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Polyamines and polyamine antagonists in cerebral ischaemia

Jun Li, M.Sc

A thesis submitted in partial fulfilment of the requirements of the University of Dublin, Trinity College for the degree of Doctor of Philosophy

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October 2004
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Summary:

Polyamines are ubiquitous components of all eukaryotic cells and are found in brain tissue. A number of studies have shown that changes in the levels of polyamines occur following cerebral ischaemia. However, whether polyamines are neurotoxic or neuroprotective in ischaemia still remains unclear, although most of the evidence points to a toxic effect. The polyamine/NMDA antagonist N\(^1\)-dansyl-spermine has been shown to be neuroprotective in a global ischaemia model. However, at the onset of the present study, the neuroprotective potential of this compound was not known in using focal ischaemia models, which are of more clinical relevance. Very recently, more novel polyamine analogues (BU31b, BU37b, BU33b, BU36b and BU43b) have been synthesized. These compounds, with the exception of BU37b, have shown some polyamine antagonist potential in vivo. Therefore these polyamine analogues are candidate neuroprotective agents. The pre-ischaemic effect of all these compounds mentioned above was investigated in the present study. This investigation also aimed to shed light on the role of the polyamines in ischaemia.

The cerebral ischaemia models used in this work were a focal permanent cerebral ischaemia model and a focal transient cerebral ischaemia model using mice. The widely used intraluminal middle cerebral artery occlusion technique was employed. A range of histological and behavioural assessments were carried out in these two models.

In the permanent ischaemia model, N\(^1\)-dansyl-spermine showed a neuroprotective effect as it reduced the %HLV (percentage hemisphere lesion volume), oedema, neurological deficit score and improved the rotarod performance of MCAO mice, when administered pre-ischaemia. These effects were comparable to MK-801, which was also investigated as a reference compound. Also in this permanent ischaemia model, drug BU36b reduced oedema and BU43b decreased %HLV and oedema. Therefore both drugs showed pre-ischaemic neuroprotection. The neuroprotective
profile of this novel polyamine analogue series observed in the present study favours the hypothesis that polyamines are neurotoxic following ischaemia.

In the transient ischaemia model, N'-dansyl-spermine was as neuroprotective as MK-801 in reducing the %HLV. N'-dansyl-spermine showed more impressive beneficial effects on oedema, rotarod performance and LMA (locomotor activity) than MK-801. Also in this transient ischaemia model, both drug BU36b and drug BU43b showed neuroprotection. Drug BU36b reduced oedema and drug BU43b decreased the %HLV and oedema and enhanced the rotarod performance and LMA.

To further investigate the role of polyamines in ischaemia, the change in the profile of the polyamines in homogenates of various brain regions was measured. In addition, the change in the levels of extracellular polyamines in cerebral cortex was also studied using microdialysis. The study showed that the levels of both putrescine and N¹-acetyl spermidine were increased following focal permanent ischaemia or transient ischaemia. Reduction of tissue spermidine and spermine levels was seen in the cerebral cortex and a decrease in the spermidine level occurred in the hippocampus following permanent ischaemia. Further more, cortical extracellular spermidine and spermine levels were found to be elevated following focal permanent ischaemia, suggesting that extracellular polyamines may participate in ischaemic damage through a stimulatory effect at the NMDA receptor or other mechanisms such as the activation of calcium channels.

Collectively, this work suggests that an effective polyamine antagonist may have potential as a neuroprotective agent for stroke.
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Section 6 Change of polyamine profiles following cerebral ischaemia
List of abbreviations

AMPA: a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BBB: blood brain barrier
MCAO: middle cerebral artery occlusion
MPT: mitochondrial permeability transition
NMDA: N-methyl-D-aspartate
NO: nitric oxide
NOS: nitric oxide synthase
ODC: ornithine decarboxylase
ROS: reactive oxygen species
SSAT: spermidine/spermine N\textsuperscript{1}-acetyltransferase
Section 1 Introduction

1.1 POLYAMINES

1.1.1 Introduction

Polyamines, the naturally occurring di-, tri-, and tetra- amines, are constituents of all eukaryotic cells, including the cells of the vertebrate nervous system (Seiler, 1994). They share this ubiquity with amino acids, proteins and nucleic acids. Structures of most commonly occurring polyamines are shown in Figure 1.1.

![Chemical structures of three polyamines (putrescine, spermidine and spermine).](image)

Polyamines have a long history. The initial discovery of the polyamines was made by Antonie van Leeuwenhoek in 1678 when he isolated some ‘three-sided’ crystals from human semen (Wallace et al., 2003). However, it was not until 1924 that the empirical formula of the crystals was deduced and it was a further 2 years before the products were synthesized chemically (Dudley et al., 1926). The names spermidine and spermine therefore reflect the original discovery. Putrescine was first isolated from Vibrio cholerae, but its name was derived based on the large quantities found in
putrefying flesh. Today, polyamines are considered critical regulators of cell growth, differentiation and cell death (Wallace, et al., 2003).

Tissue concentrations of the polyamines vary greatly throughout the body. Rapidly proliferating cells and tissues (embryos, tumours, etc) usually have higher putrescine and spermidine concentrations than the corresponding non-growing tissues. In normal rat tissues, putrescine concentrations range from less than 10 nmol g\(^{-1}\) (brain) to 44 nmol g\(^{-1}\) (spleen) (Seiler, 1991). Spermidine and spermine concentrations range from 70 nmol g\(^{-1}\) (skeletal muscle) to 1600 nmol g\(^{-1}\) (spleen) (Seiler, 1991). Secretory organs (pancreas, prostate, hypophysis, lactating mammary gland etc.) have spermidine concentrations of 5000-9000 nmol g\(^{-1}\). There are species differences, but the order of magnitude of the polyamine concentrations is the same in all vertebrates (Seiler, 1991).

Due to the existence of the blood-brain barrier, the exchange of polyamines between blood and brain is limited (Shin et al., 1985). The vertebrate brain is an autonomous, nearly closed system with regard to polyamine metabolism, although this may not be true in pathological conditions such as cerebral ischaemia, where there is an impaired blood-brain barrier function (Seiler, 1994). As shown in table 1.1, brain concentrations of polyamines vary from region to region and putrescine levels are much lower than those of spermine and spermidine. It is thought that spermidine may be a constituent of myelin and it is found at its highest concentration in areas of the brain consisting primarily of white matter such as the medulla oblongata and the midbrain. Observations of decreased spermidine levels in myelin deficient strains of mice have been reported (Russell & Meier, 1975).
<table>
<thead>
<tr>
<th>Brain area</th>
<th>Putrescine nmol/ g wet</th>
<th>Spermidine nmol/ g wet</th>
<th>Spermine nmol/ g wet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>weight</td>
<td>weight</td>
<td>weight</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>9.4±1.3</td>
<td>235 ±22</td>
<td>221±20</td>
</tr>
<tr>
<td>Rear Cortex</td>
<td></td>
<td>368±56</td>
<td>324±50</td>
</tr>
<tr>
<td>Olfactory Bulb</td>
<td>5.5±0.4</td>
<td>446±81</td>
<td>506±88</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>7.1±1.2</td>
<td>420±107</td>
<td>334±64</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>22.9±2.0</td>
<td>591±109</td>
<td>253±54</td>
</tr>
<tr>
<td>Striatum</td>
<td></td>
<td>420±107</td>
<td>285±38</td>
</tr>
<tr>
<td>Midbrain</td>
<td>6.2±1.1</td>
<td>884±176</td>
<td>273±61</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>3.7±0.8</td>
<td>1016±77</td>
<td>157±33</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>13.0±1.3</td>
<td>674±58</td>
<td>381±47</td>
</tr>
</tbody>
</table>

Table 1.1 The variation (mean ± SD) in the distribution of polyamines in different regions of rat brain. Putrescine data (n=3) adapted from Seiler and Schmidt-Glenwinkel, 1975. Spermidine and spermine data (n=40) adapted from AL-Deen, 1982.

At physiological pH, the amino groups of the polyamines are protonated. The charge on the polyamines is distributed along the entire length of the carbon chain, making them unique and distinct from the point charges of the cellular bivalent cations. Their positive charge allows polyamines to interact electrostatically with polyanionic macromolecules within the cell (Wallace, 2003). Binding energy increases with the number of charges (putrescine < spermidine < spermine) (Seiler, 1991). In addition to electrostatic interactions, polyamines form covalent bonds with amino acids and proteins. Covalently bound polyamines represent only a small proportion of the total polyamines in vertebrate tissue.

Electrostatically bound and free polyamines are in a dynamic equilibrium. The intracellular concentration of the free polyamines is difficult to assess due to the
limitations of present day techniques. However, Kroigaard et al (1992) have detected significant levels of polyamines in nerve terminals and secretory granules from ox neurohypophysis. The concentration of polyamines they detected were in pmol (microgram protein)$^{-1}$ levels. There is no doubt that the intracellular concentration of the free polyamines constitutes only a small fraction of total polyamines (Seiler, 1991).

1.1.2 Polyamine homeostasis

Intracellular levels of polyamines are maintained within very narrow limits as the levels are controlled in a very fast, sensitive and precise manner. This control can be achieved through a harmony of four different mechanisms: de novo synthesis, interconversion, terminal degradation and transport of polyamines (Urdiales, 2001).

1.1.2.1 Synthesis of polyamines

Synthesis of polyamines in vertebrate organisms starts with ornithine. Putrescine is formed from ornithine by decarboxylation in a reaction catalysed by the enzyme ornithine decarboxylase (ODC) (Grillo, 1985). Spermidine and spermine are formed by the addition of an aminopropyl group from decarboxylated S-adenosylmethionine to putrescine and spermidine, respectively. In order to produce the aminopropyl group, which is necessary for the formation of spermidine and spermine, methionine first reacts with ATP to form S-adenosylmethionine. This is decarboxylated to decarboxy-S-adenosylmethionine by the enzyme SAMDC (S-adenosylmethionine decarboxylase). In a final step, a transferase enzyme, spermidine synthase or spermine synthase, catalyses the transfer of the aminopropyl group to the primary amine groups of putrescine or spermidine, respectively (Seiler, 1990; Seiler, 1991; Urdiales, 2001).
Figure 1.2 Pathways of polyamine metabolism showing the synthesis and interconversion of polyamines. Abbreviations: MAT, methionine adenosyltransferase; ODC, ornithine decarboxylase; PAO, polyamine oxidase; SAMDC, S-adenosylmethionine decarboxylase; SMO, spermine oxidase; SSAT, spermidine/spermine N\(^1\)-acetyltransferase. Figure adapted from Wallace et al. (2003).
The by-product of decarboxy-S-adenosylmethionine (5-methylthioadenosine) following the removal of the aminopropyl group during the production of spermidine and spermine can be reused for the formation of ATP (Seiler, 1990). ODC and SAMDC are the key enzymes in polyamine biosynthesis. Like other rate-limiting enzymes, both have short half-lives, their basal activities are low and they are rapidly induced by different stimuli (Urdiales, 2001). In particular, ODC is an interesting enzyme with several novel regulatory features. It is a highly inducible cytosolic enzyme that responds to a range of stimuli (Heby & Persson, 1990).

1.1.2.2 Polyamines interconversion and terminal degradation

The polyamines are interconverted in a cyclical process which allows metabolism of polyamines from spermine to spermidine and spermidine to be metabolized to putrescine. The initial reaction of this pathway is an acetylation, catalysed by the enzyme spermidine/spermine acetyltransferase (SSAT) yielding N\(^\text{1}\)-acetyl spermine or N\(^\text{1}\)-acetyl spermidine from spermine or spermidine respectively (Figure 1.2). These acetyl derivatives undergo an oxidative splitting by polyamine oxidase (PAO). Spermidine or putrescine, the products of these reactions, can be used again for the formation of new spermidine or spermine (Urdiales et al., 2001). The intermediate products of polyamine catabolism by interconversion, N\(^\text{1}\)-acetyl spermidine and N\(^\text{1}\)-acetyl spermine, are found in higher concentrations in cancer cells than normal cells, providing a link between alterations in polyamine metabolism and carcinogenesis (Wallace et al., 2000). 3-acetamidopropanol and H\(_2\)O\(_2\) are produced in the oxidation of acetylated polyamines. Both have been shown to be cytotoxic (Parchment et al., 1989). Thus it has been suggested that the metabolism of the higher polyamines could generate a self-sustaining cell death cycle (Wallace et al., 2000). As shown in Figure 1.3, SSAT acetylates the higher polyamines; PAO then oxidizes the acetyl derivative, generating H\(_2\)O\(_2\) with each oxidation. H\(_2\)O\(_2\) is able to induce the activity of SSAT, thereby perpetuating the cycling. The high local concentrations of H\(_2\)O\(_2\) produced could then lead to oxidative stress and cell death (Chopra & Wallace, 1998). The rate-limiting enzyme of polyamine interconversion is SSAT (Casero & Pegg, 1993; Urdiales et al., 2001).
Apoptosis

Figure 1.3 Polyamine metabolism and the potential for cell death. Metabolism of polyamines via SSAT (spermidine/spermine N\textsuperscript{1}-acetyltransferase) and PAO (polyamine oxidase) produces H\textsubscript{2}O\textsubscript{2}. H\textsubscript{2}O\textsubscript{2} induces both SSAT and cell death, thus causing a positive cell death signal-generating cycle. Figure adapted from Wallace et al. (2003).

In addition, there are reports that PAO (polyamine oxidase) could also act directly on spermidine to produce putrescine (Ivanova et al., 1998) although it has been suggested that contribution of the direct oxidation by PAO of spermidine to putrescine appears to be minimal (Rao et al., 2000). In 2001, a further oxidase was cloned that converts spermine back into spermidine without the need for an acetylation step (Wang et al., 2001). This enzyme has now been named ‘spermine oxidase’ (SMO) (Vujcic et al., 2002). However, so far there is little evidence that spermine oxidase (SMO) is of significant importance in the polyamine interconversion pathway.
The terminal degradation of polyamines is a third regulatory pathway of polyamine metabolism. In this procedure, the primary (terminal) amino groups are oxidised to produce polyamine derivatives that cannot be cycled into polyamines again (Seiler, 1985). Polyamines are oxidized by various oxidases classified as amine oxidase FAD-dependent; amine oxidase Cu²⁺-dependent; putrescine oxidase; and polyamine oxidase (PAO). Catabolism by all of these enzymes leads to the production of ammonia, hydrogen peroxide and aldehyde. The aldehydes formed are rapidly transformed into amino acids and lactams by intracellular aldehyde dehydrogenase activities, thus avoiding toxic effects (Urdiales et al., 2001). It is thought that the terminal catabolic reactions do not participate in the regulation of cellular polyamine levels under normal conditions (Seiler, 1990a; Seiler, 1990b).

1.1.2.3 Polyamine transport

In mammalian cells, transport into and out of the cell also contributes to polyamine homoeostasis (Wallace, 1996).

It is thought that polyamine transport is carrier-mediated, energy dependent and a saturable process (Pegg, 1988; Seiler, 1990). The polyamine uptake system transports molecules as diverse as paraquat, MGBG [methyglyoxal bis(guanylhydrazone)] and polyamine analogues (Wallace et al., 2003). This lack of specificity has been used to advantage in the design of potential inhibitors of polyamine metabolism, which may have cytotoxic benefits in cancer treatment (Wallace et al., 2003). Recently it has been shown that putrescine and spermidine transport in cerebellar granule cells is voltage dependent, whereas spermine uptake is not a voltage dependent process (Dot et al., 2004).

Polyamines can also be transported out of cells. Polyamine export is probably mediated by different transporters to those involved in uptake (Byers et al., 1994). Using selective uptake inhibitors, it has been shown that the inward and outward transporters are separate and distinct, since the inhibitors of uptake had no effect on
polyamine export (Wallace et al., 2003). Export is a selective process that is regulated by the growth status of the cell. Polyamine export is stimulated by a decrease in cell growth rate and turned off in response to a growth stimulus (Wallace & Keir, 1981; Wallace & Micharel, 1998). It is thought that the major polyamines released from the cell are N\(^1\)-acetylspermidine and putrescine (Wallace & Mackarel, 1998). This is in contrast with the normal intracellular polyamine pool, where the predominant polyamine, at least in human cells, is usually spermine (Wallace et al., 2003). This evidence shows that export is a selective and regulated process, with metabolism required before efflux (at least in the case of N\(^1\)-acetylspermidine). Thus the enzymes involved in polyamine catabolism and the outward transporter may be regulated by the same signals (Wallace et al., 2003).

1.1.2.4 Abnormalities of polyamine homeostasis

As described above, under physiological conditions, the polyamine pool is precisely and rapidly controlled within a narrow range of levels by the harmony of four different mechanisms. However, in various pathological states, polyamine homeostasis was found to be sharply altered. For example, following cerebral ischaemia, putrescine levels in the brain were found to be consistently increased (Baskaya et al., 1997). Spermine and spermidine concentrations in regions of brain were also decreased after the ischaemic insult (Sauer et al., 1992). It is thought that the alteration of polyamine homeostasis contributes to the neuronal damage in these diseases (Paschen, 1992). Intratumour polyamine concentrations are increased in a large number of solid tumours (Kingsnorth et al., 1984; Nowels et al., 1986; Loser et al., 1990) in comparison with the control groups. It is most likely that the high concentrations of polyamines found in cancer cells are the result of several changes in polyamine metabolism. For example, in some tumours, the regulation of ODC is altered which results in increased ODC expression (Wallace et al., 2003). Several studies have confirmed that an increase in ODC activity and the subsequent increase in intracellular polyamine concentrations are early events in carcinogenesis (Wallace et al., 2003). The change of the polyamine levels in cancer cells could also be
responsible for the development of the disease. The biological effects of polyamines have been investigated extensively and are discussed below.

1.1.3 Biological effects of polyamines

1.1.3.1 Polyamines as regulators of cell growth/cell death

As discussed in the previous section, cells have developed a complex system of regulation for polyamine homeostasis. Polyamines have been long been known to act as intracellular growth factors, increasing the rate of cell growth. Normal cell growth is regulated in a cyclical manner by increases and decreases in specific proteins known as kinases, cyclins and cyclin-dependent kinases (cdks) (Pines, 1994). Appropriate activation of the cdks and their partner cyclins is required for continual progression through the cell cycle. The cyclin/cdks exhibit cycle-specific regulation, and both polyamines and cyclin/cdks show phased changes throughout the cell cycle (Wallace et al., 2003). Intracellular polyamine concentrations have been reported to regulate both the up- and down-regulation of important cellular checkpoints within the cell cycle, and this may, in part, explain why their concentrations are controlled throughout the cycle (Kramer et al., 2001). Direct binding of polyamines to DNA and their ability to modulate DNA-protein interactions has been suggested to be important in the molecular mechanisms of the polyamine action in cell growth (Thomas & Thomas, 2001). In animal cells, the initiation of the growth always involves the stimulation of ornithine decarboxylase (Janne et al., 1991). Treatment of cells with a polyamine synthesis inhibitor depletes the polyamine pool and slows the cell growth (Kramer et al., 2001).

As mentioned above, there is amounting evidence of the marked increase of polyamine levels in tumours. Increased ODC activity and the subsequent increase in intracellular polyamine concentrations are early events in carcinogenesis. Therefore it is likely that ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC) and spermidine and spermine synthase are promising targets for anticancer therapy. Two enzyme inhibitors showed therapeutic promise—the ODC
inactivator, 2-(difluoromethyl) ornithine (DFMO) and the competitive inhibitor of SAMDC, methylglyoxal-bis(guanylhydrazone) (MGBG). However clinical trials using MGBG failed owing to its toxicity (Seiler et al., 1998). DFMO, which was the first effective, rationally designed antiproliferative drug aimed at depleting polyamines from cells, failed to demonstrate lasting antitumour effects clinically, although there is a resurgence of interest in using DFMO as a drug for cancer prevention (Wallace et al., 2003). Interestingly, DFMO has, however, been successful as an anti-parasitic agent. It has been shown to cure acute infections of *Trypanosoma brucei brucei* in mammals (Giffin et al., 1986). It has also shown promise in the cure of African sleeping sickness caused by *T. brucei gambiense* and *T. brucei rhodesiense* (Milord et al., 1992; Bacchi et al., 1993).

Polyamines can also regulate cell death, particularly the cellular suicide known as apoptosis (Nitta et al., 2002). The hallmarks of apoptosis include chromatin condensation, nuclear segmentation, cytoplasmic shrinkage, blebbing, and formation of apoptotic bodies (Endres & Dimagl, 2002). It has been suggested that spermine and spermidine are involved in programmed cell death in mammalian embryos through their oxidative catabolism. As previously described, catabolism of these polyamines produces hydrogen peroxide, which is extremely toxic to cells (Coffino & Poznanski, 1991). However, it is thought that the effects of the polyamines are far from simple with both induction and inhibition of biosynthetic and catabolic enzyme activities involved in increased and decreased apoptosis (Schipper et al., 2000). Packham and Cleveland (1994) demonstrated that enforced expression of c-Myc caused increased ODC levels and apoptosis, both of which could be prevented by the ODC inhibitor - DFMO. However, exogenous polyamines have also been shown to prevent cell death induced by high KCl concentrations in rat cerebellar granule neurons, implicating a protective role for polyamines (Harada & Sugimoto, 1997; Redman et al., 1997). The protective effects of the polyamines may be afforded through DNA stabilization (Basu et al., 1997) protection of DNA from oxidative stress by binding to DNA strands (Muscari et al., 1995) or by inhibition of endonucleases (Ribeiro & Carson, 1993).
1.1.3.2 Polyamines as cations

As described in section 1.1.1, the polyamines are positively charged molecules at physiological pH, their cationic nature allows them to bind to negatively charged molecules, such as the highly acidic phosphate groups of nucleic acids, and therefore polyamines can mediate effects at the genomic level (Tabor & Tabor, 1964). Through their electrostatic interactions, spermidine and spermine can bridge the major and minor grooves of DNA, thereby acting as a clamp, holding together either two different molecules or two distant parts of the same molecule (Matthews, 1993). It has also been suggested that polyamines not only alter the structure, but also the function of the DNA (Hampel et al., 1991). Research on these interactions may provide further information on how polyamines regulate the growth regulatory genes such as c-myc (Wallace et al., 2003).

In addition to their interaction with DNA, polyamines have been found to interact with acidic phospholipids in membranes (Shaw, 1994). It has been demonstrated that, by binding to the acidic phospholipids of the membrane, polyamines can influence the intracellular second messenger system based on phosphoinositide metabolism (Shaw, 1994). Polyamines are also thought to be involved in the regulation of several membrane-bound enzymes, including adenylate cyclase, and tissue transglutaminase (Wallace et al., 2003).

In addition, the polyamines are thought to regulate several ion channels. The interaction of polyamines with ion channels such as Kir (inwardly rectifying K⁺), voltage-activated Ca²⁺ channels, some non-NMDA ionotropic receptors (N-methyl-D-aspartate) and NMDA receptors, are discussed below in detail.

1.1.4 Polyamine as regulators of ion channels

1.1.4.1 Inward rectifier K⁺ channels (Kir channels)
Various K⁺-selective ion channels have been identified, including Kir channels (Doupnik et al., 1995). The term 'inward rectifier' refers to the capacity of the channel to conduct ions in the inward direction at negative membrane potentials, these channels show a greatly decreased outward conductance at membrane potentials positive to the potassium equilibrium potential (Ek) (Williams, 1997). Kir channels are present in both excitable and non-excitable cells and are crucial for maintaining the resting membrane potential close to Ek (Williams, 1997). Intracellular magnesium ions are responsible for blocking the inward rectifier potassium channels. However, block by Mg²⁺ cannot account for all of the gating and rectification properties of Kir channels, and may only count for a minor component of rectification in strong Kir channels (Williams, 1997). It has been demonstrated that there is another rectification component, called 'intrinsic gating'. Intrinsic gating is thought to be a major contributor to rectification (Williams, 1997). Polyamines may be the intracellular factors responsible for intrinsic gating and rectification at strong Kir channels (Williams, 1997; Fakler et al., 1995), although there may be another component of intrinsic gating that is not mediated by polyamine block (Shieh et al., 1996). Mg²⁺ and polyamines have been suggested to mediate their effects on Kir channels via different binding sites (Shieh et al., 2000). Thus, intracellular polyamines, by blocking Kir channels, may play an important role in controlling the resting membrane potential and the excitability threshold for the initiation of action potentials. Therefore, a rise in the concentration of intracellular polyamines would tend to increase the rectification of Kir channels and hence cause an increase of cellular excitability (Johnson, 1998).

1.1.4.2 Polyamines and voltage-activated Ca²⁺ channels

An elevation of calcium levels is an essential trigger for a wide variety of physiological, as well as pathophysiological events in neurons (Seiler et al., 1994). A major mechanism causing a rise in cytoplasmic Ca²⁺ is depolarization-induced Ca²⁺ influx from the extracellular space, and this is mediated by voltage-activated Ca²⁺ channels in the cell membrane (Seiler et al., 1994). Polyamines can interact with voltage-activated Ca²⁺ channels (Scott et al., 1994). It has been proposed that there
are a number of intracellular and extracellular sites of action for polyamines to inhibit and/or enhance the voltage-activated Ca\(^{2+}\) channels (Scott et al., 1994). Electrophysiological and pharmacological studies have identified four major classes of voltage-gated Ca\(^{2+}\) channels, the L-, T-, N-type channels and P-type channels (Nowycky et al. 1985; Llinas et al., 1989). For example, T-type Ca\(^{2+}\) channels have a low threshold for activation and rapidly inactivate. L-type Ca\(^{2+}\) channels have a high threshold for activation and do not readily inactivate. N-type Ca\(^{2+}\) channels, like L-type Ca\(^{2+}\) channels, have a high threshold but readily inactivate (Scott et al., 1994).

According to the new nomenclature system where voltage gated Ca\(^{2+}\) channels are classified on the basis of the α1 subunit gene subfamily, the Ca\(_{v}1\) (Ca\(_{v}1.1\) to Ca\(_{v}1.4\)) subfamily includes L-type Ca\(^{2+}\) channels; the Ca\(_{v}2\) (Ca\(_{v}2.1\) to Ca\(_{v}2.3\)) subfamily includes P and N-type channels; the Ca\(_{v}3\) (Ca\(_{v}3.1\) to Ca\(_{v}3.3\)) subfamily includes T-type channels (Catterall et al., 2003). Pullan et al. (1990) reported that polyamines inhibit N-type Ca\(^{2+}\) channels. Herman et al. (1993) demonstrated that putrescine increases L-type Ca\(^{2+}\) channel currents through the action of protein kinase C, but both spermidine and spermine are unable to produce this action. Polyamines have been shown to inhibit diltiazem binding to L-type calcium channels in the brain and allosterically inhibit [3H]nitrendipine (a dihydropyridine) binding (Schoemaker, 1992). Polyamines have also been shown to block an inward current activated by the dihydropyridine agonist BAYK 8644 in a guinea-pig intestinal smooth muscle (Gomez & Hellstrand, 1995). Dihydropyridines bind to a site on the α1 subunit of the L-type Ca\(^{2+}\) channel and prevent Ca\(^{2+}\) influx. Therefore, polyamines may have an interaction with the α1 subunit of the L-type Ca\(^{2+}\) channels. It has also been demonstrated that polyamines interact at the α2δ subunit of the L-type calcium channels. Spermine has been shown to inhibit the binding of [3H]gabapentin in detergent solubilised porcine cerebral cortical membranes by interacting with an allosteric site on the α2δ subunit (Dissanayake et al., 1997). The authors suggest that the α2δ subunit of L-type Ca\(^{2+}\) channels bears one or more modulatory spermine sites. Thus, polyamines are thought to function as endogenous positive modulators of voltage-dependent Ca\(^{2+}\) channels.
1.1.4.3 Block of AMPA and kainate receptors

Glutamate receptors have been categorized into a family of metabotropic or ionotropic receptors on the basis of pharmacological, electrophysiological and biochemical studies (Scatton, 1993). The metabotropic receptors are coupled to phospholipase C or adenylate cyclase via pertussis toxin sensitive G proteins and control the production of intracellular messengers. The ionotropic receptors contain integral cation-specific ion channels (Scatton, 1993). Based on agonists that selectively activate them, ionotropic glutamate receptors include the N-methyl-D-aspartate (NMDA), a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (Collingridge et al., 1989). Similar to Kir channels, AMPA and kainate receptors show inward rectification, which requires intracellular components (Isa et al., 1995). It has been demonstrated that the polyamines can cause rectification of AMPA and kainate receptors through blocking the pore of the receptor channel and thus preventing the flux of Na$^+$ (and Ca$^{2+}$) (Isa et al., 1995; Kamboj et al., 1995). A subset of native receptors of AMPA and kainate receptors that show inward rectification, and are controlled by intracellular polyamines have been shown to be Ca$^{2+}$ permeable (Williams, 1997). Therefore changes in levels of intracellular polyamines could alter Ca$^{2+}$ flux and the excitability threshold at synapses containing polyamine-sensitive AMPA and kainate receptors. The block of AMPA and kainate channels would be reduced by a decrease in the concentration of polyamines, thereby increasing cell Ca$^{2+}$ influx and excitability.

1.1.4.4 Polyamines and NMDA receptors

The NMDA receptor is the most extensively investigated glutamate receptor. The NMDA receptor is important in a variety of physiological processes. NMDA receptor activation is essential in several systems to achieve synaptic plasticity. For example, activation of NMDA receptors in the CA1 region of hippocampus is necessary for the increase of synaptic efficiency following tetanic stimulation, which has been termed long-term potentiation and is suggested to be a cellular correlate of learning and memory (Nicoll et al., 1988). NMDA receptor activation plays an
important role in pathophysiological changes in the CNS such as seizures and ischaemia (Murphy, 2003; Kirby & Shaw, 2004). The NMDA receptor may also be of importance in neurotoxicity following the development of AIDS (Lipton et al., 1992) and in various psychiatric disorders (Olney et al., 1990).

The NMDA receptor complex contains an integral ion channel that gates K^+, Na^+ and Ca^{2+} ions, but is blocked by Mg^{2+} and other divalent cations in a voltage-dependent manner (Nowak et al., 1984; Westbrook, 1987; MacDonald et al., 1990). This block can be relieved by depolarization, allowing the activation of the NMDA receptor by NMDA or glutamate. On activation, the NMDA receptor allows the Ca^{2+} and Na^{+} entry and K^+ efflux (Figure 1.4). The activity of NMDA ion channels is highly regulated. Figure 1.4 shows the binding sites for NMDA receptor regulation. Glutamate (NMDA) is necessary for activation of the NMDA receptor. A second agonist, a co-agonist, glycine, must also be bound to allow opening of the channels (Johnson et al., 1987). Similarly to Mg^{2+}, the divalent cation Zn^{2+} can act as a fast open channel blocker, but it can also reduce the open duration and frequency of channel opening by causing voltage-independent changes in gating properties of NMDA receptor single channels (Christine et al., 1990). Dissociative anaesthetics—such as dizocilpine (MK-801) and phencyclidine (PCP) (Fagg et al., 1987)—reduce the current through the NMDA receptor by binding to a site within the open channel of the receptor. MK-801 binding is greatly enhanced in the presence of glutamate and glycine (Foster et al., 1987; Reynolds et al., 1987; Huettner et al., 1988).
For this reason, binding of PCP-like dissociative anaesthetics has been used as a quantitative measure for activation of the NMDA receptor.

Ransom and Stec first described the effect of polyamines on the NMDA receptor. In 1988, they reported that spermine and spermidine increased the binding of MK-801 in the presence or nominal absence of glutamate and glycine (Ransom & Stec, 1988). It was proposed that this enhancing effect of the polyamines was mediated at a novel site associated with the NMDA receptor (Ransom & Stec, 1988). Since then, and before the cloning of NMDA receptor subunits, investigations were carried out in a number of laboratories on the effects of extracellular spermine on NMDA-induced whole-cell currents in cultured neurons in vitro. It has been shown that spermine has stimulatory effects on the NMDA receptor, although the degree of potentiation by spermine varied greatly between individual neurons, suggesting NMDA receptors expressed on different neurons are differentially sensitive to spermine (Rock et al.,
1992; Araneda et al., 1993; Benveniste et al., 1993). It is thought that this variability is due to the existence of different molecular forms of the NMDA receptor and partly to the multiple effects of spermine on the NMDA receptors. Spermine can potentiate NMDA currents in the presence of saturating concentrations of glycine (glycine independent stimulation) (Figure 1.4). It involves an increase in the frequency of channel opening and a decrease in the desensitisation of NMDA receptors (Lerma, 1992; Rock & Mcdonald, 1992). Spermine also increases the affinity of the NMDA receptor for the co-agonist glycine (Glycine-dependent stimulation) (Figure 1.4) (Benveniste & Mayer, 1993). Spermidine has been shown to potentiate the seizure activity of NMDA in an apparently selective manner (Singh et al., 1991) and the intraventricular injection of spermine causes tonic convulsions, which are mediated, at least in part, by the activation of the NMDA receptor (Doyle & Shaw, 1996). When heteromeric NMDA receptors expressed from cloned subunits were studied in Xenopus oocytes, NR2 subunits were found to control the stimulatory effects of spermine at NMDA receptors (Williams, 1994).

In addition to stimulation, voltage-dependent inhibition by extracellular spermine was seen at native NMDA receptors in the absence of extracellular Mg$^{2+}$, possibly representing a direct block of the ion channel by spermine (Rock et al., 1992; Araneda et al., 1993; Benveniste et al., 1993). Conceptually, this is similar to the block of Kir channels and AMPA channels by intracellular polyamines. However, it is thought that in the case of NMDA receptors, it is extracellular spermine and not the intracellular polyamines that block the channels (Williams, 1997). The voltage dependent inhibitory effects may include the interaction of spermine with negatively charged residues on the NMDA receptor, possibly close to the channel mouth, which impedes ion flow and thus reduces currents through NMDA channels (Rock et al., 1992). The voltage-dependent block by extracellular spermine is weak and develops much more slowly than the block caused by physiological concentrations of extracellular Mg$^{2+}$, and therefore this action is thought to have negligible effect under physiological conditions. Indeed, using the NMDA receptors expressed in Xenopus oocytes, Williams (1994) found that NR1A/NR2B controls both the stimulatory and inhibitory effects of spermine at the NMDA receptors but in the presence of
extracellular Mg$^{2+}$, only stimulation by spermine was observed at NR1A/NR2B. It is thus suggested that stimulation, seen in the presence of physiological concentrations of Ca$^{2+}$ and Mg$^{2+}$, may be the predominant effect of polyamines at NMDA receptors in the intact nervous system (Williams, 1994).

