THE ROLE OF THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS IN THE CARDIOVASCULAR RESPONSES TO ELEVATIONS IN BODY TEMPERATURE.

A thesis submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy.

Joo Lee Cham
B.Sc. (Hons)

School of Medical Sciences
RMIT University
Australia

October 2007
DECLARATION

To the best of my knowledge, this thesis does not contain material that has been accepted for the award of any other degree or diploma in any university. I certify that the work is that of the author alone and does not contain any material which has been published or written by another person, except where due reference is made in the text of the thesis. The content of this thesis is the result of work that has been carried out since the official commencement date of the approved research program and any editorial work, paid or unpaid, carried out by a third party is acknowledged.

............................................................
Signature

JOO LEE CHAM

............................................................
Name

17th October 2007

............................................................
Date
I Dedicate this Thesis to My Family and Husband

For Believing in Me
ACKNOWLEDGEMENTS

To my supervisor, Associate Professor Emilio Badoer, I express gratitude for his patience, guidance and support all throughout the course of my PhD. Emilio gave his time generously and is always there for me both as a supervisor and also as a friend. I am also sincerely grateful to Emilio for his dedication and commitment that he showed in nurturing and guiding my passion for research.

To my second supervisor, Dr Neil Owens, I would like to express thanks for all his assistance, patience and support during the earlier years of my candidature. I am also grateful to Neil for his friendship, advice and moral support throughout these long years. Neil assisted in the technical production of some of the figures presented in this thesis, and was essential to this project in many ways IT. His calculated persistence gave me the strength to continue after his departure from the group.

I would like to acknowledge the opportunity given to me by the folk in the Anatomy Department who allowed me to teach during the latter stages of my PhD and who were most considerate of the many hats that I wore during my innings at RMIT. Their tolerance allowed me to work simultaneously as lecturer, demonstrator and student to complete my PhD. In particular I would like to thank Dr Rudi Klein and Dr Richard Guy for providing me with the opportunity to develop my communication skills by giving me the opportunity to lecture and demonstrate. To Gayle Johnstone and Romy Valdes, many thanks for their patience and assistance for making it all possible.
To Susan Mooney, I sincerely thank for all her help for all her support and help throughout my PhD. Working as a casual staff at the RMIT Animal Facility, I have learnt other things that are beyond that of the scope of my PhD and for that I am grateful.

To Chi Wai Ng, Aristotle Kantzides and Ms Hala Raghib: for their encouragement and support. Their presence through the early years of my candidature has made it an enjoyable and memorable experience for me.

I would also like to thank all of our group members: Dr Martin Stebbing, Dr Feng Chen, Dr Tien Huynh, Nilanka, Melissa, Jennifer, Louie and Melvin. To them, I extend a big thanks for their support and for making me feel like I have a family right here at university.

To my family, I express my deepest appreciation. To my dearest Dad and Mum, I thank for unconditional love, support, blessings and encouragement. To my wonderful and understanding husband, thank you for the years of immense support, love and kindness. To Genie, Bubble and Teddy, I thank them for keeping me company. To my best friends Roy, Shirley, Christabel and Jane, thank you for believing in me.

Acknowledging the various contributions of those that have made this work possible is the final task on my to-do list and signals a long-awaited event in my life! After many long nights this moment of sheer relief and happiness has dawned. Yet, sadness confounds this occasion of bliss, as I part from the work that has feverishly preoccupied the past few years and close the pages of another chapter in my life.
PUBLICATIONS

Publications arising from this thesis:


COMMUNICATIONS

Communications to scientific meetings during candidature:


Cham, JL and Badoer E (2007). Activation of RVLM-projecting neurons following heat exposure. IBRO World Congress of Neuroscience (Melbourne) (POS-FRI-177).
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Title</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declaration</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Publications</td>
<td>vi</td>
</tr>
<tr>
<td>Communications</td>
<td>vii</td>
</tr>
<tr>
<td>Table of contents</td>
<td>viii</td>
</tr>
<tr>
<td>List of tables and figures</td>
<td>xvi</td>
</tr>
<tr>
<td>Abbreviations and acronyms</td>
<td>xx</td>
</tr>
<tr>
<td>Summary</td>
<td>2</td>
</tr>
</tbody>
</table>

CHAPTER 1

Introduction

1.0  INTRODUCTION                                    7

1.1  THE PARAVENTRICULAR NUCLEUS (PVN)              9

1.1.1  Anatomical location of the PVN

1.1.2  Structural organization of the PVN

1.1.2.1  Magnocellular PVN neurons

1.1.2.2  Parvocellular PVN neurons

1.1.3  Neuronal connections of the PVN

1.1.3.1  Afferent projections

1.1.3.2  Efferent projections

1.1.3.3  PVN connections to the IML

1.1.3.4  PVN connections to the RVLM

1.1.4  Role of the PVN in integration of cardiovascular reflexes and sympathetic outflow.

1.2  TEMPERATURE REGULATION AND THE PVN              27
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 MICROINJECTIONS OF RETROGRADELY TRANSPORTED TRACER INTO THE RVLM</td>
<td>58</td>
</tr>
<tr>
<td>2.6 POST-OPERATIVE CARE</td>
<td>62</td>
</tr>
<tr>
<td>2.7 EXPERIMENTAL DAY</td>
<td>62</td>
</tr>
<tr>
<td>2.8 PERFUSION</td>
<td>63</td>
</tr>
<tr>
<td>2.8.1 Fixing the rat brain</td>
<td>63</td>
</tr>
<tr>
<td>2.8.2 Removing the rat brain and spinal cord</td>
<td>65</td>
</tr>
<tr>
<td>2.9 SECTIONING OF THE RAT BRAIN AND SPINAL CORD</td>
<td>66</td>
</tr>
<tr>
<td>2.10 STAINING PROTOCOLS</td>
<td>66</td>
</tr>
<tr>
<td>2.10.1 Immunohistochemical detection of Fos</td>
<td>67</td>
</tr>
<tr>
<td>2.10.2 Nicotine Adenine Dinucleotide Phosphate-diaphorase (NADPH-d)</td>
<td>68</td>
</tr>
<tr>
<td>2.11 ANALYSIS</td>
<td>68</td>
</tr>
<tr>
<td>2.11.1 Histological analysis</td>
<td>68</td>
</tr>
<tr>
<td>2.11.2 Statistical analysis</td>
<td>70</td>
</tr>
<tr>
<td>2.12 MAPPING</td>
<td>71</td>
</tr>
<tr>
<td>2.13 PHOTOMICROSCOPY</td>
<td>71</td>
</tr>
<tr>
<td>2.14 BLOOD FLOW EXPERIMENTS</td>
<td>72</td>
</tr>
<tr>
<td>2.14.1 Surgical preparation of animals used in blood flow experiments</td>
<td>72</td>
</tr>
<tr>
<td>2.14.2 Microinjection into the hypothalamic PVN</td>
<td>73</td>
</tr>
<tr>
<td>2.14.3 Renal blood flow measurement</td>
<td>73</td>
</tr>
<tr>
<td>2.14.4 Experimental protocol</td>
<td>76</td>
</tr>
<tr>
<td>2.14.5 Brain histology</td>
<td>76</td>
</tr>
<tr>
<td>2.14.6 Statistical analysis</td>
<td>79</td>
</tr>
</tbody>
</table>
CHAPTER 3
Activation of spinally-projecting and nitrergic neurons in the PVN following heat exposure.

3.0 INTRODUCTION

3.1 EXPERIMENTAL PROCEDURES
3.1.1 Animals and housing
3.1.2 Microinjections of retrogradely transported tracer into IML
3.1.3 Experimental day
3.1.4 Detection of Fos by immunohistochemistry
3.1.5 Nicotine Adenine Dinucleotide-diaphorase (NADPH-d) staining
3.1.6 Analysis
   3.1.6.1 Rats with intraspinal – injections
   3.1.6.2 Rats without intraspinal – injections
3.1.7 Statistical analysis
3.1.8 Mapping
3.1.9 Photomicroscopy

3.2 RESULTS
3.2.1 Effect of heating on Fos expression in the PVN
3.2.2 Distribution of spinally-projecting neurons in the PVN
3.2.3 Distribution of spinally-projecting neurons in the PVN containing Fos
3.2.4 Distribution of neurons that contained NADPH-d
3.2.5 Distribution of spinally-projecting neurons containing NADPH-d
3.2.6 Distribution of neurons containing NADPH-d and Fos
3.2.7 Distribution of triple-labeled neurons
3.2.8 Behavioural responses to heat exposure

3.3 DISCUSSION

3.4 PERSPECTIVE
**CHAPTER 4**

Exposure to a hot environment can activate spinally-projecting neurons and nitricergic neurons in the brainstem.

| 4.0 | INTRODUCTION | 113 |
| 4.1 | EXPERIMENTAL PROCEDURES | 116 |
| 4.1.1 | Animals and housing | 116 |
| 4.1.2 | Microinjections of retrogradely transported tracer into IML | 116 |
| 4.1.3 | Experimental day | 117 |
| 4.1.4 | Detection of Fos by immunohistochemistry | 117 |
| 4.1.5 | Nicotine Adenine Dinucleotide-diaphorase (NADPH-d) staining | 118 |
| 4.1.6 | Analysis | 118 |
| 4.1.7 | Statistical analysis | 119 |
| 4.1.8 | Mapping | 119 |
| 4.1.9 | Photomicroscopy | 120 |

| 4.2 | RESULTS | 121 |
| 4.2.1 | Midline brainstem | 121 |
| 4.2.1.1 | Distribution of Fos positive neurons | 121 |
| 4.2.1.2 | Distribution of NADPH-d positive neurons | 121 |
| 4.2.1.3 | Distribution of neurons containing NADPH-d and Fos | 122 |
| 4.2.1.4 | Distribution of spinally-projecting neurons | 122 |
| 4.2.1.5 | Distribution of spinally-projecting neurons that also contained Fos | 123 |
| 4.2.1.6 | Distribution of spinally-projecting neurons that also contained Fos and NADPH-d | 123 |

| 4.2.2 | Ventromedial brainstem | 123 |
| 4.2.2.1 | Effect of heating on Fos expression | 123 |
| 4.2.2.2 | Distribution of NADPH-d positive neurons | 124 |
4.2.2.3 Distribution of neurons containing NADPH-d and Fos 124
4.2.2.4 Distribution of spinally-projecting neurons 125
4.2.2.5 Distribution of spinally-projecting neurons that also contained Fos 125
4.2.2.6 Distribution of spinally-projecting neurons that also contained Fos and NADPH-d 125

4.2.3 Ventrolateral brainstem 126
4.2.3.1 Distribution of Fos positive neurons after heat exposure 126
4.2.3.2 Distribution of NADPH-d positive neurons 126
4.2.3.3 Distribution of neurons containing NADPH-d and Fos 127
4.2.3.4 Distribution of spinally-projecting neurons 127
4.2.3.5 Distribution of spinally-projecting neurons that also contained Fos 128
4.2.3.6 Distribution of spinally-projecting neurons that also contained Fos and NADPH-d 128

4.3 DISCUSSION 135
4.4 CONCLUSIONS 140

CHAPTER 5

Exposure to a hot environment can activate RVLM-projecting neurons in the hypothalamic PVN in conscious rats.

5.0 INTRODUCTION 145

5.1 EXPERIMENTAL PROCEDURES 149
5.1.1 Animals and housing 149
5.1.2 Microinjections of retrogradely transported tracer into RVLM 149
5.1.3 Experimental day 150
5.1.4 Detection of Fos by immunohistochemistry
5.1.5 Nicotine Adenine Dinucleotide-diaphorase (NADPH-d) staining
5.1.6 Analysis
5.1.7 Statistical analysis
5.1.8 Mapping
5.1.9 Photomicroscopy

5.2 RESULTS
5.2.1 Effect of heating on Fos expression in the PVN
5.2.2 Distribution of RVLM-projecting neurons in the PVN
5.2.3 Distribution of RVLM-projecting neurons that contained Fos
5.2.4 Distribution of neurons that contained NADPH-d
5.2.5 Distribution of neurons containing NADPH-d and Fos
5.2.6 Distribution of RVLM-projecting neurons containing NADPH-d
5.2.7 Distribution of RVLM-projecting neurons that contained Fos and NADPH-d

5.3 DISCUSSION

5.4 CONCLUSIONS

CHAPTER 6

The hypothalamic paraventricular nucleus is critical for renal vasoconstriction elicited by elevations in body temperature.

6.0 INTRODUCTION

6.1 EXPERIMENTAL PROCEDURES
6.1.1 Animals and housing
6.1.2 Surgical preparations
6.1.3 Microinjection into the hypothalamic PVN
6.1.4 Renal blood flow measurement
### CHAPTER 7

**General discussion**

General discussion 196

### APPENDIX

**Appendix 1 - Construction of water-circulating jacket** 209
**Appendix 2 - Phosphate Buffer (PB)** 211
**Appendix 3 - Phosphate buffer saline (PBS)** 211
**Appendix 4 - Tris buffer** 211
**Appendix 5 - Paraformaldehyde** 211
**Appendix 6 - 3,3’-diaminobenzidine hydrochloride** 212
**Appendix 7 – Preparation of subbed slides** 212

### BIBLIOGRAPHY

Bibliography 214
# LIST OF TABLES AND FIGURES

## Chapter 1

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1: Photomicrograph of a coronal section through the rat hypothalamus showing the paraventricular nucleus (PVN)</td>
<td>10</td>
</tr>
<tr>
<td>1.2: Diagrammatic illustration of major sub-divisions of the PVN</td>
<td>12</td>
</tr>
<tr>
<td>1.3: Main afferent projections to the PVN</td>
<td>16</td>
</tr>
<tr>
<td>1.4: Main efferent projections from the PVN</td>
<td>19</td>
</tr>
</tbody>
</table>

## Chapter 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1: Photo of a typical blood vessel cannula</td>
<td>53</td>
</tr>
<tr>
<td>2.2: Photo of rat positioned on a Stoelting stereotactic frame</td>
<td>60</td>
</tr>
<tr>
<td>2.3: Mean arterial pressure (MAP) trace following injection of L-glutamate into the RVLM</td>
<td>61</td>
</tr>
<tr>
<td>2.4: Photo of the temperature chamber</td>
<td>64</td>
</tr>
<tr>
<td>2.5: Diagram of a renal flow probe positioned around the renal artery</td>
<td>75</td>
</tr>
<tr>
<td>2.6: Photomicrograph of coronal section through the hypothalamus showing the injection site in the PVN</td>
<td>78</td>
</tr>
</tbody>
</table>

## Chapter 3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1: Photomicrograph of a transverse section of a rat spinal cord showing the site of injection of retrogradely-transported tracer into the IML</td>
<td>95</td>
</tr>
<tr>
<td>3.2: Average number of Fos-positive cell nuclei, spinally-projecting neurons and double-labeled neurons counted in the PVN</td>
<td>96</td>
</tr>
<tr>
<td>3.3: Diagrammatic illustration of the distribution of Fos-positive cell nuclei, spinally-projecting neurons and of spinally-projecting neurons containing a Fos-positive cell nucleus in the subnuclei of the PVN.</td>
<td>97</td>
</tr>
<tr>
<td>3.4: Photomicrographs of the PVN showing the distribution of Fos-positive cell nuclei following heating and in a control animal at low magnification. Also shown are high magnification photomicrograph of double-labeled neuron from a rat that had undergone heating.</td>
<td>98</td>
</tr>
<tr>
<td>3.5: Photomicrographs of the PVN showing distribution of Fos-positive</td>
<td></td>
</tr>
</tbody>
</table>
cell nuclei from rats exposed to a hot environment between control
groups and group where hypotonic saline was administered.

Figure 3.6: Average numbers of NADPH-d-positive neurons, NADPH-d-
positive neurons that also project to the spinal cord panel, NADPH-
d-positive neurons that also contained a Fos-positive nucleus and
triple-labeled neurons in the PVN

Figure 3.7: Diagrammatic illustration of the distribution of NADPH-d-positive
neurons, NADPH-d-positive neurons that project to the spinal cord,
NADPH-d-positive neurons containing a Fos-positive nucleus and
triple labeled neurons in the PVN

Figure 3.8: Photomicrographs of coronal section through the PVN showing
distribution of Fos-positive cell nuclei and NADPH-d positive cells
between control and heated groups and a triple labeled neuron

---

**Chapter 4**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 4.1</td>
<td>Schematic diagram of coronal section from the brainstem illustrating midline brainstem, ventromedial brainstem and ventrolateral brainstem</td>
<td>129</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Average number of Fos-positive cell nuclei in the midline brainstem, ventromedial brainstem and the ventrolateral brainstem between control and warmed groups.</td>
<td>130</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>Average number of NADPH-d-positive neurons in the midline brainstem, ventromedial brainstem and the ventrolateral brainstem between control and warmed groups.</td>
<td>131</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Average number of spinal-projecting neurons in the midline brainstem, ventromedial brainstem and the ventrolateral brainstem between control and warmed groups.</td>
<td>132</td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>Diagrammatic illustration of the distribution of Fos-positive cell nuclei, NADPH-d positive neurons and spinally-projecting neurons of the rat brainstem.</td>
<td>133</td>
</tr>
<tr>
<td>Figure 4.6</td>
<td>High magnification photomicrographs of the coronal section of rat brainstem illustrating Fos-positive nuclei and NADPH-d positive neurons in the midline brainstem, ventromedial brainstem and ventrolateral brainstem</td>
<td>135</td>
</tr>
</tbody>
</table>
Figure 4.7: Average numbers of NADPH-d-positive neurons that also contained a Fos-positive nucleus and spinal-projecting neurons that also contained a Fos-positive nucleus in the midline, ventromedial and ventrolateral brainstem

<table>
<thead>
<tr>
<th>Chapter 5</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 5.1: Photomicrographs of transverse sections of the RVLM injection site</td>
<td>158</td>
</tr>
<tr>
<td>Figure 5.2: Average numbers of Fos-positive cell nuclei, RVLM-projecting neurons and double-labeled neurons counted in the PVN</td>
<td>159</td>
</tr>
<tr>
<td>Figure 5.3: Diagrammatic illustration of the distribution of Fos-positive cell nuclei, RVLM-projecting neurons and of NADPH-diaphorase – positive neurons (NADPH-d) in the subnuclei of the PVN</td>
<td>160</td>
</tr>
<tr>
<td>Figure 5.4: Average numbers of NADPH-diaphorase – positive neurons (NADPH-d), Fos-positive NADPH-d – positive neurons, RVLM-projecting neurons containing NADPH-d counted in the PVN</td>
<td>161</td>
</tr>
<tr>
<td>Figure 5.5: Photomicrographs of the PVN showing Fos-positive, NADPH-d positive and RVLM-projecting neurons</td>
<td>162</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 6</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 6.1: Basal MAP, HR, renal blood flow and renal conductance prior to microinjections of muscimol (1nmol/side) into the hypothalamus</td>
<td>182</td>
</tr>
<tr>
<td>Figure 6.1: Increase in tail skin temperature following an increase in core body temperature in anaesthetized rats</td>
<td>183</td>
</tr>
<tr>
<td>Figure 6.2: Responses of mean arterial pressure (MAP) and heart rate (HR) following an increase in core body temperature</td>
<td>184</td>
</tr>
<tr>
<td>Figure 6.3: Responses of renal blood flow and renal conductance following an increase in core body temperature in anaesthetized rats</td>
<td>185</td>
</tr>
<tr>
<td>Figure 6.4: Schematic transverse sections of the rat hypothalamic PVN</td>
<td></td>
</tr>
</tbody>
</table>
showing the centre of the microinjection sites in which muscimol or saline were microinjected into the PVN

Figure 6.5: Photomicrograph of a bilateral microinjection in the PVN taken using fluorescent lighting conditions

Figure 6.6: Schematic transverse sections of the rat hypothalamic PVN showing the centre of the microinjection sites in which muscimol were microinjected out of the PVN

<table>
<thead>
<tr>
<th>Chapter 7</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 7.1: Schematic diagram summarizing findings of the present work on the involvement of the PVN in the regulation of renal blood flow</td>
<td>205</td>
</tr>
<tr>
<td>Figure 7.2: Diagrammatic illustration of neuronal populations activated by elevations in body temperature.</td>
<td>207</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Appendix</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure A.1: Schematic diagram showing the position of input and output tubes for the manufacture of the water-circulating jacket.</td>
<td>210</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Ap</td>
<td>Area postrema</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>Bpm</td>
<td>Beats per minute</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin releasing hormone</td>
</tr>
<tr>
<td>CVLM</td>
<td>Caudal ventrolateral medulla</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-Diaminobenzidine hydrochloride</td>
</tr>
<tr>
<td>Fx</td>
<td>Fornix</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>HTS</td>
<td>Hypertonic saline</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IEG</td>
<td>Immediate early gene</td>
</tr>
<tr>
<td>ION</td>
<td>Inferior olivary nucleus</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MnPO</td>
<td>Median preoptic nucleus</td>
</tr>
<tr>
<td>NADPH-d</td>
<td>Nicotine Adenine Dinucleotide Phosphate-diaphorase</td>
</tr>
<tr>
<td>NHS</td>
<td>Normal horse serum</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NSPV</td>
<td>Spinal trigeminal nucleus</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus of the solitary tract</td>
</tr>
<tr>
<td>OT</td>
<td>Optic tract</td>
</tr>
<tr>
<td>OVLT</td>
<td>Organum vasculosum of the lamina terminalis</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Polyethylene</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl chloride</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>RSNA</td>
<td>Renal sympathetic nerve activity</td>
</tr>
<tr>
<td>RVLM</td>
<td>Rostral ventrolateral medulla</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SFO</td>
<td>Subfornical organ</td>
</tr>
<tr>
<td>SNA</td>
<td>Sympathetic nerve activity</td>
</tr>
<tr>
<td>SON</td>
<td>Supraoptic nucleus</td>
</tr>
</tbody>
</table>
DECLARATION

To the best of my knowledge, this thesis does not contain material that has been accepted for the award of any other degree or diploma in any university. I certify that the work is that of the author alone and does not contain any material which has been published or written by another person, except where due reference is made in the text of the thesis. The content of this thesis is the result of work that has been carried out since the official commencement date of the approved research program and any editorial work, paid or unpaid, carried out by a third party is acknowledged.

..........................................................  
Signature

..........................................................
JOO LEE CHAM

......................................................  
Name

......................................................  
17th October 2007

......................................................  
Date
I Dedicate this Thesis to My Family and Husband

For Believing in Me
ACKNOWLEDGEMENTS

To my supervisor, Associate Professor Emilio Badoer, I express gratitude for his patience, guidance and support all throughout the course of my PhD. Emilio gave his time generously and is always there for me both as a supervisor and also as a friend. I am also sincerely grateful to Emilio for his dedication and commitment that he showed in nurturing and guiding my passion for research.

To my second supervisor, Dr Neil Owens, I would like to express thanks for all his assistance, patience and support during the earlier years of my candidature. I am also grateful to Neil for his friendship, advice and moral support throughout these long years. Neil assisted in the technical production of some of the figures presented in this thesis, and was essential to this project in many ways IT. His calculated persistence gave me the strength to continue after his departure from the group.

I would like to acknowledge the opportunity given to me by the folk in the Anatomy Department who allowed me to teach during the latter stages of my PhD and who were most considerate of the many hats that I wore during my innings at RMIT. Their tolerance allowed me to work simultaneously as lecturer, demonstrator and student to complete my PhD. In particular I would like to thank Dr Rudi Klein and Dr Richard Guy for providing me with the opportunity to develop my communication skills by giving me the opportunity to lecture and demonstrate. To Gayle Johnstone and Romy Valdes, many thanks for their patience and assistance for making it all possible.
To Susan Mooney, I sincerely thank for all her help and support throughout my PhD. Working as a casual staff at the RMIT Animal Facility, I have learnt other things that are beyond that of the scope of my PhD and for that I am grateful.

To Chi Wai Ng, Aristotle Kantzides and Ms Hala Raghib: for their encouragement and support. Their presence through the early years of my candidature has made it an enjoyable and memorable experience for me.

I would also like to thank all of our group members: Dr Martin Stebbing, Dr Feng Chen, Dr Tien Huynh, Nilanka, Melissa, Jennifer, Louie and Melvin. To them, I extend a big thanks for their support and for making me feel like I have a family right here at university.

To my family, I express my deepest appreciation. To my dearest Dad and Mum, I thank for unconditional love, support, blessings and encouragement. To my wonderful and understanding husband, thank you for the years of immense support, love and kindness. To Genie, Bubble and Teddy, I thank them for keeping me company. To my best friends Roy, Shirley, Christabel and Jane, thank you for believing in me.

Acknowledging the various contributions of those that have made this work possible is the final task on my to-do list and signals a long-awaited event in my life! After many long nights this moment of sheer relief and happiness has dawned. Yet, sadness confounds this occasion of bliss, as I part from the work that has feverishly preoccupied the past few years and close the pages of another chapter in my life.
PUBLICATIONS

Publications arising from this thesis:


COMMUNICATIONS

Communications to scientific meetings during candidature:


Cham, JL and Badoer E (2007). Activation of RVLM-projecting neurons following heat exposure. IBRO World Congress of Neuroscience (Melbourne) (POS-FRI-177).
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Title</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declaration</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Publications</td>
<td>vi</td>
</tr>
<tr>
<td>Communications</td>
<td>vii</td>
</tr>
<tr>
<td>Table of contents</td>
<td>viii</td>
</tr>
<tr>
<td>List of tables and figures</td>
<td>xvi</td>
</tr>
<tr>
<td>Abbreviations and acronyms</td>
<td>xx</td>
</tr>
<tr>
<td>Summary</td>
<td>2</td>
</tr>
</tbody>
</table>

CHAPTER 1

Introduction

1.0  INTRODUCTION 7

1.1  THE PARAVENTRICULAR NUCLEUS (PVN) 9

1.1.1  Anatomical location of the PVN 9

1.1.2  Structural organization of the PVN 9

1.1.2.1  Magnocellular PVN neurons 11

1.1.2.2  Parvocellular PVN neurons 11

1.1.3  Neuronal connections of the PVN 14

1.1.3.1  Afferent projections 14

1.1.3.2  Efferent projections 17

1.1.3.3  PVN connections to the IML 18

1.1.3.4  PVN connections to the RVLM 20

1.1.4  Role of the PVN in integration of cardiovascular reflexes and sympathetic outflow 21

1.2  TEMPERATURE REGULATION AND THE PVN 27
<table>
<thead>
<tr>
<th>1.3</th>
<th>NITRIC OXIDE</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3.1</td>
<td>NO and temperature regulation</td>
<td>37</td>
</tr>
<tr>
<td>1.3.2</td>
<td>Distribution of NO in the central nervous system</td>
<td>39</td>
</tr>
<tr>
<td>1.3.3</td>
<td>NO in the PVN</td>
<td>41</td>
</tr>
<tr>
<td>1.3.4</td>
<td>The role of NO within the PVN in the regulation of cardiovascular responses and temperature regulation</td>
<td>42</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1.4</th>
<th>FOS, A MARKER OF INCREASED NEURONAL ACTIVITY</th>
<th>44</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4.1</td>
<td>The fos-oncogene</td>
<td>44</td>
</tr>
<tr>
<td>1.4.2</td>
<td>Regulation of c-fos</td>
<td>45</td>
</tr>
<tr>
<td>1.4.3</td>
<td>Methodological considerations.</td>
<td>46</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1.5</th>
<th>SUMMARY</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6</td>
<td>AIMS OF PRESENT STUDY</td>
<td>49</td>
</tr>
<tr>
<td>1.7</td>
<td>THESIS OUTLINE</td>
<td>49</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 2</th>
<th>Materials and methods</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>MANUFACTURE OF INSTRUMENTS</td>
<td>52</td>
</tr>
<tr>
<td>2.1.1</td>
<td>Manufacture of blood vessel cannulae</td>
<td>52</td>
</tr>
<tr>
<td>2.1.2</td>
<td>Manufacture of Glass Micropipettes</td>
<td>52</td>
</tr>
<tr>
<td>2.2</td>
<td>SURGICAL PREPARATION OF ANIMALS</td>
<td>54</td>
</tr>
<tr>
<td>2.2.1</td>
<td>Animals and housing</td>
<td>54</td>
</tr>
<tr>
<td>2.2.2</td>
<td>Surgical preparation of animals in neuroanatomical studies</td>
<td>55</td>
</tr>
<tr>
<td>2.3</td>
<td>CANNULATION OF FEMORAL VESSELS</td>
<td>56</td>
</tr>
<tr>
<td>2.4</td>
<td>MICROINJECTIONS OF RETROGRADELY TRANSPORTED TRACER INTO THE SPINAL CORD</td>
<td>57</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>2.5 MICROINJECTIONS OF RETROGRADELY TRANSPORTED TRACER INTO THE RVLM</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>2.6 POST-OPERATIVE CARE</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>2.7 EXPERIMENTAL DAY</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>2.8 PERFUSION</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>2.8.1 Fixing the rat brain</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>2.8.2 Removing the rat brain and spinal cord</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>2.9 SECTIONING OF THE RAT BRAIN AND SPINAL CORD</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>2.10 STAINING PROTOCOLS</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>2.10.1 Immunohistochemical detection of Fos</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>2.10.2 Nicotine Adenine Dinucleotide Phosphate-diaphorase (NADPH-d) staining</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>2.11 ANALYSIS</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>2.11.1 Histological analysis</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>2.11.2 Statistical analysis</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>2.12 MAPPING</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>2.13 PHOTOMICROSCOPY</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>2.14 BLOOD FLOW EXPERIMENTS</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>2.14.1 Surgical preparation of animals used in blood flow experiments</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>2.14.2 Microinjection into the hypothalamic PVN</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>2.14.3 Renal blood flow measurement</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>2.14.4 Experimental protocol</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>2.14.5 Brain histology</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>2.14.6 Statistical analysis</td>
<td>79</td>
<td></td>
</tr>
</tbody>
</table>
## CHAPTER 3
Activation of spinally-projecting and nitrergic neurons in the PVN following heat exposure.

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>INTRODUCTION</td>
<td>81</td>
</tr>
<tr>
<td>3.1</td>
<td>EXPERIMENTAL PROCEDURES</td>
<td>84</td>
</tr>
<tr>
<td>3.1.1</td>
<td>Animals and housing</td>
<td>84</td>
</tr>
<tr>
<td>3.1.2</td>
<td>Microinjections of retrogradely transported tracer into IML</td>
<td>84</td>
</tr>
<tr>
<td>3.1.3</td>
<td>Experimental day</td>
<td>85</td>
</tr>
<tr>
<td>3.1.4</td>
<td>Detection of Fos by immunohistochemistry</td>
<td>86</td>
</tr>
<tr>
<td>3.1.5</td>
<td>Nicotine Adenine Dinucleotide-diaphorase (NADPH-d) staining</td>
<td>87</td>
</tr>
<tr>
<td>3.1.6</td>
<td>Analysis</td>
<td>87</td>
</tr>
<tr>
<td>3.1.6.1</td>
<td>Rats with intraspinal – injections</td>
<td>87</td>
</tr>
<tr>
<td>3.1.6.2</td>
<td>Rats without intraspinal – injections</td>
<td>88</td>
</tr>
<tr>
<td>3.1.7</td>
<td>Statistical analysis</td>
<td>88</td>
</tr>
<tr>
<td>3.1.8</td>
<td>Mapping</td>
<td>88</td>
</tr>
<tr>
<td>3.1.9</td>
<td>Photomicroscopy</td>
<td>89</td>
</tr>
<tr>
<td>3.2</td>
<td>RESULTS</td>
<td>90</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Effect of heating on Fos expression in the PVN</td>
<td>90</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Distribution of spinally-projecting neurons in the PVN</td>
<td>91</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Distribution of spinally-projecting neurons in the PVN containing Fos</td>
<td>92</td>
</tr>
<tr>
<td>3.2.4</td>
<td>Distribution of neurons that contained NADPH-d</td>
<td>92</td>
</tr>
<tr>
<td>3.2.5</td>
<td>Distribution of spinally-projecting neurons containing NADPH-d</td>
<td>92</td>
</tr>
<tr>
<td>3.2.6</td>
<td>Distribution of neurons containing NADPH-d and Fos</td>
<td>93</td>
</tr>
<tr>
<td>3.2.7</td>
<td>Distribution of triple-labeled neurons</td>
<td>93</td>
</tr>
<tr>
<td>3.2.8</td>
<td>Behavioural responses to heat exposure</td>
<td>94</td>
</tr>
<tr>
<td>3.3</td>
<td>DISCUSSION</td>
<td>103</td>
</tr>
<tr>
<td>3.4</td>
<td>PERSPECTIVE</td>
<td>109</td>
</tr>
</tbody>
</table>
# CHAPTER 4

Exposure to a hot environment can activate spinally-projecting neurons and nitrergic neurons in the brainstem.

