Hypertension in an animal model of polycystic kidney disease is not dependent on the short-term actions of endogenous tumour necrosis factor-α in the subfornical organ

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Originality Statement:

This work is an original piece of research that has been written by me and has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

This research was approved by the Macquarie University Animal Ethics Committee under the Animal Research Authority 2018/016.

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Declaration of Contributions:

CM Hildreth and JK Phillips contributed to the conception and design of the study. CM Hildreth assisted with the review of the thesis. CU Underwood performed the in vivo microinjections of LPK animals in the isoguvacine only cohort.

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Abstract:

Introduction | The development of hypertension in an animal model of polycystic kidney disease, the Lewis polycystic kidney (LPK) rat, is caused, in part, by the overactivation of the subfornical organ (SFO). Circulating proinflammatory cytokines, namely tumour necrosis factor-α (TNFα), are suggested to act in the central nervous system to produce an increase in neuronal excitability. Therefore, we hypothesised that TNFα acts on the SFO to increase neuronal activity, therefore contributing to the development of hypertension.

Methods | Urethane-anaesthetised Lewis control (*n*=20 total) and LPK (n=19 total) animals underwent microinjections of TNFα or tumour necrosis factor receptor 1 antibody (TNFRI Ab) or minocycline, an inhibitor of microglial activation, followed by a $GABA_a$ agonist into the SFO. Microglial activation in the SFO was assessed by staining with an anti-Iba1 antibody (*n*=2 Lewis and *n*=3 LPK).

Results | Microinjection of TNFα into the SFO led to a dose-dependent increase in mean arterial pressure (MAP) in Lewis animals ($P<0.01$) but not in LPK animals ($P=0.32$). Microinjection of TNFRI Ab did not reduce MAP in Lewis or LPK animals (1 ± 1 mmHg vs -1 ± 1 mmHg, Lewis vs LPK, $P>0.05$). Minocycline microinjection did not reduce MAP in Lewis or LPK animals (-1 ± 1 mmHg vs -1 ± 1 mmHg, Lewis vs LPK, $P>0.05$). Microinjection of minocycline in LPK animals significantly increased the tonic activation of the SFO, indicated by the increased depressor response to $GABA_a$ agonist microinjection (-12 ± 2 mmHg vs -23 ± 4 mmHg, no inhibitor vs prior minocycline microinjection, $P=0.04$). Preliminary observation suggests that LPK animals have considerably more activated microglia than Lewis animals.

Conclusions | Overall, these findings demonstrate that proinflammatory cytokines in the SFO do not contribute to the short-term control of BP in normotensive conditions and do not contribute to the overactivation and therefore the development of hypertension in LPK animals.

Key words:

Hypertension, proinflammatory cytokines, tumour necrosis factor-α, subfornical organ, polycystic kidney disease

Abbreviations:

ADPKD	Autosomal dominant polycystic kidney disease
ARPKD	Autosomal recessive polycystic kidney disease
BP	Blood pressure
BSA	Bovine Serum albumin
CKD	Chronic kidney disease
DBP	Diastolic blood pressure
$GABA_a$	Gama-aminobutyric acid receptor a
HR	Heart rate
Iba1	Ionised calcium binding adaptor molecule 1
LPK	Lewis polycystic kidney disease rat
MAP	Mean arterial pressure
PKD	Polycystic kidney disease
PBS	Phosphate buffered saline
PVN	Paraventricular Nucleus
RSNA	Renal sympathetic nerve activity
SBP	Systolic blood pressure
SFO	Subfornical Organ
TNFRI	Tumour necrosis factor receptor I
TNFRI Ab	Tumour necrosis factor receptor 1 antibody
TNFRII	Tumour necrosis factor receptor II
TNFα	Tumour necrosis factor-α
TPBS	Tris-phosphate buffered saline
TPBSm	Tris-phosphate buffered saline with methionine

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1. Introduction

Hypertension is a common comorbidity in polycystic kidney disease (PKD) (Chapman et al., 2010; Marlais et al., 2016; Karava et al., 2018). Despite the high prevalence and mortality rate associated with hypertension in PKD, current antihypertensive treatments are not effective in many patients with PKD (De Beus et al., 2015; Thomas et al., 2016). Furthermore, the underlying pathophysiological mechanism(s) behind the development of hypertension in these patients is not fully understood, therefore limiting the potential for researchers to develop new treatment targets. In an animal model of PKD, the Lewis polycystic kidney disease (LPK) animal, a main contributor of the hypertension observed is an increase in activity in the paraventricular nucleus (PVN) of the hypothalamus, that is driven by an increase in glutamatergic input from the subfornical organ (SFO) (Underwood et al., 2019). However, what is driving this increase in activity of the SFO is unknown. Systemic inflammation is present from an early stage in the pathogenesis of PKD and is, in part, a key contributor to development of many of the underlying symptoms of this disease including cystogenesis and a decline in renal function (Karihaloo, 2015). Furthermore, many proinflammatory cytokines, such as tumour necrosis factor-α (TNFα), act in the SFO to increase the excitability of neurons and causes a significant increase in blood pressure (BP) in normotensive Sprague-Dawley animals (Wei et al., 2015; Simpson and Ferguson, 2017). Therefore, the purpose of this thesis was to 1) establish if proinflammatory cytokines in the SFO contribute to the control of blood pressure (BP) in normotensive conditions and from that 2) identify if proinflammatory cytokines act in the SFO to increase its activity in LPK animals, and therefore contributes to the development of hypertension.

1.1. Determinants of BP

BP is constantly regulated and altered to maintain an adequate BP through the diverse range of physiological, pathological and environmental challenges. This constant and tight regulation of BP is important, as chronically low BP can disrupt cellular functioning due to a lack of perfusion, while chronically high BP can lead to severe end organ damage (Raven and Chapleau, 2014). Cardiac output and total peripheral resistance are the main determinants of BP (Feher, 2012). Cardiac output refers to the amount of blood pumped out of the left ventricle of the heart over a certain period of time. It is determined by a range of factors that work together to produce changes in BP including; heart rate (HR), stroke volume, end diastolic volume, venous return, cardiac contractibility and autonomic nervous system input (Gordan et al., 2015; Crystal et al., 2019). Total peripheral resistance refers to the resistance of the blood vessels that has to be overcome in order to pump blood through the circulatory system and is determined by a range of factors including; inherent myogenic tone of the vasculature, vasoactive stimuli and hormones (Johansson, 1989; Quyyumi, 1998). Overall, there are a range of complex mechanisms that act in unison to regulate BP in response to a changing pressure requirement of the animal.

1.2. The role of the central nervous system in BP regulation

The central nervous system is a major centre of integration of peripheral signals of BP, including stretch sensors in the systemic blood vessels and heart, metabolic signals and hormonal signals (Chopra et al., 2011). This information is detected from multiple regions of the brain and integrated, allowing for a suitable response to return BP back to homeostatic levels. One such integration region of the brain is the hypothalamus, a collection of nuclei that integrate peripheral signals to produce endocrine, autonomic and behavioural responses (Swanson and Sawchenko, 1983; Hughes et al., 1995). One hypothalamic nuclei in particular, the PVN, is an important coordinator for autonomic and neuroendocrine mediated cardiovascular responses. The PVN increases BP by altering the sympathetic outflow to a range of end organs through direct connections to the spinal cord and indirect connections via other key autonomic centres including the rostral ventrolateral medulla (Pyner and Coote, 2000). Furthermore, via magnocellular neural connections to the pituitary gland, the PVN regulates BP by stimulating the release of vasopressin which act to raise BP by increase water reabsorption in the kidneys and by inducing arteriole constriction (Bankir, 2001). The PVN also stimulates the release of adrenocorticotropic hormone from the anterior pituitary gland through the release of corticotrophin releasing hormone, both of which have been shown to increase BP (Lorenz et al., 2008; Sabban et al., 2009; de Kloet et al., 2017; Wang et al., 2018).

1.3. The role of the SFO in BP regulation

The SFO is one of the small, specialised neuronal populations known as the circumventricular organs of the brain (Gross and Weindl, 1987). The SFO is found on the dorsal border of the third ventricle below the fornix and, unlike most cortical brain regions, lacks an intact blood brain barrier, as the capillaries in the SFO are highly permeable due to the presence of numerous fenestrae and absence of tight junctions normally found between endothelial cells (Gross, 1991; Petrov et al., 1994). Furthermore, the SFO has an extensive vascular system with many capillary loops and large perivascular spaces surrounding the capillaries, all of which create a large area for substances to diffuse into the SFO and therefore be detected by neurons (Gross, 1991). As the blood brain barrier acts to tightly control the movement of circulating substances into the brain, the lack of an intact blood brain barrier allows the SFO to detect, and respond to, a large range of circulating substances that are not usually detected by or exposed to neurons, demonstrated by the range of receptors expressed on SFO neurons that are not found on neurons protected by the blood brain barrier (Gross and Weindl, 1987; Smith and Ferguson, 2010). This has important implications for diseases like PKD, as a dysregulation in circulating substances is evident from the early stages of the disease which, in turn, may affect the functioning of this brain region and therefore drive, or exacerbate, the underlying symptoms of this disease.

The SFO plays a major role in many key homeostatic functions including osmoregulation, cardiovascular regulation and integration of autonomic function (Smith and Ferguson, 2010). The role that the SFO plays in fluid balance and osmoregulation has been well established, with landmark studies of this brain region identifying that an increase in total water consumption was elicited in rats when angiotensin II, a hormone released in response to low blood volume, was microinjected into the SFO (Simpson and Routtenberg, 1973; Mangiapane and Simpson, 1980a). Furthermore, lesioning of the SFO led to diminished drinking responses in rats exposed to hypertonic saline, angiotensin II and polyethylene glycol solutions (Hosutt et al., 1981); physiological challenges that elicit drinking behaviour in SFO intact animals.

In addition to controlling drinking behaviour, the SFO can monitor plasma sodium levels (Anderson et al., 2000), allowing for this brain region to alter its firing rate in response to changes in plasma osmolarity. The SFO then projects this information to the PVN and the supraoptic nucleus in the hypothalamus, brain regions that are responsible for the release of vasopressin (Gutman et al., 1988). This finding is supported by the observations that chemical and electrical stimulation of the SFO leads to an increase in circulating vasopressin (Ferguson and Kasting, 2017). This demonstrates that the SFO is an important brain region for maintaining the blood

volume and osmolarity of an organism through altering the activity of downstream brain regions, inducing the release of hormones responsible for maintaining blood volume and eliciting drinking behaviour.

It is evident that, in addition to the regulation of blood volume and osmolarity, the SFO plays a role in regulating BP with five key experimental observations supporting this: (1) chemical and electrical stimulation of the SFO induces a significant pressor response in rats (Ishibashi and Nicolaidis, 1981); (2) electrical stimulation of the SFO causes an activation of the sympathetic nervous system, a key regulator of BP (Ciriello and Gutman, 2011); (3) ablation of the PVN, an important brain region in BP regulation, abolishes the pressor response observed upon stimulation of the SFO (Ferguson and Renaud, 1984); (4) microinjection of angiotensin II into the SFO to elicits a significant pressor response (Lind et al., 1983); and (5) lesion of the SFO and the SFO's efferent connections to other brain regions attenuates the pressor response to systemic infusion of angiotensin II (Mangiapane and Simpson, 1980b). Therefore, it is clear that the SFO plays a major role in the control of BP due to its ability to detect a range of circulating substances and project that information through efferent connections to other brain regions that maintain BP. Overall, the SFO is an important centre for integration of peripheral signals, allowing for an adequate response by the autonomic nervous and neuroendocrine system to maintain BP and blood volume homeostasis.

1.3.1. The role of the SFO in hypertension

It is well established that the SFO is an important brain region of integration of central and peripheral signals to help coordinate a physiological response to restore homeostasis. However, disruption to normal functioning of this brain region can lead to perturbations in the homeostasis of the organism. This is evident in the contribution that a dysfunctional SFO has to the development of multiple forms of hypertension as evident in the results of six studies: (1) selective silencing of angiotensin type II receptors in the SFO of two kidney one clip model of renovascular hypertension reduced mean arterial pressure (Rossi et al., 2019); (2) chronic lesioning of the SFO led to a small reduction in BP in the deoxycorticosterone acetate-salt model of hypertension (Osborn et al., 2006), (3) chronic silencing of angiotensin II receptors in the SFO led to a significant decline in BP in the deoxycorticosterone acetate-salt model of hypertension (Hilzendeger et al., 2013); (4) chronic electrolytic lesion of the SFO significantly reduced BP in the angiotensin II induced hypertension model (Hendel and Collister, 2005; Osborn et al., 2011); (5) targeted knockdown of prorenin receptor in the SFO, an important receptor involved in the production of angiotensin II in the brain, elicited a decrease in BP likely due to a reduction in the

number of angiotensin type 1a receptors and circulating levels of vasopressin (Li et al., 2013); and (6) knockdown of angiotensin type 1A receptors in the SFO decreased mean arterial pressure in the chronic intermittent hypoxia animal model (Saxena et al., 2015). Pathological changes in the SFO has also been shown to play a role in the development of hypertension in humans as there is a positive correlation between the level of immunolabelling for prorenin receptors in the SFO in post-mortem brain samples and systolic blood pressure (SBP) in human patients with essential hypertension when compared to normotensive controls (Cooper et al., 2018). Overall, it is apparent that a dysfunction of the SFO can lead to inappropriate responses to circulating indicators of blood volume and pressure, therefore contributing to the development of hypertension. This makes this the SFO a prime candidate for studying the aetiology of hypertension in other diseases.

1.4. TNFα

TNFα is a proinflammatory cytokine that plays a role in regulating the immune response, maintaining homeostasis from the cellular to the organism level and driving pathological processes (Probert, 2015). Under normal conditions, the production and activity of TNFα is highly regulated (Vassilli, 1992; Montgomery and Bowers, 2012; Probert, 2015) with excess TNFα being released in response to an immunological threat, injury or underlying pathological process by multiple types of cells including immune cells, endothelial cells, neurons and glial cells (Vezzani and Viviani, 2015). Once released, TNFα acts on two receptors in the body: tumour necrosis factor receptor 1 and 2 (TNFRI and TFRII, respectively; Probert, 2015).

Activation of cells by TNFα leads to a range of effects, largely dependent on which receptor and cell type is activated (Montgomery and Bowers, 2012). TNFRI is found on many cell types in the body and when activated by TNFα, it leads to a range of inflammatory processes including apoptosis and fever (Probert, 2015). Furthermore, activation of TNFRI in pathological processes is associated with poor disease outcomes, as the activation of TNFRI has been shown to play a major role in driving neurodegeneration and neuronal cell death (Probert, 2015). Of particular interest to this thesis, TNFα leads to an alteration to normal neuronal transmission and activity levels as in the central nervous system, as TNFα acts on TNFRI on neurons and glia to alter their functioning due to the initiation of an alteration to glutamate transmission, the upregulation of excitatory markers and increases in the expression of surface receptors involved in neuronal excitability (Bezzi et al., 2001; Beattie et al., 2002; Han and Whelan, 2010; Oshima et al., 2015; Wei et al., 2015; Colonna and Butovsky, 2017).

On the other hand, TNFRII is sparsely expressed throughout the body as it is largely found to only be expressed on T-cells (Zheng et al., 1995; Aspalter et al., 2003). The physiological role of TNFRII is still not clear, as historically activation of TNFRII by TNFα was largely thought to be protective, as activation of TNFRII receptors has been shown to limit the progression of many diseases including heart disease and neurodegeneration (Montgomery and Bowers, 2012). Although it is becoming clearer that the activation of TNFRII can also lead to proinflammatory processes and apoptosis (Depuydt et al., 2005), the role of TNFRII plays is still yet to be completely understood.

1.4.1. Association with the development of hypertension

Although TNFα is important for restoring homeostasis and promoting recruitment of the immune system following an acute injury or infection, sustained increases in TNFα have detrimental effects in the central nervous system including inducing cellular dysfunction, the deterioration of the blood brain barrier and the development of multiple forms of hypertension (Montgomery and Bowers, 2012). Experimentally this has been demonstrated in the spontaneously hypertensive rat, where chronic PVN inhibition of TNFα or nuclear factor-κβ, a transcription factor activated by TNFα, results in the significant reduction in BP (Song et al., 2014; Hayden and Ghosh, 2015; Yu et al., 2015). Similarly, in mice with deoxycosterone acetate salt associated hypertension, which exhibit increased TNFα release by T cells, chronic inhibition of TNFα by the TNFα antibody etanercept reduces BP (Guzik et al., 2007); an effect that has been replicated in many other forms of hypertension including the fructose-fed rats (Tran et al., 2009) and systemic lupus erythematosus (Venegas-Pont et al., 2010). These data demonstrate that an imbalance of TNFα can lead to an alteration of normal physiological functioning and an increase in BP across a range of different pathologies. Of particular relevance to this thesis, in the two kidney one clip model of renovascular hypertension, acute inhibition of TNFα in the area postrema, a circumventricular organ, led to the normalisation of BP (Korim et al., 2019). This demonstrates not only that circulating cytokines have access to the brain through the circumventricular organs, but that they are able to alter the functioning of brain regions resulting in a sustained increase in BP. Overall, TNFα is an important factor that drives the development of hypertension in a range of diseases, however it is yet to be determined if it plays a role in the development of hypertension in PKD.

1.4.2. Activity in the SFO

Under normal conditions, TNFRI and the mRNA encoding for the receptor is expressed in the SFO (Nadeau and Rivest, 1999). In the presence of high levels of circulating TNFα, the levels of TNFRI and TNFRI mRNA within the SFO significantly increase, suggesting that circulating TNFα

acts to alter the short- and long-term functioning of the SFO (Hindmarch et al., 2008). Accordingly, intravenous infusion of TNFα produces a pressor response that can be attenuated by lesioning of the SFO (Wei et al., 2013), while microinjection of TNFα into the SFO results in a significant pressor response and an increase in the expression of associated inflammatory and excitatory markers within the SFO (Wei et al., 2015). This demonstrates that TNFα acts within the SFO to not only increase BP, but to increase the excitability of this brain region. Supporting this, Simpson and Ferguson (2017) demonstrated that exposing patch-clamped SFO neurons to TNFα increases the activity of SFO neurons in a dose dependant manner for up to 30 minutes. Furthermore, they identified that incubating neurons extracted from the SFO in TNFα (10ng/ml) for 24 hours led to a sustained increase in the neuron's membrane excitability and basal firing rate when compared to non-treated SFO neurons (Simpson and Ferguson, 2017). Overall, this research demonstrates that circulating TNFα can access the SFO, resulting in a pressor response and an alteration of the functioning of neurons in the SFO through the upregulation of excitatory markers and mechanisms resulting in overexcitation of this brain region. As the SFO plays a major role in body fluid homeostasis and BP regulation, this has a range of implications as many diseases present with an increase in circulating TNFα, making it likely that TNFα is acting on the SFO to cause some of the pathologies observed in these diseases. However, the link between TNFα and its actions in the SFO to drive hypertension in any disease context has yet to be explored.

1.5. PKD

PKD is the most common form of inherited chronic kidney disease (CKD) as it affects an estimated 1 in 1,000 to 2,500 individuals (Willey et al., 2017; Lanktree et al., 2018). The most common form of PKD is autosomal dominant PKD (ADPKD), however there are many forms of more uncommon ciliopathies including autosomal recessive PKD (ARPKD) and nephronophthisis (Braun and Hildebrandt, 2017; Willey et al., 2017). The presentation of ADPKD and ARPKD are very similar as both forms involve an increase in renal tubular proliferation and apoptosis, causing affected individuals to develop renal cysts leading to a decline in kidney function which ultimately declines into end stage renal disease (Grantham et al., 2006). However, the progression of ARPKD is significantly more rapid as most individuals progress to end stage renal disease in the first decade of life, compared to individuals with ADPKD who typically progress to end stage renal disease in the fifth to sixth decade of life (Chebib and Torres, 2016; Sweeny and Avner, 2019). In addition to the development of renal cysts, patients with PKD can also experience hypertension, hepatic cysts, infection or rupture of renal cysts, cardiovascular disease and diverticular disease (Chebib and Torres, 2016).

1.5.1. Hypertension in PKD

Unlike many other forms of CKD, patients with PKD commonly develop hypertension before a significant decline in their renal function is evident (Chapman et al., 2010; Marlais et al., 2016; Karava et al., 2018). Hypertension in PKD significantly increases the mortality rates of patients through increasing their risk of cardiovascular disease (Perrone et al., 2001). Moreover, since the development of dialysis, which has significantly reduced the number of deaths due to renal failure, the most common cause of death in PKD patients is cardiovascular disease which is exacerbated by the presence of hypertension (Perrone et al., 2001; Rahman et al., 2009). Furthermore, hypertension in PKD is associated with an increase in the risk of PKD patients progressing to end stage renal disease (Idrizi et al., 2007), demonstrating that it is imperative to reduce BP in these patients to reduce the significant morbidity and mortality associated with uncontrolled BP in these patients.

There have been some clinical insights into the pathophysiological mechanisms underpinning the development of hypertension in PKD including the enlargement of the renal cysts causing renal ischemia, attenuation of the renal arterioles and activation of the renin-angiotensin aldosterone system (Gabow et al., 1990; Wang and Strandgaard, 1997), a hormonal system which increase BP through increased vasoconstriction and water retention through the release of angiotensin II and aldosterone. This leads to a continual cycle of renal cyst growth and the triggering of the activation of the renin angiotensin aldosterone system, which in turn raises BP and promotes further cyst growth (Chapman et al., 2010). However, as many patients do not respond to treatment targeting this system, including angiotensin converting enzyme inhibitors and angiotensin II receptor blockers, this continual cycle is not solely responsible for the development of hypertension in PKD (Chapman et al., 2010). An increase in the activity of the sympathetic nervous system may also contribute to the development of hypertension in individuals with PKD, as when compared to controls hypertensive PKD patients have been shown to have higher levels of noradrenaline, indicative of a prolonged activation of the sympathetic nervous system (Cerasola et al., 1998). However, the role that the sympathetic nervous system plays in the aetiology of hypertension in PKD is unclear as, contrastingly, some studies have not found an increase in sympathetic nervous system activity in human hypertensive PKD patients (Bell et al., 1988) and research in our lab indicates that an increase in sympathetic nervous system activity does not contribute to the development of hypertension in LPK animals (Underwood et al., 2019). This indicates that alterations of the sympathetic nervous system may not drive an increase in BP, but rather may be another underlying symptom of PKD.

Despite these clinical insights and the development of more advanced antihypertensive medication, many PKD patients have hypertension that is resistant to currently available antihypertensive medication, which in turn significantly increases their risk of death due to cardiovascular disease and end stage renal disease (Thomas et al., 2016). Furthermore, researchers still lack a complete understanding of the pathophysiological mechanisms behind the development of hypertension in PKD. Therefore, further studies into the pathophysiology behind the development of hypertension are necessary, as new treatment methods to tackle the high levels of treatment resistant hypertension in patients with PKD cannot be developed without a complete understanding of the pathophysiology underlying the development of hypertension in PKD.

1.5.2. Inflammation in PKD

Although PKD is not typified as an inflammatory disease, inflammation is present, occurring very early in the disease progression and is a known driver of the wider disease pathology in PKD (Karihaloo, 2015). Four important pieces of evidence supports the assertion that inflammation is a major pathological contributor to PKD including: (1) PKD patients early in their disease progression were found to have significantly higher levels of proinflammatory biomarkers and demonstrated vascular abnormalities (increased arterial stiffness and pulse wave velocity) that are indicative of systemic subclinical inflammation when compared to nonaffected individuals (Kocyigit et al., 2012); (2) presence of proinflammatory cytokines within cystic fluid from PKD patients (Gardner et al., 1991); (3) TNFα induces cystogenesis in $Pkd2^{+/-}$ mouse kidney embryonic cells by disrupting proteins involved in the development of cilia and renal tubules (Li et al., 2008); and (4) high levels of circulating cytokines, namely TNFα and interleukin-6, are associated with a more rapid loss of kidney function (Menon et al., 2011).

Inflammation has also been linked to the development of hypertension in the development of treatment resistant hypertension and the decline in renal function in CKD patients (Amdur et al., 2016; Chen et al., 2019), however as PKD patients were excluded from the study cohort in this study, it remains to be determined if inflammatory biomarkers are associated with these poor outcomes in PKD. Although, Menon et al. (2011) found a correlation between inflammation and renal decline in a separate cohort of PKD patients, demonstrating that some of the research regarding inflammatory biomarkers in CKD is replicable in patients with PKD, therefore making inflammation a prime candidate for driving the development of hypertension in patients with PKD.

Although treatment studies aimed at reducing TNFα and other proinflammatory cytokines in animal models of PKD have shown conflicting responses with a significant improvement in

symptoms being reported (Wu et al., 2016) and no improvement reported (Roix and Saha, 2013), these studies failed to measure BP during these treatments. Therefore, the role that inflammation plays in the development of hypertension in PKD that has still yet to be explored.

