Respiratory sympathetic coupling in chronic kidney disease

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Declaration of originality

I hereby declare that the work presented in this thesis entitled "Respiratory sympathetic coupling in chronic kidney disease" has not previously been submitted for a higher degree to any other university or institution. To the best of my knowledge this submission contains no material previously published or written by another person, except where due reference is stated otherwise. Any contribution made to the research by others is explicitly acknowledged.

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Candidate contributed to experimental design, performed all experiments, analysed data, and interpreted results. Candidate was the major contributor to the manuscript. Jacqueline Phillips, Qi-Jian Sun, and Cara Hildreth contributed to interpretation of results and editing, revision and final approval of manuscript.

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Candidate performed all experiments, analysed data and interpreted results. Candidate was the major contributor to the manuscript. Cara Hildreth, Jacqueline Phillips and Qi-Jian Sun contributed to conception and design of experiments, data analysis, interpretation of results and editing, revision and final approval of manuscript.

Chapter 5

Candidate performed all experiments, analysed data and interpreted results. Candidate was the major contributor to the manuscript. Jacqueline Phillips, Qi-Jian Sun and Cara Hildreth contributed to conception and design of experiments, data analysis, interpretation of results and editing, revision and final approval of manuscript.

This thesis is dedicated to my beloved wife, daughter, mother, sister and father

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Summary

Chronic kidney disease (CKD) is a major public health problem, resulting in significant morbidity and mortality. Among the different causes and comorbidities of CKD, hypertension draws special attention as it is often difficult to treat and increases cardiovascular risk in these patients. It has been established that sympathetic nerve activity (SNA) is increased in hypertension and CKD. Recent works suggest that respiratory modulation of SNA [respiratory sympathetic coupling (respSNA); the coordination between breathing and SNA] is a driving factor behind increased SNA in a number of diseased states including hypertension; however, the pattern of respSNA with its role in underlying mechanism in CKD is not yet known.

Accordingly, the aims of the thesis were to (i) determine if respSNA is altered in a classical rat model of CKD, the Lewis Polycystic Kidney (LPK) rat and determine if this is associated with hypersensitivity of peripheral chemoreceptors; (ii) identify if the pattern of respSNA along with its association with peripheral chemoreceptors is different between male and female animals with CKD; (iii) examine if respSNA contributes to baroreflex dysfunction in CKD and (iv) to investigate if bilateral carotid sinus (CSN) transection, a method by which to prevent peripheral chemoreceptors input, results in reduction of respSNA and blood pressure in the LPK rat.

The main finding was that the male LPK rats had amplified respSNA compared to Lewis control rats since very early age (5 weeks). Notably, peripheral chemoreceptor stimulation revealed more enhanced respSNA in both juvenile and adult LPK rats. The female LPK rats also exhibited amplified respSNA compared to female Lewis rats. Another significant finding was that inhibition of altered respSNA selectively improved the baroreflex response of SNA in LPK rats. Furthermore, transection of CSN decreased blood pressure in the LPK model of CKD, although respSNA was elevated in LPK rats that had undergone CSN transection compared to controls.

The results help us to understand the basic linking between SNA and respiration, with the goal of targeting peripheral chemoreceptors and respSNA for novel therapies for hypertension associated with CKD.

Publications arising from this thesis

Journal Publications

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List of abbreviations

% - Percent
α receptor - Alpha adrenergic receptor
ACE - Angiotensin-converting enzyme
ACEI - Angiotensin-converting enzyme inhibitors
ACh - Acetylcholine
ACTH - Adrenocorticotrophic hormone
ADN - Aortic depressor nerve
ADPKD - Autosomal dominant polycystic kidney disease
Ang II - Angiotensin II
ANOVA - Analysis of variance
ANP - Atrial natriuretic peptide
ANS - Autonomic nervous system
AP - Arterial pressure
ARB - Angiotensin receptor blockers
ARPKD - Autosomal recessive polycystic kidney disease
AT1 - angiotensin type 1 receptors
ATP - Adenine triphosphate
AUC - Area under the curve
AV - Atrioventricular
β receptors - Beta adrenergic receptors
BötC - Bötzinger complex
BPM - Beats per minute
BRS - Baroreflex sensitivity
BW - Body weight
BZJ - Bezold-Jarisch reflex
ºC - Degree Celsius
Ca^{2+} - Calcium
cAMP - Cyclic adenosine monophosphate
CIH – Chronic intermittent hypoxia
CKD - Chronic kidney disease
cm - Centimetre
CNS - Central nervous system
CO_2 - Carbon dioxide
COPD – Chronic obstructive pulmonary disease
CPAP – Continuous positive airway pressure
CSN - Carotid sinus nerve
CVD - Cardiovascular disease
CVLM - Caudal ventrolateral medulla
cVRC - Caudal ventral respiratory column
CVPN - Cardiac vagal preganglionic neurons
DBP - Diastolic blood pressure
DMNV - dorsal motor nucleus of the vagus
DRC – Dorsal respiratory column
EAA - Excitatory amino acid
EDTA - Ethylenediaminetetraacetic acid
ESRD - End-stage renal disease
g - Gram
GABA - Gamma amino butyric acid
GFR - Glomerular filtration rate
h - Hour

HF - High frequency
HR - Heart rate
HRV - Heart rate variability
5 HT - 5-hydroxytryptamine
Hz - Hertz
i.p. - Intraperitoneal
i.v. - Intravenous
IML - intermediolateral cell column
jck - Juvenile cystic kidney
K^+ - Potassium
2K-1C - 2 kidney-1 clip
KDOQI - Kidney Disease Outcomes Quality Initiative
kHz - Kilohertz
L - Litre
L-NAME – N(G)-nitro-L-arginine methyl ester
lSNA - Lumbar sympathetic nerve activity
LVH - Left ventricular hypertrophy
µV - Microvolt
M - Muscarinic cholinergic receptors
m^2 - Square meter
MAP - Mean arterial pressure
MAP_{50} - Mean arterial pressure at midpoint of the curve
MAPsat - Saturation mean arterial pressure
MAPthr - Threshold mean arterial pressure
min - Minute
ml - Millilitre
mmHg - Millimetre of mercury
ms - Millisecond
MSNA - Muscle sympathetic nerve activity
N_2 - Nitrogen
NA - Noradrenaline
Na^+ - Sodium
NADPH - Nicotinamide adenine dinucleotide phosphate
Nek8 - Never in mitosis gene A-related kinase 8
NIMA - Never in mitosis gene A
NMDA - N-methyl-D-aspartate
NO - Nitric oxide
NOS - Nitric oxide synthase
NPHP - Nephronophthisis
NPY - Neuropeptide Y
ns No - significance
NTS - Nucleus tractus solitarius
O_2 - Oxygen
P - Pressure
PCO_2 - Carbon dioxide partial pressure
PE - Phenylephrine
PKD - Polycystic kidney disease
PO_2 - Oxygen partial pressure
PP - Pulse pressure
pre-BötC - Pre-Bötzinger complex
PSN - Presympathetic neurons
PVN - Paraventricular nucleus

RAAS - Renin-angiotensin-aldosterone system
respSNA - Respiratory sympathetic coupling
RMSDD - Square root of the mean squared differences of successive normal-to-normal intervals
rSNA - Renal sympathetic nerve activity
RVLM - Rostral ventrolateral medulla
rVRC - Rostral ventral respiratory column
s - Second
s.c. - Subcutaneous
SA - Sinoatrial
sat - Saturation
SBP - Systolic blood pressure
SBPV - Systolic blood pressure variability
SDANN - Standard deviation of the average normal-to-normal intervals
SDNN - Standard deviation of normal-to-normal intervals
SEM - Standard error of the mean
SHR - spontaneously hypertensive
SLE - Systemic lupus erythematosus
SNA Sympathetic nerve activity
sSNA - Splanchnic sympathetic nerve activity
SNP - Sodium nitroprusside
SPN - Sympathetic preganglionic neurons
thr - Threshold
UCr Urinary creatinine
UPC - Urinary protein:creatinine ratio
UPro - Urinary protein
v/v Volume by volume
VLF - Very low frequency
VRC - Ventral respiratory column

1. Literature review

1.1. Introduction

The incidence of chronic kidney disease (CKD) is increasing worldwide at an alarming rate (Ayodele & Alebiosu, 2010; Couser *et al.*, 2011). Approximately 16% and 14% of the Australian and US adult population, respectively, are affected by kidney diseases (White *et al.*, 2010; Saran *et al.*, 2018). The morbidity and mortality of patients with CKD is largely related to hypertension and heart disease (Go *et al.*, 2004; Schiffrin *et al.*, 2007; ANZDATA Registry, 2018; USRDS, 2018), and intriguingly, the process of cardiovascular damage starts in the very early stages of CKD (Vanholder *et al.*, 2005). In many patients with CKD, it is often difficult to control blood pressure in spite of multi drug treatments, with a human study showing that 22.9% patients with CKD have true resistant hypertension and 42.9% have sustained hypertension (De Nicola *et al.*, 2013b). As a result, CKD itself is recognised as a common cause of resistant and sustained hypertension. Given that resistant hypertension and sustained hypertension are associated with high cardiovascular and renal mortality (De Nicola *et al.*, 2013b), and that traditional management of hypertension associated with CKD is difficult to resolve, research into the underlying mechanisms is critical.

The sympathetic nervous system is a major regulator of blood pressure contributing to both cardiac output and total peripheral resistance, the major determinants of overall blood pressure. Various studies suggest that sympathetic nervous activity (SNA) is increased in essential hypertension in both humans and animals (Lundin *et al.*, 1984; Grassi, 1998; Esler, 2000; Lambert *et al.*, 2007). Moreover, in CKD, it has been found that the onset of altered SNA occurs early in the disease process, often before the onset of hypertension (Korner *et al.*, 1993; Phillips *et al.*, 2007). Direct sympathetic nerve recordings and plasma catecholamines levels have provided evidence of an overactive sympathetic nervous system in association with the hypertension of kidney disease (Klein IH, 2003; Phillips, 2005; Schlaich *et al.*, 2009b; Campese *et al.*, 2011; Grassi *et al.*, 2012). Furthermore, treatment with centrally acting sympatholytic agents or renal nerve ablation reduces hypertension in renal failure patients (Campese & Massry, 1983; Levitan *et al.*, 1984; Schlaich *et al.*, 2009b; Hering *et al.*, 2012) and neonatal sympathectomy attenuates hypertension in the bilateral 5/6 nephrectomized rat (Augustyniak *et al.*, 2010). As a result, there is a growing consensus that increased SNA plays an important role in development and /or maintenance of hypertension associated with kidney disease (Hausberg *et al.*, 2002; Phillips *et al.*, 2007; Schlaich *et al.*,

2009b). However, we still do not understand the underlying mechanisms driving this increase in activity.

Currently, one mechanism for the elevation of SNA that has drawn the attention of scientists is respiratory sympathetic coupling, whereby respiration synchronously modulates SNA. Changes in sympathetic nerve discharge have been found to be associated with different phases of respiration both in animal and humans (Adrian *et al.*, 1932; Badra *et al.*, 2001; Dick *et al.*, 2004). The harmonious alterations in blood pressure (Traube-Hering waves) associated with cycles of respiration are essential for adequate tissue perfusion (Simms *et al.*, 2010). It is, therefore, plausible that alteration of respiratory modulation of SNA could contribute to hypertension. Accordingly, it has been shown in the spontaneously hypertensive rat (SHR) model of essential hypertension that respiratory modulation of SNA is augmented, and that enhanced respiratory–related bursts of sympathetic activity may be responsible for development of hypertension (Simms *et al.*, 2009). This was corroborated with an earlier study showing an altered pattern of respiratory-modulated sympathetic discharge in the SHR (Czyzyk-Krzeska & Trzebski, 1990).

However, the contributory role of augmented respiratory modulation of SNA in hypertension was challenged by a study conducted in humans which suggested that increased respiratory modulation of SNA did not underlie the elevation in muscle SNA (MSNA) seen in hypertensive humans (Fatouleh & Macefield, 2011). Taken together, these studies do not provide definitive evidence for or against the notion that augmented respiratory modulation contributes to the elevated SNA observed in essential and secondary hypertension. Rather, they highlight the fundamental gaps in knowledge regarding how the respiratory and sympathetic nervous systems interact in pathological states.

This project therefore aims to assess the hypothesis that increased respiratory modulation of SNA is a feature of hypertension associated with CKD. This chapter substantially will review the key factors regulating respiration and blood pressure with involvement of autonomic pathways in these physiological processes and their role in hypertension related to CKD.

1.2. Central control of respiration

Respiration can be broken into three main phases: inspiration, post inspiration and expiration. Each of these phases are regulated by different types of central respiratory neurons. Most are inspiratory and expiratory neurons, along with other central respiratory neurons, with their output mediated by respiratory motor neurons in the spinal cord. The brainstem is the main

location of the central respiratory generators and contains ventral respiratory column (VRC), dorsal respiratory column (DRC) and pontine nuclei. Central respiratory neurons are clustered in these areas and classified according to their firing patterns (e.g. augmenting or decrementing) in relation to the different phases of the respiratory cycle (Bianchi *et al.*, 1995; Smith *et al.*, 2007; Smith *et al.*, 2009; Simms *et al.*, 2010). Accordingly, they are classified as pre-inspiratory (pre-I), early inspiratory (early-I), augmenting inspiratory (aug/ramp-I), post-inspiratory (post-I) and augmenting and decrementing expiratory (E) neurons. The respiratory motor neurons in the spinal cord driving respiratory activity are the phrenic motor neurons (C3-C5) for the diaphragm, intercostal motor neurons (T1-T2) for the intercostal muscles and abdominal motor neurons (T4-L3) for the abdominal muscles (Bianchi *et al.*, 1995; Smith *et al.*, 2007; Smith *et al.*, 2009).

1.2.1. Ventral respiratory column

The VRC can be divided into several domains and is the main regulator of the different phases of respiratory activity. The main domains are: the Bötzinger complex (BötC), the pre-Bötzinger complex (pre-BötC), and the rostral (rVRC) and caudal (cVRC) parts of the VRC (Bianchi *et al.*, 1995; Smith *et al.*, 2007; Smith *et al.*, 2009; Simms *et al.*, 2010). Most recently, Anderson et al have described a group of post inspiratory neurons (PiCO) that provide excitatory signals to induce post inspiratory activity (Anderson *et al.*, 2016).

a) The BötC mainly regulates expiration and mostly consists of post-I neurons, which are essential for the transition between inspiration and expiration. It also contains E-augmenting and E-decrementing neurons that are crucial for the basic patterns of normal expiration (Ezure, 1990; Bianchi *et al.*, 1995; Smith *et al.*, 2007; Smith *et al.*, 2009). The BötC neurons are usually inhibitory in nature and release glycine when stimulated (Ezure, 1990; Ezure *et al.*, 2003).
b) The pre-BötC is the rhythm generator of inspiration, situated caudal to the BötC complex. It mostly consists of pre-I neurons providing rhythmic inspiratory impulse before the onset of inspiration (Smith *et al.*, 1991; Rybak *et al.*, 2004; Rybak *et al.*, 2007a; Smith *et al.*, 2009). Most of these neurons are excitatory neurons and non-N methyl D aspartate (non-NMDA) in nature. Within the pre-BötC, there are some neurons that have pacemaker like activity which generate the definite pattern of inspiration (Koshiya & Smith, 1999). There are some glycinergic inhibitory inspiratory neurons also present in this region that generate inspiratory inhibition thereby coordinating the rhythmic inspiration expiration cycle (Winter *et al.*, 2009).

However, pacemaker neurons are not always necessary for respiratory rhythm generation, with data to suggest that excitatory synaptic interactions within the brain homeobox protein 1 (DBX1) neurons of the pre-BötC may contribute to the respiratory rhythm (Del Negro *et al.*, 2018).

c) The rVRC mostly consists of aug/ramp-I neurons projecting to motor inspiratory neurons in the spinal cord. These are mostly pre-motor neurons of the phrenic nerve and intercoastal nerve (Bianchi *et al.*, 1995; Smith *et al.*, 2009; Simms *et al.*, 2010). These neurons are inspiratory-excitatory in nature, and do not possess any pace maker activity. They modulate the control and shape of the inspiration output with help of excitatory neurons of the BötC complex, and inhibitory neurons of the pre- BötC complex (Bianchi *et al.*, 1995; Smith *et al.*, 2007; Smith *et al.*, 2009).

d) The cVRC are mostly E neurons located in the retroambigual area, and their function limits the augmenting and decrementing activity of expiration with projections to spinal expiratory motor neurons (Ezure, 1990; Bianchi *et al.*, 1995; Smith *et al.*, 2009; Simms *et al.*, 2010). These are pre-motor neurons of the intercostal and abdominal muscles. The cVRC, receives inputs from the BötC complex and controls the expiratory drive (Bianchi *et al.*, 1995; Smith *et al.*, 2009).

1.2.2. Retrotrapezoid nucleus

The retrotrapezoid nucleus (RTN), located below and around the facial nucleus, is emerging as a rhythm generator for active expiration and works with the pre-BötC to coordinate rhythmic patterns of breathing (Janczewski & Feldman, 2006; Smith *et al.*, 2007; Abdala *et al.*, 2009a; Smith *et al.*, 2009). There is another theory that the parafacial respiratory group (pFRG), a component of RTN, has pre-I neurons which may generate rhythmic excitatory drive to the pre- BötC (Onimaru *et al.*, 2006). As well, the RTN is believed to be a central chemosensitive area responsive to biochemical changes in brain pH/ CO_2 and receives input from oxygen-dependent peripheral chemoreceptors. Moreover, it controls reciprocal inspiratory and expiratory activity through a feedback mechanism involving the medullary respiratory neurons rather than from intrinsic neuronal mechanisms (Guyenet *et al.*, 2005; Onimaru *et al.*, 2006; Guyenet *et al.*, 2008). Emerging evidence suggests that two major rhythm generators are present in the medulla, with the pre-BötC complex generating inspiration and, under conditions of increased respiratory drive, the RTN generating active expiration (Janczewski & Feldman, 2006; Smith *et al.*, 2009).

1.2.3. Pontine respiratory neurons

The pontine respiratory group, which is composed of the Kölliker-Fuse nucleus and the medial and lateral parabrachial nuclei, is situated in the rostral dorsolateral and ventrolateral pons. By virtue of its extensive connections with medullary respiratory neurons, this group regulates the rhythmic pattern of respiration by controlling post inspiratory activity, the transition between the inspiratory phase and the expiratory phase which coordinates with the upper airway muscles (Dutschmann & Herbert, 2006; Smith *et al.*, 2007; Dutschmann *et al.*, 2008; Smith *et al.*, 2009).

1.2.4. Dorsal respiratory column

The DRC is located within the nucleus of the solitary tract (NTS) and contains both inspiratory and expiratory neurons. The ventrolateral part of the DRC provides inspiratory impulses whereas the medial part controls expiratory activity (Bianchi *et al.*, 1995; Subramanian *et al.*, 2007). These neurons also regulate the Hering-Breuer reflex (Bonham & McCrimmon, 1990; Bianchi *et al.*, 1995).

1.3. Central control of sympathetic activity by presympathetic neurons

Sympathetic preganglionic neurons (SPNs) located in the intermediolateral (IML) nucleus of the spinal cord regulate vascular tone and arterial blood pressure in response to input from presympathetic neurons (PSNs). These neurons are responsible for the generation and maintenance of sympathetic outflow. The PSNs are distributed in five key areas: the rostral ventrolateral medulla (RVLM), the rostral ventromedial medulla (RVMM), the caudal raphe nuclei, the A5 non-adrenergic cell group in the caudal ventrolateral part of the pons, and the paraventricular nucleus of the hypothalamus (PVN) (Strack *et al.*, 1989a; Dampney, 1994).

1.3.1. Rostral ventrolateral medulla

Among all areas, the RVLM plays a lead role in the regulation of sympathetic vasomotor activity. Several animal studies on inactivation of cells of the RVLM or inhibition of transmission of impulse caudal to facial nerve nucleus showed the evidence of reduction of SNA and blood pressure in those animal models similar to the effect of transaction of spinal cord (Guertzenstein & Silver, 1974; Feldberg & Guertzenstein, 1976). Accordingly, the role of the PSNs of the RVLM is important to dominate the sympathetic out flow. The PSNs of the RVLM that directly regulate blood pressure project monosynaptically to SPNs of the spinal cord (Haselton & Guyenet, 1990; Dampney *et al.*, 2002; Guyenet, 2006). These PSNs are regulated by different neurotransmitters like glutamate, Gamma aminobutyric acid (GABA),

acetylcholine, opiates, catecholamines, 5-hydroxytryptamine (5-HT), and angiotensin. This regulation makes it possible to control the vasoactive activity of these neurons (Dampney, 1994). Among those transmitters, glutamate and GABA regulate and counter regulate the activity of RVLM neurons, thereby maintaining resting vasomotor tone. Moreover, both anatomical and electrophysiological studies from animal models provided evidence of direct projections from the PSNs of the RVLM to the SPNs of the spinal cord (Amendt *et al.*, 1979; Ross *et al.*, 1981; Ross *et al.*, 1984; Morrison & Gebber, 1985; McAllen, 1986).

1.3.2. Rostral ventromedial medulla

The PSNs of the RVMM are situated just lateral to the pyramidal tract and directly innervate the SPNs, projecting to the adrenal medulla and all the major sympathetic ganglia (Strack *et al.*, 1989a; Strack *et al.*, 1989b). Animal studies provide evidence that the pressor effect of these neurons on the RVMM is mediated by 5-HT neurons (Loewy & McKellar, 1981; Steinbusch, 1981; Aiyagari *et al.*, 2010; Cersosimo & Benarroch, 2013), however, the knowledge about the role of RVMM in sympathetic outflow is currently limited..

1.3.3. Caudal raphe nuclei

The caudal raphe nuclei, situated in the midline of medulla, projects to the SPNs of the spinal cord. Its exact function has not been identified yet, however, it is believed that it has both excitatory (serotonergic) and non-serotonergic inhibitory neurons, projecting to the SPNs (Morrison & Gebber, 1982, 1985; Barman & Gebber, 1989; Morrison, 2001; Kung *et al.*, 2010; Depuy *et al.*, 2011).

1.3.4. A5 cell group

The A5 cell group, composed of non-noradrenergic neurons situated in the pons is an important sub class of the PSNs in the brain (Byrum *et al.*, 1984; Strack *et al.*, 1989a; Strack *et al.*, 1989b). In most studies, it has been suggested that the A5 cell group is sympathoexcitatory with baroreceptor mediated inhibition present (Andrade & Aghajanian, 1982; Byrum *et al.*, 1984; Guyenet, 1984; Dampney, 1994; Kanbar *et al.*, 2011; Abbott *et al.*, 2012) and that this group does not show auto-rhythmicity. However, only a few studies have suggested the presence of sympathoinhibitory neurons in it (Neil & Loewy, 1982; Loewy *et al.*, 1986).

1.3.5. Paraventricular nucleus

The hypothalamic PVN innervates SPNs in the spinal cord with collateral projections to other autonomic nuclei in the brain including the mid brain including periaqueductal gray (PAG) nucleus and parabrachial region, RVLM, NTS, dorsal vagal nucleus, and nucleus ambiguous (Lundin *et al.*, 1984; Dampney *et al.*, 1987; Dampney, 1994; Allen, 2002; Chen & Toney, 2010). Different subgroups of PVN neurons interact with different organ specific SPNs (Coote, 2005; Ramchandra *et al.*, 2006) that may result in opposite or different actions of sympathetic outflow such as an increase in adrenal nerve activity and a decrease in renal nerve activity (Katafuchi *et al.*, 1988). Accordingly, the net effect of the PVN on blood pressure depends on the collective activation of the PVN, SPNs and collateral connections with other regions.

The other PSNs are located in the midbrain PAG, the lateral hypothalamic area, and the zona incerta, projecting to the mid thoracic spinal cord with connections to other autonomic nuclei (Strack *et al.*, 1989a). They may coordinate sympathetic outflow directly and indirectly to regulate respiratory and cardiovascular function.

1.3.6. Nucleus of the solitary tract in autonomic control

The NTS is an important area of the brain stem located in the dorsolateral medulla extending from the level of the caudal portion of the facial nucleus to the caudal portion of the pyramids. It functions to process, integrate and incorporate monosynaptic input from visceral sources including cardiopulmonary receptors like arterial baroreceptors, pulmonary stretch receptors, peripheral chemoreceptors and volume receptors as well as polysynaptic inputs from sympathetic and somatic sources, before transmitting and communicating these afferent inputs to other autonomic nuclei in the brain, especially the RVLM and spinal cord (Dampney, 1994; Blessing, 1997; Potts *et al.*, 2003). Among different subgroups of neurons in the NTS, the intermediate NTS (iNTS) and the caudal NTS (cNTS) are responsible for integrating cardiorespiratory information (Kumada *et al.*, 1990; Loewy, 1990; van Giersbergen *et al.*, 1992; Zoccal *et al.*, 2014). Furthermore, these sub groups of neurons of the NTS which are activated by specific visceral afferent information, process and transmit impulses to different specific groups of neurons such as PSNs of the RVLM, parasympathetic neurons and SPNs of the spinal cord to produce specific efferent activities (Aicher *et al.*, 1996; Bailey *et al.*, 2006; Alheid *et al.*, 2011; Song *et al.*, 2011). Therefore, variation in the processing in different subgroups of the NTS may contribute to the alteration of autonomic reflexes and cardiorespiratory function.

1.4. Sex variations in baseline cardiorespiratory functions, sympathetic activity and autonomic reflexes

Several studies suggest that the resting blood pressure and heart rate are comparable between healthy male and female (Sevre *et al.*, 2001; Shoemaker *et al.*, 2001; Hogarth *et al.*, 2006; Kim *et al.*, 2011), whereas, indices of respiratory function show contrast findings between male and female. In animal experiments of mice, respiratory burst frequency is similar between males and females (Garcia *et al.*, 2013), however, tidal volume is smaller in females when compared to males (Bellemare *et al.*, 2003). In reference to sympathetic activity, resting plasma noradrenaline level is comparable between male and female in a human study (Del Rio *et al.*, 1993); however, SNA studies have shown that men exhibit higher or similar baseline levels in comparison to woman (Ng *et al.*, 1993; Dart *et al.*, 2002). There is also conflicting data around sex differences in autonomic reflex function. Relative to females, young male Sprague-Dawley rats have lower tidal volume and respiratory frequency in normoxia and hypoxia, with a longer duration of post hypoxia recovery (Holley *et al.*, 2012). Accordingly, the authors of the study suggested that females might be protected from respiratory insult. On the other hand, it has been suggested that females have an increased chemoreflex response to hypoxia as reflected by a stronger ventilatory effort of pre-menopausal females with altitude hypoxia compared to males (Joseph *et al.*, 2005; Holley *et al.*, 2012). The parasympathetic component of the baroreflex has been shown to be similar, or reduced (Crofton *et al.*, 1988; Tank *et al.*, 2005), or augmented (Chen & DiCarlo, 1996; Kim *et al.*, 2011) in females compared with males, whereas the sympathetic component has been found to be the same (Tank *et al.*, 2005) or increased (Hogarth *et al.*, 2007) in the same sex.

Regardless of the conflicting data, the gonadal hormones do play an important role in sex dimorphism in the autonomic regulation of the respiratory and cardiovascular systems. For example, oophorectomy induced altered heart rate baroreceptor sensitivity (BRS), and augmented sympathetic activation can be corrected by oestrogen replacement therapy (Mohamed *et al.*, 1999; Fadel *et al.*, 2003) and hormone replacement therapy recovers baroreflex dysfunction with reduced sympathetic activity in postmenopausal women (Huikuri *et al.*, 1996; Hunt *et al.*, 2001). However, limited information is available regarding the exact physiological role of gonadal hormones in integration and processing of sympathetic outflow. To explore further, studies reveal that several neurons of the brain have express oestrogen receptors and application of oestrogen affects parasympathetic and sympathetic activity in rats (Saleh & Connell, 2000; Dart *et al.*, 2002). Oestrogen acting on oestrogen receptors in the

brainstem increases BRS (Goldman *et al.*, 2009; Brooks *et al.*, 2012), whereas, progesterone causes a decrease in BRS by modulating neurons in the rostral ventrolateral medulla (Brooks *et al.*, 2010; Brooks *et al.*, 2012). In addition, testosterone potentiates baroreflex control of heart rate in male rats (El-Mas *et al.*, 2001). Apart from a central effect, gonadal hormones also have an effect on peripheral neurons (McEwen, 1992) and nerves (Du *et al.*, 1994), being able to influence signal transmission pathways by modulating presynaptic (Roberts *et al.*, 1981; Du *et al.*, 1991) and post synaptic neurotransmitter release and uptake (Hamlet *et al.*, 1980). As a result, physiological dissimilarities between the sexes in cardiorespiratory control, from central to peripheral autonomic pathways, may cause expression of disease differently in male and female.

1.5. Concept of respiratory sympathetic coupling and blood pressure

The circulatory system serves to distribute fuel (O_2) and food products in molecular form to all cells throughout the body, and reciprocally works as a medium to remove end products of fuel and metabolic waste from these cells. The heart has pacemaker activity, which drives contractile function and hence delivery of blood to the periphery. The vascular system circulates blood and also carries it back to the heart from the periphery by virtue of skeletal muscle pumps and pressure gradients along the vessels. The respiratory system works to provide O_2 to the circulation and to eliminate end-products, mostly CO_2, in two different phases of the respiration. The respiratory centres in the brainstem exert excitatory/inhibitory input to respiratory motor neurons in the spinal cord, resulting in contraction/relaxation of the diaphragm and intercostal muscles to produce different phases of respiration. However, these two systems, cardiovascular and respiratory, need to work synchronously to maintain homeostasis and each interacts to regulate the function of the other. The phases of respiration are synchronised with pulse wave movements, termed Traube-Hering waves, in blood vessels, contributing to blood flow and blood pressure (Killip, 1962). Adrian et al were the first to show that the different phases of the respiratory cycle have a rhythmic relationship with excitation and inhibition of SNA (Adrian *et al.*, 1932). This demonstration of a harmonious relationship between SNA and the different phases of breathing, termed respiratory sympathetic modulation or coupling, has since been echoed in different animal models (Haselton & Guyenet, 1989; Czyzyk-Krzeska & Trzebski, 1990; Habler *et al.*, 1994; Dick *et al.*, 2004; Simms *et al.*, 2009), and in human studies (Eckberg *et al.*, 1985; Badra *et al.*, 2001; Dempsey *et al.*, 2002; Fatouleh & Macefield, 2011).

Absence of input from peripheral reflexes like baroreceptors and pulmonary stretch receptors by bilateral vagotomy in anaesthetised animal models (Barman & Gebber, 1980; Haselton & Guyenet, 1989; Czyzyk-Krzeska & Trzebski, 1990; Habler *et al.*, 1994) and bilateral pneumonectomy in an in-situ rat model (Pickering & Paton, 2006; Simms *et al.*, 2009) did not affect respiratory sympathetic coupling. As a result, there is growing consensus that a central interaction persists in the respiratory neurons and sympathetic motor neurons of the brainstem that regulates sympathetic outflow activity to produce respiratory sympathetic coupling. Respiratory modulation of SNA is believed to contribute to the cyclic wave pattern of vasoconstriction and vasodilation of arterioles, as mediated by the muscle vasoconstrictor class of sympathetic fibres (Habler *et al.*, 1994; Jänig, 2006). From this evolved the theory of respiratory sympathetic coupling as an independent concept for regulation of blood pressure. The adjacent existence of respiratory neurons and autonomic cardiovascular neurons in the brainstem and their close synaptic interactions, plus several common neurons having both respiratory and vasomotor functions convert the cardiovascular and respiratory systems to a combined unit that enables respiratory sympathetic coupling, rather than acting two separate units. It is therefore able to coordinate systemic and regional blood flow and breathing activity, maintaining systemic and regional blood flow and breathing activity in order to preserve internal homeostasis and blood pressure.

1.6. Interactions between respiratory neurons and presympathetic neurons

There are synaptic connections between respiratory neurons and presympathetic neurons in the brainstem. Sun et al provided a direct evidence that neurons in the BötC complex project to PSNs in the RVLM and are critical for respiratory modulation of SNA, being responsible for inhibitory input to PSNs (Sun *et al.*, 1997). However, inhibition of glycinergic transmission, a potential neurotransmitter of the BötC neurons to PSNs of the RVLM does not affect respiratory modulation of sympathetic outflow (Guyenet *et al.*, 1990). The mapping of input to PSNs of the RVLM in the animal model has discovered monosynaptic connections with respiratory neurons of brainstem. Interestingly, no glycinergic neural input had been identified, rather glutamatergic inspiratory neurons of the pre-BötC complex were demonstrated (Guyenet & Wang, 2001; Stornetta *et al.*, 2003; Dempsey *et al.*, 2017). Therefore, it is conceivable that the respiratory-related excitation can influence the activity of PNSs of the RVLM (McAllen, 1987; Moraes *et al.*, 2013a) and sympathetic outflow (Habler *et al.*, 1994; Pilowsky *et al.*, 1996) and the connections between respiratory neurons and PSNs in the RVLM is the anatomical basis of respiratory sympathetic coupling.

1.6.1. Central mechanism of respiratory sympathetic coupling

It is evident that most of the respiratory sympathetic modulation originates from central interactions between respiratory neurons and sympathetic neurons (Richter, 1990). However, the complexity of the central interactions required for respiratory sympathetic coupling is only beginning to be established.

Broadly we could illustrate the origin of the central mechanism of respiratory sympathetic coupling at two different levels: one in the brain and another in the spinal cord.

1.6.1.1. Respiratory sympathetic coupling in the brain

In the brain, the RVLM is the main area where PNSs are distributed to regulate sympathetic output (Reis *et al.*, 1989; Guyenet *et al.*, 1990; Habler *et al.*, 1994). Haselton and Guyenet et al initially identified three different patterns of respiratory modulation among PSNs in the RVLM from single unit recordings in anaesthetised animals. These are inspiratory-inhibited or depressed, inspiratory-activated or peak, and early inspiratory-inhibition or depression with post-inspiratory peak (Haselton & Guyenet, 1989). Later on, other neurons with expiratory depression and no modulation were also identified (Miyawaki *et al.*, 1995). However, whole-cell patch recordings of barosensitive, bulbospinal PSNs of the RVLM using a working heart-brain preparation in the rat without anaesthesia exhibited 3 types of respiratory modulated respiratory neurons and one type of non-respiratory modulated neurons (Moraes *et al.*, 2013b).

1. Group I, tyrosine hydroxylase (TH)-positive glutamatergic respiratory non-modulated neurons with low spike frequency. They did not show any specific change during different phases of the respiratory cycle and also did not display sympathetic hyperactivity after chronic intermittent hypoxia (CIH).
2. Group II, TH-positive glutamatergic inspiratory modulated neurons that showed an increase in spike frequency during the inspiratory phase of the respiratory cycle. Similar to group 1 cells, they may not be involved in sympathetic hyperactivity after CIH.
3. Group III, TH-negative glutamatergic post-inspiratory modulated neurons whose spike discharge frequency increased during the post-inspiratory phase. These post inspiratory modulated PSNs seemed to be responsible for increased respiratory sympathetic coupling in different phases, specifically in the expiratory phase with

increased SNA in CIH rats, probably via excitatory input from expiratory neurons of the RVLM.

4. Group IV, TH-negative glutamatergic inspiratory inhibited neurons. They revealed a great inhibition during the inspiratory phase of the respiratory cycle. However, they were not involved in increased SNA after CIH.

The caudal ventrolateral medulla (CVLM) is another site of brainstem thought to play an important role in respiratory sympathetic modulation. Barosensitive GABAergic neurons of this area also exhibited 4 different types of respiratory-related activity: inspiratory peak (type I), inspiratory depression (type II), inspiratory peak with post-inspiratory depression (type III), and post-inspiratory peak (type IV) (Mandel & Schreihofer, 2006). It has been suggested that these neurons work with PSNs RVLM and modify the respiratory pattern of sympathetic outflow by providing inspiratory and expiratory related inhibitions and limit of post inspiratory peaks of SNA.

The pons, mainly its dorsolateral region and Kölliker-Fuse neurons, may also play a role in central mechanism of respiratory sympathetic coupling (Dutschmann & Herbert, 2006; Rybak *et al.*, 2007b; Smith *et al.*, 2007). Studies in anaesthetised cats and *in situ* prepared rats with bilateral vagotomy showed that an intact pons is essential for respiratory sympathetic modulation as ponto-medullary transection results in loss of respiratory modulation of thoracic (Baekey *et al.*, 2008; Dick *et al.*, 2008), and splanchnic nerves and post inspiratory activity of the vagus nerve (Morrison *et al.*, 1994; Morrison, 1996). However, attenuated inspiratory vagus nerve activity persisted in these animals. The nucleus ambiguous, a regulator of heart rate, exhibits respiratory modulation and expresses highest activity during the post inspiratory or early expiratory period and lowest activity during inspiration (Rentero *et al.*, 2002; O'Leary & Jones, 2003; Evans *et al.*, 2005). Pontine input controls parasympathetic outflow to regulate sinus arrhythmia. In addition to regulating medullary sympathetic activity, pontine nucleus stimulates cardiac vagal neurons, decreasing heart rate in the post-inspiratory period or expiratory period and ponto medullary transection removes this impulse and silences the respiratory sympathetic coupling (Dick *et al.*, 2009). Therefore, pontine nuclei regulate central respiratory sympathetic coupling while activating the nucleus ambiguous.

The PVN of the hypothalamus also takes part in central mechanisms of respiratory sympathetic modulation. The neurons of parvocellular regions of the PVN connect to sympathetic neurons of the spinal cord and vasomotor PSNs of the RVLM (Coote *et al.*,

1998; Pyner & Coote, 1999; Badoer, 2001; Cruz *et al.*, 2008). Moreover, spinally projecting neurons also send collaterals to PSNs of the RVLM (Badoer, 2001; Moon *et al.*, 2002). Similarly, the neurons of the PVN directly connect to phrenic motor neurons. These PVN neurons exhibit some form of phasic activity like respiration (Kastella *et al.*, 1974; Kristensen *et al.*, 1997; Yeh *et al.*, 1997). Therefore, the neurons of the PVN may influence respiratory modulation of sympathetic outflow.

1.6.1.2. Respiratory sympathetic coupling in the spinal cord

The PSNs in the IML nucleus of the spinal cord carry information for cardiorespiratory function. These PSNs show respiratory modulation however, it is not obvious if they are directly linked to the alteration of central respiratory sympathetic coupling transmitted from the brain stem region. Notably, it has been found that differences in intrinsic excitability of PSNs as evidenced by respiratory modulation with late inspiratory peak of muscle vasoconstrictor PSNs and gap junctional coupling of cutaneous vasoconstrictor PSNs may play a key role in shaping sympathetic outflow (Stalbovskiy *et al.*, 2014). Similarly, the intrinsic excitability of PSNs with amplified respiratory modulation may be one of the underlying mechanisms of increased SNA in hypertension of SHR rats (Briant *et al.*, 2014; Briant *et al.*, 2015).

1.6.2. Respiratory sympathetic modulation in different diseases

The respiratory modulation of sympathetic activity is thought to be altered and related to the underlying mechanisms of different diseases. Sleep apnoea is a classic example where altered breathing pattern with intermittent hypoxia results in sympathetic hyperactivity and is considered an important cause of secondary systemic and pulmonary hypertension and cardiovascular diseases including chronic heart failure and arrhythmias (Golbin *et al.*, 2008; Somers *et al.*, 2008). Moreover, in a human study of patients with obstructive sleep apnoea (OSA), it has been suggested that the temporal position of respiratory sympathetic coupling shifts without any increase in respiratory sympathetic coupling in OSA and this alteration is reversed after long-term continuous positive airway pressure (CPAP) treatment (Fatouleh *et al.*, 2014). The respiratory modulation of MSNA was shown to be altered in patients with chronic heart failure (Goso *et al.*, 2001). Moreover, heart failure patients with breathing abnormalities have a poor prognosis and are prone to higher risk of mortality (Garde *et al.*, 2010; Costanzo *et al.*, 2015). In the SHR rat model, the temporal position of respiratory sympathetic coupling is altered and amplified and this alteration contributes to sympathetic

hyperactivity and hypertension (Simms *et al.*, 2009). It is also evident that altered breathing patterns such as slow breathing reduces blood pressure in patents with primary hypertension (Joseph *et al.*, 2005). In contrast, however, Fatouleh et al showed that the respiratory sympathetic coupling was not altered in patients with either chronic obstructive pulmonary disease (COPD) or persistent hypoxia (Fatouleh & Macefield, 2011). Collectively, these demonstrate that further investigations are required to explore the contributory role of respiratory sympathetic coupling in diseases.

1.6.3. Respiratory sympathetic coupling in increased sympathetic nerve activity and hypertension

It is evident that sympathetic overactivity is one a key feature of both primary and secondary hypertension in both humans and animals (Lundin *et al.*, 1984; Korner *et al.*, 1993; Grassi, 1998; Esler, 2000; Lambert *et al.*, 2007; Phillips *et al.*, 2007). Czyzyk-Krzeska & Trzebski first described an altered pattern of respiratory modulated sympathetic discharge that shifted a peak from the post inspiratory phase towards inspiratory phase and its association with hypertension in the SHR under anaesthesia (Czyzyk-Krzeska & Trzebski, 1990). Later on, in the working heart brain preparation, without the confounds of anaesthesia, the temporal position of respiratory sympathetic coupling was shifted towards the inspiratory phase and respiratory sympathetic coupling was also amplified in juvenile SHR, indicating that respiratory modulation plays a role in the development of sympathetic hyperactivity and hypertension in this rat model (Simms *et al.*, 2009). In the CIH rat model which mimics the pathological condition of sleep apnoea, augmented expiratory modulation of SNA and hypertension are evident (Zoccal *et al.*, 2008). Similarly, chronic infusion of angiotensin II (Ang II) in rats induces altered respiratory sympathetic coupling, sympathetic overactivity and hypertension (Toney *et al.*, 2010). Moreover, in the Goldblatt (two kidneys, one clip) kidney model, which is a renin-angiotensin model of secondary hypertension, animals exhibit amplified inspiratory-related SNA that contributes to renovascular hypertension (Oliveira-Sales *et al.*, 2016). Therefore, it is plausible that altered respiratory sympathetic coupling may contribute to increased SNA and hypertension in CKD.

1.6.4. Sex difference in respiratory sympathetic coupling:

It has been previously mentioned that there are sex differences in lung structures, pulmonary functions and respiratory networks. Males usually have larger lung volumes including tidal volume, higher expiratory flow rates and larger diffusion areas due to in part of their larger body surface area and structure (Mead, 1980; McClaran *et al.*, 1998). However, sex

differences in lung function persist even after adjusting for body surface area and weight (Kilbride *et al.*, 2003) suggesting sex variation in respiration depends on multiple factors. Sex hormones play a definite role in sex differences of respiratory functions (Wenninger *et al.*, 2009; Holley *et al.*, 2012). In addition, a study in an animal model of mice showed that after acute episodes of hypoxia, brain slices containing male respiratory neurons recovered later than its female counterpart (Garcia *et al.*, 2013). Accordingly, it is suggested that central respiratory neurons, especially the pre-BötC neurons with autorhythmicity, may have some sex differences in their intrinsic properties. Souza at al linked increased sympathetic activity and hypertension after CIH in female rats to inspiratory sympathetic modulation, whereas it was linked to expiratory sympathetic modulation in male rats (Souza *et al.*, 2016; Souza *et al.*, 2017).

1.6.5. Respiratory sympathetic coupling and baroreflex function

The NTS is the key brain region that integrates and initiates the central component of the baroreflex. In the classical baroreflex pathway, high blood pressure stimulates baroreceptors to sends impulses to the NTS, activating 2nd order NTS neurons. From the NTS, impulses travel to the CVLM to activate GABAergic neurons to send inhibitory input to PSNs in the RVLM, and results in sympathoinhibition with activation of the parasympathetic pathway (Guyenet *et al.*, 1990; Dampney, 1994). This direct pathway does not involve respiratory generators and is independent of respiratory modulation. There is, however, a parallel pathway in baroreflex arc, described by Baekey et al, involving the VRC of the brainstem and the pons. This baroreflex arc depends on respiratory sympathetic coupling as stimulation of post-I neurons of VRC prolongs expiration and inhibits PSNs in the RVLM, thereby reduces SNA. (Baekey *et al.*, 2008; Baekey *et al.*, 2010; Molkov *et al.*, 2014)

In the working heart brainstem prepared rat model, stimulation of baroreceptors during the post inspiratory period results in a reduction of thoracic SNA and prolongation of the expiratory period when the pons is intact. However, removal of pontine input resulted in similar baroreceptor induced inhibition of thoracic SNA with loss of the respiratory modulation of SNA (Baekey *et al.*, 2008). Accordingly, it has been proposed that barostimulation results in activation of the NTS that sends input to the post-I neurons of the VRC which transmits inhibitory signals to PSNs in the RVLM, therefore reducing sympathetic outflow. Whereas barosensitive neurons are not evident within respiratory networks in the anaesthetised rat preparation. However, a 30% increase was seen in the expiratory period without any changes in phrenic amplitude following aortic depressor nerve

(ADN)-induced baroreflex stimulation in similar rat preparation (Kanjhan *et al.*, 1995; McMullan *et al.*, 2009). Moreover, a study in the animal model suggests that in response to CIH, rats exhibited larger respiratory-related tSNA and RVLM PSNs activities during expiration. This study also showed the expiratory-related baroreceptor inputs to the respiratory modulated bulbospinal RVLM PSNs selectively amplified following CIH (Moraes *et al.*, 2016). Therefore, it is plausible that respiratory neurons may be involved in baroreflex pathways by modulating PSNs.

1.6.6. Peripheral Chemoreceptors in sympathetic overactivity in different diseases including hypertension:

The carotid body is the main hub of peripheral chemoreceptors and is situated in the bifurcation of the common carotid artery. It is a highly vascularised organ rich in sympathetic innervation (Barnett *et al.*, 1988). The carotid sinus nerve carries the input from activated peripheral chemoreceptors of the carotid body to the NTS (Donoghue *et al.*, 1984). The carotid body contains two types of cells: Type I (glomus cells) and Type II (supporting cells). Glomus cells originate from neural crest cells, and are large, spherical cells that are responsive to hypoxia, hypercapnia, acidosis, hypotension, sodium cyanide and inorganic phosphate (Marshall, 1994; Paton *et al.*, 1999). In response to any of above stimuli, cells within the carotid body release different mediators such as noradrenaline, dopamine, substance P that act on chemoreceptors and increase carotid sinus nerve input to the NTS (Fitzgerald *et al.*, 2009). From the NTS, an efferent stimulus directly and indirectly via respiratory generators, activates PSNs of the RVLM to produce a sympathetic response (Molkov *et al.*, 2014). Activation of peripheral chemoreceptors results in a primary response of hyperventilation, vasoconstriction and bradycardia. However, activation of pulmonary stretch receptors from hyperventilation contributes to a secondary response, that being tachycardia withdrawal of vagal stimuli and vasodilatation withdrawal of sympathetic input (De Burgh Daly & Scott, 1962; Daly & Scott, 1963). However, the ultimate effect of peripheral chemoreceptor stimulation depends on strength of each response, ventilator response and strength and type of stimulus. In response to hypoxia, activation of peripheral chemoreceptors usually leads to tachypnoea, sympathetic over activity and increased blood pressure. Repeated hypoxia contributing to increased sympathetic activity may result in further reduced blood flow in the carotid body (Zoccal *et al.*, 2008; Moraes *et al.*, 2016). As a result, it is plausible that hypoxia with sympathetic overactivity acts as a stimulus for

activation of chemoreceptors of the carotid body that may contribute to hypersensitivity of the peripheral chemoreceptors.