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>Introduction</td>
<td>113</td>
</tr>
<tr>
<td>4.1</td>
<td>Experimental Procedures</td>
<td>116</td>
</tr>
<tr>
<td>4.1.1</td>
<td>Animals and housing</td>
<td>116</td>
</tr>
<tr>
<td>4.1.2</td>
<td>Microinjections of retrogradely transported tracer into IML</td>
<td>116</td>
</tr>
<tr>
<td>4.1.3</td>
<td>Experimental day</td>
<td>117</td>
</tr>
<tr>
<td>4.1.4</td>
<td>Detection of Fos by immunohistochemistry</td>
<td>117</td>
</tr>
<tr>
<td>4.1.5</td>
<td>Nicotine Adenine Dinucleotide-diaphorase (NADPH-d) staining</td>
<td>118</td>
</tr>
<tr>
<td>4.1.6</td>
<td>Analysis</td>
<td>118</td>
</tr>
<tr>
<td>4.1.7</td>
<td>Statistical analysis</td>
<td>119</td>
</tr>
<tr>
<td>4.1.8</td>
<td>Mapping</td>
<td>119</td>
</tr>
<tr>
<td>4.1.9</td>
<td>Photomicroscopy</td>
<td>120</td>
</tr>
<tr>
<td>4.2</td>
<td>Results</td>
<td>121</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Midline brainstem</td>
<td>121</td>
</tr>
<tr>
<td>4.2.1.1</td>
<td>Distribution of Fos positive neurons</td>
<td>121</td>
</tr>
<tr>
<td>4.2.1.2</td>
<td>Distribution of NADPH-d positive neurons</td>
<td>121</td>
</tr>
<tr>
<td>4.2.1.3</td>
<td>Distribution of neurons containing NADPH-d and Fos</td>
<td>122</td>
</tr>
<tr>
<td>4.2.1.4</td>
<td>Distribution of spinally-projecting neurons</td>
<td>122</td>
</tr>
<tr>
<td>4.2.1.5</td>
<td>Distribution of spinally-projecting neurons that also contained Fos</td>
<td>123</td>
</tr>
<tr>
<td>4.2.1.6</td>
<td>Distribution of spinally-projecting neurons that also contained Fos and NADPH-d</td>
<td>123</td>
</tr>
<tr>
<td>4.2.2</td>
<td>Ventromedial brainstem</td>
<td>123</td>
</tr>
<tr>
<td>4.2.2.1</td>
<td>Effect of heating on Fos expression</td>
<td>123</td>
</tr>
<tr>
<td>4.2.2.2</td>
<td>Distribution of NADPH-d positive neurons</td>
<td>124</td>
</tr>
</tbody>
</table>
CHAPTER 5
 Exposure to a hot environment can activate RVLM-projecting neurons in the hypothalamic PVN in conscious rats.
5.1.4 Detection of Fos by immunohistochemistry 151
5.1.5 Nicotine Adenine Dinucleotide-diaphorase (NADPH-d) staining 151
5.1.6 Analysis 152
5.1.7 Statistical analysis 153
5.1.8 Mapping 153
5.1.9 Photomicroscopy 153

5.2 RESULTS 154
5.2.1 Effect of heating on Fos expression in the PVN 154
5.2.2 Distribution of RVLM-projecting neurons in the PVN 154
5.2.3 Distribution of RVLM-projecting neurons that contained Fos 155
5.2.4 Distribution of neurons that contained NADPH-d 155
5.2.5 Distribution of neurons containing NADPH-d and Fos 156
5.2.6 Distribution of RVLM-projecting neurons containing NADPH-d 156
5.2.7 Distribution of RVLM-projecting neurons that contained Fos and NADPH-d 157

5.3 DISCUSSION 163

5.4 CONCLUSIONS 169

CHAPTER 6
The hypothalamic paraventricular nucleus is critical for renal vasoconstriction elicited by elevations in body temperature.

6.0 INTRODUCTION 172

6.1 EXPERIMENTAL PROCEDURES 174
6.1.1 Animals and housing 174
6.1.2 Surgical preparations 174
6.1.3 Microinjection into the hypothalamic PVN 175
6.1.4 Renal blood flow measurement 176
6.1.5 Experimental protocol
6.1.6 Brain histology
6.1.7 Statistical analysis

6.2 RESULTS
6.2.1 Resting levels
6.2.2 Effect of increases in temperature on cardiovascular variables
6.2.3 Responses in rats microinjected with saline into the PVN
6.2.4 Responses in rats microinjected with muscimol into the PVN
6.2.5 Responses in rats microinjected with muscimol out of the PVN
6.2.6 Histological analysis of microinjection sites

6.3 DISCUSSION

CHAPTER 7
General discussion

APPENDIX
Appendix 1 - Construction of water-circulating jacket
Appendix 2 - Phosphate Buffer (PB)
Appendix 3 - Phosphate buffer saline (PBS)
Appendix 4 - Tris buffer
Appendix 5 - Paraformaldehyde
Appendix 6 - 3,3’-diaminobenzidine hydrochloride
Appendix 7 – Preparation of subbed slides

BIBLIOGRAPHY
# LIST OF TABLES AND FIGURES

## Chapter 1

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Photomicrograph of a coronal section through the rat hypothalamus showing the paraventricular nucleus (PVN)</td>
<td>10</td>
</tr>
<tr>
<td>1.2</td>
<td>Diagrammatic illustration of major sub-divisions of the PVN</td>
<td>12</td>
</tr>
<tr>
<td>1.3</td>
<td>Main afferent projections to the PVN</td>
<td>16</td>
</tr>
<tr>
<td>1.4</td>
<td>Main efferent projections from the PVN</td>
<td>19</td>
</tr>
</tbody>
</table>

## Chapter 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Photo of a typical blood vessel cannula</td>
<td>53</td>
</tr>
<tr>
<td>2.2</td>
<td>Photo of rat positioned on a Stoelting stereotactic frame</td>
<td>60</td>
</tr>
<tr>
<td>2.3</td>
<td>Mean arterial pressure (MAP) trace following injection of L-glutamate into the RVLM</td>
<td>61</td>
</tr>
<tr>
<td>2.4</td>
<td>Photo of the temperature chamber</td>
<td>64</td>
</tr>
<tr>
<td>2.5</td>
<td>Diagram of a renal flow probe positioned around the renal artery</td>
<td>75</td>
</tr>
<tr>
<td>2.6</td>
<td>Photomicrograph of coronal section through the hypothalamus showing the injection site in the PVN</td>
<td>78</td>
</tr>
</tbody>
</table>

## Chapter 3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Photomicrograph of a transverse section of a rat spinal cord showing the site of injection of retrogradely-transported tracer into the IML</td>
<td>95</td>
</tr>
<tr>
<td>3.2</td>
<td>Average number of Fos-positive cell nuclei, spinally-projecting neurons and double-labeled neurons counted in the PVN</td>
<td>96</td>
</tr>
<tr>
<td>3.3</td>
<td>Diagrammatic illustration of the distribution of Fos-positive cell nuclei, spinally-projecting neurons and of spinally-projecting neurons containing a Fos-positive cell nucleus in the subnuclei of the PVN.</td>
<td>97</td>
</tr>
<tr>
<td>3.4</td>
<td>Photomicrographs of the PVN showing the distribution of Fos-positive cell nuclei following heating and in a control animal at low magnification. Also shown are high magnification photomicrograph of double-labeled neuron from a rat that had undergone heating.</td>
<td>98</td>
</tr>
<tr>
<td>3.5</td>
<td>Photomicrographs of the PVN showing distribution of Fos-positive</td>
<td></td>
</tr>
</tbody>
</table>
cell nuclei from rats exposed to a hot environment between control groups and group where hypotonic saline was administered.

**Figure 3.6:** Average numbers of NADPH-d-positive neurons, NADPH-d-positive neurons that also project to the spinal cord panel, NADPH-d-positive neurons that also contained a Fos-positive nucleus and triple-labeled neurons in the PVN

**Figure 3.7:** Diagrammatic illustration of the distribution of NADPH-d-positive neurons, NADPH-d-positive neurons that project to the spinal cord, NADPH-d-positive neurons containing a Fos-positive nucleus and triple labeled neurons in the PVN

**Figure 3.8:** Photomicrographs of coronal section through the PVN showing distribution of Fos-positive cell nuclei and NADPH-d positive cells between control and heated groups and a triple labeled neuron

<table>
<thead>
<tr>
<th>Chapter 4</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 4.1: Schematic diagram of coronal section from the brainstem</td>
<td>129</td>
</tr>
<tr>
<td>illustrating midline brainstem, ventromedial brainstem and ventrolateral brainstem</td>
<td></td>
</tr>
<tr>
<td>Figure 4.2: Average number of Fos-positive cell nuclei in the midline</td>
<td>130</td>
</tr>
<tr>
<td>brainstem, ventromedial brainstem and the ventrolateral brainstem</td>
<td></td>
</tr>
<tr>
<td>between control and warmed groups.</td>
<td></td>
</tr>
<tr>
<td>Figure 4.3: Average number of NADPH-d-positive neurons in the midline</td>
<td>131</td>
</tr>
<tr>
<td>brainstem, ventromedial brainstem and the ventrolateral brainstem</td>
<td></td>
</tr>
<tr>
<td>between control and warmed groups.</td>
<td></td>
</tr>
<tr>
<td>Figure 4.4: Average number of spinal-projecting neurons in the midline</td>
<td>132</td>
</tr>
<tr>
<td>brainstem, ventromedial brainstem and the ventrolateral brainstem</td>
<td></td>
</tr>
<tr>
<td>between control and warmed groups.</td>
<td></td>
</tr>
<tr>
<td>Figure 4.5: Diagrammatic illustration of the distribution of Fos-positive cell nuclei, NADPH-d positive neurons and spinally-projecting neurons of the rat brainstem.</td>
<td>133</td>
</tr>
<tr>
<td>Figure 4.6: High magnification photomicrographs of the coronal section of rat brainstem illustrating Fos-positive nuclei and NADPH-d positive neurons in the midline brainstem, ventromedial brainstem and ventrolateral brainstem</td>
<td>135</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>page</td>
</tr>
<tr>
<td>-----------</td>
<td>-------</td>
</tr>
<tr>
<td>Figure 5.1: Photomicrographs of transverse sections of the RVLM injection site</td>
<td>158</td>
</tr>
<tr>
<td>Figure 5.2: Average numbers of Fos-positive cell nuclei, RVLM-projecting neurons and double-labeled neurons counted in the PVN</td>
<td>159</td>
</tr>
<tr>
<td>Figure 5.3: Diagrammatic illustration of the distribution of Fos-positive cell nuclei, RVLM-projecting neurons and of NADPH-diaphorase – positive neurons (NADPH-d) in the subnuclei of the PVN</td>
<td>160</td>
</tr>
<tr>
<td>Figure 5.4: Average numbers of NADPH-diaphorase – positive neurons (NADPH-d), Fos-positive NADPH-d – positive neurons, RVLM-projecting neurons containing NADPH-d counted in the PVN</td>
<td>161</td>
</tr>
<tr>
<td>Figure 5.5: Photomicrographs of the PVN showing Fos-positive, NADPH-d positive and RVLM-projecting neurons</td>
<td>162</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 6</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 6.1: Basal MAP, HR, renal blood flow and renal conductance prior to microinjections of muscimol (1nmol/side) into the hypothalamus</td>
<td>182</td>
</tr>
<tr>
<td>Figure 6.1: Increase in tail skin temperature following an increase in core body temperature in anaesthetized rats</td>
<td>183</td>
</tr>
<tr>
<td>Figure 6.2: Responses of mean arterial pressure (MAP) and heart rate (HR) following an increase in core body temperature</td>
<td>184</td>
</tr>
<tr>
<td>Figure 6.3: Responses of renal blood flow and renal conductance following an increase in core body temperature in anaesthetized rats</td>
<td>185</td>
</tr>
<tr>
<td>Figure 6.4: Schematic transverse sections of the rat hypothalamic PVN</td>
<td>186</td>
</tr>
</tbody>
</table>
showing the centre of the microinjection sites in which muscimol or saline were microinjected into the PVN

Figure 6.5: Photomicrograph of a bilateral microinjection in the PVN taken using fluorescent lighting conditions

Figure 6.6: Schematic transverse sections of the rat hypothalamic PVN showing the centre of the microinjection sites in which muscimol were microinjected out of the PVN

<table>
<thead>
<tr>
<th>Chapter 7</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 7.1: Schematic diagram summarizing findings of the present work on the involvement of the PVN in the regulation of renal blood flow</td>
<td>205</td>
</tr>
<tr>
<td>Figure 7.2: Diagrammatic illustration of neuronal populations activated by elevations in body temperature.</td>
<td>207</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Appendix</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure A.1: Schematic diagram showing the position of input and output tubes for the manufacture of the water-circulating jacket.</td>
<td>210</td>
</tr>
</tbody>
</table>
# ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Ap</td>
<td>Area postrema</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>Bpm</td>
<td>Beats per minute</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin releasing hormone</td>
</tr>
<tr>
<td>CVLM</td>
<td>Caudal ventrolateral medulla</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-Diaminobenzidine hydrochloride</td>
</tr>
<tr>
<td>Fx</td>
<td>Fornix</td>
</tr>
<tr>
<td>GABA</td>
<td>$\gamma$-aminobutyric acid</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>HTS</td>
<td>Hypertonic saline</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IEG</td>
<td>Immediate early gene</td>
</tr>
<tr>
<td>ION</td>
<td>Inferior olivary nucleus</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MnPO</td>
<td>Median preoptic nucleus</td>
</tr>
<tr>
<td>NADPH-d</td>
<td>Nicotine Adenine Dinucleotide Phosphate-diaphorase</td>
</tr>
<tr>
<td>NHS</td>
<td>Normal horse serum</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NSPV</td>
<td>Spinal trigeminal nucleus</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus of the solitary tract</td>
</tr>
<tr>
<td>OT</td>
<td>Optic tract</td>
</tr>
<tr>
<td>OVL T</td>
<td>Organum vasculosum of the lamina terminalis</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Polyethylene</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl chloride</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>RSNA</td>
<td>Renal sympathetic nerve activity</td>
</tr>
<tr>
<td>RVLM</td>
<td>Rostral ventrolateral medulla</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SFO</td>
<td>Subfornical organ</td>
</tr>
<tr>
<td>SNA</td>
<td>Sympathetic nerve activity</td>
</tr>
<tr>
<td>SON</td>
<td>Supraoptic nucleus</td>
</tr>
</tbody>
</table>
The hypothalamic paraventricular nucleus (PVN) is known to be a major integrative region within the forebrain. It is composed of functionally different subgroups of neurons, including the parvocellular neurons that project to important autonomic targets in the brainstem e.g. the rostral ventrolateral medulla (RVLM) and the intermediolateral cell column (IML) of the spinal cord, where the sympathetic preganglionic motor-neurons are located. These regions are critical in cardiovascular regulation; hence, these projections are likely to mediate the effects of the PVN on sympathetic nerve activity and hence may contribute to the cardiovascular changes induced by physiological stimuli such as elevations in body temperature.

The neurotransmitter such as nitric oxide (NO) is important in cardiovascular regulation and it is now emerging as a major focus of investigation in thermoregulation. One of the most striking accumulations of NO containing-neurons is in the PVN where it appears to be playing an important role in cardiovascular regulation and body fluid homeostasis.

The aims of the work described in this thesis were; (i) to investigate whether elevation of core body temperature activated “autonomic” pathways emanating from the PVN such as those projecting to the IML of the spinal cord and the RVLM, (ii) to establish whether hypothalamic PVN nitrergic neurons were activated by acute hyperthermia, and (iii) to determine if the PVN plays an important functional role in the cardiovascular response elicited by the elevation in body temperature.
The results of the work show:

(1) Compared to controls, rats exposed to an environmental temperature of 39°C for one hour, had a tenfold increase in the number of cells producing Fos in the PVN. Of the spinally-projecting neurons in the PVN of heated rats, approximately 22% expressed Fos. Additionally, of the nitrergic neurons located in the parvocellular PVN, approximately 40% also expressed Fos. Furthermore, there was a significant increase in the number of spinally-projecting neurons in the PVN that were nitrergic and expressed Fos following heat exposure (12%) compared to controls (0.1%). These results suggest that spinally-projecting and nitrergic neurons in the PVN may contribute to the central pathways activated by exposure to a hot environment.

(2) There was a significant increase in activated neurons in the midline (by five-fold), ventromedial (by eight-fold) and in the ventrolateral (by nine-fold) brainstem. Some of these neurons were nitrergic, particularly in the ventromedial brainstem (5% of the nitrergic neurons in this region). A small proportion of activated neurons were spinally-projecting neurons (2-3% of spinal projecting neurons). There were no triple-labeled neurons at any level of the brainstem examined. These findings suggest that nitrergic neurons and spinally-projecting neurons in the brainstem may make a small contribution to the central pathways mediating the reflex responses initiated by hyperthermia.
(3) Compared to controls, exposure of conscious rats to a hot environment of 39°C also significantly increased the number of activated RVLM-projecting neurons (approximately 8% in the heated group versus 1.5% in the controls). Although heating significantly increased the number of activated nitrergic PVN neurons, triple labeled neurons (i.e. activated, nitrergic and RVLM - projecting) in the PVN were rarely observed. Thus, the present study illustrates that these PVN neurons projecting to the RVLM may make a smaller contribution than the spinal-projecting neurons in the PVN to the cardiovascular responses initiated by heat.

(4) The GABA receptor agonist, muscimol was used to inhibit neuronal activity to investigate the involvement of the PVN in the physiological response to an increased body temperature. Under normal conditions, changes in body temperature elicit reflex cardiovascular responses which affect blood flow distribution and this includes a reduction in the renal blood flow to help re-direct blood from the internal organs to the peripheral vasculature.

The results of my studies showed that the microinjection of muscimol to inhibit the neuronal activity in the PVN abolished the reflex decrease in renal blood flow following an elevation of core body temperature. In addition, this effect was specific to the PVN, since microinjections of muscimol into areas outside the PVN were not effective. These findings demonstrate that the PVN is critical for this reflex cardiovascular response initiated by hyperthermia.
In conclusion, PVN is critical for the reflex decrease in renal blood flow during elevations in core body temperature. We hypothesise that projections from the PVN to the spinal cord and the RVLM contribute to the reflex cardiovascular responses. Additionally, nitrergic neurons in the PVN may contribute but the physiological role of those neurons in the reflex responses elicited by hyperthermia needs to be investigate.
CHAPTER 1.

INTRODUCTION
INTRODUCTION

The paraventricular nucleus (PVN) of the hypothalamus is a major integrative region within the forebrain. There is a growing body of evidence in the literature suggesting the PVN contributes to the regulation of metabolic rate, food intake, the responses to stress, body fluid homeostasis, endocrine function, sympathetic nerve activity, thermoregulation and the cardiovascular system (Badoer et al., 1993; Badoer et al., 2003; Bamshad et al., 1999; Benarroch, 2005; Bratinesak and Palkovits, 2004; Coote et al., 1998; Coote, 2005a; Coote, 2005b; Dampney et al., 2005; Duan et al., 1997; Guo and Moazzami, 2004; Kantzides and Badoer, 2003; McKinley et al., 2001; Ng et al., 2004; Patel, 2000; Polson et al., 2002; Romanovsky, 2007; Sakamoto et al., 2004; Shih et al., 2003; Stocker et al., 2004; Swanson and Sawchenko, 1980; Yang and Coote, 2007). Of particular interest for this thesis is the role of the PVN in cardiovascular responses to temperature regulation.

At present, there is strong functional evidence indicating the PVN is important in the cardiovascular responses to changes in body fluid homeostasis e.g. blood volume and osmolality (Badoer et al., 1993; Kantzides and Badoer, 2003; Ng et al., 2004; Polson et al., 2002; Stocker et al., 2004). Whilst the role of the PVN in modulating the sympathetic nervous system and cardiovascular response to body fluid homeostasis have been extensively studied (Badoer et al., 1993; Kantzides and Badoer, 2003; Ng et al., 2004; Polson et al., 2002; Stocker et al., 2004), less research has focused on the role of the PVN in the cardiovascular responses elicited by an elevation of body core temperature. This is
surprising given the circumstantial evidence suggesting that it plays an important role in the regulation of body temperature.

Changes in body temperature elicit reflex cardiovascular effects which affect blood flow distribution and this involves altered sympathetic nerve activity to different vascular beds. Although it is well known that the PVN can influence blood flow via the sympathetic nervous system, a role for the PVN in the sympathetic nerve responses elicited following a change in body temperature remains unknown. Is it possible the PVN also contributes to the cardiovascular changes elicited by changes in body temperature?

The hypothalamic PVN is composed of functionally different subgroups of neurons, including the parvo cellular neurons that project to important autonomic targets in the brainstem and spinal cord that are critical in cardiovascular regulation, such as the intermediolateral cell column (IML) and rostral ventrolateral medulla (RVLM) (Pyner and Coote, 1999; Pyner and Coote, 2000; Sawchenko and Swanson, 1982; Shafton et al., 1998; Swanson and Kuypers, 1980). These projections are likely to mediate the effects of the PVN on sympathetic nerve activity and hence may contribute to the cardiovascular changes induced by elevations in body temperature. However, this has not been examined to date. Therefore, the aims of this thesis were (i) to investigate whether elevation of core body temperature activated “autonomic” pathways emanating from the PVN such as those projecting to the IML of the spinal cord and the RVLM and (ii) to determine if the PVN plays an important functional role in the cardiovascular response elicited by the elevation in body temperature.
1.1 THE PARAVENTRICULAR NUCLEUS (PVN)

1.1.1 Anatomical location of the PVN

The PVN is an important integrative nucleus located within the hypothalamus. The PVN is located bilaterally between the fornix and the third ventricle (Figure 1.1). On the dorsal aspect of the PVN lies the thalamus whilst on the anterior and ventral aspect is the anterior hypothalamic nucleus. In the rat, the PVN extends approximately 1 mm in the rostral-caudal direction and is approximately 1 mm at its most lateral extension; occupying an area which is less than a third of a cubic millimetre.

1.1.2 Structural organization of the PVN

The PVN is comprised of magnocellular and parvocellular neurons. Using Nissl stained preparations, the magnocellular and parvocellular neurons within the PVN range in size from small (6-10 μm), medium (10-13 μm) to large (13-19 μm) and these can be readily distinguished (Armstrong et al., 1980; Kiss et al., 1991; Swanson and Kuypers, 1980). In addition, immunohistochemical and anatomical studies have enabled the identification of several cytologically and neurochemically distinct magnocellular and parvocellular PVN sub-divisions (Figure 1.2) (Swanson and Sawchenko, 1983a). These sub-nuclei may have different connections and hence they are likely to subserve diverse functions (Sawchenko et al., 1996; Swanson and Sawchenko, 1980; Swanson and Sawchenko, 1983a). The current thesis adheres to the anatomical sub-divisions proposed originally by Swanson and Kuypers (Swanson and Kuypers, 1980) which was later modified by Swanson and Sawchenko (Swanson and Sawchenko, 1983a).
Figure 1.1

Photomicrograph of a coronal section through the rat hypothalamus showing the paraventricular nucleus (PVN) (both sides). The section is counterstained with cresyl violet. The schematic diagram outlines the distinct position of the magnocellular and parvocellular PVN sub-divisions. Abbreviations: III, third ventricle.
1.1.2.1 Magnocellular PVN neurons

The magnocellular neurons can be sub-divided into the anterior, medial and posterior groups (Figure 1.2) and are composed mostly of large neurons (13 to 19 µm in diameter) \((\text{Armstrong et al., 1980; Kiss et al., 1991; Swanson and Kuypers, 1980; Swanson and Sawchenko, 1983a})\). The axons of magnocellular neurons leave the nucleus either ventro-medially or ventro-laterally to course above or below the fornix and then arch postero-ventromedially toward the median eminence.

The majority of the magnocellular PVN neurons synthesise and store anti-diuretic hormone (ADH) and oxytocin and release these hormones via the hypothalamo-neurohypophyseal tract via projections to the posterior pituitary gland \((\text{Cunningham and Sawchenko, 1991; Flament-Durand, 1980; Ju et al., 1986; Kovacs, 2002; Miselis, 1981; Richard et al., 1997})\). Nevertheless, it should be noted that not all neurons located in the magnocellular subdivisions are neurosecretory; in fact some of the neurons located in these regions of the PVN are known to project to the brainstem and also the spinal cord \((\text{Nilaver et al., 1980; Nylen et al., 2001})\).

1.1.2.2 Parvocellular PVN neurons

The parvocellular PVN neurons are sub-divided into several sub-nuclei; namely, the periventricular, anterior, medial, ventral, lateral and dorsal subdivisions which consist predominantly of small to medium sized neurons (6 to 10 µm and 10 to 13 µm in
Figure 1.2

Diagrammatic illustration of major sub-divisions of the PVN. Five different rostral (A) – caudal (E) levels are shown and the approximate anterior – caudal levels caudal to the bregma in mm is indicated. Abbreviations: III, third ventricle; ap, anterior parvocellular; dp, dorsal parvocellular; Fx, fornix; lp, lateral parvocellular; mp, medial parvocellular; pv, periventricular; pm, posterior magnocellular. (Redrawn and modified from (Sawchenko and Swanson, 1983b).)
diameter, respectively) (Figure 1.2) (Armstrong et al., 1980; Kiss et al., 1991; Swanson and Kuypers, 1980; Swanson and Sawchenko, 1983a).

The majority of the anterior, medial and periventricular parvocellular neurons are involved in the hypothalamic-pituitary adreno-cortical axis and these neurons project to the external part of the median eminence (Wiegand and Price, 1980). Through the median eminence, projections from these parvocellular neurons can also influence and affect the secretion of trophic hormones from the anterior pituitary gland (adenohypophysis). Thus, these neurons regulate the release of hormones; which affect the functions of various peripheral organs including the adrenal cortex, the thyroid gland, gonads, as well as body growth.

The parvocellular neurons located in the dorsal, ventral and lateral sub-divisions of the PVN (Figure 1.2), on the other hand, are known to send projections to the various autonomic nuclei that are critical for autonomic and cardiovascular regulation (Saper et al., 1976b; Swanson and Kuypers, 1980; Swanson et al., 1980). Some of these important autonomic nuclei are the nucleus tractus solitarius (NTS), the rostral ventrolateral medulla (RVLM), and IML of the spinal cord where the sympathetic pre-ganglionic motor neurons are situated (Schwanzel-Fukuda et al., 1984; Swanson et al., 1980). The latter is of particular interest as PVN neurons that directly project to the sympathetic pre-ganglionic motor neurons located in the IML provides the anatomical framework that enables the PVN to directly regulate sympathetic nerve activity (Coote et al., 1998; Strack et al., 1989a; Strack et al., 1989b; Swanson and Kuypers, 1980). Furthermore,
these spinally-projecting neurons in the PVN are also known to send collaterals to the RVLM (Shafton et al., 1998; Yang and Coote, 1998), an area that is important for the tonic generation of sympathetic nerve activity. Additionally, the RVLM is also known to contain neurons that project to the IML of the spinal cord (Strack et al., 1989a). Hence, the projection of PVN neurons to the RVLM provides the PVN with the ability to indirectly influence sympathetic nerve activity. Such organisational complexity of the anatomical connections suggests that the PVN has the capacity to both directly and indirectly influence sympathetic nerve activity.

1.1.3 Neuronal connections of the PVN

1.1.3.1 Afferent projections

The PVN is strongly innervated by afferent projections from various brain regions including other neighbouring hypothalamic nuclei, the forebrain and the brainstem (Figure 1.3) (Armstrong, 1995; Korf, 1984; Liposits, 1993). From within the hypothalamic nuclei, the PVN receives projections from the preoptic area, the arcuate nucleus, the ventromedial and dorsomedial nuclei, the anterior and lateral hypothalamic areas, the ventral pre-mammillary nucleus and the suprachiasmatic nucleus (SCN) (Berk and Finkelstein, 1981a; Berk and Finkelstein, 1981b; Bernardis and Bellinger, 1998; Buijs et al., 1993; Larsen et al., 1994; Saeb-Parsy et al., 2000; Saper et al., 1979; Silverman et al., 1981; Swanson and Cowan, 1975; Swanson, 1976; Tanaka et al., 1986a; Uschakov et al., 2006). Moreover, both the magnocellular and parvocellular neurons of the PVN are targeted by the projections from the subfornical organ (SFO), organum
vasculosum of the lamina terminalis (OVLT) and median preoptic nucleus (MnPO) (Bains and Ferguson, 1995; Sawchenko and Swanson, 1981; Silverman et al., 1981; Tanaka et al., 1985; Tanaka et al., 1986).

Additionally, the PVN also receives inputs converging from several forebrain structures, including parts of the sepal region, the amygdaloid complex and the hippocampal formation, as well as the bed nucleus of the stria terminalis (NTS), the medial pre-optic area and each of the major parts of the cerebrum, including the limbic system (Berk and Finkelstein, 1981a; Krettek and Price, 1978; Ono et al., 1985; Swanson and Cowan, 1977).

Autonomic regions within the brainstem which are involved in the regulation of the cardiovascular system also project to the PVN. These regions include the NTS, RVLM, caudal ventrolateral medulla (CVLM), area postrema (AP), parabrachial nucleus, locus coeruleus and the raphe nuclei (Berk and Finkelstein, 1981a; Fontes et al., 2001; Hardy, 2001; Kantzides et al., 2005; Svensson and Thorén, 1979; Torvik, 1956; Tribollet and Dreifuss, 1981).
Figure 1.3

Main afferent projections to the PVN. A1, noradrenergic cell group 1; ARC, arcuate nucleus; BNST, bed nucleus of the stria terminalis; C1, adrenergic cell group 1; DM, dorsomedial nucleus; LC, locus coeruleus; NTS, nucleus of the solitary tract; PM, pre-mammillary nucleus; PB, parabrachial nucleus; SCN, suprachiasmatic nucleus; SFO, subfornical organ; IX, glossopharyngeal nerve; X, vagal nerve (modified from Liposits, 1993).
Based on the intricate anatomical connections, including those from the hypothalamus it is not surprising that the PVN is an important integrative nucleus. Of particular interest are the afferent projections from the pre-optic nuclei to areas known to be important in thermoregulation (Bachtell et al., 2003; Boulant, 2000; Bratincsak and Palkovits, 2004; Brown et al., 2005; Chen et al., 1998; Ishiwata et al., 2001; Ishiwata et al., 2002; Ishiwata et al., 2005; Kazuyuki et al., 1998; Konishi et al., 2007; Maruyama et al., 2003; McAllen, 2004; Nakamura et al., 2002; Osaka, 2004; Ray et al., 2005; Srividya et al., 2006; Taniguchi et al., 2003; Yoshida et al., 2002). This raises the possibility that the PVN may play a role in the responses elicited by temperature challenge.

1.1.3.2 Efferent projections

The extensive network of efferent projections from the PVN emphasises the role of the PVN in the regulation of the cardiovascular system. The PVN projects to many brain regions including the brainstem and spinal cord (Figure 1.4) (Armstrong, 1995; Korf, 1984; Liposits, 1993). Many of these brain nuclei, to which the PVN projects, are known to be involved and are important in the regulation of the cardiovascular system. These include the RVLM, NTS, AP, CVLM, parabrachial nucleus, locus coeruleus, periaqueductal gray matter and the amygdala (Dampney et al., 1987; Luiten et al., 1985; Motawei et al., 1999; Pyner and Coote, 2000; Ranson et al., 1998; Reyes et al., 2005; Saper et al., 1976a; Swanson and Kuypers, 1980).
1.1.3.3 PVN connections to the IML

The projections from the PVN to the spinal cord are directed to the intermediolateral (IML) cell column and the adjacent dorso-lateral funiculus regions of the thoracic and upper lumbar segmental levels of the spinal cord, where the sympathetic preganglionic motor neurons are located (Hosoya et al., 1991; Luiten et al., 1985; Motawei et al., 1999; Ranson et al., 1998; Sawchenko and Swanson, 1982; Strack et al., 1989a). Of particular interest for this thesis are the PVN neurons projecting directly to the IML. The density of axonal terminals in the spinal cord arising from axons whose cell bodies are within the parvocellular PVN, is the greatest at three regions of the spinal cord: segments T₁ – T₃, T₉ – T₁₁, and T₁₃ – L₂ (Swanson and Sawchenko, 1980). Injections into these regions of the spinal cord with conventional monosynaptic neuroanatomical tracers that travel retrogradely to the cell body clearly highlight neurons in the PVN that project to the IML.

More recently, transsynaptic viral tracers have been introduced to study anatomical pathways emanating from the PVN. These viral tracers, travel across synapses and thereby are capable of identifying poly-synaptic pathways. These tracers have been used to show connections from the PVN to specific organs. For example, injections of transsynaptically transported viruses into the kidney have demonstrated that the PVN indeed projects directly onto the sympathetic pre-ganglionic motor neurons that mediate the sympathetic nerve activity to the kidney (Huang and Weiss, 1999; Schramm et al., 1993; Weiss et al., 2001).
Figure 1.4

Main efferent projections from the PVN. AP, area postrema; Ap and Pp, anterior and posterior pituitary; DX, dorsal nucleus of the vagus nerve; IML, intermediolateral column; ME, median eminence; MZ, marginal zone; OVLT, organum vasculosum laminae terminalis; LC, locus coeruleus; NTS, nucleus of the solitary tract; PAG, periaqueductal gray matter; PB, parabrachial nucleus; PPN, ponto-peduncular nucleus; PVN, paraventricular nucleus; SFO, subfornical organ; VLM, ventro-lateral medulla; X, vagal nerve (modified from Liposits, 1993).
The work described in this thesis will provide insight into the role of PVN neurons projecting to the IML and investigate whether such inputs may play an important role in the cardiovascular changes induced by elevations in body temperature.

1.1.3.4 PVN connections to the RVLM

The projections of the PVN to medullary centres may also play an important role in the regulation of the cardiovascular system and sympathetic nerve activity (Hardy, 2001). The PVN projects directly to the pressor region of the RVLM, a critical region in the tonic generation of sympathetic nerve activity (Dampney, 1994; Yardley et al., 1989). Electrical or chemical activation of the RVLM dramatically increases blood pressure and sympathetic nerve activity, whilst destruction of the RVLM causes a profound fall in blood pressure and sympathetic nerve activity (Dampney, 1994; Dampney et al., 2000; Guertzenstein and Silver, 1974; McAllen, 1986). Thus, it is believed that the RVLM is crucial for the tonic nature of resting sympathetic vasomotor tone (Dampney et al., 2000; Hardy, 2001; Kiely and Gordon, 1994; Schreihoffer et al., 2000). Hence any inputs into the RVLM are, therefore, likely to be very important in cardiovascular regulation. Indeed, chemical and electrical stimulation of the PVN activates the bulbo-spinal vasomotor neurons found in the RVLM (Coote et al., 1998; Dampney et al., 1982; Dampney et al., 2000; Dampney et al., 2003; Yang and Coote, 1998). Thus, this pathway is likely to contribute to the influence of the PVN on sympathetic nerve activity.
The PVN also contains neurons that not only project directly to the RVLM, but also collateralise to the sympathetic pre-ganglionic neurons in the IML of the spinal cord (Pyner and Coote, 2000; Shafton et al., 1998). Thus, this pathway emanating from the PVN may have an important influence on sympathetic nerve activity by influencing the RVLM pre-motor neurons, as well as directly influencing sympathetic pre-ganglionic motor neurons.

Since the RVLM plays a critical role in the regulation of sympathetic nerve activity, inputs from the PVN to the RVLM suggest that the PVN can influence vasomotor tone by affecting the RVLM pre-motor neurons. Is it possible that this pathway contributes to the central pathway mediating reflex cardiovascular responses to disturbances in body temperature? One of the goals of the present work tests the hypothesis that PVN neurons projecting to the RVLM form part of the neural circuitry activated in response to an elevation in body temperature.