1.5.3. Animal Models of PKD

The first studies into the pathophysiology underlying the development of hypertension in PKD were made possible in the mid-1900's when chemically induced models of PKD were first developed (Lager et al., 2001). Although many of these early animal models of PKD were well characterised and allowed researchers to gain important insights into the pathophysiological mechanisms underlying the development of PKD, these animals did not present with all of the pathological features of PKD, required a long treatment period for renal cysts to develop and varied in effectiveness and reproducibility (Leadbetter and Burkland, 1938). As our knowledge in genetics and genetic modification advanced, this allowed for the development of newer animal models of PKD that more accurately and consistently mimic the pathology due to either the identification of spontaneous genetic mutations or through genetic modification. Although many of these alternative animal models of PKD have allowed for valuable insights into the pathophysiology underpinning the disease and the testing of potential treatments, the LPK animal model is the most extensively characterised and studied in regards to its BP and autonomic nervous system profile (Table 1.1), making this model the ideal model to study the pathogenesis underpinning the development of hypertension in PKD.

Table 1.1 A comparison of the disease-causing mutation, disease progression and blood pressure of a range of animal models commonly used to study polycystic kidney disease.

Animal Model	Disease-Causing Mutation	Disease Progression	Blood Pressure	References
Lewis polycystic kidney rat	Nonsynonymous mutation in the Nek8 gene	Moderate	Hypertensive from a young age	(Phillips et al., 2007; McCooke et al., 2012)
Han:SPRD-cy rat	Missense mutation in the ANKS6 gene	Slow (heterozygous animals), lethal at a young age (homozygous animals)	Mildly hypertensive late in disease progression	(Schäfer et al., 1994; Bihoreau et al., 1997)
Pkd2$^{Ws25/-}$ mouse	One null allele and one unstable allele of the Pkd2 gene	Fast	Not measured	(Wu et al., 1998, 2000)
Jck mouse	Mutation in the Nek8 gene	Slow	Not measured	(Atala et al., 1993)

ANKS6: ankyrin repeat and sterile alpha motif domain containing 6, Jck: juvenile cystic kidney, Nek8: never in mitosis related kinase 8, PKD2: polycystin-2.

1.5.4. LPK animal model

The LPK animal model was first described in 2007 at the Animal Resource Centre in Perth, Australia. As a result of a spontaneous nonsynonymous mutation in the never-in-mitosis related kinase 8 (Nek8) gene, the LPK develop nephronophthisis, a form of ARPKD (McCooke et al., 2012). LPK animals have renal tubule dilations that develop into renal cysts from three weeks of age leading to a marked decline in renal function by 12 weeks of age and end stage renal failure from 18-20 weeks of age (Phillips et al., 2007). In addition to the development of cysts, these animals experience anaemia (Phillips et al., 2015) and multiple signs of cardiovascular disease including left ventricular hypertrophy (Phillips et al., 2007), aortic remodelling and arterial stiffness (Ng et al., 2011; Ameer et al., 2014b). Furthermore, these animals also exhibit early signs of hypertension at three weeks of age, well before their renal function declines and progressively become severely hypertensive as they age (Phillips et al., 2007). However, the underlying pathophysiology behind the development of hypertension in these animals is not fully understood.

It is suggested that human patients with PKD experience autonomic dysfunction and an overactivation of the sympathetic nervous system which in turn contributes to the development of hypertension (Klein et al., 2001, 2003). Like human patients with PKD, it has been identified that LPK animals also experience autonomic dysfunction under anaesthetic (Harrison et al., 2010; Yao et al., 2015) and in conscious conditions (Hildreth et al., 2013). Furthermore, these animals experience an increase in sympathetic vasomotor tone (Ameer et al., 2014a) and an increase in sympathetic nerve activity (Salman et al., 2015a). However, as the autonomic dysfunction observed in these animals occurs after the development of hypertension, it can be determined that it is unlikely that autonomic dysfunction contributes to the development of hypertension.

Previous research has identified that, compared to controls, LPK animals have a higher level of activity in the PVN as indicated by increased Fos and Fra immunostaining, suggesting a potential role of this nucleus in the hypertension observed in LPK animals (Ang et al., 2007). The role that the PVN plays in the development of hypertension in LPK animals was further elucidated when it was identified that an increase in the activity of the PVN, in conjunction with the independent activation of vasopressin type 1a receptors, was responsible for the development of hypertension in LPK animals (Underwood et al., 2019). This increase in activity of the PVN was found to drive hypertension through mechanisms that were independent of the sympathetic nervous system, as there was no major changes in the sympathetic nerve activity measured from renal, splanchnic or lumbar sympathetic nerves (Underwood et al., 2019). This indicates that in LPK animals the PVN drives hypertension by other downstream effectors which are yet to be identified. Further studies identified that the overactivity in the PVN originates from an increase in glutamatergic input from the SFO (Underwood et al., In Preparation). This overactivity of the SFO was not ameliorated by chronic angiotensin II receptor I antagonism or lowering the plasma osmolarity through high water intake treatment, demonstrating that angiotensin II and an increase in plasma osmolarity are unlikely to be the underlying mechanism driving the SFO overactivity (Underwood et al., In Preparation). As such, what is acting on the SFO to cause this increase in glutamatergic activity to the PVN is yet to be determined.

1.6. Thesis aims

Overall, an increase in glutamatergic input to the PVN from the SFO contributes to the development of hypertension in the LPK model of PKD (Underwood et al., 2019), however what is driving this increase in activity of the SFO is unknown. Proinflammatory cytokines, in particular TNFα, have been shown to act to increase the excitability of neurons in the SFO and cause a significant pressor response in normotensive Sprague-Dawley animals (Wei et al., 2013, 2015), therefore the actions of TNFα were focused upon in this study. The first aim of this thesis was to determine if the short-term actions of endogenous proinflammatory cytokines in the SFO contribute to the control of BP in normotensive Lewis animals by measuring responses to microinjection of TNFα, TNFRI Ab and minocycline, a microglial activation inhibitor. The second aim of this study was to identify if the short-term actions of proinflammatory cytokines drive the increase in activity of the SFO in LPK animals and therefore contribute to the development of hypertension in this animal model. This was examined by measuring the responses to microinjection of TNFα, TNFRI Ab and minocycline into the SFO of LPK animals. We elected to examine the role of microglial activation in the SFO, in addition to the role of TNFα, as inflammation is present in PKD (Karihaloo, 2015), making it likely that the microglia in these animals are activated. Therefore, as chronically activated microglia have been shown to alter neuronal function (Wolf et al., 2017), we hypothesised that they may play an underlying role in the development of the overactivity of the SFO in the LPK animals. Finally, microglial activation was evaluated by immunohistochemistical staining for ionised calcium binding adaptor molecule 1 (Iba1).

2. Methods

2.1. Animals

All experiments were undertaken on adult (13-14 weeks) male Lewis (n=26) and LPK (n=22) animals obtained from the Animal Resource Centre Perth, Western Australia. All animals were group-housed in a standard 12hr light/dark phase (lights on at 6:00am) at the Central Animal Facility, Macquarie University with standard laboratory rat chow and water available ad libitum. All experiments were approved by the Macquarie University Animal Ethics Committee (Animal Research Authority 2018/016) and were undertaken in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th Edition, National Health and Medical Research Council, 2013.

2.2. Confirmation of renal disease pathology

At least 24 hours prior to the surgery all animals were placed in metabolic cages for approximately six hours to collect a urine sample. During surgery, 0.2ml of arterial blood was collected and centrifuged to separate the plasma (IDEXX StatSpin, IDEXX laboratories Pty Ltd., NSW, Australia). All urine and plasma samples were stored at -20°C until they were analysed. A dipstick test (URS-10T Urinalysis Strips, Henso Medical, HK) was used to measure the levels of protein in the urine and a VetTest chemistry analyser was used to measure plasma urea concentration.

2.3. Surgical preparation

Anaesthesia: Animals were anaesthetised with urethane (1.3g/kg, intraperitoneally, Sigma Aldrich, Australia). Adequate depth of anaesthesia was determined by a lack of withdrawal in response to a hindpaw pinch, additional doses of urethane (130mg/kg, intraperitoneally or intravenously) were administered as required. Body temperature was monitored by a digital rectal thermometer (Harvard Apparatus, MA, USA) and maintained at 37±0.5 ˚C using a heating mat (Harvard Apparatus, MA, USA) and a manual heat lamp.

Arterial and Venous Cannulation: The right femoral artery and vein were cannulated to allow for the measurement of BP and administration of fluids and drugs, respectively. Briefly, an incision was made along the upper inner hind leg and the superficial adipose tissue excised to expose the femoral artery and vein, the fascia overlying the femoral artery and vein were removed allowing them to be separated from the saphenous nerve. The femoral artery was cannulated with polyethylene tubing (inner diameter 0.58mm and outer diameter 0.8-0.9mm) prefilled with

heparinised saline (5IU/ml 0.9% saline) and connected to a pressure transducer. Arterial pressure was then acquired with a CED 1401 plus (Cambridge Electronic Designs Ltd., Cambridge, UK) and recorded using Spike2 software (v7, Cambridge Electronic Designs Ltd., Cambridge, UK). The femoral vein was cannulated with polyethylene tubing (inner diameter 0.58mm and outer diameter 0.96mm) prefilled with 0.9% saline.

Tracheotomy: A midline incision was made along the ventral surface of the neck and the sternohyoid muscles were retracted to visualise the trachea. A small hole was made in the trachea and a 14G cannula was inserted and secured in place with surgical suture (2-0). Animals were mechanically ventilated with oxygen enriched air (10ml/kg body weight at 65 ± 5 breaths per minute). Animals were paralysed with Cisatracurium (6 mg/kg/hr intravenously GlaxoSmithKline Pty Ltd, Victoria, Australia). After the animals were mechanically ventilated and paralysed, 0.2ml of arterial blood was collected and blood gas parameters were measured by a VetStat blood gas analyser (IDEXX laboratories Pty Ltd.) and artificial ventilation was altered to maintain arterial pCO_2 at 40 ± 5 mmHg and pH of 7.4 ± 0.5.

Renal Nerve Dissection and Recording: A dorsal flank incision was made, the overlying fascia was incised, and the muscle and fat were separated to allow for access to the retroperitoneal cavity. The left kidney was exposed by retracting back the muscle on either side of the incision and the fascia around the aorta and left renal artery was carefully resected. A branch of the left renal nerve was separated from the fascia, cut at the distal end, placed on a silver bipolar recording electrode and immersed in paraffin oil. The nerve signal was band pass filtered (30-1000Hz), passed through a bioamplifier (2000x; CWE Inc., Ardmore, PA, USA) and sampled at 5kHz with a CED 1401 plus and Spike2 software. The validity of all nerves were verified by confirming the presence of pulse modulation (Salman et al., 2015a). A representative trace of a recording from a renal nerve of a Lewis control animal is presented in Figure 2.1.

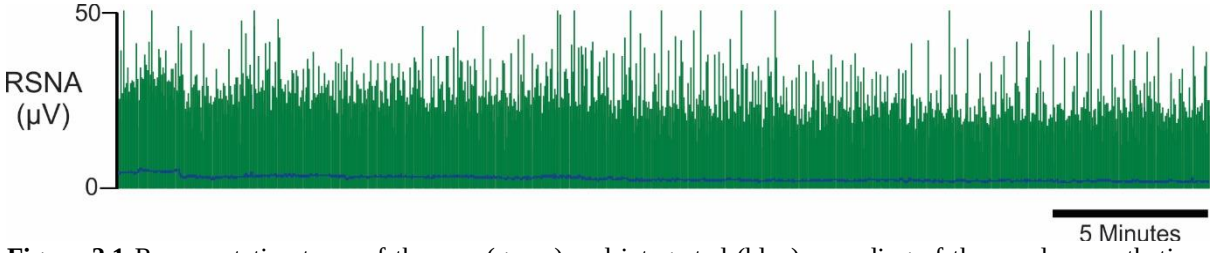

Figure 2.1 Representative trace of the raw (green) and integrated (blue) recording of the renal sympathetic nerve activity (RSNA) in a Lewis animal. μV: microvolts.

Craniotomy: Animals were placed into a stereotaxic frame and a midline incision was made along the dorsal surface of the skull and the skin retracted back. The dorsal-ventral coordinates of bregma and lambda were taken to ensure that the head was flat in the horizontal plane. A burr hole of approximately 10mm in diameter was made over the location of the SFO.

Rest period: After the completion of the surgical preparatory procedures, animals recovered for a minimum of thirty minutes to allow for all measured parameters to return to baseline before any further experimentation was undertaken.

2.4. SFO microinjections

Microinjection Method: The stereotaxic coordinates for the SFO used in this thesis were 0.7mm caudal from bregma, 0mm lateral from midline and 5.4mm ventral from the surface of the brain. As the sagittal sinus is found along the midline, micropipettes were inserted into the brain 0.5mm lateral to the midline and moved to midline coordinates once inserted to ensure that the sagittal sinus remained intact throughout the experiment. For each study, 50-100nl of the respective drug was microinjected into the SFO using a micropipette (inner diameter 0.25mm, outer diameter 1mm) that was connected to a syringe with silastic tubing.

Drugs for Microinjection: Drugs were prepared for microinjection as outlined in Table 2.1.

Table 2.1 Drug preparation for microinjection studies.

Drug	*Company*	*Dose*	*Diluent*	*Visualising Agent*
Vehicle (0.1% BSA 1X PBS)	PBS - ThermoFischer Scientific, CA, USA BSA – Sigma Aldrich, NSW, Australia	-	-	FluoroMax fluorescent polystyrene microbeads (1/1,000; Thermo Fisher Scientific, CA, USA)
Recombinant Rat TNFα	PeproTech, NJ, USA	1-300pg/50nl	0.1% BSA PBS	
Vehicle (1X PBS)	ThermoFischer Scientific, CA, USA	-	-	
TNFRI Ab	ab19139, Abcam, Cambridge, UK	1ng/50nl	PBS	
Minocycline	Sigma Aldrich, NSW, Australia	0.5µg/50nl	PBS	
Muscimol	In Vitro Technologies, VIC, Australia	10mM	PBS	
Isoguvacine	Sigma Aldrich, NSW, Australia	10mM	PBS	Chicago Sky Blue 6B dye (1:100; Sigma Aldrich, NSW, Australia).

BSA: bovine serum albumin, PBS: phosphate buffered saline, TNFα: tumour necrosis factor-α, TNFRI Ab: tumour necrosis factor receptor 1 antibody.

Histological Analysis of Microinjection Sites: At the termination of each experiment the animal was euthanised with 0.5ml of 3M potassium chloride and the forebrain was collected and post-fixed in 10% neutral buffered formalin for a minimum of 12 hours. The brain was then sliced at 80µm with a vibrating microtome (Lieca VT1200ss, Lieca Microsystems Pty Ltd., NSW, Australia) and was wet-mounted onto a microscope slide and cover slipped with an antifade mounting medium (Fluorescence Mounting Medium, Aligent, VIC, Australia). To allow for identification of the microinjection sites, all of the drugs microinjected into the SFO were combined with a visualising agent as outlined in Table 2.1. Sections were than imaged with a fluorescent microscope (Axioimager, Carl Zeiss Microscopy, Germany) and mapped onto the rat brain atlas (Paxinos and Watson, 2006) with CorelDRAW Graphics Suite X7 software (Corel Corporation, Ontario, Canada).

2.5. Study 1: Do proinflammatory cytokines contribute to the control of BP in normotensive conditions?

2.5.1. TNFα microinjections

To determine the HR and BP responses elicited by microinjection of TNFα into the SFO, vehicle (0.1% bovine serum albumin, BSA PBS) and increasing doses of TNFα (1pg-300pg/50nl) were consecutively microinjected into the SFO of Lewis animals (n=5) as described above. When all measured parameters returned to baseline for a minimum of five minutes, the next dose was microinjected to ensure that all responses to TNFα were captured. For this study, renal sympathetic nerve activity (RSNA) was not recorded.

2.5.2. TNFRI Ab and minocycline microinjections

To determine the effect of inhibiting the actions of endogenous TNFα in the SFO on BP under normotensive conditions, vehicle (PBS), anti-TNFRI antibody (1ng/50nl), TNFα (300pg/50nl) and a Gama-aminobutyric acid receptor a ($GABA_a$) agonist (10mM muscimol and 10mM isoguvacine) were microinjected into the SFO of Lewis animals (n=5), with a 30 minute recording period in between each microinjection as outlined in Figure 2.2. In n=2 Lewis animals with the TNFRI Ab microinjection RSNA was recorded.

Next, to determine the effect of inhibiting the activation of microglia on BP under normotensive conditions, vehicle (PBS), minocycline, TNFα (300pg/50nl) and $GABA_a$ agonist (10mM muscimol or 10mM isoguvacine) were microinjected in to the SFO of Lewis animals (n=5) with a

30 minute recording period in between each microinjection as outlined in Figure 2.2. In n=3 Lewis animals with minocycline microinjection RSNA was recorded.

Figure 2.2 Flowchart illustrating the method for the microinjections into the subfornical organ in Study 1 and Study 2. HR: heart rate, MAP: mean arterial pressure, PBS: Phosphate buffered saline, RSNA: renal sympathetic nerve activity, TNFα: tumour necrosis factor-α, TNFRI Ab: tumour necrosis factor receptor I antibody.

2.6. Study 2: Do proinflammatory cytokines act in the SFO to increase its activity and therefore contribute to the development of hypertension in LPK animals?

2.6.1. TNFα microinjections

To determine the HR and BP responses caused by microinjection of TNFα into the SFO in hypertensive conditions, vehicle (0.1% BSA PBS) and the increasing doses of TNFα (1pg-300pg/50nl) were consecutively microinjected into the SFO of LPK animals (n=4) as described above. When all parameters returned to baseline for a minimum of five minutes, the next dose was microinjected to ensure all responses to TNFα were captured. For this study, RSNA was not recorded.

2.6.2. TNFRI Ab and minocycline microinjections

To determine the effect of inhibiting the actions of endogenous TNFα in the SFO on BP under hypertensive conditions, vehicle (PBS), anti-TNFRI antibody (1ng/50nl), TNFα (300pg/50nl) and a $GABA_a$ agonist (10mM muscimol and 10mM isoguvacine) were microinjected into the SFO of LPK (n=4) animals, with a 30 minute recording period in between each microinjection as outlined in Figure 2.2. In n=2 LPK animals with TNFRI Ab microinjection the RSNA was recorded.

Next, to determine the effect of inhibiting the activation of microglia on BP in hypertensive conditions, vehicle (PBS), minocycline, TNFα (300pg/50nl) and $GABA_a$ agonist (10mM muscimol or 10mM isoguvacine) were microinjected in to the SFO of LPK animals (n=5), with a 30 minute recording period in between each microinjection as outlined in Figure 2.2. In n=3 LPK animals the RSNA was recorded.

2.7. Immunohistochemical analysis of microglial activation in the SFO

2.7.1. Perfusion

Lewis (n=2) and LPK (n=3) animals were deeply anaesthetised with an injection of sodium pentobarbital (intraperitoneally, 100mg/kg) and transcardially perfused with 250ml 0.9% saline followed by 250ml 4% formaldehyde solution. The brain was removed and fixed in 4% formaldehyde for five hours at room temperature.

2.7.2. Immunohistochemistry method

After being removed from the fixative, the brains were washed in TBPS (3x30min) and the brains were cut coronally (50µm) on a vibrating microtome (Leica VT1200ss, Leica Microsystems Pty Ltd., NSW, Australia) and stored in Tris-phosphate buffered saline with methionine (TPBSm) until further analysis. Sections containing the SFO were washed with 0.1M PBS (3 x 30min) and then incubated in 10% normal donkey serum in TBPS for one hour at room temperature. The sections were then incubated with the primary antibody, goat anti-Iba1 (1:1000, Wako 019-1974, Osaka, Japan), for 48hrs at 4˚C. The sections were then washed with 0.1M PBS (3 x 30min) and incubated with the secondary antibody, donkey anti-goat Cy3 (1:500, Jackson Immunoresearch, PA, USA) at 4˚C in darkness for 8hrs. The sections were washed with Tris-phosphate buffered saline (TPBS) (3 x 30min) before being mounted on microscope slides and cover slipped with an antifade mounting medium (Fluorescence Mounting Medium, Aligent, VIC, Australia) and stored at 4˚C until further analysis. To validate the specificity of the anti-Iba1 antibody to microglia, slices were incubated with the primary antibody only and the secondary antibody only and examined under the microscope. In these negative controls there was no unspecific binding or fluorescence (Data not shown).

2.8. Data analysis

All data was analysed offline using Spike2 software. From the arterial pressure waveform, SBP and diastolic blood pressure (DBP) were determined from the peak and trough amplitude, respectively. The following equation was input into Spike2 software to manually determine mean arterial pressure (MAP);

$$\frac{(SBP + 2\ x\ DBP)}{3}$$

HR was determined from the frequency of peaks in the arterial pressure waveform in Spike2 software.

2.8.1. Baseline definition

Baseline measurements was defined as the average MAP, SBP, DBP and HR over the 1-minute (for TNFα microinjections) or 5-minute (for TNFRI Ab and minocycline microinjections) period immediately before each microinjection.

2.8.2. Combination of vehicle data

In this thesis, the vehicle responses from the animals treated with TNRI Ab and minocycline were combined. As these animals were age matched, had the same surgical preparation prior to the vehicle microinjection and responses were combined within the same strain, it is unlikely that there would be significant differences in response to the vehicle within strains. This lack of difference between the responses to vehicle microinjection in the different cohorts was confirmed by two-way ANOVA analysis which indicated that there was no treatment (P=0.15), time (P=0.20) or treatment by time interaction effect (P=0.51).

2.8.3. Statistical analysis

All data is expressed as mean±standard error of the mean (SEM) and was analysed using Graphpad Prism software (Graphpad Prism Software v8.1.2 Inc., La Jolla, CA, USA). Significance was set at $P \leq 0.05$. An unpaired t-test was used to identify differences in the baseline parameters between the two strains. For the SFO microinjection data recordings, data was exported from Spike2 software and averaged 5-minutes which were analysed in GraphPad Prism software. One-way or two-way multiple comparisons ANOVA with a post-Hoc Dunnett's correction was used to determine if there was an effect of the SFO microinjections or a strain effect, respectively. To determine the effectiveness of the inhibitory actions of TNFRI Ab and minocycline microinjection, the response at 10-minutes post-microinjection was compared between treatments with one-way ANOVA's and unpaired t-tests. To identify any pre-treatment effects on isoguvacine microinjection data, the peak response within five minutes of microinjection were extracted and compared by a one-way ANOVA and unpaired t-tests.

2.9. Microscopy analysis

Sections stained with anti-Iba1 which contained the SFO were visualised and captured with a fluorescent microscope (Axioimager, Carl Zeiss Microscopy, Germany). Images of the SFO were captured at 5x and 20x magnification, stitched and adjusted for brightness and contrast in an identical manner using Zen 2 Pro Software (Carl Zeiss Microscopy, Germany). Criteria for categorising microglia as surveillant was the presence of multiple branching processes with a small, but not prominently stained, cell body and the criteria for categorising microglia as activated

was the lack of multiple long processes and a prominent amoeboid shaped cell body (Hovens et al., 2014). A representative fluorescent image of a surveillant and activated microglia is provided in Figure 2.3.

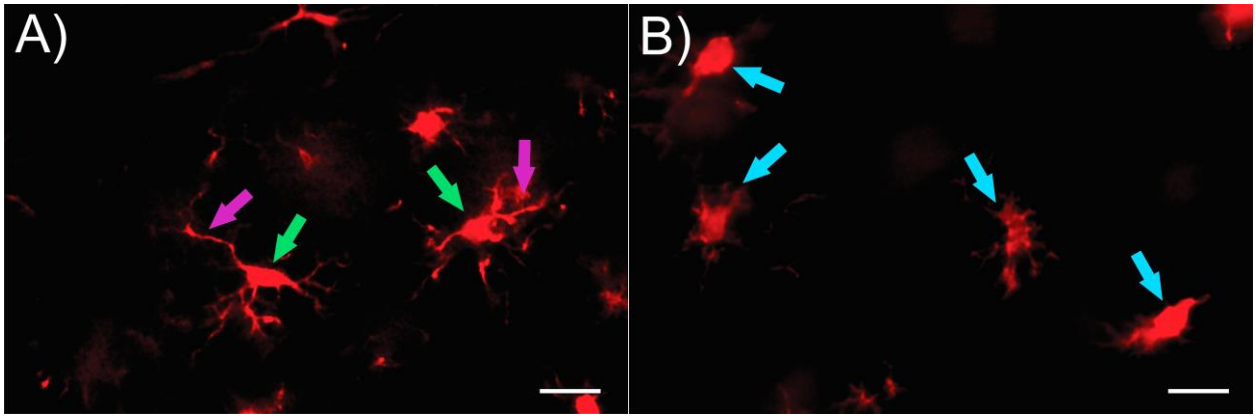

Figure 2.3 Representative fluorescent image (40x magnification) of a (A) surveillant microglia in the subfornical organ (SFO) of a Lewis animal, as indicated by the small cell body (green arrows) and numerous processes (purple arrows), and (B) activated microglia in the SFO of a Lewis polycystic kidney disease animal, as indicated by the prominent cell body (blue arrows) with few processes, as stained with ionised calcium binding adaptor molecule 1 (Iba1). Scale bar = 20µm.

