2019

THE OREXIN SYSTEM IN DOCA-SALT HYPERTENSION: REGULATION OF VASOPRESSIN

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By

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A THESIS

Submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

In Kinesiology

MICHIGAN TECHNOLOGICAL UNIVERSITY

2019

This thesis has been approved in partial fulfillment of the requirements for the Degree of MASTER OF SCIENCE in Kinesiology.

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Acknowledgements

I would first like to thank my advisor, Dr. Zhiying Shan, for her continued guidance and full support in everything that I do, both in and out of lab. Dr. Shan's lessons and teachings have helped me develop both as a researcher, as well as an individual, and I cannot thank her enough for all she has done. She has made research both exciting and interesting, and has only given me a greater outlook as I move forward in graduate school and life. I could not have asked for a better mentor.

I would also like to thank all my committee members, Dr. Qinghui Chen, Dr. Jason Carter, as well as Dr. Feng Zhao, for their willingness to help me through my Master's degree and all the assistance they have provided in writing my thesis.

I owe an enormous amount of gratitude to Dr. Erica Wehrwein, without whom I would not be in graduate school in the first place. She instilled in me a desire to always strive to learn more, and to take life head on without any hesitation. She has shown me how to remain optimistic and curious, and has offered a model of who I hope to resemble in the future. She has helped shape me into the individual I am today, and I will forever be grateful.

I would also like to thank my friends and fellow graduate students for all of the support they have given me during my time as a Master's student. Namely, I'd like to thank Dr. Andrew Chapp for teaching me many of the procedures utilized in this study.

I am also very grateful for the generosity of Dr. Matthew and Mrs. Laura Songer, whose monetary award, the *Songer Research Award for Human Health Research,* has provided the primary source of funding for this project.

Last, but certainly not least, I would like to thank my family, especially my parents, who from a young age taught me the importance of working hard and

following whatever aspirations I may have. Most importantly, they taught me to not turn away in the face of adversity, but rather work to overcome it and in doing so, achieve my goals. I love you both very much!

List of abbreviations

ANGII Angiotensin II

ANS Autonomic Nervous System

AVP Arginine Vasopressin

CO Cardiac Output

CVD Cardiovascular Disease

CVLM Caudal Ventrolateral Medulla

CVO Circumventricular Organs

DOCA Deoxycorticosterone Acetate

GABA Gamma Aminobutyric Acid

HS High Salt

ICV Intracerebroventricular

IML Intermediolateral Cell Column

MAP Mean Arterial Pressure

NTS Nucleus Tractus Solitarius

PNS Parasympathetic Nervous System

PVN Paraventricular Nucleus

RAAS Renin Angiotensin Aldosterone System

RVLM Rostral Ventrolateral Medulla

SD Sprague Dawley

SFO Subfornical Organ

SNS Sympathetic Nervous System

SON Supraoptic Nucleus

SSH Salt Sensitive Hypertension

TPR Total Peripheral Resistance

Abstract

Orexin is a neuropeptide with a large range of functions, with a recently discovered role in blood pressure (BP) regulation. Although the role of brain orexin system in hypertension has been investigated in several hypertensive animals, it remains unclear whether activation of the orexin system contributes to the development of Deoxycorticosterone-acetate (DOCA) hypertension, an animal model of human salt sensitive hypertension. In this study, we investigated the hypothesis that Orexin-1 receptor (OX1R) expression is increased in the paraventricular nucleus (PVN), a critical brain area controlling cardiovascular function, which subsequently increases vasopressin (AVP) expression and peripheral secretion, resulting in hypertension development in this model. Seven to eight-week-old male Sprague Dawley (SD) rats were split into three groups including DOCA-salt, untreated controls, and OX1RshRNA-DOCA rats. Following knockdown of OX1R in the PVN via viral infection in the OX1RshRNA-DOCA rats, they, as well as the DOCA-salt group, were implanted with a 75mg DOCA pellet and treated with 1%NaCl/0.2%KCl drinking water, while the control group remained untreated. Blood pressure of each rat was measured using tail-cuff plethysmography. Three weeks following DOCA-salt or sham treatment, all rats were sacrificed, and brains were subjected to either real-time PCR or immunostaining to assay mRNA level and protein expression of Orexin A, OX1R, and AVP in the PVN. Their blood was collected for plasma AVP measurement, and their hearts were weighed for measurement of their heart weight/body weight (BW/HW) ratio. Our results showed that chronic knockdown of the PVN OX1R effectively attenuated hypertension induced by DOCA-salt treatment (control: 107.91±5.99 vs. DOCA-salt: 142.43±7.73 vs. DOCA+OX1RshRNA: 115.69±8.23 mmHg; $P<0.01$) PCR data showed the mRNA levels of OX1R was increased by 23%, while vasopressin showed more than a 2-fold increase in the PVN of DOCA-salt rats compared to controls. Immunostaining data showed a dramatic increase in OX1R as well as vasopressin expression within the PVN following DOCA-salt

treatment, and both increases were attenuated following PVN OX1R knockdown. Plasma AVP levels also showed a significant increase following DOCA-salt treatment (control: 9.04±2.57 vs. DOCA-salt: 37.94±8.66 pg/ml; $P<0.05$), and PVN OX1R significantly attenuated this (DOCA-OX1RshRNA: 0.644±0.281, $P<0.05$). The heart weight to body weight ratio was also measured to be larger in the DOCA-salt group when compared to the control group (Control: 0.31±0.011%, vs DOCA-salt: 0.40±0.017%, $P<0.005$), while the OX1RshRNA injection appeared to mitigate this increase (OX1RshRNA: 0.35±0.019%, vs DOCA-salt: DOCA-salt: 0.40±0.017%). The combination of this data shows a potential role for orexin in the pathology of salt-sensitive hypertension development.

1 Introduction

1.1 Overview

Hypertension is a major pathological condition that affects millions of individuals, and approximately one-third of adults in the United States alone (Centers for Disease and Prevention 2011). Along with the various pathophysiological traits that accompany the onset of hypertension, individuals who are affected are also at an increased risk for devastating cardiovascular diseases, such as cardiac ischemia, hearty failure, and stroke (Klungel, Kaplan et al. 2000, Drazner 2011, Turin, Okamura et al. 2016), both of which raise the likelihood of death due to cardiovascular complications. A plethora of studies have found that the majority of individuals who are diagnosed with primary hypertension are often known as salt-sensitive, meaning that sodium handling and pressor responses following a salt challenge are faulty, leading to an increase and maintenance of an abnormally high blood pressure (Weinberger 1996, Whelton, Appel et al. 2012, O'Donnell, Mente et al. 2015). Although it is estimated that slightly more than half of individuals diagnosed with high blood pressure are considered salt-sensitive (Weinberger, Miller et al. 1986), very little is known about the molecular mechanism underlying the abnormal response to sodium intake. Because of this, it is important to produce new knowledge and deeper insight into the mechanism underlying Salt-Sensitive Hypertension (SSH) to decrease its prevalence in the general population, thus decreasing cardiovascular disorders, as well as the associated mortality rate.

1.2 Blood Pressure Regulation

Current American Heart Association guidelines for hypertension state that a blood pressure of 120/80 mmHg is classified as normotensive (Whelton, Carey et al. 2018). To maintain a relatively constant blood pressure, the body has employed various means of regulating this stable environment. Mean arterial

pressure (MAP) is defined as the average blood pressure experienced by an individual over one cardiac cycle. MAP is the product of both cardiac output (CO) and total peripheral resistance (TPR). This offers two variables that must be in working condition to efficiently regulate blood pressure. However, there are numerous ways in which the body can modify CO as well as TPR, specifically through autonomic and neural-hormonal signaling, which act as short-term and long-term means of blood pressure management.

1.2.1 Short Term Blood Pressure Regulation: Autonomic Influences

As the heart pumps blood throughout the body, the cardiovascular system is notified on a beat-by-beat basis of any abnormalities in blood flow, primarily by the action of the two branches of the autonomic nervous system (ANS): the parasympathetic nervous system (PNS) and the sympathetic nervous system (SNS). The PNS and SNS have antagonistic actions in relation to one another, although both are intrinsically active (Swenne 2013). The PNS serves as the mediator of action during resting phases, while the SNS elicits excitation and increased activity. In blood pressure regulation, the SNS innervates the sinoatrial (SA) node, also known as the pacemaker, in the right atrium of the heart, as well as the myocardium. Because of this, when activated, the SNS can increase heart rate, as well as cardiac contractility, thus effecting stroke volume. The PNS opposes this action, and innervates the SA node through the vagus nerve, which, when activated, causes a decrease in heart rate. Along with this, innervation of blood vessels by the SNS results in vasoconstriction, which can also increase TPR.

The PNS and SNS are activated and regulated through the actions of baroreceptors. Baroreceptors function as mechanoreceptors, and are primarily located in both the carotid sinus and the aortic arch. These mechanoreceptors respond to any changes in the pressure against blood vessel walls that may indicate a dysfunction in the regular flow of blood through the cardiovasculature

(Charkoudian and Rabbitts 2009). When a change is observed by the mechanoreceptors, they activate the body's intrinsic baroreflex mechanism (Charkoudian and Rabbitts 2009). When the mean arterial pressure is increased, the increase in blood volume stretches vessel walls and is sensed in both the aortic arch, as well as the carotid sinus, which are innervated by the vagus nerve and the glossopharyngeal nerve (Benarroch 2008), respectively. This immediately increases afferent signal firing frequency from the baroreceptors to the cardiovascular control center located within the medulla oblongata of the brainstem, specifically the nucleus tractus solitarius (NTS) (Guyenet 2006, Dampney 2016). The NTS acts as a key cardiovascular integration center, and functions to activate inhibitory interneurons through glutamate release within the caudal ventrolateral medulla (CVLM). The CVLM elicits an inhibitory effect through release of the neurotransmitter Gamma-Aminobutyric Acid (GABA) on the rostral ventrolateral medulla (RVLM) (Kumagai, Oshima et al. 2012), the location of many pre-sympathetic neurons and a key center for sympathetic output (Pilowsky and Goodchild 2002). This pathway causes a cessation of sympathetic efferent output, and an increase in PNS activity. This effectively decreases both heart rate and stroke volume, decreasing cardiac output, and causes vasodilation of the vasculature, reducing the total peripheral resistance, allowing the blood pressure to fall back to its normotensive state. If an individual becomes hypotensive, the opposite occurs, and sympathetic nerve activity is increased through a reduced inhibition of the RVLM, resulting in subsequent glutamatergic activation of sympathetic preganglionic neurons within the intermediolateral cell column (IML) (Guyenet 2006, Kumagai, Oshima et al. 2012), resulting in an increase in cardiac output as well as vasoconstriction, effectively raising blood pressure. Although this mechanism allows an efficient means of monitoring blood pressure on a short-term basis, it is not meant to be a long-term solution to blood pressure dysregulation.

1.2.2 Long Term Blood Pressure Regulation: Neural-Hormonal Influences

To compensate for long term adaptations to blood pressure changes, the human body produces multiple hormones in response to changes in blood pressure, which can carry out their function over a much longer period of time. The Renin-Angiotensin-Aldosterone-System (RAAS) is the predominant controller of this hormonal response, and acts as the primary controller of long-term blood pressure regulation through modulation of both natriuresis and diuresis within the kidney tubules. When blood pressure lowers to a level that is classified as hypotensive, or the osmolality of the blood reaches a lower level than the norm, the kidneys will release renin, an important catalytic enzyme that facilitates the transformation of circulating Angiotensinogen to Angiotensin I (ANGI). Angiotensin Converting Enzyme (ACE) then turns Angiotensin I into Angiotensin II (ANGII). ANGII is arguably one of the most important, and potent hormones involved in blood pressure regulation. ANGII carries out its primary activity through two receptors, AT1 and AT2 receptors. Although the actions of ligand binding to AT2 receptors are not as well understood, binding of ANGII to its AT1 receptor facilitates an increase in sympathetic tone as well as vasoconstriction. ANGII also heavily influences the synthesis and subsequent release of two major hormones, aldosterone and vasopressin (AVP) (**Fig. 1.1**).

Further research has observed that ANGII may also have influences on brain function and subsequent synaptic activity through interaction with the central nervous system. All RAAS components have been found within the brain (McKinley, Allen et al. 2001, McKinley, Albiston et al. 2003, Pan 2004), indicating an intrinsic central mechanism for RAAS functioning. It is also worth mentioning that the bulk concentration of AT1 receptors, are found in major cardiovascular areas such as the PVN and Supraoptic Nucleus (SON) (Pan 2004). Studies have shown that an ANGII brain injection, whether it be intracerebroventricular (ICV) or through direct microinjection into the PVN, elicits an increase in blood pressure,

and antagonism of the AT1 receptor within the brain causes a dampening of the blood pressure increase (Jensen, Harding et al. 1992, Bahner, Geiger et al. 1995). Interestingly, it has also been observed that subcutaneous and peripheral injections of ANGII have shown an increase in AT1 receptors within the PVN, despite the presence of the blood brain barrier, which ANGII cannot readily cross (Wei, Yu et al. 2009). Although it cannot directly elicit a response on major brain areas, ANGII acts on AT1 receptors within Circumventricular Organs (CVO) such as the vascular organ of lamina terminalis (OVLT), subfornical organ (SFO), the median eminence, and the area postrema, all of which lack a blood brain barrier (Sunn, McKinley et al. 2003, Pan 2004). These brain regions have high concentrations of AT1 receptors (Pan 2004), as well as multiple projections to other brain areas, namely, projections from the SFO to the PVN (Kawano and Masuko 2010). This offers an explanation as to why peripheral actions of ANGII in response to lowered blood pressure, or changes in blood osmolality, can elicit a centrally mediated response in cardiovascular control centers, such as the PVN.

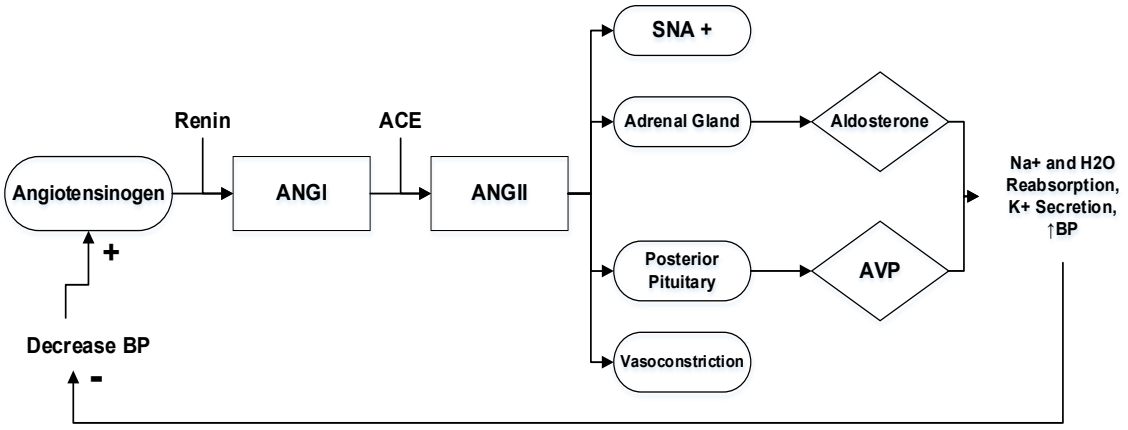

Figure 1.1: Diagram representing function of the Renin-Angiotensin-Aldosterone system (RAAS) in blood pressure regulation. Angiotensin I (ANGI), Angiotensin-Converting Enzyme (ACE), Angiotensin II (ANGII), Sympathetic Nerve Activity (SNA), Arginine Vasopressin (AVP), Blood Pressure (BP).

1.2.2.1 Aldosterone and Arginine Vasopressin in Blood Pressure Regulation

Aldosterone was first discovered in 1953, and since that time, it has been recognized as a major regulator of fluid and electrolyte balance within the kidney natriuresis/diuresis system. Aldosterone is a mineralocorticoid that is produced by the adrenal cortex upon stimulation from various factors, such as circulating ANGII, increased serum potassium levels, among others (Briet and Schiffrin 2010). Upon activation and subsequent release from the adrenal glomerulosa, aldosterone acts on both glucocorticoid as well as mineralocorticoid receptors within the cytoplasm of target tissues, with a higher affinity for the latter (Muto 1995). After binding to its receptor, the hormone-receptor complex will relocate to the nucleus of the cell, and mediate regulation of gene transcription (Muto 1995). These genes will then lead to the production of proteins that work to preserve fluid homeostasis within the body, specifically through regulation of electrolyte (sodium and potassium) levels within the bloodstream (Muto 1995, Summa, Mordasini et al. 2001). One of the downstream transcription regulation pathways occurs in cells of the distal convoluted tubule of the kidney, where binding of aldosterone causes downstream activation of epithelial sodium channels (ENaC) on the apical membrane of the epithelial cells, as well as Na^+/K^+ ATPase on the basolateral side, resulting in a net sodium, and subsequent water reabsorption, as well as potassium excretion into the urine (Summa, Mordasini et al. 2001, Briet and Schiffrin 2010). This process effectively increases blood osmolality as well as volume, making it essential to proper functioning of the complete RAAS system (**Fig. 1.2**).

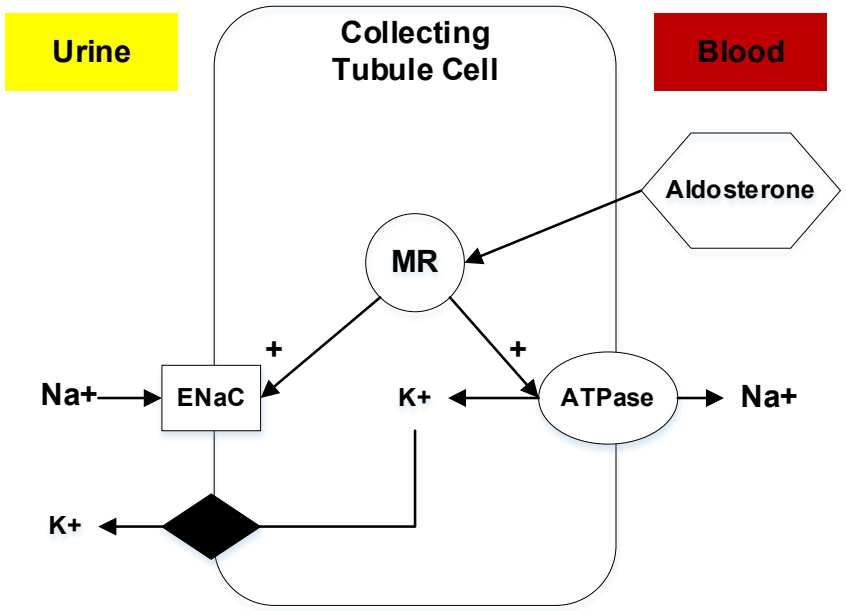

Figure 1.2: Representation of aldosterone action on cells of the distal collecting tubule of the kidney. Mineralocorticoid receptor (MR), Sodium/Potassium ATPase (ATPase), endothelial sodium channel (ENaC), sodium (Na+), potassium (K+).

Another key hormone in cardiovascular and fluid homeostasis is Arginine Vasopressin (AVP). AVP is produced in both magnocellular as well as parvocellular neurons located in both the (PVN) of the Hypothalamus as well as the SON (Treschan and Peters 2006). It is primarily activated by an increase in plasma osmolality, decreased blood volume, as well as decreased arterial pressure (Treschan and Peters 2006). Through its action on its three receptor types (V1, V2, V3), AVP can elicit multiple physiological responses. Worth noting, AVP specifically causes vasoconstriction through its interaction with V1 receptors on smooth muscle cells, and causes water reabsorption through relocation of aquaporins on the collecting ducts of the kidney through its interaction with V2 receptors (Thibonnier, Berti-Mattera et al. 1998, Bankir, Fernandes et al. 2005, Treschan and Peters 2006). The V1 and V2 receptors are two different types of G-protein Coupled Receptors (GPCRs), with differing downstream signaling cascades. The V1 cascade involves interaction with phospholipase C (PLC) following conformational change of the GPCR upon substrate binding. PLC then

acts to facilitate the cleavage of inositol 4,5 – bisphosphate (PIP2) into inositol 1,4,5 – trisphosphate (IP3) and diacyglycerol (DAG) (Thibonnier, Berti-Mattera et al. 1998, Treschan and Peters 2006). IP3 is a secondary messenger that, upon diffusion through the cell, binds to a calcium channel located within the endoplasmic reticulum (ER). Upon binding, calcium is released into the cytosol, which mediates activation of various further calcium regulated intracellular signals. These secondary messengers then act on other enzymes, resulting in vasoconstriction (Thibonnier, Berti-Mattera et al. 1998, Treschan and Peters 2006). V2 receptors act through a slightly different mechanism, stimulating the adenylate cyclase pathway, resulting in activation of various protein kinases via activation by the secondary messenger cAMP, eventually resulting in aquaporin-2 transcription and protein formation followed by relocation into the membrane of the collecting ducts of the kidneys, allowing water reabsorption (Treschan and Peters 2006). This process effectively decreases blood osmolality, while increasing plasma volume (**Fig.1.3**).

