BLOOD PRESSURE REGULATION BY THE ENDOCANNABINOIDS SYSTEM IN CONDITIONS ASSOCIATED WITH HYPERTENSION

By

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Aunt Linda, Uncle Paul, cousins Michael and David, and grandparents Jimmy and Maria:
this is all for you. Thank you for always challenging me to do better.
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<tbody>
<tr>
<td>2-AG</td>
<td>2-arachidonoylglycerol</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>Ang I</td>
<td>angiotensin I</td>
</tr>
<tr>
<td>Ang II</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>Ang-(1-7)</td>
<td>angiotensin-(1-7)</td>
</tr>
<tr>
<td>AT1</td>
<td>Ang II type I receptor</td>
</tr>
<tr>
<td>AT2</td>
<td>Ang II type II receptor</td>
</tr>
<tr>
<td>AV3V</td>
<td>anteroventral third ventricle area</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Ca-NAT</td>
<td>Ca$^{2+}$-dependent (N)-acyltransferase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CVLM</td>
<td>caudal ventrolateral medulla</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DAGL</td>
<td>DAG lipase</td>
</tr>
<tr>
<td>dmnX</td>
<td>dorsal motor nucleus of the vagus</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAAH</td>
<td>fatty acid amide hydrolase</td>
</tr>
<tr>
<td>GABA</td>
<td>(\gamma)-aminobutyric acid</td>
</tr>
<tr>
<td>MAGL</td>
<td>monoacylglycerol lipase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NAE</td>
<td>(N)-acylethanolamine</td>
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<tr>
<td>NAPE</td>
<td>(N)-acylphosphotidylethanolamine</td>
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<tr>
<td>NAPE-PLD</td>
<td>NAPE phospholipase D</td>
</tr>
<tr>
<td>NMDA</td>
<td>(N)-methyl-D-aspartate</td>
</tr>
<tr>
<td>NTS</td>
<td>solitary tract nucleus</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositoide-3 kinase</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>RAS</td>
<td>renin-angiotensin system</td>
</tr>
<tr>
<td>RVLM</td>
<td>rostral ventrolateral medulla</td>
</tr>
<tr>
<td>SFO</td>
<td>subfornical organ</td>
</tr>
<tr>
<td>SHR</td>
<td>spontaneously hypertensive rat</td>
</tr>
<tr>
<td>THC</td>
<td>(\Delta^9)-tetrahydrocannabinol</td>
</tr>
</tbody>
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ABSTRACT

Christopher Lee Schaich

BLOOD PRESSURE REGULATION BY THE ENDOCANNABINOID SYSTEM IN CONDITIONS ASSOCIATED WITH HYPERTENSION

Dissertation under the Direction of
Debra I. Diz, Ph.D., Professor

Hypertension is associated with various conditions including obesity, type II diabetes and aging. Despite decades of research, the mechanisms involved in the development of hypertension are still poorly understood. Emerging evidence suggests hypertension is mediated by an imbalance in autonomic nervous system activity. Baroreflex sensitivity for control of heart rate, an important index of vagus nerve function as well as general morbidity and mortality, is often impaired in conditions associated with hypertension. Restoration of baroreflex function is now recognized as a therapeutic strategy for prevention of cardiovascular diseases. Therefore, it is crucial to identify key factors that regulate brainstem regions controlling autonomic outflow in conditions associated with baroreflex dysfunction.

Mounting evidence suggests a functional role for the endocannabinoid system in the impairment of baroreflex sensitivity during hypertension, which may be related to interactions with the renin-angiotensin system (RAS). Our goal is to determine if the brain endocannabinoid system contributes to baroreflex dysfunction and elevated blood pressure in conditions associated with altered RAS activity.
Specifically, we determine whether CB$_1$ cannabinoid receptor tone is present at the level of the brainstem solitary tract nucleus (NTS) in transgenic rats with high or low brain RAS activity. Data from these studies demonstrate that blockade of NTS CB$_1$ receptors in anesthetized hypertensive (mRen2)27 rats with high brain RAS expression dose-dependently improves the blunted baroreflex sensitivity in this strain. However, blockade of CB$_1$ receptors in the NTS of anesthetized ASrAOGEN rats with low brain RAS expression has opposite effects, dose-dependently reducing the enhanced baroreflex sensitivity in this strain. In contrast, there is no effect of NTS CB$_1$ blockade in normotensive Sprague-Dawley rats. Biochemical data indicate higher medullary production of the endocannabinoid 2-arachidonoylglycerol (2-AG) in (mRen2)27 rats compared to lower 2-AG production in ASrAOGEN rats. Together, these observations suggest a differential role for the endocannabinoid system in the modulation of baroreflex sensitivity in conditions associated with altered brain RAS activity.

We then determine whether altered endocannabinoid tone is present at the level of the NTS of aged Sprague-Dawley rats. Microinjection studies in older animals demonstrate that blockade of NTS CB$_1$ receptors normalizes baroreflex sensitivity only in animals that exhibit age-related impaired baseline baroreflex function. However, biochemical data indicate significantly higher 2-AG content in the dorsal medulla of aged relative to young adult Sprague-Dawley rats, and also show altered expression of CB$_1$ and CB$_2$ receptor mRNA expression with age in this region. These studies suggest a role for the endocannabinoid system in the impairment of baroreflex sensitivity associated with age.
Finally, we evaluate the hemodynamic and metabolic effects of systemic administration of a CB₁ receptor antagonist in (mRen2)27 rats, which feature increased body weight and insulin resistance in addition to angiotensin II-dependent hypertension. Results from these studies indicate that systemic blockade of CB₁ receptors significantly reduces blood pressure in this strain while having no effect on blood pressure in normotensive control rats. The blood pressure-lowering effect is associated with significant improvement in indices of conscious autonomic outflow following chronic treatment with the CB₁ antagonist. Furthermore, animals receiving chronic systemic CB₁ blockade exhibit an improved metabolic profile compared to animals treated with the vehicle.

In summary, the present studies suggest that upregulation of the endocannabinoid system contributes to the maintenance of hypertension, impaired baroreflex sensitivity, and symptoms of metabolic syndrome associated with overactivity of the RAS. These results are consistent with an interaction between the endocannabinoid system and RAS that may promote the development of hypertension associated with aging and obesity. Therefore, we conclude that targeting the endocannabinoid system for blockade may confer therapeutic benefits during hypertension associated with an activated RAS.
CHAPTER ONE

INTRODUCTION

Cardiovascular disease remains the leading cause of morbidity and mortality for both men and women in the United States. Hypertension, diagnosed when systolic blood pressure is greater than 140 mmHg and/or diastolic blood pressure is greater than 90 mmHg, is the single most important risk factor for the development of cardiovascular disease and currently affects 1 in 3 U.S. adults. The incidence of hypertension rises substantially with increases in body mass index, a unit of relative body fat calculated from the weight and height of patients. For example, values of body mass index greater than 40 kg/m$^2$, a level classified as “extreme obesity” and representing 6-8% of U.S. adults, are associated with a 7-fold increase in the incidence of hypertension compared to the lean population. In addition, the incidence of hypertension rises with age, in part due to the increased prevalence of obesity in aged populations. However, while there are several effective drug therapies to treat patients with hypertension, many antihypertensive medications have reduced efficacy and produce undesirable side effects in aged and obese patients. Therefore, research into the relationships among hypertension, aging and obesity is needed to gain better insights into therapeutic treatments for these conditions and their related pathologies.

Despite decades of research, the mechanisms involved in the development of hypertension are not fully understood. While several interacting factors likely contribute to its onset and progression, emerging evidence suggests that an imbalance in autonomic
nervous system activity is integral to the development of hypertension associated with aging, obesity and type II diabetes.\textsuperscript{10, 11} The autonomic imbalance observed in these conditions is linked to alterations in various biochemical factors associated with the onset of hypertension, including the metabolic hormones insulin\textsuperscript{12} and leptin,\textsuperscript{13, 14} catecholamine signaling in the brain,\textsuperscript{15, 16} and components of the RAS.\textsuperscript{17} The studies contained in this volume present evidence that altered central endocannabinoid system signaling is an additional mechanism that may contribute to autonomic imbalance in conditions associated with hypertension, providing novel insights for the preservation of autonomic function and blood pressure in hypertension, aging and obesity.
1. The Autonomic Nervous System

The autonomic nervous system is a part of the peripheral nervous system that controls cardiovascular, digestive and respiratory homeostasis, as well as a multitude of other functions associated with the viscera. The autonomic nervous system is divided into two opposing branches, the sympathetic and parasympathetic nervous systems, which regulate visceral functioning through reflex arcs between sensory and motor neurons in the periphery and autonomic brainstem nuclei. The cardiovascular system is regulated by a balance of output from both the sympathetic and parasympathetic nervous systems, which must work together to maintain cardiovascular homeostasis and correct short-term disturbances in the circulation. Activation of the sympathetic nervous system is associated with energy mobilization in response to environmental stress. For control of the cardiovascular system, the sympathetic nervous system mediates increases in cardiac output and vasoconstriction, primarily through noradrenergic neurotransmission acting at β1 receptors in the heart and α1 receptors in arterioles to elevate cardiac output, heart rate and thus blood pressure. Opposing the sympathetic nervous system, the parasympathetic nervous system regulates vegetative and restorative functions. During cardiovascular regulation, the parasympathetic nervous system exerts tonic inhibitory control over the heart to lower heart rate to decrease cardiac output and blood pressure. These actions are mediated primarily by cholinergic neurotransmission through efferent neurons of the vagus nerve that originate in the brain and innervate nodal pacemaker cells in the heart. Parasympathetic activity is often assessed by activity of the vagus nerve, which is considered a marker of general morbidity and mortality. Heart rate variability, a
measure of vagal tone to the heart, and baroreflex sensitivity for control of heart rate in response to increases in arterial pressure are often reported as indices of vagus nerve function.\textsuperscript{19}

\textit{The Arterial Baroreflex}

Arterial blood pressure is regulated by autonomic feedback control systems that work in concert over varying time frames to determine the prevailing level of pressure and its variability.\textsuperscript{21} The arterial baroreflex is the primary control system for regulation of blood pressure in the short-term time frame. The effectors of the baroreflex are stretch-sensitive baroreceptors concentrated in the walls of the aortic arch, carotid sinus, and auricles of the heart and vena cava that are capable of detecting peripheral changes in blood pressure or volume. Although baroreflexes also exist for control over the vasculature, renal function and fluid intake,\textsuperscript{22} the studies presented in this volume focus on the baroreflex for control of heart rate. The baroreflex for control of heart rate is a negative feedback loop in which changes in blood pressure produce immediate, transient compensatory changes in heart rate to return blood pressure back to the operating point of the system.

High pressure baroreceptors located in the aortic arch and carotid sinus are innervated by the vagus and glossopharyngeal nerves, respectively, and initiate autonomic feedback loops when activated by increases in pressure. Baroreceptor stimulation produces excitation along vagal and glossopharyngeal afferents which make their first synapse in the NTS of the dorsomedial medulla. The NTS is a major site of autonomic integration and receives input from chemosensitive neurons\textsuperscript{23} and the gut,\textsuperscript{24} in addition to baroreceptor neurons. Lesions to this region eliminate baroreflex responses,\textsuperscript{25}
illustrating the importance of the NTS to the integrity of the baroreflex arc. The NTS integrates information from afferent baroreceptor fibers and sends input to second order brainstem nuclei involved in the modulation of sympathetic and parasympathetic nervous system activity.

To effectively minimize variability in blood pressure during its activation, the baroreflex concomitantly modulates outflowing sympathetic and parasympathetic pathways, illustrated in the figure below. Excitation of baroreceptor afferents causes the NTS to transmit excitatory glutamatergic input to the caudal ventrolateral medulla (CVLM), a vasodepressor region that inhibits sympathetic activity, and preganglionic neurons in the dorsal motor nucleus of the vagus (dmnX) and nucleus ambiguus. Concurrent stimulation of these brain sites represents the diverging sympathetic and parasympathetic branches of the baroreflex arc. Neurons of the CVLM transmit inhibitory γ-aminobutyric acid (GABA) input to the rostral ventrolateral medulla (RVLM), a pressor region containing pre-motor neurons that project to sympathetic preganglionic neurons along the interomediolateral cell column of the spinal cord. Inhibition of the RVLM by the CVLM thus reduces sympathetic outflow to the heart and vasculature to lower heart rate and blood pressure. Concurrently, activated neurons of the dmnX are joined by efferent neurons from the nucleus ambiguus to reduce blood pressure by lowering heart rate and cardiac output. Alternatively, decreases in blood pressure silence baroreceptor activity, leading to disinhibition of the RVLM and inactivation of the dmnX and nucleus ambiguus, to elevate blood pressure back to temporal operating levels.
Adapted from Arnold (2009). Glu refers to glutamate; BP to blood pressure; HR to heart rate; + indicates excitatory input via glutamate; - indicates inhibitory input via GABA.

Two distinct components of the baroreflex that are independently regulated are the set-point and the sensitivity (occasionally called the baroreflex gain). The set-point represents the pressure at which the baroreflex operates, typically near the level of resting blood pressure, but will change or reset itself rapidly following sustained changes in cardiac output, as during exercise. Baroreflex sensitivity represents the effectiveness for correction of heart rate or sympathetic nerve activity. Baroreflex sensitivity for control of heart rate influences the ability to maintain arterial pressure within a narrow range by increasing heart rate variability. It is also an important index of vagus nerve function and is often impaired in conditions associated with hypertension including aging, obesity and type II diabetes.

**Autonomic Imbalance**

Activity of the sympathetic and parasympathetic nervous systems is normally in dynamic balance and rapidly adjusts based on changing environmental or physiological
circumstances.\textsuperscript{18} However, an imbalance in the autonomic nervous system, typically in which sympathetic nervous system predominates due to increased activity or decreased parasympathetic activity, is associated with various pathological conditions and may be an important mechanism promoting the development of age- and obesity-related hypertension.\textsuperscript{11, 33, 34}

Independent of hypertension, aging and obesity are associated with increased activation of the sympathetic nervous system in both humans and animals.\textsuperscript{11, 34, 35} Although increased sympathetic activation is hypothesized to be a beneficial compensatory mechanism in obesity to increase resting energy expenditure,\textsuperscript{36} elevations in sympathetic outflow are also associated with the development of hypertension.\textsuperscript{18, 37} The observation that pharmacological blockade of adrenergic receptors yields greater decreases in blood pressure in obese relative to lean hypertensive patients confirms the importance of sympathetic activation to obesity-related hypertension.\textsuperscript{38}

Reduced vagus nerve-mediated parasympathetic function also contributes to the pathogenesis of hypertension, independently of increased sympathetic activity.\textsuperscript{19} In fact, all established risk factors for cardiovascular disease are associated with impaired indices of parasympathetic function, including heart rate variability and baroreflex sensitivity for control of heart rate.\textsuperscript{19, 32} Reduced baroreflex sensitivity is associated with cardiovascular diseases including stroke, atherosclerosis and end organ damage, and is believed to precede and contribute to the onset of hypertension.\textsuperscript{19, 32, 39} For this reason, identification of factors that contribute to reductions in baroreflex sensitivity will be critical for understanding the development of hypertension associated with various conditions.
To date, the majority of research focuses on the contribution of the RAS in maintaining baroreflex function and other indices of autonomic activity, but additional factors are being discovered. For instance, a role for leptin in the modulation of central nervous system (CNS) activity and impairment of baroreflex sensitivity has recently emerged,\textsuperscript{40, 41} following previous demonstrations of insulin-mediated sympathetic activation and baroreflex impairment.\textsuperscript{42, 43} There are a few studies evaluating the role of the endocannabinoid system in sympathetic baroreflexes. Cannabinoid receptor activation at brain sites involved in autonomic regulation elicit cardiodepressor effects and prolong baroreflex-evoked sympathetic nerve inhibition,\textsuperscript{44, 45} implicating the ability to modulate parasympathetic function. Curiously, the latter effect is absent in an animal model of chronic hypertension,\textsuperscript{46} suggesting differential modulation of autonomic reflexes in hypertensive versus normal states. However, the contribution of the endocannabinoid system to modulation of parasympathetic activity in either the normal or hypertensive state has yet to be considered.
2. The Classical and Brain RAS

The RAS is the predominant biological signaling system for regulation of blood pressure and volume homeostasis in vertebrates. The description of the circulating, or classical, RAS is of an endocrine cascade in which active peptide hormones, synthesized from enzymes and precursors secreted from various systemic tissues, bind to receptors in heart, vasculature and kidney to exert their actions. As illustrated above, angiotensinogen, an inert glycoprotein derived from the liver, is the precursor of the RAS and is cleaved by the kidney-derived enzyme renin to form the inactive decapeptide angiotensin (Ang) I. Ang I may then be processed by lung-derived angiotensin converting enzyme (ACE) into the octapeptide hormone Ang II. Ang I is also cleaved by various endopeptidases including neprilysin to form the heptapeptide hormone Ang-(1-7). Alternatively, Ang II may be cleaved by an ACE homologue, ACE2, to form Ang-(1-7).

Ang II is the primary effector of the RAS and exerts physiological actions by binding to G protein-coupled Ang II type I (AT1) or type II (AT2) receptors. The ubiquitously expressed AT1 receptor mediates the majority of pressor actions attributed to Ang II, which include vasoconstriction, renal sodium and water reabsorption, adrenal aldosterone and epinephrine release, production of reactive oxygen species, cell proliferation and fibrosis, angiogenesis and suppression of baroreflex function. ACE inhibitors or AT1 receptor antagonists abolish these actions by preventing the formation or receptor-mediated actions of Ang II, respectively. As a receptor coupled to $G_{q/11}$,
activation of AT1 is typically associated with inhibition of adenyl cyclase and activation of phospholipase C (PLC) to initiate membrane-derived phosphoinositide turnover and intracellular calcium mobilization. Most of the actions mediated by the AT2 receptor are depressor effects in opposition to the AT1 receptor, and include vasodilation and inhibition of cellular proliferation.

The hormone Ang-(1-7) opposes the pressor actions of Ang II. Primary actions of Ang-(1-7) include vasodilation, antiproliferation and facilitation of baroreflex sensitivity for control of heart rate. However, Ang II and Ang-(1-7) can both elicit similar pressor or depressor actions in certain brain nuclei and stimulate vasopressin release through actions in the hypothalamus. The actions of Ang-(1-7) are blocked by [D-Ala\textsuperscript{7}]-Ang-(1-7), but not AT1 or AT2 receptor antagonists, indicating a unique receptor through which Ang-(1-7) exerts its effects. In 2003, Santos and colleagues identified Ang-(1-7) as an endogenous ligand of the G protein-coupled mas receptor, which stimulates prostaglandin and nitric oxide release in target cells, among other actions.

The classical RAS plays an essential role in blood pressure regulation through actions on the cardiovascular system and various brain nuclei lacking a blood-brain barrier, called circumventricular organs. Historically, it was presumed that the endocrine RAS was physically separated from the brain by the blood-brain barrier. The discovery of the brain circumventricular organs, which have direct access to cerebrospinal fluid and contain fenestrated capillaries to allow access to large molecular weight molecules, challenged this presumption and sparked debate and research into the actions of circulating Ang II inside the brain. Even before their discovery it was already known that central infusion of Ang II increases blood pressure, suggesting the presence of
specific receptors for Ang II in the brain. Further observations of normal or even low plasma renin and ACE activity in animal models of hypertension, coupled with clinical data showing beneficial effects of ACE inhibitors and AT1 blockers in hypertensive patients with low plasma Ang II levels, suggests the existence of an extra-circulatory RAS. All major components of the RAS are now known to coexist in various tissues, including the heart, adipose tissue, vasculature and brain. Thus, both the classical and brain RAS participate in blood pressure regulation by exerting actions on specific brain nuclei.

The Brain RAS and Blood Pressure Regulation

As reviewed in detail by others, Ang II and Ang-(1-7) are produced in the brain and AT1, AT2 and mas receptors are expressed beyond the blood-brain barrier in many brain regions known to influence cardiovascular function. Ang II alters blood pressure when microinjected into various CNS sites, elevating it from within the hypothalamic paraventricular nucleus, NTS and interomediolateral spinal cord column. In contrast, Ang II decreases blood pressure when microinjected into the dmnX, CVLM and RVLM. Importantly, microinjection of AT1 receptor antagonists into several of these listed regions produces changes in blood pressure that are opposite to those elicited by Ang II. Pressor effects of peripheral or central administration of Ang II are abolished by sympathetic nerve incision, implying that sympathetic activation underlies central Ang II-mediated increases in blood pressure. Indeed, AT1 receptors are abundant at each synaptic relay of the sympathetic and parasympathetic nervous systems, as well as in sympathetic preganglionic neurons of the interomediolateral cell column, paravertebral sympathetic ganglia, sympathetic nerve terminals and brain sites involved
in control of sympathetic outflow such as the hypothalamus, dmnX, CVLM and RVLM. Collectively, Ang II mediates changes in blood pressure through actions at many specific brain sites, although the molecular and neurotransmitter mechanisms involved in the alteration of neuronal activity at these sites is unknown. The central pressor actions of Ang II may be mediated in part by interactions with other neurotransmitter systems. For example, Ang II stimulates release of dopamine, substance P and norepinephrine from CNS neurons, all of which can contribute to elevations in blood pressure.  

Similar to Ang II, Ang-(1-7) is synthesized in the circumventricular organs and other brain nuclei involved in blood pressure regulation. Microinjection of Ang-(1-7) into these sites generally produces blood pressure effects that are opposite to those of Ang II, with the notable exceptions of in the dmnX, CVLM and RVLM. In these regions Ang II and Ang-(1-7) elicit similar responses, decreasing blood pressure in the dmnX and CVLM, and increasing pressure in the RVLM. The mechanisms underlying these actions appear to differ, however, as Ang-(1-7)-mediated reductions in blood pressure are attenuated by endothelial and neuronal nitric oxide synthase blockers, which have no effect on Ang II responses. Furthermore, in contrast to Ang II, Ang-(1-7) reduces neuronal catecholamine release.

The distribution of AT1 and mas receptors is consistent with the ability of Ang II and Ang-(1-7) to modulate baroreflex function. Intracerebroventricular infusion or NTS microinjection of Ang II impairs baroreflex sensitivity for control of heart rate in response to increases in arterial pressure, demonstrating an inhibitory central effect of the peptide on vagal tone. Furthermore, NTS administration of a selective AT1 receptor
antagonist improves baroreflex sensitivity in anesthetized Sprague-Dawley rats, suggesting that endogenous Ang II in this region exerts tonic influence over baroreflex function. The mechanism of Ang II impairment of baroreflex sensitivity is unclear, but likely involves facilitation of sympathetic outflow based on the ability of Ang II to modulate release of neurotransmitters that mediate sympathetic responses.

In contrast to Ang II, central infusion or NTS microinjection of Ang-(1-7) improves baroreflex sensitivity for control of heart rate in normotensive and hypertensive rats, in addition to eliciting depressor and bradycardic responses. The mechanism by which Ang-(1-7) enhances baroreflex sensitivity may be related to its ability to reduce sympathetic tone and modulate local effects of norepinephrine in the brain, as Ang-(1-7) decreases norepinephrine release and Ang II-mediated norepinephrine release from neurons. An alternative mechanism may involve synergistic effects with bradykinin, another peptide known to increase baroreflex sensitivity, suggesting these two peptides can interact in the NTS to modulate baroreflex function. In addition, [D-Ala\textsuperscript{7}]-Ang-(1-7) alone in the NTS impairs baroreflex sensitivity, revealing that like Ang II, endogenous Ang-(1-7) in the brain exerts a tonic influence within the NTS to modulate baroreflex function. Together, these observations suggest that a balance in RAS peptide actions in brain nuclei mediating the baroreflex is important to maintaining normal baroreflex function. Since the prevailing level of baroreflex sensitivity in part depends on the endogenous balance of these two peptides, an increase in Ang II or a decrease in Ang-(1-7) activity in the NTS may result in baroreflex dysfunction and subsequent blood pressure instability.
There is substantial evidence to associate dysfunction of the brain RAS with the development and maintenance of hypertension. For instance, animal models of hypertension such as the spontaneously hypertensive rat (SHR), DOCA and Dahl salt-sensitive rats and the transgenic (mRen2)27 rat, exhibit hyperactivity of the brain RAS with elevations in sympathetic nerve activity. Blockade of central ACE or AT1 receptors by RAS inhibitors reduces blood pressure in SHR and (mRen2)27 rats at doses that have no effect in their normotensive controls, supporting a neural basis for the development of hypertension in these animals. Central and systemic blockade of Ang II also improves the set-point and sensitivity of the baroreflex in hypertensive patients and animal. These data suggest that disruption of Ang II signaling within the brain restores autonomic balance and control of blood pressure in hypertensive populations.

Finally, altered central Ang-(1-7) signaling may also contribute to impaired baroreflex function and the progression of hypertension. Microinjection of [D-Ala$^7$]-Ang-(1-7) into the CVLM improves the blunted sympathetically-mediated baroreflex sensitivity found in two-kidney one-clip hypertensive rats, while AT1 blockade in this area has no effect, suggesting that Ang-(1-7) in the CVLM may contribute to impaired baroreflex function in hypertensive animals. Furthermore, restoration of Ang-(1-7) in the NTS, but not blockade of AT1 receptors, improves baroreflex sensitivity in aged Sprague-Dawley and young (mRen2)27 rats.

Collectively, these observations confirm the importance of the balance between central Ang II and Ang-(1-7) for normal baroreflex modulation. The mechanisms involved in modulation of baroreflex sensitivity and blood pressure by the brain RAS are still unclear, especially in light of additional factors that are known to influence blood
pressure regulation through central mechanisms. Therefore, the studies contained in this volume examine the contribution of one potential factor, the endocannabinoid system, to central blood pressure regulation.

*Central Ang II Pathways in Hypertension*

Systemic and centrally generated Ang II activates AT1 receptors located along specific brain pathways that contribute to hypertension, including in the circumventricular organs and brainstem nuclei involved in regulating sympathetic outflow. Three of the circumventricular organs straddling the third ventricle—the subfornical organ (SFO), median preoptic nucleus and organum vasculosum of lamina terminalis—form a continuum of AT1 receptor-expressing neurons called the anteroventral third ventricular (AV3V) area that plays an important role in mediating effects of the brain RAS on fluid homeostasis and blood pressure regulation. Lesions to the AV3V area dramatically attenuate the development of Ang II-dependent hypertension in the two-kidney one-clip, renal wrap, and chronic Ang II infusion animal models, indicating that elevated systemic Ang II acts through this region to promote the development of hypertension. The area postrema, situated on the fourth ventricle in close association with the NTS, is an additional circumventricular organ implicated in the central actions of Ang II. Lesions to the area postrema attenuate pressor effects of chronic systemic Ang II as well as the development of hypertension in young SHR.

Direct efferent projections from the SFO terminate in the anterior hypothalamus, the paraventricular nucleus and the RVLM. Electrophysiological studies demonstrate that AT1 receptor antagonists inhibit activation of these nuclei in response to stimulation of the SFO, suggesting that some SFO neurons use Ang II as an excitatory
neurotransmitter to activate downstream regions involved in modulation of sympathetic nerve activity. Furthermore, microinjection of AT1 receptor antagonists into each of these nuclei lowers resting blood pressure in hypertensive animals. In addition to the SFO, the RVLM also receives input from the paraventricular nucleus, and cardiovascular effects of paraventricular nucleus stimulation in anesthetized rats are blocked by AT1 receptor blockade in the RVLM. Activation of the RVLM directly stimulates the interomediolateral cell column of the spinal cord and thus enhances sympathetic outflow to promote or maintain hypertension.

In summary, these observations provide a multisynaptic neuroanatomical model for the actions of central and circulating Ang II in the development of hypertension driven by increased sympathetic activity. However, despite a known role for Ang II, the molecular mechanisms of chronic sympathetic stimulation in hypertension are unknown, creating the need for research into factors that mediate this Ang II-related activity.
3. The Endocannabinoid System

The discovery of an endogenous receptor as the primary site of action for Δ⁹-tetrahydrocannabinol (THC), the principal psychoactive compound in cannabis, has intensified research into the endocannabinoids. As reviewed by others\textsuperscript{101-103}, the endocannabinoid system is a paracrine cellular signaling system comprising the biologically active endocannabinoids anandamide and 2-AG, the G protein-coupled receptors mediating their effects, and their respective biosynthetic and degradative enzymes. The actions of the endocannabinoids are exerted predominantly through CB₁ and CB₂ cannabinoid receptors. The CB₁ receptor is widely expressed in the CNS and peripherally, whereas the CB₂ receptor is primarily concentrated in immune tissues. CB₁ receptors in the CNS are predominantly expressed on presynaptic neuronal terminals. Their activation at these sites by anandamide or 2-AG synthesized and released from postsynaptic neurons directly modulates voltage-gated ion channels to repolarize the cellular membrane and decrease intracellular Ca\textsuperscript{2+} concentrations, temporarily reducing release of neurotransmitter into the synaptic cleft. Thus, a primary function of endocannabinoid signaling in the CNS is to modulate neurotransmission at the level of the synapse.\textsuperscript{104}

Cannabinoid receptors in the periphery, quiescent under normal conditions, become activated and upregulated during cardiovascular disease and its metabolic risk factors.\textsuperscript{105} Two distinct cannabinoid receptors, CB₁ and CB₂, sharing approximately 44% sequence homology, have been identified to date.\textsuperscript{101, 106} Like the RAS, the near ubiquity of the endocannabinoid system makes it inevitable that its physiological actions are complex and often tissue-, context- or even ligand-specific. The emergence of a role for
the endocannabinoid system in the pathogenesis of cardiovascular diseases and metabolic syndrome makes it critical to understand its integrative signaling mechanisms in order to gain novel insights into the relationship between the endocannabinoid system and these pathological conditions.

The relevance of the CB2 receptor to autonomic, cardiovascular and metabolic homeostasis is still emerging. Expression was once thought to be restricted to immune tissues and glial cells, although there is now some conflicting evidence of its expression in the brainstem. Further recent evidence implicates a cardioprotective role for peripherally upregulated CB2 receptor expression in atherosclerosis and ischemia. As more emphasis is placed on the inflammatory process in chronic disease states, a functional role for the CB2 receptor in cardiovascular or metabolic disease is likely to emerge. However, due to the present lack of clarity surrounding the functional significance of brainstem CB2 receptors, as well as preliminary data illustrating CB1-mediated responses in the NTS, the studies in this volume primarily focus on actions of the CB1 receptor.

Biosynthesis and Degradation

Anandamide and 2-AG, the two best characterized endocannabinoid ligands, are synthesized upon demand from membrane phospholipids and then degraded shortly after release. Endocannabinoids in the CNS contrast with classical neurotransmitters because they are not stored in vesicles, but rather released immediately upon synthesis from postsynaptic neurons to retrogradely activate CB1 receptors expressed on presynaptic terminals. In the periphery, endocannabinoids act as paracrine and autocrine mediators. The biosynthesis and degradation of both molecules is governed by multiple
parallel enzymatic pathways to ensure tight temporal and spatial control over their signaling functions. The biosynthetic pathways of anandamide and 2-AG illustrate this concept.

