HYPO-OSMOTIC EFFECTS IN CARDIOVASCULAR CONTROLS: HEPATIC AND RENAL MECHANISMS UNDERLYING THE OSMOPRESSOR RESPONSE

By

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DEDICATION

To my father, Dr. Mai Hoàng Anh and my mother, Dr. Huỳnh Kim Gàn,

who made my dream come true...

"Công cha như núi Thái Sơn
Nghĩa mẹ như nước trong nguồn chảy ra"

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LIST OF ABBREVIATIONS

-/-	Homozygous knockout
4α-PDD	4α-phorbol ester
Ang	Angiotensin
AT1	Angiotensin receptor 1
AVP	Arginine vasopressin
ANS	Autonomic nervous system
BP	Blood pressure
BW	Body weight
CGRP	Calcitonin-gene-related peptide
CO	Cardiac output
CVLM	Caudal ventrolateral medulla
CGX	Celiac ganglionectomy
CNS	Central nervous system
DHPG	Dihydroxyphenylglycine
DA	Dopamine
Dbh	Dopamine-β-hydroxylase
DMNX	Dorsal motor nucleus of the vagus
DRG	Dorsal root ganglia
ENS	Enteric nervous system
HR	Heart rate
i.v	Intravenous
JG	Juxtaglomerular
MSA	Multiple system atrophy
NaCl	Sodium Chloride
NTP	Nitroprusside
NE	Norepinephrine
NA	Nucleus ambiguous
PNS	Parasympathetic nervous system
PAF	Pure autonomic failure

RAAS	Renin-angiotensin-aldosterone system
SAD	Sino-aortic denervation
SNS	Sympathetic nervous system
TRP	Transient receptor potential
TRPV4	Transient receptor potential 4
RVLM	Ventrolateral medulla
WT	Wild type

CHAPTER I

BACKGROUND AND INFORMATION

1. The Role of the Autonomic Nervous System in Cardiovascular Control

The autonomic nervous system (ANS), with the somatic nervous system, makes up the peripheral nervous system. Sensory neurons carry information from internal organs, such as the heart, blood vessels, lungs, and gastrointestinal tract to the hypothalamus and brainstem, homeostasis control centers of the body, via cranial nerves to regulate and monitor activities of the preganglionic autonomic neurons (McCorry 2007). All efferent nerves coming from the central nervous system (CNS), except for the ones that innervate the skeletal muscles, are considered autonomic. The ANS is often divided into three elements: the sympathetic (SNS), the parasympathetic (PNS) and the enteric nervous systems (ENS).

The SNS is traditionally known as the system that mediates the fight-or-flight response. It has short myelinated pre-ganglionic fibers that synapse onto long, small-diameter (<5 μm), unmyelinated post-ganglionic fibers in the sympathetic ganglion chains that run parallel to the spinal cord. These post-ganglionic nerves then innervate effector organs such as: smooth muscle and cardiac muscle, liver, kidneys, reproductive organs and others (Hamill, Shapiro, and Vizzard 2012). The principal neurotransmitter released at the endings of sympathetic post-ganglionic nerves is norepinephrine (except many fibers that innervate sweat glands release acetylcholine). Activation of receptors

by norepinephrine (NE) can increase BP, HR, and cardiac output (CO). Vascular smooth muscle tone and sweating are primarily controlled by the SNS. Pre-ganglionic nerves can also bypass the sympathetic chain to directly innervate the adrenal medulla to regulate the release of NE and epinephrine (McCorry 2007).

The PNS neurons emerge from the brainstem and the sacral region of the spinal cord (S2-S4). The majority (75%) of all PNS fibers are in the vagus nerve.
They have long preganglionic axons that synapse onto short postganglionic neurons next to or directly embedded in effector tissues. Because of this, the effects of the PNS are usually localized and discrete (McCorry 2007). The neurotransmitter released at parasympathetic nerve endings is acetylcholine. At rest, the PNS is responsible for energy conservation of the body by reducing BP and HR. It is also activated during digestion and nutrient absorption (Waxman et al. 1989) (Figure 1).

The ENS is made up of a highly dense neuronal network that runs along the entire length of the gut. The neurons of the ENS form an interconnected circuit and are grouped into ganglia in the myenteric and the submucosal plexus (Sasselli, Pachnis, and Burns 2012). It plays an important role in controlling mucosal secretion and absorption, and immune function as well as the local blood flow (Vermeulen et al. 2014). There are sensory neurons in the ENS that are sensitive to mechanical, chemical and osmotic stimuli that can send either excitatory or inhibitory signals to the enteric neural circuits and affect local processes such as vasoconstriction/vasodilation of blood vessels in the gut area (Costa, Brookes, and Hennig 2000).

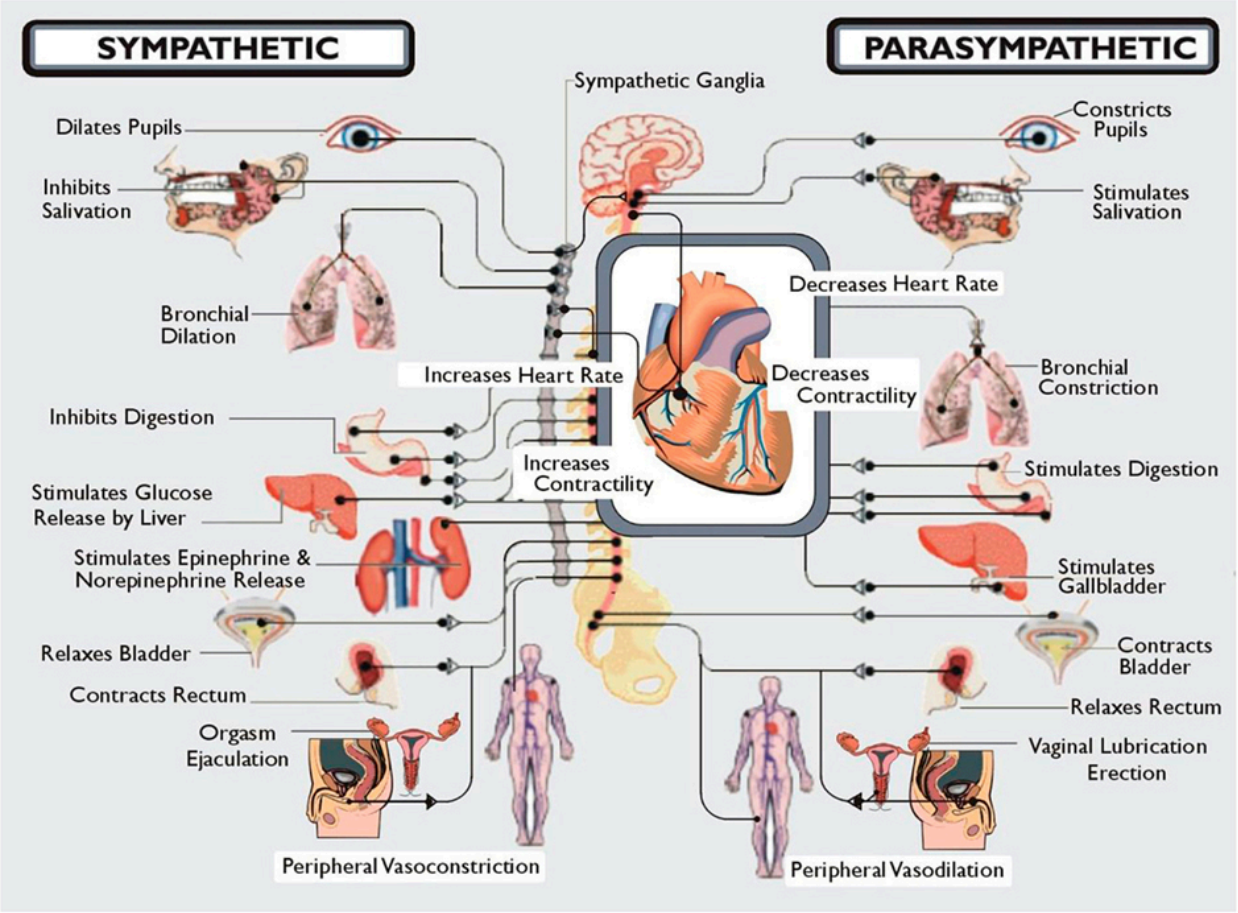

Figure 1: Actions of sympathetic and parasympathetic nerves (Vinik 2012).

1.1. Baroreceptor reflex

The baroreflex is a mechanism that monitors beat-to-beat changes of BP and HR. The arterial baroreceptors are located in the carotid sinuses and the aortic arch and transmit afferent information via the vagal and the glossopharyngeal nerves to the nucleus tractus (NTS) solitarii of the medullary brain stem. Baroreceptors can detect mechanical deformation of the vessels when BP rises and send a signal to the brainstem. The NTS sends excitatory glutamateric signals to the nucleus ambiguous

(NA), the dorsal motor nucleus of the vagus (DMNX) and the caudal ventrolateral medulla (CVLM). While the CVLM then transmits inhibitory GABA-mediated messages to the rostral ventrolateral medulla (RVLM) to decrease sympathetic efferent output, the NA and DMNX release excitatory signals to increase parasympathetic tone (Figure 2) (Kirchheim 1976, Robertson, Diedrich, and Chapleau 2012). The cardiopulmonary baroreceptors are present in the heart, vena cava and the pulmonary vasculature and are sensitive to blood volume changes. These project into the nodose ganglia (Robertson, Diedrich, and Chapleau 2012). When blood pressure rises, baroreceptor activity increases and activates three responses: 1) Increases parasympathetic signals 2) Reduces sympathetic signals and 3) Decreases arginine vasopressin secretion from the posterior pituitary. These effects tend to bring BP back to normal level.

Baroreflex sensitivity decreases with aging and also in several diseases such as hypertension, heart failure, autonomic failure, and obesity. In patients where the baroreflex is impaired, the ability to buffer changes in BP decreases, and therefore, the responses to either extrinsic or intrinsic stimuli are magnified. Recent studies hypothesize that carotid body denervation may possibly reduce blood pressure (McBryde et al. 2013, Ribeiro et al. 2013, Abdala et al. 2012). However, it is an invasive procedure and even though it exerts short-term lowering of BP, the long-term effects on cardiovascular events are still to be determined.

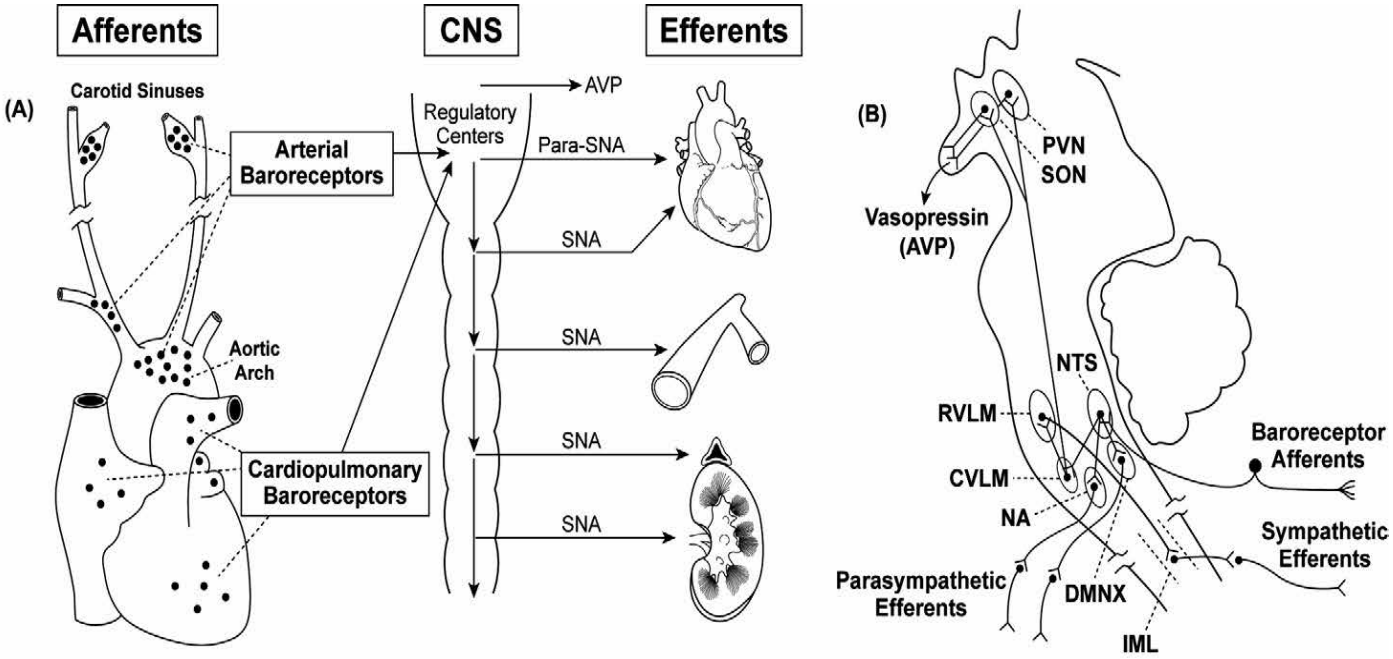

Figure 2: Baroreceptors detect changes in BP and send signals to the central nervous system to adjust parasympathetic and sympathetic nervous signals to bring BP and HR back to normal (Robertson, Diedrich, and Chapleau 2012). (NA: nucleus ambiguous, DMNX: the dorsal motor nucleus of the vagus, CVLM: the caudal ventrolateral medulla, RVLM: ventrolateral medulla, PVN: paraventricular nucleus, SON: supraoptic nucleus)

1.2. Autonomic control of the splanchnic circulation

The splanchnic organs include the stomach, small intestine, large intestine, colon, liver, spleen and pancreas. These organs receive ~25% of cardiac output. Blood vessels serving these organs make up the splanchnic circulation and contain about 25% of total blood volume. Because of its powerful capacitance for blood storage, the splanchnic circulation plays a major role in blood pressure regulation. There are two mechanisms by which the splanchnic circulation could affect systemic blood pressure:

1) constriction of splanchnic arteries can dramatically increase BP and the total peripheral resistance and 2) cardiac preload increases because of venous constriction. Extrinsic neural and hormonal inputs such as catecholamines, angiotensin II and vasopressin, etc. have the most impact on these two mechanisms (King, Osborn, and Fink 2007). The veins and arteries in the splanchnic region are heavily innervated. There are three main sensory nerves that receive information from the splanchnic region and convey it to the CNS: the vagal, the splanchnic and the pelvic nerves. While the vagus transmits signals through the nodose ganglia to the solitary tract nucleus, the splanchnic and pelvic nerves have neuron cell bodies in the celiac and dorsal root ganglia (DRG) that project into the spinal cord (McBride et al. 2001) (Figure 3).

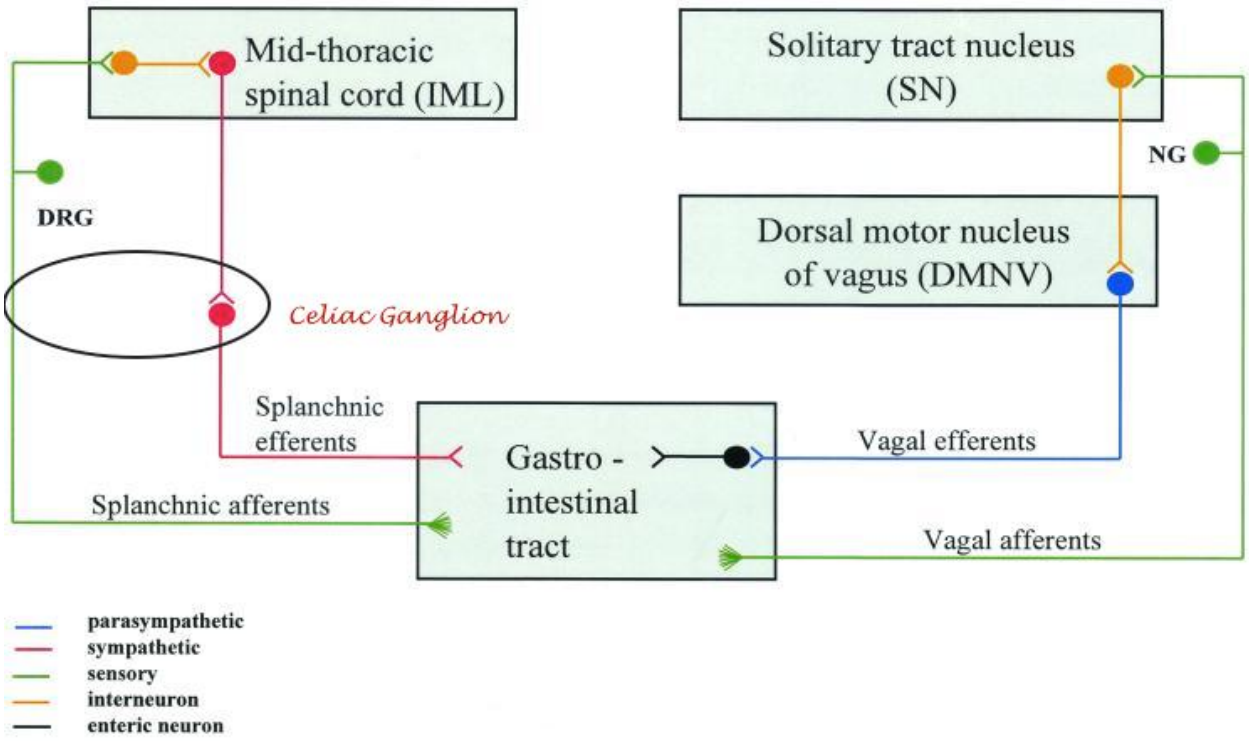

Figure 3: Afferent and efferent nerves that innervate the splanchnic region. Modified from (McBride et al. 2001).

Both vagal and splanchnic nerves are sensitive to mechanical and chemical stimuli in the gastrointestinal tract due to a variety of receptors sensitive to these stimuli. Among these receptors are the non-specific Ca^{2+} ion channels in the transient receptor potential (TRP) family (Brierley et al. 2009). These channels play an important role in maintaining mucosal integrity, osmolality, blood pressure, and intestinal motility, among other functions (Sipe et al. 2008).

1.3. Autonomic control of the kidneys

The renin-angiotensin-aldosterone system (RAAS) interacts with the autonomic nervous system to control blood pressure, blood volume and sodium/fluid balance. This control is primarily sympathetic (Jackson and Raghvendra 2004). When a decrease in blood volume is sensed by the cardiopulmonary baroreceptors, antidiuretic hormone secretion from the posterior pituitary increases and so does the sympathetic signal in the renal nerves. Norepinephrine released from the sympathetic nerve can trigger the release of renin in juxtaglomerular (JG) cells exclusively via β1-adrenergic receptors. In addition, a decrease in blood flow in renal arterioles and /or in NaCl flow can also increase the rate of renin release (Castrop et al. 2010). Renin converts angiotensinogen into angiotensin I (Ang), and then the angiotensin converting enzyme converts Ang I into Ang II. Ang II can directly increase BP by acting on angiotensin receptor 1 (AT1) to increase vasoconstriction, elicit baroreflex dysfunction by reducing the baroreceptor reflex in the NTS, and further increase sympathetic activation to release even more renin in a positive feedback loop (Fyhrquist and Saijonmaa 2008, McKinley et al. 2003). Ang II also can stimulate aldosterone release from the adrenal cortex to increase water

reabsorption and sodium retention from the collecting duct (Diz et al. 2011). All of these effects of Ang II would increase BP back to normal (Figure 4). Afferent renal nerves feed directly into the DRG and the efferent nerves run through both the celiac and mesenteric ganglia (Castrop et al. 2010) (Figure 3). Unlike the immediate control of BP by the baroreflex, the RAAS regulates BP over a longer period of time.

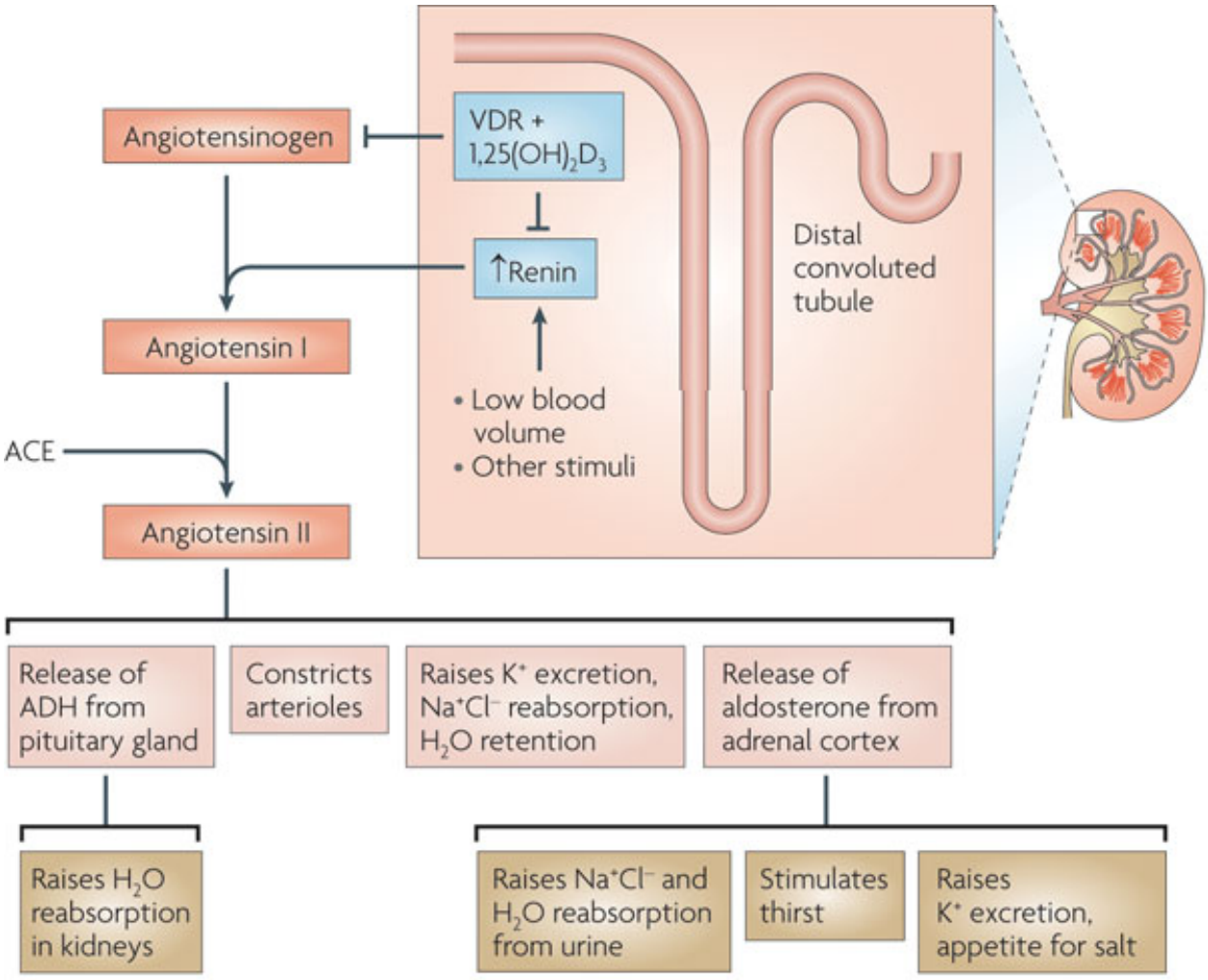

Figure 4: The RAAS system (Plum and DeLuca 2010).

2. Autonomic Nervous System Dysfunction and Blood Pressure Control

Autonomic failure patients commonly experience baroreflex impairment (Heusser et al. 2005). In multiple system atrophy (MSA) patients, the impairment occurs centrally. On the other hand, patients with pure autonomic failure (PAF) have defects in the peripheral efferent nerves (Robertson, Diedrich, and Chapleau 2012). Because of baroreflex impairment, patients with MSA and PAF can no longer buffer changes in BP and HR due to endogenous or exogenous stimuli. As a result, orthostatic hypotension, a condition in which systolic BP drops at least 20 mmHg (or diastolic BP reduces ~10 mmHg) when standing, is observed in both populations even though ~50% of patients are hypertensive when in the supine position (Figure 5). Patients with autonomic failure present the perfect opportunity to study the fundamental determinants of autonomic cardiovascular regulation that are normally masked by baroreflex function.

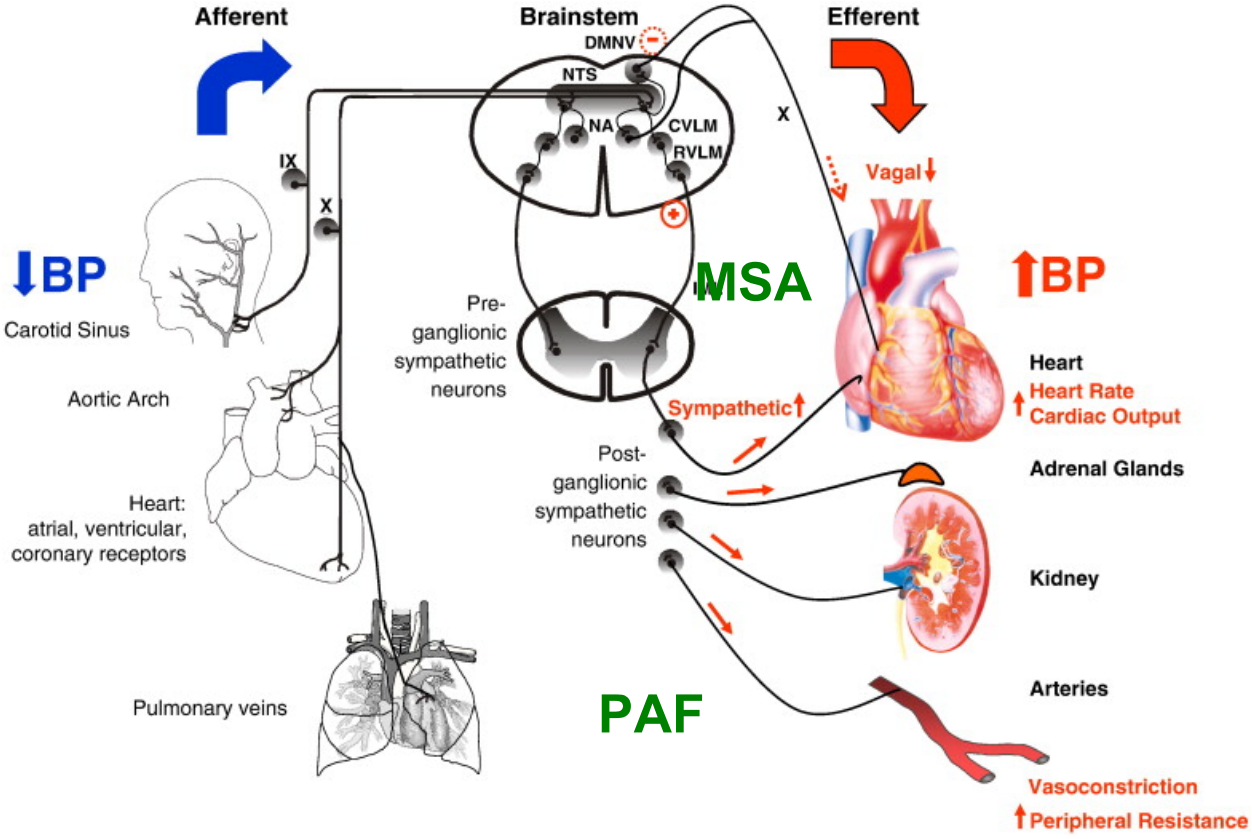

Figure 5: Autonomic controls of cardiovascular function. MSA and PAF patients have impairment in the efferent arch of the baroreflex (Robertson, Diedrich, and Chapleau 2012).

2.1. The effect of water drinking in patients with dysautonomias

Water makes up the majority of body weight (Slaughter et al. 1993, Knechtle et al. 2010). The ratio between free water molecules and solute in the intracellular and extracellular compartment defines osmolality and deviation of osmolality as little as 3% can be lethal (Bourque 2008) (Liamis et al. 2008, Saly and Andrew 1993). Therefore, the regulation of water content in the body is tightly regulated. The central osmosensors

are reportedly located in the organum vasculosum of the lamina terminalis and subfornical organ (Geerling and Loewy 2007, Hollis et al. 2008). There are also osmosensors in the periphery, one of which is the transient receptor potential vanilloid 4 (TRPV4) channel.

In patients whose baroreflex is compromised, ingestion of 480 ml of water causes an acute increase in BP that peaks after around 15-20 minutes and lasts for more than 40 minutes. The average response is 33 mmHg in MSA patients and 37 mmHg in PAF patients. An attenuated response occurs in healthy elderly individuals (~11mmHg) whereas the response is absent in healthy young individuals (Jordan et al. 2000, Jordan et al. 1999, Cariga and Mathias 2001) (Figure 6). When these patients were given the same volume of 0.42 % NaCl solution, the pressor effect paradoxically decreased (Raj et al. 2006). Also, an intravenous infusion of 5% dextrose in water could not produce as dramatic a response as water drinking. Plasma volume also did not increase after water ingestion (Jordan et al. 2000). These data all suggest that the pressor effect of water is not due to volume expansion. Because osmolality was the determining factor in the initiation of the response, this pressor effect of water was named the “osmopressor response (OPR).” The OPR is dose-dependent; 120 ml, 240 ml and 480 ml of water increase BP by up to 9 mmHg, 29 mmHg and 44mmHg, respectively, in autonomic failure patients (data not published).

