Spinal Regulation of Sympathetic Nerve Activity and Arterial Pressure Under Conditions of Increased Plasma Osmolality

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Dr. John Osborn, Advisor

September 2012

Acknowledgements

I thank Dr. John Osborn for his excellent mentorship, patience, encouragement, and advice. His support throughout my graduate career has been invaluable. I also thank the members of my thesis committee (Dr. William Engeland, Dr. Scott O'Grady, Dr. Martin Wessendorf, and Dr. Chris Honda) for their valuable feedback and suggestions.

In the years of work that went into this thesis, I also received continual support from the following individuals: Pilar Ariza Guzman, Marcos Kuroki, Joanna Abrams, Jason Foss, and other past and present members of the Osborn laboratory; Dr. Gregory Fink; my fellow students and faculty of the GPN - particularly Nancy Day, Stacy Decker, Dr. George Wilcox, Dr. Virginia Seybold, and Dr. Janet Dubinsky. Finally, this work would not have been possible without the unending support and encouragement of my friends and family, particularly Scott Veitenheimer, Ellie Tome, and Christopher Rupp.

Dedication

To my daughter, Malena.

"May you build a ladder to the stars
and climb on every rung"
-Bob Dylan

Abstract

Hypertension, or chronic high blood pressure, is a major health concern worldwide. Increased dietary salt may contribute to high blood pressure through the activation of the sympathetic nervous system. Indeed, more than half of the cases of human hypertension show evidence of elevated sympathetic nerve activity. Key brain regions that activate sympathetic activity in response to increased osmolality have been identified, but regulation at the level of the spinal cord - the final point of sympathetic outflow from the central nervous system - is much less understood. The experiments in this thesis were designed to examine the spinal neurotransmitters and sympathetic nerves that may increase arterial pressure in response to elevated plasma osmolality. The first major finding of this thesis suggests that the arterial pressure response to increased osmolality relies on spinal glutamate, not vasopressin as previously hypothesized. The second major finding suggests that elevated arterial pressure during water deprivation - a condition of chronic hyperosmolality - is sympathetically mediated and may be dependent on the adrenal cortex, but is *not* due to increased sympathetic activity to a specific vascular bed. The discoveries described in this thesis contribute to the overall knowledge of spinal regulation of osmotically driven sympathoexcitation and suggest new areas of focus for future studies. Moreover, these findings may lead to a better understanding of the etiology of salt-sensitive hypertension and development of novel antihypertensive therapies.

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List of Abbreviations

ACh	acetylcholine
aCSF	artificial cerebrospinal fluid
AngII	angiotensin II
AVP	vasopressin
bpm	beats per minute
CNS	central nervous system
CVO	circumventricular organ
DOCA	deoxycorticosterone acetate
Glut	glutamate
HR	heart rate
HS	hypertonic saline
IML	intermediolateral cell column
IT	intrathecal
IV	intravenous
MAP	mean arterial pressure
mmHg	millimeters of mercury
NE	norepinephrine
PVN	paraventricular nucleus
RVLM	rostral ventrolateral medulla
SNA	sympathetic nerve activity
SNS	sympathetic nervous system
SPN	sympathetic preganglionic neuron
WD	water deprivation

Contributions

The following contributions were made by individuals other than Britta Veitenheimer Rupp or John Osborn:

Chapter 4: Drs. William Engeland and Gregory Fink both contributed valuable suggestions and feedback to this study. Additionally, Dr. Engeland contributed resources towards the plasma corticosterone/ACTH assay (Figure 6), which was performed by Marina Yoder in his laboratory. Likewise, Dr. Fink provided the plasma norepinephrine analysis (Table 3), which was measured at Michigan State University. Finally, Pilar Ariza Guzman performed the surgeries and ran most of the trials in Experiment #6 (Figure 7).

Chapter 1

Introduction

Central Questions

The overall goal of this thesis was to expand the current knowledge about osmotic regulation of sympathetic nerve activity and arterial pressure *at the level of the spinal cord* in *conscious* rats. Specifically, we aimed to 1) identify the spinal neurotransmitter(s) required for the pressor response to increased plasma osmolality and to 2) investigate the role of global and regional sympathetic nerve activity in elevating arterial pressure during one condition of chronic osmotic stress, water deprivation.

Background and Significance

Primary Hypertension and Salt-Sensitivity

Hypertension, or chronic high blood pressure, is a major health concern in developed countries around the world. It is known as the "silent killer" due to its lack of symptoms and its link to a variety of high-mortality conditions such as atherosclerosis, heart attack, heart disease, stroke, aneurism, and kidney disease (Lopez, Mathers et al. 2006). In fact, the World Health Organization predicts hypertension to be the greatest risk factor for death and disability worldwide by 2020 (Murray and Lopez 1996). Currently, it affects approximately 30% of adults worldwide and is responsible for 7 million premature deaths each year (WHO 2002). Despite the major health concerns associated with hypertension, the Center for Disease Control recently reported that only 46% of hypertensive patients are effectively treating their disease (CDC 2011).

Ninety percent of hypertensive patients have primary (or essential) hypertension, which means the cause is unknown (Oparil, Zaman et al. 2003). Genetics undoubtedly contribute to the development of hypertension, but environmental factors like exercise, lifestyle, and diet also play an important role in blood pressure control. In particular, there is overwhelming evidence that high salt consumption contributes to elevated blood pressure (He and MacGregor 2009; He and MacGregor 2010). Unfortunately, in most countries in the modern world, humans eat an excessive amount of salt - approximately 40 times more than what our evolutionary ancestors consumed and 4 times the daily recommended amount (He and MacGregor 2009). In fact, studies suggest that a modest

reduction in population-wide sodium intake would save thousands of lives and billions of dollars in medical costs (Bibbins-Domingo, Chertow et al.).

Although it is clear that dietary salt can affect blood pressure, there is great variability in the blood pressure response to salt intake among individuals. Salt-sensitivity is loosely defined as a greater-than-normal blood pressure response to fluctuations in salt intake. Weinberger et al. found that 51% of hypertensive patients are salt-sensitive compared to only 26% of normotensive individuals (Weinberger 1996); additionally, salt-sensitivity may indicate a high risk for developing hypertension and may be a better indicator of future morbidity than blood pressure itself (Weinberger, Fineberg et al. 2001). Clearly, understanding how dietary salt intake affects blood pressure is crucial to developing effective anti-hypertensive therapies for salt-sensitive populations.

The etiology of hypertension is complex but likely involves some combination of influences from the kidneys, blood vessels, and nervous system. For example, a shift in the pressure-natriuresis relationship, which determines the amount of sodium excreted by the kidneys at a given pressure, could affect blood pressure through changes in blood volume (Guyton 1991). Additionally, changes in blood vessels in response to chronic high pressure and aging can affect vascular resistance and thereby perpetuate elevations in blood pressure. Finally, the nervous system can affect blood pressure through changes in sympathetic regulation of cardiac output, renal function, and peripheral vasoconstriction. The remainder of this thesis focuses on the sympathetic nervous system component of blood pressure regulation.

Sympathetic Nervous System in Hypertension

In recent decades, accumulating evidence has led to the theory that the sympathetic nervous system contributes to the development and maintenance of many cases of hypertension (Guyenet 2006). Evidence in support of this "neurogenic hypertension" hypothesis includes both animal and human data, and it clearly indicates that sympathetic activation occurs during hypertension. Nevertheless, the ongoing debate

continues about the extent to which sympathetic activation *causes* hypertension (Esler, Lambert et al. 2010).

Plenty of data from animal studies demonstrates that sympathetic nerve activity affects arterial pressure. For example, in the spontaneously hypertensive rat, which is considered to be a comparable model to primary human hypertension, sympathetic responses are exaggerated (Judy, Watanabe et al. 1979), and sympathectomy or renal denervation attenuates the hypertension (Winternitz, Katholi et al. 1980). Additionally, selective sympathetic denervations (Jacob, Clark et al. 2005; King, Osborn et al. 2007); and blockade of epithelial sodium channels (Abrams, Engeland et al. 2010), angiotensin II receptors (Ito, Hiratsuka et al. 2003), or excitatory amino acid receptors (Ito, Komatsu et al. 2001) *in the brain* decreases arterial pressure in salt-sensitive rat models of hypertension.

Evidence is equally convincing in humans that hypertension is associated with elevated sympathetic nerve activity: 1) Plasma norepinephrine, albeit a rough indicator of sympathetic activation, has been found to be 25-30% higher in hypertensive patients (Grassi, Seravalle et al. 2010). 2) Regional norepinephrine spillover studies have suggested that hypertensive patients have increased SNA to the heart and kidneys (Esler, Jennings et al. 1986). 3) Microneurography recordings of muscle sympathetic nerve activity have demonstrated increased SNA to skeletal muscle in most forms of human hypertension (Schlaich, Lambert et al. 2004; Wallin and Charkoudian 2007). Overall, it is estimated that at least 50% of hypertensive individuals have hypertension that is associated with sympathetic excitation (Esler 2010).

Antihypertensive therapies that target the sympathetic nervous system provide additional support for the idea of neurogenic hypertension and suggest a causal link between sympathetic activation and hypertension. For example, centrally acting sympatholytic drugs, like clonidine, are well-known to lower blood pressure by inhibiting sympathetic outflow (Vongpatanasin, Kario et al. 2011). Additionally, a recently developed treatment, chronic stimulation of the carotid baroreceptors, has been found to attenuate hypertension (Wustmann, Kucera et al. 2009) by modulating sympathetic outflow. Finally, another promising antihypertensive therapy developed recently is a

renal denervation procedure using radiofrequency ablation, which chronically lowers blood pressure in humans by destroying the efferent and/or afferent renal sympathetic nerves (Schlaich, Sobotka et al. 2009; Schlaich, Sobotka et al. 2009).

Osmotic Regulation of Sympathetic Nerve Activity

Sympathetic activation may be responsible for the effects of salt on arterial pressure and may be important in the etiology of salt-sensitive hypertension and other cardiovascular diseases (Brooks, Haywood et al. 2005; Toney and Stocker 2010). Indeed, elevated plasma norepinephrine levels in salt-sensitive hypertensive patients compared to salt-resistant patients suggest sympathetic activation (Campese, Romoff et al. 1982), and evidence suggests that salt-sensitive rat models of hypertension, such as the Angiotensin II - salt hypertensive rat (King, Novotny et al. 2008), the deoxycorticosterone acetate (DOCA)-salt rat (Jacob, Clark et al. 2005), or the Dahl salt-sensitive rat (Ito, Hiratsuka et al. 2003) are dependent on elevated sympathetic nerve activity.

Sympathetic activity may be affected by changes in plasma osmolality (Stocker, Osborn et al. 2008), which is defined as the total amount of solutes dissolved in the body fluids. Since NaCl (salt) makes up more than 80% of the total body fluid solutes (Rhoades and Tanner 2003), plasma NaCl concentration is the primary determinant of plasma osmolality. Indeed, increased dietary salt leads to elevations in plasma sodium/osmolality in rats (Habecker, Grygielko et al. 2003) and humans (He, Markandu et al. 2005) of approximately 1-4%; importantly, this is above the thirst-triggering threshold, suggesting that it is a sufficient amount to activate omosensitive neurons (Fitzsimons 1998).

Many studies indicate that elevations in plasma osmolality contribute to sympathetic activation, both acutely and chronically (Stocker, Osborn et al. 2008). Acutely, central osmotic activation increases SNA, for example, to the renal (Chen and Toney 2001; Shi, Stocker et al. 2007; Shi, Martinez et al. 2008) or lumbar (Weiss, Claassen et al. 1996; Brooks, Freeman et al. 2004; Antunes, Yao et al. 2006) vascular beds in animals and to skeletal muscle in humans (Farquhar, Wenner et al. 2006). Chronic osmotic stimulation, such as water deprivation (Kiss, Jezova et al. 1994; Scrogin,

Grygielko et al. 1999; Stocker, Keith et al. 2004) and DOCA-salt hypertension (O'Donaughy and Brooks 2006), also appears to increase sympathetic outflow. Importantly, these sympathetic and arterial pressure responses are due to increased osmolality acting in the *brain*, which was demonstrated by Brooks et al. by delivering intracarotid hypotonic saline to reduce SNA and arterial pressure during water deprivation (Brooks, Qi et al. 2005) and DOCA-salt hypertension (O'Donaughy, Qi et al. 2006). Additionally, it has been proposed that even very small changes in osmolality may influence sympathetic activity when facilitated by inappropriately high plasma angiotensin II or aldosterone, for example (Brooks, Haywood et al. 2005).

A growing body of research has focused on identifying the brain regions and mechanisms involved in sympathetic activation in response to increased salt intake (reviewed in (Toney, Chen et al. 2003; Brooks, Haywood et al. 2005; Stocker, Osborn et al. 2008; Toney and Stocker 2010)). Strong evidence suggests that increased plasma osmolality is detected by osmosensory neurons in the circumventricular organs (CVOs) of the lamina terminalis (McKinley, Gerstberger et al. 1999; Toney and Stocker 2010). The organum vasculosum of the lamina terminalis (OVLT) is thought to be particularly important for detecting osmotic changes (Ramsay, Thrasher et al. 1983; Toney and Stocker 2010). It is unclear precisely how plasma osmolality changes are detected, and the process may differ between acute and chronic osmotic stimuli. Acutely, it may involve cell shrinkage as water moves extracellularly along the osmotic gradient, but responses to chronic osmotic challenges may involve other, still unresolved mechanisms (Toney and Stocker 2010). Regardless, changes in these osmosensitive neurons in the lamina terminalis causes depolarization and neuronal firing that activates downstream brain pathways, leading to increased drinking, vasopressin secretion from the pituitary, and sympathetic activation (Brooks, Haywood et al. 2005).

A major downstream target of CVO neurons is the paraventricular nucleus of the hypothalamus (PVN) (Toney, Chen et al. 2003). The PVN is not intrinsically osmosensitive (Antunes, Yao et al. 2006), but rather relies on synaptic transmission, possibly involving angiotensin II (Chen and Toney 2001; Cato and Toney 2005) or glutamate (Antunes, Yao et al. 2006). However, the PVN appears to be essential for

osmotic-induced activation of SNA and blood pressure. Stimulation of PVN neurons increases SNA and arterial pressure (Martin, Haywood et al. 1993), and numerous studies have found that inhibition of the PVN greatly attenuates the SNA and arterial pressure responses to osmotic activation of neurons in the lamina terminalis (Ferguson and Renaud 1984; Gutman, Ciriello et al. 1985; Stocker, Keith et al. 2004; Stocker, Hunwick et al. 2005; Antunes, Yao et al. 2006).

In terms of pathways involved in SNA responses to hyperosmolality, there are two main PVN projections of interest to this thesis. One is a direct projection from the PVN to the intermediolateral cell column (IML) of the spinal cord; the other is a projection to the rostral ventrolateral medulla (RVLM) in the brain stem, which also innervates the IML. Evidence suggests that both the direct PVN-IML (Bains and Ferguson 1995; Toney, Chen et al. 2003) and PVN-RVLM-IML (Stocker, Cunningham et al. 2004; Stocker, Simmons et al. 2006) pathways are activated during conditions of increased osmolality.

The RVLM is thought of as the primary brain region involved in blood pressure control, so it is likely that it is also important in mediating SNA and blood pressure responses to increased osmolality. PVN activation of RVLM neurons likely involves glutamate (Yang, Bertram et al. 2001; Brooks, Freeman et al. 2004; Brooks, Freeman et al. 2004). Like the PVN, inhibition of the RVLM attenuates the SNA and/or blood pressure responses during both acute (Brooks, Freeman et al. 2004; Stocker, Osborn et al. 2008) and chronic (Ito, Hiratsuka et al. 2003; Brooks, Freeman et al. 2004) hyperosmotic challenges.

