THE MECHANISMS OF NEUROPROTECTION BY MELATONIN IN CEREBRAL ISCHAEMIA REPERFUSION

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A thesis submitted for the degree of Doctor of Philosophy in Pharmacology at the University of Otago, Dunedin, New Zealand.

Date: 21st April 2011
This thesis is dedicated to the memory of

DR IAN APPLETON

2nd July 1966 – 3rd October 2007
ABSTRACT

Stroke is a major cause of death and the highest cause of morbidity worldwide. In NZ, the elderly and Maori and Pacific Island ethnicities are most affected. With failure of many neuroprotectant agents, current clinical therapy is limited to the use of thrombolytics to treat ischaemic stroke. Inflammation-dependent neurodegeneration occurs over a prolonged period after stroke. Melatonin has been shown to have anti-inflammatory, anti-oxidant, and neuroprotective effects after cerebral ischaemia reperfusion (IR) injury. This thesis focuses on the mechanisms of neuroprotection conferred by melatonin application after cerebral IR injury. Transient (2 hours) cerebral IR was achieved using the filament insertion model of middle cerebral artery occlusion (MCAO). The animals were first dosed (5 mg/kg i.p.) 1 hour after MCAO and two further doses administered over the next 48 hours. Melatonin administration was confirmed to reduce infarct size and be non-toxic. Immunohistochemistry was used to localise endogenous melatonin and its receptors (MT₁ and MT₂) in cerebral IR in MCAO and control animals. The majority of cells expressing melatonin and its receptors were found within the hypothalamus. The presence of melatonin and its receptors in the blood vessels, may suggest a role for melatonin in controlling immune cell infiltration after cerebral IR. Following MCAO, cellular immunoreactivity to melatonin receptor antibodies increased. Within the infarct, infiltrating inflammatory cells expressed melatonin and MT₂ receptor. Following MCAO, major inducible enzymes such as nitric oxide synthase (iNOS), and cyclooxygenase (COX-II) were stimulated. Melatonin administration resulted in a significant decrease in iNOS activity as well as total NOS activity and a consequent decrease in nitrite levels. Melatonin administration also attenuated both the MCAO-induced increase in COX expression and activity. HT-1080 human fibrosarcoma fibroblasts was utilised to probe the effects of melatonin on arginase enzymes. Melatonin treatment decreased cell viability at high concentrations, and this effect was attributed to its pro-oxidant effect present at these concentrations in cancer cell lines. The pro-oxidant effect was associated with increased total NOS activity. On the other hand, both arginase II expression and activity were increased with higher concentrations of melatonin treatment. These results highlighted the possibility that melatonin may be able to stimulate eNOS and arginase enzymes, both of which are beneficial after cerebral IR. Inflammation occurring after cerebral IR has been linked closely to mitochondria-driven apoptosis. Increased oxidative stress was seen as indicated by inhibition of aconitase enzyme activity after MCAO. Consequently, most of the electron transport chain complexes measured were significantly impaired. Melatonin administration led to protection of electron transport chain complexes, thus providing evidence of mitochondrial protection after
MCAO. In conclusion, this thesis has highlighted the multifaceted action of melatonin in attaining neuroprotection after stroke. This presents an exciting possibility of the use of melatonin in stroke treatment.
ACKNOWLEDGMENTS

Undertaking this PhD as part of the MBChB/PhD programme has been the greatest challenge of my life. Doing a revise and resubmission for this work was a true character building exercise. I have been through some humbling experiences in my life, with the Christchurch quake being one of them. It reminds us that we are part of something bigger, and that it is up to Him to determine our destiny. We should simply cherish the journey. Some destinations require a lot more perseverance and doing this research project over the past decade has definitely one of the most difficult endeavours I have taken. This work would never have been completed without the support and belief of several key people in my life. This thesis is dedicated to my parents to whom I owe everything. I thank them wholeheartedly for instilling good principles and values to pursue success in life, whilst always standing by my side. Despite living several thousand miles apart I always knew I could rely on them. I would also like to acknowledge my younger sister, Supriya Nair, for being my best friend in childhood and helping me develop a great sense of humour, one which proved vital in undertaking this daunting programme.

Firstly, I would like to acknowledge Dr Mohit Kapoor for introducing me to the Appleton laboratory group, which was synonymous to a family and for helping me settle into a totally different environment from medical school. This PhD would not have been possible if it weren’t for my primary supervisor, Dr Ian Appleton. His brilliant ideas formed the basis of this thesis and I am forever indebted to his trust in my capabilities as a researcher, especially since I started the PhD without any research experience. I especially thank his patience and extensive supervision during the experimental period of this PhD. I have not seen a supervisor as dedicated to his students. It is really unfortunate that he had suffered from a terminal illness and a promising research career was cut far too short. It would have been priceless to see the satisfaction on his face on being awarded this degree. We are always thinking of you, Ian, wherever you are.

In addition, I sincerely thank Dr Rosanna Rahman who as an elder sister literally held my hand and helped me during the early stages of this project. She also established the MCAO model at the University of Otago and helped me develop skills as a researcher and a writer. I would like to show my heartfelt gratitude towards Prof. Paul Smith for providing me with supervision during the writing phase of the first submission of this PhD. Special acknowledgement to Dr Ivan Sammut, who helped plan the initial mitochondrial experiments, assisted immensely in manuscript publication and revised form of the thesis. My supervisory panel, that has been changed several
times, Professor George Lees, Dr Greg Giles, and Dr John Ashton, has to be commended for being so tolerant and preserving with this project. I also thank Dr Andrew Clarkson and Dr Alexandra Tramaoundamas for their help with the mitochondrial assays. I would like to extend my gratitude to Dr Rhonda Rosengren and her students for their assistance in evaluating the possible toxic effects of melatonin. I would like to acknowledge the help of Dr Brad Sutherland who was been a great mate, in addition to being there for me to brainstorm ideas, assisting with publication and revision of the thesis. Dr Matthew Goddard, who has been one of my best mates, flatmates and colleague and has always been available to help me out in times of need, whether it is pertaining computers, statistics or life in general. I am always grateful to meet him through this PhD and his continued friendship. I would like to thank Mr Andrew Gray from the Department of Preventive and Social Medicine, for providing sound statistical guidance throughout this project. To my colleagues Odette Shaw, Dr Kamali Pugazhenti, Sharleen Irvine, Dr Kelly McKelvey and Dr Ian Winburn, thank you for being so supportive and making this experience so memorable and rewarding. I would also like thank Kevin Markham, Anne-Marie Olsen, Irene Hall, Sue Johnstone, Murray Craig, Toro Hill, Sonya Hart and Andrea Hessian for their continued assistance and support. 

My special thanks for the various funding providers of this project. The Health Research Council of New Zealand for funding the first year of this project via the Pacific Health Research Masters Awards, and the University of Otago for providing me Special Health Division PhD Scholarship for the remainder. I would also like to acknowledge the assistance and support of the various Deans of the Faculty of Medicine, Prof. John Campbell, Prof. Linda Holloway and Prof. Don Roberton.. In addition, I would like to thank Dr Charles Tustin, Prof. Christine Winterbourn, Eddy van de Pol and Bruce Smith for their administrative assistance.

I wish to thank my friends for their continued support and friendship. I would especially like to mention the invaluable support and encouragement of Dr Leo Schep from the National Poisons Centre. Lastly but definitely not the least I would like to thank my soul-mate and beautiful wife, Suprita Nair. She has been absolutely awesome in supporting me to finish this project. I would like to thank her for her continued assistance with the PhD in addition to being so understanding and sacrificing. Getting to know her has been one of the best things that has ever happened to me and with her by my side I am confident that I will attain any goal I set in life. I will forever be indebted to you for allowing me to pursue this revision, in lieu of my personal commitments..
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedication</td>
<td>ii</td>
</tr>
<tr>
<td>Abstract</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xv</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xix</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xx</td>
</tr>
</tbody>
</table>

## 1 CHAPTER 1

### INTRODUCTION

1.1 Stroke

1.1.1 Definition
1.1.2 Epidemiology of stroke
1.1.3 Aetiology
1.1.4 Pathogenesis of stroke

1.2 Inflammation and stroke

1.2.1 Inflammation in stroke
1.2.2 The possible beneficial role of inflammation in stroke
1.2.3 Cytokines and chemokines as mediators of inflammation

1.3 Inducible enzymes in inflammation post stroke

1.3.1 L-arginine metabolism
1.3.2 Contribution of NOS enzyme isoforms
1.3.3 Contribution of arginase enzymes
1.3.4 The role of polyamines in stroke
1.3.5 Contribution of agmatine
1.3.6 Arachidonic acid metabolism
1.3.7 Contribution of COX enzymes
1.3.8 Contribution of prostaglandins

1.4 Mitochondria in stroke

1.4.1 Normal electron transport chain function
1.4.2 The formation of ROS in mitochondria
1.4.3 Mitochondria mediated apoptosis in stroke

1.5 Pharmacological interventions of stroke
   1.5.1 The Stroke Therapy Academic Industry Roundtable (STAIR) committee
   1.5.2 The therapeutic time window
   1.5.3 Targeting inflammation after stroke
   1.5.4 Utilising current interventions of stroke
   1.5.5 Future directions of acute stroke therapy

1.6 Animal models of stroke
   1.6.1 Use of animals in stroke research
   1.6.2 Animal models of stroke
   1.6.3 Focal ischaemia by MCAO
   1.6.4 MCAO by intraluminal filament

1.7 Melatonin
   1.7.1 Synthesis of melatonin
   1.7.2 Physiology of melatonin
   1.7.3 Melatonin receptors
   1.7.4 Antioxidant properties of melatonin
   1.7.5 Anti-inflammatory properties of melatonin
   1.7.6 The role of melatonin in neurodegeneration

1.8 Thesis objectives

2 CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

2.2 Animal maintenance

2.3 MCAO model (Longa et al., 1989)
   2.3.1 Preparation and allocation of animals
   2.3.2 Preparation of intraluminal occlusion threads (Belayev et al., 1996)
   2.3.3 Equipment preparation
   2.3.4 Animal anaesthesia
   2.3.5 Blood pressure and gas sampling
   2.3.6 Neck incision and exposure of the carotid artery
   2.3.7 Insertion of the intraluminal thread
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3.8 Blood sampling and closure of wounds</td>
<td>77</td>
</tr>
<tr>
<td>2.3.9 Reperfusion surgery</td>
<td>78</td>
</tr>
<tr>
<td>2.3.10 Experimental inclusion criteria</td>
<td>79</td>
</tr>
<tr>
<td>2.4 Immunohistochemical studies</td>
<td>79</td>
</tr>
<tr>
<td>2.4.1 Tissue extraction and fixation</td>
<td>79</td>
</tr>
<tr>
<td>2.4.2 Poly-L-lysine coating of slides</td>
<td>80</td>
</tr>
<tr>
<td>2.4.3 Tissue fixation for wax embedded microtomy</td>
<td>80</td>
</tr>
<tr>
<td>2.4.4 Immunolabelling</td>
<td>80</td>
</tr>
<tr>
<td>2.4.5 3’3’ Diaminobenzidine tetrahydrochloride (DAB) labelling</td>
<td>81</td>
</tr>
<tr>
<td>2.4.6 Harris’ Haematoxylin</td>
<td>81</td>
</tr>
<tr>
<td>2.4.7 Nuclear counter staining</td>
<td>81</td>
</tr>
<tr>
<td>2.4.8 Mounting of slides</td>
<td>82</td>
</tr>
<tr>
<td>2.4.9 Preabsorption analysis</td>
<td>82</td>
</tr>
<tr>
<td>2.4.10 DAB saturation curve</td>
<td>82</td>
</tr>
<tr>
<td>2.4.11 Morphometric analysis</td>
<td>83</td>
</tr>
<tr>
<td>2.5 Dosing regimen of melatonin for the MCAO experiment</td>
<td>84</td>
</tr>
<tr>
<td>2.5.1 Preparation and administration of melatonin</td>
<td>84</td>
</tr>
<tr>
<td>2.6 The effects of melatonin on infarct size after MCAO</td>
<td>85</td>
</tr>
<tr>
<td>2.6.1 Tissue extraction and fixation</td>
<td>85</td>
</tr>
<tr>
<td>2.6.2 2, 3, 5 – Triphenyltetrazolium chloride staining (Bederson et al., 1986)</td>
<td>85</td>
</tr>
<tr>
<td>2.6.3 Quantification of the relative infarct size</td>
<td>86</td>
</tr>
<tr>
<td>2.7 Biochemical analysis of the effects of melatonin on MCAO</td>
<td>86</td>
</tr>
<tr>
<td>2.7.1 Tissue extraction</td>
<td>86</td>
</tr>
<tr>
<td>2.7.2 Analysis of possible toxic effects of melatonin</td>
<td>86</td>
</tr>
<tr>
<td>2.7.3 ALT measurement</td>
<td>87</td>
</tr>
<tr>
<td>2.7.4 Tissue homogenisation</td>
<td>87</td>
</tr>
<tr>
<td>2.7.5 Protein determination by Bradford assay (Bradford, 1976)</td>
<td>87</td>
</tr>
<tr>
<td>2.7.6 Measurement of nitrite levels by Griess Reaction (Huygen, 1970)</td>
<td>88</td>
</tr>
<tr>
<td>2.7.7 Measurement of arginase activity</td>
<td>88</td>
</tr>
<tr>
<td>2.7.8 Measurement of NOS activity</td>
<td>89</td>
</tr>
<tr>
<td>2.7.9 Measurement of COX activity</td>
<td>90</td>
</tr>
<tr>
<td>2.8 Western blot analysis</td>
<td>91</td>
</tr>
<tr>
<td>2.8.1 Sample preparation</td>
<td>91</td>
</tr>
<tr>
<td>2.8.2</td>
<td>Preparation of gels</td>
</tr>
<tr>
<td>2.8.3</td>
<td>Electrophoresis and blotting</td>
</tr>
<tr>
<td>2.8.4</td>
<td>Immunolabelling and Hyperfilm development</td>
</tr>
<tr>
<td>2.8.5</td>
<td>Stripping and reprobing</td>
</tr>
<tr>
<td>2.8.6</td>
<td>Analysis</td>
</tr>
<tr>
<td>2.9</td>
<td><strong>In vitro effects of melatonin on L-arginine metabolism</strong></td>
</tr>
<tr>
<td>2.9.1</td>
<td>Cell culture</td>
</tr>
<tr>
<td>2.9.2</td>
<td>Passaging of cells</td>
</tr>
<tr>
<td>2.9.3</td>
<td>Cell viability by Trypan blue exclusion</td>
</tr>
<tr>
<td>2.9.4</td>
<td>Sulforhodamine B (SRB) assay</td>
</tr>
<tr>
<td>2.9.5</td>
<td>MTT assay</td>
</tr>
<tr>
<td>2.9.6</td>
<td>Nitrite level determination (cell extract supernatants)</td>
</tr>
<tr>
<td>2.9.7</td>
<td>Collection of cell samples for biochemical assays</td>
</tr>
<tr>
<td>2.10</td>
<td><strong>Effects of melatonin on mitochondrial complexes</strong></td>
</tr>
<tr>
<td>2.10.1</td>
<td>Preparation of mitochondrial samples</td>
</tr>
<tr>
<td>2.10.2</td>
<td>Mitochondrial complex I (NADH-ubiquinone oxidoreductase) assay</td>
</tr>
<tr>
<td>2.10.3</td>
<td>Mitochondrial complex II-III (succinate-ubiquinone/ubiquinol-cytochrome c reductase) assay</td>
</tr>
<tr>
<td>2.10.4</td>
<td>Mitochondrial complex V (ATP hydrolase activity) assay</td>
</tr>
<tr>
<td>2.10.5</td>
<td>Citrate synthase assay</td>
</tr>
<tr>
<td>2.10.6</td>
<td>Ratio of ETC activity assays to citrate synthase activity</td>
</tr>
<tr>
<td>2.10.7</td>
<td>Aconitase [citrate(isocitrate) hydro-lyase] assay</td>
</tr>
<tr>
<td>2.11</td>
<td><strong>Statistical analyses</strong></td>
</tr>
<tr>
<td>2.11.1</td>
<td>Effects of melatonin administration on infarct volume</td>
</tr>
<tr>
<td>2.11.2</td>
<td>Localisation of melatonin and its receptors post MCAO</td>
</tr>
<tr>
<td>2.11.3</td>
<td>Biochemical study of the effects of melatonin administration</td>
</tr>
<tr>
<td>2.11.4</td>
<td><strong>In vitro effects of melatonin administration</strong></td>
</tr>
<tr>
<td>2.11.5</td>
<td>Effects of melatonin administration on mitochondrial function after MCAO</td>
</tr>
</tbody>
</table>

### Chapter 3

**THE EFFECTS OF MELATONIN ON NEURAL INJURY POST ISCHAEMIA**

103
3.1 Introduction
  3.1.1 Hypothesis and aims 104
  3.1.2 Experimental approach 104

3.2 Results
  3.2.1 Inclusion of animals 105
  3.2.2 Physiological parameters of animals 106
  3.2.3 Histological analysis of infarction 107
  3.2.4 Toxic effects of melatonin administration 110

3.3 Discussion
  3.3.1 Inclusion of animals and physiological parameters 111
  3.3.2 Infarct size analysis following melatonin administration 111
  3.3.3 Toxic effect of melatonin administration 117

3.4 Conclusion 118

4 CHAPTER 4
IMMUNOHISTOCHEMICAL LOCALISATION OF MELATONIN AND ITS RECEPTORS

4.1 Introduction
  4.1.1 Hypothesis and aims 120
  4.1.2 Experimental approach 121

4.2 Results
  4.2.1 Inclusion of animals 121
  4.2.2 Optimal dilutions for antibodies used 121
  4.2.3 Specificity of antibodies 123
  4.2.4 Verification of the method of identifying the brain regions 125
  4.2.5 Melatonin antibody binding distribution in normal brains 127
  4.2.6 Melatonin antibody binding distribution after MCAO 131
  4.2.7 Distribution of the MT_1 receptor in normal brains 131
  4.2.8 Distribution of the MT_1 receptor after MCAO 132
  4.2.9 Distribution of the MT_2 receptor in normal brains 134
  4.2.10 Distribution of the MT_2 receptor after MCAO 135

4.3 Discussion
  4.3.1 Melatonin antibody labelling 137
Table of Contents

4.3.2 Melatonin membrane receptors distribution 138
4.3.3 Limitations of this study 140
4.4 Conclusion 141

5 CHAPTER 5
MECHANISMS OF NEUROPROTECTION BY MELATONIN IN CEREBRAL ISCHAEMIA REPERFUSION INJURY 142
5.1 Introduction 143
5.1.1 Hypothesis and aims 143
5.1.2 Experimental approach 143
5.2 Results 144
5.2.1 Inclusion of animals 144
5.2.2 Optimization of antibody concentrations for inducible enzymes 144
5.2.3 Effect of melatonin on NOS enzymes following MCAO 145
5.2.3 Effect of melatonin on arginase activity 149
5.2.4 Effect of melatonin on COX enzymes 152
5.3 Discussion 154
5.3.1 Melatonin and the NOS enzyme system after MCAO 154
5.3.2 Melatonin and the arginase enzyme system after cerebral IR injury 158
5.3.3 Melatonin and the COX enzyme after MCAO 159
5.4 Conclusion 162

6 CHAPTER 6
IN VITRO EFFECTS OF MELATONIN ON L-ARGININE METABOLISM 163
6.1 Introduction 164
6.1.1 Hypothesis and aims 164
6.1.2 Experimental approach 164
6.2 Results 165
6.2.1 Optimization of antibody concentrations for inducible enzymes 165
6.2.2 Effects of melatonin on the cell viability 165
6.2.3 Effects of melatonin on NOS 167
6.2.3 Effects of melatonin on the arginase enzyme system 170
<table>
<thead>
<tr>
<th>6.3</th>
<th>Discussion</th>
<th>172</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.3.1</td>
<td>Effects of melatonin on cell proliferation</td>
<td>172</td>
</tr>
<tr>
<td>6.3.2</td>
<td>Effects of melatonin on L-arginine metabolism in vitro</td>
<td>173</td>
</tr>
<tr>
<td>6.4</td>
<td>Conclusion</td>
<td>175</td>
</tr>
</tbody>
</table>

7 | CHAPTER 7 | 176 |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>EFFECTS OF MELATONIN ON MITOCHONDRIAL FUNCTIONING FOLLOWING STROKE</td>
<td>177</td>
<td></td>
</tr>
<tr>
<td>7.1</td>
<td>Introduction</td>
<td>178</td>
</tr>
<tr>
<td>7.1.1</td>
<td>Hypothesis and aims</td>
<td>178</td>
</tr>
<tr>
<td>7.1.2</td>
<td>Experimental approach</td>
<td>178</td>
</tr>
<tr>
<td>7.2</td>
<td>Results</td>
<td>178</td>
</tr>
<tr>
<td>7.2.1</td>
<td>Inclusion of animals</td>
<td>178</td>
</tr>
<tr>
<td>7.2.2</td>
<td>Effects of melatonin on mitochondrial complex I</td>
<td>179</td>
</tr>
<tr>
<td>7.2.3</td>
<td>Effects of melatonin on mitochondrial complex II-III</td>
<td>179</td>
</tr>
<tr>
<td>7.2.4</td>
<td>Effects of melatonin on mitochondrial complex V</td>
<td>180</td>
</tr>
<tr>
<td>7.2.5</td>
<td>Effects of melatonin on citrate synthase activity</td>
<td>181</td>
</tr>
<tr>
<td>7.2.6</td>
<td>Effects of melatonin on aconitase activity</td>
<td>182</td>
</tr>
<tr>
<td>7.3</td>
<td>Discussion</td>
<td>183</td>
</tr>
<tr>
<td>7.3.1</td>
<td>Impairment of mitochondrial complexes and enzymes after MCAO</td>
<td>183</td>
</tr>
<tr>
<td>7.3.2</td>
<td>Effect of melatonin on mitochondrial ETC complexes following stroke</td>
<td>185</td>
</tr>
<tr>
<td>7.3.3</td>
<td>Effect of melatonin on the preservation of mitochondria following stroke</td>
<td>186</td>
</tr>
<tr>
<td>7.4</td>
<td>Conclusion</td>
<td>187</td>
</tr>
</tbody>
</table>

8 | CHAPTER 8 | 188 |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>GENERAL DISCUSSION</td>
<td>189</td>
<td></td>
</tr>
<tr>
<td>8.1</td>
<td>Summary of major findings</td>
<td>189</td>
</tr>
<tr>
<td>8.2</td>
<td>Significance of findings</td>
<td>190</td>
</tr>
<tr>
<td>8.3</td>
<td>Future research</td>
<td>192</td>
</tr>
<tr>
<td>8.4</td>
<td>Clinical significance</td>
<td>193</td>
</tr>
<tr>
<td></td>
<td>Chapter</td>
<td>Section</td>
</tr>
<tr>
<td>---</td>
<td>-----------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>9</td>
<td>CHAPTER 9</td>
<td>REFERENCES</td>
</tr>
<tr>
<td>10</td>
<td>CHAPTER 10</td>
<td>PUBLICATIONS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.1 Research articles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.2 Review articles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.3 Refereed conference proceedings</td>
</tr>
<tr>
<td>11</td>
<td>APPENDICES</td>
<td>A.1 List of equipment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A.2 Immunohistochemistry analysis – validation of regions</td>
</tr>
<tr>
<td>Number</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.1</td>
<td>Focal ischaemia caused by blockage of middle cerebral artery (MCA)</td>
<td>4</td>
</tr>
<tr>
<td>1.2</td>
<td>The glutamate transporter reversal after anoxic depolarisation</td>
<td>6</td>
</tr>
<tr>
<td>1.3</td>
<td>The NMDA receptor activation</td>
<td>6</td>
</tr>
<tr>
<td>1.4</td>
<td>The complex primary excitotoxic cascade in the acute phase of cerebral ischaemia</td>
<td>9</td>
</tr>
<tr>
<td>1.5</td>
<td>An overview of the inflammation cascade following IR</td>
<td>10</td>
</tr>
<tr>
<td>1.6</td>
<td>A cascade of detrimental events in cerebral IR injury</td>
<td>10</td>
</tr>
<tr>
<td>1.7</td>
<td>The metabolism of L-arginine by two major metabolic pathways, NOS and arginase</td>
<td>14</td>
</tr>
<tr>
<td>1.8</td>
<td>The paradoxical role of NO in the cerebral IR</td>
<td>18</td>
</tr>
<tr>
<td>1.9</td>
<td>Effect on AA metabolism after cerebral IR injury with a focus on PGE$_2$ production</td>
<td>24</td>
</tr>
<tr>
<td>1.10</td>
<td>The electron transfer through electron transport chain</td>
<td>29</td>
</tr>
<tr>
<td>1.11</td>
<td>Pathway detailing mechanisms of actions for the increased risk of ICH associated with NSAIDS treatment</td>
<td>38</td>
</tr>
<tr>
<td>1.12</td>
<td>The proposed, ideal primary therapeutic regime with current successful interventions for acute stroke</td>
<td>40</td>
</tr>
<tr>
<td>1.13</td>
<td>The chemical structure of melatonin</td>
<td>48</td>
</tr>
<tr>
<td>1.14</td>
<td>The circuitous pathway from the hypothalamus to pineal gland showing the control of light stimulus on melatonin production</td>
<td>49</td>
</tr>
<tr>
<td>1.15</td>
<td>The mechanism of synthesis of melatonin in the pinealocyte</td>
<td>50</td>
</tr>
<tr>
<td>1.16</td>
<td>The subtypes of melatonin receptors and its classifications</td>
<td>53</td>
</tr>
<tr>
<td>1.17</td>
<td>The activation of melatonin membrane receptors</td>
<td>56</td>
</tr>
<tr>
<td>1.18</td>
<td>One of the possible mechanisms of direct scavenging of free radicals by melatonin</td>
<td>61</td>
</tr>
<tr>
<td>1.19</td>
<td>The proposed mechanism of anti-inflammatory role of melatonin</td>
<td>63</td>
</tr>
<tr>
<td>1.20</td>
<td>The possible mechanisms of melatonin in achieving neuroprotection</td>
<td>66</td>
</tr>
<tr>
<td>2.1</td>
<td>The aerial view of the surgical setup for MCAO</td>
<td>75</td>
</tr>
</tbody>
</table>
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2</td>
<td>A schematic diagram showing the insertion of the nylon filament into the MCA, via the ECA</td>
<td>78</td>
</tr>
<tr>
<td>2.3</td>
<td>The extracted rat brain</td>
<td>85</td>
</tr>
<tr>
<td>3.1</td>
<td>A TTC-stained section at 5 mm from the frontal pole exhibiting an ICH</td>
<td>105</td>
</tr>
<tr>
<td>3.2</td>
<td>TTC staining at 5 mm from frontal pole of ischaemic and normal brain</td>
<td>108</td>
</tr>
<tr>
<td>3.3</td>
<td>Neuroprotective effects of melatonin treatment following MCAO</td>
<td>109</td>
</tr>
<tr>
<td>3.4</td>
<td>Effects of melatonin treatment after MCAO in cortex and striatum</td>
<td>110</td>
</tr>
<tr>
<td>4.1</td>
<td>DAB saturation curve for MT&lt;sub&gt;1&lt;/sub&gt; antibody</td>
<td>122</td>
</tr>
<tr>
<td>4.2</td>
<td>DAB saturation curve for MT&lt;sub&gt;2&lt;/sub&gt; antibody</td>
<td>123</td>
</tr>
<tr>
<td>4.3</td>
<td>Contrasting results of the preadsorption study for melatonin antibody</td>
<td>124</td>
</tr>
<tr>
<td>4.4</td>
<td>Specificity of MT&lt;sub&gt;1&lt;/sub&gt; antibody shown by use of a negative control</td>
<td>124</td>
</tr>
<tr>
<td>4.5</td>
<td>Specificity of MT&lt;sub&gt;2&lt;/sub&gt; antibody elucidated by use of a negative control</td>
<td>125</td>
</tr>
<tr>
<td>4.6</td>
<td>A representative illustration of a section corresponding to the relevant neuroanatomy atlas plate</td>
<td>126</td>
</tr>
<tr>
<td>4.7</td>
<td>An illustration of identifying a region (cerebellum) after matching the section to the relevant neuroanatomy atlas plate</td>
<td>127</td>
</tr>
<tr>
<td>4.8</td>
<td>Photomicrographs of positive immunoreactivity to melatonin antibody</td>
<td>129</td>
</tr>
<tr>
<td>4.9</td>
<td>Photomicrographs of MT&lt;sub&gt;1&lt;/sub&gt; receptor expression</td>
<td>132</td>
</tr>
<tr>
<td>4.10</td>
<td>Photomicrographs of MT&lt;sub&gt;2&lt;/sub&gt; receptor antibody labelling</td>
<td>134</td>
</tr>
<tr>
<td>4.11</td>
<td>Photomicrographs of MT&lt;sub&gt;2&lt;/sub&gt; receptor expression after MCAO</td>
<td>137</td>
</tr>
<tr>
<td>5.1</td>
<td>Effects of melatonin on administration nNOS expression after MCAO</td>
<td>145</td>
</tr>
<tr>
<td>5.2</td>
<td>Effects of melatonin on eNOS protein levels following stroke</td>
<td>146</td>
</tr>
<tr>
<td>5.3</td>
<td>Effects of melatonin on iNOS expression following stroke</td>
<td>147</td>
</tr>
<tr>
<td>5.4</td>
<td>Effects of melatonin on iNOS activity after MCAO</td>
<td>147</td>
</tr>
<tr>
<td>5.5</td>
<td>Effects of melatonin on total NOS activity after stroke</td>
<td>148</td>
</tr>
<tr>
<td>5.6</td>
<td>Effects of melatonin on nitrite levels following stroke</td>
<td>149</td>
</tr>
<tr>
<td>5.7</td>
<td>Effects of melatonin on arginase I expression following stroke</td>
<td>150</td>
</tr>
<tr>
<td>5.8</td>
<td>Effects of melatonin on arginase II expression following stroke</td>
<td>151</td>
</tr>
<tr>
<td>5.9</td>
<td>Effects of melatonin on arginase activity after stroke</td>
<td>151</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>5.10</td>
<td>Effects of melatonin on COX-I expression following MCAO</td>
<td>152</td>
</tr>
<tr>
<td>5.11</td>
<td>Effects of melatonin on COX-II expression following stroke</td>
<td>153</td>
</tr>
<tr>
<td>5.12</td>
<td>Effects of melatonin on COX activity following MCAO</td>
<td>153</td>
</tr>
<tr>
<td>5.13</td>
<td>Schematic diagram showing temporal profile of key enzyme isoforms</td>
<td>155</td>
</tr>
<tr>
<td>6.1</td>
<td>Effects of melatonin on cell viability determined by the MTT assay</td>
<td>166</td>
</tr>
<tr>
<td>6.2</td>
<td>Effects of melatonin on cell viability determined by the SRB assay</td>
<td>166</td>
</tr>
<tr>
<td>6.3</td>
<td>Effects of melatonin on the eNOS expression in HT-1080 fibroblasts</td>
<td>167</td>
</tr>
<tr>
<td>6.4</td>
<td>Effects of melatonin on the iNOS expression in HT-1080 fibroblasts</td>
<td>168</td>
</tr>
<tr>
<td>6.5</td>
<td>Effects of melatonin on the iNOS activity in HT-1080 fibroblasts</td>
<td>168</td>
</tr>
<tr>
<td>6.6</td>
<td>Effects of melatonin on the total NOS activity in HT-1080 fibroblasts</td>
<td>169</td>
</tr>
<tr>
<td>6.8</td>
<td>Effects of melatonin on arginase I expression in HT-1080 fibroblasts</td>
<td>170</td>
</tr>
<tr>
<td>6.9</td>
<td>Effects of increasing melatonin concentrations on arginase II expression</td>
<td>171</td>
</tr>
<tr>
<td>6.10</td>
<td>Effects of melatonin on arginase activity in HT-1080 fibroblasts</td>
<td>171</td>
</tr>
<tr>
<td>7.1</td>
<td>Effects of melatonin on complex I activity following MCAO</td>
<td>179</td>
</tr>
<tr>
<td>7.2</td>
<td>Effects of melatonin on complex II-III activity following MCAO</td>
<td>180</td>
</tr>
<tr>
<td>7.3</td>
<td>Effects of melatonin on complex V activity following MCAO</td>
<td>181</td>
</tr>
<tr>
<td>7.4</td>
<td>Effects of melatonin on mitochondrial integrity following MCAO</td>
<td>181</td>
</tr>
<tr>
<td>7.5</td>
<td>Effects of melatonin on tissue injury by oxidative stress</td>
<td>183</td>
</tr>
<tr>
<td>A.1</td>
<td>An illustration of identifying the olfactory region after matching the section to the relevant neuroanatomy atlas plate</td>
<td>282</td>
</tr>
<tr>
<td>A.2</td>
<td>An illustration of identifying the cerebral cortical region after matching the section to the relevant neuroanatomy atlas plate</td>
<td>283</td>
</tr>
<tr>
<td>A.3</td>
<td>An illustration of identifying the basal ganglia region after matching the section to the relevant neuroanatomy atlas plate</td>
<td>284</td>
</tr>
<tr>
<td>A.4</td>
<td>An illustration of identifying the septal and forebrain region after matching the section to the relevant neuroanatomy atlas plate</td>
<td>285</td>
</tr>
<tr>
<td>A.5</td>
<td>An illustration of identifying the hippocampal region after matching the section to the relevant neuroanatomy atlas plate</td>
<td>286</td>
</tr>
<tr>
<td>A.6</td>
<td>An illustration of identifying the epithalamic region after matching the section to the relevant neuroanatomy atlas plate</td>
<td>287</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>A.7</td>
<td>An illustration of identifying the thalamic region after matching the section to the relevant neuroanatomy atlas plate</td>
<td>288</td>
</tr>
<tr>
<td>A.8</td>
<td>An illustration of identifying the subthalamic region after matching the section to the relevant neuroanatomy atlas plate</td>
<td>289</td>
</tr>
<tr>
<td>A.9</td>
<td>An illustration of identifying the hypothalamic region after matching the section to the relevant neuroanatomy atlas plate</td>
<td>290</td>
</tr>
<tr>
<td>A.10</td>
<td>An illustration of identifying the amygdala region after matching the section to the relevant neuroanatomy atlas plate</td>
<td>291</td>
</tr>
<tr>
<td>A.11</td>
<td>An illustration of identifying the midbrain and pons region after matching the section to the relevant neuroanatomy atlas plate</td>
<td>292</td>
</tr>
<tr>
<td>A.12</td>
<td>An illustration of identifying the medulla region after matching the section to the relevant neuroanatomy atlas plate</td>
<td>293</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Number</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Major phase III clinical trials for acute stroke therapy</td>
<td>34</td>
</tr>
<tr>
<td>1.2</td>
<td>Most of the commonly used animal models of ischaemia</td>
<td>44</td>
</tr>
<tr>
<td>1.3</td>
<td>The effects of melatonin mediated by its membrane receptors in various physiological and pathological systems</td>
<td>57</td>
</tr>
<tr>
<td>2.1</td>
<td>List of chemicals and their sources</td>
<td>69</td>
</tr>
<tr>
<td>2.2</td>
<td>Ordinal scale used for single labelling IHC</td>
<td>83</td>
</tr>
<tr>
<td>2.3</td>
<td>The resolving gel compositions used for Western blotting</td>
<td>92</td>
</tr>
<tr>
<td>3.1</td>
<td>Body weights of animals in both treatment groups</td>
<td>106</td>
</tr>
<tr>
<td>3.2</td>
<td>Physiological parameters from blood samples</td>
<td>107</td>
</tr>
<tr>
<td>3.3</td>
<td>Statistical analysis examining the difference between melatonin and vehicle treatment groups at each slice</td>
<td>109</td>
</tr>
<tr>
<td>3.4</td>
<td>Effects of melatonin on wet organ weights after MCAO</td>
<td>110</td>
</tr>
<tr>
<td>4.1</td>
<td>The specifications and optimal concentrations of each primary antibody used in the immunohistochemical analysis</td>
<td>121</td>
</tr>
<tr>
<td>4.2</td>
<td>The specifications and optimal concentrations of each secondary antibody used in the immunohistochemical analysis</td>
<td>122</td>
</tr>
<tr>
<td>4.3</td>
<td>The immunohistochemical profile of melatonin in stroke-induced and non-intervention control brains</td>
<td>130</td>
</tr>
<tr>
<td>4.4</td>
<td>MT₁ distribution throughout the brain in neuronal and non-neuronal cells</td>
<td>133</td>
</tr>
<tr>
<td>4.5</td>
<td>MT₁ distribution throughout the brain in naïve and MCAO animals</td>
<td>136</td>
</tr>
<tr>
<td>5.1</td>
<td>Optimal dilutions of primary antibodies used for Western blot analysis</td>
<td>144</td>
</tr>
<tr>
<td>7.1</td>
<td>The ratios of ETC complexes against citrate synthase</td>
<td>182</td>
</tr>
<tr>
<td>A.1</td>
<td>List of equipment and their sources</td>
<td>279</td>
</tr>
</tbody>
</table>
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AA-NAT</td>
<td>Arylalkylamine-N-acetyltransferase</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylate cyclase</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>AFMK</td>
<td>$N^1$-acetyl-$N^2$-formyl-5-methoxykynuramine</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase activity</td>
</tr>
<tr>
<td>AMK</td>
<td>$N^1$-acetyl-5-methoxykynuramine</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variances</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ASL</td>
<td>Argininosuccinate lyase</td>
</tr>
<tr>
<td>ASS</td>
<td>Argininosuccinate synthase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BI</td>
<td>Barthel Index</td>
</tr>
<tr>
<td>BK$_{Ca}$</td>
<td>Calcium activated potassium channel</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAST</td>
<td>Chinese Acute Stroke Trial</td>
</tr>
<tr>
<td>CCA</td>
<td>Common carotid artery</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanine monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COOL AID</td>
<td>Cooling for Acute Ischaemic Brain Damage</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>Complex I assay</td>
<td>NADH-ubiquinone oxidoreductase</td>
</tr>
<tr>
<td>Complex II-III assay</td>
<td>Succinate-ubiquinone/ubiquinol-cytochrome c reductase</td>
</tr>
<tr>
<td>Complex IV assay</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>Complex V assay</td>
<td>ATP synthase</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP responsive element binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Cu</td>
<td>Redox copper centres</td>
</tr>
<tr>
<td>DAB</td>
<td>3’3’ Diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>d.f.</td>
<td>Degrees of freedom</td>
</tr>
<tr>
<td>DG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DPX</td>
<td>Di-n-butyl phthalate in xylene</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EAA</td>
<td>Excitatory amino acid</td>
</tr>
<tr>
<td>EAIS</td>
<td>Edaravone Acute Infarction Study</td>
</tr>
<tr>
<td>ECA</td>
<td>External carotid artery</td>
</tr>
<tr>
<td>ECL™</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FADH₂</td>
<td>Flavin adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>Fe-S</td>
<td>Iron-sulphur cluster</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>GABA_A</td>
<td>γ-aminobutyric acid receptor (A subtype)</td>
</tr>
<tr>
<td>Glu</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>Guanosine monophosphate</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotrophin-releasing hormone</td>
</tr>
<tr>
<td>GOS</td>
<td>Glasgow Outcome Score</td>
</tr>
<tr>
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<td>Glutamate semi-aldehyde</td>
</tr>
<tr>
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<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>HIOMT</td>
<td>Hydroxyindole-O-methyltransferase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IBMX</td>
<td>Isobutlymethlyxantine</td>
</tr>
<tr>
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<td>Internal carotid artery</td>
</tr>
<tr>
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<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
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<td>Intracerebral haemorrhages</td>
</tr>
<tr>
<td>IFN γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
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<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
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<td>Inositol phosphate</td>
</tr>
<tr>
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<td>Inositol 1,4,5 trisphosphate</td>
</tr>
<tr>
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</tr>
<tr>
<td>ISPF</td>
<td>Isonitrosopropiopheanone</td>
</tr>
<tr>
<td>Kir3</td>
<td>G protein gated K⁺ channel</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase/extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>MERCI</td>
<td>Mechanical Embolus Removal in Cerebral Ischaemia</td>
</tr>
<tr>
<td>mPTP</td>
<td>Mitochondrial permeability transition pores</td>
</tr>
<tr>
<td>mRS</td>
<td>Modified Rankin Scale</td>
</tr>
<tr>
<td>mtNOS</td>
<td>Mitochondrial nitric oxide synthase</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide dinucleotide dehydrogenase</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Nicotinamide adenine phosphate reduced form</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NIHSS</td>
<td>National Institutes of Health Stroke Scale</td>
</tr>
<tr>
<td>NINDS</td>
<td>National Institute of Neurological Disorders and Stroke</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>NZ</td>
<td>New Zealand</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>OAT</td>
<td>Ornithine aminotransferase</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>ODC</td>
<td>Ornithine decarboxylase</td>
</tr>
<tr>
<td>PARS</td>
<td>Poly (Adenine dinucleotide phosphate-ribose) synthetase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
</tr>
<tr>
<td>PEEP</td>
<td>Positive end expiratory pressure</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phosphatidylinositol 4,5-biphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipases C</td>
</tr>
<tr>
<td>PT</td>
<td>Pars tuberalis</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>ROR</td>
<td>Retinoid orphan receptors</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rt-PA</td>
<td>Recombinant tissue plasminogen activator</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>RZR</td>
<td>Retinoid Z receptors</td>
</tr>
<tr>
<td>SCN</td>
<td>Suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>sICH</td>
<td>Symptomatic intracranial haemorrhage</td>
</tr>
<tr>
<td>SS</td>
<td>Spermine synthase</td>
</tr>
<tr>
<td>SSS</td>
<td>Scandinavian Stroke Scale</td>
</tr>
<tr>
<td>STAIR</td>
<td>Stroke Therapy Academic Industry Roundtable</td>
</tr>
<tr>
<td>STAT</td>
<td>Stroke Treatment with Ancrod Trial</td>
</tr>
<tr>
<td>TBH&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N', N'-tetramethylenediamine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Th-2</td>
<td>T-helper 2</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>TTBS</td>
<td>0.1% Tween 20 in Tris buffered saline</td>
</tr>
<tr>
<td>TTC</td>
<td>2,3,5 – triphenlytetrazolium chloride</td>
</tr>
<tr>
<td>TTT</td>
<td>Time to treat</td>
</tr>
<tr>
<td>TX</td>
<td>Thromboaxane</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular adhesion molecule-1</td>
</tr>
<tr>
<td>VDCC</td>
<td>Voltage-dependent K⁺ channel</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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</tbody>
</table>
CHAPTER 1

INTRODUCTION
1.1 STROKE

1.1.1 Definition

The World Health Organisation (WHO) defines a stroke as “a clinical syndrome typified by rapidly developing signs of focal or global disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than that of vascular origin” (WHO MONICA Project Principal Investigators, 1988). This definition also includes the ischaemic damage caused by subarachnoid haemorrhage.

The two major stroke categories are haemorrhagic and ischaemic strokes. A haemorrhagic stroke results from breach of the blood brain barrier, whereby the blood constituents come in direct contact with neuronal cells. The barrier normally comprises a complex cellular system of endothelial cells, astroglia, pericytes, perivascular macrophages and a basal lamina (Bradbury, 1985). The violation of the barrier leads to disturbance of an otherwise strict homeostatic neuronal environment (de Vries et al., 1997). Further cell death is caused by lipid peroxidation and DNA degradation from haemoglobin and DNA degradation from senescent haeme release of catalytically active iron (Matz et al., 1996). Ischaemic stroke, which accounts for 80% of all strokes, arises from lack of oxygen availability to neuronal cells (Mergenthaler et al., 2004). Neuronal cells unable to cope without oxygen begin to die by necrosis, leading to a cascade of complex events resulting in infarction (Mergenthaler et al., 2004). In this thesis, the focus will be on ischaemic stroke and thus will be exclusively referred to as ‘stroke’, unless otherwise stated.

1.1.2 Epidemiology of stroke

Globally, stroke is the leading cause of morbidity and third leading cause of death. Incidence of stroke in 2002 was 20.5 million cases, eventuating in 5.5 million mortalities (World Health Organisation, 2002). The incidence of stroke is higher in males across all age groups, even though mortality is higher amongst females. Increased mortality was shown to be positively associated with several factors: patients who are elderly (> 65 years), previous history of stroke, severity of stroke, and impact on daily functioning of the patient (Anderson et al., 2004). Within New Zealand (NZ), it has been estimated that annual stroke has an incidence of 7,600 cases, with highest rates reported amongst the Maori and Pacific Island ethnicities.
(Stroke Foundation of NZ Inc., 2011). In addition, stroke incidence in NZ is higher in the elderly, with average age of first stroke onset being 75 (Bonita et al., 1993).

Furthermore, stroke has recently received increased recognition in infants as an important and common neurological disorder, with a peak in the perinatal period. It was found that neonatal stroke had an incidence of 26.4/100,000 live births per year, with rates of 17.8 for ischaemic stroke (Lynch et al., 2002). The disease outcome and long term effects are usually moderate to severe. A high proportion (two thirds) of patients with neonatal stroke have long term neurological deficits (Ashwal et al., 2001).

1.1.3 Aetiology

There are two categories of predisposing factors which increase the risk of stroke, lifestyle factors (modifiable) and non-modifiable. The factors that cannot be changed are: age greater than 55 years, gender, family history of stroke or heart attack and previous history of stroke or transient ischaemic attack. There are several modifiable factors for stroke. Hypertension is a well established risk factor (Goldberg et al., 1962), which when aggressively treated has been known to decrease incidence of stroke (Chalmers et al., 2003). On the other hand, alcohol consumption as a lifestyle factor has a complex association with stroke aetiology. There is evidence of a protective effect with light to moderate alcohol consumption, which may be achieved by increasing high density lipoprotein cholesterol levels (Thornton et al., 1983) and decreasing fibrinogen levels and platelet aggregation (Pellegrini et al., 1996). However, with excessive usage alcohol elevates the risks of stroke (Gill et al., 1986).

Various different observational studies have found cigarette smoking to be an independent risk factor for stroke (Abbott et al., 1986; Bonita et al., 1986; Colditz et al., 1988; Gorelick, 1989). Cigarette smoking accelerates atherosclerosis predisposing individuals to thrombus formation leading to ischaemic stroke (Howard et al., 1998; Wolf, 1986). High plasma cholesterol levels further promote atherosclerosis and treatment with statins has shown decreased incidence of cardiovascular events (Bucher et al., 1998; Plehn et al., 1999). Furthermore, obesity (Rexrode et al., 1997; Rodriguez et al., 2002; Walker et al., 1996) and diabetes mellitus (Goldstein et al., 2001; Mokdad et al., 2003) have been known to be independent risk factors for stroke. Lifestyle factors, such as poor diet and lack of exercise, by contributing to increased likelihood of obesity and diabetes mellitus would also increase risk of stroke (Fletcher, 1994).
Even though there are developments in treating acute stroke, the best treatment will always be prevention. This can be attained by minimising risk from modifiable factors (for reviews on prevention of stroke refer to Adams et al., 2003; Gorelick et al., 1999; Hankey et al., 1999).

1.1.4 Pathogenesis of stroke

Ischaemic stroke is mostly caused by arterial occlusion. The most common mechanism is the rupture of an atherosclerotic plaque, leading to formation of a mural thrombus (Figure 1.1). The second common cause of obstruction is cardiogenic embolus, whereby a blood clot formed in the circulation embolises to one of the cerebral arteries (for classification of ischaemic stroke, see Adams et al., 1993). In both scenarios, the occlusion is not permanent and subsequent reperfusion occurs, thereby subjecting the cells to ischaemia reperfusion (IR) injury.

Figure 1.1 Focal ischaemia caused by occlusion of a branch of the middle cerebral artery (MCA). The atherosclerotic plaque leads to thrombus formation and subsequent blockage of blood flow. The ischaemic area is highlighted by the darker contrast, however, with no differentiation between the ischaemic core and penumbra. (Reproduced and modified with permission from Stroke Foundation of NZ Inc.)
Occlusion of blood vessel leads to decreased blood flow and oxygen supply. When the blood flow drops below < 15% of normal (Nedergaard et al., 1986), necrotic cell death of neurons and glial cells occurs (Qin, 1998). This forms the core of the infarct and is the region furthest away from blood supply. The core is enclosed by an ischaemic penumbral region (Astrup et al., 1981). Here the blood flow is < 40% of normal (Back et al., 1995) and cell death may be characterized by apoptosis (programmed cell death). Increased duration of insult results in permanent damage within the penumbra (Zhao et al., 1997). Even though, the necrotic core of the infarct has incurred irreversible damage, the prolonged apoptotic process and inflammation in the penumbra, makes it salvageable (Mergenthaler et al., 2004). However, morphological studies focusing on cell death in the penumbral region have failed to find evidence of apoptosis (Garcia et al., 1997; Garcia et al., 1993). Using electron microscopy studies, the investigators found evidence of cell death by delayed necrosis and not by apoptosis. This lack of apoptotic bodies in the penumbra could be attributed to the short timespan of cell death. Conversely, pharmacological intervention studies examining cell death in the penumbra have found that blockade of apoptotic pathways has been associated with reduced neuronal damage (for review see, Yuan, 2009). Neuronal cell death in the penumbra may consequently be attributed to a combination of necrotic and apoptotic process. Thus, all putative therapeutic agents for stroke treatment are directed at preventing the progression of cell death in the penumbral region (Dirnagl et al., 1999; Ginsberg, 2003; Ovbiagele et al., 2003; Rahman et al., 2005a).

