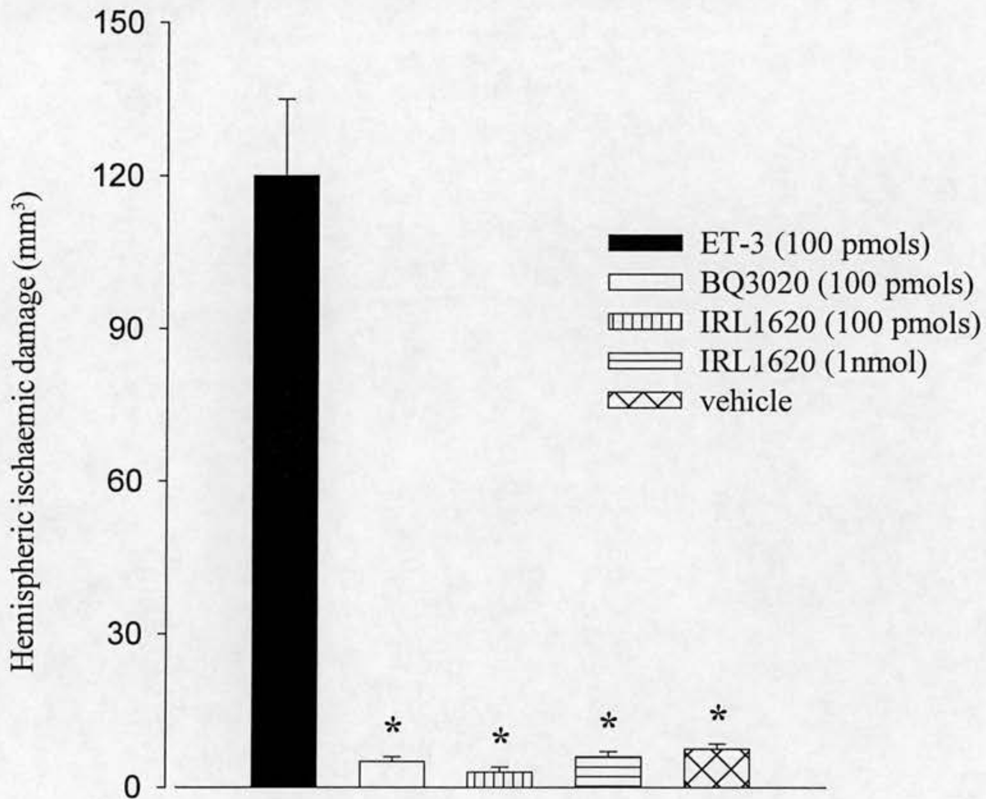


Figure 31. Effects of intracerebral ET_B receptor agonists. 100 pmols ET-3 (n=13), 100 pmols BQ3020 (100 pmols), 100 pmols IRL1620 (n=8), 1nmol IRL1620 (n=8) or vehicle (n=5) were injected. ET_B receptor agonists did not produce significantly more damage than vehicle injected controls. Data are presented as mean ± s.e.mean. **P*=<0.05 compared to ET-3 control group.

Effects of pretreatment with FR139317 on ET-1 and ET-3-induced MCA occlusion

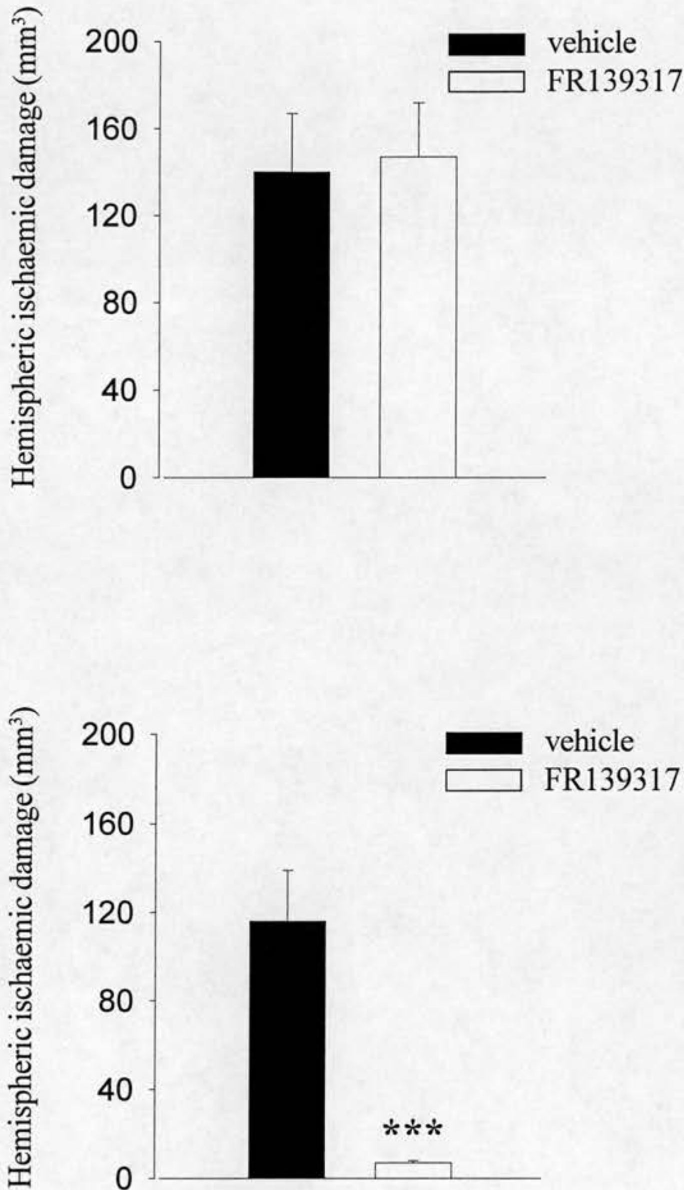


Figure 32. Effects of intracerebral FR139317 10 minutes before ET-1 (top) and ET-3 (bottom) -induced MCA occlusion. 3nmols of FR139317 (n=12) or vehicle (n=12) were injected 10 minutes before ET-1 (100 pmols), and 3nmols of FR139317 (n=10) or vehicle (n=10) before ET-3 (100 pmols). Data are presented as mean ± s.e.mean. FR139317 pretreatment had no effect on ET-1-induced MCA occlusion, whilst FR139317 blocked ET-3-induced MCA occlusion. *P=<0.001.

6.4. DISCUSSION

6.4.1. ET-3 vs ET-1 induced MCA occlusion

Quantitative analysis of ischaemic brain damage determined that 33-300 pmols of ET-3 produced a consistently large ischaemic brain lesion which was restricted to the vascular territory of the MCA. This MCA occlusion-like pattern of damage was lost when using 10 pmols ET-3, with animals displaying only needle tract damage in the cortex. ET-3 was effective over the same dose range as previously reported for ET-1 (33-300 pmols) (see section 2.3.2.) and isopeptides shared the same minimal effective dose of 33 pmols. Neither 10 pmols ET-1 or ET-3 was capable of producing more ischaemic damage than that found in sham operated animals. Comparison of the lesion volume produced by 100 pmols of ET-1 or ET-3 confirmed that the isopeptides are equipotent in this model.

LDF recordings in the present studies determined that ET-1 and ET-3 produce similar reductions in cortical flow velocity which were sustained for 3 hours. The magnitude of the reduction in perfusion following both ET-1 and ET-3-induced MCA occlusion, and the subsequent temporal profile of perfusion deficit, is consistent with findings in other models of MCA occlusion (Dirnagl *et al.* 1989).

Comparing these LDF results of ET-1-induced MCA occlusion with the LDF study in Chapter 3 demonstrates a difference in the temporal profile of ischaemia. In the former studies, the LDF signal showed a degree of recovery over the three hour recording period, whilst in these studies there was no such recovery. This difference may be due to a number of factors. Possibly the most important is the positioning of the LDF probes between these studies. Whilst both studies sampled from parietal

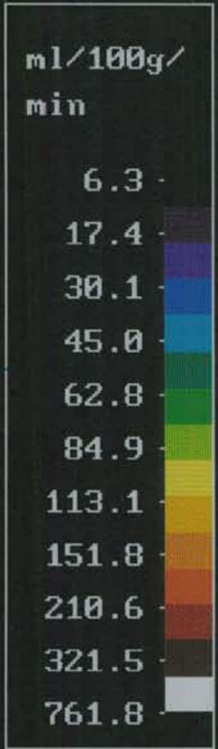
cortex, the former study sampled a dorsal region compared to the more ventral site chosen for these studies. The absence of LDF signal recovery in the present studies may reflect a recording site lying within the core zone of the ischaemic territory, whilst the earlier studies sampled from the area of the penumbra. This difference is reflected in the pseudocolour transform of local CBF following [^{14}C]-iodoantipyrine autoradiography taken 3 hours after ET-1-induced MCA occlusion, shown in figure 33. This illustrates the relative positions of LDF probes between the previous and present studies. An area of higher perfusion is evident at the level of prior probe placement, whilst local CBF at the probe site chosen in the current study remains ischaemic even at three hours. Such differences may explain the proportionally greater recovery seen in the earlier study. Thus both histopathology and LDF recordings suggest that ET-1 and ET-3 are equipotent in the ischaemic insult produced by MCA occlusion.

6.4.2. ET_B agonist effects

The equipotency of ET-3 with ET-1 in our model was unexpected because ET-3 is less potent than ET-1 at the ET_A receptor (Goto *et al.* 1996). Consequently, the possibility that ET-3 might be acting via a non-ET_A receptor was examined. BQ3020 and IRL1620 are potent and selective ET_B receptor agonists both *in vitro* and *in vivo* (James *et al.* 1993; Battistini *et al.* 1994; Ishikawa *et al.* 1994; Nambi & Pullen, 1995; Widdowson & Kirk, 1996). Since the *in vitro* and *in vivo* potency of these drugs and the endothelin isopeptides is equivalent (James *et al.* 1993; Bacon &

Figure 33.