The PVN and RVLM project to cell bodies of the sympathetic preganglionic neurons (SPNs), which are located primarily in the IML of the thoracic and upper lumbar spinal cord. The axons of these neurons comprise the sympathetic nerves that ultimately innervate the vasculature, heart, and kidney to affect blood pressure. The classic understanding is that these nerves project from the spinal cord to autonomic ganglia, where they release acetylcholine (ACh) onto post-ganglionic cells. The post-ganglionic neurons then release norepinephrine (NE) onto the target end-organ or vascular bed. An exception is the adrenal medulla, which secretes the catecholamines epinephrine and

norepinephrine into the circulation in response to pre-ganglionic release of acetylcholine (Rhoades and Tanner 2003).

An oversimplified illustration of the neural pathways that mediate SNA and MAP responses to hyperosmolity is presented in Figure 1. Since this thesis focuses on the regulation at the level of the spinal cord, it is worth mentioning that in addition to the PVN and RVLM, the neurons in the IML receive descending inputs from the lateral hypothalamus, the rostral ventromedial medulla, and the A5 region of the pons (Guyenet 2006). However, since overwhelming evidence indicates that the PVN and RVLM are important sources of inputs that regulate SNA and blood pressure during conditions of increased osmolality, we have focused on these regions.

Spinal Neurotransmitters Mediating Sympathetic Nerve Activity During Osmotic Stress

As reviewed by Stocker et al. (Stocker, Osborn et al. 2008), questions remain regarding the pathways involved in osmotic-induced SNA, and the relevance to salt-sensitive hypertension remains to be determined. One of the largest knowledge gaps in the topic of osmotic activation of SNA and arterial pressure involves regulation at the level of the spinal cord. For example, the neurotransmitters involved in activating SPNs during conditions of increased osmolality remain unresolved (Llewellyn-Smith 2009). A wide variety of neurotransmitters - including excitatory amino acids, catecholamines, and neuropeptides (McCall 1988; Dun, Karczmar et al. 1993) - have been implicated in SPN regulation in anatomical, pharmacological, and electrophysiological studies over the last thirty years (Dampney 1994; Llewellyn-Smith 2009). However, taking into account the fact that the PVN and RVLM are the primary brain sites innervating IML neurons during osmotic stimulation, it is helpful to examine the likely neurotransmitters used by neurons in those regions.

The direct PVN-IML projection terminates near (or synapses onto) SPNs in the IML (Saper, Loewy et al. 1976; Sawchenko and Swanson 1982; Hosoya, Sugiura et al. 1991), and studies have shown these neurons contain a number of neurotransmitters, including vasopressin, oxytocin, and dopamine (Pyner 2009). Of all of these, vasopressin is the most often cited as the probable neurotransmitter in the PVN-IML pathway

(Riphagen and Pittman 1985; Gruber and Eskridge 1986; Antunes, Yao et al. 2006; Stocker, Osborn et al. 2008; Toney and Stocker 2010). Anatomically, vasopressinergic fibers from the PVN descend to the spinal cord (Riphagen and Pittman 1989; Hallbeck, Larhammar et al. 2001), 40% of PVN neurons contain vasopressin mRNA (Hallbeck and Blomqvist 1999), and V1a receptors exist in the neurons of the IML (Tribollet, Barberis et al. 1997; Sermasi, Howl et al. 1998). Administration of intrathecal vasopressin (Porter and Brody 1986) and chemical stimulation of the PVN (Malpas and Coote 1994) increase arterial pressure, which both can be blocked with vasopressin receptor antagonists (Martinez-Arizala, Holaday et al. 1989; Malpas and Coote 1994). Additionally, a small number of studies suggest that hyperosmotic conditions, such as DOCA-salt hypertension (Abrams, Engeland et al. 2010), water deprivation (Stocker, Cunningham et al. 2004) or intravenous (IV) hypertonic saline (Antunes, Yao et al. 2006), may activate a vasopressinergic pathway from the PVN to the IML. Of these studies, the one by Antunes et al. (Antunes, Yao et al. 2006) was the most convincing that this pathway was involved. In that study, administration of an intrathecal V1a receptor antagonist blocked the increase in lumbar SNA that accompanied acute administration of IV hypertonic saline. However, this study was done in an *in situ* rat preparation in which it was impossible to measure arterial pressure. Therefore, despite the often-cited hypothesis that a direct vasopressinergic pathway from the PVN mediates the sympathetic response to hyperosmolality, this has not been examined in conscious rats or under other conditions of osmotic stimulation.

Like the PVN, RLVM projections innervate the IML and synapse onto SPNs, but the primary neurotransmitter of these neurons is likely the excitatory amino acid, glutamate (Morrison 2003). Glutamatergic nerve terminals from the RVLM have been identified in the IML (Morrison, Callaway et al. 1991), SPNs likely express both NMDA and non-NMDA ionotropic glutamate receptors (Inokuchi, Yoshimura et al. 1992), and two-thirds of the synaptic inputs to SPNs contain glutamate (Llewellyn-Smith, Phend et al. 1992; Llewellyn-Smith 2009). Functionally, intrathecal glutamate receptor antagonists block the SNA and arterial pressure responses to RVLM stimulation (Mills, Minson et al. 1988; Bazil and Gordon 1991; Bazil and Gordon 1993), and glutamate

receptor agonists depolarize SPN neurons (Bazil and Gordon 1991; Morrison 2003). Although glutamate is the most obvious candidate (Dampney 1994) because of its powerful and ubiquitous effects throughout the central nervous system, to our knowledge, no studies have directly assessed the role of spinal glutamate controlling arterial pressure under conditions of hyperosmolality.

Differential Regulation of Sympathetic Nerve Activity

In addition to the lack of knowledge regarding the spinal neurotransmitters that regulate SNA and MAP during conditions of osmotic stress, the pattern of sympathetic outflow mediating osmotic-induced elevations in blood pressure is also unknown. Classically, the sympathetic nervous system was thought of as an all-or-none system, with sympathetic activity increasing or decreasing in all nerves simultaneously (Cannon 1937). However, it is now well-known that differential regulation of sympathetic activity occurs, in which SNA is increased/decreased only to *specific* vascular beds (Morrison 2001). Furthermore, the precise pattern of sympathetic outflow, or "sympathetic signature" (Osborn and Kuroki 2012) appears to vary among different states of sympathetic activation. For example, heart-failure appears to be accompanied by an increase in SNA to the heart, while renal SNA remains unchanged (Esler 2010; Ramchandra, Hood et al. 2012); and the rat AngII-salt model of hypertension seems to rely on an increase in splanchnic SNA while renal and lumbar SNA are decreased or unchanged (King, Osborn et al. 2007; Yoshimoto, Miki et al. 2008; Osborn and Kuroki 2012). In order to begin understanding the spinal pathways involved in regulating SNA and arterial pressure in response to changes in osmolality, it is imperative to identify the sympathetic signatures activated by hyperosmotic conditions.

There are several approaches that can be used to investigate the sympathetic signature of a given condition. For example, sympathetic nerve activity can be measured directly to determine which nerves increase their firing in response to a stimulus. However, although this is now possible in conscious rats in chronic experiments (Miki, Kosho et al. 2002), it is a difficult approach and often has low success rates. Alternatively, selective sympathetic vascular beds can be surgically denervated. This

approach measures the functional outcome of eliminating specific sympathetic nerves, which is important because changes in sympathetic nerve activity may not always correspond to changes in arterial pressure. Ideally, one would combine direct sympathetic nerve recordings with functional measurements involving denervations; however, practical time restraints are prohibitive of this approach. Therefore, selective denervations were used in this thesis to assess the *functional* sympathetic signature of water deprivation.

Regionally specific denervations are possible because the spinal cord and sympathetic nerves are organized somewhat viscerotopically – meaning that the SPNs projecting to a specific vascular bed are grouped together in specific segments of the spinal cord, although some overlap between targets occur (Figure 2). Therefore, the SPN axons projecting to the periphery are grouped into discrete sympathetic nerve bundles targeting specific vascular beds (Strack, Sawyer et al. 1988). For example, the vasomotor sympathetic nerves involved in systemic blood pressure regulation include nerves to the heart (cardiac), kidney (renal), mesenteric vascular beds (splanchnic), and skeletal muscles of the hindlimb (lumbar).

Experimental Models of Increased Osmolality

Three different conditions of increased osmolality were used throughout this thesis: i) an acute, bolus administration of intravenous (IV) hypertonic saline, ii) 48-hours of water deprivation, and iii) 4 weeks of deoxycorticosterone acetate (DOCA)-salt hypertension. All three conditions are accompanied by elevations in plasma osmolality and increases in arterial pressure which are likely due, in part, to sympathetic activation (Brooks, Freeman et al. 2004; O'Donaughy and Brooks 2006).

Because the primary interest of our laboratory and this thesis is chronic regulation of arterial pressure, only one experiment (in chapter 2) utilized IV hypertonic saline as an osmotic stimulus. It was used in chapter 2 to replicate the stimulus used by Antunes et al (Antunes, Yao et al. 2006), in which they demonstrated that intrathecal blockade of vasopressin (V1a) receptors attenuated the lumbar SNA response to IV hypertonic saline. This was important to our conclusions of chapter 2, since the neural mechanisms may

differ between acute stimuli such as IV hypertonic saline and more chronic osmotic stimuli.

Forty-eight hours of water deprivation was the primary osmotic stimulus used throughout this thesis. Consistent with previous studies in the literature (Brizzee, Harrison-Bernard et al. 1988; Russ, Brizzee et al. 1992), we repeatedly found that arterial pressure increased by approximately 15mmHg and plasma osmolality increased by approximately 13 mOsm/kg when rats were denied water for 48 hours. Additionally, forty-eight hour water-deprived rats lose approximately 10-15% of their body weight (Kiss, Jezova et al. 1994; Blair, Woolf et al. 1997), about half of which is due to aphagia (Blair, Woolf et al. 1997). Water deprivation has been used frequently to study the sympathetic and arterial pressure responses to chronic increases in osmolality (Scrogin, McKeogh et al. 2002; Brooks, Freeman et al. 2004; Stocker, Osborn et al. 2008), so much of the literature on the topic is in regards to water deprivation. Additionally, water deprivation is a good model to work with because it relies on physiological mechanisms (as opposed to exogenous factors) and can be completed in a relatively short time frame. However, it is important to keep in mind the potentially complicating factor that in addition to increasing plasma osmolality, water deprivation also decreases blood volume.

In addition to water deprivation, we used the DOCA-salt rat model of hypertension as a longer chronic osmotic stimulus. In this model, rats chronically receive DOCA - an exogenous precursor to the sodium-retaining mineralocorticoid, aldosterone - along with a high salt solution to drink (Jacob, Clark et al. 2005). The combination of high salt intake with artificially elevated DOCA/aldosterone leads to the development of sustained hypertension. The DOCA-salt experiments were included in Chapters 2 and 3 of this thesis for several reasons: a previous study in our lab suggested a possible activation of the direct vasopressinergic PVN-IML pathway in DOCA-salt rats (Abrams, Engeland et al. 2010); DOCA-salt hypertension is a salt-sensitive model of hypertension and is thus of interest to our laboratory; and we believe that using more than one model of chronic osmotic stimulation strengthens our findings and challenges any generalizations about pathways activated by increased osmolality.

Relevance to Human Hypertension and Potential Clinical Application

The purpose of these studies is not to mimic dehydration in humans, but rather to investigate the physiological pathways that increase arterial pressure in response to dehydration-induced hyperosmolality. These pathways may be common throughout evolution and therefore may play a role in salt-sensitive hypertension in humans. Since dietary salt was a scarcity through most of evolutionary history, dehydration was likely the only situation in which plasma osmolality was elevated in our evolutionary ancestors. Therefore, it is possible that the mechanisms that have evolved to respond to osmolality increases during water deprivation are also involved in the response to the high salt diets of the modern world and the development of salt-sensitive hypertension.

The rationale behind the experiments in this thesis was based on the idea that the spinal cord may be a potential target for novel antihypertensive therapies. Despite the abundance of drugs that exist to treat hypertension, the disease remains unmanaged in the majority of people with hypertension (CDC 2011). While some forms of hypertension are drug-resistant, many of the treatment problems arise because of compliance issues (Elliott 2008). This is unsurprising considering that hypertension itself is asymptomatic and many of the antihypertensive drugs, particularly the ones that target the central nervous system, are commonly associated with unpleasant side effects. The potential to specifically target regions of the spinal cord involved in salt-sensitive hypertension could lead to much smaller drugs doses and less non-specific activation of brain regions that cause the side effects. Therefore, by investigating the main neurotransmitters that may be involved and examining the specific contribution of individual sympathetic nerves, the experiments in this thesis were designed to lay the foundation to identify potential spinal targets for future antihypertensive therapies.

Summary

Hypertension is a growing problem worldwide, and despite the numerous antihypertensive drugs available, the condition remains untreated in many people. A high salt diet is a major factor contributing to the rise in hypertension, but much is still unknown about the mechanisms by which salt chronically affects blood pressure. It is

now clear that many forms of hypertension involve activation of the sympathetic nervous system, which is also thought to play a role in elevating arterial pressure in response to increased plasma osmolality. Investigating the pathways involved in osmotic regulation of blood pressure may lead to a better understanding of salt-sensitive hypertension and potential novel targets for improved antihypertensive therapies.

Key brain regions that activate sympathetic activity in response to increased osmolality have been identified, but regulation at the level of the spinal cord - the final point of sympathetic outflow from the central nervous system - is much less understood. In the brain, changes in plasma osmolality are detected by neurons in circumventricular organs, which activate neurons in the PVN. PVN neurons are thought to activate SNA through two possible pathways: a direct vasopressinergic projection to the IML of the spinal cord, or a projection that synapses onto neurons in the RVLM, which likely use glutamate as their primary neurotransmitter. The contributions of spinal vasopressin and glutamate to osmotic-induced arterial pressure changes, however, have not been investigated in conscious rats. In addition to identifying the spinal neurotransmitters involved, it is important to understand the pattern of sympathetic outflow in response to increased osmolality, since sympathetic nerves can be differentially regulated.

The goal of this thesis was to examine the spinal neurotransmitters and sympathetic nerves that may increase arterial pressure in response to elevated plasma osmolality. Specifically, the studies were designed to examine the roles of spinal vasopressin and glutamate and to identify the sympathetic signature involved in elevating arterial pressure during hyperosmotic conditions, particularly water deprivation.

Thesis Overview

Chapter Two examines the effects of blocking spinal vasopressin (V1a) receptors on arterial pressure responses to three conditions of osmotic stress: IV hypertonic saline, 48 hours of water deprivation, and 4 weeks of DOCA-salt hypertension. These studies were designed to test the hypothesis that spinal V1a receptors are required for the pressor response during these conditions. We reasoned that if spinal V1a receptors were required, the intrathecal V1a receptor antagonist would decrease arterial pressure.

Experiments in Chapter Three use a similar approach to test the hypothesis that ionotropic glutamate receptors in the spinal cord are involved in the pressor responses to 48 hours of water deprivation and 4 weeks of DOCA-salt hypertension. Additionally, in order to approximate whether the site of action of the intrathecal antagonist was primarily in the spinal cord or in the brain, we compared the responses to administration near the base of the thoracic region to those near the rostral end of the thoracic spinal cord.

