

**CARDIOVASCULAR EFFECTS OF WATER INGESTION: AN OSMOSENSITIVE
PRESSOR RESPONSE**

By

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LIST OF ABBREVIATIONS

-/-	Homozygous knockout
ACE	Angiotensin converting enzyme
ANS	Autonomic nervous system
BP	Blood pressure
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CVLM	Caudal ventrolateral medulla
DBH	Dopamine β hydroxylase
GI	Gastrointestinal
i.p.	Intraperitoneal
i.v.	Intravenous
MSA	Multiple systems atrophy
NaCl	Sodium chloride
NE	Norepinephrine
NK ₁	Neurokinin 1 receptor
NTS	Nucleus tractus solitarius
OI	Orthostatic intolerance
PAF	Pure autonomic failure
PE	Phenylephrine (α_1 -agonist)
RAAS	Renin-angiotensin-aldosterone system
RVD	Regulatory volume decrease

RVLM	Rostral ventrolateral medulla
SNS	Sympathetic nervous system
TRP	Transient receptor potential
TRPV	Transient receptor potential vanilloid

CHAPTER I

INTRODUCTION AND BACKGROUND

Autonomic Nervous System and BP Control

The visceral, or autonomic nervous system (ANS) is a branch of the nervous system that regulates individual organ function and maintains homeostasis within the body.

All efferents from the central nervous system (CNS) are considered autonomic nerves with the exception of those fibers that innervate skeletal muscle. The efferent arm of the ANS is responsible for transmitting signals from the CNS to peripheral organs. Important functions include control of heart rate and force of contraction, vessel diameter (constriction/dilation), visual accommodation, pupillary size, and secretions from various endocrine and exocrine glands. Autonomic afferents transmit information regarding visceral sensation to the CNS as well as facilitate vasomotor, respiratory, and cardiovascular reflexes. (Barrett et al., 2010b)

The ANS is divided into sympathetic and parasympathetic branches on the basis of anatomical and functional differences. Both branches consist of myelinated preganglionic fibers which synapse onto unmyelinated postganglionic fibers innervating the effector organ. The parasympathetic branch consists of outputs from motor nuclei of cranial nerves III (oculomotor), VII (facial), IX (glossopharyngeal), X (vagus), and sacral segments of the spinal cord. Many

parasympathetic outputs conserve energy and are associated with the body at rest in that they reduce heart rate (HR) and blood pressure (BP), and aid in digestion and absorption of nutrients. Acetylcholine is the neurotransmitter at postganglionic parasympathetic endings. (Waxman 2010)

The sympathetic branch consists of output from neurons with cell bodies in the lateral horn region of T1-L2 segments of the spinal cord. Most preganglionic fibers synapse onto sympathetic ganglia along the paravertebral chains on either side of the vertebral bodies. Some fibers bypass paravertebral ganglia to synapse onto prevertebral ganglia (celiac and mesenteric). Other fibers travel via splanchnic nerves to directly synapse with cells in the medulla of the adrenal gland. The sympathetic system is associated with fight or flight responses. Its activation results in increased HR, BP, cardiac output and contractility, as well as bronchiolar dilation and diversion of blood flow away from the splanchnic vessels to those supplying skeletal muscle. Noradrenaline is the principal neurotransmitter at postganglionic sympathetic endings with the exception of fibers innervating sweat glands, which release acetylcholine. (Westfall and Westfall 2010) (Figure 1).

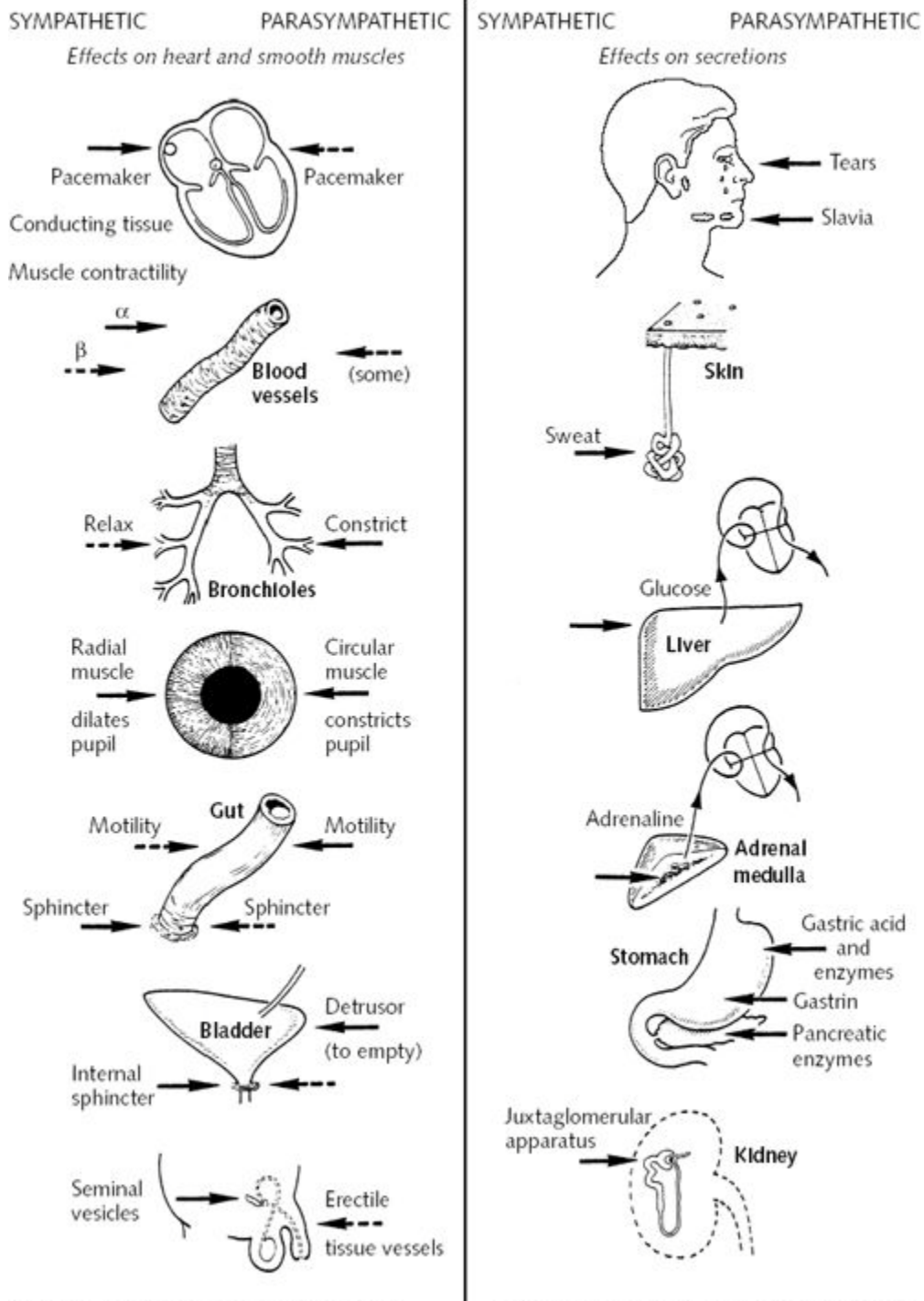


Figure 1: Actions of autonomic nerves. Solid arrows represent stimulation; broken arrows represent inhibition. (Jannett 1989)

Many reflex pathways are facilitated by the ANS. Autonomic reflexes modulate the function of visceral organs and are responsible for regulating heart rate, respiratory rate, digestive function, vascular tone, and many other bodily functions. Stimuli acting on pain, mechano-, or chemo- receptors travel through spinal or vagal afferents and affect the firing of efferent nerves that ultimately control end-organ function (Figure 2) (Barrett et al., 2010b).

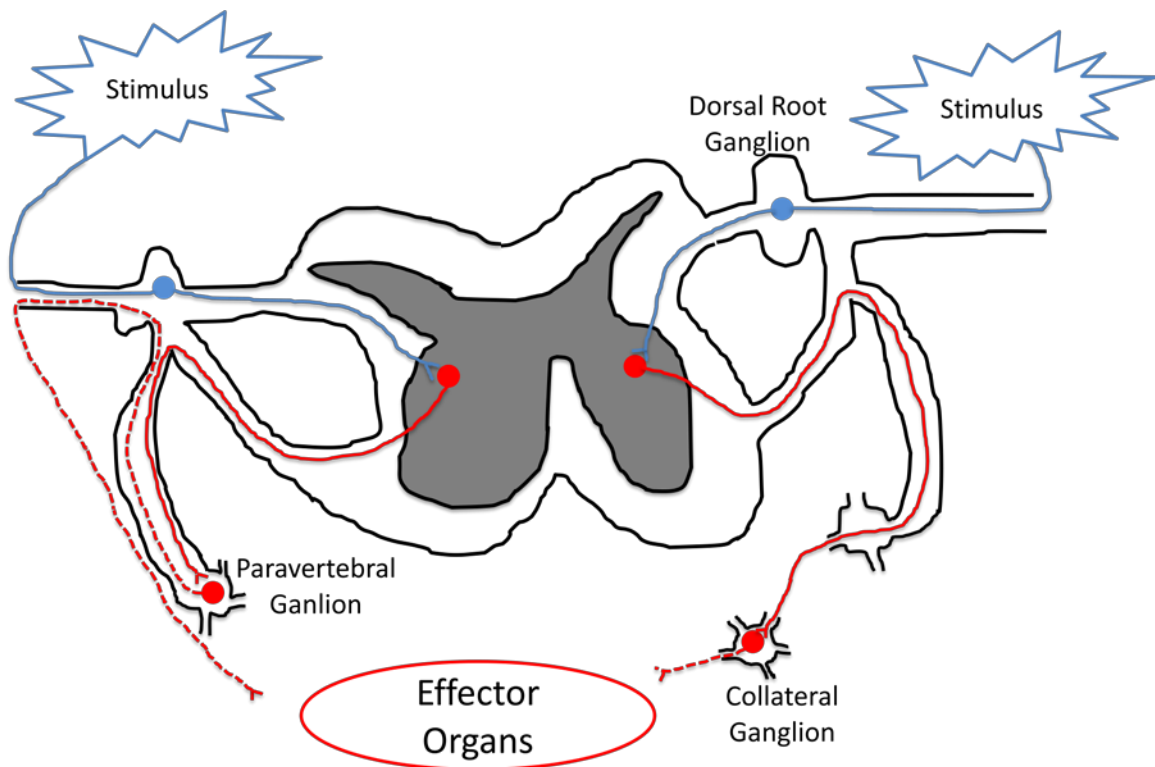


Figure 2: Autonomic reflex. Blue represents afferent fibers and red represents efferent fibers. Efferents may synapse at one of the paravertebral ganglia, or pass through the sympathetic trunk to synapse at a collateral ganglion.

The baroreflex is one example of an autonomic reflex. Baroreceptors (mechanoreceptors) sense the degree of stretch along the aortic arch and carotid sinuses and relay this information via vagal and glossopharyngeal afferents to the nucleus tractus solitarius (NTS) in the medulla. NTS sends excitatory

(glutamatergic) inputs to the caudal ventrolateral medulla (CVLM), which in turn sends inhibitory (GABAergic) inputs to the rostral ventrolateral medulla (RVLM). The RVLM sends excitatory signals (glutamatergic) to the spinal cord (intermediolateral nucleus) and is the main regulator of the sympathetic nervous system. In addition to reducing sympathetic output through indirect inhibition of the RVLM, the NTS also sends excitatory projections to the nucleus ambiguus, increasing parasympathetic efferent activity (Kirchheim, 1976). Therefore, when baroreceptors become activated by stretch induced by increased BP, projections from the NTS both decrease sympathetic output and increase parasympathetic output, returning BP and HR to normal values (Figure 3). It is through the baroreflex that the body avoids acute fluctuations in BP and HR.

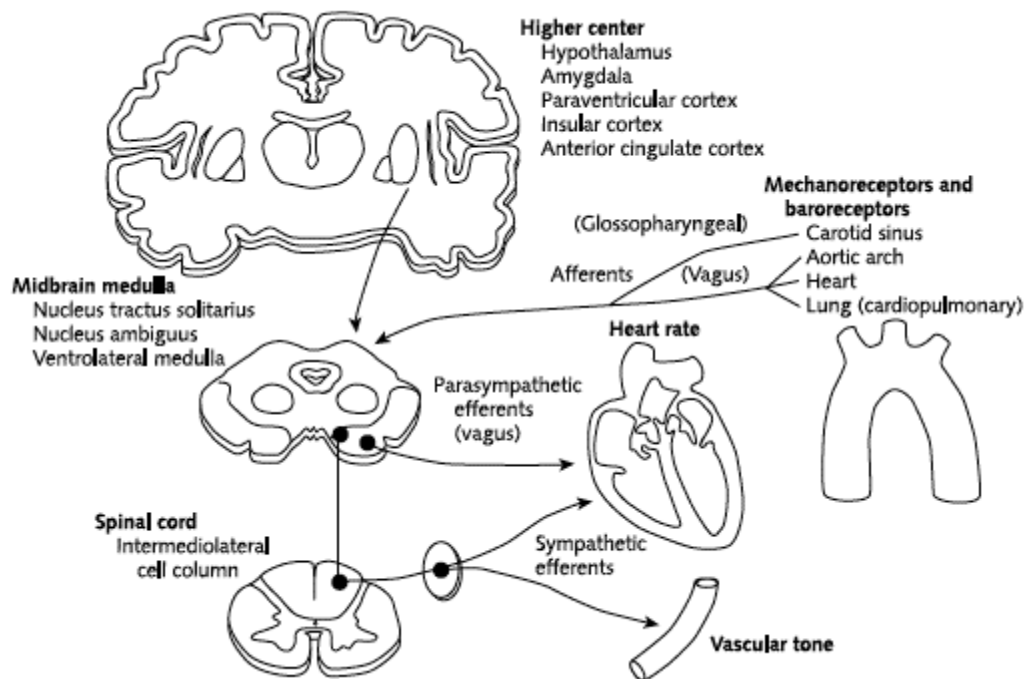


Figure 3: Baroreflex pathways. (Fenton et al., 2000)

The renin-angiotensin-aldosterone system (RAAS) is another pathway by which the autonomic nervous system controls BP (and sodium/fluid balance). This system is activated during periods of hyponatremia, hypovolemia or hypotension. Activation occurs via three mechanisms: (Sherwood 2004)

1. Reduced renal afferent arteriole pressure activates intrarenal barosensitive juxtaglomerular (JG) cells, which synthesize and release of renin.
2. Reduced NaCl flow activates macula densa cells, which stimulate renin release from JG cells
3. Reduced systemic BP activates the sympathetic system, which induces JG cells to release renin

Renin, together with angiotensin converting enzyme (ACE), produces angiotensin II (from cleavage of angiotensinogen and angiotensin I). Angiotensin II is a potent vasoconstrictor, about 4-8 times as active as norepinephrine by weight (Barrett et al., 2010a). Angiotensin II facilitates the release of norepinephrine from postganglionic sympathetic neurons, increases arteriolar vasoconstriction, as well as acts directly on the adrenal cortex to increase secretion of aldosterone (increasing renal NaCl reabsorption). Additionally, angiotensin II stimulates pituitary release of vasopressin (ADH), which increases water reabsorption in renal collecting ducts (Figure 4). Overall, the actions of the renin/angiotensin system increase circulating volume via

mechanisms that promote water and salt retention. In contrast to the baroreflex's buffering of acute BP perturbations, the renin-angiotensin system acts to maintain blood pressure homeostasis over a longer period of time.