Pseudocolour transform of local CBF determined by [^{14}C]iodoantipyrine autoradiography three hours after intracerebral injection of 100 pmols ET-1 adjacent to the MCA. Arrows delineate (a) approximate site of LDF probe placement for dorsal parietal cortex recordings in chapter 3 & (b) approximate site of LDF probe placement for "core" recordings in chapter 6. Note the "penumbral" hyperperfusion lies within the area of tissue commonly sampled in chapter 3 studies, whilst local CBF is still markedly reduced within the region sampled in the later studies.


(a)
(b)
ml/100g/
min
6.3
17.4
30.1
45.0
62.8
84.9
113.1
151.8
210.6
321.5
761.8

Davenport, 1996), the dose range employed in this study would be expected to elucidate any ET_B receptor-mediated response.

In the present studies, injection of either drug did not produce ischaemic brain damage, with only needle tract damage within the cortex, that was not significantly different from vehicle-injected animals. This suggests that an ET_B receptor does not mediate the vasoconstrictor effects of ET-3. This finding confirms previous studies using ET_B receptor agonists *in vitro*, in which ET_B receptor agonists did not cause vasoconstriction (Willette *et al.* 1994; Kitazono *et al.* 1995a; Schilling *et al.* 1995).

6.4.3. FR139317 effects

In the final studies addressed in this chapter, a specific ET_A receptor antagonist was employed to determine if ET-3-induced MCA occlusion could be blocked by FR139317. Intracerebral injection of FR139317 (3 nmols) 10 minutes before ET-3, abolished ET-3-induced MCA occlusion. By contrast, ET-1-induced MCA occlusion was not blocked by intracerebral injection of the same dose of FR139317, 10 minutes before ET-1. Whilst some previous studies have shown ET_A receptor antagonists can block the effects of ET-1 on the cerebrovasculature (Salom *et al.* 1993; Feger *et al.* 1994; Patel *et al.* 1996b), other studies have reported relative ineffectiveness of ET_A antagonists at putative ET_A receptors (Sudjarwo *et al.* 1993; Yoneyama *et al.* 1995; Patel *et al.* 1995b).

6.4.4. Evidence for a non ET_A/atypical ET receptor on the rat MCA

The present data are consistent with the presence of an atypical ET receptor on the rat MCA. A selective antagonist for the ET_A receptor would be expected to have similar potency against ET isopeptides if they are equipotent. However, whilst ET-1 and ET-3 were equipotent in this model, only ET-3-induced MCA occlusion was blocked by FR139317. Since ET_B receptor agonists were ineffective in this model, an atypical ET receptor may be involved. Whilst no functional studies have directly addressed the effects of ETs on the rat MCA, the effects of ETs on the rat basilar artery have been studied (Feger *et al.* 1994), as well as the MCA of a number of other species including the cat (Jansen *et al.* 1989; Saito *et al.* 1989), goat (Salom *et al.* 1993), and dog (Asano *et al.* 1990). The findings from these studies have suggested that ET_A receptors mediate constriction (Salom *et al.* 1993; Feger *et al.* 1994), whilst a population of ET_B receptors are also present and mediate vasodilatation (Feger *et al.* 1994; Schilling *et al.* 1995), although ET_B-mediated dilatation was not noted in the study by Saito *et al.* (1989). Whilst only the basilar artery of the rat has been studied in any detail in vitro, the ET receptor pharmacology on the dog basilar artery was the same as on the dog MCA (Asano *et al.* 1990), although extrapolation of this trend to the rat MCA may not be legitimate.

An alternative explanation may be derived from the irreversibilty of ET-1 binding. If ET-1 to ET_A receptor association is irreversible, then receptor activation could still occur in the presence of a competitive antagonist that displayed rapid dissociation from the receptor. However, FR139317 binds to the ET_A receptor with very high affinity. In the study by Bacon & Davenport, (1996) the K_D for FR139317 displacement of $[^{125}I]$ET-1 binding to the ET_A receptor was subnanomolar, which

suggests the dissociation rate is not only very slow for ET-1 but also for FR139317. Furthermore, Wu-wong *et al.* (1994) reported pseudo-irreversible binding for FR139317 as well as ET-1. These findings suggest that the lack of effect of FR139317 against ET-1 is unlikely to be a consequence of such pharmacokinetics.

There are some discrepancies in the ET receptor pharmacology of the cerebrovasculature, and the existence of an atypical ET_A receptor, and ET_A receptor subtypes, have been proposed following receptor binding and functional responses of the cerebrovasculature to ETs (Salom *et al.* 1993; Feger *et al.* 1994; Patel *et al.* 1995b). Studies in other tissues support the existence of further subtypes of endothelin receptors that do not comply to the ET_A/ET_B nomenclature (Emori *et al.* 1990; Summner *et al.* 1992; Sudjarwo *et al.* 1994; Yoneyama *et al.* 1995), although the existence of an ET_C receptor is still a matter of debate (Masaki, 1995). Further studies might elucidate as to whether the endothelin receptor mediating vasoconstriction of the rat MCA is an atypical ET_A or non ET_A/ET_B receptor.

6.4.5. Conclusions

The studies described in this chapter document a number of novel findings in regard to this model of stroke. First that ET-3 is shown to produce ischaemic brain damage by occlusion of the rat MCA, following intracerebral injection. ET-3 has not been studied as extensively as ET-1 in its effects on the cerebrovasculature, and this is the first demonstration that ET-3 is capable of producing ischaemic brain damage by occlusion of a major cerebral artery.

Second, is the finding that ET-3 is equipotent with ET-1 in the ischaemic insult it produces. This finding was determined by both histopathological end-point, and by examining the temporal profile of ischaemia using LDF. Further studies determined that this equipotency was not a consequence of an action at the ET_B receptor. Finally the disparate effects of FR139317 against ET-1 and ET-3-induced MCA occlusion in combination with these findings, suggest the presence of an atypical ET_A or non ET_A/ET_B receptor on the rat MCA.

CHAPTER SEVEN

CHAPTER 7. ET-3-INDUCED MCA OCCLUSION WITH CONTROLLED REPERFUSION

7.1. INTRODUCTION

7.1.1. Background

The two previous chapters have demonstrated two important points with respect to modifying the ET-1 model to produce a controlled reperfusion model. First, ET-1-induced MCA occlusion is not effectively interupted by an ET_A receptor antagonist. Second, the ET isopeptide ET-3 is capable of producing a large ischaemic brain lesion by occlusion of the rat MCA. ET-3 was equipotent with ET-1 in this respect, being effective over the same dose range, producing a similar volume of ischaemic brain damage and possessing the same LDF temporal profile of ischaemia, and furthermore, it was demonstrated that ET-3-induced MCA occlusion could be effectively blocked by pre-treatment by the ET_A receptor antagonist FR139317. In these studies, we address whether ET-3-induced MCA occlusion can be manipulated by the specific ET_A receptor antagonist FR139317 to produce a model of MCA occlusion with controlled reperfsuion.

7.1.2. Apoptosis component in focal cerebral ischaemia

As has been addressed in preceeding sections, focal cerebral ischaemia that encompases a phase of reperfusion has a different pathophysiology to models of stroke in which arterial occlusion is permanent. A further characteristic of stroke models is the mechanism of cell death. As was mentioned in section 1.3.2., cells may die by two distinct processes, necrosis and apoptosis, and both these forms of cell

death have been identified following focal cerebral ischaemia. Identification of the contribution of apoptosis to stroke in which reperfusion may affect pathophysiological events may have important consequences for the treatment of stroke pateints. For example, cells within the region of salvage, the penumbra, have been shown to be effectively salvaged by agents such as MK801, but if cells were to die by apoptosis-like mechanisms it is possible that intervention could be modelled on our growing understanding of what controls this cell death.

Linnik *et al.* (1993) first identified evidence of programmed cell death (apoptosis) in a model of focal cerebral ischaemia using the intraluminal filament technique. Furthermore a number of recent studies support these findings. Li *et al.* (1995d) produced MCA occlusion using an intraluminal filament in Wistar rats. A two hour occlusion was followed by 22 hours of reperfusion prior to histological examination, and identification of apoptotic cells by 3'-OH DNA end labelling. They reported apoptotic cells throughout the MCA territory although the majority were localised to the inner boundary zone of infarcted tissue (216±43 per section), which suggests apoptosis contributes to infarct development after focal cerebral ischaemia with reperfusion. Whilst a number of studies have provided evidence for apoptosis in models of focal cerebral ischaemia with reperfusion (Charriaut-Marlangue *et al.* 1995; 1996; Li *et al.* 1995a; 1995b; 1995c), the number of studies comparing to permanent MCA occlusion are limited. Linnik *et al.* (1995) addressed whether apoptotic cells contribute to the infarct after permanent focal cerebral ischaemia, whereby gel electrophoresis of cortical DNA 24 hours post permanent MCA

occlusion revealed characteristic DNA laddering in the "penumbral" region. Analysis further showed that DNA fragmentation occurred as soon as 1 hour post-occlusion.

Du *et al.* (1996) identified a model of stroke in which apoptosis is the dominant mechanism by which cells may die. Using an intraluminal filament model of MCA occlusion, they demonstrated that a 30 minute MCA occlusion did not produce a large ischaemic lesion when assessed three days following occlusion. However, they demonstrated that if the survival period was extended to 14 days, then a large volume of ischaemic cell death was found which was not significantly different to that produced by a 90 minute MCA occlusion. They concluded that this "very delayed cell death" is due to apoptosis of cells injured by the brief ischaemic challenge that consequently triggered the process of programmed cell death. One aspect of the ET model of MCA occlusion addressed using a model of controlled reperfusion was whether there was a significant component of very delayed ischaemic cell death.