The process of necrotic cell death is mainly due to a decline in adenosine triphosphate (ATP) levels from aerobic metabolism. The cells switch to anaerobic metabolism, but are unable to accommodate the resultant lactic acid generation and are consequently exposed to an increasingly acidotic environment. Concurrently, the decrease ATP production jeopardises ATP-dependent membrane ionic channels. Failure of these pumps leads to a plethora of events. Firstly, there are changes in ionic gradients and intracellular pH. The loss of $K^+$ gradient disrupts the cellular electric membrane potential (for review, see Lipton, 1999). The pathological alterations of the cellular electric membrane potential results in a burst of action potentials from the neurons, termed anoxic depolarisation (Hansen, 1985; Szatkowski et al., 1994). The anoxic depolarization, which occurs only in the core, precipitates excessive excitatory amino acid (EAA) release such as glutamate (Barone et al., 1995; Chen et al., 1993). The glutamate transporter, coupled to the Na$^+$/K$^+$ pump, allows expulsion of glutamate and both Na$^+$ and K$^+$ to diffuse by osmosis (Figure 1.2). The increased glutamate concentration in the extracellular space activates the N-methyl-D-aspartate (NMDA) receptor, facilitating increased Ca$^{2+}$ (intracellular influx) and decreased K$^+$ (intracellular efflux; Figure
1.3). These ionic changes act as a positive feedback on the glutamate release, thereby causing further rises in extracellular glutamate levels (for overview, see Szatkowski et al., 1994). Activation of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors by increased extracellular glutamate, recruits more NMDA receptors to be active, further perpetuating the glutamate increase. In addition, intracellular Na$^+$ and Cl$^-$ concentrations are also increased. Conversely, the decrease in intracellular glutamate concentration activates metabotropic receptors (Lipton, 1999). This leads to release of Ca$^{2+}$ storage from the endoplasmic reticulum (Petito, 1979). The increase in intracellular cytosolic Ca$^{2+}$ is exacerbated by opening of the voltage sensitive Ca$^{2+}$ channels (due to pH changes and membrane depolarisation). Ca$^{2+}$ dependent enzymes, such as phospholipases and nitric oxide synthase (NOS) are consequently activated (Ciani et al., 1996; Lipton, 1999). The role of NOS, inflammation and mitochondria are discussed in later sections. Figure 1.4 illustrates the initial cascade of events due to ischaemia in the necrotic core.

Figure 1.2 The glutamate transporter reversal after anoxic depolarisation. The diffusion of ions and glutamate lead to an increase in extracellular concentration of glutamate and loss of ionic gradient (adapted from Szatkowski et al., 1994).

Figure 1.3 NMDA receptor activation leads to diffusion of Na$^+$, K$^+$ and Ca$^{2+}$ by osmosis. There are a number of factors influencing NMDA receptor activation. In cerebral ischaemia the initial cause of NMDA receptor activation is by glutamate from anoxic depolarisation (adapted from Szatkowski et al., 1994).
Furthermore, recent evidence suggests that mechanism of necrosis is more complicated than previously thought. Necroptosis is defined as programmed cell death via necrosis (for reviews, see Vandenabeele et al., 2010; Yuan, 2009). This pathway is caspase independent and involves stimulation of death receptors. Stimulation of these receptors lead to activation of death domain containing kinases present within receptor interacting protein-1 (Degterev et al., 2008; Holler et al., 2000). This kinase activation may in turn lead to necrotic damage of mitochondria and other cellular organelles.

Tissue injury is further exacerbated with reperfusion. The morphological studies show immediate neuronal necrosis in the core followed by delayed cell death in the penumbral region. This process is different to permanent ischaemia without reperfusion (Garcia et al., 1997). The majority of the structural damage may occur at reperfusion (Carden et al., 2000) with an influx of oxygen radicals as well as activated leukocytes (Jenkins et al., 1981; Kumar et al., 1987). Reperfusion into the ischaemic core leads to increased free fatty acid metabolism and superoxide formation (Krause et al., 1988). There is release of iron from storage proteins, mediated by superoxide (Krause et al., 1987). Reduction of ferric iron (in ferritin and transferrin) results in formation of ferrous iron. This acts as a catalyst for lipid peroxidation chain reactions. Nitric oxide (NO) formation (see Section 1.3.2), is also involved leading to generation of peroxynitrite and subsequent lipid peroxidation and protein nitrosylation (Tanaka et al., 1997). The lipid peroxidation process has been shown to be sustained for up to 72 hours after reperfusion, resulting in cellular membrane damage and subsequent cell death (Bromont et al., 1989). This pathology is compounded by inhibition of lipid repair enzymes after reperfusion (Das et al., 1986). Decreased Ca\(^{2+}\) in endoplasmic reticulum and inhibition of eukaryotic initiation factors, required for translation of proteins during ischaemia results in the subsequent inhibition of protein synthesis (for review see, White et al., 2000). This inhibition of protein synthesis is absent in ischaemia without reperfusion (Nowak et al., 1985). The penumbral region characterised by the inhibition of protein synthesis occurring prior to ATP depletion can be predicted to demarcate the extent of infarction (Hata et al., 2000). Even after the rapid recovery of ATP production (due to reavailability of oxygen from reperfusion) there are still lower protein levels, indicating cellular energy may be used to fuel biochemical processes which maintain inhibition of protein synthesis (Cooper et al., 1977). Conversely, the availability of ATP in the penumbra after reperfusion may fuel the delayed neuronal death process (Sims et al., 2010). Since, the oxidative stress is greater following reperfusion, there is greater mitochondrial damage and subsequent activation of apoptotic signalling pathways (for review, see Sims et al., 2010). For example, activation of caspase-dependent apoptosis plays a significant role in the evolution of the infarct following reperfusion (see Section 1.4.3).
Inhibition of caspase activity in transient ischaemia has provided neuroprotection, but failed to do so in permanent ischaemia studies (Endres et al., 1998; Gill et al., 2002). This implies that processes involved in delayed neuronal death are different after reperfusion of the ischaemic brain (for review on ischaemia reperfusion injury, see Carden et al., 2000; White et al., 2000).

The substantial ionic changes in the core of the infarct result in a loss of cellular homeostasis. Influxes of Na\(^+\) and Cl\(^-\) via osmosis lead to increased cellular volume due to higher water content. Together with damage from reperfusion, the cellular membrane is unable to contain the cellular oedema, resulting in osmotic lysis (Dirnagl et al., 1999; Mergenthaler et al., 2004). The decrease in ATP production, cell death in the necrotic core and the excess free radical production causes activation of inflammation (Figure 1.5, Han et al., 2003; Kroemer et al., 1998; Walton et al., 1998).

Concurrently to excitotoxic depolarisations in the necrotic core, a phenomenon known as peri-infarct depolarization occurs in the penumbra (for overview, see Mergenthaler et al., 2004). Briefly, the excess glutamate from the necrotic core diffuses to the penumbra, setting off a chain of depolarisations of neurons. This process is more marked toward the centre of the core where the concentration of glutamate is higher (Back et al., 1996; Back et al., 1994; Nedergaard et al., 1993). It also results in release of glutamate and potassium ions from neurons in the penumbral region, thereby causing a series of depolarisations. Peri-infarct depolarisations have been confirmed by in vivo observations following cerebral ischaemia (Busch et al., 1996; Wolf et al., 1997). Cells in the penumbral region are already under mild oxidative stress and have compromised oxygen availability. The repolarisation of neurons after each depolarization is an energy-dependent process, resulting in exacerbation of stress on these cells. Hence, the growth of the infarct beyond the necrotic core is partly driven by the peri-infarct depolarization. (Figure 1.6, Back et al., 1996; Back et al., 1994; Fabricius et al., 2006; Mergenthaler et al., 2004; Mies et al., 1993).

The excitotoxicity damage is the first step of the pathogenesis of stroke, present in the necrotic core. This is followed immediately by peri-infarct depolarisations. Other processes such as inflammation and apoptosis occur more slowly but over a greater length of time (Figure 1.6, Dirnagl et al., 1999). Thus, the loss of non-ischaemic penumbra occurs over a period of days. Inflammation and apoptosis is discussed thoroughly in the following sections.
Figure 1.4 The complex primary excitotoxic cascade in the acute phase of cerebral ischaemia (adapted from Ovbiagele et al., 2003).
Chapter 1: Introduction

Figure 1.5 An overview of the inflammation cascade following IR. The vicious cycle progresses further with time corresponding to increased tissue damage (adapted from Han et al., 2003).

Figure 1.6 Representation of the cascade and relative time-line of detrimental events occurring in cerebral IR injury. The primary and most damaging event is the excitotoxicity, which results in the majority of cell death, and it triggers the secondary damage. Tissue damage in the penumbra is attributed to peri-infarct depolarisations, inflammation and apoptosis. The ordinate-axis gives a relative impact of each factor on the eventual outcome and the evolution of the mechanisms of damage over time are represented along the abscissa (Adapted from Dirnagl et al., 1999).
1.2 INFLAMMATION AND STROKE

1.2.1 Inflammation in Stroke

Initially, it was generally thought that the inflammation associated with cerebral ischaemia was a normal response to cellular damage and did not affect the evolution of tissue injury (Tan et al., 2003b). This is now known not to be the case, as a reduction in pro-inflammatory cytokines such as tumour necrosis factor α (TNFα) and interleukin-6 (IL-6) levels post-ischaemic insult is correlated with improved outcomes in in vivo animal and clinical studies (Clarkson et al., 2004b; Dirnagl et al., 1999; Jeong et al., 2002; Jordan et al., 2008; Lakhan et al., 2009; Suzuki et al., 1999). This finding is further supported by the fact that upregulation of these very same inflammatory markers, including a sustained inflammatory response monitored by C-reactive protein and white blood cell levels is correlated with increased mortality and neurological deterioration (Di Napoli, 2001; Kazmierski et al., 2004; Vila et al., 2000). In contrast to the ischaemic cascade, the inflammatory response to neural tissue injury has been shown in vivo to be initiated as early as 1 hour post-onset of ischaemia (Jander et al., 2000). The inflammation persists with an upregulation of inflammatory mediators [e.g. cyclooxygenase-II (COX-II) and inducible nitric oxide synthase (iNOS)] up to 3 months post-ischaemic insult in the brain (see Section 1.3, Clarkson et al., 2005; Iadecola et al., 2001a). Supporting evidence has already begun to accumulate as to the role of inflammation in ischaemia induced neurodegeneration (for reviews see Becker, 2001; Danton et al., 2003; del Zoppo et al., 2000), and there is a host of experimental drugs with anti-inflammatory capabilities that have been recently developed and have clinical potential (Barone et al., 1999; Khan et al., 2005; Liao et al., 2004; Meller et al., 2005; Sironi et al., 2005; Watanabe et al., 2004). As the inflammatory reaction occurs over a long period (up to months), it seems a logical progression to alter our focus on therapeutic interventions to anti-inflammatory mediators, as it is more realistic that they could be administered clinically within their therapeutic time window and ameliorate neuronal injury. The neural inflammatory reaction post-stroke, including the role of cytokines and chemokines, has previously been reviewed (see Section 1.2.3, Clarkson et al., 2004b; Han et al., 2003; Jordan et al., 2008; Lakhan et al., 2009; Tan et al., 2003b).

The inflammation may also exacerbate tissue damage by increasing local metabolic demand, which in turn increases local temperature. The increase in temperature further activates the inflammatory process, but more critically, alterations in cerebral temperature have been shown
to profoundly affect the outcome of focal cerebral ischaemia in laboratory animals (Xue et al., 1992) and patients (Reith et al., 1996). Hyperthermia exacerbates ischaemic neuronal injury and physiological dysfunction (Chopp et al., 1992), whereas mild to moderate decrements in brain temperature have been shown to decrease post-ischaemic neuronal necrosis (Ginsberg et al., 1998). In an attempt to isolate and treat this inflammation related symptom, a clinical trial inducing cerebral hypothermia was initiated (Krieger et al., 2001). However, the results were inconclusive due to patients developing complications such as pneumonia, bradycardia and infections. Meta-analysis examining hypothermia in acute stroke found no significant neuroprotection and even though efficacious in cardiac arrest, it is not recommended for use in acute stroke management (Adams et al., 2007; Correia et al., 2000). Currently, there are efforts underway to conduct a larger Phase III trial (Macleod et al., 2010). Future trials may be successful if treatment is limited to lowering cerebral temperature as opposed to core body temperature, and to younger (< 60) patients who are less likely to develop co-morbid conditions. To this end, inflammation is a detrimental process that can be targeted by therapeutic interventions.

1.2.2 The possible beneficial role of inflammation in stroke

The inflammatory process is a complex pathophysiological process involving different cells, enzymes and various mediators such as cytokines. Although, it is generally accepted that inflammation has been harmful in progenerating the tissue injury, there is evidence that it may be beneficial in stroke.

It has been argued that extremely high doses of putative neuroprotectants (with anti-inflammatory properties) are required to target inflammatory response post-stroke to have an effect. Furthermore, inhibiting cytokines may not be always desirable (Feuerstein et al., 2001). Inflammatory cells, such as macrophages and T lymphocytes are beneficial in models of neuronal damage (Moulem et al., 1999; Rapalino et al., 1998). Inhibition of pro-inflammatory cytokines such TNFα, IL-1β, and IL-6 have been detrimental in stroke (Bruce et al., 1996; Ohtsuki et al., 1996; Scherbel et al., 1999; Stahel et al., 2000; Tasaki et al., 1997). Thus, inflammation in stroke may be having some beneficial effects yet to be fully understood. Hence, despite a minority of reports suggesting a possible beneficial effect of inflammation, overwhelming evidence exists of the detrimental role of inflammation in stroke.
Chapter 1: Introduction

1.2.3 Cytokines and chemokines as mediators of inflammation

Production of oxidative stress and increased Ca$^{2+}$ from cerebral IR cause release of pro-inflammatory cytokines from immune cells as well as neuronal and glial cells (Lakhan et al., 2009). Cytokines are chemicals, which facilitate communication between the immune cells, while chemokines are stimulatory messengers from the immune cells directed towards other cells. These inflammatory mediators lead to recruitment of inflammatory cells into the infarct. The role of each cell type varies according to time course since cerebral IR. Thus, the recruitment of inflammatory cells triggers both the innate and adaptive immune responses. Some of the cells such as T helper cells are beneficial in production of anti-inflammatory cytokines (for review, see Jin et al., 2010). Both cytokines and chemokines are pivotal in directing the inflammatory process following stroke (for review, see Clarkson et al., 2004b; Jordan et al., 2008; Lakhan et al., 2009). Inhibiting the chemokine response with a broad non-specific chemokine receptor antagonist, such as NR58–3.14.3 and viral macrophage inflammatory protein-II proved to be beneficial in stroke models (Beech et al., 2001; Minami et al., 2003). The balance of pro-inflammatory and anti-inflammatory cytokines is shifted toward promotion of inflammation in stroke, as it would in any other inflammatory pathology (for an overview on the role of cytokines in stroke, see Kim, 1996). The majority of the experimental and clinical studies indicate that suppressing the inflammatory response, via modulation of cytokines, is favourable in stroke (Clarkson et al., 2004b, Jordan et al., 2008; Lakhan et al., 2009).

1.3 INDUCIBLE ENZYMES IN INFLAMMATION POST STROKE

1.3.1 L-arginine metabolism

The major amino acid of a healthy adult diet is L-arginine (reviewed by Raghavan et al., 2004). Physiologically, there are many roles of L-arginine, including that of a pivotal carrier of nitrogen in the urea cycle (Braissant et al., 1999). Astroglial cells in the brain sequester L-arginine and store it for potential use by surrounding cells. The astroglial cells may release large amounts of L-arginine following a stimulus such as cerebral IR injury (for overview see Wiesinger, 2001). Furthermore, in inflammation, it is the most common amino acid used
(Gartner et al., 1991). This makes L-arginine metabolism an important contributor to inflammation post stroke, and thus it is critical to analyse its metabolites.

The substrate, L-arginine, can be metabolised by 3 enzymes, namely NOS, arginase and arginine decarboxylase, resulting in a wide array of products such as polyamines (spermine, spermidine, putrescine), NO, glutamate and agmatine (Figure 1.7). NOS and arginase are the major enzymes shown so far to be involved in inflammation. The NOS enzymes have a 1000 fold higher binding capacity for L-arginine than arginase. The $K_m$ (Michaelis-Menton constant) for NOS and arginase was found to be 2-20µM and 2-20 mM, respectively (Buga et al., 1996; Griffith et al., 1995; Grody et al., 1987; Stuehr et al., 1991). This implies that if both enzymes were co-expressed at the same levels in the same cellular compartment, i.e. competing for same substrate pool, the NOS enzymes would be favoured and predominant. Hence, modulation of the NOS enzyme, as opposed to arginase, should result in greater effects on the overall outcome of inflammation.

**Figure 1.7** The metabolism of arginine by two major metabolic pathways, NOS and arginase, leading to different outcomes on the overall ischaemic damage. Most NOS (apart from eNOS) isoforms exacerbate damage via NO production. Production of agmatine by arginine decarboxylase is not shown for simplicity. ASS – Argininosuccinate synthase, ASL – argininosuccinate lyase, Glu – glutamate, Gsa – glutamate semi-aldehyde, NADP – nicotinamide adenine phosphate, NADPH – nicotinamide adenine phosphate reduced form, NOS – nitric oxide synthase, ODC – ornithine decarboxylase, OAT – ornithine aminotransferase, Pc – pyrroline 5 carboxylate, SS – spermine synthase, $TBH_4$ - tetrahydrobiopterin (adapted from King et al., 2004; Li et al., 2001).
1.3.2 Contribution of NOS enzyme isoforms

The NOS enzymes (EC 1.14.13.39) metabolise L-arginine into NO and L-citrulline. The enzymes are the major source of NO, though not the only source (Lundberg et al., 2010). NO can be produced in neutrophils via anti-neutrophil cytoplasm antibodies (Braissant et al., 1999). Reduction of nitrite and nitrate under ischaemic conditions can also result in NO production (for review, see Lundburg et al., 2010). NO has a plethora of physiological and pathological actions largely determined by its concentration and the isoform of NOS by which it is produced (Vannucchi et al., 2005). NO can have varying effects on the same organelle depending on concentration. For example, NO may stimulate mitochondrial energetic at low concentrations, whilst at higher concentration it may be disruptive (for review see Poderoso, 2009). Thus, in low concentrations, NO tends to have physiological functions such as maintaining vascular homeostasis (for review on physiological role of NO, see Guix et al., 2005). Conversely, higher concentrations are prevalent in inflammation, whereby NO reacts with superoxide to form peroxynitrite. Peroxynitrite levels have been found to be elevated in stroke patients compared with normal individuals (Taffi et al., 2008). This cytotoxic free radical causes widespread damage to cellular enzymes, lipid membranes and DNA (for overviews see Iadecola, 1997; Moro et al., 2004; Murphy, 2000).

There are 4 major isoforms of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS), mitochondrial NOS (mtNOS) and iNOS (reviewed in Guix et al., 2005). All of the isoforms except iNOS are constitutively expressed and therefore have physiological functions. NO produced by iNOS has a pathophysiological role and for example, is useful in neutralizing invading bacteria (for review, see Guix et al., 2005). After ischaemia, iNOS was localized to neurons, astrocytes, endothelial cells and neutrophils (Endoh et al., 1994; Iadecola et al., 1996; Iadecola et al., 1995; Moro et al., 1998; Wallace et al., 1994). In humans the expression was limited to cells within the lesion (Forster et al., 1999). iNOS is also the only isoform which produces NO independent of Ca\(^{2+}\)/calmodulin, and is normally induced by pro-inflammatory cytokines, such as interferon-\(\gamma\) (IFN \(\gamma\)), TNFa, and IL-1\(\beta\) (for reviews see Iadecola, 1997; Moro et al., 2004). These cytokines have been shown to increase following IR injury (see Section 1.2.1). The enzyme may also be induced by nuclear factor kappa B (NF-kB, (Xie et al., 1994), which is upregulated in ischaemia (see Section 1.1.4). Increased glutamate levels may be able to induce iNOS expression via augmenting TNFa and NF-kB levels (Guerrini et al., 1995; Hurtado et al., 2001; Kaltschmidt et al., 1995). The increased iNOS mRNA, protein expression and consequent enzyme activity have been shown in models of transient and permanent ischaemia (Grandati et al., 1997; Iadecola et al., 1995). In addition, iNOS can...
produce larger quantities of NO compared with constitutive isoforms (Raghavan et al., 2004; Suzuki et al., 2002). Apart from peroxynitrite formation, NO produced by iNOS can result in a variety of tissue damage, such as mitochondrial dysfunction (see Section 1.4) and vasoparalysis (for reviews see Iadecola, 1997; Moro et al., 2004). Since iNOS seems to be predominantly detrimental to tissue injury, inhibiting iNOS should be beneficial. This was supported by neuroprotection conferred by iNOS inhibitors in experimental models (De La Cruz et al., 2004; Kidd et al., 2005; Moro et al., 2000; Park et al., 2004; Pereira et al., 2005). On the other hand, a recent study using transgenic mice without iNOS gene showed no difference in infarct volumes after transient MCAO (Pruss et al., 2008). Thus, the iNOS isoform may be a major contributor to inflammatory damage following cerebral IR (Figure 1.8).

The nNOS isoform is exclusively expressed by neurons, and the NO produced by nNOS may be physiologically used as a neurotransmitter and synaptic plasticity (for review, see Guix et al., 2005). There are five splice variants of nNOS, of which nNOSα and nNOSβ are predominant in the brain (Corso-Diaz et al., 2010). The constitutive isoform functions after calmodulin binds to the enzyme in presence of Ca$^{2+}$. Thus, the rise in intracellular Ca$^{2+}$ levels during ischaemia would stimulate nNOS enzyme activity (Guix et al., 2005; Moro et al., 2004). Moreover, it has been postulated that the excess level of NO is from the increased stimulation of nNOS due to the EAA, such as glutamate (Dawson et al., 1991; Nowicki et al., 1991). However, nNOS activity has been shown to be down regulated after an ischemic event (De Alba et al., 1999; Iadecola et al., 1995). The nNOS activity initially escalates from 10 minutes post ischaemia and declines to normal levels at 60 minutes (Kader et al., 1993). This may be either due to the inhibition of protein synthesis (as explained in Section 1.1.4), or a direct inhibition effect of the excessive NO. There is evidence that NO directly inhibits NOS activity, in a negative feedback mechanism (Griscavage et al., 1993; Rogers et al., 1992). Even though the nNOS levels may return to baseline an hour after the IR injury, it still contributes NO to the pathogenesis. This detrimental effect of nNOS was confirmed with transgenic mice lacking the gene, showing reduced cerebral damage in permanent (Ferriero et al., 1996) and transient (Huang et al., 1994) models of ischaemia. Furthermore, selective nNOS inhibitors were found to be neuroprotective in experimental models (Chabrier et al., 1999; Goyagi et al., 2001; O'Neill et al., 2000; Yoshida et al., 1994). The NO-derived from nNOS appears to be neurotoxic and contributes toward the inflammatory damage (Figure 1.8).

The only isoform expressed in endothelial cells is eNOS, which is exclusive to this cell type (Knowles et al., 1994). NO production from eNOS has been shown to prevent atherosclerosis
Chapter 1: Introduction

(for review, see Toda et al., 2009). eNOS, similar to nNOS, is upregulated by greater intracellular concentrations of Ca\(^{2+}\). The upregulation of eNOS activity during initial stages of ischaemia occurs prior to induction of nNOS activity (Nagafuji et al., 1994; Zhang et al., 1993). This may be a defence mechanism for lack of blood supply. NO derived from eNOS relaxes the vascular smooth muscle, causing vasodilatation and increased blood flow (reviewed by Guix et al., 2005; Moro et al., 2004). This implies that the role of eNOS is beneficial by maintaining tissue perfusion (Huang et al., 1994). Various types of evidence support this theory. Firstly, administration of nitrite protected the brain against IR injury via vasodilation (Jung et al., 2006). Secondly, eNOS-lacking transgenic mice were more prone to tissue damage following cerebral IR (Huang et al., 1996). Moreover, increasing the eNOS activity by L-arginine administration in first 30 minutes was neuroprotective (Morikawa et al., 1994). Replicating this effect of eNOS by NO donors (given soon after induction of ischaemia) resulted in better cerebral perfusion and decreased infarct sizes (Salom et al., 2000; Zhang et al., 1994). Conversely, eNOS inhibition can lead to worsened outcomes (de la Torre et al., 2005). Furthermore, therapeutic agents such as statins (cholesterol lowering drugs), exhibited neuroprotection via selectively up regulating eNOS (for review on eNOS in stroke, see Endres et al., 2004). Interestingly, NO donors can be beneficial even without affecting cerebral blood flow (for review, see Toda et al., 2009). This may be attributed to angiogenesis effects of eNOS. Recently, it was shown that eNOS inhibition led to decreased rehabilitative outcome after MCAO, due to lack of angiogenesis long term (Gertz et al., 2006). Therefore, eNOS (unlike the other isoforms) confers neuroprotection (Figure 1.8).

The existence of a fourth isoform, mtNOS, is controversial. Of all the isoforms, this was most recently identified and isolated (Ghafourifar et al., 1997; Tatoyan et al., 1998). However, new data disputes the existence of mtNOS, as no isoform of NOS was isolated from heart mitochondria in humans (Csordas et al., 2007). Furthermore, investigators have indicated that mtNOS may be a post translational variant of nNOS\(\alpha\), thus disputing the notion of a fourth isoform (for review, see Poderoso et al., 2009). Physiologically, mtNOS-derived NO may modulate mitochondrial respiration, mitochondrial membrane potentials and act as a signal for mitochondrial proliferation (Dedkova et al., 2004; Kanai et al., 2001; Lopez-Figueroa et al., 2000; Navarro et al., 2005). Mitochondrial NO may also play a part in mitochondrial Ca\(^{2+}\) uptake thus leading to mitochondrial-linked apoptosis (Dedkova et al., 2004; Navarro et al., 2005).
Figure 1.8 The paradoxical role of NO in the cerebral IR. NO produced by eNOS stimulation leads to neuroprotection via vasodilation. However, NO produced by iNOS and nNOS activation is neurodegenerative. The putative effect of mtNOS contribution is not shown (adapted from Moro et al, 2004).

The mtNOS isoform has been indicated to be both constitutive and inducible (Lopez et al., 2006; Zanella et al., 2004). The role of mtNOS in inflammation is unclear. Lopez et al. (2006) found mtNOS activity to be induced in a model of inflammation, in mice expressing iNOS. As expected, mtNOS contributed to mitochondrial respiratory dysfunction. Surprisingly, without iNOS expression in mice, the mtNOS activity remained unchanged (Lopez et al., 2006). These studies show the complexity of the role of mtNOS. The full mechanism of action of mtNOS, if it exists, in inflammation is far from being fully elucidated.
The role of NO in inflammation post stroke can be both detrimental and beneficial (Figure 1.8), depending on the source of NO and concentration of NO. Both iNOS and nNOS derived NO have detrimental effects on tissue injury mainly through formation of peroxynitrite. An alternate theory has been suggested that peroxynitrite may be beneficial by nitrosylating apoptotic proteins (for review, see Calabrese et al., 2007). However, there is overwhelming evidence for the contrary. eNOS confers neuroprotection by promoting vasodilation and cerebral perfusion acutely, and angiogenesis long term (for reviews on roles of NOS isoforms in stroke, see Guix et al., 2005; Iadecola, 1997; Moro et al., 2004). Promoting eNOS whilst inhibiting iNOS and nNOS would be ideal. This has been shown with studies that found selective inhibition of iNOS and nNOS to be neuroprotective, but non-selective NOS inhibitors were not effective (reviewed by Willmot et al., 2005). Currently, the role of mtNOS in inflammation is not yet clear.

1.3.3 Contribution of arginase enzymes

Arginase (EC 3.5.3.1), also known as L-arginine hydrolase, metabolises L-arginine to urea and L-ornithine (Figure 1.7). L-ornithine is then further metabolised to glutamate, proline and the polyamines: spermine, spermidine and putrescine (for overview see Meijer et al., 1990; Wu et al., 1998). There are two isoforms of arginase, namely arginase I and arginase II, with arginase I being mainly expressed in the liver (reviewed by Jenkinson et al., 1996; Wiesinger, 2001), and the predominant form in the rodent brain (Yu et al., 2001). Within the brain, high protein expression of arginase I was found within regions of the cerebral cortex, midbrain, pons, cerebellum and medulla (Yu et al., 2001). Arginase I is cystolic and abundant in the liver as part of the urea cycle. Arginase II is predominantly located in the mitochondrial matrix (for reviews on arginase isoforms, see Jenkinson et al., 1996; Wiesinger, 2001; Wu et al., 1998), and has been localised in various tissues including brain (Vockley et al., 1996; Yu et al., 2001). The expression of arginase isoforms is not mutually exclusive in the same cell type, as individual cells have been known to express both isoforms (Buga et al., 1996; Louis et al., 1998; Morris et al., 1998).

In experimental models of inflammation, it is debatable which arginase isoform is predominant. Previously, in activated macrophages arginase II was found to be predominant (Wang et al., 1995). Conversely, in wound derived fibroblasts arginase I expression was induced without detection of arginase II (Witte et al., 2002). More recently, researchers using an in vivo model of inflammation found an upregulation of arginase I. This model highlighted
potential involvement arginase I in T-cell mediated responses (Xu et al., 2003). Regardless of which isoform is predominant, the induction of both arginase I and II seems to be beneficial in inflammation post stroke, for two major reasons. Firstly, induction of arginase leads to increased substrate availability for polyamine production. Polyamines may play a significant part in inflammation (see Section 1.3.4).

As both NOS and arginase use a common substrate, they are intrinsically linked and inhibitors of one enzyme would be indirectly inhibiting the other in the same cell. This theory has been supported by various experimental in vitro and in vivo models (for review, see Munder, 2009). In macrophages arginase induction occurred with NOS inhibitors, interleukin-4 (IL-4), IL-10, and prostaglandin E \(_2\) (Corraliza et al., 1995), and transforming growth factor-\(\beta\) (Boutard et al., 1995). Similarly, upregulation of NOS activity occurs with inhibition of arginase by alpha-amino acid N (omega)-hydroxy-nor-1-arginine (Tenu et al., 1999) and IFN \(\gamma\) (Hesse et al., 2001). The findings of these experimental models are consistent with clinical findings. In a physiological state of sexual arousal, an increase in NOS enzymes corresponded with lower arginase activity (Christianson, 2005). Likewise, in asthma, NOS and arginase activities were inversely correlated (Maarsingh et al., 2005). Arginase expression has been associated with the Th2 inflammatory response whilst Th1 response induces iNOS (for review see, Munder, 2009). Recently, there is evidence to suggest that in a model of inflammation (parasitic infection with \(S.\) mansoni) arginase I deletion led to decreased Th2 response (Herbert et al., 2010; Pesce et al., 2009). This suggests a complex role of arginase in inflammatory responses which seem to be pathology specific. The role of NOS is mainly detrimental in inflammation post stroke (as discussed in Section 1.3.2), and thus competitive inhibition by arginase is neuroprotective.

The arginase isoforms are pivotal in the inflammation following stroke, by their interactions with NOS enzyme system and the production of polyamines.

### 1.3.4 The role of polyamines in stroke

Polyamines are synthesized from \(L\)-ornithine by ODC. Each of the polyamines can also be converted to another by enzyme driven reactions, and metabolised into physiologically active compounds (for review on polyamine metabolism, see Kim et al., 2009). Polyamines play a host of physiological roles, including regulation of mitochondrial Ca\(^{2+}\) homeostasis, mitochondrial membrane stability, ion channel functions, cellular signalling and proliferation.
(for overview on physiological roles of polyamines, see Igarashi et al., 2000). However, unlike arginase distribution in the brain, the spermine/spermidine like immunoreactivity is very selectively localized. It is generally higher in the hypothalamus and brainstem regions (Laube et al., 2002). The polyamines have been implicated in IR injury (Kleihues et al., 1975), and animal models of inflammation (Bird et al., 1983). Generally, polyamines have been known to be direct anti-oxidants and free radical scavengers (Chattopadhyay et al., 2003; Farbiszewski et al., 1996; Ha et al., 1998; Lovaas et al., 1991; Schuber, 1989). This property would allow them to protect against reactive oxygen species (ROS) induced damage to cells following inflammation. In addition polyamines exert anti-inflammatory properties (Lovaas et al., 1991), partly through inhibition of NOS (Hu et al., 1994; Kapoor et al., 2005a). Thus, polyamines should be generally neuroprotective, given the abovementioned properties. This is confirmed in mice with over expression of ODC, whereby polyamine production is higher, and neuroprotection present after middle cerebral artery occlusion (Lukkarinen et al., 1999; Lukkarinen et al., 1998). Administration of polyamines is neuroprotective in in vitro and in vivo models of IR (Abe et al., 1993; Clarkson et al., 2004a; Farbiszewski et al., 1996; Farbiszewski et al., 1995; Ferchmin et al., 2000; Gilad et al., 1991).

However, the role of polyamines in inflammation post stroke is much more complicated. Inhibition of ODC, which should be harmful in MCAO, also led to neuroprotection (Baskaya et al., 1996b; Muszynski et al., 1993; Sparapani et al., 1997). Furthermore, elevated levels of polyamines were associated with greater neuronal tissue injury (Baskaya et al., 1996a; Dogan et al., 1999; Henley et al., 1997; Rao et al., 1995; Rao et al., 2000). Upon closer examination, it appears that putrescine (the precursor polyamine for spermidine and spermine), promotes cellular damage from cerebral IR (Paschen et al., 1988). After cerebral IR, increased ODC activity led to increased putrescine levels (and lower spermine/spermidine levels) correlating to worsened outcome (Kim et al., 2009). Putrescine is implicated in exacerbating excitatory damage, blood brain barrier dysfunction, resulting in vasogenic oedema (Lee et al., 2003; Rao et al., 1995; Rao et al., 2000). The effect on oedema formation post stroke is in contraindication to the general property of polyamines. Polyamines were previously shown to suppress oedema formation via increasing vasoregulin synthesis (Oyanagui, 1984). Moreover polyamine antagonists blocking the actions of putrescine has been shown to be effective against neuronal damage after IR (for review, see Li et al., 2007). However, spermine levels have been shown to alter following cerebral IR injury (Baskaya et al., 1997; Koenig et al., 1990; Paschen et al., 1991; Paschen et al., 1992). Furthermore, spermine (and not spermidine and putrescine) administration in vivo led to neuroprotection in cerebral IR (Clarkson et al., 2004a). The beneficial effects were present via a multitude of actions. Spermine suppressed
the induced arginase activity, and most likely increased eNOS activity. Mitochondrial energetics (respiratory chain enzymes and complexes) and the mitochondrial membrane integrity were preserved following the insult (Clarkson et al., 2004a). Further anti-inflammatory properties of spermine and spermidine may be due to their ability to suppress lymphocyte proliferation (Theoharides, 1980). Regardless of these advantageous effects, high concentrations of spermine have been demonstrated to be neurotoxic (Webber et al., 1980). The neuroprotective effects of spermine only appear to be present at lower concentrations, i.e. <50nM (Munir et al., 1993).

In inflammation post stroke, putrescine seems detrimental while spermine, in lower concentrations, is neuroprotective. Spermidine, although able to suppress lymphocyte proliferation, was not advantageous. Thus, the role of polyamines in stroke appears to be complex. For example, the enzymes involved in polyamine metabolism have been recently shown to be regulated by inflammatory mediators, such as cytokines (for review on role of polyamine catabolism in inflammation, see Babbar et al., 2007).

1.3.5 Contribution of agmatine

Arginine decarboxylase, a mitochondrial bound enzyme, produces agmatine and CO₂. Agmatine has been proposed to be a modulator of both arginase and NOS enzyme systems depending on the physiological state of the cell (for review see Satriano, 2004). Arginine decarboxylase has been found to be most abundant in liver and kidney (Lortie et al., 1996; Morrissey et al., 1995). Under physiological conditions, agmatine induces antizyme (Satriano et al., 1998), thus inhibiting polyamine biosynthesis via degradation of ODC (Mitchell et al., 1994; Suzuki et al., 1994). Agmatine increased spermine/spermidine N-acyltransferase activity (involved in converting spermine back to putrescine). Since spermine is much more readily exported out of the cell than putrescine, the intracellular concentration of this polyamine is thus decreased (Vargiu et al., 1999). As explained in Section 1.3.4, polyamines are vital for cellular proliferation, and agmatine would thus be inhibiting this effect. Agmatine administration suppressed ODC activity (via antizyme induction) and decreased cellular proliferation (Eto et al., 2006; Satriano et al., 1998). These effects have been proposed to prevent abnormal cellular proliferation (Satriano, 2004).

In the context of inflammation, it was originally thought that agmatine was a direct inducer of NOS, causing vasodilation (Gao et al., 1995; Ishikawa et al., 1995; Lortie et al., 1996).
However, it was also found agmatine inhibited NOS (Auguet et al., 1995; Feng et al., 2002; Galea et al., 1996). These contrasting results were highly dependent on the experimental conditions used (overviewed in Satriano, 2004). It was later found that a metabolite of agmatine, agmatine aldehyde (guanidinobutyraldehyde), inhibits NOS (Satriano et al., 2001). Agmatine aldehyde is metabolised from agmatine by amine oxidases, such as diamine oxidase (Holt et al., 1995). Satriano (2004) proposed that during inflammation, there is an induction of such an enzyme. This would inhibit the NOS enzyme system, and since there is less agmatine available (after metabolism), promote arginase enzyme system (for review see Satriano, 2004). An in vitro model of inflammation showed similar effects of agmatine when compared with iNOS inhibition in other studies (Satriano, 2004; Satriano et al., 2001; Schwartz et al., 1997). Thus, it seems that agmatine would be anti-inflammatory, and so neuroprotective. This hypothesis is supported by neuroprotective effects of agmatine in in vivo models of neuronal injury involving ischaemia and subsequent inflammation (Fairbanks et al., 2000; Feng et al., 2002; Gilad et al., 2000; Kim et al., 2006; Kim et al., 2004; Yu et al., 2000). Kim et al., (2006) found administration of agmatine at 100 mg/kg i.v. following MCAO to be neuroprotective. There was decreased oedema and lower iNOS expression, leading to less neuronal cell death when agmatine was given after MCAO (Kim et al., 2010; Wang et al., 2010). Therefore, it appears that agmatine, as modulator of L-arginine metabolism, may be beneficial in inflammation post-stroke.

1.3.6  Arachidonic acid metabolism

Another essential amino acid that is pivotal in inflammation is arachidonic acid (AA). AA is bound mostly to phospholipids in the cell membrane (Anggard et al., 1965). Cell injury, for example from cerebral ischaemia, leading to a release of phospholipids from cell membrane leading to activation of phospholipase A$_2$, generating AA (Piper et al., 1971). In addition, cerebral ischaemia results in influx of intracellular Ca$^{2+}$ (as discussed in Section 1.1.4), resulting in activation of phospholipases, thus increasing the availability of AA (Chan et al., 1985; Hirabayashi et al., 2004; Mattson, 1998). The free AA is further metabolized into eicosanoids by three enzyme systems, namely: COX, lipoxygenase enzymes and P450 epoxygenase enzymes. The COX enzyme produces PGs and thromboxanes (TXs) whilst generating superoxide. PGs include PGE$_2$, PGD$_2$, PGF$_{2\alpha}$ and PGI$_2$, whereas TXA$_2$ and functional antagonist of TXA$_2$ are major TX products (Figure 1.9, for review see Appleton et al., 1996).
Figure 1.9 Effect on AA metabolism after cerebral IR injury with a focus on PGE<sub>2</sub> production. ROS from cerebral IR damages the cellular membrane, concurrently increased intracellular Ca<sup>2+</sup> and cytokines lead to activation of phospholipases. Cytokines also induce COX enzyme. For simplification, only COX pathway is shown, minor enzymes are deleted and receptors for other PGs and TXs not shown (adapted from Appleton et al., 1996).

1.3.7 Contribution of COX enzymes

There are two well known isoforms of COX (EC 1.14.99.1) in the brain, COX-I and COX-II (Deininger et al., 2000) while a third form COX-3 is disputed (Chandrasekharan et al., 2002; Dinchuk et al., 2003). COX-I and COX-II isoforms are 60% homologous in protein structure (Vane et al., 1998), even though they are encoded by different genes (O'Banion et al., 1991). COX-I is the constitutive isoform and mainly expressed in the endoplasmic reticulum (reviewed in Appleton et al., 1996; Ren et al., 1995). Its many physiological roles include: protection of gastric mucosa, cellular homeostasis, reproduction and cardiovascular
homeostasis (for overview, see Sellers et al., 2010; Vane et al., 1998; Vanhoutte, 2009). COX-I has been found in neurons and microglia (for review, see Candelario-Jalil et al., 2008). Conversely, COX-II is associated with the nuclear membrane (Vane et al., 1998). Its basal levels of expression are extremely low (Yamagata et al., 1993), and it plays a physiological role in renal blood flow (Dinchuk et al., 1995; Morham et al., 1995). Constitutive expression of COX-II has been found in the brain, in neuronal and glial cells. The presence of COX-II in postsynaptic dendritic spines, has been implicated in synaptic signalling, memory consolidation and neuronal plasticity (Canderlario-Jalil et al., 2008; Chen et al., 2002).

COX-II protein expression and enzymatic activity are rapidly induced by a number of factors, such as activation of NMDA receptors (Candelario-Jalil et al., 2008). Most significant of these are inflammatory mediators such as cytokines and platelet activating factor (reviewed by Appleton et al., 1996; Iadecola et al., 2005). Thus, COX-II plays a vital role in inflammation and associated symptoms of fever and pain (Vane et al., 1998).

Data on the role of COX-I in inflammation is not consistent. Even though COX-I is constitutively expressed in brain, it has been disputed whether it is upregulated post cerebral IR or remains unchanged (Candelario-Jalil et al., 2008; Nogawa et al., 1997; Schwab et al., 2001). Schwab et al. (2001) discovered that in humans COX-I expression by microglial cells was elevated well into the remodelling phase of the inflammation, suggesting a possible role of COX-I in later stages. It seems that upregulation of COX-I may be beneficial. The induction of COX-I in cerebral IR injury led to neuroprotection and these beneficial effects were attenuated with by the inclusion of a COX-I inhibitor (Lin et al., 2002). Similarly, COX-I knockout mice suffered a greater infarct volume after distal MCAO (Iadecola et al., 2001c). There were however no differences in neurological function and infarct between COX-I knockout animals and wild type in other models of MCAO (Cheung et al., 2001; Zou et al., 2006). These studies show that if COX-I does have a role in inflammation, it is more likely to be beneficial.

The role of COX-II in inflammation post stroke is more clearly defined. In animals and humans, upregulation of COX-II expression and activity is seen in glia, neurons, vascular cells and inflammatory cells after cerebral IR injury (reviewed by Iadecola et al., 2005). Activation of COX can arise from the presence of the inflammatory cytokines (reviewed by Appleton et al., 1996; Iadecola et al., 2005). COX-II induction leads to mainly the production of pro-inflammatory PGE$_2$ (Appleton et al., 1996), however, it may also lead to production of anti-inflammatory PGD$_2$ (Gilroy et al., 1999). The role of COX metabolites is discussed in Section 1.3.8. Numerous animal studies of cerebral IR have shown that COX-II inhibition is
neuroprotective (Candelario-Jalil et al., 2005; Govoni et al., 2001; Nogawa et al., 1997; Sugimoto et al., 2003; Yagami et al., 2005). Conversely, overexpression of COX-II led to poorer outcomes in focal cerebral IR (Dore et al., 2003). Moreover, COX-II knockout mice were more resistant to excitotoxicity mediated neuronal damage (Iadecola et al., 2001b). More interestingly, in a model of global ischaemia, it was found that there was less proliferation of neuronal progenitor cells in COX-II knockout mice. Thus, it has been suggested that COX-II has an important mediator role in the enhancement of neuronal plasticity (Sasaki et al., 2003). Furthermore, recent data shows that adverse effects of COX-II induction require production of NO by iNOS (Nagayama et al., 1999). This shows vital interplay of COX and NOS as two major enzymes in inflammation (Nogawa et al., 1998). It is probable that COX-II products, such as PGE$_2$ may play a part in induction of iNOS. The COX-II enzyme seems only to play a role in neuronal death in the penumbra rather than the infarct core. Suggested mechanisms include: production of free radicals, via neurotoxic action of prostaglandins, promotion of cell cycle activity, and metabolism of endocannabinoids (for review, see Candelario-Jalil et al., 2008). Therefore, the plethora of evidence indicates that COX-II is vital in the detrimental effects of inflammation post cerebral IR injury.

### 1.3.8 Contribution of PGs

Prostaglandins are one of the two groups of metabolites produced by the COX enzyme (the other being TXs). Briefly, TXA$_2$ is involved in platelet aggregation and vasoconstriction and is a mediator of inflammation (for review on TXs, see Tilley et al., 2001). These effects would favour atherosclerosis and thrombosis leading to arterial occlusion. PGE$_2$, the major metabolite derived from AA during inflammation, has both pro- and anti-inflammatory effects (Appleton et al., 1996). PGE$_2$ causes vasodilation and contributes to erythema and oedema in inflammation (Juhlin et al., 1969; Vane, 1976). In addition, PGE$_2$ activates caspase-2 (Nencioni et al., 2002) thereby potentiating apoptosis (processes in apoptosis are discussed in Section 1.4.3). PGE$_2$ levels have been correlated with worse outcomes in cerebral IR injury. The inhibition of COX-II with a consequent decrease in PGE$_2$ concentration was associated with neuroprotection (for review see Iadecola et al., 2005). Furthermore, inhibition of PGE$_2$ synthases (enzymes involved in synthesizing PGE$_2$ from PGH$_2$) has been found effective in decreasing inflammation (for review on PGE synthases, see Samuelsson et al., 2007). However, the effects of PGE$_2$ are anti-inflammatory as well. It modulates the immune system by inhibiting lymphocyte proliferation (Ruggeri et al., 2000). PGE$_2$ prevented ROS formation by activated lymphocytes (Appleton et al., 1996). These properties would lead to
neuroprotection. Moreover, PGE$_2$ increased ODC levels as well as putrescine and spermine, whilst decreasing spermidine (Ruggeri et al., 2000). The implication of this result on neuroprotection is unclear, as spermine is beneficial as opposed to putrescine (see Section 1.3.4).

The effects of PGE$_2$ are diversified when examining neurodegeneration post cerebral IR. These broad range effects of PGE$_2$ depend on the activation of its receptors, of which there are 4: EP$_1$, EP$_2$, EP$_3$ and EP$_4$ (Coleman et al., 1994). The receptors, differentially localized within the body, have varied functions. For example, EP$_1$ and EP$_3$ activation led to pyrexia whereas EP$_4$ activation leads to decreased body temperature (for overview see Oka, 2004). In the context of cerebral ischaemic injury, activation of EP$_1$ has been neurodegenerative (Ahmad et al., 2006a; Kawano et al., 2006), as opposed to EP$_3$/EP$_4$ activation leading to neuroprotection (Ahmad et al., 2006b; Echeverria et al., 2005). Stimulation of EP$_1$ receptor worsened stroke outcomes (Ahmad et al., 2007) and EP$_3$ knockout mice had lower infarct volumes after cerebral IR (Saleem et al., 2009b), however these results have been disputed (for review, see Andreasson, 2010). The effect of EP$_3$ is debatable with reports claiming neuroprotection (Liu et al., 2005; McCullough et al., 2004) and neurodegeneration (Takadera et al., 2006) in cerebral IR injury. Therefore, although PGE$_2$ may have been previously believed to be detrimental in inflammation post stroke, evidence that is more recent demonstrates its putative neuroprotective role.

Prostacyclin or PGI$_2$, is produced by endothelial cells and vascular smooth muscle cells (Ali et al., 1980; Weksler et al., 1977). In addition to being a potent vasodilator, it inhibits platelet aggregation, platelet adhesion, leukocyte activation and leukocyte-endothelial interaction (for review on prostacyclins, see Moncada, 1983). PGI$_2$ analogues led to reduced cerebral damage (via these mechanisms), whilst also providing direct neuroprotection (Cui et al., 1999; Matsuda et al., 1997). Likewise deletion of PGI$_2$ receptor, IP, led to worse neurological outcome in models of cerebral IR (Saleem et al., 2010; Wei et al., 2008). Thus, PGI$_2$ would be reducing the impact of inflammation on tissue injury post cerebral IR.

PGD$_2$ is produced mainly by mast cells (Lewis et al., 1982). It also inhibits platelet aggregation and mediates allergic reactions. Furthermore, it is converted into the J series of PGs which are key anti-inflammatory mediators (Kapoor et al., 2005b). Inhibition of PGD$_2$ and deletion of PGD$_2$ receptor, DP1, led to increased infarct size in models of stroke (Ahmad et al., 2010; Liu et al., 2009). PGD$_2$ as well would be beneficial in inflammation post stroke.
Physiologically, PGF$_{2\alpha}$ is involved in reproduction and smooth muscle contraction (Anderson, 1973; Ishizawa, 1983; Weems et al., 1991). It is a vasoconstrictor and thus able to prevent oedema formation (Willoughby, 1968). Prevention of platelet aggregation is another beneficial effect of the PG (reviewed by Appleton et al., 1996). PGF$_{2\alpha}$ has been shown to have anti-inflammatory properties, which may make it beneficial (for review, see Kapoor et al., 2005b). Following IR injury PGF$_{2\alpha}$ was found to be activating neutrophil adhesion, without affecting monocyte or lymphocyte adhesion (Fontana et al., 2001). Moreover, in knockout mice deficient in PGF$_{2\alpha}$ receptor, FP, infarct volumes were significantly smaller (Saleem et al., 2009). Thus, this outlines a harmful role of the prostaglandin in inflammation after stroke.

PGI$_2$, and PGD$_2$ are beneficial, PGF$_{2\alpha}$ detrimental, whilst the effects of PGE$_2$ are paradoxical. The PGE$_2$ effects outweigh those of any other PG as they are most abundant in inflammation (Appleton et al., 1996). Since neuroprotection is seen with inhibition of PGE$_2$, it may be assumed that its most dominant effect is via activation of its neurodegenerative receptors, such as EP$_1$.

