Renal denervation does not reduce blood pressure in an animal model of polycystic kidney disease

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This thesis is dedicated to my late mother, my beloved husband, my dearest father and my siblings.

Declaration of originality

I certify that the work in this thesis entitled “Renal denervation does not reduce blood pressure in an animal model of polycystic kidney disease”, has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University. I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. The research presented in this thesis was approved by Macquarie University Ethics Review Committee, reference number: 2015/036

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Conflict of interest statement

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Declaration of experimental contributions

The candidate contributed to the conception and design of experiments, assisted with all surgical intervention procedures undertaken and cared for the animals both prior to and after the surgical procedures. She was primarily responsible for development of the protocol for selective afferent nerve denervation and the telemetry data collection, its analysis, interpretation and presentation and undertook all the biochemical assays for urine and plasma urea and creatinine levels for the animals used in this study. She developed the protocol for the determination of renal sensory and sympathetic nerve markers and urinary sodium concentration levels (chapter 6) and undertook RNA extractions as well as assisted with the RT-PCR experiments for kidney tissue samples analysed in Chapter 6.

In addition to the contributions of the candidate, Jacqueline Phillips contributed to the conception and design of experiments, data analysis, interpretation and presentation of results. Cara Hildreth undertook surgical denervation, telemetry probe implantation and contributed to the conception and design of experiments, methodology for the telemetry data analysis, interpretation and presentation of results. Ahmed Rahman and Vikram Tallapragada undertook the surgical procedures for telemetry probe implantations and denervation procedures. Plasma and kidney samples were assayed for renin content by Mr. David J Casley from Prosearch (Melbourne, Australia) (Chapter 6). Sean Barton undertook RT-PCR experiments and preliminary data analysis (Chapter 6).

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Publications arising from this thesis

The results presented in this thesis have been published in part in conference abstract form.

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1. Yao, Y.; Hildreth, C.M.; Li, S.; Boyd, R.; Kouchaki, Z.; Butlin, M.; Avolio, A.P.; Pilowsky, P.M.; Phillips, J.K. Increased arterial stiffness does not respond to renal denervation in an animal model of secondary hypertension. 2017 39th Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBC); 2017. p. 258-261.

Abstract

Hypertension is a highly prevalent co-morbidity for patients with polycystic kidney disease (PKD), but difficult to treat. As a novel therapeutic strategy, renal denervation (RDN) offers an alternative approach; however, its efficacy in this patient cohort is not clear. There is evidence that the mechanism by which the removal of the renal nerves acts to reduce blood pressure could be mediated by both the renal sympathetic and sensory nerves and there is also a strong likelihood of the procedure impacting other homeostatic pathways regulated by the kidney such as the renin-angiotensin-aldosterone system (RAAS) and sodium balance. The issue of reinnervation is also still an open question, given that in humans there are proposed long-term benefits despite anatomical and functional evidence of reinnervation from experimental animal models. The overall aim of this thesis is therefore to investigate the effect of RDN on hypertension secondary to PKD, the individual contribution of renal sensory and sympathetic nerves to the RDN-induced effect, the potential effect of RDN on the RAAS and renal handling of sodium, and the potential impact of renal reinnervation. We addressed these questions employing a well-established rodent model of juvenile onset PKD, the Lewis polycystic kidney (LPK) rat in a series of three studies.

In Study 1 **(Chapter 3),** we aimed to validate the effectiveness of two RDN procedures, being total RDN by stripping of the renal nerves and periaxonal application of phenol to destroy both renal sensory and sympathetic nerves in the two strains and the relatively new procedure of selective afferent RDN using periaxonal application of capsaicin. Experiments were undertaken in 6-week old LPK and their normotensive Lewis control strain. This study revealed that at one-week post total RDN, both the renal sympathetic nerve marker tyrosine hydroxylase (TH) and the sensory nerve marker calcitonin gene-related peptide (CGRP) were essentially absent, while after afferent RDN, CGRP immunoreactivity was significantly abolished while levels of the sympathetic nerve marker TH remained intact.

Study 2 aimed to examine the effect of total and selective afferent RDN on cardiovascular, renal **(Chapters 4)** and autonomic function **(Chapter 5)** in Lewis and LPK rats over an 8-week follow up. To our surprise, neither total or afferent RDN affected the level of systolic (SBP), mean (MAP) and diastolic (DBP) blood pressure in the LPK while total, but not afferent RDN, caused a significant reduction in SBP, MAP and DBP in Lewis rats. The renal function of both strains as determined by plasma urea, creatinine, urine protein/creatinine ratio (UPC) and creatinine clearance rate (CCR) was not affected by either total or afferent RDN.

Regarding the effect of RDN on autonomic function as determined by spectral analysis, in the Lewis animals, total RDN significantly reduced systolic blood pressure variability (SBPV) parameters but did not affect heart rate variability (HRV) or baroreceptor sensitivity (BRS) parameters. Afferent RDN increased the overall level of low frequency (LF) components of HRV, decreased very low frequency (VLF) of SBPV but did not affect BRS parameters. In the LPK animals, total RDN lowered the overall level of VLF HRV but did not affect SBPV and BRS parameters in LPK. Afferent RDN increased the HF components of SBPV but did not affect HRV and BRS parameters in LPK. The overall interpretation is that the RDN procedure did not provide any beneficial effect on autonomic function in the LPK.

The impact of RDN on the RAAS was assessed by examining circulating and intra-renal RAAS components and determining any impact of the procedures on urine sodium excretion in 10 weeks old Lewis and LPK **(Chapter 6).** Plasma and kidney renin content as determined by radioimmunoassay were significantly lower in the LPK relative to Lewis animals and were not affected by RDN in either strain. Intra-renal gene expression levels of RAAS components in the LPK vs. Lewis were significantly lower for renin, angiotensinogen, angiotensin converting enzyme 2 (ACE2) and angiotensin type one receptor (AT1αR), except for ACE1, which was significantly higher in the LPK, however RDN had no impact on the intra-renal RAAS gene expression. With regard to sodium excretion, urine sodium concentration was significantly lower in the LPK relative to Lewis, and not impacted by either total or afferent RDN in both

strains. When normalised to the 24h urine volume, the level was still significantly lower in the LPK relative to Lewis. There was an overall treatment effect (P=0.04) on 24h sodium excretion with *post hoc* analysis within each strain indicating no difference between treatment groups.

In the course of study 2, substantial renal reinnervation of both renal sensory and sympathetic nerves was demonstrated immunohistochemically at 4 and 8 weeks post-denervation. Study 3 **(Chapters 4 and 5)** therefore aimed to assess the impact of a repeat RDN procedure. Only total RDN was performed given the lack of effect seen in response to afferent denervation. Cardiovascular, renal and autonomic function parameters were compared between animals that had two (one at 6 weeks, the other at 10 weeks) vs single (6 weeks only) total RDN procedures. In the Lewis, repeat total RDN had no additional impact on SBP, MAP or DBP, while in the LPK rats, repeat total RDN caused a transient increase in blood pressure. In the Lewis, the repeat procedure did not have any treatment effect on renal function while in the LPK rats, a repeat RDN was associated with worsening renal function. Repeating total RDN had a negative effect on autonomic function parameters in the LPK, evidenced by an overall decrease in HRV and BRS. In Lewis, a repeat total RDN caused a decrease in SBPV and an increase in BRS, consistent with improved cardiac autonomic control.

Collectively, these studies indicated that neither total or afferent RDN has a beneficial effect in attenuating hypertension, renal dysfunction or autonomic dysfunction in a rodent model of PKD, and that the procedure has limited impact on circulating or intra-renal RAAS or urine sodium excretion that could explain the decrease in blood pressure seen in the Lewis animals. These results suggest that as a patient cohort, RDN in conditions such as juvenile onset PKD is not a recommended procedure.

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List of abbreviations

2K1C	Two-kidney-one-clip
ACE	Angiotensin-converting-enzyme
ACEIs	Angiotensin-converting enzyme inhibitors
ADPKD	Autosomal dominant polycystic kidney disease
AGT	Angiotensinogen
Ang I	Angiotensin I
Ang II	Angiotensin II
AP	Arterial pressure
ARBs	Angiotensin receptor blockers
ARPKD	Autosomal recessive polycystic kidney disease
AT_1R	Angiotensin type 1 receptor
BP	Blood pressure
BRS	Baroreceptor sensitivity
CCR	Creatinine clearance rate
CGRP	Calcitonin gene-related peptide
CKD	Chronic kidney disease
DBP	Diastolic blood pressure
DOCA	Deoxycorticosterone acetate
ESRD	End-stage renal disease

GFR	Glomerular filtration rate
HF	High frequency
HR	Heart rate
HRV	heart rate variability
IHC	Immunohistochemistry
JGA	Juxtaglomerular apparatus
LF	Low frequency
LPK	Lewis polycystic kidney
MAP	Mean arterial pressure
Nek 8	Never in mitosis gene a-related kinase 8
NPHP	Nephronophthisis
NTS	The nucleus of the solitary tract
OCT	Optimal cutting temperature compound
PBS	Phosphate buffered saline
PC-1	Polycystin 1
PC-2	Polycystin 2
PKD	Polycystic kidney disease
PRA	Plasma renin activity
PVN	Paraventricular nucleus
RAAS	Renin-angiotensin-aldosterone system

RDN	Renal denervation
RVLM	Rostral ventrolateral medulla
SBP	Systolic blood pressure
SBPV	Systolic blood pressure variability
SHR	Spontaneously hypertensive rat
SNA	Sympathetic nerve activity
TH	Tyrosine hydroxylase
TP	Total power
TRPV1	Transient receptor potential (TRP) V1 receptor
UPC	Urine protein to creatinine ratio
VLF	very low frequency

Chapter 1 Literature review

The global prevalence of hypertension is high and adequate control of blood pressure in the general population as well as patients with specific diseases such as diabetes, heart failure or chronic kidney disease remains a great challenge. This literature review first provides a detailed description of the mechanisms involved in normal blood pressure control and the pathology of hypertension, followed by an overview of available pharmaceutical therapies as well as proposed novel non-pharmaceutical techniques with a focus on renal denervation, identifying a lack of understanding of the mechanisms by which renal denervation acts to reduce blood pressure. A summary of the treatment of hypertension associated with chronic kidney disease is provided, including the nature of polycystic kidney disease and the use of animal models to study these conditions. Finally, the thesis aims are outlined.

1.1 Normal Control of blood pressure

Blood pressure is the measurement of the pressure of the blood in the artery, which is positively related to cardiac output and peripheral vascular resistance. Factors that affect cardiac output such as myocardial contractility and heart rate, and factors that affect peripheral resistance such as sympathetic nerve activity and vascular responsiveness are shown to vary in their contributions to the changes in blood pressure depending on the physiological situation (Joyner and Limberg 2014). In other words, the blood pressure is regualted through an intergaration of multiple control systems, which include the autonomic nervous system, the kidney and hormones. During a cardiac cycle, blood pressure peaks during the systole when the heart muscle contracts and pumps blood, and falls during the diastole when the heart relaxes and refills with blood. The highest and lowest blood pressure in a cycle are referred to systolic (SBP) and diastolic blood pressure (DBP), respectively. Pulse pressure (PP) is the difference between SBP and DBP, while mean arterial pressure

(MAP) is the average pressure for the entire cycle and is near to one-third of SBP plus two-thirds of DBP.

1.1.1 Autonomic nervous system

The autonomic nervous system consists of a somatic afferent pathway, a central nervous system integrating complex (brain and spinal cord), and two efferent limbs, being the sympathetic and the parasympathetic nervous systems (Hall 1990, Guyenet 2006). The two efferent arms consist of parallel and differentially regulated pathways made up of preganglionic neurons located in the central nervous system and postganglionic motor neurons innervating target organs or tissues (Guyenet 2006).

1.1.1.1 Sympathetic nervous system

The sympathetic nervous system plays an important role in blood pressure regulation. The sympathetic outflow is regulated by key central cardiovascular regions including the rostral ventrolateral medulla (RVLM), the paraventricular nucleus (PVN) and the nucleus of the solitary tract (NTS) (Guyenet 2006), which transmit sympathetic flow to preganglionic neurones located in the intermediolateral cell column of the spinal cord. The axons of these preganglionic neurons then synapse with postganglionic neurons in sympathetic ganglia, after which the postganglionic sympathetic nerve fibres project to the target organs, including the heart, the blood vessels, the kidney and the adrenal gland (Guyenet 2006). The activation of sympathetic nerves will trigger release of adrenal neurotransmitters (noradrenaline, adrenaline) into the circulation, which will then act through beta-adrenergic receptors located in the heart to cause an inotropic effect (increase in heart rate and strength of heart contraction), and through alpha-adrenergic receptors located in the blood vessel to cause vasoconstriction (Touyz 2014). Hence, activation of the sympathetic nervous system exerts a direct effect on the two major parameters determining blood pressure, namely, cardiac output and peripheral resistance. In addition, activation of the sympathetic nervous system also

influences the function of the kidney, another important organ in blood pressure regulation, which will be discussed later.

1.1.1.2 Parasympathetic nervous system

The regulatory effect of the sympathetic system on the circulation involves both the heart and the resistance vessels, while the parasympathetic system affects mainly the heart (Mancia 2018). The predominate preganglionic parasympathetic nerve that innervates the heart is the vagus nerve, which originates in the medulla in the nucleus ambiguus (Chen and Dilsizian 2017). The vagal nerve fibres synapse with postganglionic parasympathetic neurons located within the cardiac plexus and walls of the atria, which then innervate the sinoatrial node and the atrioventricular node (Chen and Dilsizian 2017). Efferent vagus nerve activation can cause acetylcholine release from postganglionic parasympathetic nerve terminals, which acts to reduce the heart rate by inhibiting the activity of sinoatrial and atrioventricular node (Chen and Dilsizian 2017). Thus, parasympathetic nervous activity exerts an effect on blood pressure through regulating the heart rate. Under normal conditions, the sympathetic and parasympathetic nervous system work collectively to maintain cardiovascular homeostasis.

1.1.1.3 Baroreflex control

Typically, the cardiovascular autonomic nervous system modulates heart rate and peripheral vascular tone continuously through the baroreflex arc, to maintain a relatively stable level of blood pressure (Guyenet 2006, Palma and Benarroch 2014). The baroreflex arc ensures that changes in blood pressure result in the activation of compensatory mechanisms thereby restoring blood pressure to normal limits (Kaur *et al.* 2016). For instance, when arterial pressure rises, pressure sensors, i.e. baroreceptors, which are located primarily in the arterial walls of the carotid artery sinus and aortic arch detect the rise in arterial pressure. Once a rise in arterial pressure is detected, afferent signals are generated in proportion to changes in arterial pressure and relayed via the glossopharyngeal nerve and the vagus nerve to the NTS (Kaur *et al.* 2016). This will, in turn, change the sympathetic and parasympathetic outflow to

the heart and resistance vessels. A rise in blood pressure ultimately results in a decrease in heart rate and vasodilatation, and hence restoration of the previous level of blood pressure. Baroreflex arc activity is an important indicator of the integrity of cardiovascular homeostatic regulation.

Methodologically, baroreflex sensitivity (BRS) can be assessed based on baroreflex-mediated changes in pulse interval following artificially imposed changes in arterial BP via pharmacological manipulation, for example, using vasoconstrictor (phenylephrine) or vasodilators (nitroprusside) to produce rise or fall in blood pressure (Hart *et al.* 2013, Khan *et al.* 2014, Chen *et al.* 2016). Consecutive SBP values are plotted against the simultaneously recorded inter-beat or pulse intervals with one beat delay to fit the linear regression line between the two variables, and the slope of this line is a measure of BRS (Smyth *et al.* 1969).

Alternatively, BRS can be assessed by computer-based analysis of spontaneous oscillations in blood pressure and heart rate interval obtained using non-invasive beat to beat blood pressure monitors in humans or invasive radio telemetry device in experimental animals (Grassi *et al.* 1997, Hart *et al.* 2013, Hildreth *et al.* 2013). One way to calculate BRS is the sequence method, which computes the slope of the linear regression between the blood pressure and pulse intervals from specific "sequences". These sequences should meet the criteria that blood pressure shows a continuous increase (or decrease) for at least three consecutive beats that is accompanied by lengthening (or shortening) of consecutive pulse intervals with zero to two beats delay. The other way to calculate BRS is the frequency domain method which first assesses the SBP variability and heart rate variability as spectral powers at low and high-frequency bands, followed by calculation of the square root of the ratio of powers of pulse interval to SBP, the α-index (Robinson and Carr 2002).

1.1.2 The kidneys and blood pressure regulation

While the autonomic nervous system can regulate blood pressure through rapid alterations in cardiac output and/or total peripheral resistance, the kidney is important in long-term control

of blood pressure, as it regulates the overall amount of sodium and water in the body, which in turn impacts on the extracellular fluid volume, a key factor influencing both intracellular fluid volume and blood pressure (Wadei and Textor 2012).

The pressure-natriuresis theory, as first proposed by Guyton (Guyton 1990) explained how the kidney works to maintain a steady blood pressure level. When blood pressure increases, renal perfusion pressure will increase. The kidney will respond by decreasing tubular reabsorption of sodium and increasing sodium and water excretion. The resulting reduction in the extracellular fluid volume will return the blood pressure to normal (Guyton 1990). In contrast, a decrease in blood pressure will lead to a decrease in renal perfusion pressure, resulting in a decrease in sodium excretion and an increase in tubular sodium reabsorption. The resulting increase in the extracellular fluid volume will return the blood pressure to normal This feedback loop functions to reset the blood pressure to a normal level. Thus, according to this theory, the set point for long-term blood pressure control is the arterial pressure at which sodium and water intake and output are at equilibrium. The pressure natriuresis mechanism can be influenced by various neurohormonal systems with studies demonstrating that during high salt intake, decreased angiotensin II (AngII) and aldosterone formation enhance the effectiveness of pressure natriuresis, allowing sodium balance to be maintained with minimal changes in blood pressure as long as the renin-angiotensin-aldosterone system (RAAS) is functioning normally (Hall *et al.* 1992, Ivy and Bailey 2014). Excessive RAAS activation or sympathetic nerve activation, which acts to reduce glomerular filtration rate or increase tubular reabsorption, can reduce the effectiveness of pressure natriuresis, thereby necessitating greater increases in blood pressure to maintain sodium balance (Hall *et al.* 2012, Hall 2016, Lohmeier and Pruett 2016). The disturbance of renal pressure natriuresis by intrinsic or extrinsic factors will ultimately affect the arterial pressure level (Wadei and Textor 2012).

1.1.3 Hormonal control of blood pressure

1.1.3.1 Renin-angiotensin-aldosterone system

The RAAS system plays an essential role in regulating blood pressure and fluid balance in the body (Beevers *et al.* 2001). The classic RAAS components include renin, angiotensin-converting enzyme 1 (ACE1), angiotensinogen (AGT), angiotensin I (Ang I), angiotensin II (Ang II), Ang II type 1 receptor (AT_1R) and aldosterone (Hall 2003). When renal perfusion pressure or tubular sodium concentration decreases, the renin-secreting cells located in the juxtaglomerular apparatus of the kidney will increase the synthesis and section of renin (Hackenthal *et al.* 1990, Hall 2003), the rate-limiting enzyme for the RAAS pathway that converts AGT, which is produced in the liver (Hall 2003), to the hormone Ang I. Ang I will be converted to Ang II by ACE1, which is produced by endothelial cells (in particular in the lungs) (Corvol *et al.* 1995, Costerousse *et al.* 1998). Through binding to the specific AT_1R located in the blood vessels, Ang II causes vasoconstriction and consequently blood pressure increases (Hollenberg and Williams 1979). Ang II also stimulates the release of the hormone aldosterone in the adrenal glands (Hollenberg and Williams 1979). Aldosterone can alter the renal-pressure natriuresis relationship via its sodium-retaining actions on the renal tubules. Therefore, the RAAS system exerts its effect on blood pressure via vasoconstriction and renal sodium reabsorption.

In addition to the classical RAAS pathway, increasing evidence suggests the existence of a local RAAS in several tissues including the heart, the brain and the kidney (Campbell 2014, de Morais *et al.* 2018). A key feature of local RAAS is the RAAS components including AGT and enzymes such as renin are synthesised independently of the circulating RAAS. The tissue RAAS can act locally as a paracrine and/or autocrine factor in meeting specific needs for individual tissues, and it can operate, in whole or in part, independently of the circulating counterpart (Campbell 2014, de Morais *et al.* 2018). The clinical relevance of local RAAS is under investigation.

1.1.3.2 Vasopressin

Vasopressin is a nonapeptide produced by the neurons located in the PVN, median preoptic and supraoptic nuclei (Japundzic-Zigon 2013). It has two distinct functions, being pressor and antidiuretic in effect, respectively (Japundzic-Zigon 2013). The vasoconstrictive effect is mediated by vasopressin V1a receptors expressed in vascular smooth muscle cells, while the antidiuretic effect is mediated by vasopressin V2 receptors on the cortical and medullary collecting kidney tubules (Deen *et al.* 1994, Japundzic-Zigon 2013). The secretion of vasopressin is mainly triggered by an increased plasma osmolality, which then acts on the kidney to preserve more water, in turn reducing plasma osmolality to normal levels. Vasopressin release can also be triggered by the stimulation of peripheral receptors located in the thoracic vessels that monitor changes in blood pressure (Japundzic-Zigon 2013). Increased release of vasopressin from the PVN acts to target arterial blood vessels and increase peripheral resistance, in turn changing blood pressure (Japundzic-Zigon 2013).

1.1.4 Renal innervation

Having listed the mechanisms for blood pressure regulation, an important link between these factors are the renal afferent sensory and efferent sympathetic nerves, which connects the activity of the kidney to that of the autonomic nervous system and the RAAS (DiBona and Kopp 1997).

1.1.4.1 Renal sensory innervation

The majority of renal sensory nerves are located in the renal pelvic area, where they function to sense stretch of the renal pelvic wall and/or changes in the chemical composition of the urine (Marfurt and Echtenkamp 1991). The renal sensory nerves exit the kidney via the adventitia of the renal artery then reach the spinal cord and synapse with interneurons projecting to sites within the central nervous system, many of which have been mapped following stimulation of the renal sensory nerve through either detection of Fos-like proteins (Solano-Flores *et al.* 1997) or extracellular recording of neurons (Xu *et al.* 2015). These

centres include the PVN, organum vasculosum laminae terminalis, subfornical organ, and the NTS (Solano-Flores *et al.* 1997) (Figure 1.1). The PVN is a critical region that regulates sympathetic outflow after the integration of signals from the renal sensory nerves, baroreceptors, and cardiac afferent nerves (Caverson and Ciriello 1988, Ferguson *et al.* 2008). It has been shown that 84% of RVLM-projecting pre-autonomic PVN neurons are activated by afferent renal nerve stimulation, indicating the importance of PVN in the integration of renal afferent information (Xu *et al.* 2015). The NTS is also an important site for the homeostasis of autonomic function, acting to relay afferent information carried by the vagus and glossopharyngeal nerves, which is then presented to other brain regions including the ventrolateral medulla, and PVN (Ciriello *et al.* 1993). Also, neurons containing Fos-like immunoreactivity after renal sensory nerve stimulation were found in the NTS (Solano-Flores *et al.* 1997), indicating a role of NTS in the processing of renal sensory signals.

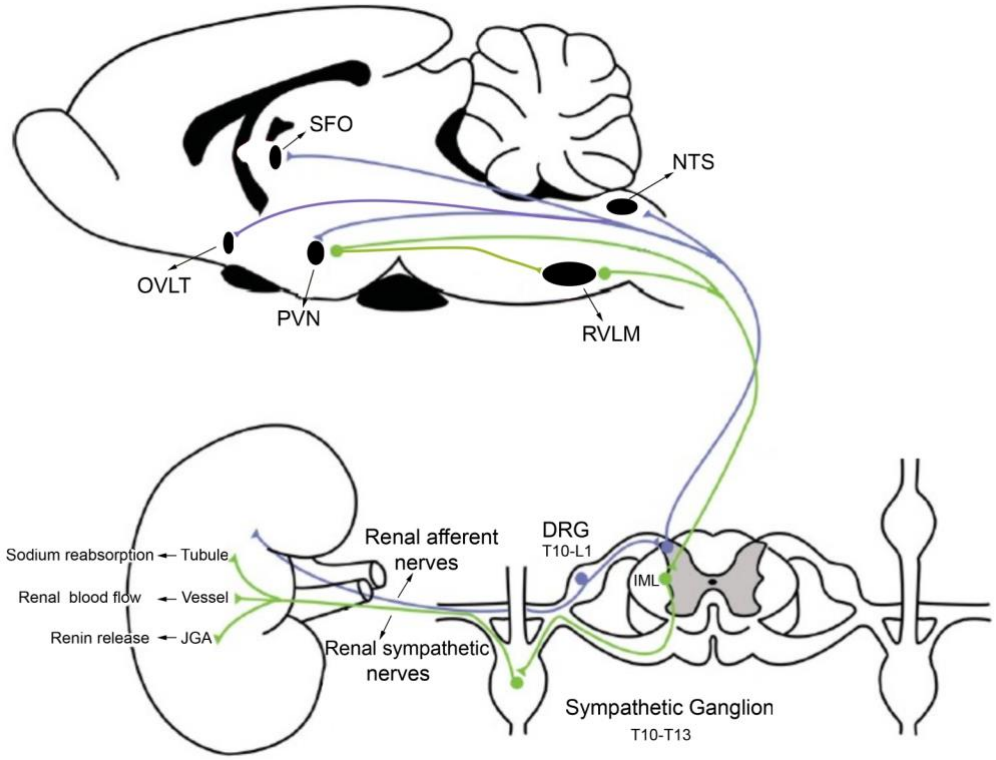


Figure 1.1 Schematic representation of the integration of renal sensory and sympathetic nerves in the central nervous system and kidneys.

Renal afferent sensory nerves sense changes in the kidney and provide afferent information to the central nervous system. First-order neurons are located in the dorsal root ganglion (DRG) and project to the dorsal horn of the spinal cord, where they synapse with the neurons that project to the cardiovascular nuclei involved in blood pressure and homeostasis regulation, such as the paraventricular nucleus of the hypothalamus (PVN), organum vasculosum laminae terminalis (OVLT), subfornical organ (SFO), and the nucleus tractus solitaries (NTS). Pre-sympathetic neurons from the PVN and the rostral ventrolateral medulla (RVLM) project to the intermediolateral cell column (IML) and synapse with preganglionic neurons. Within the sympathetic ganglion, these fibres synapse with the renal postganglionic (sympathetic) nerves that innervate the renal tubules, vasculature and juxtaglomerular apparatus (JGA) [Modified from Figure 1 (Nishi *et al.* 2015)]

1.1.4.2 Renal sympathetic innervation

Renal sympathetic nerves fibres originate from the intermediolateral column of the spinal cord, from T10 to T13, which receives projecting neurons from several brain nuclei, e.g., the

RVLM, and the PVN (DiBona and Kopp 1997). These fibres pass to the aorticorenal, splanchnic, celiac and superior mesenteric ganglia, where they make contact with many postganglionic neurons innervating the kidney. The postganglionic sympathetic nerves reach the kidney through the adventitia of the renal artery and innervate all parts of the renal vasculature, renal tubules and cells in juxtaglomerular apparatus (JGA) with the greatest density of innervation found along afferent arterioles (Barajas *et al.* 1992) (Figure 1.1). Activation of renal sympathetic nerves results in frequency-dependent renin secretion from JGA, sodium and water reabsorption from renal tubules and renal vasoconstriction (Johns *et al.* 2011, Osborn and Foss 2017).

It is evident that there is a close interaction between the renal sympathetic nerves and the RAAS. In the kidney, Ang II has an important tonic presynaptic action on renal sympathetic nerve terminals present on both renal tubular epithelial cells and vessels to facilitate the release of noradrenaline (DiBona 2000). The function of Ang II to increase renal chloride reabsorption from the proximal tubules largely depend on intact sympathetic innervation as after renal denervation the increase in chloride reabsorption is decreased by 75% (DiBona 2000). Circulating Ang II could also influence peripheral (including renal) sympathetic nerve activity (SNA) by acting on central nervous system sites including the subfornical organ (Rossi *et al.* 2019) and area postrema (Collister *et al.* 1996), wherein the lack of a blood-brain barrier enables ready access by circulating Ang II. There is also evidence suggesting angiotensin peptides of brain origin may elicit a local paracrine or autocrine action on sites that regulate renal SNA and arterial baroreflex control of renal SNA (de Morais *et al.* 2018).

Therefore, circulating and local RAAS interact with the sympathetic nervous system to regulate blood pressure long-term via their effect on resistance vessels and renal tubules.

1.1.4.3 Reno-renal reflex

Renal sensory and sympathetic nerves have a close interaction with each other. In healthy normal animals, an increase in ipsilateral renal afferent nerve activity due to stretching of the

renal pelvic wall triggers a bilateral decrease in efferent renal sympathetic activity and an increase in urinary sodium excretion (Kopp *et al.* 1984, Kopp 2011). This inhibitory reflex is termed the reno-renal reflex and is crucial in the regulation of total body sodium and fluid volume homeostasis. A part of this reflex is a negative feedback loop whereby changes in efferent renal SNA modulates afferent renal nerve activity, by the release of noradrenaline, which activates α2- adrenoceptors on renal sensory nerves, leading to decreases in afferent renal nerve activity (Kopp *et al.* 2007). A high sodium diet enhances and a low-sodium diet reduces the efferent renal SNA-induced increases in afferent renal nerve activity (Kopp *et al.* 2009).

1.2 Hypertension

Elevated blood pressure or, hypertension, is a significant global public health issue, affecting around 40% of the general population (WHO 2013). It is a significant risk factor for cardiovascular diseases including stroke, heart failure, coronary artery disease (Kjeldsen 2018) and a leading cause of cardiovascular and overall mortality, responsible for around 9.4 million deaths every year (WHO 2013).

Hypertension occurs most often without a specific cause and is generally named essential or primary hypertension. Hypertension caused by or linked to a specific diagnostic entity is named identifiable or secondary hypertension. Common diseases that can cause hypertension include diabetes, primary aldosteronism, sleep apnea syndrome and various forms of chronic kidney disease (CKD) including those due to for example proteinuric nephropathies, renal vascular disease and genetic kidney disease.

In essential hypertension or hypertension caused by diabetes, sleep apnea syndrome or CKD, several systems seem to interact with each other, contributing to the development of hypertension, which will be discussed in detail in the following section.

1.2.1 Mechanisms of hypertension

1.2.1.1 Sympathetic overaction

Compelling evidence exists that in the early stages of hypertension, the sympathetic nervous system is activated. In classic studies by Julius et al. (Julius *et al.* 1991), young subjects with borderline hypertension had increased heart rate which was accompanied by increased plasma noradrenaline levels. Meanwhile, increased sympathetic activity was also demonstrated in young adults with borderline hypertension using microneurography (Anderson *et al.* 1989) or measurements of noradrenaline spillover (Esler *et al.* 1988). In middle age and elderly patients with an established hypertensive state, sympathetic overdrive still exists (Greenwood *et al.* 2001, Smith *et al.* 2002, Schlaich *et al.* 2003, Burns *et al.* 2007, Grassi *et al.* 2009, Lambert *et al.* 2011, Mancia and Grassi 2014). Grassi G et al. (Grassi *et al.* 1998, Grassi *et al.* 2000) showed that muscle SNA exhibited a progressive and significant increase from normotension to moderate and more severe essential hypertension, paralleling the progressive increase in blood pressure values. Smith et al. (Smith *et al.* 2004) also reported that individuals with essential hypertension had higher muscle SNA than individuals with normal or high normal pressure. This evidence suggests that sustained sympathetic overdrive may contribute to the development and progression of essential hypertension (Grassi *et al.* 2018).

Sympathetic overdrive not only exists in essential hypertension, but is also a feature in several forms of secondary hypertension, including renal artery stenosis (Miyajima *et al.* 1991), primary aldosteronism (Kontak *et al.* 2010), polycystic kidney disease (PKD) (Klein *et al.* 2001) and chronic kidney failure (Grassi *et al.* 2011, Grassi *et al.* 2012), variably demonstrated by measurements of plasma noradrenaline level, noradrenaline spillover rates or muscle SNA. Increased sympathetic tone is also a feature in experimental hypertension with studies demonstrating an augmentation in systemic or local sympathetic activity in spontaneous hypertensive rat (SHR) (Cabassi *et al.* 2002), obesity induced-hypertension

(Carlson *et al.* 2000, Armitage *et al.* 2012), Ang II hypertension (Guild *et al.* 2012), and CKD-associated hypertension (Ye *et al.* 2002, Salman *et al.* 2014).

In summary, direct evidence that sympathetic activation exists in the early as well as established stages of essential and secondary hypertension supports the concept that the sympathetic overdrive is a critical contributor to the development and maintenance of hypertension.

1.2.1.2 The kidney and salt

A functional pressure natriuresis indicates that the kidney can adequately balance sodium and water in the body with blood pressure in a steady state. Any perturbations to pressure natriuresis, which impairs the kidney's ability to maintain sodium and water balance can result in hypertension, most notably salt-sensitive hypertension and hypertension secondary to kidney disease. In patients with salt-sensitive hypertension, it has been shown that a high sodium loading could not be buffered by the kidney, leading to a significant increase (>10%) in mean arterial pressure compared with low sodium intake (Kawasaki *et al.* 1978). Experimentally, such a situation is mimicked by the Dahl-salt hypertension rat model (Dahl *et al.* 1962, Bashyam 2007). When the kidney from a salt-sensitive rat was transplanted into a normotensive histocompatible rat, the recipient developed hypertension. Conversely, when a kidney from a normotensive control rat was transplanted into a salt-sensitive rat, hypertension resolved, indicating that an intrinsic genetic defect in the renal excretion of sodium was the cause of this hypertension (Dahl *et al.* 1974, Rettig and Grisk 2005, Hall 2016).

In patients with various kidney diseases due to vascular, inflammatory, metabolic or genetic disorders, hypertension could develop partly due to salt and water retention following a decline in glomerular filtration rate (GFR), an overall index of renal function (Mancia 2018). Experimentally, the 5/6 nephrectomy model, where one kidney is removed and 2/3 of the remaining kidney is ablated, mimics the progressive renal failure after the loss of renal mass in humans (Yang *et al.* 2010). It is reported that in early stages of kidney disease, when the

GFR remains normal, patients with kidney disease have already developed sensitivity in blood pressure to a sodium-restricted diet, i.e. a reduction in blood pressure in response to low sodium, suggesting an early impairment in kidney's ability in sodium handling (Konishi *et al.* 2001).

1.2.1.3 RAAS

Dysregulation of the RAAS is involved in the pathogenesis of several hypertensive disorders, most notably renovascular hypertension (Textor 2006). Unilateral renal artery stenosis reduces renal perfusion pressure, leading to the release of renin and widespread activation of the RAAS and elevation of blood pressure. Experimentally, this situation is mimicked by the two-kidney-one-clip (2K1C) hypertension model (Goldblatt *et al.* 1934, Reinhold *et al.* 2009).

In addition to renovascular hypertension, there is evidence that perturbations of the RAAS are also involved in essential hypertension. Measurements of plasma renin activity (PRA) in patients with essential hypertension revealed that approximately 15% of patients have mild to moderate increases in PRA, 50% of patients have PRA within the "normal" range and 25% of patients have decreased PRA (Mulatero *et al.* 2007), although it has been argued that a normal renin level in the face of hypertension (which ought to suppress renin secretion) may be inappropriate (Flack *et al.* 2007). The low level of circulating renin might not necessarily reflect the renin activity in tissues, especially the kidney. Moreover, more and more studies using hypertensive animal models support an important role of intrarenal RAAS in hypertension, especially in the SHR, which mimics essential human hypertension. Kobori et al. (Kobori *et al.* 2005) showed that SHR had enhanced intrarenal angiotensinogen production that contributed to increased Ang II levels, and Lee et al. (Lee *et al.* 2012) revealed that SHRs showed higher expressions of ACE1 mRNA and protein than normotensive Wistar-Kyoto (WKY) rats. Intrarenal RAAS is also augmented in several other animal models, including 2K1C hypertension (Kim *et al.* 2016) and Dahl-salt hypertension (Kobori *et al.* 2003). This inappropriately activated intrarenal RAAS could lead to increased sodium and fluid retention

and eventually hypertension (Carey 2015). Activated intrarenal RAAS is also thought to induce renal organ damages including renal vascular, glomerular, tubular, and interstitial inflammation and fibrosis (Carey 2015).

1.2.1.4 Vascular system

Peripheral vascular resistance, which is a major determinant for blood pressure, is elevated in hypertension (Cooper and Heagerty 1997). This is mainly due to functional and structural abnormalities in small arteries (lumen diameters <300 μm), which contributes most to total peripheral resistance. The structural changes in resistance arteries include thickening of the media and narrowing of the vessel lumen (Cooper and Heagerty 1997). Remodelling of these vessels contributes to high blood pressure and its associated target organ damage (Folkow 1982, Mulvany and Aalkjaer 1990).

In addition to vascular remodelling in small arteries, arteriosclerotic changes in the large conduit arteries can result in reduced elasticity. As a consequence, their ability to buffer blood flow is reduced, leading to an increase in PP and SBP. Arteriosclerotic changes can be attributed to deposition of collagen, smooth muscle cell hypertrophy, in addition to thinning, fragmenting, and fracture of elastin fibres in the media (O'Rourke 2000).

1.3 Treatment of hypertension

1.3.1 Pharmacological therapy

1.3.1.1 Sympatholytic drugs

Sympatholytic drugs act to oppose the influence of the sympathetic nervous system on blood pressure. Depending on the target, they can be characterised into centrally acting and peripherally acting drugs. Centrally active drugs were widely used in the earliest decades of antihypertensive treatment when other treatments were not available, but are less frequently used now, principally because of their poorer tolerability relative to the newer major classes of drugs (Sica 2007). Common side effects of this type of drugs include somnolence and dry

mouth, resulting in a high rate of discontinuation (Sica 2007). Among the peripherally acting drugs, beta-blockers, which act on postsynaptic beta-adrenergic receptors in the heart and vascular smooth muscle are considered a major class of drugs to treat hypertension based on its proven ability to lower blood pressure and reduce cardiovascular morbidity and mortality through their action of lowering inappropriately high sympathetic activity (Thomopoulos *et al.* 2015). This is particularly relevant for younger/middle-aged hypertensive subjects (less than 60 years old), who demonstrate increased sympathetic activity. Meta-analysis has shown that beta-blocker use reduces the risk of myocardial infarction by 35-50% and stroke by 50-55% in young/middle aged non-smoker men compared with placebo or diuretics (Cruickshank 2017). Whether patients will response to beta-blockers or not may be associated with certain single nucleotide polymorphisms (SNP) as a missense SNP rs28404156 in BST1 predicted better response (Singh *et al.* 2019) whereas a SNP in the gene ALDH1A2 was associated with uncontrolled BP following treatment with a thiazide diuretic/beta-blocker combination (Magvanjav *et al.* 2017). Interestingly, beta-blocker usage has been shown to produce a better improvement in clinical parameters and left ventricular function in elderly heart failure patients with reduced left ventricular ejection fraction (LVEF) compared with those with preserved LVEF, suggesting it has significant cardioprotective effects (Edelmann *et al.* 2016). Another class of peripherally acting drugs, alpha blockers which act on postsynaptic alpha adrenergic receptor in the vascular smooth muscle, are also effective at lowering blood pressure through decreasing vascular resistance (Chapman *et al.* 2008, Williams *et al.* 2015), but are associated with increased adverse cardiovascular outcomes such as new-onset heart failure when compared to a diuretic for example (Einhorn *et al.* 2007). Therefore, they are not recommended as first-line therapy for hypertension.

1.3.1.2 Diuretics

Diuretics act on different parts of the renal tubules to promote urine production. Depending on the target, they can be characterised into different types, including loop diuretics (such as

furosemide), thiazides (such as hydrochlorothiazide), carbonic anhydrase inhibitors (such as acetazolamide), and aldosterone receptor antagonists (such as spironolactone). Among them, thiazides are the most widely used antihypertensive drugs with proved efficacy in lowering blood pressure and preventing cardiovascular morbidities and mortality (Thomopoulos *et al.* 2015). However, a common complication with the use of thiazides is hyperkalaemia (Corrao *et al.* 2008). Studies also have shown that thiazides can have an adverse effect on glucose metabolisms, such as insulin resistance and high risk of new-onset diabetes, which might be reduced by the addition of a potassium-sparing diuretic (Brown *et al.* 2016). It needs to be noted that thiazides agents have less or limited effect in patients with a reduced renal function. In such circumstances, loop diuretics such as furosemide are used to replace thiazides to achieve an antihypertensive effect (Thomopoulos *et al.* 2015).

1.3.1.3 RAAS inhibitors

Drugs targeting the RAAS system include angiotensin-converting enzyme inhibitors (ACEIs), AT_1R blockers (ARBs) and direct renin inhibitors (DRIs). Large clinical trials in hypertension have demonstrated that ACEIs had comparable or even superior effects compared to conventional therapy with sympatholytic agents or diuretics (Hansson *et al.* 1999, Hansson *et al.* 1999). ACEIs and ARBs also have other beneficial effects in hypertensive patients, which includes reducing albuminuria, delaying the progression of diabetic and non-diabetic CKD and reducing cardiovascular morbidity and mortality (Yusuf *et al.* 2008). The renin inhibitor aliskiren has been shown to be as effective as ARBs (Stanton *et al.* 2003), ACEIs (Andersen *et al.* 2008), or hydrochlorothiazide (Schmieder *et al.* 2009) in blood pressure reduction. However, simultaneous dual RAAS blockade either provides no additional benefit (Yusuf *et al.* 2008) or can cause more adverse events (Parving *et al.* 2009) compared to monotherapy. Current guidelines of management of hypertension in the United States (James *et al.* 2014) and Europe (Williams *et al.* 2018) recommend monoACEIs and ARBs as first-line antihypertensive agents.

1.3.1.4 Calcium channel blockers (CCBs)

CCBs act on the calcium channels embedded on the membrane of different cells to prevent the extracellular calcium from entering the cell. When acting on vascular smooth muscle, they reduce the contraction of arteries and lower blood pressure by vasodilation. Therefore, CCBs is particularly effective in conditions of large vessel stiffness. Comparing to other classes of antihypertensive drugs, CCBs are non-inferior in terms of lowering blood pressure and preventing cardiovascular events and mortalities (Tsioufis *et al.* 2015, Ettehad *et al.* 2016). CCBs are superior to ACEIs in terms of stroke reduction (Leenen *et al.* 2006). CCBs are also recommended as first-line antihypertensive drugs by current guidelines in the United States (James *et al.* 2014) and Europe (Williams *et al.* 2018).

1.3.2 Non-pharmacological therapy

Despite the use of multiple antihypertensive drugs, about 10% of patients with essential hypertension do not achieve the target blood pressure levels (Noubiap *et al.* 2019). Several non-pharmacological therapies are currently being trailed to reduce blood pressure in this patient cohort.

1.3.2.1 Carotid baroreceptor stimulation

Carotid baroreceptor stimulation is being trialled to treat patients with resistant hypertension based on the following concepts: 1) baroreceptors play an important role in blood pressure regulation (McCubbin *et al.* 1956, Bristow *et al.* 1969, Thrasher 2004); 2) a significant blood pressure reduction was seen after carotid baroreceptor stimulation in experimental studies (Bilgutay and Lillehei 1965, Bilgutay and Lillehei 1966, Lohmeier *et al.* 2004, Lohmeier *et al.* 2005); 3) marked technical progress has been made in implantable devices for carotid baroreceptor stimulation, which can be achieved either by implantation of a pulse generator in the pectoral region with or a stent-like device into the carotid artery sinus to stimulate the baroreceptors. Despite that a sustained blood pressure-lowering efficacy was seen with the first generation of implantable pulse generators, the treatment was associated with a high

incidence of procedural adverse events including chest pain and nerve injuries (Bisognano *et al.* 2011). More advanced endovascular baroreceptor stimulation devices are being investigated for their efficacy and safety in resistant hypertension (Spiering *et al.* 2017) (NCT03179800, NCT02804087).

1.3.2.2 Renal denervation (RDN)

RDN has recently developed significant interest as a therapy for resistant hypertension.

1.3.2.2.1 Renal nerves and hypertension

After the discovery of the importance of the sympathetic nervous system in contributing to hypertension in the early 20th century, the medical community treated patients with severe hypertension by non-selective surgical sympathectomy, a procedure that removes the thoracic and lumbar ganglia (Peet 1948) and markedly reduces high blood pressure. However, this procedure was associated with a high rate of peri-operative morbidity, mortality and long-term complications including hypotension, bowel and bladder problems (Smithwick and Thompson 1953, Evelyn *et al.* 1960), and was abandoned with the advent of effective antihypertensive drugs. Nevertheless, the investigation of the important role the renal nerves play in blood pressure control did not stop, and studies continued to reveal their importance in several experimental hypertension studies (Gattone *et al.* 1984, Campese *et al.* 1995). In the SHR, which mimics the human essential hypertension, bilateral RDN delayed the onset of hypertension but did not alter the final level of blood pressure, suggesting renal nerves play a critical role in the early phase of hypertension in this model (Kline *et al.* 1978, Winternitz *et al.* 1980, Pires *et al.* 2015). In obesity or hyperinsulinemia induced hypertension, RDN prevented the appearance of hypertension possibly via the attenuation of sodium retention (Kassab *et al.* 1995, Huang *et al.* 1998). In the 2K1C hypertensive rat, RDN of the clipped kidney resulted in a reduction in SBP and the depressor effect was associated with a decrease in SNA, specifically to the lumbar and renal vascular beds (Lincevicius *et al.* 2017), while RDN of the unclipped kidney produced no such an effect, suggesting involvement of renal

sensory nerve abnormality in the development of hypertension in this model (Katholi *et al.* 1982). In the deoxycorticosterone acetate (DOCA) salt rat, which is induced by a combination of administration of DOCA, reduced kidney mass, and increased salt intake, removal of the renal sensory nerves was shown to attenuate the level of hypertension to a similar degree as removal of both sensory and sympathetic nerves (Banek *et al.* 2016). In this model, resting renal sensory nerve activity was elevated (Banek *et al.* 2016).

As for 5/6 nephrectomy model, which mimics secondary hypertension after renal failure, both renal sensory nerve ablation by bilateral dorsal rhizotomy and total RDN could prevent the development of hypertension (Campese and Kogosov 1995, Chen *et al.* 2016). These studies provide a strong experimental rationale for RDN as a means by which to treat hypertension.

1.3.2.2.2 Catheter-based RDN

In addition to studies in animal models, renal sympathetic activity, as determined by renal noradrenaline spillover, was elevated in patients with essential hypertension compared with normotensive controls (Esler *et al.* 1988), further providing a convincing theoretical background and impetus for targeting renal nerves to treat hypertension in humans. As the renal nerve bundle travels through the adventitia of the renal artery, it can be easily accessible to radiofrequency energy emitted via a catheter in the artery lumen. The technical development of interventional angioplasty for cardiovascular disease such as acute myocardial infarction facilitated the development of devices suitable for RDN. The radiofrequency catheter for RDN was first developed by Ardian and later was put in a clinical trial under the executive of Medtronic (Krum *et al.* 2009). The procedure is performed by advancing a radiofrequency generating catheter into the renal artery via the femoral artery, which is then used to ablate the renal nerves.

1.3.2.2.3 RDN efficacy in resistant hypertension

In 2009, the first proof-of-concept study of catheter-based RDN showed that the procedure could produce a blood pressure lowering effect in a small cohort of patients with resistant

hypertension (Krum *et al.* 2009). Moreover, it did not seem to cause serious adverse consequences (Krum *et al.* 2009). A flurry of clinical trials were subsequently published between 2010 and 2013, supporting the use of the procedure in producing a sustainable blood pressure reduction in resistant hypertension (Masuo *et al.* 2010, Sadowski *et al.* 2011, Ahmed *et al.* 2012, Esler *et al.* 2012, Krum *et al.* 2012, Prochnau *et al.* 2012, Prochnau *et al.* 2012, Kaltenbach *et al.* 2013). However, the first double-blinded, sham-controlled, randomised trial Simplicity III showed no beneficial effect after RDN compared with the sham procedure, evoking widespread discussion and re-examination of RDN as a valid and appropriate surgical intervention (Bakris *et al.* 2014, Bakris *et al.* 2015). It has since been proposed that the discrepancy between the Simplicity III and previous studies might be due to the patient selection, denervation inadequacy associated with operator experience and variation in adherence to medication (Reddy and Olin 2014, Mahfoud and Luscher 2015). A new proof-of-concept trial, aiming to determine the actual effect of RDN in moderately hypertensive patients was published in 2017 and provided further evidence demonstrating that RDN could reduce blood pressure in mildly hypertensive patients who are not on antihypertensive medications (Townsend *et al.* 2017). A parallel study in patients with moderate hypertension on commonly prescribed antihypertensive drugs also showed a significant and clinically relevant reduction in blood pressure after RDN, compared with a sham procedure (Kandzari *et al.* 2018). However, it is of note that there was a relatively high non-responder rate in these trials, indicating that the selection of patients who will benefit from the procedure still needs to be resolved.