It is evident that peripheral chemoreceptors become hypersensitive in disease conditions like primary hypertension and heart failure (Ponikowski *et al.*, 2001; Sinski *et al.*, 2012; Paton *et al.*, 2013b). A hypersensitive peripheral chemoreflex is also observed in SHR rats. Another striking finding in relation to this is that amplified peripheral chemoreceptor activity and sympathetic overactivity occurs before the development of hypertension in the SHR (Tan *et al.*, 2010).

As noted above, recurrent hypoxic exposure may result in hypersensitive peripheral chemoreceptors of the carotid body, which has been linked to sympathetic hyperactivity and hypertension in normotensive animal models (Fletcher *et al.*, 1992; Zoccal *et al.*, 2008). Similarly, in patients with OSA, it has been found that peripheral chemoreceptor hypersensitivity resulting from repeated anoxic episodes contributes to sympathetic hyperactivity and hypertension. Importantly, this sympathetic hyperactivity is reduced by CPAP treatment of the hypoxia (Narkiewicz *et al.*, 1998; Narkiewicz & Somers, 2003). Examination of the carotid body from hypertensive patients and animal models reveals an enlarged carotid body with histological evidence of hypertrophied glomus cells and angiogenesis (Heath *et al.*, 1985; Clarke *et al.*, 1999). The mechanisms of these microscopic changes and chemoreceptor hypersensitivity are still not well understood and may depend on the underlying disease processes. For example, in the SHR, amplified sensitivity of glomus cells to pH is dependent on the over expression of two voltage gated channels: ASIC3 and TASK1 (Tan *et al.*, 2010), whereas studies of the animal models of heart failure suggest reduced nitric oxide synthetase levels, increased ang II receptors, reduced blood flow, and the inflammatory process underlie stimulated carotid chemoreceptors (Li *et al.*, 2004; Ding *et al.*, 2011; Schultz, 2011; Lam *et al.*, 2012).

1.6.7. Peripheral chemoreceptors in respiratory sympathetic coupling

In experiments on normotensive animal models, it has been shown that recurrent stimulation of peripheral chemoreceptors in the carotid body by CIH results in amplified respiratory-related sympathetic burst and increased SNA, with the development of hypertension in these rats (Zoccal *et al.*, 2008). Similarly, it is credible that activated peripheral chemoreceptors contributes to increased respiratory sympathetic modulation and sympathetic overactivity in humans with hypertension (Trzebski *et al.*, 1982; Narkiewicz & Somers, 2003; Sinski *et al.*, 2012). By way of this mechanism, it has been suggested that recurrent activation of peripheral

carotid body chemoreceptors results in the production of adrenomedullin in the RVLM which may result in long term potentiation of sympathetic outflow (Paton *et al.*, 2013a). However, carotid body removal prevents the development of hypertension and also reduces established hypertension with a reduction of respiratory sympathetic coupling and sympathetic hyperactivity in the SHR (Abdala *et al.*, 2012; McBryde *et al.*, 2013). This is consistent with the finding that in patients with hypertension, blood pressure was significantly reduced after ablation of the carotid body (Winter & Whipp, 2004; Narkiewicz *et al.*, 2016). Therefore, these observations suggest that peripheral chemoreceptors in the carotid body may regulate central respiratory sympathetic coupling in different diseases.

1.7. Sympathetic regulation of blood pressure

Peripheral vascular resistance, one of the determinants for blood pressure regulation, is largely contributed to by noradrenaline alongside humoral agents and local mechanisms, whereas cardiac output, the other determinant of blood pressure, is regulated by the balance between the sympathetic and parasympathetic nerve activity. It has been well established that acute derangement of blood pressure due to different types of stressors like fear, exercise, and temperature abnormalities is buffered by the immediate actions of the autonomic nervous system, either through reflex pathways or release of humoral agents. The long-term regulation of blood pressure is additionally contributed to by the pressure-natriuresis concept, with a key role played by the kidneys.

The arterial system of the body, and mostly arterioles, receive the majority of vascular sympathetic innervation and is the major contributor to peripheral vascular resistance, whereas the venous system receives less sympathetic innervation and works as a reservoir for the vascular system (Bevan & Su, 1971; Nilsson *et al.*, 1986; Thomas, 2011). Overall the vascular system receives minimal parasympathetic innervation (Duke, 2011; Dampney, 2016).

The axons of postganglionic neurons that constitute sympathetic efferent fibres innervate and form a plexus between the adventia and media of the vasculature supplying the organs of the body (Nilsson *et al.*, 1986; Thomas, 2011). Cardiovascular sympathetic efferents can be classified into three categories according to their functional status: thermosensitive, glucosensitive and barosensitive (Guyenet, 2006). The glucosensitive sympathetic fibres innervate the adrenal medulla and regulate glucose metabolism by releasing adrenaline from the adrenal gland. The thermosensitive efferents mostly supply the skin of humans while in the rat the tail artery is a critical target. In addition to being regulated by temperature, they are

also responsive to changes in emotional factors and increased ventilation. Both glucosensitive and thermosensitive sympathetic neurons play a minor role in blood pressure regulation (Morrison & Cao, 2000; Janig & Habler, 2003; Guyenet, 2006; Jänig, 2006).

1.7.1. Direct sympathetic nerve involvement in blood pressure regulation

The barosensitive sympathetic efferent fibres are the major contributor to sympathetic regulation of blood pressure. These neurons supply the blood vessels of the major organs including heart, muscle and kidney and are essential to maintain resting vascular tone. Moreover, their activities are modulated by both the cardiac and respiratory cycles, and are actively involved in different autonomic reflex pathways and thereby play a key determinant role in regulation of blood pressure (Dampney *et al.*, 2002; Ootsuka *et al.*, 2002; Janig & Habler, 2003; Jänig, 2006).

Adrenergic post ganglionic sympathetic axons release mostly noradrenaline as their main neurotransmitter, with neuropeptide Y (NPY) and adenosine tri phosphate (ATP) as co-transmitters, that in the vasculature results in vasoconstriction and maintenance of sympathetic tone. Noradrenaline binds with G-protein and subsequently stimulates adrenergic receptors that ultimately activates a series of cascade reactions including activation of enzymes such as phosphorylase and myosin light chain kinase, increased concentration of calcium, phosphorylation of myosin and increased tension of myosin and induction of vascular tone (Sweeney *et al.*, 1993; Guimaraes & Moura, 2001). Both NPY and ATP can cause vasoconstriction directly by stimulating vascular NPY Y1 receptors or purinergic P2X receptors, or enhance the function of noradrenaline indirectly (Pablo Huidobro-Toro & Veronica Donoso, 2004).

The sympathetic vasomotor response is not only dependent on the types of sympathetic neurons involved but also influenced by the organ specific location of arterioles and the type of stimulus. It has been suggested that the cerebral and coronary circulations are less responsive compared to cutaneous, muscular, renal and splanchnic circulations to sympathoexcitatory stimuli like anxiety (Brown *et al.*, 2012), exercise (Ichinose *et al.*, 2006b) increased body temperature, sodium intake (DiBona & Jones, 2003) and hypoxia (Silva & Schreihofer, 2011). Moreover, there are differential responses of individual sympathetic nerves to a specific stimulus (Morrison & Cao, 2000; Yoshimoto *et al.*, 2010; Ramchandra *et al.*, 2012; Turner *et al.*, 2013). For example, Yao et al showed impaired baroreflex responses in the renal and splanchnic nerves but an intact response in the lumbar nerve in an animal model of CKD (Yao *et al.*, 2015).

1.7.2. Indirect sympathetic involvement in blood pressure regulation

There are indirect effects of sympathetic innervation that also play key roles in the regulation of blood pressure, especially long-term regulation.

1.7.2.1. Adrenaline

The preganglionic sympathetic neurons innervate chromaffin cells of the adrenal gland, which are themselves modified postganglionic sympathetic neurons, and synthesize and release mostly adrenaline (Reid, 1992; Morrison & Cao, 2000). The systemic release of adrenaline, along with noradrenaline released directly from the sympathetic nerve terminals, are the key regulators controlling cardiovascular homeostasis of the body (Guimaraes & Moura, 2001).

1.7.2.2. Angiotensin II

The juxtaglomerular cells in the walls of the renal afferent arterioles, stimulated by sympathetic efferent nerves through the activation of β1 receptor, release renin in the circulation. The renin converts angiotensinogen, a globulin formed in liver, to angiotensin I. The angiotensin- converting enzyme (ACE), situated mainly in the endothelium of lung and kidney transforms angiotensin I to ang II, one of the main bio products of this pathway found in endothelial cells of capillaries of the whole body. Ang II directly activates angiotensin receptors which results in both systemic and localised vasoconstriction, increased peripheral resistance and hence increased blood pressure. Moreover, it stimulates the adrenal gland to release aldosterone thereby promoting sodium reabsorption from renal tubular cells, leading to water retention and an increase in blood pressure (DiBona & Sawin, 1994; DiBona, 2000a; Brewster & Perazella, 2004; Peti-Peterdi & Harris, 2010). There is also evidence that Ang II regulates the secretion of noradrenaline from axon terminals of postganglionic sympathetic neurons, promotes synaptic transmission and controls central neural processing via the circumventricular organs, especially the subfornical organ (Wang *et al.*, 2013). The renin-angiotensin-aldosterone system (RAAS) therefore has multiple mechanisms by which it can influence the sympathetic nervous system to regulate blood pressure (Reid, 1992; Dampney, 1994; DiBona, 2000b). Other indirect actions of sympathetic activity, as mediated by the RAAS, includes the release aldosterone, which promotes sodium reabsorption from renal tubular cells and contributes to high blood pressure. Aldosterone also acts centrally to stimulate mineralocorticoid receptors in NTS neurons and increase sodium appetite that may contribute to hypertension (DiBona, 2000b).

1.7.3. Reflex regulation of blood pressure

Normal blood pressure is essential to maintain perfusion of cells, tissues and organs that helps the body to get energy and oxygen and remove waste products and carbon dioxide. Any abnormalities of blood pressure upset this internal homeostasis, and activates different reflex pathways to reverse the deviations of blood pressure immediately and prevent further long-term effects.

1.7.3.1. Baroreflex pathways in regulation of blood pressure

The baroreflex is one of the key reflex mechanisms to maintain blood pressure within a normal range in spite of a wide range of challenges. Both sympathetic and parasympathetic components work together to produce a reflex to neutralize the upset of heart rate and blood pressure.

Baroreceptors are stretch receptors situated mostly in the tunica adventia and connected to the smooth muscle of the tunica media of the arterial wall. Various intracellular mechanisms like activation of voltage-gated ion (Na^+, K^+ or Ca^{2+}) channels (Tu *et al.*, 2010), transient receptor potential vanilloid 1 (TRPV1) (Sun *et al.*, 2009) or P2 purinoceptors (Song *et al.*, 2012) may be responsible for impulse generation at the baroreceptor level, with the main stimulus for activation of baroreceptor being stretching of the vessel wall containing these receptors.

The impulse from the baroreceptors is carried by afferent sympathetic fibres, characterized as low-pressure threshold myelinated A-fibres and high-pressure threshold unmyelinated C-fibres (Kumada *et al.*, 1990; Fan & Andresen, 1998; Fan *et al.*, 1999). The fibres from the aortic arch travel via the vagus nerve in human and the aortic depressor nerve (ADN) in rats, with neurons located in the nodose ganglion (Sapru & Krieger, 1977; Sapru *et al.*, 1981; Kumada *et al.*, 1990; Dampney *et al.*, 2002; Thomas, 2011). Afferent input from the carotid body run in the carotid sinus nerve in both humans and rats with neurons located in the petrosal ganglion (Sapru & Krieger, 1977; Kumada *et al.*, 1990; Dampney *et al.*, 2002; Thomas, 2011).

The NTS plays a vital role to integrate baroreflex function directly and indirectly via activation of respiratory neurons. Moreover, hypothalamic vasomotor centers such as the PVN send regulatory stimuli to the NTS (Bailey *et al.*, 2006) and thereby influence baroreflex function. Glutamate and GABA are two counter-regulatory neurotransmitters involved in central integration of the baroreflex at the NTS level (Talman *et al.*, 1980; Leone & Gordon, 1989).

Sympathetic contributions of baroreflex function are contributed by mainly the CVLM, RVLM and NTS. After integration of baroreflex input in NTS, impulse is carried to the CVLM via glutamatergic axons (Miyawaki *et al.*, 1997). Then the inhibitory GABAergic projection from the CVLM activates PSNs of the RVLM (Dampney *et al.*, 2002; Pilowsky & Goodchild, 2002; Schreihofer & Guyenet, 2002; Guyenet, 2006). The signals from PSNs of the RVLM directly and bilaterally stimulate SPNs of IML nucleus of the spinal cord (Dampney, 1994; Jeske *et al.*, 1995; Pilowsky & Goodchild, 2002), resulting in inhibition of SNA and reduction of blood pressure.

The parasympathetic component of the baroreflex pathway involves NTS activation of central vagal preganglionic neurons (CVPN) situated in the nucleus ambiguous, and the dorsal motor nucleus of the vagus (DMNV) (Nosaka *et al.*, 1979; Stuesse, 1982; Izzo *et al.*, 1993). The GABAergic input along with activation of excitatory N methyl D aspartate (NMDA) and non-NMDA receptors contributes to the cardiac response to blood pressure changes (Neff *et al.*, 1998; Wang *et al.*, 2001). The ultimate effect of vagal mediated response to reduce heart rate is the main determinant of parasympathetic arm of baroreflex control of blood pressure.

All segments including receptors, afferent pathways, central integrated system and efferent pathways of baroreflex pathways have a unique property, being able to modify themselves and work in a changing system at a new base line of blood pressure (McCubbin *et al.*, 1956; Sapru & Wang, 1976; Andresen *et al.*, 1978; Wallin & Sundlof, 1979; Gonzalez *et al.*, 1983; Andresen, 1984). This “baroreceptor resetting” characteristic of the baroreflex pathway has the ability to readjust its target of operating blood pressure at a new base line. As a result, any persistent change by high or low blood pressure rewrites the afferent pathways target range, with modification of the central component, and the resetting of the sympathetic and parasympathetic components with varying degree of adjustments in target organs. This resetting of the baroreflex upsets the normal long-term control of blood pressure and contributes to hypertension. Temporary elevation of blood pressure during pain stimuli, emotional stimuli and exercise, or reduction of blood pressure during shock (Hatton *et al.*, 1997; Ichinose *et al.*, 2006a; Kanbar *et al.*, 2007) results in transient resetting of the baroreflex to keep SNA, heart rate, and blood pressure at the altered level to maintain optimum circulation in vital organs. These acute changes return to the baseline following cessation of the stimuli.

However, a persistent or recurrent stimulus resulting in continued increased blood pressure for more than several weeks or months contributes to chronic resetting of baroreflex that is

associated with hypertension (Andresen, 1984; Ligtenberg *et al.*, 1999b; Head & Burke, 2001; Huber & Schreihofer, 2010). Under such conditions, the baroreflex works to a new higher resting blood pressure. Moreover, the sensitivity of the baroreflex is gradually altered. As a result, it is conceivable that the baroreflex is activated at an abnormally high threshold level of blood pressure rather than a safe and normal level of blood pressure, and becomes less responsive in an acute situation. The mechanism of chronic resetting of the baroreflex is not well known; however, central mechanisms involving reduced NTS feedback input to baroreceptors, and altered input from the hypothalamus or inhibition of circulating peptides, may play crucial role in chronic resetting (Andresen *et al.*, 2001; Paton *et al.*, 2001a; Potts *et al.*, 2003).

1.7.3.2. Chemoreflex pathways to regulate blood pressure

The chemoreflex is another important reflex mechanism by which blood pressure is regulated, and that also provides simultaneous control of respiration. The common triggers for a chemoreflex response are hypoxia, hypercapnia and acidosis (Cao & Morrison, 2001; Braga *et al.*, 2006; Silva & Schreihofer, 2011; Wenker *et al.*, 2013). Among them, hypoxia (low PO_2/SO_2 in arterial blood) stimulates mostly peripheral chemoreceptors located mainly in the carotid body, and hypercapnia (high PCO_2 in arterial blood) mostly activates central chemoreceptors in different areas of brain including the medulla, pons and lateral hypothalamus (Chapleau *et al.*, 1989; Marshall, 1994; Vasquez *et al.*, 1997; Dampney *et al.*, 2002; Guyenet *et al.*, 2010; Thomas, 2011). Lack of O_2 and hence hypoxia triggers peripheral chemoreceptors in either the carotid body or aortic body through an underlying O_2 sensing mechanism. PaO_2 and SaO_2 are indirect parameters of O_2 concentration in the blood and positively corelated. Lahiri et al 1981 proposed aortic chemoreceptors sense O_2 saturation, whereas carotid bodies monitor PO_2, based on only the finding that carboxyhemoglobinemia caused marked stimulation of aortic bodies, whereas it has no effect on the carotid body sensory activity in cats (Lahiri *et al.*, 1981). However, this hypothesis has not been clearly established by further studies and the underlying O_2 sensing mechanism of the carotid body and aortic body are not yet well understood (Prabhakar & Peng, 2004). Therefore, both of these parameters (PaO_2 and SaO_2) are used equally in clinical or experimental settings to monitor the oxygenation of blood (Czyzyk-Krzeska & Trzebski, 1990; Hering *et al.*, 2007).

After activation of peripheral chemoreceptors, the impulse is transmitted by the carotid sinus nerve in human and rat, and additionally by the vagus nerve in human, and then stimulates the NTS (Sapru & Krieger, 1977; Dampney *et al.*, 2002; Timmers *et al.*, 2003; Guyenet *et al.*,

2010). From the NTS, the impulse activates sympathetic outflow by stimulating PSNs in RVLM via glutamatergic and non-glutamatergic pathways and parasympathetic outflow by CVPNs in the nucleus ambiguus and/or the DMNV with connecting respiratory neurons of the brain stem. As a result, following activation by hypoxia, the initial cascade of chemoreflex containing sympathetic vasoconstriction and vagal bradycardia initiates restoration of blood pressure and maintenance of perfusion of the vital organs with hyperventilation to increase oxygen saturation in the blood. Later on, hyperventilation overrides the vagal activity towards the heart that results in tachycardia after stabilization (De Burgh Daly & Scott, 1962; Marshall, 1994; Paton *et al.*, 2001b; Dampney *et al.*, 2002; Thomas, 2011) .

Neurons with the functional characteristics of central chemoreceptors have been seen in the different areas of brain including the raphe, the RTN, the NTS and the locus coeruleus (Dean *et al.*, 1989; Richerson *et al.*, 2005; Nattie & Li, 2008, 2009), with the list also including orexinergic neurons in the hypothalamus and the fastigial nucleus (Martino *et al.*, 2006; Deng *et al.*, 2007; Williams *et al.*, 2007). Among them, the RTN is the important one that integrates peripheral and central chemoreceptor activity. Acidity in brainstem interstitial tissue activates central chemoreceptors to stimulate respiratory neurons with the result being hyperventilation. In addition, this impulse excites PSNs of the RVLM which receives excitatory RTN input (Guyenet *et al.*, 2008) thereby producing vasoconstriction and high blood pressure. However, chemoreflex does play a minor role in regulation of blood pressure when blood pressure is within physiological level whereas it dominates to upregulate blood pressure during hypotension directly or by modifying the baroreceptor activity (Thomas, 2011).

1.8. Measurement of sympathetic nerve activity, autonomic reflexes and respiratory sympathetic coupling

To characterise abnormality in sympathetic activity, reflexes and respiratory pattern in different pathological conditions, measurement of SNA and respiratory sympathetic coupling is very important. There are different ways to assess the abnormality of these parameters in humans and animal models.

1.8.1. Urine and plasma noradrenaline level and noradrenaline spill over rate

Sympathetic activity has been assessed by measuring plasma or urinary noradrenaline and their metabolites levels in animal experiments and human patients for a long period of time (Grassi & Esler, 1999; Sinski *et al.*, 2006; Vink *et al.*, 2013). Given that plasma noradrenaline level represents only one tenth of the neurotransmitter secreted from nerve endings and that

there is regional variation in sympathetic activity, plasma noradrenaline level does not really reflect total or generalised sympathetic activity (Grassi & Esler, 1999; Esler *et al.*, 2003). Furthermore, plasma noradrenaline level is inconsistent because of its presynaptic uptake or release (Grassi & Esler, 1999). In spite of these limitations, measurement of plasma noradrenaline level is more advantageous than urinary noradrenaline level (Sinski *et al.*, 2006), as immediate changes in SNA are better reflected in plasma noradrenaline rather than in urinary noradrenaline level.

Noradrenaline spill over rate is another method of assessment of sympathetic activity which estimates the rate of removal of noradrenaline from the system by calculating the clearance of noradrenaline from a region or system after infusion of radiolabelled noradrenaline (Esler *et al.*, 2003; Sinski *et al.*, 2006). With this specific method, it is possible to estimate both total body noradrenaline and regional or systemic noradrenaline spill over rates including heart, kidney, brain, splanchnic circulation and skeletal muscle (Esler *et al.*, 1989; Wallin *et al.*, 1996; Mitchell *et al.*, 2009; Yoshimoto *et al.*, 2010).

1.8.2. Direct measurement of sympathetic nerve activity

While the underlying mechanisms such as reuptake and clearance of neurotransmitter can cause variability in the indirect measurement of sympathetic activity, direct measurement of sympathetic nerves provides a more definitive definite measurement of systemic sympathetic activity (Grassi & Esler, 1999; Sinski *et al.*, 2006; Chapleau & Sabharwal, 2011).

Microneurography is a direct method of measuring SNA in humans, recording multi-unit SNA from superficial peripheral nerves by inserting tungsten electrodes into muscle or skin fascicles. The common nerves used for microneurography are the superficial peroneal nerve, tibial nerve and median nerve (Esler *et al.*, 2003; Grassi *et al.*, 2011a; Brown *et al.*, 2012; Grassi *et al.*, 2012; Vink *et al.*, 2013). The MSNA and skin SNA can be assessed separately by microneurography. MSNA is regulated by the baroreflex as it is responsive to the alterations of blood pressure whereas skin SNA is not responsive to blood pressure changes (Hagbarth *et al.*, 1972; Sinski *et al.*, 2006). Using microneurography in a single limb in the human model which is representative of whole body is a specific, reliable and sensitive procedure with minimal side effects (Grassi *et al.*, 1997). However, it is an invasive procedure that requires proper training and time. It has been shown to correlate with noradrenaline spill over rate and is therefore considered one of the ideal techniques to assess sympathetic activity along with noradrenaline spill over (Wallin *et al.*, 1996; Vink *et al.*, 2013). MSNA is calculated as bursts per minute and/or bursts per 100 heart beats and/or each burst peak

amplitude. Peripheral nerve measurements using microneurography have been used in different disease conditions to determine sympathetic activity, reflexes and to study respiratory sympathetic coupling (Wallin & Sundlof, 1979; Wallin *et al.*, 1992; Grassi *et al.*, 1997; Grassi & Esler, 1999; James *et al.*, 2013a; James *et al.*, 2013b; Vink *et al.*, 2013).

Single-unit SNA recording that measures sympathetic discharge from single muscle vasoconstrictor neuron is another direct measure of SNA. The firing frequency, firing probability and number of spikes per cardiac interval can be calculated from recording of single-unit SNA recording, to assess SNA (Macefield *et al.*, 1994; Greenwood *et al.*, 1999; Burke *et al.*, 2011; Hering *et al.*, 2013). Single unit SNA recordings are believed to be better able to differentiate severity of disease process (Burke *et al.*, 2011). For example, in different stages of hypertension single-unit recording shows significant differences across different levels of hypertension whereas multi-fibre units did not reveal any significant differences in SNA across the different levels of hypertension (Greenwood *et al.*, 1999).

In animal models, whole nerve activity, a technique of multifibre recordings is typically used to assess sympathetic function as multiple axons are clustered into fascicles and fire equally in multifibre recordings (Montano *et al.*, 2009). The whole nerve recording for a long period of time at variable sampling rates is bandpass filtered, rectified and integrated for analysis offline. Experimental preparations including anaesthetised, in-situ working heart preparations and conscious studies have utilised different sympathetic nerves to measure directly sympathetic nerve activity. The renal, greater splanchnic and lumbar nerves are often used in conscious and unconscious preparations while the thoracic splanchnic nerve is used to obtain nerve recording in working heart brain preparations.

1.8.2.1. Thoracic sympathetic nerve

Thoracic sympathetic nerve (tSNA), arising from sympathetic trunk (T5-T12) as thoracic splanchnic nerve in thorax has three branches named greater splanchnic nerve, lesser splanchnic nerve and least splanchnic nerve (Naidoo *et al.*, 2001). This nerve, being the largest splanchnic nerve in sub-diaphragmatically bisected animal, is widely used to measure SNA in working heart brain preparation (Paton, 1996). There is evidence where tSNA is increased with enhanced respiratory sympathetic coupling in pathological conditions like hypertension (Zoccal *et al.*, 2008; Simms *et al.*, 2009).

1.8.2.2. Greater splanchnic sympathetic nerve

The greater splanchnic nerve, a branch of thoracic splanchnic nerve carries post ganglionic axons of IML nucleus of T5-T9 of the spinal cord, and innervates the gastrointestinal organs and splanchnic circulation (Naidoo *et al.*, 2001). Here the greater splanchnic nerve is mentioned as the splanchnic nerve. There are many examples where the splanchnic nerve has been used in conscious, anaesthetised and working heart preparations. In the anaesthetised rat, recording of the splanchnic nerve has been used to study diseases models including the SHR, Lewis polycystic kidney (LPK) rats and Obese Zucker rats, and served to reveal increased SNA and altered baroreflex and chemoreflex function (Czyzyk-Krzeska & Trzebski, 1990; Montano *et al.*, 2009; Yao *et al.*, 2015). Similarly, splanchnic nerve recordings in conscious rats have been used to demonstrate increased SNA and impaired baroreflex response in hypertensive model of rats including SH rats (Ricksten *et al.*, 1984).

1.8.2.3. Renal sympathetic nerve

The renal nerve originates from IML nucleus of T9-T13 of the spinal cord and consists of post ganglionic axons (Scislo *et al.*, 1998). It supplies the kidney nephrons including renal tubules, intrarenal circulation and juxtaglomerular apparatus (Johns *et al.*, 2011). Salman et al in both anaesthetised and conscious rats provided evidence for increased baseline renal SNA (rSNA) in a secondary hypertension model of CKD (Salman *et al.*, 2014; Salman *et al.*, 2015a; Salman *et al.*, 2015b). Moreover, evidence of impaired baroreflex function and altered respiratory sympathetic modulation has been obtained from recordings of rSNA in hypertension and CKD (Czyzyk-Krzeska & Trzebski, 1990; Vitela *et al.*, 2005; Toney *et al.*, 2010; Yao *et al.*, 2015).

1.8.2.4. Lumbar nerve

The lumbar nerve primarily innervates the skeletal muscles of the hind limb and consists of pre and post-ganglionic sympathetic neurons (Scislo *et al.*, 1998). It also supplies the skin of the hindlimb. Lumbar nerve recordings have been used in anaesthetised rat models to assess SNA and baroreflex (Scislo *et al.*, 1998; Toney *et al.*, 2010; Yao *et al.*, 2015). As an example of direct nerve recordings being able to demonstrate regional or target organ specific differences in sympathetic outflow, Yao et al showed differential baroreflex responses in the sympathetic nerves in the LPK animal model of CKD, with baroreflex function of both renal SNA (rSNA) and splanchnic SNA (sSNA), but not lumbar SNA being impaired (Yao *et al.*,

2015). Similarly, stimulation of RVLM results in less increase in lumbar SNA compared to renal and adrenal SNA in anesthetized rats (Mueller *et al.*, 2011).

There are several instances where other sympathetic nerves such as adrenal and cardiac nerves are used to assess SNA and baroreflex function (Scislo *et al.*, 1998; Ramchandra *et al.*, 2012; Turner *et al.*, 2013).

1.8.3. Pharmacological agents to assess sympathetic activity

Pharmacological agents can also be used to assess the level of sympathetic activity in both animals and humans. For example, ganglionic blockers such hexamethonium (Phillips *et al.*, 2007; Burke *et al.*, 2008), α1-adrenoceptor blockers like prazosin (Oates *et al.*, 1977) and acting α2adrenoceptor agonists (e.g., clonidine and moxonidine) (Iriuchijima, 1997; Neumann *et al.*, 2004) are commonly used pharmacological agents. Typically, the blood pressure response after administering these agents is used as an indirect estimate of sympathetic activity. The advantage of the use of pharmacological agents is that they are less costly and easy to execute as compared to direct estimation of sympathetic activity.

1.8.4. Heart rate and blood pressure variability

The application of spectral analysis to determine heart rate and blood pressure variability in different frequency domains is a commonly used method to assess sympathetic activity. These methods are less costly, non-invasive and simple to apply in both humans and animal models, noting that SNA cannot be assessed directly, but indirect evidence of SNA can be determined (Head, 2003; Zygmunt & Stanczyk, 2010; Hildreth *et al.*, 2013a).

1.8.4.1. Heart rate variability

Analysis of physiological variation of duration of beat to beat (R-R) intervals is the main principle of assessment of heart rate variability (HRV) as this variation depends on sympathetic activity. There are two ways to measure HRV: namely time domain and frequency domain analysis. The recommended methods for time domain HRV assessment for estimation of overall HRV are the standard deviation of normal-to-normal intervals (SDNN) and HRV triangular index. The others are the standard deviation of the average normal-to-normal intervals (SDANN) for estimation of long-term components of HRV and the square root of the mean squared differences of successive normal-to-normal intervals (RMSSD) for estimation of short-term components of HRV (Zygmunt & Stanczyk, 2010; Hildreth *et al.*, 2013a).

Among various methods of frequency domain analysis of HRV, power spectral density (PSD) analysis is the common one. The components for frequency domain analysis depends on length of recording. Very low frequency (VLF) band (≤ 0.04 Hz) connected to hormonal and thermoregulatory influences on the heart, low frequency (LF) band (0.04–0.15 Hz) associated with baroreceptor activity, high frequency (HF) band (0.15–0.4 Hz) modulated by the parasympathetic nervous system and related with respiratory and blood pressure changes, and LF /HF ratio considered as a measure of sympathovagal balance, are regularly used in short term recording in humans. In addition to these spectral components, ultra-low frequency (ULF) band (≤ 0.003 Hz) component has also been used for long term recording (Zygmunt & Stanczyk, 2010; Hildreth *et al.*, 2013a).

1.8.4.2. Systolic blood pressure variability

Systolic blood pressure variability (SBPV) is also used as a measure of sympathetic activity. The spectral components for SBPV analysis are VLF (0.02–0.07Hz in humans), LF (0.077–0.15Hz in humans), HF (0.15 –0.40Hz in humans) and LF/HF. Among them LF and LF/HF are used to assess sympathetic activity on vessels (Stauss, 2007; Hocht, 2013)

1.8.5. Assessment of baroreflex function

Baroreflex function can be assessed by invasive and non-invasive method.

1.8.5.1. Invasive methods

1.8.5.1.1. Drug induced baroreflex response assessment

This method, named the Oxford method is one of the common methods to assess baroreflex function in both animal and human models. This method determines the changes in heart rate and/or SNA resulting from blood pressure changes in response to bolus injections or infusions of a vasoconstrictor like Phenylephrine (PE) and vasodilator like Sodium nitroprusside (SNP) (Smyth *et al.*, 1969; Parati *et al.*, 2000; Chapleau & Sabharwal, 2011). BRS can be calculated from the linear regression between heart rate/SNA and blood pressure in response to vasoconstrictor provoked increased blood pressure contributing to decreased heart rate and inhibition of SNA. Similarly, SNP-induced tachycardia and decreased blood pressure in response to low blood pressure determines baroreflex response of heart rate and/or SNA. However, alternate uses of vasoconstrictor and vasodilator agent with combined effect in heart rate/SNA helps to build a sigmoidal baroreflex curve, from where, gain, range, threshold and saturation point heart rate / SNA versus blood pressure can be calculated accurately (Kent *et al.*, 1972; Head & McCarty, 1987; Hunt & Farquhar, 2005).

1.8.5.1.2. Stimulation of baroreceptor afferents

Baroreflex function can be more precisely estimated by electrical stimulation of baroreceptors afferent fibres coming from the carotid body or the aortic arch as this method is able to avoid stimulation of mechanoreceptors in the carotid body and aortic arch induced by blood pressure changes (De Paula *et al.*, 1999). There is evidence of use of this technique to assess baroreflex function in both anaesthetised and conscious rats (DiBona, 2000b; Chapleau & Sabharwal, 2011).

1.8.5.1.3. Carotid sinus nerve method

This method, also called Moisejeff preparation involves isolation of carotid sinus nerve from circulation for more control of stimulus that applied to the baroreceptors. It has been used in experiments of both anaesthetised and *in situ* animal experiments (Chapleau *et al.*, 1989; Pickering *et al.*, 2008).

There are other invasive methods to assess baroreflex functions like occlusion of the common carotid artery, anaesthesia of the carotid sinus nerve and vagus nerve, and the neck chamber technique.

1.8.5.2. Non-invasive methods

The BRS can be determined by using modern computerised techniques to calculate natural changes in heart rate in relation to blood pressure changes. These spontaneous BRS techniques can investigate day to day changes in BRS in addition to point analysis of BRS at rest. Among the techniques to calculate spontaneous BRS, the sequence technique, α coefficient technique, and autoregressive and moving average (ARMA) techniques are used commonly. Other useful methods to assess BRS are valsalva manoeuvre, head up lifting, lower-body negative pressure application, carotid sinus massage.

1.8.6. Measurement of respiratory sympathetic coupling

Different methods can be used to assess respiratory sympathetic coupling based on the experimental approach and model.

1.8.6.1. Respiratory sympathetic coupling assessment in anaesthetised and *in situ* preparations

In animal models, the phrenic nerve is recorded as a means by which to assess respiratory drive and the splanchnic nerve, renal nerve or thoracic nerves are commonly used to concurrently determine SNA. For analysis of respiratory sympathetic coupling parameters,

phrenic triggered (end of inspiratory burst) averaging of rectified and smoothed SNA recordings from the onset of PNA is usually performed offline. The phrenic cycle and corresponding SNA are divided into three phases: inspiratory (I), post-inspiratory (PI) and expiratory (E) based on phrenic inspiratory discharge (from the onset to the end of augmentation of phrenic bursts (Xing & Pilowsky, 2010), or four phases: I, PI, mid E and late E from the onset of inspiration (Simms *et al.*, 2009). Measures of respiratory sympathetic coupling can then be assessed in different ways from the phrenic triggered integrated SNA, for example, examining the temporal position of the sympathetic excitation peak or inhibition trough in relation to the different phases of respiration, or measuring the size of the respiratory related sympathetic burst activity such as the peak amplitude (μV), the maximum amplitude of the SNA burst coincident with inspiratory/post-inspiratory phase, the duration (from onset of activity to return to baseline (sec), peak trough amplitude (μV) and area under curve of peak (μV.s) (Figure 1) and mean sympathetic nerve activity in different phases of respiration (Czyzyk-Krzeska & Trzebski, 1990; Habler *et al.*, 1994; Dick *et al.*, 2004; Zoccal *et al.*, 2008; Simms *et al.*, 2009; Toney *et al.*, 2010; Xing & Pilowsky, 2010). In *in situ* experiments in SHR and WKY rats, Simms et al showed that peak of respiratory related tSNA shifted from the PI phase to the I phase with increasing age and development of hypertension in the SHR whereas the peak of tSNA remains in PI phase in normotensive rat model, WKY rats (Simms *et al.*, 2009). Moreover, SHR showed larger respiratory related sympathetic burst (peak trough amplitude) compared to WKY rats in all age groups. After CIH, rat models demonstrated increased expiratory related tSNA (Zoccal *et al.*, 2008). Similarly, in anaesthetised SHR, peak of respiratory related both sSNA and rSNA shifted left compared to normotensive rats (Czyzyk-Krzeska & Trzebski, 1990).

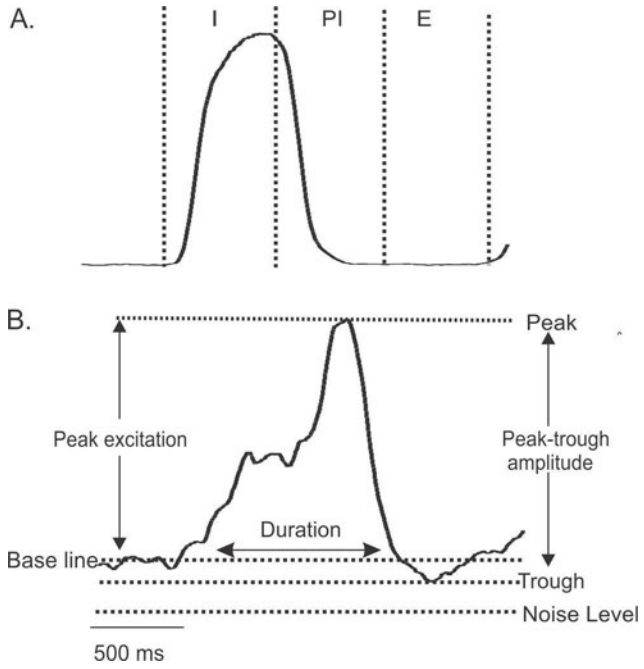

Figure 1.1 Different phases of respiratory cycle in integrated phrenic and sympathetic nerve recordings

Panel A illustrates the phases of the respiratory cycle: (I) inspiratory, (PI) post-inspiratory and (E) expiratory based on phrenic nerve activity (PNA) discharge. Panel B illustrates corresponding variables used for measurement of respiratory sympathetic coupling in a representative trace of phrenic triggered average of integrated SNA; PA (peak amplitude), peak excitation, peak-trough amplitude, and duration of respiratory related SNA (from onset to end of activity at baseline). The level of SNA after euthanasia was considered as the 0 level (noise) and the mean level of SNA as measured from -200ms to 0ms before the onset of the phrenic burst was considered the basal level.

1.8.6.2. Respiratory sympathetic coupling assessment in humans

In human studies, respiratory parameters are usually obtained by recording from a strain-gauge transducer enclosed around the chest while muscle fascicles supplied by the common peroneal nerve are an often-used site of recording for MSNA. Respiratory modulation of MSNA can then be assessed using modulation index and MSNA in different phases of respiration (Eckberg *et al.*, 1985; Badra *et al.*, 2001; Dempsey *et al.*, 2002; Fatouleh & Macefield, 2011). Modulation index can be calculated by subtracting the number of spikes at trough from the number of spikes at peak and expressed at percentage of spike number at peak (Fatouleh & Macefield, 2011; Fatouleh *et al.*, 2014). There are different forms of respiratory modulation of MSNA in normal humans, including within-breath modulatory effects, inhibitory effects of lung inflation, muscle metaboreceptor influences and chemoreceptor effects/after-effects, and with little apparent effect of central respiratory motor output, per second (Dempsey *et al.*, 2002). Fatouleh et al used modulation index to quantify the respiratory modulation of MSNA in human patients with different diseases including OSA, essential hypertension and COPD, demonstrating the efficacy of modulation index to assess respiratory modulation of MSNA (Fatouleh & Macefield, 2011; Fatouleh *et al.*, 2014).

1.9. Chronic kidney disease

Chronic kidney disease is a public health problem affecting 5-7% of the global population (Couser *et al.*, 2011). The annual incidence of CKD is increasing at a rate of 3% (Collins *et al.*, 2009). Morbidity and mortality in patients with CKD are mostly dependent on cardiovascular events and has been shown to be increasing rapidly in recent years (Go *et al.*, 2004; Tonelli *et al.*, 2006; ANZDATA Registry, 2018; USRDS, 2018). Cardiovascular events are more common in late stages of CKD, especially end stage kidney disease, and the presence of cardiovascular events are prognostic markers of CKD (Go *et al.*, 2004) . As a result, the World Health Organization considers CKD one of the emerging non-communicable disease and urges early detection and management of CKD (National Kidney Foundation, 2012).

CKD is characterised by progressive deterioration of renal function and renal structures, regardless of presentation, over a period of months and years. According to the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF-KDOQI) guidelines, CKD is defined as abnormalities of kidney structure or function, present for >3 months, with negative implications for health (Table 1).

Table 1.1 Criteria for diagnosis of CKD

Criteria for CKD (either of the following present for >3 months)	
Markers of kidney damage (1 or more)	Albuminuria
	Urine sediment abnormalities
	Tubular disorders
	Structural abnormalities by imaging and histopathology
	Kidney transplantation
Decreased GFR	GFR < 60ml/min/1.73 m2

CKD; chronic kidney disease, GFR; glomerular filtration rate (KDIGO : Kidney Disease: Improving Global Outcomes CKD Work Group, 2013; Inker *et al.*, 2014)

1.9.1. Staging of chronic kidney disease

CKD is classified based on cause, glomerular filtration rate (GFR) category, and albuminuria category (KDIGO : Kidney Disease: Improving Global Outcomes CKD Work Group, 2013)

1.9.1.1. Causes of chronic kidney disease

Diabetes, hypertension and glomerulonephritis are the three main causes of CKD. Other factors including environmental, genetic, autoimmune disease, vasculitis, and drugs are identified as risk factors for CKD (Matovinović, 2009; Levey & Coresh, 2012; KDIGO : Kidney Disease: Improving Global Outcomes CKD Work Group, 2013; Goddard, 2014; Tomino, 2014). There are also influencing clinical and sociodemographic factors.

Clinical Factors

- Diabetes
- Hypertension
- Autoimmune diseases
- Systemic infections
- Urinary tract infections
- Urinary stones
- Lower urinary tract obstruction
- Neoplasia
- Family history of chronic kidney diseases
- Recovery from acute kidney failure
- Reduction in kidney mass
- Exposure to certain drugs
- Low birth weight

Sociodemographic Factors

- Older age
- US ethnic minority status: African American, American Indian, Hispanic, Asian or Pacific Islander
- Exposure to certain chemical and environmental conditions
- Low income/education

1.9.1.2. Staging according to glomerular filtration rate category

Glomerular filtration rate, one of the main ways to measure renal function, estimates the rate of fluid passes through the kidneys. Measurement of clearance of compounds that are 100% filtered through the glomerulus and neither reabsorbed nor secreted by the tubules of nephron determines the direct GFR (Goddard, 2014). These compounds are inulin, radiolabelled ethylene diamine tetra acetic acid (EDTA). Inulin clearance is the gold standard to measure GFR (Goddard, 2014). The stages of CKD according to GFR are illustrated in table 2.

Table 1.2 Staging of CKD according to GFR

GFR category	GFR	Terms
G1	>90	Normal
G2	60-89	Mildly decreased
G3a	45-59	Mild to moderately decreased
G3b	30-44	Moderately to severely decreased
G4	15-29	Severely decreased/ prepared for RRT
G5	<15	ESRD/ RRT

In the absence of evidence of kidney damage neither GFR category G1 nor G2 fulfil the criteria for CKD. ESRD; end stage renal disease, RRT; renal replacement therapy. (KDIGO : Kidney Disease: Improving Global Outcomes CKD Work Group, 2013)

1.9.1.3. Staging of albuminuria

Albuminuria is another marker of CKD. The rate of excretion of albumin directly correlates with the degree of CKD (Table 3) (KDIGO : Kidney Disease: Improving Global Outcomes CKD Work Group, 2013).

Table 1.3 Staging of CKD according to ACR

Category	AER	ACR		Stage
	(mg/24 hours)	(mg/mmol)	(mg/g)	
A1	<30	<3	<30	Normal to mildly increased
A2	30-300	3-30	30-300	Moderately increased
A3	>300	>30	>300	Severely increased

ACR; albumin creatinine ratio, AER; albumin excretion rate. (KDIGO : Kidney Disease: Improving Global Outcomes CKD Work Group, 2013)

1.9.2. Measures of renal function

Clinical symptoms of CKD are not evident until renal function deteriorates to the level of GFR stage G4. To detect the progression of renal disease, measurement of renal function is essential.

While GFR is the gold standard, it is not carried out routinely in clinical medicine as it is relatively expensive and time consuming and is usually reserved for special circumstances (e.g. for potential live kidney donors) (Parmar, 2002; KDIGO : Kidney Disease: Improving Global Outcomes CKD Work Group, 2013).

For many years, estimation of serum levels of endogenous compounds of protein origin such as blood urea and serum creatinine have been used to assess renal function (Goddard, 2014), however their interpretation must be made in association with other clinical factors. For example, blood urea level depends on other factors including dehydration, gastrointestinal bleeding, level of protein intake and liver disease (Goddard, 2014). Serum creatinine estimation is more accurate to assess renal function because its production from muscle is consistent unless there is muscle loss or gain and it is nearly 100% filtered by the kidney. (Goddard, 2014). Creatinine clearance rate is also a measure of GFR. Ideally, creatinine clearance rate should be estimated from 24 hours collection of urine however this is often impractical for day to day use and estimated GFR (eGFR) is determined from equations such as the Crock Croft and Gault equation, Modification of Diet in Renal Disease Study (MDRD) equation or the CKD epidemiology collaboration (CKD EPI) equation (Parmar, 2002; KDIGO : Kidney Disease: Improving Global Outcomes CKD Work Group, 2013; Goddard, 2014). KDOQI guidelines prefer to use CKD EPI equation to determine eGFR in adults. As serum creatinine value depends on muscle mass, eGFR is proportionate to body surface area

mL/min/1.73 m2. To assess eGFR from serum creatinine is not accurate for children or the older population (Inker *et al.*, 2014). A new marker (Cystatin C) is being used to assess renal function particularly in the older population (Inker *et al.*, 2014).

The kidney normally excretes some protein in urine, though usually less than < 80 mg/24 hour. Any disease affecting the kidney may result in increased protein excretion and therefore, persistent proteinuria is a marker of kidney damage (Levey & Coresh, 2012; Goddard, 2014; Inker *et al.*, 2014). It is also a prognostic marker for cardiovascular morbidity and mortality (Iseki *et al.*, 1996). Types of proteinuria depends on the pattern of kidney disease. Albuminuria is the most sensitive marker for CKD as it is common in hypertension, diabetes and glomerular inflammation of primary or secondary origin (KDIGO : Kidney Disease: Improving Global Outcomes CKD Work Group, 2013) and the severity of albuminuria (micro/macro albuminuria) is helpful for staging and prognosis of CKD. To assess proteinuria, 24 hr urinary protein estimation is ideal however as noted above 24 hr samples are problematic and estimates of proteinuria can be determined using urinary protein creatinine ratio from spot urine sample, which have been shown to correlate with 24 hr protein results (KDIGO : Kidney Disease: Improving Global Outcomes CKD Work Group, 2013). Albumin to creatinine ratio can also be determined from spot urine samples and is recommended by the KDOQI guidelines as one of the markers of CKD (Inker *et al.*, 2014).

Urine can also be used to screen for nitrate, glucose, ketone, red blood cell (RBC), and white blood cells (WBC) with urine microscopy undertaken to confirm the presence of RBC, WBC, cast and microorganisms (Goddard, 2014). Electrolytes (serum and urinary) and ADH (vasopressin) can be tested to assess tubular function. Imaging technologies (X-ray, ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), or radionuclide studies) are used to detect structural abnormalities and stone diseases of urinary system and determine split of kidneys (Goddard, 2014).

1.9.3. Chronic kidney disease and hypertension

In spite of a complex interaction between hypertension and chronic kidney disease, it is clear that that impaired renal function leads to hypertension (Schiffrin *et al.*, 2007; Tedla *et al.*, 2011; Morgado, 2012). Furthermore, hypertension, in itself one of the leading causes of CKD, contributes to gradual worsening of renal disease and ultimately leads to end stage renal disease (ESRD) (Parmar, 2002; Young *et al.*, 2002; Matovinović, 2009; Tedla *et al.*, 2011; Inker *et al.*, 2014; Tomino, 2014). In addition to its effect on CKD, hypertension is a predisposing and independent risk factor for cardiovascular events like arrhythmias, acute

coronary syndrome cardiomyopathy and left ventricular hypertrophy (LVH) in patients with CKD (Locatelli *et al.*, 2001; Locatelli *et al.*, 2003; Schiffrin *et al.*, 2007; Whitman *et al.*, 2012; Rifkin *et al.*, 2013). Most of the patients with stage 5 CKD have high blood pressure which is often very difficult to manage despite numerous types of antihypertensives (Ridao *et al.*, 2001; De Nicola *et al.*, 2013b; De Nicola & Zoccali, 2016). Accordingly, it is challenging for physicians to manage resistant hypertension specially in patients with end stage renal disease. Further investigations to determine the underlying mechanisms causing hypertension in CKD are required if new therapeutic interventions are to be developed.

