The role of PACAP in central cardiorespiratory control

Melissa Ann Inglott
BSc (Hons 1)

Australian School of Advanced Medicine,
Faculty of Human Sciences,
Macquarie University
Sydney, Australia

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Supervisor
Professor Paul M. Pilowsky

Co-supervisor
Dr Melissa M.J. Farnham

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Abstract

The autonomic nervous system is essential for the tonic and reflex control of the cardiovascular system. Specifically, the rostral ventrolateral medulla (RVLM) is critical in maintaining sympathetic vasomotor tone and mean arterial pressure during rest, and in response to external stimuli. The RVLM may also be implicated in the aeitology of essential/neurogenic hypertension. Presympathetic RVLM neurons directly innervate sympathetic preganglionic neurons (SPN) in the intermediolateral cell column of the thoracic spinal cord, controlling the level of sympathetic outflow to the periphery. RVLM neurons are a heterogenous cell population that contain many metabotropic neurotransmitters, which act to modulate sympathetic outflow and therefore blood pressure. Pituitary adenylate cyclase activating polypeptide (PACAP) is one example of an RVLM neuropeptide. PACAP is an excitatory neuropeptide known to be involved in the central control of blood pressure; PACAP and its receptors are found in the RVLM, SPN and other in important cardiovascular regions within the brain and spinal cord. Intrathecal administration of PACAP causes marked sympathoexcitation, but no change in mean arterial pressure. The aims of this thesis were to further investigate the role of PACAP within the tonic and reflex control of the cardiorespiratory system, at the level of the spinal cord, with a particular interest in determining the mechanistic cause underlying the lack of blood pressure response following intrathecal PACAP.

The work presented here finds that 1) intrathecal PACAP causes a prolonged, widespread sympathoexcitation, and does not differentially affect sympathetic outflows, resulting in no net change in blood pressure. 2) That the intrathecal PACAP response is due to neuronal activation at the level of the spinal cord, and not due to activation of higher order brainstem centers through the use of spinal cord transection. 3) That intrathecal PACAP-38 increases metabolic rate and is likely to increase brown adipose tissue thermogenesis. 4) That the PACAP antagonist did not affect mean arterial pressure, heart rate or splanchnic sympathetic nerve activity, suggesting that PACAP is not tonically active in blood pressure control. 5) The vast differences seen in the blood pressure responses to maxadilan (PAC_1 receptor activation) and vasoactive intestinal polypeptide ($VPAC_1$ and $VPAC_2$ receptor

activation) suggest that the PAC_1 and VPAC receptor subtypes produce opposing blood pressure effects when activated by intrathecal PACAP, producing a cancellation effect that results in no net blood pressure change. 6) Intrathecal PACAP administration increased phrenic nerve amplitude and phrenic nerve frequency, demonstrating a role for PACAP in central respiratory control. 7) That intrathecal PACAP and catestatin alone did not effect the cardiovascular reflex responses, demonstrating that PACAP and catestatin are not involved in the reflex control of blood pressure in this preparation. 8) Finally, pre-treatment with catestatin (90-minute duration; intrathecal injection) before intrathecal PACAP injection causes a significant decrease in mean arterial pressure and phrenic nerve frequency, and enhances sympathetic barosensitivity and chemosensitivity.

The data indicates that the responses to intrathecal PACAP are receptor dependant and may be due to differential activation of adrenaline and noradrenaline secreting chromaffin cells. Collectively, the results suggest that PACAP plays an important excitatory role in central cardiorespiratory and metabolic control in the urethane-anaesthetised, vagotomised, paralysed and artificially ventilated rat. The stimulus for the release of PACAP remains unknown, however, PACAP may be involved in another aspect of central cardiovascular control, such as the stress response.

Declaration

I certify that the work in this thesis entitled **"The role of PACAP in central cardiorespiratory control"** has not previously been submitted for a higher degree, nor has it been submitted as part of the requirements for a degree to any other university or institution.

I also certify that this thesis is an original piece of research and that I am the author. Any help and assistance that I received in my research, and in the preparation of this thesis, have been appropriately acknowledged. Specifically, I acknowledge Dr. Melissa Farnham for conducting the SHR/WKY experiments presented in **Chapter 4** and Dr Andrea Gaede for her assistance with the design and experiments described in **Chapter 7**. In addition, I certify that all information sources and literature used are indicated within this thesis, a full reference list can be found in **Chapter 9**. All results chapters are published in their entirety (**Appendix 1**).

The research presented in this thesis was approved by the Macquarie University Animal Ethics Committee, protocol numbers: 2007/022, 2008/015, 2009/011 and 2010/055 (**Appendix 3**).

Melissa Ann Inglott, BSc (Hons)
Student ID: 40724336
20th December 2012

Publications and Communications arising from this thesis

Publications

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Inglott MA, Lerner EA, Pilowsky PM & Farnham MMJ. (2012). Activation of PAC_1 and VPAC receptor subtypes elicits differential physiological responses from sympathetic preganglionic neurons in the anaesthetised rat. *Brit J Pharmacol* **167,** 1089-1098.

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*Equal first authors

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"Real knowledge is to know the extent of one's ignorance"
~ Confucius

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Abbreviations

AC	Adenylate cyclase
ACh	Acetylcholine
Ad	Adrenaline
ANS	Autonomic nervous system
AP	Arterial blood pressure
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BBR	Boehringer blocking reagent
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BötC	Bötzinger complex
cAMP	Cyclic adenosine monophosphate
CgA	Chromograinin A
CNS	Central nervous system
CPG	Central pattern generator
$_C$SNA	Cervical sympathetic nerve activity
CVLM	Caudal ventrolateral medulla
DβH	Dopamine-β-hydroxylase
ECG	Electrocardiogram
EPSP	Excitatory post-synaptic potential
GABA	Gamma-aminobutyric acid
GPCR	G-protein coupled receptor
HR	Heart rate
i.c.v	Intracerebroventricular
IHC	Immunohistochemistry
IML	Intermediolateral cell column
IP3	Inositol-3-phosphate
i.p.	Intraperitoneal
ISH	*In situ* hybridisation
i.v.	Intravenous
KCL	Potassium chloride
$_L$SNA	Lumbar sympathetic nerve activity
LTF	Lateral tegmental field
MAP	Mean arterial pressure
MCPA	Medullo-cervical pressor area
nAChR	Nicotinic acetylcholine receptor

NAd	Noradrenaline
NBT	Nitroblue tetrazolium
NTS	Nucleus of the solitary tract
PAC$_1$	PAC$_1$ receptor
PACAP	Pituitary adenylate cyclase activating polypeptide
PaCO$_2$	Partial arterial pressure of CO_2
PAG	Periaqueductal grey
PaO$_2$	Partial arterial pressure of O_2
PBS	Phosphate buffered saline
PBT	PBS with 0.1% Tween-20
PC	Prohormone convertase
PCR	Polymerase chain reaction
PE	Phenylephrine
PKA	Protein kinase A
PLC	Phospholipase C
PLD	Phospholipase D
PMN	Phrenic motor nucleus
PNA	Phrenic nerve activity
PNAmp	Phrenic nerve amplitude
PNf	Phrenic nerve frequency
PNMT	Phenylethanolamine-N-methyltransferase
PNS	Peripheral nervous system
PreBötC	PreBötzinger complex
PSNS	Parasympathetic nervous system
PVC	Polyvinyl chloride
PVN	Paraventricular nucleus
$_R$SNA	Renal sympathetic nerve activity
RTN	Retrotrapezoid nucleus
RVLM	Rostral ventrolateral medulla
RVMM	Rostral ventromedial medulla
SD	Sprague-Dawley
SEM	Standard error of the mean
SHR	Spontaneously hypertensive rat
SNA	Sympathetic nerve activity
SNP	Sodium nitroprusside
SNS	Sympathetic nervous system

SPN	Sympathetic preganglionic neuron
SSC	Sodium citrate
$_s$SNA	Splanchnic sympathetic nerve activity
SSR	Somatosympathetic reflex
TH	Tyrosine hydroxylase
VIP	Vasoactive intestinal polypeptide
VPAC$_1$	VPAC$_1$ receptor
VPAC$_2$	VPAC$_2$ receptor
VRC	Ventral respiratory column
WKY	Wistar-Kyoto

Chapter 1

Literature Review

Chapter 1: Literature Review

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1.1 Introduction

Cardiorespiratory function is achieved through complex interactions between peripheral receptors, central neural networks and efferent neurons, which link the central nervous system (CNS) with the peripheral visceral effectors. The cardiovascular system is regulated by the two branches of the autonomic nervous system (ANS); the sympathetic nervous system (SNS) and the parasympathetic nervous system (PSNS). The SNS and PSNS differentially innervate and regulate the organs of the cardiovascular system, adapting the system's function to the body's metabolic and environmental needs. The respiratory system enables the transfer of O_2 and CO_2 between the air and the blood stream (respiration), via air movement through the lungs. Respiration is driven by the metabolic needs of the body, which are sensed by central and peripheral chemoreceptors. This thesis investigates the function of the SNS in central cardiovascular control.

Sympathetic outflow to the cardiovascular system is generated by dedicated cardiovascular nuclei present in the medulla oblongata (Dampney, 1994; Guyenet, 2006). In particular, the rostral ventrolateral medulla (RVLM) is crucially implicated in both the tonic and reflex maintenance of sympathetic outflow to the cardiovascular system and possibly in the etiology of essential hypertension (Guertzenstein & Silver, 1974; Feldberg & Guertzenstein, 1976; Pilowsky & Goodchild, 2002; Sved *et al.*, 2003). The RVLM generates tonic sympathetic outflow to the cardiovascular system via neurons termed bulbospinal presympathetic RVLM neurons. Excitation of bulbospinal RVLM neurons generates sympathetic outflow to the target organs of the SNS, via sympathetic preganglionic neurons (SPN) and subsequent sympathetic postganglionic neurons, maintaining/regulating basal arterial blood pressure (AP) and vasomotor tone over both short- and long- term time frames (Dampney, 1994; Pilowsky & Goodchild, 2002; Guyenet, 2006). A number of neurotransmitters/neuropeptides operate in the RVLM to mediate this pathway and aid in central cardiovascular control (Aicher *et al.*, 2001; Miyawaki *et al.*, 2002a; Pilowsky & Goodchild, 2002; Makeham *et al.*, 2005; Pilowsky, 2008). Pituitary adenylate cyclase activating polypeptide (PACAP) is one example of a RVLM neuropeptide (Légrádi *et al.*, 1994; Dun *et al.*, 1996a; Dun *et al.*, 1996b; Hannibal, 2002; Farnham *et al.*, 2008).

PACAP is an excitatory neuropeptide that was first discovered for its ability to stimulate adenylate cyclase (AC; Miyata *et al.*, 1989). PACAP mRNA and receptors are found in SPN (Arimura, 1998; Vaudry *et al.*, 2009), PACAP mRNA is also present in the ventral medulla (Légrádi *et al.*, 1994; Dun *et al.*, 1996a; Dun *et al.*, 1996b; Hannibal, 2002). In particular, PACAP mRNA is present in bulbospinal presympathetic RVLM neurons (Farnham *et al.*, 2008). The location of PACAP within important sympathetic premotor nuclei and SPN indicates that PACAP may play a role in the central autonomic control of AP. In a previous study intrathecal PACAP caused tachycardia and increased sympathetic nerve activity (SNA; Farnham *et al.*, 2008) when injected at T5/6. Interestingly, AP remained constant, despite the expectation that AP would increase due to an increase in SNA (Farnham *et al.*, 2008). Current understanding of the mechanisms by which PACAP causes sympathoexcitation without an accompanying AP increase remains unknown, and is one of the questions addressed in this thesis. The aims of this thesis are to further investigate the role of PACAP within tonic and reflex cardiorespiratory control, and to determine the mechanism of action and pharmacology of PACAP in the cardiorespiratory system.

1.2 The autonomic nervous system

The central and peripheral branches of the vertebrate nervous system act together to maintain bodily homeostasis, through their effects on the somatic, behavioral, endocrine and autonomic nervous systems. The ANS is a functional branch of the vertebrate CNS that controls involuntary body systems such as the cardiorespiratory and gastrointestinal systems. The ANS also regulates other autonomic functions including: micturition, defecation, accommodation (of the eye's lens), temperature, osmolarity and the stress response (mediated by cortisol, adrenaline (Ad) and noradrenaline (NAd) released from the adrenal glands; Brooks & Seller, 1981; Gershon, 1981; Axelrod & Reisine, 1984; Guyton & Hall, 2006). The ANS is classically subdivided into three parallel, but differentially regulated, efferent limbs; the SNS, PSNS and enteric nervous system. The SNS and PSNS generally have antagonistic effects on the same organ/system; with the SNS traditionally regarded as the stimulatory system, and the PSNS as the inhibitory system (Wang *et al.*, 1995; Iverson & Saper, 2000).

1.2.1 The parasympathetic nervous system

The PSNS is primarily concerned with energy conservation, and is generally active in periods of 'rest and digest'. The functions of the PSNS include: slowing heart rate (HR), constricting airways and stimulating salivation and digestion (Wang *et al.*, 1995; Iverson & Saper, 2000; Freeman, 2006). Pelvic parasympathetic outflow originates from preganglionic nuclei residing in the intermediolateral (IML) cell column of the sacral spinal cord (S2-S4). Cranial PSNS activity originates from the dorsal motor nucleus of the vagus, nucleus ambiguus, salivatory nucleus and Edinger-Westphal nucleus in the brainstem; these nuclei give rise to the PSNS fibres in cranial nerves III, IV, IX and X (Jones, 2001). The outflow from the parasympathetic preganglionic nuclei listed above results in distinct and varied functionalities, which contribute to the overall functions of the PSNS (Wang *et al.*, 1995). The PSNS has preganglionic neurons originating from these CNS nuclei which synapse in the periphery with postganglionic neurons, to innervate the system's target organs (**Figure 1.1**; Guyenet, 2006). The majority (75%) of PSNS outflow is derived from the vagus nerve, which carries the parasympathetic efferent neurons that innervate the heart (McAllen & Spyer, 1978; Takanaga *et al.*, 2003).

1.2.2 The sympathetic nervous system

The other arm of the ANS, the SNS, is concerned with the expenditure of energy and is commonly known as the 'flight or fight' component of the ANS (Wang *et al.*, 1995; Iverson & Saper, 2000). The functions of the SNS include: increasing HR, modulating vasomotor tone, stimulation of the adrenal medulla, sweating, and inhibition of digestion (Clutter *et al.*, 1980; Cryer *et al.*, 1980; Pyner & Coote, 1994). Sympathetic outflow is mediated by presympathetic neurons originating from brainstem and hypothalamic nuclei (see **section 1.4**). Tonically active presympathetic neurons innervate SPN originating in the IML of the thoraco-lumbar spinal cord (T1-L2; Petras & Cummings, 1972), maintaining the functionality of the SNS. Axons of SPN exit the spinal cord through the ventral roots of spinal nerves. Preganglionic fibres join the white rami communicans and enter the sympathetic chain ganglion. SPN synapse with sympathetic postganglionic neurons in sympathetic chain ganglia at either the same segmental level, or by travelling rostro-caudally. Sympathetic postganglionic neurons form the peripheral nerves that travel throughout the body to innervate the target organs of the SNS, however the

adrenal and extra-adrenal chromaffin cells are directly innervated by SPNs (**Figure 1.1**; Kesse *et al.*, 1988; Guyenet, 2006; Llewellyn-Smith, 2009).

1.3 Arterial blood pressure

AP is the pressure exerted by the circulating blood volume on arterial walls. A sufficient AP is necessary to ensure the functions of the cardiovascular system (adequate perfusion, oxygenation, nutrient supply and waste removal) are properly carried out. An AP of ~120/80 mmHg is considered sufficient and normal.

1.3.1 The determinants of arterial blood pressure

AP is determined by the product of cardiac output and total peripheral resistance, two factors that are influenced by autonomic innervation. Cardiac output is determined by HR, end-diastolic volume and myocardial contractility; total peripheral resistance is determined by the diameter of the arterioles (Guyenet, 2006). The SNS exerts it's control over AP via its innervation of the heart, adrenal glands and arterioles; affecting cardiac output and total peripheral resistance. The PSNS effects AP via its innervation of the heart; affecting cardiac output (Dampney, 1994; Guyenet, 2006). Despite the importance of the PSNS in the tonic and reflex control of AP, this study is only concerned with the SNS which will be discussed for the remainder of this review.

Total peripheral resistance is generated by constriction of the arterial resistance vessels (arterioles); this constriction is known as vasomotor tone. Sympathetic activity regulating vasomotor targets is tonically active, maintaining resting AP, and aiding in the reflex control of AP. Basal vasomotor activity is maintained by the tonic SPN activity, which is sustained by supraspinal excitatory drive (Dembowsky *et al.*, 1985; Meckler & Weaver, 1985; Strack *et al.*, 1989a) and mediated by spinal afferents carrying information from the periphery (Dampney, 1994; Sun, 1995; Guyenet, 2006). Acute C1 spinal transaction abolishes SNA and leads to a fall in AP to ~50 mmHg, the 'spinal level' (Guertzenstein & Silver, 1974; Dampney & Moon, 1980; Dampney, 1994). Circulating catecholamines released by the adrenal medulla upon SNS stimulation, and other local factors such as nitric oxide, hormones and neuropeptides within the SNS, may also function to affect the determinants of AP.

A

Sympathetic Nervous System

Parasympathetic Nervous System

B
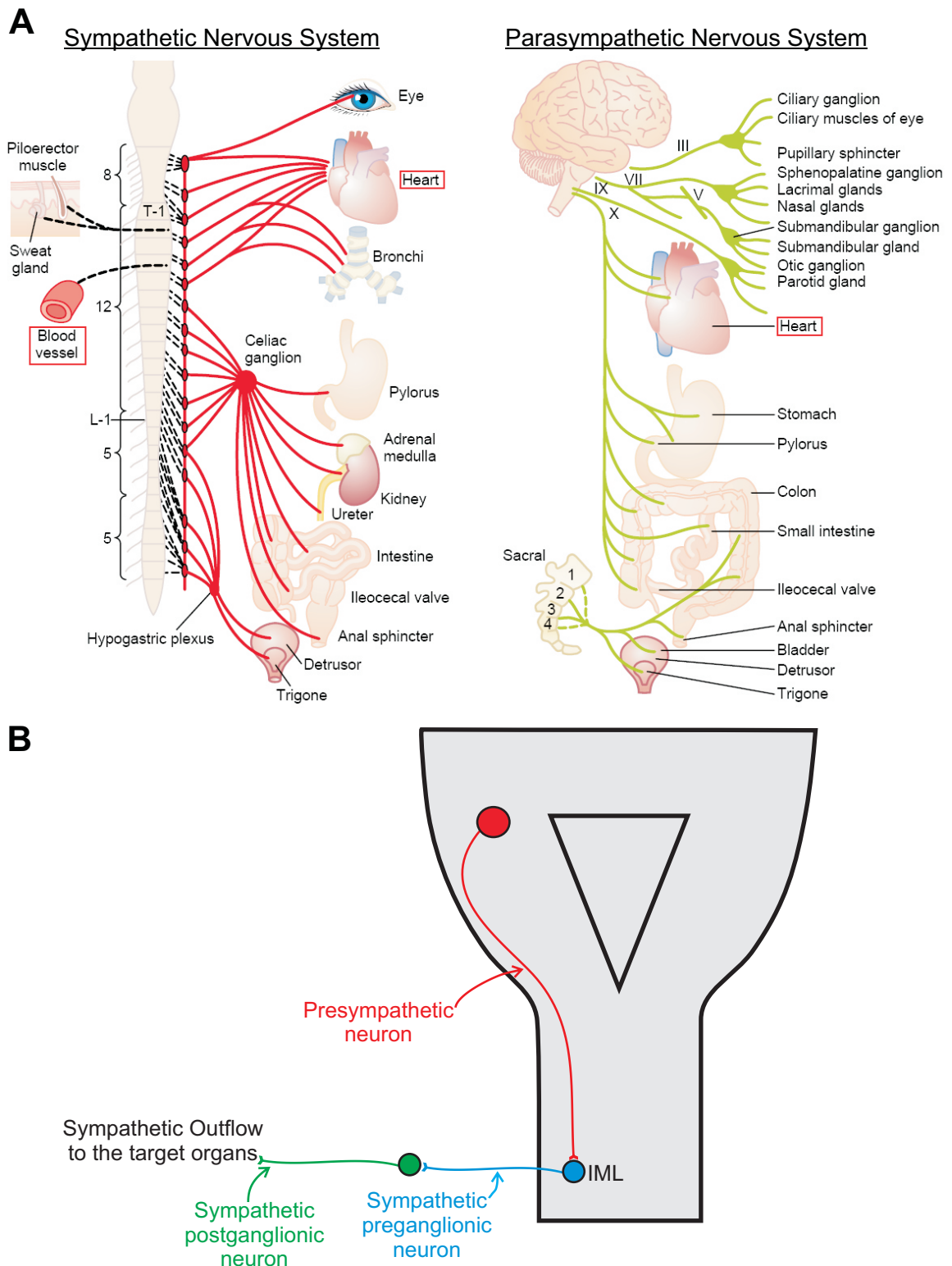
Figure 1.1 The target organs of the autonomic nervous system and arrangement of the sympathetic nervous system
(**A**) The actions of the autonomic nervous system occur as a result of target organ innovation by either the sympathetic and/or parasympathetic nervous systems (Adapted from (Guyton and Hall, 2006). Communication within the sympathetic nervous system (**B**) occurs via action potential propagation through a series of neurons. Presympathetic neurons originating from brainstem and hypothalamic nuclei conduct action potentials to sympathetic preganglionic neurons. Sympathetic preganglionic neurons originate in the intermediolateral (IML) cell column of the spinal cord and conduct information to postganglionic neurons in the periphery, which innervate the system's target organs, causing the desired effect.

1.3.2 Short- and long- term control of arterial blood pressure

The branches of the ANS are key regulators of AP homeostasis, with hormones, neuropeptides and local factors also contributing to the regulation of this vital sign (Dampney, 1994; Habecker *et al.*, 2008). Whilst the SNS is responsible for maintaining basal AP through its tonic drive, the body must also compensate for, and rectify, any changes to AP. The body has developed feedback mechanisms to maintain a designated 'set-point', around which AP is maintained in both the short- and long- term. Altering vasomotor tone is the principal method for maintaining/modulating AP. In the short-term the ANS maintains AP through its effects on cardiac function and vascular smooth muscle, whilst in the long term neural, renal and hormonal control act to maintain the AP 'set-point'.

AP may fluctuate in the short-term (seconds to hours) in order to satisfy the demands of physiological/environmental/emotional stressors (Seagard *et al.*, 1987; Morrison, 2001; Mueller & Hasser, 2006). Adjustments to these stimuli are made by autonomic reflex arcs, a key mechanism employed by the ANS to maintain AP homeostasis (Blessing, 1997). Critical homeostatic reflexes include the baroreceptor, chemoreceptor (hypoxia, or hypo- and hyper- capnia), and the somatosympathetic reflexes (see **section 1.5.5**). Autonomic reflexes are triggered by stimuli that move AP away from the 'set-point'; the reflexes initiate rapid and significant changes in AP to restore homeostasis. By doing so, adaptive reflexes maintain the functionality of the cardiovascular system and prevent adverse events in the short-term (Pilowsky & Goodchild, 2002; Morrison *et al.*, 2008; Kc & Martin, 2010).

Disregarding the normal circadian rhythm present in healthy individuals, AP remains relatively stable over the long-term (<24 hours). Historically, there is considerable debate concerning the function of the ANS in long-term AP regulation. The current prevailing view indicates that the ANS is involved in this process (Kishi *et al.*, 2001; Madden & Sved, 2003). Originally, the baroreceptors (Kirchheim, 1976; Head, 1994; Dampney *et al.*, 2002) were thought to be unimportant in determining long-term AP levels, as they acutely reset to the current AP, and total barodenervation does not affect average daily AP (Conway *et al.*, 1984a; Conway *et al.*, 1984b; Dorward & Korner, 1987; Head, 1994). However, recent studies suggest that baroreceptors may chronically reset to a higher operating AP 'set-point', within

48 hours. This occurs in salt, or other pathologically induced, hypertensive states where the baroreceptors act to permit hypertension by increasing basal vasomotor tone. Barosensitivity is also decreased in this case, hindering recovery of AP to control (Thrasher, 2004, 2005a, b). Baroreceptor-mediated activation and up regulation of the renin-angiotensin-aldosterone system, via the renal sympathetic nerve, can also cause long-term increases in AP by chronically increasing sympathetic outflow (Lohmeier *et al.*, 2005; Mueller, 2008). Despite the evidence, the role for the baroreceptors in the long-term control of AP is still controversial.

Long-term AP regulation is mainly concerned with salt and blood volume regulation by the kidneys. Blood volume correlates directly with AP (Lohmeier *et al.*, 2005; Nguyen Dinh Cat & Touyz, 2011), and is regulated by hormones and the SNS. Upon detecting low renal blood flow (low AP) or low salt concentration (excess fluid loss), the kidneys activate the renin-angiotensin-aldosterone system (Guyton & Hall, 2006; Burke *et al.*, 2008b), resulting in increased angiotensin II. The actions of angiotensin II are multifaceted, all with the aim of increasing AP. Angiotensin II directly increases SNA, resulting in vasoconstriction of the blood vessels, to increase AP (Buckley & Jandhyala, 1977; Miller, 1981). Angiotensin II also prompts sodium reabsorption, and therefore water retention within the kidney tubules, subsequently increasing blood volume and AP. Indirectly, angiotensin II acts through the release of aldosterone and vasopressin to retain water (Williams & Williams, 2003). This hormone system is inactive when AP is too high, and must be decreased. Chronic up-regulation of angiotensin II may also alter the baroreceptor 'set-point' (Bishop *et al.*, 1995; Brooks, 1995), potentially resulting in hypertension due to a reduction in renal medullary blood flow and increased responsiveness of renal medullary blood flow to renal sympathetic nerve stimulation (Evans *et al.*, 2010).

1.3.3 Hypertension

A dysfunction in the regulation of the cardiovascular system may cause hypertension, and subsequent target organ damage, or persistent hypotension that may cause ischemia. Hypertension is clinically defined as an increase in AP (an AP $\geq$ 140/90; Briganti *et al.*, 2003; Carrington *et al.*, 2010). It is a common disorder whose etiology involves complex interactions between environmental and genetic

factors (Folkow, 1982; Mahmud & Feely, 2007; Messerli *et al.*, 2007). Hypertension is a major underlying cause of morbidity and mortality in the western world, and affected 44% of Australians in 2010 (Carrington *et al.*, 2010). Target organ damage caused by hypertension results in coronary heart disease, stroke, congestive heart failure, renal insufficiency, peripheral vascular disease and other adverse outcomes (Stamler, 1991). Hypertension can be classed as either essential (of unknown cause) or secondary (due to underlying disease; Chiong *et al.*, 2008); majority of cases are essential (~90%). There is increasing evidence to suggest that elevated sympathetic tone contributes to the development of essential hypertension and heart failure (Esler *et al.*, 2001; Guyenet, 2006; Malpas, 2010; Henry *et al.*, 2012). Vasomotor tone is under the control of the SNS; therefore, sympathetic dysfunction probably contributes to the development of these conditions (Malpas *et al.*, 2001). It is for this reason that investigation into the central control of vasomotor tone and AP is important, is of interest to basic science and clinical researchers and is the focus of this thesis.

1.4 Cardiovascular sympathetic premotor nuclei

Supraspinal inputs to SPN arise from six nuclei in the lower brainstem and hypothalamus; the paraventricular nucleus (PVN) of the hypothalamus, A5 noradrenergic cell group of the ventral pons, caudal raphé nuclei, medullo-cervical pressor area (MCPA), rostral ventromedial medulla (RVMM) and RVLM (**Figure 1.2**). Identified with retrograde labeling of SPN (Strack *et al.*, 1989a; Strack *et al.*, 1989b; Jansen *et al.*, 1995; Seyedabadi *et al.*, 2006), these nuclei are the cardiovascular sympathetic premotor nuclei. The premotor nuclei are topographically organised, and form extensive ipsilateral and contralateral branches (Guyenet *et al.*, 1989; McAllen *et al.*, 1997). These six nuclei differentially regulate sympathetic outflows (for review see (Morrison, 2001; Guyenet, 2006; Coote, 2007; Malpas, 2010)), with some nuclei, more than others, influence sympathetic functions such as; thermoregulation though control of cutaneous vasculature and brown adipose tissue (BAT) metabolism (Blessing & Nalivaiko, 2000; Ootsuka *et al.*, 2004; Nakamura & Morrison, 2007), vasomotor tone, HR and blood volume regulation (Brown & Guyenet, 1985; Pilowsky & Goodchild, 2002). Neurons in the C2 and C3 cell groups also project to the IML, however their cardiovascular function is not well understood (Minson *et al.*, 1990; Sevigny *et al.*, 2012).

Bulbospinal presympathetic neurons, originating from the six premotor nuclei, synapse with SPN in the IML (**Figures 1.1** and **1.2**), and exert crucial effects on the tonic and reflex control of AP. However, only the RVLM is tonically active (Ross *et al.*, 1984b; Dampney, 1994). Presympathetic neurons use a variety of neurotransmitters and neuropeptides to modulate the level of sympathetic outflow from SPN to the peripheral target organs (**section 1.5.3**; Dampney, 1994; Guyenet, 2006). The activity of presympathetic neurons is affected by input from higher cortical areas, parasympathetic premotor neurons and other presympathetic neurons (McKitrick & Calaresu, 1996; Kubo *et al.*, 2000; Saha *et al.*, 2005). For example, the amygdala projects to the RVLM potentially mediating the cardiovascular response to emotional stress (Saha *et al.*, 2005). Although there are specific areas of the brainstem and hypothalamus that regulate sympathetic outflow, sympathetic outflow is ultimately controlled by a complex neural network.

1.4.1 Paraventricular nucleus of the hypothalamus

The PVN is located bilaterally along the midline, lateral to the third ventricle (**Figure 1.2**), and plays an important role in autonomic and neuroendocrine control of AP (Coote, 2005) and blood volume (Deering & Coote, 2000). The PVN contains two functionally distinct cell populations; the magnocellular and parvocellular hypothalamic neurons (Herman *et al.*, 1996). Magnocellular PVN neurons project to the posterior pituitary and secrete vasopressin or oxytocin directly into the vascular system (Swanson & Kuypers, 1980). The parvocellular PVN neurons, and some vasopressinergic magnocellular neurons, are involved in ANS and AP regulation via spinal cord projections (Swanson & Kuypers, 1980; Sawchenko & Swanson, 1982; Ranson *et al.*, 1998). Neurons in the PVN project to the RVLM (Coote *et al.*, 1998), nucleus ambiguus, nucleus of the solitary tract (NTS), A1 and A5 cell groups and dorsal vagal nucleus (Swanson & Kuypers, 1980; Geerling *et al.*, 2010). The PVN receives input from the A1 nucleus (Sawchenko & Swanson, 1982; Day *et al.*, 1984), but most importantly from the RVLM (Card *et al.*, 2006; Das *et al.*, 2007).

Activity of RVLM-projecting PVN neurons is increased by hemorrhage, but not by mild hypotension (Badoer & Merolli, 1998). Stimulation of the parvocellular neurons in the dorsal cap of the PVN increases AP, barosensitive RVLM neuron firing (Coote *et al.*, 1998), and increases SNA to the splanchnic, adrenal and cardiac
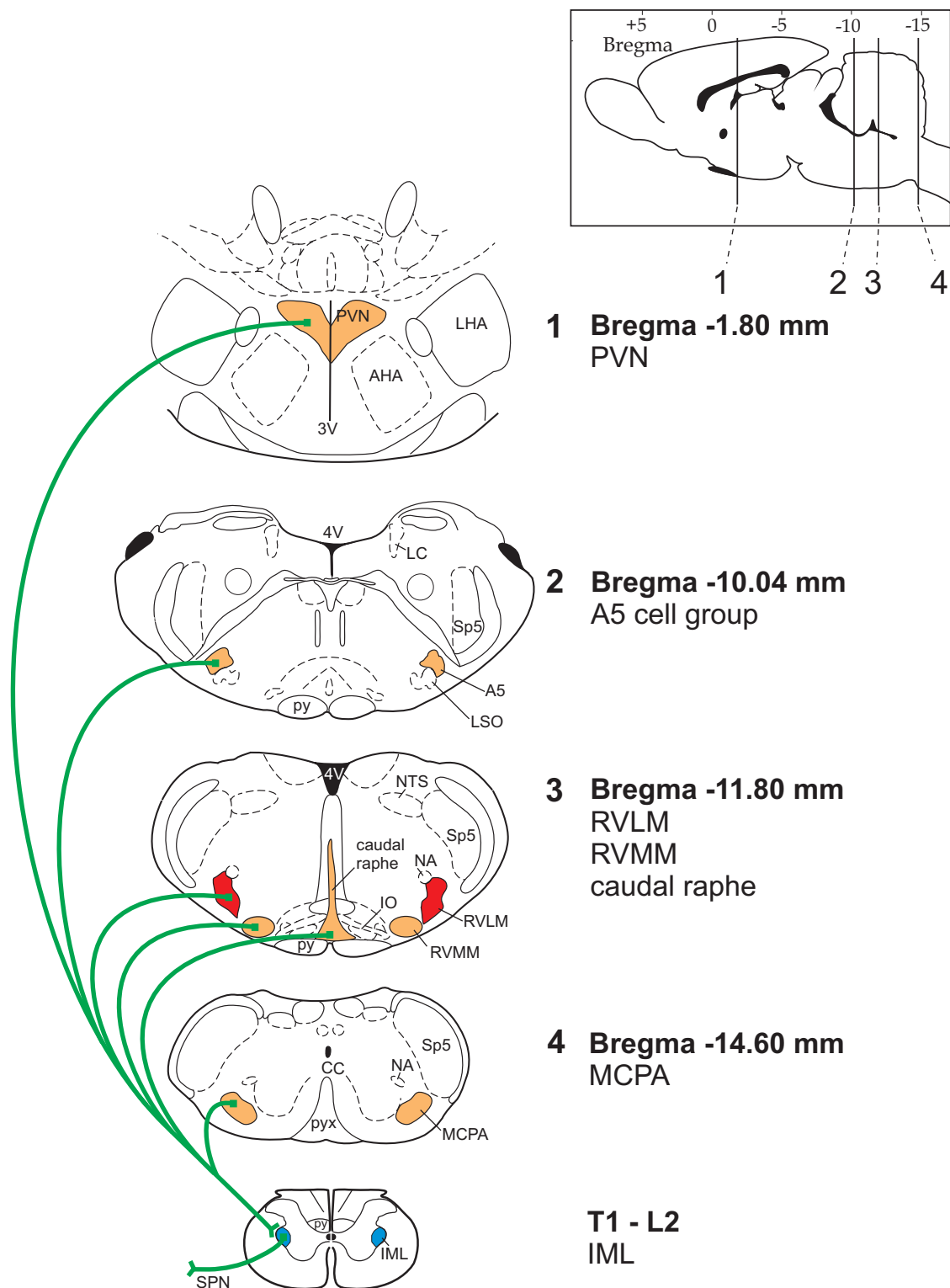
Figure 1.2 Sympathetic premotor nuclei and descending sympathetic pathways
Six sympathetic premotor nuclei (shown in orange, except the RVLM in red) project directly to SPN in the IML (blue) of the thoracolumbar spinal cord (T1-L2). Saggital orientation of the above coronal sections are shown in the top right. Abbreviations: 3V, third ventricle; 4V, fourth ventricle; AHA, anterior hypothalamic area; CC, central canal; IML, intermediolateral cell column; IO, inferior olive; LC, locus coeruleus; LHA, lateral hypothalamic area; LSO, lateral superior olive; MCPA, medullo-cervical pressor area; NA, nucleus ambiguus; NTS, nucleus of the solitary tract; PVN, paraventricular nucleus of the hypothalamus; py, pyramidal tract; Pyx, pyramidal decussation; RVLM, rostral ventrolateral medulla; RVMM, rostral ventromedial medulla; Sp5, spinal trigeminal tract; SPN, sympathetic preganglionic neuron (Adapted from (Strack *et al.,* 1989))

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sympathetic beds, whilst decreasing renal sympathetic nerve activity ($_R$SNA; Coote *et al.*, 1998; Deering & Coote, 2000). Increases in $_R$SNA following PVN stimulation have also been reported (Tagawa & Dampney, 1999). Inhibition of PVN neurons with muscimol (gamma-aminobutyric acid (GABA) agonist) decreased AP and SNA, an effect at least partially mediated by an RVLM relay (Deering & Coote, 2000; Allen, 2002; Akine *et al.*, 2003). Together with the knowledge that RVLM inhibition results in AP and SNA falling to spinal levels (Guertzenstein & Silver, 1974; Dampney & Moon, 1980; Dampney, 1994), this evidence suggests that the parvocellular PVN is necessary but not sufficient for maintaining basal sympathetic tone and may differentially regulate SNA. The PVN contributes more to state-dependant adjustments in AP, such conditioned fear (Carrive & Gorissen, 2008) or heart failure (Coote, 2005; Xu *et al.*, 2012).

1.4.2 A5 noradrenergic cell group of the ventral pons

The A5 group is a tyrosine hydroxylase (TH) positive cell group located in the ventrolateral pons, at bregma -10.04 mm (**Figure 1.2**), between the facial nucleus and the superior olive (Clark & Proudfit, 1993). The A5 nucleus is a major source of noradrenergic input to SPN (Byrum *et al.*, 1984) and also projects to the NTS (Loewy *et al.*, 1979b; Byrum & Guyenet, 1987). The A5 nucleus receives afferent inputs from major cardiovascular centres such as the ventrolateral medulla, PVN and NTS (Byrum & Guyenet, 1987; Li *et al.*, 1992). Despite the anatomical evidence, the role of the A5 region in cardiovascular contol is not clear. A5 neurons are inhibited by baroreflex activation (Guyenet, 1984), while glutamatergic stimulation of the A5 in anaesthetised rats decreased AP, HR, stroke volume and cardiac output (Neil & Loewy, 1982; Stanek *et al.*, 1984). This decrease is likely to be mediated via direct or interneuron GABA-ergic projections to SPN (Hara *et al.*, 1997). However, chemical stimulation of the A5 nucleus, in rabbits, decreases (Drye *et al.*, 1990) or does not alter AP (Maiorov *et al.*, 1999). Electrical stimulation of the A5 region increases AP in rats (Loewy *et al.*, 1979a) and rabbits (Woodruff *et al.*, 1986; Drye *et al.*, 1990), possibly due to activation of both neurons and fibres of passage (Maiorov *et al.*, 1999). Glutamate microinjection into the A5 nucleus increases $_R$SNA and splanchnic sympathetic nerve activity ($_S$SNA), whilst decreasing lumbar sympathetic nerve activity ($_L$SNA) in the anaesthetised rat (Huangfu *et al.*, 1992), implicating the A5 region in the differential control of SNA to various vascular beds.

However, inactivation/ablation of the A5 results in little change to AP or SNA in anaesthetised animals (Huangfu *et al.*, 1992; Koshiya & Guyenet, 1994; Maiorov *et al.*, 2000). Indicating that the A5 group is not involved in the tonic maintenance of AP, however its effects may be more profound in reflexive adjustments (such as fear; Carrive & Gorissen, 2008).

1.4.3 Caudal raphé of the medulla

The raphé nuclei are a complex neural network, spanning the entire length of the midline brainstem. They are divided into two groups at the mid-pons, the rostral raphé group and the caudal raphé group (Hornung, 2003), the caudal raphé group is of interest here. The caudal raphé is located at bregma -11.80 mm, and extends ventrally towards the pyramids (**Figure 1.2**) as collections of scattered neurons grouped into three nuclei: the raphé magnus, raphé pallidus and raphé obscures (Hornung, 2003). Many raphé neurons are serotonergic, with many functions and diverse projections throughout the entire brain and spinal cord (Holstege & Kuypers, 1987; Hornung, 2003), including both the IML and RVLM (Bacon *et al.*, 1990; Smith *et al.*, 1998; Heslop *et al.*, 2004). Chemical/electrical stimulation of the caudal raphé nuclei results in an increase or decrease in AP, depending on the location of the stimulation (McCall, 1984; Minson *et al.*, 1987; Haselton *et al.*, 1988; Coleman & Dampney, 1995). The depressor sights are thought to mediate their effects through GABA (McCall & Humphrey, 1985; McCall, 1988). Chemical inhibition demonstrates that the caudal raphé is not tonically active (Coleman & Dampney, 1995), however, some bulbospinal raphé neurons are inhibited by baroreceptor activation, implicating the caudal raphé in the reflex control of AP (Pilowsky *et al.*, 1995b). The precise role for the caudal raphé in cardiovascular control remains uncertain despite anatomical evidence suggesting varied autonomic roles (Pilowsky *et al.*, 1995b). The caudal raphé appears to have a role in chemoreception (Bernard *et al.*, 1996), hemorrhage (Dean & Woyach, 2004; Heslop *et al.*, 2004), differential sympathetic control and temperature regulation through its influence on SNA to cutaneous vasculature and BAT (Morrison, 1999; Morrison *et al.*, 1999; Rathner *et al.*, 2001; Cao & Morrison, 2003; Nakamura & Morrison, 2007; Madden, 2012).

1.4.4 Rostral ventromedial medulla

The RVMM (**Figure 1.2**) pressor region is located caudally from the rostral half of the facial nucleus to the rostral pole of the inferior olivary nucleus, extending from 0.5-1.5 mm lateral to the midline (Cox & Brody, 1989). The RVMM plays role in nociception, by integrating pain signals from the periphery, spinal cord and higher brain structures (Dampney, 1994). The RVMM has two distinct cell populations, neurons that either rapidly increase or decrease their activity in response to nociceptive stimuli (Heinricher *et al.*, 2009). A portion of the RVMM, parallel to the facial nucleus (Varner *et al.*, 1992), is involved in cardiovascular function. The cardiovascular subdivision of the RVMM receives inputs from the periaqueductal grey (PAG; Morgan *et al.*, 2008), and projects to the IML, NTS, caudal ventrolateral medulla (CVLM), RVLM and nucleus ambiguus (Smith *et al.*, 1998; Babic & Ciriello, 2004; Babic *et al.*, 2008). Chemical/electrical activation of RVMM neurons increases AP and SNA (Pilowsky *et al.*, 1986; Minson *et al.*, 1987; Zhuo & Gebhart, 1990), while lignocaine microinjection decreases AP and SNA (Cox & Brody, 1989). The effects of the RVMM are thought to occur via CVLM inhibition causing RVLM dis-inhibition (Babic & Ciriello, 2004). Inhibition of the RVMM results in little change to AP or SNA in anaesthetised animals (Henderson *et al.*, 1998), indicating that the RVMM is not involved in the tonic control of AP. Its effects may be more profound in responses to nociceptive or other stimuli, or may be impaired under anaesthesia. RVMM neurons also play a role in thermoregulation (Smith *et al.*, 1998; Blessing & Nalivaiko, 2000; Ootsuka *et al.*, 2004; Nakamura & Morrison, 2007; Morrison *et al.*, 2008), and chemoception (Miura *et al.*, 1996).

1.4.5 Medullo-cervical pressor area

In 1997, retrograde tracing studies identified novel neurons in the lateral cervical nucleus, lateral spinal nucleus and the lateral funiculus innervating SPN (Jansen & Loewy, 1997), neurons that could potentially influence vascular function. Functional testing of these neurons resulted in the discovery of the MCPA (see **Figure 1.2**; Seyedabadi *et al.*, 2006). Glutamate microinjections made from bregma -14.6 mm to the caudal portion of the third cervical segment evoked significant hypertension (>20 mmHg) and sympathoexcitation (Seyedabadi *et al.*, 2006). The MCPA contributes bulbospinal projections to SPN, establishing the MCPA as a potentially important area in central cardiovascular control (Seyedabadi *et al.*, 2006).

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The MCPA functions independently of the RVLM and the caudal pressor area, since RVLM blockade and transection below the caudal pressor area did not affect the responses of the MCPA to glutamatergic stimulation (Seyedabadi *et al.*, 2006). Despite this, RVLM blockade and transection below the caudal pressor area both resulted in AP and SNA falling to spinal levels. Therefore, the MCPA is not involved in tonic vasomotor tone maintenance. The role of the MCPA in cardiovascular regulation remains unknown; however, it is activated by visceral pain and may be involved in an autonomic response to such stimuli (Menetrey & De Pommery, 1991; Clement *et al.*, 2000).

1.4.6 Rostral ventrolateral medulla

From the evidence above, we can see that the non-RVLM presympathetic nuclei are not involved in maintaining sympathetic tone and basal AP levels. The exact role of these nuclei remains unknown or controversial. However, the RVLM plays an essential role in the tonic and reflex control of the cardiovascular system, by setting basal vasomotor tone and AP. The RVLM, and its tonic outflow to SPN, is the focus of this thesis and is discussed further in **section 1.5**.

1.5 The rostral ventrolateral medulla

The RVLM describes a functional and anatomical region located 0-1000 μm (concentrated to the first 400 μm) directly caudal to the caudal border of the facial motor nucleus, ventral to the compact formation of the nucleus ambiguus, medial to the spinal trigeminal nucleus and lateral to the inferior olivary nucleus/pyramidal tract (**Figure 1.3**; Ruggiero *et al.*, 1985; Card *et al.*, 2006). Of the sympathetic premotor nuclei (**section 1.4**), the RVLM is most crucial in the tonic and reflex control of the cardiovascular system as vasomotor tone cannot be maintained without a functional RVLM. (Pilowsky & Goodchild, 2002; Guyenet, 2006). Although originally identified during the 1870's, the importance of the RVLM in maintaining vasomotor tone was not established until the 1970-1980's when AP and SNA fell to spinal levels following chemical/electrical lesioning of the RVLM (Guertzenstein & Silver, 1974; Feldberg & Guertzenstein, 1976; Dampney & Moon, 1980; Reis *et al.*, 1984). An effect not replicated by the lesioning/destruction of the other premotor nuclei (see (Dampney, 1994) for review). Stimulation of the RVLM causes increases in SNA, plasma catecholamines and AP (Goodchild *et al.*, 1982; Brown & Guyenet,

A

4V

NTS

caudal raphe

Sp5

NA

RVLM

IO

RVLM

py

B

PVN SON

Other decending inputs e.g. DMH

RVLM

Somatosympathetic

Intramedullary respiratory inputs

CVLM

Chemoreceptors

Baroreceptors

NTS

A1

CPA

Sympathetic Outflow to blood vessels, heart, adrenals, etc.

IML

Legend

● Inhibitory
● Excitatory
----◁ Inhibitory
——◁ Excitatory
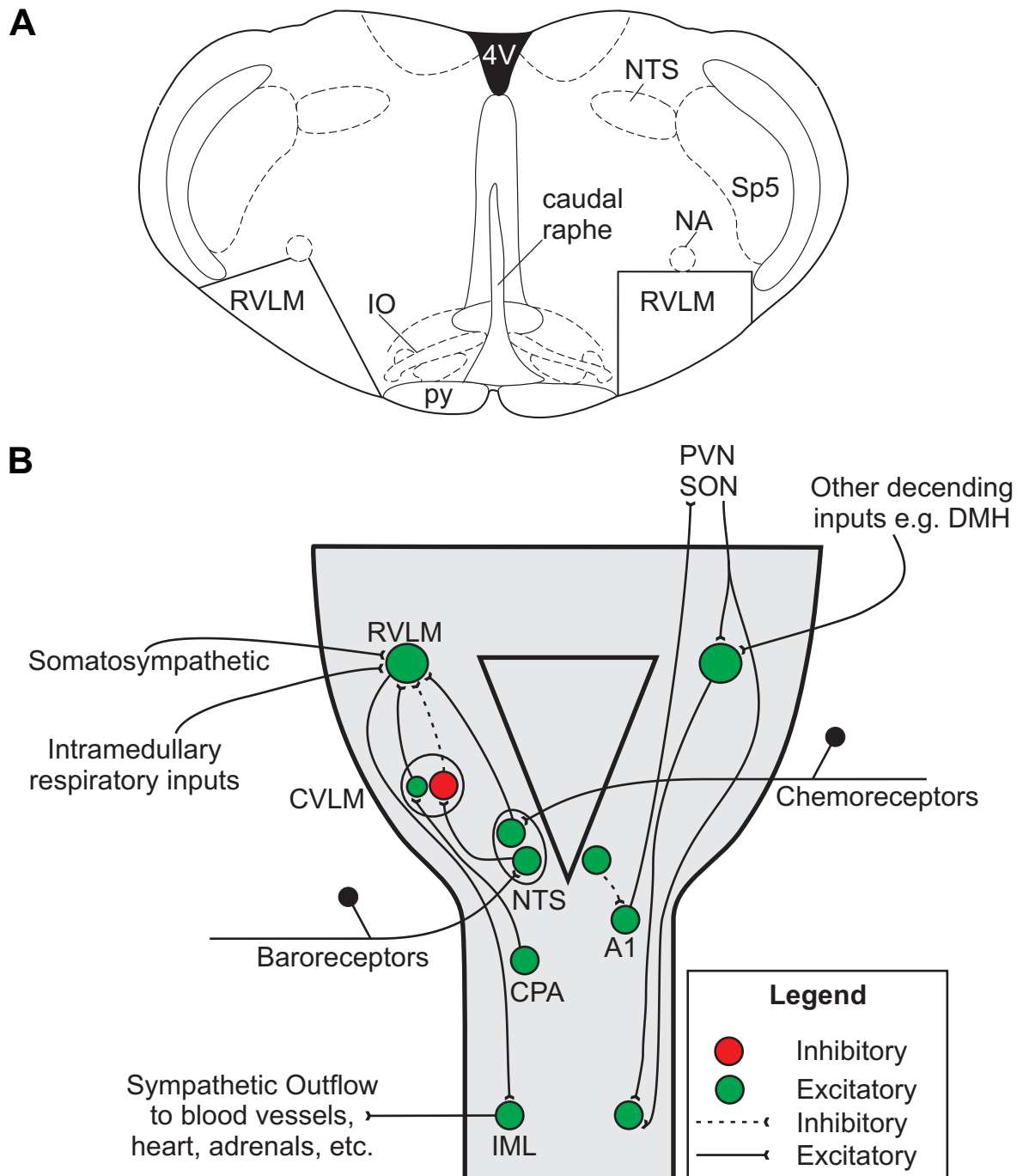
Figure 1.3 Anatomy of the RVLM and it's afferent and efferent projections
(**A**) The anatomical region containing the RVLM can be defined differently in the literature. The coronal brainstem section shows two alternative definitions; the RVLM can be defined as a triangular (left) or rectangular (right) area located ventral to the NA, lateral to the IO/Py and medial to the Sp5. The afferent and efferent projections of the RVLM are extensive and complex. Here, some of the numerous pathways that influence the main nuclei involved in the tonic and reflex control of blood pressure are shown in diagrammatic form (**B**). Inhibitory pathways are indicated by dashed lines and excitatory pathways are indicated by continuous lines. Red circles indicate inhibitory neurons and green circles indicate excitatory neurons (Adapted from (Pilowsky *et al.,* 2002)). Abbreviations: 4V, fourth ventricle; A1, A1 cell region; CPA, caudal pressor area; CVLM, caudal ventrolateral medulla; DMH, dorsal medial hypothalamus; IML, intermediolateral cell column; IO, inferior olive; NA, nucleus ambiguus; NTS, nucleus of the solitary tract; PVN, paraventricular nucleus of the hypothalamus; py, pyramidal tract; RVLM, rostral ventrolateral medulla; SON, supraoptic nucleus; Sp5, spinal trigeminal tract.

1984; Goodchild *et al.*, 1984; Ross *et al.*, 1984b; Morrison *et al.*, 1988). Furthermore, anatomical studies identified the RVLM as the primary source of supra-spinal innervation to SPN (Amendt *et al.*, 1979; Ross *et al.*, 1981; Dampney *et al.*, 1982; Caverson *et al.*, 1983).

The RVLM includes a functionally and neurochemically heterogenous neuronal population; including Supraspinal, interneurons and presympathetic neurons involved in cardiovascular regulation, but also includes non-cardiovascular neurons, which include central chemoreceptors (Mulkey *et al.*, 2004; Stornetta *et al.*, 2006), respiratory interneurons (Kanjhan *et al.*, 1995; Sun *et al.*, 1998) and other functionally ambiguous neurons. The RVLM is not only involved in the tonic control of AP, but also differentially controls SNA to the various vascular beds, is important in the reflex control of AP and modulates respiratory function. Moreover, the RVLM has also been implicated in the etiology of neurogenic hypertension (Sved *et al.*, 2003). The function, projections, cells groups and neurochemical content of the RVLM will be discussed in the following sections.

1.5.1 The cardiovascular neurons of the RVLM

The neurons essential for the generation of vasomotor tone and expression of sympathetic reflexes are the 'cardiovascular' RVLM neurons. Cardiovascular RVLM neurons represent only a subpopulation of the neuronal phenotypes present in the RVLM; these neurons are defined as those that are spontaneously active, are inhibited by baroreflex activation (pulse modulated) and have modest respiratory modulation. This population includes bulbospinal (presympathetic) and non-bulbospinal neurons, as well as mixed collaterals (Caverson *et al.*, 1983; Ross *et al.*, 1984b; Brown & Guyenet, 1985; Tucker *et al.*, 1987; Morrison *et al.*, 1988; Barman & Gebber, 1989; Stornetta *et al.*, 1999; Oshima *et al.*, 2006). The response of cardiovascular RVLM neurons to the activation of visceral, somatic and supra-medullary inputs mirrors the effects of these inputs on peripheral SNA (Brown & Guyenet, 1985; Morrison *et al.*, 1988) with varying magnitudes (Brown & Guyenet, 1985; McMullan *et al.*, 2008). Therefore, cardiovascular RVLM neurons are thought to be intimately involved in the regulation of SPN and SNA, thereby maintaining vasomotor tone. Mention of the RVLM or RVLM neurons in this thesis refers to the cardiovascular RVLM neurons.

Intracellular recordings from bulbospinal RVLM neurons *in vivo* shows that they have an irregular firing pattern (evident in SPN also; **Figure 1.4**), as their membrane excitability is regulated by ongoing excitatory and inhibitory inputs (Lipski *et al.*, 1996b). The basal activity of these neurons was found to be 5-40 Hz, and was negatively correlated to AP. The spontaneous activity of RVLM neurons and the characteristic bursting pattern of SNA is absent above ~150 mmHg (Brown & Guyenet, 1985; Morrison *et al.*, 1988; Haselton & Guyenet, 1989b; Lipski *et al.*, 1995b). This basal activity also exhibits strong pulse modulation (also present in SPN (**Figure 1.4**), this is evident as increased firing during the falling phase of the AP wave (Brown & Guyenet, 1985; Lipski *et al.*, 1996b). Pulse modulation is lost following acute barodenervation or pharmacological baroreceptor unloading (McAllen & Spyer, 1978; Thrasher, 2005a), confirming that this firing pattern is due to ongoing baroreceptor input. RVLM neurons also exhibit firing patterns related to respiration (**Figure 1.4** and **section 1.7.3**), the magnitude of which is related to central respiratory drive. *In vivo* respiratory modulation of RVLM neurons is characterised either by an inspiratory depression, inspiratory activation, early inspiratory depression or unclear modulation (McAllen, 1987; Haselton & Guyenet, 1989a; Miyawaki *et al.*, 1995).

1.5.2 Afferent and efferent RVLM projections

The extent and complexity of the networks involving the RVLM (**Figure 1.3**) is a testament to its importance within AP control. The activity of RVLM neurons and its efferent projections are modulated by input from many areas. Retrograde tracing studies in the rat, rabbit and cat have shown that these areas include; the caudal pressor area (Campos & McAllen, 1999; Campos *et al.*, 2008), NTS (Aicher *et al.*, 1996; Koshiya & Guyenet, 1996a), PVN (Coote *et al.*, 1998), somatosympathetic afferents (Morrison & Reis, 1989), CVLM (Li *et al.*, 1992), supraoptic nucleus, RVMM, caudal raphé and PAG (Chen & Aston-Jones, 1995; Farkas *et al.*, 1998; Sartor & Verberne, 2003).

Anterograde (Ross *et al.*, 1984a; Pyner & Coote, 1998; Card *et al.*, 2006) and retrograde (Ross *et al.*, 1984a; Minson *et al.*, 1990; Minson *et al.*, 1991; Jeske & McKenna, 1992; Pyner & Coote, 1998) tracing studies established that many RVLM neurons project to the IML of the spinal cord (T1-L2), projections were confirmed

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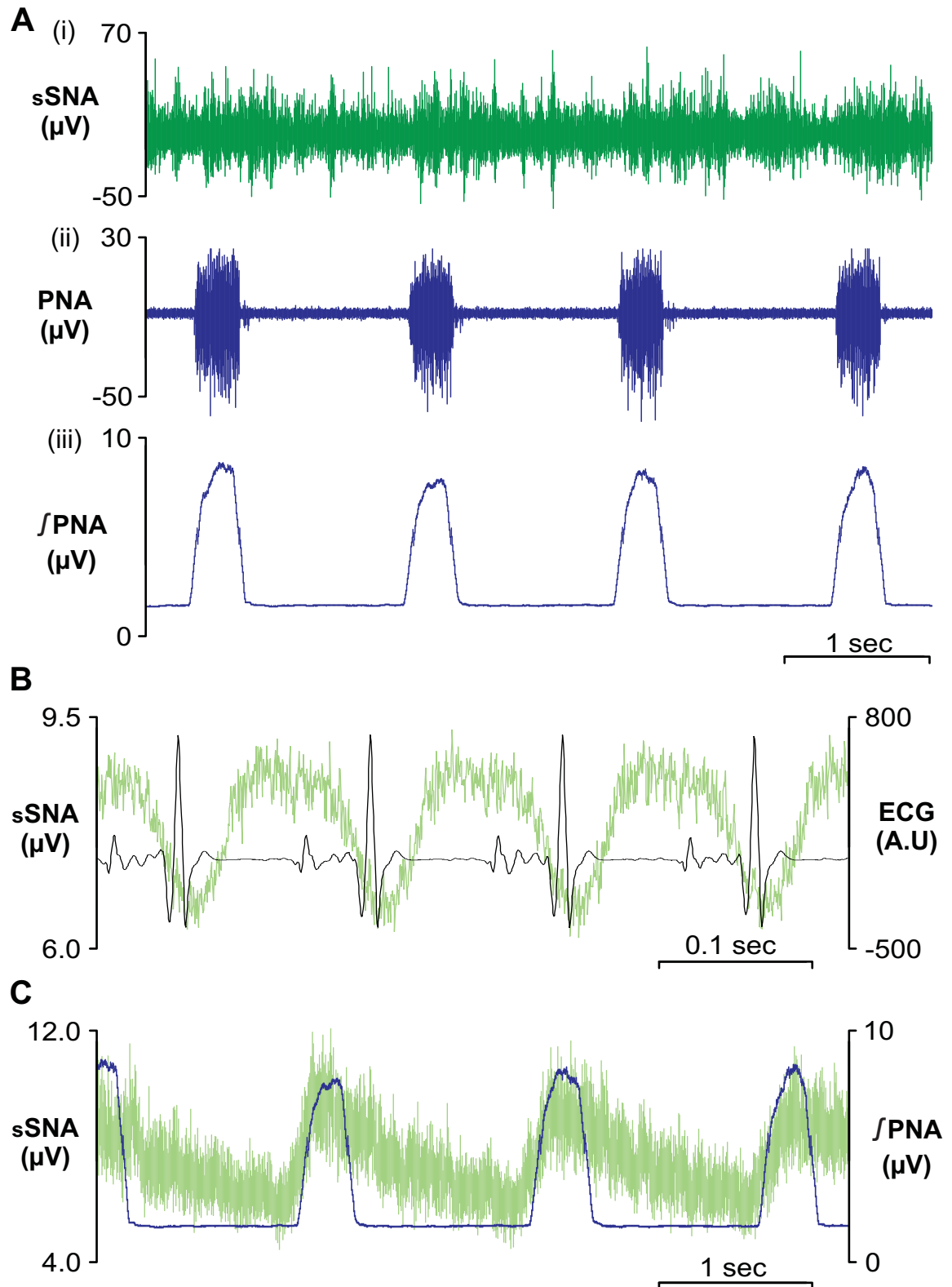
Figure 1.4 Representative nerve recording traces
(**A**) Representative traces showing recordings of sSNA (i; green) and PNA (ii; blue). SNA regulating sympathetic vasomotor tone displays a characteristic irregular rhythmic bursting pattern that is tonically active. PNA controls the contraction of the diaphragm. The integrated PNA trace (iii) was smoothed with a 50 msecond time constant. Basal SNA exhibits strong pulse modulation (**B**) and also is synchronised with respiratory discharge (**C**). Abbreviations: ECG, electrocardiogram; PNA, phrenic nerve activity; sSNA, splanchnic sympathetic nerve activity.

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with antidromic activation of RVLM neurons (Brown & Guyenet, 1985; Lipski *et al.*, 1995b; Lipski *et al.*, 1996a). Projections from the RVLM to SPN are highly specific and predominantly ipsilateral (~63%; Jeske & McKenna, 1992; Moon *et al.*, 2002), with no projections to thoracic dorsal or ventral horns. Bulbospinal RVLM neurons originate from the rostral RVLM (within 400 μm caudal to the facial nucleus in rat), and innervate all levels of the thoracic IML corresponding to SPN distribution (Strack *et al.*, 1989a; Jansen *et al.*, 1995; Pyner & Coote, 1998). Recently, Oshima and colleagues combined whole-cell patch clamp recording of SPN with extracellular recording of RVLM units, to confirm monosynaptic coupling between RVLM neurons and vasomotor SPN (Oshima *et al.*, 2008).

RVLM neurons project dorsomedially for roughly 1.5-2 mm before turning sharply caudal to project to the IML (Schreihofer & Guyenet, 1997). Axonal collaterals arise from the dorsomedially-projecting stem in 66% of neurons and project to several brainstem regions including: the RVLM, CVLM, A5 and dorsomedial medulla (Haselton & Guyenet, 1990; Lipski *et al.*, 1995b; Schreihofer & Guyenet, 1997; Agassandian *et al.*, 2012). Most barosensitive, non-bulbospinal neurons also coursed dorsomedially before turning rostrally; few neurons exhibited axonal bifurcations adjacent to the cell body, projecting rostrally and caudally (Lipski *et al.*, 1995b; Schreihofer & Guyenet, 1997).

The RVLM also contains neurons with dedicated discrete projections throughout supraspinal areas such as the PVN, supraoptic nucleus, arcuate nucleus, raphé, PAG, locus coeruleus (Tucker *et al.*, 1987; Card *et al.*, 2006) and to the contralateral RVLM (McMullan & Pilowsky, 2012). Supraspinally projecting RVLM neurons are concentrated in the caudal RVLM (600-800 μm caudal to the facial nucleus in rat; Verberne *et al.*, 1999). Approximately half of these neurons co-express catecholamines and neuropeptide Y (**Figure 1.5**; Sawchenko *et al.*, 1985; Stornetta *et al.*, 1999). These neurons have slow axonal conduction (<1 millisecond/m) and low basal discharge rates (Verberne *et al.*, 1999), illustrating that RVLM neurons form distinct populations, based on neurochemical content and connectivity, which probably pertain to a specific function. The function of the supraspinal RVLM projections is not well understood, they may drive aspects of central cardiovascular control that are regulated by the hypothalamic-pituitary axis,

including; blood volume, feeding, circadian rhythm, thermogenesis and pain (Tucker *et al.*, 1987; Pieribone & Aston-Jones, 1991; Herbert & Saper, 1992; Verberne *et al.*, 1999; Coote, 2005; Card *et al.*, 2006; Madden *et al.*, 2006).

1.5.3 Neurochemical phenotype of RVLM neurons

The RVLM is a highly heterogeneous cell population that contains with many neurotransmitters, neuropeptides and receptors (**Figure 1.5**). A number of chemical lesioning, electrophysiological, pharmacological and immunohistochemical studies contribute to the large body of work dedicated to determining the phenotype of RVLM neurons (Tucker *et al.*, 1987; Schreihofer & Guyenet, 1997; Madden & Sved, 2003). This information is important, as it provides some insight into the potential function and downstream targets of the RVLM.

Bulbospinal RVLM neurons involved in the tonic and reflex control of AP are excitatory. Majority of cardiovascular RVLM neurons express the vesicular glutamate transporter (VGLUT2; Stornetta *et al.*, 2002a; Stornetta *et al.*, 2002b), indicating that glutamate is the primary neurotransmitter used in neurotransmission between RVLM neurons and SPN (Morrison *et al.*, 1989a; Morrison *et al.*, 1989b; Morrison *et al.*, 1991; Llewellyn-Smith *et al.*, 1992). In support of this, blockade of ionotropic glutamate receptors (kynurenic acid) abolishes SPN responses to RVLM stimulation (Morrison, 2003). Not all RVLM neurons contain glutamate, there is a population of ill defined inhibitory neurons (GABA-ergic and glycinergic), that are not thought to be intimately involved in SPN regulation; they may represent inhibitory respiratory neurons (Stornetta *et al.*, 2004). Subpopulations of glutamatergic RVLM neurons are co-localised with other neurotransmitters such as Ad (C1 cell group; contains both TH and phenylethanolamine-N-methyltransferase (PNMT)), NAd (A1 cell group; contains TH only), and various other metabotropic neurotransmitters (**sections 1.5.3.2** and **1.5.3.3**; Pilowsky & Goodchild, 2002).

1.5.3.1 The C1 adrenergic cell group of the RVLM

The best characterised subpopulation of RVLM neurons is the C1 adrenergic cell group, a cell group overlapping with the anatomical location of the RVLM. By definition, C1 neurons contain all the necessary enzymes for Ad production, these include TH, L-amino-acid-decarboxylase, dopamine-β-hydroxylase (DβH; converts

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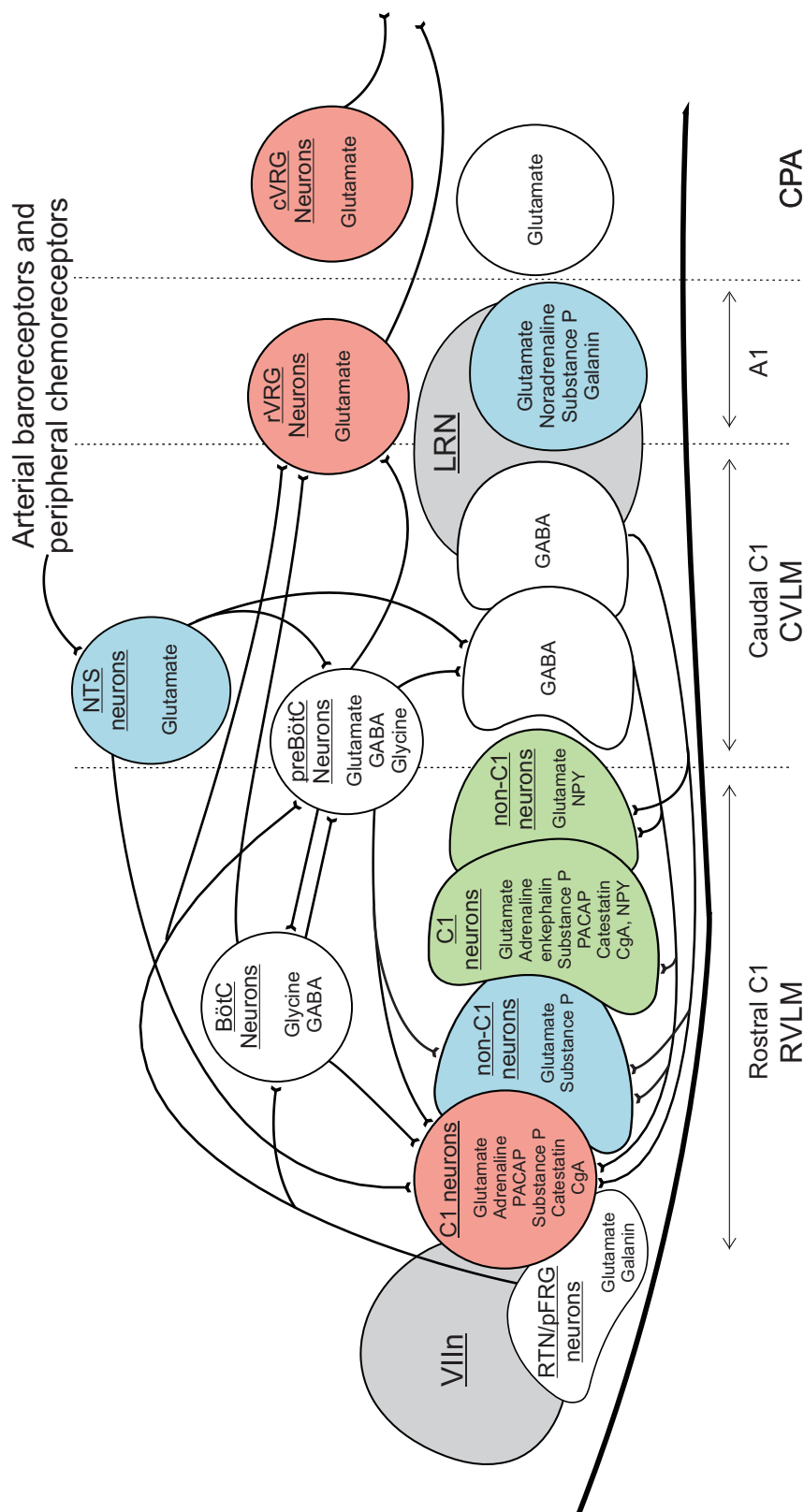
Figure 1.5 The neurochemical phenotype of neurons in the ventral medulla

A schematic illustration of a parasagittal section through the ventral medulla indicating some of the diverse neuronal subtypes involved in central cardiorespiratory control. Neuronal subtypes are identified on the basis of their neurotransmitter content, efferent projection and/or function. An indication of the rostrocaudal positions of the neurons is given with respect to other neurons (not to scale). Note the functionally diverse cell groups that can be identified within each defined region. Intermedullary connections are indicated above, while neurons with spinal projections are identified in red, those with supramedullary projections are in green and those that have both spinal and supramedullary projections are in blue (Adapted from (Pilowsky *et al.*, 2002)). Abbreviations: BötC, Bötzinger complex; CgA, Chromogranin A; CPA, caudal pressor area; CVLM, caudal ventrolateral medulla; cVRG, caudal ventral respiratory group; LRN, lateral reticular nucleus; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; preBötC, pre-Bötzinger complex; RTN/pFRG, retrotrapezoid nucleus/parafacial respiratory group; RVLM, rostral ventrolateral medulla; rVRG, rostral ventral respiratory group; VIIn, facial nucleus.

dopamine to NAd) and PNMT (Bolme *et al.*, 1974; Hokfelt *et al.*, 1974; Ross *et al.*, 1981; Ross *et al.*, 1984b; Stornetta *et al.*, 1999; Phillips *et al.*, 2001; Akine *et al.*, 2003). Around 60-80% of presympathetic bulbospinal RVLM neurons are C1 neurons (Tucker *et al.*, 1987; Lipski *et al.*, 1995b; Sved *et al.*, 2003). Many C1 neurons (~40%) are barosensitive (Tucker *et al.*, 1987; Jeske & McKenna, 1992; Verberne *et al.*, 1999; Phillips *et al.*, 2001; Sartor & Verberne, 2003), are activated by decreases in AP (Sved *et al.*, 1994) and are sympathoexcitatory (Abbott *et al.*, 2009). Quantitative ultrastructural analysis revealed that ~5% of all synapses within the IML were TH positive. However the functional influence of C1 neurons in AP control may still be large, given that only around 8% of cat lumbar SPN are vasomotor (Pilowsky *et al.*, 1994a).