Anandamide is a lipid signaling molecule belonging to a class of naturally occurring fatty acids known as the N-acylethanolamines (NAEs). The precise biosynthetic pathway of anandamide is not without controversy, but the direct synthesis pathway includes plasma membrane phospholipid precursors, the intermediate substrate $N$-acylphosphatidylethanolamine (NAPE), and the enzymes $Ca^{2+}$-dependent $N$-acyltransferase (Ca-NAT) and NAPE-hydrolyzing phospholipase D (NAPE-PLD). Typically, Ca-NAT will catalyze the transfer of arachidonic acid from a glycerophospholipid to the ethanolamine headgroup of a membrane phospholipid to form NAPE. NAPE-PLD then hydrolyzes NAPE to anandamide. The degradation of anandamide is governed by the integral membrane enzyme fatty acid amide hydrolase (FAAH), as well as by NAE-hydrolyzing acid amidase, to yield arachidonic acid and ethanolamine.

In contrast to anandamide, three major biosynthetic pathways for 2-AG are proposed. The first, which appears to dominate in the CNS, is through a two-step process involving conversion of the membrane phospholipid phosphotidylinositol 4,5-bisphosphate to a diacylglycerol (DAG) intermediate by PLC. DAG is then catalyzed by one of two isoforms of DAG lipase (DAGL) into 2-AG. The second pathway involves the conversion of a phosphatidyl lipid to the intermediate 2-arachidonoyl-lyso phosphatidyl inositol by the action of phosphatidyl lipase, and then to 2-AG by a lyso-PLC. The third pathway involves lysophosphatidic acid hydrolysis by a phosphatase
to form 2-AG. The involvement of the latter two pathways in the production of 2-AG in the CNS has not been evaluated in detail, but may explain reports of 2-AG-mediated actions that are insensitive to DAGL inhibitors. A more diverse assortment of enzymes participate in the metabolism of 2-AG than anandamide. For example, monoacylglycerol lipase (MAGL) is accepted as the dominant enzyme in the degradation of 2-AG, but a role has been found for others depending on context including FAAH, cyclooxygenase-2 and the serine hydrolases ABHD6 and ABHD12. The physiological relevance of these enzymes in 2-AG metabolism is still emerging.

**CB₁ Receptor Signaling**

The CB₁ receptor is primarily a Gᵢ/ₒ protein-coupled receptor that is widely expressed throughout the CNS, and also found in the myocardium, adipose tissue, hepatic tissue, and endothelial and smooth muscle cells of the vasculature and gastrointestinal tract. CB₁ receptor agonists include the endocannabinoids anandamide and 2-AG, the plant-derived THC and its analogs, and synthetic compounds such as CP55,940, HU210, and WIN55,212-2. Common antagonists of the CB₁ receptor include rimonabant (SR141716A), AM251 and AM281.

Prior to the characterization of the cannabinoid receptors in 1988, it was believed that the lipophilicity of THC enabled it to interact with the plasma membrane to disrupt enzyme and ion channel activity. Howlett and Fleming first observed functional inhibition of adenylyl cyclase and subsequent reduction in intracellular cAMP following application of THC to neuroblastoma cells. Adenylyl cyclase inhibition by THC was blocked by pertussis toxin (PTX), indicating the involvement of a Gᵢ/ₒ protein. The CB₁ receptor was cloned from rat cerebral cortex in 1990.
The sensitivity of most intracellular cannabinoid responses to PTX demonstrates the preference of CB1 receptors for the G_{i/o} protein family.\textsuperscript{131, 132} However, strong evidence exists that CB\textsubscript{1} receptors can stimulate cAMP accumulation under conditions that prevent interaction of the receptor with G_{i/o} proteins, such as during PTX treatment, perhaps due to disinhibition of adenylyl cyclase.\textsuperscript{133, 134} Others suggest that G protein coupling to CB\textsubscript{1} may be an agonist-specific event,\textsuperscript{133, 135} or dependent upon concurrent activation of co-expressed G protein-coupled receptors.\textsuperscript{134, 136} It is also possible that non-receptor proteins associated with the CB\textsubscript{1} receptor can direct the probability of switching signaling pathways.\textsuperscript{137}

Activation of presynaptic CB\textsubscript{1} receptors in neuronal cells temporarily reduces the amount of neurotransmitter released into the synaptic cleft.\textsuperscript{120, 138-140} The principal mechanism underlying cannabinoid-induced inhibition of neurotransmitter release is the direct modulation of voltage-gated membrane ion channels by presynaptic CB\textsubscript{1} receptors. During bursts of activity, postsynaptic neurons produce and release 2-AG or anandamide, which then bind to presynaptic CB\textsubscript{1} receptors. The G_{\beta\gamma} subunits associated with CB\textsubscript{1} receptors then directly interact with presynaptic membrane-bound ion channels. Cumulatively, this results in hyperpolarization of the plasma membrane, and reduction of the local intracellular Ca\textsuperscript{2+} concentration necessary for neurotransmitter release.

High intracellular concentration of Ca\textsuperscript{2+} is required for proper neurotransmitter vesicle fusion to the plasma membrane and subsequent exocytosis, and is supplied locally by the voltage-operated Ca\textsuperscript{2+} channels that open when the membrane is depolarized. Anandamide, WIN55,212-2 and CP55,940 each inhibit Ca\textsuperscript{2+} channels via CB\textsubscript{1} receptors in cell preparations and brain tissue slices, effects blocked by SR141716A or PTX.\textsuperscript{141, 142}
This inhibition is independent of other signaling enzymes known to interact with voltage-gated ion channels, such as the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway,\textsuperscript{141} therefore likely resulting from direct interaction with dissociated \(G_{\beta\gamma}\) subunits.

Inwardly rectifying \(K^+\) currents stabilize the membrane potential following release of neurotransmitter. WIN55,212-2 enhances inward \(K^+\) currents carried by \(G\) protein-coupled inwardly rectifying \(K^+\) channels, and is sensitive to low concentrations of \(Ba^{2+}\), a \(K^+\) channel inhibitor.\textsuperscript{142} It was later demonstrated that WIN55,212-2- and CP55,940-mediated inhibition of glutamatergic signaling in mouse nucleus accumbens is blocked by both \(Ba^{2+}\) and SR141716A,\textsuperscript{143} suggesting \(CB_1\) receptor activation of inwardly rectifying \(K^+\) channels as a mechanism for the inhibition of neurotransmitter release. \(A\)-type \(K^+\) channels, by contrast, are rapidly activating voltage-gated ion channels that mediate the deactivating inward \(K^+\) currents as part of the repolarization process following cell depolarization. WIN55,212-2 increases this current in a concentration-dependent and SR141716A- and PTX-sensitive manner.\textsuperscript{144}

In addition to voltage-gated ion channels and neurotransmission, the \(CB_1\) receptor modulates several other intracellular processes essential to normal cell function. Since Howlett and colleagues’ demonstration of \(CB_1\) receptor-mediated suppression of cAMP production in neuroblastoma cells,\textsuperscript{127, \textsuperscript{128}} functional inhibition of adenylyl cyclase by cannabinoid agonists has been identified in many other settings. These responses are generally PTX- and \(CB_1\) antagonist-sensitive and occur independently of forskolin, the universal adenylyl cyclase activator. In the CNS, cannabinoid-mediated attenuation of forskolin-stimulated cAMP accumulation has been demonstrated in slices of rat
hippocampus, striatum, cerebral cortex and cerebellum, as well as *in vivo* in rat striatum.

The complexity of cannabinoid adenylyl cyclase regulation was recognized following several reports of CB₁-mediated increases in cAMP accumulation. It is now known that adenylyl cyclase has nine distinct isozymes, activated by different stimuli, organized into six classes based on functional similarities and tissue expression. In addition to the potential dual coupling of CB₁ to Gᵢₒ and Gₛ, contrasting effects of cannabinoid agonists on functional adenylyl cyclase activity is attributed to the specific isoform present in different cellular preparations. Altered cAMP concentration can yield major changes in cellular activity due to its downstream consequences (i.e., PKA phosphorylation, A-type K⁺ channel regulation, and gene expression via cAMP response elements). Therefore, modulation of adenylyl cyclase activity must be carefully considered when interpreting intracellular cannabinoid signaling events.

The mitogen-activated protein kinase (MAPK) pathway is a major signaling mechanism that governs many cellular functions including cell growth, differentiation, adhesion and apoptosis. Activation of the MAPK signaling cascade is typically initiated by activation of a tyrosine kinase- or G protein-coupled receptor. CB₁ receptors are almost universally positively linked to the MAPK pathway. In *vivo*, CB₁ agonists increase phosphorylation of the MAPK extracellular signal-regulated kinase (ERK)1/2 in cells expressing both endogenous and recombinant CB₁ receptors. These effects are SR141716A- and PTX-sensitive. The mechanisms for MAPK activation by CB₁ receptors are not yet fully known; multiple pathways could result in
CB₁-mediated MAPK induction, and these likely depend on cell type and stimulus. For example, astrocytoma cells utilize a pathway involving phosphoinositide-3 kinase (PI3K) and Akt, which then activates Raf-1 to initiate the MAPK cascade to subsequently increase glucose metabolism. The same PI3K-Akt-Raf pathway is activated in CHO cells transfected with recombinant CB₁ receptors in response to anandamide.

MAPK activity may be regulated indirectly via CB₁ receptor stimulation through its effects on cAMP accumulation. For example, MAPK activation in MCF-7 cancer cells by anandamide is significantly attenuated by forskolin and 8-Bromo-cAMP, an analogue of cAMP. A similar effect following anandamide and 2-AG exposure was observed in rat hippocampal slices pretreated with 8-Bromo-cAMP, suggesting that a decrease in cAMP accumulation may facilitate the stimulatory effects of CB₁ activation on the MAPK pathway. In vivo, administration of cannabinoid agonists produces a progressive and transient activation of ERK1/2 in several rat and mouse brain regions that is blocked by SR141716A, suggesting involvement of the CB₁ receptor. Furthermore, the effect of cannabinoid agonists on ERK1/2 is absent in CB₁−/− knockout mice.

Role in Cardiovascular Regulation

Chronic marijuana use in humans and prolonged treatment in animals can lead to postural hypotension and bradycardia, providing the first evidence of a role for cannabinoid modulation of the cardiovascular system. In 1996, Varga and colleagues reported a prolonged blood pressure depressor effect of systemically administered anandamide in anesthetized rats. This depressor response was accompanied by bradycardia and reduced cardiac contractility, and these effects were sensitive to CB₁ antagonists. The
expression of CB₁ in central autonomic sites, myocardium and vascular walls may account for the direct cardiovascular actions of cannabinoid ligands.

In conscious normotensive animals the cardiovascular actions of CB₁ ligands are less pronounced or absent entirely, leading some to conclude that the endocannabinoid system plays a limited role in normal cardiovascular regulation. Under anesthesia, for example, autonomic function is compromised, potentially contributing to the loss of normal baroreflex function in the presence of CB₁ receptor activation. However, the importance of the endocannabinoid system in cardiovascular regulation is much more apparent when studied in experimental models of hypertension. In models of both acute and chronic hypertension, and across anesthetized and conscious animals, the cardiovascular effects of systemically administered cannabinoids are amplified compared to normotensive controls. Specifically, greater CB₁-mediated reductions in heart rate and blood pressure, as well as associated increases in vasodilation, are reported across these hypertensive models compared with normotensive animals. The mechanisms of these hypertension-specific CB₁ effects are not fully understood, but may involve modulation of sympathetic outflow from the brain by CB₁ receptors, in addition to direct vasodilator and inotropic effects in the vasculature and heart, respectively. Others find increased CB₁ receptor expression in the heart, aorta and coronary arteries in hypertension and other cardiovascular diseases. It is also possible that central CB₁ coupling to G₁ proteins is enhanced in hypertension, as recently reported in the SHR brain. Upregulation of G₁₆ protein isoforms is documented in the heart and vasculature of these and other hypertensive animals. The CB₁ receptor thus participates in
autonomic cardiovascular regulation, with greater importance to the homeostatic adjustments under pathological conditions.

The endocannabinoid system could be an attractive therapeutic target for the treatment of hypertension and other cardiovascular diseases. In fact, the use of marijuana-like substances as antihypertensive medications was contemplated as early as the 1970s because of observed long-lasting decreases in blood pressure associated with chronic marijuana use in humans and animals.\textsuperscript{171-174} However, the inability to separate the neurobehavioral, metabolic and cardiovascular effects of cannabinoid agonists led to the abandonment of this strategy. Interest in pursuing the endocannabinoid system as a target of antihypertensive therapy has since reemerged with the discovery of amplified CB\textsubscript{1}-mediated cardiovascular effects in hypertension, suggesting a role as a compensatory mechanism in some animal models.\textsuperscript{181}

CB\textsubscript{1} cannabinoid receptors are densely expressed in the NTS and are associated with vagal afferent neurons of the nodose ganglion.\textsuperscript{192, 193} It was observed as early as 1975 that prolonged THC ingestion impaired circulatory reflexes to standing and exercise in humans.\textsuperscript{168} Later tissue bathing studies show that NTS neurons are sensitive to cannabinoid ligands,\textsuperscript{194, 195} implying that the endocannabinoid system can modulate the arterial baroreflex. Importantly, both increases and decreases in the frequency of action potentials are observed in response to cannabinoid receptor stimulation in the NTS,\textsuperscript{194} suggesting the presence of CB\textsubscript{1} receptors on both glutamate- and GABA-releasing neurons. However, the precise mechanism of neuronal modulation within the NTS by the endocannabinoid system appears complex.
A series of studies over the past decade examined the effects of cannabinoid receptor activation within the NTS on modulation of the sympathetic branch of the baroreflex, and report CB₁-mediated disinhibition of baroreceptive NTS neurons. Specifically, they demonstrate that microinjection of cannabinoid agonists, including anandamide and WIN55,212-2, into the NTS prolongs baroreflex-evoked renal sympathetic nerve inhibition in anesthetized Sprague-Dawley and Wistar-Kyoto rats.⁴⁵, ⁴⁶ This effect is absent in the SHR strain,⁴⁶ suggesting differential regulation of baroreflex function in hypertensive relative to normotensive conditions. The same study also reports reduced CB₁ receptor density in the NTS of SHR, which may account for the differences in the baroreflex-evoked sympathetic response. However, these studies did not assess effects on the parasympathetically-mediated vagal branch of the baroreflex for control of heart rate.

Differential modulation of glutamate and GABA neurotransmitters in the NTS may be responsible for these effects. In fact, a preponderance of data links the altered balance of NTS neurotransmission shifted toward excessive GABAergic tone to several models of hypertension, including SHR⁹⁷, ⁹⁸ and transgenic (mRen2)27 rats.⁹⁹, ²⁰⁰ The origin of this altered inhibitory tone in hypertension and the factors that mediate it remain unclear. We propose that altered endocannabinoid system tone within the NTS may be one contributing factor based on the data contained in the present studies. Another possibility involves interactions with other factors known to regulate autonomic or cardiovascular function, including the brain RAS.
Metabolic Effects and Cardiovascular Risk Factors

An emerging model of metabolic syndrome has identified central and peripheral overactivity of the endocannabinoid system as a key participant in its pathogenesis. Metabolic syndrome refers to a cluster of risk factors for cardiovascular disease including obesity, glucose intolerance, insulin resistance, leptin resistance and dyslipidemia.\textsuperscript{201-203} Excess sympathetic nervous system activity is also typical of these conditions.\textsuperscript{11, 26} A dynamic relationship exists between the endocannabinoid system and cardiometabolic regulation as revealed by peripheral and central CB\textsubscript{1} receptor blockade in obese humans and in animal models of metabolic syndrome.

Animals administered cannabinoid agonists gain a significant amount of weight, and this weight gain is attributable not only to increased food intake\textsuperscript{204} but also to alterations in peripheral metabolic processes. Specifically, activation of CB\textsubscript{1} receptors in adipose tissue in animals fed a high-fat diet directly stimulates adipogenesis and attenuates secretion of adiponectin,\textsuperscript{205} while CB\textsubscript{1} activation in the liver directly increases gene expression of lipogenic transcription factors and their enzymatic targets, increasing the release of free fatty acids.\textsuperscript{206-208} Attenuation of adiponectin release has significant downstream consequences for metabolic balance, as it is an important modulator of numerous processes including glucose management and fatty acid catabolism. The actions of weight-independent CB\textsubscript{1} receptor overstimulation in fat and liver cells can lead to impaired glucose utilization accompanied by peripheral insulin resistance due to direct inhibition of insulin receptor activation,\textsuperscript{207-209} which in turn may contribute to the onset of type II diabetes. Increased CB\textsubscript{1}-mediated hepatic free fatty acid generation has also been shown to disrupt the balance of cholesterol, decreasing high density lipoprotein while
increasing low density lipoprotein and triglycerides in the circulation. Additionally, central CB\textsubscript{1} activation dampens insulin signaling in the brain and periphery, and is associated with altered central leptin receptor expression.\textsuperscript{211-213} Therefore, while CB\textsubscript{1} agonists may acutely reduce blood pressure in hypertension through actions in the heart and vasculature,\textsuperscript{214-216} they may adversely affect important metabolic parameters to induce a condition resembling metabolic syndrome.

CB\textsubscript{1} blockade thus has potential as a therapy for some metabolic pathologies.\textsuperscript{108,217} Indeed, CB\textsubscript{1} antagonists are now well-associated with reversal of diet-induced obesity in animals, as well as with normalization or improvement of glucose tolerance, insulin resistance, leptin resistance, and obesity-related inflammation in obese or diabetic animals.\textsuperscript{218-223} Human clinical trials confirmed that chronic treatment with SR141716A is effective for the management of obesity and type II diabetes,\textsuperscript{224-229} with a modest secondary improvement to blood pressure in hypertensive patients.\textsuperscript{230} Unfortunately, further clinical trials were halted due to reports of negative psychiatric effects of increased anxiety and depression associated with chronic CB\textsubscript{1} receptor blockade.\textsuperscript{231} Despite its withdrawal from clinical development, SR141716A remains an important pharmacological tool to study the role of the endocannabinoid system in metabolic, cardiovascular and autonomic dysfunction. It remains unclear how CB\textsubscript{1} antagonists will alter the cardiovascular system in the face of metabolic syndrome or hypertension. Recent data, however, may reveal an important mechanism for the exacerbation of hypertension and metabolic syndrome involving endocannabinoid system activation.
Interactions with the RAS

The direct effects of SR141716A on blood pressure are not clearly predictable. Reports suggest that reductions in blood pressure observed in obese humans and animals following treatment with SR141716A are not independent of general weight loss.\textsuperscript{221, 230} However, emerging evidence suggests an important interaction between the endocannabinoid system and the RAS in hypertension and other cardiovascular diseases, which may potentially be a primary mechanism underlying the pathogenic properties of Ang II or endocannabinoids.

SR141716A treatment in apolipoprotein E-deficient mice fed a high-fat diet yields decreased vascular AT1 receptor expression and reduced arterial constriction with improved endothelial function.\textsuperscript{232} At the cellular and molecular levels, a recent study by Rozenfeld and colleagues indicates that CB\textsubscript{1}-AT1 receptor interaction via dimerization enhances Ang II-mediated ERK1/2 signaling in Neuro2A and HEK293 cells expressing CB\textsubscript{1} and AT1 receptors.\textsuperscript{233} Furthermore, administration of SR141716A prevents Ang II-mediated mitogenic signaling and fibrogenic gene expression in these cells. \textit{In vivo}, CB\textsubscript{1} receptors mediate the enhancement of ERK1/2 phosphorylation and cAMP accumulation by Ang II in activated hepatic stellate cells harvested from rats treated with ethanol for 8 months. Additional CB\textsubscript{1}-AT1 receptor interactions may be through a transactivation mechanism involving the production and release of 2-AG following stimulation of AT1-G\textsubscript{q} protein signaling in target cells.\textsuperscript{234, 235}

The maintenance of hypertension in several animal models of the disease and in humans is often dependent in part on Ang II acting at AT1 receptors in vasculature and kidney to modulate vascular tone and fluid balance, as well as in the brain for impairment
of baroreflex sensitivity and facilitation of sympathetic outflow. Thus, it is possible that CB₁ receptor blockade may lower blood pressure independently of weight loss or improvement in metabolic profile. In hypertension resulting from excess action of Ang II, CB₁ receptor blockade may serve as a potentially beneficial therapy. In addition, long-term AT₁ receptor blockade has a beneficial metabolic profile in older animals and elderly human patients. While the improvement of metabolic profiles with AT₁ blockade may be interpreted as directly interfering with Ang II-mediated attenuation of signaling pathways for metabolic hormones such as insulin and leptin, we must now consider that effects on the endocannabinoid system may also contribute.

In summary, the resulting dyslipidemia, insulin resistance and obesity that can arise due to perturbations of the endocannabinoid system or the RAS are components of metabolic syndrome that represent significant risk factors for hypertension and other cardiovascular diseases. The endocannabinoid system will require more intensive study of its potential negative metabolic and cardiovascular risk factor profile, including the long term effects on baroreflex function after systemic administration, before endocannabinoid-targeted therapeutic strategies can progress.
4. The Use of Transgenic and Aged Animals to Investigate the Role of the Endocannabinoid System in Blood Pressure Regulation

Transgenic rodents with systemic alterations in components of the RAS are an important tool to study the contribution of various physiological factors to the development and maintenance of hypertension. The importance of the RAS to cardiovascular regulation is illustrated by the observation that mice with targeted deletion of renin, angiotensinogen, ACE or AT1 receptors all have decreased resting blood pressure.\textsuperscript{59} Similarly, overexpression of angiotensinogen increases blood pressure.\textsuperscript{240} Recent studies using transgenic rodents highlight the crucial role of the brain RAS in the pathogenesis of hypertension through alterations in cardiovascular and autonomic regulation, consistent with the central distribution of angiotensin receptors in brain regions that regulate these functions.\textsuperscript{56, 59} For example, central administration of RAS blockers that have no effect in normotensive animals reduces blood pressure in hypertensive SHR and transgenic (mRen2)27 rats.\textsuperscript{82} Studies in transgenic ASrAOGEN rats with low brain RAS expression extend these findings to show differential sources of angiotensinogen for Ang II and Ang-(1-7) within the brain that are involved in baroreflex modulation,\textsuperscript{241} consistent with mice overexpressing glial versus neuronal angiotensinogen or renin.\textsuperscript{242} Additional studies in aged Sprague-Dawley rats find a similar role for the brain RAS in the progression of increasing blood pressure and reduced baroreflex sensitivity with age.\textsuperscript{88} Although the contribution of the brain RAS to modulation of blood pressure and baroreflex function is well established, less is known about how the RAS may interact with various other factors present in the brain, such as the endocannabinoid system, to produce its physiological actions.
In addition to influence over blood pressure and baroreflex modulation, recent studies using transgenic rodents provide evidence that the brain RAS is involved in the regulation of energy balance and metabolic profile. For instance, (mRen2)27 rats with overactive brain RAS are heavier and exhibit insulin and leptin resistance in response to an oral glucose load relative to age-matched Sprague-Dawley rats.\textsuperscript{243, 244} In contrast, ASrAOGEN rats with low brain RAS activity are lighter and have enhanced insulin and leptin responsiveness for metabolic actions.\textsuperscript{243} Evidence linking the RAS to metabolic dysfunction includes the observations that RAS blockade in Fischer-344 rats or mice fed a high-fat diet reduces body weight and improves insulin sensitivity.\textsuperscript{245, 246} In hypertensive patients, ACE inhibitors and AT1 blockers are associated with positive metabolic effects including reduced plasma insulin and leptin levels and reduced risk for new-onset diabetes.\textsuperscript{247-249} Together, these reports suggest that disruption of brain Ang II is associated with overall improved metabolic profile with increased sensitivity to insulin and leptin.

While sensitivity to metabolic hormones is beneficial for energy and glucose homeostasis, some metabolic actions of CB\textsubscript{1} receptor blockade are attributed to increased stimulation of the sympathetic nervous system,\textsuperscript{250, 251} which may potentially elevate arterial pressure at central sites of action. Whether CB\textsubscript{1} receptor blockade also results in altered baroreflex sensitivity, blood pressure or metabolic profile in transgenic or aged animals with differential brain RAS activity has yet to be determined. Therefore, we employed transgenic ASrAOGEN and (mRen2)27 rats with low or high brain RAS expression, respectively, and aged Sprague-Dawley rats to determine interactions
between the brain RAS and the endocannabinoid system with respect to autonomic, cardiovascular or metabolic regulation.

**Transgenic (mRen2)27 Rats**

The transgenic (mRen2)27 rat strain was created in 1990 by insertion of the mouse submandibular gland *Ren2* renin gene into the genome of the Hannover Sprague-Dawley rat. Rats homozygous for the *Ren2* gene exhibit fulminant hypertension beginning around 4 to 5 weeks of age that results in a high mortality rate at a young age, making antihypertensive treatment necessary for survival. Heterozygous (mRen2)27 rats are used for most research studies and exhibit hypertensive systolic blood pressure levels ranging from 170 to 190 mmHg, similar to SHR. This strain has shown a stable expression and phenotype for approximately 24 years and is used extensively by our laboratory to evaluate the role of the brain RAS in baroreflex modulation.

In (mRen2)27 rats, the renin transgene is highly expressed in the brain and adrenal gland with reduced expression in the kidney, in contrast to non-transgenic animals which exhibit low renin expression in the brain and adrenal gland. Whether circulating levels of renin, angiotensinogen, Ang I, Ang II or Ang-(1-7) are altered is unclear as studies report reduced, similar, or modestly elevated plasma levels of these components relative to Sprague-Dawley rats. However, an 18-fold increase in brain tissue Ang II levels is reported in (mRen2)27 rats compared to Sprague-Dawley rats. Chronic oral treatment with ACE inhibitors or AT1 receptor blockers lowers blood pressure in (mRen2)27 rats, indicating that the hypertension they exhibit is dependent on actions of Ang II at AT1 receptors. Mounting evidence suggests that hypertension in (mRen2)27 rats is of central origin. For example, intracerebroventricular
administration of AT1 blockers reduce blood pressure and heart rate in these animals, suggesting that central Ang II is responsible for the maintenance of hypertension, with potential concomitant disturbances in autonomic balance.

In addition to hypertension, (mRen2)27 rats display several indices of autonomic imbalance, including increased sympathetic transmission in response to Ang II relative to Sprague-Dawley rats. This strain also exhibits an inverted circadian rhythm of blood pressure and reduced resting blood pressure under urethane-chloralose anesthesia, similar to the resting level of Sprague-Dawley rats, providing further evidence of enhanced sympathetic nervous system activity. Despite lower resting pressure under anesthesia, (mRen2)27 rats still exhibit marked impairments in baroreflex sensitivity for control of heart rate in response to increases in blood pressure, indicating reduced parasympathetic tone. The impaired baroreflex sensitivity in this strain is likely related to low brain Ang-(1-7) because microinjection of [D-Ala\(^7\)]-Ang-(1-7) in the NTS has no effect on baroreflex sensitivity in these animals, unlike the inhibitory effect of mas receptor blockade in Sprague-Dawley and ASrAOGEN rats. Moreover, NTS microinjection of an ACE inhibitor, but not an AT1 receptor antagonist, improves baroreflex sensitivity in (mRen2)27 rats, providing further evidence that reduced Ang-(1-7) tone contributes to impaired cardiovascular regulation in this strain. Finally, (mRen2)27 rats exhibit an anxiogenic behavioral profile, potentially due to hyperactivity of the hypothalamic-pituitary-adrenal axis that mediates stress responses. The anxiety-like behaviors of these rats are abolished by chronic systemic ACE inhibition, suggesting that they too are mediated by an overactive RAS underlying autonomic imbalance.
Hypertensive (mRen2)27 rats have increased body weight and higher fat mass relative to ASrAOGEN and Sprague-Dawley rats.\textsuperscript{243, 270} Although plasma levels of insulin, leptin and glucose are similar to those in ASrAOGEN and Sprague-Dawley rats at 15 weeks of age, (mRen2)27 rats display whole body and skeletal muscle insulin resistance in response to oral glucose tests. Therefore, these animals are studied as a model of both hypertension and metabolic syndrome.\textsuperscript{271} While our understanding of the cardiovascular sensitivity to metabolic hormones in this strain is still emerging, the observation that they exhibit metabolic dysfunction may imply that these animals have altered endocannabinoid tone associated with upregulated RAS expression. Determining factors that contribute to baroreflex and metabolic dysfunction in (mRen2)27 rats will be necessary to further understand the contribution of the endocannabinoid system to obesity and hypertension in the face of an overactivated brain RAS.