The experiments in Chapter Four were designed to test the hypothesis that the pressor response to 48 hours of water deprivation relies on sympathetic nerve activity and to identify its functional sympathetic signature. To do this, we conducted several experiments in which we pharmacologically blocked global sympathetic nerve activity or selectively denervated the hindlimb, renal, or mesenteric vascular beds. In two additional experiments, we performed bilateral adrenal demedullation or complete adrenalectomies in order to examine the role of the adrenal glands.

Chapter Five summarizes the key discoveries of the previous chapters and integrates the findings into the current understanding of pathways involved in osmotic-induced increases in blood pressure, particularly during water deprivation. This chapter also reports lessons learned about the intrathecal catheterization technique, explores possible roles of adrenal cortical hormones, and discusses the relevance of the findings to the development of novel antihypertensive therapies.

Figure 1
Proposed osmosensitive neural pathways involved in arterial pressure regulation. The PVN-spinal pathway (purple) is thought to release vasopressin (AVP) onto SPNs, and the RVLM-spinal pathway (green) likely releases glutamate (Glut).

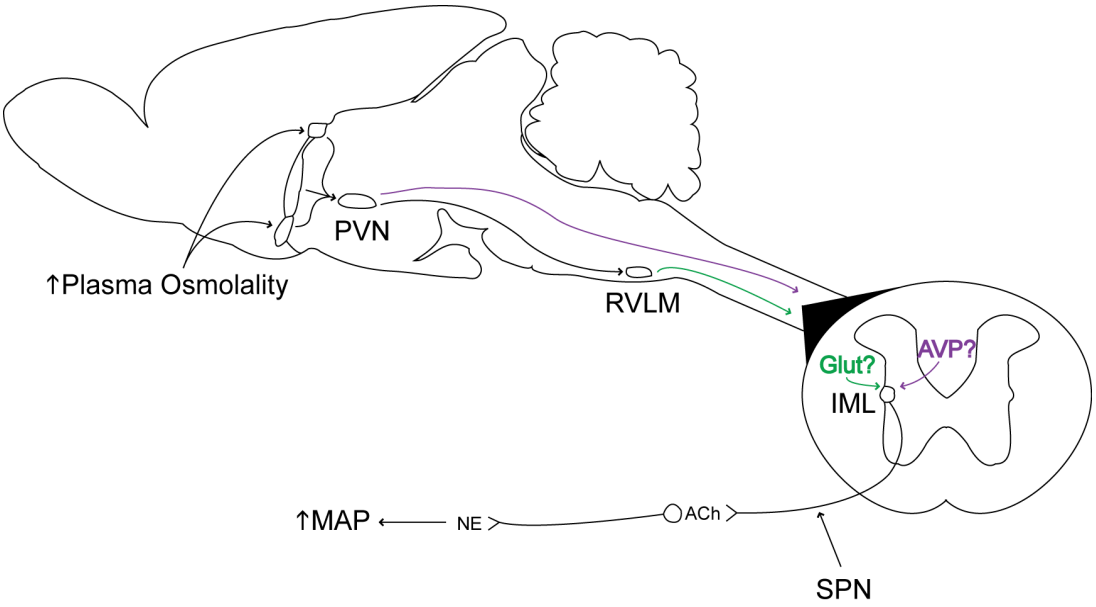
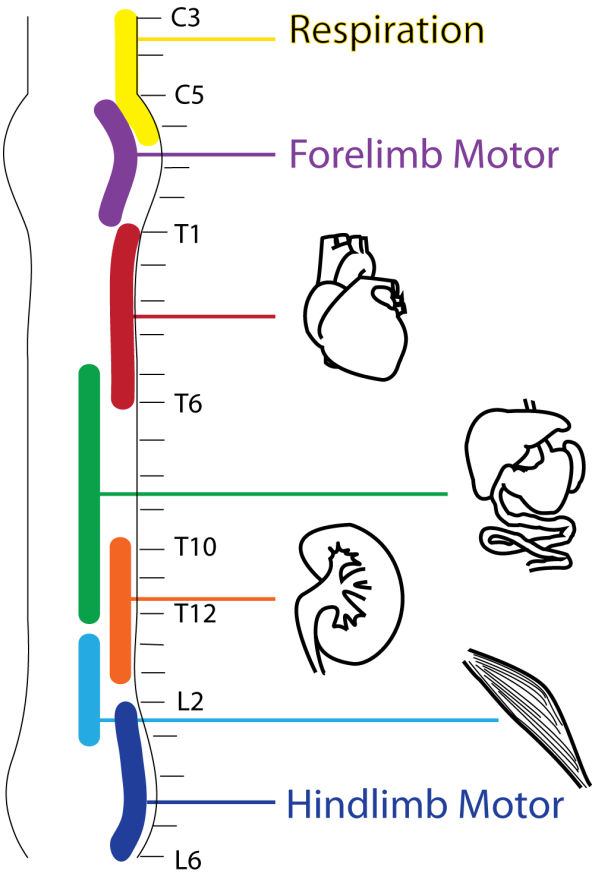


Figure 2
Schematic representation of spinal nerve targets. Spinal segments (C3-L6) are shown along with a colored bar to represent each group of cell bodies that project to a specific end organ. Sympathetic preganglionic neurons are located in segments T1-L3 and project to the heart, mesenteric organs, kidney, or hindlimb musculature.



Chapter 2

Role of spinal V1a receptors in regulation of arterial pressure during acute and chronic osmotic stress

Veitenheimer, B. and J. W. Osborn (2011).
Am J Physiol Regul Integr Comp Physiol 300(2): R460-469.

Vasopressinergic neurons in the paraventricular nucleus (PVN) project to areas in the spinal cord from which sympathetic nerves originate. This pathway is hypothesized to be involved in the regulation of mean arterial pressure (MAP), particularly under various conditions of osmotic stress. Several studies measuring sympathetic nerve activity support this hypothesis. However, the evidence that spinal vasopressin influences MAP under physiological or pathophysiological conditions in conscious animals is limited. The purpose of this study was to investigate, in conscious rats, if the increases in MAP during acute or chronic osmotic stimuli are due to activation of spinal vasopressin (V1a) receptors. Three conditions of osmotic stress were examined: acute intravenous hypertonic saline, 24- and 48-hour water deprivation, and 4 weeks of DOCA-salt treatment. Rats were chronically instrumented with an indwelling catheter for intrathecal (i.t.) injections and a radiotelemeter to measure MAP. In normotensive rats, i.t. vasopressin and V1a agonist increased MAP, heart rate, and motor activity; these responses were blocked by pretreatment with an i.t. V1a receptor antagonist. However, when the i.t. V1a antagonist was given during the three conditions of osmotic stress to investigate the role of *endogenous* vasopressin, the antagonist had no effect on MAP, heart rate, or motor activity. Contrary to the hypothesis suggested by previous studies, these findings indicate that spinal V1a receptors are not required for elevations of MAP under conditions of acute or chronic osmotic stress in conscious rats.

Key words: paraventricular nucleus, vasopressin, osmolality, sympathetic nerve activity, intrathecal

Introduction

A number of reports implicate arginine vasopressin (AVP) as a neurotransmitter involved in the regulation of spinal sympathetic preganglionic neurons (SPNs) (Coote 2005; Pyner 2009). Forty percent of the spinally-projecting neurons in the paraventricular nucleus (PVN) of the hypothalamus, a key sympathoregulatory site (Toney, Chen et al. 2003; Guyenet 2006; Stocker, Osborn et al. 2008), contain AVP mRNA (Hallbeck and Blomqvist 1999); and PVN stimulation increases the amount of AVP in spinal fluid (Pittman, Riphagen et al. 1984). Intrathecal administration of AVP causes a dose-dependent increase in arterial pressure (Porter and Brody 1986), and this increase is prevented by pretreatment with a V1-specific antagonist (Martinez-Arizala, Holaday et al. 1989). Likewise, a V1-specific antagonist is able to completely block increases in renal sympathetic nerve activity (SNA) and arterial pressure due to chemical stimulation of the PVN (Malpas and Coote 1994). Anatomically, V1a receptors have been identified in all lamina of the gray matter along the length of the spinal cord (Tribollet, Barberis et al. 1997), including the intermediolateral cell column (IML) neurons (Sermasi, Howl et al. 1998); and fibers from the PVN have been found to terminate near SPNs in the IML (Sofroniew 1980; Coote 1988; Ranson, Motawei et al. 1998; Motawei, Pyner et al. 1999). Electrophysiological studies have shown that V1a receptors in the spinal cord depolarize neurons when activated, and this is blocked with a V1a receptor antagonist (Ma and Dun 1985; Sermasi and Coote 1994; Raggenbass 2008). Although these studies suggest that spinally released AVP influences SNA and arterial pressure at the level of the spinal cord, the physiological conditions which activate spinally projecting vasopressinergic pathways have not been established.

Osmotic stress is associated with increased SNA, and previous studies suggest that either spinal vasopressin or glutamate is responsible for the elevated SNA under conditions of increased osmolality (Gruber and Eskridge 1986; Antunes, Yao et al. 2006; Stocker, Osborn et al. 2008). Osmoreceptors in the CVOs detect small changes in plasma osmolality (Bourque and Oliet 1997) and change the firing patterns of neurons that project to the PVN (Stocker, Osborn et al. 2008). PVN activation results in hormone

release from the pituitary gland and affects SNA through direct spinal projections or via the rostral ventrolateral medulla (RVLM), which sends glutamatergic projections to the spinal cord (Toney, Chen et al. 2003; Benarroch 2005; Stocker, Osborn et al. 2008). Several studies have found evidence for activation of PVN-spinal vasopressinergic neurons during various types of osmotic stress. Recent data from our laboratory suggest that vasopressinergic PVN neurons are activated in DOCA-salt hypertensive rats (Abrams, Engeland et al. 2010), which are known to have elevated plasma osmolality (O'Donaughy and Brooks 2006; O'Donaughy, Qi et al. 2006). Increased osmolality during water deprivation is thought to influence arterial pressure in part through activation of descending brain pathways (Brooks, Qi et al. 2005). Also, vasopressin mRNA within the PVN is enhanced during dehydration (da Silveira, Junta et al. 2007), and spinally-projecting PVN neurons show increased Fos labeling in water-deprived rats (Stocker, Cunningham et al. 2004). Finally, in an *in situ* rat preparation, acute intravenous infusion of hypertonic saline is accompanied by an increase in lumbar SNA which is blocked by pretreatment with intrathecal V1a antagonist (Antunes, Yao et al. 2006).

Taken together, the above studies are consistent with the hypothesis that increased plasma osmolality stimulates PVN vasopressinergic neurons to act on spinal V1a receptors on SPNs and elevate arterial pressure. However, this hypothesis has not been tested under conditions of osmotic stress in conscious animals. The present study tested this hypothesis by measuring the response of arterial pressure to intrathecal administration of a V1a antagonist in conscious rats under conditions of acute (intravenous hypertonic saline), semi-chronic (24- and 48-hour water deprivation), and chronic osmotic stress (4 weeks DOCA-salt treatment). Contrary to the hypothesis supported by earlier studies, we found that V1a receptors are not required for the pressor responses to osmotic stress.

Methods

Animals

Male Sprague Dawley rats were purchased from Charles River Laboratory (Wilmington, MA) and housed in a temperature-controlled animal room with a 12 hour light/dark cycle. Unless otherwise noted, animals ate normal rat chow (Lab Diet 5012) and drank distilled water *ad libitum*. All surgical procedures in this study were approved by the Institutional Animal Care and Use Committee.

Surgical Procedures

To continuously measure mean arterial pressure (MAP) and heart rate (HR), a telemetry transmitter (model TA11PA-C40, Data Sciences International, St. Paul, MN) was implanted into the descending aorta. At the same time, for some experiments, an intravenous (i.v.) catheter (silastic tubing, Dow Corning 508-002) was implanted for drug delivery. Rats were anesthetized with 2% isoflurane (after a brief 4% induction), given atropine sulfate (0.2 mg/kg i.p., Baxter) and gentamicin sulfate (2.0 mg i.m., Hospira), and the left femoral artery and vein were exposed. The vein was cut, and the tip of the catheter was advanced 6 cm into the inferior vena cava and tied securely into place. The catheter was tunneled under the skin to exit between the scapulae. For the telemetry device, a midline abdominal incision was made, and the body of the telemetric transmitter was placed in the abdominal cavity and sutured to the abdomen wall. The fluid-filled catheter of the transmitter was then tunneled through the abdominal wall, inserted into the femoral artery, advanced 4cm until the tip lay in the abdominal aorta caudal to the renal arteries, and tied securely into place. The femoral incision was sutured close, and the abdominal incision was closed with 9-mm surgical wound clips.

At the time of telemetry transmitter and i.v. catheter implantation, an intrathecal catheter was also implanted. A 3cm incision was made near the midline over the lumbar vertebrae, and the rat was placed in the prone position over a 150 ml beaker to slightly separate the vertebrae. A 32-gauge intrathecal catheter (CR3212 Cth RSR 32G 12 w/stylet; ReCathCo, LLC; Allison Park, PA) was threaded into a 23 gauge needle, which was inserted between L6 and S1 vertebrae until a tail flick indicated penetration of the

dura. The needle was angled along the spinal column, and the catheter was advanced slightly to check for resistance. If no resistance was felt, the catheter was advanced 7cm cranially, so the tip was positioned adjacent to spinal segments T11-T13. This position was chosen based on preliminary experiments with Evans blue dye that suggested the injectate would travel rostrally and cover the length of the thoracic cord. The needle and stylet were removed, and cyanoacrylate adhesive was applied to point of exit. A loop was made in the catheter tubing and sutured in several places to secure. The tubing was then glued to 34cm of PE10 tubing (Intramedic TM; BD, Sparks, MD) attached to 1cm of PE50 to complete the catheter. The catheter was tunneled under the skin to exit between the scapulae along with the venous catheter. The two catheters were threaded through a spring and attached to a swivel that allowed the rat to move freely. Each rat was caged individually. For all surgeries performed, amoxicillin (1mg/ml in drinking water, West-ward Pharmaceutical Co.) and the analgesic buprenorphine (0.3mg/ml in 0.02ml, PharmaForce) were administered post-operatively.

On the final day of recovery, catheter placement was checked by injecting 20μl of lidocaine (10mg/ml, Hospira) into the intrathecal space. Immediate hindlimb paralysis indicated intrathecal placement; animals showing no paralysis were eliminated from the study.

General Protocol

Each cage was placed on a receiver (model RPC1) that was connected to a computer via a Data Exchange Matrix (DSI; St. Paul, MN). Data was acquired and analyzed with Dataquest A.R.T. 4.0 software (DSI; St. Paul, MN). MAP and HR data were collected at 500Hz over 10s every 1 min, except during the 10 min before and after injection in which the sampling rate was continuous at 500Hz. Also, an index of motor activity was monitored by the DSI system by counting the number of times the signal strength fluctuated due to changes in the animal's position or orientation. The counts were summed and reported in counts per time.

Animals were allowed at least 5 days to recover from surgery before beginning the experimental protocols. All experiments were conducted in conscious rats in their

home cages, and the experiments consisted of intrathecal (i.t.) and/or intravenous (i.v.) injections. At least ten minutes of a stable MAP baseline was collected before each injection, and MAP and HR were recorded for 1 hour following the injection. When more than one compound was injected (i.e. antagonist pretreatment before agonist injection), five minutes were allowed between the two injections. At least one day was given for recovery between different injection treatments.