7.1.3. Experimental approach

In order to determine if ET-3-induced MCA occlusion could be interupted by an ET receptor antagonist the following experimental approaches were taken:

1. FR139317 was delivered 10 & 90 minutes following ET-3 and the histological outcome was assessed.
2. LDF assessment of the temporal profile of FR139317 effects on ET-3-induced MCA occlusion, and comparison to the physiological antagonist sodium nitroprusside, as performed for ET-1 in chapter 5.

3. [^{14}C]iodoantipyrine assessment of FR139317-induced reversal of ET-3-induced MCA occlusion
4. Histopathological and immunocytochemical assessment of histopathological outcome following 30 minute occlusion addressing the component of very delayed (apoptotic) cell death.

7.2. METHODS

7.2.1. Histological studies

Surgery and histological techniques employed in the assessment of ischaemic brain damage for 10, 30 and 90 minute time points have been described previously in sections 2.2.1. & 2.2.2. with the modifications outlined in section 5.2.1. Following craniotomy, a 24 gauge dual cannula was lowered 8.7mm below skull. ET-3 (100 pmols in 2μl) was injected (1μl/90 sec) via the inner (31 gauge) cannula. A period of 10, 30 or 90 minutes elapsed before delivery of FR139317 (3 nmols in 3μl) at the same rate. The cannula was left in situ for 5 minutes following the final injection before being slowly withdrawn. Following suturing of the scalp wound, animals were placed in an incubator prior to being returned to cages. Following 3 or 14 day recovery, animals were transcardially perfused, the brain removed and stored, and the volume of ischaemic brain damage quantified as previously described.

7.2.2. Immunocytohistochemistry

Representative sections from standard ET-1 lesioned brains, as well as brains from 30 minute ET-3-induced MCA occlusion animals fixed after 3 or 14 day recovery,

were processed for immunocytochemistry to determine the number of apoptotic cells within the respective ischaemic lesions. The procedure used the ApopTag® (Oncor) *in situ* apoptosis detection kit. The method uses the enzyme deoxynucleotidyl transferase (TdT) to catalytically add digoxigenin-nucleotide residues to the 3'-OH ends of double or single stranded DNA that are generated during endonucleotide (apoptotic) DNA fragmentation. An anti-digoxigenin Ab is then added that carries a conjugated peroxidase enzyme, which binds the digoxigenin-nucleotide and generates a signal (brown colour) from a chromogenic substrate (diaminobenzidene), which is clearly distinguishable from background (methyl green).

Since DNA nick-end labelling has been detected in necrotic cells (Collins et al. (1992; Columbano), identification of apoptotic cells was supported by morphological identification of apoptosis based on the light-microscopy criteria described by Wyllie et al. (1980). These criteria include chromatin aggregation, cytoplasmic condensation and rounding of the cell.

Sections were processed according to the manufacturers instructions. Briefly, perfused brains were stored for 12-24 hours in 4% paraformaldehyde in PBS, and then frozen and sectioned (20μm) on a cryostat. Sections were then collected on glass microscope slides, and processed according to the protocol in Appendix A. The apoptotic component in these three models of ET-induced MCA occlusion was determined by counting the numbers of apoptotic cells within the both ischaemic and contralteral hemispheres at the sterotaxic level of bregma.

Staining of histologically prepared sections was performed by Mr. G. Carlsson of the Fujisawa Institue of Neuroscience, University of Edinburgh.

7.2.3. LDF studies

LDF studies were performed as previously described in section 6.2.2., and animals were implanted with an indwelling cannula as described in section 3.2.1. Following a 5-10 day recovery period, animals were re-anaesthetised and a catheter inserted into the right femoral artery for blood pressure monitoring (Transonic systems Inc.) and frequent blood gas and plasma glucose analysis (Ciba Corning model 238 and Beckman Glucose analyser 2). A tracheotomy was then performed and the animal artificially ventilated (Harvard Instruments Rodent ventilator model 2).

The animal was placed in a stereotaxic frame. A craniotomy was performed over the parietal cortex and LDF probes (24 gauge) advanced by a micromanipulator to rest on the surface of the intact dura overlying the parietal cortex. Recordings were made for 15-30 minutes prior to the insertion of a 33 gauge needle into the lumen of the indwelling cannula to deliver ET-3 (100 pmols in 2μl). Ten minutes following ET-3 injection, FR139317, SNP (3nmols in 3μl) or vehicle was injected. Recordings were then made for 90 minutes. At the end of the recording period animals were killed by anaesthetic overdose (5% halothane), a zero flow recording taken which was later subtracted form LDF signal values, and the brain inspected under a microscope. Arterial blood gases (pCO_2 and pO_2), blood pH and plasma glucose concentration were determined for animals in the LDF study. Physiological variables were measured before and 15-30 minutes after ET-3 injection, and then subsequently one hour post FR139317/SNP.

7.2.4. [^{14}C]iodoantipyrine studies

[^{14}C]iodoantipyrine autoradiography was employed to assess local CBF one hour after FR139317 injection, 10 minutes following ET-3-induced MCA occlusion. Animals were prepared with an indwelling cannula 5-10 days before blood flow studies as previously described in section 3.2.1. The protocol for [^{14}C]iodoantipyrine autoradiography followed that described in section 3.2.4. with some modifications. On the day of study, rats were anaeathetised with halothane (5%) in a nitrous oxide:oxygen mixture (80/20% v/v). Both femoral arteries and veins were catheterised, the incision sites infiltrated with anaethetic gel (xylocaine 2%), and the wound sutured closed. A plaster of Paris cast was then applied, body temperature maintained by means of a rectal probe and heating lamp, and a blood pressure monitor was connected. Anaethesia was discontinued and the animal allowed to recover for at least 2 hours prior to further experimentation. Injection of drugs was performed following brief re-anaesthetisation of the animal. ET-3 (100 pmols in 2μl) was injected via a 31 gauge injection cannula inserted into the lumen of the indwelling cannula. Ten minutes following ET-3 injection, FR139317 (3nmols in 3μl) or vehicle (3μl of saline) was injected. The cannula was then withdrawn and anaesthesia discontinued. Samples of arterial blood were taken for blood gas, blood pH, and plasma glucose determination immediately before the animal was re-anaesthetised for injections, following discontinuation of anaesthesia, and then once again immediately prior to infusion of [^{14}C]iodoantipyrine. One hour following FR139317/vehicle injection, [^{14}C]iodoantipyrine autoradiography was performed according to the protocol described previously in section 3.2.4.

[^{14}C]iodoantipyrine studies were performed with the assistance of Dr. J Sharkey of the Fujisawa Institue of Neuroscience, Univeristy of Edinburgh.

7.2.5. Statistical analysis

All data are presented as mean ± s.e.mean. Analysis of data from 10 minute and 90 minute MCA occlusion were compared to vehicle injected control by ANOVA on ranks with post hoc Dunn's method. Data from the comparison of 30 minute group were compared by Mann-Whitney Rank sum test. LDF data were analysed by Two-way ANOVA with post hoc Tukey test. Data from local CBF studies were compared by Student's T test.

7.3. RESULTS

7.3.1. Histological studies

Intracerebral injection of FR139317 (3 nmols) 10 minutes following ET-3 (n=10), significantly reduced the volume of hemispheric ischaemic brain damage to 12±3mm^3, compared to 132±27mm^3 in the vehicle post treatment group (n=10) (P<0.05) (Figure 34). By contrast, the volume of hemispheric ischaemic damage in the group receiving FR139317 (3 nmols), 90 minutes following ET-3 (n=8), was 103±23mm^3 which was not signifcantly different to that seen in the control ET-3 group, but was significantly larger than in the 10 minute group (P=<0.05).

The total volume of hemispheric ischaemic damage in rats undergoing 30 minute ET-3-induced MCA occlusion was not significantly different between animals fixed after 3 day (n=10) or 14 day (n=9) recovery (P=0.903) (figure 35).

Effects of intracerebral FR139317, 10 and 90 minutes post ET-3-induced MCA occlusion

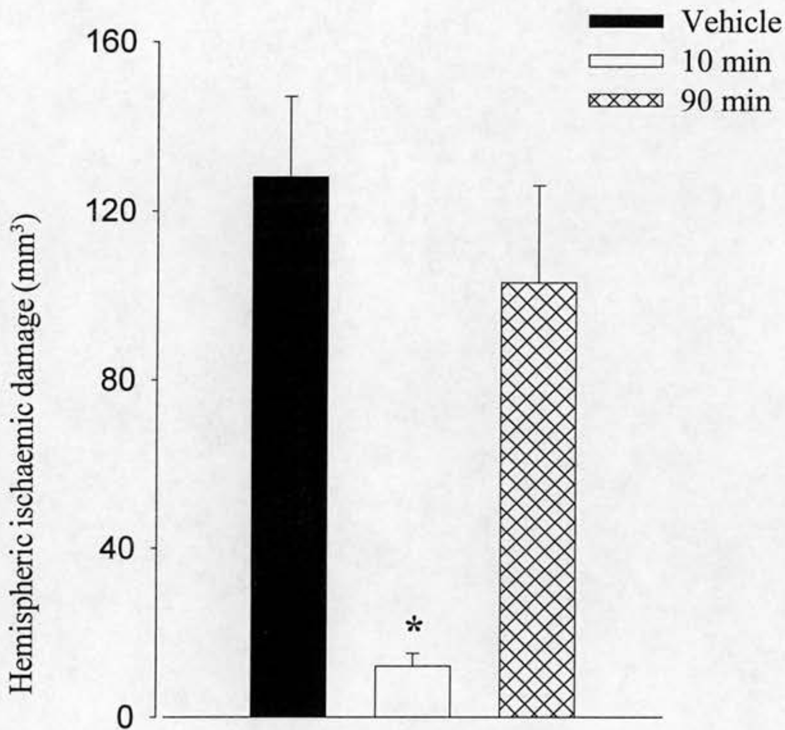


Figure 34. Effects of intracerebral FR139317 (3 nmols), injected 10 (n=9) or 90 (n=8) minutes after ET-3 (100 pmols) compared to standard ET-3-induced MCA occlusion. Data are presented as mean ± s.e.mean. Whilst FR139317 blocked ET-3-induced MCA occlusion when injected 10 minutes after ET-3, injection 90 minutes after ET-3 resulted in a volume of ischaemic damage not significantly different to a standard ET-3 infarct. *P=<0.05 compared to other groups.