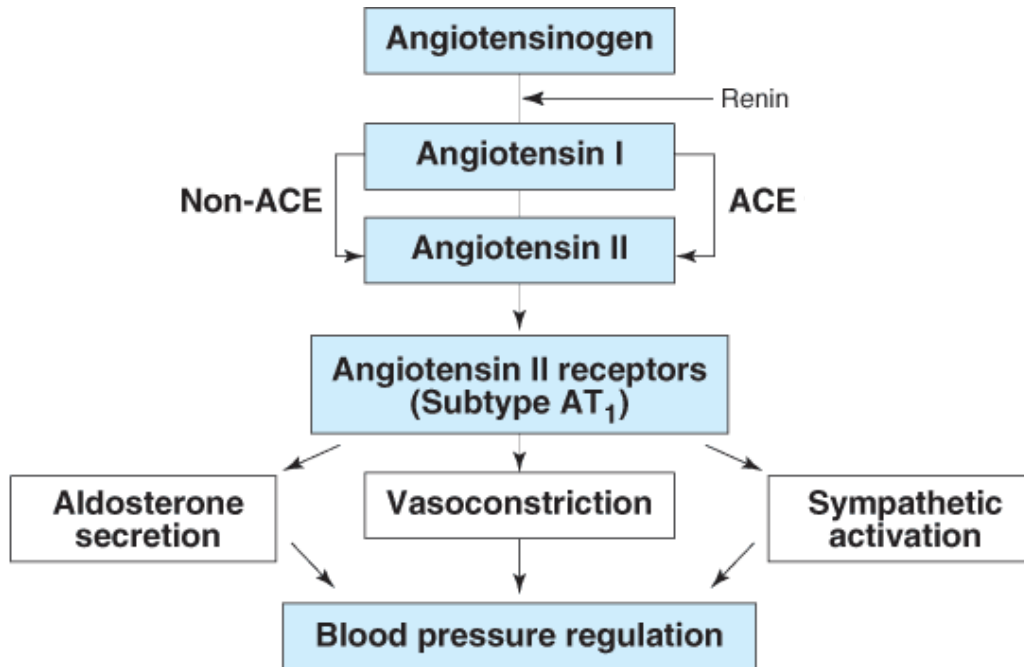


Figure 4: RAAS and the multiple effects of angiotensin II. (Don and Lo 2007)

Autonomic Nervous System Dysfunction

Autonomic failure can have devastating consequences and affect the function of many organ systems. Symptoms are wide-ranging, including problems with regulation of HR, BP, body temperature, perspiration, as well as fatigue, lightheadedness, syncope, and generalized weakness. Autonomic dysfunction can occur as a secondary condition of another disease process (such as diabetes), aging, or as a primary disorder. Lesions may be present in peripheral autonomic nerves or central nervous system.

Impaired baroreflex function is present in most forms of autonomic failure. The reduced capacity to buffer BP rises or falls allow BP effects of endogenous or environmental stimuli, that might otherwise escape detection, to emerge dramatically. Patients with baroreflex impairment have contributed to the discovery of the depressor effect of food (Hollister et al., 1986), the large depressor effect of beta-2 agonist terbutaline (Robertson et al., 1984), and the pressor effect of hypoventilation (Jordan et al., 2000b; Onrot et al., 1991). Therefore, this patient population presents a unique opportunity for discovery of fundamental determinants of autonomic cardiovascular regulation that would otherwise be masked by autonomic function.

Water-drinking in Patients with Autonomic Failure

Patients with autonomic failure respond to water ingestion with a dramatic increase in BP (Figure 5). The average pressor effect after 16 oz (473 mL) oral water is about 40 mm Hg, with occasional subjects experiencing BP increases of greater than 75 mm Hg. The effect appears within 10 minutes, is maximal at 25-40 minutes, and largely dissipates by 90 minutes after ingestion. Ganglionic blockade by trimethaphan prior to water ingestion attenuates the increase in BP, suggesting that the response is mediated by autonomic nervous system mechanisms. Additionally, the pressor effect of water appears to be independent of water temperature and bladder distension. Patients responded no differently to ingestion of water at temperatures of 9°C or 25°C, and prevention of bladder distention with a Foley catheter also had no effect on the response. (Jordan et al., 2000a)

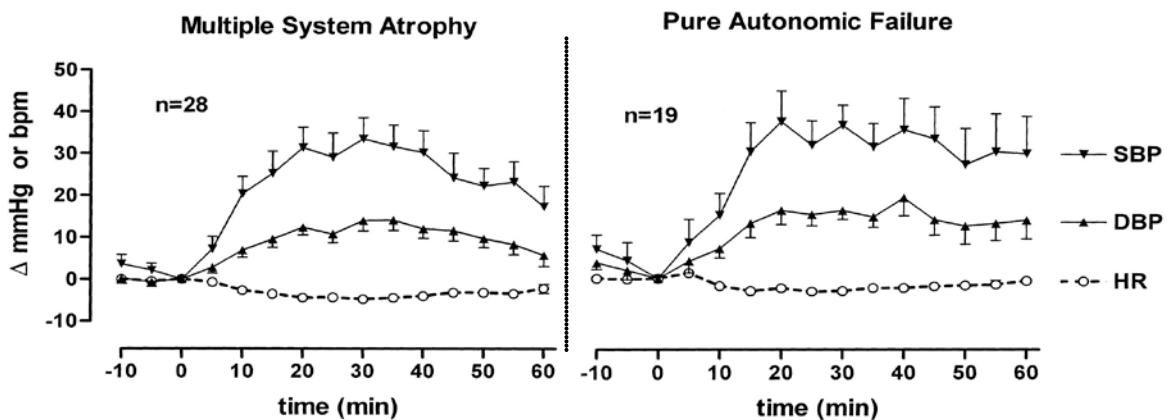


Figure 5: Pressor response to water ingestion in patients with dysautonomias. Changes in systolic BP (SBP), diastolic BP (DBP), and heart rate (HR) after patients with multiple system atrophy (left) and pure autonomic

failure (right) drank 473 ml tap water. Patients started drinking at 0 minutes. (Jordan et al., 2000a)

While water's effect is greatest in individuals with impaired baroreflex buffering, it is also present in healthy persons. In healthy young subjects with intact baroreflexes, water elicits an increase in peripheral vascular resistance without an increase in BP because of a compensatory reduction in cardiac output (Lu et al., 2003). Additionally, water ingestion raises plasma norepinephrine (NE) but not renin or vasopressin, supporting a sympathetic nervous system mechanism for the observed pressor response (Lu et al., 2003; Raj et al., 2006).

Water has also been shown to enhance tolerance of upright posture. Head-up tilt is a clinical test that assesses orthostatic tolerance. Healthy subjects given 16 oz. water 5 minutes prior to tilt testing tolerated the orthostatic stress of being tilted 26% longer than subjects not given water (Figure 6).

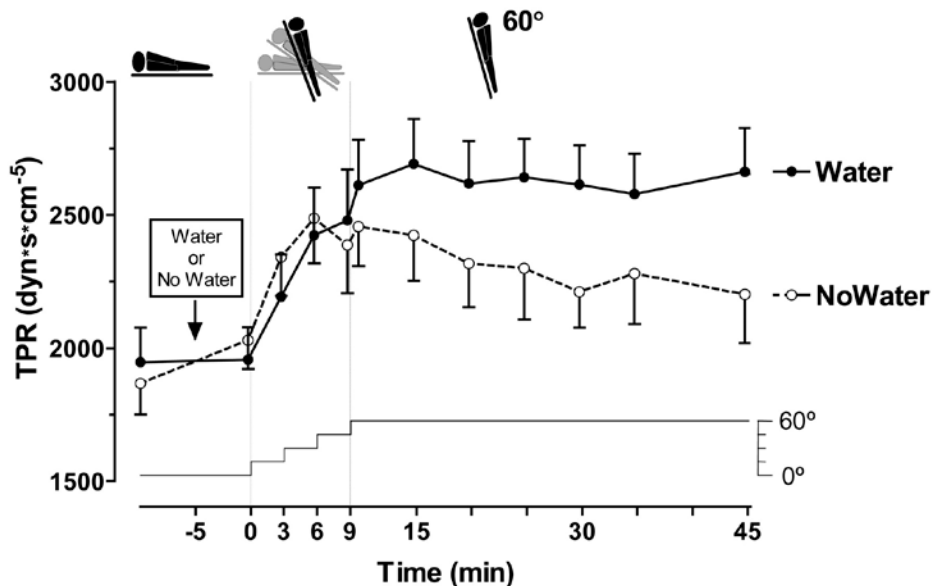


Figure 6: Total peripheral resistance (TPR) during head-up tilt. Water ingestion accentuated the increasing TPR during tilt-table testing. $P < 0.001$. (Lu et al., 2003)

Although it may seem paradoxical that the pressor effect of water could be so dramatic in patients with an impaired autonomic system if it were autonomically mediated, such observations are not unusual within this population. For example, the α_2 -adrenoreceptor antagonist yohimbine (acting centrally to increase sympathetic output and peripherally to enhance NE release), elicits an exaggerated BP response in patients with autonomic failure despite compromised efferent sympathetic output (Biaggioni et al., 1994). This may be due to 2 different mechanisms. First, since these patients have reduced sympathetic activity, their adrenoreceptors may be hypersensitive from lack of stimulation. Second, the baroreflex, which normally buffers BP in healthy individuals, is not operative in autonomic failure. Both the hypersensitivity and the lack of buffering contribute to the excessive sympathetic response of BP to yohimbine in autonomic failure. Therefore, it is plausible that with severe but incomplete autonomic failure, autonomic responses may be present and seemingly hyper-reflective.

Gastrointestinal Afferents

There are two broad categories of afferent neurons innervating the GI tract: intrinsic and extrinsic sensory neurons. Intrinsic neurons are part of the enteric nervous system and do not have a direct connection to the CNS. Extrinsic neurons sense stimuli within the GI region and transmit that information to the CNS through either vagal or splanchnic pathways (Furness et al., 1998). Vagal afferents originate from the nodose ganglia and project to the medullary region of the brainstem, whereas splanchnic afferents have cell bodies in the dorsal root ganglion and project to the dorsal spinal cord (Figure 7) (Holzer, 2001).

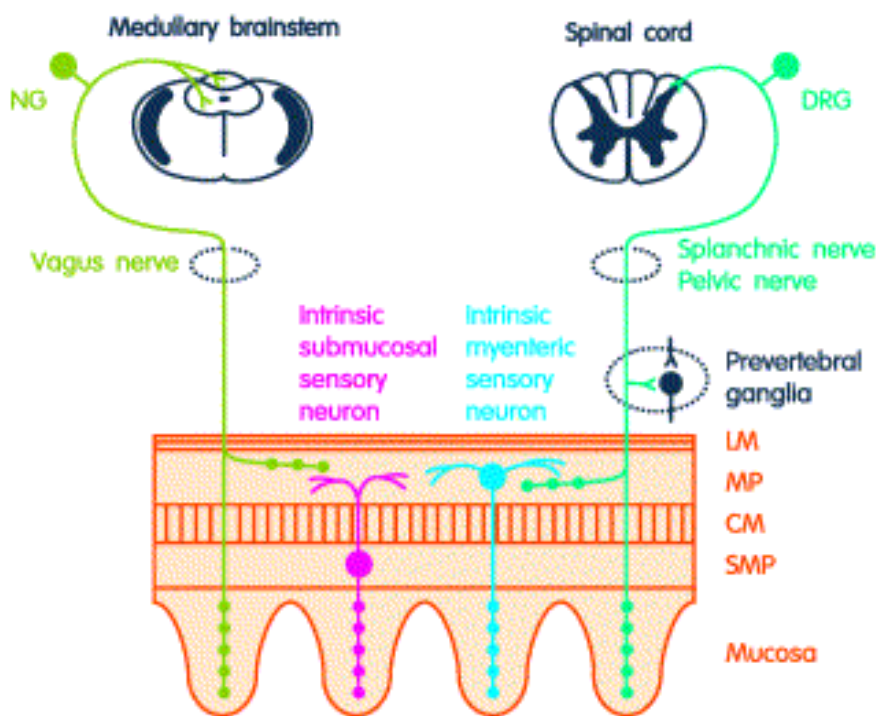


Figure 7: GI afferents. LM = longitudinal muscle; MP = myenteric plexus; CM = circular muscle; SMP = submucosal plexus; NG = nodose ganglion; DRG = dorsal root ganglion (Holzer, 2001).

Vagal and splanchnic GI afferents are sensitive to both mechanical and chemical stimuli, and express a wide variety of mechano- and chemo-sensitive ion channels. The transient receptor potential (TRP) channel represents one such group of channels expressed in a large portion of sensory/afferent fibers. These channels are capable of sensing multiple stimuli (including those from the gut) and play a role in a multitude of functions, including maintenance of mucosal integrity, intestinal motility, visceral sensation, osmotic regulation, BP regulation, among many others (Beyak et al., 2006).