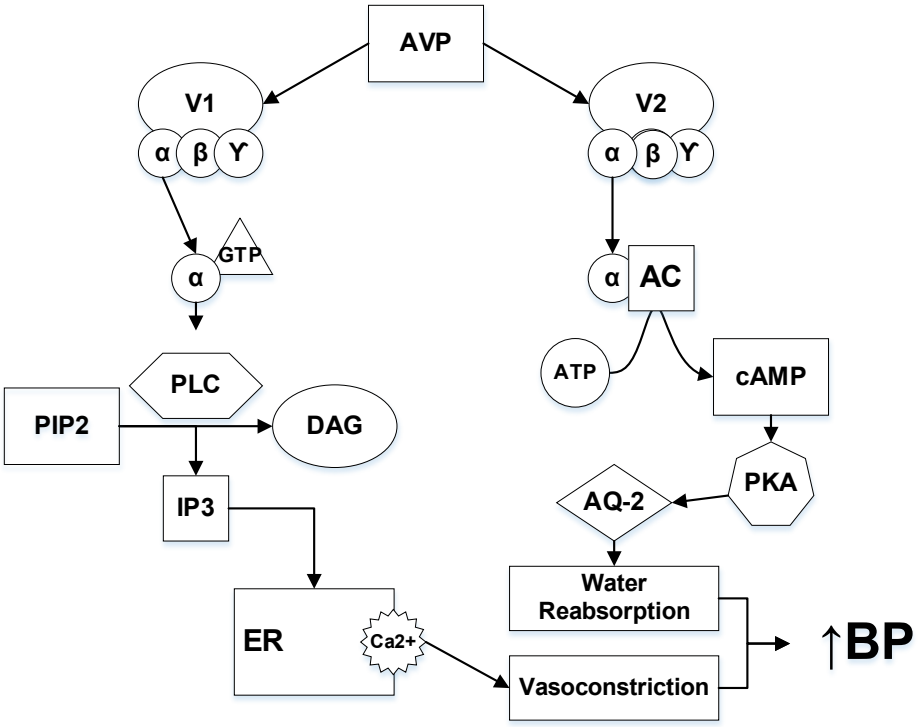

Figure 1.3: The mechanism of AVP on its two receptors, V1 (Vasopressin 1 Receptor) and V2 (Vasopressin 2 Receptor) in vascular smooth muscle cells and the collecting duct of the kidney. Arginine Vasopressin (AVP), Guanosine triphosphate (GTP), phospholipase-C (PLC), inositol 4,5 – bisphosphate (PIP2), diacyglycerol (DAG), inositol 1,4,5 – trisphosphate (IP3), endoplasmic reticulum (ER), adenylate cyclase (AC), cyclic-AMP (cAMP), phosphokinase A (PKA), aquaporin-2 (AQ-2), blood pressure (BP).

Although the implementation of these two blood pressure regulatory systems is essential in maintaining proper blood volume, osmolality, and therefore consistent pressure, any over-activation of either sympathetic activity or the RAS can result in detrimental cardiovascular complications, of which, the mechanism underlying the pathology is still poorly understood.

1.3 Hypertension

Hypertension is defined as an abnormally high blood pressure. When this abnormality becomes a chronic condition, the individual is exposed to a higher risk of further cardiovascular dysfunction, and serious disease that can result in

premature death. Not only this, hypertension also incurs a large healthcare financial burden of approximately $131 billion per year (Kirkland, Heincelman et al. 2018). The AHA's recent guidelines go further in outlining three different levels of hypertension: Elevated (120-129/80 mmHg), stage 1 (systolic anywhere between130-139 mmHg or diastolic between 80-89 mmHg), and stage 2 (systolic at least 140 mmHg or diastolic at least 90 mmHg) (Whelton, Carey et al. 2018). These modifications to the former hypertension guidelines mean that an even greater portion of the general population can be diagnosed with some variation or differing level of hypertension, and thus require health intervention. Unfortunately, there is no one known mechanism underlying hypertensive tendencies. In addition, despite the numerous means of treatment currently available, hypertension and cardiovascular disease (CVD) remain a major global issue. Depending on the cause, hypertension has been split into two subtypes: primary (essential) and secondary hypertension.

Primary hypertension is labelled such when the given hypertension has no known cause. Approximately 90% of individuals who are currently diagnosed with hypertension have primary hypertension (Carretero and Oparil 2000, Beevers, Lip et al. 2001). There is currently no known cause for primary hypertension, given the complex etiology of the disease and the differing symptoms and causative factors among those afflicted by the condition. However, it is well established that many lifestyle factors and behaviors may have a causative relation with hypertension and CVD. Some of these factors include smoking, alcohol consumption, high salt intake, sedentary lifestyle, and many more generally correctable behaviors (Carretero and Oparil 2000, Bolivar 2013). In addition to the multitude of environmental and behavioral factors associated with the pathogenesis of primary hypertension, many researchers are also searching for a common genetic abnormality among individuals with hypertension.

The remaining approximately 10% of individuals with hypertension are classified under secondary hypertension. Although the prevalence of secondary hypertension is much smaller in proportion to the number of primary hypertensive individuals, the effects of secondary hypertension are still a major health concern worldwide. Secondary hypertension is named so because the hypertension is secondary to some other abnormality, such as kidney disease, hyperaldosteronism (Douma, Petidis et al. 2008, Puar, Mok et al. 2016), obstructive sleep apnea (Pedrosa, Drager et al. 2011), obesity, and many others. Although secondary hypertension is generally curable, the underlying mechanisms are not always well defined. However, it is important to investigate the causes in order to prevent the development of resistant hypertension and further cardiac complications, which becomes a risk for individuals who are left undiagnosed.

1.4 Salt Sensitive Hypertension (SSH)

Over the years, the amount of salt used in meal preparation has drastically increased. The Center for Disease Control has established guidelines outlining how much salt should be ingested daily. Despite these guidelines, the average amount of daily salt intake continues to increase, with average daily intake in the United States alone reaching levels greater than 3200 mg/day (Bernstein and Willett 2010), which is much more than the amount necessary for normal physiological functioning. A correlation between excessive intake of dietary salt and an increase in the risk of hypertension has been well established in literature from animal and clinical studies (Whelton, Appel et al. 2012, O'Donnell, Mente et al. 2015). This susceptibility of certain individuals to an abnormal increase in blood pressure following salt intake has been termed “Salt Sensitive Hypertension” (SSH). SSH is possibly one of the most prevalent types of hypertension, and occurs when an individual’s blood pressure is abnormally increased following salt intake. It has been suggested that approximately 51% of hypertensive individuals

can be classified as salt-sensitive, while approximately 26% of normotensive individuals are also salt-sensitive (Weinberger, Miller et al. 1986, Choi, Park et al. 2015). Despite the prevalence of this disorder, the pathology and mechanism are still poorly understood, despite advancements in hypertension research.

Numerous reports have focused primarily on the effects of kidney dysfunction as a primary cause of SSH. A previously outlined mechanism of kidney control of blood pressure regulation was developed by Guyton (Coleman, Granger et al. 1971, Guyton 1991), in which it is hypothesized that an increase in salt intake, and resultant fluid ingestion, causes an increase in fluid volume within the body. This will eventually lead to pressure natriuresis within the kidneys, leading to excretion of both salt and water to enable the blood pressure to return back to equilibrium (Guyton 1991). This model has been a classic representation of what many researchers consider the primary controller of blood pressure regulation via the kidneys. Any sort of dysfunction in this system would likely lead to chronic hypertension. In a classic experiment performed by Lewis Dahl, salt-sensitive and salt-resistant rats were subjected to a bilateral nephrectomy followed by kidney transplant from either the same, or opposite strain. They were then given a normal salt diet. They found that in rats receiving a kidney from a salt-sensitive strain, the blood pressure increase was approximately 30 mmHg greater than those rats that received a kidney from the salt resistant strain (Dahl, Heine et al. 1974). This finding points to a key role of local genetics within the kidneys in blood pressure changes in response to salt loading. This finding was repeated, and a greater increase in blood pressure in resistant rats receiving kidneys from sensitive rats when compared to those who received kidneys from other resistant rats was observed (Morgan, DiBona et al. 1990). However, in the same study, when given a high salt diet (8%Nacl) as opposed to a normal salt diet (0.4%NaCl), salt sensitive rats who received kidney transplants from resistant rats as well as fellow salt sensitive rats alike responded with similar increases in blood

pressure after the salt loading, indicating that the kidneys alone may not be the only regulators of blood pressure.

Because of this discrepancy, others have argued the potential for the brain and the central nervous system as the primary source of dysfunction in SSH. Multiple studies have shown that sympathetic outflow is increased following salt intake (Leenen, Ruzicka et al. 2002, Fujita and Fujita 2013). As previously mentioned, there is also evidence for central action of the RAAS following high salt intake, which is specifically mediated through peripheral interactions with CVO, and subsequent synaptic transmission to areas such as the PVN, which are important in regulation of cardiovascular function (Pan 2004, Wei, Yu et al. 2009, Kawano and Masuko 2010).

Clearly, the etiology and pathogenesis is a rather complex system that requires a large deal of attention. Because there is no one clear mechanism underlying SSH as of right now, multiple animal models have been utilized to mimic SSH in humans, in order to find the molecular connections to SSH. Due to various reasons, the primary animal model used in current hypertension research is the rat (Pinto, Paul et al. 1998). Two of the most commonly used strains of rats used specifically for SSH research are the Dahl Salt-Sensitive (Dahl S) and Deoxycorticosterone Acetate (DOCA)-Salt rats, both of which will be the primary focus for the remainder of this study.

1.4.1 Dahl Salt-Sensitive Rat Model

The Dahl S rat model was developed by researcher Lewis K. Dahl, who has come to be known as a significant influence on salt-sensitivity research. During his research on the effects of salt ingestion, he noticed that some of the Sprague Dawley rats acquired hypertension, while some remained unaffected by the salt treatment. He began to selectively inbreed those whose blood pressures increased following salt intake with one another, as well as those who did not

respond to the salt treatment. Following a few years of breeding, Dahl found that he had successfully bred two strains of rats with statistically different reactions in blood pressure to a high salt intake, and labelled them sensitive and resistant to a high salt intake (Dahl, Heine et al. 1962). This salt sensitive strain eventually became known as the Dahl Salt Sensitive rat model, and has since been a major model used in salt sensitive hypertension research. The Dahl S rat model is generally used as a model for genetic *primary* hypertension, as this model can acquire hypertension even with normal salt intake (Pinto, Paul et al. 1998). Salt sensitivity is an additive factor in the adequacy of this model. It is an effective model because there is potential for genetic predisposition to salt-sensitivity in the 90% of individuals who can be classified under primary hypertension.

1.4.2 DOCA-salt Rat Model

The DOCA-salt rat model was originally developed in the 1970s. In this SSH model, the combination of both DOCA pellet implantation and administration of a salt drink solution (~1% NaCl) results in impaired renal handling of sodium, eventually leading to hypertension development (Basting and Lazartigues 2017). Often, this model is paired with uninephrectomy, which exaggerates the onset of hypertension (Basting and Lazartigues 2017). This rat model offers a model for primary aldosteronism, a term coined by Jerome Conn to describe excessive production of aldosterone from the adrenal glands (Conn 1955). The development of hypertension in this model is dependent on the excessive amounts of DOCA, a mineralocorticoid and precursor to aldosterone, which results in excessive sodium and water reabsorption, as well as potassium excretion in the kidneys (Yemane, Busauskas et al. 2010, Basting and Lazartigues 2017). Because increased aldosterone levels have been gaining more interest as a primary cause for hypertension (Kaplan 2004, Tomaschitz, Pilz et al. 2010), the DOCA model offers an adequate model of this pathology. The onset of hypertension with this specific model is often observed to occur in stages, with an initial spike in blood pressure

within the first couple of days, followed by a more gradual increase and maintenance of an elevated blood pressure in the weeks following administration (Yemane, Busauskas et al. 2010). This model's etiology varies greatly from the Dahl S rat model, in that it acts as a hypertensive model caused by excessive adrenal steroid release and subsequent endocrine dysfunction, as opposed to the primary genetic model observed in the Dahl S rat.

1.5 Neuro-hormonal Mechanisms in Dahl and DOCA-salt Models

Both rat models offer insight into two differing types of salt sensitive hypertension, but both have been observed to show patterns of neural as well as central hormonal mechanisms in response to a high salt intake. In the Dahl S model, dysfunctional nervous system activity as well as baroreflex dysfunction is commonly observed. Studies done on aortic nerve activation, which is part of the afferent response to baroreflex activation, and regulation of baroreflex response in prehypertensive Dahl S rats fed a low salt diet compared to Dahl Resistant (Dahl R) rats found that prehypertensive Dahl S rats showed a significantly lower baroreceptor discharge following phenylephrine infusion when compared to resistant rats (Gordon and Mark 1984). The same group also found impaired SNA activation and subsequent vascular responsiveness increase, resulting in a higher TPR in prehypertensive Dahl S rats when compared to Dahl R rats (Gordon and Mark 1983). This relationship indicates that Dahl S rats have a genetic predisposition leading to impaired baroreflex sensitivity even before hypertension is fully developed. Further evidence showed that, following artificial volume expansion of veins through the use of dextran, a high salt diet increased the neural inhibitory response and potentiated the dampening of SNA in Dahl R rats, while in Dahl S rats fed a high salt diet, cardiopulmonary baroreflex inhibition of SNA was decreased, leading to an increase in SNA outflow to the splanchnic nerve (Victor, Morgan et al. 1986). The combination of these studies shows that Dahl S

rats have a compromised baroreflex and subsequent SNA response to arterial pressure changes, most likely due to genetic predispositions.

Baroreflex dysfunction and excessive SNA have also been observed in the DOCA rat model. However, because DOCA hypertension is generally characterized by a sharp increase in blood pressure in the first few days, followed by a more gradual increase in the following weeks (Yemane, Busauskas et al. 2010), there is some discrepancy on the magnitude of SNA input on the development of hypertension as opposed to the input of hormonal signaling as the primary cause of hypertension. In a few studies performed by deChamplain (de Champlain, Krakoff et al. 1968, Bouvier and de Champlain 1986) it was discovered that DOCA rats, when given 1% NaCl for an extended period of time, are found to have reduced norepinephrine binding and storage in the heart, meaning that there is more NE in the bloodstream. This was one of the first pieces of evidence showing that sympathetic over activity played a role in DOCA-salt hypertension during the fully developed phase of hypertension. However, others have found that there was no increase in SNA during the early phases of hypertension development (Yemane, Busauskas et al. 2010). This is important in distinguishing the role that SNA plays in certain portions of hypertension development in the DOCA rat. Further research has shown, similar to Dahl S rats, that the baroreflex following aortic nerve stimulation is dampened in DOCA rats compared to their controls (Takeda, Nakamura et al. 1988, Schenk and McNeill 1992).

Along with increased SNA following a HS intake, central RAAS function has been extensively observed and studied. The Hypothalamus is an important center for RAAS function (McKinley, Allen et al. 2001, Pan 2004), and as previously stated, cardiovascular relevant areas such as the PVN have been found to hold a large concentration of AT1 receptors, as well as AVP producing neurons (Pan 2004). Because of this, the brain RAAS has become a major focus in the

pathology of Dahl S hypertension. Development of hypertension following a high salt diet in Dahl S rats has repeatedly shown evidence of mediation through the RAAS within the brain (Teruya, Muratani et al. 1995, Kubo and Hagiwara 2006). Further studies have shown that ICV of hypertonic saline elicited a greater increase in the blood pressure of Dahl S rats when compared to Dahl R or normotensive rats (Huang, Wang et al. 2001, Kubo and Hagiwara 2006). This response is thought to be mediated by an increase in cerebrospinal fluid sodium concentration, which is sensed by the CVOs and transmitted to cardiovascular relevant areas of the brain such as the PVN, activating the RAAS (Yang, Jin et al. 1992, Huang, Van Vliet et al. 2004). Inhibition of AT1 receptors has also been shown to decrease the hypertension normally observed in Dahl S rats (Yang, Jin et al. 1992, Gabor and Leenen 2012). A similar role for central RAAS activation has also been observed in the DOCA rat model. Following DOCA pellet implantation and high salt diet treatment, central RAAS has been shown to be a common regulator of the hypertension development seen in this rat model (Basso, Ruiz et al. 1981, Itaya, Suzuki et al. 1986, Gutkind, Kurihara et al. 1988). Also, similar to the Dahl S model, knockdown of ANGII function following ICV injection of captopril, an ACE inhibitor, has been shown to alleviate the elevated blood pressure found in DOCA-salt rats, with no effect on normotensive rats (Itaya, Suzuki et al. 1986). The combination of these studies in both rat models shows the importance of SNA outflow and more importantly, RAAS activity, in the pathogenesis of both forms of SSH.

1.5.1 AVP in Dahl and DOCA Models of Hypertension

Because of the importance of the RAAS in the development and maintenance of elevated blood pressure in both primary and secondary SSH, as well as the downstream effects of elevated RAAS activity on SNA outflow, it is important to produce new knowledge concerning the dysregulation of central hormonal responses following a high salt intake. One of the most important

hormones and regulatory factors of blood pressure is AVP. It has become well known that an increase in salt intake causes activation of osmoreceptors found in the circumventricular organs due to the increased blood osmolarity and mineral imbalances. The CVOs then send excitatory projections to the PVN, resulting in an increased AVP synthesis within magnocellular neurons of the PVN. This AVP is then stored within vesicles at the axon terminals that project from the PVN to the neurohypophysis of the pituitary gland. Upon further stimulation, the PVN neurons will release AVP directly from these terminals into systemic circulation. As previously stated, this increase in AVP within the bloodstream results in activation of V1 and V2 receptors, resulting in both vasoconstriction as well as water reabsorption in the kidneys. Also, the significance of AVP upregulation in both Dahl S and DOCA rats following a high salt challenge has been well documented and extensively reviewed (Berecek, Barron et al. 1982, Berecek, Murray et al. 1982, Schenk and McNeill 1992, Huber, Fan et al. 2017). Along with these studies, microinjection of AVP has been shown to elicit an increase in SNA outflow, and antagonism of the V1 receptor within the PVN reduced the SNA outflow and blood pressure in salt-loaded rats (Ribeiro, Panizza Hdo et al. 2015). Given the increased presence of AVP following salt loading in both neurogenic and secondary forms of SSH, the potent regulatory effects of AVP on long term blood pressure, the presence of elevated plasma AVP in human hypertension patients (Os, Kjeldsen et al. 1986), and the regulatory effect of AVP on SNA outflow (Ribeiro, Panizza Hdo et al. 2015), it is logical to assume that AVP over-activation within the PVN and SON and subsequent elevations in systemic circulation following release from the neurohypophysis is critical to the development and maintenance of hypertension.

1.6 Pharmaceutical Interventions

Because of the importance of RAAS activation and subsequent AVP release, many pharmaceuticals used to treat hypertension are aimed towards

decreasing RAAS function. Two of the most commonly used pharmaceutical interventions are ACE Inhibitors and Angiotensin II Receptor Blockers (Jarari, Rao et al. 2015). One study reported that over a five year period, ACE Inhibitors and ANGII Receptor Blockers represented approximately 13.3 and 28.3% of prescribed anti-hypertensive medications, respectively (Xu, He et al. 2015). In practice, these drugs are used to diminish RAAS activity. Although the use of these pharmaceutical interventions generally has a positive impact on improper blood pressure regulation, studies have shown that an increase in salt intake has an opposing action to hypertensive medications (Weinberger, Cohen et al. 1988, Calhoun, Jones et al. 2008). In addition, excessive salt intake is believed to have a direct impact on the development of *resistant hypertension* (Calhoun, Jones et al. 2008), which is characterized as persistent hypertension despite the use of 3 or more anti-hypertensive prescription drugs. The prevalence of resistant hypertension has been a point of contention, although studies generally observe a prevalence in approximately 10-30% of hypertensive individuals (Calhoun, Jones et al. 2008, Sim, Bhandari et al. 2013). Although the prevalence among the general population is under dispute, primarily due to discrepancy in measurement technique and diagnosis, it is generally shown that approximately 5-15% of the population exhibit signs of primary aldosteronism (Douma, Petidis et al. 2008), the same pathology modelled in the DOCA rat. However, in those individuals with resistant hypertension, approximately 20% can also be diagnosed with primary aldosteronism (Calhoun, Nishizaka et al. 2002, Eide, Torjesen et al. 2004). In order to combat the decreased efficacy of traditional anti-hypertensive interventions in resistant individuals, testing and use of anti-aldosterone, aldosterone synthase inhibitors, as well as mineralocorticoid receptor antagonists has gained recent attention as a possible addition to classic antihypertension drug regiments (Oparil and Schmieder 2015), although there is still uncertainty in the efficacy of these drugs. Despite new knowledge concerning regulation of blood pressure in these hypertensive individuals, an exact mechanism underlying the development of salt sensitive hypertension as well as drug resistance has not yet

been discovered, nor have the central pathways that regulate RAAS and subsequent AVP release following salt intake.