Outcome following 30 minute
ET-3-induced MCA occlusion

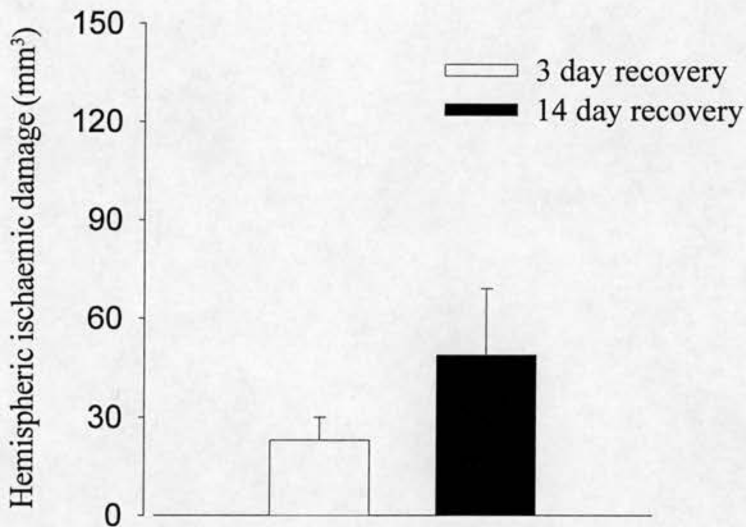


Figure 35. Histopathological outcome following 30 minute ET-3-induced MCA occlusion. FR139317 (3nmols) was injected 30 minutes after ET-3 (100 pmols), and animals were fixed after 3 (n= 10) or 14 (n=9) day recovery. Data are presented as mean ± s.e.mean. No significant differences were found between treatments.

7.3.2. Immunocytohistochemical identification of apoptosis

The infarct volume between brains processed for immunocytohistochemistry was kept within a narrow range (30-80mm^3). The number of apoptotic cells found in the contralateral hemisphere was <3 per brain between treatment groups. The number of apoptotic cells within the ischaemic hemisphere of ET-1-induced MCA occlusion rats was 50 ± 4. This was not significantly different from the number found in sections from rats that underwent 30 minute ET-3-induced MCA occlusion fixed after 3 days (49 ± 9) or 14 days (47 ± 8). Examples of apoptotic cells are shown in Figure 36.

7.3.3. LDF studies

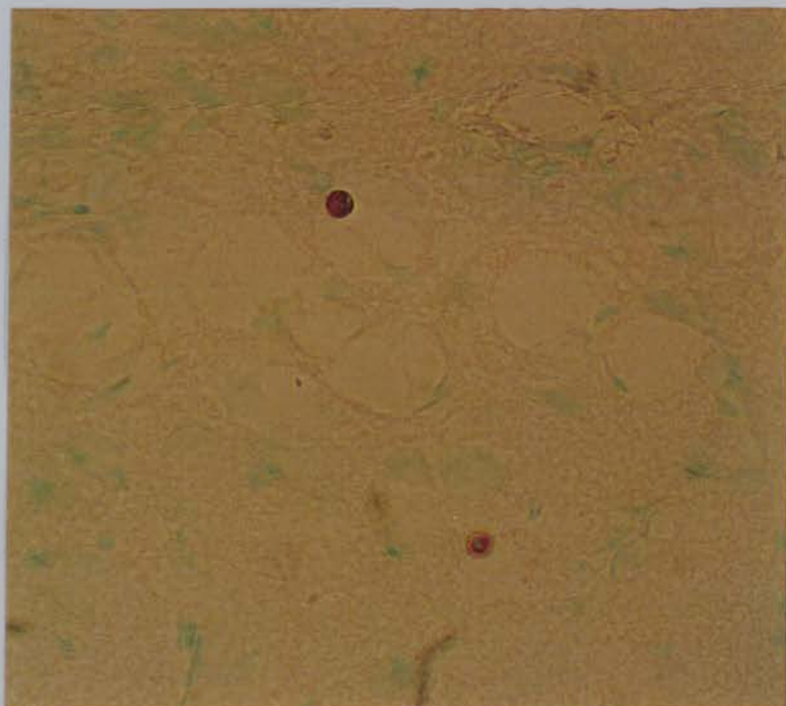
During LDF studies, physiological parameters for FR139317 and SNP effects on ET-3-induced MCA occlusion were kept within normal limits, and no significant differences were found between groups for any parameter at any time point (Tables 10 and 11 respectively).

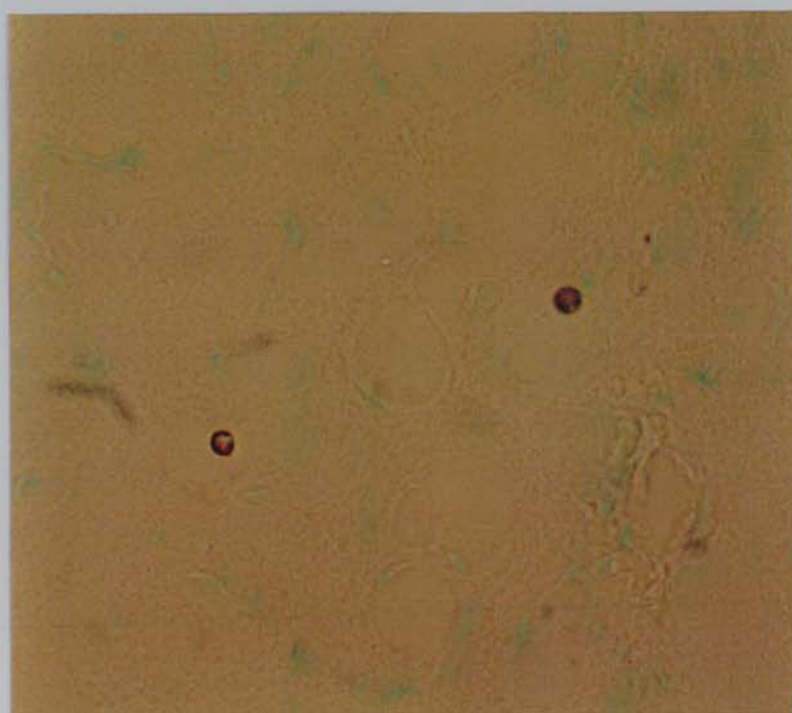
ET-3 injection resulted in a profound reduction in LDF signal relative to baseline in all studies. This reduction in FR139317 post-treated rats was 60 ± 5%, and 65 ± 5% in control animals, and was not significantly different between treatments. FR139317 completely reversed the effects of ET-3 within five minutes. The signal did not significantly change compared to baseline for the duration of the remaining recordings (Figure 37). By contrast, LDF in vehicle treated animals showed no recovery throughout recordings.

Figure 36.

High-power light microscope pictures of apoptotic cells from rats perfusion fixed three days following 30 minute ET-3-induced MCA occlusion. Both pictures illustrate profound immunohistochemical staining of individual cells that have undergone endonuclease-induced cleavage to form 3'-OH DNA end breaks, stained by ApopTag®. Morphological signs of apoptosis are also identifiable as chromatin marginalisation & clumping, and cell rounding. Magnification = x 400

TUNEL-positive staining of apoptotic cells
within ischaemic cortex





Physiological variables for LDF determination of effects of FR139317 on ET-3-induced MCA occlusion

Variable	ET-3 + vehicle			ET-3 + FR139317		
	Pre ET-3	**Post ET-3**	**Post 1 hr**	**Pre ET-3**	**Post ET-3**	**Post 1 hr**
M.A.B.P. (mmHg)	81±1	82±1	82±1	83±1	82±1	83±1
Rectal temperature (°C)	37.4±0.05	37.5±0.05	37.5±0.02	37.4±0.1	37.3±0.1	37.3±0.05
pH (units)	7.46±0.01	7.48±0.01	7.45±0.02	7.46±0.01	7.46±0.03	7.42±0.06
pCO_2 (mmHg)	39±1	38±1	38±1	39±1	36±2	37±1
pO_2 (mmHg)	100±5	104±2	107±7	102±3	108±8	105±6
plasma glucose (mM)	8.6±0.7	8.8±0.8	9.8±0.8	8.2±0.6	8.1±0.7	8.6±0.4

Table 10. Physiological parameters for LDF profile of the effects of FR139317 (3 nmols; n=4) or vehicle (n=5) injection 10 minutes after ET-3 (100 pmols). Data are shown as mean ± s.e.mean. No significant differences in physiological parameters were found between groups at any time point.