TRP channels

The TRP (transient potential vanilloid) ion channels are a ubiquitously expressed, integral part of our sensory system, crucial for sensing a wide range of stimuli including temperature, pain, touch, osmolarity, pheromones, and taste. These channels are evolutionarily conserved, and function as sensory apparatus in yeast, nematodes, arthropods, as well as mammals both at the level of single cells as well as for entire systems (Clapham, 2003).

TRP channels are grouped into 6 families based on homology: canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), polycystin (TRPP), mucolipin (TRPML), and ankyrin (TRPA). Each family is further divided into subtypes. All channels consist of 6 transmembrane subunits, which are arranged in tetramers to form a cation-permeable pore (Clapham, 2003).

Activation of TRP channels can occur through multiple mechanisms, including receptor activation through G protein-coupled receptors, ligand activation by small organic molecules (capsaicin and icilin) and endogenous products of lipid metabolism, as well as direct activation from changes in temperature, osmolality, and mechanical stimuli (Ramsey et al., 2006). The ability of TRPs to integrate multiple environmental and endogenous stimuli and their ability to modulate downstream signal transduction cascades makes these channels well suited for cellular sensation.

TRP channels hold particular interest for us in studying the mechanism of water's cardiovascular effects due to these channels' ability to sense a variety of stimuli and elicit multiple functions including regulation of BP. TRP channels are

present throughout the GI tract, portal system, sensory neurons (dorsal root ganglia), as well as osmosensitive regions of the CNS (Brierley et al., 2008; Cenac et al., 2008; Gradilone et al., 2007). Although the stimulus eliciting the pressor response to water remains unclear, likely possibilities included luminal stretch of the gastrointestinal tract, acute osmolality changes, or local changes in pH of the stomach. TRP channels are capable of sensing and transducing all of the above mentioned stimuli, making these channels prime candidates for mediating the water-induced cardiovascular response.

TRPV1

The transient receptor potential vanilloid 1 channel, also known as the capsaicin receptor, is a subgroup of TRP channels expressed predominantly in sensory nerves (Caterina et al., 1997). TRPV1 is activated by a vast range of stimuli, including noxious temperature, changes in pH, diverse lipid molecules, vanilloid compounds, as well as osmotic and mechanical stimuli (De and Di, V, 2005; Dhaka et al., 2009; Liu and Simon, 2000). Rutaecarpine, a TRPV1 agonist and major alkaloid isolated from the Chinese herbal drug Wu-Chu-Yu, has long been used for the treatment of gastrointestinal disorders in traditional Chinese medicine. Since then, several studies have shown TRPV1-positive sensory nerves throughout the GI system are responsible for maintenance of mucosal integrity, pH, intestinal motility, and visceral sensation (Ward et al., 2003). In addition to its gastroprotective properties, rutaecarpine has also been reported to play a role in blood pressure control in hypertension (Deng et al., 2004; Deng and Li, 2005; Wang et al., 2005). It is believed that the actions of rutaecarpine is mediated by the release of neurotransmitters (calcitonin gene-related peptide and substance P) upon TRPV1 activation.

More recently, TRPV1 activation has also been shown to have effects on the cardiovascular system (Hu et al., 2002; Wang et al., 2007). TRPV1, through its regulation of CGRP release, is thought to protect against cardiac injury (Rang et al., 2004; Zhong and Wang, 2008). Conversely, genetic knockout of TRPV1 in mice results in excessive cardiac inflammation and abnormal ventricular

remodeling after myocardial ischemia, providing evidence for the role of TRPV1 in cardiac adaptation to ischemic stress (Huang et al., 2009).

TRPV1 also plays a role in BP regulation, which occurs in large part due to alterations in peripheral vascular resistance. Peripheral vessels are highly innervated by sympathetic sensory nerves, many of which are also sensitive to capsaicin (characteristic of TRPV1 positive neurons). Control of vascular tone by these nerves occurs predominantly through the release of vasoactive neurotransmitters including calcitonin gene related peptide (CGRP) and substance P (SP), both of which are released upon TRPV1 activation. Activation of TRPV1 has been shown to produce antihypertensive effects through stimulation of CGRP release, whereas rats depleted of CGRP in TRPV1 sensory neurons show enhanced development of hypertension (Deng and Li, 2005).

In addition to its multiple functions in the GI and cardiovascular system, TRPV1 is also required for producing normal osmoregulatory responses. TRPV1 knockout mice (*TRPV1*^{-/-}) show impaired vasopressin release in response to hypertonic stimuli. Furthermore, vasopressin-releasing neurons in central osmosensitive regions of these knockout mice are unable to produce the expected excitatory responses to hypertonic stimuli (Sharif et al., 2006). Other reports show that *TRPV1*^{-/-} mice have impaired thirst response to systemic hypertonic challenge (Ciura and Bourque, 2006).

TRPV1's ability to sense GI stimuli coupled with its effects on the cardiovascular system makes it a candidate mediator of the pressor response to water.

TRPV4

TRPV4, like TRPV1, is considered an “osmomechano-TRP” (Liedtke and Kim, 2005). The channel is expressed in the GI tract, mesenteric vessels, liver, cholangiocytes, dorsal root ganglia, and other locations throughout the body (Brierley et al., 2008; Cenac et al., 2008; Gradilone et al., 2007), and is constitutively active at body temperature (Nilius et al., 2004). TRPV4 responds to a wide variety of stimuli, including changes in osmolality, shear stress, heat, phorbol esters (4 α -PDD), and physiologically active lipids (5'6'-EET) (Watanabe et al., 2003). Activation of TRPV4 results in release of NO, CGRP, and substance P, all of which are potent vasoactive compounds and can function as neurotransmitters (Grant et al., 2007; Kohler et al., 2006).

TRPV4 has been shown to be important in the maintenance of systemic osmotic homeostasis. Knockout animals are unable to regulate their osmolality as effectively as wild-types when exposed to systemic osmotic perturbations and have an abnormal vasopressin response to hypertonic stimuli (Liedtke and Friedman, 2003). Additionally, *Trpv4*^{-/-} mice have reduced pain response to hypotonic and hypertonic subcutaneous solutions, and dorsal root ganglion neurons isolated from these animals fail to respond to hypotonic challenge with an increase in intracellular calcium as observed in wild-types (Alessandri-Haber et al., 2005). These data suggest that TRPV4 plays an important role in transduction of stimuli elicited by changes in osmolality.

TRPV4 has also been shown to play a critical role in regulatory volume decrease (RVD) in response to hypotonicity, a mechanism for maintenance of osmotic homeostasis at the cellular level. Under hypotonic conditions, cell swelling occurs due to the influx of water down its osmotic gradient. RVD begins in response to cell swelling through an ion-transport process involving loss of ions from the cell. CHO cells, which do not express TRPV4, are unable to undergo RVD under hypotonic conditions. However, when transfected with TRPV4, CHO cells gain the ability to undergo RVD and regulate their cell volume in hypotonic conditions (Becker et al., 2005).

TRPV4 has recently been implicated in salt-sensitivity and hypertension. Rats fed a high-sodium diet showed increased expression of TRPV4 in sensory nerves and mesenteric arteries and an enhanced depressor response to synthetic agonist 4 α -PDD (Gao et al., 2009). In studies from Dahl salt-sensitive (DS) and salt-resistant rats (DR) placed on high-sodium diets, only DS rats developed hypertension. DS rats developed hypertension on high-salt diets and had reduced TRPV4 expression in DRG neurons and reduced depressor response to 4 α -PDD, whereas DR rats on the same diet had increased TRPV4 expression and augmented depressor response to 4 α -PDD, and no hypertension (Gao and Wang, 2010). These studies suggest that TRPV4 may play a role in preventing salt-induced increases in BP.

Summary and Significance

Water ingestion elicits dramatic increases in BP in individuals with baroreflex impairment. The effect appears within 10 minutes, is maximal at 25-40 minutes, and largely dissipates by 90 minutes. Although the pressor effect of water is greater in individuals with baroreflex impairment, healthy subjects increase peripheral vascular resistance and show improved orthostatic tolerance following water ingestion. Plasma NE (but not renin or vasopressin) is increased after water ingestion, and ganglionic blockade by trimethaphan attenuates the response, both suggest water is acting through autonomic mechanisms. The ability of TRP channels to sense a wide range of stimuli and to elicit multiple functions (including BP effects) and their presence in GI afferents make these channels prime candidates for mediating water's cardiovascular effects. It is possible that the gastrointestinal tract represents an important modulator of cardiovascular regulation, and this thesis project examines the potential mechanisms and stimuli central to this system. Specific aims are outlined in the next section.

Thesis Aims

It has been estimated that 1,000,000 Americans have a problem in orthostatic regulation, of which 50% present in the form of orthostatic hypotension. Although there has been much research and progress in the study of the autonomic nervous system in both health and disease, there are still unanswered questions regarding the basic determinants of autonomic cardiovascular regulation. One such determinant was uncovered during studies in patients with autonomic failure in which oral ingestion of water elicited a surprisingly large, prolonged pressor response (outlined in earlier sections). Effects of water ingestion have also been observed in healthy young and older subjects, in patients with hypertension, and in individuals with neurally mediated syncope. However, little is known about the mechanism by which water exerts this effect. The magnitude of the response suggests that water may be involved in an unrecognized mechanism involving cardiovascular regulation in both health and disease.

We do not yet understand the physiological basis underlying water's effect, or how solute content (such as NaCl) in water might affect it. Nor do we know if the response is mediated within the gut, in the portal circulation, or systemic circulation. Murine models often prove to be powerful tools in the study of mammalian physiology. We developed a mouse model that mimics human autonomic dysfunction and have used this model in elucidating the pressor response to water.

The purpose of this thesis is to analyze the pharmacology of water ingestion and to address the mechanism of its pressor effect. Where and how is this response evoked? Is it neurohumoral? Which neurotransmitters or hormones might be involved? Is it mediated by the autonomic nervous system? Is the response simply due to volume change? (Figure 8)

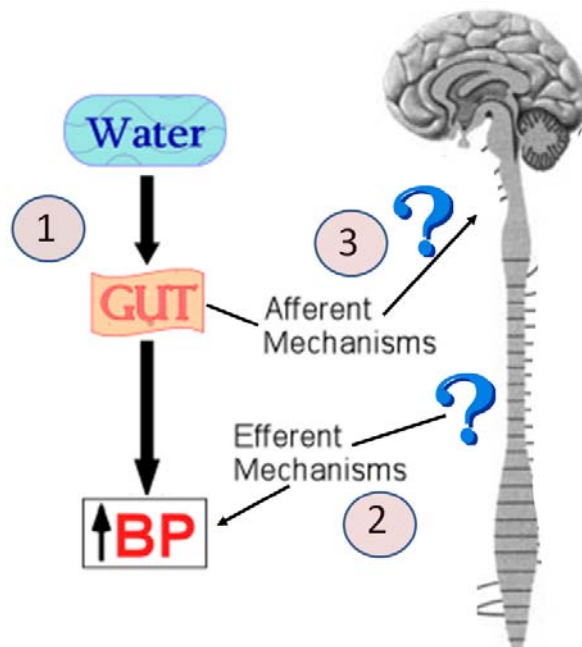


Figure 8: Thesis aims.

Specific Aim 1: Elucidate the location and stimulus eliciting the water's cardiovascular effect.

The stimulus eliciting water's pressor effect could operate in many different ways. If the stimulus were gastric stretch or increased luminal pressure, then any substance ingested of a certain volume would be expected to elicit a similar pressor response as observed with water ingestion. The location where the stimulus is being sensed is also unclear. If the stimulus were low osmolality,

then osmolyte content of the ingested liquid would determine the magnitude of the pressure response. The purpose of Aim 1 is to elucidate these mechanisms.

- 1.1 Is the pressor effect dependent on pharyngeal, esophageal, gastric, or other mechanisms?
- 1.2 Does electrolyte content alter water's pressor action?
- 1.3 Is increased intestinal luminal pressure the stimulus for water's pressor action?
- 1.4 Is plasma volume expansion responsible for eliciting the pressor response?

Specific Aim 2: Determine the nature of the efferent arm of water's pressor action.

The attenuation of the pressor response after ganglionic blockade suggests that the autonomic nervous system is essential for producing the increase in BP after water ingestion. Plasma NE also increases during water ingestion, favoring a "sympathetic" hypothesis. The purpose of Aim 2 is to test the sympathetic hypothesis and explore potential efferent mechanisms.

- 2.1 Is the pressor response prevented by α_1 -adrenoreceptor blockade?
- 2.2 Is the pressor response present in dopamine β -hydroxylase deficiency?
- 2.3 Does angiotensin converting enzyme inhibition alter pressor response to water?

Specific Aim 3: Determine the nature of the afferent arm of water's pressor action.

Aim 3 will focus on potential afferent mechanisms and neurochemical pathways which may be involved. If the water response is mediated by autonomic reflex, either vagal or sympathetic (spinal) afferents may be responsible for transmitting the stimulus to the central nervous system. There are many possible mechanisms by which the pressor response might be elicited. And since multiple mechanisms are often used by the body to modulate important physiological responses, it may be possible that more than one mechanism is involved. For this reason, a more generalized, broad-based strategy has been used to determine the leading mechanisms for initial studies.

One such candidate is substance P, a neurotransmitter in the tachykinin family present in high concentrations in the GI tract and CNS. Other candidates include osmo-sensitive gene products like transient receptor potential vanilloid receptors (TRPV) 1 and 4. Substance P, TRPV1, and TRPV4 knockout mice are used to study the role of these candidate mediators in the pressor response.

- 3.1** Is the pressor response altered in mice with bilateral subdiaphragmatic vagotomy?
- 3.2** Is the pressor response present in mice deficient in substance P (*Tac1*^{-/-}) ?
- 3.3** Is the pressor response present in mice with *Trpv1* deletion (*Trpv1*^{-/-})?
- 3.4** Is the pressor response present in mice with *Trpv4* deletion (*Trpv4*^{-/-})?

