

**Functional Neuroanatomy
of Tachykinins in
Brainstem Autonomic
Regulation**

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University of Sydney
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Doctor of Philosophy**

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*To Meredith,
Charles and Georgina*

Declaration

This thesis is submitted to the University of Sydney in fulfillment of the requirements for the degree of Doctor of Philosophy. The work presented in this thesis is, to the best of my knowledge and belief, original and has not been published or written by another person, except where due reference is made in the text of the thesis. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution. Parts of this thesis have been published in abstract form or as full papers and they are listed on page *X*.

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1.1 Summary

Little is known about the role that tachykinins, such as substance P and its receptor, the neurokinin-1 receptor, play in the generation of sympathetic nerve activity and the integration within the ventrolateral medulla (VLM) of many vital autonomic reflexes such as the baroreflex, chemoreflex, somato-sympathetic reflex, and the regulation of cerebral blood flow.

The studies described in this thesis investigate these autonomic functions and the role of tachykinins through physiological (response to hypercapnoea, chapter 3), anatomical (neurokinin-1 receptor immunohistochemistry, chapter 4) and microinjection (neurokinin-1 receptor activation and blockade, chapters 5 and 6) experiments.

In the first series of experiments (chapter 3) the effects of chemoreceptor activation with hyperoxic hypercapnoea (5%, 10% or 15% CO₂ in O₂) on splanchnic sympathetic nerve activity and sympathetic reflexes such as the baroreflex and somato-sympathetic reflex were examined in anaesthetized rats. Hypercapnoea resulted in sympatho-excitation in all groups and a small increase in arterial blood pressure in the 10 % CO₂ group. Phrenic nerve amplitude and phrenic frequency were also increased, with the frequency adapting back to baseline during the CO₂ exposure. Hypercapnoea selectively attenuated (5% CO₂) or abolished (10% and 15% CO₂) the somato-sympathetic reflex while leaving the baroreflex unaffected. This selective inhibition of the somato-sympathetic reflex while leaving the baroreflex unaffected was also seen following neurokinin-1 receptor activation in the rostral ventrolateral medulla (RVLM) (see below).

Microinjection of substance P analogues into the RVLM results in a pressor response, however the anatomical basis for this response is unknown. In the second series of experiments (chapter 4), the distribution of the neurokinin-1 receptor in the RVLM was investigated in relation to catecholaminergic (putative sympatho-excitatory “C1”) and bulbospinal neurons. The neurokinin-1 receptor was demonstrated on a small percentage (5.3%) of C1 neurons, and a small percentage (4.7%) of RVLM C1 neurons also receive close appositions from neurokinin-1 receptor immunoreactive terminals. This provides a mechanism for the pressor response seen with RVLM microinjection of substance P analogues. Neurokinin-1 receptor immunoreactivity was also seen a region overlapping the preBötzinger complex (the putative respiratory rhythm generation region), however at this level a large percentage of these neurons are bulbospinal, contradicting previous work suggesting that the neurokinin-1 receptor is an exclusive anatomical marker for the propriobulbar rhythm generating neurons of the preBötzinger complex.

The third series of experiments (chapter 5) investigated the effects of neurokinin-1 receptor activation and blockade in the RVLM on splanchnic sympathetic nerve activity, arterial blood pressure, and autonomic reflexes such as the baroreflex, somato-sympathetic reflex, and sympathetic chemoreflex. Activation of RVLM neurokinin-1 receptors resulted in sympatho-excitation, a pressor response, and abolition of phrenic nerve activity, all of which were blocked by RVLM pre-treatment with a neurokinin-1 receptor antagonist. As seen with hypercapnoea, RVLM neurokinin-1 receptor activation significantly attenuated the somato-sympathetic reflex but did not affect the sympathetic baroreflex. Further,

blockade of RVLM neurokinin-1 receptors significantly attenuated the sympathetic chemoreflex, suggesting a role for RVLM substance P release in this pathway.