3. Results

3.15. Baseline characteristics

Consistent with previous studies (Phillips et al., 2007), in this thesis LPK animals had a significantly lower body weight, significantly higher levels of plasma urea, and moderate levels of urinary protein detected when compared to Lewis animals (Table 3.1).

Table 3.1 Bodyweight and renal function assessment in Lewis and Lewis polycystic kidney (LPK) animals.

	Lewis (*n*=20)	**LPK (*n*=13)**	***P*-Value**
Bodyweight (g)	338±5	231±8	<0.0001*
Plasma Urea (mmol/L)	8.5±0.3	33.8±2.2	<0.0001*
Urinary Protein	-	++	N/A

All values are expressed as mean±SEM. * $P<0.05$. – No urinary protein detected, ++ moderate level of urinary protein detected.

Under urethane anaesthesia, LPK animals had significantly greater baseline levels of SBP and MAP but decreased levels of DBP, and consequently a widened pulse pressure, compared to Lewis animals (all $P<0.05$, Table 3.2). No significant difference was observed between the baseline levels of HR and RSNA between the two strains (all $P>0.05$, Table 3.2).

Table 3.2 Baseline blood pressure, heart rate (HR) and renal sympathetic nerve activity (RSNA) in Lewis and Lewis polycystic kidney (LPK) animals.

	Lewis (*n*=20)	**LPK (*n*=13)**	***P*-Value**
SBP (mmHg)	130±4	191±10	<0.0001*
DPB (mmHg)	75±3	62±6	0.03*
MAP (mmHg)	87±4	102±7	0.03*
PP (mmHg)	54±4	129±7	<0.0001*
HR (bpm)	385±9	369±14	0.32
RSNA (μV)[#]	7.7±1.4	6.1±0.1	0.57

All values are expressed as mean±SEM. SBP: systolic blood pressure, DBP: diastolic blood pressure, MAP: mean arterial pressure, PP: pulse pressure. *$P<0.05$. # Lewis $n=10$ and LPK $n=3$ for RSNA analysis.

3.2. Anatomical location of microinjection sites

A representative image illustrating a microinjection of Chicago blue dye into the dorsal region of SFO is provided in Figure 3.1.

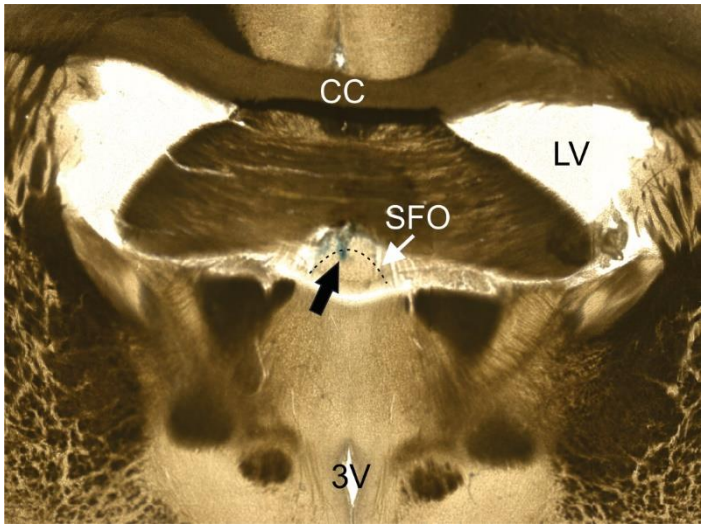

Figure 3.1 Representative image illustrating a microinjection of Chicago blue dye into the subfornical organ (SFO) as indicated by the black arrow. CC: corpus callosum, LV: lateral ventricle, 3V: third ventricle.

Figure 3.2 outlines the anatomical location of all microinjection sites performed in this thesis. For all data analysed within this thesis, the microinjection sites were positioned within the SFO between -0.60mm and -1.20mm caudal of bregma. For six Lewis animals in Study 1 (*n*=2 for TNFα microinjections, *n*=2 for TNFRI Ab microinjection and *n*=2 for $GABA_a$ microinjections) and six LPK animals in Study 2 (*n*=2 for TNFα microinjections, *n*=2 for TNFRI Ab microinjection and *n*=2 for minocycline microinjections), the microinjections were determined to be outside of the region of the SFO but in nearby brain regions that do not play a role in the control of BP and were therefore analysed as offsite controls.

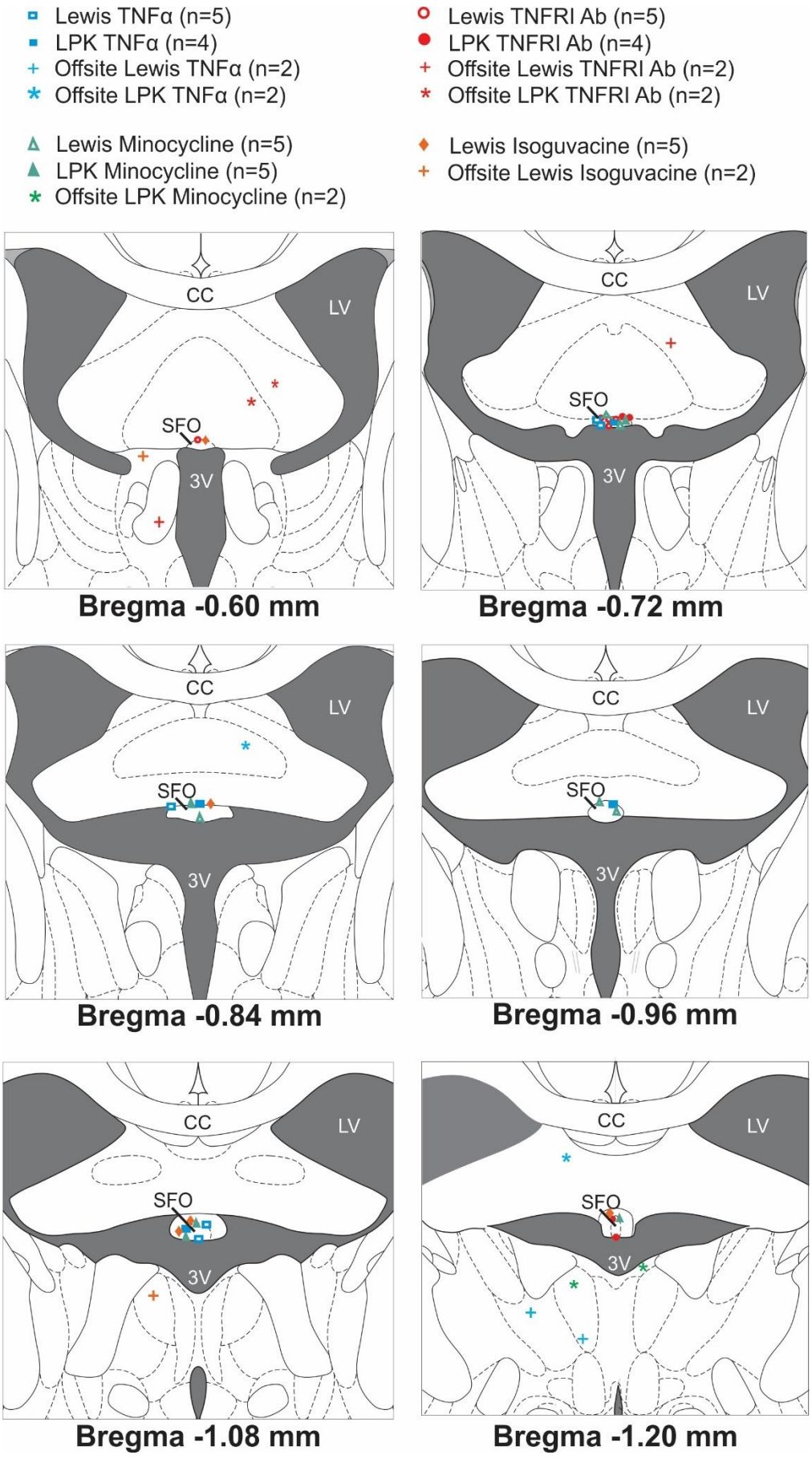

Figure 3.2 Anatomical location of microinjections made into the subfornical organ (SFO) during this study. Diagrams modified from the Rat Brain Atlas, Sixth Edition (Paxinos and Watson, 2006). CC: corpus callosum, LV: lateral ventricle, 3V: third ventricle.

3.3. Study 1: Do proinflammatory cytokines in the SFO contribute to the control of BP in normotensive conditions?

3.3.1. Microinjection of TNFα into the SFO causes a dose-dependent increase in BP, but not HR, under normotensive conditions

Previous studies have demonstrated that microinjection of increasing doses of TNFα into the area postrema, a circumventricular organ involved in BP control, results in a dose-dependent increase in BP and HR in normotensive Sprague-Dawley animals (Korim et al., 2019). However, it is currently unknown whether the same response occurs in the SFO. To address this knowledge gap, increasing doses of TNFα (1pg-300pg/50nl) were microinjected into the SFO of Lewis control animals and resulting changes in BP and HR measured. Microinjection of increasing doses of TNFα evoked a dose-dependent increase in MAP (P<0.01). The change in MAP elicited upon microinjection of the highest dose of TNFα (300pg) was significant when compared to the response to microinjection of vehicle (P<0.01, Figure 3.3A). A time-course of the MAP response to microinjection of the 300pg dose of TNFα is provided in Figure 3.3B, demonstrating that the peak response occurred at 10-minutes post-microinjection (7 ± 2 mmHg change from baseline 10-minutes post-microinjection, P=0.05, Figure 3.3B).

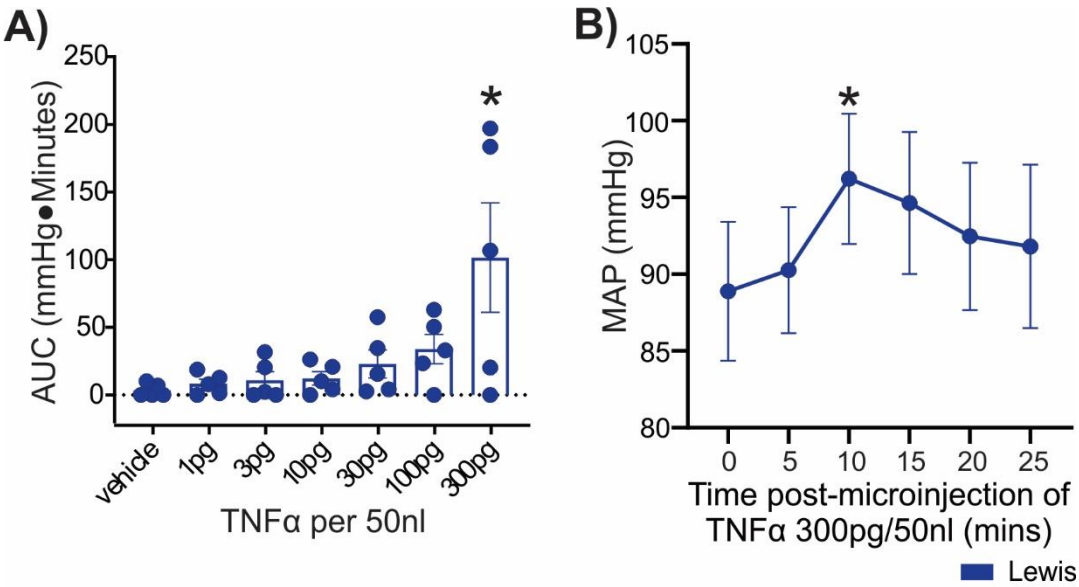

Figure 3.3 Tumour necrosis factor-α (TNFα) microinjected into the subfornical organ (SFO) caused a dose-dependent increase in (A) mean arterial pressure (MAP) in Lewis animals (n=5). Data in panel (A) are presented in area under the curve (AUC)±SEM, *P<0.05 compared to vehicle response as determined by a one-way multiple comparisons ANOVA with a post-Hoc Dunnets Test. Grouped data of the changes in (B) MAP in Lewis animals (n=5) in response to microinjection of TNFα (300pg/50nl) into the SFO. Data in panel (B) are presented as mean±SEM, *P<0.05 compared to baseline value as determined by a paired T-test.

Unlike the significant dose-dependent increase in MAP upon microinjection of TNFα into the SFO, one-way ANOVA indicated that there was no significant difference in HR (P=0.38) upon microinjection of TNFα, despite an overall tendency of HR to increase with increasing doses of

TNFα (Figure 3.4A). This is reflected in the time-course of the HR response to microinjection of 300pg dose of TNFα into the SFO of Lewis animals, provided in Figure 3.4B, which demonstrates an observable, but not significant, increase in HR (25 ± 14 bpm peak change from baseline 15-minutes post-microinjection, *P*=0.37, Figure 3.4B).

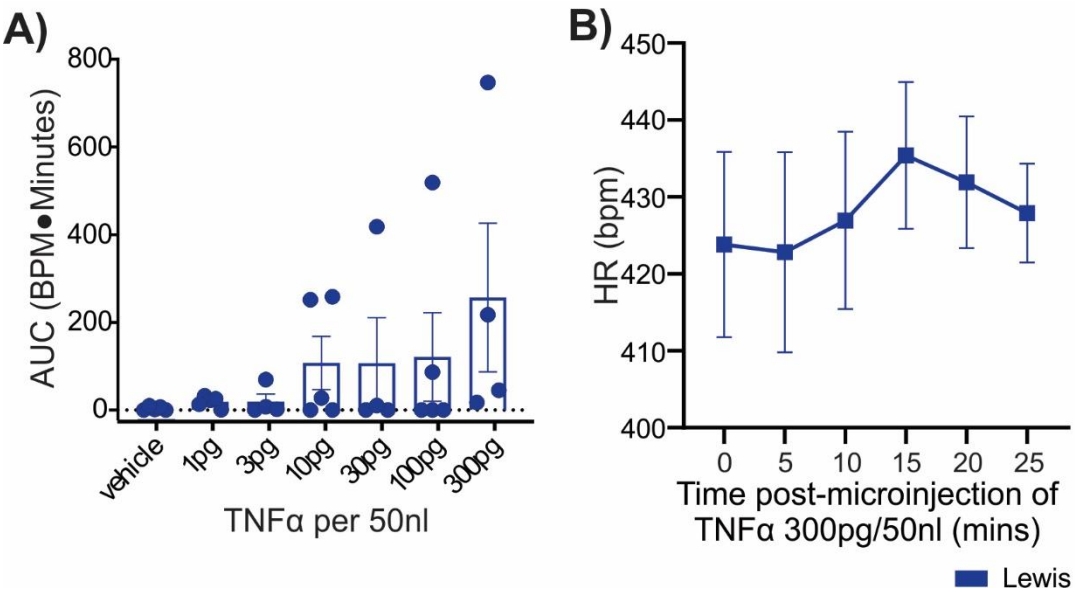

Figure 3.4 Tumour necrosis factor-α (TNFα) microinjected into the subfornical organ (SFO) caused a dose-dependent increase in (A) heart rate (HR) in Lewis animals (*n*=5). Data in panel (A) are presented in area under the curve (AUC)±SEM. Grouped data of the changes in (B) HR in Lewis animals (*n*=5) in response to microinjection of TNFα (300pg/50nl) into the SFO. Data in panel (B) are presented as mean±SEM. BPM: beats per minute.

To confirm that the pressor response observed upon microinjection of TNFα into the SFO of Lewis animals is unique to this brain region and not a response that is observed with microinjection of TNFα into any brain region, irrespective of if it plays a role in BP control, the change from baseline 10-minutes post-microinjection in offsite TNFα microinjections (*n*=2) were compared to the response observed in onsite microinjections. Unlike the marked pressor response observed at 10-minutes post-microinjection in onsite TNFα microinjections, there was no observable increase in MAP after microinjection of the 300pg dose outside of the SFO (7 ± 2 mmHg vs -3 ± 5 mmHg, onsite vs offsite TNFα microinjection change from baseline 10-minutes post-microinjection).

3.3.2. TNFRI Ab, but not minocycline, inhibits the acute pressor response to TNFα microinjection into the SFO of Lewis animals

TNFRI Ab and minocycline have been previously reported to inhibit the actions of TNFα microinjection into other brain regions in normotensive Sprague-Dawley animals (Bardgett et al., 2014; Korim et al., 2019). To validate these findings in Lewis animals, changes in MAP and HR in response to TNFα microinjected 30-minutes after the microinjection of TNFRI Ab and minocycline were recorded and compared to the uninhibited response to TNFα. Representative

traces of the changes in HR and MAP in Lewis animals in response to TNFα microinjection with no prior inhibitors, prior TNFRI Ab microinjection and prior minocycline microinjection is provided in Figures 3.5A, 3.5B and 3.5C, respectively.

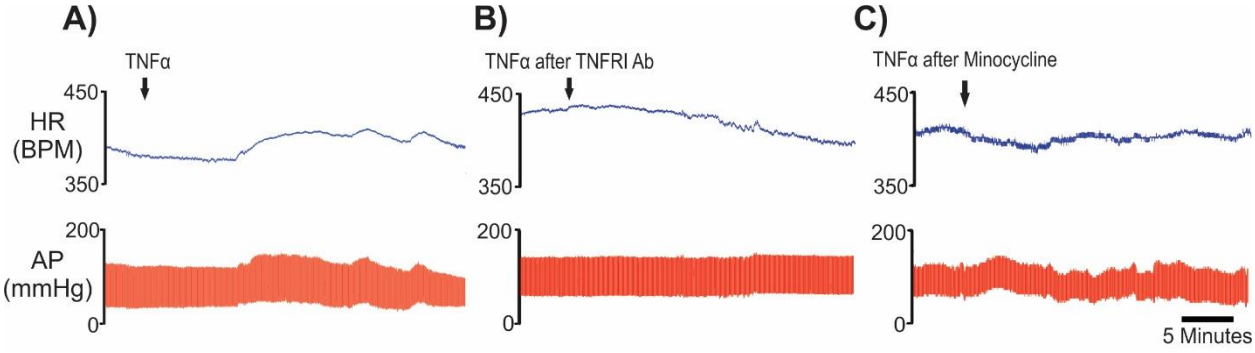

Figure 3.5 Representative traces demonstrating the effect of tumour necrosis factor-α (TNFα, 300pg/50nl) microinjection into the subfornical organ (SFO) on heart rate (HR) and mean arterial pressure (MAP) in Lewis animals with (A) no inhibitor, (B) prior tumour necrosis factor receptor 1 antibody (TNFRI Ab, 1ng/50nl) microinjection and (C) prior minocycline (0.5µg/50nl) microinjection. BPM: beats per minute.

Figure 3.6 demonstrates the change from baseline 10-minutes post-microinjection of TNFα with no prior inhibitors (*n*=5), prior TNFRI Ab microinjection (*n*=5) and prior minocycline microinjection (*n*=5), one-way ANOVA indicated that there was no overall significant difference between any of the responses (*P*=0.17). However, given the small sample sizes and the fact that the response appeared different when graphically presented, we explored this data set further. T-test comparison between the changes in MAP between microinjection of TNFα only and after TNFRI Ab microinjection demonstrates a significant difference between the responses (7 ± 2 mmHg vs 1 ± 1 mmHg, no inhibitors vs prior TNFRI Ab microinjection change from baseline 10-minutes post-microinjection, *P*=0.03). Unlike previous reports, we are unable to definitively confirm that minocycline completely inhibits the MAP responses to TNFα.

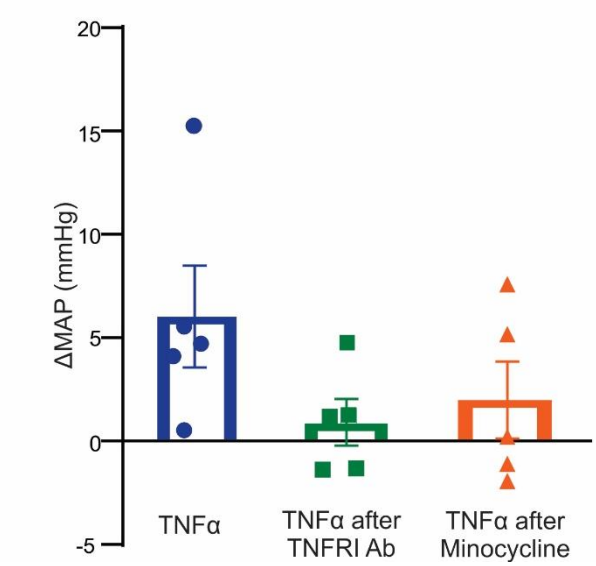

Figure 3.6 Grouped data demonstrating the change from baseline in mean arterial pressure (MAP) 10-minutes post microinjection of tumour necrosis factor-α (TNFα, 300pg/50nl) into the SFO in Lewis animals (*n*=5 per cohort) with

no prior inhibitor, microinjection of tumour necrosis factor receptor 1 antibody (TNFRI Ab, 1ng/50nl) 30-minutes prior to TNFα microinjection and microinjection of minocycline (0.5µg/50nl) 30-minutes prior to TNFα microinjection. Data is presented as mean±SEM, **P*<0.05.

3.3.3. Microinjection of TNFRI Ab and minocycline into the SFO do not acutely change BP under normotensive conditions

To determine if endogenous TNFα acts on the SFO to contribute to the control of BP under normotensive conditions, changes in BP were measured after microinjection of TNFRI Ab and minocycline into the SFO of Lewis animals. Representative traces demonstrating the acute effect of microinjection of vehicle, TNFRI Ab and minocycline into the SFO of Lewis animals on HR and MAP is provided in Figures 3.7A, 3.7B and 3.7C respectively.

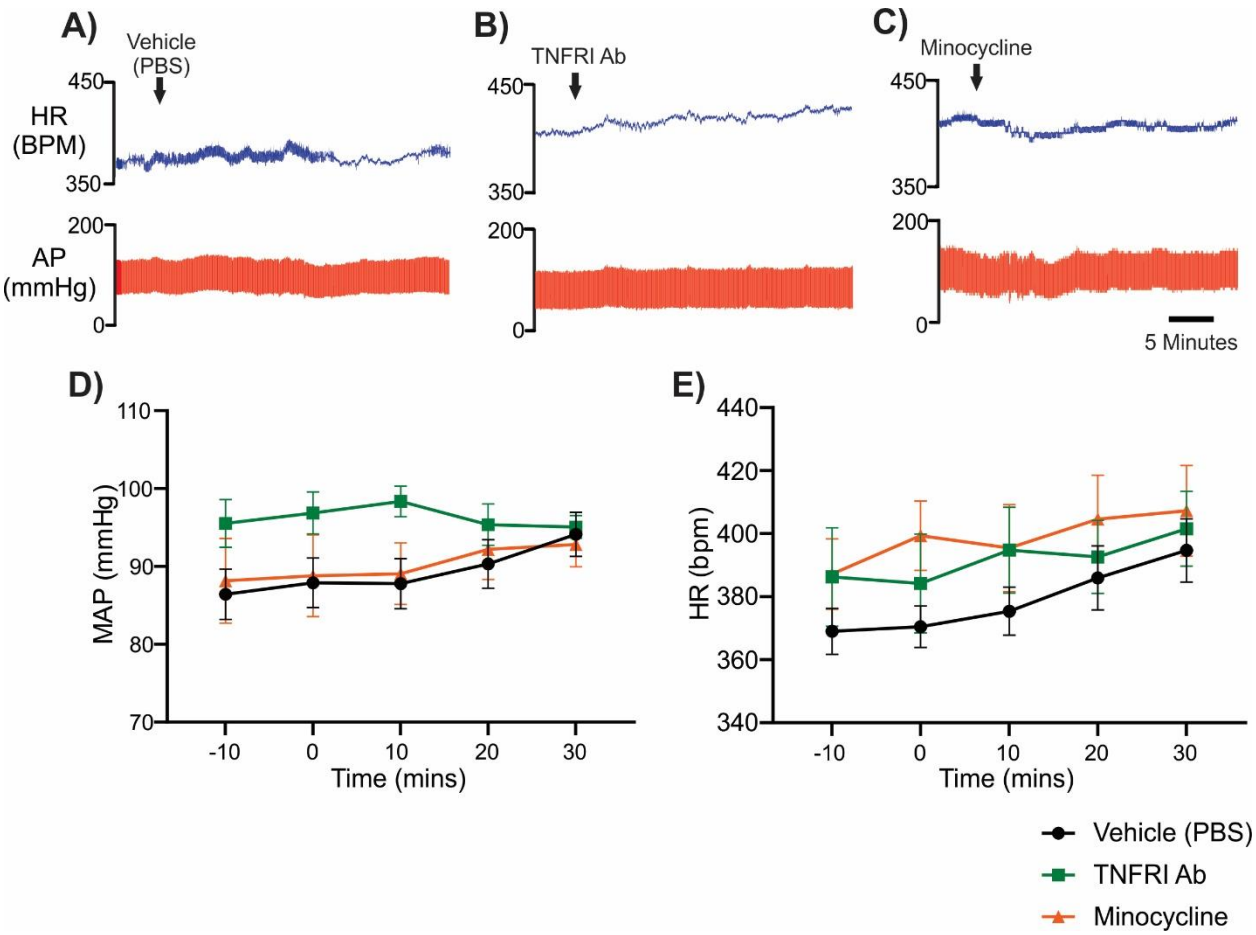

Figure 3.7 Representative traces demonstrating the effect of microinjection of (A) vehicle (phosphate buffered saline, PBS), (B) tumour necrosis factor receptor 1 antibody (TNFRI Ab, 1ng/50nl) and (C) minocycline (0.5µg/50nl) into the subfornical organ (SFO) on heart rate (HR) and mean arterial pressure (MAP) in Lewis animals. Grouped data demonstrating the change in (D) MAP and (E) HR in response to microinjection of vehicle, TNFRI Ab and minocycline into the SFO of Lewis animals (*n*=10, *n*=5 and *n*=5, respectively). Data is presented as mean±SEM. BPM: beats per minute.