1.3.2.2.4 The proposed mechanism of RDN

Although RDN has demonstrated a blood pressure lowering effect in patients with resistant hypertension, the exact mechanisms are still to be established. A commonly proposed pathway is that interruption of renal sympathetic control of the kidney might be the underlying mechanism. This could be mediated by a number of pathways. As detailed above,

the renin-producing cell is innervated by the renal sympathetic nerves and RDN would function to decrease the renin production, therefore decreasing Ang II such that the blood pressure could be lowered. However, available evidence obtained from animal studies indicates that plasma renin activity remained similar in normotensive rats that underwent bilateral RDN or sham denervation, although the blood pressure was significantly lower in bilateral RDN, compared with sham groups (Jacob *et al.* 2005). Moreover, infusion with β1-adrenergic blockers, which function to block β1-adrenergic receptor-mediated renin release, did not affect plasma renin activity or the difference in blood pressure between the bilateral RDN and sham groups, suggesting that RDN does not reduce blood pressure in these normotensive animals by reducing renin release (Jacob *et al.* 2005). In experimental models, an inconsistent impact of RDN on renin has been reported with plasma renin levels reduced in dogs with heart failure (Chen *et al.* 2017), but unaltered in the Schlager mouse model of neurogenic hypertension (Gueguen *et al.* 2018) and in the SHR (Gao *et al.* 2016). A decreased plasm renin activity was also noted in hypertensive patients at 6 month post catheter-based RDN (Hamza and Khamis 2014). It is important to note that the collection of blood samples in rodent models at study endpoint is often achieved under anaesthesia, which is known to activate the renin-angiotensin system (Petropoulos *et al.* 2000, Efrati *et al.* 2012), whereas blood collection in large animals or patients is often achieved in the unanaesthetised state. In this regard, small changes in plasma renin activity in response to RDN in rodent models may be difficult to detect.

As renal sympathetic nerves control sodium and water reabsorption, removing sympathetic denervation to the kidney might also reduce blood pressure by affecting the sodium and water reabsorption. However, Jacob et al (Jacob *et al.* 2003) showed that RDN could lower blood pressure by the same magnitude in normotensive Sprague-Dawley rats whether the animals were fed with normal (0.04%) or high sodium (4%) intake, without affecting sodium balance, suggesting that the blood pressure lowering effect was not mediated by increased sodium

excretion. Alternatively, loss of sympathetic innervation to the kidney could produce a blood pressure lowering effect through altering renal vascular resistance. This hypothesis is supported by reports of a negative correlation between renal nerve activity and renal vascular conductance (the inverse of resistance) in conscious rats (Yoshimoto *et al.* 2004).

As noted in section 1.1.4.1 (Renal sensory innervation), renal sensory nerves relay afferent information to the central nervous system, which in turn modulates sympathetic outflow. As RDN also interrupts renal afferent inputs to the central nervous system, it is likely that this could alter central pathways involved in blood pressure homeostasis (Phillips 2015). Campese and Kogosov (Campese and Kogosov 1995) have shown that dorsal root rhizotomy at T10 to L2, which involves transecting the cell bodies of renal sensory nerves, prevents both the rise in arterial pressure and increased turnover of noradrenaline in the posterior and lateral hypothalamus in the 5/6 renal ablation model of kidney failure. Dorsal root rhizotomy results in a slight but significant reduction in mean arterial pressure in SHR compared to sham controls (Janssen *et al.* 1989) and has been shown to attenuate hypertension in the unilateral nephrectomized SHR model, in accompaniment with a reduction of ACE1 and AT1 receptors mRNAs in the hypothalamus and lower brainstem (Nishimura *et al.* 2007). This evidence suggests that the removal of renal afferents could indeed contribute to the blood pressure reduction via altering central sympathetic or the RAAS activity after RDN.

1.3.2.2.5 Unresolved issues of RDN

Although RDN has been trialled in patients for close to a decade, several fundamental issues remain unsolved. The first problem is that there are no available intraprocedural methods to confirm technical success. In animal studies, the loss of renal nerve activity can be confirmed by postprocedural direct nerve recordings and lack of cardiovascular parameter (MAP and heart rate) changes upon nerve stimulation (Booth *et al.* 2015). However, this is not feasible in humans due to the inaccessibility of the renal nerves and the invasiveness of the procedure (Sobotka *et al.* 2015). A few studies reported a reduced renal noradrenaline spillover after the

procedure, which serves as a surrogate for the effectiveness of the procedure (Krum *et al.* 2009, Schlaich *et al.* 2009). However, this procedure is neither readily accessible nor practicable in a general clinical setting.

The second problem is that with emerging evidence of renal reinnervation following RDN, whether and how long after RDN these nerves resume their functionality, thus limiting the blood pressure effect post-procedure remains to be determined. Certainly reinnervation has been documented in animal studies after both surgical/chemical RDN procedures and catheter-based RDN. In the study of Mulder et al. (Mulder *et al.* 2013) after the use of periaxional application of phenol, both renal sympathetic and sensory nerves were found to regrow to 40-50% of that in innervated kidneys at 4-week post-denervation. Foss et al. (Foss *et al.* 2015) found that renal sensory marker staining was abolished at 10 days, but returned to 20% of that observed in sham controls 4 weeks after capsaicin-induced afferent denervation, providing additional evidence for the reinnervation after RDN. Functionally, renal vasoconstrictor responses to electrical renal nerve stimulation were absent during the first 2 weeks following total RDN using phenol in male Wistar rats but reoccurred at 4 weeks, though at this time renal cortical noradrenaline content was still less than 30% of that found in control kidneys (Kline and Mercer 1980). Total RDN was reported to cause a delay of onset of hypertension in SHR rats, associated with a gradual return of renal noradrenaline content following denervation (Winternitz *et al.* 1980). In a large animal sheep model, both anatomical and functional evidence of reinnervation has been documented (Booth *et al.* 2015, Singh *et al.* 2019). It needs to be noted that in normotensive animals the reinnervation was complete at 11 months post-procedure (Booth *et al.* 2015) whereas in the hypertensive animals, being a model of CKD, the reinnervation was incomplete at 30 months post-procedure, with the authors arguing that RDN may permanently correct the renal hyperinnervation that was evident in the hypertensive model (Singh *et al.* 2019). The regrowth of renal nerves in humans has been documented in one post-mortem analysis of

kidney-transplanted patients, where sympathetic nerves were evident in the donor's kidney 5 months after transplantation (Mauriello *et al.* 2017). Nerve regrowth after catheter-based RDN and its effect on the blood pressure in patients is a critical issue to be addressed in the future as this determines the longevity of the procedure effectiveness.

The third problem is that there are currently no reliable indicators by which to predict which patients will benefit from the procedure. Clinical studies that address possible predictors of the blood pressure response to RDN have identified only one generally reliable factor, namely pre-RDN blood pressure level (Ewen *et al.* 2015, Kandzari *et al.* 2015, Rohla *et al.* 2016). There is growing evidence for a few other factors which will predict individual response to RDN. Xu et al. (Xu *et al.* 2018) suggested that an increase in blood pressure during radiofrequency energy delivery, which is most likely caused by stimulation of the sympathoexcitatory renal afferent pathways, could predict the long-term procedural success of RDN. Renal nerve stimulation-induced blood pressure changes before versus after RDN were correlated with changes in 24h ambulatory blood pressure monitoring 3 to 6 months after RDN, both for SBP and DBP (de Jong *et al.* 2016). These studies suggested that patients who had greater blood pressure response during the ablation procedure might have a better outcome. In another study, nighttime systolic blood pressure and variability, which are less influenced by daily activities, had predictive value to blood pressure response to denervation (Gosse *et al.* 2017) and there is evidence to suggest that patients with less arterial stiffness show a significantly better response to denervation than those with increased stiffness (Okon *et al.* 2016). Marked endothelial dysfunction and a higher vasomotor tone are also indicators that have been proposed to identify likely responders to RDN (Steinmetz *et al.* 2018). However, these observations need to be confirmed in further research for accurate identification of patients likely to respond to RDN with a fall in blood pressure that is clinically significant in magnitude and well-maintained over time. Embedded within the question as to which specific patients will respond to RDN is the bigger question as to

whether specific patient cohorts, as defined by disease aetiology for example, are more or less likely to benefit from the procedure.

1.4 Chronic kidney disease (CKD)

CKD is a well-recognised public health problem, with an estimated prevalence of 10% and 14% in Australia and the United States, respectively (Centers for Disease Control and Prevention , Coresh *et al.* 2007). CKD results from a number of heterogeneous disorders including hypertension, diabetes, glomerulonephritis, and genetic kidney diseases that ultimately cause a structural change and functional compromise to one or both kidneys (USRDS 2017). CKD is defined as evidence of kidney damage or glomerular filtration rate (GFR) <60 ml/min per 1.73m$_2$ for ≥ 3 months, irrespective of underlying cause (Levey *et al.* 2011, Levin *et al.* 2013) and is further classified into 5 different stages based on the level of GFR (Table 1). The symptoms are variable depending on the cause, with some patients asymptomatic before diagnosis and others having symptoms due to kidney damage. As the disease progresses, patients might experience symptoms such as fatigue or nausea associated with metabolic waste accumulation, and ultimately develop complications including high blood pressure, anaemia, bone disease, and cardiovascular disease associated with the impaired renal function.

Table 1.1 Staging of CKD

Stages	GFR (ml/min per 1.73m$_2$)	Description
1	≥90	Normal or high
2	60-89	Mildly decreased
3a	45-59	Mildly to moderately decreased
3b	30-45	Moderately to severely decreased
4	15-29	Severely decreased
5	<15	Kidney failure

The medical costs attributable to CKD are substantial and increase with disease severity. In Australia in 2015 (Wyld *et al.* 2015), per-person annual direct healthcare costs by CKD stages were $2719 for stage 1 to 2, $3489 for stage 3, and $ 145455 for stage 4 to 5. This is comparable to the USA in 2013 where the annual Medicare expenses per person attributable to CKD stage 1 to 4 were 0, $1700, $3500, and $12,700, respectively (Honeycutt *et al.* 2013) and the United Kingdom in 2015 where the annual hospital care cost incurred was £1055 for CKD stage 1-3, £3694 for stage 4, £12952 for stage 5 not on dialysis, and £20511 for dialysis patients (Kent *et al.* 2015). For patients with a functioning kidney transplant, the hospital care cost was £24,602 in the year of transplantation and £1,148 annually thereafter (Kent *et al.* 2015). Thus, interventions to slow the progression of CKD are essential to reduce the economic burden on the patients and society.

1.4.1 Hypertension in CKD

Hypertension is a common comorbidity in patients with CKD (Whaley-Connell *et al.* 2008). The prevalence of hypertension is elevated in patients with kidney damage and a normal GFR and increases further as the GFR falls. Data from the US Renal Data System, for example, showed that the prevalence of hypertension rose progressively as GFR declines, being 35.8% at stage 1, 48.1% at stage 2, 59.9% at stage 3, and 84.1% at stage 4-5, respectively (System 2010). As a group, CKD patients with hypertension are at a higher risk of developing end-stage renal disease (ESRD) than those without (Hsu *et al.* 2009). Moreover, hypertension is associated with a higher risk of cardiovascular death, the most common cause of mortality in CKD patients (Fick *et al.* 1995, Neumann *et al.* 2002). Factors contributing to hypertension in CKD will be briefly discussed in the following.

Volume overload as a result of decreased GFR serves as a significant contributor to hypertension in CKD (Koomans *et al.* 1982, Vasavada and Agarwal 2003) and underlies the rationale for using diuretics to reduce high blood pressure in this patient group. Augmentation of RAAS, i.e., increased renin release due to decreased renal blood flow and subsequent

increase in Ang II, a potent vasoconstrictor, is another well documented factor underlying hypertension in CKD (Weidmann *et al.* 1971, Vaughan Jr *et al.* 1979, Siragy and Carey 2010) and the use of ACEIs and ARBs markedly improve the outcome of CKD patients (Jafar *et al.* 2003).

Sympathetic overactivation is another factor contributing to hypertension in CKD. Ishii et al. (Ishii *et al.* 1983) documented that plasma concentrations of noradrenaline, adrenaline, aldosterone and plasma renin activity were significantly elevated in hypertensive patients with primary glomerular disease compared to normotensive patients or healthy subjects. The plasma noradrenaline level was correlated positively with blood pressure in these patients, suggesting increased SNA contributing to hypertension in this patient group. Converse et al. (Converse Jr *et al.* 1992) found a higher level of muscle SNA in patients receiving haemodialysis compared to normal controls, which was normalised after bilateral nephrectomy, indicating a role for the diseased kidneys in the overactivity of sympathetic tone in ESRD. In a classic study by Klein et al. (Klein *et al.* 2001), muscle SNA was shown to be significantly higher in hypertensive autosomal dominant PKD (ADPKD) patients than their normotensive counterparts. Whatsmore, muscle SNA was higher in ADPKD patients with renal insufficiency than in hypertensive ADPKD patients with normal renal function. Using a phenol-induced kidney injury model, Ye et al. (Ye *et al.* 2002) showed an acute increase in both renal sensory nerve and sympathetic nerve activity, as well as a chronic elevation in blood pressure and plasma noradrenaline. Using direct nerve recording, our group has also demonstrated an increased SNA to various vascular beds including the kidney in a genetic CKD model, the Lewis polycystic kidney rat (LPK) (Salman *et al.* 2015, Salman *et al.* 2015, Yao *et al.* 2015). Moreover, the administration of ganglionic blockade hexamethonium significantly reduced mean blood pressure in the LPK compared with their normotensive Lewis controls (Phillips *et al.* 2007, Ameer *et al.* 2014). These observations in animal models

and humans indicate not only a generalised increase in SNA in association with CKD but that the kidney is a potential source of this drive that could be pursued clinically.

1.4.1.1 RDN in CKD

Resistant hypertension is common in CKD patients (Borrelli *et al.* 2013, Unni *et al.* 2015, Verdalles *et al.* 2016). However, due to concerns of renal safety, initial clinical trials of RDN in resistant hypertension excluded patients with estimated GFR (eGFR) < 45 ml/min per 1.73m2. As mentioned earlier, sympathetic overactivity is implicated in hypertension associated with CKD, and afferent signalling derived from the native injured kidneys may potentiate this sympathoexcitation, making CKD patients suitable candidates for RDN. Hering et al. (Hering *et al.* 2012) reported in a pilot study examining 15 patients with hypertension and moderate to severe CKD that RDN caused a marked ~30 mmHg reduction in SBP at 1, 3, 6, and 12 months of follow-up without deterioration of renal function. Several case reports and a case series study also have served to establish efficacy and safety of RDN in CKD patients (Di Daniele *et al.* 2012, Ott *et al.* 2012, Schlaich *et al.* 2013). A 29-year-old woman with ESRD on haemodialysis and resistant hypertension evaluated by 24h ambulatory blood pressure monitoring showed a remarkable blood pressure reduction of 38/30 mmHg 6 months following RDN (Ott *et al.* 2012). In another case report, RDN caused a progressive and sustained reduction of SBP by 25 mmHg at one month in a 39-year-old ESRD patient on haemodialysis, with no loss of baseline residual kidney function (daily urine output ~500ml) (Di Daniele *et al.* 2012). Schlaich et al. (Schlaich *et al.* 2013) showed that RDN significantly reduced the office blood pressure in 9 patients with ESRD at 3, 6, and 12 months after RDN. Ott et al. (Ott *et al.* 2015) found that RDN not only decreased blood pressure but also ameliorated the decline of renal function in patients with stages 3 and 4 CKD. A study published in 2017 showed that the estimated GFR decline rate in patients with CKD was slowed down by RDN as the GFR showed an annual decline by 3.5 mL/min/1.73m2 from 60 months before RDN but, did not change during the 24 months after RDN (Hering *et al.* 2017).

However, none of these clinical studies was randomised sham-controlled trials and therefore the strength of the evidence is mitigated. Whether these observed blood pressure lowering and renoprotective effect of RDN can be replicated in large randomised clinical trials needs further investigation. In addition, a mechanistic explanation explaining these observations is still not clear. Therefore, further studies are warranted to provide a better understanding of mechanisms for the RDN-induced effect in CKD.

1.5 Polycystic kidney disease: a cause of CKD

As noted in section 1.4, various types of renal disease can lead to CKD. One example, which is the focus of this thesis, is PKD. Polycystic kidney disease encompasses a group of inherited diseases characterised by the development of fluid-filled cysts in both kidneys and a progressive decline in renal function, with or without involvement in other organs such as the liver, the pancreas and the brain (Igarashi and Somlo 2002). It represents the fourth leading cause of CKD and according to Australia and New Zealand Dialysis and Transplant (ANZDATA) Registry (ANZDATARegistry 2017) accounts for 7% of patients receiving renal replacement therapy. A unifying feature of the proteins associated with PKD is that they are all normally expressed in either primary cilia or centrosomes, indicating these protein products might cause PKD through a common signalling pathway (Hildebrandt *et al.* 2009). A detailed review of different forms of PKD due to different gene mutations is presented as follows.

1.5.1 Autosomal dominant polycystic kidney disease (ADPKD)

Autosomal dominant PKD (ADPKD) is the most common human monogenic disease, with a ranging incidence between 1 in 4000 to 1 in 1000 depending on the geographic location (Ong *et al.* 2015). The causal genes for ADPKD are PKD1 on chromosome 16 and PKD2 on chromosome 4, encoding polycystin-1 (PC-1) and polycystin-2 (PC-2), respectively, which account for over 95% of all cases (Hayashi *et al.* 1997, Bycroft *et al.* 1999). In some patients

with typical clinical features for ADPKD, mutations in other genes such as GANAB which is located on chromosome 11, have recently been identified (Reddy and Chapman 2017). The mutations in PKD1 and PKD2 result in dysfunction of PC-1 and PC2, leading to dysregulation in cellular signalling pathways involved in cell growth and fluid secretion and therefore cyst formation, which can occur in multiple organs including the kidney, the liver and the pancreas (Simms 2016). It is suggested that PKD1 mutations result in a more severe form than PKD2 mutations (Simms 2016). The symptoms of ADPKD include abdominal pain in the flank or back, abdominal fullness, cyst haemorrhage, cyst infection, kidney stones, haematuria and hypertension (Torres *et al.* 2007, Lantinga *et al.* 2015). Epidemiological studies show that hypertension affects around 20% of children with ADPKD (Marlais *et al.* 2016). In adults with ADPKD, the prevalence of hypertension is 50-62% in patients with preserved renal function and almost 100% in patients with renal failure (Ecder and Schrier 2001). Hypertension in ADPKD is generally easy to treat, and resistant forms are very rare (Wuthrich *et al.* 2015). Cardiovascular diseases are common complications, with arrhythmias (25.9%), peripheral vascular disease (16.5%), heart valve problems (14.4%), cardiac enlargement (9.5%), stroke or cerebral bleeding (7.5%), myocardial infarction (6%) and brain aneurysm (5.0%) being reported (Torres *et al.* 1994, Pirson *et al.* 2002, Torres *et al.* 2007, Helal *et al.* 2012). Treatment of ADPKD had been limited to supportive measures to control disease complications including hypertension and chronic pain until recently when tolvaptan, a vasopressin receptor 2 (V2) antagonist, whose mechanism of action is to block the deleterious effect of vasopressin on cytogenesis, was proven to significantly reduce both kidney volume and decline of estimated glomerular filtration rate (eGFR) in a 3-year randomized double-blinded placebo controlled trial (Torres *et al.* 2016). The side effects of tolvaptan include marked nocturia and polyuria, which lower the adherence of the patients to the treatment and there is still ongoing research for therapeutic strategies to limit cyst development with tolerable side effects.

1.5.2 Autosomal recessive polycystic kidney disease (ARPKD)

Autosomal recessive PKD (ARPKD) is less prevalent than ADPKD with an estimated incidence of 1 in 20,000 births (Zerres *et al.* 1998, Guay-Woodford *et al.* 2014). All ARPKD cases are due to the mutations of one gene, the polycystic kidney and hepatic disease 1 (PKHD1) gene, which is located on chromosome 6 and encodes fibrocystin/polyductin (Zerres *et al.* 1998, Melchionda *et al.* 2016). Fibrocystin is thought to work with PC-1 and PC-2 in mediating its function of maintaining the structural integrity of organs such as kidney and liver, by modulating important cellular functions, including proliferation, secretion, apoptosis, and terminal differentiation (Benjamin 2018). Genetic abnormalities in PKHD1 result in proliferation in kidney epithelial cells and cyst formation in ARPKD (Yang *et al.* 2007, Benjamin 2018). Neonates often present with a deficiency of amniotic fluid, massively enlarged kidneys and pulmonary hypoplasia leading to respiratory failure and perinatal death (Hartung and Guay-Woodford 2014). Advances in neonatal supportive care have significantly improved the survival rates of ARPKD infants. The age at onset of ARPKD appears to affect the age of progression to ESRD, with one study showing that patients diagnosed before 1 year of age had a renal survival rate (that is being alive without ESRD) of 36% 20 years after diagnosis while those diagnosed at the age of 1 to 20 years had a renal survival rate of 80% (Adeva *et al.* 2006). In neonatal ARPKD suvivors, about 75% developed systemic hypertension during a mean observation period of 6 years (range 0-35 years) (Bergmann *et al.* 2005), and several classes of medication are often required for treatment as resistence to multi-drug treatment is prevalent (Bergmann 2015, Dell *et al.* 2016). Systemic hypertension is common in patients with ARPKD (Kaplan *et al.* 1989, Zerres *et al.* 1996, Roy *et al.* 1997, Capisonda *et al.* 2003, Guay-Woodford and Desmond 2003), and often develops before renal function declines. Extrarenal and systemic manifestations include biliary development defect leading to cystic liver disease, portal hypertension, growth impairment and possibly neurocognitive dysfunction.

1.5.3 Nephronophthisis

Nephronophthisis (NPHP) is another autosomal recessive form of genetic cystic kidney disease, with an estimated incidence of 1 in 1,000,000 live births in the United States (Sun *et al.* 2014). However it is the most common genetic cause of kidney failure in children (Sun *et al.* 2014). To date, more than 25 different genes have been identified to be associated with NPHP (Srivastava *et al.* 2017). NPHP1, encoding nephrocystin-1, is the most commonly reported genetic mutation in NPHP, accounting for around 20% of cases. Each of the remaining NPHP genes accounts for 3% or less of all cases of NPHP, and around two-thirds of cases remain genetically unsolved (Srivastava *et al.* 2017, Luo and Tao 2018). Three clinical forms of NPHP have been recognised according to the age of ESRD onset: infantile, juvenile, and adolescent/adult NPHP, which display ESRD at the median ages of 1, 13 and 19 years, respectively (Hildebrandt *et al.* 2009). The juvenile form is the most common variant and is associated with mutations in all the NPHP genes except NPHP2 (Niaudet 2018). In particular, patients with mutations of the NPHP1 gene, the most commonly affected gene, present with juvenile NPHP (Parisi *et al.* 2004). The infantile form is most commonly associated with mutations in the NPHP2 gene (Otto *et al.* 2003) and the adolescent form with mutations of the NPHP3 gene (Omran *et al.* 2000). Clinical manifestations include polyuria, polydipsia, isosthenuria, anaemia, impaired urinary concentrating ability, and eventually renal failure (Wolf and Hildebrandt 2011). A renal ultrasound would reveal corticomedullary cysts, leading to the nephron loss and tubulointerstitial fibrosis (Wolf and Hildebrandt 2011), with the term NPHP meaning disappearance of nephrons. Extrarenal manifestations can include retinal defects, liver fibrosis, skeletal abnormalities, and developmental brain disorders dependent upon the mutation and associated syndrome disorder (Wolf and Hildebrandt 2011). No specific therapy for NPHP is available and supportive management to control complications and slow the worsening of renal function is the primary therapy with the ultimate requirement for renal transplantation as for other forms of PKD (Luo and Tao 2018).

The cytogenesis is thought to cause the compression of the normal renal structure including vasculature, which in turn leads to intrarenal ischemia and activation of the RAAS, accelerating in the development of hypertension, and renal dysfunction (Halvorson *et al.* 2010).

1.6 Murine models of polycystic kidney disease

Several murine models have been widely used to help understand the pathogenesis and advance the treatment of PKD.

The Han: SPRD-cy rat is a well-established rat model of ADPKD, resulting from a spontaneous missense mutation in the Pkdr1 gene on chromosome 5 in the Sprague-Dawley strain (Bihoreau *et al.* 1997, Brown *et al.* 2005). The disease progression and severity are affected by genetic background and sex. In homozygous animals, renal cysts are observed in the neonatal stage. Rapid disease progression results in a short 3-week life span (Schafer *et al.* 1994). In heterozygous animals, renal cystic lesions appear mainly in the proximal tubules within the first few weeks of life , and progress slowly, with females and males having a life span of 1.5 years and 1 year, respectively (Schafer *et al.* 1994, Gretz *et al.* 1995, Nagao *et al.* 2003). This model has been widely used to evaluate therapeutic interventions for PKD (Belibi *et al.* 2011, Ibrahim *et al.* 2016).

The polycystic kidney (*pck*) rat model is characterized by a spontaneous mutation in PKHD1 gene on chromosome 9, which is the orthologous human PKHD1 gene, in the Crj: CD/SD strain (Katsuyama *et al.* 2000, Lager *et al.* 2001). In affected homozygotes, renal architecture is normal at birth. Renal cystic lesions appear after the first week of life, with cysts initially expressed in the collecting ducts and affecting whole nephron segments in the end-stage disease. Biliary ductal dilatation with fibrosis is evident in the initial stage, progresses with age, and is associated with marked hepatomegaly (Yoshihara *et al.* 2011).

The congenital polycystic kidney (*cpk*) mouse model results from a spontaneous mutation in the Cys1 gene on chromosome 12 in the C57BL/6J (B6) strain (Hou *et al.* 2002). Mutants develop massive renal cystic disease and progressive renal deficiency in a pattern that strongly resembles human ARPKD (Preminger *et al.* 1982, Fry *et al.* 1985). Death ensures by 3-4 weeks of age, presumably due to renal failure. The BALB/c polycystic kidney (*bpk*) model results from a spontaneous mutation of the gene Bicc1 located on chromosome 10 in the BALB/c strain (Ricker *et al.* 2000). Affected homozygotes develop both renal cystic disease and biliary dysgenesis. Both the Cys1 gene product cystin and Bicc1 gene product are located in the primary cilia, consistent with disease features in human PKD.

Targeted genetic deletion of human orthologs PKD genes, Pkd1, Pkd2, and Pkhd1 gene has created a number of PKD models, e.g., $Pkd1^{del34}$, $Pkd1^{null}$, Pkd1-, $Pkd2^{WS25/-}$, allowing a better understanding of the development and progression of PKD and the discovery of novel targets for therapeutic drugs (Kim *et al.* 2000, Lu *et al.* 2001, Lantinga-van Leeuwen *et al.* 2007, Nagao *et al.* 2012). Both heterozygous and homozygous Pkd1 targeted mice develop renal, biliary, and pancreatic cysts. While slow or no disease progression is observed in heterozygous Pkd1 targeting mice, almost all types of homozygous Pkd1 targeting mice demonstrate embryonic lethality. The $Pkd2^{WS25/-}$ mouse is a gene-targeting mouse for Pkd2 with an unstable allele, and an important model used for drug treatment studies (Wu *et al.* 2000). Due to the Pkd2 gene inactivation, renal cyst formation and enlargement appear, resulting in a 2-fold increase in kidney weight compared with that in wild-type mice (Stroope *et al.* 2010). In homozygous Pkhd1 gene-targeting mice, renal cysts derived from the collecting ducts and biliary ductal plate malformations are seen in adulthood, which is similar to the symptoms of the *pck* rat (Williams *et al.* 2008). In 2012, a breakthrough study reported that spontaneous mitotic recombination in induced pluripotent stem cells (iPSCs) obtained from a Pkd1+/− knockout mouse resulted in the restoration of the normal Pkd1 allele (+/repaired+) from the heterozygous Pkd1 deletion (+/−) (Cheng *et al.* 2012). This finding

suggests that research on genetic correction may enable new treatment strategies for patients carrying genetic PKD disease.

1.7 Lewis polycystic kidney disease rat model (LPK)

The LPK rat is a well-established autosomal recessive model of PKD and secondary hypertension (Phillips *et al.* 2007). This model exhibits a mutation in the never in mitosis gene a-related kinase 8 (Nek8) gene, which is orthologue to human nephronophthisis 9 (NPHP9) (McCooke *et al.* 2012). The Nek8 protein localises to the proximal region of the primary cilium, where it seems to modulate ciliary targeting of polycystin-1 and polycystin-2 (Sohara *et al.* 2008) and this localisation is disturbed in the LPK rat model (McCooke *et al.* 2012). A description of the arising disease presentations including hypertension, autonomic dysfunction and renal dysfunction and associated mechanisms is summarised below.

1.7.1 Cystic development

In this model, renal cysts are found throughout the cortex and medulla from as early as 3 weeks, and the cyst size increases progressively to 12 weeks (Phillips *et al.* 2007). These cysts originate from mainly the collecting ducts and to a lesser extent from the distal convoluted tubule and descending limbs of the Henle (Phillips *et al.* 2007). Phenotypically, this resembles the ARPKD in humans (Avner and Sweeney 2006). However, unlike human ARPKD, cysts are not evident in other organs such as liver and pancreas (Phillips *et al.* 2007).

1.7.2 Hypertension 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 and remained high till 24 weeks old (Phillips *et al.* 2007). The mechanisms for the development of hypertension have been explored. The activation of the sympathetic nervous system is thought to be involved. Salman et al. showed elevated SNA to the renal vascular bed in both conscious (Salman *et al.* 2015) and anesthetized (Salman *et al.* 2014) LPK animals, whereas Phillips et

al. (Phillips *et al.* 2007) and Ameer et al. (Ameer *et al.* 2014) showed that the LPK displayed a significantly greater reduction than the Lewis upon administration of ganglion blocker hexamethonium. This evidence suggested a systemic and localised elevation of sympathetic tone in the LPK. Inappropriate activation of the RAAS might also participate in the development of hypertension in LPK, as the administration of perindopril (Ng *et al.* 2011) and valsartan (Ameer *et al.* 2016) can attenuate hypertension in this model. Other factors contributing to hypertension in the LPK include increased excitatory regulation of the PVN (Underwood *et al.* 2019), and vascular stiffness (Ng *et al.* 2011, Quek *et al.* 2016). It has been proposed that the activation of renal sensory nerves due to local renal ischaemia in association with renal hypoxia in the LPK (Ow *et al.* 2014) might contribute to the increased sympathetic nervous system activity via central autonomic pathways such as the PVN. With both increase renal SNA and likely increased afferent activity, the model therefore could be a valuable tool to undertake further studies regarding the efficacy and mechanisms of RDN as it applies specifically to CKD. Moreover, commonly-used antihypertensive drugs do not normalise the blood pressure in LPKs and resistance to treatment is much greater as the disease progresses, which resembles resistant hypertension in humans (Ng *et al.* 2011, Ameer *et al.* 2016).

1.7.3 Renal dysfunction

Elevated serum urea is present by age 3 weeks in the LPK, with a significant elevation of serum creatinine present at age 12 weeks, at which point, isosthenuria, consistent with a loss of urine concentrating ability, is also evident (Phillips *et al.* 2007). There is also a gradual increase in urine protein to creatinine ratio in the model, consistent with a gradual loss of glomerular filtration ability. LPK animals also demonstrated a progressive normocytic normochromic anaemia from age 12 weeks, reflected by decreasing haemoglobin concentrations and haematocrit (Phillips *et al.* 2015), which is consistent the anaemia in human CKD patients (Padhi *et al.* 2015).

1.7.4 Autonomic dysfunction

Autonomic dysfunction in the LPK model first becomes apparent between 10-12 weeks old and is characterised by reduced heart rate variability and increased systolic blood pressure variability, and a reduction in heart rate baroreflex function (Hildreth *et al.* 2013). The development of autonomic dysfunction is rapid in onset at that age and appears to be independent of the development of hypertension, which is established by age 6 weeks (Hildreth *et al.* 2013). Subsequent work suggests that this was caused by a deficit in the afferent component of the baroreflex from an early age and a decrease in central processing pathway developed in older animals (Salman *et al.* 2014), though the deficit in the afferent component of the baroreflex was only present in young male not female animals (Salman *et al.* 2015). Interestingly, vascular remodelling of the aortic arch was only present in young male LPK animals, which might explain the decline in the functionality of the afferent component of the reflex in the male animals only (Salman *et al.* 2014, Salman *et al.* 2015).

1.8 Thesis objective

Cardiovascular complications are a key co-morbidity for patients with CKD (Alani *et al.* 2014) and the associated hypertension is highly prevalent and difficult to treat (Horowitz *et al.* 2015). As a novel therapeutic strategy, RDN offers an alternative approach however its efficacy in this patient cohort is not clear. There is evidence that the mechanism by which the removal of the renal nerves acts to reduce blood pressure could be mediated by both the renal afferent sensory (Banek *et al.* 2016) and efferent sympathetic (Foss *et al.* 2016) components and there is also a strong likelihood of the procedure impacting other homeostatic pathways regulated by the kidney such as the RAAS (Gueguen *et al.* 2018, Takahashi *et al.* 2018) and sodium balance. The issue of reinnervation is still an open question, given that in humans there are proposed long-term benefits (Esler *et al.* 2014) despite anatomical and function evidence from experimental animal models of reinnervation (Kline and Mercer 1980, Mulder *et al.* 2013, Booth *et al.* 2015). The overall aim of this thesis is therefore to investigate 1) the

effect of RDN on hypertension related to CKD and the individual contribution of renal sensory and sympathetic nerves to the RDN-induced effect, 2) the effect of RDN on the RAAS and renal handling of sodium, 3) the potential effect of renal reinnervation.

The specific aims of the present thesis are as follows:

Chapter 3: The protocol used for RDN in experimental animal models is either to destroy both nerves by chemical denervation with phenol (Gattone *et al.* 1984) or destroy the renal afferents by dorsal rhizotomy (Campese and Kogosov 1995). The relatively new procedure of applying capsaicin the renal nerves to selectively destroy renal sensory nerves, as compared to the dorsal root rhizotomy procedure has been proposed as an equally effective but less invasive option (Foss *et al.* 2015). Therefore, in this chapter we aimed to validate the effectiveness of the periaxonal application of capsaicin alongside that of the use of phenol to selectively destroy renal sensory nerves or both sensory and sympathetic nerves, respectively in both LPK and Lewis control rats. This serves as a fundamental step for the following studies.

Chapter 4: Experimental studies indicate that renal sensory and sympathetic nerves make a differential contribution to varying forms of hypertension (Banek *et al.* 2016, Foss *et al.* 2016). Although RDN seems to attenuate hypertension in CKD patients (Hering *et al.* 2017) and animal models (Singh *et al.* 2019), it is not clear whether the antihypertensive effect observed in these studies is primarily mediated by reduction of renal sensory input to the central nervous system, a reduction of renal sympathetic outflow or a combination of both given the fact that RDN is achieved by removal of both renal sensory and sympathetic nerves. Moreover, strong evidence exists for the regrowth of both renal sensory and sympathetic nerves after denervation in animal models (Kline and Mercer 1980, Mulder *et al.* 2013, Booth *et al.* 2015, Singh *et al.* 2019), suggesting that the regrowth of renal nerves in humans is also likely. However, human RDN studies suggest a long-term blood pressure lowering effect, indicating that re-innervation (while not yet confirmed in humans) does not affect the

observed blood pressure response. Thus, our aim was to examine the effect of selective renal sensory and total RDN on blood pressure variables over eight weeks in Lewis and LPK rats, predicting that both procedures would attenuate hypertension in this model. We also examined any impact on renal function and compared the effect of a repeat vs single total RDN to determine the potential functionality of renal reinnervation after the first procedure.

Chapter 5: Cardiovascular autonomic dysfunction is a serious complication in CKD, and associated with adverse cardiac events and increased mortality (Parati *et al.* 2012, Ryu *et al.* 2014, Almakramy *et al.* 2017). RDN appears to offer benefits in autonomic function beyond its blood pressure lowering effect (Hart *et al.* 2013). Whether RDN could also improve autonomic dysfunction in LPK is unknown. We therefore evaluated the long-term effect of selective and total RDN on the autonomic function parameters in both Lewis and LPK using spectral analysis, predicting that the autonomic function parameters in LPK could be improved after RDN procedure and that this may be independent of any effect on blood pressure.

Chapter 6: The RAAS is suggested to play a critical role in human and experimental hypertension (Yim and Yoo 2008). Renal sympathetic nerves impact the RAAS at the level of the kidney by promoting renin section, and sodium balance by acting directly on the renal tubules or indirectly by activating aldosterone production for sodium reabsorption (DiBona 2000). RDN, therefore, by removing the sympathetic innervation to the kidney, would be expected to cause alterations in RAAS components through reduction of renin production and in sodium excretion through a reduction in sympathetic nerve-mediated or aldosterone-mediated sodium reabsorption. However, available evidence is contradictory, with some studies demonstrating reduced RAAS components in the plasma, kidney or urine in several experimental models (Gueguen *et al.* 2018, Takahashi *et al.* 2018) but others showed no change (Gao *et al.* 2016, Mansuri *et al.* 2017). Inconsistent impact of RDN on sodium excretion was also reported in experimental studies, with some demonstrating an increased

sodium excretion after RDN (Winternitz *et al.* 1980, Li *et al.* 2016) but others showing no impact (Kline *et al.* 1978, Greenberg and Osborn 1994). We hypothesised that removing the sympathetic control of the kidney would result in a decrease in sodium reabsorption (increase in sodium excretion) and downregulation of the RAAS pathway in our LPK model. We therefore investigated the effect of RDN on plasma and kidney renin content, renal RAAS gene expression (AGT, renin, ACE1, ACE2 and AT_1R,) and urinary sodium. In the LPK, our previous studies have shown that relative to the Lewis, the RAAS is suppressed systemically (Phillips *et al.* 2007) however to date intra-renal RAAS has not been studied in this model, nor has sodium excretion. Therefore, we also aimed to establish if a strain difference in systemic and kidney renin content, RAAS gene expression and sodium excretion exists between Lewis and LPK rats.

Chapter 2 Materials and Methods

This Chapter included materials and methods that are relevant to thesis content across different results chapters. Methods specific to individual chapters are provided in detailed description within each chapter.

2.1 Animals

All experiments were approved by the Animal Ethics Committee of Macquarie University (ARA 2015/036) and were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council 8th Edition, 2013). Lewis and Lewis Polycystic Kidney (LPK) rats of both sexes as detailed in Table 2.1 were obtained from the Animal Resource Centre, Perth, Western Australia, Australia and housed at the Central Animal Facility of Macquarie University. All animals were allowed to acclimatise in the Central Animal Facility on a 12-hour light-dark cycle (lights on at 6 am) at 22 ± 20°C with access to food and water ad libitum for at least one week prior to experimentation.

2.2 Experimental surgical protocols

At age 6 weeks, animals were subjected to one of three surgical protocols: (i) total renal denervation (RDN) by periaxonal application of 10% phenol in ethanol (Gattone *et al.* 1984); (ii) afferent RDN by periaxonal application of 33 mM capsaicin (Foss *et al.* 2015); or (iii) sham RDN by periaxonal application of normal saline.

2.2.1 Anaesthesia

For all surgical procedures, anaesthesia was induced with 5% isoflurane in 100% O_2 using an induction chamber. Animals were then maintained on 2-3% isoflurane in 100% O_2 using a nose cone for administration. The depth of anaesthesia was assessed by loss of withdrawal reflexes and maintenance of a respiratory rate of 40-60 breaths per min. Pre-operative analgesia (carprofen; 2.5 mg/kg s.c. Norbrook Laboratories, VIC, Australia) and antibiotics (cephazolin; 50mg/kg i.m. Hospira, VIC, Australia) were administered.

2.2.2 Renal denervation

The kidneys were approached from a dorsal aspect with a midline lumbar skin incision followed by bilateral incisions which allowed exposure of the kidneys by gently retracting the abdominal muscles using sterile saline-moistened cotton swabs. Using a surgical microscope, the connective tissue surrounding the renal artery was stripped back from the renal pelvis to the abdominal aorta. For total RDN, all visible nerve bundles running along the renal arteries were dissected and cut off and the renal arteries were painted with 10% phenol in absolute ethanol to ensure the damage of the nerves; for selective afferent RDN the renal artery was painted with 33mM capsaicin (Sigma, Missouri, USA) dissolved in 5% ethanol and 5% Tween 80 in 0.9% normal saline two-three times at 2-3-minute intervals. Sham surgery entailed visualisation of the renal artery and painting with normal saline. The surgical site was kept moist with sterile saline at all times so as to reduce tissue trauma and minimise adhesion formation.

After the RDN procedure, the flank incision was sutured using 5-0Vicryl sutures (Ethicon, New Jersey, USA) and the skin incision closed using wound clips (Medicon, E.G., Tuttlingen, Germany). A bolus of warmed 0.9% saline or 5% glucose solution was administered to the animals, with the volume determined according to the duration of the surgery (10ml/kg/hr). Subsequent supportive fluid therapy and pain relief (buprenorphine 50µg /kg, s.c. Reckitt Benckiser Ltd, Auckland, New Zealand, or carprofen, 2.5mg/kg s.c.) were administered during the post-operative period as required. Wound clips were removed at 7 days post-operative.

2.2.3 Telemetry probe implantation

For studies 2 and 3 (see section 2.3), animals had telemetry pressure transmitter probes (PA-C10 or HD-X10, Data Sciences International, USA) implanted at the same time as the first denervation surgery in order to measure 24h blood pressure profiles, from which heart rate and circadian rhythms could also be assessed. The left femoral artery was exposed using a medial hind leg incision. Two 6-0 silk ties were placed underneath the femoral artery and retracted back using haemostats to occlude blood flow. A small incision in the artery was made and the tip of the probe catheter inserted and forwarded into the abdominal aorta. The ties were released and used to then secure the catheter in place. The body of the probe was placed in the subcutaneous space of the inguinal region. The leg incision was closed using wound clips which were removed 7 days postoperatively. The animals were allowed to recover for 7-10 days with monitoring of weight gain and wound healing. Data was not collected from the animals until the return of circadian rhythms, as evidenced by a higher nighttime HR.

2.3 Study design

The study design was presented in Figure 2.1

2.3.1 Study 1

The aim of study 1 was to validate the effectiveness of the two RDN procedures. After the denervation or sham procedure at age 6 weeks, animals were then euthanised at age 7 weeks (1-week post procedure). The animals' kidneys were collected post-mortem for later assessment of the degree of denervation (total n = 13 Lewis and n = 15 LPK). Urine was collected by putting the animals in a metabolic cage for 4-5 hrs within 48 hrs of euthanasia and blood samples were collected at the time of euthanasia for later analysis. Results are presented in Chapter 3.

2.3.2 Study 2

The aim of study 2 was to determine the impact of total and sensory afferent RDN on cardiovascular and renal function. After the denervation or sham procedure at age 6 weeks, telemetry-based blood pressure recordings were undertaken through to age 10 weeks (cohort 1: n = 14 Lewis, n = 14 LPK) or 14 weeks (cohort 2: n = 22 Lewis, n = 22 LPK). At the end of the study, animals were euthanised and the kidneys collected post-mortem for later assessment of the degree of re-innervation that had occurred over the 4 or 8 weeks post-RDN, respectively. Urine samples were collected by putting the animals in a metabolic cage for 4-5 hrs (fortnightly spot urine collection) or 24 hrs within 48 hrs of euthanasia (24h urine collection) and blood was taken at the time of euthanasia for later analysis. Results are presented in Chapters 4, 5 and 6.

2.3.3 Study 3

Preliminary data analysis from Study 2 indicated that the total but not selective afferent RDN procedure impacted cardiovascular parameters and there was immunohistochemical evidence of reinnervation of both sympathetic and sensory nerves in the kidney at 4 weeks post-

denervation. The aim of study 3 therefore was to assess if repeating the total RDN had any additional impact on cardiovascular parameters. Animals received a second total RDN or sham procedure at age 10 weeks (after their 10-week cardiovascular and autonomic function data had been obtained; total n = 8 Lewis and n =10 LPK). The animals were euthanised at 14 weeks of age and the kidneys collected for determination of the level of reinnervation. Urine samples were collected by putting the animals in a metabolic cage for 4-5 hrs (fortnightly spot urine collection) or 24 hrs within 48 hrs of euthanasia (24h urine collection) and blood was taken at the time of euthanasia for later analysis. Results are presented in Chapters 4 and 5.

2.4 Euthanasia and tissue collection

At the termination of the study period, animals were deeply anaesthetised with an i.p. injection of 20% (v/v) solution of sodium pentobarbital (100mg/kg, Virbac, NSW, Australia). Blood was collected via cardiac puncture into ethylenediaminetetraacetic acid (EDTA) tubes (Becton, Dickinson and Company, New Jersey, United States), spun, and plasma removed for immediate analysis or frozen at -80 °C for later assessment. The heart was collected and weighed before the left ventricle was dissected and weighed separately. Both kidneys were collected and weighed and then cut into three along the transverse axis. For immunohistochemistry studies, the central part containing the renal pelvic wall was drop-fixed in 4% formaldehyde (Ajax Finechem, NSW, Australia) or 10% neutral formalin (Sigma, Missouri, USA) for 4 to 5 hours at 4°C. After being fixed, the kidneys were then washed three times with phosphate buffered saline (PBS: 0.9%NaCl, 10 mmol/L phosphate buffer - NaH_2PO4/Na_2HPO4, pH 7.4) and stored in 30% sucrose (Sigma, Missouri, USA) for cryoprotection until processing for immunohistochemistry. The polar parts were snap frozen in dry ice for tissue measurements.

2.5 Immunohistochemistry

2.5.1 Kidney tissue processing

Sucrose-protected kidneys were embedded with optimal cutting temperature compound (OCT, Proscitech, Queensland, Australia) and frozen in the vapour phase of liquid nitrogen or on dry ice. Fourteen µm coronal sections were cut using a cryostat (CM1950, Leica, Hesse, Germany), mounted on superfrost plus slides (Lomb Scientific Pty, Ltd, NSW, Australia) and dried overnight at room temperature. The slides were then processed for immunolabelling or kept at -80 °C until further analysis.

2.5.2 Immunolabeling for renal sympathetic (TH, tyrosine hydroxylase) and sensory (CGRP, calcitonin gene-related peptide) nerve markers

The kidney slides were rehydrated for 30 mins in 0.01M Tris PBS (TPBS, 10 mmol/L Tris buffer and PBS). The sections were then incubated in blocking solution containing either 20% normal rat serum (Sigma, USA), 20% donkey serum (Sigma, USA) (for CGRP immunohistochemistry) or 10% donkey serum (for TH immunohistochemistry), as well as 0.3% Triton-X 100 for permeabilisation and 0.05% thimerosal (Sigma, USA) as a preservative agent in TPBS at room temperature for 2 hrs, followed by 48-hour incubation in the same blocking solution containing the primary antibody against CGRP (goat anti-CGRP, 1:500, AbD Serotec, serial code, 1720-9007, United Kingdom) or TH (mouse anti-TH, 1:200, Avanti Antibodies, serial code AV#1. NSW, Australia). Sections were then washed in TPBS (3×10 mins) and Cy3-conjugated species-specific secondary antibody (for CGRP, donkey anti-goat Cy3 –conjugated antibody, for TH, donkey anti-mouse Cy3-conjugated antibody, Jackson ImmunoResearch, PA, USA), diluted at 1:500 in TPBS containing 5% normal donkey serum and 0.3% Triton-X 100, was added to the sections which were then incubated in the dark at room temperature for 4 hrs. Sections were washed in TPBS (3×10 mins), coverslipped using fluorescence mounting medium (Dako, CA, USA), and dried in the dark for 12-24 hrs. Negative controls were kidney sections incubated in the sample blocking

solution omitting the primary antibody. The specificity for both CGRP and TH antibodies has been validated previously (Yasuhara *et al.* 2008, Nedoboy *et al.* 2016).

2.5.3 Image collection and analysis

Sections were viewed using a ZENPRO epifluorescence microscope (Zeiss, Gottingon, Germany). For sections stained with CGRP, three different sections from each animal containing the renal pelvic wall were viewed and three images from each section were collected using a 20x objective (341.8 µm by 1260 µm), using a comparable anatomical position within the kidney in all animals. Positive CGRP labelling within the pelvic wall in the three sections was determined using ImageJ software and averaged for each animal expressed as µm/mm$_2$. Images were thresholded to differentiate positive labelling from background staining, and the percentage of area with positive labelling measured after manually outlining the pelvic wall area.

Due to cystic lesions in LPK animals, methods of TH analysis which determine innervation density as relative area of staining (µm/mm$_2$) for kidney regions (Booth *et al.* 2015) could not be applied. Instead, the level of TH staining was quantified in the immediate vicinity of blood vessels in the cortico-medullary junction [a region well established to be high in sympathetic innervation (Johns *et al.* 2011)], using a minimum of 4 arteries from each animal. Innervation was measured no further than 30µm from the vessels, incorporating both adventitial fibres and associated perivascular nerve bundles but excluding the vascular smooth muscle. Images were thresholded to discriminate positive labelling from background staining, and the percentage of area with positive TH labelling was measured within the automatically outlined perivascular area (µm/mm$_2$ perivascular region).