Although many neurotransmitters are known to be present in C1 neurons, the role that each transmitter plays is still debated. C1 neurons are catecholaminergic (Ad/NAd) and glutamatergic, the primary neurotransmitter, and the stimuli for the release of each transmitter, is unclear. C1 neurons appear to communicate with both transmitters, as sympathoexcitation observed following electrical stimulation of the RVLM is attenuated by intrathecal glutamate and α_2 adrenergic receptor antagonists (Mills *et al.*, 1988; Morrison *et al.*, 1989a; Morrison *et al.*, 1989b; Huangfu *et al.*, 1994). However, these studies did not separate the contribution of C1 and non-C1 neurons in these effects. Recent work in brainstem slice cultures supports the hypothesis that C1 neurons primarily communicate with glutamate, conditionally releasing catecholamines depending on the degree of neuronal activation, paracrine signals and/or the local environment (Chiti & Teschemacher, 2007; Kasparov & Teschemacher, 2008).

Less is known about non-C1 RVLM neurons (lack catecholamine producing enzymes; Lipski *et al.*, 1995a; Lipski *et al.*, 1995b). When compared with C1 neurons, non-C1 neurons share similar anatomical (Ross *et al.*, 1984a; Cunningham Jr *et al.*, 1990; Schreihofer & Guyenet, 1997; Card *et al.*, 2006), electrophysiological (Lipski *et al.*, 1995a; Lipski *et al.*, 1995b) and neurochemical properties (Goodchild *et al.*, 2000; Stornetta *et al.*, 2001; Stornetta *et al.*, 2002a; Burman *et al.*, 2004; Farnham *et al.*, 2008; Gaede *et al.*, 2009; Shahid *et al.*, 2012). One point of difference between C1 and non-C1 RVLM neurons is their conduction velocities.

Bulbospinal RVLM neurons have a bimodal conduction velocity (fast conducting; 0.6-0.8m/s; slow conducting; 3.1-3.5m/s; total range; 0.4-7.0m/s), as demonstrated by antidromic stimulation (Morrison *et al.*, 1988; Schreihofer & Guyenet, 1997). The conduction velocity correlates with TH content; C1 and non-C1 neurons display fast conduction, whereas only C1 neurons conduct slowly (Schreihofer & Guyenet, 1997). C1 neurons have bimodal conduction velocities due to the presence of myelinated and unmyelinated neurons within the population (Morrison *et al.*, 1988); the significance of this is unclear.

1.5.3.2 Neurochemical heterogeneity of the RVLM

Many neuropeptides are co-expressed in subpopulations of bulbospinal C1 and non-C1 RVLM neurons (**Figure 1.5**). For example, the endogenous opiate precursor preproenkephalin is present in ~80% of non-C1 bulbospinal neurons and in ~20% of C1 bulbospinal neurons (Stornetta *et al.*, 2001). PACAP is found in 84% of C1 neurons and in 82% of bulbospinal C1 neurons; 40% of the PACAP neurons were unlabelled for either TH or cholera toxin B (retrograde tracer; Farnham *et al.*, 2008). Other neuroactive substances found in region include: cholecystokinin (Sartor & Verberne, 2002), calbindin (Goodchild *et al.*, 2000), neuropeptide Y (Minson *et al.*, 1994; Stornetta *et al.*, 1999), neurokinin 1 (Makeham *et al.*, 2005), cocaine- and amphetamine-regulated transcript (Burman *et al.*, 2004), catestatin (Gaede *et al.*, 2009), orexin (Shahid *et al.*, 2012), preprotachykinin (substance P precursor; Li *et al.*, 2005) and angiotensin II (Head, 1996; Le Mevel *et al.*, 2008; Arakawa *et al.*, 2012; Chen *et al.*, 2012; Lancien *et al.*, 2012). Differences in neurochemical phenotype may allow us to define different RVLM cell subtypes that serve different functions in regulating SNA.

1.5.3.3 Metabotropic neurotransmission within the RVLM

RVLM neurons are synaptically influenced by phenotypically diverse neurons that employ both ionotropic and metabotropic neurotransmission. Glutamate, glycine and GABA are the classic fast transmitters that act within the RVLM. Release of these fast neurotransmitters is intrinsic in the neuronal signaling involved in the tonic and reflex activity of RVLM neurons (for review see (Milner *et al.*, 1989; Sun, 1996; Guyenet *et al.*, 2002b; Milner & Pickel, 2003)). Metabotropic neurotransmitters are conditionally released from pre- and post- synaptic sites to

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modulate the excitability of RVLM neurons and SPN downstream. Many substances affect the excitability of neurons in the RVLM, and thereby alter SNA: these include: PACAP (Farnham *et al.*, 2012), galanin (Le Mevel *et al.*, 1998; Spirovski *et al.*, 2012), neuromedin U (Rahman *et al.*, 2012), somatostatin (Burke *et al.*, 2008a), catestatin (Gaede & Pilowsky, 2010), angiotensin II (Le Mevel *et al.*, 1994; Seyedabadi *et al.*, 2001; Chen *et al.*, 2012; Lancien *et al.*, 2012), orexin (Shahid *et al.*, 2012), serotonin (Pilowsky *et al.*, 1995a), opioids (Miyawaki *et al.*, 2002a), neurotensin (Zogovic & Pilowsky, 2012) and others (Pilowsky, 2008; Abbott & Pilowsky, 2009). Physiologically, the release of neuropeptides aids in the tonic and reflex control of the cardiorespiratory system. Dysfunction in a transmitter system may lead to pathological conditions, for example, over-expression of apelin mRNA and protein levels in the spontaneously hypertensive rat (SHR) results in chronic AP elevation (Zhang *et al.*, 2009b).

Neuromodulators and their receptors, such as PACAP, are expressed and located in specific populations of RVLM neurons (**Figure 1.5**); it seems probable that these different sub-populations of neurons have functional implications for sympathetic control (Milner & Pickel, 2003). Evidence suggests that the heterogeneity of the RVLM may contribute to the ability of the nuclei to differentially regulate sympathetic outflow (**section 1.5.4.2**; Krukoff *et al.*, 1985; Elfvin *et al.*, 1993; Morrison, 2001). Metabotropic neurotransmission commonly affects neuronal excitability (and/or pre-synaptic neurotransmitter or neuromodulator release) in the RVLM and IML in the short term, and may also change gene expression in the long term; assisting in the generation/maintenance of vasomotor tone (**section 1.5.4.1**). These effects are achieved through a combination of ionotropic receptors and G-protein coupled receptors (GPCR). For example, *in vivo* activation of the somatostatin 2a receptor (present in 35% of rat RVLM neurons) abolishes sympathetic tone and causes severe hypotension comparable with spinal transection (Burke *et al.*, 2008a). Whereas, PACAP receptor activation in the RVLM is sympathoexcitatory and causes a pressor effect (Farnham *et al.*, 2012). However, metabotropic neurotransmitters alone do not drive the tonic activity of the RVLM, as antagonism of many metabotropic receptors in the RVLM has little effect on AP or SNA, including angiotensin I receptors (Hirooka *et al.*, 1997; Head & Mayorov, 2001), neurokinin 1 receptors (Makeham *et al.*, 2005), PAC_1 receptors (PAC_1;

Farnham *et al.*, 2012), cannabinoid 1 receptor (Padley *et al.*, 2003) and the somatostatin 2a receptor (Burke *et al.*, 2008a). The modulatory effect of these neuropeptides also extends to homeostatic reflex responses (**section 1.5.5**), both in the RVLM and in the IML, for example catestatin (Gaede & Pilowsky, 2010) and orexin (Shahid *et al.*, 2011) increase barosensitivity in the RVLM, whereas neuromedin U decreases barosensitivity at IML (Rahman *et al.*). Centrally administered Angiotensin II also modulates the cardiac baroreflex (Gaudet *et al.*, 2000).

The prevailing view concerning the role of metabotropic neurotransmitters within the RVLM and IML is that their activation encodes different patterns of autonomic output whilst intrinsic mechanisms and fast neurotransmitters maintain basal vasomotor tone (Pilowsky, 2008; Pilowsky *et al.*, 2009). Modulation of sympathetic outflow is important in regulating AP, as such; understanding the effects of neuropeptide release in the RVLM and IML provides important insight into how the cardiovascular system is regulated. The focus of this thesis is on describing the role of the neuropeptide PACAP in the spinal cord. Specifically to investigate how PACAP release from presympathetic neurons at the IML influences the function of SPN, vasomotor tone, and the reflex control of the cardiovascular system.

1.5.4 Tonic control of the cardiovascular system

Bulbospinal C1 neurons were originally thought to be responsible for maintaining basal sympathetic outflow from the RVLM, however, their importance is now unclear. Selective bilateral destruction of ~75-85% of presympathetic C1 neurons with DβH-saporin has no effect on basal AP or SNA in anaesthetised rats, but ablates the sympathetic response to hypoxia, and attenuates baroreceptor activation and electrical stimulation of the RVLM (Schreihofer & Guyenet, 2000a, b). Microinjection of anti-DβH-saporin into the RVLM of conscious rats produced comparable effects to those seen in the anaesthetised rat; attenuation of cardiovascular reflexes and a reduction in AP (~10 mmHg; Madden & Sved, 2003; Madden *et al.*, 2006). Barosensitive non-C1 RVLM neurons remain intact following anti-DβH-saporin application, demonstrating that non-C1 neurons may be involved in maintaining resting SNA and AP. However, selective activation of C1 neurons, by photostimulation of channelrhodopsin-2 transduced RVLM neurons, increases AP

and vasomotor SNA (Abbott *et al.*, 2009). This evidence suggests that C1 neurons contribute modestly to the maintenance of vasomotor tone, but are important for the expression of sympathetic reflexes.

1.5.4.1 Origin of vasomotor tone; pacemaker theory versus network theory

The origin of the irregular firing pattern of barosensitive bulbospinal RVLM neurons (Brown & Guyenet, 1985; Morrison *et al.*, 1988) remains unclear despite extensive research (Guyenet *et al.*, 1989; Dampney *et al.*, 2007). Two major theories exist to explain how this tonic activity may be generated; 1) the pacemaker theory and 2) the network theory (Dampney *et al.*, 2000). There is still much debate regarding which theory is correct (Campard, 1997; Dampney *et al.*, 2000; Dampney, 2004; Coote, 2007). Network theory is currently the most accepted, due to a lack of evidence in support of pacemaker theory. Recent evidence shows that sympathetic tone in adult rats may, in part, result from nickel sensitive ion channels expressed by RVLM neurons (Miyawaki *et al.*, 2003). For thorough review regarding the rhythmogenicity of presympathetic RVLM neurons please see (Dampney *et al.*, 2002; Coote, 2007).

The Pacemaker Theory; suggests that RVLM neurons have the ability to generate action potentials due to an intrinsic pacemaker, akin to the sinoatrial node (Dampney *et al.*, 2000). Pacemaker-like neurons are defined as those that are able to generate their own action potentials when synaptic inputs are removed. The pacemaker theory was first proposed in 1988 when *in vitro* intracellular recordings of neonatal input-deprived RVLM neurons were found to display a pacemaker-like discharge (Sun *et al.*, 1988b). These neurons discharged regularly, with a gradual depolarisation associated with a persistent sodium conductance that preceded each action potential. Glutamate antagonism did not inhibit this pacemaker-like activity, supporting the idea that RVLM neurons do not rely on tonically active synaptic inputs (Sun *et al.*, 1988a). Sun *et al.,* (1988) also reported the absence of any TH immunoreactive pacemaker cells (Sun *et al.*, 1988c).

Intrinsic pacemaker properties were also observed in bulbospinal C1 neurons of neonatal rat slices (Kangrga & Loewy, 1995; Li *et al.*, 1995), but pacemaker cells have not been observed *in vivo* in the adult rat (Lipski *et al.*, 1996a; Lipski *et al.*,

1998). Synaptically intact, mature RVLM neurons discharge in a spontaneous irregular fashion, with no period of gradual depolarisation preceding each action potential; but with fluctuations indicative of excitatory post-synaptic potentials (EPSP) that can be slowed or stopped by induced outward currents. Furthermore, acutely dissociated RVLM neurons are not spontaneously active, despite sodium and chloride conductance during depolarisation (Lipski *et al.*, 1998). Pacemaker theory fails to account for the intrinsic fluctuations in the activity of bulbospinal RVLM neurons, which translates to similar activity in SPN and the periphery (Barman & Gebber, 1980; Gebber, 1980). It also fails to account for non-uniformity of sympathetic activity in various vasomotor nerves (Ninomiya & Irisawa, 1975; Meckler & Weaver, 1985; Janig, 1988; Michaelis *et al.*, 1993; Habler *et al.*, 1994). This evidence suggests that, under normal conditions in the mature rat, the tonic activity of RVLM neurons is primarily driven by excitatory and inhibitory synaptic inputs (network theory). However, akin to the respiratory system, it is possible that the pacemaker properties of these neurons may become apparent under abnormal conditions, where synaptic inputs become inactive.

The Network Theory; suggests that tonic RVLM activity is determined by the net summation of excitatory and inhibitory synaptic afferent inputs (Dampney *et al.*, 2000). Network theory was proposed when *in vivo* extracellular recordings of adult rats neurons, revealed the irregular firing pattern of bulbospinal RVLM neurons (Brown & Guyenet, 1985; Morrison *et al.*, 1988; Kanjhan *et al.*, 1995; Lipski *et al.*, 1996a). This output pattern, although seemingly random, displays a modulation correlated with HR and phrenic nerve activity (PNA; **Figure 1.4**; Kanjhan *et al.*, 1995), indicating that at least some synaptic input to the RVLM arises from these two events, under normal physiological conditions. Currently, it is thought that tonic RVLM neuron activity is maintained by EPSPs originating from a central neural rhythm generator (Gebber, 1980; Gebber & Barman, 1989; Lipski *et al.*, 1996a), possibly the neurons in the adjacent reticular formation in the lateral tegmental field (LTF; Gebber & Barman, 1985; Barman *et al.*, 2000). LTF neuron firing in cats (Barman & Gebber, 1987) and rats (Barman & Gebber, 1989) precedes and correlates with RVLM and postganglionic neuron discharge (Barman & Gebber, 1987; Orer *et al.*, 1999). This strong synchronicity, proximity of the LTF to the RVLM, the barosensitivity and non-bulbospinal nature of a significant proportion of

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LTF neurons supports this hypothesis. In cats and rats, cardiovascular sympathetic rhythms oscillate preferably at ~2-6 Hz (Taylor & Gebber, 1975; Barman & Gebber, 1987) and ~10 Hz (Green & Heffron, 1967; Gebber & Barman, 1989), similar frequencies to LFT neurons (Gebber & Barman, 1989). The 2-6 Hz rhythm corresponds to cardiac (Orer *et al.*, 1999) and respiratory (Bainton *et al.*, 1985; McAllen, 1987; Barman & Gebber, 1989) modulation seen in RVLM, LTF and postganglionic neurons. In cats, the parabrachial and Kölliker-Fuse nuclei may produce the 10 Hz rhythm (Zhong *et al.*, 1992).

However, the firing pattern of LTF neurons suggests participation in the rhythmicity of SNA, not in the origin of SNA. Moreover, sympathetic oscillatory networks are seen in lower neural structures, such as the spinal cord (Allen *et al.*, 1993; Gilbey, 2007). Antagonism of ionotropic excitatory and inhibitory inputs to the spinal cord does not affect the 2-6 Hz rhythm (Allen *et al.*, 1993). Although the pattern generator for the 2-6 Hz frequencies of SNA possibly lies in the spinal cord, it seems that descending projections from the medulla oblongata co-ordinate these multiple oscillators. Finally, the correlation of activities in LTF and RVLM neurons does not exclude the possibility of neurons exciting both subsets simultaneously, and independently.

1.5.4.2 Differential control of sympathetic outflow by the RVLM

Bulbospinal RVLM neurons may not uniformly control sympathetic output to different vascular beds via SPN (McAllen & Dampney, 1990). The RVLM appears to be topographically organised, with neurons controlling vasomotor output to distinct sympathetic beds being localised to subgroups within the RVLM (McAllen & Dampney, 1990; Morrison, 2001; Sved *et al.*, 2001; Stornetta, 2009; Gowen *et al.*, 2012). Chemical stimulation of discrete sub-regions within the RVLM of the cat and rabbit, differentially increases the activity of different sympathetic nerves (Dampney & McAllen, 1988; Dean *et al.*, 1992; McAllen & May, 1994; Campos & McAllen, 1997; Ootsuka & Terui, 1997). There is limited evidence to support this hypothesis in the rat (Pyner & Coote, 1998; Tanaka *et al.*, 2002), however, somatosympathetic reflex (SSR) patterning varies amongst the sympathetic innervations to the vascular beds, revealing non-uniform presympathetic drive from C1 and non-C1 RVLM neurons (Burke *et al.*, 2011). Neurons activating the renal, cardiac, lumbar and

splanchnic nerves are found in the rostromedial RVLM. Whereas, neurons controlling SNA to the blood vessels of skeletal muscle are located in the lateral and caudal RVLM. Those controlling the cutaneous SNA are located in the medial RVLM (Dampney & McAllen, 1988; Dean *et al.*, 1992; McAllen & May, 1994; Campos & McAllen, 1997; Ootsuka & Terui, 1997). Some areas in the RVLM demonstrate the ability to increase SNA to more than one target tissue, possibly due to overlapping of the neuronal subgroups within the RVLM (Campos & McAllen, 1997; McAllen *et al.*, 1997). This hypothesis is also supported by the diving reflex (O_2 conserving response; Dampney & Moon, 1980; Sun & Reis, 1994; Reis *et al.*, 1997) and the defense reaction (blood flow redistribution in preparation for exercise; Carrive *et al.*, 1989; Carrive, 1993); two states that demonstrate an immediate, powerful, differential activation of SNA.

However, the RVLM may be topographically organised in a functional (type of vascular outflow), rather than regional, manner (McAllen & Dampney, 1990; Rathner & McAllen, 1999). Vasoconstrictor drive to the forelimb and hindlimb are functionally similar and anatomically inseparable, whereas the drive to the kidney is anatomically and functionally distinct (McAllen & Dampney, 1990). The discovery that RVLM inputs excite subpopulations of bulbospinal RVLM neurons also supports this argument. For example electrical stimulation of the lateral rat PAG excites a subgroup of barosensitive bulbospinal RVLM neurons and produces profound splanchnic and renal sympathoactivation, which is withdrawn following feeding or intravenous (i.v.) cholecystokinin (Sartor & Verberne, 2003). Whereas, cholecystokinin activates RVLM neurons causing lumbar sympathoexciation and reduced skeletal muscle blood flow (Sartor & Verberne, 2002). Additionally, electrical stimulation of the posterior dorsomedial hypothalamus may excite a subpopulation of RVLM neurons that elicit the defence reaction (Wang *et al.*, 2010).

Despite the evidence suggesting topographical organisation (functional or regional) of the RVLM, it is unknown if the distinct subgroups within the RVLM are identifiable based on their neurochemical properties (Dampney, 1994; Stornetta, 2009). The neurochemical heterogeneity of the RVLM (see **section 1.5.3**) may underlie a difference in neuronal function, for example the differential control of sympathetic outflow (Krukoff *et al.*, 1985; Elfvin *et al.*, 1993; Morrison, 2001). The

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lack of AP increase, despite a large increase in regional SNA, following intrathecal PACAP (Farnham *et al.*, 2008) indicates that PACAP may have a role in the differential control of SNA. This hypothesis is supported by a study that shows PACAP differentially controls regional blood flow (relative to SNA; Minkes *et al.*, 1992b), and is investigated in this thesis (**Chapter 5** (Inglott *et al.*, 2011)).

1.5.5 Reflex control of the cardiovascular system

In addition to its role in tonic cardiovascular control, the RVLM is also crucially involved in the reflex control of AP. Autonomic reflex control of the cardiovascular system ensures adequate functioning, maintaining all parameters within their physiological ranges, despite constant environmental stimuli. The most important and extensively studied autonomic reflexes are mediated through the RVLM which receives, integrates and processes inputs from a variety of central and peripheral sensors and adjusts AP accordingly (to 'set-point'). The RVLM is involved in the baroreceptor (Pilowsky & Goodchild, 2002; Guyenet, 2006), chemoreceptor (Koshiya & Guyenet, 1996b; Nattie, 1999) and somatosympathetic reflexes (Morrison & Reis, 1989; Pilowsky & Goodchild, 2002; Guyenet, 2006), through its influence on SPN and peripheral vasomotor tone. Any neurotransmitters or neuromodulators present within this system may modulate/manipulate the reflexes (Bendle *et al.*, 1997; Burke *et al.*, 2008a; McMullan *et al.*, 2008; Gaede & Pilowsky, 2010; Shahid *et al.*, 2011; Rahman *et al.*, 2012). However, PACAP does not appear to play an important role in the reflex control of the cardiovascular system at the level of the spinal cord (**Chapter 7** (Gaede *et al.*, 2012)) or RVLM (Farnham *et al.*, 2012).

1.5.5.1 The baroreceptor reflex

Rapid increases/decreases in AP trigger a constantly active centrally-mediated negative feedback loop that regulates SNA and cardiac function in response to fluctuations in AP, to restore normal AP. This reflex loop is known as the baroreflex (**Figure 1.6**; Brown & Guyenet, 1985; Minson *et al.*, 1997; Guyenet *et al.*, 2001; Madden & Sved, 2003). There are several baroreceptor mediated reflexes including the vasopressin, HR and sympathetic baroreflexes. The focus of this thesis is the SNS, therefore, discussion of the vital parasympathetically mediated HR baroreflex and the hypothalamic vasopressin baroreflex, will cease here.

Baroreceptor input to the brainstem originates from receptors located in the tunica media and adventitia of the carotid artery and aortic arch. Baroreceptor cell bodies are located in the carotid body (petrosal ganglion) and nodose ganglion, respectively (McDonald, 1983). The baroreceptors are specialised mechanoreceptors that are activated by pressure-induced stretch of arterial walls between 50-150 mmHg (Andresen *et al.*, 1979). As the arterial walls expand in response to an increased AP, baroreceptors are activated and the reflex loop is initiated. The baroreceptors are maximally active over a limited functional range, and cause the greatest changes in SNA, per one mmHg change in AP, around normal physiological AP (90-110 mmHg), creating a sigmoidal relationship between SNA and AP. There are several baroreceptor classes with distinct pressure sensitivities, operating ranges and axonal conduction, allowing baroefferent neurons to convey transient changes in AP and absolute AP (Seagard *et al.*, 1990; Fan *et al.*, 1999). In response to baroreceptor activation, SNA to the vascular beds is inhibited, leading to restoration of normal AP. When baroreceptor input to the brainstem is withdrawn, by decreasing AP/arterial stretch, SNA is substantially increased (**Figure 1.6**). The changes in SNA are mediated by inhibition/dis-inhibition of RVLM neurons which affects the activity of the SPN in the IML (Pilowsky & Goodchild, 2002; Guyenet, 2006).

Anatomical tracing studies and chemical/electrical stimulation of the NTS, RVLM and nucleus ambiguus confirms their vital roles in the baroreflex (**Figure 1.6**; Krieger, 1964; Blessing & Reis, 1982; Pilowsky *et al.*, 1985; Sved *et al.*, 1985; Minson *et al.*, 1997; Castillo *et al.*, 2012). Activation of the baroreceptors causes mechanical-electrical coupling, generation (Brown, 1980) and propagation of an action potential, to activate the medial and dorsolateral NTS via cranial nerves IX and X (Ciriello, 1983; Housley *et al.*, 1987). Aortic baroreceptors have a dominant role over carotid baroreceptors in HR baroreflex control, as baroreflex induced bradycardia was reduced by 85% following denervation of the aortic depressor nerve (Pickering *et al.*, 2008). Baroreceptor afferent neurons are thought to communicate with NTS neurons primarily with glutamate (Zhang & Mifflin, 1998; Seagard *et al.*, 2000b; Mayorov & Head, 2003), but probably also with substances such as substance P and nitric oxide under certain conditions (Seagard *et al.*, 2000a; Pilowsky & Goodchild, 2002). Originally, it was thought that NTS neurons provided a direct GABA-ergic input to the RVLM to alter sympathetic tone (Chalmers, 1975;

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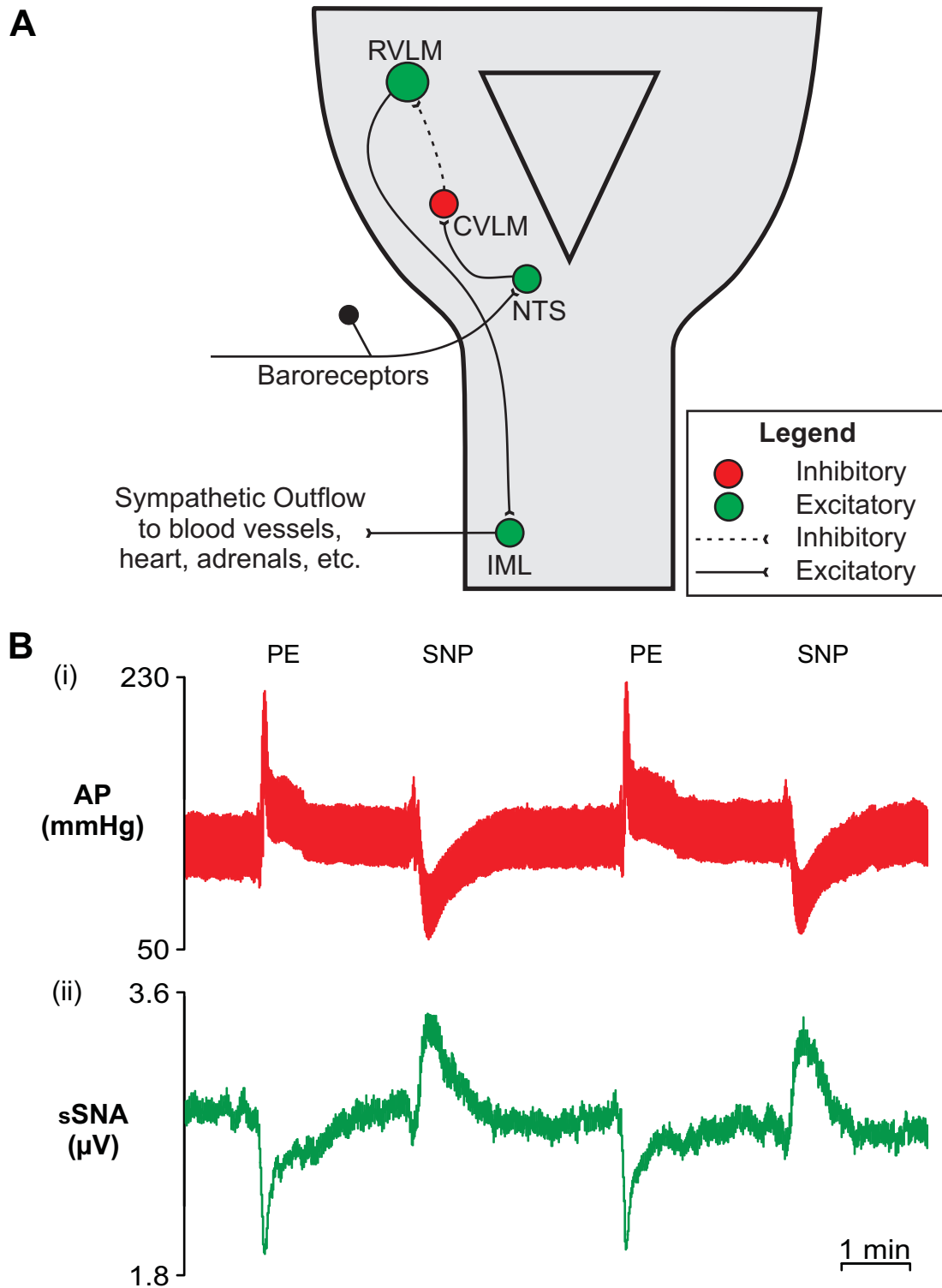
Figure 1.6 The baroreceptor reflex
(**A**) A schematic representation of the sympathetic baroreflex pathway, demonstrating the major regions and projections involved in the pathway. Inhibitory pathways are indicated by dashed lines and excitatory pathways are indicated by continuous lines. Red circles indicate inhibitory neurons and green circles indicate excitatory neurons (Adapted from (Pilowsky *et al.,* 2002)). (**B**) A representative experimental trace showing the AP and sSNA responses to vasoconstriction (PE) and vasodilation (SNP). The sympathetic baroreflex mediates opposing changes in sSNA, following rapid changes in AP, to return AP to baseline. Abbreviations: AP, arterial pressure; CVLM, caudal ventrolateral medulla; IML, intermediolateral cell column; NTS, nucleus of the solitary tract; PE, phenylephrine; RVLM, rostral ventrolateral medulla; SNP, sodium nitroprusside; sSNA, splanchnic sympathetic nerve activity.

Ross *et al.*, 1984b). However, destruction/blockade of the CVLM permanently attenuates baroreflex function (West *et al.*, 1981), altering this paradigm. The release of glutamate from excitatory NTS neurons activates GABA-ergic inhibitory CVLM neurons, which monosynaptically inhibit RVLM neurons, causing a reduction in SNA and a decrease in AP following stimulation of the reflex (Sun & Guyenet, 1985; Li & Dampney, 1992; Li *et al.*, 1992; Jeske *et al.*, 1995; Lipski *et al.*, 1996b; Minson *et al.*, 1997). Barosensitive GABA-ergic CVLM neurons are also tonically active, creating baro-dependent inhibitory synaptic tone in the RVLM (Masuda *et al.*, 1992; Koshiya & Guyenet, 1996a; Ito & Sved, 1997; Dampney & Horiuchi, 2003).

1.5.5.2 The chemoreceptor reflex

Peripheral and central chemoreceptors provide rapid, ongoing feedback to the brainstem (**section 1.7**) regarding partial arterial pressure of O_2 (PaO_2), partial arterial pressure of CO_2 ($PaCO_2$) and $[H^+]$ levels (Nattie, 1999; Makeham *et al.*, 2005; Moreira *et al.*, 2006). This enables manipulation of the cardiorespiratory system to restore adequate pH. Central chemoreceptors, located within the ventral medulla, respond to changes in the pH of cerebrospinal fluid. Severe central hypoxia can also generate cardiorespiratory adjustments, although the extent of hypoxia needed to activate central neurons is much greater than that needed to activate peripheral chemoreceptors (Kemp, 2006). Peripheral chemoreceptors are located within the carotid and aortic bodies. Activation of peripheral chemoreceptors, in response to falling PaO_2 (below 100 mmHg), causes a set of reflexive changes in cardiorespiratory function and blood chemistry. The peripheral chemoreceptors, are less sensitive to, but do respond to, increases in $PaCO_2$ (Sapru & Krieger, 1977; Marshall, 1994; Nattie, 1999). The circulatory response to peripheral chemoreflex activation includes increased vasomotor tone to the muscles, gut and kidney, to reduce organ flow and elevate AP (**Figure 1.7**; Korner & Uther, 1969; Reis *et al.*, 1997; Guyenet, 2000). Alongside simultaneous vasodilation in the brain and heart to maintain adequate perfusion/oxygenation of these vital organs; the reflex also induces bradycardia, to reduce coronary O_2 consumption. The respiratory response is biphasic; with an initial increase in central inspiratory activity, followed by a secondary depression which progresses to complete respiratory arrest with severe

hypoxia (Richter *et al.*, 1991; Richter *et al.*, 1999). This review will focus on the peripheral (hypoxic) chemoreflex, and will not discuss the central chemoreflex.

The peripheral chemoreflex arc (**Figure 1.7**) begins with stimuli triggered depolarisation of type 1 glomus cells in the carotid body or aortic body (Montoro *et al.*, 1996), releasing various excitatory transmitters, that activate terminals of the carotid sinus nerve which innervates the carotid body (McDonald & Mitchell, 1975; McDonald, 1983; Kusakabe *et al.*, 2003; Spyer *et al.*, 2004; Nurse, 2005). This information is then relayed to caudal NTS neurons, lacking in respiratory modulation (Koshiya & Guyenet, 1996b), via cranial nerves IX (carotid body) or X (aortic body) in humans. Chemoreflex afferent neurons synapse bilaterally in the medial and commissural NTS (Lipski *et al.*, 1976; Lipski *et al.*, 1977; Mifflin, 1992). Glutamate microinjection into the NTS reproduces the chemoreflex responses (Vardhan *et al.*, 1993b, a), whilst glutamate antagonism ablates such responses (Zhang & Mifflin, 1993; Sapru, 1996), indicating that NTS inputs are excitatory and are mainly mediated by glutamate (Zhang & Mifflin, 1998). However, an alternative transmitter, for example ATP (adenosine triphosphate; Antunes *et al.*, 2005), may mediate the AP response to hypoxia (Haibara *et al.*, 1995; Machado *et al.*, 2000; Machado & Bonagamba, 2005), suggesting that various neurotransmitters in the NTS may mediate various efferent responses to the reflex. NTS neurons excited by hypoxia project to the RVLM, CVLM (Koshiya & Guyenet, 1996b) and to the retrotrapezoid nucleus (RTN; Guyenet *et al.*, 2008).

Activation of bulbospinal RVLM neurons is essential in the relay of the chemoreflex (Sun & Spyer, 1991; Koshiya *et al.*, 1993; Guyenet & Koshiya, 1995) via activation of ionotropic glutamate receptors in the RVLM (Moraes *et al.*, 2012). Hypoxic activation of these neurons results in chemoreflex expression, and is closely linked with respiratory neuron activity (Sun & Spyer, 1991; McAllen, 1992; Sun & Reis, 1993). Moreover, bilateral destruction (Underwood *et al.*, 1994; Golanov & Reis, 1996) or neurochemical blockade (Koshiya *et al.*, 1993; Sun & Reis, 1995; Miyawaki *et al.*, 1996b) of the RVLM abolishes chemoreflex responses. Bilateral muscimol microinjections into the A5 attenuates the sympathetic chemoreflex by ~50%, suggesting that activation of A5 noradrenergic neurons may also be essential for full expression of the reflex (Koshiya & Guyenet, 1994; Guyenet & Koshiya,

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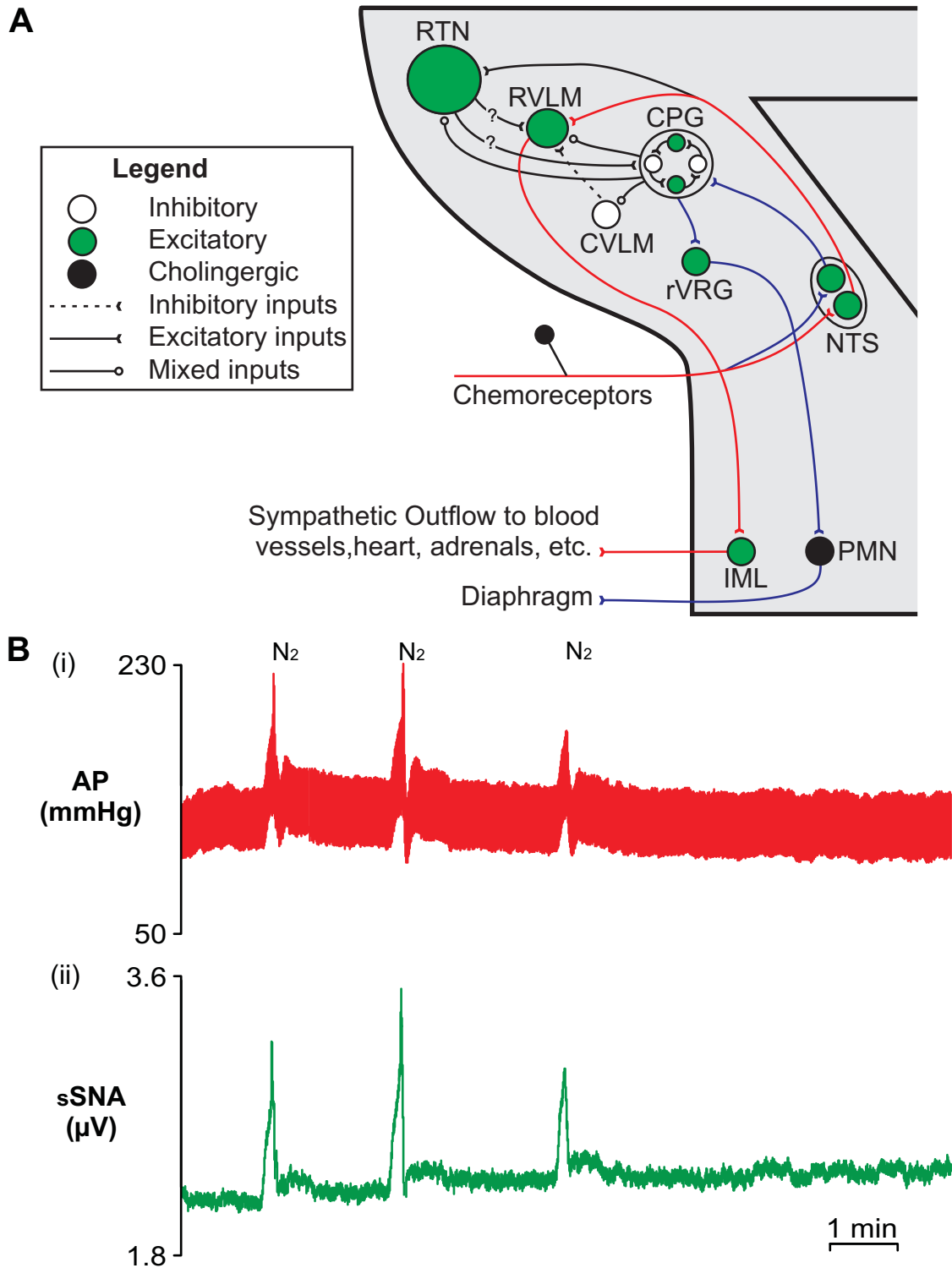
Figure 1.7 The peripheral chemoreflex

(**A**) A schematic illustrating the peripheral chemoreflex pathway. Sympathoexcitation produced by peripheral chemoreceptor activation relies on RVLM neuron activation via the NTS (pathway in red). Respiratory CPG activation makes a small contribution to the sympathoexcitatory effects of the reflex, augmenting the strength of respiratory coupling in RVLM neurons. Peripheral chemoreceptor activation drives the CPG via the NTS (pathway in blue). Chemosensitive RTN neurons are activated by the peripheral chemoreceptors via the NTS and may contribute to the effects of hypoxia. (**B**) A representative trace showing the AP and sSNA responses to hypoxia (N_2). Abbreviations: AP, arterial pressure; CPG, central pattern generator; CVLM, caudal ventrolateral medulla; IML, intermediolateral cell column; NTS, nucleus of the solitary tract; PMN, phrenic motor nucleus; RTN, retrotrapezoid nucleus; RVLM, rostral ventrolateral medulla; rVRG, rostral ventral respiratory group; sSNA, splanchnic sympathetic nerve activity.

1995). Beyond the NTS, the respiratory responses of the peripheral chemoreflex are thought to be produced by direct and indirect activation of respiratory central pattern generator (CPG; **section 1.7**) neurons from the NTS, pons and RTN (Young *et al.*, 2003; Takakura *et al.*, 2006; Song & Poon, 2009; Takakura *et al.*, 2011). Once generated, the integrated responses travel to final relay centres in the IML and phrenic motor nucleus (PMN), to execute the responses of the reflex.

1.5.5.3 The somatosympathetic reflex

Activation of the SSR (**Figure 1.8**), a defensive reflex response to noxious Aδ nociceptor stimulation, causes hyperventilation and generalised activation of the SNS (Katz & Perryman, 1965). Information conveyed by Aδ afferents includes cooling-specific thermoreceptors, muscle-mechanoreceptors and nociceptors (sharp pain). Sciatic C-fibre afferents include muscle metaboceptors, nociceptors (burning pain), warming-specific receptors, ultra-slow histamine-releasing (itch) and tactile receptors (Millan, 1999; Craig, 2003b). These fibres generally terminate in lamina I of the dorsal horn of the spinal cord. Axonal projections from these cells cross the midline and ascend in the spinothalamic tract. This ascending projection is hierarchical, initially targeting autonomic sites in the spinal cord, brainstem, midbrain, forebrain and the cortex in primates (Stornetta *et al.*, 1989; Andrew *et al.*, 2003). This organisation permits a series of input-output loops that drive somato-autonomic reflexes, such as the SSR (Sato & Schmidt, 1973; Craig, 2003a). Lamina I has major projections to spinal cord and brainstem sites that integrate homeostatic responses, including the RVLM and A1 (Craig, 1995; Craig, 2002).

Ascending pathways synapse with bulbospinal RVLM neurons to generate cardiovascular change (Morrison & Reis, 1989). Activated RVLM neurons project to the IML and elicit the SSR, which is characterised by increases in AP, HR and by distinct sympathetic nerve discharge patterns ('early' and 'late' reflex potentials); patterns that are dependent on the fibre type (Aδ or C) and origin (eg cutaneous) of the stimulated somatic afferent (Janig *et al.*, 1972; Sato & Schmidt, 1973). Low (Aδ) or high (Aδ & C) intensity activation of the sciatic nerve produces characteristic bi- and tri-phasic bursts of $_S$SNA, respectively (Miyawaki *et al.*, 2002a; Makeham *et al.*, 2005; McMullan *et al.*, 2008). So that stimulation of afferent SSR fibres (example tibial and sciatic nerves) induces co-ordinated changes in SNA and AP that are

mediated by the RVLM (Sato & Schmidt, 1973; Craig, 2003a, b; Burke *et al.*, 2008a; McMullan *et al.*, 2008). The SSR response is comprised of spinal (short-latency) and supraspinal (long-latency) components (Sato & Schmidt, 1971), with the RVLM playing a key role in the supraspinal component. Interference with communications to, or from, the RVLM significantly attenuates the SSR (Stornetta *et al.*, 1989; Verberne & Guyenet, 1992; Miyawaki *et al.*, 2002a; Makeham *et al.*, 2005).

1.5.6 Role of the RVLM in neurogenic hypertension

There is increasing evidence to suggest that elevated sympathetic tone contributes to the development of essential hypertension (**section 1.3.3**; Esler *et al.*, 2001; Guyenet, 2006; Korner, 2007; Malpas, 2010). Sympathetic tone is controlled by the SNS and is directly influenced by the RVLM and its efferent neuronal pathways. Therefore, it is reasonable that the increased vasomotor tone seen in some cases of essential hypertension may be caused by altered neurochemistry and a resultant increase in firing RVLM neurons, thereby increasing SNA to effector sights (Sved *et al.*, 2003). This hypothesis is supported by studies in the SHR and other models of neurogenic hypertension such as the Schlager genetically hypertensive mice (Davern *et al.*, 2009; Palma-Rigo *et al.*, 2011). The SHR is a genetic model for essential hypertension, SHR display a generalised increase in tonic RVLM activity, AP and SNA (Judy *et al.*, 1976; Judy *et al.*, 1979; Minson *et al.*, 1996; Ito *et al.*, 2000) and an upregulation of RVLM catecholamine gene expression (Kumai *et al.*, 1996; Xiong *et al.*, 1998; Reja *et al.*, 2002a; Reja *et al.*, 2002b). Several other mechanisms are proposed to explain the role of the RVLM in neurogenic hypertension, these include; resetting of the baroreflex (Judy *et al.*, 1979; Wolk *et al.*, 2003a), resetting/persistent chemoreceptor activation (obstructive sleep apnoea; Wolk *et al.*, 2003b; Wolk & Somers, 2003; Kishi & Hirooka, 2012), systemic inflammation (Wu *et al.*, 2012), NTS dysfunction resulting in altered reflex input (Gao *et al.*, 2004), neurovascular compression of the RVLM (Morimoto *et al.*, 1997; Pickering, 2007) or a dysfunction in one of many modulatory neurotransmitter systems present in this area (Gabor & Leenen, 2012; Parker *et al.*, 2012). This thesis, and our laboratory, focuses on the role of modulatory neurotransmitters within the RVLM and how altered function or dysfunction of a transmitter system/s may be involved in the aetiology of neurogenic hypertension.
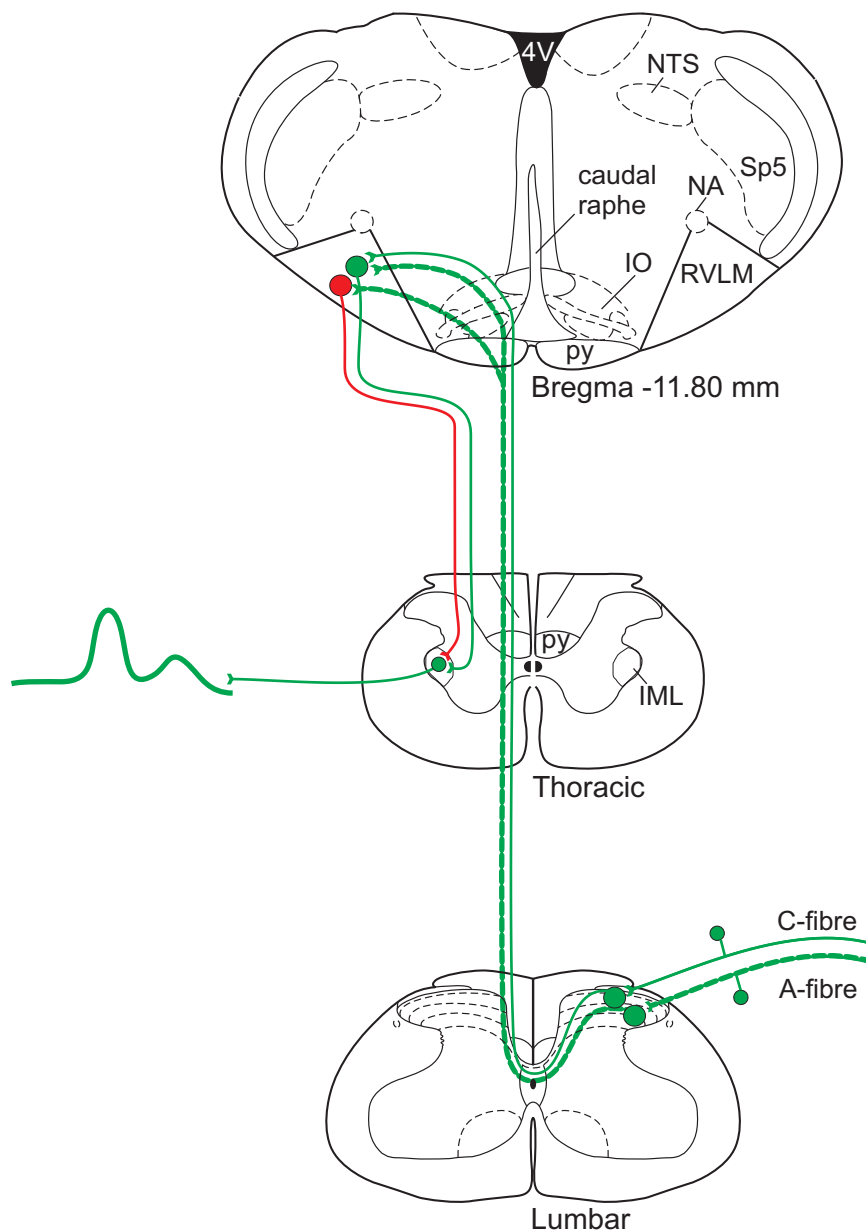
Figure 1.8 Somatosympathetic reflex

A schematic diagram illustrating a possible mechanism by which myelinated and unmyelinated nociceptive inputs may regulate sSNA (Figure and Legend adapted from (McMullan *et al.,* 2008)). Abbreviations: 4V, fourth ventricle; IML, intermediolateral cell column; IO, inferior olive; NA, nucleus ambiguus; NTS, nucleus of the solitary tract; py, pyramidal tract; RVLM, rostral ventrolateral medulla; Sp5, spinal trigeminal tract; sSNA, splanchnic sympathetic nerve activity.

1.6 Central pathways regulating arterial blood pressure

The central sympathetic pathways regulating AP consist of four categories of neurons; presympathetic neurons (see **section 1.5**), SPN, primary afferent neurons and supraspinal interneurons. Together, these neurons comprise the pathway that dictates the basal level of, and reflexive changes to, sympathetic vasomotor tone. The activity levels of these neurons, and peripheral SNA, is altered by the interplay of CNS neurons, under the influence of afferent inputs such as those from the baro- and chemo- receptors (Sved *et al.*, 2001; Pilowsky & Goodchild, 2002; Guyenet, 2006).

1.6.1 Sympathetic preganglionic neurons

SPN are responsible for integrating and conveying information from presympathetic neurons in the CNS to sympathetic postganglionic neurons in the periphery (**Figures 1.1** and **Figure 1.2**). SPN cell bodies are found in clusters within four regions of the thoraco-lumbar spinal cord: the IML, lateral funiculus, central autonomic nucleus and intercalated nucleus. Of these, the lateral IML and adjacent lateral funiculus contain the majority of vasomotor SPN cell bodies (Janig, 1985; Coote, 1988; Strack *et al.*, 1988; Pyner & Coote, 1994; Llewellyn-Smith, 2009). The activity of SPN, which are otherwise silent, is maintained by the excitatory drive provided by supraspinal inputs such as the sympathetic premotor nuclei (**section 1.4**; Strack *et al.*, 1989a; Seyedabadi *et al.*, 2006) and by a network of spinal interneurons (Poree & Schramm, 1992; Chau *et al.*, 1997; Chizh *et al.*, 1998). SPN activity primarily relies on supraspinal inputs, since the resting membrane potential of SPN displays irregular fluctuations unrelated to breathing or AP (Lewis & Coote, 2008). Spinal interneurons are unable to maintain resting vasomotor tone, since cervical cord transection eliminates efferent SNA at rest and under anaesthesia (Guertzenstein & Silver, 1974; Dampney & Moon, 1980; Dampney, 1994).

SPN predominantly receive glutamatergic input from presympathetic and spinal interneurons (Morrison *et al.*, 1991; Deuchars *et al.*, 1997; Lewis & Coote, 2008), but do receive input from a number of other classical neurotransmitters such as GABA, serotonin, NAd and glycine. A variety of metabotropic neurotransmitters (as outlined in **section 1.5.3**) also provide direct input to SPN, modulating their output (Guyenet, 2006; Vaudry *et al.*, 2009) Additionally, immunoreactive terminals

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for neurotensin (Krukoff *et al.*, 1985), oxytocin (Appel & Elde, 1988) and vasoactive intestinal polypeptide (VIP; Chiba & Masuko, 1987) are closely apposed to SPN, but synaptic connections have not been established (Dampney, 1994).

There have been many reports on the electrophysiological characteristics of SPN, *in vitro* (Coote, 1988) and *in vivo* (Lewis & Coote, 2008). The diameter of SPN cell bodies is ~26 μm, dendrites form bundles that project in the longitudinal and transverse directions. SPN have a resting potential of ~-56.8 mV and a latency of ~85 milliseconds to antidromic stimulation of the cervical sympathetic nerve. ~80% of recorded SPN have spontaneous activity and are pulse modulated (**Figure 1.4**; Lewis & Coote, 2008). Anatomically, SPN are topographically arranged in a functional rostrocaudal distribution within the IML (**Figure 1.9**), in accordance with their targets. For example, SPN innervating the cervical sympathetic nerve are found between C8-T5 and those innervating the adrenal gland between T4-T12 (Janig, 1985; Strack *et al.*, 1988). There are further functional divisions between SPN, for example, SPN innervating adrenal chromaffin cells are histologically and electrophysiologically distinct populations in the rat and cat (Edwards *et al.*, 1996; Morrison & Cao, 2000). Clusters of SPN can also be categorised by neurochemical phenotype (Edwards *et al.*, 1996; Grkovic & Anderson, 1996, 1997; Gonsalvez *et al.*, 2010; Kumar *et al.*, 2010). For example, calbindin is exclusively found in SPN that project to superior and stellate ganglia (Grkovic & Anderson, 1997). Moreover, calretinin is expressed in SPN projecting to noradrenergic chromaffin cells (Edwards *et al.*, 1996). The divisions within SPN may reflect differences in the central circuits that control SPN activity and is consistent with the notion of differential control of SNA (**section 1.5.4.2**; Morrison, 2001).

All SPN project to sympathetic postganglionic neurons that innervate the peripheral target organs of the SNS, except the adrenal medulla (**Figure 1.1**; Dampney, 1994; Murphy *et al.*, 2003). Neurotransmission between SPN and postganglionic neurons generally occurs via Acetylcholine (ACh) acting on postsynaptic nicotinic acetylcholine receptor (nAChR; Janig, 2005). However, it has been demonstrated that SPN also release neuropeptides that modulate transmission. For example neurotensin and substance P (Krukoff *et al.*, 1985), which generate
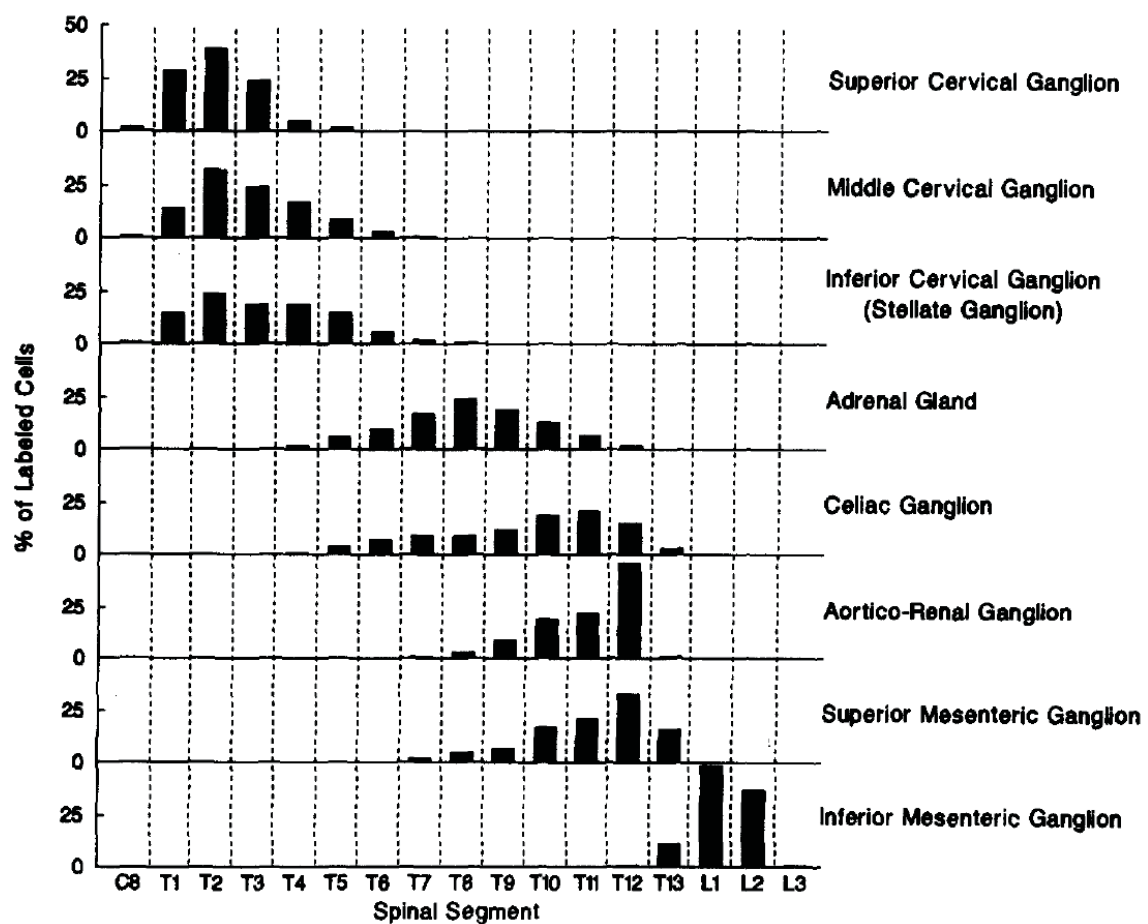
Figure 1.9 Topographical distribution of sympathetic preganglionic neurons in the rat spinal cord

Histograms illustrating the proportion of sympathetic preganglionic neurons labelled following Flurogold injections into the major sympathetic ganglia and adrenal gland in rats, as indicated on the right. The segmental distribution of sympathetic preganglionic neurons is topographically correlated with the outflow to different sympathetic target organs and sympathetic beds (Figure and legend from (Strack *et al.,* 1988)).

slow EPSPs in some postganglionic neurons (Janig, 2005). Other neuropeptides include; somatostatin, enkephalin (Krukoff *et al.*, 1985), ghrelin (Furness *et al.*, 2012), and notably PACAP (Beaudet *et al.*, 1998). PACAP mRNA and PACAP receptors are found in SPN (Arimura, 1998). PACAP is also present in RVLM neurons (Légrádi *et al.*, 1994; Dun *et al.*, 1996a; Dun *et al.*, 1996b; Hannibal, 2002; Farnham *et al.*, 2008), suggesting that PACAP may be involved in neurotransmission between RVLM neurons and SPN and also between SPN and postganglionic neurons. This evidence leads us to believe that PACAP may have a role in maintaining sympathetic outflow and AP. The actions, influence and pharmacology of PACAP at the synapse between bulbospinal RVLM neurons and SPN in the IML, is the focus of this body of work.

1.6.2 Sympathetic postganglionic neurons

SPN synapse with sympathetic postganglionic neurons, also termed ganglionic neurons, in the paired sympathetic chain ganglia and the prevertebral ganglia (Baron *et al.*, 1985; Chevendra & Weaver, 1991; Moon *et al.*, 2002). Sympathetic postganglionic neurons innervate the target organs of the SNS to elicit its functions (**Figure 1.1**). The adrenal medulla is the only tissue that receives direct input from SPN. Splanchnic nerves travel through the celiac ganglion and synapse directly onto chromaffin cells in the adrenal medulla to stimulate catecholamine release into the bloodstream (Kesse *et al.*, 1988; Burnstock, 2007). Increases in SNA result in; increasing HR, increases vasomotor tone, stimulation of the adrenal medulla and inhibition of digestion (Clutter *et al.*, 1980; Cryer *et al.*, 1980; Pyner & Coote, 1994). Sympathetic postganglionic innervation of the viscera is mostly noradrenergic; however, innervation of sweat glands is cholinergic (via muscarinic acetylcholine receptors). NAd may act on both α- and β- adrenoreceptors to facilitate the actions of sympathetic postganglionic neurons (Pincus & Magitsky, 1989). The release of Ad and NAd is differentially controlled, as these hormones serve different functions. The cardiovascular end organs of the SNS are controlled separately to non-cardiovascular end organs. This discussion is limited to the former.

1.6.3 The sympathoadrenal system

Chromaffin cells of the adrenal medulla arise from the sympathoadrenal cell lineage; a cell line arising from the neural crest. Sympathoadrenal cells migrate to

various sites, forming many cell types including sympathetic neurons and neuroendocrine chromaffin cells (Langley & Grant, 1999). Chromaffin cells are morphologically distinct from other structures formed by the sympathoadrenal cell line, with a large dense core of chromaffin granules from which it releases stored catecholamines. Adrenal and extra-adrenal Chromaffin cells are the only sympathetic target to be directly innervated by SPN (ACh via nAChR; Strack *et al.*, 1988; Murphy *et al.*, 2003). Chromaffin cells act as modified sympathetic postganglionic neurons, receiving sympathetic input and releasing NAd and Ad directly into the blood stream, rather than innervating a target organ (Burnstock, 2007 Kesse, 1988 #420). Increasing plasma catecholamines has a systemic effect on multiple organs, and modulates a variety of responses. Catecholamine release from chromaffin cells plays an essential role in the stress response by enhancing cardiorespiratory and metabolic functions. Circulating catecholamines increase metabolic rate, chronotropy, dromotropy, inotropy and cause vasoconstriction (Wendelaar Bonga, 1997; Reid *et al.*, 1998).

Ad and NAd secreting chromaffin cells are differentiated by the presence of PNMT, which allows the conversion of NAd to Ad in Ad secreting chromaffin cells. This occurs in response to activation of the PNMT promoter by a functional glucocorticoid response element (Ross *et al.*, 1990). Chromaffin cells are activated by specific stimuli, in line with their distinct physiological roles (Vollmer *et al.*, 1992). Decreased blood glucose and exercise promotes Ad secretion, whereas a reduction in AP, chemoreceptor activation, cold exposure or haemorrhage selectively releases NAd (Gagner *et al.*, 1985; Khalil *et al.*, 1986; Vollmer, 1996; Morrison & Cao, 2000; Vollmer *et al.*, 2000; Cao & Morrison, 2001). Morrison and Cao (2000) determined that this phenomenon is mediated by central sympathetic pathways that converge on specific populations of SPN, innervating either NAd or Ad secreting chromaffin cells (Morrison & Cao, 2000). This was achieved by dividing adrenal SPN according to their behaviour (i.e. responses to glucoprivation (Ad) or baroreflex stimulation (NAd); Morrison & Cao, 2000). Like other SPN, adrenal SPN use a variety of neurotransmitters and neuropeptides, co-localised with ACh, to convey and modulate information to the chromaffin cells. PACAP and enkephalin mRNA is present in ~97% and ~47% of adrenally projecting SPN, respectively (Kumar *et al.*, 2010). Suggesting that PACAP, released by SPN, modulates the activity of most

chromaffin cells; Enkephalin may also affect chromaffin cells, but in smaller numbers. With this, and previous evidence, Kumar *et al.,* (2010) hypothesise that the adrenally projecting PACAP-containing SPN regulate both NAd and Ad release from the adrenal gland, while enkephalinergic SPN control a subpopulation of chromaffin cells – possibly Ad secreting cells (Kumar *et al.*, 2010). This study shows a neurochemical basis for the differential control of SNA and for the specific activation of NAd or Ad secreting chromaffin cells. The role of PACAP in adrenally projecting SPN is outlined in **section 1.9** and is investigated in **Chapters 6 and 7**.

As well as releasing NAd and Ad, chromaffin cells manufacture, co-store and co-release a number modulatory transmitters/peptides which exert a range of autocrine/paracrine effects during the stress response. These include; PACAP (Fukushima *et al.*, 2001a; Fukushima *et al.*, 2001b; Lamouche & Yamaguchi, 2003), VIP (Wakade *et al.*, 1991; Conconi *et al.*, 2006), chromogranin A, catestatin (O'Connor & Frigon, 1984; Mahata *et al.*, 1997b; Laslop & Mahata, 2002), galanin (Holst *et al.*, 1991), substance P (Nieber & Oehme, 1987), and neuropeptide Y (Ait-Ali *et al.*, 2004).

1.7 Central control of breathing

The primary function of the mammalian respiratory system is gas exchange to maintain PaO_2, $PaCO_2$ and blood pH levels, within the narrow physiological ranges required for correct cellular functioning. In mammals, the neural circuits that control breathing are arranged in longitudinal columns within the pons and medulla oblongata (Smith *et al.*, 2009). Breathing requires the co-ordination of several muscle groups; the diaphragm is the principle muscle driving inspiration. Other muscle groups (intercostals and abdominal muscles) involved, display less activation during normal breathing but having specific activity patterns during reflex responses. The phrenic nerve innervates the diaphragm, PNA (**Figure 1.4**) is therefore commonly used as a measure of neural inspiratory drive – particularly in experiments that incorporate neuromuscular blockade and ventilation. The phrenic nerve originates from motoneurones located in the PMN (C3-C5; Bianchi *et al.*, 1995). The capacity of metabotropic neurotransmitters to alter PNA at the PMN is of interest here; as are the medullary neural circuits that regulate PNA, PMN outflow and therefore rhythmic diaphragmatic contraction. Lesioning and serial sectioning of

the brainstem has identified the functional nuclei involved in the central control of breathing and in the patterning and shaping of PNA (Rybak, 2007; Rybak *et al.*, 2007; Smith *et al.*, 2007). Each nucleus involved in respiratory control operates under the control of rostral nuclei, in a 'rostro-caudal functional hierarchy' (Smith *et al.*, 2007). Particularly, destruction of the rostral medulla results in the termination of breathing. The medulla oblongata was subsequently recognised as an essential site for respiratory control. The neural networks that control the accessory muscles of respiration will not be discussed here (for review see (Kubin *et al.*, 2006)).

1.7.1 The mammalian respiratory motor pattern

The firing pattern of rodent respiratory neurons, and the resultant motor pattern of the respiratory cycle (**Figure 1.10**), has three major phases; inspiration, post-inspiration (also referred to as early or passive expiration) and expiration (also referred to as late or passive expiration; Bianchi *et al.*, 1995; Richter & Spyer, 2001; Rybak *et al.*, 2007; Smith *et al.*, 2007; Rubin *et al.*, 2009). The co-ordinated firing of inspiratory and expiratory neurons generates rhythmic motor outflow to the airways and muscles of respiration (**Figure 1.10**). Different parts of the network determine respiratory rate (rhythm) and amplitude (McCrimmon *et al.*, 2000; Monnier *et al.*, 2003). Intrinsic chemosensitivity of the respiratory network (or some of its neurons) and afferent inputs from peripheral O_2-sensitive chemoreceptors determines the appropriate levels of neural output and ventilation (Hayashi & Fukuda, 2000; Mahamed *et al.*, 2001a; Mahamed *et al.*, 2001b; Mulkey *et al.*, 2004; Richerson, 2004).

1.7.2 Respiratory rhythm generation

Autonomic rhythmic behaviors rely on neural networks within the hindbrain to generate and co-ordinate their activity. These networks, known as CPGs, generate rhythmic output by incorporating multiple non-rhythmic inputs, to modify the motor pattern produced (Grillner, 2006; Rubin *et al.*, 2009). It is current opinion that a CPG is involved in the origin of the intrinsic rhythmicity of breathing.

