A Role for Brain Cellular Senescence in the Pathogenesis of Hypertension

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Dedication

For my sister, Danielle, whose incredible love and strength encouraged me to get to where I am today.

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Abstract of Dissertation

A Role for Brain Cellular Senescence in the Pathogenesis of Hypertension

Hypertension, or high blood pressure, affects an estimated 33% of adults worldwide. A major challenge for treating hypertensive patients is that the etiology is unclear in many cases, an alarming issue considering that hypertension is a leading risk factor for many life-threatening conditions. Over the past several decades, it has become increasingly clear that the actions of the peptide hormone angiotensin II (Ang-II) in the central nervous system (CNS) are central in driving hypertension development. However, Ang-II is too large to cross the blood-brain-barrier and instead acts at a specialized brain regions known as the circumventricular organs (CVOs). In particular, within the subfornical organ (SFO), a CVO well-recognized for its role in cardiovascular regulation, Ang-II induces cellular stressors known to mediate hypertension development, including oxidative stress, endoplasmic reticulum stress, and inflammation. However, the underlying mechanisms that integrate these stressors to drive hypertension remain incompletely defined. In this dissertation, we hypothesized that cellular senescence in the SFO may be a novel mechanism for Ang-II-induced hypertension development.

There is mounting evidence that cellular senescence is a complex, heterogeneous cellular phenotype that is strongly implicated in aging and other disease conditions closely associated with hypertension. Importantly, cellular senescence can be induced by the same pro-hypertensive stressors that occur in the SFO to mediate Ang-II-induced hypertension development. Thus, we first sought to determine if there is a role for senescent cells in Ang-II-induced hypertension development. Our data indicate that global removal of senescent

cells prevents Ang-II-induced hypertension development, and induction of CNS-specific cellular senescence results in increases in blood pressure. Building upon our initial findings, we next aimed to characterize cellular senescence specifically in the SFO and found increased gene and protein expression of cellular senescence markers in the SFO of male mice during Ang-II-induced hypertension. The increase in senescence was paralleled by the inflammatory senescence-associated secretory phenotype (SASP). Interestingly, the upregulation of senescence and SASP markers was not observed in the SFO of female mice, a finding that is consistent with literature that females are relatively protected against hypertension development. When examining cellular senescence in a cell-type specific manner, our studies indicate that senescent SFO neurons and astrocytes exhibit differing phenotypes during Ang-II-induced hypertension development. Taken together, these findings suggest that cellular senescence and SASP occurs in the SFO during Ang-II-induced hypertension development in both a cell type- and sex-dependent manner.

Ultimately, a central goal for this dissertation was to discern the contribution of SFO cellular senescence in driving hypertension development. Based on previously literature and our current findings, we expected that targeting senescent cells in the SFO would prevent Ang-II-induced hypertension development. We performed selective knockdown of the upstream senescence mediator $p19^{ARF}$ in the SFO using viral targeting in $p19^{ARF}$-floxed male mice, paired with radiotelemetry recordings. SFO-specific knockdown of $p19^{ARF}$ partially alleviated hypertension development in response to Ang-II, indicating a role for $p19^{ARF}$-$p21^{CIP1/WAF1}$-related senescence in hypertension development.

Although it is well-known that Ang-II signaling within the SFO is a key mechanism for hypertension development, our results reveal cellular senescence to be a previously unknown stressor that occurs at the SFO during Ang-II-induced hypertension development. Thus, this work holds implications for unraveling the complexity of the pathogenesis of hypertension and the potential to provide new therapeutic targets for antihypertensive therapies.

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Chapter 1

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List of Abbreviations

ACE	Angiotensin-converting enzyme
Ang-II	Angiotensin II
AP	Area postrema
ATP	Adenosine triphosphate
AT_1R	Angiotensin II type 1 receptor
Casp8	Caspase 8
Cdk	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CVO	Circumventricular organ
DNA	Deoxyribonucleic acid
eGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
FKBP	F506 binding protein
GFAP	Glial fibrillary acidic protein
ICV	Intracerebroventricular
IHC	Immunohistochemistry
IL-6	Interleukin 6
IL-10	Interleukin 10
IML	Intermediolateral nucleus
IP	Intraperitoneal

MCP1	Monocyte chemoattractant protein 1
MOC	Mander's overlap coefficient
MnPO	Median preoptic nucleus
mRNA	Messenger ribonucleic acid
NeuN	Neuron specific nuclear protein
NOX	Nicotinamide adenine dinucleotide phosphate
OVLT	Organum vasculosum of the lamina terminalis
PCC	Pearson's correlation coefficient
PVN	Paraventricular nucleus of the hypothalamus
$p16^{INK4A}$	Cyclin-dependent kinase inhibitor 2A
$p19^{ARF}$	Alternative reading frame cyclin-dependent kinase inhibitor 2A
$p21^{CIP1/WAF1}$	Cyclin-dependent kinase inhibitor 1
p27	Cyclin-dependent kinase inhibitor 1B
p53	Tumor protein p53
RAAS	Renin-angiotensin-aldosterone system
Rb	Retinoblastoma
ROS	Reactive oxygen species
RT-qPCR	Real time quantitative polymerase chain reaction
RVLM	Rostral ventrolateral medulla
SASP	Senescence-associated secretory phenotype
SATB1	Special AT-rich sequence binding protein 1
SA-β-gal	Senescence-associated beta galactosidase
SFO	Subfornical organ

SIPS	Stress-induced premature senescence
Smad2	Mothers against decapentaplegic homolog 2
SON	Supraoptic nucleus
TNFα	Tumor necrosis factor α
VMSC	Vascular smooth muscle cell

Chapter 1

Introduction

1.1 Blood pressure regulation and hypertension

Changes in blood pressure, or the amount of force being exerted by blood against the walls of the arteries, is a dynamic process. Under normal circumstances, blood pressure is tightly regulated by the combined effort of endocrine, neural, and renal mechanisms[1]. These mechanisms include the autonomic nervous system, which sends electrochemical signals to stimulate processes at various target organs that result in blood pressure changes, such as the constriction of the vasculature to control resistance to blood flow[2] and influencing sodium and water reabsorption at the kidneys to regulate blood volumes[3, 4]. Similarly, key hormones are synthesized and transported to these target organs via the circulation to regulate blood pressure responses[5]. Thus, these various systems must harmonize together for stable blood pressure control. However, when the balance within these regulatory mechanisms become misaligned, blood pressure can become persistently elevated, known as hypertension. Chronic uncontrolled hypertension is linked to an increased risk for other major cardiovascular, metabolic, and neurodegenerative diseases[6], such as heart attack[7], stroke[7, 8], Alzheimer's disease[9, 10], obesity[11], and diabetes[12]. Unfortunately, the prevalence of hypertension is alarming, with approximately 1.28 billion adults worldwide living with hypertension[13]. Within the U.S., 46% of adults have hypertension[14]. Additionally, sex differences in the prevalence of hypertension are well-established. According to the NHANE's survey period between 2017-2018, the prevalence of hypertension in men below the age of 60 was much higher (51%) compared to age-matched women (39.7%)[15].

Given the strong link between chronic hypertension and other concerning health conditions, managing hypertension through anti-hypertensive treatments is critical for the well-being of hypertensive patients. Currently available anti-hypertensive treatments include angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, beta-blockers, and diuretics[16]. However, despite the prevalence and availability of treatments among hypertensive U.S. adults, a staggering 53.5% do not have their blood pressure under control[14]. It has been estimated that 56.7% of hypertensive adults, both with uncontrolled and controlled blood pressure, were taking two or more anti-hypertensive medications[16]. The current statistics regarding hypertensive adults with uncontrolled blood pressure call into question the efficacy of currently available anti-hypertensive therapeutic options, highlighting the need to better understand the mechanisms underlying hypertension, and identify novel therapeutic targets.

1.2 The renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system (RAAS) is a key endocrine mechanism involved in blood pressure control. The RAAS is comprised of several factors that ultimately lead to the synthesis of the peptide hormone angiotensin II (Ang-II). First, juxtaglomerular cells of the kidney cleave the precursor compound prorenin into renin. Renin is then packaged and released into the circulation. Importantly, renin is the rate-limiting enzyme of the RAAS. Angiotensinogen, the precursor of Ang-II, is synthesized by the liver and released into circulation where it encounters circulating renin. Upon interaction, renin cleaves angiotensinogen into the inactive compound angiotensin I. Angiotensin I continues to circulate and meets with angiotensin-converting enzyme (ACE),

an enzyme that is mainly expressed on the plasma membrane of vascular endothelial cells, but also locally in the intestines, kidney, testes, heart, thyroid, adipose tissue, liver, lungs, and brain[17]. ACE cleaves angiotensin I into Ang-II, which is the primary effector of the RAAS.

Ang-II generally acts to increase blood pressure by modulating several processes at various organs. The majority of the known cardiovascular effects produced by Ang-II are mediated through its G-protein coupled receptor subtype, angiotensin II type 1 receptor (AT_1R)[18]. AT_1Rs are expressed throughout the body, including in the heart, vasculature, kidney, adrenal glands, and brain[19, 20]. At the vasculature, Ang-II causes vasoconstriction of vascular smooth muscle[21, 22]. Additionally, Ang-II promotes sodium and water reabsorption at the kidneys, which elevates arterial blood pressure by increasing blood volume[23, 24]. In the central nervous system (CNS), Ang-II promotes sympathetic nervous system activation and the release of vasopressin[25-27]. Ang-II also stimulates the synthesis and secretion of the final RAAS component, aldosterone, from the adrenal cortex. Both vasopressin and aldosterone contribute to blood pressure changes by influencing the vasculature to induce vasoconstriction, as well as fluid and salt balance, which impacts blood volume. Ang-II also induces the release of the catecholamine hormones norepinephrine and epinephrine from the adrenal medulla, which enter the circulation and influence additional target organs.

It has been long known that perturbations in the expression and/or activity of the RAAS components are implicated in hypertension development. For example, a large subset of hypertensive patients displays elevated levels of circulating Ang-II[28] whereas others exhibit higher plasma levels of angiotensinogen[29] or variable levels of renin[30].

Mounting evidence also indicates a genetic component to alterations in RAAS components in hypertensive patients, including variants of the renin, angiotensinogen, and AT_1R genes that are strongly associated with hypertension[31-37]. Anti-hypertensive treatments that act to reduce the production of Ang-II or block AT_1R activity, such as ACE inhibitors and angiotensin receptor blockers, further support a role for high expression or activity of the RAAS in hypertension. Ultimately, overactivity of RAAS components and subsequent elevations in Ang-II are implicated mechanisms for hypertension development.

1.3 Central regulation of blood pressure by Ang-II

As stated above, Ang-II acts in the CNS to drive sympathetic nervous system activation[38]. The sympathetic nervous system is comprised of a variety of neural networks that extends from the brain to the spinal cord and thus regulate various physiological processes at target organs. Numerous brain regions contribute to sympathetic outflow related to cardiovascular control[39], but a major site for the integration of these neural inputs is the brainstem[2, 40]. Cardiovascular sympathetic premotor neurons originate from five major cell groups in the brain: the rostral ventrolateral medulla (RVLM), rostral ventromedial medulla, caudal raphe nuclei, the A5 noradrenergic cell group of the caudal ventrolateral pons, and the paraventricular nucleus of the hypothalamus (PVN). Sympathetic premotor neurons project from these regions to the spinal cord, primarily to the intermediolateral nucleus (IML). Within the IML, sympathetic premotor neurons then innervate sympathetic preganglionic neurons, which have the majority of their cell bodies in the IML. Sympathetic preganglionic neurons in turn innervate postganglionic neurons, releasing acetylcholine onto postganglionic neuron cell bodies in ganglia located outside

of the CNS. Finally, sympathetic postganglionic neurons project to their prospective target organs, such as the vasculature, adrenal medulla, or kidneys. Upon stimulation, postganglionic neurons release norepinephrine that bind adrenergic receptors expressed in target tissues, leading to physiological effects associated with sympathetic nervous system activation.

Activation of the sympathetic nervous system driven by Ang-II appears to be particularly essential for blood pressure regulation and hypertension development. Several studies in various animal models used peripheral nerve recordings demonstrate that systemic Ang-II administration increases the activity of multiple nerves, including the renal, splanchnic, and inferior cardiac nerves[41-44]. Severing the peripheral nerves diminishes the pressor response elicited by Ang-II infusion[45]. Furthermore, Ang-II is associated with increased sympathetic nervous system activity in human hypertensive patients, measured through microneurography recordings and plasma norepinephrine concentrations[41, 46-48]. These findings collectively indicate a role for the CNS in hypertension development.

1.4 Blood-brain-barrier and circumventricular organs

Despite its ability act in the CNS to drive sympathetic outflow, Ang-II, as a peptide hormone, is too large to cross directly into the CNS due to the highly selective semipermeable blood-brain-barrier. The blood-brain-barrier is composed of endothelial and glial cells that surround blood vessels to shield the CNS from potentially detrimental factors while allowing in essential nutrients required for CNS functioning. Therefore, to exert its effects in the brain, Ang-II must act at specialized brain regions known as

circumventricular organs (CVOs). CVOs are unique structures that are characterized by a more permeable blood-brain-barrier composed of atypical ependymal cells, as well as their dense vascularization and proximity to ventricles along the midline of the brain[49]. Out of a total of seven identified CVOs in the mammalian brain, only three contain neuronal cell bodies: the organum vasculosum of the lamina terminalis (OVLT), area postrema (AP), and subfornical organ (SFO)[49]. These three brain regions were dubbed by Johnson and Gross in 1993 as "sensory" circumventricular organs because the permeable blood-brain-barrier exposes the neuronal cell bodies to circulating factors in the periphery, allowing them to "sense" peripheral factors, including Ang-II[50]. Importantly, CVOs provide input to downstream cardiovascular and autonomic brain regions, such as the PVN and RVLM, implicating them in the regulation of blood pressure-related sympathetic outflow[49, 51-56].

1.5 Cardiovascular regulation by the SFO

The SFO, a small, forebrain structure, is well recognized for its role in cardiovascular and fluid balance regulation. Sitting along the midline, the SFO protrudes from the anterior wall of the third ventricle between the columns of the fornix and hippocampal commissure. The SFO can be divided into two subregions based on collective morphological, neuroanatomical, and functional criteria defined by McKinley and colleagues: a ventromedial core and an outer shell. The dense, intricate network of fenestrated capillaries that is characteristic of CVOs is confined solely to the SFO's ventromedial core[57]. In terms of cell types, the ventromedial core is composed primarily of neurons and nerve terminals, although neurons are present throughout the SFO[58, 59]. The ventromedial core is also characterized by the high expression of AT_1Rs, the predominant

Ang-II receptor subtype in the SFO[60-63], suggesting that these neurons are readily exposed and sensitive to circulating Ang-II. Indeed, in rats infused intravenously with Ang-II, c-fos labeling of stimulated SFO neurons was greatest in the ventromedial core region[64]. Ang-II also increases SFO neuron firing *in vitro* and is blocked by application of an angiotensin receptor blocker[65], further supporting that SFO neurons are responsive to Ang-II. Astrocytes are also abundant throughout the SFO, composed of the protoplasmic and fibrous types[58, 59]. SFO astrocytes maintain close contact to surrounding capillaries, ependymal cells, and neuronal axons and dendrites[58, 59, 66, 67]. Unlike neurons, astrocytes do not appear to express AT_1R as AT_1R mRNA expression does not colocalize with astrocytic marker glial fibrillary acidic protein (GFAP)[68]. In addition to its anatomy, the role of the SFO in Ang-II-related blood pressure control is further supported by its efferent projections to prominent cardiovascular and autonomic nuclei, including the median preoptic nucleus, supraoptic nucleus (SON), and PVN[49, 52, 55, 56]. SFO neurons that are responsive to Ang-II appear to modulate the activity of vasopressin-secreting PVN and SON neurons[69-71]. Furthermore, the SFO appears to send excitatory projections to PVN neurons that extend to the IML[51]. Thus, it may be that increased activation of SFO neurons projecting to the PVN may lead to increased sympathetic outflow from sympathetic preganglionic neurons.

1.6 Hypertension development mediated by the SFO

In addition to its well-established role in cardiovascular regulation, the SFO is also recognized to contribute to hypertension development. More specifically, excess Ang-II induces various cellular stressors in the SFO that are known to mediate hypertension development, including oxidative stress, inflammation, and endoplasmic reticulum stress.