### 1.4 MITOCHONDRIA IN STROKE

#### 1.4.1 Normal electron transport chain function

The cellular mitochondrial organelle is responsible for supplying the cell with energy rich molecules, ATP, for all the cellular functioning. The organelle has two lipid bilayer membranes: a permeable outer membrane and selectively permeable inner membrane containing an electron transport chain (ETC). It also has its own double stranded circular DNA that codes for most of the mitochondrial proteins. Of the several enzymes, aconitase (EC 4.2.1.3), containing an iron-sulphur complex, and physiologically part of the citric acid cycle is present both in the mitochondria and the cytoplasm. The iron-sulphur core (of aconitase and other enzymes) is relatively sensitive to oxidation by elevated ROS (Gardner et al., 1995). Hence, the enzyme can be used as a marker of oxidative stress in mitochondria and whole cell (Clarkson et al., 2007; Clarkson et al., 2004a; Sammut et al., 2001). Citrate synthase (EC 2.3.3.1) is also located in the mitochondrial matrix, and is part of the Kreb’s cycle. This enzyme is used as a marker for mitochondrial membrane integrity and the number of intact mitochondria in the tissue (Clarkson et al., 2007; Clarkson et al., 2004a; Sammut et al., 2001).
as mitochondrial membrane damage leads to cytoplasmic leakage of this enzyme and consequent inactivity (Schild et al., 2006).

The mitochondria by having a favourable environment for metabolic reactions, are able to produce ATP most efficiently, via oxidative phosphorylation and the ETC (Figure 1.10), housed in its inner membrane. The ETC contains 5 types of enzyme super complexes with upiqinone and cytochrome $c$ acting as electron carriers. Each complex is arranged in the chain according to their redox potential. Electrons derived from co-enzymes nicotinamide dinucleotide dehydrogenase (NADH) and the reduced form of flavin adenine dinucleotide FADH$_2$, via complex I (NADH dehydrogenase, EC 1.6.5.3) or complex II (succinate dehydrogenase, EC 1.3.5.1), are included in the ETC. Electrons from NADH and FADH$_2$ are transferred by complex I and II, respectively, to ubiquinone, which diffuses into the mitochondrial space, eventually carrying the electrons to complex III (cytochrome $bc_1$ complex, EC 1.10.2.2). Concurrently, complex III transfers electrons to complex IV (cytochrome $c$ oxidase, EC 1.9.3.1) via cytochrome $c$. In turn, complex IV reduces O$_2$ to H$_2$O.

At each complex from I – IV, protons are pumped from the mitochondrial matrix into the intermembrane producing an electrochemical gradient. Complex V (ATP synthase, EC 3.6.1.34) then uses the energy from this potential gradient to produce ATP by transferring inorganic
phosphate to adenine dinucleotide phosphate (ADP). Complexes I and III, in particular, may leak electrons to oxygen forming superoxide (for review on mitochondrial energetics, see Nicholls et al., 2000).

### 1.4.2 The formation of ROS in mitochondria

Superoxide ions released as a consequence of ETC activity can then form other ROS, such as hydroxyl ion (OH\(^-\)), and peroxynitrite (ONOO\(^-\)). Peroxynitrite is formed when superoxide reacts with NO. NO has a physiological role of in controlling mitochondrial respiration and signalling (for review, see Poderoso, 2009). Under physiological conditions, the superoxide ion is converted by mitochondrial superoxide dismutase to highly toxic hydrogen peroxide (H\(_2\)O\(_2\)). This final product is scavenged by other homeostatic antioxidants, such as glutathione. Under physiological conditions, the antioxidant enzymes are able to withstand the minimal ROS formation as a by-product of ETC activity. There are several conditions when elevation of ROS occurs. Failure of the antioxidant enzymes to counter ROS sufficiently would lead to a rapid increase in ROS. Insufficiency of the ETC due to oxidative (or toxic) damage to any of its complexes will give rise to a decline in ATP generation and consequently increased ROS production (for reviews on mitochondrial ROS formation, see Adam-Vizi, 2005; Adam-Vizi et al., 2006). Furthermore, ROS formed from pathological conditions such as IR insult would be amplified by the organelle. Reperfusion causes an influx of oxygen, increased metabolism of fatty acids and thus superoxide formation, leading to increased oxidative stress (Krause et al., 1988). IR injury results in inflammation, which further generates ROS and peroxynitrite (for review on pathophysiology of ischaemia reperfusion, see Carden et al., 2000). The initial increase in ROS leads to partial damage of mitochondria and possible damage and inhibition of ETC complex I and complex III. Both these complexes then release superoxide into the mitochondria (Jezek et al., 2005). Dysfunctional mitochondria thus produce more ROS affecting the normal functioning mitochondria, causing a vicious cycle of ROS formation (for review, see Szeto, 2006). The amplification effect of mitochondria leads it to be the major source of ROS following IR (Adam-Vizi, 2005; Adam-Vizi et al., 2006; Saris et al., 1995). This is confirmed by studies showing expression of endogenous antioxidant enzymes, such as superoxide dismutase, relating to improved outcomes in stroke (for review, see Sims et al., 2010).
1.4.3 Mitochondria mediated apoptosis in stroke

Under physiological conditions, the mitochondrion plays a vital role in the cellular calcium homeostasis. Mitochondria sequester excess Ca\(^{2+}\) from the cytosol and stores it within the inner mitochondrial matrix (Bernardi et al., 1996). The mitochondria also contain mitochondrial permeability transition pores (mPTP), which span across the inter membrane space in the organelle (Bernardi et al., 1996; Crompton, 1999). The increase in ROS leads to damage to the inner mitochondrial membrane releasing high amounts of ions, such as Ca\(^{2+}\), and leading to depolarization of the membrane. The increased ROS in addition damages the complexes, changing the NAD\(^+\)/NADH ratio (Adam-Vizi, 2005; Adam-Vizi et al., 2006). The mPTP opening occurs due to a conformational change arising from several factors, such as high Ca\(^{2+}\), depolarization of the membrane, overproduction of ROS and high NAD\(^+\)/NADH ratio (Chinopoulos et al., 2006; Friberg et al., 2002; Halestrap et al., 2000; Sims et al., 2002). This mPTP opening makes the mitochondria permeable and initiates a cascade of events. Firstly, pro-apoptotic members of Bcl-2 family facilitate the release of various proteins such as cytochrome c and apoptosis inducing factor (for review of Bcl-2 proteins, see Antonsson, 2004). Factors that initiate the caspase dependent apoptosis, such as cytochrome c (Bernardi, 1999; Cai et al., 1998), and caspase independent apoptosis, such as apoptosis inducing factor (Lindholm et al., 2004; Susin et al., 1999), also leak out into the cytoplasm (for overview of caspases, see Ferrer et al., 2003; Friedlander, 2003). In addition, water diffuses into the mitochondria and causes outer membrane rupture (Cai et al., 1998; Susin et al., 1999). With the homeostatic environment for the oxidative metabolism disrupted, the mitochondria are no longer effective in producing ATP. Furthermore, the mPTP mediated efflux of mitochondrial Ca\(^{2+}\) into the cytoplasm along with ROS, damages other mitochondria within the cell (Stavrovskaya et al., 2005). All these changes lead to cell death by apoptosis (for reviews on mitochondrial mediated apoptosis, see Haeberlein, 2004; Lindholm et al., 2004; Sims et al., 2010; Szewczyk et al., 2002).

Apoptosis is a normal physiological process, whereby damaged, redundant or infected cells are removed by the body (Willis et al., 2003). However, in pathological conditions, such as cerebral IR, delayed neuronal death is a consequence of a vicious cycle of apoptosis and inflammation. Apoptosis is directly linked to inflammation, as caspases lead to activation of inflammation via maturation (proteolytic cleavage) of proinflammatory cytokines (Festjens et al., 2006). Inflammation is a process employed to remove cellular debri after cell death. However, inflammation itself leads to production of ROS and causes apoptosis. In addition, ROS leads to further inflammation by stimulating pro-inflammatory cytokines from immune
This propagates the extent of tissue injury even after the initial cause is removed. Therefore, mitochondrial driven apoptosis should be explored as an avenue for clinical benefit.

1.5 PHARMACOLOGICAL INTERVENTIONS OF STROKE

1.5.1 The Stroke Therapy Academic Industry Roundtable (STAIR) Committee

Despite the millions of dollars spent on stroke research (acute therapy) almost all phase III clinical trials of neuroprotective drugs, and many of the thrombolytic/recanalization studies conducted thus far, have been negative despite evidence of experimental success (Table 1.1, STAIR II, 2001). This fact alludes to fundamental flaws in one or more of three underlying processes: (1) the experimental evidence given to support a drug’s ability to neuroprotect; (2) how drugs are selected to proceed to clinical trial; and (3) clinical trial design. In an attempt to improve upon all three processes, academic and industry leaders in stroke research formed a review panel called the STAIR committee and published guidelines for experimental and clinical drug development with the main goal of expanding the number of effective and safe acute stroke therapies (Fisher, 2003; STAIR, 1999; STAIR II, 2001). The first STAIR meeting focused on the discrepancy between preclinical/experimental evaluations and clinical outcomes (STAIR, 1999). Their concern was that the information clinical trials were founded on was inaccurate and based on imperfect experimental results. The main experimental parameters tackled included: stroke severity (Grotta, 2002), full dose–response evaluation, drug efficacy, therapeutic time window, use of appropriate models, i.e. transient and permanent focal ischaemia (Green et al., 2003b; Hunter et al., 1995), functional behavioural testing in addition to measurement of infarct volume, measurement of physiological parameters, and reproducibility of data by external laboratories. The second meeting was on phases I and II clinical trial designs and how to ensure that the parameters and endpoints were maximized so that trials would require the fewest number of patients to detect significant differences (STAIR II, 2001). The third meeting deliberated on how to increase the percentage of patients who might be included in acute stroke therapy trials (Fisher, 2003). However, an antioxidant (NXY-059) that was developed mostly in accordance with STAIR criteria and using much higher doses than used in pre-clinical studies, recently failed phase III studies (for reviews, see Ginsberg, 2008; Green, 2008). The main reason for this failure has been...
suggested to be inclusion of patients with and without thrombolysis (Ginsberg, 2008). Thus, proving that even if stringent pre-clinical goals are adhered to, a slight flaw in trial design could lead to failure. The latest STAIR updated guidelines (from its fourth meet) recommends calculation of sample size, predefined inclusion and exclusion criteria, randomization, blinding of investigators, reporting of exclusion of animals from the study and reporting conflicts of interests (Fisher et al., 2009). One of the important issues that was raised by the STAIR group was the therapeutic time window.

1.5.2 The therapeutic time window

The therapeutic time window is defined as the longest period of time between vessel occlusion (ischaemia) and drug treatment in which the treatment will still confer a significant amount of neuroprotection. The earlier patients present for diagnosis, the more likely they will be eligible for effective treatment. Clinically, this is a particularly problematic parameter as one of the common symptoms of stroke is mild confusion and disorientation, which entails the presence of an observer who can recognize the symptoms and expedite the process of hospital admission. The therapeutic window is a key factor in choosing treatments as many patients (approximately 58%), may not be candidates for some therapies because they do not present within 24 hours (Alberts et al., 1990). For reasons such as these, preclinical criteria for putative neuroprotective drugs includes, not only exhibiting significant efficacy at a non-toxic dose, but doing so with a large therapeutic time window, one that is achievable in humans (e.g. 4 – 6 hours).
### Chapter 1: Introduction

#### Thrombolytic interventions

<table>
<thead>
<tr>
<th>Drug/Trial name</th>
<th>Mechanism of action</th>
<th>N</th>
<th>TTT</th>
<th>Trial Design</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recombinant Tissue Plasminogen activator</strong>&lt;br&gt;(rt-PA-Alteplase)&lt;br&gt;(NINDS, 1995; Kwiatkowski et al., 1999)</td>
<td>Binds to fibrin in a thrombus and converts plasminogen to plasmin which helps break the cross linked fibrin clot</td>
<td>624</td>
<td>3 hours</td>
<td>0.9 mg/kg, 10% bolus, 90% over 60 min iv</td>
<td>Sig. improv. on BI, mRS, GOS, and NIHSS at 3 months. ↑ incidence of ICH in first 10 days</td>
</tr>
<tr>
<td><strong>Ancrod</strong>&lt;br&gt;(STAT, The Ancrod Stroke Study investigators, 1994)</td>
<td>Induces rapid defibrinogenation by splitting fibrinopeptide A from fibrinogen</td>
<td>500</td>
<td>3 hours</td>
<td>72 hours i.v. 1mL (70IU)(dose dependent on prettr fibrinogen levels) + 1 hour i.v. at 96 and 120 hours</td>
<td>Sig. improve. on SSS at 90 days. No sig. diff. in mortality or morbidity. Sig. ↑ in asymptomatic ICH in ancrod group. Clinicians approv. of benefit-risk profile</td>
</tr>
<tr>
<td><strong>Aspirin</strong>&lt;br&gt;(Wilterdink et al., 2001)</td>
<td>Anti-platelet therapy that inhibits COX, and lowers TXA₂ formation, which is involved in platelet aggregation</td>
<td>1275</td>
<td>NA</td>
<td>Aspirin use prior to 7 days of stroke</td>
<td>Sig. improv. on SME but not NIHSS at 3 months</td>
</tr>
<tr>
<td><strong>Aspirin</strong>&lt;br&gt;(CAST, 1997)</td>
<td></td>
<td>21106</td>
<td>48 hours</td>
<td>160 mg/d o.s. upto 4 weeks</td>
<td>Sig. ↓ in mortality and recurrent strokes at 4 weeks Non. sig. ↑ in ICH</td>
</tr>
<tr>
<td><strong>Mechanical embolectomy</strong>&lt;br&gt;(MERCI, Smith et al., 2008)</td>
<td>Recanalisation by surgery (clot is surgically removed)</td>
<td>164</td>
<td>8 hours</td>
<td>24 mg rt-PA or 250000 U of urokinase</td>
<td>Sig. improv. on mRS at 90 days. 5.5% sig. procedural complications.</td>
</tr>
</tbody>
</table>
### Neuroprotective Interventions

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Description</th>
<th>Phase</th>
<th>Duration</th>
<th>Dose</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edaravone (MCI-186)</td>
<td>Free radical scavenger, inhibits lipid peroxidation and vascular endothelial cell injury <em>in vitro</em> as well as ameliorating brain oedema <em>in vivo</em></td>
<td>252</td>
<td>72 hours</td>
<td>30 mg bid i.v. over 30 min for 14 days</td>
<td>Dose well tolerated. Sig. improv. on mRS at 3 months. 12 months follow-up indicated a sustained benefit for treatment</td>
</tr>
<tr>
<td>Edaravone (EAIS, 2003)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lubeluzole (Lub)</td>
<td>A benzothiazole that prevents the ↑ of extracellular glutamate, normalises the ionic balance in the penumbra and inhibits glutamate-activated NO production</td>
<td>721</td>
<td>6 hours</td>
<td>7.5 mg over 1 hour + 10 mg/d (maximum of 5 days)</td>
<td>↓ mortality (21 vs 25%) and sig. ↑ on NIHSS, BI and mRS at 12 weeks.</td>
</tr>
<tr>
<td>Lubeluzole (Grotta, 1997)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minocycline (Lampl <em>et al.</em>, 2007)</td>
<td>Broad spectrum antibiotic with anti-inflammatory effects</td>
<td>152</td>
<td>24 hours</td>
<td>200mg/day for 5 days</td>
<td>Sig. improve. on NIHSS score at 7, 30, and 90 days. No sig. difference with mRS, BI.</td>
</tr>
</tbody>
</table>

| Table 1.1 Major phase III clinical trials for acute stroke therapy. To be included, trials had to be prospective, exhibit a positive clinical outcome (as determined by statistically significant improvements on neurological examinations), and have distinguished between patients with ischaemic and haemorrhagic strokes. All trials designs had additional control ‘placebo’ groups unless specified. BI—Barthel Index, CAST—Chinese Acute Stroke Trial, EAIS—Edaravone Acute Infarction Study, GOS—Glasgow Outcome Score, improv – improvement, MERCI—Mechanical Embolus Removal in Cerebral Ischemia, mRS—modified Rankin Scale, NIHSS—National Institutes of Health Stroke Scale, NINDS—National Institute of Neurological Disorders and Stroke, sICH—symptomatic intracranial haemorrhages, sig – significant, SME—Supplemental Motor Examination, SSS—Scandinavian Stroke Scale, STAT—Stroke Treatment with Ancrod Trial, TTT—time to treat. |

35
Despite numerous drugs having met this criterion, almost all have failed to show clinical efficacy due, in part, to the narrow therapeutic time window of the drugs and clinicians repeated failure to follow experimentally defined parameters (for review, see Green, 2002). For example, magnesium, which boasts a long experimental therapeutic window of 6 hours, was administered in a phase III clinical trial up to 12 hours post-onset of stroke symptoms (Muir et al., 2004). Not surprisingly, the clinical trial met with a negative outcome. Monosialoganglioside (GM₁) was also unsuccessful in a phase III clinical trial. However, post hoc analysis found that patients treated within 4 hours instead of 5 hours from stroke onset had significantly improved neurological scores at 4 months (Lenzi et al., 1994). This implies that if other trials had also more strictly adhered to the experimentally defined therapeutic time window, more may have been successful. The artificial lengthening of the therapeutic time window from the experimental to clinical phase by stroke researchers may have been due to the general assumption that the therapeutic time window was larger in humans than animals. This assumes that the human brain develops ‘damage’ at a slower rate than the analogous animal model. However, the failure of so many clinical trials prompted a re-evaluation of this theory. In clinical practice, recombinant tissue plasminogen activator (rt-PA) maintained the therapeutic time window that was observed in the corresponding animal model (Grotta, 2002; NINDS rt-PA Stroke Study Group, 1995). Initially, administration of rt-PA was limited exclusively to within 3 hours however recent meta-analysis has now led to the i.v. use of the thrombolytic up to 4.5 hours from onset of ischaemia (Lees et al., 2010; NINDS rt-PA Stroke Study Group, 1995). Treatment with the same thrombolytic after the therapeutic window post stroke symptom onset is however, adversely correlated with increased risk of ICH and mortality (Green, 2002). This strongly suggests that experimental therapeutic time windows should be strictly adhered to in clinical trials.

Additional evidence to support the maintenance of the therapeutic window from preclinical to clinical trials can be taken from the principle of ‘time is brain’ (Hill et al., 1998). Implicit in this statement is that the ischaemic cascade is a finite process. If drugs are targeting specific time sensitive mechanisms in the cascade but are given after the mechanism of ‘action’ has occurred, the drugs may not only be ineffective, but have the potential to create an additional imbalance and exacerbate the deterioration. Therapies may also be time specific, meaning that the longer the time to treat the less effective they are (reviewed in Sherman et al., 2000). An example of deleterious effects induced by inappropriate clinical protocol is ZK200775, a glutamate AMPA/kainate receptor antagonist. It was administered up to 24 hours post stroke even though experimentally it had only exhibited a 4 hour therapeutic time window (Turski et al., 1998). It not only failed to provide any clinical benefit, but treatment significantly
decreased neurological functioning as defined by the NIHSS at 1 month (Elting et al., 2002). With such unambiguous evidence that the experimentally defined therapeutic window should be upheld in clinical practice, it is incomprehensible that these breaches of protocol still occur. One explanation may be that pressure from pharmaceutical companies and their consultants, who are concerned about establishing the largest market for their drug, force researchers to disregard the laboratory data and extend the time to treatment in most clinical studies to 6 hours or more (Grotta, 2002). In light of such circumstances, a solution must be devised that does not involve an artificial expansion of the therapeutic time window, rendering the drugs ineffective. Alternate strategies for putative neuroprotective agents that exhibit a larger, clinically achievable therapeutic window must be examined. Since inflammation has been established to be a pathological process occurring for a much longer duration than acute excitotoxicity, targeting it would be most appropriate.

1.5.3 Targeting inflammation after stroke

Initial clinical trials with non-steroidal anti-inflammatory drugs (NSAIDs) proved to be unsatisfactory, mainly due to their propensity to cause increased ICH (Bak et al., 2003; Qureshi, 2003). NSAIDs inhibit TXA\(_2\) formation, which in turn inhibits blood clot formation (FitzGerald, 2002). Unfortunately, NSAIDs also disrupt the normal homeostatic balance between the formation of blood clots by TXA\(_2\) and haemorrhages by PGI\(_2\). This effectively exacerbates the effects of PGI\(_2\), increasing the risk of haemorrhaging (Clark et al., 2004). In addition, if a haemorrhage does occur, the absence of TXA\(_2\) will mean that it cannot initiate blood clot formation, thereby intensifying the damage (see Figure 1.11).
Figure 1.11 Pathway detailing mechanisms of actions for the increased risk of ICH associated with NSAIDS treatment.

Despite the lacklustre results from NSAID trials, there is no reason to assume alternate anti-inflammatory drugs would not be beneficial. Recently, putative neuroprotective drugs with anti-inflammatory capabilities have shown encouraging results in clinical trials. Edaravone (a free radical scavenger) was given up to 72 hours after stroke and significantly improved functional outcome with sustained benefit lasting up to 12 months (Edavarone Acute Infarction Study Group, 2003). Edaravone has two attributes that make it a very appealing candidate for acute therapy; one, it has a large therapeutic window (72 hours) and two, it does not increase the risk of intracerebral bleeding and may therefore be used in combination with thrombolytic therapy. In addition, ebselen, an anti-inflammatory and anti-oxidant agent, when administered within 24 hours post cerebral ischaemia, also provided significant neuroprotection at 1 month, but not at 3 months (Yamaguchi et al., 1998). Ebselen may have provided a more sustained benefit if given within a shorter therapeutic time window. Minocycline, shown to have wide range of anti-inflammatory and anti-apoptotic effects, improved neurological outcomes when administered 6 to 24 hours after onset of stroke (Lampl et al., 2007). An added benefit, is its broad spectrum antibiotic properties (similar to tetracycline in structure), and this could also reduce secondary infections that arise in stroke patients. These results and the contribution of inflammatory mediators to post-ischaemic brain
injury present a compelling argument that the inflammatory pathway may be an appropriate target for therapeutic intervention in the acute treatment of ischaemic stroke.

1.5.4 **Utilising current interventions of stroke**

An ideal treatment regime for ischaemic stroke entails laying a carefully coordinated plan that encompasses an understanding of the fundamental mechanisms underlying focal ischaemic brain injury. The course of the interventions must change and adapt with the natural progression of the disease. In acute therapy, if the blood clot remains, a thrombolytic is most appropriate. However, once re-canalization has occurred a neuroprotectant is required, as much of the neurological damage associated with cerebral ischaemia is attributed to oxidative stress caused by reperfusion (Hill *et al.*, 1998). The choice of therapy (either thrombolytic and/or neuroprotectant) should be determined by the duration of the stroke and clinical contraindications (Figure 1.12). All suggested primary and secondary therapies are based on current information about acute stroke phase III clinical trials exhibiting improved functional outcomes. Foremost, patients must be accurately diagnosed clinically and radiological imaging (such as computerized tomography or magnetic resonance imaging) to have an ischaemic stroke in which thrombolytic therapy is not contraindicated (i.e. by presence of an ICH). In terms of primary therapy, if a patient presents within 4.5 hours after onset of stroke symptoms they preferably receive either rt-PA (*Lees et al.*, 2010) or ancrod (*The Ancrod Stroke Study Investigators*, 1994). Delayed presentations of up to 8 hours of onset may be offered mechanical embolectomy in order to recanalise the artery (*Smith et al.*, 2008). Thrombolytic treatment may be followed by a neuroprotectant such as lubeluzole (within 6 hours, *Grotta*, 1997) or minocycline (6 to 24 hours, *Lampl et al.*, 2007) or edaravone (within 72 hours, *Edavarone Acute Infarction Study Group*, 2003). These agents may be given simultaneously with the thrombolytic, should they not decrease its efficacy or increase risk of ICH, such as seen with edaravone (see Figure 1.12).

Proceeding thrombolytic therapy, low dose aspirin or warfarin (for patients with atrial fibrillation) can be administered as secondary therapies as they have been proven to decrease mortality and the risk of a second stroke, although neither has shown an effect on neurological functioning (*Aronow et al.*, 2000; *Chinese Acute Stroke Trial*, 1997; *The International Stroke Trial Collaborative Group*, 1997).
Chapter 1: Introduction

Figure 1.12 The proposed, ideal primary therapeutic regime with current successful interventions for acute stroke. Arrows indicate the therapeutic time window for each drug. Therapies have been selected based on clinical trial efficacy.

Notably, aspirin should not be administered concurrently with thrombolytics as it increases the risk of ICHs (Chinese Acute Stroke Trial, 1997). This combination of primary and secondary therapies represents the most effective treatment regime offered by current clinically proven acute stroke therapies. Unfortunately, rt-PA, aspirin and warfarin are, at present, the only drugs out of the suggested therapies that are approved for use in acute ischaemic stroke. However, in future, ideal polytherapy for stroke treatment is most likely to be implemented.

1.5.5 Future directions for acute stroke therapy

Unlike many other diseases, acute stroke therapy has a limited therapeutic time window. Understanding the influence of the inflammation pathway in stroke may enable future therapeutics to target the inflammatory mediators for acute interventions and possibly extend the therapeutic window. Thus far stroke has been regarded as a progressive, irreparable disease; however, stem cell research may provide a method to induce neuronal replacement, a type of artificial neuroplasticity for the ischaemic tissue (Ourednik et al., 2002; Rothstein et al., 2004). Considerable developments in our understanding of neurodevelopmental principles and basic pathophysiological process are required before this new prospect, stem cell implantation, can become a reality (Snyder et al., 2002). The clinical application of experimental therapies can be equally problematic to developing novel therapeutics. It is
readily acknowledged that the ischaemic cascade is an incredibly complex pathophysiological process involving multiple pathways such as necrosis, apoptosis and inflammation. Ischaemia should be looked at as more than an event but as a progressive disease that evolves over time. Future strategies for therapy should recognize this and target multiple mechanisms, forming a multi-factorial approach. The therapy should also be adaptable to the clinical presentation and stage of pathophysiological pathway. Furthermore, therapies that can simultaneously target multiple facets of the neurodegenerative processes should be researched. The pharmacological interventions available should be applied clinically only to those experimental parameters, under which it was found to be efficacious. For example, if a putative neuroprotectant only attenuated infarct size in a transient model of stroke (as opposed to a permanent model), then clinically it should not be administered until a thrombolytic has been applied. A neuroprotectant investigated in a permanent model of ischaemia may be given to patients in whom reperfusion has not been achieved to minimize neurological deterioration. Targeting pathways which can extend the therapeutic time window such as inflammation and ‘neurorestoration’ would be able to further increase success clinically (Green, 2008). Ideally, treatment with neuroprotective agent could be commenced prior to hospitalisation by the paramedics. This idea is being investigated by use of hyperacute magnesium (for review, see Ginsberg, 2008). In the future, a new approach of polytherapy using neuroprotectants and thrombolytics will be most likely to be effective and would overcome the shadows of failure from previous clinical trials using a single therapeutic agent.

1.6 ANIMAL MODELS OF STROKE

1.6.1 Use of animals in stroke research

Putative therapeutic agents should be validated in animal models that replicate clinical syndromes prior to human use. Carrying out a clinical trial prior to having as much evidence as possible on the benefits of any agent could be seen as unethical and expensive. In most pathologies, biological samples are required to evaluate the mechanisms and examine effectiveness. In stroke, it is difficult to use human samples primarily to determine the effect of a drug. There are two major problems. Firstly, it is difficult to obtain the samples. Samples may be limited to peripheral blood collection, or post mortem samples for brain tissue. An alternative, which is now being more readily used, is the application of imaging in determining
infarct volumes. Thus, mechanisms of action of the putative neuroprotectant may not be easily examined in human studies.

Secondly, the homogeneity of the treatment group is difficult to obtain. The time interval between onset of stroke and patients presentation to physicians vary (usually a few hours later). Furthermore, in order to carry out an experiment or trial to examine effectiveness of a drug, all parameters must be constant. There should be only one variable between groups, i.e. the putative therapeutic. Large sample sizes are often required so that confounding variables such as co-morbidities can be factored into the final analysis. This makes clinical trials very expensive to run and impractical to carry out without strong data supporting a drug’s effectiveness. Thus, animal models can provide a cheaper alternative, in offering reproducibility and controlling confounding variables.

The experimental models of stroke in animals are used to screen putative neuroprotectants prior to possible clinical trials. This provides a more economical and ethical technique, after the initial success in vitro. Rats and mice seem to be the most highly used animals due to the availability and low maintenance with cheaper costs. The high clinical failure rate of therapeutics effective in animals has led to scrutiny of animal model use (Wiebers et al., 1990). However, most clinical trials tested efficacy on limited animal experimental data and with physiological parameters outside the testing capacity of the models (Hunter et al., 1995). Animals used in stroke models are normally young and healthy, whereas stroke occurs in patients with multiple co-morbidities. Rodents can be genetically modified to have conditions such as hypertension, hyperglycaemia and hypercholesterolemia. Ideally, animals would be older (matching human data for stroke incidence), with the pre-morbidities. This is especially important as the neuroprotectants seem to be less efficacious in older animals (Green, 2008). However, with such severe multiple co-morbidities, the animals may not have a normal life expectancy. Furthermore, a therapeutic needs to be shown to be effective in a model of stroke, which is not homogeneous in character. This would be to match the heterogeneity of stroke presentations in a clinical setting. Therefore, with greater stringency of animal models and ability to mimic as closely as possible to the clinical condition (Karpiak et al., 1989), there may be greater success of neuroprotectants clinically (for review on use of animal models in stroke, see Green et al., 2003a).

Moreover, the pathobiological events following an ischaemic event such as a stroke may be viewed as similar between the animal species used in the experimental models of stroke and humans (Hunter et al., 1995). This would be the most logical approach to develop a
reasonably efficacious neuroprotectant and to estimate the physiological parameters in which it is most efficient. The conditions under which the efficacy was attained must be applied in a clinical trial as well. Violations of such parameters may be a contributing factor to failure of putative therapeutics (for review on guidelines on preclinical stroke models, see STAIR, 1999). Interestingly, even when STAIR criteria were strictly adhered to, NXY-059 failed in a large phase III study (Green, 2008). This study used both patients with permanent and transient stroke, i.e. patients with and without rt-PA use (Shuaib et al., 2007). Even though the drug was efficacious in both permanent and transient ischaemia in animal studies the trial would have resulted in non-homogenous sample. This would make it difficult to detect a difference. In the future, the clinical studies will need to be stringent in the inclusion criteria that match the pre-clinical studies. Animal models of stroke would perhaps need to be better developed to reflect clinical presentations (Green, 2008).

1.6.2 Animal models of stroke

The animals used in the experimental model of stroke can be either large (for example, cats, dogs and monkeys) or small (mainly rodents).

In large animals, their brains are more structurally similar to humans, for example, large animals brains are gyrencephalic. A greater amount of useful data can be collected from the larger animals. It is easier to use imaging techniques such as nuclear magnetic resonance spectrometry (Chatham et al., 2001) and positron emission tomography (Cherry et al., 2001) in larger animals (although recent advances are allowing use in rodents). In addition, measurement of various physiological parameters, such as blood pressure and lactate, coupled with functional outcomes can be carried out at various time points in the same animal. The main disadvantage is the higher financial cost, labour intensive procedures and greater outcome variability. Furthermore, the use of large animals, especially dogs, cats and monkeys, are of major public animal welfare concern (for review see Traystman, 2003).

The main advantage of using small animals is the genetic homogeneity of the sample thereby considerably reducing the variability of infarct. This also allows reproduction of many transgenic animals for use in neuroprotective models, for example, the use of transgenic mice to determine effect of COX-I in stroke (Cheung et al., 2002). The size of the animal also allows utilisation of certain fixation procedures which require quick freezing of tissues (Ponten et al., 1973; reviewed in Traystman, 2003)
There are two major types of animal stroke models utilised (Table 1.2). In global ischaemia, the cerebral blood flow is reduced to almost the whole brain, mimicking a cardiac arrest. In focal ischaemia, reduction of cerebral blood flow to a specific brain region is achieved. Ischaemia can be permanent or temporary in any model. Transient focal ischaemia is most clinically relevant to stroke.

The most common model of global ischaemia currently used is the 2-vessel occlusion method. Systemic hypotension is induced in conjunction with the occlusion of the bilateral common carotid artery (CCA) to give a reversible ischaemia (Eklof et al., 1972a; Eklof et al., 1972b). The procedure is relatively simple and immediate reperfusion can be achieved. The injury characteristically occurs specifically in the selectively vulnerable regions, such as pyramidal neurons of the hippocampus (Smith et al., 1984). For review of major animal models see Traystman (2003).

<table>
<thead>
<tr>
<th>Model</th>
<th>Type of Ischaemia</th>
<th>Earliest publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unilateral CCA ligation plus hypoxia</td>
<td>Global</td>
<td>(Levine, 1960)</td>
</tr>
<tr>
<td>Blood clot embolisation</td>
<td>Focal</td>
<td>(Meyer et al., 1962)</td>
</tr>
<tr>
<td>Decapitation</td>
<td>Global</td>
<td>(Lowry et al., 1964)</td>
</tr>
<tr>
<td>Aortic arch artery branches’ occlusion</td>
<td>Global</td>
<td>(Hossmann et al., 1970)</td>
</tr>
<tr>
<td>Forebrain compression-ischaemia</td>
<td>Global</td>
<td>(Ljunggren et al., 1974a; Ljunggren et al., 1974b)</td>
</tr>
<tr>
<td>Cardiac Arrest</td>
<td>Global</td>
<td>(Safar et al., 1976)</td>
</tr>
<tr>
<td>Neck tourniquet</td>
<td>Global</td>
<td>(Siemkowicz et al., 1978)</td>
</tr>
<tr>
<td>Microsphere embolisation</td>
<td>Focal</td>
<td>(Larsen et al., 1978)</td>
</tr>
<tr>
<td>Middle cerebral artery occlusion</td>
<td>Focal</td>
<td>(Robinson et al., 1975)</td>
</tr>
<tr>
<td>Arachidonate-induced thrombosis</td>
<td>Focal</td>
<td>(Cahn et al., 1982)</td>
</tr>
<tr>
<td>Photochemically induced focal cerebral embolism and thrombosis</td>
<td>Focal</td>
<td>(Watson et al., 1985)</td>
</tr>
<tr>
<td>Endothelin-1 injection (potent vasoconstrictor)</td>
<td>Focal</td>
<td>(Macrae et al., 1993)</td>
</tr>
<tr>
<td>Uni-hemispheric forebrain compression with ipsilateral CCA occlusion</td>
<td>Focal</td>
<td>(Watanabe et al., 2001)</td>
</tr>
</tbody>
</table>

**Table 1.2** Most of the commonly used animal models of ischaemia, listed in order of first publication.
1.6.3 Focal ischaemia by MCAO

The MCAO model has been more favourable due to its clinical relevance to stroke. The MCA is the most common site of occlusion in a thromboembolic stroke (Mohr, 1986). The degree and the territory of infarct depend on the length of occlusion, amount of collateral blood flow to the region and the site of occlusion in the MCA. There have been various methods of MCAO developed and they can range from permanent or transient, proximal or distal occlusion (for overview, see Durukan et al., 2008; Sicard et al., 2009; Traystman, 2003).

Initial methods of MCAO involved craniotomy and required animals to be under constant anaesthesia if reperfusion was intended. The first method used had a fronto-parietal approach with ligation of MCA distally (Robinson et al., 1975). This method however, was not able to produce infarcts consistently. Later, modification by approaching sub-temporally and thus ligating MCA more proximally, led to reproducible infarcts (Tamura et al., 1981). Both these methods produced permanent occlusions. To overcome this, clips or ligatures were placed on the distal portions of the MCA after craniotomy (Brint et al., 1988; Shigeno et al., 1985). This method however, led to large variability in infarct characteristics depending on site of occlusion. To lessen the impact of a craniotomy, a photochemical model was developed (Watson et al., 1985). Using a small craniotomy, beams from a laser were used to permanently ligate the MCA distal branches. However, this method was restricted by its inability to allow reperfusion.

Methods were developed to avoid the surgically invasive craniotomy. A group of investigators used the principle of embolus. Initially a microsphere was introduced into the CCA embolising to the distal MCA. The size of the microsphere determined the location of the occlusion, thus the characteristics of the infarct (Hegedus et al., 1985). This model however only allowed permanent ischaemia. Blood clot embolus was an alternative that allowed reperfusion. Blood clot fragments from the same animal were introduced in either the CCA or external carotid artery (Kaneko et al., 1985). This method was modified to increase reproducibility of the location of infarct by using pre-formed clots from a different animal (Wang et al., 2001). This modification provided an excellent model for research into putative thrombolytics. However, the reperfusion time point was not clearly defined. This makes it harder to study mechanisms of putative neuroprotectants. Concurrently, another group of investigators used application of a vasoconstricting agent to MCA (Macrae et al., 1993). There was a significant occlusion of MCA after exposure to this agent and reperfusion was allowed following removal of the agent.
Although, the occlusion occurred relatively quickly after application, the reperfusion process was gradual. This again led to an unknown time to restoration of normal perfusion.

1.6.4 MCAO by intraluminal filament

The most popular method used presently is MCAO by intraluminal filament. This model was first developed in the 1980’s to allow transient ischaemia, without craniotomy (Koizumi et al., 1986). A suture was inserted into the internal carotid artery (ICA) and advanced through to the MCA. The suture end, which was thickened by silicone, was placed distally into the MCA. This prevented collateral circulation from the ICA, posterior and anterior communicating arteries. To further reduce the collateral circulation the CCA was permanently ligated (Koizumi et al., 1986). This model catered for both permanent and transient occlusion (depending on whether the intraluminal filament was removed).

Later modifications were made to this method by Longa et al. (1989). These included heat thickening the suture end and occlusion of all branches of the ECA and ICA to reduce collateral circulation. However, suture modification led to a drop in success rate from 93 % to 58 % (Koizumi et al., 1986; Longa et al., 1989). This was a huge drop compared to the original results of 100 % in the permanent model of occlusion by Tamura and colleagues (Tamura et al., 1981). An extensive study found the drop to be due to incomplete occlusion of the MCA (Laing et al., 1993). Subsequently, another modification was made. The suture was coated by poly-L-lysine, and this increased adhesion to the arterial wall, leading to a complete occlusion (Belayev et al., 1996). However, there are also higher occurrences of subarachnoid haemorrhages (Bederson et al., 1995; Laing et al., 1993). The differences within the types of the nylon 4-0 filament also significantly influences the outcome (Kuge et al., 1995). This implicates a large variability between laboratories and in addition, within the same setup if the filament is changed. It has been recently suggested that silicone-rubber coated filaments is better than heat blunted or poly-L-lysine coated sutures. The success rates range from 66% to 100% whilst the subarachnoid haemorrhage rates decrease to 8% (for review, see Liu et al., 2009). The authors also suggest optimizing the filament to the weight of the animal and obtaining these filaments commercially.

Sprague-Dawley rats have been commonly used in this model. The analysis of the MCA patterns in a large number of this species found a significant variability, even though the rats
are of an inbred strain. Its consequences are increased infarction variability (Kahveci et al., 2000). The animals also have to be of a particular age and a minimum weight, as proximal MCAOs in younger animals (less than 150 grams) do not result in infarcts (Coyle, 1982). Numerous modifications of this method can also be found in the literature indicative of the large variations between each laboratory setting (Kahveci et al., 2000).

In addition, there are other possible confounding factors. The MCAO by intraluminal filament damages the hypothalamus and other surrounding nuclei, which are essential in thermoregulation (He et al., 1999; Li et al., 1999). The resultant hyperthermic state would be neurodegenerative, since hypothermia has been shown to be neuroprotective (see Section 1.2.1). The use of anaesthetics, such as halothane or isoflurane, in the surgical procedure is a confounding variable. In rodent cerebral IR, neuroprotective effects were seen with these anaesthetics (Liu et al., 2009; Popovic et al., 2000). Most anaesthetics work via NMDA receptor and K$^+$ channel blockade. Hence, if the putative drug was acting via this K$^+$ channel mechanism then the anaesthetic agent would confound the actual effect (Liu et al., 2009). However, these effects can be normalised. For example, in an experiment testing the efficacy of a therapeutic, the different arms of the study should be subjected to the same duration of anaesthesia. Other important aspects relating to the control of surgical parameters is the regulation of mechanical ventilation and maintenance of blood pressure. Hypoxia, hypercapnia and hypotension can compromise cerebral blood flow, so parameters should be adjusted during the surgery according physiological variables (Liu et al., 2009). In addition, plasma glucose levels can be altered as a consequence of both ischaemia and anaesthesia. Both extremes of hyperglycaemia and hypoglycaemia can exacerbate neuronal damage and thus must be monitored. These confounding variables make the surgical procedure complex and lengthy.

Despite the disadvantages, this model is the most practical and clinically relevant. It is less invasive than craniotomy approaches, and it does not require a long duration of anaesthesia (between ischaemia induction and reperfusion). This makes this model the most ethically acceptable and clinically relevant. It has the flexibility of permitting either permanent or transient ischaemia protocols to be applied. Furthermore, the precise control of the reperfusion onset permits a more accurate study of the neuroprotective mechanism of putative agents. Therefore, MCAO by intraluminal filament procedure was employed to attain cerebral IR in this study.
1.7 MELATONIN

1.7.1 Synthesis of melatonin

Melatonin, a hormone, in the impure form of bovine pineal gland extract, was first reported to blanch the tadpole skin by McCord and Allen (McCord et al., 1917). Melatonin was then isolated from bovine pineal gland and shown to disperse melanin in epidermal melanocytes (Lerner et al., 1958). It was characterised as N-acetyl-5-methoxytryptamine (Figure 1.13, Lerner et al., 1959). Given its structure, it is highly permeable across lipid membranes and so is not stored for later release (Costa et al., 1995). Melatonin readily diffuses into the capillary bed (Arendt, 2000) within the pineal gland as well as into the cerebrospinal fluid of the third ventricle (Tricoire et al., 2002).

The pineal gland is the major source of melatonin. Unlike other endocrine organs, melatonin synthesis in the pineal glands is regulated by the light: dark environment, and thus is one of the end organs of the visual system (Hardeland, 2008; Reiter, 2003). Instead of the photoreceptor cells used for sight, specialised neurons containing a unique photopigment are involved in the modulation of melatonin synthesis (Brainard et al., 2001; Foster et al., 2002). Therefore, the physical (light) stimulus is converted to a neuronal signal, which via the retinohypothalamic tract is conveyed to the hypothalamus (Figure 1.14, Reiter, 2003).

![Chemical structure of melatonin](Image)

*Figure 1.13 The chemical structure of melatonin (adapted from Reiter, 2003)*
Figure 1.14 The circuitous pathway from the hypothalamus to pineal gland showing the control of light stimulus on melatonin production. The order of the neuronal pathway: retina, optic nerve, anterior hypothalamus, suprachiasmatic nucleus (SCN), paraventricular nuclei, intermediolateral cells of upper thoracic cord, preganglionic sympathetic neurons, superior cervical ganglia, postganglionic sympathetic neurons, pineal gland. In the absence of the light cues, the SCN drives the endogenous circadian rhythm of pineal melatonin production. Light modulates the SCN and suppresses the synthesis of melatonin. In the absence of photic cues, melatonin feedbacks onto the master clock to regulate circadian rhythms, via its receptors (adapted from Reiter, 2003).

Noradrenaline released from the postganglionic sympathetic neurons binds to beta-adrenergic receptors on the pinealocytes. The resultant increased intracellular cyclic adenosine monophosphate (cAMP) causes stimulation of the rate-limiting enzyme in melatonin production. Arylalkylamine-N-acetyltransferase (AA-NAT, EC 2.3.1.87) is required to acetylate serotonin to N-acetylserotonin. The second enzyme in melatonin synthesis is hydroxyindole-O-methyltransferase (HIOMT, EC 2.1.1.4), but it is not rate limiting (Figure 1.15, Reiter, 2003).
Figure 1.15 The mechanism of synthesis of melatonin in the pinealocyte. Activation of $\alpha_1$- and $\beta$- adrenoreceptors leads to increase cAMP, resulting in activation of enzymes responsible for melatonin synthesis. AC – adenylate cyclase, ATP – adenosine triphosphate, cAMP – cyclic adenosine monophosphate, DG – diacylglycerol, G – G-protein, HIOMT – hydroxyindole-O-methyltransferase, IP – inositol phosphate, NAT – N-acetyltransferase, NE – norepinephrine, PI – phosphatidylinositol-3-phosphate, PKC – protein kinase C, PLC – phospholipases C (adapted from Reiter, 2003).

The pineal gland is the major source of melatonin production, however other tissues have also been shown to synthesise the hormone. High levels of melatonin were found in retina (Cardinali et al., 1974). However, most investigators have utilised the localisation of the rate-limiting enzymes in melatonin synthesis, AA-NAT and HIOMT, as an aid in finding sites of melatonin synthesis (for review, see Simonneaux et al., 2003). The AA-NAT activity has been detected in several brain regions of the rat (Gaudet et al., 1991), while AA-NAT mRNA has been found in several regions of the brain of different species including human (Coon et al., 1996; Hamada et al., 1999; Shi et al., 2004; Uz et al., 1999). In addition, AA-NAT has a nocturnal rhythm, with the activity correlating well with melatonin levels and the mRNA expression several hours prior (for review, see Simonneaux et al., 2003). This time lag may be explained by the time required for mRNA to be expressed as a functional protein.

The second major enzyme involved in the melatonin synthesis pathway, HIOMT, seemed to be rate limiting as its activity was lower than that of AA-NAT (for review, see Simonneaux et al., 2003). The lower levels have made it harder to detect circadian rhythms, however, more recent studies have found a significant increase in activity during the dark phase (Ribelayga et
Chapter 1: Introduction

HIOMT activity has been found in retina of various species including humans (Dkhissi et al., 1998; Wiechmann et al., 1989; Wiechmann et al., 1987; Willbold et al., 2002). Other human tissues where one of these enzymes have been found are: lymphocytes (Carrillo-Vico et al., 2004a), placenta (Iwasaki et al., 2005) and gall bladder (Aust et al., 2004).

However, both HIOMT and AA-NAT have been shown to metabolise other compounds not related to melatonin synthesis (Axelrod et al., 1961). If the anabolic enzymes of melatonin were to be effectively used to determine melatonin production, both the enzymes should be localised in the same tissue. Both enzymes have been localised in several human tissues outside the central nervous system (CNS): human placenta (Iwasaki et al., 2005), lymphocytes (Lardone et al., 2009), thymus (Naranjo et al., 2007), human pituitary gland, adrenal gland, myometrium, skin and melanoma cells (Slominski et al., 2002).

This shows the diverse sources of melatonin, and indicates a paracrine/autocrine role of the compound (for review of paracrine effects of melatonin in retina, see Tosini et al., 2003). The higher levels of melatonin synthesis in the pineal gland account for the circulating melatonin. The hormonal effect of melatonin may be attributed to its high levels in the circulation.

1.7.2 Physiology of melatonin

Melatonin has a daily diurnal rhythm. This was first shown in chicken brains (Pang et al., 1974). In humans, levels are higher by 10-20 fold during night (Reiter, 2003; Wetterberg, 1978). In nocturnal animals this rise in melatonin still occurs, showing that melatonin synthesis is independent of activity, but dependent on exposure to light (Wehr, 2001). There is large inter-individual variance for melatonin levels (Macchi et al., 2004). The amplitude of the melatonin peak and the total melatonin produced seems to be genetically determined (for review, see Reiter, 2003).

Melatonin has been linked to several physiological roles. These are: circadian rhythm, sleep promotion, immune function, sexual reproduction, metabolism, depression, gastrointestinal homeostasis, bone, cardiovascular physiology and pain regulation (for comprehensive reviews on physiology of melatonin, see Pandi-Perumal et al., 2006; Reiter, 2003). Only functions of melatonin pertaining to the immune function are described.
Melatonin has been established to have immuno-modulatory functions (for thorough review, see Carrillo-Vico et al., 2005; Radogna et al., 2010). A functioning pineal gland is necessary for primary lymphoid organ development and immune function (Brainard et al., 1988; Cunnane et al., 1979; McKinney et al., 1975; Vaughan et al., 1971). Altered immune functions were seen after pinealectomy in various species, and this was normalised with melatonin administration (Carrillo-Vico et al., 2005). Furthermore, indirect evidence was gathered from correlating diurnal melatonin rhythms with immune cell proliferation and function, such as lymphocytes (Paglieroni et al., 1994; Radogna et al., 2010). Higher melatonin levels are associated with increased immune cells and this is reflected in increases in thymus and spleen (Rai et al., 2003). Melatonin has been shown to increase synthesis of major histocompatibility complex, this increases the antigen presentation to T cells (Pioli et al., 1993). In addition, melatonin directly increases gene expression of various cytokines, TGF-β, IL-1β and IFN γ (Liu et al., 2001). The effect on TNFα by melatonin is debatable, with reports suggesting both increases and decreases in its expression (Liu et al., 2001; Pioli et al., 1993; Raghavendra et al., 2001). Furthermore, chronic administration of melatonin induced a T-helper 2 (Th-2) response via increasing IL-10 and decreasing TNFα. Carrillo-Vico and colleagues have argued that effects of melatonin are seen when immune system has been artificially depressed. It has been postulated that the immunostimulatory effect of melatonin is via opiateergic mechanism, as an opioid antagonist attenuated the effect of melatonin (Maestroni et al., 1987). These effects of melatonin in vivo are not as clearly supported by in vitro studies. Stimulation and inhibition of proliferating immune cells have been noted (Drazen et al., 2000; Lopez-Gonzalez et al., 1998), whereas some studies have noted no effect (Pahlavani et al., 1997; Wolffler et al., 1998). Furthermore, melatonin inhibits cytokines and genes which are associated with the Th1 response, for example, IFN γ and TNFα (Capelli et al., 2002; Di Stefano et al., 1994). Thus, melatonin has also been implicated to induce the Th1 response (for review see Carrillo-Vico et al., 2005). To add to this complex role of melatonin, locally produced melatonin by human lymphocytes stimulated IL-2 production in an autocrine or paracrine manner (Carrillo-Vico et al., 2004a). The receptors of melatonin also play a vital role (see Section 1.7.3). Nonetheless, clinically, in human immunodeficiency virus-1 infected individuals it was found that decreased melatonin levels were correlated with impairment of Th1 immunoresponse (Nunnari et al., 2003). Melatonin has a very complicated role in the immune system (for reviews, see Carrillo-Vico et al., 2005; Radogna et al., 2010), which is yet not clearly understood and its clinical implications remain a subject of further research.
1.7.3 **Melatonin receptors**

Melatonin exerts some of its effects via activation of its receptors. The functional characterisation of receptors has led to two major categories of receptors, nuclear and membrane (Figure 1.16, Dubocovich et al., 2003).