The respiratory CPG consists of the Bötzinger complex (BötC), the pre-Bötzinger complex (preBötC), the rostral ventral respiratory group and caudal ventral respiratory group, contained within the ventral respiratory column (VRC;

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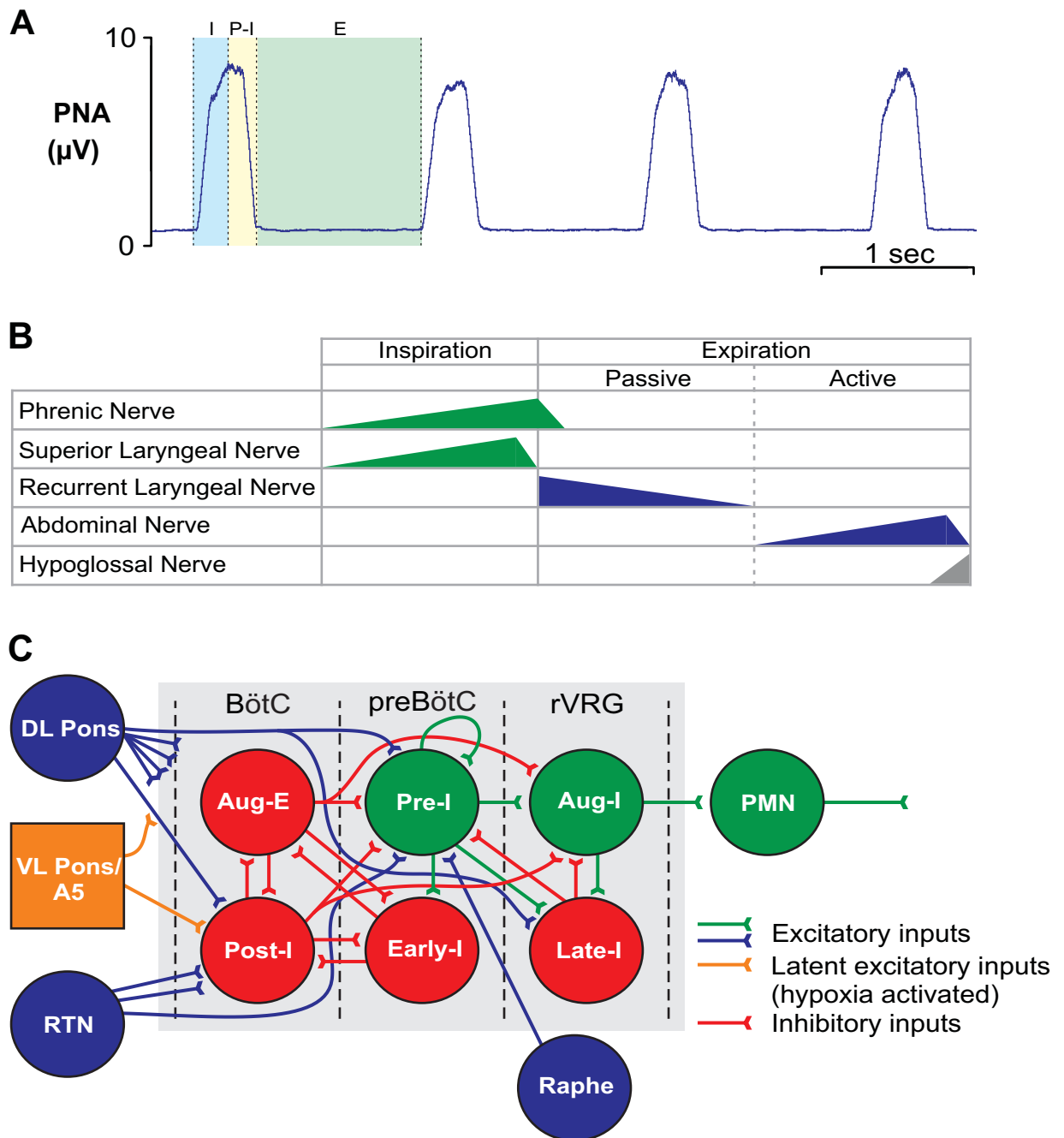
Figure 1.10 The firing patterns of respiratory neurons and the respiratory CPG
The phrenic nerve cycle (**A**) has three phases; the I phase is defined by the initiation of the phrenic burst (blue). The P-I phase describes the termination of the phrenic burst, which results from competing inhibitory and excitatory inputs (yellow). Finally, the E phase, during which there is no phrenic burst (green). (**B**) During inspiration, PNA increases, contracting the diaphragm and permitting inflation of the lungs. During the P-I phase, PNA is halted, the diaphragm recoils and deflates the lungs. In parallel, the recurrent laryngeal nerve is activated to adduct the vocal folds to regulate the rate of air outflow. During E, abdominal muscles are activated to force air from the lungs and the hypoglossal nerve is activated to depress the tongue in preparation for the next inhalation. (**C**) Diagrammatic representation of the synaptic interactions between neurons within the VRC that determine PNA. Excitatory neurons are in green/blue, latent excitatory neurons (hypoxia) activated in orange and inhibitory neurons in red (Adapted from (Smith *et al.,* 2007)). Abbreviations: Aug, Augmenting; BötC, Bötzinger complex; DL Pons, dorsolateral pons; E, expiratory; I, inspiratory; P-I, post-inspiratory; PMN, phrenic motor nucleus; PNA, phrenic nerve activity; preBötC, pre-Bötzinger complex; RTN, retrotrapezoid nucleus; rVRG, rostral ventral respiratory group; VL Pons, ventrolateral pons.

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Figures 1.5 and **1.10**; Monnier *et al.*, 2003; Spirovski *et al.*, 2012). These regions provide tonic and phasic, excitatory and inhibitory inputs to produce the innate, involuntary, rhythmic neural drive to the diaphragm and accessory muscles of respiration, resulting in co-ordinated respiration. The CPG integrates central and peripheral inputs, such as those from the chemoreceptors, pulmonary stretch receptors, vagal afferents and supraspinal sites (Rybak *et al.*, 2004; Smith *et al.*, 2007; Abdala *et al.*, 2009; Rubin *et al.*, 2009). These afferent inputs influence the CPG so that the oscillation period and amplitude of the rhythmic respiratory motor pattern is adapted to maintain O_2 and pH homeostasis (Smith *et al.*, 2009). Furthermore, the VRC are controlled by inputs from brainstem nuclei including the RTN, raphé nuclei, and other ponto-medullary circuits (Alheid & McCrimmon, 2008). Neurons in the CPG also affect the activity of non-respiratory neurons in adjacent regions, such as barosensitive neurons in the RVLM and CVLM (see **section 1.7.3** for further details), thereby influencing SPN activity. The CPG is comprised of neurons commonly subdivided into two compartments labeled the BötC and preBötC (**Figure 1.10**), although their borders are not entirely distinct (Ellenberger & Feldman, 1990; Dobbins & Feldman, 1994; Rybak *et al.*, 2008).

The BötC primarily contains inhibitory (glycinergic) neurons that are active during expiration (**Figure 1.10**; Okazaki *et al.*, 2001; Ezure *et al.*, 2003). BötC neurons provide phasic inhibition to inspiratory neurons in the preBötC, initiating and maintaining expiration (Hayashi *et al.*, 1996; Rybak *et al.*, 2004; Smith *et al.*, 2007). While BötC neurons are not rhythmogenic, they provide an essential inhibitory counter-balance that is required to produce a normal (eupneic) respiratory motor output, and are therefore considered to be essential in the CPG (Smith *et al.*, 2007). Recent studies suggest that the BötC is not exclusively responsible for expiration, with pre-inspiratory oscillatory neurons being characterised in the parafacial respiratory group. This group of neurons was thought to be responsible for the rhythmic expiratory drive that entrains the preBötC inspiratory oscillator (Onimaru *et al.*, 1988). However, other studies suggest that parafacial respiratory group neurons act as an expiratory oscillator, working in concert with the preBötC inspiratory oscillator produce co-ordinated patterns of respiratory activity (Feldman & Del Negro, 2006; Janczewski & Feldman, 2006).

The preBötC, located caudal to the BötC, contains glutamatergic, GABA-ergic and glycinergic neurons, along with interneurons that are either inspiratory or phase-spanning. The preBötC is considered to be the primary site (kernel) for the generation of central rhythmic excitatory inspiratory drive to premotor respiratory neurons (**Figure 1.10**; Rekling & Feldman, 1998; Sun *et al.*, 1998; Koshiya & Smith, 1999; Smith *et al.*, 2009; St.-John *et al.*, 2009; Morgado-Valle *et al.*, 2010). This was first demonstrated by sequential sectioning of the *en bloc* neonatal rat brainstem, showing that inspiratory-like activity was generated from within a 400 µm thick region of the VRC (Smith *et al.*, 1991). Subsequent studies revealed that rhythmic PNA persists in *in situ* preparations that preserve the preBötC and the central phrenic motor pathways (Smith *et al.*, 2007; Rybak *et al.*, 2008), indicating that this restricted area contains critical CPG machinery. *In vivo*, pharmacological inhibition (Koshiya & Guyenet, 1996b), selective destruction (McKay *et al.*, 2005; McKay & Feldman, 2008) and genetically targeted inhibition (Tan *et al.*, 2008) of preBötC neurons in anaesthetised and/or conscious animals disturbs or abolishes respiratory rhythm. Furthermore, embryonic respiratory activity is coincident with the appearance of the preBötC *in vitro* (Thoby-Brisson *et al.*, 2005; Thoby-Brisson *et al.*, 2009).

The origin of rhythmic activity in preBötC slices is not well understood. Research has targeted glutamatergic neurons characterised by early- or pre-inspiratory discharge patterns, with a ramping voltage trajectory (inspiratory drive potential) and rapid discharge preceding inspiration (Smith *et al.*, 1991; Schwarzacher *et al.*, 1995; St.-John *et al.*, 2009). A combination of intrinsic properties, recurrent glutamatergic collaterals and extrinsic drives initiates the activation pattern of these neurons. Pre-inspiratory neurons are the driver or pacemaker neurons initiating inspiration (Rekling & Feldman, 1998; Koshiya & Smith, 1999; Thoby-Brisson & Ramirez, 2001; Del Negro *et al.*, 2002; Feldman & Del Negro, 2006), although some disagree with this view (Onimaru *et al.*, 2006). The origin of rhythmic activity in the preBötC is dependent on the synaptic release of excitatory amino acids, specifically the activation of non-NMDA receptors. However, neuropeptides such as substance P, serotonin and opioids, are considered important regulators of respiratory neuron excitability and the expression of respiratory motor patterns (for review see (Pena, 2009)). Neurokinin-1 receptors

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(Guyenet & Wang, 2001; Guyenet *et al.*, 2002a), µ-opioid receptors (Gray *et al.*, 1999), and somatostatin-mRNA (Stornetta *et al.*, 2003) define a select subpopulation of PreBötC neurons. In this thesis we examine the effect of PACAP on PNA at the level of the PMN (**Chapter 7**).

1.7.3 Cardiorespiratory coupling

SNA is coupled to distinct phases of the respiratory cycle (Adrian *et al.*, 1932; Pilowsky, 1995; Pilowsky, 2009), a phenomenon that particularly applies to vasomotor SNA. Respiratory modulation persists following sensory denervation or vagotomy, suggesting a central origin (Adrian *et al.*, 1932; Numao *et al.*, 1987). The pattern of respiratory coupling depends on species, strain, pH, anaesthetic and peripheral nerve (Janig & Habler, 2003). Respiratory patterns in SNA may be produced by hypoxia/hypercapnia (Boychuk *et al.*, 2012) or by interactions between the CPG and cardiovascular neurons in the ventrolateral medulla (Pilowsky *et al.*, 1996); although pontine structures may contribute (Baekey *et al.*, 2008; Dick *et al.*, 2009). By averaging vasomotor SNA in relation to onset of inspiration, respiratory-related patterns proportional to central inspiratory drive, can be observed in vagotomised rodents (**Figure 1.4**). There are multiple respiratory-modulated firing patterns in the activity of rat SPN these patterns include: maximally excited or inhibited during either inspiration, post-inspiration or expiration, or no modulation (Zhou & Gilbey, 1992; Pilowsky *et al.*, 1994b). In adult rats, the cervical and lumbar nerves commonly exhibit a post-inspiratory respiratory modulation (Miyawaki *et al.*, 2002b), whilst $_s$SNA displays biphasic inspiratory and post-inspiratory modulation (**Figure 1.4**; Dick *et al.*, 2004). Muscle and visceral sympathetic fibres tend to be strongly modulated by respiration, whereas sympathetic fibres innervating cutaneous vasculature (Janig & Habler, 2003), BAT (Morrison *et al.*, 1999) or adrenergic chromaffin cells (Cao & Morrison, 2001) are unaffected or are weakly coupled to the respiratory cycle. Integration between respiration and regional/systemic blood flow is thought to be critical to the efficiency of O_2 uptake (Taylor *et al.*, 1999).

Neurons in presympathetic nuclei (**section 1.4**) also exhibit similar patterns of respiratory modulation, correlating with artificial ventilation in vagally-intact rats and with central respiratory activity in vagotomised rats (Haselton & Guyenet, 1989a; Miyawaki *et al.*, 1995; Miyawaki *et al.*, 2002b). It is likely that

presympathetic neurons receive inputs from respiratory neurons with different firing patterns. Expiratory and inspiratory neurons of the BötC have extensive local branches; their dendrites often project towards the ventral surface and their terminals intermingle with C1 RVLM neurons (Pilowsky *et al.*, 1990; Pilowsky *et al.*, 1994b; Kanjhan *et al.*, 1995). Additionally, some expiratory BötC neurons form close appositions with TH or spinally projecting RVLM neurons in rat (Sun *et al.*, 1997). This input may explain the respiratory modulation of some RVLM neurons, and their connecting SPN (Miyawaki *et al.*, 2002b).

Excitatory inputs to RVLM neurons are a potent source of post-inspiratory excitation of SNA (Miyawaki *et al.*, 1996a). Blockade of AMPA/kainate receptors in the RVLM with CNQX selectively abolished the post-inspiratory peak in $_S$SNA. Blockade of GABA$_A$ receptors in the RVLM with bicuculline enhanced post-inspiratory discharge in $_S$SNA and $_L$SNA, suggesting that excitatory post-inspiratory inputs are also tonically inhibited within the RVLM (Miyawaki *et al.*, 2002b). The source of these inputs in unclear. Recently, four types of respiratory modulation were demonstrated in extracellularly-recorded GABA-ergic, barosensitive CVLM neurons (Mandel & Schreihofer, 2006). These findings suggest that neurons that relay inhibitory baroreceptor input to the RVLM also transmit respiratory-related inputs. Barosensitivity is also influenced by respiratory drive (Miyawaki *et al.*, 1995); RVLM neurons display changing levels of barosensitivity that correspond to PNA. This effect likely results from respiratory-driven changes in RVLM and CVLM excitability (Haselton & Guyenet, 1989a; Mandel & Schreihofer, 2006). There is little evidence to support the existence of barosensitive respiratory neurons in the CPG, suggesting that baroreceptor dependent modulation of respiratory drive is likely regulated by VRC neurons outside the respiratory CPG (Kanjhan *et al.*, 1995).

1.8 PACAP

Two molecular forms of PACAP with 38 (PACAP-38) or 27 (PACAP-27) amino acids were originally identified from ovine hypothalamus for their ability to stimulate AC in anterior pituitary cells (Miyata *et al.*, 1989; Miyata *et al.*, 1990). Several other functions of PACAP have been identified; PACAP can act as a hormone, neurohormone, neurotransmitter or trophic factor in many systems, including the cardiorespiratory system. PACAP acts on three receptors; the PACAP

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specific receptor (PAC$_1$) and the VIP/PACAP receptors (the VPAC$_1$ receptor (VPAC$_1$) and VPAC$_2$ receptor (VPAC$_2$)) to bring about its actions (Vaudry *et al.*, 2009; Farnham & Pilowsky, 2010; Alexander *et al.*, 2011).

1.8.1 The structure of the PACAP gene and protein

Both PACAP -38 and -27 share 68% sequence homology, and structural similarities with VIP; however, PACAP is 1000 times more potent in stimulating AC (Miyata *et al.*, 1989; Miyata *et al.*, 1990). PACAP is a member of the VIP/glucagon/secretin superfamily which consists of 9 highly conserved members including; PACAP, VIP, glucagon, glucagon-like peptide-1, glucagon-like peptide-2, growth hormone-releasing hormone, peptide histidine methionine, secretin and glucose-dependent insulinotropic polypeptide. PACAP shows varying degrees of sequence and structural similarity to other peptides within the superfamily, confirming that the peptides originated from a single ancestral sequence (Mayo *et al.*, 1985; Lamperti *et al.*, 1991; Campbell & Scanes, 1992; Montero *et al.*, 2000).

The sequence and architecture of the PACAP gene is highly conserved among invertebrate and vertebrate species. The sequence of PACAP-38 is identical in all mammalian species, and when compared to 15 vertebrate species, the lowest percentage sequence homology was 89% (between the human and stingray genes; **Figure 1.11**; Montero *et al.*, 2000; Sherwood *et al.*, 2000). The high level of homology in the PACAP sequence between high divergent taxa and its conservation over >700 million years of evolution, suggests that PACAP may be the ancestral molecule of the VIP/glucagon/secretin superfamily (McRory & Sherwood, 1997; Gonzalez *et al.*, 1998; Montero *et al.*, 2000; Sherwood *et al.*, 2000). The final three-dimensional protein structure of PACAP (**section 1.8.1.3**) also shows similarities with other members of the superfamily such as VIP, glucagon and secretin (Braun *et al.*, 1983; Gronenborn *et al.*, 1987; Wray *et al.*, 1993). However, minor differences are observed between different peptides of the family, these differences may contribute to the selectivity of the peptides for their receptors and differences in their functions (Inooka *et al.*, 1992). PACAP and VIP are sufficiently similar that they both bind to VPAC$_1$ and VPAC$_2$ with equivalent efficacy (Vaudry *et al.*, 2009).

1.8.1.1 Structure of the PACAP gene

The gene encoding PACAP has been cloned and sequenced in humans (Hosoya *et al.*, 1992), mice (Yamamoto *et al.*, 1998), and in rats (White *et al.*, 2000). The PACAP genes are all organised similarly and are comprised of five exons and four introns, encoding a 176 amino acid prepro-peptide (Hosoya *et al.*, 1992). PACAP is constitutively expressed but transcription of the PACAP gene can be enhanced by PACAP itself, via cyclic adenosine monophosphate (cAMP) and phospholipase C (PLC; Hashimoto *et al.*, 2000).

1.8.1.2 Post-translational processing of prepro-PACAP

Prepro-PACAP is cleaved by prohormone convertases (PC) to produce multiple peptides (Okazaki *et al.*, 1992), including biologically active PACAP -27 and -38 (**Figure 1.11**). Prepro-PACAP is cleaved by 7 PC subtypes at 7 specific sites (Seidah *et al.*, 1998), only 3 have been studied with PACAP as the substrate. In the testis, PC4 is exclusively responsible for cleavage of prepro-PACAP (Li *et al.*, 1998; Li *et al.*, 2000a; Li *et al.*, 2000b). PC1 and PC2 are expressed intensely in hypothalamic neurons (Dong *et al.*, 1997), and both cleave prepro-PACAP into PACAP -38 and -27 in the hypothalamus and supraoptic nucleus (Li *et al.*, 1999).

The sequence of mature PACAP -27 and -38 is encoded by exon 5 of the gene at the C-terminal end (Okazaki *et al.*, 1992), while exon 4 encodes a 29 amino acid peptide known as PACAP-related peptide (not biologically active in mammals; Ogi *et al.*, 1990; Ohkubo *et al.*, 1992) and exon 2 encodes a 24 amino acid signal protein at the N terminal of prepro-PACAP (**Figure 1.11**; Hosoya *et al.*, 1992). Prepro-PACAP is cleaved at certain residues by PC to generate the above peptides and an extended form of PACAP-38 (Seidah *et al.*, 1994; Rouille *et al.*, 1995; Seidah *et al.*, 1998). The extended form of PACAP-38 is cleaved and amidated at its C-terminal to form mature PACAP-38. Mature PACAP-27 is formed by further cleavage and amidation of the C-terminal of PACAP-38 (Okazaki *et al.*, 1992).

1.8.1.3 Structure of the PACAP protein

Inooka and colleagues determined the conformational structure of PACAP-27 using nuclear magnetic resonance and circular dichroism spectroscopy. PACAP-27 was studied as the first 27 amino acid are necessary for biological activity (Miyata *et*

A

1 2 3 4 5

5' 3'

SP | PRP | PACAP

1   24   82   110   132   158 169
PC1/PC2

PACAP-27
PACAP-38

B

```
              1    5    10   15   20   25   30   35   40   45
PACAP-38    HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRVKNKa
PACAP-27    --------------------------a
VIP         ---AV---N-T-L----------NSI-Na
GHRH        YA-A---N---KVLG-LSAR-L-QDIMSRQQGESNQERGARARLa
Secretin    ----T--SEL--L-EGARLQRL-QGLVa
Glucagon    --Q-T--SD--K-LDSRRAQDFVQWLMNT
GLP-2       -A--S-SDEMNTILDNL-ARDFINWLIQTKITD
PRP         DVAH--LNEA-RKVLD-LSAG-H-QSLVAa
PHM         -A--V--SDF-KLLGLSA----ESLMa
```

C

```
               1    5    10   15   20   25   30   35   40   45
mammals      HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRVKNKa
chicken      --------------------------------------
lizard       ------------------------------.....?
frog         ---------------------------------I---a
catfish      --------------------------R--R--FR--a
salmon       ----------------------------R--YR--a
stargazer    -----------------Q-------R--R---R--a
stingray     -------------------------PK-R-SGRRVFY
sea squirt 1 --------------N----------a
sea squirt 2 --------------N------IN-L-a
```

Figure 1.11 The PACAP gene and the primary structure of PACAP
(**A**) The mammalian PACAP gene consists of five exons and four introns, the sequence of SP, PRP and PACAP are encoded for by exons 2, 4 and 5 of prepro-PACAP (176 aa), respectively. Arrows indicate the sites of cleavage by prehormone convertase 1 and 2. (**B**) The primary structures of the different members of the VIP superfamily in humans all share a degree of sequence homology. The amino acid sequence of PACAP shares 68% sequence and structural homology with the VIP gene, confirming that these genes originated from a common ancestral sequence through gene duplication. (**C**) The sequence and architecture of PACAP has been well preserved, and is identical in all mammalian species studied thus far. Strong evolutionary pressure has acted to preserve the sequence of the biologically active region of the peptide (N-terminal domain), highlighting the biological importance of PACAP. Amino acids are shown in the single letter code. Abbreviations: -, amino acid identical to that of PACAP-38; a, amidated; GHRH, growth hormone-releasing hormone; GLP-2, glucagon-like peptide-2; PC1, prehormone convertase 1; PC2, prehormone convertase 2; PHM, peptide histidine methionine; PRP, PACAP-related peptide; SP, signal peptide; VIP, vasoactive intestinal polypeptide (Adapted from Montero *et al.*, 2000)).

al., 1990). Residues 9-20 and 22-25 have defined tertiary conformations, but the residues outside of these regions appear to have an unordered conformation. The region containing residues 9-20 consists of three distinct regions of β-turn conformation (9-12), an α-helix (12-14) and loose helical regions (15-20). Residues 22-25 constitute an α-helix (Inooka *et al.*, 1992).

Gradual deletions/substitutions of the N-terminal PACAP -38 and -27 residues cause marked reduction in AC activation (Sherwood *et al.*, 2000). Removal of up to, but not more than, the first 6 N-terminal residues of PACAP is progressively antagonistic. Demonstrating that the N-terminal of the peptide is crucial for its biological activity, but is not necessary for receptor binding (Gourlet *et al.*, 1991; Vandermeers *et al.*, 1992; Bitar & Coy, 1993; Bourgault *et al.*, 2009). The N-terminal truncated form of PACAP, PACAP(6-38), is the most potent competitive PAC$_1$ antagonist (K$_d$ ~ 1.5 nM; Robberecht *et al.*, 1992; Harmar *et al.*, 1998). PACAP(6-38) does not bind to VPAC$_1$ but has debated affinity for VPAC$_2$ (Dickinson *et al.*, 1997). PACAP receptors have greater affinity for N-terminally truncated analogs of PACAP-38 than for similar analogs of PACAP-27 (Robberecht *et al.*, 1992), indicating that PACAP binds to its receptors using its C-terminal domain to facilitate the recognition of the receptor binding sites. Furthermore, the addition of residues 28-38 from PACAP-38 to VIP produces a 100 fold increase in the affinity of VIP for PAC$_1$ (Gourlet *et al.*, 1996; Gourlet *et al.*, 1997), moreover, shortening of the C-terminal end decreases the ability of PACAP to bind to PAC$_1$ (Bourgault *et al.*, 2008).

1.8.2 The PACAP receptors

Three PACAP receptors have been cloned: PAC$_1$, VPAC$_1$ and VPAC$_2$ (Ishihara *et al.*, 1992; Lutz *et al.*, 1993; Morrow *et al.*, 1993; Pisegna & Wank, 1993), all are GPCRs with 7 transmembrane regions (Pisegna & Wank, 1993). The receptors are differentiated into two classes on the basis of their relative binding affinities for PACAP and VIP (Harmar *et al.*, 1998; Harmar *et al.*, 2012). The PACAP specific receptor (PAC$_1$) was identified one year after PACAP (Buscail *et al.*, 1990). The second receptor type was identified later; a binding study found binding sites in the adrenal gland and epididymis that were selective for PACAP, and sites in lung and liver with equal affinity for PACAP and VIP (Shivers *et al.*,

1991). Following this, the PACAP specific receptors were named PACAP type I and the receptors shared between PACAP and VIP were named PACAP type II. When the receptors were identified and cloned it was discovered that there were two type II receptors. The first type II receptor was called VIP1 (Ishihara *et al.*, 1992) and the second type II receptor was called VIP2 (Lutz *et al.*, 1993). Each PACAP receptor has been known by various names (**Table 1.1**). A new standard nomenclature for the PACAP receptors was introduced in 1998 and was designed to allow for new receptors to be discovered and to reduce confusion with other receptor classes (Harmar *et al.*, 1998; Alexander *et al.*, 2011; Harmar *et al.*, 2012). Recent evidence suggests that PACAP may also directly penetrate the cell to bind intracellular receptors (Doan *et al.*, 2012).

1.8.2.1 The PAC_1 receptor

The type 1 receptor, PAC_1 (and splice variants), is specific for PACAP and exhibits equally high binding affinities for PACAP -38 and -27 ($K_d \approx 0.5$-1.0 nM), and a 1000 flow lower affinity for VIP ($K_d > 500$ nM; Cauvin *et al.*, 1990; Gottschall *et al.*, 1990; Lam *et al.*, 1990; Robberecht *et al.*, 1992; Suda *et al.*, 1992; Harmar *et al.*, 1998; Laburthe *et al.*, 2007; Harmar *et al.*, 2012). It is described as one of the most highly spliced receptors known. The multiple splice variants of PAC_1 (see **section 1.8.2.3**) result from alternative splicing of the receptor, the most common splice variants in the rat hypothalamus and brainstem are the PAC_1-null and PAC_1-hop1 variants (Spengler *et al.*, 1993). Each variant is coupled to specific secondary signaling pathways (**section 1.8.2.3** and **Figure 1.12**) and exhibits tissue or cell specific expression (Spengler *et al.*, 1993; Arimura, 1998; Abad *et al.*, 2006; Dickson & Finlayson, 2009; Vaudry *et al.*, 2009).

1.8.2.2 The VPAC receptors

The type 2 receptors, $VPAC_1$ and $VPAC_2$, exhibit equal binding affinity for PACAP-38, PACAP-27 and VIP ($K_d \approx 1$ nM; Robberecht *et al.*, 1992; Harmar *et al.*, 1998; Laburthe *et al.*, 2007; Dickson & Finlayson, 2009; Couvineau & Laburthe, 2012; Harmar *et al.*, 2012). Splice variants for $VPAC_1$ and $VPAC_2$ have also been discovered, these also appear to be coupled to specific secondary signaling pathways (see **section 1.8.2.3** and **Figure 1.12**).
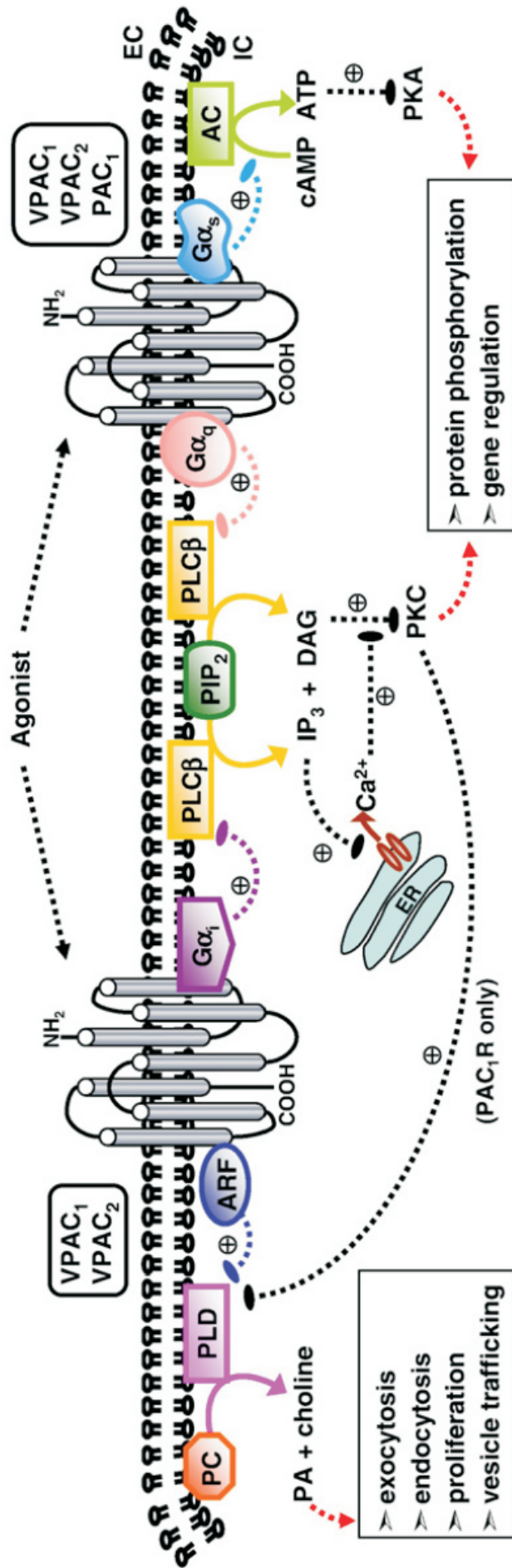
Figure 1.12 PAC₁, VPAC₁ and VPAC₂ intracellular signalling pathways

The figure highlights the principal transduction pathways activated by VPAC₁, VPAC₂ and PAC₁-null receptor coupling to G-proteins. Upon activation, all three receptors are capable of coupling to $G\alpha_s$ leading to downstream production of cAMP. In addition, the three receptors can also activate PLC leading to an increase in intracellular [Ca²⁺], via coupling to $G\alpha_q$ (all three receptors) and $G\alpha_i$ (VPAC1 and VPAC2 only). PLD activity can also be stimulated by the three receptor subtypes via ARF (VPAC1 and VPAC2) and PKC (PAC1) sensitive pathways. Abbreviations: AC, adenylate cyclase; ARF, ADP ribosylation factor; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; IC, intracellular; IP3, inositol triphosphate; PA, phosphatidic acid; PAC₁, PAC1 receptor; PC, phosphatidylcholine; PKA, protein kinase A; PKC, protein kinase C; PLCβ, phospholipase C beta; PLD, phospholipase D; R, receptor; VPAC₁, VPAC1 receptor; VPAC₂, VPAC2 receptor (Figure and legend from Dickson & Finlayson, 2009)).

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Table 1.1 Nomenclature and selective ligands for the PACAP receptors

Receptor subtype		Gene name (HUGO)	Selective agonists	Selective Antagonists
Current name	Previous names			
PAC$_1$	Type I-binding site	ADCYAP1R1	Maxadilan	PACAP(6-38)[^] M65 Max.d.4 [Ser$_4$]-PACAP-38
	PACAP Type-I receptor			
	PACAP/VIP receptor 1 (PVR 1)			
VPAC$_1$	Type II-binding site	VIPR1	[A^{11,22,28}]VIP [A^{2,8,9,11,19,22,24,25,27,28}]VIP [Lys^{15},Arg^{16},Leu^{27}]VIP-(1-7)/GRF(8-27)-NH2 [Arg16]chicken secretin[°]	PG97-269 [Y9,Dip18]-VIP(6-23)
	"Classic" VIP receptor			
	VIP-PACAP Type-II receptor (PVR I)			
	VIP1 receptor			
	PACAP/VIP receptor 2 (PVR 2)			
VPAC$_2$	Type II-binding site	VIPR2	Ro 25-1553 Ro 25-1392	PG99-465
	"Helodermin"-preferring VIP receptor			
	VIP2 receptor			
	PACAP/VIP receptor 3 (PVR 3)			

[^] Displays affinity for VPAC$_2$.

[°] Selective only in rodent tissues that do not express the secretin receptor (e.g. brain).

Adapted from (Harmar *et al.*, 1998; Sherwood *et al.*, 2000; Vaudry *et al.*, 2009; Alexander *et al.*, 2011; Harmar *et al.*, 2012).

1.8.2.3 Signal transduction through the PACAP receptors

The three PACAP receptors are GPCRs possessing 7 transmembrane domains. Ligand activation of the different PACAP receptors releases G-proteins, coupled to the receptors, which triggers the activation of receptor specific intracellular secondary messenger signaling cascades. This results in downstream cellular excitability and functionality of the peptide.

GPCRs are classically subdivided into three groups; 1) the A class containing rhodopsin receptors (~1200 members), 2) the B class or peptide class (>50 members) and 3) the C class containing metabotropic receptors (~25 members; mainly

glutamate). PACAP is a neuropeptide, therefore, its receptors are part of the B class of GPCRs. B class GPCRs all possess a >120 amino acid N-terminal domain containing both a signal peptide and hormone binding domain (Harmar, 2001). The B class of GPCRs is further divided into B1, B2 and B3 groups (Harmar, 2001), the PACAP receptors are all classed as B1 receptors, a class of classic peptide hormone receptors. All B1 category receptors are positively coupled to $G\alpha_s$ and act to increase AC activity. PACAP receptors also exert their effects through PLC (Spengler *et al.*, 1993), ligand gated calcium channels (Chatterjee *et al.*, 1996) and phospholipase D (PLD; McCulloch *et al.*, 2000).

The various PAC_1 splice variants are coupled to specific second messengers. The majority of the PAC_1 subtypes are coupled to $G\alpha_s$ that triggers cAMP and protein kinase A (PKA) production, and turnover, by activating AC (Pisegna & Wank, 1993; Arimura, 1998; Vaudry *et al.*, 1998). Others are $G\alpha_{q/11}$-coupled and cause increased inositol-3-phosphate (IP3) accumulation though PLC activation (Spengler *et al.*, 1993) and some are coupled to ADP-ribosylation factor, activating PLD (McCulloch *et al.*, 2000). The remaining PAC_1 subtype acts through a $G\alpha_s$ linked to a ligand gated calcium channel, enabling calcium influx (Chatterjee *et al.*, 1996).

Activation of $VPAC_1$ by PACAP or VIP also primarily stimulates cAMP production via $G\alpha_s$ activation of AC (Sherwood *et al.*, 2000; Vaudry *et al.*, 2009). Activation of this receptor can also have a stimulatory effect on IP3 production through PLC interacting with a $G\alpha_{i/o}$ (Van Rampelbergh *et al.*, 1997). Additionally, activation of $VPAC_1$ increases PLD activation and increases intracellular calcium, which has a multitude of downstream cellular effects (Sreedharan *et al.*, 1994; McCulloch *et al.*, 2000). $VPAC_2$ acts similarly on the AC, PLC, PLD and calcium systems (McCulloch *et al.*, 2000; Sherwood *et al.*, 2000; Vaudry *et al.*, 2009). The signal transduction mechanisms are further explained in **Table 1.2** and **Figure 1.12**.

The three PACAP receptors can have differential actions in various systems, most probably due to the different second messenger systems used by the receptors, and the tissue/cell specific expression of the receptors and their splice variants (Vaudry *et al.*, 2009). For example, the VPAC receptors, and not PAC_1, are involved

in pressure-induced vasodilation in the skin (Fizanne *et al.*, 2004), increased local pancreatic blood flow (Igarashi *et al.*, 2008) and vasodilation of the isolated perfused rat heart (Sawmiller *et al.*, 2006). Whereas, PAC_1 alone is implicated in Ca^{2+} release from adrenal chromaffin cells, causing catecholamine release (Payet *et al.*, 2003). The two receptor subtypes act synergistically to enhance neuroexcitability in intracardiac neurons (DeHaven & Cuevas, 2004). It is unknown what role PACAP or each of its 3 receptors, has in regulating tonic AP control, at the level of the spinal cord, this is addressed in **Chapter 6**.

Table 1.2 PACAP receptor variants and their signal transduction mechanisms

Receptor subtype	Splice variant	Sequence alternation	Functional consequence
PAC_1	PAC1-short	Deletion of exons 5 and 6: 21 aa deletion from N-terminal (aa 89–109)	1. Slightly increased PACAP-27 potency (PLC); 2. increased VIP potency, VIP and PACAPs equipotent; 3. increased VIP potency (cAMP, PLC), but still PACAP preferring
	PAC1-short,hop1	Deletion of exons 5 and 6: 21 aa deletion from N-terminal (aa 89–109), 28 aa insertion into 3rd IC loop	1. PACAP-27 responses larger than PACAP-38; 2. slightly increased VIP potency (cAMP, PLC)
	PAC1-TM4	Substitution and deletion of CVTV to SA—in 4th TM domain (aa 297–283), substitutions D136N (N-terminal) and N190D (2nd TM domain)	No activation of PLC or AC, stimulation of L-type calcium channels
	PAC1-vs	Deletion of exon 4: 57 aa deletion in N-terminal (aa 53–88); deletion of exons 5	1. Reduced PACAP potency, still PACAP preferring; 2. reduced VIP and PACAP potency (cAMP)
	PAC1-3a	Exon 3a inclusion: insertion of 24 aa into N-terminal	Slightly reduced PACAP potency (cAMP, PLC)
	PAC1-hip	Inclusion of exon 14: 28 aa insertion into 3rd IC loop	1. Slightly reduced PACAP potency (cAMP), no PLC activation; 2. increased VIP potency (cAMP)
	PAC1-hop1	Inclusion of exon 15: 28 aa insertion into 3rd IC loop	1. Similar to null (PLC, cAMP); 2. reduced VIP potency (cAMP)
	PAC1-hop2	Inclusion of part of exon 15: 27 aa insertion into 3rd IC	Similar to null (PLC/cAMP)

		loop	
	PAC1-hiphop1	Inclusion of exons 14 and 15: 56 aa insertion into 3rd IC loop	Reduced PACAP potency (PLC)
	PAC1-hiphop2	Inclusion of exons 14 and part of 15: 55 aa insertion into 3rd IC loop	Reduced PLC activation
	PAC1-δ5	Deletion of exon 5: 7 aa deletion from N-terminal (aa 89–96)	No clear change
	PAC1-δ5hop	Deletion of exon 5: 7 aa deletion from N-terminal (aa 89–96); inclusion of exon 15: 28 aa insertion into 3rd IC loop	No clear change
	PAC1-δ5hip	Deletion of exon 5: 7 aa deletion from N-terminal (aa 89–96); inclusion of exon 14: 28 aa insertion into 3rd IC loop	Reduced VIP and PACAP-38 potency (cAMP), reduced PACAP-38 potency (PLC)
	PAC1-δ5,6hip	Deletion of exons 5 and 6: 21 aa deletion from N-terminal (aa 89–109); inclusion of exon 14: 28 aa insertion into 3rd IC loop	Reduced PACAP-38 potency (cAMP, PLC)
	PAC1-δ5,6,16,17	Deletion of exons 5–6, 16–17: 21 aa deletion from N-terminal (aa 89–109), 118 aa deletion from TM6, 3rd EC loop, TM7 (aa 350–468) and 13 aa insertion (aa 350–361)	No signalling (cAMP)
VPAC$_1$	VPAC1-5TM	Deletion of 88 aa from 3rd IC loop, 6th and 7th TM domains	Reduced coupling to signalling pathways (cAMP)
VPAC$_2$	VPAC2-5TM	Deletion of 74 aa from 3rd IC loop, 6th and 7th TM domains	No signalling examined
	VPAC2de367-380	Deletion in exon 12: 14 aa deletion from C-terminal (aa 367–380)	Reduced coupling to signalling pathways (cAMP, IL2)
	VPAC2de325-438(i325-334)	Deletion of exon 11: 114 aa deletion from IC3 (aa 325–438), insertion of 10aa into 3rd IC loop (aa 325–334)	Reduced coupling to signalling pathways
	VIPs	2 aa change from VPAC$_2$ in N-terminal ligand binding domain (L40F, L41F)	VIP more potent than PACAP (cAMP)

Adapted from (Dickson & Finlayson, 2009; Vaudry *et al.*, 2009)

1.8.3 The distribution of PACAP and its receptors

PACAP immunoreactivity and mRNA have been localised in neurons of the CNS and peripheral nervous system (PNS; Vaudry *et al.*, 2009). PACAP-38 is the

predominant form of the peptide in both areas, and is therefore the peptide used in the experiments described here (**Chapters 3 - 7**). Centrally, PACAP-27 represents less than 10% of the total peptide content in the brain (Arimura *et al.*, 1991; Ghatei *et al.*, 1993; Masuo *et al.*, 1993; Hannibal *et al.*, 1995a; Piggins *et al.*, 1996). Peripherally, the proportions of PACAP -38 and -27 vary between different organs (Arimura *et al.*, 1991). PACAP, and its receptors, are located in the nerve fibres and ganglia of the PNS including the; eye, endocrine glands, genitourinary tract, respiratory tract, digestive system, skin, lymphoid tissues and, most notably, in the peripheral cardiovascular system (Arimura *et al.*, 1991; Vaudry *et al.*, 2009). This study concentrates on the effects of PACAP in central cardiovascular control, discussion of the peripheral locations of PACAP, and its receptors, will be limited.

1.8.3.1 Distribution of PACAP within the CNS

Since its discovery in 1989 (Miyata *et al.*, 1989), the distribution of PACAP has been extensively mapped in the CNS of numerous species. Radioimmunoassay and northern blot hybridisation techniques, were used to map PACAP immunoreactivity and PACAP mRNA in both human and rat brain (Arimura *et al.*, 1991; Ghatei *et al.*, 1993), before non-radioactive detection methods for PACAP gene expression were available in 1995. Following their introduction, immunohistochemistry and *in situ* hybridisation showed that PACAP is located extensively throughout fibres of the brain and is in major areas and nuclei of the CNS e.g. the hypothalamus, forebrain, cerebral cortex, amygdala, hippocampus, thalamus and cerebellum (Arimura *et al.*, 1991; Hannibal *et al.*, 1995a; Hannibal, 2002; Vaudry *et al.*, 2009). PACAP immunoreactivity and mRNA were also extensively mapped in the human (Dun *et al.*, 1996a) and rat spinal cord (Chiba *et al.*, 1996; Dun *et al.*, 1996b; Beaudet *et al.*, 1998; Pettersson *et al.*, 2004). For review refer to the following (Köves *et al.*, 1994; Sherwood *et al.*, 2000; Vaudry *et al.*, 2009).

PACAP immunoreactive cells and mRNA are found in areas of the medulla oblongata and spinal cord that influence SNA. This includes regions such as the dorsal motor nucleus of the vagus, the raphé nuclei (Légrádi *et al.*, 1994), the PVN (Hannibal *et al.*, 1995b; Das *et al.*, 2007), the ventromedial hypothalamus (Maekawa *et al.*, 2006) and the arcuate nucleus (Murase *et al.*, 1995; Mounien *et al.*, 2006a;

Dürr *et al.*, 2007). Most importantly, PACAP is localised to the region of the venterolateral medulla (Légrádi *et al.*, 1994), recently the presence of PACAP in the RVLM was confirmed (Farnham *et al.*, 2008). PACAP is co-localised in the RVLM with presympathetic bulbospinal neurons (82%), neurons that control sympathetic outflow to the cardiovascular system via SPN (Farnham *et al.*, 2008). PACAP is also found in the IML, and is specifically found in spinal cord neurons that are involved in the transmission of SNA to the cardiovascular system such as presympathetic neurons and SPN (Dun *et al.*, 1996a; Dun *et al.*, 1996b; Hannibal, 2002). This evidence suggests a role for PACAP in the central neural control of AP.

1.8.3.2 Distribution of the PACAP receptors within the CNS

Following the identification of the PACAP receptors, each receptor was cloned and then mapped using immunohistochemistry and *in situ* hybridisation (Buscail *et al.*, 1990; Gottschall *et al.*, 1990; Basille *et al.*, 2000). Each receptor has tissue specific distribution in the CNS and the periphery (Lam *et al.*, 1990; Arimura *et al.*, 1991; Shivers *et al.*, 1991). The PACAP receptors were identified in nerve fibres/ganglia/nuclei of the CNS (Kyeung *et al.*, 2004) and the PNS (Arimura, 1998; Dickson & Finlayson, 2009; Vaudry *et al.*, 2009). PAC_1 receptors predominate in the CNS, while VPAC receptors predominate in the PNS (Arimura & Shioda, 1995). In the CNS, the PACAP receptors were identified in the spinal cord and in many regions of the brain and brainstem such as the cerebral cortex, basal ganglia, amygdala, hippocampus, thalamus, hypothalamus, pons and the medulla oblongata (Arimura, 1998; Dickson & Finlayson, 2009; Vaudry *et al.*, 2009). PACAP receptors are most notably found in the rat brainstem, localised to the RVLM, PAC_1 is more abundant than the VPAC receptors in the RVLM (Farnham *et al.*, 2012). However, specific localisation of the receptors to other important cardiorespiratory areas remains undetermined. All three PACAP receptors are also found on SPN cell bodies (Hannibal, 2002), further implicating PACAP in central cardiovascular control.

1.8.3.3 Distribution of PACAP and its receptors in the PNS

Along with being located in important cardiovascular regions of the brain and spinal cord, PACAP and its receptors are found within the peripheral cardiovascular system. Receptors are present in the heart (Wong *et al.*, 1998), aorta (Miyata *et al.*, 1998), cardiac ganglia (Braas *et al.*, 1998) and blood vessels (Koves *et al.*, 1990;

Cardell *et al.*, 1991; Huang *et al.*, 1993; Fahrenkrug *et al.*, 2000). Most importantly, immunoreactivity and mRNA for PACAP and its receptors are found in the adrenal medulla of neonatal and adult rats (Moller & Sundler, 1996; Nogi *et al.*, 1997). PACAP and its receptors are also localised to SPN innervating Ad and NAd secreting chromaffin cells (Watanabe *et al.*, 1992; Babinski *et al.*, 1996; Tönshoff *et al.*, 1997; Morrison & Cao, 2000; Hamelink *et al.*, 2002b; Ghzili *et al.*, 2008; Kumar *et al.*, 2010). Ad release from the Ad secreting chromaffin cells is involved in the "fight or flight" stress response. Functionally distinct populations of SPN innervate Ad and NAd secreting chromaffin cells (**section 1.6.3**; Morrison & Cao, 2000; Kumar *et al.*, 2010). The role of PACAP as a co-transmitter within the sympathoadrenal system is discussed further in **section 1.9** and is investigated in **Chapters 6 and 7**.

1.8.4 The function of PACAP

PACAP acts as a hormone, neurohormone, neurotransmitter or trophic factor. The evolutionary conservation (**section 1.8.1**) and widespread distribution of PACAP and its receptors (**section 1.8.3**) suggests that PACAP is an important peptide within many biological systems. PACAP was first discovered for its ability to stimulate AC (Miyata *et al.*, 1989). Since then, many other functions of the peptide have become apparent. The systemic functions of PACAP are due to signal cascade activation; caused by PACAP binding its receptors (**section 1.8.2.3**) on neurons that innervate target organs. Discussion regarding the function of PACAP will mostly be limited to its actions within the cardiorespiratory and other systems of interest.

1.8.4.1 General actions of PACAP

The actions of PACAP are diverse and are seen in many systems including the nervous, cardiorespiratory, reproductive, endocrine, muscular and immune systems (Sherwood *et al.*, 2000; Dickson & Finlayson, 2009; Vaudry *et al.*, 2009; Brubel *et al.*, 2012). Within the CNS, PACAP is involved in;

- learning and memory (Otto *et al.*, 2001a)
- motor activity (Hashimoto *et al.*, 2001)
- fear conditioning (Nicot *et al.*, 2004)

- social behaviour (Otto *et al.*, 2001b)

- mediation of chronic pain (Ohsawa *et al.*, 2002; Davis-Taber *et al.*, 2008)

- glutamate metabolism (Zink *et al.*, 2004)

- feeding behavior and carbohydrate intake (Matsuda *et al.*, 2005; Mounien *et al.*, 2006b; Nakata & Yada, 2007; Adams *et al.*, 2008)

- retinal development (Seki *et al.*, 1998; Borba *et al.*, 2005)

- maintaining photic entrainment of circadian rhythm (Cagampang *et al.*, 1998Ehab, 2010 #1039; Hannibal *et al.*, 1998; Harrington *et al.*, 1999; Hannibal *et al.*, 2001; Harmar *et al.*, 2002; Kawaguchi *et al.*, 2003; Hannibal *et al.*, 2008; Racz *et al.*, 2008)

- neuroprotection (Vaudry *et al.*, 2000; Dejda *et al.*, 2005; Chen *et al.*, 2006; Shioda *et al.*, 2006; Brenneman, 2007; Ohtaki *et al.*, 2008; Tsuchikawa *et al.*, 2012).

Along with its role in the CNS and in the cardiorespiratory and sympathoadrenal systems, PACAP is also involved in general processes throughout the body such as cell proliferation (Suzuki *et al.*, 1993; Braas *et al.*, 1994; Tischler *et al.*, 1995; Campard, 1997; Lu *et al.*, 1998; Moroo *et al.*, 1998), cell differentiation (Chafai *et al.*, 2006), thermoregulation (Gray *et al.*, 2002) and metabolism (Jamen *et al.*, 2000; Gray *et al.*, 2001; Asnicar *et al.*, 2002; Shintani *et al.*, 2003; Shintani *et al.*, 2004).

1.8.4.2 Peripheral cardiovascular functions of PACAP

In the periphery PACAP increases both cardiac output and contractility (Minkes *et al.*, 1992b; Sawangjaroen *et al.*, 1992; Sawangjaroen & Curlewis, 1994) as well as acting on vascular smooth muscle (Abad *et al.*, 2006; Dickson & Finlayson, 2009; Vaudry *et al.*, 2009). I.v. and intra-arterial PACAP administration increases cardiac contractility (Minkes *et al.*, 1992b; Champion *et al.*, 1996) and HR (Minkes *et al.*, 1992a; Sawangjaroen *et al.*, 1992; Suzuki *et al.*, 1993; Sawangjaroen & Curlewis, 1994). Following the discovery that cAMP production in cardiac myocytes is stimulated by PACAP-38, Suzuki *et al.,* (1993) suggested that the ionotropic and chronotropic effects of PACAP may be due to direct stimulation of AC in cardiac muscle cells (Suzuki *et al.*, 1993). This theory is supported by the

presence of PACAP receptors in the heart (Wong *et al.*, 1998) and cardiac ganglia (Braas *et al.*, 1998). The effects of PACAP on the heart are mediated through PAC_1, as HR was reduced following intracoronary injections PACAP(6-27) (PAC_1 antagonist; Markos *et al.*, 2002).

The blood vessels of the vascular system, including the cerebral (Uddman *et al.*, 1993; Fahrenkrug *et al.*, 2000), gastric (Huang *et al.*, 1993), respiratory (Cardell *et al.*, 1991), reproductive and cardiac vascular beds, are richly innervated by PACAP-containing neurons (Koves *et al.*, 1990; Cardell *et al.*, 1991) and contain a high number of PACAP receptors (Huang *et al.*, 1993). The physiological effects of PACAP within peripheral vessels has predominated the research investigating the cardiovascular effects of PACAP. The first studies investigating the effect of PACAP on AP injected PACAP i.v. into anaesthetised cats and dogs. AP decreased following low doses of PACAP (0.1-0.3 nmol/kg), however, high doses of the peptide resulted in a biphasic AP response (initial decrease followed by an increase; Minkes *et al.*, 1992a; Suzuki *et al.*, 1993; Runcie *et al.*, 1995; Champion *et al.*, 1996; Erickson *et al.*, 1996). A response that may be explained by transport of PACAP across the blood brain barrier, via by peptide transport system-6 (Banks *et al.*, 1998; Somogyvári-Vigh *et al.*, 2000; Nonaka *et al.*, 2005). When AP is elevated, central venous pressure, plasma Ad and heart ionotropy/chronotropy are also increased (Minkes *et al.*, 1992a; Suzuki *et al.*, 1993). Adrenalectomy and α-adrenergic receptor antagonists block PACAP-induced pressor responses, indicating that the effects are mediated by adrenal catecholamine release (Minkes *et al.*, 1992a; Suzuki *et al.*, 1993; Runcie *et al.*, 1995; Champion *et al.*, 1996).

Peripheral PACAP administration in the guinea pig (Cardell *et al.*, 1991), rat (Huang *et al.*, 1993) and pig (Ross-Ascuitto *et al.*, 1993), causes potent vasorelaxant effects, with no pressor response. However, intra-arterial PACAP caused no significant cardiovascular effects in the trout (Le Mevel *et al.*, 2009). PACAP causes vasodilatation in the cerebral arteries of cats, rats and rabbits (Uddman *et al.*, 1993; Dalsgaard *et al.*, 2003; Syed *et al.*, 2012) and in the coronary arteries of rabbits and neonatal pigs (Tong *et al.*, 1993; Champion *et al.*, 1996; Dalsgaard *et al.*, 2003). The vasodilator effect seen in the hindquarter, renal and mesenteric vascular beds of the rat were found not to be mediated by PACAP-induced renin secretion (Gardiner *et*

al., 1994; Hautmann *et al.*, 2007). Activation of the VPAC receptors is involved in mediating pressure induced vasodilatation response, where local blood flow is transiently increased following pressure application (Fizanne *et al.*, 2004). Peripheral PACAP also has highly potent vasorelaxant affects in many organs including the eye (Dorner *et al.*, 1998), submandibular glands (Nilsson, 1994), lungs (Cheng *et al.*, 1993; Foda *et al.*, 1995) and pancreas (Ito *et al.*, 1998).

1.8.4.3 Central cardiovascular functions of PACAP

Peripheral studies have dominated the research investigating the cardiovascular effects of PACAP, while the central cardiovascular effects of PACAP remain poorly understood. Centrally administered PACAP has a sympathoexcitatory effect on the cardiovascular system (Farnham & Pilowsky, 2010). Intracerebroventricular (i.c.v; Murase *et al.*, 1993) and intracisternal (Seki *et al.*, 1995) PACAP has little effect on AP at low doses (50 pmol/rat), however higher doses (100-500 pmol/rat) increased AP, plasma Ad and plasma arginine vasopressin levels. This was recently reaffirmed by Le Mével and colleagues, who demonstrated an increase in dorsal aortic AP following i.c.v PACAP in trout (Le Mevel *et al.*, 2009), this group has subsequently shown that i.c.v. PACAP and VIP dose-dependently decrease baroreflex sensitivity in trout (Lancien *et al.*, 2011). There is a stark contrast between the central and peripheral actions of PACAP, this discrepancy may be due to differences in the central and peripheral distributions of the PACAP receptors. Centrally, PAC_1 predominates, whereas VPAC receptors are the majority in the periphery (Arimura & Shioda, 1995).

To date, within cardiovascular neurons, it is known that PACAP directly excites SPN in spinal cord slices from juvenile rats, causing intense depolarisation accompanied by large increases in neuronal discharge (Lai *et al.*, 1997). Also, presympathetic neurons and SPN both have rich PACAP immunoreactivity (Dun *et al.*, 1996a; Dun *et al.*, 1996b; Hannibal, 2002; Farnham *et al.*, 2008). Our laboratory has investigated the functional significance of PACAP within presympathetic neurons and SPN by targeting PACAP-containing SPN in the spinal cord and PACAP-containing presympathetic RVLM neurons, via intrathecal administration of PACAP and via direct microinjection into the RVLM, respectively. When directly microinjected into the RVLM, PACAP results in increased AP, HR and $_S$SNA

(Farnham *et al.*, 2012). Only one other group has microinjected PACAP into the brain, however, their main aim was to determine the effects of PACAP on gastric motor function, with secondary aims to study the effects of PACAP on cardiovascular function. Krowicki *et al.,* (1997) microinjected PACAP into the nucleus ambiguus and raphé obscures, nuclei immediately dorsal and lateral to the RVLM, respectively. They reported inconsistent AP and HR effects (Krowicki *et al.*, 1997) that could be due to injections sites overlapping the region of the RVLM. Little information is know about the role of PACAP in the reflex control of the cardiovascular system, however our laboratory has recently shown that PACAP does not affect the homeostatic cardiovascular reflexes when administrated at the level of the spinal cord (Gaede *et al.*, 2012) or RVLM (Farnham *et al.*, 2012).

Intrathecal injection of PACAP causes sympathoexcitation in multiple sympathetic beds and a marked tachycardia (Farnham *et al.*, 2008; Inglott *et al.*, 2011), confirming the SPN activation seen in the Lai *et al.,* (1997) patch-clamping study (Lai *et al.*, 1997). However, the widespread sympathoexcitation is not accompanied by an increase in AP. Following intrathecal PACAP, AP remains unchanged, an effect that is not baroreceptor mediated (Farnham *et al.*, 2008; Inglott *et al.*, 2011). The sympathoexcitatory effect following intrathecal PACAP are sustained for at least three hours (Farnham *et al.*, 2008), despite the discovery that 75% of i.v. PACAP-38 is cleared from the plasma within 6 minutes (Zhu *et al.*, 2003). The time course of the intrathecal response suggests that the PACAP effects may be a result of downstream signaling pathway activation, and not due to persistent PACAP receptor activation. The results from our laboratory conflict with a previous study that reports a pressor response, persistent for over one hour, following intrathecal PACAP injected at T2 in anaesthetised rats (Lai *et al.*, 1997). However, differences in spinal levels of injection, doses used and lower baseline parameters in the Lai *et al.,* (1997) study may account for this difference.

The role of PACAP in the regulation of vascular tone, the distribution of PACAP within the spinal cord and RVLM and the physiological effects observed in our laboratory implicate PACAP in the central neural control of AP and vasomotor tone. The response observed following intrathecal PACAP injection is paradoxical. Generally, sympathoexcitatory peptides (such as orexin (Shahid *et al.*, 2011) and

substance P (Yashpal & Henry, 1993)) also cause a pressor response, when delivered intrathecally. This thesis aims to further investigate the role of PACAP within central neural control of the cardiovascular system at the level of the spinal cord, and also aims to determine the mechanism that underlies the lack of AP response following intrathecal PACAP injection (see **Chapters 4, 5, 6 and 7**).

1.8.4.4 PACAP and hypertension

The RVLM and the many modulatory neurotransmitters found in this region are implicated in the aetiology of essential, neurogenic, hypertension (**section 1.5.6**; Pilowsky & Goodchild, 2002; Guyenet, 2006). Here, the role of PACAP in hypertension is investigated. PACAP is not involved in the etiology of hypertension in the anaesthetised SHR at the level of the IML (Farnham *et al.*, 2011) or RVLM (Farnham *et al.*, 2012), as antagonism of PACAP receptors results in little change to AP or SNA in this strain. However, the prolonged, widespread SNA increase seen upon intrathecal injection (Lai *et al.*, 1997; Farnham *et al.*, 2008; Inglott *et al.*, 2011) and RVLM microinjection of PACAP (Farnham *et al.*, 2012) suggests a role for PACAP within the pathophysiology of essential hypertension. A hypothesis supported by evidence that PAC_1 knockout mice develop pulmonary hypertension at a very young age (Otto *et al.*, 2004). This hypothesis is also supported by the widespread distribution of PACAP, and its receptors, in areas of the brain that are important in cardiovascular regulation and in the etiology/pathophysiology of hypertension. Altered function/dysfunction of the PACAP system may offer a cause or solution to the unanswered questions regarding the pathophysiology of essential hypertension. Therefore, understanding the function of PACAP within central cardiovascular control is important, and is the focus of this thesis.

1.8.4.5 Effects of PACAP within the respiratory system

I.v. PACAP causes bronchodilation and a powerful stimulation of breathing (Runcie *et al.*, 1995). The respiratory responses to i.v. PACAP may be evoked due to chemoreflex activation, as bilateral carotid sinus nerve transection abolishes the effects of PACAP (Runcie *et al.*, 1995). Peripheral PACAP also causes vasodilation of the pulmonary vasculature (Murphy *et al.*, 1992; Foda *et al.*, 1995; Chen *et al.*, 2006); as such, inhaled PACAP is currently being investigated as a possible therapy for asthma (Lindén *et al.*, 1999; Doberer *et al.*, 2007).

Despite its peripheral effects, little information is known about the central respiratory effects of PACAP, or of the distribution of PACAP, and its receptors, in central respiratory sites (Wilson & Cummings, 2008). Evidence from PACAP and PACAP receptor knockout mice, (Jamen *et al.*, 2000; Gray *et al.*, 2001; Cummings *et al.*, 2004; Wilson & Cummings, 2008), indicates that PACAP plays an important excitatory role in respiratory rhythm generation. PACAP deficient mice (prepro-PACAP knockout mice) display a reduction in tidal volume and ventilation that is sensitive to lowered temperatures (Cummings *et al.*, 2008), and a reduction in basal respiratory rate (Cummings *et al.*, 2004). In PACAP deficient mice, apnoea precedes atrioventricular block and death; most PACAP deficient mice die within the first two weeks of life. Sudden neonatal death is reported in both the PACAP and PAC_1 knockout mice (Cummings *et al.*, 2004), with the PAC_1 knockout mice having an increased incidence of unexplained neonatal death compared with wild-type controls (Jamen *et al.*, 2000; Arata *et al.*, 2012). *In vitro* evidence also suggests that PACAP is excitatory within central respiratory centres, with PACAP exciting central respiratory rhythm generation in preBöt slices (Pena, 2010). PACAP gene abnormalities may be present in some clinical respiratory conditions such as sudden infant death syndrome; however this remains unproven (Wilson & Cummings, 2008).

Despite this evidence, there is only one *in vivo* study examining the effect of central PACAP administration on respiration. In 2009, Le Mével and colleagues showed that i.c.v PACAP significantly increased ventilation rate and amplitude, with PACAP and VIP both increasing total ventilation in the trout (Le Mevel *et al.*, 2009). Intra-arterial administration of PACAP and VIP did not cause any significant respiratory effects in this species (Le Mevel *et al.*, 2009). No *in vivo* experiments have been conducted to determine the direct effect of central PACAP administration on breathing, via microinjection into brainstem nuclei or into the intrathecal space, a deficit addressed in experiments described in **Chapter 7**.

1.8.4.6 Effects of PACAP on metabolic function

PACAP neurons (Das *et al.*, 2007; Dürr *et al.*, 2007) and/or PACAP receptors (Anderson *et al.*, 2005; Mounien *et al.*, 2006a) are found in both the arcuate and raphé nuclei, two nuclei of the brain involved in feeding, glucose

metabolism and thermoregulation. The RVLM is also thought to play a role in these processes, resulting in AP effects (Madden *et al.*, 2006). The raphé and arcuate nucleus both receive efferent projections from the RVLM (Card *et al.*, 2006), while BAT thermogenesis, a major source of non-shivering heat production, is under sympathetic control from the raphé (Blessing, 2005). Recent studies have placed a significant emphasis on the involvement of PACAP in metabolism (Nakata & Yada, 2007; Adams *et al.*, 2008), this is discussed below.

Glucose metabolism; The liver is the major site of glucose metabolism, a process under hormonal control. Insulin stimulates glycogenesis, while glucagon and catecholamines stimulate glycogenolysis via activating AC. It is suggested that PACAP is involved in energy storage after feeding through insulin, adipogenesis, adipocyte differentiation and by stimulating feeding (Yamaguchi & Lamouche, 1999; Adams *et al.*, 2008; Sakurai *et al.*, 2012). When fasting, PACAP is involved in energy utilisation through catecholamine- and glucagon-mediated lipolysis (Watanabe *et al.*, 1992; Filipsson *et al.*, 1997; Hamelink *et al.*, 2002a), increased glycogenolysis (Yokota *et al.*, 1995), stimulation of AC activity in hepatocytes (El Fahime *et al.*, 1996) and also by stimulating feeding (Ghzili *et al.*, 2008). The downstream effect of this being increased glucose output from the liver (Nakata & Yada, 2007).

The importance of PAC_1 in glucose homeostasis is highlighted in PAC_1 knockout mice. The first PACAP knockout mice were of low body weight, with impaired glucose-induced insulin secretion and with increased glucose intolerance (Jamen *et al.*, 2000). PAC_1 knockout mice recover poorly from an insulin induced hypoglycaemia, suggesting an impaired secretion of counter-regulatory hormones such as glucagon and catecholamines (Hamelink *et al.*, 2002b). Long-term administration of the PACAP antagonist impaired glucose tolerance and insulin sensitivity in obese diabetic rats (Green *et al.*, 2006), confirming the results seen in the PAC_1 knockout mice. $VPAC_2$ knockout mice have reduced body fat and become progressively leaner with age than their wild-type littermates, indicating an increased basal metabolic rate (Asnicar *et al.*, 2002). It was later found that $VPAC_2$ mediates lipolytic effects in primary rat adipocites (Åkesson *et al.*, 2005).

Rats with C1 depletion have smaller compensatory responses to hypotension and glucoprivation (Madden *et al.*, 2006). It was therefore proposed that, in addition to playing a role in acute cardiovascular reflexes, C1 neurons of the RVLM play an important role in responses to homeostatic challenges such as hypotension and glucoprivation (Madden *et al.*, 2006). Since PACAP is present in C1 neurons (Farnham *et al.*, 2008), and RVLM projections to the PACAP receptor-containing raphé and arcuate nucleus are well established, the effects observed with C1 depletion may be a result of PACAP depletion. The influence of intrathecal PACAP on metabolic rate and glucose metabolism is investigated in **Chapter 5**.

Feeding; in 2004, it was first demonstrated that PACAP knockout mice had reduced carbohydrate intake, normal high-fat diet intake and reduced neuropeptide Y mRNA expression in the arcuate nucleus (Nakata *et al.*, 2004). I.c.v PACAP suppresses food intake and gut motility in goldfish (Matsuda *et al.*, 2005). While, microinjection of PACAP into the ventromedial hypothalamus also reduces food intake in rats (Resch *et al.*, 2011), complimenting the Nakata study (Nakata *et al.*, 2004). It has been suggested that the PACAP-induced increases in neuropeptide Y and decreases in corticotropin-releasing hormone mRNA expression in the PVN is the reason for reduced food intake (Hashimoto *et al.*, 2006). Most have concluded that PACAP mediates carbohydrate metabolism, is necessary for feeding, is involved in maintaining energy homeostasis but is not required for the regulation of food intake (Adams *et al.*, 2008).

Thermogenesis; The first PACAP knockout mice were cachexic with lipid accumulation in the heart, skeletal muscle and liver; majority died in the second postnatal week (Gray *et al.*, 2001). This prompted Gray and colleagues to conclude that PACAP was essential for normal lipid and carbohydrate metabolism; however the PACAP knockout pups were also temperature sensitive. Subsequent to this, the same group has showed that the majority of PACAP knockout mice survive weaning when housed in warmer conditions (24°C compared with 21°C; Gray *et al.*, 2002). The wasting originally observed in the PACAP knockout pups was also prevented when the mice were provided with a high fat diet or housed in warmer conditions (28°C versus 21°C). Adipose tissue analysis from PACAP knockout mice found that PACAP is essential for Ad release from nerves in adipose tissue and for the

stimulation of adipocyte thermogenesis (Gray *et al.*, 2002). Adams *et al.,* (2008) also showed that the temperature sensitivity of the knockout mice was not a result of altered food intake or physical activity, and concluded that PACAP was essential for energy homeostasis with a particularly important role in thermoregulation (Adams *et al.*, 2008). The role of PACAP in thermoregulation is investigated in **Chapter 5**.

1.9 PACAP, catestatin and the sympathoadrenal system

PACAP is expressed in 97% of SPN projecting to the adrenal medulla (Kumar *et al.*, 2010) and is expressed in the chromaffin cells of the adrenal medulla (Fukushima *et al.*, 2001a; Fukushima *et al.*, 2001b; Conconi *et al.*, 2006; Thouënnon *et al.*, 2010). It appears that PACAP acts as a neuropeptide in this system, modulating catecholamine release and the secretion of other neurotransmitters including PACAP itself (Lamouche & Yamaguchi, 2003; Kuri *et al.*, 2009; Smith & Eiden, 2012). Catestatin, an inhibitor of catecholamine release, is co-stored and co-released with catecholamines and the other neurotransmitters in the chromaffin cells, potentially including PACAP (O'Connor & Frigon, 1984; Mahata *et al.*, 1997b; Laslop & Mahata, 2002; Mahapatra *et al.*, 2006). Catestatin acts to modulate cardiorespiratory effects mediated by nAChR activation (Mahata *et al.*, 2010) and to inhibit the exocytosis of many other neurotransmitters from the adrenal chromaffin cells (Mahata *et al.*, 2010). As in the RVLM and SPN, co-transmission in the sympathoadrenal system will alter the net catecholamine and neurotransmitter outflow, and the physiological responses of the system. We anticipate that a co-modulatory effect may be apparent between catestatin and PACAP, due to the overlap in their expression and intracellular mechanisms of action; this hypothesis is investigated in **Chapter 7**.

1.9.1 Catestatin; a regulatory peptide derived from Chromogranin A

Catestatin is one of many biologically active chromogranin A (CgA) cleavage products. It is a cationic, amphiphilic neuropeptide discovered for its ability to inhibit nicotine-stimulated catecholamine release, through its action as an antagonist at the nAChR (Mahata *et al.*, 1997a). CgA is found in the secretory granules of chromaffin cells where it is co -stored and -secreted with catecholamines (Ghzili *et al.*, 2008). The biological effects of catestatin were first observed in 1988, CgA fragmentation resulted in a peptide that could reduce catecholamine secretion

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from chromaffin cells *in vivo* (Simon *et al.*, 1988). However, it was not until 1997 that catestatin was identified (Mahata *et al.*, 1997a). Mahata *et al.,* (1997) synthesised peptides corresponding to various sections of CgA and tested these with nicotine-stimulated catecholamine secretion in PC12 cells. Only one of these peptides (bovine $CgA_{344-364}$) inhibited catecholamine release, and was thus named "catestatin" (Mahata *et al.*, 1997a). Catestatin decreases catecholamine release by blocking nicotine-induced Na^+ and Ca^{2+} uptake; the first step of the nicotinic cationic signal transduction cascade. This effect is not observed when catecholamine secretion is stimulated by secretagogues that circumvent nAChR, and act at later stages in the signalling pathway (Mahata *et al.*, 2003; Mahapatra *et al.*, 2006).

