Angiotensin II-Driven Mechanisms of Neuroinflammation in the Maintenance of Hypertension

by

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Abstract

Hypertension, one of the most prevalent cardiovascular risk factors, exhibits numerous pathophysiologies and is typically attributed to dysregulation of the cardiovascular, renal, and/or central nervous systems (CNS). Whereas the contributions of adaptive immune system activation to high blood pressure are well documented, the molecular mechanisms that activate the innate immune system and lead to priming/activation of the adaptive immune response are not fully understood. The innate immune system identifies distinct signatures of invading microbes and damage-associated molecular patterns and triggers a chain of downstream proinflammatory signaling cascades and shapes the adaptive immune response. Accumulating evidence from the past decade indicates that a hyperactive innate immune system response, particularly via Toll-like receptor (TLR) stimulation, fosters chronic inflammation in hypertension, inducing deleterious local and systemic effects in host cells and tissues and contributing to disease progression.

A putative TLR4 ligand, angiotensin II (AngII), is the primary signaling peptide of the renin-angiotensin system (RAS). Within the CNS, AngII stimulates the production of pro-inflammatory cytokines, increases sympathetic nervous system activity, and is implicated as a driving force behind blood-brain barrier (BBB) disruption in neurogenic hypertension. Our prior work shows that AngII type 1 receptor (AT1R) blockade restores BBB integrity in CNS cardioregulatory nuclei (i.e., the hypothalamic paraventricular nucleus [PVN], rostral ventrolateral medulla [RVLM], and nucleus tractus solitarius

[NTS]), and that exogenous AngII stimulates TLR4 via AT1R in hypothalamic microglia *ex vivo*, resulting in microglial activation.

The aim of the studies herein was to determine the contribution of AT1R-TLR4 signaling interactions to BBB disruption, neuroinflammation, and autonomic dysfunction in neurogenic hypertension. Spontaneously hypertensive rats (SHRs) were treated with Losartan (AT1R inhibitor) or TAK-242 (TLR4 inhibitor) and age-matched to control Wistar Kyoto rats (WKYs). AT1R and TLR4 inhibitions normalized increased TLR4 protein density in the PVN, RVLM, and NTS of SHRs versus WKYs. Losartan and TAK-242 downregulated elevated interleukin-6 and tumor necrosis factor-α densities and abolished enhanced microglial activation in SHR central cardioregulatory nuclei. PVN, RVLM, and NTS BBB permeability analyses revealed complete restoration after TAK-242 treatment, whereas dye leakage, indicative of BBB disruption, was elevated in SHR. Elevated indirect mean arterial pressure was normalized in Losartan-treated SHRs and attenuated with TLR4 inhibition. During conscious baroreflex sensitivity assessment, TLR4 blockade rescued SHR responsiveness to vasoactive drugs. Ganglionic blockade induced a greater pressor response in SHRs compared to WKYs that was abolished by TLR4 inhibition. Overall, our data suggest a feed-forward pro-hypertensive cycle involving BBB disruption, neuroinflammation, and autonomic dysfunction driven by AngII-induced AT1R-TLR4 signaling interactions.

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I would like to express my deepest gratitude to each animal whose existence was unknowingly dedicated to the research herein and to all of the work upon which it is built. Without you, these studies would not be possible.

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I am tremendously grateful to my family for everything that they are and for all that they do. To my parents especially, Christina and Christofer, thank you. Thank you for a lifetime of love and support, for your encouragement, for never failing to catch me when I've fallen, and for helping me to stand tall in the face of any storm. To my sister, Annelise, and my brother, Andrew, thank you for being my sources of motivation in so many ways.

This work is dedicated, in loving memory,

to my four-legged shadow, my best friend, my heart,

Little Miss

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List of Abbreviations

ACE	Angiotensin-converting enzyme
ACTH	Adrenocorticotropic hormone
AD	Alzheimer's disease
Ang	Angiotensin
AT1R	Angiotensin II type 1 receptor
AV3V	Anteroventral third ventricle
BBB	Blood-brain barrier
BH4	Tetrahydrobiopterin
CNS	Central nervous system
COX	Cyclooxygenase
CREB	cAMP-responsive element protein
CRP	C-reactive protein
CVD	Cardiovascular disease
CVO	Circumventricular organ
DAMP	Damage-associated molecular pattern
DOCA	Deoxycorticosterone acetate
EC	Endothelial cell
ECM	Extracellular matrix
EPC	Endothelial progenitor cell
ETC	Electron transport chain

GABA	Gamma aminobutyric acid
HF	Heart failure
HMGB1	High-mobility group box 1
HSP	Heat shock protein
icv	Intracerebroventricular
IKK	IkB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory factor
L-NAME	L-N(G)-nitro-l-arginine methyl ester
LPS	Lipopolysaccharide
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MasR	Mas receptor
MCP	Monocyte chemoattractant protein
MD	Myeloid differentiation factor
MMP	Matrix metalloproteinase
MyD88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Norepinephrine
NF-kB	Nuclear factor kappa B

NLRP3	Nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3
NO	Nitric oxide
NOX	NADPH oxidase
NTS	Nucleus of the tractus solitarius
PAMP	Pathogen-associated molecular pattern
PG	Prostaglandin
PIC	Pro-inflammatory cytokine
PMN	Polymorphonucleocyte
PRR	Pattern-recognition receptor
PSNS	Parasympathetic nervous system
PVN	Paraventricular nucleus of the hypothalamus
RAS	Renin-angiotensin system
RIP	Adapter kinase receptor-interacting protein
ROS	Reactive oxygen species
RVLM	Rostral ventrolateral medulla
SFO	Subfornical organ
SHR	Spontaneously hypertensive rat
SNS	Sympathetic nervous system
SOD	Superoxide dismutase
TAB	Transforming growth factor-B-activated kinase binding protein
TAK	Transforming growth factor-B-activated kinase

TGF	Transforming growth factor
TIR	Toll-interleukin 1
TIRAP/MAL	TIR domain-containing adaptor/MyD88 adaptor-like protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	Tumor necrosis factor receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor inducing IFN-B
UCP	Uncoupling protein
VIPER	Viral inhibitory peptide
VP	Vasopressin
VSMC	Vascular smooth muscle cell
WKY	Wistar Kyoto rat

Chapter 1. Introduction

Defining hypertension

Hypertension is a sustained elevation in systemic arterial pressure, whether systolic blood pressure (SBP) or diastolic blood pressure (DBP). Quantitatively, BP categories vary slightly according to the clinical guidelines set forth by specific professional medical societies (**Table 1-1**). The primary difference observed in classification is that the 2017 American College of Cardiology (ACC)/American Heart Association (AHA) guidelines eliminated the category of “prehypertension,” lowering the threshold BP for Stage 1 hypertension and therapeutic intervention to an office/clinic BP of 130/80 mmHg. Conversely, BP values of 130-139/85-89 mmHg are classified as elevated/high-normal according to the 2018 European Society of Cardiology (ESC)/European Society of Hypertension (ESH) and 2020 International Society of Hypertension (ISH) guidelines, with lifestyle changes recommended and therapeutic intervention advised only in instances of high risk due to pre-existing CVDs (Bakris, Ali & Parati, 2019; Unger et al., 2020; Whelton et al., 2017).

The five major classes of antihypertensives target various aspects of the renin-angiotensin system (RAS; i.e., angiotensin-converting enzyme [ACE] inhibitors and angiotensin II receptor blockers [ARBs]), fluid homeostasis (i.e., thiazide diuretics), and the peripheral nervous system (i.e., calcium channel blockers and beta-blockers). Each of these targets, in turn, is well-documented as a primary factor in maintaining a hypertensive state. However, the recent estimate that nearly 20% of patients remain resistant to prescribed therapeutics (Carey, Sakhuja, Calhoun, Whelton & Muntner,

2019) suggests that these pharmaceutical interventions either (1) represent symptomatic, rather than causal, treatment options; or (2) fail to target one or more primary actors in the maintenance of resistant hypertension. Moreover, RAS dysregulation, imbalanced body fluid homeostasis, and inappropriate peripheral nervous system activity fail to account for the totality of resistant hypertensive pathophysiology.

SBP (mmHg)	ACC/AHA	ESC/ESH	ISH/WHO
<120	Normal	Normal/optimal	Normal
120-129	Elevated		
130-139	Stage 1	High-normal	High-normal
140-149	Stage 2	Grade 1	Grade 1
150-159			
160-169		Grade 2	Grade 2
170-179			
≥180	Hypertensive crisis	Grade 3	
Age-specific target SBP			
<65 years old	*<130*	*<130*	*<130*
≥65 years old	*<130*	*<140*	*<140*
DBP (mmHg)	**ACC/AHA**	**ESC/ESH**	**ISH/WHO**
<80	Normal	Normal/optimal	Normal
80-84	Stage 1		
85-89		High-normal	High-normal
90-99	Stage 2	Grade 1	Grade 1
100-109		Grade 2	Grade 2
110-119		Grade 3	
≥120	Hypertensive crisis		
Age-specific target			
<65 years old	*<80*	*<80*	*<80*
≥65 years old	*<80*	*<80*	*<90*

Table 1-1. Classification of arterial hypertension. Comparison of BP categories outlined by the 2017 American College of Cardiology (ACC)/American Heart Association (AHA), 2018 European Society of Cardiology (ESC)/European Society of Hypertension (ESH), and the 2020 International Society of Hypertension (ISH)/World Health Organization (WHO). SBP: systolic blood pressure; DBP: diastolic blood pressure.

Autonomic control of blood pressure and hypertension

Various experimental and clinical investigations have shown that dysfunctional autonomic control of blood pressure, mediated primarily by the sympathetic arm of the autonomic nervous system, is involved in the initiation and maintenance of hypertension. In fact, elevated sympathetic outflow from the central nervous system (CNS) is well-documented in patients and animal models of hypertension (DeQuattro & Chan, 1972; Esler, 2000; Esler, Lambert & Jennings, 1989; Grassi, 1998; Grassi, Seravalle & Quarti-Trevano, 2010; Lee, Lu & DeQuattro, 1996; Mancia, Grassi, Giannattasio & Seravalle, 1999; Rahn, Barenbrock & Hausberg, 1999). The occurrence of such sympathoexcitation to the heart and kidneys, driven by alterations in autonomic BP regulation, defines neurogenic hypertension as a classification (DiBona, 2013; Grassi, Mark & Esler, 2015; Guyenet, Stornetta, Souza, Abbott & Brooks, 2020; Mann, 2018; Stocker, Kinsman & Sved, 2017).

Central pathways modulating sympathetic outflow are governed by cardioregulatory nuclei found primarily within the hypothalamus and brainstem, including the hypothalamic paraventricular nucleus (PVN), nucleus tractus solitarius (NTS), rostral ventrolateral medulla (RVLM), and circumventricular organs (CVOs) **(Figure 1-1).** The integration of various peripheral and central signals in and among these nuclei allows for a coordinated homeostatic response to fluctuations in blood pressure in both the short-term and long-term. Conversely, idiopathic abnormalities in the afferent arm, comprised of the baroreceptors, chemoreceptors, and renal afferents, or in the central circuitry itself, result in hypertension (Guyenet, 2006; Malpas, 2010). The baroreceptor reflex is the major feedback compensatory reflex mechanisms

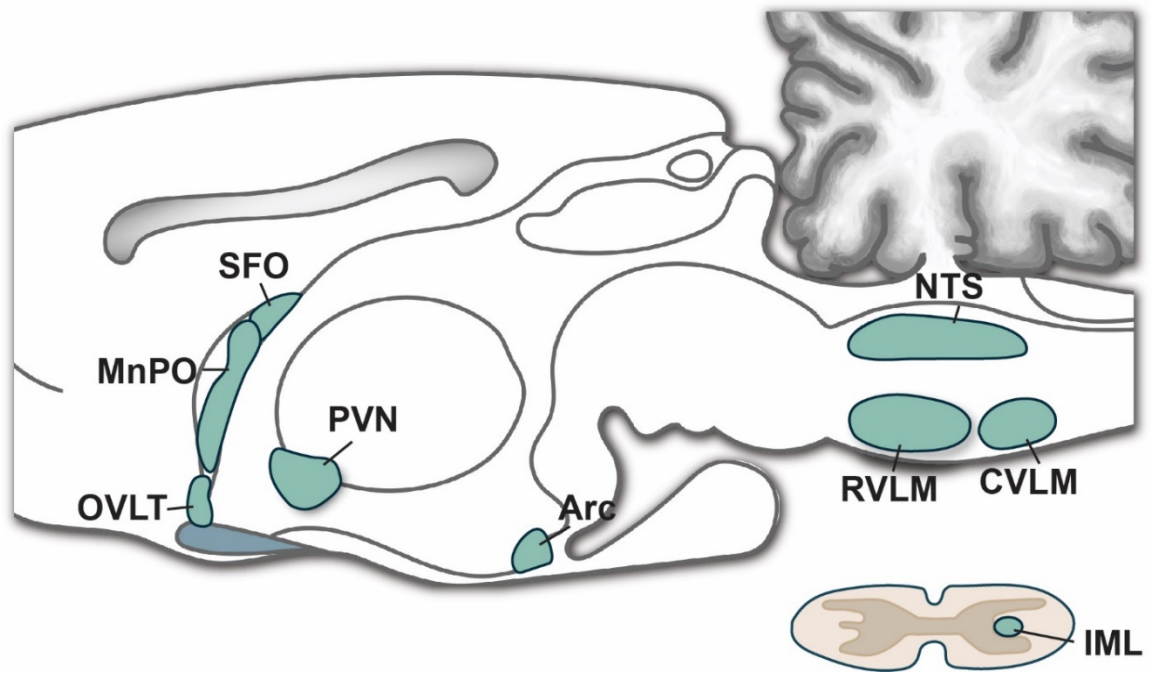

Figure 1-1. Cardioregulatory circuitry of the CNS. Simplified schematic of primary hypothalamic and brainstem regions involved in autonomic regulation of blood pressure. The nucleus of the tractus solitarius (NTS) integrates peripheral signals from arterial baroreceptors, chemoreceptors, and cardiopulmonary receptors, as well as from the amygdala and paraventricular nucleus of the hypothalamus (PVN). NTS outflow depresses activity of the rostral ventrolateral medulla (RVLM) via projections to the caudal ventrolateral medulla (CVLM), the primary inhibitory center. The RVLM also receives inputs directly from the PVN and afferent chemoreceptors, and regulates sympathetic activity through projections to sympathetic preganglionic nuclei in the intermediolateral column (IML) of the spinal cord alongside those from the PVN. The PVN integrates signals from multiple circumventricular organs (CVOs) – the median preoptic nucleus (MnPO), subfornical organ (SFO), and organum vasculosum lamina terminalis (OVLT) – as well as from the NTS and arcuate nucleus (Arc).

responsible for short-term control of arterial pressure. In brief, the systemic cardiovascular system contains two groups of baroreceptors, high-pressure (arterial baroreceptors), and low-pressure volume receptors (cardiopulmonary receptors). Both groups are mechanoreceptors stimulated by stretching in the walls of blood vessels that occurs with BP fluctuations. Functionally, the baroreceptors influence BP regulation by

informing the autonomic nervous system of beat-to-beat changes in BP within the arterial system or the blood volume within the cardiovascular system itself, respectively.

Arterial baroreceptors are located within the carotid sinuses and the aortic arch. The carotid sinus baroreceptors are innervated by a branch of cranial nerve IX (glossopharyngeal nerve), known as the sinus nerve of Hering. The aortic arch baroreceptors are innervated by the aortic nerve, which joins cranial nerve X (vagus nerve) and, together with cranial nerve IX, synapses in the nucleus of the tractus solitarius (NTS).

Baroreceptor impulses exert a tonic inhibitory influence on medullary SNS activity under normal physiological conditions. Acute hypotension reduces baroreceptor impulse firing rates, thereby reducing SNS inhibition in the medulla and indirectly increasing sympathetic outflow, whereas acute hypertension enhances the inhibitory effect and thus suppresses SNS activity. Due to the proclivity for baroreceptor “resetting” during sustained changes in MAP, the baroreflex arc is primarily a mechanism of short-term BP regulation. In neurogenic hypertension, impairment of arterial baroreceptor sensitivity is commonly observed both in experimental settings and hypertensive patients such that device-based approaches to manipulate arterial baroreflex has long been of interest for the treatment of patients with resistant hypertension (Lohmeier & Hall, 2019).

Whereas the cardioregulatory nuclei of the hypothalamus and brainstem serve as the integration center(s) for the baroreceptor reflex arc, autonomic nerves are responsible for efferent control of blood pressure (**Figure 1-2**). The primary impact of

parasympathetic activity in regulating cardiovascular function is to reduce heart rate through vagal nerve impulses. In contrast, the ability of the sympathetic nervous system to increase BP results from a combination of increasing cardiac output and peripheral resistance via heart rate acceleration, cardiac contractility enhancement, venous capacitance reduction, and vascular constriction.

Long-term autonomic control of blood pressure regulation depends on the interactions of hormones and the sympathetic nervous system, as will be discussed throughout the following chapters. Angiotensin II (AngII), commonly referred to as the primary RAS signaling peptide, regulates BP via systemic vascular resistance, fluid, and electrolyte balance. Importantly, AngII is known to have a major influence on chronic alterations in sympathetic activity through a myriad of signaling mechanisms within CNS cardioregulatory nuclei, including the PVN, RVLM, NTS, and CVOs (Dampney et al., 2002).

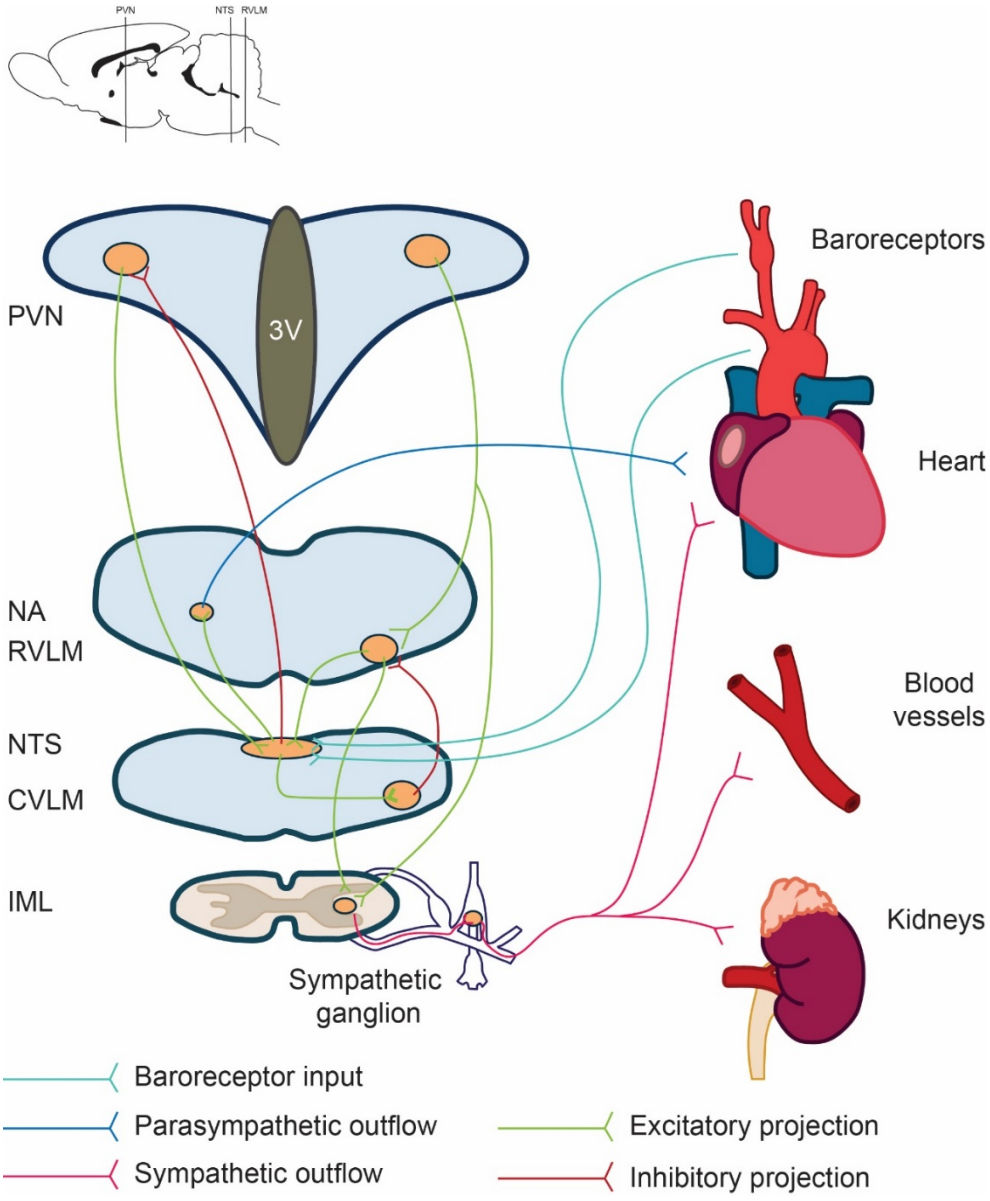

Figure 1-2. Autonomic regulation of blood pressure.

Tonic stimulation of neurons in the nucleus tractus solitarius (NTS) by arterial baroreceptors in the aortic arch and carotid sinuses regulates sympathetic outflow from the rostral ventrolateral medulla (RVLM), primarily by altering the magnitude of RVLM inhibition from the caudal ventrolateral medulla (CVLM). Projections from the hypothalamic paraventricular nucleus (PVN) and RVLM to the intermediolateral (IML) column directly influence sympathetic nervous system activity to effector organs (heart, vasculature, kidneys, etc.). Projections from the NTS also synapse in the nucleus ambiguus (NA) to influence cardioinhibitory (parasympathetic) input to the sinoatrial node (SA) to regulate heart rate.

Dissertation overview

The research described herein examines the contribution of interactions between AngII and the innate immune system to the progression of hypertensive pathophysiology. More specifically, the role of Toll-like receptor (TLR) 4 in propagating neuroinflammation, blood-brain barrier (BBB) disruption, and autonomic dysfunction driven by aberrant AngII signaling. The literature reviews presented in Chapters 2 and 3 are previously published peer-reviewed works that describe the role of various RAS peptides in modulating the neuroinflammatory process (Chapter 2) and the evidence to-date of chronic TLR4 activation as a pathophysiological process underlying hypertension (Chapter 3):

Chapter 2. Mowry FE, & Biancardi VC (2019). *Neuroinflammation in hypertension: the renin-angiotensin system versus pro-resolution pathways*. Pharmacol Res 144: 279-291. IF: 5.893 (2019/2020).

Chapter 3. Nunes KP, de Oliveira AA, Mowry FE, & Biancardi VC (2019). *Targeting toll-like receptor 4 signalling pathways: can therapeutics pay the toll for hypertension?* Br J Pharmacol 176: 1864-1879. IF: 7.73 (2019).

Together, the subsequent chapters (Chapters 4-7) present the original research article, “AT1R and TLR4 mediate blood-brain barrier disruption, neuroinflammation, and autonomic dysfunction in spontaneously hypertensive rats,” which is currently in preparation for submission.

Chapter 2. Neuroinflammation in hypertension: the renin-angiotensin system versus pro-resolution pathways

Mowry FE, & Biancardi VC (2019).

Pharmacol Res. 144: 279-291

Abstract

Overstimulation of the pro-inflammatory pathways within brain areas responsible for sympathetic outflow is well evidenced as a primary contributing factor to the establishment and maintenance of neurogenic hypertension. However, the precise mechanisms and stimuli responsible for promoting a pro-inflammatory state are not fully elucidated. Recent work has unveiled novel compounds derived from omega-3 polyunsaturated fatty acids (ω-3 PUFAs), termed specialized pro-resolving mediators (SPMs), which actively regulate the resolution of inflammation. Failure or dysregulation of the resolution process has been linked to a variety of chronic inflammatory and neurodegenerative diseases. Given the pathologic role of neuroinflammation in the hypertensive state, SPMs and their associated pathways may provide a link between hypertension and the long-standing association of dietary ω-3 PUFAs with cardioprotection. Herein, we review recent progress in understanding the RAS-driven pathophysiology of neurogenic hypertension, particularly in regards to the chronic low-grade neuroinflammatory response. In addition, we examine the potential for an impaired resolution of inflammation process in the context of hypertension.

Introduction

Hypertension is commonly denoted as the preeminent risk factor for cardiovascular disease (CVD) worldwide, with a global prevalence that currently exceeds 1.4 billion people (Mills et al., 2016). Many instances of resistant hypertension have a strong autonomic component and fall under the classification of neurogenic hypertension. These cases are often associated with a chronic elevation in sympathetic nervous system (SNS) activity that enhances sympathetic outflow to the heart and vasculature (Acelajado, Hughes, Oparil & Calhoun, 2019; Stocker, Kinsman & Sved, 2017). Underlying the heightened sympathetic tone are two primary characteristics of neurogenic hypertension: 1) dysregulation of renin-angiotensin system (RAS) components, and 2) a persistent low-grade neuro-inflammatory response.

The classic peripheral RAS is intrinsic to maintaining cardiovascular homeostasis and plays a substantial role in the development and progression of hypertension (for review, see Balakumar & Jagadeesh, 2014). While comprised of numerous signaling peptides, angiotensin (Ang) II is generally considered the major biologically active component of RAS, fulfilling many of its numerous physiological roles via AngII type 1 receptors (AT1R). In addition to the peripheral RAS, dysregulation of the tissue-specific central nervous system (CNS) RAS is well evidenced in the hypertensive state (for review, see de Morais, Shanks & Zucker, 2018). Central RAS hyperactivity, particularly of AngII and AT1R, is observed in several experimental and genetic models of hypertension, including the spontaneously hypertensive rat (SHR) and deoxycorticosterone acetate (DOCA)-salt rat, among others (Basso, Ruiz, Mangiarua & Taquini, 1981; Ganten, Hermann, Bayer, Unger & Lang, 1983; Reaux et al., 1999).

Persistent activity of AngII-AT1R signaling at the circumventricular organs (CVOs) and within cardioregulatory nuclei increases SNS outflow, dampens baroreflex sensitivity, stimulates vasopressin secretion, and contributes directly to maintaining the pro-inflammatory milieu associated with increased blood pressure (de Morais, Shanks & Zucker, 2018; Winklewski, Radkowski, Wszedybyl-Winklewska & Demkow, 2015). Beyond AngII-AT1R signaling, an alternative RAS axis, composed of angiotensin-converting enzyme (ACE) 2, Ang-(1-7), and the G protein-coupled receptor Mas (MasR), has been biologically characterized (Bader, Alenina, Andrade-Navarro & Santos, 2014; Campagnole-Santos, Diz, Santos, Khosla, Brosnihan & Ferrario, 1989; Santos et al., 1988; Santos et al., 2003; Schiavone, Santos, Brosnihan, Khosla & Ferrario, 1988). Whereas peripheral ACE2/Ang-(1-7)/MasR is thought to be protective and counteract the effects of the classical ACE/AngII/AT1R axis, evidence would suggest that central Ang-(1-7) has site-specific cardioregulatory actions within the CNS (de Morais, Shanks & Zucker, 2018; Santos et al., 2018). Nevertheless, Ang-(1-7) and MasR were shown to exert beneficial effects on neuroinflammation through modulation of T cell activation and macrophage polarization and migration, raising the possibility of therapeutically targeting this axis to counteract the pro-inflammatory effects of AngII-AT1R signaling (Hammer, Stegbauer & Linker, 2017).

In tandem with works establishing neurogenic hypertension as a neuroinflammatory disease, a *paradigm shift* has occurred within the field of immunology. The concept that the acute inflammatory response dissipates passively has been replaced with the recognition of a highly active, self-limiting, *resolution of inflammation* process, which enables the re-establishment of tissue homeostasis, as illustrated in **Figure 2-1** (Serhan

& Chiang, 2004). Pro-resolving lipid mediators are biosynthesized from ω-3 and ω-6 long-chain polyunsaturated fatty acids (PUFAs), namely eicosapentenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA). Despite ongoing debate regarding the benefits of essential ω-3 PUFA supplementation in hypertension, the

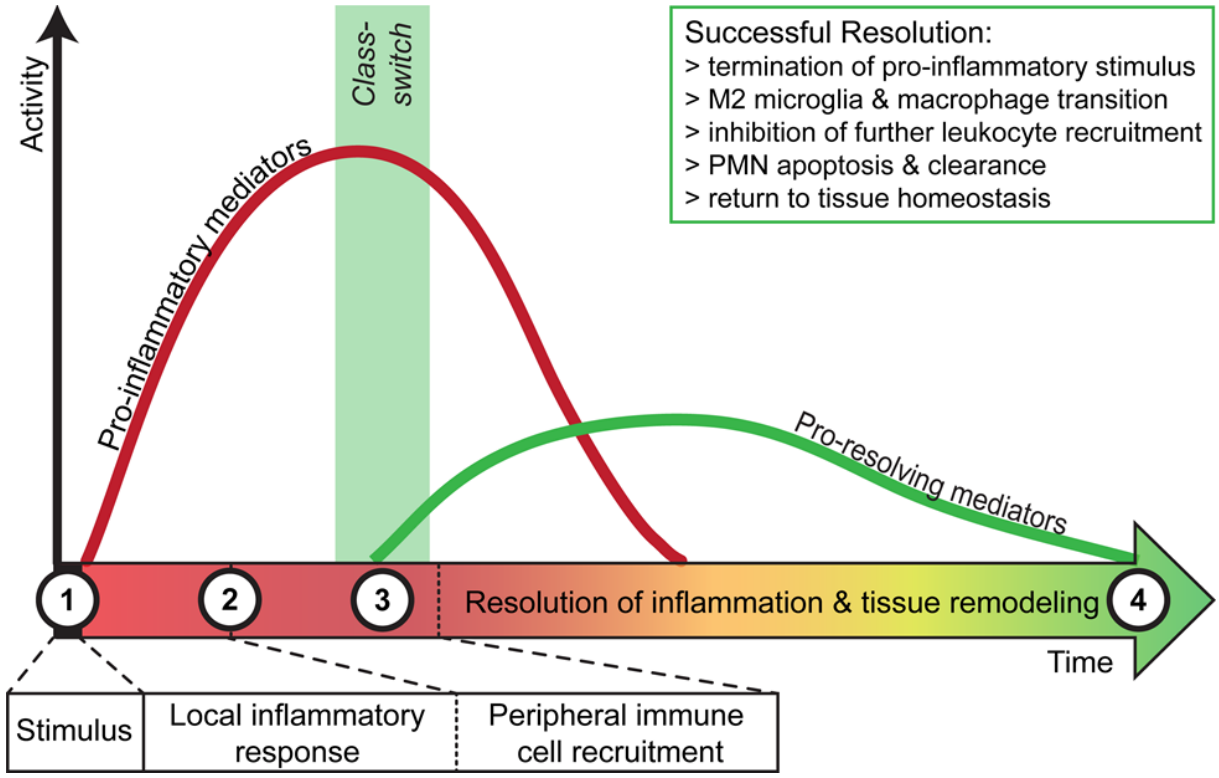

Figure 2-1. The acute inflammatory response and its resolution. In response to an acute inflammatory stimulus (**1**), pattern recognition receptors stimulate the rapid production and release of pro-inflammatory mediators (cytokines, chemokines, adhesion molecules, and lipid mediators). The local inflammatory response propagates the recruitment of peripheral immune cells (**2**), leading to elimination of the pro-inflammatory trigger. A class-switch (**3**) to the production of specialized pro-resolving mediators (SPMs) marks the beginning of the resolution phase. SPMs mediate the sequestering of pro-inflammatory cytokines and ROS, limit further leukocyte recruitment, induce polymorphonuclear (PMN) leukocyte apoptosis and efferocytosis by M2-state macrophages, and stimulate tissue remodeling, allowing for the return of tissue homeostasis (**4**).

notion that their adequate consumption improves CVD risk is long-standing (for review, see Mozaffarian & Wu, 2011). Insights into the active process of resolution of inflammation and, specifically, into the evolving class of pro-resolving mediators, have altered our understanding of the potential associations between these fatty acids and CVDs, adding another degree of complexity to deciphering the unyielding pro-inflammatory and pro-hypertensive CNS milieu.