A time-course of the MAP response to microinjection of vehicle, TNFRI Ab and minocycline into the SFO of Lewis animals is provided in Figure 3.7D. Two-way ANOVA of the changes in MAP to vehicle, TNFRI Ab and minocycline indicated an overall significant treatment effect (*P*<0.01).

Post-Hoc analysis on the treatment effect identified that there was a significant difference between the vehicle and TNFRI Ab (P<0.01), however this result was likely due to the different baseline values for MAP between the vehicle cohort and TNFRI Ab treated cohort. Furthermore, given there was no significant overall time effect (P=0.73) or treatment by time interaction (P=0.95), it can be determined that there was no significant effect of microinjection of TNFRI Ab or minocycline on MAP.

A time-course of the HR response to microinjection of vehicle, TNFRI Ab and minocycline into the SFO of Lewis animals is provided in Figure 3.7E. Two-way ANOVA of the changes in MAP to vehicle, TNFRI Ab and minocycline indicated an overall significant treatment effect (P<0.01). Post-Hoc analysis on the treatment effect identified that there was a significant difference between the vehicle and minocycline (P<0.01), however this is likely due to the different baseline values for MAP between the vehicle cohort and minocycline cohort. Furthermore, given there was no significant overall time effect (P=0.21) or treatment by time interaction (P=0.99), therefore it can be determined that although there appeared to be a steady increase in HR after microinjection of TNFRI Ab and minocycline (Figure 3.7E), this was not significant when compared to the vehicle response. As such it can be determined that there was no significant effect of microinjection of TNFRI Ab or minocycline on HR.

In a small subset of animals, RSNA was recorded. While preliminary, these recordings demonstrated that microinjection of the TNFRI Ab into the SFO causes a small decrease in RSNA (-10 ± 15% change from baseline 30-minutes post-microinjection; n=2 Lewis), whereas microinjection of minocycline produced a larger reduction in RSNA (-43 ± 29% change from baseline 30-minutes post-microinjection; n=3 Lewis).

In n=2 Lewis animals, microinjection of TNFRI Ab was determined to be outside of the SFO and were therefore analysed as offsite controls. Similar to the onsite TNFRI Ab microinjections, the offsite microinjections did not induce any observable changes in MAP during the 30-minute recording period (1 ± 1 mmHg vs -1 ± 1 mmHg, change from baseline onsite vs offsite TNFRI Ab microinjections).

3.3.4. Prior microinjection of TNFRI Ab and minocycline does not alter the response to microinjection of a $GABA_a$ agonist into the SFO under normotensive conditions

The SFO is an important brain region in the control of BP (Mangiapane and Simpson, 1980a; Ishibashi and Nicolaidis, 1981; Ciriello and Gutman, 2011) and previous data from our lab

demonstrate an acute depressor response upon microinjection of a $GABA_a$ agonist into the SFO of normotensive animals (Underwood et al., in Preparation). If TNFα or microglial activation by proinflammatory cytokines has a role in the control of BP in normotensive conditions, prior microinjection of TNFRI Ab or minocycline may alter the response to microinjection of a $GABA_a$ agonist. To test this hypothesis, we compared the changes in MAP after microinjection of a $GABA_a$ agonist into the SFO of normotensive animals without prior microinjection of inhibitor, prior microinjection of TNFRI Ab or minocycline. A representative trace showing the effect of microinjection of a $GABA_a$ agonist into the SFO on HR and MAP in Lewis animals under basal conditions or following microinjection of TNFRI Ab or minocycline is provided in Figures 3.8A, 3.8B and 3.8C, respectively.

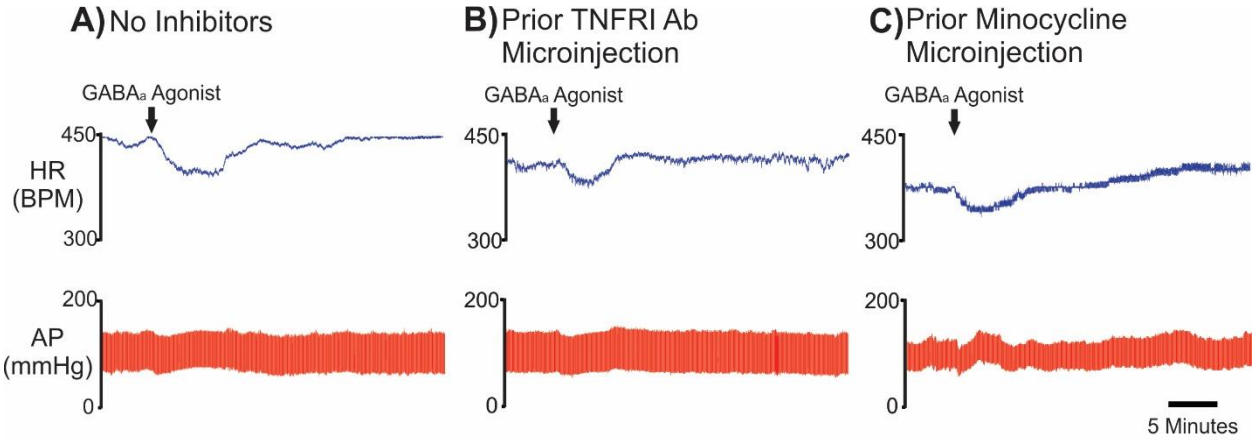

Figure 3.8 Representative traces demonstrating the effect of microinjection of a $GABA_a$ agonist (10mM isoguvacine) into the subfornical organ (SFO) on heart rate (HR) and mean arterial pressure (MAP) in Lewis animals with (A) no prior inhibitor microinjection (n=5), (B) prior microinjection of tumour necrosis factor receptor 1 antibody (TNFRI Ab, *n*=5, 1ng/50nl) and (C) prior microinjection of minocycline (*n*=5, 0.5µg/50nl). BPM: beats per minute.

In Lewis animals, the peak acute depressor response to microinjection of a $GABA_a$ agonist into the SFO was not altered by prior microinjection of either TNFRI Ab or minocycline when compared to the depressor response elicited without the presence of an inhibitor (-7 ± 2 mmHg vs -6 ± 1 mmHg vs -5 ± 1 mmHg, no inhibitor vs prior TNFRI Ab microinjection vs prior minocycline microinjection peak change from baseline 5-minutes post-microinjection, *P*=0.42, Figure 3.9), therefore confirming that it is unlikely that TNFα in the SFO contributes to the control of BP in normotensive conditions.

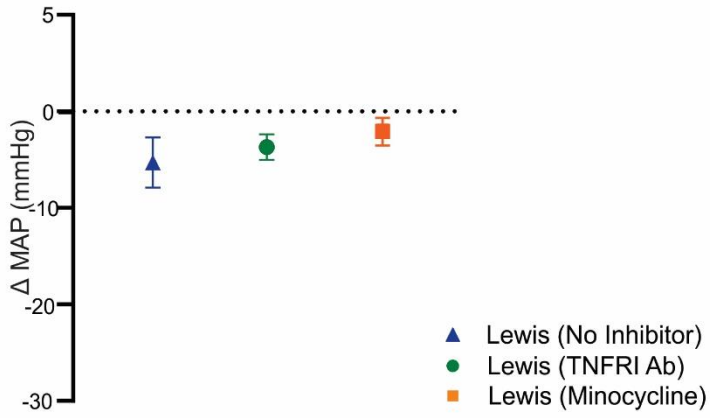

Figure 3.9 Grouped data demonstrating the acute peak change in mean arterial pressure (MAP) after microinjection of a $GABA_a$ agonist (10mM isoguvacine) into the SFO in Lewis animals with no prior inhibitor microinjection (*n*=5), prior TNFRI Ab microinjection (*n*=5, 1ng/50nl) and minocycline microinjection (*n*=5, 0.5µg/50nl)). Data is expressed in change from 5-minute baseline preceding microinjection. Values are presented as mean±SEM.

Preliminary analysis was undertaken to determine the specificity of the small depressor response to activation of $GABA_a$ receptors within the SFO by comparing the response to microinjection of a $GABA_a$ agonist within the SFO to microinjection of a $GABA_a$ agonist outside the region of the SFO. Offsite injections of a $GABA_a$ agonist were performed in two Lewis animals and preliminary comparisons show that BP increased upon offsite microinjection of a $GABA_a$ agonist, compared with the consistent and reproducible depressor response observed when the injections were targeted at the SFO (3 ± 7 mmHg vs -7 ± 2 mmHg, offsite vs onsite $GABA_a$ agonist microinjection change from baseline 5-minutes post microinjection).

3.4. Study 2: Do proinflammatory cytokines act on the SFO to increase its activity and therefore contribute to the development of hypertension in LPK animals?

3.4.1. Microinjection of TNFα into the SFO does not change MAP or HR in LPK animals

In LPK animals, microinjections of increasing doses of TNFα did not change MAP, as no dose elicited an observable or significant change in MAP (*P*=0.32, Figure 3.10A). This is in direct contrast to the actions of exogenous TNFα in the SFO in Lewis animals, as reflected by the trend towards a strain effect with TNFα microinjections on MAP (110 ± 10 mmHg•minutes vs 11 ± 6 mmHg•minutes, Lewis vs LPK average response to 300pg/50nl TNFα dose, *P*=0.06). A time-course of the MAP response in LPK animals to the highest dose of TNFα (300pg/50nl) is presented in Figure 3.10B, demonstrating only a slight decrease in MAP over the 25-minute recording period. As the peak response to TNFα was identified to occur at 10 minutes post injection in Lewis animals, we compared the change in BP at 10 minutes post TNFα microinjection between Lewis

and LPK animals. This analysis showed that TNFα microinjection in LPK animals lead to a small reduction in MAP that is significantly different from the increase in MAP in Lewis animals (-3 ± 1 mmHg vs 7 ± 2 mmHg, LPK vs Lewis change from baseline 10-minutes post-microinjection, *P*=0.03). Similar to the onsite microinjections of TNFα, in offsite controls there was a small decrease in MAP 10-minutes post-microinjection of the 300pg dose of TNFα (-3 ± 1 mmHg vs -4 ± 3 mmHg, offsite vs onsite TNFα microinjection).

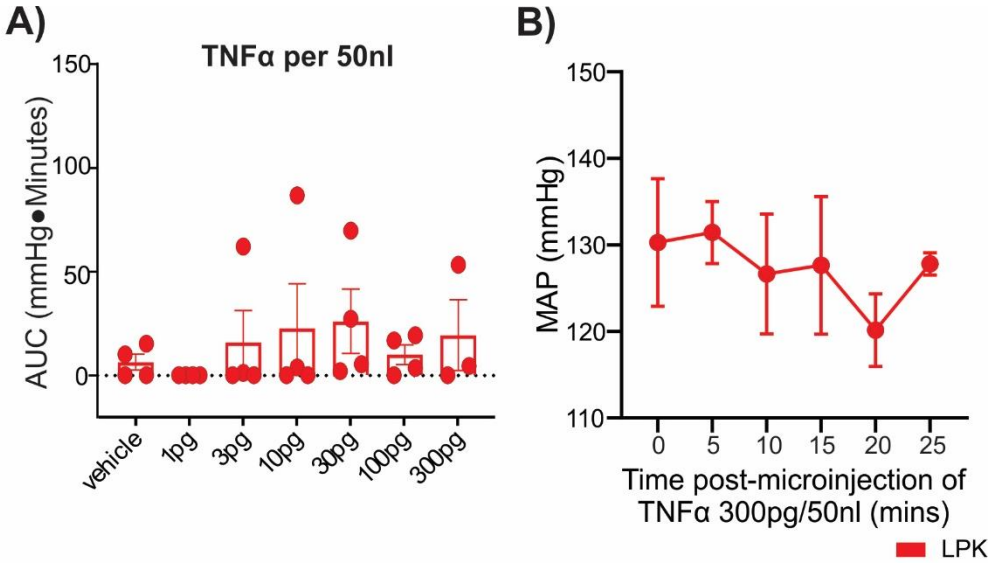

Figure 3.10 Tumour necrosis factor-α (TNFα) microinjected into the subfornical organ (SFO) did not cause a change in (A) mean arterial pressure (MAP) in Lewis polycystic kidney disease (LPK) animals (*n*=4). Data is panel (A) is presented in area under the curve (AUC)±SEM. Grouped data of the changes in (B) MAP in LPK animals (*n*=4) in response to microinjection of TNFα (300pg/50nl) into the SFO. Data in panel (B) is presented as mean±SEM. BPM: beats per minute.

Unlike the tendency towards a tachycardia response observed in Lewis animals, microinjection of increasing doses of TNFα did not cause a change in HR in LPK animals (*P*=0.30, Figure 3.11A). This is reflected in the time-course of the HR response to the highest dose of TNFα (300pg/50nl) which is presented in Figure 3.11B, demonstrating no observable changes in HR from baseline at any time point over the 25-minute recording period (*P*=0.30).

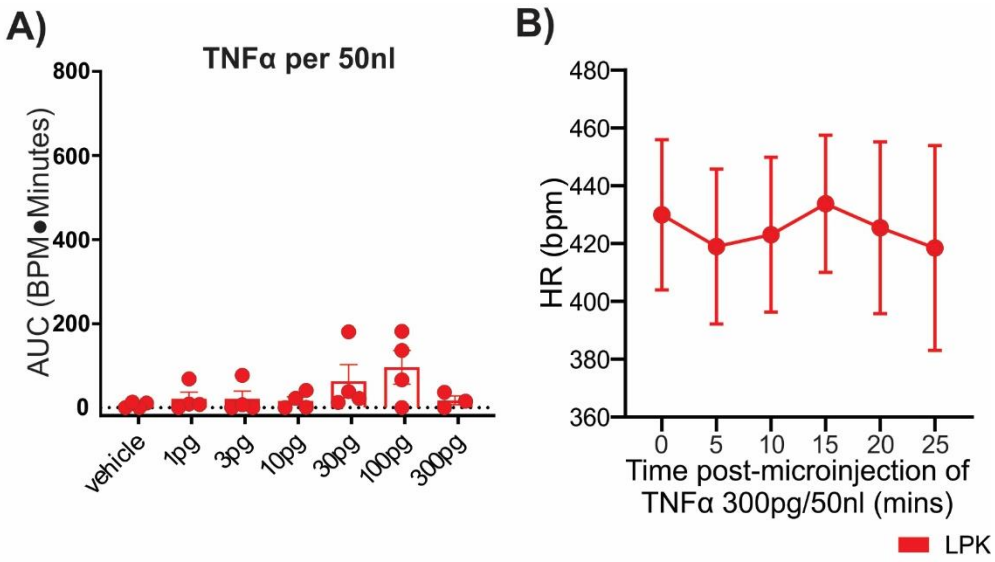

Figure 3.11 Tumour necrosis factor-α (TNFα) microinjected into the subfornical organ (SFO) did not elicit a change in (A) heart rate (HR) in Lewis polycystic kidney disease (LPK) animals (*n*=4). Data in panel (A) is presented in area under the curve (AUC)±SEM. Grouped data of the changes in (B) HR in LPK animals (*n*=4) in response to microinjection of TNFα (300pg/50nl) into the SFO. Data in panel (B) is presented as mean±SEM. BPM: beats per minute.

3.4.1. Microinjection of TNFRI Ab and minocycline into the SFO did not acutely alter MAP in LPK animals

To determine if endogenous TNFα or generalised proinflammatory cytokines act to drive the increase in activity in the SFO and therefore contribute to the development of hypertension in LPK animals, TNFRI Ab and minocycline were microinjected into the SFO. Representative traces demonstrating the acute effect in a LPK animal of microinjection of vehicle, TNFRI Ab and minocycline into the SFO on HR and MAP is provided in Figures 3.12A, 3.12B and 3.12C, respectively.

A time-course of the MAP response to microinjection of vehicle, TNFRI Ab and minocycline into the SFO of LPK animals is provided in Figure 3.12D. Two-way ANOVA of the changes in MAP to microinjection of vehicle, TNFRI Ab and minocycline indicated an overall significant treatment effect (P=0.03). Post-Hoc analysis on the treatment effect identified that there was a significant difference between the vehicle and TNFRI Ab (P=0.05), however this is likely due to the different baseline values for MAP between the vehicle cohort and TNFRI Ab treated cohort as demonstrated in Figure 3.12D. Furthermore, given there was no significant overall time effect (P=0.99) or treatment by time interaction (P=0.99), therefore it can be determined that there was no significant effect of microinjection of TNFRI Ab or minocycline on MAP in LPK animals. This result is similar to what is observed in Lewis animals, as indicated by the lack of strain effect for HR in TNFRI Ab microinjection (1 ± 1 mmHg vs -1 ± 1 mmHg, Lewis vs LPK average change from

baseline, *P*=0.95) and minocycline microinjection (-2 ± 1 mmHg vs -4 ± 1 mmHg, Lewis vs LPK average change from baseline, *P*=0.18).

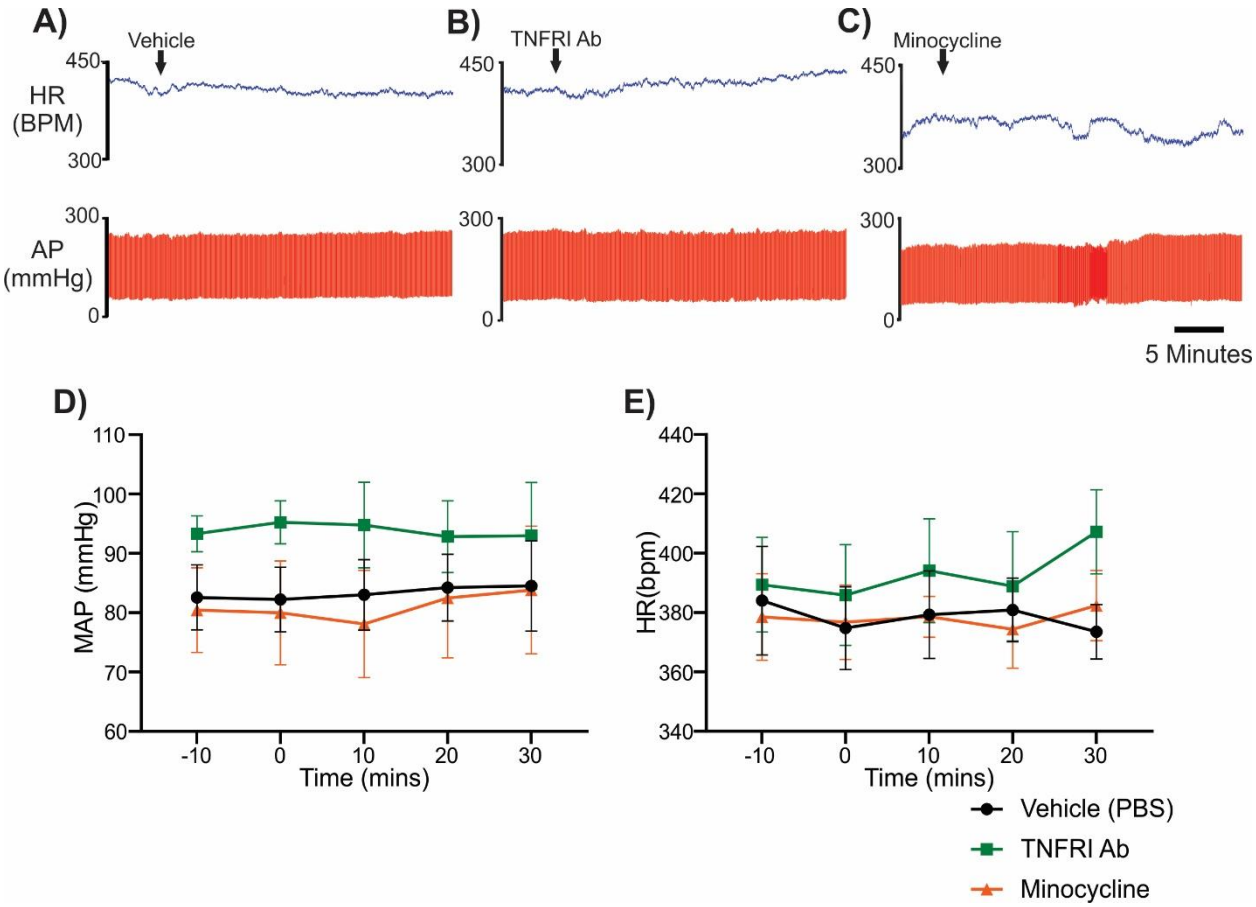

Figure 3.12 Representative traces demonstrating the effect of microinjection of (A) vehicle (phosphate buffered saline, PBS), (B) tumour necrosis factor receptor 1 antibody (TNFRI Ab, 1ng/50nl) and (C) minocycline (0.5µg/50nl) into the subfornical organ (SFO) on heart rate (HR) and mean arterial pressure (MAP) in Lewis animals (*n*=7, *n*=4 and *n*=5, respectively). Grouped data demonstrating the change in (D) MAP and (E) HR in response to microinjection of vehicle, TNFRI Ab and minocycline into the SFO. Data is presented as mean±SEM. BPM: beats per minute

A time-course of the MAP response to microinjection of vehicle, TNFRI Ab and minocycline into the SFO of LPK animals is provided in Figure 3.12D. Two-way ANOVA of the changes in MAP to vehicle, TNFRI Ab and minocycline indicated an overall significant treatment effect (*P*=0.03). Post-Hoc analysis on the treatment effect identified that there was a significant difference between the vehicle and TNFRI Ab (*P*=0.05), however this is likely due to the different baseline values for MAP between the vehicle cohort and TNFRI Ab treated cohort as demonstrated in Figure 3.12D. Furthermore, given there was no significant overall time effect (*P*=0.99) or treatment by time interaction (*P*=0.99), it can be determined that there was no significant effect of microinjection of TNFRI Ab or minocycline on MAP in LPK animals. This result is similar to what is observed in Lewis animals, indicated by the lack of strain effect for MAP in TNFRI Ab microinjection (1 ± 1 mmHg vs -1 ± 1 mmHg, Lewis vs LPK average change from baseline, *P*=0.95) and minocycline

microinjection (-2 ± 1 mmHg vs -4 ± 1 mmHg, Lewis vs LPK average change from baseline, *P*=0.18).

A time-course of the HR response to microinjection of vehicle, TNFRI Ab and minocycline into the SFO of LPK animals is provided in Figure 3.12E. Two-way ANOVA of the changes in MAP to vehicle, TNFRI Ab and minocycline indicated no significant overall treatment effect (*P*=0.23), time effect (*P*=0.97) or treatment by time interaction (*P*=0.99), therefore it can be determined that there was no significant effect of microinjection of TNFRI Ab or minocycline on HR. This result is similar to what is observed in Lewis animals, indicated by the lack of strain effect for HR in TNFRI Ab microinjection (6 ± 1 bpm vs 7 ± 3 bpm, Lewis vs LPK change from baseline, *P*=0.95) and minocycline microinjection (3 ± 3 bpm vs 14 ± 1 bpm, Lewis vs LPK change from baseline, *P*=0.18).

In a small subset of LPK animals, RSNA was recorded. While preliminary, these recordings demonstrate that microinjection of the TNFRI Ab into the SFO causes a moderate increase in RSNA (17% change from baseline 30-minutes post-microinjection; *n*=1 LPK), similarly microinjection of minocycline produces a moderate increase in RSNA (4 ± 31% change from baseline 30-minutes post-microinjection; *n*=2 LPK).