The (mRen2)27 rat showcases the powerful role of increased extrarenal renin expression in the regulation of cardiovascular homeostasis, yet many new questions have been generated as a result of this model. While several other models of chronic hypertension exist, the advantage of the (mRen2)27 rat is that a well-defined single genetic manipulation is the primary initiator of a series of consequences yielding the development of hypertension with metabolic dysfunction.\textsuperscript{272} Thus, it is possible to determine interactions between experimental manipulations in this strain and the genetic background against which they occur, in contrast to models with a broad and elusive pathophysiology, such as the SHR, exhibiting a variety of features that cannot be easily compared to outbred normotensive controls.\textsuperscript{273}
Transgenic ASrAOGEN Rats

The second model of altered brain RAS we use is the transgenic ASrAOGEN rat, a unique rodent model for under-expression of the endogenous brain RAS via targeted gene silencing created by Ganten and colleagues in 1999. Transgenic ASrAOGEN rats were created by transfection of Sprague-Dawley rats with an antisense oligonucleotide to angiotensinogen driven by a glial fibrillary acid protein promoter\textsuperscript{274} to target expression specifically to astrocytes.\textsuperscript{275} Since astrocytes are the primary source of angiotensinogen in the brain,\textsuperscript{276} the antisense oligonucleotide yields a 90% reduction in angiotensinogen protein levels in brain regions including the medulla and hypothalamus.\textsuperscript{274, 275} Therefore, as expected, ASrAOGEN rats have decreased brain tissue levels of Ang I, Ang II and Ang-(1-7) compared to Sprague-Dawley rats, but upregulated brain AT1 receptor expression.\textsuperscript{277, 278} The brain specificity of the antisense oligonucleotide is confirmed by the unaltered levels of circulating angiotensinogen in ASrAOGEN relative to Sprague-Dawley rats.\textsuperscript{274, 275} ASrAOGEN rats have maintained a stable phenotype over the 15 years since their creation and, like (mRen2)\textsuperscript{27} rats, are used extensively by our laboratory to investigate the contribution of the brain RAS to cardiovascular and metabolic regulation in pathologies related to hypertension and aging.\textsuperscript{241, 255, 279, 280}

Conscious ASrAOGEN rats exhibit lower resting blood pressure and heart rate relative to Sprague Dawley rats, with systolic pressures typically between 80 and 100 mmHg.\textsuperscript{275} The hypotension in this strain is generally attributed to reduced glia-derived angiotensin peptides, although it may also be due in part to reduced plasma vasopressin levels and resulting diabetes insipidus-like syndrome in these animals.\textsuperscript{275} Similar to (mRen2)\textsuperscript{27} rats, ASrAOGEN rats display altered circadian rhythm of blood pressure,
suggestive of autonomic imbalance. However, baroreflex sensitivity for control of heart rate and heart rate variability are significantly enhanced in these animals relative to Sprague-Dawley rats,\textsuperscript{241, 255} indicating higher resting parasympathetic activity. In line with this interpretation, ASrAOGEN rats exhibit a paradoxical elevation of resting arterial pressure while under the influence of urethane-chloralose anesthesia. This anesthesia is reported to increase sympathetic neurotransmission in the brain,\textsuperscript{180, 281} which in ASrAOGEN rats may be mediated by increased expression of AT1 receptors in brainstem regions involved in descending sympathetic pathways including the NTS and RVLM.\textsuperscript{277} Elevated blood pressure under anesthesia notwithstanding, blockade of either NTS AT1 or mas receptors decreases pressure in these animals, while only mas receptor blockade decreases baroreflex sensitivity.\textsuperscript{241} This implies a non-glial source for angiotensinogen involved in maintaining Ang-(1-7) tone over baroreflex function, providing further evidence for and possible mechanism of independent modulation of baroreflex sensitivity and blood pressure by RAS peptides. In addition to differences in blood pressure regulation, ASrAOGEN rats feature lower body weight with enhanced insulin sensitivity and increased food consumption.\textsuperscript{243, 279} Collectively, the reduced brain RAS activity exhibited by ASrAOGEN rats provides an ideal animal model in which to compare and contrast influence of the endocannabinoid system over cardiovascular and autonomic regulation relative to (mRen2)27 rats with an overactive brain RAS.

\textit{Aged Sprague-Dawley Rats}

Aging is associated with gradual increases in systolic blood pressure, as well as reductions in heart rate variability and baroreflex sensitivity for control of heart rate which may facilitate the progression of hypertension and related diseases.\textsuperscript{88, 282}
Autonomic imbalance with increased sympathetic tone is a primary cause of these cardiovascular deficits associated with aging. Treatment with ACE inhibitors or AT1 receptor blockers reduces age-related cardiovascular and metabolic dysfunction and improves lifespan in humans and animals, suggesting that Ang II plays a role in the development of adverse cardiovascular consequences during aging. In transgenic ASrAOGEN rats, blood pressure remains low while baroreflex and metabolic function are preserved over lifespan, implicating the brain RAS in age-related cardiovascular decline. Furthermore, as in young adult (mRen2)27 rats, NTS microinjection of Ang-(1-7) but not an AT1 receptor antagonist restores baroreflex sensitivity for control of heart rate in older Sprague-Dawley rats. Together, the body of evidence suggests that aging is an additional context associated with altered RAS activity that contributes to autonomic imbalance and subsequent reduced baroreflex sensitivity. The role of the endocannabinoid system in age-related cardiovascular pathologies has not yet been investigated. Therefore, older Sprague-Dawley rats represent an additional setting in which to evaluate the contribution of endocannabinoids to blood pressure regulation in a condition associated with hypertension with altered brain RAS activity.

In summary, transgenic rats with high or low brain RAS activity and aged Sprague-Dawley rats exhibit alterations in autonomic cardiovascular regulation, and thus may be powerful model systems in which to evaluate potential interactions between the RAS and the endocannabinoid system in the face of hypertension and related pathologies. The table below summarizes the features of animals used in the present studies relative to young adult Hannover Sprague-Dawley rats from our colony:
<table>
<thead>
<tr>
<th></th>
<th>ASrAOGEN</th>
<th>(mRen2)27</th>
<th>Aged Sprague-Dawley</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent Strain:</td>
<td>Hannover</td>
<td>Hannover</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Sprague-Dawley</td>
<td>Sprague-Dawley</td>
<td></td>
</tr>
<tr>
<td>Conscious Blood Pressure:</td>
<td>Hypotensive</td>
<td>Hypertensive</td>
<td>Hypertensive</td>
</tr>
<tr>
<td>Autonomic Balance:</td>
<td>↑Parasympathetic</td>
<td>↑Sympathetic</td>
<td>↑Sympathetic</td>
</tr>
<tr>
<td>Baroreflex Sensitivity:</td>
<td>Enhanced</td>
<td>Impaired</td>
<td>Impaired</td>
</tr>
<tr>
<td>Body Weight:</td>
<td>Light</td>
<td>Heavy</td>
<td>Heavy</td>
</tr>
<tr>
<td>Sensitivity to Insulin</td>
<td>Enhanced</td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>and Leptin:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain RAS Activity:</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>NTS Endocannabinoid</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Tone:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5. Rationale and Aims

Ongoing studies in our laboratory focus on understanding factors that alter baroreflex sensitivity for control of heart rate at the level of the NTS. The majority of evidence to date indicates a key role for RAS peptides in modulating baroreflex function in which the endogenous balance of Ang II and Ang-(1-7) within this brain region determines the prevailing level of baroreflex sensitivity. However, additional factors, such as leptin, have been recently identified to influence resting baroreflex sensitivity, suggesting that alterations in endogenous hormone or signaling molecule levels during conditions related to hypertension may contribute to baroreflex dysfunction.

Previous studies by others show that exogenous cannabinoids alter neuronal cell firing in the NTS as well as baroreflex-evoked sympathetic nerve activity. One report notes that the enhanced sympathoinhibition elicited by cannabinoid agonists in the NTS of normotensive animals is absent in SHR, suggesting differential effects of cannabinoids on baroreflex modulation in hypertensive relative to normotensive animals. However, the effect of exogenous cannabinoids in the NTS on baroreflex sensitivity for control of heart rate in response to increases in arterial pressure, a vagally-mediated autonomic response, is unknown. Therefore, we evaluated the effect of NTS microinjection of a non-selective cannabinoid receptor agonist, CP55,940, on baroreflex sensitivity for control of heart rate in anesthetized young adult Sprague-Dawley rats.

Preliminary studies demonstrate a dose-related biphasic effect of NTS cannabinoid receptor activation on baroreflex sensitivity, in which lower doses of CP55,940 (0.12 and 1.2 pmol/120 nL) facilitate while a higher dose (12 pmol/120 nL) impairs baroreflex sensitivity (Figure 1-1). These responses are fully prevented or...
reversed by co-administration of the selective CB$_1$ receptor antagonist SR141716A (Figure 1-1), suggesting that effects on the baroreflex are mediated by the CB$_1$ rather than CB$_2$ receptor. We then tested whether the lower doses of CP55,940, which increase baroreflex sensitivity in Sprague-Dawley rats, would similarly improve baroreflex sensitivity in hypertensive (mRen2)27 with impaired baseline baroreflex function. Similar to baroreflex-evoked sympathetic nerve activity in SHR, there is no effect of NTS cannabinoid receptor activation on baroreflex sensitivity for control of heart rate in (mRen2)27 rats (Figure 1-2), consistent with differential modulation of the baroreflex by cannabinoids in hypertension.

![Figure 1-1. Effect of CB$_1$ Agonist in NTS of Sprague-Dawley Rats.](image)

Microinjection of CP55,940 into the NTS of Sprague-Dawley rats produced a dose-related biphasic effect on baroreflex sensitivity for control of heart rate in response to increases in arterial pressure after 10 minutes. Subsequent microinjection of SR141716A (36 pmol/120 nL) reversed changes in baroreflex sensitivity elicited by CP55,940. Pooled baseline is represented in the figure (N = 16). **P < 0.01 vs. respective baselines; n = 5-6.
These data raise additional questions. Neither endocannabinoid expression nor activity has been investigated in (mRen2)27 rats, so it is unknown whether endocannabinoid actions in the NTS contribute to the suppressed baseline baroreflex sensitivity in this strain. However, the central and peripheral endocannabinoid system is overactive in other animal models of hypertension and metabolic syndrome.\textsuperscript{181, 288} Moreover, signaling interactions between CB\textsubscript{1} and AT\textsubscript{1} receptors may function to enhance the pathogenic properties of Ang II,\textsuperscript{233} previously shown to transactivate CB\textsubscript{1} receptors via AT\textsubscript{1} receptor-mediated stimulation of 2-AG production.\textsuperscript{234, 289} Therefore, we hypothesize differential roles for the endocannabinoid system in modulation of baroreflex sensitivity at the level of the NTS in rats with altered brain RAS activity, and employ transgenic (mRen2)27 and ASrAOGEN as well as aged Sprague-Dawley rats to investigate the functional role of endocannabinoids in influencing baroreflex function during these conditions. We further hypothesize a role for the endocannabinoid system in the maintenance of the Ang II-dependent hypertension and symptoms of metabolic syndrome exhibited by (mRen2)27 rats.\textsuperscript{243} To evaluate this hypothesis, we assess effects of acute and chronic systemic CB\textsubscript{1} receptor blockade on blood pressure, metabolic profile
and conscious baroreflex function in this hypertensive strain. Accordingly, the specific aims of the present studies are as follows:

**Aim 1:** Determine if endocannabinoid tone at the level of the NTS contributes to the differential resting baroreflex sensitivity for control of heart rate in rats with high or low brain RAS activity using transgenic and older animals.

**Aim 2:** Determine if systemic CB$_1$ receptor blockade alters conscious blood pressure and autonomic function, and improves metabolic profile in hypertensive (mRen2)27 rats.
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CHAPTER TWO

MEDULLARY ENDOCANNABINOIDS CONTRIBUTE TO THE DIFFERENTIAL RESTING BAROREFLEX SENSITIVITY IN RATS WITH ALTERED BRAIN RENIN-ANGIOTENSIN SYSTEM EXPRESSION

By

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Short title: Endocannabinoids and Baroreflex Sensitivity

The following manuscript is in revision for Hypertension and represents the efforts of the first author. Differences in formatting and organization reflect the requirements of the journal.
Abstract

CB₁ cannabinoid receptors are expressed on vagal afferent fibers and neurons within the solitary tract nucleus, implicating a role in modulation of the arterial baroreflex. Hypotensive ASrAOGEN rats with low glial angiotensinogen have enhanced bradycardic baroreflex sensitivity compared to Sprague-Dawley rats, while (mRen2)27 rats, a model of angiotensin II-dependent hypertension, feature impaired baroreflex sensitivity. Mass spectrometry revealed higher levels of the endocannabinoid 2-arachidonoylglycerol in (mRen2)27 rats compared to ASrAOGEN rats (2.70 ± 0.28 vs. 1.17 ± 0.09 ng/mg tissue; P < 0.01), while Sprague-Dawley rats had intermediate content (1.85 ± 0.27 ng/mg tissue). Microinjection of the CB₁ receptor antagonist SR141716A into the solitary tract nucleus did not change baroreflex sensitivity in anesthetized Sprague-Dawley rats. However, SR141716A in (mRen2)27 rats dose-dependently improved baroreflex sensitivity: 0.36 pmol/120 nL of SR141716A increased baroreflex sensitivity from 0.43 ± 0.03 to 0.71 ± 0.04 ms/mmHg (P < 0.001), and 36 pmol of SR141716A increased baroreflex sensitivity from 0.47 ± 0.02 to 0.94 ± 0.10 ms/mmHg (P < 0.01) in this strain. In contrast, both the 0.36-pmol (1.50 ± 0.12 versus 0.86 ± 0.08 ms/mmHg; P < 0.05) and 36-pmol (1.38 ± 0.16 versus 0.46 ± 0.003 ms/mmHg; P < 0.01) SR141716A microinjections reduced baroreflex sensitivity in ASrAOGEN rats. These observations reveal differential regulatory functions of the brain endocannabinoid system for modulating baroreflex function during conditions associated with altered brain renin-angiotensin system expression that may involve interactions with neurotransmitters known to influence cardiovagal baroreflex sensitivity.
Introduction

Tonic overactivation of the endocannabinoid system is implicated in the maintenance of cardiovascular diseases, including hypertension and atherosclerosis, and the metabolic syndrome.\textsuperscript{1,2} These adverse conditions are associated with arterial baroreflex dysfunction and reduced heart rate variability, themselves indices of general morbidity and mortality.\textsuperscript{3} Baroreflex sensitivity (BRS) for control of heart rate (HR), measured as the bradycardia evoked in response to increases in mean arterial pressure (MAP), is an important indicator of parasympathetic activity and vagus nerve function, and its impairment often precedes the development of cardiovascular disease.\textsuperscript{3}

The G protein-coupled CB\textsubscript{1} and CB\textsubscript{2} cannabinoid receptors mediate the biological actions of the endocannabinoid system in target cells. CB\textsubscript{1} is widely expressed in the central nervous system (CNS) and in peripheral tissues, enabling the activated receptor to regulate a variety of physiological functions including blood pressure and heart rate.\textsuperscript{4} In contrast, CB\textsubscript{2} receptors are primarily restricted to immune tissues and are expressed in the CNS at levels many-fold lower than CB\textsubscript{1}.\textsuperscript{5} CB\textsubscript{1} receptors in the CNS are predominantly expressed on presynaptic neuronal terminals, where their activation by the endocannabinoids anandamide or 2-arachidonoylglycerol (2-AG), released from postsynaptic neurons, directly modulates voltage-gated ion channels to repolarize the cellular membrane, temporarily reducing the release of neurotransmitter into the synaptic cleft (see Castillo et al. (2012)\textsuperscript{6} for review).

Presynaptic CB\textsubscript{1} receptors are densely expressed in the solitary tract nucleus (NTS) of the dorsomedial medulla, the primary site for termination of baroreceptor afferent neurons relaying sensory information from the periphery, as well as in vagal
afferent neurons originating in the nodose ganglion. Several studies show that anandamide within the NTS modulates baroreflex-evoked suppression of renal sympathetic nerve activity in rats by altering release of the inhibitory neurotransmitter GABA. Furthermore, blockade of CB1 receptors in the medial prefrontal cortex, a limbic structure involved in the modulation of autonomic responses exerting influence on the cardiovascular system, enhances spontaneous BRS for control of HR in conscious rats. Therefore, the brain endocannabinoid system likely influences BRS through the modulation of neurotransmitter release in brain sites regulating effects on the baroreflex.

There is growing evidence for signaling interactions between the endocannabinoid system and the pathogenic actions of the renin-angiotensin system (RAS). For instance, blockade of CB1 receptors in Sprague-Dawley (SD) rats treated chronically with ethanol prevents angiotensin (Ang) II-mediated mitogenic signaling and profibrogenic gene expression in hepatic stellate cells. In obese Zucker rats, longterm CB1 receptor blockade reduces the vasoconstrictor effect of acutely administered Ang II. However, no studies to date have investigated the endocannabinoid-RAS interaction in animals with altered brain RAS expression.

Transgenic (mRen2)27 rats, a monogenetic model of Ang II-dependent hypertension in which the mouse Ren2 renin gene was transfected into the genome of the normotensive SD rat, have a phenotype of chronic hypertension with markedly impaired BRS for control of HR compared to their normotensive genetic controls. In contrast, transgenic ASrAOGEN rats with low brain angiotensinogen, resulting from glial overexpression of an angiotensinogen antisense oligonucleotide, exhibit chronic hypotension with significantly enhanced resting BRS compared to SD rats. These
strains are frequently utilized to investigate the contribution of the brain RAS and its interacting factors to impaired BRS during hypertension. In this study, we provide functional and biochemical evidence for altered endocannabinoid tone at the level of the dorsomedial medulla and NTS in rats with altered brain RAS activity.
Methods

Animals

Experiments were performed in 15- to 20-week-old male Hannover SD and transgenic (mRen2)27 and ASrAOGEN rats obtained from the Hypertension & Vascular Research Center colony at Wake Forest School of Medicine. Animals were housed two per cage in a humidity- and temperature-controlled room with ad libitum access to food (standard chow) and water. The colony maintained a 12-hour light/dark cycle (lights on at 06:00). The Institutional Animal Care and Use Committee approved all experimental procedures.

Surgical Procedures and Hemodynamic Measures

As described previously, rats were anesthetized with combination urethane-chloralose (750 and 35 mg/kg, respectively) via intraperitoneal injections with supplemental intravenous doses given as needed (diluted to 3:7 with saline). Animals were instrumented with femoral artery and vein catheters and placed in a stereotaxic frame with the head tilted at a 45° downward angle for surgical exposure of the dorsal brain medulla. Rats were allowed a 30 minute resting and equilibration period before testing began. Pulsatile arterial pressure (AP) was recorded and digitized by a data acquisition system (AcqKnowledge software version 3.8.1, BIOPAC Systems, Goleta, CA), and heart rate (HR) was determined from the AP wave. After obtaining stable measures of MAP and HR, baseline BRS was established by sequential intravenous bolus injection of 3 doses of phenylephrine (PE; 2, 5 and 10 μg/kg in 0.9% NaCl) to determine the bradycardic BRS response to increases in AP. BRS for bradycardia was defined for each animal as the slope of the relationship between changes in MAP (ΔMAP; mmHg) and the pulse interval (ΔPI; ms) generated from the 3 doses of PE (mean $R^2 = 0.95$ for all
animals; N = 34). Reflex testing was repeated 10 and 60 minutes after bilateral microinjection of the CB$_1$ receptor-selective antagonist SR141716A into the NTS, and was completed within 15 minutes, with each animal serving as its own control. Maximum transient changes in MAP and HR in response to NTS microinjection of SR141716A were also recorded.

**NTS Microinjections**

SR141716A (0.36 or 36 pmol in a 120 nL volume of 10% DMSO in artificial cerebrospinal fluid vehicle) or vehicle (120 nL) was microinjected bilaterally into the NTS (0.4 mm rostral, 0.4 mm lateral to the calamus scriptorius, and 0.4 mm below the dorsal surface) using a glass micropipette connected to a hand-held syringe, as previously described.\(^{18, 20}\) Doses of SR141716A were determined based on the literature\(^{21}\) and previous experience with the drug.\(^{22}\) NTS microinjection of vehicle solution had no effect on evoked BRS, MAP or HR in SD, (mRen2)27 or ASrAOGEN rats (Table 2-1). At the end of experiments, brains were removed, frozen, and sectioned (30 µm) for localization of microinjection sites (Figure 2-1). Only data from injections within the medial NTS at the

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**Figure 2-1. Histological Analysis of Microinjection Sites**

Representative photomicrography (5X magnification) of an unstained rat medullary section (30 µm) at approximately -13.8 mm caudal to bregma showing a typical NTS microinjection site (0.4 mm rostral, 0.4 mm lateral to the calamus scriptorius [caudal tip of the area postrema] and 0.4 mm below the dorsal surface). Only data from injections within the medial NTS at rostrocaudal level -13.3 to -14.0 mm caudal to bregma were used in this study. AP = area postrema; C = central canal; dmnX = dorsal motor nucleus of the vagus; NG = nucleus gracilis; NTS = solitary tract nucleus.
rostrocaudal level -13.3 to -14.0 mm caudal to bregma were used in the analysis. The accuracy rate for injections was >90%.

**Table 2-1. Values of MAP, HR and BRS in Response to NTS Microinjection of Vehicle**

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>MAP (mmHg)</th>
<th>HR (bpm)</th>
<th>BRS (ms/mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle in SD rats</td>
<td>5</td>
<td>93 ± 2</td>
<td>330 ± 12</td>
<td>1.02 ± 0.12</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td>92 ± 3</td>
<td>319 ± 15</td>
<td>1.02 ± 0.10</td>
</tr>
<tr>
<td>Values at 10 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle in (mRen2)27 rats</td>
<td>3</td>
<td>96 ± 8</td>
<td>294 ± 20</td>
<td>0.44 ± 0.04*</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td>92 ± 4</td>
<td>296 ± 9</td>
<td>0.45 ± 0.06*</td>
</tr>
<tr>
<td>Values at 10 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle in ASrAOGEN rats</td>
<td>4</td>
<td>119 ± 8</td>
<td>360 ± 16</td>
<td>1.43 ± 0.09*</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td>121 ± 8</td>
<td>358 ± 9</td>
<td>1.46 ± 0.14*</td>
</tr>
<tr>
<td>Values at 10 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SEM and represent MAP, HR and BRS values at baseline and 10 minutes after NTS microinjection of vehicle; N = number of animals; MAP = mean arterial pressure; HR = heart rate.

*P < 0.05 vs. SD; #P < 0.01.

**Mass Spectrometry Detection of Anandamide and 2-AG**

In separate groups of naïve 15-week-old SD, (mRen2)27 and ASrAOGEN rats, brains were removed and frozen on dry ice for excision of 3-mm³ dorsal medullary sections. For the analysis of the two major endocannabinoids anandamide and 2-AG, an extraction technique was developed for rat medulla tissue. Optimal extraction conditions for the tissue were obtained using HPLC-grade acetonitrile and tissue homogenization with stainless steel beads (2.3 mm, BioSpec Products, Bartlesville, OK) that reduced enzymatic degradation and attenuated 2-AG isomerization. Tissue homogenates were fortified with isotopically-labeled internal standards for 2-AG and anandamide 2-[²H₅]2-
AG (AG-d5) and [²H₄]anandamide (AEA-d4) on a per-mg basis for sample integrity and robustness of the method. The chromatography solvents were Honeywell B&J HPLC-grade ammonium acetate and acetic acid–double distilled (Sigma-Aldrich). Stock solutions of anandamide and 2-AG and their deuterated analogs were freshly prepared and stored as recommended by the manufacturer. The study samples, quality control samples, and standards (targeted analysis) were processed using automated liquid handling to ensure the reproducibility and consistency of the sample preparation method.

A Waters Acuity ultraperformance liquid chromatography (UPLC) system (Waters, Milford, MA) equipped with an Acquity UPLC column was utilized for the separation of endocannabinoids prior to quantitative analysis by selected reaction monitoring (LC-MS/MS). A triple quadrupole mass spectrometer (API-5000; AB SCIEX, Framingham, MA) was coupled to the UPLC for quantitative analysis, and operating parameters were optimized for anandamide and 2-AG. Calibration curves spanning the range of quantitation were produced through least-squares linear regression analysis of response (analyte integrated area/internal standard integrated area) versus concentration, and weighted to improve fit, accuracy and precision. Sample content of anandamide or 2-AG is expressed as ng analyte per mg tissue wet weight.

**Quantification of CB₁, CB₂, and CRIP1a mRNA**

RT-qPCR was used to measure mRNA levels of various effector components of the brain endocannabinoid system, including CB₁ and CB₂ receptor, and the cannabinoid receptor interacting protein-1a (CRIP1a)²³ in dorsal medullary tissue from the same naïve 15-week-old rats (n = 4-6 each strain). Brains were removed and frozen on dry ice as described for endocannabinoid measurements. Isolated RNA from excised tissue was
assessed for concentration and stability. Total RNA (1 µg) was reverse transcribed using AMV reverse transcriptase in a 20-µL reaction mixture containing deoxyribonucleotides, random hexamers, and RNase inhibitor in reverse-transcriptase buffer, as described previously.\textsuperscript{18, 20, 24} For RT-qPCR, 2 µL of resultant cDNA were added to TaqMan Universal PCR Master Mix with the appropriate gene-specific primer/probe set for CB\textsubscript{1} and CB\textsubscript{2} receptors, and CRIP1a (Applied Biosystems), and amplification was performed. All reactions were performed in triplicate. 18S ribosomal RNA served as the internal control. Results were quantified as Ct values, in which Ct is the threshold cycle of PCR at which an amplified product is first detected, and was defined as relative gene expression (ratio of target:control).

**Analysis of Data**

Values are presented as mean ± SEM. Comparisons of changes in BRS over time in response to SR141716A or vehicle in the different strains were assessed using a repeated measures two-way ANOVA. Where appropriate on the basis of the two-way ANOVA, one-way ANOVA and post-hoc Tukey multiple comparisons elucidated further differences between strains or time points. Multiple regression analysis was performed on BRS scatterplots to determine slopes of fit lines. The criterion for statistical significance was P < 0.05. Statistical tests were performed using Prism 5.0 (GraphPad Software, San Diego, CA).
Results

Baseline BRS for Control of HR in Response to Increases in MAP in SD, (mRen2)27 and ASrAOGEN Rats

The pooled bradycardic BRS for control of HR at baseline for ASrAOGEN rats was significantly higher compared to SD rats (1.43 ± 0.07 vs. 1.03 ± 0.06 ms/mmHg; P < 0.001; n = 11 and 10, respectively; Figure 2-2), while baseline BRS in (mRen2)27 rats was significantly lower than in SD rats (0.44 ± 0.02 ms/mmHg; P < 0.001; n = 13; Figure 2-2). These observations are fully consistent with previous studies from our laboratory.18, 19 Furthermore, there were no differences in baseline BRS values of SD, (mRen2)27 or ASrAOGEN rat treatment groups receiving vehicle or various doses of SR141716A (Figure 2-3).

![Figure 2-2. Baseline BRS for Control of HR in SD, (mRen2)27 and ASrAOGEN Rats](image-url)

A. Pooled baseline BRS for control of HR in response to increases in AP evoked by PE prior to NTS microinjection of 120 nL of vehicle or SR141716A in anesthetized SD (n = 10), (mRen2)27 (n = 13) and ASrAOGEN (n = 11) rats. B. Scatterplot illustrates significantly different pooled baseline BRS reflex testing regression lines among ASrAOGEN (1.41 ± 0.15 ms/mmHg), SD (0.90 ± 0.09 ms/mmHg) and (mRen2)27 (0.48 ± 0.09 ms/mmHg) rats (P < 0.0001). ‡P < 0.001 vs. SD.
There were no differences in baseline BRS for control of HR in response to increases in AP evoked by PE among treatment groups of SD (n = 5), (mRen2)27 (n = 3-5) or ASrAOGEN (n = 3-4) rats receiving vehicle or various doses of SR141716A.

**Effect of CB₁ Receptor Antagonist on BRS for Control of HR in SD, (mRen2)27 and ASrAOGEN Rats**

In SD rats, NTS microinjection of 36 pmol of SR141716A had no effect on BRS for control of HR in response to increases in MAP produced by PE at 10 or 60 minutes after microinjection (Figure 2-4). However, microinjection of 0.36 and 36 pmol doses of SR141716A into the NTS of hypertensive (mRen2)27 rats significantly and dose-dependently increased BRS by 65% (P < 0.001) and 100% (P < 0.01), respectively, after
10 minutes (Figure 2-5). These effect are fully consistent with previous experiments by our laboratory showing improvement in spontaneous BRS following systemically administered SR141716A in conscious (mRen2)27 rats. Timecourse experiments showed that the BRS-enhancing effect of each dose in (mRen2)27 rats was maintained at 60 minutes post-microinjection (Figure 2-5).

In contrast to the potentiating effect found in (mRen2)27 rats, NTS microinjection of 0.36 pmol SR141716A in hypotensive ASrAOGEN rats significantly reduced BRS for control of HR by 43% (P < 0.05), while 36 pmol of SR141716A reduced BRS by 67% after 10 minutes (P < 0.01; Figure 2-6). These changes suggest a dose-dependent response to NTS microinjection of SR141716A, although the differences between the effects of the two doses did not reach statistical significance after post-hoc analysis. As in (mRen2)27 rats, timecourse experiments showed that the attenuating effect of each dose on BRS was maintained at 60 minutes after microinjection (Figure 2-6).

![Figure 2-4](image-url)

Figure 2-4. Effect of NTS Microinjection of CB₁ Receptor Antagonist SR141716A on BRS for Control of HR Evoked by PE in SD Rats. A, NTS microinjection of 36 pmol of SR141716A in SD rats did not significantly change BRS after 10 minutes, nor after 60 minutes in the same animals (n = 5). B, The slope of the relationship between the increases in MAP produced by PE and the corresponding reflex bradycardia (ΔPI) does not show change from baseline in the linear regression slope 10 or 60 minutes following NTS microinjection of 36 pmol of SR141716A (1.00 ± 0.08 ms/mmHg baseline; 1.13 ± 0.11 ms/mmHg after 10 minutes; 1.14 ± 0.14 ms/mmHg after 60 minutes; R² = 0.84 to 0.92 for pooled data).
Figure 2-5. Effect of NTS Microinjection of SR141716A on BRS for Control of HR Evoked by PE in (mRen2)27 Rats. A-B, In (mRen2)27 rats, NTS microinjection of 0.36- (n = 5) and 36-pmol (n = 5) of SR141716A significantly improved BRS after 10 and 60 minutes. C, The 0.36- and 36-pmol doses produced graded, though not statistically significant, increases in the slope of the regression line in (mRen2)27 rats (0.47 ± 0.10 ms/mmHg baseline; 0.69 ± 0.10 ms/mmHg 10 minutes after 0.36 pmol of SR141716A; 0.79 ± 0.12 ms/mmHg 10 minutes after 36 pmol SR141716A; R² = 0.44 to 0.79 for pooled data). †P < 0.01 vs. baseline; ‡P < 0.01.
MAP and HR Responses to NTS Microinjection of CB₁ Receptor Antagonist

There were no significant differences in baseline MAP or HR within groups of SD, (mRen2)27 or ASrAOGEN rats receiving vehicle or SR141716A microinjections, nor were there significant differences between pooled baseline HR of SD and (mRen2)27 rats receiving vehicle or SR141716A (Table 2-1 and 2-2; Figure 2-7). However, as reported previously, the pooled baseline MAP of anesthetized ASrAOGEN rats was significantly higher compared to SD rats (115 ± 4 vs. 94 ± 2 mmHg, respectively; P < 0.01; Figure 2-
7A), possibly due to an anesthesia-induced activation of the sympathetic nervous system observed in these animals. The pooled baseline HR was also significantly higher in ASrAOGEN rats compared to SD and (mRen2)27 rats (347 ± 7 vs. 313 ± 8 and 312 ± 6 bpm, respectively; P < 0.01; Figure 2-7B). The pooled baseline MAP of anesthetized (mRen2)27 rats (108 ± 4 ms/mmHg) was also significantly higher compared to SD rats (P < 0.05; Figure 2-7A).