1.1.4 Role of PVN in integration of cardiovascular reflexes and sympathetic outflow.

The emergence of the first study to suggest that the hypothalamus was capable of influencing the sympathetic nervous activity occurred in the early 1900s (Karplus and Kreidel, 1909). In the many reports that followed the examination of the hypothalamic areas either did not include the PVN, or it is unclear from the anatomical descriptions whether or not the PVN was stimulated. Thus it was not until the 1960s to 1970s, following observations of a short-latency cardiovascular response following electrical
stimulation of the hypothalamus (Enoch and Kerr, 1967) or anatomical work highlighting hypothalamic to spinal projections (Kuypers and Maisky, 1975; Sawchenko and Swanson, 1982; Strack et al., 1989a) that stimulated great interest in the cardiovascular effects of the hypothalamic PVN.

The first comprehensive study that examined the cardiovascular responses obtained from the electrical stimulation of the PVN was performed in anesthetized cat by Ciriello and Calaresu, where they observed the occurrence of pressor responses (Ciriello and Calaresu, 1980). Likewise, several other studies also reported similar findings, utilizing the anesthetized rat preparation and high intensity electrical stimulation (Coote et al., 1998; Kannan et al., 1987; Kannan et al., 1989; Porter and Brody, 1985). Low intensity electrical stimulation, on the other hand, appeared to elicit predominately depressor responses (Kannan et al., 1989; Yamashita et al., 1987). Therefore, both pressor and depressor responses have been observed following electrical stimulation of the paraventricular nucleus (Kannan et al., 1989; Porter and Brody, 1985; Yamashita et al., 1987).

A major disadvantage of electrical stimulation is that the stimulus activates fibers of passage that pass through the stimulated area, in addition to neuronal cell bodies (Goodchild et al., 1982). However, this problem may be overcome by utilizing chemical stimulation, which is an effective means of selectively stimulating cell bodies rather than fibres of passage (Goodchild et al., 1982). Studies utilizing the excitatory amino acid glutamate, or D, L-homocysteic acid, have reported various responses in blood pressure.
For example, in anesthetized rats micro-injection of glutamate has been reported to decrease arterial pressure (Kannan et al., 1988; Katafuchi et al., 1988; Yamashita et al., 1987). But other studies have found very little change in arterial pressure (Darlington et al., 1989), whilst others have found increases in blood pressure (Malpas and Coote, 1994). Furthermore, in another report, both increases and decreases in blood pressure and renal sympathetic nerve activities have been observed (Gelsema et al., 1989).

Similarly, stimulation of the PVN with D,L-homocysteic acid in anaesthetised rats have been reported to increase the level of renal sympathetic nerve activity (Malpas and Coote, 1994). However, in anaesthetised rabbits, PVN microinjection of D,L-homocysteic acid decreases renal sympathetic nerve discharge but increases splanchnic and adrenal nerve activity (Deering and Coote, 2000).

To some degree, the presence of anaesthesia contributes to the variable responses observed. Indeed, it has been clearly shown that these cardiovascular responses observed in the conscious animal can be reversed in the presence of anaesthesia (Baum et al., 1985; Kannan et al., 1989; Matsukawa and Ninomiya, 1989; Matsukawa et al., 1993; Vlahakos et al., 1985). Additionally, the sympathetic nerve activity responses observed after paraventricular nucleus stimulation are not generalized, for example, glutamate injected into the PVN increases splenic (Katafuchi et al., 1993), adrenal (Katafuchi et al., 1988) and interscapular brown adipose tissue (Yoshimatsu et al., 1993), but decreases renal sympathetic nerve activity in anesthetized rats (Katafuchi et al., 1988). Non-generalized responses in sympathetic nerve activity have also been observed in the anesthetized rabbit.
where Deering J and Coote JH demonstrated that they could inhibit renal sympathetic nerve activity and elicit an increase splanchnic activity upon chemical stimulation of PVN neurones (Deering and Coote, 2000). Furthermore, the precise injection site may also be important in the observed response. Thus, the findings strongly indicate that the PVN may have an important role in influencing the regulation of sympathetic nerve activity. The precise nature of the response, however, may vary with a number of factors which include the presence of anaesthesia, species and the area of the PVN stimulated.

The effect of the PVN on sympathetic nerve activity is under tonic inhibitory GABAergic influence, as evidenced by reports in which bicuculline has been used. Bicuculline, a GABAₐ receptor antagonist elicits blood pressure responses and marked changes in sympathetic nerve activity (Chen and Toney, 2003; Kenney et al., 2001b; Kenney et al., 2003; LaGrange et al., 2003; Martin et al., 1991; Martin and Haywood, 1993; Reynolds et al., 1996; Zhang and Patel, 1998). The projections from the PVN to the spinal cord and to the RVLM are likely to be the candidates that contribute to these responses (Tagawa and Dampney, 1999). Furthermore, the responses mediated via the RVLM appear to involve angiotensin II within the RVLM shown by the studies of Tagawa and Dampney (Tagawa and Dampney, 1999) who showed that the pressor and sympathoexcitatory responses were attenuated by 40-50% after microinjection of the specific AT(1) receptor antagonist losartan into the RVLM. A tonic glutamatergic input into the RVLM may also arise from the PVN (Dampney et al., 2003).
The studies involving the stimulation of the PVN have shown that the cardiovascular responses are complex and can be influenced by factors such as actual site of stimulation within the PVN, the species and also the presence of anaesthesia. Despite the variable nature of the responses reported, it is clear that the PVN can influence sympathetic nerve activity and other cardiovascular parameters and hence influence the cardiovascular system. A physiological role for the PVN in mediating the cardiovascular responses, especially the sympathetic neural components, to physiological stimuli requires much more work. However, the evidence for a key role of the PVN in the reflex nerve responses induced by disturbances in blood volume is particularly strong. Lesions of the PVN with ibotenic acid markedly attenuated the reflex renal vasodilation induced by volume expansion with rats (Lovick et al., 1993). Similarly, Haselton and colleagues have shown that the lesioning of the PVN with kainic acid can attenuate the normal renal sympatho-inhibition that is produced in response to acute volume expansion in the anaesthetised rat (Haselton et al., 1994). Further support of the PVN mediating the sympathetic neural components of cardiovascular reflexes elicited by disturbances in blood volume stems from work by Ng CW et al and colleagues who demonstrated that acute inhibition of the PVN with muscimol abolished the renal sympathetic nerve activity responses induced by volume expansion in conscious rabbits (Ng et al., 2004).

The preceding sections have highlighted that the PVN has the anatomical framework to mediate changes in sympathetic nerve activity. More recent studies have highlighted a physiological role of the PVN in the reflex sympathetic nerve responses elicited by disturbances in body fluid homeostasis (Haselton et al., 1994; Lovick et al.,
1993; Ng et al., 2004). Whilst the role of the PVN in body fluid homeostasis has been extensively studied, the role of the PVN in the cardiovascular responses that are elicited by other physiological stimuli remain poorly understood. A disturbance in body temperature is such a stimulus.
1.2 TEMPERATURE REGULATION AND THE PVN

Following a temperature challenge, homeothermic animals elicit diverse physiological and behavioural responses which are aimed ultimately at maintaining core body temperature within narrow limits (Kanosue et al., 1994a; Kanosue et al., 1994b; Kazuyuki et al., 1998). Both behavioural and autonomic responses elicited by exposure to cold or warm environments are primarily controlled by the central nervous system.

Behavioural thermoregulation is the act of finding or establishing an appropriate thermal environment and in humans it involves behaviours that include wearing appropriate clothing and seeking an appropriate environment e.g. finding a cool area when one is hot. Behavioural thermoregulation is predominantly involved in long term temperature regulation (Cabanac and Dib, 1983; Cabanac, 1996). The anatomical loci in the brain that are involved in behavioural thermoregulation are not well characterized. Interestingly, destruction of the preoptic area does not impair behavioural thermoregulation (Satinoff and Rutstein, 1970), even though the pre-optic area functions as a key thermo-sensitive site as shown by studies in which warming or cooling the pre-optic area appropriately alters behavioural thermoregulation. These findings suggest the existence of a complex organisational network (Carlisle and Ingram, 1973; Refinetti and Carlisle, 1986).
The autonomic thermoregulatory processes, on the other hand, have been more extensively studied (Kanosue et al., 2001; Nagashima et al., 2000; Nagashima, 2006; Tanaka et al., 2001). Numerous studies to date have shown that information from receptors in the skin and body core travels to the central nervous system, which integrates the information and sends efferent signals to effector organs such as brown adipose tissue, salivary glands and vessels of the skin and thereby eliciting the appropriate thermo-effector responses to allow for the rapid adjustment of body temperature (Boulant and Gonzalez, 1977; Boulant and Dean, 1986; Chen et al., 1998; Gordon and Heath, 1986; Hubschle et al., 2001; Morrison, 1999; Morrison, 2001a; Morrison, 2001b; Nakamura and Morrison, 2007; Oldfield et al., 2002; Simon et al., 1986; Simon, 1999).

In the cold, the appropriate thermo-effector responses involve mechanisms that reduce heat loss and that increase heat production. For example, regulatory adjustments to increase heat production include non-shivering thermogenesis that involves changes in the metabolism of brown adipose tissue in rodents and also in newborns (Freeman and Wellman, 1987; Holt et al., 1987; Morrison, 1999; Morrison et al., 2000; Morrison, 2001c; Perkins et al., 1981; Thornhill and Halvorson, 1994). Importantly, the act of shivering; an involuntary tremor of skeletal muscles, is the main effector response of homeothermic animals following exposure to the cold. The posterior hypothalamus plays an important role in this response (Thornhill and Halvorson, 1994).

Exposure to a hot temperature challenge elicits thermo-effector responses that are mediated in part by the autonomic nervous system to assist in the promotion of heat loss.
Such responses include sweating in humans (evaporative heat loss), panting in dogs, increased salivary secretion in rodents, an increase in heart rate, increased respiration rate, skin vasodilatation and visceral vasoconstriction (Kanosue et al., 1994a; Kazuyuki et al., 1998). The latter autonomic cardiovascular responses involve changes in sympathetic nerve activity and results in the redistribution of blood flow from the viscera to the skin.

These changes are mediated by the central nervous system (Kanosue et al., 1991; Kanosue et al., 1994a; Morrison, 2001c; Nagashima et al., 2000; Owens et al., 2002; Rathner et al., 2001; Scammell et al., 1993; Zhang et al., 1997b).

The essential role of the central nervous system in the regulation of body temperature has been recognized for many years. Insights into the means by which the central nervous system controls and regulate body temperature stem from early physiological studies using thermal stimulation and intracerebral lesions that have indicated the importance of the hypothalamus as well as medullary structures in thermoregulation (Lipton, 1971a; Lipton, 1971b; Lipton et al., 1974; Szymusiak and Satinoff, 1982). Within the hypothalamus of homeothermic animals, the pre-optic and the anterior hypothalamic area serve as the main regulatory center (Boulant, 1981; Boulant and Silva, 1988; Boulant, 2000). This has been established by studies using local heating and cooling (Hensel, 1981; Kanosue et al., 1991; Kanosue et al., 1994a), electrophysiological recording (Dean and Boulant, 1989a; Dean and Boulant, 1989b; Hellon and Taylor, 1982; Jell and Gloor, 1972) and 2-deoxyglucose autoradiography (Morimoto and Murakami, 1985; Murakami and Morimoto, 1982) to show that the pre-optic and anterior hypothalamus contains neurons that change their activity in response to
temperature challenges. Furthermore, the pre-optic area is well known to play a key role in detecting local temperature (central thermo-receptors) as well as integrating temperature signals from all over the body (peripheral thermo-receptors) (Boulant and Dean, 1986; Kanosue et al., 2001; Nagashima et al., 2000). Even though the pre-optic area is known to contain intrinsically thermosensitive neurons that may be either warm-sensitive or cold-sensitive (Hori, 1991; Nakayama, 1985), more recent studies suggest that both heat production and heat loss mechanisms are primarily controlled by signals arising mainly from the warm-sensitive neurons (Kanosue et al., 2001; Zhang et al., 1995).

In homeothermic animals, there are also several other brain regions that are likely to contribute to the central nervous system pathways that mediate the thermoregulatory responses. Studies using the marker of neuronal activation, Fos, or electrophysiological recordings have shown that several brain areas are activated following the elevation in body temperature (Bachtell et al., 2003; Boulant, 1981; Boulant, 1998; Bratincsak and Palkovits, 2004; Harikai et al., 2003; Hori et al., 1999; Kiyohara et al., 1995; Maruyama et al., 2003; McKitrick, 2000; Morimoto and Murakami, 1985; Murakami and Morimoto, 1982; Patronas et al., 1998; Scammell et al., 1993; Schmid and Pierau, 1993). Examples of these areas include the parabrachial nucleus, raphe nuclei, supraoptic nucleus, supramamillary nucleus, amygdala, the preoptic area, anterior hypothalamus and the PVN of the hypothalamus.
In contrast to the hot/warm environment, the specific means through which normothermia is attained following exposure to a cold environment particularly in rodents is maintained in part though metabolic activation of BAT i.e. non-shivering thermogenesis.

Non-shivering thermogenesis in brown adipose tissue (BAT) is a well-known heat source for the defense of the body temperature in the cold environments, especially in rodents (Cannon and Nedergaard, 2004), and the central mechanisms controlling BAT thermogenesis have been extensively studied (Morrison, 2004).

BAT thermogenesis is controlled by the sympathetic nervous system, and anatomical observations from transneuronal tracing after inoculation of pseudorabies virus (PRV) into BAT suggest that BAT-controlling neurons in autonomically related brain regions are organised in a hierarchical network in which more rostral regions, such as POA, influence the activity of increasingly caudal structures such as dorsal medial hypothalamic nucleus (DMH), periaqueductal gray (PAG), and rostral medullary raphe nuclei (Bamshad et al., 1999; Cano et al., 2003; Cao et al., 2004; Nakamura et al., 2004; Oldfield et al., 2002; Yoshida et al., 2003).

Historically, the DMH has been implicated in feeding and metabolic regulation associated with feeding behaviour (Bernardis and Bellinger, 1987), a phenomenon which is closely correlated to thermoregulation (Hamilton and Ciaccia, 1971; Rothwell and Stock, 1983). However, it was Zaretskaia and colleagues that provided the first clear
evidence that activation of the DMH could dramatically alter body temperature (Zaretskaia et al., 2002). Physiologically, they demonstrated that, disinhibition of neurons within the DMH with the GABA_A receptor antagonist, bicuculline, augments BAT SNA contributing to the evoked increases in activation of BAT thermogenesis and tachycardia (Zaretskaia et al., 2002). This work has been supported by recent work by Cao and colleagues (Cao and Morrison, 2006). In urethane anaesthetised rats, similar microinjections evoked rapid and dramatic increases in BAT temperature that preceded and exceeded the responses in core temperature (Zaretskaia et al., 2002).

Cao and colleagues have confirmed and extended these findings by characterizing the thermogenic response to disinhibition of DMH and establishing the role of the RPa (rostral) in the changes in anaesthetized preparations (Cao et al., 2004). They demonstrated that microinjection of bicuculline (a GABA antagonist) (60pmol) into the DMH can dramatically elevates BAT, renal and cardiac SNA, as well as HR and BP and that these increases were associated with marked increases in BAT temperature (reflecting the increased metabolic activity in BAT). However, following microinjection of muscimol into the RPa, these DMH-evoked increases in BAT temperature and BAT SNA were greatly attenuated or abolished (implying that activation of sympathetic premotor neurons in the RPa mediate these changes).

More recently, Cao and Morrison have provided an insight into the nature of excitatory signalling through which disinhibition of DMH activates thermogenic mechanisms in the RPa. They explored the effect of microinjection of glutamate receptor
antagonists into the RPa after they injected bicuculline into the DMH (Cao and Morrison, 2006). Microinjection of kynurenate (non-selective antagonist for inotropic glutamate receptors), rapidly and completely abolished the bicuculline-induced increases in SNA to BAT and attenuated the accompanying increases in BAT temperature. There was no effect on HR, BP and renal SNA (Cao and Morrison, 2006). Interestingly, microinjections of AP5 or CNQX (antagonists with capacity for relative selectivity for the NMDA subtype of glutamate receptors) were also shown to elicit similar effects to those of kynurenate. The authors suggested that the DMH-induced sympathetic activation of BAT is mediated at least, in part, through stimulation of ionotropic glutamate receptors in the RPa (Cao and Morrison, 2006). Thus at present, there is considerable evidence to suggest that the DMH and RPa play important roles in the activation of BAT thermogenesis following exposure to cold (Cao et al., 2004; Chen et al., 2002; Madden and Morrison, 2003; Morrison, 1999; Nakamura et al., 2004; Rathner et al., 2001; Zaretskaia et al., 2002).

More recently, there has also been emerging interest in the role of the DMH and the RPa in the thermogenic response and cardiovascular components of prostaglandin E2–evoked febrile response (Morrison, 2003; Nakamura et al., 2005). Recent observations suggest that ventromedial medullary neurons, including those in the RPa, are an essential site of synaptic integration in the central pathways mediating thermogenic and cardiovascular components of the febrile response in the rodent model (Madden and Morrison, 2003; Morrison, 2003; Nakamura et al., 2002). Inhibition of neurons within the DMH attenuates the rise in body core temperature and heart rate evoked by
microinjection of PGE$_2$ into the medial preoptic area (Zaretskaia et al., 2003). Furthermore, neurons in the raphe regions that were activated by cold exposure were also found to be activated by central administration of PGE$_2$ (Morrison, 1999; Nakamura et al., 2002) and suppression of these regions abolished sympathetic BAT thermogenesis and fever triggered by central PGE$_2$ administration (Morrison, 2003; Nakamura et al., 2002).

In contrast to the regions mentioned above (e.g. the preoptic and the anterior hypothalamic areas), the role of the PVN in thermoregulation is relatively poorly explored. This is surprising given the studies indicating that the PVN contains (i) thermosensitive neurons (Inenaga et al., 1987) and (ii) neurons that project to the spinal cord and influence sympathetic nerve activity to important thermoregulatory effector organs such as the brown adipose tissue and the vasculature of the rat tail, salivary gland as well as kidney and gut (Chen et al., 1998; Gordon and Heath, 1986; Hubschle et al., 2001; Morrison, 1999; Oldfield et al., 2002). Furthermore, increases in body temperature activates neurons within the PVN (Bachtell et al., 2003; Bratinsak and Palkovits, 2004; Harikai et al., 2003; Kiyohara et al., 1995; McKitrick, 2000; Patronas et al., 1998). Additionally, the PVN receives inputs from the preoptic area suggesting a strong interplay between the hypothalamus and the preoptic area during temperature regulation. Thus, circumstantial evidence suggests a role for the PVN however the neuronal pathways utilized, the potential neurochemical mediators and the functional role of the PVN in the responses elicited by temperature challenge have not been investigated to date.
The existence of a myriad of neurochemicals present within the PVN is a reflection of the integrative role of this nucleus in the maintenance of homeostasis (Cechetto and Saper, 1988; Liposits, 1993; Swanson and Sawchenko, 1983a). In temperature regulation, one important neurotransmitter that is emerging as a major focus of research recently is nitric oxide (Krukoff, 1999; Schmid et al., 1998; Simon, 1998; Simon, 1999).
1.3 NITRIC OXIDE

The discovery of nitric oxide (NO) as a neurotransmitter has radically altered our perception of synaptic transmission. Being a labile, free radical gas (though in most biological situations NO is in solution), NO is not stored in synaptic vesicles like other neurotransmitters. Instead NO is synthesized from its precursor L-arginine on demand by the enzyme nitric oxide synthase (NOS). NO, in turn, exerts most of its physiological actions by activating a soluble guanylate cyclase (GC) to catalyze the production of the second messenger, cyclic guanosine monophosphate (cGMP), in target cells (Arnold et al., 1977; Bredt and Snyder, 1989; Garthwaite, 1991).

NOS exists in three different isoforms namely neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) and each is present in the brain (Bredt et al., 1990; Forstermann et al., 1995; Förstermann et al., 1994; Förstermann and Kleinert, 1995; Förstermann et al., 1995; Vincent, 1994; Yang and Krukoff, 2000). Both eNOS and nNOS are Ca\(^{2+}\) and calmodulin dependent whilst iNOS is independent of Ca\(^{2+}\) and calmodulin. NOS isoforms require flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme and tetrahydrobiopterin as cofactors and display nicotinamide adenine dinucleotide phosphate (NADPH) -diaphorase activity (Bredt et al., 1991; Griffith and Stuehr, 1995; Hope et al., 1991; Nathan, 1992; Vincent and Hope, 1992).
It is now well recognized that NO is a non-conventional neurotransmitter in the central nervous system (Krukoff, 1998; Krukoff, 1999; Patel et al., 2001; Stern, 2004). In the central nervous system, the most abundant form of NOS is nNOS. Due to its gaseous nature, upon production, NO is also able to diffuse freely across the membranes of neurons. This means it can have widespread actions and act either pre-synaptically or post-synaptically as an intracellular messenger.

NO may play a diverse role in many physiological functions such as blood pressure regulation, food and water intake and also vasopressin secretion (Garthwaite, 1991; Shih et al., 2003; Snyder and Bredt, 1992). NO can also mediate smooth muscle relaxation and is released in both normal and pathological states (Cawley et al., 2007; Golder et al., 2007). More recently, the putative role of NO in temperature regulation has also received attention due to its influence on brain pathways (Gerstberger, 1999; Schmid et al., 1998; Simon, 1998).

1.3.1 NO and temperature regulation.

The role of NO in thermoregulation is emerging as a major focus of investigation (Gerstberger, 1999; Schmid et al., 1998; Simon, 1998). There is now a growing body of evidence accumulating from studies in different animal species, in which various NO donors or NOS inhibitors have been applied by different routes to study the responses of thermoregulatory effectors and core temperature and the subsequent coordinated changes under conditions of normothermia and heat stress. The effects of NO on temperature...
regulation clearly involve the central nervous system since doses given centrally that alter body temperature have no effect given peripherally (Mathai et al., 2004).

Most studies suggest NO decreases body temperature. For example, de Luca and colleagues investigated the effect of impaired brain production of NO in conscious rats following i.c.v. injection of L-NAME and observed an increase in sympathetic nerve activity to brown adipose tissue, an important thermoregulatory effector in rats (De Luca et al., 1995). Further studies by Monda and colleagues also revealed that i.c.v. injection of L-arginine or nitroprusside reduces the increases in core body temperature induced by prostaglandins (Monda et al., 1995). In another study performed on conscious rabbits by Mathai and colleagues, blockade of endogenous NO synthesis induced a sustained rise in body temperature primarily mediated by modulating the rate of respiratory heat dissipation (Mathai et al., 1997). However, NO may act through various other thermoregulatory effector mechanisms to lower heat in different species as shown by the observation that inhibition of NO production reduces saliva production in rats during warming (Damas, 1994). An elevation in body temperature induces enhanced secretion of saliva, which is spread on the fur as a means of heat defense in rats (Damas, 1994; Yanase et al., 1991). Furthermore, inhibition of NO production have been shown to augment the febrile responses elicited by endotoxin and lipopolysaccharide (Gourine, 1995; Steiner et al., 2002). In addition, interleukin-1β, a cytokine mediating endotoxin responses, is reported to cause an increase in NO production within the hypothalamus (Brunetti et al., 1996). In support of those findings, it has also been observed that the
mRNA for NOS within the hypothalamus is elevated by the presence of endotoxin (Lee et al., 1995).

The studies to date, therefore, support the consensus view that NO within the central nervous system is critical in the thermoregulatory pathways mediating heat dissipation (Eriksson et al., 1997; Garthwaite and Boulton, 1995; Gerstberger, 1999; Gourine, 1995; Mathai et al., 2004; Schmid et al., 1998; Simon, 1998).

1.3.2 Distribution of NO in the central nervous system

As with any neurotransmitters, insight into its function can come from information about its localization. In the central nervous system, the most abundant form of NOS is nNOS (Bhat et al., 1996; Bredt et al., 1990; Miyagawa et al., 1994; Yang and Krukoff, 2000). nNOS neurons are located throughout the brain in areas such as the cerebral cortex, hippocampus, amgydala, forebrain, corpus striatum, midbrain and the brainstem (Forstermann et al., 1995; Förstermann et al., 1994; Harada et al., 1999; Kantzides and Badoer, 2005; Vincent and Kimura, 1992). These nNOS neurons are scattered in no obvious pattern and display morphologic properties of medium-to-large aspiny neurons.

In the brainstem, NO-producing neurons are located in the periaqueductal gray (PAG), parabrachial nucleus, raphe nuclei, NTS, intermediate ventrolateral medulla, CVLM and RVLM (Chang et al., 2003; Dun et al., 1994; González-Hernández et al.,
1992; Johnson and Ma, 1993; Kantzides and Badoer, 2005; Lin et al., 1998; Lu et al., 1994; Ohta et al., 1993; Onstott et al., 1993; Vincent and Kimura, 1992; Wotherspoon et al., 1994). NO-producing neurons are also present in the neurons in the IML of the spinal cord and these are also cholinergic sympathetic pre-ganglionic neurons (Anderson, 1992; Blottner and Baumgarten, 1992; Bredt et al., 1991; Dun et al., 1992; Hope et al., 1991; Saito et al., 1994; Spike et al., 1993).

Within the forebrain, NOS has been localized to many discrete areas such as the medial pre-optic area, diagonal band of Broca, medial septum and also the lateral septum and the hypothalamus (Bhat et al., 1995; Doutrelant-Viltart and Poulain, 1996; Pasqualotto and Vincent, 1991; Sugaya and McKinney, 1994; Vincent and Kimura, 1992).

In the hypothalamus, perhaps one of the most striking accumulations of nNOS-positive neurons are in the PVN. In the PVN, both the magnocellular and parvocellular components contain neurons that are nNOS or NADPH-d positive (a marker of nitrergic neurons) (Arévalo et al., 1992; Cham et al., 2006; Crespo et al., 1998; Hatakeyama et al., 1996; Kantzides and Badoer, 2005). Some of these areas containing nNOS are also known to have an influential effect on sympathetic nerve activity and hence may play a profound role in the cardiovascular responses during cardiovascular regulation. Of particular interest in this thesis is the presence of nNOS within the PVN of the hypothalamus.
1.3.3 NO in the PVN

As indicated above, the PVN contains the highest concentration of nitrergic neurons. The presence of NOS or the NOS marker, NADPH-d has been shown in the PVN (Arévalo et al., 1992; Bredt et al., 1991; Calka and Block, 1993; Hatakeyama et al., 1996; Miyagawa et al., 1994; Nylen et al., 2001; Vacher et al., 2003; Vincent and Kimura, 1992). Additionally, the precursor of NO, L-arginine, and the co-products of NO production, citrulline and cGMP have been shown in the PVN (Pasqualotto and Vincent, 1991; Vacher et al., 2003).

The presence of NOS in the PVN strongly suggests that NO acts as a neurochemical transmitter within this nucleus. Indeed, electrophysiological studies provide direct evidence that NO can influence neurons in the PVN. In general, studies performed to date suggest that NO has an inhibitory role on the autonomic-related PVN neurons. For example, application of NO donors or L-arginine into the PVN have been shown to enhance the spontaneous miniature inhibitory current that was detected in spinally-projecting PVN neurons (Li et al., 2002a). Furthermore, administration of NO donors inhibited autonomic-related PVN neurons that were spontaneously active (Li et al., 2003). Conversely, the administration of a NOS inhibitor has been shown to increase the basal excitatory firing rate of these autonomic-related PVN neurons (Li et al., 2003). It should also be noted that the application of L-arginine has been reported to depolarize parvocellular neurons within the PVN (Bains and Ferguson, 1997), suggesting a possibility that NO may have an excitatory role on autonomic-related neurons.
Nevertheless, these studies indicate that endogenous NO within the PVN may affect neurons that ultimately could influence the regulation of sympathetic nerve activity.

1.3.4 The role of NO within the PVN in the regulation of cardiovascular responses and temperature regulation

Accumulating evidence suggests that NO, in addition to its actions in the periphery, can play an important role in the central nervous system in the regulation of body fluids, the cardiovascular system and body temperature. The influence of NO on body fluid homeostasis and on the cardiovascular system involves NO acting within the PVN, and the changes in the sympathetic nerve activity contribute to its actions. In anaesthetised animals, administration of NO donors and precursors into the PVN elicits decreases in blood pressure, heart rate and RSNA (Horn et al., 1994; Zhang et al., 1997a; Zhang and Patel, 1998). For example, Zhang et al. demonstrated that following microinjection of the NO donor, sodium nitroprusside (SNP), into the PVN, a significant decrease in renal sympathetic nerve activity occurred (Zhang et al., 1997a). Conversely, the application of NOS inhibitors such as L-NMMA or L-NAME, into the PVN reverses these effects i.e. it produces increases in renal sympathetic nerve activity (Li et al., 2001; Li et al., 2002b; Zhang et al., 1997a; Zhang and Patel, 1998). These data suggests that NO in the PVN has an inhibitory effect on renal sympathetic nerve activity.

The cardiovascular and sympathetic nerve activity effects of NO within the PVN may be mediated by the neurons that project to the RVLM or the spinal cord. These
neurons are influenced by NO as shown by electrophysiological studies. Following the administration of the nNOS inhibitor, 7-nitroindazole, or a NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), the firing activity of PVN neurons that project to the RVLM were potentiated (Li et al., 2003).

Furthermore, NO can be produced in a subpopulation of those PVN neurons projecting to the spinal cord and/or RVLM since those neurons contain nNOS (Hatakeyama et al., 1996; Li et al., 2002a; Li et al., 2003; Nylen et al., 2001). Some of these neurons are likely to influence renal sympathetic nerve activity directly since microinjection of the trans-synaptic neuroanatomical tracer, pseudorabies virus, into the kidney subsequently labels neurons in the PVN that contain nNOS (Weiss et al., 2001). Thus, the pathways emanating from the PVN and projecting to the RVLM and/or the spinal cord are likely to influence renal sympathetic nerve activity, and these pathways can be influenced by the effects of NO.

It is possible that nitrergic projections from the PVN that project to the spinal cord and/or the RVLM contribute to the cardiovascular responses during an elevation in body temperature. Given the role of NO in cardiovascular control and temperature regulation, one of the aims of this study was to gain an insight into the involvement of nitrergic neurons in the PVN in temperature homeostasis by determining whether they were activated by increases in body temperature and whether some of the nitrergic neurons also projected to the RVLM or the spinal cord.
1.4 FOS, A MARKER OF INCREASED NEURONAL ACTIVITY.

More than two decades ago, the first reports appeared suggesting that the protein product of the c-fos proto-oncogene could be used to detect activated neurons (Bullitt, 1989; Bullitt, 1990; Dragunow et al., 1989; Dragunow and Faull, 1989; Hunt et al., 1987; Sagar et al., 1988). Since then, Fos immuno-histochemistry has become widely accepted and used as a marker for acute neuronal activation in the central nervous system. This technique is therefore a useful tool to localize neuronal cells and cell groups involved in thermoregulation following an acute exposure of the animals to a hot environment.

1.4.1 The fos-oncogene

The c-fos proto-oncogene encodes a DNA-binding protein (Fos) that functions as a component of the mammalian transcription factor, activator protein-1 (AP-1) (Curran and Franza, 1988; Franza BR et al., 1988; Rauscher et al., 1988). AP-1 corresponds to several proteins related to Fos that form homodimeric and heterodimeric complexes through a leucine zipper structure. These proteins regulate the expression of many genes containing AP-1 DNA sequence elements.

Proto-oncogenes are normal cellular genes that play a critical role in the regulation of cell growth and differentiation (Dragunow et al., 1987; Dragunow and Faull, 1989; Sukhatme et al., 1988). They are also responsible for encoding proteins that
function as extracellular growth factors, cell surface receptors, G-proteins, protein kinases, hormone receptors, and transcription factors (Reddy and Rao, 1988).

### 1.4.2 Regulation of c-fos

Three families of signal-regulated transcription factors exist. The first type is constitutively expressed within the cell and its activity is regulated by post-translation modification, *i.e.*, phosphorylation, hence they are known as *post-translationally activated transcription factors*. The second type is the *ligand-activated transcriptional factor* and is an intracellular receptor for steroid hormones. The binding of the hormone to the receptor protein induces a conformational change that activates the receptor-ligand complex, which is then able to directly interact with DNA to regulate gene expression. The third type of transcription factor protein regulates gene expression indirectly in that these molecules can only interact with DNA once they themselves are transcribed and translated. Hence, they are called *transcriptionally activated transcription factors* (Curran and Morga, 1995; Dragunow et al., 1987; Dragunow and Faull, 1989).

The genes coding for transcriptionally activated transcription factor proteins are called immediate early genes (IEG) and include *c-fos, c-jun, fra-1* and several others. When a cell is stimulated, the first wave of gene transcription involves IEG activation. Once translated, the products of these genes (IEGPs) re-enter the nucleus and activate other late-response genes, resulting in a delayed secondary wave of gene activity. Thus,
IEGs are seen as a mechanism by which short-term cellular activation can have long-term influences. In the present study, the detection of Fos protein was used to indicate increased neuronal activity over a short term.

1.4.3 Methodological considerations

In the past, an understanding of the functional organisation of the central pathways that sub serve cardiovascular responses to homeostatic challenges and other stressors was derived almost entirely on studies performed in anaesthetised animals. More recently, however, numerous studies have utilised the method of the expression of c-fos gene, to identify populations of central neurons that are activated by similar challenges in conscious animals.

Fos expression can be increased by a variety of treatments in neurons. For example, seizure activity (chemically and electrically induced), kindling, brain injury (ischaemic or mechanical), sensory stimulation (noxious, visual, olfactory, somatosensory), stress, learning, and the induction of long-term potentiation (LTP) result in increased expression of IEGs within the nervous system (Dampney and Horiuchi, 2003; Hughes and Dragunow, 1995). As mentioned earlier, activation of specific neurotransmitter receptors results in increased immediate-early gene expression within the central nervous system. The temporal profile of the immediate-early gene protein,
Fos, shows maximum induction within 1 hour of the commencement of the stimulus and falls to baseline within 4 hours (Badoer et al., 1992; Curran and Morga, 1995).