1.9.4. Mechanisms of hypertension in CKD

The underlying mechanisms of hypertension in CKD are not simple to define because of comorbidities and the presence of often more than one aetiological factor. Patients with CKD develop hypertension mostly in glomerulonephritis, diabetes mellitus, and polycystic kidney disease (PKD) (Ridao *et al.*, 2001; Morgado, 2012). The traditional mechanisms associated with abnormalities in sodium excretion, expansion of extracellular fluid volume and activation of RAAS are not enough to explain progressive hypertension in CKD (Guyton & Coleman, 1999; Klein *et al.*, 2003b; Kashihara & Satoh, 2008; Matovinović, 2009; Tomino, 2014). Two evolving concepts are sympathetic overactivity with autonomic dysfunction (Klein *et al.*, 2001; Klein *et al.*, 2003a; Lugon *et al.*, 2003; Neumann *et al.*, 2004; Neumann *et al.*, 2007; Schlaich *et al.*, 2007; Schlaich *et al.*, 2009b; Masuo *et al.*, 2010) and imbalance between endothelial vasodilators and vasoconstrictors with vascular remodelling (Locatelli *et al.*, 2003; Passauer *et al.*, 2005; Quek *et al.*, 2018a). However, in most cases, there is overlap between the underlying mechanisms for the development and maintenance of hypertension in CKD.

1.9.4.1. Sympathetic nerve activity in hypertension and CKD

Research has focused on increased SNA as one of the underpinning mechanisms in essential hypertension (Lundin *et al.*, 1984; Grassi & Esler, 1999; Esler, 2000; Lambert *et al.*, 2007). Importantly, studies reveal that the SNA is amplified very early in the disease process, even prior to the development of hypertension (Korner *et al.*, 1993; Phillips *et al.*, 2007). Measurement of plasma noradrenaline in CKD patients in different studies revealed elevated plasma level indicating sympathetic overactivity along with an association with cardiovascular risk and poor survival among CKD patients (Schohn *et al.*, 1985; Zoccali *et al.*, 2002; Masuo *et al.*, 2010). Similarly, it has been seen that SNA level, as determined from

direct recordings, is likewise increased (Klein *et al.*, 2003a; Phillips, 2005; Schlaich *et al.*, 2009b; Campese *et al.*, 2011; Grassi *et al.*, 2011a). In addition, altered blood pressure responses to handgrip exercises or heart rate variation during deep respiration (Sahin *et al.*, 2006), or more bradycardic response to beta blocker (Furgeson & Chonchol, 2008) and amplified LF oscillations of SBPV (Lewanski & Chrzanowski, 2003) among CKD patients in clinical settings provide evidences of sympathetic overactivity in CKD. Notably, therapeutic interventions using centrally acting sympatholytic agents (Campese & Massry, 1983; Levitan *et al.*, 1984; Schlaich *et al.*, 2009b) or renal nerve ablation (Schlaich *et al.*, 2009a; Hering *et al.*, 2012; Schlaich *et al.*, 2013) improve blood pressure in patients with CKD. As such, it is credible that SNA is augmented in CKD and has a role in the development and maintenance of secondary hypertension in kidney diseases (Phillips *et al.*, 2007; Schlaich *et al.*, 2009b).

A hyperactive sympathetic system can result in worsening of hypertension and target organ damage including renal, cardiovascular and neurological changes, that ultimately progress to severe renal damage in CKD patients. Pathological changes such as glomerular podocyte injury (Rafiq *et al.*, 2012), glomerulosclerosis (Adamczak *et al.*, 2002), progressive atherosclerosis and interstitial fibrosis (Zhang & Faber, 2001; Erami *et al.*, 2002) of the kidney in CKD triggered by sympathetic hyperactivity are associated with the severity of renal failure (DiBona, 2002; Grassi *et al.*, 2011b; Kaur *et al.*, 2017). Similarly, manifestations of CKD including proteinuria are strongly correlated with increased MSNA in patients with mild to moderate CKD (Grassi *et al.*, 2011b). In addition, cardiac morphological changes (Guizar-Mendoza *et al.*, 2006) with the potential of arrhythmia (Volders, 2010) and acute coronary events (Coutsos *et al.*, 2013) may be related to increased cardiac SNA in patients with CKD. It has been suggested that the end organ changes arise either directly in response to noradrenaline which stimulates proliferation of fibroblasts and smooth muscle cells of the vascular wall (Zhang & Faber, 2001; Erami *et al.*, 2002) or proliferation of cardiac myocytes indirectly by activating of RAAS and production of Ang II with vascular and cardiac remodelling (Grisk & Rettig, 2004; Raizada *et al.*, 2012).

In contrast to sympathetic hyperactivity, the parasympathetic component of autonomic function is reduced in CKD (Hildreth, 2011; Phillips, 2012). The reduced bradycardic response to valsalva manoeuvre or positional changes or deep respiration (Agarwal *et al.*, 1991; Sahin *et al.*, 2006), decreased HF oscillations of HRV (Lerma *et al.*, 2004; Tory *et al.*, 2004), and reduced tachycardic response in response to anti muscarinic agents such as atropine (Mircoli *et al.*, 2003) in CKD patients support impaired parasympathetic nervous

system function in CKD. What is critical is to determine the underpinning mechanisms driving this autonomic dysfunction.

1.9.4.2. Mechanisms driving increased sympathetic activity in chronic kidney disease

There are a number of possible mechanisms that may underlie increased sympathetic activity in association with CKD

1.9.4.2.1. Dysregulation of autonomic reflexes

Autonomic reflexes actively coordinate sympathetic and parasympathetic activity for physiological regulation of blood pressure. As a result, any deviation of these autonomic reflexes could potentially disturb the normal blood pressure profile and contribute to hypertension.

The sympathetic component of baroreflex function works reciprocally with the parasympathetic vagal component in the long-term control of blood pressure. In several clinical studies, there is convincing evidence of impaired cardiac parasympathetic baroreflex function in CKD as assessed in response to pharmaceutical agents (Tomiyama *et al.*, 1980; Tinucci *et al.*, 2001; Studinger *et al.*, 2006) or when spontaneous function is analysed (Lacy *et al.*, 2006; Studinger *et al.*, 2006; Johansson *et al.*, 2007). Interestingly, this cardiac baroreflex defect has been linked to the magnitude of reduction of GFR (Lacy *et al.*, 2006). Sympathetic deficits in baroreflex function have also been documented in clinical studies of hypertensive CKD patients. In a study by Ligtenberg et al., a rightward shift of the baroreflex curve was seen associated with increased SNA, although without any significant change in sympathetic BRS when compared to normotensive control subjects with normal renal function. After treatment with angiotensin converting enzyme inhibitor (ACEI), the sympathetic baroreflex curve shifted left in the same group of patients (Ligtenberg *et al.*, 1999a). In another study, Tinucci at al demonstrated that sympathetic baroreflex response was diminished in hypertensive CKD patients compared to hypertensive patients with normal renal function. The most striking finding of this study was that even in patients with mild renal impairment, the sympathetic BRS was blunted (Tinucci *et al.*, 2001). In a hypertensive CKD rat model, BRS is reduced in renal and splanchnic nerves with a shifting of the baroreflex sympathetic curve to the right, reflecting resetting of blood pressure to a higher range with increased SNA compared to normotensive rats (Salman *et al.*, 2014; Salman *et al.*, 2015a; Yao *et al.*, 2015). While this impairment has been localised to being of central origin, the underlying mechanism is not yet established.

In addition to baroreflex impairment in CKD, the involvement of other autonomic reflexes as drivers of sympathetic hyperactivity in the disease process is likely. Among them is the chemoreflex (which as detailed in section 7.3.2) is an important regulator of blood pressure responsive to arterial blood oxygen, hydrogen and carbon dioxide concentration. Studies examining the chemoreflex in CKD patients have suggested that hyperactive chemoreceptors are involved, as deactivation of chemoreceptors with 100% oxygen significantly reduced MSNA in these patients with or without chronic heart failure (Hering *et al.*, 2007; Despas *et al.*, 2009). Moreover, chemoreflex sensitivity of heart rate was blunted in CKD patients (Rassaf *et al.*, 2010a). The chemoreflex in the LPK model of CKD is also reduced in response to hypoxia (10% O_2 in N_2) and associated with increased SNA (Yao *et al.*, 2015).

The volume dependent cardiopulmonary reflex [Bezold-Jarisch (BZJ)] reflex also influences the regulation of autonomic outflow; however, knowledge of changes in the function of this reflex in CKD is limited. There is some data to suggest it is impaired in CKD patients (Grassi, 1987; Frank *et al.*, 2004) and may be associated with release of adenosine in dialysis-induced hypotension among haemodialysis-dependent patients (Ligtenberg *et al.*, 1999b; Daugirdas, 2001). It has also been shown to be also blunted in Adriamycin induced nephrotic rat, an animal model of CKD (Neahring *et al.*, 1995).

Accordingly, it is clear that impairment of autonomic reflex regulation impacts the sympathetic activity in hypertension and CKD. A key focus of this thesis is the study of the interaction between autonomic reflexes and respiratory sympathetic coupling and its role in sympathetic hyperactivity and hypertension in CKD.

1.9.4.2.2. The renin-angiotensin-aldosterone system

The RAAS works synchronously both in the brain and organs to modulate SNA in CKD (Grassi, 2001; Schiffrin *et al.*, 2007). As the end product of RAAS, Ang II acts on synaptic terminals to increase the concentration of noradrenaline by augmenting noradrenaline release and decreasing noradrenaline uptake, and in the adrenal medulla facilitating the release noradrenaline/adrenaline. This drives vasoconstriction and increased vascular resistance of peripheral vessels (Reid, 1992; Saino *et al.*, 2000; Grassi, 2001). A number of different studies on CKD patients show that administration of ACEI or angiotensin receptor blockers (ARB) markedly decreases muscle SNA in these patients (Ligtenberg *et al.*, 1999b; Klein *et al.*, 2003b; Neumann *et al.*, 2007).

1.9.4.2.3. The renal afferent pathway

The primary areas of afferent innervation of the kidney are situated in connective tissues of cortico-medullary junctions, renal pelvis and around the major vessels (Barajas & Wang, 1978; Recordati *et al.*, 1981). Impulses from stretch or chemo stimulation transmit through ipsilateral afferent sensory fibres to the dorsal root ganglia and dorsal horn of the spinal cord and ultimately terminate in the supra spinal neurons of mostly the NTS, RVLM, and PVN regions of the brain (Donovan *et al.*, 1983; Solano-Flores *et al.*, 1997). Among different thoughts of triggering factors from diseased kidneys for activated renal afferent pathways contributing to sympathetic overactivity, the main stimulating variables are altered renorenal reflex with inhibition of negative feedback, uraemic toxins, renal ischemia and fibrosis (Kopp *et al.*, 1987; Kopp & Buckley-Bleiler, 1989; Ye *et al.*, 1998; Koomans *et al.*, 2004; Schlaich *et al.*, 2009b; Johns *et al.*, 2011; Underwood *et al.*, 2017). In animal studies, it has been showed that removal of afferent impulse by bilateral nephrectomy (removal of kidneys) (Bigazzi *et al.*, 1994) or inhibition of afferent transmission by dorsal rhizotomy (transection of afferent nerve at dorsal root of ganglia) (Campese & Kogosov, 1995) decreases SNA, controls high blood pressure, and halts the progression of renal diseases (Wyss *et al.*, 1986; Campese *et al.*, 1995; Ye *et al.*, 1997). Therefore, it is conceivable that stimulation from the diseased kidney activates central vasomotor areas to increase SNA and hypertension in renal disease.

1.9.4.2.4. Uraemic toxins

Uraemic toxins, a heterogenous group of endogenous compounds, accumulate in the body in acute or chronic renal failure. It has been suggested that uremic toxins may be responsible for upregulation of renal afferent pathways and contribute to increased SNA and hypertension in renal disease (Converse *et al.*, 1992; Schlaich *et al.*, 2009b). Moreover, Underwood et al. have also proposed that central interaction between uraemic toxins and neuronal cells in the brain may contribute to neurohumoral dysfunction in CKD (Underwood *et al.*, 2017). However, reduction of uraemic toxins by renal transplantation or dialysis did not reduce SNA in patients with severe CKD (Hausberg *et al.*, 2002). Hence, further investigations are required to identify the exact role of uremic toxins in autonomic dysfunction of CKD.

1.9.4.2.5. Vascular remodelling

Vascular remodelling, one of the key pathognomic features of CKD affects vascular resistance and critically, may change the transmission of afferent impulses that initiate autonomic reflex pathways. The pathological characteristics of vascular remodelling including collagen deposition and rupture of elastin with increased fibromascular

hypertrophy, fibrosis and calcification in the arterial wall , all of which are present in animal models of CKD (Ng *et al.*, 2011b; Pai *et al.*, 2011; Sutliff *et al.*, 2011) and in humans (Blacher *et al.*, 2001; Chesterton *et al.*, 2005; Temmar *et al.*, 2010). The changes in the vascular structure are also associated with endothelial damage and impaired vasodilator function through the NO pathway in CKD (Quek *et al.*, 2018b). The vascular remodelling can reduce arterial distension and progression of Hering-Traube pulse wave (Bruno *et al.*, 2012). As a result, the ability of arterial baroreceptors to transduce impulse to central vasomotor center is impaired. This is supported by studies in CKD patients with renal replacement therapy, where the baroreflex is negatively correlated with vascular stiffness (Studinger *et al.*, 2006) and calcification (Chan *et al.*, 2005; Chesterton *et al.*, 2005).

1.9.4.2.6. Other mechanisms which may cause sympathetic hyperactivity in CKD

There are other important factors that contribute to sympathetic dysfunction in CKD. Among them, renalase, a flavoprotein belonged to oxidase and monoaminoxidase family presents in kidney, heart, skeletal muscle, small intestine, brain and peripheral nervous system. However, renalase is highly expressed in glomeruli and proximal renal tubule of kidney (Xu *et al.*, 2005; Desir & Peixoto, 2014; Li *et al.*, 2014). A recent study shows that plasma levels of both renalase and catecholamines are higher in patients with hypertension and CKD (Zbroch *et al.*, 2016). Another important factor is natriuretic peptides (NP). Among NPs, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) secreted from atrial myocytes and ventricular myocytes respectively, regulate blood pressure with their vasodilator effect contributed by promoting renal excretion of sodium and inhibiting function of RAAS and SNA directly or indirectly (Anand-Srivastava, 2005; Santos-Araújo *et al.*, 2015). Another one is endothelin, a peptide formed within endothelial cells that regulates vascular resistance (Mortensen, 1999; Schneider & Mann, 2014). There is evidence in animal studies that endothelin modulates the function of autonomic reflexes like baroreflex / chemoreflex (Mortensen, 1999) and also causes sympathetic overactivity and elevated blood pressure in hypertensive subjects (Nakamura *et al.*, 1999). Although, insulin is a natural hormone that regulates the function of glucose metabolism, excess insulin in blood or insulin resistance may play a significant role in impaired renal function in CKD, especially diabetic nephropathy (Svensson & Eriksson, 2006). Indeed, it has been revealed in different studies that increased plasma levels of insulin/insulin resistance may contribute to baroreflex dysfunction (Ryan *et al.*, 2013), sympathetic overactivity (Vollenweider *et al.*, 1993) and hypertension (Hall *et al.*, 1995) directly or indirectly.

1.9.5. Sex dimorphism, cardiovascular disease and chronic kidney disease

There is sexual dimorphism in the prevalence and incidence of hypertension, autonomic function, cardiovascular disease and CKD. With regard to blood pressure and autonomic function, in experimental animal models of hypertension such as the SHR (Reckelhoff, 2001), Dahl salt-sensitive rats (Crofton *et al.*, 1993), deoxycorticosterone acetate/salt hypertensive rat (Ouchi *et al.*, 1987), renal wrap (Haywood & Hinojosa-Laborde, 1997) and Ang II hypertension animals (Xue *et al.*, 2005; Schneider *et al.*, 2010), male animals typically have higher blood pressure. Moreover, removal of ovaries results in higher blood pressure in hypertensive females, MSNA is lower in females with hypertension compared to males (Hogarth *et al.*, 2007), and HRV and heart rate BRS are markedly higher in men with hypertension compared to hypertensive women (Sevre *et al.*, 2001; Pavithran *et al.*, 2008). Similarly, female rats exhibit a lower heart rate with greater HRV and heart rate BRS, alongside a lower resting blood pressure and SBPV in Ren2 rat model of hypertension (Xue *et al.*, 2005; Johnson *et al.*, 2011). In the LPK animal model of CKD, the underpinning mechanism of baroreflex dysfunction is operates differently between male and female animals (Salman *et al.*, 2014; Salman *et al.*, 2015a).

There is also a variable prognosis in association with cardiovascular disease due to sex. For example, following cardiovascular events such as after acute myocardial infarction, the prognosis is worse for women than men (Greenland *et al.*, 1991; Marrugat *et al.*, 1994; Tunstall-Pedoe *et al.*, 1994), yet mortality rate is lower in women with advanced heart failure (Adams *et al.*, 1999). Notably, the incidence and severe course of cardiovascular disease (CVD) in women before menopause is significantly reduced compared to men of similar age (Burt *et al.*, 1995; Reckelhoff, 2001; Maric, 2005). Furthermore, the cardiovascular morbidity and mortality in CKD patients (Franczyk-Skóra *et al.*, 2012; Nitsch *et al.*, 2013) show trends of sex variations with a reduced risk of cardiovascular related mortality in female (Nitsch *et al.*, 2013)

A sex difference is also evident in the prevalence, development and progression of CKD although the studies are conflicting. (Eriksen & Ingebretsen, 2006; Norris & Nissenson, 2008; Okada *et al.*, 2014). For example, one study demonstrated that female CKD patients progress more slowly with higher renal survival compared to men (Eriksen & Ingebretsen, 2006), while in the study by (Okada *et al.*, 2014) the progression of renal disease was faster in females with CKD. Interestingly, female patients exhibit a lower incidence of renal replacement therapy for end stage renal diseases (Hecking *et al.*, 2014). Current evidence

suggests that gonadal hormones have a direct effect in the development and maintenance of glomerular/tubular structure and renal and systemic circulation (Dart *et al.*, 2002; Stringer *et al.*, 2005). However, if this is the basis for the underlying sex difference in CKD is not yet known.

1.10. Targeting the sympathetic nervous system in the treatment of hypertension in CKD

As noted above, hypertension is an independent risk factor for cardiovascular morbidity and mortality in CKD patients (Locatelli *et al.*, 2001; Locatelli *et al.*, 2003; Schiffrin *et al.*, 2007; Whitman *et al.*, 2012) that contributes to gradual worsening of renal function (Parmar, 2002; Young *et al.*, 2002; Matovinović, 2009; Tedla *et al.*, 2011). Among the different mechanisms underlying the hypertension, increased sympathetic drive plays key role (Schlaich *et al.*, 2009b; Vink *et al.*, 2013; Kaur *et al.*, 2017). Current therapeutic interventions focusing on sympathetic activity aim to restore autonomic imbalance, reduce blood pressure and slow the progression of renal disease to some extent.

1.10.1.1. Pharmacological management:

Pharmacologically, sympathetic activity can be reduced or inhibited by targeting different sites (either peripheral or central) and receptors.

1. Alpha receptor blockers: Alpha (α) receptor blockers, such as prazosin and doxazosin, are effectively used in patients to control blood pressure with CKD through blocking the direct action of noradrenaline on the vasculature. However, they may cause intradialytic hypotension in dialysis patients and as such caution in their use is required (Inrig, 2010).
2. Beta blocker: Beta (β) blockers are widely used in the management of hypertension for their potent hypotensive effects however, their effect on SNA is inconsistent, with studies variably reporting no effect (Burns *et al.*, 2004) or a reduction in SNA (Wallin *et al.*, 1984). The data of its effect on SNA in CKD is limited.
3. Centrally acting drug: Centrally acting sympathoinhibitors, used to treat hypertension have been found to be effective to attenuate sympathetic overactivity (Kaur *et al.*, 2017). Traditional α2 receptor blockers like clonidine and alpha methyl dopa and the newer I_2 imidazoline receptor agonists with central α2 receptor antagonist activity like moxonidine and rilmenidine are used alone or in combination of other agents for treatment of hypertension in CKD (Neumann *et al.*, 2004; Schlaich *et al.*, 2009b).

4. Diuretics/ Spironolactone: In patients with CKD, discontinuing diuretics is associated with an increase in body weight and blood pressure, however, MSNA and plasma renin activity (PRA) decrease (Klein IH, 2003), suggesting diuretics may have inverse relationship with sympathetic activity. This is consistent with a study that shows treatment of hypertension with spironolactone reduces blood pressure but has no effect on MSNA (Menon *et al.*, 2009).
5. ACEI/ARB: Given that the activation of RAAS is a likely underlying mechanism driving sympathetic hyperactivity in CKD (Vink *et al.*, 2013), interventions targeting RAAS are considered as a central component in the management of CKD (Weir, 2009; Masuo *et al.*, 2010). Studies in CKD patients using different classes of RAAS inhibitors like ACEI (enalapril) (Ligtenberg *et al.*, 1999a), ARB (losartan) (Klein *et al.*, 2003b) or renin inhibitors (aliskiren) (Siddiqi *et al.*, 2011) show they are able to reduce MSNA and blood pressure, in addition to their antiproteinuric effect (Klein *et al.*, 2003b). Furthermore, there is an example where RAAS inhibitor along with centrally acting sympatholytic agent like moxonidine normalizes MSNA in CKD patients (Neumann *et al.*, 2004). In regards to autonomic function, human studies do not find any effect of ACEI or ARB on baroreflex sensitivity in patients with CKD (Ligtenberg *et al.*, 1999a; Neumann *et al.*, 2007). However there is evidence from animal studies that shows baroreflex sensitivity is improved after treatment with ARBs, in models of CKD (Yao *et al.*, 2015)
6. Statin: Statins (Atorvastatin), the lipid lowering drug usually used for micro or microvascular disease to prevent or treat stroke or ischaemic heart disease has been shown to cause a reduction of sympathetic activity in renal failure patients treated with aliskiren with stable blood pressure (Siddiqi *et al.*, 2011).

1.10.1.2. Surgical interventions

1.10.1.2.1. Renal denervation

As discussed in section 9.4.2.3, the renal nerves are thought to contribute to hypertension with evidence supporting this in CKD patients (Campese & Kogosov, 1995). A trigger of sympathetic nerve hyperactivity in CKD is likely mediated by the diseased kidney via the renal afferent nerves as following renal replacement therapy, MSNA and blood pressure remain elevated in CKD patients who retain their native diseased kidney, but after bilateral nephrectomy, blood pressure and MSNA are normalised (Converse *et al.*, 1992).

In recent years, much progress has been made of the role of renal denervation in the treatment of resistant hypertension using a catheter based percutaneous technique where radiofrequency is used to destroy renal nerves in the adventia of renal arteries (Vink *et al.*, 2013), although a large clinical trial on renal denervation, SIMPLICITY 3 did not reveal any blood pressure lowering effect in patients with resistant hypertension compared to sham control after 6 months of the procedure, raising questions around the technique and which patients groups may or may not benefit (Bhatt *et al.*, 2014). Schlaich et al showed that catheter based renal denervation resulted in a reduction of blood pressure along with decreased MSNA in patients with CKD (Hering *et al.*, 2012; Schlaich *et al.*, 2013). Similarly, other studies revealed that MSNA is reduced in resistant hypertension following renal denervation (Hering *et al.*, 2013; Hering *et al.*, 2014). However, a study on a canine CKD models shows a reduction in plasma noradrenaline level without normalization following total renal denervation (Liang *et al.*, 2015). Renal denervation has also been suggested to retard the progression of renal disease by virtue of its blood pressure lowering effect (Ravera *et al.*, 2006) as well as causing a decrease in podocyte injury and glomerulosclerosis (Nagasu *et al.*, 2010; Hering *et al.*, 2012; Kiuchi *et al.*, 2013).

1.10.1.2.2. Baroreceptor stimulation:

Stimulation of baroreceptors is another surgical intervention technique being used to counteract sympathetic overactivity and control blood pressure in hypertension. A device with electrically stimulating capacity is placed within the carotid sinus to stimulate the carotid baroreceptors. In both human and animal model with hypertension it has been shown to cause sympathoinhibition along with reduction of blood pressure (Heusser *et al.*, 2010; Lohmeier *et al.*, 2010; Scheffers *et al.*, 2010). This is thought to occur without any change in renin activity and sympathetic baroreflex curve function (Heusser *et al.*, 2010). Limited studies have examined carotid baroreceptor stimulation in the CKD population, with a study by Beige et al (Beige *et al.*, 2015) having found this technique useful to control blood pressure where following one year of baroreflex activation therapy, office blood pressure significantly reduced with a decreased median number of prescribed antihypertensive drugs.

1.10.1.2.3. Carotid body denervation

Inhibition of input from peripheral chemoreceptors by carotid body ablation is another prospective technique to reduce sympathetic hyperactivity in different diseases (McBryde *et al.*, 2013; Paton *et al.*, 2013b; Iturriaga, 2018). In a study Abdala et al demonstrated that after carotid body denervation blood pressure and SNA were reduced in SHR rats (Abdala *et al.*, 2012). Similarly, in heart failure rabbit model increased respiratory sympathetic coupling with

SNA was reduced following carotid body denervation (Marcus *et al.*, 2014b). Moreover, bilateral carotid body denervation caused a reduction in 24 hr urinary noradrenaline excretion with decreased blood pressure and myocardial hypertrophy in hypertensive and heart failure rat model (Fujii *et al.*, 2017). Notably, a study in human carotid body ablation revealed a decrease in ambulatory blood pressure with two serious adverse events after 6 months of carotid body ablation in hypertensive patients (Schlaich *et al.*, 2017). Although, carotid body ablation technique has not yet been utilized in the management of hypertension in CKD, it has a great therapeutic potential in CKD, as peripheral chemoreceptors hypersensitivity has been apparent in CKD (Hering *et al.*, 2007).

1.10.1.3. Renal replacement therapy

Renal replacement therapy (RRT) is a final treatment option in the face of failing kidneys. Dialysis is the most common, used for both acute renal failure and end stage renal disease to filter blood and remove or reduce uraemic toxins to acceptable levels with maintenance of metabolic and fluid homeostasis although not replacing endocrine function (Berger *et al.*, 2009; Goddard, 2014). Kidney transplantation is most definitive treatment form of RRT, replacing all functions of the kidney with better chance of long-term survival (Wolfe *et al.*, 1999; Goddard, 2014). RRT actually offers indirect benefit of sympathetic hyperactivity in CKD by correcting volume, metabolic and acid base disorders, thereby, improves autonomic dysfunction and hypertension.

1.10.1.3.1. Dialysis

Studies on the impact of dialysis in autonomic dysfunction are based primarily on non-invasive techniques and cardiac autonomic function. BRS, HF power of HRV and LF/HF ratio of HRV have been shown to improve when patients have switched from conventional haemodialysis to frequent nocturnal dialysis (Chan *et al.*, 2004; Chan *et al.*, 2005; Chan *et al.*, 2014) as well as in patients receiving chronic peritoneal dialysis (Dursun *et al.*, 2004). A study examined MSNA, shows that MSNA was reduced in ESRD patients requiring frequent haemodialysis (Zilch *et al.*, 2007). Intradialytic hypotension, a common complication of haemodialysis resulting from rapid fluid removal from the body, may contribute sympathetic hyperactivity and parasympathetic inhibition and dialysis may therefore have mixed effects on autonomic dysfunction in CKD patients, creating a dilemma about the beneficiary role of dialysis in these patients (Agarwal *et al.*, 1991; Vita *et al.*, 1992; Vlachojannis *et al.*, 2000).

1.10.1.3.2. Renal transplantation

Renal transplantation is a definitive and sought-after treatment for terminal stage of CKD. However, its role in reducing sympathetic hyperactivity in CKD is not clear. Rubinger et al showed that following renal transplantation, SBPV was normalized at 1 year (Rubinger *et al.*, 2009), whereas others reported that MSNA was not reduced after renal transplantation in similar patients (Hausberg *et al.*, 2002). Other studies have shown no effect (Heidbreder *et al.*, 1985) or an adjusted response (Agarwal *et al.*, 1991). Parasympathetic functions do appear to be improved in ESRD patients following renal transplantation (Yildiz *et al.*, 1998; Yang *et al.*, 2010) and relevant to this thesis, chemoreflex sensitivity after deactivation with 100% O_2 is more improved in kidney transplant patients when compared to haemodialysis patients (Rassaf *et al.*, 2010b).

1.10.1.4. Limitations of the management of hypertension in chronic kidney disease

Currently the treatment strategy for patients with CKD is based on management of underlying causes and symptoms of the disease. The predominance of autonomic failure, particularly sympathetic overactivity in different forms of CKD, however, suggests a common underlying pathway driving hypertension in CKD that should be a focus of treatment strategies. Centrally acting sympatholytic medications can be used to control hypertension in CKD, however, these medications have side effects including postural hypotension and other treatment (non-pharmacological and surgical) approaches have not been yet proved their efficacy in the management of hypertension specifically for CKD patients. Accordingly, there is a real need to define the underlying mechanisms in order to develop new therapeutic options in hypertension with CKD.

1.11. Polycystic kidney disease

Polycystic kidney disease is a genetic cause of CKD resulting in severe renal failure in children and adults (Fall & Prisant, 2005). Adult onset autosomal dominant PKD (ADPKD), is the most common cause of PKD while autosomal recessive PKD (ARPKD) is less common in the general population but is an important cause of renal disease in children (Guay-Woodford, 2003; Goddard, 2014). Nephronophthisis (NPHP) is another form of autosomal recessive cystic disease of the kidney and results in severe renal failure in children and adolescents (Hildebrandt *et al.*, 1992; Hildebrandt *et al.*, 2009).

The pathological congenital renal cysts containing serosanguinous fluid develop from the nephron and in syndromic conditions can coexist with cysts in other organs especially the

liver (Nadasdy *et al.*, 1995; Igarashi & Somlo, 2002; Comperat *et al.*, 2006). The common features of PKD are abdominal discomfort, hypertension, haematuria, urinary tract infections and ultimately renal insufficiency (Fall & Prisant, 2005; Goddard, 2014). Hypertension, being common in PKD, develops often before the development of renal insufficiency (Gabow *et al.*, 1990; Capisonda *et al.*, 2003; Sweeney & Avner, 2006). A key linking molecular feature of all forms of PKD is abnormal ciliogenesis, due to with presence of disordered proteins linked to the cilia or the centromere of the renal tubular epithelium, categorising the diseases as ciliopathies (Boucher & Sandford, 2004; Sweeney & Avner, 2006; Wolf & Hildebrandt, 2011; Goddard, 2014; Srivastava *et al.*, 2017)

People with ADPKD are born with mutation of either the PKD1 and PKD2 gene in each cell, whereas, people with ARPKD have altered copies of the polycystic kidney and hepatic disease 1 (PKHD1) gene in each cell (Sweeney & Avner, 2006; Goddard, 2014; Lanktree *et al.*, 2018). While, ADPKD is more common, presents late, and is slowly progressive, ARPKD is less common, presents early, usually in infancy and childhood, and is more aggressive (Guay-Woodford & Desmond, 2003; Boucher & Sandford, 2004; Kaimori & Germino, 2008). As a form of ARPKD, defects in more than 20 different genes are critical to develop NPHP (Srivastava *et al.*, 2017). There are three different patterns of NPHP according to the onset of ESRD. These are infantile (Gagnadoux *et al.*, 1989), juvenile (Hildebrandt *et al.*, 1992), and adolescent NPHP (Omran *et al.*, 2000). Polyuria, polydipsia, impaired urinary concentrating ability, gradual renal failure, anaemia, hypertension are the main presenting features of this disease (Ala-Mello *et al.*, 1996; Hildebrandt *et al.*, 2009; O'Toole *et al.*, 2010; Wolf & Hildebrandt, 2011). Around 10% of the children affected by juvenile NPHP progress to ESRD at the median age of 13 years (Salomon *et al.*, 2009; Wolf & Hildebrandt, 2011; Srivastava *et al.*, 2017). The histological features include tubular basement membrane thickening, tubular micro cyst formation, tubulointerstitial inflammation and interstitial fibrosis (Srivastava *et al.*, 2017). Adolescent NPHP patients show a similar clinical presentation with similar histopathological features and progress to ESRD variably from 10 years of age to adulthood (Omran *et al.*, 2000). In contrast, infantile forms typically reach ESRD before 2 years of age with evidence of microcyst formation tubulointerstitial change and renomegaly though no changes in the tubular basement membrane (Gagnadoux *et al.*, 1989; Wolf & Hildebrandt, 2011).

As noted above, hypertension is a key feature of PKD and with this is evidence of increase SNA and autonomic dysfunction. Peroneal nerve recordings have shown MSNA to be

increased in hypertensive PKD patients with renal failure, and plasma catecholamine levels has also been shown to be elevated in this group of patients (Cerasola *et al.*, 1998; Klein *et al.*, 2001).

1.11.1. The Lewis polycystic kidney rat as an animal model of chronic kidney disease

Studies using animal models of CKD provide the opportunity to identify a direct relationship between aetiological factors and CKD. Moreover, animal models can also be used for pre-clinical studies to explore therapeutic interventions. There are a number of different animal models of kidney disease including surgical models such as the 5/6 nephrectomy model (Liu *et al.*, 2003) and unilateral ureteric obstructed model (Chevalier, 2006), drug or nephrotoxin (e.g., cyclosporine, adenine, streptozotocin, etc.) induced models (Kinzler *et al.*, 1991; Kelly *et al.*, 1998; Jia *et al.*, 2013), and genetic models (LPK).

We have been using the LPK rat as a model of CKD to study autonomic function. These rats, which present with cardiovascular and renal impairment similar to ARPKD of human origin (Phillips *et al.*, 2007; Hildreth *et al.*, 2013b; Ameer *et al.*, 2016; Salman *et al.*, 2017; Quek *et al.*, 2018b) ascended as a result of a spontaneous mutation in the never in mitosis gene A-related kinase 8 (Nek8), the same gene responsible for NPHP9 in humans (McCooke *et al.*, 2012a). The LPK rat shows evidence of renal cysts at the age of 3 weeks, established hypertension by the age of 6 weeks and moderate severity of renal failure by the age of 12 weeks (Phillips *et al.*, 2007; Kandukuri *et al.*, 2012; McCooke *et al.*, 2012a). This unique genetic model has a similar natural course of disease progression as seen for human PKD and as such provides a controlled model system to explore the pathogenesis of increased sympathetic activity in CKD and its role in driving hypertension, as well as studying novel therapeutic options.

1.11.1.1. Genetic and phenotypic abnormalities of the LPK rat.

Cystogenesis is the main structural abnormalities arising from abnormal signalling due to mutations in cilia-based proteins that are critically involved in epithelial cell differentiation and cell to cell adhesion (Phillips *et al.*, 2007; Salomon *et al.*, 2009).

The mutation responsible for NPHP9 is protein called Nek8, that is a member of the NimA (never in mitosis A) related serine-threonine kinase (NeK) family. Nek8 is situated in the proximal region of cilia of collecting tubules and collecting ducts (McCooke *et al.*, 2012b). NPHP9 in humans is a cause of end stage of renal disease in children (Hildebrandt & Zhou,

2007; Otto *et al.*, 2008; Frank *et al.*, 2013) and is also the gene responsible for kidney cystic disease in jck mutant mice (Atala *et al.*, 1993).

In terms of renal disease phenotype, the LPK rat shows gradual enlargement of both kidneys without any other gross organ phenotype. Renal cysts in both the cortex and medullary rays contribute to the renomegaly (Phillips *et al.*, 2007).

A. Phase 1: At week 1, no cyst is evident although there are focal areas of dilatation of proximal and distal tubules indicating precystic stage of development.
B. Phase 2: The evidence of cysts formation as reflected by presence of cuboidal and flattened epithelial lined cysts developed mostly from collecting ducts in cortex and medulla. Gradual cystic changes with interstitial inflammation are observed until the 12th week of age.
C. Phase 3: Rapid changes in size of cysts are evident at 12 weeks of age along with marked interstitial changes. The characteristics of the tubule interstitial changes include collagen deposition in renal parenchyma, tubular atrophy, glomerulosclerosis and interstitial fibrosis. These changes continue up to 24 weeks.

1.11.1.1.1. Functional changes in the kidney of the LPK rat.

Impairment of renal function is the hallmark of CKD. In the LPK, blood urea increases from the age of 3 weeks indicating early pre-renal impairment. The animals also develop polyuria, reflecting impairment of concentrating ability because of tubular defects and cyst formation (Phillips *et al.*, 2007; Ding *et al.*, 2012). The increasing blood urea level with the marked increase in serum creatinine from 12 weeks with reduced creatinine clearance rate provides the evidence of established CKD at this age in the LPK rats (Phillips *et al.*, 2007), alongside low serum albumin, high urinary protein creatinine ratio and anaemia (Phillips *et al.*, 2015). The gradual deterioration of renal function along with the progressive changes in cyst and renal parenchyma mimics the natural course of ARPKD and CKD where end stage renal failure is the end point of this disease.

1.11.1.1.2. Cardiovascular changes in LPK

Blood pressure is elevated in LPK rats, as measured both by telemetry (Kandukuri *et al.*, 2012) and tail cuff measurement (Phillips *et al.*, 2007) from 6 weeks of age. Moreover, cardiac damage as reflected by left ventricular hypertrophy and interventricular hypertrophy is from 12 -24 weeks of age (Phillips *et al.*, 2007; Jeewandara *et al.*, 2015). This is in contrast to other rat models of cystic kidney disease that exhibit normotension or mild hypertension without other target organ damage (Atala *et al.*, 1993; Braun *et al.*, 1996; Nauta *et al.*, 2000;

Lager *et al.*, 2001). The animals also demonstrate vasculopathy, with structural changes including increased medial thickness, reduction in the elastin component of the arterial wall, collagen deposition, and marked aortic arterial calcification (Ng *et al.*, 2011a; Ng *et al.*, 2011b; Salman *et al.*, 2014; Salman *et al.*, 2015a). Notably, these vascular changes cause increased arterial stiffening which is related to elevated pulse pressure (Ng *et al.*, 2011a; Ng *et al.*, 2011b).

1.11.1.1.3. Sympathetic hyperactivity, autonomic dysfunction in the LPK rat

Sympathetic hyperactivity is evident and also contributes to hypertension in LPK rat, as reflected by the hypotensive effect by administrating the ganglion blocker hexamethonium (Ameer *et al.*, 2014). Direct nerve recordings of numerous sympathetic outflows also provide evidence of increased sympathetic activity in these animals (Salman *et al.*, 2014; Yao *et al.*, 2015; Underwood *et al.*, 2019). Autonomic dysfunction is also evident as demonstrated by raised LF band of SBPV (Harrison et al., 2010, Hildreth et al., 2013b), an upward and rightward shift in the baroreflex function of sSNA and an inability to maximally suppress SNA in response to increases in blood pressure (Harrison *et al.*, 2010; Hildreth *et al.*, 2013b; Salman *et al.*, 2014; Salman *et al.*, 2015a). In addition, the LPK rats exhibit impairment of central and afferent components of the baroreflex as well chemoreflex and somatosensory reflex abnormalities (Salman *et al.*, 2014; Salman *et al.*, 2015a; Yao *et al.*, 2015).

The LPK model therefore, presenting with persistent severe hypertension sympathetic hyperactivity, reflex dysfunction and progressive renal failure and target organ damage, with a similar natural course to human disease make the LPK rat a valuable animal model of CKD in which to explore the pathogenesis hypertension, and specifically, the role of respiratory sympathetic modulation as a driver of the sympathetic overactivity.

1.12. Thesis objectives

Chronic kidney disease, a common non-communicable disease, causes significant morbidity and mortality worldwide. As a comorbid condition, hypertension plays a critical role in the pathogenesis of CKD and increases cardiovascular risk in patients with CKD. Although sympathetic hyperactivity is a driving factor for hypertension and CKD, the underpinning mechanism of increased SNA in CKD is not well understood. Studies on animal model of hypertension suggest that augmented respiratory sympathetic coupling contributes to the sympathetic overactivity in different diseases including hypertension; however, the pattern of respiratory modulation of SNA, with its role as an underlying mechanism in CKD, is not yet known. Further, while centrally originated respiratory sympathetic coupling plays a dominant role to modulate sympathetic activity, the link between peripheral chemoreceptors and respiratory centres argues in favour of an influence of peripheral chemoreceptors on respiratory sympathetic coupling in diseased condition such as CKD.

Accordingly, the first aim of this thesis was to determine if respiratory modulation of SNA is altered in a classical rat model of CKD, the LPK rat and also determine if input from peripheral chemoreceptors affects respiratory sympathetic coupling in CKD.

As detailed in this preceding literature review, sex variation exists in both the prevalence and incidence of cardiovascular disease and CKD. In this context, the second aim of this thesis was to identify if the pattern of respiratory modulation of SNA along with its association with peripheral chemoreceptors is different between male and female animals with CKD.

Baroreflex dysfunction, often associated with CKD (Tinucci *et al.*, 2001; Studinger *et al.*, 2006; Johansson *et al.*, 2007) triggers the sympathetic overactivity in this disease. We have previously shown that all components including afferent, central and efferent pathways of baroreflex are affected in LPK rat model (Hildreth *et al.*, 2013b; Salman *et al.*, 2014; Yao *et al.*, 2015). Central respiratory generators interact with the central component of baroreflex pathways to produce altered baroreflex mediated response of heart rate and SNA (Eckberg & Orshan, 1977; Eckberg *et al.*, 1980; Gilbey *et al.*, 1984; Baekey *et al.*, 2008; Baekey *et al.*, 2010). As a result, the third aim of this thesis was to examine if respiratory modulation of SNA contributes to baroreflex dysfunction in CKD.

Finally, as detailed in section 6.6, there is evidence of a decrease in MSNA in renal failure patients after inhibition of peripheral chemoreceptors (Hering *et al.*, 2007). Moreover, studies report that denervation of peripheral chemoreceptors reduces both SNA and hypertension in the SHR model (McBryde *et al.*, 2013; Pijacka *et al.*, 2016). Our understanding regarding

input of peripheral chemoreceptors in sympathetic activity and hypertension of CKD is not clear. Accordingly, the last aim of this thesis was to investigate if bilateral carotid sinus (CSN) denervation, a method by which to inhibit peripheral chemoreceptors input, results in reduction of respiratory modulation of SNA and blood pressure in the LPK rat, and thereby examine the hypothesis that activation of peripheral chemoreceptors play critical role in altered respiratory modulation of SNA and hypertension in CKD.

2. Respiratory sympathetic modulation is augmented in chronic kidney disease

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2.1. Abstract

Respiratory modulation of sympathetic nerve activity (respSNA) was studied in a hypertensive rodent model of chronic kidney disease (CKD) using Lewis Polycystic Kidney (LPK) rats and Lewis controls. In adult animals under *in vivo* anaesthetised conditions *(n*=8-10/strain), respiratory modulation of splanchnic and renal nerve activity was compared under control conditions, and during peripheral (hypoxia), and central, chemoreceptor (hypercapnia) challenge. RespSNA was increased in the LPK vs. Lewis (area under curve (AUC) splanchnic and renal: 8.7±1.1 vs. 3.5±0.5 and 10.6±1.1 vs. 7.1±0.2 μV. s, respectively, *P*<0.05). Hypoxia and hypercapnia increased respSNA in both strains but the magnitude of the response was greater in LPK, particularly in response to hypoxia. In juvenile animals studied using a working heart brainstem preparation *(n*=7-10/strain), increased respSNA was evident in the LPK (thoracic SNA, AUC: 0.86±0.1 vs. 0.42±0.1 μV.s, *P*<0.05), and activation of peripheral chemoreceptors (NaCN) again drove a larger increase in respSNA in the LPK with no difference in the response to hypercapnia. Amplified respSNA occurs in CKD and may contribute to the development of hypertension.

Keywords

Respiratory sympathetic coupling, peripheral chemoreceptors, hypertension, chronic kidney disease, working heart brainstem preparation.

2.2. Introduction

Hypertension is a major comorbidity associated with chronic kidney disease (CKD), arising early in the development of CKD and acting as a significant causal factor for the development of end-organ damage (Vanholder *et al.*, 2005). Increased sympathetic nerve activity (SNA) is believed to play an important role in the development and/or maintenance of hypertension associated with kidney disease, with direct sympathetic nerve recording and plasma catecholamines levels elevated in individuals with CKD (Klein IH, 2003; Phillips, 2005; Schlaich *et al.*, 2009b; Campese *et al.*, 2011; Grassi *et al.*, 2012). Treatment with centrally acting sympatholytic agents can ameliorate hypertension in renal failure patients (Campese & Massry, 1983; Levitan *et al.*, 1984; Schlaich *et al.*, 2009b), however, we still do not understand the mechanisms driving this increase in SNA.

Modulation of sympathetic nerve discharge occurs during the phases of respiration both in animals and humans, although the temporal relationship can differ between species and different nerve beds (Boczek-Funcke *et al.*, 1992; Ng *et al.*, 1993; Zoccal *et al.*, 2008; Simms *et al.*, 2009). This respiratory modulation of SNA (respSNA) creates synchronous alterations in blood pressure that allow optimal tissue perfusion. In experimental animals, where open-chest experiments have been performed, a component of the respSNA involves coupling between the relevant neuronal circuits within the brainstem (Simms *et al.*, 2010; Machado *et al.*, 2017). In the spontaneously hypertensive rat (SHR), a model of essential hypertension, respSNA is augmented and when compared to normotensive Wistar Kyoto control rats, the phase relationship is shifted from the post-inspiratory to inspiratory phase (Czyzyk-Krzeska & Trzebski, 1990; Simms *et al.*, 2009). This exaggerated respSNA contributes to the development of hypertension in the SHR (Menuet *et al.*, 2017). In CKD it is not known whether the phasic pattern of respSNA is altered or whether it contributes to the observed increase in SNA and the associated hypertensive state (Augustyniak *et al.*, 2002; Salman *et al.*, 2015b).

The respSNA originating from central connections of respiratory and sympathetic networks (Haselton & Guyenet, 1989; Spyer, 1993; Koshiya & Guyenet, 1996; Sun *et al.*, 1997) contributes independently to increased SNA in animal models following chronic intermittent hypoxia (Zoccal *et al.*, 2008), with evidence of input from excitable central chemoreceptors (Molkov *et al.*, 2011). The peripheral chemoreceptors may also provide a critical respiratory-related input that contributes to respSNA and, in disease states, drives the development and/or maintenance of sympatho-excitation, and in turn hypertension. This hypothesis is supported

by a large body of work showing that exposure to chronic intermittent hypoxia (CIH) is correlated with enhanced respSNA (Machado *et al.*, 2017). Patients with CKD are more vulnerable to respiratory disorders such as obstructive sleep apnoea (Hanly, 2004), and those with sleep apnoea have elevated blood pressure compared to those with CKD alone (Sekizuka *et al.*, 2010). Further, deactivation of the peripheral chemoreceptors, with hyperoxia, reduces muscle SNA in individuals with renal failure (Hering *et al.*, 2007). In this context, there is exciting therapeutic potential in the investigation of the role of central respSNA and its underlying mechanism in hypertension CKD.

