



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

An investigation of the role of the polyamines in epilepsy and cerebral ischaemia

Brian P. Kirby

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

Department of Pharmacology
School of Pharmacy
Trinity College
Dublin 2

September 2000



THESIS
6262

Declaration:

I hereby certify that the work embodied in this thesis has not been submitted as an exercise for a degree at any other University.

I also certify that the work embodied in this thesis is entirely my own work.

I also agree that the Library may lend or copy this thesis upon request.

A handwritten signature in blue ink, appearing to read "Brian Kirby", with a large, stylized flourish above the name.

Brian P. Kirby

Summary:

The polyamines, spermine and spermidine, are ubiquitous in nature but are found in especially high concentrations in brain tissue. The polyamines are required for cell growth and differentiation but in high doses are neurotoxic. A dose of 100µg spermine given by the intracerebroventricular route results in the development of CNS excitation, culminating in tonic convulsion, within 8 hours of injection.

The work presented here aimed to further characterise this CNS excitation. It was previously shown that both the NMDA receptor and calcium channels may be involved in this syndrome. Therefore, NMDA receptor antagonists, calcium channel antagonists and a novel polyamine analogue were utilised to investigate the spermine induced CNS excitation.

Spermine is known to enhance the activity of the NMDA receptor through an action on an extracellular binding site on the NMDA receptor macrocomplex. An *in vivo* model was used to investigate this spermine enhancement of NMDA activity. In an attempt to characterise the action of spermine on the NMDA receptor, a calcium channel antagonist, polyamine antagonists and a novel polyamine analogue, N¹-dansylspermine, were used.

It was shown from these studies that the most potent antagonist, N¹-dansylspermine was acting to reduce the effects of spermine on the positive polyamine site of the NMDA receptor. Similarly, the work further demonstrated that calcium channel blockers especially those with NMDA receptor antagonist activity, the polyamine antagonists, eliprodil and CP-101,606, and the NMDA channel blocker, memantine, all reduced the development of spermine induced CNS excitation.

Many studies have demonstrated a role for the polyamines in cerebral ischaemia. However, the reports are conflicting. All agree that changes in polyamine levels occur following ischaemia but some studies propose a neurotoxic role for the polyamines whereas others suggest the polyamines are neurotrophic. Further work was therefore needed to elucidate the role of the polyamines following ischaemia.

This work examined, using the gerbil model of cerebral ischaemia, the role of the polyamines and the NMDA receptor by employing an NMDA receptor antagonist, polyamine antagonists and N¹-dansylspermine. It was demonstrated that all the compounds were neuroprotective following ischaemia. This argues for a neurotoxic role for the polyamines in ischaemia. From this study it was also discovered that N¹-dansylspermine was unique in producing neuroprotection in the CA1 area of the hippocampus without restoring the ability to form spatial maps.

An image analysis system was examined as an alternative to the histological examination of the effects of putative neuroprotectants. However, it was not shown to be effective for the counting of individual cells, though would probably be useful for the measurement of areas of degeneration.

An *in vitro* model of epilepsy was used to examine the *in vitro* effects of N¹-dansylspermine on spontaneous epileptic discharges produced by NMDA receptor activity. In this model the enhancing effect of the polyamine, spermine was examined on the discharges and the effect of N¹-dansylspermine studied. N¹-dansylspermine was confirmed as an effective polyamine antagonist at low concentrations, while at higher concentrations it reduced the discharges, most likely by an agonist action at the negative polyamine site within the ion channel. This study also suggests a possible role for the polyamines in epilepsy, especially since earlier work discovered raised polyamine levels in epileptic brains.

Acknowledgements:

I would like to express my utmost thanks to Professor Graham Shaw. You have been an inspiration, mentor and friend to me during my time in the Department and it has been a pleasure and honour to work with you. Keep Fishin'!!

I would also like to thank the rest of the staff of the Department of Pharmacology, especially Dr. Neil Frankish for his photographic expertise and Alison for all the ink cartridges and paper I ever needed. Not forgetting the technical staff, Therese, Brian and Breda including those that left during my time, Adrienne and Claire.

Thanks to Professor N. Seiler and Dr. J. Renault for supplying me with N¹-dansylspermine, and to Rhona Prendergast for synthesising it thereafter. Thanks to Professor B. Leonard and Dr. M. Maginn, National University of Ireland, Galway for the help with the gerbil global ischaemia model. Thanks also to Dr. J. Davies and Wendy Marsh, University of Wales, College of Medicine, Cardiff for their help with the cortical wedges. Thanks to Dr. M. O'Regan, Department of Statistics, Trinity College for her help with the logistic regression analysis.

I would also like to thank for their technical assistance, Kathrina Doran for the image analysis, Rebecca Breslin for the calcium channel antagonist studies.

To all the postgrads and friends (especially Stephen, Sandra, JJ, Maeve, Dairine, Alan, Justin and Ollie) for their support, friendship and numerous coffee breaks.

Thanks to Enterprise Ireland and Trinity College Dublin for their financial support. Thanks also to the University of Oxford for giving me time to finish this work.

Finally, I would like to dedicate this work to my family – Mum, Dad and Ronan – without whose support, love, understanding and computer expertise I would not have succeeded.

Table of Contents

	Page:
<u>Section 1</u>	1
1.1 Polyamines	1
1.1.1 Introduction	1
1.1.2 Polyamine metabolism	3
1.1.3 The pharmacology of polyamines	8
1.1.4 Polyamine interactions with receptors and ion channels	10
1.2 The NMDA receptor	12
1.3 Epilepsy	16
1.3.1 Introduction	16
1.3.2 Mechanism of action of antiepileptic agents	17
1.3.3 Role of polyamines in epilepsy	20
1.4 Stroke and cerebral ischaemia	21
1.4.1 Introduction	21
1.4.2 Metabolic and chemical mediators of cerebral ischaemia	24
1.4.3 Polyamines and cerebral ischaemia	29
1.4.4 Apoptosis	30
<u>Section 2</u> Spermine, NMDA and CNS excitation	32
2.1 Introduction	32
2.2 Experimental procedure	37
2.2.1 Direct spermine administration	37
2.2.2 Spermine enhanced NMDA-induced convulsions	38
2.3 Antagonists	40
2.4 Treatment of results	41
2.5 Results	43
2.5.1 Direct administration of spermine	43
2.5.1.1 Memantine	43
2.5.1.2 Calcium channel antagonists	49

2.5.1.3	N ¹ -Dansylspermine	56
2.5.2	Spermine enhanced NMDA-induced convulsions	60
2.5.2.1	Nisoldipine	63
2.5.2.2	Ifenprodil and its analogues	66
2.5.2.3	N ¹ -Dansylspermine	73
2.6	Discussion	78
2.7	Conclusion	90
<u>Section 3</u>	Cerebral Ischaemia	93
3.1	Introduction	93
3.1.1	<i>in vivo</i> models	93
3.1.2	<i>in vitro</i> models	95
3.1.3	Assessment of neurodegeneration	96
3.1.4	The gerbil model of ischaemia	97
3.2	Experimental procedure	101
3.3	Materials	105
3.4	Treatment of results	106
3.5	Results	107
3.5.1	EMLA	107
3.5.2	MK-801	111
3.5.3	N ¹ -Dansylspermine	114
3.5.3.1	Effect on locomotor activity	119
3.5.3.2	Effect on temperature	122
3.5.4	Ifenprodil and eliprodil	125
3.5.4.1	Effect on locomotor activity	125
3.5.4.2	Effect on temperature	132
3.6	Discussion	136
3.7	Conclusion	148
<u>Section 4</u>	Image Analysis	150

4.1	Introduction	150
4.2	Experimental procedure	151
4.3	Results	152
4.4	Conclusion	161
<u>Section 5</u>	An electrophysiological model of epilepsy	162
5.1	Introduction	162
	5.1.1 <i>In vivo</i> models	162
	5.1.2 <i>In vitro</i> models	163
	5.1.3 Cortical wedge preparations	166
5.2	Experimental procedure	168
5.3	Treatment of results	170
5.4	Results	172
	5.4.1 Effect of N ¹ -Dansylspermine	174
	5.4.2 Action of spermine and its antagonism by N ¹ -dansylspermine	178
5.5	Discussion	183
5.6	Conclusion	188
<u>Section 6</u>	Further discussion	189
6.1	Introduction	189
6.2	The NMDA receptor macrocomplex	189
6.3	Calcium channels	196
6.4	Potassium channels	200
6.5	Conclusions	204
6.6	Further studies	205
	Bibliography	207
	Appendix I	247

SECTION 1

1.1 POLYAMINES

1.1.1 Introduction

The polyamines (putrescine, spermidine, spermine) are found in all eukaryotic cells including those of the nervous system (Seiler, 1991). They are di-, tri-, and tetra- amines and occur in high, but variable, concentrations in cells.

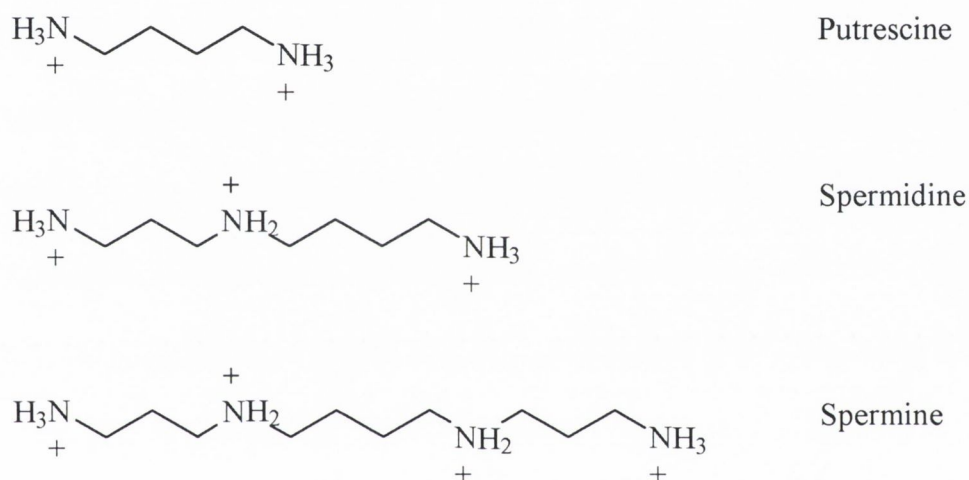


Figure 1.1: Structures of the common, naturally-occurring polyamines

It is thought that the polyamines were first discovered by Van Lewenhoeck in 1677. In his letter to the Royal Society he described the existence of spermatozoa in seminal fluid and also crystals, which were probably crystals of spermine phosphate (Tabor & Tabor, 1964). Around the turn of the twentieth century polyamines were isolated from various tissues and named according to their origin. Neuridine was isolated from the brain and musculamine from muscle. However, these two substances were later demonstrated to be identical to the previously discovered spermine and as a result this earlier name was used (Tabor & Tabor, 1964).

The tissue concentrations of the polyamines vary greatly throughout the body, though the orders of magnitude are the same in all vertebrates. Those cells growing at a rapid

rate have higher polyamine concentrations than those that are not growing. Putrescine ranges from 10nmol/g in the brain to 225nmol/g in the prostate. Spermidine varies from 70nmol/g in skeletal muscle to 8890nmol/g in the prostate and spermine varies from 240nmol/g in skeletal muscle to 5670nmol/g in the prostate (Seiler, 1994).

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

Brain area	Putrescine (nmol/g wet mass)	Spermidine (nmol/g wet mass)	Spermine (nmol/g wet mass)
Frontal cortex	9.4 \pm 1.3	235 \pm 22	221 \pm 20
Rear cortex		368 \pm 56	324 \pm 50
Olfactory bulb	5.5 \pm 0.4	446 \pm 81	506 \pm 88
Hippocampus	7.1 \pm 1.2	420 \pm 107	334 \pm 64
Hypothalamus	22.9 \pm 2.0	591 \pm 109	235 \pm 54
Striatum		420 \pm 107	285 \pm 38
Midbrain	6.2 \pm 1.1	884 \pm 176	237 \pm 61
Medulla	3.7 \pm 0.8	1016 \pm 77	157 \pm 33
Cerebellum	13 \pm 1.3	674 \pm 58	381 \pm 47

Because of the blood-brain barrier, the exchange of polyamines between the blood and the brain is limited and as a result almost all brain polyamines are synthesised in the brain itself. Brain concentrations of polyamines vary greatly from location to location as can be seen in Table 1.1. Spermidine was thought to be a constituent of myelin as a consequence of its high concentrations in areas consisting primarily of white matter. This was underlined by research by Russell and Meier (1975), who demonstrated altered polyamine distribution in myelin deficient strains of mice.

At physiological pH, the amino groups of the polyamines are protonated. This positive charge distributes along the aliphatic carbon chain and enables the polyamines to form ion pairs with negatively charged molecules. The binding energy increases with the number of charges (putrescine<spermidine<spermine). These charges allow electrostatic interactions with DNA, RNA, proteins and negatively charged membrane constituents. This is thought to be the basis for some functions of the polyamines.

Electrostatically bound and free polyamines are in dynamic equilibrium. The intracellular concentration of the free polyamines likely only forms a small fraction of the total spermidine and spermine. Due to the electrostatic binding abilities of the polyamines it is difficult to assess the free intracellular concentrations. However, Kroigaard *et al.* (1992) detected significant levels of polyamines in nerve terminals and secretory granules from ox neurohypophysis. It is most likely that the free, not the total polyamines, are essential for the regulation of biosynthesis, degradation, uptake and release.

1.1.2 Polyamine metabolism

Though cellular uptake systems exist for the polyamines, most cells synthesise their own polyamines. The only exception to this are anuclear red blood cells which have an uptake mechanism to accumulate polyamines. In the vertebrate organism polyamines appear to be synthesised by only one metabolic pathway (Seiler 1990; Seiler 1991).

Putrescine is produced by the decarboxylation of ornithine (Figure 1.2). This reaction is catalysed by the rate-limiting enzyme ornithine decarboxylase (ODC). Spermidine and spermine are formed from putrescine and spermidine, respectively, by the addition of an aminopropyl residue. This residue is produced from methionine, which reacts with ATP to produce S-adenosylmethionine. This is then decarboxylated to form decarboxy-S-adenosylmethionine. This compound then donates the

Figure 1.2 (overleaf): The reactions involved in polyamine synthesis, metabolism and exchange between intracellular and extracellular compartments. The enzymes numbered are as follows: (1) Ornithine decarboxylase (ODC). (2) S-adenosyl methionine decarboxylase (AdoMetDC or SAMDC). (3) Spermidine synthase. (4) Spermine synthase. (5) AcetylCoA: polyamine N¹-acetyltransferase (cytosolic; cSAT). (6) AcetylCoA: spermidine N⁸-acetyltransferase (nuclear; nSAT). (7) N⁸-Acetylspermidine deacetylase. (8) Polyamine oxidase (FAD-dependent). (9) Arginase. (10) Ornithine aminotransferase. (11) 5'-Methylthioadenosine (MTA) phosphorylase.

(Figure adapted from Seiler 1990).

aminopropyl residue for transfer to putrescine or spermidine. This residue transfer, to putrescine to form spermidine and to spermidine to form spermine, is catalysed by spermidine synthase and spermine synthase, respectively.

The second product of decarboxy-S-adenosylmethionine, 5'-methylthioadenosine, is formed in equimolar amounts during the production of spermidine and spermine and can be reused for the formation of ATP (Seiler, 1990; Seiler, 1991).

As can be seen from Figure 1.2, putrescine, as well as being converted into spermidine, can also be converted into acetyl putrescine. This reaction is catalysed by AcetylCoA: spermidine N⁸-acetyltransferase. Spermidine is subject to a similar reaction catalysed by the same enzyme. In these reactions a positive charge is removed from the polyamine molecules, which reduces their ability to interact with negatively charged binding sites (Seiler, 1990; Seiler, 1991). These acetyl derivatives can be transported out of the cell to the extracellular space. However, this reaction can also be reversed with the enzyme N⁸-Acetylspermidine deacetylase, releasing acetate in the process.

There are two major pathways, which are responsible for the breakdown of the polyamines. These are the retroconversion pathway and the terminal catabolic pathway (Seiler, 1990; Seiler, 1991).

The retroconversion pathway results in the production of spermidine and putrescine from spermine and spermidine, respectively. This is a two step process. The first step involves the conversion of the polyamines, spermine and spermidine, to their monoacetyl derivatives, N¹-acetylspermine and N¹-acetylspermidine. These reactions are catalysed by the enzyme AcetylCoA: polyamine N¹-acetyltransferase. This enzyme is rate-limiting and acetyl-CoA acts as the acetyl group donor.

These monoacetylated derivatives of spermine and spermidine are substrates for the enzyme polyamine oxidase. This enzyme splits the N¹-acetylpolyamines into an aldehyde, 3-acetamidopropanal. This represents the part of spermidine and spermine

that originates from methionine and this aldehyde is further oxidised to β -alanine. A by-product of the production of β -alanine is hydrogen peroxide (Coffino & Poznanski, 1991). The polyamines that are produced from this process can be re-used for polyamine synthesis, demonstrating that polyamine synthesis is a cyclic process. Due to this cyclic process, the estimate, by Shaskan and Snyder (1973), of the half-lives of polyamines in terms of days, is a massive over estimate. Similarly, this would account for the over estimate by Shaw (1979), who recorded half-lives of spermine and spermidine of 92 and 22 days, respectively. In fact, recently, daily regional variations of spermine and spermidine have been demonstrated (Al-Deen & Shaw, 1991) thus illustrating that the polyamine concentrations are in a constant state of flux and also lending support to significantly shorter duration half-lives of polyamines.

The terminal catabolic pathway involves the breakdown of polyamines to products that cannot be directly reconverted back into polyamines. The polyamines can undergo oxidative deamination by the Cu^{2+} -containing amine oxidases. This results in the production of hydrogen peroxide, ammonia and cytotoxic aldehydes. These aldehydes can undergo further metabolism by aldehyde dehydrogenases into the corresponding amino acids. However, in the absence of a suitable dehydrogenase the aldehydes deriving from spermine and spermidine will produce the toxic acrolein by β -elimination (Seiler, 1990; Seiler, 1991).

As a result of the uneven distribution of the Cu^{2+} -containing amine oxidases, it is unlikely that the terminal catabolic reactions participate in the regulation of cellular polyamine levels in general. However, these amine oxidases have been found in high concentrations in the intestines and placenta and may, therefore, have regulatory functions in these tissues (Seiler, 1990; Seiler, 1991).

Rate-limiting enzymes can be recognised by their low activity and three such enzymes are involved in polyamine metabolism. These enzymes are ornithine decarboxylase, S-adenosylmethionine decarboxylase and acetyl CoA: polyamine N^1 -acetyltransferase. However, these enzymes are rapidly inducible by both physiological and non-physiological means. Also these enzymes have very short half-

lives resulting in a high turnover rate. Rapid induction of these enzymes and a high rate of inactivation allows short bursts of polyamines to be produced. In this way, polyamines can regulate their own formation at a transcriptional level, by affecting the formation of mRNA and also at translational and post-translational levels (Seiler, 1990).

1.1.3 The pharmacology of polyamines

Since Jänne *et al.* (1978) demonstrated that an early accumulation of putrescine and spermidine was a sign of accelerated growth, the polyamines have been shown to be involved in the normal growth and differentiation of many types of cells (Schuber, 1989). Jänne and co-workers (1991) demonstrated that, in animal cells, the initiation of the growth process almost always involves the stimulation of ornithine decarboxylase. Polyamine levels have been found to be greatly elevated in malignant cells. Indeed, Moulinoux and co-workers (1991) demonstrated that levels of spermidine synthesised from radiolabelled putrescine in Lewis lung carcinoma cells increase concurrently with tumour volume, and correlate directly with tumour progression. It appeared from this study that ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC) and spermidine and spermine synthase were suitable targets for anticancer therapy. Since then, only two inhibitors of these enzymes have gained therapeutic significance: the ODC inactivator, 2-(difluoromethyl)ornithine (DFMO) and the competitive inhibitor of SAMDC, methylglyoxal-bis(guanylhydrazone) (MGBG), though the trials using MGBG have been terminated because of toxicity (Seiler *et al.*, 1998(a)).

Indeed, these inhibitors of the enzymes of polyamine synthesis have also found use in the treatment of parasitic protozoal infections. DFMO was originally developed as an antiprotozoal agent in the early 1980's when Bacchi and co workers (Bacchi, 1981; Nathan *et al.*, 1981) demonstrated that DFMO cured mice infected with *Trypanosoma brucei brucei*. The exact mechanism of action is still not known, however, development has continued and the initial clinical trials are already showing that

DFMO exerts a dramatic and rapid therapeutic effect in patients with African sleeping sickness.

As a result of stressful stimuli, polyamine synthesis in the CNS is greatly stimulated (Gilad & Gilad, 1992). These stressful stimuli include electrical stimulation, mechanical injuries and ischaemia. In cases involving ischaemia, which results in cell death, there are substantial changes in the polyamine levels (Paschen *et al.*, 1992). This will be discussed in further detail in Section 1.4.3 and Section 3.

It has been demonstrated (Anderson & Shaw, 1974) that, intracerebroventricular injections of spermidine or spermine, in doses of 250 μ g and 500 μ g in rabbits, produce a significant increase in the blood glucose level. Normal values were recovered within 3 hours. After comparison with intravenous doses it was concluded that polyamines exert their hyperglycaemic effect centrally.

Anderson *et al.* (1975) demonstrated that spermine dose-dependently produced convulsions in mice when given by the intracerebroventricular route. Low dose (10 μ g) produced convulsions 18 hours or more after injection, whereas 100 μ g spermine produced clonic convulsions within minutes. Spermidine (100 μ g) did not produce convulsions but was shown to result in paralysis that developed over four days ultimately resulting in quadriplegia.

The polyamines have been shown to have a hypotensive effect. Rossi *et al.* (1984) demonstrated that the administration of putrescine to dogs, either by the intravenous route (10mg/kg) or by microinjection (200 μ g/animal) into the third cerebral ventricle, produced a hypotensive effect. In animals pre-treated with a H1 receptor antagonist there was no hypotensive effect observed. Therefore, it was concluded that the cardiovascular effect was exerted through histamine release. Indeed, the release of histamine by polyamines was previously shown by Shaw (1972).

The polyamines have been demonstrated to have an inhibitory effect on the secretion of gastric acid. This was thought to be as a result of uncoupling of the gastric H⁺/K⁺

exchange pump in the secretory membrane, affecting the transport of H^+ (Ray *et al.*, 1982).

As previously mentioned the polyamines are positively charged at physiological pH. This cationic nature allows the polyamines to bind to negatively charged molecules, such as the acidic phosphate groups of nucleic acids (Tabor & Tabor, 1964). Therefore, the polyamines can mediate effects at the genomic level.

Through these electrostatic interactions the polyamines are able to regulate a number of membrane functions and bind to cellular components. Yoneda *et al.* (1991) reported specific, but low affinity, binding of spermidine in synaptosomal fractions from the rat brain. By binding to acidic phospholipids, the polyamines can influence the intracellular second messenger system based on phosphoinositide metabolism (Shaw, 1994). Polyamines have been shown to modulate signals transduced through G-protein-coupled receptors. This has been shown to be through stimulation of GTPase activity of purified GTP-binding proteins.

1.1.4 Polyamine interactions with receptors and ion channels

A number of receptor systems have been shown to be influenced by polyamines. These include the NMDA (N-methyl-D-aspartate) receptor macrocomplex, Ca^{2+} -channels, K^+ -channels, $GABA_A$ receptors (γ -aminobutyric acid) and adenosine receptors. The NMDA receptor will be discussed in more detail shortly.

Both ubiquitous polyamines and polyamine toxins have been shown to have an effect on voltage-activated calcium channels. Putrescine has been demonstrated to increase L-type calcium channel currents and this is mediated by protein kinase C (Herman *et al.*, 1993). A number of intracellular and extracellular sites of action have been proposed and these mediate both the inhibition and enhancement of voltage-activated calcium channels (Scott *et al.*, 1994). Pullan *et al.* (1990) demonstrated that polyamines inhibit N-type calcium channels. Schoemaker (1992) showed that the

polyamines, putrescine, spermidine and spermine, inhibit diltiazem binding, with IC_{50} values of 1053, 344 and 417 μ M respectively to L-type calcium channels in the brain. Similarly, Schoemaker (1992) demonstrated allosteric inhibition of nitrendipine binding by spermidine and spermine, but not putrescine, to L-type calcium channels (IC_{50} values of 219 and 260 μ M, respectively).

Inward rectifier potassium channels (Kir) are present in both excitable and non-excitable cells and are vital for holding the resting membrane potential close to the potassium equilibrium potential (E_K). Intracellular magnesium ions are responsible for blocking the inward rectifier potassium channels, which is responsible for inward rectification (Matsuda, 1991). However, the magnesium block cannot account for all the gating and rectification properties. Through examination of the $I-V$ relationship for strong Kir channels it was determined that there were intracellular factors responsible for the inward rectification. Spermine and spermidine have recently been shown to be the intracellular factors responsible for the intrinsic gating and rectification at strong Kir channels (Lopatin *et al.*, 1994; Lopatin *et al.*, 1995). Lopatin and co-workers (1995) proposed that two molecules of spermine line up, end to end, to block the Kir channel and stop K^+ ions moving through the pore. This demonstrates that the polyamines, particularly spermine and spermidine, have a role to play in control of the resting membrane potential and the excitability threshold for the initiation of action potentials.

AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and Kainate receptors are ionotropic glutamate receptors similar to the NMDA receptor. As for the Kir channels, the AMPA and kainate receptors show a marked inward rectification that is lost in membrane patches removed from the cell. It has been demonstrated that polyamines cause this inward rectification and probably act by blocking the pore of the receptor channel to prevent the flux of Na^+ and Ca^{2+} as the membrane is depolarised (Isa *et al.*, 1995; Bowie & Mayer, 1995). AMPA and kainate receptors actually exhibit a double rectification and the polyamines have been shown to be responsible for both components (Bowie & Mayer, 1995).

Gilad *et al.* (1992) recently demonstrated that polyamines can potentiate benzodiazepine binding in rat forebrain membranes in a concentration-dependent manner. However, after treatment of the membranes with detergent, there was an inhibition of binding in response to high polyamine concentrations, possibly due to a structural change near the receptor. Polyamines also have a modulatory effect on the binding and function of A₁ adenosine receptors in the brain (Wasserkort *et al.*, 1991) and have been shown to inhibit the binding of acetylcholine to synaptic vesicles from mouse brain at millimolar concentrations (Kuriyama *et al.*, 1968). Finally, Paul *et al.* (1990) demonstrated an inhibition by polyamines of ligand binding to sigma receptors at concentrations present in the mammalian central nervous system.

1.2 THE NMDA RECEPTOR

The NMDA receptor is one of the ionotropic glutamate receptors and is also the most widely studied of these receptors. It is voltage dependent and on activation allows the influx of Ca²⁺ and Na⁺. The native NMDA receptor has been shown to be made up of two different subunits, which accounts for the differing pharmacological responses of native NMDA receptors (Lynch *et al.*, 1995). Moriyoshi and colleagues (1991) cloned the first subunit of the NMDA receptor named NMDAR1 (NR1) from a rat brain cDNA library and this exists in eight splice variants. An additional four receptor subunits have also been cloned NMDAR2A (NR2A) – NMDAR2D (NR2D) (Ishii *et al.*, 1993). Combinations of NR1 with different NR2 subunits produce receptors with distinct properties. It was originally thought that the receptor complexes were only binary but ternary complex formation has recently been demonstrated (Dunah *et al.*, 1998).

The NMDA receptor includes a glutamate binding site, a binding site for its co-agonist glycine as well as modulatory sites for polyamines (stimulatory and inhibitory), protons, zinc and magnesium. An illustration of the NMDA receptor macrocomplex is shown in Figure 1.3 overleaf.

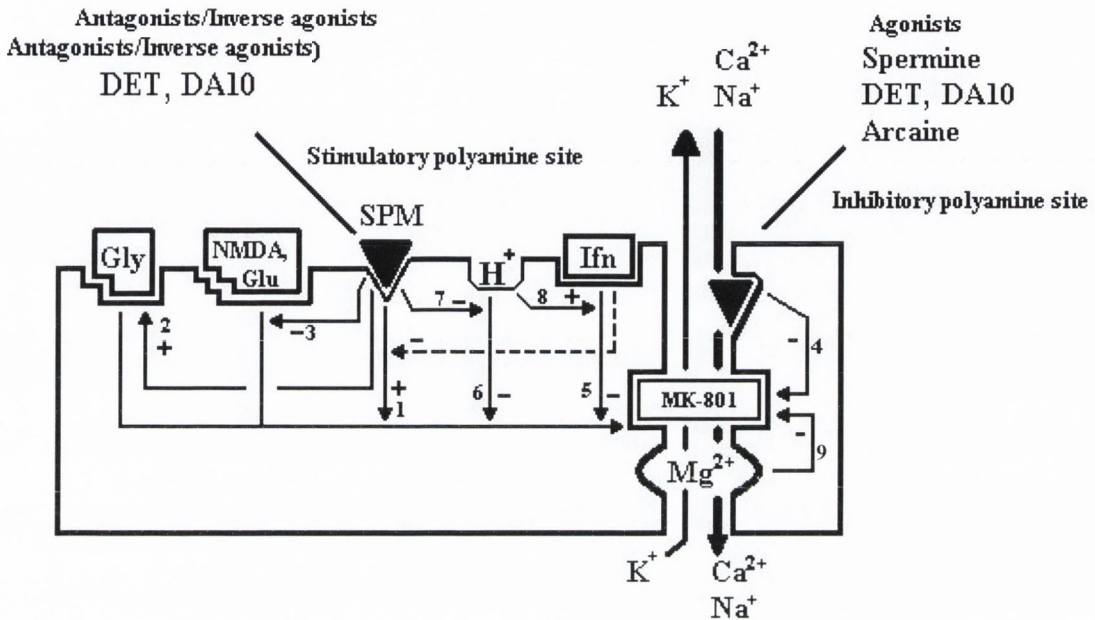


Figure 1.3: A schematic representation of the NMDA receptor. Figure modified from Kashiwagi et al. (1997).

Activation of the NMDA receptor, by NMDA or glutamate, is dependent on the presence of glycine (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988) and, on activation, the receptor allows the influx of calcium ions and, to a lesser extent, sodium ions. Radiolabelled open channel blockers of the NMDA receptor, such as [³H] MK-801 and [³H] TCP (*N*-(1-[thienyl]cyclohexyl)piperidine), have been widely used to study the properties of the NMDA receptor. Under normal physiological conditions, the NMDA receptor is blocked by magnesium ions but this block is relieved on depolarisation, allowing activation of the receptor complex by NMDA or glutamate.

The NMDA receptor macrocomplex is now thought to be involved in long-term potentiation and long-term depression in the hippocampus and neocortex which are thought to contribute to a cellular mechanism of memory formation in the mammalian brain (Collingridge & Bliss, 1987; Bliss & Collingridge, 1993; Bear & Malenka,

1994). It has also been well established that the NMDA receptor has a role in the mechanism of neuronal degeneration caused by anoxic/ischaemic conditions and seizure mediated brain damage and other neurodegenerative diseases in the brain (Rothman & Olney, 1986).

The effects of polyamines on the NMDA receptor were first reported in 1988 by Ransom and Stec. They demonstrated that the polyamines, spermine and spermidine, increased the affinity of a binding site for [³H] MK-801 in the presence or nominal absence of glutamate and glycine, and that spermine increased the potency of these amino acids for enhancing the binding of [³H] MK-801. As a result, it has been proposed that this enhancing effect of the polyamines was mediated at a novel site associated with the NMDA receptor (Ransom & Stec, 1988; Williams *et al.*, 1991). Subsequently, it was shown that high concentrations of polyamines actually inhibit the binding of radiolabelled open-channel blockers (Sacaan & Johnson, 1990).

Spermine potentiates NMDA currents in the presence of saturating concentrations of glycine. This glycine-independent stimulation (Figure 1.3, 1) involves an increase in the frequency of channel opening and a decrease in the desensitisation of NMDA receptors (Lerma, 1992; Rock & Macdonald, 1992(a); Benveniste & Mayer, 1993). Spermine also increases the affinity of the NMDA receptor for glycine (glycine-dependent stimulation, Figure 1.3, 2) (Benveniste & Mayer, 1993). It has been demonstrated that at some recombinant NMDA receptors spermine causes a decrease in the affinity for glutamate (Figure 1.3, 3)(Williams, 1994). This effect is proposed to reflect an increased rate of dissociation of glutamate from the receptor in the presence of spermine. Finally, spermine is able to inhibit the NMDA receptor through an action within the ion channel (Figure 1.3, 4). This inhibition is strongly voltage-dependent and is thought to be due to a fast open channel block similar to that caused by Mg²⁺ (Figure 1.3, 9)(Romano & Williams, 1994). It has also been suggested that spermine may interact with negatively charged residues on the NMDA receptor, close to the channel mouth, to impede ion flow and thus reduce currents through the NMDA receptor (Rock and Macdonald, 1992(a, b)).

Protons have been demonstrated to block the NMDA receptor with a tonic inhibition of approximately 50% at physiological pH (Figure 1.3, 6) (Traynelis & Cull-Candy, 1990). Interestingly, the glycine-independent form of spermine stimulation may involve relief of this proton mediated inhibition (Figure 1.3, 7). The inhibition of the NMDA receptor by ifenprodil has been proposed to be due to a polyamine antagonistic action (Figure 1.3, 5 and dotted line). However, block by ifenprodil has also recently been proposed to be dependent on extracellular pH (Figure 1.3, 8). Indeed, Pahk and Williams (1997) predicted that a decrease in proton inhibition would reduce ifenprodil inhibition. More recently, Mott *et al.* (1998) demonstrated that ifenprodil and its analogue CP-101,606, inhibit NMDA receptors by increasing the sensitivity of the receptor to inhibition by protons.

Magnesium ions, which cause a voltage-dependent block of the NMDA receptor, have recently been shown to potentiate NMDA receptor activity similar to spermine. This action is thought to be mediated through an extracellular site (Paoletti *et al.*, 1995). It was demonstrated that spermine causes stimulation in the presence of 1mM extracellular Mg^{2+} (Williams *et al.*, 1994) but not in the presence of high concentrations (Paoletti *et al.*, 1995). It has been proposed therefore that Mg^{2+} may act via the same stimulatory site as spermine on the NMDA receptor, which suggests that, in the presence of extracellular magnesium, spermine may have only moderate stimulatory effects (Williams *et al.*, 1994).

Issues relating to the role of the various transmembrane and pore-forming residues of the NMDA receptor are therefore complex and will be examined in greater detail in the discussion, together with further observations concerning the physiological relevance of the various polyamine sites.

Since the polyamines produce convulsions and interact with the NMDA receptor, it is important to consider the possibility of the polyamines having a contributory role to disorders which involve the NMDA receptor, such as epilepsy.

1.3 EPILEPSY

1.3.1 Introduction

Epilepsy is a chronic neurological disorder consisting of the unpredictable occurrence of seizures. Epileptic attacks start with an abnormal paroxysmal discharge of neurones in the brain, which can spread locally to nearby neurones or be more widespread. There are a number of different subtypes of epilepsy and the classification depends on the site of origin of the discharge, on the clinical manifestation, or on the electro-encephalogram (EEG) changes.

Partial seizures are generally localised in origin, arising from a focus in part of one hemisphere, usually the temporal or frontal lobe. If consciousness is maintained they are termed simple but if there is a loss in consciousness they are termed complex. Generalised seizures do not appear to have any local origin arising simultaneously in both hemispheres and involving loss of consciousness at the outset. Such seizures include tonic-clonic (grand mal) and absences (petit mal). A partial/focal seizure may spread to involve the whole cortex resulting in a secondarily generalised tonic-clonic seizure. Epileptic seizures are those that originate spontaneously within the brain and are not evoked by such stimuli as electroshock, alcohol or cocaine. When episodes of any form of epilepsy occur on a continuing basis, this is termed status epilepticus and is potentially life-threatening if not treated immediately.

Patients may present with more than one type of epilepsy, such as grand mal in association with petit mal and psychomotor epilepsy. As a result it is difficult to calculate the incidence of epilepsy. A recent study by Wallace *et al.* (1998) examined age-specific incidence and prevalence rates of treated epilepsy in an unselected population of over two million people. Using the General Practice Research Database, they demonstrated that the prevalence of treated epilepsy in 1995 was 5.15 per 1000 people and that the incidence of treated epilepsy was 80.8 per 100,000 people.

There are numerous potential causes of epilepsy. Neonatal asphyxia and trauma at birth may lead to brain damage and as a result give rise to epileptiform disorders. Infections, such as meningitis and viral encephalitis, may be accompanied by epilepsy or may result in brain damage, which will cause epilepsy later on in life. Drug withdrawal, alcohol or barbiturates, may precipitate convulsive episodes. Brain insults of various types (stroke and head trauma) may result in the development of post traumatic epilepsy.

There is little that can be done in these cases to prevent the development of epilepsy as the sequence of events culminating in the appearance of spontaneous fits is unclear. However, the development has a number of phases. First, the initial insult, which can last hours or days. Second, the latency period, which could be as long as months or years. Finally, recurrence of seizures, which can persist for the rest of the patient's life. There is much current debate over the prevention of the development of post traumatic epilepsy. The use of anticonvulsant prophylaxis to prevent epileptogenesis following head trauma has been investigated. However, the use of anti-epileptic drugs as prophylaxis is not generally indicated due to a lack of supporting, beneficial evidence from clinical trials (Hernandez, 1997).

1.3.2 Mechanism of action of anti-epileptic agents

Gamma-amino-butyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system. Two subtypes of receptor have been identified, GABA_A and GABA_B. GABA_A is the most abundant receptor and is a ligand-gated Cl⁻ ion channel. The GABA_B receptor is a G protein-coupled receptor. A number of the anti-epileptic drugs in use today utilise GABA to exert their effects. The benzodiazepines, such as diazepam, midazolam and clonazepam, are primarily used for status epilepticus. The benzodiazepines have the ability to enhance GABA-induced increases in the conductance of Cl⁻ ions. They stimulate GABAergic pathways and enhance GABA-induced changes in the membrane potential. The barbiturates (phenobarbitone and mephobarbitone) work in a similar way by potentiating responses to GABA through the GABA_A receptor (MacDonald & Barker,

1979). Though the net result is the same for both the benzodiazepines and the barbiturates, enhanced GABA-mediated inhibition, recent work has demonstrated that the mode of action may be slightly different. Twyman *et al.* (1989) demonstrated that diazepam binds to a site and enhances single channel burst frequency by increasing the affinity of GABA, whereas phenobarbitone may stabilise the bursting open state of the channel by binding to a different modulatory site, at or near the chloride channel.

Sodium valproate, which is used as a first line of treatment for petit mal and grand mal seizures, has a marked effect on the levels of GABA. It has been demonstrated, *in vitro*, that sodium valproate inhibits the GABA degradative enzymes, GABA transaminase and succinic semialdehyde dehydrogenase (Chapman *et al.*, 1982), and sodium valproate also increases the activity of the GABA synthesising enzyme, glutamic acid decarboxylase (Phillips & Fowler, 1982). Both of these effects result in increased GABA levels in the CNS and may contribute to the antiepileptic effects of sodium valproate.

Vigabatrin, or gamma-vinyl GABA, was designed specifically to increase the GABA-mediated synaptic inhibition. Vigabatrin works by acting as an irreversible/suicide inhibitor of the degradative enzyme GABA transaminase. As a result the action of GABA is significantly prolonged. Tiagabine has been shown to be effective for the treatment of partial seizures through an action on GABA. Tiagabine increases synaptic GABA concentrations by inhibiting the re-uptake of GABA into glial cells (Meldrum, 1996). Finally, Topiramate, which has a number of different modes of action, has been demonstrated to potentiate the effects of GABA through an interaction with GABA_A receptors (Meldrum, 1996).

Sodium channels have been shown to be involved in the production and spread of seizures in epilepsy. By altering the rate of inactivation of the sodium channels it is possible to decrease the high-frequency firing, which is characteristic of epilepsy. For example, phenytoin, one of the hydantoin, limits the repetitive firing of action potentials evoked by a sustained depolarisation in cultured neurons. This action is facilitated by a slowing of the rate of recovery of voltage activated sodium channels

from inactivation. In a similar fashion both carbamazepine and sodium valproate reduced the repetitive firing of action potential evoked by sustained depolarisation. This is thought to be mediated by slowing the recovery of voltage-activated sodium channels from inactivation (McLean & MacDonald, 1986(a & b)). Lamotrigine is also thought to exert some of its action through modulation of sodium channels (Meldrum, 1996). Topiramate has been suggested to block voltage-activated sodium channels as part of its mechanism of anti-epileptic action. Similarly, zonisamide blocks the spread of epileptic discharges through an action on sodium channels (Meldrum, 1996).

The principal drugs used to treat absence/partial seizures have a similar mechanism. These compounds include ethosuximide, trimethadione and sodium valproate. These drugs reduce the flow of Ca^{2+} ions through T-type calcium channels and as a result reduce the pacemaker current underlying thalamic rhythm in spikes and waves seen in generalised absence seizures.

Gabapentin, a novel antiepileptic, has provided a puzzle for scientists trying to elucidate its mode of action. Gabapentin was originally designed as a lipid soluble GABA-mimetic. However, this has not been shown to be its actual mode of action. Recently, a gabapentin-binding protein has been located (Gee *et al.*, 1996). It was demonstrated that gabapentin binds to an $\alpha_2\delta$ subunit of a voltage dependent calcium channel. Thus, it was proposed that the antiepileptic action of gabapentin was mediated through modulation of voltage-dependent neuronal calcium channels, possibly the L-type calcium channels. This mechanism of action was further elucidated by the work of Fink *et al.* (2000). They proposed that gabapentin inhibits neuronal calcium influx through an action on P/Q-type calcium channels. This results in reduced influx of calcium causing a drop in the release of excitatory amino acids.

The NMDA receptor may play a role in the development and expression of seizures in epilepsy. A vast number of NMDA receptor antagonists have been demonstrated to possess marked anticonvulsant properties and indeed the NMDA receptor has been proposed to be a prerequisite in epileptogenesis (Dingledine *et al.*, 1990). It has been

demonstrated that the levels of the excitatory amino acid, glutamate, are elevated in human epileptic foci (Perry & Hansen, 1981). As mentioned in Section 1.2, NMDA receptor activation involves the relief of the voltage-dependent Mg^{2+} block and a resultant influx of both Ca^{2+} and Na^{+} ions and an efflux of K^{+} ions. It is this change in ion concentrations that is thought to be involved in the seizures. The role of the NMDA receptor in the development of seizures also seems to depend on the area in question. Of the available antiepileptic compounds on the market, Lamotrigine has been shown to block the release of glutamate and topiramate blocks AMPA and Kainate receptor (Meldrum 1996). Felbamate, a novel anti-epileptic agent, has been demonstrated to exert its action through modulation of sodium channel conductance (White *et al.*, 1992). However, in the same study felbamate was demonstrated to be effective against clonus and forelimb tonic extension in mice following intracerebroventricular administration of NMDA, suggesting a mechanism of action exerted through the NMDA receptor. Subsequently, it has been demonstrated that, *in vitro*, felbamate has antagonistic activity against epileptiform activity induced by excitatory amino acids (both NMDA and non-NMDA receptors) (Domenici *et al.*, 1994 & 1996). More recently, Felbamate has been proposed to act on a unique site on the NR2B subunit of the NMDA receptor. It is thought that this site interacts allosterically with the NMDA/glutamate binding site (Kleckner *et al.*, 1999).

1.3.3 Role of polyamines in epilepsy

The polyamines have diverse properties and are involved in multiple systems throughout the body. As mentioned earlier, the polyamines have been demonstrated to cause convulsions when given by the intracerebroventricular route (Anderson *et al.*, 1975). Similarly, there is much evidence demonstrating a role for the polyamines in the enhancement of the activity of the NMDA receptor (Matsumoto *et al.*, 1993; Singh *et al.*, 1990). Morrison and co-workers (Morrison *et al.*, 1994) demonstrated an increased activity of S-adenosylmethionine decarboxylase, a regulatory enzyme in polyamine biosynthesis, in regions of active epileptogenic cortical discharge in samples from patients with intractable epilepsy. This demonstrates that the polyamines may be involved in the maintenance of hypersynchronous discharges and

this was suggested to be mediated through the NMDA receptor. Similarly, Kanai *et al.* (1992) demonstrated that 1-naphthylacetyl spermine, an analogue of the polyamine toxin, Joro spider toxin (JSTX), potently suppressed hippocampal epileptic discharges mediated through the non-NMDA receptors. It is conceivable, therefore, that the polyamines have a role in the initiation and spread of neuronal discharges during epilepsy. It was also demonstrated by Hayashi *et al.* (1993), that there are increases in brain polyamine levels following kindling (putrescine – 2.5 fold; spermidine – 1.5 fold; spermine – 1.2 fold), which lead to the suggestion that polyamine concentrations are involved in neuronal excitability in the epileptic brain.

The excitotoxic effects of the polyamines illustrated above suggest that the polyamines may play a role in other excitotoxic syndromes that involve the NMDA receptor. One such condition is cerebral ischaemia.

1.4 STROKE AND CEREBRAL ISCHAEMIA

1.4.1 Introduction

Despite a reduction in incidence and severity over the last number of years (Broderick *et al.*, 1989), stroke/cerebrovascular disease is still the third most frequent cause of death after heart disease and cancer (Mas & Zuber, 1991). In most countries the decline shown over the last number of years has been greater in women than in men. The annual incidence is about 1-2 strokes per 1000, which is equivalent to 5% of the population over 65 years of age (Mas & Zuber, 1991).

Strokes were traditionally divided into pathological groupings based on the clinical findings. These groups were haemorrhage, thrombosis and embolism. It was thought that all these conditions evoked permanent and catastrophic changes in the brain. However, clinicians became aware of different degrees of severity of strokes, such as minor strokes which were described as transient ischaemic attacks (TIA's) (Marshall, 1964). These are focal neurological deficits of a sudden onset and duration in nature (last less than 24 hours). These minor strokes are significant as they can often give

warning of an approaching major stroke. Deficits of more than 24 hours but less than 3 weeks, are referred to as reversible ischaemic neurological deficits (RINDS). An event that produces deficits lasting over three weeks is termed a stroke.

An ischaemic attack is classified using a number of criteria. These criteria are the site of the lesion (carotid or vertebrobasilar), the duration of attack (TIA or RIND) and finally its pathology (haemorrhagic, thrombotic or embolic).

The decline, over recent years, in the incidence of stroke is probably partly due to the change in classification of strokes over the years. Indeed, at the start of the twentieth century the common clinical term was apoplexy and was used to describe all forms of sudden death. As a result, it is not possible to get an accurate figure for the incidence of cerebral ischaemia in the early part of the twentieth century. Now, with the development of such techniques as computer aided tomography, it is possible to distinguish between different types of stroke. However, despite the change in classification and diagnosis of strokes the decline in incidence and mortality appears to be real. This may be due to changes in disease severity and improved stroke care. Similarly, the identification of specific risk factors for stroke have affected incidence and mortality. These risk factors include hypertension, impaired cardiac function, cigarette smoking, alcohol, diabetes mellitus, elevated plasma lipids, haemostatic/haemorrhagic factors and hormonal factors.

Since stroke has a huge human and economic impact, preventive measures must remain a priority. As a result control of hypertension and smoking have been shown to have a great effect on the stroke incidence. However, a small but significant number of cases of stroke still remain where the underlying mechanism is unidentified (Mas & Zuber, 1991).

The main principles of the present stroke treatment are two-fold. First, the neurones must be protected from cell death and second, attempts must be made to increase blood flow to the ischaemic area. At present these treatments are limited and depend on the type of stroke. One of the main criteria for a stroke treatment is its efficacy in the post-ischaemic period. Most patients will present in a hospital within minutes to

hours of suffering a stroke and as a result any treatments must be effective within this time period. The present clinical approaches concentrate on improving cerebral blood flow and decreasing cerebral metabolism or energy demand with the use of barbiturates, and lowering body temperature in order to reduce metabolism (Jabaily & Davies, 1984).

The following are the suggested general treatments (World Health Organisation (WHO), 1989; Brott & Reed, 1989; Hacke *et al.*, 1990). Pulmonary function must be maintained and the airways protected. Good oxygenation of the blood and a normal or low PaCO₂, to avoid any steal effect, are the basis of any treatment for stroke. This will have no effect on the ischaemic core but is important for preservation of the penumbral area. In many cases the patient will have to be intubated to maintain a patent airway.

Cardiac output must be optimised by maintaining a high normal blood pressure and normal heart rate. Indeed, after acute cerebral ischaemia, hypertension can persist for some days (Awad *et al.*, 1987). Cerebral ischaemia may affect cardiac function causing ECG changes, cardiac arrhythmias and occasionally raised transaminases suggesting myocardial infarction (Norris *et al.*, 1978).

Many stroke patients are diabetic, occasionally only being diagnosed on presentation of a stroke. High glucose levels are a problem in stroke as a result of poor glucose utilisation due to a lack of oxygen. Indeed, experiments have correlated high glucose levels with lactic acidosis and tissue damage (Pulsinelli *et al.*, 1983).

Water and electrolyte levels should be balanced so as to avoid dehydration, raised haematocrit and impairment of rheologic properties of the blood. Other measures include the prevention of epileptic attacks in the acute phase of ischaemic attacks.

In the acute phase of an ischaemic attack perfusion and blood flow must be maintained. Blood flow can be increased by using haemodilution (Grotta, 1987), however clinical benefit has yet to be demonstrated. Thrombolytic therapy has been used for recanalisation of a thromboembolic occluded vessel. Fibrinolysis has been

carried out with urokinase and tissue-type plasminogen activator (Hacke *et al*, 1990). Anticoagulant therapy has been used in the treatment of acute stroke to prevent both local thrombus propagation and recurrent embolism. Patients suffering from acute thromboembolic stroke are treated with heparin bolus and subsequent intravenous infusion.

Finally, attempts are made to prevent ischaemic cellular damage. Unfortunately, there are only a few therapies available to the clinician but there are a number of clinical trials underway. Naloxone, an endorphin antagonist with antioxidant properties, has shown some promise by reversing the neurological deficits following ischaemia (Jabaily & Davies, 1984). Barbiturates reduce the infarct size in experiments but have shown no clinical benefit against the neurological deficits. Calcium has an important role to play in ischaemic cell damage as described in Section 1.4.2. As a result, a number of calcium channel blockers are being investigated, such as (S)-emopamil, nimodipine and nicardipine. However, a review of clinical data by Elben and co-workers (1992) found few positive results with calcium channel blockers in humans. Most relevant to this thesis, NMDA antagonists are also under investigation in neurotrauma. The two NR2B subunit selective compounds, eliprodil and CP-101,606, have been evaluated in phase IIb and phase III trials respectively though overall conclusions are not yet available (Reinert & Bullock, 1999).

1.4.2 Metabolic and chemical mediators of cerebral ischaemia

Energy failure is the main event underlying much of the pathophysiology of ischaemia. However, there are secondary processes that occur after ischaemia and contribute to the neuronal death. These are interlinked with the energy failure and also with each other. The extent of the parts each process plays in the production of neurodegeneration depends on the aetiology, whether it is hypoglycaemia, hypoxia or ischaemia. Figure 1.4 illustrates the combination of the processes following cerebral ischaemia and their production of cell death.

The brain is critically dependent on a continuous supply of oxygen and glucose, which is regulated by cerebral blood flow. Ischaemia is said to exist when the blood supply is insufficient to meet the metabolic demands of the tissue. Oxygen and glucose are the necessary substrates for the formation of ATP. Also there are no stores of oxygen and glucose in the brain and thus, when there is a lack of these substrates and continued metabolism of ATP, there is a net loss of ATP. There are a number of consequences of this energy failure. Protein synthesis is required for maintenance of the cytoskeleton but is dependent on a supply of ATP. The lack of ATP and oxygen causes glucose to be metabolised by anaerobic metabolism rather than aerobic and this produces lactic acid.

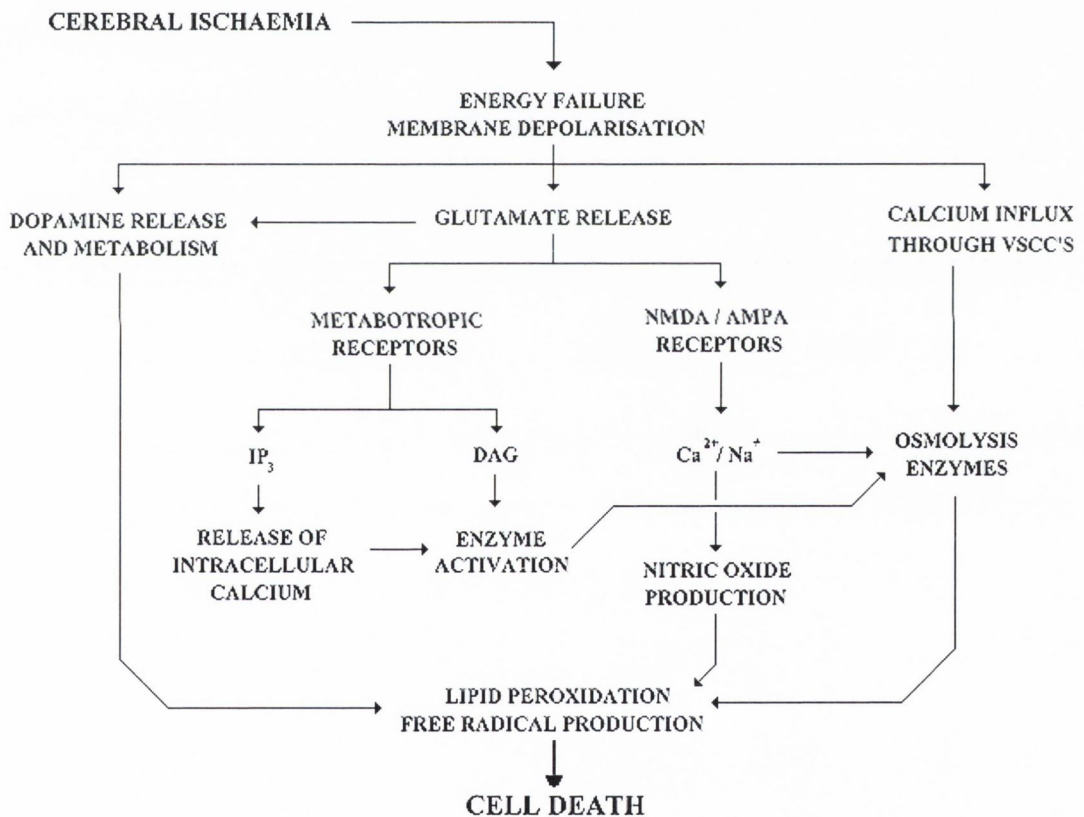


Figure 1.4: A schematic representation of the possible mechanisms involved in neuronal degeneration following cerebral ischaemia (VSCC – voltage sensitive calcium channels).

The lactic acid results in intra- and extracellular acidosis. ATP is also required to maintain ionic gradients across the cell membranes. Na^+ and Ca^{2+} are driven out of

the cell and K^+ in by ATP-dependent pumps ($Na^+ -K^+ -ATPase$ and $Ca^{2+} -ATPase$). There are also passive ion fluxes into and out of the cell following concentration gradients. During ischaemia these passive leaks are increased by membrane depolarisation and increased membrane permeability. Loss of ATP to drive the ionic pumps leads to increased influx and efflux of ions. This results in depolarisation of the cell membrane, which causes increased intracellular calcium concentrations and glutamate release. Water also flows into the cell following osmotic gradients caused by the influx of Na^+ and Cl^- , this then produces neuronal swelling.

Depolarisation of the synaptic membranes and increases in intracellular calcium results in the release of neurotransmitters. During ischaemia a nonphysiological release of glutamate takes place (Siesjö, 1992; Globus *et al.*, 1988). The release of other transmitters such as dopamine and GABA is also increased, as are the extracellular amino acid concentrations (Globus *et al.*, 1988; Andiné *et al.*, 1991). Most cells rely on uptake to terminate the action of neurotransmitters, however this is impaired in ischaemia, resulting in a prolonged action. Glutamate and aspartate are amino acid transmitters and elicit fast excitatory responses at postsynaptic receptors. Glutamate, when released, will act on numerous receptors including both the AMPA and NMDA receptors. The AMPA receptor allows Na^+ to enter the cell and this leads to depolarisation. The NMDA receptor, which, as mentioned previously, gates the transport of Ca^{2+} as well as Na^+ , is normally blocked in a voltage-dependent manner by magnesium but this is relieved by the membrane depolarisation. Since the normal termination method of this depolarisation, uptake of glutamate, is not functioning the increased intracellular calcium concentration persists resulting in cellular toxicity (Choi, 1985). The major role that this glutamate mediated toxicity plays in ischaemic brain damage has been demonstrated by the successful neuroprotective effects of various NMDA and glutamate antagonists (Siesjö, 1992).

Intracellular calcium is an important intracellular second messenger. The concentration is kept under tight control and is kept approximately 10,000 times lower than concentrations in the extracellular space. Calcium concentrations are controlled by influx and efflux across the cell membrane, release and uptake from intracellular stores and binding to intracellular proteins (Tymianski & Tator, 1996). Ischaemia

causes depolarisation of membranes and this results in the influx of calcium through NMDA receptors, as mentioned above, and also through the voltage sensitive calcium channels of the L- and T-type. Secondary to this, there is no ATP to facilitate sequestration or efflux. This nonphysiological increase in intracellular calcium concentrations can result in the activation of enzymes such as proteases, phospholipases, protein kinases and nitric oxide synthases, among others. The proteases will affect the cytoskeleton. Phospholipases such as phospholipase A₂ and phospholipase C will cause increased production of free radicals and vasoactive and inflammatory substances. Protein kinase C is responsible for normal cellular functions but may have detrimental effects when overactivated in ischaemia. Protein kinase C phosphorylates proteins including cell membrane channels, which may contribute to their dysfunction and to loss of ion homeostasis. It may also mediate some of the detrimental effects of glutamate since glutamate activation of NMDA channels may activate protein kinase C and this can increase intracellular calcium concentrations (Macdonald *et al.*, 1998).

Over recent years there has been a lot of interest in the involvement of dopamine in cerebral ischaemia. A possible interaction between the dopaminergic and glutamatergic systems has been proposed. Kornhuber & Kornhuber (1986) proposed that dopaminergic nigrostriatal fibres mediate presynaptic inhibitory responses on striatal glutamate release via D₂ receptors located on the terminals of corticostriatal neurones. There has been evidence to show that there may be a functional linkage between the dopaminergic and glutamatergic systems in the development of cell injury (Globus *et al.*, 1988). Dopamine concentrations have been shown to increase significantly as a result of an ischaemic episode (Phebus *et al.*, 1986). It has been proposed that extracellular accumulation of dopamine, or its metabolites, may have a mechanistic role in the pathogenesis of striatal neuronal injury (Phebus *et al.*, 1986; Phebus & Clemens, 1989). One possible role is that oxidation products of dopamine bind to nucleophilic sulphhydryl groups of proteins and this can result in an inactivation of enzymatic and other biological processes necessary for cell survival (Phebus *et al.*, 1986; Phebus & Clemens, 1989). Indeed, a number of positive results have been produced with compounds that affect the dopaminergic system. The

dopamine D₂ agonists, piribedil and lisuride, have been shown to be neuroprotective in rat models of cerebral ischaemia (Caldwell *et al.*, 1996).

In ischaemia there is a lack of oxygen for the Krebs's cycle and electron transport chain and as a result pyruvate is metabolised to lactate. This results in acidosis. Four mechanisms of acidosis-induced neuronal damage have been recognised by Siesjö (1992); oedema formation, inhibition of lactate oxidation, inhibition of mitochondrial respiration and inhibition of H⁺ extrusion. The severity of acidosis depends on the pre-ischaemic blood glucose levels. Hyperglycaemia significantly increases brain damage following global ischaemia in animal models (Ginsberg *et al.*, 1980; Pulsinelli *et al.*, 1982(b)). Indeed increased blood glucose levels have been shown to increase damage after a stroke in patients (Pulsinelli *et al.*, 1983).

Nitric oxide (NO), when released from endothelial cells, activates guanylate cyclase in smooth muscle cells and results in smooth muscle relaxation. Also, in neurons, nitric oxide may act as a neurotransmitter. By itself NO is not thought to be toxic, however it is involved in reactions following ischaemia that lead to free radical production. NO is thought to react with superoxide radicals to produce peroxynitrite, which is an oxidising agent. Studies have demonstrated that NO-synthesis inhibitors are neuroprotective when administered one hour prior to 5-minute bilateral carotid occlusion (BCO) in the gerbil (Caldwell *et al.*, 1994).

A free radical is a molecule with an unpaired electron in its outer shell and, as a result, it is highly reactive and can alter the chemical structure of other molecules. The fact that there are cellular enzymes and molecules whose sole function is to metabolise free radicals and make them unreactive provides evidence for their role as cellular toxins. These molecules and enzymes are required because free radicals are produced as a result of normal cellular processes such as electron transport. However, ischaemia causes an increased production of free radicals and this overproduction overwhelms the normal cell defences. Reperfusion following ischaemia leads to the production of superoxide and hydroxyl free radicals as well as hydrogen peroxide. Each free radical can participate in chain reactions that produce more radicals and in the process damage important cellular proteins, nucleic acids and lipids.