The \textit{c-fos} functional mapping technique is widely utilised because it is relatively easy to carry out, using conventional immunohistochemical procedures to reveal Fos-positive neurons. However, only activated cell bodies express Fos, thus, neurons that are inhibited by upstream events will not express Fos. In addition, not all neurons are capable of expressing Fos (Dampney et al., 1995). Despite this, the \textit{c-fos} functional mapping technique is sensitive enough to label individual neurons. The combination of this technique in conjunction with retrograde labels was used extensively throughout this work (Badoer et al., 1993; McAllen et al., 1992; McKinley et al., 1992; Polson et al., 2002).
1.5 SUMMARY

In recent years, there has been a growing body of evidence demonstrating that the PVN is important in the cardiovascular responses to changes in body fluid homeostasis. Whilst the role of the PVN in modulating the sympathetic nervous system and cardiovascular response to body fluid homeostasis have been extensively studied, it is not known, however, whether the PVN is also important in the cardiovascular responses that are normally elicited by disturbances in body core temperature. Changes in body temperature elicit reflex cardiovascular effects which affect blood flow distribution resulting from altered sympathetic nerve activity to different vascular beds. Although it is well known that the PVN can influence the blood flow via the sympathetic nervous system, a role for the PVN in the sympathetic nerve responses elicited following a change in body temperature remains unknown. This is surprising given the circumstantial evidence suggesting that the PVN plays an important role in the regulation of body temperature.

The hypothalamic PVN is composed of functionally different subgroups of neurons, including the parvocellular neurons that project to important autonomic targets in the brainstem and spinal cord that are critical in cardiovascular regulation, such as the IML and RVLM. These projections are likely to mediate the effects of the PVN on sympathetic nerve activity and hence may contribute to the cardiovascular changes induced by elevations in body temperature. However, this has not been examined to date.
1.6 AIMS OF PRESENT STUDY

The present thesis examines the role of the PVN in the cardiovascular responses elicited during exposure to a hot environment. Firstly, the role of the PVN was determined by investigating whether elevation of core body temperature activated “autonomic” pathways emanating from the PVN such as those projecting to the IML of the spinal cord and the RVLM. Secondly, the present thesis also establishes whether hypothalamic PVN neurons activated by acute exposure to heat were also capable of producing NO. Subsequently, work in this thesis also looked at whether those neurons included the sub-population of neurons in the PVN that project to the IML of the spinal cord or to the RVLM. Finally, the physiological role of the PVN in response to heat exposure was examined by inhibiting PVN neuronal function with the GABA (A) receptor agonist, muscimol.

1.7 THESIS OUTLINE

The work performed in this thesis is outlined as follows. Chapter 2 provides a detailed description of the methods, techniques and preparations used. The results are presented in Chapters 3 - 6. In Chapter 3, the first aim of this study was to determine whether spinally projecting neurons in the PVN were activated by exposure to a hot environment in the conscious rat. Additionally, it was also determined whether neurons in the hypothalamic PVN that were activated by acute heat exposure were also capable of producing NO and whether those neurons included the sub-population of neurons in the PVN that project to the IML of the spinal cord. In Chapter 4, it was determined whether
neurons in the brainstem were activated by acute hyperthermia resulting from exposure to a hot environment were also capable of producing NO. Furthermore, the study also determined whether exposure to a hot environment activated brainstem neurons that project to the spinal cord. In Chapter 5, the role of the PVN during heat exposure was further investigated by determining whether RVLM - projecting neurons in the PVN were activated by placing conscious rats in a hot environment. In this chapter, the work presented also determined whether neurons in the PVN that project to the RVLM, and were activated by a hot environment, were also nitrergic. In Chapter 6, the aim was to determine the effect of inhibition of neuronal activity within the PVN on the renal blood flow response elicited by an increased body temperature.
CHAPTER 2.

MATERIALS AND METHODS
2.1 Manufacture of Instruments

2.1.1 Manufacture of blood vessel cannulae

Two different sizes of polyvinyl chloride (PVC) tubings were utilised in the manufacture of the cannulae. The smaller tube (catalogue number 530010 - internal diameter of 0.28 mm and outer diameter of 0.61 mm; Biocorp, VIC, Australia) was inserted approximately 20 mm into a pre-cut 20 cm length of the larger bore tubing (catalogue number 530055 - internal diameter 0.80 mm and outer diameter 1.20 mm, Biocorp, VIC, Australia) and the two were connected using Araldite® Epoxy Resin (Selleys Pty Ltd; NSW, Australia) glue. The glue was moulded into a ball to assist in tying the cannula in place following implantation. In addition, the smaller tube was trimmed at a 45° angle so that it extended a minimum of 30-40 mm past the ball of glue (Figure 2.1).

2.1.2 Manufacture of Glass Micropipettes

A P-97 Flaming/Brown micropipette puller (Sutter Instrument Company, CA, USA) was used to manufacture the glass micropipettes. These glass micropipettes (Accu-fill 90®, supplied by Clay Adams, Becton, Dickson and Co., NJ, USA) were used for the spinal, intra-medullary and PVN microinjections (Refer to Sections 2.4, 2.5 and also 2.14.2 respectively). The puller was programmed (Program details: Heat 740, Pull 40, Velocity 50, Time duration 110) to produce pipettes with an external diameter of
Figure 2.1 – Photo of a typical blood vessel cannula. The tube of smaller bore is inserted approximately 20 mm into the larger bore tubing and held fast by Araldite® Epoxy Resin glue. The glue was then moulded into a ball to assist in tying the cannula in place following implantation. In addition, the smaller PVC tube was trimmed at a 45° angle so that it extended a minimum of 30-40 mm past the ball of glue.
approximately 60 μm and an internal diameter of approximately 30 μm when the shaft was trimmed down to 12 mm in length.

2.2 Surgical preparation of animals

2.2.1 Animals and housing

Male SPF (specific pathogen free) Sprague-Dawley rats obtained from Monash University Animal Services (Victoria, Australia) were used in these studies. Animals that were used in the neuro-anatomical studies (spinal and RVLM injections) typically had a body weight ranging between 200-250 grams whilst those in the physiological blood flow experiments had body weights ranging between 300-350 grams. The animals were housed in a separate, temperature-controlled room on a 12:12 hour light/dark cycle (lights on at 7:00 A.M.); in the Animal Facility (RMIT University, Victoria, Australia), where rat chow and tap water were available ad libitum. The animals were held for a minimum period of one week and handled regularly prior to undergoing any experimental procedure. Following the first experimental procedure, the animals were subsequently kept in separate cages for recovery.

All experimental protocols used in this study were performed in accordance with the Prevention of Cruelty to Animals Act 1986 and conform to the guidelines set out by the National Health and Medical Research Council (Australia) and were approved by the RMIT University Animal Ethics committee. Every attempt was made to minimize animal suffering, discomfort and reduce the number of animals needed to obtain reliable results.
Animals were also handled on a daily basis prior to the experimental day to minimize stress.

2.2.2 Surgical preparation of animals used in neuroanatomical studies

For the studies in which the animals recovered from the surgery, the surgical procedures were performed under general anaesthesia [sodium pentobarbitone 60mg/kg intraperitoneal (i.p.) (Nembutal® 60 mg/mL, Boehringer Ingelheim, NSW, Australia), with top-ups, 20 mg/kg i.p. every 50 minutes]. For the acute studies, the surgical procedures were performed under general anaesthesia with Equithesin (sodium pentobarbitone (0.5 g): chloral hydrate (2.219 g) (per 100 ml) mixture and anaesthesia was subsequently maintained with urethane (1 – 1.4 g/kg intravenously (i.v.) initially, followed by supplemental doses of approximately 0.05g/kg as required). The depth of anesthesia was maintained to ensure the absence of corneal and pedal reflexes.

At the commencement of surgery (only for groups of animals intended to recover from the surgery), Buscopan Compositum® [0.3 ml/kg subcutaneous (s.c.), consisting of a mixture of hyoscine-N-butyl bromide (12.5 mg/kg) and dipyrene (0.1 mg/kg), Boehringer Ingelheim, NSW, Australia] was administered to prevent excessive salivation. At the completion of surgery buprenorphine HCl 15μg (Temgesic® 0.3 mg/ml, Reckitt and Coleman Pharmaceuticals, N.S.W., Australia) was routinely administered intraperitoneally for analgesia. The antibiotic oxytetracyclin 200 mg/kg (200 mg/ml
Terramycin®, Provet, Victoria, Australia) was given subcutaneously to prevent infection. Finally, at the completion of surgery, if there was further signs of wheezing, Buscopan® (0.015 ml/kg s.c.) was re-administered. All surgical procedures were performed under clean, aseptic conditions. All instruments used were sterilized using a sterilizer (Vauxhall Products, NSW, Australia). The area surrounding the surgical site was covered with cotton gauze sponge and the skin was swabbed clean with 70% alcohol swabs prior to incision.

2.3 Cannulation of femoral vessels

The cannulation of the femoral artery was performed to enable continuous monitoring of blood pressure, whilst the femoral vein was cannulated if required, to enable infusion of urethane to maintain anaesthesia during the experiments. Under general anaesthesia, the right groin region was shaved and swabbed with 70% alcohol before a 5-7 mm incision was made through the skin over the femoral neurovascular bundle. The subcutaneous fascia was cleared by blunt dissection to expose the underlying bundle, and the vessels separated by clearing the femoral sheath. A few drops of the local anaesthetic, lignocaine (Sigma Aldrich, NSW, Australia), were placed onto the vessel to be cannulated to avoid vasospasm. Two lengths of fine thread (silk 5-0, Deknatel, NY, USA) were passed under the vessel and pulled tight to elevate the vessel. Using very fine McPherson-Vannas 8cm scissors (World Precision Instruments Inc., FL, USA), a small cut, about one-third through the diameter of the vessel, was made in the vessel. Closed fine tweezers # 5 (World Precision Instruments Inc., FL, USA) were
inserted into the cut and expanded slightly to enable the cannula tip to be introduced into the vessel. The tweezers were then withdrawn and the cannula, filled with heparinised saline (50 U/mL) was threaded 30-40 mm along the vessel and secured around the ball of glue of the cannula (Refer to Figure 2.1).

In cases where catheterisation was required only for the duration of a particular surgical procedure (e.g., recording blood pressure to facilitate the injection of tracer into the RVLM), the cannula was removed by passing a third length of silk (silk 5-0, Deknatel, New York, USA) under the vessel, proximal to the other two. The cannula was then slowly withdrawn until its tip could be seen distal to the third tie, at which point the vessel was tied off and the cannula withdrawn.

2.4 Microinjections of retrogradely transported tracer into the spinal cord.

For this study the surgical procedures were performed under general anaesthesia, sodium pentobarbitone [60mg/kg i.p. initially and top-ups of 20 mg/kg i.p. every 50 minutes] (Nembutal® 60 mg/mL, Boehringer Ingelheim, NSW, Australia). The head and upper back region was shaved and swabbed with alcohol. The rats were placed prone and the head was mounted and stabilised in a Stoelting stereotactic frame. The tail was taped to the surgical table to firmly secure the animal. A midline incision was made in the upper back, and the spinal cord exposed between the T2 and T3 vertebrae. T1 was identified by its large dorsal protuberance. A fine glass micropipette (tip diameter 50-70
μm) filled with the neuronal retrogradely-transported tracer, rhodamine- tagged microspheres (1:1 dilution with 0.9% sterile saline, LumaFluor, NY, USA) was inserted into the right side of the spinal cord and lowered approximately 0.7mm below the surface and microinjected. The tip of the micropipette was aimed at the IML of the spinal cord. Three unilateral injections of 250nl each were made into separate anterior-posterior sites. After each injection, the micropipette was left in place for several minutes before its removal to minimize tracer leakage along the route of the micropipette. After the injections, the muscles overlying the spinal cord were sutured and the wound closed. The animal was administered analgesic and antibiotic and placed in a warmed environment to recover. The exact locations of the spinal cord injections were verified histologically at the end of the experiment. Only animals in which the injected tracer covered the IML were used in this study (Refer to Figure 3.1).

### 2.5 Microinjections of retrogradely transported tracer into the RVLM

Under general anaesthesia, the right femoral artery was cannulated to enable blood pressure monitoring. The animals were placed prone and their head was mounted in a Stoelting stereotaxic frame such that both bregma and lambda were positioned on the same horizontal plane (Figure 2.2). A burr hole, approximately 4 to 5mm in diameter, was drilled into the occipital bone on the left hand side of the skull approximately 2mm lateral of the mid-sagittal suture and 3mm caudal of the lambdoid suture. The pressor region of the RVLM was identified functionally by the microinjection of 25 to 50 nl of L-glutamate (0.1M) using a fine glass micropipette (tip diameter of 50-70 μm), which
elicited a minimum increase of 20mm Hg in arterial pressure (Figure 2.3). The precise location of the microinjections was verified histologically at the end of the experiment (Refer to Figure 5.1). Only animals in which the injected tracer covered the RVLM were used in this study. Typically, the co-ordinates of the pressor area of the RVLM were 1.8 to 2.2mm lateral of mid-sagittal suture, 2.5 to 3.5mm caudal of the lambdoid suture and 8.9mm ventral of the cerebellar surface. After locating the pressor region, the pipette was carefully withdrawn, filled with the neuronal retrogradely-transported tracer, rhodamine-tagged microspheres (1:1 dilution with 0.9% sterile saline, LumaFluor, NY, USA) and then re-inserted into the pressor region of the RVLM. After the injection, the micropipette was then left in place for 10 minutes prior to its removal to reduce tracer spread along the route of the micropipette. After the micropipette was removed, the skin overlying the skull was sutured and the wound closed. Finally, the arterial cannula was carefully removed from the femoral artery to minimize blood loss and the wound was sutured closed. The animal was subsequently given antibiotic and then analgesic and allowed to recover.
Figure 2.2 – Rat were positioned prone and the head was mounted and stabilised in a Stoelting stereotactic frame.
Figure 2.3 – Mean arterial pressure (MAP) trace showing a minimum rise in blood pressure of approximately 20mm Hg following a 25 to 50 nL injection of L-glutamate (0.1M) into the pressor region of the RVLM.
2.6 Post-operative care

Following the surgical operation, the animals were housed individually to prevent them from removing sutures thereby diminishing the risk of infection. The animal’s recovery following all surgical procedures utilised in this study was closely monitored and recorded. The appearance and general well being of the animal was observed, with particular attention to demeanour, alertness, signs of pain (piloerection or vocalisation in extreme cases) and grooming behaviour. Furthermore, the animals posture (stress implied by lower front and high back), movement around the cage, drinking, eating, activity, bowel movements and weight changes were also monitored. Weight was recorded on a daily basis. Finally, the consistency and colouration of the faeces and urine were observed.

In addition to the above, the animals were also handled twice daily during the recovery period to reduce stress during the experimental day. Finally, approximately 2 weeks following the microinjection of the retrograde tracer, the rats were brought to the experimental room to acclimatize and adjust to their new surroundings overnight.

2.7 Experimental Day

On the day of the experimental temperature challenge, animals were randomly assigned to either a warm or control group (number of animals in each group are located in the respective chapters) and transferred, in their home cages to the temperature
chamber (Plexiglas box measuring 75cm x 60cm x 55cm with a metal mesh stage in the bottom) (Figure 2.4). In the warm group, the rats remained in the heating chamber (ambient temperature 38.9 ± 0.1 °C) for one hour during which their behavior was monitored at 5-minute intervals. Control animals were treated in the same way except the temperature chamber was maintained at room temperature (ambient temperature 23.0 ± 1.0 °C).

Immediately after the temperature challenge, the rats were removed from the chamber and were placed at room temperature (23.0 ± 1.0 °C) for one hour. Subsequently, the rats were deeply anaesthetized with sodium pentobarbitone and transcardially perfused.

2.8 Perfusion

2.8.1 Fixing the rat brain

The animal was deeply anaesthetised by administration of sodium pentobarbitone (Nembutal 0.15 g/kg i.p). The absence of pedal and corneal reflexes confirmed deep anaesthesia. The recumbent rat was then placed on a wire rack and the limbs extended and fastened to the rack. A midline incision was made and the diaphragm was dissected from the chest wall. The sternum was cut along the midline and the cut then made to extend laterally by following the inferior edge of the ribcage on the both the left and
Figure 2.4 – Photo of the temperature chamber (Plexiglas box measuring 75cm x 60cm x 55cm with a metal mesh stage in the bottom). In the warm group, the rats remained in the heating chamber (ambient temperature set at 38.9 ± 0.1 °C) for one hour. Control animals were treated in the same way except the temperature chamber was maintained at room temperature (ambient temperature set at 23.0 ± 1.0 °C).
right side to expose the beating heart. A cut, placed in the apex of the heart, enabled the perfusion needle to be passed through the left ventricle into the aorta, where it was clamped in place. Finally, a cut was made in the right atrium to allow the infusate to drain.

The brain was fixed by administering approximately 350-400 ml of heparinized phosphate buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in phosphate buffer (PB, 0.1M, pH 7.4) (Refer to Appendix 2). The perfusion pressure was maintained at about 100-120mmHg using a foot pump.

2.8.2 Removing the rat brain and spinal cord

Following a midline incision of the skin extending approximately from between the eyes to the level of T2, the muscle layers were scraped away to reveal the occipital bone and vertebrae. Using rongeurs, the spongy part of the occipital bone was carefully removed to reveal the cerebellum, and then the cerebral hemispheres and brainstem. The dura mater was peeled back from the brain surface, and two transverse cuts were made, one at the level of the olfactory bulb and the other at the caudal end of the medulla. A long thin spatula was used to gently raise the brain and the optic nerves were cut. The brains were then carefully removed from the skull and post-fixed in the fixative solution, 4% PFA, for two hours and then placed in PB containing 20% sucrose solution overnight. Similarly, the vertebrae were cut away to expose the spinal cord which was carefully
removed and post fixed for two hours before placing the tissue into PB containing 20% sucrose solution overnight.

2.9 Sectioning of the rat brain and spinal cord

In this study, forty-micron thick sections were cut using a Leica CM 1900 cryostat (Leica Microsystems Nussloch GmbH, Nussloch, Germany). For the PVN, one in three serial coronal sections were taken for immunohistochemical analysis of Fos throughout the entire rostrocaudal extent of the PVN. A total of ten sections were analysed. Similarly, one in three 40μm serial sections (total of fifteen sections) were taken for the analysis of the rostral brainstem which covered a total distance of approximately 2.4mm. This region encompassed the raphe pallidus (RPa), rostral ventromedial medulla (RVMM) and the RVLM. Forty-micron thick sections of the spinal cord and the RVLM were also sectioned to allow for the determination of the exact locations of the spinal cord and RVLM injections respectively (Refer to Figure 3.1 and Figure 5.1). The spinal cord and RVLM sections were mounted onto subbed slides, washed in distilled water, dried and cover-slipped in DePeX mounting medium before being examined under fluorescence illumination.
2.10 Staining protocols

2.10.1 Immunohistochemical detection of Fos

To identify activated neurons, Fos immunohistochemistry was performed. The sections were incubated and processed using standard immunohistochemical procedures as previously described (Kantzides and Badoer, 2003). Briefly, the floating sections underwent washes in PB prior to incubation with 10% normal horse serum (NHS) in PB for one hour at room temperature. This was followed by an overnight incubation with a primary antibody raised in rabbit against a conserved region of the human Fos (Ab5, 1:20 000; Oncogene Research Products, Cambridge, MA, USA) containing 2% NHS (JRH Biosciences, VIC, Australia) and 0.3% Triton X-100 (Sigma Aldrich, NSW, Australia). After washes in PB, the sections were incubated for one hour with biotinylated anti-rabbit secondary antibody (diluted to 1:600 in 0.1M PB, Sigma Aldrich, Australia) that was raised in goat. Following washes in PB, the sections were incubated for one hour using Extravidin (Sigma Aldrich, NSW, Australia) diluted to 1:400 in 0.1M PB. Subsequently, the sections were then washed in Tris buffer (0.05M, pH 7.6) and incubated for ten minutes in 0.05% 3,3’-diaminobenzidine hydrochloride (DAB) (Sigma Aldrich, NSW, Australia) in 0.05M Tris Buffer. The reaction was initiated by the addition of 5μl of 17.5% hydrogen peroxide (H₂O₂) (Biotech Pharm P/L, VIC, Australia) and terminated by washes with fresh 0.05 M Tris Buffer.
2.10.2 Nicotine Adenine Dinucleotide Phosphate-diaphorase (NADPH-d) staining

Immediately after the immunohistochemistry procedure to detect Fos, the sections were incubated in a mixture of 2.5mg Nitroblue Tetrazolium (Sigma Aldrich, NSW, Australia), 10mg β-NADPH (Sigma Aldrich, NSW, Australia) and 0.2% Triton X-100 in 10ml of 0.05 M Tris buffer. The reaction was then allowed to proceed for 30-40 minutes at room temperature (23°C). The intensity of staining was examined before terminating the reaction with 0.05 M Tris buffer washes.

Sections were then mounted onto gelatine-subbed slides and dried prior to a brief wash in water, and re-drying. The slides were then dipped in Xylene (Analar, Merck Pty Ltd, VIC, Australia) before being cover-slipped using DePex mounting medium (BDH Lab Supplies, Poole, UK).

2.11 Analysis

2.11.1 Histological analysis

Both Fos-positive cell nuclei and NADPH-d positive neurons were identified under normal bright field illumination. Retrogradely labeled cells were detected by using a fluorescent light source on a microscope fitted with a Rhodamine filter. Double-labeled neurons containing retrogradely-transported tracer and either a Fos-positive nucleus or NADPH-d positive cytoplasm were detected by rapidly switching between the two light sources. Double-labeled neurons containing both a Fos-positive nucleus and NADPH-d...
positive cytoplasm were detected under normal bright field illumination. Triple-labeled neurons were also detected by rapidly switching between the two light sources.

**Hypothalamic PVN**

On the side of the PVN ipsilateral to the injection site, labeled neurons were counted (using 200X magnification) in 10 sections (processed in 5 lots of 2), which represented five different levels encompassing the rostral-caudal extent of the PVN. The data were expressed as the average number per section at each level. The average values for Fos-positive cell nuclei, NADPH-d positive neurons and retrogradely labeled neurons for each group of animals were then calculated and compared between the heated and control groups. The average numbers of double-labeled and triple-labeled neurons were also calculated.

**Rostral brainstem**

Labeled neurons were counted unilaterally on the side of the brainstem ipsilateral to the injection site (using 200X magnification). These sections were grouped to represent five different rostral-caudal levels. Each level consisted of four sections, three of which were used for quantification. The levels of the brainstem examined represented approximately 1.2mm to 3.6mm caudal to the interaural line. Each level represented a rostral – caudal distance of between 0.5mm to 0.6mm. Levels 1 and 2 contained the mid to rostral parts of the medulla, whilst levels 3, 4 and 5 encompassed the pontine raphe.
For quantification purposes, the brainstem regions were subdivided into 3 areas that encompassed the midline, ventromedial and ventrolateral regions of the brainstem (Refer to Chapter 4.1). In each region, the number of Fos- positive cell nuclei, NADPH-d positive neurons and retrogradely labeled neurons was counted in each brain section in each animal. The number of multiple-labeled neurons were also counted. In each animal, the number of labeled neurons in each level of the brainstem and the total number of labeled neurons in each region of the brainstem (i.e. overall number) were calculated and averaged for the heated and the control group of animals.

2.11.2 Statistical analysis

**Hypothalamic PVN and rostral brainstem**

The overall mean values in the heated and control groups of rats were compared using the unpaired Student’s T-test. For comparisons between the groups at the five different levels of the PVN, the means were compared using Student’s T-test and applying Bonferroni’s modification to compensate for multiple comparisons. The statistical software package used was GB-STAT® version 7.0 (Dynamic Microsystems Inc., U.S.A), and the level of significance was set at p<0.05.
2.12 Mapping

For illustration of the distribution of labeled neurons in the areas of interest, maps were drawn from representative sections at different rostrocaudal levels. The digital maps were generated using the software package MD Plot version 4.0 (Minnesota Datametric Corporation, MN, U.S.A.) and a MD3 microscope digitizer stage (Minnesota Datametric Corporation, MN, U.S.A.) attached to a Leica DMLB microscope (Meyer Instruments Inc., TX, USA). By this means, it is possible to accurately pinpoint the location of labeled neurons within the section. The individual maps generated by MD Plot were then imported into CorelDraw version 9.0 (Corel Pty. Ltd, NSW, Australia) to create the final figures utilized through the experimental chapters of this thesis.

2.13 Photomicroscopy

Photographic images were acquired using a digital camera (Sensi Cam, PCO CCD Imaging, Kelheim, Germany) on an Olympus BX60 microscope (Olympus Inc, PA, USA). The digital images obtained were imported into Adobe Photoshop® 5.5 (Adobe Systems Incorporated, CA, U.S.A) and only the contrast and brightness were modified for presentation purposes.
2.14 Blood flow experiments

2.14.1 Surgical preparations of animals used in blood flow experiments.

All animals were anaesthetized initially with Equithesin (sodium pentobarbitone (0.5g): chloral hydrate (2.219g) (per 100ml) mixture and administered intraperitoneally (0.6ml/100gm]) to enable the cannulation of the femoral artery and vein (Refer to Section 2.3). Anaesthesia was then subsequently maintained with urethane (1–1.4 g/kg intravenously initially followed by supplemental doses of approximately 0.05g/kg as required). The depth of anesthesia was maintained to ensure the absence of corneal and pedal reflexes.

The femoral artery was cannulated for monitoring arterial blood pressure (Refer to Section 2.3). The signal was recorded using a Mac Lab data-acquisition System (AD Instruments, Colorado Springs, CO, USA). Mean arterial pressure (MAP) and heart rate (HR) were determined electronically from the phasic arterial pressure. The femoral vein was cannulated for the intravenous delivery of supplemental doses of urethane.

Throughout the surgical procedures, the body temperature was maintained at 37.0°C ± 1.0°C with a custom-made water-circulating blanket (Refer to Appendix 1), through which either cold water (4°C to 8°C measured directly at source) or warm water (48°C to 52°C measured directly at source) were pumped through at a rate of approximately 16-26ml/min.
A thermocouple was taped onto the base of the tail (MLT409, AD Instruments, Colorado Springs, CO, USA) to record tail temperature. A second thermocouple, (RET-2 rectal probe for rats, PhysiTemp Instruments Inc, Clifton, NJ, USA) was inserted approximately 2-3cm into the rectal cavity and connected to a Thermocouple Analog converter (MLT1101, AD Instruments, Colorado Springs, CO, USA) to enable the measurement of body core temperature.

2.14.2 Microinjection into the hypothalamic PVN

The animals were placed prone and the head was mounted in a Kopf stereotaxic frame such that both bregma and lambda were positioned on the same horizontal plane. A midline reference point was marked 2mm rostral to bregma. This was necessary because bregma was removed during the subsequent drilling procedure. To expose the dorsal surface of the brain, a hole, approximately 4mm in diameter, was drilled into the bone centered 3.5mm caudal from the reference point. Following the drilling procedure, the hole was covered with cotton wool soaked in normal saline to prevent drying of the exposed surface.

Microinjections were made bilaterally using a fine glass micropipette (with a tip diameter of 50-70 μm) into the PVN (stereotaxic coordinates: 1.5 mm caudal to bregma, 0.5 mm lateral to midline and 7.5 mm ventral to the surface of the brain) or into the
hypothalamus adjacent to the PVN (stereotaxic coordinates: 2.8 mm caudal to the bregma, 0.5 mm lateral to the midline and 7.5 mm ventral to the brain surface.

2.14.3 Renal blood flow measurement

The kidney was identified using a retroperitoneal approach. The renal artery was dissected free and carefully cleared from the surrounding tissue and from the renal vein. 

Whilst this method have also been used to renal denervate, extra care is made during the procedure to ensure that the renal nerve is left undamaged. A flow probe was positioned around the renal artery and connected to a T206 small animal blood flow meter (Transonic System Inc., Ithaca, NY, USA) to enable monitoring of the renal blood flow (Figure 2.5). Following the implantation of the renal flow probe, approximately 15 - 20 minutes were allowed to elapse to ensure that a steady basal renal blood flow was attained. Subsequently, the rat’s body temperature was lowered to 36.0° C by passing cold water through the water- circulating blanket. This occurred within 5 - 10 minutes. Renal conductance was calculated by dividing renal blood flow (ml/min) by the mean arterial pressure.
Figure 2.5

A flow probe was positioned around the renal artery and connected to a T206 small animal blood flow meter to enable monitoring of the renal blood flow.
2.14.4 Experimental protocol

When body temperature reached 36.0°C, muscimol (Sigma-Aldrich, St. Louis, MO, USA) (1nmol in 100nl, n=6) or saline vehicle (100nl, n=5) was microinjected into the PVN bilaterally. In a third group of rats, muscimol was microinjected in the hypothalamus outside the PVN (N=5). In another group of animals muscimol was injected into the PVN and the effects were followed over time. In this group of rats, core body temperature was not altered and was maintained between 37.0 - 37.5°C. To mark the injection sites, rhodamine- tagged microspheres (LumaFluor, NY, USA) were included in the microinjected solution. After the completion of the microinjections, the core rectal temperature of the animal was gradually increased to 41°C. This was performed over approximately 90-120 minutes.

2.14.5 Brain Histology

At the completion of the experiment, rats were killed with an overdose of sodium pentobarbitone (300mg/kg) (Lethaborb, Virbac Pty Ltd., NSW, Australia). The brains were then carefully removed and fixed in 4% paraformaldehyde solution for approximately 7 days, and then placed into a solution of phosphate buffer containing 20% sucrose overnight. The hypothalamus was cut on a cryostat into sections (40µm thick), and mounted onto gelatine-subbed slides. The sections were then viewed wet under fluorescent microscopy to identify the rhodamine beads at the site of injection. The sections were then dried before being counterstained with cresyl violet (9-Amino-5-
imino-5H-benzo[a]-phenoxazine salt, Sigma Chemical CO., MO, USA) and cover slipping with Depex Mounting medium (BDH Lab Supplies, Poole, UK). The sections were then examined using light microscopy to determine anatomical structure and the injection sites were subsequently mapped in relation to the anatomical structure.

An injection was categorised as ‘inside the PVN’ if it was less than 0.5 mm from the boundary of the PVN on both sides of the brain (a photomicrograph of an injection site within the PVN is shown in Figure 2.6). An injection was categorised as ‘outside the PVN’ if it was more than 0.5 mm from the boundary of the PVN on both sides of the brain. Animals that did not satisfy these criteria were excluded from the study.
Figure 2.6
Photomicrograph of a coronal section through the hypothalamus showing the injection site in the paraventricular nucleus (PVN) (one side only). The section is counterstained with cresyl violet. The schematic diagram shown on the right panel is a tracing of the section shown and outlines the position of the PVN and the injection site. Abbreviations: III, third ventricle.
2.14.6 Statistical Analysis

The basal resting MAP, HR, renal blood flow and renal conductance levels prior to microinjections into the brain were compared between the three groups (muscimol into PVN, muscimol out of PVN and saline into the PVN) using one-way ANOVA. Effects on the MAP, HR, renal blood flow and renal conductance levels as body temperature changed were compared between all groups using two-way ANOVA with repeated measures. When the overall difference was statistically significant, the following comparisons were made; (i) control versus muscimol in the PVN, (ii) muscimol in the PVN versus muscimol out of the PVN, and (iii) muscimol out of the PVN versus saline in the PVN groups, using two-way ANOVA with repeated measures.
CHAPTER 3.

ACTIVATION OF SPINALLY-PROJECTING
AND NITEROGIC NEURONS IN THE PVN
FOLLOWING HEAT EXPOSURE.
3.0 INTRODUCTION

Exposure to a hot temperature challenge elicits responses mediated in part by the autonomic nervous system to promote heat loss and maintain body fluid homeostasis. Such responses include sweating in humans, increased salivary secretion in rodents, an increase in heart rate, increased respiration rate, skin vasodilatation and visceral vasoconstriction (Kanosue et al., 1994a; Kazuyuki et al., 1998). The latter autonomic cardiovascular responses often involve changes in sympathetic nerve activity and result in the redistribution of blood flow from the viscera to the skin. These changes are mediated by the central nervous system (Kanosue et al., 1991; Kanosue et al., 1994a; Morrison, 2001c; Nagashima et al., 2000; Owens et al., 2002; Scammell et al., 1993; Zhang et al., 1997b).

It has been well established that the central nervous system is essential in the regulation of body temperature. There are several brain regions that are likely to contribute to the central nervous system pathways that mediate the thermoregulatory responses. Studies using the marker of neuronal activation, Fos, or electrophysiological recordings have shown that several forebrain areas are activated following the elevation in body temperature (Bachtell et al., 2003; Boulant, 1981; Boulant, 1998; Bratinescak and Palkovits, 2004; Hori et al., 1999; Kiyohara et al., 1995; Maruyama et al., 2003; McKitrick, 2000; Morimoto and Murakami, 1985; Murakami and Morimoto, 1982; Scammell et al., 1993; Schmid and Pierau, 1993). These forebrain areas include the preoptic area, anterior hypothalamus and the PVN of the hypothalamus. The preoptic area and anterior hypothalamus are well known key thermoregulatory sites within the brain;
however, a role of the PVN in thermoregulation has largely been ignored. This is surprising given the circumstantial evidence suggesting that it plays an important role in the cardiovascular changes elicited by the disturbances in body temperature. In particular, the PVN contains (i) thermo sensitive neurons (Inenaga et al., 1987) and (ii) neurons that project to the spinal cord and influence sympathetic nerve activity to important thermoregulatory effector organs such as the brown adipose tissue and the vasculature of the rat tail, salivary gland as well as kidney and gut (Hubschle et al., 2001; Morrison, 1999; Oldfield et al., 2002). Furthermore, increases in body temperature activates neurons within the PVN (Bachtell et al., 2003; Bratincsak and Palkovits, 2004; Harikai et al., 2003; Kiyohara et al., 1995; McKitrick, 2000).

The PVN consists of several subgroups of neurons, including those that can directly influence SNA via projections to the IML of the thoracolumbar spinal cord, where the sympathetic preganglionic motor neurons are located (Badoer, 2001; Kantzides and Badoer, 2003; Shafton et al., 1998). These projections are likely to mediate the effects of the PVN on SNA and may contribute to the cardiovascular changes induced by elevations in body temperature. However, this has not been examined to date. Therefore, the first aim of this study was to determine whether spinally projecting neurons in the PVN are activated by thermal stimulation in the conscious rat.