Several studies from the early 2000s outlined a role and mechanism for oxidative stress in the SFO related to hypertension development. Increased levels of reactive oxygen species (ROS), a common measure of oxidative stress, in the SFO mediate hypertension development in response to peripheral Ang-II administration[72]. Ang-II induces oxidative stress in neurons by activating nicotinamide adenine dinucleotide phosphate oxidase (NOX), a major source of ROS production, through a Rac1-dependent mechanism[73]. Importantly, alleviating oxidative stress by either overexpression of superoxide dismutase or knockdown of NOX2, NOX4, or NOX docking subunit $p22^{phox}$ in the brain prevents Ang-II-induced production of ROS and subsequent blood pressure increases[72-76]. Collectively, Ang-II-induced oxidative stress in the SFO is a key mediator for hypertension development.

In addition to oxidative stress, Ang-II at the SFO appears to mediate peripheral inflammatory responses that result in hypertension. Ang-II-induced increases in blood pressure involve T cells, given that mice lacking T cells are resistant to Ang-II-induced hypertension. Reintroducing T cells in these mice enabled Ang-II-induced hypertension, as well as increased circulating levels of the inflammatory factor tumor necrosis factor α (TNFα)[77, 78]. Importantly, SFO neurons are responsive to inflammatory factors, such as TNFα and interleukin 1β[79-81]. Injection of these inflammatory factors directly into the SFO induces increases in blood pressure, supporting the SFO's role in driving peripheral pro-hypertensive inflammatory responses[80-82]. Furthermore, there may be a role for the SFO in activating T cells during Ang-II-induced hypertension. Ablation of the anteroventral third ventricle, a region that receives inputs from the SFO, attenuates the activation and vascular

infiltration of T cells in response to peripheral Ang-II infusion and subsequent hypertension[55, 82]. [79-82]

Endoplasmic reticulum (ER) stress is another cellular stress induced by Ang-II within the SFO during hypertension development. First, ER stress in the brain, produced by intracerebroventricular (ICV) injections of an ER stress inducer thapsigargin, results in increases in blood pressure. Within the SFO, ER stress markers are elevated in response to increased circulating Ang-II, as well as alterations in ER morphology observed with electron microscopy. Inhibition of ER stress specifically in the SFO through a viral targeting strategy was shown to prevent Ang-II-induced increases in blood pressure[83]. These data together suggest that ER stress in SFO drives Ang-II-induced hypertension development.

Importantly, there appears to be an interweaving relationship between these three Ang-II-induced cellular stressors; oxidative stress, inflammation, and ER stress[84]. Ang-II-induced ER stress can lead to oxidative stress in the SFO during hypertension development through the production of ROS[83]. During hypertension, both Ang-II-induced ER stress and oxidative stress drive increased activity of nuclear factor-κB, a key transcription factor for mediating inflammatory responses, in the SFO[85]. These findings indicates that the cellular pathways between oxidative stress, inflammation, and ER stress coalesce in the SFO; however, the exact downstream mechanisms that integrate these stress pathways and subsequently drive hypertension development remain unclear. Intriguingly, inflammation, oxidative stress, and ER stress are also known to be inducers of a phenomenon known as cellular senescence[86].

1.7 Cellular senescence

Cellular senescence is a cell state that can be characterized by four hallmark features: cell cycle withdrawal, macromolecular damage, deregulated metabolism, and the secretion of pro-inflammatory factors[87]. Although the defining features of senescence may sound detrimental, senescence in cells is necessary for certain physiological processes, including patterning in embryonic development and wound healing[88-90]. Furthermore, in the context of cancer, cellular senescence limits tumorigenesis and tumor progression[91-94] and provides a protective effect against fibrosis during tissue remodeling[95-97]. Thus, at certain levels, senescent cells are beneficial in these physiological contexts. It is thought that senescent cells exhibit an inflammatory phenotype to attract immune cells to clear senescent cells in tissues[97, 98], which may be an important mechanism to limit the buildup of senescent cells. However, with increasing age, tissues throughout the body progressively accumulate senescent cells as immune cells become less efficient at clearing senescent cells[99], and they may become senescent themselves[100]. Furthermore, existing senescent cells may be capable of evading the immune system[101]. The accumulation of senescent cells has been implicated with adverse effects in various tissues including the kidney, heart, and white adipose tissue, along with age-related conditions[102]. Fortunately, eliminating senescent cells can improve lifespan and alleviate age-associated phenotypes[88, 103]. Thus, it is crucial to understand why senescence occurs, characterize cellular senescence in various cell types, and delineate the detrimental effects of senescent cells in disease conditions, including hypertension.

1.8 Causes of cellular senescence

Cellular senescence can be classified into two categories based on the causative mechanism: replicative senescence and stress-induced premature senescence (SIPS). Replicative cellular senescence was first described by Hayflick and Moorhead in 1961. In their study characterizing the usefulness of several human diploid cell strains for serial cultivation and virus sensitivity, they recognized that dividing cells have a limited number of replications before they enter a state of irreversible cell cycle arrest known as "senescence"[104, 105]. Hayflick and Moorhead attributed this phenomenon to the influence of intrinsic factors rather than extrinsic. Indeed, the most common cause of replicative senescence is the shortening of telomeres[106, 107]. With each cell division, telomeres become shorter until they reach a critical length that induces a deoxyribonucleic acid (DNA) damage response signaling that permanently halts cell cycle progression[108]. Inducing the expression of telomerase, an enzyme that repairs telomere length at the end of each replication cycle, in human cells can extend the number of replications a cell may undergo, supporting a role for telomere length in replicative senescence induction[109].

Since Hayflick and Moorhead's initial findings, cellular senescence has been studied in numerous cell types, and it has become increasing clear that senescence is a very heterogeneous, complex cellular phenotype. In addition to telomere shortening, the presence of chronic stressors can also drive a cell to prematurely take on a senescent state, also known as SIPS. It is noteworthy to mention that these stressors are capable of inducing senescence independently of telomere shortening[110], supporting the notion that stress-induced senescence is the premature halting of a cell's full proliferative lifespan and can also occur in fully differentiated, post-mitotic cell types. Indeed, senescent phenotypes have been observed in post-mitotic cardiomyocytes, neurons, and glia[111-114].

1.9 Defining features of cellular senescence

Senescent cells have differing expression patterns depending on the cell type and mechanism of induction[115, 116], but as previously mentioned, several recurring characteristics have been identified in both mitotic and post-mitotic cell types. For various mitotic cell types, senescence can be classically defined by a state of permanent cell cycle arrest while remaining metabolically active[87, 117]. For both mitotic and postmitotic cell types, macromolecular damage leading to the senescent state involves two major DNA damage pathways that includes the upregulation of cyclin-dependent kinase (Cdk) inhibitor 2A ($p16^{INK4A}$) and Cdk inhibitor 1 ($p21^{CIP1/WAF1}$). Both $p16^{INK4A}$ and $p21^{CIP1/WAF1}$ are Cdk inhibitors that block various Cdk-cyclin complexes, including Cdk4/cyclin D and Cdk6/cyclin D complexes for $p16^{INK4A}$ and Cdk2/cyclin E for $p21^{CIP1/WAF1}$ [118-123]. Consequently, retinoblastoma (Rb) protein family members, which are kept inactive by Cdk/cyclins, are free to downregulate the expression of genes responsible for cell cycle progression[123-125] . Thus, cell cycle progression is halted. Chronic activation of these senescence pathways in response to stress eventually results in the cell entering a senescent state. Depending on the cell type and inducing stressor, additional markers of senescent cells have been observed, including morphological changes, increased senescence-associated beta galactosidase staining (SA-β-gal), increased DNA damage foci, persistent DNA damage foci unique to senescence, decreased lamin B1, relocation and increased secretion of high mobility group box 1, and changes in cell metabolism [126-132]. The senescent cell state is also accompanied by an inflammatory cascade known as the senescence associated secretory phenotype (SASP). The SASP involves the secretion of a comprehensive range of cytokines, chemokines, and proteases[133, 134]. The exact

combination of SASP factors released depends on the stressor or inducer type[135]. Once initiated, the pro-inflammatory factors are released into the surrounding tissue where they can act in both autocrine and paracrine fashion to exacerbate and perpetuate senescence[136, 137]. It is important to note that the senescence phenotype can occur independently of the SASP. Although $p16^{INK4A}$ is a key contributor to the development of the senescent cell state and senescent cells often exhibit the SASP, the development of the SASP may not be dependent on $p16^{INK4A}$ expression[138], further adding to the complexity and heterogeneity of cellular senescence and SASP.

1.10 Cellular senescence in the central nervous system

Cellular senescence in nervous tissues has been primarily characterized in the context of neurodegenerative diseases with differing phenotypes depending on cell type. When astrocytes undergo senescence, they exhibit many of the hallmark features described above, although the exact presentation of senescent markers varies depending on the type of stressor (Table 1). Treatment of rodent and human astrocyte cultures with ROS H_2O_2 or the proteasome inhibitor lactacystin-2 induces a vacuolized, flattened morphology with increased cell size, increased SA-β-gal staining, and the upregulation of $p16^{INK4A}$, $p21^{CIP1/WAF1}$, and tumor protein p53 (p53), which activates the transcription of $p21^{CIP1/WAF1139}$ (Table 1). A similar phenotype can be observed in cultured human astrocytes treated with the herbicide paraquat, along with reduced proliferative capacity, enhanced secretion of interleukin-6 (IL-6), larger nuclear size, and increased expression of p53-binding protein 1 positive foci relative to untreated astrocytes[140] (Table 1). Ammonia-treated cultured rat astrocytes displayed reduced proliferative capacity, increased

expression of p53, $p21^{CIP1/WAF1}$, Growth Arrest and DNA Damage Inducible Alpha, and decreased expression of lamin A and B1[141] (Table 1). It has been suggested that senescent astrocytes may have an impaired capacity to clear excess glutamate, which may lead to excitotoxicity in neurons. Cultured human astrocytes, made senescent by X-ray irradiation treatment, displayed upregulation in senescence and SASP markers, but downregulation in genes associated with excitotoxicity, including excitatory amino acid transporters 1 and 2, potassium transporter Kir4.1, and water transporter AQP4 (Table 1). Furthermore, when these senescent astrocytes were co-cultured with neurons and treated with excess glutamate, fewer astrocytes and neurons survived after 24 hours compared to astrocytes and neurons that were co-cultured in regular media[142]. Taken together, this indicates that senescent astrocytes may not be able to maintain an environment adequate for neuronal survival.

Given that mature neurons have already undergone permanent cell cycle arrest, defining cellular senescence in neurons is an ongoing challenge since it requires using other common senescence markers to identify senescent neurons. One of the first studies to characterize cellular senescence in neurons found that loss of Sirtuin 1, a transcriptional regulator that counteracts oxidative stress and other cellular insults, from wake-active neurons induced the accumulation of lipofuscin aggregates, a hallmark for aging[111]. Cultured Purkinje, cortical, hippocampal, and peripheral neurons from old C57Bl/6 mice exhibited increases in senescent indicators such as γ histone family member X DNA damage foci, activated p38 mitogen-activated protein kinase, secretion of IL-6, expression of heterochromatin marker mH2A, and SA-β-gal staining. Furthermore, this neuronal senescent phenotype was exacerbated in a mouse model of accelerated telomere

shortening[112]. Others have demonstrated that cultured mouse dopaminergic neurons with a loss of special AT-rich sequence-binding protein 1 (SATB1), a transcriptional regulator, take on a senescent phenotype characterized by elevated expression of $p21^{CIP1/WAF1}$and SASP genes, enlarged nuclei, increased lysosomal accumulation and diminished activity of lysosomal enzymes, abnormal mitochondrial morphology, a decrease in basal respiration and ATP production, and lipofuscin accumulation[143]. Interestingly, across the various models that induce cellular senescence in postmitotic neurons, one recurring trend is that $p21^{CIP1/WAF1}$ appears to be necessary for these senescent phenotypes to occur. Knockout of $p21^{CIP1/WAF1}$ rescued the senescent features observed in neurons that were made senescent by accelerated telomere shortening[112]. Additionally, $p21^{CIP1/WAF1}$ appears to mediate the senescent phenotype in dopaminergic neurons lacking SATB1. The transcriptional regulator SATB1 binds to the regulatory region of *CDKN1A*, the gene encoding $p21^{CIP1/WAF1}$, thus leading to increased $p21^{CIP1/WAF1}$ expression in dopaminergic neurons lacking SATB1. Targeting $p21^{CIP1/WAF1}$ with a p21-short hairpin RNA decreased SA-β-gal staining in SATB1 knockout dopaminergic neurons[143]. Similarly, one study found that loss of growth differentiation factor 11 from mouse cortical excitatory neurons lead to an upregulation in phosphorylated Mothers against decapentaplegic homolog 2 (Smad2), leading to enhanced binding of Smad2 to the $p21^{CIP1/WAF1}$ promoter and subsequent increased $p21^{CIP1/WAF1}$ expression[144]. Furthermore, $p21^{CIP1/WAF1}$ knockout using Crispr-Cas9 also reduced SA-β-gal staining in cultured Neuro2A cells. Although there is little evidence on how cellular senescence affects excitability in neurons, Wang et al. also observed that *in vitro* deletion of growth differentiation factor 11 induced senescence in cortical excitatory neurons that resulted in increased excitability in response to a range of

current injections, higher frequency action potential, and changes in the active and passive membrane properties that were indicative of enhanced excitability in senescent neurons.

1.11 A role for brain cellular senescence in hypertension

Although cellular senescence has been extensively studied in aging and neurodegenerative diseases[86, 145-147], both conditions closely associated with hypertension, investigation into the role of cellular senescence in the context of hypertension has been extremely limited. Cellular senescence related to hypertension has exclusively been studied in peripheral tissue and cell types, including skeletal muscle feed arteries[106148], aortic tissue[149], aortic smooth muscle and endothelial cells[150-154], and heart and kidney smooth muscle cells[155]. In a study where arterial biopsies were taken from skeletal muscle in hypertensive and normotensive individuals, hypertensive adults had elevated expression of DNA damage foci γH2 localized to telomeres, although there was no difference in telomere length. Furthermore, chromatin immunoprecipitation-sequencing analysis revealed increased p53 bound to $p21^{CIP1/WAF1}$, which correlated with the level of γH2 expression localized to telomeres[148]. In rats treated with DOCA salt, a model of hypertension, $p16^{INK4A}$ expression was elevated in kidney and heart cell types[155]. A few studies that performed indirect blood pressure recordings have found that peripheral cellular senescence was associated with increases in blood pressure. One such study utilized $Ercc1^{d/-}$ mice, which have nucleotide excision repair deficiency, and found that the thoracic aorta wall and cultured lung endothelial cells from these mice displayed increased SA-β-gal staining, $p21^{CIP1/WAF1}$, and p53 expression relative to wildtype mice[151]. Endothelial progenitor cells were also found to be senescent, measured through increased SA-β-gal staining and

decreased telomerase activity in spontaneously hypertensive rats, DOCA salt rats, and human hypertensive patients[154]. Another study assessed cellular senescence in mice with collagen resistant to collagenase, which occurs with aging. After treatment with Ang-II, mice with collagenase resistance displayed upregulation of $p16^{INK4A}$ and $p21^{CIP1/WAF1}$ expression, greater levels of SA-β-gal staining in the outer medial layers of the aortic arch compared to wildtype mice[150]. Taken together, these studies set the framework for further exploration into cellular senescence in CNS cell types in the context of hypertension.

In certain contexts, aside from hypertension, it has been demonstrated that Ang-II is capable of inducing senescence in peripheral cell types, astrocytes, and possibly neurons. In the periphery, Ang-II induces cellular senescence in cultured human aortic vascular smooth muscle cells (VMSCs), mediated by the p53- $p21^{CIP1/WAF1}$ pathway. Ang-II also induces cellular senescence and SASP in aortic VSMCs in ApoE -/- mice, a model for atherosclerosis, but can be rescued by also eliminating $p21^{CIP1/WAF1}$ in a double knockout model[156]. Ang-II-induced vascular smooth muscle senescence has also been confirmed in cultured VMSCs isolated from rat thoracic aorta, mediated by Kirsten-ras2A upregulation and oxidative stress. In addition to increased SA-β-gal staining, p53, and $p21^{CIP1/WAF1}$ expression, Ang-II treatment of VMSCs led to upregulation of $p16^{INK4A}$ and cyclin-dependent kinase inhibitor 1B (p27) expression. However, the addition of the AT_1R inhibitor valsartan reduced the number of SA-β-gal positive cells and reversed elevated levels of p53, $p21^{CIP1/WAF1}$, $p16^{INK4A}$, and p27 induced by Ang-II[157]. In CNS cell types, application of Ang-II to cultured human astrocytes induces cellular senescence but can be rescued by introducing the AT_1R blocker CV11974[158]. In one transcriptomic study,

candesartan, another AT_1R blocker, negatively correlated with markers of cellular senescence in cultured rat primary neurons[159].