The number of kidney sections used was based on previous studies (Mulder *et al.* 2013, Foss *et al.* 2015). A blind analysis of these sections was not possible as the main researcher performing the analysis was also aware of the group assignment.

2.6 Renal function analysis

Renal function was determined by measurement of urine protein to creatinine ratio (UPC), plasma urea, plasma creatinine, and creatinine clearance rate (CCR), a surrogate for glomerular filtration rate (GFR). For UPC, urine samples were collected either as spot urine samples (Study 1) or a final 24h urine sample (Study 2 and 3), by placing the animals in a metabolic cage for up to 5 hrs (between 9am to 2pm) or 24 hrs (between 9am to 9am), respectively. Animals were placed in a metabolic cage for up to 2 hrs in the week prior to urine collection for familiarization with the cage conditions. Water intake and urine output were measured during urine collection. Urine samples were spun and analysed immediately or frozen at -20 °C for later assessment. For plasma urea and creatinine, blood was collected after euthanasia via cardiac puncture as detailed in Section 2.4. Plasma and urine samples were analysed using an IDEXX VetLab analyser (IDEXX Laboratories Pty Ltd., NSW, Australia). CCR was estimated using the following equation:

Creatinine clearance rate (ml/min) = (24h urine output (ml)×urine creatinine level (µmol/L))/ (plasma creatinine level (µmol/L) ×24×60)

Table 2.1 Breakdown of the total number of animals used across all studies1

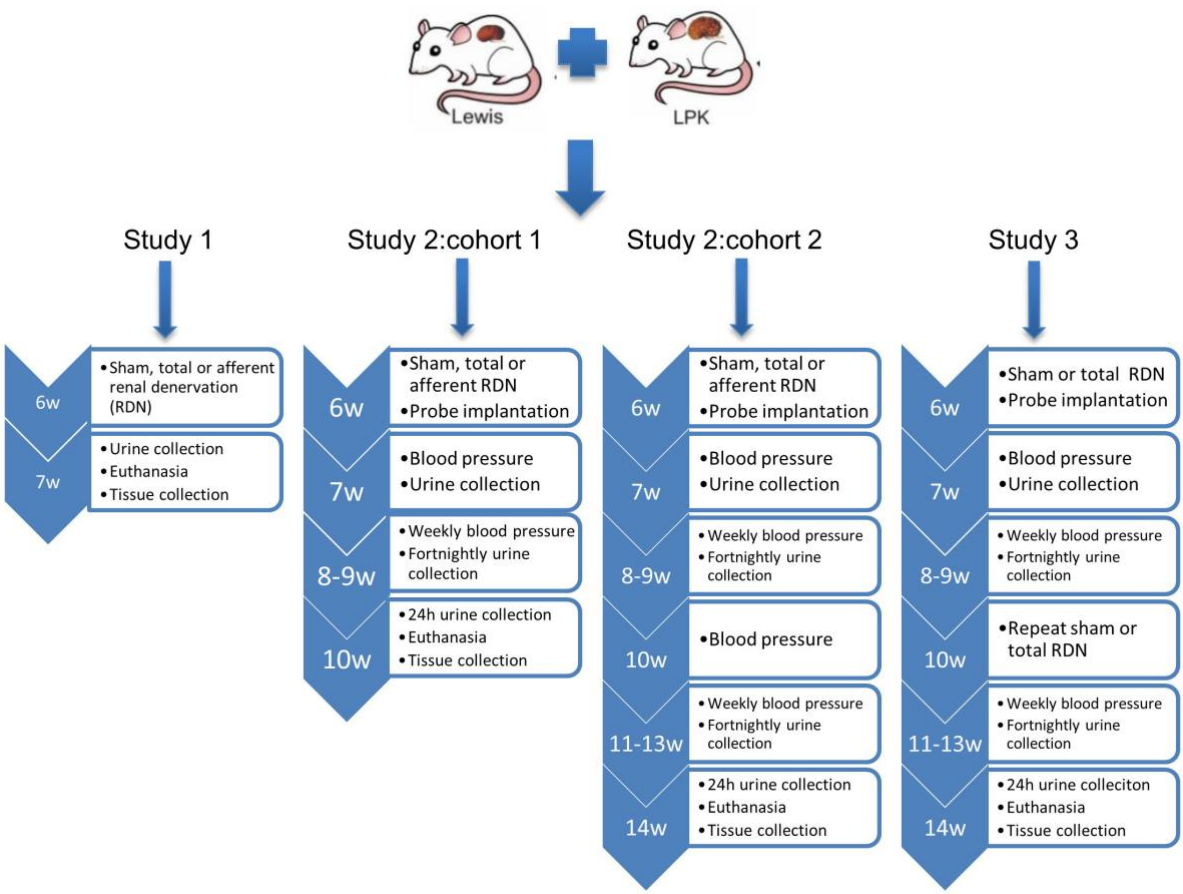
	Lewis male	Lewis female	LPK male	LPK female	Total
Study 1: 7 weeks					
Sham RDN	4	1	3	3	11
Total RDN	3	1	2	3	9
Afferent RDN	4	0	3	1	8
Study 2: Cohort 1: 10 weeks					
Sham RDN	2	3	2	2	9
Total RDN	2	3(1)	2	3(1)	10 (2)
Afferent RDN	2	2	3	2	9
Cohort 2: 14 weeks					
Sham RDN	3(2)	3(2)	4(3)	4	14 (7)
Total RDN	5(2)	2	5(2)	2	14 (4)
Afferent RDN	5(3)	4(2)	3(3)	4(1)	16 (9)
Study 3: 14 weeks					
Repeat sham RDN	2	1	2	2	7
Repeat total RDN	2	3	3	3	11
Total	34 (7)	23(5)	32(8)	29(2)	118 (22)

RDN, renal denervation. Age indicates study endpoint age. In studies 2 and 3, probe failure associated with battery life or signal loss precluded data collection across all time points from some animals in the study cohort. However, recordings from these animals were included in the data set up to the time point of probe failure. Number in brackets indicates the number of animals that were euthanised prior to study endpoint age due to probe failure.

1 Animal numbers specific to each study are also provided in the relevant chapter

2.7 Figures

Figure 2.1 Experimental design of the animal study.



Chapter 3 Validation of renal denervation procedure in a rodent model of polycystic kidney disease.

3.1 Abstract

We aimed to validate the effectiveness of two renal denervation (RDN) procedures, being total RDN by stripping of the renal nerves and periaxonal application of phenol, to destroy both renal sensory and sympathetic nerves and the relative new procedure of selective afferent RDN using periaxonal application of capsaicin, using a rodent model of polycystic kidney disease, the Lewis polycystic kidney (LPK) rat and their normotensive Lewis control strain. Experiments were undertaken in 6-week old female and male animals (Lewis n = 13, LPK n = 15) and the animals were then euthanased one week later and the kidneys removed for determination of denervation efficacy using immunohistochemistry against renal sympathetic [tyrosine hydroxylase (TH)] and sensory [calcitonin gene-related peptide (CGRP)] nerve markers. Renal function in these animals was also determined by urine protein to creatinine ratio (UPC), plasma creatinine and plasma urea. Results indicated that at one-week post total RDN, both the renal sympathetic nerve marker TH at the perivascular region and the sensory nerve marker CGRP in the pelvic wall are essentially absent, while after afferent RDN, CGRP immunoreactivity was significantly abolished while levels of the sympathetic nerve marker TH remained intact. Neither procedure acutely affects renal function in either strain. These results indicate that both total RDN and afferent RDN can effectively abolish the target nerve population without altering the animal's renal function.

3.2 Introduction

Catheter-based renal denervation (RDN) is a novel procedure currently in use to treat hypertension in patients whose blood pressure is not able to be controlled with lifestyle and pharmacological therapeutic interventions. In humans RDN involves placing a radiofrequency generating catheter in the renal artery, which is then used to emit energy to destroy the renal nerves (Krum *et al.* 2009, Masuo *et al.* 2010, Bakris *et al.* 2015). Its potential to limit hypertension has been documented in multiple clinical trials (Krum *et al.* 2009, Masuo *et al.* 2010), however, several major issues remain to be resolved. One of these issues is the relative contribution of renal sensory and sympathetic nerves to the observed antihypertensive effect of RDN given the fact that this procedure nonselectively ablates both renal nerves. As discussed in Chapter 1, section 1.1.4, renal sympathetic nerves directly regulate renal vascular resistance, renal sodium reabsorption and renin release, all key players in blood pressure regulation (DiBona and Kopp 1997), whereas activation of renal afferent sensory nerves could affect the sympathetic outflow to vascular beds (Smits and Brody 1984, Patel and Knuepfer 1986). Therefore it is logical that the removal of either sensory nerves or the sympathetic nerves could be the initial drive for the observed reduction in blood pressure in clinical trials.

In the animal models, nonselective surgical RDN is achieved by exposing the renal artery followed by periaxonal application of phenol (Gattone *et al.* 1984), whereas selective sensory denervation used to be achieved by dorsal rhizotomy through removing the dorsal roots at the T11 to L2 level (Lappe *et al.* 1985). Nevertheless, dorsal rhizotomy also destroys other visceral, somatic and cutaneous afferent inputs projecting to these levels. Besides, this procedure was associated with a risk of hindquarter paralysis (Lappe *et al.* 1985). The development of a technique to selectively destroy either renal afferent or efferent nerves would be vital to isolate the individual impact of each arm of nerves in the development of hypertension.

In 2015, a novel method was developed to selectively destroy the renal sensory nerves by topically applying capsaicin to the renal artery (Foss *et al.* 2015). The rationale for this method is that prolonged exposure to capsaicin has a neurotoxic effect on unmyelinated C-fibres of sensory nerves that express transient receptor potential cation channel subfamily V member 1 (TRPV1) (Holzer 1991). The authors confirmed that periaxonal application of capsaicin on renal artery caused a significant reduction of renal content of the afferent nerve marker, calcitonin gene-related peptide (CGRP), measured out to 10 days post-intervention, but no reduction of renal content of the efferent nerve markers tyrosine hydroxylase (TH) or neurotransmitter noradrenaline (Foss *et al.* 2015). Moreover, the cardiovascular responses to intrarenal infusions of bradykinin, which has been shown to stimulate renal sensory nerves and increase arterial pressure and heart rate, were abolished. Therefore, this novel technique might serve as an experimental alternative to bilateral dorsal rhizotomy to selectively destroy renal sensory nerves.

This thesis aims to evaluate the individual role of renal sympathetic and sensory nerves in the development of hypertension, autonomic and renal dysfunction in a rodent model of Lewis polycystic kidney disease rat (LPK). Therefore, the first series of experiments aimed to validate the efficacy of the procedures and confirm that (1) the total RDN procedure effectively removed both the sympathetic and sensory innervation to the kidney and (2) that the selective afferent RDN procedure effectively removed the sensory innervation to the kidney but left the sympathetic innervation intact. Further, we aimed to determine the otherwise overall safety of the procedure and its impact on renal function on the rodents.

3.3 Method

The protocol details and experimental design for this study (Study 1) are provided in Chapter 2 and Figure 3.1. Briefly, a total of 13 Lewis and 15 LPK rats of both sexes were subjected to one of three surgical protocols at 6 weeks old: (i) total RDN by periaxonal application of 10% phenol in ethanol; (ii) afferent RDN by periaxonal application of 33 mM capsaicin; or (iii) sham RDN by periaxonal application of normal saline as previously described (Chapter 2, section 2.2). At 7 weeks, urine samples were collected (spot urine samples) and 24-48 hrs subsequent the animals were euthanised and tissues and plasma samples collected. Urine and plasma samples were analysed as previously described (Chapter 2, Section 2.6). Immunostaining of sympathetic (TH) and sensory (CGRP) nerve markers was performed as described in Chapter 2 Section 2.5.

3.4 Results

3.4.1 The organ and total body weights in Lewis and LPK after RDN

Table 3.1 presents the organ and total body weights of both Lewis and LPK animals after sham, total or afferent RDN. Within each strain, total body weight, kidney weight (net weight and % body weight) and heart weight (net weight and % body weight) weight were not significantly different between groups (all $P > 0.05$). One-way ANOVA indicates a difference in net left ventricle weight between the three groups in the LPK ($P = 0.03$), however post hoc analysis did not differentiate between total or afferent RDN groups compared to those that underwent the sham procedure.

3.4.2 Renal function after RDN

Renal function parameters in Lewis and LPK animals after sham, total or afferent RDN is presented in Figure 3.2. One-way ANOVA indicated that within each strain the plasma creatinine or urea were comparable between three groups (all $P > 0.05$). Urinary protein was only detected in 4 of 9 Lewis rats from all groups (range 0.09 to 0.32 g/L) and 5 of 12 LPK animals from all groups (range 0.07 to 0.58 g/L). Thus, a comparison of urine protein and UPC between treatment groups was not determined. One-way ANOVA indicates a comparable level of urinary creatinine between three groups within each strain (Lewis sham 0.76 ± 0.13 vs. total 0.50 ± 0.13 vs. afferent RDN 0.57 ± 0.15 g/L, P=0.57, LPK sham 0.27 ± 0.15 vs total 0.41 ± 0.07 vs. afferent RDN 0.39 ± 0.00 g/L, $P = 0.44$).

3.4.3 Immunohistochemical validation of RDN procedures

Representative immunohistochemistry images of perivascular TH and pelvic CGRP labelling from Lewis and LPK one week after the different denervation protocols (sham, total and afferent) and quantitative analysis are provided in Figure 3.3. In both the Lewis and LPK animals, after total RDN, TH labelling was significantly reduced to ~ 4% of sham levels, and similarly, CGRP labelling was reduced to ~8% of sham levels, indicating the effectiveness of the stripping/phenol application protocol in destroying both the sympathetic and sensory innervation to the kidney. After afferent RDN, there was no significant change in the TH labelling in either the Lewis or LPK animals, while CGRP labelling was reduced to less than 5% of sham levels, demonstrating the selective destruction of only the afferent nerves within the renal nerve plexus.

3.5 Discussion

Here we have validated the use of two different methodologies (stripping and application of phenol, and periaxonal application of capsaicin) as procedures by which to achieve total RDN and selective sensory denervation, respectively, in a rodent model of polycystic kidney disease and their normotensive Lewis control strain. We show that one-week post total RDN, both TH and CGRP are essentially absent, while after sensory afferent denervation, CGRP was significantly abolished while the sympathetic nerve marker TH remained intact. Our results are consistent with previous studies undertaken to achieve total renal RDN (Mulder *et al.* 2013) and critically, for the newly established procedure using capsaicin, comparable with the findings of Foss et al. (Foss *et al.* 2015) in which selective afferent denervation using capsaicin caused significant loss of CGRP as determined 10 days after the procedure. Our additional findings include that 1) neither total or selective afferent RDN acutely affect kidney, heart or total body weight; 2) neither procedure acutely affects renal function as determined by UPC, plasma urine creatinine and plasma urea.

3.5.1 Immunohistochemical assessment of renal innervation density after denervation

In the present study, immunohistochemical labelling of renal sensory and sympathetic nerve markers was used to determine the effectiveness of both denervation procedures. The sensory nerve marker CGRP was primarily found in the renal pelvic wall in our sham-operated Lewis and LPK animals, consistent with previous reports (Kopp *et al.* 2007, Kopp *et al.* 2009), consistent with the function of sensory nerves in sensing the stretch of the renal pelvic wall and/or changes in chemical composition of the urine (Johns *et al.* 2011). Thus the renal pelvic wall area was selected to examine the effectiveness of the procedures to destroy renal sensory nerves, as previously described in the work of Booth et al. (Booth *et al.* 2015) and Singh et al. (Singh *et al.* 2019). Our results showed that both total and afferent denervation caused a substantial reduction in the expression of CGRP in the pelvic wall in both Lewis and LPK,

supportive of a marked reduction in sensory nerve innervation of the kidney, consistent with previous reports (Mulder *et al.* 2013, Foss *et al.* 2015).

The sympathetic nerve marker TH was used to determine both the degree of denervation after the total RDN procedure, as well as assessment of preservation of sympathetic innervation after the afferent RDN procedure using capsaicin. Sympathetic nerves have been shown to innervate both vascular and tubular structures throughout the kidney except in the inner medulla (Barajas and Powers 1990) with the cortico-medullary junction being a region known to possess a high degree of sympathetic innervation (Johns *et al.* 2011). Typically, when TH is used as a marker of renal innervation, researchers quantify TH innervation density as relative area of staining for kidney regions (Mulder *et al.* 2013, Booth *et al.* 2015). However, the presence of cystic lesions in LPK animals prevented us from using this methodology and the current thesis quantified the sympathetic innervation along renal vasculature in the cortico-medullary region. Consistent with previous studies (Mulder *et al.* 2013, Foss *et al.* 2015), total RDN caused a marked reduction in perivascular TH staining, while afferent RDN had no impact, confirming the effectiveness and selectivity of our denervation procedures.

3.5.2 Renal denervation did not affect animal growth or renal function in Lewis and LPK

In the present study, we report that neither total or afferent denervation affected the kidney, heart or total body weights in both strains. Renal function, as determined by plasma urea and creatinine was also unaffected by either procedure. This is of significance in terms of the relative safety of the procedures and their capacity to translate into clinical practice. Our finding in the LPK animal model is consistent with other animal studies. For example, Rudd et al. (Rudd *et al.* 1986) demonstrated that acute unilateral RDN did not change the glomerular filtration rate (GFR), a marker for renal function, in spontaneous hypertensive rat, Wistar Kyoto rat or Sprague Dawley rats. Bello-Reuss et al (Bello-Reuss *et al.* 1975) also reported that unilateral RDN did not change the whole kidney GFR on either side in Sprague

Dawley rats. Banek et al. (Banek *et al.* 2016) showed that neither total or afferent denervation, similarly using phenol and capsaicin, respectively, as in the current study, affected plasma urea, creatinine, or urine protein to creatinine ratio determined 2 weeks post-denervation in Deoxycorticosterone acetate (DOCA) salt hypertension model. In a different model of PKD, Gattone et al. (Gattone *et al.* 2008) showed that RDN by periaxional application of phenol did not affect the level of plasma urea level determined 8 weeks post-denervation. Chen et al. (Chen *et al.* 2016) demonstrated that RDN had a positive effect of significantly lowering the increases in serum creatinine, urea and urine protein to creatinine ration caused by 5/6 nephrectomy. Thus RDN, in general, has a neutral effect in animal models and a potentially beneficial effect in models with renal dysfunction. Its long-term effect on renal function in our PKD model will be presented in the following chapter.

3.5.3 Methodological considerations

This study used an immunohistochemical technique to validate the effectiveness of RDN, using stripping and periaxonal use of phenol, or periaxonal application of capsaicin, respectively, to undertake total RDN or selective renal sensory denervation. Functional validation of the loss of renal nerves after denervation is another confirmatory approach that has been used by other researchers. In an early study, Katholi et al. (Katholi *et al.* 1984) showed that after RDN, the pressor response to chemical stimulation of the afferent nerves after intrarenal infusion of adenosine in dogs was abolished, demonstrating successful removal of renal sensory nerves. Foss et al. (Foss *et al.* 2015) also validated the removal of renal sensory nerves by an absence of pressor response upon stimulation of afferent nerves by intrarenal infusion of bradykinin in rats. Booth et al. (Booth *et al.* 2015) provided functional evidence of the loss of renal sensory and sympathetic nerves after catheter-based RDN by the abolishment of cardiovascular changes upon electric stimulation of the nerves in sheep. In this study, the researchers also used immunohistochemical approaches and found that one-week post-RDN, the levels of TH and CGRP and the noradrenaline content in the kidney were

significantly reduced compared to controls, which to some extent paralleled the abolishment of functions in the acute stage (Booth *et al.* 2015).

Capsaicin is the primary active agent in chilli peppers and when applied to sensory nerves, acts at the TRPV1 cation channel which is expressed in unmyelinated C-fibres, producing an initial short-lasting stimulation, followed by desensitisation to capsaicin and other stimuli of sensory neurons (Holzer 1991). The mechanism of capsaicin-induced desensitisation is not entirely understood, but may include depletion of neuropeptides such as substance P and CGRP in the nerve fibres and an increase of intracellular calcium levels, which activates calcium-dependent proteins responsible for desensitisation of sensory nerves (Fattori *et al.* 2016). The activation of TRPV1 by capsaicin can also cause degeneration of sensory fibres (Simone *et al.* 1998), proposedly via induction of apoptosis after DNA fragmentation and reduction of the nucleus (Kim *et al.* 2004), which forms the rationale for its use for RDN. As compared to dorsal root rhizotomy for example, as a method to induce selective afferent denervation, capsaicin therefore will not target sensory fibres that lack the TRPV1 receptor (Holzer 1991). Thus TRPV1 negative sensory fibres are likely still present and functionally active. Although the exact percentage of renal sensory fibres lacking TRPV1 receptor is not known (Foss *et al.* 2015), the generally held concept is that the majority of renal sensory nerves expresses TRPV1 (Foss *et al.* 2015).

In summary, this preliminary study demonstrated that in the Lewis and LPK model, both total and afferent RDN had effectively abolished the target nerve population, confirmed by the marked loss of specific renal nerve makers using immunohistochemistry.

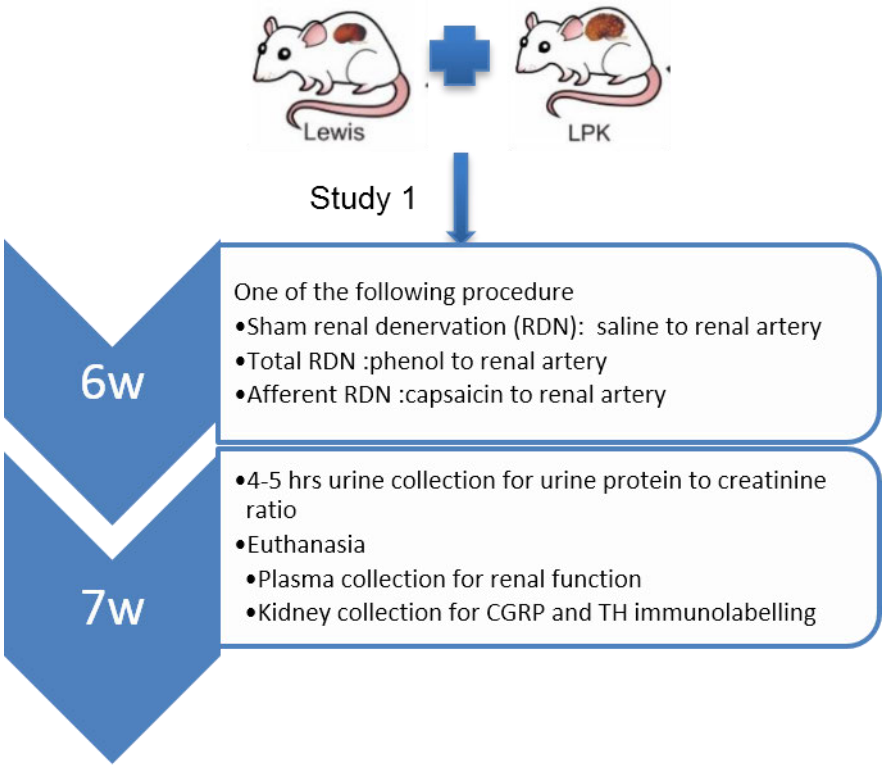
Table 3.1: Body and organ weights in Lewis and Lewis polycystic kidney disease rats from the 7-week-old cohort.

RDN procedure	Sham	Total	Afferent	P-value
Lewis (n)	(5)	(4)	(4)	
Body weight (g)	196.5 ± 11.3	206.1 ± 19.8	206.9 ± 6.2	0.82
Kidney weight (g)	1.9 ± 0.1	2.1 ± 0.2	1.9 ± 0.1	0.57
Heart weight (g)	0.78 ± 0.03	0.80 ± 0.03	0.78 ± 0.03	0.69
LV weight (g)	0.49 ± 0.02	0.50 ± 0.03	0.54 ± 0.02	0.37
Kidney, % body weight	0.84 ± 0.02	1.01 ± 0.05	0.92 ± 0.02	0.19
Heart, % body weight	0.39 ± 0.02	0.39 ± 0.04	0.38 ± 0.01	0.85
LV, % heart weight	65.0 ± 1.7	62.3 ± 2.6	69.5 ± 1.9	0.11
LPK (n)	(6)	(5)	(4)	
Body weight (g)	132.9 ± 11.6	138.7 ± 10.0	165.5 ± 18.1	0.24
Kidney weight (g)	9.2 ± 0.8	6.8 ± 0.6	8.8 ± 1.1	0.14
Heart weight (g)	0.72 ± 0.03	0.68 ± 0.04	0.78 ± 0.02	0.15
LV weight (g)	0.52 ± 0.01	0.49 ± 0.02	0.58 ± 0.03	0.03
Kidney, % body weight	7.2 ± 0.8	5.1 ± 0.7	5.3 ± 0.2	0.08
Heart, % body weight	0.56 ± 0.04	0.50 ± 0.03	0.49 ± 0.06	0.48
LV, % heart weight	72.0 ±1.3	72.7 ± 4.2	74.7 ± 2.9	0.81

RDN, renal denervation. Data is expressed as the mean ± SEM. LV, left ventricle

3.6 Figures

Figure 3.1 Experimental design of the animal study.



All Lewis and LPK animals were subjected to sham, total or afferent RDN at age 6 weeks and euthanased at 7 weeks with blood and kidney samples collected for analysis of renal function and renal denervation effectiveness. Prior to the euthanasia, a spot urine sample was collected for urine protein to creatine analysis. (w = weeks of age)

Figure 3.2 The effect of renal denervation (RDN) on plasma urea (A and C) and plasma creatinine (B and D) in 7-week old Lewis (top panels) and LPK rats (lower panels) one week after RDN procedure.

Data is expressed as mean ± SEM, analysed using one-way ANOVA. N = 4-6, per treatment group per strain.

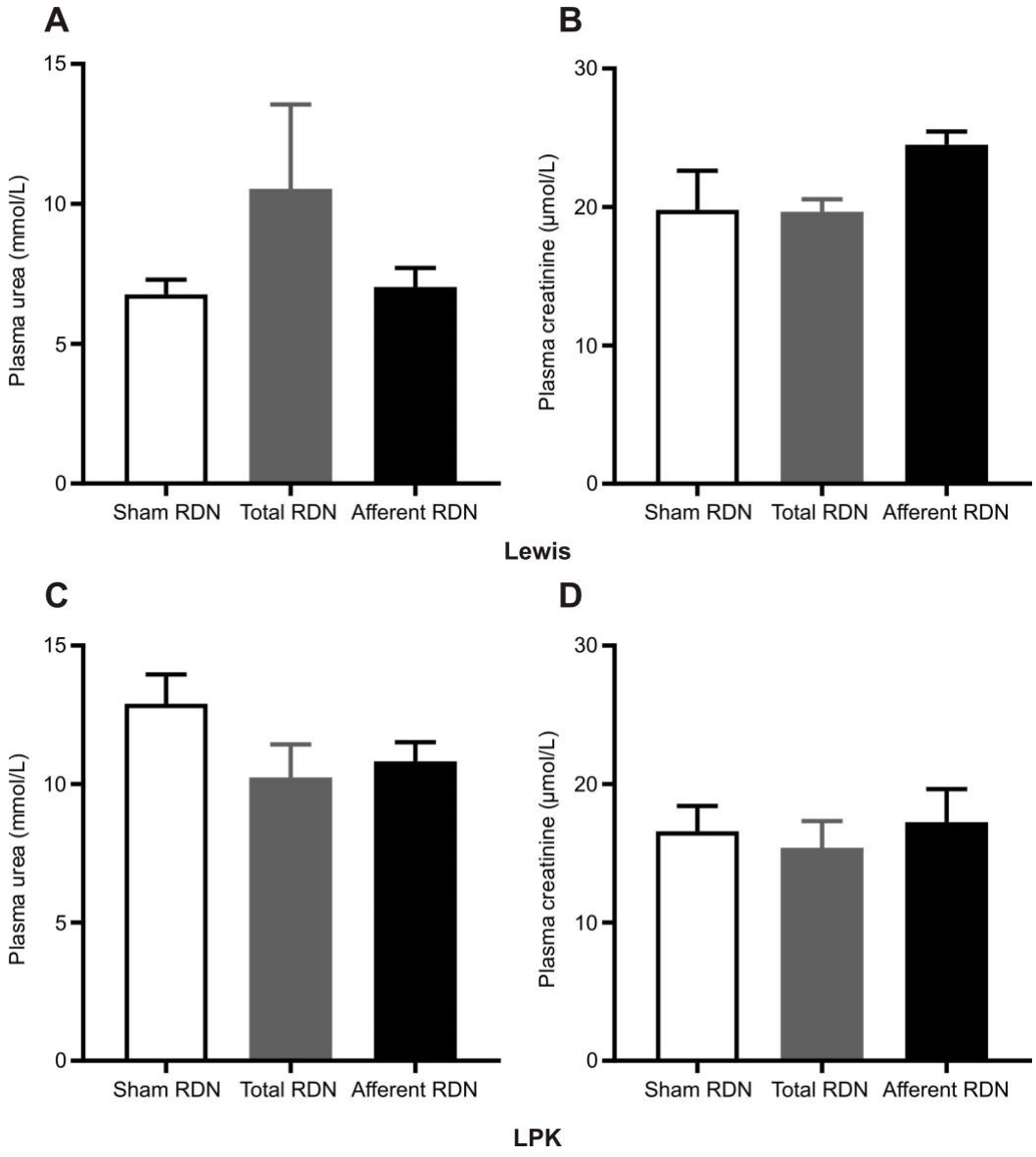
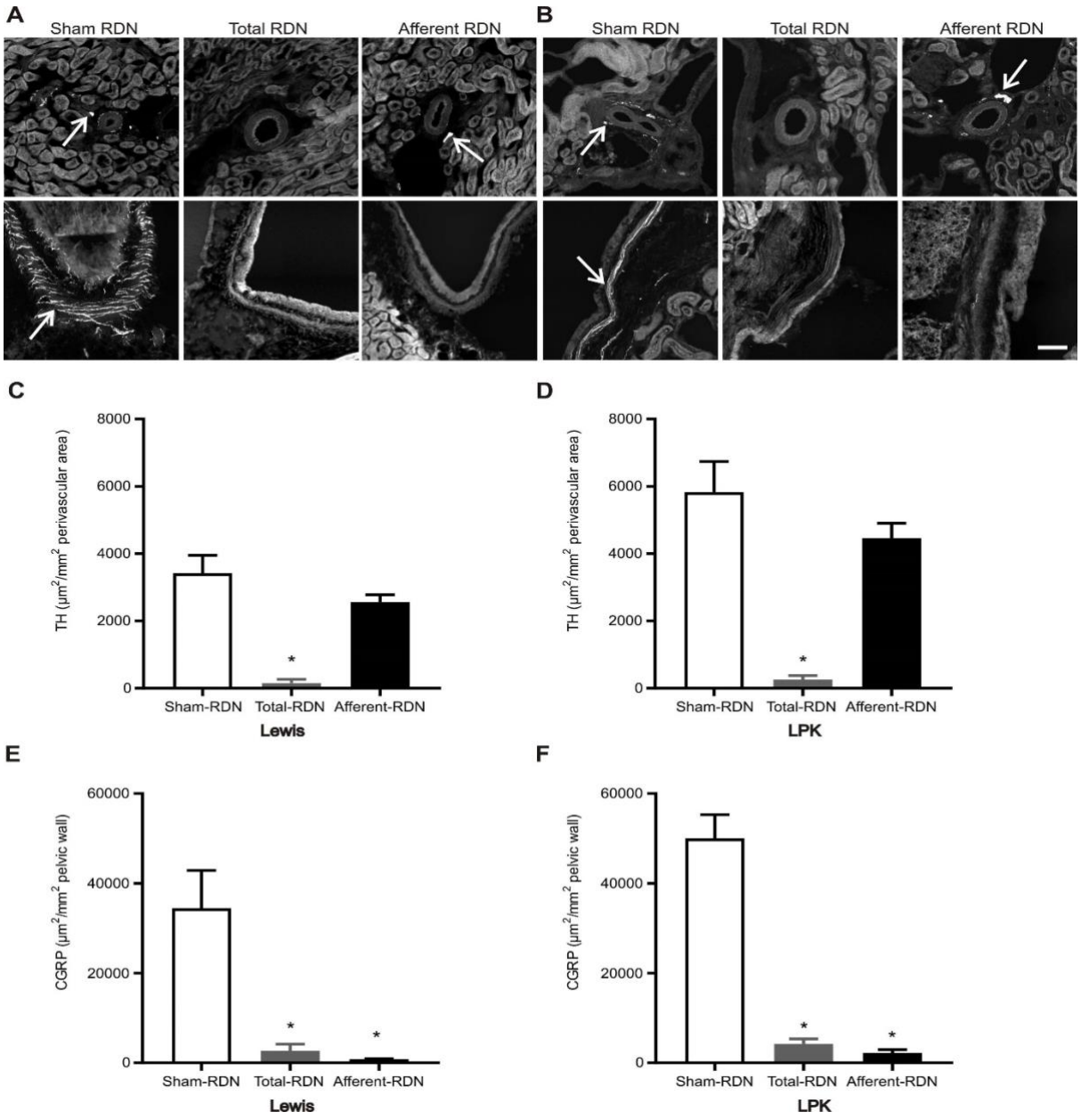


Figure 3.3 Immunohistochemical validation of renal denervation (RDN) protocols determined one-week post-denervation procedure.

Representative images of tyrosine hydroxylase (TH: top row) and calcitonin gene-related peptide (CGRP: bottom row) innervation density staining in Lewis (A) and Lewis Polycystic Kidney (LPK; B) rats after sham, total and afferent RDN, respectively. Arrow indicates positive staining, scale bar lower right panel = 100 µm for all images. Panels C-F present quantitative analysis of TH and CGRP innervation density staining. Data is expressed as mean ± SEM, * indicates $P < 0.05$ difference vs. sham RDN analysed using one-way ANOVA and Bonferroni's *post hoc* analysis. N=4-6, per treatment group per strain.



Chapter 4 Effect of renal denervation on blood pressure and renal function in LPK

4.1 Abstract

Hypertension is a highly prevalent, but poorly treatable, co-morbidity of polycystic kidney disease (PKD). As a novel therapeutic strategy, renal denervation (RDN) offers an alternative approach; however, its efficacy in this patient cohort is not clear. We examined the effect of total and afferent RDN on hypertension and renal dysfunction present in a rodent model of polycystic kidney disease. Lewis Polycystic Kidney (LPK) and Lewis control rats received total, afferent or sham RDN by periaxonal application of phenol, capsaicin or normal saline, respectively, at 6-weeks of age. Arterial pressure and heart rate (HR) were assessed using radio telemetry to age 14-weeks. Afferent RDN did not impact mean arterial pressure (MAP) in either strain while total RDN significantly reduced MAP in Lewis rats. Neither procedure affected renal function. Repeating total RDN at 10-weeks of age had no further impact on MAP or HR in Lewis but caused a transient increase in MAP in LPK rats. Structural evidence of re-innervation was evident at 4-weeks post-surgery and by 8-weeks by immunohistochemistry with sensory and sympathetic fibre innervation in denervated animals was not different from sham controls. We conclude that RDN does not limit hypertension or renal dysfunction in the LPK model and suggest RDN might not be a suitable antihypertensive strategy for individuals with PKD.

4.2 Introduction

Polycystic kidney disease (PKD) are a group of genetic diseases that present with fluid-filled cysts in both kidneys and represent the fourth leading cause for chronic kidney disease (CKD) (ANZDATARegistry 2017). As presented in Chapter 1, sections 1.4 and 1.5, hypertension is a common feature in PKD and in CKD in general, and is thought to be driven by multiple and interrelated factors (Rahbari-Oskoui *et al.* 2014), with evidence from experimental models of hypertension (Katholi *et al.* 1982, Foss *et al.* 2013, Banek *et al.* 2016) suggesting that perturbations in renal nerve function, be it increased sympathetic efferent drive and/or increased afferent signalling to the central nervous system exist in experimental hypertension including hypertension secondary to CKD (Gattone *et al.* 2008). Catheter-based renal denervation (RDN) seems to attenuate hypertension in CKD patients (Hering *et al.* 2012, Ott *et al.* 2015, Hering *et al.* 2017). However, none of these clinical studies was sham-controlled, potentially undermining this evidence. Whether the observed blood pressure lowering effect of RDN can be replicated in large randomised clinical trials needs further investigation. In addition, these studies did not provide a mechanistic explanation of these observations. Specifically, it is not clear whether the antihypertensive effect observed is primarily mediated by reduction of renal sensory input to the central nervous system, or reduction of renal sympathetic outflow or a combination of both. An understanding of the contribution of renal sympathetic and sensory nerves to the antihypertensive effect of RDN is critical in determining if in the future afferent- or efferent- specific RDN should be developed to optimise the treatment efficacy for patients.

The Lewis polycystic kidney disease rat (LPK) is a well-established PKD model arising from a non-synonymous mutation in the never in mitosis gene a -related kinase 8 (Nek 8) gene, homologous to human nephronophthisis (NPHP) type 9 (McCooke *et al.* 2012). The model presents phenotypically similar to human autosomal recessive PKD (ARPKD) and develops hypertension alongside a progressive decline in renal and autonomic function (Phillips *et al.*

2007, Hildreth *et al.* 2013), and renal sympathetic overactivity (Salman *et al.* 2014, Salman *et al.* 2015), suggesting that heightened renal sympathetic nerve activity may play a pathological role in the disease process. Further, the renal sensory nerves are likely activated as local renal ischaemia in association with renal hypoxia is evident in this model (Ow *et al.* 2014). Based on this evidence, we hypothesised that both the renal sensory and sympathetic nerves play a critical role in the development of hypertension in this model of PKD and that both total RDN, ablating renal sympathetic and sensory nerves, and selective sensory only RDN, would have an impact on established hypertension in this animal model. In Chapter 3, it was confirmed in the LPK and the Lewis normotensive control strain that the surgical exposure of the renal nerves and stripping/topical application of phenol (Gattone *et al.* 1984) was an effective procedure which to achieve ablation of both the sympathetic and sensory innervation to the kidney, and that topical application of capsaicin (Foss *et al.* 2015) was able to effectively remove the sensory innervation to the kidney, while leaving the sympathetic innervation intact, providing a solid experimental foundation by which to be able to dissect out the individual contribution of renal sympathetic and sensory nerves in hypertension in this animal model. Therefore, in this Chapter, we undertook a longitudinal study, examining the long-term (8 weeks) effect of total and selective afferent RDN on blood pressure and renal function in Lewis and LPK rats. Noting the controversy around reinnervation of the kidneys (Chapter 1, section 1.3.2.2), we also aimed to determine the course of reinnervation of the renal sensory and sympathetic nerves after the RDN procedure in the rodents.

4.3 Methods

4.3.1 Experimental design

The protocol details and experimental design for this study (Study 2 and Study 3) are provided in Figure 4.1. Briefly, all animals assigned to Study 2 (n = 36 Lewis, n = 36 LPK) received one of three surgical protocols: (i) total RDN; (ii) afferent RDN; or (iii) sham RDN, as well as telemetry probe implantation as described in Chapter 2, section 2.2. Cohort 1 and 2 animals were monitored to and euthanased at age 10 weeks (cohort 1: n = 14 Lewis, n = 14 LPK) or 14 weeks (cohort 2: n = 22 Lewis, n = 22 LPK), respectively. Preliminary analysis of the data from Study 2 indicated total but not selective afferent RDN procedure impacted cardiovascular parameters and there was immunohistochemical evidence of reinnervation of both sympathetic and sensory nerves in the kidney at 4 weeks post-denervation. Therefore, an additional study was undertaken to test if repeating total RDN procedure had any additional impact on cardiovascular parameters. In Study 3, all animals (n = 8 Lewis, n = 10 LPK) underwent a total or sham RDN at 6 weeks of age, which was repeated at 10 weeks of age. These animals were also instrumented with telemetry probes at 6 weeks old. The animals were then euthanised at 14 weeks old. In all animals, blood pressure recordings were collected weekly with telemetry, while urine samples were collected either as spot urine sample fortnightly during the course of the study or a final 24h urine sample, by placing the animals in a metabolic cage for up to 5 hrs or 24 hrs, respectively. After euthanasia, blood and kidney samples were collected and analysed as detailed in Chapter 2 (Section 2.5 and 2.6).

4.3.2 Cardiovascular parameter analysis

Arterial pressure (AP) waveform and activity data during the night (6 pm to 6 am) and day (6 am to 6 pm) periods were collected over a 5 min timeframe every 15 min for 48h once a week with the animal in their home cage (i.e.192 recordings in total). During the recording period, human interaction was kept at a minimum level to minimise the potential effect on the

recording. From the AP waveform, systolic (SBP), diastolic (DBP), pulse pressure (PP), mean arterial blood pressure (MAP), heart rate (HR) and activity were derived offline using Dataquest ART (Data Sciences International) and exported as text into Microsoft Excel (Microsoft, Redmond, WA, USA).

In the initial data analysis, a comparison was made within each strain of the effect of a single sham (study 2, cohorts 1 and 2 data combined) vs repeat sham RDN (study 3). Figure 4.2 shows the mean SBP, MAP, DBP and HR after a single or repeat sham RDN in both strains. Consistent with the development of hypertension in the LPK reported previously (Phillips *et al.* 2007), SBP, DBP and MAP steadily increased in both LPK sham groups (Figure 4.2 E, F and G). An age effect was also noted in Lewis animals, with an increase in SBP, DBP and MAP also evident with age (Figure 4.2 A, B and C). In both strains, an age-related reduction in HR was seen (Figure 4.2 D and H).

No significant difference was noted in any blood pressure parameter between sham animals that had one surgery (study 2) or two surgeries (study 3) within each strain (All $P > 0.05$ for treatment effect). Therefore, the data from sham animals in studies 2 and 3 were combined and used as the control sham group for all subsequent data analysis.

Similarly, in the sham animals at age 14 weeks, renal function parameters for both Lewis and LPK animals after either one sham (Study 2) or two sham RDN procedures (Study 3) did not differ except for urine output, which was significantly higher in LPKs that received two sham procedures compared with those with only one procedure (Table 4.1). The data from these two sham groups were therefore combined and used as a single sham group for subsequent analysis of the 14 weeks old data (detailed in section 4.4.1.3).

For circadian rhythm analysis, all the 48h raw data of SBP, HR and activity were modelled using a non-linear regression (Cosine fitting analysis: a + b *cos(2 * pi * (x—c)/d); where a = baseline level, meaning the value around which the curve oscillates, b = amplitude of the oscillation, meaning the difference between the maximum and the baseline of each parameter,

c = phase shift, meaning first time in a cycle at which the cycle peaks and d=wavelength, meaning the time it takes for a complete cycle).

4.3.3 Statistical analysis

Data were analysed using GraphPad Prism (v7.02, GraphPad Software, La Jolla, USA) and IBM Statistical Package for the Social Sciences (v25, SPSS; Chicago, IL). All results are expressed as mean ± SEM (standard error of the mean). The strain differences in many of the parameters studied have already been established previously and hence the goal of the analysis was within strain treatment effects. For Study 2, for comparison of body and organ weight parameters, immunohistochemical labelling, renal function parameters including plasma urea, plasma creatinine, urine protein, urine creatinine and urinary protein/creatinine ratio (UPC) between the RDN groups within each strain, a one-way analysis of variance (ANOVA) was used. If a treatment effect was noted, the data were further analysed using a Bonferroni's *post hoc* test to determine group differences. For both studies 2 and 3, for all cardiovascular function parameters, a two-way ANOVA with Bonferroni's *post hoc* analysis was used to identify if (and at what age) these parameters were different between treatment groups (sham vs. total vs. afferent RDN). For Study 3, comparison of renal function, organ and body weights, and immunohistochemical labelling between single and repeat total or sham RDN groups, an unpaired t-test was performed between groups. For statistical analysis of the circadian rhythm data, extra sum-of-squares F test was used to compare if the parameters for the circadian rhythm differ between groups. Significance was defined as a $P < 0.05$ for all analysis.

4.3.4 Power analysis

To determine the effect of animal numbers, a power analysis was performed on some data sets through an online calculator (HyLown Consulting LLC, Atlanta, Georgia) (HyLown 2019) using the following formula:

$1-\beta = \phi(z - z_{1-\alpha/2\tau}) + \phi(-z - z_{1-\alpha/2\tau})$, $z = \frac{\mu_{sham} - \mu_{total}}{\sigma\sqrt{\frac{2}{n}}}$ (Chow *et al.* 2007)

(n is sample size, σ is standard deviation, ϕ is the standard Normal distribution function, ϕ^{-1} is the standard Normal quantile function, α is Type I error, 0.05, β is Type II error, meaning $1-\beta$ is power, τ is the number of comparison to be made, μ is the group mean),.

To further investigate the required number of animals to provide a significantly different mean between groups the following formula was used:

$n = 2\left(\sigma\frac{z_{1-\alpha/2\tau} + z_{1-\beta}}{\mu_{sham} - \mu_{total}}\right)^2$ (Chow *et al.* 2007).

4.4 Results

Study 2:

4.4.1 Effect of single total or afferent RDN procedure on body weights, cardiovascular parameters and renal function at 10 and 14 weeks

4.4.1.1 Body and organ weights.

Table 4.2 and 4.3 presents the effect of total and afferent RDN on the organ and total body weights of both Lewis and LPK animals at 10 or 14 weeks old, respectively. One-way ANOVA did not show any effect of total or afferent RDN on body weight, kidney weight (net weight and % body weight), heart weight (net weight and % body weight), and left ventricle weight (net weight and % heart weight) within each strain (all $P > 0.05$).

4.4.1.2 Cardiovascular parameters

4.4.1.2.1 Effect of RDN on the blood pressure and heart rate parameters

The whole data set was analysed to determine if there was an effect of sex or day period (daytime or nighttime) and if this interacted with any treatment effect for parameters of SBP, MAP, DBP and HR. Overall a sex effect in Lewis SBP and in all LPK variables (all $P < 0.05$) was noted, and a day/night effect for all Lewis variables and LPK HR (all $P < 0.05$) was noted (Table 4.4). These results are provided in Supplementary Figures S4.1 (sex) and S4.2 (day/night). However, no treatment*sex or treatment*day period interaction was noted for parameters of SBP, MAP and DBP, as evidenced by consistent treatment effect in males and females, and consistent treatment effect using either daytime values or nighttime values (Table 4.4). Therefore, 24h blood pressure values from animals of both sexes were pooled to test the effect of treatment within each strain. An overall treatment effect was noted for Lewis HR in females but not in males (Supplementary Figure S4.3). An overall treatment effect was noted for daytime HR in both Lewis and LPK but not for nighttime HR (Supplementary

Figures S4.4). The effect of RDN on the differences between nighttime and daytime SBP (ΔSBP), MAP (ΔMAP), DBP (ΔDBP) and HR (ΔHR) are presented in Supplementary Figure S4.5. Neither total or afferent RDN affected these parameters within each strain.

Figure 4.3 illustrates the longitudinal data for blood pressure and HR parameters for the different treatment groups for each strain. In the LPK, there was no treatment effect on SBP, MAP or DBP (all $P > 0.05$, Figure 4.3 E, F and G). In the Lewis, an overall treatment effect was noted for SBP, MAP and DBP (all $P < 0.001$, Figure 4.3 A, B and C). *Post hoc* analysis indicated that this treatment effect was driven by total RDN which produced a blood pressure lowering effect in SBP, MAP and DBP, most evident at 8, 9 and 10 weeks of age. Afferent RDN had no impact on the SBP, MAP and DBP in the Lewis relative to sham-operated animals (all $P > 0.05$). An overall treatment effect on HR was noted in both strains (both $P < 0.05$, Figure 4.3 D and H), with *post hoc* analysis showing a overall lower HR in afferent denervated Lewis ($P = 0.05$) and a overall higher level of HR in afferent denervated LPK ($P = 0.05$) relative to sham denervated animals.

Power analysis using the 8 and 11-week old SBP data from the Lewis sham and total RDN group indicated a statistical power was 0.93 and 0.83 respectively. When the 8-week old SBP from the LPK sham and total RDN group was used to determine the study power, the result was 0.13, with the required number of animals to suggest a significantly different mean between groups being 80 for each group.

4.4.1.2.2 The treatment effect of RDN on circadian rhythm

To determine the effect of RDN on the circadian rhythm in Lewis and LPK, a nonlinear regression analysis of SBP, HR and activity recordings at 10 and 14 weeks of age.

Figure 4.4 illustrates circadian rhythms of SBP (A), HR (B), and locomotor activity (C) in 10 weeks old Lewis rats after sham surgery. The parameters for the Cosine curve in Lewis and LPK at both ages are presented in Table 4.5 and Table 4.6, respectively. In Lewis, the phase shift for 10-week SBP, which represents the first time in a cycle at which the SBP peaks, was

significantly higher in total RDN group relative to sham groups ($P < 0.05$). The baseline for SBP, which represents the value around which the SBP oscillates, was significantly lower in both total and afferent RDN group at both 10 and 14 wks (all $P < 0.05$) compared with shams. The baseline for 14-week HR was significantly lower in the afferent RDN group compared with the sham RDN group ($P < 0.05$). No significant treatment effect was noted for the rest of the parameters.