Materials and Methods

All studies were conducted with an approved protocol from the Vanderbilt University Institutional Animal Care and Use Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). All efforts were made to minimize the number of animals used and their suffering.

Mice

Wild-type C57BL/6J mice (n = 53; Jackson Laboratories) were used in all of the experiments unless otherwise noted. Dopamine β -hydroxylase knockout mice (*Dbh*^{-/-}; n = 5) were developed by standard gene-targeting methods, as described by Thomas et al. (Thomas et al., 1995). *Tac1*^{-/-} mice (n = 5) and *Trpv1*^{-/-} mice (n = 6) were purchased from Jackson Laboratories. *Trpv4*^{-/-} mice (n = 21) were developed and provided by Wolfgang Liedtke (Liedtke and Friedman, 2003).

Continuous BP Recordings

Mice were anesthetized with 4% isoflurane and maintained on 1% isoflurane in oxygen delivered from a precision vaporizer. Body temperature was maintained at 36°C to 37°C with an isothermal pad (Braintree Scientific, Inc). Drugs were administered through a venous catheter in the left jugular vein, and BP was measured through a catheter in the right femoral artery (Micro-Renathane,

Braintree Scientific Inc) connected to a pressure transducer (DTX Plus-4812, Becton-Dickinson) and carrier amplifier (Gould Instruments). BP signals were recorded using a WINDAQ data acquisition system (DATAQ). Data were analyzed by PVwave (Visual Numerics).

Gastric/Duodenal Cannulation

An upper abdominal midline incision was made to expose the stomach for gastric and duodenal cannulation (Figure 9). The fundus was punctured at the greater curvature with blunt forceps. A PE-50 catheter was inserted into the gastric lumen or passed just beyond the pyloric sphincter into the duodenum. Sutures placed around the blunted and flanged end of the catheter secured the catheter in place and prevented retrograde flow of GI fluids. Normal saline or water was infused into the stomach or duodenum at a volume of 25 μ L/g of body weight over a 6-minute period.



Figure 9: Fundus of mouse stomach.

Subdiaphragmatic Vagotomy

The esophagus was isolated and secured at 2 points with silk sutures; one suture was placed where the thoracic esophagus emerges through the diaphragm and the other placed closer to the gastroesophageal junction. The sutures acted as retractors to separate the esophagus from other structures in the area, as well as to prevent leakage of GI contents into the peritoneum. The esophagus was severed just below the diaphragm, along with the dorsal and ventral vagus nerves.

Baroreflex Impairment Model Preparation

Our method is similar to previously described methods of baroreflex deafferentation (15,16). Briefly, a ventral midline incision was made in the neck, exposing the carotid bifurcation and allowing isolation of afferent components of the baroreflex. The superior cervical ganglion and superior laryngeal nerves were isolated and removed, along with the carotid sinus nerve. In addition, the adventitia and associated connective tissue were stripped from the carotid sinus regions. The vagus and glossopharyngeal nerves were left undisturbed (Figure 10). Baroreflex impairment was confirmed by comparing Δ heart rate (HR)/ Δ BP on phenylephrine challenge before and after deafferentation.

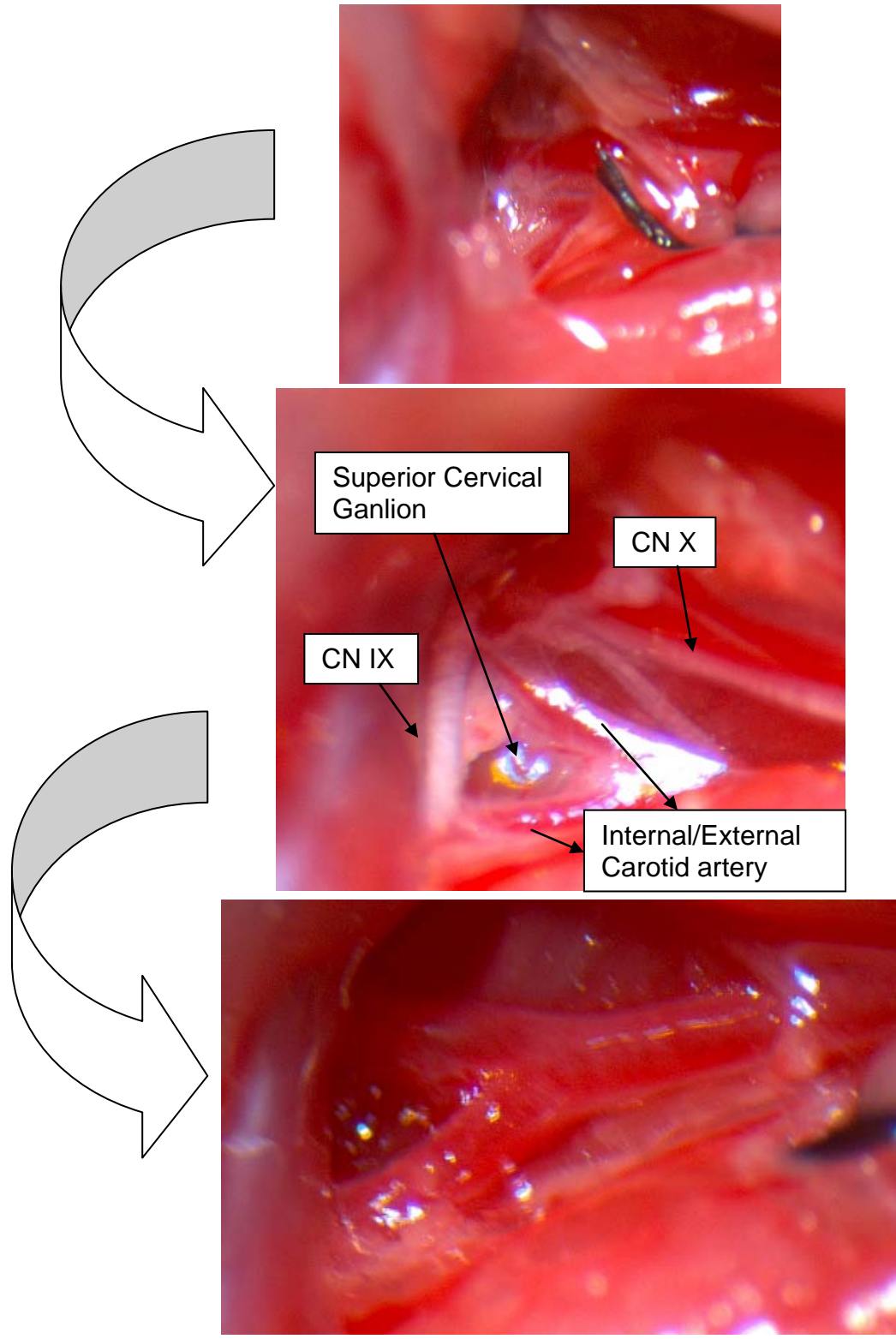


Figure 10: Baroreflex deafferentation. Images taken throughout the baroreflex deafferentation procedure.

Conscious BP Measurements for Restraint Stress Test

Mouse BP radiotelemetry (Data Sciences International) has been described previously (Mills et al., 2000). Briefly, the left carotid artery was isolated, a vessel clamp was placed 8 to 10 mm below the bifurcation to occlude blood flow, and the lumen was cut to allow insertion of the transmitter catheter to the point of the bifurcation. The transmitter body was placed in a lateral subcutaneous pocket. The overlying muscles and skin were secured via sutures.

Restraint Stress Test

A universal mouse restrainer (Braintree Scientific, Inc) was used to induce restraint stress in telemetered mice (Figure 11). Mice were placed in the restrainer for 2 minutes. Beat-to-beat BP and HR measurements were recorded using the DSI ART Gold software (Data Sciences International). Data were analyzed by PVwave (Visual Numerics).

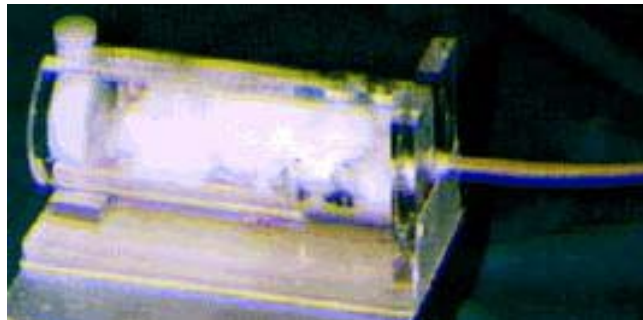


Figure 11: Restraint-stress test.

Drugs

Prazosin hydrochloride (Sigma) was dissolved in distilled water with the application of heat. Final dilutions were made with saline and given i.p. at 0.5 mg/kg in 100 μ L saline. This dose was chosen because it sufficiently blocked the α -1 pressor effect of phenylephrine. Water was infused intraduodenally 20 minutes after i.p. prazosin. Phenylephrine hydrochloride (Sigma) was dissolved in saline and given intravenously.

Osmolality Measurement

Blood was drawn from the portal vein and carotid artery 10 minutes after duodenal infusion of water or saline and centrifuged right after collection (600 g for 10 minutes) to reduce cell lysis. Plasma osmolality was measured using the Vapro Vapor Pressure Osmometer 5520 (Wescor Inc).

Statistics

The data in this study consist of multiple BP measurements before and after the intervention. We have used a response-feature approach to account for the repeated-measures aspects of our data while avoiding complex longitudinal models.¹⁸ Average BPs were derived for each mouse during the baseline and post-treatment intervals (BP_{ib} and BP_{ip} , respectively). The average change from baseline for the i^{th} mouse was $BP_i = BP_{ip} - BP_{ib}$. BPs were recorded continuously throughout the experiment. The baseline interval was from 10 minutes before the onset of treatment until the start of infusion.

Infusion lasted 6 minutes. The posttreatment interval was from the end of infusion and lasted 34 minutes. Tests of the change in BP in response to treatment within each treatment group were assessed by comparing BP_{ib} with BP_{ip} using the Wilcoxon signed-rank test. The difference in BP response to infusion between separate treatment or genetic groups was assessed by comparing ΔBP_i in the 2 groups using a Mann-Whitney U test. Data are presented as the mean \pm SD. SPSS was used for all of the statistical calculations. All probability values were derived with respect to 2-sided alternative hypotheses.

CHAPTER II

LOCATION AND STIMULUS

Baroreflex Denervation Unmasks Pressor Response

The baroreflex, which prevents acute fluctuations in BP, would tend to mask any potential changes in BP after water ingestion. For this reason, the baroreflex can be denervated to facilitate study of the cardiovascular effects of water ingestion. After deafferentation of the baroreflex, cardiovascular changes elicited by water or other stimuli become unmasked and magnified, making for easier detection and quantification.

Water infused into the stomach of baroreflex-intact mice produced almost no BP changes. After baroreflex deafferentation, infusion of the same volume of water (25 μ L/ g/ 6 min.) elicited an increase in systolic BP with a similar time profile to that observed after water ingestion in baroreflex- impaired patients (Figure 12). Unless otherwise noted, all BP recordings were obtained after bilateral baroreflex deafferentation. Phenylephrine was infused intravenously before and after denervation to test the completeness of the procedure. Effective baroreflex denervation results in reduced compensatory decreases in HR in response to phenylephrine-induced increases in BP (Figure 13).

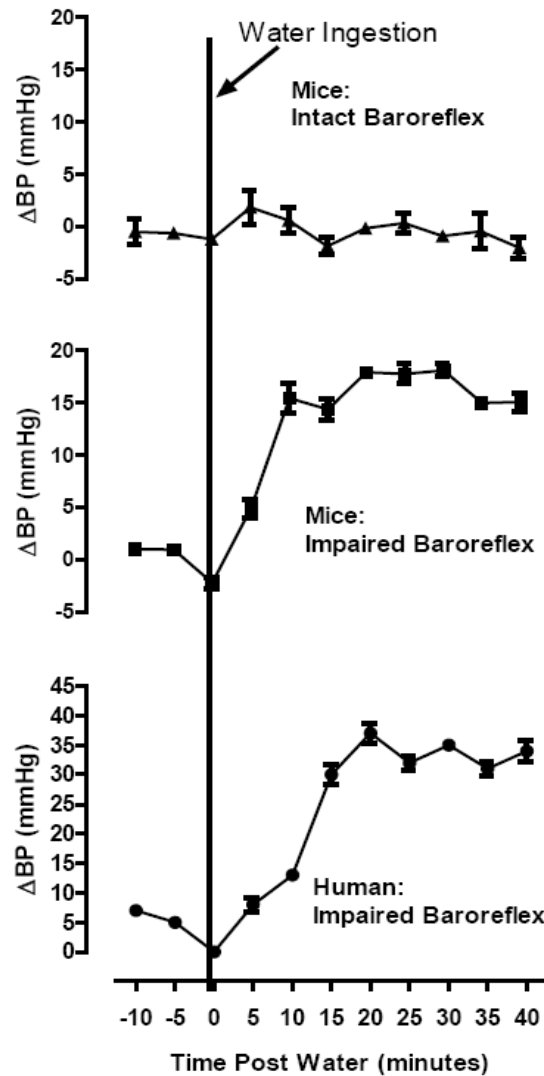


Figure 12: BP profile during water consumption in humans and mice. Mice with intact baroreflexes show little BP response to water infusion, since baroreflex buffering attenuates it. Surgically baroreflex-impaired mice, as well as patients with baroreflex impairment, show a robust increase in BP that is sustained well beyond the period of water ingestion. This enhanced pressor response facilitates mechanistic studies of water's cardiovascular effects.