Based on the current literature, cellular senescence in the brain in the context of hypertension is a novel concept. Furthermore, given the importance of the Ang-II in regulating blood pressure, the role of brain cellular senescence in the development of Ang-II-induced hypertension is an area that demands further exploration.

1.12 Summary and specific aims

In summary, hypertension is a prevalent health concern, but the underlying causes remain unclear. Ang-II is a well-recognized driver of hypertension, particularly through its sympathoexcitatory actions within the CNS. As a peptide hormone, Ang-II is too large to cross into the brain and acts at circumventricular regions lacking a blood-brain-barrier, particularly the forebrain SFO. While Ang-II has been shown to drive pro-hypertensive cellular stressors in the SFO (i.e. oxidative stress, ER stress, and inflammation), it is unclear how these mechanisms translate into long-term alterations at the cellular level. Intriguingly, these stress-associated pathways can induce cellular senescence and the SASP. Cellular senescence is induced by Ang-II in certain peripheral tissue and cell types and has been implicated in conditions closely associated with hypertension. Yet, cellular senescence in the brain during hypertension remains unexplored. Based on this, the goal of this dissertation was to evaluate cellular senescence in the SFO during Ang-II-induced hypertension and discern the contribution of SFO cellular senescence to the development and sexually dimorphic nature of hypertension. We approached this goal through the following three aims:

Aim 1: Characterize cellular senescence in the central nervous system during Ang-II-induced hypertension development.

As cellular senescence has been studied in various diseases contexts and cell types over the past few decades, it has become increasingly clear that the presentation of senescence and SASP is extremely heterogeneous[115, 116]. Given the diversity of senescent cell phenotypes and the involvement of senescence-inducing stressors that occur in the SFO during hypertension, we first aimed to determine whether cellular senescence is related to hypertension development. To achieve this goal, we utilized a transgenic mouse model coupled with an established model of hypertension and radiotelemetry recordings to evaluate the participation of senescent cells in driving a hypertensive phenotype. We also applied a pharmacological approach to induce senescence solely in the CNS and determine whether CNS cellular senescence gives rise to hypertension. We further evaluated cellular senescence specifically in the SFO, characterizing the senescence and SASP profiles through molecular and histological techniques.

Aim 2: Outline the role of brain cellular senescence in the sexual dimorphism of hypertension.

Sex differences in the prevalence and development of hypertension are well-established. Below the age of 60, women have a lower risk for hypertension than men[15]. The difference in prevalence suggests that at younger ages, women are relatively protected against hypertension. Female protection against hypertension has been confirmed in animal models, in which female mice infused with Ang-II are resistant to hypertension development while males become hypertensive in response to Ang-II infusion[160].

Importantly, the SFO appears to be involved in mediating female resistance to hypertension. SFO-specific knockdown of estrogen receptor alpha, the predominant estrogen receptor type in the SFO, results in Ang-II-induced hypertension in females[161]. These data indicate that estrogen signaling specifically in the SFO is key for female resistance to hypertension, although the exact mechanisms of female protection remain incompletely defined. Intriguingly, estrogen has been suggested to be protective against cellular senescence in peripheral tissues[162-164]. Taken together, we sought to determine whether females are protected against cellular senescence in the SFO, as opposed to males, during Ang-II-induced hypertension development. This was accomplished by comparing the presence of senescence and SASP indicators between male and female mice through real time quantitative polymerase chain reaction (RT-qPCR) during Ang-II-induced hypertension development.

Aim 3: Delineate the contribution of brain cellular senescence to Ang-II-induced hypertension.

Ang-II is known to generate various stressors in the SFO, including oxidative stress, ER stress, and inflammation that are: 1) mediators of hypertension; and 2) drivers of senescence[72, 73, 79-81, 83, 86]. Yet, the complete mechanism of hypertension development involving these stress factors remains undefined. In line with this, an overarching goal of this dissertation was to determine whether cellular senescence in the SFO facilitates Ang-II-induced hypertension development. Thus, in this aim, we tested the functional role of SFO cellular senescence in driving hypertension using a combination of selective viral targeting with a transgenic mouse model and *in vivo* cardiovascular techniques.

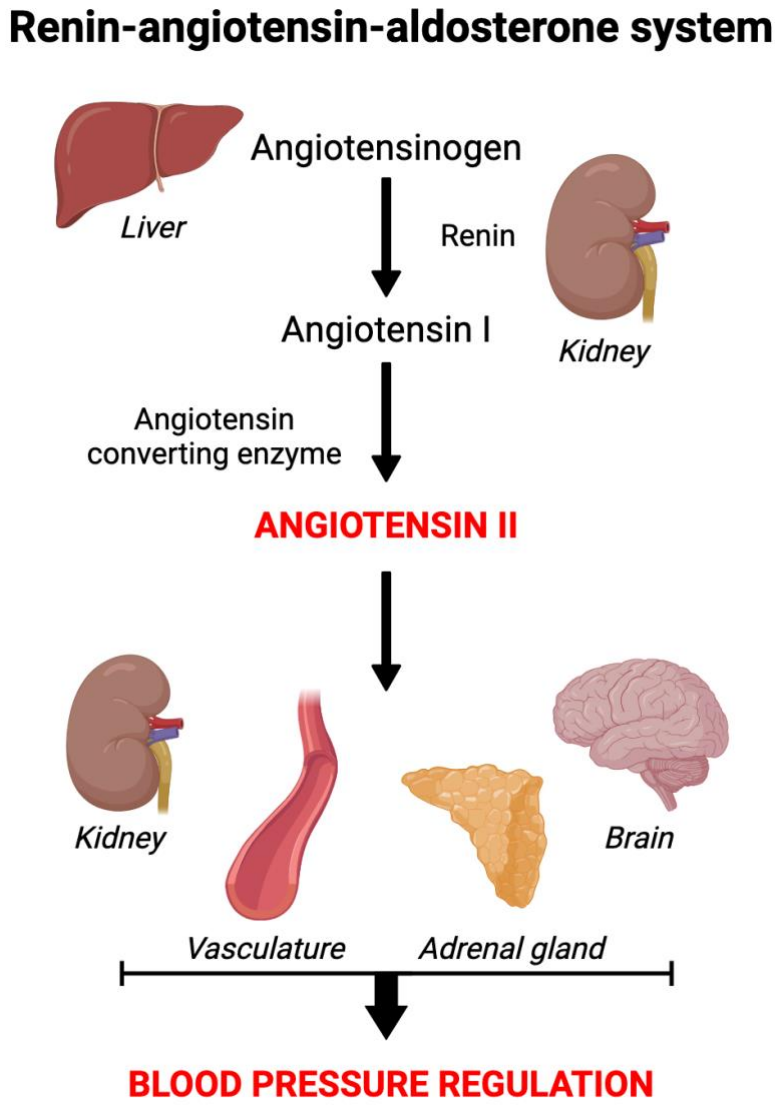

Figure 1.1 Overview of the renin-angiotensin-aldosterone system
Schematic summarizing the organization and localization of components of the renin-angiotensin-aldosterone system that result in the synthesis of angiotensin II. Angiotensinogen is synthesized and released by the liver. Renin is an enzyme produced by the kidneys. Renin cleaves angiotensinogen to produce the inactive compound angiotensin I. Angiotensin I is then cleaved by angiotensin converting enzyme, expressed in various tissues, to produce angiotensin II. Angiotensin II acts at the kidneys, vasculature, brain, and adrenal gland to regulate blood pressure. Created using biorender.com.

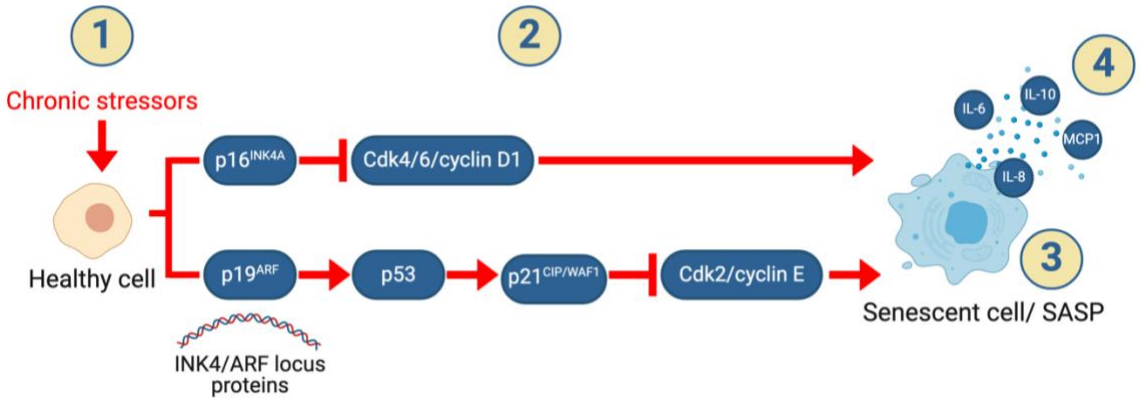

Figure 1.2 Cellular senescence signaling pathways
Cellular senescence and the senescence-associated secretory phenotype (SASP) result from the chronic activation of major senescence pathways. (1) Cells are exposed to chronic stressors, such as oxidative stress, ER stress, and inflammation. (2) DNA damage pathways become activated in response to the presence of chronic stressors. These include pathways involving the upregulation of $p16^{INK4A}$ and $p21^{CIP1/WAF1}$, two major senescence indicators. (3) Long-term activation of the pathways involving $p16^{INK4A}$ and $p21^{CIP1/WAF1}$ leads to the senescent cell state. (4) The senescent cell state is accompanied by the SASP. The SASP is comprised of the secretion of pro-inflammatory cytokines, chemokines, proteases. Created using biorender.com.

Cell type	Stressor type	Characteristics of cellular senescence
Astrocytes	Oxidative stress	• Increased SA-β-gal staining • Upregulation of $p16^{INK4A}$, $p21^{CIP1/WAF1}$, and p53 • Vacuolized, flattened morphology • Increased cell size
	Paraquat (herbicide)	• Enhanced secretion of IL-6 • Larger nuclear size • Increased expression of 53BP1 positive foci • Reduced proliferative capacity
	Ammonia	• Increased expression of p53, $p21^{CIP1/WAF1}$, and Gadd45α • Decreased expression of lamin A and B1 • Reduced proliferative capacity
	X-irradiation	• Upregulation of $p16^{INK4A}$, IL-6, IL-8, and CXCL-1 • Downregulation of LMNB1, loss of HMGB1 • Down regulation in excitatory amino acid transporters 1 and 2, potassium transporter Kir4.1, and water transporter AQP4 (genes associated with excitotoxicity)
Neurons	Aging	• Increased γH2AX DNA damage foci, activated p38MAPK, IL-6, mH2A, and SA-β-gal staining
	Loss of SIRT1	• Accumulation of lipofuscin aggregates
	Loss of SATB1	• Increased expression of $p21^{CIP1/WAF1}$ and SASP genes • Enlarged nuclei • Increased lysosomal accumulation and diminished activity of lysosomal enzymes, • Abnormal mitochondrial morphology, a decrease in basal respiration and ATP production • Lipofuscin accumulation
	Loss of growth factor	• Upregulation in phosphorylated Smad2 • Enhanced binding of Smad2 to the *CDKN1A* promoter • Increased $p21^{CIP1/WAF1}$ expression • Increased SA-β-gal staining

Table 1.1 Cell type- and stress-specific cellular senescence profiles
The specific characteristics expressed by senescent cells is dependent on the cell identity and type of inducing stressor.

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Chapter 2

Cellular Senescence in the Subfornical organ as a Potential Mediator of Angiotensin II-Induced Hypertension

2.1 Summary

Hypertension affects nearly 1.28 billion people worldwide and is a primary risk factor for other major cardiovascular, neurological, and metabolic conditions. Despite the prevalence, the underlying pathological mechanisms remain unclear. Here, we tested the hypothesis that cellular senescence in the subfornical organ (SFO), a key cardiovascular brain region, is a novel contributor to hypertension development. First, in a proof-of-principle experiment, we found that induction of brain cellular senescence through intracerebroventricular doxorubicin injections resulted in increased blood pressure in mice. Furthermore, selectively removing senescent cells using the INK-ATTAC mouse model alleviated the hypertensive response to angiotensin II (Ang-II). In the SFO specifically, cellular senescence was increased at both the mRNA and protein levels in response to Ang-II infusion. Importantly, the senescence response to Ang-II infusion occurred in male but not female mice. Further examination revealed that SFO neurons and astrocytes display differing senescent profiles during Ang-II-induced hypertension development. Finally, SFO-specific knockdown of the upstream senescence mediator $p19^{ARF}$ partially alleviated hypertension development in response to Ang-II. Taken together, these studies provide a primary characterization of cellular senescence in the SFO and suggest a role for SFO senescence in Ang-II-induced hypertension development, which may contribute to a more precise understanding of hypertension development and the expansion of antihypertensive therapeutic options.

2.2 Introduction

High blood pressure, or hypertension, is a major health concern that affects nearly half of the U.S. adult population and is a primary risk factor for other detrimental cardiovascular, neurological, and metabolic conditions[1-4]. However, up to 53% of hypertensive adults do not have their blood pressure under control[5]. Additionally, sex differences in hypertension are well-established, with a higher prevalence of hypertension in men compared to women prior to the age of 60. However, following menopause, the prevalence of hypertension between men and women equalizes[6]. Despite its prevalence, the etiology of hypertension in most cases remains unclear.

The peptide hormone angiotensin II (Ang-II) is recognized as an essential regulator of blood pressure, as well as driver of hypertension development. Excess levels of circulating Ang-II, as well as the perturbed expression of other components of the renin-angiotensin-aldosterone system, have been implicated in hypertensive patients[7-9]. Ang-II acts, in part, in the central nervous system (CNS) to drive sympathetic nervous system activity and neurotransmitter release, resulting in increases in blood pressure[10-17]. In line with this, hypertensive patients display enhanced sympathetic nervous system activity, indicating a role for the brain in hypertension development[10, 18-20].

Despite its ability to influence central mechanisms of blood pressure regulation, as a peptide hormone, Ang-II is too large to cross the protective blood-brain-barrier. Instead, Ang-II acts at specialized brain structures known as circumventricular organs, including the subfornical organ (SFO). The SFO is a forebrain circumventricular organ with a fenestrated blood-brain-barrier and an established role in cardiovascular regulation.

Importantly, the SFO is well-recognized to mediate Ang-II-induced increases in blood pressure[21-23]. During hypertension, elevated Ang-II has been shown to induce several cellular stressors in the SFO, including oxidative stress, inflammation, and endoplasmic reticulum stress[24-29]. Although the exact mechanisms by which these stressors coalesce to result in hypertension development remains unclear, these pro-hypertensive stressors are all known to be inducers of cellular senescence[30].

Cellular senescence is a cellular state induced by a variety of stressors and can be characterized by four hallmark features: cell cycle withdrawal, macromolecular damage, deregulated metabolism, and the senescence-associated secretory phenotype (SASP)[31]. Importantly. the exact senescence phenotype is dependent on the cell type and inducing stressor[32, 33]. Specifically, within the CNS, both neurons and astrocytes display senescent phenotypes that includes altered morphology, upregulation of $p16^{INK4A}$ and $p21^{WAF1/CIP1}$, increased beta-galactosidase activity, and the SASP. Moreover, it has been found that Ang-II is capable of inducing senescence in peripheral cell types including endothelial progenitor cells and vascular smooth muscle cells[34, 35]. Despite these findings, cellular senescence in the CNS has not been extensively investigated in the context of hypertension.

Based on this, we hypothesized that cellular senescence in the SFO is a novel contributor to Ang-II-induced hypertension development. To address our hypothesis, we used transgenic mice and pharmacological agents in combination with radiotelemetry to examine the role of CNS cellular senescence in hypertension. We further characterized cellular senescence in SFO cell types throughout Ang-II-induced hypertension development using molecular and immunohistological techniques. Finally, we investigated

the role of the $p19^{ARF}$-$p21^{WAF1/CIP1}$ pathway in Ang-II-induced hypertension development using viral targeting in transgenic mice along with radiotelemetry.

2.3 Materials and Methods

Animals. All mice were housed on a 12-hour light-dark cycle with ad libitum access to normal chow and water. Radiotelemetry studies were performed in singly housed adult (7-12 weeks old) male C57Bl/6J (Jackson Laboratory, Strain #: 00064), INK-ATTAC (gifted from Dr. Baker, Mayo Clinic), or $p19^{ARF}$-floxed (Jackson Laboratory, Strain #: 023323) mice. Doxorubicin studies were performed in singly housed adult C57Bl/6J male mice. All other studies characterizing SFO cellular senescence during Ang-II-induced hypertension were performed in group-housed adult male or female C57Bl/6J mice.