Norepinephrine levels in autonomic failure patients increase significantly after drinking water, indicating activation of the sympathetic nervous system (Figure 7). This

finding was surprising because the sympathetic response to stimuli such as handgrip and cold pressor testing is absent in these patients (Raj et al. 2006). When autonomic ganglia were blocked by trimethaphan, the pressor effect of water was abolished even in patients most sensitive to water (Shannon et al. 2000). It was also observed that when treated with yohimbine, an α2-adrenergic receptor antagonist, blood pressure increased in this population of patients. Interestingly, pressor responses to yohimbine and water were correlated (Biaggioni, Robertson, and Robertson 1994, Jordan et al. 2000). These data suggest that efferent sympathetic function is not completely lost in autonomic failure patients; some residual efferent nerves are present and patients may be hypersensitive to blood pressure stimuli to compensate for their loss of efferent nerves. An inability to buffer the pressor response due to an impaired baroreflex would also contribute to the OPR. Although the pressor effect of water ingestion was not present in healthy young adults, the sympathetic nervous system was still activated by water drinking. The increased total peripheral resistance observed in these subjects was not able to cause an increase in blood pressure because of the compensatory reduction of cardiac output (Lu et al. 2003).

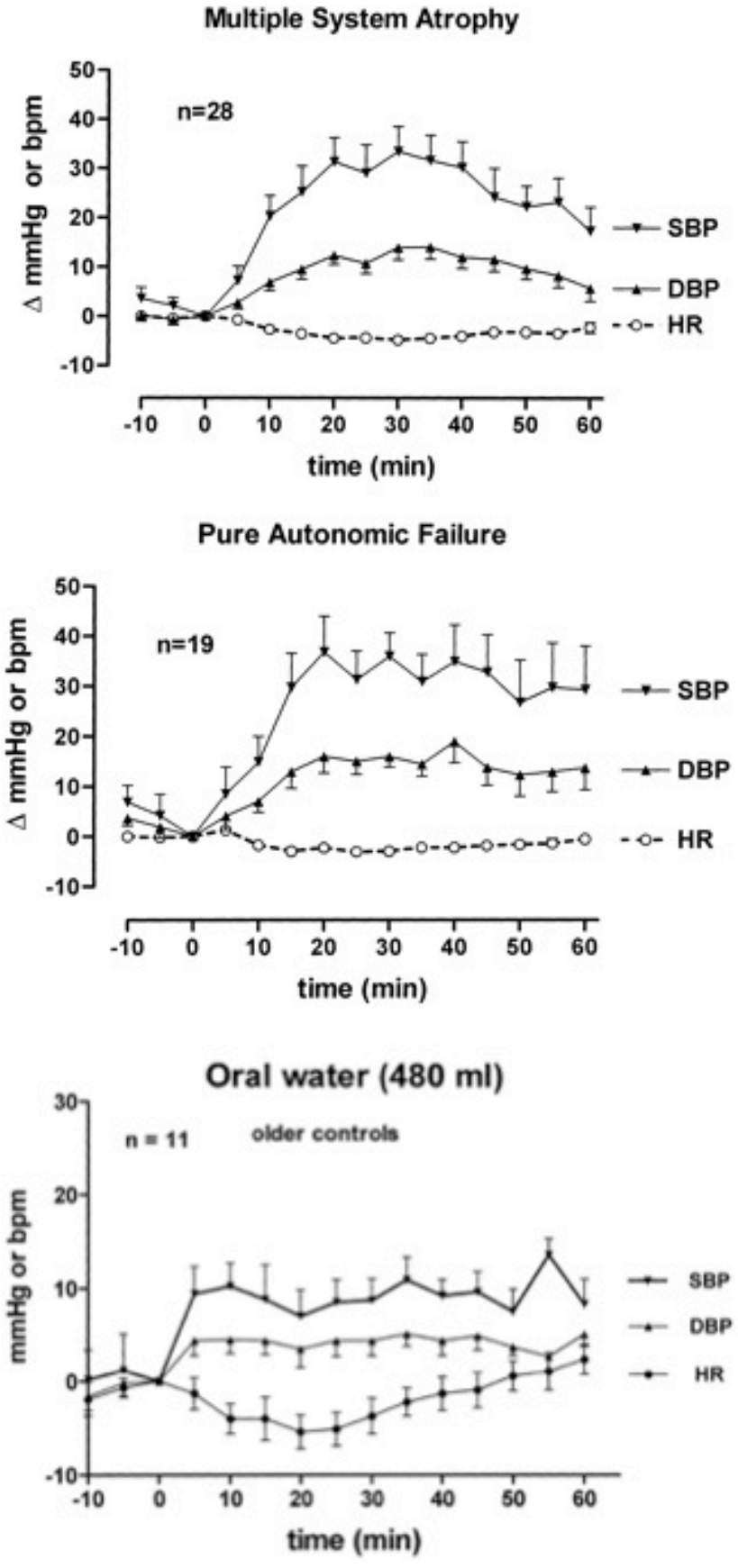

Figure 6: Water ingestion produced pressor effect in patients with MSA, PAF and healthy elderly. Modified from (Jordan et al. 2000)

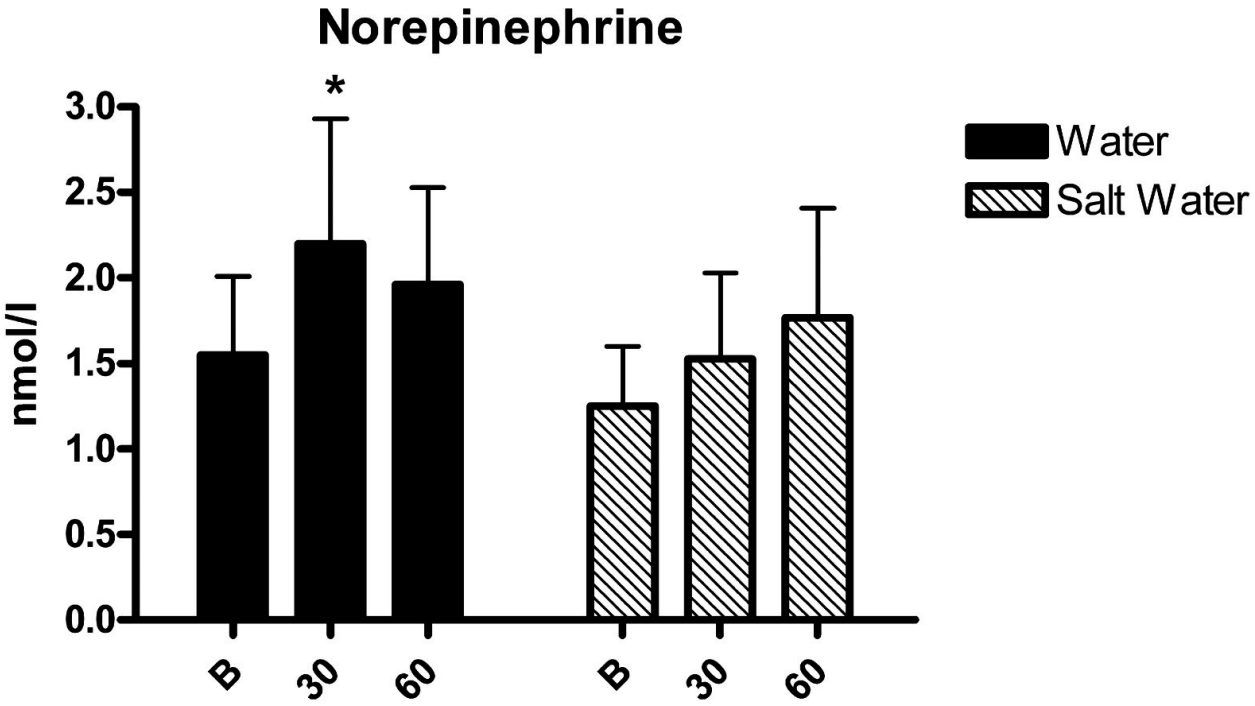

Figure 7: Plasma norepinephrine increased significantly 30 minutes after water ingestion (Raj et al. 2006).

Drinking 473 ml (16 ounces) of water before a tilt-table test helped young healthy subjects to prolong their tolerance for upright posture (Lu et al. 2003). Since the discovery of the osmopressor response, patients with autonomic failure have benefited from it as a treatment to cope with syncope (Shannon et al. 2002). Further studies to better understand the exact locations and mediators of this response could lead to development of more effective drug therapy and management of blood pressure for these patients.

2.2. Previous works in animals on the osmopressor responses

The OPR can be produced in a sino-aortic-denervation (SAD) mouse model. It has a magnitude and time-course similar to that seen in human studies. This response is present when water is infused to either the stomach or the duodenum. On the other hand, infusion of 0.9% saline into the duodenum does not elicit an osmopressor response in this mouse model (McHugh et al. 2010). This finding further confirms that the OPR is not a product of volume expansion or luminal stretch. Interestingly, osmolality in the portal vein is significantly lower than systemic osmolality at 15 minutes after water infusion, at the time of the maximal pressor response (Figure 8) (McHugh 2010). TRPV4 is considered a potential mediator of the OPR since this effect is lost in TRPV4 knockout mice. Yet the osmolality of the portal vein of these mice was also lower than systemic osmolality (Figure 9) ((McHugh et al. 2010). We therefore designed a study of the role of the portal circulation and the liver in sensing changes in plasma osmolality and the relationship between osmolality and the OPR (see chapter II).

The OPR is completely absent in dopamine-β-hydroxylase (Dbh) knockout mice which, due to their genetic defect, have undetectable norepinephrine (Weinshenker et al. 2002, Szot et al. 1999). The α1-adrenergic receptor antagonist prazosin blocks the OPR in mice. These results confirm pharmacologically and genetically the importance of norepinephrine in eliciting the OPR, as was indicated in human studies and suggest the involvement of the sympathetic nervous system in the efferent arm of the OPR (McHugh et al. 2010). McHugh *et al*. also studied the role of the vagus in the OPR by infusing water into the duodenum of mice following vagotomy. It was concluded that the vagal

nerve was not crucial for the OPR because a similar increase of BP was observed in these mice (McHugh et al. 2010).

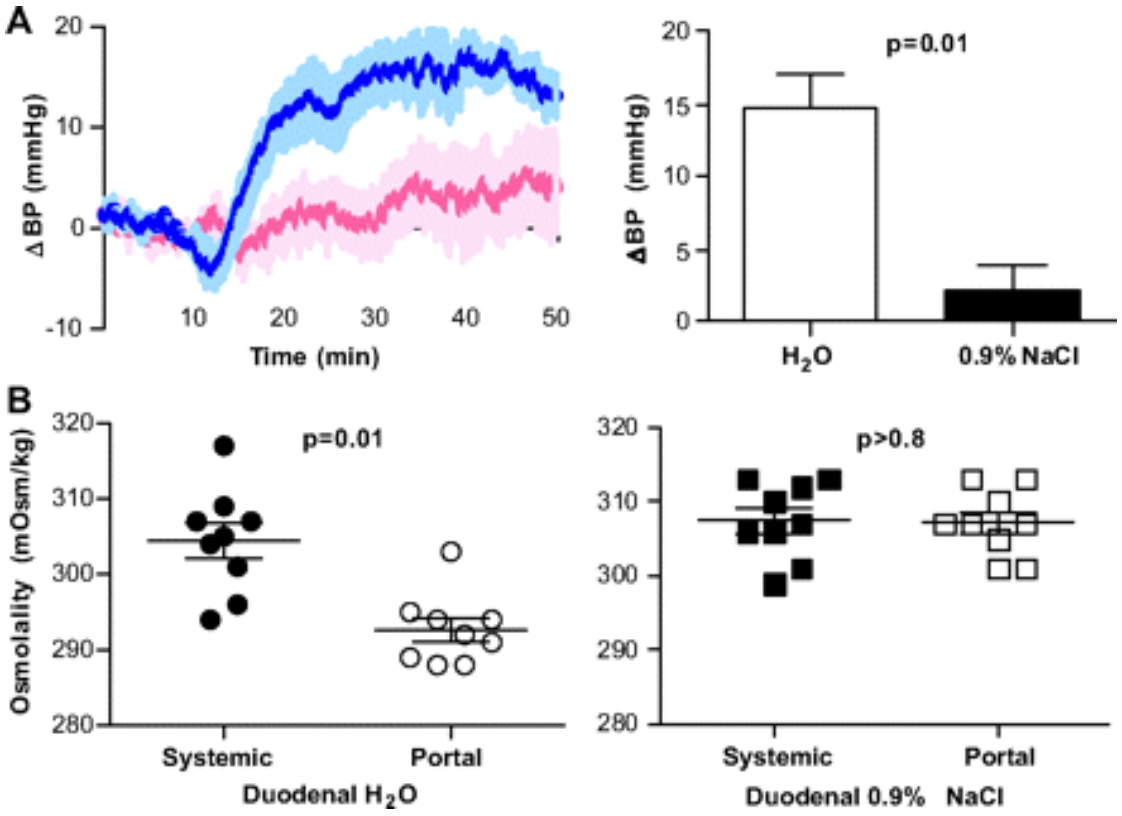

Figure 8: Osmopressor response in SAD mice A) Water (Blue) but not saline (Pink) could produce the OPR. B) After water infusion, portal osmolality was significantly lower than systemic osmolality. This difference was not observed when saline was given (McHugh 2010).

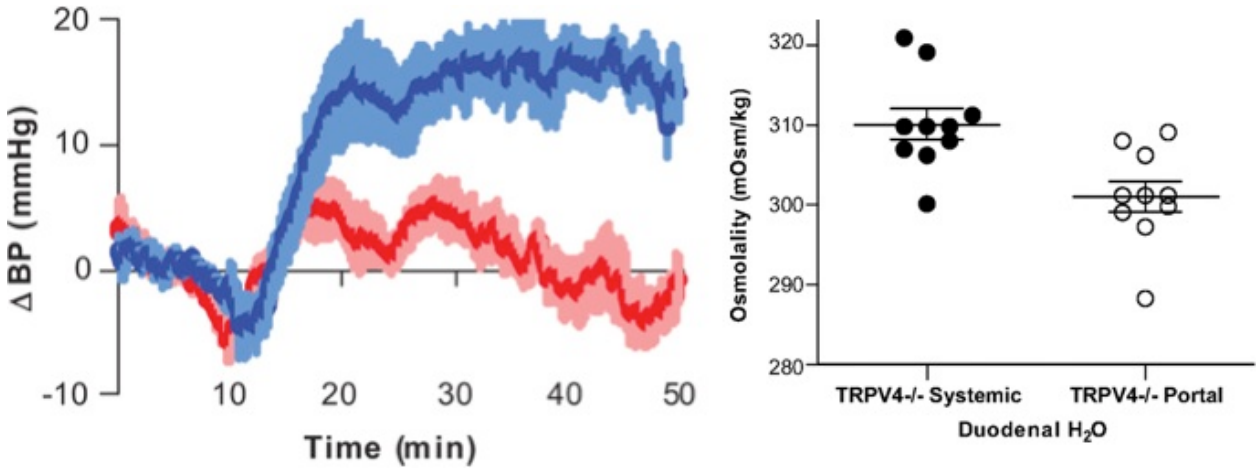

Figure 9: TRPV4$^{-/-}$ mice did not respond to water despite having a similar osmolality reduction in the portal vein. Modified from (McHugh et al. 2010).

Study of OPR in mice suggested that osmolality reduction in the portal region was the key stimulus of the pressor response. This reduction might be detected by TRPV4 channels in the portal region and the signal transferred to spinal afferent nerves to increase sympathetic signals and release norepinephrine as the result.

2.3. Potential molecular mediators of the osmopressor response

2.3.1. Transient receptor potential vanilloid 4 (TRPV4)

TRPV4 remains the most likely mediator of the OPR. These channels are expressed in the heart, brain, kidneys and the neurons of the dorsal root ganglia (Earley et al. 2005, Tian et al. 2004, Pritschow et al. 2011, Sukumaran et al. 2013, Wu et al. 2007). Previous work in TRPV4$^{-/-}$ mice showed that these knockout mice did not have increased blood pressure after water ingestion despite their portal osmolality being significantly lower than the systemic osmolality just like in WT mice

(McHugh et al. 2010). However, the exact location where TRPV4 might detect osmolality change to convey the signals to the spinal cord remains to be determined. TRPV4 channels can be activated by hypo-tonicity, heat, mechanical and chemical stimuli such as 4α-phorbol ester (4α-PDD) and arachidonic acid (Watanabe et al. 2003). Once activated, TRPV4 channels are ~10x more permeable to Ca^{2+} than to Na^{+} (Watanabe et al. 2003). Ca^{2+} entry to cells can result in neurotransmitter releases such as calcitonin-gene-related peptide, substance P or nitric oxide (NO) (Kohler and Hoyer 2007, Grant et al. 2007, Zhang and Gutterman 2011, Rath, Dessy, and Feron 2009).

In $TRPV4^{-/-}$ mice, hepatic afferent nerves no longer respond to a hypo-osmotic stimulus, suggesting the importance of TRPV4 channels in the hepatic region (Lechner et al. 2011). TRPV4 also plays a critical role in regulating volume decrease and salt-sensitive hypertension (Gao and Wang 2010a, b, Gao et al. 2009). High sodium intake presents as a risk factor for hypertension and is observed in >60% of essential hypertension patients (Rodriguez-Iturbe, Romero, and Johnson 2007). When rats were fed 3% high salt diet, TRPV4 expression in the DRS and the mesenteric resistant arteries was significantly upregulated (Gao et al. 2009). On the other hand, TRPV4 expression in the Dahl salt-sensitive rat is significantly lower than in the salt-resistant rat after high-salt diet (Gao and Wang 2010b). These results suggest that TRPV4 not only responds to hypo-tonicity but might counteract salt-induced hypertension also.

2.3.2. Calcitonin-gene-related-peptide

Calcitonin-gene-related peptide (CGRP) is a 37-amino acid neuropeptide currently viewed as perhaps the most potent known vasodilator (Brain and Williams 1985). It is 100-1000 times more potent than substance P or acetylcholine (Asimakis et al. 1987). CGRP exists in two forms, α-CGRP and β-CGRP, that share more than 90% homology (Steenbergh et al. 1986). While α-CGRP is the result of tissue-specific alternative splicing from the calcitonin gene, β-CGRP is the product of translation from a completely different gene. Both genes are located on chromosome 11 (Alevizaki et al. 1986). The majority of CGRP is α-CGRP, which is expressed widely in both central and peripheral nervous systems (Brain and Grant 2004). In the periphery, α-CGRP is found mainly in sensory neurons that are closely associated with blood vessels, at the junction of the adventitia and the media passing into the muscle layer, where CGRP release is localized appropriately to act as a potential vasodilatory agent (Holzer 1988, Uddman et al. 1986). CGRP has been known to co-express with TRPV1 and TRPV4 channels in sensory neurons (Vergnolle et al. 2010, Brierley et al. 2008). Activation of TRPV channels leads to the release of downstream signaling chemicals such as CGRP and substance P (Grant et al. 2007, Chan et al. 2003). CGRP is also expressed in other organs and tissues important for autonomic cardiovascular regulation, such as the sinoatrial and atrioventricular nodes, *nucleus tractus solitarii*, the vagal nerve and the hypothalamus (Zaidi et al. 1985, Hokfelt et al. 1992). The administration of CGRP systemically causes a dose-related decrease in blood pressure (BP) in both normotensive and spontaneously hypertensive rats, as well as in human subjects (Wimalawansa 1996, 1997).

Patients with secondary hypertension are reported to have elevated plasma CGRP as a compensatory mechanism for high blood pressure (Masuda et al. 1992). Interestingly, in essential hypertension subjects, plasma CGRP is reduced compared to healthy volunteers (Portaluppi et al. 1992, Wang et al. 2007). In mice more than 80% of gastric spinal afferents contain CGRP (Chan et al. 2003). Due to its neurotransmitter properties, presence in spinal and vagal afferents, and association with the TRP family CGRP may be a potential mediator in the pressor effect.

3. Thesis Aims

Input from the autonomic nervous system is constantly required to maintain the blood pressure (BP) during upright posture and normal daily activities. It is a major challenge for the body to sustain cardiovascular homeostasis because even mild perturbation of this fine-tuning control mechanism can impair orthostatic tolerance. As many as 1,000,000 Americans are reported to have problems with orthostatic regulation, of which 25% present with orthostatic hypotension. Since orthostatic hypotension causes significant symptoms and a substandard quality of life, much effort has been invested to study the autonomic nervous system in health and diseases. However, fundamental determinants of autonomic cardiovascular regulation are yet to be determined.

One influence on autonomic cardiovascular regulation was unexpectedly discovered during studies in patients with efferent autonomic failure, in which oral ingestion of water elicited a robust pressor response. Since these initial observations, effects of water ingestion have also been observed in healthy young and old subjects, in patients with hypertension, and individuals with neurally mediated syncope, though responses are of smaller magnitude. This water response is now widely recognized and confirmed. Results from this clinical research led to the suggestion to the Red Cross that water could be given prior to blood donation to prevent fainting (van den Berg et al. 2012).

Little is yet known about the mechanism by which water exerts this pressor effect. The magnitude of the response suggests that water may play a role in an unrecognized mechanism involving cardiovascular regulation in both heath and disease. To study this phenomenon, a mouse model that mimics human baroreflex dysfunction has been used in studies described in chapters II, III and IV. Previous mouse studies showed that both gastric and duodenal infusion of water produce a robust pressor response, placing the location of water's actions at or distal to the duodenum and independent of oral, esophageal, and gastric mechanisms. The stimulus for this response is osmolality reduction in the portal region. The sympathetic nervous system is crucial for the osmopressor effect.

The nature of the afferent limb in the OPR and the exact location where hypo-osmolality is detected to initiate the response is yet to be determined. Several potential molecular mediators of this response (TRPV1, Substance P, TRPV4) have been studied in genetically altered knockout mice and only TRPV4 has been implicated thus far. In the TRPV4 knockout mice, the water effect is significantly diminished. The role of CGRP in the OPR is be explored in studies described in chapter V.

The purpose of this dissertation is to study the afferent arm of the OPR by investigating the roles of the portal circulation and the splanchnic nerve. The kidneys are an important factor in BP and osmolality homeostasis. Therefore, this project also aims to study the role of the kidneys in the OPR. The knowledge gained from this project may lead to identification of novel gene product targets for which agonist and

antagonist drugs can be developed as new tools with implications for cardiovascular regulation (Figure 10).

In an attempt to study the mechanisms underlying the OPR, two side projects were carried out to further understand the hemodynamic and autonomic properties of the CGRP$^{-/-}$ and TRPV4$^{-/-}$ mice (Chapters V and VI).

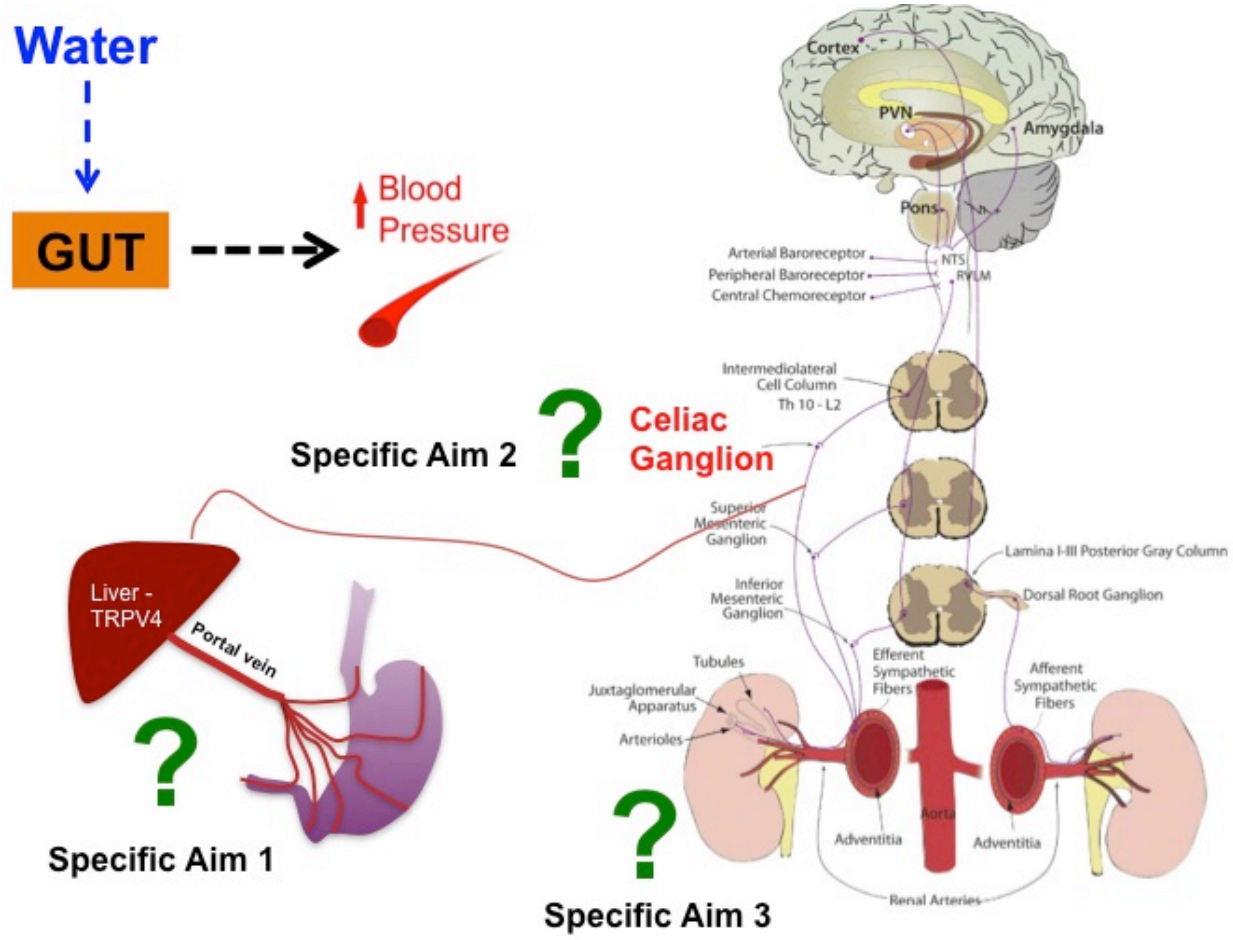

Figure 10: Thesis aims.

3.1. Specific Aim 1: To study the role of osmolality and the portal circulation in initiating the osmopressor response

Water infusion into the duodenum stimulates transport of NaCl from blood to lumen, resulting in the absorption of water against its osmotic gradient in the lower small intestine. During this process, alteration in portal blood osmolality occurs. After water infusion, portal osmolality is lower than systemic osmolality (Figure 8). On the other hand, no change is observed when normal saline is infused.

However, the sites of action, as well as the mechanism that could detect changes in osmolality and elicit the OPR are still unclear. The purpose of this aim is to investigate the role of the portal region in detecting changes in plasma osmolality and initiating the OPR.

To address this aim, the four following questions were answered in chapter II:

1) Is OPR present when hypo-tonicity is introduced directly into the portal vein?

2) Is OPR present when hypo-tonicity is introduced directly into the jugular vein?

3) TRPV4$^{-/-}$ mice did not have OPR when water was infused into the duodenum. However, can OPR be elicited when hypo-tonicity is infused directly in the portal vein?

4) Would hypertonic solution elicit the OPR when directly infused into the portal vein?

3.2. Specific Aim 2: To study the role of gastrointestinal afferent nerves in the OPR

There are two known afferent nerves that send signals from the splanchnic area to the central nervous system: the vagal and splanchnic nerves (Figure 3). The role of the vagal nerve in OPR was previously eliminated. TRPV4 has been shown to be a key player in the OPR. This gene product is localized throughout the mesenteric vessels and the dorsal root ganglia (DRG). The splanchnic nerve innervates the liver and projects into the DRG (Lechner et al. 2011). Therefore, this aim will focus on studying the involvement of the splanchnic nerve in the OPR.

To address this aim, the following two questions were answered in chapter III:

1) Is OPR present in mice that have celiac ganglionectomy when water is infused into the duodenum?

2) Is OPR present in mice that have both celiac ganglionectomy and vagotomy?

3.3. Specific Aim 3: To study the involvement of the renal system in OPR

The kidneys have long been recognized as the principal effector for systemic osmoregulation and chronic blood pressure control. Increased sympathetic efferent activities triggered by water ingestion can also lead to increased renin secretion in the kidneys. As renin release increases via sympathetic activation, angiotensin II (Ang II) in plasma increases as a consequence. Angiotensin II receptors are present throughout the vascular system, and more recently, can be found inside the blood brain barrier. Besides being the regulator for BP, the Ang II system has been reported to regulate the central and peripheral sympathoadrenal systems, as well as water and sodium intake (Saavedra 2005). Therefore, Ang II might be important for the pressor effect of water. Moreover, the renal nerves recently have been recognized as a key regulator of hypertension in human (Polimeni, Curcio, and Indolfi 2013, Abdulla et al. 2011, Salman et al. 2010). This aim will study the necessity of the renal system in the OPR.