Acute NTS microinjection of either 0.36 or 36 pmol of SR141716A did not significantly change resting MAP in SD or ASrAOGEN rats, nor HR in SD, (mRen2)27 or ASrAOGEN rats at the time of reflex testing 10 and 60 minutes after microinjection. While 0.36 pmol of SR141716A did not have a significant effect on resting MAP in (mRen2)27 rats over the duration of testing, the 36 pmol dose produced a modest yet statistically significant decrease in MAP at 10 and 60 minutes post-microinjection (P < 0.001 vs. baseline; Table 2-2). There were no significant effects of NTS microinjection of vehicle on MAP or HR in any strain (Table 2-1). These responses indicate that differences in BRS after administration of SR141716A are generally not attributable to differences in resting hemodynamics, confirming that the set point of the baroreflex is regulated independently from the sensitivity. Differences in MAP or HR within groups of transgenic animals relative to SD rats at individual timepoints are noted in Table 2-1 and Table 2-2.

Microinjection of 36 pmol of SR141716A yielded a modest yet statistically significant potentiation of intravenous PE-induced MAP increases in SD rats after 10 and 60 minutes (P < 0.05; Figure 2-8), consistent with sympathetic activation associated with SR141716A. However, there were no differences in MAP responsiveness to PE
injections among groups of (mRen2)27 or ASrAOGEN rats receiving various doses of SR141716A.

Table 2-2. Values of MAP and HR in Response to NTS Microinjection of SR141716A

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>MAP (mmHg)</th>
<th>HR (bpm)</th>
</tr>
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<tr>
<td></td>
<td></td>
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<tr>
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<td>Values at 10 minutes</td>
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<td></td>
<td></td>
<td>Values at 60 minutes</td>
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<tr>
<td>SD 36 pmol SR141716A</td>
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<td>297 ± 6</td>
</tr>
<tr>
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<td></td>
<td>90 ± 3</td>
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<tr>
<td></td>
<td></td>
<td>91 ± 4</td>
<td>291 ± 11</td>
</tr>
<tr>
<td>(mRen2)27 0.36 pmol SR141716A</td>
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<td>103 ± 5</td>
<td>319 ± 5</td>
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<tr>
<td></td>
<td></td>
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<td>308 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96 ± 4</td>
<td>326 ± 9</td>
</tr>
<tr>
<td>(mRen2)27 36 pmol SR141716A</td>
<td>5</td>
<td>119 ± 6</td>
<td>316 ± 5</td>
</tr>
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<td></td>
<td>109 ± 7‡</td>
<td>318 ± 10*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>105 ± 7‡</td>
<td>325 ± 10*</td>
</tr>
<tr>
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<td>347 ± 12*</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
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<td>360 ± 5‡</td>
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<td>107 ± 5</td>
<td>335 ± 7</td>
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<tr>
<td></td>
<td></td>
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<td>98 ± 6</td>
<td>345 ± 15*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM and represent baseline MAP and HR and values at 10 or 60 minutes after the initial SR141716A NTS microinjection; N = number of animals.

‡P < 0.001 vs. baseline.

*P < 0.05 vs. SD; †P < 0.01; ‡P < 0.001.
Figure 2-7. Baseline MAP and HR of SD, (mRen2)27 and ASrAOGEN Rats
A-B, Pooled baseline MAP (A) and HR (beats per minute [bpm]) (B) prior to NTS microinjection of 120 nL of vehicle or SR141716A (0.36 or 36 pmol) in anesthetized SD (n = 10), (mRen2)27 (n = 13) and ASrAOGEN (n = 11) rats. *P < 0.05 vs. SD; †P < 0.01 vs. SD; #P < 0.01 vs. (mRen2)27.
Figure 2-8. MAP Responsiveness in SD, (mRen2)27 and ASrAOGEN Rats at Baseline and in Response to NTS Microinjection of SR141716A

Changes in MAP in responsiveness to intravenous graded doses of PE were assessed in anesthetized SD, (mRen2)27 and ASrAOGEN rats at baseline and in response to NTS microinjection of 0.36 or 36 pmol of SR141716A. A, Baseline MAP responsiveness to 2 µg of PE was significantly greater in ASrAOGEN and (mRen2)27 rats compared to SD rats (P < 0.05). B, 36 pmol of SR141716A in SD rats significantly potentiated PE-induced increases in MAP at 10 and 60 minutes after NTS microinjection (P < 0.05; n = 5). C-D, There were no significant differences in PE-induced increases in MAP within groups of (mRen2)27 (C; n = 5 per group) or ASrAOGEN rats (D; n = 3-4 per group).

Dorsal Medullary 2-AG and Anandamide Content in SD, (mRen2)27 and ASrAOGEN Rats

Mass spectrometry revealed that levels of 2-AG were lowest in dorsal medulla of ASrAOGEN rats and highest in (mRen2)27 rats (1.17 ± 0.09 vs. 2.70 ± 0.28 ng/mg tissue, respectively; P < 0.01; n = 4-5; Figure 2-9A). SD rats had intermediate medullary 2-AG content (1.85 ± 0.27 ng/mg tissue; n = 5), falling just short of statistical significance compared to (mRen2)27 rats (P = 0.069) and ASrAOGEN rats (P = 0.052).
Of note, the relative 2-AG levels parallel the trend in resting conscious MAP and are inversely related to the baseline BRS for control of HR across rat strains. Dorsal medullary anandamide was found in levels 1000-fold lower than 2-AG and there were no significant differences among rat strains (Figure 2-9B).

**Figure 2-9. 2-AG and Anandamide Content in Dorsal Medulla of SD, (mRen2)27 and ASrAOGEN Rats.**
A, Mass spectrometry revealed levels of 2-AG were significantly higher in the dorsal medullary tissue of 15-week-old (mRen2)27 rats (n = 5) relative to ASrAOGEN rats (n = 4), and trended higher (P = 0.069) in (mRen2)27 rats compared to SD rats (n = 5). In addition, there was a trend (P = 0.052) for higher 2-AG levels in dorsal medulla of SD rats relative to ASrAOGEN rats. B, Anandamide was detected at levels 1000-fold lower than 2-AG in the same tissue samples, and there were no significant differences among strains. Representative chromatograms of 2-AG (A) and anandamide (B) are shown next to respective figures. †P < 0.01 vs. ASrAOGEN.

**CB₁, CB₂ and CRIP1a mRNA in SD, (mRen2)27 and ASrAOGEN Rats**
Relative gene expression of CB₁ and CB₂ receptors, and CRIP1a was measured in dorsal medullary tissue of naïve SD (n = 5), (mRen2)27 (n = 4) and ASrAOGEN (n = 6) rats at 15 weeks of age (Figure 2-10). CB₁ receptor mRNA was approximately 0.25-fold lower in the dorsal medulla of ASrAOGEN rats relative to SD (P < 0.01) and (mRen2)27 (P <
0.05) rats (Figure 2-10A). There were no differences in relative mRNA levels of CB$_2$ receptor or CRIP1a among the same rat groups (Figure 2-10B and 2-10C).

![Graphs showing relative gene expression of CB$_1$, CB$_2$, and CRIP1a](image)

**Figure 2-10. CB$_1$, CB$_2$, and CRIP1a mRNA in Dorsal Medulla of SD, (mRen2)$_{27}$ and ASrAOGEN Rats.** A, Relative gene expression of CB$_1$ receptor was lower in the dorsal medullary tissue of 15-week-old ASrAOGEN rats (n = 6) compared to SD (n = 5) and (mRen2)$_{27}$ (n = 4) rats. B-C, No significant differences in relative gene expression of CB$_2$ receptor (B) or CRIP1a (C) were found among strains. *P < 0.05 vs. (mRen2)$_{27}$; †P < 0.01 vs. SD.
Discussion

In this study, we report for the first time the endocannabinoid content and relative mRNA levels of proteins associated with the endocannabinoid system in the dorsal medulla of (mRen2)27 and ASrAOGEN rats compared to SD rats. Of note, the relative levels of dorsal medullary 2-AG paralleled brain RAS expression and resting conscious blood pressure, and were inversely related to baseline BRS for control of HR across the hypotensive, normotensive and hypertensive rat strains. We also determined the effects of exogenous CB1 cannabinoid receptor blockade by SR141716A on cardiovagal BRS, an index of parasympathetic activity, at the level of the NTS in anesthetized rats with differential brain RAS expression profiles. The improvement in BRS in response to SR141716A in (mRen2)27 rats was associated with increased 2-AG content in the dorsal medulla, suggesting that long-term increases in brain angiotensin peptides may be related to upregulation of 2-AG release that contributes to the blunted baseline BRS in this hypertensive strain, consistent with Ang II-mediated vascular endocannabinoid release.27 In ASrAOGEN rats, however, medullary 2-AG content was significantly lower compared to (mRen2)27 rats, and CB1 receptor mRNA was significantly lower relative to SD and (mRen2)27 rats. Therefore, the BRS-suppressing effect of SR141716A in the NTS of ASrAOGEN rats suggests differential regulatory functions of the brain endocannabinoid system in rats with high or low brain RAS expression.

Evidence suggests that exogenous cannabinoids in the NTS modulate baroreflex function by interacting with local excitatory or inhibitory neurotransmitter systems. Exogenous anandamide in the NTS of anesthetized SD rats prolongs PE-evoked inhibition of renal sympathetic nerve activity,9 indicating an increase in sympathetic
BRS. In addition, anandamide shortens the duration of sympathetic nerve inhibition following blockade of NTS GABA receptors, revealing that inhibition of excitatory glutamate release by anandamide is obscured by a more dominant GABA-mediated effect. The synthetic cannabinoid agonists WIN55,212-2 and CP55,940 in the NTS of anesthetized SD rats suppress sympathetic nerve discharge. These effects are prevented by NTS microinjection of the CB₁ receptor-selective antagonist AM281, which had no effect alone in the NTS. Therefore, our current study investigating the role of endocannabinoids in the parasympathetically-mediated cardiovagal branch of the baroreflex potentially reveals evidence for altered NTS glutamate or GABA neurotransmission through CB₁ receptor activation in the context of altered brain RAS activity. Results showing improved BRS in (mRen2)27 rats after SR141716A microinjection in the NTS are bolstered by observations of direct and indirect inhibition of GABA release in rat arcuate nucleus by SR141716A. In ASrAOGEN rats with increased NTS glutamate content, suppression of glutamate may be the predominant effect of NTS CB₁ receptor blockade, which may explain the inhibition of BRS by CB₁ receptor blockade in these animals. More research is needed to clarify this mechanism, for which a magnetic resonance spectroscopy technique previously used in our laboratory to assess glutamate and GABA content in the NTS of these rats may be useful.

Whole-cell patch clamp recordings in rat brainstem slices reveal that WIN55,212-2 and exogenous anandamide in the NTS suppress synaptic input to neurons in the dorsal motor nucleus of the vagus (dmnX), an important second-order nucleus in the parasympathetic baroreflex arc. Therefore, upregulation of NTS or dmnX endocannabinoids or cannabinoid receptors would likely contribute to the suppression of
visceral autonomic responses, such as BRS for control of HR. However, transient receptor potential vanilloid type 1 (TRPV1) channels in the NTS and dmnX\textsuperscript{31} activated by anandamide\textsuperscript{32} may also regulate synaptic activity in brainstem nuclei involved baroreflex mediation. Activation of TRPV1 channels triggers spontaneous glutamate release in the NTS, opposing the actions of CB\textsubscript{1} receptors\textsuperscript{33} while exerting a tonic influence over inputs to the dmnX in rats and mice.\textsuperscript{34, 35} The presence of TRPV1 in brainstem nuclei in the baroreflex arc thus provides an additional role for endocannabinoids in modulating baroreflex function, and may contribute to the actions of exogenous anandamide and WIN55,212-2, reported to inhibit neuronal TRPV1,\textsuperscript{36} in the studies discussed above.\textsuperscript{9, 30} Although brainstem endocannabinoids may modulate BRS via TRPV1 channels, we show only the contribution of endocannabinoid modulation of BRS through actions at CB\textsubscript{1} receptors because SR141716A is a selective CB\textsubscript{1} receptor antagonist that is not reported to interact with TRPV1 or other receptors, including CB\textsubscript{2} receptors.\textsuperscript{37, 38}

Our present study presents evidence for a direct action of endocannabinoids to modulate baroreflex function in transgenic rats over- or underexpressing components of the brain RAS. Microinjection of the selective CB\textsubscript{1} receptor antagonist SR141716A (0.36 and 36 pmol) into the NTS differentially altered BRS measured as the bradycardic response to PE-evoked increases in AP in (mRen2)27 rats with Ang II-dependent hypertension and in hypotensive Ang-deficient ASrAOGEN rats. In (mRen2)27 rats, the 0.36-pmol dose of SR141716A significantly improved BRS, and the 36-pmol dose improved BRS from baseline to a greater degree, indicating a dose-response relationship. In fact, the 36-pmol dose improved BRS in these animals to \(\approx1.05\) ms/mmHg, a level
comparable with baseline BRS of SD rats and suggesting normalization of BRS for control of HR in (mRen2)27 rats. In ASrAOGEN rats, however, SR141716A significantly impaired BRS to ≈0.46 ms/mmHg, a level often observed in hypertension and comparable to baseline BRS of (mRen2)27 rats. The differential BRS responses to CB1 receptor antagonism in the NTS of these strains with altered brain RAS expression support the interpretation that the brain endocannabinoid system exerts a tonic influence over baroreflex function via NTS CB1 receptors which may contribute to suppressed baseline cardiovagal BRS in (mRen2)27 rats and enhanced baseline BRS in ASrAOGEN rats.

In contrast to the transgenic animals, there was no effect of CB1 receptor blockade by 36 pmol of SR141716A in the NTS on BRS for control of HR in SD rats. This is in line with previous observations by others that NTS microinjection of SR141716A alone in dogs did not affect BRS, nor did the CB1-selective antagonist AM281 by itself alter sympathetic nerve discharge in SD rats. This may indicate minimal or absent tonic influence of the endocannabinoid system over baroreflex function in these normotensive animals, or that baseline CB1 receptor modulation of the baroreflex in SD rats reflects balanced effects on GABA and glutamate release. It is also possible that this reflects a compensatory balance of additional factors in the NTS, such as endovanilloids, leptin, serotonin, and nonesterified fatty acids that may potentially influence baroreflex function. We did not evaluate the effect of NTS CB1 receptor blockade on the sympathetically-mediated tachycardic BRS response to reductions in AP, so we cannot yet determine if the endocannabinoid system selectively influences the parasympathetic over the sympathetic baroreflex in our study paradigm. However, NTS microinjection of
0.36- or 36-pmol of SR141716 in SD, (mRen2)27 and ASrAOGEN rats did not alter depressor and bradycardic responses to cardiac chemosensitive vagal fiber activation induced by intravenous phenylbiguanide (Figure 2-11). The absence of alterations in CVA responses supports the specificity of SR141716A actions on BRS because these responses are mediated by chemoreceptor fibers that converge with baroreceptor inputs within the NTS.42

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**Figure 2-11. Effects of Chemoreflex Activation on MAP and HR Induced by Intravenous Phenylbiguanide in SD, (mRen2)27 and ASrAOGEN Rats**

No change in depressor or bradycardic responses to chemosensitive vagal fiber activation following NTS microinjection of SR141716A in SD, (mRen2)27 or ASrAOGEN rats (n = 3-5 all treatment groups; pooled baselines are represented in figures).
We found that 2-AG content was highest in dorsal brainstem of (mRen2)27 rats and lowest in ASrAOGEN rats, which may be a potential mechanism to explain differential resting BRS among SD, (mRen2)27 and ASrAOGEN rats. Anandamide may also play an important role in this region, but its detection at 1000-fold lower levels without significant differences among strains indicates that 2-AG is likely the more functionally significant endocannabinoid with respect to BRS modulation at the level of the NTS in the face of altered brain RAS activity. We further observed a significantly lower expression of CB₁ receptor mRNA in dorsal medullary tissue of ASrAOGEN rats relative to SD and (mRen2)27 rats. Together, these observations suggest differential regulation of the endocannabinoid system in dorsal medulla of (mRen2)27 and ASrAOGEN rats, respectively, paralleling the relative expression of RAS components in these animals. In (mRen2)27 rats, the higher dorsal medullary 2-AG levels may reflect increased release, increased activity of enzymes involved in its biosynthesis, such as diacylglycerol lipase, or downregulation of its primary degradative enzyme monoacylglycerol lipase as the principal drivers of upregulated CB₁ receptor tone in this region. Our data are consistent with evidence for upregulated endocannabinoid tone that is widely reported in acute and chronic animal models of cardiovascular disease, including atherosclerosis and hypertension.

Although dorsal medullary gene expression of CB₁ receptor in (mRen2)27 rats did not differ from SD rats, enhanced sensitivity of the receptor cannot be ruled out as an additional mechanism for upregulated CB₁ receptor tone in this strain because Ang II-induced increased expression of G₁ protein isoforms is reported in SHR and one-kidney one-clip hypertension. It is also possible that a signaling interaction between CB₁ and
Ang II type 1 (AT1) receptors enhances the pathogenic signaling of Ang II, as recently described. In ASrAOGEN rats, lower levels of 2-AG and CB₁ receptor mRNA suggest a more general downregulation of the dorsal medulla endocannabinoid system that may contribute to the enhanced baseline BRS for control of HR in these animals. Whether the differential expression of the CB₁ receptor and production of 2-AG in the dorsal brainstem of (mRen2)27 and ASrAOGEN rats is attributable to a direct interaction with the RAS or an indirect effect is currently unknown; however, transactivation of CB₁ by AT1 receptors and direct stimulation of 2-AG production by Ang II has been observed in vitro. While we detected CB₂ receptor mRNA in the NTS, it is likely not involved in BRS modulation because previous studies in our laboratory with the non-selective cannabinoid receptor agonist CP55,940 show all effects on BRS in SD rats are completely blocked or reversed by the CB₁ antagonist SR141716A. It is also unlikely that the CB₁-associated protein CRIP1a, believed to serve an autoinhibitory function of CB₁ receptors, is a primary contributor to differential modulation of BRS because we did not detect significant differences in mRNA expression among the three rat strains.

We cannot exclude the possibility that the spread of the SR141716A injection may have accessed neighboring brainstem nuclei, such as the area postrema or the dmnX, for effects on BRS. However, microinjection of ¹²⁵I-Sar-Thr Ang II at this volume was mostly confined to the NTS, and functional assessments showed that 50 nL of an AT1 receptor antagonist injected into the dmnX did not alter responses to NTS microinjection of Ang II. The injection of SR141716A accessed NTS neuronal cell bodies as well as presynaptic vagal afferents and other terminals for projecting pathways within the NTS, so it is not clear which elements mediate the effects on BRS. However, Ang II peptides
are believed to exert actions on BRS at neuronal cell fibers, glial and vascular elements in the NTS.\textsuperscript{53}

We also cannot exclude the possibility that weak inverse agonist properties of SR141716A are reflected in our present data.\textsuperscript{54} Maximal inhibition of constitutive G-protein activity by SR141716A ranged from 20-40\% at micromolar concentrations depending on brain region in rats, although effects in brainstem were not assessed.\textsuperscript{55} However, it is unclear if this property of SR141716A yields functionally significant actions \textit{in vivo}. For example, SR141716A by itself only slightly reduced neuronal discharge in rat locus coeruleus.\textsuperscript{56} In the NTS of dogs SR141716A had no effect on baseline AP or resting sympathetic BRS,\textsuperscript{39} fully consistent with our present data in SD rats. Finally, in the periphery SR141617A did not alter basal constriction of rat gracilis arterioles.\textsuperscript{27} Potential inverse agonism effects notwithstanding, the increased levels of 2-AG we detected in dorsal brainstem of (mRen2)27 rats are consistent with a competitive antagonist effect of SR141716A in NTS to improve BRS, while the impairments to BRS in ASrAOGEN rats by SR141716A are too large to attribute to an inverse agonist effect.

We investigated changes in cardiovagal BRS, MAP and HR in response to acute, site-specific CB\textsubscript{1} receptor blockade in normotensive, hypertensive, and hypotensive rat strains. Our results support our hypothesis for differential expression of brain endocannabinoid system components paralleling the trend for brain RAS expression in these animals. The novel finding that NTS CB\textsubscript{1} receptor blockade produces opposite effects in rats over- or under-expressing components of the brain RAS may have implications for understanding the mechanisms of autonomic reflex control of HR and blood pressure in pathological conditions that are in part dependent on an activated brain
RAS, such as hypertension. Effects of chronic peripheral or central CB₁ receptor blockade will need to be studied to further evaluate the role of endocannabinoid-mediated impairments to BRS in pathophysiologies associated with elevated or diminished circulating, cerebrospinal fluid, or brain tissue RAS.
Perspectives

The impairment of BRS for control of heart rate, an index of vagus nerve function, often precedes the onset of hypertension and stroke\textsuperscript{3} and is a common feature of cardiovascular risk factors, including obesity and aging.\textsuperscript{57} Identifying factors that modulate baroreflex function may therefore be important for understanding predisposition or progression of these negative conditions. The present data suggest an upregulated brain endocannabinoid system in Ang II-dependent hypertension may contribute to the impaired BRS typical of these conditions, and that blockade of CB\textsubscript{1} receptors improves BRS. Therefore, CB\textsubscript{1} receptor-mediated impairments in BRS may contribute to the progression of increasing AP in populations with elevated Ang II or endocannabinoid production. The positive metabolic effects of chronic systemic CB\textsubscript{1} receptor blockade in obese or diabetic humans and animals are well known.\textsuperscript{58} The results of our present study suggest that chronic systemic administration of a CB\textsubscript{1} receptor antagonist may have additional, positive autonomic or cardiovascular effects in hypertension accompanied by impaired metabolic function. Understanding the consequences and mechanisms of an upregulated endocannabinoid system in hypertension will be important for therapeutic targeting of all features of cardiovascular disease.
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Disclosures

None.
References


58. Van Gaal LF, Rissanen AM, Scheen AJ, Ziegler O, Rossner S. Effects of the cannabinoid-1 receptor blocker rimonabant on weight reduction and

**Novelty and Significance: 1) What Is New, 2) What Is Relevant?**

**What Is New?**

- Blockade of NTS CB₁ receptors produces opposite effects on baroreflex sensitivity for control of heart rate in animals with differential expression of the brain RAS.
- Transgenic (mRen2)27 rats have increased production of 2-AG in dorsal medulla compared to ASrAOGEN rats; ASrAOGEN rats have decreased CB₁ receptor gene expression relative to Sprague-Dawley and (mRen2)27 rats.

**What Is Relevant?**

- Results indicate novel mechanisms for preservation of baroreflex function involving the endocannabinoid system in conditions associated with altered brain RAS activity.

**Summary**

Altered brain RAS activity is associated with differential expression and function of the dorsal medullary endocannabinoid system with respect to blood pressure regulation. Increased endocannabinoid production in this region may maintain impaired resting baroreflex sensitivity in conditions associated with upregulated brain RAS expression.
CHAPTER THREE

ALTERATIONS IN THE MEDULLARY ENDOCANNABINOID SYSTEM CONTRIBUTE TO AGE-RELATED IMPAIRMENT OF BAROREFLEX SENSITIVITY

By

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Abstract

As they age, Sprague-Dawley (SD) rats develop elevated systolic blood pressure associated with impaired baroreflex sensitivity (BRS) for control of heart rate and metabolic dysfunction. An imbalance in the angiotensin (Ang) peptides Ang II and Ang-(1-7) in the brain solitary tract nucleus (NTS) also emerges with age and contributes to the age-related decline in BRS. We previously demonstrated in young (mRen2)27 hypertensive rats featuring low medullary Ang-(1-7) relative to Ang II that impaired BRS in this strain is restored by CB₁ cannabinoid receptor blockade in the NTS, consistent with elevated content of the endocannabinoid 2-arachidonoylglycerol (2-AG) in dorsal medulla relative to SD rats. We now report that in older SD rats, dorsal medullary 2-AG levels were two-fold higher at 70 versus 15 weeks of age (3.89 ± 0.66 vs. 1.85 ± 0.27 ng/mg tissue; P < 0.05; n = 4-6). Furthermore, relative expression of CB₁ receptor mRNA was significantly lower in aged rats, while CB₂ mRNA was significantly higher. Microinjection of the CB₁ receptor antagonist SR141716A (36 pmol) into the NTS of older SD rats normalized BRS only in animals that exhibited impaired baseline BRS relative to younger animals (0.56 ± 0.06 ms/mmHg baseline vs. 1.06 ± 0.05 ms/mmHg after 60 min; P < 0.05; n = 4). In contrast, there was no effect of SR141716A in older SD rats with normal baseline BRS (1.14 ± 0.17 ms/mmHg baseline vs. 1.29 ± 0.23 ms/mmHg after 60 min; n = 4), consistent with previous observations in younger SD rats. Therefore, the present study provides evidence for altered endocannabinoid tone within the NTS of older SD rats that may contribute to age-related impairment of BRS.
Introduction

Aging is associated with changes in autonomic regulation of blood pressure that may facilitate the progression or maintenance of hypertension and related diseases.\(^1\) An imbalance in the autonomic nervous system, typically in which the sympathetic nervous system predominates over the parasympathetic, is a primary cause of increased systolic blood pressure in aged individuals with hypertension.\(^2\) Declining baroreflex sensitivity (BRS) for control of heart rate (HR), an index of vagus nerve function and general mortality,\(^3\) is permissive toward reduced heart rate variability, increased sympathetic outflow, and impaired ability to correct subsequent elevations in blood pressure during aging.\(^4\) Therefore, identification of central nervous system factors that contribute to age-related reductions in BRS is required to gain a greater understanding of the development of hypertension in the worldwide aging population.

Alterations in the brain endocannabinoid system that accompany age-related cognitive decline,\(^5\) cardiovascular diseases and metabolic disorders\(^6\) may contribute to the modulation of baroreflex function during aging. The endocannabinoid system is a central and peripheral cellular signaling system that comprises the endogenous cannabinoids anandamide and 2-arachidonoylglycerol (2-AG), their G protein-coupled CB\(_1\) and CB\(_2\) receptor subtypes, and enzymes regulating their biosynthesis and degradation. Upregulated endocannabinoid tone is reported in hypertension and its metabolic risk factors,\(^7,\,8\) suggesting a pathogenic or compensatory role in these negative conditions.

Mounting evidence links upregulated endocannabinoid tone with imbalances in the two active peptides of the renin-angiotensin system (RAS), angiotensin (Ang) II and Ang-(1-7).\(^13,\,14\) Ang II exerts a widespread influence over the sympathetic nervous
system and impairs BRS during aging, and may stimulate production of endocannabinoids through actions at Ang II type 1 (AT1) receptors. We previously demonstrated that blockade of CB1 receptors within the solitary tract nucleus (NTS) of the dorsomedial medulla, the primary site for termination of baroreceptor afferent neurons, improves the blunted BRS for control of HR in young adult (mRen2)27 rats with Ang II-dependent hypertension, with no effect in normotensive Sprague-Dawley (SD) rats with normal baroreflex function. The impaired BRS in (mRen2)27 rats is also associated with loss of Ang-(1-7) facilitation of the BRS, resembling the brain RAS profile of aged SD rats. To date there has been no investigation into the role of the NTS endocannabinoid system in impaired BRS associated with aging.

In the present study, we evaluate the effect of the CB1 receptor antagonist SR141716A on BRS for control of HR when microinjected into the NTS of older SD rats. We also employ mass spectrometry to measure levels of dorsal medullary endocannabinoids, and RT-qPCR to measure mRNA levels of cannabinoid receptors (CB1 and CB2), and cannabinoid receptor interacting protein (CRIP)1a in the NTS of younger and older SD rats. Based on evidence for decreased NTS Ang-(1-7) tone in aging and the association between augmented Ang II and endocannabinoid tone in hypertensive rats with impaired BRS, we hypothesized a similar trend for endocannabinoid levels in aged rats. We now provide functional and biochemical evidence that components of the endocannabinoid system within the NTS change during normal aging and may contribute to age-related declining baroreflex function.
Methods

Animals

Experiments were performed in 15-, 25-, and 60- to 78-week-old male Hannover SD rats obtained from the Hypertension & Vascular Research Center colony at Wake Forest School of Medicine. Rats were housed two-per-cage in a temperature- and humidity-controlled room that maintained a 12-hour light/dark cycle (lights on at 06:00), with free access to food (standard chow) and water. All procedures were approved by the Institutional Animal Care and Use Committee.

Surgical Procedures and Hemodynamic Measures

As described previously, aged SD rats (60- to 69-weeks-old; mean age = 64 ± 1 weeks for all rats; N = 8) were anesthetized with combination urethane-chloralose (750 and 35 mg/kg, respectively) via intraperitoneal injections with supplemental doses given intravenously as needed (diluted to 3:7 with saline). Polyethylene catheters (Clay Adams) were inserted into the femoral artery and vein, with the venous catheter positioned near the right atrium. Rats were then placed in a stereotaxic frame with the head tilted at a 45° downward angle for surgical exposure of the dorsal medulla, and were provided a mixture of room air and oxygen to breathe. A 30 minute resting and equilibration period was allowed before testing began. Pulsatile arterial pressure (AP) and mean AP (MAP) were recorded by data acquisition software (AcqKnowledge version 3.8.1, BIOPAC Systems, Inc., Goleta, CA), and HR was calculated from the AP wave. After obtaining stable measures of MAP and HR, baseline BRS was established by sequential bolus intravenous injection of 3 doses (2, 5 and 10 μg/kg in 0.9% NaCl) of phenylephrine (PE) to determine the bradycardic BRS response for increases in AP. BRS
for bradycardia was defined as the slope of the relationship between changes in MAP (ΔMAP; mmHg) and the pulse interval (ΔPI; ms) generated from the 3 doses of PE (mean $R^2 = 0.92$ for all animals) in units of ms/mmHg. Reflex testing was repeated 60 minutes after bilateral microinjection of the CB$_1$ receptor-selective antagonist SR141716A (obtained from the National Institute on Drug Abuse) into the NTS, and was completed within 15 minutes, with each rat serving as its own control. The doses and timecourse of the study were chosen based on previous experience with SR141716A microinjections in our laboratory$^{12, 13}$ in which the effect of the drug on BRS was immediate and sustained over 60 minutes without changing resting MAP or HR.

**NTS Microinjections**

SR141716A (36 pmol in a 120 nL volume of 10% DMSO in artificial cerebrospinal fluid vehicle) was bilaterally microinjected into the NTS (0.4 mm rostral, 0.4 mm lateral to the calamus scriptorius [caudal tip of the area postrema], and 0.4 mm below the dorsal surface) using a glass micropipette connected to a hand-held syringe, as reported previously.$^{15}$ Previous studies in older SD rats from a similar age range in our laboratory revealed NTS microinjection of the vehicle solution has no significant effect on evoked BRS, MAP or HR at any timepoint studied.$^{15, 17}$ At the end of testing, brains were removed, frozen, and sectioned (30 μm) for localization of microinjection sites. Only data from injections within the medial NTS at the rostrocaudal level -13.3 to -14.0 mm caudal to bregma were used in the analysis.