1.7 Orexin System

Orexin is a small neuropeptide that was discovered in 1998 by two different labs concurrently (de Lecea, Kilduff et al. 1998, Sakurai, Amemiya et al. 1998). De Lecea and his colleagues reported that following tag PCR identification of multiple mRNA sequences, one was specifically located within the lateral hypothalamus (LH). Following cDNA isolation, they found that this prepro-peptide encodes the mRNA sequence necessary for the production of two similar proteins, specifically in the LH. Following western blot analysis as well as immunohistochemistry, they discovered that the protein was indeed localized within the LH. De Lecea named these neuropeptides hypocretins, due to their position in the Hypothalamus, as well as their similarity to secretin (de Lecea, Kilduff et al. 1998). Simultaneous research performed by Sakurai, who was searching for potential ligands for receptors whose ligand specificity was still unknown, found similar results and named the same proteins Orexin A (OXA) and Orexin B (OXB), while their mRNA precursor was named prepro-orexin (Sakurai, Amemiya et al. 1998). It was then discovered that the orexins carry out their function through interaction with two GPCRs: Orexin Receptor 1 (OX1R) and Orexin Receptor 2 (OX2R) (de Lecea, Kilduff et al. 1998, Sakurai, Amemiya et al. 1998). Despite similar properties, the two receptor types exhibit differing affinities. While OX2R can bind with either OXA or OXB with nearly equal affinity between subtypes, OX1R has a much higher affinity for OXA (Sakurai, Amemiya et al. 1998). Sakurai also found that, following ICV injection of OXA or OXB, food intake increases in a dose dependent manner, and in a fasted state, prepro-orexin is elevated in the LH, indicating an initial role for orexin in appetite (Sakurai, Amemiya et al. 1998).

Despite the initial idea that orexin primarily controls appetite regulation, it was discovered that although orexin is only produced in the LH, its projections reach multiple parts of the brain. Axonal projections from LH orexin neurons reached important areas such as the hypothalamus, brain stem, limbic system, as well as the circumventricular organs (Peyron, Tighe et al. 1998, Nambu, Sakurai et al. 1999, Kilduff and Peyron 2000). Because of its extensive projections throughout the brain, orexin has been implicated in multiple physiological processes such as sleep (Kilduff and Peyron 2000), arousal, appetite (Sakurai, Amemiya et al. 1998), and in recent years, regulation of blood pressure and sympathetic outflow (Li, Hindmarch et al. 2013, Li and Nattie 2014) (**Fig. 1.4**).

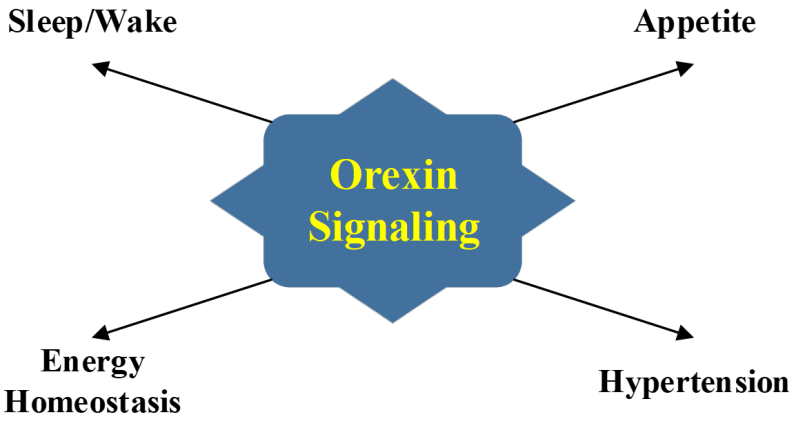

Figure 1.4: Although orexin production only occurs within neurons located in the Lateral Hypothalamus, vast axonal projections to other brain areas allow orexin to carry out functions in multiple physiological processes.

1.7.1 Orexin Effect on Blood Pressure

Many orexin projections from the LH reach important cardiovascular regulatory areas, indicating the potential for orexin action on cardiovascular function. Along with the localized orexin neurons far reaching axonal projections, many of the areas of innervation coincide with concentration of both OX1R and OX2R in these cardiorespiratory areas. Similar to the OX projections, OX1R and OX2R mRNA, the precursor to protein formation, are present in areas of the

hypothalamus such as the PVN, in Sprague Dawley rats (Trivedi, Yu et al. 1998, Li and Nattie 2014), as well as areas of SNA control such as the NTS (Marcus, Aschkenasi et al. 2001, Li and Nattie 2014). OX1R and OX2R containing cell bodies were also found to be localized in similar areas (Li and Nattie 2014).

Because of the co-localization of both orexin hypothalamic neuron projections and their receptors in major cardiovascular areas of the brain, researchers began to study the potential effect of orexin on cardiovascular regulation. Samson et al. found that upon ICV administration of OXA and OXB in doses of 1 and 5 nmol in normal conscious SD rats, mean arterial pressure significantly increased in a dose dependent manner (Samson, Gosnell et al. 1999). Interestingly, these reported increases in MAP were similar to those elicited by ICV injection of 0.1 nmol ANGII under the same conditions (Samson, Gosnell et al. 1999). Following the established role of orexin in blood pressure regulation, electrophysiological recording showed that administration of OXA and OXB to primary neuron cultures of the PVN and RVLM elicited dose dependent depolarization of the neurons, a response that was effectively mitigated following administration of both OX1R and OX2R antagonists together (Shirasaka, Miyahara et al. 2001, Huang, Dai et al. 2010).

Further studies in rats showed that lack of orexin function following genetic prepro-orexin knockout showed that normal rats lacking the prepro-orexin gene exhibited a significantly lower resting blood pressure when compared to their normotensive wild-type littermates (Schwimmer, Stauss et al. 2010). Similar to Samson et al., others found that central administration of both orexins via ICV in rats elicited a dose dependent response in not only MAP, but also SNA outflow in conscious and anaesthetized animals alike (Shirasaka, Nakazato et al. 1999, Li and Nattie 2014). However, since the lateral ventricles allow the orexin to diffuse to various brain areas, it makes it hard to pinpoint an exact area of orexin influence on SNA outflow and cardiovascular influence. More localized microinjections of

OXA in varying doses into the RVLM of SD rats showed a significant increase in both SNA as well as MAP (Shahid, Rahman et al. 2012), and this response was attenuated following prior administration of an OX1R antagonist (Shahid, Rahman et al. 2012). The combination of these studies outlines the extensive role that orexin plays, when administered exogenously, in various parts of the brain, leading many researchers to believe that orexin system dysfunction may play a role in the development and maintenance of hypertension.

In BPH/2J mice, a model for essential hypertension, Marques et al. (Marques, Campain et al. 2011) found that there is an increase in the gene that encodes for orexin within the hypothalamus of these mice when compared to controls in both early and established hypertension (Marques, Campain et al. 2011, Marques, Campain et al. 2011), indicating an intrinsically higher orexin activity in the hypothalamus of rat models of primary hypertension. Similar studies were then carried out in Spontaneously Hypertensive Rats (SHR), a commonly used rat model of primary/neurogenic hypertension that mimics age related progression of hypertension development often observed in humans. Li et al. found that decreasing orexin activity through oral administration of almorexant, a dual orexin receptor antagonist, resulted in a decrease in blood pressure of approximately 30mmHg in SHR (Li, Hindmarch et al. 2013). The same study showed a large increase in RVLM orexin mRNA expression, although this finding did not reach statistical significance (Li, Hindmarch et al. 2013). A subsequent study concerned with the specific effects of OX2R found that upon central blockade of OX2R function via ICV of an OX2R antagonist as well as microinjection into the RVLM of SHR caused a significant decrease in blood pressure as well as HR (Lee, Dai et al. 2013), but found a decrease in RVLM OX2R expression, in opposition to previous studies. A follow-up study performed by the same group showed an increase in hypothalamic OXA and OXB as well as RVLM OXA in SHR compared to WKY rats (Lee, Tsai et al. 2015), and also used retrograde labelling to discover that SHR have more orexin projections to the

RVLM than do WKY rats (Lee, Tsai et al. 2015). Further immunohistochemical studies in SHR and normal Wistar Kyoto (WKY) rats showed that SHR showed a greater number of orexin expressing neurons in the medial hypothalamus compared to WKY rats (Clifford, Dampney et al. 2015), indicating that central orexin system overactivation may exacerbate or potentially be a cause for neurogenic hypertension observed in the SHR.

1.7.2 Orexin in Salt Sensitive Hypertension

Despite the importance of orexin function in the regulation of blood pressure as well as in the pathology of hypertensive animal strains such as the BPH/2J mouse and SHR, little is known about the implications of orexin in SSH. However, there is evidence that the orexin system directly impacts the pathogenesis of SSH. It has been extensively reviewed and established that increased SNA as well as RAAS activity and subsequent AVP release are essential to the development of SSH in both DOCA and Dahl S rats, two models of SSH (de Champlain, Krakoff et al. 1968, Berecek, Barron et al. 1982, Gordon and Mark 1983, Gordon and Mark 1984, Victor, Morgan et al. 1986, Gutkind, Kurihara et al. 1988, Yang, Jin et al. 1992). Exogenous orexin administration in normotensive rats has also been shown to cause a drastic increase in blood pressure (Shirasaka, Nakazato et al. 1999, Li and Nattie 2014), while orexin knockdown via differing mechanisms such as oral or central receptor blockade as well as genetic manipulations of prepro-orexin activity mitigate the increase in blood pressure (Schwimmer, Stauss et al. 2010, Lee, Dai et al. 2013, Li, Hindmarch et al. 2013). In addition, orexin has been shown to play a role in the SHR and the development of hypertension.

Implications for mediation of SSH through the PVN specifically through AVP production and secretion have been developed. AVP within the PVN following salt loading has been shown to increase blood pressure as well as SNA in SSH (Ribeiro, Panizza Hdo et al. 2015). Recent research done on Dahl-S rats fed a HS diet has shown that PVN orexin signaling effects the development of hypertension

in this salt sensitive model (Huber, Fan et al. 2017). Polymerase Chain Reaction (PCR) studies conducted on the PVN of Dahl S rats following a HS diet show that mRNA levels of OX1R, OX2R, as well as AVP are all significantly increased in Dahl S rats fed a HS diet when compared to their normal salt counterparts (Huber, Fan et al. 2017), while immunostaining showed a greater concentration of OX1R overexpression in the PVN following HS intake. The same study shows that, in primary hypothalamic cultures, AVP mRNA is increased in a dose dependent manner following incubation in OXA, and this effect is diminished following application of an OX1R antagonist (Huber, Fan et al. 2017). Furthermore, PVN OX1R blockade following microinjection decreased the elevated BP normally observed in this model (Huber, Fan et al. 2017). This data indicates the essential role that orexin, specifically OXA and its interaction with OX1R, plays in the maintenance of SSH in the Dahl S rat through modulation of AVP release and SNA outflow.

Despite the implications for orexin system over-activation in the pathogenesis of *Dahl Salt Sensitive* SSH, there is no data addressing its potential role for it in other SSH models, specifically the DOCA rat. To date, only one paper has been published concerning orexin activity in the DOCA model. However, this paper was primarily concerned with diurnal influences on orexin function in DOCA-salt hypertension (Hernandez, Watkins et al. 2018). In this study, they found that OX1R, OX2R, and OXA are increased within the hypothalamus following DOCA Salt treatment (Hernandez, Watkins et al. 2018). However, many questions remain concerning the role of the orexin system in the DOCA rat model, and other salt sensitive models of hypertension.

1.8 Hypothesis

In both Dahl S and DOCA rats, AVP is a primary influence on the development and maintenance of hypertension (Berecek, Barron et al. 1982, Berecek, Murray et al. 1982, Schenk and McNeill 1992, Huber, Fan et al. 2017) . AVP is primarily

produced within the PVN and SON (Treschan and Peters 2006). The PVN is also an area of integration of blood osmolality signals through projections stemming from the CVOs (Sunn, McKinley et al. 2003, Pan 2004). Upon HS intake, AVP is upregulated in the PVN, an area of major cardiovascular integration within the brain. Orexin system over activation has been implicated in primary hypertension development in both BPH/2J mice (Marques, Campain et al. 2011) as well as SHR (Li, Hindmarch et al. 2013). Ablation of orexin function both centrally and through peripheral means has been shown to cause a decrease in the normal elevation of BP (Schwimmer, Stauss et al. 2010, Lee, Dai et al. 2013, Li, Hindmarch et al. 2013). Furthermore, OXA has been shown to directly affect SSH in the Dahl S rat model following HS intake, specifically through interaction with the OX1R within the PVN, and subsequent release of AVP to the periphery (Huber, Fan et al. 2017). Lastly, the only study concerned with orexin function in the DOCA rat model shows an increase in OXA as well as OX2R within the hypothalamus following DOCA-HS treatment. However, this study does not observe the PVN specifically, nor does it directly address the effects that the orexin system may play on the development of hypertension (Hernandez, Watkins et al. 2018).

Due to this evidence for orexin function in SSH development, we hypothesize that, following DOCA-HS treatment in SD rats, the DOCA pellet induced renal sodium and water reabsorption, as well as HS intake will cause PVN OX1R signaling to be upregulated via projections of osmoreceptors detecting blood osmolality from the CVOs. We also hypothesize that OXA projections to the PVN, as well as cell body concentration within the LH will be increased in the DOCA rats. Following the interaction between OXA and OX1R in the PVN, we believe an increased AVP production and subsequent secretion into peripheral circulation will cause an increase in both vasoconstriction as well as water reabsorption within the kidneys, resulting in an increased blood volume, and resultant hypertension. Furthermore, we hypothesize that PVN microinjection of AAV2-OX1R-shRNA, a virus causing downregulation of OX1R, will result in a decrease

in OX1R activation and AVP release from the PVN, as well as decreased plasma AVP and levels. We believe that reducing OX1R function within the PVN of DOCA rats will alleviate, at least in part, the development of hypertension in this model. A visual representation of our hypothesis can be observed in **figure 1.5**. The results of this study may offer insight into the role of orexin in SSH, and may produce evidence offering a new potential pharmaceutical target for hypertension treatment.

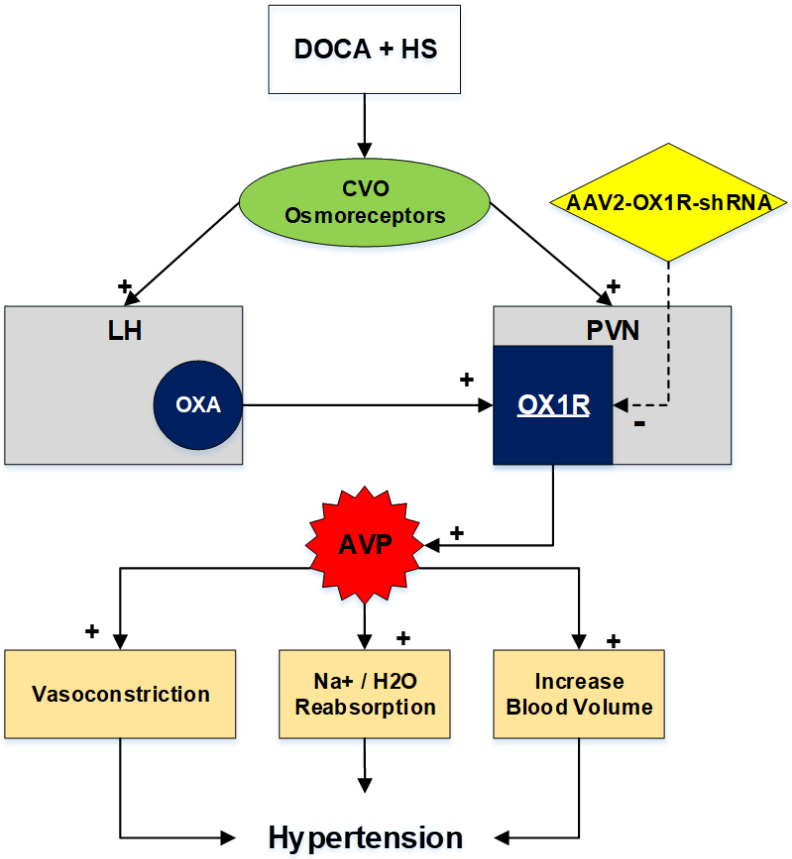

Figure 1.5: Representative model of the hypothesized mechanism underlying orexin control of blood pressure in the DOCA-rat model. Circumventricular organs (CVO), lateral hypothalamus (LH), paraventricular nucleus (PVN), orexin A (OXA), orexin-1 receptor (OX1R), arginine vasopressin (AVP), adeno-associated virus – OX1RshRNA (AAV2-OX1R-shRNA).

2 Methods

2.1 Animals

All animal breeding was performed and overseen by our lab with the help of the Animal Care Facility (ACF). Adult male rats (200-350g) were placed into three groups: DOCA-Salt, DOCA-Salt + OX1RshRNA, or untreated control. The DOCA-Salt group received implantation of a 21-day release DOCA pellet (75mg, Innovative Research of America, FL, USA), and saline drinking solution (1% NaCl and 0.2% KCl) with normal chow for 21 days. DOCA-Salt + OX1RshRNA rats received a PVN microinjection of AAV2-OX1R-shRNA (University of Florida) two weeks prior to DOCA and high salt diet administration, identical to the DOCA-Salt group. The untreated controls were given sham surgeries, and normal water and chow. During the 3 weeks, blood pressure measurements were taken on DOCA-Salt, DOCA-Salt + OX1RshRNA, and control rats. At the end of the three-week DOCA release, rats were placed in metabolic cages for 3 consecutive days. Following this, rats were euthanized and used for PCR, immunostaining, plasma, and heart weight measurements. The time of euthanization was confined to early afternoon, between approximately 2:00-5:00pm, in order to mitigate the chance of any variability in measured physiological parameters due to the natural fluctuations of orexin activity throughout the day. All rats were housed at a constant temperature and a 12:12 hour light dark cycle, and given their respective diets ad libitum. All animal experiments were performed in adherence to protocols approved by the Michigan Technological University Institutional Animal Care and Use Committee (IACUC).

2.2 Hypothalamic Paraventricular Nucleus Microinjections

Prior to any diet or DOCA treatment, DOCA-Salt + OX1RshRNA rats were subjected to bilateral PVN microinjection of AAV2-OX1R-shRNA, a viral vector that carries a small hairpin RNA that specifically targets the gene encoding for

OX1R, effectively knocking down OX1R function. Rats were anaesthetized using 5% isoflurane for induction, and 2-3% isoflurane exposure for maintenance of anesthesia. Following complete anesthesia, rat heads were fixed in a stereotaxic frame, so that bregma and lambda were level with one another. Two holes were drilled through the skull at coordinates of the PVN so that a single glass microinjector pipette could be lowered into the PVN area. The coordinates for the PVN (in mm) were as follows: -1.6 caudal to bregma, 0.5-0.7 lateral to the midline, and 7.2 deep. Once the microinjector was in place, 200 nl of AAV2-OX1R-shRNA was injected bilaterally into the PVN. Approximately 10-12 minutes was taken between injections to allow for diffusion of the viral vector. Following injection, the wound was sutured, and rats were given a subcutaneous injection of a cocktail solution of meloxicam, penicillin, and sterile 0.9% saline the day of injection, as well as two days after in accordance with IACUC and ACF standards. DOCA-Salt OX1RshRNA rats were given 2 weeks to recover as well as to allow full viral expression, before any other procedures were performed.

2.3 DOCA Pellet Implantation

DOCA-Salt and DOCA-Salt + OX1RshRNA groups were subjected to subcutaneous implantation of a DOCA pellet (75mg, 21-day release, Innovative Research of America, FL, USA) prior to beginning the high salt drink treatment. Rats were given 5% isoflurane for anesthesia induction followed by 2-3% isoflurane to maintain adequate anesthesia during the procedure. A subcutaneous incision was made in the retro-scapular region, and the DOCA pellet was placed under this layer. The wound was sutured, and rats were given the same post-operative care as above. However, directly following the procedure, all rats' drinking water was switched to a saline solution (1%NaCl and 0.2%KCl) for the remainder of the 21 days. As previously mentioned, DOCA treatment is usually paired with uninephrectomy to exacerbate the development of hypertension. However, we decided to exclude the kidney removal following a previously

established model (Kandlikar and Fink 2011) to obtain a more gradual progression of hypertension development that more closely mimics that seen in a humans, as well as to eliminate many of the adverse effects that kidney removal has, and their causative effects on hypertension (Kandlikar and Fink 2011). This allows us to more confidently assume that any results from this study occur because of the DOCA pellet and high salt administration and its interaction with the orexin system.

2.4 Blood Pressure Measurement

A subset of each group was subjected to blood pressure measurement during their treatment period using tail plethysmography (Kent Scientific, CT, USA). DOCA-Salt, DOCA-Salt +OX1R-shRNA, and control rats were all acclimated to the procedure for a week before measurements began through everyday blood pressure measurements. Briefly, rats were placed in a plastic cylinder with a dark nose-cone, to reduce vision and anxiety. After 10 minutes acclimation, the tail cuff apparatus including the occlusion cuff and volume pressure recording cuff were placed on the animal for an additional 5-10 minutes. Artificial heating was also applied to maintain adequate blood flow to the tail. Following this, blood pressure recording began, and 10 acclimation cycles followed by 20 measurement cycles were conducted. Following blood pressure recording, averages of the 20 measurement cycles were taken and paired with their group to obtain a group mean, which was reported. Rats maintained acclimation during the 18-day treatment by daily 20-minute sessions in the rat holder/tail cuff apparatus for every rat, to reduce variability and anxiety among the rats.