The fourth series of experiments (chapter 6) investigated the role of neurokinin-1 receptors in the RVLM, caudal ventrolateral medulla (CVLM), and nucleus tractus solitarius (NTS) on regional cerebral blood flow (rCBF) and tail blood flow (TBF). Activation of RVLM neurokinin-1 receptors increased rCBF associated with a decrease in cerebral vascular resistance (CVR). Activation of CVLM neurokinin-1 receptors decreased rCBF, however no change in CVR was seen. In the NTS, activation of neurokinin-1 receptors resulted in a biphasic response in both arterial blood pressure and rCBF, but no significant change in CVR. These findings suggest that in the RVLM substance P and the neurokinin-1 receptor play a role in the regulation of cerebral blood flow, and that changes in rCBF evoked in the CVLM and NTS are most likely secondary to changes in arterial blood pressure. Substance P and neurokinin-1 receptors in the RVLM, CVLM and NTS do not appear to play a role in the brainstem regulation of tail blood flow.

In the final chapter (chapter 7), a model is proposed for the role of tachykinins in the brainstem integration of the sympathetic baroreflex, sympathetic chemoreflex, cerebral vascular tone, and the sympatho-excitation seen following hypercapnoea. A further model for the somato-sympathetic reflex is proposed, providing a mechanism for the selective inhibition of this reflex seen with hypercapnoea (chapter 3) and RVLM neurokinin-1 receptor activation (chapter 5).

In summary, the ventral medulla is essential for the generation of basal sympathetic tone and the integration of many vital autonomic reflexes such as the baroreflex, chemoreflex, somato-sympathetic reflex, and the regulation of cerebral blood flow. The tachykinin substance P, and its receptor, the neurokinin-1 receptor, have a role to play in many of these vital autonomic functions. This role is predominantly neuromodulatory.

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Publications and Communications

Publications (see appendix)

Pilowsky P.M. and **Makeham J.** 2001. Juxtacellular labelling of identified neurons: Kiss the cells and make them dye. *Journal of Comparative Neurology* 433:1-3.

Makeham JM, Goodchild AK, and Pilowsky PM. 2001. NK1 receptor and the ventral medulla of the rat: bulbospinal and catecholaminergic neurons. *Neuroreport* 12:3663-3667. **(cover illustration)**.

Makeham JM, Goodchild AK, Costin NS, and Pilowsky PM. 2004. Hypercapnia selectively attenuates the somato-sympathetic reflex. *Respiratory Physiology and Neurobiology* 140:133-143.

Makeham JM, Goodchild AK, and Pilowsky PM. 2005. NK1 receptor activation in rat rostral ventrolateral medulla selectively attenuates somato-sympathetic reflex while antagonism attenuates sympathetic chemoreflex. *American Journal of Physiology* 288:R1707-R1715.

Communications

Makeham JM, Miyawaki T, Goodchild AK, and Pilowsky PM. 2001. Stimulation of substance P receptors in the Rostral Ventrolateral medulla. 34th Congress of the International Union of Physiological Sciences, Sydney. Poster presentation.

Makeham JM, Goodchild AK, and Pilowsky PM. 2001. NK1R immunoreactive varicosities closely appose RVLM C1 neurons. 21st Annual Meeting of the Australian Neuroscience Society, Brisbane. Poster presentation.

Makeham JM, Goodchild AK, and Pilowsky PM. 2002. NK1 receptor stimulation in the Rostral Ventrolateral Medulla. 22nd Annual Meeting of Australian Neuroscience Society, Sydney. Poster presentation.

Reja V., **Makeham JM**, Goodchild AK, and Pilowsky PM. 2003. AT1 Receptors in the RVLM do not play a significant role in maintaining high blood pressures observed in renovascular hypertensive rats. *Journal of Hypertension* 24 (suppl. 4):S196.