Protons have been demonstrated to block the NMDA receptor with a tonic inhibition of approximately 50% at physiological pH (Figure 1.4) (Traynelis & Cull-Candy, 1990). It is interesting that stimulation by spermine may involve relief of tonic inhibition by protons (Pahk and Williams, 1997). The block of NMDA receptor activity by ifenprodil is thought to be due to polyamine antagonism action (Figure 1.4). This block has been proposed to be dependent on extracellular pH (Figure 1.4) (Pahk & Williams, 1997). Further, Ifenprodil and its analogue CP-101 were found to inhibit NMDA receptors by increasing the sensitivity of the receptor to inhibition by protons (Mott et al., 1998).

As previously described, spermine and spermidine are present in the nervous system and uptake and depolarization-induced release of polyamines from brain slices has been demonstrated (Harman & Shaw, 1981b; Fage et al., 1992). Thus, it is likely that sufficient polyamines could be released from neurons or glia into the synaptic cleft to influence the activation of NMDA receptors. Indeed, extracellular polyamine levels (as measured e.g. in microdialysis studies) are reported to be in the sub to low micromolar range (Fage et al., 1992; Fage et al., 1993). Very recently it has been demonstrated that low μM concentrations of spermine potentiated a guanidinosuccinate-evoked current through the action of spermine on the polyamine binding site of the NMDA receptor complex (D’Hooge et al., 2003). Therefore polyamines could enhance the activation of NMDA receptors following ischaemia.
1.2 CEREBRAL ISCHAEMIA

1.2.1 Introduction

1.2.1.1 Stroke disease

After cardiovascular disease and cancer, stroke is the third most common cause of death in industrialized countries (Bogousslavsky et al., 2000). Each year, stroke occurs in 31 million individuals worldwide and is responsible for ~4 million deaths (Mancia et al., 2004). Stroke is defined as a sudden neurological deficit owing to central nervous ischaemia or haemorrhage (EUSI, 2003). Ischaemic stroke, which is the most frequently occurring type and accounts for about 75% of all strokes (Mancia et al., 2004), is discussed below.

Ischaemic stroke is induced by focal vessel occlusion, which causes cessation of the oxygen and glucose supply to an area of brain. This triggers breakdown of the metabolic processes in the affected territory (EUSI, 2003). Neuronal cell death takes place in the core of the infarcted area within minutes after cessation of the blood supply following occlusion or hypoperfusion of a cerebral vessel (EUSI, 2003). The ischaemic penumbra, the area surrounding the core and supplied with blood from collateral vessels, contains functionally impaired, but still viable brain tissue. This area may suffer secondary neuronal damage through the activation of deleterious biochemical cascades resulting in cytotoxic effects (EUSI, 2003).

There are many different causes of ischaemic stroke. (1) Atherosclerotic and atherothrombotic stenotic lesions of extracranial cervical and large basal cerebral arteries leading to critical hypoperfusion distal to high-grade stenoses; (2) arterio-arterial emboli from atherothrombotic lesions leading to intracranial vessel occlusion; (3) Systemic embolism (cardiac sources such as prosthetic valves, atrial fibrillation, cardiac thrombi, dilated cardiomyopathy, recent myocardial infarction, or intracardiac shunts); (4) Lipohyalinosis of small vessels, leading to microangiopathic
lacunar lesions; (5) Less common causes also include cervical artery dissection, vasculitis, or thrombosis due to coagulopathies (EUSI, 2003).

A range of risk factors for stroke have been identified or proposed. Age, sex, ethnicity, and heredity are among the nonmodifiable risk factors. Among the modifiable risk factors are hypertension, cardiovascular (CV) disease, diabetes mellitus, hyperlipidemia, cigarette smoking, and alcohol abuse (Rosamond et al., 1999; Benson et al., 2000; Sacco et al., 2001). Cigarette smoking is an independent risk factor for stroke and increases the risk in a dose-dependent manner (Shinton et al., 1989). There is extensive evidence that hyperlipidemia represents a modifiable risk factor for stroke (Ingall et al., 2000). Physical inactivity increases the risk of stroke among both men and women. There is overwhelming evidence to indicate that prolonged hypertension significantly increases the risk for stroke (Mancia et al., 2004). Recently, inflammatory markers, infection, homocysteine and sleep-disordered breathing have been ranked as the four most important new risk factors or markers of cerebral atherosclerosis (Mancia et al., 2004).

The greatest burden of stroke, apart from death, is the serious long-term physical and mental disability, and thus stroke constitutes an enormous economic burden on individuals, family, and institutions (Robinson et al., 1997; Haring et al., 2002). Despite intensive research efforts, there are only limited effective treatments once a stroke attack has occurred; therefore, stroke prevention is considered to be a primary focus for health care providers (Mancia et al., 2004). As mentioned above, control of hypertension and smoking has been shown to have a great effect on the stroke incidence, however, a significant number of cases of stroke still remain where the underlying mechanism is unidentified.

1.2.1.2 Acute treatment of stroke

During the acute phase of an ischaemic attack, early restoration of circulation in the affected territory can preserve reversibly damaged neuronal tissue in the penumbra (Heiss et al., 1999). There are currently few therapeutic options for the acute
treatment of an ischaemic stroke event. Intravenous tissue plasminogen activator (t-PA) is an agent approved for acute ischaemic stroke treatment (Mancia et al., 2004). The recovery of neuronal function reduces clinical neurological disability. t-PA has produced a 12% increase in the number of patients with normal neurological function 3 months after a stroke in comparison to placebo (Alberts et al., 1997). The clinical experience with t-PA in stroke treatment is largely favorable, although nonspecific factors such as the need to treat concomitant infections or to correct electrolyte disturbances may influence treatment success. t-PA has a narrow therapeutic window (3 hours) and may increase the risk of symptomatic hemorrhage (Alberts et al., 1999).

Aspirin and heparin or heparinoids are the other agents used in the treatment of acute ischaemic stroke (Mancia et al., 2004). Aspirin was suggested to produce modest benefits in acute stroke in the Chinese Acute Stroke Trial (1997) and the International Stroke Trial (1997). The effects of heparin are less clear. A meta-analysis of 21 clinical trials found that heparin reduced the rate of recurrent ischaemic stroke, although this benefit was negated by an increase in the rate of cerebral hemorrhage (Gubit et al., 2000).

As only at most 5% of patients are eligible for intravenous thrombolysis, it remains of foremost importance to develop efficacious neuroprotective agents for ischaemic stroke. Neuroprotective treatment, designed to interrupt the cellular, biochemical, and metabolic elaboration of injury during, or following exposure to ischaemia would save viable brain tissue surrounding the irreversibly damaged ischaemic core. To date, neuroprotective therapy has definite benefits in animal models, but has not made it to the clinic, due to side effects or lack of efficacy. There is, however, substantial optimism for the development of neuroprotective therapies to improve outcome in stroke patients (Chua et al., 2001).

1.2.2 Pathophysiology of focal cerebral ischaemia
1.2.2.1 Energy depletion and excitotoxicity

Metabolism in the brain depends exclusively on oxygen and glucose (Endres & Dimagl, 2002). The weight of the brain accounts for 2% of the total body weight but it claims 20% of total body perfusion and 20% of oxygen consumption (Endres & Dimagl, 2002). Moreover, to generate energy, the brain is exclusively dependent on oxidative phosphorylation to produce ATP, which is essential for maintaining ionic gradients across cell membranes. Ischaemia causes a profound depletion of both oxygen and glucose, thereby reducing the production of ATP. Within minutes, the depletion of ATP needed to drive the ionic pumps such as Na\(^+\)-K\(^+\) ATPase, leads to increased influx and efflux of ions as the energy-dependent ion pumps become inactivated, leading to the loss of the resting membrane potential (Martin et al., 1994). This causes the depolarization of neuronal cells. The depolarization of the synaptic membrane induces the activation of voltage-dependent Ca\(^{2+}\) -channels, which contribute to the increase of intracellular Ca\(^{2+}\). The depolarization due to depletion of ATP also causes the release of the neurotransmitter excitatory amino acid (such as glutamate) from presynaptic axon terminals into the synaptic cleft (Choi et al., 1996). Most cells rely on an uptake mechanism to terminate the action of neurotransmitters; however, this mechanism fails during ischaemia. This subsequently causes accumulation of the excitatory amino acids in the synaptic cleft (Gagliardi, 2000). The released glutamate acts on receptors including NMDA and non NMDA receptors (AMPA receptors and kainate receptors). As mentioned in 1.1.4.3, the AMPA and kainate receptors allow Na\(^+\) influx and a subset, allow Ca\(^{2+}\) influx. Cerebral ischaemia induces membrane depolarization, which releases the block of the NMDA receptor channel by Mg\(^{2+}\), and allows glutamate and glycine to activate the NMDA receptors. The accumulation of excitatory amino acids subsequently induces prolonged activation of these ligand-gated Ca\(^{2+}\)- permeable channels, leading to the enhancement of the intracellular increase of Ca\(^{2+}\).
The most important trigger for all the subsequent events leading to cellular disruption and cell death is the intracellular increase of $\text{Ca}^{2+}$. Under physiological conditions, the intracellular $\text{Ca}^{2+}$ concentration is kept under tight control and is approximately 10,000 times lower than extracellular levels (Tymianski & Tator, 1996). Cerebral ischaemia causes an elevation of the intracellular $\text{Ca}^{2+}$ concentration. Acting as second and third messenger, $\text{Ca}^{2+}$ triggers multiple cytoplasmic and nuclear cascades via enzyme induction. Some important enzymes activated by $\text{Ca}^{2+}$ are: (1) proteolytic enzymes which subsequently degrade the cytoskeleton and structural proteins (Chen et al., 1992). (2) xanthine oxidase and phospholipases (such as phospholipase A2 and C). Phospholipase A2 and C will cause increased production of free radicals and vasoactive and inflammatory substances. (3) cyclooxygenase, leading to free radicals.
radical generation. (4) protein kinase C (PKC). In a normal situation, PKC is inactive in the cytosol; but in the presence of excessive $\text{Ca}^{2+}$, membrane bound PKC is activated by diacylglycerol and phosphatidylycerine, PKC phosphorylates proteins which are responsible for mechanisms of extrusion of $\text{Ca}^{2+}$. (5) neuronal type nitric oxide synthase (nNOS) leading to the production of nitric oxide (NO) and NO-derived radical peroxynitrite by reaction with superoxide (Beckman & Koppenol, 1997; Iadecola, 1997).

Nitric oxide is of great importance during cerebral ischaemia. NO may be beneficial as well as deleterious depending on the compartment of its production and the time-point during the evolution of the ischaemia. There has been much confusion in the literature on its role during ischaemia and it is described as a "double-edged sword". NO is synthesized by NO synthases (NOS). There are two constitutive isoforms, neuronal (nNOS, type I) and endothelial (eNOS, type III), which are calcium/calmodulin-dependent. In addition, there is one inducible isoform (iNOS, type II). In physiological conditions, NO synthesized by constitutive NOS regulates a wide range of functions such as blood pressure, vascular tone, permeability and neurotransmission (Dalkara & Moskowitz, 1994). The iNOS is expressed in pathophysiological conditions; it is continuously active and leads to high production of NO, causing cytotoxicity and inflammatory actions (Endres & Dimagl, 2002). Similar to iNOS, nNOS can also produce a high quantity of NO and can cause cytotoxicity in cerebral ischaemia following the rise of intracellular $\text{Ca}^{2+}$, as described previously (Dawson et al., 1996). NO neurotoxicity is thought to be mediated by peroxynitrite formed by the reaction of NO with superoxide, a complex that rapidly decomposes into hydroxyl radicals, which are a highly reactive oxygen species (ROS) (Endres & Dimagl, 2002). These reactive oxygen species can damage proteins, lipids, and DNA, and lead to cell injury, necrosis and apoptosis (Rego et al., 2003). On the other hand, NO produced by the eNOS may have a protective effect, through augmenting cerebral blood flow, inhibiting platelet aggregation and activating leukocytes (Endres & Dimagl, 2002). eNOS knockout animals, which lack expression of the eNOS subtype, have been found to develop enlarged cerebral lesion size following middle cerebral ischaemia occlusion (Endres & Dimagl, 2002). The
neuroprotection of eNOS indicated here may be exerted through its function to increase the blood flow at the margin of an ischaemic lesion and to decrease the platelet and neutrophil adhesion (Huang et al., 1996).

Mitochondria are another important source of free radicals. Mitochondria may be damaged by reactive oxidative species (ROS) thereby initiating a vicious cycle (Dugan & Choi, 1994). Specifically, following cerebral ischaemia, the inner mitochondria membrane may be severely damaged by the generation of free radicals and also by the formation of a so-called mitochondrial permeability transition pores (MPT), which lead to organ swelling, generation of additional ROS and finally cessation of ATP production (Fujimura et al., 1998).

Following oxygen deprivation, glucose is metabolized through anaerobic glycolysis leading to lactate accumulation and tissue acidosis (Endres & Dirnagl, 2002). Acidosis has been documented as a major pathomechanism of tissue injury following stroke (Endres & Dirnagl, 2002). Lactic acidosis exerts its lethal effect in several ways. Mitochondrial respiration is depressed at low pH, inhibiting further ATP formation. Further, low tissue pH enhances free radical formation by causing the release of organic bound iron, which, when released, is able to catabolize the formation of free radicals (Zheng et al., 2003).

1.2.2.2 Oedema

As mentioned above, following ischaemia, K⁺ ions leak out of cells and Na⁺, Cl⁻, and Ca²⁺ ions influx. Following that, there is an almost instantaneous increase in tissue water, which is cytotoxic. This causes cytotoxic oedema. The cytotoxic oedema refers to cell swelling without any loss of the normal impermeability of the BBB while another type of oedema, vasogenic oedema, involves overall brain swelling due to fluid entry from the vasculature because of openings in the blood-brain barrier (BBB) (Kimelberg et al., 1995). Vasogenic oedema follows the cytotoxic oedema, secondary to breakdown of the blood brain barrier, which is also impaired by the
energy failure and hydrostatic force created by the blood pressure. By compressing capillaries, the resultant brain oedema may further decrease regional blood flow into the ischaemic zone and inhibit the reperfusion which may follow the relief of arterial obstruction (Klatzo, 1985; Anderson & Cranford, 1979). Oedema formation is also shown to involve multiple pathways, including glutamate receptor activation, mitochondrial dysfunction, elevated intracellular Ca\(^{2+}\) and oxidative stress (Hossmann et al., 1998).

1.2.2.3 Inflammation

It is now well established that cerebral ischaemic injury triggers a robust in situ inflammatory reaction. Brain ischaemia turns on various inflammatory genes that result in the generation of a wide variety of inflammatory mediators (Zheng et al., 2003). These local factors interact with residential brain cells. One of the resultant events is the immigration of peripheral circulating leukocytes into the area of injury, which leads to an amplification of the intricate signaling cascade of inflammatory mediators and contributes to the exacerbation of brain damage (Dimagl et al., 1999; del Zoppo et al., 2001). However, although the literature is in favour of a destructive effect, whether inflammation after cerebral ischaemia is good or bad has not unequivocally been resolved.

1.2.2.4 Apoptosis

As previously mentioned, apoptosis is a form of naturally occurring cell death. Some of the characteristic features include cell shrinkage, blebbing, DNA fragmentation, chromatin condensation and formation of apoptotic bodies. Ischaemic cell death was considered to be necrotic cell death. Necrosis is also called accidental cell death, describing the swelling of cells with disruption of subcellular organelles (Endres & Dirnagl, 2002). However, the possibility that apoptosis may also be another form of ischaemic brain cell death was proposed in 1993 (Linnik et al., 1993). Now much biochemical, histochemical, molecular and genetic data supports the proposal that apoptosis is an additional mechanism of cell death in animal models of cerebral
ischaemia. Cysteine proteases termed caspases act as the executioners of apoptosis. During ischaemia, activated caspases dismantle the cell by cleaving multiple substrates including cytoskeletal proteins and enzymes essential for cell repair, leading to apoptosis (Schulz et al., 1999). Also, as mentioned previously, the NO and free radical overproduction during ischaemia can trigger the cascades of apoptosis (Rego et al., 2003).

1.2.2.5 Reperfusion injury

In the treatment of acute cerebral ischaemia, one of the two primary therapeutic strategies is to limit the cerebral ischaemia by early reperfusion after cerebral ischaemia (Heiss et al., 1999). And as mentioned previously, t-PA is a proved drug for this approach. Early reperfusion can prevent infarct expansion, but can also aggravate oedema formation, depending on the severity and duration of prior ischaemia and efficiency of reperfusion (Schaller & Graf, 2004). In rodent models, the initiation of reperfusion within the first 1 to 2 hours after focal ischaemia generally results in smaller infarct volume when assessed several hours or days later (Heiss et al., 1997). However, delayed reperfusion can precipitate a progressive destruction of reversibly damaged neuronal cells, leading to paradoxical tissue dysfunction, cellular necrosis or apoptosis (Schaller & Graf, 2004). During reperfusion, high levels of oxygen result in the generation of various reactive species that can potentiate secondary ischaemic injury (Chan et al., 2001). Mitochondria which are injured during ischaemia increase the accumulation of reactive oxygen species (Fujimura et al., 1998). As we have mentioned previously, these reactive oxygen species can damage tissues directly and initiate necrosis as well as programmed cell death (Endres & Dirnagl, 2002). In addition, reperfusion may enhance ischaemic damage by inducing a secondary increase in the release of excitatory amino acids possibly due to secondary mitochondrial function failure and secondary microvessel deterioration (Yang et al., 2001) and further exacerbate excessive Ca$^{2+}$ influx into cells (Schaller & Graf, 2004).
It is of great importance to stress the effect of reperfusion on oedema formation. Ischaemia increases vascular permeability (the break down of the blood brain barrier). When reperfusion is induced in already irreversibly damaged areas bordering the infarct core, it leads to increased brain oedema as well as parenchymal hemorrhage (Schaller & Graf, 2004). Brain oedema is a major contributing factor to morbidity and mortality in stroke (Davalos et al., 1999; Rosenberg et al., 1999; Kasner et al., 2001; Steiner et al., 2001). It is maximal by 24 to 72 hours after the ischaemic event. Brain swelling in patients with large cerebral infarctions, can increase intracranial pressure, followed by herniation, loss of consciousness, and death: in patients with such rapidly progressing (‘malignant’) oedema, the mortality rate is up to 80% (Hacke et al., 1996). Even with smaller infarcts, the resulting vasogenic and cytotoxic oedema can cause an increase in intracranial pressure that can lead to additional ischaemia (Sherman & Easton, 1980). In particular, reperfusion can markedly increase the vasogenic oedema following the breakdown of the blood brain barrier, leading to increased mortality rate.

1.2.3 Potential neuroprotective strategies in cerebral ischaemia

1.2.3.1 NMDA antagonists

As previously described, glutamate release and accumulation of glutamate in the extracellular space due to cerebral ischaemia produces prolonged activation of glutamate receptors, particularly NMDA receptors, leading to excitotoxicity. Antagonism of NMDA receptors provides potential for neuroprotection. NMDA antagonists can act at several distinct sites within the NMDA receptor complex (a) within the ion channel of the receptor, producing a dose and activity dependent non-competitive blockade, (b) competitively, at the glutamate or glycine recognition site, or (c) at modulatory sites, such as the sites where polyamines act to enhance NMDA receptor function (Hargreaves et al., 1994). The polyamine sites on the NMDA receptor complex offer a therapeutic target for focal ischaemia, potentially devoid of most side effects associated with competitive NMDA receptor antagonists and noncompetitive open channel blockers such as MK-801 (Dizocilpine). The non-
competitive polyamine site NMDA antagonists, ifenprodil and eliprodil, have shown pre-clinical neuroprotective efficacy with a good side effect profile (Muir & Lees, 1995; Gagliardi, 2000). Historically, there has been a lack of effective competitive polyamine antagonists to study, but recently, a number of polyamine analogues, that are putative competitive polyamine antagonists, have been developed.

1.2.3.2 Other major potential neuroprotective strategies

a. AMPA antagonists; As AMPA receptors also play an important role in increasing the level of $\text{Ca}^{2+}$ inside the cells, AMPA antagonists provide another possible route to neuroprotection. NBQX is one example of a drug in this group, but unfortunately it has been shown to be very nephrotoxic and the outcome of clinical studies is not optimistic (Meldrum, 1994).

b. Interference with NO production; Selective inhibition of nNOS and iNOS may offer protection in neuronal tissues during stroke. Conversely, selective upregulation of eNOS activity has been demonstrated to be neuroprotective in animal models (Endres et al., 1998).

c. Glutamate release inhibitors; the synthetic toxin SNX-111 has shown neuroprotective promise, but unfortunately it blocks adrenaline release leading to severe hypotension (Gagliardi, 2000).

d. Reduction of intracellular $\text{Ca}^{2+}$ mobilization; this is another possible route to oppose excitotoxicity. GM-1 that inhibits the translocation of CPK (creatine phosphokinase) and some drugs that inhibit $\text{Ca}^{2+}$ dependent enzymes such as protein kinase C (AT 877) have been studied (Nagumo et al., 1998; Gagliardi, 2000).

e. Inhibition of inflammation; Induction of neutropenia and inhibition of several inflammatory mediators (such as IL-1b) or adhesion molecules may significantly protect cells from death and improve outcome after stroke (Endres & Dinnagl, 2002).

f. Blocking apoptosis; Strategies that inhibit caspase activity block apoptosis in experimental models of mild ischaemia, and preserve neurological function (Schulz et al., 1999).
g. Hypothermia; Hypothermia provides neuroprotective effect. Hyperthermia has an opposite effect (Gagliardi, 2000).
1.3 POLYAMINES AND ISCHAEMIA

1.3.1 Change of polyamine profile following cerebral ischaemia

Following cerebral ischaemia, there are marked changes in polyamines levels. 30 min of transient focal cerebral ischaemia in rats produced increases in cortical putrescine levels at 4 and 24 hours with a significant three fold increase achieved at the latter time. Significant increases in striatal putrescine were also reported at both 4 and 24 hours reperfusion following 30 minutes focal cerebral ischaemia in rats in the same study (2.2 and 4 fold respectively) (Paschen et al., 1991). Baskaya et al. (1997) reported a two fold increase in putrescine in the penumbra after 6 h occlusion in a focal permanent cerebral ischaemia model in cats. In the gerbil global ischaemia model, putrescine levels were shown to be largely increased throughout the brain after 3 bouts of 5 min ischaemia (Paschen et al., 1992). Increase of putrescine has been reported in cortex and striatum at both 6 (4.6 and 2.1 fold increase for cortex and striatum respectively) and 24 hours (5 and 2.2 fold for cortex and striatum respectively) in a transient focal cerebral ischaemia model in rats (Adibhatla et al., 2002). In the same study, a significant increase of putrescine was observed in both the cortex (6.9 fold) and hippocampus (4.7 fold) 24 hours following transient forebrain ischaemia in gerbils (Adibhatla et al., 2002).

It has been proposed that the overshoot of putrescine may be due to an increase of ODC activity and/or the inhibition of the S-adenosylmethionine decarboxylase activity (Paschen, 1987). However, it has been estimated that ODC accounts for 30% of putrescine whereas the remaining 70% is formed through SSAT/PAO pathway (Seiler & Bolkenious, 1985; Seiler, 1995). Inhibition of PAO with MDL 72527 significantly attenuated the putrescine levels at day 1 after transient forebrain ischaemia in gerbil cortex and hippocampus (Rao et al., 2000). Although there is recent evidence demonstrating that under certain conditions PAO could directly act on spermine and spermidine to generate putrescine (Ivanova et al., 1998), it also has been demonstrated that the contribution of PAO via the direct oxidation of
spermidine to putrescine may be minimal (Rao et al., 2000) and putrescine is largely formed by PAO actions on N\textsuperscript{1}-acetylspermidine. Therefore the induction of SSAT could also account for, at least partly, the increase in putrescine following cerebral ischaemia.

Despite the consistently observed increases in putrescine levels following ischaemia, changes in spermine and spermidine levels vary considerably depending on the types of ischaemia and possibly the animal species. This may reflect the fact spermine and spermidine synthesis requires the use of adenosylmethionine derived from ATP. Furthermore, the breakdown of spermine and spermidine by the interconversion via acetylation involves the use of acetyl CoA (Seiler, 1991). Both ATP and acetyl CoA are of course affected under ischaemic condition, but may be variously affected at different times and in different types of ischaemia (Seiler, 1991). A 27% and 22% decrease in spermidine was observed in the cortex and striatum respectively of rats following 30 min of focal cerebral ischaemia (Paschen et al., 1991). Spermine levels were also reduced in these tissues during the ischaemic period by 14% in the striatum and 22% in the cortex although the statistical significance was only seen in the latter structure. However, in the same study, no alterations in spermine or spermidine concentrations were observed at any of the recirculation times (2, 4, and 24 h) (Paschen et al., 1991). Following permanent focal ischaemia in rats, both spermine and spermidine levels were decreased 48 hours after permanent MCA occlusion (Sauer et al., 1992). In a permanent focal ischaemia model in cat, a non-significant decrease of spermidine levels (32%) was reported in the densely ischaemic core, while no alteration of spermine levels were observed (Baskaya et al., 1997). In a study using a gerbil global ischaemia model, Paschen et al. (1992) reported that spermine levels were significantly reduced in the hippocampal CA1-subfield after 5 min of ischaemia. Spermine levels were also reduced in the striatum and thalamus after 3-bouts of 5 min ischaemia in this model (Paschen et al., 1992). However, spermine and spermidine have also been reported to be increased following ischaemia. A small and non-significant increase in spermidine (20%) and spermine (11%) appeared 1-2 minutes after the occlusion and spermidine and spermine showed a modest increase during the 15 minutes recirculation (with significance seen
with the increase of the spermidine) in a gerbil global ischaemia model (Koenig et al., 1990).

1.3.2 The role of the polyamines in cerebral ischaemia: a neuroprotective role?

Although the majority of evidence points to a role for the polyamines and polyamine metabolism in enhancing the neurodegeneration following cerebral ischaemia, there is still confusion over the role of polyamines because some studies have demonstrated that they, particularly spermine, are neuroprotective after cerebral ischaemia. Spermine has been reported to be neuroprotective in a model of forebrain ischaemia. Hippocampus and striatal cell loss in gerbils was significantly decreased by the intra-peritoneal administration of spermine (Gilad and Gilad, 1991). Farbiszewski et al. (1995) demonstrated that, in a rat model of forebrain ischaemia, spermine significantly reversed the decrease in superoxide dismutase (SOD) activity in the cortex. Spermine when given 30 min prior to the ischaemia, showed a dose-dependent neuroprotective effect in a rat reversible focal cerebral ischaemia model (Coert et al., 2000). Spermine has been shown to be of significant importance in the Ca^{2+}-buffering capacity of mitochondria (Rottenberg & Marback, 1990). Spermine can increase the rate of calcium uptake by mitochondria by increasing the affinity of the uniporter for calcium, therefore regulating the intracellular Ca^{2+} levels (Jensen et al., 1987). Also spermine can stabilize nucleic acids by its interaction with endonuclease or its substrate, DNA (Brune et al., 1991). All of these factors could contribute to the neuroprotection observed in ischaemia with the exogenous polyamines and some novel polyamine derivatives, which are thought to function in a similar manner (Gilad & Gilad, 1999). More recently, a tentative antioxidant mechanism of spermine neuroprotection in ischaemia has been proposed (Adibhatla et al., 2002). Iron released from ferritin during ischemia–reperfusion promotes the formation of OH• by catalyzing the OH• generation, which contributes to ischaemic injury (Ying et al., 1999; Chan, 2001). Spermine has been demonstrated to act as an antioxidant by scavenging oxygen radicals possibly through chelation of Fe (Ha et al., 1998). Therefore the authors suggested that spermine could afford its neuroprotection through this mechanism (Adibhatla et al., 2002). However, despite
these few reports of neuroprotection by polyamines, the well-known stimulatory action of the extracellular polyamines on the NMDA receptor would suggest that polyamines would actually enhance the neurotoxicity following ischaemia (see section 1.1.4.4 for review). Therefore the idea that exogenous polyamines are neuroprotective is far from convincing. Most evidence points towards a role of polyamines and polyamine metabolism in neurodegeneration following ischaemia and is described below.

1.3.3 The role of the polyamines in cerebral ischaemia: a neurotoxic role?

It has been suggested that higher polyamines could be released from necrotic neurons into the extracellular space and cause multiple effects (Paschen et al., 1992). This may also explain the decrease of spermine and/or spermidine observed in some studies, following cerebral ischaemia, as polyamines released into the extracellular space then may be cleared by the blood circulation. Following ischaemia, the polyamines released from necrotic neurons may diffuse to intact neurons in the vicinity and bind to the polyamine recognition site at the NMDA-receptor (Paschen et al., 1992). This binding could enhance the activational state of the NMDA receptor complex, potentiating the intracellular signal mediated by the NMDA receptor, and thereby increase excitotoxicity (Ransom et al., 1988). In fact, potassium-stimulated depolarization induces a calcium-dependent release of polyamines from the cell, the re-uptake of which is nearly completely inhibited in the presence of high potassium concentration (Harman & Shaw, 1981a; Harman & Shaw, 1981b). This observation indicates a release of polyamines from cells during conditions of energy depletion (Paschen et al., 1992). Indeed, Djuricic and co-workers observed that in hippocampal slices, polyamines are released from cells into extracellular compartments during a period of energy depletion produced by incubation in the absence of oxygen and glucose (Paschen et al., 1991). Carter et al. (1995) reported that cortical spermidine, although not spermine was released into the extracellular space following permanent focal cerebral ischaemia in rats. The amount of spermidine released was thought to be sufficient enough to enhance the activity of the NMDA receptor (Carter et al., 1995). Further more, the NMDA antagonists,
ifenprodil and eliprodil (SL 82.0715), are believed to produce their neuroprotection through blocking the polyamine recognition site (Carter et al., 1990). Spermidine, a polyamine modulatory site agonist, potentiated NMDA- and glutamate-induced cytotoxicity in cultured rat cortical neurons (Tamura et al., 1993). In the same study, spermidine also significantly reduced the protective effects of ifenprodil against NMDA- and glutamate-induced cytotoxicity (Tamura et al., 1993).

The marked post-ischaemic rise in putrescine levels may activate Ca^{2+} fluxes at the cell membrane, through the voltage-activated Ca^{2+} channels, as mentioned previously in section 1.1.4.2, although there is little evidence that putrescine potently activates the Ca^{2+} channels. Within a given brain structure, the postischaemic putrescine levels were found to correlate closely with the density of ischaemic cell injury and the time period of cerebral ischaemia (Paschen et al., 1988). The ODC inhibitor, DFMO, has been shown to reduce the brain infarction volume by 57% after middle cerebral artery occlusion in rats (Muszynski et al., 1993). The PAO inhibitor, MDL 72527 which blocks the overshoot of putrescine by 45% following cerebral ischaemia, has also been shown to have a neuroprotective effect against neuronal damage in a focal cerebral ischaemia model in rats (Dogan et al., 1999).

In addition, polyamines have been found to influence the integrity of blood-brain barrier (Koenig et al., 1989). Alteration of polyamine metabolism after cerebral ischaemia, particularly the increase of putrescine is an important factor in blood-brain barrier dysfunction and in the development of vasogenic oedema (Baskaya et al., 1997). The breakdown of the blood-brain barrier and vasogenic oedema can be reversed by blocking ODC using DFMO in a focal cerebral ischaemia model in cats. Putrescine abolished the protective effect of DFMO (Schmitz et al., 1993). In another study, DFMO treatment reduced both the ODC activity and oedema formation in a gerbil global cerebral ischaemia model, also indicating a role for polyamines in postischaemic oedema formation (Rao et al., 1995).

During ischaemia, the change in polyamines may also have an effect on K^{+} homeostasis. Intracellular polyamines, by blocking Kir channels, are of importance
in controlling the resting membrane potentials and the excitability threshold for the initiation of action potentials as previously described. Johnson (1998) proposed that the increase of intracellular putrescine, which is a hallmark of cerebral ischaemic insult, would potentiate the polyamine-dependent Kir channel rectification resulting in an increased cellular excitability.

Also, as mentioned previously in section 1.3.1, the interconversion pathway of polyamines is proposed to be actively involved in the metabolism of polyamines following ischaemia. The increase of putrescine levels, the decreases of spermine/spermidine levels often observed, and recently, the increase of the N\textsuperscript{1}-acetylspermidine reported (Rao \textit{et al.}, 2000), following ischaemia may implicate the activation of the interconversion pathway of polyamine metabolism. As previously described, oxidation of spermine or spermidine by tissue PAO leads to the formation of spermidine or putrescine respectively. Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and 3-aminopropanal are also produced; both of which may induce cell death (Wallace \textit{et al.}, 2003). PAO metabolism of spermine/spermidine was shown to have cytotoxic effect in human umbilical vein vascular endothelial cell cultures (Morgan \textit{et al.}, 1987). Cerebral infarction volume, largely limited to the cerebral cortex, was significantly attenuated by i.p. administration of aminoguanidine in a three-vessel occlusion model of focal cerebral ischaemia in rats (Cockroft \textit{et al.}, 1996) and the neuroprotection by aminoguanidine (83% reduction in infarct volume) was suggested to be due to its PAO inhibitory activity (Cockroft \textit{et al.}, 1996). Intracortical injection of either spermidine or spermine, but not putrescine, produced cerebral necrosis, which was inhibited by either systemic or cortical administration of aminoguanidine (Johnson, 1998). The neuroprotective effect of the PAO inhibitor MDL 72527 observed in cerebral ischaemia models also supports the possibility that the cytotoxic intermediates produced by the polyamine interconversion pathway metabolism may be responsible for the observed cellular damage following cerebral ischaemia (Dogan \textit{et al.}, 1999).

As described before (See section 1.1.4.3 for review), changes in levels of intracellular polyamines could alter Ca\textsuperscript{2+} flux and the excitability threshold at
synapses containing polyamine-sensitive AMPA and kainate receptors. A decrease in the concentration of intracellular polyamines following ischaemia would reduce the block of AMPA and kainate channels, allowing more Ca\(^{2+}\) influx, resulting in increased excitability. As mentioned in section 1.3.2, spermine has been shown to be of significant importance in the Ca\(^{2+}\)-buffering capacity of mitochondria (Rottenberg & Marback, 1990; Jensen et al., 1987). Therefore during ischaemia, in regions where spermine levels are reduced, the Ca\(^{2+}\) buffering capacity of mitochondria may be disturbed, which could also contribute to the cellular damage (Paschen et al., 1992).