Pharmacological agents. Ang-II (SigmaAldrich, A9525) was diluted in 0.1% Bovine Serum Albumin. Phenylephrine (SigmaAldrich, PHR1017) was diluted in saline. Continuous, controlled infusions of Ang-II (600 ng/kg/min) and phenylephrine (30 μg/kg/day) were achieved through subcutanous mini-osmotic pump (Alzet, Model 1002) implantation. AP20187 (APExBIO, B1274) was diluted in 0.1% DMSO (0.2 μg/g) and administered intraperitoneally (IP). Chlorisondamine (SigmaAldrich, C5366) was diluted in 0.1% DMSO (12 μg/g) for IP injections. Doxorubicin (SigmaAldrich, D1515) was diluted in artificial cerebrospinal fluid (0.0125 mg/μL) and injected at 1 μL per day intracerebroventricularly (ICV).

Stereotaxic surgeries. Mice were anesthetized with isoflurane using an isoflurane vaporizer (2.5% in 100% O_2, flow rate 2.0 L/min) and received subcutaneous injection of ketofen (5 mg/kg) for pain management prior to surgical procedures. Heads were then shaven, and mice were placed into the ear bars of a stereotaxic apparatus. ICV cannulas were implanted in the lateral ventricle using the following coordinates: -0.3 mm caudal, 1.0 mm lateral (right), 3.1 mm ventral from bregma. For stereotaxic SFO injections, 50-100 μL of viral vectors AAV-CMV-Cre-GFP (2.47E+13 vg/mL, University of Iowa Viral Vector Core) or AAV-CMV-GFP (1.63E+13vg/mL, University of Iowa Viral Vector Core) were injected into the SFO using stereotaxic pressure microinjection using glass micropipettes (an inner tip diameter of ~40 μm). The following coordinates were used: -0.55 mm caudal, 0.0 mm lateral (midline), 3.0 mm ventral from bregma. Viruses were injected over a 5-minute period. Mice were provided with ketofen 24 hours following surgical procedures, and experiments were performed two weeks following injection to allow for viral incorporation and surgical recovery.

Radiotelemetry recordings. For conscious blood pressure recordings, mice were instrumented with radiotelemeters (Data Sciences International, HDX-10 and HDX-11). Mice were anesthetized with isofluorane (2.5% in 100% O_2, flow rate 2.0 L/min), and their thoraxes were shaved. Mice received ketofen (5 mg/kg) for pain management prior to surgery. An incision was made just below the jaw through the skin and muscle, and the right carotid artery was isolated from the surrounding tissue and vagus nerve. After tying off the carotid artery just below the bifurcation, a small notch was made to insert the radiotelemetric catheter into the right carotid artery and secured with two additional

sutures. The battery was placed subcutaneously on the lower right side of the abdomen. Immediately following the radiotelemetry surgery, mice then received SFO injections of viral constructs as applicable (see above). Mice were provided with ketofen 24 hours following surgical procedures and recovered from surgery for two weeks before starting baseline recordings. Using Data Sciences International's Ponemah software, systolic pressure, diastolic pressure, mean arterial pressure, and heart rate were recorded for a three-day baseline. After recording on the third baseline day, if applicable, mice received subcutaneous implantation of mini-osmotic pumps for Ang-II infusion for 14 days (see above). Radiotelemetry recordings were performed at the same time each day throughout a 14-day period.

Ganglionic blockade. Ganglionic blockade was performed by injecting chlorisondamine IP, then performing radiotelemetry recordings for two hours to monitor blood pressure changes.

Real time-quantitative PCR. Following each of the respective timepoints, brains were collected and immediately frozen on dry ice. Single micropunches of cardiovascular and autonomic regions were then collected for each animal. mRNA from single brain punches was isolated using a TRIzol (Invitrogen, 15596018)-chloroform extraction method, as previously described[36, 37]. Isolated mRNA was converted to cDNA using Quanta cDNA SuperMix (QuantaBio, 101414-108). The resulting cDNA (25 ng) was then used for RT-qPCR along with target gene primers and SSoFast EvaGreen (BioRad, 1725202). Primer

sequences used were derived from *mus musculus* by using the NCBI Reference Sequence Database as follows:

β-actin	*F*: GGCTGTATTCCCCTCCATCG	*R*: CCAGTTGGTAACAATGCCATGT
$p16^{INK4A}$	*F*: CGAACTCGAGGAGAGCCATC	*R*: TACGTGAACGTTGCCCATCA
$p19^{ARF}$	*F:* CTCACCTCGCTTGTCACAGT	*R:* CGTGAACGTTGCCCATCATC
p53	*F*: CCCAAACTGCTAGCTCCCAT	*R*: ACTACTCAGAGAGGGGGCTG
$p21^{CIP1/WAF1}$	*F*: TAAGGACGTCCCACTTTGCC	*R*: CTGAGGATCACCCCCAGGTA
IL-6	*F*: GCCTTCTTGGGACTGATGCT	*R*: TGTGACTCCAGCTTATCTCTTGG
IL-8	*F*: CTAGGCATCTTCGTCCGTCC	*R*: AGCCCATAGTGGAGTGGGAT
IL-10	*F*: TAAGGCTGGCCACACTTGAG	*R*: GTTTTCAGGGATGAAGCGGC
MCP1	*F*: CAGGTCCCTGTCATGCTTCT	*R*: GTGGGGCGTTAACTGCATCT

β-actin was used as the reference gene in all experiments. For each target gene set, the average expression of each target within each experimental group was calculated relative to the reference gene, and the relative fold-change compared to the reference gene was calculated according to the ΔΔCt method[38].

Immunohistochemistry. Mice used for immunohistochemistry were transcardially perfused with 4% paraformaldehyde. Brains were collected and post-fixed for 24 hours in 4% paraformaldehyde, followed by immersion in 20% sucrose for three days. Fixed brains were then frozen in Optimal Cutting Temperature compound for cryosectioning. 10μm sections containing the SFO were washed four times for 5-minutes in 1x PBS followed by a 2-hour incubation with a blocking buffer (1% Bovine Serum Albumin, 1% DMSO, 0.3% Triton-X, 97.7% 1x PBS). Sections were then incubated with a primary antibody for

$p16^{INK4A}$ (1:500; Proteintech, 10883-1-AP) overnight at 4°C. Sections were then washed four times for 5-minutes in 1x PBS and incubated in Alexa-Fluor-conjugated antibodies (1:1000, Invitrogen) in blocking buffer. DAPI was used as a counterstain, and slides were mounted with coverslips. For $p21^{WAF1/CIP1}$ immunohistochemistry staining, antigen retrieval was first performed by incubating the 10um sections in sodium citrate buffer (10mM sodium citrate, 0.05% Tween-20, pH 6.0) for 15 minutes at 80°C. Following antigen retrieval, the same immunohistochemical procedures described for $p16^{INK4A}$ were followed using a $p21^{WAF1/CIP1}$ primary antibody (1:500; Proteintech, 28248-1-AP). For double immunohistochemistry, staining for GFAP (1:1000; Abcam, ab4674) and NeuN (1:500; Abcam, ABN90) was performed following $p16^{INK4A}$ staining procedures (1:500). For $p21^{WAF1/CIP1}$ double immunohistochemistry, the antigen retrieval step was performed first, followed by $p21^{WAF1/CIP1}$ staining (1:250) with either GFAP (1:1000) or NeuN (1:500) labeling. Immunohistochemistry images were obtained using a Carl Zeiss Cell Observer Spinning Disk Confocal Microscope. Immunohistochemistry staining was quantified as integrated density, measured with ImageJ software. Colocalization was analyzed using Pearson's correlation coefficient and Mander's overlap coefficient with IMARIS software.

Quantification and statistical analysis. Data are expressed as mean ± SEM. Students two-tailed unpaired *t* tests were used for comparisons between two groups. Multiple group analyses were performed using a one-way or two-way repeated measures ANOVA followed by a Bonferroni *post-hoc* test. Significance was set at $p < 0.05$.

2.4 Results

Selectively eliminating senescent cells throughout the body prevents Ang-II-induced hypertension. Targeting senescent cells has been shown to increase lifespan and improve age- and disease-related phenotypes[33, 39-43]. Since hypertension is closely associated with aging and neurodegenerative diseases, we sought to determine whether Ang-II-induced hypertension could be alleviated by removing senescent cells. We utilized the INK-ATTAC mouse model, designed to allow for selective elimination of senescent cells in response to the inducing drug AP20187[39]. In short, the INK-ATTAC mouse encodes a fusion protein under the $p16^{INK4A}$ promoter that includes a caspase 8 flag. Administration of AP20187 facilitates dimerization between fusion proteins, activating caspase-8 and triggering a pro-apoptotic cascade in senescent cells with high expression of $p16^{INK4A}$. To induce hypertension in INK-ATTAC mice, low dose Ang-II subcutaneous infusion over a 14-day period was used[26, 44, 45]. Over the 14-day Ang-II infusion period, mice received daily IP injection of either AP20187 or vehicle control. In response to Ang-II, vehicle-treated demonstrated a progressive increase in blood pressure, reaching peak hypertension at 14 days of infusion (Figure 2.1). However, mice treated with AP20187 to remove senescent cells were resistant to Ang-II-induced hypertension development (Figure 2.1). These data indicate that cellular senescence is obligatory for Ang-II-mediated hypertension development.

Induction of CNS cellular senescence elevates blood pressure via sympathetic nervous system activation. The use of the INK-ATTAC mouse model revealed the importance of senescent cells in the development of Ang-II-induced hypertension; however, this model only allows for non-specific removal of senescent cells throughout the body. To delineate

whether cellular senescence specifically in the CNS contributes to hypertension development, we performed a proof-of-principle experiment to induce CNS cellular senescence. 7-week-old C57BL/6 male mice were implanted with radiotelemeters and also fitted with ICV cannulas to administer either the senescence-inducing agent doxorubicin or vehicle control for three days[46-48]. Within the first 48 hours, doxorubicin-treated mice displayed a clear increase in blood pressure that was not present in the vehicle treated mice. Moreover, this elevation in blood pressure was sustained throughout the three-day period (Figure 2.2A). At the end of the three days, ganglionic blockade was performed by IP injection of chlorisondamine (12 μg/g) to evaluate the potential role of the autonomic nervous system. In response to ganglionic blockade, doxorubicin-treated mice exhibited a greater fall in blood pressure relative to vehicle control mice, which suggests that the doxorubicin-induced increases in blood pressure were mediated by sympathetic nervous system activation (Figure 2.2B). Together, these data indicate that CNS senescence is capable to drive increases in blood pressure.

Cellular senescence occurs in the SFO during Ang-II infusion. Given the SFO's role in mediating Ang-II-induced increases in blood pressure, the SFO could be a site for the accumulation of senescent cells that is observed during elevations in blood pressure. Thus, we aimed to assess CNS cellular senescence in the SFO during hypertension development. Studies of neurodegenerative diseases have shown that cellular senescence in CNS cell types are characterized by upregulation of $p16^{INK4A}$, $p21^{WAF1/CIP1}$, and SASP indicators[41, 43, 49-55]. Whether Ang-II drives similar changes in the SFO in response to Ang-II remains unknown. Using the low dose Ang-II-infusion model in C57Bl/6J male mice, RT-qPCR

revealed a marked increase in the expression of senescence indicator *p16*^{INK4A} in the SFO at 14 days of Ang-II infusion compared to day 0 controls (Figure 2.3A). Additionally, *p21*$^{WAF1/CIP1}$, another well-accepted senescence indicator and cyclin-dependent kinase inhibitor, was upregulated by more than two-fold in the SFO following 14 days of Ang-II infusion (Figure 2.3B). Another detrimental effect of senescence is the accompanying SASP phenotype. The SASP is particularly concerning because it can act in either autocrine fashion to exacerbate existing senescence or in a paracrine manner to perpetuate senescence induction in the local tissue[56-58]. Interestingly, Ang-II-induced senescence in the SFO was paralleled by the upregulation of the pro-inflammatory SASP markers *interleukin* (*IL*)*-6* following 14 days of Ang-II infusion (Figure 2.3C-F). Additionally, there was an upward trend for the SASP markers *IL-8* and *monocyte chemoattractant protein 1* (*MCP1*) in the SFO. Taken together, our data indicates that Ang-II-induced hypertension is associated with the activation of key senescent genes *p16*^{INK4A} and *p21*$^{WAF1/CIP1}$ in the SFO, accompanied by an elevation in proinflammatory SASP markers.

Ang-II-induced cellular senescence in the SFO is sexually dimorphic. Hypertension is known to occur in a sexually dimorphic manner. Notably, female mice are resistant to Ang-II-induced hypertension development[59]. Importantly, the sex difference in the response to Ang-II infusion appears to be mediated by the SFO[60-62]. Building upon this, we examined whether females experience the same upregulation in SFO senescence in response to Ang-II infusion that males do. Interestingly, female mice did not exhibit an increase in *p16*^{INK4A} or *p21*$^{WAF1/CIP1}$ expression following 14 days of Ang-II infusion (Figure 2.4A-B). Similarly, Ang-II-induced changes in the expression of proinflammatory SASP markers *IL-6*, *IL-10*,

IL-8, and *MCP1* were not observed in the SFO of females (Figure 2.4C-F). Thus, females do not develop cellular senescence (Figure 2.5A) or SASP (Figure 2.5B) in the SFO to the same extent as males during Ang-II-induced hypertension.

Upregulation of cellular senescence in the SFO is specific to Ang-II-induced hypertension. To confirm that the increase in cellular senescence and SASP observed in the SFO is not due to peripheral increases in blood pressure, we utilized another hypertensive model of phenylephrine infusion that is not centrally mediated[26, 63]. 14 days of subcutaneous phenylephrine infusion did not lead to elevations in the senescence indicators $p16^{INK4A}$ or $p21^{WAF1/CIP1}$ in the SFO (Figure 2.6). Thus, our findings suggest that SFO cellular senescence and SASP is not induced by increases in blood pressure, but rather by elevated levels of Ang-II acting at the SFO.

SFO cellular senescence is cell type heterogeneous. To further characterize senescence in the SFO during hypertension development, we performed immunohistochemistry analysis following 14 days of Ang-II infusion and found a marked increase in $p16^{INK4A}$ expression throughout the rostral to caudal extent of the SFO compared to the day 0 control group (Figure 2.7). We also assessed $p16^{INK4A}$ expression in the median preoptic nucleus (MnPO), another brain region with a fenestrated blood-brain-barrier, the paraventricular nucleus of the hypothalamus, a cardiovascular brain region that receives inputs from the SFO, and the cortex. Importantly, $p16^{INK4A}$ expression was not enhanced in these brain regions following 14 days of Ang-II infusion (Figure 2.8), indicating that Ang-II-induced increases in $p16^{INK4A}$ may be specific to the SFO. Similarly, $p21^{WAF1/CIP1}$ expression was increased

following 14 days of Ang-II infusion (Figure 2.9). Collectively, these findings indicate that senescence occurs in the SFO at both the mRNA and protein level during Ang-II-induced hypertension development.

We next aimed to characterize the SFO cell types affected by Ang-II-induced senescence. To examine specific SFO populations, double immunohistochemistry was performed for either the astrocytic marker GFAP or neuronal marker NeuN with $p16^{INK4A}$ (Figure 2.10A-B) and $p21^{WAF1/CIP1}$ (Figure 2.11A-B). Colocalization analyses indicated that there was extremely low to no colocalization between $p16^{INK4A}$ and SFO neurons. However, $p16^{INK4A}$ was highly colocalized with astrocytes. In contrast, $p21^{WAF1/CIP1}$ moderately colocalized with both neurons and astrocytes. In other words, p16-dependent senescence occurs primarily in SFO astrocytes while $p21^{WAF1/CIP1}$ -dependent senescence may affect both SFO neurons and astrocytes following Ang-II infusion. Taken together, these findings indicate that cellular senescence in the SFO is heterogeneous depending on cell type.