1.4.3 Polyamines and cerebral ischaemia

A number of contradictory theories for the role of polyamines in cerebral ischaemia have been proposed by different laboratories. Following cerebral ischaemia there are marked changes in the polyamine levels. During postischaemic recirculation, putrescine levels dramatically increase, whereas the levels of spermidine and spermine do not change. This overshoot of putrescine concentration (16 fold, 17 fold and 13 fold in the cerebral cortex, caudoputamen and hippocampus respectively) is due to an increase in ornithine decarboxylase activity and an inhibition of S-adenosylmethionine decarboxylase (Paschen *et al.*, 1987). Polyamines are also proposed to be released from injured neurons after prolonged reperfusion in severely damaged areas (Paschen *et al.*, 1992). More recently, Baskaya and co-workers (1997(a)) demonstrated that tissue putrescine is increased, two fold, in the penumbra region, an area of incomplete ischaemia that is developing brain oedema. It was originally suggested that the accumulation of putrescine during postischaemic recirculation may be responsible for the delayed neuronal death occurring after ischaemia (Paschen *et al.*, 1987). However, more recently this has been modified and now it has been concluded that the polyamines, specifically spermine, released from necrotic neurons, may bind to the NMDA receptor at intact neurons in the vicinity and thus enhance the intracellular response to signals mediated through the NMDA receptor (Paschen *et al.*, 1992).

Zoli *et al.* (1996) demonstrated an increase in spermidine/spermine N¹-acetyltransferase in rat CNS following ischaemia and suggested that this may contribute to the observed increase in putrescine and decrease in spermidine levels. This lends support to the notion that the whole polyamine interconversion pathway is actively involved in the changes in polyamine levels after cerebral ischaemia. More recently, a specific polyamine oxidase inhibitor, MDL 72527, was shown to be neuroprotective against neuronal cell damage after temporary focal cerebral ischaemia (Dogan *et al.*, 1999). This demonstrates that the polyamine interconversion pathway may also play a role in neuronal damage through the by-products of polyamine metabolism, such as hydrogen peroxide (Seiler, 1995), which has been implicated in cell damage.

1.4.4 Apoptosis

There is mounting evidence to suggest that there are two forms of cell death taking place following cerebral ischaemia, primarily necrosis but also a contribution from apoptosis. Recently, Martin *et al.* (2000) demonstrated that in dogs, using a transient partial global ischaemic model, damage in the CA1 pyramidal neurons and cerebellar Purkinje cells was slow to develop and was due to necrosis but the damage to granule neurons in the dentate gyrus and cerebellar cortex developed quickly and was as a result of apoptosis. Furthermore, Sparapani *et al.* (1997) demonstrated that the polyamines are involved in excitotoxic neuronal death and that the neuronal death caused by spermine occurred, at least in part by apoptosis. Apoptosis is a form of naturally occurring cell death. Some of the characteristic features include cell shrinkage, membrane blebbing and convulsion, DNA fragmentation and the chromatin becomes pyknotic and condenses in patches against the nuclear membrane and it finally breaks down to dense spheres called apoptotic bodies. Initiation of apoptosis can be through a number of mechanisms such as a genetic signal, calcium, hydrogen peroxide and anticancer drugs. Due to the polyamines positive charge they are able to interact with negatively charged DNA strands. The observation that polyamines are responsible for cell growth and differentiation (Jänne *et al.*, 1978; Schuber, 1989), has lead to the suggestion that increased catabolism of polyamines are involved in cell death. Increased activity of spermidine/spermine N¹-acetyltransferase correlates with growth inhibition (Porter *et al.*, 1985) and with cytotoxicity and apoptosis (Casero *et al.*, 1995; McCloskey *et al.*, 1996). More recently, Lindsay and Wallace (1999), using HL-60 leukaemia cells, demonstrated that polyamine oxidation, which produces hydrogen peroxide, may be associated with the initiation of apoptosis, whereas the activation of acetyltransferase and loss of intracellular polyamines may be involved in the final stages of cell death. However, in a subsequent study, Nairn *et al.* (2000) demonstrated that increased polyamine catabolism and spermidine/spermine N¹-acetyltransferase induction was not essential for the initiation of apoptotic cell death. They suggested that since cytotoxic polyamine analogues seem to work by ultimately increasing hydrogen peroxide production, it might be more useful to target polyamine oxidase rather than spermidine/spermine N¹-acetyltransferase, which indirectly increases hydrogen

peroxide production. Obviously, further investigations need to be carried out to elucidate the possible role of polyamines in apoptosis, though the initial work is promising.

Spermine has been shown to play a significant role in the Ca^{2+} -buffering capacity of mitochondria (Rottenberg & Marbach, 1990). As a result, in brain regions where, through transient cerebral ischaemia, there are reduced spermine levels, the Ca^{2+} -buffering capacity of mitochondria may be impaired. However, it has yet to be demonstrated if this is of physiological significance.

Finally, it has been demonstrated that the polyamines may influence the integrity of the blood-brain barrier following cold injury of the cerebral cortex (Koenig *et al.*, 1989). This was demonstrated using fluorescein dye uptake into the cerebral microvasculature following intravenous administration. These effects on the blood-brain barrier can be reversed by blocking ODC with 2-(difluoromethyl)ornithine (DFMO), as shown by Schmitz and co-workers (1993).

However, there is still some confusion over the role of the polyamines as a number of studies have shown that they are neuroprotective after cerebral ischaemia. Gilad *et al.* (1985) originally showed that treating newborn rats with polyamines, for a limited time, prevents nerve cell loss during this period of programmed cell death. Subsequently they have shown that, in the gerbil model of global cerebral ischaemia, daily polyamine treatment (10mg/kg, ip) can protect a wide variety of central and peripheral neurons from natural or induced degeneration (Gilad & Gilad, 1991). These results should be interpreted with caution, though, as the polyamines do not readily cross the blood-brain barrier. As a result of this and other work doubt has been cast on the role of polyamines in cerebral ischaemia. However, the majority of evidence points to a role for the polyamines in enhancing the neurodegeneration following cerebral ischaemia.

SECTION 2

Spermine, NMDA and CNS excitation

2.1 INTRODUCTION

As previously mentioned in Section 1 the polyamines are a family of amines found in all cells in the body. They have a diverse pharmacology with numerous roles in different areas of the body (see Section 1.1.3).

When the polyamines, spermine and spermidine, are administered by the intraperitoneal route sedation and hypothermia are produced, whereas intravenous administration resulted in convulsions (Shaw, 1972). However, the doses required to produce these effects were quite large (15 – 100mg/kg), whereas the doses required for direct injection into the brain are orders of magnitude smaller. This is due to the existence of a blood-brain barrier to the polyamines.

The first description of the effects of spermine and spermidine on the central nervous system was by Anderson *et al.* (1975). In their studies Anderson and co-workers injected both spermine and spermidine, by the intracerebroventricular route, to both mice and rabbits. Both spermine and spermidine produced marked sedation and hypothermia. Convulsions occurred, in mice, after the administration of 100µg spermine but after administration of the same dose of spermidine the mice developed quadriplegia over the course of four days. The potency of the polyamines is underlined by the fact that more than 80% of the administered dose immediately exits the brain after direct intracerebroventricular injection in mice (Shaw, 1974).

Spermine induced CNS excitation is split into two distinct phases. The first phase commences after injection and lasts for approximately 40 minutes. The second phase commences after two hours and lasts up to 7.5 hours from injection. This is described in greater detail in Section 2.2.1. In an attempt to determine the origin of this

spermine induced CNS excitation a number of studies have been carried out to assess the affect of various potential antagonists.

Doyle and Shaw (1996) investigated the role of the NMDA receptor macrocomplex. They demonstrated that on their own, the glutamate antagonist, D-CPP, the glycine antagonist, 7-chlorokynurenate or the polyamine antagonist, ifenprodil had an antagonistic effect on the first phase of effects but not on the second phase. However co-administration of ifenprodil with either CPP or 7-chlorokynurenate resulted in antagonism of the second phase of effects. Also investigated was MK-801, an open channel blocker of the NMDA receptor. However, only repeated dose administration was sufficient to inhibit the second phase of effects. It was suggested from this study that the two phases of the spermine-induced effects were mediated by different pharmacological mechanisms but that the NMDA receptor had a role to play in both phases (Doyle & Shaw, 1996).

These studies point to the possibility of the NMDA receptor being involved in both phases of the spermine-induced CNS excitation, but do not rule out other mechanisms. A number of other systems have also been proposed to be involved. GABA might play a role, as GABA potentiating anticonvulsants such as benzodiazepines and phenobarbitone were shown to antagonise the first phase spermine induced convulsions (Doyle & Shaw, 1992). In the same study, anticonvulsants that work through sodium channel antagonism (phenytoin and carbamazepine) were only effective at very high doses.

In theory, a polyamine-like molecule should provide a suitable competitive antagonist. Doyle and Shaw (1998) investigated the effect of the putative polyamine antagonists (all polyamine analogues), arcaine, 1,10-diaminodecane and diethylenetriamine, on the spermine-induced behavioural changes. It was demonstrated that arcaine and 1,10-diaminodecane appeared to act as partial polyamine agonists and not as pure antagonists, inhibiting the second phase effects at low doses but causing potentiation at higher doses. However, diethylenetriamine was ineffective at inhibiting the second phase of effects.

The delay in onset of the second phase of spermine's action is a puzzle. It is possible that a polyamine metabolite, produced after the administration of spermine, causes the excitation and convulsions, hence the delay in onset. It is also possible that after administration of spermine a sequence/chain of events is initiated which culminates in overactivity in a number of systems resulting in convulsions.

As previously mentioned in Section 1.3.2, the NMDA receptor is almost certainly involved in some forms of epilepsy, as indicated by numerous studies. Moreau *et al.* (1989) demonstrated that the central administration of NMDA caused wild running, clonic convulsions and tonic seizures. They developed this model for the identification and classification of competitive and non-competitive NMDA antagonists. Himi *et al.* (1990) examined convulsions produced by the glutamate agonists, NMDA, quisqualate and kainate and the effect of Joro spider toxin (JSTX-3) on these. The intracerebroventricular injection of NMDA produced an almost immediate tonic convulsion depending on dose. Mathis and Ungerer (1992) made a comparative analysis of the seizures produced by the intracerebroventricular injection of NMDA, kainate and quisqualate in mice. They demonstrated that the seizures are different in each case due to different anatomical pathways. Finally, Turski *et al.* (1990) examined the effects of antiepileptic drugs on the seizures produced by intraventricular administration of excitatory amino acids, including NMDA.

NMDA-induced convulsions have also been examined after administration by the intraperitoneal route. Czuczwar & Meldrum (1982) first demonstrated that NMDA and NMDLA (N-methyl-DL-aspartate) produced severe convulsions in mice after i.p. injection, and also demonstrated its antagonism by 2-amino-7-phosphonoheptanoate, a competitive NMDA antagonist. Subsequently, Czuczwar and co-workers (1985) examined the antagonism, by antiepileptic agents, of NMDLA-induced convulsions. NMDLA was administered by either the subcutaneous or intravenous route. This work demonstrated the antagonism of NMDLA-induced convulsions through direct antagonism of the excitation, enhancement of GABA-mediated inhibition and activation of central α_2 -adrenoceptors.

Following on from these studies, a number of workers used the NMDA-induced convulsions to examine the effects of putative antagonists. Leander *et al.* (1988) used NMDA induced lethality to investigate the effects of phencyclidine-like drugs. They demonstrated that the NMDA induced lethality was antagonised by both phencyclidine-like drugs and NMDA receptor antagonists. Similarly, Ferkany *et al.* (1988) demonstrated the inhibition, by dextromethorphan and by a selection of competitive and non-competitive NMDA antagonists, of NMDA-induced convulsions.

The NMDA receptor is thought to be involved in the seizures following ethanol withdrawal after chronic ethanol treatment. This is thought to be due to up-regulation of the NMDA receptor. The NMDA involvement in the seizures was examined by Grant *et al.* (1990). They demonstrated that the non-competitive NMDA antagonist, MK-801, decreases the occurrence of ethanol withdrawal seizures whereas they are exacerbated by the administration of NMDA. This underlines the role of the NMDA receptor in these seizures.

It has been demonstrated that the polyamines, spermine and spermidine, enhance the binding of [³H]-MK-801 to the NMDA receptor, demonstrating the existence of a distinct polyamine site linked to the NMDA receptor macrocomplex (Ransom & Stec, 1988). This led to a number of investigations of the interaction of the polyamines with the NMDA receptor *in vivo*. Singh *et al.* (1990) investigated the ability of spermidine to modulate NMDLA-induced tonic seizures in mice. They showed that spermidine dose-dependently decreased the dose of NMDLA required to induce seizure activity in 50% of mice, demonstrating an interaction of the polyamine, spermidine, with the NMDA receptor. Matsumoto and co-workers (1993) examined the effect of ethanol treatment on polyamine-enhanced NMDA receptor activity. They demonstrated that spermidine administration by the icv route prior to ip administration of NMDA results in a reduction in latency to tonic-clonic convulsions and in a significant induction in *c-fos* expression, one of the immediate early genes. This was only susceptible to inhibition at high doses of ethanol demonstrating that the polyamine enhancement of NMDA receptor function is relatively insensitive to the

inhibitory effects of ethanol. Chu *et al.* (1994) examined the facilitation by the polyamines of NMDLA-induced seizures in mice. Both spermidine and spermine, but not putrescine, shortened the latency to production of clonic convulsions induced by NMDLA. They also demonstrated that N-(3-Aminopropyl)cyclohexylamine (APCHA), a spermine synthase inhibitor and polyamine antagonist, when co-administered with the spermidine antagonised the facilitatory effect of the polyamine.

Given the likely importance of the NMDA receptor macrocomplex to the epileptogenic action of spermine, it was decided to investigate the action of potential antagonists both against the direct effects of spermine and against its potentiation of NMDA-induced seizures. The direct effects of spermine administration *in vivo* were examined as described by Doyle and Shaw (1996 & 1998). A modification of the model used by Singh *et al.* (1990) was employed to investigate the potentiation of NMDA. Spermine was used rather than spermidine as, from earlier studies (Anderson *et al.*, 1975), spermine's action is more directly related to the production of convulsions. Thus, the spermine was administered icv before intraperitoneal doses of NMDA.

2.2 EXPERIMENTAL PROCEDURE

2.2.1 Direct spermine administration

The mice used were female *Laca* mice, weighing 20 – 25g and were obtained from the Bioresources Unit, Trinity College. The mice were housed in groups of 4 – 6 under a 12 hour light/dark cycle (light: 7am – 7pm) with food and water freely available. Spermine or saline (control) was administered into the left cerebral ventricle of the mouse by freehand injection as described by Brittain in 1966. The injection was made using a Hamilton microlitre syringe, which was calibrated to administer exactly 20µl. A 27-gauge needle was used on the syringe and was inserted to the required depth of 3mm. This depth was achieved by attaching a length of PVC tubing to the needle so it was only able to penetrate the skull to the correct depth. The injection was made 1mm to the left of the midline and 2mm rostral to a line joining the anterior base of the ears. It was possible to inject directly through the skull as, in mice of the weight range used, the point of injection on the skull is not completely calcified and a “soft” spot is present in the elective area. The microlitre syringe was held in a vertical position in the clamp of a retort stand and the mouse was raised up onto the needle and held in position on the needle while the solution was injected slowly. The mouse was then replaced in the cage and allowed to recover.

Spermine-induced behavioural changes

Directly after injection of spermine the mice were sedated, hypothermic and mildly anoxic. This was thought to be due to the injection process rather than the polyamines themselves as the same reaction was seen after administration of saline. Within a few minutes the first phase of the polyamine-induced behavioural changes commenced. This first phase consisted of scratching, grooming and face washing and occasionally, clonic convulsions. The face washing and scratching varied in intensity with the most intense, frenzied scratching or face washing manifested before a clonic convulsion. Therefore, the face washing was possibly a displacement reaction. This first phase of effects usually lasted for up to 40 minutes but could occasionally last for 1 – 2 hours.

The second phase of effects commenced after approximately 2 hours and manifested as a mild body tremor which increased in intensity with time culminating, within 8 hours, in a tonic convulsion. The second phase of effects was used for assessment as all antagonists tested to date blocked the first phase and also the majority of mice injected developed the second phase of effects whereas not all the first phase effects appeared in a consistent manner.

The method of assessment used was that of Doyle and Shaw (1996 & 1998). The tremor was assessed by lifting the mouse by the tail and feeling the degree of tremor. The tremor is initially very slight but, as the CNS excitation develops the tremor can be easily assessed and can even be seen visually. A scoring system was used for the assessment of the tremor and is shown below.

Scoring system:

1. Slight tremor.
2. Moderate tremor.
3. Severe tremor.
4. Tonic convulsion – survived.
5. Fatal tonic convulsion.

Only three grades of tremor were used so as to minimise the experimental error. These tremor values were recorded every 30 minutes from 2 hours after injection to the termination of the experiment 7.5 hours after injection.

2.2.2 Spermine enhanced NMDA-induced convulsions

The second experimental procedure involved the enhancement of NMDA-induced convulsions (Singh *et al.*, 1990). However, as previously mentioned (Section 2.1), spermine pretreatment was used rather than spermidine as spermine has a more pronounced CNS excitatory action. Female *Laca* mice, weighing 20 – 25g, were obtained from the Bioresources Unit, Trinity College and were housed 5 to a cage,

under a 12 hour light/dark cycle (light: 7am – 7pm), with food and water freely available.

A sub-convulsive dose of spermine (25µg) was administered by an intracerebroventricular injection, as detailed in Section 2.2.1, thirty minutes or three hours before an intraperitoneal injection of NMDA (i.e. active D enantiomer). The mice were then observed for thirty minutes after injection of the NMDA and the number of mice producing an extensor tonic convulsion recorded together with the latency to this convulsion. Those mice not producing a tonic convulsion within the thirty minutes were given a latency score of 30. Potential antagonists were either co-administered with the spermine or given by an intraperitoneal injection thirty minutes before spermine.

NMDA-induced behavioural changes

When given by the intraperitoneal route, NMDA produced a characteristic pattern of behaviour. The mice were initially sedated but over 5 minutes developed an abnormal gait, face washing and grooming, scratching, tail biting, jumping, wild running and clonic convulsions. Ultimately, at sufficiently high doses, a full extensor tonic convulsion was produced. This was used as the end point as it was noted that none of the other behavioural effects was consistently produced in every case. It was also noted that if the tonic convulsion had not been produced by the 30-minute cut-off point, it was not produced afterwards (based on observation for up to 2 hours after injection of NMDA).

All procedures underwent local ethical review and were carried out in accordance with guidelines laid down under the relevant legislation.

2.3 ANTAGONISTS

In the investigation of the direct effects of spermine three classes of drugs were used, the non-competitive NMDA antagonist, memantine, various calcium channel antagonists and a novel polyamine analogue, N¹-Dansylspermine. Memantine was used in these studies to confirm earlier findings with the NMDA antagonist, MK-801 (Doyle, 1993) and as further evidence of the role of the NMDA receptor in spermine's excitatory effects. Since calcium channels are involved in excitotoxicity and as a result may be involved in the CNS excitation produced by spermine, a variety of calcium channel antagonists were investigated. Finally, N¹-Dansylspermine is a novel polyamine analogue (Seiler *et al.*, 1998). It has a bulky dansyl residue attached to a polyamine moiety and has already been shown to stimulate the negative polyamine site (Chao *et al.*, 1997). Since a structure of this type may also possess polyamine antagonist activity, this was investigated in these studies.

Memantine was administered, by the intraperitoneal route, dissolved in 0.9% sterile saline. The Ca²⁺ channel antagonists used were verapamil, nisoldipine and nitrendipine and all were administered by the intraperitoneal route. Verapamil was dissolved in 0.9% sterile saline. Nisoldipine was dissolved in a solution of alcohol/saline (35:65) which was then diluted with 0.9% sterile saline (1 in 10), to yield a solution containing 3.5% alcohol. As a result the control used for Nisoldipine was a saline solution with 3.5% alcohol. Nitrendipine was dissolved in 0.2ml ethanol, placed in a sonic bath for 15 minutes and then added to 0.9% sterile saline to produce a 5% ethanol solution. Solubilisation was produced by adding the minimal amount of Tween 80 (2-3 drops). Due to their photosensitivity, the dihydropyridine calcium channel antagonists were protected from light at all times. N¹-Dansylspermine, dissolved in 0.9% saline, was co-administered with the spermine in the icv injection.

In order to examine the enhancing effects of spermine on NMDA-induced convulsions, a number of classes of compounds were again used. The calcium channel antagonist, nisoldipine, was administered (ip) in a 3.5% ethanol solution. It was of interest to examine ifenprodil and some of its analogues because of the well established polyamine/NMDA antagonist action of these substances. Ifenprodil,

eliprodil and CP-101,606 were dissolved in 0.9% sterile saline with the minimal amount of Tween 80 required to produce solubilisation and administered by the intraperitoneal route. N¹-Dansylspermine was dissolved in 0.9% saline and either co-administered with the spermine in the icv injection or given 30 minutes before spermine by the intraperitoneal route. The structures of these compounds and all compounds used are illustrated in Appendix 1.

2.4 TREATMENT OF RESULTS

In the investigation of the CNS excitatory effects of spermine, the median second phase CNS excitation scores of the spermine control group and test groups were calculated. The results were presented as graphs of the median CNS excitation scores versus time. Statistical significance of the difference between test and control subjects was calculated by the Mann-Whitney U-test. The effect of the drugs on the total number of mice reaching tonic convulsion was also assessed.

For the assessment of the effect of spermine on NMDA-induced convulsions, the ED₅₀ and 95% confidence intervals for the amount of NMDA required to produce convulsions was calculated. To calculate this, the guidelines set out by Weil (1952) were followed. The raw data was also analysed using Minitab. The data was analysed in a logistic regression model and also in a regression with censored life data model to assess statistically significant differences between groups. This test was devised with the assistance of Dr. M. O'Regan, Department of Statistics, Trinity College Dublin. Four test groups were required as detailed below.

	Time = -30	Time = 0	Time = +30min or +3hrs
Group 1:	Test drug (ip)	spermine (icv)	NMDA (ip)
Group 2:	Test drug (ip)	saline (icv)	NMDA (ip)
Group 3:	Vehicle (ip)	spermine (icv)	NMDA (ip)
Group 4:	Vehicle (ip)	saline (icv)	NMDA (ip)

Normally, only three groups were used. However, if there was a statistically significant difference as a result of the administration of the test compound on

spermine's effect on NMDA-induced convulsions, an extra group (No. 2) was required. This was necessary to show there was no difference between groups two and four. This would determine if the effect was due to polyamine or NMDA antagonism.

When the N¹-Dansylspermine was co-administered with the spermine three groups were required. These groups are shown below.

	Time=0	Time= +30min or +3hrs
Group 1	Spermine + N ¹ -Dansylspermine (icv)	NMDA (ip)
Group 2	Spermine + 0.9% saline (icv)	NMDA (ip)
Group 3	0.9% saline + N ¹ -Dansylspermine (icv)	NMDA (ip)

Statistical significance in all tests was accepted at the $p < 0.05$ level, with the actual p values shown on the Figures and Tables.

2.5 RESULTS

2.5.1 Direct administration of spermine

The direct administration of spermine (100 μ g), as described previously, produced the characteristic behavioural syndrome. This characteristic spermine-induced effect is quantified in Figure 2.1. It can be seen that the median excitation score for spermine increases with time and by 6.5 hours after icv injection the median score has reached a maximum score of 5, which represents a fatal tonic convulsion.

2.5.1.1 Memantine

The noncompetitive NMDA antagonist, memantine, was examined for any effect on the spermine induced CNS excitation. The noncompetitive NMDA antagonist, MK-801, has previously been examined in this laboratory (Doyle, 1993) and was demonstrated to have a marked effect on the development of spermine induced CNS excitation, but only when administered thirty minutes before and thirty minutes after spermine, and not as a single dose. Thus, memantine was examined both as a single and double dose. Memantine (15mg/kg) was administered by the intraperitoneal route either 30 minutes before spermine or thirty minutes before and after spermine.

As can be seen in Figure 2.1 and Table 2.1, a single dose of memantine (15mg/kg) had no effect on the development of CNS excitation when administered thirty minutes before spermine. Indeed, the development of CNS excitation for memantine was slightly increased relative to the control but this is not statistically significant and both reach a median score of 5 after 6 hours.

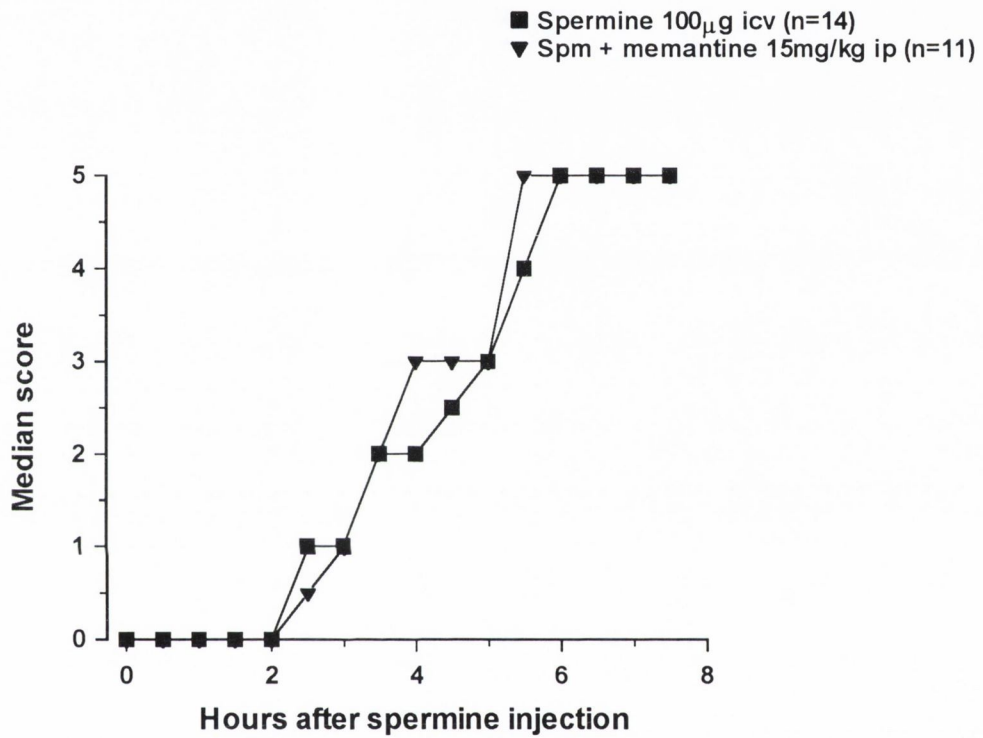


Figure 2.1: Effect of Memantine 15mg/kg (thirty minutes before spermine) on median CNS excitation score.

Table 2.1: The effect of memantine on median CNS excitation scores (plain text) and interquartile ranges (IQR) (italic) recorded over the time range in spermine convulsion experiments. Test groups have been compared to spermine control group for statistical significant differences at individual time points using the Mann-Whitney U-test (**p<0.01).

Drugs:	0.0 hr	0.5 hr	1.0 hr	1.5 hr	2.0 hr	2.5 hr	3.0 hr	3.5 hr	4.0 hr	4.5 hr	5.0 hr	5.5 hr	6.0 hr	6.5 hr	7.0 hr	7.5 hr
Control Spermine	0.0	0.0	0.0	0.0	0.0	1.0	1.0	2.0	2.0	2.5	3.0	4.0	5.0	5.0	5.0	5.0
100µg icv	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>1.0</i>	<i>0.75</i>	<i>1.75</i>	<i>1.75</i>	<i>1.0</i>	<i>3.0</i>	<i>2.75</i>	<i>2.0</i>	<i>2.0</i>	<i>2.0</i>	<i>2.0</i>
Memantine 15mg/kg 30 min before Spm	0.0 <i>0.0</i>	0.0 <i>0.0</i>	0.0 <i>0.0</i>	0.0 <i>0.0</i>	0.0 <i>0.0</i>	0.5 <i>1.0</i>	1.0 <i>1.5</i>	2.0 <i>1.0</i>	3.0 <i>1.0</i>	3.0 <i>0.5</i>	3.0 <i>2.0</i>	5.0 <i>2.0</i>	5.0 <i>1.0</i>	5.0 <i>1.0</i>	5.0 <i>2.0</i>	5.0 <i>2.0</i>
Control Spermine	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0	2.0	2.0	3.0	3.0	3.0	3.0	4.0	5.0
100µg icv	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>1.0</i>	<i>2.0</i>	<i>3.0</i>	<i>2.0</i>	<i>4.0</i>	<i>4.0</i>	<i>4.0</i>	<i>4.0</i>	<i>4.0</i>	<i>3.0</i>	<i>3.0</i>
Memantine 30 min before and after Spm	0.0 <i>0.0</i>	0.0 <i>0.0</i>	0.0 <i>0.0</i>	0.0 <i>0.0</i>	0.0 <i>0.0</i>	0.0 <i>1.0</i>	0.0 <i>1.0</i>	1.0 <i>1.0</i>	1.0 <i>1.0</i>	2.0 <i>2.0</i>	2.0 <i>2.0</i>	2.5 <i>3.0</i>	3.0 <i>4.0</i>	3.0 <i>4.0</i>	3.0** <i>4.0</i>	3.0** <i>4.0</i>

However, when the memantine is administered both before and after spermine there is a significant reduction in the development of CNS excitation. As can be seen in Figure 2.2, there is a statistically significant difference between the control score and the memantine score at time points 7 and 7.5 hours. Ultimately, 7.5 hours after the injection of spermine the control score has reached a value of 5 whereas the treated score has only reached a median value of 3. This difference is statistically significant at the $p < 0.01$ level. This is also reflected in Table 2.2, which shows that two doses of 15mg/kg memantine resulted in a statistically significant reduction in the number of mice undergoing tonic convulsions by 7.5 hours. The lack of effect of the single dose of memantine on the number of animals undergoing tonic convulsions is also illustrated in Table 2.2.

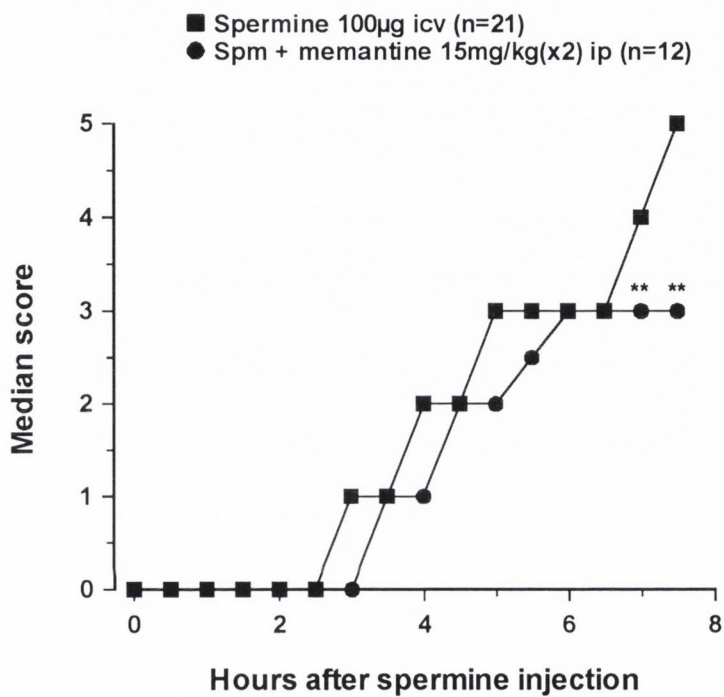


Figure 2.2: Effect of Memantine 15mg/kg (thirty minutes before and thirty minutes after spermine) on median CNS excitation score. $**p < 0.01$ Vs spermine control (Mann-Whitney U-test).

Table 2.2: The effect of memantine on the percentage of mice developing tonic convulsions within 7.5 hours of spermine injection. Statistical significance was assessed using a proportionality test (*Primer of Biostatistics, McGraw-Hill, 1992*)(* $p < 0.1$).

Drug treatment	n	% of animals showing tonic convulsions by 7.5 hours after spermine injection
Spermine 100 μ g (icv) + saline (ip, -30min)	14	64.3
Spm + memantine 15mg/kg (ip, -30min)	11	81.8
Spm + saline (ip, 30min before and 30min after)	21	57
Spm + memantine 15mg/kg (ip, 30min before and 30min after)	12	16.7*

2.5.1.2 Calcium channel antagonists

Figure 2.3 and Table 2.3 show the effect of administration of verapamil, 20mg/kg, by the intraperitoneal route, thirty minutes before direct cerebroventricular administration of spermine. As can be seen, the CNS excitation develops almost in tandem with that from spermine alone but there is a small reduction in the spermine-induced CNS excitation at three time points and the differences are statistically significant. From Table 2.4, it can be seen that there is a small difference between spermine and verapamil for the total number of mice showing tonic convulsions by 7.5 hours. However, this difference is not statistically significant.

Figure 2.4 and Table 2.3 show the effect of nisoldipine, 2mg/kg, given by the intraperitoneal route, thirty minutes before spermine, on the development of the CNS excitation. The spermine-induced CNS excitation develops, as before, up to a maximum score of 5. The median score for nisoldipine treated animals, however, does not increase as rapidly as that of spermine, representing a delayed development of the CNS excitation. The maximum score reached is 3.5. This demonstrates that nisoldipine has an effect on the development of CNS excitation. However, there is only a statistically significant difference at one point (6.5 hours).

As with verapamil, there is a difference between spermine and spermine with nisoldipine for the number of mice showing a tonic convulsion at 7.5 hours after injection. As can be seen in Table 2.4, there is a difference of 12.5% between the number of mice showing tonic convulsions for each treatment. However, this difference is not statistically significant. The control value for nisoldipine is lower than either of the other controls. Even though this is most likely due to the small numbers examined the nisoldipine test group was compared to the spermine with saline group for completeness. However, no statistically significant difference was found.

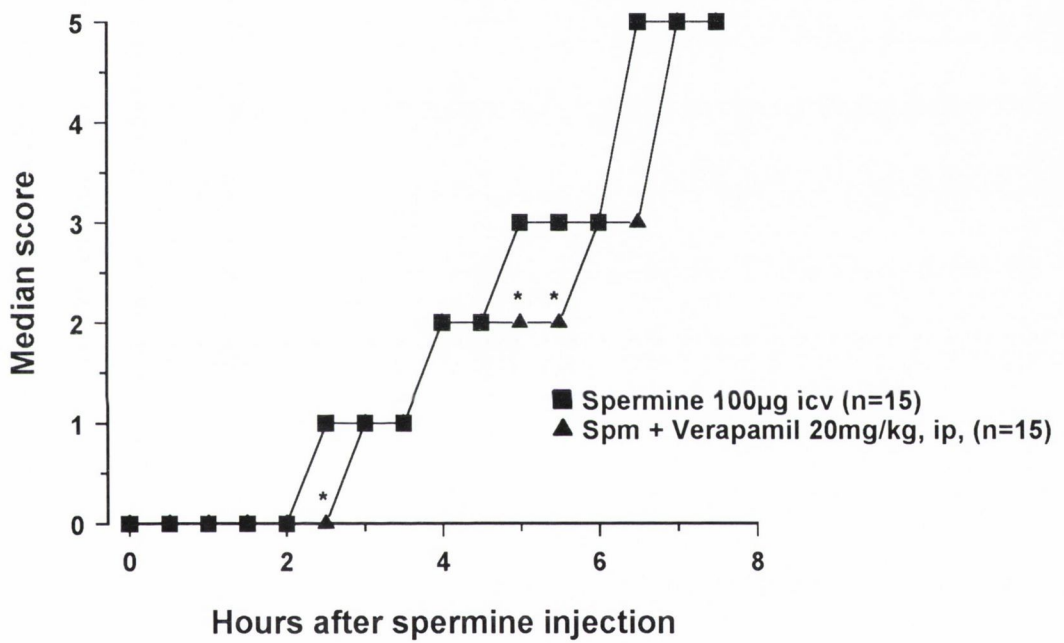


Figure 2.3: The effect of verapamil 20mg/kg on median CNS excitation score.
 * $p < 0.05$ Vs spermine control (Mann-Whitney U-test).

Table 2.3 : The effect of various calcium channel antagonists on median CNS excitation scores (plain text) and interquartile ranges (IQR) (italic) recorded over the time range in spermine convulsion experiments. Test groups have been compared to spermine control group for statistical significant differences at individual time points using the Mann-Whitney U-test (* $p < 0.05$, ** $p < 0.01$).

Drugs:	0.0 hr	0.5 hr	1.0 hr	1.5 hr	2.0 hr	2.5 hr	3.0 hr	3.5 hr	4.0 hr	4.5 hr	5.0 hr	5.5 hr	6.0 hr	6.5 hr	7.0 hr	7.5 hr
Spermine 100 μ g (icv)	0.0	0.0	0.0	0.0	0.0	1.0	1.0	1.0	2.0	2.0	3.0	3.0	3.0	5.0	5.0	5.0
+ saline (ip)	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>1.0</i>	<i>0.0</i>	<i>0.0</i>	<i>1.0</i>	<i>0.0</i>	<i>0.5</i>	<i>1.0</i>	<i>2.5</i>	<i>2.0</i>	<i>2.0</i>	<i>1.0</i>	<i>1.0</i>
Spm + verapamil	0.0	0.0	0.0	0.0	0.0	0.0*	1.0	1.0	2.0	2.0	2.0*	2.0*	3.0	3.0	5.0	5.0
20mg/kg (ip)	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.5</i>	<i>0.5</i>	<i>0.0</i>	<i>1.0</i>	<i>1.0</i>	<i>1.5</i>	<i>1.0</i>	<i>3.0</i>	<i>3.0</i>	<i>3.0</i>	<i>2.5</i>
Spm + nisoldipine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0	2.0	2.5	2.5	3.0	4.0	4.0	5.0
vehicle (ip)	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.25</i>	<i>0.25</i>	<i>0.25</i>	<i>1.0</i>	<i>2.0</i>	<i>1.25</i>	<i>1.25</i>	<i>1.5</i>	<i>2.0</i>	<i>2.0</i>	<i>2.0</i>
Spm + nisoldipine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	1.0	1.0	1.5	2.0	2.0	2.5*	3.0	3.5
2mg/kg (ip)	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.75</i>	<i>1.0</i>	<i>0.75</i>	<i>1.0</i>	<i>1.0</i>	<i>1.0</i>	<i>1.75</i>	<i>1.0</i>	<i>1.0</i>	<i>2.75</i>
Spm + nitrendipine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0	1.0	1.0	2.0	3.0	5.0	5.0	5.0
Vehicle (ip)	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.5</i>	<i>1.0</i>	<i>1.5</i>	<i>0.5</i>	<i>1.0</i>	<i>1.0</i>	<i>1.5</i>	<i>1.5</i>	<i>2.0</i>	<i>1.5</i>	<i>1.5</i>
Spm + nitrendipine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0*	0.5*	0.5*	1.0*	1.0*	1.0**	1.0*	1.0*
15mg/kg (ip)	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>1.0</i>	<i>1.0</i>	<i>1.0</i>	<i>1.0</i>	<i>1.0</i>	<i>1.0</i>	<i>1.0</i>	<i>1.5</i>	<i>1.75</i>

Table 2.4: The effect of various calcium channel antagonists on the percentage of mice developing tonic convulsions within 7.5 hours of spermine injection. Statistical significance was assessed using a proportionality test (Primer of Biostatistics, McGraw-Hill, 1992)(* $p < 0.05$).

Drug treatment	n	% of animals showing tonic convulsions by 7.5 hours after spermine injection
Spermine 100 μ g (icv) + saline (ip)	15	73.3
Spm + verapamil 20mg/kg (ip)	15	66
Spm + nisoldipine vehicle (ip)	8	62.5
Spm + nisoldipine 2mg/kg (ip)	14	50
Spm + nitrendipine Vehicle (ip)	7	71
Spm + nitrendipine 15mg/kg (ip)	14	14*

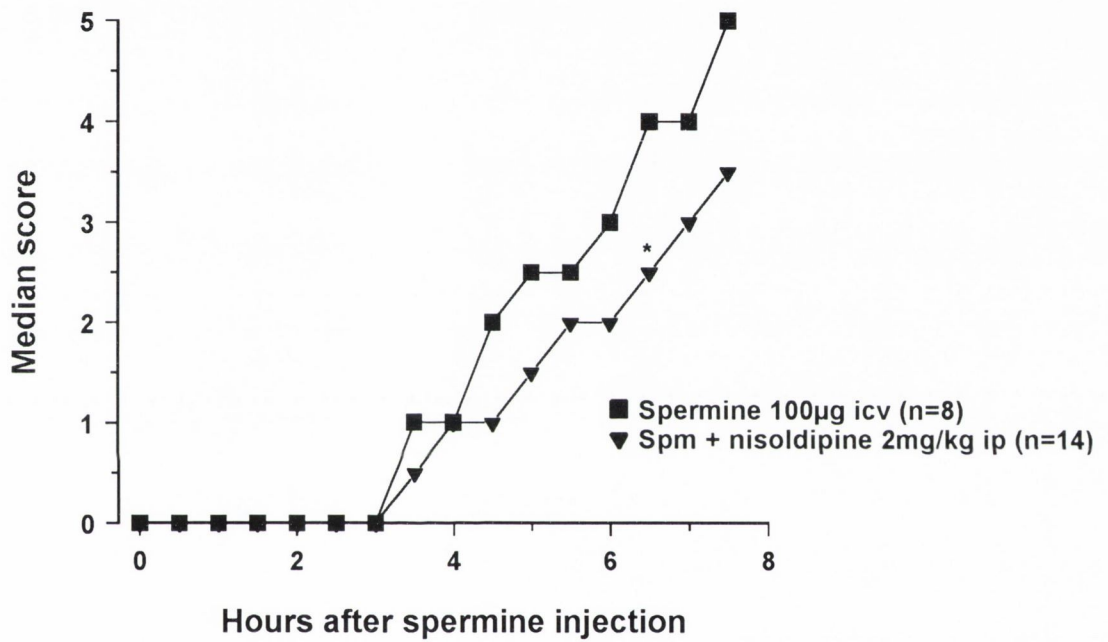


Figure 2.4: Effect of nisoldipine 2mg/kg on median CNS excitation score. * $p < 0.05$ Vs spermine control (Mann-Whitney U-test).

The effect of Nitrendipine, 15mg/kg, by the intraperitoneal route, thirty minutes before spermine is illustrated in Figure 2.5 and Table 2.3. There was a substantial reduction in development of CNS excitation when nitrendipine was administered before spermine. In the nitrendipine group, the CNS excitation induced by spermine only becomes apparent after 4.5 hours and only increases to a maximum median score of 1 representing a mild tremor. This difference is statistically significant at all points from 4 hours onwards. Indeed at 6.5 hours the difference is statistically significant at $p < 0.01$.

Accordingly, the number of animals reaching the tonic convulsion stage was greatly reduced. The percentage of spermine controls showing a tonic convulsion after 7.5 hours was 71% whereas in the nitrendipine and spermine group only 14% reached this stage. This difference is statistically significant ($p < 0.05$).

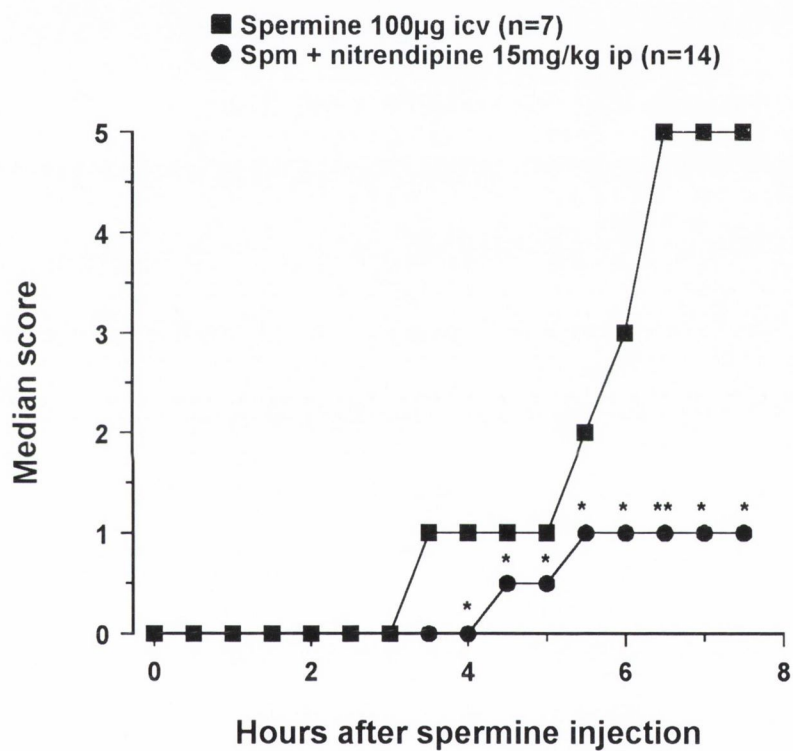


Figure 2.5: Effect of Nitrendipine 15mg/kg on median CNS excitation score.
 * $p < 0.05$, ** $p < 0.01$ Vs spermine control (Mann-Whitney U-test).

2.5.1.3 *N*¹-dansylspermine

Figure 2.6 and Table 2.5 show the effect of co-administration of *N*¹-dansylspermine, at various doses, with spermine. *N*¹-dansylspermine (2µg) appears to produce a slight but statistically insignificant reduction in the development of CNS excitation caused by spermine.

At the higher dose of 5µg, *N*¹-dansylspermine, causes a notable reduction in the development of CNS excitation. This is seen in Figure 2.6 by the levelling off of the median score at a value of 1 only rising to 2, 7.5 hours after injection of spermine. This difference is statistically significant. A similar pattern is also seen at the dose of 10µg and in this case the median score reached at 7.5 hours after injection was 1, compared to 5 in the case of the spermine control. Yet again, as for 5µg *N*¹-dansylspermine, this difference is statistically significant.

These findings are also illustrated in Table 2.6. Here it is shown that both 5µg and 10µg *N*¹-dansylspermine cause a statistically significant reduction in the number of mice showing tonic convulsions by 7.5 hours. Indeed the value for 10µg *N*¹-dansylspermine is significant at the 99.9% level.

*N*¹-dansylspermine has previously been shown to have mild effects on behaviour when administered by the intracerebroventricular route (S. Ryder, in preparation). *N*¹-dansylspermine (10µg, icv) caused a decrease in locomotor activity 5 and 10 minutes after administration when compared with saline control but the levels recovered to those of the control thereafter. *N*¹-dansylspermine (20µg, icv) caused a more marked and prolonged reduction in locomotor activity though this did not reach statistical significance. Similar effects were seen on temperature measurements. *N*¹-dansylspermine (10µg) produced mild hypothermia (35.35°C) whereas 20µg *N*¹-dansylspermine produced marked hypothermia (34.17°C) though these differences did not reach statistical significance.

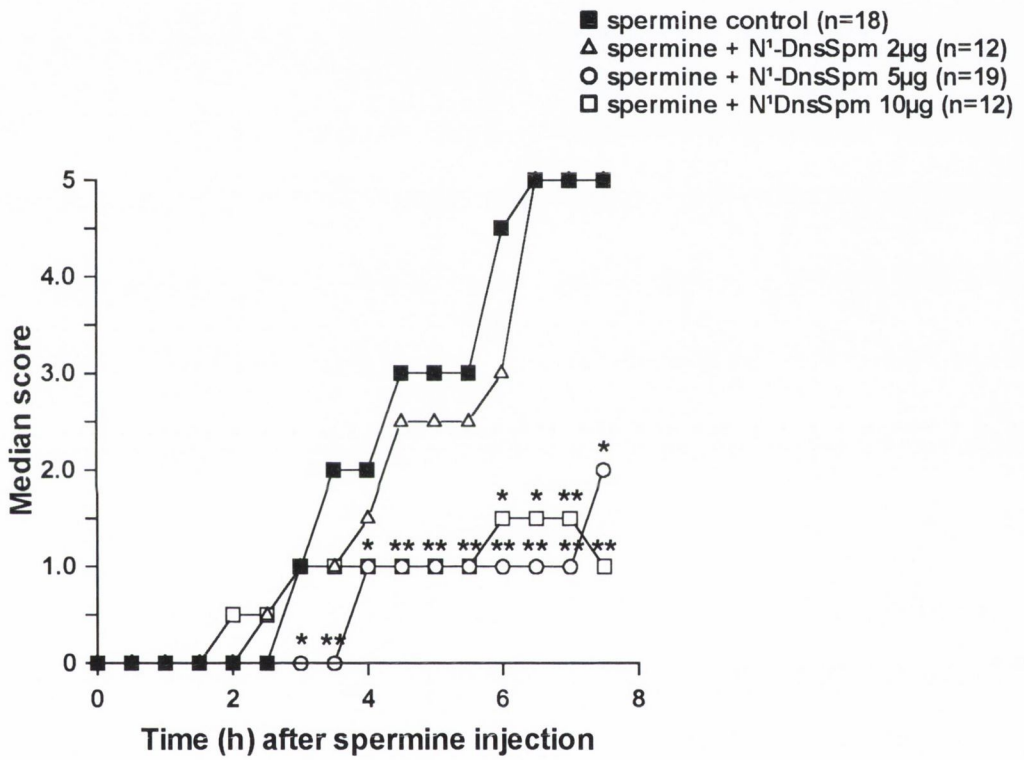


Figure 2.6: The effect of N¹-dansylspermine on the spermine induced CNS excitation.
 * $p < 0.01$; ** $p < 0.001$ (Mann-Whitney U-test).

Table 2.5 : The effect of N^1 -dansylspermine on median CNS excitation scores (plain text) and interquartile ranges (IQR) (italic) recorded over the time range in spermine convulsion experiments. Test groups have been compared to spermine control group for statistical significant differences at individual time points using the Mann-Whitney U-test (* $p < 0.01$, ** $p < 0.001$).

Drugs:	0.0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5
	hr	hr	hr	hr	hr	hr	hr	hr	hr	hr	hr	hr	hr	hr	hr	hr
Spermine 100 μ g	0.0	0.0	0.0	0.0	0.0	0.0	1.0	2.0	2.0	3.0	3.0	3.0	4.5	5.0	5.0	5.0
Icv	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>1.0</i>	<i>0.5</i>	<i>1.25</i>	<i>1.25</i>	<i>2.0</i>	<i>2.0</i>	<i>3.0</i>	<i>3.0</i>	<i>3.0</i>	<i>3.0</i>	<i>3.0</i>
Spm + N^1 -DnsSpm	0.0	0.0	0.0	0.0	0.0	0.5	1.0	1.0	1.5	2.5	2.5	2.5	3.0	5.0	5.0	5.0
2 μ g	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>1.0</i>	<i>1.0</i>	<i>2.75</i>	<i>2.0</i>	<i>3.25</i>	<i>2.5</i>	<i>2.5</i>	<i>3.0</i>	<i>2.75</i>	<i>2.25</i>	<i>2.25</i>
Spm + N^1 -DnsSpm	0.0	0.0	0.0	0.0	0.0	0.0	0.0*	0.0**	1.0**	1.0**	1.0**	1.0**	1.0**	1.0**	1.0**	2.0*
5 μ g	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>1.0</i>	<i>1.0</i>	<i>1.0</i>	<i>2.0</i>	<i>2.0</i>	<i>3.0</i>	<i>3.0</i>	<i>2.0</i>	<i>2.0</i>	<i>4.0</i>
Spm + N^1 -DnsSpm	0.0	0.0	0.0	0.0	0.5	0.5	1.0	1.0	1.0*	1.0**	1.0**	1.0**	1.5*	1.5*	1.5**	1.0**
10 μ g	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>1.0</i>	<i>1.0</i>	<i>0.75</i>	<i>1.0</i>	<i>0.75</i>	<i>1.5</i>	<i>1.0</i>	<i>1.0</i>	<i>1.0</i>	<i>1.0</i>	<i>1.0</i>	<i>1.75</i>

Table 2.6: The effect of N¹-dansylspermine on the percentage of mice developing tonic convulsions within 7.5 hours of spermine injection. Statistical significance was assessed using a proportionality test (*Primer of Biostatistics, McGraw-Hill, 1992*)(* $p < 0.1$; *** $p < 0.01$).

Drug treatment	n	% of animals showing tonic convulsions by 7.5 hours after spermine injection
Spermine 100 μ g (icv)	18	72.2
Spm + 2 μ g N ¹ -DnsSpm (icv)	12	75
Spm + 5 μ g N ¹ -DnsSpm (icv)	19	36.8*
Spm + 10 μ g N ¹ -DnsSpm (icv)	12	16.7***

2.5.2 Spermine enhanced NMDA-induced convulsions

Spermine (25 μ g) was administered, by the intracerebroventricular route, either thirty minutes or three hours before NMDA, and a dose response curve to NMDA produced. Table 2.7 shows the effect of these administrations of spermine on the ED₅₀ for NMDA induced convulsions. In animals, which received saline (icv), the ED₅₀ for NMDA induced convulsions was 118.9mg/kg. When spermine (25 μ g, icv) was administered 30 minutes before NMDA the resulting ED₅₀ was 113.7mg/kg. This small difference was not statistically significant. However, when spermine (25 μ g, icv) was administered 3hours before the NMDA there was a highly statistically significant ($p < 0.001$) reduction in the ED₅₀ for NMDA to 13.57 mg/kg.

The effect on latencies to first tonic convulsion can be seen in Figure 2.7. When spermine was given 30 minutes before the NMDA there was no significant change in the log dose-latency curve. However, when spermine was administered 3 hours before the NMDA there was a large shift in the curve to the left representing a reduced latency to tonic convulsion.

Table 2.7: The effect of time of administration of spermine on the ED₅₀ for NMDA-induced convulsions. *** $p < 0.001$ Vs Saline control, Binary logistic regression.

Drug	Dose	NMDA ED ₅₀ (mg/kg)	95% Confidence Intervals (mg/kg)
Saline control (n=40)	20µl icv	118.9	96.7 – 146.3
Spermine (n=40) 30 min before NMDA	25µg icv	113.7	90.27 – 143.21
Spermine (n=20) 3hrs before NMDA	25µg icv	13.57***	8.4 – 21.9

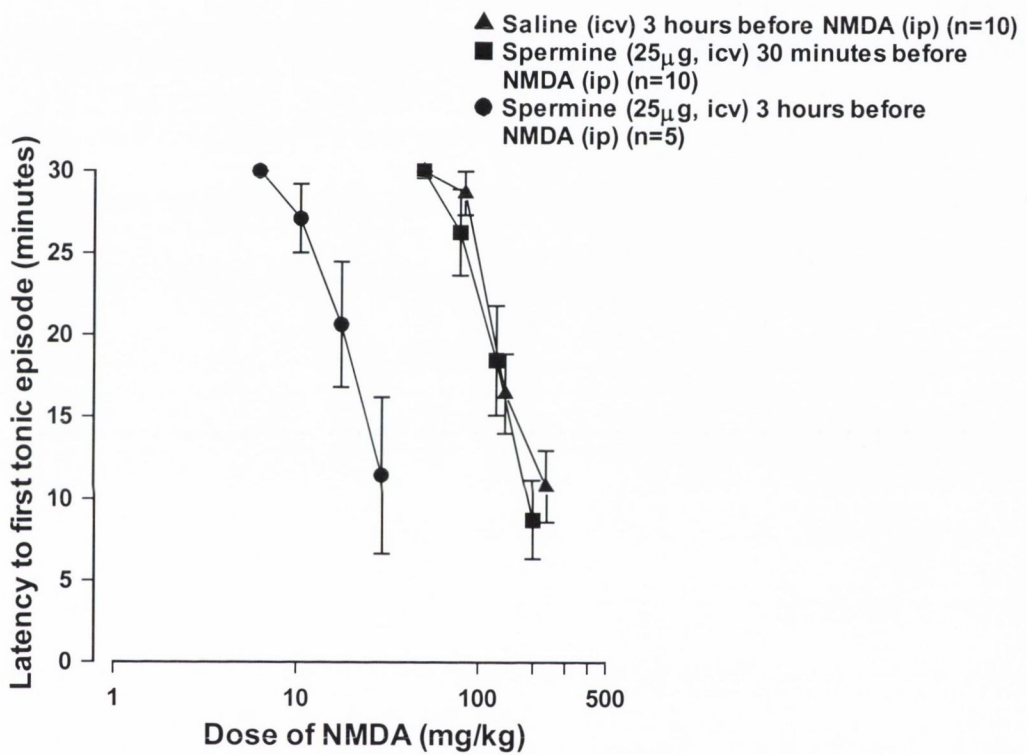


Figure 2.7: The effect of time of administration of spermine (25 μ g, icv) on the latency to first tonic convulsion induced by NMDA (ip). Each point represents a mean \pm standard error of the mean (SEM).

2.5.2.1 Nisoldipine

Nisoldipine, a Ca^{2+} channel antagonist, was investigated at a dose of 2mg/kg, by the intraperitoneal route, thirty minutes before administration of spermine (icv), NMDA was then administered (ip) three hours after spermine. From Table 2.8, it can be seen that there is little difference between the ED_{50} values for NMDA for either the spermine or spermine and nisoldipine treatments (14.03 mg/kg compared to 14.55 mg/kg). This slight difference is not statistically significant. Similarly, from Figure 2.8, it can be seen that nisoldipine has no effect on the latency to first tonic episode. The familiar left shift was seen as a result of administration of spermine. However, this was not reversed by the administration of nisoldipine thirty minutes before spermine.

Table 2.8 : The effect of Nisoldipine, 2 mg/kg, on the ED₅₀ for spermine enhancement of NMDA-induced convulsions.

Drug	Dose	NMDA ED ₅₀ (mg/kg)	95% confidence interval (mg/kg)
Saline + 3.5% EtOH (-30min), saline (0), NMDA (3hrs) (n=40)	0.1ml/10g ip 20µl icv	100	76.37 – 130.94
Saline + 3.5% EtOH (-30min), spermine (0), NMDA (3hrs) (n=40)	0.1ml/10g ip 25µg icv	14.03	10.61 – 18.56
Nisoldipine (-30min), spermine (0), NMDA (3hrs) (n=40)	2mg/kg ip 25µg icv	14.55	11.25 – 18.8

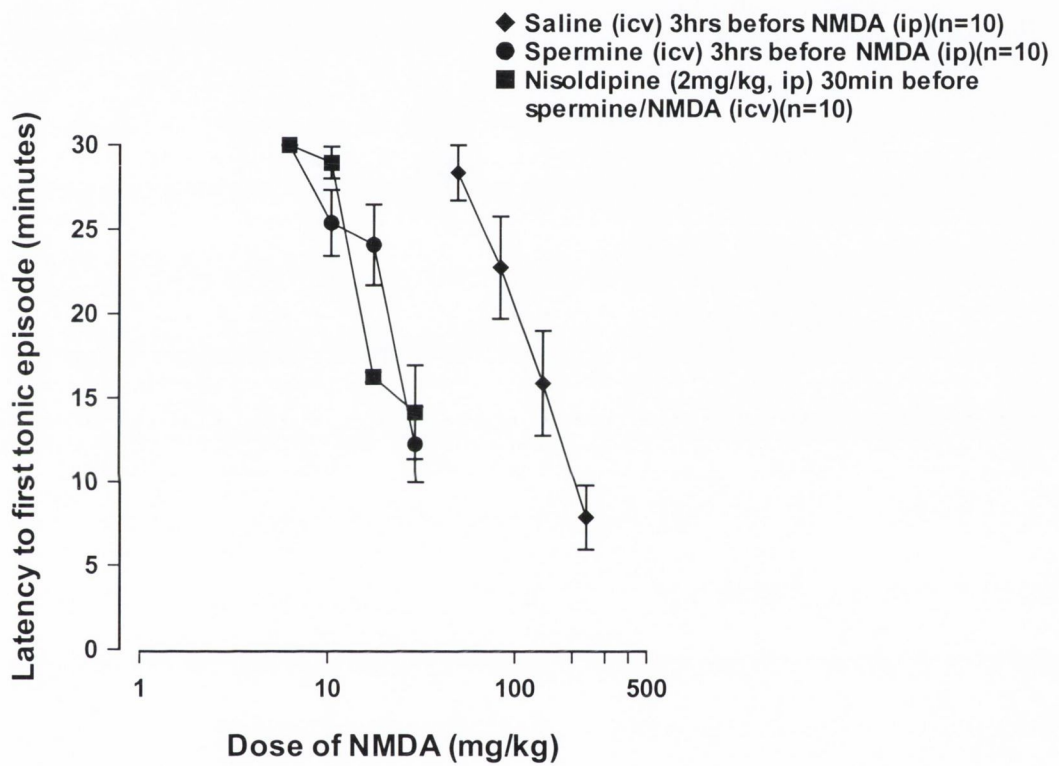


Figure 2.8: The effect of administration of Nisoldipine (2mg/kg, ip) thirty minutes before spermine (25 μ g, icv) on the spermine enhancement of NMDA-induced convulsions. Each point represents mean \pm SEM.

2.5.2.2 Ifenprodil and its analogues

Ifenprodil, eliprodil and CP-101,606 are all related phenylethanolamines with polyamine antagonist activity. Doyle and Shaw (Doyle & Shaw, 1996; Doyle, 1993) previously demonstrated the polyamine antagonist activity of high doses of ifenprodil and eliprodil in the spermine-induced CNS excitation model. Therefore, it was of interest to investigate them in this experimental model. Ifenprodil was administered by the intraperitoneal route, thirty minutes before spermine (icv), at a dose of 30 mg/kg. Similarly, eliprodil was administered by the intraperitoneal route at doses of 30 mg/kg and 60 mg/kg and CP-101,606 at a dose of 30mg/kg.

The results of these experiments are shown in Table 2.9 and Figures 2.9, 2.10, 2.11 & 2.12. As can be seen, ifenprodil (30mg/kg) had no effect on either the ED₅₀ value for NMDA-induced convulsions or on the latency to first tonic episode. As would be expected there was the normal shift to the left for the NMDA doses to produce tonic episodes after the administration of spermine. This, however, was not reversed after pretreatment with ifenprodil.