Catestatin does not have any specific receptors, it affects multiple receptor types and intracellular mechanisms in order to exert its effects; catestatin interacts with nAChR, β-adrenergic receptors, H1 histamine receptors, and the $β_2$-adrenergic receptor-$Gα_i/_o$ protein signalling pathway (Mahata *et al.*, 1997a; Kennedy *et al.*, 1998; Angelone *et al.*, 2008; Mazza *et al.*, 2008; Zhang *et al.*, 2009a). One mechanism that catestatin relies on to exert its action is antagonism of the nAChR. When nAChR on chromaffin cells are activated, the exocytosis of catestatin, catecholamines, and other co-transmitters is stimulated. The catestatin released inhibits these nAChR in a negative-feedback loop, thus inhibiting CgA gene transcription and the further release of its co-transmitters (Mahata *et al.*, 1997a; Mahata *et al.*, 2010). Alternatively/additionally, catestatin may alter the activity of intracellular mechanisms that are coupled to $Gα_s$ including AC and PLC (Mahata *et al.*, 2003; Aung *et al.*, 2011). It is also thought that catestatin may act as a cell penetrating peptide, influencing Ca^{2+} signalling. Evidence supporting this hypothesis includes the sequence homology of catestatin and penetratin ($≥ 60\%$ homology), another well known cell penetrating peptide (Henriques *et al.*, 2006; Sugawara *et al.*, 2010). Catestatin rapidly penetrates the cell membrane to inhibit calmodulin binding, activating Ca^{2+}-independent phospholipase A_2, leading to the production of lysophospholipids that induce the opening of store-operated Ca^{2+} channels (Shivers *et al.*, 1991; Bolotina & Csutora, 2005; Zhang *et al.*, 2009a).

Along with its effect on adrenal catecholamine secretion and intracellular signalling pathways, catestatin modulates CgA gene transcription (Mahata *et al.*,

2003), stimulates histamine release (Kennedy *et al.*, 1998), has potent antimicrobial activity (Briolat *et al.*, 2005) and has a role in the induction of chemotaxis (Egger *et al.*, 2008; Aung *et al.*, 2011). Most notably, peripheral catestatin has significant cardiorespiratory functions. In the Langendorff-perfused heart preparation, catestatin caused tachycardia, hypotension and had negative ionotropic and lusitropic effects (Angelone *et al.*, 2008), catestatin is vasodilatory when administered i.v. (Kennedy *et al.*, 1998). Catestatin may also be involved in central cardiorespiratory control. Catestatin (Gaede & Pilowsky, 2010) and CgA (Gaede *et al.*, 2009) are co-localised with 88% and 85% of TH-containing RVLM neurons, respectively. Microinjection of catestatin in the RVLM is sympathoexcitatory, acutely increases AP and PNA, whilst also increasing barosensitivity, presumably via SPN activation (Gaede & Pilowsky, 2010). Moreover, microinjection of catestatin into the CVLM is sympathoinhibitory and attenuates baro- and chemo- reflex sensitivity (Gaede & Pilowsky, 2012). However, intrathecal application of catestatin did not affect basal cardiovascular parameters, but did attenuate the effects of nicotine and isoproterenol, most likely through interactions with nAChR and β_2-adrenergic receptors (Gaede *et al.*, 2009).

1.9.2 PACAP and the sympathoadrenal system

The sympathoadrenal system relies on external neurotropic factors and components of the extracellular matrix for its development, maintenance, function and neurotransmitter expression (Cowen & Gavazzi, 1998), PACAP is an integral regulator in this system (Smith & Eiden, 2012). PACAP is present in the embryo, where it enhances the proliferation and survival of sympathetic nerve cells, provokes neuronal differentiation (DiCicco-Bloom *et al.*, 2000; Salvi *et al.*, 2000) and regulates the activity of foetal chromaffin cells via PAC$_1$ (Payet *et al.*, 2003). PACAP is also present in the sympathoadrenal system of adult animals (Beaudet *et al.*, 1998). Specifically, PACAP mRNA is present in 97% of adrenally projecting SPN (Kumar *et al.*, 2010) where it exerts autocrine effects on sympathetic neurons to regulate their synaptic output by stimulating TH expression. *In vivo* studies show that i.v. injection of PACAP provokes tachycardia (Whalen *et al.*, 1999) and BAT thermogenesis (Gray *et al.*, 2002) due to catecholamine release from cardiac and BAT sympathetic fibres, respectively. PACAP also promotes the expression of other neuropeptides including VIP, neuropeptide Y, substance P, CgA and PACAP itself

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via activation of multiple signal transduction pathways such as PLC, cAMP, IP3 and others (Taupenot *et al.*, 1998; Girard *et al.*, 2002; Vaudry & Taupenot, 2002; Ghzili *et al.*, 2008).

Within the sympathoadrenal system, PACAP also regulates the excitability of sympathetic neurons and induces neuronal depolarisation by increasing sodium entry and inhibiting potassium current via mechanisms dependant on PLC and IP3 (Beaudet *et al.*, 2000; Hill *et al.*, 2012; Smith & Eiden, 2012). The neuronal depolarisation induced by PACAP increases gene expression, production and secretion of the peptide (Brandenburg *et al.*, 1997). The effects of PACAP within the sympathoadrenal system are most likely mediated by PAC_1, which is expressed in sympathetic neurons (Braas & May, 1999). PACAP positively modulates ACh release at nicotinic neuronal synapses (Pugh *et al.*, 2010), potentially adding to its influence over neuronal transmission in the sympathoadrenal system, specifically at the synapse joining adrenally projecting SPN and chromaffin cells (ACh via nAChR).

As well has being localised with adrenally projecting SPN, several studies have found PACAP to be stored in and released from chromaffin cells of the adrenal medulla (Fukushima *et al.*, 2001a; Fukushima *et al.*, 2001b; Lamouche & Yamaguchi, 2003; Conconi *et al.*, 2006). Several studies have demonstrated PACAP immunoreactvity in the adrenal medulla and chromaffin cells (Tabarin *et al.*, 1994; Frödin *et al.*, 1995; Shiotani, 1995; Moller & Sundler, 1996; Shioda *et al.*, 2000). Within the adrenal gland PACAP functions as a neurotransmitter to regulate catecholamine secretion from chromaffin cells (Turquier *et al.*, 2001; Hamelink *et al.*, 2002a; Hamelink *et al.*, 2002b; Payet *et al.*, 2003), and as such has been named a potent secretogogue of catecholamines from the adrenal gland (Kuri *et al.*, 2009). In chromaffin cells, the effect of PACAP on catecholamine release is associated with the increased expression/activity of catecholamine synthesizing enzymes; TH, DβH, and PNMT (Tönshoff *et al.*, 1997; McKenzie & Marley, 2002). There has been considerable study on the influence of PACAP on TH. PACAP regulates the gene expression and activity of TH in several areas including the adrenal medulla (Tönshoff *et al.*, 1997; Hong *et al.*, 1998; Hamelink *et al.*, 2002a; Hamelink *et al.*, 2002b; Wong *et al.*, 2002), hypothalamus (Anderson *et al.*, 2005) and nucleus

accumbens (Moser *et al.*, 1999). It is thought that this up regulation is due to phosphorylation of TH at serine 40 (Bobrovskaya *et al.*, 2007a; Bobrovskaya *et al.*, 2007b). By stimulating TH activity and expression, the rate limiting enzyme in the pathway to Ad and NAd synthesis (Zigmond *et al.*, 1989), PACAP increases catecholamine synthesis and release from the chromaffin cells of the adrenal gland. Interestingly, PACAP augments CgA mRNA expression by approximately four- to five- fold in rat PC12 cells (Taupenot *et al.*, 1998). However, it was later reported that PACAP does not affect CgA expression in bovine chromaffin cells, but does increase the expression of other members of the chromogranin/secretogranin family (Turquier *et al.*, 2001).

Controversy over the downstream mechanisms of activation continues, with some reports suggesting that both PKA and PKC pathways are required for TH phosphorylation (Choi *et al.*, 1999) and others reporting that only PKA is involved in TH phosphorylation and gene transcription (Muller *et al.*, 1997; Corbitt *et al.*, 1998; Corbitt *et al.*, 2002; Bobrovskaya *et al.*, 2007a). Other studies suggest that PACAP acts via L-type Ca_{2+} channel opening (Geng *et al.*, 1997; Fukushima *et al.*, 2001a; Fukushima *et al.*, 2001b; Morita *et al.*, 2002) as PACAP activates calcium entry, ensuing long-lasting increases in intracellular calcium levels (Morita *et al.*, 2002). This is linked to persistent catecholamine release via voltage-dependent and store-operated calcium channel pathways. Despite the contention, it is agreed that PACAP induced TH phosphorylation and gene transcription are mediated through PAC_1 (Payet *et al.*, 2003). In spite of the relationship between PACAP and catecholamines, it is noteworthy that monoaminergic development is unaffected in PACAP knock-out mice (Hannibal, 2002; Ogawa *et al.*, 2005).

Chapter 2

General Aims

Chapter 2: General Aims

Central control of vasomotor tone, and hence AP, involves the action of multiple neurotransmitters and modulatory neuropeptides within each of the key cardiovascular nuclei and within the major regulatory neurons of the SNS (**Chapter 1**). The role of PACAP in central and peripheral cardiorespiratory function, the distribution of PACAP within the spinal cord and RVLM and the physiological effects observed in our laboratory, implicate PACAP in the central neural control of AP and vasomotor tone (Farnham *et al.*, 2008; Farnham & Pilowsky, 2009; Farnham & Pilowsky, 2010; Farnham *et al.*, 2011; Inglott *et al.*, 2011; Inglott *et al.*, 2012). The response observed following intrathecal PACAP injection is particularly paradoxical, with AP not rising in accompaniment with HR and SNA, which increase dramatically following intrathecal PACAP (Farnham *et al.*, 2008; Inglott *et al.*, 2011). Overall, this thesis aims to further investigate the role of PACAP within central tonic and reflex control of the cardiorespiratory system at the level of the spinal cord, with a particular interest in determining the mechanistic cause underlying the lack of AP response following intrathecal PACAP injection.

Specifically, the aims of this thesis are as follows;

Chapter 4: Intrathecal PACAP-38 causes increases in sympathetic nerve activity and heart rate but not blood pressure in the spontaneously hypertensive rat

1. To determine any difference in the proportion of RVLM catecholaminergic neurons containing PACAP mRNA in SHR and Wistar-Kyoto (WKY) rats.
2. To establish intrathecal dose response curves for PACAP-38 and the PACAP antagonist (PACAP(6-38)).
3. To test whether the spinal PACAP receptors differed in number or activity between hypertensive and normotensive rat models by investigating responsiveness to intrathecal injection of PACAP-38.

4. Intrathecal pre-treatment with PACAP(6-38) was used to determine whether the effects seen on mean arterial pressure (MAP), HR and SNA with PACAP-38 were attributable to effects at PAC_1 and $VPAC_2$.

5. Intrathecal injection of PACAP(6-38) was used to investigate a possible role for tonic activation of PAC_1 and $VPAC_2$ in SHR and WKY rats.

   (**Appendix 1**, Farnham *et al.,* 2011)

Chapter 5: Intrathecal PACAP-38 causes prolonged widespread sympathoexcitation via a spinally mediated mechanism and increases basal metabolic rate in the anaesthetised rat

1. To determine the cardiovascular effects of intrathecal PACAP-38 administration.

2. To investigate whether or not intrathecal PACAP, differentially or globally, affects sympathetic outflows by recording from multiple sympathetic nerves.

3. To determine whether the responses to intrathecal PACAP are due to a spinally mediated mechanism by intrathecal injection of PACAP-38 in C1 spinally transected rats.

4. To determine whether intrathecal PACAP affects metabolic function through the measurement of end-tidal CO_2, pH, $PaCO_2$, PaO_2, core temperature, and glucose levels.

   (**Appendix 1**, Inglott *et al.,* 2011)

Chapter 6: Activation of PAC_1 and VPAC receptor subtypes elicits differential physiological responses from sympathetic preganglionic neurons in the anaesthetised rat

1. To determine the involvement of PAC_1 in the intrathecal PACAP response with maxadilan (PAC_1 selective agonist).

2. To determine the combined involvement of $VPAC_1$ and $VPAC_2$ in the response to intrathecal PACAP using VIP ($VPAC_1$ and $VPAC_2$ selective agonist).

3. To determine the sole involvement of $VPAC_1$ in the intrathecal PACAP response with PACAP(6-38), the PAC_1 and $VPAC_2$ antagonist and PACAP

4. To investigate the role of PAC_1 and $VPAC_2$ in tonic MAP control in the Sprague-Dawley (SD) rat using PACAP(6-38).

(**Appendix 1**, Inglott *et al.,* 2012)

Chapter 7: Catestatin has an unexpected effect on the normally isotensive peptide PACAP, causing a dramatic fall in blood pressure

1. To determine the role of catestatin in central cardiorespiratory modulation of the intrathecal PACAP-38 response, and to determine if co-release of PACAP and catestatin within the circuitry controlling sympathetic vasomotor tone may be critical in mediating essential hypertension.

2. To determine the separate effects of intrathecal catestatin and PACAP-38 on basal cardiorespiratory parameters and reflex control of the cardiovascular system.

(**Appendix 1**, Gaede *et al.,* 2012)

Chapter 3

General Methods

Chapter 3: General Methods

Chapter 3: General Methods

This chapter describes the common methods used in the subsequent results chapters. Please refer to the methods section of each results chapter (**Chapters 4, 5, 6** and **7**) for the specific methods used in the individual studies. Suppliers of equipment, chemicals/reagents, drugs, software and other materials used in this thesis are listed in **Appendix 2**.

3.1 Animal preparation

3.1.1 Ethics approval

All experiments were carried out with strict accordance to the guidelines set in the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (NSW Animal Research Act 1985) and were approved by the Animal Care and Ethics Committee of Macquarie University (protocol numbers: 2007/022, 2008/015, 2009/011 and 2010/055; see **Appendix 3**). Experimentation was reviewed annually by the Animal Care and Ethics Committee of Macquarie University and progress was subject to approval by the committee. All experiments were also approved by the Macquarie University Biosafety Committee (protocol number: ASAM/SOP/002; see **Appendix 3**).

3.1.2 Animals

Male Sprague-Dawley (SD) rats, weighing between 350g and 550g, were used in the majority of this work. Wistar, Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR; 350g-550g) were used in **Chapter 4** only. SHR and WKY rats were age matched and used after 18 weeks of age, including phenotyping (**section 3.11.1**). All animals were supplied by Animal Resources Centre, Perth, Australia, a registered breeding facility.

The animals were housed at the Macquarie University Animal House in high-top cages (n ≤ 6) with environmental enrichment and access to food and water *ad libitum*. The environment was conditioned to have fixed daily 12 hour light/dark cycles and temperature was maintained at 21°C ± 2°C.

3.2 Pre-operative procedures for electrophysiological experiments

Individual rats were collected from the Macquarie University Animal House on the day of the experiment and transported to the laboratory. Animals were handled carefully to minimise stress prior to anaesthesia and surgery.

3.2.1 Anaesthesia

Anaesthetic administration procedures were designed and conducted to minimise stress to the animal and avoid injury. All experiments were performed under surgical anaesthesia; surgical procedures were not commenced until adequate anaesthetic depth was achieved. Surgical anaesthesia was determined in the unparalysed condition by the absence of reflex responses to nociceptive stimuli (e.g. paw/tail pinches), or alternatively, by the absence of the corneal touch reflex. In the paralysed condition, anaesthetic depth was confirmed by a mean arterial pressure (MAP) rise of <10 mmHg in response to noxious paw/tail pinch. All rats were anaesthetised with urethane (ethyl carbamate). Urethane provides long-lasting, stable surgical anaesthesia with a large therapeutic window and with minimal cardiorespiratory depression, allowing for stable nerve recordings (Field *et al.*, 1993).

Surgical anaesthesia was induced via intraperitoneal (i.p.) injection of urethane (1.3 g/kg; 10% w/v in 0.9% physiological saline) using a 26G needle. The injection was given in 2 bolus doses, separated by 20 minutes, to minimise the risk of anaesthetic overdose. This injection was supplemented with atropine sulfate (50 µg i.p.) to reduce bronchial secretions. Once anaesthetised, regions of the rat were shaved to expose skin for surgical incision sites. Temperature monitoring (see **section 3.2.2**) was also commenced at this time. Maintenance of urethane-induced anaesthesia during surgical procedures was achieved by additional intravenous (i.v.) doses of urethane, up to a total dose of 1.5 g/kg as required (i.e. positive reflex responses to paw/tail pinch or corneal touch, or a MAP rise of >10 mmHg in response to these stimuli). Sodium pentobarbital (3 g/kg; 10% w/v in 0.9% physiological saline i.v.) was administered if further anaesthetic was necessary. In some cases, skin incision sites were pre-treated with an injection of the local anaesthetic Xylocaine (1% w/v in 0.9% saline).

3.2.2 Temperature monitoring

Core body temperature was monitored throughout surgery using a rectal probe thermometer. Temperature was maintained between 36.5°C and 37.5°C by a thermoregulated heating pad, and if required, with an infra-red lamp. Core temperature was recorded during all experiments, but is only reported in **Chapter 5**.

3.3 General surgical procedures for electrophysiological experiments

3.3.1 Arterial and venous cannulations

In all experiments cannulation of the right common carotid artery and jugular vein were performed to enable the recording of arterial blood pressure (AP) and the administration of fluids and drugs, respectively. These vessels were exposed by a ventral midline incision from the mandible to the supra-sternal notch, and by dissection through the superficial fascia and deeper encasing muscles. Each vessel was isolated from surrounding tissues, and a silk suture (5/0) was tied distally to occlude blood flow. Occlusion of blood flow in the artery was further assisted with the use of an artery clip at the proximal end of the isolated artery. A small incision was then made in the vessel wall to allow insertion of a polyvinyl chloride cannula (PVC; OD: 0.96 mm x ID: 0.58 mm). Cannulas were prepared using PVC tubing attached to an 18G needle and 3-way tap using silastic tubing. Cannulas were advanced ~1 cm towards the heart and secured in place with silk sutures (5/0). The venous cannula was filled with 0.9% physiological saline, while the arterial cannula was filled with heparinised (~10 IU heparin/ml) 0.9% saline to prevent blood clotting in the cannula.

3.3.2 Tracheotomy

A tracheotomy was performed on all animals to ensure airway patency and to facilitate artificial ventilation. The trachea was exposed through the ventral midline incision made for the cannulations, by dissection through superficial fascia and deeper encasing/insulating laryngeal muscles. A small incision was made in the trachea below the larynx, to allow insertion of the plastic sheath from a 14G catheter. The catheter sheath was secured in place with braided silk sutures (2/0). Rats then continued to breathe freely until such time that a bilateral vagotomy was performed (see **section 3.3.3**), and mechanical ventilation commenced (see **section 3.3.4**). **Figure 3.1** illustrates the procedures and cannulations required for experiments.

3.3.3 Vagotomy

A bilateral vagotomy was performed in all experiments to abolish the input of the parasympathetic nervous system. This creates a 'purely' sympathetic system and eliminates afferent feedback from slowly adapting lung receptors during mechanical ventilation. The right vagus nerve was separated from the carotid sheath and transected at the time of carotid artery cannulation. The left vagus nerve was isolated from the carotid bundle and transected either after the tracheotomy, or after phrenic nerve or cervical sympathetic nerve dissection (see **section 3.3.5**), depending on the nerves chosen for recording in a single experiment. Once located, the left vagus nerve was transected only after all surgery was complete, and the rat was ready for artificial ventilation. Following the vagotomy all animals were secured in a stereotaxic frame.

3.3.4 Ventilation

Immediately following vagotomy, all animals were artificially ventilated with oxygen (100% O_2)-enriched room air using a rodent respirator connected to the tracheal cannula. The volume (1-1.2 ml/100g) and rate (60-80 cycles/second) of the respirator were then adjusted. To ensure correct ventilation, end-tidal CO_2 was monitored and maintained between 3.5-4.5%, with a CO_2 analyser connected to the expiratory ventilator line, close to the rat.

In some cases, the arterial pH was also monitored and maintained around 7.4 by intermittent blood gas analysis. The normal ranges for measured variables in an arterial blood gas analysis were; pH (7.35-7.45), PaO_2 (>200 mmHg as ventilated with hyperoxic/room air mixture), $PaCO_2$ (35-45 mmHg) and HCO_3 (22-28 mM). Following arterial blood gas analysis, ventilation parameters (rate and/or volume) were modified accordingly, if necessary to keep blood gas and electrolyte concentrations within their optimum ranges. In some cases, blood collected for arterial blood gas analysis was also used to perform blood glucose measurements. See **Chapter 5** for further information regarding the sampling of arterial blood for blood gas and blood glucose analysis, and data recorded from this analysis.

Following the commencement of mechanical ventilation, all animals were paralysed by i.v. administration of a 0.4 ml (0.8 mg) bolus of the neuromuscular

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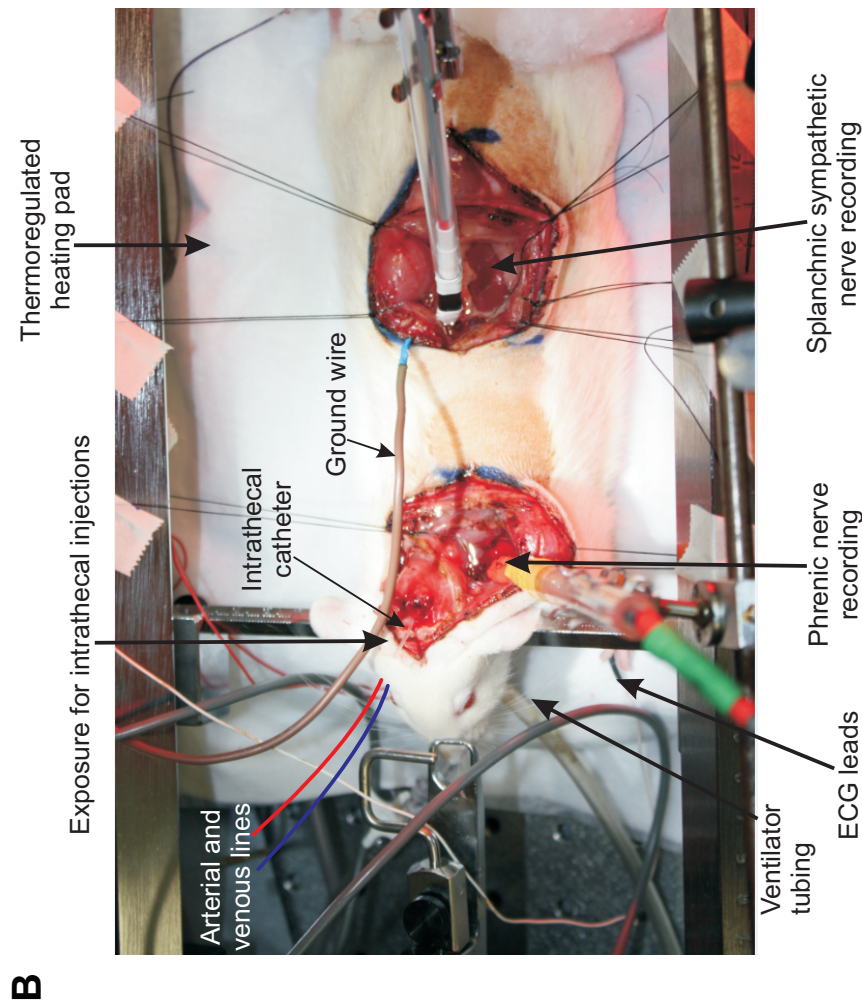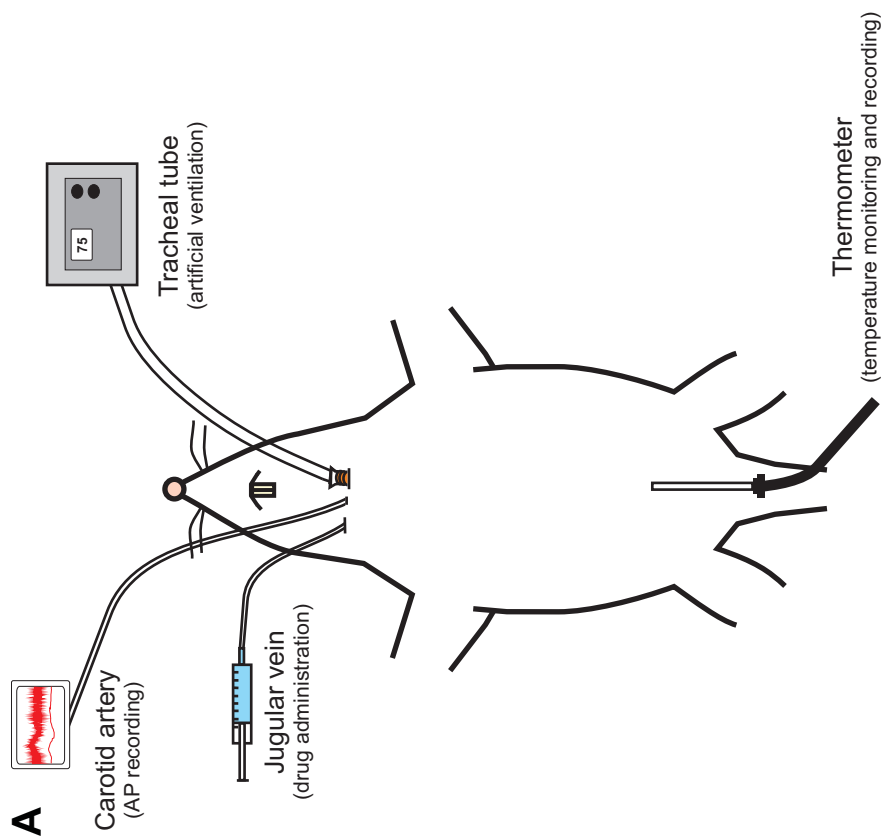
Figure 3.1 Surgical Preparation

An illustration (**A**) and a photograph (**B**) demonstrating the ventral and dorsal views of the general surgical preparation used in electrophysiological studies. In each experiment, the rat is secured in a stereotaxic frame, the arterial (red) and venous (blue) cannulas enable the recording of AP and the administration of fluids and drugs, respectively. The tracheal tube allows for artificial ventilation throughout the experiment. A rectal probe thermometer coupled to a thermo-regulated heating pad is used to maintain/record the core body temperature of the rat. Here, the phrenic and splanchnic sympathetic nerves are exposed, and positioned on silver bipolar electrodes for recording. An intrathecal catheter has been inserted at the atlanto-occipital junction, through the dura mater, for drug injection. Abbreviations: AP, arterial pressure; ECG, electrocardiogram.

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blocking agent, pancuronium bromide. This prevents muscle twitches and prevents the animal from becoming ventilator-entrained. Neuromuscular blockade was maintained with continuous infusion of 10% pancuronium bromide in a 5% w/v glucose in 0.9% saline solution, infused with an automated infuser at a rate of 2 ml/hr. Continuous infusion with fluids helped support hydration, energy production and electrolyte balance, along with maintaining neuromuscular blockade.

3.3.5 Nerve dissection for recording

Nerves isolated for recording included the left cervical sympathetic, phrenic, splanchnic sympathetic and lumbar sympathetic nerves (**Figures 3.1** and **3.2**). Sympathetic nerve recordings provide a good measure of sympathetic nerve activity (SNA) to a vascular bed; centrally mediated changes to SNA can be observed in these recordings. Vascular beds measured include; the head and neck (cervical sympathetic nerve activity; $_C$SNA), gastric region (splanchnic sympathetic nerve activity; $_S$SNA) and hind-limb musculature (lumbar sympathetic nerve activity; $_L$SNA). Respiratory activity was quantified by recording phrenic nerve activity (PNA). More specifically, phrenic nerve amplitude (PNAmp) and phrenic nerve frequency (PNf) were used as a measure of inspiratory drive. Note; different combinations of nerves were recorded in different experiments; see **Chapters 4, 5, 6** and **7** for details of the nerves isolated in each case.

3.3.5.1 Cervical sympathetic nerve

The left cervical sympathetic nerve was approached dorsally with a midline incision from the top of the skull to the mid thoracic level. Muscles lying between the scapula and the midline vertebrae were cauterised and separated, and the carotid artery was exposed. The cervical nerve, found running along the carotid artery, within the carotid sheath, was carefully separated from surrounding fascia, tied with 5/0 silk sutures caudal to the cervical ganglion and transected rostral to the tie. The nerve was then covered with saline-soaked cotton wool until required for recording.

3.3.5.2 Phrenic nerve

The left phrenic nerve was exposed with the same dorsal approach as used above (**section 3.3.5.1**). Lateral retraction of the scapula exposed the brachial plexus; superomedially directed blunt dissection, distal to the brachial plexus, exposed the

phrenic nerve. The phrenic nerve was carefully separated from surrounding fascia, tied distally with 5/0 silk sutures and cut distal to the tie. The nerve was then covered with saline-soaked cotton wool until required for recording.

3.3.5.3 Splanchnic and lumbar sympathetic nerves

A retroperitoneal/dorsolateral approach was used to expose these nerves via a midline incision from the lower thoracic level to the pelvis. The underlying muscle layers were cauterised and the fascia was separated by blunt dissection to expose the retroperitoneal cavity. The muscles and skin were retracted by ties to expose the aorta in the midline, and the left kidney and adrenal gland. The celiac ganglion, and then the splanchnic nerve were located. The left lumbar postganglionic fibres were identified by their characteristic diagonal crossing of the aorta caudal to the renal artery; these fibres were then tracked back to the sympathetic chain, to ensure their origin was correct. The fibres of the sympathetic nerves were carefully separated from surrounding fascia, isolated, tied distally with 5/0 silk sutures and cut distal to the tie. Both nerves were protected with saline soaked cotton wool prior to recording.

3.4 Additional surgical procedures for electrophysiological experiments

In some cases, the following surgical procedure was performed in addition to the general surgical procedures described in this Chapter.

3.4.1 Surgical spinal transection

C1 surgical spinal transection was completed by removal of the overlying back muscles, exposure and laminectomy of the C1 vertebra to expose the corresponding spinal segment. The dura was removed and the spinal cord was then carefully transected. Please refer to **Chapter 5** for further details on this surgical method, and for details regarding verification of complete/successful surgical spinal transection.

3.5 Electrophysiological recordings

All signals were digitised (A/D Converter 1401 Cambridge Electronic Design Ltd., UK) and recorded in real time on a computer using Spike2 acquisition and analysis software (Cambridge Electronic Design Ltd., UK). **Figure 3.2** demonstrates the set-up used to record MAP, heart rate (HR), end-tidal CO_2 and SNA.

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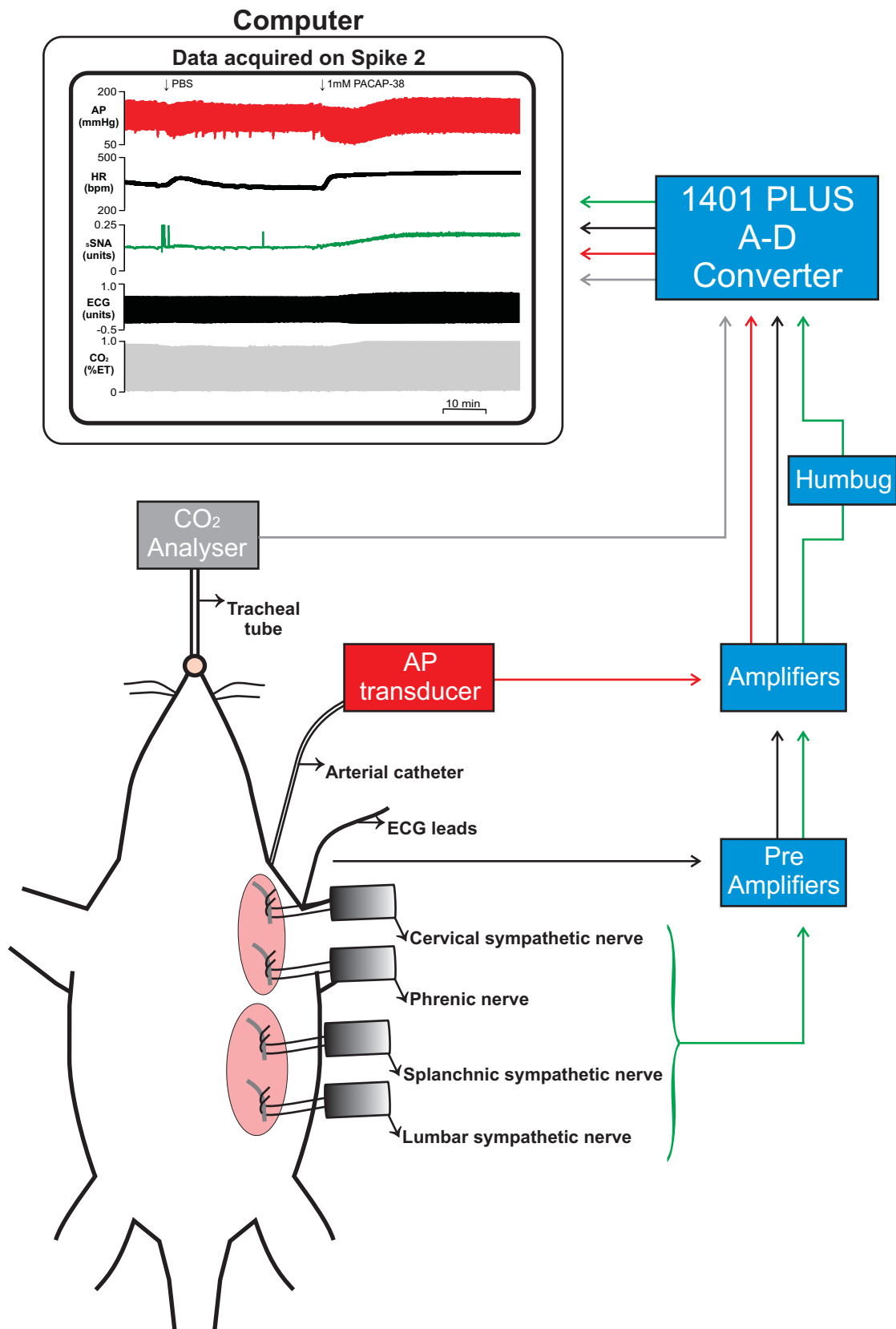
Figure 3.2 Electrophysiological recordings
Schematic representation of electrophysiological recordings. Here we show how MAP, ECG, SNA, PNA and end tidal CO_2 are recorded, amplified, digitised and displayed. Original trace shows the MAP, HR, sSNA, ECG and end tidal CO_2 responses to an intrathecal injection of PBS and 1mM PACAP-38. NB: Core temperature is recorded in the same way as end tidal CO_2. Abbreviations: AP, arterial pressure; ECG, electrocardiogram; HR, heart rate; PACAP, pituitary adenylate cyclase activating polypeptide; PBS, phosphate buffered saline; PNA, phrenic nerve activity; SNA, sympathetic nerve activity; sSNA, splanchnic sympathetic nerve activity.

3.5.1 Arterial pressure and heart rate recordings

The arterial cannula was connected directly to a pressure transducer, which converted AP to an analogue signal. This signal was amplified 1000 fold by a bridge amplifier, digitalised and recorded on computer using Spike2 software at a sampling rate of 200 Hz. The pressure transducer was periodically, manually calibrated with a sphygmomanometer to ensure accuracy.

Electrocardiogram (ECG) was recorded using a custom made three-lead sliver needle electrode. The ground lead was inserted into exposed muscle, as close as possible to nerve recording sites, while the positive and negative leads were inserted into the front paws of the rat. The ECG signal was amplified 10-fold by a preamplifier, band pass filtered between 1 and 100 Hz and amplified a further 1000 times by a scaling amplifier (Bioamplifier). This signal was then digitalized, sampled at a rate of 1 kHz and displayed/recorded as a waveform using Spike2 software. HR was then derived on Spike2 as an event channel, triggered by the QRS complex of the ECG or the systolic peaks in AP.

3.5.2 Nerve recordings

Upon completion of surgery, the nerves were prepared for recording as follows. A well was created for each nerve by securing the surrounding tissues and skin to the stereotaxic frame. Custom built, silver wire, bipolar recording hook electrodes were carefully positioned in the wells, and the isolated nerves (see **section 3.3.5**) were laid/suspended across the two poles of the electrodes. The wells were then filled with paraffin oil or Silgel, creating a hydrophobic medium that electrically isolated the nerve. Nerve recordings were grounded by the ECG grounding wire (see **section 3.5.1**) and were improved by several steps taken to minimise electrical nose. These include: pre-amplification close to the signal, passing signals via a 50/60 Hz line frequency filter ('Humbug') and by grounding/earthing of electrical/metal equipment. The recorded nerve activity was amplified 10-fold by a preamplifier, then band pass filtered (100 Hz-2 kHz) and amplified a further 1000 times by a scaling amplifier (Bioamplifier), 50/60 Hz line frequency filtered, digitalised, sampled at a rate of 2 kHz, and recorded/displayed as a waveform on a computer using Spike2 software (in arbitrary units or in μV).

Following the commencement of recordings, physiological functioning of the sympathetic nerves was tested by hypoxic challenge (induced by turning the ventilator off for 10 seconds, stimulating a breath hold), or by activating the baroreceptor reflex using vasoactive drugs, (phenylephrine (PE; 10 µg/kg) and/or sodium nitroprusside (SNP; 10 µg/kg)) administered i.v. Waveform averages of all SNA and PNA recordings were generated by averaging 60 seconds of data triggered by the onset of the QRS complex of the ECG (SNA) or by the onset of the phrenic burst, also confirming the physiological functioning of the nerves by allowing the bursting pattern of the nerves to be viewed in real time.

3.5.3 End-tidal CO_2 and core temperature

End-tidal CO_2 was monitored by a CO_2 analyser, core temperature was monitored and maintained by a rectal probe thermometer connected to a thermoregulated heating pad. Both signals were then digitalised in real time, and recorded/displayed on computer using Spike2 software as a waveform.

3.6 Intrathecal drug administration

All experiments described in this body of work investigate the effects of pituitary adenylate cyclase activating polypeptide (PACAP) on cardiorespiratory parameters when injected into the intrathecal (subarachnoid) space. Following the surgical preparation described above, an intrathecal catheter was inserted into the intrathecal space.

3.6.1 Atlanto-occipital junction exposure and intrathecal catheterisation

A skin incision was made from the top of the skull to the level of T2. The overlying occipital musculature and connective tissue were exposed and removed, while carefully controlling any bleeding. The muscle layer directly attached to the occipital bone was removed via gentle scraping to reveal the occipital bone and to expose the atlanto-occipital membrane (dura mater and the underlying arachnoid mater). A slit was then made in the membrane overlying the fourth ventricle, using a custom made fine scalpel (26 G needle attached to a long-stemmed cotton bud). Leakage of cerebrospinal fluid from the slit indicated that the membrane was completely cut and that the correct space was found. A catheter (PVC; OD: 0.61 mm x ID: 0.28 mm), with a dead space of ~6 µl, was cut to the appropriate size by

measuring the length between the membrane and T6 (identified by counting the spinous processes). A small piece of silastic tubing was attached to the external end of the catheter to allow for syringe attachment. The catheter was then inserted into the intrathecal space through the slit in the atlanto-occipital membrane and advanced caudally to the level of T5/6. Positioning of the catheter within the intrathecal space was confirmed by drawing cerebrospinal fluid up the catheter with a glass Hamilton syringe.

3.6.2 Intrathecal drug administration protocol

All intrathecal injections were made using a 25 µl glass Hamilton syringe connected to the catheter tubing. Volumes of 10 µl, washed in by 6 µl of 10 mM phosphate buffered 0.9% saline (PBS), were injected. All injections were done over a 10 to 15 second period. All rats received control injections of the vehicle, PBS (10 µl of PBS washed in with 6 µl PBS), 30 minutes prior to their drug treatments. Drugs were dissolved in 10 mM PBS, aliquoted and immediately frozen at -20°C until needed. Drugs injected into the intrathecal space are listed in **Table 3.1**.

Table 3.1 Drugs injected into the intrathecal space

Drug	Description	Concentration	Volume
PBS	Vehicle	10 mM	10 µl
PACAP-38	Agonist at the PAC_1, $VPAC_1$ and $VPAC_2$ receptors	0.03, 0.1, 0.3, 1 mM	10 µl
Vasoactive intestinal polypeptide (VIP)	Agonist at the $VPAC_1$ and $VPAC_2$ receptors	0.03, 0.1, 0.3, 1 mM	10 µl
Maxadilan	Agonist at the PAC_1 receptor	0.03, 0.1, 0.3, 1 mM	10 µl
Catestatin	Acetylcholine nicotinic receptor antagonist and β-adrenergic receptor antagonist	0.1 mM	10 µl
PACAP(6-38)	Antagonist at the PAC_1 and $VPAC_2$ receptors	0.1, 0.3, 1 mM	10 µl
M65	Antagonist at the PAC_1 receptor	1, 3 mM	10 µl
[D-p-Cl-Phe^6, Leu^{17}] - VIP	Antagonist at the $VPAC_1$ and $VPAC_2$ receptors	0.03, 0.1, 0.3, 1 mM	10 µl

Refer to **Appendix 2** for company/supplier information. Note; PACAP-38, and not PACAP-27, was chosen for use in this body of work as PACAP-38 is the predominant form of PACAP found in the central nervous system (Arimura *et al.*, 1991).

Specific drug treatments given in experiments varied depending on the study and its aims; please refer to **Chapters 4, 5, 6** and **7** for the specifics of the treatments used in each study. Dose response curves were constructed for PACAP, PACAP(6-38), Maxadilan and VIP; please see **Chapters 4** and **6** for details regarding dose response curve construction and results.

3.7 Activation of sympathetic reflexes

3.7.1 Baroreceptor reflex

Stimulation of the sympathetic baroreflex is used to measure the ability of an organism to respond to instantaneous changes in AP, and by extension it is used as an indicator of cardiovascular health (see **section 1.5.5.1**). Sympathetic baroreflex sensitivity was assessed chemically, using 0.1 ml bolus i.v. injections of PE (10 µg/kg), in conjunction with 0.1 ml bolus i.v. injections of SNP (10 µg/kg) to generate baroreflex curves. PE is an α1-adrenergic receptor agonist that induces a rapid increase in AP due to vasoconstriction, causing a reflex decrease in SNA. SNP is a nitric oxide (NO^-) donor that induces a rapid decrease in AP due to vasodilatation, causing a reflex increase in SNA. AP and SNA return to baseline within 2-3 minutes. The baroreflex was repeated 2 to 3 times during the control and experimental periods to obtain an average response. Each repeat of the challenge was conducted only after all measures had returned to baseline (pre-challenge) levels (**Chapter 7**).

3.7.2 Chemoreceptor reflex

The hypoxic chemoreflex is used to demonstrate alterations in cardiovascular responsiveness to changes in PaO_2 (see **section 1.5.5.2**). Peripheral chemoreceptors were activated by ventilating with 100% N_2 for 12 seconds (hypoxia). This was repeated 2 to 3 times during the control and experimental periods to obtain an average response. Each repeat of the challenge was conducted only after all measures had returned to baseline (pre-challenge) levels (**Chapter 7**).

3.8 Euthanasia and confirmation of injection site

At the conclusion of the experiments, all rats were euthanised using 0.5ml of 3M potassium chloride (KCl) i.v. This dose was sufficient to instantly stop the heart.

Recordings of all parameters were continued for at least 5 minutes after death to obtain the background level of noise (death level) from the nerve recordings.

Postmortem confirmation of the location of the intrathecal catheter was achieved by injecting 10μl of India ink washed in with 6μl of PBS into the intrathecal catheter and then exposing the spinal cord. The spinal segment level of the catheter was recorded as the level where the tip of the catheter was observed or where the blue/black spot appeared most intensely on the spinal cord. Data failing to meet the T5/6 anatomical criteria were excluded from analysis.

3.9 Data analysis

Data was analysed off-line using Spike2 software and was either entered into Microsoft Excel for further calculations to be performed, or was exported directly into GraphPad Prism software. Data analysis varied depending on the experiment; please refer to **Chapters 4, 5, 6** and **7** for the specifics of the data analysis used in each study.

3.9.1 Mean arterial pressure

AP was always analysed *post hoc* as MAP. MAP was obtained by applying a 1 second smoothing function to the raw AP trace and analysed in two ways. Reflex (fast) responses were recorded as a peak change from a 5 minute averaged baseline. Slow/sustained responses were analysed by averaging 5 minute blocks of data every 10 minutes, up to 60 minutes, and every 30 minutes thereafter, or by averaging 1 minute blocks of data every 10 minutes for the duration of the experimental period (up to 180 minutes).

3.9.2 Heart rate, end-tidal CO_2 and core temperature

HR was derived from the ECG recording and displayed as beats/minute (see **section 3.5.1**). Both HR and the digitised CO_2 waveform (see **section 3.5.3**) were analysed in the same way as AP, using peak changes and 1 or 5 minute averages.

3.9.3 Sympathetic nerve activity

All raw SNA data (2 kHz, 1k-100k x gain, 0.1-2 kHz filtering, 50/60 Hz line frequency filtered) was rectified and smoothed/averaged to 2 seconds. This trace of

averaged SNA was normalised to the residual death level activity (noise) 5 minutes after death, and then analysed in the same manner as MAP and HR.

3.9.4 Sympathetic reflexes

The sympathetic reflexes evoked by hypoxic chemoreflex challenge and vasoactive drug baroreflex challenge were compared before and after intrathecal drug administration. In all cases with reflex analysis, $_s$SNA and MAP were analysed from their respective averaged traces, as derived above, both traces were smoothed/averaged to 1 second for reflex analysis.

3.9.4.1 Baroreceptor reflex

Baroreflex curves ($_s$SNA versus MAP) were plotted using a first order (linear) polynomial ($Y=B_0 + B_1*X$) fitted to the steepest portion of the curve. The slope value (B_1) was used to estimate and compare sympathetic barosensitivity.

3.9.4.2 Chemoreceptor reflex

A Boltzmann sigmoid curve was fitted to the MAP and $_s$SNA responses to hypoxic challenge in order to measure chemosensitivity. The range and slope of the curves after vehicle and drug treatment (catestatin, PACAP, or catestatin+PACAP) were analysed.

3.9.5 Phrenic nerve activity

PNA raw data (2 kHz, 1k-100k x gain, 0.1-2 kHz filtering, 50/60 Hz line frequency filtered) was rectified and smoothed/averaged to 1 second. From this trace of averaged PNA, the following phrenic variables were measured; PNf and PNAmp. This averaged trace was normalised to the residual death level activity (noise) 5 minutes after death. PNAmp was measured directly from this trace, PNf was derived from the un-normalised averaged PNA trace; PNf was triggered by the onset of the phrenic burst, in the same way HR was derived from the QRS complex of the ECG recording (see **section 3.5.1**). Both PNAmp and PNf were then analysed in the same manner as MAP, HR and SNA, using peak changes and 1 minute averages.

3.10 Statistical analysis

All statistical analysis and graphical representations were carried out using GraphPad Prism software. Graphs were aesthetically altered in Corel Draw software for presentation in figures. Statistical analysis varied depending on the experiment, but included the use of paired and unpaired Student's t-tests, one- and two- way ANOVAs, along with various *post hoc* tests, to compare baseline, control and drug responses. All data are expressed as mean ± standard error of the mean (SEM); results are displayed as either vehicle control versus drug treatment, or as a treatment pre-stimulus versus post-stimulus. In all cases, responses and differences were considered statistically significant if $P < 0.05$. Please refer to **Chapters 4, 5, 6** and **7** for the specifics of the statistical analysis used in each study.

3.11 Additional methods

The following procedures were not performed by the candidate and are included for completeness, with respect to papers published or manuscripts in preparation.

3.11.1 Phenotype validation of SHR and WKY

Systolic blood pressure phenotype was determined by tail cuff plethysmography for the SHR and their normotensive controls, the WKY rats, before experimentation. This non-invasive technique works by detecting the return of peripheral blood flow following tail artery occlusion (Bunag & Butterfield, 1982).

Rats were transported to the laboratory and then left undisturbed for a minimum of 1 hour, under a heating lamp, in order to reduce stress levels and ensure accuracy of measurements. An individual rat was removed from the cage and allowed to crawl into a cone-shaped piece of material; the rat was placed on a heating blanket while still in the material cone. An inflatable cuff (sphygmomanometer, attached to a blood pressure transducer) was applied to the base of the tail to occlude peripheral blood flow in the conscious rats, and systolic blood pressure measurements were taken. Rats were then returned to the Macquarie University Animal House for a minimum of 1 day prior to any further experimentation that included; electrophysiological methods (described above) or molecular biology procedures (described below).

The criterion required was a systolic blood pressure ≥150 mmHg (hypertension) in the SHR and a systolic blood pressure ≤140 mmHg (normotension) in the WKY. All animals used in further study met these criteria. See **Chapter 4** for further details regarding this methodology and the systolic blood pressure sampling.

3.11.2 Anaesthesia, transcardial perfusion and tissue preparation for molecular biology procedures

Rats perfused for *in situ* hybridisation (ISH) and immunohistochemistry (IHC) studies were anaesthetised (overdose) with sodium pentobarbital (80 mg/kg i.p.) injections. Once anaesthetised, rats were transcardially perfused through the left ventricle/ascending aorta to allow for tissue fixation and harvesting for use in combined ISH/IHC studies. An 18G drawing up needle connected to a peristaltic pump was inserted through the left ventricle and advanced into the ascending aorta. Heparin (1 ml of 5000 IU/ml) containing 10% sodium nitrate was administered to simultaneously prevent coagulation and cause vasodilatation. Rats were then perfused with ~300 ml of ice cold saline (0.9% w/v, pH 7.4), the right atria was cut to allow blood and fluid to exit the system. Once the fluid exiting the right atria was clear, saline perfusion was ceased, this was followed by ~400 ml of ice cold 4% paraformaldehyde in PBS (pH 7.4) for fixation. The brainstem was then removed and stored in the same fixative overnight at 4°C on an orbital shaker.

Once fixed and ready for processing, the brainstem was rinsed in PBS. The meninges were removed, the brainstem was cut down to size and then mounted on the stage of a vibrating microtome with 1.7% agar. Brainstems were sectioned coronally (40 μm) and collected sequentially into four containers containing PBS with 0.1% Tween-20 (PBT). Sections were then used in combined ISH/IHC studies.

3.11.3 *In situ* hybridisation for PACAP mRNA combined with immunofluorescence

Sense and antisense probes for PACAP mRNA were synthesised. A DNA fragment of the PACAP gene was amplified by polymerase chain reaction (PCR) from rat brain cDNA using forward and reverse primers with SP6 and T7 promoters attached at the 5'end, respectively (PACAP-ISH f:GGATCCATTTAGGTGACACT-ATAGAAGTTACG-<u>ATCAGGACGGAAACC</u>; PACAP-ISH r: GAATTCTAATA-

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CGACTCACTATAGGGAGATGC-<u>ACGCTTATGAATTGCTC</u>). PCR was run in the Hybaid PCR Express Gradient Thermal Cycler. The PCR products showed a single band of the correct size when run on a 2% agarose gel, and the purified PCR products were then directly purified using the QIAquick PCR Purification Kit or purified from the excised gel band using the QIAquick Gel Extraction Kit. The purified PCR products were subjected to automated sequencing at the DNA Sequencing Facility in the Department of Biological Science, Macquarie University. The output sequence was then aligned with the template mRNA sequence, and a 100% match of the two sequences confirmed the specificity of the designed primers. Riboprobe synthesis could then proceed.

Sense and anti-sense riboprobes were transcribed *in vitro* using digoxigenin-11-UTP and an Sp6 or T7 RiboMAX large scale RNA production system. The specificity of the riboprobe was confirmed with a dot blot and by running it on a 1.2% (w/v) agarose and 0.67% (w/v) formaldehyde gel, which showed a single band of the correct size.

For combined ISH and fluorescence IHC, free-floating sections of rat brain were pre- hybridised for 3 h at 37°C in pre-hybridisation buffer (50% formamide, 5 x sodium citrate (SSC), pH 7.0, 250 µg/ml herring sperm DNA, 100 µg/ml yeast tRNA, 100 µg/ml heparin, 5% dextran sulphate, 1 x Denhardt's solution, 0.1% Tween-20) followed by 30 minutes at $58°$C. The PACAP probe was then added to the buffer to a final concentration of 100 ng/ml and sections were incubated at 58°C overnight, on an orbital shaker. Subsequently, the sections were washed 2 x 30 minutes in 2 x SSC, 0.1% Tween-20 at 58° C, followed by 2 x 30 minutes in 0.1 x SSC, 0.1% Tween-20 at 58°C. To reveal digoxigenin-labelled (mRNA) neurons simultaneously with immunohistochemically labelled neurons, sections were rinsed in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, 0.1% Tween-20) 2 x 15 minutes at room temperature and incubated for 3 h in maleic acid buffer containing 2% Boehringer blocking reagent (BBR) and 10% normal horse serum. Primary antibodies were added to the buffer and incubated for 48 h at 4°C. Sections were then washed 3 x 30 minutes in TPBS buffer (Tris-HCl 10 mM, sodium phosphate buffer 10 mM, 0.9% NaCl, pH 7.4). Secondary antibodies were then added to the sections

in 1 ml of TPBS with 0.1% methiolate (TPBSm) with 2% normal horse serum and incubated overnight at room temperature. See table (**Table 3.2**) below for a complete list of primary and secondary antibodies used. To reveal digoxigenin-labelled neurons, the sections were then washed 3 x 1 h in maleic acid buffer with 2 mM levamisole. Following 2 x 15 minutes equilibration in the alkaline buffer NTMT (0.1 M NaCl, 0.1 M Tris-HCl, pH 9.5, 0.1 M $MgCl_2$, 0.1% Tween-20, 2 mM levamisole), a colourimetric reaction using nitroblue tetrazolium (NBT 4.5 µg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP 3.5 µl/ml) salts in NTMT revealed digoxigenin-labelled neurons (PACAP-containing neurons) as those containing dark purple precipitants. The reaction was stopped by washing 3 x 15 minutes in 1 x Stop Solution (0.1 M Tris-HCl, 1 mM ethylenediamine tetraacetic acid [EDTA], pH 8.5). Sections were mounted sequentially on glass slides, cover-slipped with Vectashield and sealed with nail polish.

Viewing, image capturing and counting was conducted on a Zeiss Z1 epifluorescence microscope under both brightfield and fluorescence conditions. ISH labeling was visualised using brightfield. Tyrosine hydroxylase (TH)-immunoreactive (Cy3-labelled) neurons were visualised using a Cy3-4040B filter set. Images were captured in greyscale with an Axiocam MR3 digital camera. Pseudo-colouring was applied to the fluorescence images for better visualisation of distribution and colocalisation. The images were adjusted individually for brightness and contrast with Axiovision software to best reflect the appearance of the original images. Labelled cells were counted within the rostral ventrolateral medulla (RVLM) when a distinct cell body could be visualised. The RVLM was defined as a triangular area ventral to the nucleus ambiguus, medial to the spinal trigeminal tract, and lateral to the inferior olive or the pyramidal tracts. Bilateral cell counts were taken from the section with the rostral pole of the inferior olive (bregma 11.96 mm). Counts were expressed as total counts of single populations and also as percentages of populations for colocalisation studies. Calculations were performed in Microsoft Excel before being exported to GraphPad for statistical analysis and graphical representation. The proportion of PACAP mRNA-containing TH neurons within the RVLM was compared between the rat strains with a one-way ANOVA.

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Table 3.2 IHC primary antibodies and fluorescent secondary antibody

IHC primary antibodies		
Antibody	**Species raised in**	**Concentration**
Alkaline phosphatase conjugated anti-digoxigenin	Sheep	1:1000
Anti-tyrosine hydroxylase	Mouse	1: 2000
Fluorescent secondary antibody		
Antibody	**Conjugation**	**Concentration**
Donkey anti-mouse IgG	Cy3	1: 500

Refer to **Appendix 2** for company/supplier information

Chapter 4

Results I

Chapter 4: Intrathecal PACAP-38 causes increases in sympathetic nerve activity and heart rate but not blood pressure in the spontaneously hypertensive rat

Chapter 4: Intrathecal PACAP-38 causes increases in sympathetic nerve activity and heart rate but not blood pressure in the spontaneously hypertensive rat

The work in this chapter has been published (**Appendix 1**): Farnham MMJ, Inglott MA, & Pilowsky PM. (2011). Intrathecal PACAP-38 causes increases in sympathetic nerve activity and heart rate but not blood pressure in the spontaneously hypertensive rat. *Am J Physiol* **300,** H214-H222. Abbreviations and formatting have been edited from the published paper.

Contributions: Conception and design of experiments: MAI, MMJF, PMP; Experimental procedures: MAI (novel Sprague-Dawley experiments only), MMFJ (all other experiments); Analysis and interpretation of data: MAI, MMFJ, PMP; Manuscript preparation: MMFJ, MAI; Critical revision of manuscript: MAI, MMFJ, PMP.

4.1 Abstract

The rostral ventrolateral medulla (RVLM) contains presympathetic neurons that project monosynaptically to sympathetic preganglionic neurons (SPN) in the spinal cord, and are essential for the tonic and reflex control of the cardiovascular system. SPN directly innervate the adrenal medulla and, via postganglionic axons, affect the heart, kidneys and blood vessels, to alter sympathetic outflow and hence blood pressure. Over 80% of bulbospinal, catecholaminergic (C1) neurons contain pituitary adenylate cyclase activating polypeptide (PACAP) mRNA. Activation of PACAP receptors with intrathecal injection of PACAP-38 causes a robust, prolonged elevation in sympathetic tone. Given that a common feature of most forms of hypertension is elevated sympathetic tone, this study aimed to determine in the spontaneously hypertensive rat (SHR) and the Wistar-Kyoto (WKY) rat (normotensive control): 1) the proportion of C1 neurons containing PACAP mRNA; and 2) responsiveness to intrathecal PACAP-38. We further investigated whether intrathecal injection of the PACAP antagonist, PACAP(6-38), reduces the hypertension in the SHR. The principal findings are that: 1) the proportion of PACAP mRNA containing C1 neurons is not different between normotensive and hypertensive rats; 2) intrathecal PACAP-38 causes a strain dependent, sustained sympathoexcitation and tachycardia with variable effects on mean arterial pressure (MAP) in normotensive

and hypertensive rats and 3) PACAP(6-38) effectively attenuated the effects of intrathecal PACAP-38, but had no effect alone, on any baseline variables. This finding indicates that PACAP-38 is not tonically released in the spinal cord of rats. A role for PACAP in hypertension in conscious rats remains to be determined.

Keywords: blood pressure, sympathetic activity, SHR, heart rate, spinal cord

4.2 Introduction

The RVLM is crucial for the tonic and reflex control of the cardiovascular system (see **Chapter 1** and (Pilowsky & Goodchild, 2002; Guyenet, 2006) for review). RVLM presympathetic neurons are commonly defined as being inhibited following baroreceptor activation and having a spinal axon (Brown & Guyenet, 1985; Lipski *et al.*, 1995; Lipski *et al.*, 1996). Neurochemically, 60-80% of presympathetic neurons are 'C1' neurons (Lipski *et al.*, 1995; Stornetta *et al.*, 1999; Phillips *et al.*, 2001; Stornetta *et al.*, 2002), having all of the enzymes required for adrenaline synthesis. Presympathetic RVLM neurons project monosynaptically to SPN in the intermediolateral (IML) cell column of the spinal cord (**section 1.6.1**; Moon *et al.*, 2002; Oshima *et al.*, 2008). SPN, in turn, regulate the activity of the heart, kidneys, blood vessels and adrenal chromaffin cells, thereby determining sympathetic outflow and ultimately, blood pressure.

PACAP (**section 1.8**) is an excitatory 38-amino acid peptide originally identified in ovine hypothalamus (Miyata *et al.*, 1989) that also exists as a truncated 27 amino-acid form (Miyata *et al.*, 1990). The distributions of PACAP mRNA and PACAP peptide in the central nervous system (CNS) have been mapped using *in situ* hybridisation (ISH) and immunohistochemistry (IHC; **section 1.8.3**; Hannibal *et al.*, 1995; Hannibal *et al.*, 1997; Hannibal, 2002; Pettersson *et al.*, 2004; Dürr *et al.*, 2007). In particular, PACAP mRNA is present in over 80% of catecholaminergic RVLM neurons that project to the thoracic IML (Farnham *et al.*, 2008). PACAP elicits its effects via three G-protein coupled receptors, PAC_1, $VPAC_1$ and $VPAC_2$, which primarily act to increase adenylate cyclase activity (see (Vaudry *et al.*, 2000; Dickson & Finlayson, 2009; Vaudry *et al.*, 2009) for review). The PAC_1 receptor is specific for PACAP (Buscail *et al.*, 1990) and is the primary form in the CNS (Cauvin *et al.*, 1990; Kyeung *et al.*, 2004). Injection of PACAP-38 into the

intrathecal space has sympathoexcitatory effects (Lai *et al.*, 1997; Farnham *et al.*, 2008), although the pressor effects reported by Lai *et al.,* (1997) contrast with the findings of Farnham *et al.,* (2008) who reported no change in blood pressure.

A common feature associated with essential hypertension in human, is elevated sympathetic tone (**section 1.3.3**; Schlaich *et al.*, 2004). The SHR, which was developed in 1963 (Okamoto & Aoki, 1963), is a model of neurogenic or essential hypertension (Krieger, 1964) and is similar to essential hypertension in humans (**section 1.5.6**; Minson *et al.*, 1996; Ito *et al.*, 2000). Sympathetic tone is elevated in young SHR well before the onset of hypertension. Both the SHR and its genetic normotensive control, the WKY rat, were derived from the normotensive Wistar strain (Okamoto & Aoki, 1963). The WKY was the primary normotensive control for this study, with Sprague-Dawley (SD) rats having been studied previously (Farnham *et al.*, 2008). Where a significant difference in responses existed between the two inbred normotensive rat models (SD and WKY) in the present study, the outbred Wistar was also tested to determine if the observed differences had a genetic basis.

Here we test the hypothesis that increased spinal PACAP activity contributes to sympathoexcitation and hypertension in the SHR. The first aim of this study was to determine any difference in the proportion of RVLM catecholaminergic neurons containing PACAP mRNA in SHR and WKY rats. The second aim tested whether the spinal PACAP receptors differed in number or activity between hypertensive and normotensive rat models by investigating responsiveness to intrathecal injection of PACAP-38. Intrathecal injection of the PACAP antagonist, PACAP(6-38), was used to determine if the effects seen on MAP, sympathetic nerve activity (SNA) and heart rate (HR) with PACAP-38 were due to effects at the PAC_1 and the $VPAC_2$ receptors. Finally, injection of PACAP(6-38) was used to investigate a possible role for tonic activation of the PAC_1 and $VPAC_2$ receptors.

4.3 Methods

4.3.1 Animals

All procedures and protocols were approved by the Animal Care and Ethics Committee of Macquarie University. Experiments were conducted on adult male SD,

SHR, WKY and Wistar rats (350-500 g; Animal Resource Centre, Perth) in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

4.3.2 Tail-cuff blood pressure

The blood pressure phenotype of hypertensive and normotensive rats was confirmed at >18 weeks of age by tail cuff sphygmomanometry. Hypertension was defined as a tail-cuff systolic pressure ≥150 mmHg and normotension as ≤140 mmHg.

Briefly (see **Chapter 3** for greater detail), an inflatable cuff was placed around the base of the tail and a pressure transducer was secured over the tail artery, immediately caudal to the cuff. The signal from the pressure transducer was amplified, digitised and recorded on a computer (CED, Cambridge, UK; Spike 2). The cuff was inflated to a pressure sufficient to occlude the artery (~160 mmHg for WKY rats and ~200 mmHg for SHRs) then released. The highest tail cuff pressure, at which a pulse could be detected, was recorded as the systolic blood pressure. Measurements were repeated ≥5 times and averaged. All SHR in this study had a systolic blood pressure ≥150 mmHg (mean: 183 ± 2 mmHg; range: 171-195 mmHg) and the WKY had a systolic blood pressure ≤140 mmHg (mean: 124 ± 2 mmHg; range: 113-136 mmHg).

4.3.3 Combined *in situ* hybridisation and fluorescence immunohistochemistry

Experiments were conducted as described previously (Li *et al.*, 2005; Padley *et al.*, 2007; Farnham *et al.*, 2008). Three SHR and three WKY rats were examined to identify neurons in the RVLM that expressed PACAP mRNA and were also immunoreactive for tyrosine hydroxylase (TH), a marker for catecholaminergic neurons.

Rats were deeply anaesthetised with sodium pentobarbital (intraperitoneal (i.p.); 80 mg/kg) and perfused through the left ventricle/ascending aorta with heparinised 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer saline (PBS, pH 7.4). The brain was post-fixed overnight in the

same fixative. Brainstems were sectioned coronally (40 μm) and collected sequentially into 4 containers containing PBS with 0.1% Tween-20 (PBT).

Sense and antisense probes were synthesised using an amplified DNA fragment of the PACAP gene from rat brain cDNA using forward and reverse primers with SP6 and T7 promoters attached at the 5′ end, respectively (PACAP-ISH f: GGATCCATTTAGGTGACACTATAGAAGTTACG-ATCAGGACGGAAACC; PACAP-ISH r: GAATTCTAATACGACTCACTATAGGGAGATGC-ACGCTTA-TGAATTGCTC). Sense and anti-sense riboprobes were transcribed *in vitro* using digoxigenin-11-UTP and an Sp6 or T7 RiboMAX large scale RNA production system (Promega). The specificity of the riboprobe was confirmed with a dot blot and by running it on a 1.2% agarose gel which showed a single band.

For combined ISH (for PACAP mRNA) and fluorescence IHC (for TH), free-floating sections of rat brain were hybridised overnight before addition of the primary antibodies, alkaline phosphatase conjugated sheep anti-digoxigenin (1:1000, Roche, Australia) and mouse anti-TH (1:2000; Sigma Aldrich, Australia). After 48h, TH immunoreactivity was subsequently revealed by incubation overnight with Cy3-conjugated donkey anti-mouse IgG- (1:500, Jackson, USA). A colourimetric reaction using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) salts in NTMT (0.1 M NaCl, 0.1 M Tris.HCl, pH9.5, 0.1 M $MgCl_2$, 0.1% Tween-20, 2 mM levamisole) revealed DIG-labelled neurons as those containing dark purple precipitants. Sections were mounted sequentially on glass slides and cover-slipped with Vectashield (Vector Laboratories, Australia).

4.3.3.1 Imaging and analysis

All image capture and cell counts were conducted on an epifluorescence microscope (AxioImager Z1, Zeiss, Germany). The RVLM was defined as a triangular area ventral to the nucleus ambiguus, medial to the spinal trigeminal tract and lateral to the inferior olive or the pyramidal tracts. Bilateral cell counts were taken from the section with the rostral pole of the inferior olive (~Bregma -11.96 mm). The proportion of PACAP mRNA containing TH neurons within the RVLM was compared between SHR, WKY and SD with a one-way ANOVA.

4.3.4 Intrathecal administration of PACAP-38 and PACAP(6-38)

PACAP-38 was administered intrathecally to SHR, WKY and Wistar rats to investigate any differences in responsiveness to PACAP-38 between normotensive and hypertensive rat strains. The PACAP antagonist, PACAP(6-38) was administered intrathecally to investigate whether PACAP is tonically released in the spinal cord and if it contributes to the hypertension of the SHR. PACAP-38 was also administered intrathecally to SD rats 15 minutes after intrathecal PACAP(6-38) to determine the extent to which the antagonist was effective in attenuating the PACAP response. A PACAP-38 dose-response curve was constructed by infusing 10 µl of 100 and 300 µM (n = 3) concentrations in SD rats and comparing the results with the previously reported responses to a 1000 µM solution (Farnham *et al.*, 2008). An antagonist dose-response curve was constructed in a similar manner by administering 10 µl of 100 (n = 4), 300 (n = 3) and 1000 µM (n = 6) concentrations of PACAP(6-38) 15 minutes before administering 10 µl of 1000 µM (1 mM) PACAP-38.

4.3.4.1 Anaesthesia and surgical preparation

All rats (n = 12 SHR, n = 9 WKY, n = 7 Wistar, n = 16 SD) were anaesthetised with 10% urethane (1.0-1.5 g/kg; i.p.). Atropine sulphate (100 µg/kg; i.p.) was administered in the same injection to reduce bronchial secretions prior to vagotomy. Surgical level of anaesthesia was defined as the absence of any withdrawal reflex to nociceptive or tactile stimuli, such as tail pinch or corneal touch. Whilst under neuromuscular blockade, a rise in blood pressure (>10 mmHg) in response to a tail or paw pinch indicated a need for an anaesthetic top-up. Additional anaesthetic (30-40 mg intravenous (i.v.) urethane) was administered as required. Complete details of surgical preparation and data acquisition methods are as described previously (Farnham *et al.*, 2008).