The nuclear receptors for melatonin belong to the subfamily of ROR (retinoid orphan receptors)/ RZR (retinoid Z receptors). The double nature type of the ROR/RZR is constitutive and ligand-dependent (for review on nuclear receptors of melatonin, see Smirnov, 2001). This double nature may explain the difficulty in examining the roles of these receptors and the lack of the research in the area. The subfamily of the ROR/RZR nuclear receptors includes the products of three genes: variants of RORα (RORα1, RORα2, RORα3, RZRα) differing in the N-terminal domain, RZRβ, and RORγ, which have a domain organisation typical for nuclear receptors (Figure 1.16, Giguere et al., 1994; Hirose et al., 1994).

Originally melatonin membrane receptors were classified according to the $^{125}$I-iodomelatonin binding affinities, with ML1 being in the picomolar range and ML2 in the nanomolar range (Dubocovich, 1995; Dubocovich, 1988). ML1 has two subclasses, MT1 and MT2, whilst ML2 melatonin binding site is known as MT3 (Figure 1.16, Dubocovich et al., 2003). This thesis will focus on the MT1 and MT2 subclasses.

![Diagram](image)

**Figure 1.16** The subtypes of melatonin receptors and its classifications. There are two major categories, nuclear and membrane, with each having sub-classes. The denotation MT in the membrane receptors implicates its presence in mammals.
In order to maintain timely and efficient cellular responses to maintain homeostasis, it is essential to regulate signal transduction events via these receptors. Melatonin has been demonstrated to regulate its own receptors, both positively and negatively. Radioligand binding studies have found an inverse correlation with maximal available binding sites and plasma melatonin concentrations (de Reviers et al., 1991; Gauer et al., 1993; Gauer et al., 1992; Guerrero et al., 1999; Masson-Pevet et al., 2000b). Melatonin administration can also lead to receptor ‘supersensitisation’. Masana et al., achieved supersensitisation of hMT$_1$ receptors expressed in Chinese Hamster Ovary cells, after a limited exposure (8 hours) to a physiological concentration of melatonin (400 pM) followed by a 16 hour withdrawal (Masana et al., 2003). Thus, melatonin has the ability to directly modulate its receptor expression.

The inverse relationship between melatonin levels and its receptors seem to be more related to MT$_1$, and not MT$_2$. In some animal species, the MT$_1$ mRNA has been shown to have a circadian rhythm and to be inversely related to melatonin levels (Guerrero et al., 2000; Neu et al., 1997; Poirel et al., 2002; Sallinen et al., 2005; Schuster et al., 2000). Conversely, with MT$_2$ expression there is no variation or rhythm of expression (Gillespie et al., 2003; Natesan et al., 2002; Sallinen et al., 2005). However, in animals like the Wistar rat, no variation in MT$_1$ was found (Sugden et al., 1999). This is supported by other studies that conclude a variation in binding profiles exists between species (for review, see Stankov et al., 1991). Differences in expression of receptors have been seen in hemispheres of the same animal (Wiechmann et al., 1992), sexes of same species (Guerrero et al., 1994) and ages (Laitinen et al., 1992). There seems to be a general decline with age in the receptor expression (Duncan et al., 1993; Laitinen et al., 1992; Pang et al., 1997; Wan et al., 1997). The binding sites in the PT have also been shown to be sensitive to sex steroids (Bentley, 2003; Gauer et al., 1993; Gilad et al., 1997; Soares et al., 2003). This interaction may play a part in the role of melatonin in reproductive physiology. Melatonin receptors seem to be part of an intricate system involving actions of melatonin.

The development of the high affinity 2-$[^{125}\text{I}]$-iodomelatonin allowed the identification of the binding sites in discrete neuronal tissues (Dubocovich et al., 1987). Although the radioligand binds to both recombinant MT$_1$ and MT$_2$ receptors, it appears to be restricted to MT$_1$ in the native mammalian tissues (Dubocovich et al., 1998; Liu et al., 1997). This is shown by its absence in the SCN and thalamic areas of transgenic mice with MT$_1$ receptor deletion (Liu et al., 1997). However, studies have shown that radioligand binding sites may not correspond to the MT$_1$ and MT$_2$ expressions (Mazurais et al., 1999). Furthermore, results from autoradiography has been shown to be difficult to replicate and reliable (for review, see
Stankov et al., 1991). Therefore, in order to study MT₁ and MT₂ distribution, either mRNA or its protein structure (by immunohistochemistry) must be localised. Using reverse transcriptase-polymerase chain reaction (RT-PCR), in situ hybridization and immunohistochemistry (with specific MT₁ and MT₂ antibodies), both MT₁ and MT₂ receptors have been localised in different species including humans (for recent review on melatonin receptor distribution, see Dubocovich et al., 2010). This widespread distribution implicates a widespread role of melatonin via the receptors’ activation.

Both MT₁ and MT₂ receptors have been shown to interact with different G proteins depending on availability of each G protein subtype in host cells (Brydon et al., 1999). The MT₁ and MT₂ melatonin receptors have been shown to have distinctive structural features, which potentially leads to unique binding pockets for ligand recognition (Dubocovich et al., 2003). Thus, the activation of each receptor leads to different signalling pathways (Figure 1.17).

Activation of the MT₁ receptors also potentiates PGF₂α and ATP – mediated stimulation of phospholipase C, via the release of the βγ subunit (Godson et al., 1997; Roka et al., 1999). The mechanism of MT₁ receptor stimulation increasing potassium conductance by the activation of G protein-coupled inwardly rectifying potassium channel, may also involve activation of the βγ subunit (Nelson et al., 1996). Furthermore, activation of the MT₁ melatonin receptor also increases phosphorylation of extracellular-signal-regulated kinase (ERK) 1 and 2, and mitogen-activated protein kinase/ERK 1 and 2, (Chan et al., 2002; Witt-Enderby et al., 2000) and increases phosphoinositide hydrolysis (MacKenzie et al., 2002). In addition, there is increased intracellular Ca²⁺ and decreased phosphorylated CREB (Figure 1.17, for review, see Dubocovich et al., 2003).

Activation of MT₂ receptors inhibits forskolin-stimulated cAMP formation (Petit et al., 1999; Reppert et al., 1995) and cGMP accumulation (Petit et al., 1999), and increases phosphoinositide hydrolysis (MacKenzie et al., 2002). Like MT₁, activation of MT₂ also leads to increased intracellular Ca²⁺ and cyclic guanine monophosphate levels and decreased phosphorylated CREB (Figure 1.17, for review, see Dubocovich et al., 2003).
Figure 1.17 The activation of A) MT₁ and B) MT₂ receptors by melatonin binding. The activation eventually leads to increased levels of intracellular Ca²⁺ and decreased P-CREB. ATP – adenosine triphosphate, BKCa – calcium activated potassium channel, cAMP – cyclic adenosine monophosphate, cGMP – cyclic guanine monophosphate, CREB – cAMP responsive element binding protein, DAG – diacylglycerol, ER – endoplasmic reticulum, ERK – extracellular-signal-regulated kinase, GTP – guanosine triphosphate, GMP – guanosine monophosphate, IBMX – isobutylmethlyxantine, IP₃ – inositol 1,4,5 trisphosphate, Kir3 – G protein gated K⁺ channel, MEK – mitogen-activated protein kinase, Mel – melatonin, PGF₂α – Prostaglandin F₂α, PIP₂ – phosphatidylinositol 4,5-biphosphate, PKA – protein kinase A, PLC – phospholipase C, VDCC – voltage-dependent K⁺ channel (adapted from Dubocovich et al., 2003).
Development of receptor ligands has led to discovery of a wide variety of functions exhibited by melatonin membrane receptors (Table 1.3, Dubocovich *et al.*, 2010). Recently, there has been an increasing number of non-selective MT$_1$/MT$_2$ receptor agonists being either trialled or approved for clinical use. Some of the prominent ones are Agomelatine® for depression, Ramelteon® and Circadin® for insomnia and circadian sleep disturbances (for review, see Dubocovich *et al.*, 2010) Therefore, the melatonin receptors highlight a possible avenue for therapeutic intervention.

<table>
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<tr>
<th>System</th>
<th>Function</th>
<th>Receptor</th>
<th>Reference</th>
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<tr>
<td></td>
<td>Inhibition of neuronal firing in the SCN</td>
<td>MT$_1$</td>
<td>(Jin <em>et al.</em>, 2003; Liu <em>et al.</em>, 1997)</td>
</tr>
<tr>
<td></td>
<td>Inhibition of dopamine release from rabbit retina</td>
<td>MT$_2$</td>
<td>(Dubocovich <em>et al.</em>, 1997)</td>
</tr>
<tr>
<td></td>
<td>Prevention of depression (MT$_1$ knockout mice more depressed and poor sensorimotor gating), + MT$_1$/MT$_2$ agonist clinically effective against major depressive disorders*</td>
<td>MT$_1$</td>
<td>(den Boer <em>et al.</em>, 2006; Kennedy <em>et al.</em>, 2006; Loo <em>et al.</em>, 2002; Pjrek <em>et al.</em>, 2006; Rouillon, 2006; Weil <em>et al.</em>, 2006)</td>
</tr>
<tr>
<td>Hypothalamic-hypophyseal-gonadal axis</td>
<td>Depression of GABA$_A$ receptor function</td>
<td>MT$_2$</td>
<td>(Wan <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td></td>
<td>Stimulation of neurite (cytoskeleton) formation in neuroblastoma cells</td>
<td>MT$_1$</td>
<td>(Bordt <em>et al.</em>, 2001)</td>
</tr>
<tr>
<td></td>
<td>Pain modulation</td>
<td>MT$_1$/MT$_2$</td>
<td>(Ray <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td></td>
<td>Inhibition of prolactin secretion</td>
<td>MT$_1$</td>
<td>(von Gall <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td></td>
<td>Regulation of <em>Per1</em> gene expression</td>
<td>MT$_1$</td>
<td>(von Gall <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td></td>
<td>Suppression of GnRH mRNA expression and release</td>
<td>MT$_1$/MT$_2$</td>
<td>(Roy <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td>System</td>
<td>Effect</td>
<td>Type</td>
<td>Reference</td>
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<tr>
<td>-----------------------------</td>
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</tr>
<tr>
<td>Inhibition of cortisol</td>
<td>MT&lt;sub&gt;1&lt;/sub&gt; (Torres-Farhan et al., 2003)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>secretion</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Inhibition of testosterone</td>
<td>MT&lt;sub&gt;1&lt;/sub&gt; (Frungieri et al., 2005)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>secretion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>Vasoconstriction</td>
<td>MT&lt;sub&gt;1&lt;/sub&gt; (Vishwanathan et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>Vasodilatation</td>
<td>MT&lt;sub&gt;2&lt;/sub&gt; (Doolen et al., 1998;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Masana et al., 2002; Roka et al., 1999)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune</td>
<td>Enhancement of splenocyte proliferation</td>
<td>MT&lt;sub&gt;2&lt;/sub&gt; (Drazen et al., 2001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Attenuation of PGE&lt;sub&gt;2&lt;/sub&gt; inhibition on IL-2 production</td>
<td>MT&lt;sub&gt;1&lt;/sub&gt; (Carrillo-Vico et al., 2003)</td>
<td></td>
</tr>
<tr>
<td>Metabolism</td>
<td>Increase of leptin expression in adipocytes</td>
<td>MT&lt;sub&gt;1&lt;/sub&gt; (Alonso-Vale et al., 2005)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inhibition of insulin secretion</td>
<td>MT&lt;sub&gt;1&lt;/sub&gt; (Kemp et al., 2002)</td>
<td>MT&lt;sub&gt;2&lt;/sub&gt; (Muhlbauer et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Decrease glucose uptake in adipocytes</td>
<td>MT&lt;sub&gt;2&lt;/sub&gt; (Brydon et al., 2001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Promotion of lipolysis in adipocytes</td>
<td>MT&lt;sub&gt;1&lt;/sub&gt;/MT&lt;sub&gt;2&lt;/sub&gt; (Zalatan et al., 2001)</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>Increase duodenal mucosal secretion of bicarbonate</td>
<td>MT&lt;sub&gt;2&lt;/sub&gt; (Sjöblom et al., 2001)</td>
<td></td>
</tr>
<tr>
<td>Carcinogenesis</td>
<td>Oncostatic effect on breast cancer cells</td>
<td>MT&lt;sub&gt;1&lt;/sub&gt; (Collins et al., 2003; Yuan et al., 2002)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oncostatic effect on murine melanoma cells</td>
<td>MT&lt;sub&gt;1&lt;/sub&gt; (Kadekarok et al., 2004)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oncostatic effect on androgen sensitive prostate cancer cells</td>
<td>MT&lt;sub&gt;1&lt;/sub&gt; (Xi et al., 2001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inhibition of rat hepatoma cells</td>
<td>MT&lt;sub&gt;2&lt;/sub&gt; (Dauchy et al., 2003)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.3** The effects of melatonin mediated by its membrane receptors in various physiological and pathological systems. * denotes currently in clinical use. GABA<sub>A</sub>, γ-aminobutyric acid receptor (A subtype).
1.7.4 Antioxidant properties of melatonin

Ianas et al. (1991) first showed the antioxidant property of melatonin. They found melatonin to be prooxidative in lower concentrations (< 0.25 µmole/mL), while antioxidant at higher concentrations in vitro (Ianas et al., 1991). Many different investigators have confirmed melatonin to be an effective free radical scavenger and a general antioxidant (Figure 1.18, Reiter, 2003). Toxic free radicals such as the hydroxyl radical (‘OH), hydrogen peroxide (H₂O₂), peroxynitrite anion (ONOO⁻), hypochlorous acid (HOCl) and the singlet oxygen (¹O₂), have been shown to be scavenged by melatonin (Allegra et al., 2003; Tan et al., 2002).

In addition, to its direct scavenging properties, melatonin is an antioxidant by indirect effects on other enzymes. Levels of antioxidant enzymes, such as glutathione, are increased with melatonin-stimulated increase of mRNA (Franceschini et al., 1999). The glutathione recycling is promoted by melatonin, which ensures that it is predominantly in its reduced form (Reiter et al., 2000). Apart from increasing the levels of superoxide dismutase mRNA (Antolin et al., 1996; Garcia-Maria et al., 2011), melatonin also maintains its functional integrity (Reiter, 2003).

Concurrently, the levels of pro-oxidant enzymes are down regulated, these include 5- and 12-lipo-oxygenases (Manev et al., 1998; Uz et al., 1998; Zhang et al., 1999). The inhibitory effects of melatonin on NOS (Bettahi et al., 1996; Gilad et al., 1998; Pozo et al., 1994; Storr et al., 2002), also overlap with its role as an anti-inflammatory (see Section 1.7.5). Moreover, melatonin improves oxidative phosphorylation in the mitochondria and thus reduces free radical generation (Acuna-Castroviejo et al., 2007).

Melatonin via a series of free radical detoxification gets converted to cyclic 3-hydroxymelatonin \( \rightarrow \) N¹-acetyl-N²-formyl-5-methoxykynuramine (AFMK) \( \rightarrow \) N¹-acetyl-5-methoxykynuramine (AMK, see Figure 1.18). The conversion of AFMK to AMK seems to be predominantly enzymatically converted (Pandi-Perumal et al., 2006). The metabolism of melatonin to AFMK can consume up to 10 free radicals (reviewed in Tan et al., 2007), highlighting the potency of melatonin as a direct scavenger. Furthermore, AFMK has been shown to be the major metabolite in non-hepatic tissues (Hardeland et al., 2005; Tan et al., 2003a) and in unicellular organisms (for review see Tan et al., 2007). AFMK has been shown to scavenge up to four free radicals on its own (Rosen et al., 2006; Tan et al., 2001). AFMK matches melatonin in the capacity to scavenge O₂⁻⁻ (Maharaj et al., 2002). In a variety of models, AFMK has been demonstrated to prevent oxidative stress, for example by preventing
lipid peroxidation (Burkhardt et al., 2001; Liu et al., 2002; Onuki et al., 2005; Tan et al., 2001). These studies prove that AFMK is most likely to be a biologically active antioxidant. Moreover, AMK has greater antioxidant capabilities when compared with AFMK (Ressmeyer et al., 2003). Both AFMK and AMK have anti-inflammatory properties, in addition to their anti-oxidant properties (see Section 1.7.5, for review on properties of melatonin metabolites, see Hardeland et al., 2009; Tan et al., 2007).

Furthermore, melatonin has been shown to be catabolised in liver by cytochrome P450 to 6-hydroxymelatonin. This enzymatic conversion has also been seen in non-hepatic tissues such as cerebral cortex of higher vertebrates (for review see Tan et al., 2007). 6-hydroxymelatonin also has been shown to be an antioxidant (Liu et al., 2002; Maharaj et al., 2003; Matuszak et al., 2003). The metabolite was shown to prevent lipid peroxidation caused by oxidative stress in various tissues of rats (Hara et al., 1997). Studies using other models of oxidative stress, have also found protection of DNA by the metabolite (for review see Tan et al., 2007). Hence, regardless of the source/pathway of metabolism of melatonin, the metabolites seem to be having antioxidant properties as well.

Melatonin has been well established to be a potent antioxidant. However, recent evidence shows that its metabolites have equally, if not more potent, antioxidative effects. Different metabolites from these free radical scavenging properties of melatonin have now been referred to as “scavenging cascade reaction of melatonin” (Tan et al., 2007). This highlights the importance of melatonin as a highly potent physiological antioxidant.
Figure 1.18 One of the possible mechanisms of direct scavenging of free radicals by melatonin, leading to formation of N¹-acetyl-N²-formyl-5-methoxykynuramine (AFMK). This conversion step consumes up to 4 free radicals (adapted from Vijayalaxmi et al., 2004).

1.7.5 Anti-inflammatory properties of melatonin

As discussed in Section 1.7.2, melatonin has a physiological role in the immune system. Cytokines such as TNFα, IL-1β and IFN-γ have been shown to be modulated by melatonin
Chapter 1: Introduction

(Carrillo-Vico et al., 2005). In addition, melatonin has an effect on nuclear transcription factors. For example, melatonin directly inhibits NF-κB (Bruck et al., 2004; Mazzon et al., 2006). Melatonin, with regulation of NF-κB is able to modulate expression of adhesion proteins on leukocytes (Radogna et al., 2010). NF-κB is also a transcription factor necessary for iNOS expression (Kim et al., 1995; Vila-Del Sol et al., 2007). There is overwhelming evidence of inhibition of iNOS by melatonin (for review see Cuzzocrea et al., 2002). Melatonin by directly inhibiting NF-κB expression and binding has been shown to decrease iNOS protein levels in in vitro and in vivo models of inflammation (Alonso et al., 2006; Gilad et al., 1998; Mazzon et al., 2006; Mohan et al., 1995). Furthermore, activation of NF-κB leads to upregulation of COX-II expression and subsequent COX activity (Chen et al., 2000; Rivest, 1999; Slogoff et al., 2004). Therefore, melatonin would be able to inhibit COX systems as well via modulation of NF-κB. This is supported by in vivo models showing inhibition of PG levels and COX-II expression by melatonin (Cuzzocrea et al., 1999). Moreover, melatonin is able to inhibit other arachidonic acid metabolism pathways such as phospholipase A via activation of its receptors (for review, see Radogna et al., 2010). Hence, it seems that the majority of the anti-inflammatory role of melatonin can be attributed to modulation of cytokines and nuclear transcription factors (see Figure 1.19).

Moreover, other effects of melatonin on inflammation have been documented (for reviews on anti-inflammatory effects of melatonin, see Cuzzocrea et al., 2001; Cuzzocrea et al., 2002). Poly (ADP-ribose) synthetase activity (PARS) is also attenuated by melatonin administration. PARS is activated after the damage of DNA, and leads to severe energy depletion and necrotic cell death (Cuzzocrea et al., 2002). Finally, melatonin reduces the neutrophil infiltration into the inflammatory site (Figure 1.19). However, this effect may be secondary to intact endothelial cells, due to reduction of the antioxidant damage (Cuzzocrea et al., 2001).

Furthermore, metabolites of melatonin have most recently been shown to be anti-inflammatory (for reviews, see Hardeland et al., 2009; Tan et al., 2007). Like melatonin, the metabolites have anti-inflammatory effects at different levels of the inflammatory process. AFMK was able to inhibit TNFα and IL-8 production by LPS-induced neutrophils and lymphocytes (Silva et al., 2004). AMK was shown to inhibit NOS activity and NO levels (Entrena et al., 2005; Hardeland, 2005; Leon et al., 2006). The anti-inflammatory effects of the metabolites are extended to COX enzymes as well. Initially, AMK was shown to inhibit PGs levels (Kelly et al., 1984). Later experiments showed that both AFMK and AMK were able to inhibit mRNA levels of COX-II (Mayo et al., 2005). Hence, in inflammation, the increase in ROS levels
would be scavenged by melatonin, which in turn leads to anti-inflammatory metabolites, AFMK and AMK.

Figure 1.19 The proposed mechanism of anti-inflammatory role of melatonin. Melatonin acts as an inhibitor at various levels of the inflammation cascade as shown. The effects of metabolites of melatonin are not shown for simplicity (adapted from Cuzzocrea et al., 2001).

Melatonin has multiple anti-inflammatory effects. These anti-inflammatory effects are present even after melatonin has been consumed by its free radical scavenging. The studies on its metabolites have been fairly recent, and as more research is done, there seems to be accumulating evidence of the potency of melatonin on inflammation.

1.7.6 The role of melatonin in neurodegeneration

Melatonin has two major properties, antioxidant and anti-inflammatory, which make it a viable candidate for treating various pathologies, including neurodegeneration. There are three major neurodegenerative disorders associated with oxidative stress and inflammation: Alzheimer’s Disease (AD), Parkinson’s Disease (PD) and stroke. The most widely researched is the effect of melatonin on AD, followed by stroke and PD.
AD is manifested clinically by progressive loss of cognitive function, memory and higher cerebral capacity. Pathologically, it is characterized by cerebral atrophy with β amyloid plaques and neurofibrillary tangles, resulting in increased oxidative stress in the CNS (reviewed by Pappolla et al., 2000). The amyloid β peptide leads to mitochondrial initiated apoptosis (reviewed by Takuma et al., 2005). Melatonin has been shown to be beneficial in pathological models of AD via multiple mechanisms. A number of in vitro studies showed the antioxidant and anti-inflammatory properties of melatonin to be beneficial (for review, see Srinivasan et al., 2010). In addition, melatonin was able to prevent neurotoxic effects of β amyloid independent of its antioxidant actions (Benitez-King et al., 2003; Pappolla et al., 1997; Wang et al., 2004b). Melatonin also attenuated the formation of fibrils, an indicator of pathological progression of AD (Pappolla et al., 1998). Furthermore, melatonin membrane receptors may also play a role, as indicated by receptor changes in post mortem human samples (Brunner et al., 2006; Savaskan et al., 2007; Savaskan et al., 2001; Savaskan et al., 2002; Wu et al., 2007). Not surprisingly, melatonin administration in early onset AD not only decreased oxidative stress, but improved cognition and increased survival rates (Feng et al., 2004; Matsubara et al., 2003). Interestingly, protection afforded by melatonin may be limited in early AD. In mice with end stage AD, i.e. with well established amyloid plaques, chronic melatonin treatment was unable to prevent oxidative stress and β amyloid levels (Quinn et al., 2005).

A major problem with AD patients is the occurrence of disruption of sleep rhythms and worsening of symptoms, such as increased agitation, restlessness and confusion in evenings (known as sundowning). These effects may be due to disruption of melatonin rhythm as shown in a number of clinical studies (Liu et al., 1999; Mishima et al., 1999; Skene et al., 1990; Weldemichael et al., 2010; Zhou et al., 2003). These abnormalities in melatonin physiology have been accredited to high monoamine oxidase-A activity, decreasing precursors for melatonin synthesis (Wu et al., 2003). Two modes of therapeutic intervention have been used in AD. Firstly, exogenous melatonin in a number of small clinical trials was found to effective in reversing sleep disturbances and sundowning symptoms (Asayama et al., 2003; Cardinali et al., 2002; Fainstein et al., 1997; Mahlberg et al., 2004; Singer et al., 2003). Two trials with more than 100 patients have examined effects of melatonin on cognitive impairment. Both found improvement of cognitive symptoms and quality of life (Riemersma-van der Lek et al., 2008; Wade et al., 2007). Another treatment avenue used has been bright light therapy. Trials found similar results with elimination of sundowning symptoms and restoration of sleep cycle (Brusco et al., 2000; Satlin et al., 1992; Yamadera et al., 2000). Maximal benefit has been observed when combining bright light therapy and exogenous melatonin (Riemersma-van der
Thus, melatonin is beneficial clinically in improving circadian rhythms and mild cognitive impairment. Larger trials may be conducted in the future for evaluating efficacy in preventing cognitive decline and sleep cycle disturbances using melatonin receptor agonists (for review, see Srinivasan et al., 2010).

Similarly, to AD, there are numerous pathophysiological processes involved in cerebral IR (see Section 1.1.4). These include excitotoxicity, peri-infarct depolarisations, inflammation and apoptosis. Melatonin may have several different mechanisms of action in conferring neuroprotection post cerebral IR (Figure 1.20). Firstly, melatonin by activating the MT$_2$ receptors in the cerebral blood vessels would lead to vasodilation (see Section 1.7.3). This may be able to aid in normalising the perfusion of the ischaemic region. Secondly, melatonin has been shown to prevent neurons from excitotoxic damage (Cazevieille et al., 1997; Cheung, 2003; Giusti et al., 1996a; Giusti et al., 1996b; Husson et al., 2002; Kim et al., 1999). This action may involve membrane receptors, as activation of MT$_2$ receptors in neurons led to K$^+$ current inhibition and prevented subsequent apoptosis (Jiao et al., 2004). More importantly, it was shown in vivo that melatonin was able to prevent both NMDA and non-NMDA glutamate receptor-mediated excitotoxicity (Kim et al., 1999). Glutamate receptor mediated excitotoxicity is the initial cause of neurodegeneration, post cerebral IR. The resultant ROS production from excitotoxicity contributes to the tissue injury process (see Section 1.1.4). Melatonin and its metabolites have been well established as potent antioxidants (see Section 1.7.4). This antioxidant effect of melatonin would be contributing to a large part of its neuroprotective effects. Furthermore, melatonin and its metabolites have immune modulatory (see Section 1.7.2) and anti-inflammatory effects (see Section 1.7.5). These effects would combat the resultant inflammatory cascade which would have been initiated after cerebral IR. Mitochondria are pivotal in the apoptosis which occurs concurrently with inflammation, thus contributing to the neurodegeneration (see Section 1.4.3). Melatonin prevents ROS induced mitochondrial damage, via its antioxidant properties. In addition, melatonin has several effects on the mitochondrial homeostasis and prevents mPTP opening (for reviews see León et al., 2005; León et al., 2004). Its metabolites, AMK and AFMK, have been shown to protect mitochondria from oxidative damage, while also influencing homeostasis of the organelle (for review, see Acuña-Castroviejo et al., 2007; Cervantes et al., 2008). These prove that melatonin and its metabolites would be able to prevent mitochondrial damage in cerebral IR and thus able to offer neuroprotection. Taken together, there is overwhelming evidence of various neuroprotective mechanisms of melatonin in cerebral IR (see Figure 1.20).
Figure 1.20 The possible mechanisms of melatonin in achieving neuroprotection. The major facets of action are via the anti-oxidant and anti-inflammatory properties of the putative neuroprotectant (adapted from Pandi-Perumal et al., 2006).

These neuroprotective mechanisms of melatonin have been proven in experimental models of cerebral IR. Initially, it was found that pinealectomy led to greater infarcts in global ischaemia (Manev et al., 1996). A recent meta-analysis of 42 in vivo studies found an overall significant neuroprotective effect after cerebral IR with acute therapy of melatonin. Multiple doses were significantly more effective than single dose of melatonin (O'Collins et al., 2010). These studies confirm the actions of melatonin on the inflammatory and apoptosis cascades, as they occur over a longer duration. Moreover, chronic prophylactic treatment (4 mg/kg/day for 9 weeks) with melatonin was also found to be effective in cerebral IR (Kilic et al., 2004a).

While clinical efficacy of melatonin in stroke has not yet been tested, the effects of stroke on melatonin physiology have been studied. Clinically, the melatonin rhythms (determined by measuring urine metabolites) were impaired from 3 days up to 14 days after stroke. This disruption to physiological levels of melatonin was correlated with disturbed sleep rhythms and moods of stroke patients (Beloosesky et al., 2002; Fiorina et al., 1999). Whether this decrease in urinary metabolites of melatonin results from consumption of melatonin by the pathological processes or decreased secretion is not known. Given the overwhelming evidence
of neuroprotection attained by melatonin after cerebral IR in experimental models, it may be reasonable to assume that melatonin would be able to confer similar effects clinically.

To summarise, melatonin has been acknowledged to have neuroprotective effects. Future trials using larger cohorts are expected to test the efficacy of melatonin in improving functional outcomes in stroke.

1.8 THESIS OBJECTIVES

This study hypothesises that melatonin administration after stroke injury is neuroprotective through a number of different pathways. Thus, the general objective was to look at multiple facets of melatonin-induced neuroprotection such anti-inflammatory and anti-apoptotic mechanisms as well as melatonin membrane receptor involvement. Specific aims were based around this major objective:

1) To confirm the effectiveness of melatonin treatment when administered after onset of stroke. A modified dosing regime with 5 mg/kg i.p. was used. Toxic effects of melatonin were also evaluated.

2) To investigate the change in distribution of melatonin and its membrane receptors after stroke. Using immunohistochemistry techniques the protein distribution was assessed using an ordinal scale.

3) To assess the effect of melatonin on major inflammatory enzyme systems, NOS, arginase and COX, when melatonin is administered after the onset of stroke. Animals were subjected to 2 hours of ischaemia and multiple doses of melatonin or vehicle was given.

4) To examine the effects of melatonin on L-arginine metabolism in vitro in a high arginase expressing cell line (human fibrosarcoma HT-1080 fibroblasts). Key enzymes were studied after unstimulated HT-1080 fibroblasts were treated with melatonin for 72 hours.

5) To assess the effects of post-treatment with melatonin on mitochondria after MCAO. Key mitochondrial ETC complexes, citrate synthase and aconitase enzymes were examined.
CHAPTER 2

MATERIALS AND METHODS
2.1 MATERIALS

All inorganic materials were obtained from BDH, New Zealand, organic from Sigma, New South Wales, Australia, and chemical gases from BOC Gases, Dunedin, NZ. Antibodies were from Santa Cruz Biotechnology, Inc., California, USA, and small surgical equipment from Harvard Apparatus, USA. The sources of other reagents are listed in Table 2.1, whilst the equipment has been listed in Appendix (Table A.1).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Source</th>
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<tr>
<td>Alanine Aminotransferase Diagnostic Kit</td>
<td>Medica Pasifica Ltd., NZ</td>
</tr>
<tr>
<td>Alexa Fluor® 488 rabbit anti-goat antibody</td>
<td>Molecular Probes, USA</td>
</tr>
<tr>
<td>Alexa Fluor® 555 goat anti-rabbit antibody</td>
<td>Molecular Probes, USA</td>
</tr>
<tr>
<td>Ammonium persulphate (APS)</td>
<td>Biorad, NZ</td>
</tr>
<tr>
<td>Betadine antiseptic</td>
<td>Faulding Pharmaceuticals, NZ</td>
</tr>
<tr>
<td>Biodegradable scintillation fluid</td>
<td>Amersham, UK</td>
</tr>
<tr>
<td>Biotinylated rabbit anti-sheep antibody</td>
<td>Zymed, USA</td>
</tr>
<tr>
<td>Bis Acrylamide (1:29)</td>
<td>Biorad, NZ</td>
</tr>
<tr>
<td>Blotto, non-fat milk powder</td>
<td>Biorad, NZ</td>
</tr>
<tr>
<td>Bupivacain 0.5 – 20%</td>
<td>Healthcare Otago, NZ</td>
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<tr>
<td>Dulbecco’s Modified Eagle’s Medium (DMEM)</td>
<td>Invitrogen Life Technologies, NZ</td>
</tr>
<tr>
<td>Enhanced chemiluminescence (ECL™) reagents</td>
<td>Amersham, UK</td>
</tr>
<tr>
<td>Foetal calf serum (FCS)</td>
<td>Invitrogen Life Technologies, NZ</td>
</tr>
<tr>
<td>[^3H] L-arginine</td>
<td>Amersham, UK</td>
</tr>
<tr>
<td>[^3H] PGE₂</td>
<td>Amersham, UK</td>
</tr>
<tr>
<td>[^3H] Thymidine</td>
<td>Amersham, UK</td>
</tr>
<tr>
<td>Halothane</td>
<td>Rhodia, NZ</td>
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<tr>
<td>Heparin sodium multiparin</td>
<td>CP Pharmaceuticals Ltd, UK</td>
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<tr>
<td>Hibitane gluconate 20%</td>
<td>Multichem, NZ</td>
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<td>Hoescht stain</td>
<td>Molecular Probes, USA</td>
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<tr>
<td>Immersion oil, Immersol (518 N), non-fluorescent</td>
<td>Carl Zeiss Ltd., Germany</td>
</tr>
<tr>
<td>Immersion oil, Immersol (518 F), fluorescent</td>
<td>Carl Zeiss Ltd., Germany</td>
</tr>
<tr>
<td>Kaleidoscope prestained broad range MW markers</td>
<td>Biorad, NZ</td>
</tr>
<tr>
<td>Liquid nitrogen</td>
<td>Electron Microscope Unit, University of Otago, NZ</td>
</tr>
<tr>
<td>N, N, N’, N’-tetramethylenediamine (TEMED)</td>
<td>Biorad, NZ</td>
</tr>
</tbody>
</table>
Neutral buffered formalin (10%) | Histology Services Unit, University of Otago, NZ
---|---
Photographic developer | Kodak, NZ
Photographic fixer and replenisher | Kodak, NZ
Photographic stop bath | Kodak, NZ
Polyclonal rabbit anti-human MT2 antibody | Lifespan Biosciences, USA
Polyclonal rabbit anti-human spermine antibody | QED Biosciences, USA
Polyclonal sheep anti-human melatonin antibody | Biogenesis, UK
Potassium aluminium sulphate dodecahydrate | Fluka, Switzerland
Rabbit anti-human prostaglandin E2 | Sigma, USA
Swiss 3T3 fibroblasts | Department of Physiology, University of Otago, NZ
Vectorstatin ABC Elite® Kit | Vector Laboratories, USA

Table 2.1 List of chemicals and their sources.

## 2.2 ANIMAL MAINTENANCE

Male Sprague Dawley rats (250 ± 20 g) were obtained from the Hercus Taieri Resource Unit, University of Otago under the Animal Ethics Committee guidelines (number 26/03). The animals were then housed in the Adams Building Animal Care Facility, Department of Pharmacology & Toxicology, University of Otago until sacrifice. Animals were normally housed communally, unless being fasted (which was just prior to surgery). The animals were also kept in isolation immediately after the surgery for monitoring. All animals were allowed to acclimatize in a 12-hour light/dark cycle (0700/1900 hours), at 25°C in a humidity controlled environment for at least three days. Around 1600 hours daily, the body weight was measured and the environmental conditions were checked. Standard rat diet and water were available *ad libitum*, except overnight prior to surgery when the rats were fasted and only water was available.
2.3 MCAO MODEL (LONGA et al., 1989)

2.3.1 Preparation and allocation of animals

There were two groups for the histological studies: 1) MCAO melatonin treated group (5 mg/kg i.p., see Section 2.5.1), and 2) a MCAO DMSO vehicle treated group. For the biochemical study, an additional group of naïve non-intervention controls, was included. The immunohistochemical study compared non-intervention controls and non-treated MCAO rats. The animals were overnight fasted at a set weight of 285 ± 10 grams, overnight to surgery. The animals were randomly assigned to each treatment/control group. Animals with sudden weight gains and body weights over the pre-fasting weight criterion were chosen as non-intervention (controls).

The use of sham MCAO untreated control animals in this series of experiments was not included. For a true sham controlled experiment, there would be a need to have two groups whereby one group is just to control for anaesthesia and the other for the surgery without occlusion of MCAO. The project used a negative control with a complete absence of neuronal damage (non-intervention control) and a positive control where there would be maximal impact of the IR injury (MCAO + vehicle treatment). The extent to which surgery should be carried out in the MCAO sham is arguable. It can be argued that a true sham would include insertion of an intraluminal thread into MCA, without occluding the artery. A second sham protocol would employ a neck midline incision followed by blunt dissection and manipulation of the ECA and ligation of its branches. The extent of vascular ligation of (as done prior to the filament insertion) in the sham control is therefore variable. There is also the need to take into consideration whether cannulation of femoral artery should take place with blood pressure monitoring and blood gas sampling in the sham control. Furthermore, MCAO is a common model used to explore ischaemic neuronal injury and studies have already previously compared sham and non-interventional controls. Recently, Mariucci and colleagues (2007) investigated 72 kDa heat-shock protein and protein synthesis in synaptosomal fractions in rats following permanent (irreversible) MCAO. The study used four groups in this particular study: MCAO, sham controls, anaesthesia controls and passive (non-interventional) controls. They used chloral hydrate as their anaesthetic and found there were no differences between any of the control groups for both the end points (heat shock protein levels or protein synthesis) measured. This would be expected as sham controls in MCAO, hypothetically, should not have any neuronal injury. It is indeed a common practise when
investigating efficacy of melatonin in the MCAO model to not use a sham control and instead to compare differences between melatonin treated MCAO and vehicle treated MCAO groups (Koh, 2008a; Koh, 2008b; Koh, 2008c; Koh, 2008d; Koh, 2008e; Kondoh et al., 2010; Pei et al., 2004; Pei et al., 2003a; Pei et al., 2002a; Pei et al., 2003b; Pei et al., 2002b) The objectives of these studies were to investigate the neuroprotective effects of melatonin after MCAO. Hence these studies did not use a sham MCAO control with or without melatonin treatment. The main objective of this study is to investigate the effects of melatonin after cerebral IR injury, and thus the main comparison for treatment efficacy is between MCAO + melatonin (treatment) and MCAO + vehicle groups (positive control). The sham control + melatonin treatment group was consequently not used, as this particular study does not intend to study the physiological effects of melatonin nor compare the difference in response of melatonin with and without MCAO.

Nonetheless, it is important to consider factors in the experimental protocol that could potentially confound the results. Firstly, the use of anaesthetics during the surgical procedure has been shown to have a neuroprotective effect. In an in vitro model of cerebral IR injury it was found that halothane (the gaseous anaesthetic used in MCAO procedure) was neuroprotective through a preconditioning pathway (Popovic et al., 2000). Halothane, however, seems to produce less cerebral protection than other agents such as isoflurane (Xiong et al., 2003) and desflurane (Haelewyn et al., 2003). When comparing the MCAO animals to controls, the cerebral infarct is reduced with the use of halothane. This may make differences from MCAO more difficult to detect when compared to controls. In addition, the use of halothane or similar anaesthetics reduces the blood pressure of the animal. This reduced blood pressure may lead to decreased cerebral perfusion thereby leading to neurological deficits. To minimise the possible effects of decreased blood pressure, continuous monitoring of this physiological parameter was carried out via a femoral artery catheter (see Section 2.3.5). Halothane levels were changed to maintain the blood pressure at normal levels throughout the procedure. In addition, the lengthy surgical procedure required prolonged mechanical ventilation. Blood gases were measured at two time points: prior to neck incision and after intraluminal thread insertion (see Sections 2.3.5 and 2.3.8). The variables on the ventilation machine were adjusted to maintain normal blood gas physiology. The second blood gas pO₂ measurements were generally lower than the first readings, but always within the normal range. In addition, the stress of the surgical procedure may cause release of corticosteroids in the animal. This may subsequently affect the blood glucose levels, and higher glucose levels are known to exacerbate neurological damage following stroke. Blood glucose levels were measured at the end of the surgical procedure as an indicator of surgical stress on the animal.
(see Section 2.3.8), but were not elevated in any of the animals. Moreover, the infarct area may include the hypothalamus and other surrounding nuclei, which are essential in thermoregulation (He et al., 1999; Li et al., 1999). The resultant hyperthermic state would be neurodegenerative, since hypothermia has been shown to be neuroprotective (see Section 1.2.1). Core body temperature was continually monitoring using a rectal probe linked to a homeothermic blanket allowing body temperature to be maintained at 37°C (see Section 2.3.5).

2.3.2 Preparation of intraluminal occlusion threads (Belayev et al., 1996)

The 4-0 Dafilon, polyamide, monofilament sutures were used to make the intraluminal threads. The nylon suture was straightened with gentle heating followed by creation of a 3 mm thickened end with a lighter flame. The 30 mm thread was then coated in a 0.1% poly-L-lysine solution (prepared before and stored at -30°C) and incubated at 60°C for approximately 60 minutes. Just prior to the neck incision (see Section 2.3.4), the threads were removed from the oven and painted with liquid non-toxic paper, 17 mm from the tip of the thickened end to mark the distance to which the thread was inserted into the MCA.

2.3.3 Equipment preparation

The surgical area was set up as shown in Figure 2.1. The conditions were made aseptic with disinfectant (virulex) and the surgical drapes (clear plastic sheets) were rinsed with chlorohexane. The surfaces, which were to be in contact with the hands of the surgeon during the surgery, were wrapped in tin foil and sprayed with 70% ethanol. In addition, the surgical equipment and other instruments were sterilized by washing in pyroneg solution.

A small animal ventilator was set at approximately 97.5 breaths/minute with a 300 cubic centimetres/minute flow rate and 0.4 seconds as inspiratory time, while the flow rates of N₂O and O₂ were set to 500 and 150 cubic centimetres/minute, respectively.

2.3.4 Animal anaesthesia

The intubation area was set up with the following equipment: an endotracheal tube with a guide wire insert, cotton tips, one pair of large forceps, a light source with a long curved glass end and a thin piece of gauze. The barrel of a 10 mL syringe was kept for resuscitation when
necessary. To straighten the rat’s trachea during intubation, a mobile loop of silk was taped on the side of the table.

The rat was placed under light anaesthesia, with 5 – 8 mL of halothane in a large dessicator jar. The preferred level of anaesthesia was established when the respiration rate of the animal (observed by its diaphragm movement) was slower than that of the ventilator. The rat was then removed and placed ventrally on the table and the trachea was straightened by securing the incisors with the loop of silk. The flashlight, that replaced the large forceps used initially to move aside the tongue, was then inserted into the oral cavity as far as permissible. After the clearing of mucus (or blood during intubation) with cotton tips, the endotracheal tube with the wire insert was slotted in between the oscillating vocal cords, which provided a slight resistance. The guide wire was removed and the correct insertion of the endotracheal tube was reconfirmed by placing a piece of gauze at the entrance of the tube. Reintubation was required if the animal had stopped breathing or with the incorrect insertion of the endotracheal tube (into the oesophagus).

The intubated animal was then moved to the surgical slate and the endotracheal tube connected to the ventilation machine. To begin with, the halothane was administered at 3% and was continuously decreased at a rate of 0.5% every 2 minutes until reaching the maintenance level of 0.75%. The halothane rate was continuously readjusted according to the rat’s level of anaesthesia and blood pressure, throughout the course of the surgery. The ventilator machine outlet was linked to a scavenging system via the positive end expiratory pressure (PEEP) system (the outlet was submerged 2 cm below water in a closed conical flask). This system prevented air entering the lungs through the endotracheal tube (the bubbles from the submerged tube exited in phase with the ventilation rate). The rat’s synergic breathing with the ventilation machine and the lack of its pedal reflex (of the left leg) indicated the correct level of anaesthesia. Three of the limbs and the ventilation machine connection were then taped onto the slate.
Figure 2.1 The aerial view of the surgical setup for MCAO. \(O_2\) oxygen, \(N_2\)O nitrous oxide, \(H_2O\) water and PEEP positive end expiratory pressure. The blood pressure was monitored by the Mac Lab setup. Figure created and reproduced with permission of Karere Inc.

2.3.5 Blood pressure and gas sampling

A K-Y jelly lubricated rectal probe was inserted and secured with tape. The rectal probe was connected to the homeothermic control unit and a warming blanket (placed under the animal, see Figure 2.1). The incision areas were shaved with clippers and sterilized with Betadine soaked gauze swabs. The surgical drape with openings for the incision sites was then placed on the rat and secured. The area along an approximate length of 25 mm of the femoral artery was uncovered by blunt dissection of skin and connective tissue using blunt curved forceps and dissecting scissors. Fine dissecting forceps were then used to isolate the femoral artery from other structures in the femoral sheath. The distal end was first permanently ligated, with a braided suture, while the proximal end was temporarily clamped with a pair of micro serrefines. 0.5% Bupivacaine was used to dilate the incision site in the lumen of the artery, which was made medially with a pair of microscissors. The arterial catheter was connected to a 1 mL syringe filled with 0.9% saline (pH 7.4) and flushed to remove air. The smaller end of
Chapter 2: Materials and Methods

the catheter was then inserted as far proximal as possible (after removal of the temporary clamp), and secured with a knot on the artery. The middle section of the catheter was taped on the slate in such a manner, maintaining it at the same elevation as the insertion site.

The first blood gas sample was then obtained in a 70 μl heparinized capillary tube, which was then mixed. The 1 mL syringe was connected back to the catheter to flush away the blood (blood remnants in the line would clot and cause blockage). The cleared line was attached to the physiological pressure transducer, which was connected to an Apple computer via a MacLab and Bridge Preamplifier. The blood pressure measurements were taken from the MacLab Chart v 3.5 program. The normal blood pressure range was 80 – 130 mmHg.

Arterial blood gas data (pCO₂, pO₂ and pH) were obtained from Otago Diagnostic Laboratories (Dunedin Public Hospital, NZ). Ventilation rate and halothane administration was adjusted according to the blood gas data and the blood pressure readings. Normal blood physiology was pH 7.40 – 7.45, pCO₂ 30 – 40 mmHg and pO₂ 100 – 150 mmHg.

2.3.6 Neck incision and exposure of the carotid artery

Following an incision (approximately 40 mm) along the midline of the neck, the connective tissue between the left and right salivary glands was blunt dissected to uncover the trachea. Similarly, dissection between the right sternocleidomastoid muscle and trachea exposed the carotid arteries. Claw retractors, used to allow access to the arteries, were secured on the slate. Using blunt forceps, the connective tissue around the right CCA was separated and a thread, looped around the CCA, was clamped to the secured haemostats. This thread was unrestricting and used only in cases of accidental bleeding during surgery. Upon separation of connective tissue around the ECA, two ligatures were placed, one as superior as possible and the second slightly inferior. To free the ECA ‘stump’, the electrocautery pen was used to cauterize between the two ligatures; the isolated occipital and superior thyroid branches of the ECA. The ICA was also temporarily ligated in a manner similar to that of the CCA. The pterygopalatine artery was also isolated and permanently ligated. In addition, any further connective tissue that could hinder the path of the occlusion thread was separated with caution, especially around the Vagus nerve.
2.3.7 **Insertion of the intraluminal thread**

The carotid arteries were secured and the temporary ligatures were tightened to minimize the blood flow within the ICA. A small incision (< 1 mm) was made on the ECA stump as far distal to the CCA bifurcation as possible, with the microscissors. The occlusion thread (for preparation see Section 2.3.2) was introduced with the thickened end first into the incision site, maneuvered around the CCA bifurcation, and fed up until the ICA ligature. The temporary constriction was removed and the thread was gently inserted until resistance was felt or the 17 mm paint mark reached the CCA bifurcation (refer to Figure 2.2). The thread was then secured and any excess thread was removed. The tension on the CCA was then removed, allowing restoration of normal blood flow. The wound was closed last, to allow monitoring of any possible bleeding at the site.

2.3.8 **Blood sampling and closure of wounds**

The second blood gas sample was taken (as outlined in Section 2.3.5), and a further drop was extracted to measure the blood glucose levels via the Accu-Chek Advantage II test strips and glucose meter (normal for rats is 2.75 – 7.4 mM). The femoral artery catheter was removed and both wound sites were cleaned of any debris before closure (neck wound closed by continuous stitch, while femoral wound by multiple surgeon’s knots). 0.5% Bupivacaine was applied at the sites for temporary local anaesthesia. After the rat was able to breathe independently, the endotracheal tube was removed and the animal was kept in isolation in the surgical suite. In addition, all major time points during the surgery and any significant events were recorded. This data was continuously reviewed and variance between each group was measured.
Chapter 2: Materials and Methods

2.3.9 Reperfusion surgery

The start of the reperfusion surgery was timed in a fashion that would allow the reperfusion of the ischaemic area precisely at 2 hours post occlusion. The animal was anaesthetised (see Section 2.3.4) and placed on the surgical slate. Instead of the endotracheal tube, the animal was ventilated via a nose cone apparatus with 2% halothane, 1000 mL/min of O₂ and 2000 mL/min N₂O. The neck wound site was unstitched and the carotid arteries were exposed using the claw retractors. The occlusion thread was carefully withdrawn until the thickened end was near the insertion point. The ligature was then retightened and the wound was closed after all debris had been removed. The animal was placed on its right side until complete...
Chapter 2: Materials and Methods

consciousness was reached. The animal was isolated and monitored occasionally over a 2-hour period post-surgery.

2.3.10 Experimental inclusion criteria

Animals were included only in the study if they met the behavioural criterion (unable to turn clockwise pre-reperfusion, i.e. exhibiting damage to the relevant motor cortex), and were able to survive until scheduled sacrifice at 3 days post reperfusion. In addition, all the physiological parameters measured were to be within two standard deviations of the mean and there were no signs of haemorrhage at sacrifice (after extraction of brain). Since haemorrhagic strokes have a different aetiology (see Section 1.1.1), they were not included in the study.