Eliprodil was given at two doses, 30 or 60 mg/kg, thirty minutes before the spermine. As can be seen from Table 2.9 and Figure 2.10, the lower dose of eliprodil had no effect on the ED₅₀ value for NMDA-induced convulsions. There was no statistically significant difference between the two values produced, 13.57mg/kg for spermine compared to 20.13mg/kg after administration of eliprodil (30mg/kg). However, after administration of eliprodil (60 mg/kg) there was a statistically significant difference between the ED₅₀ values produced, 13.57mg/kg for spermine alone compared to 29.73mg/kg for spermine with eliprodil pretreatment. This difference can also be seen in Figure 2.11, which shows the effect of eliprodil on the latency to the first tonic episode.

As a result of the significant effect on the ED₅₀ value for spermine enhanced NMDA induced convulsions, eliprodil (60mg/kg) was examined for NMDA antagonist activity in this model. Eliprodil was administered (ip) 30 minutes before saline (20µl,

icv), that was given 3 hours before NMDA. The result in this case can be seen in Table 2.9. The ED₅₀ value for NMDA induced convulsions produced in eliprodil treated animals was 109.05 mg/kg which was not statistically significantly different from the saline and Tween 80 control (118.9 mg/kg). This demonstrates that the effect of eliprodil was not exerted by a direct action on NMDA-induced convulsions. Eliprodil has been demonstrated in many other models to have NMDA antagonist activity.

Like eliprodil, CP-101,606 had a significant effect on the ED₅₀ value for spermine enhancement of NMDA induced convulsions, without antagonising NMDA alone. The ED₅₀ value for spermine enhanced NMDA induced convulsions after the administration of CP-101,606 was 43.9 mg/kg which was statistically significantly different when compared to 13.57 mg/kg for spermine alone. This can also be seen in Figure 2.12. A shift to the right, away from spermine alone, in the latency curve for CP-101,606 could be seen representing a reversal of the spermine enhancement.

Table 2.9 : The effect of ifenprodil and its analogues on the ED₅₀ for spermine enhancement of NMDA-induced convulsions. ** $p < 0.01$ Vs Spermine icv control, Binary logistic regression.

Drug	Dose	NMDA ED ₅₀ (mg/kg)	95% confidence interval (mg/kg)
Saline + Tween80 (-30min), saline (0), NMDA (3hrs) (n=40)	0.1ml/10g ip 20µl icv	118.9	93 – 152.3
Saline + Tween80 (-30min), spermine (0), NMDA (3hrs) (n=20)	25µg icv	15.75	12.15 – 20.4
Ifn. (-30min), spermine (0), NMDA (3hrs) (n=40)	30mg/kg ip 25µg icv	20.14	14.75 – 27.5
Elip. (-30min), spermine (0), NMDA (3hrs) (n=40)	30mg/kg ip 25µg icv	20.1	15.3 – 26.5
Elip. (-30min), spermine (0), NMDA (3hrs) (n=40)	60mg/kg ip 25µg icv	29.73**	21.2 – 41.7
Elip. (-30min) saline (0), NMDA (3hrs) (n=40)	60mg/kg ip 20µl icv	109.05	81.03 – 146.78
CP-101,606 (-30min), spermine (0), NMDA (3hrs) (n=40)	30mg/kg ip 25µg icv	43.9**	21.4 – 89.95
CP-101,606 (-30min), saline (0), NMDA (3hrs) (n=20)	30mg/kg ip 20µl icv	132.52	93.8 – 187.2

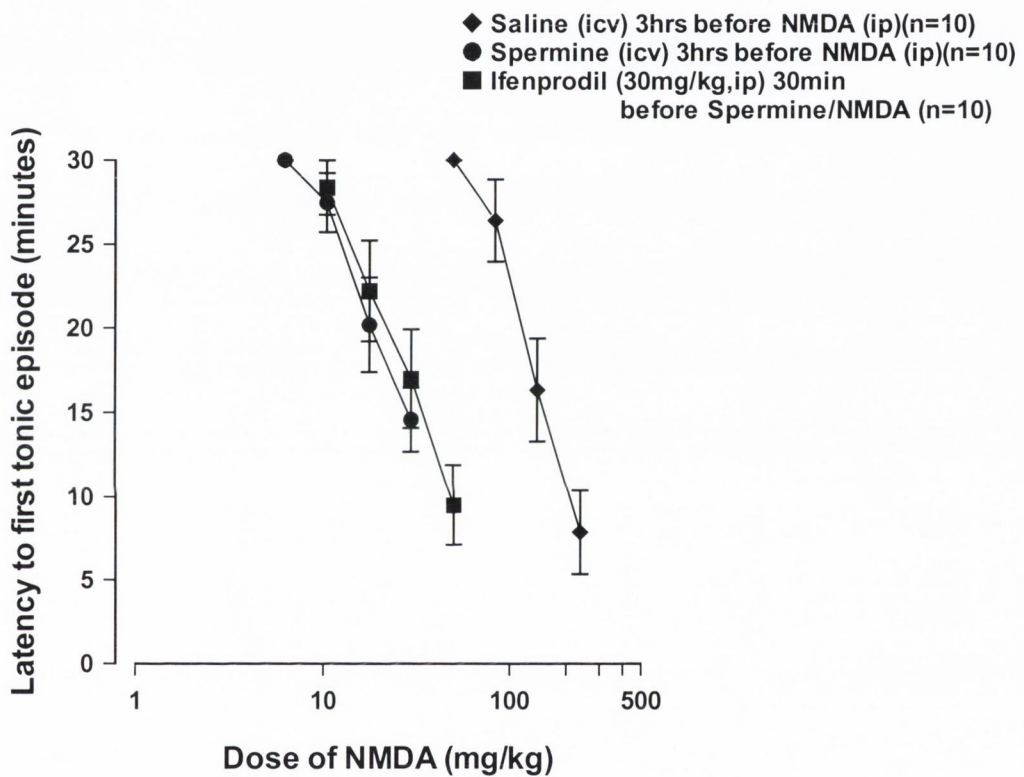


Figure 2.9: The effect of administration of ifenprodil (30mg/kg) thirty minutes before spermine (25 μ g, icv) on the spermine enhancement of NMDA-induced convulsions. Each point represents mean \pm SEM.

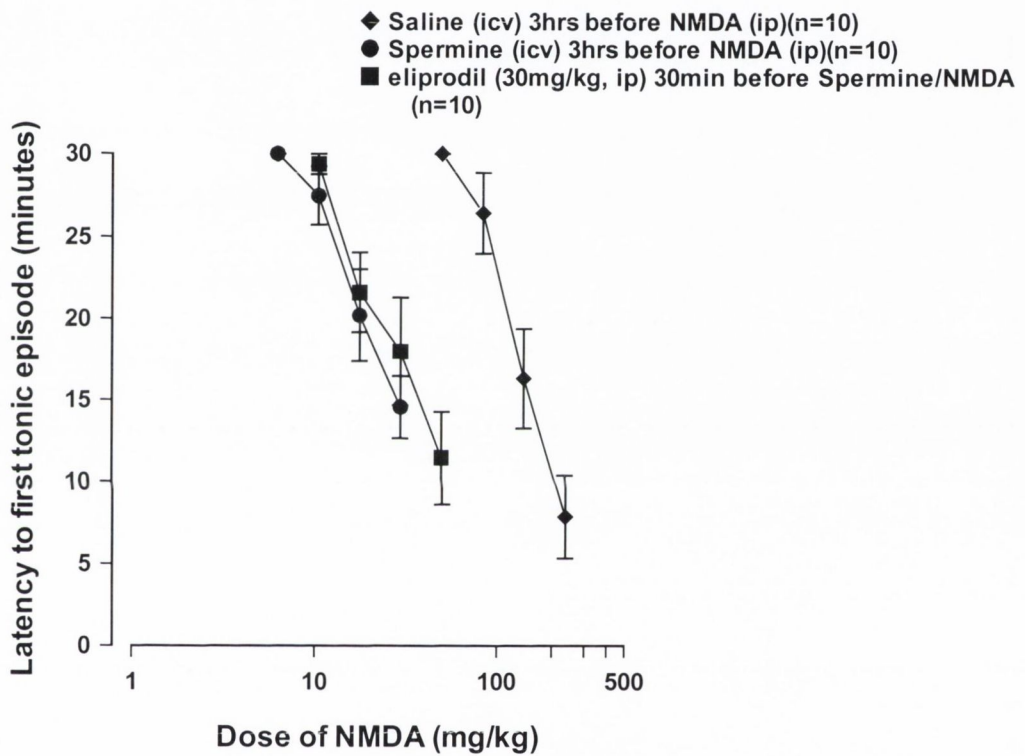


Figure 2.10: The effect of administration of eliprodil (30mg/kg) thirty minutes before spermine (25 μ g, icv) on the spermine enhancement of NMDA-induced convulsions. Each point represents mean \pm SEM.

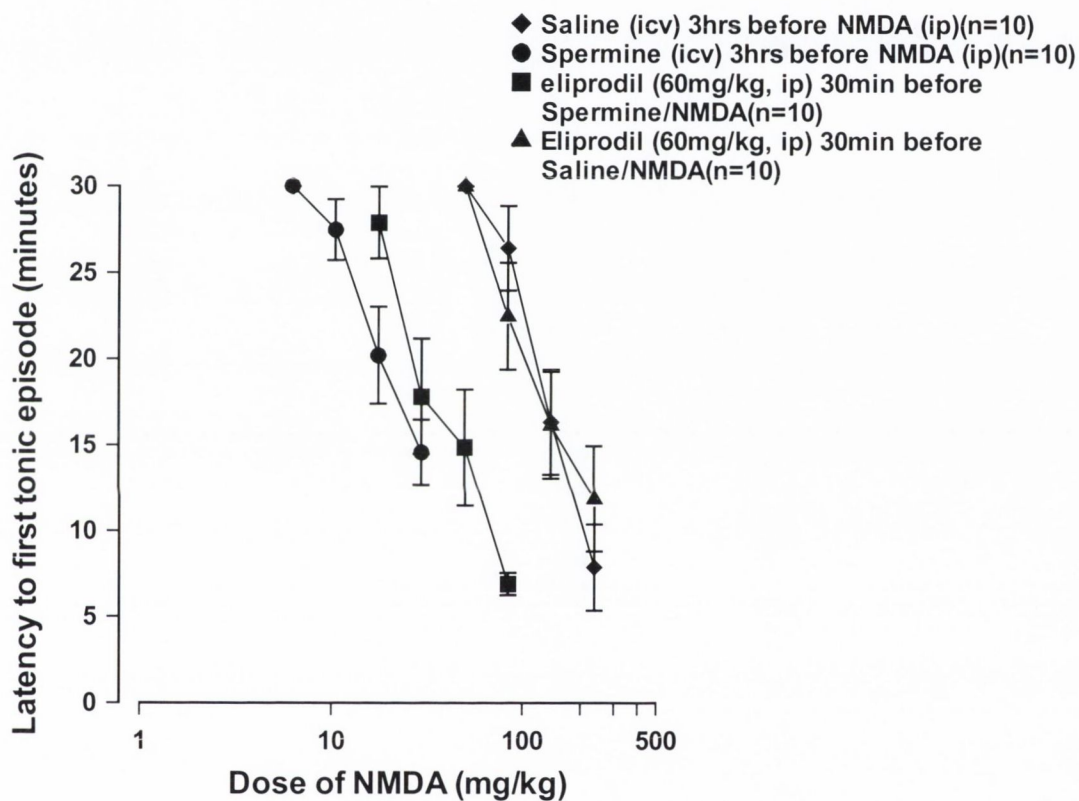


Figure 2.11: The effect of administration of eliprodil (60mg/kg) thirty minutes before spermine (25 μ g, icv) on the spermine enhancement of NMDA-induced convulsions. Each point represents mean \pm SEM.

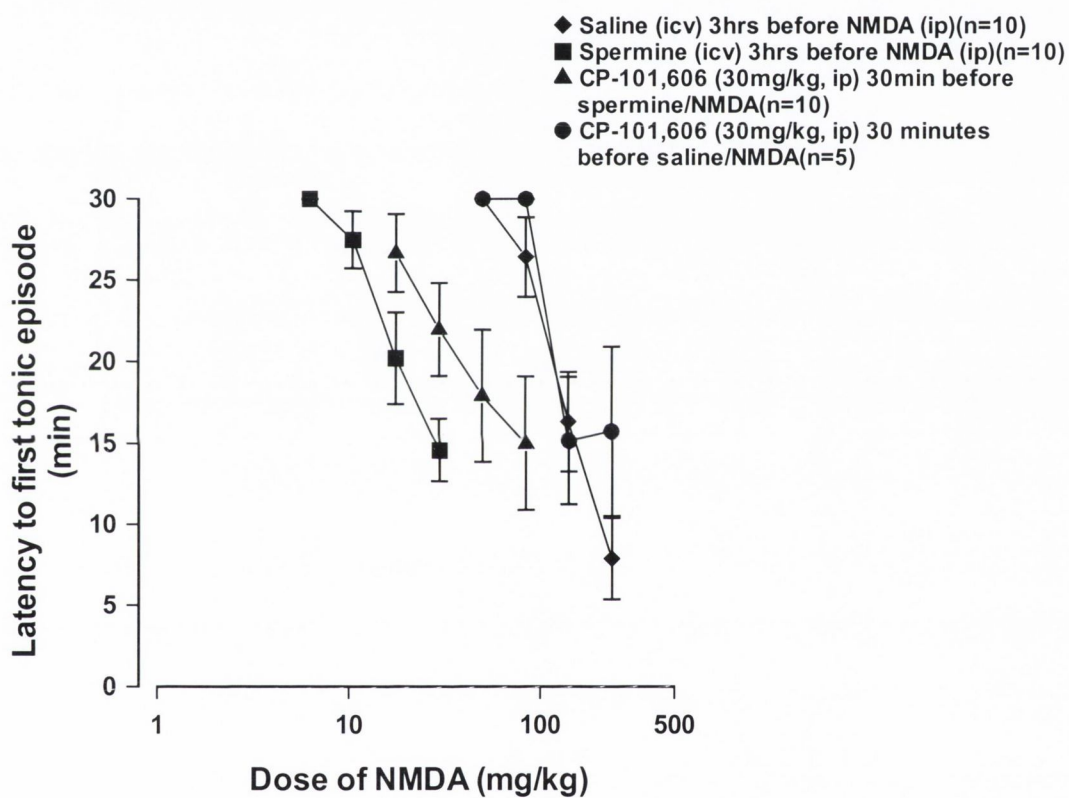


Figure 2.12: The effect of administration of CP-101,606 (30mg/kg, ip) thirty minutes before spermine (25 μ g, icv) on the spermine enhancement of NMDA-induced convulsions. Each point represents mean \pm SEM.

2.5.2.3 *N*¹-Dansylspermine

*N*¹-Dansylspermine (5µg) was co-administered with the spermine in the intracerebroventricular injection. The usual dose volume, 20µl, was used. Despite the earlier results (Figure 2.7), which demonstrated that the dose of spermine given 30 minutes before NMDA had no potentiating effect, *N*¹-Dansylspermine was given, with spermine, both 30 minutes and 3 hours before NMDA. When given 30 minutes before NMDA, *N*¹-Dansylspermine had no effect on either the ED₅₀ for NMDA-induced convulsions (Table 2.10) or the latency to first tonic episode (Figure 2.13).

However, when *N*¹-Dansylspermine (5µg) was co-administered with the spermine (25µg, icv), three hours before NMDA (i.p.) there was a significant reversal of spermine's potentiation. This is illustrated in Figure 2.14. The familiar left shift of the latency curve was seen, caused by the potentiating effect of spermine on NMDA. However, when *N*¹-Dansylspermine (5µg) was co-administered there was a shift back to the right, towards the control value. This was also demonstrated in the ED₅₀ value for NMDA induced convulsions. Spermine (25µg) administered 3 hours before NMDA reduced the ED₅₀ value to 13.57 mg/kg. However, when *N*¹-Dansylspermine (5µg) was co-administered this was reversed and the resultant ED₅₀ value was 78.8 mg/kg (Table 2.10).

To determine whether *N*¹-dansylspermine was active when administered by a peripheral route, it was given by the intraperitoneal route, at a dose of 10mg/kg, thirty minutes before the icv administration of spermine. This dose was shown to have little or no behavioural effects. However, as can be seen from Table 2.10 and from Figure 2.15, *N*¹-Dansylspermine given at this dose had no effect on the ED₅₀ value for the NMDA induced tonic convulsions or on the latency to first tonic episode.

Table 2.10: The effect of N¹-Dansylspermine on the ED₅₀ for spermine enhancement of NMDA-induced convulsions. *** p<0.001 Vs Spermine control, Binary logistic regression.

Drug	Dose	NMDA ED ₅₀ (mg/kg)	95% confidence interval (mg/kg)
Saline control (n=40)	20µl icv	118.9	96.7 – 146.3
Spermine 3hrs before NMDA (n=20)	25µg icv	13.57	8.4 – 21.9
N ¹ -DnsSpm + Spermine (n=40) (3hrs before NMDA)	5µg + 25µg icv	78.8***	57.7 – 107.7
N ¹ -DnsSpm (n=40) (3hrs before NMDA)	5µg icv	141.42	101.5 – 197.1
Saline 30mins before NMDA (n=40)	20µl icv	124.19	90.33 – 170.74
Spermine 30mins before NMDA (n=40)	25µg icv	113.7	90.27 – 143.21
N ¹ -DnsSpm + Spermine (n=40) (30mins before NMDA)	5µg + 25µg icv	102.19	75.9 – 137.58
N ¹ -DnsSpm (n=40) (30mins before NMDA)	5µg icv	156.91	91.68 – 268.58
Saline (-30min), spermine (0), NMDA (+3hrs) (n=20)	0.1ml/10g ip 25µg icv	16.2	8.08 – 32.56
N ¹ -DnsSpm (-30min), spm (0), NMDA (+3hrs) (n=40)	10mg/kg ip 25µg icv	14.54	10.87 – 19.45
Saline (-30mins), saline (0), NMDA (+3hrs) (n=40)	0.1ml/10g ip 20µl icv	118.92	93 – 152.29

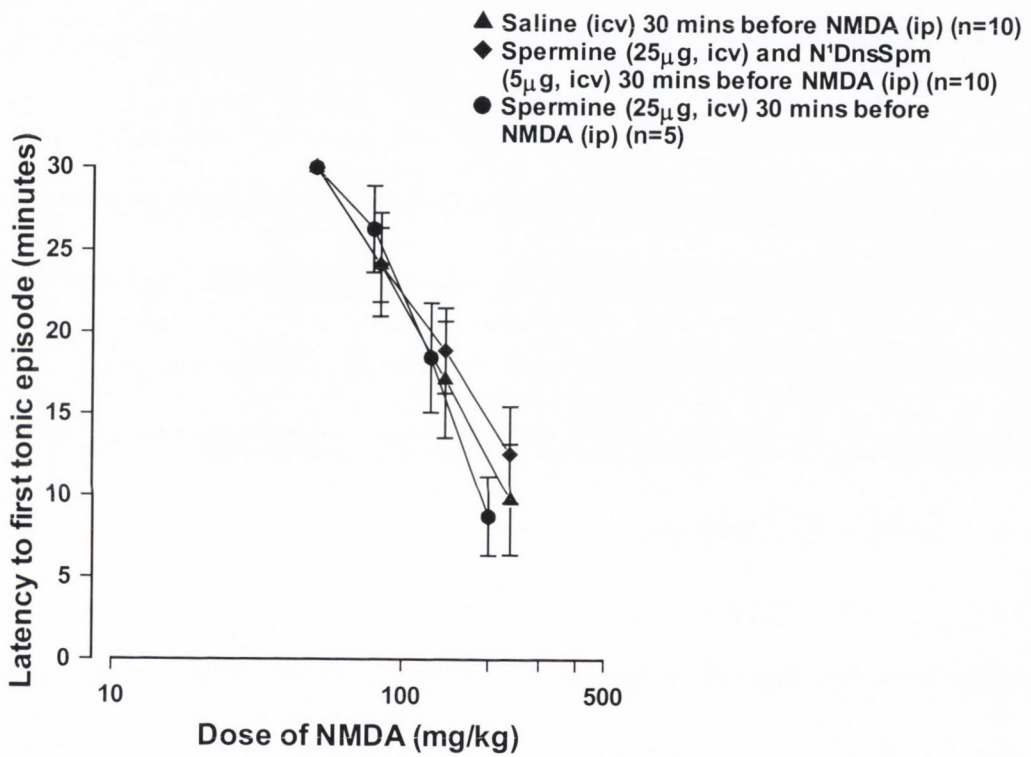


Figure 2.13: The effect of *N*¹-Dansylspermine (5 μ g), co-administered with spermine, thirty minutes before NMDA. Each point represents mean \pm SEM.

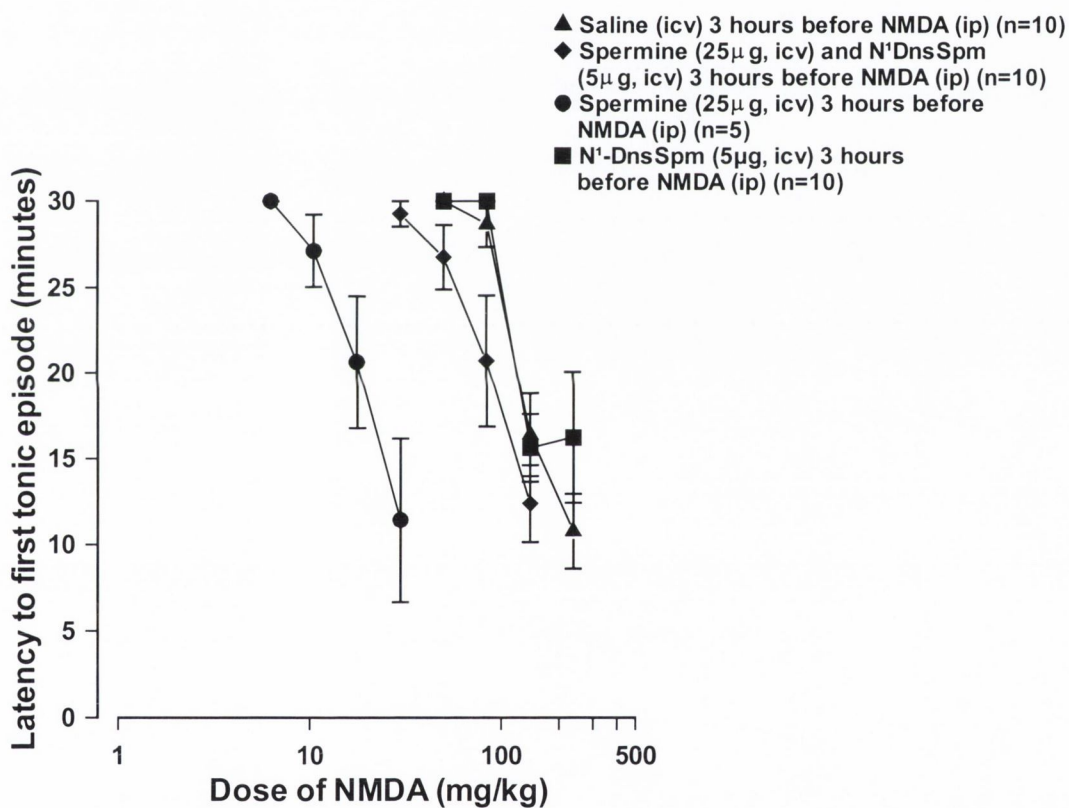


Figure 2.14: The effect of N¹-Dansylspermine (5µg), co-administered with spermine, three hours before NMDA. Each point represents mean ± SEM.

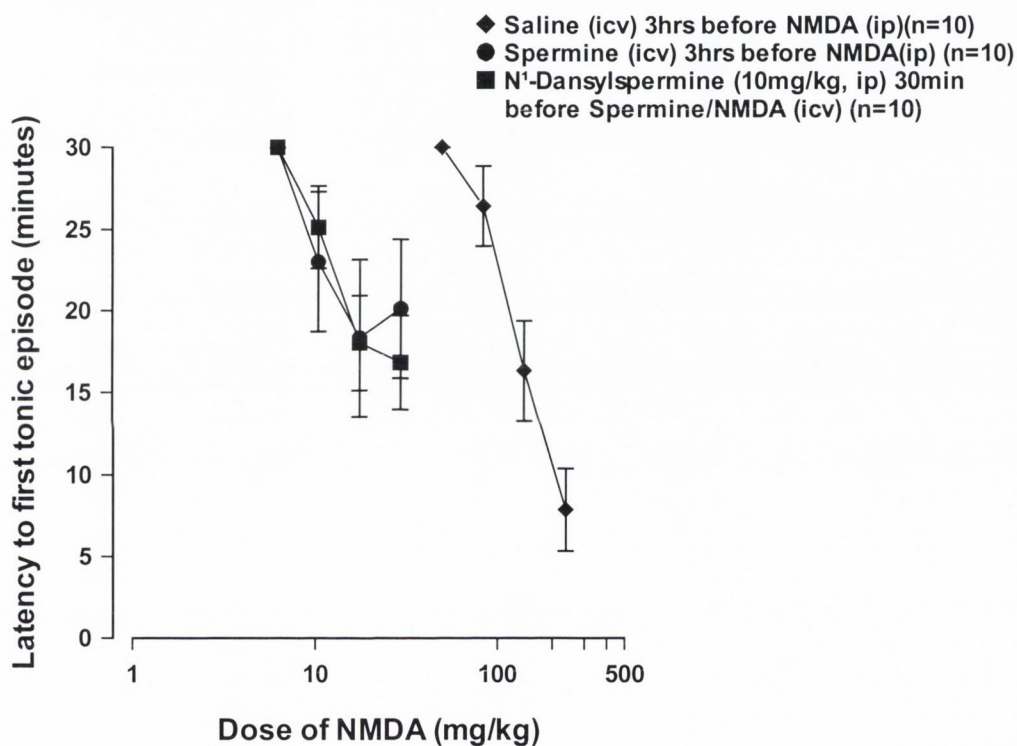


Figure 2.15: The effect of administration of N¹-Dansylspermine (10mg/kg, ip) thirty minutes before spermine (25 μ g, icv) on the spermine enhanced NMDA-induced convulsions. Each point represents mean \pm SEM.

2.6 DISCUSSION

Anderson *et al.* (1975) demonstrated the existence of the two phases of spermine-induced CNS excitation. However, for reasons stated earlier, only the second phase is examined in detail here. Spermine caused an increase in the CNS excitation score with time until a maximum median excitation score of 5 is reached 6 hours after the initial icv injection of 100 μ g spermine. This illustrates the response to a high dose of icv spermine and correlates well with previous studies carried out in this laboratory (Doyle & Shaw, 1996; Doyle & Shaw, 1998). One of the conclusions drawn from the previous studies conducted in this laboratory was that the NMDA receptor appears to be, at least partly, involved in the development of spermine-induced CNS excitation.

Memantine is a 1-amino adamantane derivative and has been demonstrated to have numerous activities. Most important for this work however is the action of memantine on the NMDA receptor. Memantine was first demonstrated to have NMDA antagonistic activity in 1989 when Bormann demonstrated its ability to block NMDA-evoked currents in embryonic mouse spinal neurons. Also demonstrated, in the same year, was the ability of memantine to displace [³H]-MK-801 from its binding sites (Kornhuber *et al.*, 1989). This led to increased interest in this compound and it was subsequently shown to be neuroprotective. Seif el Nasr *et al.* (1990) demonstrated both *in vivo* and *in vitro* that memantine is neuroprotective, though it is less potent than MK-801. Similarly, Block and Schwarz (1996) used the four-vessel occlusion model of global ischaemia in rats to demonstrate that memantine is neuroprotective. They also demonstrated a reduction in functional sequelae induced by ischaemia.

Over time, the exact mechanism of action of memantine was gradually elucidated. In 1997, Chen and Lipton demonstrated that the predominant mechanism of open-channel blockade by memantine was noncompetitive. One of the major problems with the clinical use of NMDA antagonists is their ability to inhibit long-term potentiation (LTP). However, memantine has been demonstrated to be less potent in blocking LTP than MK-801 (Frankiewicz *et al.*, 1996) and this leads to a more

suitable clinical profile for the treatment of neurodegenerative disorders (Chen *et al.*, 1998).

Recently Apland and Cann (1995) demonstrated anti-epileptogenic properties of memantine in guinea pig hippocampal slices, though this activity was shown to be 10-100 fold less potent than that of MK-801. From the results illustrated in Section 2.5, it is evident that memantine inhibits the development of spermine-induced CNS excitation, though it is necessary to administer the memantine twice. The lack of effect of memantine (15mg/kg) when administered thirty minutes before spermine could be due to the reduced potency of memantine as a blocker of the NMDA receptor and this is underlined by the effect when administered before and after spermine. However, this exact result has been previously demonstrated by Doyle (1993) for MK-801. Therefore it is unlikely that the result is due to a reduced NMDA receptor affinity. The spermine-induced CNS excitation manifests as two distinct phases. The single administration of memantine may not be sufficient to counteract the spermine administration. However, it is effective when administered as two doses, reducing the development of the second phase CNS excitation. This demonstrates a role for the NMDA receptor in the development of spermine-induced CNS excitation and tonic convulsion. However, complete protection against excitation was not attained. It is likely, therefore, that there is more than one group of receptors involved in the development of spermine-induced CNS excitation, not just the NMDA receptor, but possibly calcium channels (Section 2.5 and discussed below) and even other receptors with an, as yet, unidentified role.

The influx of calcium into a cell, through calcium channels, causes an increase in cell excitability. It is possible therefore that overactivity of calcium channels in the brain may contribute to the production of convulsions. It has also been shown that the polyamines inhibit diltiazem binding to L-type calcium channels in the rat brain (Schoemaker, 1992). Also, Doyle (1993) demonstrated that nisoldipine had a mild effect against spermine induced CNS excitation. Therefore, it was of interest to further examine and expand on this work. As a result, a number of different calcium

channel antagonists were investigated for an effect on this spermine induced CNS excitation.

Calcium channel antagonists have already been demonstrated to possess anticonvulsant properties. Meyer *et al.* (1986) demonstrated the anticonvulsant effects of nimodipine, a dihydropyridine calcium channel blocker. Ots *et al.* (1987) also demonstrated the efficacy of nimodipine on pentylenetetrazole induced seizures.

Verapamil is a group II (phenylalkylamine) calcium channel blocker and is selective for the L-type calcium channels. The L-type calcium channels are voltage activated calcium channels found predominantly in muscles and neurons. As was seen earlier, verapamil given at a dose of 20mg/kg (ip) produced a small reduction in the development of spermine induced CNS excitation. The only differences were shown at three time points and the final outcome of CNS excitation was not affected. This may suggest that the L-type calcium channels are not fundamental to the production of the CNS excitation. Several studies have failed to demonstrate an effect of verapamil on the NMDA receptor. Lucke & Speckmann (1993) demonstrated that verapamil (60-100 μ mol/l) had no effect on NMDA induced cortical field potentials in neocortical slices of guinea pigs. Indeed, Filloux *et al.* (1994) demonstrated that verapamil, at doses of up to 10^{-5} M, has no effect on the binding of [3 H]MK-801 to mouse brain sections demonstrating a lack of effect on the NMDA receptor. Other studies also reported a lack of effect of verapamil. De Sarro *et al.* (1988) demonstrated that verapamil (19 – 57mg/kg, ip) had no effect on sound-induced seizures in DBA/2 mice, which are genetically epilepsy prone. Likewise, in the study by Ots *et al.* (1987), in which it was shown that nimodipine was effective against pentylenetetrazole-induced seizures, verapamil (allegedly in the highest non-cardiotoxic dose) was shown to be ineffective. However, a subsequent study by Palmer *et al.* (1993) demonstrated a moderate ability of verapamil to antagonise NMDA-induced convulsions (ED_{50} =14.2mg/kg, ip). The dose used in the present study (20mg/kg) is greater than the ED_{50} of 14.2mg/kg for inhibition of NMDA-induced convulsions shown by Palmer *et al.* (1993) and this may therefore suggest that the mild effect seen for verapamil is due to NMDA receptor antagonism. This

study does show however, that, similar to the previous studies, verapamil does not possess potent anticonvulsant effects.

Nitrendipine was also examined for its effect on spermine-induced CNS excitation. Nitrendipine is a dihydropyridine calcium channel blocker, also selective for the L-type calcium channel. Nitrendipine has been extensively studied as it is a highly potent calcium channel antagonist. In fact, in the study by Morón *et al.* (1990), nitrendipine was found to have a highly potent antiseizure activity against pentylenetetrazole-induced seizures ($ED_{50}=167\mu\text{g}/\text{kg}$, icv). In the study mentioned above, by De Sarro *et al.* (1988), nitrendipine (30 and 45mg/kg, ip) showed significant protection against all phases of audiogenic seizures. Dolin *et al.* (1986) examined the effect of nitrendipine (100mg/kg, ip) against a number of proconvulsant treatments. It was demonstrated that nitrendipine was effective against pentylenetetrazole and high pressure-induced seizures but not against strychnine and NMDA-induced convulsions. It was also demonstrated that nitrendipine was effective versus pentylenetetrazole at brain concentrations in the low micromolar range. This suggests that nitrendipine has no effect on the NMDA receptor. Surprisingly, however, Skeen *et al.* (1993) demonstrated the ability of nitrendipine (0.1 - $1\mu\text{M}$) to suppress NMDA/glycine-mediated calcium influx by an interaction with the NMDA receptor macrocomplex. Subsequent to this Skeen *et al.* (1994) demonstrated that, in rodent cortical neurons, nitrendipine ($1\mu\text{M}$) reduced NMDA evoked currents. This was shown to be due to a direct interaction with the NMDA receptor macrocomplex. They then proposed, from this, a kinetic model of interaction between nitrendipine and the NMDA receptor, similar to the interaction of MK-801. Subsequently, work by Filloux *et al.* (1994) demonstrated inhibition by nitrendipine of MK-801 binding to mouse brain slices. This was a unique result not shown by any other dihydropyridine L-type calcium channel blockers investigated, including nimodipine, nifedipine, BAY K8644 and verapamil. This was as a result of a direct interaction between nitrendipine and the NMDA receptor.

Obviously nitrendipine is an important L-type calcium channel antagonist in relation to the present study since the consensus in the literature is that it demonstrates high

potency for both NMDA and L-type calcium channels. Palmer *et al.* (1993) demonstrated ED₅₀ values for nitrendipine's inhibition of both NMDA- and BAY K 8644 (a calcium channel agonist) induced seizures of 1.6mg/kg and 0.3mg/kg, respectively. This is relevant when taken in context with the observations presented earlier. Here nitrendipine, 15mg/kg, has a very marked effect on the development of spermine-induced CNS excitation. A statistically significant difference is seen between the spermine alone and that with nitrendipine pretreatment at every time point from 4 hours after injection onwards. Indeed the median CNS excitation score does not rise above 1, which represents a mild tremor. As mentioned earlier, and shown by the effect of memantine on the excitation, the NMDA receptor is, at least partially, involved in the production of spermine-induced CNS excitation. Therefore, nitrendipine may be exerting its effect on spermine-induced CNS excitation through the NMDA receptor or calcium channels or a combination of both.

Nisoldipine is a group I calcium channel blocker, the dihydropyridine group, and is also selective for the L-type calcium channel. Nisoldipine has been shown to have greater potency than the commonly used dihydropyridine, nifedipine, at antagonising pentylentetrazole-induced seizures (Morón *et al.*, 1990). Indeed, in the same study nisoldipine was shown to be only slightly less potent than nitrendipine (ID₅₀ - 167µg/kg icv compared to 219µg/kg icv). Nisoldipine (2mg/kg) has previously been investigated in the present spermine-induced seizure model (Doyle, 1993). It was demonstrated that nisoldipine produced a statistically significant decrease in the CNS excitation score at a number of time points through out the study. In the present study, the development of CNS excitation is also retarded, though this only becomes statistically significant at one time point. This is probably due to the small numbers used in the study. Nisoldipine has also been demonstrated to antagonise NMDA-induced seizures (ED₅₀=0.36mg/kg; Palmer *et al.*, 1993). Indeed, it was found to be more potent than either MK-801 (ED₅₀=1.06mg/kg) or nitrendipine (1.6mg/kg). Therefore, nisoldipine, because of its potent dual NMDA/calcium channel activity, would have been expected, at 5-6 times its supposed ED₅₀ versus NMDA, to produce a significant inhibition of spermine-induced CNS excitation, whereas only a small effect was seen.

Spermine has long been known to enhance NMDA induced effects and in general increase the activity of the NMDA receptor. Initially spermine was shown to increase the binding of [³H]MK-801 to NMDA receptors, representing an increased open state/activation of the NMDA receptor (Ransom & Stec, 1988). As previously mentioned, Singh *et al.* (1990) demonstrated an enhancement by the polyamine, spermidine, of NMDLA induced tonic convulsions. Similarly, Matsumoto *et al.* (1993) demonstrated spermidine enhancement of NMDA induced convulsions. This work has also held up to electrophysiological examination. Sprosen and Woodruff (1990) showed the potentiation by polyamines of NMDA-induced currents in cultured striatal neurons.

Because it is recognised that there are two distinct phases to the direct actions of spermine, as previously detailed in Sections 2.1 and 2.2.1, the effect of administration of spermine (icv) before NMDA at two different times was examined. A subconvulsive dose of spermine (25µg) was used so that the mice had not developed CNS excitation by the time the NMDA was administered. However, spermine (25µg) will still produce death due to tonic convulsions, but this can take a day or more (Anderson *et al.*, 1975). As mentioned in Section 2.2.2 above, tonic convulsions were used as the end point of the study as none of the other behavioural effects appeared reliably in each animal. This is in contrast to a study by Moreau *et al.* (1989) where NMDA administered by the intracerebroventricular route produced the characteristic behavioural effects (wild running, clonic and tonic convulsions) in each case. However, the difference here is probably due to the different route of administration.

The effect of 30 minute pretreatment with spermine falls within the first phase of spermine's action, whereas the effect of 3 hours pretreatment of spermine, falls in the second phase. There is a marked difference in response between the two times of administration of spermine. Spermine given 30 minutes before NMDA had no effect on the convulsions. However, when spermine was given three hours before NMDA there was a substantial reduction in the latency to tonic convulsion and the ED₅₀ for tonic convulsion. This serves to underline the proposal that the two phases of spermine action are mediated by two different mechanisms of action as has been

proposed by Doyle & Shaw (1996 & 1998). They showed that most anticonvulsants were able to antagonise the first phase of effects but relatively few had an effect on the second phase, suggesting a different mechanism of action. There is no easy explanation of the delay associated with the second phase effects. With three hours pretreatment, it is possible that the spermine is able to bring about changes in the brain, which could possibly be metabolic in origin. It could be a metabolite of spermine that results in enhanced activity but this is unlikely since it is known that spermine itself enhances NMDA receptor activity (Sprosen & Woodruff, 1990). Finally, it could take the pre-treatment time for the spermine to reach its site of action, the NMDA receptor (and other receptors) in a specific area. As a result of the lack of enhancing effect of spermine when administered thirty minutes before NMDA, in most subsequent studies, spermine was only administered three hours before NMDA.

Nisoldipine was also examined in the spermine-enhanced NMDA-induced seizure model. Nisoldipine did not have any effect on either spermine's reduction in ED₅₀ for NMDA-induced convulsions or on its reduction of latency to first tonic episode. This may demonstrate a lack of involvement of the L-type calcium channels in this experimental paradigm. As, if the spermine's enhancing effect was mediated through L-type calcium channels, a reversal of this enhancement would be expected to be seen in this instance, but is not.

Since nisoldipine has no effect, at 2mg/kg (ip), on the spermine-enhanced NMDA-induced convulsions, it can also be concluded that nisoldipine, certainly at this dose and under these experimental conditions, does not possess potent NMDA antagonist activity. This contrasts with the findings of Palmer *et al.* (1993). This apparent lack of effect of nisoldipine has implications for direct spermine-induced CNS excitation, in that the effect seen after nisoldipine may not be as a result of NMDA antagonist activity but rather from L-type calcium channel antagonist activity. However, this action appears to play no part in the spermine enhancement of NMDA induced convulsions. So activity of calcium channels downstream of NMDA receptors may be ruled out.

The phenylethanolamines (ifenprodil, eliprodil, CP-101,606) have long been known to exert their action through the NMDA receptor. However, the exact mechanism of this action has been much debated. Both ifenprodil and eliprodil were originally proposed to act as competitive inhibitors of the polyamine site on the NMDA receptor macrocomplex. Ifenprodil was also proposed to act through the stimulatory polyamine site, as a competitive antagonist (Carter *et al.*, 1990) and subsequently via an allosteric mechanism through a separate ifenprodil binding site on the NMDA receptor (Kew & Kemp, 1998). Both ifenprodil and eliprodil have been shown to block voltage dependent calcium channels in a number of *in vitro* studies using cultured cells.

Ifenprodil has previously been examined for activity versus direct spermine-induced CNS excitation. Doyle and Shaw (1996) examined its effects alone and in combination with the glycine site antagonist, 7-chlorokynurenate, or the glutamate antagonist, D-CPP. As monotherapy ifenprodil antagonised the first phase of effects but had no effect on the second phase. However, when in combination as described above there was a dose-dependent antagonism of the development of the second phase of spermine-induced effects.

In the present study, ifenprodil, given alone also, had no effect on the spermine-enhancement of NMDA-induced convulsions. Administration of ifenprodil had no effect on either the latency to first tonic episode or on the ED₅₀ for NMDA-induced tonic convulsions. In the present study, ifenprodil was not investigated in combination with glutamate or glycine site antagonists but the results here correlate with the first part of the study by Doyle and Shaw (1996). It is possible that the ifenprodil is not sufficiently potent to effect a polyamine antagonistic action in the model used here. It is also possible the enhancement of NMDA-induced convulsions during the second phase of spermine's effects is due to a metabolite of spermine which ifenprodil is unable to antagonise. Recently it has been demonstrated that ifenprodil, administered by the intraperitoneal route, as monotherapy, had no effect on fully amygdala-kindled rats (Ebert *et al.*, 1997). However, when ifenprodil was administered with a glycine_B receptor antagonist there was a pronounced

anticonvulsant effect. This demonstrated synergism between NMDA receptor antagonists and supports the findings of Doyle and Shaw (1996) and the present studies.

Eliprodil is a structural analogue of ifenprodil and has a similar mode of action (see above and Section 3.6), though it is more potent as it more effectively penetrates the CNS (C. Carter, personal communication). As with ifenprodil, eliprodil has been examined for effects against direct spermine-induced CNS excitation. At low doses of 15mg/kg, eliprodil was ineffective against spermine-induced CNS excitation. However, there was a slight reduction in the development of CNS excitation when eliprodil was administered at 30mg/kg and a marked reduction when administered at 60mg/kg (Doyle, 1993). It was concluded from this that eliprodil was an effective polyamine antagonist in the absence of glutamate or glycine receptor antagonists, unlike ifenprodil. Recently, Wlaz *et al.* (1999) demonstrated that a combination of eliprodil and a glycine site antagonist on the NMDA receptor showed significant anticonvulsant activity in the amygdala kindling model in rats. This underlines the thought that a combination of a polyamine and glycine site antagonists are effective NMDA receptor antagonists and could be useful in the treatment of therapy-resistant complex partial seizures.

In the present study, eliprodil was examined for antagonist activity in the spermine-enhanced NMDA-induced convulsion model. Two different doses were investigated based on the previous work by Doyle (1993). Eliprodil, 30mg/kg, was administered by the intraperitoneal route, thirty minutes before direct administration of spermine. No statistically significant difference was found between eliprodil administration and control values. There was no change in the ED₅₀ for NMDA-induced convulsions or in the latency to first tonic episode. However, when administered at 60mg/kg, the effective dose in previous studies (Doyle, 1993), eliprodil produced a statistically significant ($p < 0.01$) reversal of spermine's enhancement of NMDA-induced convulsions and of its reduction in latency to first tonic episode. This effect could in theory be caused by an NMDA or polyamine antagonistic effect. However, eliprodil (60mg/kg) had no effect on the ED₅₀ for NMDA or latency in the control, with saline

given icv instead of spermine. This demonstrates that the effect seen is due to a polyamine antagonist action at the NMDA receptor complex. This difference in apparent potency between the ifenprodil and eliprodil could be due to a slight difference in NMDA receptor subset selectivity between ifenprodil and eliprodil (Avenet, 1997). However, despite results demonstrating a higher NMDA receptor potency for ifenprodil (Chenard & Menniti, 1999), as mentioned earlier, eliprodil has a superior bioavailability to that of ifenprodil illustrated by its better anti-ischæmic effects when administered orally (Gotti *et al.*, 1988). Similarly, eliprodil has been shown to have a more potent effect than ifenprodil on NMDA-evoked release of striatal acetylcholine, spermidine, GABA and dopamine (Nankai *et al.*, 1996), suggesting that better potency *in vivo* may account for eliprodil's superiority.

CP-101,606 is a novel phenylethanolamine and a derivative of ifenprodil and eliprodil, which is selective for the NR2B subunit, as was demonstrated by its localisation in the forebrain and structural similarity to ifenprodil (Menitti *et al.*, 1997). CP-101,606 has already been shown to be a more potent NMDA antagonist than either ifenprodil or eliprodil (Chenard & Menniti, 1999). Similarly, CP-101,606 has been shown to possess antinociceptive activity (Taniguchi *et al.*, 1997; Boyce *et al.*, 1999) and to be selectively neuroprotective for forebrain neurons (Menitti *et al.*, 1997). Due to the similarity in structure between CP-101,606 and both ifenprodil and eliprodil it is reasonable to assume that it possesses some polyamine antagonistic activity.

Therefore, CP-101,606 was examined in the same model as ifenprodil and eliprodil. The dose used, 30mg/kg, was that used to demonstrate antinociceptive activity (Taniguchi *et al.*, 1997), although it was administered by the intraperitoneal route in the present study, rather than subcutaneously. CP-101,606 reverses the enhancing effect of spermine on NMDA as there is an increase in the ED₅₀ for spermine enhanced NMDA-induced convulsions from 15.75mg/kg to 43.9mg/kg, in the presence of CP-101,606, and also in the latency to first tonic episode. This effect was shown to be highly statistically significant when analysed using logistic regression ($p < 0.01$). Indeed it can be seen that this effect of CP-101,606 is greater than any of

the other phenylethanolamines investigated in this study. This may be as a result of increased potency of this novel compound for the NMDA receptor. However, as for the high dose of eliprodil, CP-101,606 had no effect, as monotherapy on either the ED₅₀ for NMDA-induced convulsions or on the latency to first tonic episode and this therefore discounts any significant NMDA antagonistic activity. As a result the activity of CP-101,606 is most likely to be from a polyamine antagonistic action on the NMDA receptor macrocomplex.

N¹-Dansylspermine is a structural analogue of spermine, consisting of a spermine backbone and a large, bulky group on one end of the molecule. This structure suggests that N¹-dansylspermine may possess polyamine antagonist activity. As mentioned previously (Section 1.2), there are numerous proposed sites of action of the polyamines on the NMDA receptor macrocomplex. These sites can be either stimulatory or inhibitory, though the stimulatory site is the more relevant at physiological concentrations. Chao *et al.* (1997) examined the effects of N¹-dansylspermine on cloned NMDA receptors, looking specifically at the inhibitory polyamine site, and found that N¹-dansylspermine was a potent blocker of the NMDA receptor through an agonist action at the inhibitory polyamine site.

N¹-Dansylspermine dose-dependently inhibits spermine induced CNS excitation. The dose of 2µg N¹-dansylspermine produces a slight retardation of the development of CNS excitation, shown by the rightward shift of the graph. However, the net end result is similar in both cases and not statistically different. In contrast, both 5µg and 10µg N¹-dansylspermine result in a marked inhibition of development of the second phase of spermine-induced effects. This demonstrates a powerful inhibitory action by N¹-dansylspermine on the central effects of spermine.

As with other analogues, N¹-dansylspermine could be acting via either an inhibitory or stimulatory polyamine site, both of which would produce the same net result. It is possible that, in accordance with the observations of Chao *et al.* (1997), N¹-dansylspermine is acting on the inhibitory site by stimulating it and as a result reduces the activation of the NMDA receptor, which manifests as reduced spermine-induced

CNS excitation. However, the stimulatory polyamine site is the most relevant physiologically as substantially larger amounts of spermine are required to stimulate the negative site than the positive site.

When activated, the stimulatory polyamine site on the NMDA receptor macrocomplex produces an increased NMDA receptor activation. Antagonism of this site will result in decreased activation of the NMDA receptor and this is the likely mode of action of N¹-dansylspermine. Supportive evidence for this view was garnered from the effects of N¹-dansylspermine in the spermine-enhanced, NMDA-induced convulsion model and is detailed below.

The key factor here is that when N¹-dansylspermine (5µg) is co-administered with the spermine there is a reversal of the leftward shift of the latency curve for NMDA induced convulsions caused by spermine and the ED₅₀ for action is raised to 78.8 mg/kg compared to 13.57 mg/kg for spermine alone. This is not a complete reversal of the spermine effect but it is substantial. This could again be as a result of antagonising the action of spermine at the stimulatory polyamine site or by a stimulatory effect at the inhibitory polyamine site. However, the icv administration of N¹-dansylspermine, alone, three hours before NMDA, has no effect on either the latency to tonic episode or on the ED₅₀ for NMDA induced convulsions. This illustrates that the action of N¹-dansylspermine is an antagonist action at the stimulatory polyamine site. If the action of N¹-dansylspermine had been mediated through the negative polyamine modulatory site then an inhibitory action on NMDA would be expected and this is not seen. It is most likely that the type of antagonistic action of N¹-dansylspermine is competitive. The molecule has a spermine backbone and should therefore bind in the same way as spermine itself. However, the large dansyl group at the end of the spermine chain would interfere with the approach of spermine to the binding site and also prevent a stimulatory action of N¹-dansylspermine. Unfortunately, from the scope of this study it was not possible to say whether the activity of N¹-dansylspermine was competitive or not.

The effect of N¹-dansylspermine was investigated when spermine was administered thirty minutes before NMDA. As was previously shown, the administration of spermine had no effect on the latency or the ED₅₀ for NMDA-induced convulsions and as a result no effect was seen for the N¹-dansylspermine. However, the lack of effect of N¹-dansylspermine when administered alone, thirty minutes before NMDA illustrates again that the N¹-dansylspermine is exerting its action through the stimulatory polyamine modulatory site and not acting as an NMDA receptor antagonist

When N¹-dansylspermine was administered, by the intraperitoneal route, at a dose of 10mg/kg, thirty minutes before spermine (icv). It had no effect on the spermine enhancement of NMDA-induced convulsions. The ED₅₀ obtained was 14.54mg/kg compared to 16.2mg/kg for the saline control. This could be due to a lack of penetration of the dose into the brain because of the blood brain barrier to polyamines. Sufficient N¹-dansylspermine may be unable to cross the blood brain barrier to act on the NMDA receptor.

2.7 CONCLUSION

The results presented here have attempted to elucidate the chain of events that result from direct cerebroventricular administration of spermine and the role and mechanism by which spermine enhances NMDA-induced convulsions. There are a number of different ways of producing convulsions *in vivo*. Such methods include amygdala kindling in rats, electroshock and a number of chemical methods such as induction by pentylenetetrazole or NMDA treatment. A number of the compounds examined here have been shown to be effective in one or more of these models (detailed above). An attempt was made to investigate the role of different receptors and channels in the production of CNS excitation by spermine and the role of the polyamines in NMDA induced convulsions.

It can be concluded from the above results that both the NMDA receptor and L-type calcium channels are involved to some extent, in spermine's production of CNS excitation. This is demonstrated by the reduction in the development of CNS excitation, though to different extents, by the L-type calcium channel antagonists, verapamil, nisoldipine and nitrendipine, all of which have some activity for the NMDA receptor as well as L-type calcium channels. Similarly, the ability of memantine to partially protect against the development of CNS excitation underlines the role of the NMDA receptor. The NMDA antagonist activity of the calcium channel antagonists examined may suggest that the effects seen are as a result of this action rather than calcium channel activity.

The second model, however, focussed on the effect of spermine on the NMDA receptor, whereas the first model examines the direct effects of spermine, which could be mediated through an action at multiple sites. Nisoldipine did not show any effect in this model. From this two conclusions can be drawn. First, the lack of NMDA antagonist action of nisoldipine, at a dose several times greater than its proposed ED₅₀ versus NMDA (Palmer *et al.*, 1993), suggests that the effect seen in the first model was due to calcium channel antagonism. This suggests a role for both L-type calcium channels and NMDA receptors in the production of spermine induced CNS excitation. The dose of nisoldipine used (2mg/kg) is greater than its ED₅₀ for antagonising calcium induced seizures (0.2mg/kg; Palmer *et al.*, 1993). This also suggests that the calcium channels are not involved in the production of seizures downstream of the NMDA receptor in the spermine enhancement of NMDA-induced seizures.

Doyle (1993) demonstrated that high doses of eliprodil (30 and 60mg/kg) produced a statistically significant inhibition of spermine-induced CNS excitation. This is demonstrated here in a different model. Eliprodil (60mg/kg) reduces the spermine enhancement of NMDA-induced convulsion. However, the lack of effect versus NMDA alone suggests that the effect of eliprodil is mediated through a polyamine antagonist action at the NMDA receptor macrocomplex. Similarly, CP-101,606 reduces the spermine enhancement of NMDA-induced convulsions most likely through a polyamine antagonist action, though the lower dose required (30mg/kg)

probably reflects its greater affinity for the NMDA receptor macrocomplex (Chenard & Menniti, 1999).

Perhaps of greatest significance is the finding that N¹-dansylspermine, a novel compound, has been demonstrated here to have potent polyamine antagonistic activity. It is the most potent compound yet examined in this laboratory for antagonism of the spermine-induced CNS excitation and it has been shown to act through the positive polyamine modulatory site on the NMDA receptor macrocomplex.

SECTION 3

Cerebral Ischaemia

3.1 INTRODUCTION

In order to understand the processes that occur following a stroke a reliable model must be used. The mechanisms of cerebral ischaemia are not yet fully understood and research is not possible in human subjects, so animal models must be used. There are a number of advantages to using animal models in stroke research. First, control is maintained over the intensity and duration of the ischaemic insult. As standard animal strains are used there is a fairly uniform cerebrovascular anatomy. Finally, the assessment of damage can be carried out by either biochemical or histological analysis. This, of course, is not possible in the human subject and the clinician must rely on functional or behavioural outcomes or on the use of an MRI scan.

The ideal animals to carry out investigations into possible stroke therapies would be non-human primates as they resemble most closely the human condition. However, in addition to the ethical problems that might arise from using primates, there are also economic ones when the purchase and breeding are taken into account. As a result, compounds are generally screened on smaller, cheaper animal models and if they are shown to be effective they can then be used in primate models. There are a number of different small animal models in regular use, and these are described below.

3.1.1 *In vivo* models

There are a number of different *in vivo* models of cerebral ischaemia and they can be broadly grouped into global and focal ischaemia models. Global ischaemia models are stated to be models of cardiac arrest producing selective necrosis, whereas focal models are more akin to acute ischaemic stroke (Hunter *et al.*, 1995; Macrae, 1992). In practical terms, however, there is little difference between them as regards

outcome, as essentially the same mechanisms of neurodegeneration are at work in both cases.

Global ischaemia occurs as a consequence of a reduction in or complete cessation of blood flow to the brain. Global ischaemia models study the vulnerability of the brain to cardiocirculatory failure and subsequent reperfusion. Complete ischaemia can be brought about by compressing the blood vessels in the neck by inflating a pneumatic cuff or by increasing the intracranial pressure (Mas & Zuber, 1991). In these models, care must be taken to avoid collateral supply of blood to the ischaemic area, as the brain circulatory system will try to compensate for the lack of blood in a region through the collateral arteries in the circulatory system (Nordstrum *et al.*, 1978).

The gerbil model of global cerebral ischaemia is one of the most widely used global ischaemia models because of the relative ease of the surgical techniques involved and there is delayed and selective neurodegeneration which can be reliably measured. However, as a consequence of the small size of the animals involved it is difficult to make any physiological measurements. There are also minor variations in cerebral circulation, which result in a variable outcome. There are also two models of global ischaemia in the rat, the two vessel and four vessel occlusion models. Both of these have disadvantages in that the two-vessel occlusion model requires the production of hypotension to ensure ischaemia and the four-vessel model involves major surgery.

Focal models of cerebral ischaemia can be divided into two types, permanent and reversible. Permanent focal ischaemia causes an ischaemic core from which the damage seems to spread out. The aim therefore is to minimise the damage to this surrounding area (penumbra) and reduce the spread of damage. However in humans the interruption in blood flow only appears to be temporary and as a result reversible focal ischaemia models have been developed. The most common form of focal ischaemia is the middle cerebral artery (MCA) occlusion model. There are a number of surgical techniques used to produce this, all of which are extensive and this can limit subsequent behavioural studies. In the most sophisticated techniques an occluding device can be implanted and then vascular occlusion can be performed in

unanaesthetised animals. In some cases this occluding device is removable allowing the study of reversible focal ischaemia.

Reversible cerebral ischaemia has also recently been produced by the abluminal application of endothelin-1, which is a 21 amino acid peptide which produces profound vasoconstriction in cerebral vessels (Giaid *et al.*, 1989). This results in reproducible, dose related ischaemia within the MCA territory followed by a gradual reperfusion.

A number of thromboembolic models have also been developed. There are three main categories, autologous blood clot embolisation, microsphere embolisation and photochemical. The autologous blood clots are clotted outside the animal and then reintroduced into the cerebral circulation. Calibrated microspheres have been widely used as embolic material to penetrate the terminal vessels. Finally, photochemically induced focal cerebral thrombosis involves infusing the animal with a photosensitive dye and irradiating the area with light of a specific wavelength. The reaction of the light with the dye produces oxygen radicals and this results in platelet aggregation and thrombosis.

3.1.2 *In vitro* models

As an alternative to *in vivo* models of cerebral ischaemia, there are a number of *in vitro* models available. In these models, brain tissue slices are incubated in a medium free of oxygen and glucose so as to mimic the conditions of ischaemia. There are, however, a number of disadvantages. First, the preparation of the tissue is traumatic and the tissue is subject to a period of ischaemia during preparation. Also the medium used is different to that seen in the physiological environment. As a consequence of this any results obtained with *in vitro* models should be interpreted carefully.

3.1.3 Assessment of neurodegeneration

The best method to assess neurodegeneration, in this or any case, is by using histology. However, this is not possible for the clinician who must rely on functional impairment. Consequently, in animal models, some measure of behavioural or functional impairment must be built in when assessing neuroprotection. This has recently been shown to be important with the demonstration that neurological deficits and lesion size do not necessarily correlate (Rogers *et al.*, 1992).

Magnetic resonance imaging and spectroscopy have also been used in the assessment of stroke damage and in the effectiveness of drug treatment of stroke. These methods are non-invasive and a rapid assessment can be made. They can also be used in both small animals and humans. A method for the volumetric assessment of early cerebral infarction was developed by Osborne *et al.*, in 1987, and was shown to be very reliable.

A number of issues must be taken into account when, having chosen the model to use, the experiment is designed. Any potential therapeutic agent would be administered to a patient after the patient has suffered a stroke. Therefore, to show real potential, a novel agent must be administered after the ischaemic insult in the animal model. There are two important reasons for this. First, if administered before the insult, the compound is present when the tissue undergoes ischaemia and it does not then have to penetrate the ischaemic area. Second, the drug, if given before the ischaemic insult may alter the cerebral blood flow, oxygen utilisation or any other number of mechanisms all of which could alter the degree of ischaemia.

Also of great importance in animal experiments is the control of physiological variables, which could have an influence on the outcome of the experiment. One such factor is blood pressure as this would affect the peripheral tissue damage, the degree of collateral blood supply and the rate of tissue reperfusion.

Another physiological parameter, which it is important to control, is cerebral temperature, especially in the global ischaemia models. Evidence has come to light recently that hypothermia confers a degree of neuroprotection (Busto *et al.*, 1987). It was proposed by Busto *et al.*, in 1989, that one mechanism, by which small variations in brain temperature exert their protective effect, may be through an action on neurotransmitter release. Indeed, they demonstrated that rats, whose brain temperatures were held at 36°C, exhibited a 500-fold and seven-fold increase in release of dopamine and glutamate, respectively. However, in those rats whose brain temperatures were held at 33 °C or 30 °C, the release of glutamate was completely inhibited and the release of dopamine was significantly attenuated (by 60%).

The decision to use a particular model for the investigation of cerebral ischaemia must depend on the ease of surgery and on the reproducibility of the neuronal damage produced. Therefore, because of the straightforward surgery, the delayed, selective neurodegeneration and the ability to conduct large studies, the gerbil model of cerebral ischaemia was used in this study. The model was used to investigate the hippocampal damage due to cerebral ischaemia and the effect of potential neuroprotective agents on this damage.

3.1.4 The gerbil model of ischaemia

The Mongolian gerbil (*Meriones unguiculatus*) is a small rodent, which is found in the dry regions and deserts of Asia. The gerbil was first introduced as an experimental model of cerebral ischaemia in 1966 by Levine and Payan. The animal is unique in that it has an incomplete circle of Willis. This means that the gerbil has no connection between the carotid and vertebro-basilar circulation. This is depicted in Figure 3.1 below.