Briefly, rats were secured in a stereotaxic frame and temperature was maintained at 37 ± 0.5°C using a rectal probe connected to a homeothermic heating blanket (Harvard Apparatus, USA). The right carotid artery and jugular vein were cannulated for the measurement of arterial blood pressure and administration of drugs and fluids, respectively. A tracheotomy was performed and the vagi cut to permit artificial ventilation. Electrocardiogram (ECG) leads were placed in forepaws to record HR. The left splanchnic sympathetic nerve was dissected, cut at the celiac

ganglion and prepared for recording (2 kHz sampling rate, 1k-100k x gain, 0.1-2 kHz filtering). The rat was artificially ventilated with O_2-enriched room air and paralysed with pancuronium bromide (0.4 mg given as a 0.2 ml bolus i.v., then an infusion of 20% pancuronium in 0.9% saline at a rate of 1 ml/h).

The atlanto-occipital junction was exposed and a catheter (polyvinylchloride tubing, id 0.2 mm; od 0.5 mm) with a dead space of ~6 µl was inserted into the intrathecal space through the slit in the dura and advanced caudally to the level of T5/T6.

4.3.4.2 Intrathecal drug administration protocol

A vehicle injection of 10 µl of 10 mM PBS was washed in with 6 µl of 10 mM PBS and MAP, HR and splanchnic sympathetic nerve activity ($_s$SNA) were recorded for 35 minutes. 10 µl of 1 mM PACAP-38 or PACAP(6-38) was then administered and flushed in with 6 µl of PBS. In the SD rats, 10 µl of 1 mM PACAP-38 was infused 15 minutes after injection of 10 µl of 1 mM PACAP(6-38). All injections were made over a 10-15 second period. MAP, HR and $_s$SNA responses were recorded for up to 125 minutes following the final drug injection. At the conclusion of the experiments the rats were euthanised with 0.5 ml of 3M potassium chloride (KCl, i.v.). Post-mortem verification of the location of the catheter tip was achieved by injecting 10 µl of India ink and recording the vertebral segments where both the tip of the catheter and the ink stain were observed.

4.3.4.3 Data acquisition and analysis

Prior to analysis, raw $_s$SNA (2 kHz sampling rate, 1k-100k gain, 0.1-2 kHz filtering; a 50/60 Hz line frequency filter (Humbug; Quest Scientific) was also used) was rectified and a 2 second smoothing function applied. This resulted in a trace of mean SNA that was analysed in the same manner as HR and MAP with an additional step of normalising the signal to "0" by subtracting residual activity 5 minutes after death. Five minute time periods of MAP, HR and $_s$SNA were analysed prior to, and following, intrathecal injections of PBS (up to 35 minutes) or PACAP-38/PACAP(6-38; up to 125 minutes).

Analysis was conducted with GraphPad Prism. Two-way ANOVA with repeated measures for time was used to examine the effects of drug over time. Two-way ANOVA with Bonferroni's correction was used to compare the drug responses between the strains for both PACAP-38 and PACAP(6-38) compared to PBS unless otherwise stated. A P value < 0.05 was taken as a significant difference.

4.4 Results

4.4.1 PACAP mRNA colocalisation with TH in the RVLM of normotensive and hypertensive rat models

In the SHR (n = 3), $85.1 \pm 1.5\%$ of TH-immunoreactive neurons within the RVLM (Bregma -11.96 mm) contained PACAP mRNA, compared with $89.3 \pm 1.5\%$ in the WKY (n = 3). Similarly, $84.4 \pm 4.2\%$ colocalisation of TH-immunoreactive and PACAP mRNA-containing neurons over the entire RVLM was reported in the SD rat (**Figure 4.1**; Farnham *et al.*, 2008). There was no difference in the proportion of PACAP mRNA containing TH-immunoreactive neurons between the three strains ($P > 0.05$) nor in the number of TH-immunoreactive neurons between the SHR (81 ± 13 neurons) when compared to the WKY (73 ± 7 neurons; $P > 0.05$).

4.4.2 Dose-response curves for PACAP-38 and PACAP(6-38)

Dose-response curves were generated for both intrathecal PACAP-38, and PACAP(6-38) followed by intrathecal PACAP-38, to determine the most effective dose for use in this study. The PACAP-38 dose-response curve was generated with 100 (n = 3), 300 (n = 3) and 1000 μM (n = 6) solutions (**Figure 4.2**). One-way ANOVAs of the peak responses revealed the 1000 μM concentration to be effective in significantly elevating HR (66 ± 9 bpm; $P < 0.0001$) and $_s$SNA ($93.2 \pm 26.5\%$; $P < 0.0001$), none of the concentrations tested significantly altered MAP. To test the effectiveness of the antagonist *in vivo*, a PACAP(6-38) dose-response curve was generated by administering 1 mM PACAP-38 15 minutes after 100 (n = 3), 300 (n = 3) and 1000 μM (n = 6) solutions of PACAP(6-38). The results were compared to SD rats that were only treated with 1000 μM intrathecal PACAP-38 (Farnham *et al.*, 2008). The 1000 μM dose of PACAP(6-38) was the only dose that attenuated the $_s$SNA (Δ -72%; $P < 0.05$) and HR (Δ -73%; $P < 0.05$) responses to 1000 μM intrathecal PACAP-38 significantly (**Figure 4.2**). Administration of PACAP(6-38)
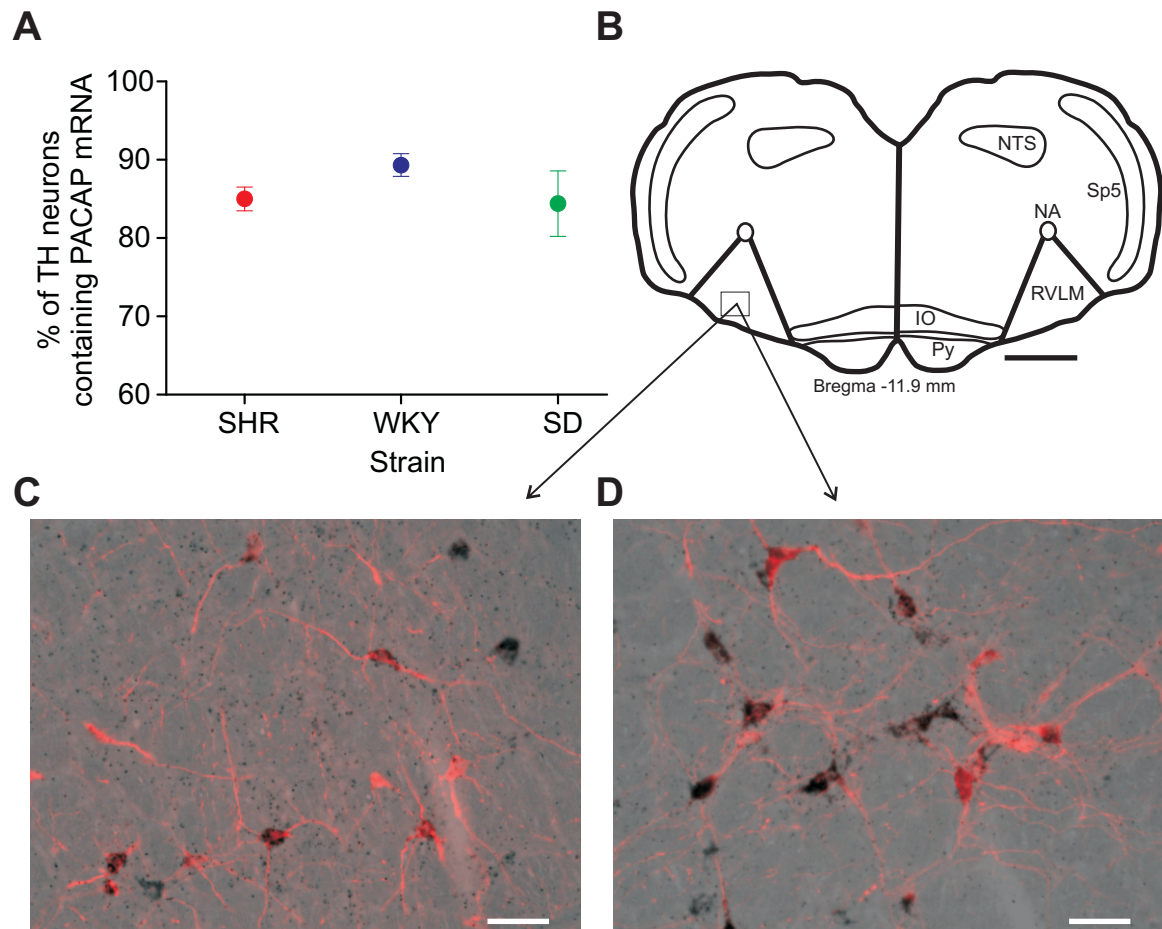
Figure 4.1 Proportion of PACAP mRNA containing TH-immunoreactive neurons in the RVLM of SHR and WKY rats

(**A**) The mean ± SEM proportion of PACAP mRNA containing TH-immunoreactive neurons in the RVLM at Bregma -11.96 mm is plotted for SHR (n = 3; red) and WKY rats (n = 3; blue). The mean ± SEM proportion of PACAP mRNA positive and TH-immunoreactive neurons over the entire RVLM (Bregma -11.6 to -12.6 mm) is plotted for the SD rat (n = 3; Farnham et al., 2008) for comparison. There was no difference in the proportion of PACAP mRNA containing TH-immunoreactive neurons between the three strains ($P > 0.05$). (**B**) Shows a diagram of a coronal section of rat brainstem. The small box within the RVLM represents the area from which the photos in (**C**) and (**D**) were taken. (**C**) Is a photo taken from the RVLM of an SHR, while (**D**) is a photo taken from the RVLM of a WKY rat. The red label is for TH-immunoreactive neurons, while the black neurons are positive for PACAP mRNA. The scale bars in both (**C**) and (**D**) represent 50 µm. Abbreviations: IO, inferior olive; NA, nucleus ambiguus; NTS, nucleus of the solitary tract; PACAP, pituitary adenylate cyclase activating polypeptide; py, pyramidal tract; RVLM, rostral ventrolateral medulla; SD, Sprague-Dawley; SHR, spontaneously hypertensive rat; Sp5, spinal trigeminal tract; TH, tyrosine hydroxylase; WKY, Wistar-Kyoto.

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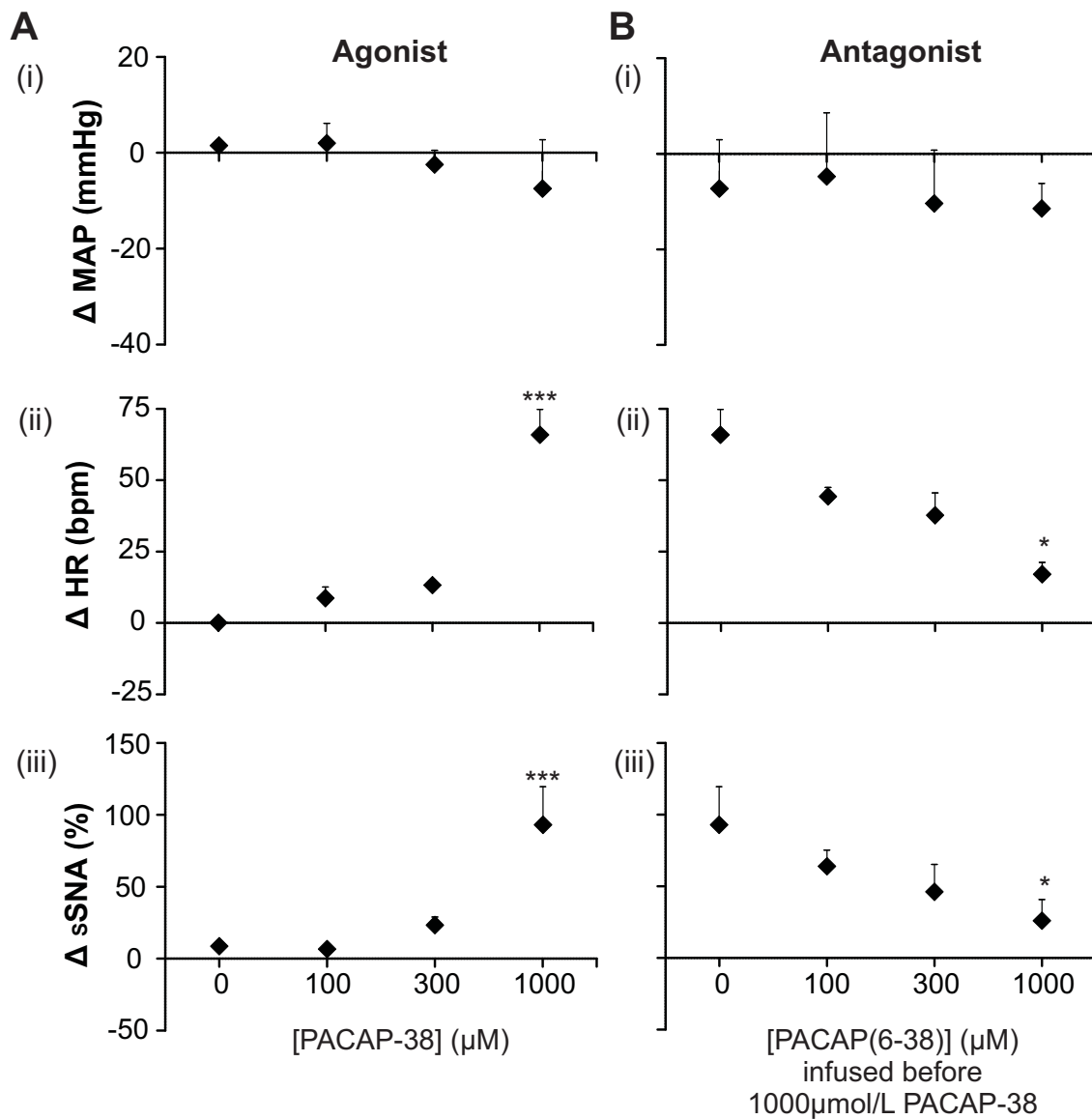
Figure 4.2 Dose-response curves for PACAP-38 and PACAP(6-38)
Changes in: (**i**) mean arterial pressure (MAP), (**ii**) heart rate (HR), and (**iii**) percentage splanchnic sympathetic nerve activity (sSNA) before and following, intrathecal administration of 10 µl of increasing concentrations of: (**A**) PACAP-38 or (**B**) PACAP(6-38) 15 minutes before 10 µl of 1000 µM PACAP-38. 0 µM is a vehicle control infusion of PBS (**A**). The data for 1000 µM PACAP-38 in (**A**) is taken from (Farnham *et al.,* 2008). This data is also used in (**B**) for 0 µM PACAP(6-38) before 1000 µM PACAP-38. *** - $P < 0.0001$ and * - $P < 0.05$ compared to '0'.

had no effect on MAP (**Figure 4.2**). PACAP-38 and PACAP(6-38) were therefore used at 1000 μM (1 mM) for the remainder of this study.

4.4.3 Effects of intrathecal PACAP-38 on MAP in normotensive and hypertensive rat models

Baseline MAP under anaesthesia was significantly higher in SHR (116 ± 5 mmHg; n = 6) when compared to WKY (90 ± 2 mmHg; n = 5) and Wistar rats (96 ± 4 mmHg; n = 7; $P < 0.005$; 1-way ANOVA with a *post hoc* Bonferroni's correction). Intrathecal injection of PACAP-38 had a significant effect on MAP over time in all strains ($P < 0.0001$), and while strain itself was not a significant factor, there was a significant interaction of strain over time ($P < 0.05$). In all strains MAP decreased ($P < 0.05$) 5 minutes after intrathecal injection of PACAP-38. Following this decrease, MAP in the Wistar and WKY remained depressed, while in the SHR MAP returned to control levels within 10-20 minutes, before steadily decreasing again to a level similar to that of the Wistar (**Figures 4.3**, **4.4** and **4.5**). While the time-course response to PACAP-38 was not strain dependent, the peak response of MAP was. **Figure 4.6** compares the peak responses to PACAP-38 in all three strains. PACAP-38 significantly decreased MAP compared to PBS in all strains ($P < 0.01$; **Figure 4.6**). This decrease was significantly lower in the Wistar compared to both the SHR and the WKY ($P < 0.001$; **Figure 4.6**).

4.4.4 Effects of intrathecal PACAP-38 on HR in normotensive and hypertensive rat models

The baseline HR was 433 ± 4 bpm in the WKY (n = 5), 395 ± 9 in the SHR (n = 6) and 438 ± 11 in the Wistar (n = 7). The baseline HR of the SHR was significantly lower than that of the Wistar ($P < 0.05$; 1-way ANOVA with a *post hoc* Bonferroni's correction). Intrathecal injection of PACAP-38 significantly increased HR over time in all strains ($P < 0.0001$) compared to PBS (**Figures 4.3**, **4.4** and **4.5**). This effect was strain dependent ($P < 0.05$), the tachycardic response to PACAP-38 persisted until the end of the experimental period (125 minutes post injection). The peak HR response after PACAP-38 was significantly increased compared to PBS in the SHR ($P < 0.001$) and the Wistar ($P < 0.05$; **Figure 4.6**). The peak PACAP-38 HR response of the SHR ($\Delta 54 \pm 11$ bpm) was significantly greater than that of the WKY ($\Delta 16 \pm 13$ bpm) and Wistar ($\Delta 30 \pm 9$ bpm) responses ($P < 0.01$; **Figure 4.6**).

4.4.5 Effects of intrathecal PACAP-38 on $_{s}$SNA in normotension and hypertension

Not all strains responded to PACAP-38 in the same way, since the time by strain effect was highly significant. Intrathecal injection of PACAP-38 significantly and differentially altered $_{s}$SNA over time in the three strains ($P < 0.0001$; **Figures 4.3**, **4.4** and **4.5**). This effect was strain dependent ($P < 0.05$), reached its maximum change ~60 minutes following PACAP-38 injection, and remained at the maximum level until the end of the experimental period (125 minutes post injection).

The overall increase in Wistar $_{s}$SNA in group analysis was smaller than that of the SHR and WKY however, individual Wistar responses were not homogenous. In 5 Wistars, intrathecal PACAP-38 increased $_{s}$SNA ($46.6 \pm 15.6\%$); while in 2 Wistars, $_{s}$SNA decreased (-84.11% & -39.54%).

The peak $_{s}$SNA response to PACAP-38 was significantly greater than the PBS response in the SHR only ($P < 0.001$; **Figure 4.6**). The peak $_{s}$SNA response to PACAP-38 in the SHR was significantly greater than that of the WKY ($P < 0.05$) and the Wistar ($P < 0.001$).

4.4.6 Effects of intrathecal PACAP(6-38) on MAP, HR and $_{s}$SNA in normotension and hypertension

The PACAP antagonist, PACAP(6-38), was administered intrathecally to test the hypothesis that PACAP has greater tonic activity in the SHR. The effects of PACAP(6-38) on cardiovascular parameters were tested in the SHR (n = 6) and WKY (n = 4), but not the Wistar since there was no difference in effect between the SHR and WKY.

Intrathecal PACAP(6-38) had no effect on HR or $_{s}$SNA in either strain compared to PBS ($P > 0.05$; **Figure 4.6**). MAP, on the other hand, was significantly increased 5 minutes after PACAP(6-38) administration in the SHR ($\Delta 11 \pm 3$ mmHg; $P < 0.01$; **Figure 4.6**). The response returned to baseline within 60 minutes but did not fall below the baseline level at any time during the test period.
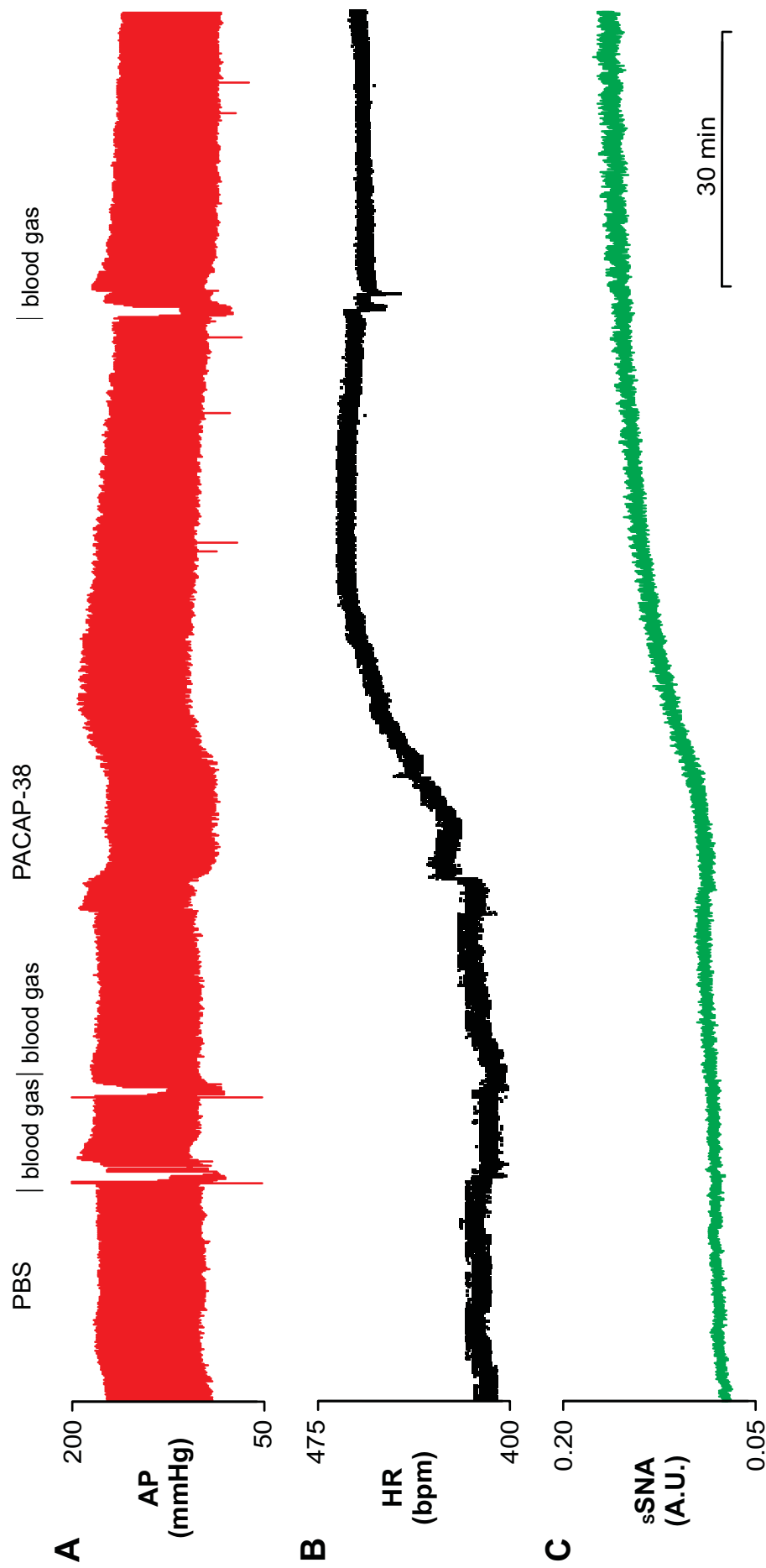
Figure 4.3 Effect of intrathecal PACAP-38 infusion in an SHR

A trace from an individual spontaneously hypertensive rat (SHR) showing the effects of intrathecal administration of vehicle (phosphate buffered saline; PBS) and PACAP-38 on: (**A**) arterial pressure (AP), (**B**) heart rate (HR) and (**C**) splanchnic sympathetic nerve activity (sSNA). Times of administration of PBS and PACAP-38 are marked with arrows. 'Blood gas' indicates the time at which 0.2 ml of arterial blood was withdrawn to ensure $PaCO_2$, PaO_2 and pH were within the normal physiological range. Scale bar represents 30 minutes.
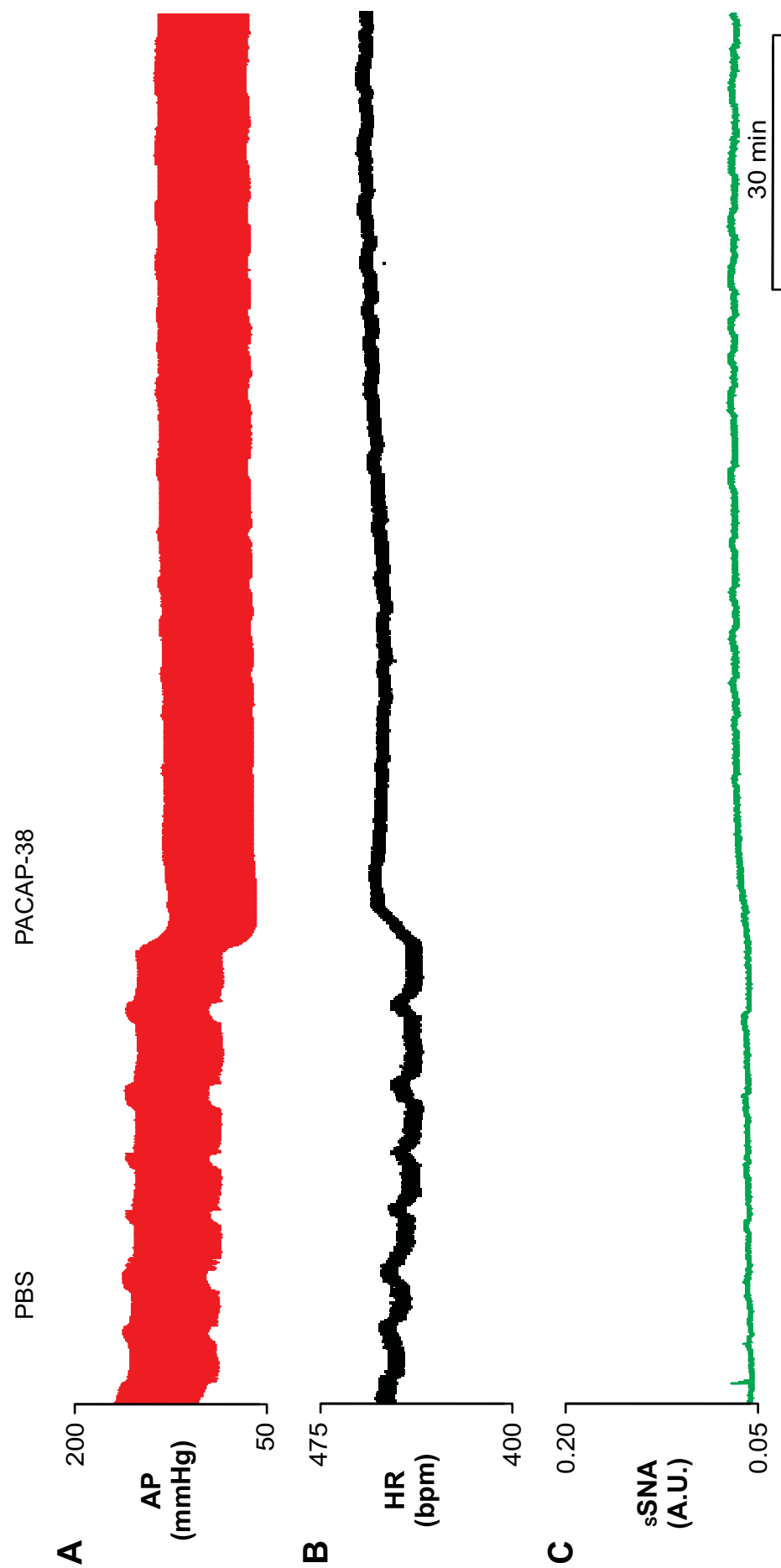
Figure 4.4 Effect of intrathecal PACAP-38 infusion in a WKY rat

A trace from an individual Wistar-Kyoto (WKY) rat showing the effects of intrathecal administration of vehicle (phosphate buffered saline; PBS) and PACAP-38 on: (**A**) arterial pressure (AP), (**B**) heart rate (HR) and (**C**) splanchnic sympathetic nerve activity (sSNA). Times of administration of PBS and PACAP-38 are marked with arrows. Scale bar represents 30 minutes.
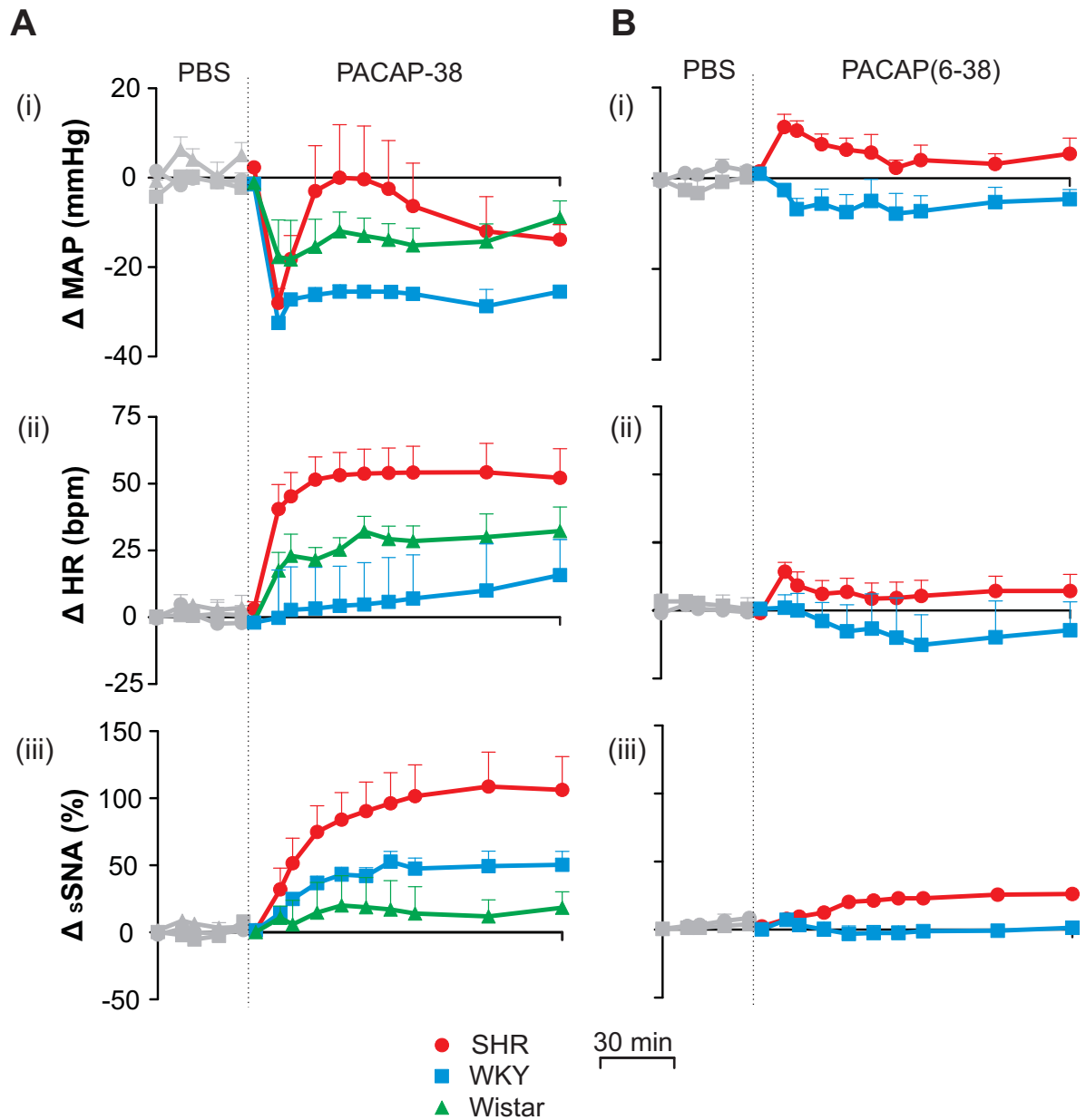
Figure 4.5 *In vivo* effects of intrathecal PACAP-38 and PACAP(6-38) in SHR, WKY and Wistar rats

Change in: (**i**) mean arterial pressure (MAP), (**ii**) heart rate (HR), and (**iii**) percentage splanchnic sympathetic nerve activity (sSNA) before and following, administration of: (**A**) PACAP-38 or (**B**) PACAP(6-38). Arrow indicates time of drug infusion. 'PBS' is the period after intrathecal infusion of phosphate buffered saline (PBS), 'PACAP-38' is the period after intrathecal infusion of PACAP-38 and 'PACAP(6-38)' is the period after intrathecal infusion of PACAP(6-38). PACAP-38 was administered to spontaneously hypertensive rats (SHR; $n = 6$; red; circle), Wistar-Kyoto (WKY; $n = 5$; blue; square) and Wistar rats ($n = 7$; green; triangle). PACAP(6-38) was administered to SHR ($n = 6$; red; circle) and WKY rats ($n = 4$; blue; square). Scale bar represents 30 minutes.
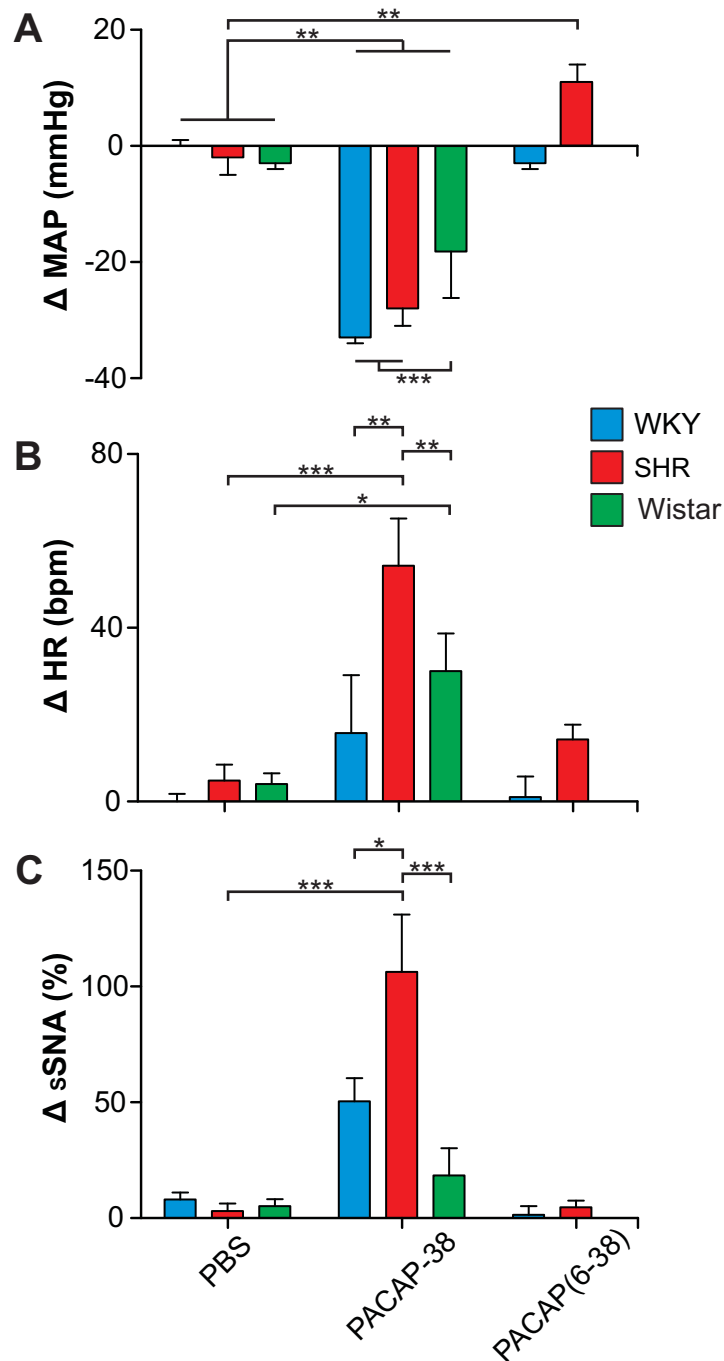
Figure 4.6 Peak responses following intrathecal PACAP-38 or PACAP(6-38) in Wistar, WKY and SHR rats

Peak changes in: (**A**) mean arterial pressure (MAP), (**B**) heart rate (HR), and (**C**) percentage splanchnic sympathetic nerve activity (sSNA) before and following, administration of vehicle (phosphate buffered saline; PBS), PACAP-38 and PACAP(6-38). PACAP-38 was administered to spontaneously hypertensive rats (SHR; n = 6; red), Wistar-Kyoto (WKY; n = 4; blue) and Wistar (n = 7; green) rats. PACAP(6-38) was administered to SHR (n = 6; red) and WKY rats (n = 5; blue). * - $P < 0.05$; ** - $P < 0.01$; *** - $P < 0.001$

4.5 Discussion

The novel findings of this study are first, that the proportion of TH-immunoreactive neurons within the RVLM, that also contain PACAP mRNA, is not different between SD, WKY and SHR strains. Secondly, intrathecal PACAP-38 causes sympathoexcitation and tachycardia in Wistar, WKY and SHR strains, as well as in SD rats (Farnham *et al.*, 2008). The sympathoexcitation and tachycardia observed varies in magnitude, and is unrelated to the resting MAP prior to PACAP injection in the four strains examined. Finally, intrathecal administration of PACAP(6-38), an antagonist at PAC_1 and $VPAC_2$, did not affect HR or SNA suggesting that PACAP is not tonically released in the spinal cord of SHR or WKY rats; at least in the anaesthetised preparation used here. Thus, we conclude that PACAP is not likely to be the underlying cause of hypertension in SHR.

To date, the extent of PACAP mRNA expression in the RVLM of hypertensive rats is unknown. In the normotensive SD, PACAP mRNA is present in ~82% of TH-immunoreactive RVLM neurons that project to the spinal cord (**section 1.8.3**; Farnham *et al.*, 2008) and also in phenylethanolamine-N-methyltransferase (PNMT)-immunoreactive RVLM neurons that project to the paraventricular nucleus (PVN; Das *et al.*, 2007). The present study, together with our earlier work, enables a comparison of brainstem PACAP mRNA expression in hypertensive and normotensive strains. Although the proportion of PACAP mRNA containing TH-immunoreactive neurons within the RVLM is not different between the strains, a caveat to our findings is that the approach does not measure the total amount of mRNA; only its presence or absence. ISH is not quantitative and does not accurately measure PACAP mRNA content of RVLM sympathetic premotor neurons. Furthermore, mRNA detection may not reflect its translation into, and thus the expression of, PACAP peptide.

To investigate the role of spinal cord PACAP in hypertension, PACAP-38 and its antagonist (PACAP(6-38)) were infused intrathecally in SHR and WKY rats; clear differences were observed between the two strains in the MAP, HR and $_S$SNA responses. The responses to PACAP-38 were compared to those seen in SD rats in our previous study (Farnham *et al.*, 2008). Interestingly the responses observed in SD and SHR were of a similar magnitude (eg. SNA increased >100%) while the

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WKY responses appeared blunted (SNA increased by 53%; **Figures 4.2**, **4.3**, **4.4**, **4.5** and **4.6**). The unexpected difference between the SD and WKY prompted an investigation in the Wistar rat, the outbred normotensive parent strain of the WKY and SHR, with the intention of revealing which normotensive strain, SD or WKY, was atypical in its responses. Instead, the Wistar responses to intrathecal PACAP-38 were intermediate in magnitude between the SHR and WKY for MAP and HR. On the other hand, the $_S$SNA response of the Wistar was less than that of the WKY. These conflicting results have complicated a straightforward answer to the aforementioned question. One important factor may be that the Wistar is an outbred rat strain compared to the inbred SD and WKY strains. The Wistar also showed greater variation in responses, particularly in the $_S$SNA responses. In 5 animals, the $_S$SNA increased in response to intrathecal PACAP but in 2 animals, there was a decrease of a similar magnitude. This heterogeneity in response was not observed in any of the other 3 strains studied.

Our results have highlighted the need for investigation into the complexity of cardiovascular differences between strains. The significance of these differences in responsiveness between strains is also difficult to interpret, since the cellular distribution of the PACAP receptors in the spinal cord is currently unknown. One possible explanation is that an as yet unknown difference in spinal cord PACAP receptor expression exists between the strains. It is known that splice variants of PAC_1 receptors exist, but their expression and functional significance in the spinal sympathetic column remains unexplored at this stage (Spengler *et al.*, 1993; Dickson & Finlayson, 2009). Another important caveat to note is that the observed differences in effects between the strains may not be strictly cardiovascular in nature, but may be due to confounding effects of PACAP receptor differences in other systems activated by intrathecal PACAP, such as the dorsal horn. Questions could also be raised about the accessibility of PACAP to the SPN. First, there is evidence that some dendrites of SPN reach the surface of the spinal cord (Pilowsky *et al.*, 1994). Secondly, while the SPN may indeed be less accessible to intrathecal injection of PACAP, the time course of the responses following PACAP administration is consistent with this fact. The responses are not immediate, nerve activity starts to rise 5-10 minutes after PACAP administration. We suggest that this

time reflects the amount of time that it takes for infused PACAP to fully occupy the available receptors on SPN.

The final aim of this study was to determine if endogenous PACAP-38 contributes to the hypertension observed in SHR. PACAP(6-38) is an antagonist specific for the PAC_1 receptor, which is the predominant form in the CNS (Cauvin *et al.*, 1991; Kyeung *et al.*, 2004), but also acts at the $VPAC_2$ receptor, albeit with a 10 times lesser affinity (Robberecht *et al.*, 1992; Dickinson *et al.*, 1997; Harmar *et al.*, 1998). PACAP(6-38) did not reduce any measured parameter from baseline in either the SHR or the WKY. Unexpectedly, PACAP(6-38) did cause a small initial increase in MAP (~10 mmHg, $P < 0.05$) in the SHR. The results from this study indicate that PACAP-38 is probably not tonically released in the spinal cord and does not contribute to the hypertension of SHR in this preparation. It is possible that the $VPAC_1$ receptors may compensate in the case of $PAC_1/VPAC_2$ antagonism, but this is unlikely since the results show absolutely no reduction in activity. The question of SPN accessibility also arises for PACAP(6-38), but we believe the suggestion given for PACAP-38 is also applicable here. The dose-response curve in **Figure 4.2** provides evidence that the antagonist is able to access much the same receptor sites as PACAP-38 itself.

The decreases in MAP following intrathecal PACAP-38 are paradoxical given the sympathoexcitatory effects observed; these responses are not baroreflex mediated as determined previously in the SD (Farnham *et al.*, 2008). One possible explanation is that PACAP differentially affects sympathetic outflows to various vascular beds (**Chapter 5**). PACAP activating the splanchnic sympathetic bed may cause vasoconstriction in the mesenteric vascular bed but inhibition of the lumbar sympathetic bed, or a large vasodilatation in the tail vascular bed. While the responses of vasomotor nerves, other than the splanchnic sympathetic nerve, are yet to be studied in rat, PACAP-induced differential regional blood flow was observed previously in the hindquarters and pulmonary circulation of the cat (Minkes *et al.*, 1992).

The contradictory MAP response observed in this study may also be driven by differential actions of the three PACAP receptors (**Chapter 6**). The different

PACAP receptors have had specific actions demonstrated in several systems. The PAC_1 receptor is important in renin secretion (Hautmann *et al.*, 2007) and in PACAP-induced depolarisations in superior cervical ganglion neurons (Beaudet *et al.*, 2000). The $VPAC_1$ receptor on the other hand, is necessary for pressure-induced vasodilatation (Fizanne *et al.*, 2004), evidence from our current study suggests that $VPAC_1$ may primarily be involved in the MAP response observed after intrathecal injection of PACAP-38.

PACAP(6-38) is an antagonist of the PAC_1 and $VPAC_2$ receptors, and attenuated the HR and $_sSNA$ effects of intrathecal PACAP-38 in the SD by over 70%, but the MAP response remained unaffected. These results provide evidence that PACAP(6-38) is an effective antagonist and also suggests a differential role for the receptors in the spinal cord. PACAP(6-38) was reported to have slight agonistic properties at high doses (Ozawa *et al.*, 1997), which could explain the small significant increase in MAP and small insignificant increases in $_sSNA$ and HR of the SHR following intrathecal PACAP(6-38). If the hypothesis that PACAP receptors have differing actions in the spinal cord is true, it could mean that the $VPAC_1$ receptor has opposing vasodilatory effects on blood pressure and predominates over the vasoconstrictor actions of the $PAC_1/VPAC_2$ receptors. The $_sSNA$ and HR responses, on the other hand, appear to be dependent on all 3 receptors for full expression of the PACAP response.

It is still unknown which stimuli cause the release of PACAP-38 physiologically from presympathetic neurons. PACAP is implicated in glucose metabolism (Hamelink *et al.*, 2002; Green *et al.*, 2006; Nakata & Yada, 2007) and thermoregulation (Gray *et al.*, 2002; see (Vaudry *et al.*, 2000; Dickson & Finlayson, 2009; Vaudry *et al.*, 2009) for review) and is frequently implicated in responses to other stressors such as adrenaline release and haemorrhage (Murase *et al.*, 1993; Kuri *et al.*, 2009). The increased HR and $_sSNA$ responses to intrathecal administration of PACAP are consistent with an enhanced metabolic response and may also contribute to the "fight or flight" response.

In summary, three major findings are reported: First, the proportion of PACAP mRNA containing TH-immunoreactive neurons is similar within the RVLM

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of normotensive and hypertensive rats. Second, intrathecal PACAP-38 causes strain dependent, sustained sympathoexcitation and tachycardia, with variable effects on MAP in both normotensive and hypertensive rats. The MAP effects may be primarily mediated via the $VPAC_1$ receptor. Lastly, intrathecal administration of PACAP(6-38), an antagonist at PAC_1 and $VPAC_2$, did not affect HR or SNA, suggesting that PACAP-38 is not tonically released in the spinal cord of SHR or WKY rats. We conclude that PACAP in the spinal cord is not an underlying cause of the hypertension in the SHR, at least in our urethane-anaesthetised, vagotomised, paralysed and artificially ventilated preparation.

4.6 Perspectives

The potent excitatory peptide PACAP is present in catecholaminergic bulbospinal neurons in the RVLM (Farnham *et al.*, 2008), in SPN in the spinal cord (Ghzili *et al.*, 2008) and in the adrenal medulla (Kuri *et al.*, 2009), and also in chromaffin cell carcinomas (Ghzili *et al.*, 2008). PACAP receptors are also present on blood vessels and in many other sites throughout the body (Vaudry *et al.*, 2000; Vaudry *et al.*, 2009). This widespread distribution suggests a crucial role for this pleiotropic polypeptide in regulating the cardiovascular system. While the precise roles played by PACAP in RVLM neurons in regulating SPN still remains a topic for future study, the findings here suggest that PACAP can differentially regulate blood pressure via PAC_1, $VPAC_1$ and $VPAC_2$ receptors. Additional specific agonists and antagonists for each of the receptors are needed to further explore this hypothesis.

Chapter 5

Results II

Chapter 5: Intrathecal PACAP-38 causes prolonged widespread sympathoexcitation via a spinally mediated mechanism and increases in basal metabolic rate in the anaesthetised rat

Chapter 5: Intrathecal PACAP-38 causes prolonged wide-spread sympathoexcitation via a spinally mediated mechanism and increases in basal metabolic rate in the anaesthetised rat

The work in this chapter has been published (**Appendix 1**): Inglott MA, Farnham MMJ & Pilowsky PM. (2011). Intrathecal PACAP-38 causes prolonged widespread sympathoexcitation via a spinally mediated mechanism and increases in basal metabolic rate in anaesthetised rat. *Am J Physiol* **300,** H2300-H2307. Abbreviations and formatting have been edited from the published paper.

Contributions: Conception and design of experiments: MAI, MMJF; Experimental procedures: MAI; Data analysis: MAI; Interpretation of data: MAI, MMFJ, PMP; Manuscript preparation: MAI; Critical revision of manuscript: MAI, MMFJ, PMP.

5.1 Abstract

The rostral ventrolateral medulla (RVLM) differentially regulated sympathetic output to different vascular beds possibly through the release of various neurotransmitters and peptides that may include pituitary adenylate cyclase activating polypeptide (PACAP). Intrathecal administration of PACAP increases splanchnic sympathetic nerve activity ($_s$SNA) and heart rate (HR), but not mean arterial blood pressure (MAP), the mechanism behind this response is unknown, but may be due to differential control of sympathetic outflows. In this study we sought to: 1) investigate whether intrathecal PACAP differentially affects sympathetic outflow, 2) determine if the intrathecal responses to PACAP are solely due to a spinally mediated mechanism and 3) determine if intrathecal PACAP affects metabolic function. Experiments using urethane-anaesthetised, vagotomised, ventilated and paralysed adult male Sprague-Dawley (SD) rats were conducted in this study. Intrathecal injections of PACAP-38 were given, and MAP, HR, the activity of regional sympathetic nerves, end tidal CO_2 and core temperature were recorded. The novel findings of this study are that; 1) intrathecal PACAP-38 causes a prolonged widespread sympathoexcitation in multiple sympathetic beds, 2) that this widespread sympathoexcitation is mediated within the spinal cord itself, since spinal transection does not abrogate the response and 3) that intrathecal PACAP-38 increases basal metabolic rate. Therefore, we conclude that intrathecal PACAP acts in the spinal cord to cause a prolonged widespread sympathoexcitation and, that PACAP also causes

an increase in basal metabolic rate, that includes an increase in brown adipose tissue (BAT) thermogenesis, in our rat preparation.

5.2 Introduction

The RVLM is critical for the tonic and reflex maintenance of the cardiovascular system (Guertzenstein & Silver, 1974; Feldberg & Guertzenstein, 1976; Sved *et al.*, 2003), and possibly in the genesis of hypertension (**section 1.5.6**; Jannetta & Gendell, 1979; Morimoto *et al.*, 1999; Nakamura *et al.*, 2007). The RVLM generates sympathetic outflow to the cardiovascular system via presympathetic neurons that project to sympathetic preganglionic neurons (SPN) in the intermediolateral (IML) cell column of the spinal cord (**Chapter 1**; Pilowsky & Goodchild, 2002; Guyenet, 2006). Stimulation of distinct sub-regions within the RVLM causes differential changes in sympathetic nerve activity (SNA; Dampney & McAllen, 1988; Dean *et al.*, 1992; McAllen & May, 1994; Campos & McAllen, 1997; Ootsuka & Terui, 1997). Some areas in the RVLM increase SNA to more than one target tissue when activated (McAllen & Dampney, 1990; Dampney, 1994; McAllen & May, 1994; Morrison, 2001; Sved *et al.*, 2001). The cause of this differential control remains unknown. The neurochemistry of RVLM neurons is heterogeneous (**section 1.5.3**), with all neurons containing at least one amino acid neurotransmitter and at least one other neurotransmitter. Generally, there are many other co-localised metabotropic neurotransmitters, including: enkephalin (Stornetta *et al.*, 2001), neuropeptide Y (Minson *et al.*, 1994) and PACAP (Farnham *et al.*, 2008), that act with glutamate and GABA to excite or inhibit RVLM neurons (Pilowsky & Goodchild, 2002). This broad spectrum of co-localised neurotransmitters in RVLM neurons may explain its ability to differentially control SNA (**section 1.5.4.2**; Krukoff *et al.*, 1985; Elfvin *et al.*, 1993; Morrison, 2001; Pilowsky *et al.*, 2009). Previous work from our laboratory, and others, indicates that PACAP may be involved in this process (Minkes *et al.*, 1992; Farnham *et al.*, 2008; Farnham *et al.*, 2011).

PACAP is a 38 amino acid, excitatory neuropeptide (Miyata *et al.*, 1989) that exerts its effects on three receptors: the PACAP specific receptor (PAC_1 receptor), and two other receptors that have an equal affinity for vasoactive intestinal polypeptide and PACAP ($VPAC_1$ and $VPAC_2$ receptors; **section 1.8**; Dickson & Finlayson, 2009; Vaudry *et al.*, 2009). PACAP mRNA and PACAP peptide are present within the central nervous system. (Légrádi *et al.*, 1994; Dun *et al.*, 1996a; Dun *et al.*, 1996b; Hannibal, 2002). In the brainstem, PACAP mRNA is found in 82% of C1 (adrenaline (Ad) synthesising) presympathetic bulbospinal neurons within the RVLM (**section 1.8.3.2**; Farnham *et al.*, 2008). Intrathecal administration of PACAP, to examine the cardiovascular effects of activating SPN, results in tachycardia and sympathoexcitation in normotensive (Farnham *et al.*, 2008) and hypertensive (Farnham *et al.*, 2011) rats. Paradoxically, the sympathoexcitation observed following administration of intrathecal PACAP, is not accompanied by an increase in MAP (Farnham *et al.*, 2008). This response is not altered in SD rats that have been surgically barodenervated (Farnham *et al.*, 2008).

PACAP is also implicated in the maintenance of normal energy homeostasis, including BAT thermogenesis and glucose metabolism (Adams *et al.*, 2008; Vaudry *et al.*, 2009); processes that the RVLM has a role in and are known to affect MAP (**section 1.8.4.6**; Madden *et al.*, 2006).

Therefore, the aims of this study are 1) to investigate whether or not intrathecal PACAP, differentially or globally, affects sympathetic outflows by recording from multiple sympathetic nerves, 2) to determine if the previously reported (Farnham *et al.*, 2008) responses to intrathecal PACAP are due to a spinally mediated mechanism by intrathecal injection of PACAP-38 in C1 spinally transected rats and 3) to determine if intrathecal PACAP affects metabolic function, through measurement of end-tidal CO_2, pH, partial arterial pressure of CO_2 ($PaCO_2$), partial arterial pressure of O_2 (PaO_2), core temperature, and glucose levels.

5.3 Methods

5.3.1 Ethics approval

All experiments were conducted on adult male SD rats (350-500g; Animal Resources Centre, Perth, Australia) in accordance with the guidelines set out by the

Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and were approved by the Animal Care and Ethics Committee and the Biosafety Committee of Macquarie University.

5.3.2 Surgical preparation

General surgical methods (**Chapter 3**) were carried out as previously described (Farnham *et al.*, 2008). All rats (n = 21) were anaesthetised with urethane (ethyl carbamate; 1.3-1.5 g/kg intraperitoneal (i.p.); Sigma-Aldrich Pty. Ltd., Australia). Anaesthetic depth was monitored by observing reflex responses to nociceptive and tactile stimuli (periodic tail/paw pinches), and the corneal touch reflex. Additional anaesthetic was administered (30-40 mg intravenous (i.v.) urethane), as required to maintain adequate anaesthesia. Atropine sulfate (100 µg/kg, i.p.; Astra Pharmaceuticals) was administered to reduce bronchial secretions. All rats were secured in a stereotaxic frame. A rectal probe, connected to a heating blanket (Harvard Apparatus, USA), allowed core body temperature to be measured and maintained between 36.5°C and 37.5°C over the experimental period.

5.3.3 General surgery

In all rats, the right carotid artery and jugular veins were cannulated for the recording of blood pressure and administration of drugs and fluids, respectively. A tracheal cannula was inserted to permit artificial ventilation (rodent ventilator; UGO Basile, Biological Research Apparatus, Italy) and CO_2 monitoring (Capstar-100 CO_2 analyser; CWE Inc. USA). HR was derived from the electrocardiogram (ECG) recording. The rats were bilaterally vagotomised, artificially ventilated and paralysed with pancuronium bromide (0.4 mg given as a 0.2 ml bolus i.v. followed by an i.v. infusion of 10% pancuronium in 0.9% saline at a rate of 2 ml/h; Astra Pharmaceuticals). The left cervical, splanchnic and/or lumbar sympathetic nerves were isolated, dissected and prepared for recording (2 kHz, 1k-100k X gain, 0.1-2 kHz filtering); a 50/60 Hz line frequency filter (Humbug; Quest Scientific) was also used.

5.3.4 Intrathecal administration of PACAP-38

A catheter (polyvinyl chloride (PVC); OD, 0.61 mm; ID, 0.28 mm, Critchley Electrical Products, Australia) with a dead space of ~6 µl was inserted into the

intrathecal space of all rats through a slit in the dura at the atlanto-occipital junction and advanced caudally to the level of T5/6. A control injection of 10 μl of 10 mM phosphate buffered 0.9% saline (PBS) was washed in with 6 μl PBS. Next, 10 μl of 1 mM PACAP-38 (Auspep Pty. Ltd., Australia) was administered and flushed in with 6 μl PBS. Injections were done over a 10 to 15 second period, as previously described (Farnham *et al.*, 2008). Responses were recorded for 30 (PBS) and 90 minutes (PACAP-38). Six rats also had respiratory blood gas (O_2 and CO_2), pH (electrolyte and blood gas analyser; IDEXX Laboratories, USA) and blood glucose analysis (blood glucose meter; Roche Diagnostics, USA) conducted during the recording periods. At the conclusion of the experiments, all rats were euthanised with 0.5 ml of 3M potassium chloride (KCl; i.v.). Postmortem verification of the location of the intrathecal catheter was achieved by injecting 10 μl of India ink washed in with 6 μl of PBS into the intrathecal catheter and exposing the spinal cord. The spinal segment level of the catheter was recorded as the level where the tip was observed or where the black spot appeared most intensely on the spinal cord. If the catheter was not found at T5-6, the results were not included in the present study.

5.3.5 Intrathecal administration of PACAP with surgical spinal transection

In a separate group of 5 rats (instrumented as described above), C1 surgical spinal transection was completed by exposure and laminectomy of the C1 vertebra to expose the corresponding spinal segment. The dura was removed and the spinal cord was carefully transected. To ensure complete transection, sympathetic baroreflex testing (phenylephrine (PE; 10 μg/kg, 0.1ml bolus; ICN Biomedicals Inc, Australia), i.v.) was conducted before and after transection, confirming elimination of neural transmission. The lesion was closely inspected at the time of transection, and following death, to ensure no tracts remained intact. Data collection was only conducted after successful verification of spinal transection (baroreflex abolition). Following the baroreflex tests, all parameters were recorded until readings returned to baseline. PBS and PACAP-38 were then injected intrathecally as described in the protocol above (**section 5.3.4**). After the responses of MAP, HR and $_S$SNA to PBS (30 minutes) and PACAP-38 (90 minutes) were recorded, the animal was killed, and the location of the catheter tip verified as above (**section 5.3.4**).

5.3.6 Data acquisition and analysis

Data were acquired using a CED 1401 ADC system (Cambridge Electronic Design, UK) and Spike 2 acquisition and analysis software (Cambridge Electronic Design, UK). Cervical sympathetic nerve activity ($_c$SNA), lumbar sympathetic nerve activity ($_L$SNA) and $_s$SNA raw data were rectified and smoothed/averaged to 2s and normalised to '0' by subtracting residual activity 5-10 minutes after death ($_s$SNA data from spinally transected animals were not normalised, as baseline activity before data collection was equal to '0'; **section 5.4.2**). MAP, HR, $_c$SNA, $_s$SNA, $_L$SNA, end-tidal CO_2 and core temperature were analysed from 5 minute blocks taken 10 and 5 minutes prior to and 5, 10, 20, 30, 40, 50, 60 and 90 minutes after intrathecal injections of PBS (up to 30 minutes) or PACAP-38 (up to 90 minutes). Arterial blood gas (O_2, CO_2, pH) and arterial blood glucose levels were measured 10 and 5 minutes prior to and 30 minutes after intrathecal injections of both PBS and PACAP-38. Statistical analysis was carried out in GraphPad Prism software. Statistical significance was determined using one- or two- way ANOVAs with Bonferroni's corrections, unless otherwise stated. $P < 0.05$ was considered to indicate a significant difference between the means.

5.4 Results

5.4.1 *In vivo* effects of intrathecal PACAP-38 on SNA

Intrathecal PACAP-38 significantly increased the activity of the cervical, splanchnic and lumbar sympathetic nerves, over time ($P < 0.0001$; **Figure 5.1**). All three nerve activities doubled (~100% increase from baseline; $P < 0.05$) ~30 minutes post PACAP-38 injection (**Figure 5.1D**). Intrathecal PACAP-38 increased $_c$SNA by 100 ± 35 %; n = 4 (**Figure 5.1A and C**), $_L$SNA by 96 ± 18 %; n = 6 (**Figure 5.1B and C**) and $_s$SNA by 105 ± 10 %; n = 6 (**Figure 5.2C**). $_L$- $_s$- and $_c$- SNA remained markedly increased for the remainder of the experimental period (90 minutes; **Figures 5.1** and **5.2C**).

As was previously reported (Farnham *et al.*, 2011), we found that MAP was not affected (Δ -12 $\pm$ 4 mmHg; n = 6; $P > 0.05$), and HR was increased significantly from baseline (Δ 48 $\pm$ 5 bpm; n = 6; $P < 0.0001$) following intrathecal injection of PACAP-38 (**Figure 5.2A** and **B**).
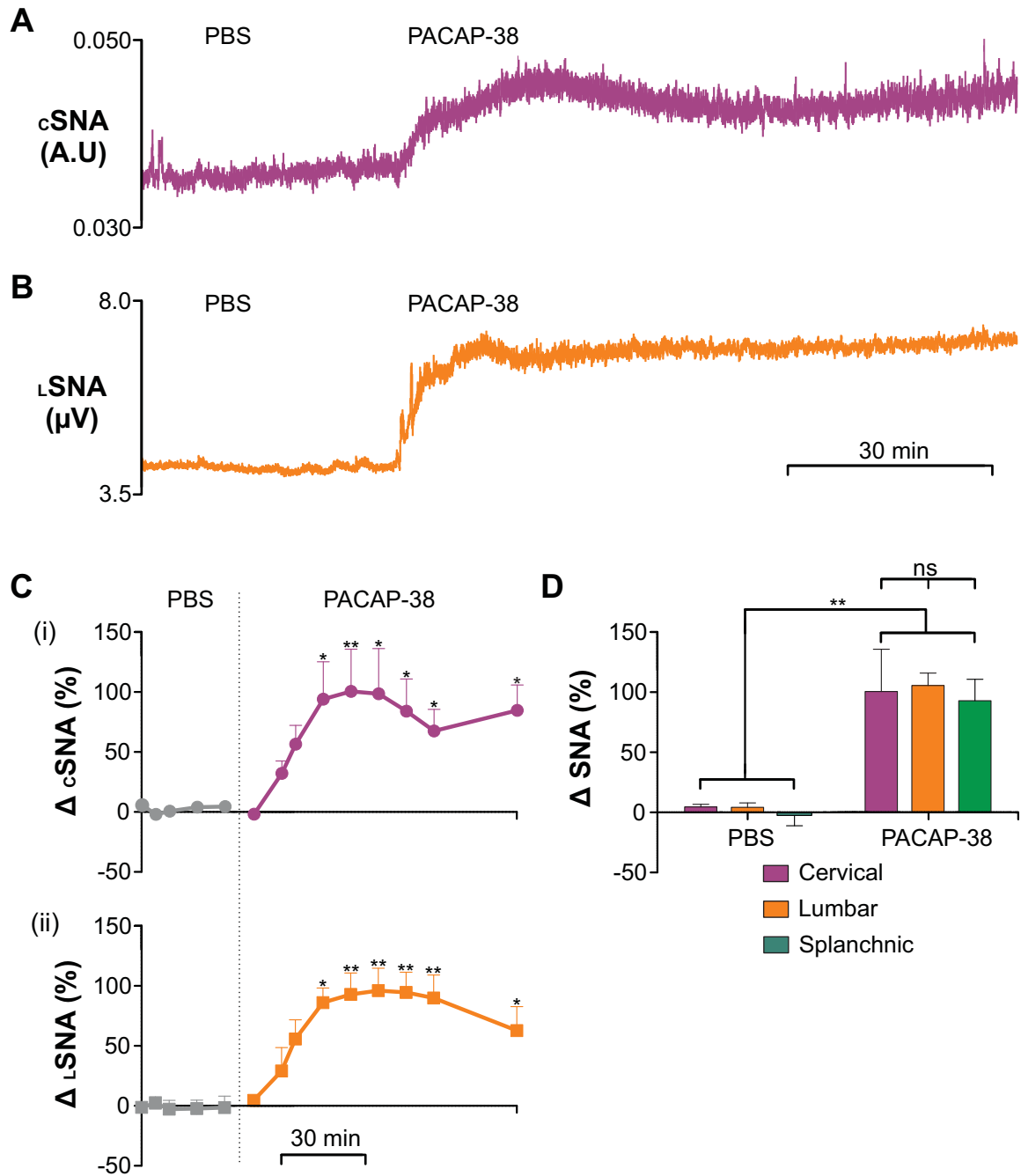
Figure 5.1 *In vivo* effects of intrathecal PACAP-38 administration on sympathetic nerve activity

Examples of traces from 2 SD rats showing the effects of intrathecal vehicle (PBS) and PACAP-38 injection on (**A**) cervical sympathetic nerve activity (cSNA) and (**B**) lumbar sympathetic nerve activity (LSNA). Arrows indicate times of PBS and PACAP-38 administration. (**C**) Grouped data showing the time-course of responses in: (**i**) LSNA (n = 6) and (**ii**) cSNA (n = 4) following PBS and PACAP-38 injection. Arrow indicates time of PACAP-38 injection. 'PBS' is the period after intrathecal injection of PBS, 'PACAP-38' is the period after intrathecal administration of PACAP-38. (**D**) 30 minute peak responses in cSNA (Δ 100 ± 35 %; $P < 0.0001$; n = 4), LSNA (Δ 105 ± 10 %; $P < 0.0001$; n = 6) and sSNA (Δ 96 ± 18 %; $P < 0.0001$; n = 6) following administration of PBS and PACAP-38. There was no difference between the responses of the nerves ($P > 0.05$) after PACAP-38 injection. Scale bars represent 30 min. * - $P < 0.05$; ** - $P < 0.001$; ns - not significant
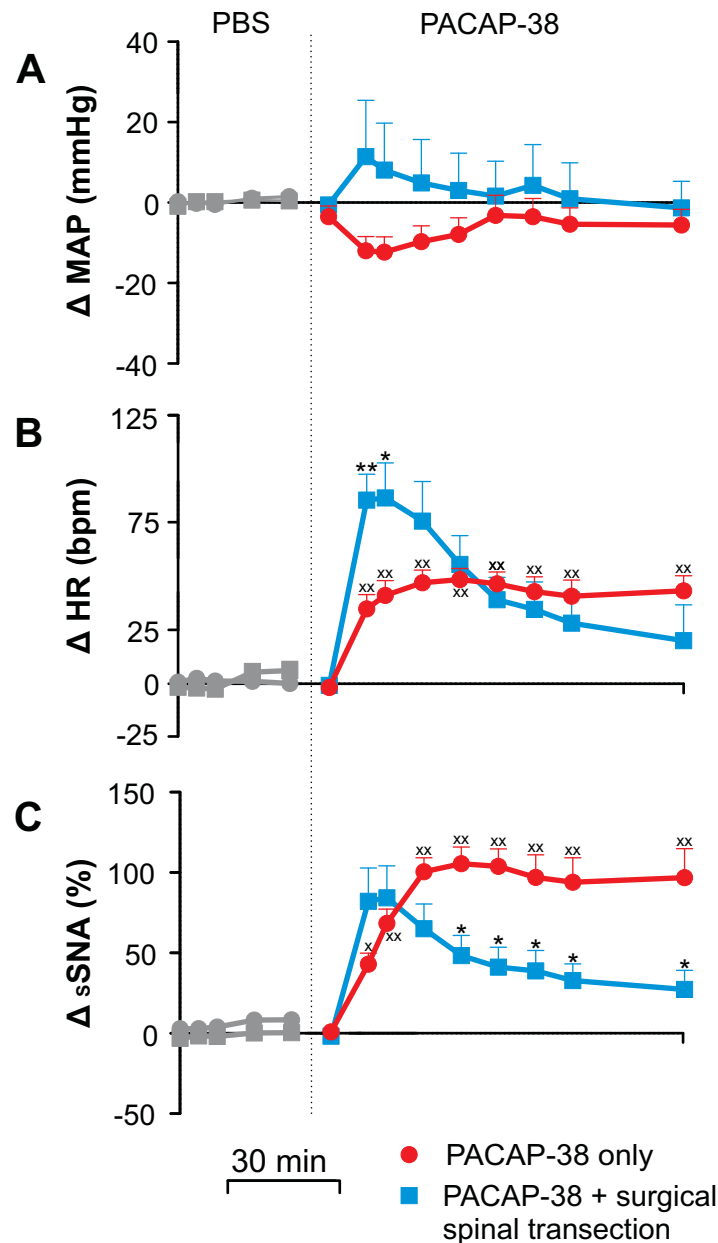
Figure 5.2 *In vivo* **effects of intrathecal PACAP-38 administration in spinally transected and spinally intact SD rats**

Grouped data showing the changes in: (**A**) mean arterial pressure (MAP), (**B**) heart rate (HR), and (**C**) splanchnic sympathetic nerve activity (sSNA) following PBS and PACAP-38 injection in spinally transected rats (blue; square; n = 5) and in rats with an intact spinal cord (circle; red; n = 6). PACAP-38 caused an increase in HR (Δ 48 ± 5 bpm; n = 6; $P < 0.0001$) and sSNA (Δ 105 ± 10 %; n = 6; $P < 0.0001$), but did not change MAP (Δ -12 ± 4 mmHg; n = 6; $P > 0.05$), in the rats with an intact spinal cord (circle). x – $P < 0.05$; xx - $P < 0.001$. The MAP and HR responses, as a whole, were unaffected by spinal transection ($P > 0.05$; square), however, the peak of the HR response was significantly higher in the transected animals when compared to the spinally intact rats, for the first 5 and 10 minutes of the response ($P < 0.05$). In spinally transected rats the sSNA response to PACAP-38 was similar to that of the intact rats for the first 20 minutes ($P > 0.05$), after which activity declined compared with spinally intact animals ($P < 0.05$), but still remained elevated compared to baseline. * - $P < 0.05$; ** - $P < 0.001$. Arrow indicates time of PACAP-38 injection. 'PBS' is the period after intrathecal infusion of PBS, 'PACAP-38' is the period after intrathecal infusion of PACAP-38. Scale bar represents 30 min. Note; cervical and lumbar sympathetic nerve activities were not recorded in spinally transected rats.