All intrathecal injections were administered with a 50µl Hamilton syringe in a volume of 10µl and a rate of ~0.5µl/sec. Each intrathecal injection was followed by a 25µl flush with vehicle solution in order to empty the 23µl dead space of the catheter. Intravenous injections were weight-dependent and were followed by a flush of 0.2ml of 0.9% saline. The following drugs were administered intrathecally or intravenously: artificial cerebrospinal fluid (aCSF; Harvard Apparatus, i.t.); V1a agonist (Phe2,Ile3,Orn8-Vasopressin, American Peptide Company, 10ng i.t.); V1a antagonist [(B-mercapto-B, B-cyclopentamethylene-propionlyl1, O-Me-Tyr 2, Arg 8)-vasopressin, Sigma-Aldrich, 100ng i.t., 100µg/kg i.v.]; [Arg8]-vasopressin (Sigma, 0.1ng i.t., 10 µg/kg i.v.).

When the experiments were complete, the rats were euthanized with isoflurane and the spinal cord was dissected to determine the precise location of the intrathecal catheter tip. Approximately 1/3 of the rats in the study were also given an intrathecal injection of 10µl saturated Evans blue dye to analyze injectate spread.

Specific Experimental Protocols

Effect of an Intrathecal V1a Antagonist on the Pressor Responses to V1a Agonists

The purpose of this study was to determine the MAP and HR responses to intrathecal V1a agonists and confirm that these responses could be blocked by pretreatment with the selected dose (100ng) of the V1a antagonist. Normotensive rats (225-325g) with *ad libitum* water and normal rat chow were instrumented as described above. After at least 5 days recovery, the responses to the following were measured on

separate days: 1) artificial cerebrospinal fluid (aCSF); 2) V1a agonist; and 3) the V1a agonist injected 5 minutes after pretreatment with the V1a antagonist. In a separate group of rats, intrathecal [Arg8]-vasopressin was administered with and without pretreatment 5 minutes prior with the V1a antagonist. A third group of rats was used to verify that the intrathecal V1a antagonist effectively blocked V1a receptors at a site distant from the tip of the catheter. Rats in these experiments were implanted with a second intrathecal catheter via the atlanto-occipital membrane, and the catheter was advanced caudally until the tip lay at segment T1. These rats received the V1a antagonist (or vehicle) via the lumbar catheter, followed 5 minutes later by the V1a agonist via the atlanto-occipital catheter.

Effect of Blockade of Spinal V1a Receptors on the Acute Pressor Response to Intravenous Hypertonic Saline

The purpose of this study was to determine if spinal V1a receptors mediate the acute pressor response to i.v. hypertonic saline (HS). Rats (250-400g) were instrumented with transmitters and catheters as described above and recovered for five days before injections began. Intravenous HS was administered by injection of 3M saline in a volume of 0.14ml/100g over a 40 second period. The same volume of isotonic saline was administered as a control.

Since cardiovascular responses to i.v. HS may be mediated by spinally released AVP or increases in plasma AVP, the following treatment groups were studied: 1) i.v. HS alone; 2) i.v. isotonic saline alone; 3) i.v. HS 5 minutes after *intrathecal* administration of V1a antagonist; 4) i.v. HS 5 minutes after *intravenously* administered V1a antagonist. This i.v. dose of the V1a antagonist was shown in a pilot study to completely block the pressor response (50 ± 5 mmHg) to i.v. injection of 100μg/kg AVP.

The response of plasma osmolality to HS or isotonic saline was also measured in a separate study. Briefly, blood was collected with heparinized syringes at the following times: 10 and 5 minutes before the i.v. hypertonic or isotonic injection; every minute for 5 minutes after the injection; and at 30, 60, 90, 120, and 180 minutes. The volume of plasma collected (200μl/sample) was replaced with isotonic saline at each sample point.

Samples were centrifuged at 4°C at 5000 RPM for 10 minutes, and the plasma osmolality was determined using a freezing-point micro-osmometer (Advanced Instruments, model 3320).

Effect of Blockade of Spinal V1a Receptors on Arterial Pressure in Water Deprived Rats

The purpose of this study was to determine if spinal V1a receptors are responsible for the sustained pressor response during water deprivation. Rats (250-275g) were instrumented with transmitters and catheters as described above. Six days later, their water bottles were removed for 48 hours. Intrathecal injections of the V1a antagonist were given at 24 hours and 48 hours of water deprivation; plasma osmolality was measured at those times in a separate group of rats. Water bottles were returned approximately one hour after the injection at the 48-hour time point. Rats were given 3 days to recover from water deprivation and then received the following injections a day apart to measure the response to i.t. V1a antagonist in rehydrated rats and confirm effectiveness of the antagonist: 1) i.t V1a antagonist; 2) i.t. V1a antagonist 5 minutes before i.t. V1a agonist; 3) and i.t V1a agonist alone.

Effect of Blockade of Spinal V1a Receptors on Arterial Pressure in DOCA-salt Hypertensive Rats

The purpose of this study was to determine if spinal V1a receptors are involved in the chronic elevation of pressure in DOCA-salt hypertensive rats. Rats (200-225g) were anesthetized as described above and received a left nephrectomy and subcutaneous implantation of 50 mg of deoxycorticosterone acetate (DOCA) pellets as previously described (Osborn, Jacob et al. 2006). After the surgery, the animals were housed individually and were given 0.1% sodium chloride food and 0.9% saline to drink. Sham animals received a left nephrectomy and 0.1% sodium food, but their implanted pellets contained no DOCA and they were given *ad libitum* access to distilled drinking water.

Three weeks later, the rats were instrumented with transmitters and intrathecal catheters as described above. The experimental protocols began the following week, so all rats received 4 weeks of DOCA-salt treatment at the time of the experimental

injections. The following injections were given on separate days: 1) i.t. V1a antagonist; 2) i.t. V1a antagonist 5 minutes prior to i.t. agonist; and 3) i.t. aCSF 5 minutes prior to i.t. agonist. Plasma osmolality was also assessed after 4 weeks of DOCA-salt or sham treatments.

Data Analysis and Statistics

MAP and HR data were plotted as 2 minute averages, and activity was plotted in 5 minute averages. All data are shown as means $\pm$ standard errors. Two-way repeated measures analysis of variance (ANOVA) was used to determine differences between and within treatments. The Bonferonni's post hoc test of Multiple Comparisons versus Control was used to identify differences between and within groups. The aCSF data were used as the control between groups, and measurements 6 minutes prior to drug treatments (t = -6 min) was used as the control within groups. A p value of <0.05 was defined as statistically significant.

Results

Effect of an Intrathecal V1a Antagonist on the Pressor Responses to V1a Agonists

The MAP, HR, and activity responses to 10µl intrathecal injections are shown in Figure 1. MAP, HR, and motor activity were unaffected by vehicle (aCSF) injection (Fig 1a). Intrathecal injection of the V1a agonist increased MAP (23 $\pm$ 5 mmHg), HR (105 $\pm$ 18 bpm), and motor activity (Fig 1b). MAP and HR both remained elevated 60 minutes following injection. Intrathecal pretreatment with 100ng of a V1a antagonist blocked all of these responses (Figure 1b), but the antagonist had no effect on its own. Therefore, this dose of V1a antagonist was selected for the remainder of the experiments.

Intrathecal injection of 0.1ng of AVP increased MAP and HR by approximately the same amount as the V1a agonist, although all three measured variables returned to control levels within approximately 30 minutes (Fig 1c). Pretreatment with 100ng of intrathecal V1a antagonist also blocked the increases in MAP, HR, and motor activity in response to this dose of intrathecal vasopressin.

In another group (n=3), we investigated whether administration of the V1a antagonist to the lower thoracic cord was effective in blocking responses to administration of the V1a agonist administered to the upper thoracic cord. Rats were instrumented with two intrathecal catheters: one advanced from the lumbar region with the tip at T11 – T13; one advanced from the atlanto-occipital region with the tip near T1. ACSF was delivered via the lumbar catheter and 5 minutes later, administration of the V1a agonist via the atlanto-occipital catheter increased MAP (16$\pm$6 mmHg at 10 min) and HR (107 $\pm$ 21 bpm at 10 min). The next day, pretreatment with the V1a antagonist via the lumbar catheter abolished the MAP (1 $\pm$ 2 mmHg at 10 min) and HR (9 $\pm$ 10 bpm at 10 min) responses to the V1a agonist delivered via the atlanto-occipital catheter.

Effect of Blockade of Spinal V1a Receptors on the Acute Pressor Response to Intravenous Hypertonic Saline

Plasma osmolality was increased to 315 $\pm$ 4 mOsm/kg after intravenous hypertonic saline but was unaffected by intravenous isotonic saline (Fig 2). Intravenous

isotonic saline also had no effect on MAP, HR, or motor activity (data not shown). Intravenous hypertonic saline increased MAP by 20 ± 3 mmHg (Fig 3) but caused no change in HR or motor activity (data not shown). This pressor response was attenuated, but not abolished, by *intravenous* V1a antagonist (Fig 3a). Pretreatment with an *intrathecal* V1a antagonist had no effect on the pressor response to i.v. hypertonic saline (Figure 3b).

Effect of Blockade of Spinal V1a Receptors on Arterial Pressure in Water Deprived Rats

Rats deprived of water for 24 - 48 hours showed elevated daytime arterial pressure (Table 1). When these animals were given intrathecal injections of the V1a antagonist at 24 hours (Fig 4a) and 48 hours (Fig 4b) of water deprivation, there was no change in arterial pressure or heart rate. At the end of the experiment, the effectiveness of the antagonist used was verified with a challenge injection of the V1a agonist (data not shown). In a separate group of rats (n=6), plasma osmolality was significantly elevated after 48 hours of water deprivation (308 ± 1 mOsm/kg) compared to euhydrated levels (298 ± 1).

Effect of Blockade of Spinal V1a Receptors on Arterial Pressure in DOCA-salt Hypertensive Rats

Rats that received 4 weeks of DOCA-salt treatment developed sustained hypertension (arterial pressure daytime average: 129 ± 10 mmHg; heart rate daytime average: 392 ± 8 bpm) A separate group of rats showed elevated plasma osmolalities (299 ± 1 mOsm/kg) compared to sham rats (294 ± 1 mOsm/kg). After 4 weeks of DOCA-salt treatment, intrathecal injections of the V1a antagonist resulted in no change in arterial pressure or heart rate (Figure 5). At the end of the experiment, the effectiveness of the antagonist used was verified with a challenge injection of the V1a agonist (data not shown).

Discussion

The PVN is a key brain region involved in regulating the central nervous system's response to osmotic stress (46, 49). It is also the site of origin of vasopressinergic neurons that terminate near SPNs (Ranson, Motawei et al. 1998; Sermasi, Howl et al. 1998; Coote 2005; Pyner 2009), and it plays a role in regulating SNA (Malpas and Coote 1994; Guyenet 2006). Therefore, we hypothesized that the pressor responses to osmotic stress are due to the activation of a PVN-to-spinal cord vasopressinergic pathway. To our knowledge, this is the first study to investigate spinal control of MAP under conditions of both acute and chronic osmotic stress in conscious intact animals. We found that spinal V1a receptors are not required for the pressor responses during intravenous hypertonic saline, 24- and 48-hour water deprivation, or DOCA-salt hypertension. Our findings do not support the hypothesis that spinal V1a receptors are involved in the regulation of MAP under conditions of acute or chronic osmotic stress in conscious rats.

Cardiovascular and Motor Responses to Intrathecal Injections of V1a Agonists in Normal Rats

Before injecting the intrathecal V1a antagonist under conditions of osmotic stress, it was necessary to verify that the dose of antagonist was sufficient to prevent increases in MAP in response to activation of spinal V1a receptors. In normotensive rats, pretreatment with the V1a antagonist blocked the pressor response to intrathecal administration of both the V1a agonist and AVP. This demonstrated that the antagonist dose was sufficient to block the pressor response and, since AVP can act on both V1 and V2 receptors, that the response to AVP is mediated exclusively by V1a receptors. This is in agreement with the findings of Porter and Brody, who first demonstrated that intrathecal vasopressin increases MAP in conscious rats and that the response relies on V1 receptors (Porter and Brody 1986).

One novel aspect of these control experiments was the discovery that the responses to the intrathecal V1a agonist followed a different time course than AVP. The AVP responses lasted approximately 20 minutes, while the responses to the V1a agonist lasted more than 50 minutes. A possible explanation may be that AVP and the V1a

agonist are metabolized differently. Both are peptides, but they differ in three amino acids, including the site of action of an aminopeptidase primarily responsible for breaking down AVP. The rate-limiting step of AVP metabolism in the brain involves cleaving the Cys-Tyr bond (Burbach, Schoots et al. 1998), which is replaced with Cys-Phe in the V1a agonist.

Another result that requires speculation is the fact that heart rate increased along with MAP. Typically, a baroreflex-mediated bradycardia accompanies pressor responses. Instead, a profound tachycardia ($\Delta HR=105\pm18$ bpm) occurred, although it was slightly delayed in comparison to the MAP response (MAP peaked within four minutes while HR took at least ten minutes to reach its maximum). Previous studies have reported variable effects on HR in response to i.t. AVP in both anesthetized (Riphagen and Pittman 1985; Tan and Tsou 1986) and conscious rats (Porter and Brody 1986; Martinez-Arizala, Holaday et al. 1989). Activation of cardiac sympathetic nerves, either directly or via activation of ascending neurons or interneurons, is one possible explanation of the tachycardia shown here. The delay in HR response could be due to diffusion time for the injectate to reach the cardiac SPNs; however, it could also be due to an initial offsetting by the baroreflex before it is overridden by sympathetic activation. Another possibility is that the tachycardia is due to activation of adrenal nerves that cause epinephrine release into the circulation. However, Riphagen *et al*. found no change in systemic epinephrine levels in response to intrathecal AVP (Riphagen, Bauce et al. 1986).

Finally, intrathecal V1a agonist and AVP cause an increase in motor activity, which was prevented by V1a antagonist pretreatment. It was previously shown that i.t. V1 antagonist can block the scratching behavior to i.t. AVP (Thurston, Campbell et al. 1992), but the current study extends the finding to show that MAP and HR responses follow the same time course as motor activity. The injections seem to cause some change in somatosensation - the behavioral response consists primarily of scratching, biting, or licking at the hind limbs. However, it is unclear whether the change in somatosensation involves pain or other sensory circuits. AVP is thought to be antinociceptive at the spinal cord (Watkins, Suberg et al. 1986; Thurston, Campbell et al. 1992), the antinociception

and scratching behavior are thought to involve separate mechanisms (Thurston, Culhane et al. 1988), and AVP-induced scratching behavior continues after morphine pretreatment (Thurston, Campbell et al. 1992). Intrathecal applications of other compounds, such as morphine, are known to produce pruritus (Kuraishi, Yamaguchi et al. 2000), but this remains to be investigated for intrathecal AVP.

Role of Spinal V1a Receptors in Mediating the Cardiovascular Responses to Acute Administration of Hypertonic Saline

Consistent with previous studies, acute intravenous injection of hypertonic saline increased plasma osmolality and MAP. Since the pressor response could be due to a combination of AVP release into the plasma and activation of SNA, we examined the effect of both intravenous and intrathecal V1a antagonist on this response. We found that systemic blockade of V1a receptors attenuated the pressor response but did not block it completely, suggesting that SNA might also contribute to the elevated MAP. However, i.t. V1a antagonist pretreatment had no effect on the pressor response. This does not support our hypothesis that spinal V1a receptors are involved in the pressor response to i.v. hypertonic saline.