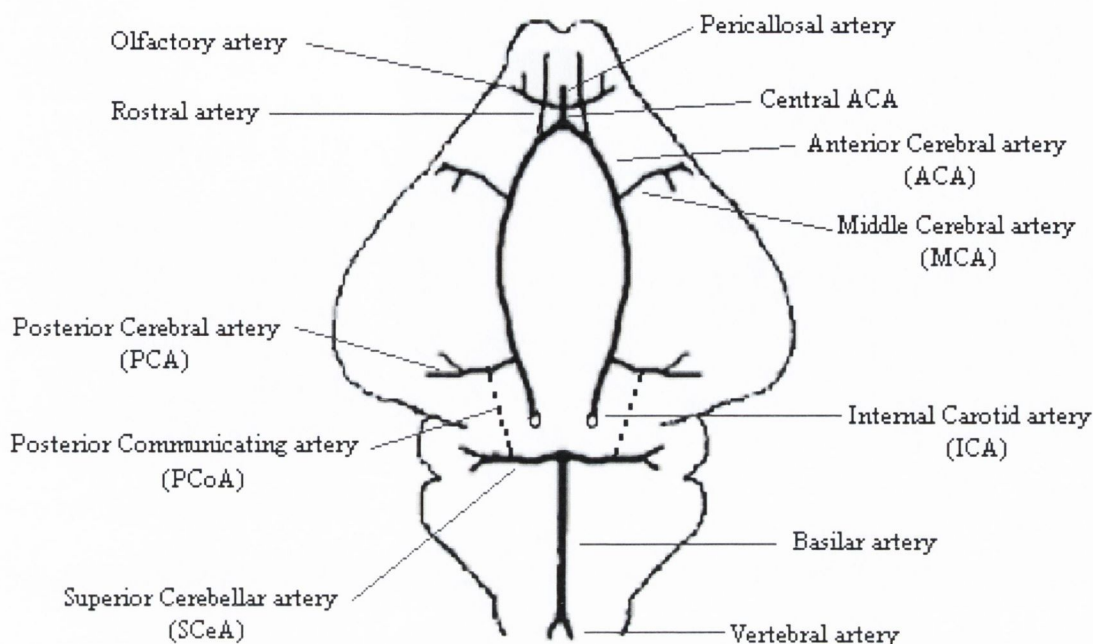


Figure 3.1: The incomplete circle of Willis found in the Mongolian gerbil. Figure adapted from Mayevsky & Breuer, 1992.

The normal connection between the carotid and vertebro-basilar circulation, the posterior communicating artery, is shown in Figure 3.1 as a dotted line. Therefore, global ischaemia can be achieved by occluding the common carotid arteries, of which the internal carotid arteries are an extension. It has been demonstrated that, in approximately 70% of gerbils, the posterior communicating arteries are not present resulting in the incomplete circle of Willis (Breuer & Mayevsky, 1992). However, in practice, occluding the common carotid arteries produces global ischaemia in over 90% of the gerbils (Kirino *et al.*, 1986). As a result of this, the gerbil model has been extensively used as a model of both cerebral ischaemia and transient forebrain ischaemia which can be produced by occlusion of the common carotid arteries (Levine & Payan, 1966; Kirino *et al.*, 1982; Gill *et al.*, 1987).

Cerebral ischaemia causes a selective pattern of neurodegeneration (Brierley, 1976). The pyramidal neurons of the CA1 subfield of the hippocampus are the most vulnerable to cerebral ischaemia, whereas the CA3 and CA4 are relatively unaffected (Crain *et al.*, 1988). Of animals subjected to 5-minute bilateral carotid artery occlusion (BCO), over 90% develop uniform destruction in the striatum, cortex and especially the CA1 pyramidal cells of the hippocampus. This damage develops slowly over time until extensive cell death is observed 4 days following ischaemia. This has been termed “delayed neuronal death” (Kirino, 1982; Kirino *et al.*, 1986).

There have only been limited behavioural studies on gerbils after cerebral ischaemia, despite the need to examine a behavioural parameter with neurological deficits. Many investigators have reported the presence of ischaemia-induced hyperactivity. Chandler *et al.* (1985) reported hyperactivity 1 day after 5 and 20 minute occlusion. Also Maginn *et al.* (1995) demonstrated increased locomotor activity 72 hours after 5 minute BCO. Katoh *et al.*, (1992) reported that after 5 minute bilateral carotid occlusion there were increases in locomotor activity 1 and 3 days post-surgery but that these increases returned to control values 5 and 7 days post-occlusion. However, hyperactivity has been reported up to 28 days after 5-minute BCO (Mileson & Schwartz, 1991). A possible explanation of the difference observed here could be habituation of the animals to the environment, resulting from repeated exposure to the locomotor activity apparatus. Indeed, Babcock *et al.* (1993) demonstrated that hyperactivity of 5 minute BCO animals returned to control values on days 13 and 14 when the animals were tested every day for 14 days. Whereas, in animals receiving the same treatment, but tested only on days 13 and 14, hyperactivity was still present demonstrating the involvement of habituation/memory in ischaemic damage. It has been shown that 5 days pre-exposure to the open field test can block the development of hyperactivity (Wang & Corbett, 1990). It was concluded from this that cerebral ischaemic damage did not just cause a simple increase in motor activity, but rather caused a loss in the animal’s ability to form spatial maps.

In 1988, Gerhardt and Boast demonstrated that the degree of hippocampal damage was positively correlated with the increase in motor activity. An earlier study carried out by Araki *et al.* (1986) made similar findings. They showed that the hippocampal damage, especially abnormalities in the CA1 neurons, may be related to deficits in memory following bilateral common carotid artery occlusion.

3.2 EXPERIMENTAL PROCEDURE

Male Mongolian gerbils (*Meriones unguiculatus*) at least three months old and weighing in excess of 50g were obtained from the Medical Biology Centre in The Queen's University of Belfast. The gerbils were housed three or four to a cage for at least one week before commencement of the experiment. Standard laboratory food and water were available *ad libitum*. The gerbils were maintained at an ambient temperature of 21 ± 1 °C under a standard 12-hour light/dark cycle (light: 7am – 7pm).

On the day of commencement of the experiment the gerbils were anaesthetised, in a chamber, with the gaseous anaesthetic halothane (5% halothane in 95%O₂/5%CO₂), and maintained with 2-3% halothane delivered via facemask throughout the experiment.

A ventral midline cervical incision was made and the connective tissue was separated until the common carotid arteries could be seen. These were then freed from the surrounding connective tissue taking care to avoid damaging the vagus or sympathetic nerves, which run alongside the carotid arteries. In those animals undergoing carotid occlusion, a short length of silastic tubing was carefully inserted under each artery and twisted three times to cause occlusion of the artery.

After 5 minutes of occlusion, the blood flow was restored by untwisting the tubing and removing it from under the arteries. The wound was then sutured and the gerbil allowed to recover.

The body temperature of the animal was maintained throughout the experiment by using a thermostatically controlled heating blanket and by heating the room to an ambient temperature of 30°C. Rectal temperatures were taken during the experiment and for up to 3 hours afterwards where appropriate to the experimental design.

When it was applicable to the experiment, locomotor activity was recorded 72 hours after 5 minutes bilateral carotid artery occlusion. The activity monitors were supplied

by Benwick (AM 1051) and consisted of a perspex cage (10" x 7.5") crisscrossed by infrared light beams to produce a grid. The fine beam setting was used which produces a grid of 12 beams by 7 beams. There were two levels of beams and 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. The gerbils were placed singly in the activity monitors and the activity recorded over a thirty-minute period.

After 96hours the gerbils were anaesthetised with chloral hydrate (350mg/kg i.p.) in saline. The animals were then transcardially perfused with 20ml of a heparinised saline solution followed by 100ml of 5% sucrose/10% formalin mixture (phosphate buffered pH 7.0). After perfusion, the brains were removed and placed in a scintillation vial containing 8ml of the fixative formalin solution (5% formalin/30% sucrose, pH 7.0) (NaH₂PO₄ 3.5g, Na₂HPO₄ 6.5g, sucrose 300g, formalin 50ml, distilled water up to 1L).

The brains were removed from the formalin solution before processing and cut into smaller sections containing the hippocampus. These were then placed in cassettes for processing. The processing was carried out in the Aquatic Veterinary Group, which is part of the National Diagnostics Centre in the National University of Ireland, Galway. The process was carried out using an automated, computerised tissue processor (Citadel 2000). The tissue processing involved a 24-hour programme, involving a slow dehydration of the sample and embedding in wax. The protocol is detailed below:

Alcohol 70% -	1.5 hr
Alcohol 90% -	1.5 hr
Alcohol 100% -	1.5 hr
Alcohol 100% -	2.0 hr
Alcohol 100% -	2.0 hr
Alcohol 100% -	2.0 hr
Xylene -	2.5 hr

Xylene	-	2.5 hr
Xylene	-	2.5 hr
Xylene	-	2.0 hr
Paraffin wax	-	1.5 hr
Paraffin wax	-	2.5hr

The tissue was placed using forceps in a mould and filled with wax; the wax and tissue were kept warm during the orientation procedure. The mould was then placed on a cold surface and the tissue was held in position. A cassette was placed on top of the mould, filled with hot wax and the mould was placed on a cold tray to set. It is important that the wax used had not been repeatedly melted and set as this would effect the consistency of the paraffin wax thereby affecting the absorption of wax into the specimen. The mould was removed when the wax had set, excess wax was removed and the block stored at 4⁰C. Prior to cutting sections, the wax was trimmed down to the cutting face of the tissue on the microtome (Leica RM2135). Sections (5µm) were then cut, floated out on a water bath of distilled water (46⁰C), picked up on a clean glass microscope slide and allowed to dry.

Cresyl violet was used to stain the tissue. Cresyl violet (0.4g) was dissolved in 400ml of distilled water. The solution was heated to 100⁰C, filtered using Whatman filter paper and allowed to cool. A 1% acetic acid solution was also used and was made by adding 1ml glacial acetic acid to 99ml of distilled water. All volumes used were 400ml.

The staining procedure was as follows:

1. Xylene (10 min to remove wax)
2. 100% ethanol (5sec, to remove xylene)
3. 100% ethanol (5sec)
4. 70% ethanol (5sec)
5. 30% ethanol (5sec)
6. Rinse in flowing water
7. Cresyl violet (10 min., to stain nuclei)

8. Rinse in flowing water
9. 30% ethanol (5sec, dehydration)
10. 70% ethanol (5sec, dehydration)
11. 95% ethanol (5sec, dehydration)
12. 95% ethanol + 1 drop 1% acetic acid (5sec, removes excess stain)
13. 100% ethanol (5sec, dehydration)
14. Xylene (5 min., remove alcohol)
15. Xylene (20sec, clear section)
16. Xylene (5 min, clear section)

The slides were then removed and a cover slip was placed on each one using a few drops of neutral mounting medium (DPX mountant, BDH laboratory supplies). The slides were then allowed to dry at room temperature for 24 hours, sorted and placed in a slide box.

Each section was examined under high magnification using a microscope with a graticule (Graticules, UK) inserted, containing grid lines (2mm x 2mm). The number of intact pyramidal cells in the CA1 region of the hippocampus are counted four times (at successive places along the CA1) and the neuronal density expressed as the number of cells per millimetre. The quantification of neuronal damage was performed totally blind. The results were then plotted on a graph of neuronal density per 1mm versus treatment.

Photographs were taken of the hippocampal slices using tungsten balanced, Fujichrome 64T, colour film. The slide was focussed on an inverted microscope with a camera attached and photographs were taken at bracketed exposures at plus or minus one f-stop.

All procedures underwent local ethical review and were carried out in accordance with guidelines laid down under the relevant legislation.

3.3 MATERIALS

Five different compounds were used in this series of experiments. Firstly EMLA (eutectic mixture of local anaesthetics (lignocaine and prilocaine)) was applied to the incision made during the surgery. This was assessed for possible use during future experiments, as pain relief for the gerbils after the surgery. This particular method of pain relief was assessed due to the fact that most of the substances commonly used for pain relief after surgery interact in some way with one or more of the systems involved in the damage due to cerebral ischaemia.

MK-801 (dizocilpine), a potent, non-competitive, antagonist of the NMDA receptor, has been demonstrated to protect against ischaemia-induced brain damage (Gill *et al.*, 1987; Gill *et al.*, 1988; O'Neill *et al.*, 1994). MK-801 was administered at a dose of 1mg/kg by the intraperitoneal route, 30 minutes after 5 minutes bilateral carotid artery occlusion.

N¹-Dansylspermine is a novel analogue of the polyamine, spermine. N¹-dansylspermine has been shown to be active at voltage-dependent, recombinant NMDA receptors (Chao *et al.*, 1997). It has also been demonstrated to antagonise the CNS excitation produced by direct intracerebroventricular administration of spermine and to antagonise the spermine enhancement of NMDA-induced convulsions as described earlier (Section 2). N¹-Dansylspermine was administered at doses of 2, 5 and 10mg/kg (i.p.) 30 minutes after 5-minute bilateral carotid artery occlusion. It was also administered 30 minutes after and 24 hours after occlusion.

Ifenprodil and eliprodil are polyamine antagonists that have been demonstrated to be effective neuroprotective agents, when given before and after occlusion in multiple doses (Bath *et al.*, 1996; Gotti *et al.*, 1988). There has, however, been some conflicting evidence. Some investigators have shown ifenprodil and eliprodil to be effective when given post-ischaemically, either by the intraperitoneal route or by intravenous infusion (Gotti *et al.*, 1988 and 1990; Scatton *et al.*, 1991; Bath *et al.*, 1996; Dogan *et al.*, 1997). However, others have shown ifenprodil to be ineffective

(Araki *et al.*, 1990; Sheardown *et al.*, 1993). Indeed the study carried out by Sheardown *et al.* (1993) showed that administration of ifenprodil (15mg/kg, ip), 60, 90 and 240 minutes after occlusion (total dose 45 mg/kg) gave no protection. Also the doses used vary greatly from small doses of ifenprodil of 7mg/kg ip (Gotti *et al.*, 1990) to doses of 30mg/kg or 45mg/kg (Araki *et al.*, 1990; Sheardown *et al.*, 1993). In this study, however, ifenprodil 30mg/kg(i.p.) was administered 30 minutes after occlusion and eliprodil 10mg/kg (i.p.) was also administered 30 minutes after occlusion.

3.4 TREATMENT OF RESULTS

All results were plotted as mean neuronal density per mm (\pm SEM) versus the specific treatment. The treatment groups were compared using one-way ANOVA with post-hoc Newman-Keuls to determine if there was a statistically significant difference between the treatments. The neuronal densities are presented in a single table at the end of the results section (Table 3.1). The rectal temperatures of the animals were plotted as mean temperature \pm SEM (standard error of the mean) versus time. The locomotor activity was presented as graphs of slow mobile count \pm SEM versus time again in both cases the differences between groups were assessed using one-way ANOVA with repeated measures followed by subsequent analysis of simple main effects. Between group differences were analysed with further ANOVA followed by post-hoc Newman-Keuls test. Statistical significance in all tests was accepted at the $p < 0.05$ level, with the actual p values shown in the Figures and Table 3.1.

The design of the experiments followed the general structure shown below. For the evaluation of a drug A in vehicle, the following dosing groups were used.

- Group 1 : BCO + drug A
- Group 2 : BCO + vehicle
- Group 3 : Sham operated + drug A
- Group 4 : Sham operated + vehicle

This design covers all eventualities and, as a result, statistical tests can easily be used to determine if there are any significant differences between the groups.

3.5 RESULTS

3.5.1 EMLA

All of the pyramidal cell densities calculated in the following experiments are shown in Table 3.1 at the end of Section 3.5. EMLA was administered to the wound on the gerbils' neck after the incision had been sutured. This was to provide some measure of post-operative pain relief for the gerbils. The neuronal density was plotted versus the treatment groups. Figure 3.2 demonstrates the difference in neuronal density between a sham operated group of gerbils and those that underwent bilateral carotid artery occlusion. This shows that occluding the carotid arteries for 5 minutes causes an approximate 65% reduction in the pyramidal cell density in the CA1 layer of the hippocampus. Figure 3.2 also shows that the application of EMLA does not provide any measure of neuroprotection, as there is no significant difference between the BCO + EMLA group and the BCO + vehicle group. Figures 3.3 & 3.4 show photographs taken of hippocampal coronal slices. Figure 3.3 shows a photomicrograph of a section taken from a sham operated gerbil. At low magnification, a dark purple line can be made out clearly, which represents the pyramidal cell line. The higher magnification picture is the magnification of a section of the CA1 and the individual pyramidal cells can be clearly seen as dark purple shapes. Figure 3.4 shows a photograph of a section taken from a gerbil that underwent 5-minute bilateral carotid artery occlusion. The formerly intact CA1 layer has now almost entirely disappeared. On the higher magnification, this is even clearer as there are only a few pyramidal cells left intact.

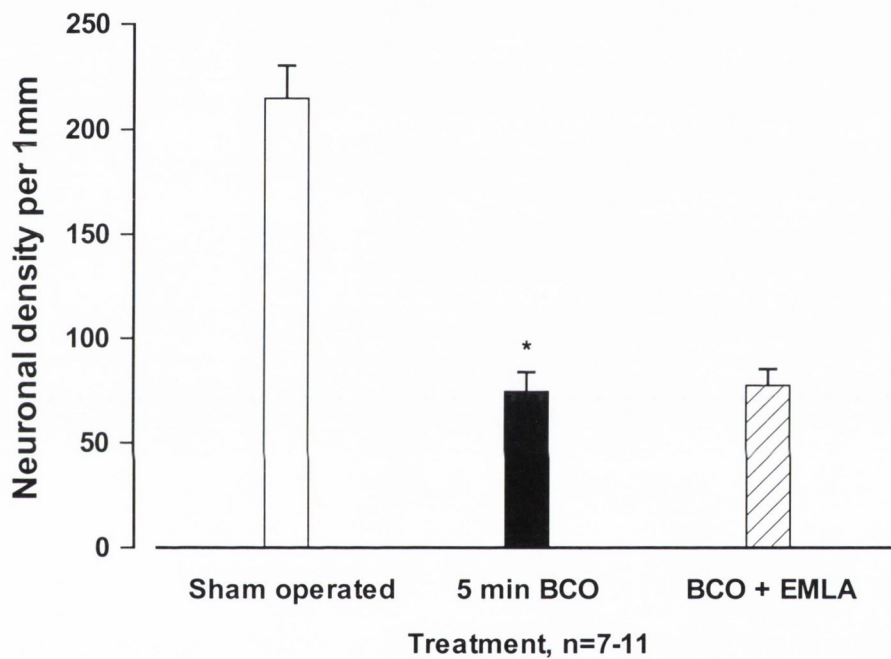


Figure 3.2: Effect of 5-minute bilateral carotid artery occlusion and EMLA applied after occlusion on neuronal density in the CA1 region of the hippocampus in the Mongolian gerbil. * $p < 0.001$ Vs sham operated (one-way ANOVA). Results are presented as means \pm SEM (standard error of the mean).

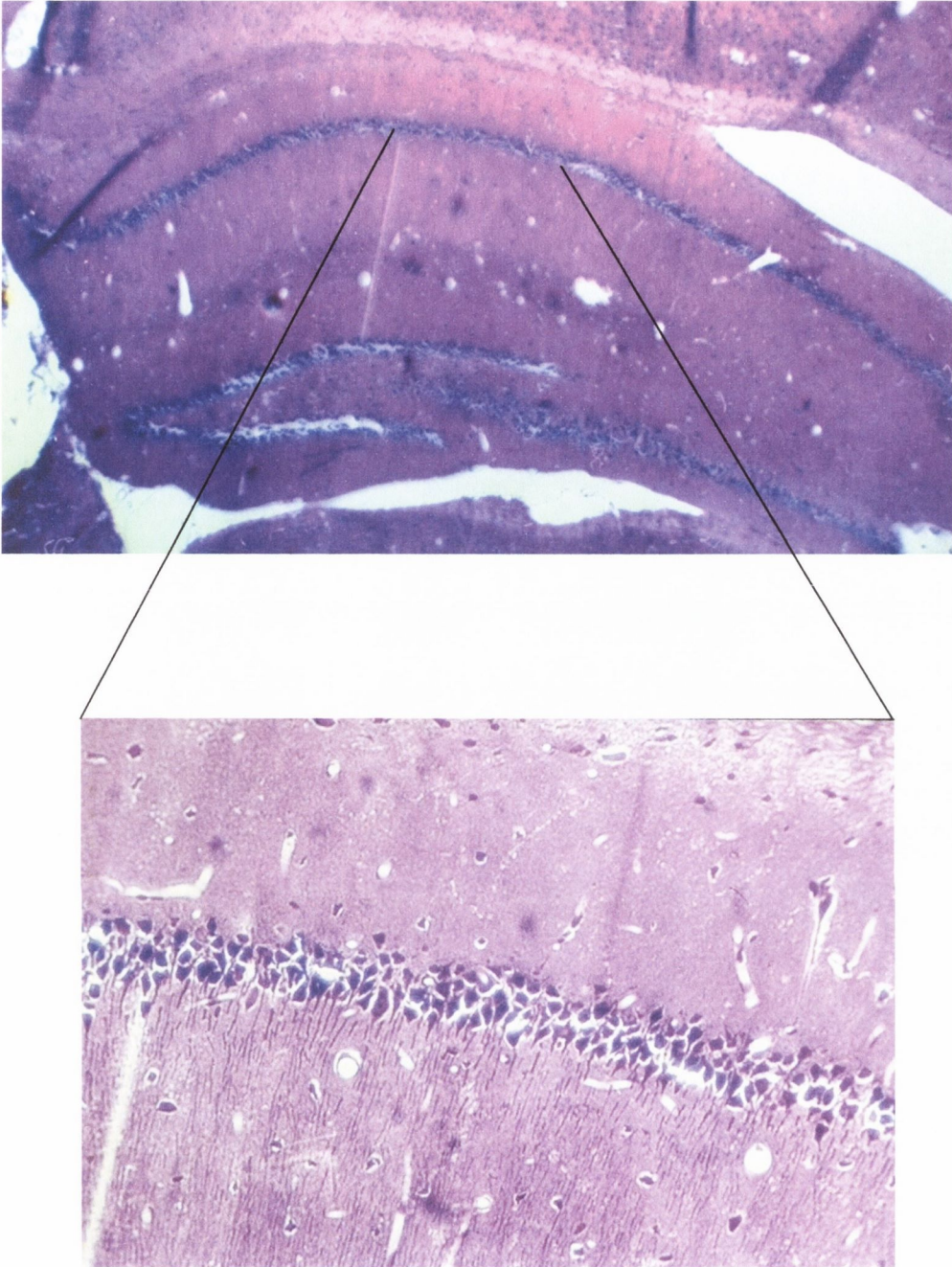


Figure 3.3: Low and high magnification photomicrographs of the hippocampus of a sham operated gerbil, showing the intact CA1 pyramidal cell layer.

_____ = 0.5/0.1mm (low/high magnification)

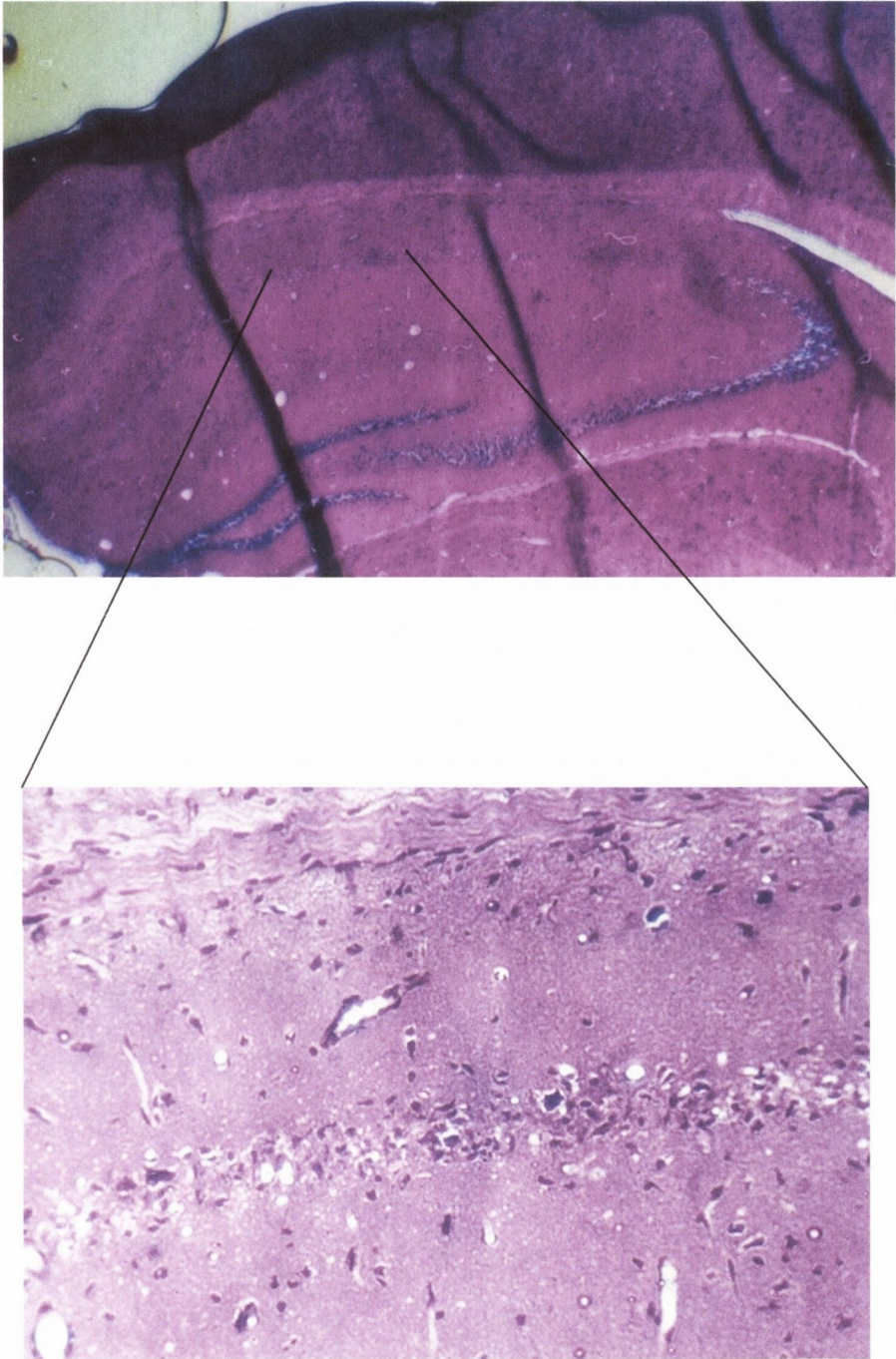


Figure 3.4: Low and high magnification photomicrograph of the hippocampus of a gerbil that has undergone 5-minute bilateral carotid artery occlusion, demonstrating the reduced pyramidal cell density in the CA1 region.

_____ = 0.5/0.1mm (low/high magnification)

3.5.2 MK-801

MK-801 (dizocilpine) was administered, by the intraperitoneal route, in a 0.9% saline solution at a dose of 1mg/kg. This dose has previously been demonstrated to be neuroprotective in work in other laboratories. The dose of MK-801 was given 30 minutes after 5-minute bilateral carotid artery occlusion. As can be seen from Figure 3.5, this dose of dizocilpine caused significant neuroprotection. A photograph of a section from a gerbil that has been treated with the neuroprotective dose of MK-801 is shown in Figure 3.6. From this figure it can be seen that the CA1 layer is more intact than that from a BCO operated animal. At higher magnification this is clearer still. It is possible to make out the intact pyramidal cells quite clearly. When this is compared to the slice from an untreated gerbil (Figure 3.4) it can be seen that MK-801 is indeed neuroprotective as the CA1 layer has been comparatively restored.

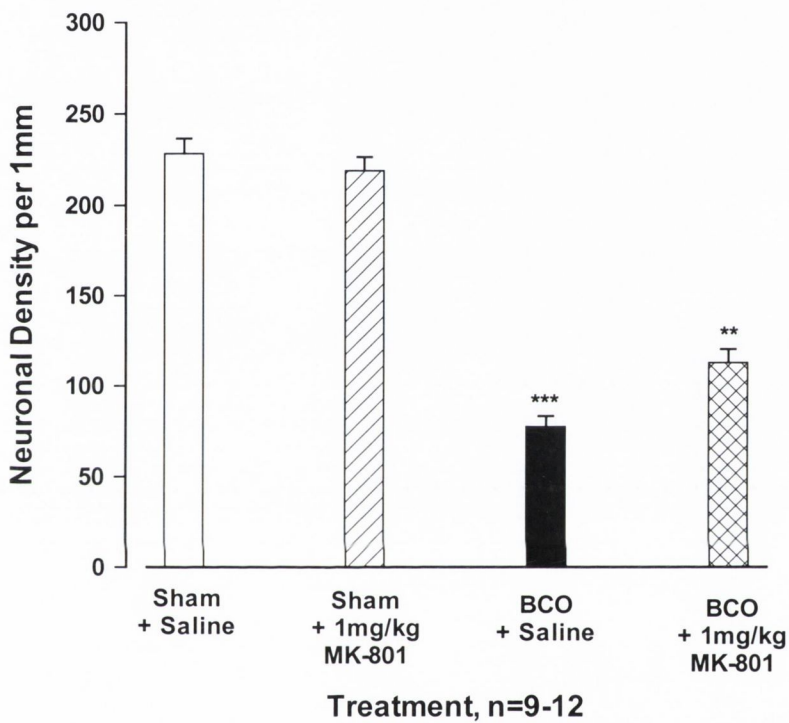


Figure 3.5: The effect of MK-801 administered 30 minutes after 5min BCO on the neuronal density of the CA1 region of the hippocampus in the Mongolian gerbil, measured 96h post surgery. $**p < 0.01$ Vs BCO + saline (one-way ANOVA); $***p < 0.001$ Vs Sham operated (one-way ANOVA). Results are presented as means \pm SEM.

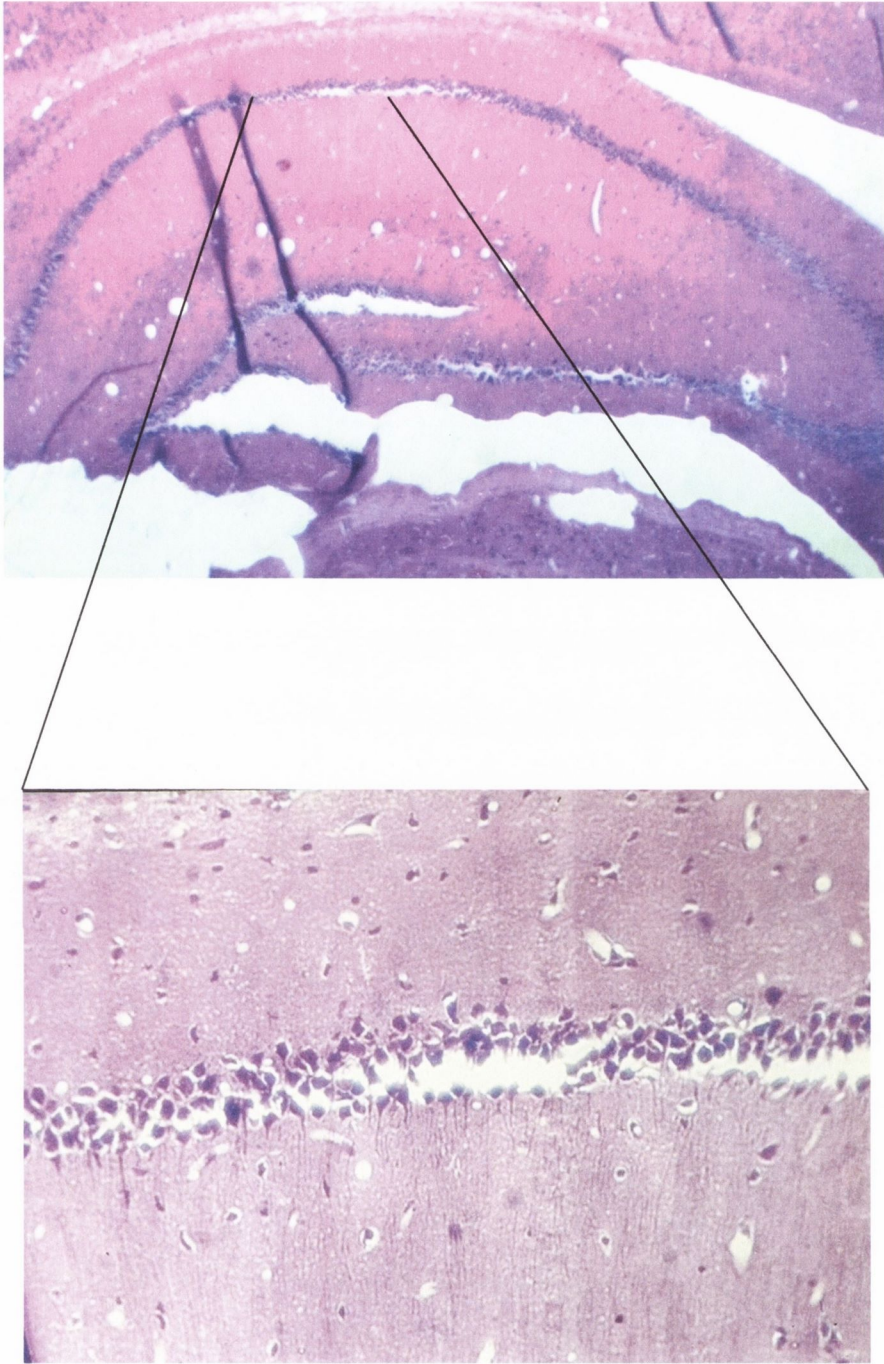


Figure 3.6: Low and high magnification photomicrograph of the hippocampus of a gerbil that has undergone 5-minute bilateral carotid artery occlusion, demonstrating the neuroprotective effect of 1mg/kg MK-801.

_____ = 0.5/0.1mm (low/high magnification)

3.5.3 N¹-Dansylspermine

N¹-Dansylspermine was administered at doses of 2mg/kg, 5mg/kg or 10mg/kg in a 0.9% saline solution. The dose of 2mg/kg caused a slight degree of neuroprotection after cerebral ischaemia. This was demonstrated by the increase in pyramidal cell density in the CA1 layer of the hippocampus when compared to a gerbil that was treated with vehicle. The increase is of approximately 12%, as can be seen from the figures included in Table 3.1. This increase, however, was not statistically significant when examined by one-way analysis of variance.

At the higher doses of 5mg/kg and 10mg/kg, N¹-dansylspermine showed significant neuroprotection on the pyramidal cell population. It produced increases in neuronal density, compared to 5-minutes bilateral carotid occlusion, of 51% and 83% respectively. These results are illustrated in Figures 3.7 and 3.8. On analysis with ANOVA these differences were both shown to be statistically significant ($p < 0.01$).

Figure 3.9 shows a photograph of a hippocampal section from a gerbil that was treated with 5mg/kg N¹-dansylspermine. This is quite similar to that from the gerbil treated with MK-801 (Figure 3.6) and demonstrates, visually, the neuroprotective properties of N¹-dansylspermine when compared to an untreated occluded gerbil (Figure 3.4).

N¹-dansylspermine, 10mg/kg, was given 30 minutes after and 24 hours after occlusion. An increase in pyramidal cell density, relative to 5-minute BCO, of 43% was found and was significant at the 95% level ($p < 0.05$). This is illustrated in Figure 3.10.

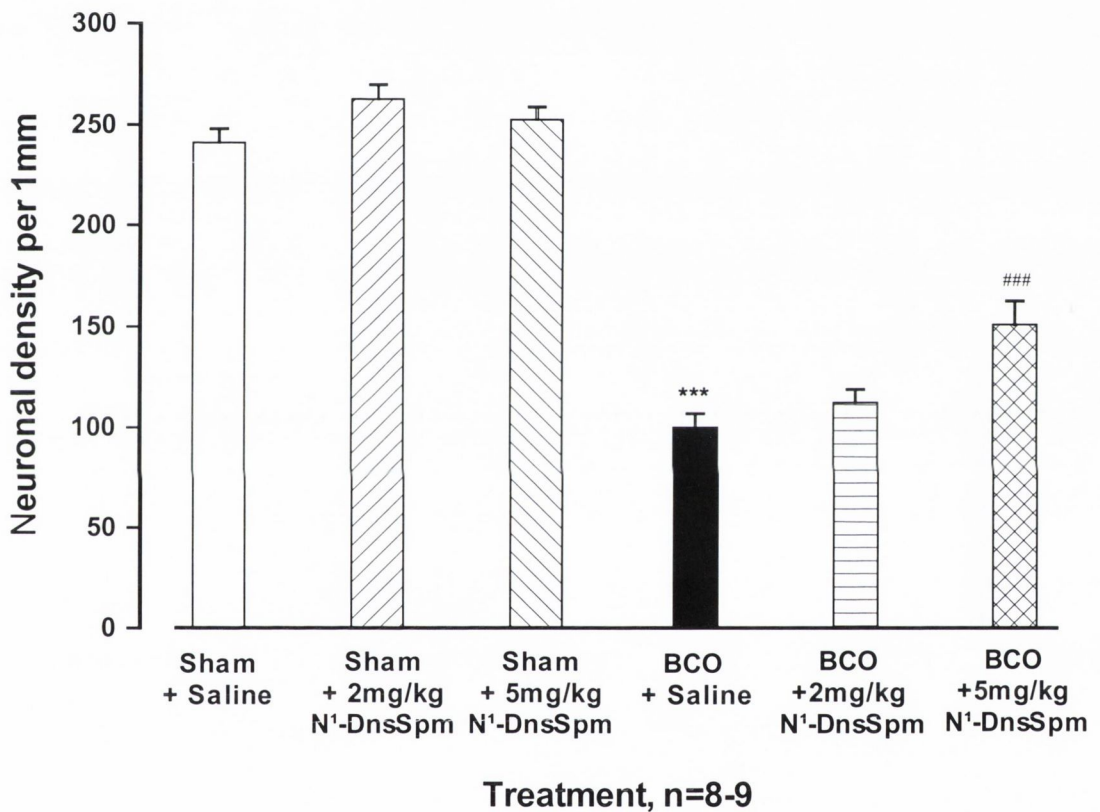


Figure 3.7: The effect of N¹-dansylspermine administered 30 minutes after 5min BCO on the neuronal density of the CA1 region of the hippocampus in the Mongolian gerbil, measured 96h post surgery. ###*p*<0.01 Vs BCO + saline (one-way ANOVA); ****p*<0.001 Vs Sham operated (one-way ANOVA). Results are presented as means±SEM.

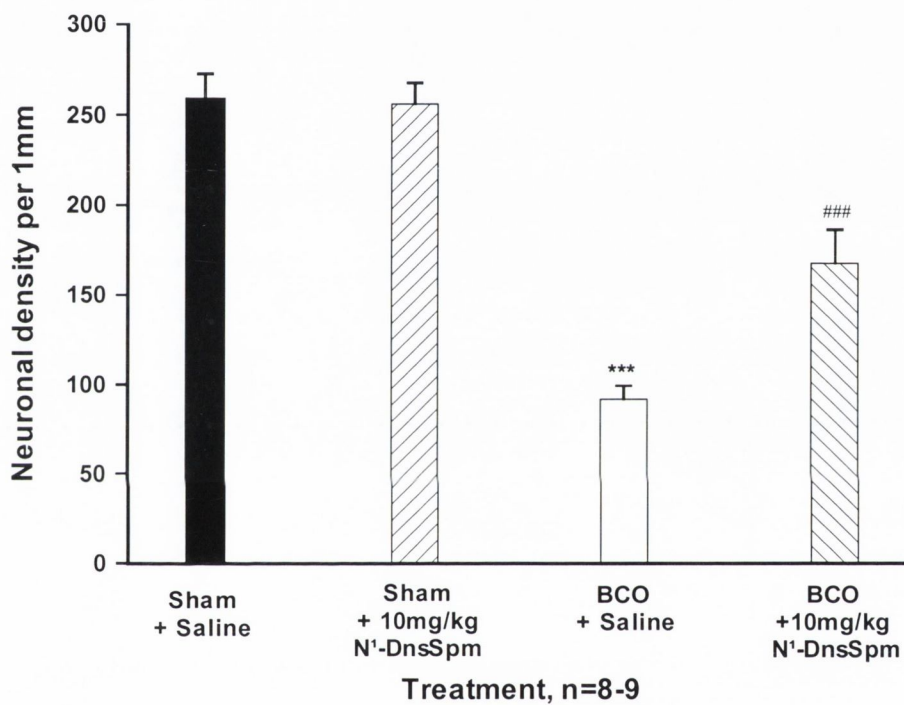


Figure 3.8: The effect of N^1 -dansylspermine administered 30 minutes after 5min BCO on the neuronal density of the CA1 region of the hippocampus in the Mongolian gerbil, measured 96h post surgery. ### $p < 0.001$ Vs BCO + saline (one-way ANOVA); *** $p < 0.001$ Vs Sham operated (one-way ANOVA). Results are presented as means \pm SEM.

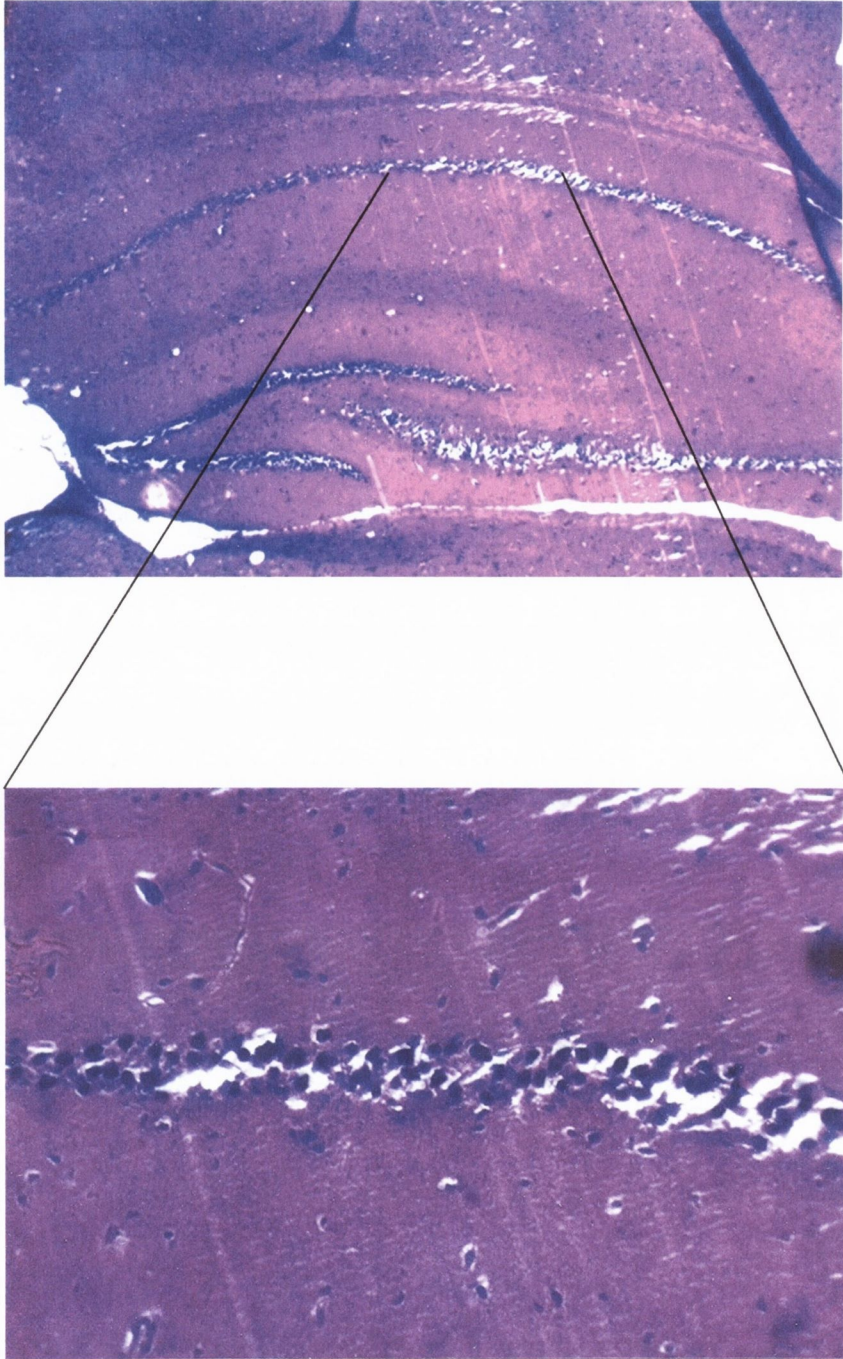


Figure 3.9: Low and high magnification photomicrograph of the hippocampus of a gerbil that has undergone 5-minute bilateral carotid artery occlusion, demonstrating the neuroprotective effect of 5mg/kg N^1 -dansylspermine.

_____ = 0.5/0.1mm (low/high magnification)

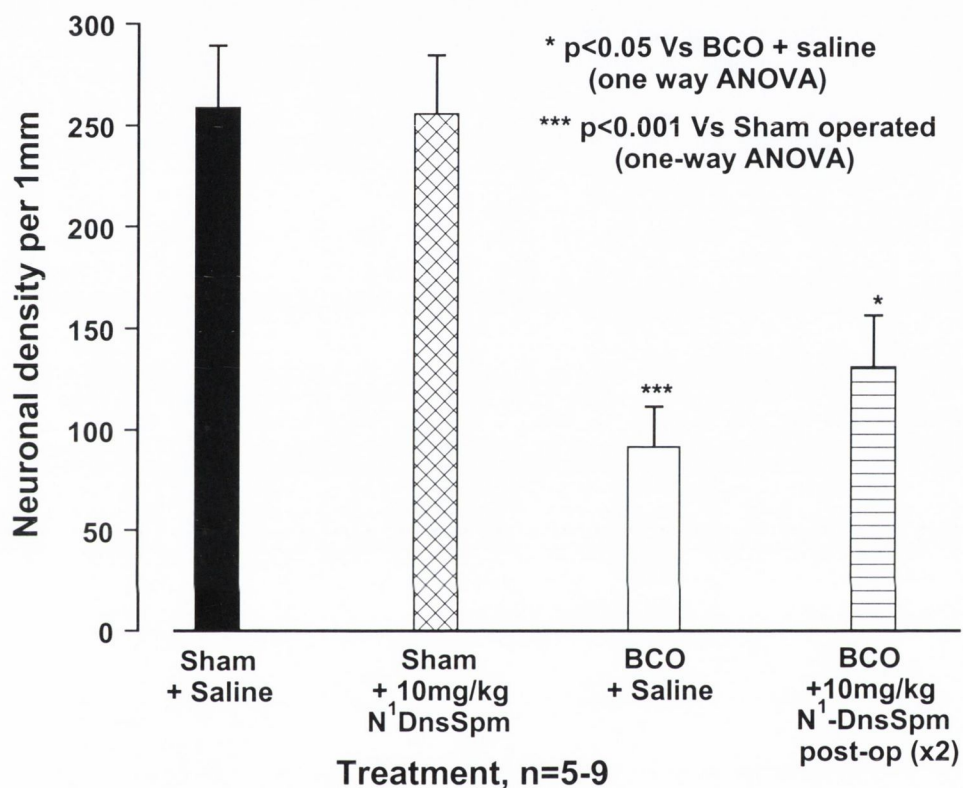


Figure 3.10: The effect of N¹-dansylspermine administered 30 minutes and 24 hours after 5min BCO on the neuronal density of the CA1 region of the hippocampus in the Mongolian gerbil, measured 96h post surgery. *p<0.05 Vs BCO + saline (one-way ANOVA); ***p<0.001 Vs Sham operated (one-way ANOVA). Results are presented as means±SEM.

3.5.3.1 Effect on Locomotor activity

Activity measurements were recorded for N¹-dansylspermine at 5mg/kg and at 10mg/kg, administered by the intraperitoneal route, 30 minutes after occlusion. These results are illustrated in Figures 3.11 & 3.12. As can be seen from both figures, the sham-operated and sham-operated with N¹-dansylspermine animals have an initial high activity count which, over the 30-minute observation time, decreases to a low level of activity. Bilateral carotid artery occlusion (5 minutes) results in a very high initial activity and this only slowly decreases over time and is statistically significantly different from the sham-operated between the 95% and 99.9% levels. When N¹-dansylspermine is administered at 5mg/kg, there is an increase in the activity relative to the BCO group but the difference is not statistically significant. This shows a lack of ability of N¹-dansylspermine to reverse the increase in activity due to occlusion (Figure 3.11). Similarly, at the 10mg/kg dose N¹-dansylspermine shows an inability to reverse the hyperactivity produced by 5-minute bilateral carotid occlusion (Figure 3.12). Analysis of the simple main effects showed no effect of administration of either dose of N¹-dansylspermine.

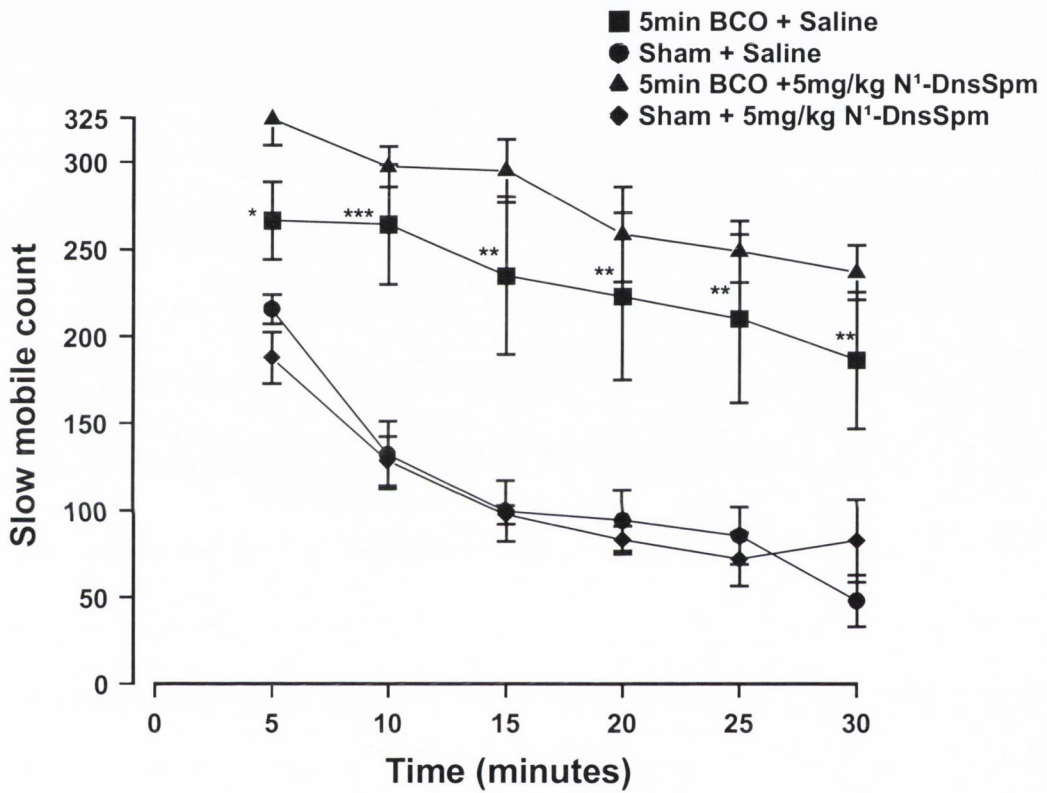


Figure 3.11: Effect of 5mg/kg N¹-dansylspermine on ischaemia induced hyperactivity in the Mongolian gerbil, measured 72hr after bilateral carotid occlusion. * $p < 0.05$ Vs sham + saline (one-way ANOVA); ** $p < 0.01$ Vs sham + saline (one-way ANOVA); *** $p < 0.001$ Vs sham + saline (one-way ANOVA). Results are presented as means \pm SEM.

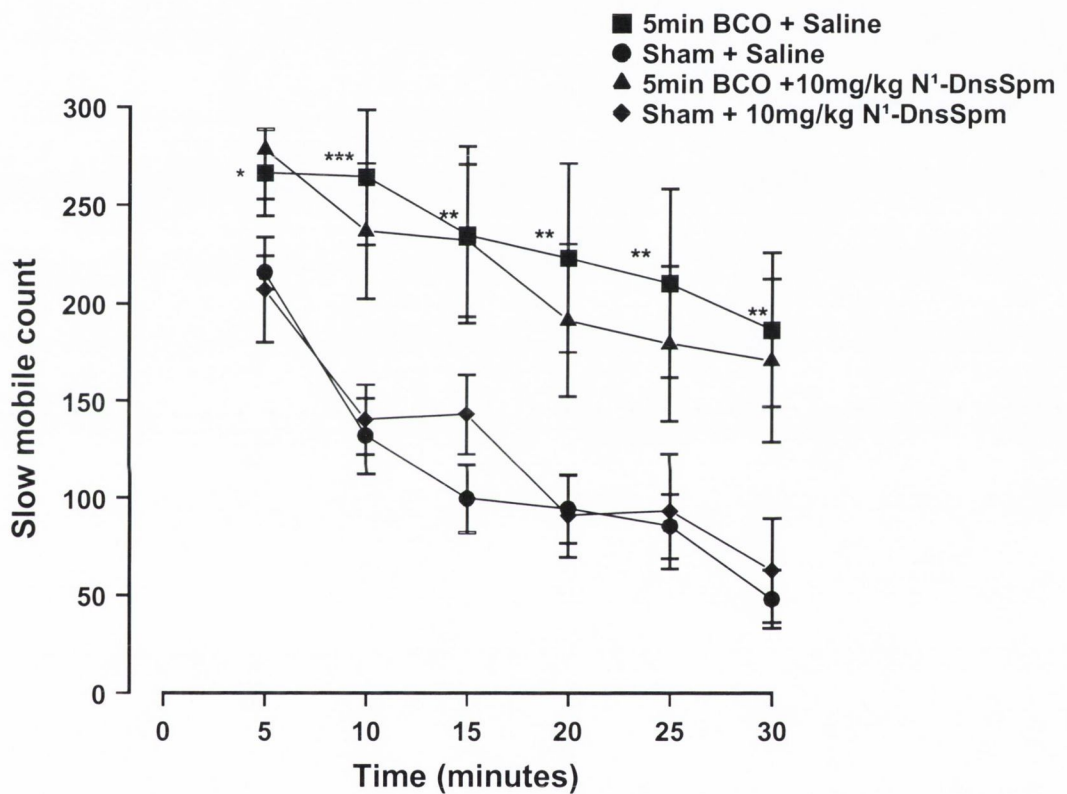


Figure 3.12: Effect of 10mg/kg N¹-dansylspermine on ischaemia induced hyperactivity in the Mongolian gerbil, measured 72hr after bilateral carotid occlusion. * $p < 0.05$ Vs sham + saline (one-way ANOVA); ** $p < 0.01$ Vs sham + saline (one-way ANOVA); *** $p < 0.001$ Vs sham + saline (one-way ANOVA). Results are presented as means \pm SEM.

3.5.3.2 *Effect on temperature*

The temperature measurements were commenced before the 5-minute occlusion and were then recorded at 30 minutes after occlusion, one, two and three hours after occlusion. From Figures 3.13 & 3.14, it can be seen that 5-minute bilateral carotid artery occlusion results in an increase in body temperature of approximately 1-1.5 degrees celsius. However, this was not a statistically significant increase as there was large variation in the body temperature readings. However, this does demonstrate that the measures taken to ensure that the gerbils did not become hypothermic while under anaesthesia were indeed effective. When N¹-dansylspermine was administered at 5mg/kg there was no significant change in the body temperatures recorded over the 3-hour period (Figure 3.13). However when the 10mg/kg dose was used there was a drop in body temperature compared to the normal body temperature and this reached statistical significance at the 120 minute mark ($p < 0.01$) (Figure 3.14). However, this reduction is unlikely to exert a neuroprotective effect (See Section 3.6). When given at 10mg/kg in the sham operated animals there was a significant reduction in body temperature when compared to the sham operated with saline. The analysis of simple main effects showed an effect of N¹-dansylspermine on temperature but as mentioned above this was only statistically significantly different to the sham operated with saline at one time point.

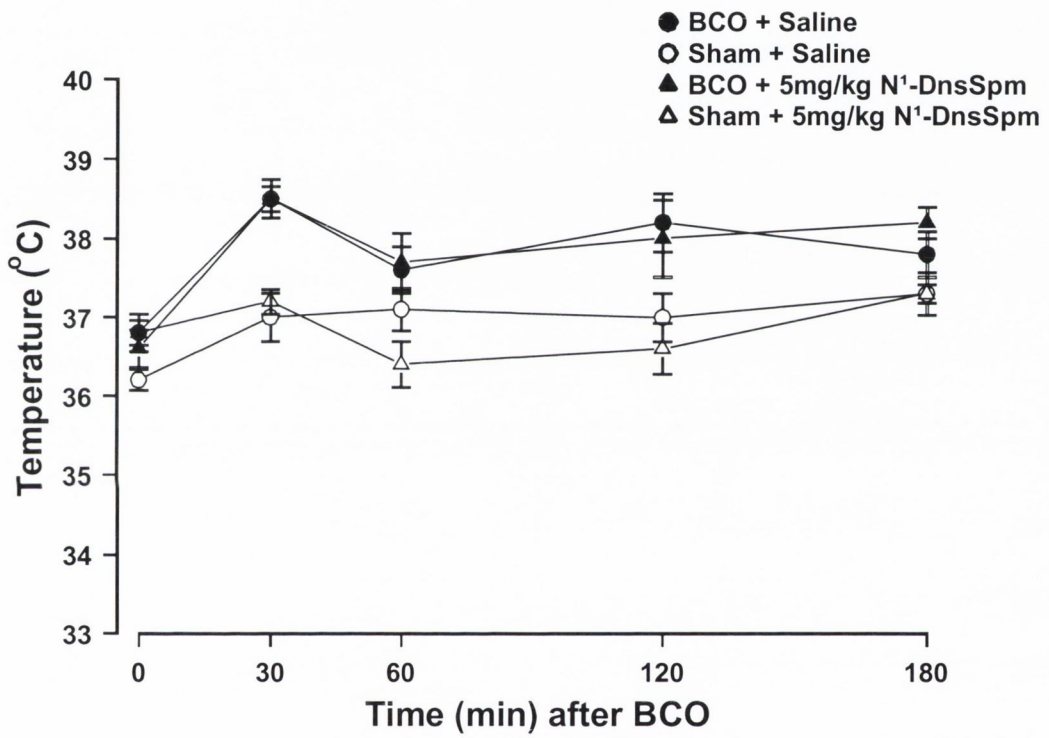


Figure 3.13: Effect of 5mg/kg N¹-dansylspermine on body temperature in the Mongolian gerbil following bilateral carotid occlusion. Results are presented as means \pm SEM.

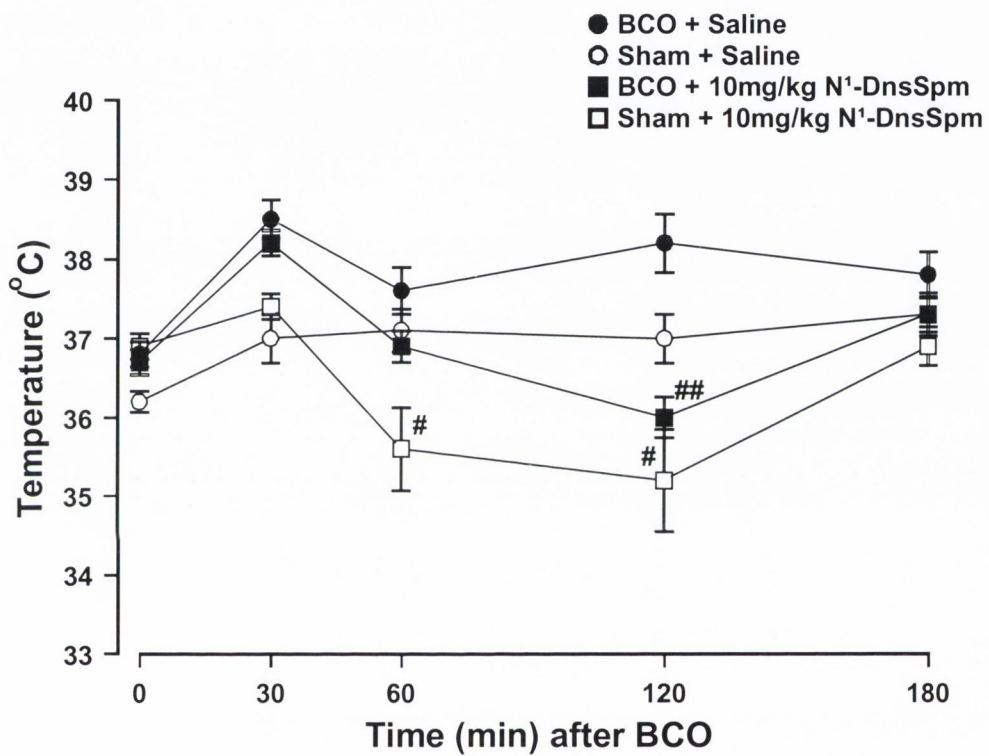


Figure 3.14: Effect of 10mg/kg N¹-dansylspermine on body temperature in the Mongolian gerbil following bilateral carotid occlusion. # $p < 0.05$ Vs sham + saline (one-way ANOVA); ## $p < 0.01$ Vs sham + saline (one-way ANOVA). Results are presented as means \pm SEM.

3.5.4 Ifenprodil and eliprodil

Ifenprodil and eliprodil are both putative polyamine antagonists and were administered to the gerbils, by the intraperitoneal route, 30 minutes after occlusion, at doses of 30mg/kg and 10mg/kg respectively. As can be seen from both Figure 3.15 and Figure 3.16, both ifenprodil and eliprodil caused a marked neuroprotection of the pyramidal cells of the hippocampal CA1 layer. Ifenprodil caused an increase in pyramidal cell density of 59% (Table 3.1) which is statistically significant at the 99.9% level, compared to 5-minute BCO. Similarly, eliprodil was found to be neuroprotective in the gerbil model of cerebral ischaemia co-incidentally resulting in an increase of 59% in neuronal density, compared to 5-minute BCO. Hippocampal coronal sections are shown in Figures 3.17 and 3.18.