Tail-cuff plethysmography can be attributed to stress-related fluctuations in blood pressure, due to confinement in a small cylinder for extended periods of time, which may lead to variability of results. However, all necessary precautions were taken to mitigate this effect. Namely, acclimation to the cylinders as well as

the occlusion/volume pressure recording apparatus began two weeks prior to any actual blood pressure recording was performed. Following one week of acclimation, blood pressure measurements were taken to identify when the blood pressure reached a normotensive level in all treatment groups. Once the blood pressure appeared to maintain a consistent, normotensive pressure, the experiment was initiated. In addition, blood pressure recording sessions were done during the same time every day (12:00-4:00pm), to mitigate circadian influences on orexin system function, and thus to alleviate any fluctuations in blood pressure that may naturally occur with differential orexin system activity throughout the day. Furthermore, during the three weeks of blood pressure recording, acclimation was maintained on days when blood pressure measurements were not taken by daily exposure to both confinement in the cylinder as well as attachment of the occlusion/volume pressure recording apparatus for 20 minutes. A diagrammatic representation of this process can be observed below **(Fig. 2.1)**:

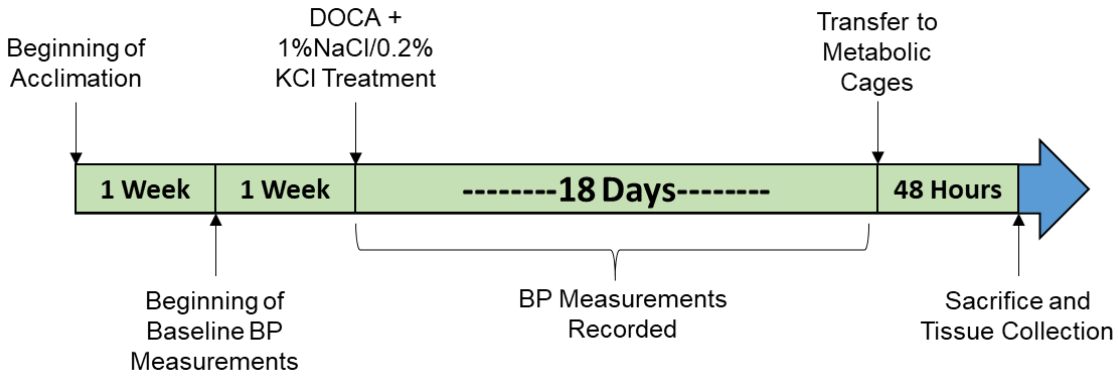

Figure 2.1: Representation of Tail-Cuff Plethysmography procedural acclimation and subsequent BP measurements. Two weeks of acclimation were performed before BP measurements were measured, mitigating any stress-induced variability caused by the recording.

2.5 Metabolic Measurements

During the last 3 days of treatment, DOCA-Salt, DOCA-Salt + OX1RshRNA, and control rats were placed in metabolic cages, where food and water intake, as

well as urine and fecal output were measured. The first day in the metabolic cages was discarded as the results were likely to be affected by the new change of environment for the rats. Body weights were also taken weekly for all rats. Following euthanization, hearts were removed and weighed, and presented as a heart weight to body weight ratio (HW:BW).

2.6 Real Time PCR and Immunostaining

Following three weeks of treatment, rats were euthanized and subjected to either real time polymerase chain reaction (PCR) analysis to observe mRNA levels or immunostaining to visualize protein levels. For PCR, brains were removed and immediately flash frozen in liquid nitrogen to mitigate RNAse activity. Following flash freezing, all brains were placed into a -80 degrees Celsius freezer, where they would remain until needed for mRNA analysis. Upon removal from the freezer, the PVN area was punched. RNA isolation was performed using RNeasy plus Mini kits (Qiagen, CA, USA) following packaged instructions. Following isolation, the RNA was converted to complementary DNA (cDNA) using iScript cDNA synthesis kits (Bio-Rad), and cDNA was used as a template for real time PCR, which was performed to analyze mRNA levels of OXA, OX1R, and AVP using gene specific Taqman primers and probes. The results were normalized to mRNA expression of the housekeeping gene, GAPDH.

Immunostaining was used to analyze OX1R, OXA, and AVP protein expression within the PVN, as well as OXA within the lateral hypothalamus. 20-µm coronal brain sections containing the PVN or LH using the following procedure. Rats were anesthetized under heavy isoflurane. Once under deep anesthesia, cold phosphate buffer saline (1xPBS) followed by 4% paraformaldehyde (PFA) in 1xPBS was used to transcardially perfuse the animal. Following perfusion, the brain was removed and kept in 4% PFA overnight in 4°C. The next day, the brains were transferred and kept at 4°C in 30% sucrose until they sank to the bottom. They were then cut in 20-µm coronal sections using a

cryostat. Areas containing the PVN area as well as the LH were then subjected to immunostaining. Following wash in 1xPBS 3 times for 10 minutes each, brain sections were incubated with either rabbit anti-OX1R antibody (Alomon Laboratories, Jerusalem, Israel, 1:300 dilution), rabbit anti-AVP antibody (1:400 dilution), or mouse anti-OXA antibody (1:300 dilution) in PBS containing 0.5% Triton X-100 and 5% horse serum for 4 days at 4°C. Following this incubation and 1xPBS washing, they were incubated overnight in secondary antibodies Alexa fluor 488 goat anti rabbit IgG (1:500), Alexa fluor 594 goat anti rabbit IgG (1:500), or Alexa fluor 594 donkey anti mouse IgG (1:500). Images representing immunofluorescence were taken with a Leica DMIL microscope.

2.7 Plasma AVP and CSF Orexin A ELISA Testing

Rats were placed under heavy anesthesia using 5% isoflurane. They were then decapitated, and blood was collected in tubes coated with 5.4mg EDTA (BD Vacutainer, NJ, USA) to collect plasma for measurement. These tubes were then centrifuged at 4°C using an Eppendorf 5804 R Centrifuge at 1300 RPM for 35 minutes. The supernatant was extracted, and stored in -80ºC until use. ELISA testing was used to analyze both plasma concentration of AVP (Arg8-Vasopressin Kit, Enzo Life Sciences, NY, USA) as well as cerebrospinal fluid Orexin A concentrations (Orexin-A ELISA, Fujuifilm, Tokyo, Japan) using the manufacturer's provided instructions.

2.8 Intracerebroventricular Injections

Male adult SD rats were subjected to ICV injections of either saline control or OXA (Sigma Aldrich, MO, USA). Rats were anesthetized under 5% isoflurane, and maintained at 2-3% isoflurane. Rats were set up in a stereotaxic frame as in PVN injections. A hole was made in the skull to allow penetration of a Hamilton syringe into the left lateral ventricle. The coordinates for injection were as follows: -0.8 mm caudal to bregma, 1.6 mm lateral to the midline, and 3.6 mm deep. For

the ICV injection, saline (0.9%) control was injected at a volume of 4µl over the course of 4 minutes on only one side. Rats that received OXA (2nmol) also received 4µl over the course of 4 minutes**.** Approximately 10 minutes following injection brain and plasma were collected and used for PCR as well as ELISA testing.

2.9 Data Collection and Analysis

All rats subjected to PFA perfusion and subsequent immunostaining were unable to be used for any physiological analysis other than protein immunofluorescence staining. However, all other animals subjected to brain, blood, and CSF collection were utilized for multiple tests, including PCR and ELISA. Because of the overlap, and the multiple utilizations of one sample for various tests, some discrepancies in sample size may be present. In general, an arbitrary sample size of 4-5, which is normally observed in other experiments performed in rodent models, was chosen as a goal for each treatment group, although this was not always the case due to the additional accumulation of new results on the same physiological parameters tested throughout the progression of the project. Furthermore, results from multiple experiments **(Fig. 2.2)** were combined over the course of the project when the same parameters were measured. However, to simplify the number of rats subjected to immunostaining as well PCR and ELISA, **Fig. 2.2** shows a representation of the number of rats subjected to each treatment, and what physiological parameters were eventually measured, excluding the six rats used during the ICV injection. In addition, all animals that were lost during surgery, before any measurements were taken, were not recorded, and were simply replaced, eliminating any effect on sample size.

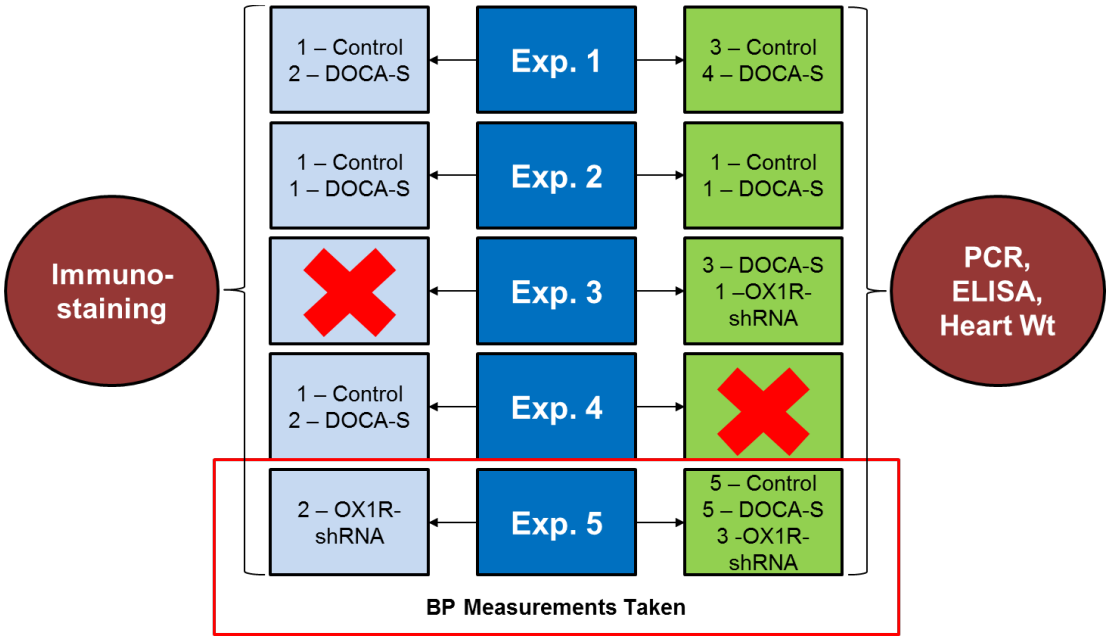

Figure 2.2: Representative diagram showing the number of rats from each experiment (Exp. 1-5) during the project and what physiological analysis was performed on each. Note: Only rats during experiment 5 were subjected to blood pressure measurements over the course of the three-week treatment.

All data is expressed as Mean ± SEM unless otherwise noted. Individual blood pressure means were observed, and then combined with other subjects within their given treatment group to find a group mean and SEM. To test significance unpaired one-tailed and two-tailed T-tests, as well as One-way ANOVA testing was performed using Graphpad Prism-6 software. If significance was found using the ANOVA, a Tukey HSD test was performed. For blood pressure and heart rate data, a two-way ordinary ANOVA was performed, and if significance was found, a Tukey multiple comparison test was performed between time points. All values with a $P<0.05$ were considered significant.

3 Results

The classic model for DOCA-salt hypertension generally utilizes kidney removal (uninephrectomy) to exacerbate the development and severity of hypertension in the animal. However, for our model, we chose to omit the kidney removal, allowing a more gradual and moderate increase in blood pressure, which more closely mimics hypertension development observed in humans. However, kidney removal affects renin levels, and thus AVP secretion and RAAS activity. It has been extensively shown that AVP secretion is essential to hypertension development in the classical DOCA-salt model, so we first assessed whether our model would produce adequate central AVP production, as well as parallel increases in OX1R levels. Furthermore, we wished to observe whether central AVP increases correlated with peripheral secretion in the plasma, using this as evidence of downstream AVP activity on vasoconstriction and renal water reabsorption. Lastly, we assessed metabolic measurements due to AVP and orexin regulation of metabolic activity.

3.1 DOCA-salt Treatment Increases PVN AVP and OX1R Expression

The efficacy of our modified DOCA-salt model in producing a similar increase in central AVP production as the classical uninephrectomized DOCA-salt rats was tested following 3-weeks of DOCA-salt treatment. Following treatment, real time PCR was used to analyze PVN AVP mRNA expression, and immunostaining was used to visualize AVP protein expression within the PVN.

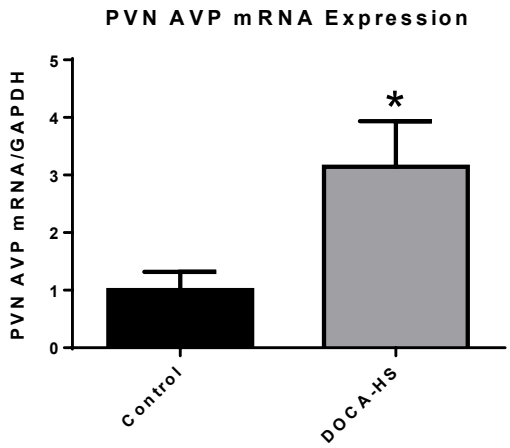

Figure 3.1: PVN AVP mRNA expression following 3-weeks of DOCA-salt treatment. DOCA-salt (n=9) treatment significantly increased PVN AVP mRNA expression compared to control rats (n=8). *P<0.05 using an unpaired two-tailed T-test.

A significant increase in PVN AVP mRNA expression was observed in DOCA-salt rats (n=9) when compared to control rats (n=8) (control: 1±0.319 vs. DOCA-salt: 3.142±0.7914, P<0.05), which was to be expected, as it is generally observed in the classic DOCA-salt model **(Fig. 3.1)**. Further immunostaining assessment also showed a large increase in PVN AVP protein expression when compared to control rats **(Fig. 3.2).** The combination of these results shows that DOCA-salt treatment results in significantly increased AVP production within the PVN, inferring that overproduction of AVP is still present in our modified DOCA-salt model.

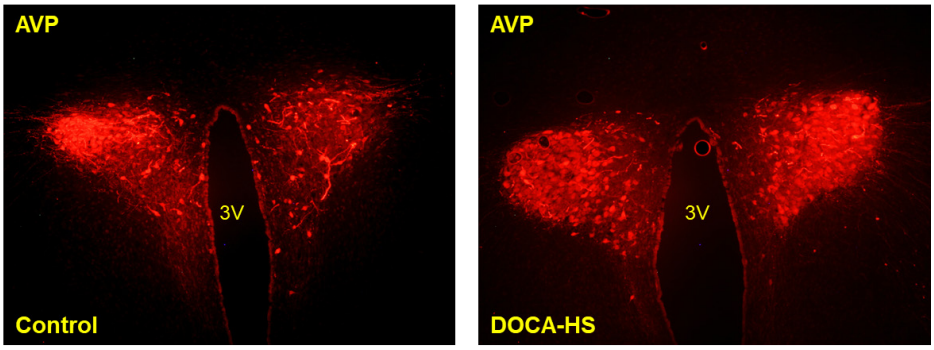

Figure 3.2: Representative immunostaining images of AVP protein expression in control (left) and DOCA-salt (right) rats.

After observing an increase in central AVP production within the PVN of DOCA-salt rats, we then analyzed whether an increase in OX1R receptor expression occurs in parallel with PVN AVP increases following DOCA-salt treatment. To assess this, PCR and immunostaining were once again utilized to assess PVN mRNA and protein expression. PCR results indicated a significant increase of approximately 23% in PVN OX1R mRNA levels following DOCA-salt treatment (n=4) compared to control (n=3) (control: 1.00±0.049 vs. DOCA-salt: 1.231±0.08; P<0.05) **(Fig. 3.3)**.

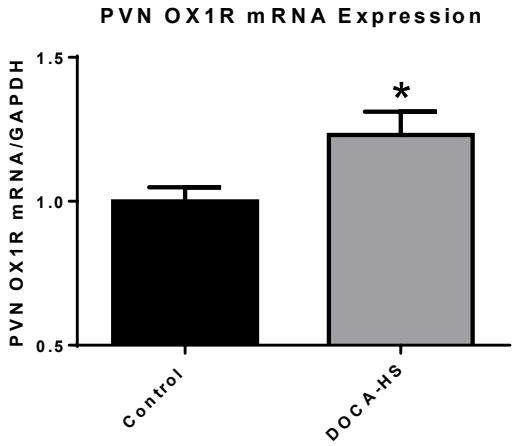

Figure 3.3: PVN OX1R mRNA expression following 3-weeks of DOCA-salt treatment. DOCA-salt (n=4) treatment significantly increased PVN OX1R mRNA expression compared to control rats (n=3). *P<0.05 using an unpaired one-tail T-test.

To affirm whether increased OX1R mRNA levels resulted in increased OX1R protein expression, immunostaining was performed. Immunostaining images show a large increase in OX1R expression and concentration in the PVN area **(Fig. 3.4).** The significant increase in both AVP as well as PVN OX1R expression is indicative of a relationship between the parallel increase observed and DOCA-salt treatment.

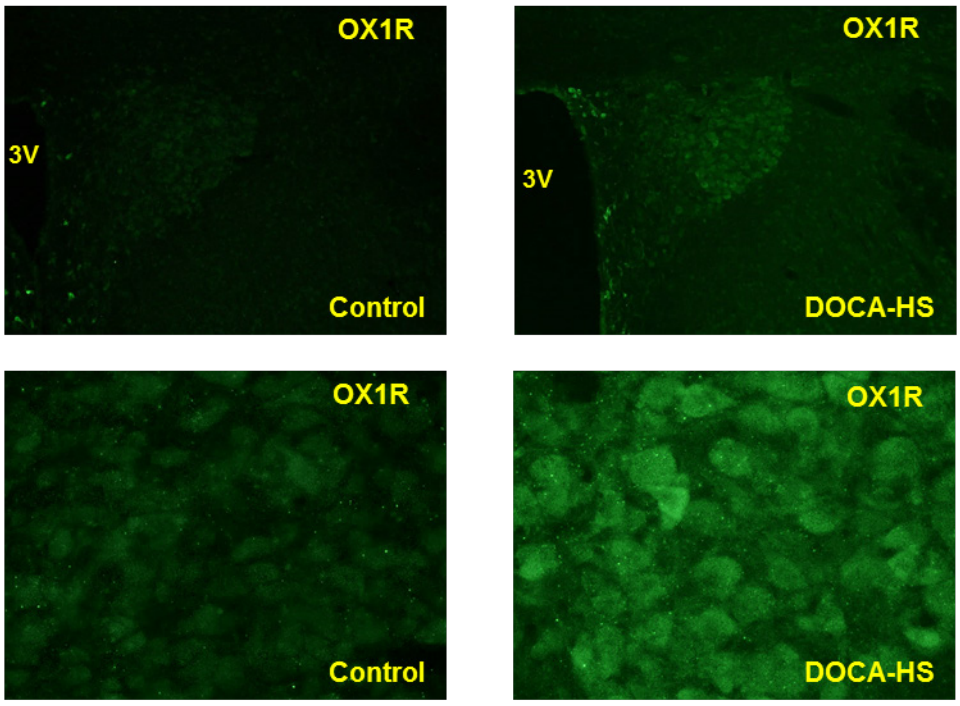

Figure 3.4: Representative immunostaining images showing expression of the orexin-1 receptor (OX1R) in the PVN in both control (left) and DOCA-salt (right) rats. Third ventricle (3V).

3.2 DOCA-salt Treatment Increases Plasma AVP Concentration

The DOCA-salt model is generally characterized by elevated plasma AVP concentrations. Since an increase in PVN AVP expression was expressed, we used ELISA to test plasma AVP concentrations in control rats as well as DOCA-salt treated rats, to observe whether the central increase in AVP production results in an increase in peripheral circulation. Following 3 weeks of treatment, DOCA-

salt treated rats (n=8) showed a significantly higher plasma AVP concentration when compared to control rats (n=4) (control: 9.04±2.57 vs. DOCA-salt: 37.94±8.66 pg/ml; P<0.05) **(Fig. 3.5)**. This result agrees with previous observations showing similar increases in peripheral AVP levels (Lariviere, St-Louis et al. 1988, Saravia, Grillo et al. 1999).

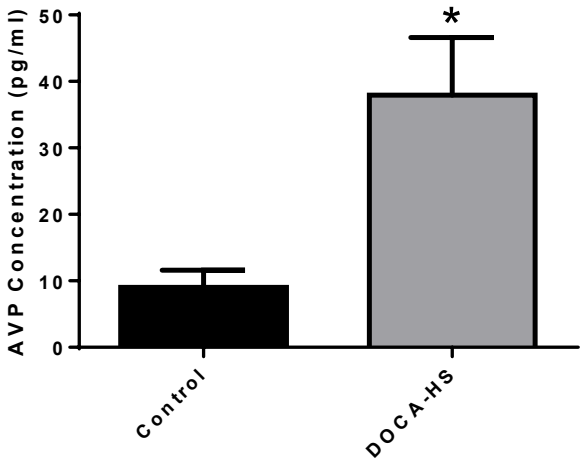

Figure 3.5: Plasma AVP concentrations (pg/ml) in control (n=4) and DOCA-HS (n=8) treatment groups. DOCA-salt treatment resulted in a significant increase in plasma AVP concentration. *P<0.05 vs. control using an unpaired two-tail T-test.