Given the relatively recent characterization of hypertension as a chronic low-grade inflammatory disease, coupled with recognition of the novel active resolution process, research to-date regarding particular pro-resolution mediators in the hypertensive state is, admittedly, rather limited. Yet, a review of the literature reveals a large body of work that can now be appreciated within the context of inflammation-resolution. Moreover, previous studies demonstrating an imbalance amid pro-inflammatory and anti-inflammatory factors have unwittingly generated ample data suggesting an impaired resolution process. In the simplest sense, chronic inflammation results from a failure of the acute inflammatory response to resolve. Successful resolution involves termination of the pro-inflammatory stimulus, a class-switch to production of anti-inflammatory and pro-resolving mediators, inhibition of further polymorphonuclear (PMN) leukocyte recruitment, PMN apoptosis and efferocytosis by recruited M2 macrophages, tissue regeneration, and the return of tissue homeostasis (Fullerton & Gilroy, 2016; Serhan & Savill, 2005). In the hypertensive setting, however, AngII appears to act as the primary pro-inflammatory stimulus, triggering production of reactive oxygen species (ROS), pro-inflammatory cytokines (PICs), chemokines, and adhesion molecules, stimulating NF-κB and protein kinase signaling cascades, activating and recruiting immune cells, and

promoting degradation of the extracellular matrix (ECM) and fibrosis. Thus, the perpetual inflammation observed in hypertension is likely, in part, a product of insufficient pro-resolution machinery in the face of overwhelming RAS-mediated stimulation. To this end, we will explore the evidence for impaired resolution in hypertension, examining recent advances in our understanding of the ongoing neuroinflammatory component of this disease and evaluating the potential link to a dysregulated resolution process.

RAS-mediated mechanisms of neuroinflammation

The acute inflammatory response is a complex process in which coordinated activities of signaling pathways orchestrate inflammatory mediators in resident and recruited immune cells. While these processes vary based upon the nature of the insult, the pro-inflammatory phase of the response involves stimulation of cell-surface pattern recognition receptors (PRRs), activation of pro-inflammatory signaling pathways, production and secretion of various inflammatory mediators, and recruitment of immune cells (for review, see Medzhitov, 2008). The innate immune system is a primary modulator of the inflammatory response, both initiating acute pro-inflammatory states to combat infections and signaling for the clearance of apoptotic cells and debris to pave the way for repair and homeostatic restoration. However, an exaggerated or prolonged response through persistent activation of central and peripheral immune cells can become pathological.

The ongoing neuroinflammatory response observed in neurogenic hypertension can be considered both a causative and consequential factor within an intricate pro-

hypertensive signaling web comprised of RAS and neurotransmitter dysregulation, blood-brain barrier (BBB) disruption, abnormal immune cell activation, and oxidative stress, among other factors. In this sense, ROS and various PICs within the paraventricular nucleus of the hypothalamus (PVN), including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, have been implicated in the development of hypertension through interactions with central RAS components (Cardinale, Sriramula, Mariappan, Agarwal & Francis, 2012; Kang et al., 2014). Whereas AngII-infusion elevates mean arterial pressure (MAP), promotes PIC and ROS production, increases ACE and AT1R expression, and decreases ACE2-MasR expression in the PVN, inhibition of PVN-specific NF-κB attenuates these deleterious effects (Cardinale, Sriramula, Mariappan, Agarwal & Francis, 2012). Importantly, alleviation of CNS inflammation attenuates BP in slow pressor AngII-infusion models of hypertension and in SHRs, highlighting the pathophysiological role of neuroinflammation in this disease state (Bai et al., 2017; Cheng et al., 2010; Kang et al., 2009; Shi et al., 2010). It should be noted that a comprehensive discussion regarding factors underlying the pro-inflammatory milieu and autonomic dysfunction in neurogenic hypertension is outside the scope of this review. Many of these mechanisms have recently been addressed and we will refer the interested reader to relevant reviews where possible. In the following sections, rather, we aim to provide a framework for understanding the potential of an impaired inflammation-resolution process within the context of neurogenic hypertension.

Disrupted immune privilege of the CNS

The BBB forms a physical, as well as biochemical, wall between the central and systemic circulations. Among its various roles, the BBB is the primary point of CNS homeostatic maintenance and protects the neural environment from potentially toxic substances (for review, see Obermeier, Daneman & Ransohoff, 2013). The physiological integrity of the BBB is tightly regulated by the various components of the neurovascular unit, i.e., neurons, pericytes, astrocytes, microglia, endothelial cells (ECs), cerebral vascular smooth muscle cells (VSMCs), perivascular macrophages, and the ECM (Erickson & Banks, 2018; Sweeney, Zhao, Montagne, Nelson & Zlokovic, 2019).

In a healthy state, the BBB prevents AngII from directly accessing cardioregulatory nuclei. Instead, the large AngII peptide acts via AT1R at BBB-lacking CVOs, which relay peripheral signals to hypothalamic and brainstem autonomic centers (Erickson & Banks, 2018; Ferguson, 2009; McKinley et al., 2003; Sweeney, Zhao, Montagne, Nelson & Zlokovic, 2019). In the developed hypertensive state, however, disruption of the BBB is observed. For example, BBB disruption in SHRs allows circulating AngII entry into typically protected nuclei, such as the PVN, rostral ventrolateral medulla (RVLM), and nucleus tractus solitarii (NTS) (Biancardi, Son, Ahmadi, Filosa & Stern, 2014). Importantly, BBB breakdown is prevented by chronic blockade of AT1R with losartan but not by treatment with a peripheral vasodilator (i.e., hydralazine), indicating that circulating AngII may facilitate its own access by altering BBB permeability (Biancardi, Son, Ahmadi, Filosa & Stern, 2014). The ‘leaky’ BBB can no longer maintain precise control of CNS ion, water, and electrolyte balances, nor guard against intrusion by

neurotoxic substances and circulating pro-inflammatory factors (Biancardi, Stranahan, Krause, de Kloet & Stern, 2016; Setiadi, Korim, Elsaafien & Yao, 2018; Yu, Zhang, Wei, Serrats, Weiss & Felder, 2010). As a result, systemic fluctuations in these variables can induce sizeable neuronal and glial disturbances, gaining access to the CNS through leaky vasculature in regions where the BBB has been disturbed.

Conversely, circulating PICs can act directly on cells of the BBB to impact protected regions. For example, TNF-α and IL-1β increase cyclooxygenase (COX)-2 activity in perivascular macrophages of the PVN, resulting in prostaglandin (PG) E_2 production (Yu, Zhang, Wei, Serrats, Weiss & Felder, 2010). Through its stimulation of PVN neuronal activity, PGE_2 enhances adrenocorticotropic hormone (ACTH) release, sympathetic outflow, and blood pressure (Felder, 2010; Schiltz & Sawchenko, 2003). Of note, perivascular macrophages, which are situated between the endothelial basement membrane and glia limitans, appear to play a key role in cerebrovascular and neurocognitive dysfunction during hypertension through AT1R- and NADPH oxidase (NOX) 2-mediated ROS production (Faraco et al., 2016; Williams, Alvarez & Lackner, 2001). Alongside circulating factors, an increase in PIC production within the cardioregulatory nuclei is closely associated with hypertension (Benicky et al., 2011; Benicky, Sanchez-Lemus, Pavel & Saavedra, 2009; Qi et al., 2013; Shi et al., 2010; Shi, Jiang, Wang, Xu & Guo, 2014; Zubcevic, Waki, Raizada & Paton, 2011). In that sense, bilateral inhibition of NF-κB within the PVN diminishes AngII-induced hypertension through reduction of ROS and PIC levels, as discussed in the following sections (Cardinale, Sriramula, Mariappan, Agarwal & Francis, 2012).

Innate immune cells of the CNS

Together, microglia and astrocytes represent the primary population of resident immune cells in the CNS, playing crucial roles in homeostatic maintenance throughout life (Ransohoff & Brown, 2012; Schwartz, Kipnis, Rivest & Prat, 2013). Recent reviews have provided excellent discussions of microglia and astrocyte cell physiology (see refs. Sofroniew, 2015; Verkhratsky & Nedergaard, 2018; Wolf, Boddeke & Kettenmann, 2017). In brief, the initial phase of microglial activation following CNS insult, referred to as the classical/pro-inflammatory M1 activation state, involves upregulation of various PICs (e.g., TNF-α, IL-1β, IL-6, IL-12, and IL-23), cytotoxic substances (e.g. ROS and quinolinic acid), chemokines, PGE_2, nitric oxide (NO), and L-glutamate. The M1 phenotype subsequently shifts to an anti-inflammatory and pro-resolving M2 state, wherein debris clearance, ECM deposition, and angiogenesis are promoted. Thus, the inflammatory microglial response, culminating in injury resolution and tissue repair, is considered self-limiting. The transition between M1 and M2 activation states appears to be disrupted in many psychiatric and neurological diseases, resulting in chronic pro-inflammatory microglial activation and neuroinflammation that may lead to cell death and further tissue damage (Dantzer, O'Connor, Freund, Johnson & Kelley, 2008; Doorduin, de Vries, Willemsen, de Groot, Dierckx & Klein, 2009; Glass, Saijo, Winner, Marchetto & Gage, 2010; Gonzalez, Elgueta, Montoya & Pacheco, 2014; Heneka et al., 2015). Notably, the pro-inflammatory CNS milieu is posited to be a crucial link between hypertension and the development of Alzheimer's disease (AD)—anti-hypertensive therapeutics, including candesartan (AT1R inhibitor), captopril (ACE inhibitor), and perindopril (ACE inhibitor), are demonstrated to exert neuroprotective effects by

increasing neurogenesis, dampening microglial activation, and reducing β-amyloid plaque deposition (Bhat, Goel, Shukla, Shukla & Hanif, 2018; Carnevale et al., 2012; Carreno-Muller et al., 2003; Torika, Asraf, Roasso, Danon & Fleisher-Berkovich, 2016).

In hypertension, multiple studies report increased microglial activation within cardioregulatory nuclei. For instance, in a chronic AngII-induced rat model of hypertension, long-term intracerebroventricular (*ICV*) infusion of the microglia inhibitor, minocycline, abolished increased IL-1β, IL-6, and TNF-α mRNA within the PVN, accompanied by BP attenuation (Shi et al., 2010). Activation of PVN microglia and their associated pro-inflammatory profile were shown to result from direct stimulation by prorenin in SHRs (Shi et al., 2014). Upon targeted microglial deletion via *ICV* administration of diphtheria toxin in AngII- and l-N(G)-nitro-l-arginine methyl ester (L-NAME)-induced hypertension mouse models, Shen *et al.* (2015) observed a significant drop in BP, expression of TNF-α and IL-1β, renal norepinephrine (NE), PVN glutamate receptor expression, and circulating vasopressin (AVP) (Shen et al., 2015). Alongside microglia, astrocytes are known contributors to neuronal activation and resident immune mechanisms, capable of producing numerous PICs upon stimulation, including IL-1β, IL-6, and TNF-α (Sofroniew, 2015). Furthermore, astrocytes have been shown to modulate AngII-stimulated neuronal activity and sympathetic outflow from the PVN (Stern, Son, Biancardi, Zheng, Sharma & Patel, 2016). Hence, RAS-driven neuroinflammation is undoubtedly a consequence of both microglial and astrocytic activation within the context of hypertension.

Toll-like receptor 4

The initial neuroinflammatory process requires a combination of cytokines, chemokines, and adhesion molecules that are produced following microglial and astrocytic PRR stimulation (Heneka et al., 2015; Morales, Guzman-Martinez, Cerda-Troncoso, Farias & Maccioni, 2014). There are two general classes of PRR ligands: 1) exogenous pathogen-associated molecular patterns (PAMPs) from invading microorganisms, such as bacteria and some viruses; and 2) endogenous damage-associated molecular patterns (DAMPs) released upon cell damage and/or stress (Kawai & Akira, 2010a; Matzinger, 2002). For instance, Toll-like receptor (TLR) 4 is classically activated by lipopolysaccharide (LPS), a PAMP derived from the cell wall of Gram-negative bacteria. Conversely, endogenous TLR4-specifc DAMPs include C-reactive protein (CRP), several heat shock proteins (HSPs), and high-mobility group box 1 (HMGB1), and, compellingly, AngII is known to stimulate the TLR4 signaling cascade (Biancardi, Bomfim, Reis, Al-Gassimi & Nunes, 2017b; Kawai & Akira, 2011). The cascade downstream of TLR4 involves both myeloid differentiation primary response 88 (MyD88)-dependent and MyD88-independent (TRIF-dependent) arms (Akira & Takeda, 2004b). Once activated, TLR4 upregulates the production and secretion of various pro-inflammatory mediators, primarily via MAPKs and NF-κB pathways (Biancardi, Bomfim, Reis, Al-Gassimi & Nunes, 2017b; Nunes, de Oliveira, Mowry & Biancardi, 2019).

TLR4 is widely expressed throughout the body by a variety of cell types, including peripheral immune cells, ECs, VSMCs, and CNS cells (Tartey & Takeuchi, 2017). Although neurons and astrocytes express low levels of TLR4 and respond to LPS stimulation, microglia are considered the primary TLR4-expressing CNS cells (Hanke &

Kielian, 2011; Lehnardt, 2010). A chief mechanisms by which the AngII-AT1R axis contributes to chronic BP elevations in neurogenic hypertension appears to be through stimulation of microglial ROS and PIC production. We have demonstrated a clear role for TLR4 in causally linking AngII-AT1R-induced microglial activation with CNS oxidative stress (Biancardi, Stranahan, Krause, de Kloet & Stern, 2016). Moreover, we showed that hypothalamic microglial ROS production in response to AngII is dependent upon the presence of functional TLR4 (Biancardi, Stranahan, Krause, de Kloet & Stern, 2016). This is in keeping with previous work showing that AT1R inhibition abolishes LPS-induced neuroinflammation in normotensive animals, both in the PVN and the subfornical organ (SFO) (Benicky et al., 2011; Benicky, Sanchez-Lemus, Pavel & Saavedra, 2009). TLR4 protein and mRNA expression are increased within the PVN in SHRs and AngII-induced hypertension models, and chronic inhibition of central TLR4 with viral inhibitory peptide (VIPER) attenuates MAP, PIC production, and levels of inducible NO synthase (iNOS) and NF-κB in hypertensive animals (Dange et al., 2014b; Dange, Agarwal, Teruyama & Francis, 2015b). Furthermore, blockade of PVN AT1R was shown to decrease activity of TLR4 and the MyD88-dependent signaling pathway in SHRs (Li et al., 2016a). Whether considered within the CNS or the periphery, TLR4 is evidenced to play a chief role in the pathophysiology of hypertension, providing a mechanistic link between the dysregulated RAS and activation of the innate immune system (Biancardi, Bomfim, Reis, Al-Gassimi & Nunes, 2017b; Nunes, de Oliveira, Mowry & Biancardi, 2019). Of note, additional members of the PRR family, such as TLR2, TLR9, and the NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 OR Nod-like receptor protein 3) inflammasome, are

associated with hypertension, further demonstrating the considerable contribution of innate immunity to this disease state (Ahmad et al., 2012; Singh, Chapleau, Harwani & Abboud, 2014).

RAS-driven oxidative stress in hypertension

There appears to be a causal association between excessive ROS production and hypertension, at least in animal models, based on studies showing that antioxidants and ROS scavengers lower BP, whereas pro-oxidants elevate BP (Bhatia, Elmarakby, El-Remessy & Sullivan, 2012; Jia, Aoyagi & Yang, 2010; Lob, Vinh, Li, Blinder, Offermanns & Harrison, 2011). Indeed, studies employing treatment with antioxidant vitamins, free radical scavengers, superoxide dismutase (SOD) mimetics, and tetrahydrobiopterin (BH4) have shown attenuation or complete prevention of hypertensive responses and organ damage (Rey, Cifuentes, Kiarash, Quinn & Pagano, 2001; Wilcox, 2010). In models of neurogenic hypertension, neuroinflammation, and heart failure (HF), markers of oxidative stress (e.g. Nox and xanthine oxidase activity, $F_{2\alpha}$-isoprostanes, and tissue ROS concentrations) are elevated, while levels of NO and antioxidant enzymes, such as MnSOD and SOD3, are reduced (Case, Tian & Zimmerman, 2017; Dikalova et al., 2010; Dominiak et al., 2017; Kishi, Hirooka, Kimura, Ito, Shimokawa & Takeshita, 2004; Kishi, Hirooka, Konno & Sunagawa, 2010; Motaghinejad, Motevalian & Shabab, 2016; Savalia et al., 2014; Su et al., 2016; Torok, 2008; Zhang, Liu, Tu, Muelleman, Cornish & Li, 2014; Zimmerman, Lazartigues, Sharma & Davisson, 2004). Within the CNS, PVN AT1R activation stimulates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to produce superoxide,

which enhances sympathetic tone and inhibits gamma aminobutyric acid (GABA) release within the nucleus, furthering sympathoexcitation (Chen & Pan, 2007; Yu et al., 2007). In the same vein, oxidative stress in the RVLM is a potent deregulator of SNS outflow and accompanies the progression of hypertension in SHRs (Kishi, Hirooka, Kimura, Ito, Shimokawa & Takeshita, 2004; Nishihara, Hirooka, Kishi & Sunagawa, 2012; Nishihara, Hirooka, Matsukawa, Kishi & Sunagawa, 2012). Both superoxide scavenging and deletion of NADPH oxidase components significantly reduce AngII-induced increases in BP. Conversely, inhibition of RAS in hypertensive patients impairs NADPH oxidase activity and ameliorates endothelium-dependent vasodilation. Mice that are deficient in NADPH oxidase have reduced BP relative to their wild-type counterparts, and infusion of AngII fails to induce hypertension in these mice (Grote et al., 2006; Haque & Majid, 2011).

It should also be considered that cellular redox homeostasis is tightly coupled to the maintenance of mitochondrial bioenergetics. In the RVLM, disruption of mitochondrial bioenergetics results in ROS production and oxidative stress, which diminishes both expression and functionality of mitochondrial biogenesis/bioenergetic regulators, thereby propagating the initial disruption and ROS generation (Wu, Wu, Chao, Hung & Chan, 2016). Experiments employing pharmacological agents and genetic manipulations to cultivate mitochondrial biogenesis in models of neurogenic hypertension have resulted, not only in reducing neural oxidative stress, but also in attenuating BP elevations (Chan, Wu, Wu, Ho, Chang & Chan, 2009; Chan, Wu, Chang, Hsu & Chan, 2010; Chan, Wu, Chang, Tai & Chan, 2009; Li, Case, Yang, Schultz & Zimmerman, 2013; Nozoe et al., 2008; Zimmerman, Lazartigues, Sharma & Davisson,

2004). Neural mitochondrial dysfunction and accumulation of tissue ROS have been detected in cardioregulatory nuclei of both pre-hypertensive SHRs and prior to hypertension onset in AngII-infusion models (Chan, Wu, Chang, Tai & Chan, 2009; Wang, Huang, White, Chen, Ahmad & Leenen, 2016; Young, Li, Dong, Horwath, Clark & Davisson, 2015). Deletion of central antioxidants in AngII-infusion models greatly sensitizes BP responsiveness to the peptide administration (Case, Tian & Zimmerman, 2017; Lob et al., 2010). Mechanistically, reduced electron transport chain (ETC) protein expression and enzymatic activity, suppressed inner membrane electron transport capacity, and downregulated mitochondrial uncoupling protein (UCP) appear to contribute to CNS oxidative stress during inflammation and AngII stimulation (Chan & Chan, 2013; Chan, Wu, Wu, Ho, Chang & Chan, 2009; Chan, Wu, Chang, Tai & Chan, 2009; Chan, Wu, Kung & Chan, 2010; Lu, Sun, Qiao, Liu, Ding & Hu, 2014; Nozoe et al., 2008).

Furthermore, the association between oxidative stress and hypertension is long-evidenced in peripheral immune cells. In humans with essential hypertension, circulating PMNs tend to produce higher levels of superoxide (Mehta, Lopez, Chen & Cox, 1994). Similarly, PMNs from SHRs exhibit enhanced function (i.e. superoxide production) and are present in higher numbers than those observed in age-matched normotensive rats (Wistar Kyoto rats; WKYs) (Schmid-Schonbein, Seiffge, DeLano, Shen & Zweifach, 1991; Shen, Sung, Whittemore, DeLano, Zweifach & Schmid-Schonbein, 1995). On the other hand, PMN superoxide production in DOCA-salt and L-NAME hypertensive rats is not increased relative to WKY controls (Ohmori, Kitoh, Harada, Sugimoto & Fujimura, 2000). This would indicate that augmented PMN activity

is not due to increases in BP, per se. Rather, given that heightened PMN function was apparent in 4 week-old SHRs, prior to the onset of hypertension, PMN-driven oxidative stress may be a genetic pre-determinant in this model of neurogenic hypertension (Ohmori, Kitoh, Harada, Sugimoto & Fujimura, 2000).

The neuro-immune axis in hypertension

Bidirectional interactions between the nervous and immune systems appear to be mediated, in part, by the SNS (Dantzer, 2018; Elenkov, Wilder, Chrousos & Vizi, 2000; Zlokovic, 2008). Interestingly, CNS AngII signaling is implicated in regulating peripheral inflammation. Central administration of AngII increases splenic IL-1β, IL-2, and IL-6 expression, likely via splenic sympathetic nerve activation (Marvar et al., 2010). Lesions in the anteroventral third ventricle (AV3V) were shown to prevent infiltration of the peripheral vasculature by activated T cells in AngII-induced hypertension (Marvar et al., 2010). In SHRs, bone marrow-derived pro-inflammatory cells migrate into the PVN and, in AngII infusion models of hypertension, activated T cells infiltrate the SFO (Pollow et al., 2014; Santisteban et al., 2015). Selective SFO deletion of extracellular SOD3 in AngII-induced hypertension enhances circulating levels of activated T cells, as well as vascular infiltration by immune cells (Lob et al., 2010). Acute SFO microinjections of PICs elevate renal sympathetic nerve activity alongside arterial BP, whereas blockade of RAS or COX-2 prevents these effects (Wei, Yu, Zhang & Felder, 2015). Of note, SFO PIC microinjections increased RAS and COX-2 activity in the PVN, as well as the SFO, suggesting that neuroinflammation facilitates central AngII's pro-hypertensive actions (Wei, Yu, Zhang & Felder, 2015). Additionally, *ICV* treatment with the SOD mimetic,

mitochondrial-targeted antioxidant (mitoTEMPO), prevents the effects of chronic AngII-infusion on bone marrow-derived circulating inflammatory and endothelial progenitor cells (EPCs) (Jun et al., 2012). In SHRs, enhanced SNS input to bone marrow is observed in concert with increased numbers of circulating inflammatory cells and decreased EPCs (Zubcevic et al., 2014). Notably, EPCs are necessary for adequate repair of damaged vasculature and the reduction in EPC numbers reflects dampened activity of the parasympathetic nervous system (PSNS) and/or an imbalance in the relative SNS-PSNS activity levels (Zubcevic et al., 2014). Together, these studies link central AngII to peripheral inflammatory responses via sympathetic bone marrow and splenic efferents. AngII-driven elevations in central ROS appear to contribute to peripheral inflammatory responses in hypertension and, concurrently, to inhibit vascular repair processes, suggesting the existence of a positive feedback loop wherein AngII stimulates inflammatory cascades in the brain that increase circulating levels of activated inflammatory cells, which then further central inflammatory processes.

The resolution of inflammation: an overview

The progression from acute to chronic inflammation is prevented by active resolution that, ultimately, inhibits additional tissue damage and restores tissue homeostasis. The resolution process is orchestrated both spatially and temporally by pro-resolving mediators (see **Figure 2-1**). Sehran *et al.* introduced this process under the name ‘Resoleomics,’ describing an inflammatory reaction regulated by the interactions between the innate immune system, sympathetic nervous system, and hypothalamic-pituitary-adrenal axis (Serhan & Chiang, 2004). Inflammation-resolution

prevents excessive leukocyte trafficking to the damage site, inhibits signals for PIC production and leukocyte survival, induces apoptosis of recruited proinflammatory cells, and subsequently promotes clearance of apoptotic leukocytes through efferocytosis (Schwab, Chiang, Arita & Serhan, 2007; Serhan, 2014). Dysregulation, or failure, of the resolution process is associated with the pathogenesis of chronic inflammatory diseases, including neurodegenerative diseases, such as AD and multiple sclerosis (MS), as well as atherosclerosis (see refs. Fredman & Tabas, 2017; Serhan, Chiang & Dalli, 2015). It is likely that similar impairments in resolution processes contribute to the chronic neuroinflammation observed in neurogenic hypertension, as depicted in **Figure 2-2**.

During the initiation phase of the inflammatory response, AA-derived pro-inflammatory lipid mediators, such as PGE_2 and leukotriene (LT) B_4, are up-regulated and contribute to increased vascular permeability and PMN leukocyte recruitment (Levy, Clish, Schmidt, Gronert & Serhan, 2001). A lipid mediator *class-switch* occurs during the time-course of inflammation wherein pro-inflammatory mediators induce translation of the mRNA necessary for production of specialized pro-resolving mediators (SPMs). For example, PGE_2, thromboxane (TX) A_2, and LTB_4, while initially pro-inflammatory, are necessary for the *class-switch* to production of pro-resolving lipoxins (Levy, Clish, Schmidt, Gronert & Serhan, 2001). Together, PUFA-derived lipoxins, maresins, protectins, and resolvins, comprise the SPM family that appears to dominate the switch towards resolution (Serhan, 2014; Serhan, Clish, Brannon, Colgan, Chiang & Gronert, 2000; Serhan et al., 2002). A number of endogenous molecules in addition to SPMs have been categorized as pro-resolving mediators, including annexin A1, IL-10, carbon

monoxide, hydrogen sulfide, adenosine, inosine, acetylcholine, and even, in some instances, ROS (Brinkmann et al., 2004; Cronstein, Montesinos & Weissmann, 1999; Gagliani et al., 2015; Perretti et al., 2002; Shinohara & Serhan, 2016; Wallace, Ianaro, Flannigan & Cirino, 2015).

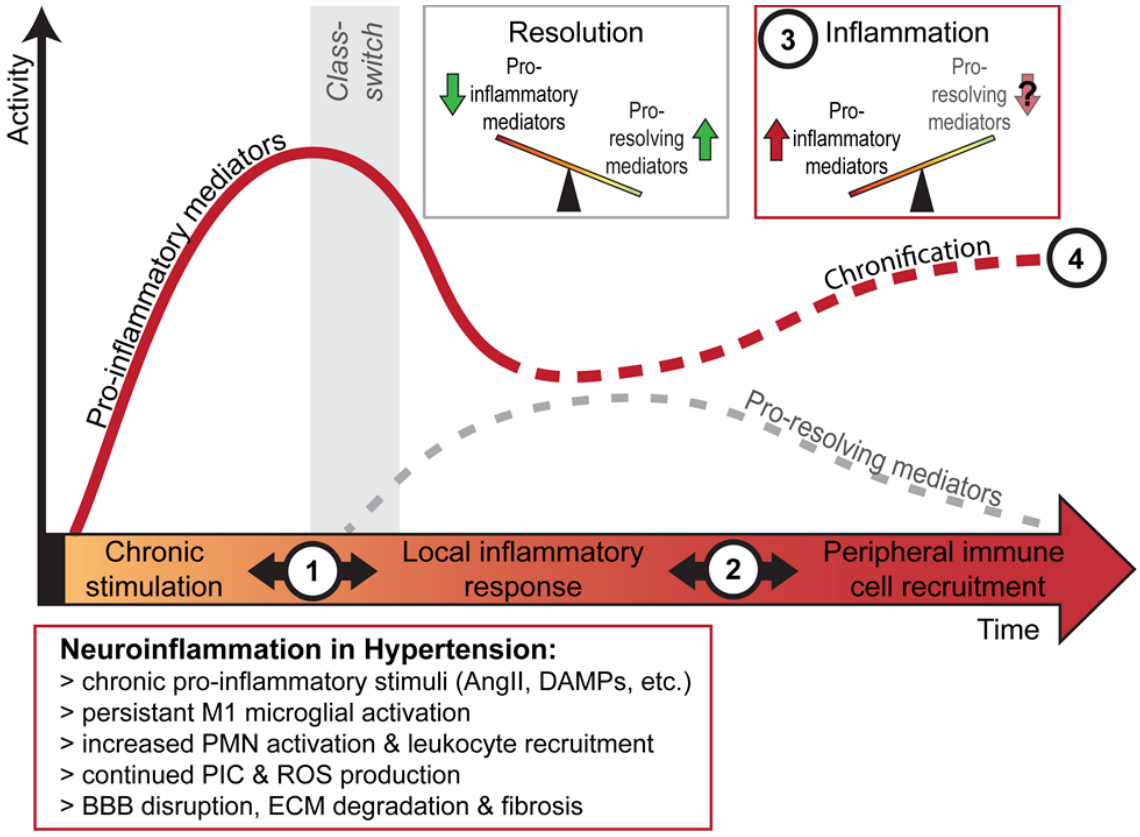

Figure 2-2. A potential schematic of the chronic low-grade neuroinflammatory response in hypertension as a product of impaired resolution. Whereas successful resolution of the acute inflammatory response involves cessation of the initial trigger, elevations in AngII provide a chronic pro-inflammatory stimulus (**1**), driving the local/innate immune response and continual activation and recruitment of immune cells (**2**). An incomplete lipid mediator class-switch and/or insufficient pro-resolution machinery (dashed grey lines) may contribute to the observed imbalance between pro-inflammatory and anti-inflammatory/pro-resolving mediators (**3**). Over time, the imbalance in inflammatory mediators leads to chronification of the inflammatory response (**4**), with failure of the M1 to M2 phenotypic switch in microglia and degeneration of tissue integrity (i.e., blood-brain barrier [BBB] breakdown, extracellular matrix [ECM] degradation, and fibrosis).