3.5.4.1 Effect on locomotor activity

Activity counts were recorded during the study. As before, the gerbils that had undergone 5-minute bilateral carotid artery occlusion, showed a raised activity count that did not reduce significantly when compared with the sham operated. However, unlike the N¹-Dansylspermine, ifenprodil (30mg/kg) reduced the hyperactivity caused by occlusion and this reduction was statistically significant ($p < 0.01$) (Figure 3.19). Eliprodil (10mg/kg) also reduces the ischaemia-induced hyperactivity (Figure 3.20). However, at this dose the reduction did not reach statistical significance.

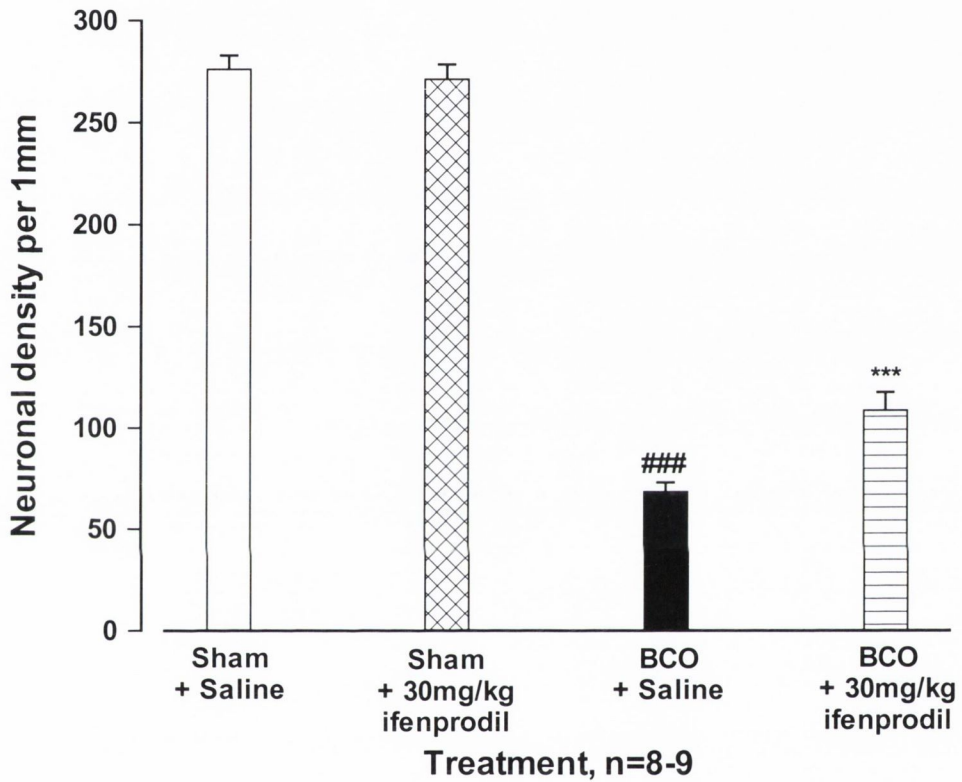


Figure 3.15: The effect of ifenprodil, administered 30 minutes after 5min BCO, on the neuronal density of the CA1 region of the hippocampus in the Mongolian gerbil, measured 96h post surgery. ### $p < 0.001$ Vs Sham operated (one-way ANOVA) *** $p = 0.001$ Vs BCO + saline (one-way ANOVA). Results are presented as means \pm SEM.

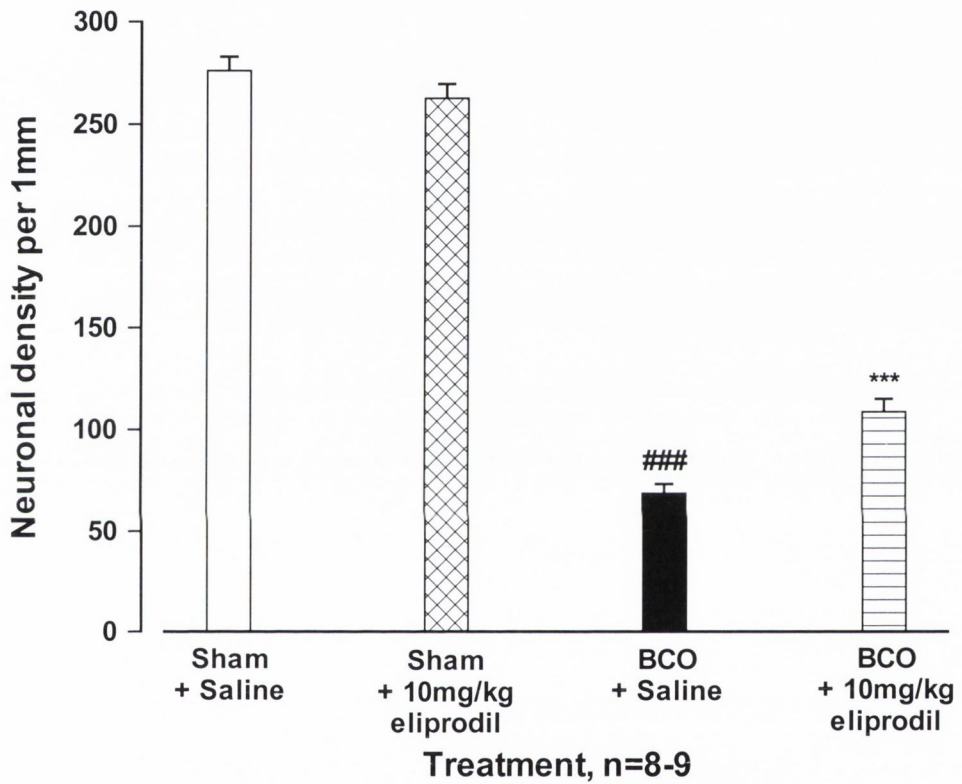


Figure 3.16: The effect of eliprodil, administered 30 minutes after 5min BCO, on the neuronal density of the CA1 region of the hippocampus in the Mongolian gerbil, measured 96h post surgery. ### $p < 0.001$ Vs Sham operated (one-way ANOVA) *** $p = 0.001$ Vs BCO + saline (one-way ANOVA). Results are presented as means \pm SEM.

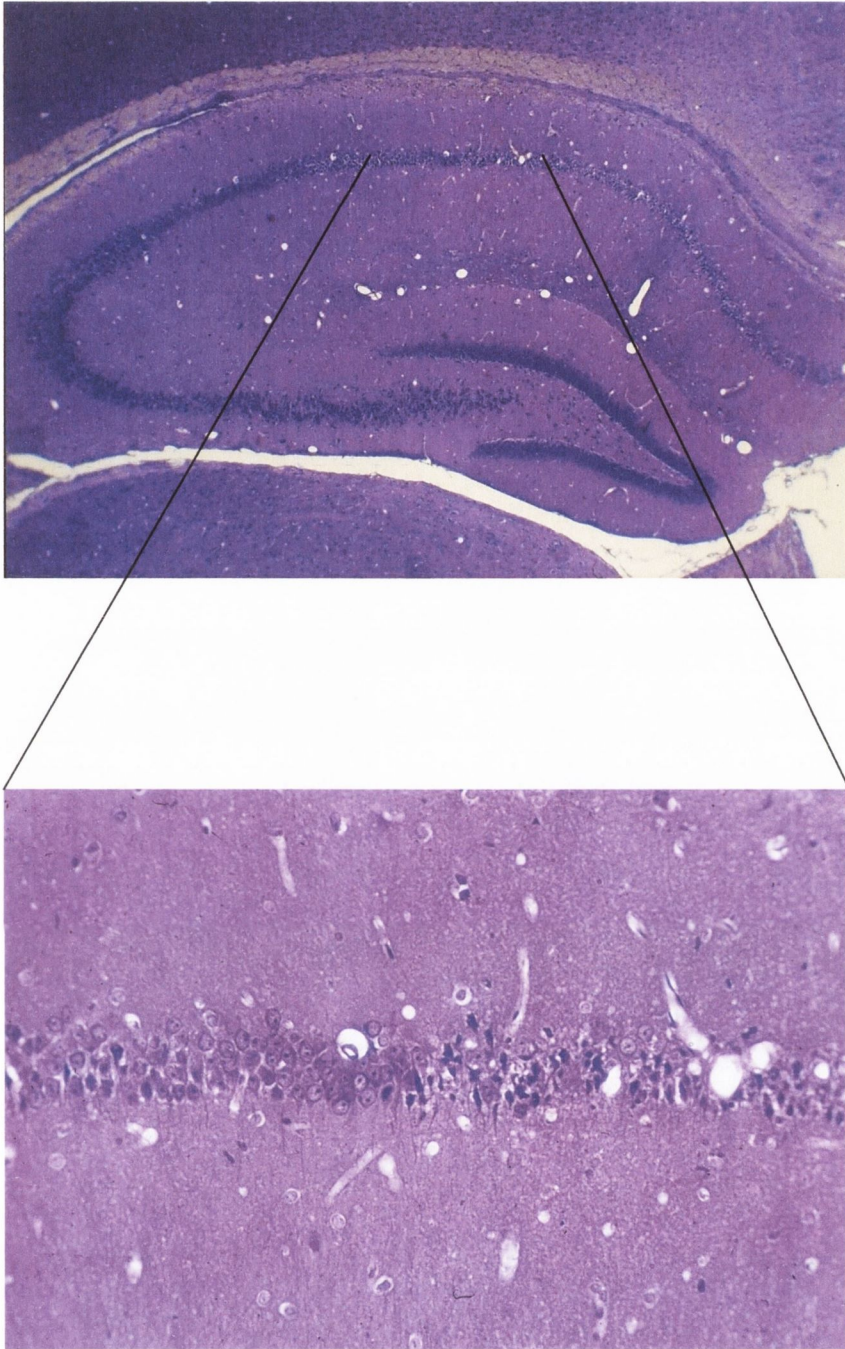


Figure 3.17: Low and high magnification photomicrograph of the hippocampus of a gerbil that has undergone 5-minute bilateral carotid artery occlusion, demonstrating the neuroprotective effect of 30mg/kg ifenprodil.

_____ = 0.5/0.1mm (low/high magnification)

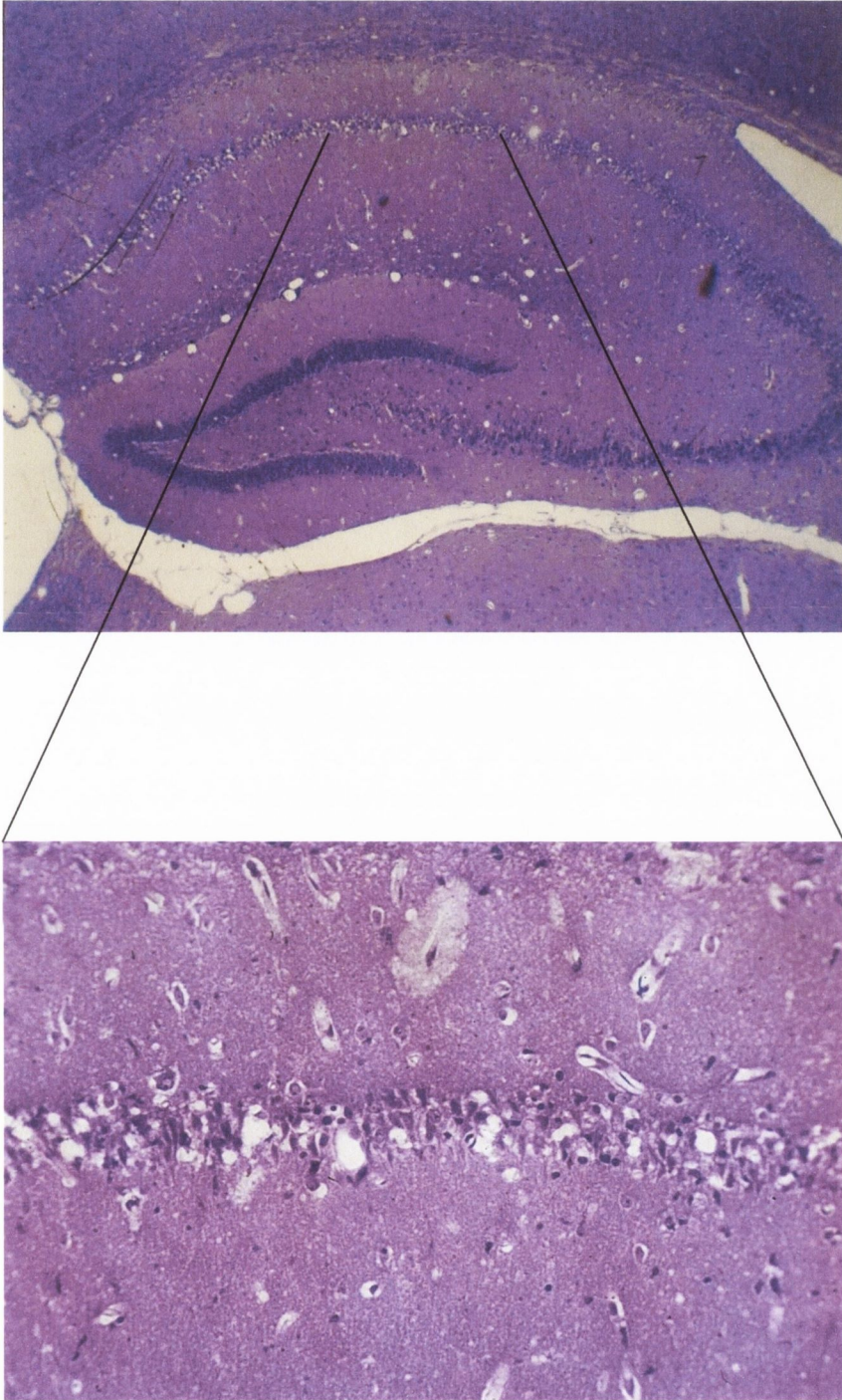


Figure 3.18: Low and high magnification photomicrograph of the hippocampus of a gerbil that has undergone 5-minute bilateral carotid artery occlusion, demonstrating the neuroprotective effect of 10mg/kg eliprodil.

_____ = 0.5/0.1mm (low/high magnification)

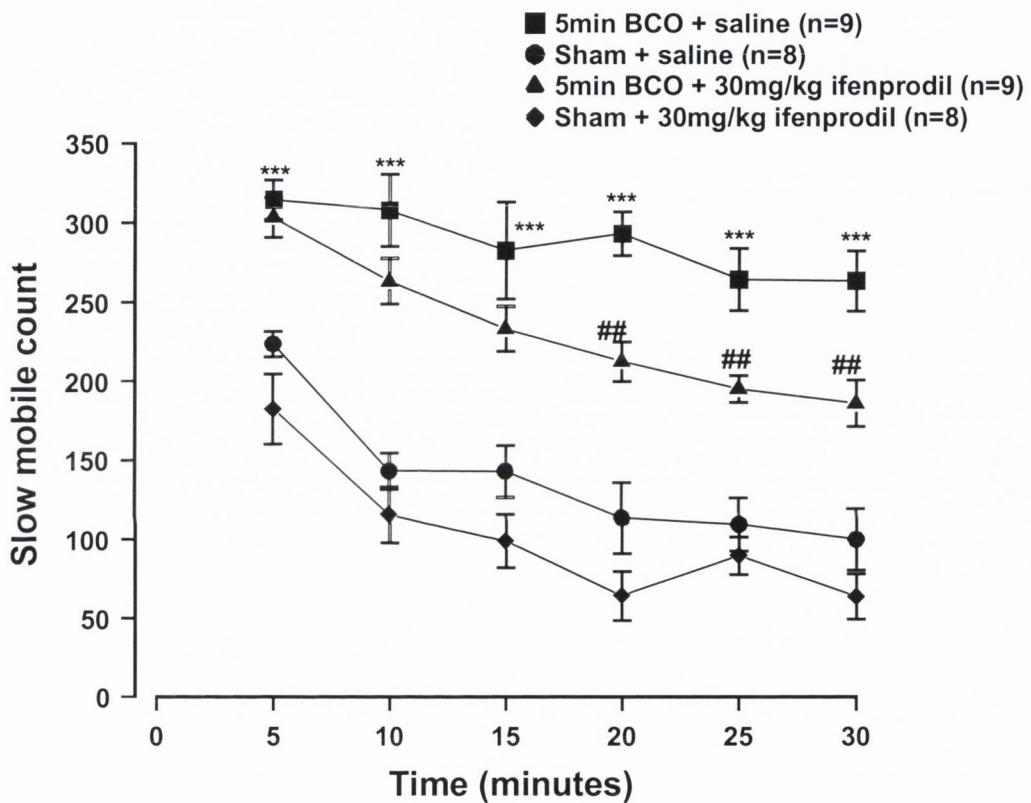


Figure 3.19: Effect of ifenprodil on ischaemia induced hyperactivity in the Mongolian gerbil, measured 72hr after bilateral carotid occlusion. *** $p < 0.001$ Vs Sham + saline (one-way ANOVA). ## $p < 0.01$ Vs BCO + saline (one-way ANOVA). Results are presented as means \pm SEM.

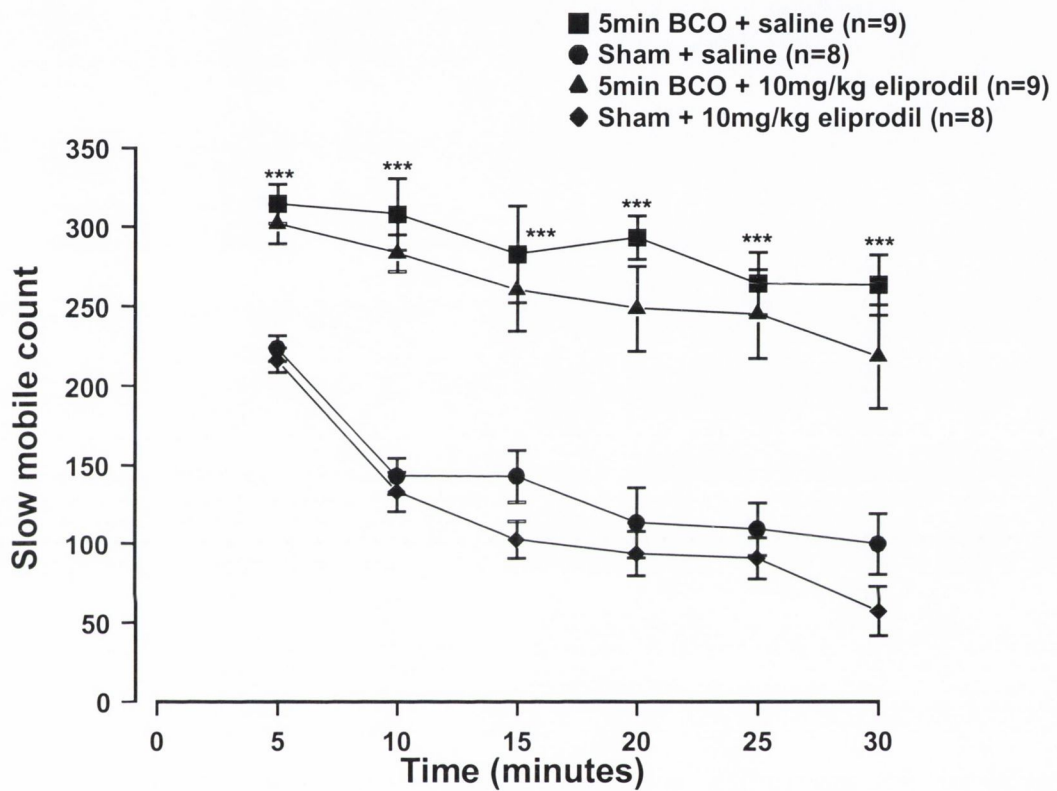


Figure 3.20: Effect of eliprodil on ischaemia induced hyperactivity in the Mongolian gerbil, measured 72hr after bilateral carotid occlusion. *** $p < 0.001$ Vs Sham + saline (one-way ANOVA). Results are presented as means \pm SEM.

3.5.4.2 Effect on temperature

The temperature measurements obtained are shown in Figures 3.21 & 3.22. It can be seen that, as previously described, there was an immediate rise in body temperature, after occlusion, of approximately 1 degree celsius, and the body temperature remained elevated relative to normal body temperature (sham operated with saline) throughout the experiment. When either ifenprodil or eliprodil was administered to the BCO gerbils there was a slight drop in the body temperature, however, at no stage did this temperature drop below that of normal body temperature represented by the sham + saline trace. The simple main effect of ifenprodil was to reduce the temperature slightly, irrespective of the treatment, however as mentioned above this did not drop below normal body temperature (sham + saline).

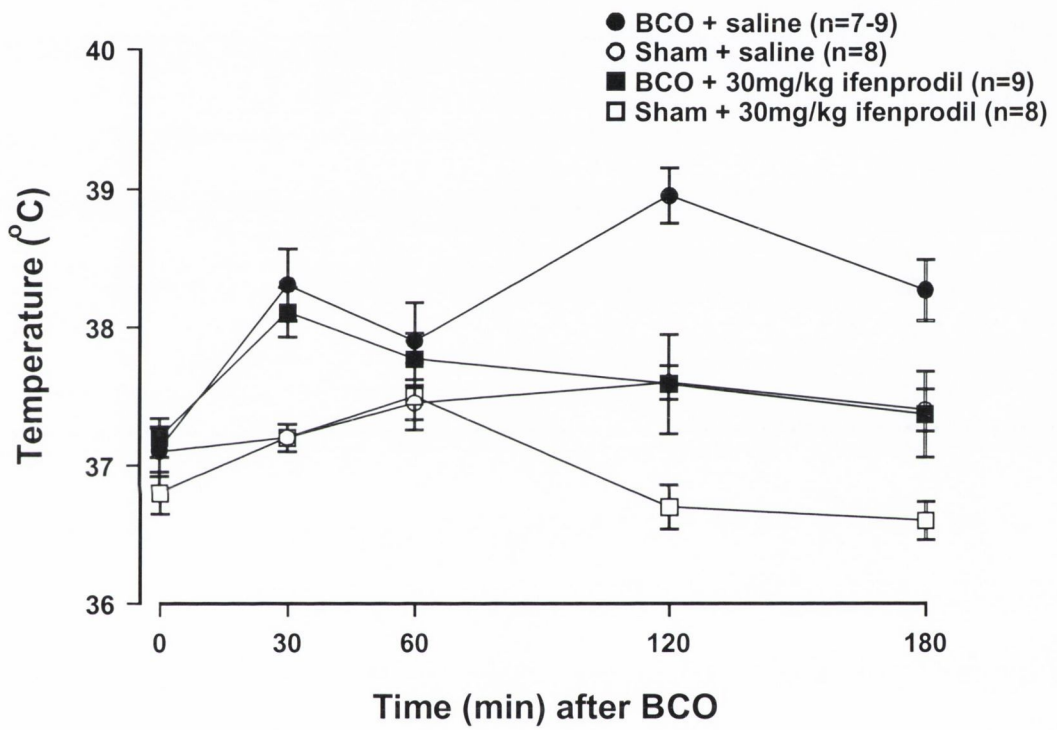


Figure 3.21: Effect of 30mg/kg ifenprodil on body temperature in the Mongolian gerbil following bilateral carotid occlusion. Results are presented as means \pm SEM.

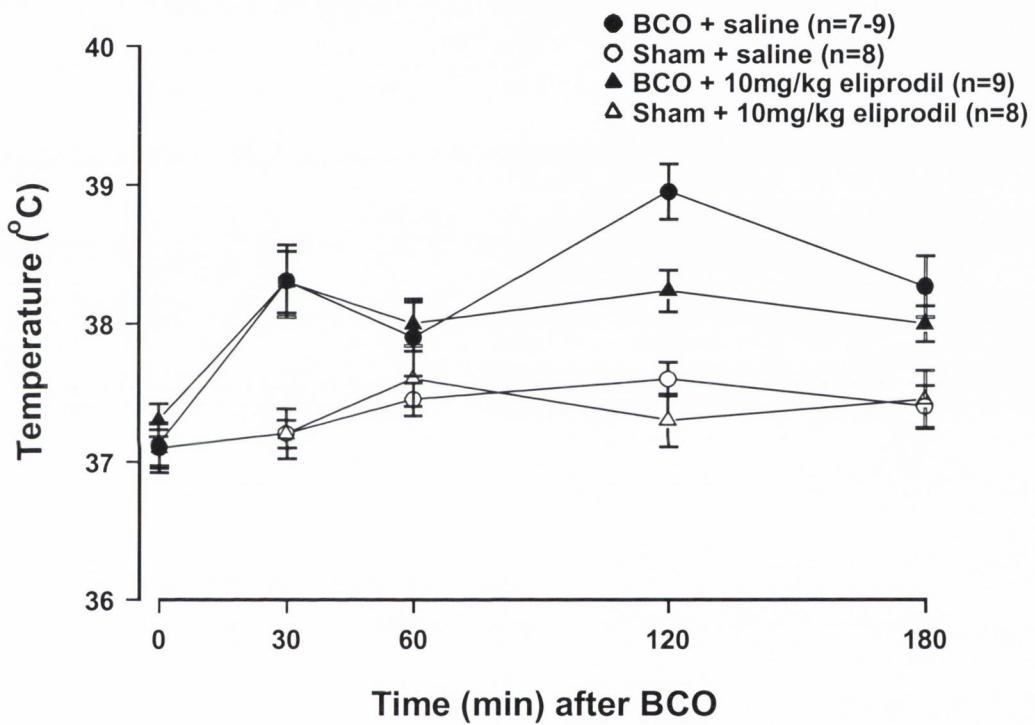


Figure 3.22: Effect of 10mg/kg eliprodil on body temperature in the Mongolian gerbil following bilateral carotid occlusion. Results are presented as means \pm SEM.

Table 3.1: the pyramidal cell densities per millimetre following different treatments. *** $p < 0.001$ Vs Sham operated (one-way ANOVA); # $p < 0.05$ Vs BCO (one-way ANOVA); ## $p < 0.01$ Vs BCO (one-way ANOVA); ### $p < 0.001$ Vs BCO (one-way ANOVA)

<u>Treatment</u>	Dose (mg/kg)	Time of administration (BCO time=0min)	Neuronal density per 1mm CA1 region (mean \pm SEM)
Sham control	-	-	214.82 \pm 15.52
5min BCO	-	-	74.56 \pm 9.25***
EMLA	Topical	0	77.58 \pm 7.8
Sham control	-	-	228.33 \pm 8.27
5min BCO	-	-	77.61 \pm 5.86***
MK-801	1.0 (i.p.)	+30	112.92 \pm 7.66##
Sham control	-	-	240.94 \pm 6.98
5min BCO	-	-	100 \pm 6.92***
N ¹ -Dansylspermine	2.0 (i.p.)	+30	112.19 \pm 6.91
N ¹ -Dansylspermine	5.0 (i.p.)	+30	150.97 \pm 11.72###
Sham control	-	-	258.75 \pm 13.71
5min BCO	-	-	91.6 \pm 7.52***
N ¹ -Dansylspermine	10.0 (i.p.)	+30	167.5 \pm 18.68####
N ¹ -Dansylspermine	10.0 (i.p.)	+30, +24hr	131 \pm 11.36#
Sham control	-	-	276.25 \pm 6.822
5min BCO	-	-	68.611 \pm 4.63***
Ifenprodil	30.0 (i.p.)	+30	108.75 \pm 8.93###
Eliprodil	10 (i.p.)	+30	108.89 \pm 6.34###

3.6 DISCUSSION

Bilateral carotid artery occlusion (5 minutes) caused severe neurodegeneration in the CA1 region of the hippocampus of the gerbil, 96 hours after surgery. Despite this severe damage to the hippocampus, all of the animals recovered from the surgery quickly and no adverse behavioural effects (e.g. abnormal gait, increased sedation, excess grooming or scratching) were observed over the four days post-occlusion, aside from the aforementioned hyperactivity. Kirino *et al.* (1982) showed, using neuropathological studies, that there are three types of changes that occur in the gerbil hippocampus following ischaemia. In the CA4 region the change was rapid (an ischaemic change); in the CA2 a relatively slow change was observed (reactive change). A very slow change in the CA1 pyramidal cells started at two days after ischaemia and by four days almost all the cells in the CA1 were destroyed, this was called “delayed neuronal death”. The mechanism of this neuronal degeneration has not yet been fully elucidated but the N-methyl-D-aspartate (NMDA) receptor is thought to play a major role by allowing the influx of ions, primarily calcium, into the cells (Siesjö, 1992). This calcium overload then sets off a chain reaction ultimately causing cell death (see Section 1.4.2).

The method of assessment of damage used here is histological examination and quantification of the surviving pyramidal cells. This has been widely used in previous studies to demonstrate neuroprotective properties of novel compounds (Contreras *et al.*, 1992; Kato, *et al.*, 1995; Maginn *et al.*, 1995; O’Neill *et al.*, 1994). From this study, the destructive effect of 5-minute bilateral carotid occlusion is apparent, reducing the neuronal density per 1mm to 40% or less of the sham operated pyramidal cell density in each case. The histological photographs taken of the 5µm coronal sections demonstrate this destructive effect very clearly. In the low magnification photomicrograph of a sham operated gerbil, a complete and well defined CA1 layer is visible and on the higher magnification the individual pyramidal cells are intact and easily counted for quantification. However, in the coronal slice from a gerbil that has undergone 5-minute carotid occlusion there are marked differences from the sham operated gerbil. The formerly intact CA1 pyramidal cell layer has almost completely disappeared, as has the CA2. On higher magnification only a few pyramidal cells are

still visible. This pattern of neuronal damage correlates well with previous studies (Kirino, 1982; Gill *et al.*, 1987 & 1988; O'Neill *et al.*, 1994).

In the first experiment, EMLA (eutectic mixture of local anaesthetics) was applied topically to the incision made in the experiment after suturing so as to provide some measure of pain relief to the animals. Since it was applied topically, no effect was expected to be seen on the neuronal density. EMLA had no effect on the pyramidal cell density. EMLA contains a mixture of local anaesthetics and acts as a membrane stabiliser and prevents the conduction of action potentials. Local anaesthetics also work by blocking sodium channels and as a result could conceivably have had an effect on pyramidal cell density. EMLA was used for pain relief in the experiment, however its use was not continued after the initial study as there was no appreciable difference between the behaviour of treated and untreated gerbils. All of the gerbils displayed normal behavioural characteristics with no differences between the groups. The standard experimental protocol does not involve the use of any analgesics and in view of the behavioural findings it was decided not to use EMLA

Local anaesthetics have, however, previously been shown to be neuroprotective, though these were not applied topically. Mizunuma *et al.* (1996) showed lidocaine to be neuroprotective, following subarachnoid administration, in rat forebrain ischaemia. Also Chen *et al.* (1998) demonstrated neuroprotective effects of intracerebroventricular procaine in the gerbil model, though this was thought to be primarily as a result of procaine's ability to inhibit release of Ca^{2+} from intracellular stores.

The majority of other pain killing drugs are either neuroprotective or interact in some way with the systems involved in producing the post-ischaemic damage. The NSAID's (non-steroidal anti-inflammatory drugs) inhibit prostaglandin synthesis from arachidonic acid and inhibit the action of arachidonic acid on tissues. It has been demonstrated that chronic administration of ketoprofen is protective against ischaemia-induced hippocampal late-onset reduction of muscarinic acetylcholine receptors, a sequela of cerebral ischaemia (Asanuma *et al.*, 1997). Narcotic

analgesics have been shown to have an effect on cerebral ischaemia (Kofke *et al.*, 1999) as have agonists at the sigma receptor, one of the opiate receptors (Contreras *et al.*, 1992; Poignet *et al.*, 1992; O'Neill *et al.*, 1994, 1995 & 1996; Earley *et al.*, 1996). Therefore, none of these substances could be used for analgesia in the procedure.

MK-801 (dizocilpine) is an orally active anticonvulsant, which was first described by Clineschmidt *et al.* in 1982. MK-801 has been demonstrated to act as an antagonist of the NMDA receptor and this antagonism has been shown to be non-competitive (Kemp *et al.*, 1986; Aram *et al.*, 1987). Drugs acting as antagonists at the NMDA receptor have proved to be beneficial against cerebral ischaemia/hypoxia (Gill *et al.*, 1987; Gill *et al.*, 1988; Meldrum *et al.*, 1989). MK-801 has been shown to provide neuroprotection against ischaemia-induced brain damage (Gill *et al.*, 1987; O'Neill *et al.*, 1994). Other work also supports the role of the NMDA receptor in ischaemia mediated cell death. Globus and co-workers (1988) found a seven-fold increase in striatal extracellular glutamate release, during 20-minute global forebrain ischaemia in the rat (four vessel occlusion model), which returned to control values following 30 minutes reperfusion. In the gerbil model of forebrain ischaemia, there was a 12-fold increase in glutamate striatal extracellular release during 5 minutes of global ischaemia, which returned to control levels following 30 minutes of reperfusion (Mitani *et al.*, 1992).

In the present study, the role of MK-801 was investigated in the gerbil model of cerebral ischaemia. MK-801 (1mg/kg, i.p.), administered 30 minutes after 5-minute bilateral carotid artery occlusion, significantly attenuated the cell loss in the CA1 layer of the hippocampus. This result is in agreement with previously reported findings (Gill *et al.*, 1987), in which it was demonstrated that, at doses of 0.3, 3 and 10 mg/kg, administered i.p., one hour before 5-minute carotid occlusion, MK-801 was neuroprotective. Pre-administration of 0.3 mg/kg MK-801 provided some neuroprotection and the pre-administration of 3 and 10 mg/kg provided complete neuroprotection.

However, other studies have cast doubt on the mechanism by which MK-801 causes neuroprotection in ischaemia/hypoxia. It has been demonstrated that lowering the body temperature, during ischaemia, by as little as 3°C, reduces the amount of glutamate released during ischaemia and greatly improved the number of neurons surviving in the CA1 region of the hippocampus (Mitani & Kataoka, 1991). Some studies have suggested that MK-801 protects by inducing mild hypothermia (Corbett *et al.*, 1990). They demonstrated that in gerbils, MK-801 produced hypothermia and that, following bilateral carotid occlusion, in gerbils kept normothermic, MK-801 (3mg/kg, ip) was not neuroprotective. In a second study in which the temperature was closely monitored to prevent intra-ischaemic hypothermia, MK-801 was not neuroprotective and did not protect against decreased receptor binding in the hippocampus 7 days after ischaemia (Hara *et al.*, 1990). The neurochemical mechanism by which MK-801 induces hypothermia is not known but Gandolfi and co-workers (1992) suggested MK-801 may have an indirect stimulatory effect on dopaminergic neurons. However, in contrast to the findings of Corbett *et al.* (1990) and Hara *et al.* (1990), Gill and Woodruff (1990) showed MK-801 (1mg/kg and 10mg/kg, ip) to be neuroprotective when the body temperature was maintained at 37°C for 24 hours following ischaemic insult.

Given these findings, in the present study it was considered important to maintain the body temperature of the animals at the normal physiological value. The body temperature of the animals was, therefore, maintained using a heated blanket and the ambient temperature was maintained between 31 and 33°C so as to counteract any possible hypothermic effect of the MK-801.

As stated earlier, the method of assessment of neuronal damage is histological. This is the ideal method of quantification of damage as the surviving intact pyramidal cells in the CA1 layer of the hippocampus can each be individually counted. This particular method of assessment has been widely used and with great success (Contreras *et al.*, 1992; Kato *et al.*, 1995; Maginn *et al.*, 1995; O'Neill *et al.*, 1994). Other methods of histological examination have been used such as that employed by

Pulsinelli *et al.* (1982(a)) in which the neuronal damage was graded according to a scale (Yamamoto *et al.*, 1993).

N¹-Dansylspermine is a potent polyamine antagonist with a likely action at the positive polyamine modulatory site on the NMDA receptor macrocomplex (see Section 2). In the present experiments it has been shown that N¹-Dansylspermine is a potent neuroprotective agent in the gerbil model of global ischaemia. At a dose of 2mg/kg N¹-Dansylspermine caused a slight increase in neuronal density after 5-minute bilateral carotid artery occlusion but this was not statistically significant when compared with the 5-minute BCO control. However, when N¹-Dansylspermine was given at the doses of 5 and 10 mg/kg there was a highly statistically significant increase in pyramidal cell density.

As mentioned previously, there is conflicting evidence with regard to the role of polyamines in the damage caused by cerebral ischaemia. Markwell *et al.* (1990) demonstrated that α -difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase, blocks NMDA-induced neurotoxicity in cultured cortical neurons. Paschen and co-workers have produced a number of reports on the neurotoxic effects of polyamines after cerebral ischaemia. They have demonstrated that there is an increase in putrescine levels after ischaemia and that this is due, at least in part, to an increase in ornithine decarboxylase (ODC) activity and they also demonstrated that there is a slight decrease in spermine levels in certain brain regions (Paschen *et al.*, 1987; Paschen *et al.*, 1988(a); Paschen *et al.*, 1988(b)). In 1991, Paschen proposed that, during ischaemia, polyamines are released from neurons into the extracellular compartment and cleared into the blood. In the follow-up study, Paschen and co-workers proposed that the spermine that is released, binds to the NMDA receptor of cells located in close vicinity and thus may render the neurons vulnerable to otherwise subtoxic levels of excitotoxins (Paschen *et al.*, 1992). More recently Zoli *et al.* (1996) demonstrated an increase in spermidine/spermine N¹-acetyltransferase which may contribute to the rise in putrescine and decrease in spermidine levels observed after ischaemia. Furthermore Dogan *et al.* (1999) showed that MDL 72527, a polyamine oxidase inhibitor, caused a reduction in the increase in

putrescine content and reduced oedema formation and ischaemic injury volume after transient cerebral ischaemia. This demonstrates that, as Paschen and co-workers postulated (1988), ODC is not the only enzyme involved in the overshoot in putrescine content. Rather the entire polyamine interconversion pathway is involved.

Furthermore, it has been demonstrated that the polyamines may influence the integrity of the blood-brain barrier (Koenig *et al.*, 1989). These effects on the blood-brain barrier can be reversed by blocking ODC with 2-(difluoromethyl)ornithine (DFMO), as shown by Schmitz and co-workers (1993).

Seiler *et al.* (1998(b)) demonstrated that N¹-Dansylspermine was a potent calmodulin antagonist and cytotoxic agent. It has been previously demonstrated that the calmodulin antagonist, W-7, is ineffective in the gerbil bilateral carotid occlusion model (Hara *et al.*, 1990). Hara *et al.* demonstrated that antagonists of cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase or calmodulin were ineffective at preventing ischaemia-induced neuronal damage in the gerbil model of global ischaemia. Therefore, the calmodulin antagonist action of N¹-Dansylspermine was not thought to be involved in its neuroprotective action.

It has been shown that N¹-Dansylspermine is a potent neuroprotective agent and that it is a polyamine antagonist at the positive polyamine modulatory site of the NMDA receptor. From these observations we can postulate that the neuroprotective properties are due to a protective action against the mechanism of damage proposed by Paschen *et al.* (1992) where spermine, released after ischaemia, increases the vulnerability of NMDA receptors to excitotoxins.

As mentioned above, hypothermia has been demonstrated to protect the brain from damage after an ischaemic insult (Busto *et al.*, 1987). Indeed the extent of hypothermia has been shown to decrease the release of neurotransmitters after an ischaemic insult and this is thought to be the cause of the reduced neuronal damage (Busto *et al.*, 1989). Therefore, the animals body temperature was monitored for up to three hours after 5-minute occlusion.

N¹-dansylspermine (10mg/kg, ip), when administered to the sham operated animals, causes a small, but statistically significant, drop in body temperature compared to that of sham controls given saline demonstrating that N¹-dansylspermine can cause hypothermia. However, when given in the occluded animals N¹-dansylspermine causes a reduction in body temperature compared with BCO and saline group but this reduction is significant at the 120 minute time point when compared with the sham operated group (normal body temperature). This slight drop in body temperature could contribute to the neuroprotection produced. However, this is unlikely, when compared to the extent of hypothermia in previously published work. Busto and co-workers (1989) used a control temperature of 36°C (which is the temperature that was reached) and had hypothermic animals held at 33°C and 30°C. Also, MK-801, which has been proposed to produce its neuroprotective effects through hypothermia (Corbett *et al.*, 1990), caused a reduction in body temperature of approximately 4°C.

In the data presented here for N¹-dansylspermine, the body temperature does not drop sufficiently to produce neuroprotection through hypothermia. Even if it was argued that N¹-dansylspermine at 10mg/kg causes sufficient hypothermia to protect the brain from damage despite the evidence to the contrary, the same cannot be said at 5mg/kg. N¹-Dansylspermine, 5mg/kg, caused significant neuroprotection but did not produce any hypothermia whatsoever. In fact the temperature trace for 5mg/kg maps almost exactly to the trace for the BCO + saline group in which there is marked neuronal damage in the CA1. This proves that N¹-dansylspermine does not produce its neuroprotection by causing hypothermia and this lends support to the polyamine antagonist mechanism proposed above.

As previously mentioned, 5-minute bilateral carotid artery occlusion causes an increase in locomotor activity which conventional wisdom attributes to the inability of the gerbils to form spatial maps after an ischaemic insult (Maginn *et al.*, 1995; Katoh *et al.*, 1992; Wang and Corbett, 1990). The locomotor activity was measured in this study over a thirty-minute period, 72 hours after carotid occlusion. The normal response for an intact animal is a high initial exploratory activity, which decreases significantly over time. This pattern was indeed seen. The traces for the sham

operated animals follow the normal pattern and decrease over time to only small exploratory movement by thirty minutes. However, the trace for the 5-minute BCO gerbils is quite different. There is an initial high value but this is significantly higher than the control and it only decreases slightly over time showing the increased locomotor activity. It has been shown previously that this ischaemia-induced hyperactivity is dose-dependently reversed by neuroprotective agents (Maginn *et al.*, 1995). However, this is not the case here. In fact, at 5mg/kg N¹-Dansylspermine, the locomotor activity is increased even further. However, this is not a statistically significant increase. There is certainly no reduction induced in the N¹-dansylspermine treated animals.

This result casts doubt on some previous studies. In 1988, Gerhardt and Boast showed that, in the gerbil carotid occlusion model, the motor activity changes were positively correlated with the degree of neuronal degeneration in the CA1 layer of the hippocampus. Following on from this, Mileson and Schwartz (1991) proposed using locomotor activity as a behavioural screen for the neuronal damage following transient forebrain ischaemia in gerbils. They showed that even though the hyperactivity did not correlate with the striatal or cortical damage it did correlate well with the damage to the CA1. In the results presented here there is no correlation between CA1 damage and hyperactivity as N¹-Dansylspermine reversed the hippocampal damage but not the hyperactivity. Perhaps, therefore, the hyperactivity is not a result of the hippocampal damage but due to damage in another as yet unidentified area.

The study by Maginn and co-workers (1995) examined amino-3-phosphonopropionate (AP-3), a metabotropic glutamate receptor antagonist. They showed that at low dose it was neuroprotective but did not reduce the ischaemia-induced hyperactivity. However, a higher dose was effective in reversing hyperactivity. This would suggest that a higher dose of N¹-dansylspermine could be even more neuroprotective and reverse the hyperactivity. However, if the surviving/protected cells are examined as a percentage of sham operated, the dose of AP-3 that did not affect hyperactivity only resulted in 34% of the cells surviving

compared to 63% for 5mg/kg N¹-dansylspermine. This, therefore, argues against the lack of effect of N¹-Dansylspermine being due to too small a dose being used.

Multiple doses of N¹-Dansylspermine were also investigated for enhanced neuroprotective activity. N¹-Dansylspermine was administered either 30 minutes before and 30 minutes after 5-minute occlusion or 30 minutes and 24 hours after occlusion. When N¹-dansylspermine is administered post-operatively only (i.e. +30 minutes and +24 hours) it is still neuroprotective against the delayed neuronal death though it is not as effective as the single dose at +30 minutes.

When N¹-dansylspermine was given 30 minutes before and 30 minutes after the 5-minute occlusion it had no neuroprotective effect. However, none of the control/sham-operated animals survived the dual doses of N¹-dansylspermine and the number of BCO gerbils was small. Therefore, a statistical analysis could not be correctly performed.

After administration of the second dose of N¹-dansylspermine, all the animals displayed severe behavioural effects. Within minutes of the second injection the animals became markedly sedated with the appearance of shallow, laboured breathing. The gerbils also appeared to be hypothermic to touch. It was felt that their condition was too fragile to permit the handling associated with temperature monitoring. Subsequently, the sham-operated animals rolled onto their side and within 15 minutes displayed a fatal extensor tonic convulsion. It is likely that this relates to partial agonist activity of N¹-dansylspermine. This is logical because the consequence of a high dose of either spermine or N¹-dansylspermine is a tonic convulsion. A possible explanation for the observation that convulsions did not occur in the BCO gerbils could be that the metabolic changes that occurred after cerebral ischaemia prevented their production. However, these BCO gerbils did appear to take longer to recover from the anaesthesia than the rest of the gerbils. Therefore, the anaesthetic may have also played a role, with the high dose of N¹-dansylspermine, in the observations described above.

Ifenprodil, and its derivative eliprodil, are phenylethanolamines, which have been shown to be allosteric inhibitors of polyamines at the NMDA receptor. Ifenprodil has been widely investigated as a possible regulator of NMDA activation and both ifenprodil and eliprodil have been reported to reduce the ischaemic injury and brain damage resulting from global or permanent focal cerebral ischaemia (Baskaya *et al.*, 1997(b); Gotti *et al.*, 1990). Also ifenprodil has been shown to be effective against reperfusion injury after transient focal cerebral ischaemia (Dogan *et al.*, 1997). The exact mechanism by which ifenprodil causes neuroprotection has not yet been fully elucidated. However, spermidine has been shown to reverse the ifenprodil-induced protection against glutamate and NMDA cytotoxicity (Tamura *et al.*, 1993). This suggests that the neuroprotective effects of ifenprodil may be caused by an antagonistic effect on polyamine modulatory sites of the NMDA receptor.

There are, however, a number of other theories regarding the mode of action of ifenprodil and of eliprodil. Eliprodil has been shown to block voltage-dependent calcium channels in cultured rat cortical neurons (Biton *et al.*, 1994) and ifenprodil has been shown to block voltage-dependent calcium channels in rat and mouse hippocampal pyramidal neurons in vitro (Church *et al.*, 1994). Bath *et al.* (1996) showed that both ifenprodil and eliprodil were neuroprotective when administered post-occlusion and that they also block N and P-type voltage-dependent calcium channels. Both ifenprodil and eliprodil have been shown to have an affinity for σ receptors (Poignet *et al.*, 1992; Contreras *et al.*, 1990) and some σ receptor ligands have been reported to have neuroprotective actions in animal models of cerebral ischaemia (Contreras *et al.*, 1992; O'Neill *et al.*, 1996). More recently, it has been shown that ifenprodil affects polyamine biosynthesis by inhibiting ornithine decarboxylase (Badolo *et al.*, 1998). Because of these and other effects, such as ifenprodil's α -adrenergic blocking properties which may cause vasodilation (Gotti *et al.*, 1988), it is hard to draw firm conclusions on the mechanism of ifenprodil and eliprodil's neuroprotective action.

There are also a number of theories regarding the mode of action of ifenprodil on the NMDA receptor. The NR2B subunit of the NMDA receptor must be present for

ifenprodil to produce high-affinity inhibition (Williams, 1993). This suggests that NMDA receptors containing this subunit contribute to excitotoxic damage. Ifenprodil has been proposed to work by stabilising a desensitised state of the receptor (Reynolds & Miller, 1989), to block the spermine binding site as previously mentioned (Carter *et al.*, 1990), to allosterically inhibit glycine binding (Williams, 1993; Legendre & Westbrook, 1991), to be a weak open channel blocker (Williams, 1993) or to promote transition to a non-conducting state of the channel (Legendre & Westbrook, 1991). More recently a unique mechanism of action has been proposed. Mott *et al.* (1998) demonstrated that ifenprodil rendered the NMDA receptor more sensitive to inhibition by protons. Kew and Kemp (1998) demonstrated an allosteric interaction between the polyamine binding site on the NMDA receptor and a proposed ifenprodil binding site on the NMDA receptor.

It is demonstrated in this study that, as expected, ifenprodil is neuroprotective. There is an increase in neuronal density over the BCO group of 59%, at a dose of 30mg/kg. This can also be seen from a coronal section from an ifenprodil treated gerbil. The restored CA1 layer can be clearly seen in both the high and low magnification. The same is seen for eliprodil. It too produces an increase in neuronal density of 59% but at the lower dose of 10mg/kg, which demonstrates its greater potency. This similarity was to be expected, as eliprodil has also been shown to be neuroprotective and to act selectively on the NR2B subunit of the NMDA receptor. Both of these results are in line with previously published studies that demonstrated the neuroprotective effects of both ifenprodil and eliprodil (Gotti *et al.*, 1988; Scatton *et al.*, 1991; Poinet *et al.*, 1992).

The gerbil body temperatures were recorded as before and, it can be seen here that, in the case of ifenprodil (30mg/kg), there is no hypothermia. The temperature in the BCO with ifenprodil does drop compared to the BCO and saline but this difference is not significant and more importantly, there is no difference between BCO and ifenprodil and the sham operated with saline (normal body temperature). This demonstrates that ifenprodil does not produce its neuroprotective effects by causing hypothermia.

There is a slight drop in temperature between the BCO with saline and with eliprodil. However, this decrease is not significant. Also, similar to ifenprodil, there is no difference between the BCO and eliprodil group and the sham operated with saline group. This shows that eliprodil, like ifenprodil, does not cause neuroprotection by producing hypothermia. This was to be expected as eliprodil is an analogue of ifenprodil and both produce their effects in a similar manner. Indeed, Bath and co-workers (1996) showed that there were no changes in rectal temperatures following administration of ifenprodil or eliprodil, in this case multiple doses of ifenprodil or eliprodil were used and temperatures were recorded for up to 6 hours after occlusion.

As previously conducted for N¹-dansylspermine, the locomotor activity of the gerbils was recorded 72 hours after 5-minute bilateral carotid artery occlusion. As expected, in the sham operated animals there was a high locomotor activity at the start of the test, which decreased with time (30 minutes) to almost zero. However, with the BCO operated animals (saline treated) there was a large increase in locomotor activity compared with the sham operated group but this declined only slightly over the 30-minute observation period. In the BCO animals treated with either ifenprodil or eliprodil, there is a reduction of this ischaemia-induced hyperactivity. This is what would be expected and has been demonstrated in previous papers (Maginn *et al.*, 1995). It would be expected that if the CA1 hippocampal damage is reversed then there should be a reversal in the hyperactivity. This is demonstrated here in the case of ifenprodil and is significant at the 99% level. However, eliprodil does reduce the hyperactivity but this does not reach significance. This is probably as a consequence of the dose used and if a higher dose was used the difference in locomotor activity would be expected to be greater. The results correlate well with other established neuroprotectants such as MK-801. Despite the finding that MK-801 produces hyperactivity itself on administration (this effect is thought to be due to indirect dopaminergic activation in the striatum (Loscher *et al.*, 1991)), MK-801 (1mg/kg, ip) has been shown to reduce the ischaemia-induced hyperactivity (Nakanishi *et al.*, 1994).

3.7 CONCLUSION

As has been previously shown, it was demonstrated here that MK-801 is neuroprotective. Doubt has been cast on the mechanism of this neuroprotection when it was shown that MK-801 produced hypothermia and that hypothermia is itself neuroprotective. Here a number of measures were taken to guard against hypothermia. The ambient temperature of the laboratory was maintained between 31°C and 33°C, a heating pad was used and the animals remained in the heated environment for two hours after the occlusion. As a result hypothermia was unlikely to occur and therefore the neuroprotective effect was due, certainly in part, to MK-801's non-competitive antagonist action on the NMDA receptor.

It has also been shown here that polyamines are involved in the neurodegeneration following an ischaemic insult. There are two schools of thought of the role of polyamines after an ischaemic insult. One suggests that the polyamines are neuroprotective (Gilad & Gilad, 1991) whereas the other suggests that they are, at least in part, involved in producing the neuronal damage after the ischaemic insult (Paschen *et al.*, 1991, 1992; Dogan *et al.*, 1997, 1999). Indeed, recent work has suggested that the neurotoxicity of polyamines was mediated through the NMDA receptor (Sparapani *et al.*, 1997). N¹-Dansylspermine has been shown to be a potent blocker of the NMDA receptor and a polyamine antagonist. In these studies it has been shown to be a potent neuroprotective agent and this lends support to the theory that polyamines are involved in the damage after an ischaemic insult. However there is evidence that N¹-Dansylspermine is possibly a partial agonist rather than a true antagonist at the positive polyamine modulatory site on the NMDA receptor.

Also shown, in these studies, are the neuroprotective effects of ifenprodil and eliprodil. These are proposed to act as polyamine antagonists to produce their neuroprotective effects. However they both have numerous other effects which are detailed above. This may explain the difference between the activity results for N¹-dansylspermine and for ifenprodil and eliprodil. Both N¹-dansylspermine and either ifenprodil or eliprodil protect the hippocampus from damage after ischaemia but

unlike the ifenprodil, N¹-dansylspermine does not reverse the ischaemia induced hyperactivity. Therefore, the proposed use of general locomotor activity as a screen for hippocampal damage and presumably as a screen for neuroprotective agents (Mileson & Schwartz, 1991) is not reasonable.

There are a number of possible explanations for the present observations with N¹-dansylspermine. It is possible that the polyamines affect a discrete population of pyramidal cells within the CA1 layer that are not responsible for spatial mapping. Also, the observation that ifenprodil reduces the ischaemia-induced hyperactivity may be due to an action by ifenprodil on a discrete population of cells, not within the CA1 layer and as yet unidentified, that are not affected by N¹-dansylspermine. This may suggest a reduced role for the hippocampus in memory than previously recognised.

It has come to light recently that the hippocampus actually has a smaller role to play than previously thought in the formation and storage of memories. The hippocampus appears to still be involved in memory acquisition and consolidation but the cortex is also involved. Bontempi *et al.* (1999) demonstrated a time-dependent reorganisation of brain circuitry, which underlies long-term memory storage. They demonstrated high activity for the hippocampus in memory acquisition and early retention. However, over time there is a reduced activity in the hippocampus on retention and increased activity in the cortex. This suggests that the hippocampus does not have as great a role as previously thought in the storage of memories. However, further studies need to be carried out to determine the time required for the change in activity from the hippocampus to the cortex.

SECTION 4

Image analysis

4.1 INTRODUCTION

Visual assessment of neuronal damage is the easiest and most straight-forward method available. However, it is not necessarily objective and with the continuous development of more powerful computers and image-analysis systems it is now possible to assess neuronal damage, using a computer, in animals as small as mice and this has recently become more affordable.

There are a number of different methods for assessing the neuronal damage resulting from an ischaemic insult. Osborne *et al.* (1987) used an image analysis system, Quantimet 720, to assess the area of damage following middle cerebral artery occlusion. Similarly, Smith *et al.* (1998) used the SeeScan image analyser to measure the lesion area resulting from middle cerebral artery occlusion in rats. These are the standard methods for measuring the area of infarction following occlusion. Also used are magnetic resonance imaging and spectroscopy. However these methods are only suitable for assessment of damage to large areas of the brain but not for counting individual cells in specific areas such as the pyramidal cells in the hippocampal CA1 area.

The methods used for assessment of damage to the CA1 layer of the hippocampus are varied. Manual counting, with the aid of a microscope, of the surviving CA1 pyramidal cells is the standard technique and was used in these studies (Section 3). However, there are a number of disadvantages to manually counting the pyramidal cells. First, the process is very tiring and time consuming and second, manual counting could be affected by bias if the sections are not randomised and counted blind. Therefore, a computerised method of assessment of CA1 damage would be very advantageous.

Such methods have been successfully used to measure hippocampal CA1 damage. Gill *et al.* (1987 & 1988) quantitatively measured the area of degeneration from the hippocampal fissure to the alveus in a number of different planes. This was carried out using an image analysis system from Cambridge Instruments. Using this they measured the area of degeneration of the CA1 and CA2 at three points, 1.5mm, 1.7mm and 1.9mm caudal to the Bregma in both hippocampi. This yielded a total area of degeneration in six hippocampal planes and these were compared for different experimental groups.

Scion Image is an image analysis program based on NIH image for Macintosh by Wayne Rasband and modified for Windows by Scion Corporation. This program is available free of charge and easily accessible and it was therefore assessed for its value for measuring the pyramidal cell population in the CA1 layer of the hippocampus.

4.2 EXPERIMENTAL PROCEDURE

The pyramidal cell population was counted in sections from representative animals (Section 3). Photographs were then taken of these sections. The photographic procedure is detailed in Section 3.2.

The photographs were then scanned into a Pentium 133 IBM compatible PC using a Microtek Scanmaker X6 flatbed 36-bit colour scanner and Microtek ScanSuite software. The images were transferred to Scion Image (version Beta 3b). The program was downloaded from the internet from the following web site:

<http://www.scioncorp.com>

This package was then used to calculate the neuronal density in the scanned images of hippocampal slices. A number of processes had to be carried out before the calculation could begin. First, a scale for the photograph had to be entered. This was determined using the microscope graticule and the scale entered into the computer package. Second, the size range of particles to be measured had to be entered. The size of the smallest pyramidal cells was determined using the microscope graticule.

Entering the minimum size data ensures that the computer only counts intact pyramidal cells and not cell debris from damaged cells that may have retained some of the cresyl violet stain. A maximum size also had to be entered. However, the value for this did not matter so long as it was greater than the largest pyramidal cells in the area to be counted. The reason for this was that there was nothing in the field of view that was larger than the pyramidal cells and that had retained the cresyl violet stain.

The area to be analysed was selected with the freehand selection tool and the whole image converted from colour to black and white. This was carried out using the "threshold" command. This had to be done as the package would only count particles in black and white format and not in colour. The level of thresholding was then altered to attempt to screen out any extraneous images and to separate any joined cells. Finally, the number of pyramidal cells were counted and this was repeated for a number of areas along the CA1. The areas measured were not identical to those counted manually as that would have entailed taking a number of photographs along the CA1. As a result the same area was photographed in each slide for the image analysis. This was to provide some level of consistency and rule out bias. The number of pyramidal cells per 1mm of the CA1 was then calculated and compared to the results obtained by manual counting using the microscope and grid.

4.3 RESULTS

There were a number of problems that came to light when using the image analysis system. First of all, the photomicrographs of the hippocampal slices had to be in perfect focus. This was not easily achieved as the system used to photograph the slides, an inverted microscope with a camera attached, had to be focussed through the camera rather than the microscope, which would have given a clearer picture. There

are computer programs available, which can be used to try to correct the focus on the picture but they are not always successful. Indeed, Ulead PhotoImpact was used to try to sharpen images but generally this had the effect of adding unwanted extra pixels, and merged images.

One of the principal ideas for using the image analysis system was to avoid operator bias. However, the operator still had to pick the area of CA1 layer to photograph thus adding an element of bias. One area may have a denser pyramidal cell population than another, which in the manual count would be averaged out but for image analysis a smaller area is photographed and counted. In an attempt to avoid this, as mentioned previously, the same section of the CA1 layer was photographed from each slide.

The computerised image analysis system was used to assess the results from the experiment using MK-801 (dizocilpine), which was known to ameliorate hippocampal damage. Each individual image was assessed by counting the number of "objects" in the selected area (CA1). These counts were presented in graphical form as for the manual counts and were compared to the manual counts obtained for the MK-801 experiment. The results of this comparison are shown in Figure 4.1. As can be seen, the computer-aided count is usually higher than the manual count. This is particularly marked for the counts when the gerbil has undergone bilateral carotid occlusion. As there were not sufficient numbers of readings in the computer-aided count group a statistical analysis could not be undertaken between the groups.