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5.4.2 Effects of spinal transection on the intrathecal PACAP-38 response

As expected, following spinal transection, the baseline values of MAP (109 ± 12 mmHg; n = 5), HR (438 ± 11 bpm; n = 5) and $_s$SNA (3.5 ± 0.2 µV; n = 5) were significantly reduced ($P < 0.05$) to 59 ± 7 mmHg, 358 ± 15 bpm and 1.6 ± 0.2 µV, respectively (**Figures 5.3** and **5.4**). Following spinal transection, the $_s$SNA response to PE is eliminated (**Figure 5.4A** and **B**), as is the pulse modulation (**Figure 5.4C**) and the 8 Hz HR peak within the $_s$SNA power spectrum (**Figure 5.4D**). Abolition of all three of these indices of supraspinal sympathetic activity confirms the effectiveness of the transection. Following surgical spinal transection, $_s$SNA fell to a level that was not different to that observed 5-10 minutes after death (1.2 ± 0.2 µV; n = 5; $P > 0.05$; **Figure 5.4B** and **C**).

Intrathecal injection of PACAP-38, following spinal transection, significantly increased HR (Δ 86 ± 16 bpm; $P < 0.0001$; n = 5) and $_s$SNA (Δ 84 ± 20 %; $P < 0.0001$; n = 5) over time compared to PBS (**Figures 5.2B, 5.2C** and **5.3**). The HR and $_s$SNA responses peaked ~10 minutes post PACAP-38 injection then slowly declined, but both remained above baseline for the duration of the experimental period (90 minutes; **Figures 5.2B, 5.2C** and **5.3**). On average, no significant effect on MAP was observed over time (Δ 11 ± 14 mmHg; $P > 0.05$; n = 5; **Figures 5.2A and 5.3**). When compared to the 6 spinally intact rats treated with intrathecal PACAP-38 (**Figure 5.2**), the MAP and HR responses were unaffected by spinal transection ($P > 0.05$). However, the peak of the HR response was significantly higher in the transected animals when compared to the intact rats for the first 5 and 10 minutes of the response ($P < 0.05$). In spinally transected rats the $_s$SNA response to PACAP-38 was similar to that of the intact rats for 20 minutes ($P > 0.05$), after which activity declined compared with the spinally intact animals ($P < 0.05$), but still remained elevated compared to baseline (**Figure 5.2**).

5.4.3 *In vivo* effects of intrathecal PACAP-38 on end-tidal CO_2, pH, core temperature $PaCO_2$ and glucose levels

The metabolic effects of PACAP-38 were assessed by recording changes in end-tidal CO_2, pH, core temperature, $PaCO_2$ and blood glucose levels (**Figure 5.5**). Intrathecal PACAP-38 significantly increased end-tidal CO_2 (Δ 0.67 ± 0.05 %ET; n = 6; $P < 0.0001$). The end tidal CO_2 response peaked ~30 minutes post PACAP-38

injection and remained elevated for the rest of the recording period (90 minutes; **Figure 5.5A**). PACAP-38 also significantly increased $PaCO_2$ (Δ 7 ± 1 mmHg; n = 6; $P < 0.05$) and significantly decreased pH (Δ -0.9 ± 0.1; n = 6; $P < 0.05$) 30 minutes post PACAP-38 administration, when compared to control (period prior to injection of vehicle) and vehicle (PBS; **Figure 5.5B**). Blood glucose decreased significantly (Δ -1.7 ± 0.6 mmol/L; n = 6; $P < 0.05$) 30 minutes after PACAP-38 administration, when compared to control and vehicle (**Figure 5.5B**). Core temperature was significantly increased 30, 40, 50, 60 and 90 minutes after PACAP-38 injection when compared to PBS (Δ 0.4 ± 0.2°C; n = 6; $P < 0.0001$; unpaired t-test; **Figure 5.5A**).

5.5 Discussion and conclusion

This is the first study to investigate possible mechanisms by which intrathecal PACAP exerts potent sympathoexcitatory effects, with no corresponding change in MAP (Farnham *et al.*, 2008). The original findings of Farnham *et al.,* (2008) are confirmed in this study (Farnham *et al.*, 2008). In addition, the novel findings of the present study are: first, that despite the lack of MAP response, intrathecal PACAP causes a prolonged sympathoexcitation in all measured sympathetic beds. Secondly, the response to intrathecal PACAP is mediated within the spinal cord, since C1 spinal transection does not abrogate the response. Finally, that intrathecal PACAP causes an increase in metabolic rate as suggested by the effects on end-tidal CO_2, pH, core temperature, $PaCO_2$ and glucose levels. Therefore, we conclude that intrathecal PACAP acts via a spinally mediated mechanism to cause a prolonged widespread sympathoexcitation, and that intrathecal PACAP causes an increase in metabolic rate (Madden & Morrison, 2006; Fan *et al.*, 2007; Nakamura & Morrison, 2007).

To date it is known that PACAP excites SPN in spinal cord slices from juvenile rats (Lai *et al.*, 1997) and that intrathecal administration of PACAP results in a prolonged tachycardia and increases $_sSNA$ by 100% (Farnham *et al.*, 2008). However, contradictory effects on MAP have been reported. Lai *et al.,* (1997) described a pressor response, while Farnham *et al.,* (2008) reported no MAP effect following intrathecal PACAP-38 (Lai *et al.*, 1997; Farnham *et al.*, 2008). The present study confirms the findings observed by Farnham *et al.,* (2008), showing
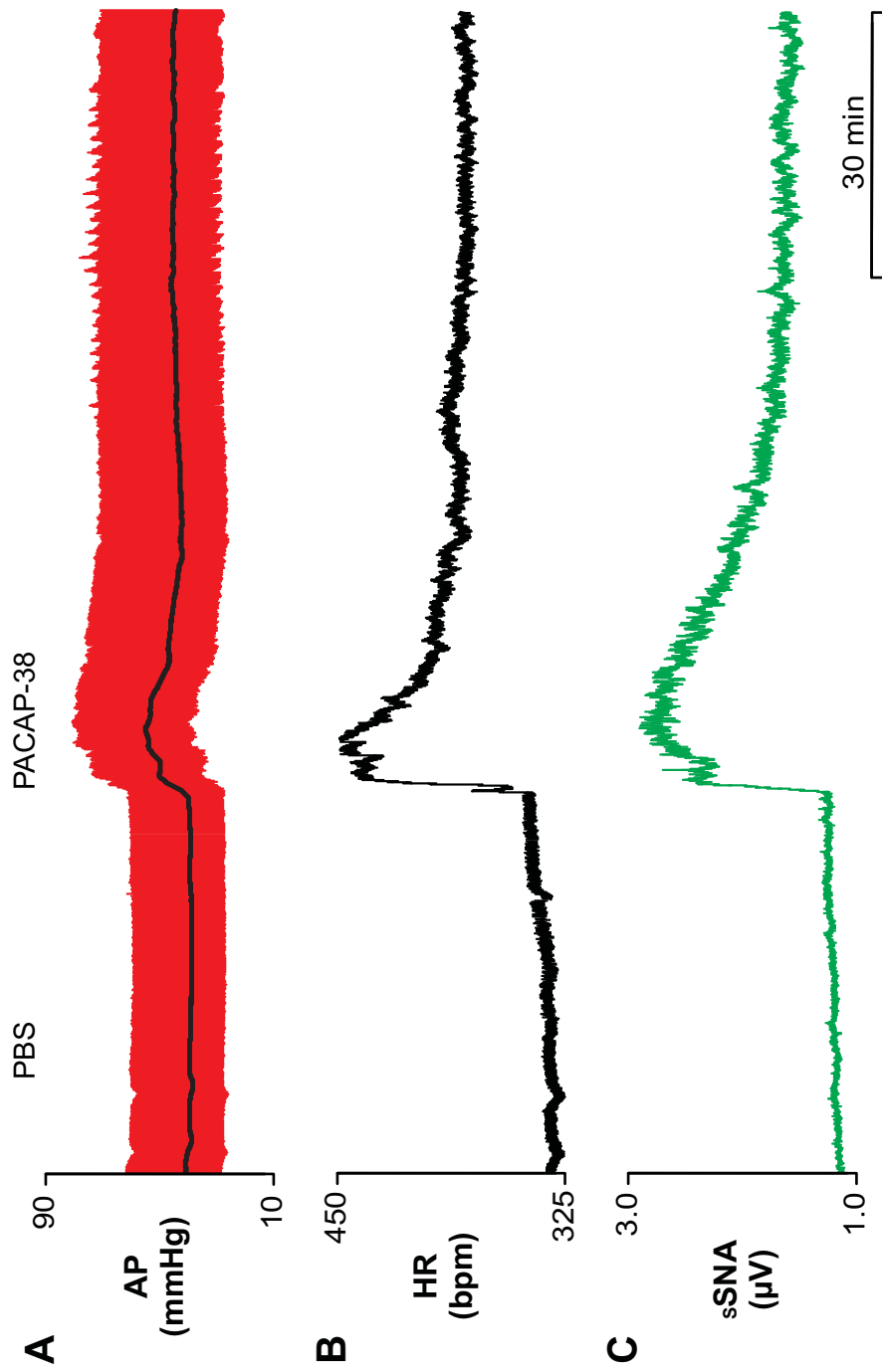
Figure 5.3 Effect of intrathecal PACAP-38 injection following spinal transection

An example of a trace from 1 spinally transected SD rat showing the effects of intrathecal vehicle (phosphate buffered saline; PBS) and PACAP-38 injection on: (**A**) arterial pressure (AP); mean arterial pressure is represented by the black line on the AP trace, (**B**) heart rate (HR) and (**C**) splanchnic sympathetic nerve activity (sSNA). Arrows indicate times of PBS and PACAP-38 administration. Scale bar represents 30 minutes.
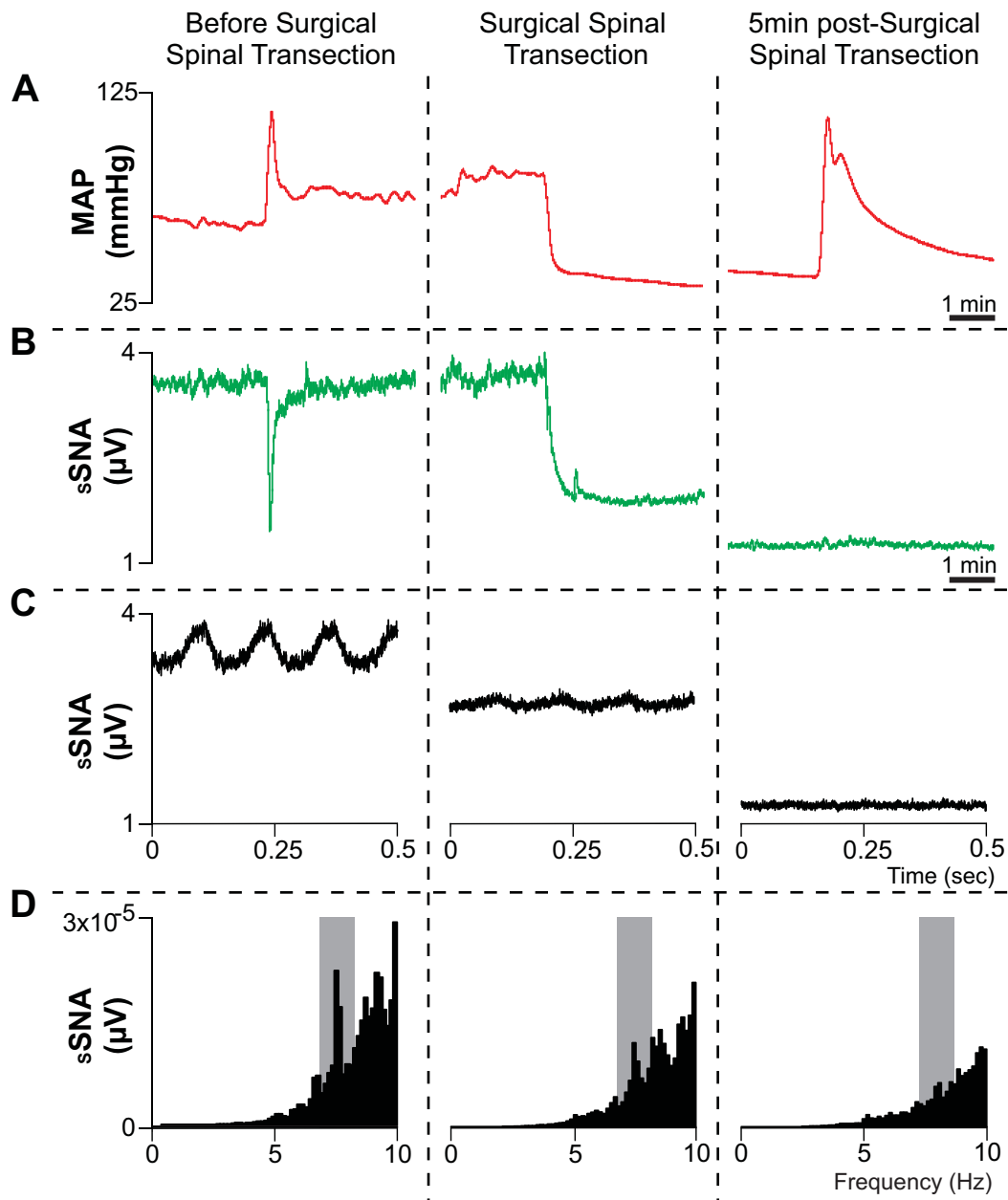
Figure 5.4 Spinal transection in a single SD rat
(**A**) mean arterial pressure (MAP) and (**B**) splanchnic sympathetic nerve activity (sSNA) traces during a sympathetic baroreflex challenge (phenylephrine) before spinal transection (**i**), during spinal transection (**ii**) and during a phenylephrine challenge 5 minutes after spinal transection (**iii**). Spinal transection reduced MAP and sSNA to spinal levels and abolished the sympathetic baroreflex. (**C**) Waveform averages of sSNA, from the 2 minutes prior to the baroreflex challenges and over the course of spinal transection, triggered from heart rate, show the pulse modulation of the nerve. This pulse modulation is reduced during the period of spinal transection and is completely abolished 5 minutes post-spinal transection. (**D**) Power spectra of sSNA over the same time periods as in (**C**). The 8 Hz heart rate peak (contained within the grey shading), present in the spectrum before spinal transection, is also reduced during the period of spinal transection and is completely abolished 5 minutes post-spinal transection. Following surgical spinal transection, sSNA was reduced to same level observed 5 minutes after death ($P > 0.05$). Scale bars represent 1minute.

Figure 5.5 Effect of intrathecal PACAP-38 injection on end-tidal CO2, pH, core temperature, PaCO2 and blood glucose levels

(A) Grouped data showing the changes in: (i) end-tidal CO2 (Δ 0.67 ± 0.05 %ET; n = 6; P < 0.0001) and (ii) core temperature (Δ 0.4 ± 0.2°C; n = 6; P < 0.0001) following vehicle (PBS) and PACAP-38 injection. Arrow indicates time of PACAP-38 injection. 'PBS' is the period after intrathecal injection of PBS, 'PACAP-38' is the period after intrathecal injection of PACAP-38. Scale bar represents 30 minutes. (B) Grouped data showing the changes in; (i) partial pressure of CO2 (PaCO2; Δ 7 ± 1 mmHg; n = 6; P < 0.05), (ii) pH (Δ -0.9 ± 0.1; n = 6; P < 0.05) and (iii) blood glucose levels (BGL; Δ -1.7 ± 0.6 mmol/L; n = 6; P < 0.05), during control (light green), 30 minutes following PBS (green) and 30 minutes following PACAP-38 (dark green) administration. * - P < 0.05; ** - P < 0.01.

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increases in $_S$SNA and HR, but no significant change in MAP (**section 1.8.4.3**).

In this study we measured the outflow from multiple sympathetic nerves, in response to intrathecal PACAP-38, to determine if the lack of MAP response following intrathecal PACAP is due to differential activation of sympathetic beds. Our data reveals that intrathecal PACAP-38 injection at the level of T6 increased $_C$SNA, $_L$SNA and $_S$SNA (by ~100%), all of which are known to contain vasoconstrictor fibres. The results indicate that intrathecal PACAP causes a widespread prolonged excitation of sympathetic vasoconstrictor pathways, and that the unaltered MAP following intrathecal PACAP is not due to a differential sympathetic outflow to these vascular beds. These findings contradict a previous study which found that intra-arterial injections of PACAP produced differential effects in the perfusion pressures of the pulmonary and hindquarter vascular beds of the cat (Minkes *et al.*, 1992). Perhaps the major reason for this difference is that in the cat there are substantial intra-thoracic pulmonary vascular noradrenergic innervations, whereas in rat there are none (Barnes & Liu, 1995). Apart from the differences in dose, species and injection method, the measures used by Minkes *et al.,* (1992) to infer centrally mediated changes to sympathetic vasomotor tone may explain the differences observed. Perfusion pressure is an indirect measurement of changes in vasomotor tone. Our study used recordings from barosensitive, pulse modulated sympathetic nerves to obtain direct, more reliable measures of changes in vasomotor SNA. The vascular isolation of the pulmonary bed and sympathetic denervation of the hindquarter vascular bed could also account for the difference observed between this and the Minkes *et al.,* (1992) study.

C1 spinal transection allowed us to confirm that the responses to intrathecal PACAP-38 observed in this and the previous study (Farnham *et al.*, 2008) are due to activation of SPN in the IML of the spinal cord, and not due to activation of presympathetic neurons in the brainstem. C1 spinal transection did not prevent the response to intrathecal PACAP-38 from occurring, confirming that intrathecal PACAP-38 acts via a spinally mediated mechanism to cause the increases in HR and $_S$SNA observed in this and the previous study (Farnham *et al.*, 2008). The peak HR response to intrathecal PACAP-38 in the spinalised rats was significantly larger than that seen in the spinally intact rats. A technical caveat to our study, and this

preparation, is that the spinal cord and sympathetic nervous system is greatly damaged by surgical spinal transection and therefore, the responses seen may be influenced by this. The difference seen between the HR responses in the spinalised and spinally intact rats may be due to this. The HR and $_s$SNA responses to intrathecal PACAP-38 observed in spinalised animals were not as long-lasting as those seen in spinally intact rats. In spinalised rats, the MAP is reduced to around 50 mmHg, and the resting level of SNA is greatly reduced. The lack of normal resting sympathetic tone present in the spinalised rats, may account for the discrepancy seen between the HR and $_s$SNA responses of the spinalised and spinally intact rats. The normally prolonged response may not be maintainable in this preparation.

Previous work in knockout mice (preproPACAP gene absent) suggests that PACAP plays a role in normal energy homeostasis, glucose metabolism and in thermogenesis (**section 1.8.4.6**; Adams *et al.*, 2008; Dickson & Finlayson, 2009; Vaudry *et al.*, 2009). However, this is the first study to show that intrathecal injection of PACAP-38 significantly increases end-tidal CO_2, $PaCO_2$ and core temperature, and decreases pH and blood glucose levels. The results indicate that intrathecal PACAP causes an increase in metabolic rate. The increase in end-tidal CO_2 may be representative of a decrease in SNA (vasodilation) to the pulmonary vascular bed (Minkes *et al.*, 1992). This is unlikely since, as noted above, rats lack an intrathoracic sympathetic innervation (Barnes & Liu, 1995). The concurrent increase in $PaCO_2$, decrease in pH and decrease in blood glucose suggests that the increase in expired CO_2 occurs as a result of increased metabolic rate. Increased metabolism is commonly associated with an increase in BAT thermogenesis. Increased SNA to BAT causes an increase in BAT thermogenesis and increases BAT temperature, end-tidal CO_2 and core body temperature. Although changes in BAT temperature are mirrored by changes in core body temperature, the response observed in core body temperature is always smaller than in BAT temperature (Madden & Morrison, 2006; Fan *et al.*, 2007; Nakamura & Morrison, 2007). The coupled increases observed in end-tidal CO_2 and core body temperature points to a possible role for PACAP in BAT thermogenesis. In one experiment, (data not shown) bupivacaine, a local anaesthetic, was injected into the intra-scapular brown fat after PACAP was injected, resulting in a fall in core body temperature.

The initiation of BAT thermogenesis may amplify, or cause a decrease in blood glucose levels following intrathecal PACAP. Nakata and Yada (2007) suggest that PACAP potentiates many metabolic processes (**section 1.8.4.6**; Nakata & Yada, 2007). It is suggested that PACAP is involved in energy storage after feeding through insulin release and action, adipogenesis, adipocyte differentiation, and by stimulating feeding (Adams *et al.*, 2008). However in the fasting state, PACAP is involved in energy utilisation through catecholamine and glucagon mediated lipolysis (Hamelink *et al.*, 2002), increased glucose output from the liver and also by stimulating feeding. The significant decrease in blood glucose levels observed in this study indicates that energy utilisation, and therefore metabolic rate, has increased in response to intrathecal PACAP. The work presented here suggests that the balance of glucose production vs. glucose utilisation tips towards a lower blood glucose level following intrathecal PACAP injection.

Taken together, the data illustrate an increase in SNA to the heart, splanchnic bed, hind limbs, head and neck, BAT and Ad-secreting chromaffin cells. The release of Ad from the adrenal medulla would have the effect of increasing HR, and mobilising glucose from glucagon in the liver. In this preparation we have performed a cervical vagotomy bilaterally. This means that any effects observed can only be due to sympathoactivation, sympathoinhibition or release of hormones from the adrenal gland or hypophysis. Plasma noradrenaline was not measured in these experiments, but activation of baroreceptors always causes a complete inhibition of SNA in the splanchnic nerve, indicating activation of fibres supplying noradrenergic chromaffin cells in the adrenal medulla. Ad secreting chromaffin cells are not innervated by barosensitive preganglionic neurons (Ootsuka & Terui, 1997), but, the fact that glucose and HR were increased supports the idea that this pathway is also activated.

The results of the present study do not enable us to account for the lack of MAP response observed following intrathecal PACAP-38 injection (Farnham *et al.*, 2008). One possibility is that PACAP-38 activation of other output pathways determines the overall effect on MAP. For example, if there is a withdrawal of sympathetic activity to the capacitance system (veins), then the overall effect on

blood pressure may be one of little, or no, change in spite of a marked increase in sympathetic activity to resistance arteries in the mesenteric or muscle beds.

Finally, PACAP is present in the preganglionic fibres that innervate the adrenal gland (Arimura, 1998; Hamelink *et al.*, 2002; Kumar *et al.*, 2010) and is a potent secretogogue of catecholamines from the adrenal medulla (Kuri *et al.*, 2009; Vaudry *et al.*, 2009). Future studies using adrenalectomy, plasma catecholamine measurement and pharmacological investigations using specific receptor agonists/antagonists (**Chapter 6**) and inhibitors specific to different intracellular transduction mechanisms, may help to clarify the paradoxical MAP response observed following intrathecal injection of PACAP-38. The results of the present study also indicate a role for PACAP in BAT thermogenesis; a suggestion supported by the finding that mice lacking PACAP-38 find it difficult to maintain normal core body temperature in a cold environment (Gray *et al.*, 2002). Experiments that include measurement of BAT temperature and BAT SNA in response to central administration of PACAP-38 (Madden & Morrison, 2006), and the effects of PACAP on cold- and chemically- evoked BAT thermogenesis, may help to further resolve the role of PACAP thermoregulation.

In summary, this study reports three major findings; first, that the paradoxical MAP response seen after intrathecal PACAP injection (Farnham *et al.*, 2011) cannot be attributed to the differential control of sympathetic outflows at the level of the spinal cord, as measured here. Secondly, the responses to intrathecal PACAP are spinally mediated, and are not a result of activation of higher presympathetic areas such as the RVLM. Thirdly, intrathecal PACAP causes an increase in metabolic rate. We conclude that intrathecal PACAP acts via a spinally mediated mechanism to cause a prolonged widespread sympathoexcitation, and that PACAP causes an increase in metabolic rate that includes an increase in BAT thermogenesis, in the urethane-anaesthetised, vagotomised, paralysed and artificially ventilated male SD rat.

Chapter 6

Results III

Chapter 6: Activation of PAC$_1$ and VPAC receptor subtypes elicits differential physiological responses from sympathetic preganglionic neurons in the anaesthetised rat

Chapter 6: Activation of PAC_1 and VPAC receptor subtypes elicits differential physiological responses from sympathetic preganglionic neurons in the anaesthetised rat

The work in this chapter has been published (**Appendix 1**): <u>Inglott MA</u>, Lerner EA, Pilowsky PM & Farnham MMJ. (2012). Activation of PAC_1 and VPAC receptor subtypes elicits differential physiological responses from sympathetic preganglionic neurons in the anaesthetised rat. *Br J of Pharmacol* **167,** 1089-1098. Methods, abbreviations and formatting have been edited from the published paper.

Contributions: Conception and design of experiments: MAI; Experimental procedures: MAI; Provision of materials for experimental procedures: EAL; Analysis and interpretation of data: MAI, PMP; Manuscript preparation: MAI; Critical revision of manuscript: MAI, EAL, MMFJ, PMP.

6.1 Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) is an excitatory neuropeptide with central and peripheral cardiovascular actions. Intrathecal PACAP increases splanchnic sympathetic nerve activity ($_s$SNA) and heart rate (HR), but not mean arterial pressure (MAP). We hypothesise that the 3 PACAP receptors (PAC_1, $VPAC_1$ and $VPAC_2$) have differential actions in central cardiovascular control, and that their summed effect results in the lack of MAP response observed following intrathecal PACAP injection. The effects of the PACAP receptors on baseline cardiovascular parameters was investigated using selective agonists and antagonists administered into the intrathecal space of urethane-anaesthetised, vagotomised and artificially ventilated male Sprague-Dawley (SD) rats (n = 41). The novel findings of this study are: first, selective activation of the PACAP receptors has differential actions on MAP: PAC_1 increases MAP, when activated by maxadilan; the VPAC receptors decrease MAP, when both are activated with vasoactive intestinal polypeptide (VIP) or when $VPAC_1$ alone is activated. Secondly, PACAP(6-38; the PAC_1 and $VPAC_2$ antagonist) did not cause any significant cardiovascular effects, suggesting that PACAP is not tonically released in the rat spinal cord. The results demonstrate that PACAP-neurotransmission is not responsible for moment-to-moment tonic regulation of central cardiovascular control mechanisms. Nevertheless, the data reveal that PACAP release within the spinal cord

may have pleiotropic effects on sympathetic outflow depending on the postsynaptic receptor type. Here we find that PAC_1 and VPAC receptor subtypes produce opposing blood pressure effects when activated by intrathecal PACAP-38 in the anaesthetised SD rat, resulting in no net MAP change.

Keywords: PACAP, vasoactive intestinal polypeptide, maxadilan, PACAP(6-38), blood pressure, sympathetic, adrenaline, noradrenaline, adrenal medulla

6.2 Introduction

PACAP, a 38 amino acid excitatory neuropeptide, was first discovered in ovine hypothalamic tissue for its ability to stimulate adenylate cyclase. Previous immunohistochemical and *in situ* hybridisation studies reveal that PACAP is distributed in important cardiovascular regions of the medulla oblongata and spinal cord, indicating a possible functional role for PACAP in central cardiovascular control (**section 1.8.3.2**). Physiological studies support these anatomical findings, with significant cardiovascular effects reported following central administration of PACAP, at different sites in the brainstem and spinal cord (Murase *et al.*, 1993; Uddman *et al.*, 1993; Seki *et al.*, 1995; Krowicki *et al.*, 1997; Lai *et al.*, 1997; Farnham *et al.*, 2008; Farnham *et al.*, 2011; Inglott *et al.*, 2011).

A recent study demonstrated PACAP mRNA within bulbospinal, C1 neurons of the rostral ventrolateral medualla (RVLM; >80%; Farnham *et al.*, 2008); a crucial region in both the tonic and reflex control of the cardiovascular system. The functional significance of these PACAP mRNA positive bulbospinal C1 neurons was tested by activation of PACAP receptors in the spinal cord, resulting in tachycardia and sympathoexcitation in normotensive and hypertensive rats (**Chapter 4**; Farnham *et al.*, 2008; Farnham *et al.*, 2011; Inglott *et al.*, 2011). Paradoxically, the sympathoexcitation was not accompanied by an increase in MAP. Thus far, a mechanism to explain this paradox is lacking. The observed sympathoexcitation is prolonged, and is found in multiple sympathetic beds, so that the MAP effect cannot be attributed to differential control of sympathetic outflow (**Chapter 5**; Inglott *et al.*, 2011).

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There are three known PACAP receptors (**section 1.8.2**): the PACAP specific, PAC_1 receptor (PAC_1; K_d ~0.5-1 nM), and two other receptors that have equal binding affinity (K_d ~1 nM) for VIP and PACAP, the $VPAC_1$ receptor ($VPAC_1$) and the $VPAC_2$ receptor ($VPAC_2$). Differential responses to PACAP are known to occur in peripheral sites depending on the post-synaptic receptor complement (**section 1.8.2.3**). Here we test the hypothesis that the different PACAP receptors have differential actions on central cardiovascular control. Specifically, that the response to PACAP-38 (from here on referred to as PACAP) depends on the post-synaptic receptor(s) present on different populations of sympathetic preganglionic neurons (SPN); for example, those adrenal chromaffin cells that secrete adrenaline (Ad), compared with those secreting noradrenaline (NAd; Payet *et al.*, 2003; DeHaven & Cuevas, 2004; Fizanne *et al.*, 2004; Sawmiller *et al.*, 2006; Igarashi *et al.*, 2008). The aims of this study were to determine the contribution of each of the 3 PACAP receptors, alone and in combination, on cardiovascular parameters, at the level of the spinal cord. Specifically: 1) to determine the involvement of PAC_1 in the intrathecal PACAP response with maxadilan (PAC_1 selective agonist), 2) to determine the combined involvement of $VPAC_1$ and $VPAC_2$ in the response to intrathecal PACAP using VIP ($VPAC_1$ and $VPAC_2$ selective agonist), 3) to determine the sole involvement of $VPAC_1$ in the intrathecal PACAP response with PACAP(6-38; PAC_1 and $VPAC_2$ antagonist) and PACAP and 4) to investigate the role of PAC_1 and $VPAC_2$ in tonic blood pressure control in the SD rat using PACAP(6-38) alone. The results demonstrate that PACAP is not involved in the tonic regulation of blood pressure, at the level of the spinal cord. However, activation of the PAC_1 and VPAC receptor subtypes produce opposing effects on blood pressure when activated by their specific agonists/antagonists.

6.3 Methods

6.3.1 Ethics approval

All Experiments were carried out on adult male SD rats (n = 48; 350-500 g; Animal Resources Centre, Perth, Australia), with the approval of the Animal Care and Ethics Committee of Macquarie University. Experiments were conducted with strict accordance to guidelines set by the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

6.3.2 General preparation

The animals were housed at the Macquarie University Animal Facility in high-top cages ($n \leq 6$) with environmental enrichment and access to food and water *ad libitum*. The environment was conditioned to have fixed daily 12 hour light/dark cycles and temperature was maintained at 21°C ± 2°C. Anaesthesia was induced by bolus injection of urethane (ethyl carbamate; Sigma-Aldrich, Sydney, NSW, Australia; 10% urethane w/v in saline; 1.3-1.5 g/kg i.p.). Anaesthetic depth was monitored by observing reflex responses to nociceptive stimuli (e.g. regular paw/tail pinches) and constant monitoring of blood pressure. Additional doses (30-40 mg; i.v.) of urethane were administered to suppress nociceptive or hypertensive reflex responses (>10 mmHg) to pinches. Atropine Sulfate (100 µg/kg i.p.; Astra Pharmaceuticals, Australia) was administered to reduce bronchial secretions. All rats were secured in a stereotaxic frame. The use of a rectal probe, connected to a heating blanket (Harvard Apparatus, Holliston, MA, USA), allowed core body temperature to be maintained between 36.5°C and 37.5°C throughout the experiment.

6.3.3 Surgical preparation

In General surgical preparation was carried out on all animals as previously described (**Chapter 3**; Farnham *et al.*, 2008; Gaede *et al.*, 2009; Inglott *et al.*, 2011). The right common carotid artery and external jugular vein were cannulated for the recording of blood pressure and administration drugs and fluids, respectively. The trachea was intubated to permit artificial ventilation with O_2-enriched air (rodent ventilator; UGO Basile, Biological Research Apparatus, Italy) and CO_2 monitoring (Capstar-100 CO_2 analyser; CWE Inc. USA). HR was derived from an electrocardiogram (ECG) recording. The rats were bilaterally vagotomised, connected to a ventilator, and subsequently paralysed with pancuronium bromide (0.8 mg given as a 0.4 ml bolus i.v. followed by an i.v. infusion of 10% pancuronium in 0.9% saline at a rate of 2 ml/hr; Astra Pharmaceuticals, Australia). The left splanchnic sympathetic nerve was isolated, dissected and prepared for recording on a silver bipolar electrode (2 kHz sampling, 1k-100k x gain, 0.1-2 kHz filtering); a 50/60 Hz line frequency filter (Humbug; Quest Scientific) was also used.

An intrathecal catheter (polyvinyl chloride (PVC); OD, 0.61 mm; ID, 0.28 mm, Critchley Electrical Products, Australia) with a dead space of 6 µl, was inserted

into the intrathecal space, through a slit in the dura at the atlanto-occipital membrane and advanced caudally to the spinal level of T5/6. All intrathecal injections (10 µl of drug washed in with 6 µl of 10 mM phosphate buffered 0.9% saline (PBS)) were made using a Hamilton syringe (Hamilton Company, USA) connected to the catheter tubing. All injections were done over a 10 to 15 second period. All rats received control injections of the vehicle, PBS (10 µl of PBS washed in with 6 µl PBS), 30 minutes prior to their treatments as described below (**section 6.3.4**).

At the conclusion of the experiments, all rats were euthanised with potassium chloride (0.5 ml 3M KCl i.v.). Postmortem verification of the location of the intrathecal catheter was achieved by injecting 10 µl of India ink washed in with 6 µl of PBS into the catheter and exposing the spinal cord. The spinal segment level of the catheter was recorded as the level where the tip was observed or where the blue/black spot appeared most intensely on the spinal cord, if the catheter was not found at T5/6, the results were not included in the present study.

6.3.4 Intrathecal drug administration
6.3.4.1 Role of PAC_1, $VPAC_1$ and $VPAC_2$ in the response to PACAP

PAC_1 involvement; Recombinant maxadilan, was used as a selective PAC_1 agonist (K_d ~0.5 nM; Lerner & Shoemaker, 1992; Moro & Lerner, 1997; Vaudry *et al.*, 2009). A maxadilan dose response curve was constructed by injecting PBS, and 30 µM, 100 µM, and 300 µM concentrations of maxadilan cumulatively (n = 3), responses were observed for 30 minutes (or until parameters returned to baseline) before the next consecutive dose was injected. These results were then compared to a 1000 µM dose injected intrathecally into a separate group of rats (n = 5; 90 minute recording period; the peak responses within the first 30 minutes post-injection were used for the dose response curve analysis). The 1000 µM dose of maxadilan was excluded from the cumulative dose response curve due to a limited supply of the peptide.

The VIP ($VPAC_1$ and $VPAC_2$) antagonist, [D-*p*-Cl-Phe[6], Leu[17]]-VIP, was also used in pilot studies (n = 7), as a method for isolating the actions of PAC_1 in the intrathecal PACAP response. A dose response curve was constructed for the VIP antagonist (PBS, 30, 100, 300 and 1000 µM), which was then injected (1000 µM) 15

minutes prior to 1000 μM VIP in separate animals. The VIP antagonist was ineffective at antagonising the effects of intrathecal VIP. The concentration of the VIP antagonist needed to maximally inhibit the response of VIP is estimated to be >1000 times greater than the VIP dose used (Pandol *et al.,* 1986). The use of this reagent was therefore discontinued, as its use with PACAP was unachievable in this preparation.

VPAC$_1$ and VPAC$_2$ involvement; A preliminary cumulative dose response study was conducted to determine the appropriate intrathecal dose of VIP (Auspep, Melbourne, VIC, Australia), the VPAC$_1$ and VPAC$_2$ selective agonist (K_d = 1 nM). Injections of PBS and, 30 μM, 100 μM, 300 μM and 1000 μM VIP were administered to 5 rats and responses were observed for 30 minutes (or until parameters returned to baseline) before the next consecutive dose was injected. The 1000 μM dose was then administered to a separate set of rats (n = 6), responses were recorded for 90 minutes.

The PAC$_1$ antagonist, M65, was also used in pilot studies (n = 6), as a method for isolating the actions of the VPAC receptors in the intrathecal PACAP response. A dose response curve was constructed for M65 (PBS, 1000 and 3000 μM), which was then injected (3000 μM) 15 minutes prior to 500 and 1000 μM maxadilan in separate animals. M65 was ineffective at antagonising the effects of intrathecal maxadilan. 1 μM M65 only partially inhibits the effects of 1 nM maxadilan (Uchida *et al.,* 1998). Therefore, the concentration of M65 needed to maximally inhibit the responses to maxadilan is estimated to be ~1000 times greater than the maxadilan dose used. The use of this reagent was therefore discontinued, as its use with PACAP was unachievable in this preparation.

VPAC$_1$ involvement; Rats were pretreated with 1000 μM PACAP(6-38; Auspep, Melbourne, VIC, Australia), a competitive PAC$_1$ and VPAC$_2$ antagonist (K_d = 1.5 nM; Robberecht *et al.*, 1992; Vaudry *et al.*, 2009; Farnham *et al.*, 2011), for 15 minutes (n = 6) and 30 minutes (n = 4) prior to PACAP-38 (1000 μM; Auspep, Melbourne, VIC, Australia) administration. Responses were recorded for 60 minutes following PACAP injection.

6.3.4.2 Role of PAC$_1$ and VPAC$_2$ in tonic blood pressure control

In 6 animals, 1000 μM PACAP(6-38) was administered alone, responses were recorded for 30 minutes.

6.3.5 Data acquisition and analysis

Data was acquired using a CED 1401 ADC system (Cambridge Electronic Design, UK) and Spike 2 acquisition and analysis software (Cambridge Electronic Design, UK). $_s$SNA raw data was rectified and smoothed/averaged to 2 seconds and normalised to '0' by subtracting residual activity 5-10 minutes after death. MAP, HR and $_s$SNA were analysed from 5 minute blocks taken 10 and 5 minutes prior to and 5, 10, 20, 30, 40, 50, 60 and 90 minutes after intrathecal injections of PBS (up to 30 minutes), PACAP(6-38; up to 30 minutes), maxadilan (up to 90 minutes), PACAP(6-38) + PACAP (up to 60 minutes) or VIP (up to 90 minutes). Statistical analysis was carried out in GraphPad Prism software. Statistical significance was determined using one- or two- way ANOVAs with Bonferroni's corrections, unless otherwise stated. $P < 0.05$ was considered to indicate a significant difference between the means.

6.4 Results

6.4.1 Dose response effects of maxadilan and VIP

Dose response curves were generated for both maxadilan and VIP (**Figure 6.1**), to determine an effective dose for use in this study. The maxadilan dose response curve was generated by injecting 30, 100 and 300 μM concentrations of maxadilan cumulatively into one group of rats (n = 3), and then by injecting 1000 μM maxadilan into a separate group of 5 rats. Maxadilan dose response curve data was recorded for 30 minutes. Of the four doses used, only the 1000 μM concentration of maxadilan was effective in significantly elevating both HR (Δ 83 ± 7 bpm; $P < 0.0001$) and $_s$SNA (Δ 70 ± 9%; $P < 0.0001$; **Figure 6.1**). The 300 μM concentration of maxadilan also increased $_s$SNA (Δ 41 ± 9%; $P < 0.01$; **Figure 6.1C**). MAP was unaffected by any of the maxadilan concentrations used within the first 30 minutes post-injection (**Figure 6.1A**), but was increased at 90 minutes by the 1000 μM dose (results described below; **section 6.4.2**). The VIP dose response curve (**Figure 6.1**) was generated by injecting 30, 100, 300 and 1000 μM doses of VIP cumulatively in 5 rats. VIP dose response curve data was recorded for 30 minutes.

Only the 1000 µM concentration of VIP increased HR (Δ 20 ± 4 bpm; $P < 0.01$) and $_s$SNA (Δ 32 ± 9%; $P < 0.01$), and decreased MAP (Δ -36 ± 5 mmHg; $P < 0.0001$; **Figure 6.1**). Therefore, given the similar K_d values of PACAP, maxadilan and VIP (for the specific target receptors), and the dose response data, the 1000 µM concentration of maxadilan and VIP were used in the remainder of this study.

6.4.2 Cardiovascular effects of PAC_1, $VPAC_1$ and $VPAC_2$ activation in the spinal cord

PAC_1 involvement; Activation of PAC_1 with maxadilan, a novel PAC_1 selective agonist, caused a pressor response after 40 minutes (**Figures 6.2** and **6.3**), and a prolonged tachycardia and sympathoexcitation (**Figures 6.2** and **6.3**). The responses to the 1000 µM dose of maxadilan were recorded for 90 minutes. Over the 90 minute recording period, 1000 µM intrathecal maxadilan significantly increased MAP (Δ 17 ± 5 mmHg; $P < 0.01$; n = 5), HR (Δ 99 ± 8 bpm; $P < 0.0001$; n = 5) and $_s$SNA (Δ 236 ± 44%; $P < 0.0001$; n = 5) when compared to vehicle (**Figures 6.2** and **6.3**). The HR and $_s$SNA responses to 1000 µM maxadilan increased over the experimental time period (**Figures 6.2** and **6.3**), whereas, the MAP response remained unchanged for the first 30 minutes of response, and then increased above baseline after this time (**Figures 6.2** and **6.3**).

$VPAC_1$ and $VPAC_2$ involvement; Activation of the $VPAC_1$ and $VPAC_2$ receptors with VIP, the endogenous $VPAC_1$ and VPAC2 agonist, caused hypotension with increases in HR and $_s$SNA. The responses to VIP (1000 µM) were recorded for 90 minutes; there was a significant decrease in MAP (Δ -51 ± 4 mmHg; $P < 0.0001$; n = 6), but significant increases in HR (Δ 30 ± 6 bpm; $P < 0.05$; n = 6) and $_s$SNA (Δ 30 ± 6%; $P < 0.01$; n = 5), when compared to PBS (**Figures 6.3** and **6.4**). HR and $_s$SNA responses peaked ~10-15 minutes following VIP administration, and remained above baseline for the remainder of the experimental period (**Figures 6.3** and **6.4**). MAP rapidly decreased in response to VIP, reaching its lowest level only 5-10 minutes after VIP injection. MAP returned to baseline after 50 minutes and then decreased again for the remainder of the experimental period (**Figures 6.3** and **6.4**).
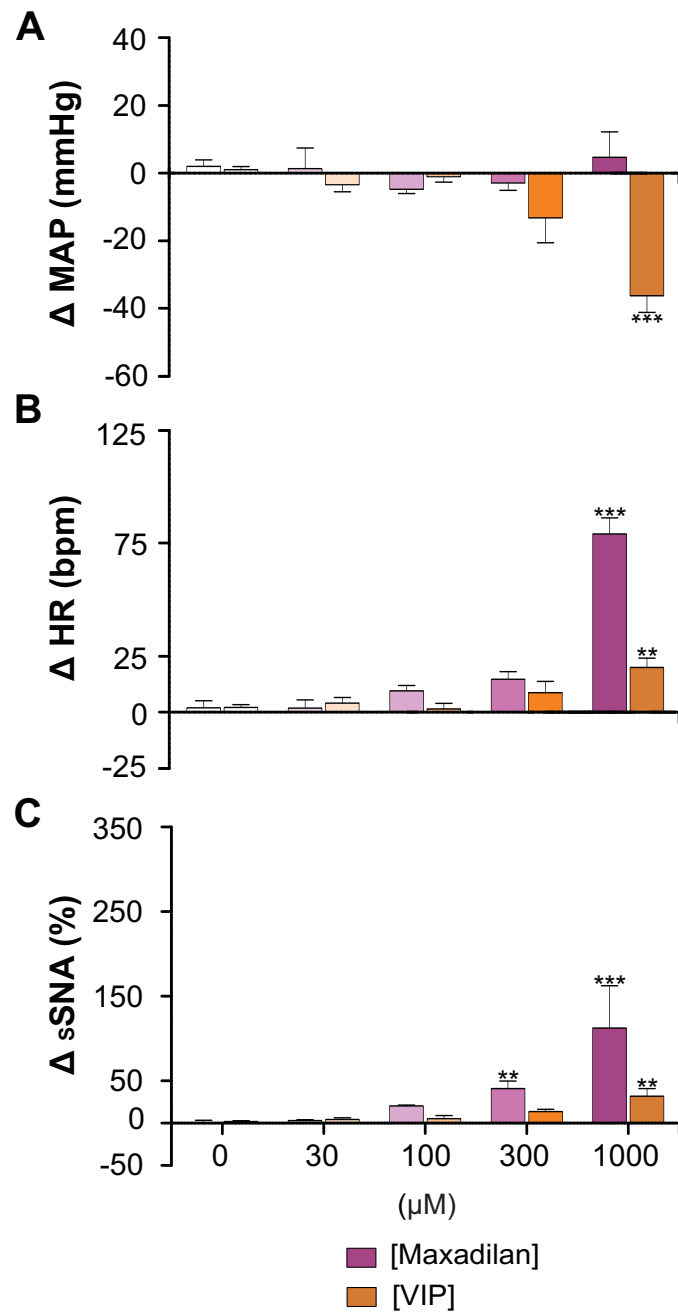
Figure 6.1 Maxadilan and VIP dose response curves
Showing the changes in (**A**) mean arterial pressure (MAP), (**B**) heart rate (HR) and (**C**) splanchnic sympathetic nerve activity (sSNA) before (0 μM is a control injection of the vehicle, PBS) and following administration of 10 μl of increasing concentrations of maxadilan or VIP.
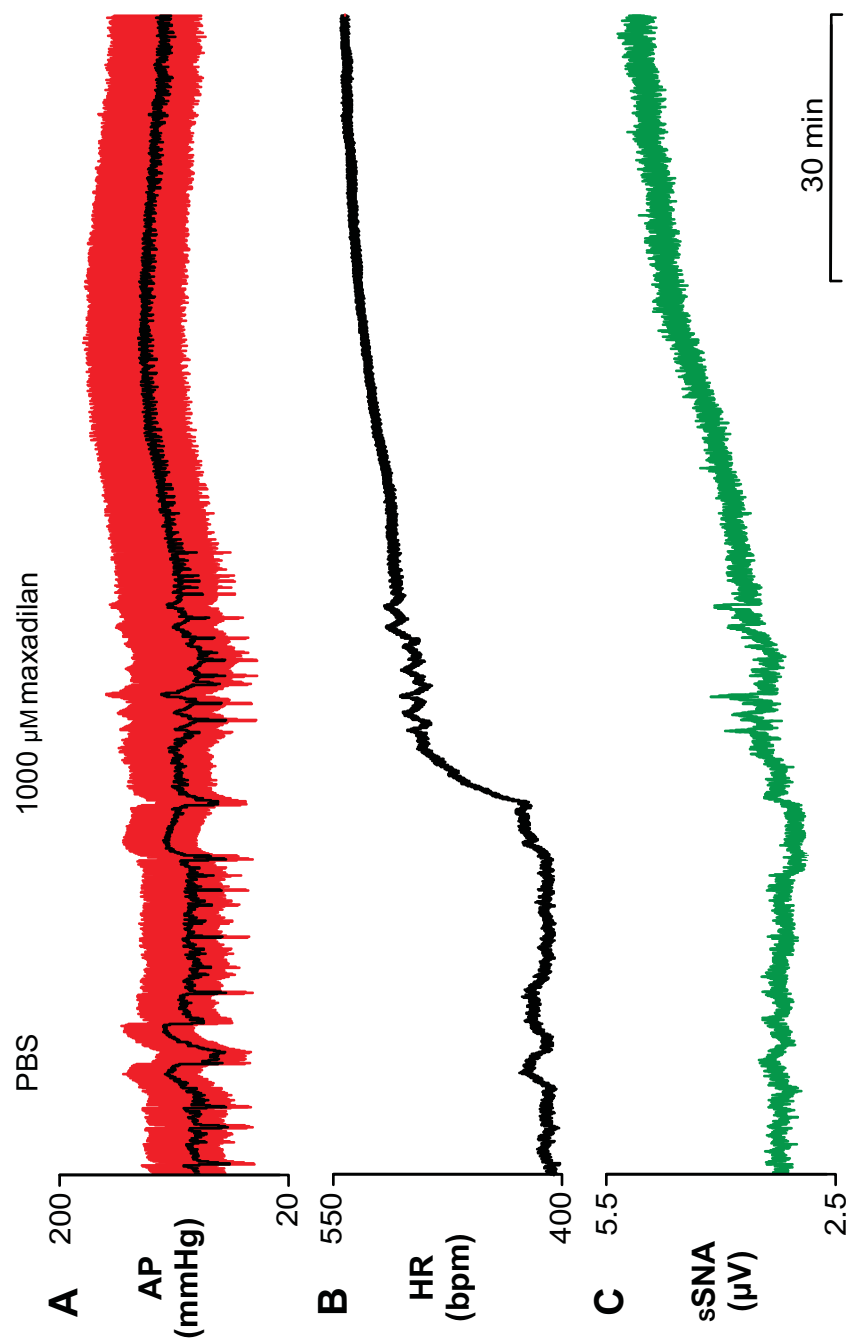
Figure 6.2 *In vivo* **effects of intrathecal PAC₁ receptor activation with maxadilan**

An example trace showing the effects of intrathecal vehicle (PBS) and PAC₁ receptor activation with maxadilan on (**A**) arterial pressure (AP); mean arterial pressure is represented by the black line on the AP trace, (**B**) heart rate (HR) and (**C**) splanchnic sympathetic nerve activity (sSNA) over a 90 minute period. Arrows indicate times of PBS and maxadilan administration. Scale bar represents 30 minutes.
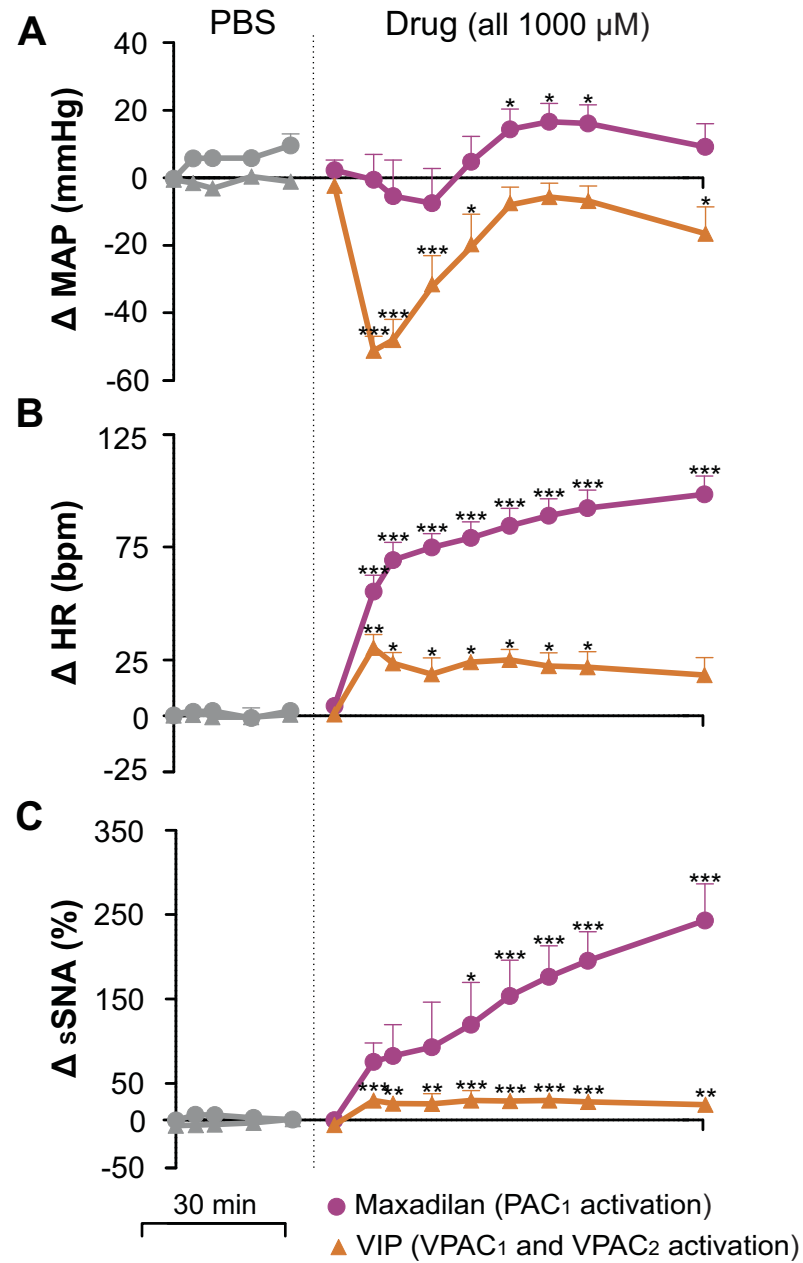
Figure 6.3 Effects of intrathecal PAC1 and VPAC receptor activation with maxadilan and VIP
Time-course changes in (**A**) mean arterial pressure (MAP), (**B**) heart rate (HR) and (**C**) splanchnic sympathetic nerve activity (sSNA) following 1000 μM maxadilan (circle; n = 5) or 1000 μM VIP (triangle; n = 6). Maxadilan significantly increased MAP (Δ 17 ± 5 mmHg; $P < 0.01$; n = 5), HR (Δ 99 ± 8 bpm; $P < 0.0001$; n = 5) and sSNA (Δ 236 ± 44%; $P < 0.0001$; n= 5) when compared to vehicle. VIP caused a significant decrease in MAP (Δ -51 ± 4 mmHg; $P < 0.0001$; n = 6), but significantly increased in HR (Δ 30 ± 6 bpm; $P < 0.05$; n = 6) and sSNA (Δ 30 ± 6%; $P < 0.01$; n = 5), when compared to PBS. Arrow indicates time of maxadilan or VIP injection. 'PBS' is the period after intrathecal infusion of PBS. 'Drug" is the period after intrathecal injection of maxadilan (circle) or VIP (triangle). Scale bar represents 30 minutes.
For both (**A**) and (**B**); * - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.0001$ (compared to PBS control).
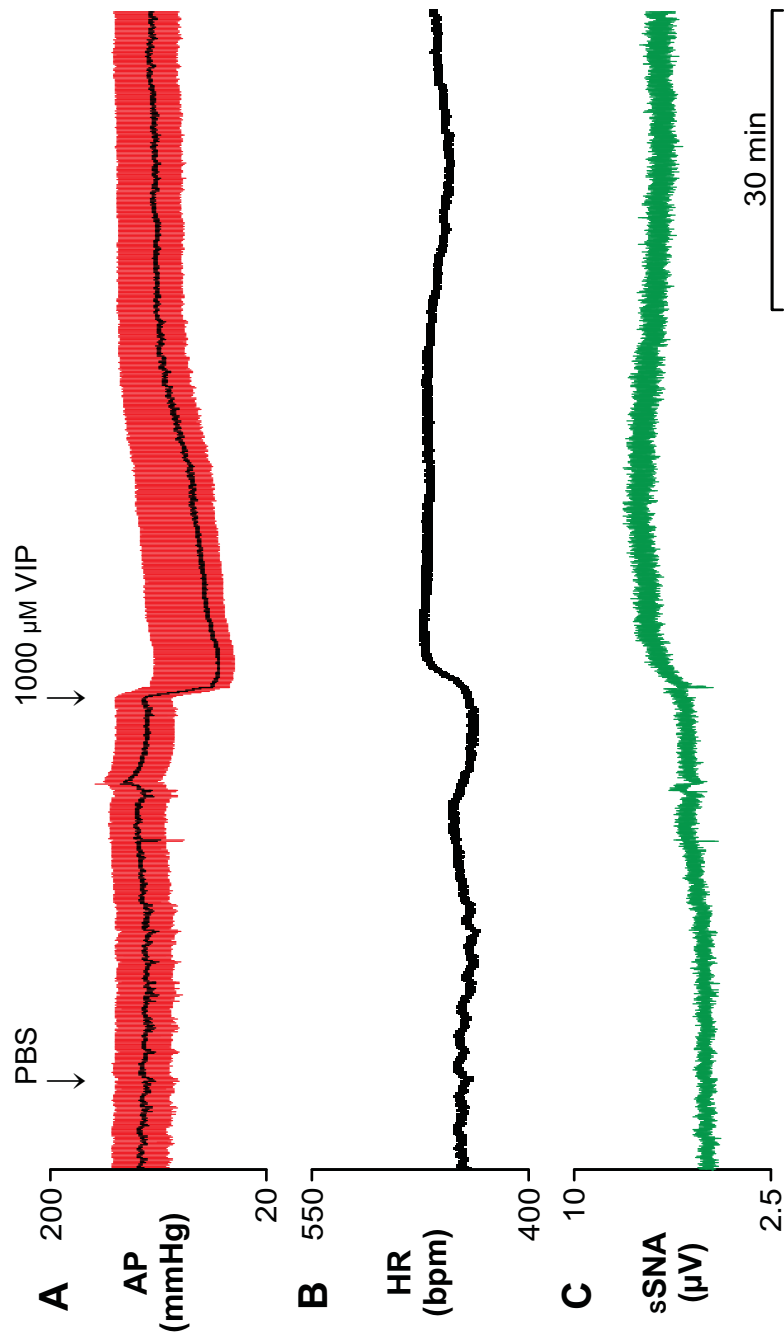
Figure 6.4 *In vivo* effects of intrathecal VPAC receptor activation with VIP
An example trace showing the effects of intrathecal vehicle (PBS) and VPAC receptor activation with VIP on (**A**) arterial pressure (AP); mean arterial pressure is represented by the black line on the AP trace, (**B**) heart rate (HR) and (**C**) splanchnic sympathetic nerve activity (sSNA) over a 90 minute period. Arrows indicate times of PBS and VIP administration. Scale bar represents 30 minutes.

VPAC$_1$ involvement; The VPAC$_1$ was activated with an intrathecal injection of 1000 µM PACAP 15 minutes after intrathecal injection of the PAC$_1$ and VPAC$_2$ antagonist, PACAP(6-38; 1000 µM). The responses to VPAC$_1$ activation were recorded for 60 minutes and caused a significant decrease in MAP (Δ -20 $\pm$ 7 mmHg; $P < 0.01$; n = 6), and significant increases in both HR (Δ 17 $\pm$ 4 bpm; $P < 0.0001$; n = 6) and $_S$SNA (Δ 32 $\pm$ 14%; $P < 0.01$; n = 6) when compared to vehicle (**Figures 6.5** and **6.6**). All parameters reached a plateau ~10-15 minutes after VPAC$_1$ activation (PACAP(6-38) + PACAP) and stayed elevated for the remainder of the experimental period (**Figures 6.5** and **6.6**). The HR and $_S$SNA responses to VPAC$_1$ activation (PACAP(6-38) followed by PACAP), were significantly ($P < 0.05$) blunted at all time points, when compared with activation of all 3 receptors, with PACAP alone (Farnham *et al.*, 2008; Inglott *et al.*, 2011; **Figure 6.7**). A separate group of 4 rats were pretreated with PACAP(6-38) for 30 minutes prior to PACAP administration, this did not produce significant attenuation of the PACAP response (data not shown).

6.4.2.1 Role of PAC$_1$ and VPAC$_2$ in tonic blood pressure control

In another group of 6 rats, PACAP(6-38) alone was administered to test the hypothesis that activation of PAC$_1$ and VPAC$_2$ is involved in tonic blood pressure control in the SD rat. Intrathecal injection of 1000 µM PACAP(6-38) had no significant effects on MAP, HR or $_S$SNA when compared to PBS ($P > 0.05$; n = 6; **Figures 6.6** and **6.7**).

6.4.3 Comparisons of receptor subtype responses

Comparison between individual receptors; When comparing PAC$_1$ activation (maxadilan) and VPAC$_1$ and VPAC$_2$ activation (VIP), the MAP responses are significantly different ($P < 0.0001$), with PAC$_1$ activation causing an increase and VPAC$_1$ and VPAC$_2$ activation causing a decrease (**Figure 6.7**). The HR and $_S$SNA responses are significantly augmented by PAC$_1$ activation ($P < 0.0001$), when compared with VPAC$_1$ and VPAC$_2$ activation.

When the results of VPAC$_1$ activation are compared to the results of maxadilan (PAC$_1$ activation), the MAP, HR and $_S$SNA responses are significantly different ($P < 0.0001$). VPAC$_1$ activation decreases MAP while PAC$_1$ activation

increases MAP (**Figure 6.7**). PAC_1 activation caused significantly higher ($P >$ 0.0001) HR and $_sSNA$ responses compared to $VPAC_1$ activation (**Figure 6.7**).

When the results of $VPAC_1$ activation are compared to the results of VIP ($VPAC_1$ and $VPAC_2$ activation), only the MAP response was significantly different ($P < 0.0001$). The decrease in MAP following $VPAC_1$ activation was significantly blunted ($P < 0.0001$) when compared to the MAP response following $VPAC_1$ and $VPAC_2$ activation (**Figure 6.7**). There was no significant difference between the HR and $_sSNA$ responses of $VPAC_1$ activation compared with $VPAC_1$ and $VPAC_2$ activation ($P > 0.05$; **Figure 6.7**).

Individual receptor subtype responses compared to overall PACAP response; PAC_1 activation caused greater changes in MAP, HR and $_sSNA$ ($P < 0.0001$) when compared to the previously reported PACAP response (**Figure 6.7**). While, $VPAC_1$ and $VPAC_2$ activation caused a significantly greater fall in MAP ($P < 0.0001$), attenuated ($P < 0.05$) the $_sSNA$ response, but had no effect on HR ($P > 0.05$) when compared to PACAP alone (**Figure 6.7**).

When comparing the MAP responses of PACAP alone and $VPAC_1$ activation, PACAP alone did not cause a significant change in MAP (Farnham *et al.*, 2008; Inglott *et al.*, 2011), whereas, in this study we show that $VPAC_1$ activation results in a significant ($P < 0.01$), ~20 mmHg, drop in MAP (**section 6.4.2**). As stated above (**section 6.4.2.1**), HR and $_sSNA$ responses to $VPAC_1$ activation, were significantly ($P < 0.05$) blunted at all time points, when compared to PACAP alone (**Figure 6.7**).

6.5 Discussion and conclusions

This is the first study to investigate the pharmacological mechanisms by which intrathecal PACAP exerts potent sympathoexcitatory effects with no corresponding MAP change, and to investigate a possible role for PACAP in the tonic control of blood pressure in the SD rat (Farnham *et al.*, 2008; Inglott *et al.*, 2011). The novel findings of this study are: first, that the three PACAP receptor subtypes have distinct actions when selectively activated. PAC_1 increases MAP, while the VPAC receptors decrease MAP. This study is also the first to report the
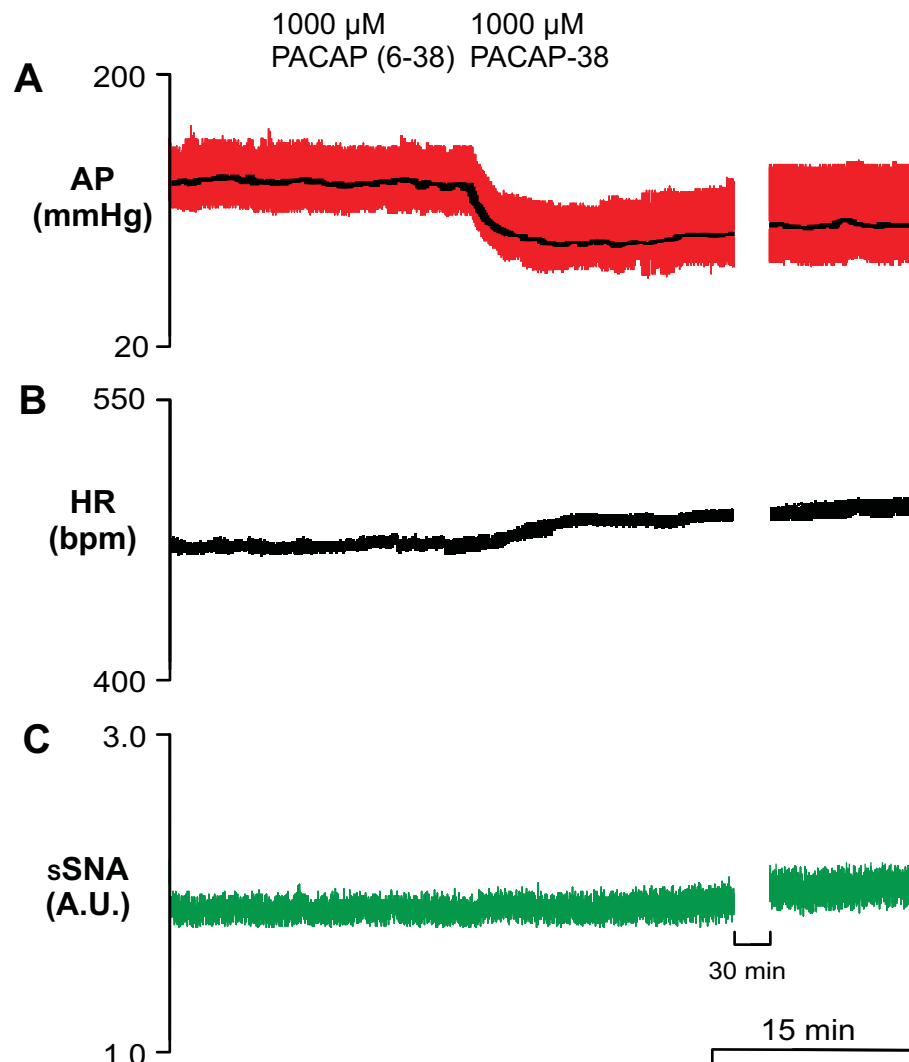
Figure 6.5 *In vivo* effects of VPAC₁ activation with 15 minutes of PACAP(6-38) followed by PACAP

An example trace demonstrating the effect of VPAC₁ activation, following intrathecal PACAP(6-38) followed by PACAP, on (**A**) arterial pressure (AP); mean arterial pressure is represented by the black line on the AP trace, (**B**) heart rate (HR) and (**C**) splanchnic sympathetic nerve activity (sSNA) over 15 minute and one hour recording periods, respectively. Arrows indicate times of 1000 μM PACAP(6-38) and 1000 μM PACAP administration. Note the lack of effect of PACAP(6-38). Scale bar represents 15 minutes.