To address this aim, the following three questions were answered in chapter IV:

1) Can the angiotensin II receptor antagonist losartan block the OPR?

2) Are intact kidneys required for initiating and maintaining of OPR?

3) Can renal nerve denervation abolish the OPR?

4. Materials and Methods

All protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC) and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition, 2011).

4.1. Mice

Wild type C57BL/6 mice (n=146; in-house breeding) were used in all of the experiments unless otherwise noted. TRPV4$^{-/-}$ mice (n = 23) with C57/BL6 background were developed and provided by Wolfgang Liedtke (Liedtke and Friedman 2003) from Duke University. Global knockout αCGRP/calcitonin mice (*CGRP*$^{-/-}$; n=33) were generated by Robert F. Gagel, MD from MD Anderson Cancer Center, Texas as previously described (Gangula et al. 2000) and re-derived by Charles River on a C57BL/6 background (Glaser et al. 2007). Briefly, exons 2 through 5 of the calcitonin I gene were replaced with PGK neoBPA to generate CGRP$^{-/-}$ homozygous deficient mice. Animals were 4-5 months and age-matched at the time of all experiments.

4.2. Sino-aortic denervation model

Mice was anesthetized with 4% isoflurane and kept at 2% during surgery. A ventral midline incision of ~1cm was made in the neck to allow access to both carotid bifurcations as previously described (Schreihofer and Sved 1994, Guo, Thames, and Abboud 1982, McHugh 2010). The submandibular glands were carefully separated to expose the carotid arteries and the afferent components of the baroreflex. All connective tissue associated with the carotid sinus region was removed. The superior

cervical ganglia, as well as the carotid sinus nerve, were isolated and removed. To ensure complete denervation, the adventitia in the bifurcation area was also stripped. Lack of bradycardia after injection of phenylephrine (20µg/Kg, 10µl/30gram body weight-BW) was used to confirm baroreflex impairment (Figure 11).

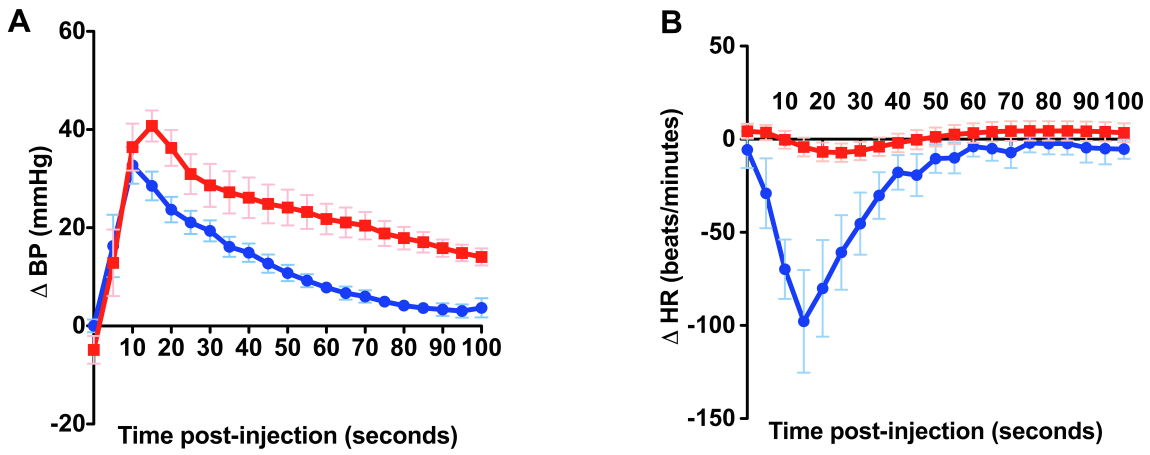

Figure 11: HR response to Phe was blunted after SAD surgery**.** A) Blood pressure response to Phe before (Blue) and after (Red) SAD, n=5. B) HR response to Phe was blunted (Red) after SAD compared to before (Blue), n=5. Phenylephrine was used to validate the completeness of SAD.

4.3. Continuous BP recordings in acute studies

Mice were anesthetized with 4% isoflurane, maintained at 2% during all surgeries, and then kept at 1% isoflurane in oxygen during measurements. An isothermal pad (Braintree Scientific, Inc.) was used to keep body temperature constant at 36^{o}C to 37^{o}C. All drugs were administered via a venous catheter in the left jugular vein. BP was measured through a left femoral artery catheter (PE-20, Micro-Renathane, Braintree Scientific Inc.) connected to a pressure transducer (DTX Plus-6), which was

then connected to a carrier amplifier (Gould Instruments). ECG leads were inserted under the skin for heart rate (HR) measurement. BP and HR signals were recorded using a WINDAQ data acquisition system (DI720, DATAQ, Akron, OH) and analyzed by Physiowave software written by Dr. Diedrich in PV-Wave (Visual Numerics, Boulder, Co.)

4.4. Gastric/Duodenal cannulation

An upper abdominal medial incision approximately ~1cm was made on the left side to expose the stomach. Blunt forceps were used to puncture the fundus and for access of the PE-50 catheter to the stomach. The catheter was passed beyond the pyloric sphincter into the duodenum and secured in place by silk suture. This would also prevent any reflux of GI fluids. Water was infused into the duodenum at 125µl/ minute at a volume of 25 µl/g of BW.

4.5. Portal vein cannulation preparation

This procedure was similar to previously described methods of portal vein cannulation (Chueh, Malabanan, and McGuinness 2006) but applied in an acute setup. A midline laparotomy of approximately ~2.5cm was made and the intestine was moved aside with saline-soaked Q-tips to expose the portal vein. A catheter constructed by connecting Silastic (0.025 in. OD) tubing to a 3mm tip of PE-10 was used to puncture the portal vein and anchored by topical tissue adhesive (World Precision Instruments, Inc.). The abdominal incision was closed with suture and secured by the same adhesive. Either half normal saline or normal saline was infused directly into the portal

vein at 100µl/minute (10% of the venous blood flow speed) at a volume of 12.5 µl/g of BW. Infusion of 0.45% saline would not cause hemolysis (Bruno, Cuppini, and Valora 1979) yet could significantly alter the osmolality of the portal circulation.

4.6. Celiac ganglionectomy procedure

The procedure was adapted from a method previously described by Li *et al*. (Li et al. 2010). A midline laparotomy incision ~2cm exposed the celiac ganglion area. All visible nerves on the aorta, celiac artery and mesenteric artery, as well as the celiac plexus, were removed by stripping with blunt forceps. The abdominal wall was closed in two layers with silk suture. In sham animals, the ganglion plexus was exposed for ~5 minutes but otherwise untouched. All surgeries were performed using aseptic techniques. All mice were given Cefazolin (1.6mg/g BW) intramuscularly (IM) for 3 days post-op and allowed to recover for one week before the water study. Completeness of the CGX surgery was confirmed by the significantly lower level of norepinephrine in the spleen, liver and small intestine.

4.7. Bilateral nephrectomy

Bilateral nephrectomy was performed 15 minutes before water was infused into the duodenum of SAD mice. We adapted the nephrectomy surgery procedure described in Skrypnyk, 2013 #137}. Incision of ~0.5cm were made bilaterally in the back to expose the kidneys. Silk suture (4.0) was used to occlude the renal artery and renal vein. The kidney capsules was carefully removed to leave the adrenal glands intact and untouched. Both kidneys were removed by scissors.

4.8. Bilateral renal denervation preparation

All surgeries were aseptic. Mice were anesthetized with 4% isoflurane and maintained at 2% during surgery. Similar to procedures previously described (Gava et al. 2012), incisions of ~0.5cm were made bilaterally in the back to expose the kidneys and their renal arteries. Silk sutures (4.0) soaked in a solution composed of 10% phenol and 70% ethanol were used to wrap around and rub on the arteries. Sham mice underwent the same procedure except that no perturbation of the arteries occurred to eliminate any damage to the nerve simply due to friction. All animals were given Cefazolin (1.6mg/g BW) IM for three days post-op and allowed at least 7 days to recover before the water study. Renal denervation was confirmed by measuring norepinephrine, epinephrine and dopamine in the kidneys at the end of the water experiment.

4.9. Telemeter implantation and recordings

Mice were anesthetized with 4% isoflurane and maintained on 2% isoflurane in oxygen during the surgery. An isothermal pad (Braintree Scientific, Inc.) maintained body temperature at 36^{o}C to 37^{o}C. Before surgery, buprenorphine hydrochloride (4ul/g body weight (BW) and Cefazolin (1.6mg/g BW) were administered intramuscularly. Following aseptic techniques, a telemeter model TA11PA-C10 from Data Science International (DSI) was implanted with the tip of the device inserted into the left carotid artery. A subcutaneous pocket was made on the left side of the body to house the device. Incisions were closed with sterile suture from Ethicon^{TM} and the mice given Ringer solution (1ml/25 gram BW) from Baxter, Inc. intraperitonealy. All animals were

monitored for at least 1 week before telemetry recording. The telemeter signal was processed using DataQuest ART (Edition 4.3) developed by DSI. The data were continuously sampled at 500Hz processed in 10 sec blocks during day (light on period 06:00-18:00) and night (light off period 18:00-6:00). Analysis was performed when heart rate and blood pressure rhythms normalized after surgery. For statistical analysis, the values for 7 consecutive days were analyzed to assess MAP, HR and activity levels. Means of the 24hr as well as 12hr for the day period and for the night period for each day were obtained. Mean MAP, HR and locomotor activities were also calculated for the whole period of seven days. The analysis program was written in MatLab by Dr. Andre' Diedrich.

4.10. Catecholamine measurements

Urine and plasma catechols were assayed using the method previously described by Eisenhofer *et al* (Eisenhofer 1986) with variations. Briefly, samples were extracted with alumina, and 3,4-dihydroxybenzylamine was added as an internal standard. Eluate was run on an ESA HPLC system consisting of an ESA 542 Autosampler, ESA pump, Axxi-chrom column (Thompson Instruments, Clearbrook Va.), ESA5011 analytical cell, and Coulochem II detector. Both urine and plasma controls were run in sample sets.

Tissue samples were prepared on ice by adding 0.1N perchloric acid. An Omni International TH homogenizer with disposable probes was used to homogenize the tissues. After homogenization, the samples were sonicated for a minimum of 20

seconds. Finally, they were centrifuged twice at 3000 rpm for 20 minutes; supernatant aliquots were transferred to microfuge tubes and centrifuged at 9500 rpm for 5 min to a clear liquid that was run on the HPLC for catechols.

4.11. Baroreflex sensitivity assessment

To determine the baroreflex sensitivity in the two genotypes, acute dose response drug studies were done. Mice were under isoflurane anesthesia (1%). Phenylephrine (PHE) and nitroprusside (NTP) were administered through a venous catheter in the left jugular vein. BP was measured through a left femoral artery catheter connected to a pressure transducer (DTX Plus-6), which was then connected to a carrier amplifier to measure heart rate. BP and HR signals were recorded using a WINDAQ data acquisition system (DATAQ) and analyzed by Physiowave software written by Dr. Diedrich in PV-Wave (Visual Numerics). For each drug, 5 different doses (0, 5, 10, 20, 30 and 40 μg/kg) were administered to study the changes in HR in response to change of BP. The sensitivity to PHE and NTP was calculated as the slopes of the dose response curve to each drug using Prism (Graphpad). The baroreflex sensitivity was calculated as the ratio of maximal R-R interval change to the bolus induced MAP change greater than 20 mmHg.

4.12. Determination of aortic compliance

Segments of thoracic aorta were mounted onto 0.7 mm cannulas in a pressure-myograph system (Danish Myograph Technologies, Model 110P) and extended to their *in situ* length. Calcium-free buffer was used to eliminate any contribution of active tone

of the vessels. A video microscope traced the outer (OD) and inner diameter (ID) of these blood vessels with step-wise increases of intraluminal pressure. Diameters were recorded with every increment of 25 mmHg from 0-200 mmHg. At 200 mmHg, both OD and ID reach a plateau. A vascular compliance curve was constructed by plotting the increment of blood vessel diameters against the corresponding intraluminal pressure applied. A downward shift of the compliance curve indicates aortic stiffening. Stress-strain relationship was also determined (Baumbach, Siems, and Heistad 1991). Circumferential stress (σ) was calculated from the following formula:

Formula 1: $\sigma=(P \times ID) / [2(OD-ID)]$, P = intraluminal pressure.

Intraluminal pressure was converted from millimeters of mercury to dynes per square centimeter (1 mmHg = 1.334×10^3 dynes/cm^2). Circumferential strain (ε) was calculated from the following formula:

Formula 2: $\varepsilon = \Delta OD/OD_0$

OD_0 is the original outer diameter, defined as diameter at 0 mmHg. ΔOD was calculated as the increase of outer diameter from OD_0 at each applied pressure.

4.13. Measurement of aortic collagen and elastin

Masson's trichrome staining and Verhoeff staining were used to visualize aortic collagen and elastin respectively. In separate samples, aortic collagen was quantified by measurement of tissue hydroxyproline as previously described (Hofman et al. 2011). In additional vessels, elastin content was determined as described by Mecham *et al.*,

with modification (Mecham et al. 1995, Starcher 2001). Briefly, insoluble elastin was separated from all other soluble proteins by hydrolysis in 0.1N NaOH at 90°C for 45 minutes. Both the soluble and insoluble fractions were further digested in 6N HCl at 105°C for 48 hours, neutralized and assayed for ninhydrin content.

4.14. Drugs

Phenylephrine hydrochloride (Sigma) was dissolved in saline and given intravenously. In the Phe challenge to validate the completeness of SAD, a dose of 20 μg/Kg was used. Saline (0.9% NaCl) was diluted 50:50 with distilled water to make a 0.45% NaCl solution. The osmolality of 145mOsm was confirmed with Advanced Instrument Micro-Osmometer Model 3320. Losartan (Sigma) was dissolved in distilled water and given intravenously via the jugular vein at the dose of 1mg/Kg to completely block Ang II receptors. High salt diet (8%) (Test Diet Lab) was given to both WT and TRPV4$^{-/-}$ mice for four weeks after baseline recording of BP and HR. This diet was chosen because previous studies showed that the diet alone could increase BP in mouse models that were sensitive to high salt diet (Johansson et al. 2009, Ma et al. 2010, Daumerie et al. 2010, McGuire, Van Vliet, and Halfyard 2008, Bernberg et al. 2009, Kim et al. 2008).

4.15. Plasma osmolality measurement

Blood (~200 μL) was collected from either the femoral artery or the portal vein. Right after collection, all tubes were put on ice for 1hr then centrifuged at 10000rpm for 10 minutes. Plasma was collected for osmolality measurement by the Micro-

Osmometer. Each sample was measured in triplicates and data was present as the mean of three measurements.

4.16. Statistics

A response-feature approach was used to avoid complex longitudinal models for our repeated-measures data (Dupont 2009) in water studies. Average change in BP for each mouse was derived using this formula: $\Delta \boldsymbol{BP} = \boldsymbol{BP}_{pi} - \boldsymbol{BP}_{b}$, in which BP_{pi} was the BP post intervention and BP_b was the baseline BP. Baseline BP was recorded for 10 minutes before the start of infusion. Area under the curve (AUC) was calculated from T_0-T_{35} where T_0 was the beginning of the baseline recording and T_{35} was when the experiment was terminated. The on-rate slope was calculated from the beginning of infusion to the maximum change in BP. Mann-Whitney U test was used to assess both the difference in $\Delta \boldsymbol{BP}$ response between separate treatments or genetic groups and to examine the difference in the AUC and the slopes.

To assess genotype differences in hemodynamic variables, we used 2-way ANOVA for repeated measurements. Tests of the change in BP in response to treatment within each treatment group were assessed by comparing Δ BP and Δ HR using a Mann-Whitney U test. The Mann-Whitney test was also used to assess differences in catechols. The Mann-Whitney U test was chosen to eliminate any potential of unusual outliners. Genotype-related differences in aortic stiffening were evaluated by two-way ANOVA. Statistical significance was accepted when $P < 0.05$. Data are presented as Mean ± SE. Prism 5.0 was used for all statistical analyses.

CHAPTER II

THE ROLE OF THE PORTAL CIRCULATION IN THE OSMOPRESSOR RESPONSE

1. Changes of Osmolality Sensed in the Liver is the Key in OPR Initiation

Previous studies in the lab showed that after water infusion into the duodenum, the portal osmolality is significantly reduced compared to the systemic osmolality (McHugh et al. 2010). Therefore, to investigate whether hypotonicity sensed in the liver initiates the pressor effect of water, either saline or half normal saline (12.5 µl/g of BW at 100 µl/minute) was infused directly into the portal vein of WT mice (Figure 12). Hemolysis of red blood cells only occurs at 0.42% NaCl or lower (bolus injection) (Makroo et al. 2011). Infusion of a 0.45% NaCl solution directly into the portal vein at the speed of roughly 10% of the blood flow therefore should not cause hemolysis.

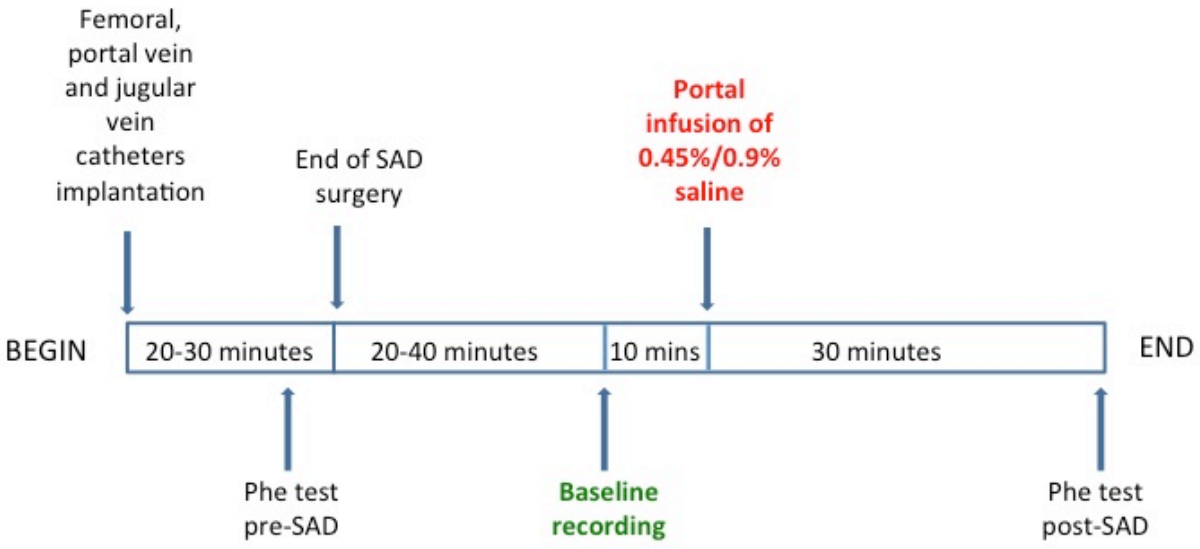

Figure 12: Protocol to study the role of portal circulation in initiating the osmopressor response

Direct infusion of half normal saline but not saline was able to trigger a robust and rapid increase of blood pressure similar in magnitude to that following water infusion into the duodenum (Figure 13A). Area under the curve (AUC) (0.45%: 149.9±99.9, n=7 vs. 0.9%: -74.0±59.6 mmHg.minute, n=5, p=0.003) and the maximum response after fluid infusion (0.45%: 14.67±13, n=7 vs. 0.9%: -7.45±2 mmHg, n=5, p=0.003) differed between saline and 0.45% saline (Figure 13B and 13C).

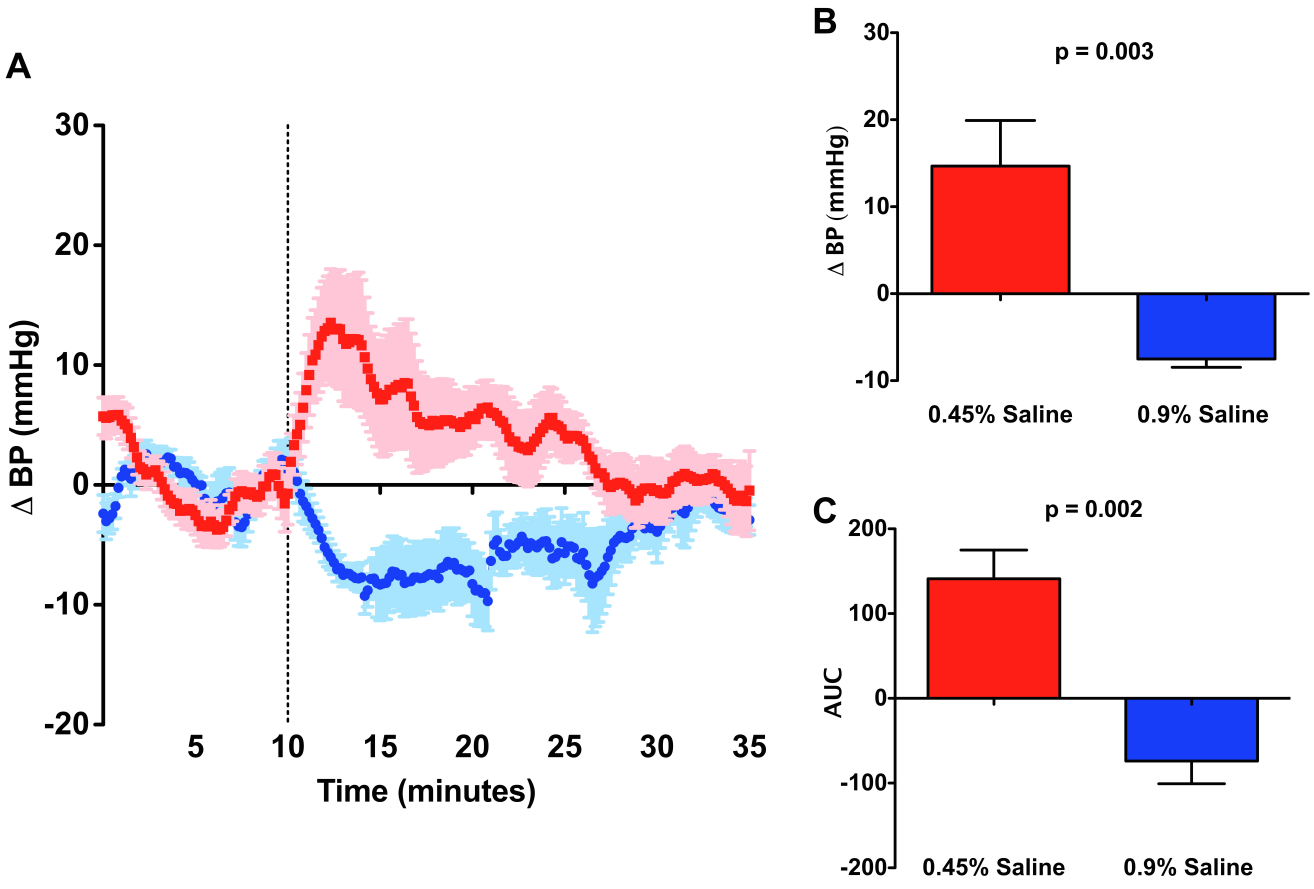

Figure 13: Hypo-osmotic fluid infused into the portal vein (Red, n= 7) can initiate the osmopressor response. This response was not observed when infusing normal saline (Blue, n=5).

To test the hypothesis that the hypotonicity is sensed specifically in the portal region rather than in the systemic circulation, the same volume of 0.45% saline was given directly into the jugular vein of WT mice at the same speed. The procedure was described previously in Figure 12 with the infusion directly into the jugular vein instead of the portal vein. Hypo-osmotic fluid in the jugular vein did not produce a pressor response (Figure 14A). The maximum change in blood pressure after infusion (portal: 14.67±13 mmHg, n=7 vs. jugular: -5.0±5.8 mmHg, n=7, p=0.002) and the area under the curve (portal: 149.9±99.9, n=7 vs. -59.0±46.7 mmHg.minute, n=7, p=0.0006) were significantly different between portal vein and jugular vein infusions, and the OPR was absent after infusion of 0.45% saline into the jugular vein. These results suggest that a decrease in osmolality is sensed specifically in the portal region to initiate the osmopressor response (Figure 14B and 14C).

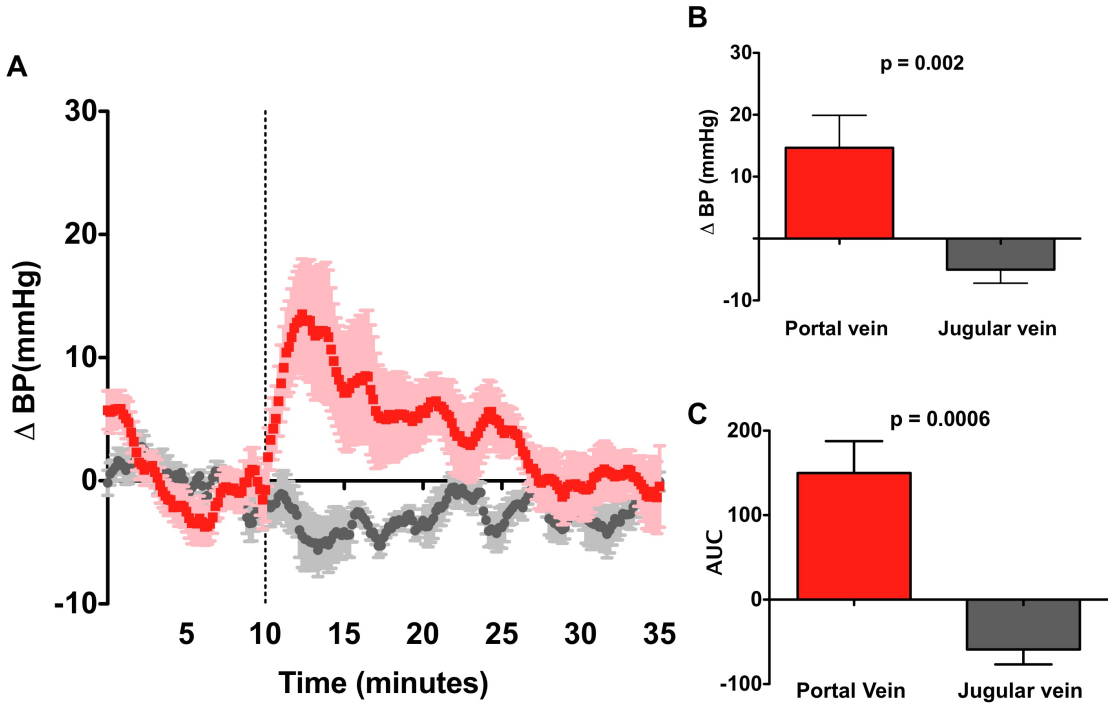

Figure 14: Infusion of 0.45% NaCl solution directly into the jugular vein (Grey, n=7) of WT mice failed to produce the pressor effect that was observed when infusing the same solution into the portal vein (Red, n=7).

2. TRPV4$^{-/-}$ Mice Did Not Display OPR When Hypo-tonicity Was Introduced Into the Portal Area

Water infusion into the duodenum of TRPV4$^{-/-}$ mice does not produce the osmopressor response observed in WT mice (McHugh et al. 2010), indicating that TRPV4 channels are essential for the osmopressor response. Given our evidence that hypotonicity in the portal circulation acts as a trigger for the osmopressor response, we determined whether we could bypass the need for TRPV4 channels by infusing 0.45% saline directly into the portal vein of TRPV4$^{-/-}$ mice (Figure 13). Hypo-osmotic fluid infusion directly into the portal vein of TRPV4$^{-/-}$ did not provoke an increase in blood pressure (ΔBP_{TRPV4}: -1.89 ± 4.9 mmHg, n=8, p = 0.009; $AUC_{TRPV4-/-}$: -74.0 ± 59.6 mmHg.minute, n=8, p=0.003, compared to WT data above) (Figure 15). These results suggest that global expression of TRPV4 channels is important for the osmopressor response.