SFO-specific $p19^{ARF}$ knockdown does not affect circadian patterns of blood pressure and heart rate. Our results thus far align with previous findings that cellular senescence in postmitotic SFO neurons may be driven primarily by $p21^{WAF1/CIP1}$ [43, 52, 54]. Given that SFO neurons are central to blood pressure regulation and undergo $p21^{WAF1/CIP1}$-dependent senescence, we tested the contribution of cellular senescence in SFO neurons to Ang-II-induced hypertension development by selectively removing the senescence mediator $p19^{ARF}$ from the SFO during Ang-II infusion. We chose to focus on $p19^{ARF}$ as a target for eliminating senescence because: 1) similar to $p16^{INK4A}$, $p19^{ARF}$ is encoded by the INK4a-

ARF genetic locus upstream of the $p21^{WAF1/CIP1}$-containing branch of the senescence signaling pathways (Figure 1.2); and 2) $p19^{ARF}$ has been predicted to be the most significant contributor to the expression profile of senescence genes in excitatory neurons related to AD pathology[64]. $p19^{ARF}$-floxed male mice received SFO injections of either control virus AAV-CMV-GFP or AAV-CMV-Cre-GFP to selectively remove $p19^{ARF}$ from the SFO, allowing for specific knockdown of $p19^{ARF}$ while leaving the $p16^{INK4A}$ protein fully intact. Following SFO injections, these mice were also implanted with radiotelemeters for continuous blood pressure monitoring. We first sought to evaluate whether knockdown of $p19^{ARF}$ in the SFO influenced basal blood pressure. No differences were observed in systolic (Figure 2.12A), diastolic (Figure 2.12B), or mean arterial blood pressure (Figure 2.12C), as well as heart rate (Figure 2.12D), between the control and $p19^{ARF}$ knockdown groups during either the light cycle or dark cycle, indicating that $p19^{ARF}$ knockdown in the SFO did not disrupt circadian patterns of blood pressure or heart rate.

Knockdown of the upstream senescence mediator $p19^{ARF}$ in the SFO partially alleviates Ang-II-induced hypertension. As expected, Ang-II infusion resulted in a progressive and sustained increase in systolic (Figure 2.13A), diastolic (Figure 2.13B), and mean arterial pressure (Figure 2.13C) throughout the 14-day recording period in the control (AAV-CMV-GFP) group. Interestingly, Ang-II infusion also induced increases in systolic, diastolic, and mean arterial blood pressure in the $p19^{ARF}$ knockdown (AAV-CMV-Cre-GFP) group. However, two-way ANOVA analysis revealed that relative to control mice, systolic pressure (Figure 2.13A) and mean arterial pressure (Figure 2.13C) were diminished in $p19^{ARF}$ knockdown mice during Ang-II infusion, but diastolic blood pressure did not differ

between the two groups (Figure 2.13B). In other words, systolic and mean arterial pressures in the $p19^{ARF}$ knockdown group did not increase to the same extent as control mice in response to 14 days of Ang-II infusion. Heart rate also differed between the control and $p19^{ARF}$ knockdown groups with 14 days of Ang-II infusion (Figure 2.13D). These findings suggest a role for the $p19^{ARF}$-$p21^{CIP1/WAF1}$ senescence pathway in the SFO in mediating Ang-II-induced hypertension development.

Given the increase in blood pressure in the $p19^{ARF}$ knockdown mice, we wanted to confirm that $p19^{ARF}$ was knockdown in the SFO and assess the expression of the other senescence markers. Following recordings, we collected micropunches of the SFO to confirm $p19^{ARF}$ knockdown using RT-qPCR analysis. We observed that $p19^{ARF}$ expression was indeed decreased in the SFO (Figure 2.13E). We also assessed the downstream mediators in the $p19^{ARF}$ signaling pathway, p53 and $p21^{CIP1/WAF1}$. As expected after knocking down $p19^{ARF}$, there was a trend for a decrease in both p53 (Figure 2.13F) and in $p21^{CIP1/WAF1}$ (Figure 2.13G) expression in the SFO. Given that $p19^{ARF}$ is encoded by the same gene as the other senescence mediator $p16^{INK4A}$, we wanted to verify that SFO expression of $p16^{INK4A}$ was not disrupted. Indeed, SFO expression of $p16^{INK4A}$ was not decreased to the same degree as $p19^{ARF}$ (Figure 2.13H). Finally, we assessed SFO expression of SASP indicators IL-6 and IL-10. Interestingly, IL-10 (Figure 2.13J), but not IL-6 (Figure 2.13I), expression was decreased following $p19^{ARF}$ knockdown in the SFO. Taken together, our findings indicate that SFO-specific $p19^{ARF}$ knockdown does not influence Ang-II-induced hypertension development.

2.5 Discussion

A significant concern for hypertensive patients is the prevalence of chronic uncontrolled hypertension, which increases their risk for additional cardiovascular, metabolic, and neurological conditions[1-4]. It has been estimated that 56.7% of hypertensive patients had to take two or more antihypertensive medications, which may or may not be successful in controlling their blood pressure[65]. These findings clearly outline the need to further our understanding of the underlying mechanisms of hypertension development and provide effectual therapeutic targets for antihypertensive treatments.

Intriguingly, cellular senescence has emerged as an important therapeutic target in various models of aging, injury, and diseases. Selective elimination of senescent cells improves measures of aging- and stress-related conditions[39, 40, 42, 43, 66]. Thus, we rationalized that targeting senescent cells may be beneficial in the context of hypertension as well. The current findings indicate that selectively removing senescent cells throughout the body using the INK-ATTAC mouse model was sufficient to prevent Ang-II-induced hypertension, indicating a role for senescent cells in hypertension development. To further delineate the mechanism of senescent cell accumulation in mediating hypertension, we selectively induced cellular senescence in the CNS via ICV doxorubicin injections, and radiotelemetry recordings revealed that senescence specifically in the CNS can drive increases in blood pressure.

The SFO is well-recognized for its role in cardiovascular regulation and hypertension development, and the SFO is anatomically equipped to be responsive to peripheral Ang-II and relay these inputs to downstream brain regions[21-23]. Based on this, we hypothesized that the SFO could be a potential site for senescent cell accumulation during Ang-II-induced hypertension development. The present findings demonstrate that

cellular senescence and the SASP is indeed upregulated in the SFO of male mice following 14 days of Ang-II infusion. As far as we know, our studies are the first to characterize CNS cellular senescence, specifically in the SFO, in the context of Ang-II-induced hypertension development.

Although our data indicates that senescence and SASP in the SFO are associated with Ang-II-induced hypertension, it remains unclear what the increase in senescence/SASP means for SFO neuron and astrocyte functioning or how the downstream neural circuity may be affected. The current literature on how senescence affects neuron excitability is extremely limited, but some anatomical and electrophysiological data suggests that senescence may disrupt synaptic plasticity and excitability in neurons[53, 67-71]. Furthermore, senescent astrocytes appear to have impaired capacity to clear excess glutamate, leading to excitotoxicity and increased cell death in cultured neurons[51].

An ongoing challenge in the field of cellular senescence is defining senescence in postmitotic cell types. Senescence can be classically defined as a state of permanent cell cycle arrest, as initially identified by Hayflick and Moorhead in 1961. However, postmitotic cell types, such as neurons, exit the cell cycle once differentiated, eliminating a key characteristic of senescent cells. Thus, there is a growing, but still limited, body of literature demonstrating the heterogeneity of cellular senescence in different cell types, as well as uncovering a more detailed profile of senescence in neurons and astrocytes[32, 64]. The present findings help to unravel the complexity of senescent phenotypes by providing an important characterization of senescent SFO neurons and astrocytes in the context of hypertension.

The mechanisms underlying the sexually dimorphic nature of hypertension remain incompletely defined but could be potentially explained by our finding that SFO senescence/SASP occurs in a sexually dimorphic manner during Ang-II-induced hypertension. In other words, female mice appear to be protected against the accumulation of senescent cells and the accompanying SASP in the SFO during Ang-II-induced hypertension development. Intriguingly, this finding is in line with the existing literature that Ang-II seems capable of inducing senescence in peripheral cell types, and female mice are resistant to hypertension development in response to Ang-II infusion[34, 35, 59]. However, the mechanism of protection against senescent cell accumulation and subsequent hypertension remains to be determined. Estrogen is a likely candidate given that 1) female risk for hypertension drastically increases following menopause; 2) estrogen signaling at the SFO specifically appears to mediate female protection against Ang-II-induced hypertension development in mice[60]; and 3) estrogen appears to be protective against cellular senescence in peripheral cell types[72-75]. Further work is required to determine whether estrogen prevents cellular senescence and SASP in SFO of females during Ang-II infusion.

Delineating the mechanisms and sex differences of hypertension development is crucial for both men and women across ages given the prevalence of chronic uncontrolled hypertension. Several studies have demonstrated that targeting $p16^{INK4A}$ as a means for selective removal of senescence improves disease states such as aging, neurodegenerative disease, nonalcoholic steatohepatitis, and spinal cord injury[33, 39, 42, 66]. The $p21^{CIP1/WAF1}$ pathway has been less explored as a potential therapeutic target; however, recent studies have uncovered a growing number of senescence-related signaling pathways aside from

$p16^{INK4A}$ that improve subsequent symptoms of aging and diabetes, such as the anti-apoptotic protein B-cell lymphoma-extra-large, growth differentiation factor 11/Mothers against decapentaplegic homolog 2, cyclin dependent kinase 2/cyclin-dependent kinase inhibitor 1B, and phosphoglycerate dehydrogenase/pyruvate kinase M2/histone 3 at threonine 11[40, 43, 76, 77]. Given the literature and our findings that SFO neurons are affected by $p21^{CIP1/WAF1}$-dependent senescence, we chose to evaluate the $p19^{ARF}$-$p21^{CIP1/WAF1}$ senescence pathway in the SFO as a potential route for alleviating hypertension. We found that selectively eliminating SFO $p19^{ARF}$ partially alleviated Ang-II-induced hypertension development. Thus, SFO cellular senescence may be a potential therapeutic target for hypertension. However, selective elimination of SFO $p19^{ARF}$ did not fully prevent hypertension development, suggesting that alternative senescence mechanisms may also be at play. In our immunohistological studies, we observed that astrocytes are also affected by $p16^{INK4A}$-dependent cellular senescence. Given that cellular senescence can be perpetuated through the SASP[56, 57], targeting both the $p19^{ARF}$-$p21^{CIP1/WAF1}$ and $p16^{INK4A}$-related senescence pathways in the SFO may be required for full prevention of senescence/SASP and subsequent hypertension development. Furthermore, the temporal dynamics of the onset of senescence have yet to be explored. For example, it may be that neurons develop the senescent state initially, causing astrocytes to become senescent through the SASP, or vice versa.

As previously mentioned, targeting senescent cells has proven to be beneficial for a number of conditions, including aging[39, 43], neurodegenerative disease[78], diabetes[40], nonalcoholic steatohepatitis[33], and spinal cord injury[42]. Thus, there is great interest in identifying drugs that eliminate senescent cells - a class of drugs known as senolytic

drugs[79]. Since the discovery of the first senolytic drugs dasatinib and quercetin[80], there is a growing list of senolytic drugs to target senescent cells, including FDA-approved senolytic drugs[81, 82]. Furthermore, the first clinical trial for senolytic drugs in human patients was conducted recently[83], and there are currently about 20 clinical trials that are ongoing or completed[82]. Our findings have implications for cellular senescence to be a potential therapeutic target for hypertension and for potentially repurpose FDA-approved senolytic drugs as antihypertensive treatments.

In summary, these findings reveal cellular senescence in the SFO during hypertension development. More specifically, CNS cellular senescence appears to mediate increases in blood pressure, and Ang-II infusion induces senescence in the SFO in a heterogeneous, cell-type specific manner in males. Collectively, these studies highlight cellular senescence as a new stress pathway that may integrate with currently known stressors and provide additional therapeutic targets for antihypertensive treatments.

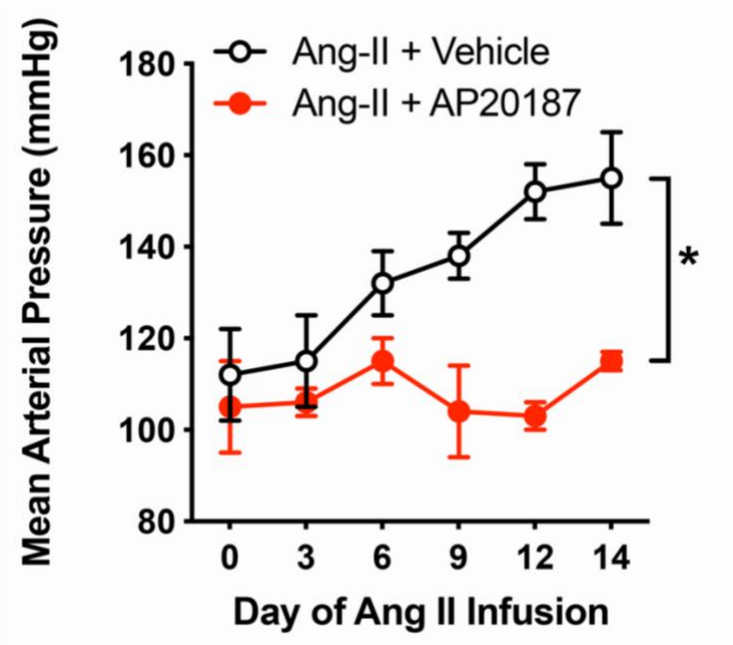

Figure 2.1. Selective elimination of senescent cells prevents Ang-II-induced hypertension development.
Radiotelemetry recordings of mean arterial pressure in INK-ATTAC male mice that received Ang-II infusion for 14 days along with daily IP injection of either the inducing agent AP20187 (red) or vehicle control (black). n=3-4/group. *p<0.05.

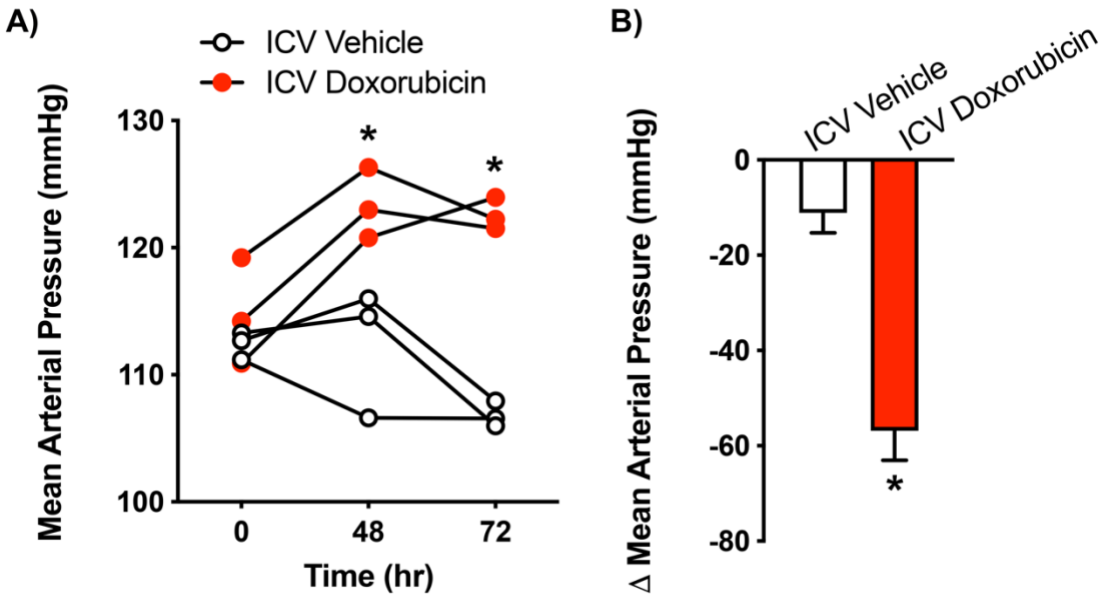

Figure 2.2 Cellular senescence in the central nervous system elevates blood pressure, mediated by sympathetic nervous system activation.
(A) Radiotelemetry recordings for mean arterial pressure in response to 3-day ICV administration of either doxorubicin (red) or vehicle (αCSF, white) injections. **(B)** Change in mean arterial pressure after ganglionic blockade using IP chlorisondamine in doxorubicin- (red) or vehicle-treated (white) mice. n=3/group. *p<0.05 vs ICV vehicle.