1.3.4 Polyamine antagonists and ischaemia

As described previously, whether polyamines serve a neuroprotective or a neurodegenerative role remains unclear, although most evidence points towards a role in neurodegeneration. The polyamine antagonists, ifenprodil and its derivative eliprodil, have been shown to be effective neuroprotective agents in cerebral ischaemia models and the neuroprotection was afforded through, at least partly, their action via the polyamine site at the NMDA receptor (Cater et al., 1997) although their other effects such as calcium antagonism and affinity for \(\sigma\) receptor, could also contribute their neuroprotection (Honda et al., 1989; Poignet et al., 1992; Biton et al., 1994; O’Neill et al., 1996). This has opened the possibility that polyamine antagonists could be neuroprotective following cerebral ischaemia. Historically, there was a lack of effective polyamine antagonists to study for neuroprotection in ischaemia. It is interesting that some old polyamine antagonists have been shown to have some agonist-like effects in vivo. Arcaine, 1, 10-diaminocadecane and DET (diethylenetriamine) were proposed as polyamine antagonists through the results of binding studies. All of these compounds reduced the increase in \([\text{H}]\)-dizocilpine binding produced by polyamines (Williams et al., 1989; Reynolds, 1990a, Williams et al., 1990). Subsequently, those polyamine antagonists were shown to have agonist effects. Arcaine and 1, 10-diaminodecane have been suggested to be inverse agonists of the polyamine site (Reynolds, 1990b; Williams et al., 1990). DET was found to increase \([\text{H}]\)-dizocilpine binding in rat brain membrane, when tested under basal conditions (Reynolds, 1990b). Both arcaine and 1, 10-diaminodecane was found to
potentiate the first stage of the spermine-induced effects but inhibited the development of the second stage effects (Doyle & Shaw, 1998). Conversely, DET was effective against the development of the first stage of spermine-induced effects, but potentiated the second stage of effects (Doyle & Shaw, 1998). Recently, a novel polyamine analogue, N\textsuperscript{1}-dansyl-spermine, has been shown to be an effective polyamine antagonist and interest in this compound has been increasing. Chao et al. (1997) demonstrated that N\textsuperscript{1}-dansyl-spermine was active at the recombinant NMDA receptor. Later, this compound was shown to be a polyamine antagonist at the NMDA receptor as N\textsuperscript{1}-dansyl-spermine suppressed spermine-induced CNS excitation and antagonised the spermine enhancement of NMDA-induced convulsions in mice (Kirby et al., 2004). More recently, N\textsuperscript{1}-dansyl-spermine has been reported to be neuroprotective in a global cerebral ischaemia model and its neuroprotective effect has been suggested to be produced through the action at the polyamine site on the NMDA receptor (Kirby & Shaw, 2004). The research on N\textsuperscript{1}-dansyl-spermine further confirms the neuroprotective possibilities of polyamine antagonists, particularly the polyamine antagonists at the NMDA receptor.

Generally speaking, mounting evidence suggests that polyamines are involved in neurodegeneration through mechanisms such as (1) the enhancement of NMDA receptor activation; (2) the activation of voltage-dependent calcium channel activity; (3) the reduction in the integrity of blood-brain barrier. Therefore it is likely that an effective polyamine antagonist could provide neuroprotection.
1.4 AIMS OF THE PRESENT STUDY

1. To shed light on the role of polyamines in ischaemia, whether they are neuroprotective or neurotoxic.

2. To investigate the neuroprotective potential of $N^1$-dansyl-spermine in a permanent and a transient focal cerebral ischaemia model (middle cerebral artery occlusion model) in mice.

3. Very recently more novel polyamine analogues have been synthesized (BU31b, BU37b, BU33b, BU36b and BU43b; their full chemical names are described in section 2). These compounds with the exception of BU37b have shown some polyamine antagonist potential in vivo. Therefore these polyamine analogues are candidate neuroprotective agents. In the present study, their neuroprotective potential was studied in both a permanent and a transient focal cerebral ischaemia model in mice.

4. To study the changes in the profiles of tissue polyamine in various brain regions including the cortex, hypothalamus, hippocampus and midbrain following ischaemia. The polyamines measured including putrescine, spermidine, spermine and one metabolite, $N^1$-acetylspermidine. The study of the polyamine profile could also help to explain the observations on the neuroprotective potential of the novel polyamine antagonists tested in this study.

5. To measure the change in brain extracellular polyamine levels to correlate the possible change with infarct following cerebral ischaemia using microdialysis.
Section 2 Materials and methods

2.1 MODELS OF CEREBRAL ISCHAEMIA

2.1.1 Introduction

The development of experimental models of cerebral ischaemia has allowed for better understanding of the pathophysiology of stroke and for the assessment of therapeutic strategies. Animal models of cerebral ischaemia are aimed at developing an experimental situation that mimics aspects of the human condition and allow for the study of the pathogenesis of lesions and the assessment of therapeutic strategies prior to their test on humans. Stroke is a highly variable clinical situation. The location, cause, severity and reversibility often coupled with pre-existing disease, can all contribute to variability in outcome and confound interpretation (Wiebers et al., 1990; Millikan, 1992). The advantage of animal models is that the cerebral ischaemia can be induced under standard conditions and some variables which may modify the results, such as severity, duration and location of ischaemia can be controlled. Therefore a large range of the variables seen in the clinical situation can be eliminated.

An animal model of stroke must be simple, and similar to the clinical condition it is intended to mimic. In addition, the model must be easily reproducible to allow for the testing of given hypothesis. There are numerous experimental models of global and focal cerebral ischaemia. They are described in section 2.1.2 and 2.1.3.

In vitro models may be useful for exploring the mechanisms of drugs which have been previously shown to protect against ischaemia. However, the in vitro models have little relevance to the screening of the efficacy of anti-ischaemic drugs. Due to the basic pathophysiological differences between ischaemia produced in vivo and in vitro, in vitro screening may lead to false positive or false negative results.
2.1.2 Global models

Global ischaemia takes place as a consequence of a reduction in, or complete cessation of blood flow to the brain. Compressing the blood vessels in the neck through inflating a pneumatic cuff or increasing the intracranial pressure can bring about complete global ischaemia (Mas & Zuber, 1991). Animals used in global models are gerbils and rats. The gerbil bilateral carotid occlusion model has enjoyed wide popularity because the surgical techniques involved are relatively easy. As shown in Figure 1.6, in the circle of Willis at the base of brain, the gerbil has no connection between the carotid and vertebro-basilar circulation, which means the global ischaemia can be achieved by occluding the two common carotid arteries, of which the internal carotid arteries are an extension. In this model, neurodegeneration in the hippocampus develops over several days and is often referred to as delayed neuronal death (Kirino, 1982). Histologically, neurodegeneration observed is obvious.

Figure 2.1 The incomplete circle of Willis found in the Mongolian gerbil. Figure adapted from Mayevsky & Breuer, 1992.
and easily analyzed (Osborne et al., 1987; Gill et al., 1987). Furthermore, the histopathology observed in the gerbil model is similar to that seen in the hippocampal CA1 region of human brain following a cardiac arrest (Zola-Morgan et al., 1986; Petito et al., 1987). There are two models of global ischaemia in rats, a two and a four vessel occlusion model. However, the two vessel occlusion model requires the production of hypotension and the four vessel occlusion model involves a two-stage major surgical procedure and both models produce significant variability in outcome (Hunter et al., 1995).

Global models are said to be models of cardiac arrest which produce selective necrosis, while focal models are stated to have more relevance to acute ischaemic stroke since most strokes are focal (Fakler et al., 1995).

2.1.3 Focal models

Focal ischaemia can be generally divided into two types, permanent and transient. Permanent focal ischaemia produces a dense region of ischaemic damage (the core) and degenerative changes spreading out from the core as mentioned in section 1.2.11. Therefore the aim of therapeutic treatment is to protect the “at risk” penumbral area, thereby reducing the spread of the damage. Clinically, some patients have spontaneous recanalization after a cerebral ischaemia insult (Saito et al., 1987) and the use of thrombolysis therapy also induces the recovery of the blood flow. Therefore transient occlusion models have also been developed to mimic the result of both the ischaemia and the consequences of reperfusion. There are many different focal ischaemia models. It is suggested that the middle cerebral artery (MCA) occlusion models are of particular relevance because this is the vessel most commonly affected in stroke victims (Hunter et al., 1995). Also MCA occlusion techniques have been incorporated with reperfusion and therefore, it is suggested more closely mimic the clinical situation. The MCA can be clipped or ligated after it is exposed by transcranial, post-orbital or transorbital approaches under the operating microscope (Hossmann, 1998). Occlusion of the MCA is also possible by local application of the potent vasoconstrictive agent endothelin-1 which causes
severe vasospasm for a half maximal restoration time of up to 1 hour, followed by gradual reperfusion (Macrae, 1992).

More recently, the surgical exposure of the MCA for occlusion, which is invasive and therefore poorly physiological, has lost popularity and has been widely replaced by the placement of flow-obstructing devices intraluminally (Hossmann, 1998). Initially, attempts were made with permanent or retractable macroemboli consisting of silicon rubber or metal balls (Hossmann, 1998). Later, fine nylon threads have been used, particularly in models using rats and mice. These threads have several advantages: they avoid the need of craniotomy because they can be inserted from the common carotid artery or external carotid artery, and they are easily withdrawn to produce reperfusion (Hossmann, 1998). Placement of threads can also be carried out under remote control which enables continuous recordings of electrophysiological or even nuclear magnetic resonance tomographic and spectroscopic data (Gyngell et al., 1995). An intraluminal suture model was used in the present study.

There are also many models of arterial occlusion by embolism. Basically, they can be divided into three major types. (1) Embolism with Microspheres. This involves injection of microspheres of e.g. coal into the internal carotid artery. The infarction produced in this model has high variability and it is impossible to predict the size and location of infarct in different animals (Ginsberg & Busto, 1989). (2) Embolism with heterologous blood clots. The blood clots used in this method are made from human blood. This model has been used to assess the thrombolytic effect of t-PA (Calandre et al., 1998). (3) Embolism with autologous blood clots. The blood clots used are obtained from the same animal, which is subsequently used as a subject of the model. This mimics the clinical situation. However, high variability at the arterial occlusion site, and therefore in the size of the lesion is still the major disadvantage of this model.

Finally, a model of occlusion by photochemically induced focal thrombosis has been developed. This involves intravenous administration of the photosensitive dye, Rose Bengal, followed by irradiation of a specific area of the brain with a focused light
beam of specific wavelength. The circulating dye reacts with the light and generates free radicals, resulting in platelet aggregation and thrombosis (Watson, 1985). This was claimed to be of value because it produced a lesion and oedema at a controllable site by a non-invasive procedure. However, no penumbral region is produced in this model, unlike other models that have a closer clinic correlation. As a result, this model is little used (Hunter et al., 1995).

2.1.4 Intraluminal suture MCAO model

As previously mentioned the intraluminal suture MCAO model has many advantages and has gained high popularity. It was initially described by Koizumi et al. (1986) and later modified to reduce the incidence of complications such as arterial tear and thrombotic occlusion due to endothelial damage (Longa et al., 1989). For rodents such as rats and mice, which have complete circle of Willis at the base of brain and thereby an excellent contralateral blood flow supply, the MCA territory is perfused by blood sources from the anterior cerebral artery, posterior cerebral artery, and internal carotid artery, as shown in Figure 2.2. Figure 2.2 also shows that the intraluminal placement of the surgical suture blocks all blood flow into the MCA. The surgical suture can be introduced from the common carotid artery (CCA) or from the external carotid artery (ECA). After the period of ischaemia, the suture can be withdrawn to allow reperfusion. Following the withdrawal of the suture, in the case where the surgical suture is introduced from the CCA, the ischaemic MCA territory will be perfused by blood sources from contralateral arteries through the posterior cerebral artery (PCA) and anterior cerebral artery (ACA). In the case where the suture is introduced from the ECA, the reperfusion blood sources are supplied from the collateral CCA as well as from the contralateral arteries through the PCA and ACA.

The diameter of the surgical suture employed to block the MCA is a crucial determinant of the extent of the reduction of cerebral blood flow (CBF) in the MCA territory, and thereby the size of the ischaemic lesion (Takano et al., 1997). To avoid tearing the arteries and a subsequent subarachnoid haemorrhage during the
procedure, the tip of the surgical suture must be blunted by heating near a flame. As a result, the tip has a larger diameter than that of suture body, which is not heated. After the intracranial placement of the suture, the position of the tip in the arterial lumen will greatly influence the results. Also the difference in the diameter and quality of commercially available sutures significantly affects the lesion size postmortem (Laing et al., 1993). It is suggested by Takano et al. (1997) that in the rat MCAO model, the silicone-coating can ensure the consistency of the diameter of the suture, therefore reducing the variability of the lesion size. However, there is concern that the use of silicone coated suture (using 8-0 suture) in the mouse model has not been well standardized due to high variability in the lesion size produced (Tsuchiya et al., 2003).

Initially rats were used to set up this model since rats have a larger body size than mice, and the middle cerebral artery occlusion (MCAO) surgery is easier and intravenous dosing and observations of arterial blood flow are readily possible. In this model, the rounded and heat-enlarged tip of the surgical suture should be blocking the origin of MCA. However, the rats used in this study were shown have a secondary MCA and an extra small artery paralleling the MCA (Figure 2.3), which led to massive variation in outcome. As a result, a reproducible MCA occlusion model could not be established on those rats and therefore, CD-1 mice, which were found to have suitable cerebrovascular anatomy, as shown in Figure 2.2, were chosen and used throughout this study.

As mentioned before, the silicone-coating method to prepare the occluder is not applicable for the mouse model, therefore, in the present study, a special monofilament (occluder) with a long blunted tip, which was of 4 mm length and consistently 0.15 mm diameter, was made to ensure the consistency of the mouse model.
Figure 2.2 Diagram of cerebrovascular anatomy in rats and mice illustrating the extracranial and intracranial vasculature exploited in this method of focal middle cerebral artery occlusion. Vessel size is disproportionately enlarged for clarity. Common, external and internal carotid arteries and their branches are shown. Mid., middle; a., artery; Com., common; Ext., external; Int., internal. Figure modified from Longa et al. (1989).
Figure 2.3 Low and high magnification photograph of the cerebrovascular anatomy surrounding the base of the brain of a Wistar rat weighing 310 g from Bioresources Unit Trinity College Dublin. A: small extra artery paralleling the MCA, B: first MCA, C: secondary MCA.
2.1.5 Factors modulating the severity of ischaemic brain injury in focal models of cerebral ischaemia

As previously mentioned, one of the main requirements of a given focal cerebral ischaemia model is the reproducibility, thereby the variables that could modify the effects of ischaemia must be monitored and kept constant as far as possible.

2.1.5.1 The location of an occlusion

The location and size of the lesion produced depends on the artery occluded and the site where the blood flow is stopped. The residual cerebral blood flow (CBF), which is dependent on collateral replacement arteries, is also important. In the MCA occlusion model in rodents, which have an excellent Willis' circle and highly effective collateral circulation, the site of the occlusion is particularly vital (Lecinana et al., 2001). If the flow is stopped in the distal portion of the ICA, there is no infarction, since the MCA territory can be supplied by the contralateral carotid artery through the ACA (Figure 2.2). Occlusions of proximal or distal portions of the MCA cause infarctions to a various extent mainly depending on the location of the occlusion (Chen et al., 1986; Brint et al., 1988). It is therefore of great importance to ensure consistency of the occlusion site in this model.

2.1.5.2 The duration of ischaemia

The brain lesion occurs after the regional CBF is stopped and the size of the lesion is related to the duration of the ischaemia. Although most of the tissue damage occurs within 6-7 hours, spread of the lesion continues to progress for up to 7 days after the CBF is stopped (Lecinana et al., 2001). Therefore, the animals were always killed after the same survival time to ensure consistency in the focal cerebral ischaemia models in the present study.

2.1.5.3 Temperature
Temperature has been shown to have an effect on the extent of the ischaemic brain injury. Hypothermia is widely reported to exert neuroprotection, experimentally (Busto et al., 1989). Small differences in intraischaemic brain temperature critically determine the extent of ischemic neuronal injury (Busto et al., 1987). Clinical trials demonstrated benefit from even mild hypothermia in patients with hypoxic-ischaemic encephalopathy (Hypothermia after Cardiac Arrest Study Group, 2002). On the other hand, hyperthermia has an aggravating effect since it increases the metabolic demand of the tissue and local acidosis. Therefore efforts were made to keep body temperature constant, remaining within normal ranges in the present study.

2.1.5.4 Blood glucose

It is well known that during ischaemia, glucose is metabolized anaerobically, which leads to lactic acid accumulation. Ischaemic brain injury can be increased by hyperglycemia through exacerbating tissue acidosis. The harmful effect of hyperglycemia has been demonstrated in animal models of focal ischaemia (Nedergaard, 1987).

2.1.5.5 Arterial blood gases and Arterial pressure

Oxygen (PaO₂) and carbon dioxide (PaCO₂) partial pressure values in arterial blood have an influence on the size of the lesion caused (Watson et al., 1997; Siesjo, 1992). During focal cerebral ischaemia, the functional disorder triggered by ischaemia in the arterioles impairs the mechanisms regulating the regional CBF. Therefore the local CBF becomes directly dependent on perfusion pressure. If there is a decrease of the arterial blood pressure, the residual pressure in the penumbra area is compromised, leading to extension of the infarction (Shima et al., 1983). Therefore measurement of arterial blood gases and arterial blood pressure are recommended in experimental focal cerebral ischaemia models in animals where it is applicable, such as in rats and cats.
2.1.6 Assessments of the ischaemic brain injury in the focal ischaemia models

In a given experimental model, there are many methods available to assess the effect of focal cerebral ischaemia. The methods most commonly used are histological assessments and functional assessments.

2.1.6.1 Histological assessments

The extent of tissue affected by ischaemia can be identified by different staining techniques including hematoxylin-eosin, 2, 3, 5-triphenyltetrazolium chloride (TTC), cresyl violet, and immunohistochemistry. It can also be identified by other techniques not requiring brain samples for histological processing, such as magnetic resonance imaging (Tatlisumak et al., 1996).

The 2% TTC method is a well-established and widely used staining technique in experimental focal ischaemia models. It is convenient, reliable, inexpensive, and easy. The infarct volumes detected by the use of TTC staining have been tested against a wide range of more traditional histological markers of infarction (Bederson et al., 1986; Lundy et al., 1986; Hatfield et al., 1991; Lin et al., 1993). As described by Lippold (1982) and his co-workers, when TTC is applied, it is reduced to red formazan by mitochondrial enzymes (specifically succinate dehydrogenase). As the mitochondrial enzymes in living brain tissue reduce TTC to red formazan, the color of the intact brain tissue rapidly (in minutes only) is converted into red whilst already infarcted (dead) brain tissue cannot reduce TTC and remains unstained (pale). The border between stained and unstained tissues is well demarcated and can be identified easily by visual inspection. This sharp colour contrast allows for photographic procedures and computer-based measurements of brain infarct volumes. In addition, the amount of brain oedema can easily be calculated from the same brain slices without dry-weight or other complicated methods (Swanson et al., 1990; Tatlisumak et al., 1998). Therefore the TTC staining was used in the present study.
2.1.6.2 Functional assessments

The majority of cerebral ischaemia research has focused on preventing or reducing morphological and physiological damage subject to ischaemia insult. Usually, the protocol of experimental stroke investigations involves histological examination of the post-stroke damage in animal models, with or without a pharmacological treatment. Recently, many stroke researchers have expanded their techniques to assess the behavioural profiles in animal models and examine the correlation of histologically-determined stroke damage in animal models. This represents an important step forward, because a potentially beneficial compound should maintain or restore brain function after stroke but have few side effects. Therefore a range of behavioural tests were used in the present study.

A very commonly used method is to assess neurological deficits by using a scoring system. Such scales typically involve assigning each animal a score from zero to 4 (no deficit to severe deficit, respectively) based on performance in a series of tasks that assess posture, and circling behavior (Hatcher, 2002). For example, when suspended by the tail, a normal animal will typically hang straight down and extend both forelimbs toward the ground. An animal that has been subjected to MCAO may flex the forelimb contralateral to the infarct toward its abdomen or in some cases rotate the contralateral shoulder or limb medially. Contralateral forelimb flexure caused by MCAO was found to correlate with the extent of cortical damage (Aronowski et al., 1996). Using the neurological scoring system to assess neurological deficits following MCAO has at least two advantages, including: (1) the technique is not time consuming in general, and (2) it is simple and does not require special equipment or extensive personnel training.

The rotarod test is a well-established procedure for testing balance and coordination aspects of motor performance in rats and mice. Evidence has indicated that the rotarod task is a more sensitive index for the assessment of motor impairment induced by traumatic brain injury in rodents than both the beam-walking and beam-balancing tasks (Hamm et al., 1994). Deficits in motor performance on the rotarod
task have been observed from 24 hours to 2 months after the induction of focal cerebral ischaemia and increasing infarct volume was thought to be significantly correlated with impaired rotarod performance in a rat focal ischaemia model (Rogers et al., 1997).

Locomotor activity has also been used in the assessment of cerebral ischaemia. In global ischaemia models, ischaemia – induced hyperactivity was reported by many researchers. This hyperactivity in the global models was linked with hippocampal damage (Wang & Corbett, 1990). In focal cerebral ischaemia models in rats, permanent occlusion of the middle cerebral artery of rats increases locomotor activity in an open field test. Locomotor activity as measured by wheel running is also elevated during the first seventeen days following permanent occlusion of the right MCAO, then returns to control value (Robinson et al., 1979). However, using a photocell apparatus, there is no significant difference in locomotor activity reported between the control group and animals subjected to MCAO (Grabowski et al., 1991). In contrast to rats, mice subjected to permanent MCAO exhibit a significant decrease in locomotor activity 24 h after surgery (Hunter et al., 2000; Hatcher et al., 2002). Various neurotransmitter systems have been implicated in locomotor activity, including the glutamatergic, dopaminergic, serotonergic & noradrenergic systems (Irifune et al., 1995; Ouagazzal et al., 1995; Yan et al., 1997). Assessment of a drug’s effect on altered LMA in animals subjected to cerebral ischaemia is of importance for evaluating their neuroprotective potential.

2.1.6.3 Mortality rate

As a result of the severity of the lesion produced, animal models of focal cerebral ischaemia show mortality. If a given drug can reduce the mortality due to brain damage, this may suggest that damage severity is decreasing and the compound has a protective action against cerebral ischaemia.
2.2 PERMANENT FOCAL CEREBRAL ISCHAEMIA MODEL

2.2.1 Animals

Studies were carried out on male CD-1 mice weighing 35 g to 40 g. Mice were obtained from Bioresources Unit, Trinity College Dublin (used in all of the work on N\(^1\)-dansyl-spermine and MK-801 in the permanent focal cerebral ischaemia model) and later from University College Dublin (used in the rest of the work). Mice were housed in groups of 5-10 for at least a week before commencement of the experiment. Standard laboratory water and food were available ad libitum. The mice were maintained at an ambient temperature of 21 ± 1 ° centigrade under a standard 12-h light: 12-h dark cycle (light on 07:00h). Following surgery, animals were individually housed. All experiments were conducted according to the requirements of Cruelty To Animals Act, 1876, European Community Directive, 86/609EC.

2.2.2 Preparation of intraluminal monofilament

To prepare the intraluminal monofilament, a 4 mm portion of a 6-0 surgical suture (Ethicon, UK) (diameter of 0.10 mm), was inserted into a 26 G needle, and then the needle with the suture was placed near a heated soldering iron for 20 seconds thus enlarging the diameter of the 4 mm portion of the suture. Under an operation microscope, the diameter of suture along the heated 4 mm portion was checked using a stage micrometer and it was consistently found to be around 0.15 mm owing to the special nature of the type of suture and heating method. The very end of the 4 mm tip was carefully blunted again by heating near the soldering iron under the microscope to reduce the incidence of tearing arteries when used. This later heating procedure did not change the diameter of the tip.

2.2.3 Permanent middle cerebral artery occlusion procedure

The anaesthetic used was 12% chloral hydrate given at a dose of 400 mg kg\(^{-1}\), i.p. It took around 5 to 10 minutes for the animals to become surgically anesthetized.
Middle cerebral artery occlusion was then performed using an intraluminal monofilament. Under an operating microscope (World Precision Instrument INC, USA), the right common carotid artery (CCA) and the right external carotid artery (ECA) were exposed through a ventral midline neck incision, isolated from the surrounding nerves and fascia, and ligated proximally and permanently using a silk suture (6-0). Next, the internal carotid artery (ICA) was dissected carefully from the adjacent vagus nerve and silk sutures were looped around the CCA and the ICA. While gently pulling the loop of the silk suture around the ICA to prevent bleeding, an arteriectomy was made in the CCA near the carotid bifurcation. The monofilament was inserted through the arteriectomy and the silk suture looped around the CCA was tightened slightly around the monofilament to prevent bleeding. The silk suture around the ICA was removed, and then the surgical suture was advanced into the ICA to a point approximately 10mm distal to the carotid bifurcation. Mild resistance indicated that the suture entered the anterior cerebral artery (ACA), thus occluding the origin of the middle cerebral artery, blocking all sources of blood flow from ICA, ACA, and PCA (the posterior cerebral artery). The suture was firmly secured in place by tightening again the silk suture around the CCA, the wound was cleaned using cotton wool and saline, and the incision was closed. The sham-operated group underwent the same surgical procedure but the monofilament was not inserted. Body temperature was maintained at 36.5 to 37.5° centigrade by using a thermostatically controlled heating blanket (Harvard Homoeothermic Blanket Control Unit) throughout the procedure from the start of the surgery until the animals recovered from the anaesthesia. Following surgery, the animals were kept in a cage with a heating lamp, which maintained the cage temperature between 29 and 30° centigrade for another 2 hours to counteract any possible hypothermic effect of the drugs.

2.2.4 Compounds

MK-801 was obtained from Tocris, U.K. and N'-dansyl-spermine was synthesized in-house (TCD), as described by Seiler (1998). The 5 novel polyamine analogues
used were a gift from Brock University, Canada. The full names of the 5 polyamine analogues are as follows:

BU31b, 
2(E)-N-(12-aminododecyl)-3-(4-hydroxyphenyl)-prop-2-enamide

BU37b, 
2(E)-N-{3-[4-(3-aminopropyl)piperazin-1-yl]-propyl}-3-(4-hydroxyphenyl)prop-2-enamide

BU33b, 
(2E)-N-{3-[4-(3-aminoproxy)-butoxy]propyl} -3-(4-hydroxyphenyl)prop-2-enamide

BU36b, 
2(E)-N-{3-[4-aminocyclohexyl]amino}-propyl]-3-(4-hydroxyphenyl)prop-2-enamide

BU43b, 
2(E)-N-[3-({4-[(3-aminopropyl)amino]-cyclohexyl} amino) propyl]-3-(4-hydroxyphenyl)prop-2-enamide

Drug BU31b and BU37b were dissolved in 0.9% sterile saline, and then placed in an ultra-sonic bath (Decon FS4006) at room temperature for 10 minutes. 2 drops of Tween 80 were added to aid solubilisation before final administration. The rest of the compounds were dissolved in 0.9% sterile saline. All compounds or saline were administered intraperitoneally, in a dose volume of 10 ml kg⁻¹, 30 minutes before the MCA (middle cerebral artery) occlusion.

2.2.5 Assessments

2.2.5.1. Neurological deficit scoring
Neurological deficits were measured according to the following graded scoring system 24 hours after the MCAO. The scoring system was based on an equivalent system used by Hatcher et al, 2002. Mice were held gently by the tail, suspended one meter from the floor, and observed for the forelimb flexion. Normal mice extended both forelimbs towards the floor. Mice that extended both forelimbs towards the floor and had no other neurological deficit were assigned grade 0; mice with infarction consistently flexed the forelimb contralateral to the injured hemisphere; posture varied from mild wrist flexion and shoulder adduction with extension at the elbow to severe posturing with full flexion of wrist, elbow, and adduction with internal rotation of the shoulder. Mice with any amount of consistent forelimb flexion and no other abnormality were graded 1; mice were then placed on the floor, while held by the tail with feet on the floor, to observe the circling behaviors. If the mice circled to the contralateral side consistently, then the mice were graded 2; then the mice were released and allowed to move freely on the floor to continue the observation of the circling behaviors. Mice with consistent spontaneous circling towards the paretic side were graded 3; mice without spontaneous motor activity were graded 4.

2.2.5.2 Spontaneous locomotor activity (LMA) test

Approximately 23.5 hours after the occlusion, mice were placed in an activity monitor (AM1051 Activity Monitor, Benwick Electronics, Essex, UK), which consisted of 5 clear perspex cages (42 x 21 x 20 cm) each positioned within a frame equipped with infrared beams along its length and width. The fine beam setting was used which produces a grid of 12 beams by 7 beams. When a beam is broken, depending on the speed and how long it remains broken, events are added to different count registers. The parameter used in this case was slow mobile activity. Total beam breaks associated with slow mobile activity were recorded in 5 min time bins over a period of 30 minutes.

2.2.5.3 Rotarod assessment
Following LMA assessment, rotarod performance was assessed to test balance and coordination. The rotarod comprised of a drum that rotated at two speeds, 2 or 4 revolutions per minute. Mice were placed individually on the rotating drum (Palmer Recording Drum, U.K.). Once they were balanced, the drum was switched on at a speed of 2 rotations per minute. The time in seconds at which each animal fell from the drum was recorded for up to 60 seconds using a stopwatch. If the mouse could stay on the drum at the first speed for 60 seconds, the second speed was used for up to 60 seconds. The two time totals were added as the final result for the mouse.

2.2.5.4 Histological assessments

Hemisphere lesion volume (% HLV) and oedema assessments

24 hours after the occlusion, the animals were killed by cervical dislocation; their brains were removed, washed in saline solution and frozen at –18 ° centigrade for 5 minutes. Then each brain was placed on a calibrated scale with 1.7 mm divisions. Each mouse cerebrum had a coronal length of approximately 8.5 mm. The cerebrum was cut into 5 individual 1.7 mm slices using a surgical blade under a microscope. The brain slices were moved into 2% TTC solution and incubated for 10 minutes at 37 ° C in the dark. After staining, the slices were submerged in 10% formalin solution over night and fixed. To calculate infarct volume, the slices were photographed using a microscope with a digital camera and the digital images were analyzed using image analysis software (UTHSCSA, image Tool, downloaded from http://ddsdx.uthscsa.edu/dig/itdesc.html). The hemispheric lesion volume was calculated by multiplying the area by the thickness of slices. Finally, %HLV and oedema were calculated by the following equations, based on that used by Tatlisumak et al., 1998. %HLV = {([Total infarct volume- (Right Hemisphere Volume-Left hemisphere Volume)]/Left hemisphere volume} x 100%; Oedema = (Right Hemisphere Volume-Left hemisphere Volume).

2.2.5.5 Mortality rate

The number of animals which underwent successful MCAO surgery but died within 24 hours after the MCAO was recorded. Animals died during surgery or found to
have subarachnoid haemorrhage due to the rupture of arteries were excluded from
the study, but a record of number of cases was kept.

2.2.6 Statistical analysis

Data was expressed as the mean ± s.e.mean, except for the neurological deficit score
and the mortality rate. The neurological deficit score was shown as median ± IQR
(Inter quartile range). Statistical analysis of the neurological deficit score data was
carried out using the Mann-Whitney U-test. The proportionality test was used to
analyse the mortality rate. Statistical analysis of differences between control and
treated groups for all other parameters was carried out using ANOVA with
Bonferroni post-hoc analysis in the first instance, and later using Student's t-test
(two-tailed, unpaired). This study was designed to examine the effect of pre-
ischaemic administration of drugs at a range of doses. The big number of groups led
to difficulties with statistical analysis using ANOVA. Student's t-test was used to
assess the significance of the effect of individual doses of each drug versus control.
A probability value of less than 0.05 was considered statistically significant.
2.3 TRANSIENT FOCAL CEREBRAL ISCHAEMIA MODEL

2.3.1 Animals

Animals used were CD-1 mice, obtained from University College Dublin, weighing 35g - 40g. Mice were housed in groups of 5 - 10 for at least a week before commencement of the experiment. Standard laboratory water and food were available ad libitum. The mice were maintained at an ambient temperature of 21 ± 1 ° centigrade under a standard 12-h light: 12-h dark cycle (light on 07:00h). Following surgery, animals were individually housed. All experiments were conducted according to the requirements of Cruelty To Animals Act, 1876, European Community Directive, 86/609EC.

2.3.2 Transient middle cerebral artery occlusion procedure

The intraluminal monofilament was made in the same way as described in section 2.2.2. The anaesthetic used was 12% chloral hydrate given at a first dose of 400mg kg⁻¹, i.p. To meet the demand of the long-term anaesthesia in this procedure, a further quarter of the first dose of chloral hydrate was administered during the procedure as required to maintain anaesthesia (Approximately speaking, the further dose was given 40 minutes after the first dose). The surgical procedure was the same as that described in the permanent middle cerebral artery occlusion procedure (see section 2.2) with the following amendments. After the insertion of the monofilament (thereby the start of the MCA occlusion), the animals were kept under surgical anaesthesia for a period of 30 minutes. After that period of MCAO, the silk suture tightened around the CCA with the monofilament was released and the monofilament was withdrawn to allow reperfusion into the MCA. The silk suture around the CCA was then firmly tightened to prevent bleeding. The wound was cleaned using cotton wool and saline, and the incision was closed. Body temperature was maintained at 36.5 to 37.5° centigrade by using a thermostatically controlled heating blanket throughout the procedure from the start of the surgery, during the period of MCA occlusion, until the animals recovered from the anaesthesia. Following surgery, the
animals were kept in a cage with a heating lamp, which maintained the cage temperature between 29 and 30° centigrade for another 2 hours to counteract any possible hypothermic effect of the drugs.

2.3.3 Compounds

The compounds used in the transient focal cerebral ischaemia model were MK-801, N\(^1\)-dansyl-spermine, BU31b, BU37b, BU33b, BU36b and BU43b. They were dissolved in the same way as that described in the permanent focal cerebral ischaemia model (section 2.2.4). All the compounds or saline were administered intraperitoneally, in a dose volume of 10 ml kg\(^{-1}\), 30 minutes before the MCA (middle cerebral artery) occlusion.

2.3.4 Assessments

Assessments performed were the spontaneous locomotor activity (LMA) test, rotarod assessment, mortality rate, histological assessments including hemisphere lesion volume (% HLV) and oedema assessments. The procedure of each assessment was the same as that described in the permanent focal cerebral ischaemia model (section 2.2.5).

2.3.5 Statistical analysis

The statistical methods were the same as those used in permanent model experiments. See section 2.2.6.
2.4 TISSUE POLYAMIN PROFILE IN ISCHAEMIA

2.4.1 Animals

The animals used were the same as described in section 2.3.1.

2.4.2 Permanent and transient MCAO

Permanent MCAO was carried out on in a group of 9 animals using the same procedure as that described in section 2.2.2 and another group of 9 animals underwent transient MCAO with 23.5 reperfusion 30 minutes after the occlusion (see section 2.3.2).