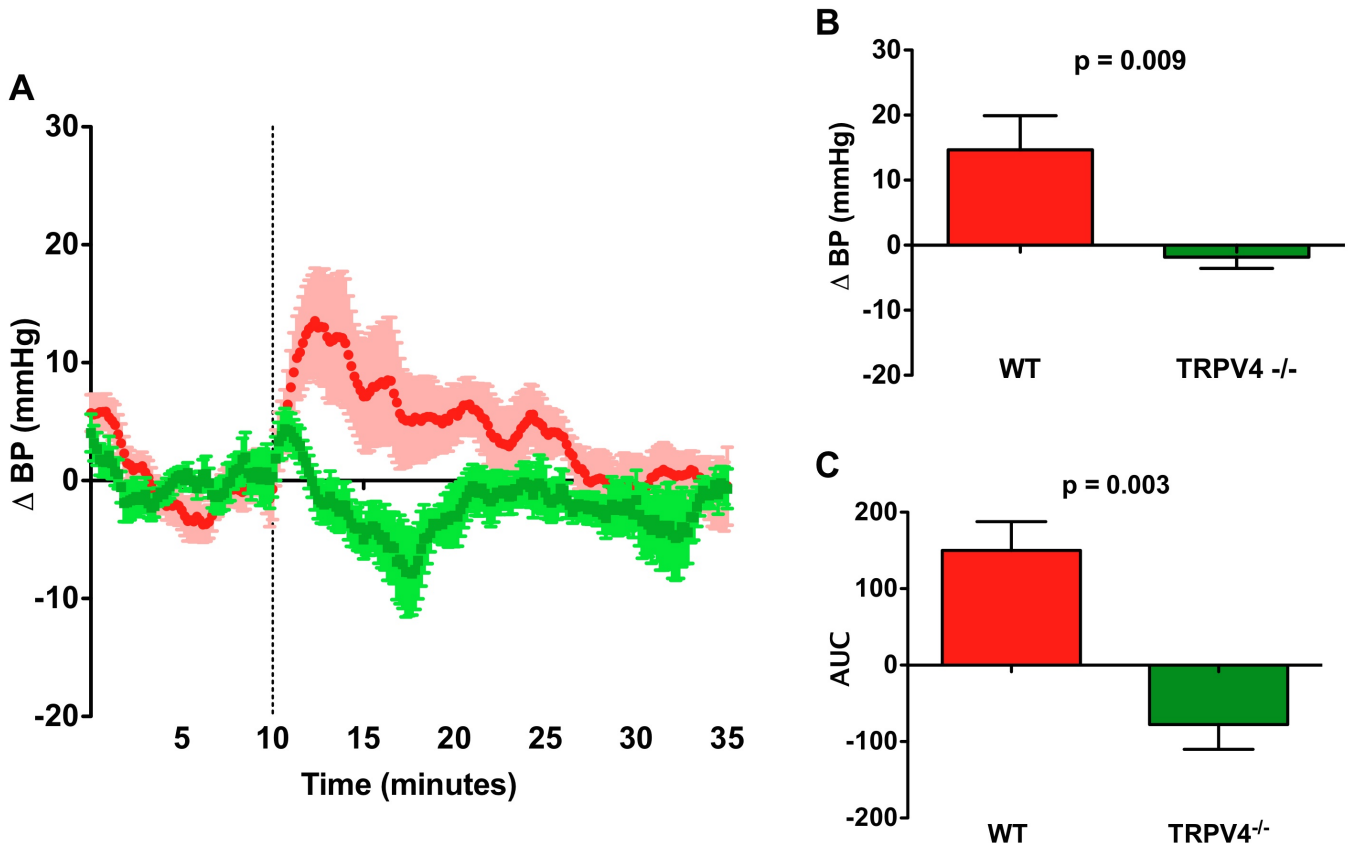

Figure 15: Hypotonic fluid infusion into the portal vein of TRPV4$^{-/-}$ mice (Green, n=8) did not produce the OPR

To study the portal and systemic plasma osmolality at baseline, blood was collected from the portal and jugular veins, and plasma osmolality was measured by an osmometer. Neither portal nor systemic osmolality differed between TRPV4$^{-/-}$ and WT mice. Therefore, the absence of OPR in KO mice was not related to baseline difference in osmolality (Figure 16).

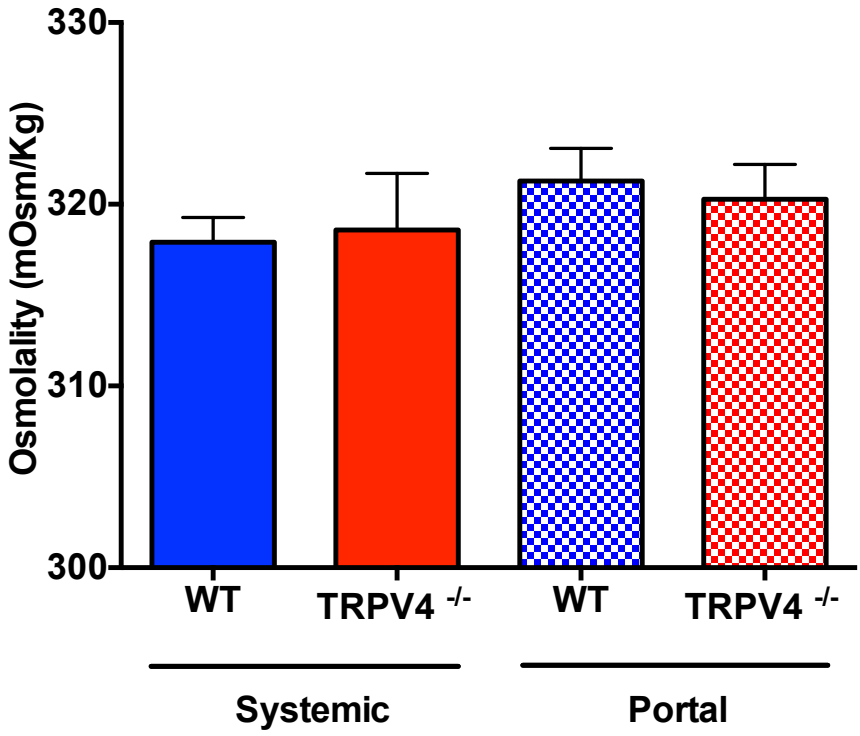

Figure 16: Portal and systemic osmolality at baseline in TRPV4$^{-/-}$ (Red, n=5) and WT mice (Blue, n=5).

3. Hyper-osmotic Fluid Produced a Transient Depressor Effect

At the other end of the spectrum, to investigate whether increasing plasma osmolality in the liver also raises blood pressure; infusion of a solution with substantially increased osmolality (6% NaCl) was given directly into the portal or jugular vein of WT mice. The result showed that after hyperosmotic challenge in both locations, blood pressure dropped dramatically but recovered quickly when infusion stopped (~3-4 minutes) (AUC_{Portal}: -128.5 ± 142.8 vs. $AUC_{Jugular}$: -244.9 ± 211.7 mmHg.minutes, n=8, p= 0.33) (Figure 17). This response was not related to the OPR that we were investigating but it showed that effects of hypertonicity on blood pressure are not specific to the portal region.

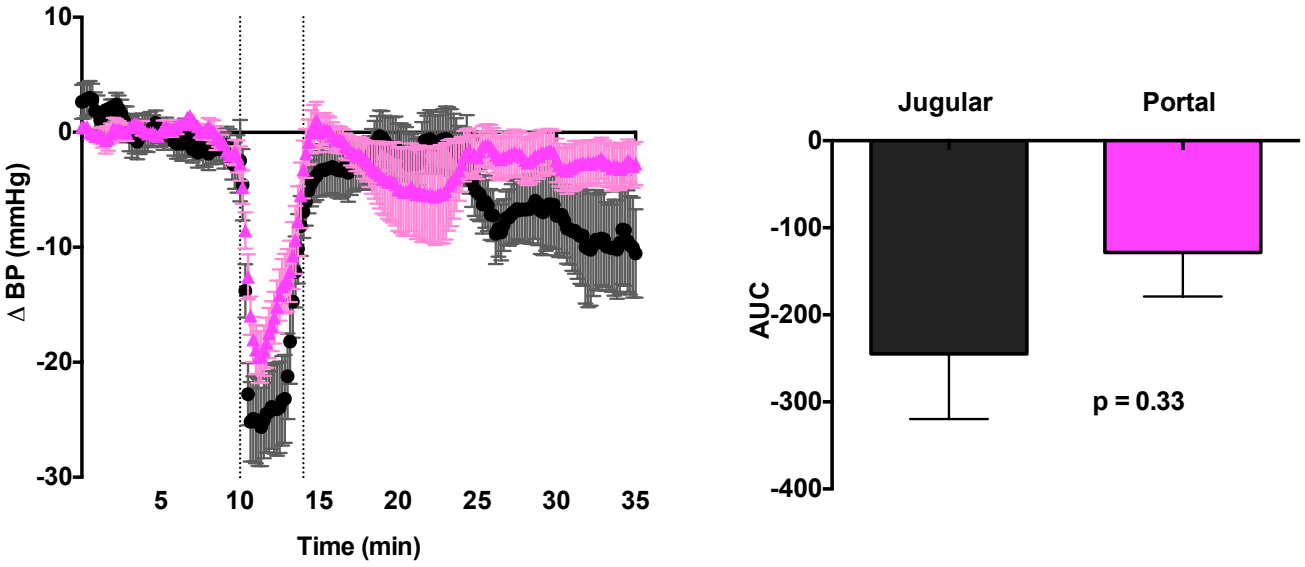

Figure 17: Hypertonicity sensing mechanism was similar in portal vein (Pink, n=8) and in jugular vein (Black, n=8).

4. Discussion

The pressor response to water ingestion provided insight into the previously overlooked contribution of water in autonomic cardiovascular regulation. In this chapter, mechanisms underlying this effect were characterized. The data suggested that: 1) The decrease in osmolality after water drinking was sensed and the OPR was initiated in the portal region; 2) hypotonicity when introduced to the systemic circulation did not provoke the OPR; 3) the OPR was absent in TRPV4$^{-/-}$ mice not only when water was given into the duodenum but also when a hypo-osmolar solution was introduced directly into the liver; and 4) hyper-osmolality caused a transient depressor effect when infused into either the portal or jugular vein.

The exact location of action of the OPR remains to be clarified. A pressor response occurs when water is infused at steady state into the stomach or the duodenum (McHugh et al. 2010). This response is absent when an isotonic solution is infused, indicating that the OPR is not in response to a mechanical stimulus (McHugh 2010, Raj et al. 2006). It was possible that a decrease in osmolality was detected in the mesenteric region. Portal osmolality is significantly lower than systemic osmolality after water infusion (McHugh et al. 2010). This led to the hypothesis that hypo-osmolality is detected in the portal region and initiates the OPR.

Figure 13 shows that hypo-osmotic fluid infusion, but not saline infusion, directly into the portal vein stimulates an immediate pressor effect. It is similar to the OPR data where either water or normal saline was given into the duodenum in mice (McHugh et

al. 2010). This further confirmed hypo-osmoticity as the stimulus of this pressor effect. Normal saline infusion directly into the portal vein of WT mice caused a drop in BP. This could be explained as the compensatory effect to counteract sudden volume increase in the circulation. This effect was overdriven by the OPR when hypo-osmotic fluid was infused.

It could be argued that adding ~8.5% of the blood volume directly into the circulation might increase BP regardless of the location as a result of the increase in blood volume. As shown in Figure 14, however, the pressor response occurred only after infusion of 0.45% NaCl solution into the portal vein and not after infusion into the jugular vein. The osmopressor response was therefore not related to an increase in blood volume. This confirmed the critical role of the liver in the OPR. Hypo-osmotic induced pressor response was specific to the portal region and not a systemic effect. Both human and mice studies have demonstrated that sympathetic nervous system activation is responsible for the rise of BP (Jordan et al. 2000, Lipp et al. 2005, Lu et al. 2003, McHugh et al. 2010, Schroeder et al. 2002, Shannon et al. 2002). Patients with a liver transplant hardly respond to water (May et al. 2011). These data suggested that liver innervation is required for transducing the osmolality signal in the OPR.

One attractive candidate for the osmosensor mechanism in the liver is TRPV4. These channels are highly expressed in the liver and sensitive to hypo-osmotic stimuli (Lechner et al. 2011, Benfenati et al. 2011, Chen, Liu, and Liu 2009, Chen et al. 2009). TRPV4$^{-/-}$ mice did not respond to water during the duodenal infusion. However, it was

possible that TRPV4 channels detected hypo-osmolality in the mesenteric vessels and sent the signal via sensory afferents that innervated the GI tract to initiate the response. The inability of TRPV4$^{-/-}$ mice to raise BP when 0.45% NaCl solution was infused directly into the portal vein (Figure 15) indicated that TRPV4 in this region is required for the OPR.

We have now demonstrated that not only is plasma osmolality in the portal vein significantly lower than systemic osmolality in both TRPV4$^{-/-}$ and WT mice after water infusion into the duodenum (McHugh 2010), but also baseline portal vain and systemic osmolality do not differ between TRPV4$^{-/-}$ and WT mice (Figure 16). These data suggest that it was a defect in the osmolality sensing mechanism, rather than an abnormality in osmolality, in the portal region of TRPV4$^{-/-}$ mice that was responsible for the lack of response to hypo-osmotic stimulus.

It is well-known that pressor hormones such as arginine vasopressin increase in states of dehydration and hyperosmotic stress (Stewart et al. 2011, Kim et al. 2013, Coiro et al. 2011, Yokoyama et al. 2010, Yamaguchi and Yamada 2008, Ivanova, Kochkaeva, and Melidi 2007, Ho et al. 2007, Szmydynger-Chodobska, Chung, and Chodobski 2006). Surprisingly, hyper-osmotic fluid infusion into the portal or jugular vein did not provoke an increase but a dramatic drop in BP. This effect was transient, persisting only during the infusion period (3-4 minutes) (Figure 17). This might be a sudden shock and compensatory response and was unrelated to the OPR that was studied.

Overall these data suggested that the pressor effect was specific to hypo-osmotic stimulus. This hypo-osmotic stress was detected and initiated in the portal region and TRPV4 channels were essential for the osmopressor response.

CHAPTER III

STRUCTURE AND FUNCTION OF THE OSMOPRESSOR RESPONSE

1. The Role of the Splanchnic Nerve in the Pressor Response

McHugh *et al.* showed that the vagus does not play an essential role in the osmopressor response. Mice underwent subdiaphragmatic vagotomy surgery in which the vagal nerves were cut right below the diaphragm still had the pressor response to water (McHugh et al. 2010). Besides the vagal nerve, the liver is also innervated by the sympathetic nervous system via the splanchnic nerve. This nerve has cell bodies in the dorsal root ganglia and projects to the spinal cord (Lechner et al. 2011). Here we investigated the role of the splanchnic nerve in the osmopressor response by performing the water study on mice that had either a celiac ganglionectomy (CGX) or sham surgery (Figure 18).

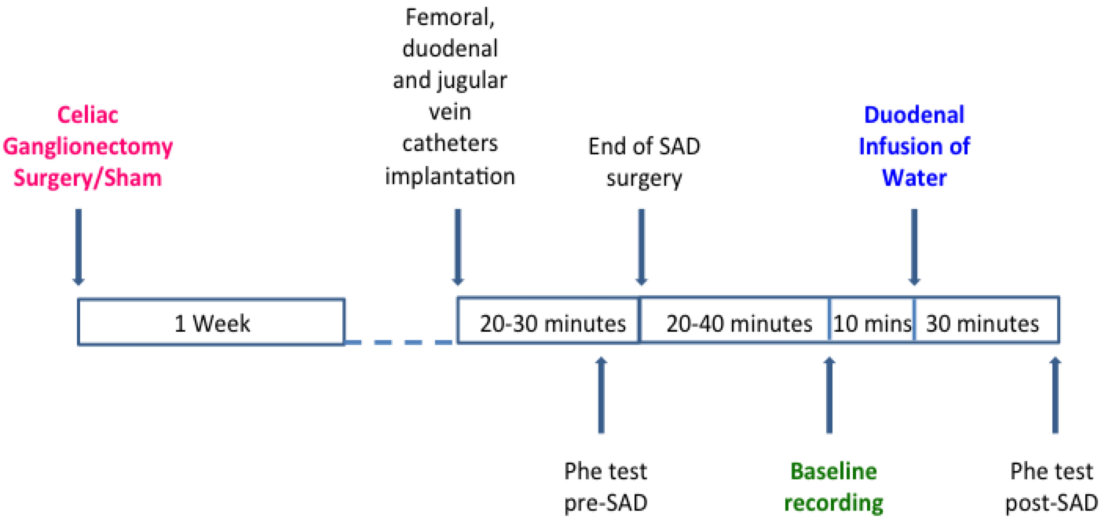

Figure 18: Study protocol to investigate the role of the splanchnic nerve in the OPR

The effectiveness of the CGX surgery was reflected in the significant reduction of NE content in the right kidneys (CGX: 245.4 ± 87.0, n=12 vs. Sham: 322.0 ± 24.8 ng/g tissue, n=14, p=0.02), duodenum (CGX: 23.7 ± 12.3, n=12 vs. Sham: 264.2 ± 118.5 ng/g tissue, n=14, p<0.0001), and spleen (CGX: 63.5 ± 39, n=12 vs. Sham: 715.2 ± 235.0 ng/g tissue, n=13, p<0.0001) (Figure 19). It is notable that following CGX, the reduction in norepinephrine in the kidneys was less than in the duodenum and spleen. This was because the renal plexus is formed not only by fibers from the celiac ganglia but also from the aorticorenal ganglia and aortic plexus (Bishop and Senesac 2010).

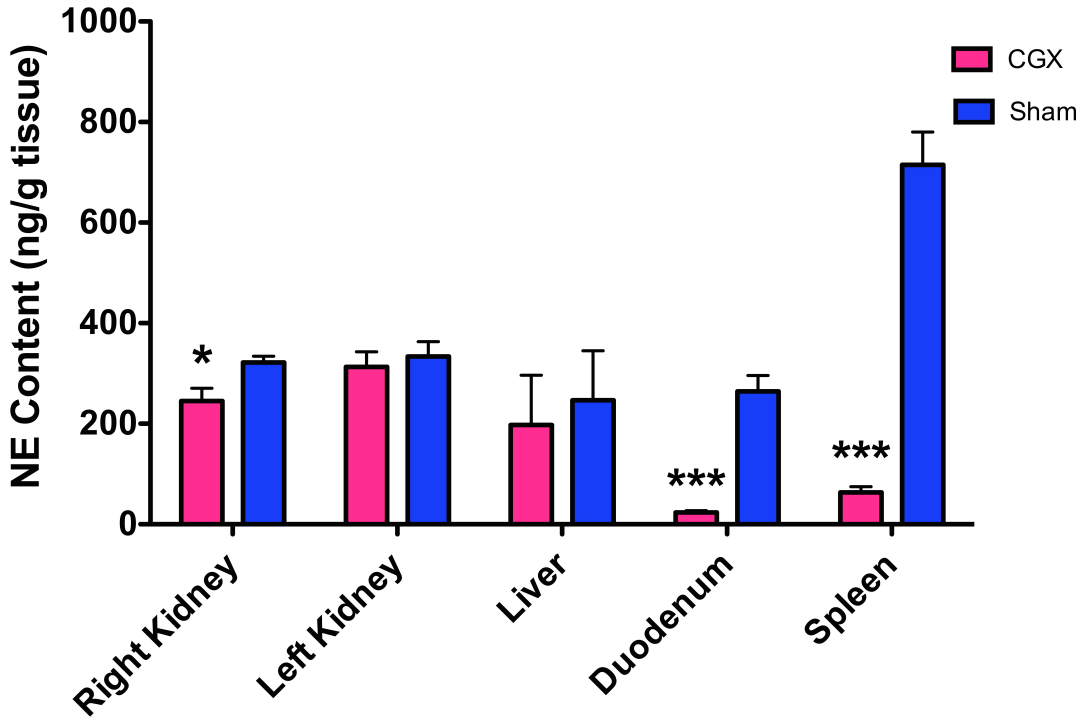

Figure 19: Norepinephrine in the splanchnic organs decreased significantly after CGX (Pink, n=12) compared to WT mice (Blue, n=13).

The osmopressor response was still present in the CGX mice; maximum ΔBP was similar to that in the sham controls (CGX: 14.6 ± 10.9, n=11 vs. Sham: 15.7±6.0 mmHg, n=12, p=0.34) (Figure 20A and 20B). However, the response did not last as long as it did in the sham animals. At 20 minutes after the start of infusion, BP dropped back to baseline value for the CGX mice while remaining elevated for sham mice (CGX: 2.2 ± 8.5, n=11 vs. Sham: 12.7 ± 6.1 mmHg, n=12, p=0.02) (Figure 20C). The AUC for sham mice was also significantly higher than for the CGX mice (CGX: 3.9 ± 161.8 n=11 vs. Sham: 175.8 ± 145.3 mmHg.minute, n=12, p= 0.02) (Figure 20D). The off-rate slope calculated from the maximum ΔBP to the end of the experiment (T=35 minutes) was calculated for each individual mouse. The off-rate slopes in CGX mice were significantly steeper compared to the sham controls, indicating a faster decrease in BP after the initiation of the OPR (CGX: -2.5 ± 1.2, n=11 vs. Sham: -0.4 ± 0.3 mmHg/minute, n=12, p<0.0001) (Figure 20E).

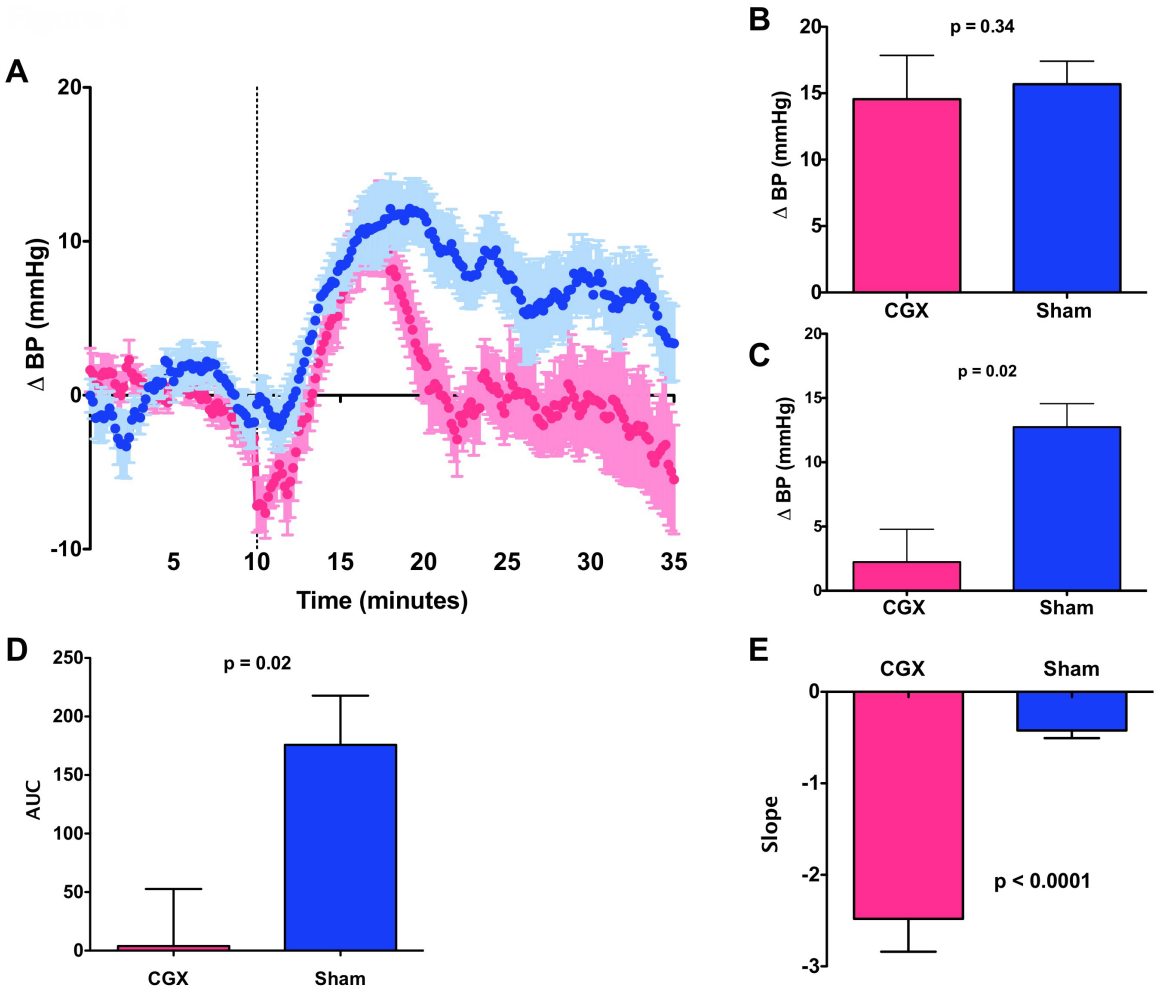

Figure 20: A) Water infusion into the duodenum of CGX mice (Pink, n=11) produced similar OPR to Sham mice (Blue, n=12). B) The maximal change of BP in response to water was similar between the two groups. C) However, the duration of the response was significantly shorter. At T=20 minutes after duodenal water infusion, BP stayed elevated in sham controls while came back down to baseline in CGX mice. D) The AUC was significantly larger in the control groups, indicating a larger OPR E) The recovery slope was significantly steeper in the CGX mice, showing a faster decrease in BP after the initiation of the OPR.

2. The Role of Gastrointestinal Afferent Nerves in the Osmopressor Response

It is possible that if either one of the gastrointestinal afferent nerves no longer functions, the other compensates and therefore, the OPR would still occur. To test the hypothesis that both the vagal and splanchnic nerves contribute to the OPR, the water study was performed in WT mice according to the protocol in Figure 18, but with mice that underwent both vagotomy and celiac ganglionectomy. After water infusion into the duodenum, OPR was still present in mice that underwent both procedures (ΔBP_{Sham}: 30.19 ± 13.9, n=3 vs. ΔBP_{CGV+V}: 16.9 ± 10.8, n=3, p= 0.4) (Figure 21). Double sham mice had full response to water (McHugh et al. 2010).

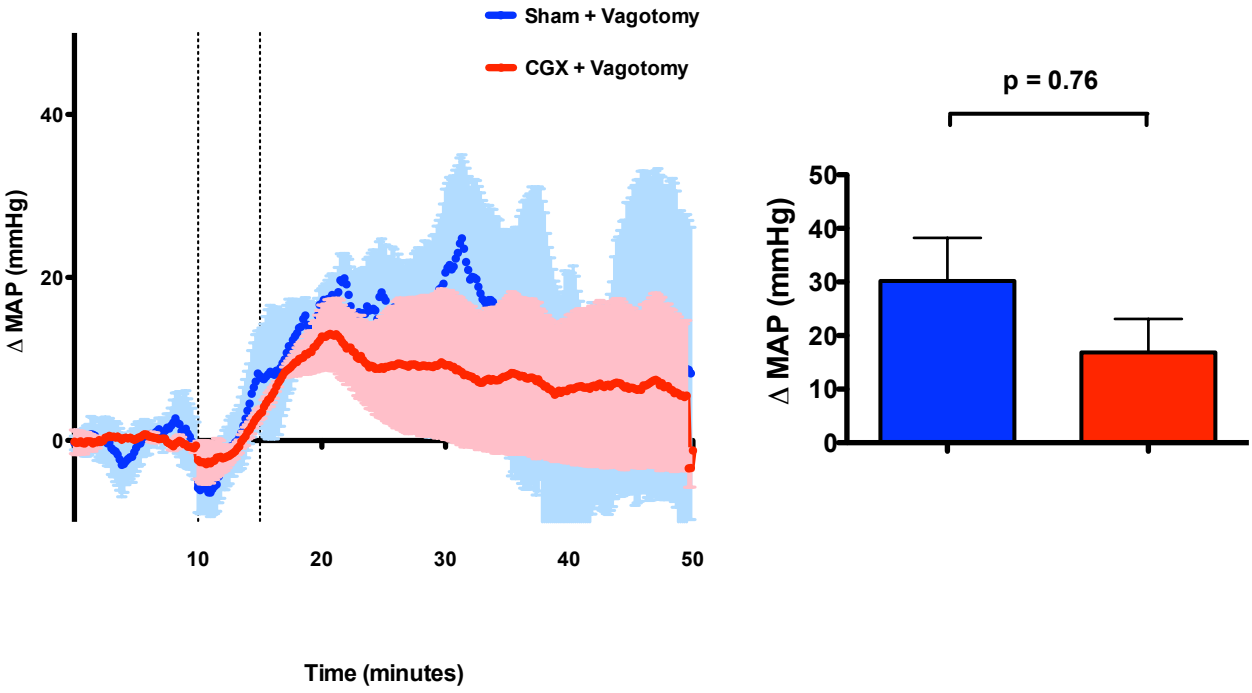

Figure 21: Water study in mice that had both bilateral vagotomy and celiac ganglionectomy (Red, n=3) and controls (Blue, n=3).

3. Discussion

Results presented in the previous chapter suggested that liver innervation is essential to convey osmotic signals. In this chapter, the role of the splanchnic nerve in the OPR was investigated. The data showed that after celiac ganglionectomy, WT mice still experienced a blood pressure increase after water infusion into the duodenum. In mice that underwent both vagotomy and celiac ganglionectomy, OPR still existed.