Ω-3 PUFAs and their derivatives

The essential fatty acids, linoleic acid (LA) and α-linoleic acid (ALA), are dietary precursors of ω-6 and ω-3 long-chain PUFAs, respectively. Clinical studies have long suggested that adequate consumption or supplementation with ω-3 PUFAs may exert cardioprotective benefits and mitigate elevated BP in hypertensive patients (Mozaffarian & Wu, 2011). However, the precise mechanism(s) underlying the anti-hypertensive and anti-inflammatory properties of ω-3 PUFAs have remained enigmatic. Thus, the recent discovery and characterization of SPMs derived from ω-3 PUFAs may provide a heretofore "missing link." It may be postulated that insufficient levels of pro-resolving machinery relative to the chronic pro-inflammatory stimulus provided by AngII results in failure of the resolution program and, thus, the ongoing low-grade inflammation associated with hypertension. Along this line of reasoning, a plausible explanation for the beneficial effects of ω-3 PUFAs is through their metabolism to SPMs, whereby the essential fatty acids may enhance pro-resolution signaling and dampen the inflammatory milieu.

Through a series of elongation, desaturation, and β-oxidation reactions, LA and ALA can be metabolized to the long chain PUFAs: AA from LA, and both EPA and DHA from ALA (Joffre et al., 2016; Kitajka et al., 2004). Metabolism of the free forms of PUFAs produces key mediators of the inflammatory response—eicosanoids, SPMs, and endocannabinoids (eCB) (Buckley, Gilroy, Serhan, Stockinger & Tak, 2013; Calder, 2015; Di Marzo, Stella & Zimmer, 2015; Lukiw & Bazan, 2000; Serhan, 2014; Witkamp, 2016). The CNS is highly enriched in DHA and AA, both of which are esterified to phospholipids critical for the integrity and functions of cell membranes (Bazinet & Laye,

2014). Three main enzymatic groups are responsible for PUFA turnover from the cell membrane: 1) group VI calcium-independent phospholipase, which releases esterified DHA; 2) group IV phospholipase A2 (PLA_2), which releases AA; and 3) acyl-CoA lysophospholipid transferases. The various inflammatory mediators are generated through COXs, lipoxygenases (LOXs), and cytochrome P450 (CYP450) monoxygenases. AA-derived mediators, include the 2-series PGs and TXs, the 4-series LTs and lipoxins, hydroxyeicosatetraeonic acids (HETEs), and hydroperoxyeicosatetraenoic acids (HPETEs). EPA is the precursor for the 5-series LTs and 3-series TX, and both EPA /and DHA give rise to 3-series PGs. Apart from the lipoxins, which are formed from all three long chain PUFAs, SPMs are derived from EPA and DHA: E series resolvins from EPA; D series resolvins, protectins/neuroprotectins, and maresins from DHA (**Figure 2-3**) (Serhan, 2007; Serhan, 2014).

Of note, in the presence of aspirin, acetylation of COX-2 leads to the production of AA-derived aspirin-triggered lipoxins (AT-LXs; 15-epi-lipoxins), as well as AT epimeric forms (17R) of protectins and D series resolvins from DHA (Serhan, 2005). Khan *et al.* (2019) recently reported on the dose-dependent effects of aspirin treatment in stroke-prone SHRs. While high-dose treatment (100 mg/kg/day) attenuated vascular dysfunction and cardiac hypertrophy, it also exacerbated renal fibrosis, BP elevations, and renal T-cell infiltration. Conversely, low-dose (10 mg/kg/day) aspirin treatment reduced renal fibrosis, as well as vascular dysfunction and cardiac hypertrophy (Khan et al., 2019). Based on the loss of aspirin's COX-1 inhibition specificity at high doses, these findings are in accordance with known pro-hypertensive effects of non-specific

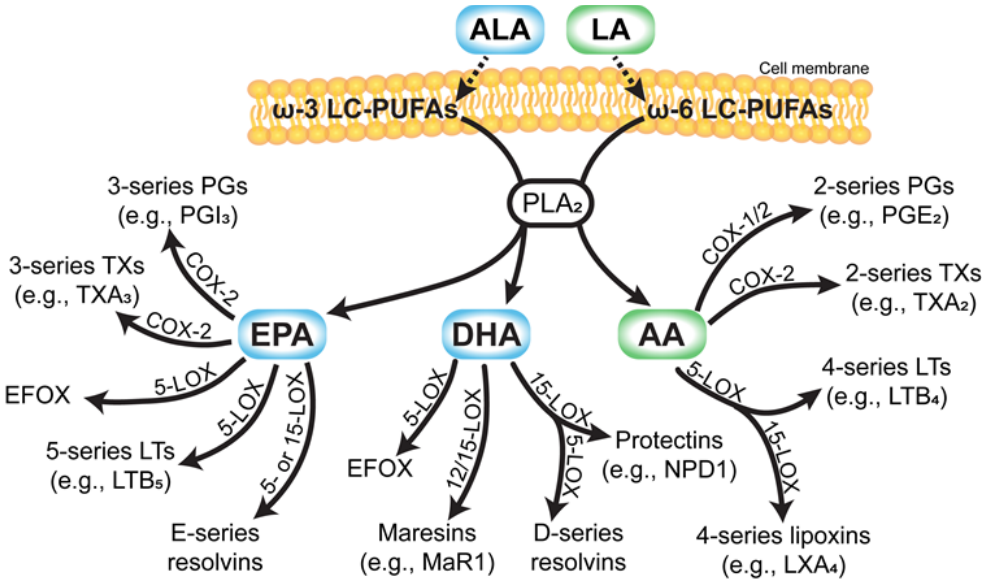

Figure 2-3. Biosynthesis of pro-inflammatory and pro-resolving lipid mediators from α-linoleic acid (ALA) and linoleic acid (LA). AA: arachidonic acid; COX: cyclooxygenase; DHA: docosahexaenoic acid; EFOX: electrophilic fatty acid oxo-derivative; EPA: eicosapentaenoic acid; LC-PUFAs: long-chain polyunsaturated fatty acids; LOX: lipoxygenase; LT: leukotriene; PG: prostaglandin; PLA_2: phospholipase A_2; TX: thromboxane.

COX and COX-2 inhibitors (i.e., NSAIDs) (Bally et al., 2017; Cheng & Harris, 2005; Rovati, Sala, Capra, Dahlen & Folco, 2010). For example, COX-1 inhibition reduces BP and increases natriuresis in AngII-infusion models, whereas COX-2 blockade has opposite effects, increasing BP and promoting renal sodium reabsorption (Qi et al., 2002). Blockade of COX-2 disrupts the TXA_2/PGI_2 (prostacyclin) balance and inhibits production of PGE_2 and PGD_2, ultimately suppressing the class-switch towards resolution (Gilroy, Colville-Nash, Willis, Chivers, Paul-Clark & Willoughby, 1999; Levy, Clish, Schmidt, Gronert & Serhan, 2001). Conversely, the benefits of selective COX-1 inhibition are likely attributable, in part, to subsequent COX-2 acetylation and generation of AT epimeric SPMs. In addition, both lipoxins and AT-LXs inhibit production of IL-6, IL-

8, and TNF-α, among other PICs, and inactivate NF-κB and AP1 (Corminboeuf & Leroy, 2015). Given the importance of AngII-mediated COX-1 stimulation, PIC production, and NF-κB activation in the maintenance of hypertension, it follows that anti-hypertensive effects of low-dose aspirin may be attributable to AT SPM epimer metabolism.

Evidence for an impaired resolution process in hypertension

Dietary ω-3 PUFA supplementation reduces BP in multiple models of hypertension, including SHRs, Dahl salt-sensitive rats, DOCA-salt hypertensive rats, and slow pressor AngII-infusion rats (Bayorh et al., 1996; Bellenger-Germain, Poisson & Narce, 2002; Bond et al., 1989; Huang, Jin & Yu, 2017; Hui, St-Louis & Falardeau, 1989; Mano et al., 1995; Mozaffarian, 2007; Rousseau-Ralliard, Moreau, Guilland, Raederstorff & Grynberg, 2009; Schoene & Fiore, 1981; van den Elsen et al., 2014; Yin, Chu & Beilin, 1990; Yin, Chu & Beilin, 1991). Similarly, dietary ω-3 PUFA deficiency increases BP in (originally) normotensive rats and exacerbates hypertension in (mRen-2)27 transgenic rats (Ren-2), an AngII-dependent model know to exhibit high brain RAS activity (Armitage, Pearce, Sinclair, Vingrys, Weisinger & Weisinger, 2003; Begg et al., 2012; Callahan, Li, Ferrario, Ganten & Morris, 1996; Campbell, Rong, Kladis, Rees, Ganten & Skinner, 1995; Weisinger, Armitage, Sinclair, Vingrys, Burns & Weisinger, 2001). Additionally, when Ren-2 rats are treated with the ACE inhibitor, perindopril, systolic BP normalizes to the same extent regardless of dietary ω-3 PUFA content, suggesting a RAS-dependent mechanism of ω-3 PUFA's anti-hypertensive actions (Jayasooriya et al., 2008). It has generally been thought that the beneficial effects of ω-3 PUFAs result from their antagonistic actions vis-à-vis AA metabolite formation, suppressing COX and

NF-κB activity, and inhibiting production of CRPs, IL-1β, IL-6, and TNF-α (Adler, Schoeniger & Fuhrmann, 2018; Faber et al., 2013; Langerhuus, Tonnesen, Jensen, Damgaard, Halekoh & Lauridsen, 2012; Mocellin et al., 2013; Ramirez-Ramirez et al., 2013; Siriwardhana et al., 2012; Takase et al., 2011). Omega-3 PUFAs are demonstrated to suppress ACE activity and AngII generation, increase endothelial NO production, and activate the PSNS, leading to improved vasomotor tone and arterial compliance (Cicero, Ertek & Borghi, 2009; Kumar & Das, 1997). Conversely, ω-6 PUFAs, such as AA, generally appear to be more active in the initiation phase of the inflammatory response, as well as in elevating RAS activity. To that end, evidence suggests that ω-3 PUFA derivatives may counteract AngII through vasodilatory effects of eicosanoids and/or attenuation of AngII-induced oxidative stress (De Caterina, Liao & Libby, 2000; Juan & Sametz, 1986; Juan, Sutter & Sametz, 1987; Polizio, Gironacci, Tomaro & Pena, 2007). For example, AT-LXA_4 was shown to abolish AngII-induced NADPH oxidase activation and ROS production in endothelial cells by inhibiting phosphorylation and translocation of $p47^{phox}$ and NF-κB (Nascimento-Silva, Arruda, Barja-Fidalgo & Fierro, 2007). Furthermore, production of AA-derived 20-HETE is upregulated by, among other autocoid factors, AngII (Gehling et al., 2000; Imig, Zou, Stec, Harder, Falck & Roman, 1996). Recently, 20-HETE was demonstrated to act as a transcriptional regulator of ACE expression in ECs (Cheng et al., 2012; Cvetkovic & Sigmund, 2000). In SHRs, the elevations in vascular 20-HETE are RAS-dependent and contribute to the progression of vascular dysfunction and hypertension (Dunn, Renic, Flasch, Harder, Falck & Roman, 2008; Sacerdoti, Escalante, Abraham, McGiff, Levere & Schwartzman, 1989). Additionally, neither AT1R blockade (losartan) nor vascular

endothelial ACE deletion prevent vascular dysfunction or remodeling in 20-HETE-dependent hypertension (Garcia et al., 2015).

Promoting the resolution of neuroinflammation through glial cells

Within the CNS, ω-3 PUFAs and their derivatives have gained attention in mediating neuroinflammation, particularly in regard to glial cell activation status. Identification of the LXA_4 receptor in astrocytes and microglia has raised the possibility of employing lipoxin analogs, specifically of LXA_4, as novel therapeutics for neuroinflammatory diseases. In 1321N1 human astrocytoma cells exposed to IL-1β, LXA_4 inhibits increases in IL-8 and ICAM-1 (intercellular adhesion molecule 1) expression, IκBα degradation, and activation of an NF-κB reporter gene, suggesting that the anti-inflammatory effects of LXA_4 are mediated, at least in part, through inhibition of NF-κB activation (Decker, McBean & Godson, 2009). Omega-3 PUFA microglial-modulation is associated with a transition from an M1 to an M2 phenotype, as indicated by lower cluster of differentiation (CD) 40 and CD86, and increased CD206 expression (Chen et al., 2014; Chhor et al., 2013; Ebert et al., 2009; Hjorth et al., 2013). Moon *et al.* (2007) were the first to demonstrate a modulatory role for ω-3 PUFAs in microglial activation status using BV2 cells treated with EPA (60 min. incubation) prior to LPS stimulation. This study showed that EPA inhibits iNOS and COX-2 expression in a dose-dependent manner, thereby suppressing NO and PGE_2 generation, and reducing IL-1β, IL-6, and TNF-α production (Moon et al., 2007). Subsequent studies, both *in vivo* and *in vitro*, have demonstrated an association between high levels of brain DHA and reduced PIC, ROS, and NO generation by microglia (for review, see Antonietta Ajmone-Cat et al.,

2012; Chen et al., 2014; Corsi, Dongmo & Avallone, 2015; Lu, Tsao, Leung & Su, 2010; Pettit, Varsanyi, Tadros & Vassiliou, 2013; Trepanier, Hopperton, Orr & Bazinet, 2016; Zendedel et al., 2015).

The evidence of ω-3 PUFA-mediated TLR4 inhibition in LPS-stimulated microglia suggests an intriguing avenue by which pro-resolving mediators may protect against the progression of hypertension. Liuzzi *et al.* (2007) reported a dose-dependent attenuation of LPS-induced matrix metalloproteinase (MMP)-9 expression in primary cultures of rat microglia by simultaneous incubation with a mixture of EPA and DHA (Liuzzi, Latronico, Rossano, Viggiani, Fasano & Riccio, 2007). MMP-9 is an endopeptidase that contributes to ECM degradation and is upregulated downstream of multiple pathways implicated in hypertension. Importantly, MMP-9 production is increased by TLR4 stimulation and is involved in BBB disruption. To that end, exogenously applied DHA impairs LPS-induced recruitment of CD14 and TLR4 to the cell membrane and, through this mechanism, reduces production of IL-1β and TNF-α by microglia (De Smedt-Peyrusse, Sargueil, Moranis, Harizi, Mongrand & Laye, 2008). Moreover, DHA is shown to affect the activation of several transcription factors that are generally regarded as proinflammatory, such as NF-κB, MAPK p38, and c-Jun N-terminal kinases in microglia (Chang, Khatchadourian, McKinney & Maysinger, 2015; De Smedt-Peyrusse, Sargueil, Moranis, Harizi, Mongrand & Laye, 2008; Ma et al., 2009). Wu *et al.* (2012) reported reduced BBB disruption and MMP-9 expression in a middle cerebral artery occlusion (MCAO) model following *ICV* injection of an LXA_4 analog (LXA_4-ME) (Wu et al., 2012). Similarly, *i.p.* IL-6 post-ischemia mitigated BBB leakage and decreased brain IL-1β and TNF-α in MCAO rats (Feng, Wang & Yang, 2015). While neither study examined

microglial alterations, the dampened CNS inflammatory status suggests that both LXA_4-ME and IL-6 attenuated microglial activation. This would be in line with a recent report from Hawkins *et al.* (2017) showing decreased microgliosis in MCAO rats treated with the LXA_4 analog, BML-111 (*i.v.*), for one week after occlusion (Hawkins et al., 2017). In addition, BML-111 treatment reduced TNF-α, interferon (IFN)-γ, MIP-1α (macrophage inflammatory protein-1α), and MCP-1 (monocyte/macrophage chemoattractant protein-1) production, enhanced IL-10 and IL-4 levels, and decreased the proportion of $CD40^+$ macrophages at the infarct site (Hawkins et al., 2017). Given our preliminary work showing that TLR4 is necessary for M1 microglial activation and BBB disruption in key cardioregulatory nuclei of SHRs in neurogenic hypertension, it is plausible that treatment with ω-3 PUFA-derivatives or LXA_4 analogs would promote resolution of CNS inflammation in hypertension via TLR4 inhibition (Mowry, Silva-Cutini, Peaden & Biancardi, 2019).

There is less evidence available, however, for the role played by non-lipoxin SPMs in the CNS. Resolvin D1 (RvD1) is synthesized from DHA through 5- and 15-LOX, and acts through GPR32 and LXA_4/formyl peptide receptor 2 (ALX/FPR2) (Krishnamoorthy et al., 2010). EPA-derived RvE1 is formed via CYTP450 and 5-LOX, and binds to chemoattractant receptor 23 (ChemR23) (Arita et al., 2005; Samson et al., 1998; Serhan, Clish, Brannon, Colgan, Chiang & Gronert, 2000). RvE1 blocks LPS-induced microgliosis and TNF-α production in primary microglial cell culture (Xu, Berta & Ji, 2013). In a midline fluid percussion model of traumatic brain injury, RvE1 was demonstrated to reduce the pro-inflammatory activation profile of microglia (Harrison et al., 2015). While both RvD1 and RvE1 pre-treatment decreases LPS-induced PIC gene

expression in BV2 microglia cell culture, RvD1 was shown to impact regulation of miRNA expression, whereas RvE1 mediates NF-κB signaling (Rey et al., 2016). Beyond the resolvins, neuroprotection D1, a DHA-derived SPM, reduces brain leukocyte infiltration, COX-2 activity, PIC production, and microglial activation (Hong, Gronert, Devchand, Moussignac & Serhan, 2003; Lukiw et al., 2005; Marcheselli et al., 2003; Marcheselli et al., 2010; Orr et al., 2013; Orr, Trepanier & Bazinet, 2013).

Endocannabinoids in hypertension

Endocannabinoids (eCBs) comprise an additional class of ω-3 PUFA derivatives, and a clear role for the eCB system in BP regulation has been well evidenced. In brief, eCBs include anandamide (AEA), 2-arachidonoylglycerol (2-AG), eicosapentaenoyl ethanolamide (EPEA), and docosahexaenoyl ethanolamide (DHEA/synaptamide). Within the brain, DHEA inhibits LPS-induced inflammation by stimulating cAMP/PKA activity and suppressing that of NF-κB (Park, Chen, Kevala, Lee & Kim, 2016). In macrophages, DHEA lowers production of MCP-1, NO, and eicosanoids (Meijerink et al., 2011; Meijerink et al., 2015). Furthermore, oxidation of DHEA yields anti-inflammatory derivatives (Kuda, 2017; Shinohara, Mirakaj & Serhan, 2012; Yang et al., 2011b). eCBs bind to the cannabinoid receptors, CB1 and CB2, which are expressed by neurons and glia (Piomelli & Sasso, 2014; Stella, 2009). Importantly, elevations in SNS activity have been linked with CB1 receptor-mediated inhibition of GABAergic signal transmission in the brainstem (Ibrahim & Abdel-Rahman, 2011). Release of GABA following exogenous application of the CB1 receptor agonist, anandamide, within the NTS dampens the activity of sympathetic neurons in the RVLM (Brozoski, Dean, Hopp

& Seagard, 2005; Seagard et al., 2004). Moreover, there is increasing evidence for interactions between RAS and the eCB system. In the RAS-dependent Ren-2 model of hypertension, chronic systemic CB1 receptor blockade reduces BP and improves baroreflex sensitivity (Schaich, Shaltout, Brosnihan, Howlett & Diz, 2014). Additionally, the impaired baroreflex sensitivity observed following NTS AngII injection in Sprague-Dawley rats is attenuated by prior NTS-specific CB1 receptor blockade. Wang *et al.* (2017) have recently shown that RVLM expression of CB1 is significantly increased in SHRs compared to WKYs, in line with previous reports that CB1 receptor expression is elevated in SHR cardiac and aortic tissue, and that the pressor response to CB1 receptor agonists is magnified in SHRs (Wang, Li, Zhang, Zhang & Li, 2017). Interestingly, the group found no alterations in RVLM AEA or 2-AG content in hypertensive rats.

Dysregulation of the eCB system is evident in chronic inflammatory diseases, ranging from cancer and atherosclerosis to metabolic and neuroinflammatory diseases (Maccarrone, 2008; Mazier, Saucisse, Gatta-Cherifi & Cota, 2015; Steffens & Pacher, 2015; Velasco, Sanchez & Guzman, 2015). Based on the ability of AEA and 2-AG to enhance resolution of the neuroinflammatory state, as well as the role of 2-AG in promoting efferocytosis, eCBs have gained attention as pro-resolving mediators (Impellizzeri et al., 2017; Impellizzeri et al., 2019; Mecha, Carrillo-Salinas, Feliu, Mestre & Guaza, 2016; Mecha et al., 2015; Skaper et al., 2015). From this perspective, various components of the eCB system are shown to attenuate ROS and PIC production, tissue leukocyte infiltration, and immune cell activation (Aizpurua-Olaizola, Elezgarai, Rico-Barrio, Zarandona, Etxebarria & Usobiaga, 2017; Cabral, Ferreira & Jamerson, 2015;

Chiurchiu et al., 2013; Chiurchiu, Lanuti, Catanzaro, Fezza, Rapino & Maccarrone, 2014; Lipina & Hundal, 2016). Given the abundant evidence for the eCB system in modulating BP and pro-resolution pathways, paired with the consistency of eCB-dysregulation across chronic inflammatory diseases, the therapeutic potential of targeting eCBs in hypertension certainly merits further exploration, particularly in regard to subduing RAS-driven neuroinflammation.

ACE2/Ang-(1-7)/MasR as a pro-resolving mediator

Since the first report of Ang-(1-7) in 1988 by Dr. Robson Santos and colleagues, the ACE2/Ang-(1-7)/MasR axis has been demonstrated to counterbalance the actions of AngII-AT1R by suppressing fibrosis, hypertrophy, inflammation, and cell proliferation, as well as by promoting vasodilation (McCollum, Gallagher & Ann Tallant, 2012; Santos, 2014a; Santos et al., 1988). Of note, another angiotensin peptide, alamandine [Ala^1-ANG-(1-7)], which can be metabolized from Ang-(1-7), appears to exert similar effects as Ang-(1-7) through Mas-related G protein-coupled receptor, member D (MrgD) (Lautner et al., 2013). However, in contrast to the peripheral antagonistic effects of Ang-(1-7) in relation to AngII, the role of the ACE2/Ang-(1-7)/MasR axis in CNS remains unclear (de Morais, Shanks & Zucker, 2018; Santos et al., 2018). Downregulation of central ACE2 has been reported in several models of hypertension, and neuron-specific ACE2 overexpression attenuates high BP in SHRs, DOCA-salt, and AngII-induced hypertension models (Deshotels, Xia, Sriramula, Lazartigues & Filipeanu, 2014; Feng et al., 2008; Xia et al., 2015; Xia, Feng, Obr, Hickman & Lazartigues, 2009; Yamazato, Yamazato, Sun, Diez-Freire & Raizada, 2007). Conversely, Ang-(1-7) microinjection

within the RVLM elevates MAP and renal sympathetic nerve activity (RSNA), and blockade of endogenous PVN Ang-(1-7) reduces MAP, RSNA, and NE in salt-induced hypertension (Yu et al., 2018; Zhou et al., 2010).

Nonetheless, a new story has arisen regarding Ang-(1-7) as an inflammatory mediator in several pathologies. Mas-deficient mice display exaggerated and prolonged inflammatory responses (da Silveira et al., 2010; Magalhaes et al., 2016). Treatment with Ang-(1-7) suppresses markers of inflammation in experimental colitis, lung fibrosis, ovalbumin-induced chronic asthma, atherosclerosis, acute lung injury, arthritis, and in a high-fat diet model of hepatic steatosis (da Silveira et al., 2010; Feltenberger et al., 2013; Khajah, Fateel, Ananthalakshmi & Luqmani, 2016; Li et al., 2016c; Li et al., 2016e; Magalhaes et al., 2018; Magalhaes et al., 2015; Meng et al., 2015; Rodrigues-Machado et al., 2013; Shenoy et al., 2010). The neuroprotective effects of Ang-(1-7) infusion were shown to occur through inhibition of NF-κB in a rat model of ischemic stroke (Jiang, Gao, Guo, Lu, Wang & Zhang, 2012). NF-κB activation is a primary survival pathway in leukocytes, and inhibition of NF-κB is associated with the resolution of inflammation through leukocyte apoptosis (Fujihara, Jaffray, Farrow, Rossi, Haslett & Hay, 2005; Sousa et al., 2009; Sousa et al., 2010). To this point, Barroso *et al.* (2017) demonstrated the ability of Ang-(1-7) to suppress NF-κB activity in neutrophils, induce leukocyte apoptosis, and promote neutrophil efferocytosis by macrophages in an antigen-induced murine model of arthritis (Barroso et al., 2017). Magalhaus *et al.* (2018) found similar effects in eosinophils from a mouse model of asthma, where Ang-(1-7) was further shown to decrease pulmonary ECM deposition (Magalhaes et al., 2018).

Senescence-accelerated mouse prone 8 (SAMP8) mice, a model of accelerated aging, have elevated brain levels of PICs (e.g. IL-1β, IL-6, and TNF-α) and increased M1 microglial activation (Grinan-Ferre et al., 2016; Jiang et al., 2018; Jiang et al., 2014). Interestingly, SAMP8 mice are reported to have reduced CNS Ang-(1-7) without alterations in either ACE2 or MasR expression, and treatment with the non-peptide Ang-(1-7) analogue, AVE0991 (*i.p.*), attenuates neural PIC production (Jiang et al., 2018; Jiang et al., 2016). AVE0991 pre-treatment of primary microglia cell culture from SAMP8 mice dampens LPS-induced increases in *Il1b*, *Il6*, and *Tnf* mRNA, while simultaneously enhancing *Arg1*, *Il10*, and *Retnla* mRNA, which are considered markers of the M2 activation state (Jiang et al., 2018). Based on the observation that suppression of LPS-stimulated PIC production by AVE0991 is abolished in the presence of A-779, a MasR agonist, AVE0991 appears to counter the pro-inflammatory microglial response by enhancing MasR activity (Jiang et al., 2018). Coupled with the upregulation in markers of M2 activation with AVE0991 pre-treatment, the findings of Jiang *et al.* (2018) suggest that, rather than inhibiting microglial activation altogether, the Ang-(1-7) analogue promotes a switch in these innate immune cells from pro-inflammatory to anti-inflammatory and pro-resolving (Jiang et al., 2018). Compellingly, there appears to be a parallel between the RAS and the inflammatory response wherein the AngII-AT1R axis takes up the role of stimulus and pro-inflammatory mediator, while the Ang-(1-7)/MasR axis exhibits the principle characteristics of a pro-resolution signaling pathway.

Concluding remarks

Neuroinflammation is generally accepted as holding a central role in the pathophysiology of essential hypertension, and intensive research efforts are focused on elucidating the molecular mechanisms underpinning this chronic inflammatory response. Recent advances within the field of immunology have brought pro-resolution pathways to the forefront of efforts to combat chronic inflammatory diseases. However, at a base level, it must be recognized that research into disease-specific resolution impairment is still very much in its infancy. Nonetheless, several aspects of the resolution process have been extensively studied outside of the context of pro-resolving pathways. In this sense, the various AngII-driven pathological processes discussed—BBB disruption, microglial activation, oxidative stress, and upregulation of pro-inflammatory mediators — mirror impairments in the resolution process (**Figure 2-4**). It is tempting to think that this combination of failed tissue repair, enhanced immune cell activation, and persistent pro-inflammatory stimulation in hypertension, which manifests as chronic low-grade inflammation, may be largely dependent upon insufficient pro-resolution activities.

In discussing the potential of targeting resolution pathways, it is important to delineate between the anti-inflammatory and pro-resolving classifications. Anti-inflammatory therapeutic strategies act by inhibiting the initial inflammatory response. This is achieved through blockade of pro-inflammatory mediator production, action and/or tissue infiltration by PMNs (e.g. NSAIDs, anti-TNF-α drugs). Conversely, pro-resolution therapeutics are designed to target resolution pathways, thereby promoting the restoration of tissue homeostasis (Perretti, Leroy, Bland & Montero-Melendez,

2015). In addition, it is pertinent to consider that the majority of research to-date regarding the resolution process has examined these pathways in periphery. Whereas the acute peripheral inflammatory response has been well characterized, particularly as occurs during bacterial infection, much less is known regarding this process within the CNS or within the context of a chronic sterile response.

Given the wealth of information regarding neuroinflammation as a primary pro-hypertensive pathophysiological mechanism, there must exist some impairment in the resolution of said inflammation. Yet, at least within the context of neurogenic hypertension, research specifically examining pro-resolution mediators is extremely scarce. Thus, the precise nature of resolution impairment in hypertension, and whether it is due to a dysfunctional resolution process or is a consequence of insufficient pro-resolution signaling, is unknown. Additionally, one must inquire as to cause and effect, i.e., is impaired resolution an underlying cause leading to the development and/or maintenance of neurogenic hypertension or does resolution fail due to the presence of chronic pro-inflammatory stimuli? These questions, among others, remain to be answered.

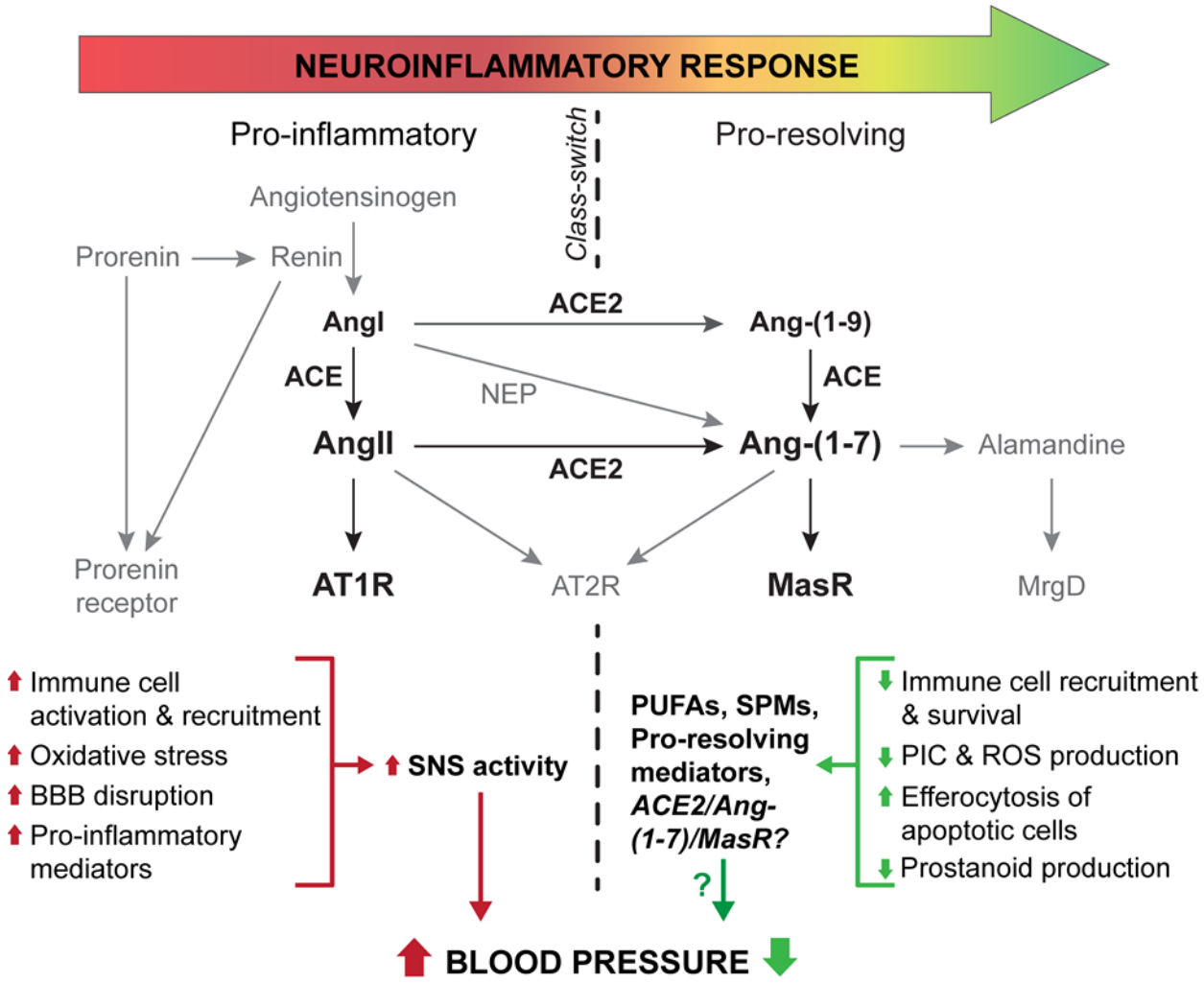

Figure 2-4. Schematic representation of the pro-inflammatory and pro-hypertensive processes driven by the ACE/AngII/AT1R axis as compared to the potential pro-resolution role of the ACE2/Ang-(1-7)/MasR axis. ACE: angiotensin converting enzyme; Ang: angiotensin; AT1R: AngII type 1 receptor; AT2R: AngII type 2 receptor; BBB: blood-brain barrier; MasR: G protein-coupled receptor Mas; MrgD: Mas-related G protein-coupled receptor, member D; NEP: neutral endopeptidase; PIC: pro-inflammatory cytokine; PUFAs: polyunsaturated fatty acids; ROS: reactive oxygen species; SNS: sympathetic nervous system; SPMs: specialized pro-resolving mediators.