2.4.3 Dissection of mouse brain and tissue preparation

24 hours after the start of the MCAO, the animals (8 in each group) were killed by cervical dislocation followed by decapitation. After the scalp and the skull were carefully removed to reveal the brain, a small spatula was placed under the brain and agitated in such a manner as to sever all the neuronal connections but without causing any lesions or damage to the brain. Each brain was then removed and placed into a petri dish and kept in the freezer for 10 minutes to make it hard and easier for handling. A surgical blade was used to separate the ischaemic hemisphere and non-ischaemic hemisphere. From each hemisphere, the following 4 regions – hypothalamus, hippocampus, cerebral cortex and midbrain were carefully removed and the rest of the regions were discarded (see figure 2.4). The method of dissection was adapted from Glowinski & Iversen (1966).
Figure 2.4 Diagrammatic representation of the procedure for the dissection of the mouse brain, adapted from Glowinski & Iversen (1966).
Each brain region was placed into a 1 ml Eppendorf tube and then homogenized with 50 μl of 0.4 M perchloric acid and 450 μl HPLC grade distilled water. The samples were then placed in an ultra-sonicator for 15 minutes and then vortexed for 1 minute before being centrifuged at 4,000 rpm for 15 minutes. 200 μl of the clear supernatant (100 μl for the analysis of putrescine, spermidine and spermine; 100 μl for the analysis of N1-acetylspermidine) was carefully removed and frozen until dansylation.

2.4.4 Measurement of tissue polyamines

The methods of polyamine HPLC assay used in the present study were based on published methods (Kabra et al., 1986; Hunter, 1998).

2.4.4.1 Standard curves preparation

A standard series of polyamine solutions containing 1.25, 2.5, 5, 7.5, 10, 12.5, 25, 37.5 and 50 μM putrescine, spermidine and spermine were made. A standard series of N1-acetylspermidine solution (0.0625, 0.125, 0.25, 0.375, 0.5, 0.625 and 1.25 μM) was made. 100 μl of each was used for dansylation. For the blank, HPLC water was used in place of the polyamine standards.

2.4.4.2 Dansylation

The 100 μl of supernatant or standard polyamine solution or N1-acetylspermidine solution was placed into a cryovial and 150 μl of borax buffer, pH 10.2 (Boric acid was dissolved in HPLC grade distilled water and spiked with 0.04 M NaOH), 50 μl of internal standard (1, 7-diaminoheptane, the concentration used was higher for the analysis of putrescine, spermidine and spermine than for N1-acetylspermidine) and 25 μl of dansyl chloride were added. The cryovial was vortexed and placed in a water bath at 60 ° centigrade in the dark for 30 minutes. 10 μl of proline (100 mg ml⁻¹) was added to each cryovial. The cryovials were then incubated for a further 30 minutes at 60 ° centigrade in the dark.
The cryovials were then left at room temperature for 10 minutes before 200 μl of toluene was added to each cryovial. The samples were then vortexed thoroughly and 100 μl of the top yellow layer was removed and placed into new vials. A vacuum centrifuge (Harrier model 15/80) was used to remove the toluene at 50 ° centigrade for 20 minutes. The dry samples left were immediately dissolved in 0.5 ml acetonitrile and 6 μl was injected into the HPLC system (for detail of this system, see section 2.4.4.3) for analysis, or otherwise the dry samples were stored at -18 ° centigrade for assaying later.

2.4.4.3 HPLC analytical technique

The equipment used and the corresponding parameters are, system controller: SCL-10A VP SHIMADZU HPLC; HPLC pumps: 2 X LC-10A VP SHIMADZU; auto injector: SIL-10AD VP SHIMADZU; Fluorescence detector: RF-10A XL SHIMADZU (Excitation = 340 nm, Emission = 510 nm).

The stationary phase and elution system were different for the three main polyamines (putrescine, spermidine, and spermine) and N1-acetylspermidine. They are described separately below.

For the analysis of putrescine, spermidine and spermine, the stationary phase used was a Phenomenox LUNAR C18 (33mm x 3.3 mm) with Phenomenox guard column. The mobile phase consisted of water and solvent B (acetonitrile) and the gradient elution system used was as follows, 59% solvent B for 2 minutes, 59% solvent B to 100% solvent B over 3.40 minutes, 100% solvent B for 2 minutes, 100% solvent B to 59% solvent B over 0.6 minute, 59% solvent B for 2 minutes.

For the analysis of N1-acetylspermidine, the stationary phase used was a Phenomenox LUNAR C18 (250 mm x 4.6 mm). The mobile phase consisted of water and solvent B (acetonitrile) and the Gradient Elution System used was as follows, 60% solvent B for 12 minutes, 60% solvent B to 80% solvent B over 1 minute, 80%
solvent B for 13 minutes, 80% solvent B to 60% solvent B over 1 minute, 60% solvent B for 9 minutes.

2.4.4.4 Data calculation

Levels of putrescine, spermidine and spermine were calculated with reference to known standards of putrescine, spermidine, and spermine and corrected with reference to the internal standards. From the polyamine and the internal standard chromatograph, the response factor (R) was calculated. (Response factor) \( R = \frac{C}{A} / \frac{Ci}{Ai} \). \( C \) = concentration of polyamine standard (\( \mu M, \text{nmol ml}^{-1} \)); \( A \) = peak area of polyamine standard; \( Ci \) = concentration of internal standard (\( \mu M, \text{nmol ml}^{-1} \)); \( Ai \) = peak area of internal standard. The concentration of the polyamine in the 100 \( \mu l \) supernatant (for tissue polyamines) was determined. \( Cm = Am \times R \times \frac{Ci}{Ai} \). \( Cm \) = concentration of polyamine in the 100 \( \mu l \) supernatant (\( \mu M, \text{nmol ml}^{-1} \)); \( Am \) = peak area for supernatant; \( Ci \) = concentration for internal standard (\( \mu M, \text{nmol ml}^{-1} \)); \( Ai \) = peak area for internal standard. Finally, the tissue polyamine concentration (\( Ct, \text{nmol g}^{-1} \)) was calculated. \( Ct = \frac{(Cm \times V)}{W} \). \( V = 0.5 \text{ ml} \) (volume of the tissue homogenate); \( W = \) the weight of the tissue (g).

For the analysis of N\(^1\)-acetyl spermidine, the level of N\(^1\)-acetyl spermidine was calculated only with reference to the known standard. The internal standard was not used as reference since it was found to be not well separated from unknown components in the sample by the HPLC system. The calculation was as follows. \( Cm = (C / A) \times Am \). \( Cm \) = concentration of N\(^1\)-acetyl spermidine in the 100 \( \mu l \) supernatant (\( \mu M, \text{nmol ml}^{-1} \)); \( C \) = concentration of standard N\(^1\)-acetyl spermidine (\( \mu M, \text{nmol ml}^{-1} \)); \( A \) = peak area of standard N\(^1\)-acetyl spermidine; \( Am \) = area of supernatant. Finally, the tissue N\(^1\)-acetyl spermidine concentration (\( Ct, \text{nmol g}^{-1} \)) was calculated. \( Ct = \frac{(Cm \times V)}{W} \). \( V = 0.5 \text{ ml} \) (volume of the tissue homogenate); \( W = \) tissue weight (g).

2.4.5 Statistical analysis
Data was expressed as the mean ± s.e.mean. The difference of polyamine levels between the ischaemic hemisphere and the non-ischaemic hemisphere was analyzed by two-tailed paired Student’s t-test. A probability value of less than 0.05 was considered statistically significant.
2.5 EXTRACELLULAR POLYAMINE PROFILE FOLLOWING PERMANENT CEREBRAL ISCHAEMIA: A MICRODIALYSIS STUDY

2.5.1 Preparation of animals

Animals used were the same as described in section 2.3.1. Body temperature was maintained at 36.5 to 37.5° centigrade by using a thermostatically controlled heating blanket (Harvard Homoeothermic Blanket Control Unit) throughout the procedure from the start of the surgery until the end of the microdialysis procedure.

2.5.2 Permanent focal cerebral artery occlusion

Similarly to the procedure described in section 2.3.2, 12% chloral hydrate was used as the anaesthetic. The anaesthetic was given at a first dose of 400mg kg⁻¹, i.p. however after that, one fifth of the first dose of chloral hydrate was repeatedly administered during the whole procedure (including the permanent focal cerebral artery occlusion and the microdialysis procedure when it was required) (Approximately speaking, 40 minutes after the first dose, one fifth of the first dose was repeatedly given every 20 minutes). The middle cerebral artery occlusion procedure was the same as described in section 2.2.3. After the occluder was inserted and secured, the wound was cleaned and the incision was closed, the animal was immediately moved to a stereotaxic frame for the microdialysis procedure.

2.5.3 Procedure for microdialysis

Microdialysis probes (MAB 4, PES, membrane length 2 mm, outer diameter of the membrane 0.24 mm, Microbiotech) were perfused continuously with a physiological salt solution (Baskaya et al., 1997) (NaCl 147.1 mM, KCl 4.0 mM, CaCl₂ 2.25 mM) at a constant rate of 2.0 μl min⁻¹ using a micro-infusion pump (Model CMA/100, Biotech Instrument Limited). The mouse subjected to the middle cerebral artery occlusion was immediately mounted in the stereotaxic frame. Two holes were drilled using a dental drill through the skull (from bregma: 3.8 mm lateral, 0.5 mm
posterior), one on the right and the other on the left. Two microdialysis probes were separately placed to a depth of 2 mm into either the ischaemic cortex or the non-ischaemic cortex using the stereotaxic micro-manipulator arm. After 30 minutes of the middle cerebral artery occlusion, collection of dialysate samples was started and samples were collected using the sample collector (Model CMA/142 Microfraction Collector, Biotech Instrument Limited) every half an hour for 2.5 hours (5 fractions for every probe). After that, Gentian Violet dye was injected into the probes, the animal was killed by cervical dislocation and the brain was checked to confirm the position of the probes.

To measure the recovery rate of polyamines across the dialysis membrane at the perfusion rate 2.0 µl min⁻¹, the probe was placed in a 0.5 µM stock polyamine solution (containing putrescine, spermidine and spermine 0.5 µM each). Three probes were tested. 30 minute sample fractions were collected. The average of the recovery rate of each polyamine was calculated from the results of the three probes.

2.5.4 Measurement of the extracellular polyamines

2.5.4.1 Standard curves preparation

A standard curve was prepared as described in 2.4.4.1. However, a standard series of much higher sensitivity polyamine solutions containing 0.0625, 0.125, 0.25, 0.375, 0.5, 0.625 and 1.25 µM was made. 60 µl of each was used for dansylation. For the blank, HPLC water was used in place of the polyamine standards.

2.5.4.2 Dansylation

The 60 µl of dialysate or standard polyamine solution was dansylated using the same procedure as described in section 2.4.2, except that the volume of every reagent used in the procedure was adjusted by a ratio of 60/100 according to the sample volume difference between the supernatant of the tissue samples (100 µl) and the dialysate samples (60 µl). The details are described below.
The 60 µl of dialysate or standard polyamine solution was placed into a cryovial and 150 µl of borax buffer pH 10.2 (Boric acid was dissolved in distilled water and spiked with 0.04 M NaOH), 30 µl of internal standard (1, 7-diaminoheptane) and 15 µl of dansyl chloride were added. The cryovial was vortexed and placed in a water bath at 60 ° centigrade in the dark for 30 minutes. 6 µl of proline (100 mg ml\(^{-1}\)) was added to each cryovial. The cryovials were then incubated for a further 30 minutes at 60 ° centigrade in the dark.

The cryovials were then left at room temperature for 10 minutes before 120 µl of toluene was added to each cryovial. The samples were then vortexed thoroughly and 60 µl of the top solvent layer was removed and placed into new vials. Then a vacuum centrifuge (Harrier model 15/80) was used to remove the toluene at 50 ° centigrade for 20 minutes. The dry samples left were immediately dissolved by 300 ml acetonitrile and 6 µl was injected into HPLC system for analysis or otherwise the dry samples were stored at -18 ° centigrade for assay later.

2.5.4.3 HPLC analytical technique

Equipment used was the same as that used for polyamines analysis in brain areas in section 2.4.4.3. However, the stationary phase used was a Phenomenox LUNAR C\textsubscript{18} (250 mm x 4.6 mm). The mobile phase consisted of water and solvent B (acetonitrile) and the Gradient Elution System used was as follows, 60% solvent B for 12 minutes, 60% solvent B to 65% solvent B over 1 minute, 65% solvent B to 96% solvent B over 11 minutes, 96% solvent B to 70% solvent B over 2 minutes, 70% solvent B for 2 minutes.

2.5.4.4 Data calculation

Polyamine levels were calculated with reference to known standards of putrescine, spermidine, and spermine and corrected with reference to the internal standards. From the polyamine and the internal standard chromatograph, the response factor (R)
was calculated. (Response factor) \( R = \frac{C}{A} / \frac{Ci}{Ai} \). \( C \) = concentration of polyamine standard (\( \mu \text{M} \)); \( A \) = peak area of polyamine standard; \( Ci \) = concentration of internal standard (\( \mu \text{M} \)); \( Ai \) = peak area of internal standard. Subsequently, the concentration of the polyamine in the 60 \( \mu \text{l} \) dialysate was determined. \( Cm = Am \times R \times \frac{Ci}{Ai} \). \( Cm \) = concentration of the polyamine in the 60 \( \mu \text{l} \) dialysate (\( \mu \text{M} \)); \( Am \) = peak area for microdialysate; \( Ci \) = concentration for internal standard (\( \mu \text{M} \)); \( Ai \) = peak area for internal standard.

2.5.5 Statistical analysis

Data are shown as mean ± s.e.mean. The difference of polyamine levels between the ischaemic cortex and the non-ischaemic control cortex was analyzed by one-way ANOVA. A probability value of less than 0.05 was considered statistically significant.
Section 3 Pre-ischaemic effect of MK-801 and $N^1$-dansyl-spermine in a permanent focal cerebral ischaemia model

3.1 INTRODUCTION

NMDA over activation during cerebral ischaemia contributes to cellular damage (Gagliardi, 2000). NMDA receptor antagonists at polyamine recognition sites could offer neuroprotection, avoiding the adverse effects associated with competitive NMDA receptor antagonists and noncompetitive open channel blockers such as MK-801 (Dizocilpine). Recently, a number of new polyamine analogues have been synthesized. One such novel compound is $N^1$-dansyl-spermine. $N^1$-dansyl-spermine is a structural analogue of spermine with a dansyl group attached to the spermine backbone. $N^1$-dansyl-spermine has been shown to inhibit NMDA receptor activity in vitro (Chao et al., 1997) and to be a potent polyamine antagonist in vivo (Kirby et al., 2004). More recently, the neuroprotective effect of this compound in a gerbil global ischaemia model has been reported (Kirby & Shaw, 2004).

In this section, the effect of $N^1$-dansyl-spermine in a permanent focal cerebral ischaemia model in mice was investigated. The model used was the widely employed intraluminal suture MCAO model. As described in section 1.3, focal models producing brain infarction are said to have more relevance to acute ischaemic stroke since most strokes are focal. The middle cerebral artery (MCA) occlusion models have been suggested to be of particular relevance because this is the vessel most commonly affected in stroke victims. Furthermore, the intraluminal suture MCAO model does not require the invasive surgical exposure of the MCA for occlusion therefore it has gained high popularity.

For comparison, the effect of MK-801, a potent non-competitive antagonist of the NMDA receptor by an open-channel blocking action (Hargreaves et al., 1994) and gold standard of neuroprotective NMDA antagonists, was also investigated.
3.2 METHODS AND MATERIALS

The effects of $N^1$-dansyl-spermine or MK-801, when administered preischaemia, on %hemispheric lesion volume, oedema, neurological deficit scoring, rotarod performance, spontaneous locomotor activity and mortality rate were assessed. Only statistically significant data is graphed. The methods and materials used in this section were described in detail in Section 2.2.

3.3 RESULTS

3.3.1 Effect of MK-801 or $N^1$-dansyl-spermine in MCAO animals

3.3.1.1 Histological assessments

MK-801, dosed at 1 mg kg$^{-1}$ or 3 mg kg$^{-1}$, significantly reduced the %HLV from 48.7± 4.9 (MCAO control) to 30.3±7.0 and 29.1± 7.7, respectively ($P<0.05$ in both cases). MK-801 at the lowest dose of 0.3 mg kg$^{-1}$ did not significantly decrease the %HLV (Figure 3.1; Figure 3.5). The oedema volume in the saline treated MCAO group was 28.3±2.4 mm$^3$. Treatment with 0.3 mg kg$^{-1}$ or 3 mg kg$^{-1}$ of MK-801 did not significantly reduce the oedema, whilst the 1 mg kg$^{-1}$ dose had a significant effect (18.7±3.7 mm$^3$, $P<0.05$) (Figure 3.2).

$N^1$-dansyl-spermine significantly reduced the %HLV to 33.1±5.4 ($P<0.05$), 31.0±6.1 ($P<0.05$) or 30.3± 4.4 ($P<0.05$) when administered at 1 mg kg$^{-1}$, 2 mg kg$^{-1}$ or 5 mg kg$^{-1}$ respectively. At the lowest dose of 0.5 mg kg$^{-1}$, $N^1$-dansyl-spermine did not significantly decrease the %HLV. Similarly, no significant neuroprotection was afforded by the highest dose (10 mg kg$^{-1}$) of $N^1$-dansyl-spermine (Figure 3.3; Figure 3.5). The oedema in the 0.5 mg kg$^{-1}$, 1 mg kg$^{-1}$ and 2 mg kg$^{-1}$ dosing groups was significantly reduced (19.9±2.9 mm$^3$, $P<0.05$; 17.7±3.1 mm$^3$, $P<0.05$; and 18.8±2.3 mm$^3$, $P<0.05$ respectively) in comparison to the saline treated MCAO group. Also
interestingly, N\textsuperscript{1}-dansyl-spermine, administered at the highest doses of 5 mg kg\textsuperscript{-1} or 10 mg kg\textsuperscript{-1} did not significantly decrease the oedema (Figure 3.4).
Figure 3.1 Effect of MK-801 (i.p.) administered 30 min before the occlusion in MCAO mice on percentage hemisphere lesion volume (%HLV). Data are expressed as mean±se.mean (n=10-14). *P<0.05 versus (saline treated) MCAO control (two-tailed, unpaired Student’s t-test).
Figure 3.2 Effect of MK-801 (i.p.) administered 30 min before the occlusion in MCAO mice on oedema. Data are expressed as mean±se.mean (n=10-14). *P<0.05 versus (saline treated) MCAO control (two-tailed, unpaired Student’s t-test).
Figure 3.3 Effect of N\textsuperscript{1}-dansyl-spermine (i.p.) administered 30 min before the occlusion in MCAO mice on percentage hemisphere lesion volume. Data are expressed as mean±se.mean (n=14-15). *P<0.05 versus (saline treated) MCAO control (two-tailed, unpaired Student's t-test).
Figure 3.4 Effect of N\textsuperscript{1}-dansyl-spermine (i.p.) administered 30 min before the occlusion in MCAO mice on oedema. Data are expressed as mean±se.mean (n=14-15). *P<0.05 versus (saline treated) MCAO control (two-tailed, unpaired Student's t-test).
Figure 3.5 Typical Images of TTC-derived brain slices of mice after 24 hours middle cerebral artery occlusion indicating MK-801 and N\textsuperscript{1}-dansyl-spermine (given 30 min prior to occlusion) induced a neuroprotective effect on infarction volume. (A) MCAO Control animal; (B) MCAO animal administered 3 mg kg\textsuperscript{-1} of MK-801; (C) MCAO animal administered 2 mg kg\textsuperscript{-1} of N\textsuperscript{1}-dansyl-spermine. Slices are at 1.7 mm intervals in descending order from frontal pole (anterior to posterior). Scale bar= 3.5 mm.
3.3.1.2. Neurological deficit scoring

Neurological deficit score was significantly decreased at the highest dose (3 mg kg\(^{-1}\)) of MK-801 (2.0±1.0), versus saline treated MCAO controls (3.5±1.0; \(P<0.05\)), although MK-801 had no significant effect at 0.3 mg kg\(^{-1}\) or 1 mg kg\(^{-1}\) (Table 3.1).

A significant decrease in the neurological deficit score was observed in mice administered 1 mg kg\(^{-1}\) N\(^1\)-dansyl-spermine (2.5±0.29; \(P<0.05\)), whilst the 0.5, 2, 5 mg kg\(^{-1}\) and 10 mg kg\(^{-1}\) dosing did not afford a significant decrease (Table 3.2).
Table 3.1 The neuroprotective effect of MK-801 (i.p.) administered 30 min before the occlusion in permanent MCAO mice on neurological deficits scoring. n, animal number; Scores, neurological deficit scores. *P<0.05 versus (saline treated) MCAO control (Mann-Whitney U-test).

<table>
<thead>
<tr>
<th>Drug and doses treated</th>
<th>Saline</th>
<th>MK-801 (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>Scores</td>
<td>3.5±1.0</td>
<td>3.0±1.25</td>
</tr>
<tr>
<td>n=18</td>
<td>n=10</td>
<td>n=11</td>
</tr>
</tbody>
</table>

Table 3.2 The neuroprotective effect of N¹-dansyl-spermine (i.p.) administered 30 min before the occlusion in permanent MCAO mice on neurological deficits scoring. n, animal number; Scores, neurological deficit scores. *P<0.05 versus (saline treated) MCAO control (Mann-Whitney U-test).

<table>
<thead>
<tr>
<th>Drug and doses treated</th>
<th>Saline</th>
<th>N¹-dansyl-spermine (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>Scores</td>
<td>3.5±1.0</td>
<td>2.5±4.0</td>
</tr>
<tr>
<td>n=18</td>
<td>n=14</td>
<td>n=14</td>
</tr>
</tbody>
</table>
3.3.1.3. Spontaneous locomotor activity (LMA) test

MCA occlusion caused a significant decrease in spontaneous locomotor activity (LMA) (saline treated MCAO group 132±49) versus sham-operated controls 584±111; \( P<0.05 \). MK-801 did not significantly reverse the MCAO induced hypoactivity at any dose tested. In fact, MK-801 significantly reduced locomotor activity at the lowest dose tested (0.3 mg kg\(^{-1}\)) (40.3±9.7; \( P<0.05 \)) (Figure 3.6).

\( \text{N}^1 \)-dansyl-spermine had no significant effect on locomotor activity following MCA occlusion. However, there was a slight trend towards improvement with the three lowest doses tested (150±36; 172±35; 198±62; for 0.5, 1 and 2 mg kg\(^{-1}\) doses respectively) when compared with the saline treated MCAO group (Figure 3.7).
Figure 3.6 Effect of MK-801 (i.p.) administered 30 min before the occlusion in MCAO mice on locomotor activity. Data are expressed as mean±se.mean (n=6-10).* P<0.05 versus (saline treated) MCAO control, +P<0.05 versus (saline treated) sham-operated control (two-tailed, unpaired Student’s t-test).
Figure 3.7 Effect of N1-dansyl-spermine (i.p.) administered 30 min before the occlusion in MCAO mice on locomotor activity. Data are expressed as mean±se.mean (n = 4-14). *P<0.05 versus (saline treated) MCAO control, +P<0.05 versus (saline treated) sham-operated control (two-tailed, unpaired Student’s t-test).
3.3.1.4 Rotarod performance assessment

Following MCA occlusion, performance of the saline treated animals in the rotarod test was significantly impaired when compared with the sham-operated group (saline treated MCAO group 6.63±2.2 sec, versus sham-operated 110±9.0 sec; \( P<0.05 \)). MCAO animals treated with 1 or 3 mg kg\(^{-1}\) of MK-801 performed significantly better (44.4±14 sec, \( P<0.05 \); 52.5±14 sec, \( P<0.05 \) respectively) than the saline treated MCAO animals, although at 0.3 mg kg\(^{-1}\), MK-801 did not afford a beneficial effect (Figure 3.8).

\( N^1 \)-dansyl-spermine treated MCAO groups showed significantly improved performance in the rotarod test at all doses (0.5, 1, 2, 5 and 10 mg kg\(^{-1}\)), when compared with the saline treated MCAO group (54.6±14 sec, \( P<0.05 \); 69.3±10 sec, \( P<0.05 \); 43.0±12 sec, \( P<0.05 \); 36.5±13 sec, \( P<0.05 \) and 35.5±15 sec, \( P<0.05 \) respectively) (Figure 3.9).

3.3.1.5 Effect of MK-801 and \( N^1 \)-dansyl-spermine on mortality rate

The mortality rate in the saline treated MCAO control was 4 out of 22. For MK-801 treated groups, it was 0 out of 10, 2 out 13, 1 out 11 respectively for 0.3, 1, 3 mg kg\(^{-1}\) doses. There was no significant difference in the mortality rate when compared with saline treated MCAO control. Administration of \( N^1 \)-dansyl-spermine in the MCAO mice did not significantly affect the mortality rate (0 out of 14, 1 out of 15, 1 out of 16, 2 out of 20 and 3 out of 18 respectively for 0.5, 1, 2, 5, 10 doses).
Figure 3.8 Effect of MK-801 (i.p.) administered 30 min before the occlusion in MCAO mice on rotarod performance. Data are expressed as mean±se.mean (n= 6-10).* P<0.05 versus (saline treated) MCAO control, +P<0.05 versus (saline treated) sham-operated control (two-tailed, unpaired Student’s t-test).
Figure 3.9 Effect of N₁-dansyl-spermine (i.p.) administered 30 min before the occlusion in MCAO mice on rotarod performance. Data are expressed as mean±se.mean (n= 4-14). *P<0.05 versus (saline treated) MCAO control, +P<0.05 versus (saline treated) sham-operated control (two-tailed, unpaired Student’s t-test).
3.3.2 Effect of MK-801 or \( N^1 \)-dansyl-spermine in sham-operated animals

Sham-operated animals administered MK-801 at 0.3, 1 or 3 mg kg\(^{-1}\) showed a significant decrease in locomotor activity (181±28, \( P<0.05 \); 170±33, \( P<0.05 \) and 168±38, \( P<0.05 \) respectively), when compared to sham-operated controls administered saline (584±111) (Figure 3.10). Similarly, in the rotarod test, MK-801 dosed at 0.3, 1 or 3 mg kg\(^{-1}\) caused a significant decrease in rotarod performance compared with the sham-operated controls (61.5±14, \( P<0.05 \); 58.2±18, \( P<0.05 \) and 53.0±15, \( P<0.05 \) respectively), compared to (110 ±9.9 for controls) (Figure 3.11).

In contrast, \( N^1 \)-dansyl-spermine produced no significant effect in locomotor activity at any dose tested, although, a non-significant decrease was apparent at the highest dose, 10 mg kg\(^{-1}\), (322±38; \( P=0.08 \)) compared with the sham-operated controls (Figure 3.12). In the rotarod test, \( N^1 \)-dansyl-spermine did not affect the animals' performance at any dose tested, compared with the sham-operated group (Figure 3.13).
Figure 3.10 Effect of MK-801 (i.p.) in sham-operated mice on locomotor activity counts. Data are expressed as mean±se.mean (n=6-10). +P<0.05 versus (saline treated) sham-operated control (two-tailed, unpaired Student’s t-test).
Figure 3.11 Effect of MK-801 (i.p.) in sham-operated mice on rotarod performance test. Data are expressed as mean±se.mean (n=6-10). +P<0.05 versus (saline treated) sham-operated control (two-tailed, unpaired Student’s t-test).
Figure 3.12 Effect of N\textsuperscript{1}-dansyl-spermine (i.p.) in sham-operated mice on locomotor activity. Data are expressed as mean±se.mean (n=4-10). *P<0.05 versus (saline treated) sham-operated control (two-tailed, unpaired Student's t-test).
Figure 3.13 Effect of N'-dansyl-spermine (i.p.) in sham-operated mice on rotarod performance test. Data are expressed as mean±se.mean (n=4-10). +P<0.05 versus (saline treated) sham-operated control (two-tailed, unpaired Student’s t-test).
3.4 DISCUSSION

3.4.1 The model

Animal models of focal cerebral ischemia are used to mimic the pattern of ischaemic brain damage observed in many human ischemic stroke patients (Ginsberg & Busto, 1989). The intraluminal suture MCAO model used in the present study has the advantage of not requiring craniotomy with its associated operative trauma.

As described in section 1, there are many factors modulating the extent of ischaemic damage in the MCAO model. It is important to ensure the reproducibility of the model. In the MCA occlusion model in rodents, which have an excellent circle of Willis and highly effective collateral circulation, the occlusion site on the MCA is vital. Distal and proximal occlusion can cause infarction to a variable extent. In our intraluminal suture model, care was taken to ensure that the location of the occlusion was always the origin of the MCA. As described in section 2, by selecting the special type of raw surgical suture and by the supplication of the heat, the diameter of the occluder used (the surgical suture) in this study, was ensured to be consistently 0.15 mm at the origin of the MCA. In the rat MCAO model, Takano et al., (1997) suggested that silicone-coating can ensure the consistency of diameter of the suture, therefore reducing the variability of the lesion size. However, there is concern that the use of the silicone coated suture (using 8-0 suture) in a mouse model has not been well standardized due to high variability in the lesion size produced (Tsuchiya et al., 2003). Studies with an uncoated occluder have shown the proportion of achieving infarction can be as low as 56% (Longa et al., 1989) in a study by Matsuo et al.(1994), in which the silicone-coated suture was used, approximately 30 of the experimental animals had to be excluded for a variety of reasons. Unsuccessful outcomes cited consisted of animals without infarction and animals in which subarachnoid hemorrhage was caused by rupture of the intracranial ICA. In the present study, 100% of animals subjected to MCAO showed infarction. Furthermore infarction size was consistent, confirming reproducibility of the model. The occluder
employed in this study rarely caused subarachnoid hemorrhage (only 3 animals with hemorrhage were excluded from the entire study, data not shown). %HLV in the present study was around 50% in the saline treated MCAO control; many other studies in rats cited a %HLV of around 20%-35% (Ozyurt et al., 1988; Gotti et al., 1990; Frazzini et al., 1994; Oliff et al., 1996; Tatlisumak et al., 1998a). HLV% at 60% was reported in a model using a 5-0 (maximum diameter 0.14 mm) monofilament (Wexler et al., 2002), which seems to be similar to the present study. In a study using a 0.18 mm diameter occluder, 24 hours of MCAO produced only a 28 mm³ infarct volume in mice (data was not presented in %HLV in that study) (Hatcher et al., 2002), which is far less than the lesion volume seen in the present study (102 mm³).

Hypothermia is widely reported to exert neuroprotection, experimentally (Busto et al., 1987) and clinically (Hypothermia after Cardiac Arrest Study Group, 2002). In the present study, several precautions were taken to reduce the likelihood of hypothermia. During surgery, body temperature was maintained at 36.5-37.5 °C by means of a heating blanket, and following surgery, the animals were kept at an ambient temperature of 29-30 °C for 2 hours to counteract any possible hypothermic effect of the procedure and the drugs.

The anesthetic used during the MCAO surgery was chloral hydrate (400 mg kg⁻¹, i.p.). It has been used as an anesthetic in a number of studies in focal cerebral ischaemia model (Kano et al., 1991; Yang et al., 1994a; Yang et al., 1994b; Gadamski et al., 2001; Lin et al., 2002). It does not need special equipment and therefore is simpler to use than gaseous anesthetics such as halothane. Ketamine and pentobarbital are two other commonly used anaesthetics. However both ketamine and pentobarbital have been reported to exert neuroprotective effects (Lees, 1989; Araki et al., 1990) and may therefore interfere with the testing of the potential neuroprotective drugs in cerebral ischaemia.

In the present study, N¹-dansyl-spermine was administered 30 min prior to MCAO. In fact, pre-ischaemic prevention is rarely necessary and may be limited to patients undergoing procedures such as carotid stenting, carotid endarterectomy, and cerebral
artery aneurysm or arteriovenous malformation operations. A post-ischaemic study would be more relevant to clinical conditions. However, the aims of the present study was to investigate (1) whether N⁰-dansyl-spermine is neuroprotective in the focal ischaemia model or not; (2) and if so, at what doses. Pre-ischaemic administration of the drug would give the best chance of seeing a neuroprotective effect of this drug in the focal ischaemia model, if indeed a neuroprotection occurred. With the best doses found in the present study, a study for the time window of opportunity could be carried out.

3.4.2 Histological and behavioural assessments used in this model

TTC staining was used in this study to identify the extent of tissue involved in the ischaemia. As mentioned before, TTC staining has been used widely in previous studies to demonstrate neuroprotective properties of novel compounds (Tatlisumak et al., 1998; Yan et al., 2001). Typical images of TTC-derived mice brain coronal slices at 1.7 mm intervals after 24 hours middle cerebral artery occlusion showed that after the TTC staining, the border between stained (life) and unstained (infarcted) tissues is well demarcated and this allowed computer-based calculation of the infarct volume in the present study. The results obtained from images of TTC-stained brain slices showed that 24 hours of MCAO produced consistent extensive cerebral infarction involving both cortical and subcortical regions. In the present study, the data of oedema, which is a major contributing factor to morbidity and mortality in stroke (Davalos et al., 1999; Rosenberg et al., 1999) was also easily obtained using the TTC-staining and subsequent computer-based calculation. In the present study, the data of %HLV and oedema showed the neuroprotection of both N⁰-dansyl-spermine and MK-801.

As described in section 2.1.6, it is important to assess the behavioural profile in the stroke model. Therefore, in addition to the histological assessments, a range of behavioural assessments were also employed in this study. The neurological deficit scoring system was successfully used. 24 hours of MCAO in this study caused neurological deficits to a median score of 3.5 which is similar to the other studies.
(Doerfler et al., 2001) and drugs tested reduced the score, showing their neuroprotection. In the present study, the rotarod performance test appeared to be particularly sensitive in detecting deficits after MCAO and the neuroprotective properties of the drugs, which is in agreement with other studies (Hamm et al., 1994). The locomotor activity test was found to be very useful in highlighting the possible adverse effect of the drugs. This will be discussed in more details in section 3.4.3.2 & 3.4.4.2.

3.4.3 MK-801

3.4.3.1 Histological effect of MK-801

The NMDA antagonist MK-801 has been used widely in models of focal cerebral ischaemia, and in most of these studies, the drug has been found to reduce the infarct volume markedly (Frazzini et al., 1994; Bertorelli et al., 1998; Gorgulu et al., 2000) and to reduce oedema formation in focal cerebral ischaemia models at doses of 0.5 mg kg\(^{-1}\) i.p. (Oh & Betz, 1991), 1 mg kg\(^{-1}\) i.v. (Yang et al., 1994) and 1 mg kg\(^{-1}\) i.p. (Gorgulu et al., 2000). The results of the present experiment are consistent with these studies. Although the lower doses tested worked, the highest dose of MK-801 used in the present study, showed no significant benefit against oedema formation. MK-801 is known to induce a dose-dependent reversible swelling and vacuolation in neurons (Olney et al., 1989; Auer & Coulter, 1994; Hargreaves et al., 1994) and this may have contributed to the oedema observed in ischaemic conditions with the highest dose in this study.