Sympathetic and parasympathetic nerves innervate the liver, and they contain both afferent and efferent fibers (Yi et al. 2010). The parasympathetic nerve, the vagus, originates in the nodose ganglia and projects to the brainstem (Holzer 1988). McHugh *et al.* showed that the vagus did not play an essential role in osmopressor response initiation, though the duration of the response was much shorter in mice with vagotomy (McHugh et al. 2010). The liver is also innervated by the splanchnic nerve, mediating sympathetic activity. This nerve has cell bodies in the dorsal root ganglia and projects to the spinal cord (Lechner et al. 2011). Figure 20B showed that the initial BP increase is similar in both CGX and sham mice after duodenal water infusion. Therefore, the splanchnic nerve did not play an important role in the initiation of the OPR. However, at 15 minutes after the infusion, BP had already returned to the baseline level in CGX mice while it stayed elevated in the sham controls (Figure 20C). The rate at which BP decreased in CGX mice was significantly higher, indicating a faster drop. AUC was also notably greater in the sham mice (Figure 20E). These data suggested that the splanchnic nerve might play a crucial role in the maintenance of BP elevation. It is worth noting that in this experiment, the celiac ganglion that contained both splanchnic

afferent and efferent nerves was severed. A study by Li *et al*. suggested that celiac ganglionectomy significantly reduces sympathetic innervation in splanchnic organs. The vasoconstrictor response of the mesenteric vessels is abolished in CGX animals (Li et al. 2010). The mesenteric vessels hold a significant proportion (~25% at rest) of the body's blood volume (Tu et al. 2013). As a result of the loss of mesenteric vasoconstriction after CGX, the BP increase of the OPR might be reduced. This might explain the shortened duration of the OPR in CGX mice. Patients with complete cervical spinal cord transaction had a full osmopressor response. In these patients, spinal sympathetic neurons are still intact even when they are not connected to the brain stem (Tank et al. 2003). MSA patients who had efferent neuronal lesion occur in the brain stem also had full response to water. Therefore, it is a possibility that the OPR is the result of a spinal reflex.

We tested the hypothesis that one of the two nerves that innervate the liver would compensate for the loss of the other and still produce the OPR. As shown in Figure 21 both sham mice and mice undergoing subdiaphragmatical vagotomy plus CGX had the OPR. The variability was large in the latter group due to the major interruption of the animals' physiology and the small number of mice that were studied. However, the OPR was still clearly present.

The enteric nervous system (ENS) is another important component that innervates the splanchnic area (Coruzzi et al. 2007, Ohlsson et al. 2007). Although the ENS is usually considered as an independent structure, it can also relay signals to the

central nervous system by interacting with the vagal nerve (McBride et al. 2001). The role of the ENS in the OPR cannot be eliminated and might provide more insight into the role of this nervous system in blood pressure regulation.

CHAPTER IV

INVOLVEMENT OF THE RENAL SYSTEM IN THE OSMOPRESSOR RESPONSE

1. Losartan Could Not Block the OPR

To test the hypothesis that Ang II is essential for the OPR, a water infusion study was performed in mice that were treated with losartan or vehicle 30 minutes before the water infusion. This time interval provides complete blockade of Ang II receptors when losartan is injected intravenously (Sugawara et al. 2002). A separate group of mice were first administered Ang II before and after losartan treatment to confirm the lack of an Ang II-mediated pressor effect 30 minutes after losartan. The protocol is described in Figure 22.

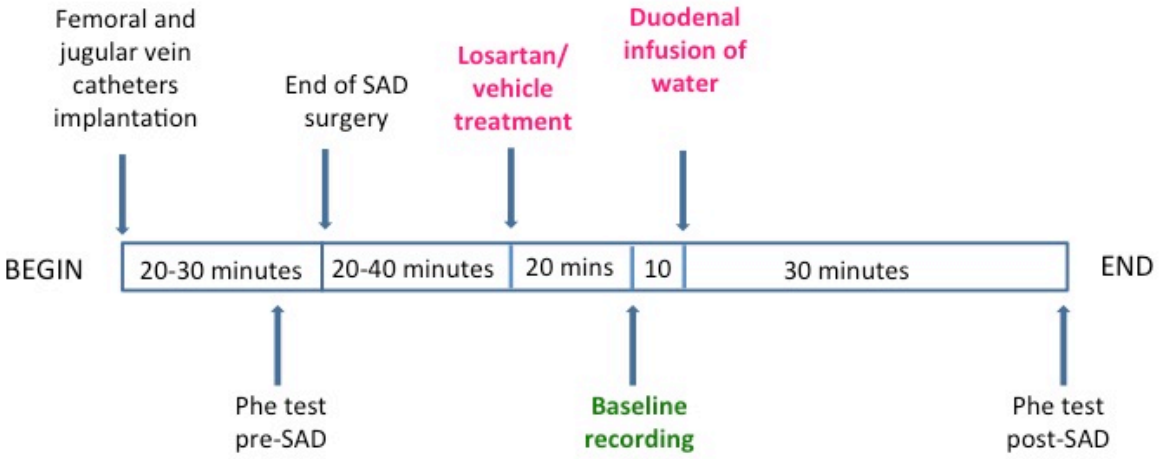

Figure 22: Water study with losartan protocol.

Losartan decreased baseline BP immediately after jugular vein infusion (~5-6 mmHg). However, baseline BP still stayed in the 70-90 mmHg range. After water

infusion into the duodenum, both groups treated with losartan or vehicle displayed the pressor response ($\Delta BP_{Losartan}$: 24.2±5.7 vs. $\Delta BP_{vehicle}$: 23.8±8.5 mmHg, n=5, p= 0.84; $AUC_{Losartan}$: 197.8±39.9 vs. $AUC_{Vehicle}$: 228.8±78.6 mmHg.minute, n=5, p= 0.53). BP started to increase right after water infusion and maximized around 25 minutes, which was similar to the characteristic of OPR observed in human.This indicated that activation of Ang II type 1 receptors is not essential in the OPR (Figure 23).

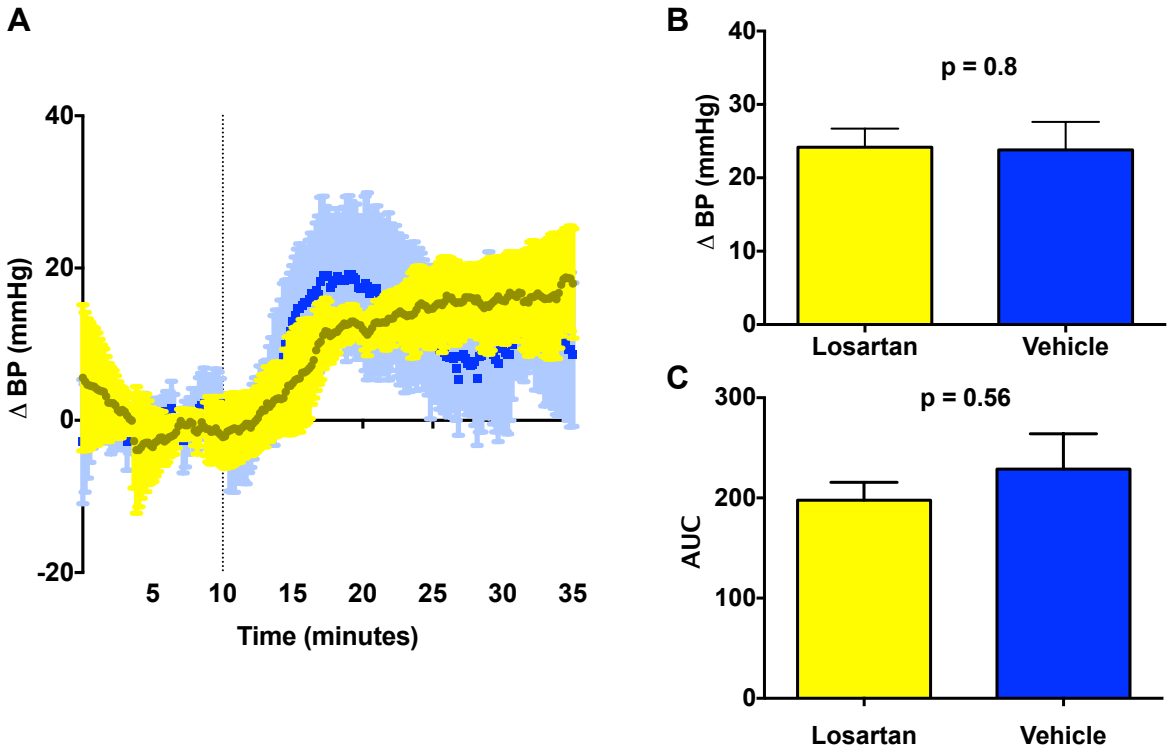

Figure 23: Losartan had no effect on the OPR. A) Mice treated with losartan (Yellow, n=5). i.v 20 minutes before water infusion still experienced the OPR similar to the vehicle-treated controls (Blue, n=5). B +C) Maximal changes in BP and AUC were similar between the two groups.

2. Bilateral Nephrectomy Abolished the Pressor Response

To test the hypothesis that the kidneys are essential for the OPR, a water infusion study was performed in bilaterally nephrectomized and sham-operated mice. The protocol is described in Figure 24. To minimize the possibility of changes in levels of hormone such as renin and angiotensin II, nephrectomy surgery was performed 5 minutes before the baseline recording of BP.

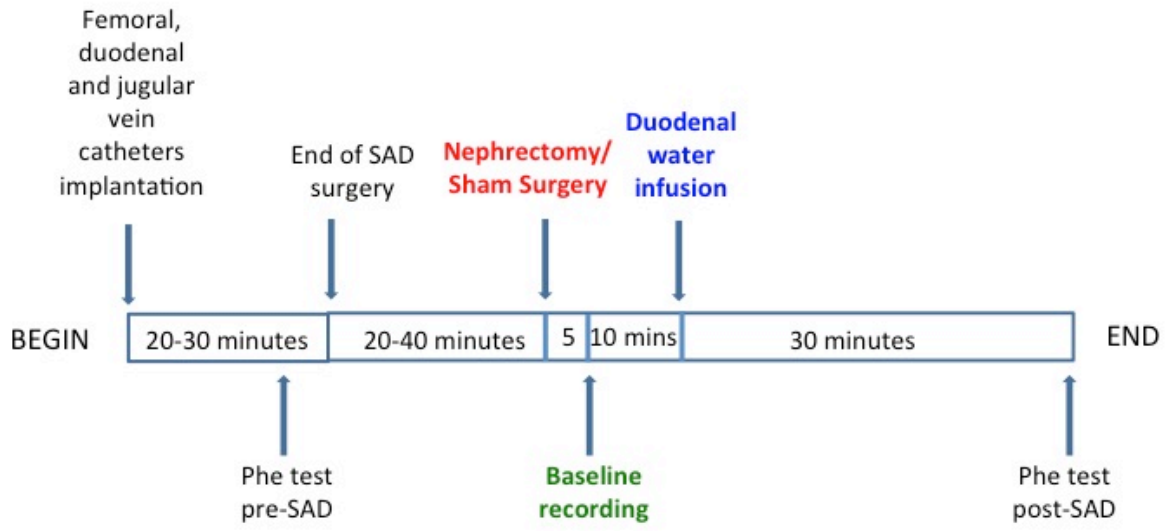

Figure 24: Protocol to study the effect of bilateral nephrectomy in the OPR

In contrast to sham controls, nephrectomized mice experienced no OPR when water was infused into the duodenum at T= 15 minutes after surgery ($\Delta BP_{nephrectomized}$: 0.7 ± 11.9 vs. ΔBP_{Sham}: 20.3 ± 8.5 mmHg, n=5, p= 0.03; $AUC_{nephrectomized}$: 1.7±258.9 vs. AUC_{Sham}: 304.9±111.1 mmHg.minute, n=5, p= 0.05) (Figure 25).

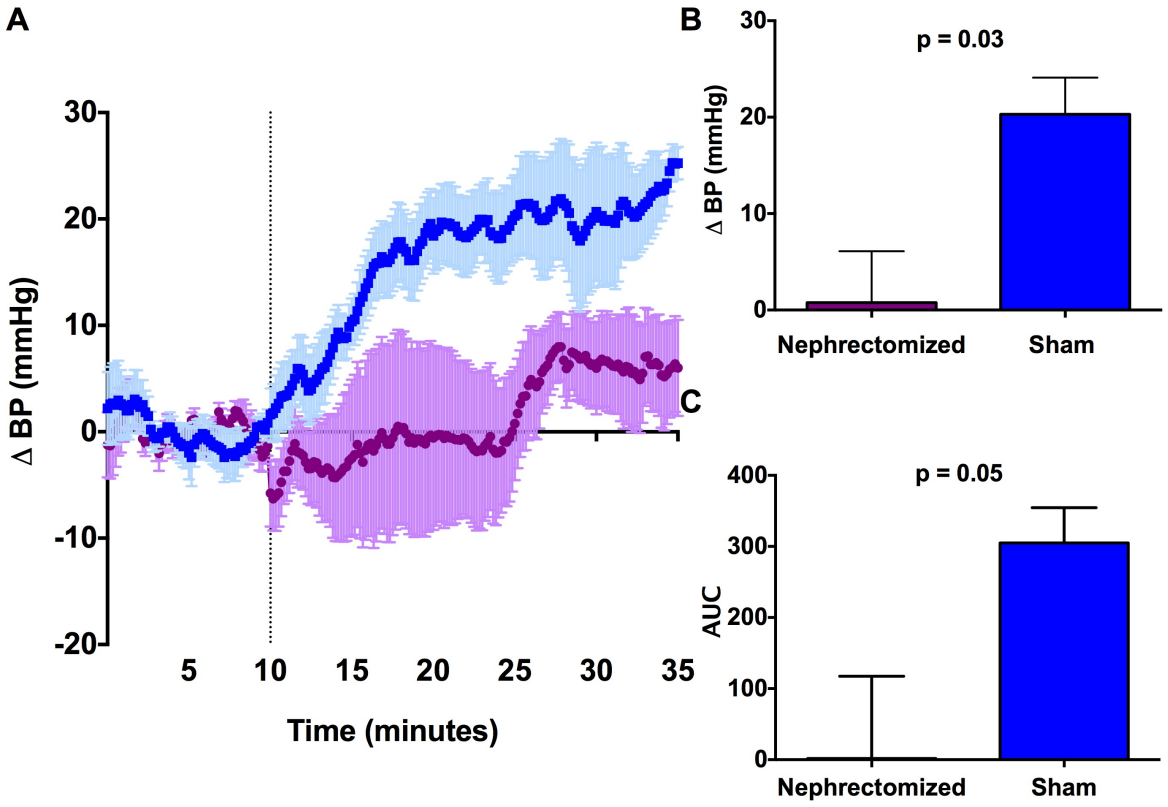

Figure 25: OPR was significantly reduced in nephrectomized mice (Purple, n=5) compared to WT mice (Blue, n=5)

3. Bilateral renal denervation diminished the osmopressor response

To test the hypothesis that a renal neuronal pathway plays an important role in the OPR, the water study was carried out in mice following bilaterally renal-denervation (RD) and in sham-operated mice. The completeness of the renal denervation surgery was confirmed by a significant reduction in the NE and dopamine but not epinephrine content (Consigny et al. 2014) of the kidneys (RD_{NE}: 39.9±28.4 vs. $Sham_{NE}$: 306.3±43.6 ng/g tissue, p<0.0001; RD_{Epi}: 6.6±2.5 vs. $Sham_{Epi}$: 11.7±7.5 ng/g tissue, p=0.08; RD_{DA}: 11.3±4.5 vs. $Sham_{DA}$: 47.6±19.3 ng/g tissue, p=0.0006) (Figure 26).

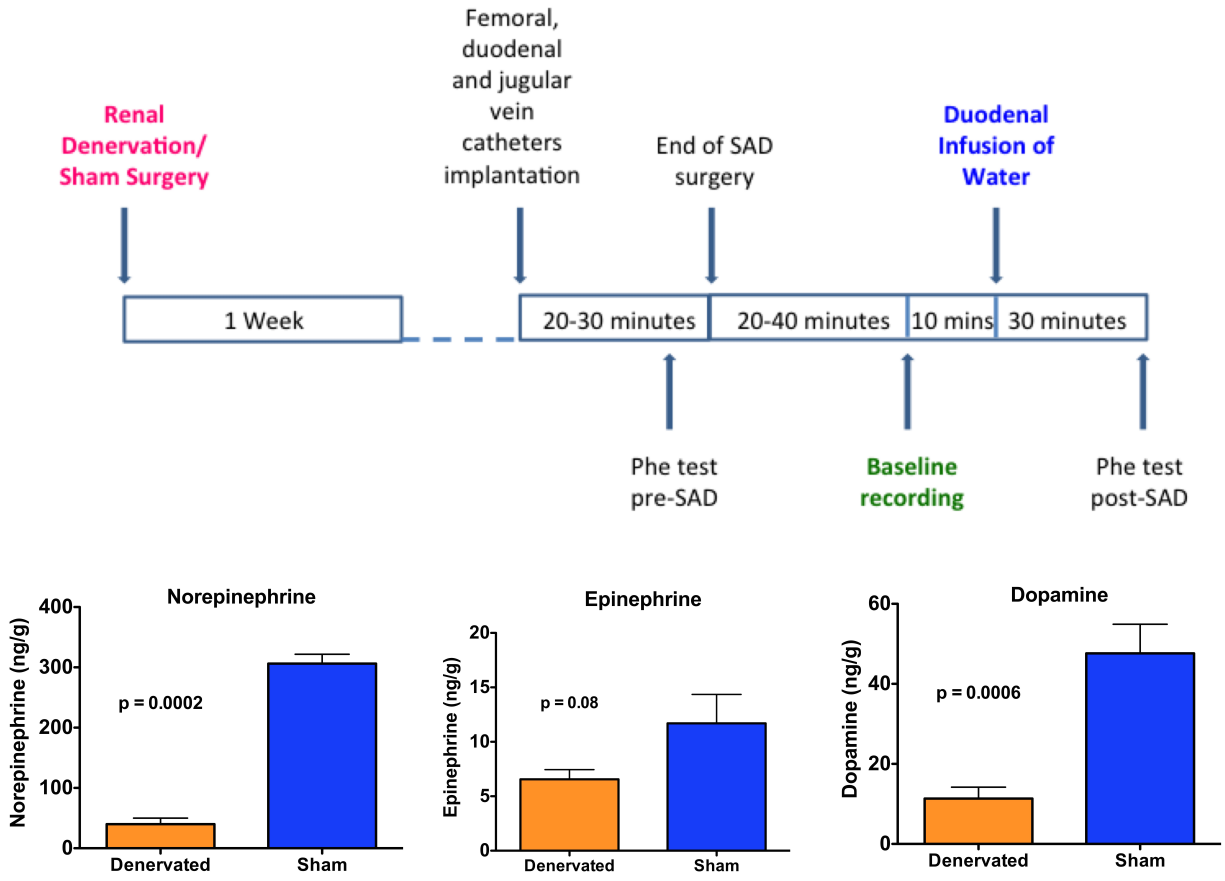

Figure 26: Protocol to study the OPR in mice that underwent bilateral renal denervation (Orange, n=5) and sham surgery (Blue, n=5). Kidney catecholamines were measured at the end of the experiment to confirm the denervation.

However, plasma and 24 hr-urine norepinephrine levels were not altered due to bilateral renal denervation surgery (Plasma: RD: 2761±618.5 vs. Sham: 2758±1637 pg/ml, n=7, p=0.54; Urine: RD: 404.4±162.3 vs. 407.3±135.5 pg/ml, n=7, p=0.9) (Figure 27). Bilateral renal denervation significantly diminished the osmopressor response in mice (ΔBP_{RD}: 9.0±3.9 vs. ΔBP_{Sham}: 28.9±13.7 mmHg, n=5, p= 0.008; AUC_{RD}: 3.7±45.3 vs. AUC_{Sham}: 277.2±129.3 mmHg.minute, n=5, p=0.008). This indicated that a kidney with intact renal nerves is required for the OPR (Figure 28).

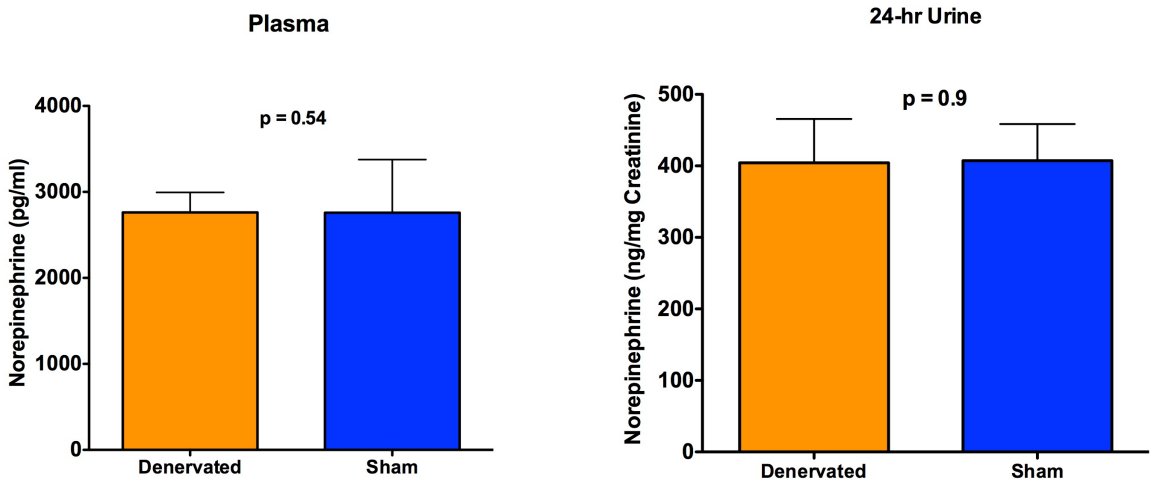

Figure 27: Plasma and 24 hr-urine norepinephrine levels were similar between the renal denervated (Orange, n=7) and sham (Blue, n=7) groups. A) Plasma norepinephrine B) 24 hr-urine norepinephrine.

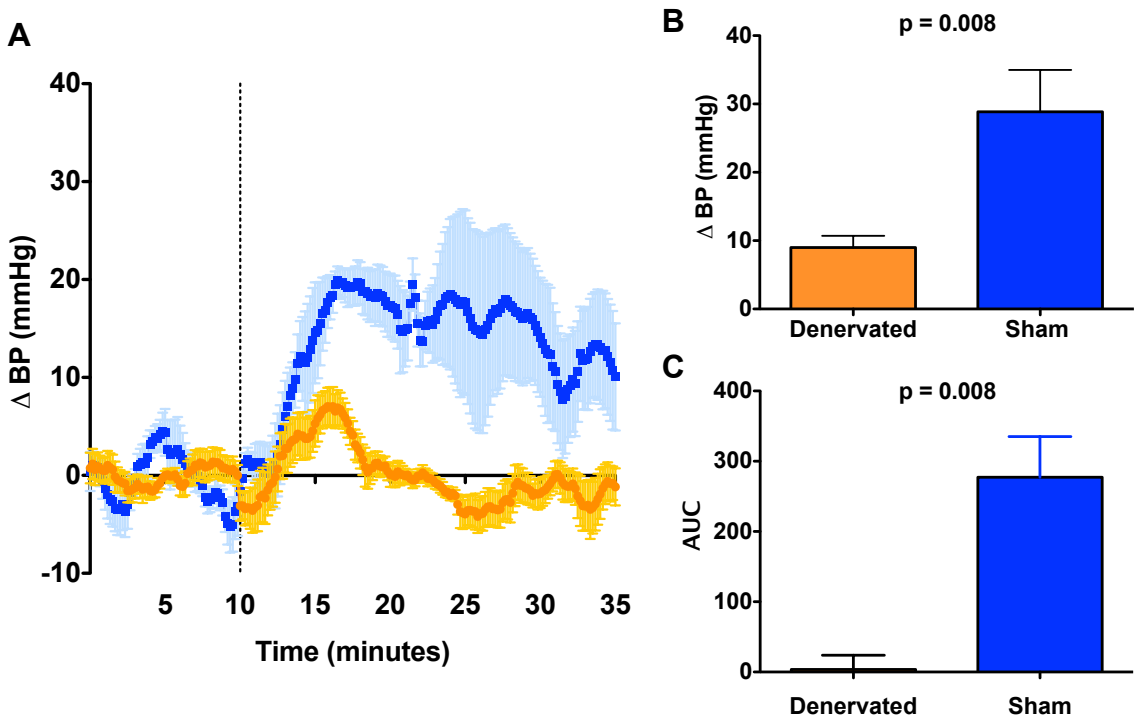

Figure 28: Bilateral renal denervation significantly attenuated the OPR in WT mice. A) Mice with bilateral renal denervation (Orange, n=5) had significantly diminished OPR compared to sham controls (Blue, n=5). B) Maximal chance in BP was significantly higher in the sham controls, indicating a higher initiation of the OPR. C) AUC was significantly smaller in the renal denervated mice, showing a smaller response to water in these mice.

4. Discussion

The kidneys have long been known to be a key regulator of blood pressure (Demerath et al. 2014, Tanimoto et al. 2014, Bernstein et al. 2014). However, the role of the kidneys in the OPR had not been studied. In this chapter, the main findings are: 1) Angiotensin II receptor blockade could not reduce the OPR; 2) Bilateral nephrectomy abolished the OPR; 3) Bilateral renal denervation significantly diminished the OPR. Therefore, the renal nerves might play an important role in the pressor effect of water.

It was shown both in mice and humans that water ingestion could increase sympathetic nervous system activity (McHugh et al. 2010, Boschmann et al. 2007, Jordan et al. 2000). Increased sympathetic signals can elevate renin levels via β1 adrenergic receptors and subsequently angiotensin II increases (Osborn, DiBona, and Thames 1981). Ang II increases BP through several mechanisms, including vasoconstriction and a further increase of sympathetic drive (Anning et al. 2005, Olson et al. 1994). When exogenous Ang II was infused intravenously into WT mice, BP immediately increased. This effect could be blocked completely by losartan (Sugawara et al. 2002). Twenty minutes after treatment with losartan, when global Ang II receptors were completely blocked, water was still able to elicit an increase in blood pressure that was similar to the vehicle control treatment. This finding suggests that Ang II is not an important mediator of the OPR.

Mice that underwent 5/6 nephrectomy in a chronic study demonstrated increased blood pressure (Gava et al. 2012). These mice had increased sympathetic activity that

led to elevation of peripheral vascular resistance. The renin-angiotensin system (RAS) was also stimulated (Campese et al. 2002). Intrarenal Ang II concentration is 3-5 nM and ~50-100 times higher than systemic Ang II. AT1 receptors are widely expressed in the vasculature and tubular components of the kidneys via which Ang II can regulate renin secretion directly (Kobori et al. 2007). Although stimulation of Ang II type 1 (AT1) receptors was not necessary for the OPR, intact kidneys might still be essential for the initiation and maintenance of the response. In order to eliminate long-term effects that might surface due to bilateral nephrectomy (Prieto-Carrasquero et al. 2005, Moriguchi et al. 2011), the kidneys were removed only 15 minutes before water was infused into the duodenum. Results (Figure 25) suggested that intact kidneys were needed for the OPR. However, it was noteworthy that removing both kidneys would also cause significant loss of blood (the kidneys hold ~25% of the blood at any given time and ~8L is filtered by both kidneys per hour (Johns 2013)), which might affect the pressor effect of water. Moreover, one of the important mediators of the OPR, TRPV4, plays dual roles, sensing both mechano- and osmotic signals in renal epithelial cells (Wu et al. 2007). The loss of TRPV4 in the kidneys could affect the OPR. At around 30 minutes after water, BP started to rise. However, comparing the time-course of OPR in sham mice, this response was not specific to the water effect.

Besides an effect on the renin-angiotensin system, the sympathetic nervous system also has a direct effect on BP via the renal nerves. Renal nerve denervation recently was proposed to reduce BP in resistant hypertensive patients (Kowalski et al. 2012, Sans Atxer and Oliveras 2013, Polimeni, Curcio, and Indolfi 2013, Kandlikar and

Fink 2011, Abdulla et al. 2011). In mice that underwent bilateral renal denervation, norepinephrine and dopamine levels in the kidneys were significantly reduced compared to the sham controls, proving the completeness of the procedure (Yoshida, Yoshida, and Satoh 1995) (Figure 26). Because 90% of renal dopamine is synthesized in the kidneys (Chugh, Pokkunuri, and Asghar 2013), the reduction of kidney dopamine after renal denervation did not reflect a decrease in circulating dopamine. Renal denervation with phenol in 70% EtOH destroyed both afferent and efferent renal nerves (Consigny et al. 2014, Salman et al. 2010).

Norepinephrine is increased in the OPR (Shannon et al. 2002, Boschmann et al. 2007, Jordan et al. 2000, Jordan et al. 1999); therefore, it was possible that the lack of OPR in renal denervated mice was due to the inability to increase renal norepinephrine after water infusion (Figure 26). However, plasma and 24hr-urine norepinephrine were similar between the two groups, indicating that systemic norepinephrine and clearance were not altered by renal denervation (Figure 27). We cannot conclude from this experiment whether the afferent or efferent renal nerve contributes to the OPR.

These data suggested that the renin-angiotensin-aldosterone system did not play an important role in the OPR, however, intact kidneys were required and the renal nerves were important in the pressor effect of water.

CHAPTER V

SALT STUDY IN TRPV4$^{-/-}$ MICE

1. Salt Sensitivity of TRPV4$^{-/-}$ Mice

In this chapter, TRPV4$^{-/-}$ and WT mice were challenged with a 4-week high salt diet (8% NaCl). BP, HR and activity levels were measured via telemetry (C-10, DSI Inc.). Baseline mean BP of TRPV4$^{-/-}$ mice recorded over 1 week was significantly lower compared to their WT counterparts (TRPV4$^{-/-}$: 105.2 ± 3.7, n=10 vs. WT: 114.5 ± 13.9, n=9, p=0.03). By the third and fourth weeks of high-salt diet, BP was significantly increased over baseline in TRPV4$^{-/-}$ but not in WT mice (Table 1, Figure 29), rising to the same levels observed in the WT mice.