**Mass Spectrometry Detection of Anandamide and 2-AG**

*A detailed protocol can be found in Chapter Two of this dissertation.* Medullary tissue was obtained from separate age groups of naïve SD rats: 15-, 25-, and 67- to 78-weeks-
old (mean age of older animals = 70 ± 2 weeks; n = 4-6; P < 0.05 vs. age of rats utilized in the microinjection studies). Brains were removed and frozen on dry ice for excision of 3-mm³ dorsal medullary sections. For analysis of the endocannabinoids anandamide and 2-AG, an extraction technique was developed for rat medulla tissue as previously described. Study samples, quality control samples, and standards (targeted analysis) were processed using automated liquid handling to ensure the reproducibility and consistency of the sample preparation method. Sample content of anandamide or 2-AG is expressed as ng analyte per mg tissue wet weight.

**Quantification of CB₁, CB₂, and CRIP1α mRNA**

RT-qPCR was used to measure mRNA levels of various effector components of the brain endocannabinoid system, including CB₁ and CB₂ receptor, and CRIP1α, in dorsal medullary tissue from the same 15- and 70-week-old rats in which anandamide and 2-AG were measured. Brains were removed and frozen on dry ice as described above. Isolated RNA from excised tissue was assayed for concentration and stability. As described previously,¹⁵ total RNA (1 µg) was reverse transcribed using AMV reverse transcriptase in a 20-µL reaction mixture containing deoxyribonucleotides, random hexamers, and RNase inhibitor in reverse-transcriptase buffer. For RT-qPCR, 2 µL of the resultant cDNA was added to TaqMan Universal PCR Master Mix with the appropriate gene-specific primer/probe set for CB₁ and CB₂ receptors, and CRIP1α (Applied Biosystems), and amplification performed. All reactions were performed in triplicate. 18S ribosomal RNA served as the internal control. Results were quantified as Ct values, in which Ct is the threshold cycle of PCR at which an amplified product is first detected, and was defined as relative gene expression (ratio of target:control).
Analysis of Data

Values are presented as mean ± SEM. Comparisons of changes in BRS from baseline in response to SR141716A in older SD rats were analyzed by Student’s paired t-test. Multiple regression analysis was performed on BRS scatterplots to determine slopes of fit lines. One-way ANOVA and post-hoc Tukey multiple comparisons were used to analyze differences in medullary endocannabinoid content between age groups. Differences in relative mRNA expression between young and older rats were analyzed by unpaired t-tests. The criterion for statistical significance was P < 0.05. Statistical tests were performed using Prism 5.0 (GraphPad Software, San Diego, CA).
Results

CB₁ Receptor Blockade in the NTS of Aged SD Rats: BRS for Control of HR, MAP, HR, and MAP Responsiveness

In 60- to 69-week-old SD rats used in our study for assessment of cardiovagal baroreflex function, we divided animals into two groups based on baseline BRS values. One group of animals exhibited normal baseline BRS for control of HR similar to younger SD rats¹⁵ whereas the others exhibited lower baseline BRS typical of hypertensive rats in our laboratory¹⁴ (1.14 ± 0.17 ms/mmHg Normal vs. 0.56 ± 0.06 ms/mmHg Impaired; P < 0.05; n = 4; Figure 3-1). The cutoff for assignment was 0.80 ms/mmHg and the mean ages of the Normal and Impaired baseline BRS groups were both 64 ± 1 weeks (Table 3-1). There was no effect of SR141716A (36 pmol) microinjection in aged SD rats with normal baseline BRS (Figure 3-2A and 3-2B). However, the CB₁ antagonist improved BRS by approximately 89% to 1.06 ± 0.05 ms/mmHg in aged SD rats with impaired baseline BRS (P < 0.05; Figure 3-2C and 3-2D). There was a significant negative correlation between baseline BRS and change in BRS after SR141716A microinjection ($R^2 = 0.54$; P < 0.05; Figure 3-2E).

There were no differences in baseline MAP or HR between Normal and Impaired baseline BRS groups, nor were values significantly changed by NTS microinjection of SR141716A after 60 minutes (Table 3-1). In addition, there were no differences in MAP responsiveness to intravenous PE injections during reflex testing by SR141716A in either group (Figure 3-3).
Table 3-1. Mean Age and Values of MAP and HR in Response to NTS Microinjection of SR141716A

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Age (weeks)</th>
<th>MAP (mmHg)</th>
<th>HR (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>64 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Values at 60 minutes</td>
<td>89 ± 8</td>
</tr>
</tbody>
</table>

Values are mean ± SEM and represent MAP and HR values at baseline and 60 minutes after NTS microinjection of 36 pmol of SR141716A; MAP = mean arterial pressure; HR = heart rate; n = 4 both groups.
Figure 3-2. Effect of NTS Microinjection of SR141716A (36 pmol) in Aged SD Rats with Normal or Impaired Baseline BRS for Control of HR. A-B, SR141716A had no effect in aged SD rats with normal baseline BRS. C, In aged SD rats with impaired baseline BRS, SR141716A significantly improved BRS to a level comparable with normal baseline BRS. D, SR141716A increased the slope of the BRS regression line in rats with impaired baseline BRS (0.50 ± 0.11 ms/mmHg baseline; 1.00 ± 0.13 ms/mmHg 60 minutes after 36 pmol of SR141716A; P < 0.01; \( R^2 = 0.67 \) to 0.87 for pooled data). E, Magnitude of SR141716A effect on BRS was negatively associated with baseline BRS among all animals (\( R^2 = 0.54; P < 0.05; N = 8 \)). *P < 0.05 vs. baseline.
Figure 3-3. MAP Responsiveness to Intravenous PE in Aged SD Rats with Normal or Impaired Baseline BRS, Before and After NTS Microinjection of SR141716A

A. There were no differences in MAP responsiveness between Normal and Impaired BRS groups of aged SD rats at baseline reflex testing. B-C. There was not a significant change 60 minutes after NTS microinjection of SR141716A in either group (n = 4 per group).
Dorsal Medullary 2-AG and Anandamide Content in Young Adult, Middle Aged and Older SD Rats

Levels of 2-AG were lowest in 15-week-old and highest in 70-week-old SD rats (1.17 ± 0.09 vs. 3.89 ± 0.66 ng/mg tissue, respectively; P < 0.001; n = 4; Figure 3-4A). Middle aged SD rats (25 weeks) had intermediate medullary 2-AG content (3.13 ± 0.46 ng/mg tissue; n = 4), falling just short of statistical significance compared to 15-week-old rats (P = 0.052; Figure 3-4A). Of note, the relative 2-AG levels parallel the trend in resting conscious MAP and are inversely related to the baseline BRS for control of HR reported across the SD rat lifespan. In contrast, dorsal medullary anandamide was found in levels 1000-fold lower than 2-AG and there were no significant differences among age groups (Figure 3-4B).

Figure 3-4. 2-AG and Anandamide Content in the Dorsal Medulla of 15-, 25- and 70-Week-Old SD Rats. A, Mass spectrometry revealed levels of 2-AG were significantly higher in the dorsal medullary tissue of 70-week-old relative to 15-week-old SD rats. In addition, 2-AG levels trended higher in 25-week-old relative to 15-week-old rats (P = 0.052). B, Anandamide was detected at levels 1000-fold lower than 2-AG in the same tissue samples, and there were no significant differences among age groups. *P < 0.05 vs. 15 weeks; n = 4-6 all groups.
CB₁, CB₂ and CRIP1a mRNA in Young and Older SD Rats

CB₁ receptor mRNA was approximately 0.45-fold lower in the dorsal medulla of 70-week-old SD rats relative to 15-week-old rats (P < 0.001; n = 5-6; Figure 3-5A), while medullary CB₂ receptor mRNA was approximately 0.73-fold higher in the older rats compared to young rats (P < 0.05; n = 5-6; Figure 3-5B). There was no difference in relative mRNA levels of CRIP1a between the same age groups (Figure 3-5C).

Figure 3-5. Expression of CB₁, CB₂ and CRIP1a mRNA in the Dorsal Medulla of 15- and 70-Week-Old SD Rats. A, RT-qPCR determined that relative gene expression of CB₁ receptor was significantly lower in dorsal medullary tissue of 70- versus 15-week-old SD rats. B, Relative gene expression of CB₂ receptor was significantly higher in the same tissue of 70- versus 15-week-old rats. C, There was not a significant difference in relative gene expression of CRIP1a in the dorsal medulla between 15- and 70-week-old rats. *P < 0.05 vs. 15 weeks; ***P < 0.001; n = 5-6 all groups.
Discussion

In this study we report the following: (1) blockade of CB₁ receptors in the NTS of aged SD rats normalizes cardiovagal BRS for control of HR only in animals that exhibit age-related impairment in baseline BRS but has no effect in aged animals with normal BRS; (2) 2-AG content in the dorsal medulla increases over the lifespan of SD rats; and (3) aging in SD rats is associated with significantly decreased CB₁ receptor mRNA expression and significantly increased CB₂ receptor mRNA expression in the dorsal medulla relative to younger animals. Collectively, these results suggest that alterations in the components of the dorsal medullary endocannabinoid system may contribute to age-related decline in baroreflex function.

Aging, independent of resting blood pressure or weight gain, is associated with physiological changes that can reduce baroreflex function over the lifespan of humans and animals. Progressive autonomic imbalance with blunted sympathetic or parasympathetic reflex control over the heart and vasculature is accepted as a primary contributor to elevated blood pressure during aging. Several factors may play a major role in the development of age-related autonomic dysfunction, including the brain renin-angiotensin system (RAS), and metabolic hormones insulin and leptin. The balance of factors involved in impaired baroreflex function during aging remains unclear. However, reported signaling interactions between the endocannabinoid system and the RAS, insulin and leptin may illuminate some of the mechanisms underlying age-related reductions in BRS for control of HR.

The RAS peptides Ang II and Ang-(1-7) have opposite effects on baroreflex modulation, with Ang II in the NTS reducing and Ang-(1-7) facilitating BRS, but the
provenance of alterations in the components of the RAS associated with aging are unknown. AT1 receptor mRNA in rat brain either declines with age or does not change, and Ang-(1-7) mas receptor mRNA expression is no different in older versus younger rats. However, a shift in balance between the content or actions of the counterbalancing Ang peptides occurs during aging that results in predominance of Ang II tone in the NTS, leading to age-related impairment of BRS. This is illustrated by previous studies in our laboratory in which AT1 blockade in the NTS by candesartan improved BRS for control of HR in both young and older SD rats, but mas receptor blockade by d-Ala\textsubscript{7}-Ang-(1-7) following candesartan treatment impaired BRS only in young rats. The same study also reported that mRNA expression of neprilysin, an endopeptidase that cleaves angiotensinogen to form Ang-(1-7), was reduced in dorsal medulla of older rats, in line with earlier reports of decreased neprilysin activity in forebrain and plasma of older animals. Collectively, the role of the BRS-enhancing peptide Ang-(1-7) in the tonic regulation of baroreflex function in the NTS is abrogated via downregulated formation to the BRS-attenuating peptide Ang II during aging.

We previously reported increased dorsal medullary 2-AG content in young transgenic (mRen2)27 hypertensive rats, which feature upregulated brain Ang II tone with markedly impaired BRS for control of HR, compared to young SD rats and transgenic Ang-deficient ASrAOGEN rats with enhanced baseline BRS. The dorsal medulla of aged SD rats therefore represents the second in vivo setting associated with increased Ang II-to-Ang-(1-7) RAS imbalance with impaired BRS in which we found increased levels of 2-AG. Functional data from our current study support our hypothesis that enhanced NTS endocannabinoid tone contributes to defective baroreflex
function in aging because blockade of NTS CB$_1$ receptors in aged SD rats normalized BRS for control of HR only in animals with impaired baseline BRS; there was no effect of NTS CB$_1$ blockade in aged rats with preserved BRS. These results are similar to our previous observations that CB$_1$ blockade in the NTS of younger SD rats had no effect on BRS but dose-dependently improved BRS in (mRen2)27 rats. We further previously demonstrated a dose-related biphasic effect of NTS CB$_1$ receptor activation on BRS, in which low doses of a CB$_1$ agonist enhanced whereas a high dose reduced BRS in young SD rats, revealing that elevated CB$_1$ receptor signaling in the NTS can impair BRS in healthy animals. Half of the aged animals used in the microinjection experiments of our current study had normal baseline BRS whereas the others exhibited impaired BRS at baseline. This may be a reflection of the younger age range of rats used in our current study for the microinjection experiments compared to previous studies in our laboratory, as well as those exhibiting elevated 2-AG. Therefore, rats from our current microinjection studies may have been aged close to the transitional period when age-related impairment in BRS first manifests.

Evidence of signaling interactions between the endocannabinoid system and RAS is mounting and may be a potential mechanism of action for the effects of NTS CB$_1$ receptor blockade on blunted BRS for control of HR associated with aging. In addition to direct endocannabinoid-RAS interactions in the NTS, it is conceivable that CB$_1$ receptors may facilitate the AT1 receptor-mediated activation of sympathetic baroreflex pathways from the hypothalamus by disinhibition of hypothalamic neurons. Although our current study does not directly address this intriguing interaction, it
would be interesting to investigate whether co-microinjection of CB₁ and AT1 or mas ligands in the NTS yields additive or synergistic effects on BRS.

In addition to the RAS, endocannabinoids are also known to interact with central insulin^{22} and leptin^{23} signaling pathways. Insulin resistance in the periphery and central nervous system is a cardinal risk factor for age-related diseases including Alzheimer’s dementia, obesity, type II diabetes, and cardiovascular disease.^{37} Direct CB₁ receptor-mediated inhibition of insulin receptor signaling has been shown in the periphery,^{38} and endocannabinoids modulate insulin signaling pathways in the brain.^{22} Defective leptin signal transduction in the brain is associated with aging and age-related obesity,^{39} and accompanies overactive endocannabinoid signaling independent of obesity or aging.^{23} Importantly, deficient central leptin receptor signaling during aging directly influences CB₁ receptor mRNA expression in the dorsal medulla.^{40} Both insulin^{41} and leptin^{42} decrease BRS for control of HR through actions in the NTS, so it is certainly possible that endocannabinoids may interact with either hormone to suppress BRS in hypertension or aging. Other factors involved in BRS modulation at the level of the dorsal medulla during aging and known to interact with the endocannabinoid system include serotonin,^{43} catecholamines,^{44} TRPV1 ion channels,^{45} and the inhibitory neurotransmitter γ-aminobutyric acid (GABA).^{46} We cannot discount that endocannabinoid interactions with any of these factors are reflected in our present functional data, particularly interactions with GABA because GABA_A receptor-mediated inhibitory responses in neurons become less sensitive to cannabinoids with age.^{47}

Expression of CB₁ receptors in dorsal medullary sites along the baroreflex arc, including in terminals of nodose ganglion vagal afferent neurons,^{48} the NTS,^{49} and dorsal
motor nucleus,\textsuperscript{50} has been previously documented in younger rats. Reduced CB\textsubscript{1} mRNA in this region is consistent with increased 2-AG production, although we did not previously detect a difference in CB\textsubscript{1} mRNA expression in young (mRen2)27 rats that also exhibited higher medullary 2-AG relative to SD rats.\textsuperscript{51} In contrast to receptor mapping studies over lifespan in humans, which find no changes in CB\textsubscript{2} receptor distribution with age in healthy individuals,\textsuperscript{52} we report that dorsal medullary CB\textsubscript{2} mRNA is increased in aged relative to younger SD rats. Peripheral expression of CB\textsubscript{2} receptors is upregulated in atherosclerotic and ischemic animals, and their activation confers cardioprotective effects in these settings.\textsuperscript{53, 54} The relevance of the CB\textsubscript{2} receptor to autonomic, cardiovascular, and metabolic homeostasis is still emerging,\textsuperscript{55} however, and less is known about its role in aging. Furthermore, the presence of CB\textsubscript{2} receptors on neurons in this region is disputed because previous studies, including at least one that utilized RT-qPCR, failed to detect evidence of CB\textsubscript{2} receptors in the dorsal medulla.\textsuperscript{50, 56} Nevertheless, on the basis of our own data we cannot exclude a role for upregulated CB\textsubscript{2} receptor expression in the modulation of cardiovagal BRS in aged rats. However, our current study examines the functional role of only CB\textsubscript{1} receptors in age-related BRS impairment because SR141716A is a selective antagonist of CB\textsubscript{1} that is not reported to interact with any other receptors, including CB\textsubscript{2}.\textsuperscript{57}

Peripheral functional or structural changes in the vasculature associated with aging likely further contributes to diminished BRS.\textsuperscript{58} Older SD rats have an attenuated vascular response to intravenous PE, an index of sympathetic activity at vascular $\alpha_1$-adrenergic receptors, as well as a lower resting HR compared to younger rats.\textsuperscript{15} The attenuated pressor response to PE in older rats may elicit inadequate carotid sinus stretch,
which in turn may reduce baroreceptor afferent input to the NTS to yield reduced BRS. Lower HR in aging may be associated with altered cardiac pacemaker cell function or density or responsiveness of cardiomyocyte β-adrenergic receptors, both of which would reduce BRS for control of HR. The extent to which either of these factors is reflected in animals with impaired baseline BRS in our current study is unknown, although normalized BRS following NTS CB₁ receptor blockade in these animals suggests their contribution is minimal.

We did not evaluate changes in the sympathetically-mediated tachycardic BRS before and after NTS CB₁ blockade, so we do not know if the endocannabinoid system selectively influences the parasympathetic bradycardic over the tachycardic BRS for control of HR in aged SD rats. However, NTS microinjection of SR141716A did not alter depressor or bradycardic responses to intravenous phenylbiguanide while assessing cardiac chemosensitive vagal fiber activation (CVA), nor were there differences in CVA responses between normal and impaired BRS rat groups at baseline (Figure 3-6). Responsiveness of carotid chemoreceptors can be either attenuated or enhanced by Ang II actions in the brain or periphery. Thus, signaling differences in central and peripheral autonomic pathways may be reflected in the responses of animals used in our study. However, unaltered CVA responses after NTS microinjection of SR141716A indicates specificity of actions on BRS because these responses are mediated by sensory inputs that converge with baroreceptor inputs in the NTS.

At present, the actions of the endocannabinoid system during aging are understudied relative to its body of literature. An understanding of how the endocannabinoid system contributes to age-related cardiovascular diseases is lacking.
However, evidence we present for altered endocannabinoid tone in the dorsal medulla of aged SD rats suggests that upregulated endocannabinoid signaling in this region contributes to impaired cardiovagal BRS for control of HR during aging. More research is necessary to determine if endocannabinoids influence BRS via direct regulation of NTS neurotransmitter pathways or indirectly through interactions with other autonomic regulatory systems such as the brain RAS.

![Graphs showing effects of chemoreflex activation on MAP and HR induced by intravenous PBG in aged SD rats.](image)

**Figure 3-6. Effects of Chemoreflex Activation on MAP and HR Induced by Intravenous PBG in Aged SD Rats**

A, No difference in depressor or bradycardic responses to CVA between aged SD rats with normal or impaired baseline BRS. B-C, No changes in either group following NTS microinjection of SR141716A (n = 3-4 all groups).
Perspectives

Baroreflex function declines with age in healthy human populations, which can contribute to the self-perpetuating progression of hypertension target organ damage and other cardiovascular diseases. The importance of addressing blunted BRS during treatment for hypertension is now recognized, creating the need for research into factors that may influence BRS. Blockade of the endocannabinoid system in conditions associated with upregulated RAS activity, including in hypertension, obesity, and during aging, with co-administration with a RAS inhibitor may yield positive synergistic effects in these conditions, with a concomitant positive influence over autonomic reflexes. If further research uncovers more evidence for endocannabinoid-mediated contributions to age-related pathologies, our current study may advance support for the endocannabinoid system as a therapeutic target in older populations with impaired cardiovagal BRS for control of HR.
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Disclosures

None.
References


Novelty and Significance: 1) What Is New, 2) What Is Relevant?

What Is New?

- Blockade of NTS CB₁ receptors in aged Sprague-Dawley rats enhances the parasympathetic baroreflex sensitivity only in rats that exhibit impaired baseline baroreflex function.
- Older Sprague-Dawley rats have increased production of 2-AG and altered cannabinoid receptor expression in dorsal medulla compared to younger animals.

What Is Relevant?

- Results indicate novel mechanisms for preservation of baroreflex function involving the endocannabinoid system during normal aging.

Summary

Normal aging is associated with upregulated expression and tone of the dorsal medullary endocannabinoid system with respect to central blood pressure regulation. Increased endocannabinoid production in this region may contribute to impaired resting baroreflex sensitivity in older rats.
CHAPTER FOUR

ACUTE AND CHRONIC SYSTEMIC CB₁ CANNABINOID RECEPTOR BLOCKADE IMPROVES BLOOD PRESSURE REGULATION AND METABOLIC PROFILE IN HYPERTENSIVE (mRen2)27 RATS

By

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Short title: CB₁ Receptor Blockade in Hypertension

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Abstract

We investigated acute and chronic effects of CB₁ cannabinoid receptor blockade in renin-angiotensin system-dependent hypertension using rimonabant (SR141716A), an orally active antagonist with central and peripheral actions. In transgenic (mRen2)27 rats, a model of angiotensin II-dependent hypertension with increased body mass and insulin resistance, acute systemic blockade of CB₁ receptors significantly reduced blood pressure within 90 minutes but had no effect in Sprague-Dawley rats. No changes in metabolic hormones occurred with the acute treatment. During chronic CB₁ receptor blockade, (mRen2)27 rats received daily oral administration of SR141716A (10 mg/kg/day) for 28 days. Systolic blood pressure was significantly reduced within 24 hours, and at Day 21 of treatment values were 173 mmHg in vehicle- vs. 149 mmHg in drug-treated rats (P < 0.01). This accompanied lower cumulative weight gain (22 vs. 42 g vehicle; P < 0.001), fat mass (2.0 vs. 2.9% of body weight; P < 0.05), and serum leptin (2.8 vs. 6.0 ng/mL; P < 0.05) and insulin (1.0 vs. 1.9 ng/mL; P < 0.01), without sustained changes in food or water consumption. Conscious hemodynamic recordings indicate two-fold increases occurred in spontaneous baroreflex sensitivity (P < 0.05) and heart rate variability (P < 0.01), measures of cardiac vagal tone. The beneficial actions of CB₁ receptor blockade in (mRen2)27 rats support the interpretation that an upregulated endocannabinoid system contributes to hypertension and impaired autonomic function in this angiotensin II-dependent model. We conclude that systemic CB₁ receptor blockade may be an effective therapy for angiotensin II-dependent hypertension and associated metabolic syndrome.
Introduction

Hypertension, diagnosed when arterial pressure (AP) exceeds 140/90 mmHg, is the single most important risk factor for the development of cardiovascular disease and currently affects 1 in 3 U.S. adults.¹ While the mechanisms involved in the development of hypertension are still not fully understood, the widespread use of medications that inhibit the formation of the peptide hormone angiotensin (Ang) II or its actions at the ubiquitously expressed Ang II type 1 (AT1) receptor as first-line treatments implicates disturbances in the renin-angiotensin system (RAS) in the maintenance of the disease.² RAS blockers have a beneficial profile in hypertension associated with metabolic syndrome, unlike many antihypertensive drugs that have reduced efficacy in obese or diabetic patients³ or produce undesirable metabolic side effects.⁴ However, there is need for further research into the mechanisms linking the RAS with the metabolic syndrome in hypertension.

The central and peripheral endocannabinoid system has emerged as a target for the treatment of conditions associated with the metabolic syndrome, which include the cluster of risk factors for cardiovascular disease, obesity and insulin resistance.⁵ The endocannabinoid system comprises a spectrum of fatty acids, including the endogenous cannabinoids anandamide and 2-arachidonoylglycerol (2-AG), which exert their paracrine-mediating effects through the widely expressed G protein-coupled CB₁ and CB₂ receptor subtypes.⁶ Systemic blockade of CB₁ receptors by selective, brain-penetrating antagonists such as rimonabant (SR141716A) confers beneficial antiobesity and antidiabetic effects in humans and animals with metabolic syndrome [see Kirilly et al. (2012)⁷ for review], implicating overactivity of the endocannabinoid system in the
pathogenesis or maintenance of these conditions. The precise role of the endocannabinoid system in promoting cardiovascular diseases associated with the metabolic syndrome, including hypertension, is less clear in part due to the inability to differentiate direct effects on AP from indirect effects mediated by weight loss or normalization of metabolic factors such as insulin or leptin sensitivity.⁸

Evidence for signaling interactions between the endocannabinoid system and the pathogenic actions of Ang II is mounting. For example, Ang II transactivates CB₁ receptors by stimulating production of the endocannabinoid 2-arachidonoylglycerol (2-AG) in cell culture⁹ and in rat arteriole beds.¹⁰ Blockade of CB₁ receptors in Sprague-Dawley (SD) rats exposed to chronic ethanol treatment prevented Ang II-mediated mitogenic signaling and profibrogenic gene expression in hepatic stellate cells,¹¹ and in obese Zucker rats long-term systemic CB₁ blockade normalized the pressor effect of acutely administered Ang II.¹² However, no studies to date have investigated the effect of systemic CB₁ receptor blockade on blood pressure in animals with altered brain RAS expression.

Transgenic (mRen2)27 rats, a monogenetic model of Ang II-dependent hypertension in which the mouse Ren2 renin gene was transfected into the genome of the SD rat, have a phenotype of chronic hypertension with markedly impaired baroreflex control over heart rate (HR), increased body weight, and reduced insulin and leptin sensitivity by 16 weeks of age compared to their normotensive genetic controls.¹³⁻¹⁵ In anesthetized (mRen2)27 rats blockade of CB₁ receptors in the brain nucleus of the solitary tract (NTS) via microinjection of SR141716A dose-dependently improves baroreflex sensitivity (BRS) for control of HR,¹⁶ an index of parasympathetic vagus
nerve function often impaired in hypertension.¹⁷ These results prompted us to study the effects of acute and chronic systemic CB₁ blockade by SR141716A on blood pressure, metabolic profile and conscious baroreflex function in (mRen2)27 rats. Based on the evidence we found for an upregulated endocannabinoid system in the brain of this Ang II-dependent model of hypertension with metabolic syndrome,¹⁶ we hypothesized that chronic systemic blockade of CB₁ receptors would improve cardiometabolic function in this strain, possibly by disrupting the interactions between the endocannabinoid system and RAS.
Methods

Animals
All experiments were performed in male 15- to 20-week-old hypertensive (mRen2)27 rats or normotensive SD rats obtained from the Hypertension & Vascular Research Center colony at Wake Forest School of Medicine. Animals were housed two per cage in a humidity- and temperature-controlled room with free access to standard chow (ProLab, PMI Nutrition International, Brentwood, MO) and water. The colony maintained a 12-hour light/dark cycle (lights on at 06:00). All experimental procedures were approved by the Institutional Animal Care and Use Committee.

Experimental Protocol
Acute experiments were performed in (mRen2)27 or SD rats aged 18- to 20-weeks. Animals were trained for per os (p.o.) daily oral gavage treatment with the vehicle (0.1% Tween-80 in double-distilled water) for seven days prior to testing, and acclimated to metabolic cages (Allentown Caging Equipment, Allentown, NJ) and the tail cuff blood pressure monitoring system (NIBP-8, Columbus Instruments, Columbus, OH) 48 hours prior to testing. On the testing day, baseline systolic blood pressure (SBP) and HR (in beats per minute [bpm]) were measured by tail cuff and recorded as the average of a minimum of 10 individual readings over a period of approximately 15 minutes per animal. Animals were then treated with either SR141716A (10 mg/kg p.o.; obtained from RTI International [Durham, NC] via the National Institute on Drug Abuse; n = 5 per strain) or its vehicle (n = 5 per strain). After 90 minutes SBP and HR were reassessed in the same manner. Animals were then placed individually in metabolic cages overnight. Rats treated with SR141716A had ad libitum access to food and water, while rats treated
with vehicle were food restricted (FR) to 13 g of food, based on previous observations of the acute hypophagic effect of SR141716A in both strains in our preliminary studies and by others,\textsuperscript{18} to control for changes in food consumption that may contribute to acute differences in SBP.\textsuperscript{19} Twenty-four hours after dosing, SBP and HR were again measured. Animals were then sacrificed by decapitation and trunk blood collected for analysis of RAS components, insulin and leptin levels. All SPB recordings were obtained between 13:00 and 16:00.

Chronic experiments were performed in separate groups of (mRen2)27 rats beginning at 15 weeks of age. As in our acute studies, animals were trained for p.o. injections with daily administration of the vehicle for 7 days and were acclimated to the tail cuff apparatus and metabolic cages 48 hours prior to the commencement of testing. Baseline values for SBP, HR, and food and water consumption were obtained on Day 0 of the study when animals were 16-weeks-old. Daily oral treatment with SR141716A (10 mg/kg/day; n = 7) or vehicle (n = 8) began on Day 1 and continued for 28 days. Body weight was recorded daily through Day 25, while SPB, HR, food and water consumption, urine volume and fasting blood glucose were measured on treatment Days 7, 14 and 21 (see Figure 4-1). Overnight food and water intake and SBP were measured in subgroups of SR141716A-treated (n = 3) and vehicle-treated (n = 4) animals on Day 2 to assess acute feeding effects of systemic CB\textsubscript{1} receptor blockade. Fasting blood glucose measurements were taken using a Freestyle glucose meter (Abbott Diabetes Care Inc., Alameda, CA) from a \textasciitilde 10 µl blood sample obtained from tail pricks at least 60 minutes after tail cuff recordings, between 15:00 and 17:00. Urine was flash-frozen over dry ice on Days 0 and 21 and collected for analysis of osmolality and vasopressin content.
Day 25 animals were surgically instrumented with a catheter in the femoral artery to record conscious hemodynamic measures including BRS, HR variability (HRV) and blood pressure variability (BPV) on Day 28. After testing was completed on Day 28, animals were sacrificed by decapitation and trunk plasma and serum collected for analysis of RAS components, insulin, and leptin levels. Fat mass index was calculated from white adipose tissue collected from retroperitoneal, inguinal and epididymal stores and weighed as a percentage of total body weight. As in our acute studies, all tail cuff SBP, HR, and conscious hemodynamic recordings were obtained between 13:00 and 16:00 and approximately 90 minutes after dosing.

**Figure 4-1.** Design and timeline of experiments investigating the effect of daily systemic CB1 receptor blockade in (mRen2)27 rats.