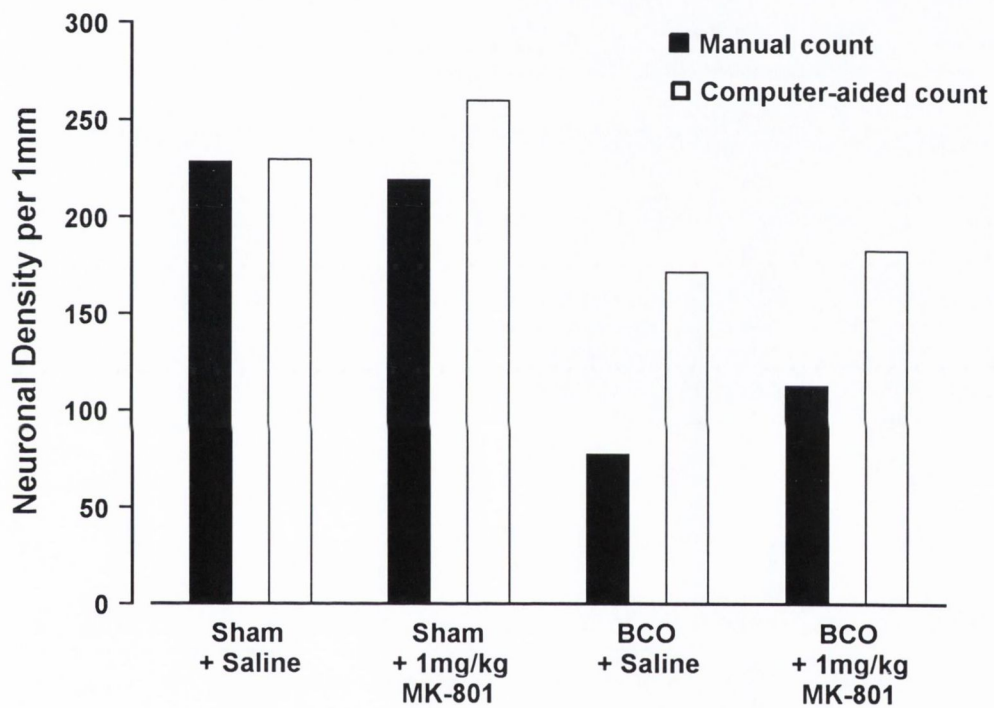
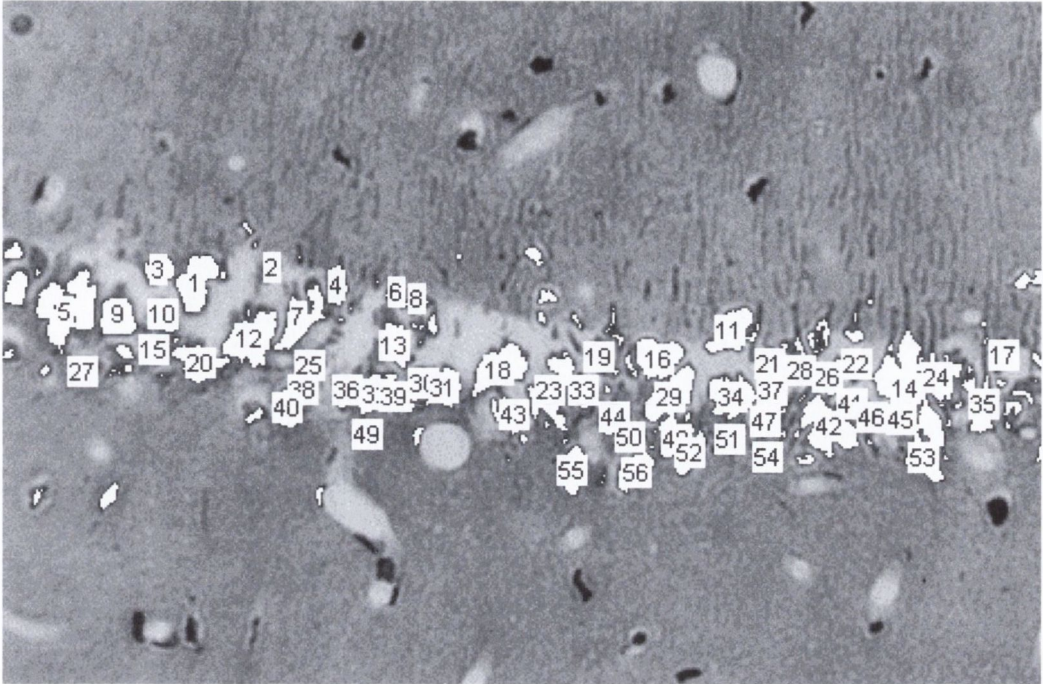


Figure 4.1: A comparison between manual method of counting and computer-aided counting of the intact pyramidal cells in the hippocampal CA1 layer.

Figure 4.2(a) shows a typical result from a counted section as produced by Scion Image. The cells counted are numbered and displayed. However, this also demonstrates the inability of the package to distinguish between touching cells. When compared to the actual picture of the section in Figure 4.2(b), it can be seen that the cells numbered 1, 5, 7, 12, 14, 16, 18, 23, 29, 34 and 42 were all counted as one cell each but are in fact two or three cells overlapping. Figures 4.3(a) and (b) also demonstrate this effect.

This was possibly the most important problem in using the package. The fact that the system could not distinguish between two, three or four cells that may be in contact and, as a result, counted them as one, results in counts that differ from the manual ones. This problem would lead to counts that are lower than the manual counts. However, in addition, the system counted items, which were not, in fact, pyramidal cells. This would have been determined on microscopical analysis but the computer package could not make that determination. The threshold command can, again, be used in this instance. The higher the level it was set at, the greater the spacing between cells but some of the smaller cells would disappear. Conversely, the lower the threshold the greater the number of cells that were counted as one.

(a)



(b)

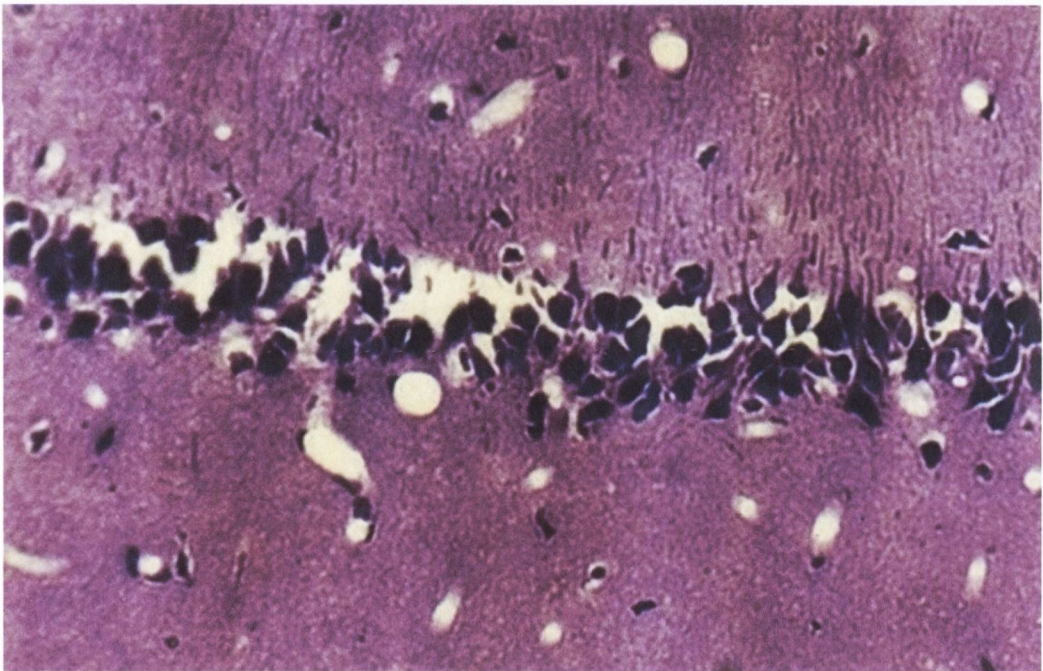
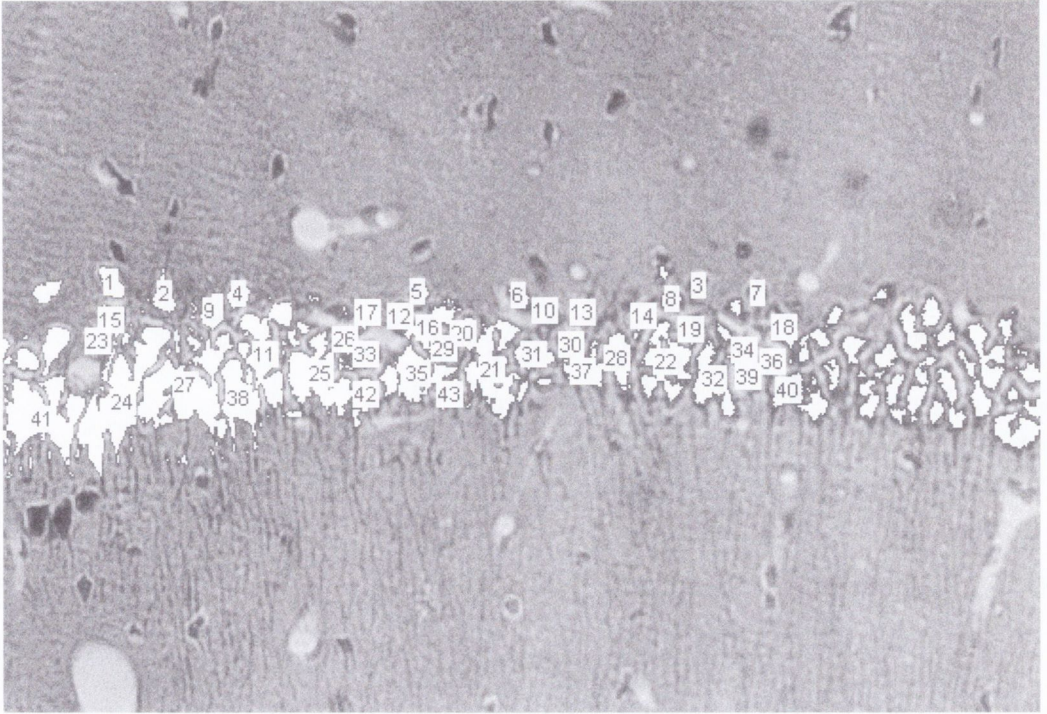


Figure 4.2(a) and (b): Photomicrographs showing the result of a count performed by Scion Image and the original scanned image.

(a)



(b)

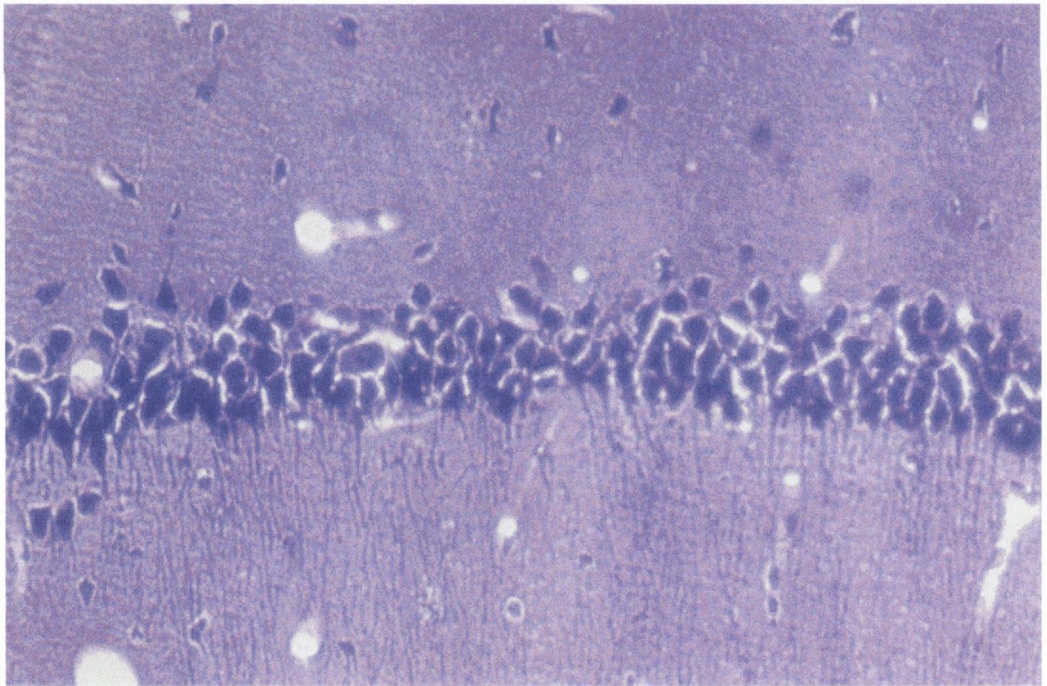
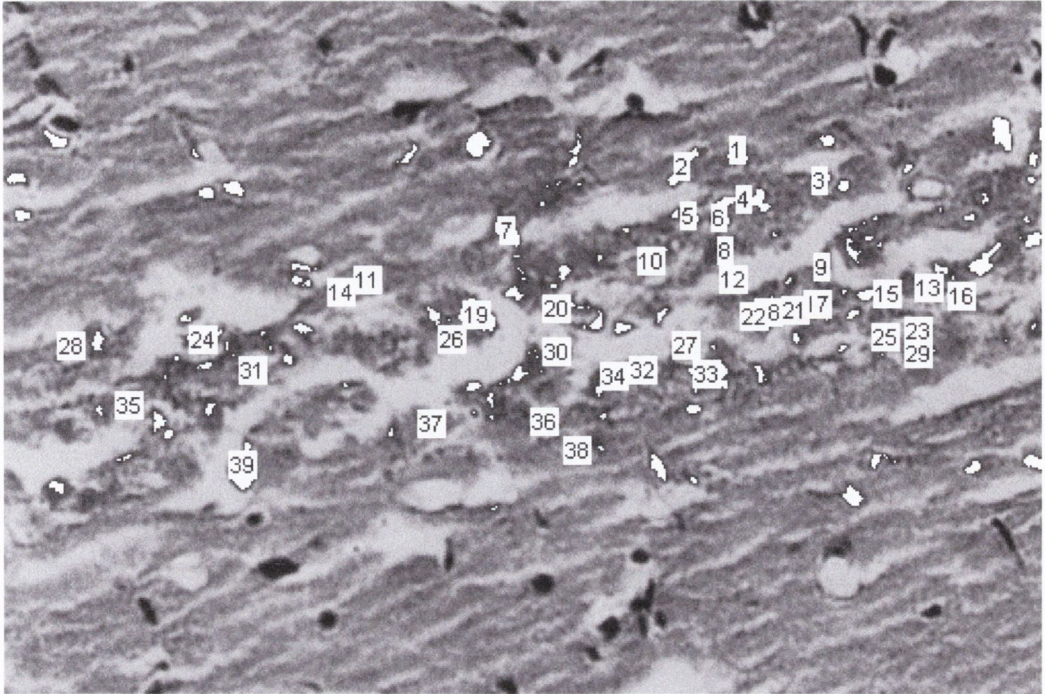


Figure 4.3(a) and (b): Photomicrographs showing the result of a count performed by Scion Image and the original scanned image.

Figures 4.4 (a) and (b) demonstrate the problem associated with choosing different levels of thresholding. The same areas were highlighted and counted but at different threshold levels. As can be seen this resulted in two different counts of 39 cells and 63 cells. To overcome this a standard threshold figure was chosen by comparing the cells that were counted and determining whether or not they are actually pyramidal cells. Another problem that arose from this was that the intensity of the cresyl violet staining varied from slide to slide and this would affect the setting of the threshold figure.

Finally, Figure 4.5 demonstrates another potential problem associated with computer aided counting. This figure shows a photograph of a section of hippocampus, which could not be counted by the computer due to the excess tissue tears. The CA1 can be seen running from the middle on the left hand side, diagonally down to the bottom right hand corner. It was possible to count this manually because the microscope could be focussed in and out so as to make out all the pyramidal cells. This, however, was not possible with Scion Image, even with the use of the threshold command.

(a)



(b)

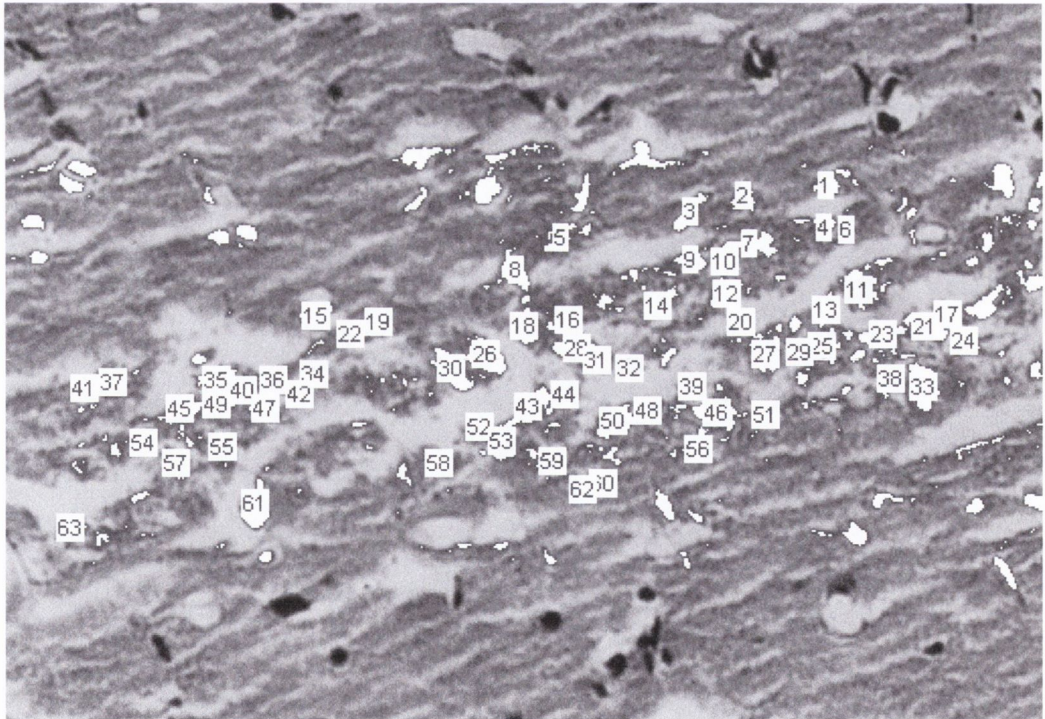


Figure 4.4: Photographs demonstrating the effect of different levels of threshold on the final pyramidal cell count.



Figure 4.5: Photograph of a hippocampal section with excessive tissue tears.

4.4 CONCLUSION

It was concluded from this that the Scion Image image analysis system was not suitable for counting the number of intact pyramidal cells in the CA1 layer because of the problems detailed above. Though manual counting can be very time consuming, for this type of analysis it is more suitable. The Scion Image package would be more effective for the analysis of area/volume of infarction in an entire brain section as has previously been shown with other, similar packages (Kawasaki-Yatsugi *et al.*, 1998; Dogan *et al.*, 1999).

SECTION 5

An electrophysiological model of epilepsy

5.1 INTRODUCTION

There are a large number of models available to explore the processes involved in epilepsy. These include intact animals, *in vitro* brain slices and cultures, and animal models that are produced *in vivo* but examined *in vitro*. To some extent these can then be subdivided into models for the different types of epilepsy.

5.1.1 *In vivo* models

A number of different whole-animal models are available presently to examine epilepsy. Two important whole-animal models are the kindling model (thought to be representative of complex partial seizures in humans) and genetic models, where the animal is bred to be susceptible to a certain type of stimulus.

Kindling is a process whereby the animal is exposed to repeated, focal application of initially subconvulsive electrical stimulations to a particular brain structure, such as the amygdala, which ultimately results in intense clonic motor seizures. This enhanced seizure sensitivity may then persist for the life of the animal. The stimulus need not be electrical. Pentylentetrazol has been successfully used (Cain 1981), as has carbachol (Wasterlain & Jonec, 1983). Once the animal has been kindled and is sensitive to a certain stimulus, various drugs can be administered in an attempt to block the seizures. Also drugs can be administered during the kindling process in order to elucidate the mechanisms at work during the development of epileptogenic activity. Hence both the process of epileptogenesis and the kindled state can be investigated with this model. There are a number of advantages to this type of a seizure model including the ability to produce consistent seizures when the researcher wants them, the ability to study the pathways responsible for the development of

kindling and the expression of a kindled seizure and also the ability to analyse distinct components of this pathway. There are of course disadvantages such as the substantial time and effort required to produce kindled animals.

Kindling has been used to screen existing anti-convulsant agents for anti-epileptogenic activity. Silver *et al.* (1990) compared valproate with both carbamazepine and phenobarbital. They demonstrated that valproate exhibited powerful antiepileptogenic effects, phenobarbitone was shown to be less effective and carbamazepine was devoid of activity. This underlines the need to investigate compounds such as valproate in post-traumatic clinical trials as prophylaxis for epileptogenesis.

There are a number of genetic animal models of epilepsy. Some human sufferers from epilepsy are susceptible to epileptic attack from stimuli that would not affect non-sufferers. This also applies in some genetic animal models of epilepsy. The genetically epilepsy prone rats (GEPR) are useful as they are a model for epilepsy involving convulsions, such as tonic-clonic epilepsy. They exhibit a number of types of seizure predisposition. They will show spontaneous seizures, seizures in response to convulsive stimuli, though at lower doses than normal rats and they also show audiogenic seizures. Similarly, the genetically absence epilepsy rat is used as a model for epilepsy, which does not involve convulsions. There are also populations of epileptic mice available. These include the DBA/2 mouse that is susceptible to audiogenic seizures (Chapman & Meldrum, 1987) and the *quaking* mouse, in which handling induces seizures (Cherbat *et al.*, 1979).

5.1.2 *In vitro* models

In vitro models are now being widely used and are particularly useful for the examination of the role of specific neural pathways in both the development of epilepsy and in the expression of seizures.

Brain slices are widely used as *in vitro* preparations for the examination of epileptiform activity. Essentially, this model uses a slice of a particular brain area freshly prepared with either a tissue chopper or vibrotome and then mounted in an electrophysiology recording apparatus. The tissue is kept constantly perfused with artificial cerebrospinal fluid (aCSF). The tissue is then usually stimulated electrically (chemical stimulation can also be used) to produce epileptiform bursts. For example Stasheff *et al.* (1985) showed that giving strong stimulus trains to the CA3 region of the hippocampus produced spontaneous epileptiform bursting. This is thought to be the *in vitro* version of *in vivo* kindling.

Recently much attention has been focussed on the NMDA receptor and its potential role in the development of epileptic discharges. Herron *et al.* (1985) demonstrated an NMDA receptor mediated component of epileptiform activity evoked by stimulation of the Schaffer collateral-commissural pathway in the presence of convulsant agents. This was performed using hippocampal slices and recording spikes in the CA1 region. Similarly, Ross *et al.* (1998) used hippocampal brain slices to examine the modulation of epileptiform activity in the CA3 region by adenine nucleotides. These studies among others have helped to elucidate the different neural pathways involved in normal synaptic transmission within the hippocampus and other brain structures and indicate how they can contribute to epileptiform activity. These methods can, of course, be used to investigate existing compounds as well as discover new ones. Sagratella (1998) used the model of the epileptic hippocampal slice to characterise the mechanism of both old and new anticonvulsant drugs.

Other methods have also been used to simulate epileptiform activity in brain preparations. Connors *et al.* (1982) have used neocortical slices from rat and guinea pig to investigate the contribution of local neocortical circuits to epilepsy and epileptiform discharge. Also patch-clamp experiments have been used to examine specific cell populations and their contribution to epilepsy.

Some of the advantages of using *in vitro* preparations are obvious. The perfusion medium of the preparation can be quickly and easily changed and this effect

examined. There is no interference with access to the site of action by the blood brain barrier (of course, this means that to be clinically useful the compound must be shown to cross the blood brain barrier). Also, it is possible to look at isolated areas of the brain without interference from other regions.

There are, however, a number of disadvantages of using brain slices rather than whole animal models. There is an unavoidable loss of neuronal circuitry during the slicing process. Extrinsic inputs to an area are lost as are any modulatory actions that these have on the area in question. The brain slice itself may be damaged in the slicing procedure, affecting the intrinsic circuitry. Finally the removal of the brain, resultant ischaemia and slicing process will all set off events characteristic of cell death, which will have effects on the tissue integrity. The slices do recover and demonstrate relatively normal electrophysiological responses. However, any results obtained must be interpreted in the context of a limited brain circuitry due to the trauma of preparation.

Although it is convenient to use *in vitro* preparations because the whole animal models require lengthy preparation, the context of *in vitro* models must always be kept clear. There is a trend now towards using cell lines and brain slices to elucidate the cellular and molecular mechanisms of epilepsy but the information garnered from these studies is only useful when applied to whole animal models of epilepsy.

It was of interest to examine the polyamines and N¹-dansylspermine in an electrophysiological model, which was both an *in vitro* model for epilepsy and displayed primarily NMDA-mediated effects. As a result of its relative ease of preparation and the comparative simplicity of recording field potentials, the cortical wedge preparation was considered the most suitable model for use in the present study.

5.1.3 Cortical wedge preparations

The strain of mice normally used for the preparation of cortical wedges is the previously mentioned DBA/2 mouse. This mouse is genetically epilepsy prone and young animals will exhibit audiogenic seizures following intense auditory stimulation. This strain has been widely used to examine the underlying causes of audiogenic seizures and the neural pathways involved. The NMDA receptor has been demonstrated to be involved in the production of audiogenic seizures (Chapman *et al.*, 1996), but is not unique in this regard.

Cortical wedge preparations allow epileptiform activity to be examined within a controlled environment. Essentially, the method involves the slicing of the brain and a wedge being cut from this slice. The wedge is then mounted in a recording chamber and the potential difference across the wedge recorded. The details of this preparation are provided in greater detail in Section 5.2. The procedure used is a modification of that used originally by Harrison & Simmonds (1985) as devised by Hu and Davies (1997). Harrison and Simmonds invented the preparation in order to obtain a brain slice, that contained cortical efferents, which was set up in such a way that depolarisations of individual neurones could be recorded as a population response. They examined some NMDA antagonists, such as ketamine and 2-amino 5-phosphonovalerate (AP5), and were able to show different mechanisms and sites of action of these antagonists. Burton *et al.* (1987) also used this cortical wedge preparation but used TO mice and examined the responses obtained to NMDA, quinolinate, kainate and quisqualate. They demonstrated the lack of effect of both tetrodotoxin and bicuculline on the responses illustrating a lack of contributing effect of interneurones (tetrodotoxin) or GABA (bicuculline) on the recorded responses, demonstrating that the responses are due to the NMDA receptor. Similarly, Lancaster and Davies (1992) demonstrated the inhibition by carbamazepine of NMDA-induced depolarisations in cortical wedges from DBA/2 mice. They proposed, therefore, that the anticonvulsant action of carbamazepine is due to an antagonistic action at the NMDA receptor.

These studies investigated the effects of exogenously applied agonists but a number of studies have also examined spontaneous depolarisations induced by perfusing the cortical side with magnesium free aCSF. This relieves the block on the NMDA receptors and results in spontaneous epileptiform depolarisations. Hu and Davies (1995) examined the effect of the desglycyl metabolite of remacemide on both these spontaneous depolarisations and direct administration of NMDA. They demonstrated that this metabolite has a specific NMDA antagonist action. They also proposed that this non-competitive inhibition was mediated through the phencyclidine site in the ion channel. The DBA/2 cortical wedge has also been used in release experiments. Rowley *et al.* (1993) demonstrated the release of NO and glutamate from cortical wedges after the application of NMDA. In this study they also demonstrated age-related effects on this release which may account for the change in sensitivity with age of DBA/2 mice to audiogenic seizures. Hu and Davies (1997) examined the effects of NMDA antagonists and proved that they decreased the number of spontaneous depolarisations suggesting a role for NMDA receptors in the production of these spontaneous bursts.

It was of interest to examine the effect if any of N¹-dansylspermine on the frequency of spontaneous depolarisations in cortical wedges. It is well established that the polyamine spermine will enhance the effects of NMDA *in vitro* (Sprosen & Woodruff, 1990). This enhancement has been examined using cortical wedges from a rat. Robichaud and Boxer (1993) demonstrated an enhancement of spontaneous discharge frequency following spermine treatment though higher concentrations inhibited spontaneous discharges. This was similar to the findings of Sacaan and Johnson (1990), who demonstrated stimulatory (low dose) and inhibitory (high dose) effects of spermine on antagonist binding to the NMDA receptor. Therefore, the effect of N¹-dansylspermine was examined on the spermine enhancement of spontaneous epileptiform discharges.

5.2 EXPERIMENTAL PROCEDURE

Male and female DBA/2, genetically epilepsy-prone, mice were obtained from Harlan, UK and subsequently bred in the Bioresources unit, Trinity College. Stock mice were housed five to a cage with standard laboratory food and water available *ad libitum*. The mice were maintained at an ambient temperature of 21 ± 1 °C under a standard 12-hour light/dark cycle (light: 7am – 7pm). When used the mice were aged between 21 and 42 days.

On the day of use the animals were killed by cervical dislocation, decapitated and the brain was quickly removed to ice-cold artificial cerebrospinal fluid (aCSF), which washed off surplus blood and cooled the tissue. The aCSF contained in mM: NaCl 124, KCl 5, NaH_2PO_4 1.25, CaCl_2 2, MgSO_4 2, NaHCO_3 26, glucose 10 and was maintained at pH 7.4 by gassing with a 95% oxygen/5% carbon dioxide mix. The brain was then transferred to the cutting stage of a McIlwain tissue chopper and secured in place using super glue (Loctite). Coronal slices, 500 μm thick, were cut using the tissue chopper and placed in gassed aCSF at room temperature (20-22°C). The slices were separated using fine brushes. From these slices, small wedges of tissue containing cerebral cortex, corpus callosum and striatum were prepared. The wedges were approximately 4mm in length and 2mm in diameter at the cortex and reducing to 1mm at the striatum. The wedges were placed in two compartment baths, with a silicone grease seal (DC4 electrical compound, Dow Corning) isolating the grey cortical matter from the callosum. The two-compartment baths had previously been set up with full thickness (four strips) nappy liner (Terry Mates, UK) on the channel (cortical) side, which was pre-moistened with gassed aCSF at room temperature. The two compartment baths were attached to perspex sheets and held at an angle of approximately 15° to allow drainage of the artificial cerebrospinal fluid (aCSF). The compartment containing the callosal side of the wedge was filled with aCSF and the channel on the cortical side was continuously perfused with gassed aCSF at a flow rate of 2ml min⁻¹. One hour was allowed for the tissue to equilibrate at room temperature. Perfusion of the cortical side of the tissue was then continued

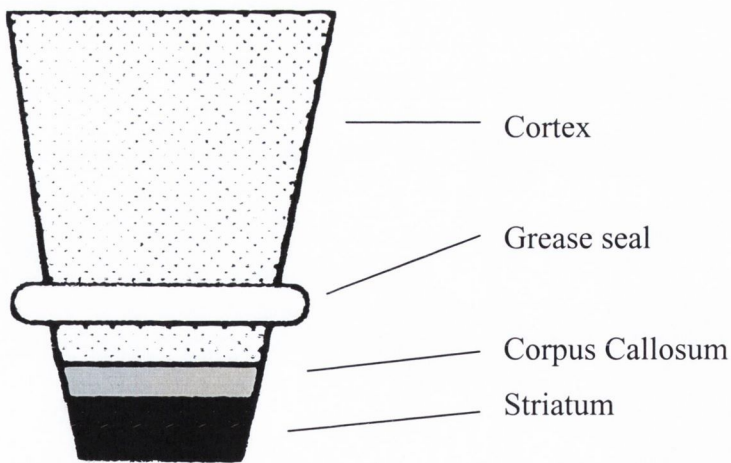
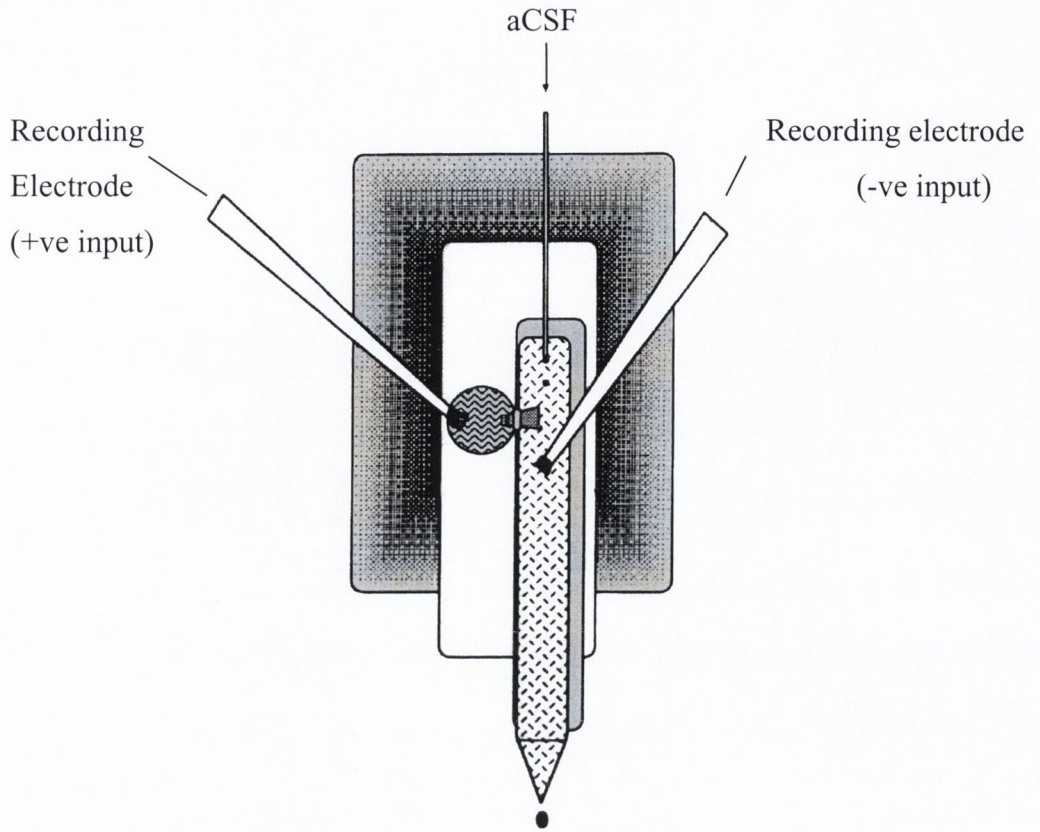


Figure 5.1: Representation of the cortical wedge apparatus showing an ideal cortical wedge and position of grease seal. After Hu and Davies, 1997.

with Mg^{2+} -free aCSF (with a corresponding isosmotic increase in Na^+) to facilitate NMDA receptor activation. The direct current (DC) potential between the two compartments was continuously monitored using silver/silver chloride electrodes. This potential was amplified (Fylde 2601A or ETH-250 from CB Sciences) and recorded on a MacLab computer system.

Drugs were only applied to the cortical side of the tissue and were administered dissolved in Mg^{2+} -free aCSF.

5.3 TREATMENT OF RESULTS

Magnesium-free aCSF-induced depolarisations became stable after approximately two hours. Thereafter, the number of depolarisations in the wedge preparation were counted in blocks of 5 minutes over three periods: 20 minutes immediately prior to drug perfusion (first period), 20-minute drug perfusion (second period) and then 20 or 40 minutes of drug free perfusion.

Mean values and standard error of the means were calculated and the results presented in graphical form as the number of spontaneous epileptiform discharges per 5 minutes versus time. Differences between groups were assessed using one-way ANOVA with repeated measures followed by subsequent analysis of simple main effects. Between group differences were analysed with further ANOVA followed by post-hoc Newman-Keuls test. Statistical significance in all tests was accepted at the $p < 0.05$ level, with the actual p values shown in the Figures.

The amplitude of the individual spikes was also examined. A section of trace (~1 minute) was examined every 10 minutes (or 20 minutes in the case of spermine traces) and the mean amplitude of the spikes in that selection calculated. This mean is presented as a percentage of control (before addition of either drug (N^1 -dansylspermine) or spermine). Each trace was examined separately. This was because of the different amplifiers used in recording, the spike amplitudes could not

be combined with similar treatment spikes unless presented as a percentage. Graphs were then plotted illustrating the percentage spike amplitude versus time for each of the different treatments.

5.4 RESULTS

After the wedges were placed in the perfusion apparatus, they were perfused with normal aCSF for approximately one hour. This was to allow them to come to room temperature and to equilibrate to the new environment. No spontaneous epileptiform depolarisations were seen at this point, though Hu and Davies (1997) demonstrated that a few depolarisations are occasionally seen at this point.

Figure 5.2 illustrates a trace obtained from one of the wedges and shows the changes that occur when the medium is changed from normal aCSF to Mg^{2+} -free aCSF. After approximately 20 minutes the first small depolarisation (spike) can be seen and the number increases from there, with time. After two hours, or more, perfusion with Mg^{2+} -free aCSF, the trace shows numerous spikes. Hu and Davies (1997) demonstrated that after two hours the number of spikes in a five minute period remained relatively constant (30 – 40 depolarisations per 5 minutes). This was also the finding in the present study.

Figure 5.2 also shows the effect of reversing the solutions. When 2mM Mg^{2+} is re-introduced to the perfusion solution, the spontaneous depolarisations cease almost immediately as the NMDA receptor is blocked by the Mg^{2+} ions.

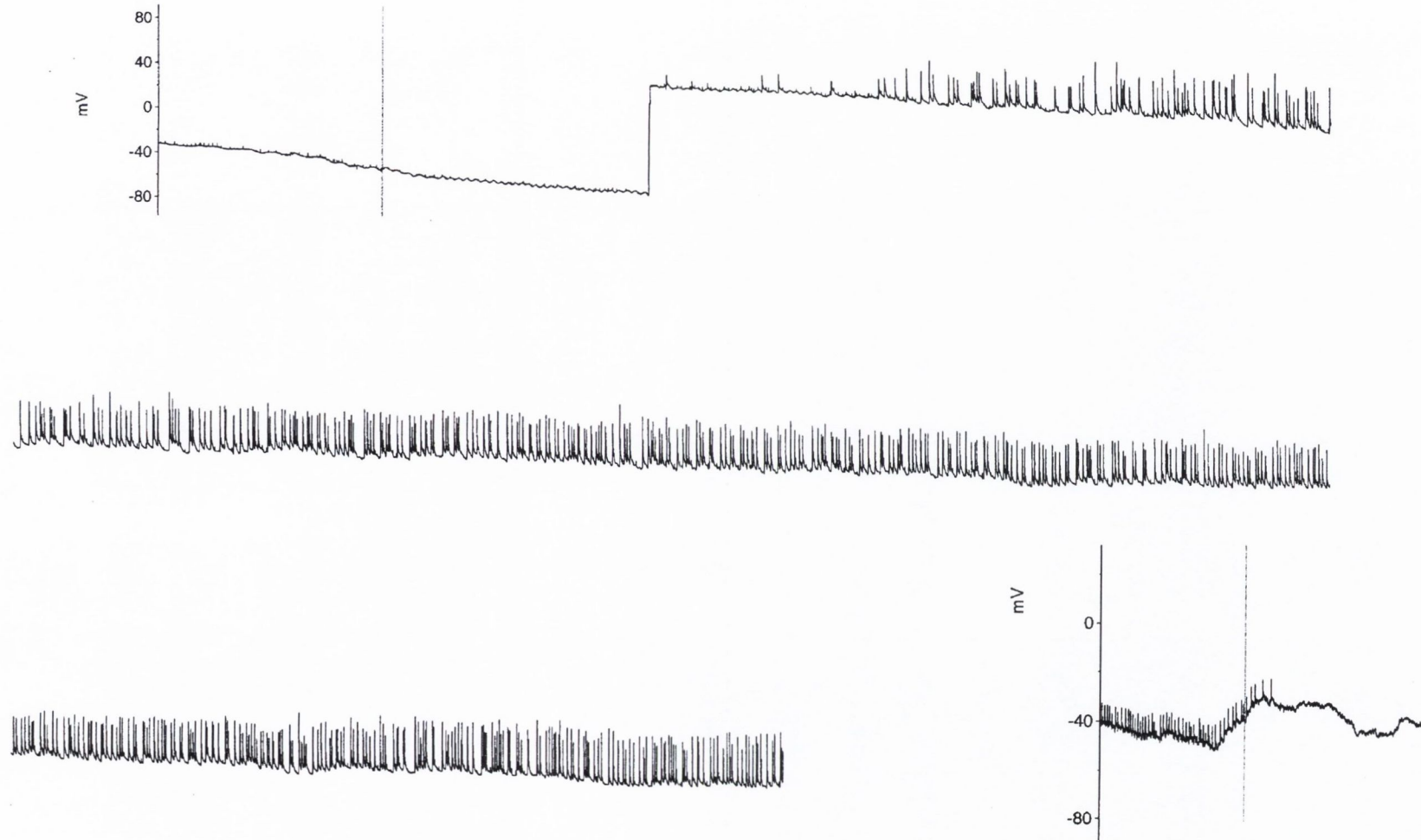


Figure 5.2: Effect of changing perfusion solution from normal aCSF to Mg^{2+} -free aCSF. Also shown (inset) is the effect of changing back to normal aCSF containing magnesium, this is from a different wedge, hence the different size spikes.

5.4.1 Effect of N¹-dansylspermine

Figure 5.3(a, b) shows traces illustrating the effect of administration of N¹-dansylspermine at two doses (50 μ M and 100 μ M). The dotted lines on the traces illustrate the points of addition and washout of N¹-dansylspermine. As can be seen, 50 μ M N¹-dansylspermine seems to have little effect on either the number of spikes or on the amplitude. However, 100 μ M N¹-dansylspermine caused a pronounced reduction of both the number of depolarisations and their amplitude.

Figures 5.4 and 5.5 show the graphs of depolarisations per 5 minutes versus time and the amplitude as a percentage of control versus time. The dotted lines again illustrate the addition and washout of N¹-dansylspermine (where applicable). From Figure 5.4, it can be seen that, as shown by Hu and Davies (1997), the number of spikes per 5 minutes remains constant after approximately 2 hours. This is shown by the control trace on the graph, which shows only slight variation. The quantitative process confirms that the lower dose of N¹-dansylspermine (50 μ M) does not have any effect on the number of depolarisations per 5 minutes, nor does it have an effect on the amplitude of the depolarisations (Figure 5.5).

However, 100 μ M N¹-dansylspermine causes both a reduction in the number of spontaneous depolarisation and a reduction in the amplitude of depolarisations. The effect in both cases is statistically significant. Also, from both of these graphs and from the trace from MacLab (Figure 5.3b), it can be seen that the effect of N¹-dansylspermine develops slowly and is prolonged, lasting long into the washout period.

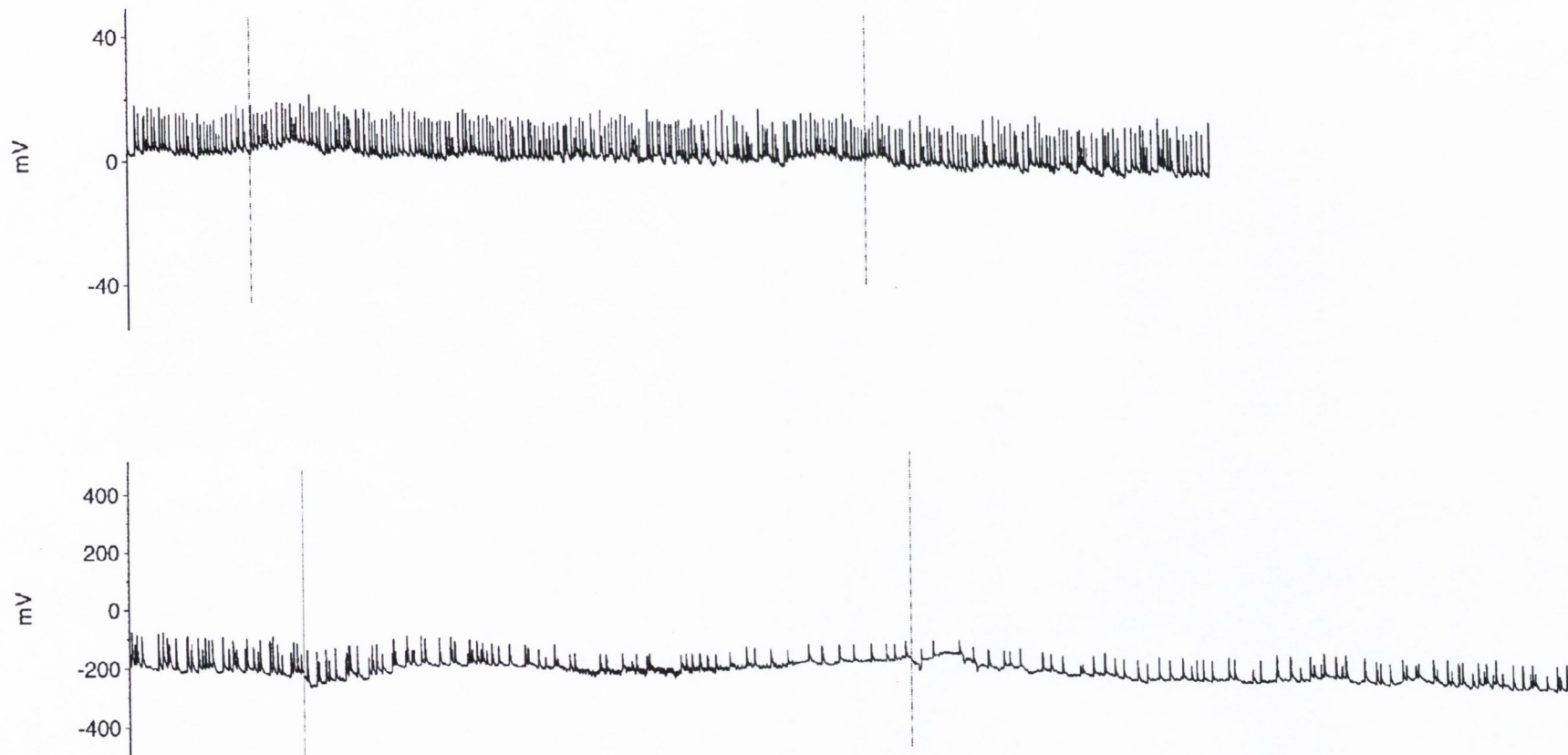


Figure 5.3: Effect of addition of either 50 μM (top trace) or 100 μM N¹-dansylspermine (bottom trace) on spontaneous epileptiform discharge production. The lines in each trace represent addition and then washout of N¹-dansylspermine.

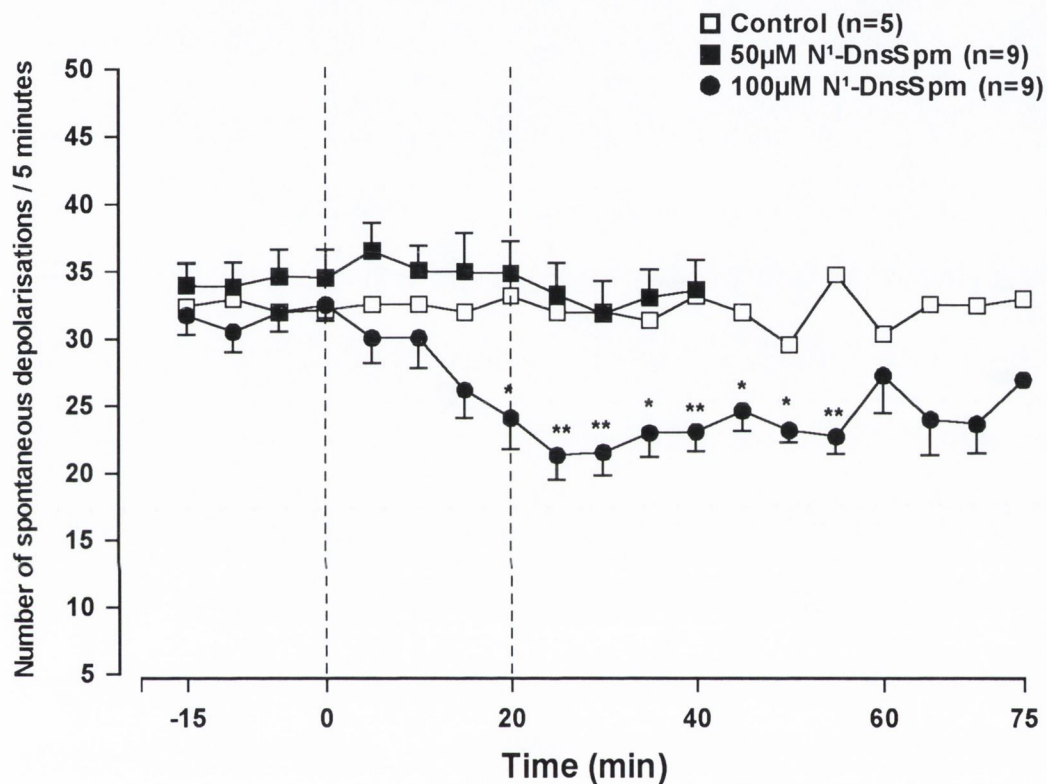


Figure 5.4: The effect of treatments of N^1 -dansylspermine on the number of spontaneous depolarisations per 5 minutes. The dotted line at time=0 marks the addition of N^1 -dansylspermine. The second line marks the washout with Mg^{2+} -free aCSF. * $p < 0.05$, ** $p < 0.01$, one-way ANOVA Vs control. Vertical bars indicate standard error of the means (SEM).

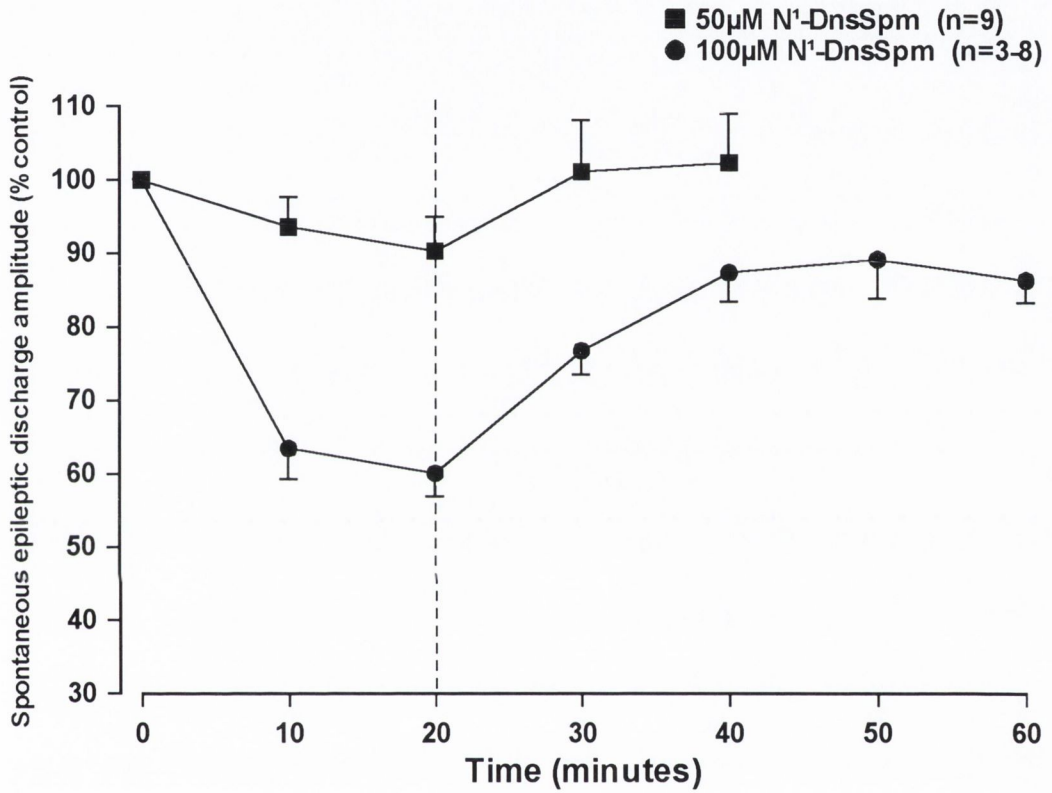


Figure 5.5: Effect of 50µM and 100µM N¹-dansylspermine on the amplitude of spontaneous epileptiform depolarisations. N¹-Dansylspermine is added to the perfusion solution at time zero and washed out at 20 minutes (dotted line). Vertical bars indicate SEM.

5.4.2 Action of spermine and its antagonism by N¹-dansylspermine

Figure 5.6 illustrates the effect of perfusion of the wedge with 300 μ M spermine. Spermine was perfused for 170 minutes and it is evident from this trace that the spermine increases the number of spikes per 5 minutes. Also as the perfusion of spermine is continued the amplitude of the depolarisations decreases. Figure 5.7 illustrates the effect of administration of 50 μ M N¹-dansylspermine on the enhancing effect of spermine. N¹-Dansylspermine decreases the number of spontaneous depolarisations despite the continued presence of spermine.

These observations are illustrated more clearly in the quantitative data expressed in Figure 5.8. From Figure 5.8 it can be seen that, after the initiation of perfusion with Mg²⁺-free aCSF containing 300 μ M spermine, there is a steady increase in the number of spontaneous depolarisations per 5 minutes up to the 90 minute time point, when N¹-dansylspermine was added. There was no difference between the test and control discharge rate when the N¹-dansylspermine was added. However, after the addition of 50 μ M N¹-dansylspermine, which on its own had no effect, there is a substantial and statistically significant reduction in the frequency of spontaneous discharges. This reduction persists for the duration of the drug treatment and continues well into the washout period.

The amplitude of spontaneous depolarisations shows a slightly different story (Figure 5.9). As the spermine is perfused the amplitude gradually decreases. This is in accordance with a previous study (Robichaud & Boxer, 1993), which also showed a decrease in amplitude with time. After 90 minutes perfusion the amplitude has decreased to 65% of the original amplitude. When N¹-dansylspermine is added to the perfusion solution at 90 minutes there is no effect on the steady decrease in amplitude.

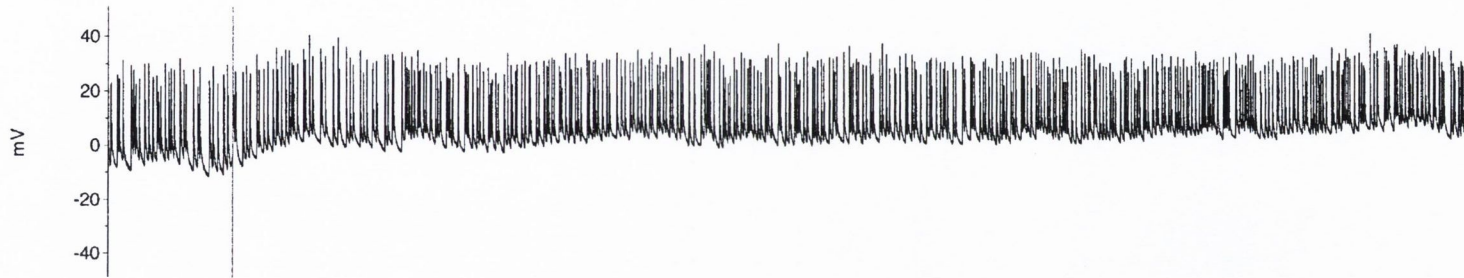


Figure 5.6: Effect of 300 μ M spermine on the frequency of spontaneous epileptiform discharges. Vertical line at the start of trace shows the point of addition of spermine.

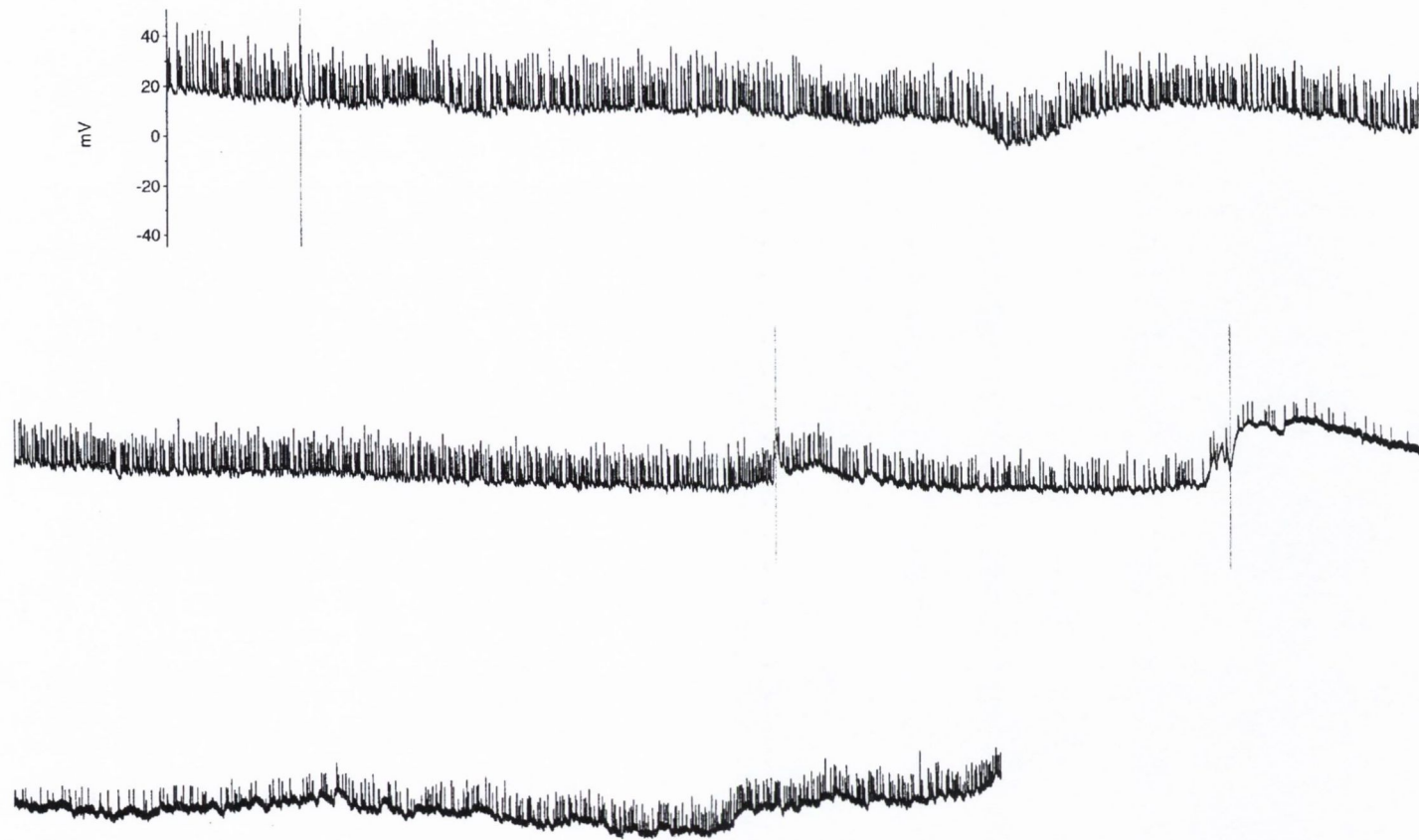


Figure 5.7: Effect of $50\mu\text{M}$ N^1 -dansylspermine on spontaneous epileptiform discharges in a cortical wedge treated with $300\mu\text{M}$ spermine. The line at the start shows the addition of spermine and the two lines in the middle illustrate the addition and washout of N^1 -dansylspermine.

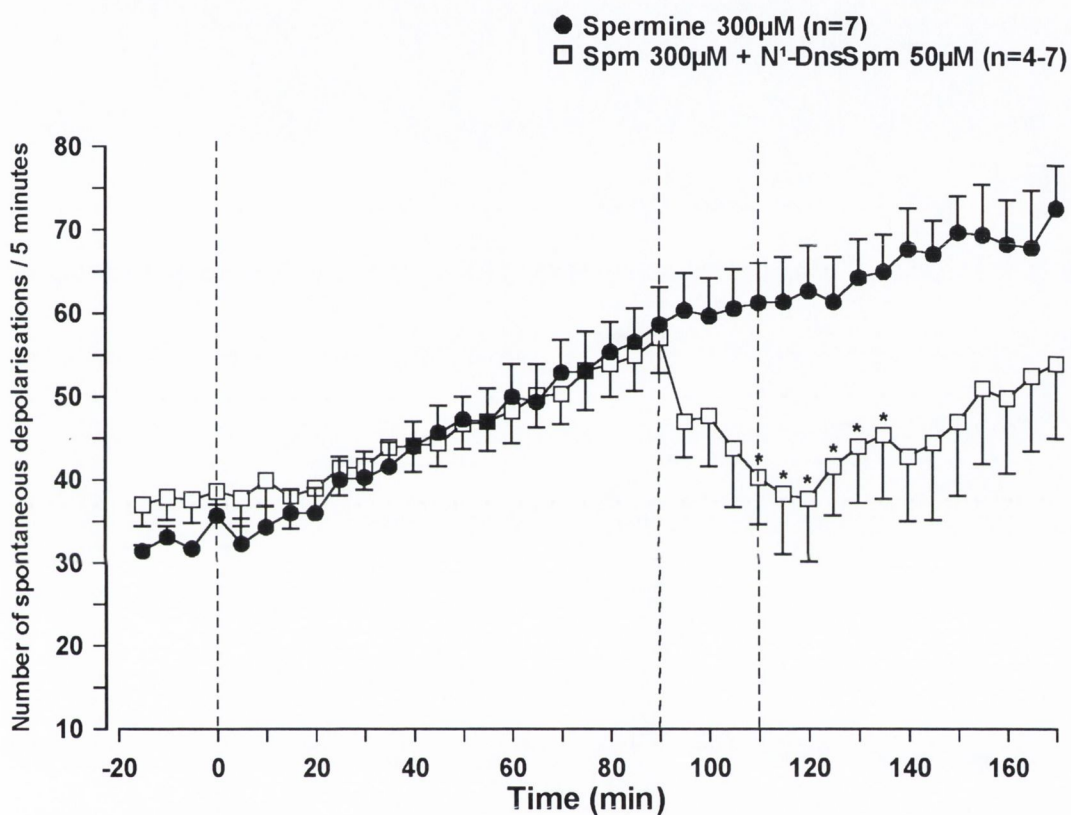


Figure 5.8: Effect of 300µM spermine and 50µM N¹-dansylspermine on the number of spontaneous epileptiform depolarisations per 5 minutes. The dotted line at time=0 represents the initiation of perfusion with Mg²⁺-free aCSF containing spermine. The lines at 90 and 110 minutes represent the start and end of drug treatment. *p<0.05 one-way ANOVA Vs 300µM Spm alone. Vertical bars indicate SEM.