Reja, V, **Makeham JM**, Goodchild AK, and Pilowsky PM. 2003. AT1 receptors in the RVLM do not play a significant role in maintaining high blood pressures observed in renovascular hypertensive rats. 13th European Meeting on Hypertension, Milan. Poster Presentation.

Makeham JM, Goodchild AK, Costin NS, and Pilowsky PM. 2003. Hypercapnia selectively attenuates the somato-sympathetic reflex. 23rd Annual Meeting of Australian Neuroscience Society, Adelaide. Poster presentation.

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Abbreviations

ABP	Arterial blood pressure
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
CART	Cocaine and amphetamine related transcript
rCBF	Regional cerebral blood flow
CGRP	Calcitonin-gene-related peptide
CPA	Caudal pressor area
CNQX	6-cyano-7-nitroquinoxaline-2, 3-dione
CVLM	Caudal ventrolateral medulla
CVR	Cerebral vascular resistance
cVRG	Caudal ventral respiratory group
DAG	Diacylglycerol
DAMGO	[D-Ala ² ,N-Me-Phe ⁴ ,Gly-ol ⁵]-enkaphalin
DLH	DL-homocysteic acid
DNQX	6,7-dinitroquinoxaline-2,3-dione
DPDPE	[D-Pen ^{2,5}]-enkaphalin
DRG	Dorsal respiratory group
DRN	Dorsal raphe neuron
EAA	Excitatory amino acid
E-AUG	Augmenting expiratory neurons
EC	Excitatory concentrations
E-DEC	Decrementing expiratory neurons
EPSP	Excitatory post synaptic potentials
GABA	Gamma aminobutyric acid
GDP	Guanosine diphosphate
GIRK	G-protein coupled inward rectifier K ⁺
GTP	Guanosine triphosphate
HK-1	Haemokinin-1
I-AUG	Augmenting inspiratory neurons
IC	Inhibitory concentration
I _{CAN}	Voltage insensitive cation current
I-CON	Constant inspiratory neurons
I-DEC	Decrementing inspiratory neurons
IML	Intermediolateral
I _{NaP}	Persistent sodium current
IP ₃	Inositol-1,4,5-triphosphate
I-peak	Inspiratory peak
IPSP	Inhibitory post synaptic potentials
MAP	Mean arterial pressure
MCVA	Medullary cerebral vasodilator area
NK	Neurokinin
NMDA	N-methyl-D-aspartate

NTS	Nucleus tractus solitarius
pFRG	Parafacial respiratory group
PIP ₂	Phosphatidyl inositol biphosphate
PI-peak	Post-inspiratory peak
PKC	Protein kinase C
PLC	Phospholipase C
PNA	Phrenic nerve activity
PPT-A	Pre-protachykinin-A
PPT-B	Pre-protachykinin-B
PPT-C	Pre-protachykinin-C
preBötC	Pre-Bötzinger Complex
PVN	Paraventricular nucleus
rCBF	Regional cerebral blood flow
RTN	Retrotrapezoid nucleus
RVLM	Rostral ventrolateral medulla
RVMM	Rostral ventromedial medulla
rVRG	Rostral ventral respiratory group
SAH	Subarachnoid haemorrhage
SIDS	Sudden Infant Death Syndrome
SP	Substance P
SPNs	Sympathetic preganglionic neurons
SNA	Sympathetic nerve activity
sSNA	Splanchnic sympathetic nerve activity
SVA	Subthalamic cerebrovasodilator area
TBF	Tail blood flow
TH	Tyrosine Hydroxylase
TM I-VII	7 hydrophobic transmembrane domains
TTX	Tetrodotoxin
TVR	Tail vascular resistance
UCINs	Upper cervical inspiratory neurons
VGLUT	Vesicular glutamate transporter
VLM	Ventrolateral medulla
VRG	Ventral respiratory group
5-HT	5- Hydroxy Tryptamine, serotonin
8-OH-DPAT	8-hydroxy-di- <i>n</i> -propylamino tetralin