3.3 DOCA-salt Treatment Effects on Metabolism

Following 3 weeks of treatment, all rats were placed in metabolic cages for 72 hours to assess urine and fecal output, food and water intake, and body weight. Following 24-48 hours of acclimation to the environment, food intake (g), fecal output (g), water intake (mL), as well as urine output (mL) were measured over a 24-hour period to analyze the effects that our modified DOCA-salt model elicited on metabolism.

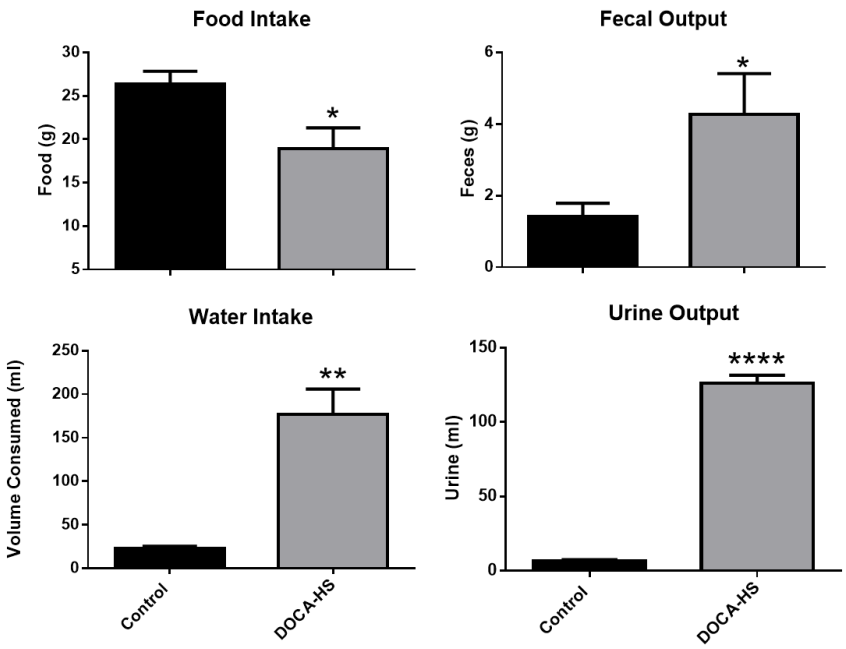

Figure 3.6: 24-hour metabolic data of control (n=4) and DOCA-salt (n=3) treated rats following 3-weeks of treatment. DOCA-salt treatment significantly reduced food intake when compared to control rats, but significantly increased water intake, fecal output, and urine output when compared to control rats. *P<0.05, **P<0.01, ****P<0.0001 vs. control using an unpaired two-tailed T-test.

Following 3-weeks of treatment, DOCA-salt rats (n=3) showed significant differences in metabolism when compared to controls (n=4). DOCA-salt treatment resulted in a significant decrease in food intake (control: 26.39±1.45 vs. DOCA-salt: 18.91±2.42 g, P<0.05), as well as a significant increase in fecal output (control: 4.26±1.15 vs. DOCA-salt: 1.41±0.38 g, P<0.05) when compared to control rats (n=4) **(Fig. 3.6)**. Also, DOCA-salt treatment resulted in a significant increase in water intake (control: 22.72±2.719 vs. DOCA-salt: 176.7±29.24 ml, P<0.01) and urine output (control: 6.8±0.578 vs. DOCA-salt: 126.1±5.346 ml, P<0.0001) compared to controls **(Fig. 3.6).** A decrease in weight gain was also observed following DOCA-salt (n=7) treatment when compared to control rats (n=6) (control: 129±6.429 vs. DOCA-salt: 81.71±8.626 g, P<0.01) **(Fig. 3.7).**

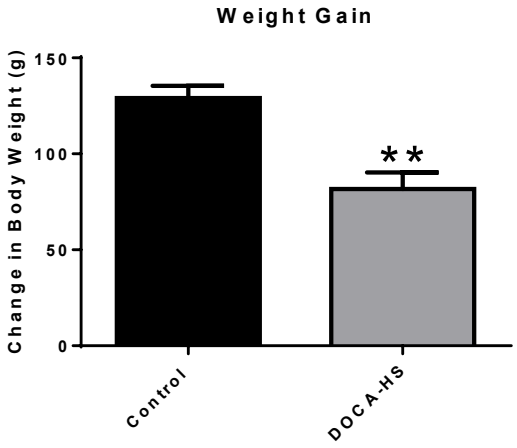

Figure 3.7: Difference in body weight from beginning and end of treatment. DOCA-salt (n=7) treatment results in a significant attenuation of weight gain compared to control rats (n=6). **P<0.01 using an unpaired two-tailed T-test.

3.4 Central Orexin-A Administration Increases Plasma AVP Concentration in Normal SD rats

Following affirmation that plasma AVP levels are indeed increased following DOCA-salt treatment, we then wished to find out whether orexin system activity was a primary regulator of AVP release peripherally. Previous research done in our lab showed a significant increase in PVN AVP protein expression following acute OXA ICV injection in normal rats (Huber, Fan et al. 2017). Because centrally administered OXA results in increased PVN production of AVP, we then tested whether central administration of OXA causes a parallel increase in AVP secretion into peripheral circulation. To test this, six rats were given ICV injections of either 0.9% saline (n=3) or OXA (2nmol) (n=3). Ten minutes following injection, animals were sacrificed and plasma was collected for AVP ELISA measurement.

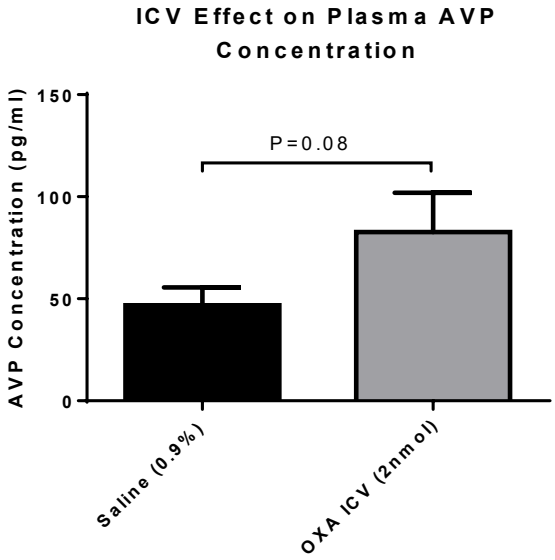

Figure 3.8: Plasma AVP concentrations (pg/ml) following ICV injection of saline (0.9%) (n=3) or OXA (2nmol) (n=3). OXA ICV elicited a noticeable increase in AVP plasma levels, although this did not reach significance (P=0.08) when using an unpaired one-tailed T-test.

Although significance was not reached (P=0.08), an obvious trend towards significance was observed as OXA ICV (n=3) elicited an increase in plasma AVP levels when compared to saline controls (n=3) (saline: 46.86±8.73 vs. OXA: 82.69±19.23 pg/ml) **(Fig. 3.8)**. This relationship indicates that acute central injection of the OX1R agonist, OXA, results in an increase in peripheral AVP circulation, leading us to believe that long-term orexin system over-activation may lead to a chronic increase in plasma AVP often observed in the DOCA-salt model.

All data collected has shown that DOCA-salt treatment increases not only central and peripheral AVP expression, but also increases PVN OX1R expression. Furthermore, acute injection of the OX1R agonist, OXA, results in a near significant increase in peripheral AVP in the plasma. However, these results do not prove an interaction between orexin system activation and regulation of AVP release in the DOCA-salt model, nor do they give any insight into long-term orexin mediated AVP release. To analyze the chronic impact of the orexin system

on DOCA-salt hypertension, rats were given bilateral PVN injections of AAV2-OX1R-shRNA, a viral vector carrying a short hairpin RNA that specifically targets and knocks down the gene that encodes for OX1R, two-weeks prior to DOCA-salt treatment.

3.5 Lateral Hypothalamic OXA Neuron Expression Remains Elevated Following Chronic PVN OX1R Knockdown

To investigate the relative concentration of protein expression of orexin related proteins, immunostaining was performed, which allows visualization of target protein expression within the relevant brain regions.

We first chose to investigate whether orexin producing neuron expression within the LH, as well as axonal projection density in the PVN is increased following DOCA-salt treatment. To test this, following three weeks of DOCA-salt treatment, animals were transcardially perfused with cold 4% PFA, and brains were removed. Coronal sections of the brain were then taken in areas that corresponded to positions of the LH and PVN. These sections were subjected to immunostaining, specifically targeting OXA. Following DOCA-salt treatment, it appears that OXA expressing cell bodies within the LH were increased when compared to SD untreated control rats **(Fig. 3.9A)**. Interestingly, there did not appear to be any major differences between PVN axonal projections in the DOCA-salt rats when compared to the controls **(Fig. 3.9B)**.

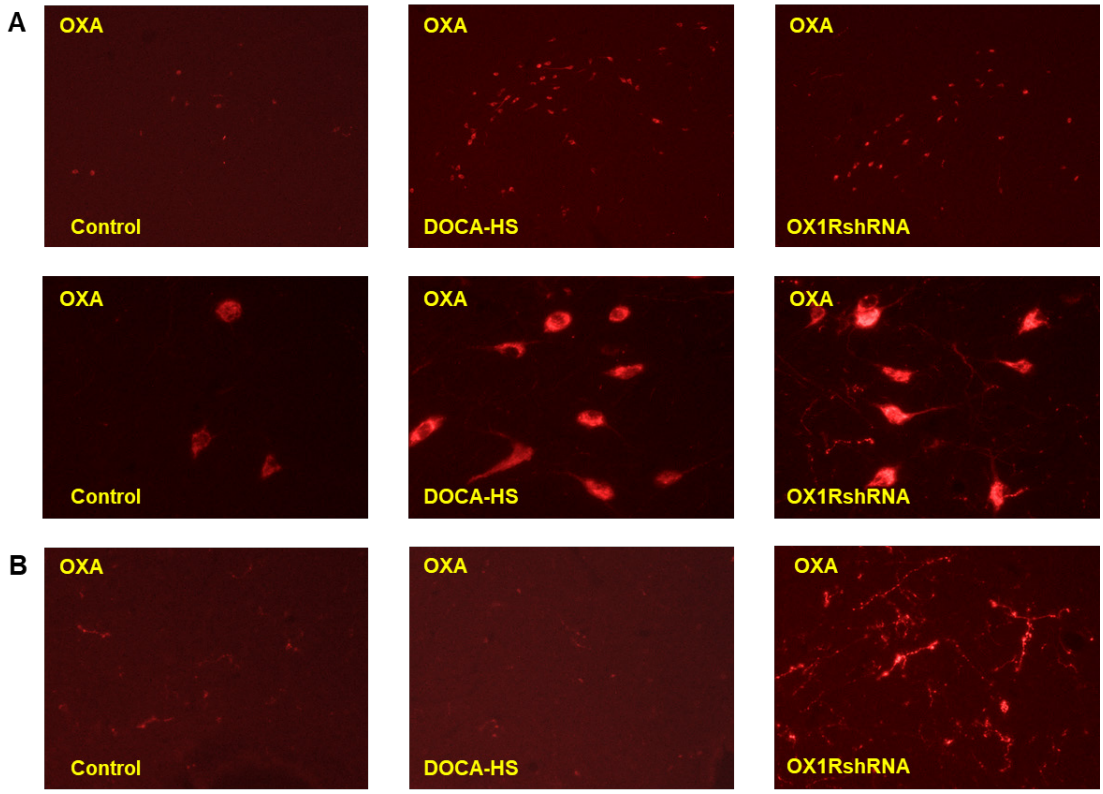

Figure 3.9: Representative immunostaining images showing expression of orexin-A (OXA) within (A) the lateral hypothalamus (LH) as well as (B) the Paraventricular Nucleus of the Hypothalamus. The first column shows untreated control rats. The second column shows DOCA pellet (75mg, 21-day release) and high salt (1%NaCl/0.2%KCl) treated rats (DOCA-HS). The third column shows rats that received prior orexin-1 receptor viral antagonist bilateral microinjection into the PVN two weeks prior to the DOCA-salt treatment (OX1RshRNA).

A similar increase in OXA cell body expression within the LH can be observed in rats that received AAV2-OX1R-shRNA (OX1R antagonist) prior to DOCA-salt treatment (**Fig. 3.9A**) when compared to untreated controls. However, in contrast to the DOCA-salt rats, PVN OXA axonal projections from the LH appear to have a greater expression and density in rats who received bilateral PVN OX1R antagonist injections (**Fig. 3.9B**).

3.6 Chronic PVN OX1R Knockdown Decreases OX1R Expression

The above results show that DOCA-HS treatment increases OXA neuron cell body immunoreactivity, indicating an intrinsic increase in LH orexin production and activity. However, we also wanted to see how the treatment would affect PVN OX1R, the receptor for OXA, expression and immunoreactivity.

PCR analysis of the PVN area showed that OX1R mRNA expression is increased by approximately 35% following DOCA-salt treatment (1.348±0.109; n=10) when compared to control rats (1±0.087; n=7) **(Fig. 3.10).** However, PVN OX1R knockdown significantly decreases OX1R mRNA expression (0.8537±0.072; n=5) when compared to DOCA-salt treated rats (P<0.05) **(Fig. 3.10)**, indicating that viral vector application to the PVN effectively reduces OX1R function within the PVN to normal levels.

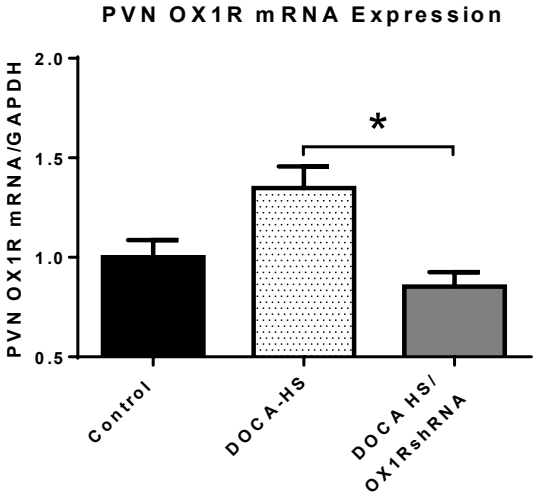

Figure 3.10: PVN OX1R mRNA levels of expression normalized to GAPDH expression. DOCA-salt treated rats (n=10) show an increase of approximately 34% PVN OX1R expression compared to control rats (n=7), and this increase is significantly reduced following OX1R knockdown (n=5) (P<0.05) using a one-way ANOVA and Tukey post-hoc analysis.

Immunostaining analysis showed an increase in OX1R expression within the PVN of DOCA-salt rats when compared to control rats (**Fig. 3.11**), in agreement with our previous results **(Fig. 3.4)**. We then wished to test whether bilateral injection of AAV2-OX1R-shRNA into the PVN of DOCA-salt rats reduces the density of OX1R within the PVN. As shown above, OX1R expression is increased in the DOCA-salt rats when compared to the controls (**Fig 3.4**). However, following injection of AAV2-OX1R-shRNA prior to DOCA-salt treatment, OX1R expression is successfully knocked down in the PVN (**Fig 3.11**), as evidenced by the lack of co-localization of OX1R and GFP expressing neurons.

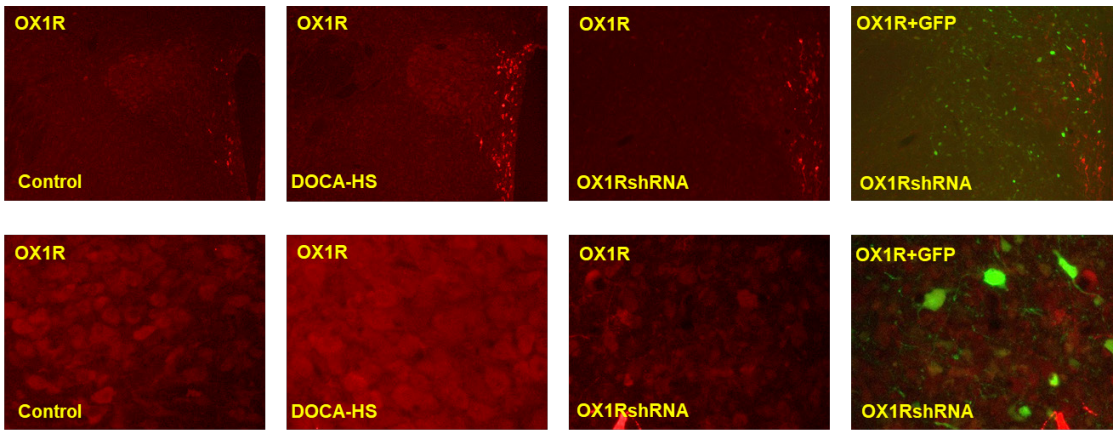

Figure 3.11: Representative immunostaining images showing orexin-1 receptor (OX1R) expression within the PVN of control (far left), DOCA-salt (middle left), and DOCA-salt rats injected with AAV2-OX1R-shRNA into the PVN (middle right). The farthest image on the right shows co-immunostaining of both OX1R as well as green fluorescence protein (GFP) that is intrinsically present in the viral vector used for OX1R knockdown. The lack of OX1R expression in areas of high GFP expression is indicative of successful OX1R knockdown following viral vector injection.

3.7 Chronic PVN OX1R Knockdown Reduces Central AVP Production

The above combination of results showing an increase in PVN OX1R mRNA and protein levels, as well as an increase in LH OXA protein expression indicate over activation of the orexin system as a key component in the development of

DOCA-salt hypertension. To test whether the decrease in OX1R function following viral injection resulted in a parallel decrease in central AVP mRNA expression, PVN tissue was punched and subjected to PCR assessment. PVN AVP mRNA levels were significantly increased following DOCA-salt treatment when compared to controls (control: 1±0.319 vs. DOCA-salt: 3.142±0.791, P<0.05) **(Fig. 3.12)**. However, following chronic knockdown of PVN OX1R, AVP mRNA levels were significantly decreased when compared to DOCA-salt (DOCA-salt: 3.142±0.791 vs. DOCA-OX1RshRNA: 0.644±0.281, P<0.05) **(Fig. 3.12)**. There was no significant difference observed between control and DOCA-OX1RshRNA groups.

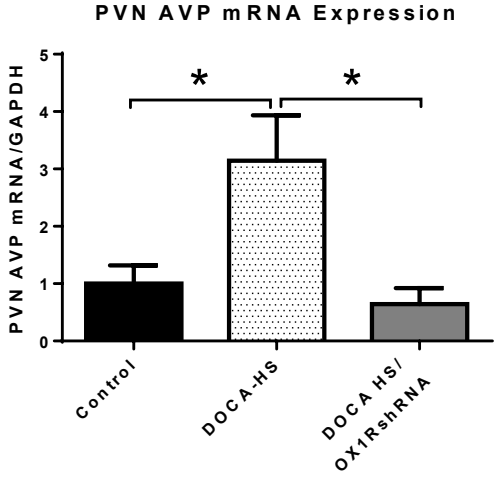

Figure 3.12: PVN AVP mRNA levels of expression normalized to GAPDH expression. DOCA-salt treated rats (n=10) have a significant increase in PVN AVP expression when compared to control (n=7) (*P<0.05) as well as when compared to DOCA-HS/OX1RshRNA (n=5) (*P<0.05) rats using a one-way ANOVA and Tukey post-hoc analysis.

Knockdown of OX1R results in a decrease in mRNA levels of OX1R as well as AVP within the PVN, along with subsequent OX1R protein immunofluorescence, indicating that the orexin system over activation can be corrected via bilateral microinjection of an OX1R antagonist into the PVN of DOCA-salt rats. We then wished to elucidate whether chronic OX1R knockdown would result in a subsequent decrease in AVP protein expression within the PVN.

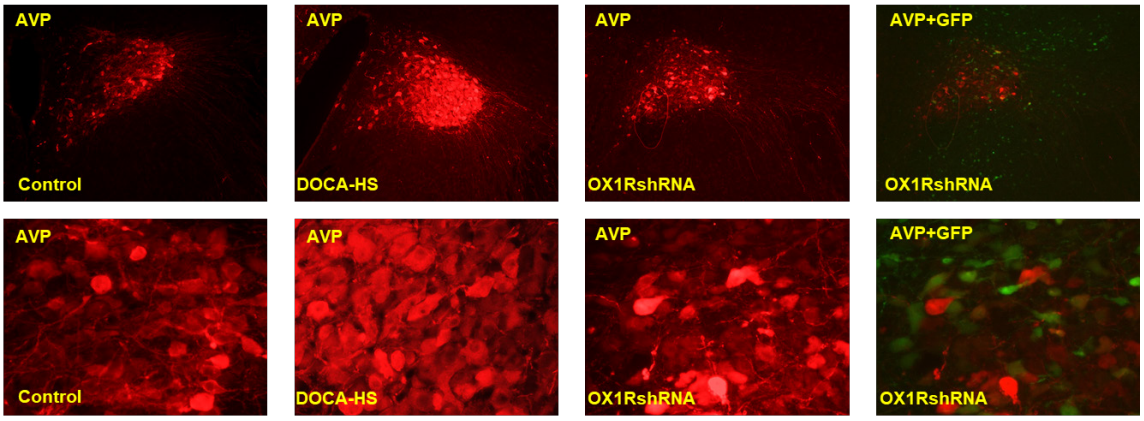

Figure 3.13: Representative immunostaining images showing arginine vasopressin (AVP) expression within the PVN of control (far left), DOCA-salt (middle left), and DOCA-salt rats injected with AAV2-OX1R-shRNA into the PVN (middle right). The farthest image on the right shows co-immunostaining of both AVP as well as green fluorescence protein (GFP) that is present in the viral vector used for OX1R knockdown. The lack of AVP expression in areas where GFP is high is indicative of successful decrease in AVP expression following OX1R knockdown.