In *n*=2 LPK animals, microinjection of TNFRI Ab was determined to be outside of the SFO and were therefore considered to be offsite controls. Similar to the onsite TNFRI Ab microinjections, the offsite microinjections did not induce any observable changes in MAP during the 30-minute recording period (-1 ± 1 mmHg vs -1 ± 2 mmHg, change from baseline onsite vs offsite TNFRI Ab microinjections). Similarly, in *n*=2 LPK animals, microinjection of minocycline was determined to be outside of the SFO and were therefore considered to be offsite controls. Similar to the onsite minocycline microinjections, the offsite microinjections did not elicit any observable changes in MAP during the 30-minute recording period (-4 ± 1 mmHg vs -2 ± 1 mmHg, change from baseline onsite vs offsite TNFRI Ab microinjections).

3.4.1 Microinjection of TNFRI Ab and minocycline did not alter the tonic activation of the SFO in LPK animals

A representative trace illustrating the effect of a $GABA_a$ agonist microinjection into the SFO on HR and MAP in LPK animals with no prior inhibitors, prior microinjection of TNFRI Ab and prior microinjection of minocycline is provided in Figures 3.13A, 3.13B and 3.13C, respectively. Analysis of previous data from our research group demonstrated that microinjection of a $GABA_a$

agonist leads to a significant depressor response (-12 ± 2 mmHg peak change from baseline post-microinjection of $GABA_a$ agonist, *P*=0.02), confirming the findings of previous studies that have identified that in LPK animals, the SFO is tonically active and contributing to the hypertension present (Underwood et al., In Preperation).

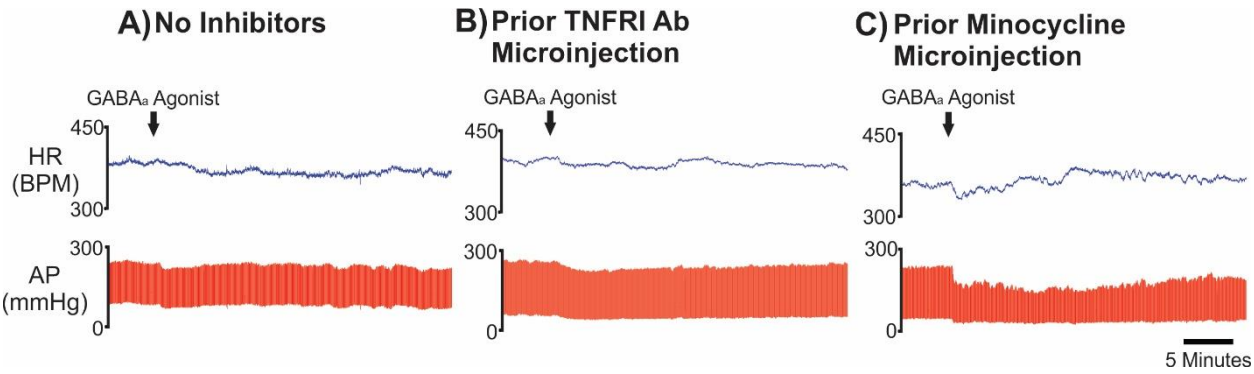

Figure 3.13 Representative traces demonstrating the effect of microinjection of a $GABA_a$ agonist (10mM isoguvacine) into the subfornical organ (SFO) on rate (HR) and mean arterial pressure (MAP) in Lewis polycystic kidney (LPK) animals with (A) no prior inhibitors, (B) prior microinjection of tumour necrosis factor receptor 1 antibody (TNFRI Ab, 1ng/50nl) and (C) prior microinjection of minocycline (0.5µg/50nl). BPM: beats per minute.

To determine if pre-microinjection with either TNFRI Ab or minocycline alters the tonicity of this brain region, the peak depressor response to microinjection of a $GABA_a$ agonist into the SFO after either TNFRI Ab or minocycline was compared to the acute response without prior microinjection of an inhibitor. One-way ANOVA indicated that the acute depressor response to microinjection of a $GABA_a$ agonist into the SFO in LPK animals was not altered by prior microinjection with TNFRI Ab or minocycline (-12 ± 2 mmHg vs -13 ± 5 mmHg vs -23 ± 4 mmHg, no inhibitor vs prior TNFRI Ab microinjection vs prior minocycline microinjection, *P*=0.11, Figure 3.14). However, given the small sample size and as the depressor response between LPK animals with no prior inhibitor and LPK animals with prior minocycline microinjection appeared to be different, we examined this data set further. T-test analysis demonstrates that prior minocycline microinjection significantly increased the depressor response when compared to the response observed with no prior inhibitors (*P*=0.04), therefore demonstrating that acute minocycline administration increased the tonicity of the SFO in LPK animals.

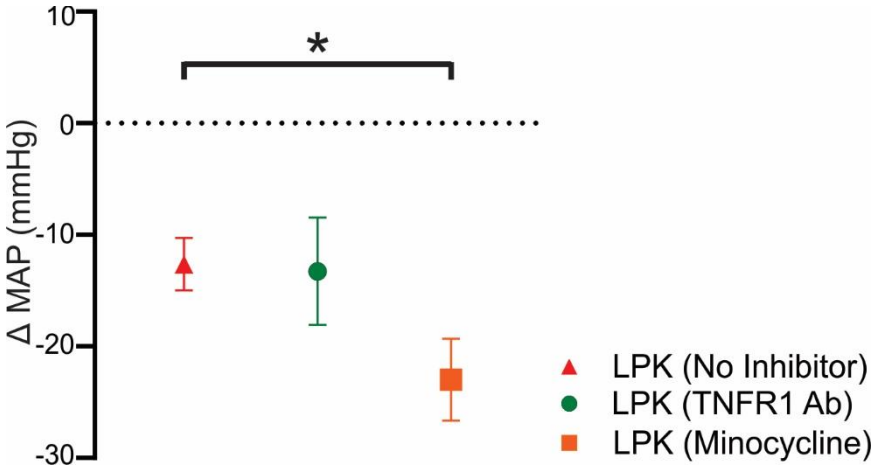

Figure 3.14 Grouped data demonstrating the acute peak change in mean arterial pressure (MAP) after microinjection of a $GABA_a$ agonist (10mM isoguvacine) into the SFO in Lewis Polycystic Kidney (LPK) animals without prior microinjection of inhibitors (*n*=6), with prior tumour necrosis factor receptor 1 antibody (TNFRI Ab, 1ng/50nl) microinjection (*n*=4, 0.5µg/50nl)) and minocycline microinjection (*n*=5). Data is expressed in peak change from baseline preceding microinjection. Values are presented as mean±SEM. **P*<0.05.

Finally, preliminary analysis was undertaken to determine the specificity of the small depressor response to activation of $GABA_a$ receptors within the SFO by comparing the response to microinjection of a $GABA_a$ agonist within the SFO to microinjection of a $GABA_a$ agonist outside the region of the SFO. Offsite microinjections of a $GABA_a$ agonist were performed in two LPK animals and preliminary comparisons show that there is no observable decrease in MAP in these animals compared with the consistent and reproducible depressor response observed when the microinjections were targeted at the SFO in LPK animals (-1 ±2 mmHg vs -12 ± 2 mmHg, offsite $GABA_a$ agonist microinjection vs onsite $GABA_a$ agonist microinjection peak change from baseline 5-minutes post microinjection).

3.5 Preliminary analysis indicated that microglial activation is present in LPK but not Lewis control animals

A representative image of an SFO from a Lewis and LPK stained with anti-Iba1 antibody is provided in Figures 3.15A and 3.15B, respectively. Preliminary observation of the microglia in the SFO of both animals suggests that LPK animals may have more activated microglia than Lewis animals, however further analysis is required to confirm this.

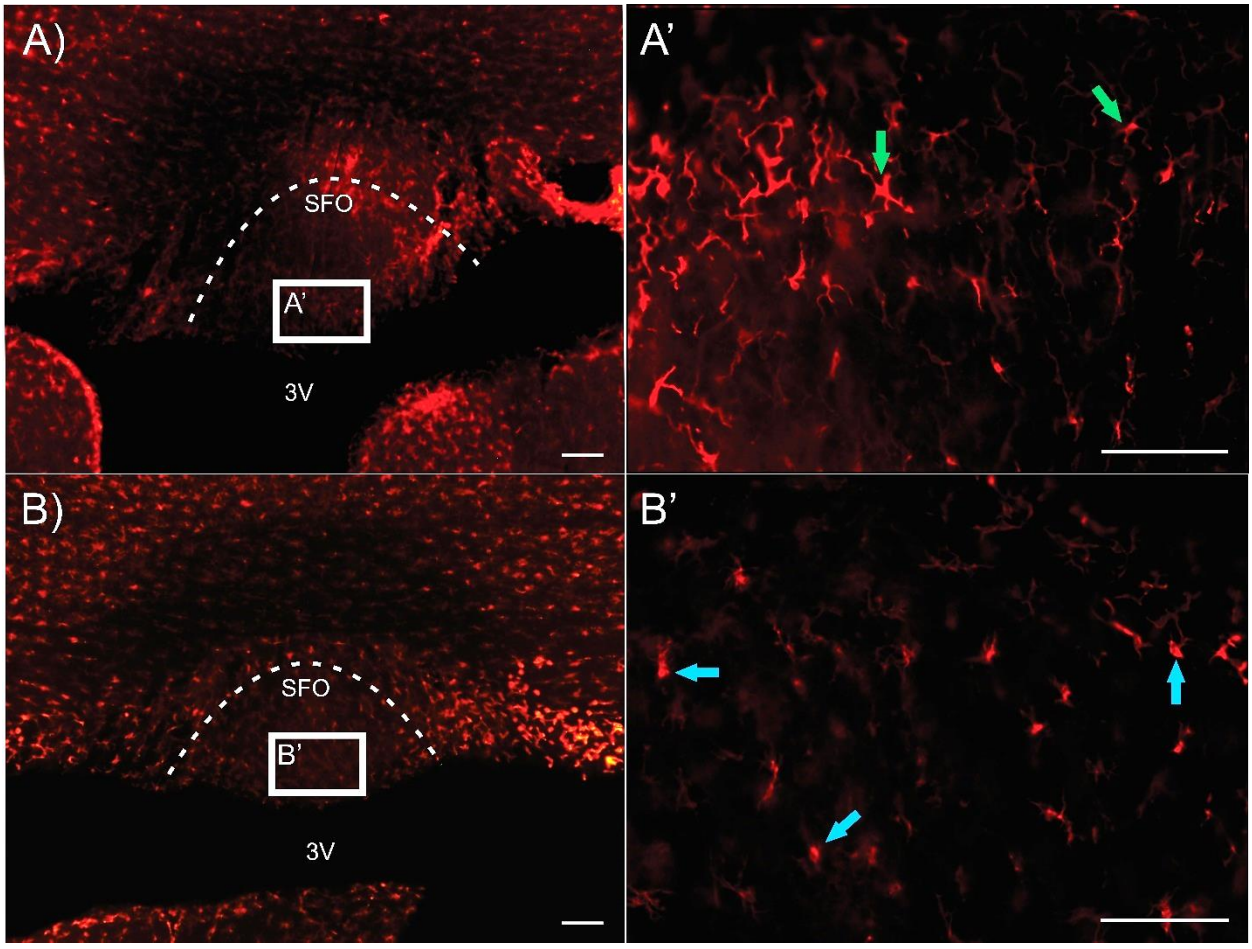

Figure 3.15 Representative microscopy images (5x magnification) of the subfornical organ (SFO) of a (A) Lewis animal and a (B) Lewis polycystic kidney disease animal stained with anti-Iba1 antibody. Surveillant microglia are indicated by green and activated microglia are indicated by blue arrows. 3V: third ventricle. Scale bar = 100µm.

4. Discussion

An increase in glutamatergic input from the SFO to the PVN is, in part, responsible for the development of hypertension in the LPK model of PKD (Underwood et al., 2019, In Preparation), however what is driving this increase in activity of the SFO is unknown. Proinflammatory cytokines, namely TNFα, increase neuronal excitability in the SFO (Wei et al., 2015; Simpson and Ferguson, 2017). As such, we hypothesised that endogenous TNFα may be driving this increase in activity of the SFO in LPK animals and thus contributes to the development of hypertension. The purpose of this thesis was to 1) identify the role that endogenous proinflammatory cytokines play in the control of BP under normotensive conditions and from that 2) examine the hypothesis that endogenous proinflammatory cytokines act on the SFO to increase its neuronal excitability in LPK animals.

The major findings of this thesis are: (1) TNFα produces a dose-dependent increase in MAP in Lewis but not LPK animals; (2) acute specific blockade of TNFα does not change MAP in either strain; (3) general blockade of all microglial activation does not change MAP in either strain; (4) microinjection of minocycline reveals an increase in tonicity of the SFO in LPK animals; and (5) preliminary observation suggests that LPK animals have considerably higher levels of microglial activation in the SFO when compared to Lewis animals. These data suggest that the short-term actions of endogenous proinflammatory cytokines in the SFO do not contribute to the control of BP in normotensive conditions or the SFO overactivity in LPK animals. However, this thesis cannot exclude the role that proinflammatory cytokines may play in the long-term control of BP, therefore further long-term inhibition of proinflammatory cytokines is warranted.

4.1. Study 1: Proinflammatory cytokines in the SFO do not contribute to the control of BP in normotensive conditions

4.1.1. Microinjection of TNFα into the SFO causes a dose-dependent increase in BP, but not HR, in normotensive animals

Microinjection of TNFα into the SFO elicits an increase in BP and HR in normotensive animals (Wei et al., 2015), however if this response to TNFα is dose-dependent and how this contrasts to actions immediately outside of the SFO is unknown. As such, changes in MAP and HR in response to microinjection of increasing doses of TNFα into the SFO and cortical regions outside the SFO were recorded. In Lewis animals, microinjection of increasing doses of TNFα causes a dose-dependent increase in MAP that was not observed in offsite controls. Upon microinjection of the highest dose of TNFα (300pg/50nl), the peak MAP response was observed approximately 10-minutes post-microinjection, a response consistent with the onset observed in previous studies (Wei et al., 2015). This rapid or acute response to TNFα is likely triggered by an alteration in glutamate transmission in the SFO, as in other brain regions TNFα causes an increase in the release of glutamate from neurons and microglia (Bezzi et al., 2001; Takeuchi et al., 2006) and a decrease in astrocytic uptake of glutamate from the synaptic cleft (Han and Whelan, 2010). Thus, to clarify the underlying mechanisms of this rapid pressor response, further experiments involving the microinjection of kynurenic acid, a non-specific ionotropic glutamate receptor antagonist, prior to TNFα microinjection are required to determine if it is mediated by glutamate transmission.

Despite the similar onset of the pressor response, the duration and magnitude observed in this thesis varies considerably to Wei et al. (2015). We observed a peak increase of approximately 7mmHg that lasted 20-30minutes before returning to baseline, whereas Wei et al. (2015) observed an increase of approximately 20mmHg that was reported to remain above baseline for more than five hours post-microinjection. This longer response may be explained by a range of longer-term adaptive changes induced by TNFα microinjection. Four hours post-microinjection of TNFα in the SFO, Wei et al. (2015) demonstrated an upregulation in mRNA expression of angiotensin converting enzyme, angiotensin II type 1 receptor and cyclooxygenase-2 which may make the SFO more susceptible to excitatory inputs. Prior microinjection with the angiotensin II type 1 receptor inhibitor losartan, angiotensin converting enzyme inhibitor captopril or the cyclooxygenase-2 inhibitor NS-398 were all shown to blunt the response to microinjection of TNFα individually (Wei et al., 2015). However Wei et al. (2015) did not examine if this pressor response is mediated by the activation of TNFRI by TNFα. As such, further studies involving the microinjection of TNFRI Ab and combinations of the range of inhibitors are required to elucidate the contributions of each mechanism to this longer-term pressor response and if these mechanisms

are combinatory. Additionally, it is possible that this pressor response could be contributed to by off-target actions of angiotensin II. Therefore, future studies with systemic inhibition of the renin-angiotensin system are required to definitively confirm if this response is contributed by the local actions of angiotensin II (Lind et al., 1983) and not systemic effects that act to raise BP (Fyhrquist et al., 1995).

These data suggests that there might be two types of changes that occur within the SFO in response to TNFα microinjection; (1) acute changes in glutamate transmission that bring about the rapid but short-lasting pressor response that was observed in this thesis and (2) longer term adaptive changes that trigger the sustained pressor response observed in the study by Wei et al. (2015). One difference that may account for the conflicting results is that Wei et al. (2015) used a substantially larger concentration of TNFα (25ng of TNFα per microinjection) when compared to the dose of TNFα used in this thesis (300-600pg of TNFα per microinjection). We elected to use a lower dose range of TNFα (1pg to 300pg) for microinjection as research has already been undertaken in normotensive animals at the nanogram dose range (Wei et al., 2015) and studies that examine the role of TNFα in other circumventricular organs have shown success in the lower concentration ranges (Korim et al., 2019). Furthermore, this lower dose range was chosen as it more accurately represents the levels of TNFα in the bloodstream as PKD patients typically have a blood concentration of TNFα in the picogram range (Gardner et al., 1991; Kocyigit et al., 2012). As this thesis has demonstrated that TNFα increases MAP in a dose-dependent manner, this could explain this discrepancy between the two studies. Furthermore, this indicates that higher doses of TNFα may be required to induce the longer-term adaptive changes. Therefore, future studies could dissect the mechanisms behind the pressor response observed at each dose through the microinjection of inhibitors of the actions of TNFα (TNFRI Ab or etanercept), glutamate transmission (kynurenic acid), the renin angiotensin system components (losartan and captopril) and cyclooxygenase-2 (NS-398). Understanding the underlying mechanisms by which TNFα acts in the SFO to increase BP is important as it may reflect the mechanisms by which hypertension develops in the presence of high plasma TNFα.

Another difference between this thesis and the study by Wei et al. (2015) is that this thesis used a smaller volume of the vehicle (100nl vs 200nl) to deliver the drug to the SFO. However, as this thesis demonstrates that off target microinjection of low dose TNFα does not trigger a significant pressor response, therefore even if the drug did spread to offsite cortical regions, it is unlikely that this would account for the difference in responses. Furthermore, it is unlikely that the difference in response is a result of diffusion through the ventricular system as, although Wei et al. (2015)

used a larger volume, the volume required for intracerebroventricular microinjections is considerably larger (Emanueli et al., 1999; Miller et al., 2002; DeVos and Miller, 2013). Finally, Wei et al. (2015) did not provide an anatomical map of their distribution of microinjection sites, therefore it remains a possibility that these differences in response might be due targeting of different regions of the SFO, as it has been suggested that there may be different subpopulations of neurons in the SFO that each play a distinct physiological role (Hindmarch and Ferguson, 2016).

The 300pg dose was chosen for further experiments in this thesis as, unlike lower doses, it led to a significant and reproducible response that returns to baseline in the acute setting. We opted to not use a higher dose as Korim et al. (2019) reported a plateau in the response to TNFα in the area postrema at similar doses to that used in this thesis and, as such, higher doses would not be necessary to demonstrate the acute response of TNFα in the SFO. Although, future dose-curve studies with the inclusion of higher doses will be required to further elucidate the mechanisms underlying the pressor response at all doses and to understand our findings better within the context of what has already been discovered around the role of TNFα in the SFO on cardiovascular control.

Unlike previous reports that demonstrate a significant increase in HR after microinjection of TNFα into the SFO (Wei et al., 2015) or the area postrema (Korim et al., 2019), this thesis did not identify a significant dose-dependent increase in HR after TNFα microinjection. Although these results need to be validated in a larger cohort, the differences in dose of TNFα (Wei et al., 2015) likely account for the lack of significant tachycardia response as the dose of TNFα used in this thesis may not have been high enough to elicit a significant increase in HR, especially if changes in HR are brought about only by the longer term adaptive changes.

The SFO is a key brain region that is more involved in the control of BP than HR, likely due to its direct neural connections to the PVN, an important hypothalamic nuclei involved in the control of BP via the autonomic nervous system and endocrine system (Ishibashi and Nicolaidis, 1981; Lind et al., 1983; Ferguson and Renaud, 1984; Ciriello and Gutman, 2011). On the other hand, the area postrema has direct neural connections to the nucleus of the solitary tract, a key nuclei that directly alters HR, and the rostral ventrolateral medulla, a nuclei that has a small role in indirectly controlling HR (Shapiro and Miselis, 1985; Tan et al., 2007; Kumagai et al., 2012). Therefore, this difference in the roles and afferent connections between the SFO and the area postrema may account for these differences observed.

Additionally, differences in surgical preparation in regards to the vagus nerve may account for the discrepancy in HR responses to TNFα microinjection, as the vagus nerve supplies parasympathetic innervation to the heart acting to reduce HR (Liu et al., 2012). This thesis used a surgical preparation where the vagus nerve remained intact and used a non-vagolytic paralytic agent (cisatracurium) (Kandukuri et al., 2018). Therefore, in this thesis the vagus nerve would be able to blunt any changes in HR elicited upon TNFα microinjection. On the other hand, Korim et al. (2019) used pancuronium bromide, a paralytic agent that has been shown to have a significant vagolytic action (Punnen et al., 1984). Therefore, this would allow for the sympathetic nervous system to more easily drive an increase in HR upon TNFα microinjection as there was no opposing parasympathetic input to the HR via the vagus nerve. However, it is not clear if Wei et al. (2015) used vagolytic paralysing agents or if they disrupted the vagal input into the heart in their study, as such this makes it challenging to compare results. Therefore, further studies are required to determine the sympathetic contribution to tachycardia response upon TNFα microinjection, especially as it is speculated that the response observed upon TNFα microinjection is mediated, in part, by the activation of the sympathetic nervous system. However, this is based only upon the observation of a significant increase in RSNA (Wei et al., 2015). As such, future studies should aim to record sympathetic nerve activity from multiple beds including the renal nerve and administer hexamethonium to ensure that it attenuates the pressor response to microinjection of TNFα, before we can definitively confirm that this response is sympathetically mediated.

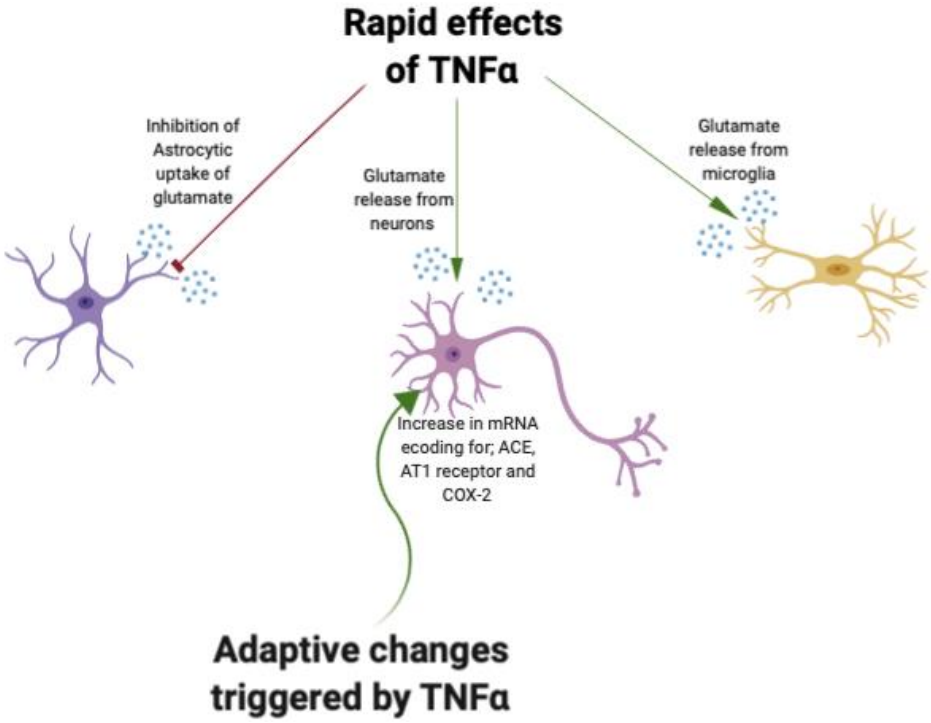

Figure 4.1 Tumour necrosis factor-α (TNFα) in the central nervous system leads to a range of physiological changes in neurons, microglia and astrocytes that increases neuronal excitability. ACE: angiotensin converting enzyme, ATI: angiotensin II receptor type 1, COX-2: cyclooxygenase-2. Created with Biorender.com.

4.1.2. TNFRI Ab, but not minocycline, inhibits the acute pressor response to TNFα microinjected into the SFO of Lewis animals

Here we extend on previous observations that TNFα acts on the TNFRI subtype to increase BP in other brain regions (Bardgett et al., 2014; Korim et al., 2019), however this thesis is the first to identify that the pressor response elicited by TNFα in the SFO is driven by TNFRI. This is consistent with the observation that TNFRI is the only TNFα receptor subtype present in the SFO (Nadeau and Rivest, 1999; Hindmarch et al., 2008) and whilst this needs to be confirmed in a larger sample size, this demonstrates that our observations of a dose-dependent increase in BP are driven by the actions of TNFα on TNFRI rather than off-target actions, such as an upregulation of angiotensin II signalling.