Physiological variables for LDF determination of effects of SNP on ET-3-induced MCA occlusion

Variable	**ET-3 + vehicle**			**ET-3 + SNP**		
	Pre ET-3	**Post ET-3**	**Post 1 hr**	**Pre ET-3**	**Post ET-3**	**Post 1 hr**
M.A.B.P. (mmHg)	82±2	81±1	81±1	81±1	82±1	81±1
Rectal temperature (°C)	37.5±0.1	37.4±0.1	37.5±0.1	37.4±0.1	37.4±0.05	37.4±0.1
pH (units)	7.48±0.02	7.47±0.02	7.46±0.01	7.48±0.01	7.46±0.01	7.45±0.01
pCO_2 (mmHg)	39±1	39±1	39±2	38±1	38±1	38±1
pO_2 (mmHg)	102±15	99±9	99±9	100±2	100±1	101±5
plasma glucose (mM)	8.3±0.7	7.7±0.6	8.4±0.2	9.0±0.2	9.2±0.2	9.4±0.1

Table 11. Physiological parameters for LDF profile of the effects of SNP (3 nmols; n=4) or vehicle (n=5) injection 10 minutes after ET-3 (100 pmols). Data are shown as mean ± s.e.mean. No significant differences in physiological parameters were found between groups at any time point.

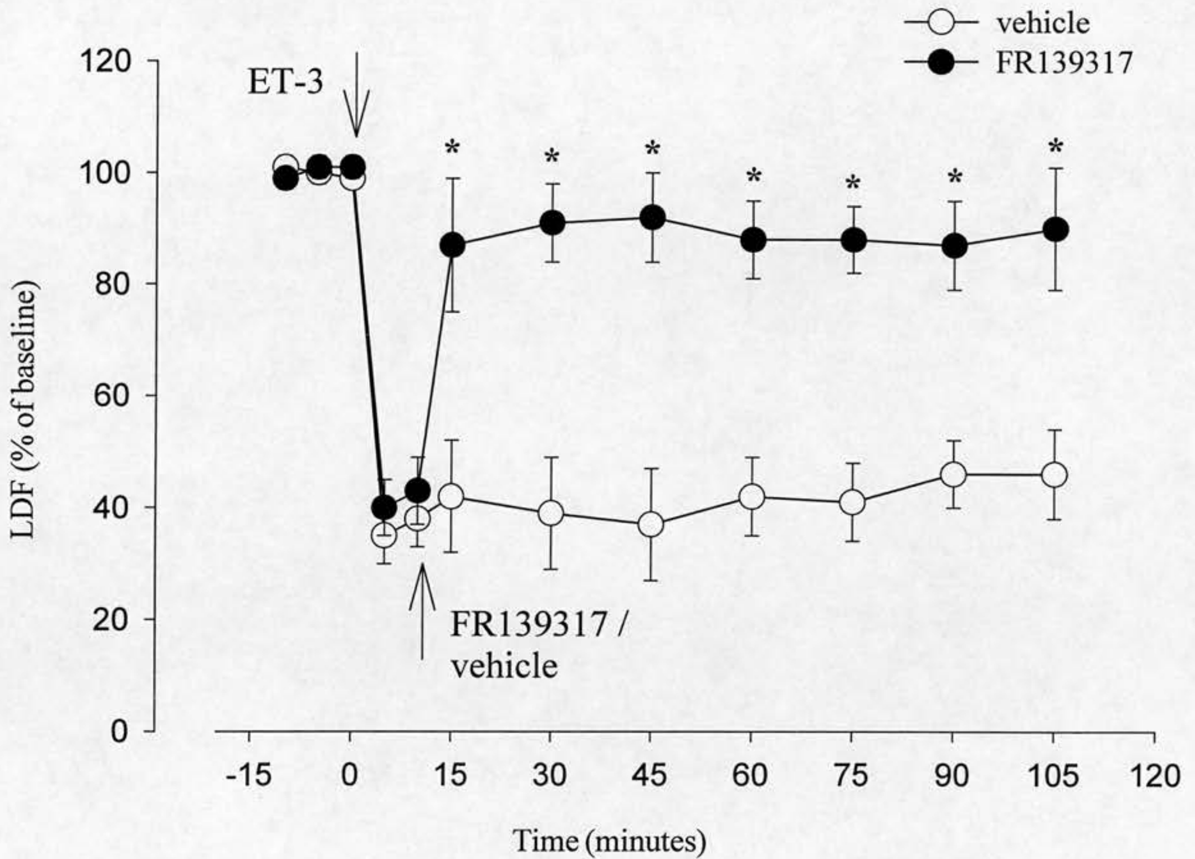


Figure 37. LDF recordings of the effects of FR139317 (3nmols; n=4) and vehicle (n=5) on ET-3-induced MCA occlusion. Data are presented as mean ± s.e.mean. LDF signal fell to ~40% of baseline following ET-3 (100 pmols). LDF recovered to ~85% within 5 minutes of FR139317 injection and fully recovered after 20 minutes, remaining stable for the duration of the remaining recordings. By contrast, LDF in vehicle controls showed no significant signal recovery during the recordings. *P=<0.05 compared to vehicle controls.

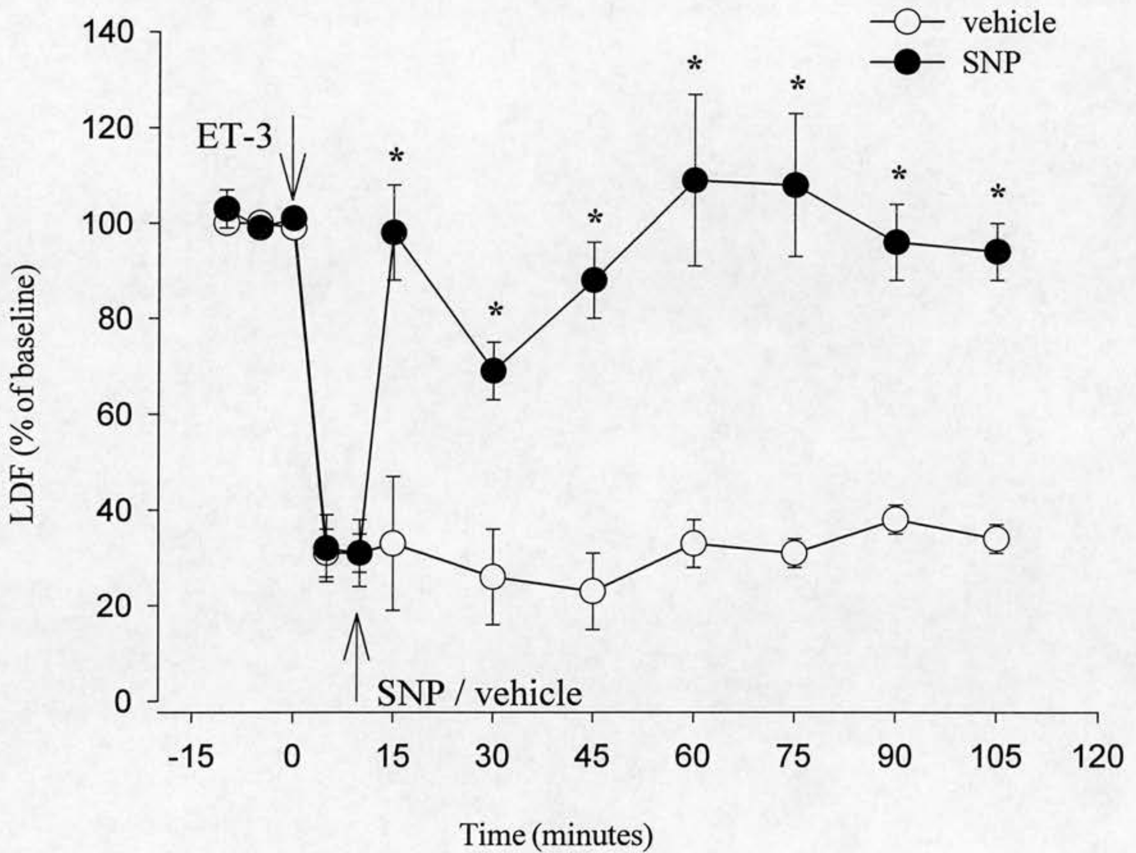


Figure 38. LDF recordings of the effects of SNP (3nmols; n=4) or vehicle (n=5) on ET-3-induced MCA oclusion. Data are presented as mean ± s.e.mean. LDF signal fell to ~30% of baseline following ET-3 (100 pmols). LDF recovered to ~100% (baseline) within 5 minutes of SNP injection, although a secondary, non-significant LDF drop 20-35min after SNP occcured before remaining stable for the duration of the remaining recording period. By contrast, LDF in vehicle controls showed no significant signal recovery during the recordings. *P=<0.05 compared to vehicle controls.

In SNP post-treated rats, ET-3 injection resulted in a reduction of 68 ± 7% in the LDF signal, compared to 69 ± 5% in vehicle post-treated animals, which was not significantly different between treatments. SNP rapidly reversed the ET-3-induced LDF signal reduction within 5 minutes. By contrast to FR139317, the signal following SNP injection showed a secondary reduction which later recovered to baseline (Figure 38). Microscopic inspection of the vascular territory of brains showed no evidence of tissue palor within the MCA territory for either group.

7.3.4. [^{14}C]iodoantipyrine autoradiography studies

Physiological parameters taken immediately prior to [^{14}C]iodoantipyrine administration were within normal limits and showed no significant differences between groups for any parameter (Table 12). Analysis of local CBF as measured by [^{14}C]-iodoantipyrine autoradiography determined that local CBF was significantly reduced in 10 of 29 brain regions compared to contralateral hemisphere in animals treated with ET-3 plus vehicle 10 minutes post ET-3. By contrast, in animals treated with FR139317 10 minutes following ET-3, ipsilateral local CBF was not significantly different to the contralateral hemisphere, or the contralteral hemisphere of control (ET-3) treated animals in any brain region studied (Table 13). The reductions in local CBF in ET-3 + vehicle and ET-3 + FR139317 are illustrated in the pseudocolour transform autoradiograms in Figure 39 which contrast control ET-3-induced MCA occlusion sections at two stereotaxic levels to FR139317 post-treated animals in which local CBF is essentially normal within the ipsilateral hemisphere.