In LPK, the baseline for 10-week SBP was significantly higher in total ($P < 0.05$) and afferent ($P < 0.05$) RDN group compared with shams. The baseline for 10-week HR was significantly higher in the afferent RDN group compared with shams ($P < 0.05$). The baseline for 14-week HR was significantly lower in total ($P < 0.05$) but higher in afferent ($P < 0.05$) RDN group compared with shams. No significant treatment effect was noted for the rest of the parameters.

4.4.1.3 Renal function

In animals that were euthanised at 10 weeks of age, no treatment effect was detected for plasma creatinine or urea (all $P > 0.05$, Figure 4.5). The 24h water intake, urine output and urinalysis for animals at age 10 weeks are shown in Table 4.7. In both Lewis and LPK, one-way ANOVA did not indicate any treatment effect in any of these parameters (all $P > 0.05$, Table 4.7).

In animals that were euthanised at 14 weeks of age, no treatment effect was detected for plasma creatinine or urea (all $P > 0.05$, Figure 4.6). The 24h water intake, urine output and urinalysis for animals at age 14 weeks are shown in Table 4.8. In both Lewis and LPK, one-way ANOVA did not indicate any treatment effect in any of these parameters (all $P > 0.05$).

4.4.1.4 Innervation profile

Representative images of perivascular tyrosine hydroxylase (TH) and pelvic calcitonin gene-related peptide (CGRP) labelling from Lewis and LPK at age 10 weeks (4 weeks after the different denervation protocols) are shown in Figure 4.7. After total RDN in the Lewis, at 10 weeks, while there was a trend for a reduction in both TH and CGRP nerve density compared

to sham (~42% of sham and ~60% of sham, respectively), this was not statistically significant (P = 0.14 and 0.15 respectively). After afferent RDN in the Lewis at 10 weeks, there was no significant difference from sham animals for TH, as predicted, but also for CGRP levels (~66% of sham levels, P = 0.15), indicating significant sensory reinnervation had occurred after this procedure. In the LPK, after total RDN, there was no significant difference between sham and denervated animals for TH at age 10 weeks (~65% of sham levels). However, CGRP levels were significantly less (~42% of sham, P = 0.03). After afferent RDN, there was no significant difference from sham animals for TH, again as predicted, but also for CGRP levels (~67% of sham, P = 0.19), indicating significant sensory reinnervation having occurred as seen in the Lewis.

A power analysis was performed based upon the CGRP labelling data and the statistical power for the Lewis and LPK strains was 0.63 and 0.85, respectively.

Representative images of perivascular TH and pelvic CGRP labelling from Lewis and LPK at age 14 weeks (8 weeks after the different denervation protocols) are shown in Figure 4.8. In the Lewis animals, there was no significant difference in TH or CGRP innervation density after either total or afferent RDN when compared to sham animals. In the LPK, there was a trend towards reduced TH immunostaining after total RDN (~ 61% of sham), but this was not significant (P = 0.11). There was no significant difference in TH innervation density after afferent RDN compared to sham animals, as anticipated (P = 0.11). CGRP was not significantly different from sham levels 8 weeks after either procedure (P > 0.05). These results are consistent with the significant reinnervation that was evident by 4 weeks post either denervation procedure.

A comparison of TH labelling at 10 and 14 weeks between sham Lewis and sham LPK animals indicated that there was an overall higher level of TH labelling in the LPK compared with the Lewis animals (P = 0.04).

4.4.2 Effect of repeat total RDN procedure on body weights, cardiovascular parameters and renal function at 10 and 14 weeks

Given the significant blood pressure lowering effect seen after total RDN in the Lewis animals alongside evidence of significant re-innervation by 4 weeks post-intervention, we sought to identify if repeating the RDN procedure at 10 weeks of age, and thereby counteracting regrowth of the renal nerves would (a) enhance the blood pressure lowering effect seen in the Lewis animals and (b) produce a treatment effect in the LPK model (Study 3).

4.4.2.1 Body and organ weights

Table 4.9 presents the effect of single or repeat total RDN on the organ and total body weights of both Lewis and LPK animals. An unpaired t-test did not show any treatment effect on body weight, kidney weight (net weight and % body weight), heart weight (net weight and % body weight), and left ventricle weight (net weight and % heart weight) within each strain (all $P > 0.05$).

4.4.2.2 Cardiovascular parameters

4.4.2.2.1 Effect of repeating total RDN on blood pressure and heart rate parameters.

Figure 4.9 illustrates the longitudinal data for blood pressure and heart rate parameters from 11-14 weeks of age for animals that received a single total RDN at 6 weeks of age (data presented above) compared with those of animals that received a repeat total RDN at 10 weeks of age. In the Lewis, repeat total RDN had no additional impact on SBP, MAP or DBP (all $P > 0.05$). In the LPK, two-way ANOVA indicates an interaction between age and treatment in SBP, MAP or DBP (Figure 4.9 E, F and G). *Post hoc* analysis showed this was due to an initially lower BP at 11 weeks of age in those animals that had a repeat denervation, but a subsequent increase in BP such that it was higher by age 13 weeks. In both strains, repeating the total RDN procedure resulted in an overall significantly higher level of HR (all $P < 0.05$).

In LPK animals that underwent two sham procedures, the increase in SBP during 7-10 weeks, i.e., following the first procedure, was comparable to that that during 11-14 weeks i.e., following the second procedure (49.7 ± 3.7 vs. 30.9 ± 11.4mmHg, n=4, *P* = 0.17). The increase in SBP during 7-10 weeks was not significantly different from that during 11-14 weeks in LPKs receiving two total RDN procedures (59.2 ± 3.5 vs. 48.8 ± 4.5mmHg, n=6, *P* = 0.10).

4.4.2.2.2 The effect repeating total RDN on circadian rhythm

The parameters for the circadian rhythms in 14-week-old animals after single or repeat total RDN are presented in Table 4.10. In Lewis, compared to single total RDN, the baseline SBP was significantly lower, while the baseline HR was higher after repeating total RDN. No significant difference was noted for the rest of the parameters.

In LPK, repeat total RDN caused an increase in amplitude and baseline and a delay in phase shift of SBP. Repeat total RDN also caused an increase in baseline HR and a shortening in wavelength for activity. No significant difference was noted for the rest of the parameters.

4.4.2.3 Renal function

The effect of a repeat total RDN procedure on renal function at age 14-weeks is shown in Table 4.11. In Lewis, the repeat procedure did not have a treatment effect on any of the measured parameters. In LPK, a repeat RDN was associated with an increase in the level of plasma urea and UPC, while CCR was reduced, suggestive of worsening renal function.

4.4.2.4 Innervation profile

In both Lewis and LPK animals, there were no difference in TH or CGRP nerve densities at age 14-weeks after a single vs. repeat sham RDN procedure (Table 4.12). When comparing Lewis animals that had a single total RDN with those that had a repeat total RDN at 10 weeks of age, there was also no significant difference in TH or CGRP nerve densities 4 weeks later at age 14 weeks. When comparing LPK animals that had a single total RDN with those that had a repeat total RDN, TH nerve density was significantly reduced (~23% of that in animals

that had had a single procedure). Regarding CGRP nerve density, while there was a trend towards a lesser innervation profile after repeating the total RDN (~50% of that in animals that had had a single procedure), it did not reach significance ($P = 0.06$).

4.5 Discussion

This study provides a comprehensive description of the impact of RDN on the development and maintenance of hypertension in a rodent model of PKD. Our major findings are: (1) neither total or afferent RDN reduces BP in the LPK rat, (2) total RDN, but not selective sensory afferent ablation, produces a small BP reduction in Lewis control animals, reflecting the ongoing role of the renal sympathetic efferent nerves in the control of blood pressure under normotensive conditions; and 3) there is significant regrowth of the renal sensory and efferent nerves within 4 weeks of the procedure as detected using immunohistochemistry, however limiting this reinnervation by repeating the RDN procedure did not produce any additional positive effect on cardiovascular parameters in either strain, and worsened renal function in the LPK animals, suggesting that repeating renal nerve ablation has no therapeutic benefit.

4.5.1 Total RDN

There is an ongoing role for the renal sympathetic efferent nerves in the regulation of blood pressure under normotensive conditions (Johns *et al.* 2011) and consistent with this, in the present study total RDN produced a reduction of ~6 mmHg in blood pressure in the Lewis that was of comparable magnitude to that observed in other normotensive animal models after RDN (Jacob *et al.* 2003, King *et al.* 2007). The blood pressure lowering effect was not sustained throughout the whole study period. One possible explanation for this is the reduction of animal numbers reduced the power of the analysis. However, power analysis suggested a power of 0.83 at 11 weeks old, when the difference in SBP between sham and total group became non-significant. Another possible reason is reinnervation by the nerves. However, a repeat procedure failed to further reduce SBP in the Lewis strain compared with a single procedure. This would suggest that the loss of blood pressure lowering effect was not mediated by the reinnervation and may involve mechanisms that are independent of the renal nerves.

Previously we have demonstrated increased renal sympathetic efferent activity in the LPK model from an early age (Salman *et al.* 2015), suggesting that renal sympathetic overactivity may contribute to the development and or/maintenance of hypertension in this model. However, we did not observe a significant reduction in blood pressure following total RDN in the LPK, despite the evidence we had successfully denervated the kidney at day 7 postoperatively with our experimental protocol. In the LPK, sympathetic vasomotor tone is elevated as ganglionic blockade with hexamethonium reduces blood pressure to within normotensive levels (Phillips *et al.* 2007, Ameer *et al.* 2014, Salman *et al.* 2014) and we have noted increased sympathetic outflow to non-renal vascular beds (Yao *et al.* 2015). Alongside clinical observations that hypertensive PKD patients have increased muscle sympathetic nerve activity (MSNA) (Klein *et al.* 2001) and plasma noradrenaline levels (Cerasola *et al.* 1997), our results indicate that in the LPK model, elimination of sympathetic outflow to one vascular bed is not sufficient to reduce blood pressure.

Our findings in the LPK model of CKD contrast previous work conducted in the Han: SPRD-Cy/+ model (Gattone *et al.* 2008) of PKD and the fetal uninephrectomised CKD sheep model (Singh *et al.* 2019), where total RDN produced a reduction in blood pressure. Several important points of difference exist between our work and these findings. Firstly, the phenotype of the Han: SPRD-Cy/+ is more consistent with autosomal dominant PKD (ADPKD), with later onset of disease and less severe hypertension, and CKD sheep model manifests as mild hypertension, whereas the LPK model presents with a phenotype more consistent with ARPKD, with hypertension from a young age and relatively rapid progression of renal disease. Secondly, a reduction in cystic volume was observed in the Han: SPRD-Cy/+ rat in response to total RDN and a reduction in urinary albumin was observed in CKD sheep. These are both consistent with an improvement in renal function which may have been a contributor to the reduction in blood pressure. The underlying mechanism by which RDN reduced the cystic volume in the Han: SPRD-Cy/+ rat was not established, with the authors

hypothesising potential roles for Ang II, sympathetic activity or direct haemodynamic effects (Gattone *et al.* 2008). In our study, we observed no difference in the kidney to body weight ratio suggesting cystic volume was not reduced and we did not observe a difference in urine protein to creatinine ratio. Within the context of PKD, therefore, the ability of RDN to limit disease progression may be dependent on the specific disease phenotype, including the age of onset and disease severity.

4.5.2 Afferent RDN did not affect blood pressure

Consistent with the lack of effect of total RDN on the development of hypertension in the LPK model, selective RDN also did not attenuate observed hypertension. These results suggest that either the renal sensory nerves are not overactive in PKD or the brain regions that are suggested to receive renal sensory nerve input and drive hypertension in disease states (Calaresu and Ciriello 1981, Campese and Kogosov 1995) are not perturbed in PKD. We did not record the activity of the renal sensory nerves in the present study; however, our previous work has demonstrated that renal tissue hypoxia is evident in the LPK (Ow *et al.* 2014) and other stimuli known to drive renal afferent nerve activity such as inflammatory processes (Frame *et al.* 2016) have also been demonstrated in the LPK (Phillips *et al.* 2007). Stimulus for renal sensory nerve activation is likely present and so it is probable that the lack of blood pressure response to selective renal sensory nerve ablation in the LPK, regardless of the level of renal sensory afferent activity, is a result of the denervation procedure not having a functional impact on the central pathways and subsequent outflows involved in driving hypertension in PKD.

In the present study, afferent RDN had no effect on the blood pressure regulation in the normotensive Lewis group, which is consistent with previous reports (Foss *et al.* 2015). This suggests that renal sensory nerves do not play a primary role in the regulation of blood pressure under normal physiological conditions. Afferent RDN does however reduce blood pressure in the DOCA salt rat, where renal nerve activity has been shown to be elevated, as

evidenced by direct nerve recording (Banek *et al.* 2016). Afferent RDN has also been shown to attenuate hypertension in the 5/6 renal ablation model of kidney failure (Campese and Kogosov 1995) and the unilateral nephrectomised SHR model (Nishimura *et al.* 2007), possibly by altering the sympathetic or RAAS activity in the hypothalamus, respectively. This would suggest that the removal of renal afferents could indeed contribute to the blood pressure reduction in certain pathophysiological conditions which involve overactivation of peripheral or central renal sensory pathways.

4.5.3 Total and afferent RDN had minimal effect on circadian rhythms

In addition to the effect on the overall development of hypertension, we also examined the effect of total or afferent RDN on the circadian rhythms of SBP, HR and activity. Both total and afferent RDN caused a reduction in baseline SBP in the Lewis but an increase in baseline SBP in LPK at 10 weeks old. The afferent RDN procedure caused a reduction in baseline HR in Lewis but an increase in baseline in LPK at 14 weeks old. Total RDN caused a delay in the peak of SBP in 10-week old Lewis. We previously reported that total RDN produced a reduction in SBP across 7-14 weeks old using 24h means. Here the observation that total RDN lowered baseline SBP at 10 weeks old in Lewis further confirmed this effect. In Lewis, we also observed that total RDN caused a rightward phase shift in SBP, suggesting a delay in the SBP peak, which may indicate that renal sympathetic nerves are involved in the rhythmic cardiovascular fluctuations. The observation that total and afferent RDN caused an increase in baseline SBP in 10 weeks old LPK, which was not evident in the 24h data, may be explained by the greater number of data points (48 fold increase) used for the circadian rhythm analysis. Nevertheless, other circadian rhythm parameters for SBP in LPK were not impacted by either procedure. There are limited studies evaluating the effect of RDN on circadian rhythms per se. One recent study in ET_B (endothelin-1)-deficient rats showed total RDN reduced the baseline MAP but had no effect on other circadian rhythm parameters including amplitude

and phase shift (Becker *et al.* 2017). Our findings in LPK are consistent with this, suggesting that the rhythmic cardiovascular fluctuations were not mediated by renal nerves.

Another way of describing the circadian pattern of cardiovascular parameters in clinical studies is "dipping patterns". Dipping is defined as a nighttime drop in blood pressure of no less than 10% of daytime values; patients with a non-dipping profile are at higher risk for cardiovascular mortality. A few clinical studies examined the impact of RDN on the dipper vs. non-dipper pattern. For example, in the SYMPLICITY HTN-3 trial (Bakris *et al.* 2014), RDN did not significantly affect the percentage of patients converting from nondippers to dippers. Tuohy et al. (Tuohy *et al.* 2016) also reported that RDN did not significantly affect the nocturnal dipping status in SBP in resistant hypertension. Our LPK model exhibits non-dipping hypertension as the day and night blood pressure values are identical and the night-day difference in blood pressure in LPK was not impacted by total or afferent denervation in our study. These results suggest that the renal nerves make a limited contribution to circadian rhythms in cardiovascular parameters under disease conditions.

4.5.4 Reinnervation after RDN.

Debate exists regarding the impact of renal reinnervation following RDN. In the present study, we noted significant reinnervation at 4 weeks post-procedure in both Lewis and LPK, consistent with work by Kline and Mercer and Mulder et al. (Kline and Mercer 1980, Mulder *et al.* 2013) who showed that after total RDN in rats, both anatomical and/or functional reinnervation of the kidneys was partially returned 4 weeks post denervation. Based on our experimental data obtained from Chapter 3, which demonstrated a significant loss of renal nerve markers one week post the denervation procedure, the presence of renal nerves seen in this study was regarded as reinnervation instead of incomplete denervation. As in longitudinal studies with humans, reliable markers are lacking to ascertain the effectiveness of renal denervation without tissue collection. We therefore relied on the evidence obtained from acute studies to ascertain that our denervation procedure was effective. Whether structural

evidence of reinnervation seen in the LPK model corresponds to the return of the functionality was not clear. We attempted to shed some light on this by repeating total RDN at 4 weeks post the initial procedure. Notably, a repeat RDN procedure did not impact blood pressure response in the Lewis animals, and there was a transient increase in SBP and MAP in the LPK. Our finding is consistent with pre-clinical work conducted in the SHR model, where a second RDN procedure likewise did not further reduce blood pressure (Kline *et al.* 1980).

The repeated denervation procedure in itself could have caused an inflammatory response that negated the effect of the RDN (Norlander *et al.* 2018). While we did not ascertain the systemic or renal inflammatory reaction following the repeat denervation, our study indicated a comparable magnitude of increase in SBP in LPKs following either the first or second total RDN procedure. This would suggest that the presence of an inflammatory response, which would presumably be more marked after the second procedure, did not impact blood pressure. Further investigation to determine the systemic or renal inflammatory response would be of value.

While direct evidence of nerve regrowth after RDN in humans is lacking, renal sympathetic nerve regrowth is observed at 5 months post kidney transplantation, supporting a notion of nerve regrowth after RDN in humans (Mauriello *et al.* 2017) and in normal sheep, both anatomic and functional evidence of reinnervation is documented at 5 ½ months post catheter-based RDN (Booth *et al.* 2015), with full functionality returned at 11 months post-procedure. So does renal re-innervation dampen the blood pressure lowering effect of RDN or does the initial procedure induce a long-term effect despite regrowth? This is directly relevant to observations from clinical studies that purport a sustained response to catheter-based RDN 3 years after the initial procedure (Sadowski *et al.* 2011, Esler *et al.* 2014). Our findings in the Lewis animals would suggest that transaction of the renal nerves is responsible for the initial reduction in blood pressure following RDN, but the sustained effects on blood pressure are subsequently mediated by mechanisms independent of the renal nerves. In contrast to this

hypothesis however is a recent study in sheep with CKD, where catheter-based RDN produced a sustained reduction in blood pressure that was associated with only partial regrowth and return of function at 30 months post-procedure (Singh *et al.* 2019) while control animals showed complete anatomical and functional recovery in the same time frame. Critically though, this reset the CKD animals to a level of innervation comparable to the controls, suggesting hyper-innervation of the kidneys was a driving factor for hypertension. Although an overall higher level of perivascular TH labelling was observed in sham LPK animals, due to the cystic lesions in the LPK kidney, it was essentially not possible to make an overall assessment of hyperinnervation in the LPK animals.

4.5.5 Reno and cardioprotective effects of RDN

There is evidence that RDN can slow the progression of CKD in patients (Ott *et al.* 2015, Kiuchi and Chen 2016, Hering *et al.* 2017). In our study, neither single total or afferent RDN improved plasma urea or creatinine in the LPK model, nor did we observe any improvement in creatinine clearance or urinary protein excretion. Attenuation of proteinuria after RDN in other animal models was often in parallel with a reduction in blood pressure (Hamar *et al.* 2007, Nishimura *et al.* 2007). Thus, the lack of a decrease in arterial pressure by total or afferent RDN in the LPK model may explain our data. Of note is that we observed a worsening of renal function after a second total RDN in LPK, which was not seen in the sham animals, suggesting a detrimental impact induced by the repeat procedure. From a translational perspective, our results indicate that in patients, exposure to a repeat RDN surgical procedure is not supported.

Cardiac hypertrophy is among the strongest risk factors for cardiovascular mortality in the general (Okwuosa *et al.* 2015) and CKD patient population (London *et al.* 2001, Zoccali *et al.* 2004). In SHR, improvements in cardiac fibrosis have been reported after RDN (Linz *et al.* 2014) and Kiuchi et al. (Kiuchi *et al.* 2016) found a reduction in left ventricle mass indexed for body weight and an increase in ventricular ejection fraction 6 months after RDN in

hypertensive CKD patients. Reduced left ventricle mass and increased ventricular ejection fraction after RDN was also reported in patients with resistant hypertension (Brandt *et al.* 2012, Mahfoud *et al.* 2014, de Sousa Almeida *et al.* 2016). The observed cardioprotective effects are associated with a lowering in BP in some patients (Brandt *et al.* 2012) but are independent of blood pressure changes in other patients (Mahfoud *et al.* 2014). While cardiac hypertrophy is present in the LPK (Phillips *et al.* 2007, Quek *et al.* 2016), we did not see any improvements in this variable with RDN, partly due to a lack of BP reduction, suggesting that in this model, ablating renal nerves is not sufficient to prevent cardiac hypertrophy.

4.5.6 Limitations

The primary limitation of this study is that the sample size for some experimental groups was quite small. This is partly due to the telemetry probe failure, which resulted in a decreasing number of animals in each group with age. Power analysis however indicated that the blood pressure lowering effect in the Lewis group, and the loss of this effect with age was not associated with the decrease of animal numbers. Furthermore, our lack of treatment effect in the LPK strain was unlikely to be due to the small sample size as it would have required a 4-fold increase in animal numbers to detect any significant difference in blood pressure. From an ethical and logistically perspective such an increase in animal numbers was not possible.

Additionally, the validation of renal denervation procedure could not be assessed in the live animal. Therefore, the possibility of incomplete denervation exists. However, given the proven effectiveness of the denervation procedure demonstrated in Chapter 3, we are confident that the procedures employed were effective. Finally, the reinnervation of renal nerves was assessed solely by immunohistochemistry, which at best serves as a semi-quantitative method. The application of quantitative measures such as Western-blot or high-performance liquid chromatography (HPLC) in future studies would be of great benefit in validating our findings.

4.5.7 Summary and perspectives

Key questions in our understanding of RDN include determination of which patient populations will benefit from the approach, what the relative contribution of renal sensory and sympathetic nerves is in mediating any procedure-induced effect, and how is the effect sustained in the face of functional reinnervation. Our study indicates that neither total or afferent RDN has a beneficial effect on hypertension, autonomic function or renal function in a rodent model of PKD, suggesting that this procedure might not benefit patients with comparable PKD conditions. Our finding that total but not afferent RDN could lower blood pressure in normotensive rats does, however, support the critical role of renal sympathetic nerves in the long-term regulation of blood pressure. The observation that a repeat total RDN did not provide any additional beneficial effect alongside structural evidence of re-innervation suggests that the primary impact of RDN is associated with the initial procedure. From a mechanistic perspective, further studies in both the pre-clinical and clinical research are needed to determine the relative contribution of renal sensory and sympathetic nerves of the blood pressure lowering effect of RDN observed in clinical trials. From a translational perspective, this is critical in order to determine if afferent- or efferent- specific RDN could optimise the treatment efficacy in specific patient cohorts.

4.6 Tables

Table 4.1 Renal function parameters in animals received single or repeat sham renal denervation (RDN)

	Single sham RDN	Repeat sham RDN	Unpaired T-test
Lewis (n)	(2)	(3)	
Kidney, %total weight	0.7 ± 0.0	0.8 ± 0.0	0.77
Plasma urea (mmol/L)	8.9 ± 1.1	12.6 ± 5.1	0.62
Plasma creatinine (μmol/L)	33.0 ± 2.0	24.7 ± 1.8	0.055
24h water intake (ml)	24.5 ± 3.5	18.1 ± 3.8	0.33
24h urine output (ml)	12.2 ± 0.4	9.1 ± 1.9	0.29
Urine protein (g/L)	0.2 *	0.4/0.1*	NA
Urine creatinine(g/L)	0.9 ± 0.2	1.1 ± 0.1	0.35
UPC	0.2 *	0.4/0.1*	NA
CCR (ml/min)	2.5 ± 0.6	3.3 ± 0.8	0.53
LPK (n)	(5)	(4)	
Kidney, % total weight	8.4 ± 0.3	9.0 ± 0.4	0.33
Plasma urea (mmol/L)	31.3 ± 7.2	31.2 ± 3.3	0.98
Plasma creatinine (μmol/L)	85.2 ± 23.2	84.8 ± 14.2	0.98
24h water intake (ml)	32.6 ± 6.1	46.8 ± 5.5	0.14
24h urine output (ml)	21.7 ± 3.2	39.7 ± 0.4	0.002
Urine protein (g/L)	1.5 ± 0.4	1.3 ± 0.2	0.74
Urine creatinine(g/L)	0.2 ± 0.1	0.2 ± 0.0	0.24
UPC	8.8 ± 3.2	8.4 ± 1.7	0.92
CCR (ml/min)	0.5 ± 0.1	0.6 ± 0.0	0.58

RDN, renal denervation. UPC, urine protein to creatinine ratio. CCR, creatinine clearance rate. *Urine protein was detected in 1 out of 2 Lewis in single RDN and 2 out of 3 Lewis in those animals that had a repeat procedure and values above were determined from those animals. NA, not applicable due to a low number of animals with detectable protein level. Data is expressed as the mean ± SEM.

Table 4.2 Body and organ weights in Lewis and Lewis polycystic kidney disease rats from the 10-week-old cohort.

RDN procedure	Sham	Total	Afferent	One-way ANOVA
Lewis (n)	(5)	(4)	(4)	
Body weight (g)	243.3 ± 25.5	253.0 ± 29.3	264.4 ± 31.1	0.87
Kidney weight (g)	2.0 ± 0.2	2.1 ± 0.3	2.1 ± 0.2	0.96
Heart weight (g)	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.55
LV weight (g)	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.83
Kidney, % body weight	0.8 ± 0.02	0.8 ± 0.0	0.8 ± 0.0	0.68
Heart, % body weight	0.33± 0.01	0.33 ± 0.01	0.35 ± 0.02	0.46
LV, % heart weight	68.8 ± 1.8	67.6 ± 3.1	65.1 ± 2.3	0.56
LPK (n)	(5)	(5)	(3)	
Body weight (g)	185.0 ± 22.5	201.0 ± 22.3	187.9 ± 17.5	0.85
Kidney weight (g)	15.2 ± 1.3	14.6 ± 1.6	16.1 ± 0.9	0.68
Heart weight (g)	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.0	0.25
LV weight (g)	0.7 ± 0.0	0.7 ± 0.1	0.7 ± 0.1	0.89
Kidney, % body weight	8.3 ± 0.5	7.4 ± 0.7	8.8 ± 0.8	0.42
Heart, % body weight	0.54 ± 0.03	0.46 ± 0.05	0.57 ± 0.04	0.20
LV, % heart weight	73.4 ± 0.6	75.5 ± 1.4	62.6 ± 11.7	0.48

RDN, renal denervation. LV, left ventricle.

Table 4.3 Body and organ weights in Lewis and Lewis polycystic kidney disease rats from the 14-week-old cohort.

RDN procedure	Sham	Total	Afferent	One-way ANOVA
Lewis (n)	(5)	(5)	(4)	
Body weight (g)	321.4 ± 35.9	304.7 ± 36.0	287.9 ± 37.1	0.82
Kidney weight (g)	2.4 ± 0.3	2.3 ± 0.3	2.2 ± 0.3	0.81
Heart weight (g)	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.74
LV weight (g)	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.86
Kidney, % body weight	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	0.93
Heart, % body weight	0.30 ± 0.02	0.29 ± 0.03	0.33 ± 0.03	0.10
LV, % heart weight	70.0 ± 1.9	71.8 ± 2.8	65.8 ± 2.0	0.21
LPK (n)	(5)	(5)	(3)	
Body weight (g)	184.1 ± 12.2	235.0 ± 26.2	169.4 ± 2.2	0.09
Kidney weight (g)	15.6 ± 1.5	20.2 ± 2.1	15.6 ± 1.2	0.15
Heart weight (g)	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.0	0.40
LV weight (g)	0.8 ± 0.0	0.9 ± 0.1	0.7 ± 0.0	0.31
Kidney, % body weight	8.4 ± 0.4	8.6 ± 0.2	9.2 ± 0.6	0.37
Heart, % body weight	0.56 ± 0.12	0.50 ± 0.04	0.58 ± 0.03	0.30
LV, % heart weight	73.6 ± 1.9	76.5 ± 2.1	73.0 ± 2.7	0.50

RDN, renal denervation. LV, left ventricle

Table 4.4 Sex, day period effect and their interaction with treatment effect in Lewis and LPK

Parameters and statistical analysis performed	Lewis	LPK
Systolic blood pressure (SBP)		
Sex effect	0.02	0.001
Rx effect in males	0.0006	0.12
Rx effect in females	<0.0001	0.96
Day period effect	<0.0001	0.67
Rx effect using day values	<0.0001	0.9
Rx effect using night values	0.0005	0.57
Mean arterial pressure (MAP)		
Sex effect	0.13	0.005
Rx effect in males	0.0007	0.12
Rx effect in females	<0.0001	0.94
Day period effect	<0.0001	0.74
Rx effect using day values	<0.0001	0.78
Rx effect using night values	<0.0001	0.43
Diastolic blood pressure (DBP)		
Sex effect	0.07	0.04
Rx effect in males	0.0002	0.12
Rx effect in females	<0.0001	0.85
Day period effect	<0.0001	0.71
Rx effect using day values	<0.0001	0.54
Rx effect using night values	<0.0001	0.29
Heart rate (HR)		
Sex effect	0.89	<0.0001
Rx effect in males	0.24	0.29
Rx effect in females	0.04	0.1
Day period effect	<0.0001	<0.0001
Rx effect using day values	0.01	0.009
Rx effect using night values	0.15	0.09

Rx, treatment, the value displayed in the table are the P value for the indicated analysis.

Table 4.5 Cosine curve parameters for SBP, HR and activity in Lewis at age 10 and 14 weeks

		10w				14w			
Parameters \RDN procedure		Sham	Total	Afferent	P value	Sham	Total	Afferent	P value
n		11	13	10		5	4	4	
SBP	Amplitude	2.9 ± 0.4	3.0 ± 0.4	2.9 ± 0.5	0.96	3.7 ± 0.6	3.6 ± 0.7	3.1 ± 1.1	0.83
	Phaseshift	8.7 ± 0.9	11.1 ± 0.6 a	8.6 ± 1.0	0.04	5.9 ± 1.4	6.8 ± 1.3	7.6 ± 2.2	0.77
	Wavelength	24.6 ± 1.2	23.22 ±0.8	24.0 ± 1.2	0.59	27.4 ± 1.7	25.7 ± 1.6	25.5 ± 2.8	0.76
	Baseline	130.1 ± 0.3	122.3 ± 0.3 a	126.6 ± 0.4 a	<0.0001	133.4 ± 0.5	131.8 ± 0.5a	129.4 ± 0.8 a	<0.0001
HR	Amplitude	35.3 ± 2.5	27.8 ± 2.4	31.4 ± 2.6	0.09	36.2 ± 3.3	32.7 ± 3.6	35.3 ± 3.8	0.76
	Phaseshift	6.3 ± 0.5	7.3 ± 0.5	6.3 ± 0.6	0.31	6.4 ± 0.6	6.0 ± 0.8	6.4 ± 0.8	0.92
	Wavelength	25.0 ± 0.6	25.1 ± 0.7	24.9 ± 0.6	0.97	25.2 ± 0.7	25.0 ± 0.9	25.5 ± 0.9	0.93
	Baseline	384.8 ± 1.8	382.9 ± 1.7	379.3 ± 1.8	0.11	361.0 ± 2.4	358.7 ± 2.6	345.6 ± 2.8 a	<0.0001
activity	Amplitude	1.3 ± 0.2	1.0 ± 0.1	1.3 ± 0.2	0.27	1.3 ± 0.2	1.0 ± 0.2	1.4 ± 0.3	0.44
	Phaseshift	7.5 ± 0.9	9.9 ± 0.8	8.7 ± 0.8	0.18	4.9 ± 1.5	6.8 ± 1.5	5.3 ± 1.6	0.75
	Wavelength	25.4 ± 1.1	24.1 ± 1.1	24.4 ± 1.0	0.61	28.0 ± 1.8	26.0 ± 1.9	28.7 ± 2.0	0.65
	Baseline	3.5 ± 0.1	3.2 ± 0.1	3.4 ± 0.1	0.15	2.8 ± 0.2	2.6 ± 0.2	2.8 ± 0.2	0.65

RDN, renal denervation, SBP, systolic blood pressure. HR, heart rate. Amplitude defines as the difference between the maximum and the baseline of each parameter. Phase shift defines as the first time in a cycle at which the cycle peaks. Wavelength defines as the time it takes to complete a cycle. Baseline defines as the y value around which the curve oscillates. "a" denotes $p<0.05$ difference vs sham RDN.

Table 4.6 Cosine curve parameters for SBP, HR and activity in LPK at age 10 and 14 weeks

		10w				14w			
Parameters\RDN procedure		Sham	Total	Afferent	P value	Sham	Total	Afferent	P value
n		11	12	8		9	4	3	
SBP	Amplitude	6.6 ± 1.6	6.7 ± 1.1	4.0 ± 2.4	0.48	4.0 ± 2.2	8.0 ± 3.2	7.6 ± 2.1	0.48
	Phaseshift	13.2 ± 1.3	12.7 ± 0.9	8.8 ± 3.6	0.45	14.0 ± 3.1	11.4 ± 2.7	14.5 ± 1.4	0.68
	Wavelength	25.1 ± 1.8	23.7 ± 1.2	27.9 ± 4.9	0.57	27.0 ± 4.8	33.8 ± 5.3	23.8 ± 1.9	0.28
	Baseline	218.7 ± 1.2	225.8 ± 0.9 [a]	226.2 ± 1.7 [a]	<0.0001	238.9 ± 1.7	239.1 ± 2.3	234.0 ± 1.5	0.94
HR	Amplitude	22.1 ± 2.4	24.5 ± 2.5	24.8 ± 3.6	0.74	16.5 ± 3.2	20.4 ± 3.4	15.9 ± 5.5	0.71
	Phaseshift	4.9 ± 0.8	5.2 ± 0.7	4.4 ± 1.1	0.78	4.7 ± 1.4	4.8 ± 1.4	3.5 ± 3.1	0.87
	Wavelength	24.6 ± 0.8	24.4 ± 0.7	24.8 ± 1.1	0.95	24.4 ± 1.3	26.2 ± 1.5	26.7 ± 3.3	0.55
	Baseline	384.7 ± 1.7	387.5 ± 1.8	396.1 ± 2.6[a]	<0.001	361.9 ± 2.3	352.7 ± 2.5 [a]	372.1 ± 4.1 [a]	<0.001
activity	Amplitude	1.2 ± 0.2	1.4 ± 0.2	1.2 ± 0.2	0.59	0.5 ± 0.2	1.0 ± 0.3	0.6 ± 0.3	0.43
	Phaseshift	6.6 ± 1.0	7.6 ± 0.6	6.0 ± 1.4	0.44	3.5 ± 3.6	5.9 ± 2.6	3.3 ± 5.3	0.77
	Wavelength	26.7 ± 1.3	24.4 ± 0.8	25.8 ± 1.7	0.30	28.2 ± 4.1	28.5 ± 3.3	29.7 ± 6.2	0.97
	Baseline	3.0 ± 0.1	2.8 ± 0.1	3.1 ± 0.2	0.23	2.5 ± 0.1	2.6 ± 0.2	2.9 ± 0.2	0.21

RDN, renal denervation, SBP, systolic blood pressure. HR, heart rate. Amplitude defines as the difference between the maximum and the baseline of each parameter. Phase shift defines as the first time in a cycle at which the cycle peaks. Wavelength defines as the time it takes to complete a cycle. Baseline defines as the y value around which the curve oscillates. a, $p<0.05$, difference vs sham RDN.

Table 4.7 Renal function parameters in animals at age 10-weeks.

RDN procedure	Sham	Total	Afferent	One-way ANOVA
Lewis (n)	(5)	(4)	(4)	
24h water intake (ml)	18.2 ± 4.1	21.2 ± 2.6	23.8 ± 2.3	0.50
24h urine output (ml)	8.5 ± 1.7	10.2 ± 0.9	10.4 ± 1.8	0.65
Urinary protein(g/L)	0.15 ± 0.02	NA	NA	NA
Urinary creatinine (g/L)	0.88 ± 0.09	0.91 ± 0.061	0.76 ± 0.10	0.45
UPC	0.17 ± 0.02	NA	NA	NA
CCR (ml/min)	2.8 ± 0.6	2.1 ± 0.3	2.3 ± 0.5	0.62
LPK (n)	(4)	(4)	(3-4)	
24h water intake (ml)	40.5 ± 1.0	39.1 ± 3.4	35.8 ± 4.6	0.61
24h urine output (ml)	27.4 ± 1.7	26.6 ± 2.8	23.6 ± 5.2	0.74
Urinary protein(g/L)	0.84 ± 0.08	0.66 ± 0.13	0.59 ± 0.15	0.36
Urinary creatinine (g/L)	0.20 ± 0.06	0.24 ± 0.03	0.30 ± 0.02	0.36
UPC	5.21 ± 1.51	2.73 ± 0.37	1.96 ± 0.39	0.12
CCR (ml/min)	1.2 ± 0.4	1.6 ± 0.3	1.0 ± 0.3	0.53

RDN, renal denervation. UPC, urine protein to creatinine ratio. CCR, creatinine clearance rate. Data is expressed as the mean ± SEM.*In the Lewis, 2 animals in the total RDN and 2 animals in the afferent RDN group had protein levels below the level of detection. Thus the mean value for urine protein and UPC is not provided. NA, not applicable.

Table 4.8: Renal function parameters in animals at age 14-weeks.

RDN procedure	Sham	Total	Afferent	One-way ANOVA
Lewis (n)	(3-5)	(4-5)	(4)	
24h water intake (ml)	20.7 ± 2.8	20.8 ± 1.6	20.0 ± 4.2	0.98
24h urine output (ml)	10.4 ± 1.3	9.0 ± 1.3	10.0 ± 3.1	0.87
Urinary protein(g/L)	0.23 ± 0.07	0.16 ± 0.05	NA	NA
Urinary creatinine (g/L)	1.02 ± 0.11	1.14 ± 0.08	1.10 ± 0.17	0.76
UPC	0.23 ± 0.07	0.13 ± 0.04	NA	NA
CCR (ml/min)	3.0 ± 0.5	3.2 ± 0.6	2.8 ± 0.3	0.82
LPK (n)	(8-9)	(5)	(3)	
24h water intake (ml)	39.7 ± 4.7	49.4 ± 6.9	38.9 ± 5.2	0.41
24h urine output (ml)	28.6 ± 3.3	38.6 ± 4.7	28.5 ± 4.9	0.20
Urinary protein(g/L)	1.41 ± 0.25	1.07 ± 0.28	1.60 ± 0.91	0.68
Urinary creatinine (g/L)	0.21 ± 0.03	0.17 ± 0.02	0.19 ± 0.08	0.83
UPC	8.66 ± 1.85	6.38 ± 1.58	13.39 ± 8.94	0.45
CCR (ml/min)	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.2	0.73

RDN, renal denervation. UPC, urine protein to creatinine ratio. CCR, creatinine clearance rate. Data is expressed as the mean ± SEM. NA, not applicable due to no animals having detectable protein in the urine samples.

Table 4.9 Body and organ weights in Lewis and Lewis polycystic kidney disease rats after a single or repeat total RDN

Total RDN procedure	Single	Repeat	Unpaired t-test
Lewis (n)	(5)	(5)	
Body weight (g)	304.7 ± 36.0	288.1 ± 40.4	0.77
Kidney weight (g)	2.3 ± 0.3	2.5 ± 0.3	0.66
Heart weight (g)	0.9 ± 0.1	0.9 ± 0.1	0.97
LV weight (g)	0.6 ± 0.1	0.6 ± 0.1	0.61
Kidney, % body weight	0.7 ± 0.0	0.8 ± 0.0	0.12
Heart, % body weight	0.3 ± 0.0	0.3 ± 0.0	0.34
LV, % heart weight	71.8 ± 2.8	68.9 ± 1.6	0.36
LPK (n)	(5)	(6)	
Body weight (g)	235.0 ± 26.2	199.5 ± 13.4	0.24
Kidney weight (g)	20.2 ± 2.1	17.5 ± 0.9	0.24
Heart weight (g)	1.2 ± 0.1	1.1 ± 0.1	0.58
LV weight (g)	0.9 ± 0.1	0.8 ± 0.1	0.64
Kidney, % body weight	8.6 ± 0.2	8.8 ± 0.2	0.56
Heart, % body weight	0.5 ± 0.0	0.5 ± 0.0	0.50
LV, % heart weight	76.5 ± 2.1	77.4 ± 0.6	0.65

RDN, renal denervation. LV, left ventricle

Table 4.10 Cosine curve parameters for SBP, HR and activity at 14 weeks old in Lewis and LPK after single or repeat total RDN.

Parameters		Single total RDN	Repeat total RDN	P value
Lewis(n)		4	5	
SBP	Amplitude	3.6 ± 0.7	2.2 ± 0.8	0.20
	Phaseshift	6.8 ± 1.3	7.0 ± 2.9	0.96
	Wavelength	25.7 ± 1.6	27.7 ± 3.5	0.56
	Baseline	131.8 ± 0.5	126.1 ± 0.6	<0.0001
HR	Amplitude	32.7 ± 3.6	27.6 ± 4.2	0.37
	Phaseshift	6.0 ± 0.8	4.4 ± 1.5	0.32
	Wavelength	25.0 ± 0.9	28.0 ± 1.7	0.08
	Baseline	358.7 ± 2.6	369.4 ± 3.2	0.01
Activity	Amplitude	1.0 ± 0.2	1.1 ± 0.2	0.84
	Phaseshift	6.8 ± 1.5	4.7 ± 2.2	0.44
	Wavelength	26.0 ± 1.9	30.6 ± 2.5	0.14
	Baseline	2.6 ± 0.2	3.0 ± 0.2	0.09
LPK (n)		4	6	
SBP	Amplitude	8.0 ± 3.2	22.5 ± 1.5	<0.0001
	Phaseshift	11.4 ± 2.7	12.4 ± 0.6	0.0015
	Wavelength	33.8 ± 5.3	46.5 ± 1.5	0.08
	Baseline	239.1 ± 2.3	248.6 ± 1.0	<0.0001
HR	Amplitude	20.4 ± 3.4	14.1 ± 2.9	0.16
	Phaseshift	4.8 ± 1.4	5.7 ± 1.4	0.63
	Wavelength	26.2 ± 1.5	24.3 ± 1.4	0.29
	Baseline	352.7 ± 2.5	376.6 ± 2.1	<0.0001
Activity	Amplitude	1.0 ± 0.3	0.9 ± 0.3	0.93
	Phaseshift	5.9 ± 2.6	18.8 ± 0.6	0.15
	Wavelength	28.5 ± 3.3	11.6 ± 0.5	0.002
	Baseline	2.6 ± 0.2	3.0 ± 0.2	0.23

RDN, renal denervation, SBP, systolic blood pressure. HR, heart rate. Amplitude defines as the difference between the maximum and the baseline of each parameter. Phase shift defines as the first time in a cycle at which the cycle peaks. Wavelength defines as the time it takes to complete a cycle. Baseline defines as the y value around which the curve oscillates. a, $p<0.05$, difference vs sham RDN.

Table 4.11: Comparison of renal function parameters in animals that received single vs. repeat total RDN

Total RDN procedure	Single	Repeat	Unpaired T-test
Lewis (n)	(4-5)	(4-5)	
Plasma urea (mmol/L)	8.6 ± 0.7	8.1 ± 0.5	0.60
Plasma creatinine (µmol/L)	27.8 ± 5.3	30.2 ± 2.6	0.69
24h water intake (ml)	20.8 ± 1.6	26.0 ± 2.7	0.12
24h urine output (ml)	9.0 ± 1.3	11.5 ± 1.8	0.29
Urinary protein (g/L)	0.2 ± 0.0*	1.3 ± 0.7*	0.17
Urinary creatinine (g/L)	1.1 ± 0.1	0.8 ± 0.2	0.11
UPC	0.1 ± 0.0*	2.7 ± 1.6*	0.17
CCR (ml/min)	3.2 ± 0.6	2.3 ± 0.3	0.22
LPK (n)	(5)	(6)	
Plasma urea (mmol/L)	27.4 ± 2.3	36.0 ± 2.3	0.03
Plasma creatinine (µmol/L)	84.2 ± 15.1	119.8 ± 13.6	0.11
24h water intake (ml)	49.4 ± 6.9	49.0 ± 3.3	0.95
24h urine output (ml)	38.6 ± 4.7	41.6 ± 2.0	0.55
Urinary protein (g/L)	1.1 ± 0.3	1.6 ± 0.3	0.22
Urinary creatinine (g/L)	0.2 ± 0.0	0.1 ± 0.0	0.007
UPC	6.4 ± 1.6	15.0 ± 2.8	0.03
CCR (ml/min)	0.7 ± 0.2	0.3 ± 0.0	0.02

RDN, renal denervation. UPC, urine protein to creatinine ratio. CCR, creatinine clearance rate. Data is expressed as the mean ± SEM. *Urine protein was detected in 4 out of 5 Lewis in single RDN and 4 out of 5 Lewis in those animals that had a repeat procedure and values above were determined from those animals.

Table 4.12. Comparison of innervation density in animals that received single vs. repeat RDN at 14 weeks of age.

	Single	Repeat	Unpaired T-test
Lewis sham RDN (n)	(2)	(3)	
TH ($\mu m^2/mm^2$ perivascular	4531 ± 1922	3984 ± 966	0.79
CGRP ($\mu m^2/mm^2$ pelvic wall)	39120 ± 2288	32629 ± 5142	0.19
LPK sham RDN (n)	(5)	(4)	
TH ($\mu m^2/mm^2$ perivascular	6947 ± 829	5082 ± 983	0.41
CGRP ($\mu m^2/mm^2$ pelvic wall)	45597 ± 4631	42874 ± 6940	0.75
Lewis total RDN (n)	(5)	(5)	
TH ($\mu m^2/mm^2$ perivascular	2269 ± 1085	564 ± 296	0.17
CGRP ($\mu m^2/mm^2$ pelvic wall)	34739 ± 14416	17700 ± 3797	0.29
LPK total RDN (n)	(5)	(6)	
TH ($\mu m^2/mm^2$ perivascular	3449 ± 1224	785 ± 292	0.046
CGRP ($\mu m^2/mm^2$ pelvic wall)	37162 ± 7953	18639 ± 4658	0.06

RDN, renal denervation. Data is expressed as the mean ± SEM. TH, tyrosine hydroxylase CGRP, calcitonin gene-related peptide.

4.7 Figures

Figure 4.1 Experimental design of animal studies

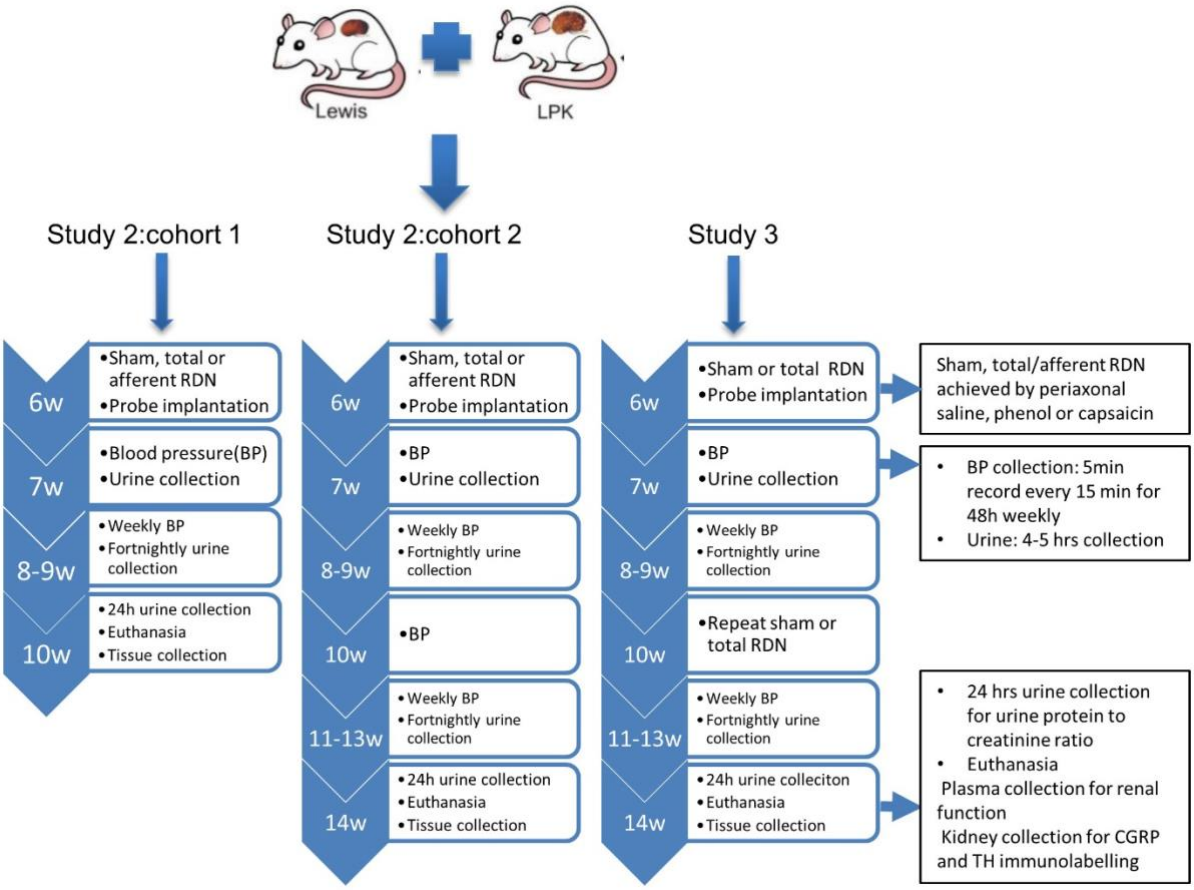


Figure 4.2 The effect of a single vs repeat sham renal denervation (RDN) on systolic (SBP; A,E), mean (MAP; B, F), diastolic blood pressure (DBP;C, G) and heart rate (HR; D, H) in Lewis (A, B, C and D) and LPK (E, F,G and H) rats between 7-14 weeks of age.