Our results are supported by findings by Liu *et al,* who administered intracerebroventricular (i.c.v.) vasopressin receptor antagonists and found no effect on the pressor response to i.v. hypertonic saline (Liu, Wang et al. 1996). However, our findings differ from those of the recent study by Antunes and Paton (Antunes, Yao et al. 2006). They reported that lumbar SNA was increased in response to either intravenous hypertonic saline or intrathecal V1a agonist, and this response was blocked with intrathecal V1a antagonist pretreatment or chemical inhibition of neurons in the PVN (Antunes, Yao et al. 2006). These data imply that the PVN releases vasopressin in the spinal cord to mediate lumbar SNA responses to hypertonic saline. Our study differs from Antunes *et al.* in the preparation employed to test this hypothesis. The present study was conducted in conscious intact rats, in contrast to the study by Antunes *et al.* which used an *in situ* rat preparation that did not allow measurement of MAP. Another study in anesthetized rats also showed elevation of lumbar SNA in response to i.v. hypertonic

saline, along with elevations in MAP (Weiss, Claassen et al. 1996). The reasons for the differences between these results and those of our study in conscious rats are not clear. Possibly, lumbar SNA is not elevated in response to i.v. hypertonic saline in conscious rats, or perhaps another neurotransmitter is responsible for its elevation. Another explanation could be that the lumbar SNA increases by the same amount (~30%) as the *in situ* or anesthetized rats (Weiss, Claassen et al. 1996; Antunes, Yao et al. 2006), but the increase in lumbar SNA does not cause the elevation in MAP. This possibility is supported by a discrepancy in timing between MAP and lumbar SNA during a 30-minute infusion of hypertonic saline in anesthetized, baroreceptor-intact rats (Weiss, Claassen et al. 1996). Weiss *et al.* found that MAP was significantly increased 5 minutes into the infusion, but lumbar SNA remained at baseline levels until 25 minutes of hypertonic saline infusion had occurred (Weiss, Claassen et al. 1996).

Role of Spinal V1a Receptors in Mediating the Pressor Response to Water Deprivation

Previous findings are consistent with the hypothesis that water deprivation increases SNA and this response may be due to activation of spinally projecting vasopressinergic neurons. Scrogin and co-workers measured lumbar SNA in water-deprived rats and concluded that it was increased as a result of increased plasma osmolality (Scrogin, Grygielko et al. 1999). Others found that water deprivation increased Fos expression, an indicator of neuronal activity, in PVN neurons that contain vasopressin (da Silveira, Junta et al. 2007); and another report demonstrated increased Fos expression in spinally-projecting PVN neurons (Stocker, Cunningham et al. 2004).

Although our findings show that MAP was increased in response to 24 and 48 hours of water deprivation, intrathecal administration of the V1a antagonist had no effect on MAP in these rats, suggesting that spinal vasopressin does not increase MAP during water deprivation. How does this relate to previous studies? Although individual Fos studies support the idea of osmotic activation of a PVN-spinal vasopressinergic pathway, to our knowledge there is no report in which PVN neurons activated by water deprivation were *both* vasopressinergic and spinally-projecting, so it is possible that the spinally-

projecting neurons activated by water deprivation use neurotransmitters other than vasopressin. Further studies are needed to answer this question.

Role of Spinal V1a Receptors in Mediating DOCA-salt Hypertension

Several studies suggest that DOCA-salt hypertension involves activation of both vasopressin and the sympathetic nervous system. For example, DOCA-salt hypertension does not develop in rats lacking vasopressin (Berecek, Murray et al. 1982), and vasopressin neurons in the PVN show Fos expression with DOCA-salt hypertension (Abrams, Engeland et al. 2010). Also, acute, central injection of hypotonic saline in DOCA-salt rats causes a fall in MAP (O'Donaughy, Qi et al. 2006) and lumbar SNA (O'Donaughy and Brooks 2006), and this is blocked by a combination of ganglionic blockade and systemic V1a antagonist (O'Donaughy, Qi et al. 2006). Together, these data suggest that DOCA-salt hypertension is due to a combination of systemic vasopressin release and activation of the sympathetic nervous system. In the present study we tested the hypothesis that *spinal* vasopressin was partly responsible for increased MAP in DOCA-salt rats. However, our findings were inconsistent with our hypothesis in that intrathecal administration of a V1a antagonist had no effect on MAP in DOCA-salt hypertensive rats. We conclude that spinal V1a receptors are not required for sustained elevations in MAP in DOCA-salt hypertension.

Implications of Negative Findings

Most of the previous studies investigating spinally-released vasopressin have led to the conclusion that vasopressinergic neurons in the PVN activate sympathetic nerves in the spinal cord to increase MAP (Riphagen and Pittman 1986; Tan and Tsou 1986; Riphagen and Pittman 1989; Malpas and Coote 1994; Yang, Wheatley et al. 2002). However, the physiological conditions under which this pathway is activated have not been investigated. We tested the hypothesis that vasopressin acts on spinal V1a receptors to mediate pressor responses under conditions of acute and chronic osmotic stress – specifically, intravenous hypertonic saline, 24 and 48 hours of water deprivation, and 4 weeks of DOCA-salt treatment. Under all three conditions, intrathecal V1a antagonist

had no effect on MAP. These findings do not support our hypothesis, suggesting that spinal V1a receptors do not support SNA control of MAP under these conditions.

An alternate explanation is that the intrathecally administered V1a antagonist did not reach the SPNs, where the endogenous vasopressin supposedly is released during osmotic stress. However, in control rats (Figure 1), the intrathecal V1a antagonist was able to block the response of exogenous intrathecal vasopressin or a V1a agonist. Additionally, the V1a antagonist delivered through the lumbar i.t. catheter was able to block the MAP and HR responses to the V1a agonist delivered through the atlanto-occipital catheter. This implies that the antagonist delivered via the lumbar i.t. catheter during hypertonic saline, water deprivation, and DOCA-salt hypertension likely blocks V1a receptors along the entire length of the thoracic spinal cord.

While the antagonist clearly reaches the site of action of exogenously administered agonist, it remains possible that the exogenous agonist activates V1a receptors somewhere other than on the SPNs. V1a receptors in the periphery, brain, dorsal horn, ventral horn, and spinal vasculature all must be evaluated as potential sites responsible for increased MAP in response to exogenous intrathecal vasopressin (or the V1a agonist).

We believe it is unlikely that the pressor response to intrathecal AVP is due to leakage of the agonist into the periphery causing direct peripheral vasoconstriction. Pressor responses to intrathecal AVP are twice as large as the same intravenous dose in conscious rats (Martinez-Arizala, Holaday et al. 1989), and ganglionic blockade abolishes the response to intrathecal AVP (Riphagen and Pittman 1989). Additionally, i.v. administration of a V1a antagonist has no effect on the response to intrathecal vasopressin (Riphagen and Pittman 1985), and intrathecal injection of tritiated AVP shows that only very small amounts get into the plasma (Riphagen and Pittman 1989).

Another possibility is that intrathecal AVP could diffuse to the brain stem to activate descending pathways that affect SNA and elevate MAP. Indeed, faint traces of Evans blue dye injected at the end of our experiments appeared in the brainstem of all of the rats that were examined. However, a previous study that also showed MAP responses to i.t. AVP (in a volume of 3μl) concluded that, under these conditions, the

spread of dye extended only to upper thoracic levels of the cord (Porter and Brody 1986). Additionally, a study comparing responses of i.t. AVP with those of i.c.v. AVP demonstrated greater sensitivity to AVP in the spinal cord (Tan and Tsou 1986).

It is also possible that AVP acts in spinal regions other than the IML. V1a receptors have been localized in both dorsal and ventral horn neurons (Liu, Tribollet et al. 2003). If activation of V1a receptors in the ventral horn are responsible for the increased motor activity, perhaps MAP responds as an exercise pressor reflex (Mitchell, Kaufman et al. 1983). We believe this is unlikely because intrathecal vasopressin also increases MAP in anesthetized rats (Martinez-Arizala, Holaday et al. 1989) that lack motor movement; however, further investigation is necessary to rule out the involvement of the exercise pressor reflex in conscious rats. On the other hand, if dorsal horn neurons are activated, they could then activate SPNs directly or via activation of spinal interneurons or a spinal-bulbo-spinal pathway.

Indeed, the behavioral response to i.t. AVP seems to indicate a change in either somatosensation or motor neuron activation. Injection of i.v. phenylephrine to increase MAP the same amount as i.t. AVP does not cause scratching behavior (unpublished observations), suggesting that the motor response is due to activation of V1a receptors and not due to the increase in MAP. Intrathecal injection of substance P causes a similar scratching response to i.t. AVP, and this is not thought to be due to perception of pain (Hassessian and Couture 1989). Instead, it appears that substance P induces convulsant-like behavior - likely through activation of motor neurons (Bossut, Frenk et al. 1988) - which can be attenuated with anti-convulsant drugs (Frenk, Bossut et al. 1988). This is also true for morphine, strychnine, and kainic acid (Frenk, Bossut et al. 1988). In contrast, anticonvulsants were shown to have no effect on the behavioral response to i.t. AVP (Thurston, Campbell et al. 1992). Finally, even if i.t. AVP causes changes in sensation through a morphine-insensitive mechanism as suggested by Thurston *et al.* (Thurston, Campbell et al. 1992), sensory and cardiovascular responses to i.t. injections may be due to separate mechanisms, as demonstrated in Khan *et al.*'s investigation of i.t. nicotinic agonists (Khan, Wart et al. 2008).

Finally, another possibility is that the pressor response to i.t. AVP is secondary to spinal ischemia caused by vasopressin acting on the spinal vasculature (Riphagen and Pittman 1989). V1a receptors have been identified on the blood vessels and capillaries of the spinal cord gray matter (Sermasi, Howl et al. 1995), and intrathecal vasopressin has been shown to cause reductions in blood flow in spinal vessels (Long, Martinez-Arizala et al. 1989). However, this was seen with higher, paralysis-inducing doses of AVP; the dose used in this study showed no reduction in blood flow (Long, Martinez-Arizala et al. 1989). Additionally, Riphagen *et al.* (Riphagen and Pittman 1989) discuss a variety of reasons to conclude that spinal ischemia is an unlikely cause of the responses to i.t. AVP: AVP applied to the outside of intact pial vessels had no effect (Lassoff and Altura 1980); vasodilators do not reduce the pressor effects of i.t. AVP; and it is unlikely that spinal ischemia would affect SNA selectively, as has been demonstrated by i.t. AVP (Riphagen, Bauce et al. 1986; Riphagen and Pittman 1989).

While it remains possible that i.t. AVP acts in one or more of the above locations to ultimately increase MAP, the most likely site of action is in the IML. The spread of intrathecal AVP, specifically, has not been investigated; however, radiolabeled substance P - a peptide made of 11 amino acids - has been shown to spread far enough into the spinal cord to reach the IML one minute after injection (Cridland, Yashpal et al. 1987). It is very likely that vasopressin, a smaller peptide, is able to reach the IML with a similar efficiency. Therefore, the most probable conclusion for this study is that intrathecal vasopressin and the V1a agonist acted on the SPNs to elevate MAP, and this was blocked by pretreatment with the V1a antagonist. This means that it is likely that the antagonist effectively blocked receptors on the SPNs during i.v. hypertonic saline, water deprivation, and DOCA-salt hypertension, but the V1a receptor blockade did not reduce the elevated MAP during these conditions. Consequently, we can conclude that spinal V1a receptors are not required for the pressor responses under these acute and chronic conditions of osmotic stress.

Perspectives

Since the 1980's, a variety of neuropeptides and amino acids have been localized in descending fibers from supraspinal sites and proposed as likely putative neurotransmitters and/or neuromodulators of SPNs and therefore arterial pressure (Helke, Charlton et al. 1985; Krukoff 1987; Coote 1988; McCall 1988; Duggan 1995; Pyner 2009). However, at the present time there are very few studies in which spinal neurotransmitter systems have been studied in conscious animals under physiological conditions. Since the spinal cord is the final point of integration in the CNS, it is a promising site for novel therapies aimed at modulation of sympathetic nervous system activity. Targeted treatment at the level of the spinal cord could eliminate side effects that are associated with drugs that target brain neurotransmitter systems. In this study, despite anatomical and neurophysiological studies supporting the central hypothesis, intrathecal blockade of spinal V1a receptors had no effect on pressor responses to the three tested conditions of osmotic stress. Further studies will be needed to investigate the role of other spinal neurotransmitters, particularly glutamate, in regulating MAP during osmotic stress in conscious animals.

Grants

This study was supported by NIH grant R01 HL64176 to J.W.O.

Table 1 – Twelve-hour daytime averages of MAP, HR, and motor activity for rats in water deprivation experiment. Data is reported as mean ± standard error. * = $p < 0.05$ vs. control.

Table 1. Effect of Water Deprivation (WD) on Baseline Parameters

	MAP (mmHg)	HR (bpm)	Activity (counts)
Control	105 ± 2	426 ± 5	1.4 ± 0.3
24-hour WD	114 ± 2*	404 ± 10*	1.4 + 0.3
48-hour WD	119 ± 1*	420 ± 8	2.2 + 0.3*

Figure 1 – Effect of Intrathecal Injections in Normotensive Rats
A) MAP, HR, and motor activity responses to intrathecal injection of artificial cerebrospinal fluid (aCSF); B) MAP, HR, and motor activity effects of intrathecal injection of a V1a receptor agonist, with and without pretreatment (5 min prior) with intrathecal V1a receptor antagonist; C) MAP, HR, and motor activity effects of intrathecal injection of AVP, with and without pretreatment (5 min prior) with intrathecal V1a receptor antagonist. The dashed line indicates time=0, at which time the V1a agonist (or aCSF in Fig. 1a) was injected. The solid line indicates t= -5, at which time the V1a antagonist was injected. # = $p < 0.05$ within group for 0-60 min vs. t = -6 min. * = $p < 0.05$ V1a ago/AVP vs. antagonist-pretreated injection.

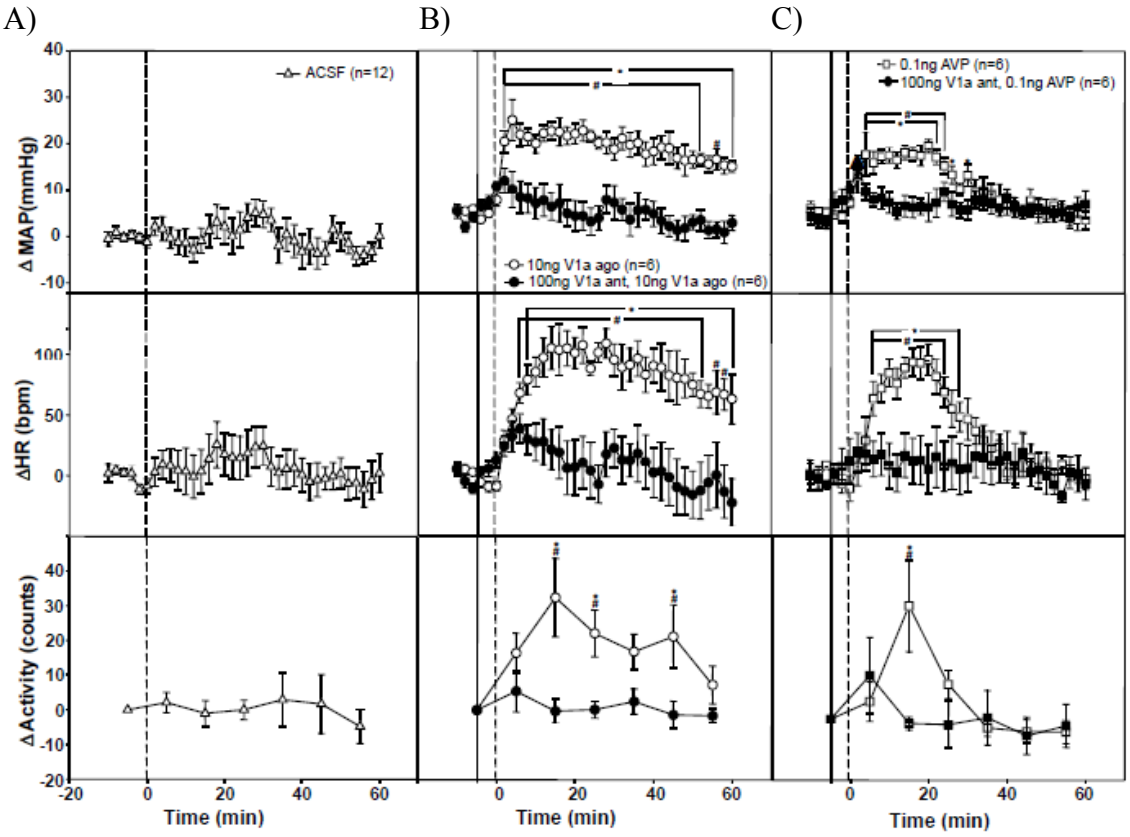


Figure 2 – Effect of Intravenous Hypertonic Saline on Plasma Osmolality
Plasma osmolality changes in response to intravenous injection of hypertonic saline or isotonic saline. # = $p < 0.05$ within group for 0-60 min vs. t = -5 min. * = $p < 0.05$ hypertonic saline vs. isotonic saline.