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Chapter 3. Targeting TLR4 signaling pathways: can therapeutics pay the toll for hypertension?

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Abstract

The immune system plays a prominent role in the initiation and maintenance of hypertension. The innate immune system, via Toll-like receptors (TLRs), identifies distinct signatures of invading microbes and damage-associated molecular patterns, and triggers a chain of downstream signal cascades, leading to secretion of pro-inflammatory cytokines and shaping the adaptive immune response. Over the past decade, a dysfunctional TLR-mediated response, particularly via TLR4, has been suggested to support a chronic inflammatory state in hypertension, inducing deleterious local and systemic effects in host cells and tissues, and contributing to disease progression. While the underlying mechanisms triggering TLR4 need further research, evidence suggests that sustained elevations in BP disrupt homeostasis, releasing endogenous TLR4 ligands in hypertension. In this review, we discuss the emerging role of TLR4 in the pathogenesis of hypertension and whether targeting this receptor and its signaling pathways could offer a therapeutic strategy for management of this multifaceted disease.

Introduction

Hypertension, one of the most prevalent cardiovascular risk factors, can exhibit numerous pathophysiologies and is typically attributed to dysregulation of the cardiovascular, renal, and central nervous systems (CNS) (Dominiczak & Kuo, 2018). Such dysregulation has been known to be associated with abnormal immune system activity for more than a half-century. A proper immune response requires precise coordination of the innate and adaptive elements of immunity. The contributions of aberrant activation of adaptive immunity to high BP have been well documented in various models of hypertension (Harrison, Vinh, Lob & Madhur, 2010; Idris-Khodja, Mian, Paradis & Schiffrin, 2014; Lopez Gelston & Mitchell, 2017; Norlander, Madhur & Harrison, 2018). However, the molecular mechanisms that activate the innate immune system and lead to priming/activation of the adaptive immune response in hypertension are still not fully understood.

Recent work reveals a core and pathogenic role for innate immune system activity in hypertension (Bomfim, Rodrigues & Carneiro, 2017; Lopez Gelston & Mitchell, 2017; Norlander, Madhur & Harrison, 2018). Evidence suggests that the receptors of innate immune cells act as gateways in hypertension, promoting end-organ damage through the propagation of chronic inflammation, oxidative stress, and vascular remodeling (Abais-Battad, Dasinger, Fehrenbach & Mattson, 2017; Bomfim, Rodrigues & Carneiro, 2017; Nosalski, McGinnigle, Siedlinski & Guzik, 2017). The initial response to potential pathogens and tissue damage is determined by the innate immune cells through pattern-recognition receptors (PRRs), which include the Toll-like receptors (TLRs) (Kawai & Akira, 2010b). In the absence of stimulation, TLRs are primarily expressed at either the cell

membrane (TLR1, TLR2, TLR4 – TLR6, TLR9 – TLR12) or within endosomes (TLR3, TLR7, TLR8, and TLR9) (McGettrick & O'Neill, 2010). TLR activation occurs in response to both exogenous pathogen-associated molecular patterns and endogenous molecules released by cells following tissue damage, termed damage-associated molecular patterns (DAMPs) (Kawai & Akira, 2010b; Matzinger, 2002). DAMP-stimulated TLR activation has gained much attention as a central component of hypertension pathogenesis (McCarthy, Goulopoulou, Wenceslau, Spitler, Matsumoto & Webb, 2014). In particular, persistent activation of TLR4, resulting in low-grade chronic inflammation, has been linked to significant kidney, cardiovascular, and CNS tissue damage within the context of hypertension (**Figure 3-1**) (Biancardi, Bomfim, Reis, Al-Gassimi & Nunes, 2017a; McCarthy, Goulopoulou, Wenceslau, Spitler, Matsumoto & Webb, 2014). Thus, the TLR4 signaling pathway may allow pharmaceutical targeting of the innate immune system through direct TLR4 modulation and/or signal transduction inhibitors. In this review, we will focus on recent findings regarding the TLR4 signaling pathways, discussing the putative role for their various components in hypertension as well as advancements in potential-target discovery within TLR4-mediated hypertension.

DAMP-mediated TLR4 activation in hypertension

When homeostatic disturbances inflict tissue insult, whether via cellular stress or direct damage, DAMPs are released from the site of insult and act as PRR ligands. Among the molecules classified as DAMPs are cell-derived nucleic acids, fatty acids, heat shock proteins (HSPs), and high-mobility group box-1 (HMGB1), as well as components of the extracellular matrix (ECM), such as proteoglycans, hyaluronic acid, and fibronectin

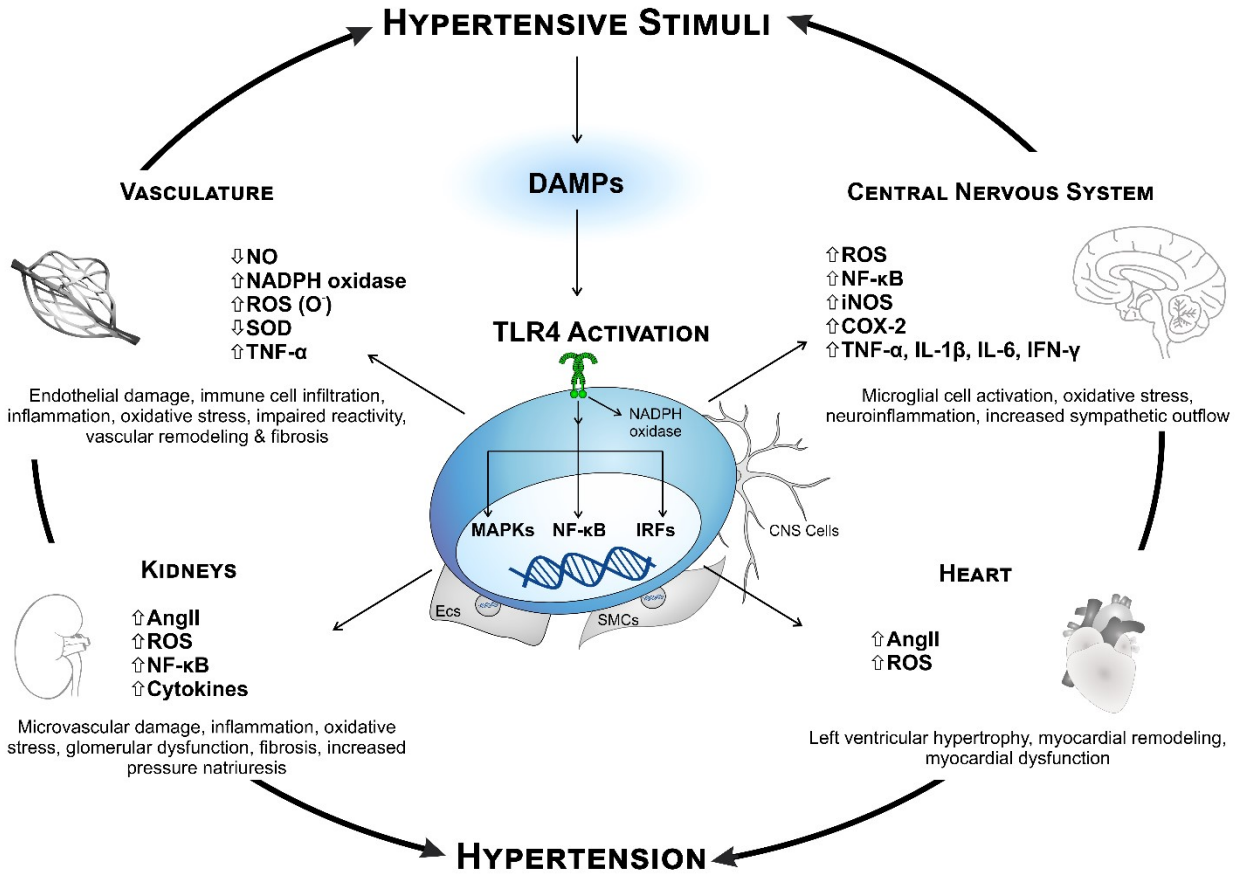

Figure 3-1: Summary of TLR4 overactivation on the vasculature, central nervous system, kidneys, and heart. Upregulation of TLR4 in the depicted systems increases pro-inflammatory mediator levels, leading to deleterious effect in hypertension. ECs: endothelial cells; SMCs: smooth muscle cells.

(Kawai & Akira, 2011). The activation of TLRs by DAMPs plays an inherently protective role, alerting cells to damage for its resolution and repair. However, excessive or prolonged DAMP-mediated stimulation of these innate immune system receptors provokes a chronic inflammatory state that contributes to the maintenance of hypertension (McCarthy, Goulopoulou, Wenceslau, Spitler, Matsumoto & Webb, 2014). Through intracellular adaptor protein-dependent signal cascades, DAMP-induced TLR activation increases the expression of pro-inflammatory genes (Akira & Takeda, 2004a). TLR4 is unique in this sense, being the only TLR known to recruit four adaptor molecules

and signal through two distinct pathways to produce pro-inflammatory cytokines and chemokines (Kawai & Akira, 2010b). Of particular interest, a variety of cell types that have long-been associated with cardiovascular diseases have been found to express TLR4, such as macrophages, renal epithelial cells, cardiomyocytes, vascular smooth muscle cells (VSMCs), endothelial cells (ECs), glial cells, and neurons (Vaure & Liu, 2014). Indeed, a myriad of studies show that abnormal activation of TLR4, primarily by DAMPs, contributes to cardiovascular dysfunction and remodeling, kidney disease, and CNS dysregulation. These studies form the basis of the suggestion that DAMP-induced TLR4 stimulation may be the missing link between inflammation and hypertension.

In this review, we will examine a variety of organs and systems known to be impacted by DAMP-mediated TLR4 activation during hypertension. Of note, it is not the purpose of this review to provide an in-depth discussion of TLR4 endogenous ligands in hypertension, but rather to concisely list those ligands specifically related to induction of TLR4's downstream pathways. **Table 3-1** contains a summary of hypertension-related DAMPs that are proposed to modulate TLR4.

TLR4 signal transduction in hypertension

TLR4 contains an extracellular domain of leucine-rich repeats and an intracellular Toll-interleukin 1 (TIR) domain that is responsible for signal transmission. Once activated, TLR4 signal transduction occurs through both myeloid differentiation primary response 88 (MyD88)-dependent and MyD88-independent (TRIF-dependent) pathways (Akira & Takeda, 2004a). Together, the two arms of TLR4's signaling cascade induce the production and release of pro-inflammatory cytokines, chemokines, and costimulatory

Table 3-1. Damage-associated molecular patterns (DAMPs) that can activate Toll-like receptor 4 (TLR4) in hypertension.

DAMP	Cell type/Tissue and References
AngII	Arteries (Bomfim et al., 2012; De Batista et al., 2014; Hernanz et al., 2015), VSMCs (De Batista et al., 2014), PVN (Dange et al., 2014a; Dange, Agarwal, Teruyama & Francis, 2015a; Li et al., 2016a), corpus cavernosum (Nunes, Bomfim, Toque, Szasz & Webb, 2017), mesangial cells (Wolf, Bohlender, Bondeva, Roger, Thaiss & Wenzel, 2006), tubular epithelial cells (Nair, Ebenezer, Saini & Francis, 2015), cardiomyocytes (Eißler et al., 2011)
ADMA	Adipocytes (Yang et al., 2009b)
C-reactive protein	VSMCs (Liu, Liu, Ji & Lu, 2010; Liu, Liu, Ji, Lu, Wang & Guo, 2011)
Fibrinogen	Cardiomyocytes (Li et al., 2009), monocytes (Smiley, King & Hancock, 2001)
Fibronectin-EDA	Aorta (Doddapattar et al., 2015)
HMGB1	ECs (Szasz, Ogbi & Webb, 2016), macrophages (Park et al., 2004)
HSPs	VSMCs (Zhao et al., 2015), cardiomyocytes (Kim et al., 2009)
Hyaluronan	Endothelial cells (Taylor, Trowbridge, Rudisill, Termeer, Simon & Gallo, 2004)
Oxidized LDL	Macrophages (Miller, Chang, Binder, Shaw & Witztum, 2003)
Uric acid	Macrophages (Liu-Bryan, Scott, Sydlaske, Rose & Terkeltaub, 2005)

AngII: Angiotensin II; ADMA: Asymmetric dimethylarginine; ECs: Endothelial cells; HMGB1: High mobility group box 1; HSPs: Heat-shock proteins; VSMCs: Vascular smooth muscle cells; PVN: Paraventricular nucleus of the hypothalamus.

factors (pro-inflammatory profile). For an overview of TLR4 signaling in hypertension, please refer to **Figure 3-2**.

Following activation, TLR4 recruits TIR domain-containing adaptor/MyD88 adaptor-like protein (TIRAP/MAL), which connects MyD88 to the TIR domain and initiates the MyD88-dependent pathway (Yamamoto et al., 2002). MyD88 then recruits and activates IL-1 receptor-associated kinase (IRAK4) 4, forming the myddosome (Suzuki et al., 2002). IRAK4 participates in the recruitment, phosphorylation, and degradation of IRAK1 and

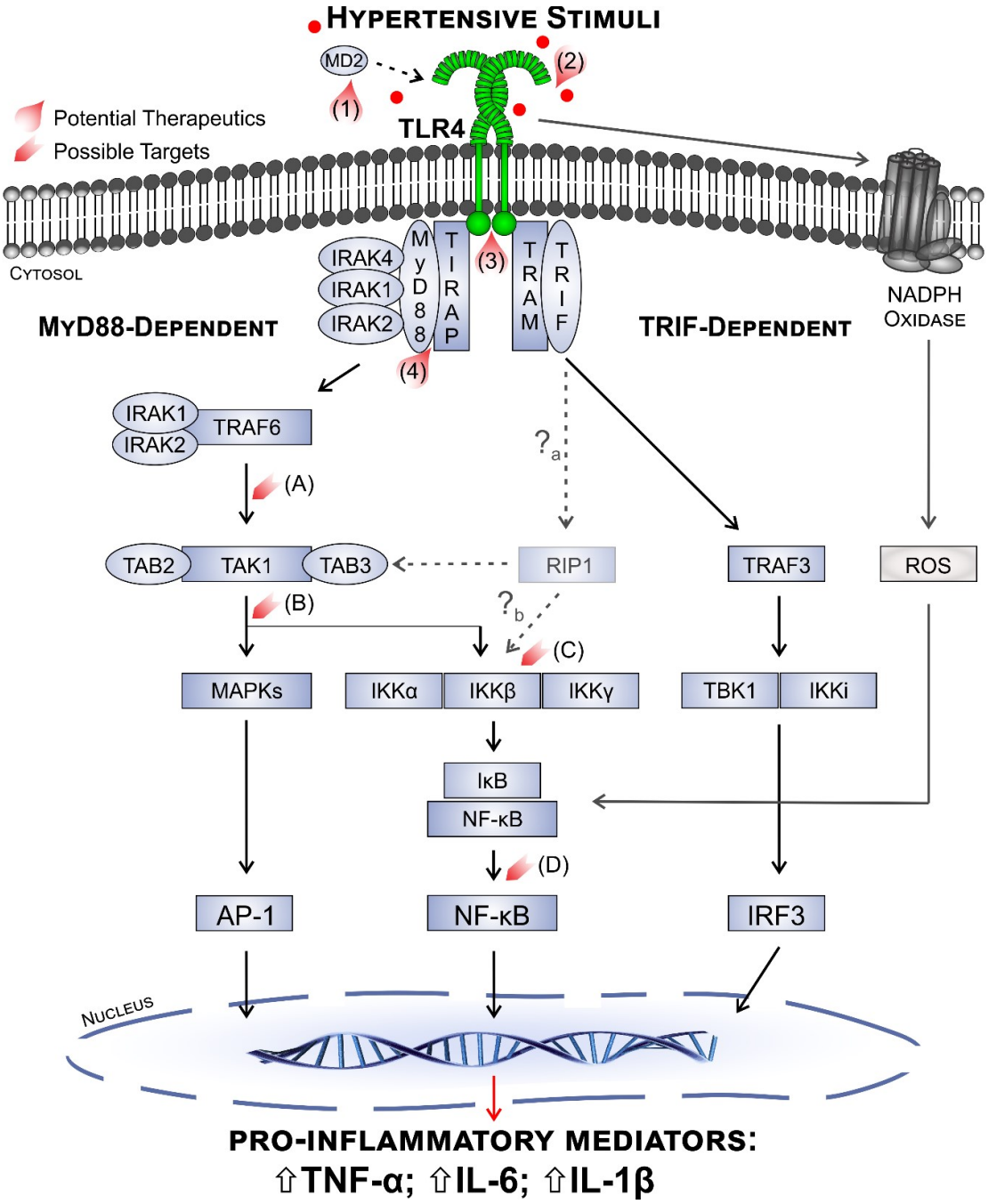

Figure 3-2: Overview of TLR4 signaling in hypertension and potential therapeutic targets. Activation of TLR4 by hypertensive stimuli, such as DAMPs, may involve a primary interaction with MD-2. Stimulated TLR4 initiates the early MyD88-dependent phase and late TRIF-dependent phase cascades. In the MyD88-dependent pathway, TIRAP associates with the receptor TIR domain, facilitating MyD88 association. MyD88 then recruits and activates IRAKs, leading to sequential stimulation of TRAF6 and TAK1. The downstream MAPK and IKK pathways ultimately result in the nuclear translocation of AP-1 and NF-κB transcription factors and production of pro-inflammatory mediators. In the delayed TRIF-dependent phase, recruitment of TRAM to

the TIR domain allows TRIF binding, which upregulates IRF3 expression through TRAF3. TRAM recruitment also leads to NF-κB activation through RIP1. Though NF-κB expression is shown to be increased in animal models of hypertension, it has not yet been determined whether RIP1 activation is involved in hypertension (a), nor is the exact mechanism of RIP1-mediated NF-κB activation fully understood (b). TLR4 stimulation also activates NADPH-oxidase, increasing ROS production and thus ROS-induced NF-κB translocation. As a result of these signaling pathways, TLR4 stimulation causes the upregulation of pro-inflammatory mediators, such as TNFα, IL-6, and IL-1β. Several molecules have been developed that inhibit TLR4 signaling and may be beneficial in hypertension management. Eritoran, tested in sepsis patients, binds to the pocket of the MD-2 adaptor protein (1). NI-0101 inhibits both exogenous and endogenous binding and is currently in a phase III clinical trial for rheumatoid arthritis (2). TAK-242 binds to the TIR domain of TLR4, and has been tested in clinical trial for sepsis (3). ST2825 inhibits the dimerization of MyD88, thus halting the MyD88-dependent pathway (4). Other drugs (not shown here) have been developed that inhibit TLR4 binding of AngII, while several decrease TLR4 signaling through unknown mechanisms. Suggested therapeutic targets within the TLR4 signaling cascades are highlighted above (A-D) and discussed throughout the review.

IRAK2, after which these IRAKs dissociate from MyD88 to bind tumor necrosis factor receptor-associated factor (TRAF) 6 (Kawagoe et al., 2007). TRAF6 is crucial for signal transduction downstream of IRAK4 and IRAK1/2 (Li, Strelow, Fontana & Wesche, 2002). In combination with TGF-β-activated kinase 1 (TAK1) binding protein (TAB) 2 and 3, TAK1 triggers activation of the IκB kinase (IKK) complex and MAPK pathways (Kawai & Akira, 2011). Within the IKK pathway, two catalytic subunits, IKKα and IKKβ, along with one regulatory subunit (IKKγ/NEMO) degrade IκB proteins through phosphorylation and ubiquitination (Israël, 2010). This TLR4-mediated activation of the IKK pathway results in early nuclear translocation and activation of NF-κB (Oeckinghaus & Ghosh, 2009).

The influence of TLR4 upon downstream MAPKs may be a critical component of cytokine and chemokine production. The highly-conserved MAPK family is typically

divided into three subfamilies: a) ERKs, b) JNKs, and c) p38/SAPKs (stress-activated protein kinases) (Morrison, 2012). Downstream of TLR4, the primary MAPKs appear to be JNKs and p38/SAPKs (Küper, Beck & Neuhofer, 2012). MAPK-stimulated transcriptional factors, activator protein (AP)-1 and cAMP-responsive element protein (CREB), are intrinsically involved in cytokine and chemokine production, and TLR4-induced transcriptional activation of cyclooxygenase (COX)-2 is shown to depend upon MAPK signaling in cells of the renal collecting ducts (Küper, Beck & Neuhofer, 2012).

Conversely, signal transduction through MyD88-independent pathway involves recruitment of TIR-domain-containing adaptor inducing IFN-β (TRIF/TICAM-1) via TRIF-related adaptor molecule (TRAM/TICAM-2) (Yamamoto et al., 2003). TRIF interacts with TRAF3 to activate IKK pathways, resulting in translocation of NF-κB, via adapter kinase receptor-interacting protein (RIP) 1 and interferon regulatory factor (IRF) 3 stimulation (Kawai & Akira, 2010b). Phosphorylated IRF3 translocates to the nucleus and modulates the expression of type I IFNs, which are crucial to viral defense (Chen et al., 2017; Kumar, Kawai & Akira, 2011).

Understanding TLR4 in BP regulation through hypertensive animal models

Animal models are an essential tool for understanding the pathophysiology of diseased states and for exploring new pharmacological interventions. Different rodent models have been used to study the role of TLR4 and its downstream signaling mechanisms in inflammation, oxidative stress, vascular remodeling, sympathetic over activity, and renal injury in the context of hypertension. The studies discussed herein were primarily conducted using animal models, particularly rodents, and, as such, care is

required in translating their results to human applications. However, such animal-based research continues to allow for the development of new pharmacological approaches to prevent and/or manage hypertension. In regards to TLR4, rodents and humans share approximately 60-70% similarity in the amino acid sequence of TLR4's extracellular and transmembrane domains (Vaure & Liu, 2014).

While the influence of TLR4 on BP control is not completely elucidated, TLR4 upregulation is suggested to contribute to the pathogenesis of hypertension in animal models. Spontaneously hypertensive rats (SHR), a genetically hypertensive model, have proved crucial to evaluating TLR4 expression and cytokine profiles. SHRs are characterized by age-dependent elevations in BP, reaching approximately 175-200 mmHg during the established phase of hypertension at 10-15 weeks of age (Okamoto & Aoki, 1963). SHRs are also susceptible to multiple types of organ damage, including cardiac hypertrophy and failure, impaired endothelium-dependent vascular relaxation, increased sympathetic drive, and renal dysfunction (Leong, Ng & Jaarin, 2015). In these animals, *in vivo* treatment against TLR4 has shown attenuation of some of the organ damage observed. For instance, systemic long-term treatment with a TLR4 antibody lowered BP and decreased both cardiac hypercontractility and remodeling in SHRs (Bomfim et al., 2012; Bomfim et al., 2015). Furthermore, a study investigating TLR4 modulation of hypertension via CNS cardioregulatory centers showed that TLR4 inhibition in the paraventricular nucleus of the hypothalamus (PVN) decreases BP in SHRs (Dange, Agarwal, Teruyama & Francis, 2015a; Wang et al., 2018) .

Conversely, systemic TLR4 blockade in the AngII-infusion model of hypertension results in less pronounced BP changes as compared to SHR. Systemic infusion of AngII

progressively enhances BP, mainly due to increases in oxidative stress and vascular remodeling (Lerman, Chade, Sica & Napoli, 2005). However, in AngII-infused mice with a TLR4 deficiency, despite significant inhibition of vascular remodeling through reduced levels of ROS, TLR4 deficiency did not impact AngII's effects on BP (Nakashima et al., 2015). Corroborating with these results, AngII-infused mice treated with a TLR4 antibody had superior vascular function but no difference in BP compared to untreated animals (Nunes, Bomfim, Toque, Szasz & Webb, 2017). Based on the literature, there is a consensus that, in response to higher levels of AngII, TLR4 is an important contributor to vascular dysfunction and oxidative stress, both hallmarks of hypertension. Interestingly, systemic blockade of TLR4 does not affect BP in AngII-infused animals in the same manner as observed in SHR. Zhang and collaborators showed that after 4 weeks of treatment with TAK-242, a specific TLR4 inhibitor, not only was a reversal in high BP observed in aldosterone-induced hypertensive animals, but renal and cardiac inflammation were also inhibited (Zhang et al., 2015). Aldosterone, the primary human mineralocorticoid, is significantly involved in cardiovascular morbidity and hypertension (Freel & Connell, 2004). The main regulators of aldosterone production are plasma levels of potassium and AngII. Thus, in hypertension, plasma levels of aldosterone and AngII positively correlates. However, in animals where hypertension is induced by external aldosterone administration, the plasma levels of AngII are not modified. Because the levels of AngII remain within normal ranges, the aldosterone model of hypertension might be considered an AngII-independent model. This could explain the controversial results regarding BP changes in hypertensive animals with higher circulating levels of AngII.

In order to target TLR4, however, the following question remains to be elucidated: why do the BP-lowering effects of TLR4 blockade differ between animal models while appearing to modulate similar events during the progression of hypertension? At this point, we can speculate that blockade of TLR4 may minimize the end-organ damage triggered by hypertension; however, it is not yet clear whether such blockade would be enough to abrogate this pathology.

TLR4 effects in specific organs and systems during hypertension

Kidneys

The kidneys, in combination with the renin angiotensin system (RAS), play central roles in BP regulation (Yim & Yoo, 2008). AngII has powerful control of sodium uptake in proximal tubules and affects glomerular filtration rate, which increases water reabsorption in a process responsible for maintaining homeostatic blood pressure levels. However, prolonged increases in circulating AngII levels contribute to the etiology of hypertension (Crowley et al., 2006). Likewise, there is substantial evidence supporting the hypothesis that increased natriuresis, with a right-ward shift in the sodium retention curve, contributes to the maintenance of a hypertensive state. In the kidneys, AngII, via its Angiotensin type 1 receptor (AT1r), has the potential to damage the renal microvasculature and is posited to be associated with fibrosis, vascular rarefaction, and glomerular dysfunction (Xu et al., 2017). Additionally, AngII has been demonstrated to be a strong inflammatory mediator and is suggested to act in concert with TLR4 pathways to promote inflammation (Biancardi, Bomfim, Reis, Al-Gassimi & Nunes, 2017a; Phillips & Kagiyama, 2002; Xu et al., 2017). In fact, AngII is among the most-commonly investigated possible endogenous

ligands of TLR4 during hypertension. Still, while we have compiled extensive knowledge regarding AngII's actions in the kidneys as a long-term mechanism of BP regulation, the implications of AngII-mediated TLR4 activation in the renal system is not completely understood.

TLR4 is expressed in renal epithelial cells and its over activation is implicated in the nephropathy associated with various diseases (Souza et al., 2015; Zhang, Ramesh, Uematsu, Akira & Reeves, 2008). In the context of the hypertensive kidney, current literature points to AngII as the main mediator of TLR4 activation. Corroborating with this statement, it has been reported that myeloid differentiation factor 2 (MD2)-deficient mice were protected from renal inflammatory injury and fibrosis (Xu et al., 2017). The adaptor protein MD2 is known to play a role in lipopolysaccharide (LPS) recognition by promoting TLR4-LPS-MD2 complex dimerization (Park, Song, Kim, Choi, Lee & Lee, 2009). More specifically, Han *et al.* have demonstrated that direct hydrogen bond interactions may occur between MD2 and AngII in a manner similar to that of MD2 and LPS interaction (Han et al., 2017). Based on this evidence, it is reasonable to speculate that AngII could mediate the activation of TLR4 within the kidneys, thereby contributing to inflammation and oxidative stress, both intrinsic factors for the development of end-organ damage in hypertensive patients.

TLR4-mediated renal damage in hypertension plays a key role in the development and progression of microvascular complications and may represent a new treatment target. Diuretics are one of the main anti-hypertensive drug classes targeting the kidneys, favoring water and sodium excretion to help relieve tubular pressure and lower BP. However, as AngII is produced systemically, it may continue to activate TLR4 in the face

of diuretic therapy, thereby propagating renal microvascular damage. Supporting this notion, combining diuretics with ACE inhibitors appears to produce better outcomes in hypertensive patients (Ruoff, 1989). A plausible explanation is that inhibiting AngII production may decrease the extent of TLR4 activation, ameliorating renal oxidative stress and inflammation.

Another cause of renal damage in hypertension comes from the high BP itself, which injures the endothelial layer and contributes to elevated ROS generation. While the precise molecular mechanisms underlying the damage-induced ROS generation are not completely elucidated, recent findings showing an association between increased renal TLR4 activation and worsened outcomes in hypertensive model point to DAMP-mediated TLR4 activation (Pushpakumar, Ren, Kundu, Gamon, Tyagi & Sen, 2017). In the kidneys, increased levels of ROS are associated with dysfunctional glomerular and tubular cells (Araujo & Wilcox, 2014). Importantly, as NF-κB is mediated downstream of TLR4 activation and is described as a source of pro-inflammatory cytokines, this may be one mechanism by which TLR4 contributes to renal dysfunction and end-organ damage in hypertension. Renal parenchymal TLR4 was shown to mediate inflammation and tissue damage following cisplatin exposure in a murine model of nephrotoxicity (Zhang, Ramesh, Uematsu, Akira & Reeves, 2008). In a mouse model of hypertension, TLR4 deficiency protected against renal oxidative damage and was further found to increase antioxidant capacity (Pushpakumar, Ren, Kundu, Gamon, Tyagi & Sen, 2017). Taken together, the aforementioned data implicate TLR4 activation in tying inflammation to kidney dysfunction in hypertension.