MK-801 is a potent non-competitive antagonist of the NMDA receptor by an open-channel blocking action (Wong et al., 1986; Gill et al., 1991). MK-801 is thought to exert its neuroprotective effect through its NMDA receptor action (Gill et al., 1991; Arias et al., 1999). It has been reported that MK-801 (1 mg kg\(^{-1}\) and 10 mg kg\(^{-1}\), i.p.) was neuroprotective when the body temperature was maintained at 37 °C for 24 hours in a transient forebrain ischaemia model in gerbils (Gill & Woodruff, 1990). However, MK-801 neuroprotection was found to be associated with hypothermia in a focal cerebral ischaemia model in rats (Hanon & Klitgaard, 2001). Corbett et
al., (1990) demonstrated that in gerbils, MK-801 produced hypothermia and that following bilateral carotid occlusion, in gerbils kept normothermic, MK-801 (3 mg kg\(^{-1}\)) was not neuroprotective. Given these findings, in the present study, it was considered important to maintain the normal body temperature of the mice (section 2.2).

3.4.3.2 Behavioural effects of MK-801

Conflicting reports have shown that MK-801 can have beneficial or detrimental effects on sensorimotor function in animal stroke models (for review, see Barth et al., 1990). In the present study, MK-801 had beneficial effects on neurological deficit scores and rotarod performance in MCAO animals. However, MK-801 had no beneficial effect on locomotor activity, and in fact, at the lowest dose, significantly reduced locomotor activity in MCAO animals. The present results show that MK-801 impaired rotarod performance in sham-operated control mice, and this finding is consistent with others studies (O'Neill & Bolger, 1989; Grant et al., 1992). Furthermore, all doses of MK-801 significantly reduced locomotor activity in control animals. MK-801 has been shown many times to increase locomotor activity within minutes of administration, but locomotor activity has also been shown to be lowered 24 hours following MK-801 (0.5 mg kg\(^{-1}\)) (Haggerty & Brown, 1996). This delayed effect correlates with the results observed in the present study (Kundrotiene et al., 2002).

3.4.4 \(N^1\)-dansyl-spermine

3.4.4.1 Histological effect of \(N^1\)-dansyl-spermine

\(N^1\)-dansyl-spermine was as effective as MK-801 at reducing the %HLV and oedema, suggesting that \(N^1\)-dansyl-spermine has some neuroprotective effect. As a polyamine analogue, \(N^1\)-dansyl-spermine could be modulating activity of the NMDA receptor, thereby reducing the excessive activation of the NMDA receptor that is known to occur in ischaemia. As previously mentioned, \(N^1\)-dansyl-spermine has been shown to act as a potent blocker of recombinant NMDA expressed in oocytes (Chao et al.,
In addition, N\textsuperscript{1}-dansyl-spermine has been shown to potently antagonize spermine induced CNS excitation in the form of tonic convulsions (Kirby et al., 2004). This spermine induced CNS excitation has previously been shown to involve, at least in part, activation of the NMDA receptor (Doyle & Shaw, 1996). Therefore, it is likely that the neuroprotective effects of N\textsuperscript{1}-dansyl-spermine are mediated by interaction with a polyamine modulatory site on the NMDA receptor.

N\textsuperscript{1}-dansyl-spermine has also been shown to act as a potent calmodulin antagonist (Seiler et al., 1998), which might also contribute to its neuroprotective effect, although there is evidence that calmodulin antagonists are ineffective in cerebral ischaemia models (Hara et al., 1990). It is also possible that N\textsuperscript{1}-dansyl-spermine may exert neuroprotection through hypothermia. In a recent report from this laboratory, N\textsuperscript{1}-dansyl-spermine was shown to cause a mild reduction in body temperature in the gerbil at a neuroprotective dose of 10 mg kg\textsuperscript{-1} i.p., although a lower dose of N\textsuperscript{1}-dansyl-spermine (5 mg kg\textsuperscript{-1} i.p.,) was not associated with hypothermia, but was neuroprotective (Kirby & Shaw, 2004). As mentioned before, it has been extensively reported that hypothermia can exert neuroprotection. Therefore several precautions were taken to reduce the likelihood of hypothermia. Further studies investigating the mechanism of action of N\textsuperscript{1}-dansyl-spermine are needed, but it is clear from the results of this study that it has potential as a neuroprotective agent.

Interestingly, N\textsuperscript{1}-dansyl-spermine was most neuroprotective at lower doses. The best neuroprotective effects were observed with the 1 and 2 mg kg\textsuperscript{-1} doses, and at higher levels, the beneficial effects declined. In a recent paper, describing the effects of N\textsuperscript{1}-dansyl-spermine in a transient global ischaemia model in gerbils, N\textsuperscript{1}-dansyl-spermine (2, 5, 10 mg kg\textsuperscript{-1}) afforded a dose-dependent neuroprotection, with the highest dose being most effective (Kirby et al., 2004). In the present study, the 10 mg kg\textsuperscript{-1} dosing was not effective, and in fact, seemed to cause some worsening of condition. It is likely that the difference in effect of N\textsuperscript{1}-dansyl-spermine in the two models may be related to species differences between the mouse and the gerbil. Furthermore, a transient ischaemia model was used in that study and this opens up two possible differences: less ischaemic damage may occur in the transient model.
than in the permanent mouse ischaemia study, and secondly, the transient model will be subject to the effect of changes due to reperfusion (Hossmann, 1998).

Older analogues of polyamines that are known to have some competitive antagonist activity, such as arcaine and 1,10-diaminodecane, were subsequently shown to act as partial agonists, rather than true competitive antagonists (Doyle & Shaw, 1998). As previously mentioned, in vitro evidence has suggested that \( N^1 \)-dansyl-spermine is an antagonist at the NMDA receptor, as it can block responses to glutamate in recombinant receptors expressed in oocytes at low \( \mu \)M levels (Chao et al., 1997). However, spermine also has an inhibitory effect in this model, albeit at high \( \mu \)M levels and mM levels (Chao et al., 1997). Preliminary studies in this laboratory have suggested that \( N^1 \)-dansyl-spermine may also act as a partial agonist, rather than as a pure competitive antagonist. \( N^1 \)-dansyl-spermine at 20 mg kg\(^{-1}\) can induce spermine-like CNS excitation effects in gerbils \textit{in vivo} (unpublished data), similar to those effects previously described in mice (Doyle & Shaw, 1996; Doyle & Shaw, 1998).

Polyamines have been implicated in the pathophysiology of stroke for some time, as levels have been shown to change following ischaemia (Paschen et al., 1992). Whether polyamines serve a neuroprotective or a neurodegenerative role remains unclear, although most evidence points towards a role in neurodegeneration. It is possible that \( N^1 \)-dansyl-spermine has low efficacy at the polyamine binding sites of the NMDA receptor, and therefore causes a physiological antagonism at low doses. However, at higher doses, the neuroprotection afforded by this drug may be lost due to cumulative polyamine agonist effects. Investigation of the receptor interactions, pharmacokinetics and pharmacodynamics of this drug is needed to further unravel its profile of actions. In addition, very little is known about this novel compound and it is possible that the toxic effect at higher doses could be due to an interaction at an, as yet, unknown site. However, the beneficial effect of low doses of this drug suggests that \( N^1 \)-dansyl-spermine, or an analogue of it, may prove to be a useful therapeutic agent for stroke.

\textit{3.4.4.2 Behavioural effects of \( N^1 \)-dansyl-spermine}
N\textsuperscript{1}-dansyl-spermine is a novel compound, and at present, very little is known about its pharmacokinetic or pharmacodynamic profile. To ensure that any behavioural effects observed in N\textsuperscript{1}-dansyl-spermine treated MCAO animals were not due to any long-lasting effects of N\textsuperscript{1}-dansyl-spermine on motor centres of the brain, the effect of the drug in sham operated control animals was observed. No effect on LMA or rotarod performance was observed 24 hours after administration in sham operated animals. However, in MCAO mice, while N\textsuperscript{1}-dansyl-spermine had no significant effect on locomotor activity, a significant improvement in the rotarod performance was observed in MCAO animals. This beneficial effect of N\textsuperscript{1}-dansyl-spermine on rotarod performance, 24h following MCAO, probably reflects a neuroprotective effect of the drug.

In addition, N\textsuperscript{1}-dansyl-spermine also significantly reduced the neurological deficit scores. It appears as though the neuroprotective effect produced with lower doses of N\textsuperscript{1}-dansyl-spermine is reversed at higher levels. This is also apparent in the rotarod performance test, oedema results and to a lesser extent, in the %HLV data as discussed previously.

Various neurotransmitter systems have been implicated in locomotor activity, including the glutamatergic, dopaminergic, serotonergic & noradrenergic systems (Irifune \textit{et al.}, 1995; Ouagazzal & Amalric, 1995; Yan \textit{et al.}, 1997). Locomotor dysfunction can be used as an index of CNS dysfunction and can highlight adverse effects of CNS active drugs (Dawson \textit{et al.}, 2001). The data presented in this study suggests that the rotarod test may be a particularly sensitive behavioural indicator of neuroprotection in this model.

In the present experiments, N\textsuperscript{1}-dansyl-spermine had no negative effects on motor function at neuroprotective doses, either in MCAO mice, or in sham-operated control mice 24 hours following administration. In contrast, MK-801 did produce motor impairment at neuroprotective levels, which is in agreement with published literature.
The different profile of side effects could be due to differences in the duration of action of the two drugs. However, no gross behavioural changes in animals administered N¹-dansyl-spermine were noted throughout the entire duration of the study (unpublished observations and see (Kirby et al., 2004)). The lack of motor effects induced by neuroprotective doses of N¹-dansyl-spermine opens the possibility that N¹-dansyl-spermine could be a promising therapeutic candidate for stroke with a good side effect profile.

3.5 CONCLUSION

The present data shows that N¹-dansyl-spermine is a good neuroprotective agent when administered 30 mins pre-ischaemia, in a permanent focal cerebral ischaemia model in mice. N¹-dansyl-spermine was as effective as MK-801 at reducing %HLV, oedema and neurological deficit score and improving the rotarod performance in MCAO mice. N¹-dansyl-spermine was more neuroprotective at lower doses (1 and 2 mg kg⁻¹). It is suggested that N¹-dansyl-spermine mediates its effect through a polyamine modulatory site on the NMDA receptor, although further investigation is needed to confirm its mechanism of action. Moreover, in direct contrast to the effect of MK-801, N¹-dansyl-spermine had no adverse effects on motor function at neuroprotective doses in sham-operated animals 24 h following administration. N¹-dansyl-spermine, or a derivative of this compound, may have great potential as a therapeutic agent for stroke.
4.1 INTRODUCTION

As found in Section 3, the polyamine analogue, N\(^1\)-dansyl-spermine is a good neuroprotective agent in the permanent focal cerebral ischaemia model in mice. More recently several novel polyamine analogues were developed. Their brief names are BU31b, BU37b, BU33b, BU36b and BU43b and their full chemical names are described in section 2.

These compounds have been shown *in vitro* to have inhibitory effect at the mammalian NR1/NR2B NMDA receptor subunit, which contains the polyamine site (Williams, 1994; Fixon-Owoo *et al.*, 2003). In an *in vivo* study, BU31b, BU33b, and in particular BU36b and BU43b were demonstrated to be effective at antagonizing the polyamine-induced CNS excitation in the form of tonic convulsions in mice (Murphy *et al.*, 2003). The spermine-induced CNS excitation observed in Murphy and co-workers’ study (2003) has previously been shown to involve, at least in part, activation of the NMDA receptor (Doyle & Shaw, 1996). These observations *in vitro* and *in vivo* suggest that these polyamine novel analogues may be effective polyamine antagonists and negative modulators of the NMDA receptor. As previously mentioned, there are conflicting thoughts on how polyamines are involved in cerebral ischaemia, namely whether they are neuroprotective or neurotoxic, and through what mechanism they mediate their effects. As suggested by Paschen *et al.* (1992) polyamines, particularly spermine could be released from neurons into the extracellular compartment and bind to the NMDA receptors of cells located in close vicinity and through this action, polyamines may thus render neurons vulnerable to otherwise subtoxic levels of glutamate. The non-competitive polyamine antagonists, ifenprodil and its derivative eliprodil, have been demonstrated to be neuroprotective in cerebral ischaemia models and the neuroprotection was afforded through, at least partly, their action via the polyamine site at the NMDA receptor (Cater *et al.*, 1997).
Therefore these novel polyamine analogues may also show some neuroprotection against a cerebral ischaemia insult.

Thus, in this section, the potential neuroprotection of these novel compounds was studied in the intraluminal permanent MCAO model in mice. A battery of histological and behavioural assessments was used to screen their neuroprotection in this model.

4.2 METHODS AND MATERIALS

In this section, the model used was the intraluminal suture permanent MCAO model. Studies were carried out using CD-1 mice weighting 35-40g. Compounds or vehicle were given 30 minutes prior to the MCAO. Only statistically significant data is graphed. For detailed methods and materials used see section 2.2.
4.3 RESULTS

4.3.1 BU43b

4.3.1.1 Histological assessments

24 hours of permanent MCA occlusion caused a %HLV of 50.4±3.1% and oedema was 26.5±2.3 mm$^3$ in the saline treated MCAO control group.

BU43b significantly reduced the %HLV to 37.8±3.8% ($P<0.05$ versus saline treated MCAO control group) when administered at 30 mg kg$^{-1}$ and, it also decreased the %HLV at dose of 20 mg kg$^{-1}$ (42.9±4.1%), although the decrease was not significant (Figure 4.1; Figure 4.2). Treatment with BU43b at both doses significantly reduced the oedema in comparison with the saline treated MCAO control group (20.2±1.3 mm$^3$; 19.5±2.1 mm$^3$, respectively, $P<0.05$ for 20 and 30 mg kg$^{-1}$ groups) (Figure 4.3).
Figure 4.1 Effect of BU43b (i.p.) administered 30 min before the occlusion in MCAO mice on percentage hemisphere lesion volume (%HLV). Data are expressed as mean±se.mean (n=10). *P<0.05 versus (saline treated) MCAO control (two-tailed, unpaired Student’s t-test).
Figure 4.2 Typical Images of TTC-derived brain slices of mice after 24 hours middle cerebral artery occlusion indicating BU43b (given 30 min prior to occlusion) induced a neuroprotective effect on infarction volume. (A) MCAO Control animal; (B) MCAO animal administered 30 mg kg$^{-1}$ of BU43b. Slices are at 1.7 mm internals in descending order from frontal pole (anterior to posterior). Scale bar = 3.5 mm.
Figure 4.3 Effect of BU43b (i.p.) administered 30 min before the occlusion in MCAO mice on oedema. Data are expressed as mean±se.mean (n=10). *P<0.05 versus (saline treated) MCAO control (two-tailed, unpaired Student's t-test).
4.3.1.2 Neurological deficit scoring

Treatment with BU43b did not significantly reduce the neurological deficit score at doses administered (3.0±1.25; 3.0±3.0, for 20 and 30 mg kg⁻¹ respectively versus 4.0±1.25, saline treated MCAO control group).

4.3.1.3 Spontaneous locomotor activity (LMA) test

MCAO induced a significant decrease on spontaneous locomotor activity (saline treated MCAO group 32.5±12) versus sham-operated controls 397±57, P<0.05).

BU43b only slightly altered the LMA at (34.4±10; 28.7±8.9 for 20 mg kg⁻¹ and 30 mg kg⁻¹ respectively) and neither the decrease nor the increase was significant compared to the saline treated MCAO control group.

4.3.1.4 Rotarod performance assessment

Rotarod performance of the saline treated MCAO animals was significantly impaired when compared with the sham-operated group (saline treated MCAO group 15.8±5.1 sec versus sham-operated 114±13 sec, P<0.05).

There was some improvement on the rotarod performance in the BU43b treated MCAO groups (19.2±5.1 sec and 21.8±7.6 sec for 20 and 30 mg kg⁻¹ respectively), although no significant effect was observed.

4.3.1.5 Mortality rate

In the BU43b treated MCAO groups, the mortality rates were 0 out of 10 and 1 out of 11 for 20 mg kg⁻¹ and 30 mg kg⁻¹ respectively. Neither was significantly different from the saline treated MCAO group (1 out of 11).
4.3.1.6 Summary of BU43b

BU43b reduced %HLV in a dose-dependent manner in this model and a significant reduction was seen at 30 mg kg\(^{-1}\). Oedema was also significantly decreased by BU43b at both 20 and 30 mg kg\(^{-1}\) doses. No significant effect of BU43b was seen on neurological scores, LMA, rotarod performance and mortality rate.

4.3.2 BU36b

4.3.2.1 Histological assessments

BU36b did not significantly reduce the %HLV at 20 or 30 mg kg\(^{-1}\) (46.0±5.0 and 44.7±4.4 respectively versus saline treated MCAO control 50.4±3.1%). However, at dose of 30 mg kg\(^{-1}\), it produced a significant reduction of the oedema (19.5±2.3 mm\(^3\), \(P<0.05\) versus saline treated MCAO control group 26.5±2.3 mm\(^3\)) although the reduction of oedema at 20 mg kg\(^{-1}\) was not significant (23.9±0.33 mm\(^3\)) (Figure 4.4).
Figure 4.4 Effect of BU36b (i.p.) administered 30 min before the occlusion in MCAO mice on oedema. Data are expressed as mean±se.mean (n=10-11). *P<0.05 versus (saline treated) MCAO control (two-tailed, unpaired Student’s t-test).
4.3.2.2 Neurological deficit scoring

BU36b did not afford a significant reduction on the neurological deficit score at doses of 20 or 30 mg kg$^{-1}$ (3.0±2.0; 3.0±2.0 for 20 and 30 mg kg$^{-1}$ respectively when compared with saline treated MCAO group 4.0±1.25).

4.3.2.3 Spontaneous locomotor activity (LMA) test

BU36b at the higher dose 30 mg kg$^{-1}$ decreased the LMA, however the decrease was not significant (17.9±3.0). Whilst dosed at 20mg kg$^{-1}$, it increased the LMA to 39.8±12 although a significance was not achieved when compared with the saline treated MCAO control group (32.5±12).

4.3.2.4 Rotarod performance assessment

Rotarod performance of MCAO animals treated with BU36b at 30 mg kg$^{-1}$ was not significantly better than the saline treated MCAO group (16.3±14 sec versus saline treated MCAO control 15.8±5.1 sec). The dosing at 20 mg kg$^{-1}$ of BU36b did not improve the rotarod performance of MCAO animals (13±4.3 sec) when compared with the saline treated MCAO control.

4.3.2.5 Mortality rate

The mortality rate was 1 out of 10 and 1 out of 11 for 20 or 30 mg kg$^{-1}$ BU43b treated MCAO groups, which was not significantly different from the saline treated MCAO group (1 out 11).

4.3.2.6 Summary of BU36b

BU36b significantly reduced oedema at 30 mg kg$^{-1}$ although oedema was not significantly reduced at lower dose 20 mg kg$^{-1}$. No significant reduction was seen on %HLV or any behavioural assessments at either dose.
4.3.3 BU33b

4.3.3.1 Histological assessments

BU33b did not afford a significant reduction of the %HLV at 20 mg kg\(^{-1}\) (48.0±4.2\%), while at the higher dose of 30 mg kg\(^{-1}\) it increased the %HLV slightly (not significantly) (52.6±6.0\%) in comparison with the saline treated MCAO group (50.4±3.1\%). No significant decrease of the oedema was achieved by BU33b (24.2±1.7 mm\(^3\); 22.6±1.6 mm\(^3\) for 20 and 30 mg kg\(^{-1}\) respectively) when compared with saline treated MCAO group (26.5±2.3 mm\(^3\)).

4.3.3.2 Neurological deficit scoring

BU33b did not reduce the median of the neurological deficit score at 30 mg kg\(^{-1}\) (4.0±1.75), however, a significant reduction was seen at the lower dose of 20 mg kg\(^{-1}\) (2.0±1.75, \(P<0.05\) versus saline treated MCAO control) (Table 3.3).

<table>
<thead>
<tr>
<th>Drug and doses treated</th>
<th>Saline</th>
<th>BU33b (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Scores</td>
<td>4.0±1.25</td>
<td>2.0±1.75*</td>
</tr>
<tr>
<td></td>
<td>n=10</td>
<td>n=8</td>
</tr>
</tbody>
</table>

Table 3.3 The neuroprotective effect of BU33b (i.p.) administered 30 min before the occlusion in permanent MCAO mice on neurological deficits scoring. n, animal number; Scores, neurological deficit scores. \(*P<0.05\) versus (saline treated) MCAO control (Mann-Whitney U-test).
4.3.3.3 Spontaneous locomotor activity (LMA) test

Treatment with BU33b slightly although not significantly reversed the decrease in LMA caused by MCAO (40.8±12; 34.5±11 respectively for 20 and 30 mg kg$^{-1}$ doses versus saline treated MCAO group 32.5±12).

4.3.3.4 Rotarod performance assessment

BU33b at the lower dose of 20 mg kg$^{-1}$ improved the rotarod performance while the improvement was not significant (26.3±8.8 versus saline treated MCAO control 15.8±5.1 sec). Administered at 30 mg kg$^{-1}$, BU33b did not affect the rotarod performance (15.2±5.3) when compared with saline treated MCAO control group (15.8±5.1).

4.3.3.5 Mortality rate

The mortality rate was 2 out of 10 for BU33b at both 20 mg kg$^{-1}$ and 30 mg kg$^{-1}$ doses, which were not significantly different from the saline treated MCAO group (1 out of 11).

4.3.3.6 Summary of BU33b

BU33b significantly reduced the neurological deficit scores at the lower dose of 20 mg kg$^{-1}$, although significant effect was not seen at higher dose of 30 mg kg$^{-1}$. No significant effect of this drug was observed on the other parameters.

4.3.4 BU37b

4.3.4.1 Histological assessments
BU37b at 20 mg and 30 mg kg\(^{-1}\) produced a non-significant increase of the %HLV (56.8±10; 55.5±4.3 respectively in contrast with saline treated MCAO group 50.4±3.1%). Treatment with BU37b did not significantly reduce the oedema (26.8±6.0 mm\(^3\); 25.0±3.9 mm\(^3\) for 20 mg and 30 mg kg\(^{-1}\) respectively versus saline treated MCAO group 26.5±2.3 mm\(^3\)).

4.3.4.2 Neurological Deficit Scoring

The neurological deficit score was not significantly reduced by the BU37b at 20 or 30 mg kg\(^{-1}\) (3.0±1.5; 3.0±2.0 for 20 and 30 mg kg\(^{-1}\) respectively).

4.3.4.3 Spontaneous locomotor activity (LMA) test

Dosed at 20 and 30 mg kg\(^{-1}\), BU37b caused a non-significant decrease on the LMA (30.2±7.0; 26.4±13, for 20 and 30 mg kg\(^{-1}\) respectively) compared to control (32.5±12).

4.3.4.4 Rotarod performance assessment

The rotarod performance of the MCAO mice treated with BU37b (16.0±11; 15.8±8.0, for 20 or 30 mg kg\(^{-1}\) respectively) was not significantly different from that of the saline treated MCAO animals (15.8±5.1 sec).

4.3.4.5 Mortality rate

BU37b produced a significant increase of mortality rate when compared to the saline treated MCAO group (5 out of 10; 6 out of 10 for 20 or 30 mg kg\(^{-1}\) respectively versus saline treated MCAO 1 out of 11, \(P <0.05\) for both cases).

4.3.4.6 Summary of BU37b
BU37b significantly increased the mortality rate at both doses tested and did not significantly alter the histological assessments and behavioral assessments.

4.3.5 BU31b

4.3.5.1 Histological assessments

BU31b did not reduce the %HLV caused by MCAO, in fact, it slightly although not significantly increased the %HLV at 20 mg kg$^{-1}$ and 30 mg kg$^{-1}$ (52.4±6.3; 51.7±9.8 respectively in contrast with saline treated MCAO group 50.4±3.1%). It also induced a non-significant increase of the oedema at those two doses tested (29.4±2.8 mm$^3$; 29.3±2.6 mm$^3$ for 20 mg kg$^{-1}$ or 30 mg kg$^{-1}$ respectively versus saline treated MCAO group 26.5±2.3 mm$^3$).

4.3.5.2 Neurological deficit Scoring

BU31b did not significantly decrease the neurological deficit score induced by the MCA occlusion (4.0±2.0 for 20 mg kg$^{-1}$; 4.0±3.0, 30 mg kg$^{-1}$ versus saline treated MCAO control group 4.0±1.25).

4.3.5.3 Spontaneous locomotor activity (LMA) test

BU31b administered at 20 mg kg$^{-1}$ did not significantly reverse the decrease in LMA caused by MCAO compared with the saline treated MCAO group (44.2±14 for 20 mg kg$^{-1}$ versus saline treated MCAO control 32.5±12). Administered at 30 mg kg$^{-1}$, it slightly decreased the LMA although this decrease is not significant (26.6±13).

4.3.5.4 Rotarod performance assessment

Dosing of BU31b impaired the rotarod performance of the MCAO mice although the effect was not significant (9.0±3.0 sec and 9.0±4.6 sec respectively for 20 or 30 mg
kg\textsuperscript{-1} doses) in comparison with the saline treated MCAO control animal group (15.8±5.1 sec).

4.3.5.5 Mortality rate

BU31b significantly increased the mortality rate at 30 mg kg\textsuperscript{-1} (7 out of 10, \textit{P}<0.01, when compared with the saline treated MCAO group, 1 out of 11). There was a non significant increase of mortality rate at 20 mg kg\textsuperscript{-1}(3 out of 8).

4.3.5.6 Summary of BU31b

A significant increase of mortality was seen at the dose of 30 mg kg\textsuperscript{-1} of BU31b and histological and behavioural assessments were not significantly affected by BU31b.

4.4 DISCUSSION

4.4.1 BU43b

As the images of TTC-derived brain slices at 5 levels showed, 24.5 hours of MCAO produced extensive focal cerebral infarction in the mice. The %HLV and oedema were similar to the control used in section 3. There seems to be differences, particularly in the rotarod performance and locomotor activity counts between the two MCAO control groups, however, none of the differences were statistically significant.

BU43b reduced %HLV in a dose-dependent manner in this model and a significant reduction was seen at 30 mg kg\textsuperscript{-1}. Oedema was also significantly decreased by BU43b at both 20 and 30 mg kg\textsuperscript{-1} doses. Those results indicate that the polyamine analogue BU43b is neuroprotective. The results may indicate that reduction in oedema is a more sensitive indicator of neuroprotection than %HLV.
In a spermine model, BU43b was shown to be the most potent antagonist against the spermine-induced CNS excitation (Murphy et al., 2003). BU43b (10μg, i.c.v) decreased the median score, which was used to grade the spermine-induced excitation, from 5 (control) to 1. In addition, when it was administered intraperitoneally (5, 10 or 20mg/kg), it highly significantly reduced the median to 3, 2 or 2 respectively. In the present study, BU43b has been shown to be the most effective in reducing the cerebral ischaemic damage following permanent MCAO. Doyle and Shaw (1996) have suggested that spermine induced CNS excitation may involve, at least in part, activation of the NMDA receptor. In an in vitro study using recombinant mammalian glutamate receptors expressed in Xenopus oocytes, BU43b at 10 μM was found to be a potent NMDA receptor inhibitor at the NR1/NR2B subunit (95%), which contains the polyamine site (Fixon-Owoo et al., 2003). Therefore it is likely that BU43b could be an effective polyamine site NMDA antagonist. BU43b, in the present study may have afforded its neuroprotection by reducing the overactivation of the NMDA receptor through its possible action at the polyamine recognition site.

As previously described in section 1.3.3, there are conflicting reports with regard to the role of polyamines in the cellular damage induced by cerebral ischaemia. The present results support the view that the polyamines may contribute to the cellular damage following cerebral ischaemia.

BU43b exerted a 24% reduction on the %HLV in present study at 30 mg kg\(^{-1}\), which is modest. The polyamine recognition site antagonist Eliprodil can provide 40-60% protection in focal cerebral ischaemia models (Gotti et al., 1988; Gotti et al., 1990) and Ifenprodil was also reported to afford a 40% reduction on the infarction size (Gotti et al., 1988). In the model used in the present study, the %HLV was 50% in the saline treated MCAO control, which is much more severe than those used in many others’ studies where the %HLV was around 20%-35% (Gotti et al., 1990; Ozyurt et al., 1988; Frazzini et al., 1994; Oliff et al., 1996; Tatlisumak et al., 1998a). It is likely that BU43b at these doses could have a higher rate of reduction on the lesion size in a less severe model. Secondly, the results showed that the effect of
BU43b was dose-dependent in the present study. The greatest reduction in %HLV for N\textsuperscript{1}-dansyl-spermine or MK-801 was approximately 40% in the present study (section 3). It is possible that BU43b could have had a greater effect if a dose higher than 30 mg kg\textsuperscript{-1} had been used.

As described previously, hypothermia has been widely reported to protect the brain from damage after an ischaemia insult (Busto et al., 1987). Another polyamine analogue, N\textsuperscript{1}-dansyl-spermine was found to cause hypothermia, although it was not associated with its neuroprotection (Kirby & Shaw, 2004) (see section 3.4.4.1). Therefore, in this study, precautions, as described in section 3.4.1, were taken to avoid neuroprotective hypothermia in present study. In addition, no significant change of body temperature was noted within 24 hours following the administration of BU43b to mice during the present study (unpublished observations). Thus, it is not likely that hypothermia contributed to the neuroprotection observed with BU43b in present study.

4.4.2 BU36b

BU36b significantly reduced oedema at 30 mg kg\textsuperscript{-1} although oedema was not significantly reduced at lower dose 20 mg kg\textsuperscript{-1}. In addition, a reduction (although not significant) was seen on %HLV and neurological deficit scores at both doses. Therefore the present study showed that BU36b has some neuroprotective effect but it is not as effective as BU43b.

In the spermine model, BU36b (20 and 30mg kg\textsuperscript{-1}, i.p.) dose-dependently reduced the median CNS excitation convulsion score caused by spermine. It was concluded that BU36b is one of the two most potent polyamine antagonists in this drug series (Murphy et al., 2003). BU36b has also been shown to have inhibitory effects (26%) on the NMDA receptor NR1/NR2B subunit in \textit{in vitro} studies (Fixon-Owoo et al., 2003). Since precautions were taken in the present study to avoid hypothermia as mentioned previously and BU36b was not found to cause a drop of body temperature (in unpublished observations, data not shown), the contribution of hypothermia to
BU36b’s neuroprotection can be ruled out. Similarly to BU43b, it can be proposed that BU36b is possibly a polyamine site NMDA antagonist and could have produced its neuroprotection against cerebral ischaemic damage through decreasing over-activation of the NMDA receptor complex.

This again, sheds light on the role of polyamines in cerebral ischaemia. Taken together with the observations of BU43b, the two most potent polyamine antagonists, and possible polyamine site NMDA antagonists in this drug series showed neuroprotection in the ischaemia model. This further supports the suggestion by Paschen et al. (1992) that polyamines are toxic following ischaemia, and polyamines released following cerebral ischaemia, could enhance the neurotoxicity induced by NMDA receptor.

The neuroprotection of BU36b was only modest in the cerebral ischaemia model tested. However, at the same doses used in the spermine model, BU36b was highly effective in blocking the spermine-induced convulsions. This may be explained by the use of different animal species in the two studies. Laca mice were used in the spermine model and CD-1 mice were used in present study. Animals of different strains could respond to the same dose of drug to a different extent. Therefore this should not cast any doubt on the suggestions for the neuroprotection of BU43b and the role of polyamines in cerebral ischaemia.

BU36b dosed at either 20 or 30 mg kg\(^{-1}\) did not significantly reverse the reduction in LMA caused by MCAO. In fact, a further non-significant decrease was seen in 30 mg kg\(^{-1}\) dosing, as mentioned previously, LMA can be used to highlight the possible adverse effect of a novel drug. However, it is hard to suggest that this drug may possess long lasting side effects since this decrease was not significant. Furthermore, in unpublished data from this lab, no abnormal behavior was noted when observations on behaviors including locomotor activity and rotarod performance were made for 5 days following administration of this drug at the same doses as tested in this study (Data not shown).
4.4.3 BU33b

Dosed at 20 mg kg\(^{-1}\), BU33b afforded a significant reduction on the neurological deficit score, although this decrease was not seen at higher dose 30 mg kg\(^{-1}\).

Murphy et al. (2003) reported that BU33b showed some effect in reducing the spermine-induced convulsions when given through the i.c.v route, but it was not a potent polyamine antagonist in that model. Furthermore, BU33b has very little inhibitory effect (3\%) on the recombinant NMDA receptor \textit{in vitro} (Fixon-Owoo et al., 2003). In the present focal cerebral ischaemia model, BU33b was not a great neuroprotectant.

It appears that BU33b was more neuroprotective in lower dose, since the higher dose 30 mg kg\(^{-1}\) did not show any beneficial effect. It is possible that BU33b, like N\(^1\)-dansyl-spermine, is not a pure polyamine antagonist and possesses some partial agonist effect. More generally speaking, BU33b may be toxic at higher doses by acting at some as yet unknown sites.

4.4.4 BU31b and BU37b

Overall, BU31b was found to be toxic in the present study. At 30 mg kg\(^{-1}\), it significantly increased the mortality rate of the MCAO mice to 7 out 10. In the 3 surviving mice, the mean %HLV and oedema were found to be even slightly higher and the rotarod performance was worse than that in MCAO controls, although none of differences reached statistical significance.

Similar to BU33b, BU31b has been demonstrated to be a less potent polyamine antagonist in the spermine model than BU36b or BU43b through the i.c.v route (Murphy et al., 2003). The in vitro study showed that BU31b, at 10 μM potently blocks responses to glutamate in recombinant NMDA receptors expressed in oocytes by 98\% (Fixon-Owoo et al., 2003). However, in the present model, no neuroprotection was seen with this drug and BU31b increased the mortality rate. A
major difference between the study using spermine model (Murphy et al., 2003) and the present study, besides the animal strain difference, is the administration routes. So far, no pharmacokinetics and pharmacodynamics information of this novel drug is available. As it was given via the i.p. route in present study, it is possible that it may have been converted into toxic metabolites before it reached the brain. This may be the underlying reason for the ineffectiveness and even toxicity seen in the present study.