N values at each week for Lewis single sham = (7, 8, 7, 8, 5, 2, 2, 2); Lewis repeat sham = (3, 3, 3, 3, 3, 3, 3, 3) respectively and for LPK single sham = (9, 7, 9, 7, 6, 5, 5, 5), LPK repeat sham= (4, 4, 4, 4, 4, 4, 4, 4) respectively. Rx, treatment effect. Data is expressed as mean ± SEM and analysed using two-way ANOVA for the treatment effect (Rx: single or repeat sham) and age effect (P value provided in each panel).

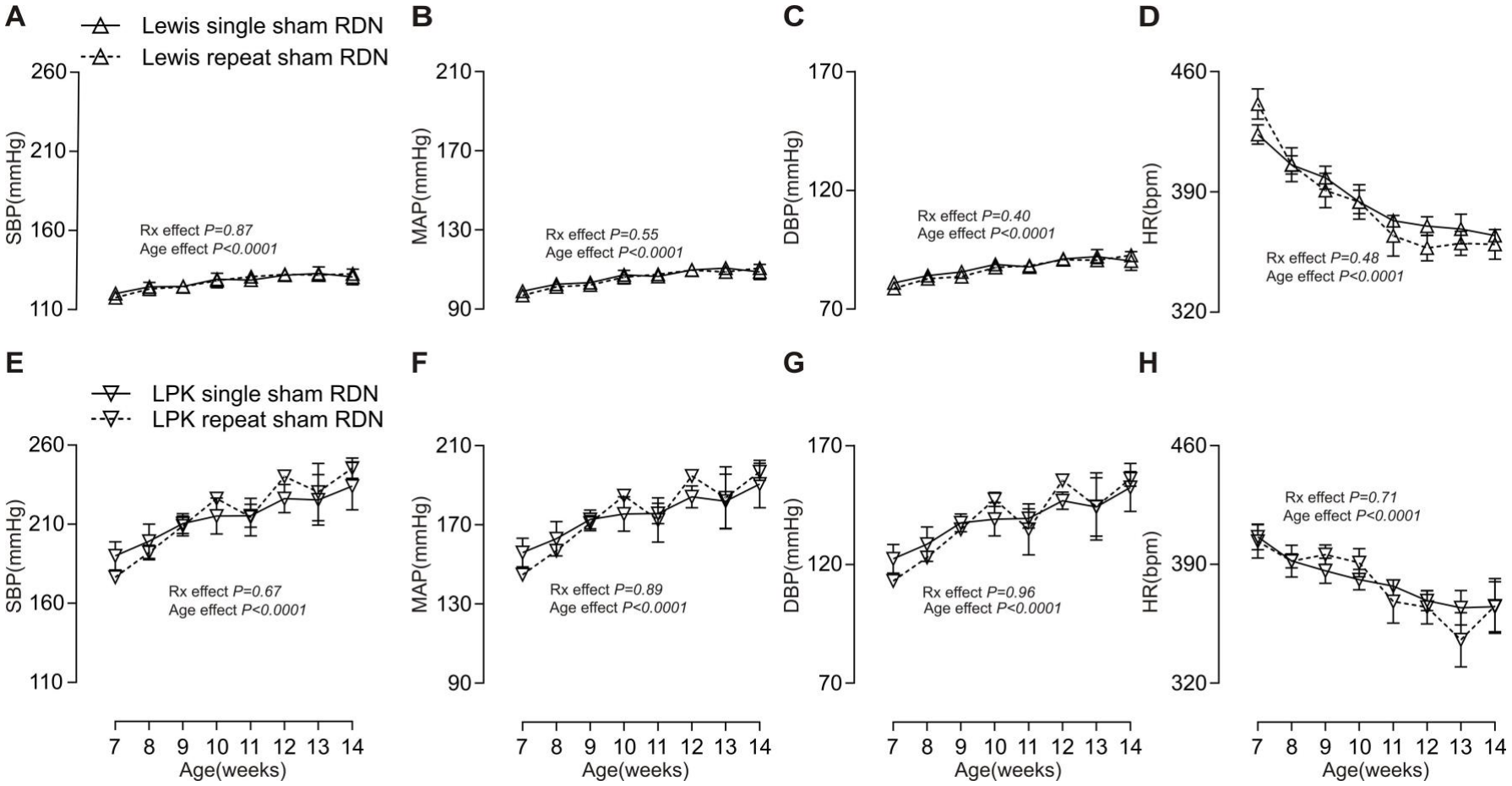


Figure 4.3 The effect of renal denervation (RDN) on systolic (SBP; A, E), mean (MAP; B, C), diastolic blood pressure (DBP;C, G) and heart rate (HR; D, H) in Lewis (A, B, C and D) and LPK (E, F, G and H) rats between 7-14 weeks old after sham, total and afferent RDN procedures.

N values at each week for Lewis sham = (10, 11, 10, 11, 8, 5, 5, 5); Lewis total = (14, 14, 14, 13, 5, 4, 4, 4); Lewis afferent = (9, 11, 11, 10, 8, 4, 4, 4) respectively and for LPK sham = (13, 11, 13, 11, 10, 9, 9, 9), LPK total = (14, 14, 12, 12, 6, 5, 4, 4) and LPK afferent = (9, 9, 8, 8, 6, 3, 3, 3), respectively. Rx, treatment effect. Data is expressed as mean ± SEM and analysed using two-way ANOVA and Bonferroni's *post hoc* analysis for the treatment effect (Rx: sham, total or afferent) and age effect (P value provided in each panel). * indicates $P < 0.05$ difference between sham and total RDN groups.

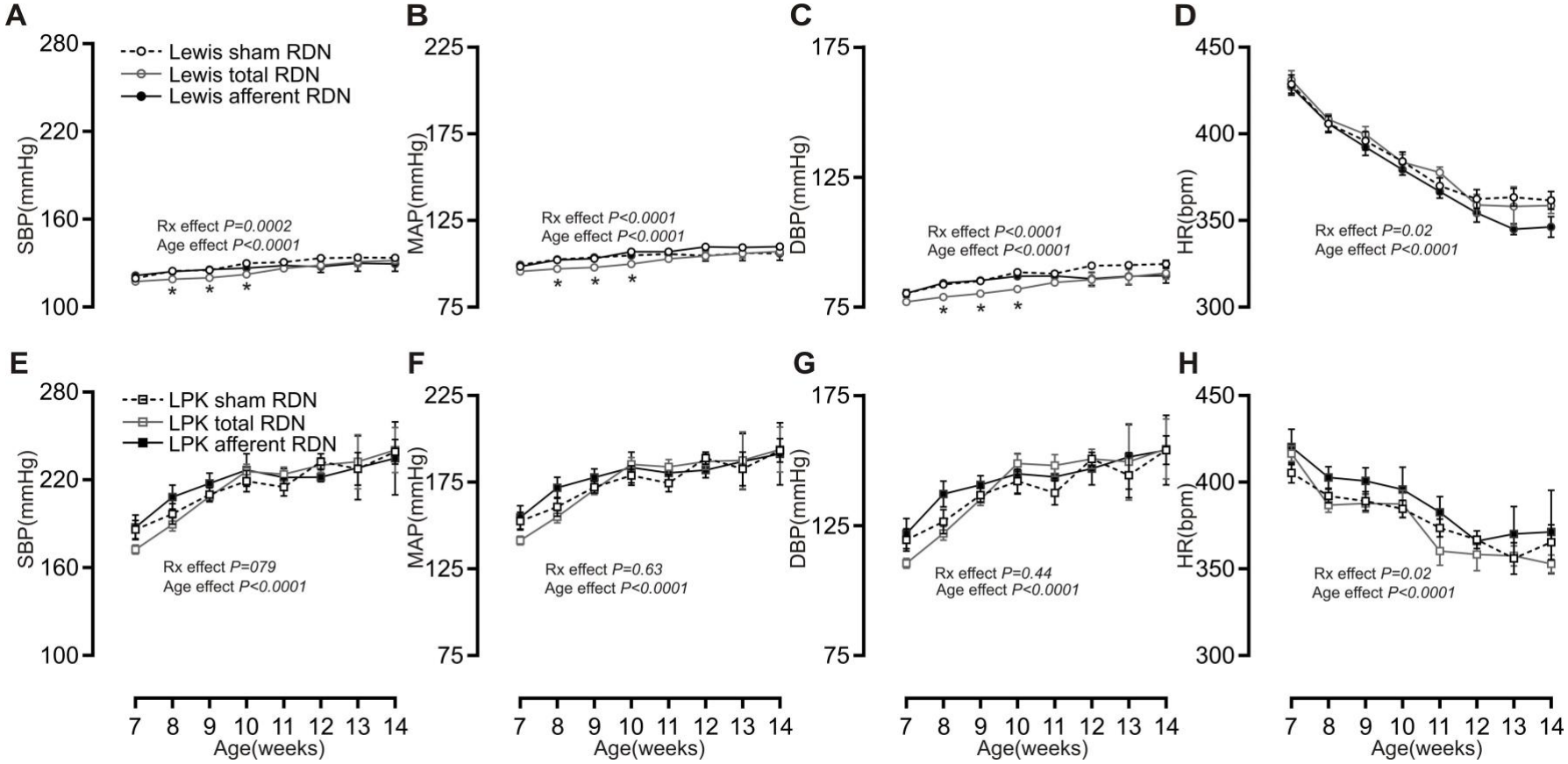


Figure 4.4 Circadian rhythms of systolic blood pressure (SBP, A), heart rate (HR, B), and locomotor activity (C) in 10 weeks old Lewis rats after sham renal denervation.

Data is expressed as mean ± SEM. Dark and white bars represent night and day periods, respectively. Fitted cosine curves overlay the data points.

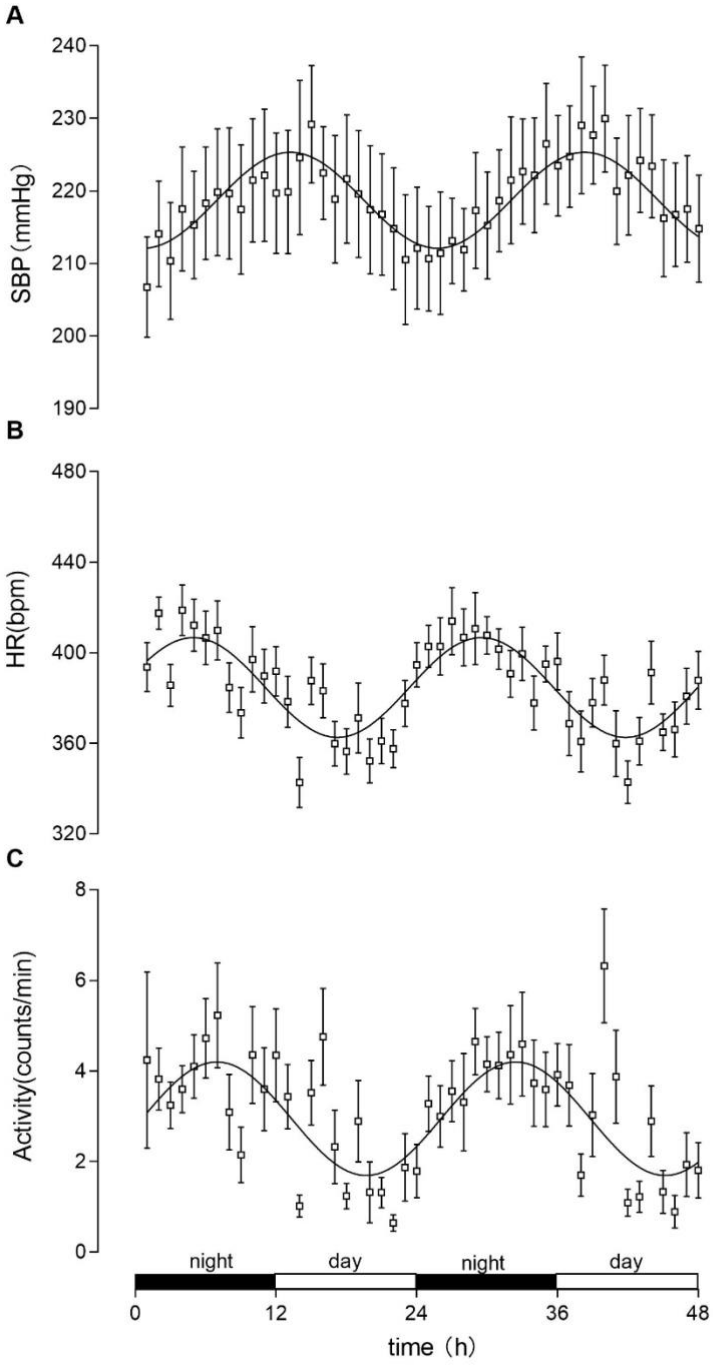


Figure 4.5 The effect of renal denervation (RDN) on plasma urea (A and C) and plasma creatinine (B and D) in 10-week old Lewis (A and B) and LPK (C and D).

Data is expressed as mean ± SEM and was analysed using one-way ANOVA. N=4-5, per treatment group per strain.

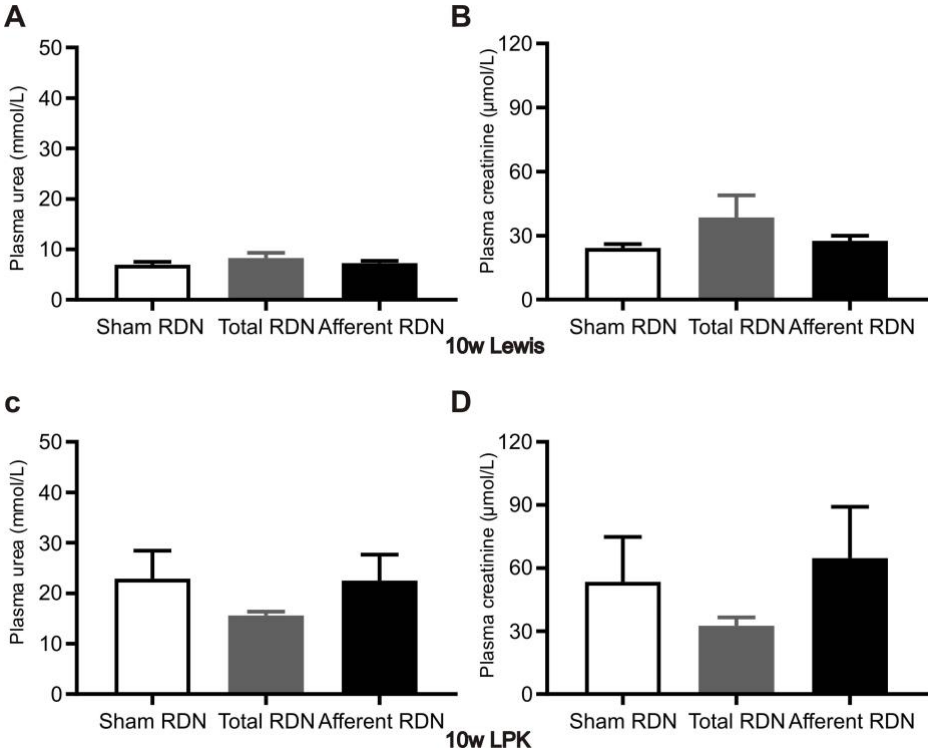


Figure 4.6 The effect of renal denervation (RDN) on plasma urea (A and C) and plasma creatinine (B and D) in 14-week old Lewis (A and B) and LPK (C and D).

Data is expressed as mean ± SEM and was analysed using one-way ANOVA. N=4-5, per treatment group per strain.

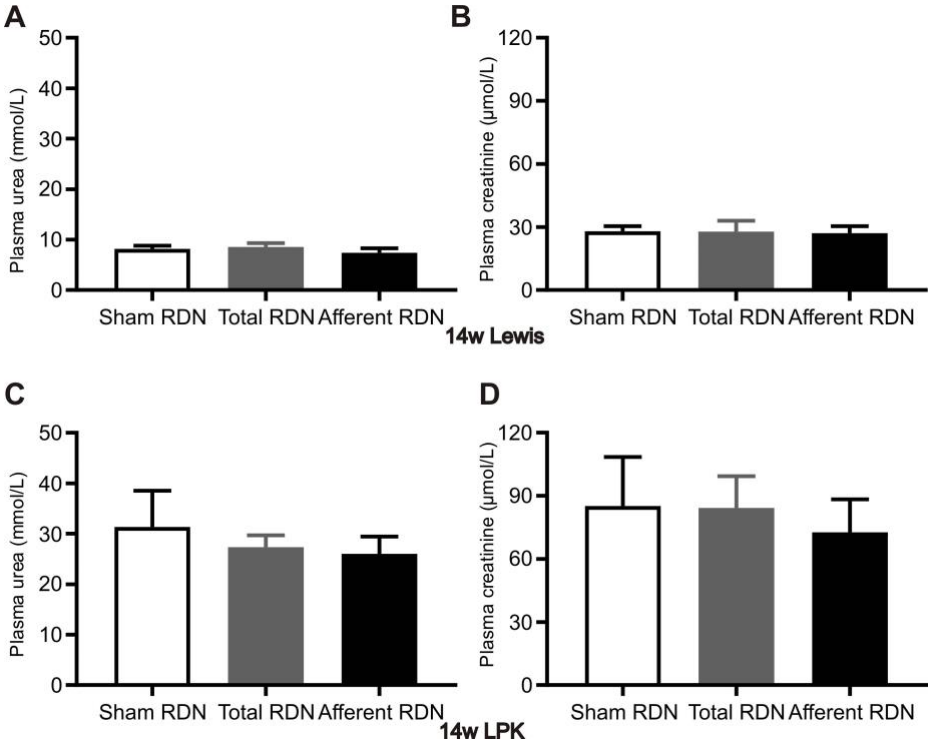


Figure 4.7 Representative images of TH (top row) and CGRP (bottom row) staining at 4 weeks post sham, total and afferent renal denervation (RDN) in the Lewis (A) and LPK (B).

Arrows indicate positive staining and scale bar in lower panel = 100µm for all images. Quantitative analysis is shown in panel C-F. Data is expressed as mean ± SEM and analysed using one-way ANOVA and Bonferroni's *post hoc* analysis. * indicates $P < 0.05$ difference vs. sham RDN. N=4-5, per treatment group per strain.

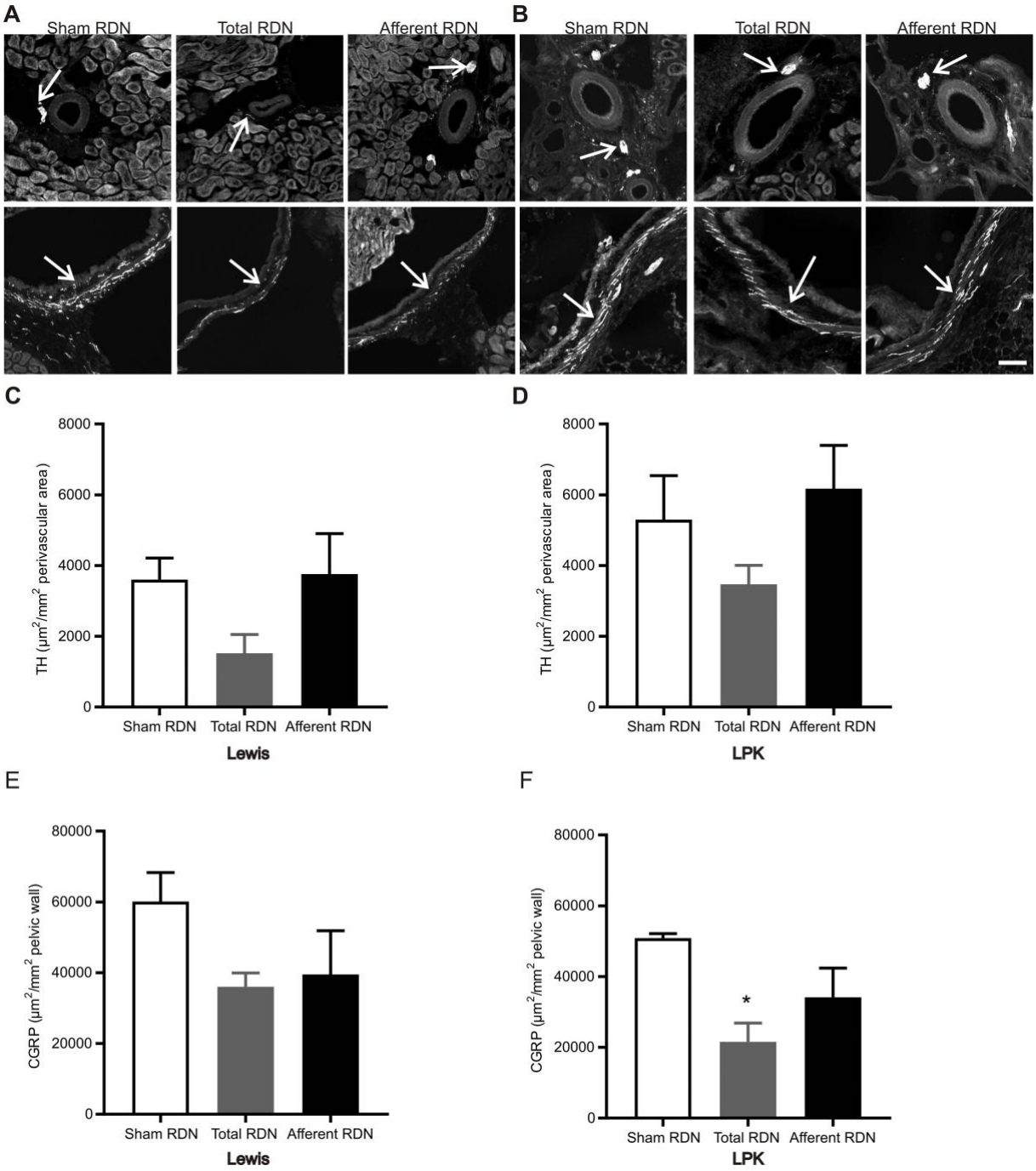


Figure 4.8 Representative images of TH (top row) and CGRP (bottom row) staining at 8 weeks post sham, total and afferent renal denervation (RDN) in the Lewis (A) and LPK (B).

Arrows indicate positive staining, scale bar in lower panel =100µm for all images. Quantitative analysis is shown in panel C-F and was analysed using one-way ANOVA. N=3-5, per treatment group per strain.

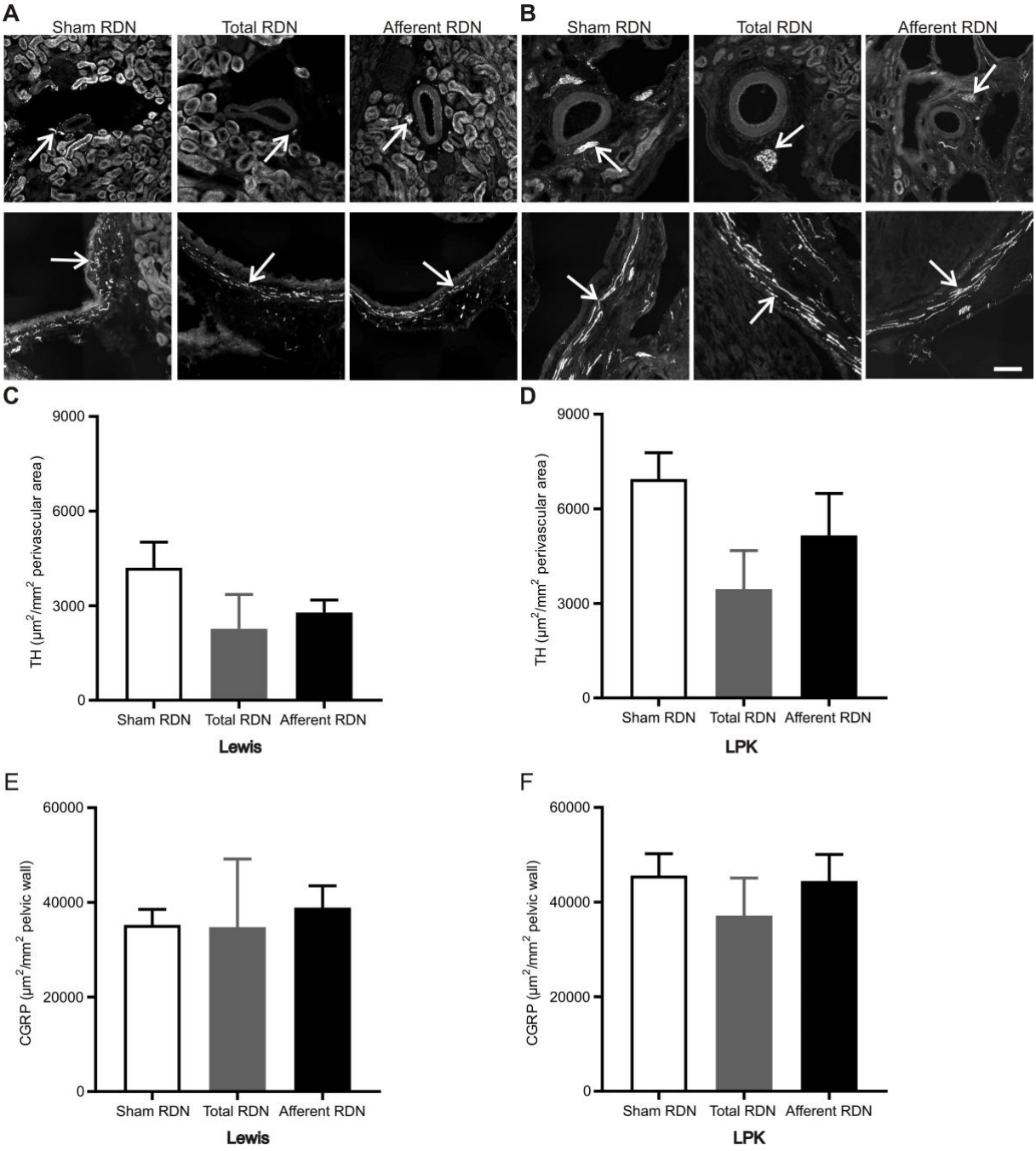


Figure 4.9 The effect of a single and repeat total renal denervation (RDN) on systolic (SBP; A, E), mean (MAP; B, F), diastolic blood pressure (DBP; C, G) and heart rate (HR; D, H) in Lewis (A, B, C and D) and LPK (E, F, G and H) rats between 11-14 weeks old.

Data is expressed as mean ± SEM and was analysed using two-way ANOVA and Bonferroni *post hoc* analysis. * indicates $P < 0.05$ vs. age-matched repeat total RDN. N values at each week for Lewis single total = (5, 4, 4, 4); Lewis repeat total= (5, 4, 5, 5) respectively and for LPK single total = (6, 5, 4, 4) and LPK repeat total = (6, 6, 6, 6), respectively.

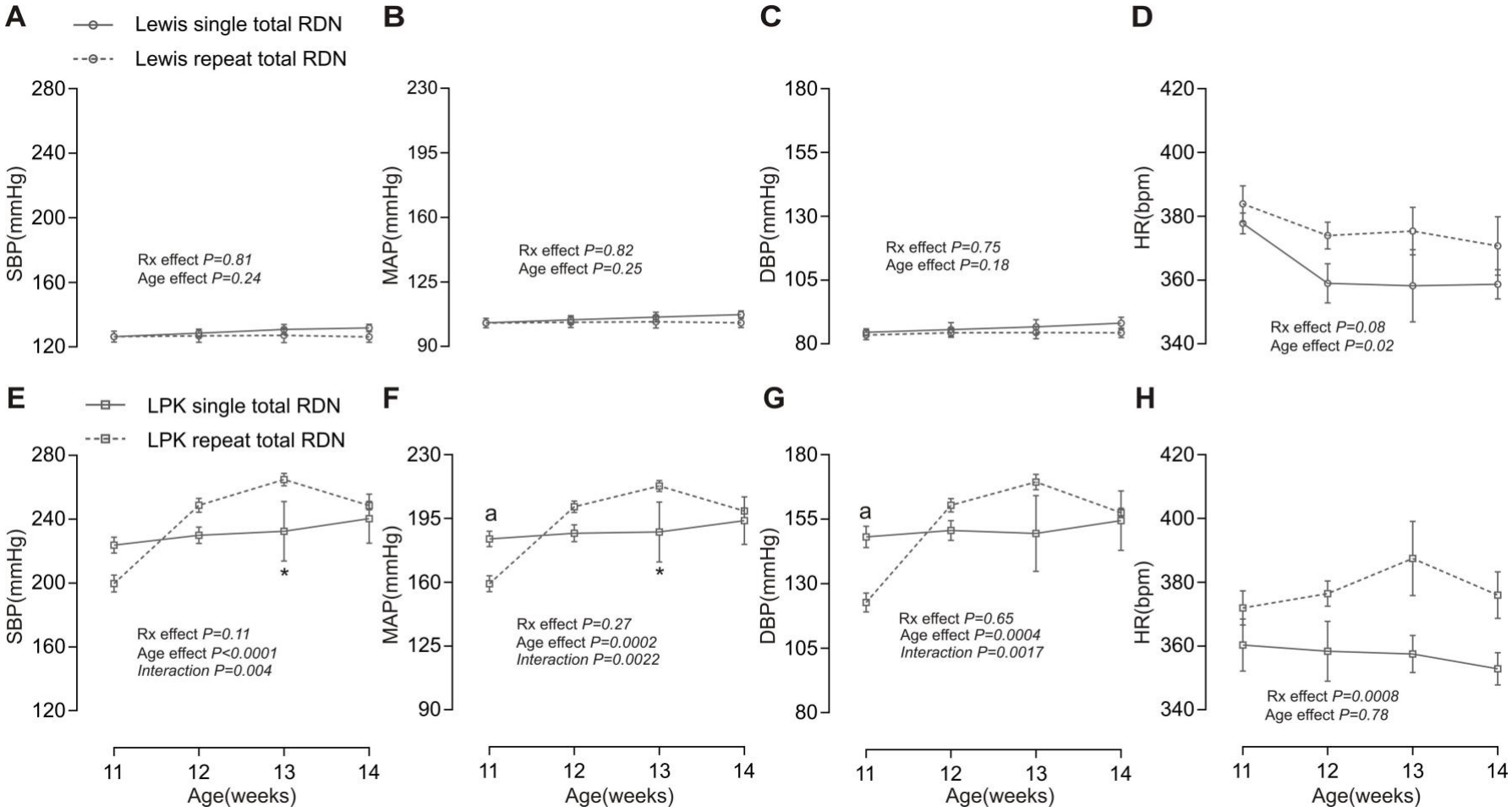


Figure S4.1 The effect of sex on systolic (SBP; A,E), mean (MAP; B, F), diastolic blood pressure (DBP;C, G) and heart rate (HR; D, H) in all Lewis (A, B, C and D) and LPK (E, F,G and H) rats between 7-14 weeks old .

N values at each week for Lewis male = (18, 19, 19, 18, 11, 7, 7, 7); Lewis female = (15, 17, 16, 16, 10, 6, 6, 6), LPK male = (20, 18, 18, 16, 10, 6, 6, 5) , LPK female = (16, 16, 15, 15, 12, 11, 11, 11) respectively. Data is expressed as mean ± SEM and analysed with two-way ANOVA for sex and age effect. Female Lewis had a significantly higher level of SBP but comparable level of MAP, DBP and HR relative to male Lewis. Male LPK had a significantly higher level of SBP, MAP and DBP but a lower level of HR compared to female LPK.

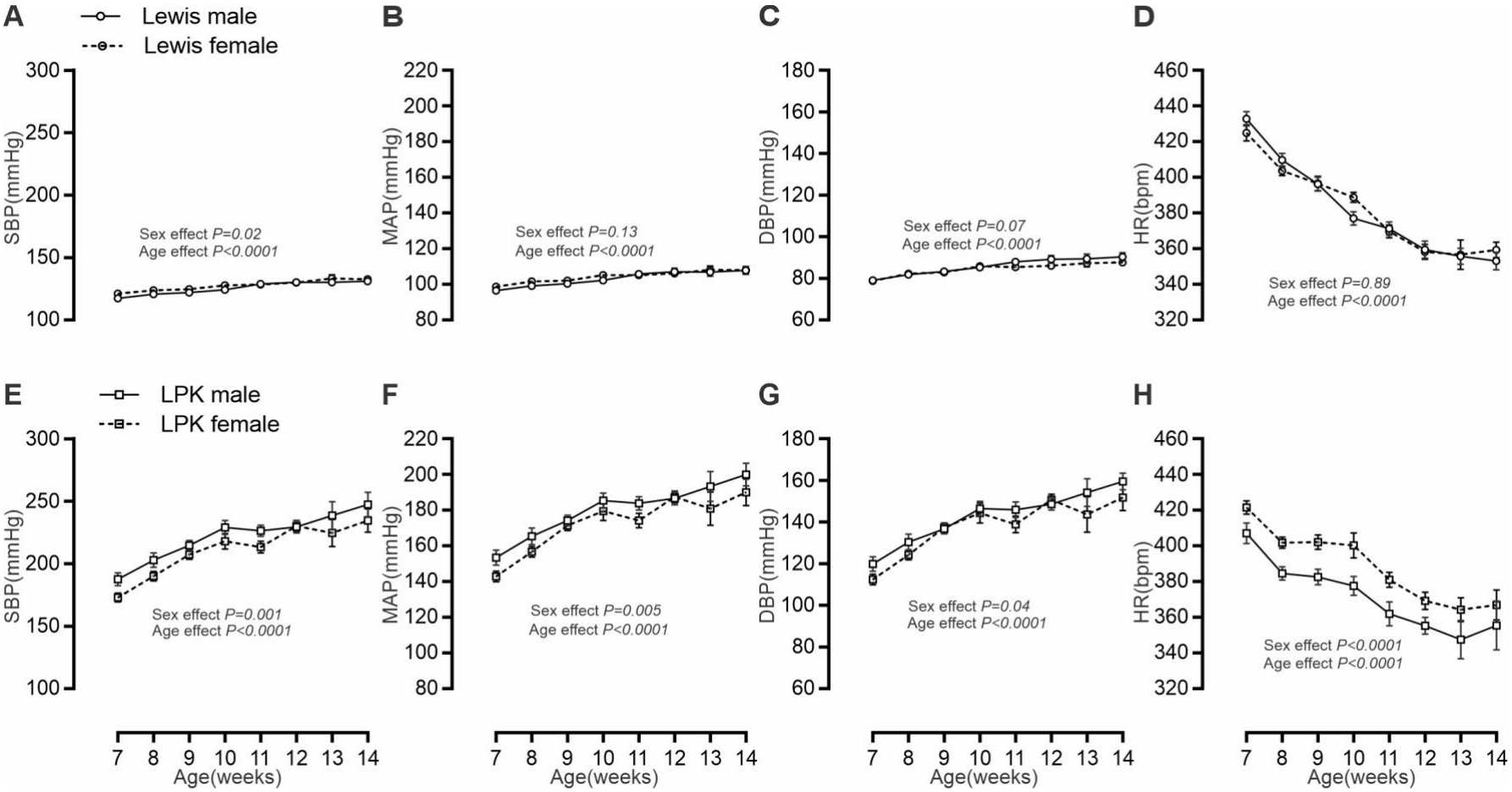


Figure S4.2 The effect of day and night period on systolic (SBP; A,E), mean (MAP; B, F), diastolic blood pressure (DBP;C, G) and heart rate (HR; D, H) in all Lewis (A, B, C and D) and LPK (E, F,G and H) rats between 7-14 weeks old.

N values at each week for Lewis = (33, 36, 35, 34, 21, 13, 13, 13); LPK = (36, 34, 33, 31, 22, 17, 16, 16) for both day and night values, respectively. D, day, N, night. Data is expressed as mean ± SEM and analysed with two-way ANOVA for day period and age effect. Lewis rats had a significantly higher level of SBP, MAP, DBP and HR during nighttime compared with daytime. LPK rats had a significantly higher level of HR during nighttime than daytime, but the level of SBP, MAP and DBP were comparable during both periods.

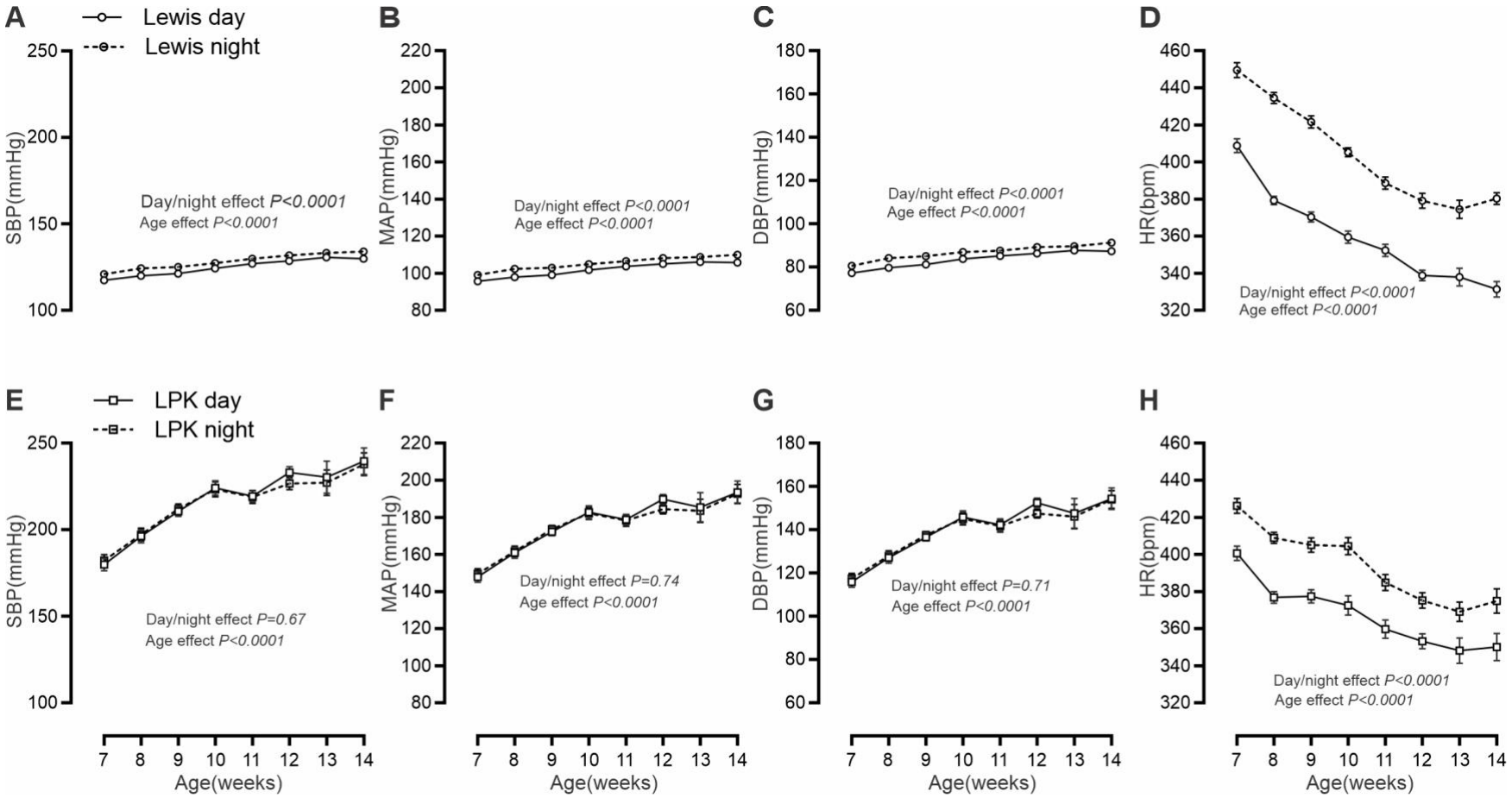


Figure S4.3 The effect of renal denervation (RDN) on heart rate in male (A and C) or female (B and D) Lewis (A and B) and LPK (C and D) rats between 7-14 weeks old.

N values at each week for Lewis male sham = (6, 6, 6, 6, 4, 3, 3, 3), male total = (7, 7, 7, 7, 3, 2, 2, 2);, male afferent = (5, 6, 6, 5, 4, 2, 2, 2), Lewis female sham = (4, 5, 4, 5, 4, 2, 2, 2) , female total = (7, 7, 7, 6, 2, 2, 2, 2), female afferent = (4, 5, 5, 5, 4, 2, 2, 2),, LPK male sham = (7, 5, 7, 5, 4, 3, 3, 3), male total =(8, 8, 7, 7, 4, 3, 2, 2), male afferent = (5, 5, 4, 4, 2, 0, 0, 0), LPK female sham= (6, 6, 6, 6, 6, 6, 6, 6) , female total= (6, 6, 5, 5, 2, 2, 2, 2), female afferent = (4, 4, 4, 4, 4, 3, 3, 3), respectively. M, male, F, female. Data is expressed as mean ± SEM and analysed with two-way ANOVA for treatment and age effect with Bonferroni's *post hoc* analysis. Texts indicate overall treatment effect and *post hoc* analysis between 7-14 weeks (Lewis male and female, and LPK female) or 7-11 weeks (LPK male, due to probe failure, no data was obtained for male LPK rats between 12-14 weeks).

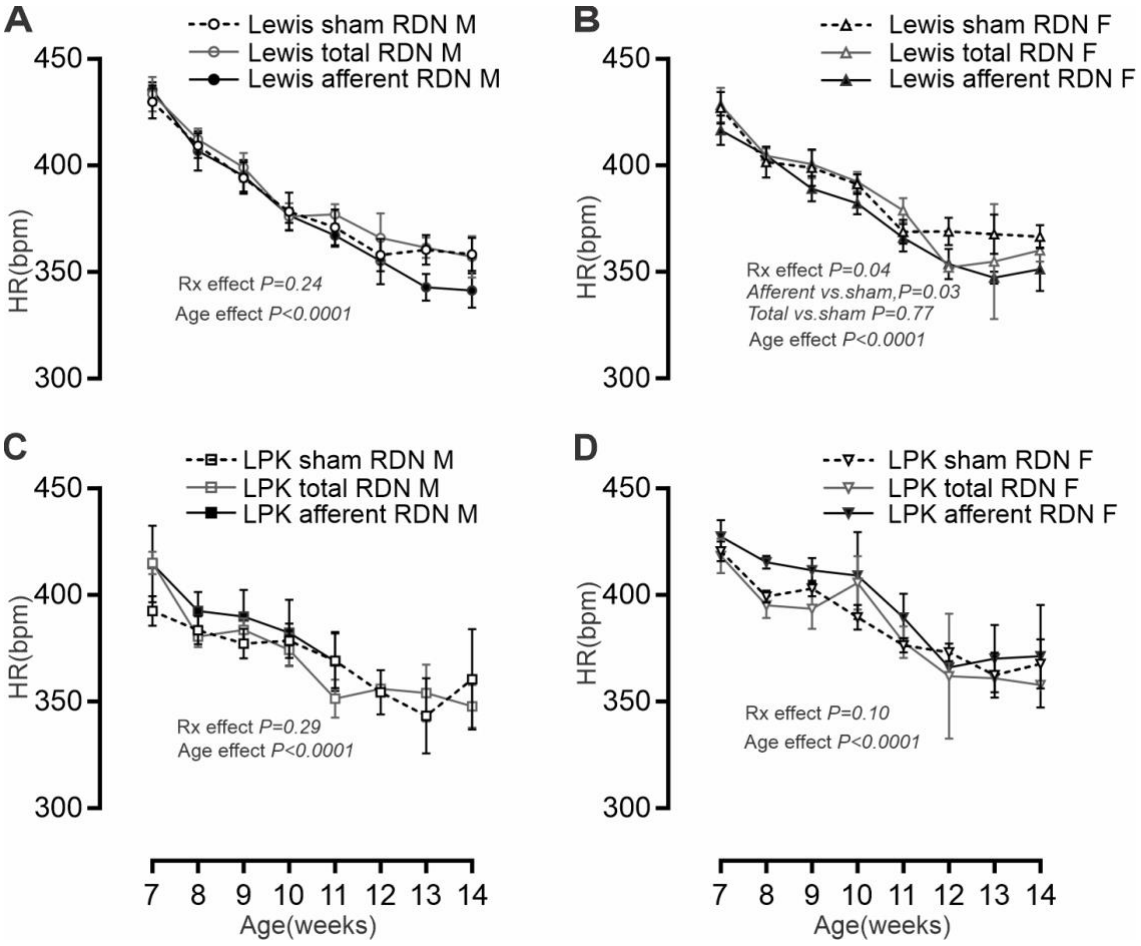


Figure S4.4 The effect of renal denervation (RDN) on daytime (A and C) or nighttime (B and D) heart rate in Lewis (A and B) and LPK (C and D) rats between 7-14 weeks old.

N values for day or night period at each week for Lewis sham = (10, 11, 10, 11, 8, 5, 5, 5); Lewis total = (14, 14, 14, 13, 5, 4, 4, 4); Lewis afferent = (9, 11, 11, 10, 8, 4, 4, 4) respectively and for LPK sham = (13, 11, 13, 11, 10, 9, 9, 9), LPK total = (14, 14, 12, 12, 6, 5, 4, 4) and LPK afferent = (9, 9, 8, 8, 6, 3, 3, 3) respectively. Data is expressed as mean ± SEM and analysed with two-way ANOVA with Bonferroni's *post hoc* analysis. Texts indicate overall treatment effect and *post hoc* analysis.