Physiological variables for [^{14}C]iodoantipyrine determination of effects of FR139317 on ET-3-induced MCA occlusion

Variable	ET-3 + vehicle			ET-3 + FR139317		
	Pre ET-1	Post ET-1	Post 1 hr	Pre ET-1	Post ET-1	Post 1 hr
M.A.B.P. (mmHg)	120 ± 2	120 ± 2	122 ± 5	119 ± 1	119 ± 2	124 ± 3
rectal temperature (°C)	37.5 ± 0.1	37.6 ± 0.1	37.7 ± 0.2	37.4 ± 0.1	37.4 ± 0.1	37.4 ± 0.1
pH (units)	7.42 ± 0.01	7.42 ± 0.02	7.43 ± 0.2	7.46 ± 0.01	7.46 ± 0.02	7.44 ± 0.01
pCO_2 (mmHg)	38 ± 1	40 ± 1	39 ± 2	37 ± 3	39 ± 4	36 ± 3
pO_2 (mmHg)	110 ± 12	97 ± 10	88 ± 4	112 ± 10	107 ± 14	108 ± 13
plasma glucose (mM)	9.4 ± 1.2	9.4 ± 0.9	8.9 ± 1.0	8.0 ± 0.2	7.9 ± 0.3	7.9 ± 0.5

Table 12. Physiological parameters for [^{14}C]iodoantipyrine determination of local CBF 1 hour following injection of vehicle (n=5) or FR138317 (n=5) 10 minutes after ET-3-induced MCA occlusion. Data are shown as mean ± s.e.mean. Physiological variables were kept within normal limits and no significant differences were found between groups at each time point, or between groups for all data for a given variable.

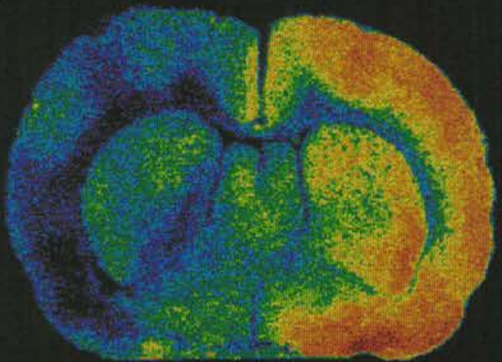
Local cerebral blood flow FR139317 reversal of ET-3-induced MCA occlusion

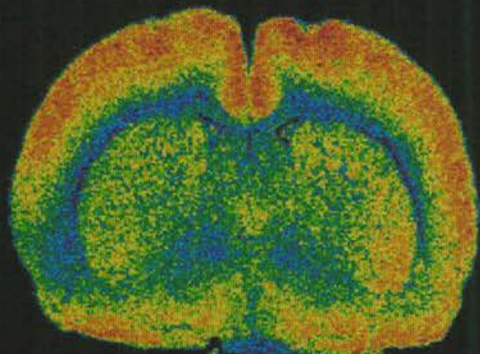
	ET-3 MCA occlusion		ET-3 + FR139317 reverse after 10 minutes	
Brain region	Blood flow ipsilateral	Blood flow contralateral	Blood flow ipsilateral	Blood flow contralateral
Superficial cerebral structures				
Medial prefrontal cortex	173±11	196±13	193±15	191±17
Frontal cortex area 8	37±10*	217±17	212±25	210±24
Frontal cortex area 10	25±7*	228±14	175±26	191±24
Piriform cortex	45±12*	235±18	219±21	226±18
Somatosensory cortex	45±11*	249±4	213±22	215±19
Cingulate cortex	234±13	236±10	235±26	233±27
Parietal cortex	42±12*	243±12	235±19	225±20
Temporal cortex	36±12*	208±17	240±28	225±19
Retrosplenial cortex	225±14	232±16	216±23	219±23
Entorhinal cortex	43±12*	130±17	153±22	148±21
Primary visual cortex	52±12*	193±16	224±31	230±26
Deep cerebral structures				
Genu of corpus callosum	46±5	48±4	48±7	47±7
Nucleus accumbens	89±20	107±20	108±14	112±13
Caudate-putamen (dorso-lateral)	58±10*	131±7	121±9	120±10
Caudate-putamen (medio-lateral)	68±10*	151±15	148±11	149±15
Septal nucleus	108±10	110±11	101±15	98±13
Anterior-medial thalamic nucleus	222±14	230±15	231±21	227±22
Ventrothalamic nucleus	151±18	175±24	155±20	159±21
Subthalamic nucleus	137±10	137±11	139±14	136±17
Globus pallidus	97±12	105±16	104±16	103±14
Hippocampal CA1	88±8	88±9	87±13	87±12
Dentate gyrus	124±10	129±10	122±11	121±9
Internal capsule	75±4	79±3	88±8	84±8
Lateral geniculate nucleus	162±12	162±10	155±10	159±11
Substantia nigra	133±13	118±7	132±21	122±16
Cerebellum & pons				
Pontine nucleus	98±6	101±7	120±11	119±12
Cerebellar white matter	40±4	42±4	42±2	41±2
Cerebellar grey (minimum)	108±3	107±3	116±9	115±10
Cerebellar grey (maximunm)	237±17	234±17	262±25	263±22

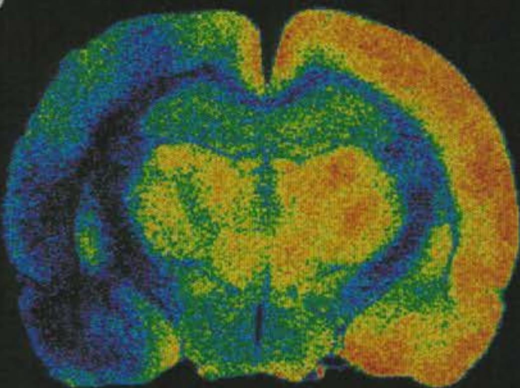
Table 13. Local cerebral blood flow (ml/100g/min) in 29 anatomically defined regions of the brain, measured 1 hour after ET-3-induced MCA occlusion or FR139317-reversal of ET-3-induced MCA occlusion. Data are presented as mean ± s.e.mean for control ET-3 (n=5) or FR139317 treated (n=5) injection. One-way ANOVA *P=<0.05.

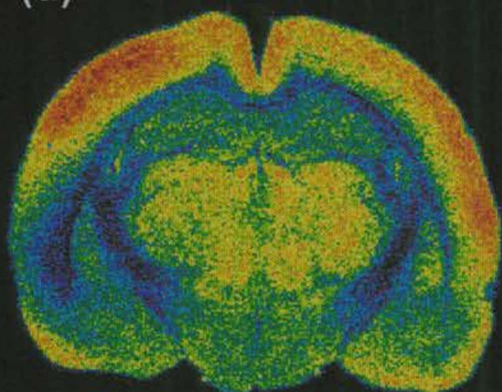
Figure 39.

Local CBF determined by [^{14}C]iodoantipyrine autoradiography one hour after co-intracerebral injection of FR139317 (3 nmols), or vehicle 10 minutes following ET-3-induced MCA occlusion. Pseudocolour transforms represent local CBF at the level of bregma in (a) ET-3 + vehilce & (b) ET-3 + FR139317 at the same level, and local CBF at the level of the hippocampus (Bregma -4.0mm) in (c) ET-3 + vehicle & (d) ET-3 + FR139317 injected animals (d). Note the profound reductions in local CBF throughout the MCA vascular territory in animals subjected to ET-3-induced MCA, whilst ipsilateral local CBF is essentially the same as the contralateral hemisphere in FR139317 post-injected animals.


(a)


(b)


(c)


(d)
ml/100g/
min
4.5
14.8
26.2
39.1
53.8
71.0
91.6
116.9
149.7
195.0
267.0
425.7

7.4. Discussion

7.4.1. 90minute vs 10 minute MCA occlusion

These data demonstrate that ET-3-induced MCA occlusion is a suitable substrate as a model of focal cerebral ischaemia with controlled reperfusion. Histopathological analysis of ischaemic brain damage, determined that injection of FR139317 (3nmols) 10 minutes after ET-3 injection interupts MCA occlusion sufficiently rapidly to effectively block any ischaemic damage. By contrast, the volume of ischaemic brain damage was not significantly different between the control ET-3 lesion and animals receiving the antagonist 90 minutes following ET-3 injection. This confirms that the cannula system only delivers the drugs at the appropriate time, rather than the antagonist diffusing out of the cannula at an earlier time point. These findings suggest that FR139317 is an effective agent with which to interupt ET-3-induced MCA occlusion. Furthermore, the duration of MCA occlusion required to produce a lesion of maximal size with ET-3 is at least 90 minutes. Indeed, the volume of ischaemic damage following 90 minute ET-3 MCA occlusion is similar to that seen following permanent MCA occlusion by electrocoagulation in the same strain in previous studies (Park *et al.* 1988; Morikawa *et al.* 1991), although studies have suggested a 3 hour MCA occlusion is required to produce maximal damage (Kaplan *et al.* 1994).

Since the volume of damage was not significantly greater in the 90 minute group, it would seem that FR139317 controlled interuption of ET-3-induced MCA occlusion does not lead to a reperfusion-induced exacerbation of damage as has been previously suggested (McAuley, 1995).