Studies investigating the nature of the neurochemical content of spinally-projecting neurons in the PVN have revealed that numerous neurochemicals may be present in this population (Cechetto and Saper, 1988; Sawchenko and Swanson, 1982).
Of interest, there is a dense concentration, in the PVN, of neurons containing NOS, the enzyme responsible for the production of NO. Current evidence suggests that NO in the central nervous system is important in the thermoregulatory pathways mediating heat dissipation (Eriksson et al., 1997; Garthwaite and Boulton, 1995; Gerstberger, 1999; Gourine, 1995; Schmid et al., 1998). For example, thermal stimulation induces enhanced secretion of saliva, which is spread on the fur as a means of heat defense in rats (Damas, 1994; Kanosue et al., 1991; Yanase et al., 1991). Inhibition of NO production reduces saliva production during body warming (Damas, 1994). Blockade of central NO production has recently been found to elevate core body temperature in the rat (Mathai et al., 2004) as well as augment the febrile response elicited by endotoxin and lipopolysaccharide (Gourine, 1995; Steiner et al., 2002). In support of those findings, it has been observed that the mRNA for NOS within the hypothalamus is elevated by endotoxin (Lee et al., 1995). Additionally, interleukin-1β, a cytokine mediating endotoxin responses, is reported to increase NO production within the hypothalamus (Brunetti et al., 1996). NO within the PVN can cause a pronounced alteration of sympathetic nerve activity (Krukoff, 1999; Patel et al., 2001), and it is likely that the PVN neurons projecting to the IML of the spinal cord contribute to those responses.

Thus, the second aim of the present work was to determine whether neurons in the hypothalamic PVN that were activated by acute thermal stimulation were also capable of producing NO, and we determined whether those neurons included the sub-population of neurons in the PVN that project to the IML of the spinal cord.
3.1 EXPERIMENTAL PROCEDURES

3.1.1 Animals and housing

Briefly, all experimental animals underwent one surgical procedure prior to the experimental day. The surgical procedure involved the placement of the retrogradely-transported tracer into the IML. At least 2 weeks separated the microinjection surgical procedure and the experimental day. Animals are transported to the experimental room 24hrs prior to allow for acclimatizing. Details of all anaesthesia are provided in Chapter 2.

3.1.2 Microinjections of retrogradely transported tracer into the spinal cord.

Under general anaesthesia, the neuronal retrogradely-transported tracer, rhodamine-tagged microspheres (1:1 dilution with 0.9% sterile saline, LumaFluor, NY, USA) were microinjected into the spinal cord. A midline incision was made in the upper back, and the spinal cord exposed between the T2 and T3 vertebrae. A fine glass micropipette (tip diameter 50-70 μm) filled with the tracer was inserted into the right side of the spinal cord and lowered approximately 0.7mm below the surface. The tip of the micropipette was aimed at the IML of the spinal cord. Three unilateral injections of 250nl each were made into separate anterior-posterior sites within the spinal segment. After each injection, the micropipette was left in place for several minutes before its removal to minimize tracer leakage along the route of the micropipette. After the injections, the muscles overlying the spinal cord were sutured and the wound closed. The exact locations of the spinal cord injections were verified histologically at the end of the experiment. Only animals in which the injected tracer covered the IML were used in this experiment.
study (see Figure 3.1 for a representative example of an injection site). The injection site also spread to parts of the tractus rubrospinalis, tractus corticothalamicus lateralis and the tractus corticostriatalis lateralis (Figure 3.1).

### 3.1.3 Experimental Day

Approximately 2 weeks after the microinjection of the retrograde tracer, to allow for the transport of the tracer, and 24 hours prior to the experiment, the rats were placed in the experimental room. On the day of the temperature challenge, animals were randomly assigned to either a heated (N=8) or control group (N=7) and transferred, in their home cages to the temperature chamber (Plexiglas box measuring 75cm x 60cm x 55cm with a metal mesh stage in the bottom). In the heated group, the rats remained in the heating chamber (ambient temperature 38.9 ± 0.1°C) for one hour during which their behavior was monitored to ensure the welfare of the animals and to observe ‘thermoregulatory’-type behavior such as saliva spreading. Control animals were treated and monitored in the same way except the temperature chamber was maintained at room temperature (ambient temperature 23.0 ± 1.0°C).

Immediately after the temperature challenge, the rats were removed from the chamber and allowed to recover for one hour at room temperature before being deeply anaesthetized with sodium pentobarbitone and transcardially perfused with approximately 350-400 ml of phosphate buffered saline (PBS), followed by 4% paraformaldehyde in phosphate buffer (0.1M, pH 7.4). The perfusion pressure was maintained at about 100-
120 mmHg. The brains and spinal cords were then carefully removed and post-fixed in the fixative solution, for two hours before being placed into PB containing 20% sucrose overnight.

In separate rats, the spinal injections were not performed. Instead, a cannula was inserted into the femoral vein of each rat, under general anaesthesia as described previously, 3-4 days prior to the experimental day. These rats underwent a similar heating protocol as described above except, in 5 rats, hypotonic saline (0.75%) was infused intravenously (4.8 ± 0.4 ml/100g body weight at a rate of 16 ml/h) during the heat exposure. This amount of fluid replacement was estimated from previous experiments and was designed to prevent the fluid loss that normally accompanied the exposure to the hot environment. The brains from these animals were only processed for Fos-immunohistochemistry. Control rats (N=5) underwent similar procedures but were not administered the hypotonic saline.

3.1.4 Detection of Fos by immunohistochemistry

Serial sections of the hypothalamus and spinal cord (40μm) were cut on a cryostat and 1:3 sections were collected. To identify activated neurons, immunohistochemistry to detect Fos was performed on sections from the hypothalamus encompassing the PVN. The sections were incubated and processed using standard immunohistochemical procedures as previously described (Kantzides and Badoer, 2003) (Refer to 2.10.1).
3.1.5 Nicotine Adenine Dinucleotide Phosphate-diaphorase (NADPH-d) staining

Immediately after Fos immunohistochemistry, the sections were incubated in a mixture of 2.5mg Nitroblue Tetrazolium (Sigma Aldrich, Australia), 10mg β-NADPH (Sigma Aldrich, Australia) and 0.2% Triton X-100 in 10ml of 0.05 M Tris buffer (Refer to 2.10.2).

3.1.6 Analysis

3.1.6.1 Rats with intraspinal-injections

Both Fos-positive cell nuclei and NADPH-d positive neurons were identified under normal bright field illumination. Retrogradely labeled neurons were detected by using a fluorescent light source on a microscope fitted with a Rhodamine filter. Double-labeled neurons containing retrogradely-transported tracer and either a Fos-positive nucleus or NADPH-d positive cytoplasm were detected by rapidly switching between the two light sources. Double-labeled neurons containing both a Fos-positive nucleus and NADPH-d positive cytoplasm were detected under normal bright field illumination. Triple-labeled neurons were detected by rapidly switching between the bright field and fluorescent light sources.

On the side of the PVN ipsilateral to the injection site, labeled neurons were counted (using 200X magnification) in 10 sections (processed in 5 lots of 2), which represented five different levels encompassing the rostral-caudal extent of the PVN. The data were expressed as the average number per section at each level. The average values for Fos-positive cell nuclei, NADPH-d positive neurons and retrogradely labeled neurons for...
each group of animals were then calculated and compared between the heated and control
groups. The average numbers of double-labeled and triple-labeled neurons were also
calculated.

3.1.6.2 Rats without intraspinal-injections

In rats that were not injected intraspinally, Fos-positive nuclei were counted
unilaterally in 2 sections of the PVN. The average values per section were determined
and compared statistically. The approximate rostral caudal level of the PVN analyzed is
shown in Figure 3.5.

3.1.7 Statistical analysis

The overall mean values in the heated and control groups of rats were compared
using the unpaired Student’s T-test. For comparisons between the groups at the five
different levels of the PVN, the means were compared using Student’s T-test and
applying Bonferroni’s modification to compensate for multiple comparisons. The
statistical software package used was GB-STAT® version 7.0 (Dynamic Microsystems
Inc., U.S.A), and the level of significance was set at p<0.05.

3.1.8 Mapping

For illustration of the distribution of labeled neurons in the different levels of the
PVN, maps were drawn from representative sections in each of the five rostral to caudal
levels. The digital files were generated using the software package MD Plot® (version
4.0) and a MD3 microscope digitizer stage (Minnesota Datametric Corporation, U.S.A.) attached to a Leica DMLB microscope.

3.1.9 Photomicroscopy

Images were acquired using a digital SPOT camera mounted on an Olympus BX60 microscope. The digital images obtained were imported into Adobe Photoshop ® (version 5.5, Adobe Systems Incorporated, U.S.A) and only the contrast and brightness were modified for presentation purposes.
3.2 RESULTS

3.2.1 Effect of heating on Fos expression in the PVN.

In the heated group of animals, the total number (unilateral) of Fos-positive cell nuclei (1336 ± 43) in the PVN was significantly elevated by tenfold compared to the control group (133 ± 23) (p<0.0001) (which is similar to the numbers found in handled animals (Krukoff and Khalili, 1997)). This increase in the production of Fos occurred throughout the rostral-caudal extent of the PVN, with the maximum number of Fos-positive cell nuclei found predominantly in the mid to caudal levels of the PVN (Figures 3.2 and 3.3). Fos-positive cell nuclei were present in both magnocellular and parvocellular regions, but only quantitated in the parvocellular region of the PVN. Within the parvocellular PVN, the Fos-positive cells were distributed in the dorsal, medial, and lateral parvocellular sub-nuclei of the PVN (Figures 3.3 and 3.4). In the control group of animals, very few Fos-positive cell nuclei were observed, and these were distributed evenly throughout the rostral-caudal extent of the PVN (Figures 3.2 and 3.4).

Following the one hour of heating, the average body weights of the rats in the heated group fell significantly to 313 ± 11 g from 324 ± 11 g prior to the heating (p<0.001). In the control animals, the body weights did not change significantly during their time in the temperature chamber. The body weights of the rats in the control group prior to entering the chamber averaged 320 ± 11 g, which was not significantly different from the respective weight in the heated group.
Since the thermal stimulation resulted in a reduction in body weight of approximately 3.5%, which reflects a loss in body fluid (and a subsequent increase in osmolality), we also investigated the effect of restoring the body fluid to pre-heating levels by infusing hypotonic saline intravenously during the heat exposure. In the rats in which fluid loss was replaced, the number of Fos-positive cell nuclei in the PVN averaged 194 ± 17 per section (counted in the plane shown in Figure 3.5, where maximal Fos-positive cell nuclei were observed in the heated rats (see Figure 3.2). This was not significantly different from controls in which 192 ± 14 Fos-positive cell nuclei per section were counted. The body weights of the rats in which fluid was infused did not change significantly during heating (average increase = 3 ± 6 g from 293 ± 23 g before heating), but fell, as expected, in the control animals by 12 ± 3 g from 329 ± 19 g. Plasma osmolality in the hypotonic saline infused group did not increase (312 ± 6 mosm/kg vs 302 ± 4 mosm/kg pre and post heating respectively).

### 3.2.2 Distribution of spinally-projecting neurons in the PVN.

Spinally-projecting neurons were observed at all rostral-caudal levels of the parvocellular PVN (Figure 3.3). The maximum number was found in the mid to caudal levels, which was similar to the distribution of Fos-positive cell nuclei. The average number of neurons per animal in the parvocellular PVN (unilateral) that projected to the spinal cord was similar in the heated (98 ± 10) and control groups (104 ± 16) (Figure 3.2).
3.2.3 Distribution of spinally-projecting neurons in the PVN containing Fos.

After the exposure of the animals to the hot environment, there was a significant increase in the number of spinally-projecting neurons that contained a Fos-positive nucleus (p<0.0001, compared to the control group). These double-labeled neurons represented 22 \pm 2 \% of all the spinally-projecting neurons in the PVN and were found in the dorsal, medial and lateral parvocellular PVN, primarily in the mid to caudal levels of the PVN (Figures 3.2 and 3.3). In the control group of animals, there were very few spinally-projecting neurons that contained a Fos-positive nucleus. These double-labeled neurons represented only 2\% of all the spinally-projecting neurons counted in the PVN (Figure 3.2).

3.2.4 Distribution of neurons that contained NADPH-d

NADPH-d positive neurons were observed throughout the rostral-caudal extent of the PVN (Figures 3.6 and 3.7). The distribution profiles of NADPH-d positive neurons in both the control and heated group of animals were similar (Figure 3.8). The total number of NADPH-d positive neurons in the heated group averaged 722 \pm 17, which was significantly greater than in the control group (542 \pm 26) (p<0.0001 between groups). This increase was predominantly due to the greater number of NADPH-d positive neurons found in the mid to caudal levels of the PVN (Figure 3.6).

3.2.5 Distribution of spinally-projecting neurons containing NADPH-d

After the exposure of the animals to the heated environment, the number of spinally-projecting neurons containing NADPH-d represented 21 \pm 2 \% of all the
spinally-projecting neurons in the PVN. These double-labeled neurons were found primarily in the mid to caudal levels of the PVN (Figures 3.6, 3.7 and 3.8). In the control group, there was a similar distribution of spinally-projecting neurons containing NADPH-d (Figure 3.6). The numbers of double-labeled neurons in the control group were not significantly different to the heated group (Figure 3.6).

### 3.2.6 Distribution of neurons containing NADPH-d and Fos

In the heated group, the average number of neurons in each animal, containing both NADPH-d and Fos in the PVN (282 ± 10) was significantly elevated by fifteen fold, compared to the control group (p<0.0001) (Figure 3.6). These double-labeled neurons represented 20 ± 1 % of all the Fos-positive cells, and approximately 40% of the NADPH-d positive neurons, counted in the PVN of the heated group. This increase occurred throughout the rostral-caudal extent of the PVN, with the maximum number found predominantly in the mid to caudal levels of the PVN (Figures 3.6 and 3.7). In the control group of animals, there was on average a total of only 19 ± 6 NADPH-d positive neurons that also exhibited a Fos-positive nucleus (Figure 3.6).

### 3.2.7 Distribution of triple-labeled neurons.

In the heated group of animals, the total number of neurons containing all three labels was significantly elevated compared to the control group (p<0.0001). These triple-labeled neurons represented 12 ± 1 % of all the spinally-projecting neurons in the PVN. This increase occurred throughout the rostral-caudal extent of the PVN, with the maximum number found predominantly in the mid to caudal levels of the PVN (Figures
3.6 and 3.7). An example of a triple-labeled neuron is shown in Figure 8. In the control group of animals, triple-labeled neurons were scarcely present in the PVN. On average, these neurons represented $0.1 \pm 0.1$ % of all the spinally-projecting neurons counted in the PVN (Figures 3.6 and 3.7).

### 3.2.8 Behavioural responses to heat exposure

In general, all animals placed in the temperature chamber usually explored the surroundings for 5 to 10 minutes. Subsequently, rats in the control group usually curled up and performed very little or no activity. In contrast, animals in the heated group continued to explore actively and also exhibit characteristic behaviors that included tunnelling into the bedding and spreading saliva on their fur, and in some cases scrotum licking, to increase their evaporative heat loss. A wet chin was commonly observed by the end of the one-hour exposure in the temperature chamber.
Figure 3.1

Photomicrograph of a transverse section of a rat spinal cord showing the site of injection (highlighted by the dotted line) of the retrogradely-transported tracer into the intermediolateral cell column. Bar = 0.3 mm
Average numbers of Fos-positive cell nuclei, spinally-projecting neurons and double-labeled neurons counted on the side ipsilateral to the spinal micro-injection site in five rostral - caudal levels of the PVN. Experiments were performed in conscious rats placed into a hot environment (39°C) for 60 minutes (solid columns) or rats left at room temperature (open columns). * P< 0.05 compared to respective controls.
Figure 3.3

Diagrammatic illustration of the distribution of Fos-positive cell nuclei, spinally-projecting neurons and of spinally-projecting neurons containing a Fos-positive cell nucleus in the subnuclei of the PVN (defined according to (Swanson and Kuypers, 1980)). Five different rostral (A) - caudal (E) levels are shown and the approximate anterior – posterior levels caudal to bregma in mm is indicated on the right. Abbreviations: III, third ventricle; ap, anterior parvocellular PVN; dp, dorsal parvocellular PVN; mp, medial parvocellular PVN; pm, magnocellular PVN; lp, lateral parvocellular PVN. For simplicity, not all cells could be represented by dots in (i) regions of high density of Fos-positive cells and (ii) in regions of high density of the spinally projecting neurons. Data is from a representative animal placed into a hot environment (39°C) for 60 minutes.
Figure 3.4

Photomicrographs of the hypothalamic paraventricular nucleus (PVN, outlined) showing the distribution of Fos-positive cell nuclei following heating (panel A) and in a control animal (panel B). Panel C shows a high magnification photomicrograph of Fos positive cell nuclei from a rat that had undergone heating. Panel D shows the same region as in C viewed under fluorescent lighting conditions to show the presence of retrogradely transported fluorescent microspheres. The microspheres had been microinjected into the spinal cord. The arrow in panels C and D highlights the same cell. Thus, this cell was activated by heat and was spinally projecting. Abbreviations: III, third ventricle. Bar =100 µm in A and B, and 20 µm in C and D.
Figure 3.5

Photomicrographs of the hypothalamic paraventricular nucleus (outlined) showing distribution of Fos-positive cell nuclei from rats exposed to a hot environment. In panel B hypotonic saline was administered intravenously during heat exposure to replace fluid loss. Panel A is from a control rat in which fluid loss was not compensated. Abbreviations: III, third ventricle. Bar =100 µm in A and B
Figure 3.6

Average numbers of NADPH-d-positive neurons (panel A), NADPH-d-positive neurons that also project to the spinal cord panel (panel B), NADPH-d-positive neurons that also contained a Fos-positive nucleus (panel C), and triple-labeled neurons (panel D) in five different rostral–caudal levels of the PVN (see text for details). Solid columns show data from rats which were placed in a heated environment for 60 minutes. Open columns represent data from controls. * P<0.05 compared to respective control level.
Figure 3.7

Diagrammatic illustration of the distribution of NADPH-d-positive neurons (left panel), NADPH-d-positive neurons that project to the spinal cord (second panel), NADPH-d-positive neurons containing a Fos-positive nucleus (third panel) and triple labeled neurons (right panel) in five different rostral (A) - caudal (E) levels of the paraventricular nucleus in warmed animals. Approximate anterior – posterior levels caudal to bregma are shown in mm on the right. Illustration of the subnuclei of the PVN is according to Swanson and Kuypers, 1980. For simplicity, not all cells could be represented by dots (i) in regions of high density of the NADPH-d-positive neurons and (ii) in regions of high density of NADPH-d-positive +Fos-positive neurons. Abbreviations: III, third ventricle; ap, anterior parvocellular PVN; dp, dorsal parvocellular PVN; mp, medial parvocellular PVN; pm, magnocellular PVN; lp, lateral parvocellular PVN.
Figure 3.8

Photomicrographs of a coronal section containing the hypothalamic paraventricular nucleus showing the distribution of Fos-positive cell nuclei and NADPH-d positive cells from a control rat (panel A) and from a rat placed in a heated environment (panel B). Panel C shows a high magnification photomicrograph of Fos positive nuclei located within the area indicated by the rectangle in B. The asterisk illustrates a single labeled cell (Fos-positive) whilst the dashed line outlines a NADPH-d positive neuron containing a Fos-positive nucleus. Panel D shows the same area as in C viewed under fluorescent lighting to highlight the presence of rhodamine-tagged microspheres in the outlined cell. This triple-labeled neuron exemplifies an activated nitrergic neuron that is spinally-projecting. Bar =100 µm in A and B, and 5 µm in C and D. Abbreviations: III, third ventricle.
3.3 DISCUSSION

In the current study we have made several novel observations. We found that following exposure of conscious animals to a heated environment of 39°C, there was; (i) a significant increase in the number of spinally-projecting neurons in the PVN that exhibited Fos, (ii) a significant increase in the number of PVN neurons that exhibited Fos and were NADPH-d positive and, (iii) a significant increase in the number of PVN neurons that contained all three markers (i.e. were Fos-positive, NADPH-d positive and spinally-projecting). Indeed, our data suggests that an environmental temperature of 39°C is a powerful stimulus that activates nitrergic neurons in the PVN as well as spinally-projecting neurons in the PVN.

The present study, for the first time, also provides a detailed quantification of Fos-positive cell nuclei and highlights their rostral-caudal distribution within the parvocellular PVN following heat exposure. In conscious rats exposed to the hot environment there was a marked increase in the number of Fos-positive cell nuclei observed in all sub-divisions of the PVN, and the numbers peaked in the mid to caudal levels of the parvocellular PVN. The increased production of Fos following heat exposure is in agreement with earlier studies (Bachtell et al., 2003; Bratincsak and Palkovits, 2004; Harikai et al., 2003; Kiyohara et al., 1995; McKitrick, 2000). In contrast, there have been previous reports that have not detected an increase in Fos production in the PVN following an elevated body temperature (Patronas et al., 1998). The contradictory nature of the observations has most likely contributed to the lack of studies investigating the role of the PVN in thermoregulation. The contrasting findings
may be reconciled, however, by the differences in species used, the duration of heat exposure and the degree to which body temperature was elevated (Bachtell et al., 2003; Bratincsak and Palkovits, 2004; Harikai et al., 2003; McKitrick, 2000; Patronas et al., 1998). In general, studies that have elicited marked activation of the parvocellular PVN have used a higher environmental temperature (Bratincsak and Palkovits, 2004; Harikai et al., 2003; McKitrick, 2000). We have previously found that the stimulus used in the present study elevates body temperature by an average of approximately 3.5 °C (Mathai et al., 2000).

One of the most important findings of the present study is that PVN neurons projecting to the spinal cord are activated following exposure to a hot environment and an increase in core body temperature. The proportion of spinally-projecting neurons that were activated following heat exposure was 22% which makes an elevated body temperature the most effective stimulus to activate this pathway observed to date. Previous stimuli that have elicited marked activation of PVN neurons include hemorrhage, dehydration, increased osmolality and hypotension (Badoer et al., 1993; Kantzides and Badoer, 2003; Polson et al., 2002; Stocker et al., 2004). These stimuli elicited as much if not more Fos production in the PVN but were not as effective as the heating stimulus used in the present study in activating spinally-projecting neurons.

In the present work we used an ambient temperature of 39 °C to elevate body temperature (Mathai et al., 2000). Under these conditions, plasma osmolality is elevated and body fluid is reduced. Thus, heat exposure involves the integration of different
afferent inputs resulting in the complex behavioural, neural and hormonal changes that characterize the response to heat defense. Since, an elevation in plasma osmolality is known to activate neurons in the PVN (Kantzides and Badoer, 2003; Stocker et al., 2004), one could argue that this stimulus is responsible for the activation of the spinally-projecting neurons in the PVN. However, we have previously shown that an intravenous infusion of hypertonic saline does not activate spinally-projecting neurons in the PVN (Kantzides and Badoer, 2003). Thus, it is unlikely that the increase in osmolality that accompanied thermal stimulation in the present study could account for the increased activation of spinally-projecting PVN neurons.

The fluid reduction accompanying heat exposure may result in a reduction in blood volume. We have previously found that haemorrhage activated spinally projecting neurons in the PVN. This suggests that the fluid loss occurring during heat exposure could contribute to the activation of spinally projecting neurons observed in the present study. It is unlikely, however, that this stimulus is solely responsible for activating those neurons because we have observed that although severe haemorrhage can elicit more Fos production in the PVN, it is not as effective as heat exposure in activating spinally-projecting neurons (Badoer et al., 1993; Kantzides and Badoer, 2003; Polson et al., 2002; Stocker et al., 2004). Furthermore, we have observed in the present study that the administration of hypotonic saline during the heat exposure to counteract the elevation in plasma osmolality and the reduction in extracellular volume, had no effect on the number of Fos-positive neurons in the PVN, suggesting that under the conditions of heating used in the present study, fluid loss and hypertonicity were not the major contributors to Fos
production. Taken together, we hypothesize that the activated spinally-projecting PVN neurons are important in the central pathways mediating the responses initiated by thermal stimulation, most likely in response to the elevation in body temperature although we cannot exclude a contribution to the response by a reduction in blood volume.

In the present study we found a significant increase in the number of activated neurons in the PVN that were NADPH-d positive following exposure to a hot environment. This could occur as a result of an increase in body temperature, a decreased blood volume, an increased osmolality or a combination of all these inputs. Previous studies have found that 24-48hrs of water deprivation, in which body weight fell by approximately 10% (after 24hr deprivation), resulted in an increase in the expression of nitric oxide synthase (the enzyme responsible for the production of nitric oxide) within the PVN (Gerstberger, 1999; Ueta et al., 1995). However, exposing the animals to a warm external environment of 34°C for 2 days did not have such an effect (Gerstberger, 1999). These results suggest that a warm external environment may not be sufficient to increase the NADPH-d positive neurons in the PVN. Thus, the reduction in body fluid and / or the accompanying increase in osmolality following exposure to a hot external environment for one hour could contribute to the increase in NADPH-d positive neurons in the PVN, observed in the present study. It needs to be kept in mind, however, that since an environmental temperature of 34°C did not increase NADPH-d expression in the PVN, it does not imply that a further increase in temperature *per se* could not contribute
to the observations we have made. This may be particularly pertinent to the activation of the spinally-projecting nitrergic neurons as discussed later.

In the present study, we found a significant increase in the number of Fos-positive, NADPH-d containing neurons in the PVN following exposure to the hot environment. Indeed almost 40% of the nitrergic neurons in the parvocellular PVN were activated. These findings suggest that NO-producing neurons in the hypothalamic PVN are activated following heating. This is in agreement with the view that NO in the CNS is important in heat dissipation (Gerstberger, 1999; Schmid et al., 1998). Thus, the PVN may be a potential site of action within the CNS through which NO may influence the redistribution of blood flow to facilitate heat dissipation. Accordingly, the PVN may be a site in the brain in which NOS inhibitors, by attenuating this action, elicit increases in body temperature (Gerstberger, 1999; Mathai et al., 1997; Mathai et al., 2004). Further investigations are needed, however, to address these issues. We also note that a reduction in blood volume and an increase in osmolality may also contribute to the observations.

Another significant finding of the present study is the increase in the number of activated PVN neurons projecting to the spinal cord which were also NADPH-d positive (12% of the spinally-projecting neurons). We hypothesize that these neurons are activated by an elevated body temperature. This view is based on our earlier discussion in which we highlight that (i) spinally-projecting neurons are not activated by an increase in plasma osmolality, and (ii) intravenous fluid replacement during the thermal stimulation did not affect the number of activated neurons in the PVN. We concede,
however, that we cannot entirely exclude hypovolemia contributing to the activation of nitrergic, spinally-projecting neurons in the PVN.

The precise function of NO in the activated PVN neurons projecting to the spinal cord is unknown. One possibility may be that NO inhibits surrounding neurons, since it diffuses easily through cell membranes, whilst its action within the activated spinally-projecting neurons is to dampen the excitatory drive that is being experienced by those neurons. Although highly speculative, this could be a way to elicit integrated responses so that during heat exposure, PVN neurons involved in vasoconstriction in the viscera are activated whilst other neurons are inhibited. For example, PVN neurons influencing the sympathetic nerves to the heart could be activated, whilst those influencing the cardiac vagal neurons could be inhibited; the PVN is well known to innervate the sites in the CNS containing the sympathetic preganglionic motor neurons and the cardiac vagal motor neurons projecting to the heart (Lawrence and Pittman, 1985). It is also of interest that there is a dense innervation of sympathetic neurons projecting to brown adipose tissue originating in the PVN, identified by studies using trans-neuronal retrograde labelling with pseudorabies virus (Cano et al., 2003; Morrison, 1999; Oldfield et al., 2002). These PVN neurons should be inhibited during heat exposure, and it could be argued that NO within the PVN plays some role in this function. This needs further investigation.

Since approximately 12% of the spinally – projecting neurons in the PVN that were activated by thermal stimulation also contained NADPH-d. Eighty-eight per cent of
the spinally-projecting neurons activated must contain some other neurochemical markers. These may include vasopressin and oxytocin, since these are present in a significant proportion of spinally-projecting neurons in the PVN (Cechetto and Saper, 1988; Sawchenko and Swanson, 1982). Indeed up to 40% of those neurons have been reported to contain mRNA for each of those neurochemicals (Hallbeck and Blomqvist, 1999; Hallbeck et al., 2001). Spinally-projecting neurons in the PVN have also been reported to contain various other neurochemicals including, enkephallin, dynorphin and corticotropin releasing factor (Cechetto and Saper, 1988; Hallbeck et al., 2001; Sawchenko, 1987). Angiotensin II might also be a possibility since inhibition of its actions in the central nervous system markedly attenuated the normal increase in mean arterial pressure, heart rate and sympathetic nerve activity to the gut observed following exposure to heat (Kregel et al., 1994).

3.4 PERSPECTIVE

The hypothalamus, particularly the rostral part, has long been recognized as a critical site in thermoregulation. The hypothalamic PVN, however, has been largely ignored, despite anatomical and electrophysiological evidence suggesting that it could contribute to the central pathways mediating thermoregulation. The present study provides evidence for a potential role of the spinally-projecting neurons of the PVN in the response to thermal stimulation. The PVN is known to contribute to the anatomical framework that influences the autonomic nervous system innervating important thermoregulatory organs such as the tail, salivary glands, BAT as well as kidney, gut and the heart (Cano et al., 2003; Hubschle et al., 2001; Morrison, 1999; Oldfield et al., 2002).
Neurons in the PVN that project to the spinal cord contribute to this anatomical framework. We hypothesize that spinally-projecting neurons in the PVN, by influencing sympathetic nerve activity, contribute to the cardiovascular responses elicited by exposure to a high environmental temperature. These responses could include an increased heart rate and visceral vasoconstriction that aids in shunting blood from the viscera to the skin. This suggestion is in agreement with studies showing that stimulation of the PVN can induce increases in heart rate and sympathetic nerve activity to the viscera (Deering and Coote, 2000).

The role of NO in thermoregulation is emerging as a major focus of investigation. The consensus view, at present, is that NO within the central nervous system is critical in heat dissipation (Gerstberger, 1999; Schmid et al., 1998). One of the highest concentrations of NOS-containing neurons within the hypothalamus occurs in the PVN, and the present findings highlight that approximately 40% of the nitrergic neurons in the parvocellular PVN are activated by thermal stimulation and a subpopulation of those neurons project to the spinal cord, suggesting an influence on sympathetic nerve activity that may contribute to the cardiovascular effects elicited by thermal stimulation. However, the ability of NO to diffuse easily across cell membranes suggests its production within the PVN may have more widespread actions during thermal stimulation.

There is increasing evidence suggesting that NO within the PVN may be important in pathological conditions like heart failure. In this condition, there is a reduced
level of NOS in the PVN (Patel et al., 1996) and this is believed to contribute to the autonomic dysfunction that is a characteristic of this debilitating condition. It is of interest to note there have been positive clinical outcomes for heart failure patients treated with thermal therapy (Tei et al., 1995). Based on the present findings, together with those from Patel’s laboratory (Patel et al., 1996; Zhang et al., 1997a; Zhang and Patel, 1998; Zhang et al., 2001) it is tempting to speculate that NO production within the PVN is enhanced by thermal therapy, and this may contribute to the positive influence of this treatment on the symptoms of heart failure.
CHAPTER 4.

EXPOSURE TO A HOT ENVIRONMENT CAN ACTIVATE SPINALLY-PROJECTING AND NITRERGIC NEURONS IN THE BRAINSTEM.
Chapter 4 – Spinally projecting and nitrergic neurons in brainstem following heat exposure.

4.0 INTRODUCTION

Exposure to a hot environment elicits responses that promote heat loss and attempt to maintain body fluid homeostasis. Such responses include an increase in heart rate, increased respiration rate, skin vasodilatation and visceral vasoconstriction, sweating in humans and increased salivary secretion and vasodilatation in the tail of rodents (Kanosue et al., 1994a; Kazuyuki et al., 1998). These responses are critical for the dissipation of heat and they involve autonomic responses mediated by changes in sympathetic nerve activity. These changes are mediated through the central nervous system (Kanosue et al., 1991; Kanosue et al., 1994a; Morrison, 2001c; Nagashima et al., 2000; Owens et al., 2002; Scammell et al., 1993; Zhang et al., 1997b).

It is well established that the central nervous system is essential in the regulation of body temperature and there are several brain regions that contribute to the central nervous system pathways that mediate the thermoregulatory responses. Following an elevation in body temperature, several forebrain areas are activated including the preoptic area, anterior hypothalamus, paraventricular nucleus of the hypothalamus and the periaqueductal gray matter (Bachtell et al., 2003; Boulant, 1981; Boulant, 1998; Bratincsak and Palkovits, 2004; Hori et al., 1999; Kiyohara et al., 1995; Maruyama et al., 2003; McKitrick, 2000; Morimoto and Murakami, 1985; Murakami and Morimoto, 1982; Scammell et al., 1993; Schmid and Pierau, 1993).

Whilst the major integrative centers for temperature regulation are in the hypothalamus and basal forebrain, specific areas of the brainstem have recently been
identified as key regions mediating the cardiovascular responses following hypothermia. In particular the rostral raphe in the midline and parapyramidal regions appear to be critical for the vasoconstriction in the rat’s tail in response to hypothermia (Nakamura et al., 2004; Owens et al., 2002; Rathner et al., 2001). By contrast, only a few studies have reported an activation of neurons in the rostral raphe and parapyramidal regions of the brainstem following heat exposure (Bratincsak and Palkovits, 2004; Kiyohara et al., 1995). Interestingly, the areas of the brainstem which are activated following heat exposure also appear to coincide with regions containing nitrergic neurons (Vincent and Kimura, 1992).

Current evidence suggests that NO in the central nervous system is important in the thermoregulatory pathways mediating heat dissipation (Eriksson et al., 1997; Garthwaite and Boulton, 1995; Gerstberger, 1999; Gourine, 1995; Schmid et al., 1998). For example, blockade of central NO production has recently been found to elevate core body temperature in the rat (Mathai et al., 2004), as well as augment the febrile response elicited by endotoxin and lipopolysaccharide (Gourine, 1995; Steiner et al., 2002). Furthermore, thermal stimulation induces enhanced secretion of saliva, which is spread on the fur to promote heat loss as a means of heat defense in rats (Damas, 1994; Kanosue et al., 1991; Yanase et al., 1991). Inhibition of NO production reduces saliva production during body warming (Damas, 1994). Additionally, we have shown that following heat exposure, approximately 40% of nitrergic neurons in the paraventricular nucleus of the hypothalamus were activated. However, it is unknown whether the nitrergic neurons present in the brainstem are also activated in response to hyperthermia (Cham et al.,
2006). Therefore, the first aim of this study was to determine whether neurons in the brainstem that are activated by acute hyperthermia resulting from exposure to a hot environment were also capable of producing NO.