### Surgical Procedures and Conscious Hemodynamic Measures

As described previously, rats were anesthetized under 2.5% to 4.0% isoflurane and instrumented with a femoral artery catheter on the afternoon of Day 25 of the study. Rats were allowed two days to recover during which they were gavaged with water to prevent...
dehydration in addition to receiving daily drug or vehicle treatment. Pulsatile pressure in conscious rats was acquired on Day 28 between 13:00 and 15:00 via strain gauge transducer connecting the arterial catheter to a data acquisition system (AcqKnowledge software version 3.8.1, BIOPAC Systems, Goleta, CA). HR was calculated from the arterial pressure wave. Indices of sympathovagal activity were calculated by spectral analysis of the time and frequency domains using software designed for rats (Nevrokard SA-BRS, Medistar, Houston, TX), as previously described. Consistent with the duration of recordings used in previous human and rodent studies, conscious BRS was determined from a minimum of 10 minutes of AP recordings obtained within 90 minutes of SR141716A or vehicle dosing. Conscious BRS was calculated in the time (Sequence [Seq] Up, Seq Down, and Seq All; in units of milliseconds per mmHg) and frequency (low-frequency [LF] and high-frequency [HF] α indices) domains. Time domain analysis was used to calculate differences in HRV, measured as the standard deviation of the beat-to-beat interval (SDRR) in milliseconds. BPV was measured in the time domain as the standard deviation of the mean AP (SDMAP) in mmHg.

**Biochemical Measurements in Plasma, Serum and Urine**

Plasma angiotensin peptides [Ang I, Ang II, Ang-(1-7)] were measured as previously reported. Serum angiotensin converting enzyme (ACE), insulin, leptin, and urine vasopressin were measured using radioimmunoassays specific for rats according to the manufacturer’s instructions (Linco, Santa Fe Springs, CA). Urine osmolality was measured using the 5004 Micro-Osmette osmometer (Precision Systems, Natick, MA) and expressed as milliOsmols per liter (mOsm/L).
Analysis of Data

Values are presented as mean ± SEM. Comparisons of changes in body weight, SPB, HR, food and water consumption, and fasting blood glucose over time between drug and vehicle groups were analyzed by repeated measures two-way ANOVA, with Bonferroni post-hoc comparisons made between drug and vehicle groups where appropriate. Within group comparisons were made by repeated measures one-way ANOVA, with post-hoc Tukey tests used to elucidate further comparisons between timepoints. Student’s unpaired two-tailed t-tests were used to analyze comparisons of fat mass index, biochemical measurements, conscious hemodynamic parameters on Day 28, and differences in food and water intake, SBP, and HR after Day 1 of chronic treatment in subgroups. Paired two-tailed t-tests were used to compare 24-hour changes in body weight from baseline in acute studies. The criterion for statistical significance was P < 0.05. Statistical tests were performed using Prism 5.0 (GraphPad Software, San Diego, CA).
Results

Acute systemic CB₁ receptor blockade in (mRen2)27 and SD rats

There were no significant differences in baseline SBP, HR (Figure 4-2) or body weight (Table 4-1) between treatment groups of (mRen2)27 or SD rats. In (mRen2)27 rats, p.o. injection of SR141716A (n = 5) lowered SBP by approximately 24%, from 176 ± 3 mmHg at baseline to 134 ± 3 mmHg, after 90 minutes (P < 0.001; Figure 4-2A). SPB remained lower 24 hours after SR141716A administration, with evidence of partial recovery to 146 ± 5 mmHg (P < 0.01 vs. baseline; Figure 4-2A). SR141716A in (mRen2)27 rats also caused a transient reduction in HR, which fell from 409 ± 17 bpm at baseline to 363 ± 15 bpm after 90 minutes (P < 0.05), but fully recovered within 24 hours (Figure 4-2B). Furthermore, (mRen2)27 rats treated with SR14171A consumed a similar amount of food overnight as those treated with vehicle and restricted to 13 g of food, which produced similar decreases in body weight in both groups (P < 0.01 vs. respective baseline body weights; Table 4-1). However, (mRen2)27 rats treated with SR141716A excreted significantly less urine overnight compared to those that received vehicle + FR (8 ± 1 vs. 14 ± 1 mL; P < 0.05), despite consuming similar amounts of water (Table 4-1). Vehicle did not significantly alter SBP or HR in (mRen2)27 rats after 90 minutes, nor did overnight FR change SBP or HR in (mRen2)27 rats 24 hours after receiving vehicle (Figure 4-2).

In contrast to (mRen2)27 rats, acute administration of SR141716A in SD rats did not significantly change SBP or HR after 90 minutes or 24 hours (Figure 4-2). As in (mRen2)27 rats, SD rats treated with SR141716A consumed similar quantities of food and water overnight as those who received vehicle + FR, producing similar changes in
body weight ($P < 0.05$ vs. respective baseline body weights; Table 4-1). Overnight water intake was similar between SD groups, but there was a trend for lower urine excretion in SR141716A-treated SD rats compared to vehicle + FR-treated rats ($6 \pm 1$ vs. $11 \pm 2$ mL; $P = 0.08$; Table 4-1). As with the SR141716A-treated group, SBP and HR was unaffected at 90 minutes and 24 hours in SD rats treated with vehicle + FR (Figure 4-2). There were no differences in circulating levels of RAS peptides or insulin or leptin between treatment groups in (mRen2)27 or SD rats (Table 4-1).

### Table 4-1. Acute Systemic SR141716A or Vehicle + Overnight FR in (mRen2)27 and SD Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SD Vehicle +FR</th>
<th>SD SR 10 mg/kg</th>
<th>(mRen2)27 Vehicle +FR</th>
<th>(mRen2)27 SR 10 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline Body Weight (g)</td>
<td>388 ± 12</td>
<td>406 ± 22</td>
<td>555 ± 19</td>
<td>555 ± 30</td>
</tr>
<tr>
<td>Body Weight at 24 h (g)</td>
<td>379 ± 12†</td>
<td>394 ± 19*</td>
<td>534 ± 16†</td>
<td>530 ± 27†</td>
</tr>
<tr>
<td>∆Body Weight (g)</td>
<td>-9 ± 1</td>
<td>-12 ± 3</td>
<td>-21 ± 4</td>
<td>-25 ± 5</td>
</tr>
<tr>
<td>Food Intake (g)</td>
<td>13</td>
<td>12 ± 1</td>
<td>13</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>Water Intake (mL)</td>
<td>25 ± 3</td>
<td>22 ± 1</td>
<td>28 ± 1</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>Urine Volume (mL)</td>
<td>11 ± 2</td>
<td>6 ± 1#</td>
<td>14 ± 1</td>
<td>8 ± 1#</td>
</tr>
<tr>
<td>Plasma Ang I (pg/mL)</td>
<td>139 ± 7</td>
<td>163 ± 19</td>
<td>139 ± 7</td>
<td>163 ± 19</td>
</tr>
<tr>
<td>Plasma Ang II (pg/mL)</td>
<td>102 ± 36</td>
<td>113 ± 43</td>
<td>102 ± 36</td>
<td>113 ± 43</td>
</tr>
<tr>
<td>Plasma Ang-(1-7) (pg/mL)</td>
<td>67 ± 10</td>
<td>58 ± 12</td>
<td>67 ± 10</td>
<td>58 ± 12</td>
</tr>
<tr>
<td>Serum ACE (ng/mL)</td>
<td>35 ± 8</td>
<td>31 ± 10</td>
<td>23 ± 2</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>Serum Insulin (ng/mL)</td>
<td>2.9 ± 0.4</td>
<td>2.7 ± 0.3</td>
<td>2.9 ± 0.4</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>Serum Leptin (ng/mL)</td>
<td>5.9 ± 1.0</td>
<td>7.1 ± 1.5</td>
<td>5.9 ± 1.0</td>
<td>7.1 ± 1.5</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. SR refers to SR141716A.

*P < 0.05 vs. baseline; †P < 0.01 vs. baseline; #P < 0.05 vs. (mRen2)27 Vehicle + FR; n = 5 all groups.
Chronic systemic CB₁ receptor blockade in (mRen2)27 rats

Body weight and fat mass

There were no baseline differences in body weight between (mRen2)27 rats in the vehicle (469 ± 10 g; n = 8) and SR141716A (469 ± 21 g; n = 7) treatment groups. By Day 25 of treatment body weights in both groups had significantly increased relative to their Day 1 baselines, to 513 ± 12 g (P < 0.001) in vehicle-treated rats and 498 ± 21 g (P < 0.001) in drug-treated rats (Figure 4-3A). The body weight values on Day 25 did not significantly differ between treatment groups. However, rats treated with SR141716A had approximately 31% lower fat composition, measured as the mass of white adipose tissue as a percentage of body weight, after Day 28 than rats treated with vehicle over the duration of the study (P < 0.05; Figure 4-3B). Furthermore, SR141716A-treated rats on average gained approximately half as much weight as rats treated with vehicle through
Day 25 of treatment (44 ± 3 g vs. 22 ± 3 g; P < 0.001; Figure 4-3C). Drug-treated rats lost approximately 12 g of body weight overnight after the Day 1 dose of SR141716A (Figure 4-3C) but thereafter steadily gained weight through Day 25 of treatment. Further statistical analysis of the weight gain curves starting on Day 2 revealed a significant interaction between treatment groups with respect to cumulative daily weight gain over the treatment period (P < 0.01), indicating a lower rate of weight gain over the duration of treatment after the initial overnight weight loss in rats that received SR141716A (regression slopes after Day 1 = 1.82 ± 0.07 g/day Vehicle vs. 1.29 ± 0.09 g/day SR141716A; P < 0.0001).

**Figure 4-3.** (mRen2)27 rats treated with chronic CB₁ receptor blockade maintained similar raw body weight (A), but had significantly lower fat mass (B) and gained significantly less weight over the treatment period (C). Fat mass reflects the sum of white adipose tissue collected after animals were sacrificed on Day 28 and expressed as percent of body weight. †P < 0.001 vs. respective baseline values; *P < 0.05 vs. Vehicle group; #P < 0.01; §P < 0.0001; n = 6-8.
Food and water intake, and urine volume

There were no differences between vehicle and SR141716A treatment groups in food intake, water intake, or urine volume at baseline. Over the duration of treatment there were no sustained differences in food intake between treatment groups; however, the weight loss exhibited after Day 1 by (mRen2)27 rats treated with SR141716A was associated with a transient reduction in food intake measured in subgroups of animals (13 ± 2 g food drug-treated vs. 26 ± 1 g food vehicle-treated on Day 2; n = 3-4; P < 0.01) that fully recovered by Day 7 of treatment (Figure 4-4A), in agreement with previous reports.18, 23 There were no significant transient or sustained differences in water intake between treatment groups (Figure 4-4B). Urine volume trended lower in rats treated with SR141716A over the duration of treatment compared to vehicle-treated rats, but there was not a significant treatment effect (Figure 4-4C).
Figure 4-4. Chronic systemic CB₁ receptor blockade produced a transient reduction in food intake (A) without sustained changes, but not in water intake (B) or urine volume (C) in (mRen2)27 rats. #P < 0.01 vs. Vehicle on Day 2; n = 7-8 on Days 0, 7, 14 and 21; n = 3-4 on Day 2.

**Blood pressure, HR and RAS components**

There were no baseline differences in SBP or HR between treatment groups. On Day 7, SBP in SR141716A-treated (mRen2)27 rats was significantly reduced from a baseline of 174 ± 3 mmHg to a new value of 151 ± 3 (P < 0.01) and remained lower through Day 21 of treatment (149 ± 6 mmHg; P < 0.01 vs. baseline; Figure 4-5A). Measurements conducted in a subgroup of rats (n = 3) on Day 2 of treatment indicated that SBP was reduced within 24 hours after receiving the Day 1 injection of SR141716A (146 ± 5
mmHg; P < 0.05 vs. baseline; Figure 4-5A). There was no effect of vehicle on SBP, nor was there a significant treatment effect on HR, over the duration of treatment (Figure 4-5A and 4-5B). Furthermore, no differences between treatment groups were found in levels of the RAS components Ang I, Ang II, Ang-(1-7) or ACE analyzed from blood collected after animals were sacrificed on Day 28 of the study (Table 4-2).

Table 4-2. Chronic Systemic SR141716A or Vehicle in (mRen2)27 Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(mRen2)27 Vehicle</th>
<th>(mRen2)27 SR 10 mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Ang I (pg/mL)</td>
<td>353 ± 112</td>
<td>302 ± 52</td>
</tr>
<tr>
<td>Plasma Ang II (pg/mL)</td>
<td>71 ± 25</td>
<td>69 ± 11</td>
</tr>
<tr>
<td>Plasma Ang-(1-7) (pg/mL)</td>
<td>45 ± 7</td>
<td>46 ± 7</td>
</tr>
<tr>
<td>Serum ACE (ng/mL)</td>
<td>26 ± 2</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>Urine Vasopressin (pg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>107 ± 12</td>
<td>104 ± 16</td>
</tr>
<tr>
<td>Day 21</td>
<td>100 ± 19</td>
<td>110 ± 31</td>
</tr>
<tr>
<td>Urine Osmolality (mOsm/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1471 ± 107</td>
<td>1528 ± 77</td>
</tr>
<tr>
<td>Day 21</td>
<td>1639 ± 110</td>
<td>1809 ± 69#</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. SR refers to SR141716A.
#P < 0.01 vs. Baseline; n = 7-8 all groups.
Leptin, insulin, fasting blood glucose, and urine vasopressin and osmolality

At the end of the study, serum collected from (mRen2)27 rats that received chronic treatment with SR141716A had significantly less leptin (P < 0.05; Figure 4-6A) and insulin (P < 0.05; Figure 4-6B) compared to rats treated with vehicle. There was no effect on fasting blood glucose over the duration of the study in either group (Figure 4-6C). Furthermore, no differences in urine vasopressin levels were found between treatment groups (Table 4-2). Urine osmolality increased in both treatment groups between baseline and Day 21 of the study, reaching statistical significance in SR141716A-treated rats (P < 0.01), but there was no effect of treatment (Table 4-2).
Following 28-day chronic systemic CB₁ receptor blockade (mRen2)²⁷ rats had lower serum leptin (A) and insulin (B) levels than rats treated with vehicle, while fasting blood glucose (C) was unaffected over the duration of treatment. *P < 0.05 vs. Vehicle; #P < 0.01; n = 7-8.

Conscious baroreflex function, HRV and BPV

Conscious AP was recorded from (mRen2)²⁷ rats treated with vehicle (n = 5) or SR141716A (n = 5) on Day 28 of the study for spectral analysis of the AP and corresponding HR waves to calculate indices of sympathovagal function. Rats that received chronic systemic treatment with SR141716A displayed a two-fold greater value in overall conscious baroreflex function (Seq All; 0.82 ± 0.13 vs. 0.41 ± 0.11 ms/mmHg; P < 0.05; Figure 4-7A), with significantly greater sympathetic (Seq Down; 0.64 ± 0.05 vs. 0.39 ± 0.09 ms/mmHg; P < 0.05; Figure 4-7B) and parasympathetic (Seq Up; 0.94 ± 0.19 vs. 0.42 ± 0.11 ms/mmHg; P < 0.05; Figure 4-7C) BRS for control of HR compared to rats treated with vehicle. In addition, HRV, a measure of cardiac vagal tone, was
significantly higher in SR141716A-treated rats relative to vehicle-treated rats (4.38 ± 0.49 vs. 2.0 ± 0.26 ms; P < 0.01; Figure 4-7D). There was not a significant difference in BPV, an index of vascular sympathetic tone, between treatment groups (Figure 4-7E).

**Figure 4-7.** Chronic systemic CB₁ receptor blockade for 28 days via SR141716A in (mRen2)27 rats improved conscious sympathetic and parasympathetic BRS for control of HR (A-C) and HRV (D) compared to vehicle-treated rats, without significantly changing BPV (E). *P < 0.05 vs. Vehicle; #P < 0.01; n = 5.
Discussion

In this study, we report for the first time effects of acute and chronic systemic blockade of CB1 cannabinoid receptors on blood pressure, body weight, fat mass, feeding, serum leptin and insulin content, and conscious baroreflex function in transgenic hypertensive rats with upregulated RAS activity. Acute oral administration of the CB1-selective antagonist SR141716A significantly lowers SBP in (mRen2)27 rats but has no effect in normotensive SD control rats. The effect is immediate, occurring within 90 minutes of administration, and sustained for 24 hours. Chronic daily treatment with SR141716A maintained the SBP-lowering effect at a similar level over the duration of the study without significantly changing HR, although the acute studies suggest a modest, transient bradycardia that recovers within 24 hours may contribute to the initial reduction in SBP after 90 minutes. Acute and chronic effects of systemic CB1 receptor blockade by SR141716A are not associated with differences in levels of circulating RAS components, or urinary vasopressin compared to vehicle treatment. Our acute studies demonstrate that the transient hypophagic effect of SR141716A alone does not significantly contribute to initial reductions in blood pressure because overnight FR did not change SBP in (mRen2)27 rats treated with the vehicle. Furthermore, the reduced SBP in rats that received chronic treatment with SR141716A is associated with significantly improved sympathovagal baroreflex function after 28 days.

In addition to these beneficial hemodynamic effects, chronic systemic SR141716A treatment significantly reduces cumulative weight gain over the treatment period, and this is associated with reduced fat mass as a percentage of body weight, and lower leptin and insulin levels in (mRen2)27 rats after the 4-week study. Taken together,
data from our current study support our hypothesis that systemic blockade of central and peripheral CB$_1$ receptors has direct beneficial effects on blood pressure with a concomitant positive influence over metabolic profile in transgenic (mRen2)27 rats with Ang II-dependent hypertension and features of metabolic syndrome.

The metabolic effects of chronic systemic SR141716A administration in (mRen2)27 rats are consistent with the reported literature in humans and animals with metabolic syndrome. In obese Zucker rats, a model of obesity with leptin receptor deficiency and insulin resistance, chronic treatment with SR141716A dose-dependently reduces weight gain, fat mass, and improves insulin sensitivity. Similar effects of SR141716A accompanied by an improved serum lipid profile are found in diet-induced obese mice and in obese and diabetic patients. Although we did not directly investigate insulin sensitivity via glucose tolerance tests, lower circulating insulin levels coupled with unchanged fasting blood glucose we observed implies that insulin sensitivity improved in rats that received chronic SR141716A treatment. However, it is unclear whether the reduced insulin levels in our drug-treated rats are a direct cause or result of the decreased weight gain and fat mass associated with SR141716A treatment.

Activation of CB$_2$ receptors in pancreatic beta cells reduces insulin release while CB$_1$ receptors are restricted primarily to glucagon-secreting alpha cells. Therefore, it is conceivable that blockade of CB$_1$ receptors could produce a shift in endocannabinoid signaling within the pancreas and other organs to favor CB$_2$ over CB$_1$ receptor activation. Peripheral expression of CB$_2$ receptors is upregulated in models of cardiovascular disease including atherosclerosis and ischemia and their activation confers cardioprotective effects in these settings. Thus, there may be a role for CB$_2$ receptors in the treatment of
the RAS-associated hypertension and metabolic syndrome in (mRen2)27 rats, which remains unaddressed by our current study.

The reduced weight gain and fat mass in (mRen2)27 rats treated with SR141716A cannot be attributed to reduced food consumption because feeding was only transiently reduced and fully recovered within 7 days, in line with other descriptions of the hypophagic effect of SR141716A.\textsuperscript{12, 25} A significant statistical interaction between the vehicle and drug treatment groups further precludes differences in weight gain and fat mass from being wholly attributed to overnight weight loss associated with hypophagia experienced by SR141716A-treated rats after Day 1. Several alternative mechanisms may contribute to the beneficial metabolic actions of CB\textsubscript{1} blockade. For instance, obesity is associated with defective leptin signaling and hyperleptinemia,\textsuperscript{30} both of which are exhibited by (mRen2)27 rats\textsuperscript{14} and may contribute to increased body weight and fat mass in this strain. Defective central leptin signaling is also associated with enhanced endocannabinoid tone independent of obesity.\textsuperscript{31} Animals that received SR141716A in our study had significantly lower serum leptin levels with reduced weight gain and lower body fat after chronic treatment, suggesting improvement in leptin sensitivity or signaling.

In addition to leptin abnormalities, metabolic syndrome is associated with depressed plasma adiponectin levels\textsuperscript{32} and dyslipidemia.\textsuperscript{33} Adiponectin released from adipocytes induces fatty acid β-oxidation and increases lipoprotein lipase activity\textsuperscript{34} with central regulatory effects on energy expenditure that complement brain actions of leptin.\textsuperscript{35} Adipocyte CB\textsubscript{1} receptors directly regulate plasma levels of adiponectin,\textsuperscript{36} and SR141716A is reported to increase release of adiponectin in obese humans,\textsuperscript{37} Zucker
rats,\textsuperscript{12} and diet-induced obese mice.\textsuperscript{38} Therefore, we cannot rule out that the reduced adiposity and weight gain we observed in (mRen2)27 rats given chronic SR141716A may in part be attributed to induced adipose tissue lipolysis or increased energy expenditure through the actions of adiponectin.

Similarly, dyslipidemia in the form of elevated triglycerides is reported in (mRen2)27 rats and may contribute to defective insulin signaling in this strain.\textsuperscript{15} Hepatocytes express CB\textsubscript{1} receptors that promote fatty acid synthesis through induction of the lipogenic transcription factor SREBP-1c and its downstream target enzyme fatty acid synthase.\textsuperscript{39} Mice fed a high-fat diet had upregulated hepatic CB\textsubscript{1} receptor expression and increased anandamide levels.\textsuperscript{39} Moreover, insulin resistance associated with a high-fat diet is linked to hepatic CB\textsubscript{1} receptor-mediated synthesis of long chain ceramides.\textsuperscript{40} All of these effects are reversed or attenuated by SR141716A\textsuperscript{39} or by JD5037,\textsuperscript{40} a peripherally-restricted CB\textsubscript{1} antagonist, as was low density lipoprotein (LDL)/high density lipoprotein cholesterol ratio by SR141716A in diet-induced obese mice.\textsuperscript{23} Therefore it is likely that SR141716A in our study exerted some of its metabolic effects through actions in the liver.

A similar physiological provenance of metabolic disturbance in (mRen2)27 rats and other animal models of metabolic syndrome, likely involving upregulated endocannabinoid tone, may be inferred because of the shared effect profile of CB\textsubscript{1} receptor blockade among these different models. Dysfunctional RAS signaling may be another shared mechanism of metabolic disturbance, as chronic blockade of AT1 receptors lowers AP and improves the principal symptoms of metabolic syndrome in obese humans,\textsuperscript{41} Zucker rats,\textsuperscript{42} spontaneously hypertensive rats (SHR) made obese by
high-fat diet,\textsuperscript{43} and (mRen2)\textsuperscript{27} rats.\textsuperscript{44} In fact, mounting evidence suggests the RAS and the endocannabinoid system may actually work in tandem to promote cardiometabolic diseases through signaling interactions that may augment the pathogenic effects of AT\textsubscript{1} or CB\textsubscript{1} receptor activation. Several observations imply that blocking the endocannabinoid system can attenuate the actions of an activated RAS. For example, chronic treatment with SR\textsuperscript{141716}A significantly reduces Ang II-mediated fibrosis in mouse liver\textsuperscript{11} and the enhanced pressor response to intravenous Ang II exhibited by Zucker rats.\textsuperscript{12} Chronic SR\textsuperscript{141716}A treatment also reduces vascular expression of AT\textsubscript{1} receptors in apolipoprotein E-deficient mice.\textsuperscript{45} However, no previous studies have investigated interactive effects of CB\textsubscript{1} receptor blockade in the context of chronic Ang II-dependent hypertension.

A compelling description of potential heteromerization mechanisms between CB\textsubscript{1} and AT\textsubscript{1} receptors\textsuperscript{11} accompanying reduced fibrosis in mouse liver led us to hypothesize that CB\textsubscript{1} receptor blockade would disrupt the Ang II-dependent hypertension in (mRen2)\textsuperscript{27} rats, and results from our current study are congruent with this hypothesis. Orally administered SR\textsuperscript{141716}A reduced SBP in (mRen2)\textsuperscript{27} rats within 90 minutes by \(\approx 43\) mmHg to a level 17 mmHg (~15\%) higher than baseline SBP of SD rats, whose SPB was unaltered by SR\textsuperscript{141716}A. The fast onset of the effect precludes changes in overnight food intake or the long-term metabolic effects of SR\textsuperscript{141716}A from contributing to the reduction of SBP in the acute setting, suggesting the effect on SBP is a direct consequence of CB\textsubscript{1} antagonism. However, whether blockade of CB\textsubscript{1} receptors directly interfered with vascular or central Ang II-AT\textsubscript{1} receptor signaling, or reduced SBP through an alternative mechanism cannot be determined from our study. CB\textsubscript{1}
receptor blockade in Zucker rats is associated with reduced sympathetic activity and improved renal function,\textsuperscript{12, 46} which could contribute to reduced SBP in (mRen2)27 rats because of the Ang II-driven increased sympathetic tone\textsuperscript{47} and impaired renal function\textsuperscript{48} in this strain. Even direct interference of AT1 receptor signaling by CB\textsubscript{1} blockade may have additional indirect effects on blood pressure because inhibition of the sympathetic nervous system following RAS blockade is documented.\textsuperscript{49} The transiently slowed HR we observed 90 minutes after treatment, which may indicate enhanced cardiac vagal tone, likely contributed to an initial reduction in SBP as well.

Spectral analysis methods for measuring indices of blood pressure regulation and autonomic tone at the conclusion of the chronic study reveal significant improvement in both sympathetic (Seq Down, Seq All) and parasympathetic (Seq Up, Seq All) conscious BRS for control of HR in SR141716A-treated rats compared to those that received vehicle. These data are fully consistent with our previous studies demonstrating increased BRS in response to increases in AP, a measure of parasympathetically-mediated cardiovagal baroreflex function, after acute NTS microinjection of SR141716A in anesthetized (mRen2)27 rats.\textsuperscript{16} Similar to conscious BRS measurements, HRV was significantly higher in drug-treated compared to vehicle-treated rats, indicating increased resting vagal tone. In fact, HRV in the drug-treated rats was comparable to values of resting HRV previously reported in conscious SD rats.\textsuperscript{50} These indices of enhanced central blood pressure regulation likely contribute to the maintenance of reduced SBP during chronic treatment with SR141716A in (mRen2)27 rats, and also provide direct evidence for central effects of systemic SR141716A in these animals.
It remains unknown to what extent these central actions of SR141716A contribute to the metabolic or blood pressure effects of the chronic treatment, because distinct central and peripheral mechanisms of cardiometabolic regulation are reported in the endocannabinoid literature.\textsuperscript{51, 52} Therefore it would be interesting to study peripherally restricted CB\textsubscript{1} antagonists in a similar experimental paradigm to distinguish peripheral from central effects of CB\textsubscript{1} receptor blockade in (mRen2)27 rats. Collectively, studies from our laboratory show SR141716A improves blood pressure regulation in (mRen2)27 rats from an impaired state typical of hypertension and obesity,\textsuperscript{17} during which the endocannabinoid system and RAS are often overactive, as assessed using several indices of autonomic function.

In the cardiovascular system, endocannabinoids mediate vasodilation and cardiomyocyte relaxation through CB\textsubscript{1} receptor-specific mechanisms,\textsuperscript{53} yielding a hypotensive effect that is enhanced in both acute hypertension and in the chronic SHR model.\textsuperscript{54, 55} Indeed, intravenous anandamide normalized while SR141716A further increased AP in SHR,\textsuperscript{56} suggesting that upregulated CB\textsubscript{1} receptor tone in this model is likely compensatory rather than pathogenic in nature. The SBP-lowering effect of CB\textsubscript{1} receptor blockade in (mRen2)27 rats may therefore seem unexpected without considering potential endocannabinoid interactions with the RAS or improvement in central blood pressure control. However, the pathogenesis of hypertension in the SHR is unclear because unlike (mRen2)27 rats, SHR have a lean phenotype and normal or subnormal RAS activity.\textsuperscript{57} Furthermore, there are conflicting reports as to whether SR141716A corrects modestly elevated, sympathetically-driven SBP in Zucker rats.\textsuperscript{12, 46} These reports are further confounded by recent evidence that at least some metabolic effects of
SR141716A are mediated by increased sympathetic activation.\textsuperscript{58} Therefore, specificity of animal models should be considered carefully when interpreting the blood pressure effects of systemic cannabinoids.

Although SBP remained suppressed at a consistent level over the chronic treatment period, it is certainly possible that more slowly manifesting metabolic effects, such as the reduction in fat mass, or decreased insulin or leptin, may contribute to lower SBP in the later stages of treatment. The potential restoration of fatty acid oxidation and reduction of fatty acid synthesis by chronic CB\textsubscript{1} blockade could yield a decrease in sympathetic tone\textsuperscript{59} and thus blood pressure. Even a RAS-mediated change in SBP could conceivably be an indirect consequence of chronic CB\textsubscript{1} blockade because correction of hypercholesterolemia may downregulate AT1 receptor expression, which is correlated with plasma LDL levels in humans and animals,\textsuperscript{60} and thus reduce intrinsic responses to Ang II. The blood pressure responses to acute and chronic SR141716A administration in (mRen2)\textsuperscript{27} rats in our study are consistent with the interpretation of disrupted Ang II signaling by CB\textsubscript{1} receptor blockade,\textsuperscript{11} but further study will be needed to elucidate the precise mechanisms of this effect and to separate direct and indirect effects of CB\textsubscript{1} blockade on blood pressure in this strain.

The results of our current study, obtained after acute and chronic systemic treatment with SR141716A in hypertensive (mRen2)\textsuperscript{27} rats, contrast with the interpretation of short-term beneficial effects of CB\textsubscript{1} receptor blockade (4-14 days) on blood pressure and metabolic profile, previously attributed to decreased food consumption and other transient actions.\textsuperscript{61} However, altered levels of angiotensin peptides or metabolic hormones at various timepoints between the timeframe of our acute
and chronic studies that we did not evaluate may certainly be a significant factor in the modulation of blood pressure and metabolism by SR141716A in (mRen2)27 rats. Nevertheless, our study showcases the (mRen2)27 rat as a unique model in which to study the hemodynamic and metabolic effects of the endocannabinoid system in a setting featuring upregulated RAS activity, and also highlights the challenges of treating diseases as multifarious as hypertension.
Perspectives

Preclinical evidence for CB₁ receptor blockade as a therapeutic strategy in cardiovascular and metabolic diseases continues to mount. Recently, SR141716A was found to act synergistically with an antidiabetic insulin sensitizing agent (BGP-15) to improve insulin signaling when co-administered in Zucker rats. This result invites the possibility that a lower dose of SR141716A used in combination therapy may yield comparable efficacy to a higher stand-alone dose, thus anticipating a lower incidence of negative psychiatric side effects associated with the drug. Combination therapy with SR141716A and a RAS inhibitor may have a similar synergistic effect in hypertension, with the risk of psychiatric side effects further lessened because blockade of Ang II formation and signaling is associated with an antidepressant and anxiolytic behavioral profile. We observed centrally mediated improvements in blood pressure regulation evidenced by increased BRS and HRV. Thus, peripherally restricted CB₁ receptor antagonists may not provide maximal cardiovascular or metabolic benefits. If further research uncovers more evidence for endocannabinoid-RAS interactions contributing to hypertension or metabolic syndrome, our current study may advance support for combination CB₁-AT₁ receptor blockade as additive or synergistic in RAS-dependent hypertension with potentially mitigated side effects of both treatments.
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Disclosures

None.
References


Novelty and Significance: 1) What Is New, 2) What Is Relevant?