Central nervous system

As in other systems, CNS alterations in TLR4 expression, ROS, and the pro-inflammatory cytokine profile are linked to the pathogenesis of hypertension. In neural tissue, innate pro-inflammatory mediators are produced primarily by the resident immune cells, microglia, and astrocytes (Ransohoff & Brown, 2012). TLR4 is constitutively expressed by microglia, while the nature of its expression in other CNS cells, including, astrocytes, and neurons, remains somewhat controversial [(Olson & Miller, 2004); for review, see: (Hanke & Kielian, 2011; Lehnardt, 2010)]. Despite low TLR4 surface expression detection, particularly in astrocytes and neurons, stimulation with LPS is shown to trigger innate immune activity in non-microglia cells through various processes.

A complex communication system exists among microglia, astrocytes, and neurons that allow the innate immune cells to sense environmental perturbations and subsequently influence neuronal activity. A primary mechanism by which such communication is achieved is through the secretion and detection of pro-inflammatory mediators. The neuro-immune communication has been implicated in the pathogenesis of hypertension, wherein DAMP/danger signal recognition by CNS cells propagates a pro-inflammatory CNS milieu, resulting in inflammation, elevated sympathetic outflow, and increased BP.

Numerous studies have characterized, at least partially, the responses of various CNS cell types to stimulation by LPS. Surface expression of TLR4 is particularly abundant in microglia and it is well-accepted that LPS stimulation activates quiescent microglia, upregulating the innate immune response and increasing cytokine and chemokine secretion (Olson & Miller, 2004). While direct activation of the MyD88-dependent pathway

is shown in astrocytes upon LPS exposure, they are also shown to have an alternate response, propagating the LPS-mediated microglial inflammatory response and furthering neurotoxic factor production (Gorina, Font-Nieves, Marquez-Kisinousky, Santalucia & Planas, 2011; Saijo et al., 2009). In neuronal cultures, LPS stimulation induces trans-endothelial migration of neutrophils and activation of cerebral ECs, the hallmarks of neuroinflammatory response (Leow-Dyke et al., 2012). Together, these studies point to TLR4 dysregulation as a key candidate for modulating neuroinflammation in hypertension. Most of the evidence implicating neural TLR4 dysregulation within the CNS in the pathogenesis of hypertension is derived from animal models which either have elevated levels of circulating AngII or are exposed to exogenous AngII (*in vivo* or *in vitro*). Within the CNS, Ang-II is intrinsic to the inflammatory process, acting as a pro-hypertensive neurotransmitter and promoting innate immune activation through AT1r (Ando, Zhou, Macova, Imboden & Saavedra, 2004; Benicky et al., 2011; Benicky, Sanchez-Lemus, Pavel & Saavedra, 2009; Harrison et al., 2011; Young & Davisson, 2015; Zhou et al., 2006; Zubcevic, Waki, Raizada & Paton, 2011).

Whether endogenous to the hypertensive model or exogenously-applied, chronic AngII elevations aggravate TLR4, activate microglia, and upregulate pro-inflammatory cytokine production (Benicky et al., 2011; Benicky, Sanchez-Lemus, Pavel & Saavedra, 2009; Biancardi, Stranahan, Krause, de Kloet & Stern, 2016; Shi et al., 2010; Zubcevic, Waki, Raizada & Paton, 2011). Benicky *et al*. found LPS-induced neuroinflammation in normotensive animals to be blocked by AT1r antagonists. This AT1r-blockade decreased production of TLR4-regulated pro-inflammatory mediators and reduced microglia activation, both *in vitro* and *in vivo* (Benicky et al., 2011; Benicky, Sanchez-Lemus, Pavel

& Saavedra, 2009). Such alterations were observed in multiple nuclei, including, of note, several associated with autonomic control such as the PVN and the subfornical organ (SFO) (Benicky et al., 2011; Benicky, Sanchez-Lemus, Pavel & Saavedra, 2009). In TLR4-competent mice, exogenous AngII applied to PVN-containing hypothalamic slices caused microglial activation and ROS production (Biancardi, Stranahan, Krause, de Kloet & Stern, 2016). TLR4-deficient mice were shown to have attenuation of such responses in the PVN, demonstrating the contribution of TLR4 to these predecessors of hypertensive autonomic dysfunction (Biancardi, Stranahan, Krause, de Kloet & Stern, 2016).

TLR4 protein and mRNA expression within the PVN are elevated in both AngII-infusion models and SHR, and chronic i.c.v. infusion of a viral TLR4 inhibitory peptide normalized these parameters in AngII-infused animals (Dange et al., 2014a; Dange, Agarwal, Teruyama & Francis, 2015a; Li et al., 2016a). Furthermore, this chronic TLR4 blockade ameliorated cardiac function, decreased the cardiac inflammatory profile, and reduced mean arterial pressure (MAP) (Dange et al., 2014a). In SHRs, inhibition of PVN TLR4 attenuates the pro-inflammatory cytokine profile as well as elevations in iNOS and NF-κB levels, which are linked to the elevated BP and circulating plasma norepinephrine characteristic of this model (Dange et al., 2014a; Dange, Agarwal, Teruyama & Francis, 2015a). Li *et al.* showed reduced MAP in SHR via chronic bilateral PVN infusion of the AT1r inhibitor, Telmisartan (Li et al., 2016a). PVN AT1r inhibition also downregulated the MyD88-dependent pathway, resulting in decreased CNS IL-1β and IL-6 levels (Li et al., 2016a).

Compellingly, these studies show that abolishment or attenuation of altered microglial and TLR4 signaling activity occurs upon blockade of TLR4 or AT1r, suggesting a role for

neural TLR4, particularly through AT1r-TLR4 crosstalk, in hypertension. The findings discussed above highlight the involvement of hypothalamic TLR4 and pro-inflammatory signal transduction in driving AngII-mediated hypertension and demonstrate the powerful cardiovascular effects of TLR4 activity in the CNS. As such, the TLR4 signaling pathways represent potential antihypertensive therapeutic targets. Importantly, CNS inflammation is a known component in numerous pathologies beyond hypertension, including neurodegenerative disorders (Appel, Beers & Henkel, 2010; Lopes Pinheiro et al., 2016; Perry, Nicoll & Holmes, 2010). Thus, elucidating the extent of TLR4's role in the hypertensive CNS may allow for an expansion of pharmaceutical targets in many neuroinflammatory diseases. On the basis of this commonality, there arises the potential that therapeutics currently employed in the management of other CNS-associated diseases may represent novel antihypertensive therapies.

Vasculature

Chronic high BP and shear stress damage the vascular endothelium overtime and contribute to migration and accumulation of both innate and adaptive immune cells in blood vessels (Goulopoulou, McCarthy & Webb, 2016). Augmented TLR4 expression and activation positively correlates with vascular inflammation, remodeling, and vasoconstriction. As in other cell types, increased TLR4 activity increases production of pro-inflammatory cytokines and ROS. Within the vasculature, TLR4 has been demonstrated to modulate nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activity, enhancing the production of ROS and free radicals (Nakashima et al., 2015). TLR4-induced ROS production decreases the availability of nitric oxide (NO), a

vasoprotective molecule imperative to the regulation of blood flow and tissue oxygenation (Nunes, Bomfim, Toque, Szasz & Webb, 2017; Schiffrin, 2008). Furthermore, it has been suggested that, following TLR4 activation, crosstalk interaction occur between ROS and NF-κB wherein ROS can influence NF-κB nuclear translocation while NF-κB may regulate ROS production via gene expression (Morgan & Liu, 2011). Additionally, secreted cytokines from individual cells can diffuse to adjacent tissue where they stimulate ROS production (Mittal, Siddiqui, Tran, Reddy & Malik, 2014). These hypertension-induced disruptions abrogate endothelial and vascular functionality, the latter of which is vital to maintaining vascular homeostasis and depends upon proper VSMC contractile responses (Biancardi, Bomfim, Reis, Al-Gassimi & Nunes, 2017a; De Batista et al., 2014; Nunes, Bomfim, Toque, Szasz & Webb, 2017; Sanchorawala & Keaney, 1997). In the hypertensive vasculature, altered TLR4 expression is found in VSMCs and ECs (De Batista et al., 2014; Hernanz et al., 2015). De Batista and collaborators reported that TLR4 mRNA levels are upregulated in VSMCs and aortae of SHRs, and that treatment with a TLR4 antibody mitigated heart rate, BP, and phenylephrine-induced contraction (De Batista et al., 2014). In a separate study, TLR4 blockade with a TLR4 antibody prevented pro-inflammatory cytokine secretion, decreased vascular structural and mechanical changes, ameliorated vascular reactivity, and increased NO production (Hernanz et al., 2015). Additionally, Bomfim *et al.* showed TLR4's pro-inflammatory actions to be ultimately mediated through the MyD88-dependent pathway in SHRs (Bomfim et al., 2015).

As previously discussed, AngII has regulatory roles in long-term BP regulation and vascular homeostasis through its interaction with AT1r. TLR4 is implicated as a key

mediator of AngII-induced vascular remodeling in hypertension through MyD88-dependent ROS generation via JNK/NF-κB activation (Hernanz et al., 2015; Nakashima et al., 2015). AngII-induced elevations in ROS have been shown to occur through TLR4-impairment of superoxide dismutase in addition to TLR4-stimulation of NADPH-oxidase (Nakashima et al., 2015). Ji and collaborators have shown that AngII stimulates TLR4 in VSMCs, triggering the production of TNF-α and MMP-9, among others mediators, and contributing to vascular dysfunction (Ji, Liu, Wang & Liu, 2009). Pharmacological inhibition of TLR4 with CLI-095 abrogated NADPH-oxidase activity, superoxide production, and decreased both cell migration and proliferation in response to AngII (De Batista et al., 2014). Importantly, antibody inhibition of TLR4 decreases MAP and vascular contractility and TLR4 protein expression in SHR mesenteric resistance arteries (Bomfim et al., 2012). In murine cavernosal smooth muscle, a highly vascularized structure, we have shown that AngII alters TLR4 expression, and that chronic TLR4 blockade rescues muscle relaxation, decreases TNF-α production, and improves NO levels (Nunes, Bomfim, Toque, Szasz & Webb, 2017). As evidenced, TLR4 enhances inflammation and contributes to vascular remodeling in models of hypertension and its inhibition appears to be protective.

In hypertension, vascular remodeling involves complex interactions between endogenous growth factors, vasoactive substances, and hemodynamic alterations (Schiffrin, 2012). As both vascular remodeling and endothelial damage progress, a positive feedback loop forms in which vasoconstriction is constantly favored over vasodilation. In this way, the physiological adaptations promoted by TLR4 in response to hypertensive stimuli ultimately turn to pathophysiological consequences. The labyrinth of

signaling pathways activated by TLR4 involve numerous overlapping mechanisms and crosstalk interactions that have been highlighted as potential therapeutic targets to combat the pathogenesis of hypertension.

Heart

Although myocardial tissue alterations underlying the transition from a healthy to a hypertensive heart are unclear, the eventual cardiac dysfunction is known to be characterized by myocardial remodeling and low-grade inflammation (Nadruz, 2015). Chronically elevated BP, coupled with factors such as altered neurohormone and cytokine levels, induces compensatory left ventricular hypertrophy (Drazner, 2011). It has been shown that cardiomyocytes express many TLRs, including TLR4, which trigger signaling pathways leading to local inflammation (Boyd, Mathur, Wang, Bateman & Walley, 2006). Consequently, during hypertension, the heart is a significant target for active immune cells.

Indeed, mRNA and protein TLR4 expression levels were found to be upregulated in cardiomyocytes of SHR (Eißler et al., 2011). Interestingly, treatment of SHRs with the ACE inhibitor, Ramipril, showed a dose-dependent response: a therapeutic dose (1 mg/kg/day) was sufficient to lower BP while a supra-therapeutic dose (10mg/kg/day) was needed to elicit reductions in the observed upregulation of cardiac TLR4 expression (Eißler et al., 2011). This indicates that, while ACE inhibitors can alleviate BP elevations, they may or may not affect the associated TLR4-driven low-grade inflammation in cardiac tissue. Thus, it is important to take into consideration that AngII is not the only endogenous ligand for TLR4 in the hypertensive heart. For example, increased circulating

levels of HSP60 positively correlate with the development of cardiovascular diseases and have been shown to modulate the TLR4/MyD88/p38/NF-κB pathway in cardiac cells (Pockley, Wu, Lemne, Kiessling, de Faire & Frostegård, 2000; Tian et al., 2013). Conversely, TLR4 knockout mice have reduced left ventricular hypertrophy after aortic banding compared to wild type mice, and animals with a dysfunctional LPS response ($TLR4^{lps\text{-}d}$ mice) show no changes in oxidative stress, ventricular hypertrophy, or cardiac dysfunction when infused with AngII (Ha et al., 2005; Matsuda et al., 2015). It has been further defined that these effects are mediated by an essential chemokine, monocyte chemoattractant protein-1 (MCP-1), which regulates macrophage tissue infiltration and is upregulated by TLR4 stimulation (Matsuda et al., 2015). These results suggest that TLR4 stimulation, whether via AngII or other ligands, contributes to cardiac damage in hypertension.

It should be noted that neural TLR4 is reported to partially mediate physiological alterations of the myocardium in AngII-infused animals. In this model, specific blockade of TLR4 in the CNS downregulates myocardial inflammation (Dange et al., 2014a). Lastly, reduced myocardial hypertrophy and remodeling were observed in $MD2^{-/-}$ mice infused with AngII and in cardiomyocyte-like H9c2 cells incubated with AngII in the presence of L6H21 (an inhibitor of MD2) (Han et al., 2017). These data indicate that MD2 may directly bind to AngII in the heart, causing dysfunction of the myocardium via TLR4 activation (Han et al., 2017). As there remains a paucity of information regarding the contribution of TLR4 to myocardial injury in response to high BP, it is unclear if blockade of TLR4 is cardioprotective in this condition. Thus, future experiments are needed to elucidate

whether the onset and development of hypertension-associated heart damage, including complications such as heart failure, would be minimized by targeting TLR4.

Translational potential of TLR4 targets in hypertension

Considering the ability of TLR4 to initiate and boost inflammation, and the literature which links TLR4 to hypertension, there is significant interest in developing novel pharmacological drugs that either target TLR4 itself or its downstream pathways in diseases associated with abnormal innate immune system over-activity. Although many mechanisms underlying disease-mediation by TLR4 have yet to be fully clarified, insights into TLR4's signal transduction have recently opened the doors to development of effective modulators. Even now, there is pre-clinical evidence of the therapeutic potential for TLR4 targeting in inflammatory diseases. **Table 3-2** summarizes pharmacological compounds that are currently being used in basic research to target TLR4 and/or its downstream signaling pathways.

Eritoran (E5564) is a synthetic TLR4 antagonist which has been well studied in inflammatory disease models and has been used in four clinical trials in the last decade (NCT00334828, NCT00756912, NCT02267317, and NCT02321111). Unfortunately, the promising results observed in animal models of inflammatory diseases were not translated to human subjects. As an example, in a phase III randomized control clinical trial that compared the efficacy of Eritoran in preventing mortality in patients with severe sepsis, human subjects receiving the drug did not have an enhanced chance of survival compared to those who received the placebo (NCT00334828). However, in the context of evaluating drug efficacy, it is important to take into consideration the etiological and

Compound	Description	Target	Mechanism of action	Disease model or cell type	Main outcomes
Eritoran (E5564)	Synthetic LPS-lipid A analog	MD2	Competitively binds a large pocket of MD2	Rat model of kidney ischemia/reperfusion	Ameliorated kidney ischemia/reperfusion related inflammatory responses (Liu, Gu, Xu, Lv, Zhang & Wu, 2010).
SPA4	Peptide	TLR4	Binds to surfactant A and blocks TLR4 activation	HEK293 cells	Decreased secretion of pro-inflammatory cytokines (Ramani, Madhusoodhanan, Kosanke & Awasthi, 2013).
TAK-242	Small molecule/ cyclohexene inhibitor	TLR4	Binds TIR domain and affects the recruitment of adapters	- Rat model of hyperaldosteronism - Rat VSMCs - Mouse model of hypertension (AngII)	- Inhibited hypertension, cardiac and renal fibrosis, and attenuates aldosterone-induced epithelial-mesenchymal transition (Zhang et al., 2015). - Decreased NADPH oxidase activity, superoxide anion production, and cell migration and proliferation (De Batista et al., 2014). - Reduced AngII-induced increase in phospho-JNK1/2 and p65 NF-κB subunit nuclear protein expression (Hernanz et al., 2015).
NI-0101	Monoclonal antibody	TLR4	Antagonist	Synovial explant culture model	Decreased pro-inflammatory cytokines secretion (TNF-α and IL-6) (Page, Buatois, Daubeuf, Chatel, Cons & Lippens, 2011).
Valsartan	AT1r - AngII receptor blocker	TLR4	Unknown	Rat model of myocardial ischemia/reperfusion	Improvement in myocardial injury, such as smaller infarct size, and decreased release of myocardial enzymes and pro-inflammatory mediators (Yang et al., 2009a).

Candesartan	AngII receptor blocker	TLR4	AngII receptor independent	Rat mesangial cells	Decreased oxidative stress and exerted anti-apoptotic effects (Lv, Jia, Yang, Zhu & Ding, 2009).
Fluvastatin	HMG-CoA reductase inhibitor	TLR4	Inhibits NF-κB activation	Rat model of myocardial ischemia/reperfusion	Decrease ischemic injury and inhibited the expression levels of TLR4, TNF-α and NF-κB (Yang et al., 2011a).
Atorvastatin	HMG-CoA reductase inhibitor	TLR4	Impairs TLR4 recruitment of lipid raft; inhibits NF-κB activation	Rabbit model of atherosclerosis	Impaired TLR4/NF-κB activation in atherosclerotic plaques which decreased inflammation (Fang, Yang, Quan, Jia, Quan & Qu, 2014).
ST2825	Peptidomimetic	MyD88	Inhibits homodimerization of MyD88	Mouse model of hypertension (AngII)	Decreased NADPH-oxidase activity (Hernanz et al., 2015).
dnMyD88	Mutated form of MyD88	MyD88	Inhibits homodimerization of MyD88	Rat model of myocardial ischemia/reperfusion	Prevented ischemia/reperfusion via inhibition of NF-κB (Ha et al., 2006).

Table 3-2. Pharmacological therapies currently in use that target TLR4 and its downstream signaling. AngII: angiotensin II; AT1r: angiotensin II type 1 receptor; HMG-CoA: hydroxymethylglutaryl coenzyme A; MD2: myeloid differentiation factor 2; MyD88: myeloid differentiation primary response 88; NADPH-oxidase: nicotinamide adenine dinucleotide phosphate oxidase; TIR: Toll-interleukin 1; TLR4: Toll-like receptor 4; VSMCs: vascular smooth muscle cells.

pathological differences among diseases, such as between sepsis and hypertension. In the same trial, it was reported that Eritoran clearance is affected by the patient's weight, high density lipoprotein levels, and age, features that are highly important factors over the course of hypertension. Eritoran inhibits MD2-mediated TLR4 stimulation by binding to a large pocket of this adaptor protein. As previously discussed, this mechanism of action is similar as one reported to be used by AngII to activate TLR4. To date, the possible benefits of TLR4 blockade by Eritoran are still unknown in hypertensive patients. Thus, it is possible to speculate that this drug might have therapeutic applications in the management of hypertension.

One of the most widely used TLR4 antagonists in basic research is TAK-242, which binds to the TIR domain at Cys747 and, consequently, inhibits TLR4's ability to recruit both adaptor proteins (TIRAP/MAL and TRAM) responsible for mediating TLR4 actions (Matsunaga, Tsuchimori, Matsumoto & Ii, 2011). Despite its consistently inhibitory effects in animal models, the drug failed to decrease sepsis symptoms when tested in human subjects (NCT00633477). Nevertheless, TAK-242 may yet have a potential application in hypertension treatment due to the different natures of these pathologies. This drug has been tested in different animal models of hypertension and has produced exciting results. TAK-242 has been reported to block NF-κB, reduce oxidative stress, decrease cell migration and proliferation, and lower BP (De Batista et al., 2014; Hernanz et al., 2015; Zhang et al., 2015). Overall, the pharmacological benefits of this inhibitor in animal models of hypertension are clear. However, it remains unknown whether the same effects would be observed in hypertensive human subjects.

Another drug used to block TLR4 signaling, NI-0101, is a monoclonal antibody currently being assessed in a clinical trial for rheumatoid arthritis (NCT01808469). In cultured cells, the drug has been shown to decrease pro-inflammatory cytokine secretion (Page, Buatois, Daubeuf, Chatel, Cons & Lippens, 2011). It is not yet known whether the drug would be effective when used systemically in animal models of hypertension. Further exploring the available data using TLR4 antibodies in basic research, there are promising outcomes regarding their abilities to ameliorate the inflammatory state and reduce oxidative stress, which, together, blunt the deleterious effects of hypertension in the major organs studied.

Repurposing drugs is a key process in unearthing new options in the treatment of a disease. In this sense, statins, which are primarily prescribed to lower cholesterol, have produced promising results in modulating the TLR4 pathway. Particularly, Atorvastatin has been shown to decrease TLR4 activation, which abolishes inflammation in atherosclerosis, and Fluvastatin is demonstrated to prevent myocardial ischemia and reperfusion (Fang, Yang, Quan, Jia, Quan & Qu, 2014; Yang et al., 2011a). Based on this data and the findings of other studies, it can be predicted that, because treated animals had decreased TLR4 levels in the Atorvastatin and Fluvastatin studies, they might also have had decreased oxidative stress. As hypertension and atherosclerosis have overlapping pathways, it would be of interest to explore whether the same outcomes would be observed in hypertensive animal models.

The pharmacological effects of the AT1r blockers, Valsartan and Candesartan, have also been examined in animal models (Lv, Jia, Yang, Zhu & Ding, 2009; Yang et al., 2009a). These drugs were shown to reduce oxidative stress and decrease cytokine

profiles. These studies were conducted before it was shown that AngII could activate TLR4 by interacting with MD2. It is still unclear whether AngII has two independent mechanisms to modulate TLR4, one through MD2 and the other via AT1r crosstalk. Additionally, it has been suggested that, when AT1r blockers are used, AngII levels might accumulate and favor TLR4 activation (Campbell, 1996). However, increased AngII levels also contribute to the formation of Ang (1-7), another important peptide of the RAS system that opposes the effects of AngII (Santos, 2014b).

The drug ST2825, a mutated form of MyD88 which inhibits MyD88 homodimerization, has also been tested. In a model of hypertension, it was observed that ST2825 decreases NADPH-oxidase activity, which in turn ameliorates oxidative stress (Hernanz et al., 2015). TWhen used in a model of myocardial infarction, ST2825 blocked NF-κB activity (Ha et al., 2006). While the effects of ST2825 differed between these studies, both showed treatment outcomes the are favorable in the management of hypertension. When comparing the results of TLR4 blockade to the use of adaptor molecule inhibitors, it must be taken into consideration that TLR4 signal transduction occurs through two arms, one led by MyD88, and the other by TRIF. This means that targeting MyD88 rather than TLR4 itself would only partially inhibit TLR4's signaling, which may prove to be an advantage depending on the clinical application.

We have presented evidence supporting the role of TLR4 in hypertension. TLR4-mediated hypertension involves many different aspects of cardiovascular, renal, and central nervous systems, much of which require further investigation. Indeed, there are many questions regarding TLR4 and hypertension that remain to be addressed. A paramount question is whether the use of immunological suppressants would produce

better results in hypertensive patients when compared to the well-established pharmacological drugs available in the market. Thus far, it is possible to speculate that such a treatment could be promising in patients who do not respond to standard pharmacotherapy and inevitably succumb to end-organ damage.

TLR4 and hypertension-associated diseases

Pulmonary hypertension

Pulmonary hypertension (PH) of different etiologies and prognoses share a central pathogenesis characterized by persistent pulmonary vasoconstriction, vascular remodeling, and thrombosis *in situ*. Pulmonary vascular remodeling, through pulmonary arterial (PA) smooth muscle cell (PASMC) proliferation and fibrosis, plays a critical role in PH development and involves a chronic imbalance in vasoactive substances. Pulmonary arterial hypertension (PAH), in which small pulmonary arteries progressively narrow, leading to increases in pulmonary vascular resistance and pressure, right heart failure, and ultimately death, is strongly associated with dysregulated immunity and pulmonary vascular inflammation largely regulated through TLR4 signaling pathways (Gerges & Lang, 2018). In response to local injury (acute lung injury) or stress (hypoxia; cold exposure), TLR4 stimulation causes PA ECs to produce and secrete fractalkine (CX3CL1), a chemokine which attracts immune cells (Amsellem et al., 2017; Florentin & Dutta, 2017). Activation of TLR4 has been demonstrated to regulate MMP-9 production in lungs after hypoxia exposure, increasing ECM degradation and PASMC migration and proliferation (Young et al., 2010). PA ECs also release TLR4 endogenous agonists, such as HMGB1, which activate platelets and stimulate their aggregation at the

injured/stressed PA sites (Bauer, Shapiro, Billiar & Bauer, 2013; Sun, 2014). The TLR4-activated platelets produce and secrete vasoactive substances (5-HT, thromboxane A2), mitogenic and growth factors (platelet-derived growth factor, TGF-β, VEGF), and pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α) (Bauer, Shapiro, Billiar & Bauer, 2013; Sun, 2014). HMGB1 has also been shown to promote both pulmonary vascular remodeling and right ventricular hypertrophy through stimulation of TLR4 (Hilbert, Dornbusch, Baumgarten, Hoeft, Frede & Klaschik, 2017). The pathogenesis of PAH has been further linked with Notch, TGF-β, PI3K/Akt, and Hippo signaling, many of which point to a dysregulation in either ligands or substrates or TLR4 signaling (Whelton et al., 2017). However, it is yet to be determined whether TLR4-induced inflammation is a primary or secondary mechanism in PAH pathogenesis, what may be the implications of these findings in other forms of PH, or how these pathways might be therapeutically targeted.

Preeclampsia

Preeclampsia (PE) is a severe complication of pregnancy characterized by the development of hypertension and proteinuria (Whelton et al., 2017). The maternal immune system, specifically maternal systemic inflammation, is strongly implicated as a key contributor to the pathogenesis of PE (Bounds, Newell-Rogers & Mitchell, 2015). The excessive pro-inflammatory response is suggested to be mediated through TLR recognition of DAMPs and other danger signals from placental dysfunction, metabolic syndrome, and/or vascular dysfunction (Bounds, Newell-Rogers & Mitchell, 2015; Yeh, Chao & Huang, 2013; Zhao, Wang, Zhang, Tian & Sun, 2017). TLR4, MyD88, and NF-κB are widely expressed in the maternal-fetal interface, and their expressions are

increased in the placenta of women with PE (Bounds, Newell-Rogers & Mitchell, 2015; Qian, Wang, Wu, Li, Chen & Lv, 2015; Xue et al., 2015; Zhu, Wu, Wu & Xia, 2013). Similarly, the cytokines TNF-α, IL-6, and MCP-1 are increased in PE patients, both systemically and within placental tissue (Xue et al., 2015). Zhao *et al.* found both TLR4 and NF-κB serum expressions to be elevated in PE patients, and demonstrated that either could be used as a serum marker for PE diagnosis (Zhao, Wang, Zhang, Tian & Sun, 2017). *In vitro* studies showed that binding of LPS by TLR4 in trophoblasts increased cytokine secretion significantly and resulted in monocyte chemotaxis (Yeh, Chao & Huang, 2013). Furthermore, TNF-α infusion in pregnant mice was associated with placental TLR4 upregulation and resulted in the development of both hypertension and proteinuria (Bomfim, Rodrigues & Carneiro, 2017). Preliminary investigations into the potential therapeutic application of immune modulation in PE have yielded promising results. Qian *et al.* showed Vitamin D3 supplementation to decrease peripheral blood monocyte TLR4 expression, serum pro-inflammatory cytokines, and PE-incidence in at-risk pregnant women (Qian, Wang, Wu, Li, Chen & Lv, 2015). Additionally, down-regulation of the TLR4 signaling pathway with curcumin attenuated both high BP and proteinuria in a rat model of PE (Gong et al., 2016). While still very few, these results highlight the potential therapeutic application of TLR4 modulation in preventing PE. Further investigation is warranted to understand whether the tested supplements would provide similar findings in different models/types of hypertension.

Obesity-associated hypertension

Development of obesity-associated hypertension has been linked with metabolic dysregulation, autonomic dysregulation, and vascular dysfunction (endothelial dysfunction and arterial stiffening) (Kang, 2013; Li et al., 2016d; Matsuda & Shimomura, 2013; Reho & Rahmouni, 2017). The chronic low-grade inflammatory state associated with obesity is characterized by elevations in cytokines, ROS production and secretion, and immune cell recruitment (Catrysse & van Loo, 2017; Kang, 2013; Matsuda & Shimomura, 2013; Schneider et al., 2015). Hypertrophy of adipose tissue induces production of inflammatory mediators (TNF-α, IL-6, MCP-1, iNOS) from adipocytes, which leads to recruitment of immune cells, especially macrophages and T lymphocytes, to the adipose mass (Catrysse & van Loo, 2017; Kang, 2013; Matsuda & Shimomura, 2013; Schneider et al., 2015). Both adipocytes and recruited cytokine-producing immune cells continue production of pro-inflammatory cytokines and chemokines, maintaining local and, eventually, systemic inflammation (Catrysse & van Loo, 2017; Kang, 2013; Matsuda & Shimomura, 2013; Schneider et al., 2015). Ahmad *et al.,* found a significant elevation in TLR2 and TLR4 expression in adipocytes and peripheral blood mononuclear cells from obese subjects in correlation with increased cytokine levels, supporting the notion that these TLRs may mediate crosstalk between metabolism and the immune system (Ahmad et al., 2012). Obesity is also linked to elevations in TLR4 ligands: Western diets provide pro-inflammatory free fatty acids and alter the composition of the gut microbiota, increasing intestinal permeability such that LPS can translocate from the gut (Schneider et al., 2015). Once in the bloodstream, these TLR4 ligands can contribute to vascular-mediated alterations (Schneider et al., 2015). Studies demonstrating elevated oxidative

stress and NADPH-oxidase subunit expression in the hypothalamus of obese rats, concurrent with antioxidant-induced reduction of arterial BP and sympathetic nerve activity in obese mice, suggest an important role for sympathetic excitation in the pathogenesis of obesity-associated hypertension (Matsuda & Shimomura, 2013). Together, oxidative stress and inflammatory signaling likely underlie vascular dysfunction and development of obesity-associated hypertension. Given that obesity-associated hypertension is also connected with low-grade chronic inflammation, the extent to which TLR4 is involved in the genesis and/or maintenance of this disease likely merits further exploration.