2.4 IMMUNOHISTOCHEMICAL STUDIES

2.4.1 Tissue extraction and fixation

The control non-intervention and the stroke animals were sacrificed at 0200 hours in a dark environment (with very low intensity red light exposure of less than 2 minutes). The numbers in each group were kept at a minimum as this was planned to be a qualitative analysis. The objective of this qualitative study was to identify gross changes and thereby act as a pilot study for quantitative studies examining smaller regions of interest. Qualitative studies have been previously used to similar effect, and are sensitive to gross changes (Savaskan et al., 2005; Savaskan et al., 2001, Sutherland et al., 2009). The initial number of MCAO animals was planned to be double that of controls to account for the relatively high rate of failure of the surgical procedure.

The brain was then extracted by using bone rongeurs. Conversely, for paraffin embedding (microtomy), the tissue was immersed in 10% neutral buffered formalin for 7 days at room temperature prior to being wax embedded by the Histology Service Unit.
2.4.2 Poly-L-lysine coating of slides

Frosted microscope slides were immersed in poly-L-lysine solution (1:10 dilution) for 5 minutes. They were then allowed to air dry at room temperature overnight and then stored for use.

2.4.3 Tissue fixation for wax embedded microtomy

A rotary microtome was used to cut 8 \( \mu \)m thick coronal sections at 1 mm intervals from the frontal lobe to give at least 15 interval samples. Each section after being cut was placed in a 30% alcohol solution for approximately 30 seconds followed by a distilled water bath at 40°C for approximately 1 minute, to flatten out the section. The section was then placed on the poly-L-lysine coated frosted slides (see Section 2.4.2) and left to air dry in a 37°C incubator overnight. The slides were then stored at room temperature until use.

2.4.4 Immunolabelling

The microtomy sections were placed in xylene to remove the paraffin wax, for 30 minutes. They were then rehydrated for 5 minutes in descending concentrations of alcohol, 100%, 90%, 70%, 50%, followed by 5 minutes in TBS (high Sodium [0.5 M] to eliminate non-specific binding). This was followed by 30 minutes in quenching solution (5 mL \( H_2O_2 \) in 495 mL ethanol) to quench endogenous peroxidase activity and then by 5 minutes in distilled water, to remove the quenching solution and 5 minutes in TBS to rehydrate the tissue. The blocking serum (200 \( \mu \)L of rabbit or goat serum in 10mL of TBS with 0.1% bovine serum albumin, BSA), was placed on the slides in a humidified chamber at room temperature for 30 minutes to block non-specific immunoglobulin G (IgG) binding. The excess liquid was wiped off and the slides were incubated with the primary antibody (diluted in TBS with 0.1% Triton X, 0.1% BSA, 0.01% sodium azide) overnight in a humidified chamber at 4°C and coverslipped. The slides were then washed three times 5 minutes each with TBS and 1% Triton-X 100. Next, the secondary antibody was added to slides, in the humidified chamber and incubated. The secondary antibody was diluted in TBS with 1% blocking serum. The slides were then washed three times with 5-minute wash cycles in TBS. The Vectastatin ABC Elite® Reagent was made up, by adding 100 \( \mu \)L of Reagent A to 10 mL of TBS, containing 0.1% Triton-X 100, followed by addition of 100 \( \mu \)L Reagent B. The solution was allowed to stand for 30 minutes.
prior to the addition to the slides in the humidified chamber. The slides were incubated with the reagent for 30 minutes at room temperature.

2.4.5 3’3’ Diaminobenzidine tetrahydrochloride (DAB) labelling

The Sigma *Fast™* DAB tablet set was dissolved in 30 mL distilled water and filtered in the dark using Grade 1 Whatman filter paper. The sections were then submerged in DAB solution in the dark for an optimal amount of time for sufficient colour development. The time of DAB exposure was optimized on each different day by visualising colour development under a Zeiss Axiostar microscope. The slides were then immersed in distilled water to stop the DAB reaction, and were given three five-minute washes in distilled water to remove any excess DAB particles.

2.4.6 Harris’ Haematoxylin

Distilled H$_2$O (500 mL) was heated to 60°C and 50 g of potassium aluminium sulphate dodecahydrate was added. The solution was then heated to 75°C and haematoxylin solution (2.5 g in 25 mL absolute ethanol) was added. The solution was boiled at 100°C for approximately 1 minute. While the solution was hot, 1.25 mg of yellow mercuric (II) oxide was added. Once the dark purple colour developed with a golden sheen, the solution was allowed to cool at room temperature. This was followed by the addition of 20 mL of glacial acetic acid once the temperature had dropped below 40°C. The solution was then filtered with a Grade 1 Whatman filter paper and stored until use.

2.4.7 Nuclear counter staining

The haematoxylin stain was used where no post DAB processing took place. For the sections, the optimum time of exposure to haematoxylin was 1 second, followed by 10 seconds in distilled water and then they were left in running tap water for 5-7 minutes, until the nuclei stained a deep blue. If the section was over counterstained, it was immersed in differentiating solution (350 mL methanol, 5 mL HCl and 145 mL distilled H$_2$O). The sections were then mounted (see Section 2.4.12).
2.4.8 Mounting of slides

Only the haematoxylin counter-stained slides were first dehydrated by immersion for 5 minutes in ascending concentrations of ethanol (50%, 70%, 90% and 100%). For both the counter-stained slides, once all residual liquid on the slide had evaporated, they were cleared in xylene for 5 minutes, in a fume cupboard. The slides were then mounted in Di-n-butyl phthalate in xylene (DPX) and cover slipped.

2.4.9 Preadsorption analysis

Slides were processed as outlined in Sections 2.4.7. However, the primary antibody step differed. To establish the specific binding of the melatonin receptor and spermine antibodies, one set of slides was incubated with the primary antibody (dilution 1:50; 1:2000 respectively), and another set with preadsorbed primary antibody overnight at 4°C in a humidified chamber. The preadsorbed primary antibody consisted of a 1:50 or 1:2000 dilution of primary antibody in high sodium TBS [0.5 M] with 1 µM of melatonin or spermine, and was incubated overnight at 4°C in a rotary shaker. Both sets of slides were then immunolabelled as described in Section 2.4.7.

2.4.10 DAB saturation curve

Slides were processed as outlined in Sections 2.4.7, for MT$_1$ and MT$_2$ antibodies. A range of DAB exposures were trialled according to previous studies (Ma et al., 2006). It was ensured that the DAB was fully saturated. Excess DAB was then washed off and sections were counterstained and mounted as previously described. All samples were then compared to the DAB saturation curve. There are multiple variables such as temperature which can affect the DAB reaction. It was not possible to run all the samples at once with all variables fixed. Thus, all the samples were compared with the DAB saturation curve, to ensure that saturation did not occur giving false positive results.
2.4.11 Morphometric analysis

Regional and Cellular Analysis

Following sectioning of the brain, each section was grossly matched and identified using a rat neuroanatomy atlas (Paxions and Watson, 1997). The macroscopic pictures of the sections were photographed with an AxioCam HR colour digital CCD camera attached to a 22 X macro lens. Representative sections are shown for an ideal animal as matched to the neuroanatomy atlas (Paxinos et al., 1997). Nuclei were grouped according to the neuroanatomical regions (Pirker et al., 2000). Verification of the location of each of the regions in the neuroanatomy atlas (Paxinos et al., 1997) is shown in Section A.2.

Immuno-DAB stained sections (8 µm) were examined under high power (63 ×) with non-fluorescent immersion oil (Immersol) using a Zeiss Axioplan Microscope and dedicated AxioCam HR colour digital camera. Immuno-positive cells were photographed in each region and identified based on overall morphology with the aid of histological reference books (Lesson et al., 1988; Rhodin, 1974; Young et al., 2000). Immuno-positive cells in each region were noted and scored using a pre-defined ordinal scale (Sutherland et al., 2008). Brain regions were scored on an ordinal scale from 0 to 4 depending on the number of positive cells (see Table 2.2)

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No positively labelled cells present in the region</td>
</tr>
<tr>
<td>1</td>
<td>Few dispersed positively labelled cells present in the region</td>
</tr>
<tr>
<td>2</td>
<td>Some positively labelled cells present in the region</td>
</tr>
<tr>
<td>3</td>
<td>Many positively labelled cells present in the region</td>
</tr>
<tr>
<td>4</td>
<td>Most cells were positively labelled in the region</td>
</tr>
</tbody>
</table>

Table 2.2 Ordinal scale used for single labelling IHC based on previous study by Sutherland et al., (2009).

Although the general shift in neuroscience has been toward fully quantitative stereological techniques, subjective ordinal scales are still being utilised (Berishvili et al., 2006; Grundtman et al., 2007; Smith et al., 2006; Spanjaard et al., 2007). The object of this study was to conduct a pilot study and observe gross changes in immunoreactivity with MCAO. Thus, while this study was qualitative in nature, semi-quantitative analysis could be carried out with its small sample size. This has been achieved previously (Sutherland et al., 2008). Ideally, quantitative methods like stereology and Western blot of particular regions would have been utilised.
However, these methods are extremely time consuming and a rationalised approach identifying the regions of interest, is necessary before carrying out such extensive quantitative methods in the entire brain. Therefore, this study was designed to carry out a qualitative assessment of the gross observable differences.

**Measurement of intensity**

Optical density (OD) was measured with the aid of Image J software (v 1.43u). For the calculation of DAB saturation curve, blood vessels were examined in sections exposed to the substrate for different periods and background intensity standardised in order to measure the actual OD. Similarly, OD of the immunobinding to the blood vessels were also computed. The values were then compared to the saturation curve.

### 2.5 DOSING REGIME OF MELATONIN FOR THE MCAO EXPERIMENT

#### 2.5.1 Preparation and administration of melatonin

Animals for the histology and biochemical studies received either melatonin at a dose of 5 mg/kg dissolved in 1 mL of TBS (pH 7.4) with 5% dimethyl sulphoxide (DMSO), or the vehicle of 1 mL TBS with 5% DMSO. The dose of 5 mg/kg was similar to previous observations (for review see Macloed *et al.*, 2005). In the meta-analysis by Macleod and co-workers, it was observed that the lowest dose of melatonin that was most consistent with neuroprotection was 5 mg/kg. There was a dose-dependent relationship to 50 mg/kg, after which the neuroprotective effect plateaued (Macleod *et al.*, 2005). Hence, it was decided to use the minimal dose at which melatonin was effective in conferring neuroprotection.

The solution of melatonin was warmed slightly to ensure higher solubility, and all preparations and dosage were carried out with minimal light exposure to melatonin. The immediate weight of the animal was used to ensure an accurate level of dosing. Intraperitoneal dosages were administered via a 27 G x 0.5” needle connected to a 3 mL syringe at 1-hour post ischaemia and 2300 hours at day 1 and 2 (with ischaemia induced on day 0). The dosages at 2300 hours were carried out in a dark environment with a low intensity red light in the background, and were aimed to boost the physiological levels of melatonin which peaked at 0200 (Laudon *et al.*, 1988).
Chapter 2: Materials and Methods

2.6 THE EFFECTS OF MELATONIN ON INFARCT SIZE AFTER MCAO

2.6.1 Tissue extraction and fixation

The animals were sacrificed without anaesthesia by guillotine 72 hours post ischaemic insult. Initially an equal number of animals (n = 11) was assigned to each treatment group. With the aid of rongeurs the brain was extracted and immediately stored in TBS on ice. In a cold room (4°C), the brain was placed in a 1 mm coronal brain matrix and a single edged scraper blade was used to cut 1 mm thick coronal sections (Figure 2.3). The sections were then placed into the individual wells of a 12 – well cell culture plate already containing the TBS solution. Animals were excluded if any sign of haemorrhage was visible after sectioning. Results (infarct sizes) outside two standard deviations of the means of each treatment group were considered outliers and excluded from the study. For example, if a smaller infarct size occurred in the control group, which made it considerably different (i.e. smaller than two standard deviations from the mean of the group), it was excluded from further analysis.

Figure 2.3 The extracted rat brain. Perpendicular lines represent the approximate 1 mm intervals of sectioning. Slice 1 was the first slice from the pole, while slice 8 was the last.

2.6.2 2, 3, 5 – Triphenyltetrazolium chloride staining (Bederson et al., 1986)

The 2,3,5 – triphenyltetrazolium chloride (TTC) solution (3% in TBS) was prepared and incubated in the dark, prior to decapitation. At room temperature and in a light deficient environment, the TBS from the well plate was replaced with TTC. The cell culture plate was
then wrapped in tin foil to maintain the activity of the light sensitive compound and was then incubated in a 37°C water bath. After 10 minutes, the slices were flipped over and incubated for a further 10 minutes prior to fixation with 10% neutral buffered formalin. This resulted in any metabolically active (i.e. viable) regions staining red with regions without active mitochondria remaining unstained (thus appearing white).

2.6.3 Quantification of the relative infarct size

The 1 mm brain slices were photographed with the AxioCam HR colour digital CCD camera attached to a 22 X macro lens. The Axiovision™ computer programme was used to take the photographs and measure the area (in mm²) of the infarct (cortex and striatum parts), the total ipsilateral and contralateral hemisphere. The results were recorded and entered into a spreadsheet for further analysis. The infarct volume was determined by calculating the area under the curve for infarct area versus slice interval.

2.7 BIOCHEMICAL ANALYSIS OF THE EFFECTS OF MELATONIN ON MCAO

2.7.1 Tissue extraction

The animals were sacrificed without anaesthesia by guillotine, at 0200 hours on day 3 following ischaemia (with ischaemia induced on day 0). Initially an equal number of animals (n = 10) was assigned to each treatment group. With the aid of rongeurs the brain was extracted and the cerebral hemispheres were separated. A series of sections were made at irregular intervals to check for intracerebral haemorrhages. On the other hand, animals with no cerebral damage were excluded on behavioural outcomes (see Section 2.3.10). After being snap frozen directly in liquid nitrogen, the brains were then stored at -80°C until further analysis.

2.7.2 Analysis of possible toxic effects of melatonin

Blood, from the decapitated animal, was collected into a heparin rinsed Eppendorf, which was then centrifuged at 1930 g for 5 minutes using a Biofuge PICO centrifuge. The isolated plasma was then stored at -20°C for alanine aminotransferase activity (ALT) measurement.
(Section 2.7.3). The major organs, that is, liver, spleen, kidneys, and testes were harvested and wet weights measured. The liver was snap frozen directly in liquid nitrogen and stored at -80°C. If animals had evident liver hepatotoxicity (abnormal ALT levels), the liver was extracted and sectioned, 20 µm thickness, with the cryostat for further examination.

### 2.7.3 ALT measurement

The ALT reagent (1 mL) from an Alanine Aminotransferase Diagnostic Kit was added to 100 µL of plasma supernatant in a glass cuvette. An absorbance count was carried out every 30 seconds for 3 minutes at 340 nm using the Spectromax spectrophotometer. Plasma ALT activity (U/L) was calculated as change in absorbance (over 3 minutes) x 1768 (a factor which took into consideration the dilution of the enzyme in the cuvette, the cuvette pathlength, the millimolar coefficient of NADH, and the conversion of mL to L). Values outside normal (25.3 to 84.2 U/L) were indicative of hepatotoxicity (Rahman et al., 2005b).

### 2.7.4 Tissue homogenisation

The individual brain hemispheres were finely diced with a scalpel blade, prior to blending with a Polytron homogeniser. The sample was kept on ice in 10 mL protease inhibitor buffer [50 mM Tris buffer (pH 7.6) with 10 µM leupeptin, 1 µM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride]. The blender head was washed with alcohol and distilled water between each sample to avoid cross contamination. The mixture was then centrifuged in 15 mL Falcon tubes at 4°C at 1040 g in an Eppendorf centrifuge for 5 minutes. The supernatant was decanted and aliquoted into cryotubes for each respective assay and stored at -80°C for later use.

### 2.7.5 Protein determination by Bradford assay (Bradford, 1976)

**Preparation of Bradford Reagent**

Coomassie brilliant blue dye (250 mg) was dissolved in 125 mL of absolute ethanol followed by addition of 250 mL of 85% orthophosphoric acid and stirred for 20 minutes. The volume was then made up to 2.5 L and left to stir overnight, followed by filtering using a grade 1 Whatman filter paper. This reagent was then stored at 4°C in a vessel covered with tin foil to prevent oxidising.
Bradford Assay
Once consistent standard curves (regression coefficient, R squared > 0.98) were attained using BSA concentrations 0 – 1 mg/mL in a 96-well microtitre plate, the homogenate samples were analysed. Two 10 µL neat concentrations were added, followed by two 10 µL aliquots of 1:10 dilution (in distilled water) in adjacent wells in the same column. The standards concentrations, which had been previously analysed, were then added in duplicate. Each well was finally topped up with 200 µL aliquots of the filtered Bradford Reagent. The absorbance of the sample was measured at 595 nm by a Spectromax spectrophotometer and protein calculations were determined from the standard curve. The results were expressed as mg protein/ml.

2.7.6 Measurement of nitrite levels by Griess Reaction (Huygen, 1970)
A standard curve using sodium nitrite concentrations 0 – 100 µM was created. 100 µL of neat sample and a 1:10 dilution (in TBS) were added adjacently to the 96-well microtitre plate. The predetermined standard samples were also aliquoted in duplicates followed by the addition of 100 µL of the Griess reagent (10 g sulphathilamid in 50 mL nitrite free H₃PO₄ with 950 mL high performance liquid chromatography (HPLC) grade water added to an equal volume of 1 g/L of naphthylethylenediamine in HPLC water). The absorbance was measured with a Spectromax spectrophotometer at 580 nm and nitrite values were calculated from the standard curve. Using the nitrite values from the assay and the protein values obtained from the Bradford assay as outlined in Section 2.7.5, results for the brain homogenates are expressed as µM nitrite/mg protein.

2.7.7 Measurement of arginase activity
The standards of urea (0 – 100 mg/mL) were used to determine the arginase activity. Sample (50 µL) was aliquoted into an Eppendorf to which 50 µL of 10 mM MnCl₂ in Tris-HCl (pH 7.5) was added. The enzyme activity was activated by a 10-minute incubation in a 55°C water bath. Two 25 µL aliquots of the mix were transferred to different Eppendorfs, while another two 10 µL aliquots of the mix were used in 1:10 dilution (with distilled water), which were further aliquoted in 25 µL to separate tubes. All four of the 25 µL sample/MnCl₂ mixtures were added to 25 µL of 0.5 M L-arginine (pH 9.7) and incubated for 60 minutes in a 37°C
Chapter 2: Materials and Methods

water bath. The reaction was stopped with the denaturing of the arginase protein accomplished by addition of 400 µL of an acid mixture (H₂SO₄, H₃PO₄, H₂O at a ratio of 1:3:7). Twenty five microlitres of 9% isonitrosopropiophaneone (ISPF) in ethanol was added to the samples and boiled in a 100°C water bath for 45 minutes. The standards were only added with the same amount of acid mix and ISPF prior to the 45 minute heating. The samples were allowed to cool in the dark for 10 minutes to accommodate colour development and the absorbance was measured in a 96-well microtitre plate with a Spectromax® spectrophotometer at 540 nm. The amount of urea calculated from the standard curve and the amount of protein (Section 2.7.5) was used to measure arginase activity. The results are expressed as µg Urea/mg protein for brain homogenates.

2.7.8 Measurement of NOS activity

The nitric oxide synthase activity was measured by its ability to convert [³H] L-arginine to [³H] L-citrulline in the presence of appropriate co-factors (Mitchell et al., 1991).

Induction of enzyme activity

Two (30 µL) different aliquots were added to separate Kimble tubes for iNOS (Ca²⁺ independent) and total NOS activity. For the iNOS Kimble tube, the following co-factors were present: 1 mM NADPH, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM L-Valine, 300 units/mL calmodulin, 0.5 mM TBH₄, 10 µL L-arginine/[³H] L-arginine (ratio of 1:1003) and 10 µL H₂O. For the total NOS activity, EGTA and H₂O was replaced by 2 mM calcium chloride. The Kimble tubes were then vortexed and incubated in a 37°C water bath for 30 minutes.

Measurement of [³H] L-citulline levels

The reaction was stopped by addition of 1 mL of the stop buffer, which comprised 20 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES, pH 5.5), 2 mM EGTA, 2 mM ethylenediaminetetraacetic acid (EDTA). The newly formed [³H] L-citrulline was separated from the [³H] L-arginine by the addition of 1 mL Dowex (50-X mesh). To two Kimble tubes labelled ‘total’ and ‘blank’, the cofactors used for total NOS activity were added and distilled water was used instead of sample. The total tube contained the maximal amount of [³H] L-arginine whereas the blank tube had the minimum. 2 mL of stop buffer was added to the total tube and the blank tube had 1 mL of stop buffer and 1 mL of Dowex (50-X mesh). From each Kimble tube, 1 mL of supernatant was decanted and aliquoted into a scintillation vial.
Chapter 2: Materials and Methods

Addition of 4 mL of biodegradable scintillation fluid was followed by vortexing. A liquid scintillation counter was used to measure the radioactivity (in counts per minute, cpm) of the samples, and hence the amount of $[^3]H$ L-citrulline present. The NOS activity was determined by calculating the final count, i.e. (sample cpm – background cpm)/total cpm/1003 (1003 is the ratio of L-arginine to $[^3]H$ L-arginine). The results were expressed as pM L-citrulline/30 min/mg protein (protein concentration determination is described in Section 2.7.5). For the in vitro studies, the results are expressed as pM L-citrulline/30 min/cell viability.

Preparation and regeneration of Dowex

The Dowex powder was dissolved in 1 M NaOH (5:1 v/v) and left to stir overnight. The Dowex was allowed to settle and the maximal amount of liquid was removed by decantation and pipetting without disturbing the Dowex. The Dowex was then rinsed with 5:1 v/v H$_2$O, followed by the adjustment of pH to 7.0 with HCl. The distilled water was then removed and the Dowex was added with 3:1 v/v of stop buffer. The suspension was stored at 4°C until further use.

The contaminated (used) Dowex was rinsed in water and then allowed to settle so that the solution was clear. The maximal amount of water was removed by decantation and pipetting without disturbing the Dowex. The Dowex was dissolved in 1 M HCl (5:1 v/v) for 6 hours. As before, the suspension was allowed to settle and excess HCl was removed without disturbance to the Dowex. The Dowex was rinsed 6 times with 5:1 v/v H$_2$O, in a similar manner to above for 30 minutes each time. Another 2 L of H$_2$O (5:1 v/v) was added and pH was adjusted with NaOH to 7.0 while the suspension was stirred. The Dowex was then rinsed with 1 M NaOH (5:1 v/v) seven times for 6 hours each time. This was followed by a final rinse with 5:1 v/v distilled water, pH adjusted to 7.0 with HCl. Finally, 3:1 v/v stop buffer was added to the Dowex and the mixture was stored at 4°C until further use.

2.7.9 Measurement of COX activity

One of the resultant products of the COX enzyme, PGE$_2$, was measured as an indicator of COX activity in homogenates. The standards of PGE$_2$ used to determine the levels of PGE$_2$ were 0 – 10 ng/mL. A 50 µL aliquot of sample was added to 450 µL of 30 µM AA (in 10 mM phosphate buffered saline, PBS, pH 7.4, containing 0.1% BSA and 0.1% sodium azide). The COX activity was facilitated by incubation at 37°C in a water bath for 30 minutes. The reaction was terminated by boiling for 10 minutes followed by centrifugation for 30 minutes at
10000 g (4°C) using an Eppendorf 5810 R centrifuge. The (100 µL) supernatant was decanted and incubated with Rabbit anti-human PGE₂ in sodium phosphate buffered saline (0.01 M, pH 7.4, containing 0.1% BSA and 0.1% sodium azide) at 4°C for 30 minutes. One hundred microlitres of [³H] PGE₂ (diluted in 0.01 M PBS, pH 7.4, containing 0.1% BSA and 0.1% sodium azide) was added and incubated at 4°C for 1 hour. A 200 µL aliquot of a cold dextran (0.1%) coated charcoal (1%) suspension was rapidly added to each Eppendorf, vortexed and incubated at 4°C for 10 minutes. The mixture was then centrifuged using an Eppendorf 5810 R centrifuge at 2000 g for 15 minutes (4°C). Six hundred microlitres of the supernatant was decanted and aliquoted into a scintillation tube, which was followed by the addition of 4 mL of biodegradable scintillation fluid. The vials were thoroughly vortexed and the levels of [³H] PGE₂ were measured with a liquid scintillation counter. Results were expressed as ng PGE₂/30 minutes/mg protein (protein concentration from Section 2.7.5).

2.8 WESTERN BLOT ANALYSIS

2.8.1 Sample preparation

All samples were first equilibrated to 2 mg/mL protein by the addition of the appropriate volume of TBS (protein concentrations were determined as outlined in Section 2.7.5). The volume of diluted homogenate (2 mg/mL) was doubled with an equal volume of sample loading buffer (9% 1.5 M Tris-HCL, pH 6.8, 22% sodium dodecyl sulfate (SDS), 44% glycerol, 1% 200 mM EDTA, 11% 14.3 M 2-mercaptoethanol, 23% distilled H₂O and 0.025% (w/v) bromophenol blue). The samples were then boiled for 3 – 5 minutes to denature the proteins and stored at 4°C until further use. For in vitro samples, the proteins were equilibrated to the lowest concentration in each group, instead of 2 mg/mL. For in vivo samples, 15 µg of protein was loaded in each well, whereas for in vitro samples 4.3 µg of protein was loaded (as this was the highest possible when samples were standardised).

2.8.2 Preparation of gels

The electrophoresis gel consisted of a 4.75% bis-Tris stacking gel and a bis-Tris resolving gel. The stacking gel consisted of 1.6 mL Bis Acrylamide (1:29), 0.12 M Tris, (pH 6.8), 0.02% SDS, 7.48 mL distilled H₂O, 0.01% APS, and 10 µL TEMED. The concentration of the Bis
Chapter 2: Materials and Methods

Acrylamide varied in the resolving gel, as the higher percentage gel was able to separate the lower molecular weight proteins and *vice versa* (Table 2.3). Each resolving gel contained 0.5 mL 50% glycerol, 0.1 µL 20% SDS. The amount of Tris HCl (pH 8.8), Bis Acrylamide and H$_2$O varied as shown in Table 2.3. The gels (without APS and TEMED) were made and left to degas overnight at 4°C. The catalysts for polymerization, APS and TEMED were added immediately prior to casting the gel.

The glass plates, separated with 0.75 mm spacers, were cleaned with methanol and placed in the casting frame. The casting frame was then placed in the casting stands, allowing the bottom portion to be sealed. After the resolving gel was poured, methanol was used to flatten the surface and was removed prior to addition of the stacking gel. A 10-well, or 15-well comb was used to create individual wells, while the stacking gel was setting. Fifteen microlitres of sample for the 15-well and 20 µL for the 10-well was loaded into the electrophoresis gel. Five microlitres of kaleidoscope prestained broad range molecular weight marker were also loaded into the gel.

<table>
<thead>
<tr>
<th>Resolving gel (%)</th>
<th>Protein size (kDa)</th>
<th>Bis acrylamide (mL)</th>
<th>Tris (pH 8.8) (mL)</th>
<th>H$_2$O (mL)</th>
<th>APS (µL)</th>
<th>TEMED (µL)</th>
</tr>
</thead>
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<td>7.5</td>
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<td>2.63</td>
<td>5</td>
<td>8.6</td>
<td>36</td>
<td>20</td>
</tr>
<tr>
<td>15</td>
<td>30 – 45</td>
<td>5</td>
<td>2.5</td>
<td>3.9</td>
<td>36</td>
<td>15</td>
</tr>
<tr>
<td>18</td>
<td>&lt; 10</td>
<td>10.5</td>
<td>4.05</td>
<td>0.6</td>
<td>115</td>
<td>10</td>
</tr>
</tbody>
</table>

*Table 2.3 The resolving gel compositions used for Western blotting.*

2.8.3 Electrophoresis and blotting

A powerpack 1000 was used to carry out the electrophoresis. The gels were first run at 90 volts (in a running buffer consisting of 0.03% Tris base, 1.44% glycine and 0.1% SDS), until the protein bands reached the interface of the resolving and stacking gels. The voltage was changed to 120 volts and left for the appropriate time, which was dependent on the resolving gel. The electrophoresed proteins were then transferred to a methanol soaked PVDF membrane using the Transblot® gel transfer system in a transfer buffer (0.3% Tris base, 1.44% glycine and 20% methanol, pH 8.3). The gel and PVDF membrane were sandwiched between the Transblot® filter paper and placed in the Transblot® cassette. The larger proteins, > 70 kDa were transferred at 90 volts for an hour using a powerpack 1000. A cooling coil, connected to a water outlet, was placed in the Transblot® gel transfer system that was
continuously being stirred. The cooling coil setup prevented overheating of the system. All other proteins were transferred at 10 volts overnight, using a powerpack 1000, without a cooling coil.

2.8.4 Immunolabelling and Hyperfilm development

Non-specific binding was reduced with an overnight incubation in blocking solution (0.1% BSA, 10% Blotto, non-fat milk powder, 0.1% Tween 20 in TBS, TTBS) at 4°C. The membranes were then rinsed with TTBS (three times) to remove excess blocking solution. Primary antibodies were incubated at 4°C overnight. The antibody diluent consisted of 0.1% BSA and 0.1% Blotto, non-fat milk powder in TTBS. Following rinsing with TTBS, the membranes were incubated overnight at 4°C with the respective secondary antibody, either donkey anti-goat horseradish peroxidase (HRP) conjugated or goat anti-rabbit HRP conjugated at a 1:3000 dilution in TTBS. The secondary antibody was rinsed off once with TTBS and twice with TBS. ECL™ reagents were added to the membrane for 60 seconds. The membranes were placed between two acetate sheets to prevent dehydrating, in the Hyperfilm™ cassette. Twenty minutes after the ECL™ addition, the ECL™ Hyperfilm™ was exposed to the membrane for 1 – 5 minutes in a dark room. The Hyperfilm™ was then placed in the photographic developer solution for 1 minute, 30 seconds in photographic stop bath and finally for 5 minutes in photographic fixer and replenisher. After fixing, the Hyperfilm™ was rinsed in tap water before being dried in an air dryer.

2.8.5 Stripping and reprobing

The PVDF membrane was stripped of the immunolabelling with a stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris HCl (pH 6.7) in H₂O). The membrane was submerged in prewarmed stripping buffer at 50°C for 30 minutes with occasional agitation. The membrane was then washed with TTBS repeatedly (four times) before immunolabelling as described above.

2.8.6 Analysis

The molecular weight markers were used to identify the target band by superimposing the Hyperfilm™ on the PVDF membrane. The optical density (OD) was analysed for each band.
using the GS-710 scanning densitometer and QuantityOne® software. The background OD for each lane was subtracted from each band, to give the final OD value. The OD was also calculated for β-actin (as loading control) for each sample. The results were then expressed as a ratio of OD of a particular protein to OD of β-actin.

2.9  **IN VITRO EFFECTS OF MELATONIN ON L-ARGININE METABOLISM**

2.9.1  **Cell culture**

Human fibrosarcoma cell line, HT-1080, was obtained from Department of Pathology (Professor Braithwaite). Initially, the cells were thawed after removal from liquid nitrogen by placing them immediately in a 37°C water bath. They were cultured in 25 cm² plastic flasks, with 3 mL of complete culture medium. The complete culture medium consisted of DMEM containing 10% FBS, 100U/mL penicillin, 100µg/mL streptomycin, 25ng/mL amphotericin B and 2.2g/L NaHCO₃. The flask was incubated at 37°C in a 5% CO₂/95% humidified air atmosphere in a Hera Cell culture incubator. The cell growth was monitored with an inverted microscope. Once approximately 95% confluent the cells were passaged and cultured in 75 cm² (T₇₅) plastic flasks with 9 mL of complete culture medium. The T₇₅ flasks were passaged (see Section 2.9.2) and split into two new flasks every 48 hours.

2.9.2  **Passaging of cells**

Cells were passaged when 90 – 95% confluency was reached. The old media were drained and the cells were rinsed with 9 mL of PBS (pH 7.4, 50 mM). Trypsin EDTA (9 mL) was added and the flask was shaken to detach the cells. Within 5 minutes, 3 mL of trypsin inhibitor was added and mixed well, to prevent cell death by the trypsin EDTA. The contents of the flask were then emptied into a 15 mL Falcon tube and centrifuged at 1600 g for 10 minutes at 4°C, using an Eppendorf 5810 R centrifuge. The supernatant was replaced with 9 mL of complete culture medium and the tube was then vigorously shaken to resuspend the cells, which had formed a pellet after centrifugation. The falcon tube was centrifuged again as before. The supernatant was removed and replaced with another 9 mL of complete culture medium. The cells were resuspended and either split into new flasks or plated out as appropriate for each assay.
2.9.3 Cell viability by Trypan blue exclusion

Trypan blue exclusion was used to determine cell numbers for further experiments. This technique is still widely used for estimation of cell viability (Heinrich et al., 2007; Kessel et al., 2007; Khan et al., 2007). Confluent T75 flask was passaged (see Section 2.9.2) and resuspended in 14 mL of complete culture medium. Aliquot (180 µL) was then pipetted into a 96-well microtitre plate. Twenty microlitres of 5% trypan blue was added to the contents of the well, to give a final concentration of 0.5%. A period of 5 minutes was allowed for the cells to uptake the dye. Twenty microlitres of the suspension was added to an Improved Neubauer haemocytometer chamber by capillarity and coverslipped until the appearance of Newton’s rings. The cells were then counted using an inverted phase contrast microscope with a 10 X objective. The viable cells had not taken the dye up and appeared transparent, whereas those retaining the trypan blue were non-viable (due to disruption of the cell membrane). The first 100 cells were counted.

2.9.4 Sulforhodamine B (SRB) Assay (Skehan et al., 1990)

The fibroblasts were seeded in 12-well plates (70000 cells/well) plates in 1mL complete media. The cells were incubated for 24 hours prior to being treated with doses of melatonin (1 nM to 1mM, in 0.1% DMSO). Control cells were treated with 0.1% DMSO. Following treatment, media was aspirated off each well. Five hundred microlitres of tetrachloroacetic acid (to fix cells) was added to each well for 30 minutes at 4°C. Excess tetrachloroacetic acid was rinsed off with tap water and the wells were dried overnight. To each well 0.4% (wt/vol) SRB dissolved in 1% acetic acid was added for 10 minutes at room temperature. The wells were then rinsed quickly with 1% acetic acid (five times). The wells were allowed to dry overnight in dark. The bound dye was solubilised with addition of 2mL Tris base (10mM, pH 10.5). A hundred microlitres of the dissolved dye were then aliquoted into a 96-well microtitre plate, in triplicate. Absorbance was measured in a 96-well microtitre plate with a Spectromax® spectrophotometer at 490 nm.

2.9.5 MTT (tetrazolium) Assay (Mossman 1983)

The fibroblasts were seeded in 96-well microtitre plates (2000 cells/well) in 200µL of complete media. The cells were incubated for 48 hours prior to being treated with doses of melatonin (1nM to 1mM in 0.1% DMSO). Control cells were treated with 0.1% DMSO.
Following treatment, media was aspirated from each well. The cells were washed once with fresh media. Sterile filtered 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) bromide in PBS (0.4mg/mL) was added to each well and incubated for 3 hours at 37°C. The MTT solution was then removed. A hundred microlitres of DMSO was then added to each well and thoroughly mixed at room temperature (to dissolve formazen crystals). Absorbance was measured in a 96-well microtitre plate with a Spectromax® spectrophotometer at 550 nm.

2.9.6 Nitrite level determination (cell extract supernatants)

The nitrite levels for HT-1080 fibroblasts were measured from the supernatant collected in Section 2.9.6. The nitrite levels were determined as outlined in Section 2.7.6. The results were expressed as µM nitrite/mg protein.

2.9.7 Collection of cell samples for biochemical assays

HT-1080 fibroblasts were seeded in 10cm culture dishes at 9 × 10⁵ cells per dish in 10 mL of culture media. Cells were incubated for 24 hours prior to being treated with different doses of melatonin (1nM to 1mM, in 0.1% DMSO) for 72 hours. The control group was treated with 0.1% DMSO.

Cells were harvested using a cell scraper and centrifuged at 1000 g for 4 minutes at 4°C, using an Eppendorf 5810 R centrifuge. The media was then removed and the cells were resuspended in PBS and re-centrifuged at 1000 g for 4 minutes at 4°C. The supernatant was removed and the cells were resuspended in 500 µl of protease inhibitors (1 mM phenylmethylsulphonyl, 10 µM leupeptin, 1 µM pepstatin) in 50 mM Tris HCl, pH 7.6, was added. Ultrasonication (amplitude 30, 3-second bursts, 5-second break) was carried out at 4°C using a high intensity ultrasonic processor to lyse the cells. The microtip head was then cleaned with alcohol and distilled water to avoid cross contamination. The suspension was then centrifuged at 10000 g for 5 minutes at 4°C and supernatant used for further analysis.
2.10 EFFECTS OF MELATONIN ON MITOCHONDRIAL COMPLEXES

2.10.1 Preparation of mitochondrial samples

The samples were removed from the -80°C freezer and thawed on ice. The whole hemisphere homogenates were then frozen in liquid nitrogen and thawed on ice twice to ensure mitochondrial lysis. Using protein concentrations previously calculated (see Section 2.7.5), the samples were diluted to 1 mg/mL with TBS and frozen at -80°C until further use.

Ideally, mitochondria would have been isolated from microdissections of the infarct regions. This would have ensured that mitochondria were not damaged due to the sample preparation technique. By isolating the infarct region, which has the most mitochondrial damage, the effects of melatonin on the impairment of mitochondria would have been accurately examined. By using the whole hemisphere homogenate, the infarct area is relatively small compared to non-infarcted areas leading to a ‘masking’ effect. However, the infarct area in the MCAO model may not always be constant, with the infarct depending on the anatomical pattern of the MCA in the particular animal (see Section 1.6.4). The only way to discern the infarcted region from normal tissue would be via staining and this process may render the sample unsuitable for mitochondrial studies. Furthermore, the mitochondrial studies carried out were done in conjunction with the biochemical assays (for which the sample preparation was optimised). Even though the mitochondrial assay used suboptimally prepared samples, it was cost-effective and involved fewer animals.

2.10.2 Mitochondrial complex I (NADH-ubiquinone oxidoreductase) assay (Ragan et al., 1987)

The NADH oxidation is catalysed by mitochondrial complex I. Hence the rotenone-sensitive decrease in NADH was used as an indicator of complex I activity. A 260 µL aliquot of the reaction mixture (25 mM phosphate buffer, pH 7.2, 10 mM MgCl₂, 5 mM NADH, 1 mM KCN and 50 mg/mL BSA) was added to each well of a 96-well microtitre plate, already containing 12 µL of the prepared sample (see Section 2.10.1). Addition of 20 µL of Ubiquinone (50 µM final concentration) initiated the reaction. The change in absorbance over 5 minutes at 340 nm was measured using a Spectramax® plate reader, to obtain a Lineweaver Burke plot. Then, 15 µL of rotenone (0.5 mM) was added and the change in absorbance over 3 minutes at 340 nm
was measured. The rotenone-sensitive complex I activity was expressed as nmol/min/mg protein (see Section 2.7.5 for protein concentration).

2.10.3 Mitochondrial complex II-III (succinate-ubiquinone/ubiquinol-cytochrome c reductase) assay (King, 1967)

Complex II – III oxidises succinate, so its activity is measured by the succinate-dependent antimycin A sensitive reduction of cytochrome c. An aliquot of 260 µL of reaction mixture (100 µM cytochrome c, 0.3 mM K\(^+\)EDTA, 1 mM KCN in 100 mM phosphate buffer, pH 7.4) was added to each well of a 96-well microtitre plate, followed by addition of 12 µL of prepared sample (see Section 2.10.1). The reaction was started with the addition of 20 µL of succinate (1 M). After the change in absorbance at 550 nm was measured over 5 minutes at 30°C using a plate reader, 10 µL of antimycin A (2 mg/mL) was added to inhibit the reaction. The change in absorbance was measured for 5 more minutes under the same conditions. The antimycin-sensitive complex II – III activity was expressed as nmol O\(_2\)/min/mg protein (see Section 2.7.5 for protein concentration).

2.10.4 Mitochondrial complex V (ATP hydrolase activity) assay (Soper et al., 1979)

The ATP hydrolase activity was measured by coupling the oxidation of NADH to ADP by lactate dehydrogenase. A 260 µL aliquot of the reaction mixture (100 mM KCl, 1 mM Rotenone, 15 mM NADH, 100 mM KCN, 100 mM phosphoenolpyruvate, 250 mM MgCl\(_2\), 200 U/mg phosphokinase, and 550 U/mg lactate dehydrogenase, in 100 mM Tris buffer, pH 8.0) was added to each well on the 96-well microtitre plate. Twelve microlitres of sample (see Section 2.10.1) was added, followed by 20 µL of ATP (300 mM) to initiate the reaction. The change in absorbance was read for 5 minutes at 340 nm using a plate reader. Twenty microlitres of oligomycin (100µg/ml in ethanol) was added to stop the reaction. The oligomycin-sensitive complex V activity was expressed as nmol O\(_2\)/min/mg protein (see Section 2.7.5 for protein concentration).

2.10.5 Citrate synthase assay (Shepherd et al., 1969)

The activity of citrate synthase was measured by its ability to catalyse the condensation of oxaloacetate and acetyl-CoA. A 260 µL aliquot of the reaction mixture (0.1 mM acetyl-CoA,
Chapter 2: Materials and Methods

0.2 mM 5,5’ dithio-bis 2-nitrobenzoic acid in 100 mM Tris-HCl with 0.1% Triton X, pH 8.0) was added to each well of a 96-well microtitre plate. To this, 12 µL of sample (see Section 2.10.1) was aliquoted followed by 20 µL of oxaloacetate (20 mM) to initiate the reaction. The absorbance was measured for 5 minutes at 412 nm with a Spectramax® plate reader. The oxaloacetate-sensitive rate was expressed in nmol O$_2$/min/mg protein (see Section 2.7.5 for protein concentration).

2.10.6 Ratio of ETC activity assays to citrate synthase activity

Citrate synthase was used in this study as a mitochondrial membrane marker, with activity of the enzyme an indicator of the number of intact mitochondria (Clarkson et al., 2007; Clarkson et al., 2004a; Sammut et al., 2001). By normalising the ETC activities to the citrate synthase, the effect on the individual ETC activity can be differentiated from merely a reduced number of intact mitochondria (Clarkson et al., 2007). Each individual ETC activity (for complexes I, II-III and V) was normalised to the respective individual citrate synthase activity. The ratios were then compared between treatment groups to examine the impact on ETC after accounting for differences in intact mitochondria.

2.10.7 Aconitase [citrate(isocitrate) hydro-lyase] assay (Kennedy et al., 1983)

Aconitase catalyses the conversion of cis-aconitase to isocitrate. A 260 µL aliquot of 120 mM Tris base in distilled water, pH 8.0 was aliquoted to each well, followed by 12 µL of sample (see Section 2.10.1). The reaction was initiated by addition of 20 µL of isocitrate (tri-sodium salt, 200 mM). The change in absorbance was measured at 240 nm for 5 minutes by a plate reader. Twenty microlitres of fluorocitrate (11 mM) was then added to stop the reaction and the absorbance measurement was repeated. The fluorocitrate inhibitable kinetic activity was expressed as nmol O$_2$/min/mg protein (see Section 2.7.5 for protein concentration).

2.11 STATISTICAL ANALYSES

Statistical tests were conducted with the help of statisticians from the Department of Social and Preventative Medicine, University of Otago, NZ. All statistical analysis was conducted using the statistical program, R (version 2.11.1) and the minimum requirement for significance
was $P < 0.05$. All results were expressed as the mean ± standard error of mean (SEM) with the exception of single labelling immunohistochemistry which was expressed as the median. Median was used as the data was ordinal (therefore, mean would be inappropriate measure of central location). Non-parametric tests (such as Mann-Whitney U test) were applied to test significant difference in the medians for the ordinal data. Continuous data were first tested for normality (with Shapiro test) and equal variance (Bartlett test). If both tests were satisfied, then the data was deemed parametric and an appropriate parametric test (such as analysis of variance, ANOVA) was used to test for significant differences of mean. However, if continuous data failed either of the Shapiro or Bartlett tests, the data was attempted to be transformed by using one of the following equations: $y = \log(x)$; $y = e^x$; $y = x^z$, where $z = -1, 0.5, 2$; or $y = (x+a)^z$. If the data was unable to be transformed, an equivalent non-parametric test was used.

### 2.11.1 Effects of melatonin administration on infarct volume

The infarct volume data collected was continuous and consisted of two treatment levels (melatonin and vehicle) and 8 levels (slices which were dependent on each other). Sample sizes were $n = 6$ and $n = 7$ for melatonin and vehicle, respectively. The data had unequal variance between the two treatment groups, which could not be transformed adequately. The slices being dependent on each other and data having unequal variances between two treatment groups violated the assumptions of ANOVA tests (Zolman, 1993). The overall volumes of both treatment groups were carried out with two sample t-test with unequal variance (also known as Welch two sample t-test). Similarly, for each slice a two sample t-test with unequal variance was conducted. Bonferroni adjustment (which adjust for multiple comparisons by appropriately reducing the alpha rate) was used to alter the significance level by $0.05/8$ comparisons (for 8 slices).

The other variables: surgical and physiological parameters, organ and body weights, were analysed using a planned parametric unpaired Students t test to detect the significant effects of Treatment (Zolman, 1993). For these variables, there was one independent factor, Treatment. Treatment consisted of two levels: Melatonin and Vehicle.
2.11.2 Localisation of melatonin and its receptors post MCAO

A ranking scale was used to assess the difference in immunoreactivity. Therefore, all the data analyses were conducted with non-parametric tests. To compare regional differences within each hemisphere of a treatment group (for example, ipsilateral hemisphere of control group), a Friedman two-way non-parametric ANOVA for dependent samples with paired Wilcoxon tests corrected for multiplicity was used. Differences between ipsilateral and contralateral hemispheres of the same animals (within each treatment group) were analyzed with the Wilcoxon Signed Rank test. Mann-Whitney U test was then used to test the significant effects of Treatment on melatonin and membrane receptor distributions in each brain region (Zolman, 1993). This involved comparing the difference in medians of the Treatment levels, MCAO and Control, for each region (i.e. for each separate antibody).

2.11.3 Biochemical study of the effects of melatonin administration

Statistically significant main effects of Treatment in inducible enzyme systems (NOS, COX and arginase) were determined using parametric one-way ANOVAs (Zolman, 1993). There was only one factor, i.e. Treatment with three levels (control, MCAO and MCAO with melatonin). The differences between each treatment group were assessed with Tukey’s pair-wise comparison post-hoc tests. This post-hoc test compared differences in means between two groups at a time in all the possible combinations (Zolman, 1993).

A linear regression model was used to determine the accuracy of the standard curves for several assays (Bradford, nitrite, arginase, and COX; Zolman 1993). A regression co-efficient (R squared) > 0.98 was considered accurate, validating the use of the standard curve in predicting the sample values. The linear regression model was also utilised in analysing the nitrite and total NOS activity data. This was used as nitrite levels are directly dependent on total NOS activity.

2.11.4 In vitro effects of melatonin administration

The in vitro data, which were normally distributed (i.e. passed Shapiro and Bartlett tests), were analysed using a parametric one-way ANOVA to test for statistically significant main effects of Treatment (Zolman, 1993). Treatment consisted of at least 8 levels: control group plus at
least 7 different melatonin concentrations. Dunnett’s Multiple Comparison tests were used to compare the effects of each concentration of treatment with the control group, where significant effects of treatment were found. The most appropriate post hoc test was Dunnett’s multiple comparison test, as this minimised the number of comparisons thereby reducing the type I error rate. Dunnett’s multiple comparison test compares only the means of the independent variables (treatment concentrations) to the mean of the control group (Zolman, 1993). When data, (for example, SRB cell viability assay), failed homogeneity of variance tests despite attempts to transform it, a non-parametric equivalent of ANOVA was used (Zolman 1993). This was Kruskal Wallis statistic with Dunn’s multiple comparison (comparing all treatment groups to control group only) was used.

In addition, a linear regression model was used to examine the dose-dependent relationship, across the different concentrations of melatonin (Zolman, 1993). The concentration-dependent relationships were examined for each of the parameters.

### 2.11.5 Effects of melatonin administration on mitochondrial function after MCAO

Data from mitochondrial function (ETC I, II/III, V; citrate synthase and aconitase activities) assays were analysed using a parametric one-way ANOVA to test for statistically significant main effects of Treatment. Treatment consisted of three levels (control, MCAO, MCAO with melatonin treatment). The differences between each treatment group were assessed with Tukey’s pair-wise comparison post-hoc tests. This post-hoc test compared differences in means between two groups at a time in all the possible combinations (Zolman, 1993).
CHAPTER 3

THE EFFECTS OF MELATONIN ON NEURAL INJURY POST ISCHAEMIA
Chapter 3: The Effects of Melatonin on Neuronal Injury Post Ischaemia

3.1 INTRODUCTION

Melatonin has been shown to have neuroprotective effects in a number of models of neurodegeneration (for review see Reiter et al., 2005). In a recent meta-analysis showed efficacy of melatonin when administered between 1.5 – 50 mg/kg (O’Collins et al., 2010). When this study was designed, Pei et al., (2003c), had just established that 5 mg/kg i.p. of melatonin given within 1 hour of ischaemia produced beneficial effects in a model of focal ischaemia. This neuroprotective effect was further amplified when multiple doses were given at 24 hours and 48 hours post induction of ischaemia. Therefore, the current study used multiple doses of 5 mg/kg i.p. melatonin to treat animals. The levels of melatonin, a physiological hormone with a rhythmic production, have been shown to peak at 0200 hours (Laudon et al., 1988; for a review of the rhythmicity of the circulating levels of endogenous melatonin refer to Section 1.7.1). Therefore, for this study a drug-dosing regime was chosen to enhance the endogenous levels of melatonin.