AVP expression within the PVN was greatly increased in DOCA-salt rats compared to control rats, as can be observed by increased density and brightness of AVP expression following immunostaining (**Fig. 3.13**). However, prior microinjection of an OX1R antagonist (AAV2-OX1R-shRNA) reduces the increased AVP expression that is normally seen in DOCA-salt rats (**Fig. 3.13**). This indicates strong evidence for orexin system modulation of AVP production and subsequent release following a DOCA-salt treatment.

3.8 Chronic PVN OX1R Knockdown Decreases Plasma AVP Concentration

After observing increases in mRNA and protein expression of both OX1R as well as AVP within the PVN of DOCA-salt rats, and the attenuation of this following chronic OX1R knockdown, we began to investigate peripheral circulation of AVP. We wished to observe whether central OX1R knockdown results in lowered plasma AVP levels. We hypothesized that OX1R PVN knockdown would

significantly decrease plasma AVP concentrations when compared to DOCA-salt treated rats.

To test the chronic effects of orexin system modulation of peripheral AVP secretion, following three weeks of DOCA-salt treatment, plasma as well as CSF were collected as previously outlined, and subjected to AVP and OXA ELISA testing.

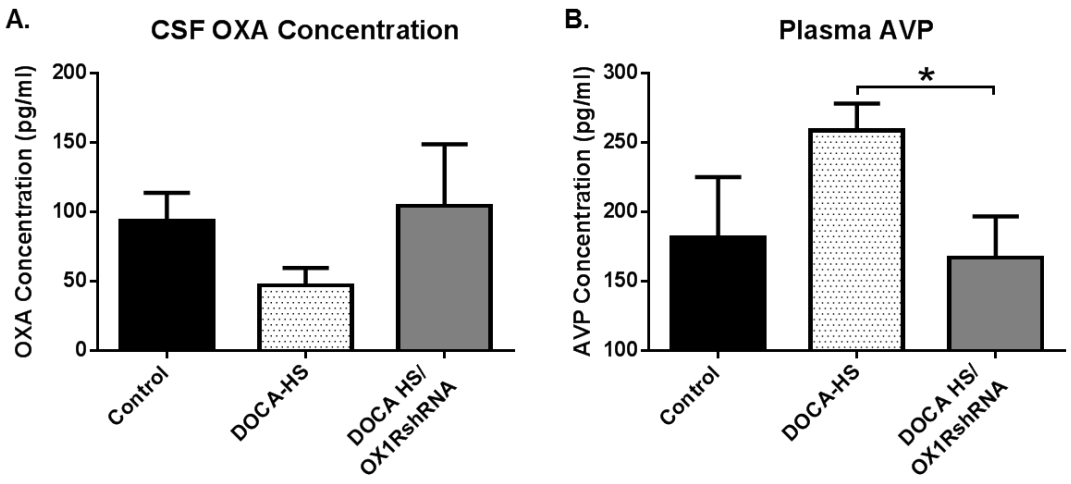

Figure 3.14: (A) Cerebrospinal fluid OXA concentration in control (n=5), DOCA-HS (n=3), and DOCA HS-OX1RshRNA rats (n=3). DOCA-salt treatment reduced circulating CSF OXA, and this is mitigated by PVN OX1R knockdown, although this relationship did not reach significance (P=0.37). (B) Plasma AVP concentration (pg/ml) in control (n=3), DOCA-salt (n=6), and DOCA HS-OX1RshRNA (n=3) rats. DOCA-salt treated rats show an increased AVP plasma concentration, but this did not reach significance (P=0.09). However, following two-tailed t-test evaluation, we observed a significant decrease in plasma AVP following chronic OX1R knockdown (*P<0.05) when compared to DOCA-salt treatment using a one-way ANOVA and Tukey post-hoc analysis.

Interestingly, we observed an apparent decrease in CSF OXA concentration in DOCA-salt treated rats (47.04±12.5 pg/ml; n=3) when compared to both control (93.62±20.25 pg/ml; n=5) and DOCA-salt/OX1RshRNA (104.6±44.34 pg/ml; n=3) rats **(Fig. 3.14A)**. Although this observation did not reach significance (P=0.37), further explanation of this result can be found in the

discussion. Furthermore, there is a strong trend towards significance (P=0.09) in the long-term increase of plasma AVP in DOCA-salt (258.9±19.5 pg/ml; n=5) when compared to control (181.7±43.6 pg/ml; n=3) and DOCA-salt/OX1RshRNA (167.2±29.8 pg/ml; n=3) **(Fig. 3.14B)**. However, because the trend was very strong, we decided to determine whether OX1R PVN knockdown significantly effects plasma AVP levels when compared only to DOCA-salt treated rats. Following two-tailed t-test evaluation of DOCA-salt and DOCA-HS/OX1RshRNA rats, we found that the OX1RshRNA treatment significantly decreased plasma AVP when compared solely to DOCA-salt treatment (P=0.035) **(Fig. 3.14B)**. This trend is indicative of long-term modulation of plasma AVP secretion by central orexin system functioning.

3.9 Chronic PVN OX1R Knockdown Effects on Metabolism

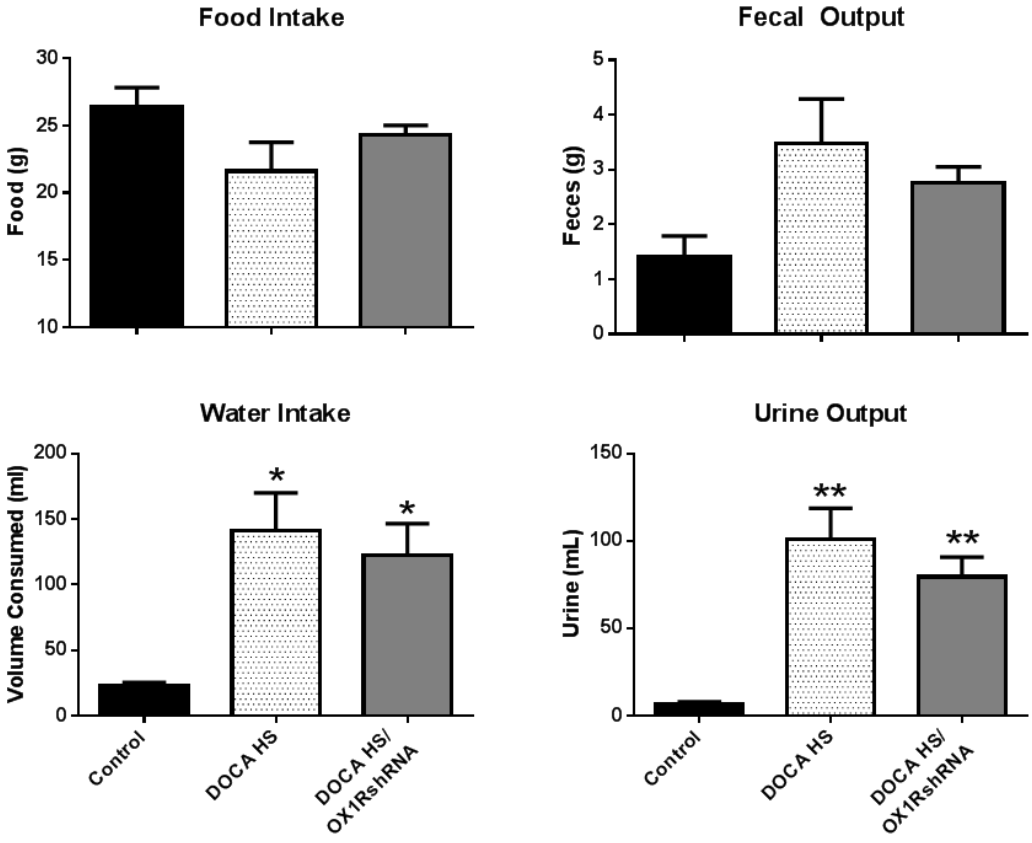

Figure 3.15: 24-hour metabolic analysis of food and water intake as well as urine and fecal output in all rats at the end of their respective treatments. DOCA-salt treatment results in a significant increase in both water intake and urine output when compared to control rats, and chronic knockdown of OX1R does not appear to alleviate this effect. Control (n=4), DOCA-salt (n=5), DOCA+OX1RshRNA (n=6). *P<0.05 vs. control, **P<0.01 vs. control using a one-way ANOVA and Tukey post-hoc analysis.

Metabolic measurements were taken following 3-weeks of DOCA-salt treatment. Over the course of 24-hours at the end of the treatment, DOCA-salt rats (n=5) experienced a significant increase in water intake when compared to control rats (n=4) (DOCA-salt: 141.3±28.89 vs. control: 22.72±2.72mL, P<0.05), as well as increased urine output (DOCA-salt: 101±17.81 vs. control: 6.8±0.578mL, P<0.01) (**Fig. 3.15**). This significant increase was also observed in DOCA-OX1RshRNA rat (n=6) water intake (DOCA-OX1RshRNA: 122.1±24.43 vs

control: 22.72±2.72mL, P<0.05) and urine output (DOCA-OX1RshRNA: 79.58±11.3 vs. control: 6.8±0.578mL, P<0.01) when compared to control rats (**Fig. 3.15**). There was no observed statistical difference between DOCA-salt and DOCA-OX1RshRNA water intake and urine output. Similarly, no significant differences were observed in food intake (control: 26.39±1.455 vs. DOCA-salt: 21.62±2.129 vs. DOCA-OX1RshRNA: 24.32±0.699g, P=0.1) or fecal output (control: 1.41±0.378 vs. DOCA-salt: 3.48±0.813 vs. DOCA-OX1RshRNA: 2.76±0.295g, P=0.07) between any of the groups (**Fig. 3.15**).

During the three-week treatment, body weight was taken once per week in control (n=10), DOCA-salt (n=17), as well as DOCA+OX1RshRNA (n=5) groups. We then assessed the difference in body weight from the beginning of treatment and the day of euthanization. This allowed us to assess the affect that DOCA-salt as well as DOCA-salt + OX1RshRNA may have on weight gain.

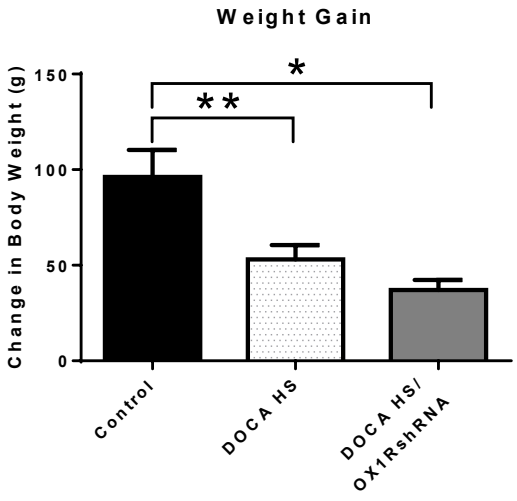

Figure 3.16: Graph of the difference in weight between the beginning and end of treatment in all groups. A significant dampening of weight gain is observed in both DOCA-salt (n=17) as well as DOCA+OX1RshRNA (n=5) rats compared to untreated controls (n=10). *P<0.05, and **P<0.01 vs. control using a one-way ANOVA and Tukey post-hoc analysis.

DOCA-Salt treatment caused a significantly lower weight gain over the course of three weeks when compared to the control rats (control: 86±16.34 vs. DOCA-salt: 53.06±7.54 g, P<0.01) (**Fig. 3.16**). DOCA-OX1RshRNA rats also had a significantly lower weight gain than control rats (control: 86±16.34 vs. DOCA-OX1RshRNA: 37.1±5.26 g, P<0.05) (**Fig. 3.16**). Although there was no significant difference between DOCA-salt and DOCA+OX1RshRNA rats, there appears to be a slight trend towards a greater attenuation of weight gain in OX1R knockdown rats (**Fig. 3.16**).

3.10 Cardiac Hypertrophy is Partially Attenuated by PVN OX1R Knockdown

Cardiac hypertrophy is often associated with hypertension development, and may cause further cardiovascular dysfunction. To test cardiac hypertrophy, rat hearts were removed and weighed following animal euthanization. Cardiac hypertrophy was quantified by measuring the heart weight to body weight ratio (HW:BW), which allows assessment of the percentage of the animal body weight that is accounted for by the heart.

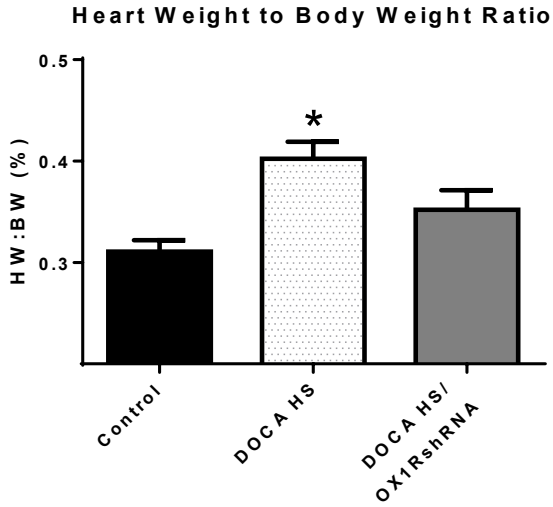

Figure 3.17: Quantification of cardiac hypertrophy through measurement of heart weight to body weight ratio (HW:BW). DOCA-salt (n=13) showed a significantly increased heart size when compared to control rats (n=8). DOCA-OX1RshRNA

(n=5) showed a slight decrease in HW:BW compared to DOCA-salt rats, but this trend did not reach significance using a one-way ANOVA and Tukey post-hoc analysis. *P<0.01 vs. control.

DOCA-salt rats (n=13) had a significantly higher HW:BW when compared to control rats (n=8) (control: 0.311±0.011 vs. DOCA-salt: 0.402±0.017%, P<0.01). DOCA-OX1RshRNA rats (DOCA-OX1RshRNA: 0.352±0.019%) showed no significant differences when compared to either control or DOCA-salt rats, although there is a slight decrease when compared to DOCA-Salt rats (DOCA-OX1RshRNA: 0.352±0.019 vs. DOCA-salt: 0.402±0.017%, P>0.05) (**Fig. 3.17**), indicating that OX1R knockdown may slightly alleviate the cardiac hypertrophy observed in DOCA-salt hypertension, even though significance was not reached.

3.11 PVN OX1R Knockdown Attenuates Elevation of Blood Pressure in DOCA-salt Rats

Because over activation of the orexin system in the PVN is observed in the DOCA-salt model, and largely attenuated following PVN OX1R knockdown, as well as AVP, we decided to analyze whether OX1R PVN knockdown alleviated DOCA-salt hypertension development. Using tail-cuff plethysmography, rats were acclimated to blood pressure measurement for one week prior to the start of the study. Control (n=5), DOCA-salt (n=4), and DOCA-OX1RshRNA (n=4) were all from the same litter and measurements were performed during the same time. The last day of the acclimation period was used as the baseline blood pressure measurement. Following acclimation, blood pressure and heart rate were taken over the course of 18 days. We did not perform BP measurements the last 3 days of treatment because the rats were in metabolic cages at this time, and transport to and from the cages to take BP measurements may have skewed the recordings. Also, to keep the rats acclimated, they were placed in the holders every day for 20 minutes on days when recordings were not measured. This was done to lower anxiety, and keep results consistent. Two rats, one from the DOCA-salt and one from the DOCA-OX1RshRNA, were removed from blood pressure

and heart rate measurements due to illness resulting in lack of food and water intake, as well as physical deformity and body weight loss, all of which are factors that may affect AVP, orexin activity, among other physiological parameters. Because of these physiological implications, they were excluded from measurement.

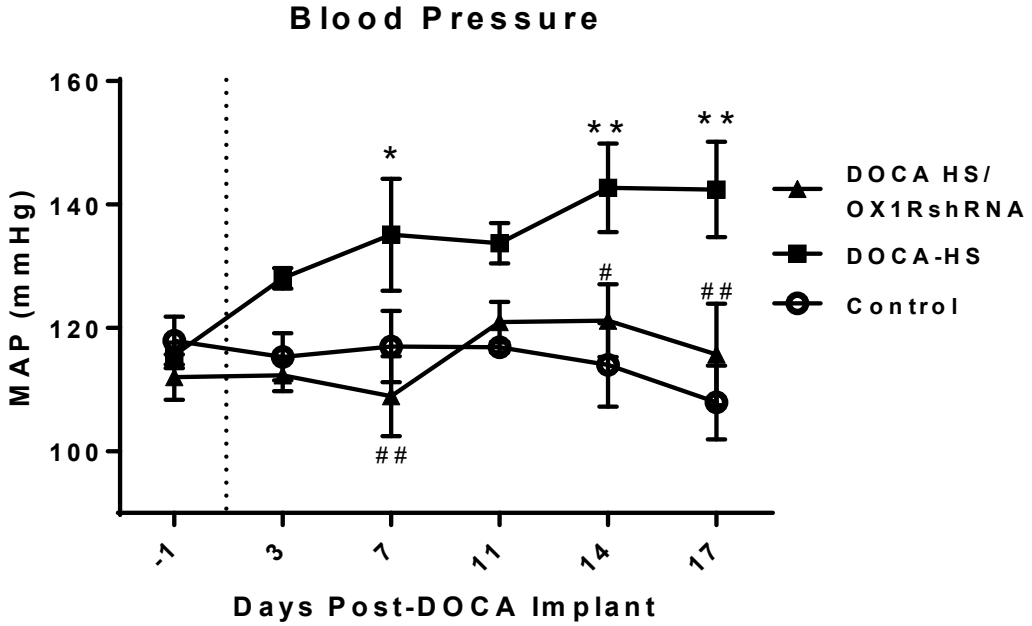

Figure 3.18: Mean arterial pressure (MAP) measurements over the course of 18 days as measured by tail-cuff plethysmography comparing control (n=5), DOCA-salt (n=4), and DOCA+OX1RshRNA (n=4) rats. Results are reported as group averages during each measurement day. Dotted line indicates when DOCA-pellet and HS diet were administered. *P<0.05, **P<0.001 vs. control. #P<0.05, ##P<0.01 vs. DOCA-salt using a two-way ANOVA and Tukey post-hoc analysis.

Approximately one week following DOCA implantation, the DOCA-salt group MAP raised to a point significantly greater than the control group (P<0.05) (**Fig. 3.18)**. This significant increase was maintained throughout treatment for the most part. Interestingly, PVN OX1R knockdown resulted in a significant, and sustained decrease in MAP when compared to the DOCA-salt rats (**Fig. 3.18)**. There was no significant difference between control and DOCA+OX1RshRNA

groups at any point during the treatment (**Fig. 3.18)**. At the end of the treatment, the final blood pressure measurements were as follows: control: 107.91±5.99 vs. DOCA-salt: 142.43±7.73 vs. DOCA+OX1RshRNA: 115.69±8.23 mmHg. Although there was a slight increase in DOCA-OX1RshRNA MAP, this was never a significant increase compared to control rats, but was significantly lower than the DOCA-salt group ($P<0.01$), indicating that OX1R knockdown within the PVN alleviates, at least in part, DOCA-salt induced hypertension, effectively reducing the normal MAP that is observed in DOCA-salt hypertension.

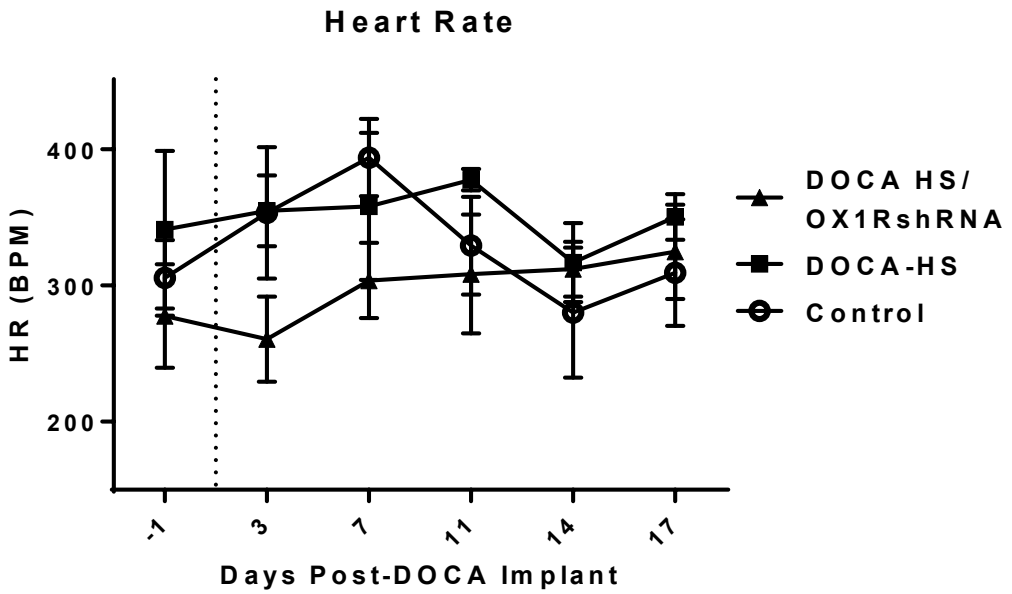

Figure 3.19: Heart rate recordings taken in the same rats that received BP measurements over the course of 18 days. The dotted line indicates beginning of DOCA-pellet and HS administration. There were no significant differences in heart rate between groups using a two-way ANOVA and Tukey post-hoc analysis.