What Is New?

- Acute and chronic systemic CB₁ receptor blockade significantly lowers blood pressure in hypertensive (mRen2)27 rats, with a concomitant positive influence over conscious autonomic blood pressure regulation and metabolic profile.

What Is Relevant?

- Results indicate novel mechanisms for maintenance of hypertension, metabolic syndrome and impaired autonomic control of blood pressure associated with upregulation of Ang II signaling.

Summary

The (mRen2)27 rat exhibits Ang II-dependent hypertension with impaired baroreflex sensitivity and features of metabolic syndrome. We demonstrate for the first time that treatment with a CB₁ receptor antagonist improves all of these parameters in this model, suggesting a role for the endocannabinoid system in the maintenance of cardiometabolic pathologies related to overactivity of the RAS.
1. Summary of Results

Hypertension is the single biggest predictor for future heart attack, stroke or congestive heart failure\textsuperscript{1} and currently affects 1 in 3 US adults,\textsuperscript{2} a figure that is expected to rise as the population ages and as the prevalence of obesity increases.\textsuperscript{3} Despite decades of research, our ability to control chronic high blood pressure is hampered by an incomplete understanding of the development of the disease. The onset of essential hypertension is often associated with reductions in baroreflex sensitivity for control of heart rate,\textsuperscript{4} an important marker of vagus nerve function, as well as imbalances in RAS activity. Reduced baroreflex sensitivity and pathogenic RAS signaling is also typical of established risk factors for hypertension, including aging and obesity.\textsuperscript{5} This implies the existence of shared physiological mechanisms contributing to the progression of cardiovascular diseases, aging, and metabolic dysfunction in part by altering baroreflex sensitivity. Therefore, the identification of factors that may influence baroreflex function in these negative conditions is imperative to advance our understanding of hypertension and its risk factors so that more effective pharmacotherapies can be developed.

The central and peripheral endocannabinoid system plays a critical role in the modulation of cellular processes governing autonomic, metabolic and cardiovascular function.\textsuperscript{6} Over the past decade, the endocannabinoid system has been the subject of intensive research in part because of its putative contributions to cardiovascular and
metabolic diseases.\textsuperscript{7, 8} Recently, signaling interactions between the endocannabinoid system and the RAS have been described in several \textit{in vitro} and \textit{in vivo} settings.\textsuperscript{9-13} However, an assessment of the endocannabinoid system in conditions associated with altered brain RAS activity is lacking. The studies contained in this volume investigate the contribution of endocannabinoids to regulation of baroreflex sensitivity and blood pressure under normal conditions and during alterations of the brain RAS. The results of these studies implicate novel mechanisms for neural control of the circulation in hypertensive, aged and obese individuals that may provide insight for preservation of baroreflex function in these conditions.

In Chapter Two we examine the contribution of dorsal medullary endocannabinoids to the differential resting baroreflex sensitivity observed in rats under- and overexpressing components of the brain RAS. Data from these studies provide functional and biochemical evidence that increased production of 2-AG at the level of the NTS and surrounding nuclei may underlie the markedly suppressed baroreflex sensitivity for control of heart rate in hypertensive (mRen2)\textsuperscript{27} rats with upregulated brain RAS activity.\textsuperscript{14} These studies also reveal that hypotensive ASrAOGEN rats underexpressing glial angiotensinogen have reduced 2-AG and CB\textsubscript{1} receptor mRNA expression in the dorsal medulla that may function to maintain enhanced baroreflex sensitivity in this strain.\textsuperscript{15} Thus, we establish a link between the endocannabinoid system and maintenance of baroreflex sensitivity in animal models featuring altered brain RAS activity.

Normal aging is characterized by progressively rising systolic blood pressure\textsuperscript{16} with declining baroreflex sensitivity\textsuperscript{17} due to autonomic imbalance driven in part by changes in the brain RAS.\textsuperscript{18, 19} A role for endocannabinoid modulation of age-related
impairment of baroreflex function has not yet been demonstrated. Therefore, in Chapter Three we report altered CB1 and CB2 receptor expression and increasing 2-AG content across lifespan in the dorsal medulla of young adult, middle aged and older Sprague-Dawley rats. We further demonstrate that the medullary endocannabinoid system functionally contributes to reduced baroreflex sensitivity associated with aging. Thus, the endocannabinoid system is linked to maintenance of baroreflex sensitivity during aging, an additional setting associated with altered brain RAS activity and hypertension.

Results from these acute, localized studies prompted us to examine systemic CB1 receptor blockade in the (mRen2)27 rat model of Ang II-dependent hypertension with metabolic syndrome.20 In Chapter Four we demonstrate positive effects of acute and chronic systemic treatment with a CB1 receptor antagonist on blood pressure, and additional positive effects of chronic treatment on conscious baroreflex sensitivity and metabolic profile. These findings may advance support for CB1 receptor blockade as a therapeutic strategy for treating RAS-dependent hypertension and metabolic dysfunction. This study further illustrates an important link between the endocannabinoid system and hypertension or obesity in the context of an altered brain RAS.

Together, these studies demonstrate important mechanisms for preservation of baroreflex sensitivity and blood pressure in hypertension, aging and obesity. The importance of pharmacological restoration of baroreflex sensitivity in these conditions is now recognized as a novel method to prevent cardiovascular diseases,21 creating the need for research into the mechanisms underlying baroreflex dysfunction. Therefore, we conclude that the endocannabinoid system may be an attractive therapeutic target for the treatment of RAS-dependent hypertension or metabolic syndrome.
2. Endocannabinoid Modulation of Baroreflex Sensitivity during Hypertension and Aging

Exogenous cannabinoids have long been known to interfere with autonomic homeostasis in humans. For example, prolonged cannabis use is associated with blunted circulatory reflexes to standing and exercising, orthostatic intolerance and reduced heart rate variability.\textsuperscript{22, 23} The identification of CB\textsubscript{1} receptors within the NTS and on terminals of vagal afferent neurons\textsuperscript{24-26} and tissue bathing studies showing that NTS neurons are sensitive to cannabinoid ligands\textsuperscript{27} suggests a role for the endocannabinoid system in baroreflex modulation.

The first studies to assess exogenous cannabinoids in baroreflex modulation at the level of the NTS in normotensive animals generally demonstrate a facilitating effect of cannabinoid agonists, including anandamide and WIN55,212, on sympathetic nerve inhibition following increases in blood pressure.\textsuperscript{28-30} Of note, prolongation of baroreflex-evoked sympathetic inhibition observed in Sprague-Dawley and Wistar-Kyoto rats is absent in SHR,\textsuperscript{31} providing the first clue for potentially altered regulation of baroreflex function in hypertension by the endocannabinoid system. However, these studies are limited in scope and do not assess dose-response relationships or effects on the vagally-mediated parasympathetic branch of the baroreflex.

Studies in our laboratory examining the effect of a non-selective synthetic cannabinoid agonist microinjected into the NTS reveal dose-related biphasic actions of cannabinoid receptor activation on baroreflex sensitivity for control of heart rate in anesthetized Sprague-Dawley rats (Figure 1-1).\textsuperscript{32} Specifically, a low dose of CP55,940 (1.2 pmol/120 nL), comparable to drug concentrations used in the studies mentioned
above, enhance baroreflex sensitivity while a 10-fold higher dose (12 pmol) significantly impairs it to a level typical of hypertension. These effects are fully prevented or reversed by microinjection of SR141716A before or after the agonist, suggesting they were mediated by activation of CB₁ receptors in the NTS. Enhanced bradycardic baroreflex sensitivity by lower doses of CP55,940 in the NTS is consistent with prolonged baroreflex-evoked sympathetic nerve inhibition by cannabinoid agonists found in normotensive animals.²⁸⁻³⁰ Furthermore, the biphasic response evoked by increasing doses of CP55,940 is consistent with biphasic actions of cannabinoids reported at many areas of the brain, attributable to their ability to modulate various competing neurotransmitter pathways.³³

As we demonstrate in Chapter Two, there is no effect of SR141716A alone in the NTS of Sprague-Dawley rats, suggesting minimal or absent tonic influence of CB₁ receptors in the modulation of baroreflex sensitivity, in line with a lack of alteration in sympathetic nerve discharge by AM281 alone in the NTS.³⁴ However, the intriguing result that a higher dose of CP55,940 impairs baroreflex sensitivity makes it conceivable that overactivation of NTS CB₁ receptors by endocannabinoids could contribute to impaired baroreflex sensitivity during certain contexts, such as onset of hypertension.

Additional studies in anesthetized (mRen2)27 rats with low baseline baroreflex sensitivity find no effect of NTS microinjection of CP55,940 at the dose that increases baroreflex sensitivity in Sprague-Dawley rats (Figure 1-2). One interpretation of this result is that CB₁ receptors in the NTS of these animals may be overactivated to suppress baroreflex function, similar to the effect of the high dose of CP55,940 in Sprague-Dawley rats. Indeed, peripheral endocannabinoid tone is increased during hypertension and
cardiovascular disease, with upregulated CB₁ receptor expression in the heart, aorta and coronary arteries reported in human coronary artery disease and aortic stenosis. To test whether increased endogenous CB₁ receptor tone contributes to suppressed baroreflex sensitivity in hypertension we microinjected SR141716A into the NTS of (mRen2)27 rats. These transgenic rats feature overactive sympathetic drive in addition to an imbalance in the brain RAS favoring Ang II signaling. Data from Chapter Two show that NTS CB₁ receptor blockade by microinjection of SR141716A (0.36- and 36-pmol/120 nL) produces dose-dependent improvement in baroreflex sensitivity for control of heart rate in these animals, increasing it to a level comparable to resting baroreflex sensitivity of Sprague-Dawley rats at the higher dose tested. Therefore, we conclude from microinjection studies in (mRen2)27 rats that altered endogenous CB₁ receptor tone at the level of the NTS functionally contributes to suppressed resting baroreflex sensitivity in this model of Ang II-dependent hypertension.

Normal aging is an additional setting characterized by excessive sympathetic nervous system activity and RAS imbalance driving reduced baroreflex sensitivity with steadily increasing systolic blood pressure. Previous studies demonstrate that similar to young (mRen2)27 rats, replacement of Ang-(1-7) rather than AT1 receptor blockade in the NTS restores impaired baroreflex sensitivity in older Sprague-Dawley rats. Furthermore, baroreflex function in older ASrAOGEN rats is preserved relative to Sprague-Dawley rats. However, mas receptor blockade by [D-Ala⁷]-Ang-(1-7) in the NTS impairs baroreflex sensitivity in these transgenic animals while having no effect in older Sprague-Dawley rats. Together, these results suggest that loss of Ang-(1-7) rather
than upregulation of Ang II tone in the NTS is a common mechanism underlying impaired baroreflex sensitivity in both hypertension and normal aging.

Based on these physiological similarities between older animals and young (mRen2)27 rats, we hypothesize that upregulated CB₁ receptor tone may also contribute to low baroreflex sensitivity in aged Sprague-Dawley rats. Data from Chapter Three support this hypothesis, as NTS microinjection of SR141716A (36 pmol) normalizes baroreflex sensitivity in anesthetized older Sprague-Dawley rats, but only in rats that exhibit impaired baseline baroreflex sensitivity. In aged Sprague-Dawley rats with preserved baroreflex function, SR141716A in the NTS has no effect. Conscious blood pressure in these animals was not measured, so it is uncertain if animals with impaired baseline baroreflex sensitivity also exhibited hypertension. However, these data are fully consistent with a role for altered brainstem endocannabinoid system activity in the suppression of baroreflex sensitivity during conditions associated with overactive brain RAS activity and consequent increased sympathetic outflow.

Effects of exogenous cannabinoids on the cardiovascular system are exaggerated in anesthetized relative to conscious animals.⁴⁶ Accordingly, the results we obtained in anesthetized (mRen2)27 and aged Sprague-Dawley rats are bolstered by data from Chapter Four in which we assess autonomic function in conscious (mRen2)27 rats following chronic systemic treatment with SR141716A (10 mg/kg/day) or its vehicle. Although these data were collected after chronic systemic treatment with the CB₁ receptor antagonist, they are fully consistent with the acute microinjection studies and demonstrate that indices of parasympathetic vagus nerve activity including heart rate variability and bradycardic baroreflex sensitivity are significantly greater in (mRen2)27
rats treated with SR141716A compared to those treated with vehicle. In fact, these indices in the drug-treated rats are comparable to values of heart rate variability and bradycardic baroreflex sensitivity recently reported by our laboratory in healthy Sprague-Dawley animals. Furthermore, conscious tachycardic baroreflex sensitivity, a functional index of the sympathetic branch of the baroreflex for control of heart rate that was not assessed in the microinjection studies, is also significantly greater in (mRen2)27 rats following chronic systemic CB1 receptor blockade. Therefore, we demonstrate in three different settings—anesthetized (mRen2)27, aged Sprague-Dawley and conscious (mRen2)27 rats—that NTS-localized or systemic blockade of CB1 receptors normalizes impaired baroreflex function in conditions associated with hypertension.

In summary, based on our acute microinjection studies in young (mRen2)27 and aged Sprague-Dawley rats from Chapters Two and Three, as well as additional studies from Chapter Four in conscious (mRen2)27 rats following chronic systemic treatment with SR14171A, we conclude that upregulated NTS endocannabinoid tone contributes to impaired baseline baroreflex sensitivity in conditions associated with autonomic imbalance or an overactive brain RAS, including hypertension and normal aging. These studies indicate novel mechanisms for preservation of baroreflex function during adverse cardiovascular conditions and suggest that medications that block central CB1 receptors, such as SR141716A, may improve blood pressure regulation in hypertensive, obese or elderly patients. However, future studies must elucidate specific mechanisms of action underlying endocannabinoid modulation of baroreflex sensitivity, which may involve altering neurotransmitter release in the NTS directly or through signaling interactions with other factors, including the brain RAS.

A primary role for endocannabinoids in the CNS is to modulate release of neurotransmitters at the level of the synapse. They do this by activating CB$_1$ receptors located on presynaptic neuronal terminals, which then stimulate heterotrimeric G proteins whose subunits directly interact with voltage-gated ion channels to both repolarize the local plasma membrane and reduce local intracellular calcium concentration.$^{48}$ We hypothesize that the immediate effects of cannabinoid ligands on baroreflex sensitivity demonstrated in the present studies are likely attributable to direct modulation of neurotransmitter release within the NTS.

Glutamate is accepted as the primary neurotransmitter released from baroreceptive afferent neurons in the NTS projecting to downstream baroreflex nuclei in the brainstem,$^{49-57}$ while GABA interneurons serve a tonic modulatory role over baroreflex function.$^{57-59}$ Several lines of evidence link altered NTS glutamate and GABA balance shifted in favor of increased GABAergic tone to impaired cardiovascular regulation in hypertension. For example, infusion of GABA-A and -B receptor agonists into the NTS of conscious normotensive Wistar-Kyoto rats reduces baroreflex sensitivity for control of heart rate in response to intravenous phenylephrine.$^{60}$ Altered GABA-A and -B receptor sensitivity is suggested to be necessary to compensate for or limit reductions in baroreflex function in Sprague-Dawley rats made hypertensive by one-kidney renal-wrap.$^{61}$ Studies in SHR reveal increased endogenous GABA release and enhanced GABA receptor sensitivity in the NTS underlying the exaggerated pressor response to GABA receptor stimulation,$^{62}$ as well as GABAergic mechanisms within the NTS acting tonically to suppress baroreflex sensitivity for control of sympathetic nerve...
activity. Finally, Ang II increases GABA-B receptor expression in cultured NTS neurons and enhances GABA-B receptor-mediated depressor responses and expression in NTS of Sprague-Dawley rats. Collectively, these findings suggest a GABAergic mechanism in the NTS that could contribute to the central nervous system actions of Ang II, yielding the impairment in baroreflex function and elevated blood pressure found in various models of hypertension, including the Ang II-dependent (mRen2)27 rat model used in our present studies.

Although magnetic resonance spectroscopy does not detect significant differences in the ratio of glutamate-to-GABA in the NTS of (mRen2)27 and Sprague-Dawley rats, enhanced NTS GABA receptor expression or sensitivity as a mechanism for blunted baroreflex sensitivity for control of heart rate in the hypertensive strain cannot be dismissed. Importantly, the same study reports that the glutamate-to-GABA ratio was significantly increased in hypotensive ASrAOGEN rats compared to Sprague-Dawley and (mRen2)27 rats. This observation implicates increased NTS glutamatergic neurotransmission as a mechanism underlying the enhanced baroreflex sensitivity and hypotension associated with this RAS-deficient strain. Indeed, glutamate released in the NTS via N-methyl-D-aspartate (NMDA) receptor stimulation or nitric oxide synthesis is associated with augmented depressor and bradycardic responses in Wistar-Kyoto rats. Furthermore, central GABA-A receptor-mediated cardiovascular responses including changes in arterial pressure, heart rate and renal sympathetic nerve activity are attenuated in ASrAOGEN rats. In summary, upregulated GABAergic neurotransmission within the NTS is associated with blunted autonomic reflexes including impaired baroreflex function and hypertension, while increased NTS glutamatergic neurotransmission is
associated with enhanced baroreflex function or hypotension. These results support our hypothesis that glutamate and GABA serve differential modulatory roles in the NTS of rats with low or high brain RAS expression.

Exogenous cannabinoids in the NTS modulate cardiovascular and baroreflex function through interactions with the local GABA or glutamate systems. Microinjection of WIN55,212-2 and CP55,940 into the NTS of anesthetized Sprague-Dawley rats suppresses sympathetic nerve discharge, and this effect is antagonized by NTS microinjection of the CB₁ receptor-selective antagonist AM281 or a non-NMDA glutamate receptor antagonist, in addition to disruption of the baroreflex arc by sinoaortic denervation. The same study reports no effect of AM281 alone in the NTS, consistent with our observations in Sprague-Dawley rats that SR141716A in the NTS does not alter resting blood pressure, heart rate or baroreflex sensitivity and suggesting absent or minimal intrinsic CB₁ receptor tone in the NTS of Sprague-Dawley rats. Another study reports that NTS microinjection of anandamide in anesthetized Sprague-Dawley rats prolongs the phenylephrine-evoked inhibition of renal sympathetic nerve activity, indicating an increase in sympathetic baroreflex sensitivity. Furthermore, anandamide shortens the duration of baroreflex-evoked sympathoinhibition following blockade of NTS GABA-A receptors, revealing the possibility that presynaptic inhibition of glutamate release by anandamide is obscured by a more dominant GABA-mediated effect. Our studies investigating the role of endocannabinoids in modulating the predominantly parasympathetically-mediated cardiovagal branch of the baroreflex are therefore consistent with evidence for the maintenance of altered NTS glutamate or
GABA neurotransmission in the context of altered brain RAS expression through activation of CB₁ receptors on these neurons.

Electrophysiological studies by others suggest a more complex relationship between the endocannabinoid and glutamate or GABA systems within the NTS. Application of WIN55,212-2 to NTS tissue slices in the presence of ionotropic glutamate receptor antagonists inhibited presynaptic GABA release to anatomically identified second-order baroreceptive neurons in the NTS, a putative mechanism to enhance baroreflex signaling in this region, although this was not tested functionally. Importantly, depolarization of second-order baroreceptive neurons decreased the frequency of miniature inhibitory postsynaptic currents, an effect blocked by the CB₁-selective antagonist AM251. This suggests that depolarization of these neurons induces endocannabinoid release and activation of presynaptic CB₁ receptors, which in turn inhibit the release of GABA. However, whole-cell patch clamp recordings in rat brainstem slices show that WIN55,212-2 and exogenous anandamide in the NTS suppress synaptic input to neurons in the dmnX, an important second-order nucleus in the parasympathetic baroreflex arc from which vagal efferents to the heart originate. Therefore, upregulation of dorsal medullary endocannabinoids or CB₁ receptors is a mechanism likely to contribute to the suppression of baroreflex sensitivity for control of heart rate, consistent with the results of our studies in anesthetized (mRen2)27 and older Sprague-Dawley rats. By implication, downregulation of endocannabinoid system expression in this region would likely increase glutamate release, contributing to enhanced baroreflex sensitivity as exhibited by young adult ASrAOGEN rats.
The contribution of central TRPV1 ion channels to regulation of synaptic activity in brainstem nuclei involved baroreflex mediation should not escape consideration. TRPV1 is found on peripheral and central neurons and is activated by endocannabinoids, among other chemical and noxious stimuli. In the brainstem, TRPV1 is expressed in the NTS and dmnX. Functionally, the activation of TRPV1 channels triggers spontaneous glutamate release in the NTS, opposing the actions of CB₁ receptors, while exerting a tonic influence over excitatory and inhibitory inputs to the dmnnX in rats and mice. These actions would likely contribute to modulation of baroreflex function, but this has not been tested directly. However, the presence of TRPV1 in brainstem nuclei in the baroreflex arc provides an additional mechanism for modulation of baroreflex function by the endocannabinoid system. It also may further explain the actions of exogenous anandamide as well as WIN55,212-2, reported to inhibit TRPV1 in trigeminal ganglion neurons, in the studies discussed above. Although brainstem endocannabinoids may modulate baroreflex sensitivity through actions at both CB₁ receptors and TRPV1 channels, in Chapters Two and Three we show only the contribution of endocannabinoid modulation of baroreflex function through actions at CB₁ receptors in our present study because SR141716A is a selective antagonist of CB₁ that is not reported to interact with any other receptors, including TRPV1 and CB₂ receptors.

In summary, data from our acute studies in transgenic rats with altered brain RAS expression are consistent with differential medullary endocannabinoid tone contributing to modulation of baroreflex sensitivity in these animals through the tonic control over excitatory or inhibitory neurotransmission in the NTS. These data are further consistent
with the interpretation that acute NTS CB\textsubscript{1} blockade is able to abrogate tonic excitatory or inhibitory neurotransmission in this region. Therefore, we hypothesize that the use of magnetic resonance spectroscopy in future experiments will detect an increase in glutamatergic or GABAergic neurotransmission after blockade of CB\textsubscript{1} receptors in the NTS of (mRen2)27 or ASrAOGEN rats, respectively. However, the physiological events that create the intrinsic balance of neurotransmission within the NTS, and whether the endocannabinoid system contributes to disruption of this balance during conditions associated with hypertension, are less clear and will be the focus of future studies.
4. CB₁ Receptor Blockade and Blood Pressure Regulation in Ang II-Dependent Hypertension

Systemically administered cannabinoid agonists generally produce a sustained hypotensive effect accompanied by bradycardia through CB₁ receptor-mediated signaling in the vasculature and heart, respectively. These depressor effects are amplified in models of acute and chronic hypertension, suggesting activation of the peripheral endocannabinoid system in response to elevated blood pressure, perhaps as a compensatory mechanism. Indeed, intravenous SR141716A further increases pressure in SHR, providing evidence of a compensatory rather than pathogenic role for the endocannabinoid system in this strain.

However, data from acute microinjection studies showing improvement in baroreflex sensitivity by CB₁ receptor blockade in the NTS prompted us to study effects of CB₁ receptor blockade in a more clinically relevant study design. We wanted to determine if chronic oral administration of SR141716A improves conscious baroreflex function in (mRen2)27 rats, in addition to whether it alters blood pressure in the conscious state. Therefore, in Chapter Four we report that systemic CB₁ receptor blockade reduces blood pressure in conscious (mRen2)27 rats. The effect occurs within 90 minutes and is sustained after 24 hours, with evidence of partial recovery to baseline. There is no evidence that tolerance develops to the blood pressure-lowering effect of SR141716A as pressure remained reduced to a similar level throughout the duration of the 28-day study. Furthermore, initial reductions in blood pressure cannot be attributed to changes in food consumption or other short-term metabolic effects. Finally, we report that at the end of the chronic treatment period, animals that received the drug had
significantly better indices of conscious autonomic control of blood pressure, including conscious baroreflex sensitivity in response to increases or decreases in blood pressure and heart rate variability, relative to animals treated with vehicle.

Collectively, these results indicate an important role for the endocannabinoid system in the maintenance of hypertension in (mRen2)27 rats, contravening the blood pressure effect of systemic CB\textsubscript{1} receptor blockade reported in SHR.\textsuperscript{35} These conflicting data may indicate differential roles for the endocannabinoid system in different forms of hypertension. For instance, endocannabinoids may play a pathogenic role in the monogenetic Ang II-dependent hypertension of (mRen2)27 rats, versus a protective or compensatory role in SHR, whose hypertension is of unknown origin.

It is unclear to what extent the centrally-mediated actions of systemic CB\textsubscript{1} blockade contribute to reducing blood pressure in (mRen2)27 rats. Evidence suggests that the hypertension in this strain may be predominantly mediated by central mechanisms because intracerebroventricular administration of RAS inhibitors normalizes blood pressure in these animals.\textsuperscript{85} Future studies will elucidate the relative contributions of the central and peripheral endocannabinoid systems in the maintenance of hypertension in (mRen2)27 rats with the use of peripherally restricted CB\textsubscript{1} antagonists. Furthermore, chronic CB\textsubscript{1} receptor blockade produced many beneficial metabolic effects in (mRen2)27 rats, which may contribute to overall enhanced blood pressure regulation in the later stages of the study. Therefore, additional timecourse experiments will be performed to clarify if different mechanisms underlie the immediate and sustained blood pressure reductions over the duration of the present study. Nevertheless, these data suggest that CB\textsubscript{1} receptor blockade may be beneficial in Ang II-dependent hypertension.
5. Chronic CB₁ Receptor Blockade in Metabolic Syndrome

Obesity is a major risk factor for hypertension and is closely associated with type 2 diabetes. Increased body fat and insulin resistance, both exhibited by (mRen2)27 rats, are together cardinal symptoms of metabolic syndrome, which also includes hyperglycemia and dyslipidemia. These features of the (mRen2)27 rat may be driven by overactivity of the RAS in various tissues, as an activated RAS promotes all characteristic properties of metabolic syndrome. Moreover, RAS inhibitors are generally associated with positive metabolic effects in humans and animals, including in (mRen2)27 rats.

Like the RAS, the endocannabinoid system is well-associated with the promotion and maintenance of metabolic syndrome. Upregulated CB₁ receptor tone is widely reported in obese or diabetic humans and animals. In animals, exogenous cannabinoid agonists directly promote overeating, fat deposition and dyslipidemia through CB₁ receptor-mediated actions in the CNS, white adipose tissue and liver, respectively. Most importantly, systemic CB₁ receptor blockade universally normalizes or improves metabolic profile in obese humans and animals. Metabolic effects of systemic CB₁ receptor blockade in (mRen2)27 rats are unknown. However, the similar beneficial metabolic profile of RAS inhibitors and CB₁ receptor antagonists in other models suggests a shared etiology of metabolic syndrome likely involving the RAS and endocannabinoid system.

The chronic study paradigm utilized in Chapter Four allows the monitoring of other parameters that may change only over a longer timeframe. Accordingly, we evaluated the effects of chronic systemic CB₁ receptor blockade on metabolic profile in
(mRen2)27 rats. Over the 28-day treatment period we tracked daily changes in body weight, weekly changes in blood glucose and food and water intake, and cumulative differences in fat mass and in circulating insulin and leptin at the end of the study. Based on the similarities between systemic CB₁ receptor and RAS blockade during metabolic syndrome reported in humans and animals, we hypothesized that the metabolic parameters we studied in (mRen2)27 rats would improve over the duration of chronic systemic treatment with a CB₁ receptor antagonist.

Data from Chapter Four demonstrate that chronic systemic treatment with SR141716A improves several indices of metabolic function in (mRen2)27 rats. Drug-treated rats gain significantly less weight over the duration of the study compared to rats treated with vehicle. This lower weight gain is not attributable to reduced food consumption alone because food intake is only transiently reduced, recovering fully within seven days of treatment. Furthermore, the difference in weight gain is associated with lower fat mass as a percentage of body weight, as well as lower serum leptin and insulin levels at the end of the study. Lower insulin content is not associated with changes in weekly blood glucose levels, which do not change over the duration of treatment and are identical between treatment groups. Although we did not directly test leptin and insulin signaling actions, reduced circulating levels coupled with lower body fat and preserved blood glucose implies enhanced sensitivity of these hormones. Collectively, these results support our hypothesis that the endocannabinoid system contributes to increased body mass and insulin resistance in the (mRen2)27 rat model of Ang II-dependent hypertension.
Questions remain regarding the timecourse of treatment and the mechanisms underlying the beneficial metabolic actions of chronic systemic CB₁ receptor blockade in these animals. For example, it is unclear whether lower insulin and leptin levels in drug-treated rats are a cause or result of decreased weight gain or lower fat mass. In addition, the contribution of other parameters that we did not examine, including plasma cholesterol and adiponectin content, and central leptin signaling, to the improved metabolic profile in drug-treated rats is unknown. Systemic CB₁ receptor blockade is associated with an improved serum lipid profile related to effects in the liver,¹⁹⁸ as well as increased release of adiponectin from white adipose tissue.⁹⁹,¹⁰⁰ Adiponectin and leptin both regulate energy homeostasis through actions in the CNS,⁹³,¹⁰¹,¹⁰² so enhanced actions of these hormones could certainly contribute to the improved metabolic profile in (mRen2)27 rats treated with SR141716A. Furthermore, it is unclear whether these metabolic effects are mediated predominantly by actions in the CNS or periphery. Peripherally restricted CB₁ receptor antagonists are available, but identification of distinct central and peripheral roles for the endocannabinoid system in maintaining energy homeostasis raises questions about the relative effectiveness of these compounds relative to brain-penetrating agents.¹⁰³ Future studies will be performed to establish a timecourse of metabolic events in (mRen2)27 rats during chronic systemic CB₁ receptor blockade, as well as investigate effects on other tissue, hormone and signaling systems that may be altered by treatment. Peripherally restricted CB₁ antagonists will also be evaluated in (mRen2)27 rats in the future.

In summary, the results from Chapter Four suggest a role for the endocannabinoid system in the pathogenesis or maintenance of hypertension and metabolic syndrome.
exhibited by (mRen2)27 rats. The effects of chronic systemic CB\textsubscript{1} receptor blockade on metabolic profile in this strain are fully consistent with the clinical and animal literature. Combined with the novel finding that systemic CB\textsubscript{1} receptor blockade in this strain significantly reduces blood pressure raises the intriguing possibility that combination treatment with a RAS blocker may produce additive or synergistic effects in certain forms of hypertension. Moreover, all of the results obtained from these studies, including data from Chapters Two and Three, are consistent with an interaction between the RAS and endocannabinoid system that may be an important mechanism contributing to hypertension and impaired baroreflex function associated with obesity and aging.
6. Interactions between the Endocannabinoid System and the Brain RAS

Baroreflex impairment during hypertension, aging and metabolic syndrome is likely the result of the disruption of a complex balance of factors within the NTS and surrounding nuclei mediating cardiovagal reflexes. Alterations in expression of brain RAS components are well-associated with these conditions\textsuperscript{19, 104, 105} and may also independently contribute to suppressed baroreflex function.\textsuperscript{106} Other factors are known to impair baroreflex sensitivity when elevated or altered during hypertension associated with aging and obesity, such as insulin\textsuperscript{107} and leptin.\textsuperscript{108} Functional and biochemical data from Chapters Two, Three and Four advance support for altered endocannabinoid system expression or activity as an additional contributing factor to declining baroreflex function during conditions associated with hypertension.