The main purpose of this study, therefore, was to test the hypothesis that respSNA is amplified in an animal model of CKD. Studies were undertaken in the Lewis polycystic kidney (LPK) rat, a genetic model of CKD presenting with kidney disease (McCooke *et al.*, 2012a) in which we have previously demonstrated hypertension, enhanced tonic SNA and perturbed reflex responses to both peripheral and central chemoreceptor stimulation (Salman *et al.*, 2014; Salman *et al.*, 2015a; Yao *et al.*, 2015). Animals were studied as both adults and juveniles using an *in vivo* and *in situ* experimental preparation, respectively.

2.3. Methods

All experimental procedures were approved by the Animal Ethics Committees of Macquarie University, NSW or the University of Melbourne, Victoria, Australia, and were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Animals arrived 2 weeks prior to experiments. During this period animals underwent the process of acclimatisation to a new housing environment and experimental procedures, including training for metabolic cage placement for 24-hour urine collection. During this period, animals were monitored for normal weight and growth for their age.

2.3.1. Study 1: Adult *in vivo* anaesthetised experiments

Adult (12-13-week-old) male LPK (n = 8) and control Lewis (n = 10) rats were used. Animals were purchased from the Animal Resources Centre, Murdoch, Western Australia.

2.3.1.1. Renal function

A 24 h urine sample was collected from all animals 48 h prior to experimentation and urine volume, urinary creatinine and protein levels examined using an IDEXX Vetlab analyser (IDEXX Laboratories Pty Ltd., Rydalmere, NSW, Australia). At the commencement of the

surgical procedure, an arterial blood sample was collected for determination of plasma urea and creatinine, and creatinine clearance calculated as described previously (Yao *et al.*, 2015).

2.3.1.2. Surgical procedures:

Animals were anaesthetised with 10% (w/v) ethyl carbamate (Urethane, Sigma Aldrich, NSW, Australia) in 0.9% NaCl solution (1.3 g/kg i.p.). Reflex responses to hind-paw pinch were assessed to determine adequate depth of anaesthesia and supplemental doses of ethyl carbamate given as required (65 mg/kg i.p. or i.v.). Body temperature was measured and maintained at 37 ± 0.5° C using a thermostatically controlled heating blanket (Harvard Apparatus, Holliston, MA, USA) and infrared heating lamp. The right femoral vein and artery were cannulated for administration of fluid (Ringer's lactate, 5 ml/kg/h) and measurement of arterial pressure (AP) and blood collection for measurement of blood gases, respectively. The AP signal was sampled at 200 Hz and acquired using a CED 1401 plus and Spike2 software v.7 (Cambridge Electronic Designs (CED) Ltd, Cambridge, UK, RRID:SCR_000903). A tracheostomy was performed and an endotracheal tube placed in situ. A bilateral vagotomy was performed to cut afferent inputs from the stretch receptors of the lung and the animal was ventilated with oxygen enriched room air (7025 Rodent Ventilator, UgoBasile, Italy) and paralysed with pancuronium bromide (2 mg/kg iv for induction, 1 mg/kg for maintenance as required; AstraZeneca, North Ryde, NSW, Australia). The left phrenic, splanchnic and renal nerves were dissected and the distal end of each nerve was tied and cut. All nerves were bathed in a liquid paraffin pool and activity was recorded from the central end using bipolar silver wire recording electrodes. The activity was 10 times amplified, band-pass filtered between 10-1000Hz by a bio amplifier (CWE Inc., Ardmore, PA, USA) and sampled at 5kHz using CED 1401 plus and Spike2 software. All recordings were made with the same bioamplifier calibrated to a pre-set setting 50 μV.

Following surgical preparation, animals were then stabilised for 30 min. Arterial blood samples were collected and analysed for pH, pCO_2, HCO_3^- and SaO_2 using a VetStat Electrolyte and Blood Gas Analyser (IDEXX Laboratories Pty Ltd., Rydalmere, NSW, Australia). SaO_2 was used to measure the oxygenation of blood instead of PaO_2 as SaO_2 was a more stable and consistent parameter to be able to monitor during the experiments and is readily interpreted relative to human studies (Hering *et al.*, 2007). After the initial blood gas measurement, arterial blood gases were then corrected by adjusting the ventilator pump rate and volume and/or slow bolus injections of 5% sodium bicarbonate (Xing & Pilowsky, 2010) as required to maintain parameters under control conditions within the following range: pH =

7.4 ± 0.5; PCO_2 = 40 ± 5 mmHg; HCO_3^- = 24 ± 2 mmol/L, SaO_2 = 100%; and end tidal (ET)CO_2 = 4.5 ± 0.5%. Repeat blood gas analysis was undertaken as required.

2.3.1.3. Experimental protocol

After the period of stabilization, the integrity of renal and splanchnic nerve recordings (rSNA and sSNA respectively) was confirmed by pulse modulation of SNA and demonstration of a baroreflex response to a bolus injection of phenylephrine (50 µg/kg iv, Sigma-Aldrich, St. Louis, MO, USA). Baseline parameters were measured under control conditions for a period of 30 min. Pilot studies indicated that hypoxic stimulation to stimulate the peripheral chemoreceptors using 10% O_2 in N_2 (Abbott & Pilowsky, 2009) could not be used for the 3 min stimulation period without affecting pH and PCO_2 in the LPK, and therefore animals were ventilated with room air, without oxygen supplementation, for a period of 3 min without any change in ventilator rate or volume. This induced a stable modest hypoxia - blood gas analysis parameters in the range of pH = 7.4 ± 0.5; PCO_2 = 43 ± 3 mmHg; HCO_3^- = 24 ± 2 mmol/L and SaO_2 = 82-84%. After a 30 min recovery period, central chemoreceptor stimulation was performed by ventilating the animals with 5% carbogen (5% CO_2 in 95% O_2) for 3 min without any change in the ventilator rate or volume, which was confirmed by blood gas analysis to produce hypercapnia with parameters in the range of pH = 7.4 ± 0.5; PCO_2 = 62 ± 7 mmHg; HCO_3^- = 24 ± 2 mmol/L and SaO_2 =100%.

At the end of experiment, all animals were euthanized with potassium chloride (3M i.v.) and the electrical noise levels for phrenic nerve activity (PNA), sSNA and rSNA recorded and later subtracted for data analysis.

2.3.1.4. Data analysis

All data were analysed offline using Spike 2 software. From the AP signal, mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic (DBP) and pulse (PP) pressure and heart rate (HR) were derived. The sSNA and rSNA were rectified and smoothed with a time constant of 0.1 s and PNA was rectified and smoothed with a time constant of 0.05 s. Baseline data was determined from a 30 s period at the end of the control ventilation period. Response to respiratory challenge was analysed over a 30 s period once PNA had stabilised for a period of 1-2 min and expressed as a change (Δ) relative to the 30 s period immediately prior to each stimulus. The PNA amplitude, frequency (number cycle.min $^{-1}$), duration (s) and minute activity (MPA=PNA amplitude x PNA frequency) were measured to quantify the changes. The phrenic cycle was divided into three phases: inspiratory (I), post-inspiratory (PI) and

expiratory (E). For SNA, the mean level was measured from the period 200 ms prior to the onset of the phrenic burst, and this was considered the baseline for all other measurements. A phrenic-triggered average of the rectified and smoothed sSNA and rSNA recordings was performed and the phases divided into I, PI, and E. From this trace the following parameters were calculated for sSNA and rSNA: the peak amplitude (PA [μV]), the maximum amplitude at the apex of the peak of the SNA burst coincident with inspiratory/post-inspiratory phase, the duration (from onset of excitatory activity to return to baseline [s]) and area under curve (AUC) of respSNA excitatory peak (μV.s) determined as the integral of the waveform. AUC of respSNA for I, PI and E phase of phrenic cycle were also calculated from the integral of the waveform.

2.3.2. Study 2: Juvenile working heart brainstem preparation

To determine respSNA in early stages of the disease process, another series of experiments were performed in juvenile rats using the working heart brainstem in-situ preparation, which while a reduced preparation has the advantage of allowing assessment of physiological patterns of central respiratory control and cardiovascular regulation in the absence of the effects of anaesthesia (Wilson *et al.*, 2001). Recordings were made in 5 week old male Lewis *(n* = 7) and LPK *(n* = 10) as described previously (Menuet *et al.*, 2014). At this age, renal function is only mildly impaired in the LPK (Phillips *et al.*, 2007). Animals were purchased from the Animal Resources Centre, Murdoch, Western Australia.

2.3.2.1. Surgical procedure

Animals were deeply anaesthetized with isoflurane until loss of the pedal withdrawal reflex and then dissected below the diaphragm, exsanguinated, cooled in Ringer solution on ice (composition in mM: 125 NaCl, 24 NaHCO3, 5 KCl, 2.5 CaCl2, 1.25 MgSO4, 1.25 KH2PO4 and 10 dextrose, pH 7.3 after saturation with carbogen gas (5% CO2, 95% O2) and decerebrated precollicularly. Lungs were removed and the descending aorta isolated and cleaned. Retrograde perfusion of the thorax and head was achieved via a double-lumen catheter (ø 1.25 mm, DLR-4, Braintree Scientific, Braintree, MA, USA) inserted into the descending aorta. The perfusate was Ringer solution containing Ficoll (1.25%) warmed to 31°C and gassed with carbogen (95% O2 and 5% CO2; closed loop reperfusion circuit). The second lumen of the cannula was connected to a transducer to monitor perfusion pressure in the aorta. Neuro-muscular blockade was achieved using vecuronium bromide added to the perfusate (2–4 μg/mL, Organon Teknika, Cambridge, UK). Simultaneous recordings of PNA,

thoracic sympathetic nerve activity (tSNA, T8-10), vagus nerve activity (VNA) and abdominal nerve activity (AbNA, T9-T12) were obtained using glass suction electrodes. The activity was amplified (10 kHz, Neurolog), filtered (50–1500 kHz, Neurolog), digitized (CED) and recorded using Spike2 (CED).

2.3.2.2. Experimental protocol

After the period of stabilization, the peripheral chemoreceptors were stimulated using sodium cyanide (NaCN; 0.05%; 100 μL bolus) injected into the aorta via the perfusion catheter (Chang *et al.*, 2015; Menuet *et al.*, 2016). Central chemoreceptors were stimulated using hypercapnic conditions by changing the level of CO_2 in the perfusate source from 95% O2 and 5% CO_2 to 90% O2 and 10% CO_2 for ~5 min. The electrical noise levels for tSNA recordings were determined at the end of experiments by sectioning the sympathetic chain at the proximal paravertebral ganglion level and were subtracted for data analysis. All chemicals were purchased from Sigma-Aldrich, Australia.

2.3.2.3. Data analysis

All data were analysed offline using Spike 2 software. The signals were rectified and integrated with a 50 ms time constant. The HR was derived using a window discriminator to trigger from the R-wave of the electrocardiogram recorded simultaneously with PNA. The PNA, and VNA signals were used to assess respiratory parameters associated with inspiration, post-inspiration and expiration, respectively. During baseline and hypercapnic conditions, phrenic-triggered (end of inspiratory burst) averaging of tSNA was used for the analysis of tSNA parameters related to the burst of respSNA tSNA and tonic non-respiratory modulated tSNA (baseline tSNA). NaCN-induced respSNA was measured as delta increase in AUC compared to a pre-stimulus control period with same duration (Menuet *et al.*, 2016), while peak activity of respSNA of tSNA was calculated as the maximum amplitude of the tSNA burst coincident with inspiratory/post-inspiratory transition.

2.3.2.4. Statistical analysis

All data are presented as mean ± SEM. A Student's t-test was used to determine baseline differences in cardiorespiratory function and respiratory sympathetic coupling between the LPK and Lewis. A two-way ANOVA with repeated measures followed by Bonferroni's post-hoc analysis was used to analyse the effect of peripheral or central chemoreceptor challenge on respiratory sympathetic coupling with strain and chemoreceptor challenge as the variables.

All analysis was performed using GraphPad Prism software v6.0 (GraphPad Software Inc., La Jolla, California, USA, RRID:SCR_002798). Differences were considered statistically significant where $P<0.05$.

2.4. Results

2.4.1. Adult baseline parameters

Adult male LPK rats showed a phenotypic elevation in blood urea (24.3 ± 2.3 vs. 5.9 ± 0.3 mmol/L) and plasma creatinine (60.6 ± 11.7 vs. 22.3 ± 4.6 μmol/L) and reduction in creatinine clearance (2.08 ± 0.4 vs. 10.1 ± 2.3 mL/min) (all LPK n=8 vs. Lewis n=10; $P<$ 0.001) reflective of impaired renal function.

During the control period, individual recordings were made of rSNA, sSNA, PNA and AP from Lewis and LPK rats (Figure 1). Consistent with our previous work (Salman *et al.*, 2014; Yao *et al.*, 2015), SBP, MAP, DBP, PP, HR and SNA (both sSNA and rSNA) were elevated in the adult LPK compared with age-matched Lewis controls (Table 1). Phrenic frequency was also significantly higher in the adult LPK, suggestive of increased central respiratory drive; however, there was no significant difference in PNA amplitude or MPA between strains (Table 1).

We examined the temporal relationship between SNA and the respiratory cycle in LPK and Lewis rats under control conditions. Both the rSNA and sSNA exhibited distinct respSNA, with a clear burst in SNA in the PI period in both the Lewis and LPK (see Figures 2 and 4, control panels A -B [renal], E-F [splanchnic]). In the Lewis rat, both sSNA and rSNA showed a tendency to increase from baseline during I, before the sharp peak occurred in PI. After the PI peak, activity fell quickly to baseline and was relatively flat during E. The LPK showed a distinct difference – with a decrease in activity below baseline during early I that was most obvious in rSNA (rSNA, I phase AUC, LPK vs Lewis: -0.07 ± 0.03 vs 0.45 ± 0.2; $P \leq 0.5$; sSNA, I phase AUC, LPK vs Lewis: 0.06 ± 0.05 vs 0.18 ± 0.08 ; $P > 0.5$). There was a gradual decline in both sSNA and rSNA during the E period which was not significantly different between the strains (rSNA, E phase AUC, LPK vs Lewis: 0.07 ± 0.04 vs 0.08 ± 0.1; $P > 0.5$; sSNA, E phase AUC, LPK vs Lewis: 0.02 ± 0.03 vs 0.04 ± 0.02; $P > 0.5$ [Figures 2 and 4, control panels A -B (renal), E-F (splanchnic)].

Quantitative parameters characterizing respSNA under control conditions for both nerves are provided in Table 2. PA and AUC for both sSNA and rSNA were significantly greater in the LPK compared with Lewis. The duration of the respSNA for both sSNA and rSNA was not

different between the Lewis and LPK indicating that the greater AUC observed in the LPK was driven primarily by the greater PA.

2.4.2. Adult responses to chemoreceptor stimulation

In response to a hypoxic challenge, a greater increase in AP was observed in the LPK compared with Lewis (Table 3). In the LPK rat this was associated with a slight, but significant, slowing of PNA frequency, but not amplitude or duration. The hypercapnic challenge produced comparable increases in AP and central respiratory drive in the two strains, with only a small difference in the HR response.

In both strains, under hypoxic and hypercapnic conditions, the respSNA curves of sSNA and rSNA retained a consistent PI peak (see Figures 2 and 4). In response to hypoxia, respSNA (sSNA and rSNA) was increased in both strains as reflected by an increase in PA in Lewis and an increase in PA and AUC in LPK (Figure 3) that was greater in LPK rats (delta AUC, sSNA: Lewis vs. LPK: 0.8 ± 0.6 vs. 5.9 ± 1.9, rSNA: 2.8 ± 0.7 vs. 7.5 ± 2.1 μV.s, both $P< 0.05$). Hypercapnia induced an increase in the respSNA of sSNA as reflected by an increase in PA in Lewis and an increase in PA and AUC in LPK. In the rSNA there was an increase in PA in the Lewis only (Figures 4, 5). However, the magnitude of the change in the respSNA of both nerves was similar between strains (Delta AUC, sSNA, Lewis vs. LPK: 1 ± 0.4 vs. 2.5 ± 0.7; rSNA, Lewis vs. LPK: 3.2 ± 0.5 vs. 2.5 ± 1.4 μV.s, both $P > 0.05$). During hypoxia, the inspiratory inhibition in the LPK increased in rSNA (I phase AUC, rSNA, control vs. hypoxia: -0.07 ± 0.04 vs. -0.9 ± 0.3 μV.s $p < 0.05$; Figure 2) and became clearly evident in sSNA (I phase AUC, sSNA, control vs. hypoxia : 0.05 ± 0.04 vs. -0.4 ± 0.1 μV.s $P < 0.05$). This increase in inspiratory inhibition did not occur in the LPK under hypercapnic conditions (I phase AUC, rSNA, control vs. hypercapnia : -0.32 ± 0.2 vs. -0.4 ± 0.2 sSNA: 0.03 ± 0.09 vs. 0.06 ± 0.1 μV.s both $P >0.05$). There was no significant change to the inspiratory phase in the Lewis animals under either condition.

2.4.3. Juvenile baseline parameters

In the working heart brainstem preparation (Figure 6), mean perfusion pressure was not different between the two strains (Lewis vs. LPK: 73 ± 4 vs. 76 ± 2 mmHg, $P = 0.55$). The HR was faster in the LPK (Lewis vs. LPK: 360 ± 13 vs. 302 ± 17 bpm, $P < 0.05$). There was no difference observed in PNA frequency (Lewis vs. LPK: 9.5 ± 0.7 vs. 10.2 ± 1.6, $P = 0.74$), although inspiratory duration of PNA was reduced in the LPK (Lewis vs. LPK: 1658 ± 147 vs. 1242 ± 79 ms, $P < 0.05$). Tonic levels of tSNA were not significantly different (Lewis vs.

LPK: 4.37 ± 0.47 vs. 4.51 ± 0.58 μV, *P = 0.85*); however, respiratory modulation of tSNA was greater in the LPK with a larger AUC observed (Lewis vs. LPK: 0.42 ± 0.13 vs. 0.86 ± 0.13 μV. s, $P < 0.05$). No differences were observed in respSNA burst duration (Lewis vs. LPK: 0.8 ± 0.1 vs. 1.4 ± 0.2 s, *P = 0.10*) or the magnitude of the peak amplitude of tSNA (Lewis vs. LPK: 6.01 ± 0.56 vs. 6.17 ± 0.85 μV, *P = 0.87*).

2.4.4. Juvenile responses to chemoreceptor stimulation

Peripheral chemoreceptor activation with NaCN produced a comparable increase in perfusion pressure in both strains (Lewis vs. LPK: 5.7 ± 1.4 vs. 6.4 ± 1.2 mmHg, *P = 0.71*). The accompanying large bradycardia was of greater magnitude in the LPK (Lewis vs. LPK: -86 ± 18 vs. -176 ± 16 bpm, $P < 0.05$). RespSNA increased in both strains in response to peripheral chemoreceptor activation (NaCN, *P<0.05*; Figure 6), as evidenced by an increase in PA and AUC that was of greater magnitude in both circumstances in the LPK. (Lewis vs. LPK: delta PA: 3.6 ± 0.6 vs. 6.5 ± 0.9 μV, delta AUC: Lewis vs. LPK: 7.0 ± 1.2 vs. 15.1 ± 2.7 μV.s, *P<0.05*).

Central chemoreceptor activation with hypercapnia produced a comparable reduction in perfusion pressure (Lewis vs. LPK: -7.9 ± 1.9 vs. -8.6 ± 1.1 mmHg, *P = 0.75*) and HR (Lewis vs. LPK: -42 ± 9 vs. -53 ± 8 bpm, *P = 0.36*) in the two strains. RespSNA increased in both strains (Figure 7) with an increase in PA in the LPK and a comparable increase in AUC in both strains (delta AUC: 0.98 ± 0.16 vs. 0.99 ± 0.29 μV. s, *P = 0.97*).

2.5. Discussion

Our study has determined the characteristic features of respSNA in a rodent model of CKD. We have directly measured the relationship between inspiratory drive and two sympathetic nerves in anaesthetized adult animals and to one sympathetic nerve in juvenile animals using the working heart brainstem preparation. The timing of the peak of respSNA was observed persistently in the PI period under all conditions tested and, from the earliest age studied (5 weeks). Adult and juvenile LPK rats demonstrated increased respSNA compared to Lewis control rats. Under control conditions, the LPK rat shows an alteration in respSNA patterning with increased inhibition of rSNA during the inspiratory period. This inspiratory inhibition is augmented in rSNA with hypoxia and becomes evident in sSNA with this respiratory challenge. In addition, we have found that peripheral chemoreceptor stimulation has an exaggerated effect on respSNA in both the juvenile and adult LPK animals, which suggests a

role for peripheral chemoreceptors in the pathophysiology of autonomic dysfunction associated with CKD.

Our finding of augmented respSNA in the LPK model of CKD is consistent with previous work in the SHR model, which also demonstrates amplified respSNA, as examined using the same methodologies in juvenile (Simms *et al.*, 2009; Menuet *et al.*, 2017) and adult SHRs (Czyzyk-Krzeska & Trzebski, 1990) that we have applied, though notably, we do not see the marked temporal shift in the peak response from post-inspiration to inspiration that is described in the SHR during the development of hypertension. Enhancement of respSNA is also a feature noted in animal models of chronic intermittent hypoxia (Zoccal *et al.*, 2008), congestive heart failure (Marcus *et al.*, 2014a); and in rats following uteroplacental insufficiency (Menuet *et al.*, 2016). Common to these diseases is autonomic dysfunction and the development of hypertension in association with increased SNA. Interestingly, we found a higher PNA frequency in adult LPK rats compared to adult Lewis rat s with a comparable PNA frequency between juvenile rats which might be due to underlying disease process and severity of renal impairment. However, this is hypothetical and would need further investigation to address.

While we did not see elevated baseline perfusion pressures or tSNA in the juvenile LPK at 5 weeks of age, we did observe increased respSNA at this early age. Our previous work in both young and adult LPK (7 and 14 weeks) show increased SNA, including recordings from conscious animals (Salman *et al.*, 2014; Salman *et al.*, 2015a), and we observe markedly elevated blood pressure in these animals from an early age (6 weeks) (Phillips *et al.*, 2007; Kandukuri *et al.*, 2012). Change in sympathetic activity is therefore a key feature of the autonomic dysfunction in the LPK model, consistent with clinical data demonstrating that in patients with polycystic kidney disease, muscle SNA is elevated very early in the disease, often prior to any significant reduction in renal function (Klein *et al.*, 2001). Our work is therefore supportive of the hypothesis that in CKD, altered interactions between central respiratory and sympathetic pathways is a contributor to the development and maintenance of hypertension.

The rostral ventrolateral medulla (RVLM) is an important site in the generation and regulation of sympathetic vasomotor tone (Guyenet, 2006), and our recent work in the SHR suggests that the inspiratory pre-Bötzinger complex is a likely site of modulatory inputs to the RVLM C1 neurons which could drive an increase in SNA (Menuet *et al.*, 2017). There is also an important contribution from the respiratory pattern generator that results in phasic

modulation of RVLM presympathetic neurons. Recordings from both anesthetised rats and juvenile rats using the working heart brainstem preparation show 3 main classes of respiratory-modulated RVLM presympathetic neurons – I activated, PI activated and I inhibited – as well as some none modulated neurons (Haselton & Guyenet, 1989; Moraes *et al.*, 2013b). Whilst not directly tested in this study, we propose that altered strength of respiratory input to these different neuron classes could underlie the different patterns of respSNA observed including the enhanced I inhibition of respSNA in the LPK rat.

While the exact mechanisms underlying amplified respSNA have not yet been elucidated, it has been suggested that interaction between central (Molkov *et al.*, 2011) and peripheral chemoreceptor pathways (Guyenet *et al.*, 2009b; Moraes *et al.*, 2015) may contribute to increased sensitivity of retrotrapezoid nucleus (RTN). The RTN has been shown to play a critical role in amplified respSNA under conditions of chronic intermittent or sustained hypoxia (Molkov *et al.*, 2014; Moraes *et al.*, 2014b). Of relevance to our work is that peripheral chemoreceptor hypersensitivity has been suggested to contribute to the development of sympathetic over activity in kidney disease (Hering *et al.*, 2007). In the current study, stimulation of peripheral chemoreceptors by NaCN and hypoxia evoked an increase in respSNA parameters in both the juvenile and adult animals, respectively, that was of a significantly greater magnitude in the LPK. In contrast, exposure to hypercapnia, which drives a centrally mediated chemoreflex and subsequent sympathoexcitatory and pressor response, produced changes which were similar between the strains. Moreover, adult LPK rats showed a higher respSNA even in control condition, and inspiratory motor activity was similar between strains after both peripheral and central chemoreceptor stimulation. Given the evidence that chronic intermittent hypoxia induces sensitization of peripheral chemoreceptors, producing an exaggerated sympathoexcitation and increased expiratory-related SNA (Braga *et al.*, 2006; Zoccal *et al.*, 2008; Moraes *et al.*, 2012), and that increased peripheral chemoreceptor sensitivity contributes to sympathetic over activity and hypertension in SHR rats (Tan *et al.*, 2010; Paton *et al.*, 2013b), we speculate that there may be at least two mechanisms working centrally in the medulla oblongata to contribute the augmented respSNA seen in association with hypertension in CKD; one dependent on peripheral chemoreceptors and another one that is independent.

Hypoxia is a common contributing factor for amplified respSNA and increased SNA in disease conditions including hypertension (Somers *et al.*, 1988; Calbet, 2003; Zoccal *et al.*, 2008), and while we did not investigate directly the mechanism behind increased respSNA in

CKD in the present study, of relevance is our previous published data that has documented vascular remodeling, hypertrophic changes and calcification in the aortic arch of LPK animals (Salman *et al*., 2014). This type of vascular remodeling is closely associated with ageing, hypertension and CKD in humans and is documented to occur in the carotid/vertebral arteries, and in association with atherosclerosis, can result in increased intima-media thickness and ultimately narrowing the lumen of the carotid and vertebral artery (Tanaka *et al.*, 2012; Yamada *et al.*, 2014). Given that the carotid body and brainstem are highly vascular organs and peripheral/central chemoreceptors are sensitive to reduced blood flow, remodeling of the carotid body/cerebral arterioles could be a persistent stimulus for central/peripheral chemoreceptor hypersensitivity (Paton *et al.*, 2013a; Moraes *et al.*, 2014b). Anaemia is also common in CKD, a feature well documented in the LPK strain (Phillips *et al.*, 2015), which in itself may be cause of persistent hypoxia. Regardless of the mechanism, we presume that in CKD, a persistent hypoxic stimulus via peripheral chemoreceptor independent and/or dependent pathways, can drive premotor sympathetic neurons and respiratory (expiratory) neurons to produce amplified respSNA and increased sympathetic tone in similar way to that of other pathological conditions (Zoccal *et al.*, 2008; Zoccal & Machado, 2010; Wong-Riley *et al.*, 2013).

In conclusion, we provide evidence that respSNA is augmented and associated with increased SNA and hypertension in CKD. We further demonstrate an exaggerated respSNA response to peripheral chemoreceptor stimulation, indicating this modulatory pathway may act as one of the driving factors of autonomic dysfunction in CKD. The pathways responsible for modulation of respSNA, from the carotid body chemoreceptors to central sites such as the RVLM, may well be valuable novel therapeutic targets in CKD. Our data also show that changes in respSNA are evident very early in the disease process, suggesting that early intervention may help to reduce the complex pathogenesis of CKD.

2.6. Acknowledgements

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2.7. Disclaimer

All authors declared no competing interests.

Table 2.1 Baseline cardiorespiratory function in adult Lewis and LPK rats

	Lewis	LPK
MAP (mmHg)	90 ± 4	125 ± 10*
SBP (mmHg)	117 ± 8	194 ± 22*
DBP (mmHg)	75 ± 3	99 ± 10*
PP (mmHg)	41 ± 6	87 ± 18*
HR (bpm)	453 ± 9	480 ± 3*
PNA amplitude (μV)	18.3 ± 2.1	24.9 ± 5.3
PNA frequency (cycles/min)	36 ± 1	45 ± 1*
PNA duration (s)	0.82 ± 0.1	0.72 ± 0.01
MPA	674.9 ± 86.03	1125 ± 232
sSNA (μV)	3.1 ± 0.5	7.9 ± 1.3*
rSNA (μV)	5.2 ± 0.4	8.9 ± 0.6*

Measures of cardiorespiratory function in Lewis and LPK rats under control conditions. MAP: mean arterial pressure, SBP: systolic blood pressure, DBP: diastolic blood pressure, PP: pulse pressure, HR: heart rate, bpm: beats per min, PNA: phrenic nerve amplitude, MPA: minute phrenic activity, rSNA: renal sympathetic nerve activity, sSNA: splanchnic sympathetic nerve activity. Results are expressed as mean ± SEM. *$P<0.05$ between the Lewis and LPK as determined by Student's t-test. n = 9 Lewis and n = 8 LPK excepting rSNA where n = 5 LPK and 6 Lewis

Table 2.2 RespSNA parameters in splanchnic and renal sympathetic nerves under control conditions.

		Lewis	LPK
PA (μV)	splanchnic	4.1 ± 0.8	8.8 ± 1.1*
	renal	8.7 ± 0.6	11.02 ± 0.8*
Duration (sec)	splanchnic	0.8 ± 0.07	0.99 ± 0.02
	renal	0.8 ± 0.05	0.96 ± 0.05
AUC (sec x (μV)	splanchnic	3.5 ± 0.5	8.7 ± 1.1*
	renal	7.1 ± 0.2	10.6 ± 1.1*

Measures of respSNA (phrenic triggered) parameters for integrated sSNA and rSNA in Lewis and LPK rats under control conditions. PA: peak amplitude, AUC: area under curve. Results are expressed as mean ± SEM. *$P<0.05$ between the Lewis and LPK as determined by Student's t-test. sSNA data: n = 9 Lewis and n = 8 LPK, excepting rSNA where n = 5 LPK and 6 Lewis**.**

Table 2.3 Effects of peripheral and central chemoreceptor stimulation on cardiorespiratory parameters in adult Lewis and Lewis Polycystic Kidney (LPK) rats

		Lewis *(n = 9)*	LPK *(n = 8)*
Hypoxia	Δ MAP (mmHg)	4 ± 4	21 ± 5 *
	Δ SBP (mmHg)	5 ± 5	34 ± 11 *
	Δ DBP (mmHg)	4 ± 4	16 ± 4
	Δ PP (mmHg)	1 ± 2	18 ± 7 *
	Δ HR (bpm)	12 ± 2	10 ± 1
	Δ PNA amplitude (μV)	10.9 ± 3.6	9.8 ± 3.9
	Δ PNA duration (sec)	0.13 ± 0.02	0.07 ± 0.01
	Δ PNA frequency (cycles/min)	5 ± 2	-2 ± 2*
	Δ MPA	536.6 ± 152.5	300.7 ± 66.9
Hypercapnea	Δ MAP (mmHg)	19 ± 3	16 ± 4
	Δ SBP (mmHg)	22 ± 4	29 ± 8
	Δ DBP (mmHg)	18 ± 3	13 ± 3
	Δ PP (mmHg)	4 ± 2	16 ± 6
	Δ HR (bpm)	-8 ± 1	-4 ± 1 *
	Δ PNA amplitude (μV)	8.7 ± 2.7	9.1 ± 1.6
	Δ PNA duration (sec)	0.15 ± 0.02	0.11 ± 0.02
	Δ PNA frequency (cycles/min)	-3 ± 1	-4 ± 1
	Δ MPA	178.5 ± 74.08	293.9 ± 71.04

Delta change in phrenic nerve activity (PNA) and blood pressure (mmHg) under hypoxic or hypercapnic conditions in adult Lewis and Lewis Polycystic Kidney (LPK) rats. MAP: mean arterial pressure, SBP: systolic blood pressure, DBP: diastolic blood pressure, PP: pulse pressure; HR: heart rate, MPA: minute phrenic activity. Results are expressed as mean ± SEM. *$P<0.05$ between the Lewis and LPK as determined by Student's t-test. Δ = Delta change in, n = number of animals per group.

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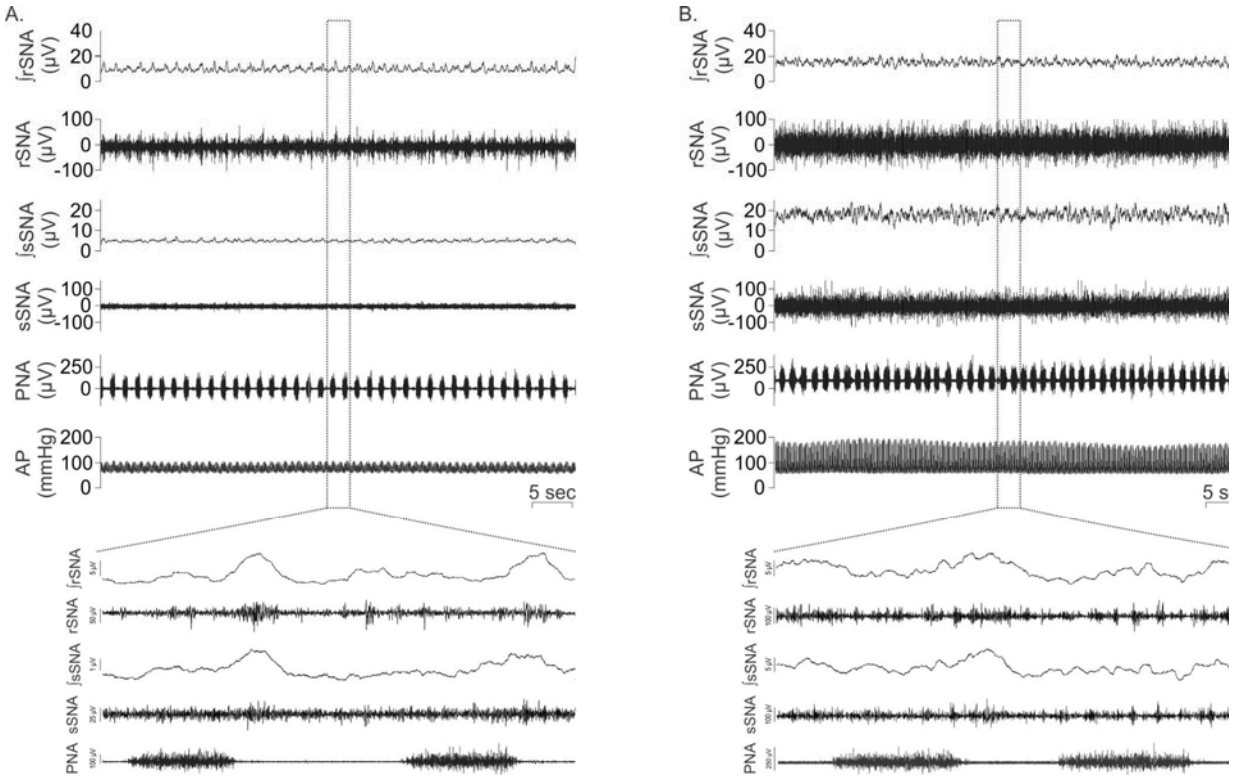

Figure 2.1 Representative traces showing higher sympathetic nerve activity during different phases of respiration in adult Lewis and LPK rats

Representative data traces showing raw and integrated splanchnic SNA (sSNA and ∫sSNA), raw and integrated renal SNA (rSNA and ∫rSNA), raw phrenic nerve activity (PNA) and raw arterial pressure (AP) in an adult Lewis (A) and LPK rat (B) under control conditions. Traces illustrate the higher rSNA, sSNA and AP including pulse pressure range in the LPK in comparison to Lewis rats. Expanded trace shows raw and integrated splanchnic and renal SNA over two respiratory cycles, noting different scale for floating scale bars for Lewis and LPK to allow for clear illustration of respSNA

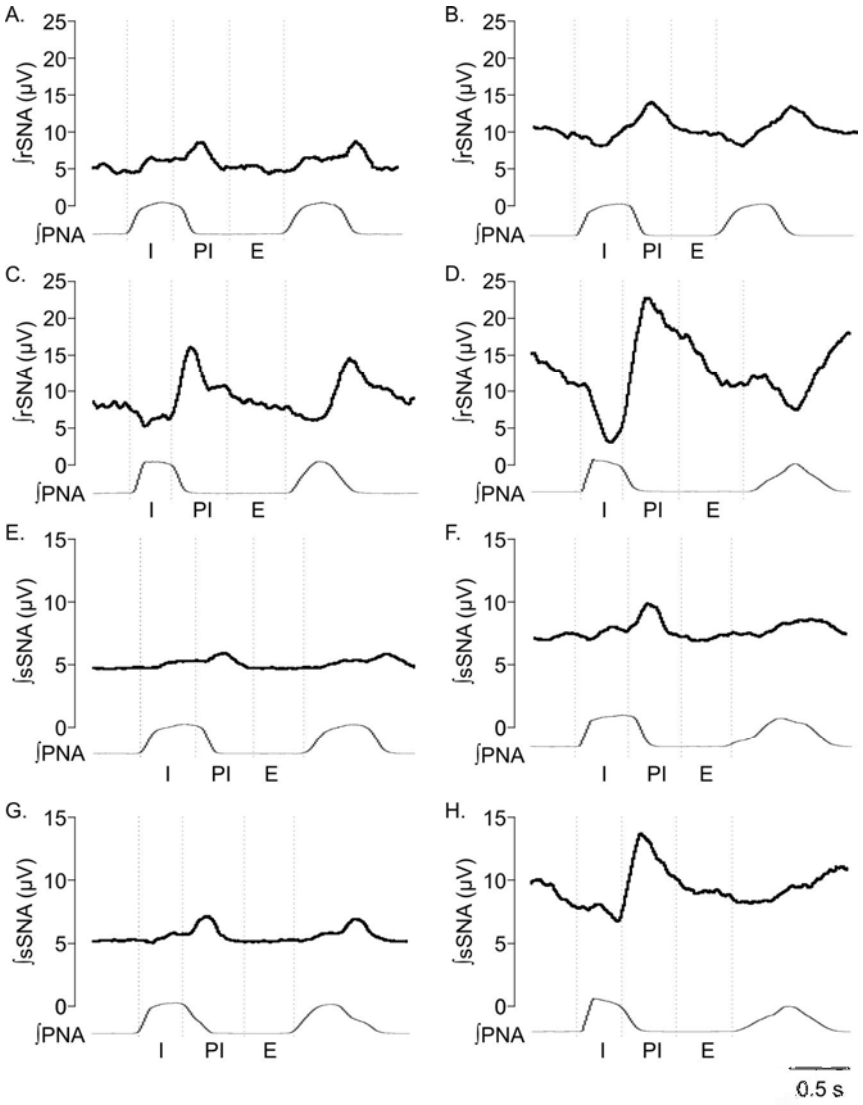

Figure 2.2 Tracings of effect of hypoxia on respiratory-related sympathetic nerve activity in adult Lewis and LPK rats.

Example figure illustrating phrenic triggered integrated rSNA (∫rSNA) and sSNA (∫sSNA) during different phases of the phrenic cycle (I, inspiration; PI, post inspiration; E, expiration within trace of phrenic nerve activity PNA) under control (panels A, B, E, F) and hypoxic conditions (panels C, D, G, H) in a Lewis rat (panels A, C, E, G) and LPK rat (panels B, D, F, H).

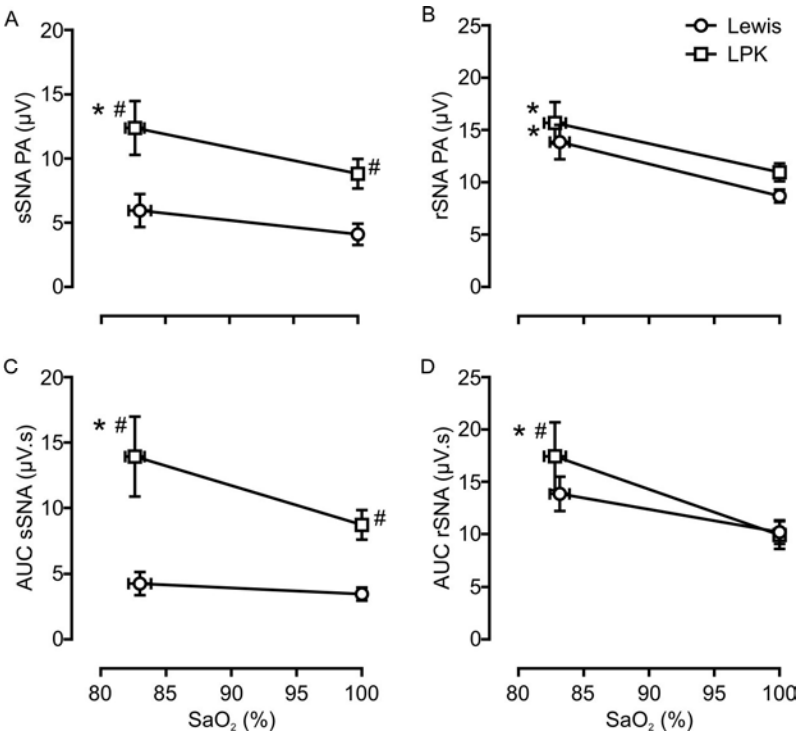

Figure 2.3 Grouped data of effect of hypoxia on respiratory-related sympathetic nerve activity in adult Lewis and LPK rats.

Grouped data is shown in panels A-D illustrating the change in peak amplitude (μV, A/B), and area under the curve ((μV.s, AUC; C/D) of the phrenic triggered ∫sSNA (panels A/C) and ∫rSNA (panels B/D) under control (SaO_2=100%) and hypoxic (SaO_2=82-84%) conditions. Data is expressed as mean ± SEM n ≥5 per group. * represents *P<0.05* versus control within each strain, # represents *P<0.05* versus treatment-matched Lewis.

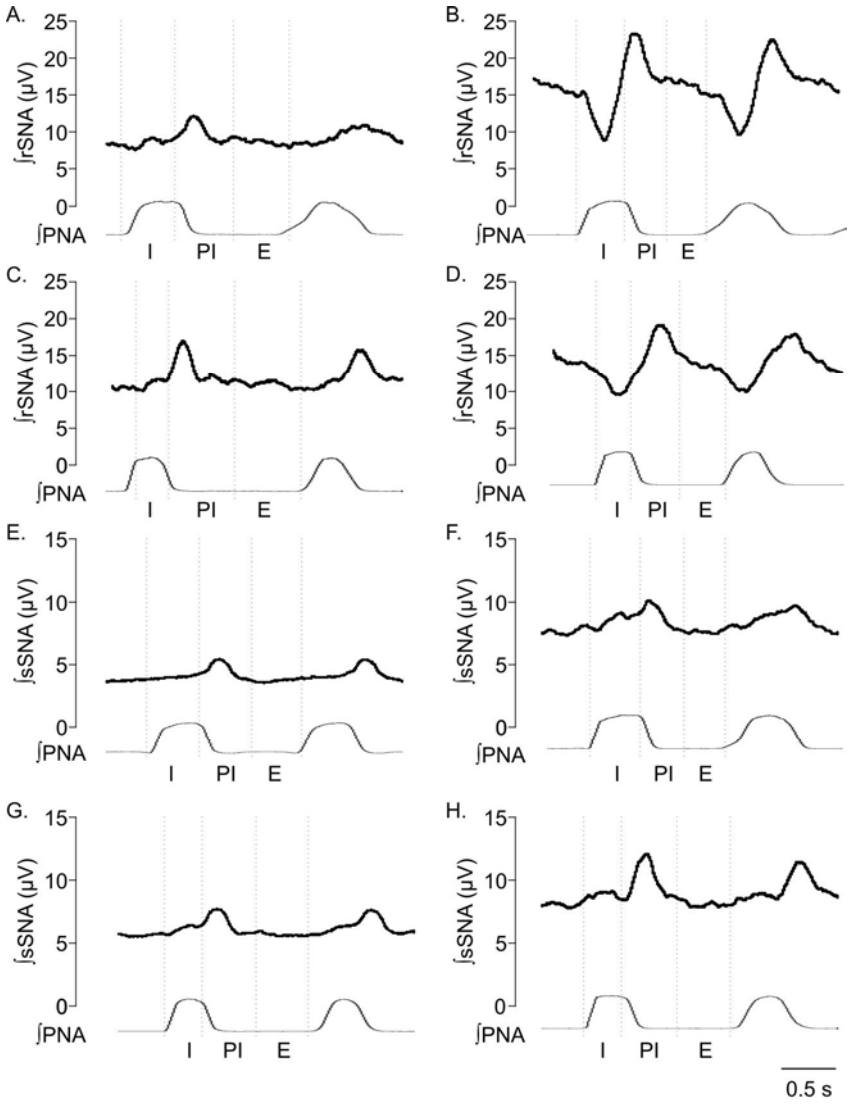

Figure 2.4 Tracings of effect of hypercapnia on respiratory-related sympathetic nerve activity in adult Lewis and LPK rats.

Example figure illustrating phrenic triggered integrated rSNA (∫rSNA) and sSNA (∫sSNA) during different phases of the phrenic cycle (I, inspiration; PI, post inspiration; E, expiration within trace of phrenic nerve activity PNA) under control (panels A, B, E, F) and hypercapnic (panels C, D, G, H) conditions in a Lewis rat (panels A, C, E, G) and LPK rat (panels B, D, F, H).

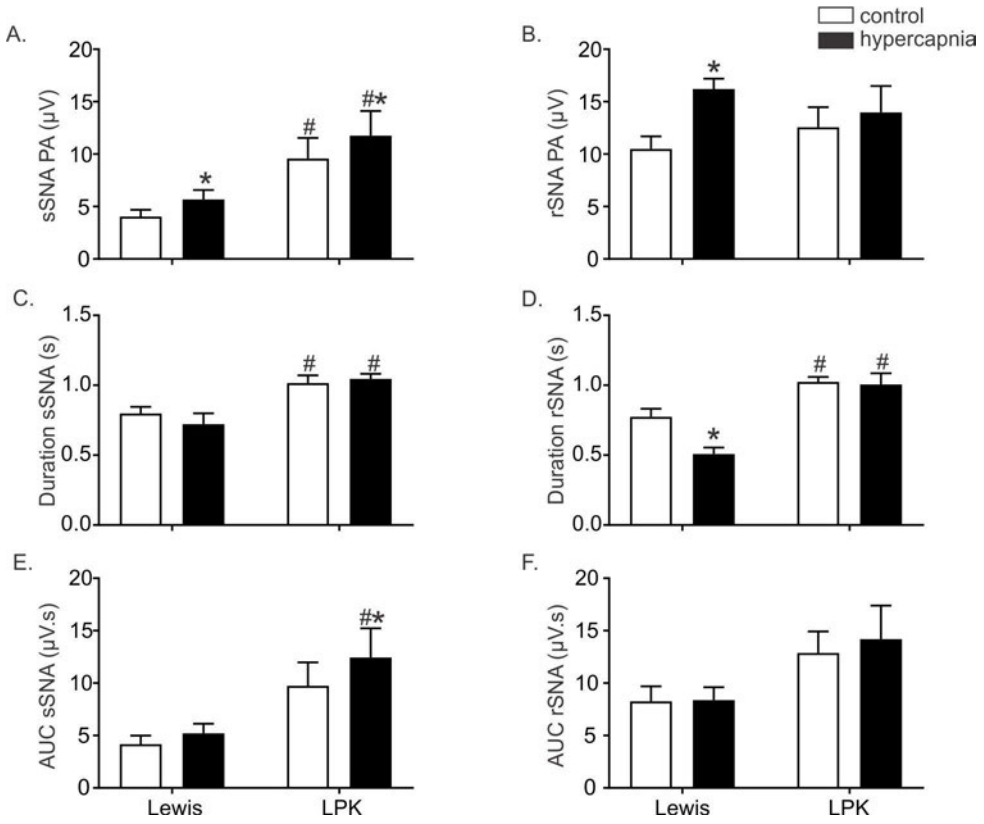

Figure 2.5 Group data of effect of hypercapnia on respiratory-related sympathetic nerve activity in adult Lewis and LPK rats.