The distribution of the nitrergic neurons, and the neurons that are activated following hyperthermia, which have been reported in the brainstem, appear to coincide with the ‘autonomic areas’ that contain spinal-projecting neurons. Neurons that project to the IML of the spinal cord, where the sympathetic preganglionic motor neurons are located, can directly influence sympathetic nerve activity and could play important roles in the re-distribution of blood flow, sweating and salivation in response to body temperature changes. Indeed, in chapter 3 we have recently found that 22% of spinally-projecting neurons in the hypothalamic paraventricular nucleus were activated by hyperthermia (Cham et al., 2006). A proportion that is greater than many stimuli examined to date including hemorrhage, and elevated osmolality (Badoer et al., 1993; Kantzides and Badoer, 2003). However, whether spinally-projecting neurons in the brainstem are activated by hyperthermia is unknown. Thus, the second aim of the present study was to determine whether exposure to a hot environment activated brainstem neurons that project to the spinal cord.
Chapter 4 – Spinally projecting and nitrergic neurons in brainstem following heat exposure

4.1 EXPERIMENTAL PROCEDURES

4.1.1 Animals and housing

Briefly, all experimental animals underwent one surgical procedure prior to the experimental day. The surgical procedure involved the placement of the retrogradely-transported tracer into the IML. At least 2 weeks separated the microinjection surgical procedure and the experimental day. Animals are transported to the experimental room 24hrs prior to allow for acclimatizing. Details of all anaesthesia are provided in Chapter 2.

4.1.2 Microinjections of retrogradely transported tracer into the spinal cord.

Under general anaesthesia, the neuronal retrogradely-transported tracer, rhodamine - tagged microspheres (1:1 dilution with 0.9% sterile saline, LumaFluor, NY, USA) was microinjected into the spinal cord. The rats were placed prone and their head was mounted in a Stoelting stereotaxic frame. A midline incision was made in the upper back, and the spinal cord exposed between the T2 and T3 vertebrae. A fine glass micropipette (tip diameter 50-70 μm) filled with the tracer was inserted into the right side of the spinal cord and lowered approximately 0.7mm below the surface. The tip of the micropipette was aimed at the IML of the spinal cord. Unilateral injections of 250nl each were made into three separate anterior-posterior sites within the spinal segment. After each injection, the micropipette was left in place for several minutes prior to its removal to minimize tracer leakage along the route of the micropipette. After the injections, the muscles overlying the spinal cord were sutured and the wound closed. The precise
location of the spinal cord injections were verified histologically at the end of the experiment. Only animals in which the injected tracer covered the IML were used in this study (Refer to Figure 3.1).

### 4.1.3 Experimental Day

On the day of the temperature challenge, animals were randomly assigned to either a heated (N=8) or control group (N=7) and transferred, in their home cages to the temperature chamber (Plexiglas box measuring 75cm x 60cm x 55cm with a metal mesh stage in the bottom). Rats in the heated group were placed in the heating chamber (ambient temperature 38.9±0.1°C) for one hour. Control animals underwent similar procedures with the exception that the temperature chamber was maintained at room temperature (ambient temperature 23±1°C).

Immediately after the temperature challenge, the rats were removed from the chamber and left at room temperature for one hour before being deeply anaesthetized with sodium pentobarbitone and transcardially perfused (Refer to 3.1.3),

### 4.1.4 Detection of Fos by immunohistochemistry

Serial sections of the medulla and pons (40μm) were cut on a cryostat and 1 in 3 sections were collected. To identify activated neurons, immunohistochemistry to detect Fos was performed. The sections were incubated at room temperature and processed...
using standard immunohistochemical procedures as previously described (Kantzides and Badoer, 2003) (Refer to 2.10.1).

4.1.5 Nicotine Adenine Dinucleotide Phosphate-diaphorase (NADPH-d) staining

Immediately after the Fos immunohistochemistry, the sections were incubated in a mixture of 2.5mg Nitroblue Tetrazolium (Sigma Aldrich, Australia), 10mg β-NADPH (Sigma Aldrich, Australia) and 0.2% Triton X-100 in 10ml of 0.05 M Tris buffer (Refer to 2.10.2).

4.1.6 Analysis

Both Fos-positive cell nuclei and NADPH-d positive neurons were identified under normal bright field illumination. Retrogradely labeled neurons were detected by using a fluorescent light source on a microscope fitted with a Rhodamine filter. Double-labeled neurons containing retrogradely-transported tracer and either a Fos-positive nucleus or NADPH-d positive cytoplasm were detected by rapidly switching between the two light sources. Double-labeled neurons containing both a Fos-positive nucleus and NADPH-d positive cytoplasm were detected under normal bright field illumination. Triple-labeled neurons were identified by rapid switching between the bright field and fluorescent light sources.

Labeled neurons were counted unilaterally on the side of the brainstem ipsilateral to the injection site (using 200X magnification). These sections were grouped to represent...
five different rostral-caudal levels covering a total distance of approximately 2.4mm. Each level consisted of four sections, three of which were used for quantification. The levels of the brainstem examined represented approximately 1.2mm to 3.6mm caudal to the interaural line. Each level represented a rostral – caudal distance of between 0.5mm to 0.6mm. Levels 1 and 2 contained the mid to rostral parts of the medulla, whilst levels 3, 4 and 5 encompassed the pontine raphe. For quantification purposes, the brainstem regions were subdivided into 3 areas that encompassed the midline, ventromedial and ventrolateral regions of the brainstem as shown in Figure 4.1. In each region, the number of Fos-positive cell nuclei, NADPH-d positive neurons and retrogradely labeled neurons was counted in each brain section in each animal. The number of multiple-labeled neurons was also counted. In each animal, the number of labeled neurons in each level of the brainstem and the total number of labeled neurons in each region of the brainstem (i.e. overall number) were calculated and averaged for the heated and the control group of animals.

4.1.7 Statistical analysis

The overall mean values in the heated and control groups of rats were compared using the unpaired Student’s T-test. If there was a significant difference overall between the heated and control groups, then comparisons between the groups at each of the five different levels of the brainstem were made using Student’s T-test and applying Bonferroni’s modification to compensate for multiple comparisons. The statistical software package used was GB-STAT® version 7.0 (Dynamic Microsystems Inc., U.S.A), and the level of significance was set at p<0.05.
4.1.8 Mapping

For illustration of the distribution of labeled neurons in the different levels of the brainstem, maps were drawn from a representative section in each of the five rostral to caudal levels. The digital files were generated using the software package MD Plot® (version 4.0) and a MD3 microscope digitizer stage (Minnesota Datametric Corporation, U.S.A.) attached to a Leica DMLB microscope. The individual maps were subsequently imported into CorelDRAW® version 9 to assemble the final figures presented.

4.1.9 Photomicroscopy

Images were acquired using a digital SPOT camera mounted on an Olympus BX60 microscope. The digital images obtained were imported into Adobe Photoshop ® (version 5.5, Adobe Systems Incorporated, U.S.A) and only the contrast and brightness were modified for presentation purposes.
4.2 RESULTS

4.2.1 Midline brainstem

4.2.1.1 Distribution of Fos positive neurons.

Following heat exposure, Fos-positive neurons were observed consistently forming a compact group located throughout the rostral-caudal extent of the raphe pallidus (RPa). Fos positive neurons were also found scattered in the ventral and dorsal parts of the raphe obscurus (ROb). The total number of Fos-positive nuclei counted within the midline of the brainstem in the heated group of animals (138 ± 4) was elevated by fivefold compared with the control group of animals (25 ± 1; P < 0.01). This increase in the production of Fos occurred throughout the rostral-caudal levels of the brainstem examined and was significantly elevated in levels 2 to 5 (Figure 4.2) i.e. the rostral medullary and pontine midline raphe. The maximum number of Fos positive neurons was located predominantly in the pontine levels (Figures 4.2 and 4.5). By contrast, only a small number of Fos-positive neurons were observed in the control group of animals and these were evenly distributed throughout the rostral-caudal extent of the midline areas (Figure 4.2).

4.2.1.2 Distribution of NADPH-d positive neurons.

NADPH-d positive neurons were observed throughout the rostral-caudal extent of the midline brainstem. Some were found in the RPa but the majority of the NADPH-d positive neurons were predominantly located in the ROb (Figures 4.5 and 4.6). The distribution profiles of NADPH-d positive neurons in both the control and the heated
group of animals were similar, with the maximum number occurring in the middle to rostral levels of the midline regions examined (Figure 4.3). On average, a total of 209 ± 18 NADPH-d positive neurons were counted in the heated group which was not significantly different to that of the control group (184 ± 8).

4.2.1.3 Distribution of neurons containing NADPH-d and Fos.

There was a small but statistically significant increase in the average number of neurons within the midline brainstem that were positive for NADPH-d and also contained a Fos-positive nucleus in the heated (3 ± 1) compared to the control group (0.3 ± 0.2; P<0.01) (Figure 4.7). In the heated group of animals, these double-labeled neurons represented less than 2 % of nitricergic neurons counted in the midline. These neurons were located dorsal to the RPa and in the ROb.

4.2.1.4 Distribution of spinally-projecting neurons.

Spinally-projecting neurons were observed in all the rostral-caudal levels of the midline brainstem (Figures 4.4 and 4.5). There was a dense concentration of spinally-projecting neurons in the RPa, whilst in the other midline raphe nuclei, the distribution of the spinally-projecting neurons was more scattered (Figure 4.5). The distribution pattern of these spinally-projecting neurons was similar between the control and the heated groups (Figure 4.4). The maximum number of retrogradely labeled neurons was found in the middle to rostral levels of the midline brainstem (Figure 4.4 and 4.5). On average, a total of 74 ± 2 neurons, were counted in the midline brainstem of the heated group, which was not significantly different from the control group of animals (62 ± 4).
4.2.1.5 Distribution of spinally-projecting neurons that also contained Fos.

After exposure of the animals to the hot environment, there was a small, but statistically, significant increase in the number of spinally-projecting neurons that also contained a Fos-positive nucleus (2 ± 1; P < 0.005, compared with the control group) (Figure 4.7). These double-labeled neurons were located exclusively in the RPa and represented about 3% of the spinally-projecting neurons in the midline brainstem. In the control group, double-labeled neurons were rarely observed (average 0.1 ± 0.1).

4.2.1.6 Distribution of spinally-projecting neurons that contained Fos and NADPH-d.

In both the heated and control group of animals, there were no triple-labeled cells observed at any level of the midline brainstem examined.

4.2.2 Ventromedial brainstem

4.2.2.1 Effect of heating on Fos expression.

Fos expression was markedly increased in the ventromedial brainstem of animals exposed to a hot environment. In the heated group of animals, Fos-positive neurons were located in the ventral gigantocellular reticular nucleus (GiV) and the ventromedial raphe magnus (RMg) (Figure 4.6). Overall, there was a significant eightfold increase in the total number of Fos-positive neurons in the heated group (267 ± 16) compared to control (34 ± 1; P< 0.0001; unilateral counts). This increase in the production of Fos was observed throughout the rostral-caudal extent of the ventromedial brainstem, and was significantly elevated in each of the rostral-caudal levels examined (Figure 4.2). The maximum
number of Fos-positive neurons was found predominantly in the rostral levels of the ventromedial brainstem (Figures 4.2 and 4.5). In the control group of animals, there were few Fos-positive neurons and these were observed scattered throughout the rostrocaudal extent of the ventromedial brainstem (Figure 4.2).

4.2.2.2 Distribution of NADPH-d positive neurons.

NADPH-d positive neurons were observed throughout the rostral-caudal extent of the ventromedial brainstem (Figures 4.3 and 4.5). The NADPH-d positive neurons were observed in a loose cluster in the GiV and became more scattered in the rostral levels of the ventromedial brainstem where the gigantocellular reticular nucleus alpha (GiA) and the RMg are located. The distribution profiles of NADPH-d positive neurons in both the control and the heated group of animals were similar with the maximum number occurring in the middle to rostral levels of the ventromedial brainstem examined (Figure 4.3). On average, a total of 271 ± 8 NADPH-d positive neurons, were counted unilaterally in the heated group of animals which was not significantly different to that of the control group (246 ± 7).

4.2.2.3 Distribution of neurons containing NADPH-d and Fos.

The average number of neurons containing both NADPH-d and Fos within the ventromedial brainstem were significantly different between the heated (12 ± 2) and the control groups (1 ± 0.3; P<0.001; unilateral counts) (Figure 4.7). The maximum number of these double-labeled neurons was located in the mid rostrocaudal level of the ventromedial brainstem. In the heated group, these double-labeled neurons were
predominantly located in the GiA and represented approximately 5% of the nitrergic neurons counted in this brainstem region.

4.2.2.4 Distribution of spinally-projecting neurons.

Spinally-projecting neurons were scattered throughout the rostral-caudal levels of the ventromedial brainstem (Figures 4.4 and 4.5). The average number of neurons in the ventral brainstem that projected to the spinal cord was 145 ± 5 in the heated group which was similar to the control group (149 ± 2; unilateral counts) (Figure 4). The maximum number of retrogradely labeled cells occurred in the rostral levels of the ventromedial brainstem (Figure 4.4).

4.2.2.5 Distribution of spinally-projecting neurons that also contained Fos.

After exposure of the animals to the hot environment, there was a small but statistically significant increase in the number of spinally-projecting neurons that contained a Fos-positive nucleus (4 ± 2; P<0.001, compared with the control group) (Figure 4.7). These double-labeled neurons were found scattered throughout the rostrocaudal extent of the ventromedial brainstem and represented less than 3% of all the spinally-projecting neurons in this region examined. In the control group, there were scarcely any double labeled neurons present (average = 0.1 ± 0.1).

4.2.2.6 Distribution of spinally-projecting neurons that contained Fos and NADPH-d.

There were no triple-labeled neurons present in the ventromedial brainstem in either the heated or control animals.
4.2.3 Ventrolateral brainstem

4.2.3.1 Distribution of Fos positive neurons after heat exposure.

Following heat exposure, the total number of Fos-positive nuclei within the ventrolateral brainstem in the heated group of animals (146 ± 5) was markedly elevated by nine-fold compared with the control group of animals (17 ± 1; P <0.001). This increase in the production of Fos occurred throughout the rostral-caudal levels of the ventrolateral brainstem examined (Figure 4.2). The maximum number of Fos-positive neurons was located predominantly in the caudal levels of the ventrolateral brainstem (Figure 4.2). By contrast, only a small number of Fos-positive neurons were observed in the control group of animals and these were evenly distributed throughout the different rostrocaudal levels (Figure 4.2).

Within the ventrolateral brainstem of the heated group of animals, the Fos-positive neurons were distributed in the lateral paragigantocellular nucleus (LPGi) and included the pressor region of the rostral ventrolateral medulla (RVLM) (Figure 4.5 and 4.6) found immediately caudal of the facial nucleus.

4.2.3.2 Distribution of NADPH-d positive neurons.

NADPH-d positive neurons were observed throughout the rostral-caudal extent of the ventrolateral brainstem (Figure 4.3 and 4.5) and were found clustered predominantly in the LPGi and RVLM (Figures 4.5 and 4.6). The distribution profiles of NADPH-d positive neurons in both the control and the heated group of animals were similar (Figure...
4.3. The total number of NADPH-d positive neurons in the heated group averaged $81 \pm 3$, which was not significantly different to that of the control group ($73 \pm 3$; unilateral counts).

4.2.3.3 Distribution of neurons containing NADPH-d and Fos.

The average number of neurons containing both NADPH-d and Fos within the ventrolateral brainstem was $3 \pm 1$ (unilateral counts) in the heated group (Figure 4.7). In contrast, the control group did not contain any neurons positive for both NADPH-d and Fos (Figure 4.7).

In the heated group, double-labeled neurons were observed in the LPGi and RVLM of the brainstem. These double-labeled neurons represented less than 4% of all the nitrergic neurons in the ventrolateral brainstem.

4.2.3.4 Distribution of spinally-projecting neurons.

Spinally-projecting neurons were distributed in all the rostral-caudal levels of the ventrolateral brainstem (Figures 4.4 and 4.5). The maximum numbers of retrogradely labeled cells were located in the mid rostrocaudal levels of the ventrolateral brainstem (Figures 4.4 and 4.5). The average number of neurons in the ventrolateral brainstem that projected to the spinal cord was $83 \pm 1$ in the heated group and $83 \pm 2$ in the control group (unilateral counts).
4.2.3.5 Distribution of spinally-projecting neurons that also contained Fos.

After exposure of the animals to the hot environment, there was a small but statistically significant increase in the number of spinally-projecting neurons that contained a Fos-positive nucleus (2 ± 2; P < 0.001, compared with the control group 0.3 ± 0.3) (Figure 4.7). These double-labeled neurons represented approximately 2% of all the spinally-projecting neurons in the ventrolateral brainstem.

4.2.3.6 Distribution of spinally-projecting neurons that contained Fos and NADPH-d.

In both heated and control group of animals, there were no triple-labeled cells observed in the ventrolateral brainstem.
Figure 4.1

Schematic drawing of a coronal section from the brainstem of a rat illustrating the three different regions, defined as midline brainstem (A), ventromedial brainstem (B) and the ventrolateral brainstem (C), in which the labeled cells were counted.

Abbreviations: NSPV, spinal nucleus of the trigeminal nerve; NA, nucleus ambiguous; PYR, pyramidal tract.
Figure 4.2

The average number of Fos-positive cell nuclei counted on the side ipsilateral to the microinjection site in five rostrocaudal levels of the midline brainstem (upper panel), ventromedial brainstem (middle panel) and the ventrolateral brainstem (lower panel). Experiments were performed in conscious rats placed into a hot environment (39°C) for 60 minutes (solid columns) or control rats left at room temperature (open columns). * P<0.05 compared to respective control.
Figure 4.3

The average number of NADPH-d-positive neurons counted on the side ipsilateral to the spinal microinjection site in five rostrocaudal levels of the midline brainstem (upper panel), ventromedial brainstem (middle panel) and the ventrolateral brainstem (lower panel). Solid columns show data from rats which were placed in a heated environment for 60 minutes. Open columns represent data from controls.
Chapter 4 – Spinally projecting and nitrergic neurons in brainstem following heat exposure.

Figure 4.4

The average number of spinally-projecting neurons counted on the side ipsilateral to the spinal microinjection site in five rostrocaudal levels of the midline brainstem (upper panel), ventromedial brainstem (middle panel) and the ventrolateral brainstem (lower panel). Solid columns show data from rats which were placed in a heated environment for 60 minutes. Open columns represent data from controls.
Chapter 4 – Spinally projecting and nitrergic neurons in brainstem following heat exposure.

133

Figure 4.5

Diagrammatic illustration of the distribution of Fos-positive cell nuclei (left panel), NADPH-d positive neurons (second panel) and spinally-projecting neurons (right panel) of the rat brainstem. Five different rostral-caudal levels are shown. These levels
correspond to those levels that are presented in Figures 4.2, 4.3 and 4.4. Data is from a representative animal placed into a hot environment (39°C) for 60 minutes. Illustrations on the right panel schematically show anatomical nuclei.

Abbreviations: NSPV, spinal nucleus of the trigeminal nerve; NA, nucleus ambiguus; PYR, pyramidal tract; ION, inferior olivary nucleus. GiV, gigantocellular nucleus – ventral; GiA, gigantocellular nucleus- alpha; RMg, raphe magnus; Rob, raphe obscurus; Rpa, raphe pallidus; LPGi, lateral paragigantocellular nucleus, RVLM, rostral ventrolateral medulla.
Figure 4.6

High magnification photomicrographs of the coronal section of rat brainstem illustrating Fos-positive nuclei (A, C, E) and NADPH-d positive neurons (B, D, F) in the midline brainstem (A + B), ventromedial brainstem (C + D), ventrolateral brainstem (E + F) respectively. The insert in each panel is a low magnification photomicrograph illustrating
the location (boxed outline) from which the respective high magnification photomicrograph was obtained. Bar = 80 μm in A, B, E, F and 40 μm in C and D.
Figure 4.7

Average numbers of NADPH-d-positive neurons that also contained a Fos-positive nucleus (top panel) and spinal-projecting neurons that also contained a Fos-positive nucleus (lower panel) in the midline, ventromedial and ventrolateral brainstem (see Figure 1 for details). Solid columns show data from rats which were placed in a heated environment for 60 minutes. Open columns represent data from controls. * P<0.05 compared to respective control.
4.3 DISCUSSION

In the present study, we have provided a detailed examination of the rostral-caudal distribution of activated neurons in the ventral brainstem following exposure to a hot environment. We have described the first detailed quantification of activated neurons (Fos-positive) in these areas examined. We found a significant increase in Fos production in the midline (by five-fold), ventromedial (by eight-fold) and in the ventrolateral (by nine-fold) brainstem. Some of these neurons were nitrergic, particularly in the ventromedial brainstem, or were spinally-projecting neurons. There were no triple labeled neurons at any level of the brainstem examined.

The significant increase in Fos production observed in the midline regions corresponded to increases in the raphe pallidus and raphe obscurus. This is in agreement with earlier studies using different heating regimes (Bratinsak and Palkovits, 2004; Kiyohara et al., 1995). Midline raphe neurons are also activated by exposure to a cold environment. Indeed, their role in cutaneous vasoconstriction has been well studied (Blessing, 2003; Ootsuka et al., 2004). Thus, the present findings together with studies investigating the effects of cooling suggest that there are neurons in the midline raphe that are activated by elevations in body temperature, and thus may contribute to the central pathways involved in the responses elicited by a hot environment, and conversely, there are neurons within the same region that may contribute to the central pathways mediating responses initiated by a cold environment. These neurons are most likely to be
distinct populations since brainstem neurons activated by heating (i.e. in the present work) would not be expected to be activated during cooling.

Fos production was significantly elevated at all rostral-caudal levels of the ventromedial brainstem which encapsulated the rostral ventromedial medulla and the raphe magnus. Our results suggest that the activated neurons were evenly spread throughout the rostral - caudal extent of this region. This contrasted with the midline where Fos production was maximal at the more rostral levels examined. Our results suggest that neurons in the raphe magnus and rostral ventromedial medulla contribute to the pathways activated by elevations in body temperature. Since cooling also activates neurons in these areas, this suggests these regions are involved in responses initiated by either decreases or increases in body temperature (Bratincsak and Palkovits, 2004; Kiyohara et al., 1995; Nakamura et al., 2004). In the ventrolateral brainstem, Fos-positive cells were predominantly located in the caudal levels examined which corresponded to the rostral ventrolateral medulla.

The function of the activated neurons in the ventral brainstem examined in the present study cannot be determined by the present work. However, it is possible that these activated neurons may contribute to the pathways mediating vasoconstriction of the blood vessels supplying the internal organs such as the gut and kidney, which redirects blood flow to the skin to enable heat dissipation. The Fos-positive neurons may also include neurons that contribute to sweating, and the profuse salivation that occurs in the rat. It is also possible that the activated neurons may represent interneurons that
ultimately contribute to the vasodilation of the skin vasculature (by inhibiting the sympathetic vasoconstrictor activity) that occurs in response to heating. Undoubtedly, it is also possible that some of the activated neurons in the ventral brainstem could also be cardioacceleratory neurons (Farkas et al., 1998). In addition, one could not exclude the likelihood that some of these activated neurons may inhibit sympathetic cutaneous activity (Stornetta et al., 2004).

NADPH-diaphorase positive neurons were found throughout the ventral brainstem examined. The neurons were found in regions that encompassed the rostral ventromedial medulla, raphe obscurus, raphe magnus and RVLM as previously described (Vincent and Kimura, 1992). The nitrergic neurons were found in areas in which activated neurons were also located. Following exposure to the hot environment we found a small but statistically significant increase in the number of activated nitrergic neurons in the midline and ventromedial brainstem. In the midline the activated nitrergic neurons represented less than 2% of the nitrergic neurons. In the ventromedial brainstem the nitrergic neurons activated by heating represented almost 5% of the nitrergic neurons counted in this region of the brainstem. Nitric oxide within the central nervous system appears to play a key role in heat dissipation (Eriksson et al., 1997; Gerstberger, 1999; Gourine, 1995; Schmid et al., 1998; Simon, 1998) and these activated nitrergic neurons in the brainstem may contribute to this. However, based on the quantitative analysis presented in the present study, the contribution is likely to be small. This is in stark contrast to the findings in the hypothalamus, in particular in the hypothalamic
paraventricular nucleus, in which we previously found almost 40% of nitrergic neurons activated by exposure to a similarly hot environment (Cham et al., 2006).

Spinally-projecting neurons were found in each region of the brainstem examined and these included those found in the midline raphe, the rostral medial raphe magnus and the RVLM. Exposure to a hot environment increased the number of spinally-projecting neurons activated. These activated neurons represented 2-3% of the spinally-projecting neurons counted in the brainstem. If these neurons contribute to the redistribution of blood flow from the internal organs to the skin that occurs during heating, the increase, though statistically significant, was not impressive. The regions of the brainstem examined included the RVLM, which is known to contain neurons that can increase sympathetic nerve activity innervating the vasculature of internal organs like the kidney and mesenteric beds. Thus, it is possible that, although small, the spinally-projecting neurons in the brainstem activated by heating may represent an important population of neurons involved in the cardiovascular responses designed to dissipate heat when body temperature rises.

The activated spinally-projecting neurons may also represent neurons that mediate the increased sympathetic nerve activity that contributes to sweating and, more importantly in rats, salivation in response to heating. Spreading saliva over the body surface is an important mechanism for dissipating heat in rats, and the rats used in the present study exhibited this response.
It is interesting that the majority of spinally-projecting neurons in the ventral medulla were not activated following the hot environment stimulus. Perhaps the spinal injections did not adequately label the spinally-projecting neurons that play a role in the vasoconstriction of the internal vasculature that contributes to the redistribution of blood flow. Alternatively, the finding could suggest that neurons in the supra-medullary / pontine regions may be more important in the heat-induced blood flow redistribution than previously realized. It is noteworthy that we have recently found that 22% of the spinally-projecting neurons in the paraventricular nucleus of the hypothalamus were activated by elevations in core body temperature (Cham et al., 2006), more than with other strong stimuli such as severe haemorrhage (Badoer et al., 1993). However, functional studies have suggested that suprapontine regions may not be required for the full expression of the increases in the renal, splanchnic, lumbar and splenic sympathetic nerve activities elicited by elevations in body temperature (Kenney et al., 2000). By contrast, in senescent rats, suprapontine regions appear to be critical contributors to the splanchnic sympathetic nerve responses elicited by heating (Kenney and Fels, 2002).

Thus, the role of suprapontine regions in the cardiovascular responses initiated by elevations in body temperature require further clarification.

Generally, spinally-projecting neurons were not nitrergic since there were only a few that contained NADPH-diaphorase. This is consistent with the finding that the spinally projecting adrenergic C1 neurons located in the RVLM are not nitrergic (Iadecola et al., 1993). Furthermore, none of the nitrergic spinally-projecting neurons in the ventral brainstem were activated by heating. Thus, the increase in activated nitrergic
neurons observed in response to the elevated body temperature occurred in neurons that projected to regions other than the spinal cord.

4.4 CONCLUSIONS

In conclusion, exposure to a hot external environment for one hour activates neurons in the medulla and pons in regions that contain nitrergic neurons or spinally-projecting neurons. Some nitrergic neurons, particularly in the ventromedial brainstem were activated. Spinally-projecting neurons in the brainstem were also activated. However, there were no nitrergic spinally-projecting neurons activated. We hypothesize that nitrergic neurons, as well as spinally-projecting (non-nitrergic) neurons in the brainstem may contribute to the cardiovascular responses elicited by an acute exposure to a hot environment, but their contribution is likely to be small and not as great as similar as that of those neurons found in the hypothalamus.
CHAPTER 5.

EXPOSURE TO A HOT ENVIRONMENT CAN ACTIVATE RVLM-PROJECTING NEURONS IN THE HYPOTHALAMIC PVN IN CONSCIOUS RATS.
5.0 INTRODUCTION

A major integrative site within the brain for autonomic function is the hypothalamic paraventricular nucleus (PVN). It is involved in regulating sympathetic nerve activity and blood flow in response to a variety of stimuli including volume expansion and haemorrhage (Badoer et al., 1993; Badoer, 1996; Kantzides and Badoer, 2003; Kantzides et al., 2005), and may play a role in regulating body temperature (Hubschle et al., 2001; Inenaga et al., 1987; Murakami and Morimoto, 1982; Oldfield et al., 2002).

Exposure to a hot environment elicits reflex responses that promote heat loss. Such responses include an increase in heart rate, increased respiration rate, vasodilatation of the skin vasculature, vasoconstriction of the visceral vasculature, sweating in humans and increased salivary secretion and vasodilatation in the tail of rodents (Kanosue et al., 1994a; Kazuyuki et al., 1998), all of which are designed to counteract the disturbance in body temperature. The redistribution of blood flow from the viscera to the skin is critical for the dissipation of heat and this involves autonomic cardiovascular responses which are mediated by changes in sympathetic nerve activity. These changes occur through the CNS (Kanosue et al., 1991; Morrison, 2001a; Nagashima et al., 2000; Owens et al., 2002; Scammell et al., 1993).

Several brain regions contribute to the thermoregulatory responses. Of particular interest is the hypothalamic PVN, which may be an important site integrating the peripheral neural components of the thermoregulatory responses (Bachtell et al., 2003;
Bratincsak and Palkovits, 2004; Harikai et al., 2003; Inenaga et al., 1987). Circumstantial evidence suggesting the PVN may contribute to the cardiovascular changes elicited by the disturbances in body temperature include; (i) thermosensitive neurons are present in the PVN (Inenaga et al., 1987) and (ii) PVN neurons project to autonomic nuclei in the CNS that influence sympathetic nerve activity to important thermoregulatory effector organs such as the brown adipose tissue and the vasculature of the rat tail, salivary gland, as well as kidney and gut (Hubschle et al., 2001; Morrison, 1999; Oldfield et al., 2002). Furthermore, neurons within the PVN are activated following an elevation in body temperature (Bachtell et al., 2003; Bratincsak and Palkovits, 2004; Harikai et al., 2003; Kiyohara et al., 1995; McKitrick, 2000).

The hypothalamic PVN is composed of functionally different subgroups of neurons such as the parvocellular neurons that project to important autonomic targets that, in addition to the spinal - projecting neurons, also include neurons that project to pressor region of the rostral ventrolateral medulla (RVLM) (Pyner and Coote, 2000; Shafton et al., 1998; Swanson and Kuypers, 1980). The RVLM is a critical autonomic region that projects directly to the sympathetic preganglionic motor neurons in the IML and is critical for the tonic regulation of sympathetic nerve activity (Dampney, 1994). Destruction or neuronal inhibition of the RVLM produces dramatic reductions in sympathetic nerve activity, whilst activation of the RVLM elicits marked increases in sympathetic nerve activity to the vasculature. Stimulating the PVN elicits marked changes in sympathetic nerve activity and the anatomical projections from the PVN to the spinal cord or to the RVLM undoubtedly contribute to those changes (Tagawa and
As indicated earlier, we have previously shown that spinal projecting neurons are activated in response to the exposure to a hot environment, however, whether neurons in the PVN that send projections to the RVLM are also part of the central pathways activated by an elevation in body temperature has not been examined to date. Therefore, the primary aim of the present study was to determine whether RVLM-projecting neurons in the PVN are activated by placing conscious rats into a hot environment.

Nitric oxide is an important neurotransmitter both in the periphery and in the central nervous system. Nitric oxide, unlike conventional neurotransmitters, does not act on membrane bound receptors, and, as it is easily diffusible through cell membranes, its actions can be widespread by acting on neurons within its diffusion range. Nitric oxide production occurs in areas known to have a thermoregulatory function such as the preoptic area, the dorsal horn of the spinal cord and in the hypothalamus and ventrolateral medulla. There is growing evidence that nitric oxide plays a role in thermoregulation but the exact function of nitric oxide may depend on its specific sites of action (Gerstberger, 1999). However, microinjection of nitric oxide donors into the third ventricle induces an integrated response involving a reduction in core body temperature and a rise in skin temperature, suggesting an important role in heat dissipation (Eriksson et al., 1997). Inhibition of nitric oxide production attenuates heat loss and results in an increase in body temperature (Mathai et al., 2004). Additionally, inhibition of NO production reduces saliva production during body warming (Damas, 1994); hyperthermia induces enhanced secretion of saliva, which is spread on the fur to promote heat loss in rats (Damas, 1994;
Kanosue et al., 1991; Yanase et al., 1991). Thus, current evidence suggests that nitric oxide (NO) in the central nervous system is important in the thermoregulatory pathways mediating heat dissipation (Eriksson et al., 1997; Garthwaite and Boulton, 1995; Gerstberger, 1999; Gourine, 1995; Mathai et al., 1997; Schmid et al., 1998; Simon, 1998).

There is a dense concentration of neurons containing nitric oxide synthase (NOS), the enzyme responsible for the production of NO, in the PVN. As discussed in Chapter 3, we have previously shown that exposure to a hot environment activates nitrergic neurons in the PVN (Cham et al., 2006). However, it is unknown whether the nitrergic neurons in the hypothalamic PVN activated in response to hyperthermia includes nitrergic neurons that project to the RVLM. Thus, the second aim of the present work was to determine whether neurons in the PVN that project to the RVLM, and were activated by a hot environment, were also nitrergic.
5.1 EXPERIMENTAL PROCEDURES

5.1.1 Animals and housing

Briefly, all experimental animals underwent one surgical procedure prior to the experimental day. The surgical procedure involved the placement of the retrogradely-transported tracer into the RVLM. At least 2 weeks separated the microinjection surgical procedure and the experimental day. Animals are transported to the experimental room 24hrs prior to allow for acclimatizing. Details of all anaesthesia are provided in Chapter 2.