The *in vitro* study showed that BU37b has some NMDA receptor inhibitory effect, as it (at 10 μM) blocked the NMDA receptor current induced by glutamate by 12% (Fixon-Owoo et al., 2003). However, BU37b was without effect in antagonizing the polyamine-induced convulsions even at a very high dosing *in vivo* (Murphy et al., 2003), indicating BU37b is not an effective polyamine/NMDA receptor blocker in vivo. Indeed, in the present study, BU37b was not neuroprotective, which is consistent with those two studies. The toxic effect of BU37b shown in the present study may due to metabolism to toxic metabolites on route to the brain or some other as yet unknown actions of this novel compound.

4.5 CONCLUSION

The present study shows that BU36b and BU43b are neuroprotective in the focal permanent cerebral ischaemia model in mice when administered pre-ischaemia and could be promising candidates for the therapeutic treatment of stroke. Also the observations of the polyamine/NMDA antagonists in the present study have suggested that polyamines are involved in the neurodegeneration following ischaemia. As described previously, there are conflicting thoughts of the role of polyamines after an ischaemic insult. Polyamines may be neuroprotective (Gilad & Gilad, 1991) while others suggested that they are involved in causing neuronal damage following cerebral ischaemia (Paschen et al., 1991; Paschen et al., 1992; Dogan et al., 1999). In the present study using the permanent MCAO model in mice, the neuroprotective property profiles of this novel polyamine analogue series are generally in line with their effect as polyamine antagonists in the spermine model.
(Murphy et al., 2003) and their effect in vitro on the NMDA receptor subunit containing the polyamine site (Williams, 1994; Fixon-Owoo et al., 2003). These results support a neurotoxic role of polyamines.
Section 5 Pre-ischaemic effect of MK-801, N\textsuperscript{1}-dansyl-spermine and novel polyamine analogues in a focal transient ischaemia model

5.1 INTRODUCTION

As mentioned in section 1.2.2.4, early reperfusion can be beneficial following cerebral ischaemia, however, delayed reperfusion can lead to cellular damage. In a preliminary study, 45 min or 60 min ischaemia with 24 hours reperfusion in mice caused a much higher mortality rate (4 out of 9 and 5 out of 8, for 45 min and 60 min respectively) than found in the 24 hours permanent MCAO mice (section 3 and 4). It is well known that delayed reperfusion can be damaging. However, in human stroke, recirculation occurs frequently after focal ischaemia, particularly in the case of cerebral embolism (Ringelstein \textit{et al.}, 1992). Also, as described in section 1.3.3, thrombolysis therapy induces the recovery of blood flow (Dogan \textit{et al.}, 1997). Therefore, the stroke injury could be the result of both ischaemia and reperfusion damage. Hence, assessment of the effect of N\textsuperscript{1}-dansyl-spermine and the series of novel polyamine analogues in a focal cerebral ischaemia model with reperfusion was important.

There are a number of mechanisms through which delayed reperfusion causes injury, such as increased accumulation of reactive oxygen species, increased release of glutamate and Ca\textsuperscript{2+} influx into cells (Chan \textit{et al.}, 2001; Schaller & Graf, 2004). As previously mentioned, the NMDA over-activation may play an important role in the development of the ischaemic damage and may also be involved in the neuronal damage taking place in the reperfusion phase (Nellgard \textit{et al.}, 1991). Polyamines are thought to be involved in the ischaemic damage but whether they are neurotoxic or neuroprotective is not clear. Paschen \textit{et al.} (1992) suggested that polyamines are neurotoxic: when released, they may bind to the stimulatory polyamine site on the NMDA receptor, thereby increasing the over-activation of the NMDA complex.
following ischaemia. Therefore, polyamine/NMDA antagonists may offer therapeutic opportunity against ischaemia-reperfusion injury (see section 4.1).

Thus in this section, the neuroprotective potential of N\textsuperscript{1}-dansyl-spermine, a polyamine/NMDA antagonist, which has been found to be effective in the permanent ischaemia model in section 3, was studied. Again, for comparison, the NMDA channel blocker, MK-801 was investigated. Also, the 5 novel polyamine analogues (BU31b, BU37b, BU33b, BU36b and BU43b), which were tested in the permanent focal cerebral ischaemia model, were investigated in the transient focal ischaemia model in mice.

The transient focal cerebral ischaemia model used in this study was the intraluminal suture transient MCAO model. It is a relatively simple model which allows occlusion without using invasive approaches to expose the MCA, and allows withdrawal of the intraluminal suture to produce reperfusion to the ischaemic territory.

5.2 METHODS AND MATERIALS

Section 2.3 describes the methods and materials in detail. Compounds or vehicle were given 30 minutes before the MCAO. Animals used were CD-1 mice. Only statistically significant data is graphed. Neurological deficit scoring was found to be not useful, therefore data are not shown (see section 5.4.1).
5.3 RESULTS

5.3.1 Results for MK-801 and N\textsuperscript{1}-dansyl-spermine

5.3.1.1 Histological assessments

Transient MCAO produced a 25.1 ± 3.0% hemisphere lesion volume and 25.0 ± 3.5 mm\textsuperscript{3} oedema in the control mouse brains.

Administered at 3 mg kg\textsuperscript{-1}, MK-801 afforded a significant reduction in the %HLV (11.7±2.7, \(P<0.05\)) in comparison with the saline treated MCAO control group and there was a trend towards a significant reduction on %HLV at 1 mg kg\textsuperscript{-1} (14.7±4.3, \(P=0.07\)) (Figure 5.1; Figure 5.3). Oedema was not significantly decreased by MK-801 (16.2±4.0 mm\textsuperscript{3}, 18.6±2.9 mm\textsuperscript{3}, for 1 or 3 mg kg\textsuperscript{-1} respectively). In fact, oedema appears to be increasing with higher doses of MK-801 (Figure 5.4).

N\textsuperscript{1}-dansyl-spermine significantly reduced the %HLV to 14.2 ± 2.6, 12.2 ± 2.9 and 13.0 ± 2.1(P< 0.05 for all cases), when administered at 1, 2 or 5 mg kg\textsuperscript{-1} respectively (Figure 5.2; Figure 5.3); Oedema was also significantly decreased by the treatment of N\textsuperscript{1}-dansyl-spermine at 1, 2 or 5 mg kg\textsuperscript{-1}(12.7 ± 2.2 mm\textsuperscript{3},; 10.6± 2.2 mm\textsuperscript{3} and 13.7 ± 2.9 mm\textsuperscript{3}, respectively, \(P<0.05\) for all the cases) (Figure 5.5).
Figure 5.1 Effect of MK-801 (i.p.) in mice subjected to 30 min transient MCAO with 24 hours reperfusion on percentage hemisphere lesion volume (%HLV). MK-801 was administered 30 min before the occlusion. Data are expressed as mean±se.mean (n=9). *P<0.05 versus (saline treated) MCAO control (two-tailed, unpaired Student’s t-test).
Figure 5.2 Effect of N\textsuperscript{1}-dansyl-spermine (i.p.) in mice subjected to 30 min transient MCAO with 24 hours reperfusion on percentage hemisphere lesion volume (%HLV). N\textsuperscript{1}-dansyl-spermine was administered 30 min before the occlusion. Data are expressed as mean±se.mean (n=9). *P<0.05 versus (saline treated) MCAO control (two-tailed, unpaired Student’s t-test).
Figure 5.3 Typical Images of TTC-derived brain slices of mice subjected to 30 min middle cerebral artery occlusion with 24 hours reperfusion indicating MK-801 and N₁-dansyl-spermine (given 30 min prior to occlusion) induced a neuroprotective effect on infarction volume. (A) MCAO Control animal; (B) MCAO animal administered 3 mg kg⁻¹ of MK-801; (C) MCAO animal administered 2 mg kg⁻¹ of N₁-dansyl-spermine. Slices are at 1.7 mm intervals in descending order from frontal pole (anterior to posterior). Scale bar = 3.5 mm.
Figure 5.4 Effect of MK-801 (i.p.) in mice subjected to 30 min transient MCAO with 24 hours reperfusion on oedema. MK-801 was administered 30 min before the occlusion. Data are expressed as mean±se.mean (n=9).
Figure 5.5 Effect of N\textsuperscript{1}-dansyl-spermine (i.p.) in mice subjected to 30 min transient MCAO with 24 hours reperfusion on oedema. N\textsuperscript{1}-dansyl-spermine was administered 30 min before the occlusion. Data are expressed as mean±se.mean (n=9). *P<0.05 versus (saline treated) MCAO control (two-tailed, unpaired Student’s t-test).
5.3.1.2 Spontaneous locomotor activity (LMA) test

30 minutes MCAO with 24 hours reperfusion caused a significant decrease on LMA (saline treated MCAO group 71.1 ± 13 versus sham-operated controls 397±57; \( P<0.05 \)) (Figure 5.6).

MK-801 did not reverse the hypoactivity caused by MCAO, in fact, it significantly decreased the LMA at both doses tested when compared with saline treated MCAO control group (26.1±8.0 and 30.7±9.3, for 1 or 3 mg kg\(^{-1}\) respectively, \( P<0.05 \) for both cases) (Figure 5.6).

N\(^{1}\)-dansyl-spermine significantly reversed the decrease of LMA caused by the MCAO at 1, 2 or 5 mg kg\(^{-1}\) (175±31, 252±50, 251±59 for 1, 2 or 5 mg kg\(^{-1}\) respectively; \( P<0.05 \) for all cases) (Figure 5.7).
Figure 5.6 Effect of MK-801 (i.p.) in mice subjected to 30 min transient MCAO with 24 hours reperfusion on locomotor activity. MK-801 was administered 30 min before the occlusion. Data are expressed as mean±se.mean (n=9). *P<0.05 versus (saline treated) MCAO control; +P<0.05 versus (saline treated) sham-operated control (two-tailed, unpaired Student’s t-test).
Figure 5.7 Effect of N\textsuperscript{1}-dansyl-spermine (i.p.) in mice subjected to 30 min transient MCAO with 24 hours reperfusion on locomotor activity. N\textsuperscript{1}-dansyl-spermine was administered 30 min before the occlusion. Data are expressed as mean±se.mean (n=9). *P<0.05 versus (saline treated) MCAO control; +P<0.05 versus (saline treated) sham-operated control (two-tailed, unpaired Student’s t-test).
5.3.1.3 Rotarod performance assessment

Following MCA occlusion, the performance of the saline treated animals in the rotarod test was significantly impaired when compared with the sham-operated group (saline treated MCAO group 49.1 ± 11 sec, versus sham-operated 114±13 sec; \( P<0.05 \)) (Figure 5.8).

Treatment with MK-801 did not improve the rotarod performance of MCAO mice at either dose in comparison to that of saline treated MCAO animals (46.2±13 sec and 44.1±15 sec for 1 or 3 mg kg\(^{-1}\) respectively).

\(N^1\)-dansyl-spermine treated MCAO groups showed significantly improved performance in the rotarod test at doses of 1 or 2 mg kg\(^{-1}\) (89.2 ± 12 sec, \( P<0.05 \), 91.3 ± 13 sec; \( P<0.05 \)), when compared with the saline treated MCAO group; However, no significant improvement at the highest dose (5 mg kg\(^{-1}\)) was observed (69.3±13 sec) (Figure 5.8).

5.3.1.4 Mortality rate

The mortality rate was 1 out of 10 in MCAO saline control group. The same mortality rate was also observed in all groups administered drugs. 1 out of 10 animals died in the MK-801 treated MCAO groups and in the \(N^1\)-dansyl-spermine treated MCAO groups.
Figure 5.8 Effect of N\textsuperscript{1}-dansyl-spermine (i.p.) in mice subjected to 30 min transient MCAO with 24 hours reperfusion on rotarod performance. N\textsuperscript{1}-dansyl-spermine was administered 30 min before the occlusion. Data are expressed as mean±se.mean (n=9). *P<0.05 versus (saline treated) MCAO control; +P<0.05 versus (saline treated) sham-operated control (two-tailed, unpaired Student’s t-test).
5.3.2 Results for novel polyamine analogues

5.3.2.1 BU43b

5.3.2.1.1 Histological assessments

BU43b markedly decreased the %HLV at both doses tested (11.8±2.7 and 14.2±3.7 for 20 or 30 mg kg$^{-1}$ respectively, $P<0.05$ for both cases, versus saline treated MCAO group 25.1 ± 3.0%) (Figure 5.9; Figure 5.10). Treatment of BU43b at 30 mg kg$^{-1}$ also significantly reduced the oedema to 12.2±2.3 mm$^3$ ($P<0.05$ versus 25.0 ± 3.5 mm$^3$ saline treated MCAO group) (Figure 5.11).
Figure 5.9 Effect of BU43b (i.p.) in mice subjected to 30 min transient MCAO with 24 hours reperfusion on percentage hemisphere lesion volume (%HLV). BU43b was administered 30 min before the occlusion. Data are expressed as mean±se.mean (n=9). *P<0.05 versus (saline treated) MCAO control (two-tailed, unpaired Student’s t-test).
Figure 5.10 Typical Images of TTC-derived brain slices of mice subjected to 30 min middle cerebral artery occlusion with 24 hours reperfusion indicating BU43b (given 30 min prior to occlusion) had a neuroprotective effect on infarction volume. (A) MCAO Control animal; (B) MCAO animal administered 30 mg kg\(^{-1}\) of BU43b. Slices are at 1.7 mm intervals in descending order from frontal pole (anterior to posterior). Scale bar= 3.5 mm.
Figure 5.11 Effect of BU43b (i.p.) in mice subjected to 30 min transient MCAO with 24 hours reperfusion on oedema. BU43b was administered 30 min before the occlusion. Data are expressed as mean±se.mean (n=9). *P<0.05 versus (saline treated) MCAO control (two-tailed, unpaired Student’s t-test).
5.3.2.1.2 Spontaneous locomotor activity (LMA) test

Bu43b significantly increased the LMA to 245±47 and 327±65 (for 20 or 30 mg kg\textsuperscript{-1} respectively, \(P<0.05\) for both cases, versus saline treated MCAO group 71.1 ± 13) (Figure 5.12).
Figure 5.12 Effect of BU43b (i.p.) in mice subjected to 30 min transient MCAO with 24 hours reperfusion on locomotor activity. BU43b was administered 30 min before the occlusion. Data are expressed as mean±s.e.mean (n=9). *P<0.05 versus (saline treated) MCAO control (two-tailed, unpaired Student’s t-test); +P<0.05 versus (saline treated) sham-operated control (two-tailed, unpaired Student’s t-test).
5.3.2.1.3 Rotarod performance assessment

BU43b, dosed at 30 mg kg\(^{-1}\), significantly improved the MCAO animals rotarod performance (87.7±10 sec) \((P<0.05\) versus saline treated MCAO group 49.1 ± 11 sec) Treatment with 20 mg kg\(^{-1}\) of BU43b did not significantly improve the rotarod performance (Figure 5.13), although there was a trend towards improvement.

5.3.2.1.4 Mortality rate

BU43b did not change the mortality rate of MCAO mice (1 out 10 for 20 or 30 mg kg\(^{-1}\)) when compared with the saline treated MCAO control (1 out 10).
Figure 5.13 Effect of BU43b (i.p.) in mice subjected to 30 min transient MCAO with 24 hours reperfusion on rotarod performance test. BU43b was administered 30 min before the occlusion. Data are expressed as mean±se.mean (n=9). *P<0.05 versus (saline treated) MCAO control; +P<0.05 versus (saline treated) sham-operated control (two-tailed, unpaired Student's t-test).
5.3.2.2 **BU36b**

5.3.2.2.1 Histological assessments

When administered at 20 or 30 mg kg$^{-1}$, BU36b did not afford a significant reduction on the %HLV (19.0±4.1 and 18.9±6.0 for 20 or 30 mg kg$^{-1}$ respectively in contrast to saline treated MCAO control 25.1 ± 3.0%) while oedema was significantly reduced at dose of 30 mg kg$^{-1}$ (12.4±2.5 mm$^3$) ($P<0.05$, versus saline treated MCAO group 25.0 ± 3.5 mm$^3$) (Figure 5.14). Oedema was reduced to 18.9±4.3 mm$^3$ at dose of 20 mg kg$^{-1}$, however, the difference was not statistically significant in comparison to the saline treated MCAO group.
Figure 5.14 Effect of BU36b (i.p.) in mice subjected to 30 min transient MCAO with 24 hours reperfusion on oedema. BU36b was administered 30 min before the occlusion. Data are expressed as mean±se.mean (n=9). *P<0.05 versus (saline treated) MCAO control (two-tailed, unpaired Student’s t-test).
5.3.2.2.2 Spontaneous locomotor activity (LMA) test

Treatment of BU36b at 30 mg kg\(^{-1}\) caused a moderate but not significant increase in LMA (137±30, \(P=0.06\) versus saline treated MCAO control group 71.1 ± 13). At the lower dose (20 mg kg\(^{-1}\)), no increase on LMA was observed (72.8±16).

5.3.2.2.3 Rotarod performance assessment

Similarly to the results in the LMA test, there was a trend towards improvement on rotarod performance in the MCAO animal group administered with BU36b at 30 mg kg\(^{-1}\) (76.0±10 sec, \(P=0.08\) versus saline treated MCAO group 49.1 ± 11 sec). No improvement was observed when animals were treated with the lower dose of BU36b (46.6±11 sec for 20mg kg\(^{-1}\)).

5.3.2.2.4 Mortality rate

The mortality rate was 2 out of 10 in animals administered the higher dose 30 mg kg\(^{-1}\) and 0 out of 10 following the 20 mg kg\(^{-1}\) dose of BU43b in MCAO groups. No significant difference in mortality from the saline treated MCAO group (1 out 10) was observed.

5.3.2.3 BU31b

(Due to scarcity of this compound only one dose (20 mg kg\(^{-1}\)) was tested. There was no more BU31b available for another dosing).

5.3.2.3.1 Histological assessments

BU31b did not reduce the %HLV; the %HLV was actually slightly but not significantly increased (27.4±12 for 20 mg kg\(^{-1}\)) when compared with saline treated MCAO control (25.1 ± 3.0%). BU31b did not significantly decrease the oedema (15.6±4.9 mm\(^3\)) in contrast to saline treated MCAO control (25.0 ± 3.5 mm\(^3\)).
5.3.2.3.2 Spontaneous locomotor activity (LMA) test

BU31b did not significantly alter the LMA at 20 mg kg\(^{-1}\) (79.8±33) in comparison to saline treated MCAO group (71.1 ± 13).

5.3.2.3.3 Rotarod performance assessment

Treatment of BU31b, slightly impaired the rotarod performance of MCAO animals at the dose administered (38.8±17 sec for 20 mg kg\(^{-1}\)), although the effects were not significant when compared with saline treated MCAO control (49.1 ± 11 sec).

5.3.2.3.4 Mortality rate

The mortality rate in MCAO control was 1 out of 10. The mortality rate in the BU31b treated group was increased (3 out 8) although the increase was not found to be statistically significant.

5.3.2.4 BU37b

5.3.2.4.1 Histological assessments

BU37b increased the %HLV to 36.8±9.8 and 29.6±9.4 (for 20 or 30 mg kg\(^{-1}\) respectively) although this increase did not reach statistical significance when compared with saline treated MCAO control (25.1 ± 3.0%). Oedema in BU37b treated groups was not significantly decreased (23.5±4.3 mm\(^3\) and 22.3±3.6 mm\(^3\) for 20 or 30 mg kg\(^{-1}\)) in comparison to saline treated MCAO control (25.0 ± 3.5 mm\(^3\)).

5.3.2.4.2 Spontaneous locomotor activity (LMA) test

The LMA counts were slightly decreased in MCAO animal groups treated with BU37b at both doses tested, although neither change was significant in comparison to the saline treated MCAO control group (71.1 ± 13). The LMA results for BU37b were 69.1±26 at 20 mg kg\(^{-1}\) and 62.4±19 at 30 mg kg\(^{-1}\) respectively.

5.3.2.4.3 Rotarod performance assessment
BU37b dosed at 20 or 30 mg kg\(^{-1}\) did not significantly change rotarod performance (46.3±15 sec at 20 mg kg\(^{-1}\), 46.6±21 sec at 30 mg kg\(^{-1}\)) in comparison to the saline treated MCAO control group (49.1 ± 11 sec).

5.3.2.3.4 Mortality rate

In BU37b treated MCAO groups, mortality rate was 2 out of 9 and 3 out of 8 for 20 or 30 mg kg\(^{-1}\) dosing groups respectively. These results were higher than the MCAO control (1 out of 10) but not statistically significantly different.

5.3.2.5 BU33b

5.3.2.5.1 Histological assessments

BU33b increased the %HLV (29.6±13 and 34.9±16 for 20 or 30 mg kg\(^{-1}\)) although not significantly when compared with saline treated MCAO group (25.1 ± 3.0%). BU33b, at the doses tested, did not significantly reduce oedema (24.0±9.3 mm\(^3\) and 23.0±8.1 mm\(^3\) for 20 or 30 mg kg\(^{-1}\) respectively) in comparison to the saline treated MCAO control (25.0 ± 3.5 mm\(^3\)).

5.3.2.5.2 Spontaneous locomotor activity (LMA) test

BU33b did not significantly change the LMA (68.7±30 for BU33b at 20 mg kg\(^{-1}\) and 70.3±34 for BU33b at 30 mg kg\(^{-1}\)) in comparison to saline treated MCAO control (71.1 ± 13).

5.3.2.5.3 Rotarod performance assessment

The rotarod performance was impaired slightly (not significantly) by the treatment of BU33b (38.3±20 sec for BU33b at 20 mg kg\(^{-1}\), and 42.0±24 sec for BU33b at 30 mg kg\(^{-1}\)) when compared with saline treated MCAO control (49.1 ± 11 sec).

5.3.2.5.4 Mortality rate
BU33b significantly increased the mortality rate to 4 out 7 in both dosing groups (20 or 30 mg kg$^{-1}$) ($P<0.05$ when compared to saline treated MCAO control 1 out of 10).
5.4 DISCUSSION

5.4.1 The model

30 minutes of middle cerebral artery occlusion with 24 hours reperfusion produced a 25% hemisphere lesion volume and 25 mm$^3$ oedema. Although the ischaemic period used in most transient ischaemia studies is from 60 to 120 minutes (Schabitz et al., 2001; Goyagi et al., 2003; Zausinger et al., 2003), there are some focal cerebral ischaemia studies which use 30 minutes (Namura et al., 2001; Tsuchiya et al., 2003). In a pilot experiment of the present study, ischaemia was induced for 45 minutes or 60 minutes with 24 hours reperfusion however, the mortality rate was found to be very high (4 out of 9 and 5 out of 8 for 45 minutes or 60 minutes respectively). In experiments using a 30 minute ischaemic period with 24 hours reperfusion a better mortality rate was seen (1 out of 10) and this duration was chosen for all the future experiments. The lesion size (25% HLV) produced in the present model is comparable to the size in most other studies using this model (20 to 35%) (Ozyurt et al., 1988; Gotti et al., 1990; Frazzini et al., 1994; Tatlisumak et al., 1998b).

The neurological deficit scoring assessment, which was used in the permanent focal cerebral ischaemia model, did not reveal any marked changes with drugs in the transient ischaemia model. Due to the small lesion size in the transient model, the median of the scores observed was 2 in the MCAO control group. As a result it was difficult to observe neuroprotection of the drugs. This assessment therefore was not very useful in the transient model.

5.4.2 MK-801

5.4.2.1 Histological effect of MK-801

In the present study, MK-801 significantly reduced the %HLV which indicates that MK-801 was neuroprotective in the transient focal cerebral ischaemia model. This is in agreement with the previous study using the permanent cerebral ischaemia model.
(Section 3) and many studies cited in the literature (Herz et al., 1998; Gorgulu et al., 2000). As previously discussed, it is likely that MK-801 exerted its neuroprotection through its inhibitory effect on the NMDA receptor.

However, in the present study, MK-801 did not reduce the oedema formation and in fact, the oedema appeared to be increasing dose-dependently. This is consistent with the observations in section 3 using the permanent model where MK-801 did not reduce the oedema at the highest dose (3 mg kg$^{-1}$). As previously mentioned, MK-801 is known to induce a dose-dependent reversible swelling and vacuolation in neurons (Olney et al., 1989; Auer & Coulter, 1994; Hargreaves et al., 1994), and this could have contributed to the oedema observed in transient ischaemic conditions in the present study.

5.4.2.2 Behavioural effects of MK-801

MK-801 did not have any beneficial effect on LMA; in fact, it worsened the condition by decreasing the LMA at both doses tested. The damaging effect of MK-801 on LMA is in agreement with the results obtained in the previous study (section 3). Locomotor dysfunction can be used as an index of CNS dysfunction and can highlight adverse effects of CNS active drugs (Dawson et al., 2001). The LMA data could reflect the pronounced side effect profile of MK-801 (Auer & Coulter, 1994). Similarly, MK-801 did not improve the rotarod performance of MCAO animals in the transient ischaemia model. This is in contrast to the effect of MK-801 on rotarod performance in the permanent model (section 3). As previously discussed, MK-801's effects on sensorimotor function in animal brain ischaemia models can be beneficial or detrimental (Barth et al., 1990). In the transient ischaemia model, the side effects of MK-801 may be enhanced by reperfusion or they may be more prominent in less-disabled animals.

5.4.3 N$^1$-dansyl-spermine
5.4.3.1 Histological effect of N<sup>1</sup>-dansyl-spermine

In this transient ischaemia model, N<sup>1</sup>-dansyl-spermine reduced the %HLV at all doses tested to a comparable extent to MK-801. In addition, N<sup>1</sup>-dansyl-spermine decreased oedema formation at all doses tested, which is impressive when compared to the ineffectiveness of the reference compound, MK-801. These results suggest that N<sup>1</sup>-dansyl-spermine is effective in reducing the ischaemic injury resulting from ischaemia, even after reperfusion. As mentioned in the introduction, NMDA over-activation plays an important role in the development of the ischaemic damage during ischaemia and also in the reperfusion phase (Nellgard et al., 1991). N<sup>1</sup>-dansyl-spermine has been suggested to act as a polyamine site NMDA antagonist and has been shown to have neuroprotective effects in the permanent focal cerebral ischaemia model (see section 3 for full discussion) and in the gerbil global ischaemia model (Kirby & Shaw, 2004).

The reduction in %HLV and oedema produced by N<sup>1</sup>-dansyl-spermine is more prominent in the present model than in the permanent model (section 3). There are possibly three main reasons for this observation. First, the models are different; the brain injury in the permanent model is caused by ischaemia alone while in the transient model, it is the result of both ischaemia and reperfusion, therefore N<sup>1</sup>-dansyl-spermine could be particularly effective in reducing reperfusion injury in brain tissue. Secondly, the transient model is less severe than the permanent model. It is likely that neuroprotection would be observed more clearly in a milder model. MK-801 also produced a more pronounced protection in the transient model than that seen in the permanent model. Thirdly, N<sup>1</sup>-dansyl-spermine was administered 30 min prior to the MCAO and it is possible that the recovery of the blood flow introduced 30 min after the occlusion in the transient model could perfuse the penumbral area with N<sup>1</sup>-dansyl-spermine from the blood circulation. However, as little is known about the pharmacodynamics of this compound, this point is speculative.

The most effective dose was 2 mg kg<sup>-1</sup>. This is consistent with the observations in the previous study using the permanent model (section 3). The results in the present
study again suggest that N\textsuperscript{1}-dansyl-spermine might have some partial agonist effect, which has been proposed previously in the study using the permanent model (see section 3 for full discussion). It has been demonstrated that N\textsuperscript{1}-dansyl-spermine produced a dose-dependent neuroprotection in a global model in gerbils with the best effect at 10 mg kg\textsuperscript{-1} (Kirby \textit{et al}., 2004). As discussed previously in section 2, it is likely that the difference in the effect of N\textsuperscript{1}-dansyl-spermine in the two models may be related to species differences between the mouse and the gerbil since animals of different species could react differently to the same level of drugs.

5.4.3.2 Behavioural effect of N\textsuperscript{1}-dansyl-spermine

In contrast to the effect of MK-801, N\textsuperscript{1}-dansyl-spermine improved the rotarod performance of the MCAO animals and markedly reversed the decrease of the LMA caused by MCAO. As previously demonstrated in section 3, N\textsuperscript{1}-dansyl-spermine does not affect the sham-operated animals' motor behaviour at these doses (1 to 5 mg kg\textsuperscript{-1}) when observed 24 hours after the administration. Therefore, the effects of N\textsuperscript{1}-dansyl-spermine on rotarod performance and LMA in MCAO animals were most likely due to its neuroprotection. The more pronounced beneficial effect of N\textsuperscript{1}-dansyl-spermine on LMA in MCAO animals observed in the transient model may be due to the previously discussed differences between the two models (section 5.1) and possibly related to the greater reduction in the lesion volume seen in the present study. The middle dose (2 mg kg\textsuperscript{-1}) was the most effective dose at enhancing rotarod performance and LMA. These results, when combined with %HLV and oedema data as previously discussed could indicate that N\textsuperscript{1}-dansyl-spermine may have, as yet unknown effects in the brain that become apparent at higher levels or may support the suggestion of a partial agonist effect of this compound.

5.4.4 BU43b

BU43b decreased both the %HLV and oedema in a dose dependent manner, showing neuroprotection in the transient model of ischaemia. BU43b also demonstrated a neuroprotective effect in the permanent cerebral ischaemia model (section 4). In that
permanent model, BU43b was also effective in reducing the %HLV and oedema. BU43b has been shown to be a potent polyamine antagonist and has a potent inhibitory effect at the NMDA receptor, so it is likely that it affords its neuroprotection by acting as a polyamine site NMDA antagonist in the permanent model (see section 4 for full discussion). As described in section 5.1, NMDA over-activation participates in the development of the ischaemic damage during ischaemia and also in the reperfusion phase (Nellgard et al., 1991). Therefore it is likely that in the transient model in the present study, BU43b protected the brain from ischaemia-reperfusion injury through an action at the polyamine site on the NMDA receptor.

The reduction in %HLV and oedema was greater in the transient model than in the permanent model. In addition, BU43b improved the rotarod performance of the MCAO mice and reversed the LMA decrease induced by MCAO in the transient model while no significant effect was seen following permanent ischaemia. These differences parallel the effect of N\textsuperscript{1}-dansyl-spermine in the two models. As discussed previously for N\textsuperscript{1}-dansyl-spermine, factors such as the involvement of reperfusion, the fact that the extent of injury following transient ischaemia was less than that following permanent ischaemia, and the possible influence of the recovery of blood flow in the transient ischaemia on drug delivery could underlie the more pronounced effect in the transient model.

5.4.5 BU36b

In the transient model, BU36b reduced oedema and there was a trend towards significant improvement in rotarod performance and LMA at the dose of 30 mg kg\textsuperscript{-1}. Therefore, BU36b showed some neuroprotection in the transient focal ischaemia model. Some neuroprotection by BU36b was also observed in the permanent ischaemia model as BU36b reduced the oedema (section 4). Similarly to BU43b, the mechanism of its neuroprotection seen in the present study could be through an action at the polyamine site on the NMDA receptor (See section 4 for detailed discussion).
5.4.6 BU31b, BU37b and BU33b

In the present study, only 20 mg kg\(^{-1}\) of BU31b was tested and the results showed that it was ineffective and possibly toxic in this transient model. This is in line with the study using the permanent model (see section 4) where BU31b was shown to be ineffective and toxic at a higher dose of 30 mg kg\(^{-1}\) (section 4). As previously discussed in section 4, BU31b has been shown to be a potent NMDA antagonist in vitro (Fixon-Owoo et al., 2003) and has some polyamine antagonist effect \textit{in vivo} when given i.c.v (Murphy et al., 2003). The ineffectiveness/toxicity seen in the present study may be related to systemic metabolism when it was administered via an i.p. route, although there is no information on its metabolism or other record of its interactions available.

BU37b appeared to be toxic in the transient model, which is similar to the findings in the permanent ischaemia model (section 4). As BU37b is a novel compound, the toxicity observed in both studies may be caused by the actions of itself or its metabolite at an unknown site.

In the present study BU33b was toxic in the transient ischaemia model as it increased the mortality rate significantly. Interestingly, in contrast, in the permanent model (section 4), it showed some neuroprotection. However, in the permanent model, the neuroprotection by BU33b was seen at the lower dose rather than the higher dose. The toxicity observed in the present study may be caused by an as yet unknown interaction.
5.5 CONCLUSION

The present study suggests that N\(^1\)-dansyl-spermine is a potent neuroprotectant in the transient focal cerebral ischaemia model in mice when administered pre-ischaemia. N\(^1\)-dansyl-spermine was comparable as MK-801 in its effectiveness at reducing the %HLV at dosed tested. Furthermore, N\(^1\)-dansyl-spermine reduced the oedema which MK-801 did not. N\(^1\)-dansyl-spermine also showed beneficial effects in all behavioral assessments, which is in contrast to MK-801. MK-801 had no beneficial effect on the sensorimotor function and even worsened the LMA. It can be suggested that N\(^1\)-dansyl-spermine produced its neuroprotective effects through a polyamine modulatory site on the NMDA receptor, although this mechanism needs to be confirmed, as discussed in section 3. Along with the observations in section 3, it is suggested that N\(^1\)-dansyl-spermine could be a promising therapeutic candidate for stroke treatment.

The present study demonstrates that BU43b and BU36b are neuroprotective agents in the focal transient ischaemia model in mice when administered preischaemia. Their neuroprotection could be afforded through an action at the polyamine site on the NMDA complex, although further studies are needed to confirm this. In addition, the observation of two polyamine analogues with neuroprotective potentials in this drug series favors the hypothesis that polyamines have a neurotoxic effect in ischaemia (see section 4 for full discussion), and a polyamine antagonist may have therapeutic potential.
Section 6 Change of polyamine profiles following cerebral ischaemia

6.1 INTRODUCTION

Polyamines are ubiquitous components of all eukaryotic cells (Seiler, 1994) and cells have developed a complex system of regulation for polyamine homeostasis (Urdiales, 2001). While it has long been recognized that changes in polyamine metabolism occur following cerebral ischaemia (Paschen et al., 1992), reports on the change of the polyamine profiles still conflict. Putrescine is consistently reported to be increased, particularly following transient ischaemia (Adibhatla et al., 2002). There is, however, much controversy about the change of spermidine and spermine levels following ischaemia, which are said to be reduced (Paschen et al., 1992), not altered (Baskaya et al., 1997), or even increased (Koenig et al., 1990) in different studies. Polyamine metabolism has been described in detail in section 1.1.2. N\textsuperscript{-}acetylspermidine is the product of a rate-limiting enzyme (SSAT) in the interconversion pathway (Wallace et al., 2003). The change in N\textsuperscript{-}acetylspermidine levels, which could reflect the change of the activity of this pathway, is rarely studied following ischaemia. One study has shown an increase of N\textsuperscript{-}acetylspermidine in a transient focal ischaemia model in rats and global ischaemia model in gerbils (Rao et al., 2000). However, the profile of N\textsuperscript{-}acetylspermidine has not been studied in a permanent focal ischaemia model. The change in the levels of extracellular polyamines in vivo following ischaemia insult remains to be investigated further.