Despite the change in blood pressure between groups, there were no significant differences in heart rate (HR) between groups at any point during the treatment (**Fig. 3.19)**. At the end of the 18-day measurements, measured heart rates were recorded as follows: controls (HR of 309.44±39.14 BPM), DOCA-salt (350.44±16.76 BPM), and DOCA-OX1RshRNA (324.82±34.59 BPM).

4 Discussion

Approximately one-third of adults in the United States have hypertension, which puts them at a much higher risk for further cardiovascular events (Centers for Disease and Prevention 2011), effectively increasing the likelihood of premature death. Of that one-third of the population affected by hypertension, it is estimated that slightly more than half of those individuals also have salt sensitive hypertension (SSH) (Weinberger, Miller et al. 1986). However, despite advancements in hypertension research, the exact mechanism underlying its pathology has yet to be fully understood. Recently, the impact that central orexin function has on blood pressure regulation in various hypertensive rat models has been gaining attention (Schwimmer, Stauss et al. 2010, Lee, Dai et al. 2013, Li, Hindmarch et al. 2013, Lee, Tsai et al. 2015), with even more recent research observing the potential role of orexin signaling in SSH (Huber, Fan et al. 2017). However, the potential role of orexin in the DOCA-salt rat, a model for salt-sensitive hypertension and hyperaldosteronism, has not yet been studied. To our knowledge, this is the first study conducted focused solely on the effect of orexin system function on the development and maintenance of hypertension in this model. Our study reports five major findings: I.) Expression of AVP and OX1R are increased in the PVN of DOCA-salt rats, and these increases are attenuated following prior PVN OX1R knockdown; II.) OXA expressing cells within the LH are increased in both DOCA-salt and DOCA-OX1RshRNA rats, and their axonal projections to the PVN are increased in the DOCA-OX1RshRNA rats; III.) Plasma AVP is increased following DOCA-salt treatment as well as OXA ICV, but is markedly decreased following PVN OX1R knockdown; IV.) Cardiac hypertrophy is present in DOCA-salt rats, and is at least partially attenuated following OX1R PVN knockdown; and lastly V.) The development of hypertension in DOCA-salt rats can be significantly attenuated following PVN OX1R knockdown. The combination of these results indicates a potential crucial role for central orexin system functioning in salt sensitive hypertension models.

The PVN is a major area of cardiovascular integration, making it critical to proper blood pressure regulation (Pan 2004, Wei, Yu et al. 2009, Ribeiro, Panizza Hdo et al. 2015). One of the primary mechanisms underlying PVN regulation of blood pressure is the production and release of AVP from magnocellular neurons. Upon stimulation from peripheral osmolality and pressure changes, the CVOs, which lack a blood brain barrier, send afferent signals to the PVN, causing the production and subsequent release of AVP to the posterior pituitary, which releases it to peripheral circulation allowing it to cause both water reabsorption as well as vasoconstriction (Sunn, McKinley et al. 2003, Pan 2004, Kawano and Masuko 2010). Due to AVP's established role in long term blood pressure regulation, as well as the presence of elevated AVP in many human hypertensive patients (Cowley, Cushman et al. 1981), RAAS activity, which can trigger AVP release, has become a major target for pharmaceutical intervention (Jarari, Rao et al. 2015). However, these pharmaceutical aids are not always effective, which can lead to the development of further complications such as resistant hypertension, making it important to find other forms of antihypertensive treatment. Previous work within our lab has found that PVN orexin functioning greatly influences both AVP release and subsequent sympathetic outflow in Dahl-S rats, a genetic model of primary hypertension (Huber, Fan et al. 2017). Because AVP is essential to the development of DOCA-salt hypertension, we hypothesized that central orexin system functioning may play a role in DOCA hypertension. In agreement with numerous other studies (Grillo, Saravia et al. 1998, Pietranera, Saravia et al. 2004), we found that mRNA and protein expression of AVP are elevated within the brain of DOCA-salt rats, specifically in the PVN **(Fig. 3.1 & 3.2)**. We also observed an increase in plasma AVP **(Fig. 3.5)**, similar to other studies (Saravia, Grillo et al. 1999). However, our study is novel in that it is the first to report elevated OX1R within the PVN **(Fig. 3.3 & 3.4)**, as well as OXA in the LH **(Fig. 3.9)** following DOCA-salt treatment. This, combined with the implications of orexin function in blood pressure regulation led us to believe that

the over active orexin system in the DOCA model may contribute to the development of hypertension in this model.

We then found that acute central administration of OXA resulted in a drastic increase in plasma AVP **(Fig. 3.8)**, showing central OXA action elicits a substantial effect on AVP release from the neurohypophysis. We chose to determine the effects that chronic knockdown of PVN OX1R may have on the development of hypertension in SSH models, to discover the long-term impact orexin played on both central as well as peripheral AVP circulation. To do this, we used an adeno-associated recombinant virus that specifically inhibits function of OX1R. Following previous models in which central knockdown of orexin receptor function resulted in attenuation of blood pressure increases (Shahid, Rahman et al. 2012, Lee, Dai et al. 2013), we performed bilateral microinjections of AAV2-OX1R-shRNA into the PVN of rats prior to DOCA pellet implantation. In agreement with our hypothesis, we found that OX1R PVN knockdown significantly reduced PVN OX1R and AVP mRNA **(Fig. 3.10 & 3.12)**, as well as PVN OX1R and AVP protein expression **(Fig. 3.11 & 3.13)**. In addition, chronic OX1R knockdown blocked the increase in plasma AVP induced by DOCA-salt treatment **(Fig. 3.14B)**. Furthermore, significant decreases in blood pressure were observed in both the development and maintenance phases of DOCA-salt hypertension development **(Fig. 3.18)**.

Our results largely affirm our hypothesis that DOCA-pellet implantation causes increased orexin release from the LH which causes excitation of PVN magnocellular neurons through interaction with OX1R, which causes release of AVP into the peripheral circulation, resulting in vasoconstriction and increased blood volume, eventually leading to the development of hypertension. However, one discrepancy was that OXA axonal projections appeared to be more dense and over-active in the DOCA-OX1RshRNA rats, as opposed to the DOCA-salt rats, who did not appear to have much difference compared to the control. In

addition, although it was not significant, DOCA-salt rats also appeared to have a lower CSF OXA concentration **(Fig. 3.14A)**, which we did not expect. However, this can be reasonably attributed to action of a feedback loop initiated through OX1R knockdown. The lack of OXA binding to OX1R in the PVN would be sensed and cause a greater input of axonal OXA to the PVN to try to elicit a response. Since the OX1R receptors were increased and abundant in the DOCA-salt rats, this same feedback loop may not have been initiated, although further studies must be conducted to adequately explain this discrepancy. Another possible explanation, more so for the decreased CSF OXA, is that the decrease of CSF OXA is indicative of a greater number of bound OXA molecules, meaning a greater increase of central orexin system function. Since the DOCA-HS/OX1RshRNA rats had fewer active binding sites on OX1R, more OXA was left unbound, unable to elicit its actions through binding with its receptor. It is important to note that OXA immunoreactivity was however increased in both DOCA-salt as well as DOCA-OX1RshRNA rats within the LH, meaning that despite OX1R knockdown in the PVN, OXA production remained elevated due to the DOCA-salt treatment. This potentially strengthens the argument that OXA production is increased in SSH, and that the increase in PVN OXA axonal projections in the DOCA-OX1RshRNA rats compared to the DOCA-salt can be contributed to receptor availability. It also offers evidence that a potential method of hypertension treatment to be explored is through modulation of central orexin receptor activity, as opposed to the receptor ligands.

There are a few reasons why we chose to use a DOCA model for salt-sensitive hypertension. Approximately 10-30% of individuals are resistant to hypertensive drug treatment, such as ACE inhibitors and ANGII receptor blockade, and are diagnosed with resistant hypertension (Calhoun, Jones et al. 2008, Lee, Dai et al. 2013, Sim, Bhandari et al. 2013). The DOCA model serves the dual purpose of allowing us to observe how orexin functions in another salt sensitive model, and more specifically allows us to accurately model

hyperaldosteronism, which is the primary cause of resistant hypertension (Mulatero, Rabbia et al. 2002, Eide, Torjesen et al. 2004, Viera and Neutze 2010). Our findings show that there is potential for orexin involvement in DOCA-salt hypertension development and maintenance due to increased presence and heightened functioning of the central orexin system, specifically through modulation of AVP production and release. We also chose to use a modified model of DOCA-salt rats to undertake this project. Namely, we omitted uninephrectomy from the protocol. We did this following a similar procedure that had been previously done (Kandlikar and Fink 2011). Excluding the uninephrectomy allows us to more accurately observe the direct effect that manipulations to orexin system function within the PVN has on hypertension development, without any adverse cardiovascular implications that kidney removal incurs (Kandlikar and Fink 2011). We believe that this model allows us to more closely mimic the development of human hypertension, which tends to occur more gradually, as opposed to the drastic, immediate spike that occurs in uninephrectomized rats. Lastly, since orexin function has been observed in primary hypertensive models such as the SHR and Dahl-S rat strains, and now has possible implications in the DOCA-salt model, this may point to an important underlying role for orexin in numerous different types of hypertension, making it more practical for human application.

4.1 Implications

Even with current hypertensive medications in use, the prevalence of hypertension and cardiovascular disease remains high. Research has been conducted that has evaluated the implications of orexin system function on blood pressure regulation, as well as SSH. The present research project offers the only look into the potential role for the orexin system in development and maintenance of DOCA-salt hypertension, primarily aldosteronism, the primary cause of resistant hypertension. This helps strengthen the notion that orexin is a major

regulator of cardiovascular function, and may help lead to future, more efficient pharmaceutical interventions targeting orexin function.

4.2 Limitations

A few limitations are present in this study. First, the use of tail-cuff plethysmography instead of blood pressure radio telemetry transducer implantation may have added more variability to the blood pressure recordings. Stress induced blood pressure changes may occur due to confinement in the plethysmography holder. However, careful consideration was taken when animals were introduced and acclimated to the procedure. This included a week of acclimation before even beginning the study, as well as daily acclimation to maintain proper comfort in the tubes. Furthermore, the efficacy of tail-cuff plethysmography has been adequately reviewed (Feng, Whitesall et al. 2008), and the blood pressure measured by tail cuff plethysmography has been shown to resemble that measured by the radio-telemetry transducer in undisturbed animals (Wilde, Aubdool et al. 2017). With all of this in mind, we do recognize the variability associated with tail-cuff plethysmography, but feel we have adequately reduced stress-induced blood pressure changes through our meticulous acclimation protocol.

We further recognize that, during PVN mRNA testing, punching of the PVN is not exact. This means that some of the brain area surrounding the PVN area may be subjected to mRNA assessment as well, which may dilute our results. It is difficult to punch the PVN alone since it is such a small area, and for this reason, some variability in mRNA levels may be present. However, when paired with the use of protein immunostaining, the mRNA results become more reliable, since parallel increases in gene expression as well as the proteins they encode for are observed.

Lastly, our project only used male rats, without any female groups. It has been observed that testosterone plays a large role in the development of hypertension, and that males are more likely to have elevated blood pressure in both animals and humans alike. The findings of this research may not encompass the effects of DOCA-salt or PVN OX1R knockdown on females. Similar studies conducted using female rats will be the goal of future studies, as will the differences in orexin system function and subsequent hypertensive tendencies between male and female animals.

Future directions to build from this study may include more chronic responses to OX1R knockdown in DOCA-salt rats, since most studies are only conducted over the course of three weeks using this model. Also, we would like to further study the peripheral effects of PVN OX1R knockdown in DOCA-salt rats, namely on kidney and heart function. Lastly, we would like to provide evidence of potential implications of orexin on adrenal gland dysfunction, which is essential to the pathogenesis of DOCA-salt development.

4.3 Conclusion

In conclusion, the present study has shown that there is potential of DOCA-salt hypertension mediation through central orexin system functioning within the PVN. DOCA-salt treatment caused a significant increase in mRNA levels of AVP and OX1R within the PVN when compared to controls, and this increase was reduced following OX1R PVN knockdown. Protein expression of AVP and OX1R showed a similar pattern of increased expression in the PVN of DOCA-salt rats, and attenuation in DOCA-OX1RshRNA rat. Plasma AVP was also significantly increased following DOCA-salt treatment. In addition, central administration of OXA via ICV injection caused a notable increase in plasma AVP. Chronic OX1R PVN knockdown resulted in a large decrease in plasma AVP concentration compared to DOCA-salt treated rats. Furthermore, DOCA-salt as well as DOCA-OX1RshRNA had increased OXA production in the LH. Also, DOCA-salt rats

showed increased cardiac hypertrophy, and this was partially attenuated by PVN OX1R knockdown. Lastly, chronic OX1R knockdown within the PVN significantly decreased the elevation in blood pressure that is commonly observed in DOCA-salt hypertension. The results of this study show evidence of orexin system regulation of AVP production and release in the DOCA-rat model, and show that knockdown of OX1R function within the PVN significantly reduces hypertension in this rat model, offering a new potential mechanism underlying the development of salt-sensitive hypertension.

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A Raw Data

Table A.1: Raw data for 24-hr fecal output (g) following 3 weeks of treatment in each group. Bolded values utilized in control vs. DOCA-salt analysis.

24-hr Fecal Output (grams)		
Control	**DOCA-salt**	**DOCA+OX1RshRNA**
1.34	2.9	3.64
2.5	1.7	2.56
0.95	**4.69**	2.3
0.85	**2.1**	1.72
-	**6**	2.84
-	-	3.47

Table A.2: Raw data for 24-hr food intake (g) following 3 weeks of treatment in each group. Bolded values utilized in control vs. DOCA-salt analysis.

24-hr Food Intake (grams)		
Control	**DOCA-salt**	**DOCA+OX1RshRNA**
22.7	25.3	25.5
29.5	26.1	24.33
27.67	**19.77**	25.9
25.7	**14.36**	24.6
-	**22.59**	21.06
-	-	24.54

Table A.3: Raw data for 24-hr water intake (mL) following 3 weeks of treatment in each group. Bolded values utilized in control vs. DOCA-salt analysis.

24-hr Water Intake (mL)		
Control	**DOCA-salt**	**DOCA+OX1RshRNA**
17.06	**232.69**	93.9
29.4	**163.31**	177.1
19.8	**134.08**	98.8

24.6	56	55.1
-	120.54	94.58
-	-	213.15

Table A.4: Raw data for 24-hr urine output (mL) following 3 weeks of treatment in each group. Bolded values utilized in control vs. DOCA-salt analysis.

24-hr Urine Output (mL)		
Control	**DOCA-salt**	**DOCA+OX1RshRNA**
7.15	36.35	75.82
7.85	90.45	91.65
7.05	**136.69**	72.85
5.15	**121.77**	32.25
-	**119.73**	89.44
-	-	115.45

Table A.5: Raw data for body weight increase (g) over the course of 3 weeks of treatment. Bolded values utilized in control vs. DOCA-salt analysis.

3-Week Change in Weight (g)		
Control	**DOCA-salt**	**DOCA-OX1RshRNA**
116	**59**	57
141	**74**	36.5
148	**74**	27
140	**52**	30
111	**98**	35
118	**110**	-
62	**105**	-
47	36	-
50	27	-
29	39	-
-	58	-
-	63	-

-	22	-
-	33	-
-	24	-
-	10	-
-	18	-

Table A.6: Raw data for mRNA levels of AVP and OX1R normalized to GAPDH expression in the PVN.

PVN AVP mRNA/GAPDH		
Control	**DOCA-Salt**	**DOCA-OX1RshRNA**
0.29861	4.075012	0.116243
0.274243	1.604951	0.182224
2.985346	3.499776	0.559579
0.441796	4.777558	0.678754
0.984581	0.469435	1.682963
0.753647	0.392013	-
0.735716	5.654253	-
1.526056	6.808918	-
-	0.999359	-

PVN OX1R mRNA/GAPDH	
Control	**DOCA-Salt**
0.963092	1.318004
0.939703	1.140277
1.096772	1.407982
-	1.056115

PVN OX1R mRNA/GAPDH		
Control	**DOCA-Salt**	**DOCA-OX1RshRNA**
1.265941	1.927575	0.946485
1.021909	1.18423	0.960295
0.703364	1.087205	0.574349
1.32161	1.474146	0.93185
0.948758	1.04782	0.855416
0.961832	1.088295	-
0.776586	1.172714	-
-	1.593106	-
-	1.875779	-
-	1.025059	-

Table A.7: Raw Data for plasma AVP concentration (pg/ml) using ELISA**.**

Plasma ELISA AVP Concentration (pg/ml)	
Control	**DOCA-salt**
14.74292	4.202917
2.488958	7.873542
8.144792	32.37125
10.78833	30.225
-	46.2918
-	50.14979
-	54.72375
-	77.54875

Table A.8: Raw Data for plasma AVP concentration (pg/ml) following OXA ICV using ELISA**.**

Plasma ELISA AVP Concentration (pg/ml)

Saline (0.9%) ICV	OXA (2nmol) ICV
45.68792	106.2921
62.52958	44.58083
32.36083	97.18917

Table A.9: Raw data for CSF OXA concentration (pg/ml) using ELISA.

CSF ELISA OXA Concentration (pg/ml)		
Control	**DOCA-salt**	**DOCA HS/ OX1RshRNA**
19.023	37.929	142.571
111.537	71.762	154.929
113.859	31.428	16.163
86.943	-	-
136.727	-	-

Table A.10: Raw data for Plasma AVP concentration (pg/ml) using ELISA following chronic OX1R knockdown.

Plasma ELISA AVP Concentration (pg/ml)		
Control	**DOCA-salt**	**DOCA HS/ OX1RshRNA**
104.6883	254.7879	137.4867
255.6463	253.6813	226.8013
184.8092	193.9575	137.1929
-	278.8671	-
-	313.2242	-
-	254.7879	-

Table A.11: Raw data for heart weight to body weight ratio presented as a percentage of body weight following 3 weeks of treatment in each group.

HW:BW (%)		
Control	**DOCA-salt**	**DOCA-OX1RshRNA**
0.334	0.518	0.390331
0.349	0.498	0.406241
0.325	0.406	0.321127
0.337	0.438	0.320442
0.266055	0.394	0.321429
0.317073	0.460252	-
0.283333	0.402793	-
0.272959	0.362155	-
-	0.359694	-
-	0.35533	-
-	0.34359	-
-	0.344262	-
-	0.346405	-

Table A.12: Raw data for MAP (mmHg) for 3 weeks of treatment in each group.

MAP (mmHg)				
Week	**Measurement Point**	**Control**	**DOCA-salt**	**DOCA-OX1RshRNA**
Week 0	Baseline	120.5	111.1818	116.6
		105.4286	116.4545	101.7
		125.5	120.8571	117.8276
		112.8182	113.7778	111.8889
		125.3571	-	-
Week 1	T1	121.375	125.3636	112.6
		119.6154	132.875	105.6154

		115.6667	127.3636	118
		119.375	126.625	113
		100.5333	-	-
	T2	134.375	127.5	101.6667
		107.333	158.75	94.58824
		108.8571	138.111	117.25
		127.375	116.1	122.25
		106.9091	-	-
Week 2	T3	116.5556	136.1429	119.7778
		115.6111	129.5	112.75
		114.75	141.75	128.4286
		118.5	127.5	122.75
		118.9231	-	-
	T4	107.875	127.2	114.125
		91.6	156.6154	120.1538
		127.75	153	138.1
		127.6364	134	112.3636
		115.1667	-	-
Week 3	T5	100.25	141.5	96.625
		90.14286	164.5	116.6154
		112.2308	133.625	136.6667
		125.6	130.0909	112.8889
		111.333	-	-

Table A.13: Raw data for HR (BPM) for three weeks during treatment in each group.