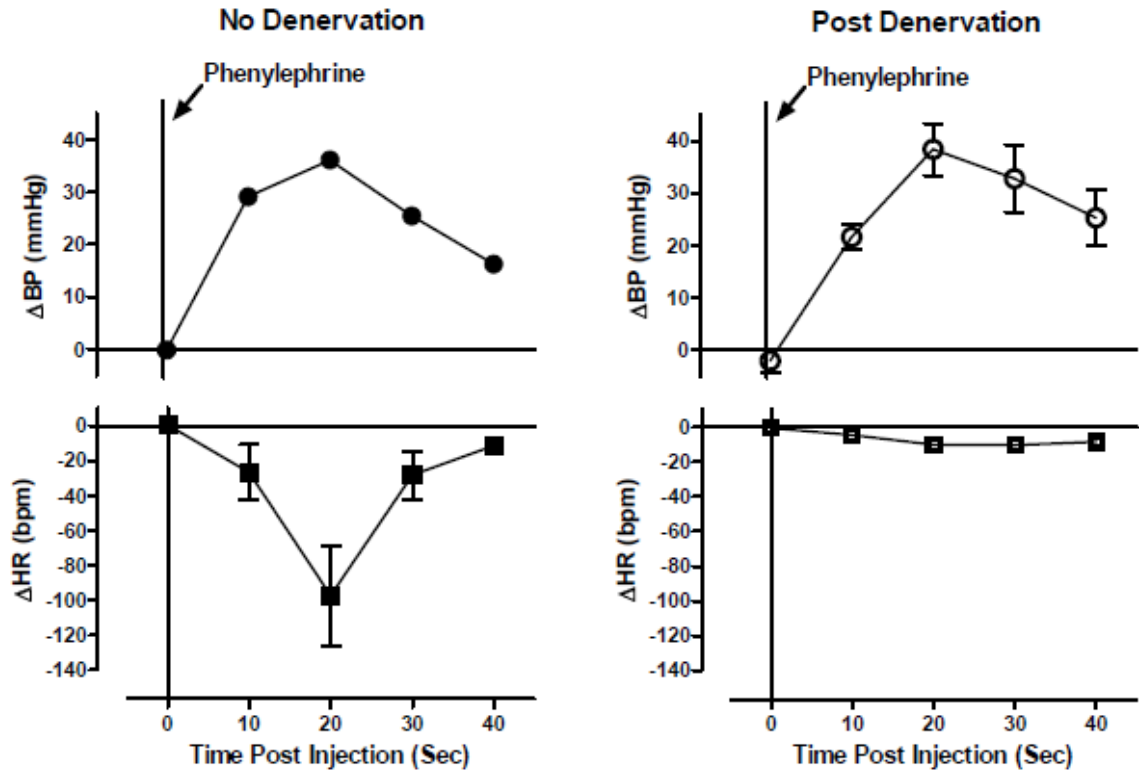


Figure 13: Blunted HR response to phenylephrine after baroreflex deafferentation. Representative BP and HR changes after i.v. phenylephrine (t = 0), pre and post baroreflex deafferentation. Phenylephrine challenge was used to validate successful baroreflex deafferentation in mice.

Anatomic Location of Water's Actions

Neurons in the pharyngeal and esophageal areas are sensitive to both mechanical and chemical stimuli (including water) and can elicit cardiovascular reflexes when activated (Cunningham et al., 1992; Remmers et al., 1986). To investigate pharyngeal and esophageal involvement in the pressor effect of water, water (25 μ L/g in 6 minutes) was infused directly into the stomach via an intragastric tube. Although both oral and esophageal regions were bypassed, water still elicited a robust increase in BP. BP began to increase near the end of water infusion, but maximal pressure was not reached until 20 minutes later (10.0 ± 13.2 mmHg; $P < 0.05$). The same experiment was repeated with direct duodenal infusion of water (catheter advanced beyond the pyloric sphincter). Intraduodenal infusion of water resulted in BP elevations of similar magnitude to intragastric infusion (14.9 ± 7.4 ; $p = 0.2$ between groups; Figure 14).

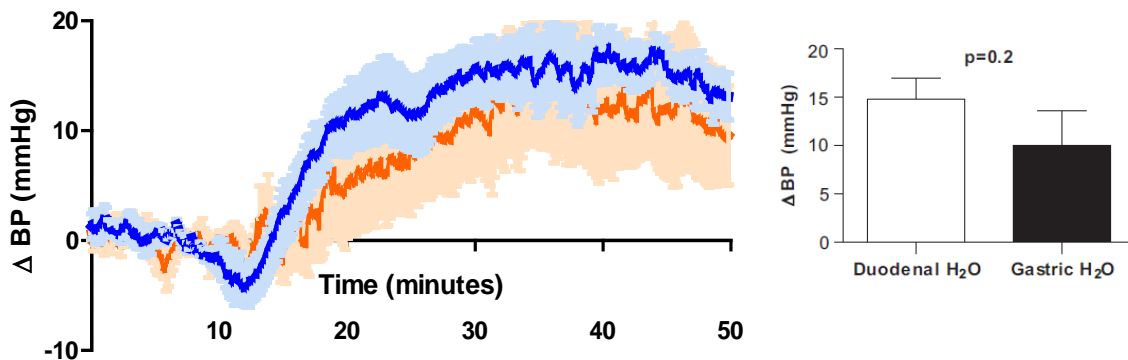


Figure 14: Location of water's action. Change in BP after intragastric or intraduodenal infusion of water (25 μ L/g). Infusions began at $t = 10$ minutes and ended at $t = 16$ minutes. Both gastric (orange; $n = 7$) and duodenal (blue; $n = 11$) infusion of water resulted in a robust increase in BP.

Stimulus Eliciting Water's Cardiovascular Effects

The stimulus exerted by water to elicit the pressor response might operate in many different ways. Stretch receptors are among the body's most widely distributed transducers of afferent inputs into the central nervous system. It is certainly possible that simple stretch of the stomach by 500 ml water could mediate or contribute significantly to the pressor response (Longhurst et al., 1981; Rossi et al., 1998). As compelling as this hypothesis is, there are nevertheless some problems with it. One might expect that stretch due to water ingestion would be maximal at the end of the ingestion and thereafter diminish as water entered the duodenum or was absorbed. However what is observed in our studies is not a maximal pressor response at the end of the infusion but a slow onset gradually building to a maximal response at about 15-20 minutes following infusion. Osmolarity is another potential means for induction of responses in the gastrointestinal tract. It is possible that water's lack of osmotic content, in striking contrast to food, conveys important information resulting in the pressor response of water.

If the stimulus were stretch, then presumably any liquid (or solid) ingested would be expected to elicit the response. If the stimulus were (hypo) osmolality, then osmolyte content of the ingested liquid would crucially determine the pressor response. To differentiate between luminal stretch and osmolality as triggers of the water response, mice were given equivolume doses (25 μ L/g) of physiologic saline (0.9% NaCl) or water. In contrast to water, intraduodenal saline infusion did not elicit an increase in BP (2.3 ± 8.1 mm Hg; $p = 0.6$; Figure

15). Hypotonicity appears to be the stimulus eliciting the water's effects, and for this reason we have termed the response the "osmopressor response".

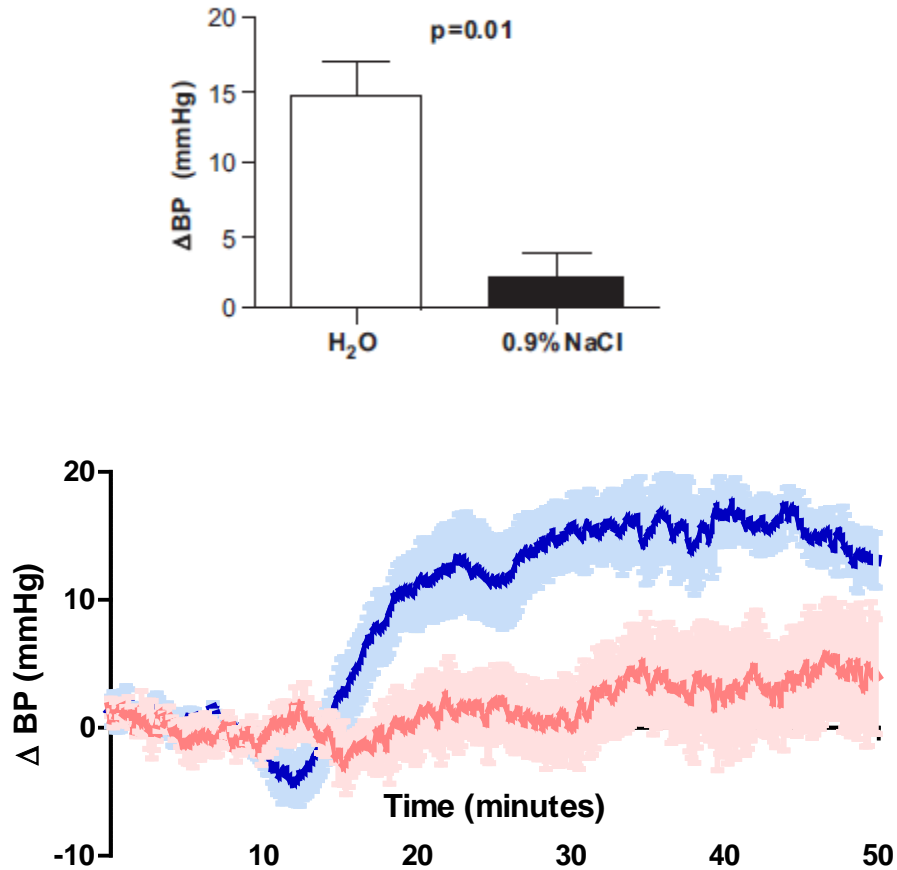


Figure 15: Effect of osmolality on BP. Change in BP after intraduodenal infusion of water (blue; n = 11) or saline (pink; n = 6; 25 μL/g). Attenuation of the pressor response during saline infusion implicates hypo-osmolality as the stimulus.

Portal Versus Systemic Osmolality After Water or Saline Infusion

As mentioned chapter 1, water absorption in the GI tract occurs through a 3-compartment mechanism. Water (or other hypotonic fluids) in the duodenum results in transport of NaCl from blood to lumen, followed by the absorption of water against its osmotic gradient in the lower small intestine. During this process, transient alterations in portal blood osmolality may be possible.

To determine the effect of intraduodenal water or saline infusion on osmolality in mice, both portal and systemic osmolality were measured 10 minutes after the infusion. Water infusion lowered portal osmolality relative to systemic osmolality (292.7 ± 4.7 and 304.4 ± 6.9 milliosmol/kg, respectively; $p = 0.01$), whereas saline infusion did not lower portal osmolality relative to systemic osmolality (307.1 ± 4.4 and 307.4 ± 1.7 milliosmol/kg, respectively; $p = 0.88$; Figure 16).

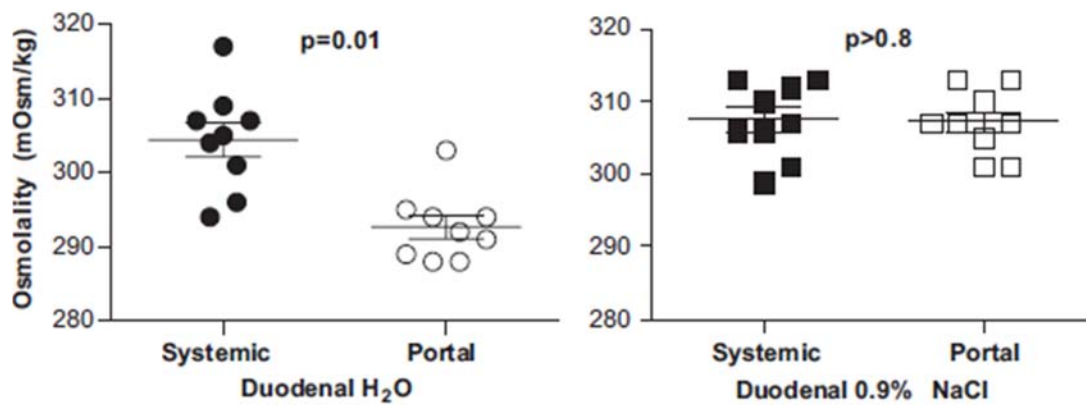


Figure 16: Systemic and portal osmolality in wild-type mice. Systemic (●, ■) and portal (○, □) osmolality 10 minutes after intraduodenal infusion of water (●, ○; n = 9) or saline (■, □; n = 9). The decrease in portal osmolality after water but not after saline infusion is consistent with portal/hepatic osmosensor involvement in the pressor response to water.

Role of Plasma Volume in Pressor Response

To address the possibility that water increases BP by increasing plasma volume, saline (150 μ L, ~10% of circulating blood volume) was infused intravenously in mice. Intravenous saline caused a small, transient spike in BP, with values returning to baseline within 2 minutes (Figure 17). This intravenous saline did not elicit the persistent pressor effect seen with water.

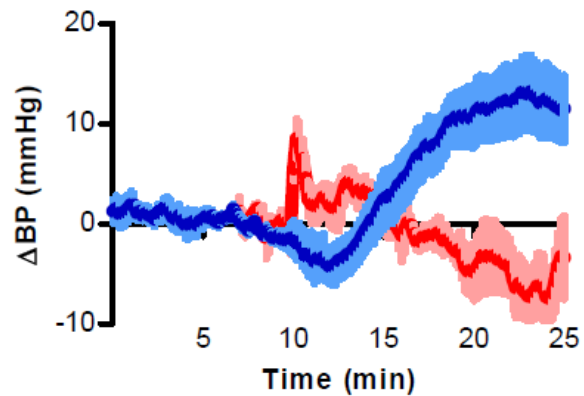


Figure 17: Role of plasma volume in pressor response. 150 μ L intravenous saline (*red*), given at 10 minutes elicited only a small, transient pressor response. Duodenal (*blue*) infusion of water is shown for comparison.

Discussion

The studies outlined in this chapter examine the location and stimulus responsible for eliciting water's cardiovascular effects. The baroreflex deafferented mouse model made the BP effects of water more easily observable without the buffering effects of an intact baroreflex system. Both gastric and duodenal infusion of water produced 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. Additionally, the stimulus eliciting the response is osmotic in nature, because only water, and not saline, produced a pressor response. Furthermore, the absence of a pressor response with saline infusion makes luminal stretch an unlikely explanation for the increase in BP, because both fluids would have induced equal duodenal stretch. However, luminal stretch may play a role during the period of infusion when the GI tract is experiencing the highest degree of stretch. The slight, transient dip in BP immediately after the start of infusion might be explained by the acute luminal stretch experienced during infusion. For these reasons, we have termed the pressor response to water the "osmopressor response".

Hydration status may also affect BP. However, we showed that acutely increasing plasma volume via saline infusion failed to elicit the pressor response seen with duodenal water administration in both magnitude and duration. Because intravenous administration would optimally increase plasma volume, these data effectively exclude an increase in plasma volume as the cause of the robust, sustained response observed after water ingestion.