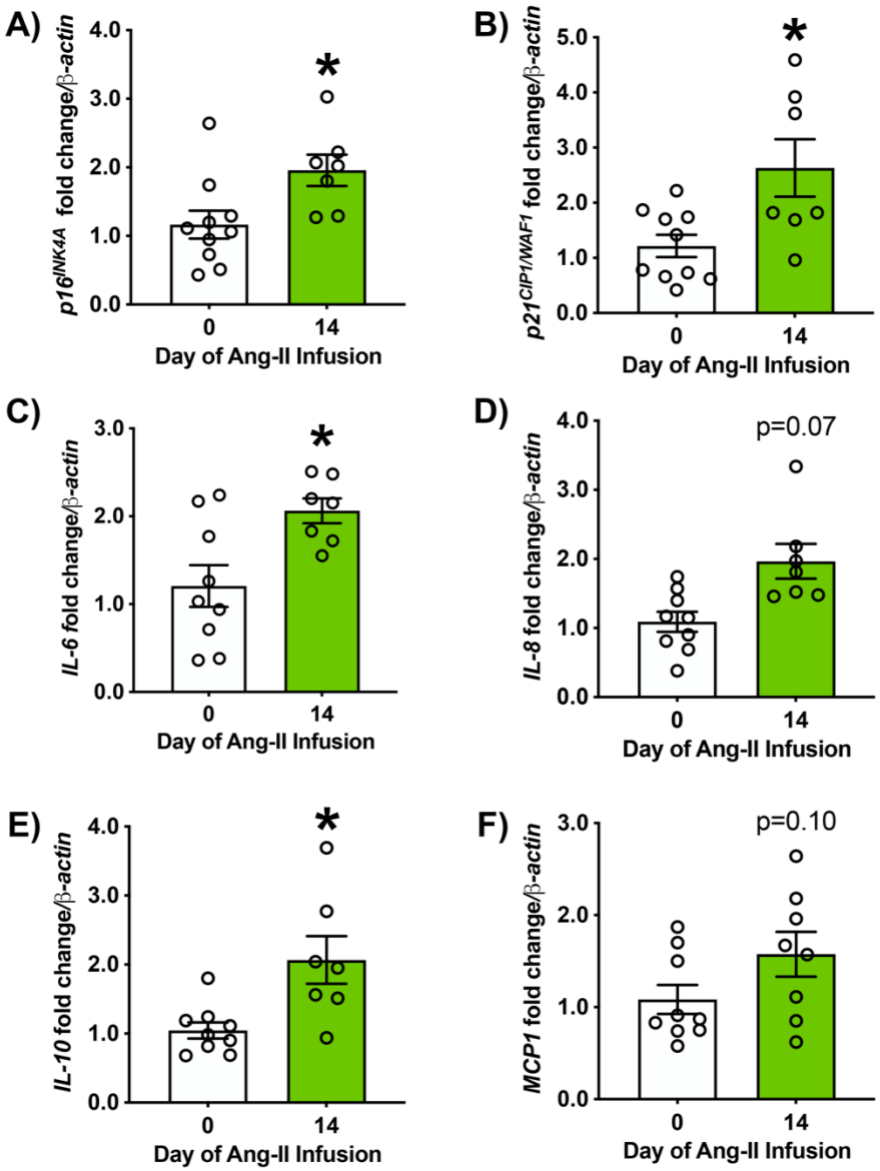

Figure 2.3. Ang-II infusion is associated with the upregulation of senescence and SASP markers in the SFO.
Quantitative-time PCR analysis for senescence markers $p16^{INK4A}$ **(A)** and $p21^{CIP1/WAF1}$ **(B)** and SASP markers IL-6 **(C)**, IL-8 **(D)**, IL-10 **(E)**, and MCP1 **(F)** in the SFO of C57BL/6J male mice that were infused with Ang-II for either 0 (white) or 14 (green) days. n=7-10/group. *$p<0.05$.

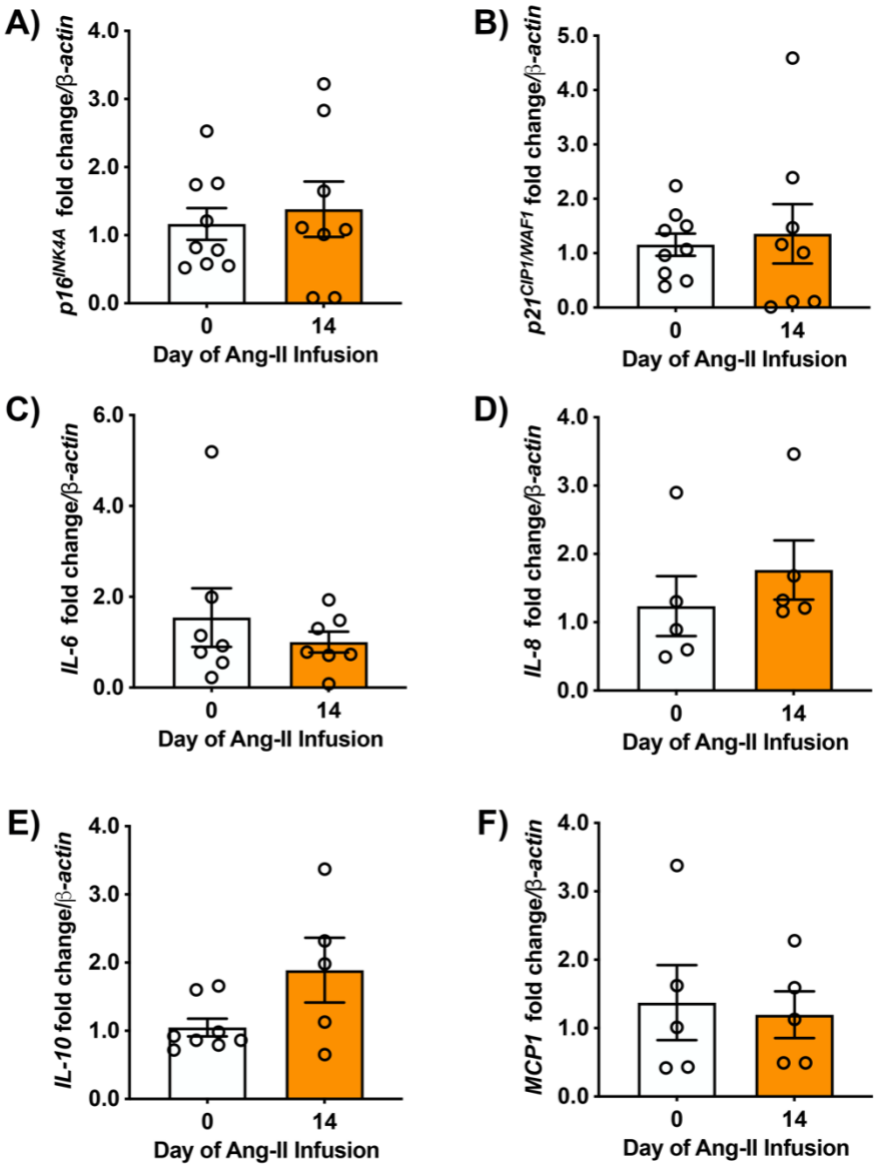

Figure 2.4. Ang-II-induced upregulation of senescence and SASP markers does not occur in the SFO of female mice.

Quantitative-time PCR analysis for senescence markers $p16^{INK4A}$ **(A)** and $p21^{CIP1/WAF1}$ **(B)** and SASP markers IL-6 **(C)**, IL-8 **(D)**, IL-10 **(E)**, and MCP1 **(F)** in the SFO of C57BL/6J female mice that were infused with Ang-II for either 0 (white) or 14 (orange) days. n=5-9/group. *$p<0.05$.

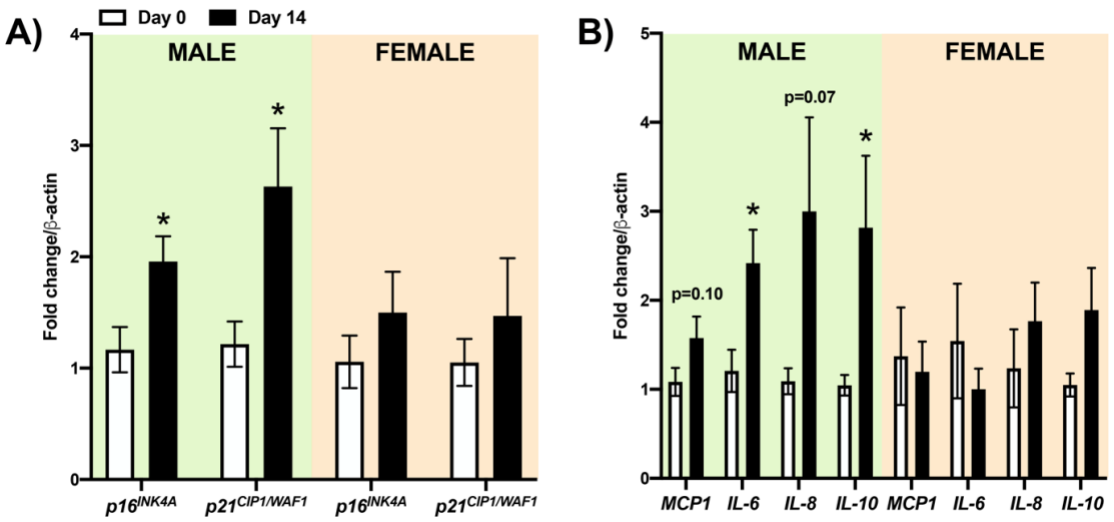

Figure 2.5. Cellular senescence and SASP in the SFO during Ang-II-induced hypertension development is sexually dimorphic.
Quantitative real-time PCR for **(A)** senescence markers $p16^{INK4A}$ and $p21^{CIP1/WAF1}$ and **(B)** SASP markers MCP1, IL-6, IL-8, and IL-10 in the SFO of male (green) and female (orange) C57BL/6J mice infused with Ang-II for either 0 (white) or 14 (black) days. n= 5-10/group. *$p<0.05$ vs. Day 0.

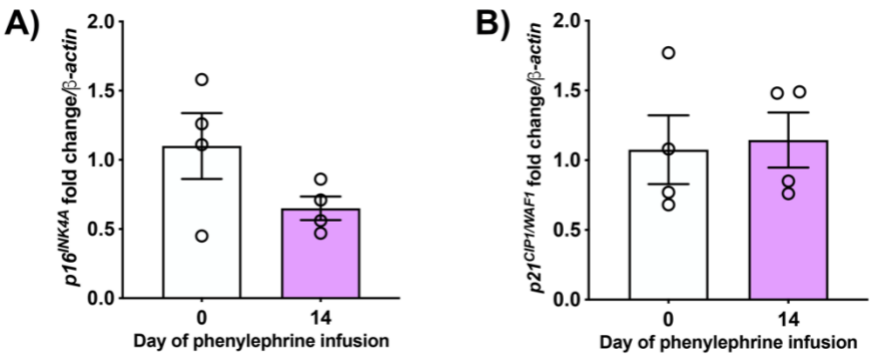

Figure 2.6. Increases in senescence in the SFO are specific to Ang-II-induced hypertension.
Quantitative real time PCR for $p16^{INK4A}$ **(A)** and $p21^{CIP1/WAF1}$ **(B)** expression in the SFO of male mice that received either 0 or 14 days of phenylephrine infusion. n=4/group.

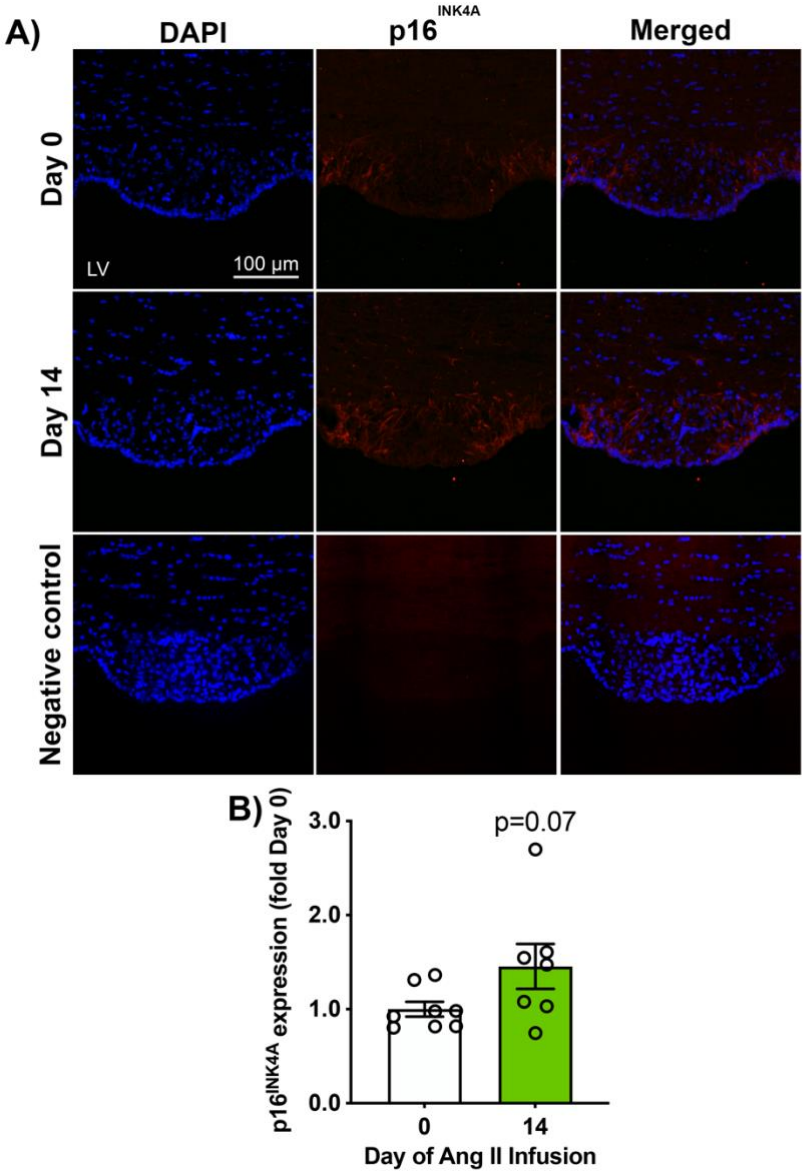

Figure 2.7. Senescence marker p16^{INK4A} expression is increased in the SFO during Ang-II hypertension.
(A) Immunohistochemistry staining for p16^{INK4A} (red) in the SFO in day 0 (top row), day 14 (middle row) mice. Negative control with no primary antibody (bottom row). **(B)** Quantification of p16^{INK4A} staining using integrated density (n=7-8/group).

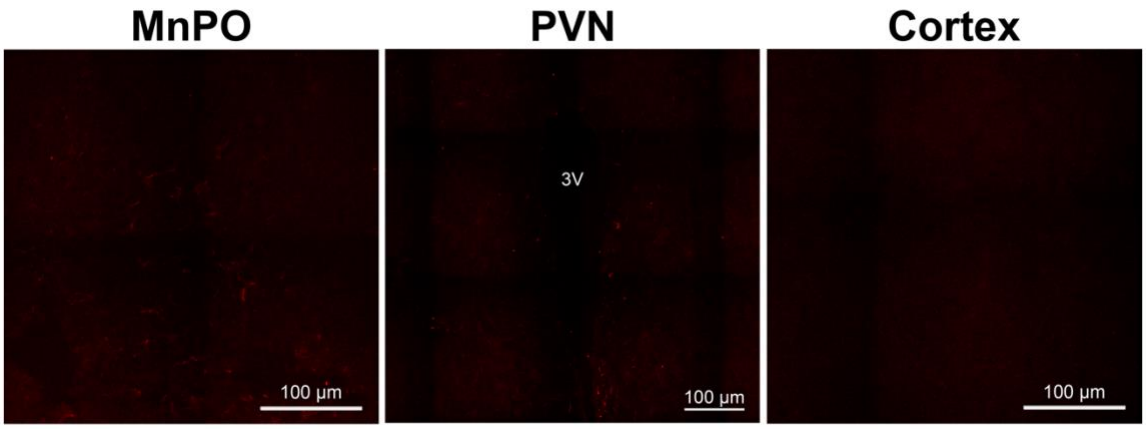

Figure 2.8. Increased p16^{INK4A} staining following Ang-II infusion is specific to the SFO.
Immunohistochemistry staining in additional brain regions, including median preoptic nucleus (MnPO), paraventricular nucleus of the hypothalamus (PVN), and cortex.