5.1.2 Microinjections of retrogradely transported tracer into the RVLM.

Under general anaesthesia, the right femoral artery was cannulated to enable blood pressure monitoring. The animals were placed prone and their head was mounted in a Stoelting stereotaxic frame such that both bregma and lambda were positioned on the same horizontal plane. A burr hole, approximately 4 to 5mm in diameter, was drilled into the occipital bone on the left hand side of the skull approximately 2mm lateral of the mid-sagittal suture and 3mm caudal of the lambdoid suture. The pressor region of the RVLM was identified functionally by microinjection of 25 to 50 nl of L-glutamate (0.1M) using a fine glass micropipette (tip diameter of 50-70 μm), which elicited a minimum increase of 20mm Hg in arterial pressure. The precise location of the microinjections was verified histologically at the end of the experiment (Figure 5.1). Only animals in which the injected tracer covered the RVLM were used in this study. Typically, the co-ordinates of the pressor area of the RVLM were 1.8 to 2.2 mm lateral of the mid-sagittal suture, 2.5 to
3.5 mm caudal of the lambdoid suture and 8.9 mm ventral of the cerebellar surface. After locating the pressor region, the pipette was carefully withdrawn, filled with the neuronal retrogradely-transported tracer, rhodamine- tagged microspheres (1:1 dilution with 0.9% sterile saline, LumaFluor, NY, USA) and then re-inserted into the pressor region of the RVLM. After the injection, the micropipette was left in place for 10 minutes prior to its removal to reduce tracer spread along the route of the micropipette. After the micropipette was removed, the skin overlying the skull was sutured and the wound closed. Finally, the arterial cannula was carefully removed from the femoral artery to minimize blood loss and the wound was sutured closed. The animal was subsequently given antibiotic and then analgesic and allowed to recover.

5.1.3 Experimental Day

Two weeks elapsed after the microinjection of the tracer to allow for its transport. The rats were then placed into the experimental room 24 hours preceding the experimental day. On the day of the temperature challenge, animals were randomly assigned to either a heated (N=6) or control group (N=6) and transferred, in their home cages to the temperature chamber (Plexiglas box measuring 75cm x 60cm x 55cm with a metal mesh stage in the bottom). Rats were placed into the heating chamber (ambient temperature 38.9 ± 0.1°C) for a duration of one hour. Control animals underwent similar procedures except the temperature chamber was maintained at room temperature (ambient temperature 23.0 ± 1.0°C).
Immediately after the temperature challenge, the rats were removed from the chamber and kept at room temperature for one hour before being deeply anaesthetized with sodium pentobarbitone and transcardially perfused with approximately 350-400 ml of phosphate buffered saline (PBS), followed by 4% paraformaldehyde in phosphate buffer (PB, 0.1M, pH 7.4). The perfusion pressure was maintained at about 100-120 mmHg. The brains were then carefully removed and stored in the fixative solution for at least two hours before being transferred into PB containing 20% sucrose solution and left overnight.

5.1.4 Detection of Fos by immunohistochemistry

Serial sections of the hypothalamic PVN (40 microns thick) were cut on a cryostat and 1 in 3 serial sections were collected. To identify activated neurons, immunohistochemistry to detect Fos was performed on the sections incubated at room temperature and processed using standard immunohistochemical procedures as previously described (Refer to 2.10.1).

5.1.5 Nicotine Adenine Dinucleotide Phosphate-diaphorase (NADPH-d) staining

NADPH-d staining was used as a marker of nitric oxide synthase in cells. Immediately after the immunohistochemistry procedure to detect Fos, the sections were incubated in a mixture of 2.5mg Nitroblue Tetrazolium (Sigma Aldrich, Australia), 10mg β-NADPH (Sigma Aldrich, Australia) and 0.2% Triton X-100 in 10ml of 0.05 M Tris buffer (Refer to 2.10.2).
5.1.6 Analysis

Both Fos-positive cell nuclei and NADPH-d positive neurons were identified under normal bright field illumination. Retrogradely labeled neurons were detected by using a fluorescent light source on a Leica DMLB microscope fitted with a Rhodamine filter. Double-labeled neurons containing retrogradely-transported tracer and either a Fos-positive nucleus or NADPH-d positive cytoplasm were detected by rapidly switching between the two light sources. Double-labeled neurons containing both a Fos-positive nucleus and NADPH-d positive cytoplasm were detected under normal bright field illumination. Triple-labeled neurons were identified by rapid switching between the bright field and fluorescent light sources.

Labeled neurons were counted unilaterally on the side of the PVN ipsilateral to the injection site (using 200X magnification), in sections which were grouped to represent five different levels encompassing the entire rostral-caudal extent of the PVN. Each level consisted of three sections, two of which were used for quantification. The data were expressed as the average number per section at each level. The overall means of Fos-positive cell nuclei, NADPH-d positive neurons and retrogradely labeled neurons for each group of animals were calculated and compared between the heated and control groups. The overall means of multiple-labeled neurons were also calculated.
5.1.7 Statistical analysis

The overall mean values in the heated and control groups of rats were compared using the unpaired Student’s T-test. If there was a significant difference overall then comparisons of each of the five different levels of the PVN were compared between the groups using Student’s T-test and applying Bonferroni’s modification to compensate for multiple comparisons. The statistical software package used was GB-STAT® version 7.0 (Dynamic Microsystems Inc., U.S.A), and the level of significance was set at p<0.05.

5.1.8 Mapping

For illustration of the distribution of labeled neurons in the different levels of the PVN, maps were drawn from representative sections in each of the five rostral to caudal levels. The digital files were generated using the software package MD Plot® (version 4.0) and a MD3 microscope digitizer stage (Minnesota Datametric Corporation, U.S.A.) attached to a Leica DMLB microscope. The individual maps were subsequently imported into CorelDRAW®9 to assemble the final figures presented.

5.1.9 Photomicroscopy

Images were acquired using a digital SPOT camera mounted on an Olympus BX60 microscope. The digital images obtained were imported into Adobe Photoshop ® (version CS, Adobe Systems Incorporated, U.S.A) and only the contrast and brightness were modified for presentation purposes.
5.2 RESULTS

5.2.1 Effect of heating on Fos expression in the PVN.

Following exposure to a hot environment, the total number of Fos-positive nuclei within the PVN in the heated group of animals (1290 ± 21, unilateral counts) was significantly elevated by twelve-fold compared with the control group of animals (105 ± 17; P<0.0001). This increase in the production of Fos occurred throughout the rostral-caudal levels of the PVN examined, with the maximum number of Fos-positive nuclei found predominantly in the middle to caudal levels of the PVN (Figures 5.2 and 5.3). Whilst Fos-positive cell nuclei were present in both magnocellular and parvocellular regions, they were only quantitated in the parvocellular region of the PVN. Within this region of the PVN, Fos-positive cells were distributed in the dorsal, medial and lateral subnuclei of the PVN (Figure 5.3).

By contrast, only a small number of Fos-positive neurons were observed in the control group of animals and these were evenly distributed throughout the rostral-caudal extent of the PVN (Figure 5.2).

5.2.2 Distribution of RVLM - projecting neurons in the PVN.

RVLM - projecting neurons were observed in all the rostral-caudal levels of the parvocellular PVN (Figures 5.2 and 5.3). The distribution pattern of these RVLM - projecting neurons was similar between the control and the heated groups (Figure 5.2). The maximum numbers of retrogradely labeled neurons were found in the middle to
caudal levels of the PVN (Figure 5.2 and 5.3). On average, a total of 155 ± 5 neurons projecting to the RVLM, were counted in the PVN of the heated group, which was not significantly different from the control group of animals (145 ± 5, unilateral counts) (Figure 5.2).

### 5.2.3 Distribution of RVLM - projecting neurons that also contained Fos.

After exposure of the animals to the hot environment, there was a significant increase in the number of RVLM - projecting neurons that also contained a Fos-positive nucleus (13 ± 1; P < 0.0001, compared with the control group, (2 ± 1)) (Figure 5.2). These double-labeled neurons represented about 8.2 ± 0.6% of the RVLM - projecting neurons counted in the PVN and were found predominantly in the middle levels of the PVN (Figures 5.2 and 5.3). In the control group, there were very few RVLM - projecting neurons that contained a Fos-positive nucleus. These double-labeled neurons represented approximately 1.5% of the RVLM - projecting neurons in the PVN.

### 5.2.4 Distribution of neurons containing NADPH-d.

NADPH-d positive neurons were observed throughout the rostral-caudal extent of the PVN (Figures 5.3 and 5.4). The distribution profiles of NADPH-d positive neurons in both the control and the heated group of animals were similar (Figure 5.4). On average, a total of 513 ± 10 NADPH-d positive neurons were counted unilaterally in the heated group which was not significantly different to that of the control group (486 ± 15).
5.2.5 Distribution of neurons containing NADPH-d and Fos.

In the heated group, the average number of neurons in each animal, containing both NADPH-d and Fos in the PVN (157 ± 6) was significantly elevated by 16-fold, compared with the control group in which there was on average a total of only 10 ± 0.5 NADPH-d positive neurons that exhibited a Fos-positive nucleus (P<0.0001) (Figure 5.4). In the heated group of animals, these double-labeled neurons represented approximately 31% of all the NADPH-d positive neurons counted in the PVN. This increase occurred throughout the rostral – caudal extent of the PVN, with the maximum number found predominantly in the middle to caudal levels of the PVN (Figure 5.4).

5.2.6 Distribution of RVLM – projecting neurons containing NADPH-d.

After the exposure of the animals to a heated environment, the number of RVLM - projecting neurons containing NADPH-d (6 ± 1) represented approximately 4% of the RVLM – projecting neurons in the PVN. These double-labeled neurons were found primarily in the middle to caudal levels of the PVN (Figure 5.4). A similar distribution profile was also observed with the control group of animals (Figure 5.4). The numbers of double - labeled neurons in the control group (6 ± 1) was not significantly different than that in the heated group of animals (Figure 5.4).
5.2.7 Distribution of RVLM - projecting neurons that contained Fos and NADPH-d.

In the heated group of animals, triple-labeled neurons were rare and the average number of triple-labeled cells in the entire PVN was 1 ± 0.5. In the control group of animals, no triple-labeled cells were observed at any levels of the PVN examined.
Figure 5.1

Photomicrographs of transverse sections of the RVLM injection site. The left panel (A) shows the section viewed with phase contrast illumination. The right panel (B) shows the same section viewed under fluorescent lighting conditions to visualise the spread of the fluorescent beads. The RVLM is outlined by the dashed lines. Bar = 0.25 mm

Abbreviations: ION, inferior olivary nucleus; NA, nucleus ambiguous; Sp5, spinal trigeminal nucleus; Sp, spinal trigeminal tract; Pyr, pyramidal tract.
Figure 5.2

Average numbers of Fos-positive cell nuclei, RVLM-projecting neurons and double-labeled neurons counted on the side ipsilateral to the RVLM micro-injection site in five rostral-caudal levels of the PVN. Experiments were performed in conscious rats placed into a hot environment (39°C) for 60 minutes (solid columns) or rats left at room temperature (open columns). * P< 0.05 compared to respective controls.
Figure 5.3

Diagrammatic illustration of the distribution of Fos-positive cell nuclei, RVLM-projecting neurons and of NADPH-diaphorase – positive neurons (NADPH-d) in the subnuclei of the hypothalamic paraventricular nucleus. Five different rostral (A) - caudal (E) levels are shown and the approximate anterior – posterior levels caudal to bregma in mm is indicated on the right. Abbreviations: III, third ventricle; ap, anterior parvocellular PVN; dp, dorsal parvocellular PVN; mp, medial parvocellular PVN; pm, magnocellular PVN; lp, lateral parvocellular PVN. For simplicity, not all cells could be represented by dots in regions of high density. Data is from a representative animal placed into a hot environment (39°C) for 60 minutes.
Figure 5.4

Average numbers of NADPH-diaphorase – positive neurons (NADPH-d), Fos-positive NADPH-d – positive neurons, RVLM-projecting neurons containing NADPH-d counted on the side ipsilateral to the RVLM micro-injection site in five rostral - caudal levels of the PVN. Experiments were performed in conscious rats placed into a hot environment (39°C) for 60 minutes (solid columns) or rats left at room temperature (open columns).

* P< 0.05 compared to respective controls.
**Figure 5.5**

Photomicrographs of the hypothalamic paraventricular nucleus (PVN). Panel A is from a control rat and shows the PVN outlined. Blue cells are NADPH-diaphorase–positive neurons. Panel B is a similar level of the PVN from a rat placed into a hot environment (39°C) for 60 minutes showing the dramatic increase in Fos–positive nuclei (brown dots). Panel C is the same section as in panel B viewed using fluorescent lighting to show neurons projecting to the RVLM. Neurons highlighted are shown in higher magnification in the insert in the right lower corner. The arrow shows a NADPH–diaphorase positive neuron containing a Fos–positive nucleus and represents an activated nitricergic neuron. The arrow head shows a RVLM–projecting neurons containing a Fos-positive nucleus and represents an activated RVLM–projecting neuron. Bar in A represents 100 microns for all panels and 30 microns for the inserts.

Abbreviations: III, third ventricle.
5.3 DISCUSSION

The present study provides two key findings. Firstly, exposure of conscious animals to a heated environment of 39°C significantly increased the number of RVLM – projecting neurons in the PVN that also contained a Fos – positive nucleus; these were predominantly located in the middle rostrocaudal levels of the PVN examined. Secondly, although there was a significant increase in the number of nitric PVN neurons that expressed Fos, triple labeled neurons (i.e. Fos-positive, NADPH-d positive and RVLM - projecting) were rare in any level of the hypothalamic PVN examined.

In the present study, we also found that following heat exposure, in conscious rats, there was a marked increase in the number of Fos-positive cell nuclei observed in all sub-divisions of the PVN. The number of activated neurons peaked in the mid to caudal levels of the parvocellular PVN which is in agreement with findings from chapter 3 (Cham et al., 2006). This increased production of Fos following heat exposure is also in agreement with earlier studies emanating from other laboratories (Bachtell et al., 2003; Bratinesak and Palkovits, 2004; Harikai et al., 2003; Kiyohara et al., 1995; McKitrick, 2000), but there have also been previous reports that have not detected an increase in Fos production in the PVN following an elevated body temperature (Patronas et al., 1998). These discrepancies may be attributed to the differences in species used, the duration of heat exposure, different heating regimes and the degree to which body temperature was elevated. In general, studies that have elicited marked activation of the neurons in the parvocellular PVN have used a higher environmental temperature (Bratinesak and Palkovits, 2004; Harikai et al., 2003).
One of the most important findings of the present study is the activation of PVN neurons projecting to the RVLM. We found that approximately 8% of those neurons were activated following exposure to a hot environment. This is considerably lower than the proportion of the spinally-projecting neurons in the PVN activated by a similar heating stimulus (Cham et al., 2006). Whilst the current study indicates that central pathways other than the spinal – projecting pathways emanating from the PVN are involved in the responses elicited by heat exposure, the relatively smaller proportion of activated PVN neurons projecting to the RVLM compared with the proportion projecting to the spinal cord could suggest that the RVLM - projecting neurons in the PVN may make a smaller contribution to the cardiovascular responses initiated by heat. Indeed, the results in Chapter 4 showed a higher proportion of Fos-positive cells in the rostral ventromedial medulla indicating the possibility that perhaps a higher proportion of Fos-positive neurons in the PVN projects to the rostral ventromedial medulla. Further experiments are necessary to elucidate this.

The RVLM is a brain nucleus that is important in the tonic regulation of sympathetic nerve activity. Present evidence suggests that the pathway from the PVN to the RVLM may be predominantly excitatory (Coote et al., 1998; Tagawa and Dampney, 1999). Since activation of the PVN can elicit sympatho – excitatory effects and elevations in blood pressure, it is possible that activation of the neurons in the PVN that project to the RVLM could mediate the increase in sympathetic nerve activity to the visceral vasculature, including the mesenteric and renal beds. This effect would
contribute to the redistribution of blood flow to the peripheral vasculature to enable heat to dissipate when body temperature rises. Similarly, heart rate is increased in response to heating, and activation of an excitatory pathway from the PVN to the RVLM could contribute to the tachycardia. Further studies are needed to explore these possibilities.

Given that only approximately 8% of PVN neurons projecting to the RVLM were activated by the heat stimulus, the results suggest that: either (i) the activated neurons, although representing a small proportion of the population of PVN neurons that project to the RVLM, may be critical contributors to the cardiovascular responses, or, alternatively, (ii) activation of this pathway is not a major requirement in mediating the reflex responses to heat, and perhaps the pathway may have a greater involvement in responses to other stimuli. Indeed, there is evidence indicating that the pathway from the PVN to the RVLM mediates reflex responses induced by haemorrhage, dehydration and simulated volume expansion (Badoer et al., 1993; Kantzides and Badoer, 2003; Stocker et al., 2004). The present results may indicate that the large majority of PVN neurons projecting to the RVLM (>90%) that do not express detectable Fos are actively inhibited by the heating stimulus.

In the present work we used an ambient temperature of 39 °C which is known to elevate body temperature and plasma osmolality and reduce body fluid. Since, an elevation in plasma osmolality is known to activate neurons in the PVN (Kantzides and Badoer, 2003; Oldfield et al., 1991; Stocker et al., 2004), one could argue that this stimulus is responsible for the activation of the RVLM - projecting neurons in the PVN.
However, we have previously shown that an intravenous infusion of hypertonic saline does not activate RVLM - projecting neurons in the PVN (Kantzides and Badoer, 2003). Thus, it is unlikely that the increase in osmolality that accompanies exposure to a hot environment could account for the increased activation of RVLM - projecting PVN neurons. Whether the reduction in body fluid per se contributes to the response is difficult to determine. Haemorrhage activates a population of PVN neurons that project to the RVLM, but as hypotension was also induced, it is not possible to differentiate the responsible stimulus (Badoer and Merolli, 1998). However, we have previously shown that replacing lost fluid during the heating stimulus does not reduce the number of activated neurons in the PVN indicating that elevating body temperature without fluid loss can activate PVN neurons (Cham et al., 2006).

The PVN is amongst the brain nuclei that have the highest concentration of neurons that contain nitric oxide synthase (NOS), the enzyme responsible for the production of the neurotransmitter NO. In the present study there was a significant increase in the number of activated neurons which were also NADPH-d positive (a marker for NOS) in the PVN following exposure to the hot environment. These activated nitrergic neurons represented approximately one-third (31%) of the nitrergic neurons in the parvocellular PVN. This suggests that exposure to a hot environment increases the production of NO within the PVN. NO in the CNS is important in heat dissipation (Gerstberger, 1999; Schmid et al., 1998), thus, the PVN may be a potential site of action within the CNS through which NO may influence the re-distribution of blood flow to facilitate heat dissipation. Our finding is in agreement with the hypothesis suggested by
Patel K and co-workers whom demonstrated that endogenous NO within the PVN have an inhibitory effect on renal sympathetic outflow (Zhang et al., 1997a) which is mediated by GABA (Zhang and Patel, 1998). Indeed NO may have extensive effects within the PVN since it easily diffuses through cell membranes.

Another significant finding of the present study was the lack of a marked activation of PVN neurons projecting to the RVLM, which were also NADPH-d positive (approximately 0.5% of the RVLM – projecting neurons) following heat exposure. Thus, although there was a marked significant increase in the number of activated nitrergic neurons following heat exposure, only very rarely do these project to the RVLM. Thus, this suggests that nitrergic RVLM - projecting neurons in the PVN do not play an important role during heat exposure. Nitrergic PVN neurons projecting to the RVLM are, however, activated by simulated volume expansion (Kantzides et al., 2005), suggesting that RVLM-projecting nitrergic neurons may be differentially activated by specific stimuli. Furthermore, since nitrergic spinally-projecting neurons but not nitrergic RVLM-projecting neurons are activated following exposure to a hot environment (Cham et al., 2006), it appears that a specific stimulus can activate specific sub-populations of nitrergic neurons in the parvocellular PVN.

The neurochemical nature of the PVN neurons projecting to the RVLM that are activated by heating requires further study, however, angiotensin II and vasopressin are possible candidates since the sympatho-excitatory and hypertensive effects of activation of the PVN can be attenuated by the blockade of angiotensin II receptors in the RVLM,
(Tagawa and Dampney, 1999) and vasopressin antagonists can reduce the excitatory effects on RVLM neurons induced by the stimulation of the PVN (Yang et al., 2001). Furthermore, it is particularly interesting to note that central angiotensin II has been found to be important in the cardiovascular response to hyperthermia (Kregel et al., 1994).
5.4 CONCLUSIONS

The forebrain, particularly the rostral part, has long been recognized as a critical site in thermoregulation. The hypothalamic PVN, however, has been largely ignored, despite anatomical and electrophysiological evidence suggesting that it could contribute to the central pathways mediating thermoregulation. The present study provides evidence that pathways that project to the RVLM from the PVN may contribute to the central pathways activated by exposure to a hot environment. Since the RVLM is critical in the tonic maintenance of sympathetic nerve activity, it is possible that the RVLM - projecting neurons in the PVN make a contribution to the cardiovascular responses elicited by exposure to a high environmental temperature. The PVN is known to contribute to the anatomical framework that influences the autonomic nervous system innervating important thermoregulatory organs such as the BAT, heart, blood vessels in the skin, kidney, and gut, as well as salivary glands and the rat tail (Cano et al., 2003; Hubschle et al., 2001; Morrison, 1999; Oldfield et al., 2002). Neurons in the PVN that contribute to this anatomical framework include neurons that project directly (i) to the IML of the spinal cord where sympathetic preganglionic motor neurons are located, (ii) to the RVLM, and (iii) to both those regions via collaterals (Pyner and Coote, 2000; Shafton et al., 1998). We hypothesise that the RVLM – projecting neurons may contribute to the cardiovascular responses elicited by an acute exposure to a hot environment, However, the spinally – projecting neurons in the PVN make a greater contribution to the responses elicited during exposure to a hot environment compared with the RVLM – projecting neurons in the PVN.
The role of NO in thermoregulation is now emerging as a major focus of investigation. Current evidence suggests that NO within the central nervous system can play a critical role in heat dissipation (Eriksson et al., 1997; Garthwaite and Boulton, 1995; Gerstberger, 1999; Gourine, 1995; Mathai et al., 1997; Schmid et al., 1998; Simon, 1998). One of the highest concentrations of NOS-containing neurons within the hypothalamus occurs in the PVN, and the present findings highlight that approximately 31% of the nitrergic neurons in the parvocellular PVN are activated by heat exposure. Since NO can diffuse easily across cell membranes, its production within the PVN may have widespread actions during heat exposure. Interestingly, activated nitrergic RVLM-projecting neurons in the PVN were rarely observed following the heating stimulus, suggesting these neurons do not influence sympathetic nerve activity that contribute to the cardiovascular effects elicited by exposure to a hot environment.
CHAPTER 6.

THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS IS CRITICAL FOR RENAL VASOCONSTRICTION ELICITED BY ELEVATIONS IN BODY TEMPERATURE.
6.0 INTRODUCTION

The hypothalamic paraventricular nucleus (PVN) is an important integrative site involved in hormonal, endocrine, and neural control. The PVN is composed of different neuronal subgroups that can influence sympathetic nerve activity and thereby contribute to cardiovascular regulation (Badoer, 1996; Swanson and Sawchenko, 1983b). There are neurons in the PVN that project to regions of the spinal cord where sympathetic preganglionic neurons are located, and thereby can directly influence sympathetic activity (Cechetto and Saper, 1988; Shafton et al., 1998; Swanson et al., 1980). Other subgroups, project to the pressor region of the rostral ventrolateral medulla, and thereby indirectly influence sympathetic nerve activity (Shafton et al., 1998). Additionally, there are subgroups that send collaterals to both autonomic regions, and therefore are capable of both direct and indirect influences on sympathetic nerve activity (Shafton et al., 1998).

Activation of the PVN can markedly alter blood pressure, sympathetic neural outflows and haemodynamic sequelae. Excitation of the PVN with excitatory amino acids or by using bicuculline to inhibit GABA-mediated inhibition of the PVN, and thereby allowing activation of the PVN via endogenous excitatory inputs, can result in increases in renal sympathetic nerve activity and a reduction in renal blood flow (Haselton and Vari, 1998; Kannan et al., 1989). The functional relevance of effects on the renal sympathetic nerve activity mediated via the PVN includes the reflex response to volume expansion. Indeed, strong evidence has been provided that the PVN is essential...
for the reflex reduction in renal sympathetic nerve activity that occurs when blood volume is elevated (Haselton et al., 1994; Lovick et al., 1993; Ng et al., 2004).

Recently we have also shown that elevations in body temperature strongly activated neurons in the PVN that project to the spinal cord (Cham et al., 2006). An elevation in body temperature induces changes in sympathetic nerve activity that result in a redistribution of blood flow from the viscera to the periphery to enable dissipation of heat from the body. The reflex responses include vasoconstriction of the renal vasculature. Several lines of evidence suggest that the PVN may contribute to the circulatory responses induced by a temperature challenge. These include: the observations that the PVN contains: (i) thermosensitive neurons (Inenaga et al., 1987), and (ii) neurons that project to the spinal cord and influence sympathetic nerve activity to important thermoregulatory effector organs such as the brown adipose tissue and the vasculature of the rat tail, salivary gland as well as kidney and gut (Hubschle et al., 2001; Morrison, 1999; Oldfield et al., 2002). Furthermore, increases in body temperature activates neurons within the PVN (Bachtell et al., 2003; Bratincsak and Palkovits, 2004; Harikai et al., 2003; Kiyohara et al., 1995; McKitrick, 2000). However, whether the PVN contributes to the decrease in renal blood flow that accompanies an increase in body temperature is not known. Thus, the aim of the present study was to determine the effect of inhibition of neuronal activity within the PVN on the renal blood flow in response to an increased body temperature and to investigate whether the effect was specific to the PVN.
Chapter 6 – PVN is critical for renal vasoconstriction elicited by elevations in body temperature. 174

6.1 EXPERIMENTAL PROCEDURES

6.1.1 Animals and housing

All experimental protocols used in this study were performed in accordance with the Prevention of Cruelty to Animals Act 1986 and conform to the *Guiding Principles for Research Involving Animals and Human Beings* (American, 2002) and to the guidelines set out by the National Health and Medical Research Council of Australia (Australian code of practice for the care and use of animals for scientific purposes) and were approved by the RMIT University Animal Ethics committee. Every attempt was made to reduce animal suffering, discomfort and reduce the number of animals needed to obtain reliable results. Male Sprague-Dawley rats (obtained from Monash University Animal Services, Victoria, Australia) weighing 300-350 grams were housed in the Animal Facility (RMIT University, Victoria, Australia) with free access to rat chow and tap water at a room temperature of 22±1°C with a 12h/12h light/dark regimen. Prior to the experimental day, animals were handled on a daily basis to minimize stress.

6.1.2 Surgical preparations

All animals were anaesthetized initially with Equithesin (sodium pentobarbitone (0.5 g): chloral hydrate (2.219 g) (per 100 ml) mixture and administered intraperitoneally (0.6ml/100gm) to enable the cannulation of the femoral artery and vein. Anaesthesia was then subsequently maintained with urethane (1 – 1.4 g/ kg intravenously initially followed by supplemental doses of approximately 0.05g/ kg as required). The depth of anesthesia was maintained to ensure the absence of corneal and pedal reflexes.
Chapter 6 – PVN is critical for renal vasoconstriction elicited by elevations in body temperature. 175

The femoral artery was cannulated for monitoring arterial blood pressure. The signal was recorded using a Mac Lab data-acquisition System (AD Instruments, Colorado Springs, CO, USA). Mean arterial pressure (MAP) and heart rate (HR) were determined electronically from the phasic arterial pressure. The femoral vein was cannulated for the intravenous delivery of supplemental doses of urethane.

Throughout the surgical procedures, the body temperature was maintained at 37.0°C ± 1.0°C with a custom-made water-circulating blanket, through which either cold water (4 to 8°C measured directly at source) or warm water (48 to 52°C measured directly at source) were pumped through at a rate of 16-26ml/min.

A thermocouple was taped onto the base of the tail (MLT409, AD Instruments, Colorado Springs, CO, USA) to record tail temperature. A second thermocouple, (RET-2 rectal probe for rats, PhysiTemp Instruments Inc, Clifton, NJ, USA) was inserted approximately 2-3cm into the rectal cavity and connected to a Thermocouple Analog converter (MLT1101, AD Instruments, Colorado Springs, CO, USA) to enable the measurement of body core temperature.

6.1.3  Microinjection into the hypothalamic PVN

The animals were placed prone and the head was mounted in a Kopf stereotaxic frame such that both bregma and lambda were positioned on the same horizontal plane. A midline reference point was marked 2mm rostral to bregma. This was necessary because
bregma was removed during the subsequent drilling procedure. To expose the dorsal surface of the brain, a hole, approximately 4mm in diameter, was drilled into the bone centered 3.5mm caudal from the reference point. Following the drilling procedure, the hole was covered with cotton wool soaked in normal saline to prevent drying of the exposed surface.

Microinjections were made bilaterally using a fine glass micropipette (with a tip diameter of 50-70 μm) into the PVN (stereotaxic coordinates: 1.5 mm caudal to bregma, 0.5 mm lateral to midline and 7.5 mm ventral to the surface of the brain) or into the hypothalamus adjacent to the PVN (stereotaxic coordinates: 2.8 mm caudal to the bregma, 0.5 mm lateral to the midline and 7.5 mm ventral to the brain surface.

6.1.4 Renal blood flow measurement

The kidney was identified using a retroperitoneal approach. The renal artery was dissected free and carefully cleared from the surrounding tissue and from the renal vein. A flow probe was positioned around the renal artery and connected to a T206 small animal blood flow meter (Transonic System Inc., Ithaca, NY, USA) to enable monitoring of the renal blood flow. Following the implantation of the renal flow probe, approximately 15 - 20 minutes were allowed to elapse to ensure that a steady basal renal blood flow was attained. Subsequently, the rat’s body temperature was lowered to 36.0° C by passing cold water through the water-circulating blanket. This occurred within 5 -
10 minutes. Renal conductance was calculated by dividing renal blood flow (ml/min) by the mean arterial pressure.

6.1.5 Experimental protocol

When body temperature reached 36.0°C, muscimol (Sigma-Aldrich, St. Louis, MO, USA) (1nmol in 100nl, n=6) or saline vehicle (100nl, n=5) was microinjected into the PVN bilaterally. In a third group of rats, muscimol was microinjected in the hypothalamus outside the PVN (N=5). In another group of animals muscimol was injected into the PVN and the effects were followed over time. In this group of rats, core body temperature was not altered and was maintained between 37.0 - 37.5°C. To mark the injection sites, rhodamine- tagged microspheres (LumaFluor, NY, USA) were included in the microinjected solution. After the completion of the microinjections, the core rectal temperature of the animal was gradually increased to 41°C. This was performed over approximately 90-120 minutes.

6.1.6 Brain Histology

At the completion of the experiment, rats were killed with an overdose of sodium pentobarbitone (300mg/kg) (Lethaborb, Virbac Pty Ltd., NSW, Australia). The brains were then carefully removed and fixed in 4% paraformaldehyde solution for approximately 7 days, and then placed into a solution of phosphate buffer containing 20% sucrose overnight. The hypothalamus was cut on a cryostat into sections (40µm thick), and mounted onto gelatine-subbed slides. The sections were then viewed wet under
fluorescent microscopy to identify the rhodamine beads at the site of injection. The sections were then dried before being counterstained with cresyl violet and cover slipping with Depex Mounting medium (BDH Lab Supplies, Poole, UK). The sections were then re-examined using light microscopy to determine anatomical structure and the injection sites were subsequently mapped in relation to the anatomical structure.

6.1.7 Statistical Analysis

The basal resting MAP, HR, renal blood flow and renal conductance levels prior to microinjections into the brain were compared between the three groups using one-way ANOVA. Effects on the MAP, HR, renal blood flow and renal conductance levels as body temperature changed were compared between all groups using two-way ANOVA with repeated measures. When the overall difference was statistically significant, the following comparisons were made; (i) control versus muscimol in the PVN, (ii) muscimol in the PVN versus muscimol out of the PVN, and (iii) muscimol out of the PVN versus saline in the PVN groups, using two-way ANOVA with repeated measures. When there was a significant difference between groups, comparisons between the groups at the different temperature points were made using Student’s unpaired t-test and applying Bonferroni’s modification to compensate for multiple comparisons.
6.2 RESULTS

6.2.1 Resting levels

Basal MAP, HR, renal blood flow and renal conductance levels prior to the microinjection of muscimol or saline were not significantly different between the three groups of animals (Table 6.1).

6.2.2 Effect of increases in temperature on cardiovascular variables.

As core body temperature increased, tail skin temperature also increased in a linear manner in each group of animals. The change was similar in each group irrespective of whether muscimol or saline was microinjected into the PVN (Figure 6.1). Further, there was no difference in the tail skin temperature response when muscimol was injected in or out of the PVN. This result suggests that blood flow to the tail increased during heating as expected.

6.2.3 Responses in rats microinjected with saline into the PVN

In animals in which saline was microinjected into the PVN, raising core body temperature from 36.0°C to 41.0°C did not greatly influence MAP or HR (Figure 6.2). However, renal blood flow was markedly altered. As core temperature increased there was a steady reduction in renal blood flow in the control animals so that by the end of the observation period, renal blood flow had fallen by 70% from the resting level (Figure 6.3). A similar response was observed in the renal conductance response (Figure 6.3).
6.2.4 Responses in rats microinjected with muscimol into the PVN

In rats administered muscimol into the PVN, there was no marked change in MAP or in HR. As core body temperature increased, the MAP and HR responses following muscimol microinjected into the PVN were not significantly different compared to the control group (Figure 6.2). Renal blood flow and conductance, by contrast, were dramatically affected. When muscimol was microinjected into the PVN, the renal blood flow did not decrease as core body temperature was elevated (Figure 6.3). This was significantly different from the control group ($F_{(1,9)} = 39.22$, $P < 0.0002$). By the end of the observation period, renal blood flow had fallen by only 8% from the resting level. Muscimol in the PVN had a similar effect on renal conductance (Figure 6.3) ($F_{(1,9)} = 20.87$, $P < 0.002$). Thus, muscimol microinjected into the PVN abolished the reduction in renal blood flow and conductance normally observed following an increase in core body temperature. When muscimol was microinjected into the PVN and the variables were monitored over time, whilst maintaining a normal core body temperature, there was no change in the renal blood flow ($5.9 \pm 1.2$ vs $5.9 \pm 1.0$ ml/min, pre and approximately 120 minutes after muscimol).