All orally ingested solids and liquids pass through the intestines and are eventually absorbed into the vessels of the splanchnic mesentery that drain into the portal vein before entering the liver. After coursing through the liver, these newly absorbed solutes enter the systemic circulation. The first internal structures to come in contact with absorbed solutes from the GI tract are the splanchnic mesentery, portal vein, and liver. Therefore, these structures represent strategic sites for peripheral osmosensors which might allow for early detection of potential osmotic disturbances elicited by ingestion of food or fluids.

Our data show that water infusion lowered portal osmolality relative to systemic osmolality and produced a pressor response, whereas saline infusion did not alter portal:systemic osmolality or raise BP. These data suggest that water may be acting through stimulation of osmosensitive mechanisms in the portal circulation or liver.

Consistent with the notion of peripheral osmosensors in the portal/hepatic region, electrophysiological studies have shown that vagal afferent activity (specifically hepatic branches of the vagus nerve) can be altered by changing the osmolality of solutions perfusing the portal vein and liver (Adachi et al., 1976; Andrews and Orbach, 1974; Niiijima, 1969). Functionally, electrical responses from hepatic vagal fibers can be stimulated by exposing regions of the portal vein to water or hypertonic solutions (Adachi et al., 1976; Andrews and Orbach, 1974). Other studies show that activation of the hypothalamic hypophyseal system following stimulation of osmoreceptors in the splanchnic region can be

blocked by anesthetics injected into the thoracic spinal cord (Vallet and Baertschi, 1982).

Although lowered portal osmolality after water infusion is suggestive of portal osmosensitive mechanisms, it cannot be excluded that there may be pre-absorptive osmosensitive mechanisms at play. Such mechanisms have been previously suggested by studies showing osmosensitive regions of the proximal GI tract affecting gastric motility and pancreatic enzyme secretions (Dooley and Valenzuela, 1984; Garnier et al., 1986; Mei and Garnier, 1986).

CHAPTER III

NATURE OF EFFERENT LIMB

Sympathetic Efferents

The increase in BP and plasma NE after water ingestion, as well as the dependence of this increase on the autonomic nervous system (shown by attenuation of response with ganglionic blockade) suggest that sympathetic efferents may be involved. Since most clinically recognized sympathetic effects are mediated by NE, an antagonist of the vasoconstrictor effect of NE should provide insights about the efferent arm of the osmopressor response.

To test the hypothesis that sympathetic activity underlay the pressor effect of water, mice were given the α -1 adrenoreceptor antagonist prazosin before water infusion. Pretreatment with 0.5mg/kg prazosin reduced baseline BP by around 30-40 mmHg, and resulted in loss of the pressor response to water (3.8 ± 2.7 versus 14.9 ± 7.4 mm Hg; $P < 0.01$; Figure 18).

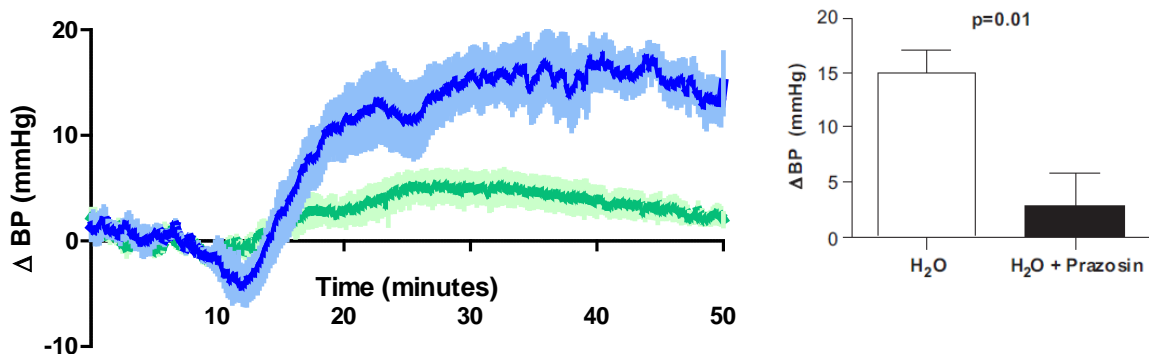


Figure 18: α 1-antagonism attenuates pressor response to water. Change in BP after duodenal infusion of water ($25 \mu\text{L/g}$) with (green; $n = 5$) or without (blue; $n = 11$) prior administration of prazosin (0.5 mg/kg, i.p.).

Dopamine β hydroxylase (DBH) is an enzyme required for conversion of dopamine to norepinephrine. The genetic deletion of this enzyme results in a complete absence of norepinephrine in the system, and essentially interrupts the efferent arm of the sympathetic nervous system in such a way that vasoconstriction in response to sympathetic nerve stimulation cannot be elicited. These qualities make DBH knockout mice a powerful model in which to examine the role of sympathetic efferents in the pressor effect of water. *Dbh* knockout mice with no detectable norepinephrine in blood, urine, or tissue did not produce an increase in BP in response to water infusion (1.3 ± 1.2 mm Hg; $P < 0.36$; Figure 19). The absence of a BP increase supports the hypothesis that noradrenergic signaling is crucial for the osmopressor effect.

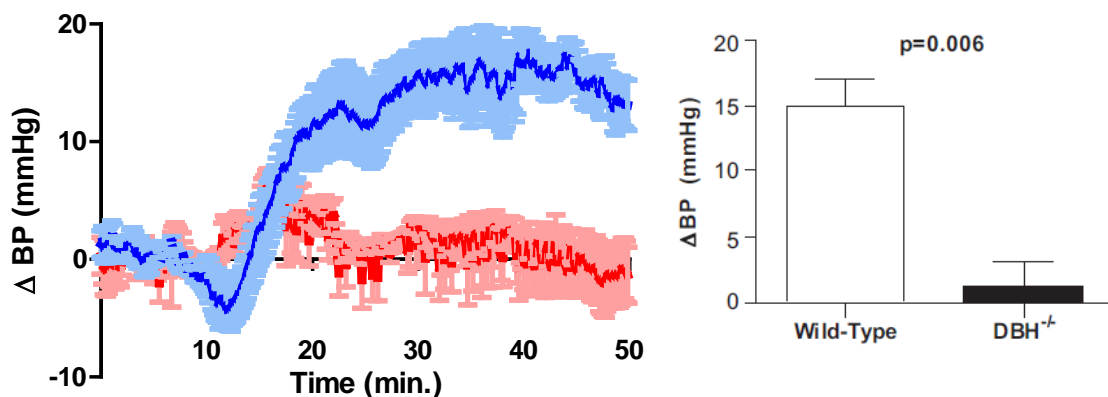


Figure 19: *Dbh*^{-/-} mice do not respond to duodenal infusion of water.

Change in BP after duodenal infusion of water (25 μ L/g) in *Dbh*^{-/-} (red; n = 5) and wild-type (blue; n = 11) mice.

Renal Mechanisms: Renin/Angiotensin

Increased sympathetic efferent activity can also lead to increased renin activity. Renin increases the levels of angiotensin I, which is converted to anangiotensin II by angiotensin converting enzyme (ACE). Angiotensin II increases BP through a variety of mechanisms, including arteriolar vasoconstriction and further increasing sympathetic drive. Captopril is a selective ACE inhibitor, and blocks the BP effects of angiotensin I. To test the role of the renin/angiotensin system on the pressor effect of water, captopril (1 mg/kg, iv.) was given prior to water infusion. Mice given captopril prior to water infusion responded with a similarly robust increase in BP as mice without captopril ($p = 0.09$ between groups; Figure 20).

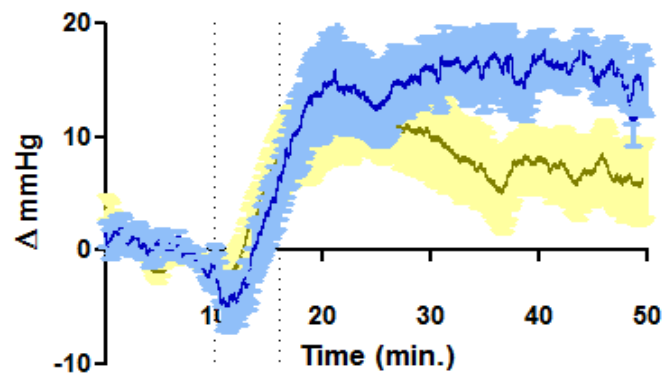


Figure 20: ACE inhibition does not block pressor response. Change in BP after duodenal infusion of water (25 μ L/g) with prior administration of captopril (yellow; $n = 10$) or water alone (blue; $n = 11$).

Discussion

Studies in humans provide indirect evidence that increased sympathetic nervous system activity underlies the pressor effect of water; plasma norepinephrine (Jordan et al., 2000a; Raj et al., 2006), muscle sympathetic nerve activity (Scott et al., 2001), and peripheral vascular resistance (Lu et al., 2003) all increase after water ingestion in human subjects. In the present study, blockade of α_1 -adrenoreceptors with prazosin attenuated the pressor response to water, implicating the sympathetic neurotransmitter norepinephrine in this response. Additional evidence strengthening the sympathetic nervous system hypothesis was obtained from *Dbh* gene knockout mice. Although *Dbh*^{-/-} mice display hypersensitivity to a variety of pressor and depressor stimuli, they were incapable of producing an increase in BP in response to water infusion. Taken together, these data show that an intact efferent sympathetic nervous system is required to elicit the pressor response of water and, specifically, that sympathetic adrenergic mechanisms are critical to this response.

Given the time frame of the response, renal mechanisms could play a role in eliciting the pressor response. The renin/angiotensin system is partly modulated by the sympathetic system and can become activated during periods of increased sympathetic activity. Although the duration of the pressor response tended to be shorter in mice given captopril (which may indicate the presence of a second, renin/angiotensin-dependent phase to water's effects), captopril did not alter the magnitude of the pressor effect. Furthermore, studies in patients show that plasma renin activity remains unchanged during the osmopressor

response (Jordan et al., 2000a). Together, these data suggest that renal mechanisms, specifically renin/angiotensin effects, are not critical for the BP increase after water ingestion.

CHAPTER IV

NATURE OF AFFERENT LIMB

Vagal involvement in Osmopressor Response

Vagal afferents and splanchnic afferents are the two major pathways that convey information about the GI environment to the CNS. To investigate vagal afferent involvement in the response to water, surgically baroreflex-impaired mice underwent bilateral subdiaphragmatic vagotomy before water infusion. The vagotomized mice responded to water infusion with a similar increase in BP as intact animals (10.5 ± 4.8 versus 14.9 ± 7.4 mm Hg; $P = 0.2$; Figure 21).

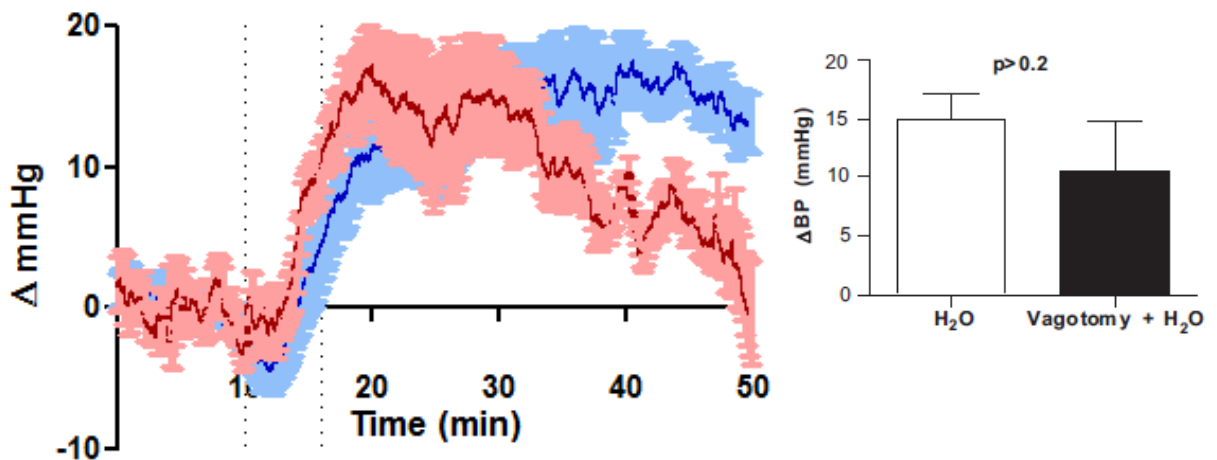


Figure 21: Vagal afferents are not essential for water's pressor effect.

Change in BP after duodenal infusion of water ($25 \mu\text{L/g}$) in vagotomized (red; $n = 6$) and intact (blue; $n = 11$) mice.

Molecular Mediators

In our search for potential mediators of the osmopressor response, we were particularly interested in neuropeptides and osmosensors present in sensory neurons serving the GI region that could also alter sympathetic drive and BP. Three such candidates were studied: Substance P, TRPV1, and TRPV4.

Substance P

Substance P is a neurotransmitter belonging to the tachykinin family. It is present in highest concentrations in the gastrointestinal tract and in the central nervous system (Hokfelt et al., 2001). In the CNS, substance P acts as a neurotransmitter with varied functions, many of which are not completely understood. Effects as diverse as nausea, emesis (Dando and Perry, 2004), pain (Boscan et al., 2002), natriuresis, respiration, and regulation of sympathetic reflexes and BP have been attributed to substance P (Boscan et al., 2002; Brattstrom and Seidenbecher, 1992; Makeham et al., 2005; Pan et al., 1995; Williams et al., 2003). Many of the effects of substance P are mediated by the neurokinin 1 (NK₁) receptor, which is the predominant tachykinin receptor in humans. Most importantly NK₁ mechanisms may be operative in afferent traffic from the gastrointestinal tract (Wu et al., 2005). Animal studies demonstrate that substance P plays a critical role in mediating baroreflexes in the nucleus tractus solitarii and associated structures (Riley et al., 2002).