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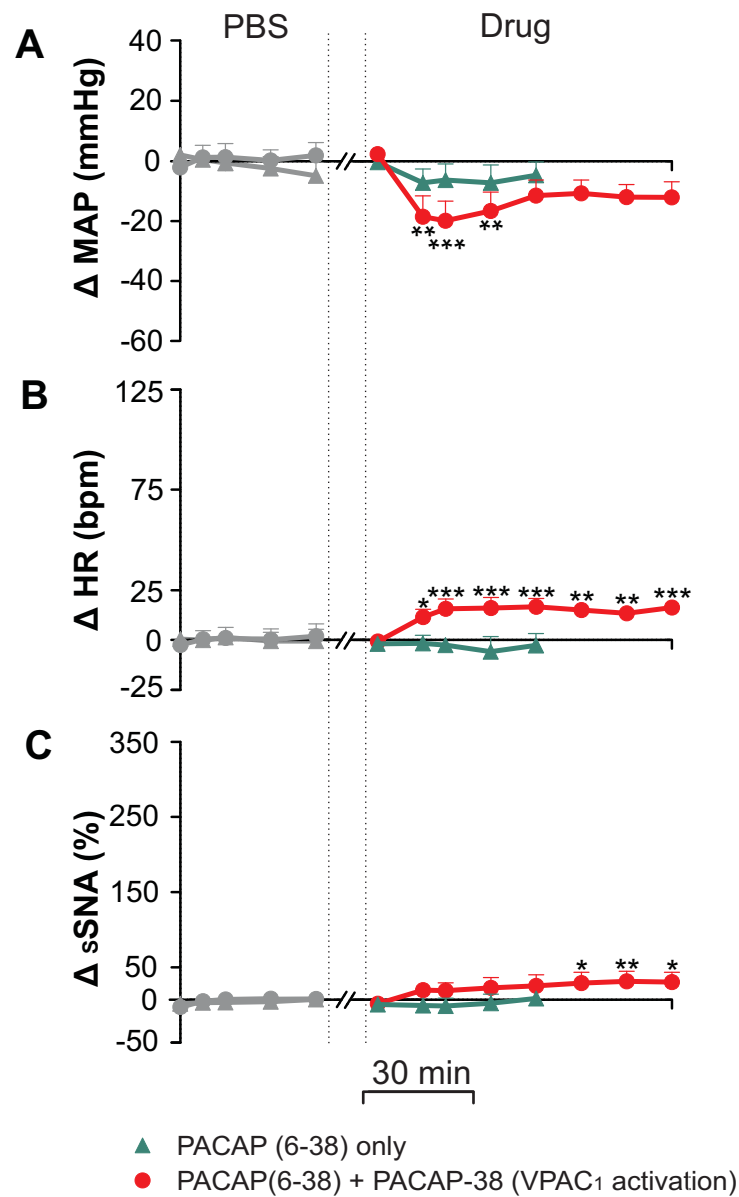
Figure 6.6 *In vivo* effects of VPAC1 activation with 15 minutes of PACAP(6-38) followed by PACAP

Grouped changes in (**A**) mean arterial pressure (MAP), (**B**) heart rate (HR) and (**C**) splanchnic sympathetic nerve activity (sSNA) after PACAP(6-38) only (triangle; n = 6) and after VPAC1 activation (circle; n = 6). PACAP(6-38) alone does not affect MAP, HR and sSNA when compared to PBS ($P > 0.05$; n = 6). PACAP(6-38) followed by PACAP (VPAC1 activation) causes a significant decrease in MAP (Δ -20 $\pm$ 7 mmHg; $P < 0.01$; n = 6), and a significant increase in both HR (Δ 17 $\pm$ 4 bpm; $P < 0.0001$; n = 6) and sSNA (Δ 32 $\pm$ 14%; $P < 0.01$; n = 6) when compared to vehicle. Arrow indicates time of PACAP(6-38) or PACAP injection. 'PBS' is the period after intrathecal infusion of PBS. 'Drug' is the period after intrathecal injection of PACAP(6-38) only (triangle) or PACAP injection following PACAP(6-38) (circle). Scale bar represents 30 minutes. For both (**A**), (**B**) and (**C**); * - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.0001$ (compared to PBS control).
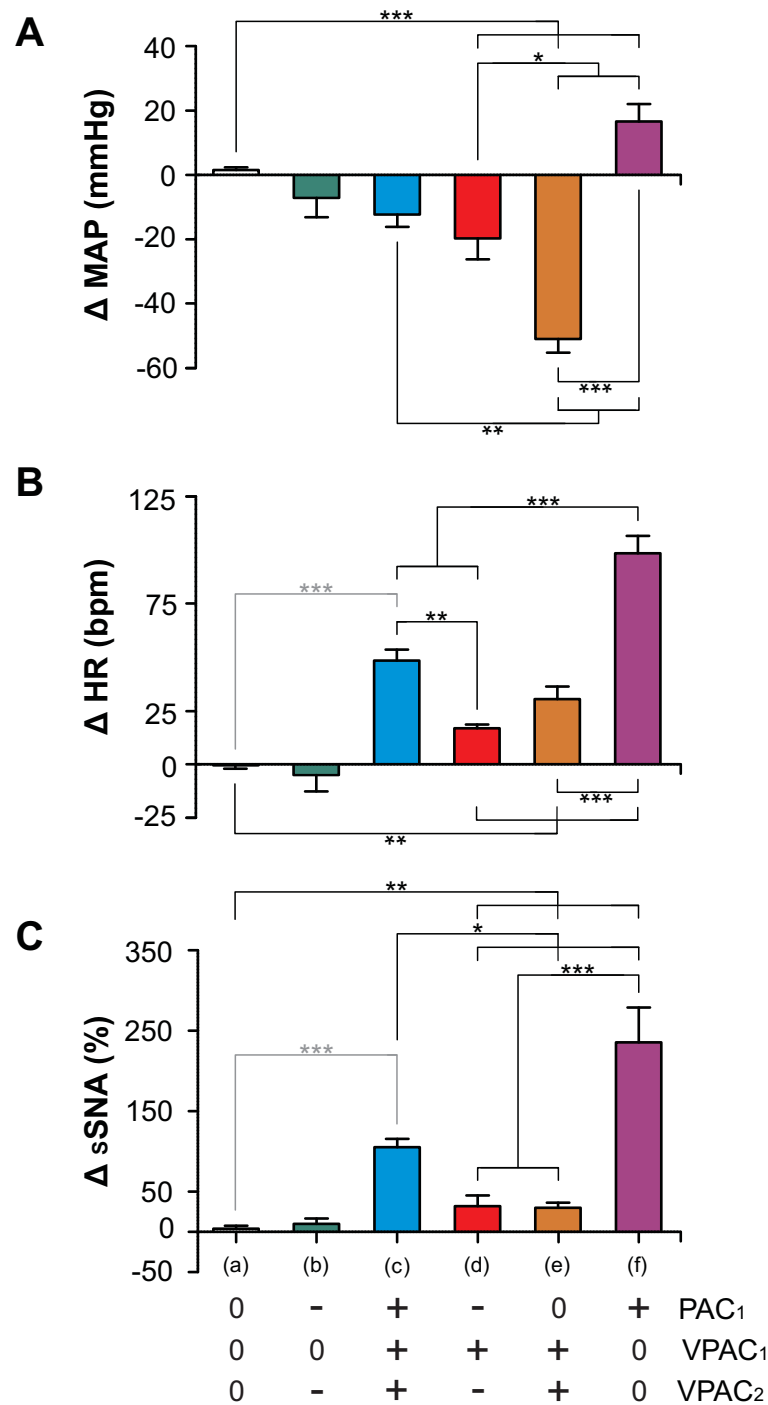
Figure 6.7 Peak responses following intrathecal activation of the PACAP receptors
Peak changes in (**A**) mean arterial pressure (MAP), (**B**) heart rate (HR) and (**C**) splanchnic sympathetic nerve activity (sSNA) following differential activation of the three PACAP receptors. In this figure, the treatment groups (abscissa) are as follows; (**a**) PBS (no PACAP receptors activated), (**b**) PACAP(6-38) only (PAC$_1$ and VPAC$_2$ antagonised), (**c**) PACAP-38 (all PACAP receptor activated), (**d**) 15 minutes of PACAP(6-38) + PACAP-38 (VPAC$_1$ activated, other receptors are antagonised), (**e**) VIP (VPAC$_1$ and VPAC$_2$ only activated) and (**f**) maxadilan (PAC$_1$ only activated). The PACAP data (blue) is taken from Inglott *et al.,* (2011), significance values of these earlier data are shown in grey. The HR and sSNA responses to PACAP (blue) are significantly ($P < 0.05$) attenuated by pretreatment with PACAP(6-38) (red).
* - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.0001$. (+) - activated (injection of agonist);
(-) - antagonised (injection of antagonist); (0) - neither activated nor antagonised

effects of intrathecal injection of maxadilan (a PAC_1 agonist) and VIP (a $VPAC_1$ and $VPAC_2$ agonist that has no effect on PAC_1; **Figures 6.2, 6.3, 6.4** and **6.7**). Secondly, intrathecal injection of PACAP(6-38; PAC_1 and $VPAC_2$ antagonist) caused no significant cardiovascular effects, suggesting that PACAP is not tonically released in the spinal cord of the SD rat (**Figure 6.7**), or if it is, it's effects are balanced by other mechanisms. Therefore, we conclude that endogenous PACAP and/or VIP, released in response to a stimulus, may differentially activate PACAP receptors in the spinal cord to modulate efferent sympathetic nerve activity. Following intrathecal injection of a high concentration of PACAP, all three receptors are activated, so that the sum of these differential effects abrogates any MAP response (Farnham *et al.*, 2008; Inglott *et al.*, 2011). We propose as a mechanism, that spinal PACAP receptors are differentially expressed on SPN that are responsible for Ad or NAd secretion from the adrenal chromaffin cells, and on SPN regulating the heart and blood vessels (**Figure 6.8**).

In this study, we measured responses to intrathecal administration of selective PACAP receptor agonists and antagonists. When compared to the previous intrathecal PACAP response (Inglott *et al.*, 2011), the HR and $_s$SNA responses to PAC_1 activation were augmented significantly, and the MAP was increased by PAC_1 activation (responses to the selective agonist maxadilan). The results demonstrate that PAC_1 primarily mediates the tachycardia and sympathoexcitation observed following intrathecal PACAP administration; PAC_1 also increases MAP, an increase that is potentially masked by the effects of activating VPAC receptors. We hypothesise that the hypertension following maxadilan injection may be due to PAC_1 mediated NAd release from adrenal chromaffin cells (Payet *et al.*, 2003).

Our study is the first to report the effects of central administration of maxadilan *in vivo*, in an anaesthetised rat preparation. The results of the present study suggest that when administered centrally, maxadilan, and therefore activation of PAC_1, has an excitatory effect on SPN, resulting in hypertension mediated by sympathetic vasoconstriction and tachycardia. Although *in vivo* activation of PAC_1 peripherally, with PACAP or maxadilan, results in potent peripheral vasodilation (Lerner *et al.*, 2007; Vaudry *et al.*, 2009), both PACAP and maxadilan increase intracellular cAMP and Ca^{2+} (Grevelink *et al.*, 1995; Jackson *et al.*, 1996;

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Eggenberger *et al.*, 1999; Pirger *et al.*, 2010), and would therefore be expected to have similar central functions.

Intrathecal PACAP administration activates all 3 receptors leading to widespread sympathoexcitation, but surprisingly, no net effect on MAP (Farnham *et al.*, 2008; Farnham *et al.*, 2011; Inglott *et al.*, 2011). We propose that this lack of a hypertensive response is due to a balance in the sympathetic efferent effects of PAC_1 compared with $VPAC_1$/$VPAC_2$ activation (**Figure 6.8**). This hypothesis is supported by the finding that activation of $VPAC_1$ and $VPAC_2$, with VIP, causes a significantly greater decrease in MAP, and markedly smaller increases in HR and $_sSNA$, when compared with PACAP (Inglott *et al.*, 2011). Furthermore, the HR and $_sSNA$ responses to activation of $VPAC_1$ and $VPAC_2$ are significantly smaller when compared with PAC_1 activation. Finally, activation of $VPAC_1$ and $VPAC_2$ (VIP) decreases MAP, while PAC_1 activation (maxadilan) causes an increase in MAP. Thus, the hypotensive response observed following VIP injection suggests that VPAC receptor activation masks the anticipated hypertensive response caused by PAC_1 activation associated with sympathoexcitation and release of NAd from the adrenal medulla (Payet *et al.*, 2003). We propose that this masking is due to a differential excitation of sympathetic pathways, with VPAC receptors activating Ad secreting chromaffin cells (**Figure 6.8**). Resulting in an Ad-mediated tachycardia and vasodilatation (Widegren *et al.*, 2010), with a subsequent fall in total peripheral resistance and net hypotension. The concept of differential regulation of chromaffin cells is supported by extensive electrophysiological (Morrison & Cao, 2000) and anatomical data (Kumar *et al.*, 2010).

Previous studies found that intrathecal VIP, administered at T2, does not affect MAP in the rat (Lai *et al.*, 1997), and that intracerebroventricular VIP does not affect MAP or HR in the trout (Le Mevel *et al.*, 2009). Apart from the differences in sites of VIP administration and species, the much lower doses used in the previous studies may account for the lack of observed MAP and HR responses. Our dose-response data suggest that the dose used in earlier studies would have been insufficient to elicit the effects seen here.
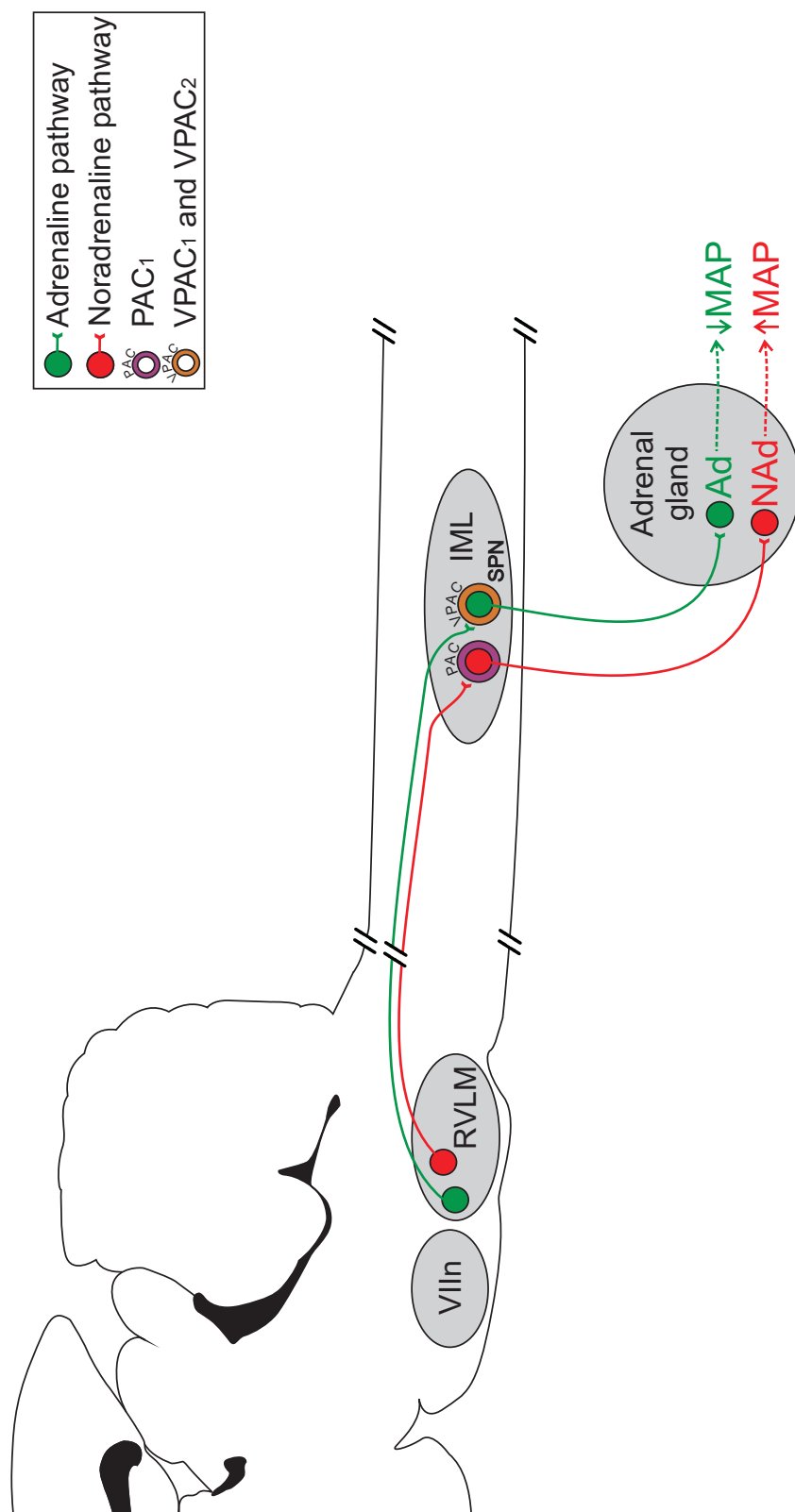
Figure 6.8 PACAP receptor subtypes differentially regulate catecholamine secretion from the adrenal medulla

Given the findings observed in the present study, we propose that PAC1 or VPAC receptors present on SPN differentially regulate noradrenaline and adrenaline secreting chromaffin cells. With PAC1 activating vasoconstrictor and cardioacceleratory pathways, causing noradrenaline release, a pressor response, and sympathoexcitation. Whilst VPAC receptors are present on SPN projecting to adrenaline secreting chromaffin cells causing adrenaline release, with direct vasodilatory depressor response, mild tachycardia and sympathoexcitation. Abbreviations: Ad, adrenaline; IML, intermediolateral cell column of the spinal cord; MAP, mean arterial pressure; NAd, noradrenaline; PAC1, PAC1 receptor; RVLM, rostral ventrolateral medulla; SPN, sympathetic preganglionic neurons; VIIn, facial nucleus; VPAC1, VPAC1 receptor; VPAC2, VPAC2 receptor.

Intrathecal injection of PACAP(6-38) which is an antagonist at PAC_1, and at $VPAC_2$, but with a 10-fold lesser affinity (Robberecht *et al.*, 1992; Harmar *et al.*, 1998; Dickson & Finlayson, 2009), did not affect MAP, HR or $_SSNA$. This finding indicates that PACAP is probably not tonically released from the spinal cord of SD rats in this preparation. It is unlikely that $VPAC_1$ compensates for the tonic activity of PAC_1 and $VPAC_2$ in this study, because the $VPAC_1$ response is different to that of PAC_1 and $VPAC_2$. The results support our previous study, which concluded that PACAP is not released from the spinal cord of the spontaneously hypertensive rats (SHR) or Wistar-Kyoto (WKY) rats (Farnham *et al.*, 2011). Farnham *et al.,* (2011) did observe an initial small increase in MAP following PACAP(6-38) injection in the SHR at the same dose. This response is likely due to a partial agonist effect that can sometimes occur at high concentrations (Ozawa *et al.*, 1997). The stimuli that evokes endogenous release of PACAP from presympathetic neurons is unknown. However, PACAP is frequently implicated in responses to stressors such as Ad release and hemorrhage (Murase *et al.*, 1993; Kuri *et al.*, 2009).

In summary, PACAP is not tonically released in the spinal cord of the anaesthetised SD rat, and therefore is not involved in the tonic control of blood pressure in this preparation, but may be involved in behavioral responses that require long-term changes in the control of the sympathetic nervous system. The absence of a pressor response following intrathecal PACAP injection, despite profound sympathoexcitation (Farnham *et al.*, 2008; Farnham *et al.*, 2011; Inglott *et al.*, 2011), may be due to differential activation of SPN that have different complements of PACAP receptors (**Figure 6.8**). Finally, activation of spinal PAC_1 alone is sympathoexcitatory and causes a significant pressor response, whereas activation of VPAC receptors results in a large depressor response and only mild sympathoexcitation. In conclusion, these data suggest that PAC_1 receptors are part of a sympathetic efferent pathway that is important in the regulation of NAd secretion from the adrenal medulla, and control of efferent sympathetic activity to the heart and blood vessels. On the other hand, we suggest that VPAC receptors play a larger role in control of Ad secretion. These findings have implications for the management of cardiovascular and metabolic disorders.

Chapter 7

Results IV

Chapter 7: Catestatin has an unexpected effect on the intrathecal actions of PACAP dramatically reducing blood pressure

Chapter 7: Catestatin has an unexpected effect on the intrathecal actions of PACAP dramatically reducing blood pressure

The work in this chapter has been published (**Appendix 1**): Gaede AH*, <u>Inglott MA*</u>, Farnham MMJ & Pilowsky PM. (2012). Catestatin has an unexpected effect on the intrathecal actions of PACAP dramatically reducing blood pressure. *Am J Physiol* **303,** R719-R726. The published paper has been altered to include respiratory data.

*Equal first authors

Contributions: Conception and design of experiments: MAI, AHG, PMP; Experimental procedures: MAI, AHG; Data analysis and interpretation of data: MAI, AHG, PMP; Manuscript preparation: MAI, AHG; Critical revision of manuscript: MAI, AHG, MMFJ, PMP.

7.1 Abstract

This study focuses on presympathetic neurons of the rostral ventrolateral medulla (RVLM) that regulate sympathetic vasomotor tone. Many neurotransmitters are co-localised in RVLM neurons and are released under specific conditions to modulate efferent homeostatic responses. Of particular interest here, are two peptides co-localised in catecholaminergic RVLM neurons: catestatin, and pituitary adenylate cyclase-activating polypeptide (PACAP). Chromogranin A (CgA) derived catestatin, is a potent endogenous non-competitive nicotinic and adrenoreceptor antagonist. Catestatin impairs adenylate cyclase and phospholipase C action; mechanisms engaged by PACAP. Although PACAP and catestatin are likely to be co-released, the possible effects of this are unknown. We aimed to determine if catestatin affects the normal sympathoexcitatory, but isotensive responses to intrathecal PACAP, and to determine the effects of intrathecal PACAP and catestatin on respiratory parameters. Urethane-anaesthetised, vagotomised, ventilated Sprague-Dawley (SD) rats (n = 22) were given an intrathecal injection of catestatin at different times prior to intrathecal administration of PACAP-38. Mean arterial pressure (MAP), heart rate (HR), splanchnic sympathetic nerve activity ($_s$SNA), phrenic nerve activity (PNA) and reflex responses to baroreceptor and chemoreceptor activation were recorded. The key findings of this study are that pre-treatment with

catestatin time-dependently enhances the PACAP-38 effect on MAP, and enhances sympathetic barosensitivity and chemosensitivity, while decreasing the effect of PACAP on phrenic nerve frequency (PNf). The time-scale of the effect of catestatin on the response to PACAP-38 strongly suggests that catestatin either causes changes in gene expression to exert its effects, or modifies intracellular signaling mechanisms normally engaged by PAC_1 receptors. The ability of catestatin pre-treatment to enhance barosensitivity and chemosensitivity after PACAP-38 injection, supports the hypothesis that catestatin manipulates the intracellular environment within sympathetic nerves in a way that increases the cardiovascular responses to PACAP.

<u>Keywords:</u> Chromogranin A, sympathetic, baroreflex, chemoreflex, adrenaline

7.2 Introduction

Hypertension is a major human health problem that is largely due to to increased sympathetic drive, emanating from the RVLM (**section 1.3.3** and **section 1.5.6**; Patel *et al.*, 2012). Central neural mechanisms maintain arterial blood pressure by integrating information from reflexes, central behavioral and emotional states to alter efferent sympathetic and parasympathetic activity according to need (**Chapter 1**; Pilowsky & Goodchild, 2002; Guyenet, 2006; Pilowsky *et al.*, 2009). The RVLM plays a critical role in this system as the final integrative region in the regulation of sympathetic tone (**section 1.5.4**) and cardiovascular adaptive reflexes (e.g. the baroreceptor and chemoreceptor reflexes; **section 1.5.5**; Pilowsky *et al.*, 2009). The RVLM contains a functionally heterogeneous cell population that includes barosensitive, presympathetic (C1) neurons that project to sympathetic preganglionic neurons (SPN) in the intermediolateral cell column of the spinal cord (**section 1.5**). SPN regulate sympathetic outflow through innervation of the adrenal medulla and sympathetic post-ganglionic neurons that project to the heart, kidneys and blood vessels. Increased excitability of RVLM neurons, SPN and by extension sympathetic drive targeting organs and blood vessels in the periphery, is critically implicated in the pathophysiology of essential hypertension, a major underlying cause of morbidity and mortality.

Many neurotransmitters are co-localised within adrenergic (C1) and non-adrenergic RVLM bulbospinal neurons (**section 1.5.3**), suggesting that interactions

occur if these neurotransmitters are released together. Such interactions will affect the excitability of SPN, and the sympathetic outflow reaching the target organs in the periphery. However, the nature and importance of such interactions remains unclear. Glutamate is the major excitatory transmitter released by these neurons, but many metabotropic transmitters are also present, and modulate SPN activity (**section 1.6.1**), including: enkephalin (Stornetta *et al.*, 2001), neuropeptide Y (Pilowsky *et al.*, 1987; Minson *et al.*, 1994) and Substance P (Li *et al.*, 2005). We hypothesise that dysfunction of, or interaction between, co-localised or temporally co-released neurotransmitters arising from the RVLM, raphé, hypothalamus or other sites, may contribute to the increased excitability of the RVLM and SPN in essential hypertension. Two vesicular peptides of interest in the current study are catestatin (human $CgA_{352-372}$), a vasoactive CgA cleavage product (Mahata *et al.*, 1997) and PACAP (Miyata *et al.*, 1989). Catestatin is contained within 88% of C1 RVLM neurons, while PACAP-38 is expressed in 85% of bulbospinal C1 RVLM neurons, indicating extensive co-localisation of these neurotransmitters in the RVLM (Farnham *et al.*, 2008; Gaede & Pilowsky, 2010). The separate effects of these peptides, on the cardiovascular system, have been studied following injection into the intrathecal space and into the RVLM (Scruggs *et al.*, 2005; Farnham *et al.*, 2008; Gaede *et al.*, 2009; Shahid *et al.*, 2011), but the physiological significance of this extensive co-localisation is unclear. The role of PACAP in central respiratory control also remains unstudied, despite evidence that it is excitatory in this system (**section 1.8.4.5**; for review see (Farnham & Pilowsky, 2010)).

Catestatin and PACAP are both vasodilator peptides in the periphery (Vaudry *et al.*, 2009; Fung *et al.*, 2010). Intrathecal PACAP increases $_sSNA$ and HR, but does not affect MAP (**section 1.8.4.3**; Farnham *et al.*, 2008). Intrathecal catestatin on its own has no effect on MAP, $_sSNA$, or HR (Gaede *et al.*, 2009), but increases MAP, SNA, PNA and PNf in the RVLM, and significantly enhances the sympathetic baroreflex; though it blunts the hypoxic chemoreflex and somatosympathetic reflexes in the RVLM (**section 1.9.1**; Gaede & Pilowsky, 2010). Catestatin is cardioprotective, and blunts the sympathoexcitatory response to spinal stimulation in the pithed rat (Kennedy *et al.*, 1998; Mazza *et al.*, 2010; Penna *et al.*, 2010). In the spinal cord, catestatin stabilises cardiovascular parameters by attenuating the responses to nicotine and isoproterenol (Gaede *et al.*, 2009). Catestatin may attenuate

the ability of adenylate cyclase and phospholipase C to act intracellularly. Since adenylate cyclase and phospholipase C are two systems engaged by PACAP through the PAC_1 receptor (**section 1.8.2.3** and **section 1.9.2**), we hypothesise that co-application of catestatin prior to PACAP would alter the ability of PACAP to influence MAP, HR $_S$SNA and PNA.

Here, we investigate two different catestatin pre-treatment regimes to examine the temporal effects of catestatin on the response to intrathecal PACAP. The principal objective of this study was to determine the role of catestatin in central cardiorespiratory modulation of the intrathecal PACAP-38 response, and to determine if co-release of PACAP and catestatin within the circuitry controlling sympathetic vasomotor tone may be critical in mediating essential hypertension. Secondly, we aimed to determine the separate effects of intrathecal catestatin and PACAP-38 on basal cardiorespiratory parameters and reflex control of the cardiovascular system.

7.3 Methods

7.3.1 Ethics approval

All procedures and protocols were approved by the Macquarie University Animal Ethics Committee in accordance with the guidelines set forth by the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*. Experiments were conducted on adult male SD rats (400-550 g; Animal Resource Centre, Western Australia) in accordance with these guidelines.

7.3.2 Surgical procedures

General surgical methods were carried out as previously described (Gaede *et al.*, 2009; Inglott *et al.*, 2011), see **Chapter 3** for greater detail. Briefly, anaesthesia was induced in male SD rats (n = 22) with a bolus of intraperitoneal (i.p.) urethane (ethyl carbamate, 10%, 1.3 g/kg in 0.9% saline w/v; Sigma-Aldrich). Additional doses of urethane (30-40 mg in 10% solution, intravenous (i.v.)) were given as needed to maintain a stable degree of anaesthesia. Absence of the withdrawal reflex or lack of arterial pressure changes (>10 mmHg) in response to a hind paw pinch were used to assess the depth of anaesthesia. The right jugular vein and common carotid artery were cannulated for administration of fluids and measurement of

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arterial blood pressure, respectively. The trachea was cannulated to allow for artificial ventilation (rodent ventilator; UGO Basile, Biological Research Apparatus, Italy). Rats were bilaterally vagotomised, paralysed (pancuronium bromide; 0.8 mg bolus, followed by an infusion of 10% pancuronium in 0.9% saline w/v, 2 ml/h; Astra Pharmaceuticals), and ventilated with 100% O_2-supplemented room air. End-tidal CO_2 was maintained between 4.0% and 5.0% by altering the stroke volume and frequency of ventilation as needed (Capstar-100 CO_2 analyser; CWE Inc. USA). Core temperature was maintained at $37\pm0.5°C$. The left phrenic and splanchnic sympathetic nerves were isolated, dissected, and prepared for recording on bipolar platinum electrodes immersed in paraffin oil. Nerve activity was amplified (1k-100k x gain), filtered (30-3,000 Hz), and sampled at 5,000 Hz using an ADC system (model 1401, CED, Cambridge, UK) and Spike2 analysis software.

An intrathecal catheter (polyvinylchloride tubing: ID, 0.2 mm; OD, 0.5 mm, Critchley Electrical Products), with a dead space of approximately 6 µL was inserted through an incision in the dura mater at the atlanto-occipital junction and passed caudally in the sub-arachnoid/intrathecal space to the level of T5/6. Intrathecal injections (PACAP-38, 1 mM, Auspep Pty. Ltd., Australia; catestatin, 0.1 mM, Phoenix Pharmaceuticals) were given in volumes of 10 µL, followed by 6 µL of vehicle (10 mM phosphate buffered saline, PBS; pH 7.4) using a 25 µL Hamilton syringe.

7.3.3 Experimental protocol

All intrathecal injections (10 µl of drug washed in with 6 µl of 10 mM PBS) were made using a Hamilton syringe. All rats received control injections of the vehicle (PBS; 10 µl of PBS washed in with 6 µl PBS) 30 minutes prior to treatment as described below. Cardiovascular reflexes, viz., the baroreflex and peripheral chemoreflex, were each evoked twice at specified times both post-vehicle and during each of the conditions described below. The sympathetic baroreflex was tested using i.v. injections of phenylephrine (PE; 10 µg/kg). Carotid chemoreceptors were activated by ventilating with N_2 for 12 seconds (isocapnic anoxia).

7.3.3.1 Catestatin pre-treatment (15 minutes) followed by PACAP

Intrathecal catestatin was administered (n = 8), and responses recorded for 15 minutes. 5 minutes post-catestatin the baro- and chemo- reflexes were evoked. At the end of the pre-treatment period, PACAP-38 was injected into the intrathecal space (referred to herein as catestatin(15)+PACAP); responses were recorded for 90 minutes. Cardiovascular reflexes were elicited at 10 minutes and 60 minutes post-PACAP-38 injection. The 15 minute pre-treatment period was chosen because intrathecal catestatin demonstrates its maximal effect on the nicotine response after 15 minutes (Gaede *et al.*, 2009).

7.3.3.2 Catestatin pre-treatment (90 minutes) followed by PACAP

In a separate group of rats (n = 4), intrathecal injections of catestatin were given and the responses recorded for 90 minutes. During the pre-treatment period, cardiovascular reflexes were elicited at 0, 20, and 75 minutes post-catestatin injection. Subsequently, PACAP-38 was injected into the intrathecal space (herein referred to as catestatin(90)+PACAP); responses were recorded for a further 90 minutes. Cardiovascular reflexes were tested at 10 and 60 minutes post-PACAP. The 90 minute pre-treatment period was used to determine whether catestatin would exert long-term effects.

7.3.3.3 Intrathecal PACAP-38 and cardiovascular reflexes

Rats (n = 5) were given intrathecal injections of PACAP-38, and responses were recorded for 90 minutes. During this period, the baro-, and chemo-, reflexes were tested at 10 and 60 minutes following PACAP-38 injection

7.3.4 Data analysis

The recording system for nerve activity was calibrated by introducing a known voltage at the amplifier headstage, and was then rectified and smoothed for analysis ($_s$SNA time constant = 1 second, phrenic nerve amplitude (PNAmp) time constant = 50 milliseconds). Postmortem background activity was subtracted from the nerve activity to normalise the data. MAP, HR, $_s$SNA, PNAmp and PNf were analysed from 1 minute averages taken 10 and 5 minutes prior to, and 5, 10, 20, 30, 40, 50, 60, 70, 80 and 90 minutes after intrathecal injections of PBS (up to 30 minutes), catestatin (up to 90 minutes) and PACAP-38 (up to 90 minutes).

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Sympathetic barosensitivity was determined by plotting the $_S$SNA versus MAP responses to i.v. PE. A first-order polynomial was fitted to the steepest portion of these curves, and the slopes were used to compare barosensitivity. The sympathetic response to activation of the peripheral chemoreflex was determined by fitting a Boltzmann sigmoid curve to the rectified, smoothed $_S$SNA response, and to the MAP response, to hypoxia. Slope and range of the fitted curve were then analysed. Statistical analysis was carried out using Prism 5 (GraphPad Software La Jolla, CA). Statistical significance was determined using one- or two- way ANOVAs with Bonferroni's correction. $P < 0.05$ was considered significant.

7.4 Results

7.4.1 In the presence of catestatin, PACAP-38 is hypotensive and sympathoexcitatory

Catestatin(90)+PACAP-38 has a significant depressor effect on MAP compared to baseline (Δ -30 ± 1 mmHg from a baseline of 115 ± 9 mmHg; $P < 0.01$; n = 4) and the untreated PACAP-38 response ($P < 0.05$). The depressor response reached a maximum approximately 10 minutes after the PACAP-38 injection, and remained below baseline for the duration of the experimental period (60 minutes; **Figures 7.1** and **7.2A**). The decrease observed following the untreated PACAP-38 injection was amplified by pre-treatment with catestatin for 90 minutes (**Figure 7.2A**). Catestatin(15)+PACAP-38 does not significantly affect MAP when compared with vehicle (Δ -12 ± 7 mmHg from a baseline of 110 ± 7 mmHg; 60 minute time course; n = 8; **Figures 7.2A** and **7.3**), or when compared with the untreated intrathecal PACAP-38 response (**Figure 7.2A**).

Catestatin(15)+PACAP-38 increased HR (Δ 31 ± 11 bpm from a baseline of 437 ± 11 bpm; $P < 0.01$; n = 8; **Figures 7.2B** and **7.3**), as did catestatin(90)+PACAP-38 (Δ 45 ± 14 bpm from a baseline of 428 ± 12 bpm; $P < 0.01$; n = 4; **Figures 7.1** and **7.2B**). Both responses reached a maximum approximately 20 minutes post-PACAP-38 injection (**Figures 7.1**, **7.2B** and **7.3**). The HR response to intrathecal PACAP-38 after 15 or 90 minute catestatin pre-treatment periods was not significantly different from the HR response to intrathecal PACAP-38 alone (**Figure 7.2B**).

Catestatin(15)+PACAP-38 augmented $_S$SNA (Δ 73 ± 27% from a baseline of 3± 0.6 μV; P < 0.001; n = 7; **Figures 7.2C** and **7.3**). Furthermore, catestatin(90)+PACAP-38 significantly increased $_S$SNA (Δ 78 ± 32% from a baseline of 3 ± 0.4 μV; P < 0.01; n = 4; **Figures 7.1** and **7.2C**) when compared to PBS. The $_S$SNA responses in both treatment groups increased gradually before reaching a maximum at approximately 50 minutes post-PACAP-38 injection (**Figures 7.1**, **7.2C** and **7.3**). The $_S$SNA response to intrathecal PACAP-38 15 or 90 minutes after catestatin pre-treatment was not significantly altered when compared to the untreated intrathecal PACAP-38 response (**Figure 7.2C**).

PNAmp increased in response to catestatin(15)+PACAP-38 (Δ 108 ± 37%; P < 0.01; n = 3; **Figures 7.2D** and **7.3**) and catestatin(90)+PACAP-38 (Δ 59 ± 9%; P < 0.01; n = 4; **Figures 7.1** and **7.2D**) when compared to PBS. However, these responses were not significantly different from the untreated PACAP-38 response (**Figure 7.2D**). The PNAmp responses to intrathecal PACAP-38 following 15 and 90 minute catestatin pre-treatment periods reached a maximum at 50 and 20 minutes, respectively (**Figures 7.1**, **7.2D** and **7.3**).

Catestatin(15)+PACAP-38 significantly increased PNf (Δ 11 ± 4 cycles/min; P < 0.05; n = 4; **Figures 7.2E** and **7.3**). The increase in frequency reached a peak approximately 30 minutes after PACAP-38 injection, and remained elevated for the remainder of the experimental period (**Figure 7.2E**). However, the PNf response to catestatin(15)+PACAP-38 was not significantly different from the PNf response to PACAP-38 after vehicle pre-treatment (**Figure 7.2E**). Catestatin(90)+PACAP-38 did not significantly affect PNf (n = 4; **Figures 7.1** and **7.2E**). Interestingly, the PNf response after catestatin(90)+PACAP-38 was significantly decreased when compared with the PNf response to untreated PACAP-38 (P < 0.05).

7.4.2 Pre-treatment with catestatin increases baro- and chemo- sensitivity after PACAP

PACAP-38 administered after pre-treatment with catestatin significantly increases barosensitivity, and is more effective than either PACAP-38 or catestatin individually (P < 0.001; **Figure 7.4**). The slopes of the baroreflex curves generated
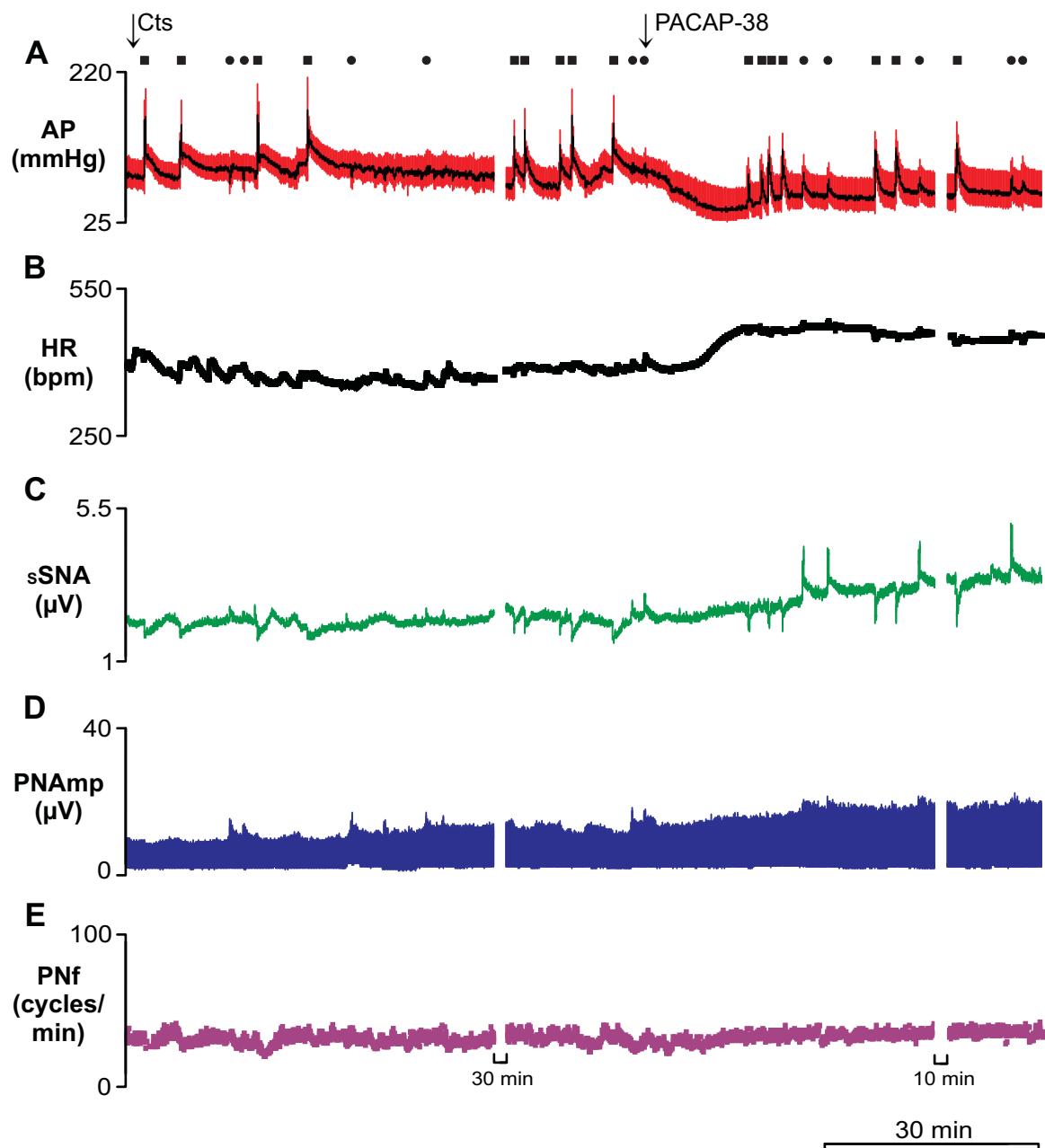
Figure 7.1 Catestatin alone caused no significant cardiorespiratory responses over a 90 minute period, but did modulate the cardiovascular responses to PACAP-38

A representative experimental trace from 1 rat showing the effects of intrathecal catestatin alone, and the effects of intrathecal catestatin(90)+PACAP-38 on (**A**) arterial pressure (AP; mean arterial pressure is signified by the black line superimposed on the AP trace), (**B**) heart rate (HR), (**C**) splanchnic sympathetic nerve activity (sSNA), (**D**) phrenic nerve amplitude (PNAmp) and (**E**) phrenic nerve frequency (PNf). Arrows indicate the times of catestatin (Cts) and PACAP-38 administration. Phenylephrine injections (i.v.) are denoted by squares; 12 second N_2 challenges are denoted by circles. Scale bar represents 30 minutes.
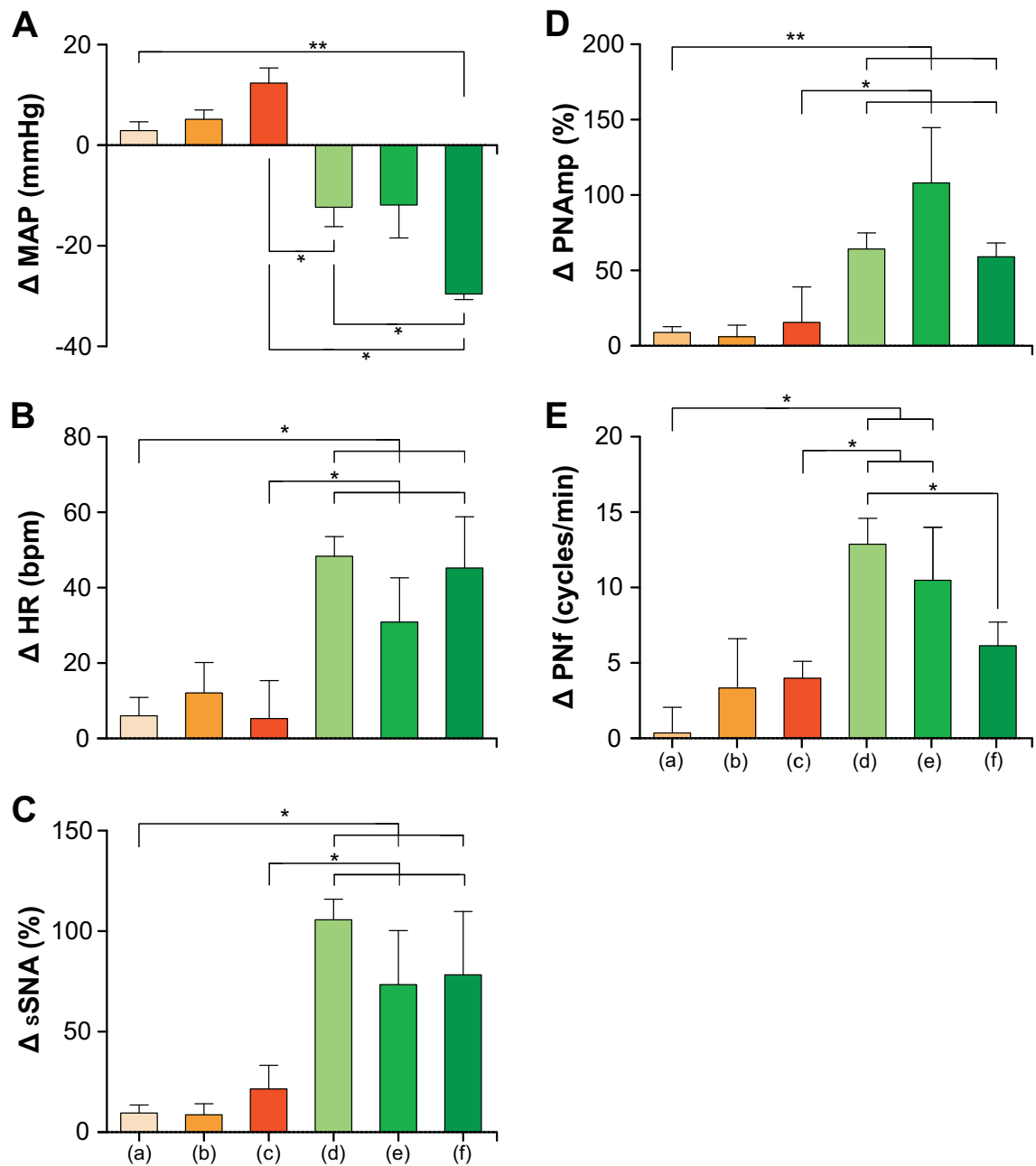
Figure 7.2 Peak responses following intrathecal drug treatments
Grouped data showing the peak changes in (**A**) mean arterial pressure (MAP), (**B**) heart rate (HR), (**C**) splanchnic sympathetic nerve activity (sSNA), (**D**) phrenic nerve amplitude (PNAmp) and (**E**) phrenic nerve frequency (PNf) following intrathecal catestatin, catestatin(15)+PACAP-38 and catestatin(90)+PACAP-38. In this figure, treatment groups (see x-axis of graphs) are as follows; (**a**) PBS, (**b**) catestatin 15 minute response, (**c**) catestatin 90 minute response, (**d**) PACAP-38 90 minute response, (**e**) catestatin(15)+PACAP-38 response and (**f**) catestatin(90)+PACAP-38 response. $* - P < 0.05$; $** - P < 0.001$.
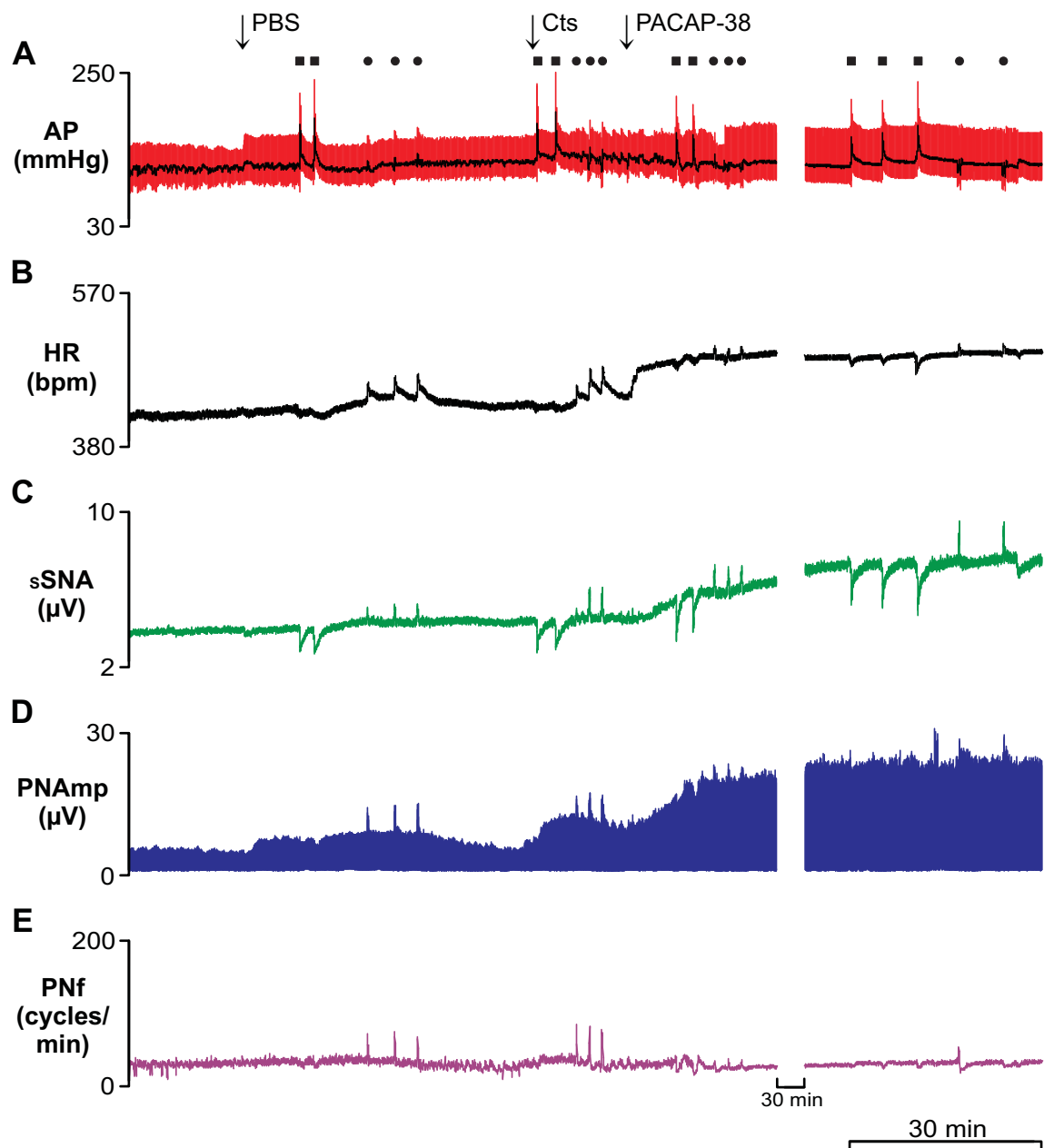
Figure 7.3 Catestatin alone caused no significant cardiorespiratory responses over a 15 minute period and had no effect on the cardiorespiratory responses to intrathecal PACAP-38 A representative experimental trace from 1 rat showing the effects of intrathecal vehicle (PBS), intrathecal catestatin and intrathecal catestatin(15)+PACAP-38 on (**A**) arterial pressure (AP; mean arterial pressure is represented by the black line superimposed on the AP trace), (**B**) heart rate (HR), (**C**) splanchnic sympathetic nerve activity (sSNA), (**D**) phrenic nerve amplitude (PNAmp) and (**E**) phrenic nerve frequency (PNf). Arrows indicate the times of catestatin (Cts) and PACAP-38 administration. Phenylephrine injections (i.v.) are denoted by squares; 12 second N_2 challenges are denoted by circles. Scale bar represents 30 minutes.

Figure 7.4 Catestatin pre-treatment increases barosensitivity after intrathecal injection of PACAP-38

(**A**) Grouped data showing the changes in the slope of the baroreflex curve in the different treatment groups. In this figure, treatment groups (see x-axis of the graph) are as follows; catestatin alone after (**a**) 0 minutes, (**b**) 20 minutes and (**c**) 75 minutes; PACAP-38 alone after (**d**) 10 minutes and (**e**) 60 minutes; catestatin(15)+PACAP-38 after (**f**) 10 minutes and (**g**) 60 minutes; catestatin(90)+PACAP-38 after (**h**) 10 minutes and (**i**) 60 minutes. * - $P < 0.05$; ** - $P < 0.001$. (**B**) Representative raw traces showing baroreflex responses after (**a**) PBS, (**b**) catestatin alone after 75 minutes, (**c**) PACAP-38 alone after 60 minutes, (**d**) catestatin(15)+PACAP-38 after 60 minutes and (**e**) catestatin(90)+PACAP-38 after 60 minutes.

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after catestatin(90)+PACAP-38 were significantly increased at both 10 ($P < 0.01$; n = 5) and 60 minutes ($P < 0.01$; n = 5) post-PACAP-38 injection (**Figure 7.4**).

The response to hypoxia following PACAP-38 administration, 90 minutes after pre-treatment with catestatin, is a significant increase in the range of the MAP response ($P < 0.01$; **Figure 7.5A**) and the slope of the $_s$SNA response ($P < 0.01$; **Figure 7.5C**). Specifically, the range of the MAP response to hypoxia is increased after catestatin (90)+PACAP, at both 10 ($P < 0.05$; **Figure 7.5A**) and 60 minute ($P < 0.05$; **Figure 7.5A**) post-PACAP-38 time points, when compared with the responses to hypoxia after catestatin(15)+PACAP-38. The slope of the $_s$SNA response to hypoxic stimuli after catestatin (90)+PACAP-38 was significantly augmented at 60 minutes post-PACAP-38 ($P < 0.05$). The slope of the $_s$SNA response to hypoxia 60 minutes after catestatin (90)+PACAP-38 was significantly greater than the response observed after catestatin(15)+PACAP-38 ($P < 0.05$; **Figure 7.5C**). The $_s$SNA range was not effected following catestatin(90)+PACAP-38 (**Figure 7.5B**), however, the $_s$SNA range was attenuated following catestatin(15)+PACAP-38 when compared to PACAP-38 alone ($P < 0.05$; **Figure 7.5B**).

7.4.3 Intrathecal catestatin does not significantly affect PNAmp, PNf, MAP, HR or $_s$SNA

Cardiorespiratory parameters were measured after intrathecal injection of catestatin in animals having the following resting levels: PNAmp 7 ± 1 µV; PNf 33 ± 2 cycles/min; MAP 110 ± 4 mmHg; HR 428 ± 8 bpm; $_s$SNA 3 ± 0.3 µV.

In four animals, PNAmp and PNf were not significantly altered in response to intrathecal administration of catestatin during 15 minute (PNAmp $\Delta 6 \pm 8\%$, $P > 0.05$; PNf $\Delta 3 \pm 3$ cycles/min, $P > 0.05$) and 90 minute (PNAmp $\Delta 16 \pm 24\%$, $P > 0.05$; PNf $\Delta 4 \pm 1$ cycles/min, $P > 0.05$) recording periods (**Figures 7.1**, **7.2** and **7.3**). Furthermore, intrathecal catestatin did not significantly ($P > 0.05$) affect MAP, HR, or $_s$SNA (MAP $\Delta 5 \pm 2$ mmHg; HR $\Delta 12 \pm 8$ bpm; $_s$SNA $\Delta 9 \pm 6\%$; n = 8; **Figure 7.2**) for up to 90 minutes after catestatin administration when compared to PBS (MAP $\Delta 12 \pm 3$ mmHg; HR $\Delta 5 \pm 10$ bpm; $_s$SNA $\Delta 22 \pm 12\%$; n = 4; **Figure 7.2**).

There was no significant difference between the peak response to catestatin and the vehicle response, at both time points, for all measured parameters (**Figure 7.2**).

7.4.4 Intrathecal catestatin does not significantly affect baro- or chemo-sensitivity

The sympathetic baroreflex was measured at three time points (0 minutes, n = 13, 20 and 75 minutes, n = 5) post-catestatin and post-vehicle. When compared to the PBS response, the slope of the sympathetic baroreflex was not significantly affected by intrathecal catestatin (**Figure 7.4**). The MAP and $_s$SNA responses to hypoxic chemoreflex stimulation were not affected by intrathecal administration of catestatin (**Figure 7.5**)

7.4.5 Intrathecal PACAP-38 increases PNAmp and PNf

In five animals (resting levels: PNAmp 14 ± 3 µV; PNf 34 ± 5 cycles/min), intrathecal PACAP-38 injection significantly augmented PNAmp (Δ 64 ± 11%; $P <$ 0.001) and PNf (Δ 13 ± 2 cycles/min; $P < 0.001$) over a 90 minute period (**Figure 7.6**). The peak PNAmp response occurred approximately 30 minutes post-injection. Despite a slight decline after the peak response, PNAmp remained significantly elevated over the entire recording period (**Figure 7.6**). The PNf response to PACAP-38 increased steadily until reaching a peak response approximately 60 minutes post-injection, at which point it stabilised for the duration of the recording period (**Figure 7.6**).

7.4.6 Intrathecal PACAP-38 increases HR and $_s$SNA but does not affect MAP

Intrathecal injection of PACAP-38 caused a significant increase in HR (Δ 59 ± 8 bpm; $P < 0.0001$; n = 6) and $_s$SNA (Δ 209 ± 90%; $P < 0.05$; n = 5), but no change in MAP (Δ -3 ± 9 mmHg; $P > 0.05$; n = 6) throughout the 90 minute recording period when compared to PBS (**Figure 7.2**).

7.4.7 Intrathecal PACAP-38 does not significantly affect baro- or chemo-sensitivity

In five animals, intrathecal PACAP-38 did not significantly change the slope of the sympathetic baroreflex when compared to PBS baroreflex responses (**Figure**
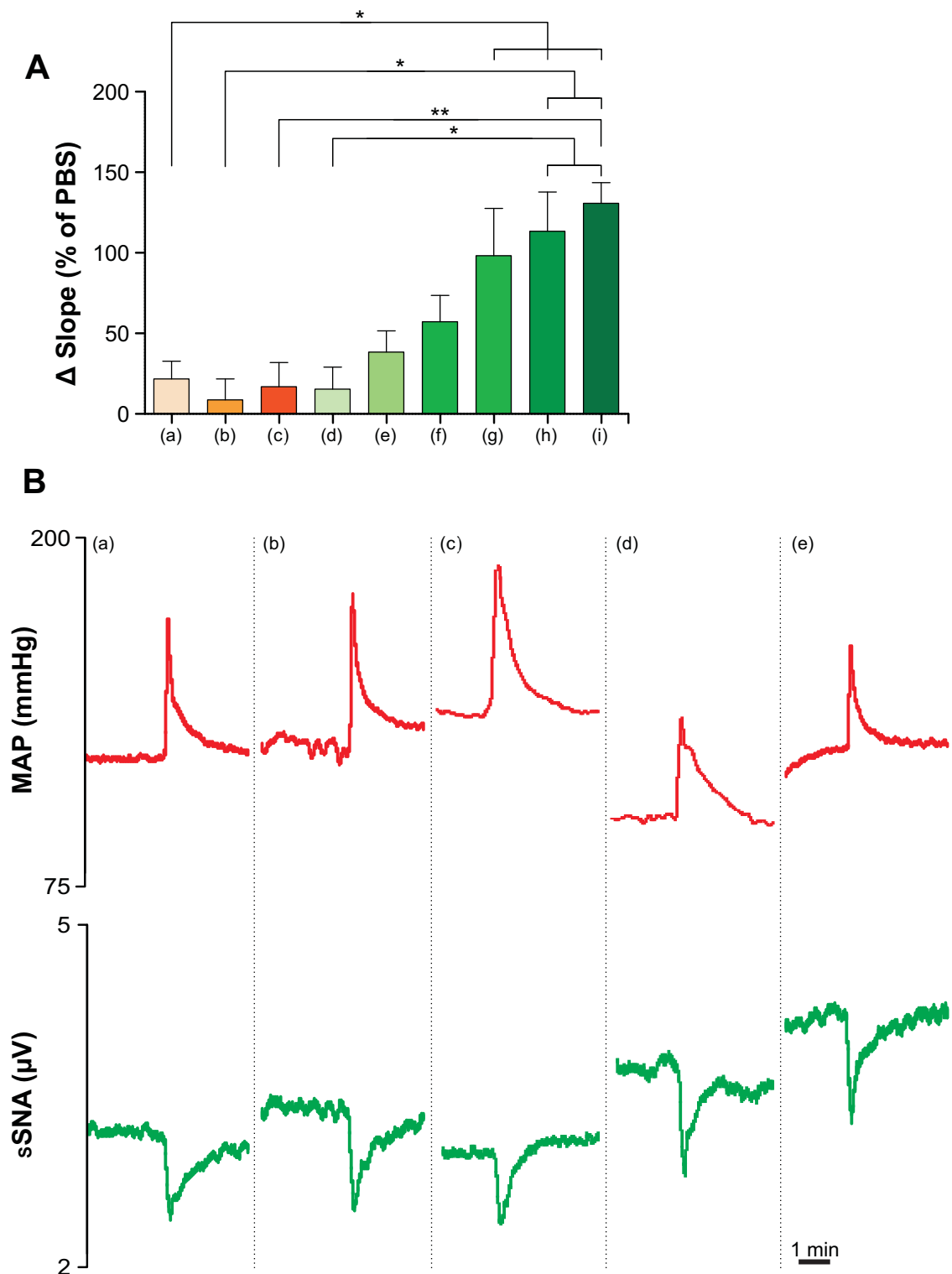
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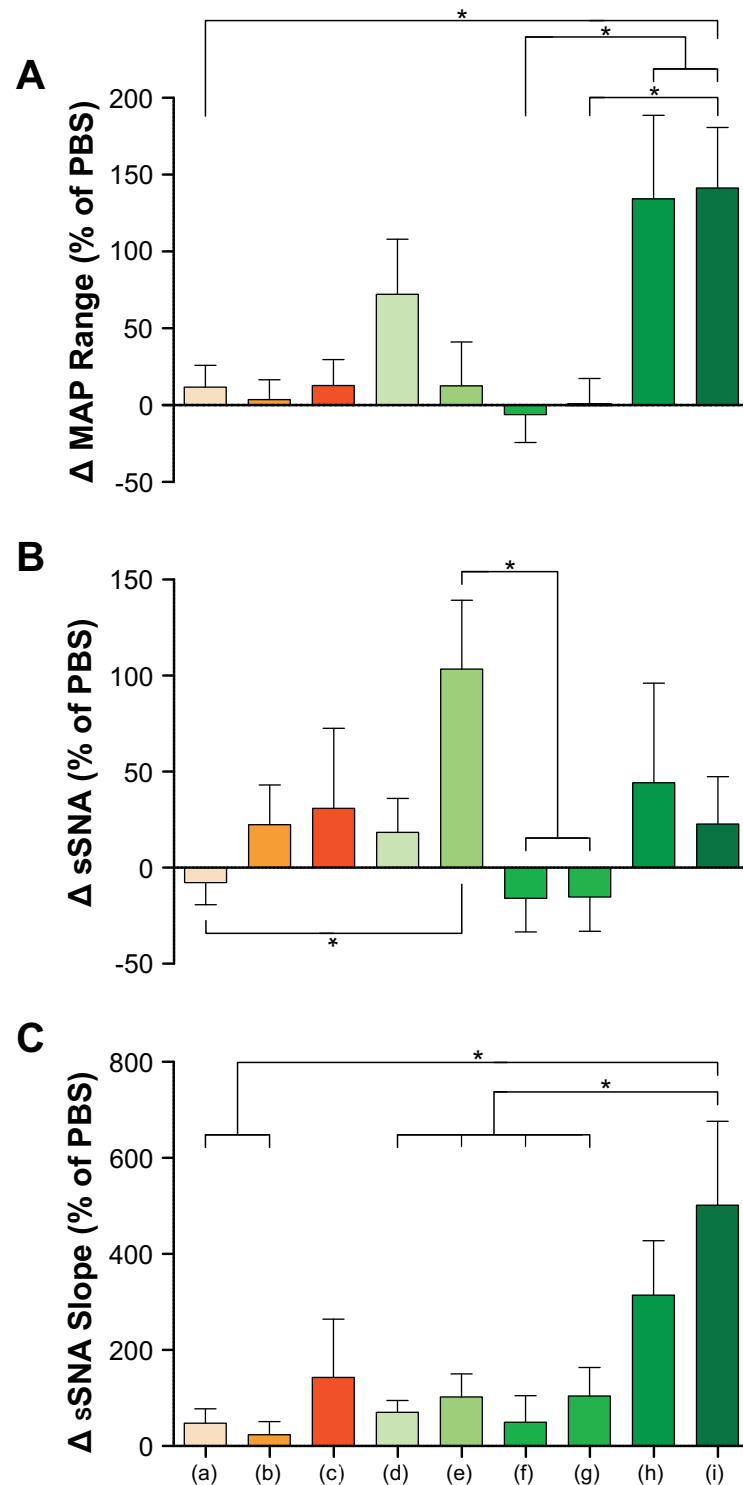
Figure 7.5 Catestatin pre-treatment increases chemosensitivity after intrathecal injection of PACAP-38

Grouped data showing the hypoxia-induced changes in (**A**) the slope of the mean arterial pressure (MAP) response, (**B**) the range of the splanchnic sympathetic nerve activity (sSNA) response and (**C**) the slope of the sSNA response. In this figure, treatment groups (see x-axis of the graphs) are as follows; catestatin alone after (**a**) 0 minutes, (**b**) 20 minutes, and (**c**) 75 minutes; PACAP-38 alone after (**d**) 10 minutes and (**e**) 60 minutes; catestatin(15)+PACAP-38 after (**f**) 10 minutes and (**g**) 60 minutes; catestatin(90)+PACAP-38 after (**h**) 10 minutes and (**i**) 60 minutes.

* - $P < 0.05$; ** - $P < 0.001$.

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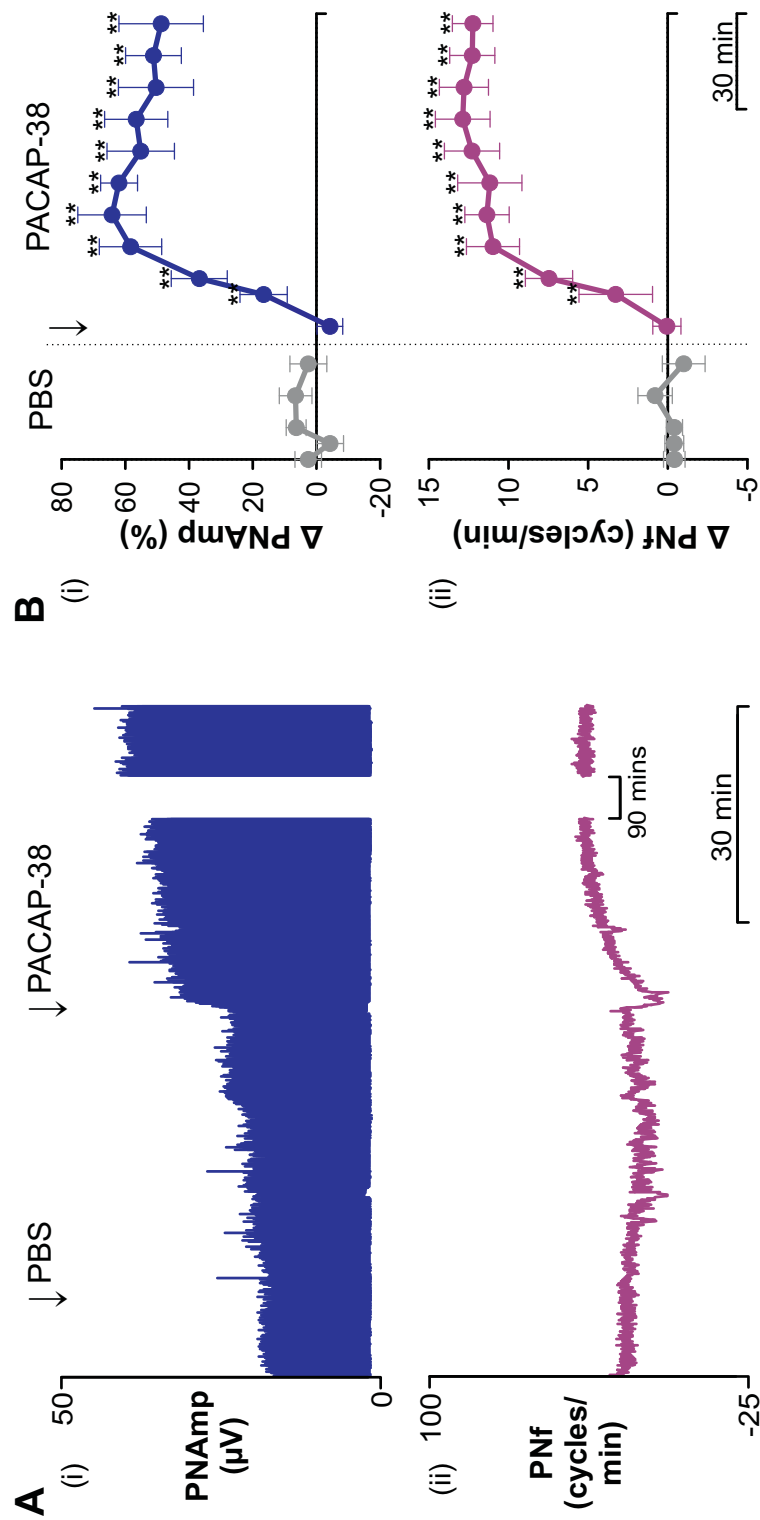
Figure 7.6 Intrathecal PACAP-38 causes a prolonged tachypnoea and increases phrenic nerve amplitude
(**A**) A representative trace from 1 rat showing the effects of intrathecal PACAP-38 on (**i**) phrenic nerve amplitude (PNAmp) and (**ii**) phrenic nerve frequency (PNf) over a 90 minute time period. Arrows indicate the times of PBS and PACAP-38 administration. Scale bar represents 30 minutes. (**B**) Grouped data showing the changes in (**i**) PNAmp (Δ 64 ± 11%; $P < 0.001$; n = 5) and (**ii**) PNf (Δ 13 ± 2 cycles/min; $P < 0.001$; n = 5) following PBS and PACAP-38 injection. 'PBS' is the period after intrathecal injection of PBS, 'PACAP-38' is the period after intrathecal injection of PACAP-38. Scale bar represents 30 minutes. * - $P < 0.05$; ** - $P < 0.001$.

7.4). Data are presented as percentage change from the vehicle (PBS) response to baroreflex stimulation.

Intrathecal injection of PACAP-38 did not significantly alter the MAP range or $_S$SNA slope responses to hypoxia when compared to the PBS responses to hypoxic chemoreflex stimulation (**Figure 7.5**). However the $_S$SNA range is increased following PACAP-38 ($P < 0.05$; **Figure 7.5B**). Data are presented as percentage change from the vehicle (PBS) response to chemoreflex stimulation.