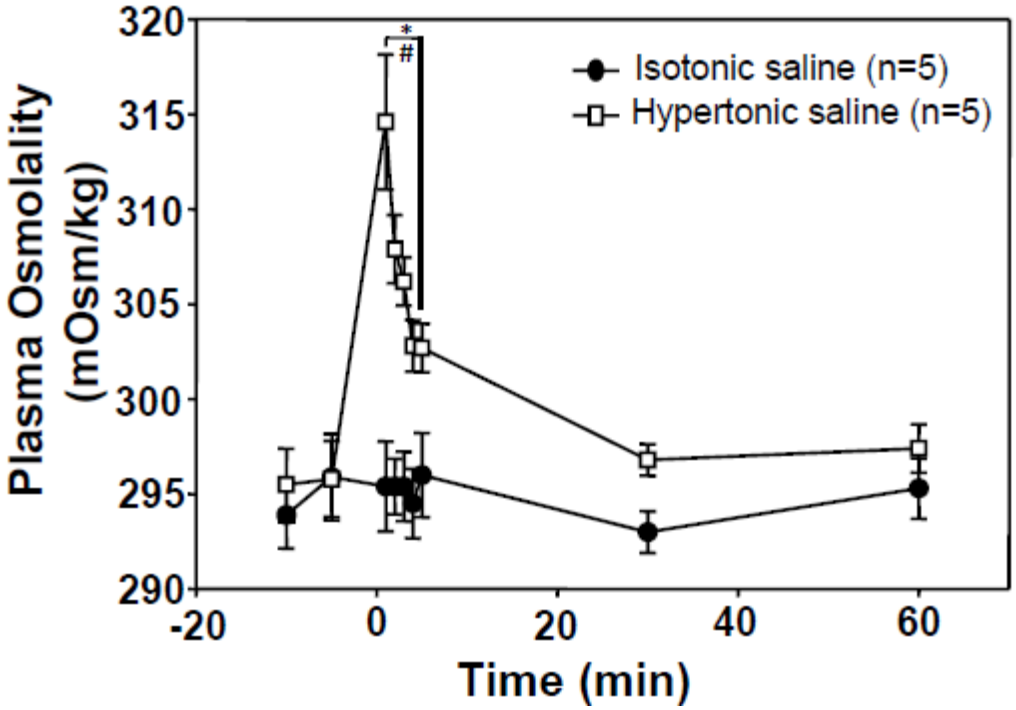


Figure 3 - Effect of Intrathecal V1a Antagonist in Rats Given Intravenous Hypertonic Saline

MAP and HR effects of intravenous hypertonic saline with and without pretreatment with an A) intravenous V1a receptor antagonist or B) intrathecal V1a receptor antagonist. Antagonist pretreatment occurred five minutes prior to hypertonic saline injection, indicated by the solid line. Hypertonic saline was administered at t = 0, indicated by the dashed line. # = $p < 0.05$ within group for 0-60 min vs. t = -6 min. * = $p < 0.05$ hypertonic saline vs. antagonist-pretreated injection.

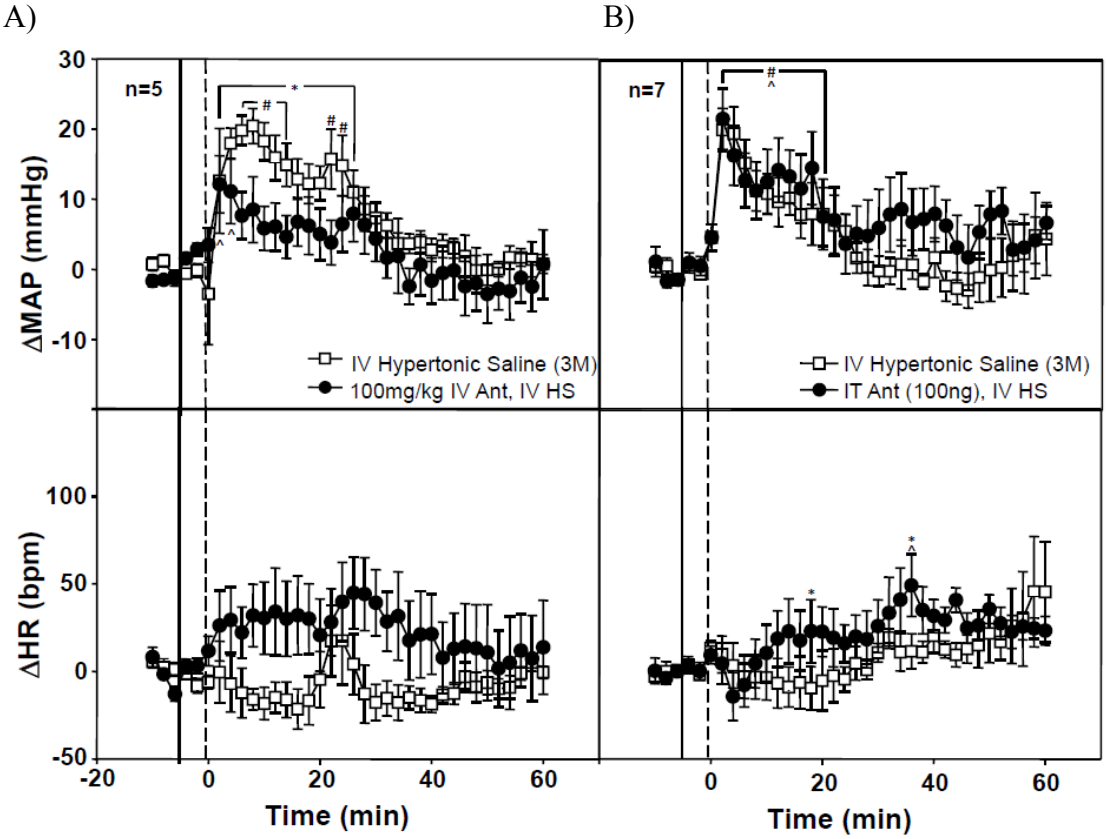


Figure 4 – Effect of Intrathecal V1a Antagonist in Water Deprived Rats
MAP and HR effects of intrathecal injection of a V1a receptor antagonist in rats deprived of water for A) 24 and B) 48 hours. There were no differences within groups.

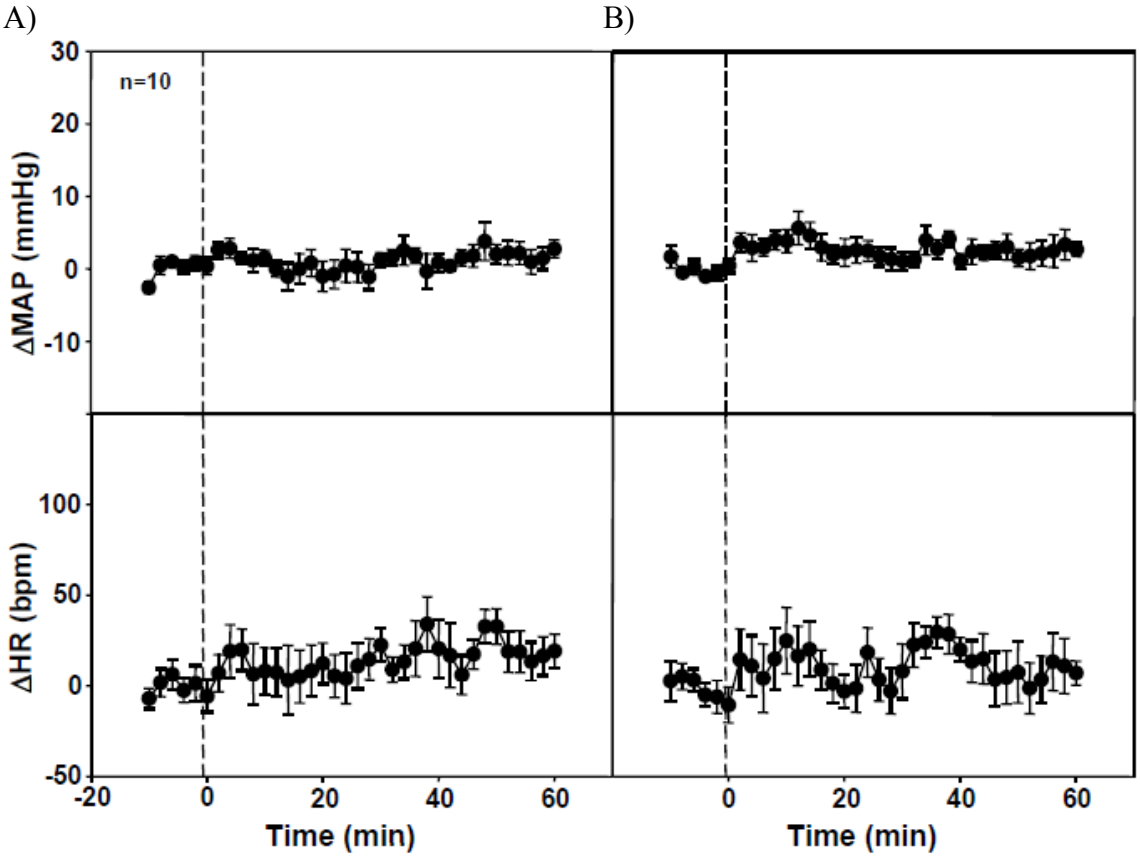
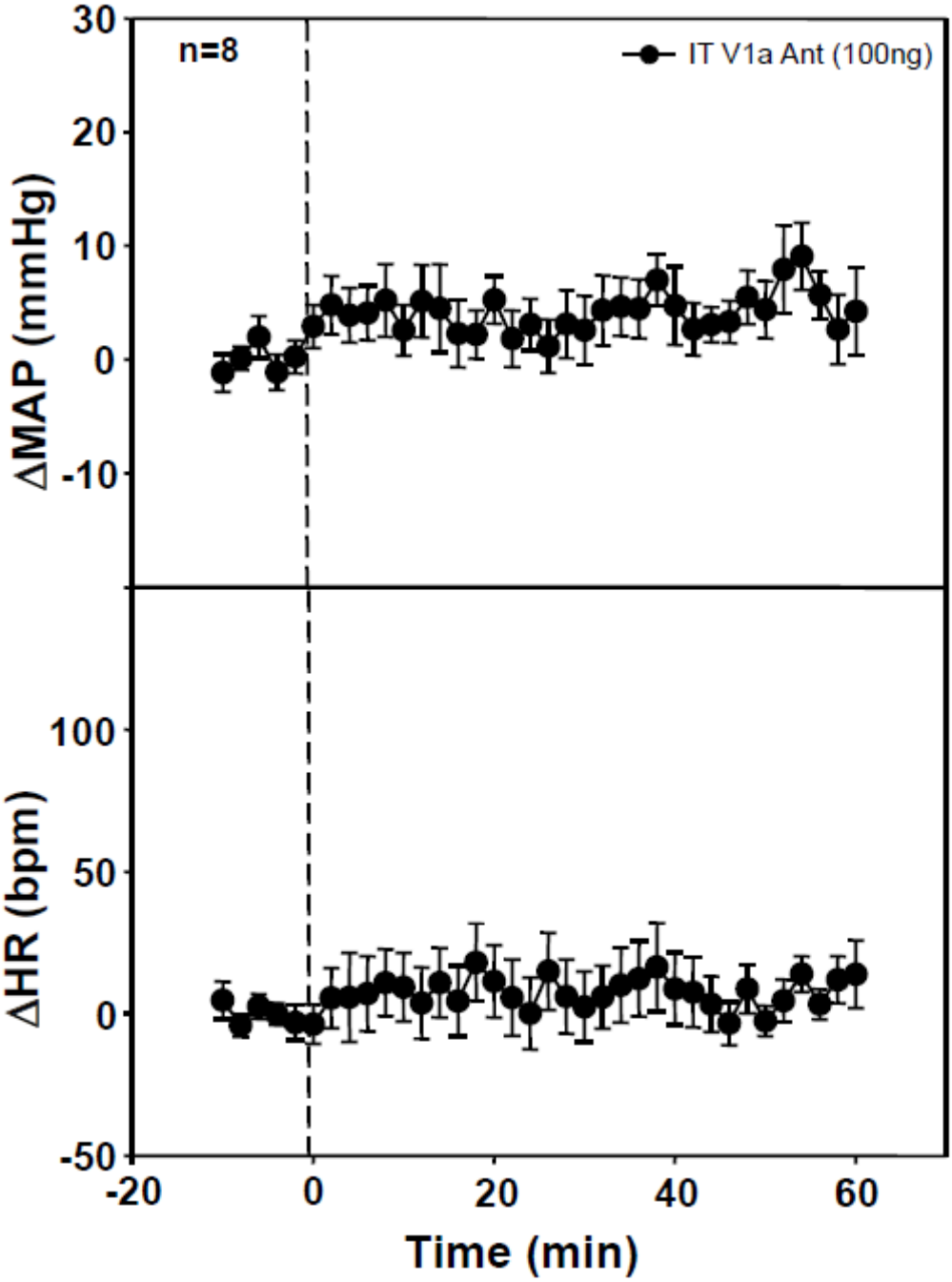


Figure 5 – Effect of Intrathecal V1a Antagonist in DOCA-salt Hypertensive Rats
MAP and HR effects of intrathecal injection of a V1a receptor antagonist in hypertensive rats after 4 weeks of DOCA-salt treatment. There were no differences within groups.