Table 1: **Blood pressure profile in 8%-salt diet challenge**

	WT (mmHg), n=9	**TRPV4$^{-/-}$ (mmHg), n=10**
Baseline	114.5 ± 13.9	105.2 ± 3.7 *
Week 1	112.3 ± 5.9	108.9 ± 4.1
Week 2	111.0 ± 2.5	108.6 ± 2.5
Week 3	111.1 ± 4.9	109.2 ± 1.5 #
Week 4	110.7 ± 5.7	110.1 ± 2.4 #

*: Statistically significant compared to WT controls (p=0.03)

#: Statistically significant compared to baseline of the same genotype ($p<0.05$)

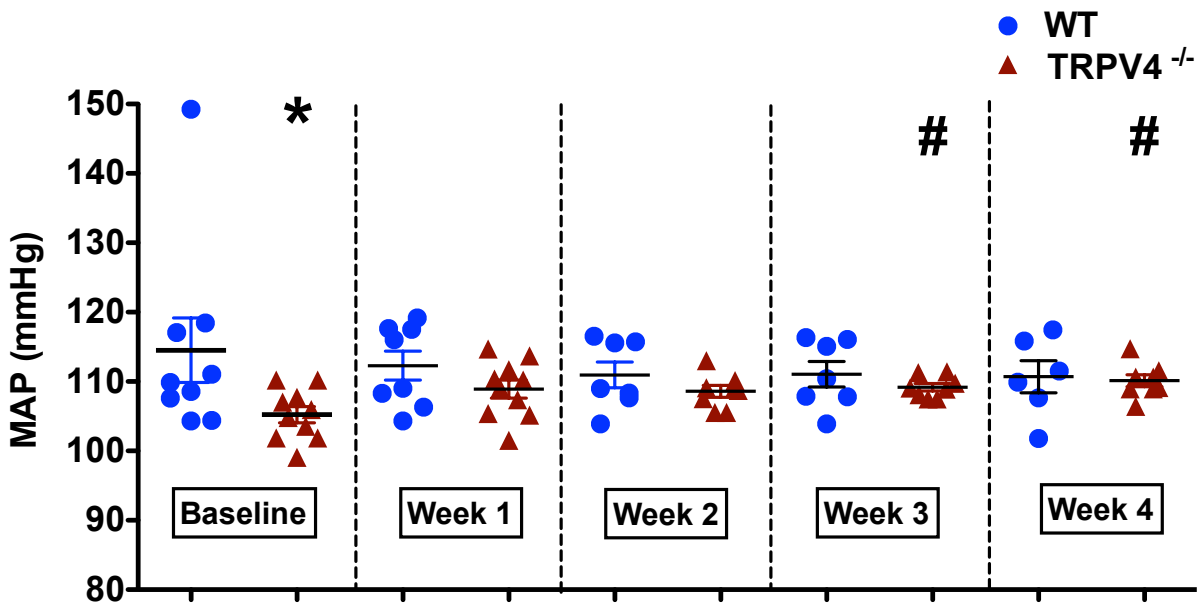

Figure 29: High-salt diet challenge. BP in TRPV4$^{-/-}$ mice at baseline was significantly lower than WT. During weeks 3 and 4 of high-salt diet, BP significantly increased compared to baseline in TRPV4$^{-/-}$ but not WT mice.

Heart rate was similar between the two genotypes at baseline (TRPV4$^{-/-}$: 574.2 ± 34.2 mmHg, n=10 vs. WT: 585.1 ± 22.2 mmHg, n=9, p= 0.54). At the end of the 4th week being on high-salt diet, HR slightly decreased in both groups (TRPV4$^{-/-}$: 566.3 ± 25.4 mmHg, n=10 vs. WT: 556.6 ± 34.2 mmHg, n=9, p= 0.39). The magnitude of the decrease in HR in WT mice tended to be greater than in TRPV4$^{-/-}$ (WT: 4.5%; TRPV4$^{-/-}$: 1.4%; p=0.05. This ratio was calculated as $\Delta HR/HR_{baseline}$ for each group, respectively) (Figure 30).

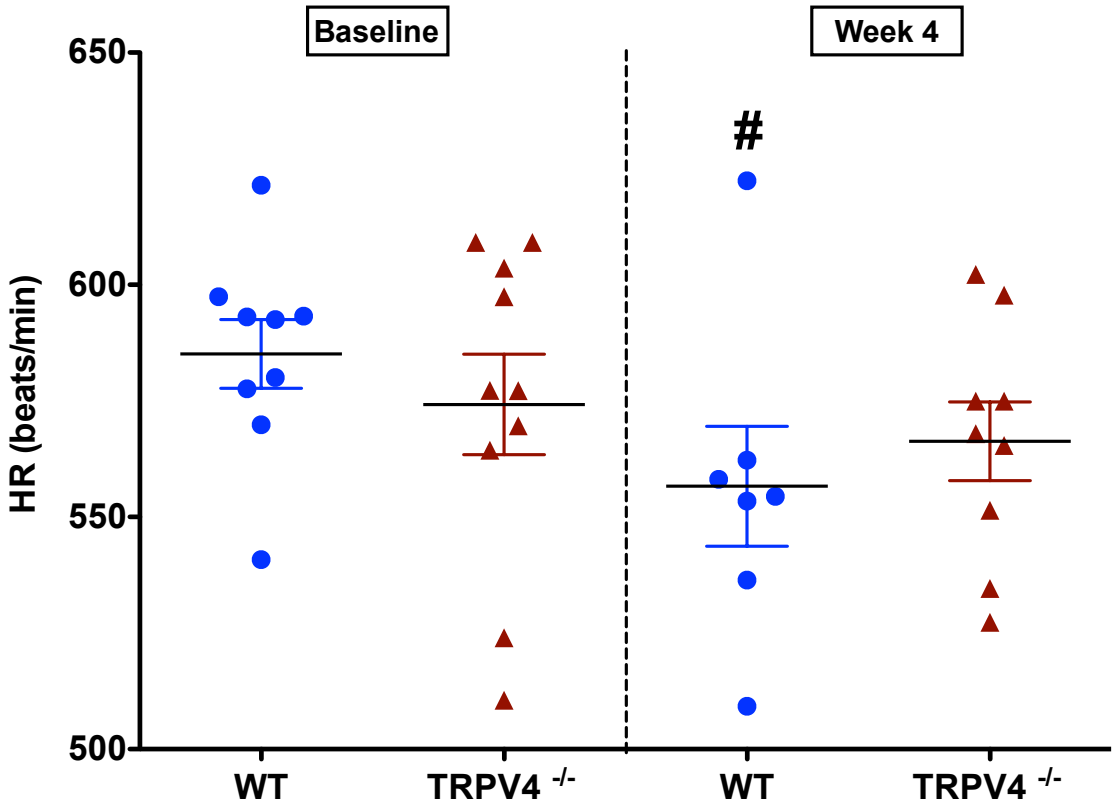

Figure 30: Heart rate in high-salt diet challenge. Though not significant, HR in both groups slightly decreased after 4 week of 8% salt diet. In WT group, when compared HR in week 4 with baseline, p=0.05 ($\Delta HR/HR_{baseline}$=4.5%).

Activity levels were also measured by telemetry. Throughout the time course of recording, TRPV4$^{-/-}$ mice showed significantly lower activity levels compared to WT mice. (Baseline: TRPV4$^{-/-}$: 4.8 ± 1.5 vs. WT: 7.8 ± 2.3 counts/minutes, p=0.01; Week 4: TRPV4$^{-/-}$: 5.6 ± 1.7 vs. WT: 10.8 ± 2.3, p=0.001) (Figure 32). There was no difference observed in feeding behavior between the two genotypes. However, TRPV4$^{-/-}$ mice were significantly heavier compared to age-matched WT controls (TRPV4$^{-/-}$: 31.6 ± 2.4, n=16 vs. WT: 27.9 ± 2.5, n=16, p=0.0005) at baseline (Figure 31). The weight data showed in this graph were collected from age-matched mice in both WT and TRPV4$^{-/-}$ groups from multiple experiments.

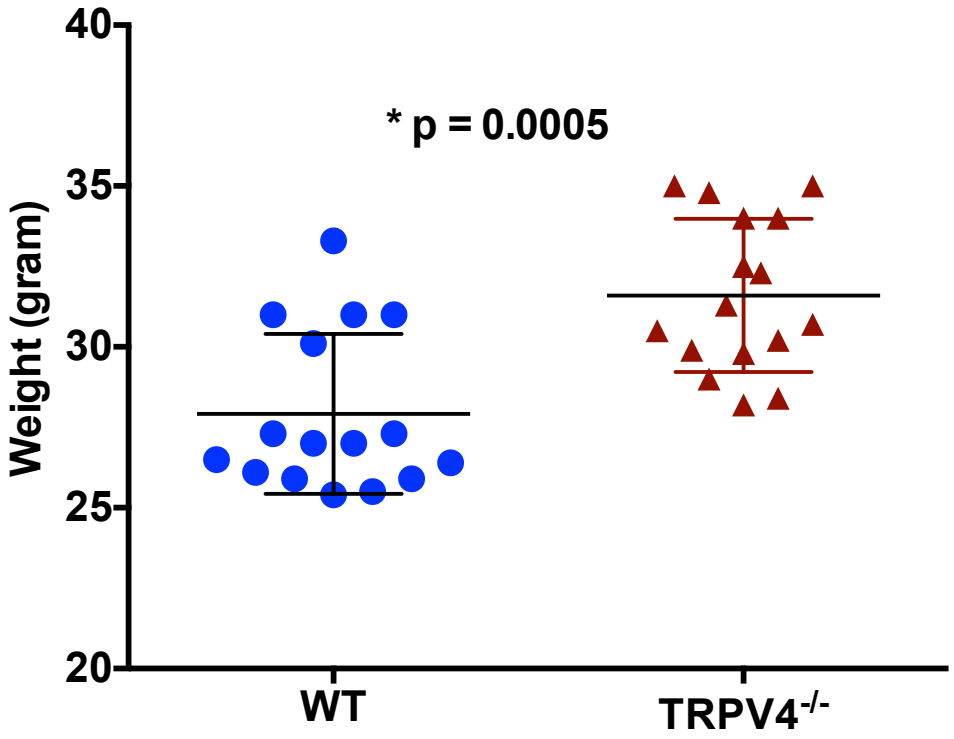

Figure 31: Body weights at baseline.

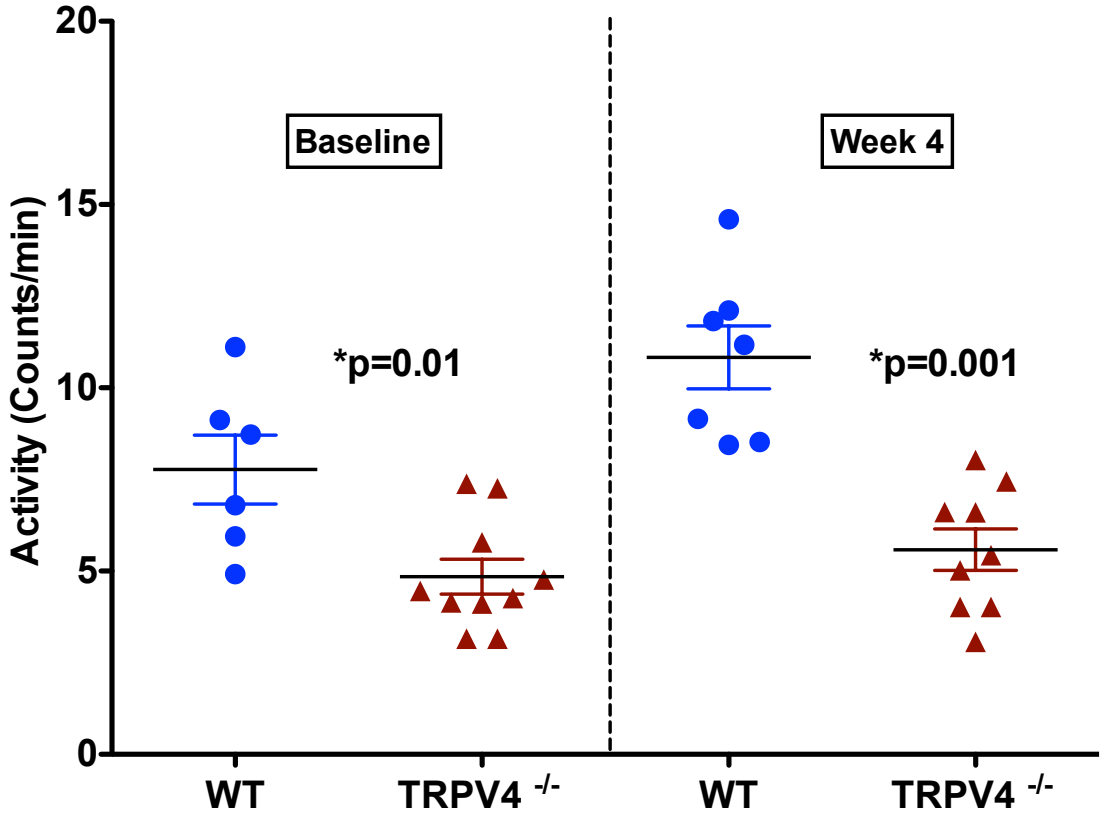

Figure 32: High-salt diet. Activity levels were consistently lower in TRPV4$^{-/-}$ mice compared to WT mice. After 4 weeks of 8% salt diet, both groups tended to be more active. *: Statistically significant compared to WT controls.

2. Catecholamine Levels in TRPV4$^{-/-}$ Mice

In a different set of animals, plasma was collected for catecholamine assessment to gain insight into the role of the sympathetic nervous system in blood pressure regulation in TRPV4$^{-/-}$ mice. Plasma norepinephrine was lower in TRPV4$^{-/-}$ mice (Table 2; Figure 33), which could at least partially explain the lower BP at baseline in the knockout mice.

Table 2: Catecholamines profile in TRPV4$^{-/-}$ mice

	WT, n=7	**TRPV4$^{-/-}$, n=5**	**p**
Norepinephrine (pg/ml)	1168 ± 259.4	476 ± 199.9	0.003 *
NE/DHPG	0.4 ± 0.3	0.2 ± 0.1	0.43
Epinephrine (pg/ml)	241 ± 140.8	123.3 ± 85.0	0.13
Dopamine (pg/ml)	70.4 ± 34.3	93.6 ± 34.4	0.34

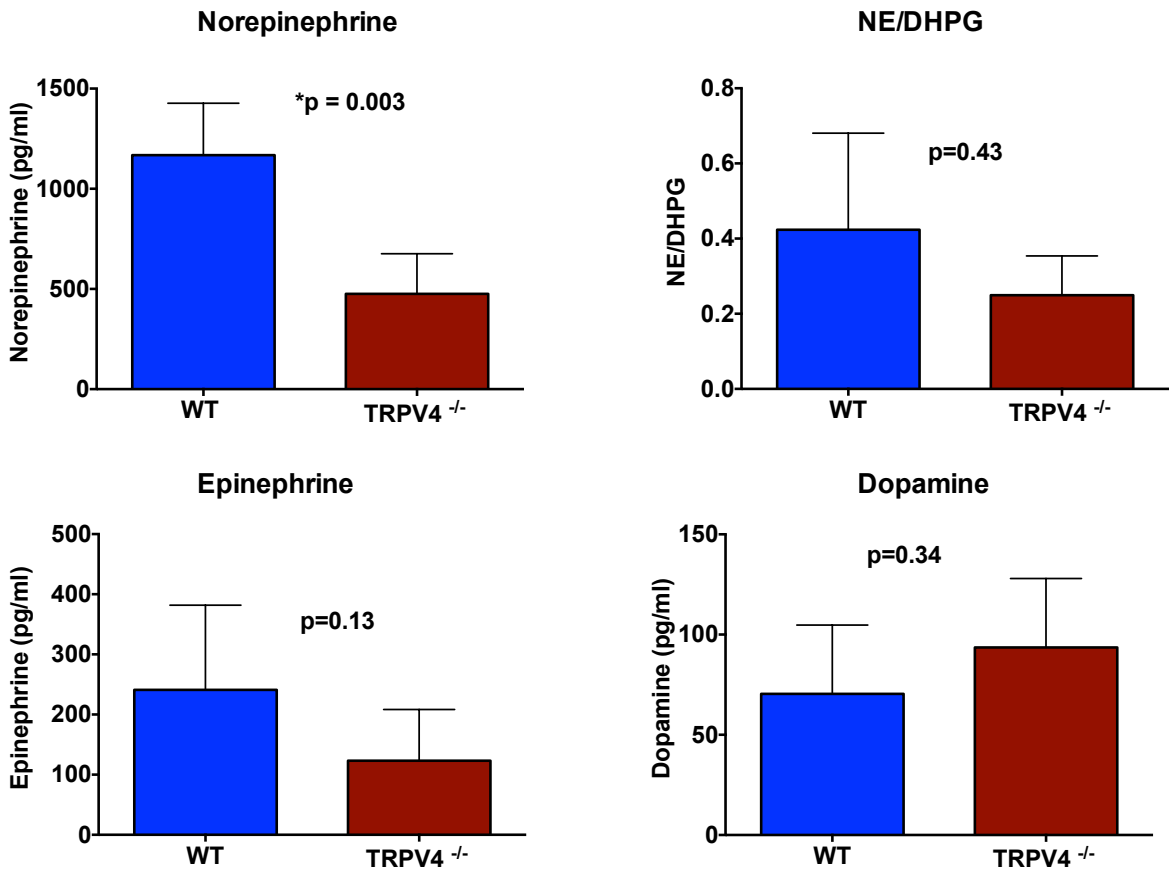

Figure 33: Plasma catecholamine profile of TRPV4$^{-/-}$ mice.

3. Baroreflex Sensitivity in TRPV4$^{-/-}$ Mice

To assess the baroreflex sensitivity, WT and TRPV4$^{-/-}$ mice were subjected to acute dose-response studies with intravenous bolus injections of Phe and NTP. Sensitivity to the two drugs was similar between the two genotypes, indicating by the similar slopes in both Phe and NTP response curves (Phe: TRPV4$^{-/-}$: 0.71 ± 0.1 vs. WT: 1.0 ± 0.2, p=5, p=0.1; NTP: TRPV4$^{-/-}$: -0.83 ± 0.2 vs. WT: -0.74 ± 0.1, n=5, p=0.7) (Figure 34).

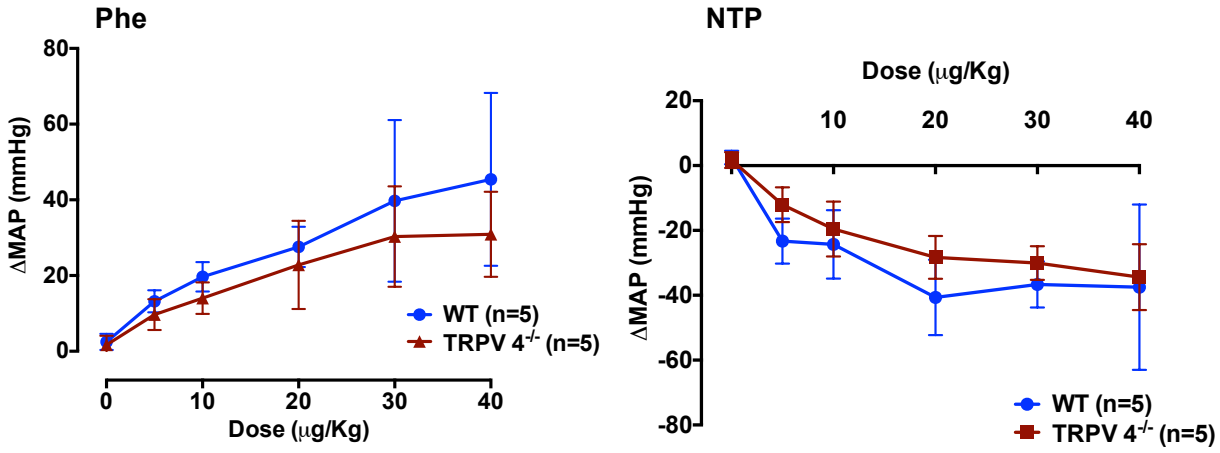

Figure 34: Sensitivity to Phenylephrine and Nitroprusside in WT and TRPV4$^{-/-}$ mice.

Baroreflex sensitivity was calculated using the same method as previously described (Chapter 1). For both drugs, a dose of 40 µg/Kg was chosen because it yielded the greatest change in BP without arrhythmia. No significant difference was observed in baroreflex sensitivity of TRPV4$^{-/-}$ and WT mice (BRS$_{Phe}$: TRPV4$^{-/-}$: 3.6 ± 4.2 vs. WT: 2.1 ± 1.1 ms/mmHg, n=5, p= 0.94; BRS$_{NTP}$: TRPV4-/-: 0.25 ± 0.1 vs. WT: 0.22 ± 0.2 ms/mmHg, n=5, p=0.90) (Figure 35).

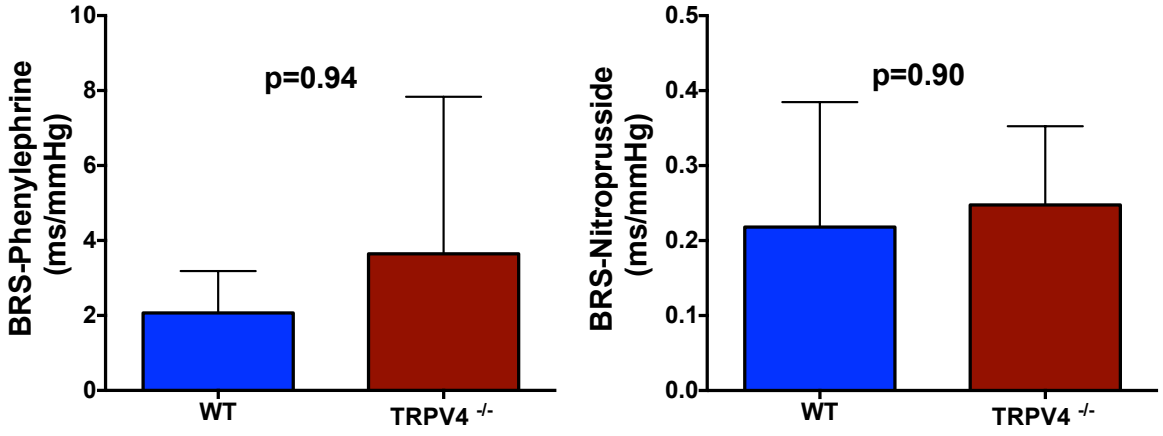

Figure 35: Baroreflex sensitivity assessment in TRPV4$^{-/-}$ (n=5) and WT (n=5) mice.

4. Discussion

This study aims to characterize the basic hemodynamic properties of TRPV4$^{-/-}$ mice. The main findings of this chapter are: 1) TRPV4$^{-/-}$ mice had significantly lower baseline BP compared to WT mice; 2) when challenged with high-salt diet, TRPV4$^{-/-}$ but not WT mice showed a significant increase in BP; 3) TRPV4$^{-/-}$ mice were significantly less active than WT mice; 3) at baseline, norepinephrine level was significantly lower in TRPV4$^{-/-}$mice; and 4) BP sensitivity to phenylephrine and nitroprusside was similar between the two groups, as was their baroreflex sensitivity.

As mentioned previously, TRPV4 is a non-selective cation channel that is sensitive to osmolality perturbations (Liedtke and Friedman 2003, Lechner et al. 2011) and is found in the mesenteric vessels, dorsal root ganglia, along the gastrointestinal tract (GI) and in the brain (Schroeder et al. 2005, Brierley et al. 2008, Lechner et al. 2011). Moreover, TRPV4 channels are highly expressed in the kidneys and play an important functional role in cellular and systemic osmoregulation (Bossus, Charmantier, and Lorin-Nebel 2011, Lechner et al. 2011). In rat kidney, TRPV4 channel expression is especially limited to the water-impermeant nephron segment (Tian et al. 2004). In addition, these channels are expressed in neurons of the organum vasculosum and lamina terminalis (Liedtke and Friedman 2003). The location of TRPV4 channels thus suggests that TRPV4 plays an important role in sodium and water homeostasis.

Our finding of lower blood pressure in TRPV4$^{-/-}$ mice compared to WT mice (Figure 29) was recently confirmed by another group (Nishijima et al. 2014). After 4

weeks on an 8% salt diet, TRPV4$^{-/-}$ but not WT mice displayed a substantial increase of BP compared to the baseline value so that by the end of the diet challenge, blood pressure in TRPV4$^{-/-}$ mice was similar to that in WT mice (Figure 29). Plasma norepinephrine is a marker of sympathetic activity (Ito et al. 2013, Ito, Hirooka, and Sunagawa 2014, Hasan, Woodward, and Habecker 2012, Chida et al. 2005). As shown in Figure 33, untreated TRPV4$^{-/-}$ mice had significantly lower plasma NE than WT mice, consistent with a lower sympathetic tone contributing to a lower baseline BP (Figure 29). It is noteworthy that NE and Epi levels were significantly high; probably due to method of collection. However, both groups were subjected to the same acclamation time and blood collection procedure. Plasma norepinephrine reuptake was similar, reflected by similar NE/DHPG ratios (Table 2). Therefore, it is possible that norepinephrine release or metabolism could be altered in TRPV4$^{-/-}$ mice.

TRPV4 was previously shown to produce a hypotensive effect during salt load to prevent increase in BP. It was proposed that TRPV4 expression was enhanced during the salt load in Dahl salt-resistant rats, leading to an increase in dilatory mediators such as substance P and calcitonin-gene-related-peptide (Gao et al. 2009). TRPV4 channels are also present in endothelial and smooth muscle cells. They contribute to the vasodilation of mesenteric arteries to counter the effects of hypertensive stimuli (Earley et al. 2009). Therefore, mice that lack TRPV4 channels could have a defect in this regulatory mechanism and be susceptible to salt-induced hypertension. On the other hand, research by Mizuno *et al*. showed that TRPV4$^{-/-}$ mice have increased arginine vasopressin (AVP) after ingestion of 2% salt water (Mizuno et al. 2003). This increase

did not happen in WT control. The baseline AVP in both groups before the hypertonic challenge was similar. AVP has long been known to be a pressor hormone. This genotype-specific response of AVP to salt might contribute to the lack of salt-induced hypertension in WT mice. These studies suggest that TRPV4 may play a role in preventing salt-induced increases in BP.

Heart rate was similar in both groups at baseline and after 4 weeks of 8% salt diet. It is noteworthy, however, that in WT mice, HR tended to decrease with high salt diet ($p=0.05$ for week 4 vs. baseline, Figure 30). This reduction in HR could happen as a compensatory response of the baroreflex to hypertensive stimulant such as salt (McNeely, Windham, and Anderson 2008). HR also decreased in TRPV4$^{-/-}$ mice but not significantly regardless of increased BP after salt diet. Further studies in heart rate variability in TRPV4$^{-/-}$ mice could give insight into the mechanism of hemodynamic regulation of salt-induced hypertension.

It is very interesting that activity levels in TRPV4$^{-/-}$ mice were consistently lower than WT mice during baseline and during week 4 of high salt diet (Figure 32). Pritschow *et al.* studied the role of TRPV4 channels in long-term maintenance of muscle contraction and proved that muscle fatigue was significantly attenuated by TRPV4 activation with 4α-phorbol-12, 13-didecanoate (4α-PDD), a TRPV4-specific agonist (Pritschow et al. 2011). This suggests that TRPV4$^{-/-}$ mice might experience muscle fatigue faster, thus reducing their movement and activity. Currently in the literature, there are contradictory results regarding obesity in TRPV4$^{-/-}$ mice (Ye et al. 2012,

O'Conor et al. 2013). Figure 31 showed that the knockout mice were significantly heavier than age-matched WT controls. Although it is difficult to conclude the cause and effect relationship between weight and locomotor activities, there is always a negative correlation between the two parameters (Basterfield, Lumley, and Mathers 2009, Jurgens et al. 2006, Dauncey and Brown 1987, He et al. 2010). TRPV4$^{-/-}$ mice in this study therefore might have lower activity levels because of their greater body weights. Further investigation of body composition and metabolism in these mice might help to better explain this observation.

Previous work by McHugh showed that TRPV4$^{-/-}$ mice had intact sympathetic efferents because their BP rose appropriately in a restraint-induced stress test (McHugh 2010). Here the afferent loop of autonomic blood pressure control was studied by measuring the sensitivity of TRPV4$^{-/-}$ and WT mice to phenylephrine and nitroprusside. Figure 34 showed that there was no significant difference between the two genotypes, indicating that sensitivity of α1-adrenergic receptors and to nitric oxide was not altered in the knockout mice. Calculation of BRS revealed that the afferent control of BP in TRPV4$^{-/-}$ mice was also normal in the absence of high salt diet. TRPV4 channels also exist in smooth muscle cells and directly contribute to the vascular tone independent of NO and SNS activity (Sukumaran et al. 2013, Pritschow et al. 2011). Future study to investigate the baroreflex sensitivity after a high-salt diet and the structure of cardiovascular vessels in TRPV4$^{-/-}$ mice will help better understanding of the role of TRPV4 channels in blood pressure regulation at baseline and in hypertonic challenge.