Grouped data is shown in panels A-F illustrating the change in peak amplitude (A/B), area under the curve (AUC; C/D) and duration (E/F) of the phrenic triggered ∫sSNA (panels A/C/E) and ∫rSNA (panels B/D/F) under control and hypercapnic conditions. Data is expressed as mean ± SEM n ≥5 per group. * represents P<0.05 versus control within each strain # represents P<0.05 versus treatment-matched Lewis.

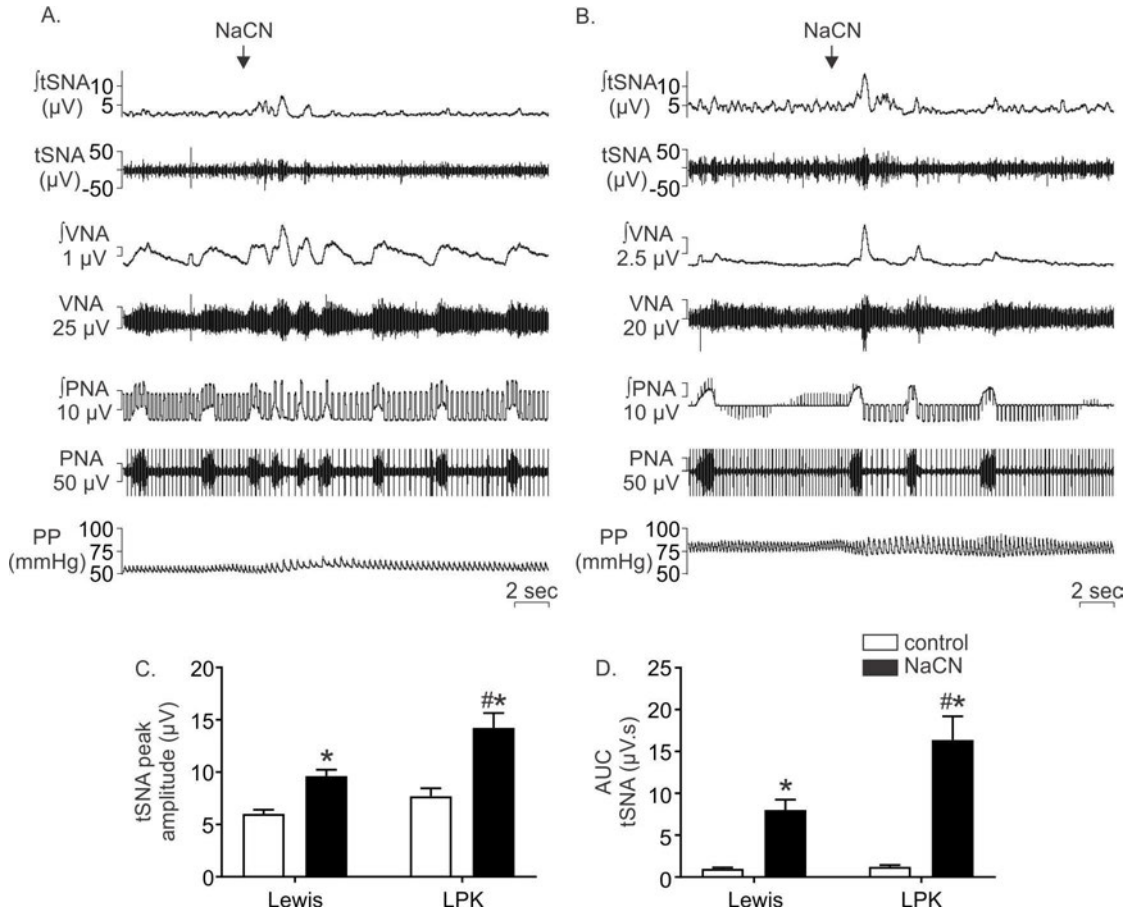

Figure 2.6 Effect of stimulation of the peripheral chemoreceptor reflex with NaCN on respiratory-related sympathetic nerve activity in juvenile Lewis and LPK rats using the working heart brainstem preparation.

Representative figure illustrating integrated tSNA (∫tSNA) over the respiratory cycle in a Lewis rat (panel A) and LPK rat (panel B), noting different floating scale bars for Lewis and LPK (∫VNA) to allow for clear illustration of nerve activity. Grouped data illustrating effect of peripheral chemoreceptor stimulation on peak amplitude (C) and area under the curve (AUC; D) of phrenic triggered ∫tSNA. Data is expressed as mean ± SEM n minimum 5 per group. * represents *P<0.05* versus control within each strain # represents *P<0.05* versus treatment- matched Lewis.

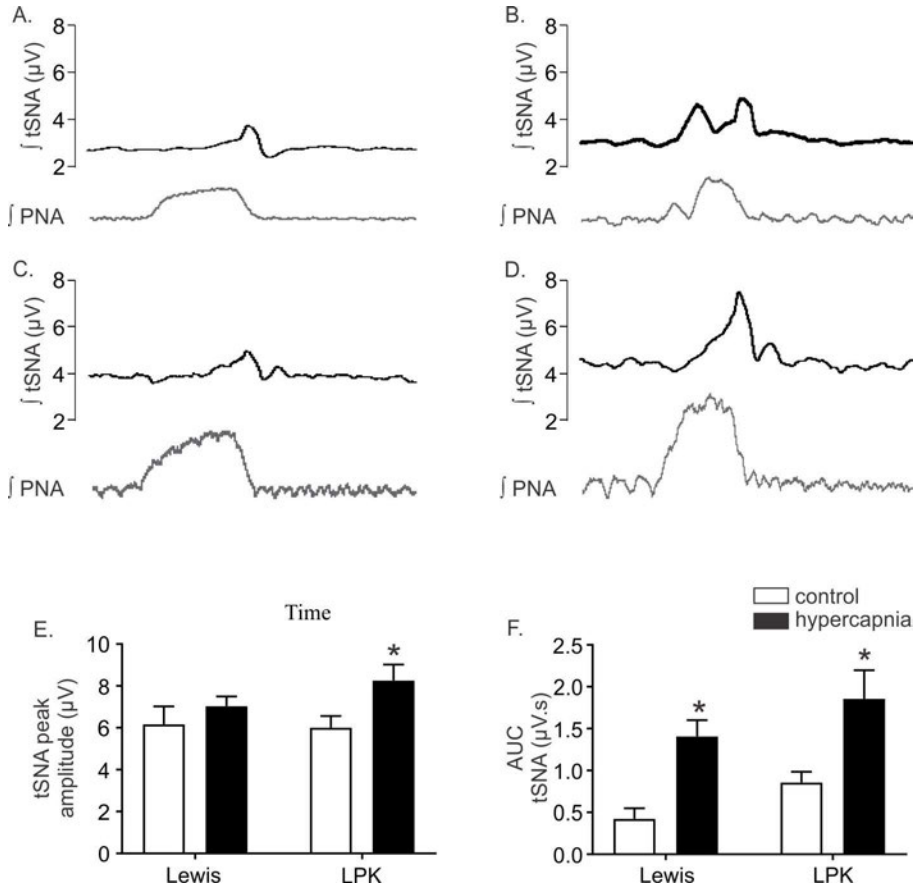

Figure 2.7 Effect of stimulation of the central chemoreceptor reflex with hypercapnia on respiratory-related sympathetic nerve activity in juvenile Lewis and LPK rats using the working heart brainstem preparation.

Representative figure illustrating phrenic triggered integrated tSNA (∫tSNA) over the respiratory cycle in a Lewis rat (panels A and B) and LPK rat (panels C and D) under control (panels A and C) and hypercapnic (panels B and D) conditions. Grouped data illustrating the effect of central chemoreceptor stimulation on peak amplitude (E) and area under the curve (AUC; F) of the phrenic triggered ∫tSNA. Data is expressed as mean ± SEM n ≥5 per group. * represents *P<0.05* versus control within each strain, # represents *P<0.05* versus treatment-matched.

3. Sex does not affect augmented respiratory sympathetic modulation in a rodent model of chronic kidney disease

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This chapter is currently in preparation for submission for publication.

3.1. Abstract

Respiratory modulation of sympathetic nerve activity (respSNA) is amplified in male rodents with chronic kidney disease (CKD) model (Chapter 2), however, it is unknown if it is also altered in female animals. Using the Lewis Polycystic Kidney rat (LPK) model, we examined if there is augmented respSNA in female rodents with CKD. In adult animals under *in vivo* anaesthetised conditions *(n*=8-9/strain), respiratory modulation of splanchnic (sSNA) and renal (rSNA) nerve activity was compared under control conditions, and during peripheral (hypoxia) and central chemoreceptor (hypercapnia) challenge. Tonic SNA and blood pressure were greater in female LPK rats vs. Lewis rats (all $P < 0.05$) and respSNA was at least 2-fold larger (area under curve (AUC), sSNA: 7.8 ± 1.1vs. 3.4 ± 0.7μV.s, rSNA: 11.5 ± 3 vs. 4.8 ± 0.7 μV.s, LPK vs. Lewis, both $P < 0.05$). Activation of peripheral chemoreceptors resulted in a larger change in respSNA in the female LPK (ΔAUC, sSNA: 8.9 ± 3.4 vs 2 ± 0.7 μV.s, rSNA: 6.1 ± 1.2 vs.3.1 ± 0.7 μV.s, LPK vs. Lewis, both $P \leq 0.05$); however, central chemoreceptor stimulation produced comparable changes (ΔAUC, sSNA: 2.5 ± 1 vs 1.3 ± 0.7 μV.s, rSNA: 4.2 ± 0.9 vs. 3.5 ± 1.4 μV.s, LPK vs. Lewis, both $P > 0.05$). These results demonstrate that the respSNA is also augmented in female rats with CKD and that sex does not provide a protective effect in this model.

Key words: Female, respiratory sympathetic modulation, chronic kidney disease, hypertension, chemoreflex, hypoxia, hypercapnia

3.2. Introduction

Chronic kidney disease (CKD), an independent risk factor for cardiovascular morbidity and mortality (Go *et al.*, 2004; Tonelli *et al.*, 2006; Santoro & Mandreoli, 2014) is associated with sympathetic dysfunction. This sympathetic dysfunction is often manifested as sympathetic hyperactivity and contributes to hypertension and underlying mechanisms of development, maintenance and progression of renal diseases (Hering *et al.*, 2007; Schlaich *et al.*, 2009b; Rubinger *et al.*, 2012; Salman *et al.*, 2014). However, we still do not clearly understand the fundamental mechanisms underlying sympathetic dysfunction in CKD.

In the Lewis Polycystic Kidney (LPK) rat, which develops nephronophthisis due to a spontaneous mutation in the Nek8 gene (McCooke *et al.*, 2012a), previously we have demonstrated a phenotypic elevation of sympathetic nerve activity (SNA) in association with impaired renal function, hypertension and impaired baroreflex function (Harrison *et al.*, 2010; Hildreth *et al.*, 2013b; Yao *et al.*, 2015). Notably however, while all afferent, central and efferent components of baroreflex control of SNA are affected in adult male LPK rats, only the central processing of baroreflex control of SNA is impaired in adult female LPK rats (Salman *et al.*, 2014; Salman *et al.*, 2015a).

Sex differences exist in cardiovascular events and progression of renal disease. A study on CKD population have demonstrated that female CKD patients have progressed slowly with higher renal survival compared to male (Eriksen & Ingebretsen, 2006). Moreover, female patients exhibit a lower incidence of renal replacement therapy for end stage renal diseases (Hecking *et al.*, 2014). There are also significant sex differences in cardiovascular morbidity and mortality in CKD patients (Franczyk-Skóra *et al.*, 2012; Nitsch *et al.*, 2013), with a reduced risk of cardiovascular-related mortality for female CKD patients (Nitsch *et al.*, 2013). Physiologically, the sympathetic component of the baroreflex has been found to be comparable (Tank *et al.*, 2005) or increased (Hogarth *et al.*, 2007) in female compared to male. Of note is that there may also be respiratory function differences, with studies revealing that pre-menopausal females have a stronger ventilatory effort at higher altitude/hypoxia compared to males (Joseph *et al.*, 2005; Holley *et al.*, 2012). The gonadal hormones have direct effect on glomerular/tubular structure and renal/systemic circulation which may give females a protection for renal diseases (Ahmed & Ramesh, 2016). Overall, in females compared to males, it would appear that the defending mechanisms against the progression of renal and cardiovascular disease are working better, however, the exact mechanisms which

alter the disease process of CKD and associated cardiovascular disease in the female population as compared to males are not well understood.

Under physiological conditions, the presence of respiratory modulation of SNA (respSNA) functions as a regulator of SNA and blood pressure, and its augmentation has been linked to sympathetic hyperactivity and hypertension in animal models of primary and secondary hypertension (Simms *et al.*, 2009; Toney *et al.*, 2010; Zoccal & Machado, 2010). Recently we showed that adult male LPK rats had greater respiratory-related sympathetic bursts compared to male Lewis rats that were associated with increased SNA and hypertension (Chapter 2). In addition to this finding, we also found that stimulation of peripheral chemoreceptors and the associated increase in magnitude of respSNA was greater in male LPK rats compared to Lewis controls. This is consistent with the work of others showing that respSNA in hypertension is associated with peripheral chemoreceptor over activity (Dick *et al.*, 2004; Zoccal *et al.*, 2008). Of note is that studies on male and female rats show different types of respSNA, such as female rats exhibited inspiratory modulation of SNA and male rats showed expiratory modulation of SNA after exposure to chronic intermittent hypoxia (CIH) (Souza *et al.*, 2016; Souza *et al.*, 2017). In addition, central respiratory neurons with spontaneous rhythm generating capacity in the pre-Bötzinger complex in female mice recovered from acute hypoxia earlier than what was seen in male mice (Garcia *et al.*, 2013). The pattern of respSNA and the response to respiratory challenge have not been previously studied for differences between the sexes in CKD.

Accordingly, in this present study, we examined whether adult female LPK rats exhibit amplified respSNA in association with sympathetic over activity and hypertension as we have previously shown in male LPK. This was undertaken by quantifying respSNA in adult female LPK rats compared to adult female Lewis rats, and determining the relative change in respiratory related sympathetic burst activity in response to hypoxia and hypercapnia.

3.3. Methods

All procedures were approved by the Animal Ethics Committees of Macquarie University, NSW and carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Adult (12-13-week-old) female LPK (total n = 8) and age-matched control Lewis (total n = 9) rats were used. The autonomic reflex alters during the reproductive cycle in female rats, however the stages of oestrus cycle was not observed in this study. Animals were purchased from the Animal Resources Centre, Murdoch, Western Australia.

3.3.1. Renal function

A 24 h urine sample was collected from all animals 48 h prior to experimentation and urine volume, urinary creatinine and protein levels examined using an IDEXX Vetlab analyser (IDEXX Laboratories Pty Ltd., Rydalmere, NSW, and Australia). At the commencement of the surgical procedure, an arterial blood sample was collected for determination of plasma urea and creatinine, and an estimate of creatinine clearance calculated as described previously (Yao *et al.*, 2015).

3.3.2. Surgical procedures

Animals were anaesthetised with 10% (w/v) ethyl carbamate (Urethane, Sigma Aldrich, NSW, and Australia) in 0.9% NaCl solution (1.3 g/kg i.p.). Reflex responses to hind-paw pinch were assessed to determine adequate depth of anaesthesia and supplemental doses given as required (65 mg/kg i.p. or i.v.). Body temperature was measured and maintained at 37 ± 0.5° C using a thermostatically controlled heating blanket (Harvard Apparatus, Holliston, MA, USA) and infrared heating lamp. The right femoral vein and artery were cannulated for administration of fluid (Ringer's lactate, 5 ml/kg/hr) and measurement of arterial pressure (AP) and blood collection for measurement of blood gases to determine any acid base disorders, respectively. The AP signal was sampled at 200 Hz and acquired using a CED 1401 plus and Spike 2 software v.7 (Cambridge Electronic Designs Ltd, Cambridge, UK). A tracheostomy was performed and an endotracheal tube placed in situ. A bilateral vagotomy was performed to prevent afferent inputs from the lung stretch receptors and the animal ventilated with room air enriched O_2 (7025 Rodent Ventilator, UgoBasile, Italy) and paralysed with pancuronium bromide (2 mg/kg iv for induction, 1 mg/kg for maintenance as required; AstraZeneca, North Ryde, NSW, Australia). The left phrenic, splanchnic and renal nerves were dissected to enable recording of phrenic nerve activity (PNA) and splanchnic and

renal SNA (sSNA and rSNA), respectively and the distal end of each nerve was tied and cut. All nerves were bathed in a liquid paraffin pool and the central end recorded using bipolar silver wire recording electrodes, 10 times amplified, band-pass filtered between 10-1000Hz by a bio amplifier (CWE Inc., Ardmore, PA, USA) and sampled at 5kHz using a CED 1401 plus and Spike 2 software v.7 (Cambridge Electronic Designs Ltd, Cambridge, UK). All recordings were made with the same bioamplifier calibrated to a presetting 50 μV.

Following surgical preparation, animals were allowed to then stabilise for 30 minutes. At the beginning of the stabilisation period, an arterial blood sample was collected and analysed for pH, pCO_2, HCO_3^- and SaO_2 using a VetStat Electrolyte and Blood Gas Analyser (IDEXX Laboratories Pty Ltd., Rydalmere, NSW, Australia). After the initial blood gas measurement, arterial blood gases were then corrected as required by adjusting the ventilator pump rate and volume and/or slow bolus injections of 5% sodium bicarbonate to maintain parameters within the following range: pH = 7.4 ± 0.5; PCO_2 = 40 ± 5 mmHg; HCO_3^- = 24 ± 2 mmol/L, SaO2 = 100%; and end tidal (ET)CO_2 = 4.5 ± 0.5%. Repeat blood gas analysis was undertaken as required.

3.3.3. Experimental protocol

After the period of stabilization, the integrity of renal and splanchnic nerve recordings (rSNA and sSNA respectively) was confirmed by pulse modulation of SNA and demonstration of a baroreflex response to a bolus injection of phenylephrine (50 μg/kg iv, Sigma-Aldrich, St. Louis, MO, USA). Baseline parameters were measured under control conditions for a period of 30 min. As we have previously documented in male animals (Chapter 2) initial pilot studies indicated that hypoxic stimulation using 10% O_2 in N_2 (Abbott & Pilowsky, 2009) for a 3 min stimulation period altered pH and PCO_2 in the LPK, and therefore animals were ventilated with room air, without oxygen supplementation, for a period of 3 min without any change in ventilator rate or volume. This induced a stable modest hypoxia with blood gas analysis parameters in the range of pH = 7.4 ± 0.5; PCO_2 = 40 ± 4 mmHg; HCO_3^- = 24 ± 2 mmol/L and SaO_2 = 82-84%. After a 30 min recovery period, central chemoreceptor stimulation was then performed by ventilating the animals with 5% carbogen (5% CO_2 in 95% O_2) for 3 min without any change in the ventilator rate or volume, which was confirmed by blood gas analysis to produce hypercapnia with parameters in the range of pH = 7.4 ± 0.5; PCO_2 = 65 ± 8 mmHg; HCO_3^- = 24 ± 2 mmol/L and SaO_2 =100%.

At the end of experiment, all animals were euthanized with potassium chloride (3M i.v.) and the electrical noise levels for PNA, sSNA and rSNA recorded and later subtracted for data analysis.

3.3.4. Data analysis

All data were analysed offline using Spike 2 software. From the AP signal, mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP) and heart rate (HR) were derived. The sSNA and rSNA were rectified and smoothed with a time constant of 0.1 s and PNA was rectified and smoothed with a time constant of 0.05 s. Baseline data was determined from a 30 s period at the end of the control ventilation period. Response to respiratory challenge was analysed over a 30 s period once PNA had stabilised for a period of 1-2 min and expressed as a change (Δ) relative to the 30 s period immediately prior to each stimulus. The PNA amplitude, frequency (number cycle.min $^{-1}$), duration (s) and minute activity (MPA=PNA amplitude x PNA frequency) were measured to quantify the changes. The phrenic cycle was divided into three phases: inspiratory (I), post-inspiratory (PI) and expiratory (E). For SNA, the mean level was measured from the period 200 ms prior to the onset of the phrenic burst, and this was considered the baseline for all other measurements. A phrenic-triggered average of the rectified and smoothed rSNA and sSNA recordings was performed and the phases divided into I, PI, and E (Xing & Pilowsky, 2010). From this trace the following parameters were calculated for sSNA and rSNA: the peak amplitude (PA [μV]), the maximum amplitude at the apex of the peak of the SNA burst coincident with inspiratory/post-inspiratory phase, the duration (from onset of excitatory activity to return to baseline [s]) and area under curve (AUC) of respSNA excitatory peak (μV.s) determined as the integral of the waveform. AUC of respSNA for I, PI and E phase of phrenic cycle were also calculated from the integral of the waveform.

3.3.5. Statistical analysis

All data are presented as mean ± SEM. An unpaired two-tailed Student's t-test was used to examine strain differences in renal function, cardiorespiratory function and SNA. A two-way ANOVA with repeated measures and Bonferroni's corrections, with strain and ventilatory condition (i.e. normoxia, hypoxia or hypercapnia) as variables, was used to examine strain differences in respiratory modulation of SNA under control conditions and in response to hypoxia or hypercapnia. An unpaired two-tailed Student's t-test was also used to determine

strain differences in delta changes from control to hypoxia or hypercapnnea in cardiorespiratory function. The magnitude of delta changes was compared with the male data presented in Chapter 2 using a two-way ordinary ANOVA and Bonferroni's corrections, with strain and sex as variables, to examine sex differences. Statistical analysis was performed using GraphPad Prism software v6.0 (GraphPad Software Inc., La Jolla, California, USA, RRID:SCR_002798. Differences were considered statistically significant where $P \leq 0.05$.

3.4. Results

3.4.1. Baseline parameters

Elevated blood urea (LPK 23.7 ± 2.7 vs. Lewis 6.7 ± 0.5 mmol/L) and plasma creatinine (44.7 ± 7.9 vs. 13.1 ± 2.6 μmol/L), and reduced creatinine clearance (1.5 ± 0.3 vs. 7.4 ± 1.2 ml/min) (LPK *n*=8 vs. Lewis *n*=9; *P<0.001*) confirmed impaired renal function in the female LPK animals.

Figure 1 illustrates individual recordings of SNA, PNA and AP from a Lewis and LPK rat under control conditions. SBP, MAP, DBP, PP, HR and SNA (both rSNA and sSNA) were elevated in the adult female LPK compared with age-matched Lewis controls (Table 1). Phrenic frequency was significantly higher in the adult female LPK suggestive of increased central respiratory drive, however, there was no significant difference in PNA amplitude (*P = 0.23*) and while minute phrenic nerve activity trended towards being different between strains this did not reach statistical significance (*P = 0.08*). The duration of the phrenic burst, however, was significantly reduced in adult female LPK (Table 1).

To identify if respSNA was altered in female LPK, we first examined the temporal relationship between SNA and the respiratory cycle. Both sympathetic nerves exhibited respiratory sympathetic harmony, with a clear burst in SNA in the post-inspiratory period observed in both the Lewis and LPK (Figure 1, Figure 2 and 4 panels A-B, E-F). In the Lewis rat, both respSNAs (rSNA and sSNA) showed a tendency to increase from baseline during I, before the sharp peak occurred in PI and after the PI peak, activity fell quickly to baseline and was relatively flat during E. In contrast, respSNA for the renal nerve in the LPK displayed inhibitory activity during early I that was most obvious in rSNA (rSNA, I AUC, LPK vs Lewis: -0.3 ± 0.1 vs 0.2 ± 0.1 μV; *P < 0.05*; sSNA, I AUC, LPK vs Lewis: 0.02 ± 0.05 vs 0.2 ± 0.07 μV; *P > 0.05*) with a persistence of excitation in both rSNA and sSNA during the E period that was not different between strains (rSNA, E AUC, LPK vs Lewis: 0.1 ± 0.04 vs -0.03 ± 0.08 μV; *P > 0.05*; sSNA, E AUC, LPK vs Lewis: 0.09 ± 0.06 vs 0.03 ± 0.07 μV; *P > 0.05*).

Under control conditions, baseline respSNA in both nerves was significantly greater in the female LPK as reflected by a larger AUC (Table 2). This amplified respSNA was associated with both a higher magnitude of PA and longer duration of respSNA in LPK compared to Lewis control rats (Table 2).

3.4.2. Responses to chemoreceptor challenge

3.4.2.1. Peripheral chemoreceptor stimulation

In response to hypoxic challenge, a greater hypertensive response was observed in the female LPK as reflected by a greater change in MAP, SBP, DBP and PP compared with Lewis, without any significant change in HR between the strains (Table 3). This was associated with a slowing of phrenic nerve discharge with reduced duration in the LPK, whereas the Lewis rats showed a reduction of phrenic duration only. The magnitude of decreased phrenic duration was greater in Lewis rats. However, there was no overall difference in the increase in MPA seen between the strains when under hypoxic conditions.

When comparing respSNA parameters after exposure to hypoxic conditions, there was an increase in PA and AUC of respSNA of both rSNA and sSNA in LPK rats, while in the Lewis there was an increase in PA and AUC of respSNA of rSNA (Figures 2 and 3). The duration of both curves in the LPK and Lewis was unaffected by hypoxia (data not provided). The increase in AUC in both rSNA and sSNA was less in the Lewis than that seen in the LPK (delta AUC, rSNA: LPK vs. Lewis: 6.07 ± 1.1 vs. 3.1 ± 0. 7μV.s, sSNA: LPK vs. Lewis: 8.9 ± 3.4 vs. 2 ± 0.7, Both $P \leq 0.05$). Of note was that under hypoxic conditions, in LPK rats, inspiratory inhibition increased significantly in rSNA (I AUC, rSNA, control vs. hypoxia: -0.25 ± 0.1 vs. -1.08 ± 0.2 μV, $P \leq 0.05$; Figure 2) and became more prominent in sSNA though the change did not reach statistical significance (I AUC, sSNA, control vs. hypoxia: 0.02 ± 0.05 vs. -0.5 ± 0.1 μV, $P = 0.08$) in LPK rats. The AUC during E in the LPK was greater during hypoxia (E AUC, sSNA, control vs. hypoxia: 0.09 ± 0.06 vs. 0.75 ± 0.3 μV, $P < 0.05$) but there was no change in E AUC in the rSNA ($P>0.05$). There was no change in I AUC or E AUC for either the renal or splanchnic nerves in the Lewis female rats ($P > 0.05$).

3.4.2.2. Central chemoreceptor stimulation

Hypercapnic stimulation produced comparable increases in blood pressure and central respiratory drive between the strains, except phrenic duration which increased in the Lewis while decreasing in the LPK (Table 3). Both LPK rats and Lewis rats exhibited increased respSNA of rSNA as evidenced by an increase in AUC in both strains and increase in PA in Lewis (Fig 4 & 5). In response to hypercapnia, the respSNA of sSNA also increased in both strains as reflected by an increase in PA in both strain and an increase in AUC in LPK rats. Hypercapnia did not alter the duration of curves in either strain (data not provided). However, the magnitude of observed changes in respSNA was comparable between the strains (Δ AUC,

rSNA: LPK vs. Lewis: 4.2 ± 0.9 vs. 3.5 ± 1.4 sSNA: LPK vs. Lewis: 2.5 ± 1 vs. 1.3 ± 0. 7μV.s, Both $P > 0.05$).

Inhibition during the inspiratory period in the LPK did not significantly change in either rSNA or sSNA (I AUC, rSNA, control vs. hypercapnia: -0.21 ± 0.1 vs. -0.34 ± 0.1 sSNA: 0.04 ± 0.07 vs. 0.08 ± 0.09 μV.s, both $P > 0.05$) and AUC in the E phase of respSNA for both nerves was comparable in response to hypercapnia in LPK rats (E AUC, rSNA, control vs. hypercapnia: 0.06 ± 0.03 vs. 0.04 ± 0.05 sSNA: 0.04 ± 0.03 vs. 0.04 ± 0.04 μV.s, both $P > 0.05$). In the Lewis, both nerves also showed no significant differences in inspiratory and expiratory period during hypercapnia (both $P > 0.05$).

The female rats exhibited a comparable cardiorespiratory response with similar magnitude compared to the male rats in response to both hypoxia and hypercapnia except for HR, which responded to a higher magnitude in male Lewis rats (Appendix, Tables 1 and 2). Similarly, there was no sex difference in the magnitude of delta change in respSNA during hypoxia and hypercapnia (Appendix, Tables 3 and 4).

3.5. Discussion

Our study has determined that in a rodent model of CKD, female animals demonstrate increased sympathetic tone and amplified respSNA when compared to female Lewis control rats. Moreover, the female LPK rat exhibits similar features like male LPK rat within their respSNA patterning including an increased inhibition of rSNA during the inspiratory period. We have further demonstrated that peripheral chemoreceptor stimulation with hypoxia increases respSNA and blood pressure more in the adult female CKD model compared to normotensive controls, while this augmented response was not seen in the response to central chemoreceptor stimulation with hypercapnia. Moreover, the magnitude of alteration of respSNA of female rats during hypoxia and hypercapnia was similar to the male rats. As a result, these results overall indicate the significance of peripheral chemoreceptors and respiratory modulation of sympathetic activity in increased sympathetic activity and hypertension in CKD.

It has been suggested in a number of different studies that sympathetic hyperactivity contributes to hypertension in both hypertensive female rat models and hypertensive women (Hart & Charkoudian, 2014; Maranon *et al.*, 2014). Our demonstration of increased SNA in adult female LPK rats corresponds with the marked elevation in blood pressure we observe in these female LPK rats from an early age (Kandukuri *et al.*, 2012; Salman *et al.*, 2015a). This

is consistent with sympathetic overactivity being an important pathological feature in female animals with PKD that is evident before renal function becomes significantly compromised (Salman *et al.*, 2015a). However, current knowledge on the underlying pathogenesis of sympathetic overactivity in female patients with kidney disease is limited.

Our present study demonstrates amplified respSNA in adult female LPK rats in association with hypertension and echoes our data from male juvenile and adult LPK, studied *in situ* and *in vivo* respectively (Chapter 2). This is consistent with the changes seen after exposure to CIH in female rats, which develop hypertension and exhibit an augmented respSNA (Souza *et al.*, 2016). The SHR model of essential hypertension also demonstrates amplified respSNA *in situ* (Simms *et al.*, 2009), as do rabbit models of congestive heart failure, that show an increased coherence between respiration and rSNA *in vivo* (Marcus *et al.*, 2014a). As we demonstrated for male animals, in the female LPK, the peak of respSNA from the onset of phrenic burst was observed persistently in the PI period under all conditions tested. This is distinct from the findings in male SHR rats where the peak of respSNA was in the inspiratory phase (Czyzyk-Krzeska & Trzebski, 1990; Simms et al., 2009). Notably, the pattern of respSNA in female LPK exhibited inspiratory inhibition during control condition, most apparent in the renal nerve, and this was exaggerated under hypoxic conditions, as we describe also for male animals (Chapter 2). The pattern of respSNA in female LPK rats is therefore not specifically distinguishable to the pattern of respSNA of male LPK rats.

Exploring respiratory pattern in context of augmented respSNA further, we did find in the anaesthetised animal that respiratory rate was higher in female LPK rats compared to Lewis controls, as also demonstrated in male LPK rats under control condition (Chapter 2). However, the duration of the PNA was significantly reduced in female LPK rats. This is in contrast to what we saw in male LPK rats, where the duration of the PNA was the same between LPK and Lewis rats. Similarly, in response to CIH, juvenile female rats exhibited a reduction in inspiratory period compared with control rats (Souza *et al.*, 2016) whereas male CIH rats showed similar phrenic frequency and duration (Zoccal *et al.*, 2008). These findings suggest that sex variation exists in different phases of respiratory cycle in disease models including CKD. Moreover, different studies suggest that variation in the respiratory phases between male and female LPK rats depends on multiple interrelated inputs at the level of rhythm generating inspiratory neurons at the brainstem (Garcia *et al.*, 2013; Garcia *et al.*, 2016). Therefore, it is conceivable that in female LPK rats, the input to respiratory neurons

causing variation in the respiratory phases of respiratory cycle may not be similar to the input of male LPK rats and may be involved in augmented respSNA by different mechanisms.

It has been suggested that increased sensitivity of the peripheral chemoreceptors contributes to increased sympathetic activity in hypertension and CKD (Hering *et al.*, 2007; Paton *et al.*, 2013b). Moreover, peripheral chemoreceptors regulate the activity of the retrotrapezoid nucleus (RTN) to modulate the central components of respSNA (Guyenet *et al.*, 2009b; Moraes *et al.*, 2015). In the present study, stimulation of peripheral chemoreceptors induced an increase in respSNA and blood pressure in both female strains, the magnitude of which was significantly greater in female LPK rats compared to Lewis rats, whereas hypercapnia produced similar changes between the strains. This is consistent with what we describe in male LPK (Chapter 2). Collectively, it would suggest that in addition of independent central mechanism of amplified respSNA, the contribution from peripheral chemoreceptors to the central components of respSNA in the brain stem through the RTN plays critical role in increased SNA and hypertension in female patients with CKD.

There are several classes of presympathetic motor neurons on the basis of their respiratory modulation including: I activated, PI activated, I inhibited and non-modulated neurons (Moraes *et al.*, 2013b). Interactions between presympathetic motor neurons and respiratory neurons in the RVLM are essential to contribute to the phasic respiratory modulation of sympathetic outflow (Haselton & Guyenet, 1989; Guyenet, 2006), with its important function in the regulation of blood pressure. From studies using male rats, it has been suggested that the hypoxic stimulus with subsequent peripheral chemoreceptor activation drives respiratory neurons and premotor sympathetic neurons to contribute to amplified respSNA during expiration, leading to increased sympathetic drive and hypertension (Zoccal *et al.*, 2008; Zoccal & Machado, 2010; Wong-Riley *et al.*, 2013) . In contrast, in studies using female rats, peripheral chemoreceptor stimulation has been shown to drive inspiratory and premotor sympathetic neurons to contribute to amplified respSNA during inspiration, leading to increased sympathetic drive and hypertension (Souza *et al.*, 2016; Souza *et al.*, 2017). In our present study in female LPK rats, the respSNA in the sSNA under hypoxic conditions showed persistence of excitation in expiration though rSNA did not show this expiratory change. This finding is in contrast to the finding of male LPK rats which did not show any excitation of respSNA during expiration. Intriguingly, our present study has also shown that in response to peripheral chemoreceptor stimulation, phrenic frequency was reduced in female LPK rats compared to control rats. This was also reduced in male LPK rats compared to male Lewis

rats. These findings are in contrast to the findings of other studies of adult female animals which demonstrate that respiratory frequency was not increased during acute hypoxia (Garcia *et al.*, 2013) and after CIH (Souza *et al.*, 2015). Given that Garcia's study was performed in neonate animals and Souza´s in pre-pubertal animals, the age and stage of development, could be one of the reasons for the contrasting result. Different forms of respiratory input to various subclasses of presympathetic motor neurons may therefore trigger this variation in the pattern of respSNA observed during hypoxia in the female LPK rats. However, we did not demonstrate any sex differences in inspiratory or expiratory related SNA under control conditions and future work is required to explore this possibility.

3.6. Conclusion

CKD is a complex progressive disease with sympathetic over-activity and secondary hypertension. Controlling hypertension through reduction of sympathetic activity should be an important focus in the management of CKD. Our present study demonstrates that amplified respSNA is associated with sympathetic overactivity and hypertension in female LPK rats, and that peripheral chemoreceptors stimulation provokes a significant increase in respSNA compared to normotensive control animals, and does not differentiate from our findings in male animals. While we did not delineate the pathways and neural mechanism underlying these changes, this novel finding of amplified respSNA indicates an inherent character of CKD that might be driving autonomic dysfunction regardless of sex.

3.7. Acknowledgements

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3.8. Disclaimer

All authors declared no competing interests.

Table 3.1 Baseline cardiorespiratory function in female Lewis and LPK rats

	Lewis	LPK
MAP (mmHg)	93 ± 4	126 ± 10*
SBP (mmHg)	125 ± 8	184 ± 18*
DBP (mmHg)	77 ± 4	99 ± 9*
PP (mmHg)	47 ± 8	84 ± 15*
HR (bpm)	427 ± 7	456 ± 37*
PNA amplitude (μV)	13.1 ± 3.5	20.9 ± 5.4
PNA frequency (cycles/min)	32 ± 1	42 ± 1*
PNA duration (s)	1.01 ± 0.05	0.69 ± 0.02*
MPA	427.2 ± 123.26	823 ± 185.4
rSNA (μV)	4.4 ± 0.8	7.5 ± 1.05*
sSNA (μV)	3.02 ± 0.5	6.4 ± 1.1*

Measures of cardiorespiratory parameters in Lewis and LPK rats. MAP: mean arterial pressure, SBP: systolic blood pressure, DBP: diastolic blood pressure, PP: pulse pressure, HR: heart rate, bpm: beats per minute, PNA: phrenic nerve amplitude, MPA: minute phrenic activity, rSNA: renal sympathetic nerve activity, sSNA: Splanchnic sympathetic nerve activity. Results are expressed as mean ± SEM. *$P<0.05$ between the Lewis and LPK as determined by Student's t-test. n = 9 Lewis and n = 8 LPK except rSNA where n = 6 Lewis and n = 5 LPK

Table 3.2 RespSNA parameters in splanchnic and renal nerves female under control conditions.

		Lewis	LPK
PA (μV)	Splanchnic	4.0 ± 0.7	7.4 ± 1.3*
	Renal	5.9 ± 0.9	9.8 ± 1.5*
AUC	Splanchnic	3.4 ± 0.7	7.8 ± 1.1*
	Renal	4.8 ± 0.7	11.5 ± 3.0*
Duration (sec)	Splanchnic	0.8 ± 0.08	1.1 ± 0.06*
	Renal	0.8 ± 0.06	1.1 ± 0.12*

Measures of respSNA (phrenic triggered) parameters for ∫sSNA and ∫ rSNA in Lewis and LPK rats. PA: peak amplitude, AUC: area under curve (sec x μV), duration (of peak) and position (time from the onset of inspiration to PA). Results are expressed as mean ± SEM. *$P \leq 0.05$ between the Lewis and LPK as determined by Student's t-test. n = 9 Lewis and n = 8 LPK for sSNA and n = 6 Lewis and n = 5 LPK for rSNA.

Table 3.3 Effects of peripheral and central chemoreceptor stimulation on cardiorespiratory pattern in adult female Lewis and Lewis Polycystic Kidney (LPK) rats

		Lewis *(n = 9)*	LPK *(n = 8)*
Hypoxia	Δ MAP (mmHg)	12 ± 6	30 ± 6 *
	Δ SBP (mmHg)	15 ± 6	41 ± 8 *
	Δ DBP (mmHg)	9 ± 5	27 ± 6*
	Δ PP (mmHg)	5 ± 1	15 ± 5 *
	Δ HR (bpm)	25 ± 6	23 ± 3
	Δ PNA amplitude (μV)	7.3 ± 1.7	11.01 ± 1.9
	Δ PNA duration (sec)	-0.2 ± 0.03	-0.07 ± 0.03*
	Δ PNA frequency (cycles/min)	5 ± 2	-10 ± 3*
	Δ MPA	161.2 ± 116.8	290.3 ± 88.6
Hypercapnia	Δ MAP (mmHg)	9 ± 5	13 ± 3
	Δ SBP (mmHg)	12 ± 6	20 ± 5
	Δ DBP (mmHg)	8 ± 4	10 ± 2
	Δ PP (mmHg)	3 ± 2	10 ± 3
	Δ HR (bpm)	-0.3 ± 1	2 ± 3 *
	Δ PNA amplitude (μV)	9.4 ± 2.8	9 ± 2.3
	Δ PNA duration (sec)	0.8 ± 0.03	-0.06 ± 0.02*
	Δ PNA frequency (cycles/min)	-0.2 ± 1	-2 ± 1
	Δ MPA	309.1 ± 113.5	274 ± 57.1

Delta change (Δ) in phrenic triggered integrated phrenic nerve activity (PNA) and blood pressure (mmHg) in hypoxia (ventilated with only room air) or hypercapnia (ventilated with 5% CO_2 with 95% O_2) when switched from control condition (ventilated with room air enriched with 100% O2) in adult Lewis and Lewis Polycystic Kidney (LPK) rats under urethane anaesthesia. MPA: minute phrenic activity, MAP: mean arterial pressure, SBP: systolic blood pressure, DBP: diastolic blood pressure, PP: pulse pressure: Results are expressed as mean ± SEM. *$P \leq 0.05$ between the Lewis and LPK as determined by Student's t-test. n = number of animals per group

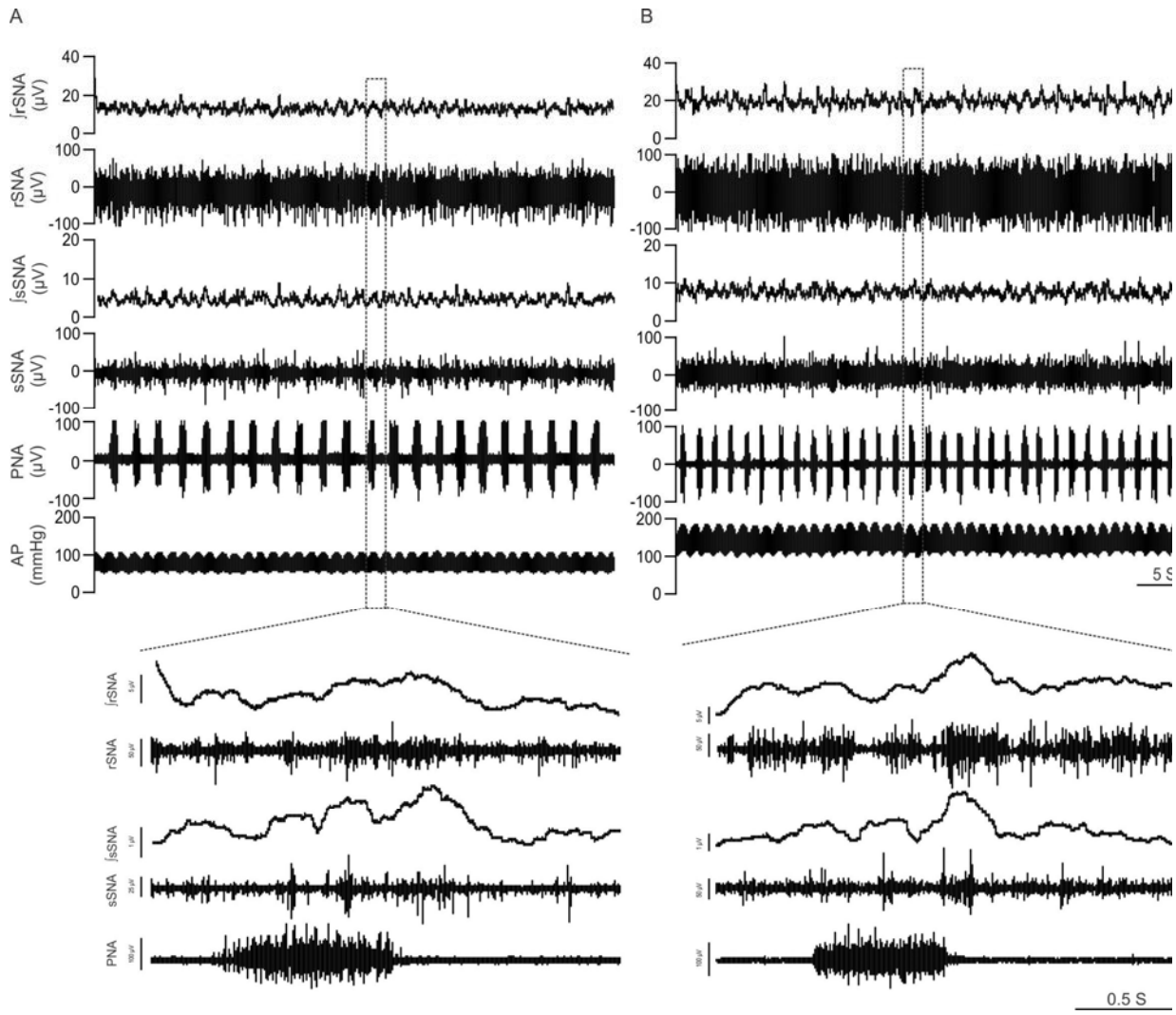

Figure 3.1 Representative traces showing higher SNA and BP in female LPK rats

Representative data traces showing raw and integrated renal SNA (rSNA and ∫rSNA), splanchnic sympathetic nerve activity (sSNA and ∫sSNA), integrated raw phrenic nerve activity (PNA) and raw arterial pressure (AP) in a 12-week-old Lewis rat (A) and 12-week-old LPK rat (B) under urethane anaesthesia. Traces illustrate the higher rSNA, sSNA and AP including pulse pressure range in the LPK in comparison to Lewis rats. Expanded traces of one respiratory cycle show raw and integrated splanchnic and renal SNA over one respiratory cycle, noting different scale for floating scale bars for Lewis and LPK.

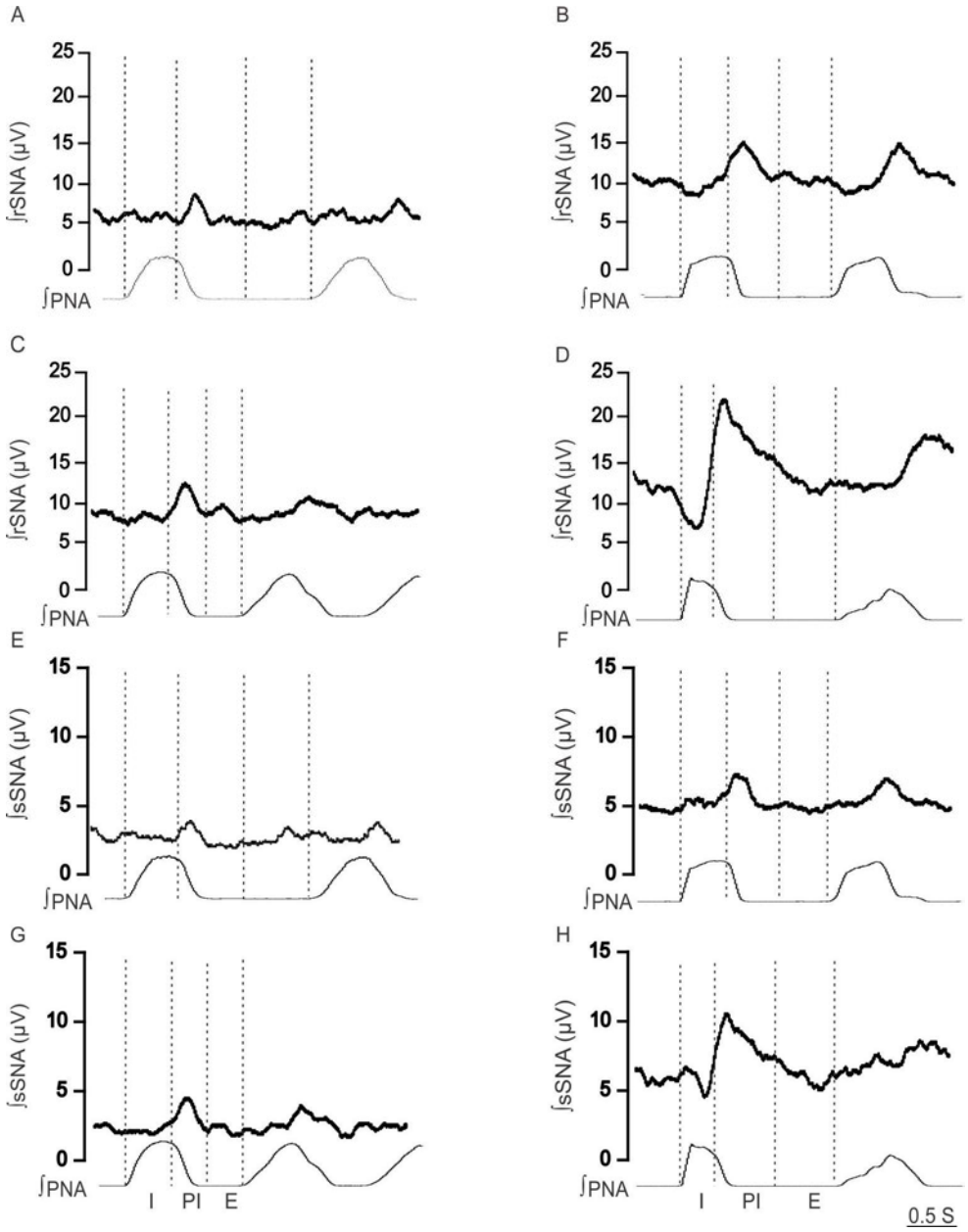

Figure 3.2 Tracings of effect of hypoxia on respiratory-related sympathetic nerve activity in adult female Lewis and LPK rats.