In this thesis, microinjection of minocycline did not completely inhibit the pressor response to TNFα. This contrasts previous reports that show the complete inhibition of the actions of TNFα by minocycline microinjection into the PVN. However it should be taken into consideration that in this thesis TNFα was microinjected 30-minutes after minocycline, whereas Bardgett et al. (2014) reported the complete inhibition of the actions TNFα when TNFα was microinjected five minutes after minocycline. As the half-life of minocycline is approximately 17 hours (Agwuh and MacGowan, 2006), it is unlikely that these differences in time frame would account for the lack of complete inhibition observed in this thesis. Interestingly, Bardgett et al. (2014) reported no significant increase in BP upon microinjection of TNFα into the PVN despite using a considerably higher dose than used in this thesis (5ng vs 300-600pg). These differences in results suggest that TNFα may have different mechanisms of action in different brain regions. This could be further examined by measuring the mRNA levels of the range of mechanisms that have been implicated in the development of the pressor response by TNFα in the PVN after microinjection of TNFα. Minocycline, an inhibitor of microglial activation, acts to inhibit protein kinases and other major downstream signalling molecules within microglia activated by proinflammatory cytokines (Pi et al., 2004; Nikodemova et al., 2006). Although microglia are therefore a major target for TNFα in the central nervous system, TNFα has also been shown to cause altered glutamate transmission in astrocytes and neurons (Takeuchi et al., 2006; Han and Whelan, 2010). As we postulate that the acute response to the 300pg dose of TNFα is mediated by this alteration in glutamate transmission, this may account for the lack of complete inhibition of this response by minocycline. This could be examined further by measuring the excitability of dissociated SFO neurons and astrocytes exposed to TNFα with and without the presence of kynurenic acid, as has been applied to identify that astrocytes and an alteration of glutamate transmission are responsible for the excitability of neurons in PVN exposed to angiotensin II (Stern et al., 2016). Overall, these data demonstrate that

the pressor response observed upon microinjection of TNFα into the SFO is likely mediated by activation of the TNFRI subtype on multiple neuronal cell types, not just microglia.

4.1.3. Ongoing activation of the TNFRI by TNFα in the SFO does not contribute to the control of BP in normotensive conditions

Basal levels of TNFα have been detected in the blood stream (Arican et al., 2005; Noori et al., 2017) and in cortical brain regions (Barichello et al., 2009), therefore it is possible that normal physiological levels of TNFα act in the SFO to contribute to the ongoing control of BP. This thesis was the first to demonstrate that ongoing activation of TNFRI in the SFO does not contribute to the control of BP in normotensive conditions, as we observed no changes in MAP over the 30-minute recording period post-microinjection of TNFRI Ab. This result is reflected in previous studies which identified that microinjection of TNFRI Ab into the area postrema (Korim et al., 2019) and etanercept, a TNFα inhibitor, into the PVN (Bardgett et al., 2014) did not change BP in normotensive animals. As the SFO has unique access to the bloodstream (Gross, 1991; Petrov et al., 1994), it is likely that there are more dominant peripheral signals, such as angiotensin II and plasma sodium (Simpson and Routtenberg, 1973; Anderson et al., 2000), that override the effects of physiological levels of TNFα acting in the SFO. Finally, this thesis can only account for the acute effects of TNFα in the SFO on the ongoing control of BP. As TNFα likely produces longer-term adaptive changes (Wei et al., 2015), further studies involving the long-term inhibition of TNFα are required to completely exclude the actions of TNFα in the SFO on the control of BP under normotensive conditions.

4.1.4. Ongoing microglial activation does not contribute to the control of BP in normotensive conditions

Microglia play an important role in ensuring that homeostasis is maintained within the central nervous system and, when a threat to homeostasis is detected, they become activated in order to remove this threat (Kreutzberg, 1996). In this process of activation they release a plethora of substances including proinflammatory cytokines, chemokines and reactive oxygen species (Colonna and Butovsky, 2017). As such, given that minocycline is an inhibitor of microglial activation we aimed to establish if microglia contribute to the SFO's ability to control BP under normotensive conditions. This thesis identified that the inhibition of the acute actions of microglial activation did not change BP. This result reflects the findings of Bardgett et al. (2014), as they also report no change in BP 30-minutes post-microinjection of minocycline into the PVN. This demonstrates that in two key BP control regions microglial activation has no major impact upon the ongoing control of BP, although long-term inhibition of microglia will be required to definitively confirm these results.

Although further replicates are required to definitively confirm this observation, preliminary data showed that microinjection of minocycline led to a large and sustained increase in RSNA despite no observable changes in either MAP or HR. As an increase in RSNA typically produces an increase in BP (DiBona and Kopp, 1997), this suggests that minocycline might cause the underlying changes in the physiological control of BP under normotensive conditions. The SFO can direct outflow to multiple blood pressure regulators including the autonomic nervous and the neuroendocrine system (Bankir, 2001; Sabban et al., 2009), as such microinjection of minocycline could lead to differential outflow in these regulators that could counteract the increase in RSNA, therefore leading to no net effect on BP. This could be examined by measuring sympathetic nervous activity from multiple nerve beds, hexamethonium administration to remove the effects of the autonomic nervous system and the systemic administration of inhibitors to the BP regulating hormones that the SFO can induce the release from the PVN (vasopressin, corticotropic releasing hormone and adrenocorticotropic hormone).

4.1.5. Prior microinjection of TNFRI Ab and minocycline do not alter the physiological response to microinjection of a $GABA_a$ agonist into the SFO under normotensive conditions

To confirm that microinjection of TNFRI Ab and minocycline did not alter the physiological functioning of the SFO, a $GABA_a$ agonist was microinjected into the SFO. Activation of the $GABA_a$ receptor subtype induces an influx of chloride ions thereby increasing the firing threshold of neurons and causing neuronal inhibition (Olsen and DeLorey, 1999). Upon microinjection of a $GABA_a$ agonist into the SFO of Lewis animals we observed a significant, but brief, decrease in MAP. A significant alteration in this acute depressor response would indicate that endogenous proinflammatory cytokines may contribute to the control of BP in the SFO. However, this depressor response observed was not altered by prior microinjection of either TNFRI Ab or minocycline. Above, we hypothesised that the acute pressor response to microinjection of TNFα is mediated by an alteration in glutamate transmission, causing an increase in excitability of the SFO (Pinheiro and Mulle, 2008), if this was the mechanism underlying the pressor response we would expect a significantly greater depressor response to $GABA_a$ activation in animals treated with TNFα only when compared to animals treated with either inhibitor, as the alteration in glutamate transmission the neurons in the SFO should make it more excitable. However, this response not observed in this thesis. An explanation of these results is that the $GABA_a$ agonist was microinjected 30-minutes after the microinjection of TNFα. As the pressor response induced by TNFα returned to baseline within 30-minutes, neuronal activity may have returned to baseline levels before the $GABA_a$ agonist microinjection was performed. As such, further studies should

microinject the $GABA_a$ agonist immediately following TNFα microinjection to confirm that the pressor response is in fact caused by an increase in neuronal excitability. Overall, these data paired with the lack of changes to BP upon microinjection of TNFRI Ab or minocycline indicate that in the short-term, proinflammatory cytokines in the SFO do not contribute to the control of BP under normotensive conditions.

4.2. Study 2: Proinflammatory cytokines do not act on the SFO in the short-term to increase its activity and therefore do not contribute to the development of hypertension in LPK animals

4.2.1. Microinjection of TNFα does not change MAP or HR in LPK animals

To the best of our knowledge, this is the first study to microinject TNFα into any brain region involved in BP control of a hypertensive animal model, as previous studies only report the responses to TNFα microinjection in normotensive animals (Bardgett et al., 2014; Wei et al., 2015; Korim et al., 2019). This thesis identifies that microinjection of TNFα into the SFO of LPK animals does not elicit any changes in BP, contrasting the dose-dependent pressor response observed in Lewis animals.

Long term incubation of TNFα has been shown to increase the basal firing rate and excitability of SFO neurons (Simpson and Ferguson, 2017), therefore one explanation for the lack of pressor response in LPK animals is that the basal firing rate of SFO neurons is already at maximal levels. Preliminary studies have identified that TNFα is elevated in the kidneys of LPK animals (Ta et al., 2016), similar to what is observed in human patients with PKD who have higher levels of TNFα in their kidneys (Gardner et al., 1991) and bloodstream (Menon et al., 2011). As such, it could be reasoned that LPK animals may have higher levels of circulating TNFα which could possibly already be acting in the SFO to increase its activity. This hypothesis could be examined by recording the effect of TNFα incubation on dissociated LPK neurons to determine if TNFα has the ability to increase their excitability.

We have already identified that the SFO is overactive in LPK animals (Underwood et al., In Preparation), which implies that there may already by an increase in excitability of this brain region. As such, it is conceivable that this increase in excitability may be driven by an increase in glutamate transmission. Therefore, as the short-term mechanisms of the pressor response of TNFα is postulated to be an alteration in glutamate transmission between neural cells, this may explain the lack of pressor response in LPK animals as the low dose of TNFα used in this thesis may not

cause a change in glutamate transmission that is large enough to overcome this alteration in glutamate transport to produce a resulting pressor response. This could be examined by measuring the glutamate levels in the SFO of LPK animals, the changes in BP in response to kynurenic acid and staining for glutamate receptor density.

4.2.2. Ongoing activation of TNFRI by TNFα does not contribute to the overactivation of the SFO in LPK animals

This is the first study to investigate the role of TNFα in the development of hypertension in a form of CKD. Although this needs to be confirmed in a larger sample size, this thesis demonstrates that acute specific blockade of TNFα did not acutely alter BP in LPK animals. We hypothesised that LPK animals may already have too much TNFα in SFO and therefore we anticipated a drop in BP upon microinjection of TNFRI Ab. One explanation that may justify these results is that TNFα possibly isn't tonically active in the SFO of LPK animals in the acute setting. This is likely, as TNFα acts in the SFO to induce long term adaptive changes that increase neuronal excitability (Wei et al., 2015; Simpson and Ferguson, 2017) and as such TNFα may be tonically active in the long-term in the SFO, therefore longer inhibition may be required to elicit a reversal of these mechanisms and therefore observe a decrease in BP. Furthermore, it has been identified that the PVN, the target of the SFO's increase in activity, is overactive in six week old LPK animals (Underwood et al., 2019), therefore it is likely that the SFO is also overactive from this young age. As such, this thesis may have inhibited TNFα too far in this disease state, therefore further experiments inhibiting the actions of TNFα in the SFO at a younger age will be required to examine the role that TNFα plays in the establishment of the disease.

This thesis cannot rule out the fact that in LPK animals TNFα may be the trigger that acts on the SFO to prime it to undergo changes that result in its overactivation. In normotensive animals, microinjection of TNFα leads to longer term adaptive changes which include an increase in the mRNA encoding for angiotensin converting enzyme, angiotensin II type 1 receptor, and cyclooxygenase-2 (Wei et al., 2015), as such similar changes could also be triggered in the SFO of LPK animals. It is unlikely that the actions of angiotensin II are the underlying causative factor of the increase in SFO activity as systemic losartan treatment did not reduce the SFO-derived glutamatergic tone in the PVN (Underwood et al., In Preparation), however long term microinjections of losartan into the SFO are required to definitively confirm this.

A currently unexplored pathway in the context of PKD is that if cyclooxygenase-2 plays a role in the increase in activity of the SFO. Cyclooxygenase-2 acts in the central nervous system to produce prostaglandins, which have been shown to increase the activity of the SFO through disinhibition (Tabarean et al., 2004), therefore it is a possibility that they may play a mechanistic role in the development of SFO overactivity. As such, future studies should examine the effect of microinjection of NS-398 into the SFO of LPK animals to determine if cyclooxygenase-2 acts within to SFO to increase its activity. Furthermore, previous studies show that the actions of TNFα are partially dependent on the actions of prostaglandins in the PVN (Zhang et al., 2003). This hints that the effects of TNFα and cyclooxygenase-2 may potentially be combinatory in causing the SFO overactivity, therefore future studies should examine the effect of inhibiting both factors by microinjections of TNFRI Ab and NS-398.

Finally, it needs to be noted that unlike the Lewis animals, we could not validate the actions of TNFRI Ab and minocycline in the SFO of LPK animals due to a lack of response to TNFα, thus limiting the conclusions that we can make from this study. Therefore, future studies should undertake immunostaining for the TNFRI Ab in the SFO of LPK animals to ensure that it is still normally expressed, as underlying pathologies can alter the expression and distribution of TNFα receptor subtypes (Nadeau and Rivest, 1999). Furthermore, future studies should examine the effect of microinjecting higher doses of TNFα into the SFO of LPK animals, as this might indicate that there may be a change in the affinity and sensitivity of TNFRI in the SFO of LPK animals. As such, if higher doses are shown to elicit a response in LPK animals, this will allow for us to validate that the TNFRI Ab and minocycline adequately inhibits the response to TNFα.

4.2.3. Ongoing microglial activation does not contribute to the overactivation of the SFO in LPK animals

This thesis identified that inhibition of microglial activation by minocycline did not reduce BP over the 30-minute recording period. As preliminary immunohistochemistry staining for microglia identified that activated microglia is present in LPK but not Lewis animals, we hypothesised a reversal of this activation may lead to a decrease in MAP as activated microglia release a range of excitatory substances that could possibly play a mechanistic role in the overactivation of the SFO. A caveat is that the responses were only recorded for a short period of time after minocycline microinjection, therefore this time period may not have been long enough to capture the reversal of microglial activation and the upregulation of excitatory pathways. As such, longer term studies are required before we can definitively confirm that microglial activation does not contribute to the overactivation of the SFO.

An alternative mechanism that may underpin the overactivation of the SFO, is that rather than just being the effect of one cytokine, it might be the effect of multiple proinflammatory cytokines acting on the SFO. Although TNFα is the most widely investigated proinflammatory cytokine, other proinflammatory cytokines may act either alone or in unison to trigger this increase in SFO activity. Other proinflammatory cytokines which are raised in PKD and have been shown to increase neuronal activity include interleukin-1β, interleukin-6 and interleukin-8 (Gardner et al., 1991; Merta et al., 1997; Viviani et al., 2003; Cui et al., 2012; Garcia-Oscos et al., 2012). Additionally, all proinflammatory cytokines, not just TNFα, have been shown to reduce the reuptake of glutamate by astrocytes (Hu et al., 2000) and therefore may be working synergistically to increase the excitability of the SFO in LPK animals. This hypothesis would explain the lack of change in BP upon inhibition of just one proinflammatory cytokine. Furthermore, as one of the major effects of proinflammatory cytokines is to increase neuronal activity by affecting not only microglia but astrocytes and neurons, this may explain the lack of change in BP in response to microinjection of minocycline. As such, further studies should examine the effects of blocking multiple cytokines at their respective receptors.

Alternative substances that may be driving the SFO overactivity are uraemic toxins, toxins that accumulate in the bloodstream as a result of the progressive renal failure in CKD (Duranton et al., 2012). Uraemic toxins have been shown to lead to a range of negative consequences in the body including a higher risk of developing cardiovascular disease and a range of neurological consequences (Moradi et al., 2013; Arnold et al., 2016). One uraemic toxin, indoxyl sulfate, increases the activity of neurons (Oshima et al., 2015) making it a likely candidate of driving the SFO overactivity. This could be examined by microinjecting indoxyl sulfate and its inhibitor, CH22319, into the SFO of LPK animals (Asai et al., 2018).

Furthermore, reactive oxidative species act within the central nervous system to control BP and consequently have been implicated in the development of multiple forms of hypertension, for example the angiotensin II infusion and Dahl-salt sensitive hypertension animal models (Datla and Griendling, 2010) and therefore may play a role in driving the SFO overactivity. Activated microglia have been shown to produce a large amount of reactive oxygen species, therefore as microglia are likely activated in the SFO of LPK animals, they may be releasing significant amounts of reactive oxygen species, which may be driving the SFO overactivity. This hypothesis could be examined by measuring the levels of reactive oxygen species in the SFO, genetic silencing of Nox2 and Nox4 genes to reduce the production of reactive oxygen species in the SFO

and the administration of tempol, a superoxide dismutase mimetic which acts to scavenge reactive oxygen species.

Although further replicates are required to confirm these preliminary results, an unexpected finding of this thesis is that microinjection of TNFRI Ab and minocycline into the SFO of LPK animals increased RSNA considerably from baseline, despite no observable changes to MAP or HR throughout the recording period. The increase in RSNA upon minocycline microinjection was also observed in the Lewis animals, therefore indicating that potentially this is a minocycline specific response in the SFO, thus suggesting that microglia may play a role in regulating the autonomic nervous system in alternative functions not related to BP. This could be examined further by long term inhibition of microglia or by genetic knockdown of microglia in the SFO, to further elucidate the SFO-specific roles of microglia.

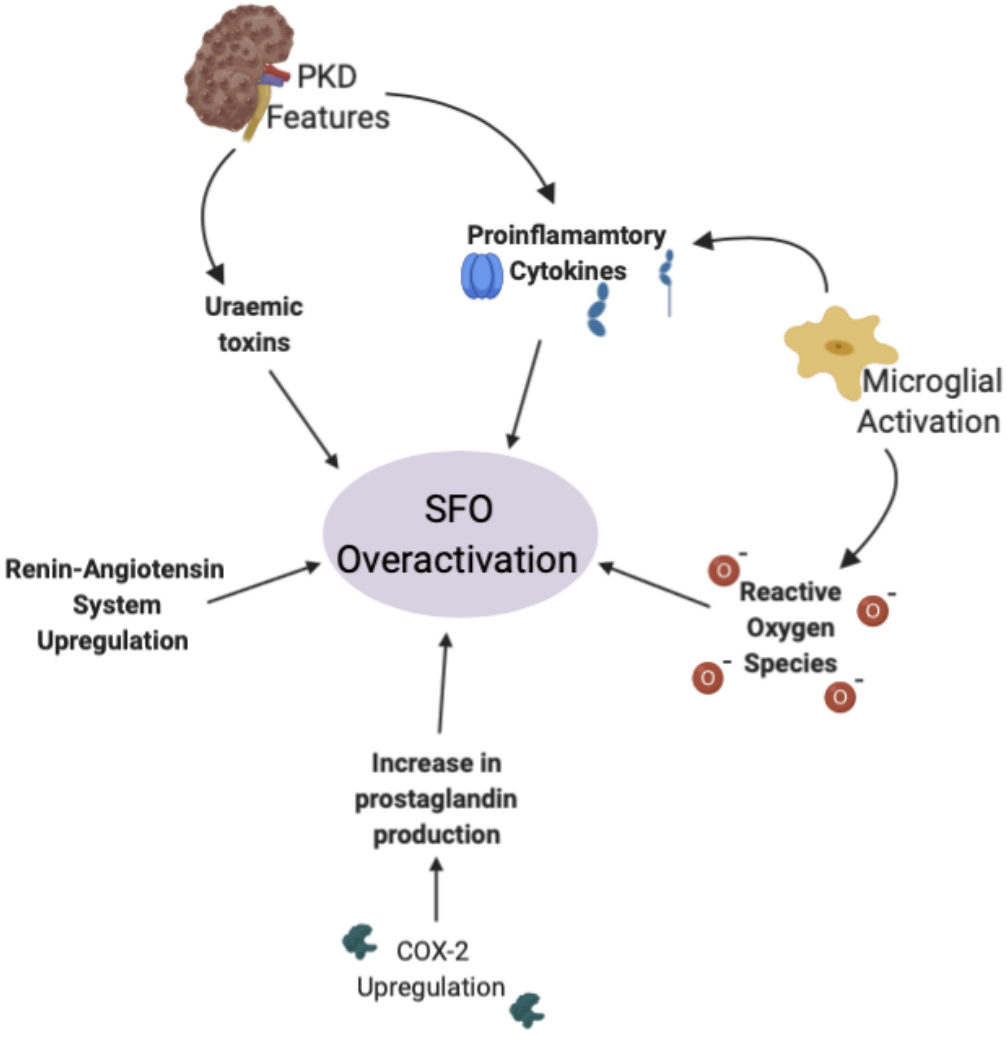

Figure 4.2 Summary of the proposed mechanisms underlying the overactivity of the subfornical organ (SFO) in Lewis polycystic kidney disease animals. COX-2: cyclooxygenase-2. Created with Biorender.com.

4.2.4. Microinjection of TNFRI Ab and minocycline does not alter the tonic activation of the SFO in LPK animals

The SFO in LPK animals is tonically activated and, in part, responsible for the development of hypertension (Underwood et al., 2019, In Preparation). This is indicated by the significant, but short lasting, depressor response observed upon microinjection of a $GABA_a$ agonist into the SFO of LPK animals. Although acute blockade of TNFα and microglial activation did not elicit an observable alteration to MAP in LPK animals, either microinjection may have altered the tonicity of this brain region, as indicated by a change in the acute depressor response upon microinjection of a $GABA_a$ agonist. This would signify that TNFα or proinflammatory cytokines may have an underlying role in the development of the overactivation of the SFO in these animals.

Microinjection of TNFRI Ab did not significantly alter the acute peak depressor observed upon microinjection of a $GABA_a$ agonist in the SFO of LPK animals. This confirms our previous results that indicate that it is unlikely that TNFα acts alone in the SFO to increase its activity. Contrastingly, prior microinjection of minocycline significantly increased the peak depressor response observed upon microinjection of a $GABA_a$ agonist in the SFO of LPK animals, therefore revealing an increase in tonicity in the SFO of LPK animals microinjected with minocycline. This may account for the lack of depressor response after minocycline microinjection, as the local neural network could have increased it's firing rate to compensate for any decrease in BP that microinjection of minocycline may cause in the SFO. Another explanation of these results is that activation of microglia in the SFO of LPK animals may be a protective adaptive change which is triggered in an attempt to reduce the tonic activation of the SFO as activated microglia can prevent excitotoxicity (Colonna and Butovsky, 2017). Activated microglia are able to detect neurons that are at risk of excitotoxicity and wrap around the cell body to reduce its excitability and the risk of damage to neurons (Kato et al., 2016). The role of microglia in the SFO could be further examined by measuring the changes in the SFO neuron activity in brain slices after the ablation of microglia. Therefore, if they do play a protective role by reducing neuronal excitability and cell death in the SFO, an increase in activity and neuronal cell death may be observed upon the removal of microglia.

4.3. Preliminary observation indicates that microglial activation is present in the SFO in hypertensive LPK animals but not normotensive Lewis animals

To determine if proinflammatory cytokines may play a role in the long term in the overactivation of the SFO, the activation levels of microglia were examined by immunohistochemistry staining for Iba1 in Lewis and LPK animals. Microglia are a major support cell of the central nervous system and are involved in a range of processes including neuronal network maintenance, removal of debris or pathogens and facilitation of the repair of neuronal tissue (Colonna and Butovsky, 2017). Although this dichotomy of microglial forms are still highly debated, microglia are generally thought to have two major forms; 1) surveillant and 2) activated. Under normal physiological conditions, microglia are surveillant, demonstrated by the ramified morphology of a small cell body with multiple long processes which allow microglia to survey their local area and thus identify threats to the central nervous system (Nimmerjahn et al., 2005; Helmut et al., 2011). In the presence of any threat to the homeostatic functioning of the central nervous system, for example infection or alteration of neuronal activity, microglia become activated, indicated by the retraction of their processes to form a prominent, or amoeboid shaped, cell body. This change in morphology allows microglia to undertake a range of processes to attempt to restore homeostasis, including removal of pathogens or debris and promoting tissue repair (Kreutzberg, 1996). Although the activation of microglia is necessary to maintain homeostasis in the brain, chronic activation of microglia can lead to a range of problems in the central nervous system, including an increase in the release of cytokines and reactive oxygen species causing the deterioration of the blood brain barrier, altered neuronal function and an increase in neuronal cell death (Wolf et al., 2017). Therefore, if microglia in the SFO of LPK animals are shown to be more activated than in the SFO of Lewis animals, this might indicate that long-term microglial activation may contribute to the SFO overactivity (Underwood et al., In Preparation).

Iba1 is a protein that is uniformly expressed on both the cell cytoplasm and processes and although the specific function of Iba1 has yet to be fully elucidated, it is thought that it has a role in the reorganisation of the cytoskeleton of microglia during activation, as indicated by the significant upregulation of mRNA encoding for Iba1 in activated microglia (Daisuke et al., 1998; Korzhevskii and Kirik, 2016). Iba1 staining for microglia is advantageous over other microglial staining antibodies as the binding of the anti-Iba1 is not affected by the fixative treatment of the tissue and the ubiquitous expression allows for the easy detection of both surveillant and activated microglia (Sukhorukova et al., 2012; Korzhevskii and Kirik, 2016).