3.1.1 Hypothesis and aims

We hypothesized that the melatonin administered after the onset of stroke would be neuroprotective without having any major toxic effects. The aim of this study was to determine whether a 5 mg/kg i.p. dose of melatonin with a different dosing regime [to that of Pei et al., (2003c)] as specified in Chapter 2, was neuroprotective in the intraluminal gold standard model of cerebral ischaemia – the filament insertion MCAO model. We also assessed for any toxic effects of melatonin.

3.1.2 Experimental approach

To investigate the efficacy of melatonin in neuroprotection following MCAO, two groups of animals were used. Each group was allocated 11 animals randomly, and animals underwent the MCAO (see Section 2.3). An hour after the onset of ischaemia (attained by thread insertion), the animals were dosed with vehicle (5% DMSO in 0.9% saline) or 5 mg/kg i.p. dose of melatonin. Following this, the intraluminal thread was withdrawn to allow reperfusion, thus allowing 2 hours of ischaemia. Animals were then dosed at 2300 hours on Day 1 and Day 2 after MCAO (see Section 2.5). Terminal studies were conducted at 72 hours from the onset of ischaemia. Coronal sections were then exposed to TTC stain to evaluate infarct size (see Section 2.6).
3.2 RESULTS

3.2.1 Inclusion of animals

The final numbers included for study were 6 (55%), and 7 (64%) animals for the melatonin and vehicle treated groups, respectively. The most common cause of exclusion was the development of ICH evident at histological analysis (Figure 3.1), with 3 animals in the melatonin and 1 animal in the vehicle treated groups being excluded. From the vehicle treated group, 3 more animals were excluded due to: death during surgery, failure to meet behavioural criteria or the absence of infarct. Similarly, 2 animals from the melatonin treated group were removed because of surgical complications and improper treatment administration. Initial statistical analysis found an outlier (greater than 2 standard deviations from the mean of the sample for infarct size) in the melatonin treatment group and this other animal was not included in the study.

Figure 3.1 TTC-stained section at 5 mm from the frontal pole exhibiting an intracerebral haemorrhage (ICH). The metabolically active, that is, normal tissue (N) is indicated by the red TTC stain. On the other hand, the non-metabolically active tissue (I) demonstrated by lack of the TTC stain is located in the region supplied by the right MCA. The region of ICH shown by the black area is in the periphery of the infarcted region.
3.2.2 **Physiological parameters of animals**

*Animal body weights*

There were no significant differences between pre-fasting weights of the rats in the vehicle group and in the melatonin treatment group. Animals in both groups showed a similar trend in body weight gains from day 1 to day 3 after an initial loss of weight post surgery (day 0; see Table 3.1).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Vehicle</th>
<th>Melatonin</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-fasting</td>
<td>289.0 ± 2.1 g</td>
<td>285.3 ± 2.0 g</td>
<td>ns</td>
</tr>
<tr>
<td>Day 0 (post-fasting)</td>
<td>267.6 ± 2.0 g</td>
<td>267.4 ± 2.3 g</td>
<td>ns</td>
</tr>
<tr>
<td>Day 1 post MCAO</td>
<td>251.3 ± 8.1 g</td>
<td>261.8 ± 8.6 g</td>
<td>ns</td>
</tr>
<tr>
<td>Day 2 post MCAO</td>
<td>253.9 ± 12.4 g</td>
<td>267.9 ± 12.6 g</td>
<td>ns</td>
</tr>
<tr>
<td>Day 3 post MCAO</td>
<td>258.1 ± 14.9 g</td>
<td>272.7 ± 14.6 g</td>
<td>ns</td>
</tr>
</tbody>
</table>

*Table 3.1 Body weights of animals in both treatment groups. There were no significant differences between the two groups at any time points.*

*Surgical parameters*

There were no differences between treatment groups in the duration of surgery (see Table 3.2). The recorded physiological parameters were the arterial blood gases, pCO$_2$ and pO$_2$, pH, mean arterial blood pressure and blood glucose levels. There were no significant differences between any of the variables, which were within the normal range. The effect of surgery on the physiological variables (differences in the two different sample points) was consistent in both groups.
Chapter 3: The Effects of Melatonin on Neuronal Injury Post Ischaemia

<table>
<thead>
<tr>
<th>Physiological Parameter</th>
<th>Treatment group</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Melatonin</td>
</tr>
<tr>
<td>1st pH</td>
<td>7.455 ± 0.013</td>
<td>7.475 ± 0.004</td>
</tr>
<tr>
<td>2nd pH</td>
<td>7.381 ± 0.017</td>
<td>7.410 ± 0.007</td>
</tr>
<tr>
<td>1st pCO₂ (mmHg)</td>
<td>35 ± 1</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>2nd pCO₂ (mmHg)</td>
<td>40 ± 2</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>1st pO₂ (mmHg)</td>
<td>145 ± 7</td>
<td>147 ± 5</td>
</tr>
<tr>
<td>2nd pO₂ (mmHg)</td>
<td>131 ± 8</td>
<td>128 ± 5</td>
</tr>
<tr>
<td>1st Blood pressure (mmHg)</td>
<td>90 ± 13</td>
<td>85 ± 10</td>
</tr>
<tr>
<td>2nd Blood pressure (mmHg)</td>
<td>86 ± 11</td>
<td>93 ± 13</td>
</tr>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>5.76 ± 0.21</td>
<td>5.65 ± 0.08</td>
</tr>
</tbody>
</table>

Table 3.2 Physiological parameters from blood samples. Independent t-tests with two-tailed significance show no differences in means between the groups.

3.2.3 Histological analysis of infarction

Histological evaluation by TTC staining revealed a significant (t = 3.17, d.f. = 6.6, P < 0.05) decrease in ischaemic infarct size in the melatonin treatment group compared to vehicle (Figure 3.2). Total infarct volume in the melatonin and vehicle treatment groups was 46 ± 8 mm³ and 155 ± 34 mm³, respectively. A statistical analysis of the ischaemic area by slice between the two groups found significant (P < 0.05) differences. The decreases in the infarct area were significant at slices 2, 3 and 4 (Table 3.3, Figure 3.3). Moreover, this neuroprotective effect of melatonin was significant in both cortical and striatal areas of the brain (Figure 3.4).
Chapter 3: The Effects of Melatonin on Neuronal Injury Post Ischaemia

Figure 3.2 Ischaemic and normal brain tissue as differentiated by TTC staining at 5 mm from the frontal pole. The area of infarct (I), marked by a lack of the TTC staining, was located in the region of the middle cerebral artery and was clearly visible in the vehicle treated group (A). Melatonin administration (B) had a substantial neuroprotective effect, which was evident by the decreased ischaemic damage when compared with the vehicle treatment group.
Chapter 3: The Effects of Melatonin on Neuronal Injury Post Ischaemia

<table>
<thead>
<tr>
<th>Slice</th>
<th>Estimated difference between treatments</th>
<th>DF</th>
<th>t value</th>
<th>P-value</th>
<th>Bonferroni adjusted P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-5.75 ± 2.37</td>
<td>11</td>
<td>-2.42</td>
<td>0.0341</td>
<td>0.27</td>
</tr>
<tr>
<td>2</td>
<td>-11.54 ± 3.14</td>
<td>11</td>
<td>-3.67</td>
<td>0.0037</td>
<td>0.03</td>
</tr>
<tr>
<td>3</td>
<td>-16.79 ± 5.02</td>
<td>11</td>
<td>-3.34</td>
<td>0.0066</td>
<td>0.04</td>
</tr>
<tr>
<td>4</td>
<td>-16.24 ± 4.89</td>
<td>11</td>
<td>-3.32</td>
<td>0.0068</td>
<td>0.04</td>
</tr>
<tr>
<td>5</td>
<td>-13.23 ± 5.59</td>
<td>11</td>
<td>-2.37</td>
<td>0.0373</td>
<td>0.29</td>
</tr>
<tr>
<td>6</td>
<td>-15.75 ± 6.59</td>
<td>11</td>
<td>-2.39</td>
<td>0.0358</td>
<td>0.28</td>
</tr>
<tr>
<td>7</td>
<td>-16.97 ± 6.20</td>
<td>11</td>
<td>-2.74</td>
<td>0.0192</td>
<td>0.15</td>
</tr>
<tr>
<td>8</td>
<td>-13.05 ± 6.23</td>
<td>11</td>
<td>-2.09</td>
<td>0.0605</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Table 3.3 Statistical analyses examining the difference between melatonin and vehicle treatment at each slice. Two sample t-test with unequal variances only found significance at slices 2, 3, 4 after Bonferroni adjustment for multiple comparisons.

Figure 3.3 The neuroprotective effects of melatonin treatment following ischaemic stroke at each slice interval. There were significant (t = 3.67 for slice 2, 3.34 for slice 3, 3.32 for slice 4, P < 0.05, slices 2-4) decreases in infarct area at each interval after administration of melatonin (continuous line) in comparison to vehicle (dashed line). Each point represents the mean ± SEM for n = at least 6 separate animals.
Chapter 3: The Effects of Melatonin on Neuronal Injury Post Ischaemia

3.2.4 Toxic effects of melatonin administration

There were no differences between the wet organ weights (liver, spleen, kidneys and testes) of the two treatment groups (Table 3.4). ALT values above 100 units per litre were considered indicative of hepatotoxicity (Rahman et al., 2005b). None of the values from either group fell above this threshold. There were also no differences in ALT values between the two groups. Therefore, no evidence of hepatotoxicity due to melatonin administration was observed.

<table>
<thead>
<tr>
<th>Physiological Parameter</th>
<th>Treatment group</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Melatonin</td>
</tr>
<tr>
<td>Kidneys (g)</td>
<td>2.41 ± 0.11</td>
<td>2.53 ± 0.17</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>10.50 ± 1.37</td>
<td>11.55 ± 0.60</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>0.60 ± 0.07</td>
<td>0.60 ± 0.06</td>
</tr>
<tr>
<td>Testes (g)</td>
<td>2.71 ± 0.07</td>
<td>2.77 ± 0.09</td>
</tr>
</tbody>
</table>

Table 3.4 Effects of melatonin on wet organ weights after MCAO when compared with vehicle administered animals. Independent t-tests with two-tailed significance showed no differences (P > 0.05) in means between the groups.
3.3 DISCUSSION

3.3.1 Inclusion of animals and physiological parameters

The inclusion rates were consistent previous publications documenting the use of the MCAO method in Sprague Dawley rats. The use of the MCAO model in Sprague Dawleys has been extensively evaluated (Kahveci et al., 2000). Kahveci and colleagues have described the major reasons for exclusion as the development of subarachnoid haemorrhages or insufficient occlusion. In this study, there was only one (8.3%) incident of incomplete occlusion. The lower rate of incomplete occlusion in comparison to previous MCAO studies may be due to the use of the improved coating of the intraluminal thread with poly-L-lysine as described by Belayev et al., (1996). The majority (18%, 4 animals) of the exclusions were due to subarachnoid haemorrhage in the current study.

There were no differences in the surgical and physiological parameters between the vehicle and melatonin treated groups. The physiological parameters recorded from the first blood gas sample were similar to that of other studies using Sprague Dawley rats (Choi et al., 2004; Hasegawa et al., 2010). The changes in physiological parameters between the first and second readings of all animals were reflective of the influence of prolonged mechanical ventilation. This trend was similar to that of another study using MCAO (Hasegawa et al., 2010). Thus, in all MCAO animals there were no notable confounding factors.

3.3.2 Infarct size analysis following melatonin administration

This has been the first study with this modified melatonin-dosing regime, which showed effective neuroprotection post ischaemia. Infarct area was significantly reduced over the eight-slice interval, particularly in the anterior sections (Figure 3.4). It is likely that rostral areas of brain are less prone to neuronal injury in MCAO model. Morphological studies have shown neuronal necrosis developing early in the striatum followed by delayed cell death in cerebral cortex (Garcia et al., 1997). The posterior sections are comprised of more striatum then cortex and thus, may encompass a greater area of irreversible damage. Consequently, melatonin treatment may have been less effective in preventing immediate necrosis occurring in striatum. There was a significant (Figure 3.4) overall reduction of cerebral damage by 70% in the melatonin treatment group as compared with vehicle treated animals,
Chapter 3: The Effects of Melatonin on Neuronal Injury Post Ischaemia

following stroke. The neuroprotection of melatonin administration is consistent with findings in the current literature (for review see, O’Collins et al., 2010). This supports the use of melatonin in acute stroke therapy.

In the new dosing regime, the animals were dosed i.p. at 1 hour post ischaemia and at 2300 hours on day 1 and day 2, in order to boost the endogenous levels of melatonin. A study by Laudon and his colleagues (1988) had measured the serum concentration of melatonin in mature and aged Wistar rats and found that regardless of the animal’s age, the serum concentration began to rise after 3 hours, peaking at 7 hours after the onset of darkness. The animals in the current study were effectively dosed at 5 hours after the onset of darkness. The new regime was more effective, since it was observed to give a 70% reduction in cerebral damage with only 5 mg/kg i.p. repeated doses. This was a much greater improvement than reported by others using the same dosage (as reviewed by Macleod et al., 2005).

The evidence of neuroprotection from melatonin administration in this study is consistent with the literature. A recent meta-analysis of the efficacy of melatonin in experimental stroke by O’Collins and her colleagues (2010), found an overall 40% reduction in damage. The study included 42 original publications on focal ischaemia, involving 758 animals. Significant benefits were found at doses of melatonin above 5 mg/kg. However, this study did not differentiate between the routes and times of administration, the duration of ischaemia and the method utilised to attain the focal ischaemia.

The present investigation was most similar in design to a study by Pei et al., (2003c). In their study, the male Sprague Dawley rats (280 – 360 g) underwent 3 hours of focal ischaemia by intraluminal filament MCAO. The animals were then administered 5 mg/kg i.p. of melatonin (dissolved in the same vehicle as this study) at 1, 24 and 48 hours post ischaemia. At 72 hours post ischaemia, analysis with TTC showed that multiple dosages of melatonin with the first dose within 2 hours of ischaemia were effective in reducing cerebral damage by 40%. Furthermore, for a single dose (5 mg/kg i.p. melatonin) to be effective, melatonin had to be administered within 1 hour of MCAO. In comparison, the results of this study showed greater neuroprotective effect with a reduction in infarct volume by 70% with multiple dosing. This may be attributed to the nocturnal dosing regime, which may have provided an additive effect. However, this has been questioned by Pei and his colleagues, who have suggested that the second and third dosage may not have any further benefit.
The abundant evidence of melatonin’s neuroprotective effect post stroke is supported by the current study. In order for melatonin to progress as a viable candidate for acute stroke therapy clinically, it is highly recommended that STAIR criteria be met (as reviewed in Section 1.5.1). The most recent meet of the STAIR Committee focused on the experimental design of pre-clinical studies (Fisher et al., 2009). Following these guidelines, the present study used and adhered to published predefined inclusion and exclusion criteria (Rahman et al., 2005b). Animals excluded in the current study have been stated in this thesis. The animals were randomly assigned to the treatment groups. Furthermore, the allocation of the animals to either the vehicle or melatonin treatment groups was concealed from the surgeon in the study. There were no conflicts of interest or bias in this report as declared in our published report of this study (Nair et al., 2011). Sample size calculation was not fully conducted however, in advance of the study as suggested in the STAIR guidelines. Even though the STAIR guidelines were published after the completion of the current study, the protocol has anticipated and complied with the majority of recommendations issued by STAIR (Fisher et al., 2009).

The present study showed a 70% reduction of infarct size in transient focal ischemia when melatonin is given after an hour of occlusion. This reduction fell within the threshold recommended by the STAIR committee (Green, 2008). There still needs to be a study showing similar efficacy in permanent focal ischaemia with post-MCAO administration of melatonin. The current study has contributed to the present literature in meeting these criteria. Previously there have been 42 other studies in models of stroke examining the benefits of melatonin administration published in peer reviewed journals (as reviewed by O’Collins et al., 2010). Most studies, such as the present one, used randomisation of animals and blinding of investigators. Taken together with the current study, these findings implicate melatonin as neuroprotective in both permanent and transient models of ischaemia and in different animal species (mainly small animals). In addition, the results have been replicated by many independent laboratory groups. The animal studies have come to a stage where only stroke models in larger animals such as primates need to be conducted to completely satisfy this portion of the STAIR criteria.

The STAIR criteria also suggested that there should be a reduction in cortical and subcortical infarct in order to establish the therapeutic effectiveness of a putative neuroprotectant (Green, 2008). In the present study, we found a reduction in infarct volume of both cortical and subcortical regions. Although, this study did not examine the effect of melatonin on white matter damage as suggested by Green (2008), there has been a previous study differentiating grey and white matter injury (Lee et al., 2005). In that
Chapter 3: The Effects of Melatonin on Neuronal Injury Post Ischaemia

study, Lee and co-workers administered melatonin an hour after onset of occlusion, leading to significant protection of white and grey matter. Thus, this criterion of the STAIR guidelines has been satisfied.

Moreover, it has been recommended by the STAIR committee that both functional and infarct volume outcomes should be measured. Functional recovery is more relevant to clinical studies when testing putative neuroprotectants. The decrease in infarct volume may not always correlate to functional recovery, as other factors such as adaptive behaviours may also contribute to the overall recovery. Measurement of small improvements in infarct volume is not generally feasible in clinical neuroprotection studies. In the present study, only infarct volume was noted to significantly decrease with melatonin administration, whereas functional outcome was not measured. This was not deemed necessary, as previous investigations have observed acute improvements of both functional and infarct volumes with melatonin treatment (as reviewed by O’Collins et al., 2010). This functional outcome should also be measured at longer survival time points. Efficacy of melatonin has not been shown at longer time points with focal ischaemia. In a model of global ischaemia, a single post-ischaemic melatonin infusion led to improvements in behaviour and infarct volumes at 120 days (Letechipia-Vallejo et al., 2007). However, these results were disputed by investigators using a different model of global ischaemia, where no significant differences in outcomes at 140 days melatonin treatment (Rennie et al., 2008). Findings from studies in global ischaemia may not be applicable to transient focal ischaemia. Therefore, there is a need to investigate the long term functional effects of acute melatonin treatment.

Another criterion that has been fulfilled is the measurement of physiological variables in animal models of stroke. In the present study, no significant differences in the physiological variables preceding the stroke induction and post stroke were found between the treatment groups. Similarly, a pharmacokinetic study using similar doses of melatonin found no significant difference in physiological variables (Cheung et al., 2006). It has been suggested that the changes in physiological variables post stroke are comparable between animals and humans (reviewed by Green et al., 2003). Therefore, we suggest that the results from the current study can be extrapolated to the clinical setting, where no effect on physiological variables is expected with melatonin treatment for stroke.

Although, the present study did not investigate the dose response curve for neuroprotection by melatonin, our observations are in agreement with findings published in the current literature. O’ Collins et al., (2010), in their meta analysis of all in vivo cerebral ischaemic models have found a significant dose dependent neuroprotection with melatonin at doses greater than 1.5 mg/kg. The maximum
neuroprotection was achieved in the 20-40 mg/kg range, with doses greater than 40 mg/kg showing lower efficacy than 5 mg/kg. In another investigation, Pei and his colleagues (2002) found doses greater than 5 mg/kg to be effective, with maximal neuroprotection at 15 mg/kg and not at 50 mg/kg. Hence, the dose-response curve for melatonin use in acute stroke in animals has been investigated.

The efficacy of a neuroprotective effect must be shown regardless of gender. The pathogenesis of stroke has been shown to vary according to gender. Both oestrogen and progesterone have been shown to have neuroprotective effects in stroke (Merchenthaler et al., 2003; Stein, 2008). One hypothesis has postulated a direct interaction of melatonin and oestrogen in neuroprotection (Harrod et al., 2005). A recent study in ovariectomised rats confirmed that melatonin and oestrogen had a synergistic neuroprotective effect after transient MCAO (Tai et al., 2011). Thus, this criterion of the STAIR committee has also been satisfied by this recent publication.

Monotherapy with melatonin has been shown to be efficacious in neuroprotection (O’Collins et al., 2010). Another STAIR criterion that has yet to be fully investigated is the use of melatonin as part of combination therapy. Firstly, melatonin therapy must be compatible with the current standard of rt-PA. Melatonin therapy after rt-PA in transient focal ischaemia led to preservation of the blood brain barrier and reduction in haemorrhagic transformations (Chen et al., 2006). Secondly, the aim of the multiple drug therapy would be to target the various pathological pathways simultaneously and hence have greater efficacy. Ideally, the treatments would work synergistically in reducing cerebral ischaemia. A recent study compared three putative neuroprotectants, melatonin, magnesium and minocycline in transient focal ischaemia (O’Collins et al., 2010). The investigators found that neither combination nor melatonin only treatment delivered 3 hours after of ischaemia onset was ineffective in aged and young animals, subjected to 2 and 3 hours of ischaemia, respectively. Similary, with smaller infarcts attained in young animals subjected to shorter ischaemic times (90 minutes), combination treatment was also ineffective when given after onset of ischaemia (O’Collins et al., 2010). The major reason for failure of this combination may be attributed to the different therapeutic time windows and administration times of each agent. It was found, that the optimal response for magnesium was obtained when administered at 2-3 hours, minocycline 4-5 hours and melatonin 1 hour after onset of ischaemia. Therefore, even though efficacious in combination with rt-PA, melatonin combination with other putative neuroprotectants needs to take into account the therapeutic window of each agent.
Chapter 3: The Effects of Melatonin on Neuronal Injury Post Ischaemia

The therapeutic window in animal models needs to be established and replicated when used in human stroke treatment. The present study did not explore the therapeutic time window as it had been shown previously. Pei et al., (2003c) found that a single dose was effective only for 1 hour from occlusion of blood supply, whereas multiple doses increased the therapeutic window to 2 hours. This is a relatively short time period, as most stroke patients may present to a clinician several hours after onset of stroke symptoms (see Section 1.5.2 for therapeutic time window). Based on these results, melatonin treatment in acute therapy as a neuroprotectant in a clinical setting would be limited to a few patients. If melatonin can be shown not to be interacting with proceeding clinical therapy, it can be administered by paramedics. Melatonin has been shown to have minimum toxicity with other benefits from its use (see Section 1.7.2 for physiological role of melatonin). In addition, due to its widespread availability and low financial cost, melatonin can be used as a prophylactic therapy.

An age related decline in endogenous melatonin production has been shown in both animals (Laudon et al., 1988) and humans (Sack et al., 1986). The prevalence of stroke has also been shown to be higher in the elderly population (see Section 1.1.2 for epidemiological data). There is therefore a possible association with premorbid melatonin levels and incidence of stroke. Melatonin may be taken as a dietary supplement to increase its circulating levels to compensate for the age related decline. To this end, animal studies have demonstrated that pre-treatment with pharmacological doses of melatonin in models of focal and global ischaemia were neuroprotective (as reviewed in Macleod et al., 2005). Furthermore, a study investigating the prophylactic treatment (4 mg/kg/day for 9 weeks) in young mice found significant neuroprotection in a model of focal ischaemia (Kilic et al., 2004). The neuroprotective effect was similar to that obtained following acute melatonin administration post stroke onset.

The administration of melatonin post stroke may also be helpful in the rehabilitation of the patient. Melatonin secretion in the urine was found to be impaired 3, 7 and 14 days post stroke, corresponding to alterations of patients’ sleep rhythms and mood (Fiorina et al., 1999). In another study, measuring urinary 6-sulfatoxymelatonin (the major excretory product of melatonin metabolism) levels, an alteration in the circadian peak was noted on days 3 and 4 post stroke. The melatonin peak was restored at 10 days post stroke (Beloosesky et al., 2002). Melatonin administration sub-acutely may play an important role in earlier restoration of physiological melatonin rhythmicity. Normalising the sleep rhythms of sleep patients would be a huge factor in their rehabilitation. Hence, melatonin may also be used sub-acutely in stroke patients.
Chapter 3: The Effects of Melatonin on Neuronal Injury Post Ischaemia

The neuroprotection attained with acute melatonin administration in cerebral ischaemia is consistent with the literature. In order for melatonin to progress as an acute stroke therapy for stroke patients, the remaining STAIR group criteria need to be met. Moreover, melatonin may be used as a prophylactic and during rehabilitation.

3.3.3 Toxic effects of melatonin administration

The potential for toxic effects with repeated doses of melatonin at 5 mg/kg i.p. were studied. With the new treatment regime, there were no toxic effects of melatonin as indicated by no differences in the organ weights or ALT measurements between the treatment groups. Melatonin is a physiological hormone (see Section 1.7.2), which is rapidly metabolised by the liver. Thus unlike exogenous compounds, the lethal dose ($LD_{50}$) was considerably higher at 1131 mg/kg (Sugden, 1983). Conversely, melatonin treatment has been shown in the literature to have a therapeutic effect on the liver. Melatonin administration at 5 mg/kg has been shown to prevent free radical induced hepatotoxicity (Basile et al., 2004). It has also been found to be beneficial in other modes of hepatic damage induced by irradiation (Koc et al., 2003), lipopolysaccharide (Sewerynek et al., 1995; Wang et al., 2004) and thioacetamide (Karabay et al., 2005). Clearly, the dose of melatonin employed in the current study has been documented to have no hepatotoxic effects.

A possible adverse outcome of the administration of melatonin may be on the reproductive system. As described in Section 1.7.2, melatonin inhibits the reproduction hormones, luteinizing hormone and follicle stimulating hormone via inhibition of GnRH. Consequently, one should anticipate a difference in the testicular weights. However, in this study there was no significant difference in testicular weights between treatment groups. The short-term dosing of melatonin may not have allowed sufficient time to observe the anti-gonadal effects of melatonin. These effects of melatonin on gross organ weights are consistent with previous findings confirming its safety in animal use (Cheung et al., 2006).

It can be concluded that 5 mg/kg i.p. has no adverse physiological effects when used post stroke in Sprague Dawley male rats.
3.4 CONCLUSION

The most prevalent cause of exclusion of animals in both treatment groups was ICH, which was confirmed on tissue extraction. There were no confounding factors due to surgery between the two treatment groups. The changes in physiological variables over the course of the surgical procedure were due to prolonged mechanical ventilation and this was consistent with the literature.

Multiple doses of melatonin at 5 mg/kg i.p. were shown to be neuroprotective post stroke. Doses were administered at 1 hour post ischaemia and at 11 pm on day 1 and day 2 following stroke induction, in order to boost the physiological levels of melatonin.

Toxicological analysis demonstrated that treatment with melatonin at 5 mg/kg i.p. produced no adverse effects.
CHAPTER 4

IMMUNOHISTOCHEMICAL LOCALISATION OF MELATONIN AND ITS RECEPTORS
4.1 INTRODUCTION

Melatonin treatment has been shown to be neuroprotective (see Chapter 3). Melatonin may exert some of its physiological functions through direct activation of its receptors (see Section 1.7.3). Thus, the rationale for this chapter was to examine a possible neuroprotective role of the melatonin receptors in cerebral IR. Radiolabelled melatonin binding has been shown to localise to several regions of the rat brain (Vanecek et al., 1987; Williams et al., 1995). However, this method did not discriminate between MT$_1$ and MT$_2$ receptor subclasses. Previous studies have localised mRNA encoding for melatonin receptors in some regions of the brain, however no study has examined the protein expression of the receptors in some regions of the brain, however no study has examined the protein expression of the receptors throughout the rat brain (see Section 1.7.3). Recently, Lee et al. (2010), showed increased expression of MT$_2$ receptors in CA1 region of hippocampus after cerebral ischaemia. This alteration in membrane receptors has also been found in other neurodegenerative pathologies, such as AD (Brunner et al., 2006; Savaskan et al., 2007; Savaskan et al., 2001; Savaskan et al., 2002; Wu et al., 2007) and PD (Adi et al., 2010).

In contrast to the thoroughness of the melatonin receptor binding studies, few studies have investigated the production of extra-pineal melatonin within the CNS. The pineal gland is the major source of melatonin production, however other tissues have also been shown to synthesise the hormone (see Section 1.7.1). Studies on the localisation of the rate-limiting enzymes in the melatonin synthesis, AA-NAT and HIOMT (see Section 1.7.1), have aided in finding melatonin synthesis at the tissue level. AA-NAT activity has been detected in several brain regions of the rat (Gaudet et al., 1991), while AA-NAT mRNA has been found in regions of the human brain (Coon et al., 1996) and rat brain (Hamada et al., 1999; Uz et al., 1999). Immunohistochemistry has been used to definitely localise melatonin in tissues (Kobayashi et al., 2005). Since, the aim of the current study was to examine the localisation of melatonin synthesis, the samples were extracted at specified times to coincide with the peak of plasma melatonin levels (Laudon et al., 1988).

4.1.1 Hypothesis and aims

We hypothesised that the distribution of melatonin and its receptors may be altered after MCAO. To this end, the aims of this study were to examine the distribution of melatonin and its receptors at the peak of plasma melatonin levels in naïve rats and to observe any changes in distribution of the hormone and its receptors as a consequence of acute stroke injury in the MCAO model.
4.1.2 Experimental approach

To investigate this hypothesis, a study was conducted to estimate any ‘gross’ difference in antibody labelling in the brain. In this study, there were two groups of animals used: a control (no intervention) and a MCAO group. Animals were randomised to the control group with \( n = 4 \) animals and to the MCAO group, \( n = 8 \) animals (see Section 2.3). The MCAO group underwent transient ischaemia for 2 hours and were not treated. On the third day post MCAO, at 0200 hours, the brains were removed and stored in formalin prior to wax embedding. Serial coronal sections (8 \( \mu \text{m} \) thick) of the brain were taken at every 1 mm. Initial optimisation and examination of specificity of antibodies was carried out. The sections were then single labelled with, either melatonin, \( \text{MT}_1 \) or \( \text{MT}_2 \) receptor antibodies. An ordinal ranking scale (as described in Section 2.4.11) was used to estimate differences in antibody labelling within and between the treatment groups.

4.2 RESULTS

4.2.1 Inclusion of animals

All 4 of the naïve control animals and 6 of MCAO group animals were included in this study. Two animals were excluded from the MCAO group due to the discovery of ICH at sacrifice (Figure 3.1). The mean actual survival time (time from ischaemia to sacrifice) for animals was 60 hours.

4.2.2 Optimisation of antibodies

The optimal conditions used in the immunohistochemical study for each primary antibody are listed in Table 4.1, whereas secondary antibody conditions are listed in Table 4.2.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specifications</th>
<th>Dilution Used</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melatonin</td>
<td>Polyclonal sheep IgG anti-human</td>
<td>1:50</td>
<td>18 hours</td>
</tr>
<tr>
<td>( \text{MT}_1 )</td>
<td>Polyclonal goat IgG anti-rat</td>
<td>1:50</td>
<td>18 hours</td>
</tr>
<tr>
<td>( \text{MT}_2 )</td>
<td>Polyclonal rabbit IgG anti-human</td>
<td>1:50</td>
<td>18 hours</td>
</tr>
</tbody>
</table>

*Table 4.1 The specifications and optimal concentrations of each primary antibody used in the immunohistochemical analysis. The sections were incubated for 18 hours at 4°C.*
Chapter 4: *Melatonin and its receptors after stroke*

<table>
<thead>
<tr>
<th>Antibody (Secondary)</th>
<th>Primary</th>
<th>Dilution Used</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotinylated rabbit anti-sheep antibody</td>
<td>Melatonin</td>
<td>1:50</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Biotinylated rabbit anti-goat antibody</td>
<td>MT&lt;sub&gt;1&lt;/sub&gt;</td>
<td>1:200</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Biotinylated goat anti-rabbit antibody</td>
<td>MT&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1:200</td>
<td>30 minutes</td>
</tr>
</tbody>
</table>

*Table 4.2* The specifications and optimal concentrations of each secondary antibody (corresponding to the relevant primary antibody) used in the immunohistochemical analysis. Sections were incubated at room temperature with the secondary antibody.

Another important part of the antibody optimisation protocol was to consider the saturation of the DAB substrate. DAB substrate is metabolised by a peroxidase based enzyme system provided with the Vectastain® kit. The avidin and biotinylated peroxidase complex binds to the biotinylated secondary antibody. With DAB substrate in excess there is a peroxidase-dependent accumulation of the DAB precipitate product. Thus, the labelling intensity reaches saturation with time, after which there is decreased specificity of results (Ma *et al.*, 2006). A quantitative DAB saturation curve was produced using optimised concentrations of MT<sub>1</sub> (Figure 4.1) and MT<sub>2</sub> (Figure 4.2) antibodies. Experimental conditions, such as room temperature can influence the DAB exposure time required to reach saturation and care was taken to standardise conditions. Thus, OD from all the samples were calculated and it was ensured that the sections used for analysis had not reached maximum saturation (based on quantitative saturation curves) for their respective antibodies.

![Figure 4.1](image_url)  
*Figure 4.1* DAB saturation curve for MT<sub>1</sub> antibody. There was a significant (*F* = 38.04, *P* < 0.001, *R<sup>2</sup> = 0.62) linear correlation of mean optical density and time of exposure of sections to DAB between 5 and 15 minutes of exposure. Saturation of DAB labelling was reached at 20 minutes, after which there was a plateau in optical density. Each point represents mean ± SEM for *n*=4-6 observations.
Figure 4.2 DAB saturation curve for MT$_2$ antibody. There was a significant ($F = 17.82$, $P < 0.001$, $R^2 = 0.42$) linear correlation of mean optical density and time of exposure of sections to DAB between 1 and 8 minutes of exposure. Saturation of DAB labelling was reached at 12 minutes, after which there was a plateau in optical density. Each point represents mean ± SEM for $n=4$-8 observations.

4.2.3 Specificity of antibodies

The preadsorption study was used to determine the specificity of the melatonin antibody labelling. Slides were incubated with either only primary melatonin antibody or the primary antibody with 1 µM melatonin. Two consecutive sections of the hypothalamus showed differences in melatonin antibody binding profiles with and without preadsorption (Figure 4.3). The preadsorption section was totally devoid of any labelling in comparison with the adjacent section. Therefore, these results demonstrated that the results of the melatonin antibody study were due to specific labelling.

To ensure specificity of MT$_1$ antibody, some slides were only incubated with primary antibody diluents (0.1% Triton X-100, 0.1% BSA and 0.01% sodium azide in 50 mM TBS) instead of primary antibody. The slides were then incubated with secondary antibody and Vectastain® ABC reagent prior to DAB exposure. There were contrasting differences between the two groups (Figure 4.4). The sections which were not exposed to primary MT$_1$ antibody were devoid of DAB labelling, confirming the specificity of the antibody.
Figure 4.3 Contrasting results of the preadsorption study for melatonin antibody immunoreactivity profile. The sections show the suprachiasmatic nucleus of hypothalamus. No DAB labelling was observed in the sections incubated with 1 µM melatonin (A). However, without melatonin preadsorption, the primary antibody binding was evident (as indicated by arrows) in cells (B). Scale bar represents 50 µm.

Similarly, MT$_2$ antibody specificity was examined with use of negative controls. The sections were incubated with either the primary antibody or its diluents. This was followed by exposure to secondary antibody and Vectastain ABC reagent prior to DAB exposure as described earlier. Obvious differences were noted with an absence of antibody labelling in the negative controls (Figure 4.5).

Figure 4.4 Specificity of MT$_1$ antibody shown by use of a negative control. No antibody labelling was seen in cerebral arterioles in absence of MT$_1$ antibody (A). However, in presence of antibody (B), DAB precipitate was found associated with the endothelial layer (as indicated by the arrows). Scale bar represents 50 µm.
Figure 4.5 Specificity of MT$_2$ antibody elucidated by use of a negative control. No antibody labelling in cerebral arteriole in absence of MT$_2$ antibody (A). However, in presence of antibody (B), labelling seemed to be in the adventitia of the arteriole (as indicated by the arrows). Scale bar represents 50 µm.

4.2.4 Verification of the method of identifying the brain regions

All the sections were matched to the closest corresponding figure in the rat neuroanatomy atlas (Figure 4.6, Paxinos et al., 1997). After the sections were matched to the corresponding figure of the atlas, regions were identified and microscopic pictures were taken (Figure 4.7). The other regions are shown in Section A.2.
Figure 4.6 A representative illustration of a section (A) corresponding to the relevant neuroanatomy atlas plate (B). The right side of the section (A) shows MCAO induced cerebral IR injury. The neuroanatomy schematic diagram is reproduced with permission from Paxinos et al., (1997). Scale bar represents 2 mm.
Chapter 4: Melatonin and its receptors after stroke

Figure 4.7 An illustration of identifying a region (cerebellum) after matching the section to the relevant neuroanatomy atlas plate. The arrows in the microscopic picture indicate the positively labelled Purkinje cells. The neuroanatomy schematic diagram is reproduced with permission from Paxinos et al., 1997. Scale bar represents 50 µM.

4.2.5 Melatonin antibody binding distribution in normal brains

Photomicrograph examination showed the presence of round bodied neurons positively labelled with melatonin antibody in the hypothalamus. Labelling was seen present in particular, in the SCN, median preoptic nucleus and median eminence with a high proportion of positive cells (see
Figure 4.3 B). In the hippocampal region, the cells were positively labelled in the CA1 and CA2/3 regions (see Figure 4.8 A). Cells were immunoreactive in the amygdala as well. Several multipolar neurons, clustered in nuclei, were positive in medulla, midbrain and pons regions. This also included several cranial nerve nuclei (Figure 4.8 B). Within the cerebellum, Purkinje cells were positively labelled (Figure 4.8 C). The optic nerve tract periphery was strongly labelled with melatonin antibody, as well as the extracellular matrix but labelling was not consistently found within these cells (Figure 4.8 D). The ependymal cells were also positively labelled (Figure 4.8 E). In most of the other regions only a few dispersed cells were immunoreactive (not shown here). The endothelial cells of venules and capillaries were strongly labelled (Figure 4.8 B, F), while melatonin antibody binding was only present in the tunica adventitia of the arterioles.

In the control brains, there were significant differences in distribution of positively labelled cells amongst different regions (see Table 4.3). There were significant differences within both the ipsilateral hemisphere ($Q = 4.27$, d.f. = 13, $P < 0.01$) and contralateral hemisphere ($Q = 4.21$, d.f. = 13, $P < 0.01$). In both hemispheres, the hypothalamus region had significantly greater number of positive cells compared to other regions (see Table 4.3).
Chapter 4: Melatonin and its receptors after stroke

Figure 4.8 Representational photomicrographs of positive immunoreactivity to melatonin antibody in the ipsilateral hemisphere. **A**, melatonin antibody binding localised to cells in the CA1 region of the hippocampus of control animals indicated by arrows. **B**, multipolar neurons were positively labelled (as indicated by arrows) within the 7th cranial nerve nuclei in a naïve animal. Endothelial cells of a blood vessel was also positively labelled (as indicated by + sign). **C**, Purkinje cells from lobe 3 of the cerebellum in a naïve animal were labelled with melatonin antibody (as indicated by arrows). **D**, the optic nerve had considerably more DAB precipitate associated (as indicated by arrow) compared to the adjacent cerebral tissue (control animal). Insert shows cells which were not labelled with antibody (indicated by * sign). **E**, Ependymal cells lining the third ventricle (of a control animal) showing higher concentration of melatonin, as indicated by arrows. **F**, melatonin immunoreactivity in different inflammatory cells (as indicated by arrows and * sign) within the infarct area of a MCAO animal. Melatonin also appeared to be expressed in the endothelial cells of a capillary within the infarct, (as indicated by + sign). Scale bars (A-H): 50 µm.
Table 4.3 The immunohistochemical profile of melatonin in stroke-induced and non-intervention control brains. Single labelling IHC analysis contains cellular immunoreactivity of melatonin antibody within neuronal and non-neuronal populations. Within the infarct area, immunoreactivity of inflammatory cells was also considered. Score values are represented by: score of 0 - no positive cells with melatonin antibody labelling were present; 1 - few dispersed positive cells in the brain region; 2 - some positive cells in the brain region; 3 - many positive cells in the brain region; 4 - most cells are positive in the brain region (Sutherland et al., 2009).

<table>
<thead>
<tr>
<th>Melatonin</th>
<th>Control</th>
<th>MCAO</th>
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<td>Contralateral</td>
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<td>Cortex</td>
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<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>1</td>
</tr>
<tr>
<td>Cerebellum</td>
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<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Midbrain &amp; Pons</td>
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<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Infarct</td>
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</table>

<sup>a</sup> $P < 0.05$, (Friedman two-way non-parametric ANOVA) revealing a significant difference between hypothalamus and specific brain regions in ipsilateral hemisphere of control animals.  
<sup>b</sup> $P < 0.05$, (Friedman two-way non-parametric ANOVA) revealing a significant difference between hypothalamus and specific brain regions in contralateral hemisphere of control animals.  
<sup>c</sup> $P < 0.05$, (Friedman two-way non-parametric ANOVA) revealing a significant difference between hypothalamus and specific brain regions in contralateral hemisphere of MCAO animals.  
<sup>d</sup> $P < 0.05$, (Friedman two-way non-parametric ANOVA) revealing a significant difference between hypothalamus and specific brain regions in ipsilateral hemisphere of MCAO animals.  
<sup>h</sup> $P < 0.05$, (Wilcoxon Signed Rank test), revealing a significant difference compared to the same region in the contralateral hemisphere of MCAO animals.  

The results shown are median scores of n=4-6 separate observations.
4.2.6 Melatonin antibody binding distribution after MCAO

Within MCAO animals, there were also significant regional differences (see Table 4.3). In the ipsilateral hemisphere there was significant difference ($Q = 4.53$, d.f. = 13, $P < 0.001$) with hypothalamus having greater number of positive cells compared to other regions. Similarly in the contralateral hemisphere, there was a significant ($Q = 4.23$, d.f. = 13, $P < 0.01$) difference in regional distribution, with hypothalamus showing greater number of immunoreactive cells compared to other regions (see Table 4.3).

Following MCAO, within the infarct area, multiple subpopulations of inflammatory cells were seen positively labelled with melatonin antibody (Figure 4.8 F). There was no change in the binding profile of blood vessels following IR.

There were significant ($V = 0$, d.f. = 6, $P < 0.01$) differences comparing ipsilateral and contralateral hemispheres of MCAO (see Table 4.3). In the ipsilateral hemisphere of MCAO animals, basal ganglia region had significantly ($W = 14$, d.f. = 1, $P < 0.05$) increased labelling of melatonin antibody compared with the same region in contralateral hemisphere. However, there were no differences detected when comparing overall hemispheres of the two treatment groups. There was also no difference in melatonin labelling in any brain regions between control and MCAO animals.

4.2.7 Distribution of the MT$_1$ receptor in normal brains

In the hypothalamus, positive labelling was localised to smaller round bodied cells (see Figure 4.9 A). The majority of Purkinje cells seen in the cerebellar region were labelled with MT$_1$ receptor antibody (see Figure 4.9 B). MT$_1$ receptor expression was localised to multipolar neurons, present in clusters, of various nuclei in the medulla, cranial nerve nuclei, midbrain and pons regions (not shown here). In most other brain regions, only a few dispersed cells were positively labelled (not shown here). The endothelial cells of blood vessels appeared to express MT$_1$ (Figure 4.4 B).
Chapter 4: Melatonin and its receptors after stroke

Figure 4.9 Representational photomicrographs of positive immunoreactivity to MT₁ receptor. A, small bodied neurons with MT₁ receptor expression in the anterior hypothalamic area of the hypothalamus in a control brain. B, expression of MT₁ receptor by Purkinje cells in lobe 4 of the cerebellum of a control animal, as indicated by arrows. Scale bars (A-D): 50 µm.

There were significant differences in distribution of positively labelled cells when comparing different regions within ipsilateral ($Q = 3.79$, d.f. = 13, $P < 0.05$) and contralateral hemispheres ($Q = 3.73$, d.f. = 13, $P < 0.05$, Table 4.4). In both the hemispheres, the hypothalamus had greater number of cells with MT₁ expression (Table 4.4).

4.2.8 Distribution of the MT₁ receptor after MCAO

Within the infarct, inflammatory cells showed no appearance of MT₁ receptor expression. In addition, the profile of MT₁ receptor expression did not change in blood vessels after MCAO from controls. Within MCAO animals, there were also significant differences in MT₁ receptor expression between ipsilateral ($Q = 4.65$, d.f. = 13, $P < 0.001$) and contralateral ($Q = 4.36$, d.f. = 13, $P < 0.001$) hemispheres, with hypothalamus and cerebellum regions having a greater number of positive cells (Table 4.4).

When comparing overall hemispheres, MCAO led to an increased number of cells with MT₁ expression in the ipsilateral hemisphere when compared to the contralateral hemisphere ($V = 152$, d.f. = 6, $P < 0.001$), whilst amongst the control animals there was no difference in hemispheres. When comparing ipsilateral hemispheres of MCAO and controls, there was also a significant ($W = 3528$, d.f. = 1, $P < 0.01$) increase in expression in MCAO group. The cerebral cortex was a region where this significant ($W = 33$, d.f. = 1, $P < 0.01$) difference was present (Figures 4.9 C and D). However, there were no differences in the contralateral hemispheres of both groups.
**Chapter 4: Melatonin and its receptors after stroke**

<table>
<thead>
<tr>
<th>MT&lt;sub&gt;1&lt;/sub&gt;</th>
<th>Control</th>
<th>MCAO</th>
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<tbody>
<tr>
<td></td>
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<td>Contralateral</td>
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<td>Olfactory System</td>
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<td>Infarct</td>
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**Table 4.4** MT<sub>1</sub> distribution throughout the brain in neuronal and non-neuronal cells. Single labelling IHC analysis contains cellular immunoreactivity of melatonin antibody within neuronal and non-neuronal populations. Within the infarct area, immunoreactivity of inflammatory cells was also considered. Score values are represented by: score of 0 - no positive cells with melatonin antibody labelling were present; 1 - few dispersed positive cells in the brain region; 2 - some positive cells in the brain region; 3 - many positive cells in the brain region; 4 - most cells are positive in the brain region (Sutherland et al., 2009).

P < 0.05, (Friedman two-way non-parametric ANOVA), revealing a significant difference between hypothalamus and specific brain regions in:

-<sup>a</sup> ipsilateral hemisphere of control animals,
-<sup>b</sup> contralateral hemisphere of control animals,
-<sup>c</sup> ipsilateral hemisphere of MCAO animals,
-<sup>d</sup> contralateral hemisphere of MCAO animals;

P < 0.05, (Friedman two-way non-parametric ANOVA), revealing a significant difference between cerebellum and specific brain regions in:

-<sup>e</sup> ipsilateral hemisphere of MCAO animals,
-<sup>f</sup> contralateral hemisphere of MCAO animals.

*, P < 0.05, **P < 0.01, (Mann Whitney U test), revealing a significant difference when a region in ipsilateral hemispheres of MCAO animal was compared with the same region in ipsilateral hemispheres of control animals.

The results shown are medians of n=4-6 separate observations.
4.2.9 Distribution of the $MT_2$ receptor in normal brains

In the hippocampus, $MT_2$ was expressed by cells in CA1 and CA2/3 regions, whilst in the dentate gyrus, cells were positively labelled. The expression of $MT_2$ was consistently found in the hypothalamus, mainly in small round bodied cells (Figure 4.10 A). In the amygdala $MT_2$ expression was mainly in pyramidal cells (Figure 4.10 B). Most Purkinje cells in cerebellum expressed $MT_2$, whilst it was also expressed by some granule cells (Figure 4.10 C). There were also occasional positively labelled cells in the molecular layer of the cerebellum. Within the brain stem (midbrain, pons, and medullar regions), various nuclei (including cranial nerve nuclei) contained positively labelled multipolar neurons (not shown here).

Figure 4.10 Representational photomicrographs of $MT_2$ receptor antibody labelling. A, $MT_2$ receptor expression by distinct small-bodied neurons of the anterior hypothalamic area of the hypothalamus in the ipsilateral hemisphere of a control brain, as indicated by arrows. B, Neurons in the anterior cortical amygdaloid nucleus of the amygdala in the contralateral hemisphere of a naïve animal, showed $MT_2$ expression (arrows). C, $MT_2$ receptor expressed by granule cells (arrow) and Purkinje cells (asterisk) in lobe 3 (cerebellum) of a control animal. D, $MT_2$ receptor expression in different cell types within an infarct area of a MCAO animal: inflammatory cells (arrow and plus sign) and endothelial cell of a capillary (asterisk). Scale bars (A-D): 50 µm.
In most other brain regions, only a few dispersed cells expressed MT$_2$ (Table 4.5). Some of the ependymal cells also expressed MT$_2$. In the blood vessels, MT$_2$ seemed to be expressed by the tunica adventitia of larger vessels, such as arteries and veins (Figure 4.5 B).

There were no significant differences in MT$_2$ expression amongst the regions of both ipsilateral and contralateral hemispheres of control animals. Similarly, there were no significant differences when comparing overall hemispheres of control animals.

### 4.2.10 Distribution of MT$_2$ receptors after MCAO

There were significant ($Q = 3.71$, d.f. = 13, $P < 0.05$) differences within the regions of each hemisphere in MCAO group (Table 2). Following MCAO, the peri-infarct and infarct areas were found to contain inflammatory cells, some of which were labelled with the MT$_2$ receptor antibody (Figure 4.10 D).

When comparing overall hemispheres, the number of MT$_2$ expressing cells were significantly ($W = 3928.5$, d.f. = 1, $P < 0.01$) higher in the ipsilateral hemisphere of MCAO when compared to their respective contralateral hemisphere. An example of this was the cerebral cortex region which showed high MT$_2$ receptor expression (Table 4.5). On the other hand, there were no significant differences when comparing overall hemispheres between MCAO and controls. When examining individual brain regions, in the MCAO ipsilateral hemisphere, the cerebral cortex and the basal ganglia (Figures 4.11 A and B) had significantly increased numbers of positive cells, compared with control ipsilateral hemispheres (Table 4.5).
Chapter 4: Melatonin and its receptors after stroke

<table>
<thead>
<tr>
<th>MT&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Control</th>
<th>MCAO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ipsilateral</td>
<td>Contralateral</td>
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<tr>
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<td>1</td>
</tr>
<tr>
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<td>1</td>
</tr>
<tr>
<td>Basal Ganglia</td>
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<td>1</td>
</tr>
<tr>
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<td>1</td>
</tr>
<tr>
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<td>1</td>
</tr>
<tr>
<td>Thalamus</td>
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<td>2</td>
</tr>
<tr>
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<td>1</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td>1</td>
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<tr>
<td>Infarct</td>
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</tr>
</tbody>
</table>

Table 4.5 MT<sub>2</sub> distribution throughout the brain in naïve and MCAO animals. Single labelling IHC analysis contains cellular immunoreactivity of melatonin antibody within neuronal and non-neuronal populations. Within the infarct area, immunoreactivity of inflammatory cells was also considered. Score values are represented by: score of 0 - no positive cells with melatonin antibody labelling were present; 1 - few dispersed positive cells in the brain region; 2 - some positive cells in the brain region; 3 - many positive cells in the brain region; 4 - most cells are positive in the brain region (Sutherland et al., 2009).