Example figure illustrating phrenic triggered integrated rSNA (∫rSNA) and sSNA (∫sSNA) during different phases of the phrenic cycle (I, inspiration; PI, post inspiration; E, expiration within trace of phrenic nerve activity PNA) under control (panels A, B, E, F) and hypoxic conditions (panels C, D, G, H) in a Lewis rat (panels A, C, E, G) and LPK rat (panels B, D, F, H).

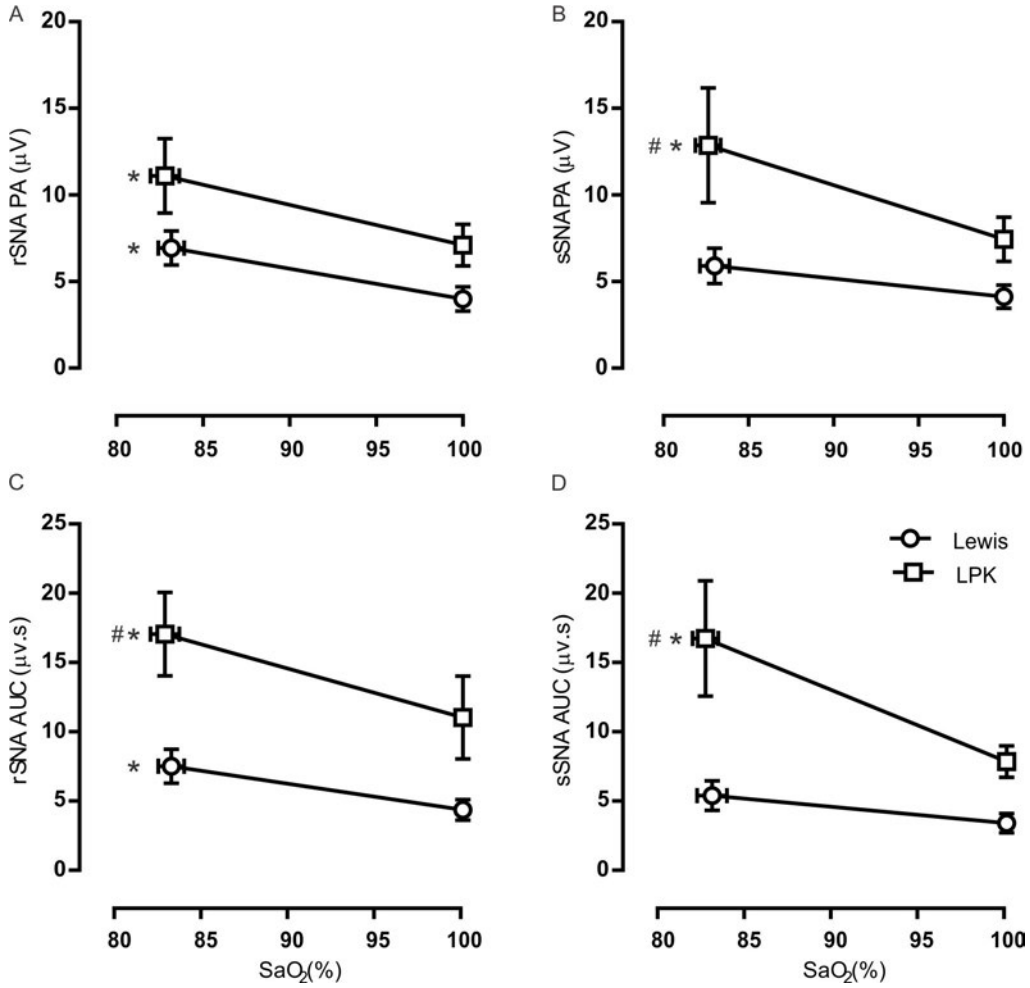

Figure 3.3 Grouped data of effect of hypoxia on respiratory-related sympathetic nerve activity in adult female Lewis and LPK rats.

Grouped data is shown in panels A-D illustrating the change in peak amplitude (A/B), and area under the curve (AUC; C/D) of the phrenic triggered ∫rSNA (panels A/C) and ∫sSNA (panels B/D) during normoxia (control) and hypoxic conditions. Data is expressed as mean ± SEM n ≥5 per group. * represents *P<0.05* versus control within each strain, # represents *P<0.05* versus treatment-matched Lewis

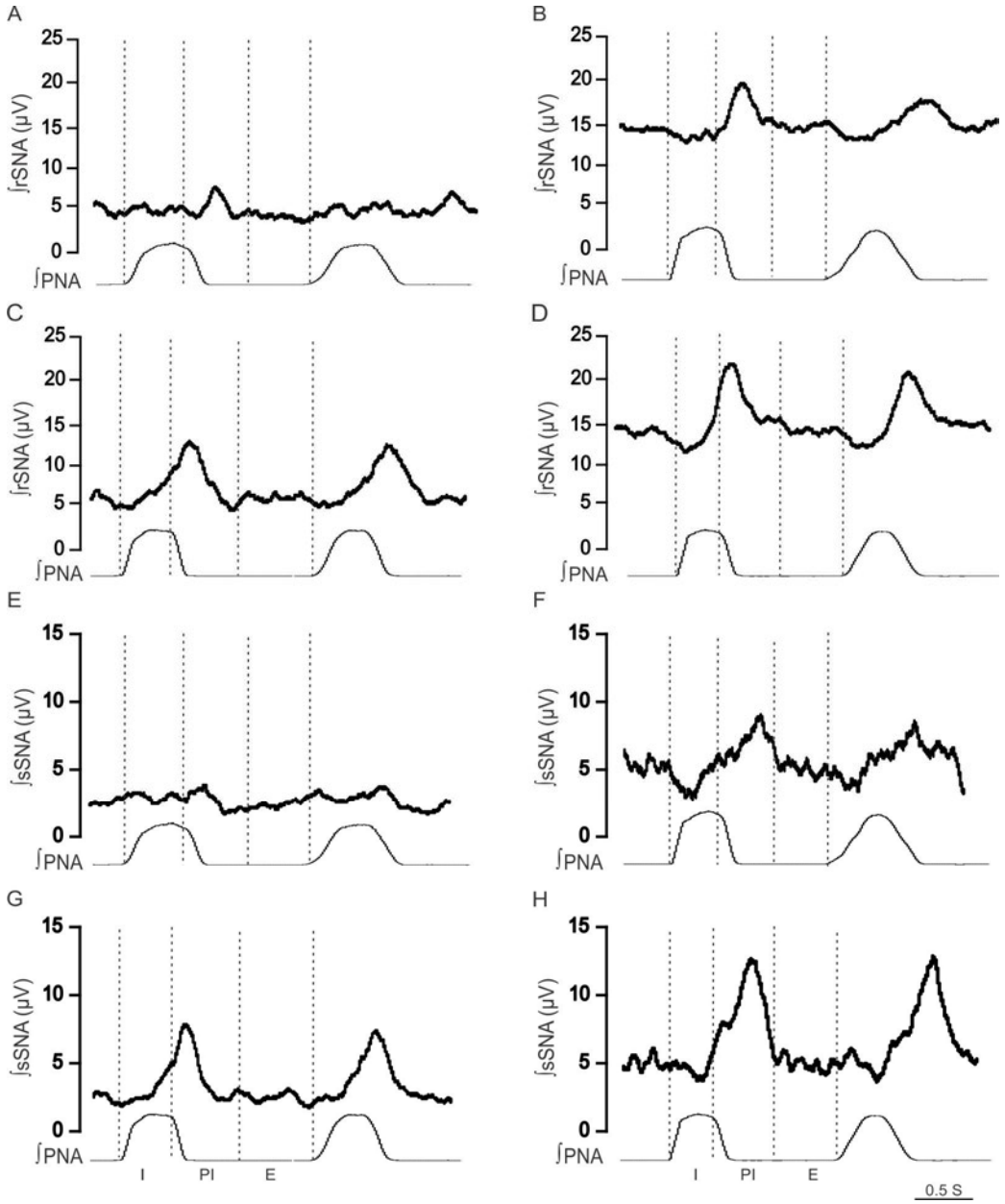

Figure 3.4 Tracings of effect of hypercapnia on respiratory-related sympathetic nerve activity in adult female Lewis and LPK rats.

Example figure illustrating phrenic triggered integrated rSNA (∫rSNA) and sSNA (∫sSNA) during different phases of the phrenic cycle (I, inspiration; PI, post inspiration; E, expiration within trace of phrenic nerve activity PNA) under control (panels A, B, E, F) and hypercapnic (panels C, D, G, H) conditions in a Lewis rat (panels A, C, E, G) and LPK rat (panels B, D, F, H).

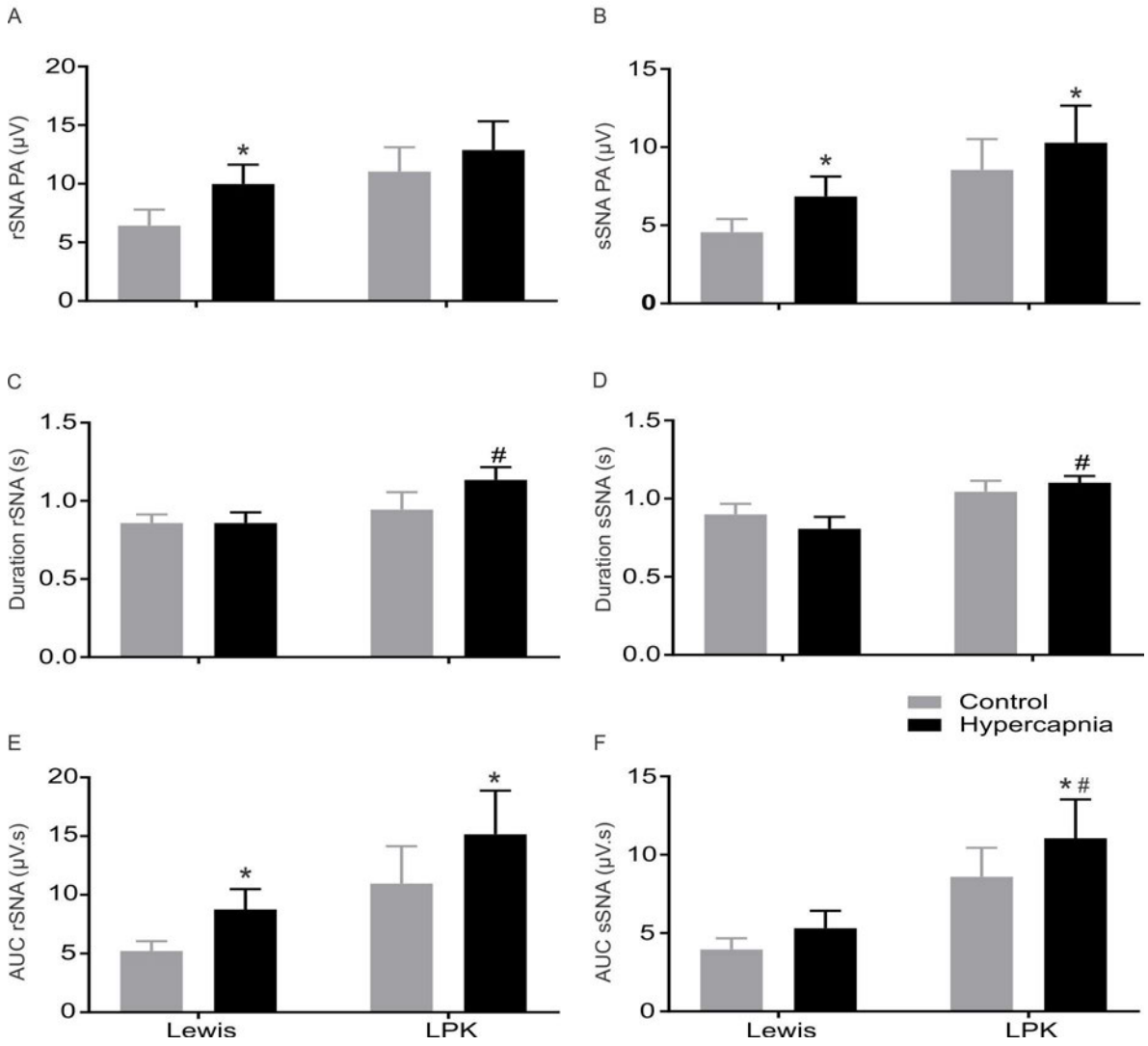

Figure 3.5 Group data of effect of hypercapnia on respiratory-related sympathetic nerve activity in adult female Lewis and LPK rats.

Grouped data is shown in panels A-F illustrating the change in peak amplitude (A/B), duration (C/D) and area under the curve (AUC; E/F) of the phrenic triggered ∫rSNA (panels A/C/E) and ∫sSNA (panels B/D/F) during normoxia (control) and hypercapnic conditions. Data is expressed as mean ± SEM n ≥5 per group. * represents *P<0.05* versus control within each strain # represents *P<0.05* versus treatment-matched Lewis.

4. Reducing respiratory sympathetic coupling selectively improves baroreflex function in a rodent model of chronic kidney disease

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4.1. Abstract

We examined the hypothesis that amplified respiratory-related control of sympathetic nerve activity is associated with baroreflex dysfunction in chronic kidney disease (CKD). Studies were performed in urethane-anaesthetised Lewis Polycystic Kidney (LPK) and Lewis rats (n=20), measuring sympathetic nerve activity (SNA). Baroreflex control of both renal SNA (rSNA) and splanchnic SNA (sSNA) was examined separately under basal eupnoeic (control) and apnoeic condition by ventilating the rats in apnoeic threshold (end-tidal CO_2, $EtCO_2$ <3%) which caused silencing phrenic nerve activity (PNA), as a mechanism by which to inhibit respiratory sympathetic coupling (respSNA). RespSNA was augmented in the LPK, as reflected by a larger area under curve (AUC), compared with Lewis rats (rSNA:13.6 ± 2.3 vs. 6.11 ± 1; sSNA: 10.25 ± 2.7 vs. 3.69 ± 0.8 μV. s, both $P < 0.05$). Under eupnoeic conditions, baroreflex regulation of both rSNA and sSNA in the LPK was shifted to the right in line with the higher blood pressure and showed a reduction in gain (rSNA: 0.8 ± 0.1 vs. 1.6 ± 0.1; sSNA: 0.9 ± 0.1 vs. 1.4 ± 0.2 %/mmHg, both $P < 0.05$). The duration and AUC of respSNA was correlated with MAP50 of baroreflex function curve of SNA ($P < 0.05$) of both nerves. Apnoea did not affect the gain of the baroreflex, which remained comparably reduced in the LPK compared with Lewis; however, it resulted in a resetting of the baroreflex to a lower blood pressure in the LPK (MAP50, rSNA: 180±10 vs. 164 ± 9; sSNA: 193±19 vs. 159 ± 10 mmHg; eupnoea vs. apnoea both $P < 0.05$) despite no effect on resting blood pressure ($P > 0.05$). This effect was also observed in sSNA of the Lewis controls (155 ± 5 vs. 132 ± 3 mmHg; eupnoea vs. apnoea $P < 0.05$). Our findings suggest that there is an ongoing interaction between baroreflex and respiratory drive that determines the operating range of the baroreceptor reflex such that under apnoeic conditions, the centring point of the reflex is closer to the threshold for initiating the baroreceptor reflex. This relationship between baroreflex and respiratory drive is preserved in CKD.

Key words: respiratory sympathetic modulation, chronic kidney disease, Baroreflex, Hypertension, respiratory drive

4.2. Introduction

Autonomic dysfunction is common in individuals with chronic kidney disease (CKD), typically manifesting as sympathetic overactivity (Klein *et al.*, 2003a; Grassi *et al.*, 2011a), impaired baroreflex (Johansson *et al.*, 2005; Johansson *et al.*, 2007) and chemoreceptor reflex function (Hering *et al.*, 2007) alongside central respiratory dysfunction (Hering *et al.*, 2007). The underlying cause for autonomic dysfunction in individuals with CKD is not clear. Given that the pathways responsible for the generation of sympathetic nerve activity (SNA), processing of both baroreceptor and chemoreceptor reflexes, and generation of central respiratory drive intersect in the brain stem (Baekey *et al.*, 2010), it is plausible that the autonomic dysfunction observed in CKD is driven by a common causal factor or by an aberrant interaction between these pathways.

Respiration strongly influences the processing of baroreceptor reflex outflow. In both humans and animals, respiration gates the central processing of the baroreceptor reflex resulting in enhanced baroreceptor-mediated changes in heart rate and SNA during expiration compared with inspiration (Eckberg & Orshan, 1977; Gilbey *et al.*, 1984; Baekey *et al.*, 2010). This gating is critically dependent on two things: (1) the intensity of baroreceptor afferent stimulation, with respiratory gating of baroreceptor reflex processing lost at high intensity baroreceptor stimulation (Baekey *et al.*, 2010) and (2) the activity of central respiratory pattern generators, with gating lost following pontine transection (Baekey *et al.*, 2008). Thus, a strong interaction exists between the ongoing processing of baroreceptor information within the medulla and the central respiratory pattern generators to produce appropriate baroreceptor-mediated responses.

Respiratory-related changes in sympathetic drive occur on an ongoing basis to facilitate synchronous changes in blood pressure that promote optimal tissue perfusion. Strong experimental evidence exists demonstrating that enhanced respiratory modulation of sympathetic vasomotor outflow may contribute to the development of hypertension across many different forms of hypertension (Czyzyk-Krzeska & Trzebski, 1990; Simms *et al.*, 2009; Toney *et al.*, 2010; Menuet *et al.*, 2017). Although short-term increases in central respiratory drive enhance respiratory sympathetic coupling without negatively impacting baroreceptor reflex function (Moraes *et al.*, 2016), it is conceivable that the pathological and chronic upregulation of respiratory modulation of sympathetic vasomotor drive associated with disease may perturb the ongoing central processing of baroreceptor reflex function and

thereby contribute to the wider autonomic dysfunction commonly associated with diseases such as CKD.

The Lewis polycystic kidney (LPK) rat model of CKD demonstrates widespread autonomic dysfunction including increased sympathetic tone and altered baroreceptor and chemoreceptor reflex function (Salman *et al.*, 2014; Salman *et al.*, 2015a; Yao *et al.*, 2015; Harrison JL, 2010). A deficit within the central component of the baroreceptor reflex plays a critical role in the development of baroreflex dysfunction in this model (Salman *et al.*, 2014; Salman *et al.*, 2015a) and based on our recent findings of enhanced coupling between respiration and sympathetic nerve activity (Chapter 2), we tested the hypothesis that enhanced respiratory sympathetic coupling (respSNA) contributes to impaired processing of the baroreceptor reflex. To test this hypothesis, we compared baroreflex control of renal and splanchnic sympathetic nerve activity (rSNA and sSNA) in the presence and absence of central respiratory drive.

4.3. Materials and Methods

4.3.1. Ethical Approval

All procedures were approved by the Animal Ethics Committee of Macquarie University and carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th edition).

4.3.2. Animals

Adult (mixed-sex, 12-13 weeks old) Lewis and Lewis Polycystic Kidney (LPK) (n = 10 per strain) rats were sourced from the Animal Resource Centre, Murdoch, Western Australia, Australia.

4.3.3. Surgical Procedures

All animals were anaesthetised with ethyl carbamate (Urethane, Sigma Aldrich, Australia; 10% w/v in 0.9% NaCl) at a dose of 1.3 g/kg (i.p.) with supplemental doses (65 mg/kg i.p. or i.v.) administered as required. Core body temperature was measured and maintained at 37 ± 0.5 ºC using thermostatically controlled heating blanket (Harvard) Apparatus, Holliston, MA, USA) and infrared heating lamp.

Following induction of anaesthesia, bilateral femoral venous and unilateral femoral arterial cannulation was performed for administration of fluid (Ringer's lactate, 5 ml/kg/hr), drugs and measurement of arterial pressure (AP) respectively. A tracheostomy was performed with endotracheal tube placed in situ. The left phrenic nerve, splanchnic nerve and renal nerve were dissected by a retroperitoneal approach and a bilateral vagotomy performed.

The animal was then placed in a stereotaxic frame, ventilated with room air enriched O_2 (7025 Rodent Ventilator, UgoBasile, Italy) and paralysed with pancuronium bromide (2mg/kg i.v. for induction and 1mg/kg i.v. for maintenance; AstraZeneca, North Ryde, NSW, Australia). Arterial blood gases and end-tidal CO_2 ($EtCO_2$) were maintained within the following ranges by regulating the ventilator pump rate and volume and/or slow bolus injections of 5% sodium bicarbonate if necessary: pH=7.4± 0.05, PCO_2= 40 ± 5 mmHg, HCO_3^- =24±2 mmol/L and $EtCO_2$= 4.5± 0.5%.

The central cut end of the phrenic (PNA), splanchnic (sSNA) and renal (rSNA) nerve were each placed on a bipolar silver wire recording electrode bathed in a pool of liquid paraffin pool, calibrated to an internal calibration of 50 μV, amplified, band-pass filtered at 10-1000Hz (CWE Inc. Ardmore, PA, USA) and sampled at 5kHz using a CED 1401 plus and

Spike 2 software v.7 (Cambridge Electronic Designs Ltd, Cambridge, UK). The arterial line was connected to a pressure transducer (Edward Life Sciences, CA, USA) and acquired using a CED 1401 plus and Spike 2 software v.7 (Cambridge Electronic Designs Ltd, Cambridge, UK). The integrity of SNA activity signal was confirmed by pulse modulation of SNA and baroreflex-mediated inhibition of SNA in response to a pressor stimulus (phenylephrine 50 µg/kg iv; Sigma-Aldrich, St. Louis, MO, USA).

4.3.4. Experimental Protocol

1. Following completion of all surgical procedures, animals were ventilated with oxygen-enriched room air and AP, $EtCO_2$ and nerve activity allowed to stabilise for a period of at least 30 min, following which, baseline levels of mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP), sSNA, rSNA and PNA were recorded over a 30s period during eupnoeic conditions.
2. The baroreceptor reflex was tested using sequential bolus injection of sodium nitroprusside (50-70µg/kg i.v., Sigma-Aldrich, St. Louis, MO, USA or phenylephrine (10-50µg/kg i.v.) as described previously (Salman *et al.*, 2014) under eupnoeic conditions for a period of 45 mins by maintaining ventilatory (both rate and volume) parameters within a range that ensured that blood pH was 7.4± 0.05, PCO_2 was 40 ± 5 mmHg and $EtCO_2$ was 4.5 ± 0.5% and where the phrenic nerve was discharging and a clear pattern of respSNA was present.
3. Thereafter, apnoeic conditions were achieved for a period of 45 mins by hyperventilating the animal with increasing ventilatory rate or volume to achieve a reduction in $EtCO_2$ to less than 3%, causing the phrenic nerve to cease firing. After a period of stabilization (30 min), cardiovascular parameters with SNA were taken over a 30s period. The baroreceptor reflex was tested again in apnoeic condition in a similar way described earlier. For technical reasons, both nerves (rSNA and sSNA) were not able to be recorded from all animals.
4. At the end of the experimental protocol the animal was euthanized (3M KCL I.V.) and background noise levels for PNA, sSNA and rSNA determined.

4.3.5. Data Analysis

All data were analysed offline using Spike 2 software. Blood pressure parameters and heart rate were derived from the AP waveform. Both sSNA and rSNA were rectified and smoothed

with a time constant of 1 sec and PNA was rectified and smoothed with a time constant of 0.05 sec. The level of nerve activity following euthanasia was subtracted from each neurogram to remove background noise.

Baroreflex Function Curves: Both rSNA and sSNA were normalised by setting the level of nerve activity over the 30s baseline period prior to drug administration to 100% and the level of activity following euthanasia as 0%. The level of nerve activity was then compared against the level of MAP over the rising or falling phase of the phenylephrine and sodium nitroprusside induced change in MAP, respectively, over a range of 50-250 mmHg in the Lewis and 50-350 mmHg in the LPK, as described previously (Salman *et al.*, 2014; Salman *et al.*, 2015a), using the following equation:

$$y = \frac{A_1}{1 + e^{A_2(MAP - A_3)}} + A_4$$

Where A_1 is the range of the curve, A_2 the gain coefficient, A_3 the midpoint of the curve and A_4 the lower plateau. The upper plateau of the SNA, range and gain of the reflex, mean arterial pressure (MAP) at the midpoint/centring of the curve (MAP_{50}), MAP threshold (MAP_{thr}), MAP saturation (MAP_{sat}) and MAP operating range were then calculated. Curves with a R^2 value less than 0.9 were not included in the data set.

RespSNA: RespSNA was analysed over the 30 sec baseline period during eupnoea. Phrenic triggered averaging of rectified and smoothed sSNA and rSNA recordings from the onset of PNA was performed offline to examine respSNA. From the phrenic-triggered integrated sSNA and rSNA, the peak amplitude (PA [μV]), the maximum amplitude at the apex of the peak of the SNA burst coincident with post-inspiratory phase, the duration (from onset of excitatory activity to return to baseline [s]) and area under curve (AUC) of respSNA excitatory peak (μV.s) were determined.

Renal function: Urine was collected from all animals over 24-hr period approximately 48-hours prior to the experiment and total urinary volume and urinary creatinine levels determined using an IDEXX Vetlab analyser (IDEXX Laboratories Pty Ltd., Rydalmere, New South Wales, Australia). An arterial blood sample was collected at the commencement of the surgical procedures and blood gas parameters and plasma creatinine and urea were measured. Creatinine clearance was calculated using the following equation:

$$\text{creatinine clearance} = \frac{\text{urinary creatinine } (\mu\text{mol. L}^{-1}) \text{ X urine volume } (\text{ml. min}^{-1})}{\text{plasma creatinine } (\mu\text{mol. L}^{-1})}$$

4.3.6. Statistical Analysis

All data are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism software v6.0 (GraphPad Software Inc., La Jolla, California, USA). Initial analysis was undertaken for each parameter comparing strain and sex using a two-way ANOVA to determine sex effects. Unless otherwise reported, sex effects were not evident, and data combined.

An unpaired two tailed Student's t-test was used to identify any statistical differences in baseline measures of renal function, cardiorespiratory function and SNA between the LPK and Lewis. A two-way ANOVA with Bonferroni's post-hoc multiple comparison test was used to analyse the effect of central respiratory drive on sympathetic baroreflex function both within and between the Lewis and LPK. A Pearson correlation was used to determine if any statistically significant relationship existed between the gain and MAP_{50} of the rSNA and sSNA baroreflex function curves and the duration, AUC and PA of the respSNA. Significance was defined as $P \leq 0.05$.

4.4. Results

4.4.1. Baseline renal function, cardiorespiratory function and respiratory sympathetic coupling

Renal function was impaired in the LPK relative to Lewis control rats as evidenced by elevated serum creatinine (35.3 ± 1.8 vs 9.1 ± 3.1 μmol/L; LPK vs Lewis $P < 0.0001$) and urea (20.8 ± 1.0 vs 6.7 ± 0.3 mmol/L; LPK vs Lewis $P < 0.0001$) and lower creatinine clearance rate (2.5 ± 0.4 vs 29.9 ± 9.7 ml/min; LPK vs Lewis $P < 0.05$). Baseline measurements of MAP, SBP, DBP, PP, heart rate, rSNA and sSNA under control (eupnoea) conditions were elevated in the LPK compared to Lewis controls ($P < 0.05$; Table 1 and Figure 1). There was a trend of higher MPA in LPK rats, however, it was not statistically significant ($P = 0.08$) between the strains. Moreover, no significant difference in other indices of respiratory drive existed with PNA amplitude, and PNA frequency comparable between the strains ($P > 0.05$; Table 1).

Consistent with our previous observations (Chapter 2) respiratory modulation of rSNA and sSNA was observed as a single burst of post inspiratory (PI) related peak activity in both adult LPK and Lewis rat under control (eupnoea) condition. Augmented respSNA was seen in LPK rats, reflected by a greater AUC, PA and duration (all $P < 0.05$; Table 2 and Figure 2 A-D).

4.4.2. Baseline sympathetic baroreceptor function

The effects of increasing and decreasing blood pressure on SNA under eupnoeic and apnoeic conditions is illustrated in Figure 1. Representative individual baroreflex function curves are shown in Figure 3 under eupnoea and the grouped functional response data presented in Figure 4. No sex difference evident when each parameter of baroreflex was compared using a two-way ANOVA (Appendix, Tables 5 and 6).

Under control (eupnoea) conditions, both rSNA and sSNA baroreflex function curves were shifted to the right in the LPK compared to Lewis as evidenced by a significantly greater MAP_{50} and MAP_{sat} for both rSNA and sSNA baroreflex function curve and MAP_{thr} for the rSNA baroreflex function curve ($P \leq 0.05$; Tables 3 and 4). In addition, the gain of the both rSNA and sSNA function curves was reduced in the LPK compared with Lewis under eupnoeic conditions ($P \leq 0.05$; Tables 3 and 4). The operating range for rSNA baroreflex function curves was greater in the LPK compared with Lewis (Table 3). While two-way ANOVA demonstrated a strain effect in the range of the rSNA baroreflex function curve ($P = 0.048$, Table 3), the SNA range for both rSNA and sSNA baroreflex function curves did not

differ under eupnoeic conditions reflected by a comparable upper and lower plateau between the LPK and Lewis for both renal and splanchnic baroreflex function curves (Tables 3 and 4).

As we observed both an increase in respSNA and reduction in SNA baroreflex function in the LPK under eupnoeic conditions, we then sought to determine if baroreceptor reflex function was associated with respSNA. To achieve this, we correlated the gain and MAP50 of both rSNA and sSNA baroreflex function curves with the duration, AUC and PA of the respSNA obtained under eupnoeic conditions across all Lewis and LPK animals (Table 5). There was a significant relationship between baroreceptor reflex function and the degree of respSNA such that for the rSNA, as the duration and AUC of respSNA increased the gain of the rSNA baroreceptor reflex function curve reduced. However, there was no relationship with PA. Additionally, an increase in rSNA respSNA, indicated by an increase in duration, AUC and PA was associated with an increase in MAP_{50}. Comparable, yet more selective, relationships were observed between sSNA baroreceptor reflex function and respSNA with an increase in the duration of respSNA associated with a reduction in reflex gain and an increase in duration and AUC associated with an increase in MAP_{50}. Examples illustrating the relationship between SNA gain, MAP_{50} and respSNA duration are shown in Figure 5.

Given the correlations that existed between respSNA and baroreceptor reflex function, it is conceivable that an increase in respSNA is contributing to the deficits in baroreceptor reflex function observed in the LPK. To examine this, we tested baroreceptor reflex function in both the Lewis and LPK that had been hyperventilated in order to suppress central respiratory drive and therefore relinquish the enhanced respSNA present in the LPK. Under apnoeic conditions, in the LPK rats, MAP_{50} of rSNA baroreceptor reflex function curve decreased as did MAP_{sat} and apnoea also shifted the operating range of rSNA baroreceptor reflex function curve with an overall shift to left, while in the Lewis, only MAP_{sat} decreased under apnoeic conditions (Table 3, Figure 4). For the sSNA baroreceptor reflex function curve, $MAP_{50,}$ $MAP_{thr,}$ and MAP_{sat} also reduced in the LPK under apnoeic conditions while in the Lewis, there was a decrease in the range, $MAP_{50,}$ MAP_{thr} and lower plateau (Table 4, Figure 4). Importantly, supressing central respiratory drive did not significantly affect resting blood pressure or heart rate in either the Lewis or LPK (Table 6) and therefore the differences in baroreceptor reflex function observed under apnoeic conditions do not relate to a change in baseline cardiovascular function. However, SNA did not exhibit any significant changes between eupnoeic and apnoeic conditions in both strains except rSNA in LPK rats which showed an increase in activity during apnoea ($P<0.05$).

4.5. Discussion

The major findings of the present study are: (1) increased respSNA is associated with both the operation of the sympathetic baroreceptor reflex at a higher resting blood pressure and a reduction in the sensitivity of the reflex and (2) supressing central respiratory drive and relinquishing the increased respSNA selectively resets the sympathetic baroreceptor reflex to a lower operating point in the LPK model. Collectively this suggests that alterations in the central networks that regulate respiration and their modulation of SNA may contribute to the baroreceptor reflex dysfunction observed in CKD.

Previous work from our laboratory has shown that a decline in the central processing of the baroreceptor reflex is a key contributor to the overall presentation of sympathetic baroreceptor reflex dysfunction (Salman *et al.*, 2014; Salman *et al.*, 2015a) in LPK rats, an animal model of CKD. Notably the onset of baroreceptor reflex dysfunction does not occur until later in the disease phase after the onset of hypertension and a decline in renal function (Hildreth *et al.*, 2013b). Interestingly, central respiratory drive in terms of respiratory frequency was comparable between LPK and Lewis rats which was contrast to our previous findings in both adult male and female LPK rats (chapter 2 and 4). Variation of phrenic discharge may depend on the severity of renal failure and between cohorts of animals, however, this is speculative and would need further investigation to address. A critical finding of the present study is our observation that respSNA correlates with sympathetic baroreceptor reflex. Incidentally, as shown in our previous study (Chapter 2), respSNA is enhanced in the LPK at an age where baroreceptor reflex dysfunction is not yet apparent. Altogether, these observations suggest that enhanced respSNA may be causally related to the sympathetic baroreceptor reflex dysfunction observed in the LPK and that alterations in central respiratory networks may contribute to sympathetic baroreceptor reflex dysfunction by perturbing the central processing of the sympathetic baroreceptor reflex. However, this possibility warrants further investigation.

In the present study we explored whether central respiratory networks might be contributing to the baroreceptor reflex dysfunction observed in the LPK by assessing sympathetic baroreceptor reflex function in the absence of central respiratory drive. We reasoned that if central respiratory networks were exerting a negative effect on baroreceptor reflex function then we would see an improvement in baroreceptor reflex function. When central respiratory drive was absent, there was no change in the gain of the sympathetic baroreceptor reflex suggesting that although a decline in the gain of the sympathetic baroreceptor reflex was

associated with increased respSNA, the reduction in the gain of the sympathetic baroreceptor reflex is not a consequence of aberrant respiratory regulation of SNA and/or interference in the central processing of the sympathetic baroreceptor reflex. This finding is aligned with recent observations in the chronic intermittent hypoxic rat model whereby early in the disease phase enhanced respSNA was associated with greater expiratory-related baroreceptor reflex sympathoinhibition and was therefore not impeding the functioning of the sympathetic baroreceptor reflex (Moraes *et al.*, 2016). It remains to be determined in the LPK if respiratory-related enhancement of sympathetic baroreceptor reflex function is also apparent in the early phases of the disease and is therefore serving to protect the baroreceptor reflex and likewise guard against the development of hypertension.

While the reduction in the gain of the sympathetic baroreceptor reflex does not appear to relate to differences in central respiratory regulation, the increase in MAP_{50} of the baroreceptor reflex does. Notably, the MAP_{50} of both rSNA and sSNA baroreceptor reflex function curves were positively correlated with respSNA, and suppression of central respiratory drive reduced the MAP_{50} of both function curves in the LPK. These findings were consistent in both male and female LPK rats. The underlying mechanism contributing to this selective improvement in sympathetic baroreceptor reflex was not explored in the present study. It is conceivable that this relationship does not reflect any association between central respiratory regulation and sympathetic baroreceptor reflex and that the association between MAP_{50} and respSNA is simply reflective of the presence of hypertension given that MAP_{50} increases as resting blood pressure increases. While this is possible, we consider this unlikely for two reasons: (1) when central respiratory drive was supressed, we observed a reduction in the MAP_{50} of the rSNA baroreceptor reflex function curve and a normalisation of MAP_{50} for the sSNA baroreceptor reflex function curve, yet resting blood pressure was not significantly reduced in the LPK; and (2) we similarly saw a marked reduction in MAP_{50} during suppression of central respiratory drive in the Lewis which does not exhibit any baroreceptor reflex dysfunction, is normotensive and did not exhibit a reduction in baseline blood pressure in response to hyperventilation. Different studies have found that the nucleus of tractus solitarius (NTS) in the dorsal medulla of the brainstem plays a central role integrating baroreflex function with its connection to presympathetic neurons of the rostral ventrolateral medulla (RVLM) and parasympathetic neurons in the brainstem, by changing SNA and parasympathetic nerve activity reflected in blood pressure and heart rate changes (Loewy, 1990; Dampney, 1994; Zoccal *et al.*, 2014). Similarly, neurons of the NTS project to the respiratory neurons, specifically post inspiratory neurons of the Bötzinger complex which

inhibit presympathetic neurons of the RVLM (Baekey *et al.*, 2010; Molkov *et al.*, 2014). Moreover, these presympathetic neurons of the RVLM lie close to respiratory neurons and an animal study on respiratory neurons provides evidence that there are synaptic connections between respiratory and presympathetic neurons (Sun *et al.*, 1997), by which respiratory neurons contribute to modulation of sympathetic nerve activity. Accordingly, the NTS, respiratory neurons and presympathetic neurons are the crucial anatomical sites for convergence of baroreflex function and the respiratory sympathetic coupling. Our observation in this study thus gives credence to the idea that central respiratory networks may be serving to gate baroreceptor input, potentially at the level of the nucleus tractus solitarius (Baekey *et al.*, 2010), and therefore in the absence of central respiratory drive and regulation over these inputs, baroreceptor-mediated changes in SNA are able to occur over a lower blood pressure range.

4.6. Conclusion

Our data indicate that amplified respSNA may serve to reduce sympathetic baroreceptor reflex in the LPK rat model of CKD. Targeting central respiratory dysfunction may serve as a novel therapeutic strategy to not only reduce sympathetic overactivity but also baroreceptor reflex dysfunction and reduce the overall cardiovascular morbidity and mortality associated with CKD.

4.7. Acknowledgements

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4.8. Disclaimer

All authors declared no competing interests.

Table 4.1 Cardiorespiratory function and sympathetic nerve activity parameters in adult Lewis and Lewis Polycystic Kidney Rats under eupnoeic conditions

Parameter	Lewis (n=10)	LPK (n=10)
MAP (mmHg)	99 ± 5	123 ± 9*
SBP (mmHg)	128 ± 5	179 ± 17*
DBP (mmHg)	82 ± 5	95 ± 8*
PP (mmHg)	45 ± 4	84 ± 14*
HR (bpm)	442 ± 5	471 ± 6*
PNA amplitude (μV)	18.5 ± 2.5	29.8 ± 4.7
PNA frequency (cycles. min^{-1})	39.8 ± 4.1	40.9 ± 1.9
MPA	669.4 ± 78.8	1180 ± 186
rSNA (μV)	5.8 ± 0.5	11.7 ± 2.7*
sSNA (μV)	3.1 ± 0.5	8.8 ± 2.5*

Parameters measured under urethane anaesthesia after 30 minutes ventilation with oxygen enriched room air. MAP: mean arterial pressure; SBP: systolic BP; DBP: diastolic BP; pulse pressure: PP, HR: heart rate, PNA: phrenic nerve activity, MPA: minute phrenic activity, rSNA, renal sympathetic nerve activity, sSNA: splanchnic sympathetic nerve activity, LPK: Lewis polycystic kidney. Results are expressed as mean ± SEM.* significantly different to Lewis ($P<0.05$), as determined using an unpaired two-tailed Student's *t*-test. *n* = number of animals per group

Table 4.2 Respiratory sympathetic coupling parameters in Lewis and Lewis Polycystic Kidney rats under eupnoeic conditions

	RespSNA (renal)		RespSNA (Splanchnic)	
	LPK (n=10)	Lewis (n=10)	LPK (n=9)	Lewis (n=9)
Duration (s)	1.09 ± 0.07 *	0.70 ± 0.06	1.06 ± 0.08*	0.73 ± 0.06
AUC (μV. s)	13.6 ± 2.3 *	6.11 ± 1	10.25 ± 2.7*	3.69 ± 0.8
PA (μV)	13.37 ± 2.3 *	8.36 ± 0.9	11.21 ± 3.1*	4.26 ± 0.7

Respiratory sympathetic coupling (RespSNA) of renal and splanchnic SNA measured in Lewis and LPK rats. LPK: Lewis Polycystic Kidney; PA: peak amplitude, AUC: area under curve. Results * significantly different to Lewis (*P<0.05*), as determined using an unpaired two-tailed Student's t-test. n = number of animals per strain.

Table 4.3 Renal sympathetic baroreceptor reflex function curves in the adult Lewis and Lewis Polycystic Kidney rat under eupnoeic and apnoeic conditions

	Lewis (n=10)		LPK (n=10)		P value	
	Eupnoea	Apnoea	Eupnoea	Apnoea	Strain	Respiration
gain (%/mmHg)	1.6 ± 0.1	1.7 ± 0.1	0.8 ± 0.1*	0.9 ± 0.1*	<0.001	0.453
range (%)	100 ± 8	93 ± 9	80 ± 10	69 ± 6	0.048	0.044
MAP_{50} (mmHg)	145 ± 6	132 ± 4	180 ± 10#*	164 ± 9*	0.005	0.002
MAP_{sat} (mmHg)	167 ± 7#	152 ± 5	214 ± 12#*	192 ± 11*	0.003	<0.001
MAP_{thr} (mmHg)	123 ± 5	113 ± 4	146 ± 8*	136 ± 7	0.015	0.051
operating range (mmHg)	44 ± 4	38 ± 3	68 ± 6#*	56 ± 6*	0.006	0.013
upper plateau (%)	117 ± 6	111 ± 6	114 ± 4	105 ± 2	0.457	0.054
lower plateau (%)	16 ± 4	17 ± 5	34 ± 7	36 ± 5*	0.026	0.547

rSNA, renal sympathetic nerve activity; MAP_{50}, mean arterial pressure at the midpoint of the curve; MAP_{thr}, threshold MAP to trigger a change in SNA; MAP_{sat}, saturation MAP at which there is no further change in SNA; LPK, Lewis polycystic kidney. The baroreflex function curve parameters were obtained from the logistic function relating normalized SNA (% baseline) to MAP. # Significantly different between before and after inhibition of central respiratory drive within same strain ($P \leq 0.05$) and * Significantly different between the strains in similar condition ($P \leq 0.05$) as determined using two-way repeated measures of ANOVA followed by Bonferroni's *post-hoc* correction. n = number of animals per group.

Table 4.4 Splanchnic sympathetic baroreceptor reflex function curves in the adult Lewis and Lewis Polycystic Kidney rat under eupnoeic and apnoeic conditions

	Lewis (n=9)		LPK (n=8)		P value	
	Eupnoea	Apnoea	Eupnoea	Apnoea	Strain	Respiration
gain (%/mmHg)	1.4 ± 0.2	1.3 ± 0.2	0.9 ± 0.1	0.8 ± 0.1	0.064	0.853
sSNA range (%)	100 ± 9#	88 ± 9	93 ± 10	88 ± 12	0.817	0.014
MAP_{50} (mmHg)	155 ± 5#	132 ± 3	193 ± 19*#	159 ± 10	0.039	<0.001
MAP_{sat} (mmHg)	182 ± 7	156 ± 5	236 ± 29#*	192 ± 14	0.049	<0.001
MAP_{thr} (mmHg)	128 ± 6#	108 ± 3	151 ± 10#	125 ± 8	0.043	<0.001
operating range (mmHg)	53 ± 6	48 ± 5	85 ± 21	66 ± 10	0.111	0.124
upper plateau (%)	115 ± 6	115 ± 6	116 ± 3	115 ± 5	0.961	0.932
lower plateau (%)	15.2 ± 6.7#	27.1 ± 6.8	22.6 ± 8.6	26.8 ± 9	0.742	0.009

sSNA, splanchnic sympathetic nerve activity; MAP_{50}, mean arterial pressure at the midpoint of the curve; MAP_{thr}, threshold MAP to trigger a change in SNA; MAP_{sat}, saturation MAP at which there is no further change in SNA; LPK, Lewis polycystic kidney. The baroreflex function curve parameters were obtained from the logistic function relating normalized SNA (% baseline) to MAP. #Significantly different eupnoeic versus apnoeic conditions within same strain($P \leq 0.05$), * Significantly different between the strains in similar condition ($P \leq 0.05$) as determined using two-way repeated measures of ANOVA followed by Bonferroni's *post-hoc* correction. n = number of animals per group.

Table 4.5 Pearson's correlation coefficients for renal sympathetic nerve activity and splanchnic sympathetic nerve activity baroreflex function curve parameters relative to respiratory sympathetic coupling in adult Lewis and Lewis Polycystic Kidney rats under eupnoeic conditions

RespSNA parameters	rSNA (*n*=20)		sSNA(*n*=17)	
	Gain (r)	MAP_{50} (r)	Gain (r)	MAP_{50} (r)
Duration (s)	-0.49 (P=0.02)	0.54 (P= 0.01)	-0.62 (P=0.008)	0.62(P=0.008)
AUC (μV. s)	-0.46 (P=0.04)	0.59 (P= 0.006)	-0.23 (P = 0.37)	0.60 (P= 0.01)
PA (μV)	-0.37 (P= 0.11)	0.50 (P= 0.02)	-0.02 (P=0.91)	0.35 (P= 0.16)

Correlation analysis of the relationship between the gain/MAP50 of rSNA and sSNA baroreflex function and respiratory sympathetic coupling. MAP50: mean arterial pressure at the midpoint of the curve, PA: peak amplitude, AUC: area under curve, r: correlation coefficients, LPK: Lewis polycystic kidney. n = number in each group.

Table 4.6 Cardiorespiratory parameters and sympathetic nerve activity in the adult Lewis and Lewis Polycystic Kidney (LPK) rat under eupnoeic and apnoeic conditions

	Lewis (n=10)		LPK (n=10)		P value	
	Eupnoea	Apnoea	Eupnoea	Apnoea	Strain	Respiration
SBP (mmHg)	128 ± 5	122 ± 11	179 ± 17*	164 ± 1*	0.005	0.283
DBP (mmHg)	83 ± 5	76 ± 6	95 ± 8	84 ± 4	0.115	0.155
MAP (mmHg)	99 ± 5	97 ± 10	123 ± 9	111 ± 4	0.030	0.424
PP (mmHg)	45 ± 3	46 ± 6	84 ± 14*	80 ± 12*	0.012	0.828
HR (bpm)	442 ± 5	433 ± 8	471 ± 6*	463 ± 12*	0.008	0.271
rSNA (μV)	5.8 ± 0.6	7 ± 0.7	10.7 ± 1.8#	12.9 ± 2.3*	0.0185	0.0145
sSNA (μV)	3.1 ± 0.1	3.5 ± 0.5	8.9 ± 2.6*	8.5 ± 2.1	0.0351	0.9227

MAP, mean arterial pressure; SBP, systolic BP; DBP, diastolic BP; pulse pressure, PP; HR, heart rate; LPK, Lewis polycystic kidney, # Significantly different between eupnoeic vs. conditions within same strain (P≤0.05) * Significantly different between the strains (P≤*0.05*) in similar condition as determined using two-way repeated measures of ANOVA followed by Bonferroni's *post-hoc* correction. n = number of animals per group.