CHAPTER VI

CARDIOVASCULAR PROPERTIES AND THE OSMOPRESSOR RESPONSE IN CALCITONIN-GENE-RELATED-KNOCKOUT MICE

1. Calcitonin Gene Related Peptide (CGRP) in Autonomic Cardiovascular Regulation and Vascular Structure

Studies of BP in CGRP knockout mouse models also have produced discordant results (Gangula et al. 2000, Kurihara et al. 2003). While Gangula's study showed that mice with α-CGRP/calcitonin knocked out had significantly elevated systolic blood pressure (SBP) and mean arterial blood pressure (MAP) compared to WT mice (Gangula et al. 2000), Lu *et al.* suggested that mice lacking the α-CGRP peptide without the disruption of calcitonin had normal cardiovascular regulation and neuromuscular development at baseline and after exercising (Lu et al. 1999). The difference in results might be due to the concurrent deletion of the calcitonin. However, later studies in mice with targeted deletion of only α-CGRP showed that these knockout mice had increased baseline MAP and heart rate (HR) even though calcitonin was intact (Kurihara et al. 2003). These data suggest that CGRP might play an important role in blood pressure regulation in both physiological and pathological conditions, yet its specific function is still debatable.

CGRP has been reported to be co-expressed with TRPV4 channels in sensory neurons (Brierley et al. 2008, Vergnolle et al. 2010). CGRP can be released as a

downstream mediator after TRPV4 activation (Gao and Wang 2010a). Previous data showed that TRPV4 was an important molecular mediator of the OPR, therefore, studying the role of CGRP in the osmopressor response might give insight into the pathway responsible for the pressor effect of water.

Given the potential importance of α-CGRP in cardiovascular regulation, the purpose of this chapter was to study the osmopressor response in $CGRP^{-/-}$ mice and to characterize hemodynamic and autonomic function of α-CGRP/calcitonin knockout mice. Also, the mechanical properties of blood vessels could contribute significantly to the hemodynamic profile. Therefore, the lack of αCGRP/calcitonin in our knockout mice would lessen relaxation of large blood vessels, leading to stiffening of the arteries, decreasing baroreflex sensitivity, and increasing blood pressure.

2. $CGRP^{-/-}$ Mice Have Full Osmopressor Response

In order to test the hypothesis that CGRP is an important mediator of the osmopressor response, water study in CGRP-/- and WT control mice was carried out as the protocol below (Figure 36). Both CGRP-/- and WT mice displayed an increase in BP after water infusion into the duodenum ($\Delta BP_{CGRP-/-}$: 42.55 ± 35.4 mmHg, n=2, ΔBP_{WT}: 27.9 ± 11.2, n=3 p = 0.53; $AUC_{CGRP-/-}$: 1214 ± 910.3 mmHg.minute, n=2; AUC_{WT}: 475 ± 81.2 mmHg.minute, n=3, p= 22) (Figure 37). This indicated that the CGRP were not involved in the pathway that initiated and maintained the OPR.

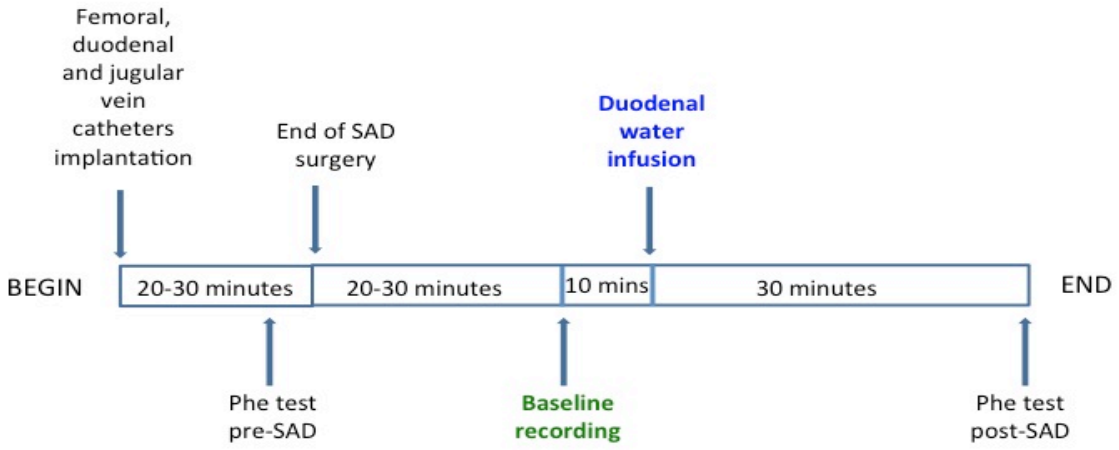

Figure 36: Water study protocol in CGRP$^{-/-}$ and WT mice.

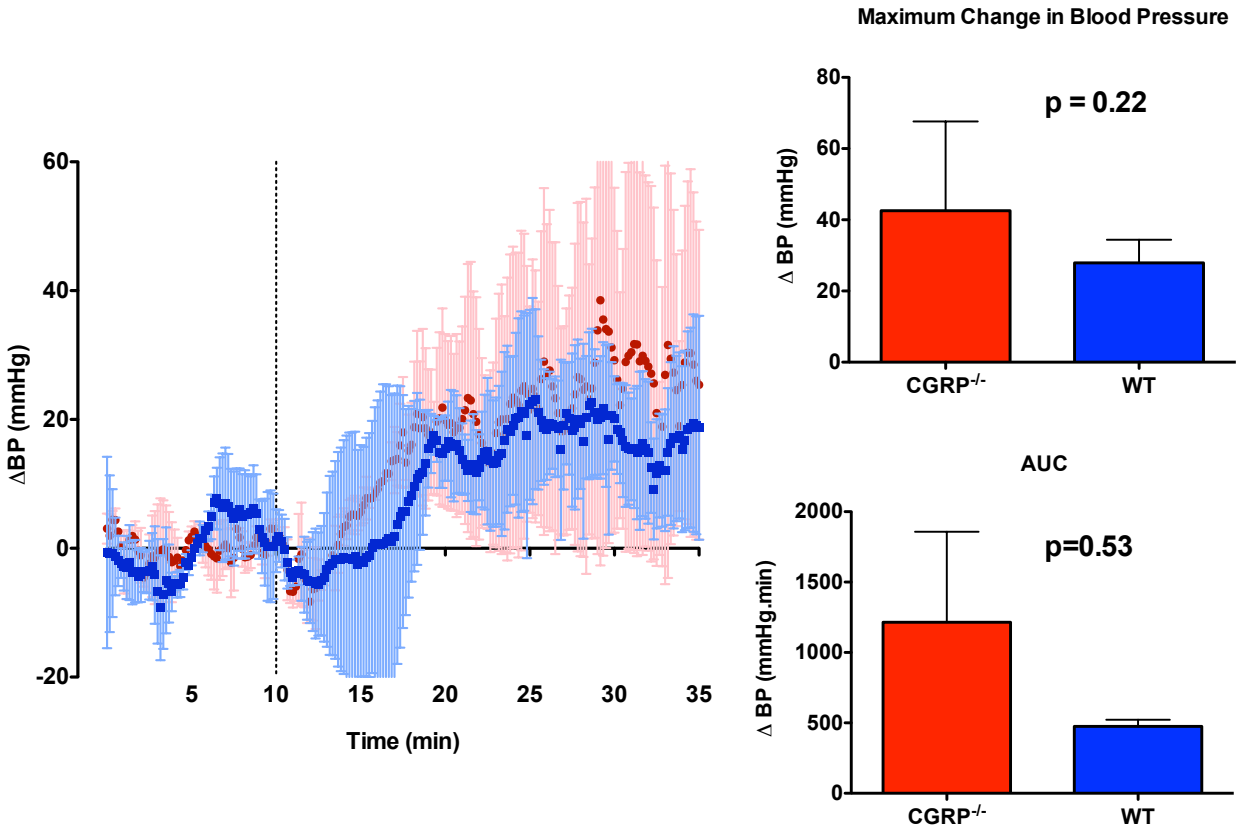

Figure 37: Water study in CGRP-/- (Red, n=5) and WT (Blue, n=4) mice. Pressor response appeared in both genotypes after water infused into the duodenum.

3. Characterizing Cardiovascular Properties in CGRP$^{-/-}$ Mice

Mice were implanted with telemeters (C10 model, Data Science International) and BP, HR and activity levels were recorded as described in the method section, chapter 1.

3.1. Effects of α-CGRP/calcitonin gene deletion on body weight, tissue weights and activity levels

CGRP$^{-/-}$ mice weighed significantly more than age-matching WT mice (CGRP$^{-/-}$: 31.9 ± 4.1 vs. WT: 26.6 ± 1.4 grams, n=16, p<0.0001) (Figure 38A). These mice also tended to be less active, indicated by the lower 24-hr locomotor activity data from the telemeter (CGRP$^{-/-}$: 6.6 ± 3.5 vs. 9.0 ± 2.7 counts/minute, n=9, p= 0.11) (Figure 38B).

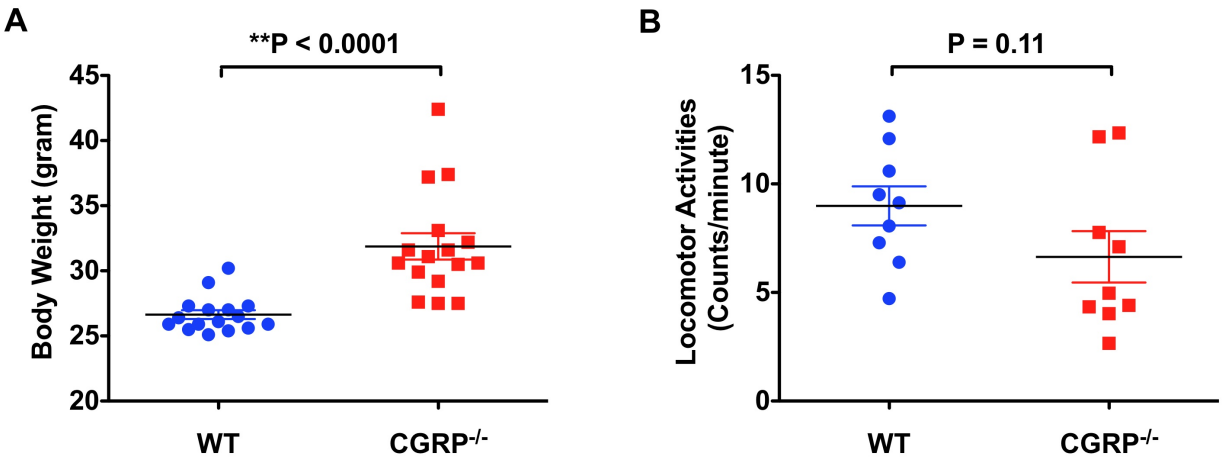

Figure 38: A) Body weight (gram, n=16 in each group, ** P < 0.0001). B) Locomotor activities measured by telemeter (counts/minute) (n=9 in each group).

To assess the possibility of organ damage such as hypertrophy, we measured the weights of the heart, kidneys and spleen and normalized them to body weight (Figure 39). There was no significant difference between the two genotypes. No difference in feeding behaviors was observed between the two groups.

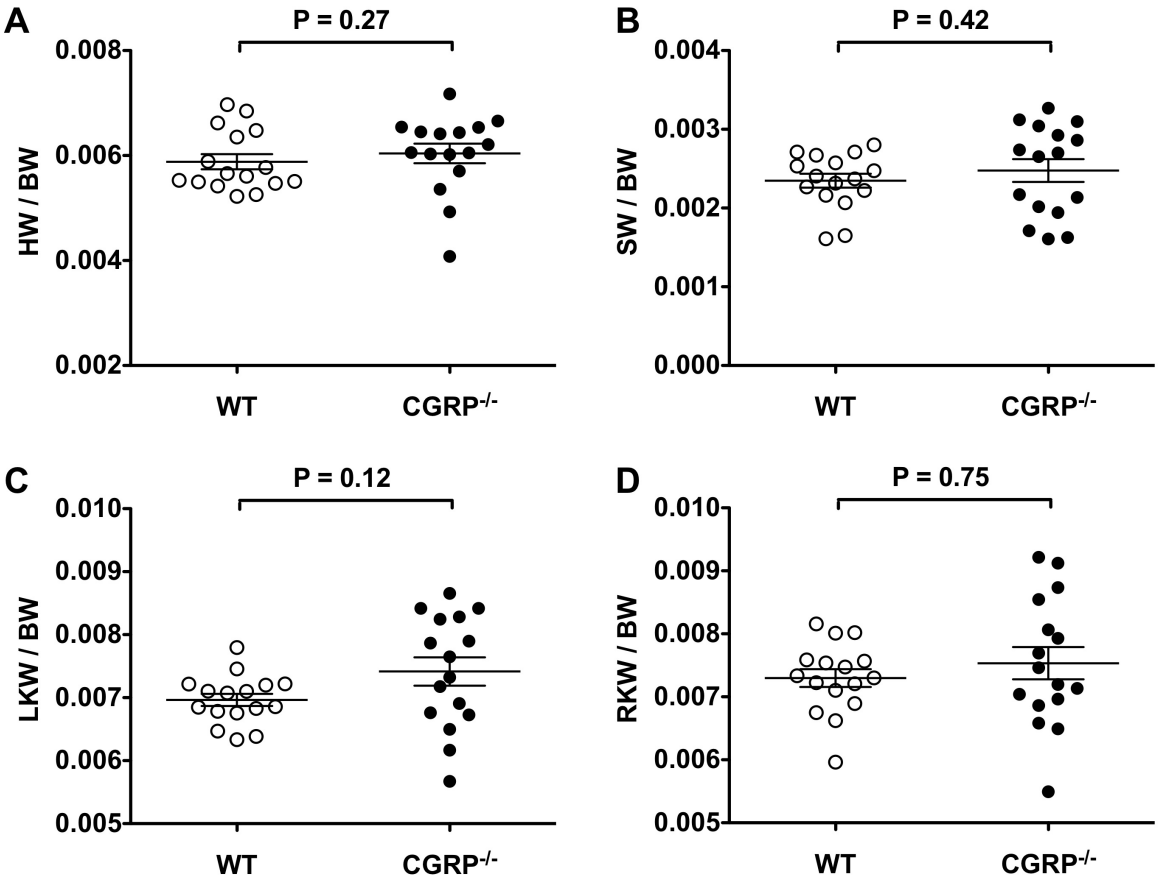

Figure 39: Normalization of tissue weights over respective body weight. A) Heart Weight (HW): Body Weight (BW) (P=0.27). B) Spleen Weight (SW): BW (P=0.42). C) Left Kidney Weight (LDW): BW (P=0.12). D) Right Kidney Weight (RKW): BW (P=0.75). n=16 in each group for all graphs.

3.2. Effect of α-CGRP/calcitonin gene deletion on blood pressure and heart rate

There was a trend for higher MAP in the CGRP$^{-/-}$ mice, but this was not significant at any individual12-hour data point (Figure 40A). However, the 7-day average daytime MAP was significantly higher in CGRP$^{-/-}$ mice (CGRP$^{-/-}$: 114.5 ± 10.6 versus WT: 104.5 ± 5.5 mmHg; P=0.04; Figure 40B). Seven-day average nighttime MAP showed a trend for being higher in CGRP$^{-/-}$ mice (CGRP$^{-/-}$: 128.0 ± 12.5 versus WT: 119.7 ± 5.9 mmHg, P=0.09) (Figure 40B). There was no difference in HR between the two genotypes, whether compared for individual 12-hour periods (Figure 40C) or 12-hour periods averaged over seven days (Figure 40D).

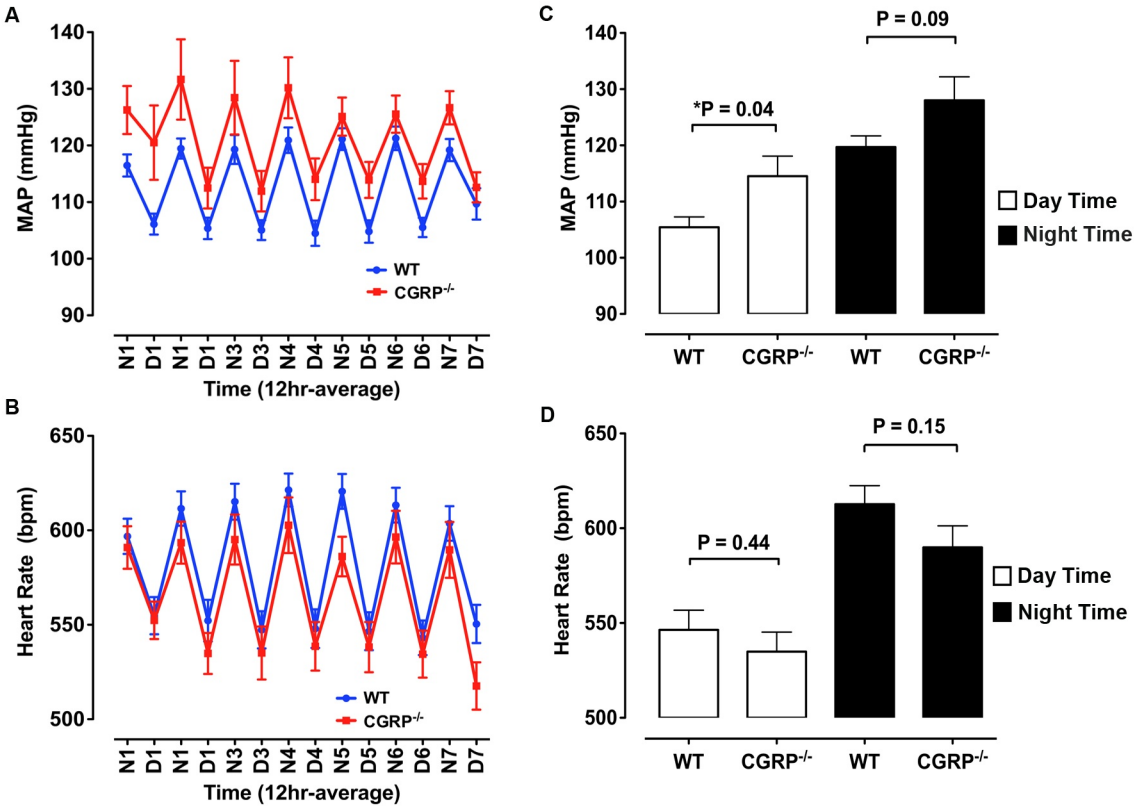

Figure 40: Mean arterial blood pressure (MAP) and heart rate (HR) in WT and CGRP$^{-/-}$ mice. A) MAP in daily 12-hour periods B) MAP in 7-day period C) HR in 12-hour periods D) HR in 7-day period. (* P=0.04, n=9 in each group)

Similarly, the 24-hr average MAP, systolic blood pressure (SYS), diastolic blood pressure (DIA) and HR calculated from the seven days of data showed no significant differences between the two groups (MAP: CGRP$^{-/-}$: 120.1 ± 10.2 versus WT: 112.9 ± 5.8 mmHg, n=9, p=0.25; HR: CGRP$^{-/-}$: 561.1 ± 36.4 versus WT: 579.8 ± 27.8 bpm, n=9, p= 0.19) (Figure 41and Table 3).

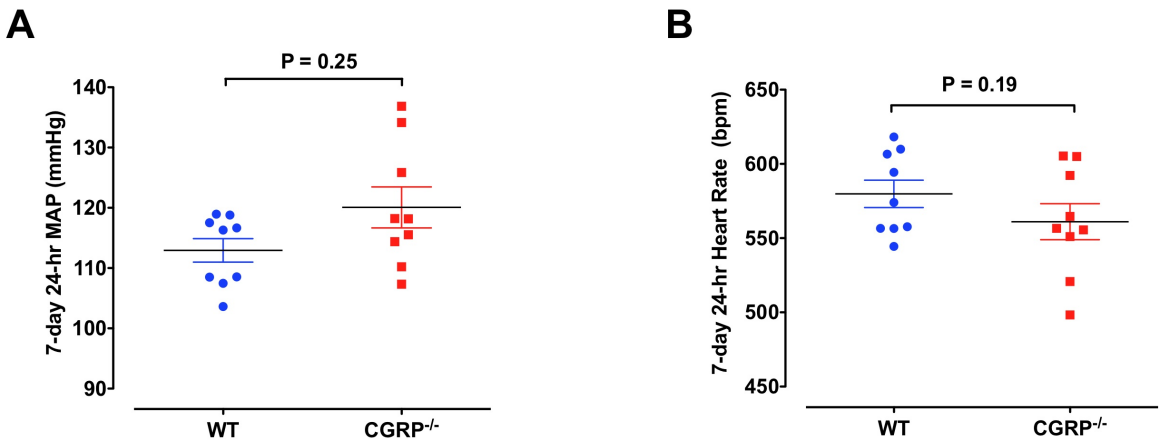

Figure 41: 24-hr recording during 7 days. A) 24-hr mean arterial blood pressure (P=0.25, n=9 in each group. B) Heart rate (p=0.19, n=9 in each group).

Table 3: Blood Pressure Values in CGRP$^{-/-}$ and WT mice recorded by telemeters.

	WT (mmHg), n=9	**TRPV4$^{-/-}$ (mmHg), n=10**
Baseline	114.5 ± 13.9	105.2 ± 3.7 *
Week 1	112.3 ± 5.9	108.9 ± 4.1
Week 2	111.0 ± 2.5	108.6 ± 2.5
Week 3	111.1 ± 4.9	109.2 ± 1.5 #
Week 4	110.7 ± 5.7	110.1 ± 2.4 #

3.3. Catecholamines profiles of CGRP$^{-/-}$ mice

Urine, plasma and tissues were collected and catecholamines were measured as described in method section, chapter 1.

3.3.1. Urine

For CGRP$^{-/-}$ mice, urinary norepinephrine was significantly higher than for their WT counterparts (CGRP$^{-/-}$: 956 ± 90 pg/ml, n=9 versus WT: 618 ± 46 pg/ml, n=9, p=0.0007)**.** Dihydroxyphenylglycol (DHPG), an intraneuronal metabolite of norepinephrine which can reflect reuptake through the norepinephrine transporter, was also higher in the knockout mouse group (CGRP$^{-/-}$: 603 ± 84 pg/ml, n=9 versus WT: 377 ± 31 pg/ml, n=9, p= 0.02) (Figure 42A). The ratio of NE/DHPG was not different (CGRP$^{-/-}$: 0.62 ± 0.03, n=9 versus WT: 0.62± 0.04, n=9, p=0.84) (Figure 42B). Epinephrine and dopamine levels did not differ between genotypes (Table 4).

3.3.2. Plasma

As was the case in urine, plasma norepinephrine was significantly higher in CGRP$^{-/-}$ compared to WT mice (CGRP$^{-/-}$: 2505 ± 596 pg/ml, n=6 versus WT: 1168 ± 98 pg/ml, n=7, p=0.02). Plasma DHPG was not significantly higher in CGRP$^{-/-}$ mice (CGRP$^{-/-}$: 4626 ± 670 pg/ml, n=6 versus WT: 3347 ± 526 pg/ml, n=7, p=0.2) (Figure 42C). The ratio of NE/DHPG was also not different between the two groups (CGRP$^{-/-}$: 0.82 ± 0.35, n=6 versus WT: 0.42 ± 0.09, n=7, p=0.53) (Figure 42D). Epinephrine was significantly higher in CGRP$^{-/-}$ than in WT mice (Table 4). There was no difference in plasma dopamine between CGRP$^{-/-}$ and WT mice (Table 4).

3.3.3. Tissues

To measure catecholamines in the tissues, mice were placed under isoflurane 2% for at least 15 minutes before tissue retrieval. There were no significant differences in tissue catechol levels between the CGRP$^{-/-}$ and the WT mice (Table 4).

Table 4: Tissue Catecholamines

Catecholamines	Norepinephrine			Epinephrine			Dopamine		
	WT	CGRP$^{-/-}$	p	WT	CGRP$^{-/-}$	p	WT	CGRP$^{-/-}$	p
Heart (ng/g)	735 ± 35	825 ± 34	0.09	23.3±3.9	11.8±3.2	0.05	37.7±4.4	36.7 ± 3.1	0.84
Spleen (ng/g)	1229±107	1097±123	0.43	18.0±1.5	10.9±4.6	0.19	86.9±12.2	78.8±14.1	0.68
L. Kidney (ng/g)	437 ± 16	417 ± 24	0.5	366 ± 89	429±177	0.74	61.0 ± 7.7	62.7 ± 8.3	0.87
R. Kidney (ng/g)	458 ± 30	455 ± 212	0.96	350 ± 89	370±207	0.57	60.6 ± 7.2	62.9 ± 7.8	0.83
Plasma (pg/ml)	1168 ±98	2505±56	**0.035**	359±128	525 ±97	**0.035**	N/A	N/A	
24-hr Urine (pg/ml)	618 ± 46	956 ± 90	**0.004**	83.6±12	103 ± 9	0.2	974 ± 98	1100±122	0.44

N/A= Not Detectable

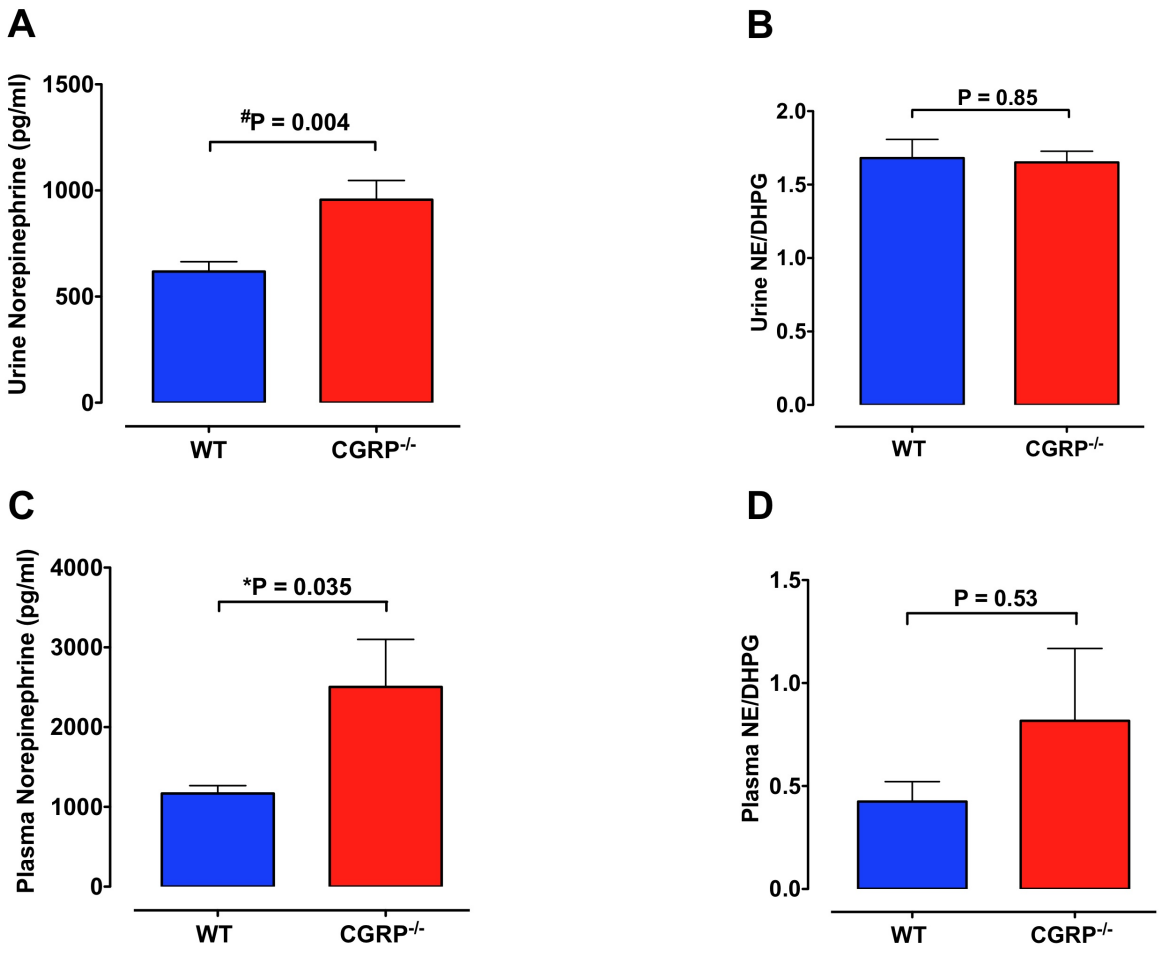

Figure 42: 24-hr urine and plasma norepinephrine levels. A) Norepinephrine concentration in 24-hr urine was significantly higher in CGRP$^{-/-}$ mice compared to WT mice ($^{\#}$P=0.004, n=9 in each group). B) NE/DHPG ratio in 24-hr urine C) Plasma norepinephrine was also higher in CGRP$^{-/-}$ mice compared to WT mice (* P=0.035, n=6 for CGRP$^{-/-}$ and n=7 for WT) D) NE/DHPG in plasma.