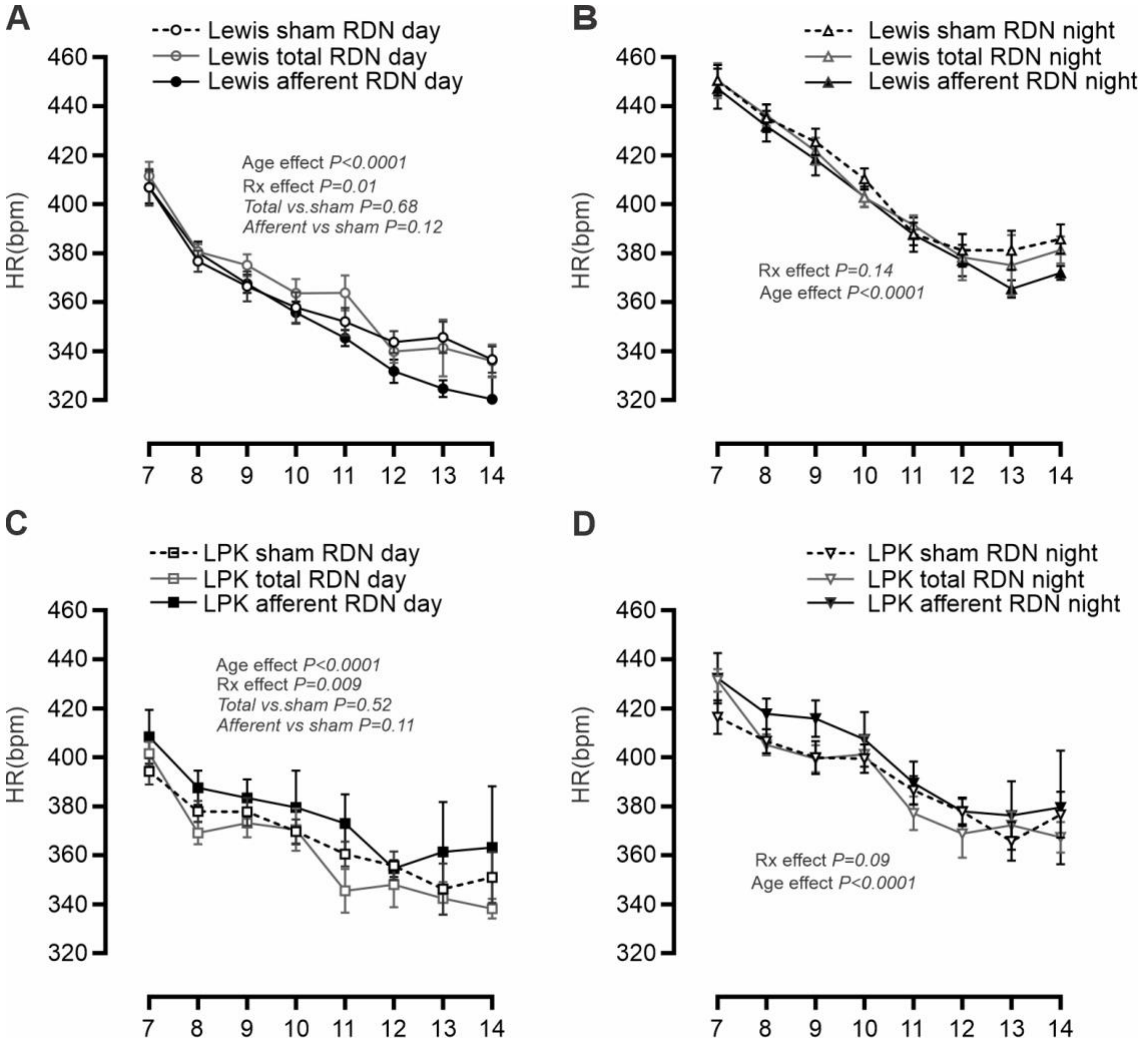
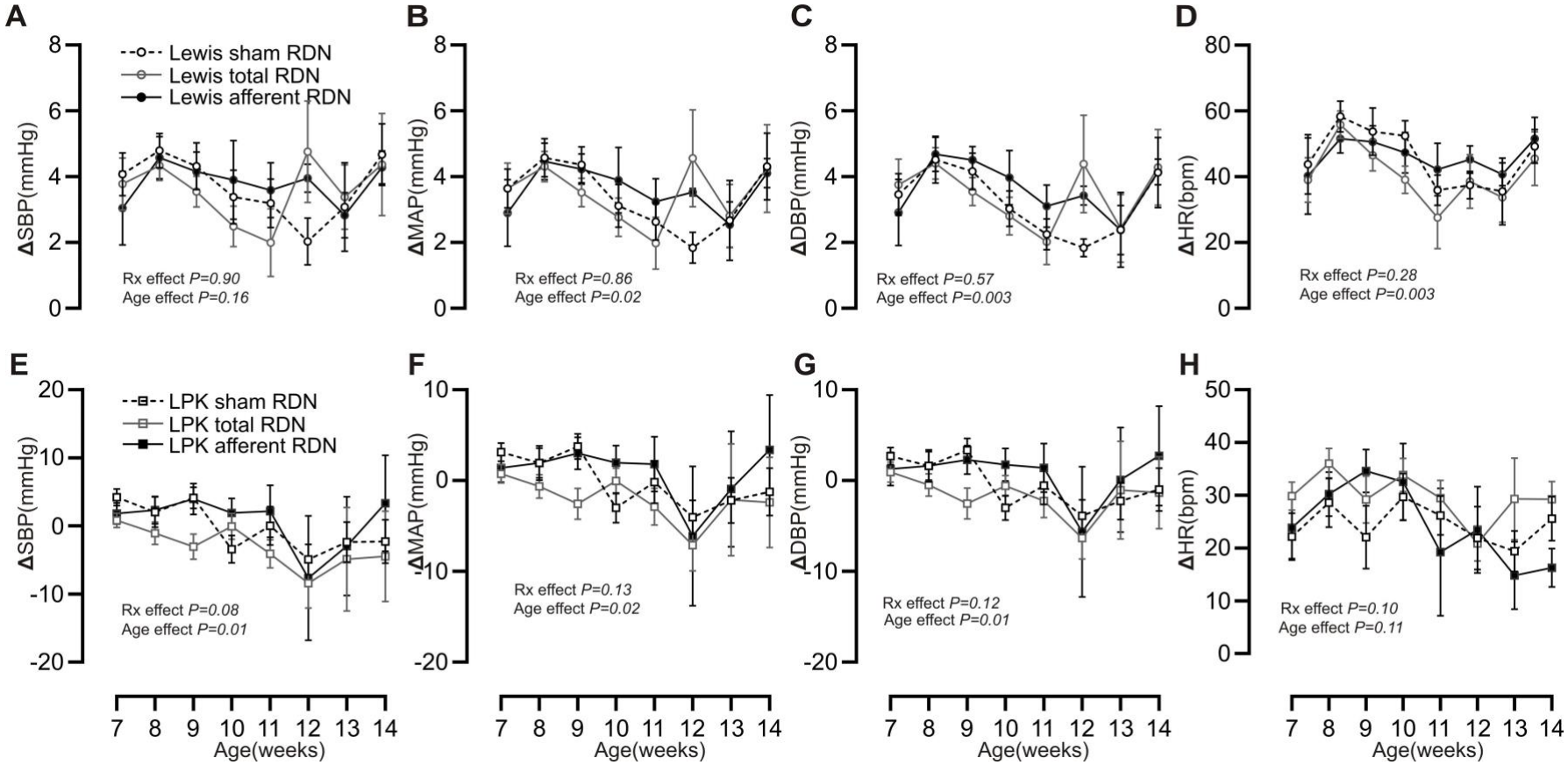


Figure S4.5, The effect of renal denervation (RDN) on the differences between nighttime and daytime SBP (ΔSBP, A and E), MAP (ΔMAP, B and F), DBP (ΔDBP, C and G) and HR (ΔHR, D and H) in Lewis (A to D) and LPK (E to H) rats between 7-14 weeks old after sham, total and afferent RDN procedures.

Rx, treatment effect. Data is expressed as mean ± SEM, analysed using Two-way ANOVA. Overall age and treatment effects are provided in each panel. The sham animals in this group include animals that underwent two sham procedures. N values at each week for Lewis sham = (10, 11, 10, 11, 8, 5, 5, 5); Lewis total = (14, 14, 14, 13, 5, 4, 4, 4); Lewis afferent = (9, 11, 11, 10, 8, 4, 4, 4) respectively and for LPK sham = (13, 11, 13, 11, 10, 9, 9, 9), LPK total = (14, 14, 12, 12, 6, 5, 4, 4) and LPK afferent = (9, 9, 8, 8, 6, 3, 3, 3), respectively.



Chapter 5 Effect of renal denervation in autonomic function in PKD

5.1 Abstract

Cardiovascular autonomic dysfunction is a serious complication in patients with chronic kidney disease (CKD) and is associated with adverse cardiac events and increased mortality. Catheter-based renal denervation (RDN), as a promising treatment for hypertension in CKD, appears to offer benefits in autonomic function beyond its blood pressure lowering effect. We investigated the impact of total and selective afferent RDN on autonomic function in Lewis and Lewis polycystic kidney disease rat (LPK) using spectral analysis (n = 64). In Lewis animals, total RDN significantly reduced systolic blood pressure variability (SBPV) (8 weeks overall level for sham 4.3 ± 0.1 vs. total 3.8 ± 0.1 vs. afferent RDN 4.1 ± 0.1 $mmHg^2$ P = 0.0002), which is indicative of a reduction in sympathetic vasomotor tone, but did not affect heart rate variability (HRV) or baroreceptor sensitivity (BRS) parameters. Afferent RDN increased the overall level of low frequency (LF) HRV (8 weeks overall level sham 3.0 ± 0.1 vs. total 2.5 ±0.1 vs. afferent RDN 3.8 ± 0.2 mec^2, P = 0.003) but did not affect SBPV BRS parameters. In the LPK animals, total RDN lowered the overall level of very low frequency (VLF) components of HRV (8 weeks overall level for sham 6.0 ± 0.2 vs. total 4.7 ± 0.2 vs. afferent 5.3 ± 0.3 mec^2 , P = 0.0025) but did not affect SBPV and BRS parameters. Afferent RDN increased the overall level of high frequency (HF) components of SBPV but did not affect HRV and BRS parameters in LPK. A repeat total RDN was associated with a lower HF HRV and lower HF BRS compared with a single procedure in LPK, suggesting a reduction in cardiac vagal tone. These results indicate that RDN overall has no beneficial effect on autonomic function in the LPK model of CKD.

5.2 Introduction

Cardiovascular autonomic dysfunction, manifesting as increased sympathetic nerve activity (SNA) and decreased parasympathetic and overall cardiac modulation, is a serious complication in chronic kidney disease (CKD) (Robinson and Carr 2002, Chou and Tsai 2016), and exposes the patients to higher risk of adverse cardiac events and increased mortality (Parati *et al.* 2012, Ryu *et al.* 2014, Almakramy *et al.* 2017). The underlying mechanisms for cardiovascular autonomic dysfunction are not clear, with evidence suggesting factors including, but not limited to, increased renin-angiotensin-aldosterone system (RAAS) activity (Grassi *et al.* 1988) and cardiovascular structural remodelling (Grassi *et al.* 1988, Salman *et al.* 2014, Salman 2015). Pharmacologic agents that directly or indirectly modulate autonomic function yield inconsistent impact. Specific and effective treatment options are needed to limit the development and progress of autonomic dysfunction.

Catheter-based renal denervation (RDN) has emerged as a promising treatment for hypertension in CKD. Beyond its blood pressure lowering effect, RDN appears to offer other benefits including improvement in autonomic function in hypertensive patients with Hart et al. (Hart *et al.* 2013) demonstrating improved cardiac spontaneous baroreceptor sensitivity (BRS) 6 months after RDN. Furthermore, this improvement in BRS was not associated with a reduction in blood pressure, suggesting that RDN might provide this beneficial effect independent of changes in blood pressure. Grassi et al. (Grassi *et al.* 2015) also documented an improvement in baroreflex control in muscle SNA in patients with resistant hypertension at both 3 and 6 months RDN. In experimental studies, RDN was shown to increase heart rate variability (HRV) without affecting BRS in diabetic-hypertensive rats (Dias *et al.* 2011), reduce systolic blood pressure variability (SBPV) in spontaneously hypertensive rats (SHR) (Gao *et al.* 2016), and improve baroreflex function in both the cisplatin-induced renal injury (Khan *et al.* 2014) and 5/6 nephrectomy CKD models (Chen *et al.* 2016). In this regard, RDN may potentially help treat cardiovascular autonomic dysfunction in CKD.

Several techniques are available to assess sympathetic and parasympathetic nervous system activity, and baroreceptor sensitivity (Robinson and Carr 2002). Among them, computer-based power spectral analysis of spontaneous oscillations in the blood pressure to determine cardiovascular and respiratory control of blood pressure and of pulse intervals to determine the cardiac sympathetic and vagal control as well as baroreflex control of heart rate (HR) is one commonly used technique. Based on a stable blood pressure recording and appropriate software, this technique can assess the number, frequency, and amplitude of the oscillatory components (frequency domain analysis) (Robinson and Carr 2002). As a result, SBPV and HRV can be expressed as spectral powers at total, very low, low and high-frequency bands (Robinson and Carr 2002). Cardiac BRS can be estimated by calculation of the square root of the ratio of powers of pulse interval to SBP, the α-index (Robinson and Carr 2002). Using this technique, an increase in SBPV and a decrease in HRV and BRS was observed in the Lewis polycystic kidney disease (LPK) model of CKD, indicating an impaired autonomic function similar to CKD patients (Hildreth *et al.* 2013). Given the benefits in the autonomic function that have been described after RDN independent of a reduction in blood pressure (Hart *et al.* 2013), we hypothesised that RDN could provide an improvement in impaired in autonomic function in the LPK even in the absence of a blood pressure effect. Therefore, the purpose of the present study was to determine the effect of total and afferent RDN on cardiovascular autonomic function in the LPK and their control strain, the Lewis rat. We compared the levels of HRV, SBPV and BRS determined by spectral analysis in Lewis and LPK after sham, total or afferent RDN over a follow-up period of 8 weeks. In Chapter 4, we noted a transient increase in arterial pressure in LPK after a repeat total RDN procedure. Therefore, we also investigated the impact of a repeat RDN procedure on the level of HRV, SBPV and BRS in both strains.

5.3 Method

5.3.1 Experimental design

The protocol details and experimental design for this study (Study 2 and Study 3) are provided in Chapter 2 (Section 2.3, Figure 2.1). Briefly, all animals assigned to Study 2 (n = 36 Lewis, n = 36 LPK) received one of three surgical protocols: (i) total RDN; (ii) afferent RDN; or (iii) sham RDN, as well as telemetry probe implantation as described in Chapter 2, Section 2.2. Cohort 1 and 2 animals were monitored to and euthanised at age 10 weeks (n = 14 Lewis, n = 14 LPK) or 14 weeks (n = 22 Lewis, n = 22 LPK), respectively. In study 3, all animals (n = 8 Lewis, n = 10 LPK) underwent two procedures: being a first and a repeat sham or total RDN at 6 and 10 weeks old, respectively. These animals were also instrumented with telemetry probes at 6 weeks old. The animals were then euthanised at 14 weeks old. In all animals, weekly telemetry-based blood pressure recordings were collected as detailed in Chapter 4 (Section 4.3.2).

5.3.2 Autonomic function analysis

The determination of autonomic function parameters is based on the arterial pressure waveforms collected as described in Chapter 4. All arterial pressure (AP) waveforms acquired during the 48-hour recording were imported into Spike 2 (version 7.02; Cambridge Electronic Designs, Cambridge, UK) and the segments between 11 am and 1 pm (day) and 11 pm and 1 am (night) during the 48 hrs period were chosen for subsequent analysis. The pulse interval (PI) was derived from the AP waveform and uniformly resampled at 10 Hz. Where possible, an 80 s period in each of the AP waveforms recorded was identified where PI was stable, and no ectopic beats were present. Power spectrums for each 80 s period were generated using a fast Fourier transformation (size 256, Hanning window, final frequency resolution of 0.04 Hz), exported as text into Microsoft Excel and total power (TP; 0–3 Hz), very low frequency (VLF; 0.04–0.2 Hz), LF (0.25–0.75 Hz), HF (1–3 Hz) power calculated for each 80s period. The LF/HF ratio was then determined. From the same segments used to estimate

HRV, systolic blood pressure (SBP) was derived, uniformly resampled at 10 Hz and a power spectrum generated (size 256, Hanning window, final frequency resolution of 0.04 Hz) with TP (0–3 Hz), VLF (0.04–0.2 Hz), LF (0.25–0.75 Hz) and HF (1–3 Hz) SBPV power calculated. BRS was estimated on the same 80 s segments of AP as HRV and SBPV, using the square root of the ratio of the power spectral density of LF (αLF) and HF (αHF) HRV and SBPV power, using the following equation (Hildreth *et al.* 2013):

$$\alpha LF=\left[\frac{POWER_{LFHRV}}{POWER_{LFsbpv}}\right]^{1/2} \text{ or } \alpha HF=\left[\frac{POWER_{HFHRV}}{POWER_{HFsbpv}}\right]^{1/2}$$

5.3.3 Statistical analysis

Data analysis was performed using GraphPad Prism (v7.02, GraphPad Software, La Jolla, USA). All results were expressed as mean ± SEM. For all autonomic function parameters, a two-way ANOVA with Bonferroni's *post hoc* analysis (if indicated) was used to identify if these parameters were different between groups.

5.4 Result

5.4.1 Effect of single total or afferent RDN procedure on autonomic function parameters

Consistent with the analysis of cardiovascular parameters in Chapter 4, no significant difference was noted in any parameter between sham animals that had one surgery (study 2) or two surgeries (study 3) within each strain (All $P > 0.05$ for treatment effect). Therefore, the data from sham animals in studies 2 and 3 were combined and used as the control sham group for all subsequent data analysis.

Heart rate variability: The effect of total or afferent RDN afferent on HRV are presented in Figure 5.1. In Lewis animals, while TP, VLF and HF power HRV were comparable across all treatment groups, LF power was greater in the afferent RDN group compared with sham controls ($P = 0.05$, Figure 5.1C). In contrast, in the LPK group, TP, HF and LF power HRV were unaffected by treatment whereas VLF was significantly lower in total RDN group compared with sham controls ($P = 0.002$, Figure 5.1F). No age effect was noted in any HRV parameters in LPKs whereas an overall age-related increase was noted in HF HRV in Lewis ($P = 0.03$, Figure 5.1).

LF/HF ratio: In the Lewis animals, the LF/HF ratio was significantly lower in total RDN group compared with sham controls ($P = 0.002$, Figure 5.2A). In LPKs, the LF/HF ratio was comparable across all treatment groups (Figure 5.2B). No age effect was noted in either Lewis or LPK strain.

Systolic blood pressure variability: The effect of total or afferent RDN on SBPV is presented in Figure 5.3. In Lewis animals, TP SBPV differed between groups with TP lower in the total RDN group compared with sham controls, being most obvious at 9–10 weeks of age ($P < 0.0001$, Figure 5.3A). VLF SBPV also differed, being lower in the afferent RDN group compared with sham controls ($P = 0.04$, Figure 5.3B). Total RDN also reduced LF SBPV

compared with sham controls, being most obvious at 8–10 weeks of age ($P < 0.0001$, Figure 5.3C). No overall treatment effect was noted for HF power (Figure 5.3D). In contrast, in the LPK animals, while TP, VLF and LF power were comparable between treatment groups, HF power was significantly higher in the afferent RDN groups compared with sham controls ($P = 0.009$, Figure 5.3H). No overall age effect in any SBPV parameters was noted in the Lewis whereas an overall age-related increase was noted in total, VLF and HF SBPV in LPK rats (all $P < 0.05$), consistent with previous reports (Hildreth *et al.* 2013).

Baroreflex sensitivity: The effect of total or afferent RDN on BRS is presented in Figure 5.4. When comparing the BRS between treatment groups, two-way ANOVA did not indicate any treatment effect in either LF or HF BRS in either strain (all $P > 0.05$, Figure 5.4). An age-related increase was noted in HF BRS ($P = 0.05$) while an age-related decrease in HF BRS was observed in the LPK, consistent with the known development of autonomic dysfunction in the LPK (Hildreth *et al.* 2013).

5.4.2 Effect of single vs repeat RDN on autonomic function parameters

Autonomic function parameters in animals aged 11-14 weeks that received sham procedure or a single total RDN at 6 weeks of age (data presented above) were compared with those of animals that received a repeat total RDN at 10 weeks of age (Figure 5.5, Figure 5.6, Figure 5.7).

Examining HRV, two-way ANOVA did not identify any treatment effect in HRV parameters in Lewis (all $P > 0.05$, Figure 5.5A-D). However, in LPK animals, an overall treatment effect was noted in all parameters, with all HRV parameters significantly lower in repeat RDN group compared with sham RDN group (all $P < 0.05$, Figure 5.5E-H), and HF power also being significantly than that of the single total RDN group ($P < 0.05$, Figure 5.5H). No age effect was noted for any HRV parameters in Lewis or LPK animals.

Examining SBPV, in the Lewis, the overall level of TP and LF SBPV was significantly lower in repeat total RDN group compared with sham controls, and VLF SBPV significantly lower in the repeat total RDN group compared with single RDN group (all $P < 0.05$, Figure 5.6A-C). The overall level of HF SBPV in Lewis was comparable between treatment groups. In LPK, no significant difference was noted for any SBPV parameters between treatment groups (Figure 5.6E-F). No age effect was noted in any SBPV parameters in Lewis while in the LPK, an age-related increase was noted in HF SBPV ($P = 0.05$).

With regard to BRS, in Lewis animals, the overall level of LF BRS was significantly higher in repeat total RDN group compared with single sham or total RDN group (both $P < 0.05$, Figure 5.7A) while there was no significant difference in the overall level of HF BRS between groups (Figure 5.7B). In contrast, In LPK animals, while the overall level of LF BRS was comparable between groups, HF BRS differed between groups, with the level significantly lower in animals after repeat total RDN procedure compared with sham controls (Figure 5.7D).

5.5 Discussion

In this study, we investigated the impact of RDN on HRV, SBPV and BRS as measures of autonomic function in both normotensive Lewis animals and the LPK model of CKD. Our major findings are: (1) in Lewis, afferent RDN increased the overall level of LF HRV whereas total RDNA reduced TP and LF SBPV; (2) In LPK, afferent RDN increased HF SBPV, while total RDN lowered the overall level of VLF HRV. (3) A repeat total RDN had no impact on HRV but produced a decrease in SBPV and an increase in BRS in the Lewis. In contrast, in the LPK a repeat total RDN produced a decrease in HF HRV and BRS.

5.5.1 Effect of RDN on HRV in Lewis and LPK

HRV is used as a measure of the balance of the activities of the sympathetic (SNS), parasympathetic nervous system (PNS) and hormonal system over the modulation of the heartbeat interval (Task force of the ESC and NASPE 1996). In general, it is proposed that SNS modulates LF oscillations, PNS affects both LF and HF oscillations, and hormonal factors modulate VLF oscillations of HR (Grassi *et al.* 2015). Changes in LF power need to be interpreted with caution given that LF HRV is considered as an index of sympathetic, parasympathetic and baroreflex control of HR (Task force of the ESC and NASPE 1996, Stauss 2007). In the Lewis, afferent RDN increased the level of LF HRV relative to sham controls, which may suggest a predominance of cardiac sympathetic inputs over vagal inputs. In support of this, LF power increases following position changes which activates arterial baroreceptors, mental stress and exercise, which can be ameliorated with beta-adrenoceptor antagonists (Pomeranz *et al.* 1985, Girard *et al.* 1995, Grillot *et al.* 1995, Kim *et al.* 2018). However, given that atropine could abolishes LF power, additional evidence of cardiac sympathetic and vagal tone following RDN is needed to make a definite conclusion.

In hypertension, typical changes in HRV are a reduced total and LF HRV and increased LF/ HF ratio, suggestive of a sympathovagal imbalance that results in additional cardiovascular

risks such as premature cardiac death for patients (Virtanen *et al.* 2003). A beneficial effect of any intervention would be reflected by an increase in TP and LF HRV and reduction in LF/HF. For example, Tsioufi et al. (Tsioufis *et al.* 2014) demonstrated an increase in all frequency components of HRV and reduction in LF/HF at 1 and 6 months post-RDN in patients with resistant hypertension. Nevertheless, in our model, neither single total or afferent RDN produced a beneficial effect. Repeating the total RDN in the LPK reduced HF HRV compared to animals that only had single denervation, suggesting a reduction in cardiac vagal tone after the repeat procedure (Hildreth *et al.* 2013). These findings indicate that RDN did not improve autonomic sympathovagal balance in the LPK. In comparison, in diabetic-hypertensive rats RDN was shown to compensate the sympathovagal imbalance by increasing total and LF HRV (Dias *et al.* 2011). Notably, the arterial pressure was not impacted by RDN procedure either, which further supported the notion that RDN might improve autonomic function independent of blood pressure changes. In patients with resistant hypertension, Peters et al. (Peters *et al.* 2017) reported that 6 months after RDN, HRV parameters were not significantly different compared to baseline or that in sham groups, as did Verloop et al. (Verloop *et al.* 2015) who showed no significant impact on HRV following RDN in patients with metabolic syndrome and mild hypertension. This is despite evidence that RDN can result in lower sympathetic autonomic balance in hypertensive patients (Tsioufis *et al.* 2014, Hoogerwaard *et al.* 2019). The discrepancy between our study and previous animal studies may be explained by disease aetiology or species difference.

5.5.2 Effect of RDN on SBPV in Lewis and LPK

Systolic blood pressure variability describes the oscillation of blood pressure in the time interval and reflects the overall influence of neural (sympathetic tone), vascular and hormonal factors over blood pressure (Stauss 2007). It is suggested that in rats the LF component of SBPV is contributed to by sympathetic modulation, that VLF components are affected by vascular function, endothelial-derived nitric oxide and RAAS, and HF components are

affected by respiratory modulation and cardiac output (Stauss 2007). In humans, while the parameters that are used to define LF, VLF and HF are different, it is proposed that they are affected by similar factors as relative to rodents. A healthy SBPV is reflected by moderate oscillations in blood pressure, while in disease, for example, in the SHR rodent model which mimics essential human hypertension, SBPV is typically increased (Zamo *et al.* 2010). A reduction in LF SBPV indicates a decrease in the sympathetic branch of the autonomic cardiovascular control (Stauss 2007) and in our normotensive Lewis animals, this was observed after total RDN compared with a sham procedure, and this was decreased further following a repeat total RDN. Importantly, in these animals, we also see a decrease in blood pressure (Chapter 4). Reduced LF SBPV has been described after RDN in spontaneously hypertensive rats (SHR), similarly correlating with a reduction in blood pressure, but the impact of RDN on LF SBPV in normotensive Wistar-Kyoto (WKY) rats was not reported (Gao *et al.* 2016). The LF SBPV reduction alongside a reduction in BP therefore suggests the reduced vasomotor tone after RDN may be responsible for the BP reduction. Afferent RDN did not alter LF SBPV in Lewis, which would support the hypothesis that the reduced sympathetic vasomotor tone after RDN was due to the removal of the sympathetic efferents, not the renal afferents. In contrast, neither total or afferent RDN had an impact on the LF component of SBPV in LPK rats, indicating no significant impact on sympathetic vasomotor tone.

Human studies that investigate the impact of RDN on blood pressure variability determined specifically by frequency-domain spectral analysis are lacking. The effect of RDN on cardiac sympathetic activity has been assessed through the uptake and washout of I-123-metaiodobenzylguanidine (MIBG), which will be actively transported into noradrenaline granules of sympathetic nerve terminals by the noradrenaline transporter. Using this method, Donazzan et al. (Donazzan *et al.* 2016) showed reduced cardiac sympathetic nerve activity 9 months after RDN in resistant hypertension. Reduced renal sympathetic

activity, measured by renal noradrenaline spillover, was also seen after RDN in patients (Krum *et al.* 2009). In contrast, van Brussel et al. (van Brussel *et al.* 2016) demonstrated no impact on the cardiac sympathetic activity at 6 weeks post-RDN when measuring MIBG uptake. While we did not undertake direct measurements of cardiac or renal sympathetic activity in this study, a reduction in both may have contributed to the reduced sympathetic vasomotor tone after total RDN in Lewis rats as measured in the LF component of SPBV.

5.5.3 Effect of RDN on BRS in Lewis and LPK

BRS reflects the effectiveness of baroreceptor activation to change heart rate in response to induced or autogenic changes in arterial pressure (Swenne 2013). A higher BRS suggests a greater ability for the autonomic nervous system to buffer blood pressure changes while a reduction in BRS suggests an impairment of the autonomic nervous system. Both LF and HF BRS correlate with BRS estimated using bolus injections of phenylephrine and the sequence method (Laude *et al.* 2004). However, the coupling between pulse interval and SBP in the LF band is highly dependent upon baroreflex inputs whereas the coupling in the HF band may be not all related to the baroreflex (Mancia *et al.* 1999, Parati *et al.* 2000). We did not observe any difference in LF BRS after single RDN between treatment groups in either stain, suggestive of a lack of impact on baroreflex function. A repeat total RDN procedure in the LPK produced a reduction in the HF BRS, but whether it is suggestive of a reduction in baroreflex function needs to be further validated using administration of phenylephrine and/or sodium nitroprusside.

The variable impact of RDN on baroreflex control has been reported in a few studies (Khan *et al.* 2014, Booth *et al.* 2015, Lincevicius *et al.* 2017). For example, RDN did not cause any significant changes in HR baroreflex curve parameters assessed by infusion of phenylephrine and sodium nitroprusside compared with sham controls in a heart failure sheep model (Booth *et al.* 2015), while Lincevicius et al. (Lincevicius *et al.* 2017) reported that total RDN of the

clipped kidney in two-kidney-one-clip rats improved the arterial BRS upon infusion of sodium nitroprusside 10 days post the procedure. Khan et al. (Khan *et al.* 2014) found that RDN did not improve impaired heart rate BRS, but did improve impaired renal SNA BRS in a cisplatin-induced renal injury model rat 1-week post-denervation. In 5/6 nephrectomy rats, another model of CKD, total RDN partially recovered baroreflex control of HR in response to phenylephrine administration 8 weeks post-denervation (Chen *et al.* 2016). In studies assessing spontaneous BRS, Hart et al. (Hart *et al.* 2013) showed that in SHR rats total RDN caused a significant albeit small increase in spontaneous cardiac BRS within 24 hrs of the RDN surgery. Hart et al. (Hart *et al.* 2013) also observed improved spontaneous BRS in patients with resistant hypertension 6 months post-RDN procedure. It is known that the NTS and RVLM receive inputs from renal afferent nerves and are directly related to the baroreflex control of heart rate and sympathetic outflow (Solanoflores *et al.* 1997, Guyenet 2006, Kumagai *et al.* 2012). In this regard, the observed effect in baroreflex function after RDN from studies mentioned above may be mediated by removal of renal afferent activity. It is also likely that the denervation mediated changes in baroreflex function is disease-dependent as renal denervation did cause improvement in cisplatin-induced renal injury and 5/6 nephrectomy model but not our PKD model.

5.5.4 Methodological consideration

In this study, we used computer-based power spectral analysis of spontaneous oscillations in the blood pressure and HR to determine the HRV, SBPV and BRS. While caution must be taken for interpretation of the parameters reported, this is an accessible method to assess the sympathetic, vagal and baroreflex control of heart rate and the neural, respiratory and/or hormonal control of blood pressure under the assumptions that during stationary periods there is a permanent linear association between systolic arterial pressure and pulse interval, albeit limited to low and high frequency bands (Panerai *et al.* 1995). Unlike spectral analysis, the sequence method suggests that activation of the baroreceptor mechanism might be adaptive

and intermittent (Panerai *et al.* 1995). Therefore, the BRS is determined from specific sequences in which blood pressure shows continuous changes that are opposed by a change in heart rate. The interpretation of the BRS determined from the sequence method needs caution. When sequences of three successive changes in blood pressure that are opposed by a change in heart rate are used, the result largely reflects baroreflex control of vagal outflow to the heart (Hildreth *et al.* 2013). Longer sequences can reflect the ability of the baroreflex to control both vagal and sympathetic outflow to the heart. It is reported that the BRS determined by these two methods is agreeable (Hildreth *et al.* 2013). Data was not further analysed using sequence method, but in the future, additional analysis could be performed to strengthen our study. Alternative method such as intravenous administration of vasoconstrictors (phenylephrine) or vasodilators (sodium nitroprusside) for the measurement of BRS, as discussed in Chapter 1 (Section 1.1.1.3), requires repeated intravenous injection in conscious rats; therefore it is not the best option to monitor long-term autonomic function.

5.5.5 Summary

Blunted baroreflex regulation, decreased HR variability, and increased BP variability have been considered enhanced risk factors for sudden cardiac death and cardiovascular mortality. These perturbations in autonomic function are all key features of the LPK model and are well described in CKD patient cohorts (Robinson and Carr 2002, Chou and Tsai 2016). Overall, our study indicates that total and afferent RDN had limited effect on autonomic function in a rodent model of PKD, suggesting that PKD patients with autonomic dysfunction will not benefit from the procedure. Our finding that total RDN reduced SBPV in normotensive rats does, however, indicate a role for the renal nerves in the control of the autonomic function, with reduced sympathetic vasomotor tone after RDN.

5.6 Figures

Figure 5.1 The effect of total and afferent renal denervation (RDN) on TP (A, E), VLF (B, F), LF (C, G) and HF (D, H) HRV in Lewis (A, B, C and D) and LPK (E, F, G and H) rats between 7-14 weeks old.

Data is expressed as mean ± SEM and was analysed using two-way ANOVA and Bonferroni's *post hoc* analysis (if required). Overall age and treatment (Rx) effects are provided in each panel, *post hoc* analysis between total or afferent vs. sham RDN (if indicated) is provided in the main text. N values at each week for Lewis sham = (10, 11, 10, 11, 8, 5, 4, 5); Lewis total = (14, 14, 14, 13, 5, 4, 4, 4); Lewis afferent = (9, 11, 11, 10, 8, 4, 4, 4) respectively and for LPK sham = (13, 11, 13, 11, 10, 9, 9, 9), LPK total = (12, 14, 12, 12, 6, 5, 4, 4) and LPK afferent = (6, 9, 8, 8, 6, 3, 3, 3), respectively. TP, total power, VLF, very low frequency, LF, low frequency, HF, high frequency. HRV, heart rate variability.

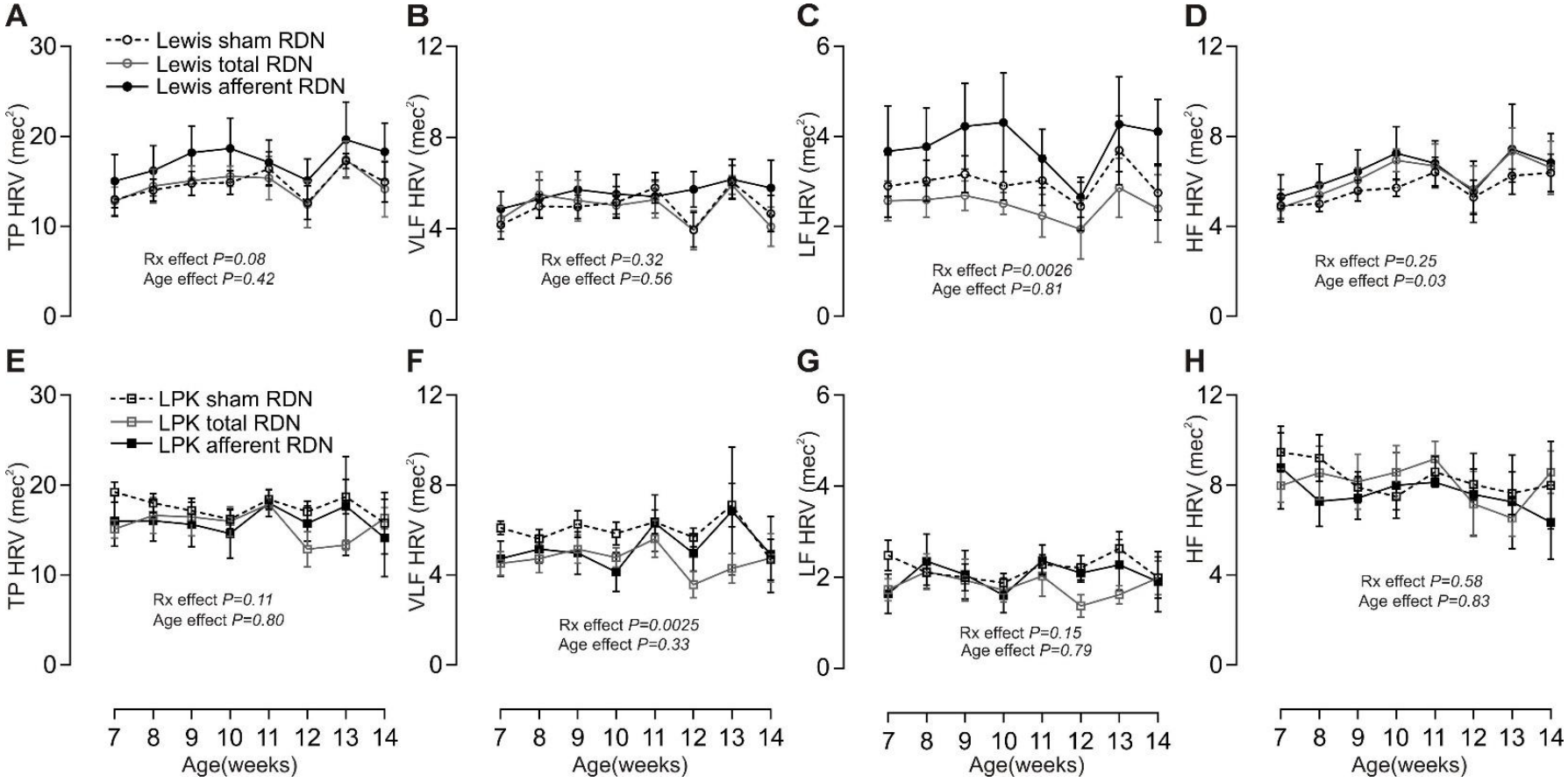


Figure 5.2 The effect of total and afferent renal denervation (RDN) on LF/HF HRV ratio in Lewis (A) and LPK (B) rats between 7-14 weeks old.

Data is expressed as mean ± SEM and was analysed using two-way ANOVA and Bonferroni's *post hoc* analysis (if required). Overall age and treatment (Rx) effects are provided in each panel, *post hoc* analysis between total or afferent vs. sham RDN (if indicated) is provided in the main text. N values at each week for Lewis sham = (10, 11, 10, 11, 8, 5, 4, 5); Lewis total = (14, 14, 14, 13, 5, 4, 4, 4); Lewis afferent = (9, 11, 11, 10, 8, 4, 4, 4) respectively and for LPK sham = (13, 11, 13, 11, 10, 9, 9, 9), LPK total = (12, 14, 12, 12, 6, 5, 4, 4) and LPK afferent = (6, 9, 8, 8, 6, 3, 3, 3), respectively.

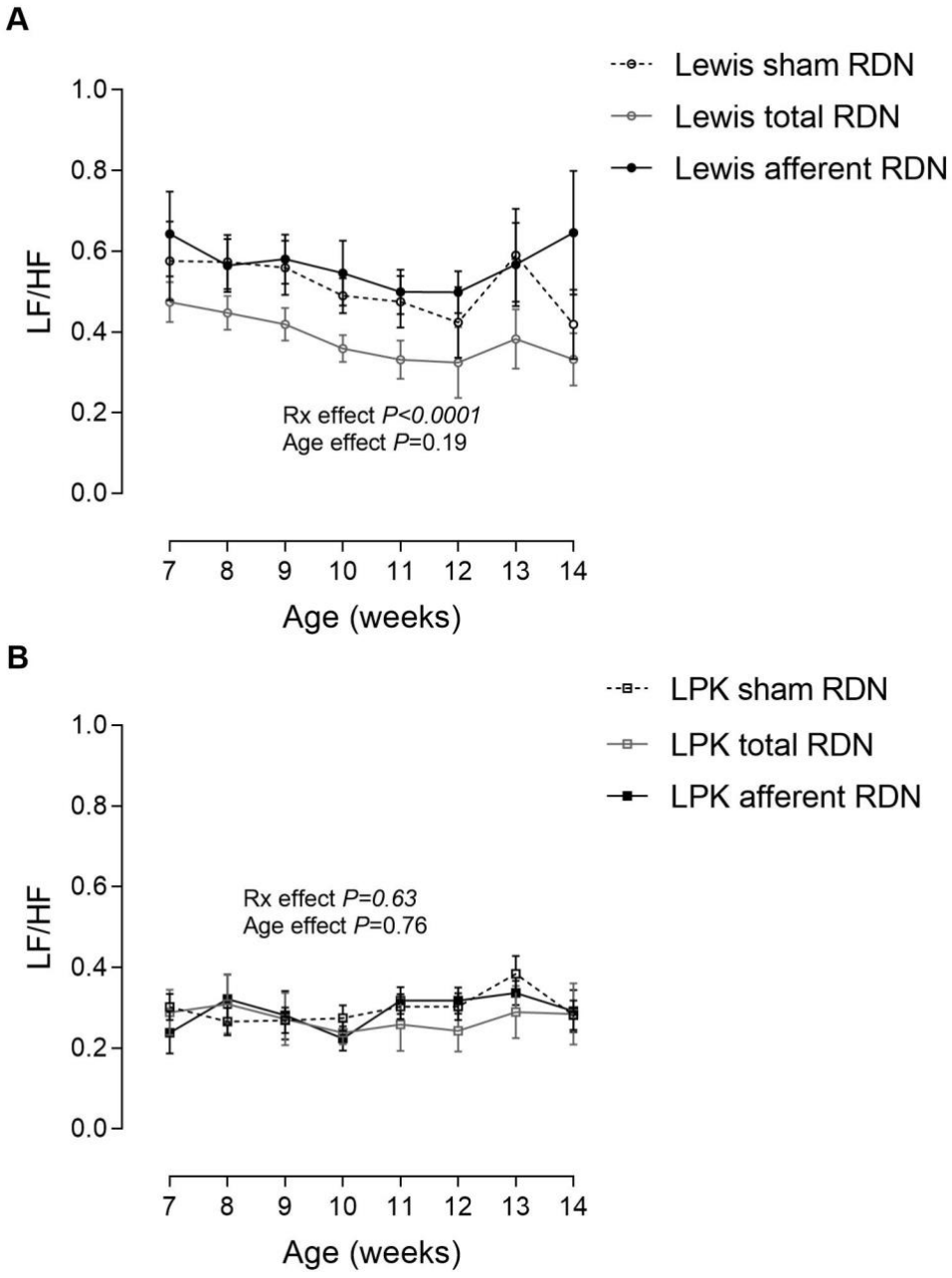


Figure 5.3 The effect of total and afferent renal denervation (RDN) on TP (A, E), VLF (B, F), LF (C, G) and HF (D, H) SBPV in Lewis (A, B, C and D) and LPK (E, F, G and H) rats between 7-14 weeks old.

Data is expressed as mean ± SEM and was analysed using two-way ANOVA and Bonferroni's *post hoc* analysis (if required). Overall age and treatment (Rx) effects are provided in each panel, *post hoc* analysis between total or afferent vs. sham RDN is provided in the main text. **P* < 0.05, vs. age-matched sham RDN. N values at each week for Lewis sham = (10, 11, 10, 11, 8, 5, 4, 5); Lewis total = (14, 14, 14, 13, 5, 4, 4, 4); Lewis afferent = (9, 11, 11, 10, 8, 4, 4, 4) respectively and for LPK sham = (13, 11, 13, 11, 10, 9, 9, 9), LPK total = (12, 14, 12, 12, 6, 5, 4, 4) and LPK afferent = (6, 9, 8, 8, 6, 3, 3, 3), respectively. TP, total power, VLF, very low frequency, LF, low frequency, HF, high frequency. SBPV, systolic blood pressure variability

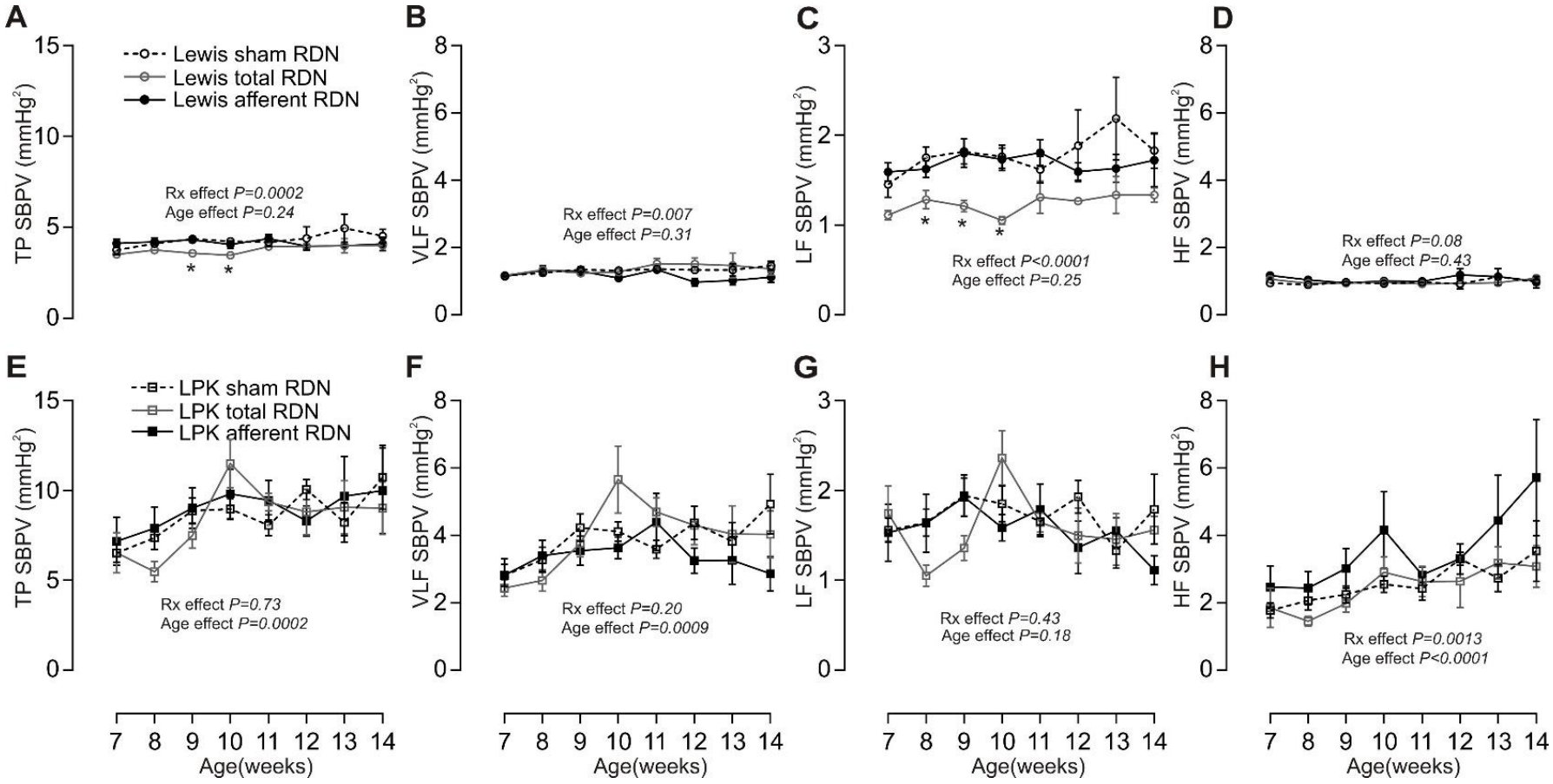


Figure 5.4 The effect of total and afferent renal denervation (RDN) on LF (A and C) and HF (B and D) BRS in Lewis (A and B) and LPK (C and D) rats between 7-14 weeks old.

Data is expressed as mean ± SEM and was analysed using two-way ANOVA. Overall age and treatment (Rx) effects are provided in each panel. N values at each week for Lewis sham = (10, 11, 10, 11, 8, 5, 4, 5); Lewis total = (14, 14, 14, 13, 5, 4, 4, 4); Lewis afferent = (9, 11, 11, 10, 8, 4, 4, 4) respectively and for LPK sham = (13, 11, 13, 11, 10, 9, 9, 9), LPK total = (12, 14, 12, 12, 6, 5, 4, 4) and LPK afferent = (6, 9, 8, 8, 6, 3, 3, 3), respectively. LF, low frequency, HF, high frequency. BRS, baroreceptor sensitivity.

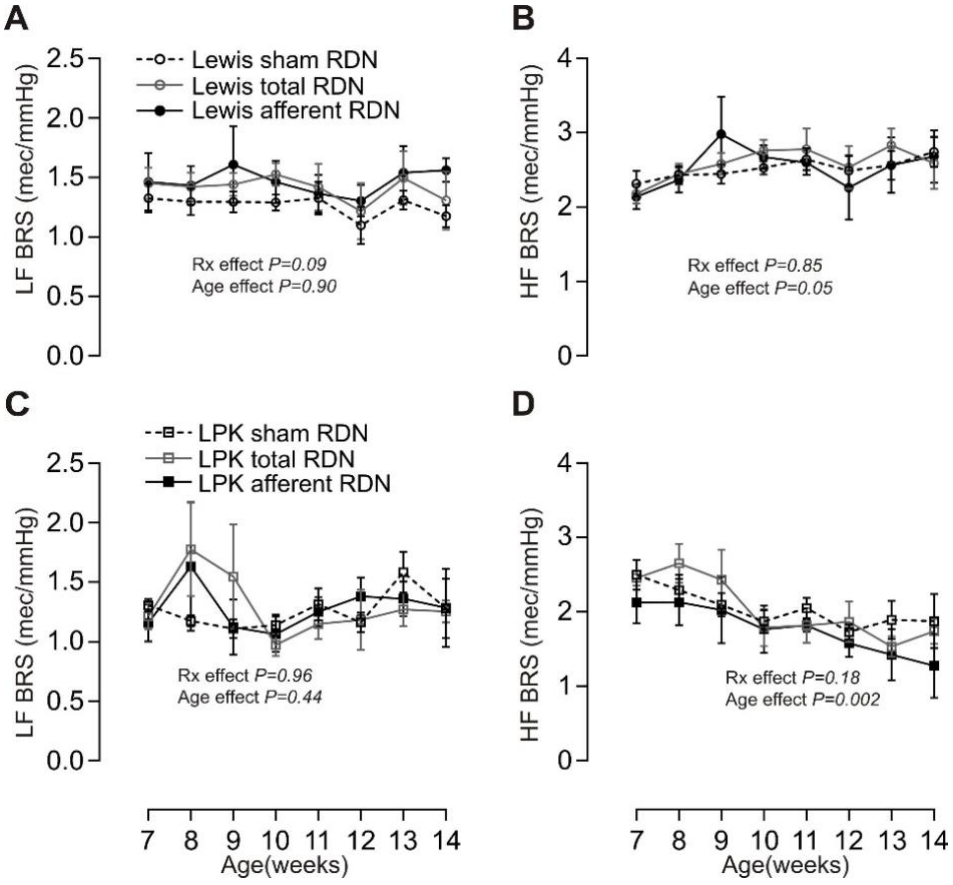


Figure 5.5 The effect of a single and repeat total renal denervation (RDN) on TP (A, E), VLF (B, F), LF (C, G) and HF HRV (D, H) in Lewis (A, B, C and D) and LPK (E, F, G and H) rats between 11-14 weeks old.