Preliminary observation indicated that LPK animals have considerably more activated microglia than Lewis animals in the SFO. Although this needs to be confirmed in a larger cohort and through semi-quantitative image analysis, this suggests that there may be a disturbance to the homeostasis of the SFO in LPK animals (Kreutzberg, 1996). Although this thesis demonstrates that microglial activation in the SFO likely doesn't contribute to the development of hypertension in the short-term, further studies are required to determine if long term inhibition of microglial activation reduces BP, as previous studies have identified that long-term inhibition of microglial activation was required to attenuate BP in the angiotensin II infusion model of hypertension (Shi et al., 2010). An alternative explanation that could account for the apparent increased activation of microglia in LPK animals is that the accumulation uraemic toxins, particularly indoxyl sulfate, have been shown to activate glial cells (Adesso et al., 2018). This could be examined further by determining if the long-term inhibition of uraemic toxins, by treatment with AST-120 or CH22319 (Vaziri et al., 2013; Asai et al., 2018), reduces the number of activated microglia in the SFO of LPK animals.

4.4. Methodological considerations

Although our preliminary results suggest that proinflammatory cytokines do not contribute to the acute control of BP in normotensive Lewis animals and to the development of hypertension in LPK animals, the small sample sizes of this study preclude definitive confirmation of our results, and, as such, further replicates will be required. Some other important methodological considerations needed when interpreting the results of this thesis include; the brain microinjection technique, immunohistochemistry technique, bias towards males and the effect of urethane anaesthesia.

4.4.1. Microinjection technique

Drug microinjection is a valuable technique that allows for the accurate delivery of a drug into specific brain regions. It has allowed researchers to evaluate the functions of brain regions and to gain valuable insights into the pathophysiological changes that occur in the brain in a range of diseases. However, a limitation of this study that cannot be overlooked is that the microinjected drug will diffuse through the brain parenchyma away from the original microinjection site. The microinjections in this thesis had a volume of approximately 50-100nl and therefore will initially spread through the extracellular space of the brain to form a sphere with a radius of approximately 485µm (Nicholson, 1985), however depending on individual factors including the individual properties of the brain parenchyma and the properties of the drug to be microinjected, the drug

may spread differentially to cover a sphere of a different size radius (Nicholson et al., 2000; Sykova and Nicholson, 2008).

Dorsally, the SFO is bordered by the ventral hippocampal commissure, a bundle of axons that connect the two halves of the hippocampus (Wyss et al., 1980). This brain region plays a role in memory rather than BP or HR (Jordan et al., 2018) therefore, even if the microinjected drugs did spread outside of the SFO to this region it is unlikely to have affected the results. Ventrally and laterally, the SFO is bordered by the third ventricle and although it is possible that a small amount of the microinjected drug could have dispersed into the third ventricle below the SFO and therefore may be detected by nuclei that border the ventricles that control BP and HR, the small volume that is used in this thesis (100nl) makes this unlikely. Studies that involve intracerebroventricular microinjections usually use a volume that is multiple magnitudes larger, ranging from 5µl up to 10µl (Emanueli et al., 1999; Miller et al., 2002; DeVos and Miller, 2013), to successfully reach brain regions that border the ventricles, thus even if the microinjected drugs did move from the SFO into the third ventricle, the small volume of the microinjection makes it is highly unlikely that the drug would reach other brain regions in a concentration large enough to alter the results.

4.4.2. Free floating section immunohistochemistry

One caveat of this study is that due to challenges encountered during the anti-Iba1 staining process, quantitative and statistical analysis of microglial activation and distribution was not possible. Whilst undertaking the free-floating brain slice immunohistochemistry method, despite reducing the agitation rate and maintaining care to ensure that the brain slices remain intact between washes, the SFO was damaged in most sections, likely due to its vulnerable location at the dorsal border of the third ventricle (Paxinos and Watson, 2006). As such, the limited number of intact SFO slices allows for only a qualitative description of microglia and, although this still provides a valuable insight into the morphological changes occurring with microglia in the SFO of both LPK and Lewis animals, further Iba1 staining using paraffin embedded slices to help maintain the integrity of the SFO will be required to definitively confirm our preliminary results through image analysis software that quantifies the amount of activated and non-activated microglia in a given area (Hovens et al., 2014). Furthermore to help with analysis, additional immunohistochemistry staining for Iba1 should utilise diaminobenzidine staining as this may allow for better visualisation of the number of dendritic processes (Hovens et al., 2014) as this was a problem encountered with the fluorescence staining, this is important as the number and length of dendritic processes from microglia is the measure used to analyse the level of microglial activation.

4.4.3. Bias towards male animals

One limitation of this thesis is that this study only included male animals. A major determining factor when considering the use of only male animals was the fact that all preceding research was undertaken only in males (Underwood et al., 2019, In Preparation), and although some of the pathophysiological changes in male LPK animals are observed in female LPK animals, previous research from our lab group has identified that the disease progression in females is moderately slower and that the autonomic control of the cardiovascular system functioning is fundamentally different when compared to male LPK animals (Phillips et al., 2007; Salman et al., 2014, 2015b). Therefore, it is likely that the underlying pathophysiological changes that contribute to hypertension in males may differ in female LPK animals, as such if the data from male and female LPK animals were combined in this thesis, this would significantly confound the interpretation of the results and may prevent the underlying pathophysiology from being uncovered.

Another reason why only male animals were chosen for this study is that the SFO is considerably affected by circulating oestrogen as activation of the oestrogen receptor-α in the angiotensin II infusion animal of hypertension, protects against the development of hypertension in female rats (Xue et al., 2007, 2015). Additionally, there are distinct sex differences that have been identified in the immune system during hypertensive states, as inflammation is more prominent in males, whereas females have more anti-inflammatory mediators and proinflammatory cytokines (Gillis and Sullivan, 2016), which may further confound the interpretation of data if both sexes were included. Therefore, future studies should investigate if the pathophysiological factors underlying the development of hypertension in female LPK animals is the same as in male LPK animals to elucidate if there are any major differences between the two sexes, which in turn could demonstrate the need to develop gender-based strategies to reduce BP.

4.4.4. Anaesthetic effects on BP, HR and RSNA recordings

Another experimental caveat that needs to be considered when interpreting these results is that this study used a urethane-anaesthetised preparation. As this thesis investigated the short-term contribution of proinflammatory cytokines to the control of BP in normotensive Lewis animals and development of hypertension in LPK animals, undertaking anaesthetised non-recovery surgeries was the logical technique to address this gap in knowledge. As although it is possible to deliver drugs to the brain under conscious conditions via the implantation of a guide cannula, the stress due to handling and constant fluctuation of BP in conscious animals would make it almost impossible to accurately capture the responses to the drug in the short term (Poole et al., 2019).

Urethane anaesthetic was used in this preparation as unlike other anaesthetics, for example pentobarbital or isoflurane, it provides long acting anaesthetic and considerable analgesic effects without a risk of severe respiratory depression (Field et al., 1993; Tremoleda et al., 2012). Previous studies have not conclusively determined the effect of urethane on MAP, HR and RSNA as studies have shown that urethane anaesthesia depresses central regulation of the cardiovascular system (Field et al., 1993; Hildreth et al., 2012; Wang et al., 2014), whilst others have demonstrated that urethane anaesthetic does not have a significant effect on these parameters (Holobotovskyy et al., 2004). Another consideration of the use of urethane anaesthesia, is that it has been shown to cause an imbalance in fluid and a decrease in circulating vasopressin (Severs et al., 1981), however as the mechanisms behind the development of hypertension occurs independently from vasopressin (Underwood et al., 2019), it is unlikely that these effects of urethane would significantly alter the results of this study. Despite this, the effects of urethane must be considered when analysing the translatability of these results to other models of PKD and the human condition.

4.5. Future directions

To allow for a more complete understanding of the role that proinflammatory cytokines have in the SFO on the control of BP of normotensive animals and the development of hypertension in LPK animals, additional replicates for each cohort are required. Furthermore, long term inhibition of TNFα and microglial activation are required to definitively determine the role of proinflammatory cytokines, as this thesis only allows for the conclusion on the role of proinflammatory cytokines in the acute setting. Long term inhibition will also help to distinguish if proinflammatory cytokines play a role in the establishment or maintenance of hypertension. This is important to examine as proinflammatory cytokines have been implicated in the establishment rather than the maintenance of the underlying pathology (Feldmann, 2002; Thomas et al., 2013). Finally, long term TNFRI Ab and minocycline administration will fill a gap in knowledge, as although studies have previously looked at this long-term inhibition in PKD, no study measured the changes observed in BP during treatment (Roix and Saha, 2013; Wu et al., 2016).

The unique anatomical properties of the blood brain barrier within the SFO make it an attractive location to target pharmacologically as, unlike other regions of the brain that control BP, drugs can easily access and act upon this brain region (Gross, 1991; Petrov et al., 1994). Therefore, if we can determine what is driving this increase in activity of the SFO and thus causing the observed hypertension, this may uncover a new method to treat hypertension in PKD. Overall, due to the complex changes observed in PKD it is likely that the overactivation of the SFO arises as a result of a combination of the proposed mechanisms throughout the discussion (Summary in Figure 4.2).

As such, further analysis looking at the changes in proteins and receptors in the SFO could identify which mechanisms are upregulated and therefore would be optimal targets in identifying what is driving the SFO overactivity.

5. Conclusion

In LPK animals, the development of hypertension is caused, in part, by the overactivity of the SFO (Underwood et al., In Preparation). The underlying cause of this overactivity, however, is unknown. This thesis investigated the hypothesis that endogenous proinflammatory cytokines are acting within the SFO to increase its activity in LPK animals. This thesis demonstrates that hypertension in LPK animals is not dependent on the short-term actions of endogenous proinflammatory cytokines or microglial activation in the SFO, despite evidence of considerable microglial activation in the SFO of LPK animals. Further studies involving the long-term inhibition of proinflammatory cytokines in the SFO are required to definitively confirm our findings.

References

Adesso S, Paterniti I, Cuzzocrea S, Fujioka M, Autore G, Magnus T, Pinto A, Marzocco S (2018) AST-120 Reduces Neuroinflammation Induced by Indoxyl Sulfate in Glial Cells. J Clin Med 7:365.

Agwuh KN, MacGowan A (2006) Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylcyclines. J Antimicrob Chemother 58:256–265.

Amdur RL, Feldman HI, Gupta J, Yang W, Kanetsky P, Shlipak M, Rahman M, Lash JP, Townsend RR, Ojo A, Roy-Chaudhury A, Go AS, Joffe M, He J, Balakrishnan VS, Kimmel PL, Kusek JW, Raj DS (2016) Inflammation and Progression of CKD: The CRIC Study. Clin J Am Soc Nephrol 11:1546–1556.

Ameer OZ, Hildreth CM, Phillips JK (2014a) Sympathetic overactivity prevails over the vascular amplifier phenomena in a chronic kidney disease rat model of hypertension. Physiol Rep 2:1–11.

Ameer OZ, Salman IM, Avolio AP, Phillips JK, Butlin M (2014b) Opposing changes in thoracic and abdominal aortic biomechanical properties in rodent models of vascular calcification and hypertension. Am J Physiol Circ Physiol 307:H143–H151.

Anderson JW, Washburn DLS, Ferguson A V. (2000) Intrinsic osmosensitivity of subfornical organ neurons. Neuroscience 100:539–547.

Ang S, Harrison J, Powers-Martin K, Reddrop C, McKitrick D, Holobotovskyy V, Arnolda L, Phillips J (2007) C-fos activation in renal hypertension. Hypertension 49:1468.

Arican O, Aral M, Sasmaz S, Ciragil P (2005) Serum levels of TNF-α, IFN-γ, IL-6, IL-8, IL-12, IL-17, and IL-18 in patients with active psoriasis and correlation with disease severity. Mediators Inflamm:273–279.

Arnold R, Issar T, Krishnan A V, Pussel BA (2016) Neurological complications in chronic kidney disease patients. J R Soc Med Cardiovasc Dis 5:1–13.

Asai H, Hirata J, Watanabe-Akanuma M (2018) Indoxyl glucuronide, a protein-bound uremic toxin, inhibits hypoxia-inducible factor–dependent erythropoietin expression through activation of aryl hydrocarbon receptor. Biochem Biophys Res Commun 504:538–544.

Aspalter RM, Eibl MM, Wolf HM (2003) Regulation of TCR-mediated T cell activation by TNF-RII. J Leukoc Biol 74:572–582.

Atala A, Freeman MR, Mandell J, Beier DR (1993) Juvenile cystic kidneys (jck): A new mouse mutation which causes polycystic kidneys. Kidney Int 43:1081–1085.

Bankir L (2001) Antidiuretic action of vasopressin: Quantitative aspects and interaction between V1a and V2 receptor-mediated effects. Cardiovasc Res 51:372–390.

Bardgett ME, Holbein WW, Herrera-Rosales M, Toney GM (2014) Ang II-salt hypertension depends on neuronal activity in the hypothalamic paraventricular nucleus but not on local actions of tumor necrosis factor-α. Hypertension 63:527–534.

Barichello T, dos Santos I, Savi GD, Florentino AF, Silvestre C, Comim CM, Feier G, Sachs D, Teixeira MM, Teixeira AL, Quevedo J (2009) Tumor necrosis factor alpha (TNF-α) levels

in the brain and cerebrospinal fluid after meningitis induced by Streptococcus pneumoniae. Neurosci Lett 467:217–219.

Beattie EC, Stellwagen D, Morishita W, Bresnahan JC, Byeong KH, Von Zastrow M, Beattie MS, Malenka RC (2002) Control of synaptic strength by glial TNFα. Science (80-) 295:2282–2285.

Bell PE, Hossack KF, Gabow PA, Durr JA, Johnson AM, Schrier RW (1988) Hypertension in autosomal dominant polycystic kidney disease. Kidney Int 34:683–690.

Bezzi P, Domercq M, Brambilla L, Galli R, Schols D, De Clercq E, Vescovi A, Bagetta G, Kollias G, Meldolesi J, Volterra A (2001) CXCR4-activated astrocyte glutamate release via TNFa: Amplification by microglia triggers neurotoxicity. Nat Neurosci 4:702–710.

Bihoreau MT, Ceccherini I, Browne J, Kränzlin B, Romeo G, Lathrop GM, James MR, Gretz N (1997) Location of the first genetic locus, PKDr1, controlling autosomal dominant polycystic kidney disease in Han:SPRD cy/+ rat. Hum Mol Genet 6:1307–1317.

Braun DA, Hildebrandt F (2017) Ciliopathies. Cold Spring Harb Perspect Biol 9:a028191.

Cerasola G, Li Vecchi M, Mulè G, Cottone S, Mangano MT, Andronico G, Contorno A, Parrino I, Renda F, Pavone G (1998) Sympathetic activity and blood pressure pattern in autosomal dominant polycystic kidney disease hypertensives. Am J Nephrol 18:391–398.

Chapman AB, Stepniakowski K, Rahbari-Oskoui F (2010) Hypertension in Autosomal Dominant Polycystic Kidney Disease. Adv Chronic Kidney Dis 17:153–163.

Chebib FT, Torres VE (2016) Autosomal Dominant Polycystic Kidney Disease: Core Curriculum 2016. Am J Kidney Dis 67:792–810.

Chen J, Bundy JD, Hamm LL, Hsu C, Lash J, Miller ER, Thomas G, Cohen DL, Weir MR, Raj DS, Chen H, Xie D, Rao P, Wright JT, Rahman M, He J (2019) Inflammation and Apparent Treatment-Resistant Hypertension in Patients With Chronic Kidney Disease. Hypertension 73:HYPERTENSIONAHA11812358.

Chopra S, Baby C, Jacob JJ (2011) Neuro-endocrine regulation of blood pressure. Indian J Endocrinol Metab 15:S281–S288.

Ciriello J, Gutman MB (2011) Functional identification of central pressor pathways originating in the subfornical organ. Can J Physiol Pharmacol 69:1035–1045.

Colonna M, Butovsky O (2017) Microglia Function in the Central Nervous System During Health and Neurodegeneration. Annu Rev Immunol 35:441–468 Available at: http://www.annualreviews.org/doi/10.1146/annurev-immunol-051116-052358.

Cooper SG, Trivedi DP, Yamamoto R, Worker CJ, Feng C-Y, Sorensen JT, Yang W, Xiong Z, Feng Y (2018) Increased (pro)renin receptor expression in the subfornical organ of hypertensive humans. Am J Physiol Heart Circ Physiol 314:H796–H804.

Crystal GJ, Assaad SI, Heerdt PM (2019) 24 - Cardiovascular Physiology: Integrative Function. In: Pharmacology and Physiology for Anesthesia (Hemmings HC, Egan TDBT-P and P for A (Second E, eds), pp 473–519. Philadelphia: Elsevier.

Cui G bin, An J ze, Zhang N, Zhao M gao, Liu S bing, Yi J (2012) Elevated interleukin-8 enhances prefrontal synaptic transmission in mice with persistent inflammatory pain. Mol Pain 8:11.

Daisuke I, Imai Y, Ohsawa K, Nakajima K, Fukuuchi Y, Kohsaka S (1998) Microglia-specific localisation of a novel calcium binding protein, Iba1. Mol Brain Res 57:1–9.

Datla SR, Griendling KK (2010) Reactive oxygen species, NADPH oxidases, and hypertension. Hypertension 56:325–330.

De Beus E, Bots ML, Van Zuilen AD, Wetzels JFM, Blankestijn PJ (2015) Prevalence of apparent therapy-resistant hypertension and its effect on outcome in patients with chronic kidney disease. Hypertension 66:998–1005.

de Kloet AD, Wang L, Pitra S, Hiller H, Smith JA, Tan Y, Nguyen D, Cahill KM, Sumners C, Stern JE, Krause EG (2017) A unique "Angiotensin-Sensitive" neuronal population coordinates neuroendocrine, cardiovascular, and behavioral responses to stress. J Neurosci 37:3478–3490.

Depuydt B, Van Loo G, Vandenabeele P, Declercq W (2005) Induction of apoptosis by TNF receptor 2 in a T-cell hybridoma is FADD dependent and blocked by caspase-8 inhibitors. J Cell Sci 118:497–504.

DeVos SL, Miller TM (2013) Direct intraventricular delivery of drugs to the rodent central nervous system. J Vis Exp:1–10.

DiBona GF, Kopp UC (1997) Neural control of renal function. Physiol Rev 77:75–197.

Duranton F, Cohen G, De Smet R, Rodriguez M, Jankowski J, Vanholder R, Argiles A (2012) Normal and pathologic concentrations of uremic toxins. J Am Soc Nephrol 23:1258–1270.

Emanueli C, Chao J, Regoli D, Chao L, Ni A, Madeddu P (1999) The bradykinin B1 receptor and the central regulation of blood pressure in spontaneously hypertensive rats. Br J Pharmacol 126:1769–1776.

Feher J (2012) 5.8 - The Cardiac Function Curve. In: Quantitative Human Physiology (Feher JBT-QHP (Second E, ed), pp 556–564. Boston: Academic Press.

Feldmann M (2002) Development of anti-TNF therapy for rheumatoid arthritis. Nat Rev Immunol 2:364–371.

Ferguson A V., Kasting NW (2017) Electrical stimulation in subfornical organ increases plasma vasopressin concentrations in the conscious rat. Am J Physiol Integr Comp Physiol 252:R425–R428.

Ferguson A V., Renaud LP (1984) Hypothalamic paraventricular nucleus lesions decrease pressor responses to subfornical organ stimulation. Brain Res 305:361–364.

Field KJ, White WJ, Lang CM (1993) Anaesthetic effects of chloral hydrate, pentobarbitone and urethane in adult male rats. Lab Anim 27:258–269.

Fyhrquist F, Metsarinne K, Tikkanen I (1995) Role of angiotensin II in blood pressure regulation and in the pathophysiology of cardiovascular disorders. J Hum Hypertens 9:S19-24.

Gabow P, Chapman A, Johnson A, Tangel D, Duley I, Kaehny W, Manco-Johnson M, Schrier R (1990) Renal structure and hypertension in autosomal dominant polycystic kidney disease. Kidney Int 38:1177–1180.

Garcia-Oscos F, Salgado H, Hall S, Thomas F, Farmer GE, Bermeo J, Galindo LC, Ramirez RD, D'Mello S, Rose-John S, Atzori M (2012) The stress-induced cytokine interleukin-6 decreases the inhibition/excitation ratio in the rat temporal cortex via trans-signaling. Biol

Psychiatry 71:574–582 Available at: http://dx.doi.org/10.1016/j.biopsych.2011.11.018.

Gardner KD, Burnside JS, Elzinga LW, Locksley RM (1991) Cytokines in fluids from polycystic kidneys. Kidney Int 39:718–724.

Gillis EE, Sullivan JC (2016) Sex Differences in Hypertension. Hypertension 68:1322–1327.

Gordan R, Gwathmey JK, Xie L-H (2015) Autonomic and endocrine control of cardiovascular function. World J Cardiol 7:204–214.

Grantham JJ, Torres VE, Chapman AB, Guay-Woodford LM, Bae KT, King BF, Wetzel LH, Baumgarten DA, Kenney PJ, Harris PC, Klahr S, Bennett WM, Hirschman GN, Meyers CM, Zhang X, Zhu F, Miller JP (2006) Volume Progression in Polycystic Kidney Disease. N Engl J Med 354:2122–2130.

Gross PM (1991) Morphology and physiology of capillary systems in subregions of the subfornical organ and area postrema ". Can J Physiol Pharmacol 69:1010–1025.

Gross PM, Weindl A (1987) Peering through the windows of the brain. J Cereb Blood Flow Metab 7:663–672.

Gutman MB, Ciriello J, Mogenson GJ (1988) Effects of plasma angiotensin II and hypernatremia on subfornical organ neurons. Am J Physiol 254:R746–R754.

Guzik TJ, Hoch NE, Brown KA, McCann LA, Rahman A, Dikalov S, Goronzy J, Weyand C, Harrison DG (2007) Role of the T cell in the genesis of angiotensin II–induced hypertension and vascular dysfunction. J Exp Med 204:2449–2460.

Han P, Whelan PJ (2010) Tumor necrosis factor alpha enhances glutamatergic transmission onto spinal motoneurons. J Neurotrauma 27:287–292.

Harrison JL, Hildreth CM, Callahan SM, Goodchild AK, Phillips JK (2010) Cardiovascular autonomic dysfunction in a novel rodent model of polycystic kidney disease. Auton Neurosci Basic Clin 152:60–66.

Hayden MS, Ghosh S (2015) Regulation of NF-κB by TNF family cytokines. Semin Immunol 26:253–266.

Helmut K, Hanisch UK, Noda M, Verkhratsky A (2011) Physiology of microglia. Physiol Rev 91:461–553.

Hendel MD, Collister JP (2005) Contribution of the subfornical organ to angiotensin II-induced hypertension. AJP Hear Circ Physiol 288:H680–H685.

Hildreth CM, Goodchild AK, Phillips JK (2012) Insight into Autonomic Nervous System Control of Heart Rate in the Rat Using Analysis of Heart Rate Variability and Baroreflex Sensitivity. In: Stimulation and Inhibiton of Neurons, pp 203–224.

Hildreth CM, Kandukuri DS, Goodchild AK, Phillips JK (2013) Temporal development of baroreceptor dysfunction in a rodent model of chronic kidney disease. Clin Exp Pharmacol Physiol 40:458–465.

Hilzendeger AM, Cassell MD, Davis DR, Stauss HM, Mark AL, Grobe JL, Sigmund CD (2013) Angiotensin type 1a receptors in the subfornical organ are required for deoxycorticosterone acetate-salt hypertension. Hypertension 61:716–722.

Hindmarch C, Fry M, Yao ST, Smith PM, Murphy D, Ferguson A V. (2008) Microarray analysis

of the transcriptome of the subfornical organ in the rat: regulation by fluid and food deprivation. Am J Physiol Integr Comp Physiol 295:R1914–R1920.

Hindmarch CCT, Ferguson A V. (2016) Physiological roles for the subfornical organ: A dynamic transcriptome shaped by autonomic state. J Physiol 594:1581–1589.

Holobotovskyy V V., Arnolda LF, McKitrick DJ (2004) Effect of anaesthetic and rat strain on heart rate responses to simulated haemorrhage. Acta Physiol Scand 180:29–38.

Hosutt JA, Rowland N, Stricker EM (1981) Impaired drinking responses of rats with lesions on the subfornical organ. J Comp Physiol Psychol 95:104–113.

Hovens I, Nyakas C, Schoemaker R (2014) A novel method for evaluating microglial activation using ionized calcium-binding adaptor protein-1 staining: cell body to cell size ratio. Neuroimmunol Neuroinflammation 1:82–88.