Chapter 3

Effects of intrathecal kynurenate on arterial pressure during chronic osmotic stress in conscious rats

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Submitted to Am J Physiol Heart Circ Physiol, August 2012

Increased plasma osmolality elevates mean arterial pressure (MAP) through activation of the sympathetic nervous system, but the neurotransmitters released in the spinal cord to regulate MAP during osmotic stress remain unresolved. Glutamatergic neurons of the rostral ventral lateral medulla (RVLM) project to sympathetic preganglionic neurons (SPNs) in the spinal cord and are likely activated during conditions of osmotic stress; however, this has not been examined in conscious rats. This study investigated whether increased MAP during osmotic stress depends on activation of spinal glutamate receptors. Rats were chronically instrumented with an indwelling intrathecal (i.t.) catheter for antagonist delivery to the spinal cord and a radiotelemetry transmitter for continuous monitoring of MAP and heart rate (HR). Osmotic stress induced by forty-eight hours of water deprivation increased MAP approximately 15 mmHg. Intrathecal kynurenic acid (KYN), a non-specific antagonist of ionotropic glutamate receptors, decreased MAP significantly more after 48 hours of water deprivation (WD) compared to the baseline response. Likewise, i.t. KYN decreased MAP more in an osmotically driven model of neurogenic hypertension - the DOCA-salt rat - compared to normotensive controls. Finally, i.t. 2-amino-5-phosphonovalerate (AP5) caused a greater fall in WD rats compared to baseline. Our results suggest that spinally released glutamate mediates increased MAP during 48-hr WD and DOCA-salt hypertension.

Key words: glutamate, kynurenic acid, intrathecal, water deprivation, DOCA-salt hypertension, sympathetic nerve activity

Introduction

Acute and chronic increases in plasma osmolality act in the forebrain to drive sympathetic outflow and affect mean arterial pressure (MAP) (for reviews, see (Toney, Chen et al. 2003; Stocker, Osborn et al. 2008; Toney and Stocker 2010). Work in recent years has identified brain pathways involved in osmotically driven increases in SNA, and the paraventricular nucleus (PVN) in particular has been identified as an important region (Stocker, Osborn et al. 2008); however, little is known about osmotically induced sympathetic activation at the level of the intermediolateral cell column (IML) of the spinal cord, where sympathetic preganglionic nerves originate. Most evidence suggests there are two primary projections from the PVN that influence sympathetic nerve activity (SNA) during osmotic stress: a direct vasopressinergic projection from the PVN to the IML, or a glutamatergic projection to the rostral ventrolateral medulla (RVLM) (Antunes, Yao et al. 2006; Stocker, Osborn et al. 2008; Veitenheimer and Osborn 2011). Therefore, the most likely neurotransmitters to drive IML neurons in response to increased plasma osmolality are vasopressin (from the PVN) and the neurotransmitters released by spinally projecting RVLM neurons.

Recently, our laboratory investigated the role of spinal vasopressin receptors during three conditions of osmotically driven increases in MAP: acute intravenous hypertonic saline, 48 hours of water deprivation, and 4 weeks of deoxycorticosterone acetate (DOCA) - salt treatment (Veitenheimer and Osborn 2011). We demonstrated that intrathecal blockade of spinal V1a receptors had no effect on MAP in any of the conditions. We concluded that spinally released vasopressin was *not* required for the pressor response to increased osmolality under the conditions we tested.

Glutamate is thought to be the principle neurotransmitter in the spinally projecting RVLM pathway involved in regulating SNA and MAP (Morrison 2003). Stimulation of the RVLM increases SNA with a timing that corresponds to fast excitatory neurotransmission like that of glutamate, and administration of glutamate receptor antagonists to the SPNs inhibits their response to the RVLM stimulation (Mills, Minson et al. 1988; Bazil and Gordon 1991; Morrison 2003). Likewise, application of glutamate

or its receptor agonists results in a depolarization and increased firing of sympathetic preganglionic neurons (SPNs) (Inokuchi, Yoshimura et al. 1992; Morrison 2003), while glutamate receptor antagonists inhibit basal SPN activity (Morrison 2003). Anatomically, glutamatergic nerve terminals from the RVLM have been localized in the IML (Morrison 2003) and there is evidence that both NMDA and non-NMDA receptors are located on SPNs (Inokuchi, Yoshimura et al. 1992). Despite the abundance of evidence to suggest glutamate as the primary neurotransmitter regulating SPNs to affect MAP, this has not been examined under conditions of increased osmolality in conscious rats.

Therefore, the present study was conducted to address the question of whether spinally released glutamate mediates osmotically driven increases in MAP in conscious rats. Our approach was similar to that of our recent report of spinally released vasopressin in rats during 48 hours of water deprivation and DOCA-salt hypertensive rats (Veitenheimer and Osborn 2011). Experiments were also conducted to confirm that the MAP responses to intrathecal delivery of antagonists were mediated by actions in the spinal cord rather than the brainstem. Finally, we examined which excitatory amino acid receptor subtypes were involved in the MAP response to osmotic stress.

Methods

Animals and General Protocols

Male Sprague Dawley rats from Charles River Laboratory (Wilmington, MA) spent the duration of the experimental protocols singly housed in their home cages in a temperature-controlled animal room with a 12-hour light/dark cycle. Unless otherwise noted, they had *ad libitum* access to normal rat chow (Lab Diet 5012) and deionized water. University of Minnesota's Institutional Animal Care and Use Committee approved all protocols in this study.

General Surgical Procedures:

All surgeries were performed under 2% isoflurane (following induction with 4%), and anesthesia depth was checked regularly by toe and tail pinch. Rats were given atropine sulfate (0.2 mg/kg i.p., Baxter Healthcare Corporation) and gentamicin sulfate (2.0 mg i.m., Hospira) on the day of surgery. For three days postoperatively, amoxicillin (1mg/ml, West-ward Pharmaceutical Co.) was added to the drinking water and either buprenorphine (0.3mg/ml in 0.02ml, PharmaForce) or ketoprofen (2.5mg/kg, subcutaneous, Fort Dodge Animal Health) were administered for analgesia.

Rats were instrumented with radiotelemetry transmitters (model TA11PA-C40, Data Sciences International, St. Paul, MN) for continuous measurement of mean arterial pressure (MAP) and heart rate (HR). Transmitter catheters were implanted into the descending aorta by way of the femoral artery. Additionally, all rats received intrathecal (i.t.) catheters to allow for drug delivery to the spinal cord. Briefly, a 32-gauge catheter (ReCathCo, LLC; Allison Park, PA) was inserted between L6 and S1 vertebrae and advanced 6.5-7 cm along the intrathecal space until the tip lay between spinal segments T10-T13. The catheter was attached to PE10 tubing (Intramedic TM; BD, Sparks, MD) and tunneled under skin to exit between the scapulae. It was then threaded through a spring and attached to a swivel above the rat's home cage to allow unrestricted movement throughout the protocol. Both the transmitter and intrathecal catheter surgeries were described previously in greater detail (Veitenheimer and Osborn 2011). The day before baseline data collection began, intrathecal catheter placement was confirmed by transient

hind limb paralysis after administration of 20µl of lidocaine (10mg/ml, Hospira) into the intrathecal space; rats in which lidocaine did not elicit paralysis were eliminated from the study.

Unless otherwise noted, the rats in this study were conscious and freely moving in their home cages. For all of the experiments conducted in conscious rats, at least 5 days of recovery were given following surgery. Each cage was placed on a receiver (model RPC1) that was connected to a computer via a Data Exchange Matrix (DSI; St. Paul, MN). At the time of intrathecal injections, MAP and HR data were collected continuously at 500Hz over 10 seconds. Otherwise, MAP and HR data were collected for 10 seconds every 4 min at 500Hz from the beginning of the protocol to the time of euthanasia. The intrathecal injections in this study were given at a rate of ~0.5µl/sec in volumes of 10µl followed by a flush of 25µl of artificial cerebrospinal fluid (ACSF). Upon completion of the study, rats were anesthetized with isoflurane, flushed with saline, and 10µl of Chicago Blue dye was injected into the i.t. catheter. The spinal cord and brainstem were exposed and the tip of the catheter and spread of dye was carefully noted in each rat.

Experimental Protocols

Effect of Intrathecal Kynurenic Acid on Arterial Pressure and Heart Rate Before and During Water Deprivation

These experiments were designed to determine the effect of the non-specific glutamate receptor antagonist, kynurenic acid (kynurenate, KYN), on the pressor response to water deprivation in conscious rats. The dose of i.t. KYN, 50mM, was based on a previously published study (Verberne, Widdop et al. 1990). KYN was dissolved in NaOH and titrated to a final pH of 7.4. In pilot studies, we established that intrathecal injection of the vehicle for KYN did not lower MAP (ΔMAP = 10$\pm$4 mmHg at t=30min) or HR (ΔHR = 31$\pm$25 bpm at t=30min), similar to what we observed following intrathecal ACSF (Veitenheimer and Osborn 2011). Additionally, systemic (intravenous) administration of 50mM KYN had no effect on MAP (ΔMAP = -1$\pm$3 mmHg at t=30min)

or HR (ΔHR = 2±21 bpm at t=30min). These results confirm that decreases in MAP or HR following i.t. KYN were not secondary to effects of vehicle or leakage into the systemic circulation.

Following recovery from surgery, MAP and HR were monitored for 2 days of baseline followed by 48 hours of water deprivation. Intrathecal KYN was administered at approximately 1200h on day 2 of baseline and day 2 of water deprivation.

Effect of Intrathecal Kynurenic Acid on Arterial Pressure and Heart Rate in DOCA-salt Rats

DOCA-sat rats were prepared as in a previous study from our group (Osborn, Jacob et al. 2006). Briefly, the left kidney was exposed via a retroperitoneal incision; the renal artery, vein and ureter were tied off and cut; and the kidney was removed. The muscle layer was closed and a silicone pellet containing 50mg of DOCA was cut into small pieces and placed subcutaneously. The skin incision was closed with surgical clips. In sham rats, the left kidney was exposed and a silicone pellet containing 0mg DOCA was implanted subcutaneously. After surgery, rats were housed individually and given 0.1% sodium food and 0.9% NaCl + 0.2% KCl solution to drink *ad libitum*. Sham rats were given deionized water *ad libitum*. Three weeks later (day 23), transmitters and intrathecal catheters were implanted as described above. On day 29, the MAP and heart rate responses to intrathecal KYN were measured.

Comparison of the Arterial Pressure and Heart Rate Responses to Intrathecal Kynurenic Acid Injected in the Lower and Upper Thoracic Cord

Since it is possible that i.t. KYN could diffuse to the brainstem, the cardiovascular responses to i.t. KYN could be due to blockade of glutamate actions on the sympathetic premotor neurons in the RVLM, rather than blockade of the spinal actions of glutamate. We reasoned that if this were the case, the MAP and heart rate responses to i.t. KYN in the upper thoracic cord would be similar to the responses to KYN injected in the lower thoracic cord.

The following protocol was conducted to address this possibility in isoflurane anesthetized rats. Telemetry transmitters and intrathecal catheters were implanted as described above. In one group of rats, the intrathecal catheter was advanced 6.5-7 cm rostrally to segments T10-T13 as described above. In another group of rats, the catheter was advanced rostrally 10 cm so the tip was in the T1-T5 region. Isoflurane was maintained at 1.75-2% for the remainder of the experiment. Anesthesia depth was verified every 15 minutes by lack of response to tail pinch. Following a 20- minute stable recording period, i.t. KYN was administered and MAP and heart rate were monitored over a 30-minute post injection period.

Contribution of Excitatory Amino Acid Receptor Sub-types to the Regulation of Arterial Pressure and Heart Rate During Water Deprivation

Since kynurenate can block both NMDA and non-NMDA receptor sub-types (Stone 1993), the goal of these experiments was to examine if NMDA and/or non-NMDA receptor blockade affected the pressor response to water deprivation. Following the same protocol as the water deprivation experiment described above, on day 2 of baseline and day 2 of water deprivation, rats received i.t. administration of the specific NMDA receptor antagonist, DL-2-Amino-5-phosphonopentanoic acid (AP5) or the specific non-NMDA receptor antagonist 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX).

Doses for AP5 (20mM) and CNQX (5mM) were based on published reports (Hong and Henry 1992; Feldman and Buccafusco 1997) and preliminary studies in 2 groups of isoflurane anesthetized rats. In one group, responses to i.t. N-methyl-D-aspartic acid (NMDA; 10mM) or α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA; 50μM) were measured five minutes after vehicle pretreatment (i.t. ACSF). In the second group, one of the antagonists (AP5 or CNQX) was administered and then, 5 minutes later, the appropriate agonist (NMDA or AMPA) was administered.

Drugs

Unless otherwise noted, the drugs used in this study were purchased from Sigma-Aldrich (St. Louis, MO). The drugs used were ACSF (Harvard Apparatus, Holliston, Massachusetts), kynurenate, NMDA, AP5, AMPA, and CNQX.

Data Analysis and Statistics

Dataquest A.R.T. 4.0 software (DSI; St. Paul, MN) was used to acquire and analyze the MAP and HR data. SigmaStat software (3.5, San Jose, CA) was used to identify statistical differences. For all comparisons, a p value of <0.05 was defined as statistically significant.

To summarize the effects of WD (Figures 1A & 4A) or DOCA-salt treatment (Figure 2A), MAP and HR were grouped by lights-on/lights-off (light/dark) phases and plotted as bar graphs. The last 8 hours of baseline (separated by light/dark phase) were averaged and compared to the last 8 hours of water deprivation (separated by light/dark phase). These data were analyzed using a two-way ANOVA and the Holm-Sidak post hoc test, with the light phase and baseline period data set as the controls between and within groups, respectively.

Experiments in water deprived rats followed a within-animal design whereas protocols in anesthetized rats and DOCA-salt rats followed a between-animal design. MAP and HR responses to i.t. injections were plotted at 2-minute intervals as the difference from baseline. The data were analyzed using a two-way repeated measures analysis of variance (ANOVA) with the Holm-Sidak post hoc test. The response during baseline was defined as the control between groups, and the t = -6 min. time point was used as the control within groups. The maximum MAP effects occurred at approximately t=30 min. for i.t. KYN, t=6 min. for i.t. AP5, and t=10 min. for CNQX. Therefore, data at these time points were reported as absolute values.

Results

Effect of Intrathecal Kynurenic Acid on Arterial Pressure and Heart Rate Before and During Water Deprivation

Figure 1A shows values for mean arterial pressure (MAP) and heart rate (HR) during the final 4 hours of the light/dark-phases for the baseline period and at the end of 48 hours of WD. MAP increased approximately 15mmHg above baseline by the end of 48 hours of water deprivation during both the dark and light phases. Heart rate decreased ~45 bpm (dark phase) and ~20 bpm (light phase) with water deprivation. These responses are in agreement with our previously published studies (Veitenheimer and Osborn 2011; Veitenheimer, Engeland et al. 2012, in press.) and were consistent across all of the water deprivation experiments in the present study. Although we did not measure plasma osmolality in this study, we have previously reported that it increases on the average of 13 $\pm$1 mOsm/kg by the end of 48 hours of WD (Veitenheimer and Osborn 2011; Veitenheimer, Engeland et al. 2012, in press.).

The MAP and HR responses to i.t. KYN before and during water deprivation are shown in figure 1B. In the water-replete state (prior to water deprivation), intrathecal kynurenate decreased MAP ~20 mmHg to an absolute value of 76 $\pm$ 4 mmHg (at t=30). HR also fell ~100 bpm to 309 $\pm$ 16 bpm (at t=30). After 48 hours of WD, intrathecal KYN decreased MAP (~50 bpm) and HR (~100 bpm) levels to 60 $\pm$ 4 mmHg and 265 $\pm$ 9 bpm (at t=30), respectively. The KYN-induced fall in MAP was significantly greater during the water-deprived state compared to the water-replete state (Fig. 1B).

Effect of Intrathecal Kynurenic Acid on Arterial Pressure and Heart Rate in DOCA-salt Rats

As shown in Figure 2A, MAP was significantly higher in DOCA-salt rats during both light and dark phases compared to sham rats. On the other hand, HR was lower than sham rats during both the light and dark phase. As shown in Figure 2B, intrathecal KYN decreased MAP significantly more in DOCA-salt rats (~50 mmHg) than the sham group (~20 mmHg). Similarly, the response of HR in DOCA-salt rats (~100 bpm) was

significantly more than sham rats. At t=30 min, MAP was 93 ± 10 mmHg in DOCA-salt rats compared to 79 ± 4mmHg in sham rats, and HR was 294 ± 22 bpm in DOCA-salt rats compared to 375 ± 18 bpm in sham rats.