It has been suggested by Paschen et al. (1992) that polyamines could be released from neurons into the extracellular space following cerebral ischaemia, thereby enhancing the neurotoxicity caused by NMDA receptor activation through binding to the polyamine site at this receptor complex. In vitro studies have shown that polyamines are released following ischaemia (Paschen et al., 1992). Ifenprodil and eliprodil have been demonstrated to produce neuroprotection through their action at the polyamine site on the NMDA receptor in animal ischaemia models (presumably by antagonising the released polyamines) (Cater et al., 1997). The novel polyamine
NMDA antagonist, N\textsuperscript{1}-dansyl-spermine has also afforded neuroprotection possibly via the same mechanism (Kirby & Shaw, 2004).

In the present study, the change in the polyamine profile (putrescine, spermidine, spermine, and N\textsuperscript{1}-acetyl spermidine) in various brain regions following permanent or transient focal cerebral ischaemia in mice was studied. In addition, the change in extracellular space polyamine (putrescine, spermidine and spermine) profile was examined following permanent focal cerebral ischaemia in mice using the microdialysis.

6.2 METHODS AND MATERIALS

For details, see section 2.4 and section 2.5. The cerebral ischaemia models used were the focal permanent and transient middle cerebral artery occlusion models. Polyamines released into the extracellular space were sampled by using microdialysis. Probes were implanted into mouse cerebral cortex (from bregma: 3.8 mm lateral, 0.5 mm posterior; a depth of 2 mm from dura). The concentration of polyamines in dialysate samples was not corrected for the recovery of the dialysis probe according to published approaches (Carter et al., 1995; Vivo et al., 2002). HPLC was used to measure the polyamine levels in the tissue homogenate and dialysate (for details of the procedure, see section 2.4 and 2.5). For the extracellular polyamine measurement, some microdialysate samples were lost during HPLC procedure, therefore the n numbers are not equal (as shown in the results).

6.3 RESULTS

6.3.1 Measurement of polyamine levels

6.3.1.1 Measurement of tissue polyamine levels

This method is useful for the determination of tissue polyamines. This is described below with standard curves and chromatograph samples shown.
Using the HPLC system described above, putrescine, spermidine, spermine and the internal standard (1, 7-diaminoheptane) were well separated from each other and unknown components. A HPLC chromatography sample is shown in Figure 6.1. The retention times were 2.66, 4.43, 5.11 and 3.77 min for putrescine, spermidine, spermine and the internal standard respectively. For the assay of N¹-dansylspermidine, a different HPLC system from that for putrescine, spermidine and spermine was used. The retention times were 9.32 and 14.3 min for N¹-acetylspermidine and the internal standard. N¹-acetylspermidine was well separated from the unknown components in the samples but the internal standard was not well separated from unknown components (Figure 6.2). So the internal standard (1, 7-diaminoheptane) was not used as reference in the N¹-acetylspermidine data calculation.

The standard curves for putrescine, spermidine and spermine were linear between 1.25 µM – 50 µM and the curve for N¹-acetylspermidine was linear between 0.0625 – 1.25 µM. Standard curves are shown in Figure 6.3.
Figure 6.1 A representative chromatograph of putrescine, spermidine, spermine and the internal standard measured from a homogenised mouse brain tissue (midbrain) sample after 30 min of middle cerebral artery occlusion with 23.5 hours reperfusion. Polyamines were determined by HPLC after dansylation. a, putrescine (3.8 μM in supernatant); b, internal standard; c, spermidine (29 μM in supernatant); d, spermine (21 μM in supernatant). The other peak(s) are unknown components in the sample.
Figure 6.2 A representative chromatograph of N\textsuperscript{1}-acetylspermidine and the internal standard measured from a homogenised mouse brain tissue (cerebral cortex) sample after 24 hours of permanent middle cerebral artery occlusion. Polyamines were determined by HPLC after dansylation. a, N\textsuperscript{1}-acetylspermidine (0.50 μM in supernatant). b, internal standard. The retention time for the internal standard was 14.3 min but the internal standard was not well separated from unknown components. The other peaks are unknown components.
Figure 6.3 Standard curves for the HPLC analysis of tissue putrescine, spermidine, spermine and N$^1$-acetylspermidine from mouse brain homogenate using dansylation. (a) Putrescine; (b) Spermidine; (c) Spermine; (d) N$^1$-acetylspermidine.
6.3.1.2 Measurement of extracellular polyamine levels

This HPLC method is useful for the determination of extracellular polyamines. The usefulness of this method is described below with standard curves and a chromatograph sample shown.

In the HPLC system described in section 2.5, putrescine, spermidine, spermine and the internal standard were well separated. Figure 6.4 shows a sample HPLC chromatograph. The retention times were 7.78, 16.5, 20.9 and 14.0 min for putrescine, spermidine, spermine and the internal standard respectively.

The standard curve for putrescine, spermidine and spermine were linear between 0.0625–1.25 μM. Standard curves are shown in Figure 6.5.
Figure 6.4 A representative chromatograph of putrescine, spermidine, spermine and the internal standard measured from a mouse brain microdialysate sample during 3 hours of permanent focal cerebral ischaemia. Microdialysis probes were implanted in cerebral cortex (from bregma: 3.8 mm lateral, 0.5 mm posterior; depth: 2 mm from dura). Polyamines were determined by HPLC after dansylation. a, putrescine (0.25 μM); b internal standard; c, spermidine (0.38 μM); d, spermine (0.40 μM). The other peaks are unknown components in the sample.
Figure 6.5 Standard curves for the HPLC analysis of extracellular putrescine, spermidine and spermine following dansylation. (a) putrescine; (b) spermidine; (c) spermine.
6.3.2 Tissue polyamine profile following cerebral ischaemia

6.3.2.1 Polyamine profile following permanent cerebral ischaemia

Putrescine levels were significantly increased in the midbrain following 24 hours of middle cerebral artery occlusion (17.3±2.4 nmol g⁻¹ wet tissue weight in the ischaemic hemisphere versus 7.38±1.4 nmol g⁻¹ in the non-ischaemic control hemisphere; \( P<0.05 \)). In the other regions, there were small increases of the putrescine levels however no statistical significance was attained (9.34±3.1 nmol g⁻¹ versus 7.33±1.5 nmol g⁻¹ in cerebral cortex, 12.6±2.5 nmol g⁻¹ versus 11.5±5.3 nmol g⁻¹ in hypothalamus, 14.2±3.6 nmol g⁻¹ versus 13.4±3.0 nmol g⁻¹ in hippocampus). Values presented compare the ischaemic hemisphere versus the corresponding non-ischaemic control hemisphere of the same animals respectively (Figure 6.6).
Figure 6.6 Regional putrescine levels in mice subjected to 24 hours of permanent focal cerebral ischaemia induced by middle cerebral artery occlusion. Data are shown as mean ±se.mean (n=8). Control: contralateral non-ischaemic hemisphere; Ischaemia: ipsilateral ischaemic hemisphere. *P<0.05 versus the control in corresponding region (two-tailed paired Student’s t-test).
Following 24 hours of middle cerebral artery occlusion, significant decreases of spermidine levels were found in the mouse cerebral cortex (134±29 nmol g\(^{-1}\) in the ischaemic hemisphere versus 334±52 nmol g\(^{-1}\) in the non-ischaemic control hemisphere; \(P<0.05\)) and hippocampus (321±80 nmol g\(^{-1}\) in the ischaemic hemisphere versus 598±83 nmol g\(^{-1}\) in the non-ischaemic control hemisphere; \(P<0.05\)). Spermidine was also reduced in the hypothalamus (225±52 nmol g\(^{-1}\) in the ischaemic hemisphere versus 309±24 nmol g\(^{-1}\) in the non-ischaemic control hemisphere) and in the midbrain (344±16 nmol g\(^{-1}\) in the ischaemic hemisphere versus 408±31 nmol g\(^{-1}\) in the non-ischaemic control hemisphere), although those reductions were not statistically significant (Figure 6.7).
Figure 6.7 Regional spermidine levels in mice subjected to 24 hours of permanent focal cerebral ischaemia induced by middle cerebral artery occlusion. Data are shown as mean ±se.mean (n=8). Control: contralateral non-ischaemic hemisphere; Ischaemia: ipsilateral ischaemic hemisphere. *P<0.05 versus the control in the corresponding region (two-tailed paired Student’s t-test).
Spermine levels were significantly reduced in the cerebral cortex of the mice subjected to 24 hours of middle cerebral artery occlusion (107±41 nmol g⁻¹ in the ischaemic hemisphere versus 321±68 nmol g⁻¹ in the non-ischaemic control hemisphere; *P*<0.05). There were non-significant decreases of the spermine levels in the ischaemic hemisphere in the other regions measured when compared to the corresponding regions in the non-ischaemic hemisphere (134±31 nmol g⁻¹ versus 213±50 nmol g⁻¹; 175±58 nmol g⁻¹ versus 244±39 nmol g⁻¹; 242±15 nmol g⁻¹ versus 313±49 nmol g⁻¹ for hypothalamus, hippocampus and midbrain respectively) (Figure 6.8).
Figure 6.8 Regional spermine levels in mice subjected to 24 hours of permanent focal cerebral ischaemia induced by middle cerebral artery occlusion. Data are shown as mean ±se.mean (n=8). Control: contralateral non-ischaemic hemisphere; Ischaemia: ipsilateral ischaemic hemisphere. *P<0.05 versus the control in corresponding region (two-tailed paired Student’s t-test).
N¹-acetyl spermidine showed significant increases in all brain regions measured; in the cerebral cortex: 5.48±1.4 nmol g⁻¹ in the ischaemic hemisphere versus 0.829±0.15 nmol g⁻¹ in the non-ischaemic control hemisphere; P<0.05. Levels in the hypothalamus: 3.54±1.7 nmol g⁻¹ in the ischaemic hemisphere versus 1.10±0.71 nmol g⁻¹ in the non-ischaemic control hemisphere; P<0.05. Levels in the hippocampus: 4.10±0.87 nmol g⁻¹ in the ischaemic hemisphere versus 1.25±0.72 nmol g⁻¹ in non-ischaemic control hemisphere; P<0.05. Levels in the midbrain: 3.83±1.0 nmol g⁻¹ in the ischaemic hemisphere versus 0.95±0.73 nmol g⁻¹ in non-ischaemic control hemisphere; P<0.05 (Figure 6.9).
Figure 6.9 Regional $N^1$-acetylspemidine levels in mice subjected to 24 hours of permanent focal cerebral ischaemia induced by middle cerebral artery occlusion. Data are shown as mean ± se.mean (n=8). Control: contralateral non-ischaemic hemisphere; Ischaemia: ipsilateral ischaemic hemisphere. *$P<0.05$ versus the control in corresponding region (two-tailed paired Student’s t-test).
6.3.2.2 Polyamine profile following transient cerebral ischaemia

Following 30 min ischaemia with 23.5 hours reperfusion, significant increases of the putrescine levels were observed in all the brain regions measured; Levels in the cerebral cortex: 29.3±3.3 nmol g\(^{-1}\) in the ischaemic hemisphere versus 11.7±2.3 nmol g\(^{-1}\) in the non-ischaemic control hemisphere; \(P<0.05\). Levels in the hypothalamus: 27.4±4.0 nmol g\(^{-1}\) in the ischaemic hemisphere versus 16.2±2.0 nmol g\(^{-1}\) in the non-ischaemic control hemisphere; \(P<0.05\). Levels in the hippocampus: 36.7±2.1 nmol g\(^{-1}\) in the ischaemic hemisphere versus 21.6±4.0 nmol g\(^{-1}\) in the non-ischaemic control hemisphere; \(P<0.05\). Levels in the midbrain: 36.4±4.8 nmol g\(^{-1}\) in the ischaemic hemisphere versus 14.2±3.0 nmol g\(^{-1}\) in the non-ischaemic control hemisphere; \(P<0.05\) (Figure 6.10).
Figure 6.10 Regional putrescine levels in mice subjected to 30 minutes of cerebral ischaemia with 23.5 hours reperfusion. Ischaemia was induced by middle cerebral artery occlusion. Data are shown as mean±se.mean (n=8). Control: contralateral non-ischaemic hemisphere; Ischaemia: ipsilateral ischaemic hemisphere. *P<0.05 versus the control in the corresponding region (two-tailed paired Student’s t-test).
Spermidine levels showed slight decreases in all the brain regions measured following the transient focal cerebral ischaemia, however, none of the decreases was statistically significant. In the cerebral cortex, the spermidine level was $183 \pm 11$ nmol g$^{-1}$ in the ischaemic hemisphere and $214 \pm 14$ nmol g$^{-1}$ in the non-ischaemic control hemisphere. In the hypothalamus the spermidine level was $356 \pm 59$ nmol g$^{-1}$ in the ischaemic hemisphere and $386 \pm 27$ nmol g$^{-1}$ in the non-ischaemic control hemisphere. In the hippocampus the spermidine level was $413 \pm 47$ nmol g$^{-1}$ in the ischaemic hemisphere and $438 \pm 63$ nmol g$^{-1}$ in the non-ischaemic control hemisphere. In the midbrain the spermidine level was $305 \pm 35$ nmol g$^{-1}$ in the ischaemic hemisphere and $347 \pm 53$ nmol g$^{-1}$ in the non-ischaemic control hemisphere (Figure 6.11).

Non-significant small reductions of spermine levels also occurred in every region measured in the transient ischaemia model. In the cerebral cortex, the level in the ischaemic hemisphere was $183 \pm 8.9$ nmol g$^{-1}$ versus control $201 \pm 11$ nmol g$^{-1}$; in the hypothalamus, the level in the ischaemic hemisphere was $202 \pm 18$ nmol g$^{-1}$ versus control $275 \pm 24$ nmol g$^{-1}$; in the hippocampus, the level in the ischaemic hemisphere was $232 \pm 12$ nmol g$^{-1}$ versus control $259 \pm 40$ nmol g$^{-1}$; in the midbrain, the level in the ischaemic hemisphere was $196 \pm 14$ nmol g$^{-1}$ versus control $213 \pm 18$ nmol g$^{-1}$ (Figure 6.12).
Figure 6.11 Regional spermidine levels in mice subjected to 30 minutes of cerebral ischaemia with 23.5 hours reperfusion. Ischaemia was induced by middle cerebral artery occlusion. Data are shown as mean±se.mean (n=8). Control: contralateral non-ischaemic hemisphere; Ischaemia: ipsilateral ischaemic hemisphere. *P<0.05 versus the control in the corresponding region (two-tailed paired Student’s t-test).
Figure 6.12 Regional spermine levels in mice subjected to 30 minutes of cerebral ischaemia with 23.5 hours reperfusion. Ischaemia was induced by middle cerebral artery occlusion. Data are shown as mean±se.mean (n=8). Control: contralateral non-ischaemic hemisphere; Ischaemia: ipsilateral ischaemic hemisphere. *P<0.05 versus the control in the corresponding region (two-tailed paired Student’s t-test).
N$_1$-acetylspermidine levels were sharply increased after the transient focal cerebral ischaemia in all the brain regions measured. Statistically significant increases were observed in the cerebral cortex (2.26±0.52 nmol g$^{-1}$ in the ischaemic hemisphere versus 0.462±0.12 nmol g$^{-1}$ in the non-ischaemic control hemisphere; $P<0.05$). Levels were also significantly increased in the hippocampus (4.6±1.0 nmol g$^{-1}$ in the ischaemic hemisphere versus 0.94±0.30 nmol g$^{-1}$ in the non-ischaemic control hemisphere; $P<0.05$) and the midbrain (1.94±0.48 nmol g$^{-1}$ in the ischaemic hemisphere versus 0.42±0.12 nmol g$^{-1}$ in the non-ischaemic control hemisphere; $P<0.05$). In the hypothalamus, there was an increase of N$_1$-acetylspermidine, although the increase did not reach statistical significance (1.86±0.83 nmol g$^{-1}$ in the ischaemic hemisphere versus 0.792±0.14 nmol g$^{-1}$ in the non-ischaemic control hemisphere) (Figure 6.13).
Figure 6.13 Regional N$^1$-acetylspetmidine levels in mice subjected to 30 minutes of cerebral ischaemia with 23.5 hours reperfusion. Ischaemia was induced by middle cerebral artery occlusion. Data are shown as mean±se.mean (n=8). Control: contralateral non-ischaemic hemisphere; Ischaemia: ipsilateral ischaemic hemisphere. *P<0.05 versus the control in the corresponding region (two-tailed paired Student’s t-test).
6.3.3 Extracellular space polyamine levels following permanent cerebral ischaemia

Cerebral ischaemia caused an increase of the extracellular spermine measured throughout the 3 hours of MCAO with microdialysis for dialysate collection. The level of extracellular spermine in the ischaemic cerebral cortex at every time point was significantly higher than that measured in the control non-ischaemic cerebral cortex at the corresponding time point. The data are shown as follows: 1 hour 0.330±0.037 μM versus 0.121±0.043 μM, 1.5 hour 0.412±0.072 μM versus 0.0648±0.039 μM, 2 hours 0.513±0.099 μM versus 0.0483±0.017 μM, 2.5 hours 0.470±0.11 μM versus 0.041±0.030 μM, 3 hours 0.372±0.039 μM versus 0.0710±0.023 μM (P<0.05 in each case) (Figure 6.14).

The spermine level in the ischaemic cerebral cortex seems to peak at 2 hours although no statistical significant difference was found when compared with the spermine levels at the other time points (1, 1.5, 2.5 and 3 hours) using one-way ANOVA.

In the non-ischaemia cerebral cortex, the highest level of spermine appears at 1 hour; however, this was not significantly different from any of the later levels (one-way ANOVA).
Figure 6.14 Extracellular space spermine levels in the contralateral non-ischaemic cerebral cortex (control) and in the ipsilateral ischaemic cerebral cortex (ischaemia) in mice subjected to 3 hours of middle cerebral artery occlusion. 30 min samples were collected and analysed. Data are shown as mean ±se.mean (n=7-10). *P<0.05 versus the control at the corresponding time point (one-way ANOVA).
Extracellular spermidine was also significantly enhanced by cerebral ischaemia 1.5 hours, 2 hours, and 3 hours following onset of the MCAO. The levels were shown as follows (0.371±0.096 μM versus 0.104±0.033 μM, 0.455±0.10 μM versus 0.134±0.068 μM and 0.239±0.045 μM versus 0.093±0.024 μM; for 1.5, 2 and 3 hours respectively; P<0.05 for each case). At the other time points measured, the spermidine levels in the ischaemic cerebral cortex were higher than that in the non-ischaemic cerebral cortex, although no statistical significance was attained (0.319±0.054 μM versus 0.196±0.064 μM, 0.338±0.15 μM versus 0.086±0.017 μM for 1 and 2.5 hours respectively) (Figure 6.15).

The spermidine level in the ischaemic cerebral cortex was highest at the 2 hours time point, although this level was not significantly different from those at the other time points (1, 1.5, 2.5 and 3 hours) using one-way ANOVA.

In the non-ischaemia cerebral cortex, the highest level of spermidine was seen at 1 hour; however, this was not significantly different from any of the later levels (one-way ANOVA).
Figure 6.15 Extracellular space spermidine levels in the contralateral non-ischaemic cerebral cortex (control) and in the ipsilateral ischaemic cerebral cortex (ischaemia) in mice subjected to 3 hours of middle cerebral artery occlusion. 30 min samples were collected and analysed. Data are shown as mean ± se.mean (n=7-10). *P<0.05 versus the control at the corresponding time point (one-way ANOVA).
The extracellular level of putrescine was not significantly changed throughout the microdialysis study following cerebral ischaemia (Figure 6.16).

In the non-ischaemia cerebral cortex, the highest level of putrescine appeared to be at 1 hour; however, this was not significantly different from any of the later levels (one-way ANOVA).
Figure 6.16 Extracellular space putrescine levels in the contralateral non-ischaemic cerebral cortex (control) and in the ipsilateral ischaemic cerebral cortex (ischaemia) in mice subjected to 3 hours of middle cerebral artery occlusion. 30 min samples were collected and analysed. Data are shown as mean ±se.mean (n=7-10). *P<0.05 versus the control at the corresponding time point (one-way ANOVA).
6.4 DISCUSSION

6.4.1 Methods used in this section

In the present study, the HPLC assay using dansylation was successfully used in the determination of polyamines. Sample preparation can be a source of imprecision. Pre-treatment steps such as chemical reactions (dansylation) and solvent extraction using toluene were required in the present study in order to achieve good separation and detection. Calibration of standard curves was employed in the present study. Since both the standards and samples were pre-treated in the same manner and the results of samples were calculated using the standard, this reduced the possibility for error. Also the internal standard was used to compensate for sample loss during preparation in the measurement of putrescine, spermidine and spermine both in the homogenate and in the microdialysate. However, the internal standard was found to be not applicable in the assay of N\textsuperscript{1}-acetylspermidine in the homogenate. The internal standard was not well separated. In the measurement of the N\textsuperscript{1}-acetylspermidine level, a different HPLC system had to be used to separate the N\textsuperscript{1}-acetylspermidine. Due to the lower concentration of the N\textsuperscript{1}-acetylspermidine level in the homogenate, a higher sensitivity in the HPLC detector had to be used. As a result, the detector picked up a lot more components and a few peaks (some of them could be putrescine, spermidine and spermine, they were not identified since it is not the focus of the work) were large. Therefore and unfortunately, the internal standard was not well separated from those components in this HPLC system.

Fluorescence detection is extremely sensitive and selective. It is typically three orders of magnitude more sensitive than UV detection. Another major advantage of fluorescence detection is its ability to discriminate analyte from interference or background peaks since few analytes possess natural fluorescences. In the present study, the pre-column dansylation was employed to endow polyamines with fluorescence to achieve high selectivity. The detector picked up polyamines at nM levels (especially from microdialysate samples), showing this method is of high sensitivity and is ideal for polyamine determination.
Homogenization of each brain region manually with micropestles and extraction of polyamines using 0.4 M perchloric acid is an established method in this laboratory. Preliminary experiments showed that this method can achieve complete polyamine extraction from the tissue into the supernatant (unpublished data). In addition, it has been shown that if protein was not precipitated completely preparation and thereby left in the supernatant, it can cause problems in the subsequent experiment: the solvent layer (yellow layer) would fail to separate from the water layer when the solvent toluene was used to extract the dansylated polyamines (see section 2.4.4). In the present study, 0.4 M perchloric acid precipitated the protein in the brain tissue well, as during the extraction of the dansylated polyamines using the solvent (toluene), the two layers (the solvent layer and water layer) were found to be distinctive and this readily allowed the removal of the solvent layer containing polyamines.

In vivo microdialysis was successfully used to collect polyamine samples from the extracellular space in the cerebral cortex. It is not complicated and allows continuous sample collection. During the microdialysis study, maintenance of the long term anaesthesia (up to 3.5 hours) was a problem to be conquered. Chloral hydrate through repeated administration was still used as the anaesthetic. Problems were encountered with the need for repeated administration. A balance had to be achieved between overdosing the animal while ensuring the animal was maintained fully under surgical anaesthesia. To overcome this problem, in the present study, each time a small dose (one fifth of the first dose) was repeatedly given approximately every 20 min and the animal was carefully monitored throughout each individual experiment.

6.4.2 Tissue polyamines

6.4.2.1 Putrescine and N\textsuperscript{1}-acetylspermidine

In the present study, following 24 hours of permanent ischaemia, a significant increase in putrescine was seen in the midbrain. In contrast, in transient ischaemia,
significant increases of putrescine levels were observed in all the brain regions measured (the cerebral cortex, hypothalamus, hippocampus and midbrain). It has been shown that both permanent and transient cerebral ischaemia cause an increase in putrescine levels (Paschen et al., 1991; Sauer et al., 1992; Baskaya et al., 1997). Similarly to the finding in the present study, a previous study also showed that the increase of putrescine was less prominent following permanent ischaemia than in transient ischaemia (Baskaya et al., 1997). Interestingly, using focal ischaemia model in cats, showed no change in putrescine levels in the densely ischaemic region, but an increase was observed in the penumbra region, the region of incomplete ischaemia (Baskaya et al., 1997). Increases of putrescine levels have also been reported only after reperfusion but not prior to reperfusion (Paschen et al., 1988). The putrescine level did not change during the ischaemia period in the same study (Paschen et al., 1988). The results in the present study are in agreement with the others in general. The putrescine basal levels in brain reported were usually from several to tens of nmol g\(^{-1}\) tissue (Rao et al., 2000; Hayashi et al., 2004), which are similar to the present study. In the present study, following transient ischaemia the putrescine levels in the contralateral hemisphere appeared to be higher than following permanent ischaemia in the corresponding regions, however, neither was significantly different.

The overshoot of putrescine following ischaemia may be due to an increase of ODC activity (Paschen, 1987). SSAT is also thought to contribute to the increase of putrescine following ischaemia (Rao et al., 2000). In the present study, the change of the levels of N\(^1\)-acetylsperrmidine, which is the direct product of the rate-limiting enzyme (SSAT) in the interconversion pathway, was investigated. Following the transient ischaemia, N\(^1\)-acetylsperrmidine levels were found significantly increased in cerebral cortex, hippocampus and midbrain (areas where putrescine also showed significant increases), indicating that the interconversion pathway is activated and could have contributed to the increase in putrescine. Therefore, SSAT may indeed play in an important role in increasing putrescine levels following ischaemia, particularly following transient ischaemia. However, following permanent ischaemia, sharp increases of N\(^1\)-acetylsperrmidine were observed in cerebral cortex,
hypothalamus and hippocampus. Conversely, putrescine showed no significant increase in those regions. Therefore, an increase of SSAT activity may not necessarily cause an increase of putrescine.

It is thought that the eventual recirculation or the continuation of blood flow, at least at the penumbra level, is necessary for the induction of the polyamine pathway (Baskaya et al., 1997). In the present study, following permanent ischaemia, no significant increase of putrescine was observed in the severely damaged areas such as the cerebral cortex and hippocampus (See Figure 3.5) where the blood flow could be severely compromised. The densely ischaemic core and the penumbra were not separated in the present experiments, there may be significant increases of putrescine in the penumbra in regions such as cerebral cortex. For the production of putrescine, both ODC and SSAT are key enzymes. The accumulation of N\(^1\)-acetylspermidine shows that SSAT was induced following permanent cerebral ischaemia in the present study. ODC activity induction may need the eventual recovery or the continuation of blood flow at necessary levels. The consistent increases of putrescine levels following transient ischaemia seen in the present study and in the literature may also support this view.

The increase in putrescine is thought to be an important factor in blood-brain barrier dysfunction and in the development of vasogenic oedema (Baskaya et al., 1997). As previously mentioned, putrescine may also activate Ca\(^{2+}\) flux at the cell membrane, through the voltage-activated Ca\(^{2+}\) channels (Herman et al., 1993). The putrescine increase observed in the present study (particularly following transient ischaemia) could have contributed to the cellular damage (Muszynski et al., 1993; Dogan et al., 1999). The effect of putrescine following ischaemia, particularly permanent ischaemia, has to be investigated further.

As previously mentioned, the present study showed the increase of N\(^1\)-acetylspermidine levels following both focal permanent and transient ischaemia in mice. Accumulation of N\(^1\)-acetylspermidine has been previously shown in global ischaemia in gerbils and transient focal ischaemia in rats (Rao et al., 2000). The
levels of N\(^1\)-acetylspermidine in the sham-operated animals and the ipsilateral hemisphere of MCAO animals reported by Rao et al. were at sub and low nmol g\(^{-1}\) levels. N\(^1\)-acetylspermidine was at an approximately 5-10 fold higher in the ischaemic hemisphere 24 hours after focal transient ischaemia than the control hemisphere. Similar observations were made in the present study. The sharp increase of N\(^1\)-acetylspermidine observed in the present study and Rao and his/her co-workers’ study (2000) implicates the activation of SSAT. Breakdown via the interconversion pathway involves the combined action of SSAT and the subsequent oxidation of acetylate derivatives by PAO (Casero & Pegg, 1993). The oxidation of acetylated polyamines by PAO produces 3-acetamidopropanol and H\(_2\)O\(_2\), both of which have been shown to be cytotoxic (Parchment et al., 1989). Therefore, the N\(^1\)-acetylspermidine increase found in both permanent and transient focal ischaemia models in the present study suggests that the activation of the interconversion pathway of polyamine catabolism may contribute to the cellular damage following ischaemia.

6.4.2.2 Spermidine and spermine

In the present study, following permanent ischaemia, both spermidine and spermine were significantly reduced in the cerebral cortex and a significant decrease of spermidine levels occurred in the hippocampus. No significant change was observed following transient ischaemia. The levels of basal spermidine and spermine levels were at hundreds of nmol g\(^{-1}\) tissue range. The results are consistent with the literature. Spermidine and spermine have been reported to be reduced following permanent focal ischaemia and during the ischaemic period in a transient ischaemia model (Paschen et al., 1991; Sauer et al., 1992) but no decrease was seen after 30 min focal ischaemia with 4 or 24 reperfusion (Paschen et al., 1991). Spermidine and spermine were also reported to be elevated following transient global ischaemia in gerbils (Koenig et al., 1990). The present study supports the majority of findings that the higher polyamines are reduced following permanent ischaemia, while following transient ischaemia, spermidine and spermine are subject to only small changes.
The decrease of spermidine and spermine levels seen following permanent ischaemia may be due to their release into the extracellular space and clearance into the blood as suggested by Paschen et al. (1992). Activation of the interconversion pathway may also contribute to the reduction of spermidine and spermine levels.

A decrease of spermine and spermidine could be harmful. During ischaemia, in regions where spermine levels are reduced, the Ca\(^{2+}\) buffering capacity of mitochondria may be disturbed (Paschen et al., 1992). A decrease in the concentration of intracellular polyamines following ischaemia could reduce the block of AMPA and kainate channels, allowing more Ca\(^{2+}\) influx, resulting in increased excitability (See section 1.1.4.3 for review). Most recently, it has been shown that intracellular spermine dose-dependently inhibits NMDA-induced responses through a mechanism which is different from spermine-induced voltage-dependent inhibition (inward rectification) of AMPA/kainate receptors (Turecek et al., 2004). A decrease in intracellular spermine may also increase the excitability of the NMDA receptor. Thus the decrease of tissue spermine and spermidine following ischaemia, particularly permanent ischaemia, could also contribute to the cellular damage through any, or all of, these mechanisms.

6.4.3 Extracellular polyamines

Polyamine levels in the dialysate have previously been shown to increase after implantation of the dialysis probe but to fall rapidly to a steady baseline within 20 minutes (Fage et al., 1992) or to stabilize within 1 hour after probe implantation (Baskaya et al., 1997). In the present study, collection of the first 30 min fraction was started approximately 15 minutes after probe implantation. Generally, in the contralateral control cerebral cortex, the highest levels of extracellular putrescine, spermidine and spermine occurred in the first fraction, thus probe implantation did appear to increase the extracellular polyamine levels in this study. The increase of polyamine efflux following probe implantation likely reflects a response to injury or traumatic brain damage (Carter et al., 1995). The implantation may also affect the polyamine release in the ischaemic cerebral cortex. However, using the extracellular
polyamine levels in the non-ischaemic cerebral cortex as a control to rule out the influence of probe implantation, the present study clearly illustrates that both the levels of extracellular spermidine and spermine, but not putrescine, were significantly increased by cerebral ischaemia. The extracellular levels of polyamines are regulated by both release and uptake mechanisms. It is said that the efficacy of glial uptake for spermidine and spermine is higher than that for putrescine (Vivo et al., 2002). In the present study, increases in extracellular spermidine and spermine were seen while extracellular putrescine levels did not change. The elevation of extracellular spermidine and spermine may reflect the enhancement of the release following ischaemia; however, this is speculative and further studies are needed to investigate this.

It has been shown that spermidine, not spermine, was released into the extracellular space in cerebral cortex following permanent focal cerebral ischaemia in rats (Carter et al., 1995). In the present study, both extracellular spermidine and spermine levels were found elevated after focal permanent cerebral ischaemia. Carter et al. (1995) reported that the increase in spermidine was observed immediately following or was initiated at any time for up to 6 hours after occlusion. Peak of the increase varied from 1.5 to 10 h in individual animals. In the present study, spermidine levels consistently started to show a significant increase from 1 hour. The elevation of extracellular spermidine was still significant at the final 3 hours collection point. The peak increase seemed to occur at 2 hours. These present results are comparable with those reported by Carter et al. (1995) while the increase of spermidine level was greater in the present study (10.6 fold basal level) than that in that study (4-5 fold basal levels). In the present study, low μM levels of spermine, comparable to spermidine levels, were also detected. Spermine levels were elevated following ischaemia in the first fraction (0.5-1 hour) of microdialysis. This increased extracellular spermine level, related to cerebral ischaemia, lasted throughout the whole microdialysis procedure. Peak level seems to be at 2 hours (3.4 fold basal level). The reduction of both tissue spermidine and spermine levels following permanent ischaemia seen in the present study and the others (Paschen et al., 1991; Saucer et al., 1992) may well reflect the increased extracellular levels of both
spermidine and spermine. In an in vitro ischaemia study using hippocampal slices, spermidine and spermine release were enhanced by ischaemia (Carter et al., 1994). As the polyamine release induced by 18 min of ischaemia was delayed until the first fraction (6 min fraction) following the reinclusion of glucose and oxygen, it is thus difficult to define whether polyamine release was delayed with respect to ischaemia, or to an immediate consequence of reperfusion (Carter et al., 1994).

It has been demonstrated that intrastriatal infusion of NMDA resulted in a marked and rapid increase in the concentration of spermine and spermidine into the dialysate in anesthetized rats (Fage et al., 1992). Spermidine and spermine can be released in vivo by potassium (Fage et al., 1992), and sodium-pump inhibition (Fage et al., 1993a). In general, none of these stimuli increases the release of putrescine (Fage et al., 1992; Fage et al., 1993a). Following ischaemia, the NMDA receptor activation is a well known event, and it is a critical determinant factor in neurotoxicity (Carter et al., 1995). Potassium accumulation occurs in the extracellular space following focal ischaemia (Hansen et al., 1985). In energetically compromised tissue, sodium-potassium ATPase inhibition (related to energy loss) or an accumulation of endogenous sodium potassium ATPase pump inhibitors is a key characteristic (Carter et al., 1995). Therefore, the release of polyamines in ischaemia is thus likely to be driven by any or all of these factors: NMDA receptor activation resulting from the release of glutamate, potassium accumulation, and sodium-pump inactivity (Carter et al., 1995). Saucer et al. have shown that the decrease in cortical tissue levels of spermidine, which is thought to probably reflect increased release, seen in focal ischaemia, is prevented by the competitive NMDA antagonist CGP 40016 (Sauer et al., 1992).