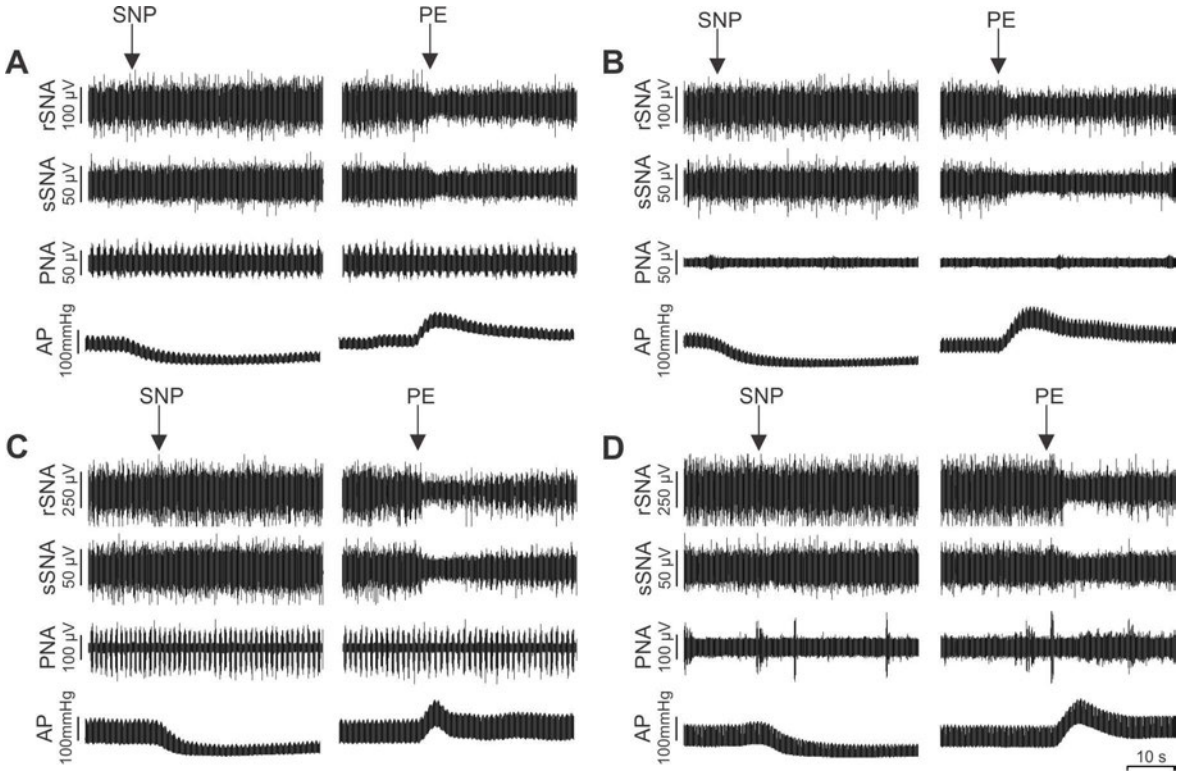

Figure 4.1 Representative traces showing baroreflex responses in Lewis and Lewis Polycystic Kidney rats

Representative raw data traces showing integrated renal sympathetic nerve activity (rSNA), integrated splanchnic sympathetic nerve activity (sSNA), integrated phrenic nerve activity (PNA) and arterial pressure (AP) in Lewis rat (A, B) and LPK rat (C, D) under urethane anaesthesia. Sympathetic nerve activity baroreflex responses evoked by phenylephrine (PE) and sodium nitroprusside (SNP) are shown under eupnoeic (left panels; A, C) and apnoeic conditions (right panels; B, D).

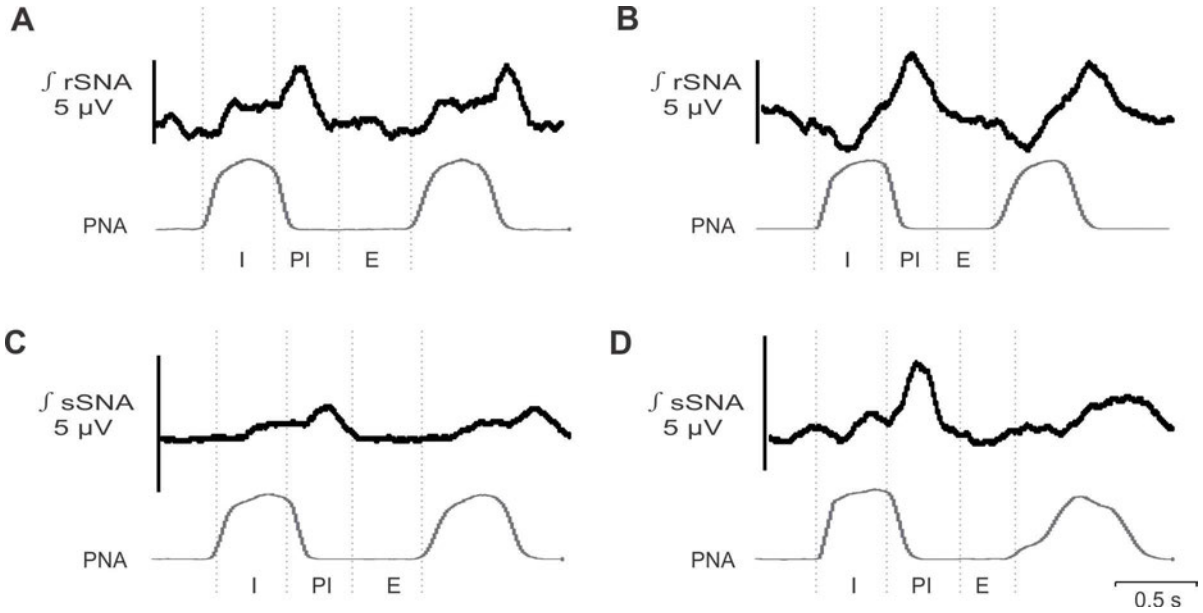

Figure 4.2 Example tracings of respiratory-related sympathetic nerve activity in Lewis and Lewis Polycystic Kidney rats

Example tracings of respiratory-related sympathetic nerve activity in Lewis and LPK rats illustrating phrenic triggered integrated rSNA (∫rSNA) and sSNA (∫sSNA) during different phases of the phrenic cycle (I, inspiration; PI, post inspiration; E, expiration within trace of phrenic nerve activity PNA) in a Lewis rat (panels A, C) and LPK rat (panels B, D)

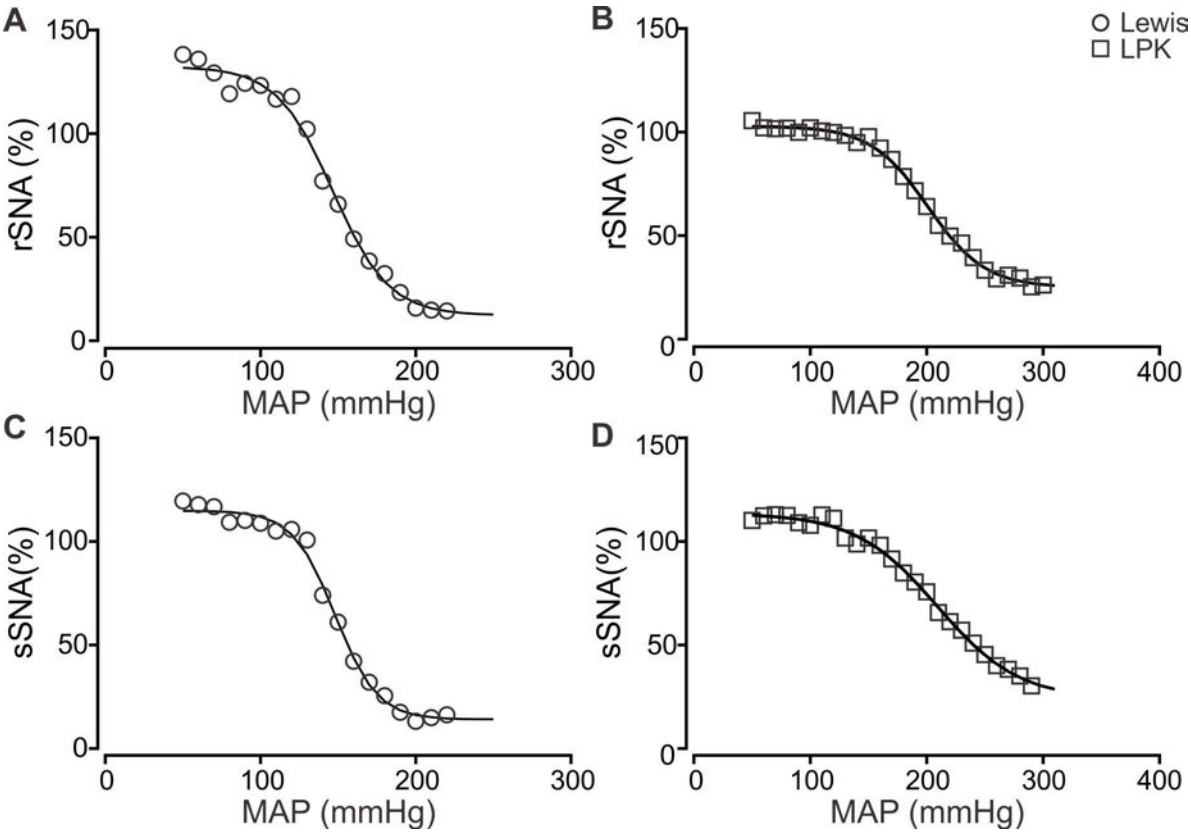

Figure 4.3 Representative individual sigmoidal curves illustrating the relationship between mean arterial pressure and sympathetic nerve activity in Lewis and Lewis Polycystic Kidney rats.

Representative individual sigmoidal curves illustrating the relationship between mean arterial pressure (MAP) and renal sympathetic nerve activity (rSNA; panels A and B) splanchnic sympathetic nerve activity (sSNA; panels C and D) in Lewis (panels A and C -circle symbols) and LPK rats (panels B and D – square symbols)) under eupnoeic conditions. $R^2 \geq 0.9$ for all curves

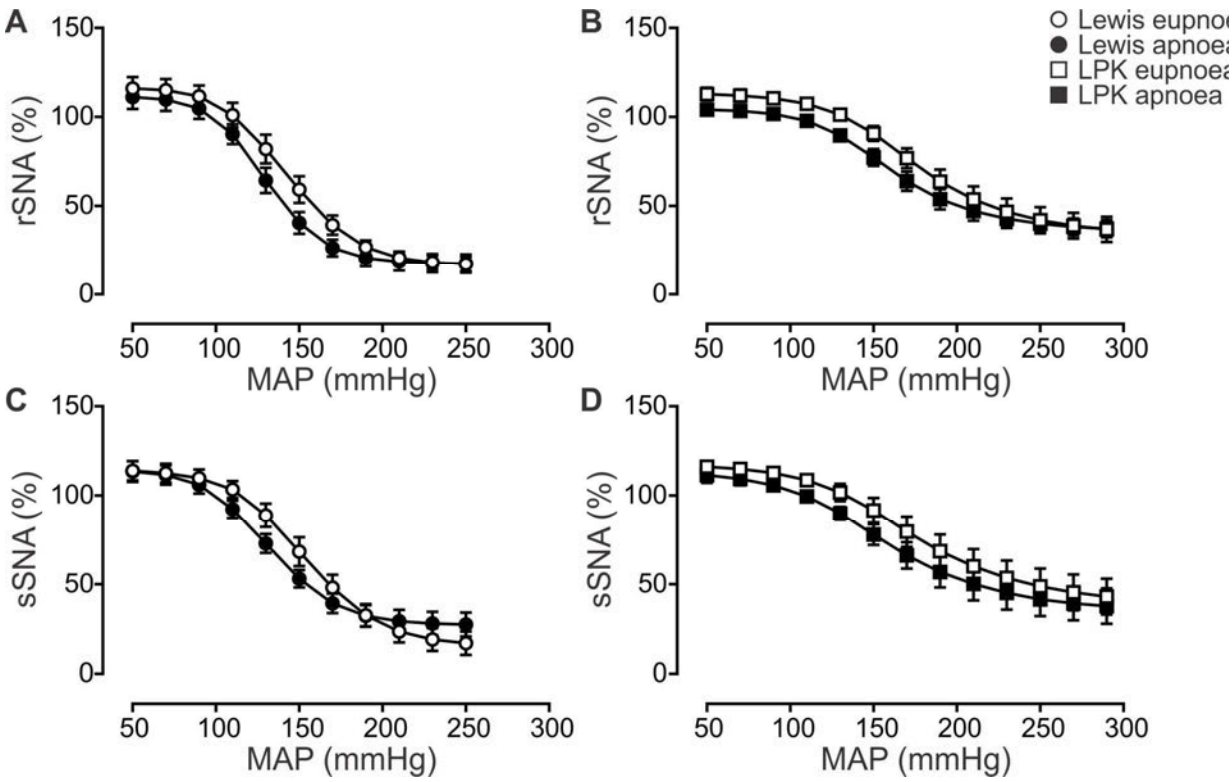

Figure 4.4 Grouped baroreceptor function curves for sympathetic nerve activity in the Lewis and Lewis Polycystic Kidney rats.

Grouped baroreceptor function curves for renal sympathetic nerve activity (rSNA) and splanchnic sympathetic nerve activity (sSNA) in the Lewis (panels A and C) and LPK (panels B and D) under eupnoeic (open symbols) and apnoeic (full symbols) conditions.

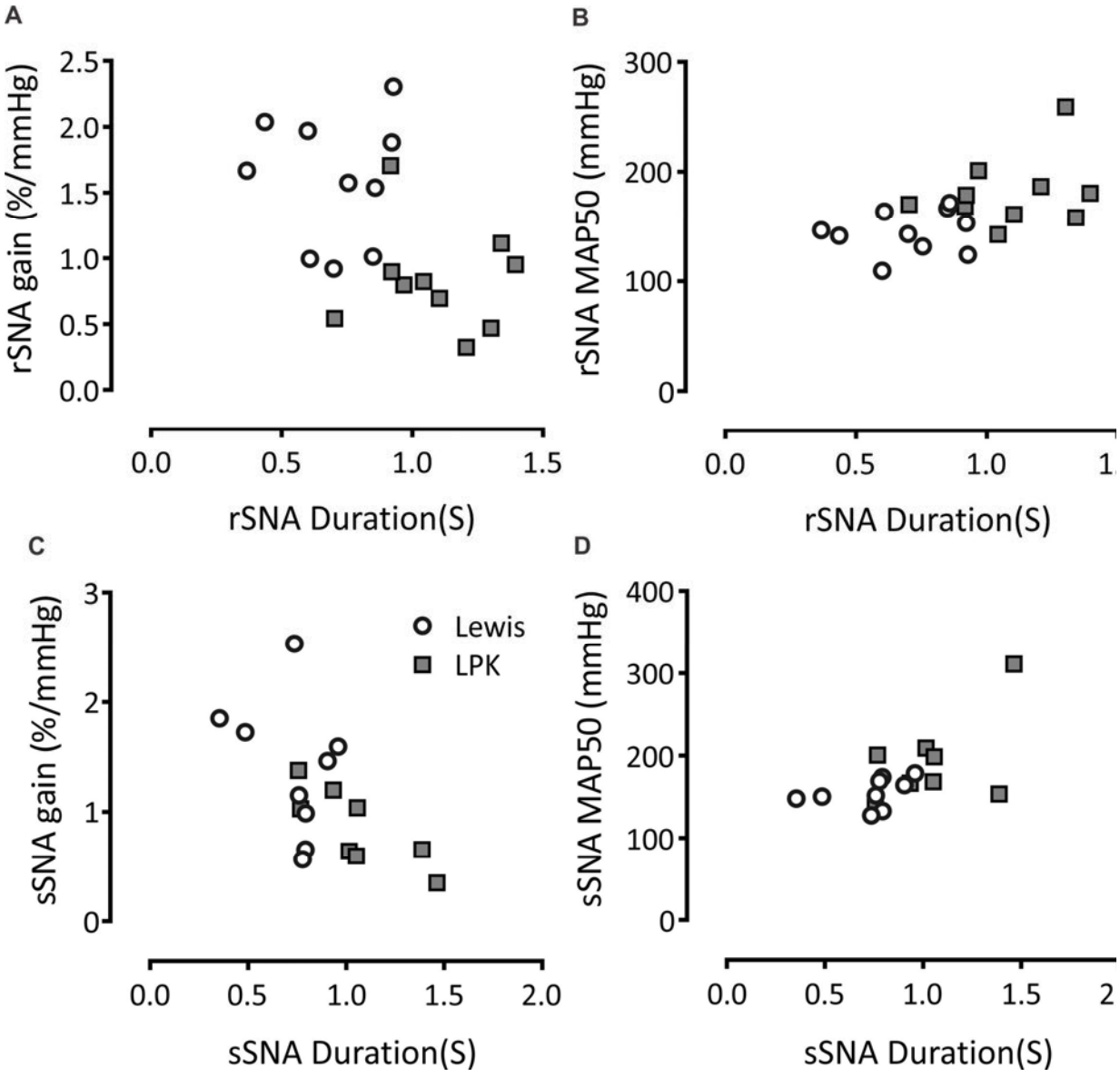

Figure 4.5 Relationship between baroreceptor reflex function and respiratory sympathetic coupling in the Lewis and LPK under eupnoeic condition.

Relationship between baroreceptor reflex function and respiratory sympathetic coupling in the Lewis (circle symbols) and LPK (square symbols) under eupnoeic condition. The relationship between the duration of the phrenic triggered integrated rSNA (∫rSNA) and sSNA (∫sSNA) and the gain and MAP_{50} of both renal (panels A and B) and splanchnic (panels C and D) baroreceptor reflex function curves are shown. rSNA, renal splanchnic nerve activity; sSNA, splanchnic sympathetic nerve activity; n= rSNA 20 (Lewis 10 + LPK 10); sSNA 17 (Lewis 9 + LPK 8) Pearson r and P values for each relationship are provided in Table 3.

5. Attenuation of high blood pressure in an animal model of chronic kidney disease after bilateral carotid sinus nerve transection

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5.1. Abstract

Peripheral chemoreceptors regulate blood pressure and their tonic activation in disease states has been proposed to contribute to increased sympathetic nerve activity (SNA) and in turn hypertension, which may be a contributor to the hypertension of chronic kidney disease (CKD). Previously we have shown that augmented respiratory sympathetic coupling (respSNA) is associated with increased SNA and hypertension in CKD, however, the role of peripheral chemoreceptors in increased respSNA and hypertension in this disease is not well understood. Accordingly, we aimed to identify if inhibition of peripheral chemoreceptors reduced blood pressure and respSNA in an animal model of CKD. Experiments were performed in anaesthetised, vagotomised, paralysed and ventilated hypertensive male Lewis Polycystic Kidney (LPK) and Lewis rats with either intact (n=6-7/strain) or transected (n=5-7/strain) carotid sinus nerves (CSN). Measurements of mean arterial pressure (MAP), renal SNA, splanchnic SNA and respSNA were obtained, noting SNA and respSNA in intact animals or after CSN transection was undertaken in two different cohorts. LPK rats were hypertensive (MAP, 140 ± 6 vs 90 ± 5 mmHg, LPK vs Lewis; $P < 0.0001$) and MAP was significantly reduced by CSN transection in LPK rats (-28 ± 9 mmHg, adjusted $P = 0.01$) however, MAP was not changed in Lewis rats following CSN transection. As we have demonstrated previously (Chapter 2), in intact LPK rats, respSNA was significantly higher than in intact Lewis. In animals that received CSN transection, respSNA remained significantly higher in LPK vs Lewis (area under the curve (AUC); sSNA: 6.7 ± 0.8 vs 3.4 ± 0.5, rSNA: 10.5 ± 2.4 vs 3.8 ± 0.5 μv. s, LPK vs Lewis; $P > 0.05$). Moreover, in the CSN transected LPK rats respSNA was not significantly different to intact LPK rats (AUC; sSNA: 7.03 ± 0.9 vs 6.7 ± 0.8, rSNA: 9.6 ± 1.9 vs 10.5 ± 2.4 μv.s, CSN intact vs CSN transected; $P > 0.05$). There was also no effect of CSN transection on respSNA in Lewis rats. Our findings indicate that removal of peripheral chemoreceptor input can reduce blood pressure in the LPK model of CKD, however this is not driven by a change in respSNA. The peripheral chemoreceptors may therefore be a therapeutic choice for hypertension in CKD however the mechanism remains to be clarified.

Key words:

Carotid sinus nerve, sympathetic overactivity, high blood pressure, respiratory sympathetic modulation, peripheral chemoreceptors

5.2. Introduction

Chronic kidney disease (CKD) is a major public health problem and increasing cause of mortality globally (GBD 2015 Mortality and Causes of Death Collaborators, 2016; Neuen *et al.*, 2017). Cardiovascular disease is a key contributor to this as it is a leading cause of morbidity and mortality in patients with CKD (Hill *et al.*, 2016). Furthermore, CKD is an independent risk factor for cardiovascular events (Go *et al.*, 2004; Weiner *et al.*, 2004). Consequently, a main component in the treatment of CKD is to treat hypertension with the aim of limiting the development of cardiovascular disease. However, this is often poorly achieved in spite of multi-drug therapy (De Nicola *et al.*, 2013a), with resistant hypertension highly prevalent within the CKD population (De Nicola *et al.*, 2013a; Rossignol *et al.*, 2015). More knowledge of the underlying mechanisms in the development of hypertension as applied to CKD specifically is required to facilitate the development of novel antihypertensive therapies that will be efficacious in CKD.

Sympathetic overactivity is common in individuals with CKD and suggested to be a contributor to the hypertension observed (Phillips, 2005; Schlaich *et al.*, 2009b; Campese *et al.*, 2011; Grassi *et al.*, 2012). The exact reasons why sympathetic overactivity occurs in CKD are unclear but may occur due to hyperactive peripheral chemoreceptor input as the peripheral chemoreceptors are reported to contribute to the sympathetic overactivity associated with essential hypertension (Sinski *et al.*, 2012; Paton *et al.*, 2013b) and heart failure (Ponikowski *et al.*, 2001). Accordingly, saturation of the peripheral chemoreceptors with hyperoxia reduces muscle sympathetic nerve activity (SNA) in patients with renal failure (Hering *et al.*, 2007). It is currently unknown, however, if tonic input from the peripheral chemoreceptors is contributing to sympathetic overdrive and hypertension in CKD.

The LPK rat, a genetic rat model of CKD resulting from a mutation of Nek8, phenotypically expresses polycystic kidney disease that resembles autosomal recessive polycystic kidney disease of human (Phillips *et al.*, 2007; McCooke *et al.*, 2012a). These rats exhibit elevated SNA, hypertension and progressive renal failure (Phillips *et al.*, 2007; Harrison *et al.*, 2010; Salman *et al.*, 2014; Salman *et al.*, 2015a; Salman *et al.*, 2015b; Yao *et al.*, 2015). Recently we have demonstrated that the LPK has increased respiratory modulation of SNA (respSNA) (Chapter 2) and proposed it may be a contributor to the observed hypertension, consistent with other studies of primary and secondary hypertension where it has been shown to contribute to the pathogenesis of these disease states (Zoccal *et al.*, 2008; Simms *et al.*, 2009; Simms *et al.*, 2010; Toney *et al.*, 2010). We further demonstrated that activation of the

peripheral chemoreceptors could increase this coupling between respiration and SNA to a greater extent in the LPK than in control Lewis animals (Chapter 2). Several studies utilising anesthetized or *in situ* rat preparations also demonstrate that peripheral chemoreceptor activation results in augmented respSNA as revealed by the emergence of higher amplitude bursts during the expiratory phase (Dick *et al.*, 2004; Zoccal *et al.*, 2008; Machado *et al.*, 2017). These observations suggest that (a) respiratory drive may be contributing to the increased SNA observed in CKD and importantly that (b) the peripheral chemoreceptors may be causally involved in the increased respSNA. In the present study we therefore sought to test the hypothesis that augmented peripheral chemoreceptor input drives the amplified respSNA and contributes to the hypertension observed in the LPK. This was achieved by examining the acute effects of peripheral chemoreceptor denervation on blood pressure and respSNA in anaesthetised LPK and control Lewis animals.

5.3. Methods

All experiments were approved by the Animal Ethics Committees of Macquarie University, NSW, Australia, and carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

12-13-week-old male LPK (n =12 and age-matched control Lewis n =14) rats were used. Animals were purchased from the Animal Resources Centre, Murdoch, Western Australia.

5.3.1. Renal function

A urine sample was collected from all animals approximately 48 hours prior to experimentation and urine volume, urinary creatinine and protein levels examined using an IDEXX Vetlab analyser (IDEXX Laboratories Pty Ltd., Rydalmere, NSW, Australia). At the commencement of the surgical procedure, an arterial blood sample was collected for determination of plasma urea and creatinine, and creatinine clearance calculated as described previously (Yao *et al.*, 2015) .

5.3.2. Surgical procedures

All animals were anaesthetised with 10% (w/v) ethyl carbamate (Urethane, Sigma Aldrich, NSW, Australia) in 0.9% NaCl solution (1.3 g/kg i.p.). Reflex responses to hind-paw pinch were assessed to determine adequate depth of anaesthesia and supplemental doses given as required (65 mg/kg i.p. or i.v.). Body temperature was measured and maintained at 37 ± 0.50 C using a thermostatically controlled heating blanket (Harvard Apparatus, Holliston, MA, USA) and infrared heating lamp. The right femoral vein and artery were cannulated for administration of fluid (Ringer's lactate, 5 ml/kg/hr) and measurement of arterial pressure (AP) and blood gases respectively. The AP signal was sampled at 200 Hz and acquired using a CED 1401 plus and Spike 2 software v.7 (Cambridge Electronic Designs Ltd, Cambridge, UK). A tracheostomy was performed and an endotracheal tube placed in situ. A bilateral vagotomy was performed to prevent afferent inputs from the stretch receptors of the lung and the animal ventilated with room air enriched O2 (7025 Rodent Ventilator, UgoBasile, Italy) and paralysed with pancuronium bromide (2 mg/kg iv for induction, 1 mg/kg iv for maintenance as required; AstraZeneca, North Ryde, NSW, Australia).

It was not possible to measure SNA and PNA in animals before CSN transection because the orientation of the animal for the CSN transection procedure was supine position, while the nerves for SNA and PNA recordings could only be securely accessed and recorded from with the animal in the prone position. Therefore, two cohorts were studied; 1) where we measured

SNA, PNA and respSNA after CSN transection and 2) a cohort of control animals that did not undergo CSN transection.

In those animals that underwent carotid sinus nerve (CSN) dissection (Cohort 1), the bifurcation of the carotid artery was then visualized and the CSN identified bilaterally. Both CSNs and their branches were dissected and cut in order to denervate the peripheral chemoreceptors. The left phrenic, splanchnic and renal nerves were then dissected to enable recording of respiratory rate [phrenic nerve activity (PNA)] and splanchnic and renal SNA (sSNA and rSNA), respectively and the distal end of each nerve was tied and cut. In those animals that did not undergo CSN transection (Cohort 2), the left phrenic, splanchnic and renal nerves were also dissected and the distal end of each nerve was tied and cut. All nerves were bathed in a liquid paraffin pool and the central end recorded using bipolar silver wire recording electrodes, 10 times amplified, band-pass filtered between 10-1000Hz by a bio amplifier (CWE Inc., Ardmore, PA, USA) and sampled at 5kHz using a CED 1401 plus and Spike 2 software v.7 (Cambridge Electronic Designs Ltd, Cambridge, UK). All recordings were made with the same bioamplifier calibrated to a pre-set setting 50 μV.

Following surgical preparation, animals were then stabilised for 30 min. At the beginning of the stabilization period, an arterial blood sample was collected and analysed. After the initial blood gas measurement, arterial blood gases were then corrected as required by adjusting the ventilator pump rate and volume and/or slow bolus injections of 5% sodium bicarbonate to maintain parameters within the following range for control conditions: pH = 7.4 ± 0.5; PCO2 = 40 ± 5 mmHg; HCO3- = 24 ± 2 mmol/L; and end tidal CO2 (EtCO2) = 4.5 ± 0.5%. Repeat blood gas analysis was undertaken as required.

5.3.3. Experimental Design

Cohort 1; CSN transection: After the initial period of stabilization AP and heart rate were recorded, prior to CSN transection. Following transection of CSN, the animal was rotated, the other nerves were dissected and prepared for recording and then 30 minutes was allowed to stabilize the AP, EtCO2 and nerve activity. The integrity of renal and splanchnic nerve recordings was confirmed by both pulse modulation of SNA, and demonstration of a baroreflex response to a bolus injection of phenylephrine (50 μg/kg i.v, Sigma-Aldrich, St. Louis, MO, USA). Completeness of CSN transection was assessed by the respiratory response (PNA) to a hypoxic stimulus of 10% O2/ N2 for 45 sec (Figure 1). The changes in AP were not assessed for completeness of CSN transection due to variability of AP changes in response to hypoxia. The recovery period from these stimuli was 30 mins. After that, animals

were ventilated with oxygen enriched room air for a period of 30 mins and AP, heart rate, PNA, sSNA and rSNA were recorded.

Cohort 2; CSN intact: CSN intact animals underwent surgical procedures to enable recording of SNA and PNA and then were allowed a period of 30 minutes to stabilize the AP, EtCO2 and nerve activity. After that, the integrity of renal and splanchnic nerve recordings was confirmed by both pulse modulation of SNA, and demonstration of a baroreflex response to a bolus injection of phenylephrine (50 µg/kg i.v, Sigma-Aldrich, St. Louis, MO, USA). The recovery period from this stimulus was 30 mins. After that, animals were ventilated with oxygen enriched room air for a period of 30 mins and AP, HR, PNA, sSNA and rSNA were recorded.

At the end of the experiments, all animals were euthanized with potassium chloride (3M i.v.) and the background noise levels for PNA, sSNA and rSNA recorded and subtracted for data analysis.

5.3.4. Data analysis

All data were analysed offline using Spike 2 software. From the AP signal, mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse pressure (PP) pressure and heart rate (HR) were derived. The sSNA and rSNA were rectified and smoothed with a time constant of 0.1 sec and PNA was rectified and smoothed with a time constant of 0.05 sec. In cohort 1, the control cardiovascular data was determined from a 30 second period at the end of initial stabilization period prior to CSN transection, and the case data was recorded during the period of 30 mins recording following CSN transection and recovery period. The control data for SNA and respSNA from animals of cohort 2 was obtained during the period of 30 mins recording following initial stabilization period.

The amplitude of PNA, frequency of PNA (number/cycle min $^{-1}$), duration of PNA (sec) and minute phrenic nerve activity (MPA=PNA amplitude x PNA frequency) were measured to quantify the phrenic changes.

For analysis of respSNA parameters, phrenic triggered averaging of rectified and smoothed sSNA and rSNA recordings from the onset of PNA was performed offline. The phrenic cycle and corresponding sSNA and rSNA were divided into three phases: inspiratory (I), post-inspiratory (PI) and expiratory (E) based on phrenic inspiratory discharge (from the onset to the end of augmentation of phrenic bursts). From the phrenic triggered integrated sSNA and rSNA the following characterizing parameters were calculated, the peak amplitude (PA [µV]),

duration (from onset of activity to return to baseline [sec]) and area under curve (AUC) of respSNA peak (μV. s) by integral of waveform of Spike 2.

5.3.5. Statistical analysis

All data are presented as mean ± SEM. A Student's t-test was used to determine baseline differences in cardiovascular and renal function between the LPK and Lewis combining control data of both cohorts. A two-way ANOVA with repeated measures followed by Bonferroni's post-hoc analysis was used to analyse the effect of CSN transection on cardiovascular changes with strain and CSN transection as the variables. A ordinary two-way ANOVA followed by Bonferroni's post-hoc analysis was used to analyse the effect of CSN transection on respiratory sympathetic coupling with strain and CSN transection as the variables. All analysis was performed using GraphPad Prism software v6.0 (GraphPad Software Inc., La Jolla, California, USA). Differences were considered statistically significant where $P \leq 0.05$.

5.4. Results

The LPK rats exhibited impaired renal function as evidenced by higher serum creatinine (77.6 ± 7.1 vs 19.9 ± 3.6 µmol/L; $P < 0.0001$), urea (26 ± 1.6 vs 6.0 ± 0.3 mmol/L; $P < 0.0001$) and lower creatinine clearance rate (1.5 ± 0.1 vs 10.9 ± 1.7 ml/min; $P < 0.001$). They also had higher MAP (129 ± 4 vs 82 ± 3 mm of Hg; $P < 0.0001$) and HR (471 ± 4 vs 450 ± 6 bpm; $P < 0.05$) compared to Lewis rats.

5.4.1. Cardiovascular response to CSN transection

To determine the effect of CSN transection on cardiovascular function we compared MAP and heart rate before and after CSN transection in both LPK and Lewis animals. Representative traces are shown in Figure 2. Two-way analysis of variance indicated that there was a significant effect of strain on MAP ($P = 0.03$), a trend towards a significant effect of CSN transection, but this did not reach significance ($P = 0.07$) and a significant interaction between strain and CSN transection (P=0.02). Further post-hoc analysis revealed that in the LPK MAP was significantly reduced by CSN transection (-28 ± 9 mmHg, adjusted $P = 0.01$). In the Lewis, however, MAP was not altered significantly by CSN transection (4 ± 8 mmHg, adjusted $P = 0.99$). A similar effect was observed for SBP (Table 1). Diastolic blood pressure was significantly higher in intact LPK rats compared to intact Lewis rats while, there was no significant difference between the strains following CSN transection. However, there was no effect of CSN transection on DBP in both Lewis and LPK rats ($P > 0.05$). In addition, PP did not exhibit any strain effect before and after CSN transection, however there was a significant increase in PP in Lewis rats following CSN transection. In contrast, two-way analysis of variance indicated that while there was an overall effect of strain on HR ($P < 0.001$), again consistent with the phenotypic elevation in HR observed in the LPK, there was no effect of CSN transection or interaction observed (both $P > 0.05$; Table 1, Figure 2).

5.4.2. Comparison of respiratory parameters and SNA between CSN intact and transected LPK and Lewis animals

In cohort 2, PNA and SNA from the CSN intact rats were determined and between group comparisons were made to identify if CSN transection altered any respiratory parameters or the level of SNA. Two-way analysis of variance demonstrated that there was no overall effect of strain or treatment group on phrenic nerve frequency (both $P > 0.05$) (Table 2) however a significant strain versus treatment effect was observed ($P = 0.03$). Post-hoc analysis revealed that this interaction was driven by a higher phrenic nerve frequency in the CSN intact LPK

animals compared with CSN intact Lewis animals ($P < 0.05$) that was not apparent between the CSN transected groups ($P = 0.81$). Phrenic nerve frequency was significantly lower in the CSN transected versus CSN intact LPK groups (-13 ± 5, adjusted $P < 0.05$; Table 2). No strain or group differences were observed for either phrenic nerve amplitude or MPA. For both rSNA and sSNA, two-way analysis of variance indicated an overall effect of strain such that SNA was elevated in LPK groups compared with Lewis controls (both $P < 0.05$, Table 2). There was no overall effect of treatment group nor was there a strain versus treatment effect. The low frequency component of SBP variability provides a surrogate measure for SNA which we have shown in the LPK to be strongly correlated with sympathetic vasomotor tone (Ameer *et al.*, 2014). We therefore analysed the low frequency component before and after CSN transection in both LPK and Lewis animals. Two-way ANOVA showed that there was no overall effect of CSN transection on the low frequency component of SBP in the two strains ($P = 0.08$, data not shown).

5.4.3. Comparison of respiratory sympathetic coupling between CSN intact and CSN transected LPK and Lewis animals

Finally, to examine the hypothesis that peripheral chemoreceptor input may be driving the augmented respiratory sympathetic coupling that we have previously reported in the LPK, we compared the AUC and PA of the respSNA for both the renal and splanchnic nerves between the CSN intact and transected groups. Example tracings of respSNA are in Figure 3.

Consistent with our previous work, two-way ANOVA showed an effect of strain on the AUC and PA of both renal and splanchnic respSNA with post-hoc analysis demonstrating that these parameters were significantly elevated in the LPK compared with Lewis (all $P < 0.05$). There was no effect of CSN transection on any of these parameters (all $P > 0.05$) and no interaction between strain and treatment (Table 3).

5.5. Discussion

Here we provide evidence showing that peripheral chemoreceptor denervation using bilateral transection of CSN reduces blood pressure in the LPK model of CKD. However, this denervation did not cause a reduction in the elevated respSNA suggesting that the origin of enhanced respiratory sympathetic coupling in the LPK is not primarily driven by the peripheral chemoreceptors and may be central in origin.

Enhanced activity of peripheral chemoreceptors is believed to contribute to hypertension in primary hypertension and other diseases including CKD, chronic heart failure (CHF) and diabetes (Phillips, 2005; Marcus *et al.*, 2014b; Machado *et al.*, 2017; Iturriaga, 2018). Our main finding that blood pressure was reduced following bilateral CSN transection in LPK rats is consistent with previous studies of bilateral CSN transection in the SHR model of essential hypertension, where blood pressure was reduced by approximately 20 mm of Hg in prehypertensive and adult SHR rats after CSN transection (Abdala *et al.*, 2012). Low frequency SBP variability was improved in CHF rabbit model after CSN transection (Marcus *et al.*, 2014a), however, we did not see a significant change in the LPK. Furthermore, the LPK rats did not show any bradycardia in response to CSN transection. Similarly, CSN transected SHR rats after 9 weeks of CSN transection exhibited a comparable response in heart rate compared to CSN transected Wistar rats (Abdala *et al.*, 2012). These particular findings would suggest that CSN transection has no direct effect on parasympathetic component of blood pressure regulatory mechanisms involving the heart in primary and secondary hypertension, including in CKD.

In our study, the observed sympathetic overactivity in LPK rats was not reduced by the acute CSN transection procedure. Reports of clinical studies in humans have shown that denervation of the carotid body (CB) contributes to a decrease in blood pressure which coincides with a reduction of SNA in responder groups (Narkiewicz *et al.*, 2016; Iturriaga, 2018). This has also been demonstrated in animal studies where ablation of the CSN results in a reduction of SNA and BP in SHR rats and Goldblatt hypertensive rats (Abdala *et al.*, 2012; Pijacka *et al.*, 2016). However, the onset of the beneficial effect of CSN ablation on SNA and blood pressure and its duration are not consistent. In a study on SHR rats, an immediate reduction in blood pressure and rSNA was observed in rats with in-situ preparation after acute CSN transection, as well as in conscious rats following acute inhibition of carotid body chemoreceptors by 100% O_2. However, SNA in fact increased immediately following CSN transection and was noted as significantly reduced chronically from day 5 of recovery post-

surgery (McBryde *et al.*, 2013). In addition, resting rSNA was also decreased in conscious CHF rabbit models after 3 days of recovery post procedure (Marcus *et al.*, 2014a). A pilot study on humans with resistant hypertension demonstrated a significant decrease in ambulatory blood pressure from baseline at six months following unilateral CSN ablation (Schlaich *et al.*, 2017). Therefore, it is credible that the effect of CNS ablation on SNA varies in different experimental conditions, species and duration from surgery.

Another key finding of our study was that respiratory frequency was improved in LPK rats with CSN transection compared to the CSN intact group, with a similar response in other variables of central respiratory drive (phrenic amplitude and phrenic duration) between the strains. These findings are consistent with reducing augmented resting respiratory responses in CHF and obstructive sleep apnoea (OSA) models of rats (Del Rio *et al.*, 2013; Del Rio *et al.*, 2016) and SHR rats (Abdala *et al.*, 2012). The reduction of ventilatory responses may provide additional symptomatic benefits in disease states including CHF. However, in a safety trial of CSN ablation on patients with resistant hypertension, unilateral carotid body resection increased apnoeic episodes in one patient with OSA and also decreased O_2 saturation levels in the blood without any change in respiratory rates (Narkiewicz *et al.*, 2016). Similarly, bilateral carotid body ablation also caused O_2 desaturation at night in patients with resistant hypertension (Niewinski *et al.*, 2017). These observations warrant further investigations to evaluate the safety profile of CSN ablation in clinical settings.

In our previous study, we demonstrated that in response to hypoxia, the magnitude of amplified respSNA and blood pressure was greater in LPK rats compared to the Lewis controls, whereas these variables were increased to a similar degree between the strains in response to central chemoreceptor stimulation (Chapter 2). These findings suggest that peripheral chemoreceptors activity may regulate respSNA, sympathetic activity and hypertension in CKD, as demonstrated in different animal models where both hypoxia induced sympathetic activity and ventilatory responses were abolished in CHF rabbits and OSA rats (Marcus *et al.*, 2014a; Shin *et al.*, 2014; Del Rio *et al.*, 2016). RespSNA was also reduced in CHF rabbits following denervation of carotid body (Marcus *et al.*, 2014a). In our present study, there was no effect of CSN transection on the tonic level of SNA and respSNA in LPK rats. Although we did not investigate the underlying mechanism of this outcome, it is plausible that alternate pathways such as central chemoreflex and atrial stretch reflex (Campagna & Carter, 2003) may be activated immediately to counteract and maintain SNA in these rats following acute CSN transection. However, the immediate blood pressure

response in LPK rats after acute CSN transection suggests that peripheral chemoreceptor inputs do make a significant contribution to short term regulation of blood pressure control, and this is not mediated by renal and/or splanchnic sympathetic outflow. Future studies under a recovery experimental protocol will be valuable to explore this further in a chronic setting.

5.6. Conclusion

Our study provides evidence that acute CSN transection immediately attenuates high blood pressure in an animal model of CKD under in-vivo experimental conditions. This mechanistic link unveils a contributory role of peripheral chemoreceptors in the genesis and maintenance of hypertension in CKD, however, further studies are required to identify the exact mechanisms by which peripheral chemoreceptors activate hypertension in this disease. Nonetheless, peripheral chemoreceptors could be a potential therapeutic target to treat resistant hypertension in CKD.

5.7. Acknowledgements

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5.8. Disclaimer

All authors declared no competing interests.

Table 5.1 Effect of carotid sinus nerve transection on parameters of cardiovascular responses in Lewis and Lewis Polycystic Kidney rat

	CSN intact		CSN transected		P value		Interaction
	LPK (6)	Lewis (7)	LPK (5)	Lewis (7)	Strain	CSN	
MAP (mmHg)	140 ± 6*#	90 ± 5	111 ± 6	94 ± 9	0.002	0.072	0.028
SBP (mmHg)	194 ± 14*#	123 ± 7	155 ± 19	140 ± 13	0.025	0.278	0.0146
DBP (mmHg)	112 ± 7*	71 ± 4	87 ± 4	73 ± 8	0.006	0.112	0.0769
PP (mmHg)	82 ± 19	53 ± 6#	69 ± 17	67 ± 6	0.328	0.592	0.0083
HR (bpm)	471 ± 6*	447 ± 5	473 ± 4*	442 ± 7	0.009	0.805	0.6226

Measures of cardiovascular function in Lewis and Lewis Polycystic Kidney (LPK) rats before and after carotid sinus nerve (CSN) transected. MAP: mean arterial pressure, SBP: systolic blood pressure, DBP: diastolic blood pressure, PP: pulse pressure, HR: heart rate, bpm: beats per min; Results are expressed as mean ± SEM. #Significantly different between before and after CSN transection within same strain ($P \leq 0.05$), * Significantly different between the strains in similar condition ($P \leq 0.05$) as determined using two-way repeated measures of ANOVA followed by Bonferroni's *post-hoc* correction. *n* = number of animals per group.

Table 5.2 Effect of carotid sinus nerve transection on parameters of ventilatory responses and SNA in Lewis and Lewis Polycystic Kidney rat

	CSN intact		CSN transected		P value		Interaction
	LPK (6)	Lewis (7)	LPK (6)	Lewis (7)	Strain	CSN	
PNA amplitude (μV)	22.9 ± 3.03	17.9 ± 5.7	22.2 ± 3.3	16.8 ± 1.6	0.19	0.820	0.9512
PNA frequency (cycles. min^{-1})	49 ± 2*#	37 ± 4	36 ± 2	41 ± 4	0.225	0.272	0.0272
PNA duration (sec)	0.68 ± 0.05	0.85 ± 0.05	0.82 ± 0.08	0.89 ± 0.07	0.073	0.176	0.4782
MPA	1107.7 ± 133.9	634.1 ± 193	806.3 ± 125.1	694.3 ± 99.3	0.577	0.407	0.2310
rSNA (μV)	8.4 ± 1.2*	4.6 ± 0.6	9.3 ± 1.7*	4 ± 0.3	0.0002	0.844	0.4692
sSNA (μV)	6.6 ± 0.9*	4.2 ± 0.5	5.9 ± 0.6*	3.4 ± 0.4	0.0006	0.277	0.9052

sSNA, splanchnic sympathetic nerve activity; rSNA, renal sympathetic nerve activity; PNA, Phrenic nerve activity; MPA, Minute phrenic nerve activity (PNA amplitude X PNA frequency), LPK, Lewis Polycystic Kidney; CSN, carotid sinus nerve; #Significantly different between the cohorts of animals with intact vs. transected CSN within same strain ($P \leq 0.05$), * Significantly different between the strains in similar condition ($P \leq 0.05$) as determined using two-way ordinary ANOVA followed by Bonferroni's *post-hoc* correction. *n* = number of animals per group.

Table 5.3 Effect of carotid sinus nerve transection on respiratory sympathetic coupling in Lewis and Lewis Polycystic Kidney rat

	CSN intact		CSN transected		P value		Interaction
	LPK	Lewis	LPK	Lewis	Strain	CSN	
rSNA (n)	6	7	6	7			
AUC	9.6 ± 1.9*	4.5 ± 0.7	10.5 ± 2.4*	3.8 ± 0.5	0.002	0.689	0.6025
PA	11.5 ± 2.5*	6.1 ± 0.6	10.9 ± 2.1*	5.7 ± 0.7	0.002	0.721	0.9568
Duration	0.88 ± 0.1	0.91 ± 0.1	0.94 ± 0.05	0.81 ± 0.1	0.589	0.850	0.3956
sSNA (n)	6	7	6	7			
AUC	7.03 ± 0.9*	4.3 ± 0.6	6.7 ± 0.8*	3.4 ± 0.5	0.0004	0.413	0.6857
PA	9.5 ± 1.2*	5.4 ± 0.5	7.4 ± 0.7	4.9 ± 0.7	0.0005	0.11	0.3506
Duration	0.89 ± 0.08	0.88 ± 0.08	1.03 ± 0.09	0.9 ± 0.1	0.422	0.46	0.5424

sSNA, splanchnic sympathetic nerve activity; rSNA, renal sympathetic nerve activity; AUC, area under curve; PA, peak amplitude; LPK, Lewis Polycystic Kidney; CSN, carotid sinus nerve; * Significantly different between the strains in similar condition ($P \leq 0.05$) as determined using two-way ordinary ANOVA followed by Bonferroni's *post-hoc* correction. *n* = number of animals per group

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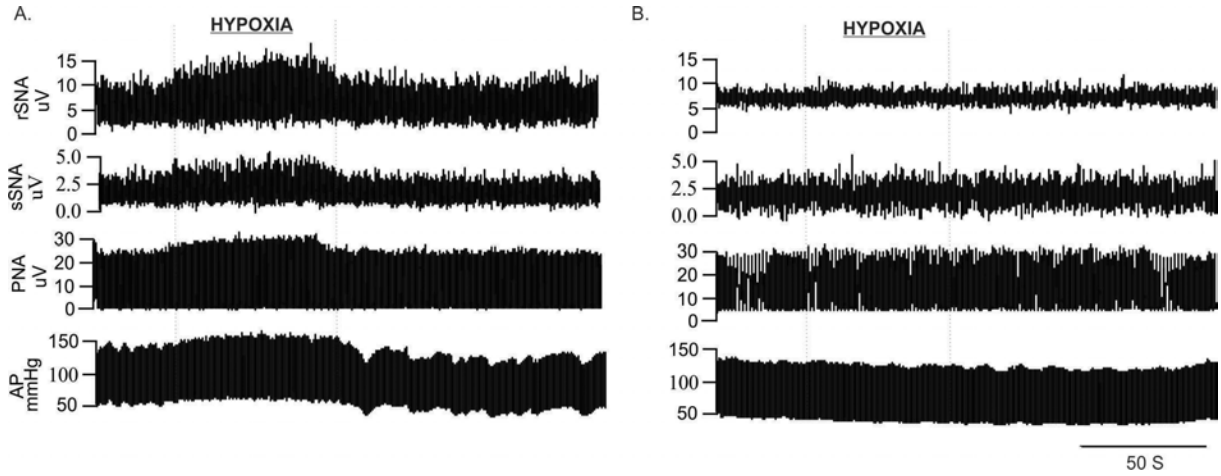

Figure 5.1. Representative traces showing sympathetic nerve activity, phrenic nerve activity and blood pressure during hypoxia in carotid sinus nerve intact and transected adult Lewis rat

Representative data traces showing integrated renal SNA (∫rSNA), splanchnic SNA (∫sSNA), phrenic nerve activity (∫PNA) and raw arterial pressure (AP) response to hypoxia (10% O2/ N2 for 45 sec) in carotid sinus nerve intact (A) and transected (B) adult Lewis rat under urethane anaesthesia.