<sup>a</sup> P < 0.05, (Friedman two-way non-parametric ANOVA) revealing a significant difference between medulla and specific brain regions in ipsilateral hemisphere of MCAO animals.

<sup>b</sup> P < 0.05, (Friedman two-way non-parametric ANOVA) revealing a significant difference between cerebellum and specific brain regions in contralateral hemisphere of MCAO animals.

<sup>c</sup> P < 0.05, (Friedman two-way non-parametric ANOVA) revealing a significant difference between hypothalamus and specific brain regions in contralateral hemisphere of MCAO animals.

<sup>d</sup> P < 0.05, (Friedman two-way non-parametric ANOVA) revealing a significant difference between amygdala and specific brain regions in contralateral hemisphere of MCAO animals.

<sup>*</sup> P < 0.05, (Mann Whitney U test) revealing a significant difference in the same region of ipsilateral hemisphere between control and MCAO animals.

<sup>#</sup> P < 0.05, (Wilcoxon Signed Rank test) revealing a significant difference in the same region between contralateral and ipsilateral hemispheres of MCAO animals.

The results shown are medians of n=4-6 separate observations.
Chapter 4: Melatonin and its receptors after stroke

4.3 DISCUSSION

4.3.1 Melatonin antibody labelling

Initially, it was thought that the lipophilic nature of melatonin would result in a uniform distribution of the immunoreactivity seen throughout the brain. However, in naïve control brains, melatonin antibody labelling was greatest in hypothalamic region. The hypothalamus has been known to play an important role in melatonin synthesis (see Section 1.7.1). The localisation of one of the rate limiting enzymes, AA-NAT, in melatonin synthesis has been described in varying brain regions, (for review, see Simonneaux et al., 2003). Co-localisation of both enzymes AA-NAT and HIOMT is necessary to localise melatonin synthesis as the presence of one does not indicate the presence or absence of the other (Aust et al., 2004; Blanc et al., 2003). No study has yet examined the distribution of HIOMT in the rat brain, and more importantly, none has determined co-localisation of both enzymes in the rat brain. However, the results from the present study correlates well with an AA-NAT activity localisation study in rat brain (Gaudet et al., 1991). However, there were differences in some regions, namely in cerebral cortex, hippocampus, thalamus and brain stem regions. This discrepancy could also be due to the difference in time of sample collection as melatonin is known to have diurnal variations.

Previously, localisation of melatonin in different tissues had led to the suggestion that melatonin has a paracrine/autocrine function (for review, see Tosini and Fukuhara, 2003; Pozo et al., 2004). The paracrine role of melatonin is most convincing in the retina. Both the AA-NAT enzyme and

![Figure 4.11 Representative photomicrographs of MT$_2$ receptor expression after MCAO. The number of cells with MT$_2$ receptor expression (as indicated by arrows) was greater within the caudate putamen (part of basal ganglia) in the ipsilateral of the MCAO brain (B), compared to control brain (A). Scale bar (A, B): 50 µm.]
melatonin receptors have been found in the retina. The retinal melatonin in mammals has been demonstrated not to enter the systemic circulation and has been implicated in aspects of retinal physiology (for review, see Tosini and Fukuhara, 2003). The distribution of melatonin seen in the present study adds to the evidence of a possible autocrine and/or paracrine role.

Similarly, the postulation of paracrine/autocrine roles of melatonin in subpopulations of human immune cells are supported by localisation of both melatonin synthesis with HIOMT and melatonin receptors in isolated populations of human immune cells (Maldonado et al., 2010; Pozo et al., 2004). The presence of melatonin antibody binding in immune cells in the current study is in agreement with these previous observations. Consequently, we suggest that this raised melatonin positive immune cell level may account for the significantly increased number of positively labelled cells in the basal ganglia. As discussed in Chapter 3, the basal ganglia (part of the striatal area) is most prone to immediate neuronal death following cerebral IR. This could thus explain the increased presence of immune cell infiltration within this region. Alternatively, it may be possible that synthesis of melatonin in affected brain regions is increased following cerebral IR. Hypothetically, melatonin could play an important paracrine role in acute oxidative stress. The increased melatonin production as a physiological response to increase ROS would also be a beneficial role. Further research would be useful to examine the role of extra-pineal melatonin in pathologies such as cerebral IR.

4.3.2 Melatonin membrane receptors distribution

The MT$_1$ receptor was expressed in all regions of the brain, with expression higher in the hypothalamus and cerebellum. The MT$_2$ receptor was present throughout the brain but was found in greater levels in the amygdala, cerebellum and hypothalamus. The present study is the first to examine melatonin receptor expression throughout the rat brain both in naïve animals and as a consequence of cerebral IR. The distribution of melatonin receptor protein expression from this study are comparable to the MT$_1$ and MT$_2$ mRNA profile recently published (Ishii et al., 2009).

The MT$_1$ receptor was localised by in situ hybridization in the SCN, and paraventricular thalamus of several species including rats (Masson-Pevet et al., 2000; Reppert et al., 1994). The MT$_2$ receptor has been localised in the hypothalamic region by in situ hybridization (Hunt et al., 2001) and rt-PCR (Ishii et al., 2009; Sallinen et al., 2005). The presence of MT$_2$ has also been well established in the hippocampal region (Hogan et al., 2001; Wan et al., 1999). Within the hypothalamus, the MT$_2$ receptor has a functional role in circadian rhythm modulation (Gerdin et
al., 2004) and in cyclic reproductive physiology (Roy et al., 2001). A functional role of MT2 with GABA\textsubscript{A} modulation has also been implicated in the hippocampus (Wan et al., 1999). Similarly, activation of MT\textsubscript{1} (and MT\textsubscript{2}) receptors in mice cerebellar granule cells has been shown to lead to neuronal ERK and Akt signaling (Imbesi et al., 2008). This suggests a role for melatonin receptors in cell signalling. The functional role of the MT\textsubscript{1} and MT\textsubscript{2} receptors in other regions remains to be investigated.

Both MT\textsubscript{1} and MT\textsubscript{2} receptors were expressed in blood vessels; postulating a vascular role for melatonin. Investigation of melatonin receptors in cerebral vessels with rtPCR found the MT\textsubscript{1} but not the MT\textsubscript{2} receptor (Chucharoen et al., 2003). Functional studies have shown that melatonin when applied to segments of cerebral artery lead to vasoconstriction (Evans et al., 1992; Viswanathan et al., 1990). This effect of melatonin is due to the involvement of G-protein coupled receptor-linked mediation of K\textsuperscript{+} and Ca\textsuperscript{2+} channels, leading to decreased NO levels in endothelial cells (Geary et al., 1998; Geary et al., 1997). Furthermore, treatment with MT\textsubscript{2} receptor antagonists, such as 4-phenyl-2-acetamidotetraline, enhanced the vasoconstrictor effects of melatonin, showing that MT\textsubscript{1} receptors have a vasoconstrictive role which is opposed by MT\textsubscript{2} receptors (Doolen et al., 1998; Viswanathan et al., 1997). Increased vascular expression of MT\textsubscript{1} receptors was seen in AD patients (Savaskan et al., 2001). However, in the present study there were no differences in expression of either receptors following MCAO. Modulation of the vascular tone by melatonin may play an important part in the chronic neurodegenerative pathologies such as AD. The actual role of melatonin on the cerebral vasculature in chronic cerebral IR injury needs to be further examined.

Inflammatory cells in the infarcted area expressed MT\textsubscript{2} receptors. Autoradiographical studies have localised melatonin binding sites to various immune cells and lymphoid organs (Garcia-Perganeda et al., 1997; Gonzalez-Haba et al., 1995; Lopez-Gonzalez et al., 1992; Maestroni et al., 2002; Yu et al., 1991). Both nuclear and membrane receptors were found in lymphocytes (Pozo et al., 2004). Our study suggests that the binding sites in the inflammatory cells demonstrated in the literature may be MT\textsubscript{2} receptors. The effects of melatonin on B lymphocytes have also been found to be mediated specifically via MT\textsubscript{2} receptors (Cernysiov et al., 2010). However, the role of melatonin in immune functions has been disputed (Maestroni, 1993; Srinivasan et al., 2005). Lymphocyte proliferation stimulated by interleukin-2 was shown to be dependent on endogenous melatonin (Carrillo-Vico et al., 2004; Zhao et al., 2005). This outlines a possible autocrine/paracrine role of melatonin in T-helper 1 (Th\textsubscript{1}) pro-inflammatory lymphocytes. The
administration of melatonin in mice has also led to an increase in interleukin-10 production (Raghavendra et al., 2001), indicating an anti-inflammatory T-helper 2 (Th2) response involvement. Receptor ligand studies showed that the anti-inflammatory effects of melatonin on lymphocytes were MT2 receptor dependent (Drazen et al., 2001; Wei et al., 2003). The current study supports the literature by localising the MT2 receptor protein in inflammatory cells.

Increased expression of both MT1 and MT2 receptors was found after cerebral IR injury. The alteration of melatonin receptor expression is consistent with a recent study showing increased MT2 receptor expression in CA1 region following global IR (Lee et al., 2010). Melatonin receptor expression is also altered in the post-mortem hippocampus of AD patients with a decrease in MT2 receptor expression (Savaskan et al., 2005), and increase in MT1 expression (Savaskan et al., 2002). However, both MT1 and MT2 expression decreased in the cortex (Brunner et al., 2006), and similarly the MT1 expression decreased in the SCN (Wu et al., 2007). Expression of both membrane receptors was also decreased with PD (Adi et al., 2010). The results from the current study provide a good basis for focusing further quantitative analysis on the changes in melatonin receptors’ localisation in specific brain regions. The variation in melatonin receptors expression, reported in different disease states and in different brain regions, has been suggested to contribute to its neuroprotective role. The exact mechanism of this has not yet been fully elucidated. Since both receptors are G-protein coupled (see Section 1.7.3), it can be hypothesised that these receptors may be involved in cell signalling pathways in neurodegenerative pathologies. Therefore, further research is required to examine mechanisms of melatonin receptor mediated neuroprotection.

4.3.3 Limitations of this study

The aim of the study was to examine differences in melatonin and its membrane receptors antibody binding profile after cerebral IR. The semi-quantitative technique was used based on previous published studies (Sutherland et al., 2009). It can be argued that stereologic analysis could be used to quantify the difference in binding profiles of the antibodies. However, such a technique is quite extensive and time consuming when applied to the whole brain. This study may thus act as a pilot, indicating the regions of interest for further quantitative studies. The extent of the change noted in the present study can be used in determining appropriate sample cohort sizes in order to generate an appropriate powered study. Moreover, expression of the receptors and even melatonin synthesis enzymes could be examined in microdissections of each region, by
measurement of mRNA or protein expression in the two groups. Microdissection could be
difficult within the infarct and penumbra regions due to the tissue morphology. The disadvantage
of such an approach lies in its inability to identify protein expression according to cell type.
Therefore, the limitations of this study was the semi-quantitative technique employed, however,
given there were no previous study to act as a pilot, it was the most feasible approach.

We also note that the strength of conclusions drawn from this study may be limited. The change in
receptor expression seen in this study could be hypothesised to play a role in possible receptor-
mediated neuroprotection afforded by melatonin. In vivo pharmacological modulation of the
melatonin receptors with selective agonists/antagonists with good bioavailability (see Section
1.7.3) could provide a more definitive answer. Another option would be to study melatonin and its
specific receptor ligands in MT\(_1\) and MT\(_2\) knockout mice after cerebral IR. It would also be
interesting to examine receptor changes when animals subjected to MCAO are treated with
melatonin. Therefore, the present study provides a reasonable foundation for further studies to
quantify the neuroprotective effects of melatonin via its membrane receptors.

This section of the study has several limitations however it can be used as a platform for further
extensive research on this subject.

4.4 CONCLUSIONS

In conclusion, the present study has shown the protein distribution of melatonin and its receptors
in normal and stroke-induced brains. The changes in MT\(_1\) and MT\(_2\) receptor profile following
MCAO indicate a possible role of melatonin receptors in cerebral IR and provide a basis for
further quantitative and ligand studies. These changes of receptor expression could hypothetically
play a role in cell signalling pathways after cerebral IR, as the membrane bound receptors are G-
protein coupled. If so, specific activation of the receptors leading to neuroprotection may be
utilised in treatment of numerous neurodegenerative diseases.
CHAPTER 5

MECHANISMS OF NEUROPROTECTION BY MELATONIN IN CEREBRAL ISCHAEMIA REPERFUSION INJURY
5.1 INTRODUCTION

In Chapter 4, the expression of melatonin membrane receptors was changed acutely post MCAO. Immune cells, immunoreactive for melatonin and MT$_2$ antibody, were present in the infarct region. These cells are known to be involved in inflammation (see Section 1.1.4), which is a major factor in tissue injury occurring in the penumbra of the infarct, well after restoration of blood flow has taken place (see Section 1.2). NOS, arginase and COX enzyme systems are pivotal in inflammation. The enzyme systems are complex in that they have both neuroprotective and neurodegenerative effects, depending on the isoforms and the metabolites, or even the presence of the receptors of the metabolites such as PGE$_2$ (see Section 1.3 for review).

Melatonin is a potent antioxidant (see Section 1.7.4, Reiter, 2003), and was shown to be beneficial in cerebral IR injury (Chapter 3). This neuroprotection afforded by melatonin may be attributed, in part, to its anti-inflammatory effects. A wide range of studies have shown its anti-inflammatory properties (for review see Section 1.7.5, Cuzzocrea et al., 2001; Cuzzocrea et al., 2002).

5.1.1 Hypothesis and aims

In the present study, it is hypothesized that melatonin administered after MCAO is neuroprotective by inhibiting iNOS, nNOS and COX enzymes, whilst increasing arginase and eNOS isoforms. To this end, the aim of the study was to examine the mechanisms of neuroprotection attained by melatonin in the transient focal ischaemia model focusing on three key enzyme systems: NOS, arginase and COX.

5.1.2 Experimental approach

To investigate this hypothesis, the study used three groups of animals: a non-intervention naïve control group (no MCAO), a MCAO vehicle control group (5% DMSO in 0.9% saline i.p.) and a MCAO melatonin treatment group (MCAO and melatonin 5 mg/kg dissolved in vehicle i.p.). The MCAO groups were assigned 9 animals each, whilst the control group was assigned 6 animals. This allotment was randomised and blinded to the surgeon. The onset of ischaemia was initiated at 1400 hours. Melatonin or vehicle was administered one hour post onset of ischaemia, followed by
2 more doses at 2300 hours on consecutive days post MCAO. Studies were terminated at 0200 hours on the third day, which equated to 60 hours post onset of ischaemia. Control animals were also sacrificed at 0200 hours. The brain tissue was extracted and individual hemispheres immediately snap-frozen in liquid nitrogen. Samples were then homogenised in 50 mM Tris buffer containing protease inhibitors and subjected to followed by specific enzyme assays and Western blot analysis (see Chapter 2).

### 5.2 RESULTS

#### 5.2.1 Inclusion of animals

The final numbers included in this study were: 6 naïve control animals, 6 animals in the MCAO vehicle group and 7 in the melatonin-treated MCAO group. The only cause of exclusion in the two MCAO groups was the development of an ICH discovered during brain extraction (Figure 3.1). The physiological parameters measured during MCAO surgery were similar between the two groups.

#### 5.2.2 Optimization of antibody concentrations for inducible enzymes

The optimised dilutions and incubation period for antibodies directed against each isoform of inducible enzyme used in Western blot analysis (see Section 2.8), are listed in Table 5.1

<table>
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<th>Antibody</th>
<th>Specifications</th>
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<th>Incubation</th>
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<td>Arginase-I</td>
<td>Rabbit polyclonal IgG anti-human</td>
<td>1:5000</td>
<td>18 hours</td>
</tr>
<tr>
<td>Arginase-II</td>
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<td>1:5000</td>
<td>18 hours</td>
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<tr>
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<td>1:1000</td>
<td>18 hours</td>
</tr>
<tr>
<td>COX-II</td>
<td>Goat polyclonal IgG anti-human</td>
<td>1:1000</td>
<td>18 hours</td>
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<td>nNOS</td>
<td>Rabbit polyclonal IgG anti-human</td>
<td>1:1000</td>
<td>18 hours</td>
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<td>iNOS</td>
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<td>18 hours</td>
</tr>
<tr>
<td>eNOS</td>
<td>Rabbit polyclonal IgG anti-human</td>
<td>1:1000</td>
<td>18 hours</td>
</tr>
</tbody>
</table>

*Table 5.1 The optimal dilutions of primary antibodies used for Western blot analysis.*
5.2.3 Effect of melatonin on NOS enzymes following MCAO

The protein levels of the three major isoforms of NOS, that is, nNOS, eNOS and iNOS, were measured using Western blot analysis (see Section 2.8). No statistically significant differences were seen in nNOS expression between treatment groups in the lesioned (right) hemisphere. Similarly, there were no differences in the contralateral hemisphere (Figure 5.1).

When examining eNOS expression, no significantly difference was found between the MCAO and naïve control groups in either hemisphere. Melatonin administration had no significant effect on the expression of eNOS protein post cerebral ischaemia (Figure 5.2).

There was a significant [F (2, 16) = 6.16, P < 0.01] change in iNOS protein expression in the ipsilateral (right) hemisphere (Figure 5.3). iNOS expression was significantly (q = 4.94, P < 0.01) upregulated in untreated MCAO animals compared to baseline (control) in the right hemisphere (Figure 5.3). The induction of iNOS expression following MCAO was normalised with melatonin.
administration (as there were no significant differences between melatonin and control group). No differences were observed in the contralateral hemisphere (Figure 5.3).

The protein expression of iNOS was mirrored by its activity profile. There were significant \( F(2, 16) = 7.34, P < 0.01 \) changes in iNOS activity in the right hemisphere. In the right hemisphere, there was significant \( q = 4.93, P < 0.01 \) induction of iNOS activity with MCAO when compared to non-infarcted animals. Melatonin administration led to a significant \( q = 4.43, P < 0.05 \) reduction in the activity of iNOS, toward baseline (control) levels (Figure 5.4). In the contralateral hemisphere, there were no statistically significant differences.

**Figure 5.2** Effects of melatonin on eNOS protein levels following stroke. No significant differences were seen between MCAO (grey) and controls (white). Melatonin treatment (black) did not significantly improve the eNOS expression after MCAO. Each point represents the mean ± SEM of at least 6 separate observations. Each graph is headed by a representative blot showing protein expression in the respective hemisphere.
**Figure 5.3** Effects of melatonin on iNOS expression following stroke. In the ischaemic hemisphere (right) there was a significant upregulation of iNOS protein levels after MCAO (grey) in comparison with controls (white). Although melatonin treatment (black) did not significantly alter iNOS protein expression, it resulted in a loss of the MCAO-induced significant increase. No significant changes in iNOS expression occurred in the left (non-infarcted) hemisphere. The graph is preceded by a representative blot. Each point represents the mean ± SEM of at least 6 separate observations. **, P < 0.01 controls compared with MCAO. Each graph is headed by a representative blot showing protein expression in the respective hemisphere.

**Figure 5.4** Effects of melatonin on iNOS activity after MCAO. Following MCAO (grey), there was a significant increase in iNOS activity when compared with controls (white). This was significantly normalised with melatonin administration (black). There were no significant changes in the contralateral hemisphere. Each point represents the mean ± SEM of at least 6 separate observations. **, P < 0.01 MCAO compared with controls; + melatonin treatment compared with MCAO.
The total NOS activity was measured to analyse the overall activities of all NOS isoforms. There was a significant \([F (2, 16) = 8.32, P < 0.01]\) difference in total NOS activity in the infarcted hemisphere (Figure 5.5). A significant \((q = 5.52, P < 0.01)\) increase in total NOS activity was seen after MCAO compared to the naïve controls. Melatonin administration also significantly \((q = 4.29, P < 0.05)\) reduced the enzyme activity to near control levels (Figure 5.5). The results obtained in the contralateral hemisphere were not statistically significant.

Nitrite levels were assessed to exhibit the cumulative NOS activity, as nitrites are stable breakdown products of NO (see Section 1.3.2). There was a significant \([F (2, 16) = 14.62, P < 0.001]\) difference in nitrite levels in the infarcted hemisphere (Figure 5.6). Nitrite levels significantly \((q = 3.74, P < 0.05)\) increased after MCAO from naïve controls. This MCAO-induced increase was significantly \((q = 4.79, P < 0.001)\) reduced with melatonin treatment. There were no significant differences noted in the contralateral hemispheres (Figure 5.6).
Figure 5.6 Effects of melatonin on nitrite levels following stroke. In the right hemisphere, a significant increase in nitrite levels occurred after MCAO (grey) from naïve control levels (white). This increase was significantly reduced with melatonin administration (black). There were no significant differences in the left hemisphere. Each point represents the mean ± SEM of at least 6 separate observations. *, P < 0.05 when compared with control; +++, P < 0.001 when compared with MCAO.

Linear regression analysis model showed a significant (F = 14.993, P < 0.01, R² = 0.577) relationship between nitrites levels and total NOS activities supporting the hypothesis that nitrite levels are an accurate indicator of the cumulative activity of NOS enzymes.

5.2.4 Effect of melatonin on arginase activity

The arginase I protein expression in the both hemispheres were not significantly altered after MCAO or melatonin treatment (Figure 5.7). Similarly, expression of arginase II was not significantly altered in both hemispheres (Figure 5.8).
ANOVA analysis of arginase activity showed a trend towards significance [F (2, 16) = 2.82, P = 0.09] in ipsilateral hemisphere following treatment (Figure 5.9). There was a small but not significant (q = 3.23, P = 0.08) restoration of arginase activity with melatonin treatment following MCAO. No significant differences were seen in contralateral hemisphere.
Figure 5.8 Effects of melatonin on arginase II expression following stroke. In both hemispheres, there were no significant differences between MCAO (grey) and controls (white) or melatonin treatment (black). Each point represents the mean ± SEM of at least 6 separate observations. Each graph is headed by a representative blot showing protein expression in the respective hemisphere.

Figure 5.9 Effects of melatonin on arginase activity after stroke. There were no statistically significant differences in either hemisphere, between any of the three groups: control (white), MCAO (grey) and melatonin administration (black). Each point represents the mean ± SEM of at least 6 separate observations.
5.2.5  Effect of melatonin on COX enzymes

COX-I protein expression in the right hemisphere was not significantly altered after stroke from baseline. Melatonin administration did not significantly alter the protein expression either (Figure 5.10). In the left hemisphere, the COX-I expressions amongst all the groups were similar.

![Figure 5.10](image)

The COX-II isoform expression in the right hemisphere was significantly altered. The expression was significantly increased after MCAO in comparison to controls. This induction in COX-II expression after MCAO was prevented in the melatonin treatment group, as there were no significant differences between melatonin and controls (Figure 5.11). No differences were seen in the contralateral hemisphere.

The COX activity was significantly altered in the ipsilateral (right) hemisphere. A significant induction of COX activity occurred following
Melatonin inhibited (q = 3.64, P = 0.05) of this MCAO-mediated increased activity.

**Figure 5.11** Effects of melatonin on COX-II expression following stroke. COX-II protein expression in the ischaemic hemispheres was significantly increased after MCAO (grey) from baseline (white). Although melatonin treatment (black) did not significantly alter COX-II expression, it prevented the MCAO-induced significant increase. No differences were seen in the left hemisphere. Each point represents mean ± SEM of at least 6 separate observations. *, P < 0.05 when compared to naïve controls. Each graph is headed by a representative blot showing protein expression in the respective hemisphere.

**Figure 5.12** Effects of melatonin on COX activity following MCAO. Following MCAO (grey), there was a significant increase in COX activity when compared with controls (white). This was normalised with melatonin administration (black). There were no significant changes in the
Chapter 5: Mechanisms of Neuroprotection in vivo

5.3 DISCUSSION

5.3.1 Melatonin and the NOS enzyme system after MCAO

The current study evaluated the effect of melatonin on the NOS enzyme systems, in particular the enzyme activity and protein expression, after MCAO. There was increased production of NO following MCAO in the ipsilateral hemisphere, and this was contributed by the iNOS isoform. Neither eNOS nor nNOS expression were significantly altered at time point of extraction. These results are consistent with published literature about detailing the role of iNOS in stroke (see Section 1.3.2, Iadecola, 1997; Moro et al., 2004). Increases in iNOS mRNA, protein and consequent activity were also seen in other studies after cerebral IR (Grandati et al., 1997; Iadecola et al., 1995). iNOS expression was noted to increase 24-48 hours after induction of MCAO and persist up to 7 days (Figure 5.13, Grandati et al., 1997; Iadecola et al., 2001). The eNOS isoform is induced at the onset of MCAO, but then declines to below normal levels (Figure 5.13, Grandati et al., 1997; Nagafuji et al., 1994; Zhang et al., 1993). This early induction may be responsible for the vasodilatory response to the initial ischaemia (see Section 1.3.2). The time point of tissue sampling in the current study was 60 hours, and thus it is unlikely that significant change in eNOS protein expression would be detected. Similarly, Kader et al. (1993), found nNOS activity to peak at 10 minutes post-ischaemia before returning to baseline (at 60 minutes). The increase in nNOS activity following cerebral IR has been attributed to its neurotoxicity (see Section 1.3.2). Another group of investigators found the nNOS levels elevated only in glial cells in penumbra for at least 3 days (Pei et al., 2004). Since the current study only utilised whole hemisphere homogenates, it may not have been able to detect the persistent present of nNOS. Thus, our current results are in consensus with the literature.
Melatonin treatment led to significant neuroprotection (Chapter 3). Melatonin treatment following MCAO ameliorated the increased nitrite levels by inhibition of iNOS expression and activity. The reduction in nitrite levels with melatonin may be attributed to its antioxidant properties (see Section 1.7.4, Reiter, 2003). It has been well established that melatonin is a potent free radical scavenger and thus should reduce the presence of nitrites. Melatonin has been shown to directly scavenge NO by undergoing nitrosation (Blanchard et al., 2000; Noda et al., 1999; Turjanski et al., 2001). A study monitoring NO levels following MCAO found significant attenuation of the NO increase with melatonin administration, confirming this hypothesis (Pei et al., 2003). Furthermore, the metabolites of melatonin are equally effective free radical scavengers and antioxidants and thus able to protect neurons from IR injury (Duan et al., 2006; Hardeland et al., 2009; Onuki et al., 2005; Rosen et al., 2006). This implies that well after catabolism of the melatonin molecule, the elimination of free radicals such as NO would still be occurring.
Chapter 5: Mechanisms of Neuroprotection in vivo

Therefore, the reduced nitrite levels by melatonin may be partly due to the antioxidant properties of melatonin and its metabolites.

The protective action of melatonin cannot be limited to the scavenging of NO produced by NOS. NO directly inhibits NOS activity in a negative feedback system (Griscavage et al., 1993). If melatonin simply eliminated NO and ROS, this feedback should result in an increase in NOS activity without a rise in nitrite levels. This was not seen. Inhibition of NOS enzyme activity occurred with a decrease in nitrite levels. This suggests that melatonin must be acting on the NOS enzyme directly in conjunction with scavenging NO. This proposed mechanism supported by the decrease in iNOS protein levels after MCAO with melatonin treatment. In a model of global cerebral IR in vivo, melatonin was shown to decrease the up regulated NOS activity (Guerrero et al., 1997). The authors measured nitrite levels as stable products of NO, as well as cyclic guanosine monophosphate (which is produced as a by-product of NOS activity). However, the isoform of NOS inhibited by melatonin was not identified. Similarly, pre-treatment with melatonin led to a lower NO level compared with vehicle after 3 hours of MCAO (Pei et al., 2003b). More recently, a study found inhibition of iNOS expression at 24 hours following pretreatment with melatonin after MCAO (Koh 2008e). Thus, this present study is the first to show that melatonin treatment after MCAO results in a direct inhibition of iNOS protein levels and consequent iNOS activity.

There is widespread consensus that melatonin has the ability to directly inhibit NOS activity. Melatonin was shown to be dose-dependently inhibiting NOS, by interacting with calmodulin in situ and in vitro (Bettahi et al., 1996; Pozo et al., 1997; Pozo et al., 1994). This evidence was supported by in vivo models. Pretreatment with melatonin led to attenuation of increased nNOS immunoreactivity in acute hypoxia and MCAO (Chang et al., 2002; Pei et al., 2004). The nNOS protein expression was shown to be similar to controls. Pei et al. (2004) found that nNOS expression in the penumbral region was upregulated at 72 hours post ischaemia. Since the present study used hemisphere homogenates, the relative contribution of the increased nNOS expression in the penumbra may not be large enough to cause overall changes detected by Western blot at time of sampling. Previous work showed that at the earlier time point of 24 hours post cerebral IR, melatonin treatment decreased overall nNOS levels compared to vehicle control (Koh 2008e). Since melatonin affects calmodulin binding to NOS, it could be hypothesised to decrease constitutive NOS (nNOS and eNOS) activities (Bettahi et al., 1996; Pozo et al., 1997; Pozo et al., 1994). However, Koh (2008e) found induction of eNOS expression compared to vehicle control at
Chapter 5: Mechanisms of Neuroprotection in vivo

24 hours after MCAO when animals were pre-treated with melatonin. This eNOS effect implies a differential effect of melatonin on the constitutive isoforms resulting in induction of the neuroprotective isoform, eNOS and inhibition of neurotoxic isoform, nNOS. The inhibition of total NOS activity seen following melatonin dosing can be mostly accounted for by the inhibition of iNOS and to a lesser extent nNOS isoforms.

The attenuation of the increase in iNOS protein expression by melatonin may occur via a multitude of mechanisms. iNOS is induced by various factors, such as IL-1β and TNFα, in inflammation (see Section 1.3.2). This induction can be inhibited by several cytokines such as TGF-β, glucocorticoids and IL-4 (see Section 1.3.2). Firstly, melatonin directly inhibits infiltration of neutrophils and other inflammatory cells into the region of inflammation (Cuzzocrea et al., 1997; Dugo et al., 2001). The modulation of the entry of the inflammatory cells into the area of inflammation may be through melatonin’s actions on its receptors (see Chapter 4). This leads to decreased exudate formation and lower levels of pro-inflammatory cytokines (as there are fewer inflammatory cells).

Concurrently, melatonin has been shown to act at the transcriptional level on iNOS. Levels of NF-κB are increased in cerebral IR (see Section 1.1.4) and in models of inflammation (for review see Cuzzocrea et al., 2002). NF-κB is a transcription factor necessary for iNOS expression (Kim et al., 1995; Vila-Del Sol et al., 2007). Melatonin by inhibiting NF-κB has been shown to decrease iNOS protein levels in in vitro and in vivo models of inflammation (Alonso et al., 2006; Gilad et al., 1998; Mazzon et al., 2006; Mohan et al., 1995). Furthermore, pro-inflammatory cytokines like TNFα have been down regulated by melatonin in models of inflammation (Gitto et al., 2004; Mazzon et al., 2006). The decrease in TNFα was also associated with inhibition of NF-κB. In addition, signal transduction pathways involved in inflammation were down regulated with melatonin (Mazzon et al., 2006). In a clinical trial of surgical neonates, melatonin decreased TNFα and subsequently lowered nitrite levels, leading to improved survival rates (Gitto et al., 2004). Moreover, the expressions of anti-inflammatory cytokines, such as TGF-β, were up regulated by melatonin, causing inhibition of NOS (Bizzarri et al., 2003; Tsai et al., 2001). Hence, both experimental and clinical data show that iNOS expression is inhibited by melatonin via modulation of cytokines and protein signal transduction pathways.
Chapter 5: Mechanisms of Neuroprotection in vivo

5.3.2 Melatonin and the arginase enzyme system after cerebral IR injury

There were no significant differences in arginase expression with stroke or after melatonin treatment. There was a tendency for arginase activity to be increased with melatonin treatment following MCAO. Previously, our research group found an increase in arginase activity at 7 days post MCAO, but not at 3 days (Rahman, 2006). Similarly, in a global model of hypoxia-ischaemia, arginase activity was only significantly induced from 7 days from time of insult (Clarkson et al., 2004; Sutherland et al., 2005). It is most likely that at the survival time point used in the present study, any increase in arginase activity may have been too early to detect (see Figure 5.13). There may even be an early decline in arginase activity secondary to the induction of NOS enzyme systems following stroke (see Section 1.3.2). Since both enzymes compete for the same substrate, L-arginine, the increased NOS enzyme activity should lead to decreased arginase activity. This effect of competitive inhibition has been shown in other in vivo studies (Buga et al., 1996; Christianson, 2005; Li et al., 2001; Maarsingh et al., 2005).

Given that arginase I is the more dominant isoform in the brain, it is most likely that induction of the arginase I isoform expression occurs after stroke. This induction was confirmed previously by our group (Rahman, 2006). It is possible that melatonin administration post MCAO may have increased arginase I expression if a later (7 days) sampling point had been chosen. The induction of arginase I isoform and subsequent activity with melatonin has been seen with another model of inflammation. In wound healing it was found that melatonin increased the arginase I isoform and activity, leading to improved scarring (Pugazhenthhi et al., 2008). Similarly, in a model of acute renal failure, 5 mg/kg melatonin increased arginase activities and ornithine levels whilst decreasing NO levels (Aydogdu et al., 2006). Furthermore, arginase activity in the liver was found to be higher nocturnally, implying the possible physiological role of melatonin (Soler et al., 1988). These findings suggest that following stroke, melatonin is most likely to stimulate the arginase system, although this was likely to occur at a later time point than used in the study.

As explained in Section 1.3.3, inducers of NOS are either direct or indirect inhibitors of arginase and vice versa. Melatonin as a potent inhibitor of the NOS enzyme system would increase L-arginine substrate availability to arginase. This would lead to increased arginase activity. However, melatonin may also be inducing the arginase I expression, at a transcriptional level. Melatonin has been shown to directly increase TGF-β levels in vitro and in vitro (Bizzarri et al.,
Chapter 5: Mechanisms of Neuroprotection in vivo

2003; Eck et al., 1998; Liu et al., 2001; Molis et al., 1995; Tsai et al., 2001). TGF-β is an inducer of the arginase enzyme system and known to increase expression of arginase I and subsequent arginase activity (Boutard et al., 1995; Witte et al., 2002). Furthermore, melatonin may be able to exert its effects on the NOS enzyme system by modulating arginase activity. Arginase has been shown to decrease NOS activity and protein levels (Chang et al., 1998; Ckless et al., 2007). Over expression of arginase I increases arginase activity, and this manipulation has been shown to decrease TNFα levels along with decreased NF-κB DNA binding and transcriptional activity (Ckless et al., 2007). This suggests that melatonin could exert its anti-inflammatory effects via increasing arginase expression.

Moreover, the downstream effects of stimulated arginase activity could contribute to the neuroprotective effects of melatonin. Increased arginase activity would imply a greater polyamine concentration, which may be beneficial (see Section 1.3.4). For example, the increased polyamine level has also been shown to directly inhibit NOS (Hu et al., 1994). In a model of kainate toxicity, it was found that melatonin provided neuroprotection by altering polyamine response (Lee et al., 2000). Lee et al. (2000) found that melatonin reduced the induction of putrescine levels, which were contributing to neurotoxicity. Melatonin administration did not alter spermine and spermidine levels, both of which may be neuroprotective (Lee et al., 2000). Melatonin may provide neuroprotection via alteration of polyamine levels following stroke.

In the present study there was no significant difference in alteration of arginase expression. It is possible that induction of the arginase enzymes by melatonin would be seen at a later time point following stroke. If so, this would add to the neuroprotective role of the indole in stroke.

5.3.3 Melatonin and COX enzymes after MCAO

The effect of melatonin on the COX enzyme system after cerebral IR was measured. There was a significant increase in COX-II expression in the right hemisphere following stroke. The upregulation of COX-II was mirrored by the significant rise in COX activity in the ischaemic hemisphere. No effect on the COX-I expression was noted with the Western blot analysis. It is likely that sampling the whole hemisphere homogenate was not sensitive enough to detect a change in COX-I expression. Previously, immunohistochemical studies have shown an increased COX-I expression was seen to be in the penumbral region as well as the necrotic core, persisting
for several months post stroke (Schwab et al., 2000). However, the contribution of the COX-I isoform in stroke is controversial (see Section 1.3.7). The absence of the COX-I isoform did not alter the extent of infarct in gene knockout mice (Cheung et al., 2002). Thus, while COX-I upregulation could not be detected by the techniques used in this study, we cannot fully exclude the occurrence of an induction of the enzyme.

The evidence of increased COX-II expression is more consistent (see Section 1.3.7). The COX-II enzyme is responsible for the majority of the increased COX activity that leads to PGE$_2$ production. Even though, PGE$_2$ can be both neuroprotective and neurodegenerative (depending on which EP receptor is activated), increased PGE$_2$ levels have generally led to greater infarcts (see Section 1.3.8). The increased COX-II expression in the study is similar to that found by another group of investigators. It was found that following transient MCAO, COX-II expression peaked at 24 hours, minimal at 48 hours and then increased again at 72 hours (Pei et al., 2004). However, another group of researchers found COX-II mRNA expression peaking between 12-24 hours and then declining at 48 hours to normal levels at 96 hours (Nogawa et al., 1997). Similarly, COX activity was found to be peaking at 24 hours whilst returning to basal levels at 72 hours (Candelario-Jalil et al., 2003). Hence, there is agreement that COX-II expression and COX activity are increased in stroke (for review see Section 1.3.7, Iadecola et al., 2005), and the data obtained at the survival point was able to detect this increase (Figure 5.13).

The present study showed for the first time that melatonin treatment after MCAO significantly decreased COX activity but not expression in MCAO animals. The contribution of COX-I toward neurodegeneration in MCAO is not universally accepted (see Section 1.3.7). The neuroprotective effect of melatonin has been shown to be independent of the presence of the COX-I isoform in MCAO (Zou et al., 2006). The results from the study by Zou et al. (2006) confirms observations from the present study, which showed no effect of COX-I expression with melatonin treatment. Conversely, pre-treatment with melatonin (5 mg/kg) in MCAO animals led to significant inhibition of COX-II expression, in the penumbral region (Pei et al., 2004). In other models of inflammation, melatonin has been shown to decrease COX-II expression and subsequent COX activity as determined by PGE$_2$ levels (Cuzzocrea et al., 1999; Dong et al., 2003). However, in a model of gastric IR, there was a decrease of PGE$_2$ levels with increased NOS activity following IR. Melatonin administration led to reduced tissue damage, whilst restoring PGE$_2$ levels and inhibiting NOS (Cabeza et al., 2003). It may very well be that PGE$_2$ via activation of its various EP receptors has a more dominant protective role in the gastric mucosa. Hence, melatonin via
Chapter 5: Mechanisms of Neuroprotection in vivo

reducing oxidative stress and the NOS enzyme system would have prevented the upregulation of the COX enzymes. It is unclear whether melatonin had directly increased COX activity in this model. The effects of melatonin on the COX enzyme system in the current study are therefore in agreement with the majority of the literature.

Modulation of the COX enzyme system is dependent on inflammatory cytokines. Pro-inflammatory cytokines such as IL-1β and TNFα induce the COX-II expression and COX activity. Conversely, anti-inflammatory cytokines TGF-β and IL-10 inhibit the COX enzyme system (for overview see Appleton et al., 1996). Furthermore, activation of NF-κB leads to upregulation of COX-II expression and subsequent COX activity (Chen et al., 2000; Rivest, 1999; Slogoff et al., 2004). By virtue of being induced by similar factors, the NOS and COX enzyme systems are mutually linked. In addition, NO derived from iNOS in inflammation stimulates COX-II activity via the NF-κB pathway (Landino et al., 1996). As explained in Section 1.7.5, melatonin may be able to inhibit the NOS enzyme system via modulating cytokines and nuclear transcriptional factors. Inhibitors of the NOS enzyme system have been shown to decrease COX-II expression and COX activity (Chen et al., 2000). In particular, in a different in vivo model of inflammation, melatonin prevented the upregulation of iNOS and COX-II expression and activities (Dong et al., 2003). Therefore, it can be concluded that the effect of melatonin on the COX enzyme system after MCAO occurs via modulation of inflammatory cytokines and nuclear transcriptional factors.

However, unlike in the NOS enzyme system, melatonin may not be having further effects downstream on the products of the COX enzyme system. Melatonin was able to inhibit lipopolysaccharide-induced hyperthermia (dependent on the activation of the COX enzyme system). This was attributed to the ability of melatonin to inhibit the COX enzyme activity. When PGE₂ itself was administered, fever in animals was not prevented with the similar dosage of melatonin (Raghavendra et al., 1999). This implies that activation of EP receptors by PGE₂ may not be affected by melatonin. The effect of melatonin may be limited to the modulation of expression of COX isoforms and COX activity.

Therefore, inhibition of COX activity by melatonin may result either through the inhibition of iNOS or NF-κB–mediated induction pathways or through a direct inhibition of the COX enzymes. This ability adds to the neuroprotective mechanism obtained by melatonin when administered after MCAO.
5.4 CONCLUSION

Following MCAO, there was a significant upregulation of iNOS protein expression, subsequently leading to significant increases in iNOS activity and total NOS activity. Cumulative NOS activity, determined by nitrite levels, was also significantly increased following cerebral IR. Melatonin administration led to inhibition of iNOS protein induction. This flow on effect was seen on iNOS activity and total NOS activity. The significant decrease in nitrite levels can thus be attributed to both its NO scavenging and NOS inhibition.

There were no significant changes in arginase isoform expression with MCAO. Melatonin treatment following MCAO was found to produce small but insignificant increases in arginase activity. It is likely that the induction of arginase enzymes at the 60 hour time point is still low as arginase expression has been shown to peak at later survival time point.

Increased COX activity after MCAO corresponded with induction in COX-II expression. Melatonin administration after MCAO resulted in decreased COX activity without significant change in expression.

Melatonin may influence these key enzyme systems via modulation of inflammatory cytokines, nuclear transcriptional factors and protein signalling pathways. Thus, the hormone is able to provide neuroprotection when administered after stroke.
CHAPTER 6

*IN VITRO EFFECTS OF MELATONIN ON L-ARGININE METABOLISM*
6.1 INTRODUCTION

The substrate L-arginine can be metabolized by either arginase, NOS or arginine decarboxylase (see Section 1.3.1 for L-arginine metabolism). NOS and arginase play a major part in inflammation while the role of arginine decarboxylase or agmatinase is not well established. As both NOS and arginase use a common substrate, they are intrinsically linked and activators of one enzyme indirectly inhibit the action of the other in the same cell. This theory has been supported by \textit{in vitro} (Boutard \textit{et al}., 1995; Corraliza \textit{et al}., 1995; Hesse \textit{et al}., 2001; Tenu \textit{et al}., 1999) and \textit{in vivo} (Aydogdu \textit{et al}., 2006; Christianson, 2005; Maarsingh \textit{et al}., 2005) observations.

In Chapter 5, melatonin was shown to produce a significant inhibition of iNOS by melatonin when administered after MCAO in animals. There were no significant differences in the protein expression of arginase isoforms, though there was a tendency in increasing arginase activity. This has been mostly attributed to the time point of collection, given the recent studies by our group show increased arginase activity at 7 days after IR. The evidence for melatonin’s actions on arginase activity is scarce.

6.1.1 Hypothesis and aims

This study hypothesised that melatonin can stimulate arginase enzyme activity. The aim of this study therefore is to confirm the effects of melatonin on L-arginine metabolism in an unstimulated cell line. Human fibrosarcoma fibroblasts (HT-1080) were chosen for this study as these cells have been shown to have high levels of arginase activity (Pohjanpelto \textit{et al}., 1983).

6.1.2 Experimental approach

To investigate this hypothesis, HT-1080 fibroblasts were treated with 1 nM to 1 mM concentrations of melatonin for 72 hours. The effect of melatonin on the cell viabilities were examined using two quantitative methods, MTT and SRB assays (see Section 2.9). The NOS and arginase enzyme systems were then studied in these treated cells (see Section 2.9.7).
Chapter 6: In vitro Effects of melatonin on L-Arginine Metabolism

6.2 RESULTS

6.2.1 Optimization of antibody concentrations for inducible enzymes

Antibodies directed against each isoform of inducible enzyme used in Western blot analysis (see Section 2.8), were applied at the optimized elicited dilutions (1:1000) and the incubation period was maintained for 18 hours at 4°C.

6.2.2 Effects of melatonin on the cell viability

Cell viability in the presence of melatonin was initially assessed by MTT assay. This assay relies on the conversion of MTT to formazan crystals by active mitochondria (Mossman 1983). Melatonin exposure had a significant effect \( F(7, 87) = 19.19, P < 0.001 \) on HT-1080 cell viability assay (Figure 6.1). Incubation with the lower concentrations of melatonin: 10 nM \( (q = 2.67, P < 0.01) \), 1 µM \( (q = 3.56, P < 0.01) \) and 10 µM \( (q = 5.14, P < 0.01) \) increased the formazan product against the vehicle control. At the higher melatonin concentrations (1 mM) cell viability was significantly reduced against vehicle controls \( (q = 5.00, P < 0.01) \). The MTT assay relies on the activity of mitochondria. It is possible that melatonin at physiological concentrations stimulates mitochondrial activity (as discussed in Chapter 7), and this effect of melatonin confounded the cell viability assay results. Thus, another cell viability assay, SRB assay was utilised.

The SRB assay is dependent on the ability of the sulforhodamine B dye to bind to the protein residues of trichloroacetic acid-fixed cells (Skehan et al., 1990). There was a significant \( (H = 20.06, \text{d.f.} = 7, P < 0.001) \) decrease in cell viability with melatonin treatment when measured by SRB assay (Figure 6.2). This effect was significant at 100 nM \( (Q = 25.25, P < 0.05) \) and 1 mM \( (Q = 31.83, P < 0.001) \).
Chapter 6: *In vitro Effects of melatonin on L-Arginine Metabolism*

**Figure 6.1** Effects of melatonin on the cell viability of HT-1080 fibroblasts as determined by the MTT assay. At lower concentrations of melatonin there was an increase in formazan product suggesting stimulation of mitochondrial activity. However at the highest (1 mM) concentration used, there was significant decrease in total mitochondrial activity most likely due to decreased number of viable cells. Each point is the mean ± SEM of at least 11 separate observations. C, control group, * P < 0.05, ** P < 0.01, *** P < 0.001 vs. controls.

Thus, the loss of cell viability seen at concentrations of 1 mM confirmed the oncostatic nature of melatonin. Since cell viability was most significantly different at 1 mM, all the assay results were also compared by standardising to cell viability. However, since there was no difference in overall
results, the assay results were normalised to amount of protein in the sample rather than to cell viability.

6.2.3 Effects of melatonin on NOS

The effects of melatonin on the NOS enzymes in HT-1080 fibroblasts were investigated. This study could not detect the presence of the nNOS isoform by WB. There were no significant differences in the expression of eNOS in HT-1080 cells after being treated with melatonin (Figure 6.3). Similarly, there were no differences in iNOS expression or activity with melatonin treatment (Figure 6.4 and Figure 6.5).

Figure 6.3 Effects of melatonin on the eNOS expression in HT-1080 fibroblasts. There were no significant differences in eNOS expression when compared to controls, C. Each point represents mean ± SEM of 6 separate observations. C, control group.
Chapter 6: *In vitro Effects of melatonin on L-Arginine Metabolism*

**Figure 6.4** Effects of melatonin on the iNOS expression in HT-1080 fibroblasts. iNOS expression in HT-1080 cells were not influenced with melatonin treatment for 72 hours. Each point represents mean ± SEM of 6 separate observations. C, control group.

**Figure 6.5** Effects of melatonin on the iNOS activity in HT-1080 fibroblasts. iNOS activity in HT-1080 cells was not significantly changed with 1 nM to 1 mM melatonin treatment for 72 hours. Each point represents mean ± SEM of 6 separate observations. C, controls.

However, there was a significant [F (7, 40) = 5.58, P < 0.001] effect on total NOS activity after treatment with melatonin (Figure 6.6). Linear regression analysis model showed the increase was concentration-dependent (F = 11.54, P < 0.01, R² = 0.22). When comparing individual concentrations of melatonin, significant differences were noted at 100 nM (q = 3.01, P < 0.05), 1 mM (q = 3.34, P < 0.05), 10 mM (q = 3.31, P < 0.05) and 1 mM (q = 4.91, P < 0.01).
Chapter 6: *In vitro Effects of melatonin on L-Arginine Metabolism*

**Figure 6.6** Effects of melatonin on the total NOS activity in HT-1080 fibroblasts. Melatonin application increased total NOS activity significantly in a concentration-dependent manner. Each point represents mean ± SEM of 6 separate observations. C, control group. * P < 0.05, ** P < 0.01 vs. controls.

Generally, increased nitrite levels are indicative of cumulative NOS activity as nitrites are stable breakdown product of NO. However, when measuring the nitrite levels in HT-1080 fibroblasts culture, there were no detectable changes in the treatment groups compared to baseline (Figure 6.7).

**Figure 6.7** Effects of melatonin on nitrite levels in HT-1080 fibroblasts tissue culture supernatants. There were no significant differences in nitrite levels with increasing melatonin concentrations. Each point represents mean ± SEM of 6 separate observations. C, control group.
6.2.4 Effects of melatonin on the arginase enzyme system

There were no significant effects of melatonin administration on the arginase I expression in HT-1080 cells (Figure 6.8).