Tac1 knockout mice were used to investigate the potential role of substance P in the osmopressor response. *Tac1*^{-/-} mice do not have any detectable levels of its protein products substance P or neurokinin A. These mice

have reduced nociceptive pain responses, but do not display any gross physical or behavioral abnormalities (Cao et al., 1998; Mazario and Basbaum, 2007).

Tac1^{-/-} mice responded to intraduodenal infusion of water with a similar increase in BP as wild-type animals ($p = 0.6$ between groups; Figure 22).

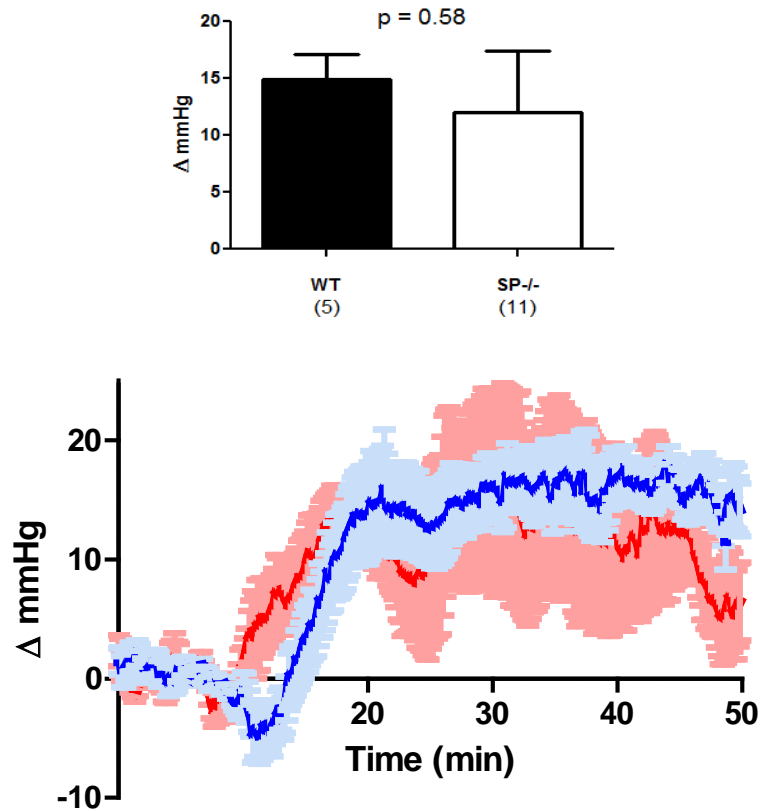


Figure 22: Absence of substance P does not alter pressor response to water. Change in BP after duodenal infusion of water (25 μ L/g) in *Tac1*^{-/-} mice (red; n = 5) vs wild-type (blue; n = 11).

TRPV1

Since TRPV1 has been implicated in CNS vasopressin control, it might have other roles relevant to osmolality, including serving as a sensor for the osmopressor response. Additionally, many sensory afferent neurons from the GI tract exhibit capsaicin sensitivity characteristic of the TRPV1 channel.

TRPV1^{-/-} mice were used to investigate the potential role of TRPV1 in the osmopressor response. Duodenal infusion of water in *Trpv1*^{-/-} mice produced a similar response as observed in wild-types (Figure 23).

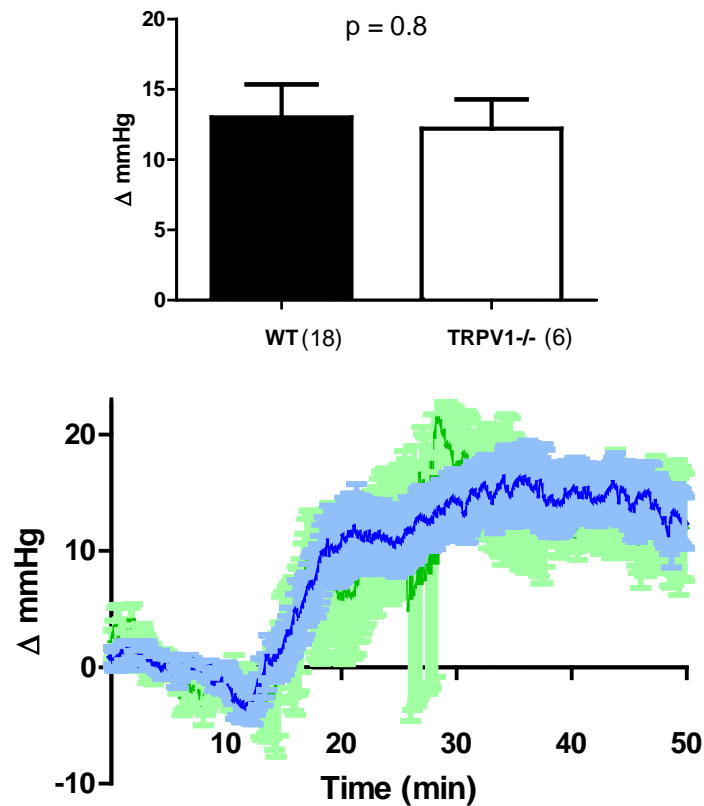


Figure 23: Absence of TRPV1 does not alter pressor response to water. Change in BP after duodenal infusion of water (25 μ L/g) in *TRPV1*^{-/-} mice (green; n = 6) vs wild-type (blue; n = 18).

TRPV4

Given its ability to respond to a diverse range of stimuli and elicit multiple functions, including effects on systemic osmolality and BP, and its expression in the GI and portal regions, TRPV4 is a strong candidate as a potential mediator of the pressor response to water.

Trpv4^{-/-} mice were used to test the importance of this channel in mediating the osmopressor response to water. When water was infused into the duodenum of these knockout mice, no pressor response was observed (1.6 ± 4.3 mm Hg, $p = 0.3$; Figure 24).

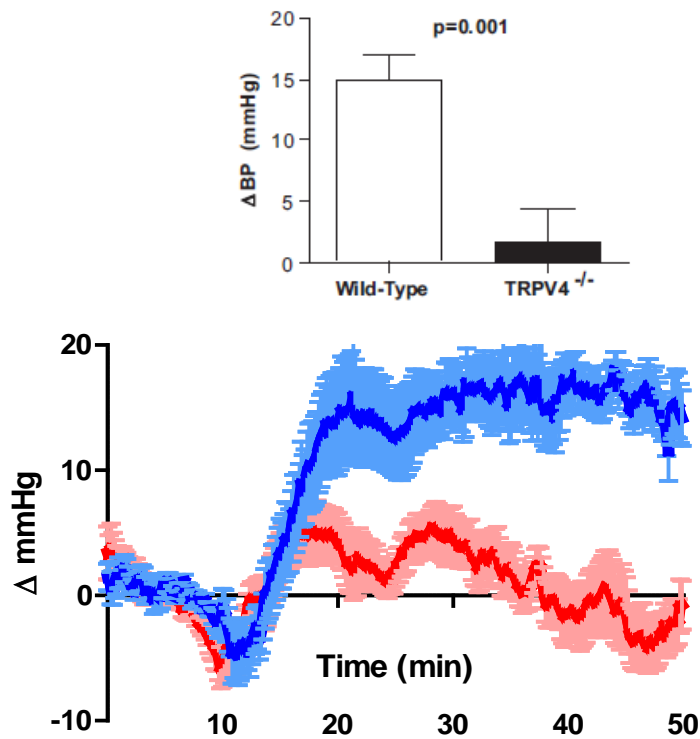


Figure 24: *Trpv4*^{-/-} mice do not respond to duodenal infusion of water. Change in BP after duodenal infusion of water (25 μ L/g) in *Trpv4*^{-/-} mice (red; n = 11) vs wild-type (blue; n = 11).

Results from studies from *Dbh*^{-/-} mice show that sympathetic efferents must be intact to produce the increase in BP observed after water ingestion. To verify that the absence of the pressor response in *Trpv4*^{-/-} animals was not because of a general abnormality in neural pathways or efferent sympathetic nerve function, the cardiovascular parameters of these mice tested with a non-osmotic stimulus known to increase sympathetic output. Mice were placed under restraint, which increases sympathetic output from the central nervous system. Both wild-type and *Trpv4*^{-/-} mice showed similar BP and HR changes when placed under restraint, whereas *Dbh*^{-/-} mice (absent sympathetic adrenergic tone) under restraint did not show increases in BP or HR (Figure 25).

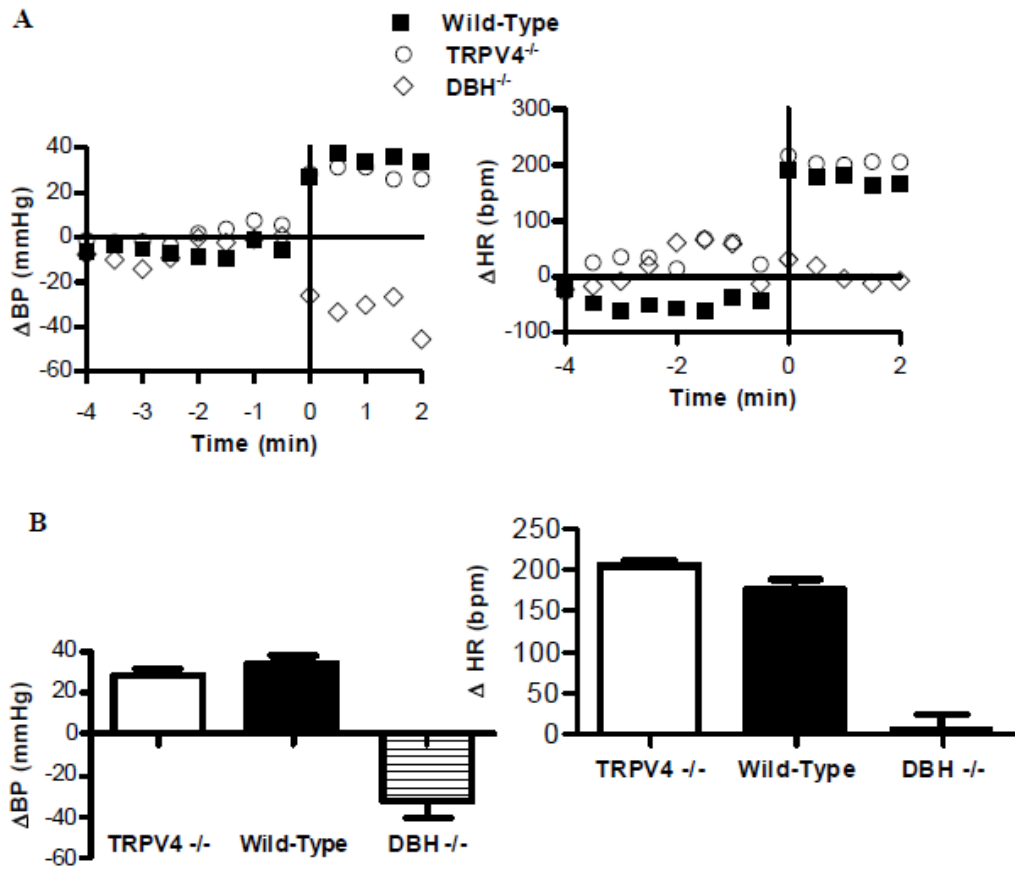


Figure 25: *Trpv4*^{-/-} have intact sympathetic efferents. A, Representative tracings of the change in BP and HR during restraint for wild-type (■), *Trpv4*^{-/-} (○), and *Dbh*^{-/-} (◇) mice. B, Average change in BP and HR during 2-minute restraint.

Portal vs. Systemic Osmolality in *Trpv4*^{-/-} after Water Infusion

Portal and systemic osmolalities were measured 10 minutes after duodenal infusion of water in *Trpv4*^{-/-} animals. Portal and systemic osmolalities in these mice were found to be 301.0 ± 6.0 and 310.2 ± 6.1 milliosmol/kg, respectively ($p = 0.003$; Figure 26).

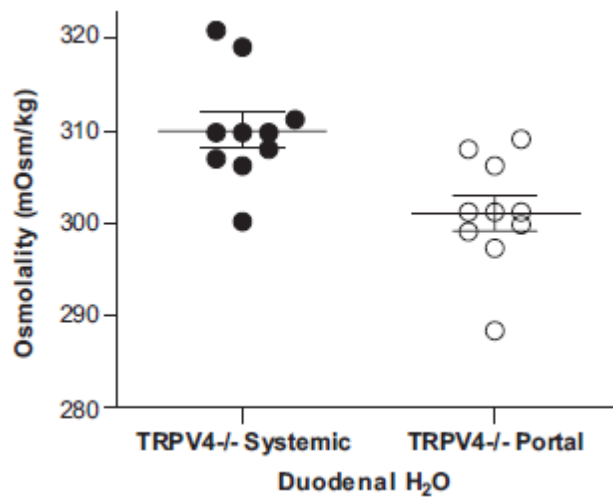


Figure 26: Portal and systemic osmolality in *Trpv4*^{-/-} mice after water infusion. Systemic (●) and portal (○) osmolality 10 minutes after duodenal infusion of water (25 μ L/g) in *Trpv4*^{-/-} mice ($n = 10$).

Direct Portal Infusion of Water

Although duodenal infusion of water produced a decrease in portal osmolality relative to systemic osmolality and elicited a pressor response like that seen after water ingestion in patients, the location where the hypo-osmotic stimulus is being sensed is still unclear. TRPV4 is present throughout the GI tract, endothelium of mesenteric vessels, as well as afferents serving portal/hepatic regions, and may be mediating the response at any or all of these locations. Additionally, there may be overlapping mechanisms and other osmo-sensitive pressor responses independent of TRPV4.

To test these possibilities, water or saline was directly infused into the portal vein through the superior mesenteric vein. Hemolysis of red blood cells normally begins at 0.42% NaCl (Kumar, 2002). By infusing water at 10% of the flow rate of the portal vein (thereby reducing osmolality by around 10%), significant hemolysis should be avoided. Water or saline was infused at 0.1 mL/min over 6 minutes. Infusion of water into the portal vein produced an immediate pressor response that tended to be slightly higher in magnitude than duodenal infusion (20.0 ± 11.9 mmHg vs 14.9 ± 7.4 mmHg, $p > 0.3$; Figure 27). The same volume and rate of saline infusion into the portal vein produced almost no pressor response (-0.6 ± 6.7 mmHg, $p = 0.8$; Figure 28). *Trpv4*^{-/-} responded to portal infusion of water with a similar increase in BP as wild-types (16.8 ± 8.9 mmHg vs. 20.0 ± 11.9 mmHg, $p > 0.6$; Figure 29).