Final remarks

As highlighted in this review, the chronic low-grade inflammation observed in hypertension is due, in large part, to activation of TLR4 and its downstream signaling pathways. Elevations in TLR4 expression have been observed in hypertensive animal models across renal, neural, vascular, and myocardial tissues. When combined with the extensive evidence of increased innate pro-inflammatory mediator profiles, it can be postulated that inhibition of TLR4 signal transduction would be globally beneficial in preventing the life-long complications associated with this disease. Furthermore, hypertensive models have proven the ability of TLR4 inhibition to ameliorate the deleterious effects of sustained hypertension. In some of the tested hypertensive models, inhibition of TLR4 was also able to normalize elevated BP. Thus, designing antagonists of TLR4 and its downstream signaling components is a compelling strategy to

pharmacologically target the dysregulated TLR4 and, feasibly, the progression of hypertension.

There is, however, much to be said for the remaining gaps in our knowledge regarding TLR4 signaling in terms of their application to therapeutic development. In this light, it must be taken into consideration that TLR4, as a facet of the innate immune system, is ultimately involved in both initial and chronic innate immune activation states. The promotion of inflammation is, first and foremost, a beneficial process, protecting against invading pathogens and responding to stress or injury. It is when this initial protective process becomes excessive or chronic that innate immune activation and, specifically, TLR4 signaling, can be calamitous. With over-activation, the disrupted immune homeostasis becomes pathogenic, manifesting as chronic inflammation, such as that observed in hypertension.

In Figure 2, we present several therapeutic targets known to modulate TLR4 signal transduction, as well as potential targets for future pharmaceutical development. As illustrated, activation of TLR4 triggers two signaling cascades, which ultimately lead to the production of pro-inflammatory cytokines. Based on studies to date, it can be argued that NF-κB is implicated as the principal downstream component, orchestrating aberrant innate immune system activity in hypertension through TLR4. With similar recent findings of NF-κB dysregulation in other inflammation-associated diseases, pharmaceutical constraint of this transcription factor undoubtedly demands further exploration, particularly as a point-of-target given the multitude of crosstalk within the TLR4 signaling web.

Multidisciplinary investigations suggest a plethora of additional crosstalk mechanisms within and between the two TLR4 pathways, and it is evident that TLR4 signal transduction is yet more intricate than we understand. While not within the scope of this review, it is of note that all TLRs have been shown to induce NF-κB activity upon stimulation, despite their extensive diversity (Singh, Chapleau, Harwani & Abboud, 2014). Thus, high specificity in an NF-kB governing agent is essential to combatting potential activation of this pro-inflammatory transcription factor either by other TLR pathways or by as-of-yet undetermined TLR4 mechanisms. On the one hand, our lack of knowledge regarding the extent of TLR4's pathways in hypertension may result in unforeseen complications with putative therapeutics. Conversely, the potential of supplementary connections could allow for fewer pharmaceutical risks—by targeting the component essential to disease propagation rather than the entire cascade, it is possible that the beneficial effects of TLR4 stimulation will be retained.

Lastly, it is of critical importance that the potential risks of targeting TLR4 in hypertension be considered. As evidenced by its vast array of ligands, the TLR4 pathway plays a substantial role in shaping the immune response, and the consequences of inhibiting this arm of the immune system are still unclear. Essential hypertension is a chronic condition that often requires lifelong treatment and, as such, the potential benefits of targeting TLR4 must be weighed against the risks and disadvantages of immunosuppressant treatment.

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Targeting toll-like receptor 4 signaling pathways: can therapeutics pay the toll for hypertension? by K. P. Nunes, A. A. de Oliveira, F.E. Mowry, and V. C. Biancardi

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Chapter 4. Project Overview

According to the 2019 Heart Disease and Stroke Statistics, approximately 46% of adult Americans have hypertension (Benjamin et al., 2019). Without proper treatment and management, chronic hypertension is liable to result in chronic heart disease, kidney disease, stroke, and/or end-organ damage. While the five major classes of antihypertensive drugs target various aspects of the renin-angiotensin system (RAS), fluid homeostasis, and the peripheral nervous system, recent estimates suggest that up to 20% of patients remain resistant to current therapeutic approaches (Carey, Sakhuja, Calhoun, Whelton & Muntner, 2019). A common observation among these patients is an elevation in sympathetic nervous system (SNS) activity (DiBona, 2013; Grassi, Mark & Esler, 2015; Guyenet, Stornetta, Souza, Abbott & Brooks, 2020; Mann, 2018; Stocker, Kinsman & Sved, 2017). Within the central nervous system (CNS), multiple cardioregulatory nuclei govern sympathetic outflow, including the hypothalamic paraventricular nucleus (PVN), rostral ventrolateral medulla (RVLM), and nucleus of the tractus solitarius (NTS). The PVN enhances sympathetic activity via projections to the intermediolateral spinal column and RVLM, the primary nucleus responsible for net sympathetic outflow (Dampney, Michelini, Li & Pan, 2018; Guyenet, Stornetta, Holloway, Souza & Abbott, 2018). Conversely, the NTS suppresses RVLM activity by way of the caudal ventrolateral medulla (Zoccal, Furuya, Bassi, Colombari & Colombari, 2014). Previous studies within these nuclei show that altered RAS mechanisms, particularly dysregulation of angiotensin II (AngII) signaling, contribute to increased sympathetic activity in hypertension (Young & Davisson, 2015). As AngII's type 1

receptor (AT1R) is expressed by multiple CNS cell types, AngII may influence neuronal activity directly by binding neuronal AT1R, or indirectly, by influencing the activity of endothelial cells, perivascular macrophages (Santisteban et al., 2020), astrocytes (Stern, Son, Biancardi, Zheng, Sharma & Patel, 2016), and microglia (Biancardi, Stranahan, Krause, de Kloet & Stern, 2016).

AngII dysregulation is strongly associated with low-grade neuroinflammation, microglial activation, and blood-brain barrier (BBB) disruption in CNS cardioregulatory nuclei (Biancardi, Son, Ahmadi, Filosa & Stern, 2014; Young & Davisson, 2015; Zubcevic, Waki, Raizada & Paton, 2011). We have shown that AngII-induced BBB breakdown facilitates entry of circulating AngII into the PVN, RVLM, and NTS of spontaneously hypertensive rats (SHRs), targeting neurons and microglia therein. Prior work consistently reports increased microglial activation and proinflammatory cytokines (PICs; i.e., tumor necrosis factor [TNF]-α, interleukin [IL]-6, and IL-1β) in the PVN of hypertensive animals due to AngII, with several studies demonstrating clear links between microglia and PIC signaling to elevated sympathetic activity in SHRs (Shi et al., 2011; Song et al., 2014), L-N^G-nitro-l-arginine methyl ester (L-NAME)- and AngII-infusion mice (Shen et al., 2015), and AngII-infusion rats (Shi et al., 2010).

Microglia are direct regulators of the neuroinflammatory response. They constitutively express AT1R and Toll-like receptor 4 (TLR4) (Biancardi, Stranahan, Krause, de Kloet & Stern, 2016; Olson & Miller, 2004), a pattern-recognition receptor implicated in multiple neuroinflammatory and cardiovascular diseases, including hypertension (Biancardi, Bomfim, Reis, Al-Gassimi & Nunes, 2017b; Nunes, de Oliveira, Mowry & Biancardi, 2019; Zubcevic, Waki, Raizada & Paton, 2011). Upon stimulation by

pathogen-associated molecular patterns (i.e., lipopolysaccharide [LPS]) or damage-associated molecular patterns (Kawai & Akira, 2011), TLR4 initiates proinflammatory signaling cascades, causing microglial activation and triggering downstream PIC production. Using an *ex vivo* preparation of PVN-containing hypothalamic slices, we have demonstrated an interaction between AT1R-dependent signaling cascade(s) and TLR4 following exogenously applied AngII, such that TLR4 becomes stimulated, leading to microglial activation and reactive oxygen species (ROS) generation (Biancardi, Stranahan, Krause, de Kloet & Stern, 2016). While various *in vivo* and *in vitro* studies have shown that AngII upregulates TLR4 expression, stimulates microglia, enhances PIC production, and disrupts the BBB (Benicky et al., 2011; Benicky, Sanchez-Lemus, Pavel & Saavedra, 2009; Biancardi, Stranahan, Krause, de Kloet & Stern, 2016; Zubcevic, Waki, Raizada & Paton, 2011), the extent to which *in vivo* AT1R-TLR4 signaling interactions contribute to the etiology and preservation of a hypertensive state remains unclear.

Hypothesis

We hypothesized that microglial AT1R-TLR4 signaling interactions represent a critical mechanistic link between AngII-mediated neuroinflammation and BBB disruption within the PVN, RVLM, and NTS, ultimately contributing to the maintenance of an inflammatory and sympathoexcitatory state in cases of chronic hypertension (**Figure 4-1**). Using SHRs, we examined the effects of systemic AT1R and TLR4 blockade on BP changes, TLR4 protein expression, microglial activation status, PIC production, BBB integrity, and autonomic regulation.

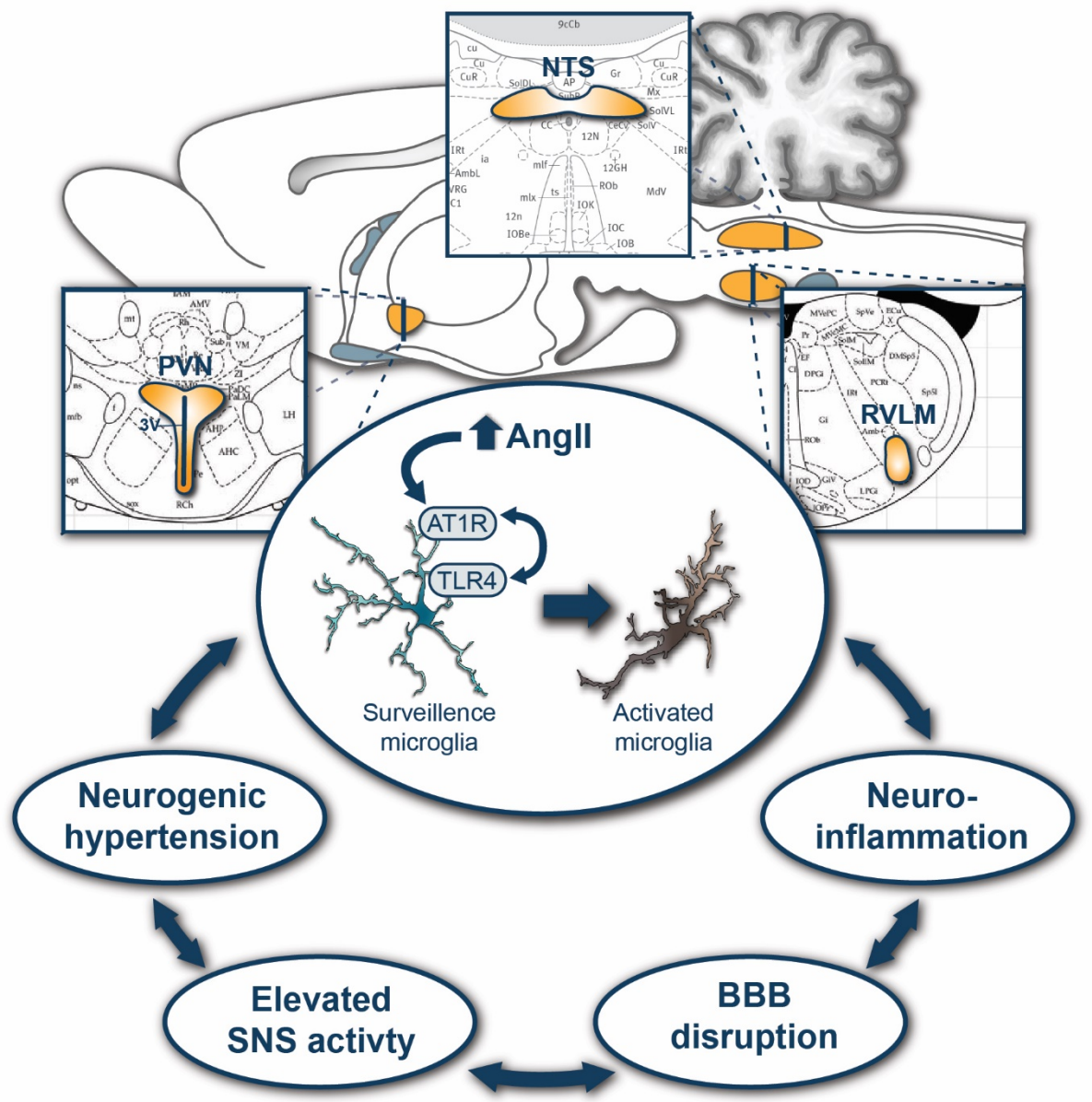

Figure 4-1. Schematic overview of working hypothesis. Increased levels of centrally-acting AngII stimulate AT1R-TLR4 signaling interactions within primary cardioregulatory nuclei of the hypothalamus and brainstem, resulting in polarization of microglial cells to a chronic neuroinflammatory phenotype and contributing to BBB breakdown, autonomic dysfunction, and progression of hypertensive pathophysiology.

Chapter 5. Methodology

Animals and experimental groups

All procedures were approved by the Institutional Committee on Animal Care (protocol 2017-2883) and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals,* as recommended by the US National Institutes of Health. Male Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) (Charles River Laboratories, USA) were housed in temperature- and humidity-controlled rooms (22±1°C; 50±5%) under a 12-12h light-dark cycle with standard rat chow and water *ad libitum*. All animals were 7-8 weeks old at the start of experiments. SHRs were randomly divided into either control or experimental groups.

AT1R blockade

As previously described (Biancardi, Son, Ahmadi, Filosa & Stern, 2014), SHRs were treated daily by oral gavage with the AT1R antagonist, Losartan (TCI America, USA) (20 mg/kg; SHR-Los) or vehicle, and matched with control WKYs (n=6/group). After 4 weeks, all animals were transcardially perfused for immunohistochemistry analyses (section **Immunohistochemistry**).

TLR4 blockade

SHRs were treated daily with TAK-242 (Apex Bio, USA; MedChem Express, USA) dissolved in 1% dimethyl sulfoxide for 2 weeks (2 mg/kg; intraperitoneal [*i.p.]*;

SHR-TAK; n=16), and age-matched with sham SHRs (n=12) and WKYs (n=11). TAK-242 is a specific TLR4 inhibitor that binds Cys747 in the receptor's intracellular domain (Takashima et al., 2009), and which is able to cross the intact BBB (Hua et al., 2015; Wang, Wang, Fang, Chen, Xiong & Yang, 2013). The dosage was selected based upon preliminary studies in rats and comparable treatments in mice (Hua et al., 2015). A subgroup of animals (n=6/group) were transcardially perfused at the end of treatment (section **Immunohistochemistry**). All other animals underwent surgical procedures (section **Surgical procedures**) for evaluation of autonomic function and BBB permeability assessment (n=5 WKY, 6 SHR, 10 SHR-TAK).

Indirect blood pressure (BP) assessment

Indirect BP measurements were performed in all animals from both cohorts using a volume-pressure recording (VPR) tail-cuff system (CODA-6, Kent Scientific Corporation, USA) (Feng, Whitesall, Zhang, Beibel, D'Alecy & DiPetrillo, 2008). Animals were acclimated to the BP recording system for three days prior to the start of experiments. For data collection, 5 acclimation and 20 regular cycles were run, the latter of which were averaged to determine mean arterial pressure (MAP) values. BP measurements were taken on a weekly basis for animals in the Losartan cohort. For TAK-242-treated animals and age-matched controls, BP was assessed on alternating days.

Surgical procedures

Animals were anesthetized with isoflurane (induction: 5%, maintenance: 1%) and/or Ketamine-Xylazine (0.1 mL of 1:1 cocktail; *i.p.*). Anesthesia levels were monitored throughout procedures by tail- and toe-pinch.

Evaluation of autonomic function

The left femoral artery and vein were catheterized for BP and HR measurement, blood sample collection, and delivery of vasoactive drugs. Catheters were tunneled subcutaneously to the mid-scapular region, and lidocaine was applied to surgical sites. Animals recovered for 24 h and given Carprofen (0.1mL/100g BW/8 h; *i.p.*) for pain. The following day, cardiac parameters were recorded from unrestrained conscious rats by a pressure transducer (model SP 844, Memscap AS, Norway) system with computer data acquisition (Bridge Amp/PowerLab 4/35, ADInstruments, Australia). After 30 min. of baseline recording, intravenous (*i.v.*) doses of phenylephrine (Phe; 20 µg/kg; MilliporeSigma, USA) and sodium nitroprusside (SNP; 25 µg/kg; Spectrum Chemical, USA) were randomly administered, followed by the ganglionic blocker, hexamethonium bromide (20 mg/kg; Sigma, USA)(Ameer, Hildreth & Phillips, 2014; Cavalcanti et al., 2016). Drug doses were separated by 10-15 min. to allow cardiac parameters to return to baseline.

Baroreceptor reflex sensitivity was determined by heart rate compensation for a given change in MAP (ΔBPM [beats per minute]/ΔMAP [mmHg]), as evaluated at the maximal response. Indirect sympathetic nervous system (SNS) activity was indexed as

the magnitude of the depressor response to hexamethonium bromide, expressed as ∆MAP, relative to the 1-min. period immediately prior to injection.

BBB permeability

BBB permeability surgeries were performed as previously described (Biancardi, Son, Ahmadi, Filosa & Stern, 2014). In short, a fluorescent dye cocktail of rhodamine B isothiocyanate-dextran (RHO70; 70kDa, 10 mg/mL; Sigma-Aldrich, USA) and fluorescein isothiocyanate-dextran (FITC10; 10kDa, 10 mg/mL; Sigma-Aldrich, USA) dissolved in sterile saline was injected through the left carotid artery (3 µL/g BW) and allowed to circulate for 30 minutes. Brains were extracted, post-fixed in 4% formaldehyde (PFA; 48 h), cryoprotected in 30% sucrose (72 h), and stored at -80°C until sectioning.

Immunohistochemistry

Brains were removed following transcardial perfusion with 150 mL 0.01M phosphate-buffered saline (PBS) and 350 mL 4% PFA. Whole brains were post-fixed in 4% PFA for 24 h, cryoprotected in a 30% sucrose solution for 72 h, and stored at -80°C prior to sectioning. Serial hypothalamic slices of 30 µm (containing the PVN) and medullary sections of 40 µm (containing the RVLM and NTS), were collected (Microm cryostat HM 525) with reference to the Paxinos and Wilson (2009) *Rat Brain* atlas (**Figure 5-1**). Slices were stored in cryoprotectant solution (200 mL glycerol [RNAse-Free; Sigma, USA], 300 mL ethylene glycol [Aldrich, USA] 450 mL dH_2O, 75 mL 0.3 M PBS) at -20°C until processing.

Slices were washed three times in PBS to remove cryoprotectant solution and incubated for 1 hr with 10% normal donkey serum (Jackson ImmunoResearch, USA). Sections were incubated in PBST (0.01M PBS, 0.1% Triton, 0.04% NaN_3) with 5% normal donkey serum and primary antibodies against mouse monoclonal IgG2bκ TLR4 (1:250, Novus Biologicals, USA, NB100-56567) and rabbit polyclonal ionized calcium binding adaptor molecule 1 (IBA1) to visualize microglia (1:1000, Wako Chemicals, USA, 016-26721). For cytokine studies, sections were incubated with either mouse monoclonal IgG1 TNF-α (1:100, Santa Cruz Biotechnology, sc-52746) or mouse monoclonal IgG2a IL-6 (1:100, Santa Cruz Biotechnology, sc-32296). Hypothalamic slices were also incubated with guinea pig polyclonal (Arg8)-vasopressin (VP, 1:1000, Peninsula Laboratories, USA, T-5048.0050) and brainstem sections were incubated with either rabbit polyclonal tyrosine hydroxylase (1:2000, EnCor Biotechnology Inc., USA, RPCA-TH) or mouse monoclonal IgG2a TH (1:1000, Santa Cruz Biotechnology, USA, sc-25270) for use as anatomical markers. In addition, antigen-retrieval (5 min. incubation in PBS containing 1% w/v SDS) was performed prior to serum blocking in tissue sections used for microglial morphological analysis. **Table 5-1** contains a summary of primary antibodies used in this study. Slices were washed in PBS and incubated with respective Alexa Fluor® AffiniPure Donkey IgG (H+L) secondary antibodies from Jackson ImmunoResearch: 488 anti-mouse (1:250), 594 anti-rabbit (1:250), 594 anti-guinea pig (1:250), or 647 anti-rabbit (1:50). Sections were washed and mounted with Vectashield Antifade Mounting Medium (Vector Laboratories, USA).

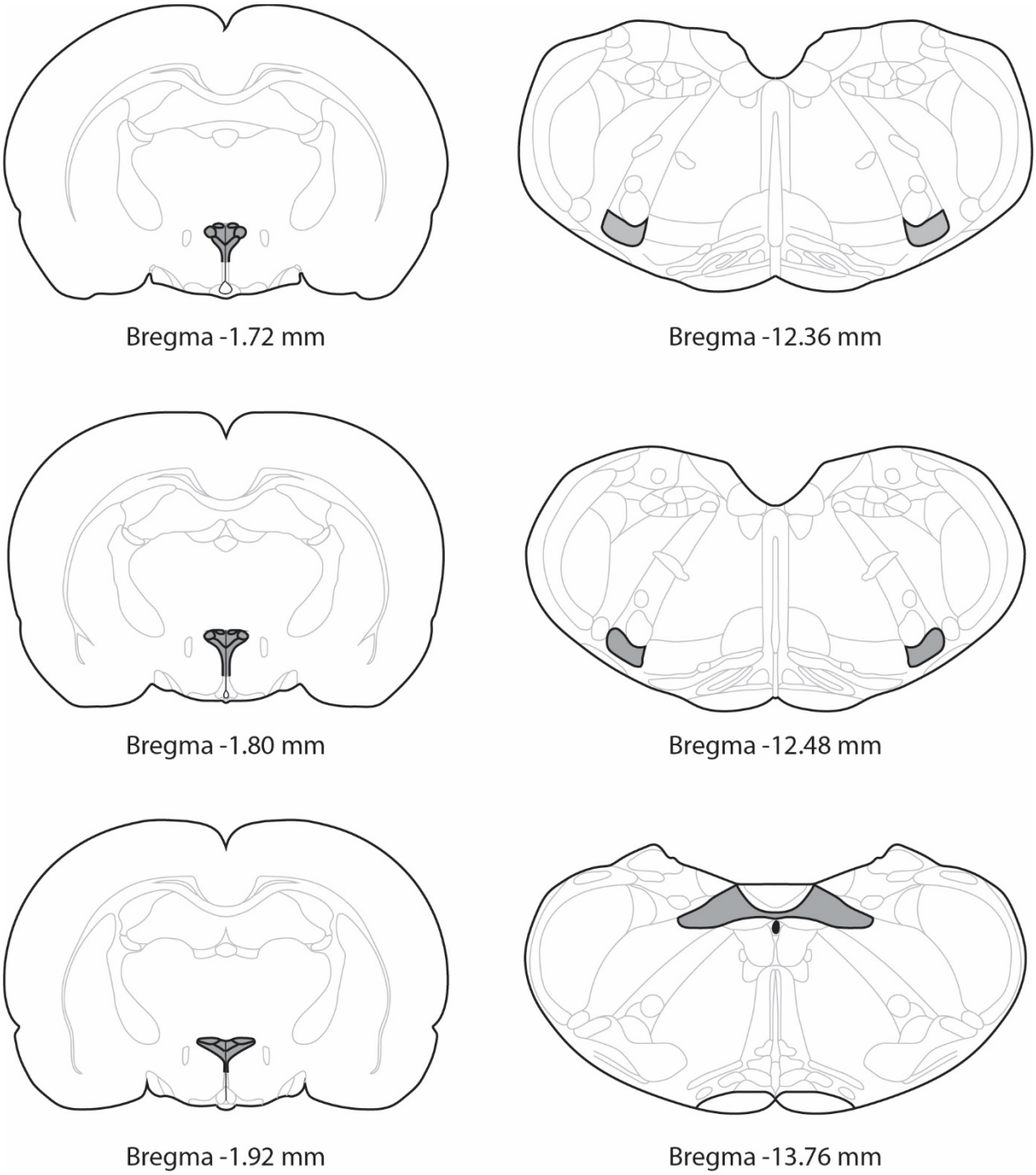

Figure 5-1. Line drawings of coronal sections collected for analysis from the hypothalamus and brainstem. Represented regions are based on the levels of the PVN (bregma -1.72, -1.80, and -1.92 mm), RVLM (bregma -12.36 and -12.48 mm), and NTS (bregma -13.76) illustrated by the *Rat Brain in Stereotaxic Coordinates* of Paxinos and Wilson (2006).

Target	Host	Clonality	Dilution	Supplier	Cat. Num.
TLR4	Mouse	Monoclonal IgG2bκ	1:250	Novus Biologicals	NB100-56567
IBA1	Rabbit	Polyclonal	1:1000	Wako Chemicals	019-19741
TNF-α	Mouse	Monoclonal IgG1	1:100	Santa Cruz Biotechnology	sc-52746
IL-6	Mouse	Monoclonal IgG2a	1:100	Santa Cruz Biotechnology	sc-32296
VP	Guinea pig	Polyclonal	1:1000	Peninsula Laboratories	T-5048.0050
TH	Rabbit	Polyclonal	1:2000	EnCor Biotechnology	RPCA-TH
TH	Mouse	Monoclonal IgG2a	1:1000	Santa Cruz Biotechnology	sc-25270

Table 5-1. Primary antibodies used in immunohistochemistry experiments.

Immunofluorescence imaging and analysis

Full-thickness confocal z-stacks (1-μm intervals) of PVN-, RVLM-, and NTS-containing brain sections were acquired with a Nikon Eclipse TE2000-E inverted microscope coupled to a Nikon A1 confocal laser and analyzed using ImageJ software (NIH; https://imagej.nih.gov/ij/index.html).

Protein density quantification

Confocal z-stacks were taken with a 20x objective in the PVN, RVLM, and NTS (1 image/nucleus/slice, 5-6 slices/animal). TLR4, IL-6, and TNF-α immunofluorescence signals (% area), expressed as % change from WKY, were quantified from maximum projection images, as previously described (Biancardi, Son, Ahmadi, Filosa & Stern, 2014) using ImageJ.

Microglia morphological analysis

Microglial activation status was examined with a skeletal analysis method adapted from Morrison and Filosa (Morrison & Filosa, 2013) using maximum projection images of IBA1 fluorescence at 60x-magnification in the PVN, RVLM, and NTS (3 images/unilateral nucleus/slice, 5-6 slices/animal). Briefly, threshold adjustments and noise reductions were applied to increase visualization of cell processes, images were converted to binary, skeletonized, and analyzed with the AnalyzeSkeleton plugin (http://github.com/fiji/AnalyzeSkeleton), as illustrated in **Figure 5-2**. The number of endpoints and total branch length per frame were used as morphological parameters reflective of relative branching complexity.

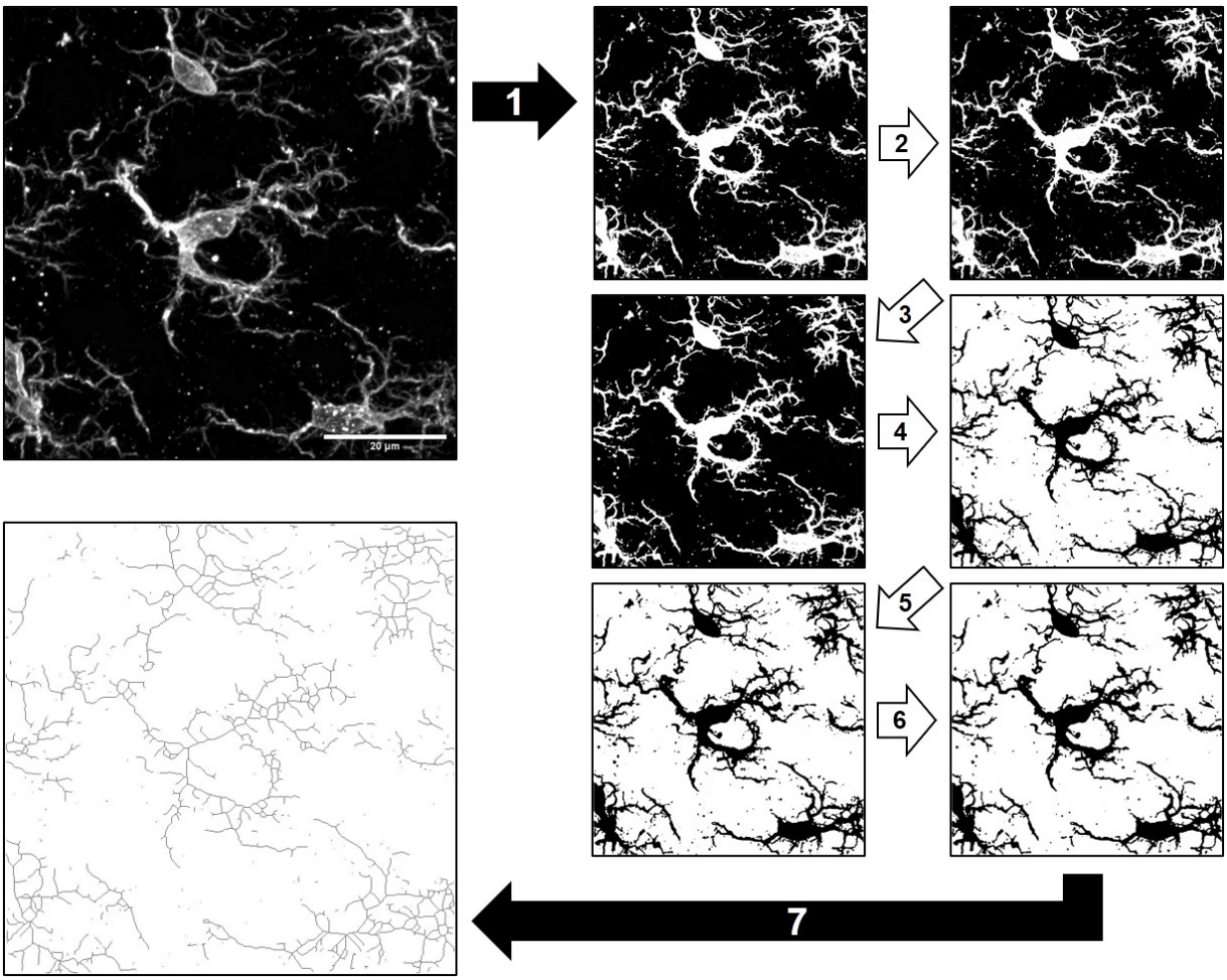

Figure 5-2. Skeletal analysis workflow for assessment of microglial morphology. Full-thickness confocal z-stack images of IBA1 immunostaining were taken with a 60x objective and converted to maximum projection images for digital processing in ImageJ. **(1)** Brightness and contrast adjustments to maximize visible branches/processes and minimize background noise; **(2)** Unsharp Mask applied to enhance visibility of details; **(3)** Despeckle filter applied to reduce noise; **(4)** image converted to binary; **(5)** binary "close-" operation applied to fill in small holes; **(6)** dark outliers removed; **(7)** final skeletonized image generated for analysis of process end points and branch length. Scale bar = 20µm.