Hu S, Sheng WS, Ehrlich LC, Peterson PK, Chao CC (2000) Cytokine effects on glutamate uptake by human astrocytes. Neuroimmunomodulation 7:153–159.

Hughes J, Ward CJ, Peral B, Aspinwall R, Clark K, Millan JLS, Gamble V, Harris PC, Hospital R, Ii HI, Ill NII (1995) The polycystic kidney disease 1 (PKD1) gene encodes a novel protein with multiple cell recognition domains. Nat Genet 10:151–160.

Idrizi A, Barbullushi M, Strakosha A, Kodra S, Thereska N, Zaimi E, Gjyzari A, Petrela E (2007) The relation of hypertension, renal function and cardiovascular events in autosomal dominant polycystic kidney disease. G Ital Nefrol 24:595–599.

Ishibashi S, Nicolaidis S (1981) Hypertension induced by electrical stimulation of the subfornical organ (SFO). Brain Res Bull 6:135–139.

Johansson B (1989) Myogenic tone and reactivity: definitions based on muscle physiology. J Hypertens Suppl 7:S5–S9.

Jordan JT, Tong Y, Pytte CL (2018) Transection of the ventral hippocampal commissure impairs spatial but not contextual memory in split-brain mice. bioRxiv:437491 Available at: http://biorxiv.org/content/early/2018/10/08/437491.abstract.

Kandukuri DS, Phillips JK, Tahmindjis M, Hildreth CM (2018) Effect of anaesthetic and choice of neuromuscular blocker on vagal control of heart rate under laboratory animal experimental conditions. Lab Anim 52:280–291.

Karava V, Benzouid C, Hogan J, Dossier C, Denjean AP, Deschênes G (2018) Early cardiovascular manifestations in children and adolescents with autosomal dominant polycystic kidney disease: a single center study. Pediatr Nephrol 33:1513–1521.

Karihaloo A (2015) Role of Inflammation in Polycystic Kidney Disease. In: Polycystic Kidney Disease (Li X, ed), pp Chapter 14. Brisbane (AU).

Kato G, Inada H, Wake H, Akiyoshi R, Miyamoto A, Eto K, Ishikawa T, Moorhouse AJ, Strassman AM, Nabekura J (2016) Microglial contact prevents excess depolarization and rescues neurons from excitotoxicity. eNeuro 3:e0004.

Klein IH, Ligtenberg G, Oey PL, Koomans HA, Blankestijn PJ (2001) Sympathetic activity is increased in polycystic kidney disease and is associated with hypertension. J Am Soc Nephrol 12:2427–2433.

Klein IHHT, Ligtenberg G, Neumann J, Oey PL, Koomans HA, Blankestijn PJ (2003)

Sympathetic Nerve Activity Is Inappropriately Increased in Chronic Renal Disease. J Am Soc Nephrol 14:3239–3244.

Kocyigit I, Kaya MG, Orscelik O, Kaya C, Akpek M, Zengin H, Sipahioglu MH, Unal A, Yilmaz MI, Tokgoz B, Oymak O, Axelsson J (2012) Early arterial stiffness and inflammatory bio-markers in normotensive polycystic kidney disease patients. Am J Nephrol 36:11–18.

Korim WS, Elsaafien K, Basser JR, Setiadi A, May CN, Yao ST (2019) In renovascular hypertension, TNF-alpha type-1 receptors in the area postrema mediate increases in cardiac and renal sympathetic nerve activity and blood pressure. Cardiovasc Res 115:1092–1101.

Korzhevskii DE, Kirik O V. (2016) Brain Microglia and Microglial Markers. Neurosci Behav Physiol 46:284–290.

Kreutzberg GW (1996) Microglia: A sensor for pathological events in the CNS. Trends Neurosci 19:312–318.

Kumagai H, Oshima N, Matsuura T, Iigaya K, Imai M, Onimaru H, Sakata K, Osaka M, Onami T, Takimoto C, Kamayachi T, Itoh H, Saruta T (2012) Importance of rostral ventrolateral medulla neurons in determining efferent sympathetic nerve activity and blood pressure. Hypertens Res 35:132–141 Available at: http://dx.doi.org/10.1038/hr.2011.208.

Lager DJ, Qian Q, Bengal RJ, Ishibashi M, Torres VE (2001) The pck rat: A new model that resembles human autosomal dominant polycystic kidney and liver disease. Kidney Int 59:126–136.

Lanktree MB, Haghighi A, Guiard E, Iliuta I-A, Song X, Harris PC, Paterson AD, Pei Y (2018) Prevalence Estimates of Polycystic Kidney and Liver Disease by Population Sequencing. J Am Soc Nephrol 29:2593–2600.

Leadbetter WF, Burkland CE (1938) Hypertension in Unilateral Renal Disease. J Urol 39:611–626.

Li W, Peng H, Cao T, Sato R, McDaniels SJ, Kobori H, Navar LG, Feng Y (2013) Brain-targeted (pro)renin receptor knockdown attenuates angiotensin II-dependent hypertension. Hypertension 59:1188–1194.

Li X, Magenheimer BS, Xia S, Johnson T, Wallace DP, Calvet JP, Li R (2008) A tumor necrosis factor-alpha-mediated pathway promoting autosomal dominant polycystic kidney disease. Nat Med 14:863–868.

Lind RW, Ohman LE, Lansing MB, Johnson AK (1983) Transection of subfornical organ neural connections diminishes the pressor response to intravenously infused angiotensin II. Brain Res 275:361–364.

Liu Q, Chen DM, Wang YG, Zhao X, Zheng Y (2012) Cardiac autonomic nerve distribution and arrhythmia. Neural Regen Res 7:2834–2841.

Lorenz JN, Loreaux EL, Dostanic-Larson I, Lasko V, Schnetzer JR, Paul RJ, Lingrel JB (2008) ACTH-induced hypertension is dependent on the ouabain-binding site of the α2-Na+-K+-ATPase subunit. Am J Physiol - Hear Circ Physiol:H237-280.

Mangiapane ML, Simpson JB (1980a) Subfornical organ: forebrain site of pressor and dipsogenic action of angiotensin II. Am J Physiol - Regul Integr Comp Physiol 8:R382-389.

Mangiapane ML, Simpson JB (1980b) Subfornical organ lesions reduce the pressor effect of systemic angiotensin II. Neuroendocrinology 31:380–384.

Marlais M, Cuthell O, Langan D, Dudley J, Sinha MD, Winyard PJD (2016) Hypertension in autosomal dominant polycystic kidney disease: A meta-analysis. Arch Dis Child 101:1142–1147.

McCooke, Appels, Barrero, Ding, Ozimek-Kulik, Bellgard, Morahan, Phillips (2012) A novel mutation causing nephronophthisis in the Lewis polycystic kidney rat localises to a conserved RCC1 domain in Nek8. BMC Genomics 13:393.

Menon V, Rudym D, Chandra P, Miskulin D, Perrone R, Sarnak M (2011) Inflammation, oxidative stress, and insulin resistance in polycystic kidney disease. Clin J Am Soc Nephrol 6:7–13.

Merta M, Tesar V, Zima T, Jirsa M, Rysava R, Zabka J (1997) Cytokine Profile in Autosomal Dominant Polycystic Kidney Disease. Biol Int 41:619–624.

Miller CC, Martin RJ, Whitney ML, Edwards GL (2002) Intracerebroventricular injection of fructose stimulates feeding in rats. Nutr Neurosci 5:359–362.

Montgomery SL, Bowers WJ (2012) Tumor necrosis factor-alpha and the roles it plays in homeostatic and degenerative processes within the central nervous system. J Neuroimmune Pharmacol 7:42–59.

Moradi H, Sica DA, Kalantar-Zadeh K (2013) Cardiovascular burden associated with uremic toxins in patients with chronic kidney disease. Am J Nephrol 38:136–148.

Nadeau S, Rivest S (1999) Effects of circulating tumor necrosis factor on the neuronal activity and expression of the genes encoding the tumor necrosis factor receptors (p55 and p75) in the rat brain: a view from the blood-brain barrier. Neuroscience 93:1449–1464.

Ng K, Hildreth CM, Phillips JK, Avolio AP (2011) Aortic stiffness is associated with vascular calcification and remodeling in a chronic kidney disease rat model. Am J Physiol Physiol 300:F1434–F1436.

Nicholson C (1985) Diffusion from an injected volume of a substance in brain tissue with arbitrary volume fraction and tortuosity. Brain Res 333:325–329.

Nicholson C, Chen KC, Hrabětová S, Tao L (2000) Diffusion of molecules in brain extracellular space: Theory and experiment. Prog Brain Res 125:129–154.

Nikodemova M, Duncan ID, Watters JJ (2006) Minocycline exerts inhibitory effects on multiple mitogen-activated protein kinases and IκBα degradation in a stimulus-specific manner in microglia. J Neurochem 96:314–323.

Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Neuroscience: Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science (80-) 308:1314–1318.

Noori NM, Shahramian I, Teimouri A, Keyvani B, Mahjoubifard M (2017) Serum levels of tumor necrosis factor-α and interleukins in children with congenital heart disease. J Tehran Univ Hear Cent 12:15–22.

Olsen RW, DeLorey TM (1999) Basic Neurochemistry: Molecular, Cellular and Medical Aspects, 6th Edition.

Osborn JW, Fink GD, Hendel MD, Collister JP, Ariza-Guzman PA (2011) The role of the

subfornical organ in angiotensin II-salt hypertension in the rat. Exp Physiol 97:80–88.

Osborn JW, Jacob F, Hendel M, Collister JP, Clark L, Guzman PA (2006) Effect of subfornical organ lesion on the development of mineralocorticoid-salt hypertension. Brain Res 1109:74–82.

Oshima N, Onimaru H, Matsubara H, Uchida T, Watanabe A, Takechi H, Nishida Y, Kumagai H (2015) Uric acid, indoxyl sulfate, and methylguanidine activate bulbospinal neurons in the RVLM via their specific transporters and by producing oxidative stress. Neuroscience 304:133–145 Available at: http://dx.doi.org/10.1016/j.neuroscience.2015.07.055.

Paxinos G, Watson C (2006) The rat brain in stereotaxic coordinates sixth edition. Acad Press.

Perrone RD, Ruthazer R, Terrin NC (2001) Survival after end-stage renal disease in autosomal dominant polycystic kidney disease: Contribution of extrarenal complications to mortality. Am J Kidney Dis 38:777–784.

Petrov T, Howarth AG, Krukoff TL, Stevenson BR (1994) Distribution of the tight junction-associated protein ZO-1 in circumventricular organs of the CNS. Mol Brain Res 21:235–246.

Phillips JK, Boyd R, Krockenberger MB, Burgio G (2015) Progression of anemia and its relationship with renal function, blood pressure, and erythropoietin in rats with chronic kidney disease. Vet Clin Pathol 44:342–354.

Phillips JK, Hopwood D, Loxley R a, Ghatora K, Coombes JD, Tan YS, Harrison JL, McKitrick DJ, Holobotvskyy V, Arnolda LF, Rangan GK (2007) Temporal relationship between renal cyst development, hypertension and cardiac hypertrophy in a new rat model of autosomal recessive polycystic kidney disease. Kidney Blood Press Res 30:129–144.

Pi R, Li W, Lee NTK, Chan HHN, Pu Y, Ling NC, Sucher NJ, Chang DC, Li M, Han Y (2004) Minocycline prevents glutamate-induced apoptosis of cerebellar granule neurons by differential regulation of p38 and Akt pathways. J Neurochem 91:1219–1230.

Pinheiro PS, Mulle C (2008) Presynaptic glutamate receptors: physiological functions and mechanisms of action. Nat Rev Neurosci 9:423–436.

Poole EI, McGavin JJ, Cochkanoff NL, Crosby KM (2019) Stereotaxic surgery for implantation of guide cannulas for microinjection into the dorsomedial hypothalamus in young rats. MethodsX 6:1652–1659 Available at: https://doi.org/10.1016/j.mex.2019.07.005.

Probert L (2015) TNF and its receptors in the CNS: The essential, the desirable and the deleterious effects. Neuroscience 302:2–22.

Punnen S, Gonzalez ER, Krieger AJ, Sapru HN (1984) Cardiac vagolytic action of some neuromuscular blockers. Pharmacol Biochem Behav 20:85–89.

Pyner S, Coote JH (2000) Identification of branching paraventricular neurons of the hypothalamus that project to the rostroventrolateral medulla and spinal cord. Neuroscience 100:549–556.

Quyyumi AA (1998) Endothelial function in health and disease: New insights into the genesis of cardiovascular disease. Am J Med 105:32S-39S.

Rahman E, Niaz FA, Al-suwaida A, Nahrir S, Bashir M, Rahman H, Hammad D (2009) Polycystic Kidney Disease : A Single Center Study. Nephrology 20:806–810.

Raven PB, Chapleau MW (2014) Blood pressure regulation XI: Overview and future research directions. Eur J Appl Physiol 114:579–586.

Roix J, Saha S (2013) TNF-α blockade is ineffective in animal models of established polycystic kidney disease. BMC Nephrol 14:233 Available at: BMC Nephrology.

Rossi NF, Zenner Z, Rishi AK, Levi E, Maliszewska-Scislo M (2019) AT 1 receptors in the subfornical organ modulate arterial pressure and the baroreflex in two-kidney, one-clip hypertensive rats. Am J Physiol Integr Comp Physiol 316:R172–R185.

Sabban EL, Schilt N, Serova LI, Masineni SN, Stier CT (2009) Kinetics and persistence of cardiovascular and locomotor effects of immobilization stress and influence of ACTH treatment. Neuroendocrinology 89:98–108.

Salman IM, Hildreth CM, Ameer OZ, Phillips JK (2014) Differential contribution of afferent and central pathways to the development of baroreflex dysfunction in chronic kidney disease. Hypertension 63:804–810.

Salman IM, Kandukuri DS, Harrison JL, Hildreth CM, Phillips JK (2015a) Direct conscious telemetry recordings demonstrate increased renal sympathetic nerve activity in rats with chronic kidney disease. Front Physiol 6:1–10.

Salman IM, Phillips JK, Ameer OZ, Hildreth CM (2015b) Abnormal central control underlies impaired baroreflex control of heart rate and sympathetic nerve activity in female Lewis polycystic kidney rats. J Hypertens 33:1418–1428.

Saxena A, Little JT, Nedungadi TP, Cunningham JT (2015) Angiotensin II type 1a receptors in subfornical organ contribute towards chronic intermittent hypoxia-associated sustained increase in mean arterial pressure. Am J Physiol Circ Physiol 308:H435–H446.

Schäfer K, Gretz N, Bader M, Oberbäumer I, Eckardt KU, Kriz W, Bachmann S (1994) Characterization of the Han:SPRD rat model for hereditary polycystic kidney disease. Kidney Int 46:134–152.

Severs WB, Keil LC, Klase PA, Deen KC (1981) Urethane anesthesia in rats. Altered ability to regulate hydration. Pharmacology 22:209–226.

Shapiro RE, Miselis RR (1985) The central neural connections of the area postrema of the rat. J Comp Neurol 234:344–364.

Shi P, Diez-freire C, Jun JY, Qi Y, Katovich MJ, Sriramula S, Francis J, Sumners C, Raizada MK (2010) Brain Microglial Cytokines in Neurogenic Hypertension. Hypertension 56:297–303.

Simpson JB, Routtenberg A (1973) Subfornical organ: Site of drinking elicitation by angiotensin II. Science (80-) 181:1172–1175.

Simpson NJ, Ferguson A V. (2017) The proinflammatory cytokine tumor necrosis factor-α excites subfornical organ neurons. J Neurophysiol 118:1532–1541.

Smith PM, Ferguson A V. (2010) Circulating signals as critical regulators of autonomic state—central roles for the subfornical organ. Am J Physiol Integr Comp Physiol 299:R405–R415.

Song XA, Jia LL, Cui W, Zhang M, Chen W, Yuan ZY, Guo J, Li HH, Zhu GQ, Liu H, Kang YM (2014) Inhibition of TNF-α in hypothalamic paraventricular nucleus attenuates hypertension and cardiac hypertrophy by inhibiting neurohormonal excitation in

spontaneously hypertensive rats. Toxicol Appl Pharmacol 281:101–108.

Stern JE, Son S, Biancardi VC, Zheng H, Sharma N, Patel KP (2016) Astrocytes Contribute to Angiotensin II Stimulation of Hypothalamic Neuronal Activity and Sympathetic Outflow. Hypertension 68:1483–1493.

Sukhorukova EG, Zakhriapin MS, Anichkov NM, Korzhevskiĭ DE (2012) [Microglia detection in the brain preparations after long-term storage in formalin]. Morfologii 142:68–71.

Swanson LW, Sawchenko PE (1983) Hypothalamic Integration: Organization of the Paraventricular and Supraoptic Nuclei. Annu Rev Neurosci 6:269–324.

Sweeny WE, Avner ED (2019) Polycystic Kidney Disease, Autosomal Recessive.

Sykova E, Nicholson C (2008) Diffusion in Brain Extracellular Space. Physiol Rev 88:1127–1340.

Ta MHT, Schwensen KG, Foster S, Korgaonkar M, Ozimek-Kulik JE, Phillips JK, Peduto A, Rangan GK (2016) Effects of TORC1 inhibition during the early and established phases of polycystic kidney disease. PLoS One 11:1–30.

Tabarean I V., Behrens MM, Bartfai T, Korn H (2004) Prostaglandin E2-increased thermosensitivity of anterior hypothalamic neurons is associated with depressed inhibition. Proc Natl Acad Sci U S A 101:2590–2595.

Takeuchi H, Jin S, Wang J, Zhang G, Kawanokuchi J, Kuno R, Sonobe Y, Mizuno T, Suzumura A (2006) Tumor necrosis factor-α induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner. J Biol Chem 281:21362–21368.

Tan PSP, Killinger S, Horiuchi J, Dampney RAL (2007) Baroreceptor reflex modulation by circulating angiotensin II is mediated by AT1 receptors in the nucleus tractus solitarius. Am J Physiol - Regul Integr Comp Physiol 67:1462–1473.

Thomas G, Xie D, Chen H-Y, Anderson AH, Appel LJ, Bodana S, Brecklin CS, Drawz P, Flack JM, Miller III ER (2016) Prevalence and prognostic significance of apparent treatment resistant hypertension in chronic kidney disease: report from the chronic renal insufficiency cohort study. Hypertension 67:387–396.

Thomas HE, Graham KL, Chee J, Thomas R, Kay TW, Krishnamurthy B (2013) Proinflammatory cytokines contribute to development and function of regulatory T cells in type 1 diabetes. Ann N Y Acad Sci 1283:81–86.

Tran LT, MacLeod KM, McNeill JH (2009) Chronic etanercept treatment prevents the development of hypertension in fructose-fed rats. Mol Cell Biochem 330:219–228.

Tremoleda JL, Kerton A, Gsell W (2012) Anaesthesia and physiological monitoring during in vivo imaging of laboratory rodents: Considerations on experimental outcomes and animal welfare. EJNMMI Res 2:1–23.

Underwood CF, Boyd R, Phillips JK, Hildreth CM (2019) Increased excitatory regulation of the hypothalamic paraventricular nucleus and circulating vasopressin results in the hypertension observed in polycystic kidney disease. J Hypertens 37:109–115.

Underwood CF, McMullan S, Goodchild AK, Phillips JK, Hildreth CM (n.d.) The Subfornical Organ Drives Hypertension in Polycystic Kidney Disese via the Hypothalamic

Paraventricular Nucleus.

Vassilli P (1992) The Pathophysiology of Tumor Necrosis Factors. Annu Rev Immunol 10:411–452.

Vaziri ND, Yuan J, Khazaeli M, Masuda Y, Ichii H, Liu S (2013) Oral activated charcoal adsorbent (AST-120) ameliorates chronic kidney disease-induced intestinal epithelial barrier disruption. Am J Nephrol 37:518–525.

Venegas-Pont M, Manigrasso MB, Grifoni SC, Lamarca BB, Maric C, Racusen LC, Glover PH, Jones A V., Drummond HA, Ryan MJ (2010) Tumor necrosis factor-α antagonist etanercept decreases blood pressure and protects the kidney in a mouse model of systemic lupus erythematosus. Hypertension 56:643–649.

Vezzani A, Viviani B (2015) Neuromodulatory properties of inflammatory cytokines and their impact on neuronal excitability. Neuropharmacology 96:70–82.

Viviani B, Bartesaghi S, Gardoni F, Vezzani A, Behrens MM, Bartfai T, Binaglia M, Corsini E, Di Luca M, Galli CL, Marinovich M (2003) Interleukin-1β enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. J Neurosci 23:8692–8700.

Wang D, Strandgaard S (1997) The pathogenesis of hypertension in autosomal dominant polycystic kidney disease. J Hypertens 15:925–933.

Wang GF, Mao XJ, Chen ZJ (2014) Urethane suppresses renal sympathetic nerve activity in Wistar rats. Eur Rev Med Pharmacol Sci 18:1454–1457.

Wang LA, Nguyen DH, Mifflin SW (2018) Corticotropin-releasing hormone projections from the paraventricular nucleus of the hypothalamus to the nucleus of the solitary tract increase blood pressure. J Neurophysiol 121:602–608 Available at: https://doi.org/10.1152/jn.00623.2018.

Wei SG, Yu Y, Zhang ZH, Felder RB (2015) Pro-inflammatory cytokines upregulate sympathoexcitatory mechanisms in the subfornical organ of the rat. Hypertension 65:1126–1133.

Wei SG, Zhang ZH, Beltz TG, Yu Y, Johnson AK, Felder RB (2013) Subfornical organ mediates sympathetic and hemodynamic responses to blood-borne proinflammatory cytokines. Hypertension 62:118–125.

Willey CJ, Blais JD, Hall AK, Krasa HB, Makin AJ, Czerwiec FS (2017) Prevalence of autosomal dominant polycystic kidney disease in the European Union. Nephrol Dial Transplant 32:1356–1363.

Wolf SA, Boddeke HWGM, Kettenmann H (2017) Microglia in Physiology and Disease. Annu Rev Physiol 79:619–643 Available at: http://www.annualreviews.org/doi/10.1146/annurev-physiol-022516-034406.

Wu G, D'Agati V, Cai Y, Markowitz G, Park JH, Reynolds DM, Maeda Y, Le TC, Hou H, Kucherlapati R, Edelmann W, Somlo S (1998) Somatic inactivation of Pkd2 results in polycystic kidney disease. Cell 93:177–188.

Wu G, Markowitz GS, Li L, D'Agati VD, Factor SM, Geng L, Tibara S, Tuchman J, Cai Y, Park JH, Van Adelsberg J, Hou H, Kucherlapati R, Edelmann W, Somlo S (2000) Cardiac defects and renal failure in mice with targeted mutations in Pkd2. Nat Genet 24:75–78.

Wu M, Gu J, Mei S, Xu D, Jing Y, Yao Q, Chen M, Yang M, Chen S, Yang B, Qi N, Hu H, Wüthrich RP, Mei C (2016) Resveratrol delays polycystic kidney disease progression through attenuation of nuclear factor κB-induced inflammation. Nephrol Dial Transplant 31:1826–1834.

Wyss JM, Swanson LW, Cowan WM (1980) The organization of the fimbria, dorsal fornix and ventral hippocampal commissure in the rat. Anat Embryol (Berl) 158:303–316.

Xue B, Pamidimukkala J, Lubahn DB, Hay M (2007) Estrogen receptor-α mediates estrogen protection from angiotensin II-induced hypertension in conscious female mice. Am J Physiol - Hear Circ Physiol 292:1770–1776.

Xue B, Zhang Z, Beltz TG, Guo F, Hay M, Johnson AK (2015) Genetic knockdown of estrogen receptor-alpha in the subfornical organ augments ANG II-induced hypertension in female mice. Am J Physiol - Regul Integr Comp Physiol 308:R507–R516.

Yao Y, Hildreth CM, Farnham MM, Saha M, Sun QJ, Pilowsky PM, Phillips JK (2015) The effect of losartan on differential reflex control of sympathetic nerve activity in chronic kidney disease. J Hypertens 33:1249–1260.

Yu XJ, Zhang DM, Jia LL, Qi J, Song XA, Tan H, Cui W, Chen W, Zhu GQ, Qin DN, Kang YM (2015) Inhibition of NF-κB activity in the hypothalamic paraventricular nucleus attenuates hypertension and cardiac hypertrophy by modulating cytokines and attenuating oxidative stress. Toxicol Appl Pharmacol 3:315–322.

Zhang ZH, Wei SG, Francis J, Felder RB (2003) Cardiovascular and renal sympathetic activation by blood-borne TNF-α in rat: The role of central prostaglandins. Am J Physiol - Regul Integr Comp Physiol 4:R916–R927.

Zheng L, Fisher G, Mlllert RE, Peschont J, Lyncht DH, Lenardo MJ (1995) By Tumour Necrosis Factor. 377:348–351.

Appendix of this thesis has been removed as it may contain sensitive/confidential content