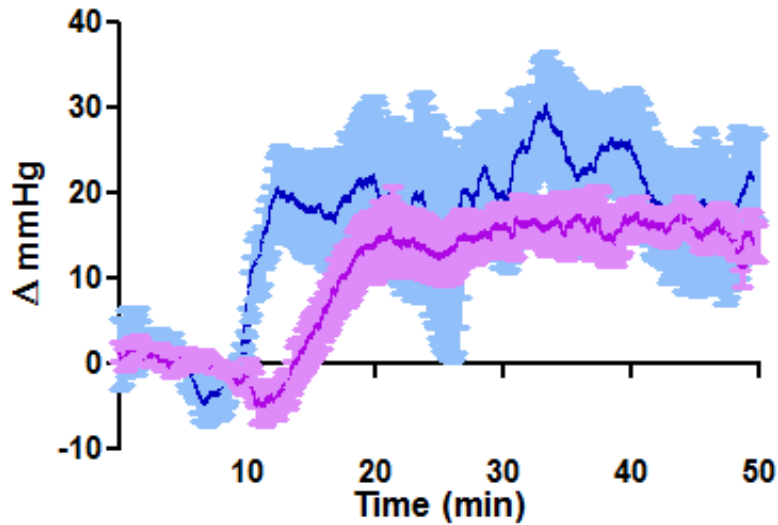


Figure 27: Direct portal vein infusion of water in wild-type mice elicits robust pressor response. Change in BP after duodenal (purple; n = 11) or portal (blue; n = 4) infusion of water at 10% hepatic venous flow (0.6 ml/ 6 min).

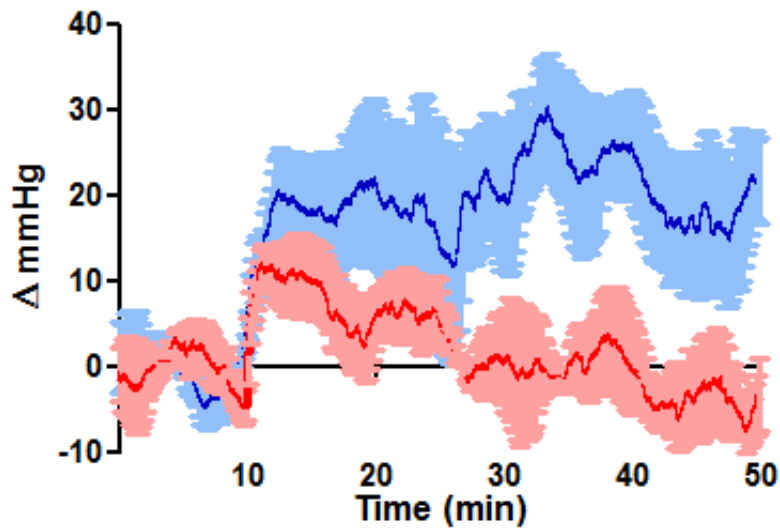


Figure 28: Direct portal vein infusion of saline in wild-type mice fails to elicit robust BP increase. Change in BP after portal infusion of water (blue; n = 4) or saline (red; n = 5).

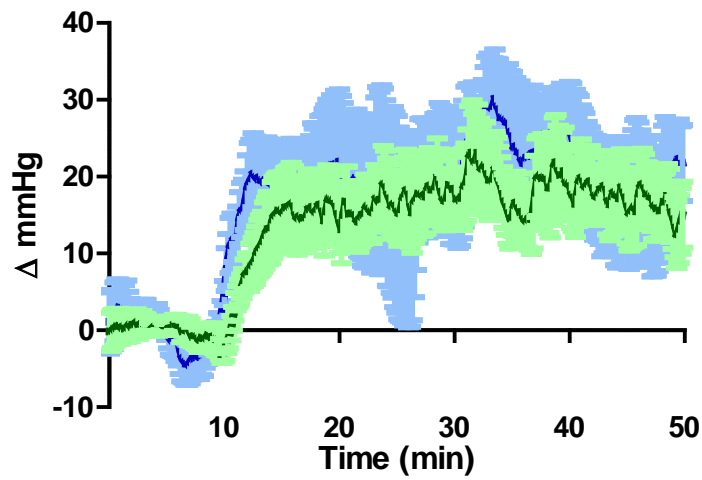


Figure 29: Direct portal vein infusion of water elicits pressor response in *Trpv4*^{-/-}. Change in BP in wild-types (blue; n = 4) and *Trpv4*^{-/-} (green; n = 6) after portal infusion of water at 10% hepatic venous flow (0.6 ml/ 6 min).

Discussion

The neural pathways that form the afferent loop of the osmopressor response might include brain stem centers, which receive input from both vagal and ascending spinal cord afferent nerves serving the gut, or could be limited to spinal afferent nerves, which can directly influence sympathetic output at the level of the spinal cord (Grundy, 2002). Although the duration of the pressor effect in subdiaphragmatically vagotomized mice tended to be slightly shorter than in intact animals, the presence of an intact response indicates that the vagus nerve is not essential for the osmopressor response. Interestingly, patients with near-complete cervical spinal cord transection also have an intact pressor response to water ingestion (Tank et al., 2003). Both of these findings suggest that the pressor response is most likely acting through a spinal reflex.

Several potential mediators of the osmopressor response were investigated in the studies outlined in this chapter. Substance P, a neurotransmitter with vasoactive properties, is responsible for modulating several autonomic reflexes including those that affect BP and HR (Cowan et al., 2000; Haeusler and Osterwalder, 1980). The localization of both substance P and its receptor NK₁ in the enteric region and CNS also made it an attractive candidate mediator. However, the presence of a pressor response in *Tac1*^{-/-} mice after water infusion suggests that substance P does not play an essential role in the osmopressor response.

TRPV1, an osmosensitive ion channel abundant in sensory afferents serving the GI tract was also studied. Deletion of TRPV1 channels in mice did not

alter their response to water infusion. Thus, the osmopressor response appears to be independent of TRPV1 mechanisms.

The location of action and osmotic nature of the stimulus eliciting the pressor response made TRPV4 one of the top candidates as a potential mediator. Its ability to react to small, physiologic changes in osmolality coupled with its expression in areas optimal for sensing these changes after water ingestion (mesenteric vessels, sensory afferents serving the GI region), made TRPV4 a promising candidate. *Trpv4*^{-/-} mice have intact sympathetic efferents and can increase BP appropriately in response to non-osmotic stimuli, such as restraint stress. The lack of a pressor response in these animals despite intact sympathetic efferents suggests that the impairment is in the afferent loop.

Direct portal infusion of water at 10% portal venous flow rate should lower portal osmolality by roughly 10%. This abrupt decrease in portal osmolality caused an immediate, robust increase in BP that tended to be greater in magnitude than after duodenal infusion. Portal infusion of the same volume of saline did not elicit this response. This suggests that there are osmosensitive pressor mechanisms present in the portal vein or liver. However, the presence of a response in *Trpv4*^{-/-} mice suggests there may be other osmosensitive mechanisms acting independent of TRPV4. It may be that abrupt change in portal osmolality, as opposed to slower and smaller changes after duodenal infusion of water, activates a second/overlapping osmosensitive response that operates independently of TRPV4. Perhaps TRPV4 is most sensitive to small, physiologic changes in osmolality and responses to larger perturbations of portal

osmolality occur through an alternate mechanism. Alternatively, TRPV4 may be sensing osmolality changes in the mesenteric venules and not the portal vein or liver. Therefore, bypassing the mesenteric venules and directly infusing into the portal vein would also bypass TRPV4-dependent osmopressor mechanisms. Lastly, we cannot exclude the possibility that the lack of a response to duodenal water in *Trpv4*^{-/-} animals might be due to their slightly higher plasma osmolality at baseline, and perhaps the existence of a threshold osmolality that must be reached for the response to be elicited. Since *Trpv4*^{-/-} animals are starting off with higher osmolalities (Liedtke and Friedman, 2003), the same magnitude decrease in portal osmolality after duodenal water infusion may not be enough to lower osmolality below a threshold needed to elicit the response. However, it should be noted that there are several overlapping portal osmolality values between *Trpv4*^{-/-} and wild-type mice after water infusion (Figures 16 and 26). And although some of the *Trpv4*^{-/-} mice achieved the same absolute osmolality as wild-types, no pressor response was observed in any *Trpv4*^{-/-} mice.

Overall, these data suggest that there are both TRPV4-dependent and TRPV4-independent osmopressor mechanisms present in the portal region. It is possible that TRPV4-dependent mechanisms are sensitive to physiologic perturbations in osmolality, whereas TRPV4-independent mechanisms respond to more dramatic disturbances in portal osmolality.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Studies in patients with impaired baroreflexes lead to the discovery of significant cardiovascular effects of water ingestion that were previously missed. In these patients, water ingestion elicits robust, sustained increases in BP. This response acts through an autonomic nervous system mechanism, as ganglionic blockade with trimethaphan has been shown to attenuate the response (Jordan et al., 2000a). Although healthy individuals with normal baroreflexes do not show large fluctuations in BP, water ingestion nonetheless causes an increase in plasma NE as well as peripheral vascular resistance (Lu et al., 2003; Raj et al., 2006).

Hypotonicity, and not luminal stretch, was found to be the stimulus eliciting water's effects, as the same volume of intraduodenal saline failed to produce the same BP increase observed with water. Additionally, plasma volume expansion was shown not to play a major role in the BP increase after water consumption. Studies in patients also show that the oral route is important for eliciting water's effects. Increases in BP were much greater after oral ingestion than after an identical volume of iso-osmotic solution (5% dextrose in water) infused intravenously (Jordan et al., 2000a). Lastly, the fact that water infusion into the duodenum produced a decrease in portal osmolality relative to systemic

osmolality and a robust pressor response, whereas the same volume of saline did not, suggests that water is acting through an osmosensitive mechanism in the portal region.

There is strong evidence that the sympathetic nervous system is crucial in facilitating the efferent arm of the osmopressor response. In addition to human data showing increased plasma NE and peripheral vascular resistance after water ingestion, data from mouse studies also strongly support the sympathetic hypothesis. Blockade of α 1-adrenergic receptors with prazosin attenuates the pressor response, and mice lacking NE (*Dbh*^{-/-}) cannot produce the response. Therefore, the cardiovascular effects of water ingestion only occur if sympathetic efferents are intact.

Afferent mechanisms responsible for sensing osmotic changes after water ingestion appear to travel via spinal nerves as part of a spinal reflex mechanism. Both bilateral subdiaphragmatically vagotomized mice as well as patients with high cervical spinal cord injuries have an intact pressor response to water.

The search for potential molecular mediators lead to the examination of three particularly promising peptides/channels: substance P, TRPV1, and TRPV4. Neither substance P nor TRPV1 appear to play an essential role in mediating the osmopressor response, as both *Tac1*^{-/-} and *Trpv1*^{-/-} mice displayed a typical pressor response after water infusion. *Trpv4*^{-/-} mice, however, did not show a pressor response to water infusion despite having intact sympathetic efferents, suggesting a defect in the afferent loop. However, the exact location of the defect remains unknown. Direct infusion of water into the portal vein of *Trpv4*

^{-/-} mice elicited a robust response similar to wild type animals, and provides evidence of additional osmosensitive pressor mechanisms in the portal system independent of TRPV4. Additionally, the presence of a response after infusion of water into the portal vein could also suggest that TRPV4 might be sensing osmolality changes at the earliest junctions between GI lumen and portal circulation: mesenteric venules. Future studies will be needed to determine more precisely where the afferent defect is located.

Although only newly recognized, the significance of the osmopressor response may be substantial. Ingestion of water is proving to be therapeutic in the relief of episodes of debilitating hypotension or inadequate sympathetic nervous activation. Interestingly, a recent American Red Cross study documented that water ingestion can prevent reactions associated with blood donations (Newman et al., 2007).

The osmopressor response and *Trpv4* are new factors now implicated in the physiology of BP regulation, particularly in hypotension and fainting. They might also represent targets for future drug development to address these problems.

Future Directions

The recent link between TRPV4 and salt-sensitive hypertension (Gao et al., 2009; Gao and Wang, 2010) merits further investigation. Gao et al. showed in a series of experiments that rats fed a high-salt diet displayed greater sensitivity to TRPV4 agonism than those on normal diets. Additionally, salt-resistant Dahl rats fed high-salt diets had increased TRPV4 expression, and did not develop hypertension. In contrast salt-sensitive Dahl rats on high-salt diets did not increase TRPV4 expression, but developed hypertension. These studies suggest that TRPV4 may play a role in preventing salt-induced increases in BP when on high-salt diets for an extended period of time. Our model addresses the effects of short-term osmolality changes and TRPV4's role in mediating the effects of these changes. We do not yet know what the long-term consequences of high salt diet are on portal osmolality nor TRPV4's role in these situations. It would be interesting to see if portal osmolalities are different between animals placed on high-salt vs. normal diets and if so, how this might affect the osmopressor response to water.

Additionally, a dose-response experiment would help further our understanding of the osmosensitive processes in the portal/hepatic region. By differing the rate and volume of hypo- or hypertonic infusion into the portal vein, a dose-response can be generated between portal osmolality and BP. This type of study would allow more insight regarding the osmolality range that TRPV4 is most sensitive in detecting, as well as a better understanding of other osmosensitive mechanisms.

Lastly, calcitonin gene related peptide (CGRP) is another potential mediator of the osmopressor response that is worth investigating. It is thought to be an important mediator of many effects of TRPV1 and TRPV4, including cardioprotective and gastroprotective properties associated with these channels, and is found in a large proportion of spinal afferent neurons (Green and Dockray, 1987). *Cgrp*^{-/-} mice have increased BP compared to wild-type counterparts, and are particularly sensitive to mechanisms that challenge BP homeostasis (Gangula et al., 2000). Studying the osmopressor response in *Cgrp*^{-/-} mice would provide more insight to the molecular mechanisms eliciting this response.

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