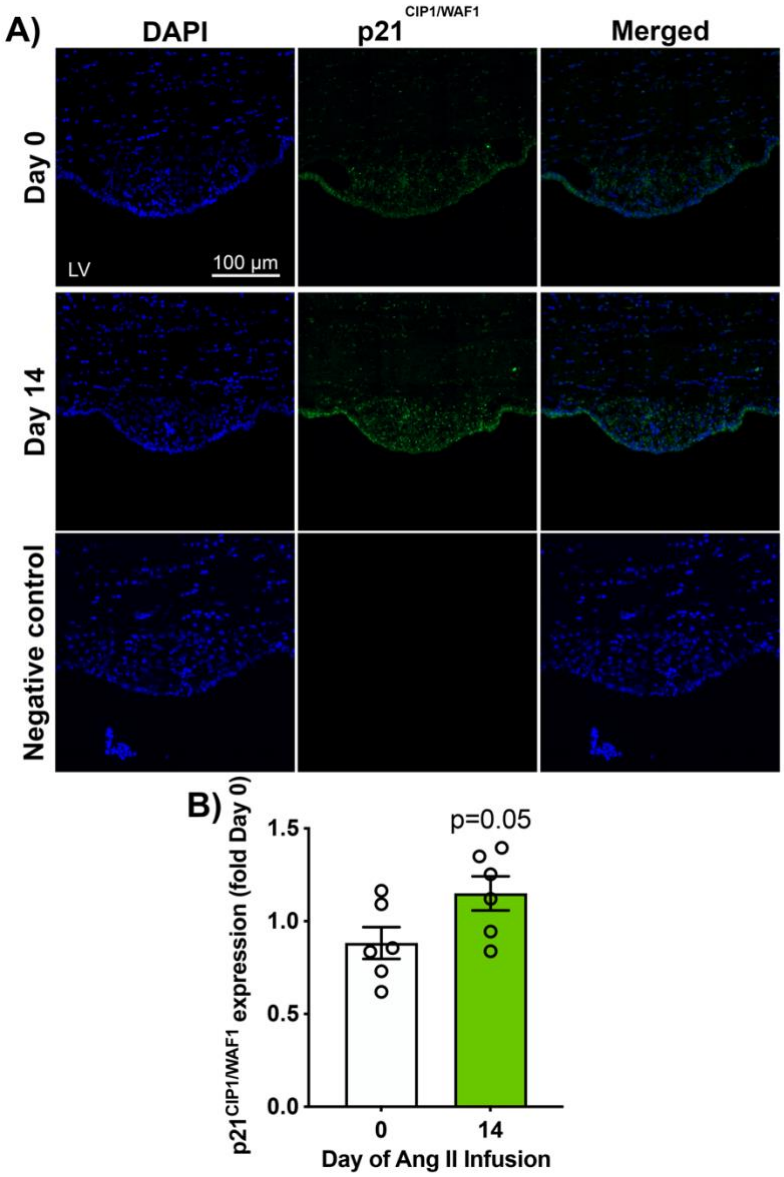

Figure 2.9. SFO expression of the senescence marker p21$^{CIP1/WAF1}$ increases during Ang-II-induced hypertension.
(A) Staining for p21$^{CIP1/WAF1}$ (green) in the SFO, nuclear marker dapi (blue) in day 0 (top row), day 14 (middle row) mice. Negative control with no primary antibody (bottom row). **(B)** Quantification of p21$^{CIP1/WAF1}$ staining using integrated density (n=6/group).

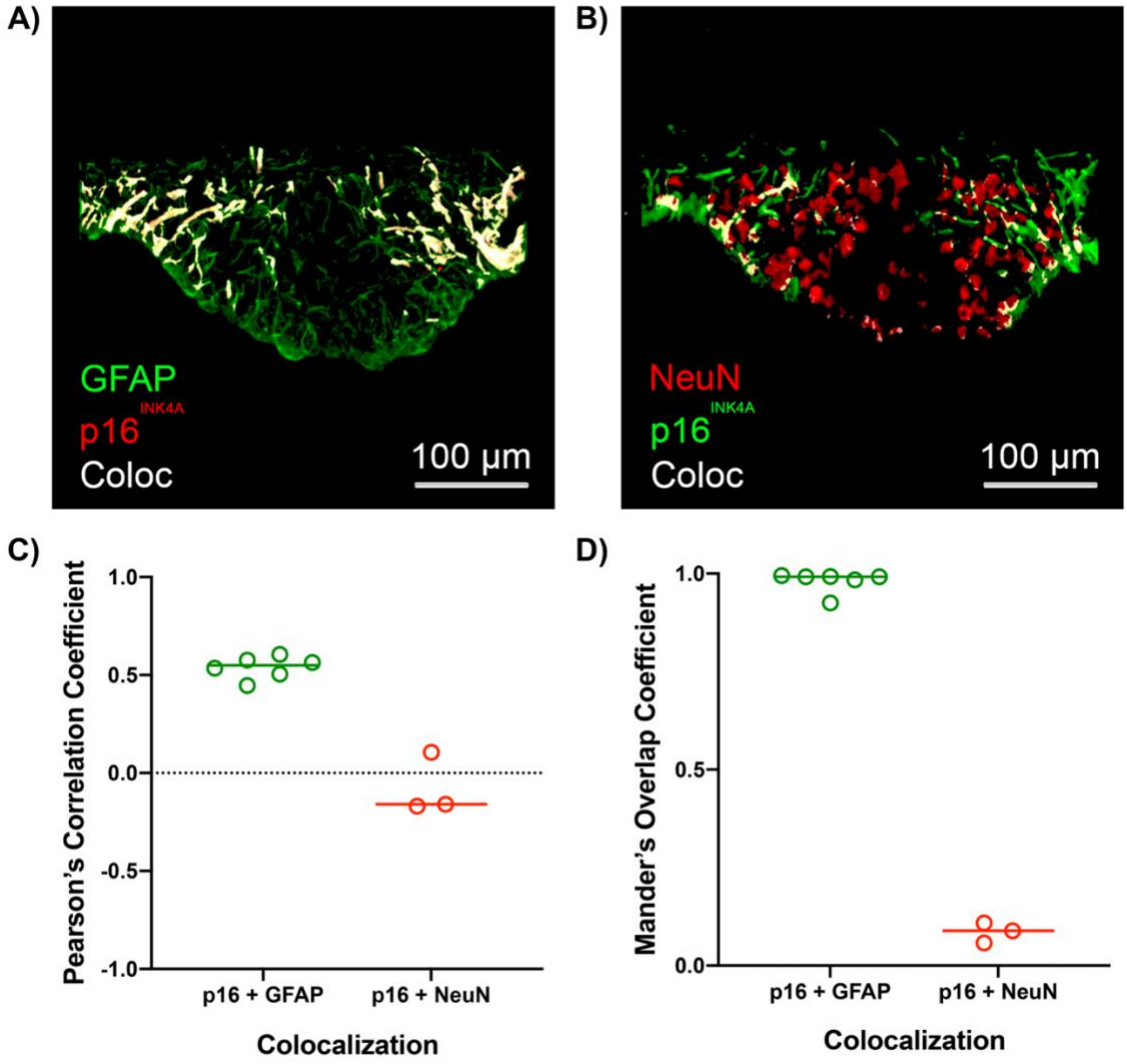

Figure 2.10. Upregulation of p16^{INK4A} expression in response to Ang-II infusion occurs primarily in SFO astrocytes.
C57Bl/6J male mice were infused with Ang-II for 14 days and stained for senescence and cell type-specific markers. **(A)** Representative image for double immunohistochemistry staining with p16^{INK4A} (red) and astrocytic marker GFAP (green). **(B)** Representative image for double immunohistochemistry staining with p16^{INK4A} (green) and neuronal marker NeuN (red). Colocalized areas are in white. **(C)** Colocalization quantification using Pearson's correlation coefficient. **(D)** Colocalization quantification using Mander's overlap coefficient.

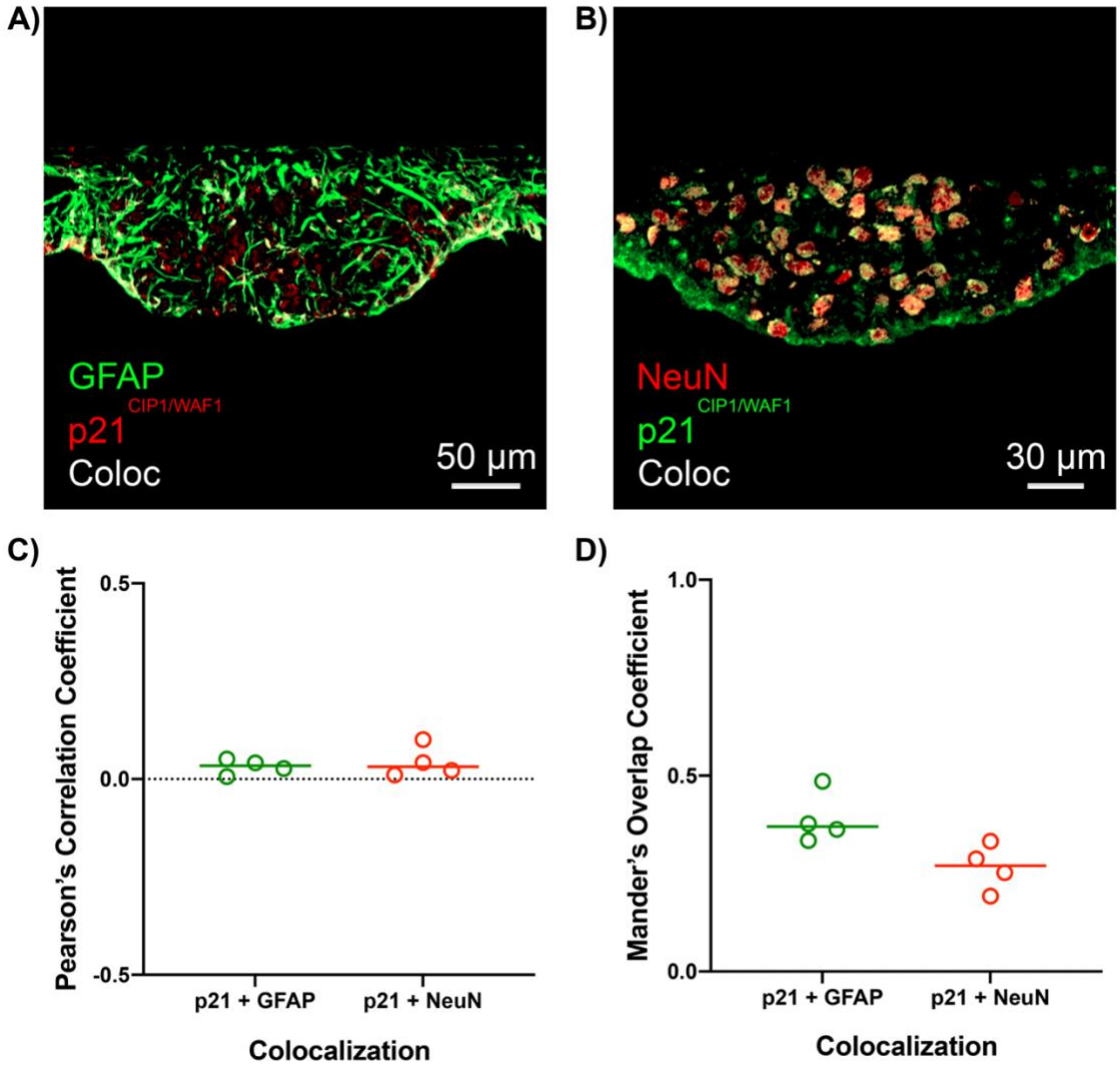

Figure 2.11. Moderate upregulation of p21$^{CIP1/WAF1}$ during Ang-II infusion occurs in both SFO astrocytes and neurons.
C57Bl/6J male mice were infused with Ang-II for 14 days and stained for senescence and cell type-specific markers. **(A)** Representative image for double immunohistochemistry staining with p21$^{CIP1/WAF1}$ (red) and astrocytic marker GFAP (green). Colocalized areas are in white. **(B)** Representative image for Double immunohistochemistry staining with p21$^{CIP1/WAF1}$ (green) and neuronal marker NeuN (red). Colocalized areas are in white **(C)** Colocalization quantification using Pearson's correlation coefficient. n=4/group. **(D)** Colocalization quantification using Mander's overlap coefficient. n=4/group.

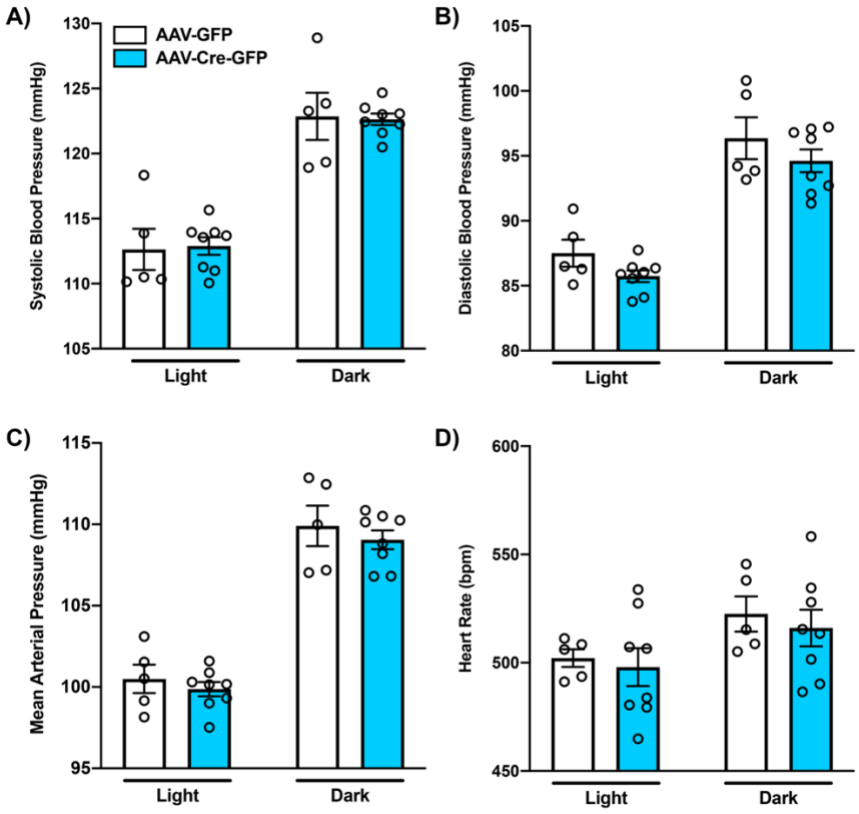

Figure 2.12. SFO-specific knockdown of p19^{ARF} does not alter circadian patterns in cardiovascular measures.

Radiotelemetry recordings to measure systolic blood pressure **(A)**, diastolic blood pressure **(B)**, mean arterial pressure **(C)**, and heart rate **(D)** in p19^{ARF}-floxed mice with SFO injection of either AAV-CMV-Cre-GFP (blue) or control viral construct AAV-CMV-GFP (white) during the light and dark cycles of the baseline period. n=5-8/group.

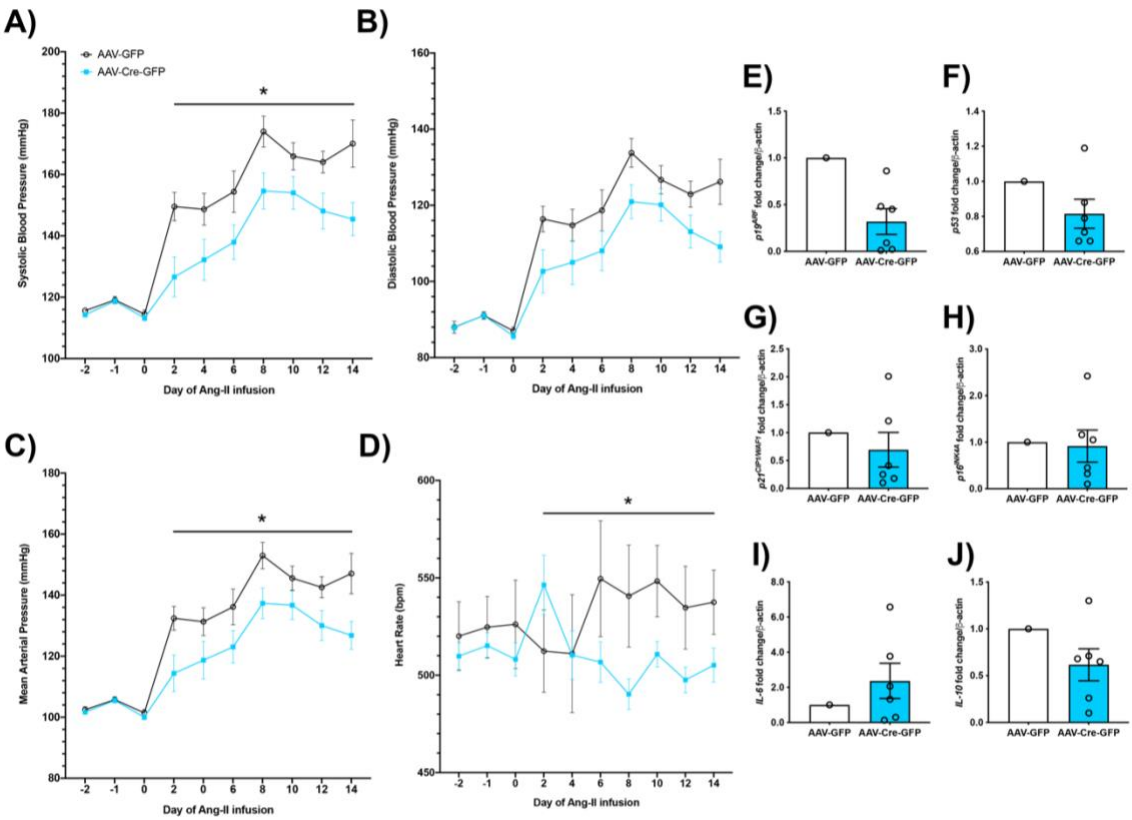

Figure 2.13. SFO-specific knockdown of senescence mediator p19^{ARF} partially alleviates Ang-II-induced hypertension development.
Radiotelemetry recordings to measure changes in systolic blood pressure **(A)**, diastolic blood pressure **(B)**, mean arterial pressure **(C)**, and heart rate **(D)** in p19^{ARF}-floxed mice with SFO injection of either AAV-CMV-Cre-GFP (blue) or control viral construct AAV-CMV-GFP (white) during 14 days of Ang-II infusion. Quantitative real time PCR analysis was performed following 14 days of Ang-II infusion for senescence pathway markers p19^{ARF} **(E)**, p53 **(F)**, p21$^{CIP1/WAF1}$ **(G)**, p16^{INK4A} **(H)**, and SASP markers IL-6 **(I)** and IL-10 **(J)**. n=1-5/group.