3.4. Effect of α-CGRP/calcitonin gene deletion on baroreflex sensitivity

To construct the dose response curves for PHE and NTP, five doses (0 – 40 ug/kg), were given in random order to each WT and $CGRP^{-/-}$ mouse. The baroreflex sensitivity (BRS) was calculated at the 40ug/Kg dose which produced a BP change greater than 20 mmHg MAP without causing arrhythmia.

As indicated by similar BP dose-response slopes (WT: 0.66 ± 0.09 versus $CGRP^{-/-}$: 0.59 ± 0.11, p=0.64), the two genotypes did not differ in sensitivity to PHE or NTP (Figure 43A and 43B). $CGRP^{-/-}$ mice had significantly higher baroreflex sensitivity compared to their WT counterparts when assessed with PHE ($CGRP^{-/-}$: 3.21± 0.46 versus WT: 1.40 ± 0.26 ms/mmHg, p=0.026; Figure 43C). However, no differences in baroreflex sensitivity were observed using NTP boluses (Figure 43D).

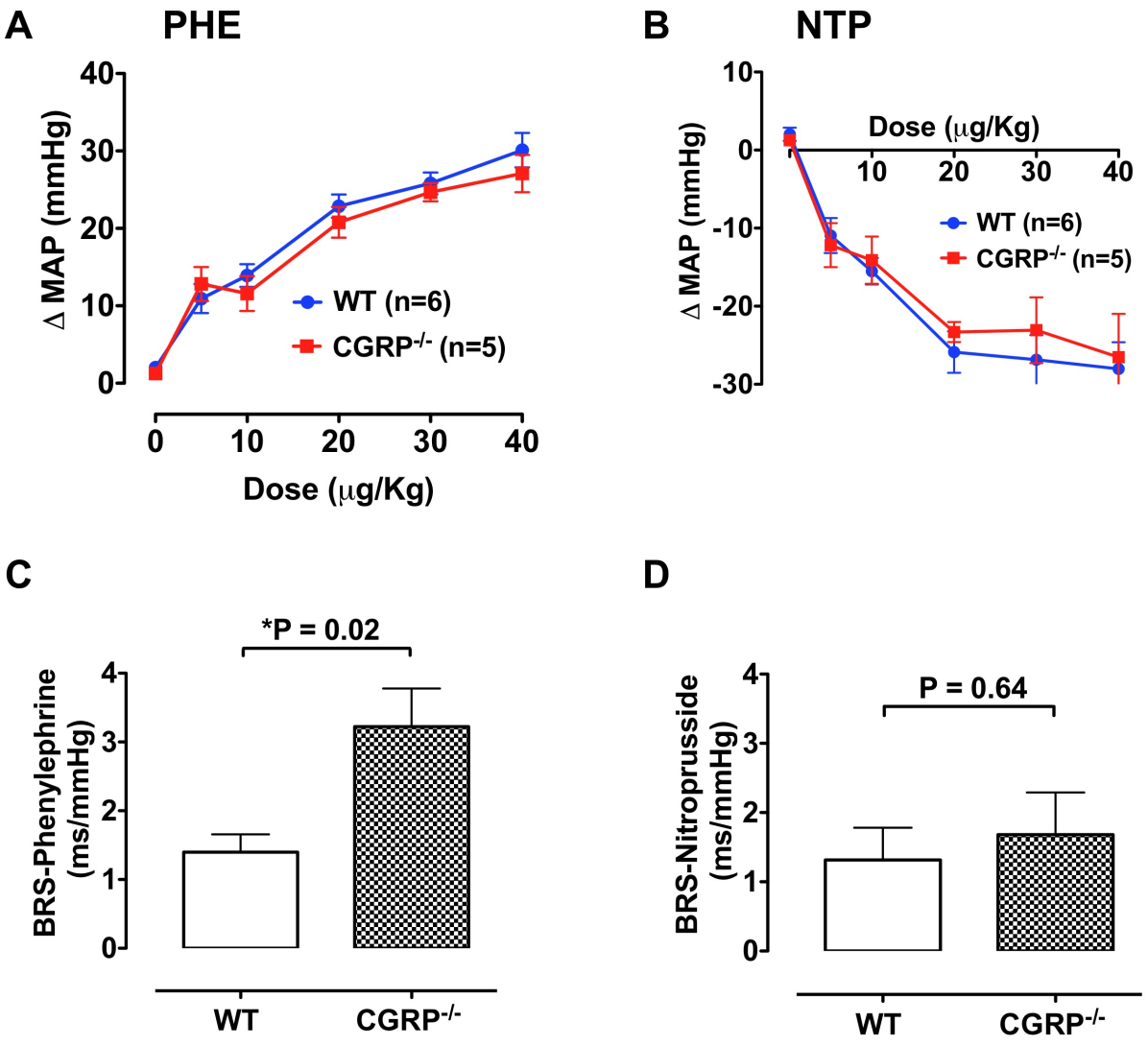

Figure 43: Sensitivity to Phenylephrine and Nitroprusside in WT and CGRP$^{-/-}$ mice. A) Phenylephrine dose response curves. B) Nitroprusside dose response curves. C) Baroreflex sensitivity calculated at 40μg/kg of Phenylephrine, representing the vagal activation and sympathetic withdrawal (*P=0.02). D) Baroreflex sensitivity calculated at 40μg/Kg of Nitroprusside, representing vagal withdrawal and sympathetic activation.

3.5. Blood vessel stiffening in CGRP$^{-/-}$ mice; increased stiffening might be indicated by decreased baroreflex sensitivity

To investigate the passive mechanical structure of the vasculature, thoracic aortas were collected and the increase in vessel diameters measured as intraluminal pressure was raised. In the physiological range of blood pressure (100-175 mmHg), the compliance curve for the CGRP$^{-/-}$ mice was markedly depressed compared to that of WT mice (Figure 44A). Similarly, a leftward shift of the stress-strain curve was observed in CGRP$^{-/-}$ mice, also indicative of aortic stiffening (Figure 44B).

To determine whether the stiffer blood vessels in CGRP$^{-/-}$ mice were related to a difference in collagen and elastin composition, the thoracic aortas were subjected to biochemical assays to quantify elastin and collagen content. Surprisingly, no difference in elastin or collagen content was seen between CGRP$^{-/-}$ and WT aortas (Figure 44C). Histological staining of collagen and elastin revealed similar distribution and content of elastin and collagen in the thoracic aortas (Figure 44D).

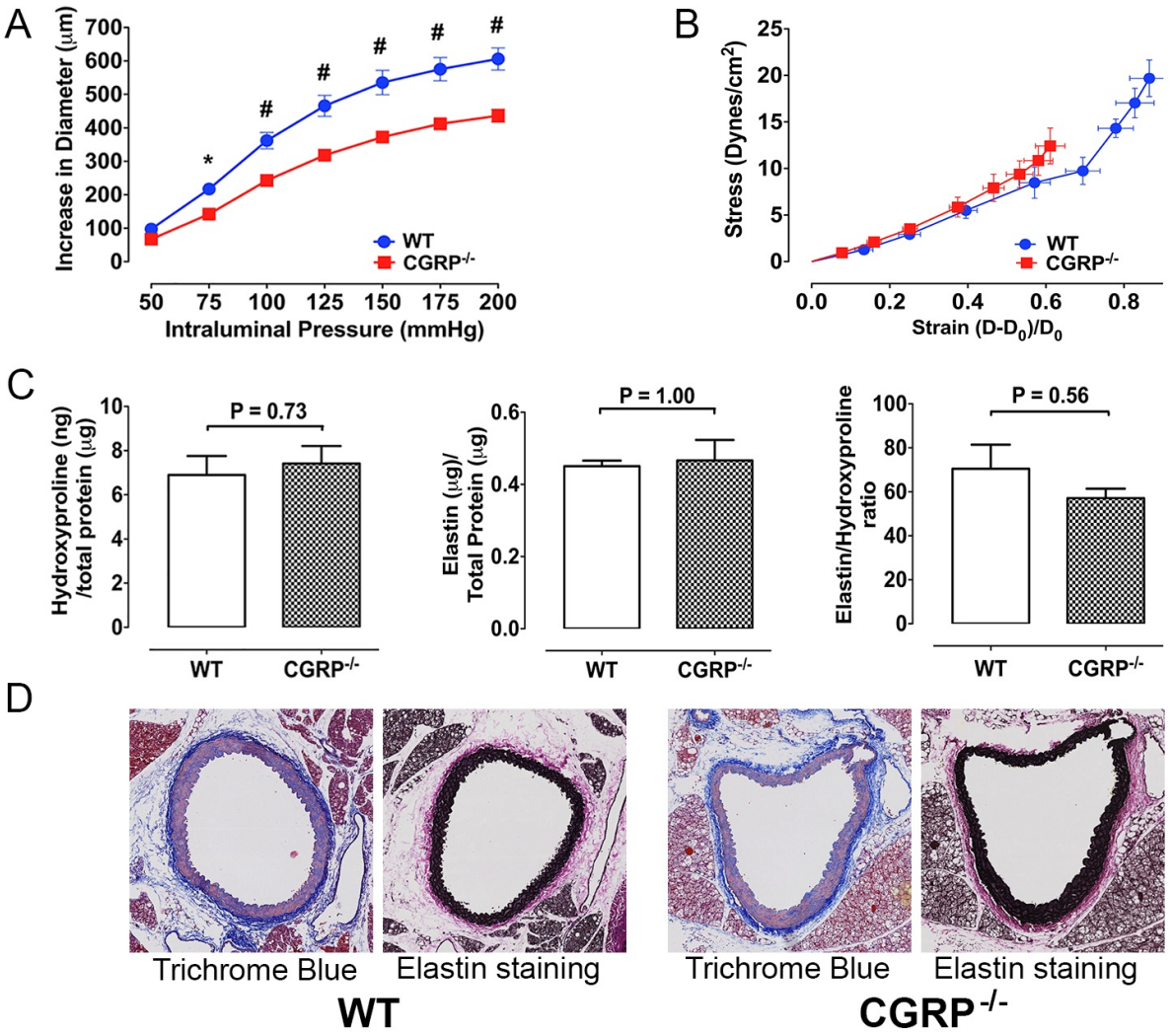

Figure 44: Blood vessel stiffening in CGRP$^{-/-}$ mice. A) Compliance curve (n=5 in each group, $^{\#}P<0.01$). B) Stress-strain curve (n=5 in each group) C) Quantification of collagen (hydroxyproline/total protein), elastin and the ratio of elastin/collagen in thoracic aortas (n=5 for each group). D) Staining for collagen (trichrome blue) and elastin in WT and CGRP$^{-/-}$ mice.

4. Discussion

The primary findings of this study are that $CGRP^{-/-}$ mice have: 1) CGRP does not play important role in the OPR 2) elevated daytime MAP; 3) higher urinary and plasma norepinephrine concentrations; 4) increased baroreflex-mediated vagal activation after α adrenergic receptor stimulation and 5) significant aorta stiffening.

CGRP is extensively distributed in the central and peripheral nervous systems and in the cardiovascular system. It is mainly synthesized in the dorsal root ganglion (DRG), which contains sensory neurons (Alevizaki et al. 1986). These neurons terminate on blood vessels and when CGRP is released in the periphery, it exerts a very potent vasodilator effect, either via the release of nitric oxide from the endothelial cells or directly through the relaxation of the smooth muscle cells. When exogenous CGRP is administered systemically, BP decreases in a dose-dependent manner (Wimalawansa 1996, Bell and McDermott 1996, DiPette et al. 1989). CGRP expression in the DRG is decreased in Dahl salt-sensitive hypertensive and spontaneously hypertensive rats (Glaser et al. 2007, Gangula et al. 2000).

There was evidence showing that CGRP expressed in afferent neurons and released after TRPV4 channels activation (Vergnolle et al. 2010, Gao and Wang 2010a). Because of earlier works and in chapter II showing that TRPV4 was an important mediator of the OPR; CGRP was hypothesized as a downstream signal following TRPV4 activation. However, Figure 36 showed full pressor response when water was infused into the duodenum of $CGRP^{-/-}$ mice that was identical to WT mice.

This indicated that CGRP did not participate in the OPR.

These data showed that age-matched $CGRP^{-/-}$ mice had higher body weight compared to the WT mice. The ratios of tissues to body weight for the heart, kidneys and spleen were not different between the two genotypes, suggesting a lack of hypertrophy (Figure 39). However, hypertensive organ damage cannot be ruled out without future histological and functional studies. $CGRP^{-/-}$ mice have been consistently reported to have higher bone mass density and up to 2-fold higher bone volume (Ballica et al. 1999, Cornish et al. 2001, Hoff et al. 2002, Ishizuka et al. 2005, Wang et al. 2010). This might explain the increase in body weight of these knockout mice. Further studies of body composition would give a more definite explanation for this interesting observation.

The finding of elevated BP in $CGRP^{-/-}$ mice is consistent with a report by Gangula *et al.* using tail cuff blood pressure measurements to show that αCGRP/calcitonin knockout mice are hypertensive (Gangula et al. 2000). This observation is also similar to Oh-hashi's blood pressure data in which BP was recorded via a catheter inserted into the femoral artery in αCGRP specific knockout mice (Kurihara et al. 2003). In the study by Lu *et al*., which found no difference in BP in αCGRP specific-knockout mice, blood pressure was assessed using a catheter inserted into the carotid artery and recorded for two minutes of integration time (Lu et al. 1999). This discrepancy in findings is not due to the additional knockout of the calcitonin but might be related to a difference in the method for blood pressure measurement and the time period of recording. This study

used telemetry, which allowed continuous beat-to-beat measurement of BP during a 7-day period. A difference between genotypes emerged only when MAP data were averaged over the seven days, and this reached significance only for daytime pressures. In Table 3, it is noted that daytime systolic and diastolic blood pressures showed a trend toward higher values in $CGRP^{-/-}$ mice (p-value = 0.06 and 0.05, respectively). Therefore, the effect on the BP of the $CGRP^{-/-}$ mice may be subtle and masked by the variability of BP. As a result, caution needs to be taken to collect data continuously and over a longer period when assessing blood pressure in the $CGRP^{-/-}$ mice.

There might be several mechanisms and contributors that explain this elevation in BP. First, plasma and urine norepinephrine were elevated in $CGRP^{-/-}$ mice. Norepinephrine can be released from the adrenal medulla into the blood as a hormone or as a neurotransmitter from sympathetic noradrenergic neurons. Significantly and sustained elevation of plasma norepinephrine to levels above 1000 pg/ml is frequently associated with sustained increases in blood pressure in man (Kjeldsen et al. 1989), in patients with pulmonary hypertension and also in animals (Hokfelt et al. 1992) (Chobanian et al. 1978, Shimada et al. 1985, Weidmann et al. 1979, Kjeldsen et al. 1989, Krakoff, de Champlain, and Axelrod 1967). However, it is noteworthy that the effect of norepinephrine depends on its origin. A plasma level of 1000 pg/ml by infusion of low doses of norepinephrine into a human subject might have little effect on BP, whereas endogenously released norepinephrine stimulated by vascular neuronal norepinephrine release mechanisms would generally produce a mild pressor effect at

this level. Though not a perfect marker, plasma norepinephrine elevation does reflect an increase in sympathetic activity when samples are carefully obtained as in these CGRP$^{-/-}$ mice. Even though the cardiac puncture method used to collect blood might raise the catecholamine levels, all mice were acclimated in isoflurane 1% for 20 minutes before the procedure. As shown in Figure 42, 24-hr urine norepinephrine concentration was also higher in CGRP$^{-/-}$ mice, indicating increased time-integrated sympathetic activity and a normal urinary clearance mechanism. Plasma norepinephrine reuptake was not different, reflected by similar NE/DHPG ratios (Figure 42). Interestingly, CGRP has been reported to inhibit norepinephrine release in rat hypothalamus (Kjeldsen et al. 1989). Therefore, it is possible that the lack of CGRP could release this inhibitory mechanism leading to an increase in plasma NE. However, norepinephrine synthesis or metabolism might also be altered in the CGRP$^{-/-}$ mice. Sympathetic input to the heart is the primary stimulator of cardiac contractility in mice (Janssen, Lukoshkova, and Head 2002). These data showed that norepinephrine had a trend to be elevated in the heart tissues of CGRP$^{-/-}$ mice (Table 4, p=0.09). This could lead to increased stimulation of the hearts in the CGRP$^{-/-}$ mice and therefore, increased blood pressure. Secondly, research by Li *et al.* showed that the renin-angiotensin system is activated in αCGRP/calcitonin knockout mice (Li et al. 2004). The activation of RAS has been well documented to raise BP in both humans and animals (McKinley et al. 2003, Fyhrquist and Saijonmaa 2008, Ito et al. 1995, Cole et al. 2000, Catanzaro and Frishman 2010) and might be contributory to the increase in blood pressure in these CGRP$^{-/-}$ mice as well.

The arterial baroreflex is the most relevant factor in adapting to acute changes in pressure (Kirchheim 1976). It senses mechanical stretch and chemical changes in the blood to either activate or reduce sympathetic tone to maintain a relatively constant blood pressure. The sensitivity of the baroreflex reflects its ability to control HR and BP variability (La Rovere et al. 1998, Tatasciore et al. 2007). Elevated plasma norepinephrine is strongly correlated with decreased baroreflex sensitivity in normal and pathological states (Dibner-Dunlap 1992, Head 1995, Zucker et al. 1995). Contrary to what expected, baroreflex sensitivity for HR control was improved in the knockout mice, with selective improvement in the vagal arm of the reflex as indicated by the baroreflex sensitivity to phenylephrine (Figure 43C).

This elevated BRS was not due to increased sensitivity of α1-adrenergic receptors since the blood pressure-dose response relationship for phenylephrine was similar for WT and $CGRP^{-/-}$ mice (Figure 43). Interestingly, the enhanced baroreflex sensitivity is consistent with findings in mice that have overexpressed human receptor activity-modifying protein (hRAMP), a component of the receptor complex that binds strongly to CGRP (Sabharwal et al. 2010). It is possible that $CGRP^{-/-}$ mice have an elevated expression or activity of RAMP as a compensatory mechanism for the loss of the peptide.

The data showed also that α1-adrenergic receptor sensitivity and sensitivity to NO are not altered in the knockout mice (Figure 43). Here it was also showed that the vessels are stiffer which could cause an increase in blood pressure (Figure 44). This

could stimulate baroreflex-mediated vagal activity to counteract increases in blood pressure. An increase in vagal activity is consistent with the trend toward a lower heart rate in the CGRP knockout mice (Figure 40 and Figure 41). Other ways in which CGRP depletion could enhance the baroreflex-mediated fall in heart rate after phenylephrine include an effect on the afferent signal of the baroreflex, such as increased responsiveness of the baroreceptors, or an effect in the brainstem that causes a change in the baroreflex sensitivity curve. As a result, the vagal increase/sympathetic withdrawal response to a vasopressor agent is enhanced. Masuki *et al*. described enhanced baroreflex sensitivity in calponin knockout mice (Masuki et al. 2003). The calponin knockout mice had reduced elasticity and an enhanced response to phenylephrine, but no change in the response to nitric oxide, a similar result to that presented in this paper. Direct measurement of sympathetic activity would provide more information that could help explain this paradoxical increase in baroreflex sensitivity.

Aortic stiffening has been shown to be associated with hypertension in humans and in animal models (Kamberi et al. 2013, Sabharwal et al. 2010). $CGRP^{-/-}$ mice had significant stiffening in the thoracic aorta without changes in collagen/elastin composition or distribution. The vascular compliance studies were performed in calcium-free buffer to eliminate vascular tone; therefore, vasoconstriction could not have contributed significantly to aortic stiffening in this model. CGRP released from damaged dorsal root ganglia suppresses the expression of transglutaminase-1 and transglutaminase-3 that form extensively cross-linked covalent bonds among matrix proteins (Gruber et al. 2009). Increased expression or activity of tissue transglutaminase causes protein cross-linking and aortic stiffening in hypertension

(Kamberi et al. 2013, Santhanam et al. 2010). In addition, the deficiency of α-CGRP in DOCA-salt hypertensive mice increases isoprostane excretion, which could also stimulate protein cross-linking (Andrade et al. 2013) Therefore, it is possible that protein cross-linking contributes to aortic stiffening in $CGRP^{-/-}$ mice. An alternative explanation for aortic stiffening relates to the sympathetic activation indicated by high levels of norepinephrine in both plasma and urine of the $CGRP^{-/-}$ mice (Figure 42 and table 3). Wang *et al*. injected mice with recombinant leptin and showed that chronic activation of the sympathetic nervous system and superoxide production likely play a role in mediating the effect of leptin to cause endothelial dysfunction with subsequent aortic stiffness (Wang et al. 2013). Therefore, it is possible that the lack of CGRP in the knockout mice caused a prolonged sympathetic activation, which led to stiffening of the blood vessels. Recently, it was suggested that smooth muscle cell stiffening might be a key contributor to aortic stiffness (Sehgel et al. 2013). CGRP inhibits the proliferation of vascular smooth muscle cells (Chattergoon et al. 2005), therefore disrupting the normal change in remodeling and reorganizing of these cells in blood vessels. Aortic stiffness has been found to precede hypertension both in animal models and in large population cohorts (Weisbrod et al. 2013, Kaess et al. 2012). Future studies are required on the mechanisms that can lead to the stiffening of blood vessels in these mice.

CHAPTER VII

CONCLUSIONS AND DISCUSSION

1. Conclusions

Although the OPR was initially only observed in baroreflex-impaired patients and elderly, water ingestion and its effect on BP might be substantial. Water ingestion beyond thirst has been used therapeutically in conditions such as: the orthostatic hypotension of autonomic neuropathy, the orthostatic hypotension in multiple system atrophy (MSA); and possibly in postprandial angina pectoris. On the other hand, water ingestion could be harmful for patients with autonomic failure at bedtime by worsening supine hypertension and in normal subjects when administered together with other pressor drugs like phenylpropanolamine (PPA, Dexatrim). Water can potentiate the pressor action of other drugs. For example, after phenylpropanolamine 50 mg treatment, BP increased by 9 mmHg (data unpublished). Water can also be used as preventive medicine such as in prophylaxis against syncope associated with blood donation or prophylaxis against needle syncope and emotional faint. Water ingestion may be contributing to the visit-to-visit variation in BP, causing diagnostic difficulty and complications in BP management in hypertension. It can also produce noise to BP assessment. Therefore, it is very important to understand precisely what physiological effects water elicits and what mechanisms underlie those effects.

Water ingestion has a surprising effect on cardiovascular regulation that was earlier missed because of the buffering effects of the baroreflex on BP and heart rate. This effect was unmasked in patients with baroreflex-impairment. After drinking ~480 ml of water, an acute pressor effect maximized around 20 minutes and lasted up to 1hr was observed in these patients. This effect is dose-dependent because 120 ml, 240 ml and 480 ml of water can increase BP up to 9 mmHg, 29 mmHg and 44mmHg, respectively. The response is mediated by the sympathetic nervous system because NE increased significantly after drinking water and ganglionic blockade with trimethaphan could block the pressor effect of water (Jordan et al. 2000). In mouse studies, sino-aortic denervated mice responded to water in a similar pattern to those seen in humans. TRPV4 channels play an important role in the initiation of the response and portal osmolality seems to be significantly lower than systemic osmolality after water consumption (McHugh et al. 2010). Hypo-osmotic fluid when introduced directly into the portal vein was able to increase BP in WT but not TRPV4$^{-/-}$ mice, suggesting that the portal circulation and the presence of TRPV4 channels there are important in the OPR. This effect was not systemic because hypotonicity was introduced into the jugular vein and no rise in BP was observed.

The neuronal afferent pathway via which the OPR was initiated is not yet determined. Although both the vagal and the splanchnic nerves innervate the liver, mice which underwent either celiac ganglionectomy, vagotomy or both still display rise in BP after water was infused into the duodenum. It was possible that the denervation was not complete or local signal transmission in the splanchnic area was sufficient enough to

initiate the OPR. A new finding seemed to implicate a role of the renal nerves in the OPR. However, it remains uncertain whether the afferent or efferents pathway of renal nerves mediated the OPR in this model. This response was likely to be neuronal and not hormonal mediated because both prazosin (McHugh 2010) and losartan could not block the OPR. Furthermore, intact kidneys seemed to be required for the OPR to occur.

In the attempt to study the mechanism and structure of the OPR, hemodynamics and autonomic properties of TRPV4$^{-/-}$ and CGRP-$^{/-}$ mice were also investigated. Both CGRP and TRPV4 were candidates as mediator of the OPR. CGRP$^{-/-}$ mice displayed full BP increase response after water infusion into the duodenum. This phenomenon was absent in TRPV4$^{-/-}$ mice (McHugh et al. 2010), suggesting TRPV4 but not CGRP as an important molecular mediator of the OPR.

Given the abundant expression of calcitonin-gene-related-peptide in the nervous system, its powerful vasodilatory effect, and the increasing number of reports on its contribution to cardiovascular disease, it is important to understand its role. Data from chapter V showed that αCGRP/calcitonin knockout mice have significantly higher blood pressure, as reflected during telemetry monitoring over a seven-day period. This difference in BP is subtle and can be missed if insufficient data is collected. The association of CGRP absence with elevation of sympathetic nervous system activity, RAS activation and aortic stiffening in the pre-hypertensive system suggests that CGRP agonists might have effects to reduce blood pressure. These mice have elevated

norepinephrine levels, enhanced baroreflex sensitivity, and aortic stiffening. Therefore, therapeutic use of CGRP antagonists in proposed treatment of migraine should take into account these effects that might accompany the loss of CGRP.

TRPV4 has been shown to contribute to salt-sensitive hypertension (Gao and Wang 2010b, Gao et al. 2009, Wu et al. 2007). TRPV4$^{-/-}$ mice had lower baseline blood pressure and subject to BP increase when challenged with 8% salt diet. They also have significantly lower NE but similar sensitivity to phenylephrine and nitroprusside to WT mice. These mice tended to weigh more and be less active than their WT counterparts. The role of TRPV4 in BP regulation during normal, low and high salt diet is still to be determined. Moreover, currently there are unsettling conclusions regarding the role of TRPV4 channels in obesity and metabolism (Ye et al. 2012, O'Conor et al. 2013). However, given how important TRPV4 is in the OPR and in salt-induced hypertension, it might be an important target for drug development, given that there is no current good TRPV4 antagonist for *in vivo* experiments.

2. Future Directions

The result in chapter II suggested that TRPV4 channel expression in the portal circulation was important to elicit the OPR. This study was done in TRPV4 knockout mice; therefore, there might be other complications and side effect arising from the global absent of the receptors. Future experiments in a local knockout mouse model of TRPV4 in the portal vein and the liver would provide a more definitive conclusion about the role of these channels in detecting changes in osmolality in the portal circulation and causing BP increase.

The renal nerves project into both the celiac and the mesenteric ganglia before entering the CNS (Johns 2013). Chapter III suggested that the celiac ganglion was not essential in the initiation of the OPR, while chapter IV showed that the renal nerves were needed for the response to occur. The role of the inferior and superior mesenteric ganglia in the OPR merits further investigation. Additionally, because renal denervation by phenol severed both afferent and efferent nerves, it was not clear which pathway was more important in the OPR. Therefore, it would be interesting if we could just denervate either efferent or afferent nerve separately to study how it might affect the OPR. Recently, it was proposed that the sensory nerves could be severed by capsaicin, a specific TRPV1 agonist (Kun et al. 2012, Vaughan and Bartness 2012). However, the data were still inconclusive.

The ENS has the ability and potential in mediating the OPR. It can regulate local vasoconstriction of splanchnic blood vessels. Because the splanchnic circulation holds

a large percentage of blood volume, vasoconstriction in this area can be sufficient enough to raise systemic BP (Sasselli, Pachnis, and Burns 2012). Studying the role of the ENS will not only increase our understanding of cardiovascular control elicited by water ingestion, but also the role of the ENS in systemic BP regulation. However, because of its structure and location, it presents difficulty in isolating and studying the ENS. Currently, there are several mouse models with denervation in the ENS being studied in Crohn's disease (Coruzzi et al. 2007, Ohlsson et al. 2007). However, the physiology of the mice is greatly compromised and not suitable for the OPR study.

New findings in the $CGRP^{-/-}$ mice leave interesting questions to be answered. Chapter V showed that $CGRP^{-/-}$ mice had significantly stiffer blood vessels compared to WT counterpart, but the mechanism underlying this effect was unclear. Collagen and elastin deposition was normal in the knockout mice. Therefore, future studies to determine the mechanisms that mediating blood vessel stiffness in these mice will give further insight into diseases such as hypertension and atherosclerosis because vascular stiffness has been associated with cardiovascular (Laurent et al. 2001, Cecelja et al. 2013, Sehgel et al. 2013, Bernberg et al. 2009).

Lastly, measuring tissue, plasma and urine catecholamines in $TRPV4^{-/-}$ mice after the high-salt diet challenge will provide information on the role of TRPV4 channels in salt-induced hypertension and the autonomic response during salt loading. Besides, currently, the contribution of these channels in metabolism and obesity is debatable (Ye et al. 2012, O'Conor et al. 2013). Data from chapter 6 suggested that $TRPV4^{-/-}$ mice

might be overweight when being on normal diet. This can be a result of lack of physical activities in these mice. Therefore, future studies to determine the exact role of TRPV4 in metabolic syndrome are desirable and might present TRPV4 as an attractive drug target in both metabolic and cardiovascular diseases.

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