Conversely, there was a significant \( F (7, 40) = 2.85, P < 0.05 \) effect exhibited by melatonin on arginase II expression in HT-1080 fibroblasts (Figure 6.9). Arginase II expression was significantly up regulated at 10 nM (\( q = 3.00, P < 0.05 \)), 100 nM (\( q = 3.60, P < 0.01 \)), 1 \( \mu \)M (\( q = 3.16, P < 0.05 \)) and 1 mM (\( q = 3.14, P < 0.05 \)) compared to control. This effect corresponded with significant \( F (7, 40) = 2.71, P < 0.05 \) induction of arginase activity (Figure 6.10). Melatonin treatment resulted in increased arginase activity in HT-1080 fibroblasts, in particular at 100 nM (\( q = 3.24, P < 0.05 \)), 1 \( \mu \)M (\( q = 2.98, P < 0.05 \)) and 1 mM (\( q = 3.90, P < 0.01 \)) concentrations versus control.
Figure 6.9 Effects of increasing melatonin concentrations on arginase II expression in HT-1080 fibroblasts. Arginase II was significantly upregulated from 10 nM to 1 mM melatonin. Each point represents mean ± SEM of 6 separate observations. C, control group. * P < 0.05, ** P < 0.01 vs. controls.

Figure 6.10 Effects of melatonin on arginase activity in HT-1080 fibroblasts. There was a significant induction of arginase activity with increasing melatonin concentrations. Each point represents mean ± SEM of 6 separate observations. C, control group. * P < 0.05, ** P < 0.01 vs. controls.
6.3 DISCUSSION

6.3.1 Effects of melatonin on cell proliferation

The effect of melatonin on cell viability was first assessed using MTT assay. This assay depends on mitochondrial activity and it assumes the agent does not influence mitochondrial activity. In this study, lower concentrations of melatonin led to increased formazan detection whilst at highest concentration there was a significant decrease. It is likely that melatonin at lower concentrations had a stimulatory effect on mitochondria (as discussed in Chapter 7). Other studies using MTT assay for studying the oncostatic effects of melatonin have found the inhibitory effect to be significant at concentrations as low as 1 nM (Cui et al., 2006; Martin-Renedo et al., 2008; Rubio et al., 2007). Another possibility is that melatonin may be stimulating the HT-1080 fibroblasts at lower concentrations. This was seen in another study using fibroblasts isolated from both healthy and systemic sclerosis volunteers (Carossino et al., 1996). To confirm this stimulatory effect, another cell viability assay, SRB was used. In this assay, there was significant inhibition of cell proliferation starting at 100 nM melatonin. Therefore, it is likely that the MTT assay results were more reflective of the mitochondrial activity stimulation by melatonin. Melatonin has been well established to be anti-proliferative in in vitro studies depending on concentration and cell type (Blask et al., 1997; Shellard et al., 1989). These studies have shown that melatonin and its metabolites have an oncostatic property and investigators have suggested its possible therapeutic use in cancer. The in vitro effect of melatonin on fibroblasts shown in the current study is consistent with literature. Carossino et al., (1996) found concentration-dependent inhibition at concentrations higher than 0.4 nM. Similarly, it was found that 1 mM melatonin significantly slowed cell cycle progression in Swiss 3T3 cells compared to untreated cells (Finocchiaro et al., 1998). Melatonin may be able to sustain the anti-proliferative effect on fibroblasts, in part, by inhibition of basic fibroblast growth factor. The induction of the basic fibroblast growth factor gene was inhibited in vitro (Graham et al., 1999). Interestingly, when normal cells are examined in presence of external stress such as oxidative stress or ultraviolet exposures, investigators have found melatonin to be protective (Choi et al., 2011; Izykowska et al., 2009). However, in the present study, using cancerous fibrosarcoma fibroblasts, HT-1080, inhibition of cellular proliferation was seen with melatonin. Therefore, the results from our study are consistent with tissue cultures using malignant cell lines.
Chapter 6: In vitro Effects of melatonin on L-Arginine Metabolism

6.3.2 Effect of melatonin on L-arginine metabolism in vitro

Increasing melatonin concentrations led to induction of total NOS activity without influencing the iNOS activity. There were no changes in expression of isoforms with melatonin. The changes in the nitrite levels of supernatant were not detectable using Griess nitrite assay. It is possible that melatonin, in the absence of oxidative stress, does not affect the expression of NOS isoforms. Previous in situ studies, examining NOS inhibition in brain homogenates, have found that co-incubation with melatonin led to inhibition of constitutive NOS (Pozo et al., 1997; Pozo et al., 1994). In animal studies, with the presence of oxidative stress, melatonin led to inhibition of iNOS expression and activity (see Chapter 5). Thus, it would have been expected to see reduction of NOS isoforms and activity by melatonin. However, no previous study has examined the effects of melatonin on NOS isoforms in unstimulated HT-1080 fibroblasts. The increase in total NOS activity without a change in iNOS activity would most likely indicate stimulation of eNOS activity, as there were no nNOS was detected using protein densitometry. Currently there are no reports of nNOS detection in HT-1080 fibroblasts using similar techniques.

Recently, a growing number of investigators have found a pro-oxidant effect of melatonin at pharmacological concentrations (> 1 µM), in cancer cell lines in vitro (Albertini et al., 2006; Bejarano et al., 2011; Buyukavci et al., 2006; Osseni et al., 2000; Radogna et al., 2009a; Radogna et al., 2009b; Wolftr et al., 2001; Zhang et al., 2011). The cell viability assessed in the current study was also consistent with these observations. This pro-oxidant effect of melatonin at higher concentrations has been attributed to its inhibitory effect on cancer cell lines, via activation of caspases leading to apoptosis. This effect may not lead to oxidative stress, as there was absence of protein carbonylation (Radogna et al., 2009a). At present, there are several mechanisms by which the pro-oxidant effect of melatonin has been shown. Firstly, the ability of melatonin to stimulate complex III in mitochondria was linked to the ability to rapidly induce ROS generation (Zhang et al., 2011). In addition, melatonin was shown to increase arachdonic acid metabolism, leading to lipoxygenase induction and subsequently free radical production (Radogna et al., 2009b). Thus, this would also lead to free radical induced apoptosis. Moreover, since the increase in ROS has been shown to be rapid and transient, it is consistent to find no change in nitrite levels in the supernatant.
Chapter 6: In vitro Effects of melatonin on L-Arginine Metabolism

In addition, it can be deduced that induction of total NOS activity was most likely due to increase eNOS activity. In cerebral IR, as discussed in Chapter 5, induction of eNOS is beneficial. Previously, it was shown that pre-treatment with melatonin after MCAO led to an early induction of eNOS expression (Koh 2008e). Since, the in vitro study findings cannot be directly translated to in vivo conditions, it can be hypothesised that melatonin when given after MCAO, may lead to an early induction of eNOS activity as well. If so, this would be a useful mechanism of neuroprotection after cerebral IR.

Fibroblasts are pivotal in extracellular matrix synthesis. Thus in fibroblasts, L-arginine would be metabolized mainly by the arginase pathway to form proline, one of the resultant amino acids of L-ornithine breakdown, essential for collagen synthesis (Meijer et al., 1990; Pohjanpelto et al., 1983, for review, see Section 1.3.3). The effects of melatonin on L-arginine metabolism should be most dominant on the levels of arginase enzymes. The current study found that high concentrations of melatonin had a significant stimulatory effect on the arginase enzyme system despite decreased melatonin-induced reduction in cell numbers. The increase in arginase activity was mirrored by higher arginase II protein expression.

The results of this in vitro study are unique in that no study had previously examined the effect of melatonin on arginase activity. Melatonin was shown to induce the arginase activity and expression in in vivo models of wound healing (Pugazhenthi et al., 2008) and acute renal failure (Aydogdu et al., 2006) leading to improved outcomes. In contrast, a previous study found that melatonin administration in a model of acute hepatic damage led to decreased arginase activity (Karabay et al., 2005). In this context, arginase activity was used as an indicator of hepatocellular damage rather than an inducible enzyme in the inflammatory cascade. Decreased arginase activity indicated protection of hepatocytes by melatonin rather than a direct inhibitory effect on the enzyme, as per se (Karabay et al., 2005).

NOS and arginase enzymes are intrinsically linked and must therefore be considered together to establish the major pathway of L-arginine metabolism in any disease process. Since NOS and arginase are known to use the same substrate, activators of one enzyme would be indirectly inhibiting the other in the same cell (i.e., may be mutually exclusive). Thus, in the HT-1080 fibroblasts used, the dominance of the arginase enzyme system would implicate minimal NOS enzyme activity. However, when the culture homogenates were incubated with abundant L-arginine in the NOS assay an induction of total NOS activity with melatonin was seen. Melatonin
also increased arginase activity and expression. These results differ from other studies which have found competitive inhibition between arginase and NOS enzyme systems (see Section 5.3.2). Melatonin has been proven to be an inhibitor of iNOS enzyme, via modulation of NF-κB, in addition to scavenging its products (as discussed in Chapter 5). However, as discussed above, melatonin may be stimulating ROS production in unstimulated cancer cell lines at pharmacological concentrations. Thus, this would imply that a reduction of arginase activity should be occurring but the results of the current study reject this hypothesis in unstimulated HT-1080 fibroblasts. Melatonin must be acting on the arginase system in a separate manner to competitive inhibition via the NOS enzyme system. One of these mechanisms may involve TGF β1 as melatonin has been well established to increase TGF β1 levels both in vivo and in vitro (Eck et al., 1998; Liu et al., 2001; Molis et al., 1995). Therefore, it is most likely that melatonin stimulates an increase in arginase protein expression via increasing TGF β1.

Stimulation of arginase system by melatonin could be beneficial to cerebral IR via several mechanisms (see Section 1.3.3 and 1.3.4). However, as stated previously, the in vitro findings of unstimulated HT-1080 fibroblasts cannot be directly translated to in vivo cerebral IR. The findings of this study raise the possibility that melatonin-induced stimulation of arginase system by melatonin after cerebral IR may provide a mechanism of neuroprotection. To examine this hypothesis further, in vitro experiments using the same cell line subjected to oxidative stress, such as oxygen-glucose deprivation, could be carried out.

6.4 CONCLUSION

Melatonin decreased cell viability on unstimulated HT-1080 fibrosarcoma fibroblasts at pharmacological concentrations, possibly via a pro-oxidant effect. To this end, an increase in total NOS activity was seen with high concentrations of melatonin. In addition, there was induction of arginase II expression and activity with melatonin incubation.

Although, the in vitro results cannot be directly translated into findings of animal model of cerebral IR, it does provide a possible hypothesis that melatonin may be neuroprotective with induction of eNOS and arginase enzymes.
CHAPTER 7

EFFECTS OF MELATONIN ON MITOCHONDRIAL FUNCTIONING FOLLOWING STROKE
Chapter 7: Effect of Melatonin on Mitochondrial Functioning

7.1 INTRODUCTION

Mitochondria are central to the development of tissue injury following an IR injury, for example, stroke. A cascade of events occurs after IR resulting in the generation of ROS and mPTP opening, eventually causing cell death by both caspase dependent and independent apoptotic and necrotic pathways (for review see, Section 1.4.3). Thus, in order to elucidate the mechanism of action for neuroprotection by melatonin, it is important to study its effect on mitochondria.

Melatonin has been extensively shown to provide protection against IR injury in a variety of tissues (Okatani et al., 2003; Ozacmak et al., 2005; Petrosillo et al., 2006; Wang et al., 2006), including brain (as discussed in Chapter 3). In addition, melatonin has been previously shown to provide protection against mitochondrial dysfunction from in vivo IR injury in liver (Okatani et al., 2003) and heart (Petrosillo et al., 2006). Several mechanisms by which melatonin offers protection to mitochondria have been noted (for review, see Acuna-Castroviejo et al., 2007; Paradies et al., 2010). Briefly, melatonin and its metabolites are potent free radical scavengers and antioxidants. Melatonin also has been shown to increase protein expression of other antioxidant enzymes (for review of antioxidant properties of melatonin see Section 1.7.4). Finally, melatonin has a direct role in mitochondrial homeostasis (Martin et al., 2000a; Martin et al., 2000b; Martin et al., 2002).

The administration of melatonin has been shown to increase mitochondrial complex I and IV activity and ATP production under basal and oxidative stress conditions (Escames et al., 2003; Martin et al., 2000b; Martin et al., 2002). This melatonin effect on super-complex IV has been suggested to result from increased gene transcription of mitochondrial and nuclear DNA and expression of its enzyme components (Acuna-Castroviejo et al., 2007). Moreover, the melatonin metabolite, AMK, was found to be more effective in increasing complex I and IV activities (Acuna-Castroviejo et al., 2007). Melatonin recently was also shown to stimulate Complex III (Zhang et al., 2011). Melatonin binding sites have been found on mitochondrial membrane preparations, however, the role of receptors on mitochondria have not been fully elucidated (Poon et al., 1992; Yuan et al., 1991). In addition, studies have found that mitochondrial membranes is permeable to melatonin and interacts directly with the mitochondrial membranes, most likely by stabilizing the inner membranes (Costa et al., 1995; Costa et al., 1997; Garcia et al., 1999). This effect may lead to increased ETC function (Acuna-Castroviejo et al., 2007). In addition, studies have shown that melatonin directly interacts with the mPTP, leading to inhibition of the mPTP-dependent cytochrome c release,
Chapter 7: Effect of Melatonin on Mitochondrial Functioning

thereby preventing apoptosis (Andrabi et al., 2004; Jou et al., 2004; Kilic et al., 2004; Wang et al., 2010; Wang et al., 2009). Despite the accumulating data on the effects of melatonin on mitochondria, no study has examined the effects in vivo using a clinically relevant model of stroke.

7.1.1 Hypothesis and aims

It is hypothesised that melatonin administration following cerebral IR injury provides protection of mitochondrial respiratory enzymes leading to attenuation of infarct. To this end, the aim of this study was to investigate the effect of melatonin on mitochondrial integrity and ETC enzyme activity post cerebral IR injury.

7.1.2 Experimental Approach

To investigate this hypothesis, the study used three groups of animals: controls (no interventions), vehicle (MCAO and 5% DMSO in 0.9% saline) and melatonin (MCAO and melatonin dissolved in vehicle). These animals were also used for biochemical analysis (Chapter 5). The animals were sacrificed at 60 hours post onset of ischaemia. Tissue was extracted, homogenised and freeze-thawed to release the mitochondrial components. These homogenates were then used for mitochondrial assays (see Section 2.10).

7.2 RESULTS

7.2.1 Inclusion of animals

As stated in Section 5.2.1, there were 6 animals as controls (which did not undergo MCAO), 6 animals as vehicle treated (MCAO and vehicle) and 7 animals as melatonin treated (MCAO and 5 mg/kg melatonin dissolved in vehicle).
7.2.2 Effects of melatonin on mitochondrial complex I

The mitochondrial complex I activity, measured by rate of rotenone sensitive NADH oxidation, was significantly [F (2, 16) = 4.58, P < 0.05] affected. Post hoc analysis found the there was significant (q = 4.19, P < 0.05) improvement of complex I activity with melatonin treatment compared to vehicle group (Figure 7.1). There were no significant differences observed in the contralateral hemisphere.

![Figure 7.1](image)

**Figure 7.1** Effects of melatonin on complex I activity following MCAO, as measured by rate of rotenone sensitive NADH oxidation. No significant differences occurred in the right hemisphere of vehicle treated animals following MCAO (grey bars) compared with control (white bars). Melatonin administration after MCAO (black bars) significantly restored activity towards normal levels, in the infarcted hemisphere. Each point represents the mean ± SEM of at least 6 separate observations. + P < 0.05 compared to vehicle treatment.

7.2.3 Effects of melatonin on mitochondrial complex II-III

The antimycin A sensitive rate of cytochrome c reduction was used to measure mitochondrial complex II-III activity. Post cerebral reperfusion injury, there was a significant [F (2, 16) = 4.64, P < 0.05] effect on complex II-III activity (Figure 7.2). There was a significant (q = 4.04, P < 0.05) impairment after cerebral IR compared to non-intervention controls. There was a trend (q = 3.31, P = 0.08) to improvement of complex II-III activity with melatonin administration (Figure 7.2). There was no significant difference in the complex II-III activity in melatonin treated animals when compared to controls showing that the IR induced damage to this linked enzyme complex was attenuated with melatonin treatment. No differences were seen in complex II-III activity in the contralateral (left) hemisphere.
Chapter 7: Effect of Melatonin on Mitochondrial Functioning

7.2.4 Effects of melatonin on mitochondrial complex V

A linked enzyme assay based on the oligomycin sensitive rate of NADH oxidation was used to measure mitochondrial complex V (ATP hydrolase) activity. There was a significant \( F(2, 16) = 5.37, P < 0.05 \) effect on complex V activity. There was a trend \( q = 3.534, P = 0.06 \) for reduction in complex V activity in the MCAO group from baseline (control) levels (Figure 7.3). The impairment in complex V activity was significantly \( q = 4.364, P < 0.05 \) normalised with melatonin administration (Figure 7.3). No changes in activity were observed in the contralateral (left) hemisphere.
Chapter 7: Effect of Melatonin on Mitochondrial Functioning

7.2.5 Effects of melatonin on citrate synthase activity

The activity of the mitochondrial enzyme, citrate synthase, was used to gauge mitochondrial membrane integrity. There were no significant differences observed between the treatment groups in the ischaemic (right) and non-ischaemic (left) hemisphere.

Figure 7.3 Effects of melatonin on complex V activity following MCAO, as measured by oligomycin sensitive rate of NADH oxidation. No significant effect on complex V activity occurred in the right (infarcted) hemisphere of vehicle treated animals following MCAO (grey bars) compared with control (white bars). Melatonin treatment after MCAO (black bars) significantly restored activity towards control, in the infarcted hemisphere. Each point represents the mean ± SEM of at least 6 separate observations. +, P < 0.05 compared to vehicle treatment.

Figure 7.4 Effects of melatonin on mitochondrial integrity following MCAO, as marked by citrate synthase activity. No significant effects were noted with vehicle treated animals following MCAO (grey bars) compared neither with control (white bars) nor with melatonin treatment (black bars) in both hemispheres. Each point represents the mean ± SEM of at least 6 separate observations.
Chapter 7: Effect of Melatonin on Mitochondrial Functioning

7.2.6 Effects of melatonin on mitochondrial complexes in comparison with citrate synthase activity

Since the samples used in this study were tissue homogenates, ratios between complexes I, II-III and V against citrate synthase were obtained (Table 7.1) to differentiate actual impairment of ETC activities from a decrease in mitochondrial numbers.

<table>
<thead>
<tr>
<th>Right Hemisphere</th>
<th>Control</th>
<th>Vehicle</th>
<th>Melatonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td>0.195 ± 0.024</td>
<td>0.198 ± 0.042</td>
<td>0.202 ± 0.031</td>
</tr>
<tr>
<td>Complex II-III</td>
<td>0.143 ± 0.026</td>
<td>0.144 ± 0.030</td>
<td>0.133 ± 0.019</td>
</tr>
<tr>
<td>Complex V</td>
<td>0.155 ± 0.017</td>
<td>0.189 ± 0.043</td>
<td>0.158 ± 0.015</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Left Hemisphere</th>
<th>Control</th>
<th>Vehicle</th>
<th>Melatonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td>0.194 ± 0.023</td>
<td>0.185 ± 0.020</td>
<td>0.177 ± 0.021</td>
</tr>
<tr>
<td>Complex II-III</td>
<td>0.155 ± 0.016</td>
<td>0.151 ± 0.029</td>
<td>0.145 ± 0.017</td>
</tr>
<tr>
<td>Complex V</td>
<td>0.171 ± 0.021</td>
<td>0.173 ± 0.015</td>
<td>0.177 ± 0.021</td>
</tr>
</tbody>
</table>

*Table 7.1* The ratios of ETC complexes against citrate synthase. No differences between treatment groups in either hemisphere were seen.

7.2.7 Effects of melatonin on aconitase activity

Aconitase activity was used as a measure of neuronal injury associated with oxidative stress, as aconitase activity is sensitive to superoxide damage. There was a significant \( F(2, 16) = 4.28, \ P < 0.05 \) effect on aconitase activity. There was a significant \( q = 3.46, \ P < 0.05 \) decrease in aconitase activity following IR (right hemisphere) compared to the control animals (Figure 7.5). No significant differences were seen with melatonin administration when compared to vehicles. However, the degree of damage with melatonin treatment after MCAO was no longer significant when compared to the non-ischaemic controls, suggesting protection of the enzyme. There were no significant differences in the contralateral (left) hemisphere.
Chapter 7: Effect of Melatonin on Mitochondrial Functioning

Figure 7.5 Effects of melatonin on tissue injury by oxidative stress following MCAO, as marked by aconitase activity. A significant reduction in activity occurred in the right (infarcted) hemisphere of vehicle treated animals following MCAO (grey bars) compared with control (white bars). Melatonin administration after MCAO (black bars) did not significantly improve the impaired aconitase activity. Each point represents the mean ± SEM of at least 6 separate observations. *, P < 0.05 compared to controls.

7.3 DISCUSSION

7.3.1 Impairment of mitochondrial complexes and enzymes after MCAO

The aim of this study was to elucidate the extent of mitochondrial protection afforded by melatonin following cerebral IR. In this clinically relevant model of stroke, MCAO, melatonin reduced damage to the mitochondrial ETC and to the ROS sensitive aconitase enzymes. We confirmed that IR injury in this model produced a significant impairment in ETC energetics in the ipsilateral hemisphere but not in the contralateral hemisphere. These results are consistent with previous findings in models of cerebral IR (Almeida et al., 1995; Clarkson et al., 2007; Clarkson et al., 2004; Jiang et al., 2010). In the current study, there was no evidence of damage to the mitochondria within the contralateral hemisphere. However, Clarkson et al. (2007) found a delayed impairment of ETC complexes in the non-lesioned hemisphere following cerebral ischaemia. The authors used a model whereby the whole hemisphere is subjected to ischaemia followed by global hypoxia, thereby producing a considerably large infarct, which could account for the trans-hemispheric lesion (Clarkson et al., 2007). Furthermore, Clarkson et al. (2007) studied the mitochondrial changes in discrete regions of the brain rather than in whole hemisphere. This allows for increased sensitivity to changes in the complexes, whereas using the whole hemisphere can effectively dilute small regional pathological changes. Therefore, overall when examining data from only the vehicle MCAO group, the data shown in this study is consistent with previous studies.
The significant decrease in aconitase activity suggested tissue damage caused by an increase in ROS. The aconitase enzyme has been shown to be sensitive to peroxynitrite and superoxide damage through oxidation of its iron-sulphur centres (Bulteau et al., 2003; Clarkson et al., 2004; Gardner et al., 1995). Increased ROS production has been well established following IR injury in a variety of tissues, including brain (for review, see Sims et al., 2002). The subsequent oxidative stress has been shown to target a wide array of sites within the mitochondria including its membranes and enzymes (Johnston et al., 2001; Powell et al., 2003; Vexler et al., 2001). Thus, data from the present study are in concord with the literature.

Increased ROS and oxidative stress has been shown to disrupt the mitochondrial membranes, and initiate Ca\(^{2+}\) influx, leading to depolarization and decreases in ETC complex activity (Lee et al., 1992). This may explain in part, the decrease in complex I activity post MCAO. The increase in ROS may also oxidise pyruvate dehydrogenase, an effect potentiated by increased Ca\(^{2+}\) from disrupted membranes (Bogaert et al., 1994). This may deprive complex I of its substrate leading to a decline in its activity. It is well known that complex I is highly susceptible to oxidative stress, more so than complex II-III (Benzi et al., 1991; Clarkson et al., 2004; Granger et al., 1985; Hillered et al., 1983). Davey et al., (1998) suggested that a decline in complex I activity by 25 % leads to a rapid decrease in oxidative phosphorylation, exacerbating oxidative stress (Davey et al., 1998).

Even though complex II-III is more robust to oxidative stress than complex I, the cytochrome centre is still prone to oxidation by peroxynitrite. In cultured neurons and astrocytes, peroxynitrite directly inhibited complexes II-III and IV (Bolaños et al., 1995). The present study was not able to examine the effects on complex IV. Based on previous observations from other models of cerebral IR, (Almeida et al., 1995; Clarkson et al., 2007; Clarkson et al., 2004), it can be postulated that complex IV activity would have been markedly decreased. This is due to its susceptibility to NO (Brown et al., 2002) and lipid peroxides (Soussi et al., 1990). Lipid peroxidation is one of the consequences of elevated ROS (Braughler et al., 1989; Gutteridge et al., 1990). Similar to the other ETC complexes, complex V is susceptible to oxidative stress, particularly peroxynitrites (Murray et al., 2003). Complex V activity is dependent on the proton gradient created by ETC and maintained by the integrity of the inner mitochondrial membrane (see Section 1.4.1). The decrease in complex V activity may be attributed to upstream ETC disruption and damage to the mitochondrial membrane by ROS. Thus, the ETC complex activities found in this study fit well with the changes expected following IR injury.
7.3.2 Effect of melatonin on mitochondrial ETC complexes following stroke

Melatonin administration at 5 mg/kg i.p. reduced levels of NO produced by inducible NOS following IR (Chapter 5). This reduction in deleterious NO levels is consistent with the loss of significant damage to aconitase enzyme activity suggesting that there was a reduction of oxidative stress with melatonin administration in the MCAO animal. Most of the impaired ETC complexes were significantly restored with melatonin treatment. The results obtained from this study are in concert with other studies examining the effect of melatonin on mitochondria following IR injury in vivo and in situ (Okatani et al., 2003; Petrosillo et al., 2009; Petrosillo et al., 2006; Wakatsuki et al., 2001; Wang et al., 2010; Wang et al., 2009). In a model of foetal rat brain IR injury, melatonin 10 mg/kg i.p. administered to pregnant rats, provided restoration of the respiratory control index and ADP/oxygen ratio (Wakatsuki et al., 2001). Mitochondrial lipid peroxidation occurring post reperfusion, was more pronounced than during ischaemia and was reduced with melatonin administration (Okatani et al., 2003). In addition, a decreased State 3 respiration rate resulting from uncoupling of mitochondria following damage, was restored with 10 mg/kg melatonin treatment (Okatani et al., 2003). Petrossilo et al., (2006) also found similar results when examining State 3 respiration and mitochondrial lipid peroxidation in rat hearts in situ. Moreover, the losses of complex I and complex III activities from IR were prevented with melatonin administration (Petrosillo et al., 2006). Melatonin administration also led to inhibition of mPTP opening after IR in hearts (Petrosillo et al., 2009). The protection afforded to mitochondria by melatonin, subsequently results in lower cytochrome c release and reduction in the apoptotic cascade (Wang et al., 2010; Wang et al., 2009). These findings are in direct agreement with the results obtained from this current study.

There are several probable aspects of protection attained by melatonin on mitochondria. Foremost is its role in reducing the production of ROS post IR. Melatonin is a well known free radical scavenger and potent antioxidant (for review on antioxidant properties of melatonin, see Reiter, 2003; Section 1.7.4). In addition, the metabolites of melatonin are at least as potent in scavenging free radicals, such as peroxynitrite (Hardeland et al., 2009; Onuki et al., 2005; Rosen et al., 2006). Furthermore, melatonin has been shown to increase protein expression of glutathione (Franceschini et al., 1999), an important homeostatic antioxidant in the mitochondria (Phung et al., 1994), and promotes it toward the reduced form (Franceschini et al., 1999; Martin et al., 2000a). The reduced form of glutathione is able to scavenge free radicals, including ROS. Melatonin has been shown to directly increase levels of superoxide dismutase mRNA (Antolin et al., 1996). Superoxide dismutase is a homeostatic enzyme
scavenger of superoxide, one of the predominant forms of ROS in the mitochondria (Mruk et al., 2002). As discussed in Chapters 5, melatonin and its metabolites are potent inhibitors of NOS. More importantly, melatonin has been shown to inhibit mitochondrial NOS and NO induced by LPS in vivo (Escames et al., 2003). The considerable potency of melatonin in inhibiting ROS via a multitude of mechanisms would have contributed largely to the restoration of the mitochondrial ETC complexes.

7.3.3 Effect of melatonin on the preservation of mitochondria following stroke

In the present study, there was a significant decrease in citrate synthase activity with IR injury. Citrate synthase, a mitochondrial matrix enzyme, has been used as a marker of mitochondrial membrane integrity (Clarkson et al., 2007; Clarkson et al., 2004; Sammut et al., 2001). The decrease in citrate synthase activity in whole tissue homogenates has been used to indicate a reduction in viable cells and a consequent drop in intact mitochondria. The ratio of complexes I, II-III and V against citrate synthase showed that the observed impairment of the activity of these complexes was mainly due to the loss of integrity of the mitochondrial membrane or decreased mitochondrial numbers. TTC staining is also dependent on mitochondria functioning (see Section 2.6.2). There was a significant decrease in TTC staining between vehicle and melatonin treated animals (Chapter 3). This further supports the theory that there was either a loss of mitochondrial numbers or decreased mitochondrial functioning. It was not determined whether an actual decrease in mitochondrial numbers had occurred. Ultramicroscopical studies using techniques such as electron microscopy could be employed to know the state of the tissue at time of sampling. Previous studies have employed this technique to examine the integrity of mitochondria and other cell organelles in situ (Garcia et al., 1997; Garcia et al., 1993; Sammut et al., 2001). This technique would be useful to delineate whether melatonin administration after stroke had led to preservation of cellular and ultrastructural morphology so as to correlate this with the functional evidence presented in this study.

Loss in mitochondrial membrane integrity may have resulted from the increased ROS following the IR insult leading to a release of mitochondrial contents into the cytoplasm as well as initiating mPTP opening. Elevated ROS in conjunction with intracellular Ca\textsuperscript{2+} overload would promote mPTP opening and initiate the apoptotic cascade. The duration of mPTP opening is important in determining the mechanism of cell death, with transient opening linked to apoptosis and prolonged opening to necrosis (for review, see Section 1.4.3).
Chapter 7: Effect of Melatonin on Mitochondrial Functioning

Melatonin administration, post IR, may lead to a significant improvement in citrate synthase activity. This could indicate a greater number of viable cells present due to neuroprotection conferred by melatonin. More importantly, the mitochondrial membrane integrity may have been preserved directly by melatonin treatment via several mechanisms. *In vitro* experiments have shown that melatonin is able to inhibit NMDA receptor induced Ca\(^{2+}\) cytosolic influx (Andrabi et al., 2004). Mitochondrial membrane depolarization associated with increased oxidative stress, is known to be one of the factors leading to mPTP opening (for review, see Section 1.4.3). Several *in vitro* studies have shown melatonin administration to decrease ROS induced elevation in intracellular Ca\(^{2+}\) and associated mitochondrial membrane depolarization (Jou et al., 2004; Kilic et al., 2004; Smith et al., 2003). Furthermore, it has been found that melatonin prevents the mPTP dependent cytochrome c release and subsequent caspase 3 activation, and therefore inhibits apoptosis (Jou et al., 2004; Kilic et al., 2004; Park et al., 2002; Wang et al., 2010; Wang et al., 2009). Andrabi and colleagues showed that melatonin directly inhibits the mPTP in a concentration-dependent manner (Andrabi et al., 2004). Furthermore, 6-hydroxymelatonin, a metabolite of melatonin, was shown to be a potent inhibitor of cytochrome c release after oxidative stress (Duan et al., 2006). Taken together, there is evidence that in addition to the ability of melatonin and its metabolites to prevent ROS induced mitochondrial membrane damage, the mitochondrial membrane is stabilised via effects of melatonin and its metabolites on mPTP. Therefore, the results from the current study showing preservation of mitochondrial function with melatonin administration after cerebral IR injury fit in excellently with the current literature on melatonin.

### 7.4 Conclusion

There was a significant decrease in aconitase enzyme activities suggesting the presence of oxidative stress post MCAO. There were also decreases in the ETC complexes measured, which correlated with this evidence of ROS-mediated damage.

Melatonin administration at 5 mg/kg i.p. post MCAO led to the attenuation of oxidative stress. In conjunction with decreased ROS, melatonin via other mechanisms also restored the functioning of ETC complexes.

Thus, the data obtained from this study extend our understanding of the mechanisms of action of melatonin in IR injury. The role of mitochondria is pivotal to IR injury and the multiple mechanisms by which melatonin affords mitochondrial protection, contributes to its overall neuroprotective effect in cerebral IR.
8.1 SUMMARY OF MAJOR FINDINGS

This thesis had several aims based on the main objective of elucidating the mechanisms of neuroprotection attained by melatonin treatment after onset of stroke. To this end, there were several different major findings:

- **To confirm the effectiveness of melatonin when administered after onset of stroke.** Melatonin treatment post MCAO was found to have a significant (70%) reduction in infarct volume. This treatment was not hepatotoxic and did not affect gross organ weights.

- **To investigate the change in distribution of melatonin and its membrane receptors after stroke.** There was differential expression of melatonin and its membrane receptors throughout the brain, with hypothalamus having significant immunoreactivity for all the antibodies examined. The cerebral vasculature was also found to have endogenous melatonin and express melatonin membrane receptors. There was a significant increase in membrane receptors following MCAO. In addition, immune cells within the infarct region were positively labelled with melatonin and MT$_2$ receptor antibodies.

- **To assess the effect of melatonin on major inflammatory enzyme systems, NOS, arginase and COX, when melatonin is administered after the onset of stroke.** Following MCAO, there was a significant (greater than 2 fold) upregulation of the iNOS protein expression, subsequently leading to significant increases in (at least 3 fold) iNOS activity and (approximately 2 fold) total NOS activity. Cumulative NOS activity, determined by nitrite levels, was also significantly increased following cerebral IR. Similarly, COX-II protein expression and activity was increased. Melatonin administration led to inhibition of both iNOS and COX enzyme systems’ induction.

- **To examine the effects of melatonin on L-arginine metabolism in vitro in a high arginase expressing cell line (human fibrosarcoma HT-1080 fibroblasts).** Melatonin decreased cell viability of unstimulated HT-1080 human fibrosarcoma fibroblasts. This effect may have been attributed to its pro-oxidant effects at pharmacological concentrations. There was increased total NOS activity (by approximately 2 fold), without change in iNOS activity or alteration of eNOS and iNOS isoform protein expression. There was a concurrent induction of arginase II expression and arginase activity (by approximately 2 fold) with melatonin treatment.

- **To assess the effects of post-treatment with melatonin on mitochondria after MCAO.** Increased tissue oxidative stress occurred following MCAO, as evidenced by the
inhibition of aconitase activity, and concurrent impairment of ETC complexes. Melatonin treatment following stroke restored these mitochondrial parameters to control levels.

8.2 SIGNIFICANCE OF FINDINGS

Cerebral IR is a complex pathophysiological process (see Section 1.1.4). In the acute neurodegenerative phase, some of the processes include excitotoxicity, peri-infarct depolarisations, inflammation and apoptosis. Several possible mechanisms have been highlighted as contributing factors for a neuroprotective role in cerebral IR (see Section 1.7.6). Firstly, melatonin may lead to vasodilation via stimulation of its membrane receptors (see Section 1.7.3). There is evidence that the BBB itself is better maintained after melatonin administration in cerebral IR, and this is associated with neuroprotection (Chen et al., 2006). Secondly, melatonin is able to counteract the excitotoxicity following immediately after onset of ischaemia. In addition, the antioxidant properties of melatonin and its metabolites would negate any ROS generated from the excitotoxic process (see Section 1.7.4).

One of the core aims of this thesis was to examine the hypothesis that melatonin provides neuroprotection through the inhibition of pro-inflammatory processes leading to apoptosis. Previous studies had demonstrated some of the anti-inflammatory properties of melatonin and its metabolites (see Section 1.7.5; Hardeland et al., 2009). The present study contributed to the literature in showing that these effects are maintained even when melatonin is administered an hour after onset of cerebral IR. The presence of melatonin and its receptors in inflammatory cells as well as blood vessels suggest that melatonin may serve to attenuate the extravasation process resulting following stroke injury. The increased number of receptor positive cells after MCAO, may also have a physiological role, though at present it is highly speculative whether this contributes significantly to neuroprotection afforded by melatonin. The anti-inflammatory effects seen in the present study mainly consisted of inhibition of key enzymes, NOS and COX, following cerebral IR. Based on other studies it is most likely that melatonin acts on the transcription factor NF-κB, thereby reducing the inflammatory response (Alonso et al., 2006; Mazzo et al., 2006). Interestingly, when examining effects of melatonin on a cancer cell line, HT-1080, without oxidative stress, pharmacological concentrations of melatonin led to a pro-oxidant effect leading to apoptosis. There was increased total NOS activity without significant change in iNOS activity or expression of eNOS or iNOS isoforms (there was no nNOS detected). This proved conceptually that eNOS stimulation was possible with melatonin. If
eNOS activity induction does occur early in cerebral IR, it can be regarded as beneficial by increasing cerebral blood flow (see Section 1.3.2). This was confirmed when animals were pre-treated with melatonin in a permanent MCAO model, leading to significant increase in eNOS expression at 24 hours (Koh, 2008e). The in vitro study also found increased arginase activity. It is likely that the effects of melatonin on L-arginine metabolism may involve pathways other than just NF-κB. Supportive evidence from the literature shows that TGF-β may be one of them (Eck et al., 1998; Liu et al., 2001; Molis et al., 1995). Stimulation of arginase system by melatonin would be beneficial in cerebral IR (see Section 1.3.3). Thus, in addition to inhibition of pro-inflammatory enzymes, melatonin treatment could selectively induce eNOS isoform and arginase enzymes.

Mitochondria are pivotal in the apoptosis which occurs concurrently with inflammation, thus contributing to the neurodegeneration (see Section 1.4.3). The impaired ETC complexes were protected with melatonin treatment after cerebral IR in the present study. This observation may be explained by three possibilities: the antioxidant properties of melatonin and its metabolites prevented ROS induced damage on the ETC complexes; there was preservation of mitochondrial numbers thus potentially confounding the results; and there was a direct stimulatory effect of melatonin on the ETC complexes. The favourable effects on ETC complexes were supported by TTC staining when investigating neuroprotection by melatonin. TTC depends on the presence of intact mitochondrial activity and melatonin treatment was shown to significantly preserve the mitochondria compared to vehicle dosing. In addition, the in vitro study examining cell viability used the MTT assay, which measures the formation of formazan by intact mitochondria. Use of the MTT assay indicated that low concentrations of melatonin may have a stimulatory effect on cellular energetics in the absence of ROS, which contrasted with the decrease in cell viability reported at the same concentrations by the SRB assay. Thus, melatonin has an effect on mitochondria even in the absence of oxidative stress, and this observation has been confirmed by other investigators. Although, the apoptotic pathway was not further investigated in the current study, it is likely that with the preservation of ETC complexes, mitochondrial induced apoptosis was inhibited. A study examining IR in vivo has shown that melatonin treatment prevented the release of apoptotic factors, such as cytochrome c, from mitochondria (Wang et al., 2010). Thus, interaction of melatonin with mitochondria adds another facet to the neuroprotective mechanism.

In conclusion, the present study has provided evidence of the neuroprotective effects of melatonin when administered after onset of cerebral IR. The study presented novel evidence of anti-inflammatory actions by inhibiting the deleterious effects of the NOS and COX enzymes.
Melatonin was also seen to attenuate the loss of mitochondrial enzyme activities suggesting that it can protect against the ischaemic injury in acute stroke. This protection was consistent with the pronounced reduction in cerebral infarct size in the right hemisphere of melatonin treated rats. These properties may also be useful in other pathologies where neuro-inflammation or even inflammation is an underlying feature.

8.3 FUTURE RESEARCH

This thesis shows the diverse mechanisms of action involved in the neuroprotective effects of melatonin within the context of delayed neurodegeneration occurring after cerebral IR injury. However, it does not show the complete mechanism of neuroprotection attained with melatonin administration after cerebral IR. Based on the results of the current study, there remains a set of challenges to be addressed for the future. Future studies that relate to the current aims of this thesis should include:

- Providing a quantitative IHC/confocal analysis with improved power to examine distribution of endogenous melatonin and its receptors after MCAO in the regions highlighted by our study. This study should also employ cellular markers to differentiate the subsets of neurons, neuroglia and inflammatory cells. Study sampling should include two different time points: mid-dark for examining melatonin distribution and mid-light for the MT$_1$ receptor.

- Examination of the effect of melatonin on metabolites of COX and its receptors, such as EP receptors for PGE$_2$.

- Examination of the effect of melatonin on downstream effects of arginase enzymes, especially polyamine levels after MCAO.

- A mitochondrial focused study on the effects of melatonin after MCAO. Utilisation of microdissection to isolate the ischaemic and penumbral regions so as reduce the volume of tissue to the region of interest. Markers of oxidative stress, such as superoxide dismutase, and downstream effects of mitochondrial driven apoptosis, (caspases-3) should also be included.

In addition, several other studies can be carried out to attain a greater understanding of the mechanistic role of melatonin and its clinical suitability:

- Profiling melatonin levels against infarct size using a longitudinal time course with a modified extended dosing regime in animals after MCAO.
• Use of higher melatonin doses (50 mg/kg i.p.), shown to confer a greater degree of neuroprotection in MCAO models (O’Collins et al., 2010). This melatonin dose may produce a more pronounced change in markers of neuroprotection. The animals used in this MCAO model should also include animal models representing: age, hypertension, hyperglycaemia, and dyslipidaemia, as this will be more reflective of stroke patients (as opposed young healthy male rats). In addition, the model should include both genders and validate the effects across females as well as males.

• Combination of melatonin within current therapeutic regimes for acute stroke, involving thrombolysis. This will be particularly important as current thrombolytic formulations such as alteplase are commonly stabilised in an L-arginine solution.

• Administration of selective melatonin receptor agonists and antagonists to animals after MCAO to examine the involvement of the individual receptor subtypes on infarct volume and to determine the putative mechanisms of action on the inflammatory cascade.

• Examination of the effect of melatonin on pro- and anti-inflammatory mediators, together with levels of enzymes pivotal in inflammation after MCAO.

• Mechanistic investigation of the expression, activity and effect of the arginine decarboxylase enzyme system after MCAO and possible effects of melatonin on its modulation.

8.4 CLINICAL SIGNIFICANCE

Acute stroke therapy is presently limited to thrombolytic principles. It is estimated that only 3% of patients presenting with ischaemic stroke get thrombolysed in New Zealand (Stroke Foundation of NZ Inc., 2011). There is consequently a high degree of morbidity associated with stroke. Thus, there is an urgent need to find agents that can improve outcomes, but are safe and can be given to most of the patients. Hundreds of agents have been tried clinically without avail. Despite strict adherence to STAIR guidelines, the recent failure of NXY-059, a free radical scavenger extensively trialled in pre-clinical studies, to meet Phase III criteria, has demonstrated the difficulty of this task (Green, 2008). One of the possible reasons for failure to show efficacy in agents could be a lack of replication of the clinical setting in vivo. Ischaemic stroke is a heterogeneous pathology and use of homogenous stroke models may not be a sufficient tool to examine clinical efficacy. Then, there is a question of accurate reporting of data. Seldom have studies accurately reported the number and reasons of excluded animals.
This is now recognised as a major concern by the recent STAIR committee meeting (Fisher et al., 2009). In addition, there should be a panel of experts from both academia and the pharmaceutical industry to objectively examine the raw data from animal studies and then plan and implement a clinical trial protocol. This however is unlikely to be achieved due to the financial interests of pharmaceutical industry.

The major benefit of studying melatonin is that its use is widespread in sleeping and circadian rhythm disorders. The agent has minimal toxic effects and lethal dose is significantly higher than used for rhythm disorders. The multiple mechanisms of action by melatonin in providing neuroprotection make it valuable for use in stroke. Recent meta-analysis of published studies has shown that the first dose of melatonin must be administered within 2 hours of stroke in order to be effective (O'Collins et al., 2010). This therapeutic time window significantly limits its potential use by clinicians in acute stroke therapy. Nonetheless, due to its non toxic effects and relatively large therapeutic index, it can be self-administered by patients prophylactically. Alternatively, it can be administered by paramedics on contact with the patient, just as aspirin is currently used for myocardial infarction, provided that no significant interactions are demonstrated with melatonin. Thus, the multi-faceted actions of melatonin post cerebral IR with minimal toxicity make it a viable candidate for further trials examining a multi-drug therapy for acute stroke treatment.
CHAPTER 9

REFERENCES


Chapter 9: References


Chapter 9: References


Chapter 9: References


CIANI, E., GRONENG, L., VOLTATTORNI, M., ROLSETH, V., CONTESTABILE, A. & PAULSEN, R.E. (1996). Inhibition of free radical production or free radical scavenging protects from the
excitotoxic cell death mediated by glutamate in cultures of cerebellar granule neurons. Brain Research, 728, 1-6.


Chapter 9: References


Chapter 9: References


DRAZEN, D.L. & NELSON, R.J. (2001). Melatonin receptor subtype MT2 (Mel 1b) and not mt1 (Mel 1a) is associated with melatonin-induced enhancement of cell-mediated and humoral immunity. Neuroendocrinology, 74, 178-84.


Chapter 9: References


Chapter 9: References


which recirculation can be introduced in the ischemia area. *Japan Journal of Stroke*, 8, 1-8.


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Chapter 9: References


motoneurons at low and high risk of degeneration in amyotrophic lateral sclerosis. *Neuroscience, 143*, 95-104.


formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), in macrophages. *Journal of Neuroimmunology, 165*, 139-49.


Chapter 9: References


Chapter 9: References


RAHMAN, R. M. Delayed neuronal damage in stroke, University of Otago, Dunedin, 2006.


RAPALINO, O., LAZAROV-SPIEGLER, O., AGRANOLOV, E., VELAN, G.J., YOLES, E., FRAIDAKIS, M., SOLOMON, A., GEPSTEIN, R., KATZ, A., BELKIN, M., HADANI, M. & SCHWARTZ, M.


Chapter 9: References


Chapter 9: References


Chapter 9: References


Chapter 9: References


cytokines in mice infected with the Venezuelan equine encephalomyelitis virus. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 96, 348-51.


References


Chapter 9: References


CHAPTER 10

PUBLICATIONS
10.1 RESEARCH ARTICLES


- Sutherland BA, Rahman RMA, Clarkson AN, Shaw OM, **Nair SM** and Appleton I. 2009. Cerebral heme oxygenase 1 and 2 spatial distribution is modulated following injury from hypoxia-ischemia and middle cerebral artery occlusion in rats. *Neuroscience Research*, 65, 326-334.


10.2 REVIEW ARTICLES


10.3 REFEREED CONFERENCE PROCEEDINGS

- **Nair SM**, Rahman RMA, Clarkson AN, Tramaoundamas AV, Sammut IA, Appleton I. (2006). The protective effects of melatonin on mitochondria function in a stroke-
Chapter 10: Publications


APPENDICES
# A.1 LIST OF EQUIPMENT

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<th>Material</th>
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A.2 IMMUNOHISTOCHEMISTRY ANALYSIS – VALIDATION OF REGIONS

The sections from each animal were matched to the most relevant plate from a neuroanatomy atlas (Paxinos et al., 1997). Once the sections from individual animals were matched to the plates from the atlas, the regions were identified (Figure A.1 – A.12).
Figure A.1 An illustration of identifying the olfactory region after matching the section to the relevant neuroanatomy atlas plate. The neuroanatomy schematic diagram is reproduced with permission from Paxinos et al., (1997). Scale bar represents 50 µm.
Figure A.2 An illustration of identifying the cerebral cortical region after matching the section to the relevant neuroanatomy atlas plate. The neuroanatomy schematic diagram is reproduced with permission from Paxinos et al., (1997). Scale bar represents 50 μm.
Figure A.3 An illustration of identifying the basal ganglia region after matching the section to the relevant neuroanatomy atlas plate. The neuroanatomy schematic diagram is reproduced with permission from Paxinos et al., (1997). Scale bar represents 50 μm.
**Figure A.4** An illustration of identifying the septal and forebrain region after matching the section to the relevant neuroanatomy atlas plate. The neuroanatomy schematic diagram is reproduced with permission from Paxinos et al., (1997). Scale bar represents 50 µm.
Figure A.5 An illustration of identifying the hippocampal region after matching the section to the relevant neuroanatomy atlas plate. Arrows indicate positively labelled cells within the hippocampus. The neuroanatomy schematic diagram is reproduced with permission from Paxinos et al., (1997). Scale bar represents 50 µm.
Figure A.6 An illustration of identifying the epithalamic region after matching the section to the relevant neuroanatomy atlas plate. The neuroanatomy schematic diagram is reproduced with permission from Paxinos et al., (1997). Scale bar represents 50 µm.
Figure A.7 An illustration of identifying the thalamic region after matching the section to the relevant neuroanatomy atlas plate. The neuroanatomy schematic diagram is reproduced with permission from Paxinos et al., (1997). Scale bar represents 50 µm.
Figure A.8 An illustration of identifying the subthalamic region after matching the section to the relevant neuroanatomy atlas plate. The neuroanatomy schematic diagram is reproduced with permission from Paxinos et al., (1997). Scale bar represents 50 µm.
Figure A.9 An illustration of identifying the hypothalamic region after matching the section to the relevant neuroanatomy atlas plate. The neuroanatomy schematic diagram is reproduced with permission from Paxinos et al., (1997). Scale bar represents 50 µm.
Figure A.10 An illustration of identifying the amygdala region after matching the section to the relevant neuroanatomy atlas plate. Arrow in the photomicrograph indicates the positively labelled cells, while the asterisk indicates a blood vessel. The neuroanatomy schematic diagram is reproduced with permission from Paxinos et al., (1997). Scale bar represents 50 µm.
Figure A.11 An illustration of identifying the midbrain and pons region after matching the section to the relevant neuroanatomy atlas plate. Arrow in the photomicrograph indicates the positively labelled multipolar neuronal cell, while the asterisk indicates a positively labelled smaller cell. The neuroanatomy schematic diagram is reproduced with permission from Paxinos et al., (1997). Scale bar represents 50 µm.
Figure A.12 An illustration of identifying the medulla region after matching the section to the relevant neuroanatomy atlas plate. Arrows indicate the positively labelled multipolar neuronal cells. The neuroanatomy schematic diagram is reproduced with permission from Paxinos et al., (1997). Scale bar represents 50 µm.