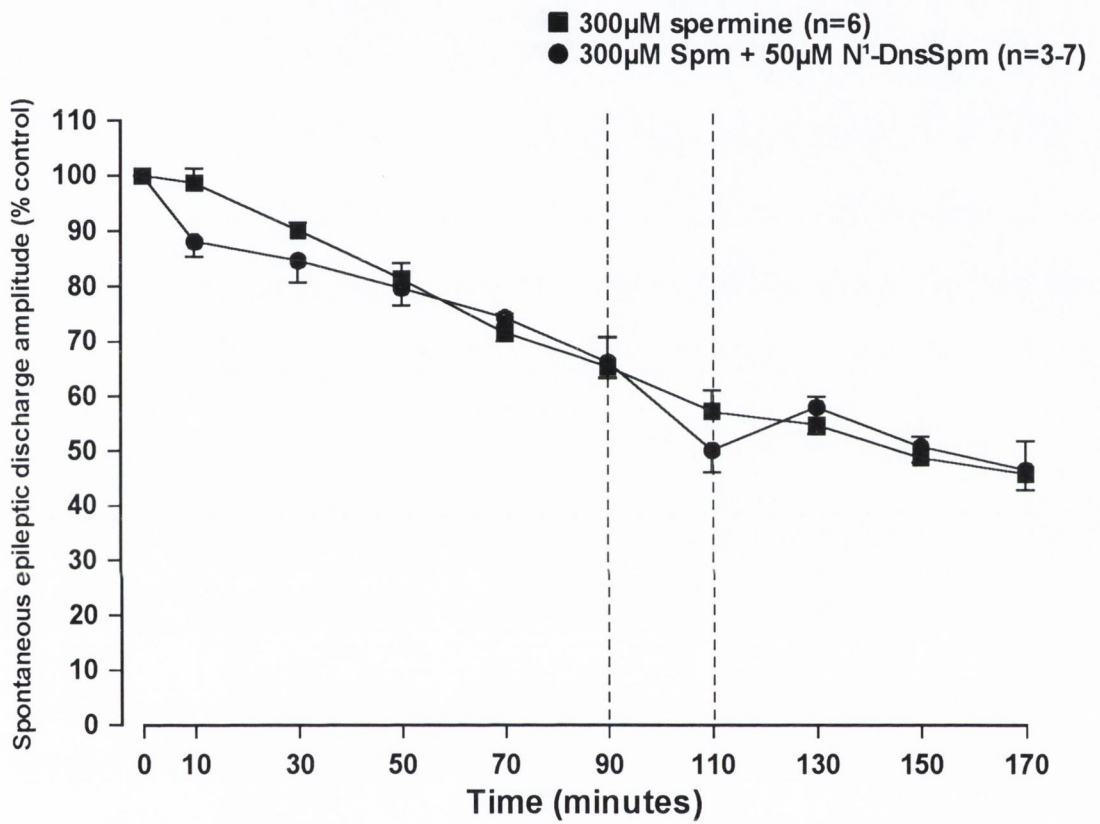


Figure 5.9: Effect of spermine and N¹-dansylspermine on the amplitude of depolarisations. Dotted lines represent addition and washout of N¹-dansylspermine. Vertical bars indicate SEM.

5.5 DISCUSSION

In the results presented here it can be seen that the removal of the 2mM Mg^{2+} from the perfusion solution has a dramatic effect on the response of the cortical wedge. It is suggested that the reason for this response is that magnesium is present in the normal extracellular medium and produces a voltage dependent blockade of the NMDA receptor macrocomplex. This implies that NMDA receptors are largely blocked in neurons at their resting membrane potential (about $-70mV$). This block is relieved on depolarisation or removal of the Mg^{2+} , which allows the glutamate to activate the NMDA receptor.

When the magnesium was removed from the perfusion solution, there was a gradual increase in the excitability of the cortical wedge. After approximately 20 minutes the first appearance of spontaneous epileptiform discharges was seen. These gradually become larger and more frequent with time. This probably arises because, though the magnesium had been removed from the perfusion solution, not all of the magnesium would have been washed off the tissue immediately. However, conversely, when all the magnesium has been washed off and the spontaneous discharges have been present for some hours, the re-addition of 2mM magnesium to the artificial cerebrospinal fluid (aCSF), causes the almost immediate cessation of depolarisations. This illustrates the crucial role of the NMDA receptor in the spontaneous depolarisations that are seen. Work by Robichaud *et al.* (1991) also came to the conclusion that the NMDA receptor was almost uniquely involved in the production of depolarisations. They demonstrated that 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[F]quinoxaline (NBQX), a potent selective AMPA receptor antagonist, had no effect on the frequency of depolarisations at doses that completely blocked AMPA and kainate induced depolarisations in the cortical wedge. Indeed Aram *et al.* (1989) demonstrated a pivotal role for NMDA receptors in epileptogenesis in neocortical slices. They showed that NMDA receptor antagonists, acting on different sites of the receptor complex, reduced burst frequency and the number of afterpotentials in the rat cortical wedge preparation. Work by Phillips *et al.* (1997) also showed that NMDA receptor antagonists affected the spontaneous

epileptiform discharge frequency but that AMPA receptor antagonists had no effect. There is, however, some conflicting evidence as Hu and Davies (1997) demonstrated that 6,7-dinitroquinoxaline-2,3-dione (DNQX), an AMPA/kainate receptor antagonist, produced a dose-dependent depression of spontaneous depolarisations. Needless to say, further study is required into the population of receptors primarily responsible for the spontaneous depolarisations.

This illustrates the role of the NMDA receptor in the production of these discharges. However, the rate of depolarisations does not depend entirely on NMDA receptor function. While the spontaneous depolarisations require glutamate or another excitatory amino acid, inhibitory processes, such as the release of GABA, also operate to stop the discharges. Strangely, in the mouse cortical wedge preparation, Davies & Shakesby (1999) demonstrated that GABA actually had a depolarising action in the slices rather than the expected hyperpolarising action. This response is also produced by inhibition of GABA uptake. This mechanism is thought to be mediated through GABA_A receptors (Phillips *et al.*, 1998; Davies & Shakesby, 1999). Despite this finding, the measurement of the spontaneous epileptiform discharge frequency has become widely used as a sensitive measure of endogenous NMDA receptor activity.

Hu and Davies (1997) demonstrated that, after approximately two hours perfusion with the Mg²⁺-free aCSF, the number of spontaneous epileptiform discharges per 5 minutes remained stable, at approximately 35 per 5 minutes, for up to 4 hours. This is illustrated here with wedges remaining viable for up to 5 hours (longest observation period).

Age-related effects have been reported for DBA/2 mice. These mice are specially bred to be susceptible to sound induced seizures. However, there appears to be an age of maximal susceptibility to audiogenic seizures (Sykes & Horton, 1982). Indeed, Rowley *et al.* (1993) investigated the effects of age on NMDA-induced release of nitric oxide and glutamate from cortical slices from DBA/2 mice of different ages. They showed that NMDA stimulated release in mice aged 21-30 days

but there was no significant release in mice both younger and older. This may suggest that the age-related susceptibility to audiogenic seizures is due to changes in sensitivity to NMDA receptor mediated effects. Accordingly, all the mice used in the present study were aged between 21 and 30 days old.

N¹-Dansylspermine was investigated for action against the spontaneous epileptiform depolarisations. Two doses were used, 50µM and 100µM, both made up in the Mg²⁺-free aCSF. The lower dose, 50µM, had no effect on either the spontaneous epileptiform discharge frequency or on the amplitude of the discharges.

However, a dose of 100µM, N¹-dansylspermine produced a statistically significant reduction in the number of spontaneous epileptiform depolarisations per 5 minutes, both during the drug treatment time and also for sometime afterwards. This is probably due to an agonist action by N¹-dansylspermine at the negative or inhibitory polyamine site on the NMDA receptor (Chao *et al.*, 1997).

In relation to the persistence of the effect, it is well known that the polyamines bind strongly to tissue and other materials. Spermine is always supplied in plastic containers as it would stick to glass. Robichaud and Boxer (1993) demonstrated that the action of spermine significantly outlasted the application of spermine to the wedge. This is possibly due to the polyamine's persistence in tissues. Therefore, it is possible that the persistent action of N¹-dansylspermine, after it has been removed from the aCSF, is as a result of a similar ability to bind to tissue.

The effect of spermine on the spontaneous epileptiform depolarisation frequency was also investigated. Robichaud and Boxer (1993) had previously investigated the effects of both spermine and spermidine. They demonstrated a gradual increase, over time, in discharge frequency for all doses of spermine up to 1mM. However, at 1mM there was an initial increase followed by a decrease in frequency below the control frequency. They also tried 3mM spermine, which only produced a reduction in frequency. These findings were thought to be as a result of an action on the positive site on the NMDA receptor at low concentrations and action at the negative

site at high concentrations, as found by Sacaan and Johnson (1990) on [³H] *N*-(1-[thienyl]cyclohexyl)piperidine (TCP) binding to the NMDA receptor.

In this study only one dose of spermine (300µM) was investigated as only the stimulatory effects were of interest. This dose of 300µM spermine was shown by Robichaud and Boxer (1993) to be stimulatory, producing a maximum response after 60 to 90 minutes. A stimulatory effect of spermine was also seen in the present study with the spontaneous epileptiform depolarisation rate being increased significantly after 90 minutes of treatment with 300µM spermine. The development of this response is slow and as suggested by Robichaud and Boxer (1993) this could be due to the spermine taking time to equilibrate with the tissue.

It was noted that as the discharge frequency increased, the amplitude of discharges gradually decreased. After 90 minutes the amplitude had decreased to only 65% of the amplitude before perfusion of spermine. This effect has been seen before, both as a result of polyamine treatment (Robichaud & Boxer, 1993) and treatment with NMDA receptor antagonists and anticonvulsants (Phillips *et al.*, 1997), but an explanation for the effect on amplitude is not forthcoming. Indeed, no one is certain of a reason for the effects on amplitude (J. Davies, personal communication). However, one possibility is that as the frequency of depolarisation increases the number of neurons firing or channels opening at any one point decreases, producing a smaller depolarisation. The reduction in the numbers firing, to produce each spike compared to before the addition of spermine is possibly due to the existence of a refractory period. Therefore, though the neurons are firing or channels opening more often, there is still a period in which they cannot produce a depolarisation, so the number of channels or neurons able to sum together to produce the spike is reduced.

N¹-dansylspermine was also investigated for activity against the spermine-induced increase in discharge frequency. It was decided that the dose of N¹-dansylspermine to be used would be 50µM as this had no effect on the spontaneous epileptiform depolarisation frequency on its own (see above). Spermine (300µM) was perfused for 90 minutes, resulting in an increase in discharge frequency, at which point N¹-

dansylspermine (50 μ M) was perfused, with spermine, for 20 minutes. N¹-dansylspermine addition resulted in an immediate onset and prolonged decrease in the discharge frequency. The decrease produced reduces the frequency to what would be a normal frequency for a wedge that had not been treated with spermine. The decrease produced here is thought to be as a result of an antagonistic action for N¹-dansylspermine at the positive polyamine modulatory site on the NMDA receptor. This is entirely consistent with the earlier finding that low doses of N¹-dansylspermine antagonise spermine induced convulsions without affecting NMDA-induced convulsions.

As in the *in vivo* situation, N¹-dansylspermine was an effective antagonist even in the continued presence of spermine. The structure of N¹-dansylspermine is such that a competitive action is the most likely. Action at the negative site can be ruled out since N¹-dansylspermine did not influence spontaneous activity at 50 μ M.

The addition of N¹-dansylspermine has no statistically significant effect on the gradual decrease in amplitude of discharges. There is an initially large drop on addition of N¹-dansylspermine, though this is not statistically significant, and on removal the trace continues the gradual decline.

An explanation put forward by Robichaud & Boxer, in 1993, for the gradual increase in the discharge frequency on addition of spermine was that the spermine was acting either intracellularly or presynaptically. Bondy & Walker (1986) demonstrated that the polyamines are involved in the regulation of the release of aspartate and could enhance release. Rao *et al.* (1990) suggested that spermine's action may be mediated through intracellular calcium mobilisation. If N¹-dansylspermine was antagonising the effects of spermine, intracellularly or presynaptically, then any effect would be delayed as the N¹-dansylspermine reaches the site of action. However, an immediate action is seen.

5.6 CONCLUSION

The results presented here provide more evidence for the mechanism of action of N¹-dansylspermine. They confirm the suggestion that at low dosage N¹-dansylspermine works through the positive polyamine modulatory site on the NMDA receptor macrocomplex to antagonise the actions of spermine. Spermine has again been demonstrated to enhance the activity of the NMDA receptor. However, the effect seen here is delayed. The delayed effect resembles that seen after intracerebroventricular injection of spermine. This may be through the spermine having to equilibrate and build up on the tissue before a marked effect is seen. This is reasonable since many other electrophysiological studies showing an immediate effect of spermine on NMDA have been carried out on single cells rather than a section of tissue. However, further investigation is required to fully elucidate the role of spermine enhancement.

SECTION 6

Further discussion

6.1 INTRODUCTION

The aim of this section is to tie together all the results and conclusions obtained from the earlier sections and to incorporate them into the present vision of existing systems. The NMDA receptor, calcium channels and potassium channels will all be discussed in greater detail than in earlier sections with mention made of sites of action and roles of modulators and reference made to earlier conclusions.

6.2 THE NMDA RECEPTOR MACROCOMPLEX

In 1954, Hayashi demonstrated the convulsive effects of L-glutamate and L-aspartate in the mammalian brain. From this beginning grew the knowledge that glutamate is the principal excitatory transmitter in the mammalian central nervous system acting on a number of different receptors. These include the ionotropic glutamate receptor, the NMDA receptor. A number of characteristics of this receptor were discovered quite early on, such as the existence of glycine as a co-agonist of the receptor (Johnson & Ascher, 1987), the antagonistic effect of Mg^{2+} ions (Evans *et al.*, 1977) and the existence of numerous other modulatory sites on the NMDA receptor macrocomplex. These include at least two polyamine sites, a proton site, a Zn^{2+} site and an ifenprodil site, all of which will be discussed below.

With the advent of molecular pharmacology and advances in gene mapping, cell line production and recombinant technology, our knowledge of the NMDA receptor and other receptors has increased dramatically. This has also had the effect of making our view of receptors and cellular mechanisms more complex.

Polyamines were originally demonstrated to have an enhancing effect on NMDA receptor channel opening (Ransom & Stec, 1988) and the following year it was

demonstrated that high concentrations produced inhibition of binding of radio-labeled open-channel blockers, MK-801 and TCP (N-(1-[thienyl]cyclohexyl)piperidine), (Sacaan & Johnson, 1990) representing a second inhibitory polyamine site. When Moriyoshi *et al.* (1991) cloned the first NMDA receptor, this opened up a whole new method of examining the NMDA receptor. They cloned the NR1 subunit and this exists in eight splice variants, the products of one gene. The NR2 subunit exists in 4 forms, NR2A, NR2B, NR2C, NR2D (Ishii *et al.*, 1993), each of which are distinct gene products having different regional and temporal patterns of expression (Hollmann & Heinemann, 1993). These two types of subunits (NR1 and NR2) combine in native receptors to produce hetero-oligomeric macrocomplexes. However the number of subunits is not known, though it is possible that both NMDA receptors and AMPA/kainate receptors contain 5 subunits (Ferrer-Montiel & Montal, 1996). It was originally thought that the heteromers contained only one type of each subunit (i.e. binary heteromers), such as NR1/NR2A or NR1/NR2B. However, recently Dunah *et al.* (1998) demonstrated the existence of ternary complexes. They showed that NR2D subunits form heteromeric complexes with NR1 as well as NR2A and/or NR2B.

As was mentioned in Section 1.2, the primary functions of polyamines are glycine dependent or independent stimulation, voltage dependent block, reduction in affinity of glutamate and relief of proton inhibition. The presence or absence of these above effects on the NMDA receptor, depend on receptor heterogeneity, as was proposed by Lynch and colleagues (1995). Indeed Williams has demonstrated that only glycine dependent stimulation and voltage dependent block are seen at receptors containing NR2A subunits (Williams *et al.*, 1994) and that none of the above described effects of spermine, all seen in NR1A and NR1A/2B receptors, were seen at NR1A/NR2C or NR1A/NR2D receptors (Williams *et al.*, 1994; Williams, 1995). This demonstrates that not only the location but also the subunit composition of NMDA receptors affects their physiological roles. Furthermore, Sharma and Reynolds (1999) suggested that the NR2 subunit influences the stimulatory effects of the polyamines and that there is no positive modulatory site on the NR1:NR2A complex.

Table 6.1: The subunit-specific effects of spermine and pH on recombinant NMDA receptors (from Johnson, 1996)

Characteristic: Effects of spermine	NMDA receptor subunit combination							
	NR1A/ NR1A	NR1A/ NR2A	NR1A/ NR2B	NR1A/ NR2C	NR1A/ NR2D	NR1B/ NR1B	NR1B/ NR2B	
Glycine independent Stimulation	+	-	+	-	-	-	-	
Glycine dependent Stimulation	+	+	+	-	-	+	+	
Voltage dependent Inhibition	+	+	+	-	-	+	+	
Decreased affinity For agonist	+	-	+	-	-	+	-	
pH sensitivity	+	+	+	+	+	-	-	

Each NMDA receptor subunit consists of a large extracellular N-terminal domain, three membrane spanning domains (M1, M3 and M4) and a re-entrant loop (M2) which is thought to form part of the channel pore. Using site directed mutagenesis it is possible to further modify the receptor subunits, altering individual amino acid residues, in order to learn more about the receptor and binding sites for modulators. In 1995, Williams *et al.* used this technique to suggest the existence of two stimulatory polyamine sites by demonstrating that a point mutation to a residue in the NR1 subunit affected glycine independent stimulation but not glycine dependent stimulation or voltage dependent block.

Kashiwagi *et al.* (1997) used site directed mutagenesis to examine block of the NMDA receptor by polyamines. They showed that mutations in the M1 and M3 transmembrane domains affected block by both spermine and N¹-dansylspermine and that a specific mutation in the pore-forming domain (M2) of a tryptophan residue at

W607 reduced block by N¹-dansylspermine and allowed it to permeate the channel. This work demonstrated that at least parts of M1 and M3 contribute to the pore or vestibule of the NMDA channel and that a tryptophan in M2 forms the narrow section of the pore.

Igarashi and Williams (1995) examined the inhibitory effects of spermine at the NMDA receptor, compared to bis(ethyl)pentaamines. At one recombinant receptor, NR1/NR2A, the pentaamines blocked the receptor but spermine did not. The block was also relieved at highly negative potentials (>-100mV). This suggested that the block by the pentaamines was different to spermine. They also suggested that, at hyperpolarised membrane potentials, the pentaamines permeated the NMDA receptor channel.

Chao *et al.* (1997) used recombinant NMDA receptors (NR1/NR2A) and site directed mutagenesis to examine the block and permeation of the NMDA receptor by N¹-dansylspermine and N¹-(n-octanesulfonyl)-spermine. The inhibition demonstrated by both of these substances was found to be 1000-fold more potent than the block by spermine. Site directed mutagenesis at NR1(N616G) or NR2A(N615G) allowed N¹-dansylspermine to permeate the pore. This was thought to be due to an increase in pore size of channels with these residues, also demonstrating that these residues are partly responsible for the pore formation.

Araneda *et al.* (1999) examined the block by spermine and arcaine of the NMDA receptor. They used patch-clamp methods to study the block. Up to potentials of -60mV both spermine and arcaine showed block of the channel. At more negative potentials however, the block was relieved demonstrating permeation of the channel. However, despite the evidence showing voltage dependent channel block by spermine and analogues, such as N¹-dansylspermine, it is not yet clear if this block has any physiological relevance, especially considering the block is weak and develops more slowly than block by physiological concentrations of extracellular Mg²⁺ (Williams *et al.*, 1994).

Zinc has been shown to produce selective and non-competitive antagonism of NMDA in cultured neurons (Westbrook & Mayer, 1987). The action appears to be different to that of magnesium as the reduction of NMDA currents is not voltage dependent but is due to reduced channel open time (Mayer *et al.*, 1988). Recently it was demonstrated, by Berger and Rebernik (1999), that the mechanism by which zinc reduces channel opening of the NMDA receptor macrocomplex, is from allosteric inhibition of a polyamine sensitive regulatory site different from that inhibited by ifenprodil. Furthermore it was shown that these two allosteric sites influence each other in a manner dependent on the brain region investigated.

The NMDA receptor has been demonstrated to be blocked by protons with an IC_{50} of pH 7.3 and this results in a tonic inhibition of approximately 50% at physiological pH (Traynelis & Cull-Candy, 1990). Stimulation by spermine has been shown to be pH sensitive and the stimulation may involve relief of the proton inhibition when responses are measured at physiological pH (Traynelis *et al.*, 1995). This was supported by work by Kashiwagi *et al.* (1996) who demonstrated that a point mutation at D699 on the NR1A subunit abolished glycine independent spermine stimulation in heteromeric NR1A/NR2B receptors. This also reduced inhibition by protons, leading to the suggestion that glycine independent stimulation resulted from the relief of proton inhibition.

As can be seen from Table 6.1, all of the receptor complexes with NR1A demonstrate sensitivity to inhibition by protons, whereas all of the complexes with NR1B were resistant to proton inhibition. This is due to the presence (NR1B) or absence (NR1A) of a 21 amino acid N-terminal loop, which is coded for by exon 5. This is predicted to form a loop on the extracellular surface of the NR1 protein. For the homomeric NR1B subunit receptors, the pH sensitivity and glycine independent stimulation by spermine are markedly attenuated compared to NR1A containing complexes. It is proposed that the N-terminal insert has a net positive charge and that this insert exerts its effect by shielding the proton sensor or may act as a spermine like modulator of the NMDA receptor (Traynelis *et al.*, 1995). It has recently been demonstrated that there is a region of the NR2B subunit, site directed mutations of which, resulted in a relief

of proton inhibition which correlated with reduction of glycine independent stimulation by spermine. This demonstrates that polyamines and protons share common NR2B determinants (Gallagher *et al.*, 1997)

Ifenprodil is an NMDA antagonist and, as mentioned previously, there have been many proposed mechanisms of action (see Sections 2.6 & 3.6). It is however, selective for NR1/NR2B receptor complexes (Williams, 1993). Williams demonstrated, using voltage clamp recording, that the ability of ifenprodil to inhibit NMDA-induced currents was 400-fold less at NR1A/NR2A receptors than at NR1A/NR2B receptors. Also illustrated, though, was the reduction of ifenprodil's inhibitory action by increasing the concentration of glycine. Thus part of the mechanism of action of ifenprodil was proposed to be through noncompetitive antagonism of the effects of glycine. Avenet *et al.* (1997) also demonstrated the NR2B subunit selectivity of ifenprodil (and its stereoisomers (Avenet *et al.*, 1996)) and also of eliprodil and suggested that this explained their favourable side effects profile when compared to channel blockers such as MK-801, memantine and phencyclidine.

Further work led to the discovery of effects of extracellular pH and protons on ifenprodil inhibition (Pahk & Williams, 1997; Mott *et al.*, 1998). Pahk and Williams (1997) demonstrated that ifenprodil inhibition was pH sensitive with a smaller inhibition at alkaline pH. This sensitivity occurs at receptors containing either the NR1A subunit or the NR1B subunit, demonstrating that exon 5 has no effect on this. Therefore, it was proposed that protons may directly affect the ifenprodil binding site. Mott *et al.* (1998) demonstrated an ifenprodil induced increase in NMDA receptor sensitivity to protons and extracellular pH. They also demonstrated this mechanism of action for the ifenprodil analogue, CP-101,606.

Recent evidence has suggested that the mechanism of action of ifenprodil is through a non-competitive, allosteric interaction with one of the spermine binding sites on the extracellular surface. This is such that the binding of spermine to the NMDA receptor causes a reduction in receptor affinity for the antagonist and vice versa (Kew &

Kemp, 1998). Allosteric inhibition was also demonstrated by Berger and Rebernik (1999), as previously mentioned, who demonstrated an allosteric inhibition of receptor channel opening and an allosteric interaction between the site for zinc and for ifenprodil.

It has been suggested by Kew and Kemp (1998) that Mg^{2+} is the physiological agonist, acting at the spermine site, mediating glycine-independent stimulation. The observation that Mg^{2+} produces similar effects to spermine on NMDA receptor affinity for ifenprodil does not necessarily imply that Mg^{2+} rather than spermine is responsible for the production of glycine-independent stimulation. This suggestion should, therefore be interpreted with caution.

Finally, a recent study by Masuko *et al.* (1999) compared a regulatory domain (R1-R2) in the amino terminus of the NMDA receptor with the bacterial leucine/isoleucine/valine binding protein. They showed using site directed mutagenesis, a similarity between the NR1 subunit and this bacterial protein, containing two regulatory domains, which they called R1 and R2. They also proposed binding sites for spermine, ifenprodil and protons within this structure, thus giving the first visual as well as positional representation of binding sites/domains in the NR1 subunit.

From the above discussions it appears that there is some way to go in the elucidation of all of the binding sites and different conformations of the NMDA receptor macrocomplex. However, it certainly appears from these studies that there are at least two positive polyamine stimulatory sites on the extracellular surface of the NMDA receptor, one of which is affected by the amino acid insert coded for by exon 5 and the other which is not. The inhibitory polyamine site has been demonstrated to be within the ion channel overlapping the magnesium site slightly. Finally, Ifenprodil, which was long thought to be an antagonist at the positive polyamine site, has been demonstrated to have its own binding site, which interacts allosterically with the polyamine binding site to inhibit it.

6.3 CALCIUM CHANNELS

Through cloning, expression and functional assessment, voltage-dependent calcium channels have been demonstrated to consist of a number of subunits, at least three. The principal subunit, $\alpha 1$, consists of four homologous transmembrane domains, each containing six transmembrane α helices surrounding a central pore. This principal subunit can carry out the basic functions of the voltage gated ion channel (Catterall, 1993). However, associated with each one are regulatory subunits ($\alpha 2$, β , γ , δ). The $\alpha 2$ and δ subunits are linked by a disulphide bond (Isom *et al.*, 1994). Most neurons coexpress multiple types of different calcium channel $\alpha 1$ ($\alpha 1A$ - $\alpha 1E$, $\alpha 1G$)(Snutch *et al.*, 1990; Perez-Reyes *et al.*, 1998). The individual components of native whole-cell calcium currents have been isolated (T-, L-, N- and P/Q-type) and these have been linked to their cloned equivalents, P/Q-type ($\alpha 1A$), N-type ($\alpha 1B$), L-type ($\alpha 1C$ and $\alpha 1D$) and T-type ($\alpha 1G$);(Sutton *et al.*, 1998).

The voltage sensitive calcium channels have been classed as above according to biophysical and pharmacological properties of the channels. The dihydropyridines have been widely used to class calcium channels as have the polyamine and polyamide toxins (Miljanich & Ramachandran, 1995)

The L-type calcium channels inactivate very slowly and are sensitive to the dihydropyridines. The N-type calcium channels inactivate more rapidly than L-type and have been demonstrated to be sensitive to ω -conotoxin GVIA. The P-type calcium channels inactivate very slowly and are not affected by either the dihydropyridines or ω -conotoxin GVIA. However, they have been shown to be sensitive to ω -agatoxin IVA and the funnel spider toxin, FTX (Llinás *et al.*, 1989).

The polyamines may possess a modulatory action at voltage activated calcium channels, which may be through effects on a second messenger system rather than direct interaction with calcium. Herman *et al.* (1993) demonstrated an increase in L-type calcium channel currents with external application of putrescine. This increase however, was blocked by the protein kinase C inhibitor, staurosporine. Indeed,

Schoemaker (1992) demonstrated that both spermine and spermidine allosterically inhibited [³H]nitrendipine binding to L-type calcium channels, suggesting that the polyamines could function as endogenous modulators of the voltage dependent calcium channels. Similarly the polyamines have been demonstrated to be involved in the regulation of neurotransmitter release, contributing to calcium stimulated release of aspartate from the brain (Bondy & Walker, 1986). Recently, Sutton *et al.* (1993) demonstrated inhibitory actions of spermine (>100 μ M) on voltage activated calcium currents recorded from dorsal root ganglion neurons.

The polyamine spider toxins and their synthetic analogues are significantly more potent than the polyamines at blocking the calcium channels. As a result some of them are now widely used to study calcium channels using site directed mutagenesis in an attempt to locate binding sites and model the optimal structure for calcium antagonism. It has been demonstrated that changing an arginine for lysine on sFTX (the synthetic analogue of FTX) abolishes its inhibitory action on calcium channels (Cherksey *et al.*, 1991). Another polyamine spider toxin, argitoxin-636, has been demonstrated to inhibit, in a use-dependent manner, voltage activated calcium currents, in the micromolar range (Sutton *et al.*, 1993).

There are a number of possible mechanisms of action on calcium channels for the polyamines and the polyamine toxins. They can bind to the calcium channel itself, lipids or other cell membrane constituents to affect the lipid microenvironment resulting in different channel properties or they can affect second messenger pathways. The polyamine toxins, including FTX, are thought to act directly on the calcium channels (Llinás *et al.*, 1989). The actions of the polyamines, including spermine, on calcium channels may involve their ability to cross-link phospholipids and stabilise membranes (Ballas *et al.*, 1983) as well as affecting second messenger systems (Herman *et al.*, 1993). It is thought that on occasion the polyamines exert their effect by acting directly on the calcium channel. Spermine and spermidine were shown to modulate ω -conotoxin GVIA binding to hippocampal synaptosomes, and thus appear to modulate its actions at N-type voltage sensitive calcium channels (Pullan *et al.*, 1990). Recently McNaughton *et al.* (1999) demonstrated that a

synthetic macrocyclic polyamine was a potent inhibitor of N-type calcium channels but also inhibited P- and L-types.

The one drawback with the study of calcium channels when compared to ionotropic glutamate channels is that there is still little understanding of the molecular mechanism of calcium channel inhibition. This is despite recent advances in molecular approaches in cloning and modeling. One recent study, however, examined inhibition of recombinant calcium channels by two well known groups of agents, benzothiazepines and phenylalkylamines (Cai *et al.*, 1997). This study demonstrated the existence of physically distinct elements, which control the drug binding and access to the binding site.

The calcium antagonists examined in Section 2, verapamil, nitrendipine and nisoldipine, are all L-type selective Ca^{2+} channel antagonists. However, they each demonstrated markedly different activities *in vivo*.

Verapamil and the phenylalkylamine class of calcium channel antagonists are intracellular blockers with a very high affinity (Hille, 1992). However, Verapamil has been demonstrated to affect ornithine decarboxylase (ODC). Hibasami *et al.* (1989) demonstrated an inhibition of methylthiopropylamine (MTPA) induced ODC activity by verapamil. Similarly, Koenig *et al.* (1989) demonstrated that the breakdown in blood brain barrier after brain injury, which is linked to stimulation of ODC activity and polyamine synthesis, is inhibited by verapamil. However, this probably reflects the possible polyamine modulatory actions at calcium channels rather than a specific polyamine antagonist activity for verapamil.

Unfortunately there are no available studies examining the possible effects of nitrendipine and nisoldipine on polyamines and the studies in general are limited to their effects in seizures as detailed in Section 2.6. Schoemaker & Langer (1989) demonstrated an allosteric interaction between both the diltiazem binding site and the verapamil binding site with nitrendipine binding. They also demonstrated that Ca^{2+} acted as a competitive antagonist of the allosteric effects of diltiazem and verapamil.

This study suggested that the diltiazem and verapamil binding sites were the same as the Ca^{2+} binding site, within the Ca^{2+} channel.

The results of this study may explain the low potency of verapamil at antagonising BAY K 8644-induced seizures ($\text{ED}_{50} > 25 \text{mg/kg}$) (Palmer *et al.*, 1993). The mechanism involved in seizure production in this case is calcium channel activation as BAY K 8644 is a calcium channel agonist. Indeed this calcium activation is thought to play a major part in most types of seizure production. The increase in Ca^{2+} ion concentration may antagonise the action of verapamil resulting in its low potency.

Nisoldipine, though the studies using this are limited, is more potent than verapamil as a Ca^{2+} antagonist (Palmer *et al.*, 1993). This may be partly due to the fact that it is a dihydropyridine and may act at a similar site to nitrendipine rather than linked to the calcium channel like verapamil. Therefore, nisoldipine would not be expected to have its action antagonised by high concentrations of Ca^{2+} ions. Similarly nitrendipine would be expected to have a greater action than verapamil in seizures involving a large influx of calcium.

Despite these findings with regard to potency at calcium channels, though it is little recognised, all the agents used in this study have been demonstrated to possess moderate to potent effects on NMDA induced seizures (Palmer *et al.*, 1993). This could mean that the antagonist action by these compounds on spermine-induced CNS excitation may be from NMDA antagonist action as it has already been shown that the NMDA receptor is involved. However, the lack of effect of nisoldipine in the spermine enhancement of NMDA induced seizures would argue against a potent NMDA antagonist effect. Therefore, it is most likely that both the NMDA receptor and L-type calcium channels are involved in spermine-induced CNS excitation.

The antiepileptic agent, gabapentin, has recently been demonstrated to act through an action on calcium channels. Gee *et al.* (1996) demonstrated gabapentin binding to an $\alpha_2\delta$ subunit of, possibly, the L-type calcium channels. However, very recently Fink *et al.* (2000) have questioned the physiological relevance of a gabapentin action through

an $\alpha_2\delta$ subunit and have proposed an action through the P/Q type channels. It was suggested that since the $\alpha_2\delta$ subunit appears to be expressed in all voltage gated calcium channels, gabapentin should have similar effects across all subtypes of calcium channels. This, however, is not the case, so it was suggested that other factors, such as different splice variants, determine its activity displayed at P/Q-type channels.

Gabapentin has not shown any effect, at doses up to 400mg/kg (ip), against the spermine induced CNS excitation and tonic convulsions (Doyle, 1993). This result fits in with the proposed role of L-type calcium channels on this excitation. Gabapentin apparently lacks L-type calcium channel activity and therefore does not affect the spermine-induced CNS excitation.

6.4 POTASSIUM CHANNELS

There are many different types of K^+ selective channels, which have been differentiated according to physical, pharmacological and molecular properties. One such type of K^+ selective channels are the inward rectifier K^+ channels. This type of channel has the ability to conduct current in the inward direction at negative membrane potentials. However, there is a greatly reduced outward flow of ions at membrane potentials positive to the potassium equilibrium potential. These inward rectifier K^+ channels are vital for controlling the resting membrane potential and as a result have the ability to control excitability by counteracting the depolarising effects of small stimuli. The conductance declines as the membrane depolarises and therefore strong stimuli find it progressively easier to depolarise the membrane and initiate an action potential.

Different types of inward rectifier K^+ channels have been described. Strong and mild rectifiers have been identified and differ in the extent of outward current (Hille, 1992). Strong rectifiers gate little or no outward current but the mild channels show some outward current. The genes responsible for these channels have been cloned

and include strong rectifiers such as Kir2.1, Kir3.1 and Kir2.3 and mild rectifiers like Kir1.1a (Doupnik *et al.*, 1995).

Magnesium ions have been recognised as contributing to the process of inward rectification (Matsuda, 1991). However, their contribution is not sufficient to account for the rectification seen in inward rectifier K⁺ channels. It has been demonstrated that the polyamines are responsible for the inward rectification seen at these channels (Lopatin *et al.*, 1994; Ficker *et al.*, 1994). It was shown, using patch clamp techniques on *Xenopus* oocytes, that there was inward rectification of the K⁺ channels. However, if the patch was removed from the oocyte, inward rectification is lost. If the patch was then moved back near to the oocyte the rectification was restored. Furthermore, it was shown that if exogenous polyamines were added to the intracellular side of the patch, rectification was restored (Lopatin *et al.*, 1994; Lopatin *et al.*, 1995). Similarly, spermine has been demonstrated to be responsible for rectification of muscarinic acetylcholine receptor-coupled strong rectifier K⁺ channels in heart myocytes (Yamada & Kurachi, 1995).

Lopatin *et al.* (1995) examined the mechanism of block of Kir2.3 channels. The model proposed for the block, that agreed with the data obtained, involved spermine, in linear formation, entering deep within the channel pore to interact with sites within the membrane spanning region of the Kir2.3 protein. Indeed, for the model to fit, two molecules of spermine are required to enter the pore sequentially.

Site-directed mutagenesis has been used to determine the important sites of the K⁺ channel. The Kir channel consists of two transmembrane segments (M1 and M2) and a re-entrant loop in the pore forming region. The second transmembrane domain (M2) forms part of the interior of the Kir channel. This segment contains an aspartate residue (D172), which appears to be very important for the polyamine-induced rectifier activity (Ficker *et al.*, 1994; Tagliatela *et al.*, 1995). Similarly, a glutamate residue (E224), in the intracellular C-terminal domain has been found to influence block by spermine (Tagliatela *et al.*, 1995). Taking into account these studies it was

proposed that D172 and E224 contribute to the formation of the binding pocket for Mg^{2+} and polyamines (Tagliatela *et al.*, 1995).

A study by Pearson and Nichols (1998) examined the effect of alkylamine analogues of spermine. This supported the earlier proposal that the polyamines enter the pore and bind to a site within the channel. From this work it was suggested that monoamines and diamines block inward rectifier potassium channels by entering deeply into a long narrow pore. However, this mechanism of action of spermine and the other polyamines was contradicted by Lee *et al.* (1999). Their study proposed that the polyamines, rather than causing open channel block, bind to a different region of the channel to form a blocking complex that occludes the pore.

Also in 1999, a study by Baukrowitz *et al.* examined cardiac and KATP (Kir6.2) channels. It was demonstrated that the polyamine mediated rectification was not fixed but changed with intracellular pH in the physiological range. At basic pH, inward rectification was prominent but it was very weak at acidic pH. This pH sensitivity is specific for KATP channels. In the same study, a C-terminal histidine residue (H215) was found to be responsible for the pH dependent rectification.

Needless to say these channels have a very important physiological role. They stabilise the resting membrane potential and excitability of many types of cells. This therefore suggests an extremely important role for intracellular polyamines. If the intracellular polyamine concentrations are increased they will result in an increase in rectification of K^+ channels, producing an increase in cellular excitability. The effects of the polyamines on the K^+ channels will depend on the proportions of polyamines present since spermine and spermidine are, respectively, 100- and 10-fold more potent blockers of Kir channels than putrescine or Mg^{2+} .

It is not yet known to what extent polyamine effects on Kir channels will have in different pathological states. However, as indicated earlier, increases in polyamine concentrations have been identified in models of seizure activity (Hayashi *et al.*, 1993) and there are marked changes in polyamine levels following cerebral ischaemia

(Paschen *et al.*, 1987; Paschen *et al.*, 1988). In cerebral ischaemia, there are increases in putrescine levels with spermidine and spermine remaining relatively steady. This increase in putrescine, though it is not very potent, may, with the other polyamines, increase rectification of Kir channels and coupled with excessive releases in glutamate could contribute to the neuronal damage.

Indeed, the experimental protocols used in Section 2 involved the intracerebroventricular administration of spermine. It is possible that this increase in spermine will increase rectification of Kir channels, causing an increase in excitability, which could manifest as the tonic convulsions seen. It would therefore be of interest to investigate the role of Kir channels in this experimental paradigm.

The calcium antagonists investigated in Section 2 have been shown to exhibit some activity on K⁺ channels. Verapamil has been shown to effect block of cardiac delayed rectifier K⁺ channels (Chouabe *et al.*, 2000; Waldegger *et al.*, 1999) but does not affect the inward rectifier K⁺ channel, Kir2.1 (Waldegger *et al.*, 1999). Similarly, Madeja *et al.* (2000) have demonstrated an effect of verapamil (at micromolar concentrations) on native and cloned hippocampal voltage-operated potassium channels. Verapamil has been demonstrated to inhibit tonic firing in rat intracardiac neurons primarily via inhibition of delayed outwardly rectifying K⁺ current (Hogg *et al.*, 1999). None of these K⁺ channels have been demonstrated to be affected by endogenous polyamines. Therefore, it is difficult to assess whether verapamil's activity on spermine-induced CNS excitation is due to an action on Kir channels, though as mentioned above this warrants further investigation.

Nisoldipine, unlike verapamil, has been shown to have no effect on the delayed rectifier potassium current (Daleau *et al.*, 1997). Indeed, it was proposed that nisoldipine could be used as a tool to inhibit inward calcium current during examination of delayed rectifier potassium currents. Nitrendipine has an effect on calcium activated K⁺ permeability (Elroy *et al.*, 1992; Benton *et al.*, 1999) but has been shown to have negligible effects on delayed rectifier K⁺ channels (Chouabe *et al.*, 2000).

In relation to known activity of calcium channel antagonists on potassium channels it seems unlikely that these agents antagonise spermine through a mechanism inhibiting potassium channels.

6.5 CONCLUSIONS

The brain is an extremely complex organ and it seems that the more that is discovered about it, the more complex the picture becomes. The preceding sections have focussed primarily on elucidating some of the functions of polyamines in two of the most destructive brain disorders, convulsions/epilepsy and cerebral ischaemia/stroke.

It was demonstrated here that the NMDA receptor is involved in the production of CNS excitation following the administration of spermine. However, the illustration that calcium channel antagonists, verapamil, nisoldipine and nitrendipine, are also effective argues a role for L-type calcium channels in the CNS excitation. These compounds, though, also possess moderate to potent NMDA antagonist activity. Therefore, it is possible that this is responsible for the effects seen. However, when the action of a low dose of nisoldipine was examined in the spermine enhancement of NMDA-induced convulsions, no antagonist action was seen. Because the same low dose of nisoldipine was effective in blocking CNS excitation produced by spermine alone, it can be concluded that the L-type calcium channels are indeed involved in the spermine induced CNS excitation.

N¹-Dansylspermine is markedly more effective and potent than any of the other compounds investigated here. Indeed, it is the most potent compound investigated in this laboratory. It was established that N¹-dansylspermine was acting through an antagonist action at the positive polyamine regulatory site, rather than at the negative site which would have, itself, affected the NMDA-induced convulsions.

Polyamines have been recognised as having an effect in the neuronal degeneration following cerebral ischaemia, whether that be through neurotoxicity or apoptosis. This is borne out in the studies presented here. N¹-dansylspermine is more potent a neuroprotectant than any of the other compounds investigated here. However, one curious result came to light, that was the effect on ischaemia-induced hyperactivity. This led to the hypothesis that there are possibly different populations of CA1 pyramidal cells with different effects on memory acquisition and recall. Also possible is that an as yet unidentified population of cells is responsible for the hyperactivity and these cells are differentially sensitive to ifenprodil/eliprodil and N¹-dansylspermine.

Finally, in an *in vitro* model of epilepsy, which was designed for examination of the role of the NMDA receptor, spermine had an enhancing effect on the spontaneous depolarisations seen. N¹-Dansylspermine antagonised the enhancing effect at a dose that did not show an effect on the spontaneous depolarisations, which are presumed to largely arise from the NMDA receptor, thus demonstrating its specific polyamine antagonist activity.

6.6 FURTHER STUDIES

With the recent demonstration by Bontempi *et al.* (1999) of a reduced role for the hippocampus and increased role for the cortex in memory acquisition, consolidation and recall, it would be important to further investigate the effects of N¹-dansylspermine and the polyamines in general in memory formation.

N¹-dansylspermine is a novel polyamine analogue and has demonstrated huge potential as a polyamine antagonist. Most of the polyamine analogues that have been used up to now as antagonists, such as arcaine, have been relatively simple molecules, many of which are available physiologically. The only other available compounds are the spider toxins, such as FTX (Funnel Web spider toxin). These compounds are very expensive and the synthesis is quite difficult. However, N¹-dansylspermine has a relatively simple synthesis and has demonstrated potent activity. Another analogue

synthesised at the same time as N¹-dansylspermine is N¹-(n-octanesulfonyl)-spermine and this too has been shown to be a potent blocker of the NMDA receptor (Chao *et al.*, 1997).

Chenard and Menniti (1999) have carried out extensive structure activity work and many of the compounds assessed possess a polyamine backbone. This work has developed a multitude of compounds, which are selective for the NR2B subunit of the NMDA receptor and may potentially be potent polyamine antagonists. Therefore, more work needs to be carried out to assess these compounds and other polyamine-like compounds for antagonist activity.

Finally, the ground breaking discovery of role of polyamines, particularly spermine and spermidine, in inward rectifier potassium channels has opened up a whole new avenue of investigation for the role of polyamines. Therefore, it would be important to examine the role of the Kir channels in spermine induced CNS excitation. Similarly, in cerebral ischaemia, excess polyamines may contribute to the neuronal damage produced by increasing rectification of the Kir channels as well as increasing the sensitivity of the NMDA receptors to excitotoxins. The increased rectification, producing increased excitability, combined with excessive release of glutamate, may play a major role in the neurodegeneration. Unfortunately, an examination of the effects of Kir channels was outside the scope of the work presented here but, nevertheless, is extremely important and should be examined as soon as possible.

BIBLIOGRAPHY

Al-Deen, I. H. S. (1982) Observations related to the possible role of spermine and spermidine in the brain. Ph.D. thesis, University of Nottingham.

Al-Deen, I. H. S. & Shaw, G. G. (1991) Circadian variation in cerebral spermine and spermidine concentrations in entrained and free running mice. *Neurochem. Int.*, **19**, 605-610.

Anderson, D. J., Crossland, J., Shaw, G. G. (1975) The actions of spermidine and spermine on the central nervous system. *Neuropharmacol.*, **14**, 571-577.

Anderson, D. J. & Shaw, G. G. (1974) Hyperglycaemia produced by the polyamines, spermine and spermidine. *Br. J. Pharmacol.*, **52**, 205-211.

Andiné, P., Orwar, O., Jacobson, I., Sandberg, M., Hagberg, H. (1991) Changes in extracellular amino acids and spontaneous neuronal activity during ischaemia and extended reflow in the CA1 of the rat hippocampus. *J. Neurochem.*, **57**, 222-229.

Apland, J. P. & Cann, F. J. (1995) Anticonvulsant effects of memantine and MK-801 in guinea pig hippocampal slices. *Brain Res. Bulletin*, **37**, 311-316.

Araki, T., Kogure, K., Nishioka, K. (1990) Comparative neuroprotective effects of pentobarbital, vinpocetine, flunarizine and ifenprodil on ischemic neuronal damage in the gerbil hippocampus. *Res. Exp. Med.*, **190**, 19-23.

Araki, H., Nojiri, M., Kawashima, K., Kimura, M., Aihara, H. (1986) Behavioral, electroencephalographic and histopathological studies on Mongolian gerbils with occluded common carotid arteries. *Physiol. Behav.*, **38**, 89-94.

Aram, J., Church, J., Davies, S. N., Martin, D., Lodge, D. (1987) Comparison of phencyclidine thienylcyclohexylpiperidine and MK-801 as NMDA antagonists on rat spinal cortical neurons. *Br. J. Pharmacol.*, **89**, 778P.

Aram, J. A., Martin, D., Tomczyk, M., Zeman, S., Millar, J., Pohler, G., Lodge, D. (1989) Neocortical epileptogenesis in vitro: studies with N-methyl-D-aspartate, phencyclidine, sigma and dextromethorphan receptor ligands. *J. Pharmacol. Exp. Ther.*, **248**, 320-328.

Araneda, R. C., Lan, J. Y., Zheng, X., Zukin, R. S., Bennett, M. V. (1999) Spermine and Arcaine block and permeate N-methyl-D-aspartate receptor channels. *Biophys. J.*, **76**, 2899-2911.

Asanuma, M., Asanuma, S. N., Gomez-Vargas, M., Yamamoto, M., Ogawa, N. (1997) Ketoprofen, a non-steroidal anti-inflammatory drug prevents the late-onset reduction of muscarinic receptors in gerbil hippocampus after transient forebrain ischaemia. *Neurosci. Lett.*, **225**(2), 109-112.

Avenet, P., Léonardon, J., Besnard, F., Graham, D., Depoortere, H., Scatton, B. (1997) Antagonist properties of eliprodil and other NMDA receptor antagonists at rat NR1A/NR2A and NR1A/NR2B receptors expressed in *Xenopus* oocytes. *Neurosci. Lett.*, **223**, 133-136.

Avenet, P., Léonardon, J., Besnard, F., Graham, D., Frost, J., Depoortere, H., Langer, S. Z., Scatton, B. (1996) Antagonist properties of the stereoisomers of ifenprodil at NR1A/NR2A and NR1A/NR2B subtypes of the NMDA receptor expressed in *Xenopus* oocytes. *Eur. J. Pharmacol.*, **296**, 209-213.

Awad, I. A., Carter, L. P., Spetzler, R. F., Medina, M., Williams, F. W. Jnr. (1987) Clinical vasospasm after subarachnoidal hemorrhage: Response to hypervolemic hemodilution and arterial hypertension. *Stroke*, **18**, 365-372.

Babcock, A. M., Baker, D. A., Lovec, R. (1993) Locomotor activity in the ischaemic gerbil. *Brain Res.*, **625**, 351-354.

Bacchi, C. J. (1981) Content, synthesis and function of polyamines in Trypanosomatids: relationship to chemotherapy. *J. Protozool.*, **28**, 20-27.

Badolo, L., Dubois, J., Helson-Cambier, M., Hanocq, M. (1998) Inhibition of ornithine decarboxylase by ifenprodil. *Eur. J. Pharmacol.*, **342**, R1-R2.

Ballas, S. K., Mohandas, N., Martin, L. J., Shoet, S. B. (1983) Stabilisation of erythrocyte membranes by polyamines. *Proc. Natl. Acad. Sci. USA*, **80**, 1942-1946.

Baskaya, M. K., Rao, A. M., Dogan, A., Donaldson, D., Gellin, G., Dempsey, R. J. (1997(a)) Regional brain polyamine levels in permanent focal cerebral ischaemia. *Brain Res.*, **744**, 302-308.

Baskaya, M. K., Rao, A. M., Donaldson, D., Prasad, M. R., Dempsey, R. J. (1997(b)) Protective effects of ifenprodil on ischaemic injury size, blood-brain barrier breakdown, and edema formation in focal cerebral ischemia. *Neurosurg.*, **40**, 364-371.

Bath, C. P., Farrell, L. N., Gilmore, J., Ward, M. A., Hicks, C. A., O'Neill, M. J., Bleakman, D. (1996) The effects of ifenprodil and eliprodil on voltage-dependent Ca²⁺ channels and in gerbil global cerebral ischaemia. *Eur. J. Pharmacol.*, **299**, 103-112.

Baukrowitz, T., Tucker, S. J., Schulte, U., Benndorf, K., Ruppersberg, J. P. (1999) Inward rectification in KATP channels: a pH switch in the pore. *EMBO J.*, **18**, 847-853.

Bear, M. F. & Malenka, R. C. (1994) Synaptic plasticity: LTP and LTD. *Curr. Opin. Neurobiol.*, **4**, 389-399.

Benton, D. C., Roxburgh, C. J., Ganellin, C. R., Shiner, M. A., Jenkinson, D. H. (1999) Differences in the actions of some blockers of calcium activated potassium permeability in mammalian red cells. *Br. J. Pharmacol.*, **126**, 169-178.

Benveniste, M. & Mayer, M. L. (1993) Multiple effects of spermine on N-methyl-D-aspartic acid receptor responses of rat cultured hippocampal neurones. *J. Physiol. (London)*, **464**, 131-163.

Berger, M. L. & Rebernik, P. (1999) Zinc and ifenprodil allosterically inhibit two separate polyamine sensitive sites at N-methyl-D-aspartate receptor complex. *J. Pharmacol. Exp. Ther.*, **289**, 1584-1591.

Biton, B. P., Granger, P., Carreau, A., Depoortere, H., Scatton, B., Avenet, P. (1994) The NMDA receptor antagonist eliprodil (SL 82.0715) blocks voltage-operated calcium channels in rat cultured cortical neurons. *Eur. J. Pharmacol.*, **257**, 297-301.

Bliss, T. V. P. & Collingridge, G. L. (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature(Lond.)*, **361**, 31-39.

Block, F. & Schwarz, M. (1996) Memantine reduces functional and morphological consequences induced by global ischaemia in rats. *Neurosci. Lett.*, **208**, 41-44.

Bondy, S. C. & Walker, C. H. (1986) Polyamines contribute to calcium-stimulated release of aspartate from brain particulate fractions. *Brain Res.*, **371**, 96-100.

Bontempi, B., Laurent-Demir, C., Destrade, C., Jaffard, R. (1999) Time-dependent reorganization of brain circuitry underlying long-term memory storage. *Nature*, **400**, 671-675.

Bormann, J. (1989) Memantine is a potent blocker of N-methyl-D-aspartate (NMDA) receptor channels. *Eur. J. Pharmacol.*, **166**, 591-592.

Bowie, D. & Mayer, M. L. (1995) Inward rectification of both AMPA and kainate subtype glutamate receptors generated by polyamine-mediated ion channel block. *Neuron*, **15**, 453-462.

Boyce, S., Wyatt, A., Webb, J. K., O'Donnell, R., Mason, G., Rigby, M., Sirinathsinghji, D., Hill, R. G., Rupniak, N. M. (1999) Selective NMDA NR2B antagonists induce antinociception without motor dysfunction: correlation with restricted localisation of NR2B subunit in dorsal horn. *Neuropharmacol.*, **38**, 611-23.

Breuer, Z. & Mayevsky, A. (1992) Brain vasculature and mitochondrial responses to ischaemia in gerbils. II. Strain differences and statistical evaluation. *Brain Res.*, **598**, 251-256.

Brierley, T. B. (1976) Cerebral hypoxia, in: Greenfield's Neuropathology, eds. W. Blackwood and A. Corsellis (Edward Arnold, London) p. 43-85.

Brittain, R. T. (1966). The intracerebral effects of noradrenaline and its modification by drugs in the mouse. *J. Pharm. Pharmac.*, **18**, 621-623.

Broderick, J. P., Phillips, S. J., Whisnant, J. P., O'Fallon, W. M., Bergstrath, E. J. (1989) Incidence rates of stroke in the eighties: The end of the decline. *Stroke*, **20**, 577-582.

Brott, T. & Reed, R. L. (1989) Intensive care for acute stroke in a community hospital setting: The first 24 hours. *Stroke*, **20**, 694-697.

Burton, N. R., Smith, D. A. S., Stone, T. W. (1987) The mouse neocortical slice: Preparation and responses to excitatory amino acids. *Comp. Biochem. Physiol.*, **88C**(1), 47-55.

Busto, R., Dietrich, W. D., Globus, M. Y.-T., Valdés, I., Scheinberg, P., Ginsberg, M. D. (1987) Small differences in intra-ischaemic brain temperature critically determine the extent of ischaemic neuronal injury. *J. Cereb. Blood Flow Metab.*, **7**, 729-738.

Busto, R., Globus, M. Y.-T., Dietrich, W. D., Martinez, E., Valdés, I., Ginsberg, M. D. (1989) Effect of mild hypothermia on ischaemia-induced release of neurotransmitters and free fatty acids in rat brain. *Stroke*, **20**, 904-910

Cai, D., Mülle, J. G., Yue, D. T. (1997) Inhibition of recombinant Ca²⁺ channels by benzothiazepines and phenylalkylamines: class-specific pharmacology and underlying molecular determinants. *Mol. Pharmacol.*, **51**, 872-881.

Cain, D. P. (1981) Transfer of pentylentetrazol sensitization to amygdaloid kindling. *Pharmacol. Biochem. Behav.*, **15**, 533-536.

Caldwell, M. A., O'Neill, M. Earley, B., Leonard, B. E. (1994) NG-Nitro-L-arginine protects against ischaemia induced increases in nitric oxide and hippocampal neurodegeneration in the gerbil. *Eur. J. Pharmacol.*, **260**, 191-200.

Caldwell, M. A., Reyman, J.-M., Bentué-Ferrer, D., Allain, H., Leonard, B. E. (1996) The dopamine agonists Lisuride and Piribedil protect against behavioural and histological changes following 4-vessel occlusion in the rat. *Neuropsychobiology*, **34**, 117-124.

Carter, C. J., Lloyd, K. G., Zivkovic, B., Scatton, B (1990) Ifenprodil and SL 82.0715 as cerebral antiischaemic agents. III. Evidence for antagonistic effects at the polyamine modulatory site within the N-Methyl-D-aspartate receptor complex. *J. Pharmacol. Exp. Ther.*, **253**, 475-482.

Casero, R. A., Mank, A. R., Saab, N. H., Ronghui, W., Dyer, W. J., Woster, P. M. (1995) Growth and biochemical effects of unsymmetrically substituted polyamine analogues in human lung tumour cells. *Cancer Chemother. Pharmacol.*, **36**, 69-74.

Catterall, W. A. (1993) Structure and function of voltage gated ion channels. *Trends Neurosci.*, **16**, 500-506.

Chandler, M. J., DeLeo, J., Carney, J. M. (1985) An unanaesthetised gerbil model of cerebral ischaemia-induced behavioural changes. *J. Pharmacol. Methods*, **14**, 137-146.

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 receptor antagonists: Block and permeation of N-Methyl-D-Aspartate receptors. *Mol. Pharmacol.*, **51**, 861-871.

Chapman, A., Keane, P. E., Meldrum, B. S., Simiand, J., Vernieres, J. C. (1982) Mechanism of anticonvulsant action of valproate. *Prog. Neurobiol.*, **19**, 315-359.

Chapman, A. G. & Meldrum, B. S. (1987) Epilepsy-prone mice: genetically determined sound-induced seizures. In *Neurotransmitters and Epilepsy*, eds. P. C. Jobe & H. E. Laird II, pgs 9-29. Clifton, NJ: Humana Press.

Chapman, A. G., Woodburn, V. L., Woodruff, G. N., Meldrum, B. S. (1996) Anticonvulsant effect of reduced NMDA receptor expression in audiogenic DBA/2 mice. *Epilepsy Res.*, **26**, 25-35.

Chen, H. S. & Lipton, S. A. (1997) Mechanism of memantine block of NMDA-activated channels in rat retinal ganglion cells: uncompetitive antagonism. *J. Physiol.*, **499**, 27-46.

Chen, H. S., Wang, Y. F., Rayudu, P. V., Edgecomb, P., Neill, J. C., Segal, M. M., Lipton, S. A., Jensen, F. E. (1998) Neuroprotective concentrations of the N-methyl-D-aspartate open-channel blocker memantine are effective without cytoplasmic vacuolation following post-ischaemic administration and do not block maze learning or long-term potentiation. *Neurosci*, **86**, 1121-1132.

Chen, J., Adachi, N., Liu, K., Nagaro, T., Arai, T. (1998) Improvement in ischaemic damage in gerbil hippocampal neurons by procaine. *Brain Res.*, **792**, 16-23.

Chenard, B. L. & Menniti, F. S. (1999) Antagonists sensitive for NMDA receptors containing the NR2B subunit. *Curr. Phar. Des.*, **5**, 381-404.

Cherksey, B. D., Sugimori, M., Llinás, R. R. (1991) Properties of calcium channels isolated with spider toxin, FTX. *Ann. N. Y. Acad. Sci.*, **635**, 80-89.

Chermat, R., Lachapelle, F., Baumann, N., Simon, P. (1979) Anticonvulsant effect of yohimbine in quaking mice: antagonism by clonidine and prazosine. *Life Sci.*, **25**, 1471-1476.

Choi, D. W. (1985) Glutamate neurotoxicity in cortical cell culture is calcium dependent. *Neurosci. Lett.*, **58**, 293-297.

Chouabe, C., Drici, M. D., Romey, G., Barhanin, J. (2000) Effects of calcium channel blockers on cloned cardiac K⁺ channels IKr and Iks. *Therapie*, **55**, 195-202.

Chu, P.-J., Shirahata, A., Samejima, K., Saito, H., Abe, K. (1994) N-(3-aminopropyl)-cyclohexylamine blocks facilitation by spermidine of N-methyl-D-aspartate-induced seizure in mice in vivo. *Eur. J. Pharmacol.*, **256**, 155-160.

Church, J., Fletcher, E. J., Baxter, K., McDonald, J. F. (1994) blockade by ifenprodil of high voltage activated calcium channels in rat and mouse cultured hippocampal pyramidal neurons: comparison with NMDA receptor antagonist actions. *Br. J. Pharmacol.*, **113**, 499-507.

Clineschmidt, B. V., Martin, G. E., Bunting, P. R. (1982) Anticonvulsant activity of (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801), a substance with potent anticonvulsant, central sympathomimetic, and apparent anxiolytic properties. *Drug Dev. Res.*, **2**, 123-134.

Coffino, P. & Poznanski, A. (1991) Killer polyamines? *J. Cellular Biochem.*, **45**, 54-58.

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

Connors, B. W., Gutnick, M. J., Prince, D. A. (1982) Electrophysiological properties of neocortical neurons in vitro. *J. Neurophysiol.*, **48**, 1302-20.

Contreras, P. C., Bremner, M. E., Gray, N. M. (1990) Ifenprodil and SL 82 0715 potently inhibit binding of [3H](+)-3-PPP to sigma binding sites in rat brain. *Neurosci. Lett.*, **116**, 190-193.

Contreras, P. C., Gray, N. M., Ragan, D. M., Lanthorn, T. H. (1992) BMY-14802 protects against ischaemia-induced damage in the gerbil. *Life Sci.*, **51**, 1145-1149.

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

Crain, B. J., Westerkam, W. D., Harrison, A. H., Nadler, J. V. (1988) Selective neuronal death after transient forebrain ischaemia in the Mongolian gerbil: A silver impregnation study. *Neurosci.*, **27**(2), 387-402.

Czuczwar, S. J., Frey, H.-H., Löscher, W. (1985) Antagonism of N-methyl-D,L-aspartic acid-induced convulsions by antiepileptic drugs and other agents. *Eur. J. Pharmacol.*, **108**, 273-280.

Czuczwar, S. J. & Meldrum, B. S. (1982) Protection against chemically induced seizures by 2-amino-7-phosphonoheptanoic acid. *Eur. J. Pharmacol.*, **83**, 335-338.

Daleau, P., Khalifa, M., Turgeon, J. (1997) Effects of cadmium and nisoldipine on the delayed rectifier potassium current in guinea pig ventricular myocytes. *J. Pharmacol. Exp. Ther.*, **281**, 826-833.

Davies, J. A. & Shakesby, A. (1999) Blockade of GABA uptake potentiates GABA-induced depolarisations in adult mouse cortical slices. *Neurosci. Lett.*, **266**, 201-204.

De Sarro, G. B., Meldrum, B. S., Nistico, G. (1988) Anticonvulsant effects of some calcium entry blockers in DBA/2 mice. *Br. J. Pharmacol.*, **93**, 247-256.