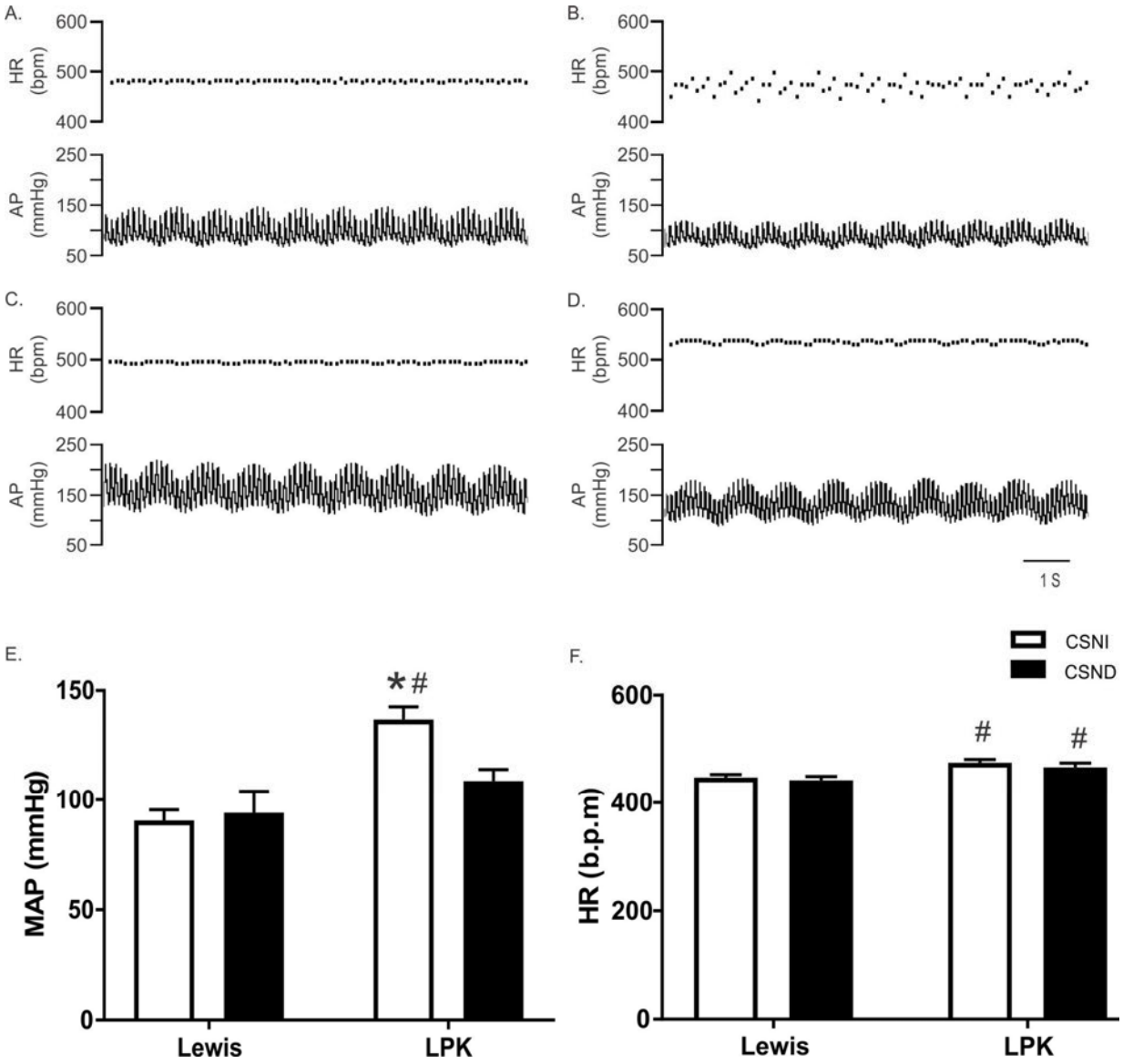

Figure 5.2 Effect of carotid sinus nerve transection on parameters of cardiovascular responses Lewis Polycystic Kidney rat

Example figure illustrating blood pressure and heart rate before and after carotid sinus nerve (CSN) transection in a Lewis rat (panels A, B) and LPK rat (panels C, D). Grouped data is shown in panels E-F illustrating the mean arterial pressure (MAP; E), and heart rate (HR; F) before CSN transection (control/intact) and after CSN transection. CSNI; carotid sinus nerve intact, CSNT; carotid sinus nerve transection. Data is expressed as mean ± SEM n ≥5 per group. * represents P<0.05 versus control (CSNI) within each strain, # represents P<0.05 versus treatment-matched Lewis.

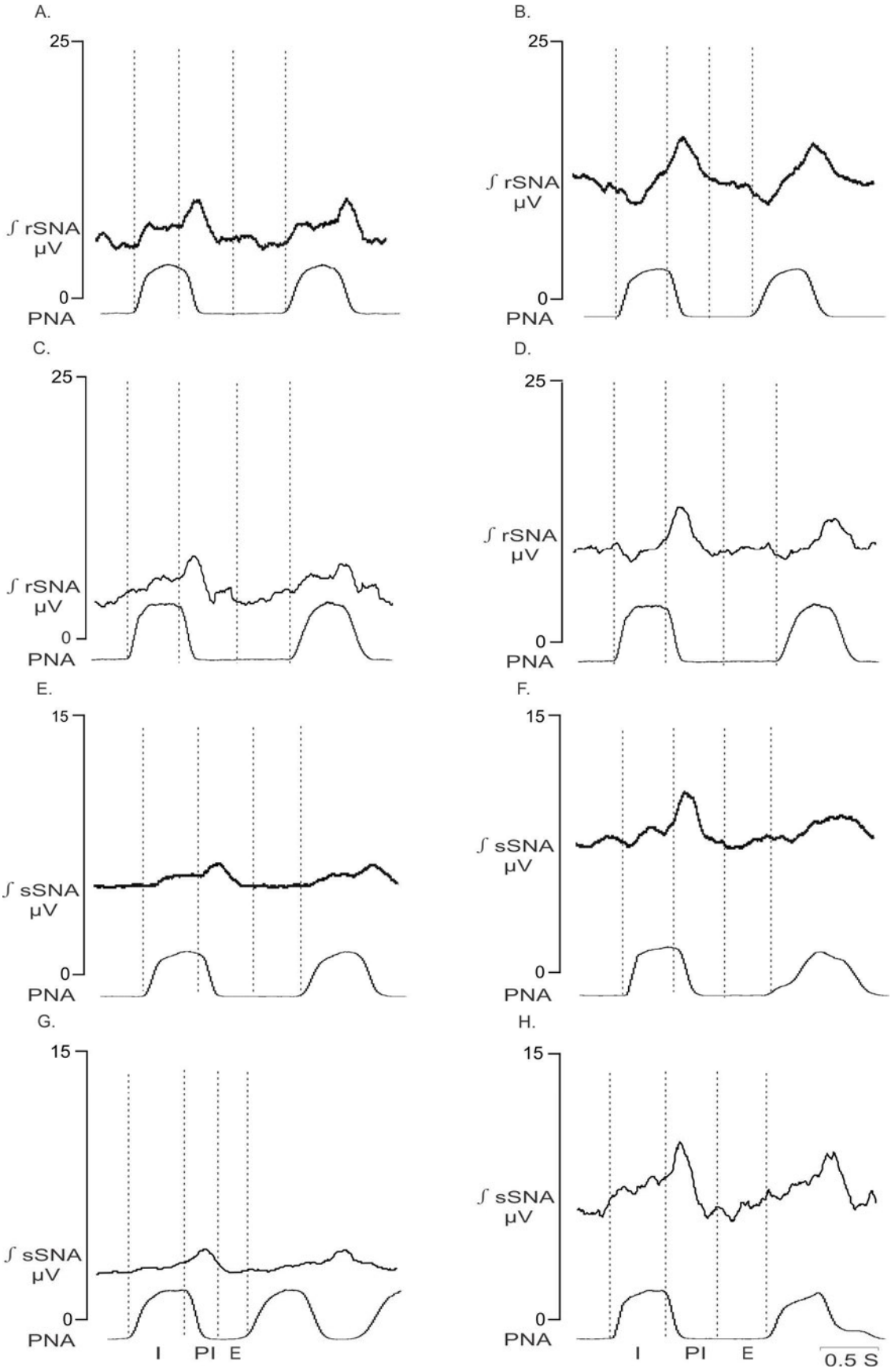
A.
25
∫ rSNA
μV
0
PNA
B.
25
∫ rSNA
μV
0
PNA
C.
25
∫ rSNA
μV
0
PNA
D.
25
∫ rSNA
μV
0
PNA
E.
15
∫ sSNA
μV
0
PNA
F.
15
∫ sSNA
μV
0
PNA
G.
15
∫ sSNA
μV
0
PNA
I PI E
H.
15
∫ sSNA
μV
0
PNA
I PI E
0.5 S

Figure 5.3 Tracings of respiratory-related sympathetic nerve activity in Lewis and LPK rats with intact and transected carotid sinus nerve

Example figure illustrating phrenic triggered integrated rSNA (∫rSNA) and sSNA (∫sSNA) during different phases of the phrenic cycle (I, inspiration; PI, post inspiration; E, expiration within trace of phrenic nerve activity (PNA) in CSN intact animals (panels A, B, E, F) and CSN transected animals (panels C, D, G, H) in a Lewis rat (panels A, C, E,G) and LPK rat (panels B, D, F, H).

6. Final Discussion

Chronic kidney disease is a progressive condition where hypertension both independently and synergistically contributes to the underlying pathogenesis and risk of severe cardiovascular events and morbidity. A comprehensive evidence-based management approach is crucial to control hypertension in CKD, aiming to limit the progression and complications of the disease. To date, our knowledge related to the factors underpinning hypertension specific to CKD is limited. Consequently, hypertension is often difficult to treat in CKD, with patients in the advanced stage of the disease requiring multiple pharmacological agents. In this context, the main focus of this thesis was to explore a novel pathophysiological concept to explain the altered regulation of blood pressure in CKD. Accordingly, the thesis delineated the following key facts:

1. Respiratory sympathetic coupling is amplified at a very early age and is associated with increased SNA and hypertension in CKD;

2. Altered respiratory sympathetic coupling influences baroreflex function in CKD and resets baroreflex target pressure to a higher level; and

3. Input from peripheral chemoreceptors is one of the contributing factors of hypertension in CKD.

6.1. Respiratory sympathetic coupling is augmented in chronic kidney disease: A new direction in chronic kidney disease research

The role of the sympathetic nervous system in the regulation of vasomotor tone is to maintain adequate blood flow and normal blood pressure. Direct recording of MSNA (Klein *et al.*, 2003a; Neumann *et al.*, 2007; Grassi *et al.*, 2011b), estimation of plasma noradrenaline (Levitan *et al.*, 1984; Masuo *et al.*, 1995; Zoccali *et al.*, 2002; Grassi *et al.*, 2011a), spectral analysis of blood pressure and heart rate (Lewanski & Chrzanowski, 2003) and inhibition of cardiovascular responses with sympatholytic agents (Levitan *et al.*, 1984; Furgeson & Chonchol, 2008; Badve *et al.*, 2011) provide clear evidence of hyperactive SNA in CKD patients with hypertension. Moreover, a hyperactive sympathetic system is causally associated with cardiovascular diseases in different stages of CKD. However, the underlying mechanisms of sympathetic overactivity in CKD are not well understood, despite a wealth of literature, both preclinical and clinical on the topic. The concept of respiratory sympathetic modulation is gaining traction as a critical factor that is altered in association with a number of different diseases, with evidence that amplified respiratory sympathetic modulation

contributes to sympathetic overactivity in primary and secondary hypertension as well, in association with other conditions such as heart failure (Goso *et al.*, 2001; Simms *et al.*, 2009; Toney *et al.*, 2010). Most striking is the link between altered respiration and sympathetic nerve activity in response to CIH, that results in a sequence of events leading to augmentation of respiratory sympathetic coupling, increased SNA and hypertension in animal models (Zoccal *et al.*, 2008). Therefore, the key aim of this thesis is to determine whether a similar relationship between breathing and SNA occurs in the secondary hypertension observed in CKD.

It has been well documented that the respiratory pattern of SNA differs among nerves, species and experimental conditions (Habler *et al.*, 1994). In this context, we tested respiratory sympathetic coupling in different preparations (*in vivo*, *in situ*), in variable conditions (hypoxia/hypercapnia) and in three different nerves in an animal model of CKD. Testing the same mechanism such as respiratory sympathetic coupling in LPK rats using the two well-established experimental protocols allowed us to validate our key question. The anaesthetised *in vivo* preparation keeps the integrity of the animal with all its complexity; however, anaesthesia does depress/modulate central respiratory control and cardiovascular regulation. While the *in situ* preparation is allowing us to examine respiratory sympathetic coupling in the LPK rats at a younger age (5 weeks), and it delivers physiological patterns of central respiratory control and cardiovascular regulation, devoid of the depressive/modulatory effects of anaesthesia, (Wilson *et al.*, 2001) however, it is a reduced, decerebrated, perfused preparation. Moreover, a number of previous studies examining respiratory sympathetic coupling similarly used the *in situ* preparation during the juvenile period to reach their conclusions (Zoccal *et al.*, 2008; Simms *et al.*, 2009). By undertaking our study under the same experimental conditions, this thesis is better placed to compare these results. Given that both hypoxia and hypercapnia induce respiratory related changes in PNA and SNA by regulating the strength of respiratory related discharges, the variable conditions (hypoxia/hypercapnia) allowed us to test respiratory sympathetic coupling rather than only tonic SNA in two different respiratory stimulating conditions and also determine the magnitude of respiratory sympathetic coupling in these conditions. Furthermore, in the Ang II-salt induced rat model of hypertension, enhanced respiratory sympathetic coupling results in increased splanchnic sympathetic tone whereas renal nerve shows reduced sympathetic response, while the lumbar nerve shows no change (Toney *et al.*, 2010). The use of different nerve beds, therefore, allowed us to establish whether or not altered respiratory sympathetic coupling occurs uniformly in different nerves in CKD. In our *in vivo* studies we examined

both the splanchnic nerve and the renal nerves. Splanchnic SNA is believed to be a critical nerve bed, and in the deoxycorticosterone acetate (DOCA) salt rat model of hypertension, has shown to contribute to the development of hypertension even in the absence of generalised sympathoexcitation as measured using whole body noradrenaline spill over (Kandlikar & Fink, 2011). Similarly, the renal nerve is essential in the regulation of GFR, renin secretion, salt/water excretion, vascular resistance and blood pressure and a well-known marker of SNA (DiBona, 2000a, b; Johns *et al.*, 2011). In our *in situ* preparation thoracic nerve was studied. For this preparation, there has been prior evidence of enhanced respiratory sympathetic coupling as demonstrated using the tSNA in the SHR model in a similar experimental preparation (Zoccal *et al.*, 2008; Simms *et al.*, 2009).

This thesis has clearly demonstrated in all nerves studied (sSNA, rSNA and tSNA) that PI peak of respiratory sympathetic coupling in LPK rats is augmented in both the *in vivo* and *in situ* preparation, as well as during peripheral and central chemoreceptors stimulation (Chapter 2-5). Moreover, the female LPK rats exhibit similar patterns of augmented respiratory sympathetic coupling like male LPK rats. This enhancement of respiratory sympathetic coupling is comparable to previous work in the SHR (Simms *et al.*, 2010; Menuet *et al.*, 2017), animal model of CIH (Zoccal *et al.*, 2008), congestive heart failure (Marcus *et al.*, 2014b); and in rats following uteroplacental insufficiency (Menuet *et al.*, 2016). However, we did not see the temporal shift in the peak response from PI phase to I phase that is described in the SHR during the development of hypertension *in situ* (Simms *et al.*, 2009; Menuet *et al.*, 2017) and in adult SHRs *in vivo* (Czyzyk-Krzeska & Trzebski, 1990). The present body of work has also revealed respiratory sympathetic coupling is altered at an early age in the LPK animal model which is consistent with our previous evidence that SNA is elevated in the early stages of disease, prior to development of overt renal failure (Klein *et al.*, 2001; Phillips *et al.*, 2007; Hildreth *et al.*, 2013a; Hildreth *et al.*, 2013b; Salman *et al.*, 2014). These results are consistent with the hypothesis that altered respiratory sympathetic coupling underpins the development and maintenance of autonomic dysfunction and hypertension in CKD, similar to what is seen in the SHR model of essential hypertension (Simms *et al.*, 2010).

The interaction between respiratory neurons and PSNs in the RVLM generate rhythmic oscillations in SNA during different phases of respiration (Habler *et al.*, 1994; Guyenet, 2006). The common feature of respiratory sympathetic coupling in rats is of an I peak, or an I inhibition with a peak in PI, which functions to provide ventilation and perfusion of the lung (Haselton & Guyenet, 1989; Habler *et al.*, 1994). Our model of CKD rats expressed a

signature respiratory sympathetic coupling of a PI peak with inhibition of SNA during the inspiratory period which was enhanced during hypoxia. This shape of respiratory sympathetic coupling is consistent between the male and female LPK rats, however, one difference we did note was that in the sSNA under hypoxic conditions the female LPK rats showed persistence of excitation in expiration, which was in contrast to the finding of male LPK rats where there was no excitation of respiratory sympathetic coupling during expiration. Given that several groups of respiratory related PSNs such as I-inhibited, I-activated, PI-activated or non-modulated exist in the RVLM (Haselton & Guyenet, 1989; Moraes *et al.*, 2013a), we assume that there was an adjustment/alteration in respiratory signals to these respiratory modulated PSNs yielding this specific signature of respiratory sympathetic coupling in CKD.

The central mechanism of the respiratory sympathetic coupling is primarily dependent on the interactions between the respiratory neurons and the PSNs in the RVLM (Haselton & Guyenet, 1989; Dampney, 1994; Sun *et al.*, 1997; Guyenet, 2006) with contributions from the CVLM (Mandel & Schreihofer, 2006), pons (Baekey *et al.*, 2008), RTN (Guyenet *et al.*, 2008; Abdala *et al.*, 2009b; Guyenet *et al.*, 2009a; Molkov *et al.*, 2011) and NTS (Costa-Silva *et al.*, 2010). However, what triggers the alteration in the central mechanism of respiratory sympathetic coupling in pathological condition, is not well understood. It has been suggested that in the CIH conditioned rat model, the plasticity of central chemoreceptors is a key underlying mechanism of altered respiratory sympathetic coupling. A reduction of the inhibitory impulse from the BötC PI neurons to the RTN following CIH may contribute to this plastic change of central chemoreceptors (Molkov *et al.*, 2010; Molkov *et al.*, 2011; Molkov *et al.*, 2014; Moraes *et al.*, 2014a). Furthermore, the respiratory sympathetic coupling may be modulated by the input from afferent receptors of autonomic reflexes like baroreceptors and pulmonary stretch receptors (Boczek-Funcke *et al.*, 1992; Habler *et al.*, 1994; Bailey *et al.*, 2001; Bernardi *et al.*, 2001; Zoccal *et al.*, 2014) and peripheral chemoreceptors can also cause plasticity in the central chemoreceptors of the RTN through the neurons of the NTS (Guyenet *et al.*, 2009a; Molkov *et al.*, 2014; Moraes *et al.*, 2015). Intriguingly, in the LPK rat, the respiratory sympathetic coupling in the vagotomised and decerebrated rats is enhanced to an even greater degree during peripheral chemoreceptor stimulation; however, this amplification of respiratory sympathetic coupling in LPK rats was not reduced after bilateral CSN transection. Zoccal et al. also reported similar persistent late E sympathetic discharge after exposure to CIH in rats with carotid body denervation (Zoccal *et al.*, 2008). Accordingly, we speculate that in CKD, central mechanisms in the medulla may be primarily involved in augmented respiratory sympathetic coupling and associated with

increased SNA; however, peripheral chemoreceptors may be indirectly involved in respiratory sympathetic coupling. Moreover, other central mechanisms in the brain stem such as hypersensitivity of central chemoreceptors could also contribute to sympathetic hyperactivity and hypertension in CKD. We acknowledge that the thesis did not explore central control of respiration or autonomic functions and agree that this is an important area of study, however, it was not possible within the scope of a PhD project and time line. Further studies in the LPK rat model are required to delineate the exact central mechanisms of augmented respiratory sympathetic coupling and to explore other central mechanism targeting central chemoreceptors. For example, a series of experiments involving simultaneous recording from PSNs and respiratory neurons in the brainstem of LPK rats are critical to elicit the central origin of respiratory sympathetic coupling in CKD.

6.2. Interaction between central respiratory networks and the baroreflex in chronic kidney disease

Central respiration has a strong impact on the ability of the baroreceptor reflex to produce changes in the autonomic outflow. There are several studies which suggest that respiration influences baroreflex responses as evidenced by variable responses of heart rate and SNA after increase in blood pressure during different phases of respiratory cycle. For example, barosensitivity of heart rate was intensified if blood pressure increased in early expiration (Gilbey *et al.*, 1984) and the sympathoinhibitory component of the baroreflex was less when the stimulus was given in inspiration compared to expiration (Baekey *et al.*, 2008). Accordingly, these observations indicate that the respiratory cycle acts as a gate within central baroreflex pathways.

The interaction between breathing and modulation of the baroreflex in disease is less known. In a recent study, it has been found that enhanced respiratory sympathetic coupling induced by CIH, improved the gain of the sympathoinhibitory component of baroreflex which prevented a further increase of blood pressure (Moraes *et al.*, 2016). Given that a deficit in central processing of the baroreflex causes the expression of sympathetic baroreflex dysfunction in the LPK (Salman *et al.*, 2014; Salman *et al.*, 2015a) and a significant correlation between amplified respiratory sympathetic coupling and sympathetic baroreflex dysfunction has been revealed in this thesis, it is plausible that amplified respiratory sympathetic coupling may regulate the sympathetic baroreflex function by modulating the central transmission process in the brain stem in CKD.

The most striking observation regarding the interaction of respiration and baroreflex dysfunction shown in this thesis is that, despite an increase in respiratory sympathetic coupling is associated with the reduction of gain and increase in MAP50/centring point of sympathetic baroreflex function curve, the reduction of amplified respiratory sympathetic coupling or inhibition of central respiratory drive does selectively reset the sympathetic baroreflex curve to operate at a lower level of blood pressure without any change in the baroreflex sensitivity. This finding suggests that alteration of central processing of baroreflex function or atypical respiratory regulation of SNA is not the prime underlying mechanism in baroreflex dysfunction in CKD. As the selective improvement of sympathetic baroreflex dysfunction curve after suppression of central respiratory drive was not accompanied with a reduction of baseline blood pressure, it is conceivable that central respiratory generator may facilitate the baroreceptor input mostly at the level of the NTS or regulate different sub-groups of neurons of the NTS to modulate the baroreflex mediated sympathetic activity (Molkov *et al.*, 2014; Zoccal *et al.*, 2014), and as such, the baroreflex may work in lower range of blood pressure in the absence of amplified respiratory sympathetic coupling in CKD.

6.3. Peripheral chemoreceptors modulate blood pressure response in chronic kidney disease

There is an indication in different studies that activated peripheral chemoreceptors may contribute to hypertension in essential hypertension (McBryde *et al.*, 2013; McBryde *et al.*, 2017) and other diseases including congestive heart failure and diabetes mellitus (Ribeiro *et al.*; Ponikowski *et al.*, 2001; Marcus *et al.*, 2014a). The activation of peripheral chemoreceptors by acute hypoxia (8% O2 for 30–45s) or acute intermittent hypoxia (10 exposures of 8% O_2 for 45s) in anaesthetised adult rats increases SNA during expiration (Dick *et al.*, 2004; Dick *et al.*, 2008). Furthermore, given the evidence that CIH induces sensitisation of peripheral chemoreceptors, producing an increased expiratory-related SNA (Braga *et al.*, 2006; Zoccal *et al.*, 2008; Moraes *et al.*, 2012), and that increased peripheral chemoreceptor sensitivity contributes to sympathetic over activity and hypertension in the SHR (Tan *et al.*, 2010; Paton *et al.*, 2013b), it is credible that stimulated peripheral chemoreceptors in the carotid body contributing to the augmented respiratory sympathetic coupling, can cause sympathetic hyperactivity and hypertension in other diseases including CKD. Notably, peripheral chemoreceptor hypersensitivity has been suggested to contribute to the development of sympathetic over activity in kidney disease as inhibition of chemoreceptors by hyperoxia reduces MSNA and hyperoxic chemoreflex sensitivity is also impaired in

patients with CKD (Hering *et al.*, 2007; Rassaf *et al.*, 2010a). In addition, renal transplantation results in improvement of hyperoxic chemoreflex sensitivity in ESRD (Rassaf *et al.*, 2010b). Accordingly, one of the main objectives of this thesis was to determine whether the link between peripheral chemoreceptors and respiratory sympathetic coupling in the development of sympathetic overactivity and hypertension in essential hypertension is similar in CKD, given we saw a greater amplification of respiratory sympathetic coupling and a higher blood pressure change in the LPK rats during peripheral chemoreceptors stimulation (Chapter 2 and 3). Interestingly, blood pressure was significantly reduced in LPK rats immediately after bilateral carotid body denervation after transecting the CSNs, however, respiratory sympathetic coupling and SNA were comparable between CSN intact and CSN transected LPK rats in the present study. This finding has significant clinical relevance in light of the recent works done showing improvement of high blood pressure and reduction of sympathetic tone by pharmacological attenuation or inactivation of peripheral chemoreceptors in the SHR and hypertensive humans in conscious and recovery experiments (McBryde *et al.*, 2013; Pijacka *et al.*, 2016), which in the SHR appears to be driven by a reduction of respiratory sympathetic coupling (McBryde *et al.*, 2013; Moraes *et al.*, 2015). The lack of effect on respiratory sympathetic coupling and SNA in the LPK rats could be due to multiple other factors including the central chemoreflex, atrial stretch reflex and a preparation involving the use of muscle relaxants all operating to counteract the inhibitory effect of CSN transection on SNA and respiratory sympathetic coupling in our acute experimental setting. Notably, initial clinical trials investigating the safety of unilateral surgical resection of the carotid body in patients with multi drug-resistant hypertension have demonstrated a 6-month reduction in daytime blood pressure in a cohort of patients (Narkiewicz *et al.*, 2016). Moreover, a recent study shows that combination of carotid body denervation and renal denervation is more effective than isolated renal denervation in cases of resistant hypertension (McBryde *et al.*, 2017). While our study does not directly pinpoint the exact contribution of the peripheral chemoreceptors to sympathetic over-activity in CKD, the reduction of blood pressure after carotid body denervation still suggests that there is a strong rationale to consider peripheral chemoreceptors as one of the drivers for hypertension in CKD. Further studies examining the longer-term impact of CSN dissection using recovery and conscious animal experiments are required to fully explore the role of the peripheral chemoreceptors in the pathophysiology of hypertension in CKD.

Given that both the peripheral chemoreceptors and altered central respiratory sympathetic coupling are working to regulate blood pressure in CKD, it is reasonable that there may be a

common trigger. In disease processes such as hypertension, hypoxia causes enhanced respiratory sympathetic coupling, activation of peripheral chemoreceptors and sympathetic overactivity (Somers *et al.*, 1988; Calbet, 2003; Zoccal *et al.*, 2008), which is proposed to be mediated by hypoxia inducible factor (HIF)-1 and HIF-2 complexes (Deng *et al.*, 2010; Greer *et al.*, 2012; Patinha *et al.*, 2017). Hence, it has been speculated that hypoxia may affect central respiratory sympathetic coupling and peripheral chemoreceptors in similar way in CKD as vascular remodelling (Tanaka *et al.*, 2012; Yamada *et al.*, 2014) and anaemia (Phillips *et al.*, 2015) contribute to persistent hypoxia to carotid body, kidney and brain in CKD.

The prevailing management of hypertension in CKD typically relies on the RAAS inhibitors and vasodilators. Increasing evidence of resistant hypertension in CKD and inadequate evidence for any substantive beneficial effect of renal nerve ablation warrants ongoing research in the search for new and effective antihypertensives. The current studies support an association between sympathetic hyperactivity and amplified central respiratory sympathetic modulation in CKD. Clinically, use of the central sympatholytic antihypertensive drug moxonidine with eprosartan, an ARB, controls blood pressure more effectively than eprosartan therapy alone in CKD patients (Neumann *et al.*, 2004) and the combination also reduces the progression of kidney disease compared to calcium channel blockers in CKD patients (Littlewood *et al.*, 2007). Nevertheless, centrally acting sympatholytic antihypertensives are not widely used in the early stages of CKD, mostly because of side effects. In this context, this thesis provides new insight into the underlying pathogenesis of autonomic failure and hypertension in CKD. The current studies suggest that augmented central respiratory sympathetic coupling potentiates autonomic failure in terms of sympathetic overactivity which causes hypertension and may well contribute to the worsening of renal function in CKD. In addition, inhibition of input from peripheral chemoreceptors causes a decrease of blood pressure in CKD. As a result, both central respiratory sympathetic coupling and peripheral chemoreceptors are important clinical targets for the development of new antihypertensive medications. It is plausible that agents which interact with central components of respiratory sympathetic coupling and thereby, reduce the augmented respiratory sympathetic coupling and play significant role to control hypertension by reducing overall sympathetic drive in CKD. However, more investigations are required to explore the underlying central mechanisms driving hypertension and cardiovascular disease in CKD, and evaluate the safe use of therapeutic interventions aiming central regions and peripheral

chemoreceptors to improve autonomic dysfunction, thereby control blood pressure and halt the progression of renal diseases.

6.4. Conclusion

CKD is a progressive and complex disease. Our current knowledge on the underlying pathogenesis of hypertension in CKD is limited which is a major barrier to the implementation of new therapeutic measures. This thesis highlights a new underlying neuronal mechanism, demonstrating that central amplified respiratory sympathetic modulation is associated with increased SNA and hypertension in CKD, and that central respiratory drive impacts autonomic function. In addition to these outcomes, this thesis suggests the therapeutic benefit of inhibition of peripheral chemoreceptors in the management of hypertension of CKD. This thesis hence provides valuable evidence to warrant targeting the central respiratory sympathetic modulatory network in the brainstem as well as peripheral chemoreceptors as therapeutic interventions.

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8. Appendix

Table 1 Effects of peripheral chemoreceptor stimulation on parameters of cardiovascular responses and ventilatory responses in both male and female Lewis and Lewis Polycystic Kidney rat

	Male		Female		P value	
	LPK (8)	Lewis (9)	LPK (8)	Lewis (9)	Sex	Strain
Δ MAP (mmHg)	21 ± 5	4 ± 4	30 ± 6	12 ± 6	0.146	0.002
Δ SBP (mmHg)	34 ± 11	5 ± 5	41 ± 8	15 ± 6	0.308	0.001
Δ DBP (mmHg)	16 ± 4	4 ± 4	27 ± 6	9 ± 5	0.135	0.007
Δ PP (mmHg)	18 ± 7	1 ± 2	15 ± 5	5 ± 1	0.9564	0.004
Δ HR (bpm)	11 ± 1	12 ± 2 #	23 ± 3	25 ± 6	0.001	0.832
Δ PNA amplitude (μV)	9.8 ± 3.9	10.9 ± 3.6	11.01 ± 1.9	7.3 ± 1.7	0.682	0.657
Δ PNA duration (sec)	0.07 ± 0.01	0.13 ± 0.02	-0.07 ± 0.03	-0.2 ± 0.03	0.095	0.053
Δ PNA frequency (cycles/min)	-2 ± 2	5 ± 2	-10 ± 3	5 ± 2 *	0.061	<0.001
Δ MPA	300.7 ± 66.9	536.6 ± 152.5	290.3 ± 88.6	161.2 ± 116.8	0.098	0.117

Delta change in phrenic nerve activity (PNA) and blood pressure (mmHg) under hypoxic or hypercapnic conditions in adult Lewis and Lewis Polycystic Kidney (LPK) rats. MAP: mean arterial pressure, SBP: systolic blood pressure, DBP: diastolic blood pressure, PP: pulse pressure; HR: heart rate, MPA: minute phrenic activity (PNA amplitude X PNA frequency). Δ = Delta change in, LPK, Lewis Polycystic Kidney; #Significantly different between the cohorts of animals with male vs. female within same strain ($P<0.05$), * Significantly different between the strains in similar sex ($P<0.05$) as determined using two-way ordinary ANOVA followed by Bonferroni's *post-hoc* correction. *n* = number of animals per group. There was significant interaction in only Δ PNA duration ($P<0.05$) between the variables.

Table 2 Effects of central chemoreceptor stimulation on parameters of cardiovascular responses and ventilatory responses in both male and female Lewis and Lewis Polycystic Kidney rat

	Male		Female		P value	
	LPK (8)	Lewis (9)	LPK (8)	Lewis (9)	Sex	Strain
Δ MAP (mmHg)	16 ± 4	19 ± 3	13 ± 3	9 ± 5	0.123	0.878
Δ SBP (mmHg)	29 ± 8	22 ± 4	20 ± 5	12 ± 6	0.098	0.237
Δ DBP (mmHg)	13 ± 3	18 ± 3	10 ± 2	8 ± 4	0.074	0.643
Δ PP (mmHg)	16 ± 6	4 ± 2	10 ± 3	3 ± 2	0.352	0.026
Δ HR (bpm)	-4 ± 1	-8 ± 1	2 ± 3	-0.3 ± 1	0.002	0.180
Δ PNA amplitude (μV)	9.1 ± 1.6	8.7 ± 2.7	9 ± 2.3	9.4 ± 2.8	0.919	0.992
Δ PNA duration (sec)	0.11 ± 0.02	0.15 ± 0.02#	-0.06 ± 0.02	0.8 ± 0.03*	<0.001	<0.001
Δ PNA frequency (cycles/min)	-4 ± 1	-3 ± 1	-2 ± 1	-0.2 ± 1	0.101	0.365
Δ MPA	293.9 ± 71.04	178.5 ± 74.08	274 ± 57.1	309.1 ± 113.5	0.514	0.635

Delta change in phrenic nerve activity (PNA) and blood pressure (mmHg) under hypoxic or hypercapnic conditions in adult Lewis and Lewis Polycystic Kidney (LPK) rats. MAP: mean arterial pressure, SBP: systolic blood pressure, DBP: diastolic blood pressure, PP: pulse pressure; HR: heart rate, MPA: minute phrenic activity (PNA amplitude X PNA frequency). Δ = Delta change in, LPK, Lewis Polycystic Kidney; #Significantly different between the cohorts of animals with male vs. female within same strain ($P<0.05$), * Significantly different between the strains in similar sex ($P<0.05$) as determined using two-way ordinary ANOVA followed by Bonferroni's *post-hoc* correction. *n* = number of animals per group. There was significant interaction in only Δ PNA duration ($P<0.05$) between the variables.

Table 3 Effects of peripheral chemoreceptor stimulation on respiratory sympathetic coupling in both male and female Lewis and Lewis Polycystic Kidney rat

	Male		Female		P value	
	LPK	Lewis	LPK	Lewis	Sex	Strain
sSNA (n)	8	9	8	9		
AUC	5.6 ± 2.1	1.8 ± 0.6	8.9 ± 3.4	2.0 ± 0.7	0.257	0.005
PA	3.9 ± 0.9	1.9 ± 0.6	5.5 ± 2.3	1.8 ± 0.6	0.540	0.028
Duration	0.07 ± 0.07	-0.19 ± 0.1	0.21 ± 0.09	0.12 ± 0.07	0.019	0.067
rSNA (n)	5	6	5	6		
AUC	7.5 ± 2.1	3.0 ± 0.8	6.1 ± 1.2	3.1 ± 0.7	0.592	0.006
PA	4.9 ± 1.2	5.2 ± 1.2	4.9 ± 1.2	3.7 ± 0.9	0.562	0.667
Duration	0.13 ± 0.07	-0.09 ± 0.07	0.09 ± 0.1	0.01 ± 0.06	0.689	0.098

sSNA, splanchnic sympathetic nerve activity; rSNA, renal sympathetic nerve activity; AUC, area under curve; PA, peak amplitude; LPK, Lewis Polycystic Kidney; #Significantly different between the cohorts of animals with male vs. female within same strain ($P<0.05$), * Significantly different between the strains in similar sex ($P<0.05$) as determined using two-way ordinary ANOVA followed by Bonferroni's post-hoc correction. n = number of animals per group.

Table 4 Effects of central chemoreceptor stimulation on respiratory sympathetic coupling in both male and female Lewis and Lewis Polycystic Kidney rat

	Male		Female		P value	
	LPK	Lewis	LPK	Lewis	Sex	Strain
sSNA (n)	8	9	8	9		
AUC	2.6 ± 0.7	1.0 ± 0.4	2.5 ± 0.9	1.3 ± 0.7	0.921	0.071
PA	2.2 ± 0.5	1.6 ± 0.4	1.7 ± 0.5	2.3 ± 0.7	0.830	0.994
Duration	0.03 ± 0.04	-0.07 ± 0.06	0.1 ± 0.07	-0.09 ± 0.07	0.707	0.024
rSNA (n)	5	6	5	6		
AUC	2.5 ± 1.4	3.2 ± 0.5	4.2 ± 0.9	3.5 ± 1.4	0.360	0.974
PA	1.6 ± 0.7 *	5.7 ± 1.3	1.8 ± 0.5	3.5 ± 0.8	0.308	0.005
Duration	-0.02 ± 0.09	-0.26 ± 0.04	0.2 ± 0.07	0.01 ± 0.09	0.009	0.014

sSNA, splanchnic sympathetic nerve activity; rSNA, renal sympathetic nerve activity; AUC, area under curve; PA, peak amplitude; LPK, Lewis Polycystic Kidney; #Significantly different between the cohorts of animals with male vs. female within same strain ($P<0.05$), * Significantly different between the strains in similar sex ($P<0.05$) as determined using two-way ordinary ANOVA followed by Bonferroni's post-hoc correction. n = number of animals per group.

Table 5 Renal sympathetic baroreceptor reflex function curves in both adult male and female Lewis and Lewis Polycystic Kidney rat

	Male		Female		P value	
	LPK (5)	Lewis (5)	LPK (5)	Lewis (5)	Sex	Strain
Gain (%/mmHg)	0.9 ± 0.2	1.6 ± 0.3	0.8 ± 0.1	1.6 ± 0.2	0.718	0.002
Range (%)	81 ± 15	114 ± 7	78 ± 11	85 ± 9	0.177	0.101
MAP_{50} (mmHg)	175 ± 9	144 ± 6	183 ± 16	145 ± 11	0.683	0.009
MAPsat (mmHg)	208 ± 13	170 ± 9	221 ± 21	163 ± 12	0.824	0.005
MAPthr (mmHg)	142 ± 8	118 ± 5	150 ± 16	128 ± 9	0.442	0.044
Operating range (mmHg)	65 ± 10	52 ± 7	72 ± 8	36 ± 3	0.516	0.005

rSNA, renal sympathetic nerve activity; MAP_{50}, mean arterial pressure at the midpoint of the curve; MAP_{thr}, threshold MAP to trigger a change in SNA; MAP_{sat}, saturation MAP at which there is no further change in SNA; LPK, Lewis polycystic kidney. The baroreflex function curve parameters were obtained from the logistic function relating normalized SNA (% baseline) to MAP. [#]Significantly different between the cohorts of animals with male vs. female within same strain (*P≤0.05*), * Significantly different between the strains in similar sex (*P≤0.05*) as determined using two-way ordinary ANOVA followed by Bonferroni's *post-hoc* correction. *n* = number of animals per group.

Table 6 Splanchnic sympathetic baroreceptor reflex function curves in both adult male and female Lewis and Lewis Polycystic Kidney rat

	Male		Female		P value	
	LPK (4)	Lewis (5)	LPK (4)	Lewis (4)	Sex	Strain
Gain (%/mmHg)	1.1 ± 0.2	1.3 ± 0.2	0.7 ± 0.1	1.5 ± 0.4	0.785	0.050
Range (%)	100 ± 10	109 ± 6	85 ± 16	88 ± 18	0.194	0.668
MAP_{50} (mmHg)	178 ± 16	151 ± 6	208 ± 35	160 ± 11	0.334	0.075
MAPsat (mmHg)	211 ± 20	183 ± 9	260 ± 55	180 ± 13	0.431	0.083
MAPthr (mmHg)	145 ± 12	119 ± 6	156 ± 17	139 ± 8	0.203	0.093
Operating range (mmHg)	66 ± 10	63 ± 9	104 ± 40	40 ± 6	0.516	0.131

sSNA, splanchnic sympathetic nerve activity; MAP_{50}, mean arterial pressure at the midpoint of the curve; MAP_{thr}, threshold MAP to trigger a change in SNA; MAP_{sat}, saturation MAP at which there is no further change in SNA; LPK, Lewis polycystic kidney. The baroreflex function curve parameters were obtained from the logistic function relating normalized SNA (% baseline) to MAP. [#]Significantly different between the cohorts of animals with male vs. female within same strain ($P \leq 0.05$), * Significantly different between the strains in similar sex ($P \leq 0.05$) as determined using two-way ordinary ANOVA followed by Bonferroni's *post-hoc* correction. *n* = number of animals per group.

MACQUARIE UNIVERSITY

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2013/016 **Date of Expiry:** 31 May 2014

Full Approval Duration: *1 June 2013 to 31 May 2016 (36 Months)*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

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

Title of the project: Does altered breathing regulation influence blood pressure - a study in rats with chronic kidney disease

Purpose: 5 - Research: Human or Animal Health and Welfare

Aims: **a. To identify if the relationship between breathing and sympathetic nerve activity is altered in chronic kidney disease**
b. To investigate the associated hypothalamic chemical changes

Surgical Procedures category: 2 - Animal Unconscious without Recovery

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age/Sex/Weight	Total	Supplier/Source
02	LPK	10-15 weeks / male / any	76	ARC Perth
02	SHR	12-24 weeks / male / any	76	ARC Perth
02	Lewis	10-15 weeks / male / any	76	ARC Perth
		TOTAL	228	

Location of research:

Location	*Full street address*
Central Animal Facility	Building F9A, Research Park Drive, Macquarie University, NSW 2109
ASAM	Level 1, F10A, 2 Technology Place, Macquarie University, NSW 2109

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

Conditions of Approval: N/A

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.

Professor Mark Connor (Chair, Animal Ethics Committee) **Approval Date:** 17 May 2013

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

MACQUARIE University

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2015/031-4 **Date of Expiry:** 01 November 2016

Full Approval Duration: *02 November 2015 to 01 November 2018 (36 months)*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

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

Title of the project: **Neuronal mechanisms underlying respiratory-sympathetic coupling in rats**

Purpose: **4 - Research: Human or Animal Biology**

Aims: **1. To study if central respiratory neurons that control the post-inspiratory phase of breathing are also responsible for respiratory sympathetic coupling.**
2. To study how respiratory sympathetic coupling is elevated in hypertensive rats.

Surgical Procedures category: 2 - Animal Unconscious without Recovery

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age/Weight/Sex	Total	Supplier/Source
02 Rattus	Lewis	14 - 18 wks /NA/Male	84	Animal Resources Centre, Perth
02 Rattus	LPK	14 - 18 wks /NA/Male	24	Animal Resources Centre, Perth
02 Rattus	SHR	14 - 18 wks /NA/Male	24	Animal Resources Centre, Perth
			132	

Location of research:

Location	*Full street address*
Central Animal Facility	Building F9A, Research Park Drive, Macquarie University, NSW 2109
Faculty of Medicine and Health Sciences	Level 1, F10A, 2 Technology Place, Macquarie University, NSW 2109

Amendments approved by the AEC since initial approval:

1. *Amendment #1 - Addition of Rahat Ul Ain Summan Toor as Associate Investigator (Exec approved 30/11/2015. Ratified by. AEC 10 December 2015).*
2. *Amendment #2 - Addition of Vikram Tallapragada as Associate Investigator (Approved by AEC 10 December 2015).*
3. *Amendment #3 - Amend administration of substances (Approved by AEC 19 May 2016).*
4. *Amendment #4(a) - Amend Technique (Executive approved. To be ratified by AEC 20 October 2016).*
5. *Amendment #4(b) - Amend administration of substances (Executive approved. To be ratified by AEC 20 October 2016).*

Conditions of Approval: N/A

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.

Associate Professor Jennifer Cornish (Chair, Animal Ethics Committee) **Approval Date: 27 September 2016**

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

MACQUARIE UNIVERSITY

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2011/044 - 9 **Date of Expiry:** 11 September 2013

Full Approval Duration: *12 September 2011 to 11 September 2014 (36 months)*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

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

Title of the project: **Do stiff blood vessels or overactive nerves cause long-term high blood pressure in kidney disease?**

Purpose: 4 – Research: human or animal biology

Aim: This project aims to use an animal model to determine if drugs which block the hormone angiotensin II therapy for hypertension reduces the stiffness of blood vessels or elevated nerve activity.

Surgical Procedures category: 3 (Minor conscious intervention)

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved:

Species	Strain	Sex	Age	TOTAL	Supplier/Source
Rat	Lewis	M/F	3-18 wks	156	ARC Perth
Rat	LPK	M/F	3-18 wks	156	ARC Perth/Westmead Hospital
Rat	SHR	M/F	4-24 wks	156	ARC Perth
Rat	WKY	M/F	4-24 wks	156	ARC Perth
		TOTAL		624	

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 Facility	Building F9A, Research Park Drive, Macquarie University NSW 2109

Conditions of Approval: N/A

Amendments approved by the AEC since initial approval:

1. Addition of new personnel to protocol: Barbara Zangerl (approved May 2012).
2. Addition of new personnel to protocol: George Lindesay (approved June 2012).
3. Addition of new personnel to protocol: Yimin Yao subject to adequate supervision (approved July 2012).
4. Addition of Melissa Farnham as Associate Investigator (approved July 2012).
5. Modification of experimental procedure (approved 16 August 2012).
6. Change of age of animals used (approved 16 August 2012).
7. Addition of new personnel to protocol: Marie-Ange Kouassi (approved September 2012).
8. Addition of new personnel to protocol: Rochelle Boyd (approved & ratified December 2012).
9. Addition of new personnel to protocol: Ko Jin Quek (approved February 2013).
10. Addition of new personnel to protocol: Manash Saha as Student (Executive approved 17 April 2013, ratified by AEC 18 April 2013).

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 supplier's license.

Prof Mark Connor (Chair, Animal Ethics Committee) **Approval Date:** 18 April 2013

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