Data is expressed as mean ± SEM and was analysed using two-way ANOVA and Bonferroni's *post hoc* analysis (if required). Overall age and treatment (Rx) effects are provided in each panel, *post hoc* analysis between repeat total vs. single total or sham RDN is provided in the main text. * indicates $P < 0.05$ vs. age-matched single total denervation. N values at each week for Lewis single total = (5, 4, 4, 4); Lewis repeat total= (5, 4, 5, 5) respectively and for LPK single total = (6, 5, 4, 4) and LPK repeat total = (6, 6, 6, 6), respectively. TP, total power, VLF, very low frequency, LF, low frequency, HF, high frequency. HRV, heart rate variability.

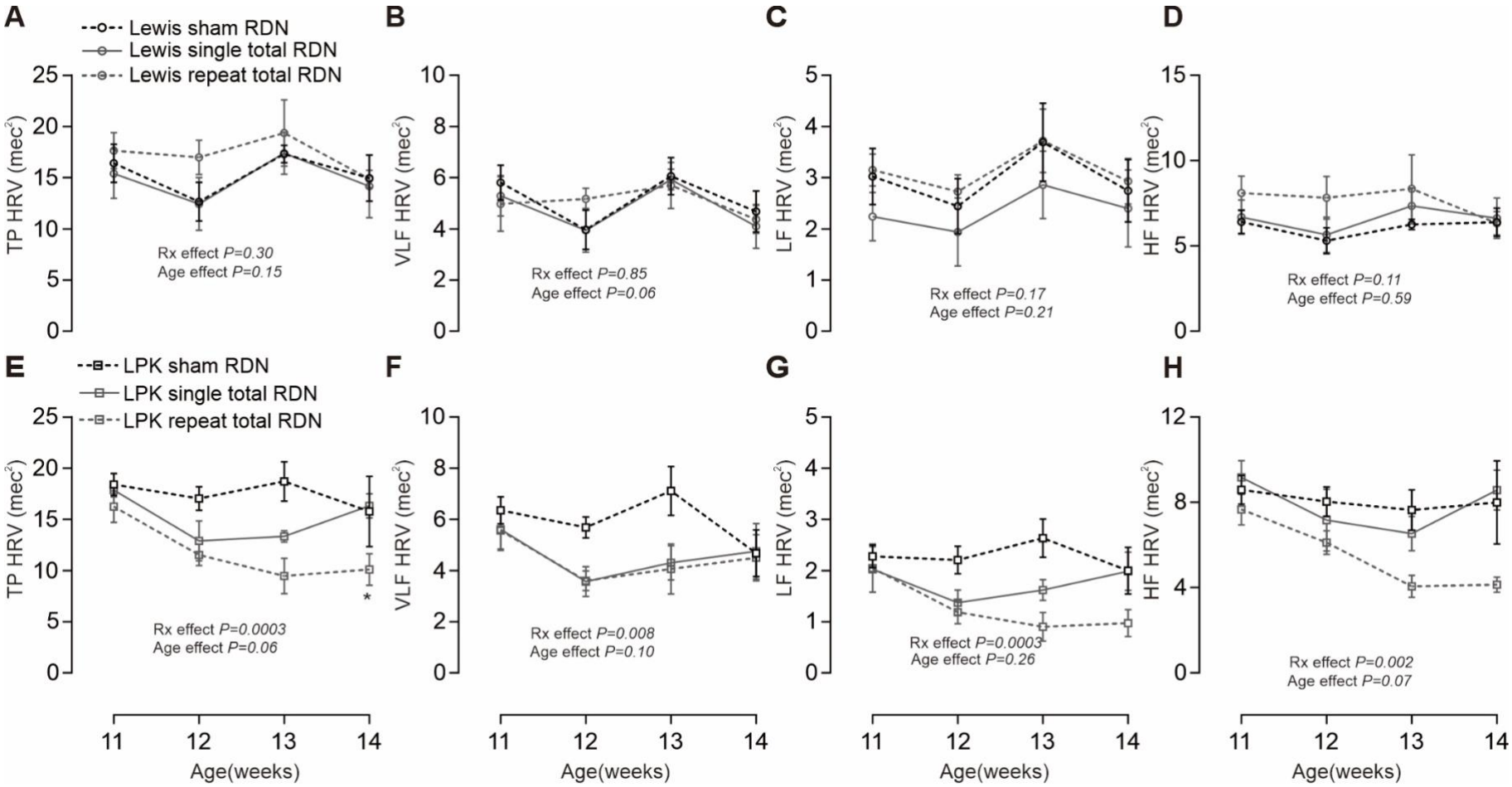


Figure 5.6 The effect of a single and repeat total renal denervation (RDN) on TP (A, E), VLF (B, F), LF (C, G) and HF SBPV(D, H) in Lewis (A, B, C and D) and LPK (E, F, G and H) rats between 11-14 weeks old.

Data is expressed as mean ± SEM and was analysed using two-way ANOVA and Bonferroni's *post hoc* analysis (if required). Overall age and treatment (Rx) effects are provided in each panel, *post hoc* analysis between repeat total vs. single total or sham RDN is provided in the main text. N values at each week for Lewis single total = (5, 4, 4, 4); Lewis repeat total= (5, 4, 5, 5) respectively and for LPK single total = (6, 5, 4, 4) and LPK repeat total = (6, 6, 6, 6), respectively. TP, total power, VLF, very low frequency, LF, low frequency, HF, high frequency. SBPV, systolic blood pressure variability.

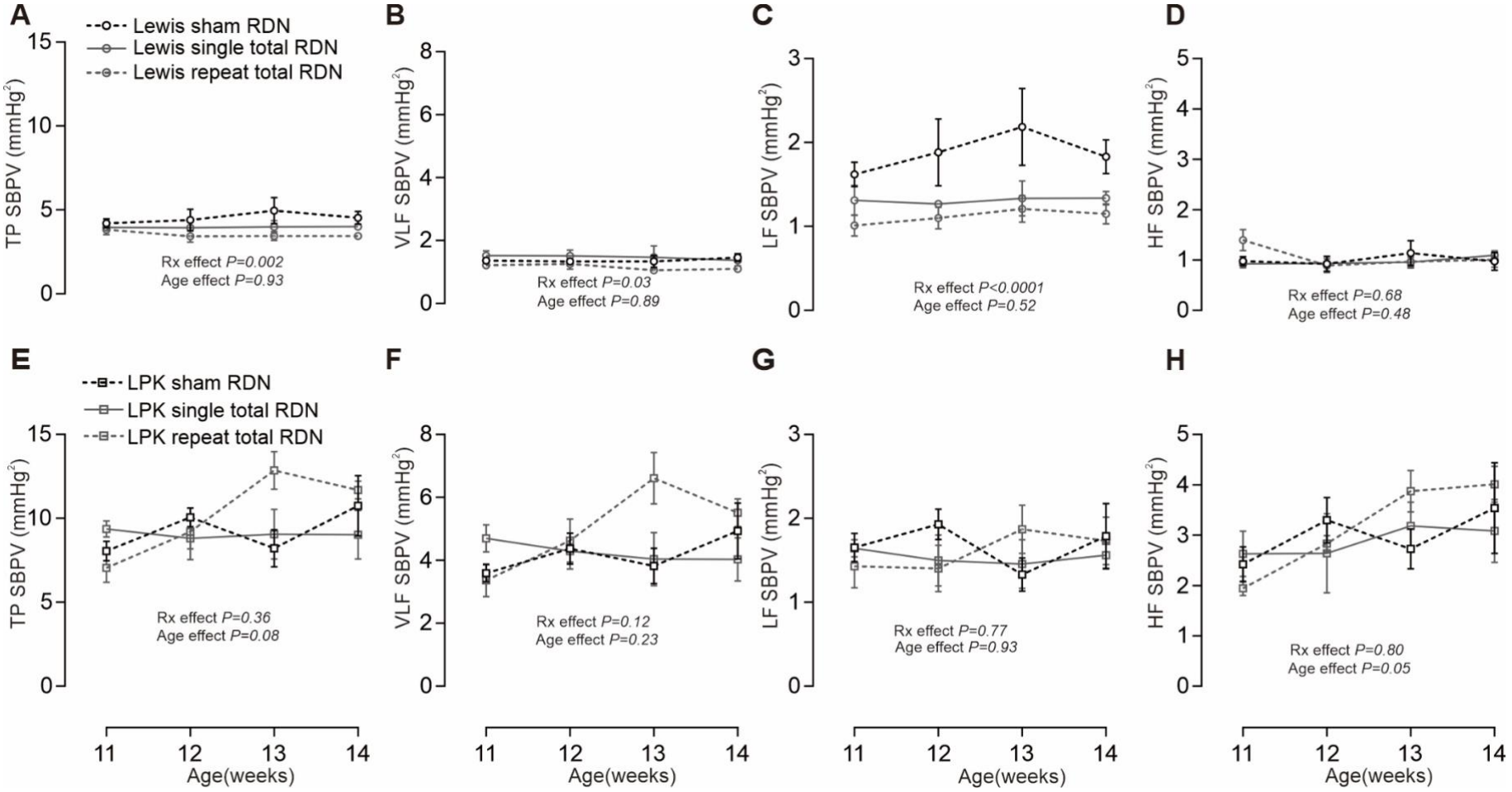
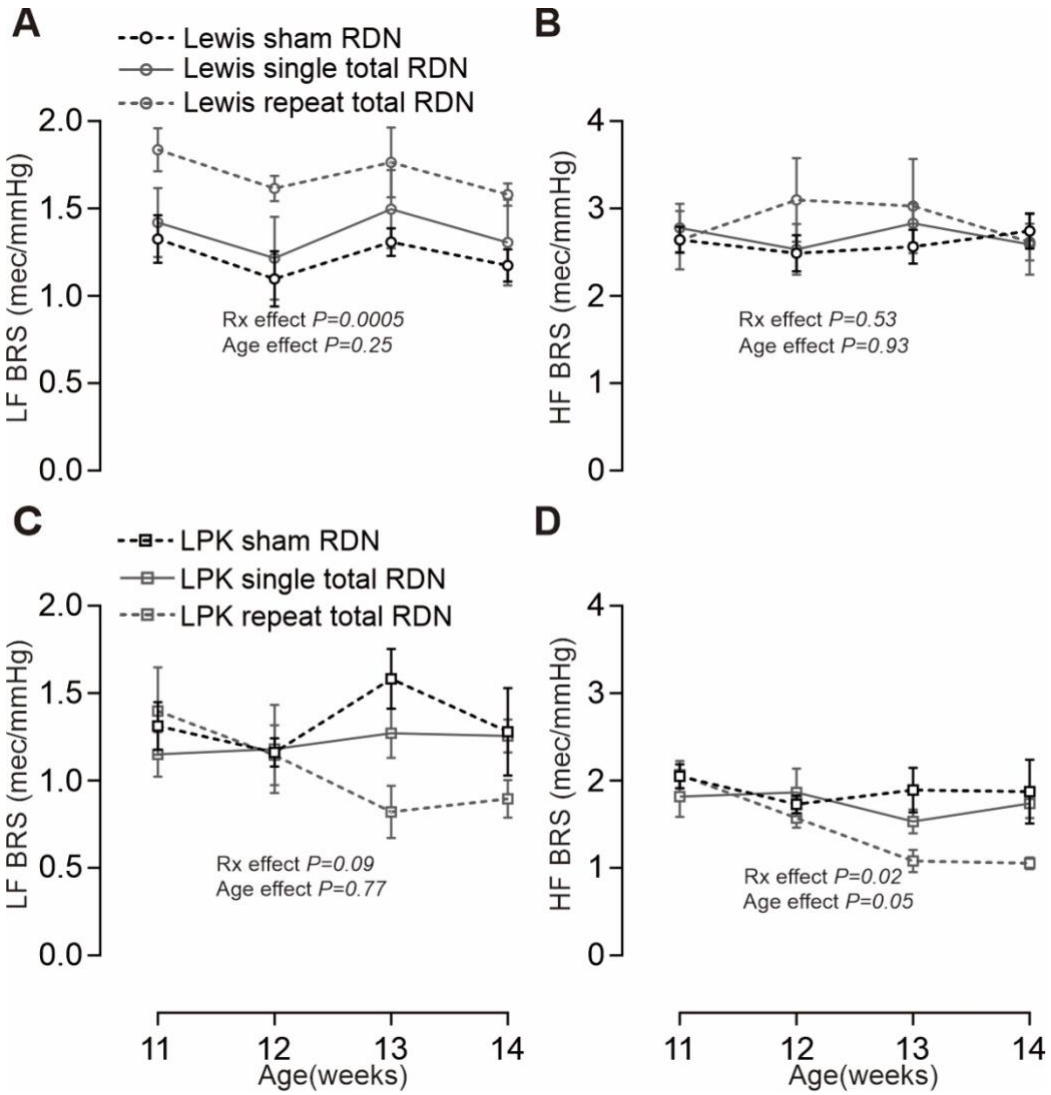


Figure 5.7 The effect of a single and repeat total renal denervation (RDN) on LF (A and C) and HF (B and D) BRS in Lewis (A and B) and LPK (C and D) rats between 11-14 weeks old.

Data is expressed as mean ± SEM and was analysed using two-way ANOVA and Bonferroni's *post hoc* analysis (if required). Overall age and treatment (Rx) effects are provided in each panel, *post hoc* analysis between repeat total vs. single total or sham RDN is provided in the main text. N values at each week for Lewis single total = (5, 4, 4, 4); Lewis repeat total= (5, 4, 5, 5) respectively and for LPK single total = (6, 5, 4, 4) and LPK repeat total = (6, 6, 6, 6), respectively. LF, low frequency, HF, high frequency. BRS, baroreceptor sensitivity.



Chapter 6 Effect of renal denervation on the renin-angiotensin-aldosterone system (RAAS) and sodium excretion

6.1 Abstract

The renin-angiotensin-aldosterone system (RAAS) and renal sympathetic nerves interact both at the level of the kidney and the central nervous system and together are crucial factors involved in the long-term regulation of blood pressure, including the regulation of sodium and water balance. Total renal denervation (RDN), which removes the innervation to the kidney may well then cause alterations in the RAAS and regulation of sodium excretion. We aimed to determine the impact of RDN on the RAAS components, both systemic and at the level of the kidney, and determine any impact of the procedures on urine sodium excretion in 10 weeks old Lewis and Lewis Polycystic Kidney (LPK) rats. Plasma and kidney renin content, as determined by radioimmunoassay, were significantly lower in the LPK compared to Lewis (Plasma renin LPK 278.2 ± 6.7 vs. Lewis 376.5 ± 11.9 ng/ml/hr; kidney renin LPK 260.1 ± 6.3 vs. Lewis 753.2 ± 37.9 ng/mg/hr). These parameters were not affected by RDN in either strain. Intra-renal gene expression levels of RAAS components (renin, angiotensinogen, ACE2 and AT1αR) were significantly lower in the LPK compared to Lewis, except for ACE1, which was significantly higher in the LPK (all $P < 0.05$). However RDN had no impact on the intra-renal RAAS gene expression. In the LPK, sodium concentration determined from 24h urine samples and 24h sodium excretion was significantly lower compared to Lewis at 10 weeks of age. There was an overall treatment effect ($P = 0.04$) on 24h sodium excretion with *post hoc* analysis within each strain indicating no difference between treatment groups. Overall, our data indicate that both total and afferent RDN has

limited impact on the RAAS, either systemic or intra-renal, or sodium excretion in the LPK model of CKD.

6.2 Introduction

The renin-angiotensin-aldosterone system (RAAS), which is involved in blood pressure and fluid balance regulation, is suggested to play a critical role in several forms of human hypertension, such as renovascular hypertension (Textor 2006), essential hypertension (Mulatero *et al.* 2007) and hypertension secondary to chronic kidney disease (CKD) (Siragy and Carey 2010). The RAAS is also implicated in experimental models of hypertension including the spontaneously hypertensive rat (SHR) (Kobori *et al.* 2005, Lee *et al.* 2012), two-kidney-one-clip (2K1C) hypertension (Kim *et al.* 2016) and Dahl-salt hypertension (Kobori *et al.* 2003). Renin-angiotensin-aldosterone system inhibitors have been shown to reduce blood pressure in hypertensive patients and are currently the first-line antihypertensive therapy recommended by current guidelines of hypertension management in the United States (James *et al.* 2014) and Europe (Williams *et al.* 2018) including for CKD patient (Levin *et al.* 2013).

The RAAS mediates its effects systemically, through the classic pathway of circulating angiotensin II (Ang II), which is the consequence of renin and ACE (angiotensin-converting enzyme) enzymic cascade on angiotensinogen and angiotensin I (Ang I) (Hall 2003). Ang II binds to the angiotensin type 1 receptor (AT1R) located in the adrenal cortex to stimulate aldosterone production, leading to water and sodium reabsorption (Hall 2003). However local RAAS may also play an important homeostatic role both in health and disease. Dysregulation of intrarenal RAAS has been described in human polycystic kidney disease (PKD) for example, as evidenced by ectopic expression of renin including in small arterioles and the interlobular artery as well as in cystic tubules (Graham and Lindop 1988, Torres *et al.* 1992, Loghman-Adham *et al.* 2004). Upregulation of RAAS components in the kidney including renin, ACE and Ang II has also been observed in rodent models of autosomal recessive PKD (ARPKD) (Goto *et al.* 2010), as demonstrated using quantitative real-time PCR and immunohistochemistry methodology.

As discussed in Chapter 1 (Section 1.1.4), there are complex interactions between the RAAS and renal sympathetic activity both at the level of the kidney (DiBona 2000) and the central nervous system (Stupin *et al.* 2017, Rossi *et al.* 2019) and they are both crucial factors in blood pressure regulation. At the level of the kidney, sympathetic nerves impact the RAAS by promoting renin release. Evidence from studies in mice showed that renal denervation (RDN) reduced intrarenal RAAS components in neurogenic hypertension (Gueguen *et al.* 2018) and hypertension secondary to chronic intermittent hypoxia (Takahashi *et al.* 2018), which suggests that total RDN, in removing the sympathetic innervation to the kidney, had the potential to cause alterations in the RAAS. In contrast, however, in prenatally programmed hypertensive models where the mother was fed with a low protein diet, RDN did not affect the RAAS (Mansuri *et al.* 2017), indicating that the renal sympathetic nerves do not drive RAAS activation in all disease states.

In the LPK, our previous studies have shown that relative to the Lewis, the RAAS is reduced systemically, evidenced by a lower level of plasma renin activity and circulating Ang II (Phillips *et al.* 2007) however to date intra-renal RAAS has not been studied in this model. In Chapter 4, our work demonstrated a blood pressure lowering effect after total RDN in normotensive Lewis rats. Downregulation of the RAAS may explain this effect and while we did not see a blood pressure lowering effect after total RDN in the LPK rats, there may still have been an effect on the RAAS but not sufficient to impact blood pressure.

Renal sympathetic nerves influence sodium excretion via both arteriolar vasoconstriction and alterations in tubular sodium reabsorption (Johns *et al.* 2011). The renal sympathetic nerves are under reno-renal reflex control by renal afferents such that activation of renal afferent nerves by chemo or mechanoreceptors results in suppression of renal sympathetic activity, which results in increased sodium and water excretion (Johns *et al.* 2011). The RAAS could also impact sodium levels in the urine, exerting its sodium-retaining actions on the renal tubules via both Ang II and aldosterone (Sparks *et al.* 2014). RDN, either total or afferents

only, could therefore be expected to impact the kidneys' ability to regulate sodium excretion. However the literature is quite inconsistent with some studies (Winternitz *et al.* 1980, Li *et al.* 2016) demonstrating an increase in urinary sodium excretion in the SHR after RDN (Winternitz *et al.* 1980, Li *et al.* 2016) while others (Kline *et al.* 1978, Greenberg and Osborn 1994) showed no impact. Levels of sodium excretion in the LPK have not been previously investigated, though it would be predicted that the sodium excretion might be reduced in LPK relative to Lewis due to decreased renal capacity to eliminate sodium (Ecder and Schrier 2001, Soi and Yee 2017). The investigation of total RDN on sodium excretion levels in the Lewis and LPK model may shed further light on both the mechanisms by which RDN is able to reduce blood pressure and possibly explain the lack of blood pressure lowering effect observed in the LPK.

The aim of this study, therefore, was to establish if there are differences in both circulating and intra-renal RAAS components between the LPK and its normotensive control the Lewis, and to determine if RDN changed the RAAS in either strain. While afferent denervation per se did not impact blood pressure (Chapter 4), we also studied tissues from those animals which underwent treatment with capsaicin, predicting that the procedure would have no impact on RAAS components and therefore serving as a control group. Animals were studied at age 10 weeks, being the time point at which a response to total RDN on blood pressure was most evident in the Lewis animals. We also investigated baseline urinary sodium concentration in the LPK animals compared to Lewis controls over the course of the disease, as well as the impact of RDN on urinary sodium excretion.

6.3 Method

The protocol details and experimental design for this study (Study 2) are provided in Chapter 2 (Section 2.3, Figure 2.1) and are illustrated schematically in Figure 6.1. Samples (plasma and kidney tissues) from animals assigned to Study 2 Cohort 1(n = 14 Lewis, n = 14 LPK) that underwent total, afferent or sham RDN protocols and were euthanased at 10 weeks of age were collected and stored at -80 °C (Section 2.4) for determination of levels of RAAS components. Animals were deeply anaesthetised with an i.p. injection of 20% (v/v) solution of sodium pentobarbital (100mg/kg, Virbac, NSW, Australia) at the termination of the study period and blood collected via cardiac puncture into ethylenediaminetetraacetic acid (EDTA) tubes (Becton, Dickinson and Company, New Jersey, United States). Sample were spun and the plasma removed, snap frozen in dry ice and stored at -80 °C until further analysis. 24h urine samples were collected from these animals 48hrs prior to euthanasia for sodium concentration analysis. Animals from study 2 cohort 2 (n = 22 Lewis, n = 22 LPK) that underwent total, afferent or sham RDN protocol have spot urine samples collected at age 7, 9 and 11 weeks of age for sodium concentration analysis.

6.3.1 Plasma and kidney renin determination

Plasma and renin content from the Cohort 1 animals were determined by radioimmunoassay (RIA) (ProSearch International Australia, Malvern, Vic., Australia).

Frozen kidney tissues were thawed and a representative sample of 0.2 to 0.5 g tissue obtained. This was homogenised in 5 mM $EDTANa_2$ (1:10, wt gram/vol mL) using a BIAOMA FJ-50 Homogenizer/blender for 30 to 60s at ½ speed at 0 °C. Homogenates were immediately centrifuged at 1000 g for 10 mins. Supernatants (SN) were collected and left at 0 °C for 1 hr and then frozen at -20 °C. A total of 0.025 uL of non-inhibited homogenate SN were made up

to 25 µL with dH_2O or 0.9% saline for subsequent incubation. For plasma samples, a total of 2.5µL of plasma was diluted to 25µL with dH_2O for subsequent analysis.

The prepared kidney or plasma samples were incubated at 37 °C with 25 µL of nephrectomised sheep plasma (Skinner 1967) and 25 µL of an angiotensinase inhibitor cocktail containing 30 mM $EDTANa_2$, 3 mM 2,3-dimercaptopropanol and 6 mM 8 hydroxyquinoline buffered with 150mM sodium phosphate buffer pH 6.2. In preliminary experiments varying volumes of tissue homogenate or plasma were incubated for varying times at 37 °C to ensure that reaction kinetics were linear and not substrate limited (Skinner 1967). Subsequent incubations at 37 °C were for 1 hr and were terminated by the addition of 925 µL water and placing the tubes into boiling water for 4 mins. Angiotensin-I (Ang I) generated was measured by RIA (Johnston *et al.* 1971) and converted to a rate of production known to be linear (zero order kinetics) and expressed as ng Ang I/mg tissue/hr (kidney) or ng Ang I /mL/hr. Intraassay variability was: Mean, SD and CV = 93 ± 10.2 ng/ml/hr, 11%. Inter-assay variability was: 91 ± 11.8, 13%. The IC 50 was 0.7 ng/ml. Cross-reactivity to Ang II and angiotensin (1-7) was less than 0.1%.

6.3.2 RNA extraction and real-time quantitative reverse transcription polymerase chain reaction (RT – qPCR)

Total RNA was extracted from left kidney tissue (30 mg) using the SV total RNA Isolation System (Promega, Wisconsin, USA) and eluted in 50 µL of water and stored at -80 °C. The RNA concentration of each sample was then determined with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, VIC, Australia). RNA (1 µg) was then reverse-transcribed with AffinityScript ® QPCR cDNA (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Quantitative-PCR was performed in 20 ul reaction volumes containing 1 ul of cDNA mix, Applied Biosystems PowerUpTM SYBRTM Green Master Mix (Thermo Fisher Scientific, VIC, Australia) and gene primers (Table 6.1) using Vii A 7 Real-time PCR system (Thermo Fisher Scientific, VIC, Australia). A

preliminary primer concentration assay was carried out to determine the optimum primer concentration (providing the lowest mean Ct value) for subsequent experiments. Preliminary analysis was also undertaken to determine the optimal pair of housekeeping genes, using NormFinder software *(*https://www.moma.dk/normfinder-software*; (Andersen et al. 2004))*, to select housekeeping genes with a stable expression between the two animal strains in kidney tissue. Conditions for qPCR were as follows: 20s at 95 °C, 20s at 60 °C, and 15s at 95 °C, 1 min at 60 °C, and 15s at 95 °C for 40 cycles, then held at 4 °C. No reverse-transcriptase cDNA mix (-RT) and water controls were also run on each plate to ensure experiment fidelity. Following qPCR cycling, the products were validated by melt curve analysis

6.3.3 Urine sodium analysis

Urine sodium was analysed using the 4100 MP-AES system (Agilent Technologies, USA), with urine samples diluted to 1:1000 (Lewis) and 1:500 (LPK) in distilled water. Data acquisition parameters of sample uptake time (20s), stabilisation time (20s) and read time (20s) were used. All parameters, including sample dilution, had been optimised in a series of preliminary experiments. The analysis consisted of washing the system with distilled water for 60 seconds and then running a series of procedural blanks, calibration sodium samples diluted to a range of 0.625 to 10 mg/L from standards (Sigma-Aldrich, USA) and the diluted urine samples sequentially. A repeat analysis of calibration samples was then performed to ensure the accuracy and precision of the procedure. For each sample, five readings were obtained and averaged to create a final concentration reading in mmol/L. Urine sodium concentration from animals at age 10 weeks and 14 weeks were then normalised using 24h urine volume and expressed as mmol/24h.

6.3.4 Data analysis

Data analysis was performed using GraphPad Prism (v7.02, GraphPad Software, La Jolla, USA) and IBM Statistical Package for the Social Sciences (v25, SPSS; Chicago, IL). For

comparison of kidney and plasma renin concentration and spot urine sodium concentrations at 7, 9 and 11 weeks, a univariate general linear model (GLM) analysis of variance was used to identify strain, sex or treatment effects followed by Tukey *post hoc* analysis as indicated. If the data displayed unequal variance (Levene's Test), Games-Howell *post hoc* analysis was used. Further analysis was undertaken on the spot urine data within each strain for the effect of age within each treatment group using a two-way ANOVA and *post hoc* Bonferroni's analysis. Due to low sample size within each treatment group for normalised 24h sodium excretion data, data was underpowered to analyse for sex as a variable and so only strain and treatment effects were tested for this specific data set. All results are expressed as mean ± SEM.

For gene expression analysis, the average threshold cycle (Ct) value of three replicates for each animal was normalised to the expression of two housekeeping genes β-ACT (β-actin) and cytochrome c1 (CYC-1). Statistical significance was determined by comparing values after normalisation to housekeeping genes (ΔCt values). ΔCt values were first analysed using a univariate GLM analysis of variance to identify strain, sex or treatment effects followed by Tukey *post hoc* analysis as indicated. If there was no sex and/or treatment effect, the data were pooled. Data that displayed unequal variance were analysed using Games-Howell *post hoc* analysis. For determination of fold difference in gene expression between treatment groups within each strain or between two strains, the $2^{-(\Delta\Delta Ct \pm \text{Standard Error (SE) of the } \Delta Ct)}$ method was used (Livak and Schmittgen 2001). To illustrate a strain effect, data was presented as a relative expression of the gene of interest in Lewis animals being equal to 1. To illustrate a sex effect, data was presented as a relative expression of the gene of interest in female Lewis animals.

Significance was defined as a P value < 0.05 for all analysis

6.4 Results

6.4.1 RAAS expression

6.4.1.1 Renin content

Univariate GLM analysis of plasma renin content in 10 weeks old Lewis and LPK animals indicated a significant strain effect between Lewis and LPK for plasma renin content with renin being lower in the LPK overall (376.5 ± 11.9 vs. 278.2 ± 6.7 ng/ml/hr; $P < 0.0001$), but no treatment ($P = 0.84$; illustrated in Figure 6.2A) or sex ($P = 0.97$) effect. With regards to kidney renin content, a significant strain ($P < 0.001$) and sex ($P < 0.001$) was present, but there was no overall treatment effect ($P = 0.31$). Figure 6.2B illustrates sex data within strain showing Lewis animals having a significantly higher level compared with their LPK counterparts, and female Lewis animals having significantly higher level compared to the males.

Kidney renin expression was also assessed immunohistochemically to determine if there were any differences in expression between the two strains. Results indicated a similar pattern of renin staining in the two strains, which was evident in both the juxtaglomerular apparatus of the afferent arteriole in close association with the glomerulus and the vascular tissue, and was not colocalised with renal tubule markers (see Supplementary methods and results in Appendix to Chapter 6).

6.4.1.2 Kidney RAAS gene expression levels

Univariate GLM analysis of RAAS gene expression in 10 weeks old Lewis and LPK animals indicated a significant strain effect (all $P < 0.05$) for all genes. There was no overall treatment effect except for ACE2 ($P = 0.04$); however, *post hoc* analysis did not distinguish further between treatment groups within each strain for this gene. The ΔCt and relative fold difference between Lewis and LPK are provided in Table 6.2. Angiotensinogen (AGT), renin, ACE2 and AT1αR were all expressed in lower levels in the LPK compared with Lewis controls, while ACE1 was higher by 3-fold in the LPK. There was also a significant sex effect

for AGT, with Lewis animals having a significantly higher level compared with their LPK counterparts, and male Lewis animals having significantly higher level compared to the females (Figure 6.3).

6.4.2 Urine sodium concentration

Univariate GLM analysis of variance indicated a significant strain effect ($P < 0.005$) but no overall treatment or sex effect on urine sodium concentration in spot samples collected from Lewis or LPK at age 7, 9 or 11 weeks, with sodium concentration being less in the LPK animals. Further analysis within each strain for the effect of age indicated a significant reduction in urine sodium concentration per L of urine in the LPK as they aged but no change in the Lewis animals. Data shown in Figure 6.4 illustrates age and treatment groups.

Urinary sodium concentrations from 24h collections, 24h urine volume and sodium concentration data after normalisation to 24h urine volumes at 10 weeks of age for each strain and treatment group are presented in Table 6.3. Univariate GLM analysis indicated a significant strain effect in all parameters ($P < 0.0001$), with the LPK again showing a lower sodium concentration alongside an increased urinary volume and overall reduced sodium excretion. After normalisation of daily sodium excretion to 24h urine volume, an overall treatment effect was also evident, with *post hoc* analysis within each strain indicating no difference between treatment groups in the LPK ($P = 0.235$) and while there was a trend towards reduced 24h sodium excretion in the Lewis animals after capsaicin RDN, this did not reach significance ($P = 0.09$).

6.5 Discussion

In this series of experiments, we aimed to determine if the blood pressure lowering effect observed in Lewis after total RDN could be explained by changes in systemic or intra-renal RAAS components or sodium excretion. We also aimed to establish the fundamental difference between Lewis and LPK regarding intra-renal RAAS and sodium excretion, which have not been examined previously. Our major finding is that while circulating and intra-renal renin, as determined by radioimmunoassay, are significantly less in LPK vs. Lewis animals, this was not altered within either strain by either total or afferent denervation procedures. There was also no evidence for ectopic expression of renin in the kidneys of LPK animals as assessed by immunohistochemistry. Renal gene expression for renin was likewise significantly reduced in the LPK animals, as was AGT, ACE2 and the AT1αR while ACE1 was significantly greater in the LPK. However there was no clear effect of total or afferent RDN in either strain. We also demonstrated that urine sodium concentration was significantly less in LPK vs. Lewis animals, and that this worsened with age. When normalised to 24h urine volumes, the overall level of urinary sodium excretion was still significantly lower in LPK compared with Lewis.

6.5.1 Systemic and local RAAS

Activation of the renal sympathetic nerves produces a frequency-dependent increase in the section of renin (DiBona 2000), the rate-limiting enzyme for RAAS cascade (Hall 2003). This leads to increased production of downstream RAAS components including Ang II, which is the active component in the regulation of blood pressure and fluid balance (Beevers *et al.* 2001) and increased RAAS activity which is implicated in renovascular hypertension in patients (Textor 2006) and animal models (Kim *et al.* 2016). Recent evidence has also revealed that inappropriate activation of tissue RAAS, i.e. intra-renal RAAS, which is independent of circulating RAAS, contributes to the pathogenesis of hypertension (Kobori *et al.* 2007, Yang and Xu 2017).

In Chapter 4, we reported a blood pressure lowering effect after total RDN in normotensive Lewis rats. We speculated that the effect produced by total RDN might have been due to changes in either systemic/intra-renal RAAS. However, total RDN did not alter plasma or kidney renin content or gene expression levels of intra-renal RAAS components in the Lewis strain. Consistent with our current findings, in a study by Jacob et al. (Jacob *et al.* 2005), plasma renin activity remained similar in male Sprague-Dawley rats that underwent bilateral RDN or sham denervation, despite these animals also demonstrating a decrease in blood pressure after total RDN. These findings indicate that the sustained decrease in blood pressure produced by total RDN in normotensive rats is not mediated by persistent alterations in systemic/intra-renal RAAS.

Even in the absence of a blood pressure response to RDN in the LPK animals, given we have previously demonstrated an increased sympathetic tone to several vascular beds including the kidney in the LPK model (Salman *et al.* 2015, Salman *et al.* 2015, Yao *et al.* 2015), we speculated that total RDN may have had a downregulating effect on the RAAS. Such an effect has been demonstrated in hypertension secondary to chronic intermittent hypoxia (Takahashi *et al.* 2018), in Schlager mice model of neurogenic hypertension, and swine model of heart failure (Sharp *et al.* 2018). There was however no significant effect on the RAAS after total RDN in our model, either in the circulation or the kidney. This may be because RAAS activity is already significantly suppressed in the LPK due to the sustained high blood pressure, in line with our previous finding of reduced RAAS markers renin and Ang II systemically (Phillips *et al.* 2007). Nevertheless, in contrast to those studies cited above where RDN has been shown to reduce the RAAS, there are contrasting examples, including a study in the SHR, where there was no effect on urinary AGT excretion rate, plasma renin activity or kidney renin content (Gao *et al.* 2016), and in human clinical studies, where RDN had no impact on circulating renin in patients with resistant hypertension (Ezzahti *et al.* 2014). RDN was also unable to normalise increased urinary AGT/creatinine ratios in a rat model of

prenatal programming, where pregnant rats were fed a 6% protein diet during the last half of pregnancy resulting in hypertensive pups (Mansuri *et al.* 2017). Taken together, these suggest that any beneficial effect of RDN may be mediated by different mechanisms in different disease states.

Another outcome of this study was a clear difference in the profile of RAAS components between the LPK and Lewis animals. The predominant feature was a much lower level of kidney renin content, intra-renal expression of renin, AGT, ACE2 and AT1αR in the LPK, excepting ACE1 mRNA expression in the kidney which was increased. This is in contrast to previous studies where increased mRNA expression of renin and Ang II staining in the kidney was observed in *pck* rat, a rodent model of ARPKD (Goto *et al.* 2010). The mechanism responsible for the suppressed RAAS both systemically and locally is not clear in our model but may be a consequence of the more severe hypertension as compared to the *pck* rat (~230 mmHg vs ~150mmHg at a similar age). Alternatively, the lower level of renin in the LPK may be due to the reduction of renal cortical mass due to cyst compression resulting in a reduction in the number of juxtaglomerular cells. Ectopic expression of renin in cysts of the kidney, has been shown by Torres et al. (Torres *et al.* 1992) and Loghman et al. (Loghman-Adham *et al.* 2004) in human autosomal dominant PKD (ADPKD) and ARPKD (Loghman-Adham *et al.* 2005) tissue samples, suggesting intra-renal RAAS overactivation may represent an essential mechanism for hypertension development in PKD. However, our examination of renin distribution in the kidney did not show an expression pattern different from that seen in the Lewis controls per se. The reasons for the difference between our study and previous studies are not clear but may be due to the differences in genetic mutations. Of note also was the marked sex effect present in the expression of AGT, being much higher in the Lewis male animals. This was seen alongside a lower level of kidney renin in male Lewis compared to their female counterparts. Yames et al. (Yanes *et al.* 2006) also observed a higher level of intra-renal AGT mRNA and protein expression in old male SHR rats compared to females,

concurrent with a relatively lower level of Ang II. The authors hypothesised that sex difference in ACE activity might account for this. We are unsure of the physiological impact of this phenomena and future studies may expand our understanding of this.

6.5.2 Sodium excretion

Physiologically, the renal sympathetic nerves act directly on the renal tubules to modulate sodium and water excretion (Johns *et al.* 2011). Renal afferent nerves are also involved in maintaining sodium and water homeostasis via the reno-renal reflex (Johns *et al.* 2011). RDN, either total or renal afferents only, could therefore have the potential to affect sodium excretion. In our study, neither of the treatments impacted the capacity of the LPK animals to excrete the sodium and there was similarly no effect on the Lewis in either spot or 24h urine samples. The amount of urine produced by the LPK animals over 24 hrs was significantly greater than that seen in the Lewis, which is consistent with PKD causing impaired urine concentrating ability (Torres *et al.* 2009), and this also was not impacted by the different treatments. Clinical observations demonstrate a decrease in 24h sodium output in patients with advancing CKD, with the kidney's capacity to excrete sodium decreasing with decreasing glomerular filtration rate (GFR) (Soi and Yee 2017), which is consistent with the decline in LPK renal function as measured by creatinine clearance (Chapter 4).

In acute studies, an instant significant increase in sodium and water excretion are seen directly after unilateral RDN (Kompanowska-Jezierska *et al.* 2001, Salman *et al.* 2010), highlighting the important role of sympathetic nerves in sodium regulation. However, over longer time frames the impact of RDN on urinary sodium levels seems inconsistent. Winternitz et al. (Winternitz *et al.* 1980) and Li et al. (Li *et al.* 2016) demonstrated increased sodium clearance after RDN in SHR for up to 2 -3 weeks, while Herlitz et al. (Herlitz *et al.* 1983) reported that after RDN, the sodium clearance rate was higher in the denervated SHR and normotensive WKY rats for only 3 days, with no significant difference in sodium clearance thereafter. Kline et al. (Kline *et al.* 1978) and Greenberg (Greenberg and Osborn 1994) report that RDN did

not affect urine sodium excretion in SHR at all during their follow up periods of 4 to 6 weeks. In normotensive rats, bilateral RDN has been shown to not affect sodium excretion in rats on normal, high or low salt diets (Bencsath *et al.* 1982, Jacob *et al.* 2003). The difference between acute and chronic studies is suggested to be that in acute studies, denervation natriuresis is attributable to the withdraw of the sympathoexcitatory effect caused by the anaesthesia and surgical stress, whereas in conscious normo- or hypertensive animals either this stimulus is absent, or compensatory intra or extra renal mechanisms are in place to maintain sodium homeostasis (Bencsath *et al.* 1982, Jacob *et al.* 2003). Our results are also consistent with the findings of Foss et al. (Foss *et al.* 2015, Foss *et al.* 2016, Foss *et al.* 2018), who reported that capsaicin does not affect sodium excretion in low, normal or high salt fed Sprague-Dawley rats, Dalt salt-sensitive rats or Sprague-Dawley rats infused with Ang II. Janssen et al. (Janssen *et al.* 1989), who used unilateral section of dorsal roots to achieve afferent denervation, also did not demonstrate any change in sodium excretion in SHR rats.

6.5.3 Limitations

There are limitations to consider in the interpretation of the data presented in this chapter. Firstly, we only looked at circulating and intra-renal renin content and we are not sure if Ang II, the active component of RAAS is altered by the RDN procedure. However, based on the effect of RDN on renin, it is unlikely that the significant changes in Ang II would be induced and our results are consistent with our previous studies showing the LPK have low levels of circulating Ang II (Phillips *et al.* 2007). As discussed in Chapter 1 (section 1.3.2.2.4), there is an inherent limitation to using blood samples collected following anaesthesia to determine plasma renin level in our study. Given the impact of stress and anaesthesia on RAS activation, it may be difficult to detect small changes in basal plasma renin levels.

Secondly, our spot urine samples were not collected during a regulated time period, which has inherent limitations due to sodium excretion having a circadian pattern, with sodium excretion being higher during the active phase and lower during the non-active phase (Nikolaeva *et al.*

2012). For this reason, 24h urine samples are considered the gold standard for the determination of sodium excretion (Amano *et al.* 2018, Swanepoel *et al.* 2018). Although we could not directly compare our spot and 24h sodium concentration data as they are not from the same cohort of animals, strain differences in sodium excretion were evident using both methods. Additionally, to determine sodium excretion, the standard method is to measure the fractional excretion of sodium, which takes into account both plasma and urine sodium, to eliminate the effect of water reabsorption (Li *et al.* 2016). We were unable to undertake plasma sodium analysis due to technical limitations, nevertheless, using normalised 24h sodium excretion to some extent overcomes this shortcoming and again supported a consistent significant difference between the strains. Further, we did not measure sodium intake and while no difference in sodium excretion was detected between sham and total RDN Lewis rats, a mismatch between sodium intake and output might have occurred following total RDN. Finally, the first urine sample collection occurred one-week post-surgery, and differences in sodium excretion between groups may have been missed as any mismatch in sodium intake and output would have been corrected by pressure-natriuresis within the first 3 days following RDN.

6.6 Summary and perspectives

In this study, we have demonstrated for the first time that circulating and intra-renal renin, renal gene expression for renin, AGT, ACE2 and AT1αR as well as urine sodium excretion were all significantly less in the LPK compared to Lewis controls. We also demonstrate that in hypertensive PKD rats, total or afferent RDN has no impact on the RAAS or sodium excretion, which may explain the lack of blood pressure lowering effect of RDN in this model. Noting however that the lack of effect on RAAS or sodium excretion was also seen in normotensive controls, despite a blood pressure lowering effect being induced by total RDN in these animals. The mechanism underlying the response in the Lewis therefore is still unclear, and may due to other factors such as reduced peripheral vascular resistance (Osborn

and Foss 2017). In summary, the current work provided mechanistic insights on the impact of RDN in homeostatic pathways involved in long-term regulation of blood pressure in a model of PKD and identified for the first time a suppressed intra-renal RAAS relative to Lewis control. Future work is needed for a better understanding of the exact mechanism of suppressed intra-renal RAAS.

Table 6.1 List of primer pairs for RT–qPCR.

Genes	NCBI Reference	Primer Sequence (5'->3')		Primer concentration	Size (bp)
		Forward	Reverse		
Renin	NM_012642.4(Williamson *et al.* 2017)	CACTCTTGTTGCTCTGGACCT	GGGGTACCAATGCCGATCTC	600nM	250
AGT	NM_134432.2 (Chung *et al.* 2010)	CACGGACAGCACCCTATTTT	GCTGTTGTCCACCCAGAACT	600nM	103
ACE 1	NM_012544.1 (Paizis *et al.* 2002)	CACCGGCAAGGTCTGCT	CTTGGCATAGTTTCGTGAGGAA	300nM	97
ACE 2	NM_001012006.1 (Anton *et al.* 2008)	CCCAGAGAACAGTGGACCAAAA	GCTCCACCACACCAACGAT	300nM	64
AT1αR	NM_030985.4 (Sanguesa *et al.* 2017)	CACAGTGTGCGCGTTTCAT	GTAAGGCCCAGCCCTATGG	300nM	63
β-ACT	NM_031144.3 (Colegio *et al.* 2014)	GGTCCACACCCGCCACCAG	CGATGGAGGGGAAGACGGC	300nM	128
CYC-1	NM_001277194.1	GCATGGCTCCTCCCATCTAC	CCCATGCGTTTTCGATGGTC	300nM	139

AGT, angiotensinogen, ACE, angiotensin-converting enzyme, AT1αR, angiotensin type 1α receptor. β-ACT (β-actin), CYC1, cytochrome c1

Table 6.2 Fold difference in RAAS gene expression in the kidney

Gene	Lewis ΔCt	LPK ΔCt	Strain effect P value	Range fold difference (LPK v. Lewis)
Renin	5.8 ± 0.4	7.4 ± 0.2	0.014	0.34(0.29~0.40)
AGT	5.4 ± 0.6	8.6 ± 0.3	< 0.001	0.11 (0.09~0.14)
ACE 1	7.3 ± 0.3	5.7 ± 0.2	< 0.001	3.08 (2.73~3.47)
ACE 2	4.0 ± 0.2	7.9 ± 0.3	< 0.001	0.06 (0.05~0.08)
AT1αR	4.6 ± 0.1	5.3 ± 0.2	0.013	0.61 (0.09~0.14)

AGT, angiotensinogen, ACE, angiotensin-converting enzyme, AT1αR, angiotensin type 1α receptor

Table 6.3 Urine sodium excretion in 10 weeks old Lewis and LPK

	Lewis			LPK			Strain	Treatment
Denervation procedure	Sham	Total	Afferent	Sham	Total	Afferent		
n	3	4	4	4	4	4		
Urine sodium (mmol/L)	228 ± 5	222 ± 8	165 ± 26	52 ± 11	67 ± 6	62 ± 10	<0.001	0.07
Urine volume (ml)	10.2 ± 1.1	10.2 ± 0.9	10.4 ± 1.8	27.4 ± 1.7	26.6 ± 2.8	23.6 ± 5.2	<0.001	0.81
24h urine sodium (mmol/24h)	2.3 ± 0.3	2.3 ± 0.2	1.6 ± 0.2	1.4 ± 0.2	1.7 ± 0.1	1.3 ± 0.1	0.002	0.04

6.7 Figures

Figure 6.1 Experimental design of animal studies.

After the denervation or sham procedure at age 6 weeks, animals were monitored to age 10 weeks (cohort 1) or 14 weeks (cohort 2). At the end of the study, cohort 1 animals were euthanased and the kidneys collected post-mortem for later assessment of kidney renin and RAAS gene expression. 24h urine samples collected from these animals within 48 hrs of euthanasia were used for sodium analysis. Blood was taken at the time of euthanasia for later analysis of plasma renin content. Fortnightly spot urine samples were from cohort 2 animals collected by putting the animals in a metabolic cage for 4-5 hrs for urine sodium analysis.

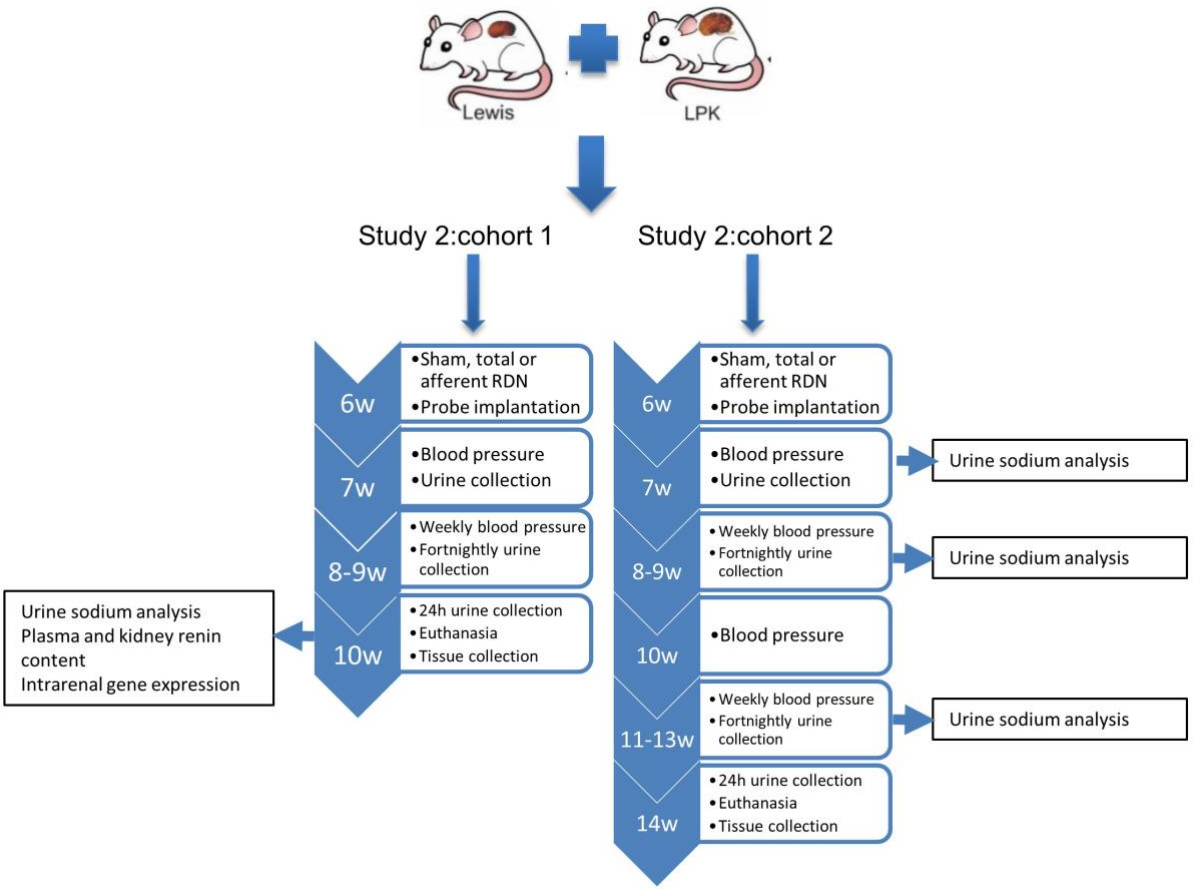


Figure 6.2 Plasma (A) or kidney (B) renin content in Lewis and LPK rats.