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Chapter 3: Conclusions and Discussion

3.1 Summary

Our findings indicate the presence of a novel stressor in the SFO that potentially mediates hypertension development (Figure 3.1). This dissertation has four major conclusions: 1) Cellular senescence occurs in the SFO at the mRNA and protein level during Ang-II-induced hypertension development in males; 2), Ang-II-induced cellular senescence in the SFO presents in a sexually dimorphic manner; 3) $p16^{INK4A}$-dependent cellular senescence occurs primarily in astrocytes whereas $p21^{CIP1/WAF1}$-dependent senescence may affect both SFO neurons and astrocytes during Ang-II-induced hypertension development; 4) SFO-specific targeting $p19^{ARF}$, the upstream mediator of a major senescence pathway, partially alleviates Ang-II-induced hypertension development.

3.2 Discussion and Future Directions

Cellular senescence in the SFO

Cellular senescence is an emerging area of interest for a number of disease states, but has yet to be explored extensively in the context of hypertension. The current work provides two novel findings: 1) A role for cellular senescence in the CNS in the development of hypertension; and 2) The first characterization of cellular senescence in the SFO. We demonstrate for the first time that cellular senescence and SASP occur in the SFO during Ang-II-induced hypertension, but the mechanism by which Ang-II induces senescence and SASP is an area for further exploration. Previous studies have indicated that Ang-II induces cellular senescence in endothelial progenitor cells via Ang-II-induced upregulation of gp91phox expression, a subunit of nicotinamide adenine dinucleotide

phosphate (NOX), which is the predominant source of Ang-II-induced reactive oxygen species (ROS)[1]. Furthermore, AT_1R signaling is linked to the activation of NOX and subsequent production of ROS in the SFO[2-4]. In other brain regions, it has been shown that Ang-II-induced NOX activation in turn leads to the activation of p38 mitogen-activated protein kinase signaling[5], a major signaling mechanism for SASP activation[6, 7]. These findings suggest the involvement of NOX-produced ROS in inducing Ang-II-induced cellular senescence and SASP. Furthermore, Ang-II induces endoplasmic reticulum (ER) stress in the SFO, which can also lead to increased ROS production[8]. Both Ang-II-induced ER stress and oxidative stress drive increased activity of nuclear factor-κB in the SFO[9], which is a mediator for inflammatory responses and another key transcription for SASP activation[6, 10]. Based on these studies, it may be that the onset of senescence is a combination of chronic exposure to these Ang-II-induced stress pathways in the SFO. In other words, cellular senescence may serve as an integrative mechanism for oxidative stress, ER stress, and inflammatory pathways in the SFO during Ang-II-induced hypertension development.

Once cellular senescence is established, the inflammatory SASP can perpetuate senescence into neighboring cell types. Our initial characterization of cellular senescence focused on SFO neurons and astrocytes, but literature supports that senescence can occur in additional cell types, such as microglia and endothelial cells[11-17]. Given that the SASP is capable of perpetuating senescence into neighboring cells and tissues, further exploration is required to better understand the temporal and spatial dynamics of the onset of cellular senescence in different cell types. In other words, cellular senescence may be initiated primarily in one SFO cell type in the short-term, then spread through the SASP to additional

cell types to induce prolonged senescence. It seems likely that Ang-II-responsive SFO neurons would be affected by senescence first given that AT_1R expression is found on SFO neurons, but not astrocytes[18-20]. Then, senescence may spread to astrocytes from the SASP activation in neurons. The temporal patterns of the onset of cellular senescence signaling pathways is an area that remains largely unexplored; however, the difference in the timing of the onset of senescence could also potentially explain the differences in $p21^{CIP1/WAF1}$ versus $p16^{INK4A}$-dependent senescence between SFO astrocytes and neurons. One transcriptomics study has suggested differing senescence and SASP profiles between early, intermediate, and late senescence in fibroblasts, melanocytes, and keratinocytes[21]. Early senescence, defined as four days of ionizing radiation treatment, was characterized by DNA damage response pathways and p53 signaling. Intermediate senescence, or 10 days of treatment, was also characterized by p53 signaling, p38-mitogen-activated protein kinase signaling, which is important for the SASP, and mitochondrial metabolic changes. Late senescence at 20 days of treatment was characterized by cell cycle arrest and chromatin remodeling. The SASP profiles at each timepoint were also heterogeneous. The involvement of p53 signaling in early and intermediate senescence might indicate that p53-$p21^{CIP1/WAF1}$ signaling is involved with the initiation of senescence. In support of this, another study found that a fibroblast cell strain with low $p21^{CIP1/WAF1}$ expression exhibited features of senescence after going through serial cultivation. However, replication and cyclin/Cdk2 activity was not blocked, suggesting that $p21^{CIP1/WAF1}$ may be responsible for the initial cell cycle arrest in senescence whereas other features of senescence could be $p21^{CIP1/WAF1}$-independent[22]. Further work is required to better understand how multiple senescence signaling pathways interact and manifest the features of senescence.

Another area in the field of cellular senescence that remains poorly understood is the functional consequences of cellular senescence, especially for neurons. Electrophysiological evidence indicates that aged neurons, made senescent with $p16^{INK4A}$ overexpression, display diminished spontaneous firing relative to control neurons[23]. The decreased spontaneous firing in senescent neurons suggests that senescent neurons may have impairments in forming synapses and plasticity. However, it remains unclear whether senescence-associated alterations in neurons enhances or diminishes neuronal excitability. Some studies have suggested that cellular senescence is associated with increases in the components of the excitatory glutamatergic system[24] and the upregulation of genes associated with excitatory signaling[25], which are changes that might shift neuron firing patterns towards enhanced excitability. In line with this, whole-cell patch clamp recordings revealed enhanced excitability of cortical excitatory neurons that were made senescent by the loss of growth differentiation factor 11[26]. On the other hand, other morphological and electrophysiological evidence point to senescent neurons having decreased dendritic spine size and density[27] and enhanced long-term depression, which would weaken synaptic strength[28], suggesting that senescence would decrease neuronal excitability. It is noteworthy to mention that most of these studies were performed in aged rodent models or cultured neurons, and more evidence is needed to understand the functional impact of stress-induced cellular senescence on neurons. Given our present findings, understanding the effect of cellular senescence on neuron excitability is crucial to understand the pathogenesis of hypertension because the SFO sends projections to numerous downstream cardiovascular and autonomic brain regions, such as the PVN[29-33]. There is also very little literature on how astrocyte function is impacted by senescence, although some studies have

suggested that astrocytes that undergo senescence may lead to astrogliosis[23], which is when astrocytes undergo significant morphological and biochemical changes, and impairments in glutamate homeostasis[34].

Sex differences in cellular senescence and hypertension

The treatment and management of hypertension is sexually dimorphic. The American Heart Association and American College of Cardiology does not recommend differential treatment based on sex for hypertension. However, hypertensive women more commonly are prescribed thiazide diuretics whereas men primarily use angiotensin-converting enzyme inhibitors or angiotensin receptor blockers[35]. Furthermore, the majority of older hypertensive patients tend to be women and are less likely to have their blood pressure under control compared to age-matched men. Thus, it is crucial to explore sex differences in hypertension to reconcile the lack of effective antihypertensive treatments for both men and women across ages by exploring sexual dimorphic mechanisms and potentially new therapeutic targets. Our findings suggest that cellular senescence may be one such sexually dimorphic mechanism. We found that female mice, who are known to be resistant to Ang-II-induced hypertension, are also resistant to SFO cellular senescence and SASP during Ang-II infusion. However, the mechanism of female protection against cellular senescence and subsequent hypertension remains unclear.

Estrogen, a hormone that is generally regarded as cardioprotective, appears to be important for female protection against hypertension. As previously mentioned, at younger ages, women are at a lower risk for hypertension than men. However, following menopause when the production of estrogen declines, the female risk for hypertensive drastically

increases to equalize with age-matched men[36]. This suggests a role for estrogen in preventing hypertension, which has been confirm in animal models where female mice infused with Ang-II are resistant to hypertension[37]. However, ovariectomized or ovotoxin-treated female mice develop hypertensive in response to Ang-II, and exogenous estrogen treatment rescues the hypertensive response[38, 39]. Estrogen signaling at the SFO seems to be particularly important for female resistance to hypertension[40]. Furthermore, estrogen is protective against cellular senescence in peripheral tissue types[41-45]. Thus, further exploration is needed to determine if estrogen signaling prevents SFO cellular senescence in females during Ang-II-induced hypertension.

Consequences of senescence as a mediator for hypertension

Many hypertensive patients must take two or more antihypertensive medications, which may or may not be successful in controlling their blood pressure[46]. Based on our findings, SFO cellular senescence could be a new potential therapeutic target for antihypertensive treatments. Targeting $p16^{INK4A}$ has been shown as a means for eliminating senescent cells, and removing cells with high $p16^{INK4A}$ expression has been shown to improve symptoms of aging, neurodegenerative disease, nonalcoholic steatohepatitis, and spinal cord injury[47-50]. Targeting the $p19^{ARF}$-$p21^{CIP1/WAF1}$ senescence pathway has been less explored, although a few studies have demonstrated beneficial outcomes of targeting $p21^{CIP1/WAF1}$, such as improved physical performance in aged mice, reduced inflammation, and reduced liver fibrosis[51-53]. Furthermore, several studies have identified that targeting additional senescence-associated pathways, including anti-apoptotic protein B-cell lymphoma-extra-large, growth differentiation factor 11/Mothers against decapentaplegic

homolog 2, cyclin dependent kinase 2/cyclin-dependent kinase inhibitor 1B, and phosphoglycerate dehydrogenase/pyruvate kinase M2/histone 3 at threonine 11, also alleviates senescence and subsequent ailments such as diabetes and aging[26, 54-56], suggesting that targeting cellular senescence should be specific to the cell type, stressor type, and disease model. Our present data also indicates that neurons are affected by $p21^{CIP1/WAF1}$-dependent senescence during Ang-II-induced hypertension development, a finding that is in line with previous literature[26, 57, 58]. Taking all this together, we sought to evaluate the role of $p19^{ARF}$-$p21^{CIP1/WAF1}$ senescence in the SFO as a potential mechanism and therapeutic target for hypertension. We found that selective elimination of $p19^{ARF}$ in the SFO partially alleviated hypertension development in response to Ang-II, indicating a role for the $p19^{ARF}$-$p21^{CIP1/WAF1}$ pathway in mediating hypertension. Although we chose to focus on $p19^{ARF}$-$p21^{CIP1/WAF1}$, there may also be a role for $p16^{INK4A}$ in the SFO in hypertension development given that our data indicated that SFO astrocytes are affected by $p16^{INK4A}$ -dependent senescence during Ang-II-induced hypertension. Thus, in future studies, targeting both $p19^{ARF}$-$p21^{CIP1/WAF1}$ and $p16^{INK4A}$-dependent cellular senescence in the SFO may lead to the full alleviation of hypertension development. Taken together, further work is needed to discern the relative contributions of the different senescence pathways in the SFO in mediating hypertension development.

Another avenue to explore senescence as a potential antihypertensive target is repurposing drugs with senolytic effects that are already FDA-approved. The most tested senolytic treatment is a combination of dasatinib and quercetin, which were the original two drugs discovered to have senolytic effects[59]. Recently, dasatinib and quercetin were tested for the first time in human patients to alleviate symptoms of idiopathic pulmonary

fibrosis[60]. Since the initial discovery of senolytics, a multitude of senolytic drugs have emerged, such as fisetin, luteolin, curcumin, navitoclax, FOXO4-related peptide,[61] and there are currently over 20 clinical trials to test the benefits of senolytics in a variety of disease conditions[62]. Collectively, our findings may have clinical implications for FDA-approved senolytic drugs to be used as anti-hypertensive treatments.

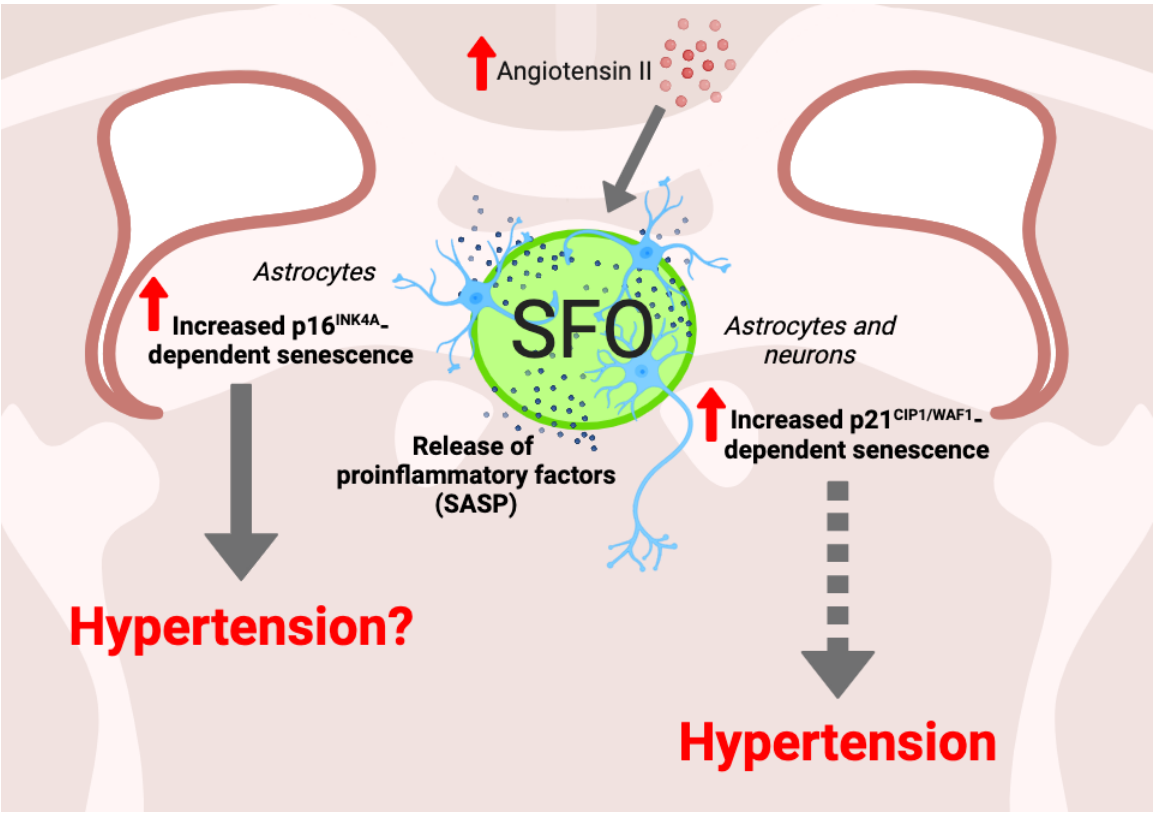

Figure 3.1. Overview of proposed role for SFO cellular senescence in hypertension development and areas for further work.
Excess levels of angiotensin II induce cellular senescence and SASP in the SFO. Neurons appear to be solely affected by $p21^{CIP1/WAF1}$-dependent senescence while astrocytes are affected by both $p16^{INK4A}$- and $p21^{CIP1/WAF1}$-dependent senescence. Cellular senescence mediated by the $p19^{ARF}$- $p21^{CIP1/WAF1}$ pathway in the SFO partially mediates hypertension development. Created using biorender.com.

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