7.4.8 Effect of catestatin versus PACAP-38 on basal MAP, HR and $_S$SNA

Catestatin and PACAP-38 alone caused significantly different cardiovascular changes in HR ($P < 0.05$) and $_S$SNA ($P < 0.01$) during the 60 minute recording period (**Figure 7.2**).

7.5 Discussion

The data demonstrate a potent synergistic effect of catestatin and PACAP-38 in the spinal cord. Both peptides are present in nerve terminals that surround SPN, and both peptides exert potent cardiovascular effects elsewhere in the brain, but have no effect on blood pressure or reflexes on their own in the spinal cord (Farnham *et al.*, 2008; Gaede *et al.*, 2009; Gaede & Pilowsky, 2010). A key difference however, is that catestatin delivered intrathecally does not affect MAP, HR, $_S$SNA or adaptive reflexes (Gaede *et al.*, 2009). PACAP on the other hand does not affect MAP, but does cause increases in HR and $_S$SNA (Farnham *et al.*, 2008).

The primary, novel findings of this study are first, that intrathecal catestatin(90)+ PACAP significantly decreases MAP and PNf, but has no effect on PNAmp or PACAP-38-induced sympathoexcitation. Secondly, treatment with catestatin(90)+PACAP enhances baroreflex and chemoreflex sensitivity. Thirdly, intrathecal catestatin alone does not significantly affect basal respiratory conditions or reflex control of blood pressure. Fourthly, intrathecal administration of PACAP-38 alone causes a prolonged tachypnoea and increases the amplitude of each phrenic burst, but minimally effects reflex control of the cardiovascular system.

7.5.1 Catestatin amplifies the MAP response to PACAP-38

Intrathecal microinjection of PACAP-38 90 minutes after catestatin injection catestatin(90)+PACAP) significantly decreased MAP, without affecting the increase in $_S$SNA observed following PACAP. Catestatin pre-treatment 15 minutes prior to PACAP-38 injection (catestatin(15)+PACAP) did not affect the cardiorespiratory responses to PACAP-38 alone. The fact that the enhanced depressor effect occurred after a 90 minute pretreatment with catestatin, strongly suggests a role for altered gene expression. Alternatively, or additionally, catestatin may alter the activity of intracellular mechanisms that are coupled to $G\alpha_s$ (Mahata *et al.*, 2003; Aung *et al.*, 2011), including adenylate cyclase and phospholipase C (Farnham & Pilowsky, 2010).

PACAP-38 activates three G-protein coupled receptors: PAC_1, $VPAC_1$, and $VPAC_2$. In human adrenal chromaffin cells, blockade of VPAC receptors does not block PACAP-mediated catecholamine release, suggesting that PAC_1 receptors are crucial for PACAP-induced catecholamine secretion (Mazzocchi *et al.*, 2002). The results reported here may be due to direct actions of catestatin that alter PAC_1 receptor function, or may be due to interactions between catestatin and other G-protein coupled receptors, either directly, or by translocating across the cell membrane (Zhang *et al.*, 2009). So that the activity of the VPAC receptors is enhanced (Vaudry *et al.*, 2009). In effect, there may be a shift in the balance of PAC_1, versus $VPAC_1$ and $VPAC_2$, function. Furthermore, the chemical phenotype of SPN that project to adrenaline (Ad)-releasing chromaffin cells is different to that of SPN regulating noradrenaline (NAd)-releasing chromaffin cells (Kumar *et al.*, 2010). Therefore, the results observed here may be due to partial blockade of PAC_1 receptors by catestatin, with a change in the balance towards excitation of Ad-releasing pathways that cause an increase in HR, a large decrease in peripheral resistance, and a subsequent fall in blood pressure (Glover *et al.*, 1962; Gardiner *et al.*, 1991). However, further study, such as catecholamine measurement, is required to support this hypothesis.

Catestatin may increase the excitability of SPN or other spinal interneurons, resulting in augmented activation of adrenal chromaffin cells and other sympathetic targets. PACAP-38-induced activation of adrenally SPN projecting may increase the

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release of catestatin, catecholamines, PACAP-38 and other co-stored substances. In particular, the hypotensive response to catestatin(90)+PACAP-38 may be a result of increased Ad release that, in turn, enhances peripheral vasodilation (Morris *et al.*, 1995; Champion *et al.*, 1996; Kennedy *et al.*, 1998).

I.v. catestatin (to reach an extracellular concentration of 6 mmol/L; 0.3 µmol/L/rat) causes histamine release-mediated hypotension following sympathoexcitation by electrical stimulation. Catestatin significantly increased plasma Ad by 11-fold, while having no effect on plasma NAd (Kennedy *et al.*, 1998); this contradicts *in vitro* studies that demonstrate the ability of catestatin to inhibit catecholamine release. Therefore, the endocrine effect of i.v. catestatin in the rat appears to be that of indirect vasodilation. In general, catestatin acts to stabilise baseline conditions in several *in vivo* and *in vitro* models (Briolat *et al.*, 2005; Rao *et al.*, 2007; Mazza *et al.*, 2008; Gayen *et al.*, 2009; Dev *et al.*, 2010; Mahata *et al.*, 2010). As such, the role of catestatin may not be apparent until an appropriate stimulus is applied. Here catestatin appears to prime the system so that PACAP-38 may induce hypotension and increase barosensitivity and chemosensitivity. As noted above, we speculate that this occurs as a result of activation of SPN that project to Ad secreting chromaffin cells.

Baroreceptor-induced sympathoinhibition is mediated by inhibition of tonically active RVLM neurons (Goodchild *et al.*, 2000; Pilowsky & Goodchild, 2002; Pilowsky *et al.*, 2009). The present study demonstrates that pre-treatment with catestatin primes this pathway so that PACAP-38 significantly increases sympathetic baroreflex sensitivity. Catestatin(90)+PACAP-38 may result in either disfacilitation or direct inhibition at the level of the spinal cord to augment baroreflex sensitivity.

Hypoxia causes a rapid and reversible excitation of bulbospinal sympathoexcitatory RVLM neurons that monosynaptically project to SPN. Sympathetic excitation elicited by hypoxia requires glutamatergic transmission (Sun & Reis, 1994). Intrathecal pre-treatment of catestatin before administration of PACAP-38 increases the range of the pressor response and markedly augments the slope and range of the $_S$SNA response to the chemoreflex.

7.5.2 Catestatin alone does not alter the tonic or reflex control of the cardiorespiratory system

Intrathecal injection of catestatin did not affect basal MAP, $_S$SNA, and HR, as previously reported (**Figures 7.1**, **7.2** and **7.3**; Gaede *et al.*, 2009). Here it is demonstrated that catestatin has no effect on PNAmp and PNf, the sympathetic baroreflex or the hypoxic chemoreflex (**Figures 7.4** and **7.5**). The mechanism by which catestatin acts in the spinal cord is unknown. Catestatin exerts divergent actions depending on the tissue being investigated (Kennedy *et al.*, 1998; Mazza *et al.*, 2008; Mahata *et al.*, 2010). In the periphery, catestatin causes vasodilation (Fung *et al.*, 2010) and has antimicrobial actions in the human epidermis, suggesting a role for catestatin in the cutaneous defense system (Radek *et al.*, 2008). In the brainstem, it is sympathoexcitatory, increases PNA and enhances sympathetic barosensitivity (Gaede & Pilowsky, 2010).

The first characterised reported action of catestatin was as a nicotinic acetylcholine receptor (nAChR) antagonist that blocks nicotine-induced catecholamine secretion from PC12 cells (Mahata *et al.*, 1999), bovine adrenal chromaffin cells (Mahata *et al.*, 1997), and rat hippocampal neurons (De *et al.*, 2003). Recent studies reported that catestatin affects many intracellular signaling pathways (Egger *et al.*, 2008; Mazza *et al.*, 2008; Zhang *et al.*, 2009). For example, catestatin inhibits the actions of nicotine and isoproterenol in the intrathecal space, most likely through interactions with nAChR and β-adrenergic receptors (Gaede *et al.*, 2009). The finding that intrathecal catestatin does not exert any effects on its own may be because constitutively active nAChR are not present on SPN or phrenic motor neurons (Chitravanshi & Sapru, 1999), the effects of catestatin are not visible under basal conditions, or that catestatin causes many opposing effects that are balanced under normotensive conditions, resulting in no net change.

Intrathecal administration of catestatin does not affect respiration, the baroreflex or the cardiovascular responses to hypoxic chemoreflex stimulation at any time point. This indicates that intrathecal catestatin on its own is not involved in the tonic or reflex control of the cardiorespiratory system. Previously, we reported that microinjection of catestatin into the RVLM increased PNA, barosensitivity and

attenuated chemosensitivity (Gaede & Pilowsky, 2010). Differences in injection method, site, and dose, may explain this discrepancy.

Many of the known actions exerted by catestatin in other systems are modulatory. For example, catestatin attenuates the pressor response to electrical stimulation in the pithed rat (Kennedy *et al.*, 1998), and levels of circulating catestatin are diminished in hypertensive and pre-hypertensive individuals (O'Connor *et al.*, 2002; Di Comite & Morganti, 2011). Furthermore, CgA knockout mice (*Chga*^{-/-}) have elevated blood pressure levels compared to their wild type counterparts. Exogenous catestatin reduces the blood pressure of *Chga*^{-/-} mice by 30 mmHg, suggesting that the elevation in blood pressure in these mice is due to the absence of catestatin (Mahapatra *et al.*, 2005). These modulatory properties likely explain why catestatin alone does not elicit any visible changes in the spinal cord, and forms the basis for our investigation into the effect of catestatin in enhancing the response to PACAP-38.

7.5.3 PACAP-38 alone alters tonic, but not reflex, control of the cardiorespiratory system

PACAP-38 is co-localised with catestatin in bulbospinal C1 (tyrosine hydroxylase-immunoreactive) neurons in the RVLM; a region of the brainstem critical in the central control of blood pressure (Farnham *et al.*, 2008; Gaede & Pilowsky, 2010). Presympathetic RVLM neurons regulate sympathetic vasomotor tone and blood pressure (Pilowsky & Goodchild, 2002; Pilowsky *et al.*, 2009). The excitatory effects of PACAP-38 are well-characterised in the cardiovascular system (Vaudry *et al.*, 2009; Farnham & Pilowsky, 2010). PACAP-38 excites SPN in spinal cord slices from juvenile rats (Lai *et al.*, 1997), and is localised in at least 82% of C1 presympathetic bulbospinal RVLM neurons (Farnham *et al.*, 2008). Functional evidence supports these findings; intrathecal PACAP-38 causes a prolonged tachycardia and sympathoexcitation in multiple sympathetic beds through activation of SPN, but causes no change in MAP (Farnham *et al.*, 2008; Inglott *et al.*, 2011). Previous studies report conflicting findings regarding the blood pressure response to PACAP-38; Lai *et al.,* (1997) found a significant increase in MAP (Lai *et al.*, 1997), while others saw no change in MAP (Lai *et al.*, 1997; Farnham *et al.*, 2008; Inglott *et al.*, 2011). This study supports the earlier findings that PACAP-38 does not affect

MAP *in vivo* (Farnham *et al.*, 2008; Inglott *et al.*, 2011). Despite the well-described effects of intrathecal PACAP-38 on the cardiovascular system, the role of PACAP-38 in reflex control of the cardiovascular system and any role of PACAP-38 within central respiratory control remains relatively unstudied (Farnham & Pilowsky, 2010).

Here we show that PACAP-38 significantly increases PNAmp and PNf, and has no effect on the sympathetic baroreflex or the hypoxic chemoreflex. Earlier studies have argued that i.v. administration of PACAP-38 increases breathing frequency through activation of peripheral chemoreceptors (Ishizuka *et al.*, 1992). In the brainstem slice preparation, PACAP-38 increases rhythmic inspiratory firing in the pre-Bötzinger complex, suggesting that it acts centrally to activate the central respiratory network (Pena, 2010). The findings of this study support the idea that PACAP-38 may act centrally to modulate inputs to the respiratory pattern generator, and ultimately PNA and respiration.

Intrathecal PACAP-38 does not affect the slope of the baroreflex or the hypoxic chemoreflex. The results demonstrate that spinal PACAP-38 appears to have little effect on adaptive cardiovascular reflexes under basal conditions. However, following pre-treatment with catestatin effects are apparent. To date, this is the only study to examine the role that PACAP-38 plays in the chemoreflex. The finding that the sympathetic baroreflex is unaffected is in contrast to a previous study examining HR barosensitivity in the unanaesthetised rainbow trout (Lancien *et al.*, 2011). Apart from the obvious differences in dose, species, and injection site, the baroreflex sensitivity was calculated using HR rather than $_{S}$SNA. PACAP-38 did not change HR in the study by Lancien *et al.,* (2011) likely explaining the observed depression in barosensitivity. Here we used direct recordings from barosensitive, pulse-modulated, sympathetic nerve fibres to obtain direct, real-time measures of nerve activity during stimulation of the baroreflex, enabling continuous measurement of changes in sympathetic baroreflex sensitivity.

7.5.4 Conclusion

In conclusion, we report for the first time that catestatin, a peptide found throughout the nervous system, including efferent cardiovascular pathways, acts in

the spinal cord as a potent negative modulator of the sympathoexcitatory effects of the neuropeptide PACAP-38. The finding that PACAP-38 augments adaptive reflexes, and causes hypotension in the presence of catestatin, suggests that catestatin may interact with adenylate cyclase or phospholipase C; two intracellular mechanisms through which both peptides are thought to act. Furthermore, this suggests that catestatin may be a useful target for the future development of cardiorespiratory therapeutic agents. We also describe the excitatory effects of intrathecal PACAP-38 on central respiratory control for the first time in this anaesthetised rat preparation. The findings of this study support the idea that PACAP-38 may be excitatory within the respiratory network, while catestatin is not involved in the tonic or reflex control of the cardiovascular or respiratory systems, but rather acts to stabilise this system.

7.6 Perspectives and significance

There is increasing evidence that elevated sympathetic tone contributes to the development of most forms of hypertension (Esler *et al.*, 2001; Pilowsky & Goodchild, 2002; Guyenet, 2006; Malpas, 2010). This may be due to increased tonic RVLM activity (Judy *et al.*, 1976; Judy *et al.*, 1979; Minson *et al.*, 1996; Ito *et al.*, 2000; Patel *et al.*, 2012). Therefore, it is reasonable to propose that the increase in vasomotor tone seen in some cases of hypertension (Patel *et al.*, 2012) may be caused by altered neurochemistry and activity of RVLM neurons; and a resultant increase in the firing of presympathetic bulbospinal RVLM neurons, SPN and therefore sympathetic outflow, at effector sites such as the blood vessels (Sved *et al.*, 2003). Given the role that catestatin has in inhibiting catecholamine release, and its effects on the actions of PACAP, the use of catestatin in the treatment of neurogenic hypertension may prove effective in reducing the significant burden of disease essential hypertension places on society.

Chapter 8

General Discussion

Chapter 8: General Discussion

Pituitary adenylate cyclase activating polypeptide (PACAP) and its receptors are found in important cardiovascular sites throughout the medulla oblongata and spinal cord (**Chapter 1**), including the rostral ventrolateral medulla (RVLM) and intermediolateral cell column (IML) of the spinal cord, which both display a high PACAP content (Légrádi *et al.*, 1994; Dun *et al.*, 1996a; Dun *et al.*, 1996b; Arimura, 1998; Basille *et al.*, 2000; Farnham *et al.*, 2008; Farnham *et al.*, 2012Hannibal, 2002 #57). Peripheral and central application of PACAP, *in vivo*, elicits various dose and site dependent cardiovascular effects (**section 1.8.4.2** and **1.8.4.3**; Minkes *et al.*, 1992; Murase *et al.*, 1993; Seki *et al.*, 1995; Lai *et al.*, 1997; Le Mevel *et al.*, 2009). Surprisingly, intrathecal PACAP injection does not affect mean arterial pressure (MAP; Farnham *et al.*, 2008), despite widespread sympathoexcitation (Inglott *et al.*, 2011). PACAP is also a potent secretagogue of catecholamines, and drives catecholamine gene expression and regulation in areas such as the adrenal gland (**section 1.9**; Légrádi *et al.*, 1994; Tönshoff *et al.*, 1997; Shioda *et al.*, 2000; Wong *et al.*, 2002; Payet *et al.*, 2003; Das *et al.*, 2007). Together, this evidence suggests that PACAP is well placed to play an important role in the central neural control of AP and vasomotor tone (Farnham *et al.*, 2008; Farnham & Pilowsky, 2009; Farnham & Pilowsky, 2010; Farnham *et al.*, 2011; Inglott *et al.*, 2011; Farnham *et al.*, 2012; Inglott *et al.*, 2012). However, the precise function/s of PACAP in central cardiovascular control remains unclear. Furthermore, no previous studies have investigated the role of PACAP in central respiratory control, in an *in vivo* preparation, despite evidence indicating that PACAP may play an important excitatory role in respiratory rhythm generation in PACAP knockout mice (**section 1.8.4.5**; Jamen *et al.*, 2000; Gray *et al.*, 2001; Cummings *et al.*, 2004; Wilson & Cummings, 2008; Arata *et al.*, 2012).

The work described in this thesis aimed to further investigate the role of PACAP within central cardiorespiratory control at the level of the spinal cord. This body of work has a particular focus on determining the mechanistic cause underlying the lack of MAP response following intrathecal PACAP injection.

Subsequent to the discovery that PACAP-38 excites sympathetic preganglionic neurons (SPN) in spinal cord slices from juvenile rates, and that intrathecal administration of the peptide has a pressor effect on MAP (Lai *et al.*, 1997). Our laboratory found that over 80% of bulbospinal, catecholaminergic RVLM neurons contain PACAP mRNA (Farnham *et al.*, 2008). The functional role of PACAP-containing bulbospinal RVLM neurons was tested using intrathecal administration of PACAP, which increased heart rate (HR) and splanchnic sympathetic nerve activity ($_s$SNA), but not MAP (Farnham *et al.*, 2008; Inglott *et al.*, 2011), a finding that contrasts with earlier reports (Lai *et al.*, 1997). There are many explanations for the differences in observed blood pressure responses as previously discussed (**Chapter 5**; Inglott *et al.*, 2011). The work described here confirms the role of intrathecal PACAP at the synapse between RVLM neurons and SPN in the IML of the spinal cord, here PACAP is sympathoexcitatory but isotensive (**Chapter 5**; Inglott *et al.*, 2011). This works also expands upon the findings of Farnham *et al.,* (2008). This body of work also finds that the responses to intrathecal PACAP are strain dependent (SD, Wistar, spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats; **Chapter 4**; Farnham *et al.*, 2011), and investigates the paradoxical MAP response to intrathecal PACAP (Farnham *et al.*, 2008), to determine the mechanism underlying this response (**Chapter 5**; Inglott *et al.*, 2011) and **Chapter 6**; Inglott *et al.*, 2012). The intrathecal PACAP response is not baroreceptor mediated (Farnham *et al.*, 2008) and as shown here, cannot be explained by differential control of sympathetic outflows to various vascular beds. As intrathecal PACAP increases nerve activity in all sympathetic beds measured, including: the splanchnic, cervical, cardiac (HR increases in the vagotomised, paralysed and ventilated rat) and lumbar sympathetic beds (**Chapter 5**; Inglott *et al.*, 2011).

One caveat to this study is the administration technique used; it could be argued that intrathecal injection may activate supraspinal pathways, thereby affecting the responses observed. However, this work found that the response to intrathecal PACAP injection is mediated at the level of the spinal cord, as cervical spinal cord transection does not affect the MAP, HR or $_s$SNA responses to PACAP. This indicates that intrathecal PACAP binds to receptors on SPN in the IML of the spinal cord to cause an effect, mimicking the release of PACAP from RVLM presympathetic neurons onto SPN at this synapse (**Chapter 5**; Inglott *et al.*, 2011).

Widespread activation of the sympathetic nervous system may also occur due to activation of PACAP-containing interspinal neurons (Dun *et al.*, 1996a; Pettersson *et al.*, 2004), resulting in activation of sympathetic neuronal networks travelling within the length of the spinal cord.

Elevated sympathetic tone is a common feature of most forms of hypertension and heart failure (**sections 1.3.3** and **1.5.6**; Esler *et al.*, 2001; Head, 2003; Guyenet, 2006; Simms *et al.*, 2009; Malpas, 2010; Patel *et al.*, 2012; Wu *et al.*, 2012; Xu *et al.*, 2012). The prolonged, widespread sympathoexcitation observed following intrathecal PACAP indicates that PACAP may be involved in the aetiology of this burdensome condition. Therefore, it was important to determine the role of PACAP in the tonic and reflex control of MAP and to determine under which physiological conditions PACAP is released. The results of this thesis show that PACAP is not tonically released on to SPN from RVLM presympathetic neurons, as basal cardiovascular parameters were not altered by intrathecal injection of the PACAP antagonist in the SD rat (PACAP(6-38) (**Chapter 6**; Inglott *et al.*, 2012). Here it is also demonstrated that PACAP is not involved in the reflex control of MAP (**Chapter 7**; Gaede *et al.*, 2012). These results are supportive of previous studies demonstrating that intrathecal PACAP is not tonically released in other normotensive rat strains (WKY or Wistar rat; Farnham *et al.*, 2011) and that antagonism of the PACAP receptors in the RVLM has little effect on MAP, HR and $_S$SNA (Farnham *et al.*, 2012). PACAP in the RVLM also did not affect sympathetic reflexes (Farnham *et al.*, 2012), although in one previous study intracerebroventricular PACAP was found to decrease barosensitivity in the trout (Lancien *et al.*, 2011). However, Lancien *et al.,* (2011) studied the HR baroreflex, which is predominantly under vagal control.

The role of PACAP in the development of essential/neurogenic hypertension (**section 1.8.4.4**) was tested in the SHR, a model for neurogenic hypertension, with a generalised increase in tonic RVLM activity, MAP and sympathetic nerve activity (SNA; Judy *et al.*, 1976; Judy *et al.*, 1979; Head & Adams, 1988; Minson *et al.*, 1996; Ito *et al.*, 2000; Malpas *et al.*, 2001). It appears that PACAP is not involved in the etiology of hypertension in the SHR at the level of the spinal cord (**Chapter 4**; Farnham *et al.*, 2011) or RVLM (Farnham *et al.*, 2012), as antagonism of the

PACAP receptors did not change MAP or SNA in this strain. Despite this, the prolonged, widespread SNA increase seen upon intrathecal injection (Lai *et al.*, 1997; Farnham *et al.*, 2008; Inglott *et al.*, 2011) and RVLM microinjection of PACAP (Farnham *et al.*, 2012) suggests a role for PACAP within the pathophysiology of essential hypertension. This hypothesis is supported by evidence that PAC_1 knockout mice develop pulmonary hypertension at a very young age (Otto *et al.*, 2004). Further investigation into this area, and into the physiological stimuli for PACAP release, is still required.

While differential control of sympathetic outflows does not account for the intrathecal PACAP response, the differential actions of the PACAP receptors (Harmar *et al.*, 2012) offers an explanation. PACAP receptors cause differential effects in many body systems (**section 1.8.2**), including the peripheral cardiovascular system (Fizanne *et al.*, 2004) and adrenal gland (Payet *et al.*, 2003), most probably via different second messenger signaling systems utilised by the different PACAP receptors. This thesis is the first to investigate the function of the different PACAP receptors in central cardiovascular control. This work demonstrates that activation of PAC_1 and VPAC receptor subtypes elicits differential physiological responses from SPN in the anaesthetised rat. The PAC_1 receptors are pressor in their function, while the VPAC receptors have a depressor function, presumably countering each other to produce no net MAP effect following activation of all three receptors with PACAP (**Chapter 6**; Inglott *et al.*, 2012). The nature of the receptor effects and the strong relationship between PACAP and the sympathoadrenal system and catecholamines (**section 1.9**; Ghzili *et al.*, 2008) indicates that NAd may be responsible for the pressor response seen upon PAC_1 activation and that Ad released causes the depressor effect following VPAC receptor activation (Inglott *et al.*, 2012). This provides physiological evidence to support neurochemical coding of adrenally projecting SPN (Kumar *et al.*, 2010). However, further work is needed to confirm this hypothesis, and better pharmacological agents are also needed to better target/block the PACAP receptors.

This body of work further investigates the co-transmitter relationship between PACAP and catecholamines via pre-treatment with catestatin, a peptide that inhibits nicotine-stimulated catecholamine release through its action as an antagonist at

nicotinic acetylcholine receptors. PACAP is a potent secretagogue of catecholamines, and drives catecholamine gene expression and regulation in the adrenal gland (**section 1.9**; Légrádi *et al.*, 1994; Tönshoff *et al.*, 1997; Shioda *et al.*, 2000; Vaudry & Taupenot, 2002; Wong *et al.*, 2002; Payet *et al.*, 2003; Das *et al.*, 2007; Ghzili *et al.*, 2008; Hill *et al.*, 2012). Long term pre-treatment (90 minutes) with catestatin acts in the spinal cord as a potent negative modulator of the sympathoexcitatory effects of PACAP. The finding that PACAP augments adaptive reflexes, and causes hypotension in the presence of catestatin (**Chapter 7**), suggests that catestatin may interact with adenylate cyclase or phospholipase C; two intracellular mechanisms through which both peptides are known to act.

This work is also the first to study the effect of intrathecal PACAP injection on respiration (**section 1.8.4.5**). Intrathecal injection of PACAP increases phrenic nerve frequency (PNf) and phrenic nerve amplitude (PNAmp), indicating that PACAP is excitatory in the respiratory system and in central respiratory rhythm generation (**Chapter 7**). This supports a previous study that investigated the role of peripheral PACAP in respiratory function; intravenous (i.v.) PACAP causes bronchodilation, a powerful stimulation of breathing (Runcie *et al.*, 1995) and vasodilation of the pulmonary vasculature(Murphy *et al.*, 1992; Foda *et al.*, 1995; Chen *et al.*, 2006). This is also seen in PACAP knockout mice, which display a temperature sensitive reduction in tidal volume and ventilation (Cummings *et al.*, 2008), and a reduction in basal respiratory rate (Cummings *et al.*, 2004). In PACAP deficient mice, apnoea precedes atrioventricular block and death. It is also hypothesised that PACAP gene abnormalities may be present in some clinical respiratory conditions, such as sudden infant death syndrome; this remains unproven (Wilson & Cummings, 2008).

While metabolic function was not a primary focus of this thesis, further evidence supporting a role for PACAP in metabolic function was recorded. PACAP is implicated in glucose metabolism, feeding and thermogenesis (**section 1.8.4.6**); processes in which the RVLM is thought to play a role (Madden *et al.*, 2006). This work reveals that activation of the PACAP receptors at the level of the spinal cord increased core body temperature, end-tidal CO_2 and $PaCO_2$, while decreasing pH and blood glucose levels (**Chapter 5**; Inglott *et al.*, 2011), indicating that PACAP plays a role in increasing basal metabolic rate. Similar effects are observed following RVLM

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microinjection of PACAP, with microinjection resulting in increased core temperature and end-tidal CO_2 (Dr. Melissa Farnham, personal communication).

This thesis provides several novel insights into the mechanisms and pharmacology underlying the action of PACAP within central cardiovascular control. PACAP is an excitatory modulatory transmitter that affects the functionality of the cardiorespiratory system. In summary, intrathecal PACAP causes prolonged widespread sympathoexcitation with no change in MAP (**Chapter 5**; Inglott *et al.*, 2011), a response that is strain dependent (**Chapter 4**; Farnham *et al.*, 2011) and is mediated at the level of the spinal cord, at the RVLM-SPN synapse in the IML (**Chapter 5**; Inglott *et al.*, 2011). The lack of MAP response following PACAP may be due to differential, receptor specific, activation of catecholamine releasing adrenal chromaffin cells (**Chapter 6**; Inglott *et al.*, 2012). Here it is also shown that PACAP is not tonically released in SD, Wistar, SHR or WKY rats (**Chapters 4** and **6**; Farnham *et al.*, 2011; Inglott *et al.*, 2012), and that PACAP does not influence reflex control of the cardiovascular system in the SD rat (**Chapter 7**; Gaede *et al.*, 2012). Intrathecal PACAP also increases basal metabolic rate (**Chapter 5**; Inglott *et al.*, 2011), interacts with catestatin in the circuitry of the system to sensitise the functioning of the system (**Chapter 7**; Gaede *et al.*, 2012) and has excitatory effects on respiratory output (**Chapter 7**; Gaede *et al.*, 2012). These findings demonstrate that PACAP is an important neuropeptide in central cardiorespiratory control. Proposed hypotheses resulting from the findings of this thesis are depicted in diagrammatic form in **Figures 8.1**, **8.2** and **8.3**.

Future studies are needed to determine, *inter alia*, the circumstances in which PACAP is released onto SPN or respiratory neurons of the phrenic motor nucleus (PMN). PACAP may potentially be released in stress-induced responses, as indicated by the strong sympathoexcitation observed after its administration. Therefore, microinjection of PACAP into the dorsomedial hypothalamus, periaqueductal grey and paraventricular nucleus, would be of interest. The intracellular signalling pathways of the PACAP receptors used in different circumstances need to be determined to identify the mechanisms underlying the effects observed following intrathecal PACAP administration. Plasma catecholamine levels following PACAP, and its agonists/antagonists, along with adrenalectomy,

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further work with catestatin and studies involving molecular biology techniques are needed to confirm the hypothesises regarding the effects of PACAP on catecholamines. The effects of PACAP on metabolism, core temperature and expired CO_2, indicate a potential role for PACAP in brown adipose tissue thermogenesis, a role that could be studied with medullay raphé microinjections of PACAP (Madden, 2012). Finally, the effects of PACAP on PNAmp and PNf suggest an excitatory role for PACAP in central respiratory control. Microinjections of PACAP into the nuclei of the ventral respiratory column and PMN of the spinal cord, coupled with combined immunohistochemistry and *in situ* hybridisation studies for PACAP and the PACAP receptors within this region, may add further evidence to that presented here.

The cardiovascular and respiratory systems maintain the most vital homeostatic functions of life; circulation and breathing. Regulation of these systems is achieved through the integrated activity of cardiorespiratory neurons within the ventrolateral medulla and spinal cord. The activity of cardiorespiratory neurons, and by extension the entire system, is modulated by excitatory and inhibitory metabotropic neurotransmitters present within the circuitry of the system. Disturbances in any one of these neurotransmitter systems, for example PACAP, or within the circuitry itself may result in cardiorespiratory dysregulation and disease, including hypertension. Through research in this area, we are increasing the knowledge of how the cardiorespiratory system is controlled in health, disease and in response to physiological stimuli, enabling us to better understand the aetiology and pathophysiology of cardiorespiratory diseases.
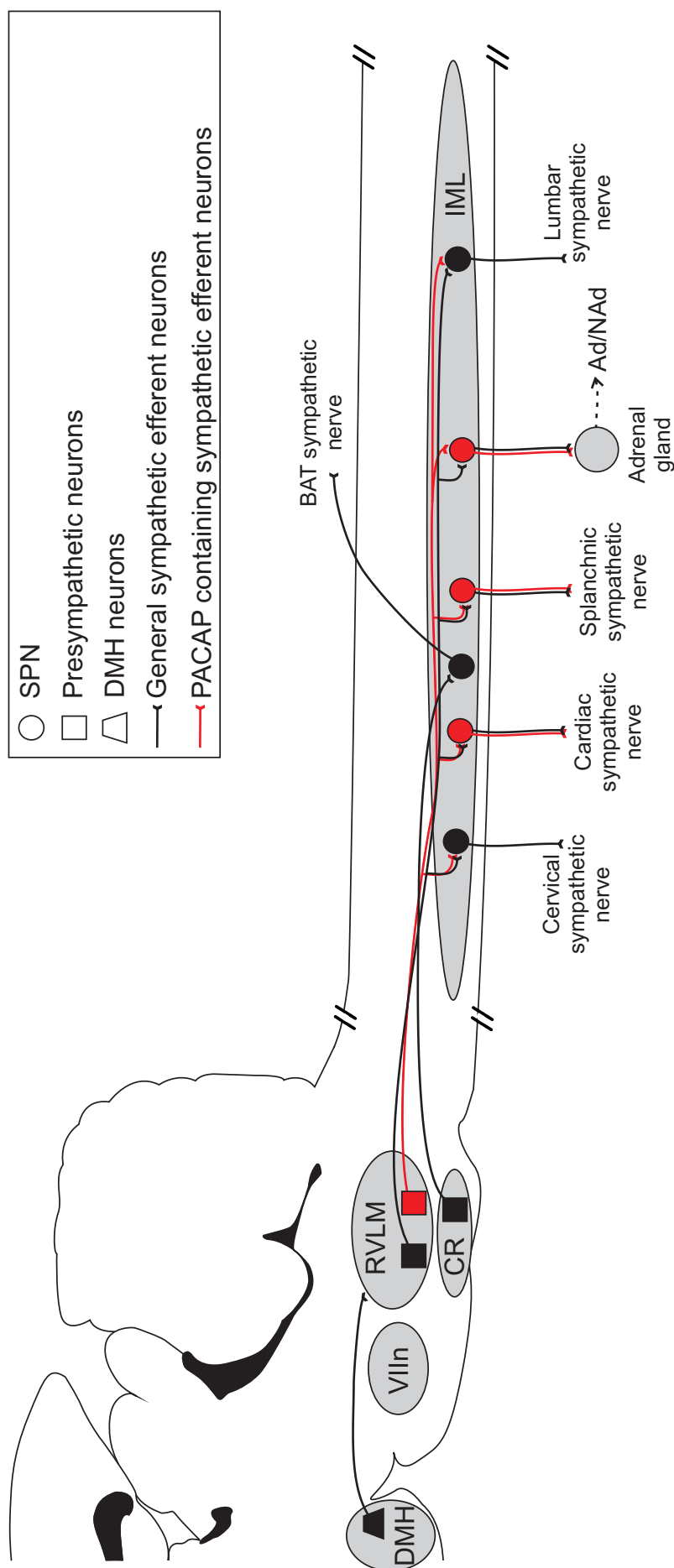
Figure 8.1 Proposed PACAP innervation and action within central cardiovascular control prior to this body of work

Molecular and *in vivo* evidence suggests an important role for PACAP within the central control of blood pressure. PACAP containing neurons are known to be present in the RVLM, IML and in SPN, *in vivo* activation of SPN with intrathecal PACAP injection increases sympathetic nerve activity and heart rate without an accompanying blood pressure increase. Prior to the studies conducted here, it was hypothesised that this paradoxical response was due to differential control of sympathetic outflows by PACAP, resulting in no net change in blood pressure. Abbreviations: Ad, adrenaline; BAT, brown adipose tissue; CR, caudal raphé; DMH, dorsal medial hypothalamus; IML, intermediolateral cell column of the spinal cord; NAd, noradrenaline; RVLM, rostral ventrolateral medulla; SPN, sympathetic preganglionic neurons; VIIn, facial nucleus.
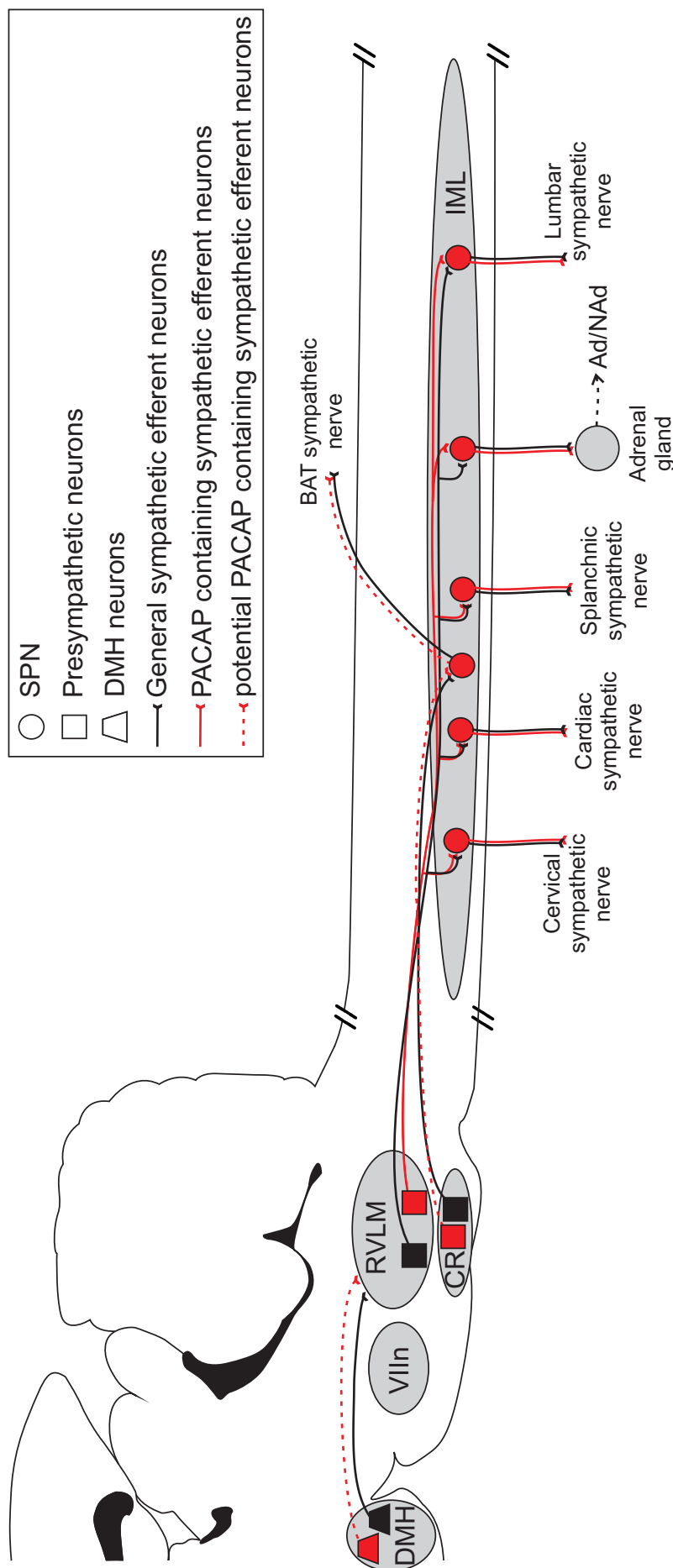
Figure 8.2 Proposed PACAP innervation and action within central cardiovascular control following the findings of this body of work

In vivo evidence presented in **Chapter 5** suggests that PACAP is not involved in the differential control of sympathetic outflow. Intrathecal PACAP injection caused sympathoexcitation in all measured sympathetic beds, indicating that the effect of PACAP is not differential, but is widespread, in the central neural control of blood pressure, as such differential control of sympathetic outflows does not account for lack of blood pressure response following intrathecal PACAP. The findings presented here indicate a role for PACAP in BAT thermogenesis and suggest that PACAP may be released in the stress response therefore, PACAP may be found in regions such as the caudal raphé or the DMH, respectively. Abbreviations: Ad, adrenaline; BAT, brown adipose tissue; CR, caudal raphé; DMH, dorsal medial hypothalamus; IML, intermediolateral cell column of the spinal cord; NAd, noradrenaline; RVLM, rostal ventrolateral medulla; SPN, sympathetic preganglionic neurons; VIIn, facial nucleus.
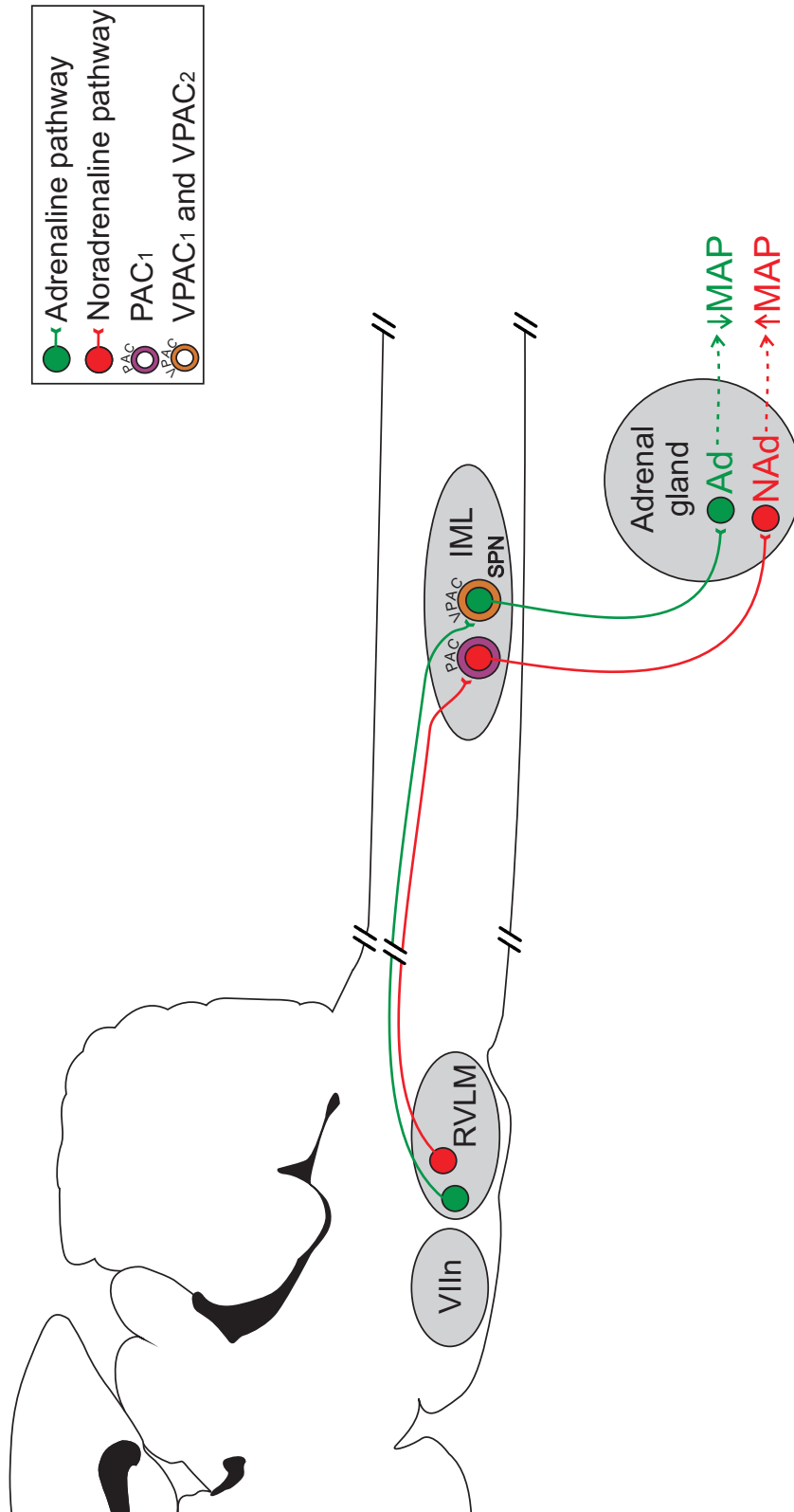
Figure 8.3 PACAP receptor subtypes differentially regulate catecholamine secretion from the adrenal medulla

Given the findings observed in **Chapter 6** of this thesis, we propose that PAC$_1$ or VPAC receptors present on SPN differentially regulate NAd and Ad secreting chromaffin cells. With PAC$_1$ activating vasoconstrictor and cardioacceleratory pathways, causing NAd release, a pressor response and sympathoexcitation. Whilst VPAC receptors are present on SPN projecting to Ad secreting chromaffin cells causing Ad release, causing a direct vasodilatory depressor response, mild tachycardia and sympathoexcitation. Together, these responses may account for the lack of blood pressure response following intrathecal PACAP. Abbreviations: Ad, adrenaline; IML, intermediolateral cell column of the spinal cord; MAP, mean arterial pressure; NAd, noradrenaline; PAC$_1$, PAC$_1$ receptor; RVLM, rostral ventrolateral medulla; SPN, sympathetic preganglionic neurons; VIIn, facial nucleus; VPAC$_1$, VPAC$_1$ receptor; VPAC$_2$, receptor VPAC$_2$.

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Appendices

Appendix 1

Publications

Due to copyright laws, the following articles have been omitted from this thesis. They appear in Appendix I. Please refer to the following citations for details.

Farnham MMJ, Inglott MA, & Pilowsky PM. (2011). Intrathecal PACAP-38 causes increases in sympathetic nerve activity and heart rate but not blood pressure in the spontaneously hypertensive rat. American Journal of Physiology. Heart and Circulatory Physiology, Vol. 300, H214-H222.

Inglott MA, Farnham MMJ & Pilowsky PM. (2011). Intrathecal PACAP-38 causes prolonged widespread sympathoexcitation via a spinally mediated mechanism and increases in basal metabolic rate in anaesthetised rat. American Journal of Physiology. Heart and Circulatory Physiology, Vol. 300, H2300-H2307

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Gaede AH, Inglott MA, Farnham MMJ & Pilowsky PM. (2012). Catestatin has an unexpected effect on the intrathecal actions of PACAP dramatically reducing blood pressure. American Journal of Physiology: Regulatory, Integrative and Comparative Physiology, Vol. 303, R719-R726.

Appendix 2

Equipment and Suppliers

Item	Description	Company/Supplier	Country
Drugs and chemicals;			
<u>Anesthetics;</u>			
Sodium Pentobarbital	Numbutal 10 % w/v in 0.9% saline	Merial Australia Pty Ltd	Australia
Urethane	10 % w/v in 0.9% saline	Sigma-Aldrich Pty Ltd	Australia
Xylocaine	Lignocaine 1% w/v in 0.9% saline	Astra-Zeneca Pharmaceuticals Pty Ltd	Australia
<u>Peptides;</u>			
PACAP-38	Agonist; all PACAP receptors	Auspep Pty Ltd	Australia
VIP	Agonist: VPAC receptors	Auspep Pty Ltd	Australia
Maxadilan	Agonist; PAC_1	Kindly provided by Professor Ethan Lerner of Harvard University	USA
Catestatin	Antagonist; AChR and β adrenergic receptor	Phoenix Pharmaceuticals Pty Ltd	USA
PACAP(6-38)	Antagonist; PAC_1 and $VPAC_2$	Auspep Pty Ltd	Australia
M65	Antagonist: PAC_1	Kindly provided by Professor Ethan Lerner of Harvard University	USA
[D-p-Cl-Phe^6, Leu^{17}]-VIP	Antagonist; VPAC receptors	Sigma-Aldrich Pty Ltd	Australia
<u>Injected drugs;</u>			
Atropine sulphate	Atropine 500 μg in 1 ml ampoules	Astra Pharmaceuticals Pty Ltd	Australia
Glucose	5% glucose solution w/v 0.9% saline	Sigma-Aldrich Pty Ltd	Australia
Heparin sodium	5000 UI in 1 ml ampoules	Mayne Pharma Pty Ltd	Australia
Pancuronium bromide	4 mg in 2ml ampoules	Astra-Zeneca Pharmaceuticals Pty Ltd	Australia
Phenylephrine	PE; α1-adrenergic receptor agonist; $C_9H_{13}NO_2$ 10 μg/kg	Sigma-Aldrich Pty Ltd	Australia
Potassium chloride	KCl 3M w/v in dH_2O	Sigma-Aldrich Pty Ltd	Australia
Sodium chloride	NaCl used as 0.9% saline	Sigma-Aldrich Pty Ltd	Australia
Sodium nitroprusside	SNP; NO^- donor; $Na_2[Fe(CN)_5NO]\cdot2H_2O$ 10 μg/kg	Sigma-Aldrich Pty Ltd	Australia

Item	Description/model	Company/Supplier	Country
Other chemicals/reagents;			
5-bromo-4-chloro-3-indolyl phosphate	BCIP	Roche	Australia
Agar		Chem supply	Australia
Agarose		Promega	Australia
Boehringer blocking reagent	BBR	Roche	Australia
Denhardts solution		Sigma-Aldrich Pty Ltd	Australia
Dextran sulphate		Sigma-Aldrich Pty Ltd	Australia
Digoxigenin (DIG)-11-UTP		Roche	Australia
Ethanol		Chem supply	Australia
Ethylenediamine tetraacetic acid	EDTA	Promega	Australia
Formaldehyde	Formalin	Univar	Australia
Formamide		Sigma-Aldrich Pty Ltd	Australia
Herring sperm DNA		Promega	Australia
India ink		Parker Ink	Australia
Iodine	Betadine	Faulding Pharmaceuticals	Australia
levomisole		Sigma-Aldrich Pty Ltd	Australia
Magnesium chloride	$MgCl_2$	Sigma-Aldrich Pty Ltd	Australia
Maleic acid		Sigma-Aldrich Pty Ltd	Australia
Methiolate		Sigma-Aldrich Pty Ltd	Australia
Nitroblue tetrazolium	NBT	Roche	Australia
Nitrogen gas (100%)	N_2	BOC Gases	Australia
Normal horse serum		Gibco	USA
Oxygen gas (100%)	O_2	BOC Gases	Australia
Paraffin oil		Faulding Pharmaceuticals	Australia
Paraformaldehyde	PFA	Sigma-Aldrich Pty Ltd	Australia
PBS	Phosphate buffered saline; 10 mM w/v in dH_2O	Sigma-Aldrich Pty Ltd	Australia
QIAquick gel extraction kit		Qiagen	Germany
QIAquick PCR purification kit		Qiagen	Germany
Silgel 612A, 612B and catalyst		Wacker	Germany
Sodium citrate	$NaH_2C_6H_5O_7$	Sigma-Aldrich Pty Ltd	Australia
Sodium nitrate	$NaNO_3$	AJAX Chemical	Australia
Tris-HCl		Sigma-Aldrich Pty Ltd	Australia
Tween-20	Detergent	Sigma-Aldrich Pty Ltd	Australia
Vectashield	Mounting Solution	Vector Laboratories Australia Pty Ltd	Australia
Yeast t-RNA		Invitrogen	USA

Item	Description/model	Company/Supplier	Country
Equipment;			
3-way taps	# 394600	BD-Connecta	Australia
50/60-Hz line frequency filter	Humbug	Quest Scientific	Canada
Analogue to Digital converting system	1401	Cambridge Electronic Design	UK
Axiocam digital camera	MR3	Zeiss	Germany
AxioImager epifluorescence microscope	Z1	Zeiss	Germany
Blood gas analyser and cartridges	VetStat, IDEXX	IDEXX Laboratories	USA
Blood glucose analyser and testing strips	Accu-Chek Performa	Roche	USA
Bridge amplifier	QA1 quad bridge amplifier	Scientific Concepts	Australia
CO$_2$ analyser	Capstar-100	CWE, Inc	USA
Combined scaling amplifier and band pass filter	BMA-400 AC/DC Bioamplifer	CWE, Inc	USA
Cutisoft cotton wool	Roll, 375 g	BSN medical	Australia
Cy3-4040B filter set		Semrock	USA
Glass hamilton micro-syringe		Hamilton Company	USA
Homeothermic heating blanket with attached rectal probe thermometer		Harvard Apparatus	USA
Hybaid PCR express gradient thernocycler		Thermo Scientific	Australia
Infrared lamp and globes		Pro-Lamps Pty Ltd	Australia
Infusion pump	11 plus	Harvard Apparatus	USA
Long stemmed cotton buds	15 cm long	BSN medical	Australia
Orbital shaker	PSU-15i	Boeco	Germany
Needles	Multiple gauge	Terumo	Australia
Perfusion/ peristaltic pump	Masterflex console drive	Cole-Palmer Instrument Company	Australia
Preamplifier	SUPER-Z	CWE, Inc	USA
Pressure transducer		Abbott Critical Care Systems	USA
Polyvinylchloride tubing	OD: 0.96 mm x ID: 0.58 mm	Critchley Electrical Products Pty Ltd	Australia

Item	Description/model	Company/Supplier	Country
Equipment (cont);			
Polyvinylchloride tubing	OD: 0.61 mm x ID: 0.28 mm	Critchley Electrical Products Pty Ltd	Australia
Rodent ventilator		UGO Basile	Italy
Scalpel blade	Blade #22	Swann-Morton Limited	UK
Silastic tubing	OD: 1.7 mm x ID: 0.8 mm	Dow Corning	USA
Silastic tubing	OD: 0.94 mm x ID: 0.51 mm	Dow Corning	USA
Silk sutures	5/0 and 2/0	Pearsalls Pty Ltd	UK
Small animal clipper (shaver)	Oster golden 2 speed A5	Harvard Apparatus	USA
Sphygmomanometer (mercury)	# 605P	Yamasu Co Ltd	Japan
Stereotaxic frame		Kopf Instruments	USA
Surgical instruments		Fine Science Tools	Canada
Surgical operating microscope	OPMI VARIO S88	Zeiss	Germany
Syringes	Multiple cc/ml	Terumo	Australia
Tracheal cannula	14G catheter plastic sheath	Johnson and Johnson Medical	Australia
Vibrating microtome	VT 1000S	Leica	Australia
Antibodies;			
Primary antibodies;			
Sheep alkaline phosphatase conjugated with anti-digoxigenin	Used at a concentration of 1: 1000	DAKO	Australia
Mouse anti-Tyrosine Hydroxylase	Used at a concentration of 1: 2000	Sigma-Aldrich Pty Ltd	Australia
Secondary antibody;			
Donkey anti-mouse IgG	Conjugated to Cy3 at a concentration of 1: 500	Jackson ImmunoResearch Laboratories	USA
Software;			
Axiovision	Version 4.7	Carl Zeiss Pty Ltd	Germany
Corel Draw	Versions X3/X4	Corel Corporation	USA
Excel	2003/2007	Microsoft Corporation	USA
GraphPad Prism	Version 5.04	GraphPad Software, Inc	USA
Spike2	Version 7.07a	Cambridge Electronic Design	UK
Word	2003/2007	Microsoft Corporation	USA

Appendix 3

Ethics Approval

ANIMAL RESEARCH AUTHORITY

AEC Reference No.: 2007/022 – 4   **Date of expiry:**   30 April 2011

Full Approval Duration: *1 November 2007 to 30 April 2011 (36 months initial approval plus 6 month extension)*

Professor Paul Pilowsky **(PI)**
Australian School of Advanced Medicine
Macquarie University NSW 2109
9812 3560

Associate Investigators
A/Prof Ann Goodchild   Phone: 0410 601 302
Ms Melissa Farnham   Phone: 0415 821 096

Other people participating
Dr Qi-Jian Sun   Phone: 0423 437 078
Dr Simon McMullan   Phone: 0402 073 146
Dr Natasha Kumar   Phone: 0413 339 954
Mr Peter Burke   Phone: 0401 497 003
Ms Cara Hildreth   Phone: 0402 836 705
Vikram Tallapragada   Phone: 0415 315 406
Mr Stephen Abbott   Phone: 0419 681 345
Mr Darko Spirovski   Phone: 0401 541 232
Ms Andrea Gaede   Phone: 0449 687 329
Ms Melissa Inglott   Phone: 0403 597 648
Mr Daniel (Tao) Xing   Phone: 0414 264 308
Eyitemi Egwuenu   Phone: 0406 777 364

In case of emergency, please contact:
Animal Welfare Officer
Dr Sally Smith: 9850 7758 / 0439 497 383
Central Animal House Manager
Christine Sutter: 9850 7780 / 0428 861 163
or the Principal Investigator / Associate Investigator named above

The above-named are authorised by MACQUARIE UNIVERSITY AEC to conduct the following research:

Title of the project: THE ROLE OF PACAP IN CENTRAL CARDIORESPIRATORY CONTROL

Type of animal research and description of project: Research (Neurophysiology / Pharmacology) – The aims of this project are to (1) identify types of neurons that contain PACAP and its receptors in the brainstem of normotensive rats (2) determine the role of PACAP and its receptors in the control of breathing and blood pressure at rest and during homeostatic reflexes (3) determine if the expression of PACAP or its receptors are altered in hypertension. *Aim 1:* anaesthesia with isoflurane; ketamine/xylazine; placed in sterwotaxic frame; local anaesthetic to skin; dorsal midline incision; sites prepared for tracer injections; incisions sutured and antiseptic applied; cephazolin administered; anaesthetic agonist administered; recovered rats housed in CAHF for up to 1 week; recovered rats overdosed with sodium pentobarbitone and perfused with saline/tissue culture or buffer followed by fixation with aldehydes; brain removed for immunohistochemistry and in-situ hybridisation. *Aim 2:* anaesthesia with isoflurane and urethane; cannulation of trachea, external jugular and carotid artery; dissection of peripheral nerves; recording and stimulation; placed in stereotaxic frame; artificial ventilation with 100% oxygen and paralysis with pancuronium; Ringer's/glucose infusion; dorsal brainstem removed; occipital craniotomy; dissected nerves placed on electrodes for stimulation and recording; experimental procedures as detailed; euthanasia at completion with i.v. KCl. *Aim3:* repeat with SHR, WKY and 2K1C (2 kidney 1 clip) rats. Renal clip applied to left kidney renal artery under anaesthesia through retroperitoneal route as detailed; recovery animals held in CAHF for three weeks. All procedures will be conducted in accordance with details provided in the original protocol approved by the AEC.

Species of animal: Sprague-Dawley rats (SD), Spontaneously Hypertensive Rats (SHR), Wistar Kyoto (WKY) Adult males 300 to 600 g.
Number: SD: 66 / SHR: 66 / WKY: 198 (Total over three years = 330)
Location: Central Animal House Facility and Ground Floor Dow Corning Building, 3 Innovation Rd, Macquarie University, NSW 2109.

Amendments approved by the AEC since initial approval:
1. Addition of Ms Andrea Gaede to the protocol
2. Addition of Ms Melissa Inglott to the protocol
3. Substitution of 12 WKY strain rats with 12 Wistar strain rats
4. Addition of Mr Tao (Daniel) Xing to the protocol
5. Substitution of 16 WKY strain rats with 16 SD rats
5. Additional procedures of acute adrenalectomy and acute spinal cord transection under anaesthesia & without recovery
6. Addition of Dr Eyitemi Egwuenu to the protocol
7. Substitution of 19 WKY strain rats with 19 SD strain rats
8. Substitution of 105 WKY strain rats with 105 SD strain rats
9. Use of two additional antagonists, available AC inhibitors and available PLC inhibitors (as per revised amendment request dated 18/01/2010)
10. Extension of approval duration for 6 months.

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.
This authority remains in force from **9 November 2010** to **30 April 2011 (6 months)**, unless suspended, cancelled or surrendered, **and is contingent upon receipt of a Final Report at the end of this period.**

Prof Michael Gillings   Date: **9 November 2010**
Chair, Animal Ethics Committee

Form C (issued under part IV of the Animal Research Act, 1985)

MACQUARIE
UNIVERSITY - SYDNEY

ANIMAL RESEARCH AUTHORITY

AEC Reference No.: 2008/015
Full Approval Duration: 25 June 2008 to 24 June 2011 (36 months)

		Associate Investigator (s)	
To:	Professor Paul Pilowsky **(PI)**	A/Prof Ann Goodchild	Phone: 0410601302
	Australian School of Advanced Medicine	Ms Andrea Gaede	Phone: 0449687329
	Macquarie University		
	NSW 2109		
	Phone:0431500553	**Other people participating**	
		Dr Qi-Jian Sun	Phone: 0413733250
		Dr Simon McMullan	Phone: 0402073146
		Dr Natasha Kumar	Phone: 0413339954
		Ms Mandy Lung	Phone: 0411698896
		Ms Melissa Farnham	Phone: 0415821096
		Mr Peter Burke	Phone: 0401497003
		Ms Cara Hildreth	Phone: 0402836705
		Mr Stephen Abbott	Phone: 0419681345
		VJ Tallapragada	Phone 0415315406

Is authorised by:

MACQUARIE UNIVERSITY to conduct the following research:

Title of the project: THE ROLE OF CHROMOGRANIN ASSOCIATED PEPTIDES IN THE CENTRAL NEURAL CONTROL OF RESPIRATION, ARTERIAL BLOOD PRESSURE AND SYMPATHETIC NERVE ACTIVITY

Type of animal research and description of project: Research (Neurophysiology / Pharmacology) – The project aims to investigate the anatomy and physiology of the large precursor protein chromogranin A and peptide cleavage products: catestatin, vasostatin and pancreastatin, examine structure and function in the spinal cord RVLM and CVLM. *Aim 1* – recovery experiments in SD rats using retrograde tracing as described in the approved protocol; *Aim 2* - Non-recovery experiments: microinjection for functional studies involving cutting of the vagus nerve, recording from the phrenic nerve, greater splanchnic nerve, lumber sympathetic trunk, stimulation of aortic depressor nerve and sciatic nerve as described in approved protocol; *Aim 3* -- Non-recovery experiments – examination of pathophysiology in SHR, WYK and OF rats.

All procedures are to be performed as described, and in accordance with, the approved protocol.

Species of animal: *Rattus* (Sprague-Dawley rats (SD), Spontaneously Hypertensive Rats (SHR) or Wistar Kyoto rats (WKY), Obese overfed rats (OF)) males, 300 – 600 gm

Number: total of 408 male rats over three years (approximately 136 rats per year for 3 years) 300 – 600 grams

Location: Central Animal House Facility, and Ground Floor Dow Corning Building, 3 Innovation Rd, Macquarie University, NSW 2109.

Amendments considered by the AEC during last period: N/A

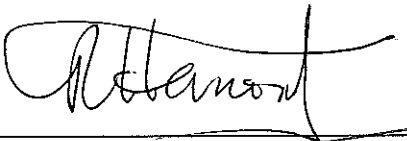As approved by and in accordance with the establishment's Animal Ethics Committee.

MACQUARIE UNIVERSITY AEC

Approval was granted subject to compliance with the following conditions: N/A

(This authority has been issued as the above condition (s) has been addressed to the satisfaction of the AEC)

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

This authority remains in force from **25 June 2008** to **24 June 2009**, unless suspended, cancelled or surrendered, **and will only be renewed upon receipt of a PROGRESS REPORT at the end of this period.**

Date: 3/7/08

A/Prof Robert Harcourt
Acting Chair of AEC, Macquarie University

Form C (issued under part IV of the Animal Research Act, 1985)

ANIMAL RESEARCH AUTHORITY

MACQUARIE UNIVERSITY

AEC Reference No.: 2009/011   **Date of expiry: 4 May 2011**

Full Approval Duration: 5 May 2009 to 4 May 2012 (36 months)

Principal Investigator	**Associate Investigators**
Professor Paul Pilowsky **(PI)**	A/Prof Ann Goodchild Phone: 0410601302
Australian School of Advanced Medicine	Ms Melissa Inglott Phone 0403597648
Macquarie University	
NSW 2109	
Phone: 0431 500 553	**Other people participating**

Other people participating:

Dr Qi-Jian Sun	Phone: 0413733250
Dr Simon McMullan	Phone: 0402073146
Dr Natasha Kumar	Phone: 0413339954
Dr Mandy Lung	Phone: 0411698896
Ms Melissa Farnham	Phone; 0415821096
Mr Peter Burke	Phone: 0401497003
Ms Cara Hildreth	Phone: 0402836705
Mr Andrea Gaede	Phone: 049687329
VJ Tallapragada	Phone 0415315406
Mr Stephen Abbott	Phone: 0419681345
Mr Darko Spirovski	Phone: 0401541232
Ms Tara Bautista	Phone 0431325479
Mr Branimir Zogovikj	Phone: 0415152413
Ms Sarah Hassan	Phone: 0410357056
Ms Belinda Bowman	Phone: 0423055789
Mr Ahmed Rahman	Phone: 0423069118
Mr Israt Shahid	Phone: 0425659036
Mr Tao Xing	Phone: 0414264308
Mr Hanafi Damanhurie	Phone: 0432353958
Mr Lindsay Parker	Phone: 0416821288

The above-named are authorised by
MACQUARIE UNIVERSITY AEC to conduct the following research:

**Title of the project: THE ROLE OF VIP AND CART IN THE CENTRAL NEURAL CONTROL OF BLOOD PRESSURE,
RESPIRATION AND SYMPATHETIC NERVE ACTIVITY**

Type of animal research and description of project:
Research (Neurophysiology / Pharmacology) – This project aims to investigate the anatomy and physiology of the peptides VIP
and CART in normal and high blood pressure conditions. Aim 1: Recovery experiments in SD rats using retrograde tracing carried
out under surgical anaesthesia and as described in to the approved protocol; Aim 2: Non-recovery experiments in SD rats carried
out under surgical anaesthesia and involving cutting of vagus nerve, recording and stimulation of selected nerves, euthanasia at
completion with 0.5ml 3M KCl; Aim 3: as per aims 1 & 2 using SHR and WKY rats.
All procedures to be performed in accordance with approved protocol.

Species of animal: *Rattus* (Sprague-Dawley rats (SD), Spontaneously Hypertensive Rats (SHR) or Wistar Kyoto rats (WKY))
Males 300 to 600 gm

Number: a total of 424 rats over three years (approx. 141 animals per year)

Location: Central Animal House Facility, and Australian School of Advanced Medicine (2 Technology Place), Macquarie
University NSW 2109

As approved by and in accordance with the establishment's Animal Ethics Committee.
MACQUARIE UNIVERSITY AEC

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection
with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

This authority remains in force from **17 June 2010** to **4 May 2011 (12 months)**, unless suspended, cancelled or
surrendered, **and will only be renewed upon receipt of a PROGRESS REPORT at the end of this period.**

_____   Date:_____23/06/2010_____

Prof Michael Gillings
Chair of AEC, Macquarie University

ANIMAL RESEARCH AUTHORITY

MACQUARIE UNIVERSITY

AEC Reference No.: 2010/055

Date of expiry: 31 December 2011

Full Approval Duration: *1 January 2011 to 31 December 2013 (36 months)*

Principal Investigator:
Prof Paul Pilowsky
Australian School of Advanced Medicine
Macquarie University NSW 2109
0431 500 553
Paul.pilowsky@asam.mq.edu.au

Associate Investigators:
Dr Melissa Farnham   0415 821 096

Other people participating:
Melissa Inglott   0403 597 648
Andrea Gaede   0449 687 329

<div style="border:1px solid red">

In case of emergency, please contact:
Animal Welfare Officer Dr Sally Smith: 9850 7758 / 0439 497 383
Central Animal House Manager Christine Sutter: 9850 7780 / 0428 861 163
or the Principal Investigator / Associate Investigator named above

</div>

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: The role of PACAP in sympathetically mediated cardiovascular, stress and temperature responses

Type of animal research and aims of project:
Research (biomedical) – This project aims to improve understanding of how the brainstem controls the cardiovascular system and may lead to the development of new therapies for high blood pressure.

Surgical Procedures category: 2 (Animal Unconscious Without Recovery); 5 (Major Surgery With Recovery)

All procedures must be performed in accordance with the AEC approved protocol.

Numbers approved:

Species	Strain	Age/Sex/Weight	Year 1	Year 2	Year 3	Total	Supplier/Source
Rat	Sprague-Dawley (SD)	Adult/male/ 300-500g	145	146	146	**437**	ARC Perth

Location of research:

Location	Full street address
Australian School of Advanced Medicine	Level 1, Clinic Building, 2 Technology Place, Macquarie University NSW 2109
Central Animal House Facility	Building F9A, Research Park Drive, Macquarie University NSW 2109

Amendments approved by the AEC since initial approval: N/A

Conditions of Approval:

1. A signed declaration that Paul Pilowsky has taken over as PI on the protocol must be supplied to the Research Office. Work may not commence until this has been received.
2. Andrea Gaede must provide her signature before commencing work on this protocol.

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

This authority remains in force from **1 January 2011 to 31 December 2011**, unless suspended, cancelled or surrendered, **and will only be renewed upon receipt of a PROGRESS REPORT before the end of this period.**

Prof Michael Gillings
Chair, Animal Ethics Committee

Date: *16 December 2010*

Adapted from Form C (issued under part IV of the Animal Research Act, 1985)

MACQUARIE UNIVERSITY
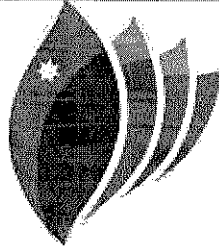
Research Office
Research Hub, Building C5C East
MACQUARIE UNIVERSITY NSW 2109

Phone   +61 (0)2 9850 8612
Fax   +61 (0)2 9850 4465
Email   ro@mq.edu.au

Biosafety Committee
Phone   +61 (0)2 9850 4194
Email   biosafety@mq.edu.au

Macquarie University Biosafety Committee

Cover Sheet for Research Conducted under the Standard Operating Procedures for Animal and Animal Tissue Handling (ASAM/SOP/002)

(July 2010 Version)

When to use this form:

USE THIS FORM if your research falls under the following category:

1. You are a researcher from the Australian School of Advanced Medicine (ASAM) and you are conducting research that is compliant with the procedures set out under the Macquarie University Biosafety Committee approved Standard Operating Procedures for Animal and Animal Tissue Handling (SOP Reference: ASAM/SOP/002).

If you are conducting research with Genetically Modified Organisms (GMOs) then you must submit a new Biosafety application. Please download an application from:
http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/biosafety_research_ethics/forms

If you are unsure about whether to submit this form then please contact the Biosafety Committee Secretariat on 02 9850 4194 or by email biosafety@mq.edu.au

Please note that if your research is covered by the above SOP and you do not notify the Committee by submitting this form, you will not be recorded as having obtained Biosafety approval for your research.

How to use this form:

1. Complete the Cover Sheet (on the following page)
2. Submit a signed hard copy of the form to the following address:

   Biosafety Secretariat
   Research Office, Level 3
   RESEARCH HUB
   Building C5C
   Macquarie University
   NSW, 2109.