Panel A illustrates plasma renin content in Lewis and LPK demonstrating levels within treatment groups for each strain, noting while there was a significant strain effect, there was no overall treatment effect. * $P < 0.05$ between strains as indicated. N= 4-5 per treatment per strain. Panel B illustrates strain and sex differences in kidney renin content. * $P < 0.05$ vs. sex-matched Lewis animals ** $P < 0.05$ between sex within strain as indicated. N =6-7 per strain per sex.

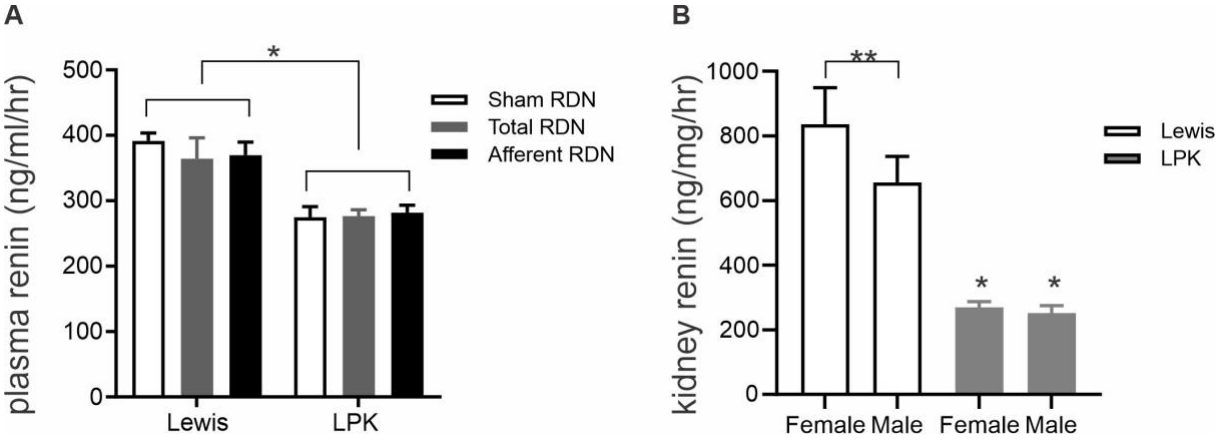


Figure 6.3 The effect of sex and strain on kidney angiotensinogen (AGT) mRNA expression.

The average threshold cycle (Ct) value for each animal was normalised to the expression of two housekeeping genes β-actin and cytochrome c1. Statistical significance between sex/strain groups was determined by comparing values after normalisation to housekeeping genes (ΔCt values), using a Tukey *post hoc* analysis after a univariate GLM analysis of variance which identified a sex and strain but not treatment effect, as illustrated in the figure. The fold range of AGT expression relative to female Lewis was determined using the $2^{-(\Delta\Delta Ct \pm \text{Standard Error (SE) of the } \Delta Ct)}$ method and presented. ** $P < 0.05$ between sex within strain as indicated. * $P < 0.05$ vs. sex-matched Lewis animals. N=6-7 per strain per sex.

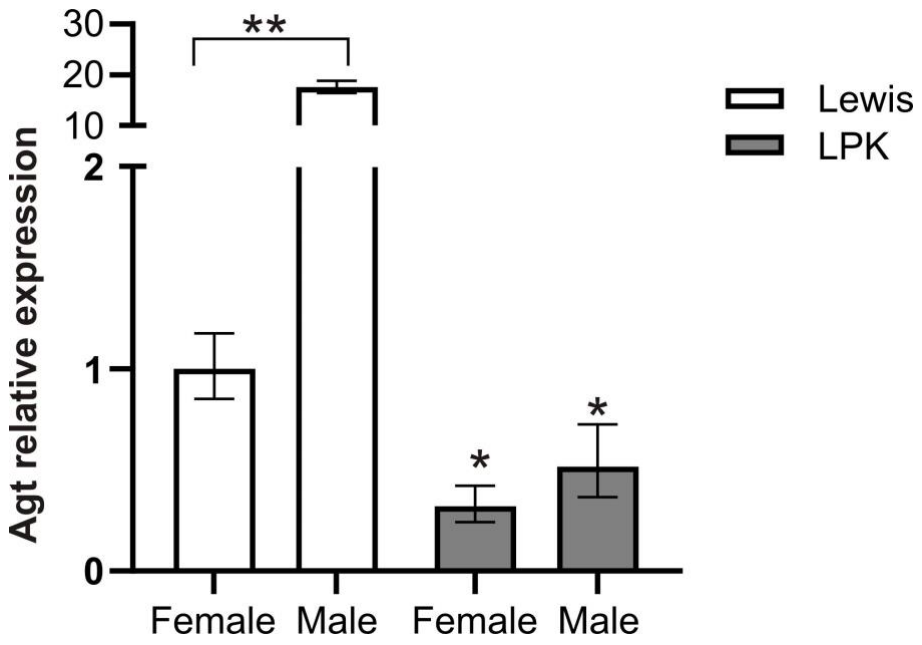
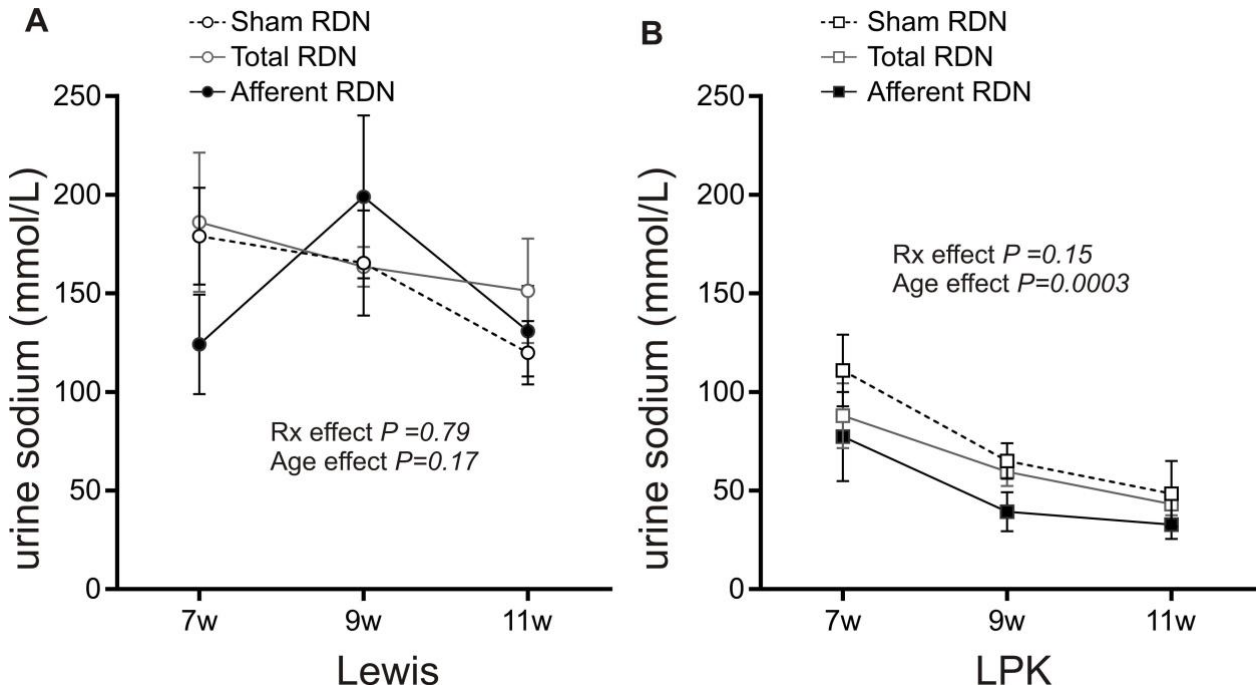


Figure 6.4 Urine sodium concentration in Lewis (A) and LPK (B) rats at 7, 9 and 11 weeks old after sham, total and afferent RDN procedures.

Rx, treatment effect. Data is expressed as mean ± SEM, analysed using two-way ANOVA and Bonferroni's *post hoc* analysis. Overall age and treatment effects are provided in each panel. N values at each week for Lewis sham = (6, 7, 7); Lewis total = (5, 5, 5); Lewis afferent = (7, 7, 7), respectively and for LPK sham = (9, 9, 5), LPK total = (10, 10, 5) and LPK afferent = (6, 6, 5), respectively.



Appendix Chapter 6

Renal renin expression localisation in the Lewis and LPK rat

S6.1 Method

To investigate the pattern of renin expression in the LPK rat and its control strain Lewis, immunohistochemistry was undertaken using renin and the renal tubule markers aquaporin-1 (AQP1), which is a marker for the proximal convoluted tubule and the thin descending limb of Henle's loop (Maunsbach *et al.* 1997); peanut agglutinin (PNA), which is expressed by distal convoluted tubules (Loghman-Adham *et al.* 2005) and aquaporin-2 (AQP2), which is a marker for cortical and medullary collecting ducts (Kishore *et al.* 2000). Based on a preliminary examination of the tissues, smooth muscle actin (SMA) was also used to confirm the expression of renin in vascular tissue (Kretschmer *et al.* 2013). Experiments were performed on tissues collected from 7- and 10-weeks old sham LPK and Lewis from study 1 and study 2 cohort 1, respectively.

The protocol for kidney collection and processing is detailed in Chapter 2, Sections 2.4 and 2.5. Two-hour blocking of kidney sections at room temperature was carried out using blocking solution of 0.01M Tris phosphate buffered saline (TPBS, 10 mmol/L Tris buffer, 0.9%NaCl, 10 mmol/L phosphate buffer -NaH2PO4/Na2HPO4, pH 7.4). The primary antibody for renin in combination with one of the markers was incubated at 4 °C for 48 hrs, followed by incubation with species-specific fluorescent-conjugated secondary antibodies for 4 hrs at room temperature. All secondary antibodies were used at a dilution of 1:500 and were from Jackson Immunoresearch Laboratories (San Francisco, Calif., USA) except for

fluorescein-labelled peanut agglutinin (PNA), which did not require a secondary antibody for signal visualisation. All primary and secondary antibodies were diluted using 0.01M TPBS with 10% donkey serum, 0.3% Triton-X 100 and 0.05% thimerosal (Sigma, USA). After incubation, sections were washed in 0.1M TPBS (3×15 mins), coverslipped using fluorescence mounting medium (Dako, CA, USA), and dried in the dark for 12-24 hrs. Negative controls were kidney sections incubated in the blocking solution omitting the primary antibody. Preliminary experiments were carried out to determine the optimal concentration of each primary antibody.

Sections were viewed using a ZENPRO epifluorescence microscope (Zeiss, Gottingon, Germany). All sections were examined under the microscope and a representative area labelled with renin and/or tubule markers imaged using either 10x or 20x objective depending on the size of the region of interest. A total of three animals for each strain each age were imaged.

Table S6.1 Primary and secondary antibodies used for kidney immunohistochemistry

Primary antibodies	Dilution and source	Secondary antibodies	Antibody target/marker
Polyclonal goat anti-renin antibody, IgG isotype	1:500, Santa Cruz, serial code, sczsc27318, California, USA	Donkey anti-goat Cy3 or 488 – conjugated antibody, Jackson ImmunoResearch, Pennsylvania, USA	Renin
Polyclonal rabbit anti-AQP1	1:100, Alomone, serial code AQP-001. Jerusalem, Israel	Donkey anti-rabbit Cy3-conjugated antibody, Jackson ImmunoResearch, Pennsylvania, USA	Proximal convoluted tubule and thin descending limb of Henle's loop (DL)
Polyclonal rabbit anti-AQP2	1:2000, Alomone, serial code AQP-002. Jerusalem, Israel	Donkey anti-rabbit Cy3-conjugated antibody, Jackson ImmunoResearch, Pennsylvania, USA	Cortical and medullary collecting ducts
Fluorescein-labelled Peanut Agglutinin (PNA)	1:2000, Vector labs, product code FL1071, California USA	NA	Distal convoluted tubule
Monoclonal mouse α-smooth muscle actin clone 1 A4, IgG2a Isotype	1:500, Sigma, product code A5228, New York, USA	Donkey anti-mouse Cy3-conjugated antibody, Jackson ImmunoResearch, Pennsylvania, USA	Vascular α-smooth muscle actin

S6.2 Results

Renin expression pattern and localisation

Renin staining in both the Lewis and LPK animals aged 7 and 10 weeks was evident in the juxtaglomerular apparatus of the afferent arteriole in close association with the glomerulus (Figure S6.1).

Based on the work of Torres et al (Torres *et al.* 1992) and Loghman-Adham et al (Loghman-Adham *et al.* 2004, Loghman-Adham *et al.* 2005), who showed ectopic expression of renin in small arterioles and the interlobular artery as well as in cystic tubules of human ADPKD and ARPKD patients, experiments were carried out double labelling for renin and specific markers against different segments of renal tubules. From these experiments it was evident that in the LPK, cysts are derived from both collecting ducts, proximal tubules and the distal convoluted tubule due to labelling of cysts with AQP2, AQP1 and PNA respectively (Figure S6.3). In both the 7- and 10-weeks old animals, there was no co-localization however of renin with any of the tubular markers in either the Lewis (Figure S6.2) or LPK (Figure S6.3). Renin was however evident in structures that were consistent with vasculature tissue, which was confirmed by double labelling using antibodies directed against α-SMA (Figure S6.4).

No staining was seen when sections were incubated with solutions omitting primary renin antibody, confirming specificity (Figure S6.1 panel c and f).

S6.3 Figures

Figure S6.1 Representative images of renin staining in 7 (a and d) and 10 (b and e) weeks old Lewis (a-c) and LPK (d-f) in the renin-producing granular cells within the afferent arteriole component of the juxtaglomerular apparatus.

Panel c and f are negative controls omitting primary renin antibody. Arrows indicate positive staining and glomeruli are indicated by (*). Scale bar in lower panel = 100 µm for all images.

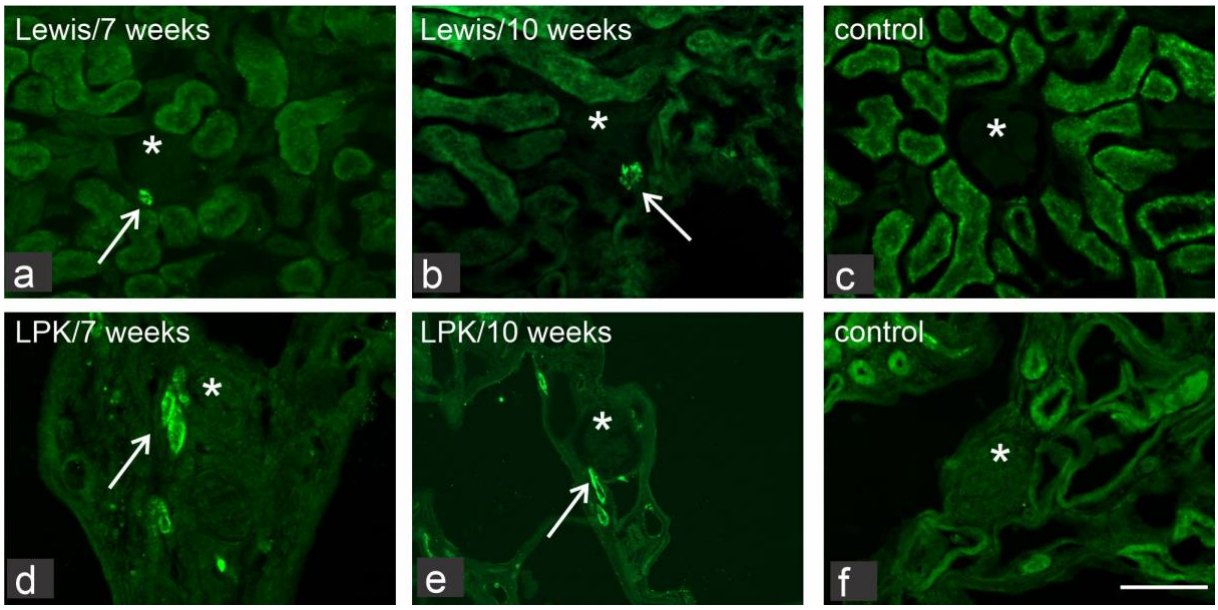


Figure S6.2 Representative images of double labelling of renin (a, d, g and j) and tubule segments marker AQP1 (b and e), AQP2 (h), PNA (k) in 7- or 10-weeks old Lewis rat kidney.

Merged images are shown in c, f, i and l. Two AQP1 images are provided due to the observation that renin was restricted to the cortical area while AQP1 staining was exclusively evident in the medullary area. Arrows indicate positive staining and scale bar in lower panel = 100 µm for all images.

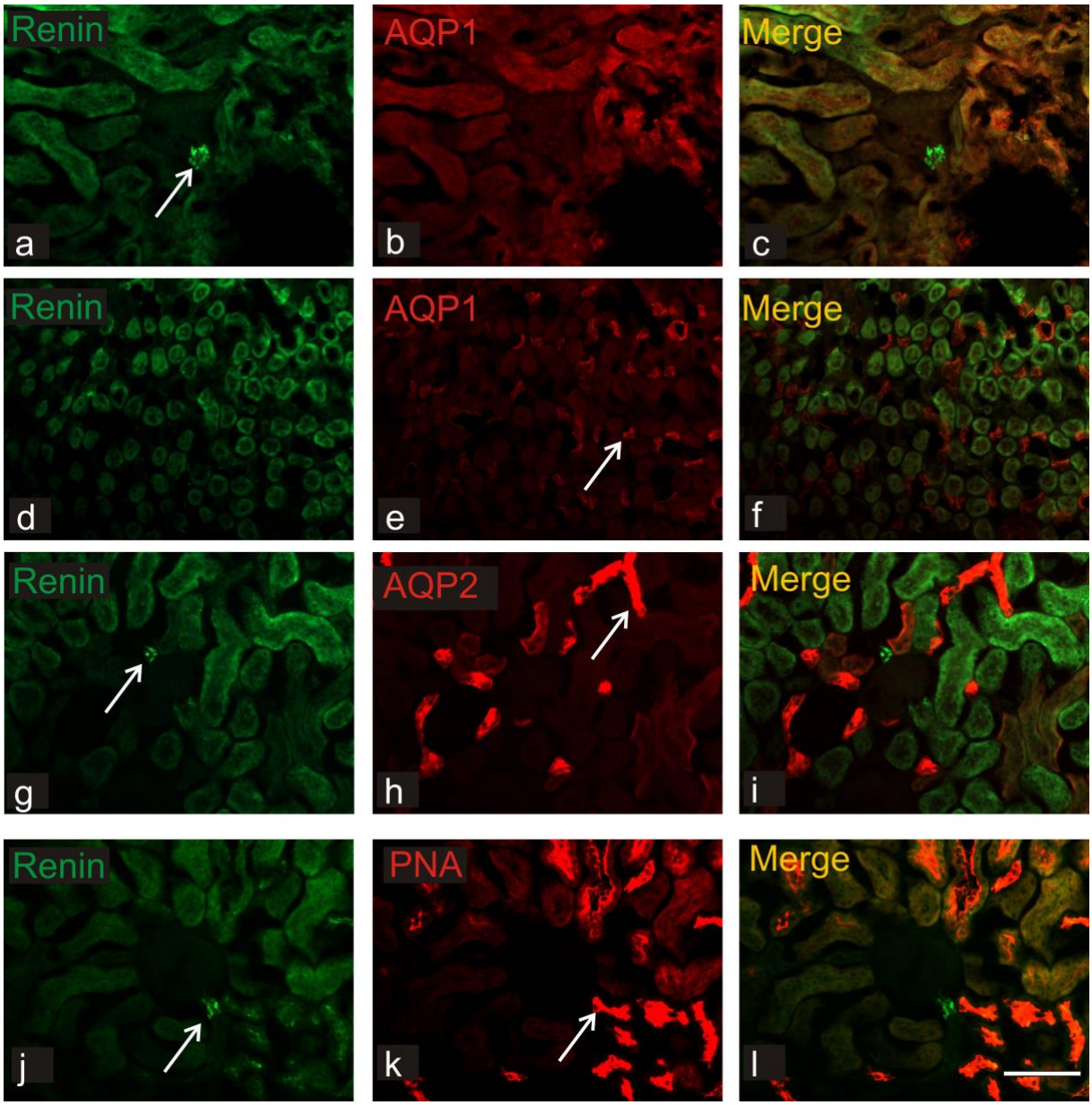


Figure S6.3 Representative images of double labelling of renin (a, d, g and j) and tubule segments marker AQP1 (b and e), AQP2 (h), PNA (k) in 7 or 10 weeks old LPK rat kidney.

Merged images are shown in c, f, i and l. Two AQP1 images are provided due to the observation that renin was restricted to the cortical area while AQP1 staining was exclusively evident in the medullary area. Arrows indicate positive staining and scale bar each panel = 100 µm.

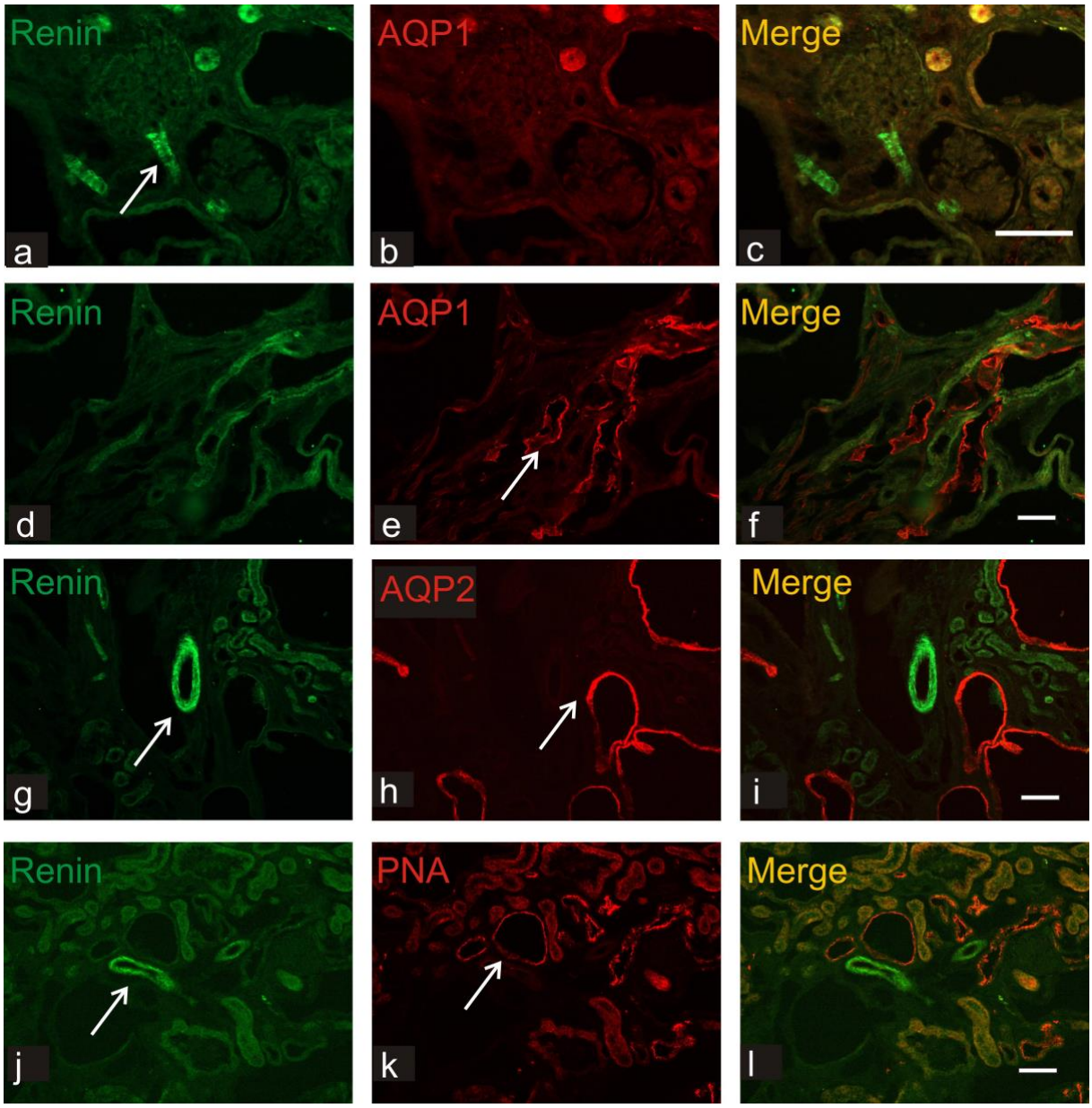
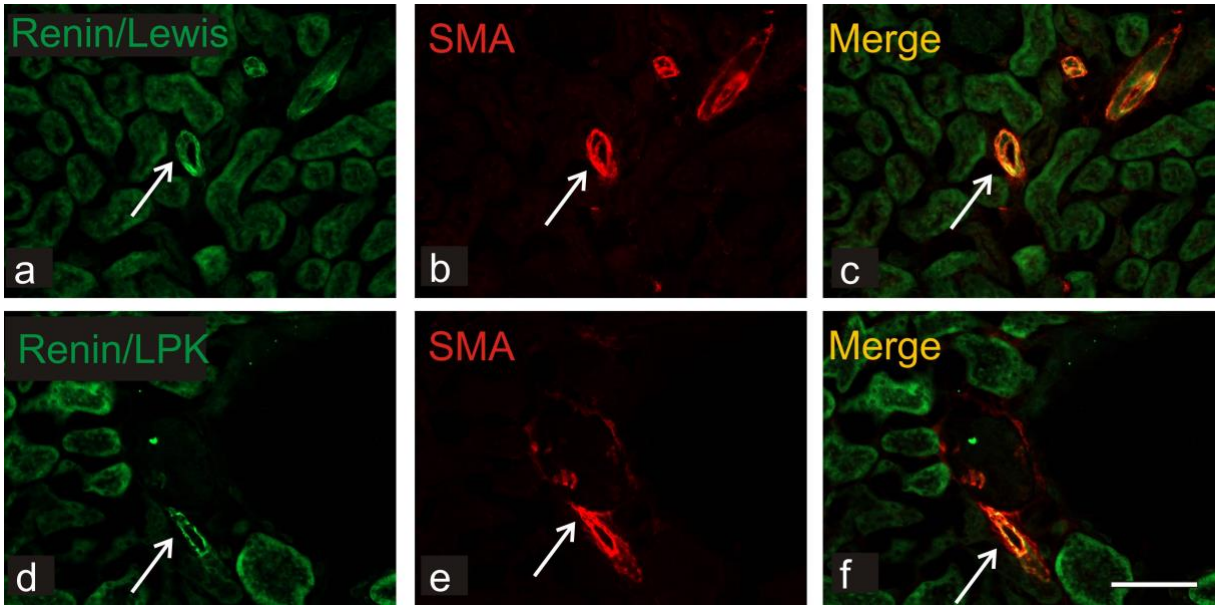


Figure S6.4 Representative images of double labelling of renin and SMA in 7w old Lewis (top row) and LPK (bottom row) in the blood vessels.

Arrows indicate positive staining and scale bar in lower panel = 100 µm for all images



Chapter 7 Final discussion

Hypertension is a common comorbidity in chronic kidney disease (CKD) (Sarafidis *et al.* 2008) and adds to the already increased risk of stroke, left ventricular hypertrophy and heart failure in this population (Go *et al.* 2004). Hypertension becomes more prevalent and challenging to control as renal function declines (Horowitz *et al.* 2015). In practice, a subset of patients with CKD suffers from resistant hypertension, whereby target blood pressure cannot be achieved with at least three different types of antihypertensive drugs, (Borrelli *et al.* 2013, Unni *et al.* 2015, Verdalles *et al.* 2016). Investigation of both the cause and more effective treatment strategies for hypertension is therefore paramount to reduce the overall risk of adverse cardiovascular and renal outcomes in this population. In view of this, the present thesis aims to address the following questions about renal denervation (RDN), a technique being trialled for hypertension management:

1. Is the destruction of either or both the renal sensory and sympathetic nerves responsible for the reported blood pressure lowering effect of RDN?
2. Does the blood pressure lowering effect persist after the nerves supplying the kidney have regrown? In other words, does renal reinnervation dampen the blood pressure lowering effect?
3. Are other health benefits, such as an improved autonomic function or renal function derived from RDN?
4. Does RDN impact homeostatic pathways involved in blood pressure regulation, such as the renin-angiotensin-aldosterone system (RAAS) and renal handling of sodium?

7.1 Renal denervation did not limit hypertension in a rodent model of polycystic kidney disease (PKD)

Renal denervation is being touted as a promising treatment for hypertension and CKD, based on the rationale from an initial series of preclinical animal studies (Winternitz *et al.* 1980,

Thukkani and Bhatt 2013) and clinical trials (Krum *et al.* 2009, Esler *et al.* 2012) that increased renal sympathetic nerve activity was involved in the development and maintenance of hypertension. Though not now without some careful considerations based on the poor outcomes of the large scale randomised clinical trial Symplicity III (Bakris *et al.* 2014, Bakris *et al.* 2015), there is consensus that specific populations of hypertensive patients may be more or less susceptible to a positive outcome. What remains unknown is the shared feature of these potential responders. For example, does the sympathetic nervous activity (SNA) in the patients have to be elevated to achieve a blood pressure reduction and if yes, how much should it be increased? Are patients with a certain type of kidney disease aetiology, for example, polycystic kidney disease (PKD), more likely to benefit from the procedure given evidence of elevated muscle SNA (Klein *et al.* 2001)? This thesis provides strong pre-clinical evidence that total RDN, as undertaken by stripping of the renal nerves followed by periaxonal application of 10% phenol, did not attenuate the sustained hypertension, as evaluated using telemetry, in a Lewis polycystic kidney disease rat (LPK) model (Chapter 4). Despite evidence of an increased sympathetic tone, especially with regard to renal sympathetic tone in this model (Phillips *et al.* 2007, Ameer *et al.* 2014, Salman *et al.* 2014, Yao *et al.* 2015), our data suggests that in this model of PKD, which is an orthologue to human nephronophthisis 9 (NPHP9) and resembles juvenile autosomal recessive PKD (ARPKD), total RDN, which eliminates sympathetic outflow to one vascular bed, is not sufficient to reduce blood pressure.

There is a general belief that in the context of CKD, the diseased kidney causes overactivation of renal afferent sensory nerves, which send inputs to the central nervous system and produces a generalised increase in sympathetic outflow promoting vasoconstriction and sodium and water retention, thus favouring the development and/or maintenance of hypertension (Campese and Kogosov 1995, Campese *et al.* 1995, Ciriello and de Oliveira 2002, Xu *et al.* 2015, Frame *et al.* 2016). The mechanism for proposed activation of renal

afferent nerves in CKD is poorly understood, with adenosine signalling (Katholi *et al.* 1985) and inflammatory signals (Frame *et al.* 2016) being potential mediators. This raises the question as to whether the removal of renal sensory nerves contributes to the blood pressure lowering effect observed in clinical trials. The current thesis, however, demonstrated that selective afferent RDN, as undertaken by the relatively new procedure of applying capsaicin to the renal nerves, had no impact on hypertension in the LPK animal model (Chapter 4). Selective interruption of renal sensory nerves reduced hypertension in a number of animal models including two-kidney-one-clip hypertension (Katholi *et al.* 1982), deoxycorticosterone acetate (DOCA) salt hypertension (Banek *et al.* 2016) and the 5/6 nephrectomy model (Campese and Kogosov 1995), proposedly by altering central pathways involved in sympathetic outflow and blood pressure regulation (Campese and Kogosov 1995, Nishimura *et al.* 2007, Frame *et al.* 2016). Therefore, the lack of blood pressure lowering effect after afferent RDN in our model may be due to an inability of the procedure to impact the central pathways and subsequent outflows involved in driving hypertension in PKD. Another possibility is that the assumed overactivation of afferent nerves as a result of renal tissue hypoxia in this model (Ow *et al.* 2014), though not yet confirmed using direct renal sensory nerve recording, doesn't feed forward to drive an increase in blood pressure. This raises the question as to to what degree the renal afferent nerves needs to be overactivated to affect the arterial blood pressure. Banek et al. (Banek *et al.* 2016) demonstrated a two-fold increase in resting renal afferent nerve activity in the DOCA salt model compared to control rats, and in this model removing the afferent nerves could attenuate ~50% of the increase in blood pressure. Therefore, it is possible that the renal afferent nerve activation in our model is not sufficient to evoke an increase in blood pressure.

Our previously identified factors that contribute to hypertension in this model include generalised overactivation of the sympathetic nervous system (Phillips *et al.* 2007, Salman *et al.* 2015) and increased excitatory regulation of the hypothalamic paraventricular nucleus

(Underwood *et al.* 2019). A lack of blood pressure lowering effect of both RDN procedures, suggests that the procedures did not have a functional impact on these central and peripheral autonomic pathways driving hypertension in PKD. We have also demonstrated previously in this model that while having marked indices of vascular stiffness (Ng *et al.* 2011, Quek *et al.* 2016), there was no impact on arterial stiffness after total RDN (Yao *et al.* 2017). The current study further demonstrated that neither procedure affected sodium excretion in LPK (Chapter 6), indicating the renal nerves do not directly influence these parameters in this model.

Another key observation in this study was that at 10 weeks of age, the LPK had a relatively lower level of circulating and intrarenal renin content compared with Lewis animals as well as lower renal mRNA expression levels of renin, angiotensinogen, angiotensin-enzyme-converting 2 (ACE2) and the angiotensin type 1 receptor (AT1R) AT1αR (Chapter 6). This raises the question as to what causes the suppression of the RAAS in LPK, particularly as increased renal SNA is elevated (Phillips *et al.* 2007, Salman *et al.* 2014, Salman *et al.* 2015), and is proposed to act to increase RAAS activity. It may be secondary to severe hypertension, which results in a higher perfusion pressure at juxtaglomerular cells which suppresses renin release (Sahay and Sahay 2012). Alternatively, in PKD it might be attributable to a reduction in the number of juxtaglomerular cells as a result of cyst formation and compression. Extending this notion, the increased renal SNA would, therefore, have a limited physiological ability to stimulate renin release due to the loss of effector cells. Therefore, it is possible to speculate that in this model either suppression by ongoing hypertension or loss of juxtaglomerular cells or both contribute to a suppressed renin release and associated low both circulating renin and angiotensin II and intra-renal RAAS activity [(Phillips *et al.* 2007)and Chapter 6], which could not be recovered by increased renal SNA. Future studies comparing the level of intra-renal renin earlier in the disease process may help determine if suppressed renin is due to severe hypertension. Additionally, investigations in the number of renin-secreting cells using immunohistochemistry or in situ hybridisation may provide further

insight into the cause of low renin. Despite a lower the circulating and intra-renal renin level, these animals were responsive to RAAS inhibition as a means by which to reduce blood pressure (Ameer *et al.* 2016, Quek *et al.* 2018), which may suggest a pathological role of the RAAS in the development of hypertension. In an early study by Chapman et al. (Chapman *et al.* 1990), plasma renin activity was higher in the supine position and upright position in the patients with autosomal dominant PKD (ADPKD) compared to patients with essential hypertension, but seemed comparable relative to normotensive PKD patients. Nevertheless, PKD and essential hypertensive patients responded to angiotensin-converting enzyme inhibitor with a similar blood pressure reduction (Chapman *et al.* 1990). Increased intra-renal RAAS was reported in *pck* rat, rodent model of ARPKD (Goto *et al.* 2010), however the hypertension in this model was of less magnitude compared to our model. The involvement of the RAAS in hypertension associated with PKD may differ between various genetic background, but the benefit of RAAS inhibitors is nevertheless universal.

7.2 Does RDN bring benefits beyond blood pressure reduction?

Apart from the blood pressure lowering effect, another commonly asked question is could RDN produce benefits beyond and possibly independent of blood pressure control? Observations from the present work showed that neither total or afferent RDN affected renal function parameters in the LPK model, as determined by plasma urea, creatinine, urine protein to creatinine ratio and creatinine clearance rate (Chapter 4). This contrasts to previous studies where RDN has been shown to slow kidney disease progression in CKD patients (Ott *et al.* 2015, Hering *et al.* 2017) and animal models (Nakashima *et al.* 1996, Nagasu *et al.* 2010, Singh *et al.* 2019). Although our work did not see a renoprotective effect from RDN, it is also important to note that we did not see a deterioration of renal function either which is an important clinical consideration.

Renal denervation has also been shown to improve cardiovascular autonomic dysfunction in hypertensive patients (Hart *et al.* 2013, Tsioufis *et al.* 2014) and animal models of CKD (Dias

et al. 2011, Chen *et al.* 2016, Lincevicius *et al.* 2017). Autonomic dysfunction, which manifests as increased systolic blood pressure variability (SBPV), a reduced heart rate variability (HRV) and impaired baroreceptor sensitivity (BRS) is present in the LPK rodent model (Hildreth *et al.* 2013), associated with impaired afferent and central baroreflex signalling pathways (Salman *et al.* 2014, Salman *et al.* 2015). Observations from the current study showed that neither total or afferent RDN produced a beneficial effect on HRV, SBPV or BRS, suggesting that renal afferent input per se does not interact with the central processing of this reflex responses. Overall, Chapter 5's findings indicate that RDN did not improve the autonomic function in the LPK rodent model.

7.3 RDN lowers blood pressure in normotensive rats

Despite a lack of treatment effect of RDN in the LPK, we observed a sustained blood pressure lowering effect in normotensive Lewis rats after total RDN, which was of comparable magnitude to that observed in other normotensive animal models (Jacob *et al.* 2003, King *et al.* 2007). The lack of blood pressure effect after afferent RDN in the Lewis is not necessarily surprising given that the kidneys in these animals are healthy and the afferents are not being subjected to ongoing abnormal stimuli. These findings support the well-established role of the renal sympathetic efferent nerves in the regulation of blood pressure under normotensive conditions (Johns *et al.* 2011). Our data would suggest the mechanism causing the reduction in blood pressure was not mediated by denervation driven natriuresis or a reduction in neurally driven renin release, as total RDN had no demonstrated impact on sodium secretion, circulating or intra-renal RAAS (Chapter 6). It has been suggested that the sustained blood pressure decrease in normotensive animals is due to a reduction in renal vascular resistance after a loss of renal vasoconstrictor tone (Osborn and Foss 2017). Future experimental work to measure the arterial blood pressure and renal blood flow before and after RDN in conscious rats can provide more insights on this. This might be achieved by implantation of a probe to measure the renal blood flow and is currently under investigation

by other researchers (Osborn and Foss 2017). The blood pressure lowering effect after total RDN in normotensive animals is also clinically relevant, as this may suggest that part of the blood pressure reduction observed in hypertensive patients may be not directly related to mechanisms that contributes to hypertension.

7.4 Renal reinnervation did not impact blood pressure lowering effect.

While direct evidence of reinnervation in humans is limited and indeed difficult to obtain, there is growing anatomical evidence indicating that the renal nerves reinnervate the kidney after RDN (Kline and Mercer 1980, Mulder *et al.* 2013, Booth *et al.* 2015), most recently demonstrated in an large animal model where catheter-based renal denervation was used (Booth *et al.* 2015, Singh *et al.* 2019). This raises an important question as to whether the renal reinnervation has restored functionality that would dampen the blood pressure lowering effect by RDN and if yes, over what time frame the reinnervation will become complete. The current thesis provides some insight on the anatomical reinnervation, with histological demonstration of both TH and CGRP nerves re-establishing their networks by 4 weeks post-RDN procedure. Our study also demonstrates that by 8 weeks post denervation,the level of renal sympathetic nerve markers after total RDN was not significantly different compared to shams, suggesting a complete reinnervation, consistent with previous reports where complete reinnervation was evident between 9-12 weeks in rats (Mulder *et al.* 2013). Nevertheless, complete reinnervation was evident at 11 months post-RDB in sheep (Booth *et al.* 2015).

To counter the impact of reinnervation, we repeated the procedure at age 10 weeks. In the Lewis rats, this had no additional impact on arterial pressure (chapter 4) compared with a single procedure however produced beneficial impacts on autonomic function. In the LPK rats, repeating total RDN caused a transient increase in arterial pressure and a worsening of autonomic function (Chapter 5). In an interesting case study by Lee et al. (Lee *et al.* 2017), it was reported that a 31-year-old female patient with resistant hypertension did not respond to either a first or second RDN, but responded to celiac plexus block. In the LPK model of CKD,

it is tempting to, therefore, speculate that the neurogenic components that contribute to hypertension in this model are not contributed to in any major fashion by the renal sympathetic nerves. Radical sympathectomy, being surgical removal of splanchnic nerves, which transmits sensory signals and regulates the functions of abdominal organs, could remarkably reduce blood pressure in patients with severe hypertension before the advent of antihypertensive drugs (Peet 1948, Smithwick and Thompson 1953, Evelyn *et al.* 1960). It is possible that this method may also work in our model but given the high rate of complications of this technique, it could only be one theoretical solution.

Findings from clinical studies showing that catheter-based RDN can produce a sustained reduction in blood pressure for up to three years (Esler *et al.* 2014, Mahfoud *et al.* 2019), suggest that reinnervation does not ameliorate the initial blood pressure lowering effect of RDN. But how this long-term effect is mediated remains unclear. In a sheep CKD model, catheter-based RDN produced a sustained reduction in blood pressure that was associated with only partial regrowth and return of function, as demonstrated by reduced nerve markers and reduced vasoconstriction to nerve stimulation 30 months post-procedure (Singh *et al.* 2019). Notably, control animals showed complete anatomical and functional recovery in the same time frame although it was shown that the initial level of both renal sensory and sympathetic innervation in the CKD animals was greater, suggesting that the RDN effectively normalised the hyperinnervation. It is possible that in patients with hypertension, a correction of hyperinnervation similar to that observed in sheep CKD model results in the sustained blood pressure lowering effect. One way to confirm this in humans is by measuring renal noradrenaline spillover with available evidence demonstrating a reduction in renal noradrenaline spillover one month after RDN in patients with resistant hypertension (Krum *et al.* 2009). In those studies where a sustained effect is evident, both clinical and preclinical, renal denervation may have permanently reset the central regulating pathways that otherwise contribute to the hypertensive state (Phillips 2015), as demonstrated in the 5/6 nephrectomy

model where increased turnover of noradrenaline in the posterior and lateral hypothalamus was prevented by afferent RDN (Campese and Kogosov 1995) or in the unilateral nephrectomized SHR model where total RDN caused a reduction of angiotensin-converting enzyme 1 (ACE1) and angiotensin type 1 receptor (AT1R) mRNA expression in the hypothalamus and lower brainstem (Nishimura *et al.* 2007). Further examination of this in hypertensive animal models which demonstrate a prolonged hypotensive effect to RDN, looking at the alterations in neurochemical, and humoral factors in the central nervous system will be important to undertake if we are to establish the mechanism by which this procedure reduces blood pressure.

7.5 Methodological considerations

Discussion associated with experimental issues is provided in each result chapter however a major consideration for this study is the effectiveness of the intervention used to denervate the kidney. Immunohistochemistry is a commonly used method to validate such an experiment with the premise that the sympathetic and sensory nerves express specific protein markers, whose absence after denervation serves to confirm the absence of that nerve sub-population. Our immunohistochemical data provides convincing data that at one-week post denervation the nerves have been damaged or removed from an anatomical perspective, however immunohistochemical methods at best provide a semi-quantitative approach, which cannot not be directly translated into a complete loss of the functionality of the nerves. Additional quantitative measurements of renal content of nerve markers by Western-blot or high-performance liquid chromatography (HPLC) and/or functional validation of the denervation would add strength of evidence. This would also be valuable when examining the kidneys for reinnervation at 4 and 8 weeks post RDN, to determine if the immunohistochemistry is reflected by an alternative quantitative measure of innervation. Noting this will be more readily applicable to the level of sympathetic innervation as compared to the levels of sensory nerve markers which are concentrated in the renal pelvis and as such tissue availability for

multiple processing methods becomes problematic. Western-blot and HPLC were attempted for this thesis however problems associated with the methodology could not be resolved prior to the end of the candidature period. Future studies using these alternative approaches will be of great value.

Our measurement of blood pressure, undertaken by telemetry recording is considered the golden standard for the determination of blood pressure, being free of many of the confounding factors associated with tail-cuff recordings such as restraint-stress and external warming (Norman *et al.* 1981, Van Vliet *et al.* 2000). Despite its advantages, probe failure due to loss of battery life or signals was seen in our study, which limited the total number of animals with complete data through to 14 weeks of age. However great efforts were made to ensure telemetry data from a minimum of 8 animals during the 7-10 week period and from a minimum of 3-4 animals during the 11-14 week period. Despite the small sample size, our power analysis provides confidence that the blood pressure lowering effect observed in the Lewis was real, and that the lack of treatment effect in the LPK was unlikely to be due to the small sample size. Hypothetically, more animals could be utilised in our study, but there are ethical considerations given our analysis indicates it would be unlikely to affect the result.

Another important consideration is the genetic PKD model, the LPK, used across all studies, which displays resistance to both total and afferent RDN intervention. This may imply a strong influence of the genetic background on the phenotype and highlight the importance to use animal models in parallel with human diseases to identify patients where this approach should be used with caution. Future experiments in animal models of CKD that arises from a different gene mutation such as (*pck* rat) or from different types of kidney damage may be undertaken to provide preclinical evidence of the efficacy of the RDN intervention.

7.6 Perspectives

The pathogenesis of hypertension in CKD is complicated, with various factors contributing or exacerbating the established hypertension, including RAAS overactivation, sympathetic

overactivation, vascular remodelling and reduced renal function (Andersen and Agarwal 2005). These factors interact with each other to result in the development of hypertension, further renal dysfunction and thus increasing the risk of cardiovascular complications. Renal nerves function to link the activity of the autonomic nervous system and the kidney and are thought to play a critical role in the development of hypertension in a number of different disease states including CKD (Osborn and Foss 2017). In that regard, this thesis has demonstrated a lack of effectiveness for RDN to attenuate hypertension in a rat model of juvenile-onset PKD, alongside no impact on other homeostatic pathways such as renal and autonomic function. This work further stresses the importance of the identification of suitable patient cohorts who will benefit from RDN. As discussed in Chapter 4, in a previous study in rodents with an adult-onset form of PKD (Gattone *et al.* 2008), beneficial effects on blood pressure were observed. It is possible that even within a family of genetic diseases such as PKD, the underlying pathology can be different and that all patients shouldn't be grouped together when considering treatment strategies. Clinical experience of treating hypertensive PKD patients by RDN is limited with inconsistent outcomes (de Jager *et al.* 2017, Pietilä-Effati *et al.* 2018). Moreover, the patients studied with PKD to date have been exclusively ADPKD patients. Taken in context with this study, utilising a juvenile-onset model of PKD, it would suggest that comparable patients – i.e. individuals with juvenile ARPKD and NPHP might not be suitable candidates for this treatment and great care should be taken to prevent unnecessary denervation procedures being performed. An important continuation of the present work is ongoing research into what are the primary mechanisms that drive an increase in blood pressure and associated cardiovascular risks this brings.

Chapter 8 Reference

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Appendix 1

MACQUARIE University **ANIMAL RESEARCH AUTHORITY (ARA)**

AEC Reference No.: 2015/036 **Date of Expiry:** 16 December 2016

***Full Approval Duration:** 17 December 2015 to 16 December 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).**

Principal Investigator:
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In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above
or Manager, CAF: 9850 7780 / 0428 861 163 and **Animal Welfare Officer:** 9850 7758 / 0439 497 383

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

Title of the project: **Investigating the role of the kidney sensory nerves in the development of high blood pressure in a rodent model of kidney disease**

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

Aims: **To Investigate:**

1. **Whether destruction of either or both the sensory or sympathetic renal nerves is responsible for the blood pressure lowering effect of renal denervation**
2. **If these results persist after the nerves supplying the kidney have regrown**
3. **Where the changes are in the brain that underlie the blood pressure response**
4. **What are the neurochemical alterations in the brain**
5. **What other health benefits are derived, such as improved autonomic outflow**

Surgical Procedures category: **5 - Major Surgery with 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 - Rat	Lewis	4-16 weeks /NA/Any	204	Animal Resources Centre, Perth
02 - Rat	Lewis Polycystic Kidney	4-16 weeks/NA/Any	204	Animal Resource Centre, Perth
			408	

Location of research:

Location	*Full street address*
Central Animal Facility	Building F9A, Research Park Drive, Macquarie University, NSW 2109
FMHS Laboratory	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 December 2015**

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