7.4.2. 30 minute MCA occlusion: Apoptosis implications

The contribution of apoptosis to cell death is currently a central topic in cerebral ischaemia research. Recently, Du *et al.* (1996) described a model of MCA occlusion in which the predominant mechanism of cell death bears many signs of apoptosis. They demonstrated that whilst a 30 minute MCA occlusion did not produce an ischaemic lesion when histopathology was determined three days following occlusion, when analysed 14 days post 30 minute occlusion, the volume of damage was the same as that seen in animals subjected to a 90 minute MCA occlusion.

Whilst evidence for the involvement of apoptosis in cell death following cerebral ischaemia cells is increasing, a number of issues remain to be resolved. Specifically, the cause of the extended time-frame for the appearance of apoptotic cells post insult, and the relative apoptotic contributions in different MCA occlusion models. It has been suggested that apoptosis may still occur rapidly, but the commitment phase may be greatly extended in the brain, whereby the final initiation of the cell to undergo apoptosis is variable (Li *et al.* 1995d).

The present study determined that numbers of apoptotic cells were not significantly different between brains from rats subjected to a standard ET-1-induced MCA occlusion, and 30 minute ET-3-induced MCA occlusion, fixed at either 3 or 14 days. Furthermore, the numbers of cells reported in the present study for all MCA occlusion groups were less than have been reported previously in models of transient MCA occlusion that employed the intraluminal filament approach (Li *et al.* 1995a; 1995d) in which ~200 or more cells were identified at a single coronal section after 2

hour MCA occlusion. The technique for labelling apoptotic cells in the present study, has previously been verified as highly specific for identifying apoptosis in ischaemic brain (Li *et al.* 1995a; 1995d). The procedure is based on immuno-labelling 3′-OH DNA strand breaks that are formed by endonucleolytic cleavage of DNA into multiple nucleosomal units of ~180 base pairs, which is a feature of apoptosis, and can be visualised by gel electrophoresis (Linnik *et al.* 1995). Since such DNA strand breaks have been reported previously following necrotic cell death (Collins *et al.* 1992; Li *et al.* 1995c), labelled cells were also examined for morphological features of apoptosis such as chromatin marginalisation and clumping.

The present data suggest that both the ET-1 model and the ET-3 reperfusion model may be associated with a differential apoptotic component to the intrluminal filament models, and conflict with the findings of Du *et al.* (1996) in which a large lesion developed between 3 and 14 days, in which cell death had features of apoptosis. This suggests that the contribution of very delayed (apoptotic) cell death is not significant following 30 minute ET-3-induced MCA occlusion. If we assume experimental conditions to be essentially the same between studies the main difference between these models is the method by which MCA occlusion is produced. The model of Du *et al.* (1996) employs the intraluminal filament technique. Consequently the large component of apoptosis in that study may be a consequence of this approach. Evidence suggests that inflammation may directly lead to apoptosis. Chopp *et al.* (1996) demonstrated that antibodies against ICAM-1 and the β2 integrin Mac-1, not only reduced leukocyte infiltration, but reduced the number of cells undergoing apoptosis in the ischaemic hemisphere. Leukocytes have

also been shown to induce apoptosis directly in cells (Squier *et al.* 1995), whilst the neutrophil is a source of oxygen free radicals which have been implicated in apoptosis (Hockenbery *et al.* 1993). These data therefore suggest that inflammatory cell infilatration may be minimal in the ET MCA occlusion models, and the potentially artifactual contribution to inflammation from cerebral vessel damage in intraluminal filament models should be addressed in detail. Further studies to identify the extent of inflammation following ET-induced MCA occlusion may clarify these discrepancies.

7.4.3. Temporal profile of reperfusion

LDF recordings determined that FR139317 rapidly reverses ET-3 induced MCA occlusion within 5 minutes. This profile is similar to that seen following clip release of the MCA (Soriano *et al.* 1997). Furthermore, perfusion returned to pre-occlusion levels without a significant hyperperfusion after FR139317, which contrasts some reperfusion models in which hyperperfusion occurs (Shigeno *et al.* 1985; Dirnagl *et al.* 1989). However, the findings after 10 minute occlusion do not necessarily extend to what would occur at later (e.g. the 90 minute reperfusion group) time points, where pathophysiological changes within the brain tissue might alter this profile. Reperfusion using SNP was employed to compare to reversal of ET-3-induced MCA occlusion by FR139317. In these studies, SNP rapidly reversed the effects of ET-3, although there was a period of hypoperfusion 15-45 minutes after addition. This may be a consequence of the continued presence of ET-3 within the tissue, whilst SNP rapidly decomposes to the short lived (~4 sec) NO molecule. Thus it is conceivable

that the effects of SNP rapidly wear off, and consequently some vasoconstriction returns as ET-3 is less rapidly metabolised. Reversal of ET-1-induced MCA occlusion by SNP did not demonstrate such a hypoperfusion phase, although the reasons for this are unclear. It is possible that ET-1 causes more rapid receptor tachyphylaxis or internalisation preventing the rebound hypoperfusion seen with ET-3.

7.4.4. Local CBF determination of reperfusion

[^{14}C]iodoantipyrine autoradiography was employed to determine local CBF in 29 brain regions 1 hour following ET-3-induced MCA occlusion, with or without FR139317 co-intracerebral injection. Local CBF within the septum, pons and white matter tracts was similar to that reported previously following permanent occlusion of the rat MCA (Tamura *et al.* 1993) and was not effected in these studies. All cortical structures, with the exception of the midline retrosplenial and cingulate cortices, ipslateral to the ET-3 + vehicle injections exhibited profound reductions in CBF. This was greatest within areas 8 & 10 of the frontal cortex, as well in the piriform and temporal cortex, where local CBF was <40 ml/100g/min. The relative reduction of cortical CBF varied between 89% in frontal cortex area 10, to 67% in the entorhinal cortex, which is only partially fed by the MCA (Tamura *et al.* 1981b).

The reduction in local CBF within the lateral caudate nucleus (55%) was less profound, with local CBF levels of 58-68ml/100g/min 1 hour post ET-3. This contrasts local CBF levels 10 minutes (Sharkey *et al.* 1993) and 30 minutes (Tamura *et al.* 1981b) following MCA occlusion, in which CBF was <30ml/100g/min, but is

lower than following 2 hours of ET-1-induced MCA occlusion by topical application (Gartshore *et al.* 1996). The explanation for higher CBF in this region at later time points in the ET-1 model has been addressed previously in chapter 3, and may be due to factors such as cerebrovascular anatomy, metabolic changes and deteriorating vasoconstrictor effects of ET-3 with time.

Ipsilateral local CBF within the thalamus, the substantia nigra and the globus pallidus were not significantly affected by ET-3-induced MCA occlusion. As has been addressed previously, there is considerable inter-study differences in the local CBF changes in these structures. Whilst local CBF in the substantia nigra and globus pallidus has been shown to increase following MCA occlusion (Tamura *et al.* 1981b; Sharkey *et al.* 1993), other groups have reported little change in these structures (Robinson *et al.* 1990) or decreases in local CBF (Gartshore *et al.* 1996). Such discrepancies may arise due to model & experimental differences, local CBF measurement time and the complexities of inter-structural neuronal and vascular innervation.

By contrast with vehicle treated rats, injection of FR139317 10 minutes following ET-3 produced a pattern of ipsilateral local CBF when assesed 60 minutes later, that was not significantly different to the contralateral hemispheres. All ipsilateral cortical structures and the caudate-putamen exhibited local CBF significantly greater than in animals injected with ET-3 followed by vehicle, and CBF was not significantly different to the contralateral hemisphere. This finding confirms previous histopathological and LDF findings that FR139317 effectively reverses ET-3-induced MCA. The protracted study time (1 hour after FR139317)

may in fact obscure any acute effects of FR139317 reversal such as hyperperfusion within the MCA territory. However, the LDF profile of reversal did not reveal any hyperperfusion within the parietal cortex, and so this may not be a feature that occurs with a short period of ischaemia. It would be interesting to examine whether reversal of ET-3-induced MCA occlusion at later time points such as 30 and 90 minutes included in the histopathological studies, led to hyperperfusion within or without the MCA vascular field.

7.4.5. Conclusions

A range of methodological approaches were utilised in these studies to characterise and evaluate the effectiveness of ET-3-induced MCA occlusion as a substrate for controlled reperfusion by the intracerebral co-injection of a specific ET_A receptor antagonist. The data demonstrate that unlike ET-1, ET-3-induced MCA occlusion is amenable to reversal by FR139317. It appears that the system can be used to produce MCA occlusion of varying duration. LDF suggests that the reversal of ET-3-induced MCA occlusion is rapid following FR139317 (within 5 minutes). Indeed the reversal followed the same time course as the physiological antagonist SNP which causes vasodilation by production of NO. These findings were supported by [^{14}C]iodoantipyrine autoradiographic mapping of local CBF, which determined that 1 hour following 10 minute FR139317-reversal of ET-3-induced MCA occlusion, local CBF is normal throughout both hemispheres, supporting the efficacy of FR139317 at interupting ET-3-induced MCA occlusion.

These data suggest that ET-3-induced MCA occlusion can be manipulated to produce a viable model of reversible focal cerebral ischaemia that does not involve physical damage to the major cerebral arteries. Furthermore, the finding of no rebound-hyperperfusion following reversal of occlusion, and the low-apoptotic component of this model suggests that this model may lack potentially artifactual components of pathophysiology associated with other models of focal cerebral ischaemia with reperfusion.

CHAPTER EIGHT

CHAPTER 8. SUMMARY AND DISCUSSION

A range of models of focal cerebral ischaemia are currently in use in stroke research, although models employing the subtemporal approach or intraluminal filament procedure, currently predominate in the literature. Whilst these models allow control over the duration of ischaemia, surgery is quite intricate, and inherently leads to damage to the vascular endothelium of cerebral arteries. Such damage has been implicated in exaggerating the inflammatory response which follows focal cerebral ischaemia, which may complicate interpretation of pathophysiological outcome. The present thesis, describes the characterisation and manipulation of an established model of MCA occlusion by intracerebral injection of ET-1, to produce a novel model of ET-induced MCA occlusion with controlled reperfusion.