In (mRen2)\textsuperscript{27} rats with overactive brain RAS we demonstrate dose-dependent improvement in baroreflex sensitivity for control of heart rate following NTS microinjection of a CB\textsubscript{1} receptor antagonist, as well as normalization of cardiac vagal nerve function following chronic systemic CB\textsubscript{1} receptor blockade. We also demonstrate that CB\textsubscript{1} receptor blockade in the NTS of ASrAOGEN rats with low brain RAS expression produces the opposite effect, dose-dependently reducing baroreflex sensitivity. These results are in contrast to effects of NTS CB\textsubscript{1} blockade in young adult Sprague-Dawley rats, which elicits no change in baroreflex sensitivity. Opposite baroreflex-modulating effects of CB\textsubscript{1} receptor blockade in the NTS of animals with differential expression of brain RAS components implies differential expression or activity of medullary endocannabinoid system components in these animals. Indeed, mass spectrometry reveals that 2-AG content in the dorsal medulla is highest in
(mRen2)27 and aged Sprague-Dawley rats and lowest in ASrAOGEN rats. Furthermore, real-time PCR reveals lower expression of CB₁ receptor mRNA in the dorsal medulla of ASrAOGEN rats relative to young adult Sprague-Dawley and (mRen2)27 rats. Thus, the pattern of medullary endocannabinoid expression parallels brain RAS activity across these strains or ages, and CB₁ receptor-mediated tonic control over baroreflex sensitivity is exhibited when endocannabinoid system components are up- or down-regulated. Therefore, we must consider potential interactions that are reported between these systems, which may functionally contribute to central regulation of blood pressure.

Signaling interactions between CB₁ and AT1 receptors are demonstrated in numerous in vitro and in vivo settings. Activity of DGL, the major enzyme involved in the biosynthesis of 2-AG in the brain, appears to play a critical role in basal or constitutive CB₁ receptor signaling, because its inhibition reduces basal activity of CB₁ receptors while increasing their expression in Chinese hamster ovary cells and cultured hippocampal neurons. Importantly, DGL activity can be stimulated by Ang II via AT1 receptor activation, and Ang II-induced G_i/o protein activation is blocked by CB₁ receptor blockade and inhibition of DGL. This observation implies a CB₁ receptor transactivation mechanism by Ang II stimulation of DGL activity and subsequent 2-AG production, a hypothesis confirmed by mass spectroscopy analyses of 2-AG content in several cell types following application of Ang II. Indeed, transactivation of CB₁ receptors may hypothetically occur by stimulation of any G_q/11-coupled receptor, including AT1 receptors, but also demonstrated by M1, M3, and M5 muscarinic, V1 vasopressin, alpha-1 adrenergic, and B2 bradykinin receptors in recombinant systems. However, AT1 receptors co-localize and co-precipitate with upregulated CB₁ receptor
expression in the CNS and periphery during Ang II-mediated pathologies.\textsuperscript{12} Moreover, CB\textsubscript{1} receptor antagonists and DAGL inhibition enhanced the vasoconstrictor effect of Ang II in rat and mouse skeletal muscle arteriole beds, demonstrating a functional \textit{in vivo} role for Ang II-stimulated production and release of 2-AG to attenuate vasoconstriction by Ang II.\textsuperscript{13} In addition to describing a potential mechanism for the differential expression of dorsal medullary 2-AG content in (mRen2)27 and ASrAOGEN rats, these data suggest a much broader role for CB\textsubscript{1} receptors in brain paracrine-mediation functions during activation of G\textsubscript{q/11} proteins by AT1 receptors.

Further evidence from bioluminescence resonant energy transfer and immunoprecipitation techniques supports direct physical interactions between CB\textsubscript{1} and AT1 receptors that function to augment Ang II-mediated signaling. As demonstrated by Rozenfeld and colleagues,\textsuperscript{12} AT1-CB\textsubscript{1} heteromers display antibody specificity and appear to signal through both G\textsubscript{i/o} and G\textsubscript{q/11} proteins to augment endocannabinoid- or Ang II-stimulated mitogenic signaling. Furthermore, antagonists of either CB\textsubscript{1} or AT1 receptors are able to block mitogenic and fibrogenic signaling in response to both CB\textsubscript{1} and AT1 receptor stimulation in \textit{in vitro} and \textit{in vivo} settings.\textsuperscript{12} A direct role for this proposed interaction in maintaining hypertension is illustrated by the observation that elevated blood pressure caused by microinjection of Ang II into the hypothalamic paraventricular nucleus is completely prevented by prior microinjection of a CB\textsubscript{1} receptor antagonist.\textsuperscript{109} More recent evidence shows that these interactions modulate further actions in the periphery, including liver fibrosis\textsuperscript{12} and gastroprotective effects in the gut,\textsuperscript{110} in addition to modulation of vasoreactivity.\textsuperscript{13} Thus, it is possible that effects of disrupting this interaction by SR141716A are reflected in data from Chapter Four showing metabolic
effects of chronic CB₁ receptor blockade in (mRen2)27 rats, especially considering increased extra-renal tissue expression of the Ren2 transgene in these animals.¹¹¹

Collectively, there is strong evidence for signaling interactions between CB₁ and AT₁ receptors through cross-talk, transactivation and heteromerization mechanisms that likely contribute to the promotion or maintenance of various pathological conditions associated with upregulated Ang II or endocannabinoid signaling. The influence of these interactions over blood pressure regulation in our experiments with transgenic animals is likely significant based on the range of contexts, including central and peripheral as well as in vitro and in vivo settings, that these interactions have been previously evaluated. Although our present studies do not directly investigate potential AT₁-CB₁ signaling interactions, our data showing enhanced baroreflex sensitivity and reductions in blood pressure by CB₁ receptor blockade in conditions associated with hypertension are consistent with disruption of enhanced Ang II-mediated signaling through interactions between CB₁ and AT₁ receptors. Future studies will establish whether interactions between the endocannabinoid system and brain RAS contribute to impaired baroreflex function, elevated blood pressure or insulin resistance and obesity during Ang II-dependent hypertension or normal aging. However, certain properties of the transgenic and aged animals utilized in our studies require us to consider additional, as-yet unexamined interactions between the endocannabinoid system and brain RAS.

As described above, exogenous Ang-(1-7) in the NTS restores baroreflex sensitivity in both young adult (mRen2)27 and aged Sprague-Dawley rats,⁴²,⁴⁴ but not blockade of AT₁ receptors,⁴³ implicating an absence of tonic Ang-(1-7) input rather than upregulated Ang II signaling in the NTS underlying impaired baroreflex function in these
animals. This further implies a potential role for Ang-(1-7) or mas receptor signaling in the modulation of endocannabinoid production, perhaps functioning to oppose the stimulating effect of Ang II on 2-AG production. Interactions between the endocannabinoid system and Ang-(1-7) are unexplored to date, but would certainly be worth investigating as a potential mechanism underlying increased 2-AG production in the dorsal medulla of (mRen2)27 and aged Sprague-Dawley rats relative to ASrAOGEN and younger Sprague-Dawley rats, respectively. The CB1 receptor is reported to interact with a wide variety of G protein-coupled receptors in the brain,\textsuperscript{112,113} so the discovery of an additional interaction with the mas receptor would be an important result.

Another potential interaction between the CB1 receptor and brain RAS during central blood pressure regulation that has not yet been investigated concerns modulation of shared downstream intracellular signaling pathways that contribute to baroreflex function. For example, activation of both CB1 and AT1 receptors is positively linked to both MAPK- and PI3 kinase-mediated signaling,\textsuperscript{114-116} which is upregulated in brainstem nuclei during hypertension\textsuperscript{117} and contributes to elevated blood pressure and suppressed baroreflex sensitivity in both SHR\textsuperscript{118} and (mRen2)27 rats.\textsuperscript{119} Therefore, future experiments will determine whether CB1 receptor blockade alters activity of shared downstream signaling targets of CB1 and AT1 receptors as a potential mechanism of action underlying modulation of baroreflex sensitivity by the endocannabinoid system.

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7. Viability of CB₁ Receptor Blockade as a Therapeutic Strategy

The results contained in the present studies support CB₁ receptor antagonists as having efficacy in conditions associated with RAS-dependent hypertension, with concomitant beneficial effects on metabolic profile and autonomic blood pressure regulation. An entire body of literature suggests that systemic CB₁ receptor blockade is an effective therapy against many disorders, including metabolic syndrome, obesity-related hypertension, cardiovascular diseases, and various drug dependence syndromes.⁹²⁰ Indeed, SR141716A was once approved by the European Medicines Agency as an obesity treatment and was the subject of several Phase III clinical trials in the U.S. However, it induces significant psychiatric side effects, primarily depression and anxiety, and was withdrawn from the market in October 2008.⁹²¹

Despite challenges associated with the endocannabinoid system as a therapeutic target, interest persists in developing novel agents that block CB₁ receptor actions. Pharmacological treatment options for obesity are limited, while antihypertensive medications lose their effectiveness in elderly and obese patients and have negative side effects of their own. Thus, there is an enormous unmet need for drugs that reduce blood pressure, body weight and associated health problems. New proposals aim to circumvent or mitigate the negative psychiatric symptoms of central CB₁ receptor blockade. Current strategies include the development of peripherally restricted CB₁ antagonists, and a personalized medicine approach to identify patients with low risk to the adverse effects of the treatment.⁹²²,⁹²³

Several CB₁ receptor antagonists that poorly penetrate the blood-brain barrier show promising results for the improvement of cardiometabolic risk factors. For
example, AM6545, a peripherally restricted analogue of SR141716A with high affinity and selectivity for the CB\textsubscript{1} receptor, improves glucose homeostasis and plasma lipid profile in mice with diet-induced obesity, without eliciting the behavioral responses of SR141716A.\textsuperscript{124, 125} However, functional data from these drugs in humans are lacking, and effects on cardiovascular functions are not yet known. Furthermore, questions about the efficacy of peripherally restricted CB\textsubscript{1} antagonists are raised by studies showing that the anti-obesity effects of these drugs are markedly reduced compared to SR141716A.\textsuperscript{103} Therefore, while blockade of CB\textsubscript{1} receptors in the periphery may improve certain cardiometabolic risk factors without central side effects, it may not be sufficient for a complete antiobesity or cardioprotective effect.

For patients with unsatisfactory therapeutic responses to peripherally restricted CB\textsubscript{1} antagonists, a brain-penetrating agent may be needed. Another strategy targeting the endocannabinoid system utilizes techniques from the field of personalized medicine to identify genetic markers in patients that indicate if they are susceptible to the adverse psychiatric effects of CB\textsubscript{1} receptor antagonists. A meta-analysis of the clinical trials studying SR141716A in the treatment of cardiometabolic risk found that 26\% of participants reported mood symptoms compared to 13\% taking placebo.\textsuperscript{127} Therefore, treatment with SR141716A may be safe and tolerable for a considerable portion of patients, making it crucial to obtain tools that can identify patients who are at a low risk of developing negative side effects during CB\textsubscript{1} receptor antagonist therapy.

One potential tool to assess patient risk to this treatment is the screening for genes or gene polymorphisms that are involved in mediating the depression-like and anxiogenic effects of CB\textsubscript{1} receptor antagonists. This requires a more complete understanding of
brain endocannabinoid signaling pathways that promote depression and anxiety, however, several close associations between the CB\(_1\) receptor and serotonergic or metabolic enzyme genes have emerged that may predict susceptibility to anxiety or depression.\(^\text{122}\) An additional clinical observation is that adverse effects of SR141716A treatment are more likely to manifest in patients with a history of depression, which suggests CB\(_1\) receptor blockade is more likely to exacerbate already existing anxiety and depressive states rather than generate new cases.\(^\text{122, 128}\) Therefore, a thorough screening process, including relevant genetic markers and history of psychiatric symptoms, may identify patients who will benefit most from CB\(_1\) receptor antagonist therapy with the lowest risk of adverse side effects.

An additional strategy for pursuing blockade of the endocannabinoid system as a pharmacotherapy involves combination treatment with agents that target systems known to interact with the endocannabinoid system. For example, SR141716A produces synergistic effects with an insulin sensitizing agent to improve insulin signaling in diabetic rats.\(^\text{129}\) A similar therapeutic effect to either agent alone is found during combination treatment with lower doses, thus inviting the possibility of reduced side effects. Based on data from the present studies, we propose a similar additive or synergistic effect may exist between CB\(_1\) receptor and RAS blockade during treatment of certain forms of hypertension. During combination treatment with a RAS inhibitor, the risk of psychiatric side effects of a CB\(_1\) receptor antagonist may be further reduced due to both a lower efficacious dose required as well as the anxiolytic and antidepressant behavioral profile of Ang II blockade.\(^\text{130, 131}\) Further study of the endocannabinoid
system-RAS interaction during hypertension will be necessary, but we conclude that the viability of CB₁ receptor blockade as a therapeutic strategy once again holds promise.
8. General Limitations of Studies

The present studies provide novel insights into the maintenance of baroreflex function and blood pressure regulation during conditions associated with hypertension. However, they do not conclusively identify molecular mechanisms underlying central blood pressure regulation by the endocannabinoid system. Thus, while these studies demonstrate a role for the endocannabinoid system in the neural control of the circulation during conditions associated with hypertension and support our primary hypotheses, they also generate new hypotheses described throughout this chapter that will drive future research. Additional limitations that should be considered while interpreting the results of our present studies include the use of anesthesia, microinjection procedures, and the transgenic animal models we utilize to investigate our hypotheses.

Anesthesia

All baroreflex measurements were obtained while animals were either under the influence of urethane-chloralose anesthesia or while conscious after recovering from surgery performed 48 hours previous under isoflurane anesthesia.

Microinjection studies are performed in the conscious state in many brain areas, however, a fixed micropipette and movement of the neck can damage medullary tissue and eliminate baroreflex responses. Therefore, the majority of microinjection experiments at the level of the NTS require the use of anesthesia. Anesthesia of any kind can significantly influence autonomic nervous system function and suppress baroreflex sensitivity for control of heart rate. Combination urethane-chloralose anesthesia is widely used to study neural control of the circulation because it preserves autonomic function to a greater extent relative to other anesthetics.
Furthermore, there is no overall effect of urethane-chloralose anesthesia on blood pressure in Sprague-Dawley rats, as demonstrated in the present as well as previous studies from our laboratory.\(^4^4\), \(^1^0^8\) However, under urethane-chloralose anesthesia, (mRen2)27 and ASrAOGEN rats exhibit altered blood pressure that suggests differential anesthesia-induced effects on the sympathetic nervous system in these animals. Blood pressure and heart rate are paradoxically increased in ASrAOGEN rats under urethane-chloralose anesthesia, suggesting activation of the sympathetic nervous system by this anesthetic.\(^1^5\) In contrast, under urethane-chloralose anesthesia, (mRen2)27 rats exhibit lower blood pressure and heart rate.\(^4^2\) Since urethane inhibits overactivity of the sympathetic nervous system,\(^1^3^5\), \(^1^3^6\) this observation provides further evidence for enhanced sympathetic activity in (mRen2)27 rats. Anesthetics also produce cardiorespiratory depression,\(^1^3^7\) so anesthetized animals used during microinjection experiments were supplemented with a mixture of room air and oxygen to stabilize ventilation. The same percentage and flow rate of oxygen was used in all microinjection studies to control for respiratory variability among animals.

Baroreflex sensitivity for control of heart rate is reduced in anesthetized relative to conscious rats,\(^1^3^5\) although as illustrated by data from Chapters Two and Three, the relative level of baroreflex function under anesthesia parallels the trend observed in conscious animals across strains or age, regardless of anesthesia-induced changes in blood pressure. For instance, relative to young adult Sprague-Dawley rats, conscious ASrAOGEN rats have higher, and aged Sprague-Dawley and (mRen2)27 rats have lower resting baroreflex sensitivity, respectively.\(^1^8\) This pattern of baseline baroreflex sensitivity is maintained in rats under the influence of urethane-chloralose anesthesia.\(^1^5\),
Moreover, the relative contributions of Ang II and Ang-(1-7) to baroreflex function at the level of the NTS are maintained in conscious relative to anesthetized Sprague-Dawley rats. These data, together with the observation that (mRen2)27 rats still exhibit suppressed baroreflex function despite blood pressure-lowering effects of anesthesia, confirm that baroreflex sensitivity is modulated independently of changes in arterial pressure. However, urethane-chloralose anesthesia induces 5-fold increases in plasma renin activity and circulation of Ang II levels, which may affect autonomic nervous system activity and cardiovascular function.

Isoflurane anesthesia was used during catheter surgery for the analysis of conscious baroreflex function in (mRen2)27 rats in Chapter Four. Similar to combination urethane-chloralose anesthesia, isoflurane also dampens baroreflex function in rats and humans, and increases circulating Ang II and vasopressin levels in patients. However, in humans under isoflurane anesthesia, baroreflex sensitivity for control of heart rate recovered to a level statistically similar to conscious baseline within 25 minutes of recovery. The recovery time of a minimum of 48 hours allowed to animals prior to the start of testing is consistent with previous studies in our laboratory.

**Microinjection Procedures**

A heterogeneous population of neurons exists within the NTS, including afferent and efferent neurons and local interneurons. Determining which neurons are accessed by the spread of injectate is a consistent problem for microinjection studies. Since injections may spread to neuronal cell bodies and presynaptic vagal afferent fibers converging on the NTS, both of which express CB₁ receptors, it is unclear which elements mediate baroreflex responses to the CB₁ receptor antagonist used in our studies. The components
mediating baroreflex function in response to \textbf{CB}_1 \text{ receptor activation are currently unknown.}

The spread of the injectate may also access other nuclei in close association with the NTS including the dmX and area postrema. Several studies address the spread of drug microinjections within the NTS. For instance, microinjection of 100 nL of a typical drug spans a 300 µm radius within the NTS with a concentration gradient radiating from the pipette tip.\textsuperscript{143} In addition, the spread of 100 nL of $[^{125}\text{I}]$Sar-Thr-Ang II is limited to an area mostly confined to within the NTS.\textsuperscript{144} Even at small volumes it is possible that the injection may diffuse beyond a single subnucleus within the NTS, potentially reaching the dmX. However, functional assessments show that a 50 nL injection of an AT1 receptor antagonist into the dmX does not alter baroreflex responses within the NTS.\textsuperscript{145} The microinjections performed in our present studies used a volume of 120 nL and thus should be confined within the NTS. Furthermore, our demonstrations of unaltered chemoreflex responses following NTS microinjections of SR141716A indicate selective actions of the drug on baroreflex modulation.

\textit{Transgenic Animal Models}

Transgenic rodents with differential expression of RAS components either systemically or in specific tissue systems are useful models to investigate the role of the RAS to physiology and pathophysiology. The (mRen2)27 rat is a model used to mimic essential hypertension in humans as well as being a practical model of the effects of high Ang II hypertension on end-organ damage. Likewise, the ASrAOGEN rat is a model used to evaluate the contribution of the glial RAS to various physiological processes. However, it is generally unknown which systems are affected by the deletion or addition of a
transgene over lifespan or during embryonic development. Therefore, the (mRen2)27 and ASrAOGEN transgenic rat models cannot be used to explore the etiology of human hypertension, and the ability to directly translate our findings to other animal models or humans may be limited.

The strength of these transgenic models is the ability to determine interactions between the singular genetic events that initiated under- or overexpression of RAS components in ASrAOGEN or (mRen2)27 rats, respectively, and the genetic background against which these interactions occur. This is in contrast to other animal models of hypertension, such as the SHR, in which the undefined genetic origin of hypertension and the difficulty of comparing these rats to outbred genetic controls results in a broad variety of effects described in this hypertensive strain. An additional difficulty in the study of the causes of human hypertension is to distinguish between effects of elevated blood pressure itself and effects that are secondary to the development of hypertension. The use of transgenic animals offers the possibility to circumvent this challenge in part, because the phenotype of the (mRen2)27 and ASrAOGEN rats must originate with the single genetic change that was introduced.

Plasma levels of insulin, leptin, IGF-1 and RAS peptides are similar at 16 weeks of age among Sprague-Dawley, (mRen2)27 and ASrAOGEN rats. In addition, the phenotype of ASrAOGEN rats resembles the phenotype of animals treated chronically with RAS inhibitors beginning at 6 months of age. Together, these data suggest that transgene insertion does not result in developmental abnormalities in (mRen2)27 or ASrAOGEN rats with respect to the variables studied in our experiments, and that the brain RAS is responsible for many of the phenotypic qualities of these animals.
9. Concluding Remarks

In summary, we demonstrate the following:

1. Dorsal medullary endocannabinoid production is increased in conditions associated with hypertension, including imbalances in the brain RAS and during normal aging.

2. Blockade of CB₁ receptors in the NTS improves the parasympathetic baroreflex sensitivity for control of heart rate in young adult hypertensive rats with an overactive brain RAS, as well as in older Sprague-Dawley rats that exhibit impaired baseline baroreflex function.

3. Acute and chronic systemic blockade of CB₁ receptors significantly lowers blood pressure in hypertensive rats with an overactive brain RAS.

4. Chronic systemic blockade of CB₁ receptors significantly improves indices of spontaneous autonomic control of blood pressure in RAS-dependent hypertensive rats.

5. Chronic systemic blockade of CB₁ receptors significantly improves the metabolic profile of RAS-dependent hypertensive rats.

Restoration of baroreflex sensitivity and blockade of the endocannabinoid system are novel targets for the treatment of cardiovascular disease and associated risk factors, including hypertension, aging and obesity.5,120 Despite controversy regarding the precise mechanisms underlying the autonomic imbalance observed in conditions associated with hypertension, the studies in this volume each provide evidence for the participation of the
endocannabinoid system in the central dysregulation of blood pressure associated with an activated Ang II versus Ang-(1-7) system. Furthermore, these studies are consistent with the novel hypothesis that interactions between the endocannabinoid system and Ang II-AT1 signaling contribute to the pathogenesis of hypertension and its related pathologies. Therefore, blockade of endocannabinoid signaling through CB$_1$ receptors may be protective against cardiovascular diseases through a variety of sites and mechanisms. Whether a direct link can be established among upregulated CB$_1$ receptor and RAS tone, baroreflex impairment, hypertension and metabolic syndrome is a subject of continued investigation.

The figure below represents our laboratory’s updated working hypothesis for modulation of sympathovagal balance at the level of the NTS during conditions associated with hypertension:
To conclude, we propose the following experiments for the future:

1. Determine if excitatory or inhibitory neurotransmitter content in the NTS is altered following microinjection of CB$_1$ receptor ligands in rats with altered brain RAS expression.

2. Determine if CB$_1$ and AT1 or mas receptors in the NTS share a common signaling pathway to modulate baroreflex sensitivity.

3. Determine if chronic systemic blockade of CB$_1$ receptors alters expression of RAS components in brain nuclei associated with neural control of the circulation.

4. Determine if combination systemic treatment with a RAS inhibitor and CB$_1$ receptor antagonist produces additive or synergistic effects on blood pressure, autonomic regulation and metabolic profile in hypertensive (mRen2)27 rats.

5. Determine if long-term systemic CB$_1$ receptor blockade extends the lifespan of (mRen2)27 rats.
References


56. Sartor DM, Verberne AJ. The role of NMDA and non-NMDA receptors in the NTS in mediating three distinct sympathoinhibitory reflexes. *Naunyn Schmiedebergs Arch Pharmacol.* 2007;376:241-252.


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SCHOLASTIC VITA

CHRISTOPHER LEE SCHAICH

EDUCATION

Ph.D., Integrative Physiology and Pharmacology (2014)
Certificate in College Level Teaching
Wake Forest University, Winston-Salem, North Carolina, USA

Advisor: Debra I. Diz, Ph.D.
Dissertation title: Blood Pressure Regulation by the Endocannabinoid System in Conditions Associated with Hypertension

B.A., Psychology, Magna Cum Laude, With Distinction (2006)
University of Connecticut, Storrs, CT
Minors in Neuroscience and Ecology & Evolutionary Biology

PROFESSIONAL EXPERIENCE

2009 – Present: Wake Forest School of Medicine, Winston-Salem, NC
Graduate Student; Predoctoral Fellow
Department of Physiology and Pharmacology; and Hypertension & Vascular Research Center

Neuroscience Department – Functional Neuroanatomy group
Research Associate

Spinal Muscular Atrophy (SMA) project
Research Associate

2004 – 2006: Neurobiology of Learning and Memory Lab, Storrs, CT
Research Assistant
University of Connecticut, Department of Psychology
MANUSCRIPTS


2. Schaich CL, Shaltout HA, Brosnihan KB, Howlett AC, Diz DI. Acute and Chronic Systemic CB\textsubscript{1} Cannabinoid Receptor Blockade Improves Blood Pressure Regulation and Metabolic Profile in Hypertensive (mRen2)\textsuperscript{27} Rats. Submitted to Journal of Pharmacology and Experimental Therapeutics. (Under review).


PUBLISHED ABSTRACTS


4. Chris L. Schaich, Hossam A. Shaltout, Allyn C. Howlett, Debra I. Diz; **Chronic Systemic CB\textsubscript{1} Receptor Blockade Reduces Blood Pressure and Weight Gain and Improves Baroreflex Function and Heart Rate Variability in Hypertensive (mRen2)\textsuperscript{27} Rats.** *Hypertension* 62:A183, 2013.

5. Chris L. Schaich, Allyn C. Howlett, Mark C. Chappell, Manisha M. Nautiyal, Debra I. Diz; **Enhanced CB\textsubscript{1} Cannabinoid Receptor Tone Contributes to Impaired Baroreflex Sensitivity in Hypertensive (mRen2)\textsuperscript{27} Transgenic Rats.** *The FASEB Journal* 27:926.13, 2013.

6. Chris L. Schaich, Allyn C. Howlett, Debra I. Diz; **Contribution of Endocannabinoids via CB\textsubscript{1} Receptors to the Impaired Baroreflex Sensitivity in (mRen2)\textsuperscript{27} Hypertensive Rats.** *Hypertension* 60:A117, 2012.

7. Chris L. Schaich, Allyn C. Howlett, Debra I. Diz; **Dose-Related Biphasic Modulation of Baroreflex Sensitivity by the CB\textsubscript{1} Receptor in Rat Solitary Tract Nucleus.** *Hypertension* 58:e105, 2011.


**SPEAKING INVITATIONS AND ORAL PRESENTATIONS**


2. Chris L. Schaich; **Chronic Systemic CB\textsubscript{1} Cannabinoid Receptor Blockade Reduces Blood Pressure and Improves Baroreflex Function in Angiotensin II-Dependent Hypertension.** Department of Physiology and Pharmacology Seminar Series, Wake Forest University School of Medicine. Winston-Salem, NC. October 14, 2013.

3. Chris L. Schaich, Allyn C. Howlett, Brian F. Thomas, Megan A. Grabenauer, Debra I. Diz; **Chronic Systemic CB\textsubscript{1} Receptor Blockade Reduces Weight Gain and Lowers Blood Pressure in Hypertensive (mRen2)\textsuperscript{27} Rats.** 23\textsuperscript{rd} Annual Symposium on the Cannabinoids, International Cannabinoid Research Society, Abstract #44. Vancouver, BC. June 22, 2013.

4. Chris L. Schaich; **Endocannabinoid Modulation of Baroreflex Sensitivity and Blood Pressure in Renin-Angiotensin System-Dependent Hypertension.**
Department of Physiology and Pharmacology Seminar Series, Wake Forest University School of Medicine. Winston-Salem, NC. March 11, 2013.


CONFERENCE PROCEEDINGS


TEACHING EXPERIENCE

Spring 2014:
DPT-6206 Pharmacology, Winston-Salem State University (NC), Department of Physical Therapy, under the direction of Allyn C. Howlett, Ph.D.
- Autonomic Nervous System Drugs (2 lecture hour)
- Cardiovascular Pharmacology (2 lecture hours)
  - Cardiac Arrhythmias
  - Treatment of Angina
Fall 2013:
  BIO-2311 Anatomy and Physiology I, Winston-Salem State University, Department of Biology, under the direction of Allyn C. Howlett, Ph.D.
  • Brain/nervous system block (4 lecture hours)

BIO-2312 Anatomy and Physiology II, Winston-Salem State University, Department of Biology
  • Endocrinology block (3 lecture hours)

Summer 2013:
  DPT-6403 Applied Physiology, Winston-Salem State University, Department of Physical Therapy, under the direction of Allyn C. Howlett, Ph.D.
  • Autonomic Nervous System (3 lecture hours)

HONORS AND AWARDS

Finalist, 3 Minute Thesis (3MT) competition, Wake Forest University (2014)

ASPET Graduate Student Travel Award (2014)
  • To attend Experimental Biology 2014, San Diego, CA, April 26 – 30, 2014.

Onsite Poster Competition Award: Council for High Blood Pressure Research (2013)
  • High Blood Pressure Research Conference, New Orleans, LA (September 11, 2013)

ICRS Travel Award (2013)
  • To attend the 23rd Annual Symposium of the International Cannabinoid Research Society, Vancouver, BC, June 21 – 26, 2013.

Finalist, Nature Careers Columnist Competition (2013)

Mary A. Bell Award, Systems Neuroscience. Western North Carolina Society for Neuroscience Research Day (December 6, 2012)

Finalist, Sixth Annual Graduate Student Poster Competition, Charlotte Life Sciences Conference (October 25, 2012)

Runner-Up Award, Integrative Sciences. Twelfth Annual Graduate Student and Postdoctoral Fellow Research Day. Wake Forest University (March 29, 2012)
Wake Forest University Alumni Travel Award (2011)
• To attend the 21st Annual Symposium of the International Cannabinoid Research Society conference, St. Charles, IL, July 5 - 10, 2011.

Wake Forest University Alumni Travel Award (2010)

Lundbeck 3R Award (2008)
• For contributions to the reduction, refinement, and replacement of wasteful resource and animal use.

PROFESSIONAL MEMBERSHIP

American Society for Pharmacology and Experimental Therapeutics (2012 – present)
Society for Neuroscience – Western North Carolina Chapter (2011 – present)
International Cannabinoid Research Society (2011 – present)
American Heart Association (2010 – present)
American Physiological Society (2010 – present)

NEWSLETTER ARTICLES