6.2.5 Responses in rats microinjected with muscimol out of the PVN

In the group of rats in which muscimol was microinjected outside the PVN, there was no marked effect on MAP and HR (Figure 6.2). Indeed, the levels of these variables observed during the time the body temperature was increasing were similar to those seen following microinjections made into the PVN. In response to an elevation in core body temperature, renal blood flow in this group of animals fell from 9.53 ml/min to 4.74
ml/min, a reduction of 50%, by the end of the observation period (Figure 6.3). This response was significantly different from the response observed when muscimol was microinjected into the PVN \((F_{(1,9)} =21.66, P<0.002)\). Furthermore, the response did not differ significantly from the control group. Renal conductance was also reduced during the rise in body temperature and this also differed significantly from the group in which muscimol was microinjected into the PVN \((F_{(1,9)} =13.29, P<0.005)\) but not the control group (Figure 6.3). Thus, muscimol microinjected into the PVN, but not out of the PVN, prevented the normal reduction in renal blood flow and conductance induced by elevating core body temperature.

### 6.2.6 Histological analysis of microinjection sites

The microinjection sites were examined histologically at the conclusion of the experiments. As shown in Figure 6.4, microinjections within the PVN were distributed at different rostral-caudal levels of the PVN ranging from the mid to caudal levels of the PVN. The rostral-caudal distribution of the microinjection sites in which muscimol was centred into the PVN was similar to the distribution of saline microinjection sites. An example of a microinjection site observed under fluorescent lighting conditions is shown in Figure 6.5. Microinjections out of the PVN were located immediately caudal to the PVN and were centred just dorsal of the dorsomedial hypothalamic nucleus (Figure 6.6).
Chapter 6 – PVN is critical for renal vasoconstriction elicited by elevations in body temperature. 182

<table>
<thead>
<tr>
<th></th>
<th>Muscimol in PVN</th>
<th>Muscimol out PVN</th>
<th>Saline Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 6)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>95.7 ± 8.1</td>
<td>100.5 ± 3.1</td>
<td>98.0 ± 1.8</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>360 ± 14</td>
<td>358 ± 14</td>
<td>372 ± 21</td>
</tr>
<tr>
<td>Renal blood flow (mL/min)</td>
<td>9.07 ± 0.56</td>
<td>9.53 ± 0.35</td>
<td>11.47 ± 1.86</td>
</tr>
<tr>
<td>Renal conductance (mL/min/mmHg)</td>
<td>0.099 ± 0.012</td>
<td>0.095 ± 0.006</td>
<td>0.118 ± 0.021</td>
</tr>
</tbody>
</table>

Table 6.1
Basal MAP, HR, renal blood flow and renal conductance prior to microinjections of muscimol (1nmol/side) into the hypothalamus.
Figure 6.1

Increase in tail skin temperature following an increase in core body temperature in anaesthetized rats. Saline (control) or muscimol (to inhibit neuronal function) were microinjected into the PVN, or muscimol was microinjected outside the PVN in separate groups. There was no significant difference between the groups.
Chapter 6 – PVN is critical for renal vasoconstriction elicited by elevations in body temperature. 184

Figure 6.2

Responses of mean arterial pressure (MAP) and heart rate (HR) following an increase in core body temperature in anaesthetized rats. Saline (control) or muscimol (to inhibit neuronal function) were microinjected into the PVN, or muscimol was microinjected outside the PVN in separate groups. There was no significant difference between the groups.
Chapter 6 – PVN is critical for renal vasoconstriction elicited by elevations in body temperature.

Figure 6.3

Responses of renal blood flow and renal conductance following an increase in core body temperature in anaesthetized rats. Left panels show absolute levels, right panels show changes from resting levels. Saline (control) or muscimol (to inhibit neuronal function) were microinjected into the PVN, or muscimol was microinjected outside the PVN in
separate groups. Microinjections of muscimol into the PVN abolished the normal reflex reduction in renal blood flow (and conductance). *P<0.001 compared to comparable time point in control group.
Chapter 6 – PVN is critical for renal vasoconstriction elicited by elevations in body temperature.

Figure 6.4

Schematic transverse sections of the rat hypothalamic PVN showing the centre of the microinjection sites in which muscimol (left panels) or saline (right panels) were
Chapter 6 – PVN is critical for renal vasoconstriction elicited by elevations in body temperature. 188

microinjected into the PVN. The rostral – caudal position of the sections drawn relative to bregma are indicted. Abbreviations: PVN, paraventricular nucleus, Fx, fornix, AHA, anterior hypothalamic area, 3V, third ventricle, OT, optic tract, AH, anterior hypothalamus, and VMH, ventromedial hypothalamus.
Figure 6.5

Photomicrograph example of a bilateral microinjection in the hypothalamic paraventricular nucleus (PVN) taken using fluorescent lighting conditions.

Abbreviations: Fx, fornix, 3V, third ventricle, OT, optic tract. Bar = 0.5mm.
Figure 6.6

Schematic transverse sections of the rat hypothalamic PVN showing the centre of the microinjection sites in which muscimol was microinjected outside the PVN. The rostral – caudal position of the sections drawn relative to bregma are indicted. Abbreviations: Fx, fornix, MMT, mammillothalamic tract, OT, optic tract, DMH, dorsomedial hypothalamus and VMH, ventromedial hypothalamus.
6.3 DISCUSSION

The present work highlights several important findings; (i) Inhibition of neuronal function, with muscimol, in the hypothalamic paraventricular nucleus prevented the normal reduction in renal blood flow elicited by raising core body temperature, (ii) this effect was specific to the PVN as microinjection of muscimol out of the PVN did not have such an effect, and (iii) the PVN does not have a tonic influence on resting renal blood flow since inhibition of the PVN did not affect this variable. The results suggest that the hypothalamic PVN is a critical central nucleus regulating reflex renal vasoconstriction in response to elevations in core body temperature.

An increase in core body temperature elicits reflex responses designed to reduce heat production and to dissipate heat so as to restore the temperature back to normal. The cardiovascular responses that are evoked are important in these thermoregulatory adjustments. The major mechanism in the cardiovascular responses involves the redistribution of blood from the hot internal environment (ie the viscera) to regions where it can be in close contact with the cooler external environment. Thus, vasoconstriction of the blood vessels supplying visceral organs and vasodilation of the skin vasculature results in a reduction in blood flow to the visceral organs and a concomitant increase in skin blood flow. In the present study, we directly investigated one component of this reflex response, the reduction in renal blood flow. We observed a dramatic 70% reduction in renal blood flow when body temperature was elevated. We also observed an increase in tail temperature, indicative of an increased flow in the tail vasculature; this
response in the rodent is equivalent to vasodilation of the skin vasculature in humans. These integrated autonomic reflex responses involve the central nervous system. The regions of the brain contributing to the cardiovascular responses must involve nuclei known to regulate sympathetic nerve activity; indeed, studies utilizing the distribution of the protein Fos, a marker of activated neurons, have highlighted such brain regions (Bachtell et al., 2003; Bratincsak and Palkovits, 2004; Cham et al., 2006; Harikai et al., 2003; Kiyohara et al., 1995; McKitrick, 2000). When core body temperature is elevated, several forebrain areas are activated, including the hypothalamic PVN. Such studies are suggestive that the PVN could contribute to the thermoregulatory responses elicited by heating. The present work is the first to provide evidence for a physiologically relevant role of the PVN in the cardiovascular responses initiated by an elevated body temperature. The present work, however, contrasts with a previous report in which midbrain transections reduced the increase in splanchnic nerve activity, but did not appear to affect the increase in renal sympathetic nerve activity elicited by heating (Kenney et al., 2000). The report suggested the renal vasoconstriction was primarily driven by medullary brain regions. Interestingly, the same laboratory subsequently reported that lesions of the PVN markedly attenuated the renal vasoconstriction elicited by an increased core body temperature in the coronary artery ligation model of heart failure in the rat (Kenney et al., 2001a), suggesting the hypothalamic PVN plays a critical role in the renal vasoconstriction, in agreement with the present hypothesis. The reasons for the contrasting findings are not clear. Perhaps, the transections used in the previous work damaged descending pathways that contribute to opposing the role of the PVN?
The efferent pathways that could contribute to the PVN’s involvement in the renal vasoconstriction include the spinal projecting neurons and/or those that project to the pressor region of the rostral ventrolateral medulla (Malpas and Coote, 1994; Shafton et al., 1998; Tagawa and Dampney, 1999). These pathways provide the anatomical framework enabling the PVN to directly and indirectly influence sympathetic nerve activity. Previous studies have shown that PVN neurons are activated by elevations in core body temperature (Bachtell et al., 2003; Bratincsak and Palkovits, 2004; Cham et al., 2006; Harikai et al., 2003; Kiyohara et al., 1995; McKitrick, 2000), and approximately 22% of those projecting to the spinal cord were also Fos-positive (Cham et al., 2006). We suggest, therefore, that an increase in body temperature activates the PVN to elicit renal vasoconstriction and we hypothesize, that the spinal projecting neurons in the PVN contribute to the central pathways mediating the response.

In the present study we found that the microinjection of muscimol into areas outside the PVN, did not significantly affect the reflex renal vasoconstriction elicited by heating. This strongly suggests that the abolition of the renal vasoconstriction following muscimol into the PVN is site specific and that the PVN is critical to the response.

We also observed that the microinjection of muscimol into the PVN had no effect on the basal level of blood pressure, heart rate, renal blood flow and conductance. Nor did the presence of muscimol in the PVN influence those variables over time, when core body temperature was kept within normal limits. This suggests that inhibiting neuronal
function within the PVN is not critical for renal blood flow when core body temperature is normal but upon elevation of body temperature, the PVN assumes an essential role.

Rat tail temperature increased linearly with the increase in core body temperature. The rat tail temperature is an indicator of tail blood flow, albeit a crude one. Nonetheless, we found that the increase in tail temperature induced by hyperthermia was virtually identical in each group of experimental animals. Thus, microinjection of muscimol into the PVN, or adjacent areas, did not affect this response. The increase in blood flow to the tail in response to heating involves sympathoinhibition, mediated by the raphe pallidus (Owens et al., 2002; Rathner and McAllen, 1999; Tanaka et al., 2002). It is tempting to speculate that the PVN may not play a critical role in the central pathways mediating tail blood flow, in contrast to the effects on the renal vasculature, however, we recognize that this is a highly speculative possibility, but we believe it warrants investigation in the future.

In conclusion, we have found that an elevation in core body temperature induces a reduction in renal blood flow in which the PVN plays an essential role. We hypothesise that the PVN receives information from the preoptic area, the major temperature sensing region within the central nervous system, and that the efferent pathways contributing to the essential role of the PVN in the response may involve neurons that project to the intermediolateral cell column in the spinal cord, thereby directly influencing sympathetic nerve activity, and neurons that project to the pressor region of the RVLM, thereby indirectly influencing the sympathetic outflow innervating the kidney.
CHAPTER 7.

GENERAL DISCUSSION
The present thesis examines the role of a key autonomic site within the diencephalon, i.e. the PVN, in the central pathways activated by, and the cardiovascular responses elicited during, an increase in body temperature. The present study demonstrated a critical role for the PVN in the reflex cardiovascular responses elicited by changes in body temperature.

The main novel findings of the present work in this thesis are; Firstly, the demonstration that elevation of core body temperature activated “autonomic” pathways emanating from the PVN and projecting to the IML of the spinal cord or to the RVLM. Secondly, the present study also established that hypothalamic PVN neurons activated by acute heat exposure were also capable of producing NO. Finally, the role of the PVN during heat exposure was examined by inhibiting PVN neuronal function by using the GABA (A) receptor agonist, muscimol, and the results showed the PVN plays an important physiological role in the cardiovascular response elicited by the elevation in body temperature.

The present work found that in conscious animals neuronal activation was markedly increased within all sub-nuclei of the parvocellular PVN following exposure of to a hot environment of 39°C. This finding is based on the increased production of Fos, a marker of neuronal activity, following heat exposure which is also in agreement with earlier studies from other laboratories (Bachtell et al., 2003; Bratincsak and Palkovits, 2004; Harikai et al., 2003; Kiyohara et al., 1995; McKitrick, 2000). Therefore, both the
present and previous studies support the view that the PVN may contribute to thermoregulatory responses.

One of the most important findings of the present study is that PVN neurons projecting to the IML of the spinal cord or to the RVLM were activated following exposure to a hot environment. The present work demonstrated that there was a significant increase in the number of spinally-projecting neurons (~22%) in the PVN that exhibited Fos following heat exposure. This proportion of activated spinally-projecting neurons observed was higher than that following many other stimuli studied to date suggesting that hyperthermia is a more effective stimulus to activate this pathway compared to other stimuli such as hemorrhage, dehydration, increased osmolality and hypotension (Badoer et al., 1993; Kantzides and Badoer, 2003; Polson et al., 2002; Stocker et al., 2004). These stimuli elicited as much if not more Fos production in the PVN but were not as effective as the heating stimulus used in the present study in activating spinally-projecting neurons.

The present work utilized an ambient temperature of 39 °C to elevate body temperature. Under these conditions, plasma osmolality is elevated and body fluid is reduced (Mathai et al., 2000). However, we have previously shown that an intravenous infusion of hypertonic saline does not activate spinally-projecting neurons in the PVN (Kantzides and Badoer, 2003); suggesting it is unlikely that the increase in plasma osmolality could account for the increased activation of spinally-projecting PVN neurons. We have also argued that it is unlikely, that reduced body fluid is solely responsible for
activating those neurons because we have observed that although severe hemorrhage can 
elicit more Fos production in the PVN, it is not as effective as elevation in body 
temperature in activating spinally-projecting neurons (Badoer et al., 1993; Kantzides and 
Badoer, 2003; Polson et al., 2002; Stocker et al., 2004). Furthermore, as discussed in 
Chapter 3 administration of hypotonic saline during the heat exposure to counteract the 
elevation in plasma osmolality and the reduction in extracellular volume, had no effect on 
the number of Fos-positive neurons in the PVN, suggesting that under the conditions of 
heating used in the present study, fluid loss and hypertonicity were not the major 
contributors to Fos production. Taken together, it is tempting to speculate that the 
activated spinally-projecting PVN neurons are important in the central pathways 
mediating the renal vasculature responses initiated by heat exposure, most likely in the 
cardiovascular response to the elevation in body temperature via the cardiac 
preganglionic neurons. It would also be very interesting indeed in future studies to 
replicate the studies with injections to identify spinal projecting neurons in the PVN that 
regulate sympathetic outflow to the mesenteric and renal beds.

It was also observed that the same heating stimulus activates a considerably 
greater proportion of spinally-projecting neurons (~ 22%) in comparison to the RVLM-
projecting neurons (~ 8%) emanating from the PVN. This suggests that perhaps the 
spinally-projecting neurons in the PVN make a greater contribution to the cardiovascular 
responses initiated by heat as compared to the RVLM-projecting neurons. Although the 
proportion of activated RVLM-projecting neurons may represent only a small proportion
of the population of identified RVLM-projecting neurons, one cannot exclude the possibility that their influence is greater than the proportion would suggest.

Since activation of the PVN can elicit sympatho-excitatory effects, it is possible that activation of the neurons in the PVN that project to the IML of the spinal cord or to the RVLM, a region critical for the tonic regulation of sympathetic nerve activity; could mediate the increase in sympathetic nerve activity to the visceral vasculature, including the mesenteric and renal beds that occurs with hyperthermia. This effect would contribute to the re-distribution of blood flow to the peripheral vasculature to enable heat to dissipate when body temperature rises. Similarly, heart rate is increased in response to heating, and activation of an excitatory pathway from the PVN could contribute to the tachycardia.

In the present work, a detailed examination of the rostral-caudal distribution of activated neurons in the ventral brainstem following exposure to a hot environment was also determined. Whilst there was a significant increase in Fos production in the midline (by five-fold), ventromedial (by eight-fold) and in the ventrolateral (by nine-fold) brainstem, the number of activated neurons in the ventral brainstem that were also spinal-projecting was very small. This is suggestive that perhaps supra-medullary spinal-projecting neurons such as those from the hypothalamic PVN may play a greater role.

In the present investigation, there was a significant increase in the number of Fos-positive and NADPH-d containing neurons in the PVN following exposure to the hot
environment. Indeed these activated nitrergic neurons in the parvocellular PVN ranges from approximately 31% to 40% of the nitrergic neurons in the parvocellular PVN. These findings suggest that NO-producing neurons in the hypothalamic PVN that are activated following heat exposure are important. This is in agreement with the observation that NO in the CNS is important in heat dissipation (Gerstberger, 1999; Schmid et al., 1998). Given that NO easily diffuses through membrane; its effects within the PVN may be quite extensive. Thus, the PVN may be a potential site of action within the CNS through which NO may influence the redistribution of blood flow to facilitate heat dissipation.

Accordingly, the PVN may be a site in the brain in which NOS inhibitors, by attenuating the production of NO, elicit increases in body temperature (Gerstberger, 1999; Mathai et al., 2004). Furthermore, NO within the PVN appears to be tonically active since blockade of its production elicits marked cardiovascular effects (Zhang et al., 1997a). The cardiovascular effects/mechanism of action of NO acting within the PVN are mediated via GABA. Thus, any role of NO in the PVN during elevations in core body temperature are likely to be complex. Perhaps, the role of NO is to act (i) as a negative feedback system that prevents activated neurons from becoming overactive and/or (ii) as an inhibitor of neurons which contributes to a complex mechanism that tunes the activity of neurons to facilitate an appropriate integrated renal response. Thus, the contribution of NO within the PVN in the responses elicited during a temperature challenge warrants further investigation.
Another significant finding of the present study is the increase in the number of activated PVN neurons projecting to the spinal cord which were also NADPH-d positive (12% of the spinally-projecting neurons), which suggests that these neurons are involved in the responses elicited during an elevation in body temperature. This finding contrasts markedly with the lack of activation of PVN nitrergic neurons projecting to the RVLM. Thus, it appears that the heating stimulus can activate specific sub-populations of nitrergic neurons in the parvocellular PVN. Furthermore, assuming that the activated, nitrergic, spinal-projecting PVN neurons are inhibitory; the question arises about their role in the cardiovascular responses following heat exposure. At present, we can only speculate; perhaps (i) these neurons inhibit inhibitory inter-neurons at the spinal level hence resulting in an increase in sympathetic nerve activity or (ii) these neurons contribute to the sympathetic nerve activity to tissues such as the brown adipose tissue and the tail vasculature which are normally inhibited by hyperthermia. The PVN is known to contain pre-motor neurons that are part of the central pathways influencing sympathetic nerve activity to these tissues, as shown by transynaptic viral tracing studies (Motawei et al., 1999; Schramm et al., 1993; Weiss et al., 2001). Any influence on the tail sympathetic nerve activity would involve an interaction, presumably at the spinal cord level, with raphe spinal projecting neurons since these are known to be essential in the sympathetic nerve activity regulating tail blood flow during thermoregulatory responses (Owens et al., 2002).

Taken together, the functional neuro-anatomical studies performed in this thesis show the “autonomic” pathways such as those projecting to the IML of the spinal cord
and the RVLM are activated by hyperthermia. The findings suggest that the PVN may contribute to the reflex cardiovascular responses elicited by an elevation of core body temperature. Thus, the physiological relevance of the PVN in hyperthermia was also investigated in this thesis.

It was found that the inhibition of neuronal activity within the PVN using the GABA receptor agonist, muscimol, abolished the renal vasoconstriction normally elicited in response to an increased body temperature. This effect appears to be specific to the PVN. This suggests that the PVN is playing an essential role in the vasoconstriction of the renal vasculature and hence crucial in regulating the re-distribution of blood flow during heating. It is likely that the PVN accomplishes this role via the efferent pathways that may include those projecting to the IML and the RVLM.

Thus we hypothesize the schema outlined in Figure 7.1 and Figure 7.2; following an elevation in body temperature heat-sensitive neurons in the pre-optic area are activated. This results in the activation of neurons in the PVN. This could be mediated via direct or indirect connections. The pre-optic area is known to project to the PVN (Sawchenko and Swanson, 1983a; Sawchenko and Swanson, 1983b; Swanson, 1976; Swanson and Sawchenko, 1983a). Within the PVN (see Figure 7.2), the activated neurons include sub-populations that (i) project to the IML of the spinal cord, (ii) project to the RVLM, (iii) are nitricergic and some of these project to the spinal cord. The activated neurons, we believe, contribute to the vasoconstriction of the renal vasculature that normally occurs during hyperthermia. Whether the PVN contributes to the
vasoconstriction occurring in other visceral vascular beds during hyperthermia requires further investigations. Certainly, the increase in splanchnic SNA elicited by hyperthermia has been reported to involve suprabulbar structures (Kenney et al., 2001a).

In addition to the findings reported in the present studies, there is further evidence to support a role of the PVN in thermoregulation including observations that the PVN contains; (i) thermo-sensitive neurons (Inenaga et al., 1987) and (ii) neurons that project to sympathetic preganglionic motor neurons that project to important effector organs such as the brown adipose tissue and the vasculature of the rat tail, salivary glands, as well as kidney and the gut (Hubschle et al., 2001; Morrison, 1999; Oldfield et al., 2002). Further support for the involvement of the PVN in heating stems from the work by Kenny and colleagues where they demonstrated that lesions of the PVN resulted in a marked attenuation in the renal sympathetic nerve discharge in response to heating in heart failure rats (Kenney et al., 2001a). This contrasts with earlier reports from that laboratory (Kenney et al., 2000).

CONCLUSION

The present study has demonstrated a key role of the PVN in the reduction in renal blood flow elicited by hyperthermia. The present work suggests PVN neurons projecting to the IML of the spinal cord or the RVLM may contribute to the reflex responses. Nitrergic neurons in the PVN may also play an important role. Future studies
will be needed to provide further insight into the role of NO within the PVN in thermoregulation and whether the PVN also contributes to the effects on the vasculature of other organs during temperature changes.
Figure 7.1

Schematic diagram summarizing the findings of the present work on the involvement of the PVN in the regulation of renal blood flow. An elevation in body temperature induces changes in sympathetic nerve activity that result in a redistribution of blood flow from the viscera to the periphery to enable dissipation of heat from the body. The reflex responses include vasoconstriction of the renal vasculature resulting in a reduced renal blood flow. The projections from the PVN to the (i) IML and (ii) RVLM are likely to mediate the
effects of the PVN on SNA and may contribute to the changes in the renal blood flow induced by elevations in body temperature.

Abbreviations: III, third ventricle; POA, pre-optic area; PVN, paraventricular nucleus; RVLM, rostral ventrolateral medulla; IML, intermediolateral cell column.
Figure 7.2
Diagrammatic illustration of neuronal populations activated by elevations in body temperature. The present work demonstrated that increased body temperature activated (i) ~22% of spinally-projecting neurons, (ii) ~31-40% of nitrergic neurons, (iii) 12% of the spinally-projecting neurons that were also nitrergic and (iv) 8% of RVLM-projecting neurons. No nitrergic RVLM-projecting neurons were activated.
Abbreviations: III, third ventricle; PVN, paraventricular nucleus; RVLM, rostral ventrolateral medulla; IML, intermediolateral cell column.
APPENDIX
Appendix 1 – Construction of the water – circulating jacket

The construction of the water-circulating jacket entails the alignment of Silastic® Laboratory Tubing (catalogue number 508-005 - internal diameter of 1.02 mm and outer diameter of 2.16 mm; Dow Corning Corporation, Midland, MI, USA) in a manner as shown in Figure A.1. The tubing is positioned such that there are input tubes and the output tubes are intertwined. Once aligned within a custom-made mould made from plasticine, Sylgard 170 (part A and B silicone elastomer; Dow Corning GmbH, Rheigaustrasse, Wesbaden, Germany) is then freshly prepared. The Sylgard 170 is supplied as a two part liquid component kits comprised of Part A and Part B which is to be mixed in a 1:1 ratio manually. This mixture is then poured and allowed to set at 70°C for a 20 minutes followed by a rest period of 1 week.
Figure A.1 – Schematic diagram showing the position of input and output tubes for the manufacture of the water-circulating jacket.
Appendix 2 - Phosphate Buffer (PB)

0.1 M PB, pH 7.4 was prepared by dissolving 77.28 gram of Stock A (NaH$_2$PO$_4$.H$_2$O) in approximately two litres of distilled water and 203.76 gram of Stock B (Na$_2$HPO$_4$) in a similar volume of distilled water. The two solutions were combined and the total volume brought up to 20 litres using distilled water. The phosphate buffer was stored at 4°C.

Appendix 3 - Phosphate Buffered Saline (PBS)

One litre of PBS was prepared by dissolving 8.87 gram of NaCl in approximately 500 mL of PB and then the volume of the solution was adjusted to 1 litre.

Appendix 4 - Tris Buffer

0.05 M Tris(hydroxymethyl)aminomethane buffer solution, pH 7.4, was prepared by dissolving 28.0 gram of Trizma base (C$_4$H$_{11}$NO$_3$) in approximately two litres of distilled water and 121.2 gram of Trizma HCl (C$_4$H$_{11}$NO$_3$.HCl) in a similar volume of distilled water. The two solutions were combined and the total volume brought up to 20 litres using distilled water. The buffer was stored at 4°C.

Appendix 5 - Paraformaldehyde

To make 1 litre of a 4 % solution (4 %= 4 g/100 mL fixative) of para-formaldehyde, 40 gram of formaldehyde powder was added to approximately 800 mL of 0.1 M phosphate buffer pH 7.4 and vigorously agitated over heat (70°C) under a fume hood until the solution turned clear. The solution was allowed to cool to room temperature before
filtering (Whatmann number 1 filter discs). The volume of the solution was then adjusted to 1 litre by the addition of 0.1 M PB.

Appendix 6 - 3,3’- diaminobenzidine hydrochloride

Preparation of 100 mL of 0.05 % DAB (3,3’-diaminobenzidine hydrochloride) solution involved dissolving 50mg of DAB powder in approximately 80 mL of 0.05M Tris buffer, pH 7.4, at room temperature under a fume hood. The solution thus prepared was filtered (Whatmann number 1 filter paper) and the volume brought up to 100 mL. If nickel intensification was required, 40 mg of (NH₄)₂Ni(SO₄)₂ was also dissolved in the 0.05 M Tris buffer prior to filtration.

Appendix 7 - Preparation of subbed slides

The slides were placed in slide holders and soaked in 80 % ethanol for 3-4 hours. The slides were then rinsed in distilled water and then soaked for a further hour in distilled water. Meanwhile, the subbing solution was prepared by the addition of 5 gram of gelatin to one litre of water. The solution was heated to 45°C and stirred vigorously until the gelatin was dissolved. The solution was removed from the heat source and 0.5 gram of chromium (III) potassium sulfate (CrK(SO₄)₂. 12H₂O dissolved with continuous stirring before filtered (Whatmann no. 1 filter paper) whilst the solution was still warm. The slides were then dipped into the subbing solution and allowed to dry overnight in an oven (60-70°C).
BIBLIOGRAPHY


Damas, J., 1994. Kallikrein, nitric oxide and the vascular responses of the submaxillary glands in rats exposed to heat. Arch Int Physiol Biochim Biophys. 102, 139-46.


Doutrelant-Viltart, O., Poulain, P., 1996. Distribution of NADPHdiaphorase and calbindin-D28k neurons in the lateral septal area of the guinea pig, with special reference to the enkephalinergic hypothalamo-septal tract. Journal of Chemical Neuroanatomy. 11, 199-207.


Horn, T., Smith, P.M., McLaughlin, B.E., Bauce, L., Marks, G.S., Pittman, Q.J., Ferguson, A.V., 1994. Nitric oxide actions in paraventricular nucleus:


Kantzides, A., Badoer, E., 2005. nNOS-containing neurons in the hypothalamus and medulla project to the RVLM. Brain Research. 1037, 25-34.


Motawei, K., Pyner, S., Ranson, R.N., Kamel, M., Coote, J.H., 1999. Terminals of paraventricular spinal neurones are closely associated with adrenal medullary sympathetic preganglionic neurones: immunocytochemical evidence for vasopressin as a possible neurotransmitter in this pathway. Experimental Brain Research. 126, 68-76.


Pyner, S., Coote, J.H., 2000. Identification of branching paraventricular neurons of the hypothalamus that project to the rostroventrolateral medulla and spinal cord. Neuroscience. 100, 549-556.

Ranson, R.N., Motawei, K., Pyner, S., Coote, J.H., 1998. The paraventricular nucleus of the hypothalamus sends efferents to the spinal cord of the rat that closely appose sympathetic preganglionic neurones projecting to the stellate ganglion. Experimental Brain Research. 120, 164-172.


Sawchenko, P.E., Swanson, L.W., 1982. Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. J Comp Neurol. 205(3), 260-72.

Sawchenko, P.E., Swanson, L.W., 1983a. The organization and biochemical specificity of afferent projections to the paraventricular and supraoptic nuclei. Prog Brain Res. 60, 19-29.

Sawchenko, P.E., Swanson, L.W., 1983b. The organization of forebrain afferents to the paraventricular and supraoptic nuclei of the rat. J Comp Neurol. 218(2), 121-44.


Strack, A.M., Sawyer, W.B., Platt, K.B., Loewy, A.D., 1989b. CNS cell groups regulating the sympathetic outflow to adrenal gland as revealed by transneuronal cell body labeling with pseudorabies virus. Brain Res. 491(2), 274-96.


Torvik, A., 1956. Afferent connections to the sensory trigeminal nuclei, the nucleus of the solitary tract and adjacent structures; an experimental study in the rat. J Comp Neurol. 106(1), 51-141.


Badoer, E., Merolli, J., 1997...


projecting neurons of the paraventricular nucleus and rostral ventrolateral medulla. Brain Research. 610, 216-223.


interscapular adipose tissue during cold exposure. The Journal of Comparative Neurology. 460, 303-326.


Doutrelant-Viltart, O., Poulain, P., 1996. Distribution of NADPHdiaphorase and calbindin-D28k neurons in the lateral septal area of the guinea pig, with special reference to the enkephalinergic hypothalamo-septal tract. Journal of Chemical Neuroanatomy. 11, 199-207.


Förstermann U, Kleinert H, Gath I, Schwarz P, Closs EI, Dun NJ. Adv 
Pharmacol. 34, 171-86.

Franza BR, J.r., Rauscher, F.J., Josephs, S.F., Curran, T., 1988. The Fos complex and 
Fos-related antigens recognize sequence elements that contain AP-1 binding 
sites. Science. 239(4844), 1150-3.

Freeman, P.H., Wellman, P.J., 1987. Brown adipose tissue thermogenesis induced by 
low level electrical stimulation of hypothalamus in rats. Brain Research 
Bulletin. 18, 7-11.

Garthwaite, J., 1991. Glutamate, nitric oxide and cell-cell signalling in the nervous 

Garthwaite, J., Boulton, C.L., 1995. Nitric Oxide Signaling in the Central Nervous 

responses to stimulation of cell bodies in the hypothalamus of the rat. Brain 
Research. 482, 67-77.

Sci. 14, 30-36.

Golder, M., Burleigh, D.E., Ghali, L., Feakins, R.M., Lunniss, P.J., Williams, N.S., 
responses to nitric oxide and contains altered levels of NOS1 and elastin in 
uncomplicated diverticular disease. Colorectal Disease. 9, 218-228.

and morphology of NADPH-diaphorase containing neurons in the superior


Kantzides, A., Badoer, E., 2005. nNOS-containing neurons in the hypothalamus and medulla project to the RVLM. Brain Research. 1037, 25-34.


Konishi, M., Kanosue, K., Kano, M., Kobayashi, A., Nagashima, K., 2007. The median preoptic nucleus is involved in the facilitation of heat-escape/cold-
seeking behavior during systemic salt loading in rats. Am J Physiol Regul
Integr Comp Physiol. 292, R150-159.

Korf, H.W., 1984. Neuronal organization of the avian paraventricular nucleus:

responses to immune challenges. Prog Brain Res. 139, 127-46.

adjustments to heat stress by central ANG II receptor antagonism. Am J

within the basal forebrain and brainstem in the rat and cat. J Comp Neurol. .
178(2), 225-54.

neurons in the rat brain. The Journal of Comparative Neurology. 377, 509-
519.

Pharmacol Physiol. . 25(6), 474-8.


peroxidase from spinal cord to brain stem cell groups in the cat. Neuroscience
Letters. 1, 9-14.

II Infusion on Responses to Hypothalamic PVN Injection of Bicuculline.
Hypertension. 42, 1124-1129.


Motawei, K., Pyner, S., Ranson, R.N., Kamel, M., Coote, J.H., 1999. Terminals of paraventricular spinal neurones are closely associated with adrenal medullary sympathetic preganglionic neurones: immunocytochemical evidence for vasopressin as a possible neurotransmitter in this pathway. Experimental Brain Research. 126, 68-76.


Pyner, S., Coote, J.H., 2000. Identification of branching paraventricular neurons of the hypothalamus that project to the rostroventrolateral medulla and spinal cord. Neuroscience. 100, 549-556.

Ranson, R.N., Motawei, K., Pyner, S., Coote, J.H., 1998. The paraventricular nucleus of the hypothalamus sends efferents to the spinal cord of the rat that closely appose sympathetic preganglionic neurones projecting to the stellate ganglion. Experimental Brain Research. 120, 164-172.


Sawchenko, P.E., Swanson, L.W., 1981. A method for tracing biochemically defined pathways in the central nervous system using combined fluorescence

Sawchenko, P.E., Swanson, L.W., 1982. Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. J Comp Neurol. 205(3), 260-72.

Sawchenko, P.E., Swanson, L.W., 1983a. The organization and biochemical specificity of afferent projections to the paraventricular and supraoptic nuclei. Prog Brain Res. 60, 19-29.

Sawchenko, P.E., Swanson, L.W., 1983b. The organization of forebrain afferents to the paraventricular and supraoptic nuclei of the rat. J Comp Neurol. 218(2), 121-44.


Strack, A.M., Sawyer, W.B., Platt, K.B., Loewy, A.D., 1989b. CNS cell groups regulating the sympathetic outflow to adrenal gland as revealed by transneuronal cell body labeling with pseudorabies virus. Brain Res. 491(2), 274-96.


Torvik, A., 1956. Afferent connections to the sensory trigeminal nuclei, the nucleus of the solitary tract and adjacent structures; an experimental study in the rat. J Comp Neurol. 106(1), 51-141.