ET-1, a 21-amino acid peptide with potent vasoconstrictor effects on cerebral arteries, was employed in the study by Robinson *et al.* (1990) to produce focal cerebral ischaemia by application to the surgically exposed MCA of the rat. Modification of this approach to inject ET-1 into the brain parenchyma adjacent to the MCA by Sharkey *et al.* (1993) led to a model that required only relatively simple, less invasive, surgery, and avoided the damage to cerebral arteries associated with the physical approach of most other stroke models. Whilst this model has subsequently been applied to neuroprotection and behavioural studies (Sharkey & Butcher 1994; Sharkey *et al.* 1995; Marston *et al.* 1995), features of this model were still unknown. Specifically, whether the ischaemic insult reflects transient or permanent MCA occlusion, and whether ET-1-induced MCA occlusion could be interupted to control reperfusion.

The optimal dose and injection volume range appear to be quite broad. Indeed, within a 10 fold dose range from 33 pmols to 300 pmols, and within a 1-3μl injection volume, a large ischaemic lesion, restricted to the vascular territory of the MCA was produced. As previously reported, the volume and extent of the infarct is comparable to that following permanent occlusion of the MCA. The ischaemic insult was characterised by measuring striatal oxygen levels, cortical tissue perfusion and local CBF. Oxygen tension recordings and [^{14}C]iodoantipyrine autoradiographical determination of local CBF suggest that the ischaemic insult is of at least three hours duration, with little evidence to suggest reperfusion occurs within a time-frame that might be of pathophysiological significance. Whilst LDF recordings during the characterisation of the ET-1 model suggested perfusion did gradually return, LDF recordings in subsequent studies from a different cortical position did not suggest any reperfusion occurs within three hours of ET-1 injection. These findings, combined with the size and distribution of the ischaemic lesion suggest that the pathophysiology of this model is representative of permanent MCA occlusion.

This model was applied to the neuroprotective and behavioural evaluation of a novel trophic peptide derived from the β-amyloid precursor protein. Intracerebroventricular infusion of the 17mer sequence was shown to confer potent neuroprotection to both the cortex and striatum following ET-1-induced MCA occlusion. By contrast to previous studies with this peptide, the 17mer peptide did not facilitate behavioural recovery in a skilled paw reach paradigm that has been shown to measure motor control disturbances following MCA occlusion.

The approach to manipulating the ET-1 model to allow reperfusion, was to inject the specific ET_A receptor antagonist FR139317 intracerebrally following ET-1. However, both histopathological and LDF assessment suggested that the effects of ET-1 are not reversed by FR139317. The isopeptide ET-3 was subsequently investigated since this peptide has been shown to have lower affinity and more reversible binding to the ET_A receptor. ET-3 was found to be equipotent in the ischaemic insult it produced, being effective over the same dose range and producing the same LDF profile as ET-1. This is the first demonstration that ET-3 is capable of producing ischaemic damage by constricting a major cerebral artery. These findings stimulated investigation of the characteristics of the receptor mediating ET-induced MCA occlusion. The isopeptide equipotency was predictive of an ET_B receptor rather than an ET_A subtype which is the ET receptor previously reported to predominate on the cerebrovasculature of the rat. However, intracerebral injection of the ET_B receptor agonists BQ3020, and IRL1620 did not produce the MCA occlusion-like pattern of ischaemic brain damage. Further to the receptor pharmacology, FR139317 pretreatment had no effect on ET-1-induced MCA occlusion but completely blocked ET-3-induced MCA occlusion. This suggests an atypical ET receptor may mediate MCA constriction in the rat.

By contrast to ET-1, ischaemic brain damage following ET-3-induced MCA occlusion was shown to be effectively blocked by injection of FR139317 10 minutes after ET-3. This was confirmed by LDF, which suggested that complete reperfusion occurs within 5 minutes with no hyperperfusion evident. [^{14}C]iodoantipyrine autoradiography confirmed that FR139317 was effective in reversing local CBF

changes throughout the ipsilateral hemisphere when injected after 10 minutes of ET-3-induced MCA occlusion. This model was subsequently employed to study the component very delayed (apoptotic) cell death. Whilst immunocytohistochemistry detected the presence of apoptotic cells, numbers were very limited and did not increase with the chronic maturation of the lesion, suggesting only a limited contribution of this form of cell death to the volume of ischaemic brain damage seen after a 30 minute ET-3-induced MCA occlusion.

The lack of an effective drug to limit the damage caused by stroke is a huge problem for medical research. This field is exhaustively researched, and our knowledge base is now considerable. That no effective treatment is established, may be a consequence of the complex pathophysiology of stroke, but is undoubtedly in part due to the experimental approaches employed. A lack of consistency in model design may prevent "overlooking" that may occur with a narrow range of models, but prevents cross-study comparison. Whether the first issue is more important than the second is unclear. However, it is likely that the continuing lack of efficacy of neuroprotectants at the clinical level is at least in part due to model design and execution. Guidelines or approved protocol for neuroprotectant study may help alleviate this, but undoubtedly pursuit of stroke models more accurately representing the clinical setting are required. The original model of stroke described within has limitations. The control over ischaemia and reperfusion is limited and penetration of brain parenchyma by a cannula is required. However, the model does have the advantage over some of the more popular models in avoiding microvascular injury, damage to structures outwith the MCA field and body & brain temperature

fluctuations. Furthermore, surgical intervention is limited by comparison to the transorbital approach. Adoption of the modifications described herein may provide a model which addresses the need for control over reperfusion, although control over the ischaemic insult itself remains limited. The present data with the reperfusion model suggest that filament and physical MCA occlusion techniques may introduce artifactual components into the maturation of an ischaemic lesion, that may not be present within the clinical setting. Clearly further studies are required to more completely address the aspects of reperfusion injury, inflammation and the contribution of apoptosis to lesion formation in both the ET models. Indeed whether this model represents a more accurate reflection of human stroke, or indeed a less accurate one, should be investigated.

Whilst characterisation of the ET-1 model is now quite extensive, the reperfusion model requires further study. Other ET receptor antagonists could be investigated to determine if FR139317 is the most suitable. A higher affinity, perhaps less selective, receptor antagonist may be capable of reversing ET-1-induced MCA, or reversing ET-3-induced MCA occlusion more rapidly. The rate of reperfusion at later time points such as 60 or 90 minutes could be addressed with LDF. Furthermore, the influx of leukocytes, and their cytotoxic effects should be addressed, as well as the neuroprotective efficacy of drugs that block inflammatory cell recruitment. In conclusion, the present thesis has described the characterisation of the established model of MCA occlusion by intracerebral ET-1 injection, and documented the development of a novel model of ET-3-induced MCA occlusion with controlled reperfusion.

APPENDIX A

PROTOCOL FOR IN SITU DETECTION OF APOPTOSIS

The procedure for labelling apoptotic cells following ET-induced MCA occlusion as described in Chapter 7, was performed according to the manufacturer's instructions for ApopTag® (Oncor).

All procedures were performed on sections from brains perfused in 4% formaldehyde in PBS, at room temperature unless otherwise specified. All washes were performed in coplin jars.

1. Sections added to coplin jar containing 2-3% hydrogen peroxide for 5 minutes, followed by two 5 min washes in PBS.
2. Excess removed and 75µl equilibration buffer added to each section, followed by 54µl of terminal deoxynucleotidyl transferase (TdT) in reaction buffer. Apply plastic cover slip and incubate in a humidified chamber at 37°C for 1 hour.
3. Remove coverslip, and add slide to coplin jar of stop/wash buffer for 10 minutes, followed by three 5 minute washes in PBS.
4. Apply 55µl of anti-digoxigenin-peroxidase to sections, and add cover slip and add to a humidified chamber at room temperature.
5. Wash in 4 changes of PBS for 5 minutes
6. Add 125µl of DAB substrate (chromogenic) and leave for 3 to 6 min.
7. Wash in 3 changes of distilled water for 1 minute each
8. Counterstain with methyl green in a coplin jar, then repeat step 7.

9. Wash slide in 100% butanol (~1 min), xylene (2 min) and mount under permanent coverslip with DPX.

Sections were subsequently examined under light microscopy for evidence of apoptosis, based on the immuno-labeling and morphological identification of features of apoptosis, as has been described in detail above.

APPENDIX B

SOLUTION FOR TRANSCARDIAC PERFUSION

Heparinised saline comprised 0.1M phosphate buffered saline (pH adjusted to 7.4) comprising di-sodium hydrogen orthophosphate and hydrogen di-sodium orthophosphate with heparin (10IU/ml).

Paraformaldehyde solution comprised 4% paraformaldehyde in 0.1M phosphate buffered saline (pH adjusted to 7.4) containing di-sodium hydrogen orthophosphate and hydrogen di-sodium orthophosphate.

Paraformaldehyde/sucrose storage solution comprised 4% paraformaldehyde in 0.1M phosphate buffered saline (pH adjusted to 7.4) containing di-sodium hydrogen orthophosphate and hydrogen di-sodium orthophosphate plus 20% sucrose.

COMPOSITION OF ARTIFICIAL CEREBROSPINAL FLUID

Composition of ACSF:

$NaCl$ = 148.2mM, $MgCl_2$ = 0.8mM, KCl = 3.0mM

$CaCl_2$ = 1.4mM, Na_2HPO_4 = 0.8mM NaH_2PO_4 = 0.2mM

pH adjusted to 7.4 at room temperature.

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