Dingledine, R., McBain, C. J., McNamara, J. O. (1990) Excitatory amino acid receptors in epilepsy. *Trends Pharmacol. Sci.*, **11**, 334-338.

Dogan, A., Rao, A. M., Baskaya, M. K., Rao, V. L. R., Rastl, J., Donaldson, D., Dempsey, R. J. (1997) Effects of ifenprodil, a polyamine site NMDA receptor antagonist, on reperfusion injury after transient focal cerebral ischemia. *J. Neurosurg.*, **87**, 921-926.

Dogan, A., Rao, A. M., Hatcher, J., Rao, V. L. R., Baskaya, M. K., Dempsey, R. J. (1999) Effects of MDL 72527, a specific inhibitor of polyamine oxidase, on brain edema, ischaemic injury volume, and tissue polyamine levels in rats after temporary middle cerebral artery occlusion. *J. Neurochem.*, **72**, 765-770.

Dolin, S. J., Grant, A. J., Hunter, A. B., Little, H. J. (1986) Anticonvulsant profile and whole brain concentrations of nitrendipine and nimodipine. *Br. J. Pharmacol.*, **89**, 866P.

Domenici, M. R., Marinelli, S., Sagratella, S. (1996) Effects of felbamate, kynurenic acid derivatives and NMDA antagonists on in vitro kainate-induced epileptiform activity. *Life Sci.*, **58**, PL391-396.

Domenici, M. R., Sagratella, S., Ongini, E., Longo, R., Scotti de Carolis, A. (1994) Felbamate displays in vitro antiepileptic effects as a broad spectrum excitatory amino acid receptor antagonist. *Eur. J. Pharmacol.*, **271**, 259-263.

Douppnik, C. A., Davidson, N., Lester, H. A. (1995) The inward rectifier K⁺ channel family. *Curr. Opin. Neurobiol.*, **5**, 268-277.

Doyle, K. M. (1993) Neurotoxic effects of the polyamines. Ph.D. thesis, University of Dublin, Trinity College.

Doyle, K. M. & Shaw, G. G. (1992) Effects of NMDA antagonists and anticonvulsant drugs on convulsions induced by spermine. *Neurosci. Lett.*, **42**, S55.

Doyle, K. M. & Shaw, G. G. (1996) Investigation of the involvement of the N-methyl-D-aspartate receptor in the development of spermine-induced CNS excitation in vivo. *Br. J. Pharmacol.*, **117**, 1803-1808.

Doyle, K. M. & Shaw, G. G. (1998) Investigation of the actions and antagonist activity of some polyamine analogues in vivo. *Br. J. Pharmacol.*, **124**, 386-390.

Dunah, A. W., Luo, J., Wang, Y. H., Yasuda, R. P., Wolfe, B. B. (1998) Subunit composition of N-methyl-D-aspartate receptors in the central nervous system that contains the NR2D subunit. *Mol. Pharmacol.*, **53**, 429-437.

Earley, B., Canney, M., Clune, B., Caldwell, M., Leonard, B. E., Junien, J. L. (1996) The effects of MK-801, ifenprodil, JO 1784, JO 1994 and JO 1997 on PK 11195 receptor binding, nitric oxide synthase (NO synthase) activity and infarct volume in a mouse model of focal cerebral ischaemia. *Neurochem. International*, **28**, 509-521.

Ebert, U., Wlaz, P., Loscher, W. (1997) Anticonvulsant effects by combined treatment with a glycine_B receptor antagonist and a polyamine site antagonist in amygdala-kindled rats. *Eur. J. Pharmacol.*, **322**, 179-184.

Elben, U., Kitter, B., Rudolphi, K. (1992) Stroke therapy research: Current trends. *Drugs Today*, **28**, 333-351.

Ellroy, J. C., Kirk, K., Culliford, S. J., Nash, G. B., Stuart, J. (1992) Nitrendipine is a potent inhibitor of the Ca^{2+} -activated K^+ channel of human erythrocytes. *FEBS Lett*, **296**, 219-221.

Evans, R. H., Francis, A. A., Watkins, J. C. (1977) Selective antagonism by Mg^{2+} of amino acid induced depolarisation of spinal neurones. *Experientia* (Basel), **33**, 489-491.

Ferkany, J. W., Borosky, S. A., Clissold, D. B., Pontecorvo, M. J. (1988) Dextromethorphan inhibits NMDA-induced convulsions. *Eur. J. Pharmacol.*, **151**, 151-154.

Ferrer-Montiel, A. V. & Montal, M. (1996) Pentameric subunit stoichiometry of a neuronal glutamate receptor. *Proc. Natl. Acad. Sci. USA*, **93**, 2741-2744.

Ficker, E., Tagliatela, M., Wible, B. A., Henley, C. M., Brown, A. M. (1994) Spermine and spermidine as gating molecules for inward rectifier K^+ channels. *Science*, **266**, 1068-1072.

Filloux, F. M., Fitts, R. C., Skeen, G. A., White, H. S. (1994) The dihydropyridine nitrendipine inhibits [^3H]MK-801 binding to mouse brain sections. *Eur. J. Pharmacol.*, **269**, 325-330.

Fink, K., Meder, W., Dooley, D. J., Göthert, M. (2000) Inhibition of neuronal Ca^{2+} influx by gabapentin and subsequent reduction of neurotransmitter release from rat neocortical slices. *Br. J. Pharmacol.*, **130**, 900-906.

Frankiewicz, T., Potier, B., Bashir, Z. I., Collingridge, G. L., Parsons, C. G. (1996) Effects of memantine and MK-801 on NMDA-induced currents in cultured neurones and on synaptic transmission and LTP in area CA1 of rat hippocampal slices. *Br. J. Pharmac.*, **117**, 689-697.

Gallagher, M. J., Huang, H., Grant, E. R., Lynch, D. R. (1997) The NR2B specific interactions of polyamines and protons with the N-methyl-D-aspartate receptor. *J. Biol. Chem.*, **272**, 24971-24979.

Gandolfi, O., Rimondini, R., Dallolio, R. (1992) The modulation of dopaminergic transmission in the striatum by MK-801 is independent of presynaptic mechanisms. *Neuropharmacol.*, **31**(11), 1111-1114.

Gee, N. S., Brown, J. P., Dissanayake, V. U. K., Offord, J., Thurlow, R., Woodruff, G. N. (1996) The novel anticonvulsant drug, gabapentin (Neurontin), binds to the $\alpha_2\delta$ subunit of a calcium channel. *J. Biol. Chem.*, **271**(10), 5768-5776.

Gerhardt, S. C. & Boast, C. A. (1988) Motor activity changes following cerebral ischaemia in gerbils are correlated with the degree of neuronal degeneration in hippocampus. *Behav. Neurosci.*, **102**, 301-303.

Giaid, A., Gibson, S. J., Ibrahim, N. B. N., Legon, S., Bloom, S. R., Yanagisawa, M., Masaki, T., Vamdell, I. M., Polak, J. M. (1989) Endothelin-1, an endothelium derived peptide is expressed in neurons of the human spinal cord and dorsal root ganglion. *Proc. Natl. Acad. Sci. USA*, **86**, 7634-7638.

Gilad, G. M., Dornay, M., Gilad, V. H. (1985) Polyamine treatment in early development leads to increased numbers of rat sympathetic neurons. *Brain Res.*, **348**, 363-366.

Gilad, G. M. & Gilad, V. H. (1991) Polyamines can protect against ischaemia-induced nerve cell death in gerbil forebrain. *Exp. Neurol.*, **111**, 349-355.

Gilad, G. M. & Gilad, V. H. (1992) Polyamines in neurotrauma, ubiquitous molecules in search of a function. *Biochemical Pharmacology*, **44**, 401-407.

Gilad, G. M., Gilad, V. H., Wyatt, R. J. (1992) Polyamines modulate the binding of GABA-A benzodiazepine receptor ligands in membranes from the rat forebrain. *Neuropharmac.*, **31**, 895-898.

Gill, R., Foster, A. C., Woodruff, G. N. (1987) Systemic administration of MK801 protects against ischemia induced hippocampal neurodegeneration in the gerbil. *J. Neurosci.*, **7**(10), 3343-3349.

Gill, R., Foster, A. C., Woodruff, G. N. (1988) MK-801 is neuroprotective in gerbils when administered during the post-ischaemic period. *Neurosci.*, **25**(3), 847-855.

Gill, R. & Woodruff, G. N. (1990) The neuroprotective actions of kynurenic acid and MK-801 in gerbils are synergistic and not related to hypothermia. *Eur. J. Pharmacol.*, **176**, 143-149.

Ginsberg, M. D., Welsh, F. A., Budd, W. W. (1980) Deleterious effects of glucose pretreatment on recovery from diffuse cerebral ischaemia in the cat. I. Local cerebral blood flow and glucose utilisation. *Stroke*, **11**, 347-354.

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

Gotti, B., Benavides, J., MacKenzie, E. T., Scatton, B. (1990) The pharmacotherapy of focal cerebral ischaemia in the mouse. *Brain Res.*, **522**, 290-307.

Gotti, B., Duverger, D., Bertin, J., Carter, C., Dupont, R., Frost, J., Gaudilliere, B., Mackenzie, E. T., Rousseau, J., Scatton, B. (1988) Ifenprodil and SL 82.0715 as cerebral anti-ischaemia agents. I. Evidence for efficacy in models of focal cerebral ischaemia. *J. Pharmacol. Exp. Ther.*, **247**, 1211-1221.

Grant, K. A., Valverius, P., Hudspith, M., Tabakoff, B. (1990) Ethanol withdrawal seizures and the NMDA receptor complex. *Eur. J. Pharmacol.*, **176**, 289-296.

Grotta, J. C. (1987) Current status of haemodilution in acute cerebral ischaemia. *Stroke*, **18**, 689-690.

Hacke, W., Hirschberg, M., del Zoppo, G. J. (eds.) (1990) Thrombolytic therapy in acute ischaemic stroke. Springer, Heidelberg.

Hara, H., Onodera, H., Yoshidomi, M. (1990) Staurosporine, a novel protein kinase C inhibitor prevents post-ischaemic neuronal damage in the gerbil and rat. *J. Cereb. Blood Flow Metab.*, **10**, 646-653.

Harrison, N. L. & Simmonds, M. A. (1985) Quantitative studies on some antagonists of N-methyl-D-aspartate in slices of rat cerebral cortex. *Br. J. Pharmacol.*, **84**, 381-391.

Hayashi, T. (1954) Effects of sodium glutamate on the nervous system. *Keio Journal of Medicine*, **3**, 183-192.

Hayashi, Y., Hattori, Y., Moriwaki, A., Lu, Y. F., Hori, Y. (1993) Increases in brain polyamine concentrations in chemical kindling and single convulsion induced by pentylenetetrazole in rats. *Neurosci. Lett.*, **149**, 63-66.

Herman, M. D., Reuveny, E., Narahashi, T. (1993) The effect of polyamines on voltage-activated calcium channels in mouse neuroblastoma cells. *J. Physiol.*, **462**, 645-660.

Hernandez, T. D. (1997) Preventing post-traumatic epilepsy after brain injury: weighing the costs and benefits of anticonvulsant prophylaxis. *Trends Pharmacol. Sci.*, **18**, 59-62.

Herron, C. E., Williamson, R., Collingridge, G. L. (1985) A selective N-methyl-D-aspartate antagonist depresses epileptiform activity in rat hippocampal slices. *Neurosci. Lett.*, **61**, 255-260.

Hibasami, H., Maekawa, S., Murata, T., Nakashima, K. (1989) Inhibition by polyamines of methylthiopropylamine-induced ornithine decarboxylase in human lymphoid leukaemia Molt 4B cells. *Biochem. Pharmacol.*, **38**, 3673-3676.

Hille, B. (1992) Ionic channels of excitable membranes, 2nd ed., Sinauer Assoc.

Himi, T., Saito, H., Kawai, N., Nakajima, T. (1990) Spider toxin (JSTX-3) inhibits the convulsions produced by glutamate agonists. *J. Neural Transm.*, **80**, 95-104.

Hogg, R. C., Trequatrini, C., Catacuzzeno, L., Petris, A., Franciolini, F., Adams, D. J. (1999) Mechanisms of verapamil inhibition of action potential firing in rat intracardiac ganglion neurons. *J. Pharmacol. Exp. Ther.*, **289**, 1502-1508.

Hollmann, M. & Heinemann, S. (1994) Cloned glutamate receptors. *Annu. Rev. Neurosci.*, **17**, 31-108.

Hu, R. Q. & Davies, J. A. (1995) The effect of the desglycyl metabolite of remacemide on cortical wedges prepared from DBA/2 mice. *Eur. J. Pharmacol.*, **287**, 251-256.

Hu, R. Q. & Davies, J. A. (1997) Glutamate receptor antagonists reduce spontaneous epileptiform activity in cortical wedges prepared from DBA/2 mice. *Exp. Brain Res.*, **115**, 311-318.

Hunter, A. J., Green, A. R., Cross, A. J. (1995) Animal models of acute ischaemic stroke: can they predict clinically successful neuroprotective drugs? *Trends Pharmacol. Sci.*, **16**(4), 123-128.

Igarashi, K. & Williams, K. (1995) Antagonist properties of polyamines and bis(ethyl)polyamines at N-methyl-D-aspartate receptors. *J. Pharmacol. Exp. Ther.*, **272**, 1101-1109.

Isa, T., Lino, M., Itazawa, S., Ozawa, S. (1995) Spermine mediates inward rectification of Ca(2+)-permeable AMPA receptor channels. *Neuroreport*, **6**, 2045-2048.

Ishii, T., Moriyoshi, K., Sugihara, H., Sakurada, K., Kadotani, H., Yokoi, M., Akazawa, C., Shigemoto, R., Mizuno, N., Masu, M., Nakanishi, S. (1993) Molecular characterization of a family of N-methyl-D-aspartate receptor subunits. *J. Biol. Chem.*, **268**, 2836-2843.

Isom, L. L., DeJongh, K. S., Catterall, W. A. (1994) Auxiliary subunits of voltage gated ion channels. *Neuron*, **12**, 1183-1194.

Jabaily, J. & Davies, J. N. (1984) Naloxone administration to patients with acute stroke. *Stroke*, **15**, 36-39.

Jänne, J., Alhonen, L., Leinonen, P. (1991) Polyamines: From molecular biology to clinical applications. *Trends in Molecular Medicine*, **23**, 241-259.

Jänne, J., Pösö, H., Raina, A. (1978) Polyamines in rapid growth and cancer. *Biochim. Biophys. Acta*, **473**, 241-293.

Johnson, J. W. & Ascher, P. (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature*, **325**, 529-531.

Johnson, T. D. (1996) Modulation of channel function by polyamines. *Trends Pharmacol. Sci.*, **17**, 22-27.

Kanai, H., Ishida, N., Nakajima, T., Kato, N. (1992) An analogue of Joro spider toxin selectively suppresses hippocampal epileptic discharges induced by quisqualate. *Brain Res.*, **581**, 161-164.

Kashiwagi, K., Fukuchi, J., Chao, J., Igarashi, K., Williams, K. (1996) An aspartate residue in the extracellular loop of the N-methyl-D-aspartate receptor controls sensitivity to spermine and protons. *Mol. Pharmacol.*, **49**, 1131-1141.

Kashiwagi, K., Pahk, A. J., Masuko, T., Igarashi, K., Williams, K. (1997) Block and modulation of N-methyl-D-aspartate receptors by polyamines and protons: Role of amine acid residues in the transmembrane and pore-forming regions of NR1 and NR2 subunits. *Mol. Pharmacol.*, **52**, 701-713.

Kato, H., Araki, T., Itoyama, Y., Kogure, K. (1995) Rolipram, a cyclic AMP-selective phosphodiesterase inhibitor, reduces neuronal damage following cerebral ischaemia in the gerbil. *Eur. J. Pharmacol.*, **272**, 107-110.

Katoh, A., Ishibashi, C., Shiomi, T., Takahara, Y., Eigyo, M. (1992) Ischaemia-induced reversible deficit of memory function in gerbils. *Brain Res.*, **577**, 57-63.

Kawasaki-Yatsugi, S., Shimizu-Sasamata, M., Yatsugi, S.-I., Yamaguchi, T., (1998) Delayed treatment with YM90K, an AMPA receptor antagonist, protects against damage after middle cerebral artery occlusion in rats. *J. Pharm. Pharmacol.*, **50**, 891-898.

Kemp, J. A., Priestley, T., Woodruff, G. N. (1986) MK-801, a novel orally-active anticonvulsant is a potent non-competitive N-methyl-D-aspartate receptor antagonist. *Br. J. Pharmacol.*, **89**, 535P.

Kew, J. N. & Kemp, J. A. (1998) An allosteric interaction between the NMDA receptor polyamine and ifenprodil sites in rat cultured cortical neurones. *J. Physiol. (Lond.)*, **512**, 17-28.

Kirino, T. (1982) Delayed neuronal death in the gerbil hippocampus following ischaemia. *Brain Res.*, **11**, 494-498.

Kirino, T., Tamura, A., Sano, K. (1986) A reversible type of neuronal injury following ischaemia in the gerbil hippocampus. *Stroke*, **17**, 455-459.

Kleckner, N. W. & Dingledine, R. (1988) Requirement for glycine in activation of N-methyl-D-aspartic acid receptors expressed in *Xenopus* oocytes. *Science (Wash., DC)*, **241**, 835-837.

Kleckner, N. W., Glazewski, J. C., Chen, C. C., Moscrip, T. D. (1999) Subtype-selective antagonism of N-methyl-D-aspartate receptors by felbamate: insights into the mechanism of action.

Koenig, H., Gladstone, A. D., Lu, C. Y. (1989) Blood-brain barrier breakdown in cold-injured brain is linked to a biphasic stimulation of ornithine decarboxylase and polyamine synthesis: both are coordinately inhibited by verapamil, dexamethasone and aspirin. *J. Neurochem.*, **52**, 101-109.

Kofke, W. A., Garman, R. H., Garman R., Rose, M. E. (1999) Opioid neurotoxicity: fentanyl-induced exacerbation of cerebral ischaemia in rats. *Brain Res.*, **818**(2), 326-334.

Kornhuber, J., Bormann, J., Retz, W., Hubers, M., Riederer, P. (1989) Memantine displaces [³H]-MK-801 at therapeutic concentrations in postmortem human frontal cortex. *Eur. J. Pharmacol.*, **166**, 589-590

Kornhuber, J. & Kornhuber, M. E. (1986) Presynaptic dopaminergic modulation of cortical input to the striatum. *Life Sci.*, **39**, 669-674.

Kroigaard, M., Thams, P., Thorn, N. A. (1992) Polyamines in nerve terminals and secretory granules isolated from neurohypophysis. *Acta. Physiol. Scand.*, **146**, 233-240.

Kuriyama, K., Roberts, E., Vos, J. (1968) Some characteristics of binding of gamma-amino butyric acid and acetylcholine to a synaptic vesicle fraction from mouse brain. *Brain Res.*, **9**, 231-252.

Lancaster, J. M. & Davies, J. A. (1992) Carbamazepine inhibits NMDA-induced depolarisations in cortical wedges prepared from DBA/2 mice. *Experientia*, **48**, 751-753.

Leander, J. D., Lawson, R. R., Ornstein, P. L., Zimmerman, D. M. (1988) N-methyl-D-aspartic acid-induced lethality in mice: selective antagonism by phencyclidine-like drugs. *Brain Res.*, **448**, 115-120.

Lee, J. K., John, S. A., Weiss, J. N. (1999) Novel gating mechanism of polyamine block in the strong inward rectifier K channel Kir2.1. *J. Gen. Physiol.*, **113**, 555-564.

Legendre, P., Westbrook, G. L. (1991) Ifenprodil blocks N-methyl-D-aspartate receptors by a two compartment mechanism. *Mol. Pharmacol.*, **40**, 289-298.

Lerma, J. (1992) Spermine regulates N-methyl-D-aspartate receptor desensitization. *Neuron*, **8**, 343-352.

Levine, S. & Payan, H. (1966) Effects of ischaemia and other procedures on the brain and retina of the gerbil (*Meriones unguiculatus*). *Exp. Neurol.*, **16**, 255-262.

- Lindsay, G. S. & Wallace, H. M. (1999) Changes in polyamine catabolism in HL-60 human promyelogenous leukaemic cells in response to etoposide-induced apoptosis. *Biochem. J.*, **337**, 83-87.
- Llinás, R., Sugimori, M., Lin, J.-W., Cherksey, B. (1989) Blocking and isolation of a calcium channel from neurons in mammals and cephalopods utilizing a toxin fraction (FTX) from funnel web spider poison. *Proc. Natl. Acad. Sci. USA*, **86**, 1689-1693.
- Lopatin, A. N., Makhina, E. N., Nichols, C. G. (1994) Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification. *Nature (London)*, **372**, 366-369.
- Lopatin, A. N., Makhina, E. N., Nichols, C. G. (1995) The mechanism of inward rectification of potassium channels: "long-pore plugging" by cytoplasmic polyamines. *J. Gen. Physiol.*, **106**, 923-955.
- Loscher, W., Annies, R., Honack, D. (1991) The N-methyl-D-aspartate receptor antagonist MK-801 induces increases in dopamine and serotonin metabolism in several brain regions of rats. *Neurosci. Lett.*, **128**, 191-194.
- Lucke, A. & Speckmann, E. J. (1993) Influence of the organic calcium antagonist verapamil on N-methyl-D-aspartate (NMDA) induced cortical field potentials (neocortical slice, guinea pig). *Neurosci. Lett.*, **161**, 179-182.
- Lynch, D. R., Lawrence, J. J., Lenz, S., Anegawa, N. J., Dichter, M., Pritchett, D. B. (1995) Pharmacological characterisation of heterodimeric NMDA receptors of NR 1a and 2B subunits: Differences with receptors formed from NR 1a and 2A. *J. Neurochem.*, **64**, 1462-1468.
- Macdonald, R. L. & Barker, J. L. (1979) Anticonvulsant and anaesthetic barbiturates: different postsynaptic actions in cultured mammalian neurons. *Neurology*, **29**, 432-447.

Macdonald, R. L. & Stoodley, M. (1998) Pathophysiology of cerebral ischaemia. *Neurol. Med. Chir. (Tokyo)*, **38**, 1-11.

Macrae, I. M. (1992) New models of focal cerebral ischaemia. *Br. J. Clin. Pharmacol.*, **34**, 302-308.

Madeja, M., Muller, V., Musshoff, U., Speckman, E. J. (2000) Sensitivity of native and cloned hippocampal delayed rectifier potassium channels to verapamil. *Neuropharmacol.*, **39**, 202-210.

Maginn, M., Caldwell, M., Kelly, J. P., Leonard, B. E. (1995) The effect of 2-amino-3-phosphonopropionic acid (AP-3) in the gerbil model of cerebral ischaemia. *Eur. J. Pharmacol.*, **282**, 259-262.

Markwell, M. A. K., Berger, S. P., Paul, S. M. (1990) The polyamine synthesis inhibitor α -difluoromethylornithine blocks NMDA-induced neurotoxicity. *Eur. J. Pharmacol.*, **182**, 607-609.

Marshall, J. (1964) The natural history of transient ischaemic cerebrovascular attacks. *Quart. J. Med.*, **33**, 309-324.

Martin, L. J., Sieber, F. E., Traystman, R. J. (2000) apoptosis and necrosis occur in separate neuronal populations in hippocampus and cerebellum after ischaemia and are associated with differential alterations in metabotropic glutamate signaling pathways. *J. Cereb. Blood Flow Metab.*, **20**(1), 153-167.

Mas, J. L. & Zuber, M. (1991) Epidemiology of ischaemic stroke. Epidemiology and clinical aspects. *Cerebrovasc. Dis.*, **1**(suppl. 1), 36-44.

- Masuko, T., Kashiwagi, K., Kuno, T., Nguyen, N. D., Pahk, A. J., Fukuchi, J., Igarashi, K., Williams, K. (1999) A regulatory domain (R1-R2) in the amino terminus of the N-methyl-D-aspartate receptor: Effects of spermine, protons and ifenprodil, and structural similarity to bacterial leucine/isoleucine/valine binding protein. *Mol. Pharmacol.*, **55**, 957-969.
- Mathis, C. & Ungerer, A. (1992) Comparative analysis of seizures induced by intracerebroventricular administration of NMDA, kainate and quisqualate in mice. *Exp. Brain Res.*, **88**, 277-282.
- Matsuda, H. (1991) Magnesium gating of the inwardly rectifying K⁺ channel. *Annu. Rev. Physiol.*, **53**, 289-298.
- Matsumoto, I., Davidson, M., Wilce, P. A. (1993) Polyamine-enhanced NMDA receptor activity: effect of ethanol. *Eur. J. Pharmacol.*, **247**, 289-294.
- Mayer, M. L., Westbrook, G. L., Vycklicky, L. Jr. (1988) Sites of action on N-methyl-D-aspartic acid receptors using fluctuation analysis and a rapid perfusion technique. *J. Neurophysiol.*, **60**, 645-653.
- Mayevsky, A. & Breuer, Z. (1992) Brain vasculature and mitochondrial responses to ischaemia in gerbils I basic anatomical patterns and biochemical correlates. *Brain Res.*, **598**, 242-250.
- McCloskey, D. E., Yang, J., Woster, P. M., Davidson, N. E., Casero, R. A. (1996) Polyamine analogue induction of programmed cell death in human lung tumour cells. *Clin. Cancer Res.*, **2**, 331-446.
- McLean, M. J. & Macdonald, R. L. (1986a) Sodium valproate but not ethosuximide, produces use- and voltage-dependent limitation of high frequency repetitive firing of action potentials of mouse cortical neurons in cell culture. *J. Pharmacol. Exp. Ther.*, **237**, 1001-1011.

McLean, M. J. & Macdonald, R. L. (1986b) Carbamazepine and 10,11-epoxy-carbamazepine produce use- and voltage-dependent limitation of rapidly firing action potentials of mouse central neurons in cell culture. *J. Pharmacol. Exp. Ther.*, **238**, 727-738.

McNaughton, N. C., White, C., Clark, B., Bath, C., Bleakman, D., Randall, A. D. (1999) Electrophysiological characterisation of the human N-type Ca^{2+} channel III: pH-dependent inhibition by a synthetic macrocyclic polyamine. *Neuropharmacol.*, **38**, 19-38.

Meldrum, B. S. (1996) Update on the mechanism of action of antiepileptic drugs. *Epilepsia*, **37**(Suppl 6), S4-S11.

Meldrum, B. S., Evans, M. C., Swan, J. H., Simon, R. P. (1989) Protection against hypoxic/ischaemic brain damage with excitatory amino acid antagonists. *Medical Biol.*, **65**, 153-157.

Menniti, F., Chenard, B., Collins, M., Ducat, M., Shalaby, I., White, F. (1997) CP-101,606, a potent neuroprotectant selective for forebrain neurons. *Eur. J. Pharmacol.*, **331**, 117-26.

Meyer, F. B., Anderson, R. E., Sundt, T. M. Jr., Sharbrough, F. W. (1986) Selective central nervous system calcium channel blockers – a new class of anticonvulsant agents. *Mayo Clin. Proc.*, **61**(4), 239-247.

Miles B. E. & Schwartz, R. D. (1991) The use of locomotor activity as a behavioral screen for neuronal damage following transient forebrain ischaemia in gerbils. *Neurosci. Lett.*, **128**, 71-76.

Miljanich, G. P. & Ramachandran, J. (1995) Antagonists of neuronal calcium channels: structure, function and therapeutic implications. *Annu. Rev. Pharmacol. Toxicol.*, **35**, 707-734.

Mitani, A., Andou, Y., Kataoka, K. (1992) Selective vulnerability of hippocampal CA1 neurons cannot be explained in terms of an increase in glutamate concentration during ischemia in the gerbil: brain microdialysis study. *Neurosci.*, **48**(2), 307-313.

Mitani, A. & Kataoka, K. (1991) Critical levels of extracellular glutamate mediating gerbil hippocampal delayed neuronal death during hypothermia: Brain microdialysis study. *Neurosci.*, **42**, 661-670.

Mizunuma, T., Ohta, S., Suzuki, M. (1996) Subarachnoid administration of lidocaine reduces delayed neuronal damage due to forebrain ischaemia in rats. *Masui – Jap. J. Anaesthesiol.*, **45**, 421-427.

Moreau, J.-L., Pieri, L., Prud'hon, B. (1989) Convulsions induced by centrally administered NMDA in mice: effects of NMDA antagonists, benzodiazepines, minor tranquilizers and anticonvulsants. *Br. J. Pharmacol.*, **98**, 1050-1054.

Moriyoshi, K., Masu, M., Ishii, T., Shigemoto, R., Mizuno, N., Nakanishi, S. (1991) Molecular cloning and characterization of the rat NMDA receptor. *Nature*, **354**, 1-36.

Morón, M. A., Stevens, C. W., Yaksh, T. L. (1990) The antiseizure activity of dihydropyridine calcium channel antagonists in the conscious rat. *J. Pharmacol. Exp. Ther.*, **252**, 1150-1155.

Morrison, L. D., Sherwin, A. L., Carmant, L., Kish, S. J. (1994) Activity of S-adenosylmethionine decarboxylase, a key regulatory enzyme in polyamine biosynthesis, is increased in epileptogenic human cortex. *Arch. Neurol.*, **51**, 581-584.

Mott, D. D., Doherty, J. J., Zhang, S., Washburn, M. S., Fendley, M. J., Lyuboslavsky, P., Traynelis, S. F., Dingledine, R. (1998) Phenylethanolamines inhibit NMDA receptors by enhancing proton inhibition. *Nature Neurosci.*, **1**(8), 659-667.

Moulinoux, J.-P., Quemener, V., Havouis, R., Guille, F., Martin, C., Seiler, N. (1991) Accumulation of polyamine analogs in red blood cells: A potential index of tumor proliferation rate. *Anticancer Research*, **11**, 2143-2146.

Nairn, L. M., Lindsay, G. S., Woster, P. M., Wallace, H. M. (2000) Cytotoxicity of novel unsymmetrically substituted inhibitors of polyamine biosynthesis in human cancer cells. *J. Cell. Physiol.*, **182**, 209-213.

Nakanishi, H., Katsuta, K., Koide, T., Ueda, Y., Shirakawa, K., Yoshida, K. (1994) Protective effect of FR115427 against ischaemic hippocampal damage in gerbils. *Jpn. P. Pharmacol.*, **64**, 189-193.

Nankai, M., Klarica, M., Fage, D., Carter, C. (1996) Evidence for native NMDA receptor subtype pharmacology as revealed by differential effects on the NMDA-evoked release of striatal neuromodulators: eliprodil, ifenprodil and other native NMDA receptor subtype selective compounds. *Neurochem. Int.*, **29**(5), 529-542.

Nathan, H. C., Bacchi, C. J., Hunter, S. H., Rescigno, D., McCann, P. P., Sjoerdsma, A. (1981) Antagonism by polyamines of the curative effects of α -difluoromethylornithine in *Trypanosoma brucei* infections. *Biochem. Pharmacol.*, **30**, 3010-3013.

Nordstrum, C. H., Siesjö, B. K. (1978) Effects of phenobarbital in cerebral ischaemia Part 1. Cerebral energy metabolism during pronounced incomplete ischaemia. *Stroke*, **9**, 327-335.

Norris, J. W., Hachinski, V. C., Meyers, M. G., Callow, J., Wong, T., Moore, R. W. (1978) Serum cardiac enzymes in stroke. *Stroke*, **10**, 548-552.

O'Neill, M. J., Caldwell, M., Canney, M., O'Halloran, A., Kelly, J., Leonard, B. E. (1995) The sigma receptor ligand JO-1784 (igmesine hydrochloride) is neuroprotective in the gerbil model of global cerebral ischaemia. *Eur. J. Pharmacol.*, **283**, 217-225.

O'Neill, M. J., Canney, M., Earley, B., Junien, J. L., Leonard, B. E. (1996) The novel sigma ligand JO 1994 protects against ischaemia-induced behavioural changes, cell death and receptor dysfunction in the gerbil. *Neurochem. International*, **28**, 193-207.

O'Neill, M., Earley, B., Leonard, B. E. (1994) MK-801 and BMY 14802 protect against ischaemia-induced cell death in the gerbil hippocampus. *Med. Sci. Res.*, **22**, 303-304.

Osborne, K. A., Shigeno, T., Balarsky, A. M., Ford, I., McCulloch, J., Teasdale, T. M., Graham, D. I. (1987) Quantitative assessment of early brain damage in a rat model of focal cerebral ischaemia. *J. Neurol. Neurosurg. Psychiatry*, **50**, 402-410.

Ots, M. E., Yaksh, T. L., Anderson, R. E., Sundt, T. M. Jr. (1987) Effect of dihydropyridines and diphenylalkylamines on pentylenetetrazole-induced seizures and cerebral blood flow in cats. *J. Neurosurg.*, **67**, 406-413.

Pahk, A. J. & Williams, K. (1997) Influence of extracellular pH on inhibition by ifenprodil as N-methyl-D-aspartate receptors in *Xenopus* oocytes. *Neurosci. Lett.*, **225**, 29-32.

Palmer, G. C., Stagnitto, M. L., Ray, R. K., Knowles, M. A., Harvay, R., Garske, G. E. (1993) Anticonvulsant properties of calcium channel blockers in mice: NMDA and Bay K9644-induced convulsions are potently blocked by the dihydropyridines. *Epilepsia*, **34**, 372-380.

Paoletti, P., Neyton, J., Ascher, P. (1995) Glycine-independent and subunit-specific potentiation of NMDA responses by extracellular Mg^{2+} . *Neuron*, **15**, 1109-1120.

Paschen, W., Csiba, L., Rohn, G., Bereczki, D. (1991) Polyamine metabolism in transient focal ischaemia of rat brain. *Brain Res.*, **566**, 354-357.

Paschen, W., Rohn, G., Meese, C. O., Djuricic, B., Schmidt-Kastner, R. (1988) Polyamine metabolism in reversible cerebral ischaemia: effect of α -difluoromethylornithine. *Brain Res.*, **453**, 9-16.

Paschen, W., Schmidt-Kastner, R., Djuricic, B., Meese, C., Linn, F., Hossmann, K.-A. (1987) Polyamine changes in reversible cerebral ischemia. *J. Neurochem.*, **49**, 35-37.

Paschen, W., Schmidt-Kastner, R., Hallmayer, J., Djuricic, B. (1988) Polyamines in cerebral ischaemia. *Neurochem. Pathol.*, **9**, 1-20.

Paschen, W., Widmann, R., Weber, C (1992) Changes in regional polyamine profiles in rat brains after transient cerebral ischaemia (single versus repetitive ischaemia): evidence for release of polyamines from injured neurons. *Neurosci. Lett.*, **135**, 121-124.

Paul, I. A., Kuypers, G., Youdim, M. B. H., Skolnick, P. (1990) Polyamines non-competitively inhibit [³H]3-PPP binding to sigma receptors. *Eur. J. Pharmac.*, **184**, 203-205.

Pearson, W. L. & Nichols, C. G. (1998) Block of the Kir2.1 channel pore by alkylamine analogues of endogenous spermine. *J. Gen. Physiol.*, **112**, 351-363.

Perez-Reyes, E., Cribbs, L. L., David, A., Lacerdd, A. E., Boudary, J., Williamson, M. P., Fox, M., Rees, M., Lee, J. H. (1998) Molecular characterisation of a neuronal low voltage activated J-type calcium channel. *Nature (Lond)*, **391**, 896-899.

Perry, T. L. & Hansen, S. (1981) Amino acid abnormalities in epileptogenic foci. *Neurology*, **31**, 872-876.

Phebus, L. A. & Clemens, J. A. (1989) Effects of transient, global, cerebral ischaemia on striatal extracellular dopamine, serotonin and their metabolism. *Life Sci.*, **44**, 1335-1342.

Phebus, L. A., Perry, K. W., Clemens, J. A., Fuller, R. W. (1986) Brain anoxia releases striatal dopamine in rats. *Life Sci.*, **38**, 2447-2453.

Phillips, I., Martin, K. F., Thompson, K. S. J., Heal, D. J. (1997) Weak blockade of AMPA receptor-mediated depolarisations in the rat cortical wedge by phenytoin but not lamotrigine or carbamazepine. *Eur. J. Pharmacol.*, **337**, 189-195.

Phillips, I., Martin, K. F., Thompson, K. S. J., Heal, D. J. (1998) GABA evoked depolarisations in the rat cortical wedge: involvement of GABA_A receptors and HCO³⁻ ions. *Brain Res.*, **798**, 330-332.

Phillips, N. I. & Fowler, L. J. (1982) The effects of sodium valproate on gamma-aminobutyrate metabolism and behaviour in naïve and ethanolamine-0-sulphate pretreated rats and mice. *Biochem. Pharmacol.*, **31**, 2257-2261.

Poignet, H., Nowicki, J. P., Scatton, B. (1992) Lack of neuroprotective effect of some sigma ligands in a model of focal cerebral ischaemia in the mouse. *Brain Res.*, **596**, 320-324.

Porter, C. W., Cavanaugh, P. F., Stolowich, N., Ganis, B., Kelly, E., Bergeron, R. J. (1985) Biological properties of N⁴- and N¹, N⁸-spermidine derivatives in cultured L1210 leukaemia cells. *Cancer Res.*, **45**, 2050-2057.

Pullan, L. M., Keith, R. A., LaMonte, D., Stumpo, R. J., Salama, A. I. (1990) The polyamine spermine affects ω -conotoxin binding and function at N-type voltage sensitive calcium channels. *J. Auton. Pharmac.*, **10**, 213-219.

Pulsinelli, W. A., Brierley, J. B., Plum, F. (1982) Temporal profile of neuronal damage in a model of transient forebrain ischaemia. *Ann. Neurol.*, **11**, 491-498.

Pulsinelli, W. A., Levy, D. E., Sigsbee, B., Scherer, P., Plum, F. (1983) Increased damage after ischaemic stroke in patients with hyperglycaemia with or without established diabetes mellitus. *Am. J. Med.*, **74**, 540-544.

Pulsinelli, W. A., Waldman, S., Rawlinson, D., Plum, F. (1982) Moderate hyperglycaemia augments ischaemic brain damage: A neuropathological study in the rat. *Neurology*, **32**, 1239-1246.

Ransom, R. W. & Stec, N. L. (1988) Cooperative modulation of [³H] MK-801 binding to the N-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine and polyamines. *J. Neurochem.*, **51**, 830-836.

Rao, T. S., Cler, J. A., Oei, E. J., Emmett, M. R., Mick, S. J., Iyengar, S., Wood, P. L. (1990) The polyamines, spermine and spermidine, negatively modulate N-methyl-D-aspartate (NMDA) and quisqualate receptor mediated responses *in vivo*: cerebellar cyclic GMP measurements.

Ray, T. K., Nandi, J., Pidhorodeckyj, N., Meng-Ai, Z. (1982) Polyamines are inhibitors of gastric acid secretion. *Proc. Natl. Acad. Sci. USA*, **79**, 1448-1452.

Reinert, M. M. & Bullock, R. (1999) Clinical trials in head injury. *Neurol. Res.*, **21**, 330-338.

Reynolds, I. J. & Miller, R. J. (1989) Ifenprodil is a novel type of N-methyl-D-aspartate receptor antagonist: interaction with polyamines. *Mol. Pharmacol.*, **36**, 758-765.

Robichaud, L. J. & Boxer, P. A. (1993) Polyamine modulation of excitatory amino acid responses in the rat cortical wedge. *Neuropharmacol.*, **32**(10), 1025-1035.

Robichaud, L. J., Malone, T. C., Boxer, P. A. (1991) NMDA antagonists but not AMPA/kainate antagonists block spontaneous epileptiform discharges in the rat cortical wedge model. *Soc. Neurosci. Abstr.*, **16**, 1168.

Rock, D. M. & Macdonald, R. L. (1992a) The polyamine spermine has multiple actions on N-methyl-D-aspartate receptor single-channel currents in cultured cortical neurons. *Mol. Pharmacol.*, **41**, 83-88.

Rock, D. M. & Macdonald, R. L. (1992b) Spermine and related polyamines produce a voltage-dependent reduction of N-methyl-D-aspartate receptor single-channel conductance. *Mol. Pharmacol.*, **42**, 157-164.

Rogers, D. C., Wright, P. W., Roberts, J. C., Reavill, C., Rothaul, A. L., Hunter, A. J. (1992) Photothrombotic lesions of the frontal cortex impair the performance of the delayed non-matching to position task by rats. *Behav. Brain Res.*, **49**, 231-235.

Romano, C. & Williams, K. (1994) Modulation of NMDA receptors by polyamines. *The Neuropharmacology of polyamines*. Editor: Carter, C. Raven Press, New York.

Ross, F. M., Brodie, M. J., Stone, T. W. (1998) Modulation by adenine nucleotides of epileptiform activity in the CA3 region of rat hippocampal slices. *Br. J. Pharmacol.*, **123**, 71-80.

Rossi, F., Nistico, G., De Marco, G., Berrino, L., Matera, C., Bile, G., Marmo, E. (1984) Cardiovascular effects of putrescine in dogs after systemic, intra-arterial vertebral and intraventricular injection. *Res. Commun. Chem. Pathol. Pharmacol.*, **46**, 43-52.

Rothman, S. M. & Olney, J. W. (1986) Glutamate and the pathophysiology of hypoxic-ischaemic brain damage. *Ann. Neurol.*, **19**, 105-111.

Rottenberg, H. & Marbach, M. (1990) Regulation of Ca²⁺ transport in brain mitochondria. I. The mechanism of spermine enhancement of Ca²⁺ uptake and retention. *Biochim. Biophys. Acta*, **1016**, 77-86.

Rowley, H. L., Ellis, Y., Davies, J. A. (1993) Age-related effects of NMDA-stimulated concomitant release of nitric oxide and glutamate in cortical slices prepared from DBA/2 mice. *Brain Res.*, **613**, 49-53.

Russell, D. H. & Meier, H. (1975) Alterations in the accumulation patterns of polyamines in brains of myelin-deficient mice. *J. Neurobiol.*, **6**(3), 267-275.

Ryder, S. (in preparation) Ph.D. thesis, University of Dublin, Trinity College.

Sacaan, A. I. & Johnson, K. M. (1990) Characterization of the stimulatory and inhibitory effects of polyamines on [³H] *N*-(1-[thienyl]cyclohexyl)piperidine binding to the N-methyl-D-aspartate receptor ionophore complex. *Mol. Pharmacol.*, **37**, 572-577.

Sagratella, S. (1998) Characterization of the *in vitro* antiepileptic activity of new and old anticonvulsant drugs. *Gen. Pharmac.*, **30**(2), 153-160.

Scatton, B., Carter, C., Benavides, J., Giroux, C. (1991) NMDA receptor antagonists: a novel therapeutic perspective for the treatment of ischaemic brain injury. *Cerebrovasc. Dis.*, **1**, 121.

Schmitz, M. P., Combs, D. J., Dempsey, R. J. (1993) Difluoromethylornithine decreases postischemic brain edema and blood-brain barrier breakdown. *Neurosurgery*, **33**, 882-7.

Schoemaker, H. (1992) Polyamines allosterically modulate [³H] nitrendipine binding to the voltage-sensitive calcium channel in rat brain. *Eur. J. Pharmac.*, **225**, 167-169.

Schoemaker, H. & Langer, S. Z. (1989) Effects of Ca²⁺ on [³H]diltiazem binding and its allosteric interaction with dihydropyridine calcium channel binding sites in the rat cortex. *J. Pharmacol. Exp. Ther.*, **248**, 710-715.

Schuber, F. (1989) Influence of polyamines on membrane functions. *Biochem. J.*, **260**, 1-10.

Scott, R. H., Sutton, K. G., Dolphin, A. C. (1994) Modulation of neuronal voltage-activated Ca²⁺ currents by polyamines. *The Neuropharmacology of polyamines*. Editor: Carter, C. Raven Press, New York.

Seif el Nasr, M., Peruche, B., Rossberg, C., Mennel, H. D., Krieglstein, J. (1990) Neuroprotective effect of memantine demonstrated in vivo and in vitro. *Eur. J. Pharmacol.*, **185**, 19-24.

Seiler, N. (1990) Polyamine metabolism. *Digestion*, **46**(2), 319-330.

Seiler, N. (1991) Pharmacological properties of the natural polyamines and their depletion by biosynthesis inhibitors as a therapeutic approach. *Progress in Drug Research*, **37**, 107-159.

Seiler, N. (1994) Formation, catabolism and properties of the natural polyamines. *The Neuropharmacology of polyamines*. Editor: Carter, C. Raven Press, New York.

Seiler, N. (1995) Polyamine oxidase, properties and functions. *Prog. Brain Res.*, **106**, 333-344.

Seiler, N., Atanassov, C., L., Raul, F. (1998(a)) Polyamine metabolism as a target for cancer chemoprevention (review). *Int. J. Oncology*, **13**, 993-1006.

Seiler, N., Douaud, F., Renault, J., Delcros, J.-G., Havouis, R., Uriac, P., Moulinoux, J.-P. (1998(b)) Polyamine sulfonamides with NMDA antagonist properties are potent calmodulin antagonists and cytotoxic agents. *Int. J. Biochem. Cell Biol.*, **30**, 393-406.

Seiler, N. & Schmidt-Glenewinkel, T. (1975) Regional distribution of putrescine, spermidine and spermine in relation to the distribution of RNA and DNA in the rat nervous system. *J. Neurochem.*, **24**, 791-795.

Sharma, T. A. & Reynolds, I. J. (1999) Characterization of the effects of polyamines on [¹²⁵I]MK-801 binding to recombinant N-methyl-D-aspartate receptors. *J. Pharmacol. Exp. Ther.*, **289**, 1041-1047.

Shaskan, E. G. & Snyder, S. H. (1973) Polyamine turnover in different regions of rat brain. *J. Neurochem.*, **20**, 1453-1460.

Shaw, G. G. (1972) Some pharmacological properties of the polyamines spermine and spermidine - a re-appraisal. *Arch. Intern. Pharmacodyn. Therap.*, **198**, 36-48.

Shaw, G.G. (1974) Some shortcomings of direct intraventricular injection in mice. *Br. J. Pharmac.*, **50**, 603-605.

Shaw, G. G. (1979) The synthesis and turnover of spermidine and spermine in mouse brain. *Neurochem. Res.*, **4**, 269-275.

Shaw, G. G. (1994) Polyamines as neurotransmitters or modulators. *The Neuropharmacology of polyamines*. Editor: Carter, C. Raven Press, New York.

Sheardown, M. J., Suzdak, P. D., Nordholm, L. (1993) AMPA, but not NMDA, receptor antagonism is neuroprotective in gerbil global ischaemia, even when delayed 24h. *Eur. J. Pharmacol.*, **236**, 347-353.

Siesjö, B. K. (1992) Pathophysiology and treatment of focal cerebral ischemia. Part I: Pathophysiology. *J. Neurosurg.*, **77**, 169-184.

Silver, J., M., Shin, C., McNamara, J. O. (1990) Antiepileptogenic effects of conventional anticonvulsants in the kindling model of epilepsy. *Ann. Neurology*, **29**, 356-363.

Singh, L., Oles, R., Woodruff, G. (1990) In vivo interaction of a polyamine with the NMDA receptor. *Eur. J. Pharmacol.*, **180**, 391-392.

Skeen, G. A., Twyman, R. E., White, H. S. (1993) The dihydropyridine nitrendipine modulates N-methyl-D-aspartate receptor channel function in mammalian neurons. *Mol. Pharmacol.*, **44**, 443-450.

Skeen, G. A., White, H. S., Twyman, R. E. (1994) The dihydropyridine nitrendipine reduces N-methyl-D-aspartate (NMDA)-evoked currents of rodent cortical neurons through a direct interaction with the NMDA receptor-associated ion channel. *J. Pharmacol. Exp. Ther.*, **271**(1), 30-38.

Smith, S. L., Martin, K. F., Heal, D. J. (1998) Demonstration of neuroprotection by the novel anticonvulsant, BTS 72 664, after permanent focal cerebral ischaemia in rats.

Snutch, T. P., Leonard, J. P., Gilbert, M. M., Lester, H. A., Davidson, N. (1990) Rat brain expresses a heterogeneous family of calcium channels. *Proc. Natl. Acad. Sci. USA*, **87**, 3391-3395.

Sparapani, M., Dall'Olio, R., Gandolfi, O., Ciani, E., Contestabile, A. (1997) Neurotoxicity of polyamines and pharmacological neuroprotection in cultures of rat cerebellar granule cells. *Exp. Neurol.*, **148**, 157-166.

Sprosen, T. S. & Woodruff, G. N. (1990) Polyamines potentiate NMDA induced whole-cell currents in cultured striatal neurons. *Eur. J. Pharmacol.*, **178**, 477-478.

Stasheff, S. F., Bragdon, A. C., Wilson, W. A. (1985) Induction of epileptiform activity in hippocampal slices by trains of electrical stimuli. *Brain Res.*, **344**, 296-302.

Sutton, K. G., Dolphin A. C., Scott, R. H. (1993) Inhibition of voltage activated calcium currents from cultured sensory neurones by spermine, argiotoxin-636, and arginine. *Mol. Neuropharmacol.*, **3**, 37-43.

Sutton, K. G., Siok, C., Stea, A., Zamponi, G. W., Heck, S. D., Volkmann, R. A., Ahlijanian, M. K., Snutch, T. P. (1998) Inhibition of neuronal calcium channels by a novel peptide spider toxin, DW13.3. *Mol. Pharmacol.*, **54**, 407-418.

Sykes, C. C. & Horton R. W. (1982) Cerebral glutamic acid decarboxylase activity and gamma-aminobutyric acid concentrations in mice susceptible or resistant to audiogenic seizures. *J. Neurochem.*, **39**, 1489-1491.

Tabor, H. & Tabor, C. W. (1964) Spermidine, spermine and related amines. *Pharmacological reviews*, **16**, 245-300.

Tagliatela, M., Ficker, E., Wible, B. A., Brown, A. M. (1995) C-terminus determinants for Mg^{2+} and polyamine block of the inward rectifier K^+ channel IRK1. *EMBO J.*, **14**, 5532-5541.

Tamura, Y., Sato, Y., Yokota, T., Akaike A., Sasa M., Takaori S. (1993) Ifenprodil prevents glutamate cytotoxicity via polyamine modulatory sites of N-methyl-D-aspartate receptors in cultured cortical neurons. *J. Pharm. Exp. Ther.*, **265**, 1017-1025.

Taniguchi, K., Shinjo, K., Mizutani, M., Shimada, K., Ishikawa, T., Menniti, F. S., Nagahisa, A. (1997) Antinociceptive activity of CP-101,606, an NMDA receptor NR2B subunit antagonist. *Br. J. Pharmac.*, **122**, 809-12.

Traynelis, S. F. & Cull-Candy, S. G. (1990) Proton inhibition of N-methyl-D-aspartate receptors in cerebellar neurones. *Nature (Lond.)*, **245**, 347-350.

Traynelis, S. F., Hartley, M., Heinemann, S. F. (1995) Control of proton sensitivity of the NMDA receptor by RNA splicing and polyamines. *Science (Wash., DC)*, **268**, 873-876.

Turski, L., Neimann, W., Stephens, D. N. (1990) Differential effects of antiepileptic drugs and β -carbolines on seizures induced by excitatory amino acids. *Neurosci.*, **39**(3), 799-807.

Twyman, R. E., Rogers, C. J., Macdonald, R. L. (1989) Differential regulation of gamma-aminobutyric acid receptor channels by diazepam and phenobarbital. *Ann. Neurol.*, **25**, 213-220.

Tymianski, M. & Tator, C. H. (1996) Normal and abnormal calcium homeostasis in neurons: A basis for the pathophysiology of traumatic and ischaemic central nervous system injury. *Neurosurgery*, **38**, 1176-1195.

Waldegger, S., Niemeyer, G., Morike, K., Wagner, C. A., Suessbrich, H., Busch, A. E., Lang, F., Eichelbaum, M. (1999) Effect of verapamil enantiomers and its metabolites on cardiac K^+ channels expressed in *Xenopus* oocytes. *Cell. Physiol. Biochem.*, **9**, 81-89.

Wallace, H., Shorvon, S., Tallis, R. (1998) Age-specific incidence and prevalence rates of treated epilepsy in an unselected population of 2052922 and age-specific fertility rates of women with epilepsy. *The Lancet*, **352**, 1970-1973.

Wang, D. & Corbett, D. (1990) Cerebral ischaemia, locomotor activity and spatial mapping. *Brain Res.*, **533**, 78-82.

Wasserkort, R., Hoppe, E., Reddington, M., Schubert, P. (1991) Modulation of A₁ adenosine receptor function in rat brain by the polyamine, spermine. *Neurosci. Lett.*, **124**, 183-186.

Wasterlain, C. G. & Jonec, V. (1983) Chemical kindling by muscarinic amygdaloid stimulation in the rat. *Brain Res.*, **271**, 311-323.

Weil, C. S (1952) Tables for convenient calculation of median effective dose (LD₅₀ or ED₅₀) and instructions in their use. *Biometrics*, **8**, 249-263.

Westbrook, G. L. & Mayer, M. L. (1987) Micromolar concentrations of Zn²⁺ antagonize NMDA and GABA responses of hippocampal neurones. *Nature*, **328**, 640-643.

White, H. S., Wolf, H. H., Swinyard, E. A., Skeen, G. A., Sofia, R. D. (1992) A neuropharmacological evaluation of felbamate as a novel anticonvulsant. *Epilepsia*, **33**(3), 564-572.

Williams, K. (1993) Ifenprodil discriminates subtypes of the N-methyl-D-aspartate receptor: Selectivity and mechanisms at recombinant heteromeric receptors. *Mol. Pharmacol.*, **44**, 851-859.

Williams, K. (1994) Mechanisms influencing stimulatory effects of spermine at recombinant N-methyl-D-aspartate receptors. *Mol. Pharmacol.*, **46**, 161-168.

Williams, K. (1995) Pharmacological properties of recombinant N-methyl-D-aspartate (NMDA) receptors containing the epsilon 4 (NR2D) subunit. *Neurosci. Lett.*, **184**, 181-184.

Williams, K., Kashiwagi, K., Fukuchi, J., Igarashi, K. (1995) An acidic amino acid in the N-methyl-D-aspartate receptor that is important for spermine stimulation. *Mol. Pharmacol.*, **48**, 1087-1098.

Williams, K., Romano, C., Dichter, M. A., Molinoff, P. B. (1991) Modulation of the NMDA receptor by polyamines. *Life Sciences*, **48**, 469-498.

Williams, K., Romano, C., Molinoff, P. B. (1989) Effects of polyamines on the binding of [³H] MK-801 to the N-methyl-D-aspartate receptor: pharmacological evidence for the existence of a polyamine recognition site. *Mol. Pharmacol.*, **36**, 575-581.

Williams, K., Zappia, A. M., Pritchett, D. B., Shen, Y. M., Molinoff, P. B. (1994) Sensitivity of the N-methyl-D-aspartate receptor to polyamines is controlled by NR2 subunits. *Mol. Pharmacol.*, **45**, 803-809.

Wlaz, P., Ebert, U., Loscher, W. (1999) Anticonvulsant effects of eliprodil alone or combined with the glycine_B receptor antagonist L-701,324 or the competitive NMDA antagonist CGP 40116 in the amygdala kindling model in rats. *Neuropharmacol.*, **38**, 243-251.

World Health Organisation (WHO) (1989) Recommendations on stroke prevention, diagnosis, and therapy. Report of the WHO Task Force on Stroke and other Cerebrovascular Disorders. *Stroke*, **20**(10), 1407-31.

Yamada, M. & Kurachi, Y. (1995) Spermine gates inward rectifying muscarinic but not ATP-sensitive K⁺ channels in rabbit atrial myocytes. *J. Biol. Chem.*, **270**(16), 9289-9294.

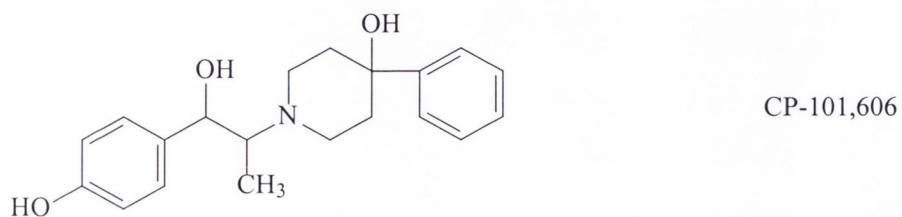
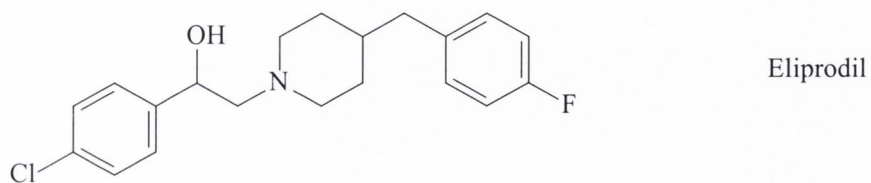
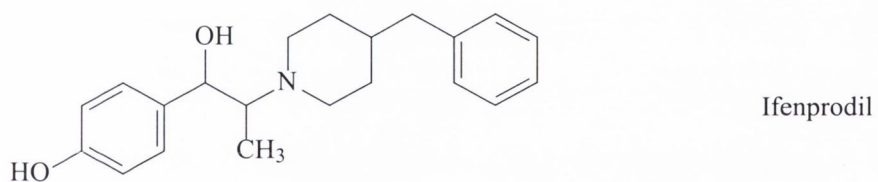
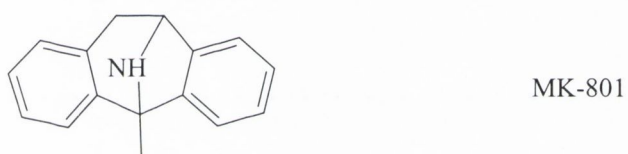
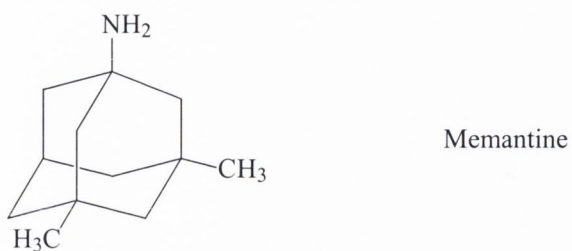
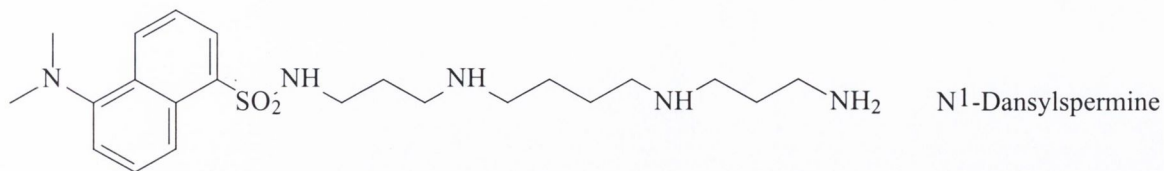
Yamamoto, M., Takahashi, K., Ohyama, M., Yamaguchi, T., Saitoh, S., Yatsugi, S., Kogure, K. (1993) Behavioral and histological changes after repeated brief cerebral ischemia by carotid artery occlusion in gerbils. *Brain Res.*, **608**, 16-20.

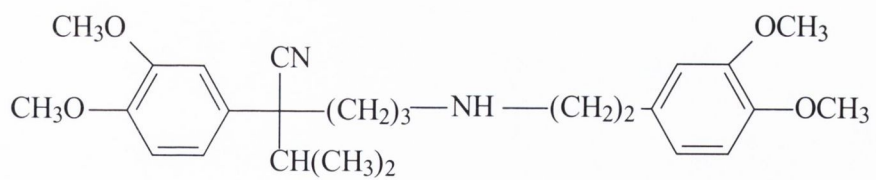
Yoneda, Y., Ogita, K., Enomoto, R., Suzuki, T., Kito, S. (1991) Identification and characterisation of specific binding sites of ^3H -spermidine in synaptic membranes of rat brain. *Brain Res.*, **563**, 17-27.

Zoli, M., Pedrazzi, P., Zini, I., Agnati, L. F. (1996) Spermidine/spermine N^1 -acetyltransferase mRNA levels show marked and region specific changes in the early phase after transient forebrain ischaemia. *Mol. Brain Res.*, **38**, 122-134.

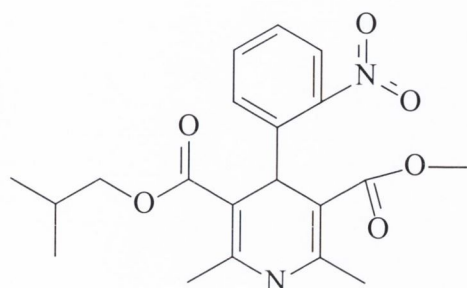
APPENDIX 1

Structural formulae of compounds used in this thesis

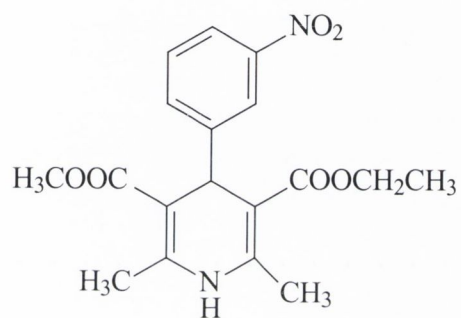




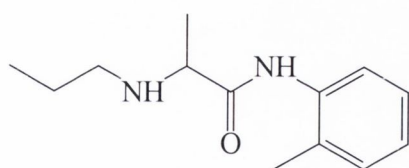
Verapamil



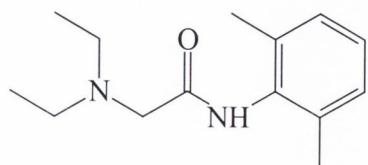
Nisoldipine



Nitrendipine



Prilocaine



Lignocaine