Heart Rate (BPM)				
Week	**Measurement Point**	**Control**	**DOCA-salt**	**DOCA-OX1RshRNA**

Week 0	Baseline	204	333	264
		327.5714	180.5455	346.5
		309.1875	418.5714	323.8966
		314.7273	431.8889	176.222
		372.6429	-	-
Week 1	T1	292.375	280.3636	221.5
		414.9231	379.375	329.5769
		193	399.4545	295.7143
		447.75	360.375	195.75
		418.6667	-	-
	T2	313.75	484.5	231.25
		395.9524	402.625	290.7059
		475.4286	308.444	339.4167
		431.625	237.2	353.4167
		353	-	-
Week 2	T3	402	385.2857	325.5556
		370.5	355.0833	188.3333
		193	379.25	397.0714
		347.375	391.375	323
		333.3846	-	-
	T4	248.875	378.1	352.125
		277.2	327.8462	255.8462
		352.58333	238.4	321.6
		400.1818	323.4615	318.5455
		121.8333	-	-
Week 3	T5	439.75	310.9286	227.75
		217.4286	347.5	323.3077
		279.6154	392.875	367.8889
		351.4	350.4545	380.3333
		259	-	-

B Statistical Analysis

Table B.1: Mean 24-hr fecal output in control and DOCA-salt groups.

Fecal Output (g)		
	Control	DOCA-salt
N	4	3
Mean	1.41	4.263
SD	0.7568	1.985
SEM	0.3784	1.146
Lower 95% CI	0.2058	-0.6669
Upper 95% CI	2.614	9.194

Table B.2: Mean 24-hr fecal output in control, DOCA-salt, and DOCA-OX1RshRNA groups.

Fecal Output (g)			
	Control	DOCA-salt	OX1RshRNA
N	4	5	6
Mean	1.41	3.478	2.755
SD	0.7568	1.818	0.7236
SEM	0.3784	0.8131	0.2954
Lower 95% CI	0.2058	1.22	1.996
Upper 95% CI	2.614	5.736	3.514

Table B.3: Mean 24-hr urine output in control and DOCA-salt treatment groups.

Urine Output (mL)		
	Control	DOCA-salt
N	4	3
Mean	6.8	126.1
SD	1.156	9.259
SEM	0.5781	5.346
Lower 95% CI	4.96	103.1
Upper 95% CI	8.64	149.1

Table B.4: Mean 24-hr urine output in control, DOCA-salt, and DOCA-OX1RshRNA groups.

Urine Output (mL)			
	Control	DOCA-salt	OX1RshRNA
N	4	5	6
Mean	6.8	101	79.58
SD	1.156	39.83	27.67
SEM	0.5781	17.81	11.3
Lower 95% CI	4.96	51.54	50.54
Upper 95% CI	8.64	150.5	108.6

Table B.5: Mean 24-hr water intake in control and DOCA-salt treatment groups

Water Intake (mL)

	Control	DOCA-salt
N	4	3
Mean	22.72	176.7
SD	5.438	50.65
SEM	2.719	29.24
Lower 95% CI	14.06	50.87
Upper 95% CI	31.37	302.5

Table B.6: Mean 24-hr water intake in control, DOCA-salt, and DOCA-OX1RshRNA groups.

Water Intake (mL)			
	Control	DOCA-salt	OX1RshRNA
N	4	5	6
Mean	22.72	141.3	122.1
SD	5.438	64.41	59.83
SEM	2.719	28.81	24.43
Lower 95% CI	14.06	61.35	59.31
Upper 95% CI	31.37	221.3	184.9

Table B.7: Mean 24-hr food intake in control and DOCA-salt treatment groups.

Food Intake (g)		
	Control	DOCA-salt
N	4	3

Mean	26.39	18.91
SD	2.91	4.182
SEM	1.455	2.415
Lower 95% CI	21.76	8.517
Upper 95% CI	31.02	29.3

Table B.8: Mean 24-hr food intake in control, DOCA-salt, and DOCA-OX1RshRNA groups.

Food Intake (g)			
	Control	DOCA-salt	OX1RshRNA
N	4	5	6
Mean	26.39	21.62	24.32
SD	2.91	4.761	1.711
SEM	1.455	2.129	0.6986
Lower 95% CI	21.76	15.71	22.53
Upper 95% CI	31.02	27.54	26.12

Table B.9: Mean 3-week body weight increases in control and DOCA-salt treatment groups.

Body Weight Change (g)		
	Control	DOCA-salt
N	6	7
Mean	129	81.71
SD	15.75	22.82

SEM	6.429	8.626
Lower 95% CI	112.5	60.61
Upper 95% CI	145.5	102.8

Table B.10: Mean 3-week body weight increases in control, DOCA-salt, and DOCA-OX1RshRNA groups.

Body Weight Change (g)			
	Control	DOCA-salt	OX1RshRNA
N	10	17	5
Mean	86	53.06	37.1
SD	54.2	31.08	11.76
SEM	16.34	7.539	5.259
Lower 95% CI	49.59	37.08	22.5
Upper 95% CI	122.4	69.04	51.7

Table B.11: Mean PVN AVP and OX1R mRNA expression normalized to GAPDH.

PVN AVP mRNA/GAPDH			
	Control	DOCA-salt	OX1RshRNA
N	8	9	5
Mean	1	3.142	0.644
SD	0.9007	2.374	0.6284
SEM	0.3185	0.7914	0.281

Lower 95% CI	0.247	1.317	-0.1363
Upper 95% CI	1.753	4.967	1.424

PVN OX1R mRNA/GAPDH		
	Control	DOCA-salt
N	3	4
Mean	0.9999	1.231
SD	0.08474	0.1609
SEM	0.04893	0.08047
Lower 95% CI	0.7893	0.9745
Upper 95% CI	1.21	1.487

PVN OX1R mRNA/GAPDH			
	Control	DOCA-salt	OX1RshRNA
N	7	10	5
Mean	1	1.348	0.8537
SD	0.2297	0.3461	0.1613
SEM	0.08683	0.1094	0.07215
Lower 95% CI	0.7875	1.1	0.6534
Upper 95% CI	1.212	1.595	1.054

Table B.12: Mean plasma AVP Concentration (pg/ml) in control and DOCA-salt groups.

Plasma ELISA AVP Concentration (pg/ml)		
	Control	**DOCA-salt**
N	4	8
Mean	9.041	37.94
SD	5.141	24.5
SEM	2.571	8.661
Lower 95% CI	0.8603	17.46
Upper 95% CI	17.22	58.42

Table B.13: Mean plasma AVP Concentration (pg/ml) following saline or OXA ICV.

Plasma ELISA AVP Concentration (pg/ml)		
	Saline (0.9%) ICV	**OXA (2nmol) ICV**
N	3	3
Mean	46.86	82.69
SD	15.12	33.31
SEM	8.729	19.23
Lower 95% CI	9.303	-0.0679
Upper 95% CI	84.42	165.4

Table B.14: Mean CSF OXA concentration (pg/ml) in all groups.

CSF OXA Concentration (pg/ml)			
	Control	DOCA-salt	OX1RshRNA
N	5	3	3
Mean	93.62	47.04	104.6
SD	45.27	21.66	76.8

SEM	20.25	12.5	44.34
Lower 95% CI	37.41	-6.755	-86.22
Upper 95% CI	149.8	100.8	295.3

Table B.15: Mean Plasma AVP concentration (pg/ml) in all treatment groups.

Plasma AVP Concentration (pg/ml)			
	Control	DOCA-salt	OX1RshRNA
N	3	5	3
Mean	181.7	258.9	167.2
SD	75.53	43.62	51.65
SEM	43.61	19.51	29.82
Lower 95% CI	-5.903	204.7	38.85
Upper 95% CI	369.3	313.1	295.5

Table B.16: Mean heart weight to body weight ratio presented as percent body weight in control, DOCA-salt, and DOCA-OX1RshRNA groups.

HW:BW (%)			
	Control	DOCA-salt	OX1RshRNA
N	4	5	6
Mean	0.3106	0.4022	0.3519
SD	0.03188	0.05974	0.04271
SEM	0.01127	0.01657	0.0191
Lower 95% CI	0.2839	0.3661	0.2989

Upper 95% CI	0.3372	0.4383	0.4049

Table B.17: Mean MAP over the course of 3 weeks in control, DOCA-salt, and OX1RshRNA rats.

MAP (mmHg)							
		Base	**T1**	**T2**	**T3**	**T4**	**T5**
Control	**N**	5	5	5	5	5	5
	Mean	117.921	115.313	116.97	116.868	114.006	107.911
	SD	8.682	8.519	12.953	1.806	15.130	13.395
	SEM	3.883	3.810	5.793	0.808	6.766	5.990
DOCA-salt	**N**	4	4	4	4	4	4
	Mean	115.567	128.057	135.115	133.723	142.704	142.429
	SD	4.131	3.317	18.140	6.503	14.326	15.468
	SEM	2.066	1.658	9.070	3.251	7.163	7.734
DOCA-OX1RshRNA	**N**	4	4	4	4	4	4
	Mean	112.004	112.304	108.939	120.927	121.186	115.699
	SD	7.331	5.091	12.975	6.526	11.759	16.454
	SEM	3.665	2.545	6.488	3.263	5.880	8.227

Table B.18: Mean heart rate over the course of 3 weeks in control, DOCA-salt, DOCA-OX1RshRNA rat groups.

HR (BPM)							
		Base	**T1**	**T2**	**T3**	**T4**	**T5**
Control	**N**	5	5	5	5	5	5
	Mean	305.626	353.343	393.951	329.252	280.135	309.439
	SD	62.041	107.702	63.583	80.473	106.894	87.523
	SEM	27.745	48.166	28.435	35.989	47.804	39.141
DOCA-salt	**N**	4	4	4	4	4	4
	Mean	341.001	354.892	358.192	377.749	316.952	350.440
	SD	115.597	52.185	108.077	15.900	57.938	33.519
	SEM	57.798	26.092	54.039	7.950	28.969	16.759
DOCA-OX1RshRNA	**N**	4	4	4	4	4	4
	Mean	277.655	260.635	303.697	308.490	312.029	324.820
	SD	76.055	62.517	55.273	87.151	40.407	69.189
	SEM	38.027	31.259	27.636	43.576	20.204	34.594

Table B.19: Unpaired two-tail t-test analysis of 24-hr fecal output between control and DOCA-salt treatment groups.

Unpaired Two-Tail T-test – Fecal Output	
P value	0.043
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.697 df=5

Table B.20: One-way ANOVA analysis of 24-hr fecal output between all treatment groups.

Fecal Output			
ANOVA Summary			
F	3.295		
P-value	0.0724		
R-square	0.3545		
ANOVA Table	**SS**	**DF**	**MS**
Treatment (Between Columns)	9.642	2	4.821
Residual (Within Columns)	17.56	12	1.463

Total	27.2	14

Table B.21: Unpaired two-tail analysis of 24-hr food intake between control and DOCA-salt treatment groups.

Unpaired Two-Tail T-test – Food Intake	
P value	0.0371
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.820 df=5

Table B.22: One-way ANOVA analysis of 24-hr food intake between all groups.

Food Intake			
ANOVA Summary			
F	2.374		
P-value	0.1353		
R-square	0.2835		
ANOVA Table	**SS**	**DF**	**MS**
Treatment (Between Columns)	51.73	2	25.87

Residual (Within Columns)	130.7	12	10.89
Total	182.5	14	

Table B.23: Unpaired two-tailed t-test analysis of 24-hr water intake between control and DOCA

Unpaired Two-Tail T-test – Water Intake	
P value	0.0015
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=6.240 df=5

Table B.24: One-way ANOVA analysis of 24-hr water intake between groups, as well as Tukey post-hoc analysis

Water Intake			
ANOVA Summary			
F	6.124		
P-value	0.0147		
R-square	0.5051		
ANOVA Table	**SS**	**DF**	**MS**

Treatment (Between Columns)	35302	2	17651
Residual (Within Columns)	34584	12	2882
Total	69886	14	

Tukey	Mean Diff.	95% CI	Significant?	Summary
Control vs. DOCA-salt	-118.6	-214.7 to -22.53	Yes	*
Control vs. OX1RshRNA	-99.39	-191.8 to -6.940	Yes	*
DOCA-salt vs. OX1RshRNA	19.22	-67.51 to 105.9	No	ns

Table B.25: Unpaired two-tail t-test analysis of 24-hr urine output between control and DOCA-salt groups.

Unpaired Two-Tail T-test – Urine Output	
P value	< 0.0001
P value summary	****
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed

t, df	t=26.36 df=5

Table B.26: One-way ANOVA analysis of 24-hr urine output between groups, as well as Tukey post-hoc analysis.

Urine Output			
ANOVA Summary			
F	12.51		
P-value	0.0012		
R-square	0.6758		
ANOVA Table	**SS**	**DF**	**MS**
Treatment (Between Columns)	21223	2	10612
Residual (Within Columns)	10180	12	848.3
Total	31403	14	

Tukey	**Mean Diff.**	**95% CI**	**Significant?**	**Summary**
Control vs. DOCA-salt	-94.2	-146.3 to -42.07	Yes	**

Control vs. OX1RshRNA	-72.78	-122.9 to -22.62	Yes	**
DOCA-salt vs. OX1RshRNA	21.42	-25.63 to 68.47	No	ns

Table B.27: Unpaired two-tail t-test analysis of 3-week weight gain between control and DOCA-salt groups.

Unpaired Two-Tail T-test – Change in Body Weight	
P value	0.0013
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.266 df=11

Table B.28: One-way ANOVA analysis of 3-week weight gain as well as HW:BW between groups, as well as Tukey post-hoc analysis.

Weight Gain			
ANOVA Summary			
F	6.843		
P-value	0.0037		
R-square	0.3206		
ANOVA Table	**SS**	**DF**	**MS**

Treatment (Between Columns)	16021	2	8011
Residual (Within Columns)	33948	29	1171
Total	49969	31	

Tukey	Mean Diff.	95% CI	Significant?	Summary
Control vs. DOCA-salt	43.14	9.467 to 76.82	Yes	**
Control vs. OX1RshRNA	59.1	12.82 to 105.4	Yes	*
DOCA-salt vs. OX1RshRNA	15.96	-27.03 to 58.95	No	ns

HW:BW	
ANOVA Summary	
F	8.547
P-value	0.0017
R-square	0.4263

ANOVA Table	**SS**	**DF**	**MS**
Treatment (Between Columns)	0.04254	2	0.02127
Residual (Within Columns)	0.05724	23	0.002489
Total	0.09978	25	

Tukey	**Mean Diff.**	**95% CI**	**Significant?**	**Summary**
Control vs. DOCA-salt	-0.09164	-0.1478 to -0.03550	Yes	**
Control vs. OX1RshRNA	-0.04136	-0.1126 to 0.02986	No	ns
DOCA-salt vs. OX1RshRNA	0.05028	-0.01547 to 0.1160	No	ns

Table B.29: Unpaired two-tail, and one-tail t-test analysis of PVN AVP and OX1R mRNA/GAPDG between groups.

Unpaired Two-Tail T-test – PVN AVP mRNA/GAPDH	
P value	0.03

P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.397 df=15
Unpaired One-Tail T-test – PVN OX1R mRNA/GAPDH	
P value	0.0383
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=2.226 df=5

Table B.30: One-way ANOVA analysis of PVN AVP mRNA/GAPDG between groups, as well as Tukey post-hoc analysis.

PVN AVP mRNA/GAPDH			
ANOVA Summary			
F	5.085		
P value	0.017		
R-square	0.3486		
ANOVA Table	**SS**	**DF**	**MS**
Treatment (Between Columns)	28.02	2	14.01

Residual (Within Columns)	52.35	19	2.755
Total	80.37	21	

Tukey	Mean Diff.	95% CI	Significant?	Summary
Control vs. DOCA-salt	-2.142	-4.191 to -0.09335	Yes	*
Control vs. OX1RshRNA	0.356	-2.048 to 2.760	No	ns
DOCA-salt vs. OX1RshRNA	2.498	0.1464 to 4.850	Yes	*

Table B.31: One-way ANOVA analysis of PVN OX1R mRNA/GAPDH between groups, as well as Tukey post-hoc analysis.

PVN OX1R mRNA/GAPDH	
ANOVA Summary	
F	6.168
P value	0.0086
R-square	0.3937

ANOVA Table	SS	DF	MS
Treatment (Between Columns)	0.9729	2	0.4865
Residual (Within Columns)	1.499	19	0.07887
Total	2.472	21	

Tukey	Mean Diff.	95% CI	Significant?	Summary
Control vs. DOCA-salt	-0.3476	-0.6992 to 0.004009	No	ns
Control vs. OX1RshRNA	0.1463	-0.2714 to 0.5641	No	ns
DOCA-salt vs. OX1RshRNA	0.4939	0.1031 to 0.8847	Yes	*

Table B.32: Unpaired T-test of plasma AVP concentration between saline and OXA ICV rats.

Unpaired One-Tail T-test – Saline ICV vs. OXA ICV Plasma AVP	
P value	0.0825

P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
t, df	t=1.696 df=4
P value	0.0825

Table B.33: Unpaired T-test of plasma AVP concentration between control and DOCA-salt rats.

Unpaired Two-Tailed T-test – Control vs. DOCA-salt Plasma AVP	
P value	0.0457
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.281 df=10
P value	0.0457

Table B.34: One-way ANOVA analysis of CSF OXA concentration (pg/ml).

CSF OXA Concentration			
ANOVA Summary			
F	1.114		
P-value	0.3744		
R-square	0.2178		
ANOVA Table	**SS**	**DF**	**MS**

Treatment (Between Columns)	5828	2	2914
Residual (Within Columns)	20932	8	2616
Total	26760	10	

Table B.35: One-way ANOVA analysis of plasma AVP concentration (pg/ml) following chronic OX1R knockdown

Plasma AVP Concentration (pg/ml)			
ANOVA Summary			
F	3.248		
P-value	0.0928		
R-square	0.4481		
ANOVA Table	**SS**	**DF**	**MS**
Treatment (Between Columns)	19776	2	9888
Residual (Within Columns)	24356	8	3044

Total	44131	10

Table B.36: Unpaired two-tail t-test of plasma AVP concentration between DOCA-salt and DOCA-HS/OX1RshRNA rats.

Unpaired Two-Tailed T-test – DOCA-salt vs. DOCA-HS/OX1RshRNA Plasma AVP	
P value	0.0354
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.704 df=6
P value	0.0354

Table B.37: Two-way ANOVA analysis of 3 week MAP between groups, as well as Tukey post-hoc analysis.

MAP				
ANOVA Summary	**%Total Variation**	**P Value**	**P Value Summary**	**Significant?**
Interaction	12.98	0.1111	ns	No
Row Factor	5.927	0.1977	ns	No
Column Factor	34.25	< 0.0001	****	Yes
ANOVA Table	**SS**	**DF**	**MS**	**P value**
Interaction	2089	10	208.9	P = 0.1111

Row Factor	953.9	5	190.8	P = 0.1977
Column Factor	5512	2	2756	P < 0.0001
Residual	7540	60	125.7	
Total	16094.9	77		

MAP					
Time Point	**Tukey**	**Mean Diff**	**95% CI**	**Significant?**	**Summary**
Base	Control vs. DOCA-HS	2.353	-15.72 to 20.42	No	ns
	Control vs. DOCA+OX1RshRNA	5.917	-12.16 to 23.99	No	ns
	DOCA-HS vs. DOCA+OX1RshRNA	3.564	-15.49 to 22.61	No	ns
T1	Control vs. DOCA-HS	-12.74	-30.82 to 5.328	No	ns
	Control vs. DOCA+OX1RshRNA	3.009	-15.06 to 21.08	No	ns

	DOCA-HS vs. DOCA+OX1RshRNA	15.75	-3.297 to 34.80	No	ns
T2	Control vs. DOCA-HS	-18.15	-36.22 to -0.07364	Yes	*
	Control vs. DOCA+OX1RshRNA	8.031	-10.04 to 26.10	No	ns
	DOCA-HS vs. DOCA+OX1RshRNA	26.18	7.127 to 45.23	Yes	**
T3	Control vs. DOCA-HS	-16.86	-34.93 to 1.217	No	ns
	Control vs. DOCA+OX1RshRNA	-4.059	-22.13 to 14.01	No	ns
	DOCA-HS vs. DOCA+OX1RshRNA	12.8	-6.253 to 31.85	No	ns
T4	Control vs. DOCA-HS	-28.7	-46.77 to -10.63	Yes	***
	Control vs. DOCA+OX1RshRNA	-7.18	-25.25 to 10.89	No	ns

	DOCA-HS vs. DOCA+OX1RshRNA	21.52	2.469 to 40.57	Yes	*
T5	Control vs. DOCA-HS	-34.52	-52.59 to -16.45	Yes	****
	Control vs. DOCA+OX1RshRNA	-7.788	-25.86 to 10.28	No	ns
	DOCA-HS vs. DOCA+OX1RshRNA	26.73	7.681 to 45.78	Yes	**

Table B.38: Two-way ANOVA analysis of 3-week HR between groups.

Heart Rate				
ANOVA Summary	**%Total Variation**	**P Value**	**P Value Summary**	**Significant?**
Interaction	7.599	0.8304	ns	No
Row Factor	4.839	0.605	ns	No
Column Factor	7.824	0.0603	ns	No
ANOVA Table	**SS**	**DF**	**MS**	**P value**

Interaction	34118	10	3412	P = 0.8304
Row Factor	21726	5	4345	P = 0.6050
Column Factor	35129	2	17565	P = 0.0603
Residual	358005	60	5967	
Total	448978	77		