BBB permeability assessment

Dye-injected brains were cut to 40 µm-thick sections containing the PVN and 50 µm-thick sections containing the RVLM and NTS, counterstained with VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, USA) or TOTO-3 Iodide (1:50,000, ThermoFisher Scientific, USA), and imaged with a 20x objective (1 image/nucleus/slice, 5-6 slices/animal). Extravasation of the low MW dye ($FITC10_{EV}$) was used as an index of BBB integrity. $FITC10_{EV}$ quantification was achieved by subtracting colocalized FITC10 and RHO70 pixels from a maximum projection of the FITC10 channel, and measuring the percent area of $FITC10_{EV}$ from the newly generated image, as illustrated in **Figure 5-3**.

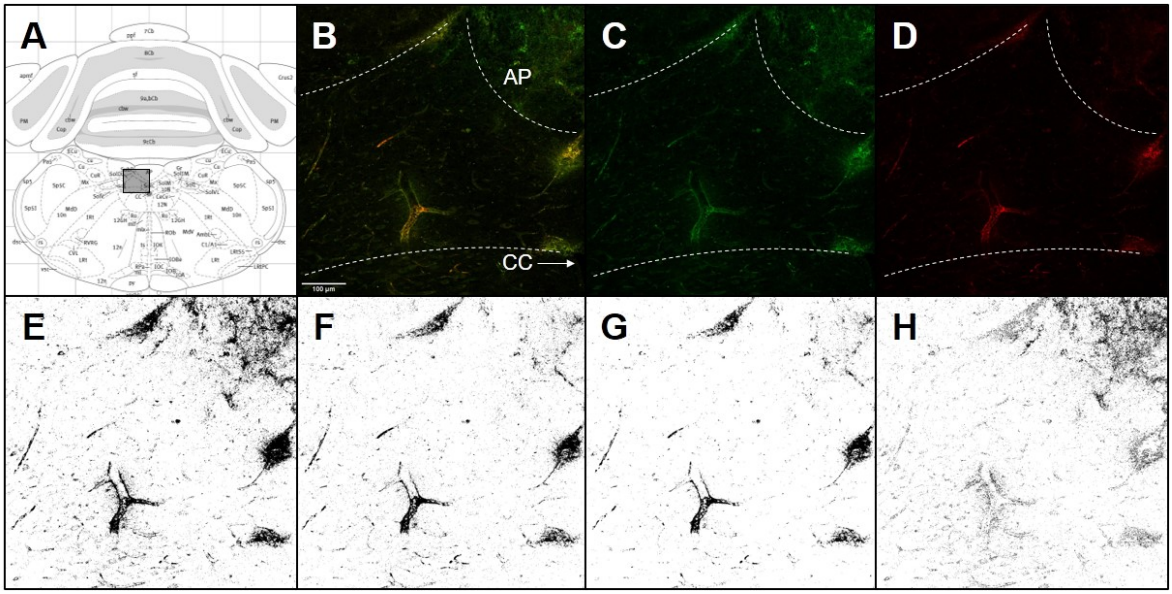

Figure 5-3. Example workflow for quantification of $FITC10_{EV}$. (A) Line drawing of coronal section containing NTS (shaded box) corresponding to image field-of-view. **(B)** Merged projection of FITC10 (green) and RHO70 (red) in the NTS. Maximum projection images of FITC10 **(C)** and RHO70 **(D)** are converted to binary (**E** and **F**, respectively) and colocalized, generating a new binary image **(G)**. Colocalized pixels are digitally subtracted from the initial FITC10 pixels (image **E**) to yield a final image containing extravasated FITC10 (i.e., non-colocalized) pixels (**H**), which are quantified to calculate % area $FITC_{EV}$. AP: area postrema; CC: central canal; scale bar = 100 µm.

Statistical Analyses

Data are reported as mean±*SEM*. Baroreceptor reflex sensitivity data was analyzed using non-linear regression. For all other analyses, groups were compared by one-way or two-way ANOVA with Tukey post-hoc tests. Analyses were run using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was considered at $p<0.05$.

Chapter 6. Results

Progression of hypertension development.

Following 4 weeks of AT1R blockade, SHR-Los MAP, as measured via indirect tail-cuff, (103.6±2.4 mmHg) was normalized to similar levels as observed in WKYs (100.0±3.0 mmHg, p=0.473), whereas SHR MAP (154.9±1.7 mmHg, p<0.0001) remained significantly elevated (12-13 weeks old, n=6/group; **Figure 6-1A**). Treatment with TAK-242 attenuated the MAP increases in SHR-TAK (n=16) that were observed in SHRs (n=12) beginning on day three (142.6±3.01 vs. 161±1.5 mmHg, p<0.0001), and persisting throughout the rest of the treatment period (day 14: 129.5±2.8 vs. 153.8±2.1 mmHg; p<0.0001). SHR-TAK MAP, while reduced, was significantly greater than MAP levels in WKYs at the end of treatment (109.2±2.9 mmHg, p<0.0001; **Figure 6-1B**). The trends observed in the TAK-242 cohort were confirmed via direct MAP recordings in unrestrained conscious animals at the end of treatment (WKY: 109.2±6.1 mmHg [n=5]; SHR: 169.4±13.5 mmHg [p<0.0001 vs. WKY, n=6]; SHR-TAK: 150.8±9.0 mmHg [p=0.003 vs. WKY, p<0.0001 vs. SHR, n=10]) (**Figure 6-1C**).

TLR4 protein expression.

To examine the role of AngII-AT1R signaling in regulating TLR4 protein expression, we performed immunofluorescence assays in the PVN, RVLM, and NTS (expressed as % change from WKY, **Figure 6-2**). Consistent with previous reports (Dange, Agarwal, Teruyama & Francis, 2015b; Li et al., 2016b; Xu et al., 2020), TLR4 protein density was increased in SHR PVN compared to WKY (+133.0±14.7%, p<0.0001)

and normalized in SHR-Los (+27.8±10.6%) and SHR-TAK (+10.7±4.9%). TLR4 expression was elevated in SHR RVLM (+107.9±6.7%, p<0.0001) and normalized in SHR-Los (-1.05±4.6%) and SHR-TAK (+11.3±3.1%). Similarly, NTS TLR4 expression was higher in SHR (+101.6±6.9%, p<0.0001), with normalization in both treatment groups (SHR-Los: +11.0±7.4%; SHR-TAK: +1.5±4.7%), suggesting feed-forward upregulation of PVN, RVLM, and NTS TLR4 in SHRs that is dependent upon AT1R and TLR4.

Pro-inflammatory cytokine expression.

Using a semi-quantitative densitometry analysis, we examined IL-6 and TNF-α immunofluorescence in the PVN (**Figure 6-3**), RVLM (**Figure 6-4**), and NTS (**Figure 6-5**). Protein density (expressed as % change from WKY) of both cytokines was increased in SHR PVN (IL-6: +46.60±4.8%, p<0.0001; TNF-α: +57.97±8.9%, p<0.0001), RVLM (IL-6: +77.44±11.6%, p<0.0001; TNF-α: +58.01±7.9%, p<0.0001), and NTS (IL-6: +49.3±4.1%, p<0.0001; TNF-α: +56.5±4.3%, p<0.0001) versus WKYs. PVN cytokine expression was normalized with Losartan (IL-6: +0.85±5.0%; TNF-α: +5.99±3.9%) and TAK-242 treatment (IL-6: +1.30±2.9%; TNF-α: +5.70±2.6%). NTS IL-6 and TNF-α were normalized in SHR-Los (IL-6: +8.8±6.1; TNF-α: +3.5±4.8%) and SHR-TAK (IL-6: +5.6±4.0%; TNF-α: +6.7±4.3%). Both treatments restored RVLM TNF-α expression to baseline (SHR-Los: +8.16±4.7%; SHR-TAK: +4.48±5.2%), and reduced IL-6 (SHR-Los: +32.37±3.9% [p=0.0011 vs. WKY, p=0.0013 vs. SHR]; SHR-TAK: +33.90±7.6% [p=0.0023 vs. WKY, p=0.0017 vs. SHR]), suggesting that both AngII and TLR4 are involved in regulating the proinflammatory profile of CNS cardioregulatory nuclei in SHRs.

Skeletal analysis of microglial morphology.

We quantified morphological changes of microglia using a skeletal analysis(Morrison & Filosa, 2013) to index activation status. The classically activated proinflammatory microglial phenotype is associated with an enlarged cell soma and overall deramification, such that a relative reduction in branching complexity is reflective of increased activation. Within the PVN, SHRs showed a significant reduction in both end points (-36.1±3.6%, p<0.0001) and branch length (-26.8±4.7%, p<0.0001) compared to WKYs, indicating a significant increase in microglial activation in hypertensive animals. These values were normalized in the PVN of SHR-Los (end points: 2.9±2.2%; branch length: 11.3±2.5%) and SHR-TAK (end points: 4.7±5.6%; branch length: 4.0±5.1%). The reduction in end points and branch length in SHR RVLM tissue (end points: -29.3±3.3%, p<0000.1; branch length: -26.9±2.9, p=0.0004) compared to WKY was restored in SHR-Los (end points: -4.3±1.5%; branch length: -2.9±4.8%) and SHR-TAK (end points: 3.8±4.8%; branch length: 8.7±3.7%) (**Figure 6-6**), with a similar trend observed in the NTS (**Figure 6-7**) of SHRs (end points: -53.1±2.0%, p<0.0001; branch length: -57.2±2.0%, p<0.0001), SHR-Los (end points: +1.7±1.7%; branch length: -7.1±1.8%), and SHR-TAK (end points: +6.8±3.2%; branch length: -3.2±3.0%). These findings confirm a regulatory role for AngII in promoting microglial activation and support a contribution of TLR4 stimulation in mediating said AngII-induced activation.

BBB disruption.

To examine the role of TLR4 in BBB permeability alterations, we quantified the degree of extravasation of a low molecular weight dextran-conjugated fluorescent dye

($FITC10_{EV}$) in the PVN, RVLM, and NTS of SHR-TAK. As previously described (Biancardi, Son, Ahmadi, Filosa & Stern, 2014), both FITC10 and the simultaneously injected high molecular weight dye, RHO70, are maintained within cerebral vasculature when the BBB is intact. Conversely, BBB disruption results in FITC10 leakage from the vasculature to the parenchyma, reflected by an increased $FITC10_{EV}$ % area. We found significant BBB disruption in the PVN (3.619±0.108% area, p<0.0001) and RVLM (3.62±0.11% area, p<0.0001) of control SHRs relative to WKYs (PVN: 1.56±0.05% area; RVLM: 1.57±0.06% area). Inhibition of TLR4 restored the barrier's integrity in the PVN (1.52±0.05% area) and RVLM (1.51±0.05% area) (**Figure 6-8**). In SHR NTS, $FITC_{EV}$ was significantly greater than that observed in WKYs (3.588±0.09% area in SHR vs. 1.800±0.04% area in WKYs, p<0.0001). As in the PVN and RVLM, we found NTS $FITC10_{EV}$ to be normalized in SHR-TAK (1.801±0.0513% area) (**Figure 6-9**). Given our prior work demonstrating a reliance of PVN, RVLM, and NTS BBB disruption upon AngII-AT1R signaling in SHRs, these data indicate that activation of TLR4 is a potential mechanism by which AngII promotes BBB disruption.

Baroreceptor reflex sensitivity.

We evaluated baroreceptor-heart rate reflex (baroreflex sensitivity) in response to TAK-242 treatment using bolus *i.v.* injections of the vasoactive drugs, phenylephrine (Phe) and sodium nitroprusside (SNP), in conscious animals during continuous direct BP and heart rate recording (Ameer, Hildreth & Phillips, 2014; Cavalcanti et al., 2016) (**Fig. 6-10**). SHRs showed a reduced HR compensation for MAP changes compared to WKYs following Phe (-1.473±0.2 vs -2.560±0.3 bpm/mmHg; p<0.0001) and SNP (-

0.9083±0.3 vs -2.390±0.7 bpm/mmHg; *p*=0.0002). TAK-242 treatment rescued SHR responsiveness to both Phe (-2.204±0.3 bpm/mmHg) and SNP (-2.061±0.4 bpm/mmHg), demonstrating a sizeable contribution of TLR4 activation to the impaired baroreflex sensitivity observed in SHRs.

Indirect SNS activity.

The magnitude of depressor response to hexamethonium bromide was used to index sympathetic activity in conscious animals (Ameer, Hildreth & Phillips, 2014) (**Fig. 6-11**). Compared to WKYs (-46.7±4.2mmHg), SHRs (-65.1±3.1mmHg, *p*=0.0396) exhibited a greater depressor response. Conversely, sympathetic activity was restored to normal values with TLR4 inhibition in SHR-TAK (-42.9±4.3mmHg).

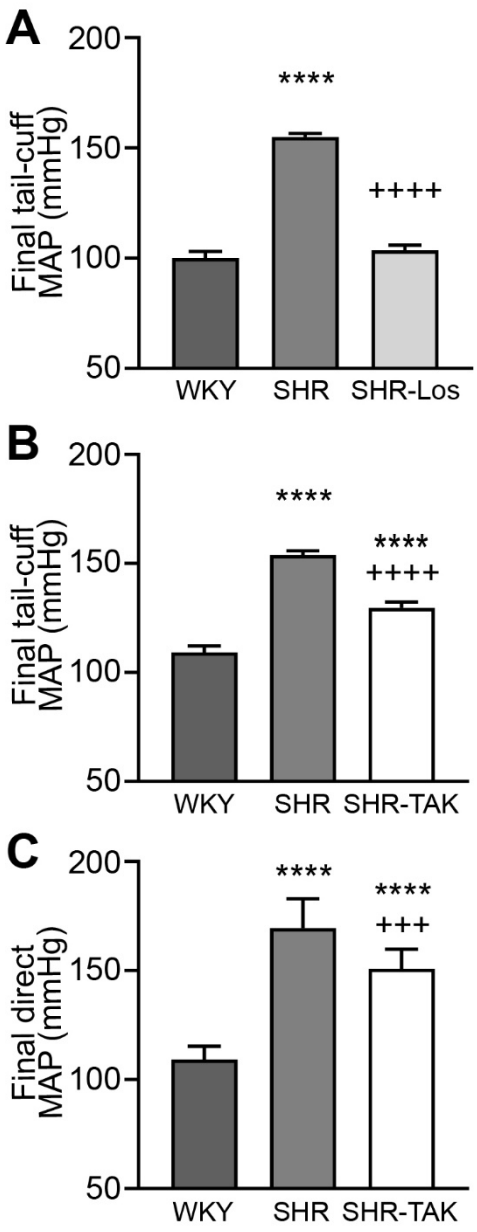

Figure 6-1. Progression of MAP elevation in SHRs is dependent upon AT1R and TLR4. Final indirect tail-cuff mean arterial pressure (MAP; mmHg) of Losartan cohort (n=6/group) (**A**) and TAK-242 cohort (n=11 WKY, 12 SHR, 16 SHR-TAK) (**B**) at conclusion of respective treatment periods. (**C**) Final direct MAP of WKY (n=5), SHR (n=6), and SHR-TAK (n=10), measured via femoral catheterization in conscious rats. Data were evaluated by one-way ANOVA with Tukey post-hoc analysis and are shown as mean±*SEM*; ****p<0.0001 vs WKY; +++p<0.001 vs SHR; ++++p<0.0001 vs SHR; MAP: mean arterial pressure.

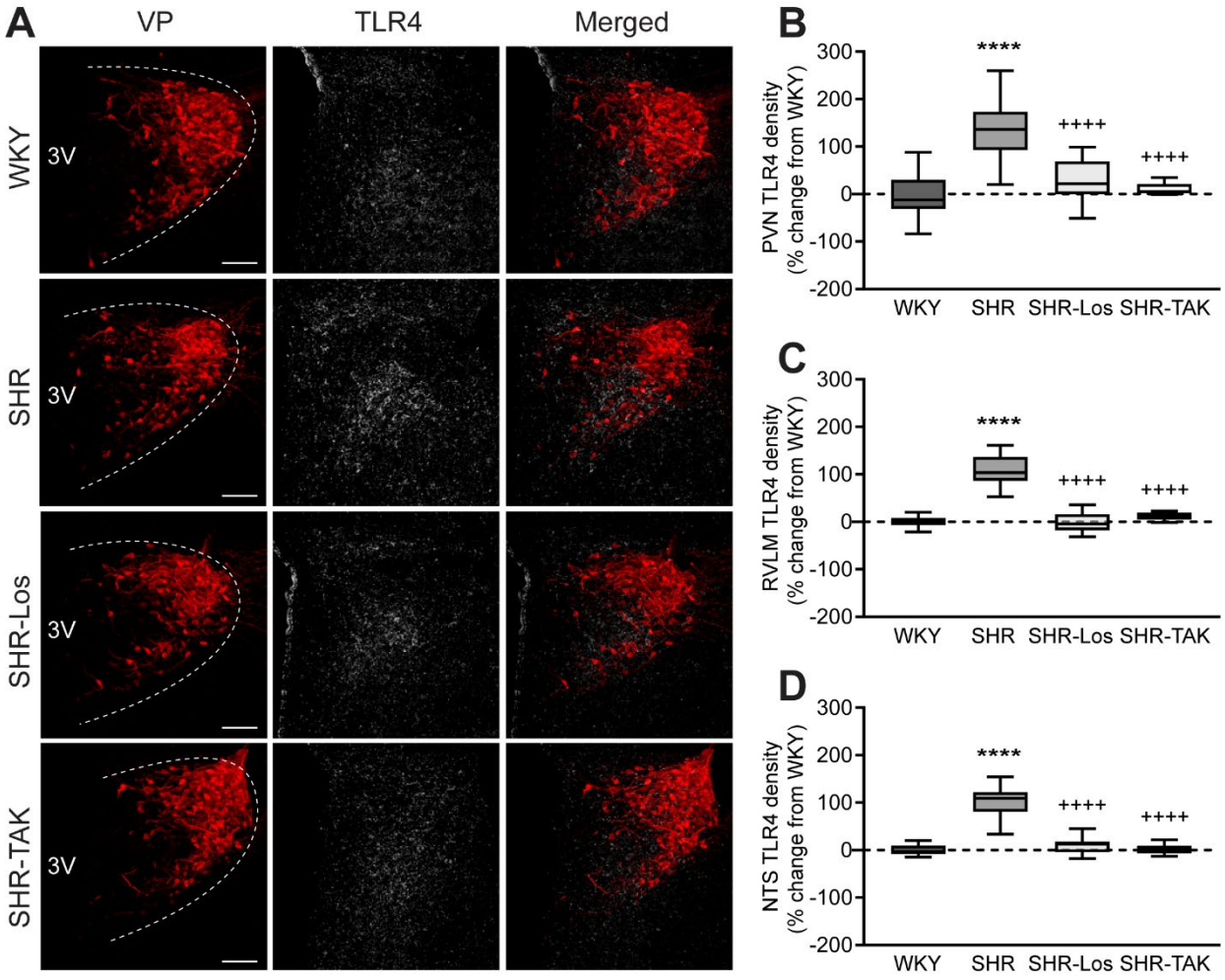

Figure 6-2. TLR4 protein expression elevations are mediated by AngII through AT1R in SHRs. Example confocal maximum projection images of PVN vasopressin (VP; red) and TLR4 (white) from WKY, SHR, SHR-Los, and SHR-TAK (n=6 animals/group) (**A**). Percent change in TLR4 staining (% area) compared to WKY in the PVN (**B**), RVLM (**C**), and NTS (**D**). Data evaluated by one-way ANOVA with Tukey post-hoc analysis; shown as mean±*SEM*; ****p<0.0001 vs. WKY; ++++p<0.0001 vs. SHR; scale bars: 100μm; 3V: third ventricle.

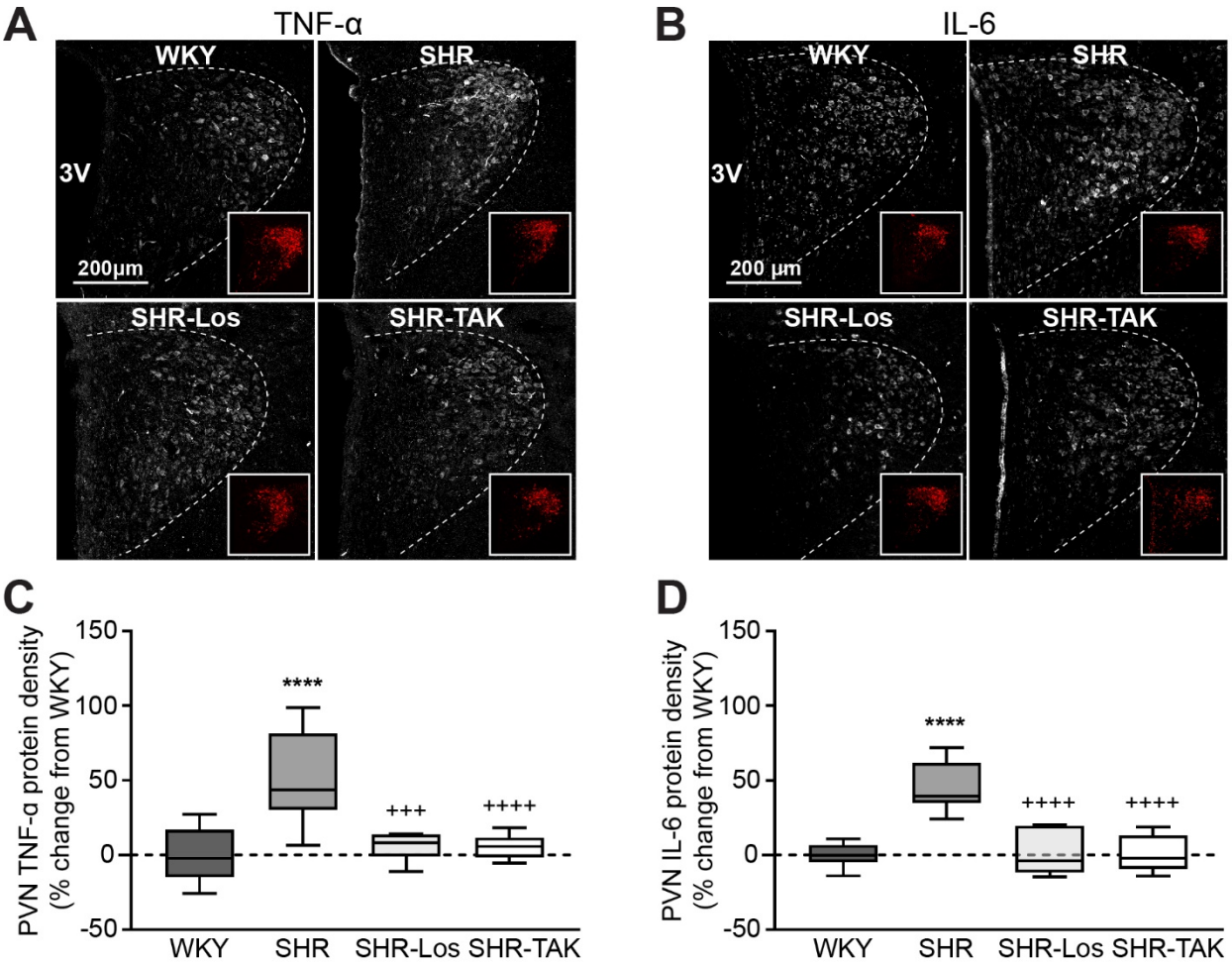

Figure 6-3. PVN pro-inflammatory cytokine density. Representative confocal maximum projection images of PVN TNF-α (**A**) and IL-6 (**B**) immunofluorescence (white) with respective anatomic marker, vasopressin (insets; red) in WKY, SHR, SHR-Los, and SHR-TAK (n=6 animals/group). Box-plot diagrams of TNF-α (**C**) and IL-6 (**D**) protein density in the PVN, calculated as change in % area relative to WKY. Data evaluated by one-way ANOVA with Tukey post-hoc analysis; shown as mean±*SEM*; ****p<0.0001 vs. WKY; +++p<0.001 vs. SHR; ++++p<0.0001 vs. SHR; scale bars: 200μm; 3V: third ventricle.

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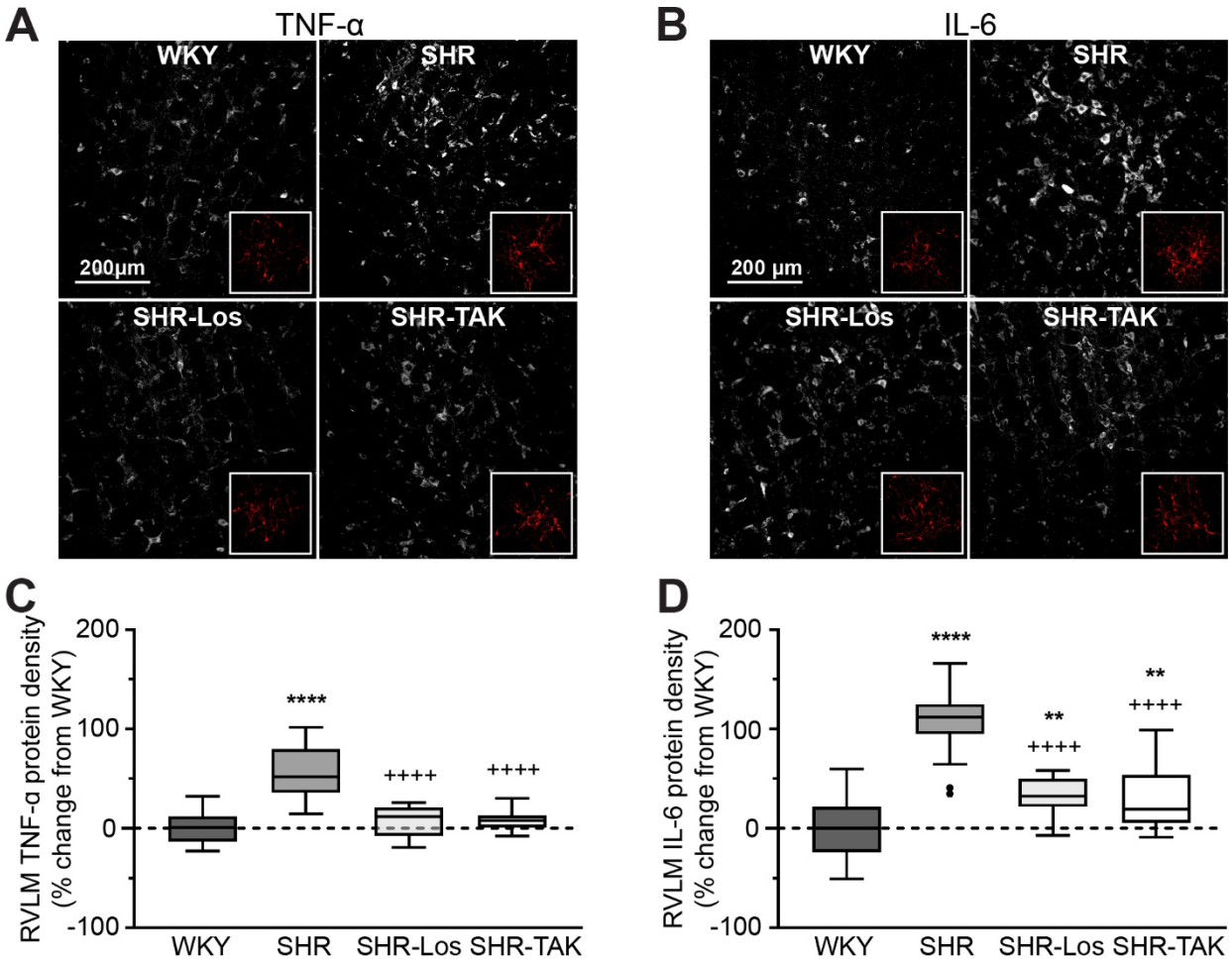

Figure 6-4. RVLM pro-inflammatory cytokine density. Representative confocal maximum projection images of RVLM TNF-α (**A**) and IL-6 (**B**) immunofluorescence (white) with respective anatomic marker, tyrosine hydroxylase (insets; red) in WKY, SHR, SHR-Los, and SHR-TAK (n=6 animals/group). Box-plot diagrams of TNF-α (**C**) and IL-6 (**D**) protein density in the RVLM, calculated as change in % area relative to WKY. Data evaluated by one-way ANOVA with Tukey post-hoc analysis; shown as mean±*SEM*; ***p*<0.01 vs WKY; *****p*<0.0001 vs WKY; ++++*p*<0.0001 vs SHR; scale bars: 200μm.

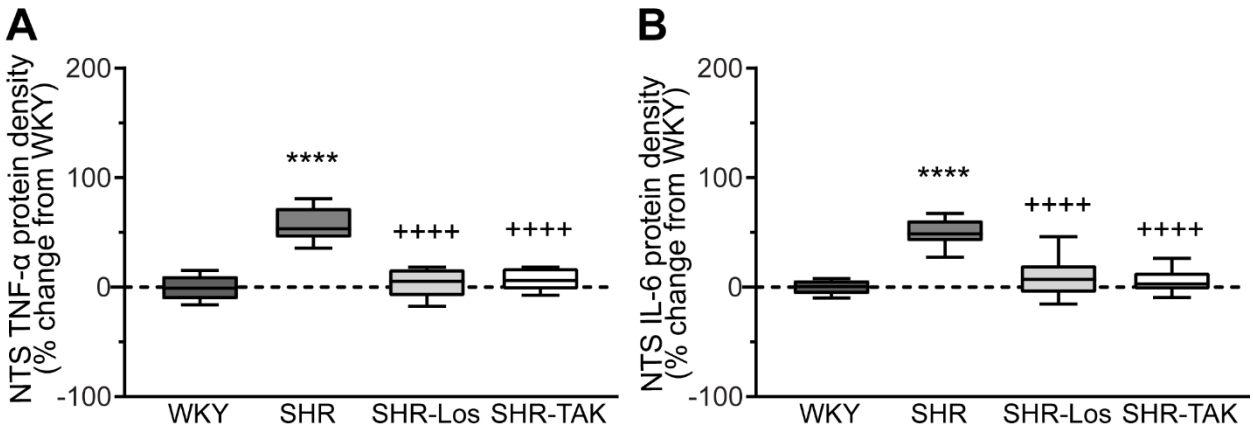

Figure 6-5. NTS pro-inflammatory cytokine density. Box-plot diagrams of TNF-α (A) and IL-6 (B) protein density in the NTS of WKY, SHR, SHR-Los, and SHR-TAK (n=6 animals/group), calculated as change in % area relative to WKY. Data evaluated by one-way ANOVA with Tukey post-hoc analysis; shown as mean±*SEM*; ***p*<0.01 vs. WKY; *****p*<0.0001 v.s WKY; ++++*p*<0.0001 vs. SHR.