Taking into account the recovery rate across the dialysis membrane (in the present study, the recovery rate for putrescine, spermidine and spermine was 41%, 28% and 21% respectively), the extracellular spermidine and spermine released in the cerebral cortex following permanent cerebral ischaemia are at low μM range. μM levels of spermidine and spermine are sufficient to increase NMDA-mediated effects in vitro. For example, spermidine potentiates the NMDA response from levels as low as 1 μM
in cultured striatal neurons (Sprosen & Woodruff, 1990). It has also been demonstrated that low μM concentrations of spermine potentiated a guanidinosuccinate-evoked current through the action of spermine on the polyamine binding site of the NMDA receptor complex in cell cultures (D'Hooge et al., 2003). Compounds that antagonize the effect of polyamines at NMDA receptors (Ifenprodil and Eliprodil) are neuroprotective in animal models of focal ischaemia (Gotti et al., 1990). Most recently, the novel polyamine/NMDA antagonist N\textsuperscript{1}-dansyl-spermine has been demonstrated to be neuroprotective in a global cerebral ischaemia model in gerbils (Kirby & Shaw, 2004). Thus it is very likely that the released spermidine and spermine could contribute to the cellular damage by enhancing the activation of the NMDA receptor following cerebral ischaemia. Polyamines are also thought to have toxic effects independent of the NMDA receptor. At levels from 50 μM, spermidine and spermine are neurotoxic in hippocampal slices and the neurotoxicity caused by these high concentrations of polyamines is not sensitive to NMDA antagonists (Carter et al., 1995). The mechanism of this NMDA-independent neurotoxicity was not clear. It has recently been shown that the current evoked by high concentrations (mM) of spermine alone involved direct activation of voltage-gated Ca\textsuperscript{2+} channels (D'Hooge et al., 2003) and there is also evidence that calcium channel activity happens at levels of polyamines from 100 μM to 1 mM (Scott et al., 1994).

6.5 CONCLUSION

(1) In the present study, tissue putrescine levels were found to be increased in permanent and transient focal ischaemia in mice. A reduction in levels of spermidine and spermine was seen in the cerebral cortex and a decrease of the spermidine levels also occurred in the hippocampus following permanent ischaemia.

(2) Consistent sharp increases of tissue N\textsuperscript{1}-acetylspermidine levels were observed following both focal permanent and transient ischaemia, indicating that interconversion pathway activation occurred.
(3) Extracellular levels of spermidine and spermine, but not putrescine, were elevated by 3 hours permanent focal ischaemia. The increase of extracellular spermidine and spermine levels suggests that the higher polyamines may play an important role in the ischaemic damage.
Section 7 Summary and further discussion

7.1 PRE-ISCHAEMIC EFFECT OF POLYAMINE ANALOGUES IN A FOCAL PERMANENT ISCHAEMIA MODEL

Polyamines have been known to be involved in cerebral ischaemia for some time since changes of levels have been reported following ischaemia (Paschen et al., 1992). Whether polyamines are neurotoxic or neuroprotective is not clear although most of the evidence points to a toxic role. It has been shown that the polyamine antagonists ifenprodil and its derivative eliprodil are neuroprotective in cerebral ischaemia models and the neuroprotection was afforded through, at least partly, their action via the polyamine site at the NMDA receptor (Cater et al., 1997). Recently a potent polyamine analogue, N\(^1\)-dansyl-spermine, has been developed and more recently more novel polyamine analogues have been synthesized (BU31b, BU37b, BU33b, BU36b and BU43b). These analogues, with the exception of BU37b have been shown to have polyamine antagonist effects (Murphy et al., 2003). Thus the neuroprotective potential of N\(^1\)-dansyl-spermine and these novel polyamine analogues was investigated in a focal permanent ischaemia model in mice.

The present study showed that N\(^1\)-dansyl-spermine has good neuroprotective effect in the focal permanent cerebral ischaemia model in mice when administered pre-ischaemia. The results are described in section 3. MK-801, which is a gold standard NMDA open channel blocker, was also studied as a reference compound. N\(^1\)-dansyl-spermine was found to have a comparable effect to MK-801 in reducing % HLV, oedema and neurological deficits in MCAO mice. As a polyamine analogue, N\(^1\)-dansyl-spermine could have modulatory effects at the NMDA receptor. N\(^1\)-dansyl-spermine has been shown to be a potent NMDA inhibitor in vitro (Chao et al., 1997). It has also been shown to be a potent polyamine antagonist in reducing spermine induced convulsions in vivo (Murphy et al., 2003). Therefore in the present study, N\(^1\)-dansyl-spermine may mediate its neuroprotection through an action at the polyamine site of the NMDA receptor. N\(^1\)-dansyl-spermine is also said to have calmodulin antagonist effect, which may also contribute to its neuroprotection,
although there is evidence that calmodulin antagonism is not neuroprotective. Further investigation is needed to study its neuroprotective mechanism. However, the present study clearly demonstrated that N\textsuperscript{1}\textsuperscript{-}dansyl-spermine provides good neuroprotection. In the present study, the results from both the histological and behavioural assessments showed that N\textsuperscript{1}\textsuperscript{-}dansyl-spermine is more neuroprotective at lower dose (1 and 2 mg kg\textsuperscript{-1}). In a preliminary experiment, spermine-like effects were seen with N\textsuperscript{1}\textsuperscript{-}dansyl-spermine at 20 mg kg\textsuperscript{-1} in gerbils, which suggests that it may possess some partial agonist effects. It is possible that, N\textsuperscript{1}\textsuperscript{-}dansyl-spermine has low efficacy at the polyamine binding site on the NMDA receptor and showed neuroprotection at lower doses, while at higher doses, the cumulative polyamine agonist effects negate its neuroprotective effect. The bell-shaped dose-responses curves observed in the present study may be due to its partial agonist effects. In addition, at neuroprotective doses, N\textsuperscript{1}\textsuperscript{-}dansyl-spermine had no negative effect on motor functions 24 hours following ischaemia in MCAO animals or sham-operated mice. In contrast, MK-801 produced motor impairment at neuroprotective levels. Therefore N\textsuperscript{1}\textsuperscript{-}dansyl-spermine may be a good therapeutic candidate for stroke.

Section 4 demonstrated the neuroprotective potential of 5 novel polyamine analogues in a permanent focal cerebral ischaemia model in mice. BU43b was shown to be a good neuroprotectant as it reduced %HLV and oedema. In addition, it has been demonstrated that administration of BU43b alone did not cause any abnormal behaviours in the rotarod performance test and locomotor activity counts in mice for up to 5 days after dosing (Data not shown). Therefore, BU43b may have therapeutic promise in the treatment of stroke. BU36b reduced oedema in the permanent model; therefore, it showed some neuroprotective effect in the present study. As polyamine antagonists in a spermine model (Murphy et al., 2003) and NMDA blockers (Fixon-Owoo et al., 2003), the neuroprotection of both BU36b and BU43b observed in the present study could be afforded through a possible action at the polyamine site on the NMDA receptor (see section 4 for discussion). Polyamines also have interactions with voltage sensitive Ca\textsuperscript{2+} channels particularly L-type Ca\textsuperscript{2+} channels (see section 1.1.4.2 for review), which are thought to play a role in the development of spermine-induced convulsions (Doyle, 1993). Therefore the novel polyamine analogues may
also have calcium antagonist effects. BU36b has only a modest inhibitory effect at the NMDA receptor in vitro. However, its effect of antagonizing spermine-induced convulsions is potent. It is possible that BU36b could have some calcium antagonist effect. The possible calcium antagonist action of polyamine antagonists could also contribute to the neuroprotection observed in the present study.

BU33b was also shown to reduce the neurological deficit scores at lower dose of 20 mg kg\(^{-1}\) in the permanent model but no beneficial effect at the higher dose. Little is known of the profile of interactions of this novel compound, and the data may reflect some, as yet unknown, mechanism of this compound. Both BU31b and BU37b were found to be toxic in the permanent focal cerebral ischaemia model at the doses tested. The lack of neuroprotection of the polyamine antagonist BU31b may be due to its metabolism following the i.p. administration. The finding that the BU37b did not show any beneficial effect in the present study is consistent with observations in the spermine model where BU37b was found to be ineffective in reducing the spermine-induced convulsion in vivo (Murphy et al., 2003). The toxic effect of BU37b in the present study may be due to its formation of a toxic metabolite peripherally on route to the brain or some other as yet unknown mechanisms of this novel compound.

As discussed in section 4, the neuroprotective property profile of the novel polyamine analogue series in the present permanent focal cerebral ischaemia model correlates with their effect as polyamine antagonists in the spermine model in vivo (Murphy et al., 2003), as the two most potent polyamine antagonists in the spermine model (BU43b and BU36b) showed neuroprotection. Also in vitro, BU43b and BU36b were shown to block the activity of the NMDA receptor subunit NR1/NR2B (BU43b very potently) (Fixon-Owoo et al., 2003). Therefore their neuroprotective profile observed in the present study is also in line with their inhibitory effect at the NMDA subunit containing the polyamine site. Hence, the activity of the series of polyamine analogues in the present study favours the toxic role of polyamines following cerebral ischaemia.
7.2 PRE-ISCHAEMIC EFFECT OF POLYAMINE ANALOGUES IN A FOCAL TRANSIENT ISCHAEMIA MODEL

A transient ischaemia model is of good clinical relevance; therefore the drugs tested in the permanent model were investigated in this model in mice.

Section 5 detailed the pre-ischaemic neuroprotective potential of N$^1$-dansyl-spermine and MK-801 in the transient model. The results showed that N$^1$-dansyl-spermine is as effective as MK-801 in reducing the %HLV. N$^1$-dansyl-spermine also reduced the oedema at all doses tested, which is impressive when compared with MK-801. The results showed that N$^1$-dansyl-spermine is neuroprotective against the ischaemic damage even after reperfusion. NMDA over-activation participates in transient ischaemic injury, both during the ischaemic phase and the reperfusion phase. The neuroprotection of the polyamine/NMDA antagonist, N$^1$-dansyl-spermine, seen in the transient ischaemia model could be mediated through blocking the NMDA receptor at the polyamine stimulatory site.

In behavioural assessments, MK-801 worsened the LMA, and had no beneficial effect in rotarod performance test in the transient model, although it did improve the rotarod performance in the permanent model. MK-801 is well known to cause side effects. The side effects of MK-801 may have been enhanced by reperfusion in the transient model, or the side effects may have been more prominent in a less disabled model. In contrast, N$^1$-dansyl-spermine consistently improved the LMA and rotarod performance. The beneficial effects of N$^1$-dansyl-spermine in behavioural assessments were more pronounced in the transient model than that seen in the permanent model.

Consistent with the observations in the permanent model, the greatest effect of N$^1$-dansyl-spermine in the transient model was not seen at the highest dose. This again suggests that N$^1$-dansyl-spermine may indeed have some partial agonist effect.
Section 5 also illustrates that BU43b at both 20 and 30 mg kg\(^{-1}\) doses is a good neuroprotective agent in the focal transient ischaemia model in mice when administered preischaemia as it reduced %HLV and oedema and enhanced LMA and rotarod performance. BU36b also reduced oedema and therefore showed some neuroprotective effect in this model. As previously mentioned, both BU43b and BU36b are potent polyamine antagonists and in vitro studies showed that they have inhibitory effect at the NMDA subunit containing the polyamine site (Fixon-Owoo et al., 2003). Their neuroprotection could be afforded through an action at the polyamine site on the NMDA receptor, although further studies are needed to confirm this.

BU33b was toxic in the transient ischaemia model as it increased mortality rate. Both BU31b and BU37b were without neuroprotection in the transient model, which is consistent with the study using permanent ischaemia model (section 4). The toxicity caused by these compounds may be related to an, as yet, unknown effects of the drugs or their metabolites.

A range of parameters were used in both the permanent and the transient models in the present study to assess the possible neuroprotection of the tested compounds. %HLV is, of course, a crucial parameter for assessing ischaemia damage. Oedema is a major contributing factor to morbidity and mortality in stroke. In the present study, using TTC staining, these two histological assessments were successfully carried out. LMA is said to be a good index of possible adverse effects of CNS drugs. In the present study, LMA revealed the side effects of the NMDA channel blocker, MK-801, in both sham-operated animals and MCAO animals. The rotarod performance test appeared to be particularly sensitive in detecting deficits after MCAO, and the neuroprotective properties of the drugs in the present study. This parameter seems to be more useful than the neurological deficit scoring system, which was not found to be useful in the transient model demonstrating less severe cerebral damage.
Section 6 presented the change in the polyamine profile in brain tissue following permanent or transient focal ischaemia in mice. In addition, a microdialysis study demonstrated the extracellular polyamine profile following up to 3 hours of focal permanent ischaemia in mice.

Following 24 hours permanent ischaemia, the change of putrescine is very modest. A significant increase was seen in the midbrain, but in the cortex, hippocampus, and hypothalamus, putrescine levels were not significantly increased. Transient ischaemia did produce prominent increases in putrescine level in every region measured (cortex, hypothalamus, hippocampus and midbrain). N\textsuperscript{-}acetylspermidine is the direct product of the rate-limiting enzyme (SSAT) in the interconversion pathway (Parchment \textit{et al.}, 1989). In the present study, N\textsuperscript{-}acetylspermidine levels were found to be elevated in almost all regions measured following both permanent and transient ischaemia. The increased activity of SSAT may have contributed to the increase in putrescine levels. Both SSAT and ODC are important in the production of putrescine (Wallace \textit{et al.}, 2003). In the present study, putrescine levels were not increased in the severely damaged regions such as hippocampus and cortex following permanent ischaemia. The induction of ODC activity may need the recovery of blood flow or the continuation of the blood flow at necessary levels (Baskaya \textit{et al.}, 1997). This may explain why less pronounced changes in the putrescine level were seen in the permanent model.

Putrescine increase has been implicated in ischaemic damage. It has been shown to induce the blood brain barrier dysfunction (Baskaya \textit{et al.}, 1997) and activate the voltage sensitive Ca\textsuperscript{2+} channels (Herman \textit{et al.}, 1993). The prominent increase seen following transient ischaemia in the present study may have contributed to the neuronal injury. However, the role of putrescine in permanent ischaemia needs to be investigated further.
It has previously been shown that N’-acetylspermidine accumulated in global ischaemia in gerbils and transient focal ischaemia in rats (Rao et al., 2000). The present study showed the increase of N’-acetylspermidine levels following focal permanent ischaemia or transient ischaemia in mice, indicating the activation of SSAT. As previously mentioned, SSAT is the rate-limiting enzyme in the polyamine interconversion pathway (Parchment et al., 1989). This pathway involves oxidation of the acetylated polyamines by PAO, which produces cytotoxic by-products including 3-acetamidopropanol and $H_2O_2$ (Wallace et al., 2003). Therefore the activation of the interconversion pathway seen in present study may participate in the cellular damage following ischaemia.

Following 24 hours permanent ischaemia, a reduction in spermine and spermidine levels occurred in the cerebral cortex and the spermidine level was reduced in the hippocampus. No significant change of spermine and spermidine levels was seen following transient ischaemia. The decreases seen in the permanent model may be due to the release of polyamines into extracellular space and clearance into the blood circulation. The activation of the interconversion pathway may also have contributed to the decrease. Reduction of spermidine and spermine could contribute to ischaemic damage by disturbing the mitochondrial $Ca^{2+}$ buffering capacity (Paschen et al., 1992) and reducing the block/inhibition of AMPA, kainate and NMDA receptors, increasing the excitotoxicity (Johnson, 1998; Turecek et al., 2004).

The microdialysis study in the section 6 showed that the extracellular levels of cortical spermidine and spermine but not putrescine were significantly increased by permanent cerebral ischaemia. This elevation of their levels reflects the decrease in the levels of cortical tissue spermidine and spermine. In vivo studies have shown that an increase of spermidine and spermine release can be caused by intrastriatal infusion of NMDA, potassium, or sodium-pump inactivity (Fage et al., 1992; Fage et al., 1993a). Following ischaemia, NMDA activation, potassium accumulation and sodium-pump inhibition are known events. Therefore in the present study, these factors or any of them may account for the increase in the extracellular levels of polyamines following ischaemia.
The levels of the released spermidine and spermine found in the present study are at low \( \mu M \) range. In vitro studies have shown that \( \mu M \) levels of spermidine or spermine are sufficient to enhance the NMDA receptor mediated neurotoxicity (Sprosen & Woodruff, 1990; D'Hooge et al., 2003). In animal models, the neuroprotection by the polyamine/NMDA antagonists, ifenprodil, eliprodil, N\(^1\)-dansyl-spermine, BU43b and BU36b also suggests that polyamines may contribute to ischaemic damage through an action at the NMDA receptor (Gotti et al., 1990; Kirby & Shaw, 2004; present study). At high levels (50 \( \mu M \) to mM), polyamines are also thought to have toxic effects independent of the NMDA receptor, which may be associated with their effects at the voltage-sensitive calcium channels (Scott et al., 1994; Carter et al., 1995; D'Hooge et al., 2003). Whether polyamine cause neuronal damage through the NMDA receptor independent mechanism or not is unclear, however, the present study clearly showed ischaemia causes increases in the extracellular spermidine and spermine, which could contribute to the ischaemic injury though the NMDA receptor.

7.4 CONCLUSIONS

The results described in this thesis demonstrated the pre-ischaemic neuroprotective effect of N\(^1\)-dansyl-spermine in both a focal permanent ischaemia model and a transient ischaemia model. It is suggested that N\(^1\)-dansyl-spermine may have afforded its neuroprotection through a polyamine modulatory site on the NMDA receptor. Future investigations such as binding studies would be interesting to confirm this. In addition, in direct contrast to MK-801, N\(^1\)-dansyl-spermine has no adverse effect in the behavioural function in sham-operated animals when assessed 24 hours following administration. The results suggest that N\(^1\)-dansyl-spermine may be a good candidate for stroke treatment. The present study has found the best doses of N\(^1\)-dansyl-spermine which offer good neuroprotection in these models. Therefore a post-ischaemic time window study using these doses to investigate further the window of opportunity for neuroprotection would be interesting in the future.
The polyamine analogues BU36b and particularly BU43b were neuroprotective in both the focal permanent ischaemia model and the transient ischaemia model when administered pre-ischaemia. The neuroprotection may be mediated through an action at the NMDA receptor polyamine site, although further investigations are needed to confirm this. BU36b and particularly BU43b may have good therapeutic potential for stroke. The neuroprotective potential profile of the novel polyamine analogue series (BU31b, BU37b, BU33b, BU36b and BU43b) observed in the present study, when combined with existing in vivo evidence of the polyamine antagonist activities (Murphy et al., 2003) and the in vitro data of NMDA receptor inhibition (Fixon-Owoo et al., 2003), favours the hypothesis that polyamines are neurotoxic following ischaemia and effective polyamine antagonists are potentially neuroprotective. Therefore the development of effective polyamine antagonists may be beneficial for the treatment of stroke.

Putrescine levels were found to be increased following ischaemia, particularly transient ischaemia. Both permanent and transient ischaemia increased the tissue N\(^1\)-acetylspermidine levels. This indicates the activation of the interconversion pathway, which may contribute to the neuronal damage following ischaemia.

The present study also showed that the extracellular levels of both cortical spermine and spermidine are elevated following permanent ischaemia, which reflects the reduction of cortical tissue spermine and spermidine levels. Polyamines may participate in ischaemic damage via a stimulatory effect at the NMDA receptor (or NMDA receptor-independent mechanisms such as calcium channel activation) and an effective polyamine antagonist could offer neuroprotection.
Bibliography


Chao, J., Seiler, N., Renault, J., Kashiwagi, K., Masuko, T., Igarashi, K., Williams, K. (1997) N¹-dansyl-spermine and N¹-(n-octanesulfonyl)-spermine, novel glutamate


Published work


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Accepted 24 September 2004

Abstract:

The polyamine sites on the NMDA receptor complex offer a therapeutic target for focal ischaemia, potentially devoid of most side effects associated with NMDA-antagonists. In this study, we investigated the effect of a novel polyamine antagonist, N\(^1\)-dansyl-spermine (0.5-10 mg kg\(^{-1}\)) in a permanent focal cerebral ischaemia model in mice, and compared its effect to that of MK-801 (0.3-3 mg kg\(^{-1}\)) following administration 30 minutes prior to ischaemia. A battery of histological and behavioural tests was employed following permanent middle cerebral artery occlusion to assess any neuroprotective effect. Following middle cerebral artery occlusion, N\(^1\)-dansyl-spermine (1-5 mg kg\(^{-1}\)) and MK-801 (1 or 3 mg kg\(^{-1}\)) caused a comparable and significant reduction in the percentage hemisphere lesion volume. Similarly, both drugs significantly reduced oedema and neurological deficit score to a similar extent. Locomotor activity in MCAO mice was not significantly improved by MK-801 or N\(^1\)-dansyl-spermine, although N\(^1\)-dansyl-spermine induced a trend towards significant improvement. Significant improvement in rotarod performance was observed at neuroprotective doses with both drugs. Upon comparison of the
profile of effects, N\textsuperscript{1}-dansyl-spermine at least matched the effectiveness of MK-801 as a neuroprotective agent in this model. In addition, in sham-operated control mice, N\textsuperscript{1}-dansyl-spermine was well tolerated, in contrast to the pronounced adverse effects of MK-801 on locomotor activity and rotarod performance. In conclusion, this study has shown that N\textsuperscript{1}-dansyl-spermine is as effective a neuroprotective drug as MK-801 in this model. Moreover, in contrast to MK-801, N\textsuperscript{1}-dansyl-spermine could be a promising therapeutic candidate for stroke as it is well tolerated at neuroprotective doses in sham operated animals.

Theme: Disorder of the nervous system
Topic: Ischaemia
Keywords: N\textsuperscript{1}-dansyl-spermine; MK-801; NMDA receptor; Polyamine; Focal cerebral ischaemia; Middle cerebral artery occlusion
### Appendix: Tables for all results

<table>
<thead>
<tr>
<th>Drugs and Doses (mg kg⁻¹)</th>
<th>%HLV</th>
<th>Oedema (mm³)</th>
<th>Rotarod</th>
<th>LMA</th>
<th>Scoring</th>
<th>MR</th>
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<tbody>
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<td>172±35</td>
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<td>31.0±6.1*</td>
<td>18.8±2.3*</td>
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<td>3.0±2.0</td>
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Table 1 Effects of MK-801 and N¹-dansyl-spermine (N¹-D) (i.p.) administered 30 min before the occlusion in permanent MCAO mice on percentage hemisphere lesion volume (%HLV), oedema, rotarod performance assessment (Rotarod in seconds), spontaneous locomotor activity (LMA), neurological deficit scoring (Scoring) and mortality rate. Data was expressed as the mean ± s.e.mean, except for the neurological deficit score and the mortality rate. The neurological deficit score was shown as median ± IQR (Inter quartile range). *P<0.05 versus (saline treated) MCAO control. Statistics used were proportionality test for mortality rate, Mann-Whitney U-test for the neurological deficit scoring and two-tailed, unpaired Student’s t-test for all the other parameters.
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<th>Drugs and doses</th>
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<th>LMA</th>
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<td>584±111 n=10</td>
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<tr>
<td>0.3 mg kg⁻¹</td>
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<td>181±28* n=6</td>
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<td>1 mg kg⁻¹</td>
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<td>3 mg kg⁻¹</td>
<td>53.0±15* n=7</td>
<td>168±38* n=7</td>
</tr>
<tr>
<td>0.5 mg kg⁻¹</td>
<td>120±0 n=8</td>
<td>500±96 n=8</td>
</tr>
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<td>1 mg kg⁻¹</td>
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<td>N⁺-dansyl-spermine</td>
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<td>2 mg kg⁻¹</td>
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<td>10 mg kg⁻¹</td>
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<td>322±38 n=7</td>
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Table 2 Effects of MK-801 and N⁺-dansyl-spermine in sham-operated mice on the rotarod performance assessment (Rotarod in seconds) and spontaneous locomotor activity test (LMA). Data are shown as mean±se.mean (n=4-10). *P<0.05 versus (saline treated) sham-operated control (two-tailed, unpaired Student’s t-test).
<table>
<thead>
<tr>
<th>Drugs and doses (mg kg⁻¹)</th>
<th>%HLV</th>
<th>Oedema mm³</th>
<th>Rotarod (seconds)</th>
<th>LMA</th>
<th>Scoring</th>
<th>Mortality Rate</th>
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<td></td>
<td>51.7±9.8 n=3</td>
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<td>37.8±3.8* n=10</td>
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<td>28.7±8.9 n=10</td>
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</table>

Table 3 Effects of Drugs BU31b, BU37b, BU33b, BU36b and BU43b (i.p.) administered 30 min before the occlusion in permanent MCAO mice on percentage hemisphere lesion volume (%HLV), oedema, rotarod performance (Rotarod), spontaneous locomotor activity (LMA), neurological deficit scoring (Scoring) and mortality rate. Data was expressed as the mean ± s.e.mean, except for the neurological deficit score and the mortality rate. The neurological deficit score was shown as median ± IQR (Inter quartile range). *P<0.05 versus (saline treated) MCAO control. Statistics used were proportionality test for mortality rate, Mann-Whitney U-test for the neurological deficit scoring and two-tailed, unpaired Student’s t-test for all the other parameters.
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<th>Drugs and doses (mg kg⁻¹)</th>
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<th>Oedema mm³</th>
<th>Rotarod</th>
<th>LMA</th>
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<td>251±59*</td>
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</tr>
<tr>
<td>BU31b</td>
<td>27.4±12</td>
<td>15.6±4.9</td>
<td>38.8±17</td>
<td>79.8±33</td>
<td>3 out of 8</td>
</tr>
<tr>
<td></td>
<td>36.8±9.8</td>
<td>23.5±4.3</td>
<td>46.3±15</td>
<td>69.1±26</td>
<td>2 out of 9</td>
</tr>
<tr>
<td></td>
<td>29.6±9.4</td>
<td>22.3±3.6</td>
<td>46.6±21</td>
<td>62.4±19</td>
<td>3 out of 8</td>
</tr>
<tr>
<td>BU33b</td>
<td>29.6±13</td>
<td>24.0±9.3</td>
<td>38.3±20</td>
<td>68.7±30</td>
<td>4 out of 7</td>
</tr>
<tr>
<td></td>
<td>34.9±16</td>
<td>23.0±8.1</td>
<td>42.0±24</td>
<td>70.3±34</td>
<td>4 out of 7</td>
</tr>
<tr>
<td>BU36b</td>
<td>19.0±4.1</td>
<td>18.9±4.3</td>
<td>46.6±11</td>
<td>72.8±16</td>
<td>0 out of 10</td>
</tr>
<tr>
<td></td>
<td>18.9±6.0</td>
<td>12.4±2.5*</td>
<td>76.0±10 (P=0.08)</td>
<td>137±30 (P=0.06)</td>
<td>2 out of 10</td>
</tr>
<tr>
<td>BU43b</td>
<td>14.2±3.7*</td>
<td>15.7±4.0</td>
<td>65.0±16</td>
<td>245±47*</td>
<td>1 out of 10</td>
</tr>
<tr>
<td></td>
<td>11.8±2.7*</td>
<td>12.2±2.3*</td>
<td>87.7±10*</td>
<td>327±65*</td>
<td>1 out of 10</td>
</tr>
</tbody>
</table>

Table 4 Effects of MK-801, N¹-dansyl-spermine, BU31b, BU37b, BU33b, BU36b and BU43b (i.p.) administered 30 min before the occlusion in mice subjected to 30 min MCAO with 24 hours reperfusion on percentage hemisphere lesion volume (%HLV), oedema, rotarod performance (Rotarod), spontaneous locomotor activity (LMA), and mortality rate. Data are expressed as mean±se.mean (n numbers are the survival animal numbers) except the mortality rate. */*<0.05 versus (saline treated) MCAO control. Statistics used were proportionality test for mortality rate and two-tailed, unpaired Student’s t-test for all the other parameters.
<table>
<thead>
<tr>
<th>Brain Regions</th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
<th>N(^1)-acetyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex Ischaemia</td>
<td>9.34±3.1</td>
<td>134±29*</td>
<td>107±41*</td>
<td>5.48±1.36*</td>
</tr>
<tr>
<td>Control</td>
<td>7.33±1.5</td>
<td>334±52</td>
<td>321±68</td>
<td>0.829±0.15</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>Ischaemia</td>
<td>12.6±2.5</td>
<td>225±52</td>
<td>134±31</td>
</tr>
<tr>
<td>Control</td>
<td>11.4±5.3</td>
<td>309±24</td>
<td>213±50</td>
<td>1.10±0.71</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>Ischaemia</td>
<td>14.2±3.6</td>
<td>321±78*</td>
<td>175±58</td>
</tr>
<tr>
<td>Control</td>
<td>13.4±3.0</td>
<td>598±83</td>
<td>244±39</td>
<td>1.25±0.72</td>
</tr>
<tr>
<td>Midbrain</td>
<td>Ischaemia</td>
<td>17.3±2.4*</td>
<td>344±16</td>
<td>242±13</td>
</tr>
<tr>
<td>Control</td>
<td>7.38±1.4</td>
<td>408±31</td>
<td>313±49</td>
<td>0.95±0.73</td>
</tr>
</tbody>
</table>

Table 5 Regional putrescine, spermidine and spermine levels (nmol g\(^{-1}\) tissue) in mice subjected to 24 hours of permanent focal cerebral ischaemia induced by middle cerebral artery occlusion. Data are shown as mean ±se.mean (n=8). N\(^1\)-acetyl: N\(^1\)-acetylspermidine; Control: contralateral non-ischaemic hemisphere; Ischaemia: ipsilateral ischaemic hemisphere. *P<0.05 versus the control in corresponding region (two-tailed paired Student’s t-test).
<table>
<thead>
<tr>
<th>Brain Regions</th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
<th>N¹-acetyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischaemia</td>
<td>29.2±3.3*</td>
<td>183±11</td>
<td>183±8.9</td>
<td>2.26±0.52*</td>
</tr>
<tr>
<td>Control</td>
<td>11.7±2.3</td>
<td>214±14</td>
<td>201±11</td>
<td>0.462±0.12</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischaemia</td>
<td>27.4±4.0*</td>
<td>356±59</td>
<td>202±18</td>
<td>1.86±0.83</td>
</tr>
<tr>
<td>Control</td>
<td>16.2±2.0</td>
<td>386±27</td>
<td>275±24</td>
<td>0.792±0.14</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischaemia</td>
<td>36.7±2.1*</td>
<td>413±47</td>
<td>232±12</td>
<td>4.60±1.0*</td>
</tr>
<tr>
<td>Control</td>
<td>21.6±4.0</td>
<td>438±63</td>
<td>259±40</td>
<td>0.94±0.30</td>
</tr>
<tr>
<td>Midbrain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischaemia</td>
<td>36.3±4.8*</td>
<td>305±35</td>
<td>196±14</td>
<td>1.94±0.48*</td>
</tr>
<tr>
<td>Control</td>
<td>14.2±3.0</td>
<td>347±53</td>
<td>213±18</td>
<td>0.42±0.12</td>
</tr>
</tbody>
</table>

Table 6 Regional putrescine, spermidine and spermine levels in mice subjected to 30 minutes of cerebral ischaemia with 23.5 hours reperfusion. Ischaemia was induced by middle cerebral artery occlusion. Data are shown as mean ± se.mean (n=8). N¹-acetyl: N¹-acetylspermidine; Control: contralateral non-ischaemic hemisphere; Ischaemia: ipsilateral ischaemic hemisphere. *P<0.05 versus the control in the corresponding region (two-tailed paired Student’s t-test).
Table 7 Extracellular space putrescine, spermidine and spermine levels (µM) in the contralateral non-ischaemic cerebral cortex (Con) and in the ipsilateral ischaemic cerebral cortex (Isch) in mice subjected to 3 hours of middle cerebral artery occlusion. Microdialysis probes were implanted into mouse cerebral cortex (from bregma: 3.8 mm lateral, 0.5 mm posterior). SPM: spermine; SPD: spermidine; PUT: putrescine. 30 min samples were collected and analysed. Data are shown as mean ± se.mean (n=7-10). *P<0.05 versus the control at the corresponding time point (one-way ANOVA).

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>1</th>
<th>1.5</th>
<th>2</th>
<th>2.5</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SPM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isch</td>
<td>0.330±0.037*&lt;br&gt;n=10</td>
<td>0.412±0.072*&lt;br&gt;n=9</td>
<td>0.513±0.099*&lt;br&gt;n=8</td>
<td>0.470±0.11*&lt;br&gt;n=8</td>
<td>0.372±0.039*&lt;br&gt;n=7</td>
</tr>
<tr>
<td>Con</td>
<td>0.121±0.043&lt;br&gt;n=10</td>
<td>0.064±0.039&lt;br&gt;n=10</td>
<td>0.048±0.017&lt;br&gt;n=9</td>
<td>0.041±0.030&lt;br&gt;n=7</td>
<td>0.071±0.023&lt;br&gt;n=7</td>
</tr>
<tr>
<td><strong>SPD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isch</td>
<td>0.319±0.054&lt;br&gt;n=10</td>
<td>0.371±0.096*&lt;br&gt;n=9</td>
<td>0.455±0.096*&lt;br&gt;n=8</td>
<td>0.338±0.15&lt;br&gt;n=8</td>
<td>0.239±0.045*&lt;br&gt;n=7</td>
</tr>
<tr>
<td>Con</td>
<td>0.196±0.064&lt;br&gt;n=10</td>
<td>0.104±0.033&lt;br&gt;n=10</td>
<td>0.134±0.068&lt;br&gt;n=9</td>
<td>0.086±0.017&lt;br&gt;n=7</td>
<td>0.093±0.024&lt;br&gt;n=7</td>
</tr>
<tr>
<td><strong>PUT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isch</td>
<td>0.267±0.066&lt;br&gt;n=10</td>
<td>0.343±0.093&lt;br&gt;n=9</td>
<td>0.231±0.079&lt;br&gt;n=8</td>
<td>0.266±0.12&lt;br&gt;n=8</td>
<td>0.206±0.091&lt;br&gt;n=7</td>
</tr>
<tr>
<td>Con</td>
<td>0.319±0.093&lt;br&gt;n=10</td>
<td>0.276±0.095&lt;br&gt;n=10</td>
<td>0.253±0.10&lt;br&gt;n=9</td>
<td>0.215±0.068&lt;br&gt;n=7</td>
<td>0.231±0.067&lt;br&gt;n=7</td>
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</tbody>
</table>