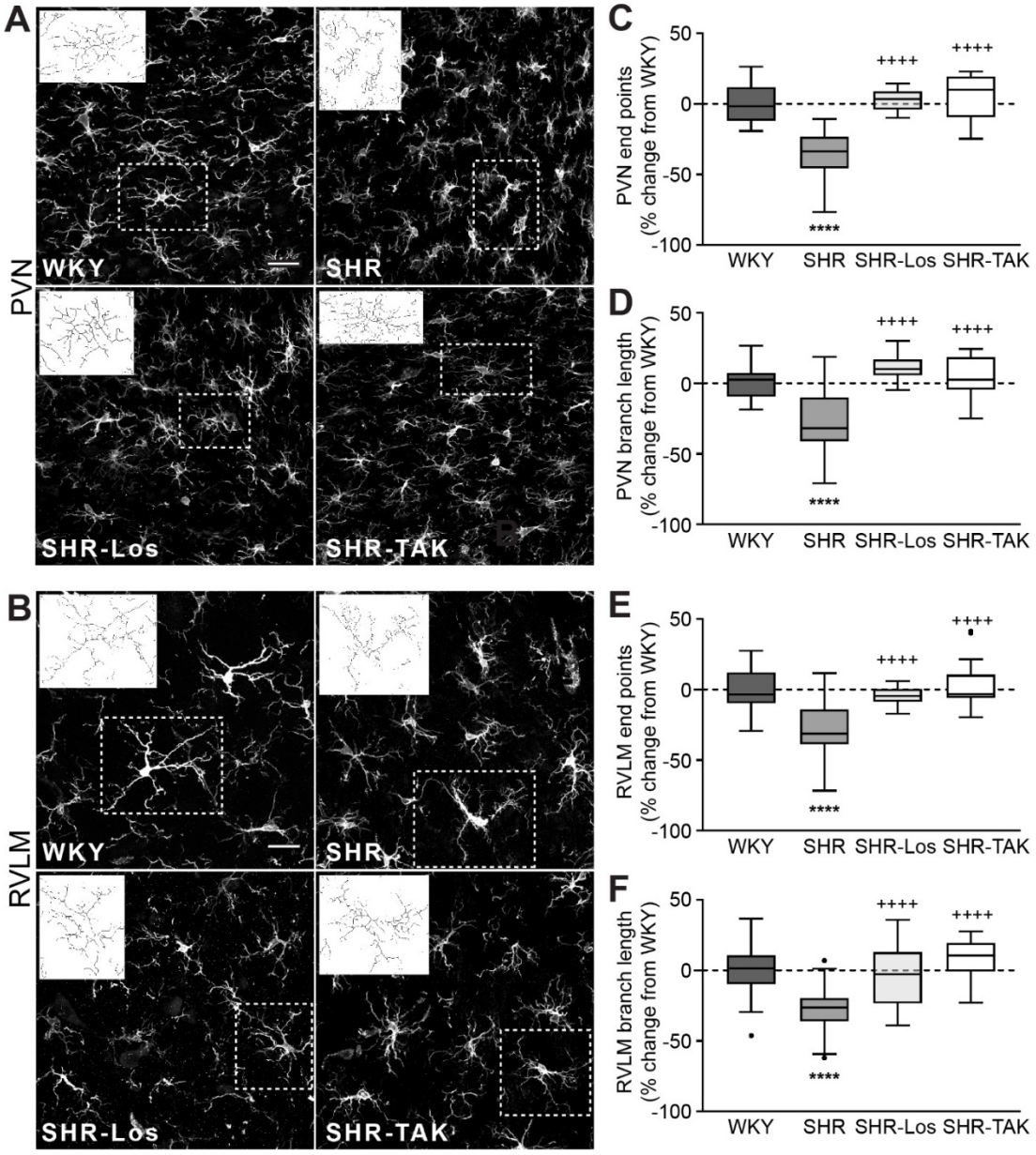

Figure 6-6. AngII mediates PVN and RVLM microglial activation through AT1R and TLR4. Representative confocal maximum projection images of IBA1 (microglial marker; white) in the PVN (**A**) and RVLM (**B**) of WKY, SHR, SHR-Los, and SHR-TAK (n=6 animals/group). Maximum projection images were converted to binary and skeletonized, as illustrated by inset skeletons of outlined microglia. Percent change in PVN end points (**C**), PVN branch length (**D**), RVLM end points (**E**) and RVLM branch length (**F**) relative to WKY. Data evaluated by two-way ANOVA with Tukey post-hoc analysis; shown as mean±*SEM*; ****p<0.0001 vs. WKY; ++++p<0.0001 vs. SHR; scale bars: 50μm.

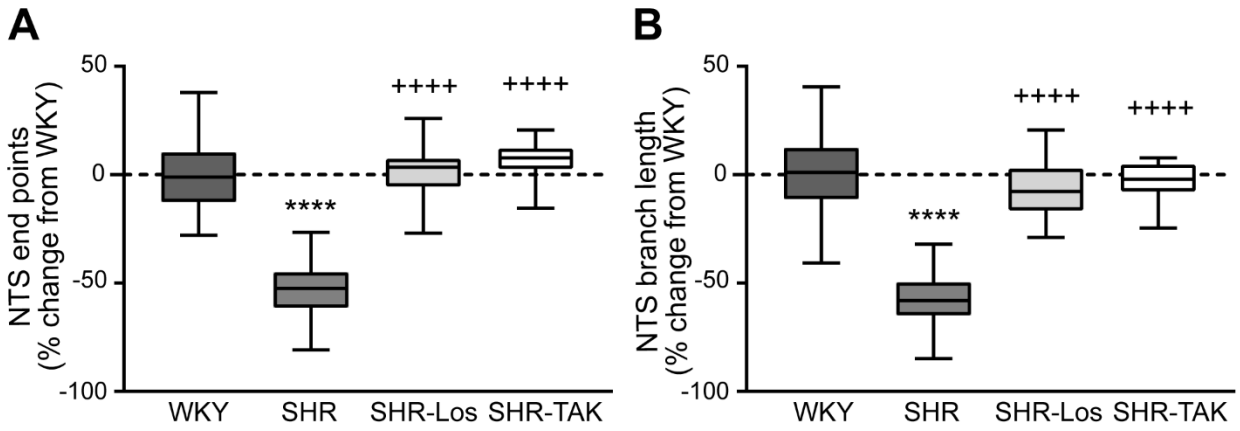

Figure 6-7. Skeletal analysis of microglial morphology in the NTS. Percent change in NTS end points (A) and branch length (B) relative to WKY in the NTS of WKY, SHR, SHR-Los, and SHR-TAK (n=6 animals/group). Data evaluated by two-way ANOVA with Tukey post-hoc analysis; shown as mean±*SEM*; ****p<0.0001 vs. WKY; ++++p<0.0001 vs. SHR.

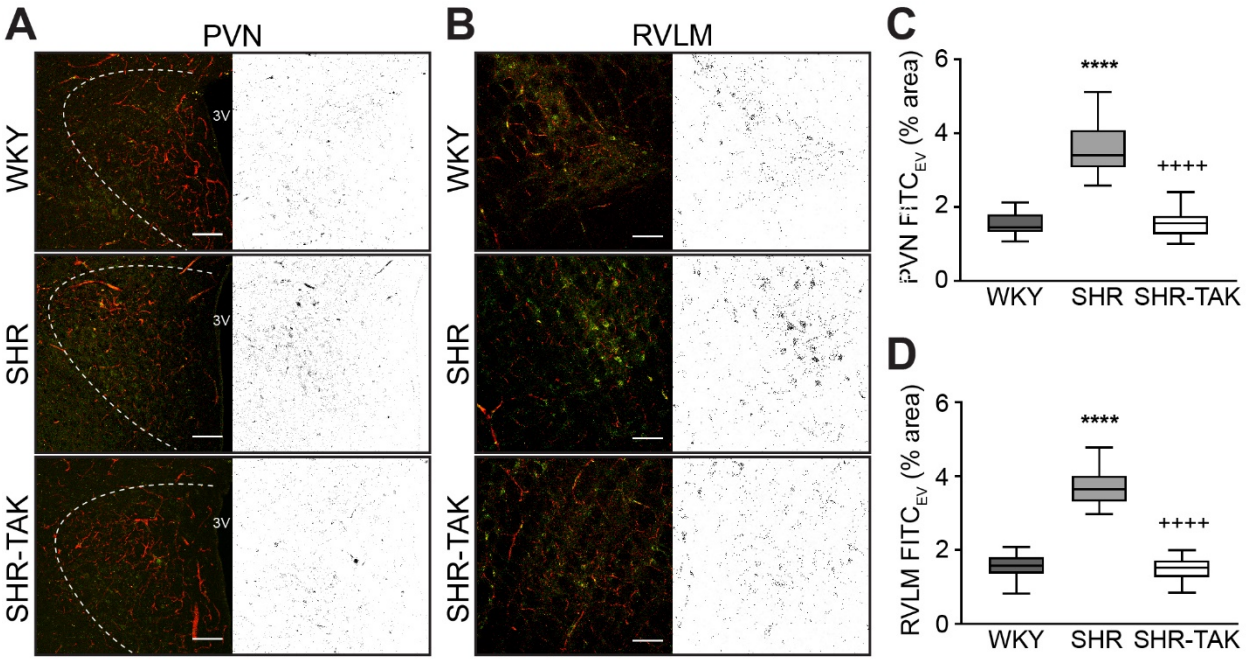

Figure 6-8. TLR4 inhibition restores PVN and RVLM BBB integrity in SHRs. Representative confocal maximum projection images showing FITC10 (green) and RHO70 (red) in the PVN (**A**) and RVLM (**B**) of WKY, SHR, and SHR-TAK (n=6 animals/group), with corresponding images of extravasated FITC10 (FITC10$_{EV}$). Box-plot diagrams of FITC10$_{EV}$ (% area) in the PVN (**C**) and RVLM (**D**). Data evaluated by one-way ANOVA with Tukey post-hoc analysis; shown as mean±*SEM*; ****p<0.0001 vs. WKY; ++++p<0.0001 vs. SHR; scale bars: 100μm; 3V: third ventricle.

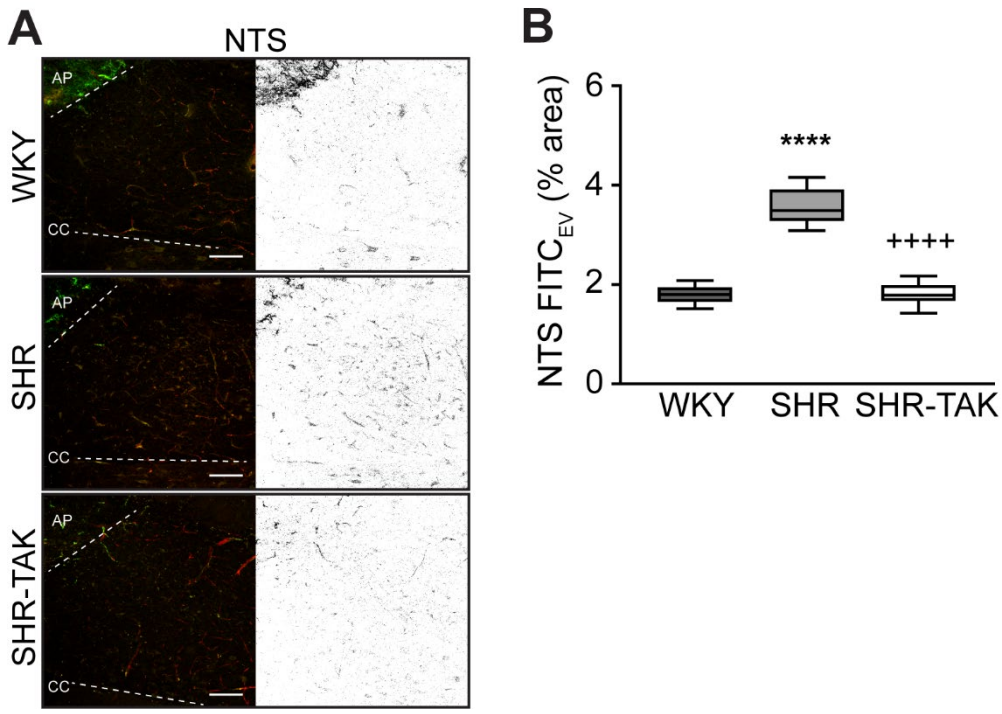

Figure 6-9. TLR4 inhibition restores NTS BBB integrity in SHRs. Representative confocal maximum projection images showing FITC10 (green) and RHO70 (red) in the NTS (**A**) of WKY, SHR, and SHR-TAK (n=6 animals/group), with corresponding images of extravasated FITC10 ($FITC10_{EV}$). Box-plot diagram of $FITC10_{EV}$ (% area) in the NTS (**B**). Data evaluated by one-way ANOVA with Tukey post-hoc analysis; shown as mean±*SEM*; ****p<0.0001 vs. WKY; ++++p<0.0001 vs. SHR; scale bars: 100μm; AP: area postrema; CC: central canal; NTS: nucleus tractus solitarius.

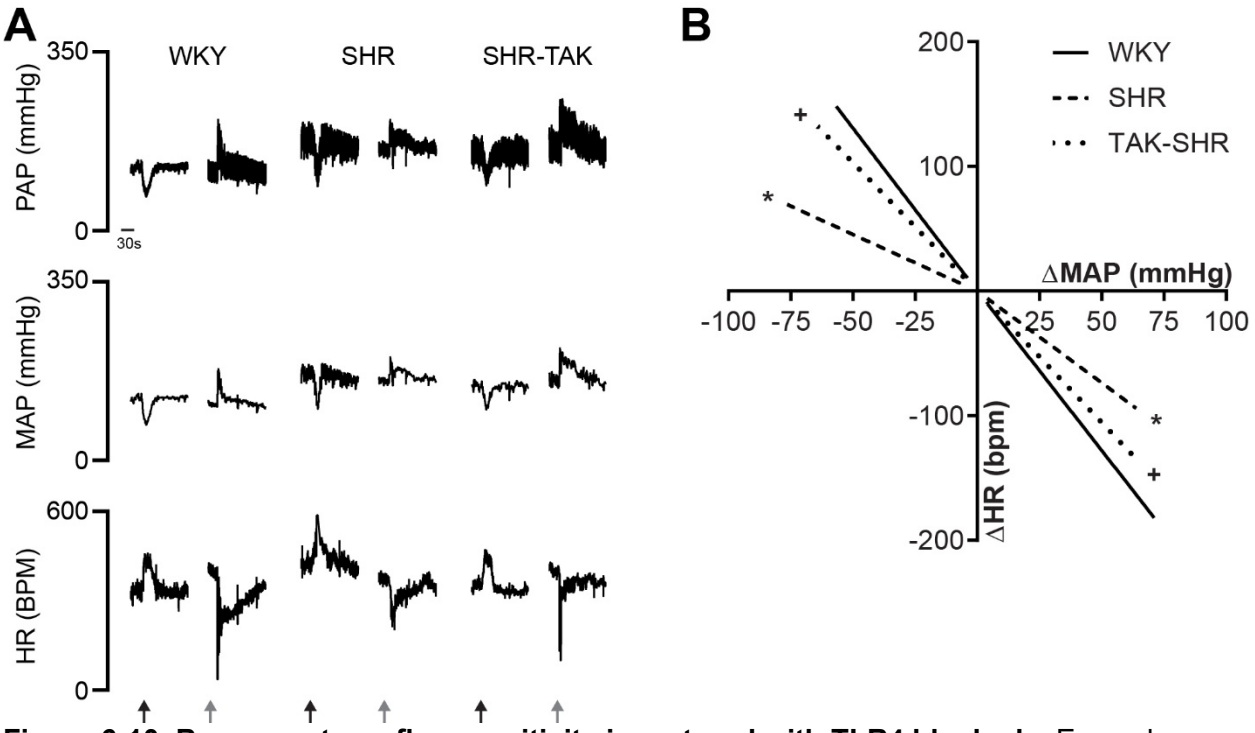

Figure 6-10. Baroreceptor reflex sensitivity is restored with TLR4 blockade. Example tracings (**A**) of pulsatile arterial pressure (PAP; mmHg), mean arterial pressure (MAP; mmHg), and heart rate (HR; BPM) showing responses to injection of sodium nitroprusside (black arrows) and phenylephrine (gray arrows) in one animal from each group. Linear regression graph (**B**) of depressor response to sodium nitroprusside (top-left quadrant) and pressor response to phenylephrine (bottom-right quadrant) in WKY (n=5), SHR (n=6), and TAK-242-treated SHR (SHR-TAK, n=10). *p<0.05 vs. WKY; +p<0.05 vs. SHR.

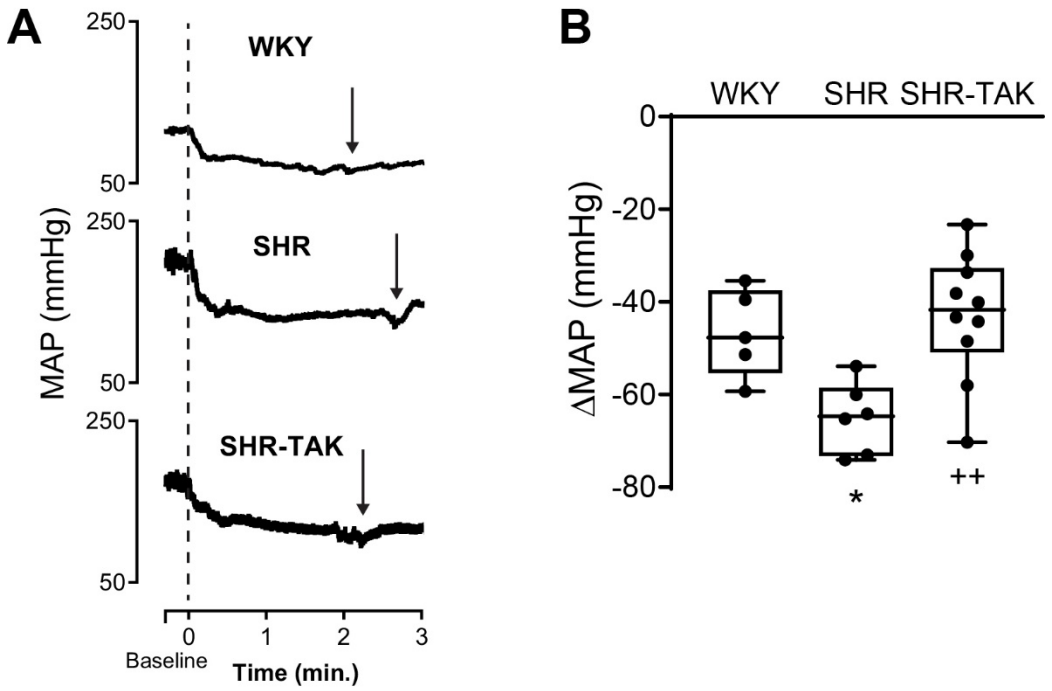

Figure 6-11. TLR4 blockade abolishes sympathoexcitation in SHRs. Example tracings (**A**) of mean arterial pressure (MAP; mmHg) showing maximal responses to ganglionic blockade with hexamethonium bromide (arrows) in one animal from each group. Box-plot diagram of response magnitude (**B**) represented as ΔMAP (mmHg) from baseline values in WKY (n=5), SHR (n=6), and TAK-242-treated SHR (SHR-TAK, n=10). Data are shown as mean±*SEM*, **p*<0.05 vs WKY, ++*p*<0.01 vs SHR.

Chapter 7. Discussion

In the present study, we investigated the relative contributions of AT1R and TLR4 activation to the maintenance of neuroinflammation, BBB disruption, and sympathoexcitation in hypertensive SHRs. Our results demonstrate that TLR4 inhibition mitigates TLR4 and PIC upregulation, combats microglial activation, and preserves BBB integrity in the PVN, RVLM, and NTS of SHRs. Likewise, AT1R blockade normalizes TLR4 expression and microglial activation in the PVN, RVLM, and NTS of SHRs, and reduces PIC expression in these nuclei to the same extent as TLR4 inhibition. Furthermore, TLR4 blockade attenuates the progression of MAP increases in SHRs and protects against autonomic dysfunction. These findings support a significant role for central TLR4 activity in the maintenance of hypertension and suggest that chronic stimulation of central TLR4 is largely dependent upon AT1R-mediated AngII signaling.

A number of studies implicate TLR4 activity throughout the body in the pathophysiology of hypertension (see(Nunes, de Oliveira, Lima & Webb, 2019; Nunes, de Oliveira, Mowry & Biancardi, 2019)). Specific to the CNS, the majority of studies have focused on alterations either within the PVN or following targeted PVN interventions. Upregulation of PVN TLR4 mRNA and protein expression has been reported in multiple models of hypertension (Dange et al., 2014b; Dange, Agarwal, Teruyama & Francis, 2015b; Li et al., 2016b; Qi et al., 2019; Xu et al., 2020; Yang et al., 2020). Blockade of PVN TLR4 reduces TNF-α and IL-1β production, mitigates sympathetic activity, and lowers BP (Dange et al., 2014b; Dange, Agarwal, Teruyama & Francis, 2015b). PVN-specific AT1R inhibition similarly decreases TLR4-dependent

TNF-α, IL-1β, and IL-6 levels in SHRs(Li et al., 2016b). Ogawa *et al.* have demonstrated that brainstem TLR4 stimulation via AT1R contributes to elevated sympathetic activity in chronic heart failure (Ogawa, Hirooka, Kishi & Sunagawa, 2011) and that silencing brain TLR4 RNA dampens sympathetic activity and ameliorates the cardiac remodeling observed in this disease pathology (Ogawa, Hirooka, Kishi, Ide & Sunagawa, 2013). However, to the best of our knowledge, the relative contribution of TLR4 activation to alterations within cardioregulatory nuclei of the brainstem has yet to be investigated in a genetic model of hypertension.

Consistent with the aforementioned studies, we found increases in TLR4, TNF-α, and IL-6 protein expressions in the PVN of SHRs that were dependent upon AT1R and TLR4 activation. Within the RVLM and NTS, we observed that TLR4 was likewise increased in an AT1R- and TLR4-dependent manner in SHRs, pointing to a feed-forward mechanism of TLR4 upregulation in each of the cardioregulatory nuclei examined. Whereas heightened TNF-α levels in the RVLM were normalized following AT1R or TLR4 blockade, IL-6 expression, while reduced relative to untreated SHRs, remained elevated compared to normotensive animals. Interestingly, while SHR TNF-α increased to a similar extent within all three nuclei, the magnitude of increased IL-6 expression was approximately 30% greater in the RVLM than in the PVN or NTS. The observation that AT1R and TLR4 blockades reduce RVLM IL-6 levels to +32.37% and +33.90% relative to WKYs, respectively, points to separate AT1R- and TLR4-independent mechanism(s) of IL-6 upregulation in the RVLM of SHRs that is absent in the PVN and NTS. Of note, we recently reported on PICs within CNS cardioregulatory nuclei of SHRs treated with a probiotic (kefir) and observed a similar trend – PVN IL-6

expression was normalized in SHRs following treatment, whereas RVLM IL-6 expression remained elevated relative to WKYs by a magnitude corresponding to the difference between RVLM and PVN IL-6 levels in the untreated SHRs (de Almeida Silva, Mowry, Peaden, Andrade & Biancardi, 2020). Based on the evidenced RAS- (Brasil et al., 2018) and TLR4- (Gomez-Guzman et al., 2015) modulatory capabilities of kefir, it is possible that a similar mechanism is responsible for the persistence of elevated RVLM IL-6 expression in kefir-, Losartan-, and TAK-242-treated SHRs.

Within the CNS, TLR4 is primarily expressed by microglia, with relatively low levels detected in astrocytes and neurons (Gorina, Font-Nieves, Marquez-Kisinousky, Santalucia & Planas, 2011; Olson & Miller, 2004). The presence of AT1R in microglia allows for AngII-mediated microglial activation (Biancardi, Stranahan, Krause, de Kloet & Stern, 2016), leading to increased ROS production and PIC synthesis. Moreover, the neuroinflammatory effects of AngII via microglial AT1R are evidenced factors underlying sympathoexcitation in neurogenic hypertension (Shi et al., 2010; Waki, Gouraud, Maeda, Raizada & Paton, 2011; Zubcevic, Waki, Raizada & Paton, 2011). Microglial inhibition in the PVN blunts the hypertensive response to AngII (Shi et al., 2010). Upon targeted microglial deletion in AngII- and L-NAME-induced hypertension, Shen *et al.* observed a significant drop in blood pressure, neuroinflammation, renal NE, and circulating arginine vasopressin (Shen et al., 2015). We have previously demonstrated that the ability of AngII to increase PVN microglial density and ROS production is dependent upon the presence of functional TLR4 (Biancardi, Stranahan, Krause, de Kloet & Stern, 2016). Herein, we found microglial activation in SHRs to be normalized in the PVN, RVLM, and NTS following either AT1R or TLR4 inhibition. A recent study by

Cohen et al. (2019) employed a similar skeletal analysis technique to examine microglia in the RVLM of 15-week-old SHRs, reporting decreased microglial density and branch length, with no differences in branch number or end points compared to WKYs (Cohen et al., 2019). One potential explanation for our observation of end point reductions is the difference in age, our studies being carried out at approximately 12-weeks of age. However, we would suggest that these differences are likely due to variations in staining and imaging techniques that allowed for enhanced visualization of fine microglial processes.

The potential contribution of astrocytic and/or neuronal TLR4 to the pro-hypertensive CNS milieu in SHRs cannot be disregarded. However, the evidenced effects of TLR4 activation in non-microglial cells of the CNS may indicate that microglial TLR4 stimulation is a primary mechanism. Interestingly, Liddelow *et al.* demonstrated that *in vivo* A1 activation of astrocytes (i.e., neurotoxic/proinflammatory activation) is absent in mice lacking microglia ($Csf1r^{-/-}$ knock-out) (Liddelow et al., 2017). Indeed, mixed reports appear regarding the expression of astrocytic TLR4 and its downstream signaling components in rodents. Whether these observations are due to differences in species and/or strain, astrocyte activation state, or regional differences in astrocyte gene profiles is unknown. However, the ability of microglia to induce A1 astrocyte activation through TNF-α, IL-1α, and complement component 1q secretion (Liddelow et al., 2017) following LPS stimulation supports the notion of astrocyte reactivity as a secondary event in neuroinflammation.

The recognition of BBB disruption as a pathological phenomenon is increasing across an array of neurological disorders associated with neuroinflammation, including

Alzheimer's disease, Parkinson's disease, and multiple sclerosis. BBB breakdown is apparent in hypertensive models (Fernandes et al., 2021; Yao & May, 2013), and our prior work with Losartan-treated SHRs demonstrated such disruption to be dependent upon AngII via AT1R (Biancardi, Son, Ahmadi, Filosa & Stern, 2014). A subsequent study by Buttler *et al.* found disruption within autonomic centers in SHRs to increase between 1 and 3 months of age – dye leakage was absent in pre-hypertensive 4-week-old animals and, in agreement with our work, apparent at 12 weeks (Buttler, Jordao, Fragas, Ruggeri, Ceroni & Michelini, 2017). Herein we report marked disruption at 9-10 weeks of age in the PVN, RVLM, and NTS of SHRs, during the establishing phase of hypertension. That SHR-TAK exhibited full barrier integrity raises the question of protection versus restoration. Moreover, whether the normalization of BBB permeability following TLR4 inhibition was due to protection against initial disruptions or a result of BBB repair processes is unknown. Given the indications of a time-course association between dye leakage and BP elevation, we suggest the latter explanation, wherein the already developing hypertension points to a loss of BBB integrity by 7-8 weeks in SHRs. While either scenario illustrates a clear role for TLR4 activation in BBB disruption, the distinction between them is particularly important from a clinical standpoint due to the evidenced disruption in cases of chronic hypertension, as well as in other neuroinflammatory diseases. To that end, further investigation regarding the timeline of BBB disruption in hypertension and the potential for BBB restoration via TLR4 inhibition is undoubtedly warranted.

Consistent with previous findings, we confirmed that 2 weeks of systemic TAK-242 reduced MAP in SHRs. Bomfim *et al.* observed reductions in MAP (approx.

20mmHg) in 15-week-old SHRs treated with an anti-TLR4 antibody (1µg/day, *i.p.*) for 15 days (Bomfim et al., 2012). Dange *et al.* reported a similar reduction following targeted bilateral PVN administration of VIPER (viral inhibitory peptide of TLR4; 40 µg/kg/day) for 14 days in 10-12-week-old SHRs (Dange, Agarwal, Teruyama & Francis, 2015b). Despite the continued elevation of BP in SHR-TAK versus WKY, the physiological relevance of the attenuated pressure should be considered. Moreover, while still presenting with elevated BP, the overwhelming majority of the other parameters examined in this study were normalized, which may suggest some "threshold BP" whose associated pathological changes are responsible for inciting neuroinflammation, BBB disruption, and autonomic dysfunction. Conversely, taking into account our prior work showing that a reduction of BP alone is insufficient to remedy these pathological alterations, it may be that their normalization occurs prior to BP reductions. As discussed above, a time-course evaluation of the pathological changes examined herein relative to the development of hypertension is necessary to address this point. Additionally, the question of long-term treatment with TAK-242 has yet to be investigated and it would be of great interest to determine whether further BP reductions occur beyond 2 weeks.

RAS-driven aberrations in baroreflex and autonomic function are well documented in hypertension. AngII increases neuronal activity within CNS cardiovascular nuclei, a process demonstrated to contribute to the maintenance of neurogenic hypertension (Kang et al., 2009; Shi et al., 2010; Waki, Gouraud, Maeda, Raizada & Paton, 2011; Zubcevic, Waki, Raizada & Paton, 2011). Prior studies show that *i.c.v.* injection of LPS induces sympathetic hyperactivity through upregulation of

PICs (Zhang, Yu, Wei & Felder, 2010), whereas PVN VIPER microinjection drastically reduces plasma norepinephrine in SHRs (Dange, Agarwal, Teruyama & Francis, 2015b). In agreement with these studies, our results indicate that autonomic dysfunction was abolished in SHR-TAK, as indexed by baroreflex sensitivity and indirect SNS activity.

Whereas systemic TLR4 blockade precludes us from identifying a specific effector location responsible for the findings herein, the administration route and pharmacological agent employed in this study are clinically significant. TAK-242 has already received FDA approval and the efficacy of less invasive treatment (i.e., *i.p.* versus *i.c.v.*) to facilitate improvements in the investigated parameters provide a clear basis for further studies regarding repurposing of TAK-242. Additionally, it should be noted that the ability of TAK-242 to cross the BBB, whether intact or disrupted, would result in non-region-specific inhibition if directly administered either *i.c.v.* or within nuclei. Moreover, regardless of the primary site of action, the physiological impact of TAK-242 administration on autonomic function and within cardioregulatory nuclei is evident.

In summary, the present work demonstrates a clear contribution of chronic AT1R and TLR4 activation in SHRs to 1) neuroinflammation via TLR4 upregulation, microglial activation, and proinflammatory cytokine production within the PVN, RVLM, and NTS; 2) BBB disruption in the PVN, RVLM, and NTS; 3) baroreflex desensitization; 4) sympathoexcitation; and 5) the progression of hypertension development. The reliance of TLR4 protein upregulation upon AT1R activation, in combination with the consistent effects of AT1R and TLR4 inhibition on those parameters investigated, provides support for TLR4 activation as a mechanism of AngII-AT1R-dependent neuroinflammation and

BBB disruption in multiple cardioregulatory nuclei, as well as autonomic dysfunction in hypertensive pathophysiology.

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