Comparison of the Arterial Pressure and Heart Rate Responses to Intrathecal Kynurenic Acid Injected at Lower and Upper Thoracic Levels

We investigated the possibility that MAP and HR responses to i.t. KYN were due to diffusion of KYN to the brainstem rather than actions in the spinal cord. We reasoned that if responses to i.t. KYN were due to actions in the brainstem, injections in the upper thoracic cord would produce responses similar to injections at the lower thoracic cord. However, we observed the opposite (Fig. 3). Rats with the catheter placed near spinal segments T1-T5 exhibited a much smaller MAP response to i.t. KYN compared to rats with catheters placed in the T10-T13 region of the cord. Although there were no significant differences in HR between groups, the decrease in HR tended to fall at an earlier time point in rats with the upper thoracic catheter. Catheter placement was confirmed post-mortem. Blue dye was seen throughout the thoracic spinal region in rats with the catheter in the lower thoracic region. In contrast, catheters placed in the upper thoracic cord showed most of the blue dye in the upper thoracic and cervical segments, with some dye in brainstem regions and only faint dye in the lower thoracic segments.

Contribution of Excitatory Amino Acid Receptor Sub-types to the Regulation of Arterial Pressure and Heart Rate During Water Deprivation

Doses of NMDA receptor and non-NMDA receptor antagonists were determined in pilot studies in anesthetized, euhydrated rats (n=2/group). Intrathecal administration of NMDA (100nmol) increased MAP 45±18 mmHg (at t=6min), and this response was prevented by pretreatment with 20mM AP5 (-15±8 mmHg at t=6min). Additionally, intrathecal administration of AMPA (0.5nmol) increased MAP by 11±2 mmHg (at t=6min), and this was prevented by pretreatment with 5mM CNQX (-1±1 mmHg at t=6min). Therefore, these doses of AP5 and CNQX were selected for administration in conscious, water-deprived rats.

Figure 4A shows the MAP and HR responses to 48 hours of WD. Similar to Figure 1A, 48 hours of WD increased MAP ~15 mmHg and decreased HR ~25 bpm. Figure 4B shows that intrathecal AP5 had no significant effect on MAP prior to WD but significantly decreased MAP after 48h of water deprivation (at t=6min, ΔMAP = -13$\pm$4 mmHg). HR was not significantly affected by AP5 under baseline or water-deprived conditions (Fig. 4B). In contrast to AP5, intrathecal CNQX unexpectedly caused an *increase* in MAP (~ 10 mmHg) and HR (~ 75 bpm) in both water-replete and water-deprived conscious rats. This response was associated with noticeable behavioral and motor responses - particularly hindlimb scratching - that compromised the interpretation of the cardiovascular effects (data not shown). There was no difference between the water-replete and water-deprived responses to i.t. CNQX (data not shown).

Discussion

Forty-eight hours of water deprivation and DOCA-salt hypertension are both accompanied by increased plasma osmolality (Brooks, Qi et al. 2005; O'Donaughy and Brooks 2006; O'Donaughy, Qi et al. 2006; Veitenheimer and Osborn 2011), which is hypothesized to increase sympathetic nerve activity (SNA) and elevate mean arterial pressure (MAP) acutely and chronically (for reviews, see (Stocker, Osborn et al. 2008; Toney and Stocker 2010)). Consistent with this hypothesis, we recently reported that the pressor response to 48 hours of water deprivation in conscious rats is prevented by chronic adrenoreceptor blockade (Veitenheimer, Engeland et al. 2012, in press.). Evidence also suggests that DOCA-salt hypertension is mediated by osmotic activation of the sympathetic nervous system (O'Donaughy and Brooks 2006; O'Donaughy, Qi et al. 2006). The central pathways and neurotransmitters mediating osmotically driven sympathoexcitation and hypertension are incompletely understood. Elucidating these mechanisms is essential to understanding the etiology of salt-sensitive hypertension and development of new therapies.

Compared to the brain and peripheral neural pathways, the *spinal* mechanisms mediating osmotically driven increases in arterial pressure are relatively unexplored. Moreover, the spinal cord has received little attention as a therapeutic target for the treatment of hypertension and other diseases associated with increased activity of the sympathetic nervous system. With this goal in mind, we have developed methods to measure cardiovascular responses to intrathecal delivery of specific neurotransmitter receptor antagonists in chronically instrumented, conscious, freely moving rats. This approach is necessary since surgical stress and anesthesia alter basal sympathetic activity, which may compromise the physiological significance some findings.

For example, based on several studies suggesting that a spinally projecting vasopressinergic pathway (Sawchenko and Swanson 1982; Ranson, Motawei et al. 1998; Hallbeck and Blomqvist 1999) from the paraventricular nucleus was activated in response to increased plasma osmolality (Antunes, Yao et al. 2006), we recently investigated the role of spinal V_{1a} receptors under conditions of acute and chronic osmotic stress in conscious rats (Veitenheimer and Osborn 2011). We found that

blockade of spinal V_{1a} receptors in conscious rats had no effect on arterial pressure under conditions of 48 hours of water deprivation or DOCA-salt induced hypertension (Veitenheimer and Osborn 2011). We concluded that, despite neuroanatomical and neurophysiological evidence for a role of spinal V1a receptors in control of sympathetic activity, this mechanism was not responsible for osmotically driven sympathetic activity and increased arterial pressure in conscious rats.

Therefore, the present study was conducted to investigate the role of another potential spinally released neurotransmitter under conditions of osmotic stress – glutamate. In this study we found that blockade of glutamate receptors with intrathecal kynurenate resulted in a significantly greater fall in arterial pressure after 48 hours of water deprivation compared to the water-replete state. Likewise, DOCA-salt rats showed a significantly greater fall in pressure in response to intrathecal kynurenate than normotensive sham rats. Finally, blockade of NMDA receptors attenuated arterial pressure in the water-deprived, but not water-replete state, suggesting the pressor response to water deprivation relies, at least in part, on NMDA receptors. A more detailed discussion of our findings and their relationship to previous studies is described below.

Effect of Intrathecal Kynurenic Acid on Arterial Pressure and Heart Rate Before and During Water Deprivation

In agreement with our previous studies (Veitenheimer and Osborn 2011) (Veitenheimer 2012), 48 hours of water deprivation increased arterial pressure approximately 15 mmHg above baseline. In contrast to our report that blockade of spinal V1a receptors had no effect on arterial pressure in water-deprived rats (Veitenheimer and Osborn 2011), we observed in the present study that intrathecal kynurenate decreased arterial pressure significantly more after 48-hours of water deprivation than in the water-replete state. Taken together these studies suggest that spinally released glutamate, but not vasopressin, is responsible for the elevation of arterial pressure during water deprivation.

One complicating factor in interpreting the magnitude of the decrease in arterial pressure as a reflection of sympathetic activity is that other factors may play a role. For example, although arterial pressure was elevated after 48 hours or water deprivation, the absolute level of arterial pressure fell to a lower level after intrathecal KYN compared to water-replete conditions. One explanation for this may be that blood volume is reduced in water-deprived rats, so vasodilation secondary to decreased sympathetic nerve activity with intrathecal KYN results in a lower arterial pressure than the water-replete state with normal blood volumes. Although this would contribute to the magnitude of the *change* in arterial pressure following intrathecal KYN, in addition to the neurogenic component, it does not change our interpretation of our findings - that the increase in arterial pressure after 48 hours of water deprivation is reversed by intrathecal KYN.

We observed that intrathecal KYN decreased arterial pressure in water replete rats approximately 20 mmHg with the maximum fall occurring at ~15min. This is a smaller and faster response than that reported by Verberne et al. (Verberne, Widdop et al. 1990) using the same dose of KYN in conscious rats. Similarly, the heart rate response we observed in the water-replete rats was smaller and faster than that reported by Verberne et al. In that study, the catheter tip was positioned at segments T8-T10, in contrast to our study in which the tip was positioned at segments T10-T13. Another possible explanation is that the injection rate may have been different which would have affected the rostral-caudal spread of injected kynurenate. A third difference between these studies was in the technique used to measure arterial pressure. Verberne et al. measured arterial pressure directly through an aortic cannula connected to an external pressure transducer after rats were moved to a separate experimental room, which may have induced a stress response. In contrast, our studies were conducted by telemetry while rats rested in their home cages.

Effect of Intrathecal Kynurenic Acid on Arterial Pressure and Heart Rate in DOCA-salt Rats

Similar to water-deprived rats, intrathecal KYN caused a greater decrease in arterial pressure in hypertensive DOCA-salt rats than normotensive sham rats.

Intrathecal KYN was administered after 4 weeks of DOCA-salt treatment - well into the established phase of hypertension (Abrams, Engeland et al. 2010) - suggesting that spinal glutamate receptors are involved in the maintenance of hypertension in DOCA-salt rats. However, whether spinally released glutamate is involved in the developmental phase of DOCA-salt hypertension remains to be determined.

In contrast, blockade of spinal V1a receptors has no effect on arterial pressure in DOCA-salt rats (Veitenheimer) despite implications that the PVN mediates increased sympathetic activity in this model (Abrams, Engeland et al. 2010). Taken together, these findings suggest that, although increased activity of the PVN in DOCA-salt hypertension may occur, increased arterial pressure in this model is not driven by spinally projecting vasopressinergic neurons from the PVN. The results of the present study are more consistent with the hypothesis that glutamatergic inputs to spinal sympathetic preganglionic neurons, perhaps from the rostral ventrolateral medulla (RVLM), mediate increased sympathetic activity in DOCA-salt rats. Further studies are needed to test this hypothesis.

It is important to note that absolute level of arterial pressure after intrathecal KYN was higher in DOCA-salt rats than in sham rats. This is consistent with the idea that DOCA-salt hypertension is not entirely mediated by increased sympathetic activity and that another mechanism, such as increased plasma vasopressin (Berecek, Murray et al. 1982; O'Donaughy, Qi et al. 2006; Abrams, Engeland et al. 2010), also contributes to maintenance of hypertension in DOCA-salt rats.

Contribution of Excitatory Amino Acid Receptor Sub-types to the Regulation of Arterial Pressure and Heart Rate During Water Deprivation

Since kynurenate is a non-specific antagonist of ionotropic glutamate receptors (Stone 1993), we attempted to investigate the contribution of NMDA and non-NMDA receptors to the pressor response to water deprivation. AP5, a specific NMDA receptor antagonist, had no effect on arterial pressure in the water-replete state, but significantly decreased arterial pressure in water-deprived rats. This finding suggests that spinal NMDA receptors are involved in the arterial pressure response to water deprivation. To

our knowledge, the cardiovascular responses to intrathecal AP5 in conscious rats have not been previously reported. In anesthetized rats, intrathecal AP5 decreases arterial pressure of approximately 10-20 mmHg (Hong and Henry 1992), which is in agreement with results of our pilot studies in anesthetized rats to establish the dose used in the present study in conscious rats.

We also investigated the cardiovascular responses to intrathecal CNQX, an antagonist for non-NMDA receptors. Preliminary experiments in anesthetized rats (n=2/group) were conducted to establish the dose of the antagonist to be used in conscious rats. This was done since intrathecal injection of agonists for non-NMDA receptors would likely produce hyperalgesia and somatic motor responses in conscious rats. The dose of CNQX used (5mM) had no significant effect on MAP or HR in isoflurane anesthetized rats, similar to findings by Feldman et al. who reported no change in MAP or HR with intrathecal CNQX (1mM and 10mM) in anesthetized rats (Feldman and Buccafusco 1997). Unfortunately, the results of experiments in conscious rats were uninterpretable due to increased motor activity and scratching, suggesting the rats may have experienced pruritis or analgesia in response to intrathecal CNQX. As a result, CNQX increased both MAP and HR during baseline and water-deprived periods. Because of these behavioral responses to intrathecal CNQX, the contribution of non-NMDA receptors to pressor response to water deprivation in conscious rats remains unclear.

Putative Sites of Action Mediating the Cardiovascular Responses to Intrathecal Kynurenic Acid

Intrathecal catheterization is a useful technique for studying functional outcomes of spinal neuronal activation or inhibition in conscious animals. However, it is not possible to know the precise site of action of the injectate, either along the rostral-caudal axis of the spinal cord, or across a single segment (i.e. IML, ventral and dorsal horns). Therefore, we cannot conclude that the effects of intrathecal KYN on MAP were due to blockade of the actions of glutamate on SPNs specifically. Indeed, spinal interneurons are thought to receive glutamatergic input, (Llewellyn-Smith, Martin et al. 2007) and

glutamate labeling remains following spinal transection (Llewellyn-Smith, Cassam et al. 1997; Llewellyn-Smith and Weaver 2001; Llewellyn-Smith, Martin et al. 2007), suggesting that not all spinally released glutamate comes from supraspinal sites. Therefore, although our results demonstrate that glutamate receptors mediate the pressor responses to water deprivation and DOCA-salt treatment, further studies are needed to identify the specific KYN-sensitive spinal neurons involved.

The rostral-caudal distribution of intrathecally administered KYN is particularly important in this study because evidence suggests that sympathetic premotor neurons in the RVLM are regulated by glutamatergic neurons from the PVN during osmotic stress (Brooks, Freeman et al. 2004; Brooks, Freeman et al. 2004; Stocker, Simmons et al. 2006). This raises the possibility that the effects of intrathecal KYN in this study were secondary to blockade of glutamate receptors in the RVLM or other brainstem nuclei, rather than the spinal cord. We addressed this possibility by comparing the cardiovascular responses in anesthetized rats to intrathecal administration of KYN in the lower thoracic cord (segments T10-T13) to those in the upper thoracic cord (segments T1-T5). We reasoned that, if the cardiovascular responses were due to blockade of the effects of glutamate in the brainstem, the MAP response to administration of KYN in the upper thoracic cord would be the same or greater than that observed in response to injections in the lower thoracic cord. However, we observed the opposite; the decrease in MAP following injection of KYN to the upper thoracic cord was significantly less compared to injections in the lower thoracic cord. In contrast, the heart rate response was similar, which is consistent with the fact that cardiac preganglionic neurons are located in T1-T5 segments. It is important to note that previous studies have shown that kynurenate administered directly into the RVLM in rats has minimal effects on MAP in normal rats (reviewed in (Sved, Ito et al. 2002; Dampney, Horiuchi et al. 2003)). Additionally, post-mortem analysis of dye spread indicated that lower thoracic injections were mostly limited to the thoracic spinal cord region, while the upper thoracic injections affected the thoracic, cervical, and possibly brainstem regions. We conclude that MAP response to intrathecal KYN in conscious rats is mostly, if not entirely, due to blockade of glutamate receptors in the *spinal cord.*

Finally, in addition to cardiovascular responses, intrathecal kynurenate caused motor paralysis, particularly in the hindlimbs. This is consistent with the findings from Verberne et al. in conscious rats and may be due to blockade of NMDA receptors in lumbar neurons (Cahusac, Evans et al. 1984; Verberne, Widdop et al. 1990). It is unlikely that the cardiovascular effects of intrathecal kynurenate are secondary to paralysis since we observed the same cardiovascular responses in anesthetized rats. Interestingly, the timing of the forelimb paralysis also serves as an indicator of the rostral spread of KYN. We observed the majority of the fall in MAP occurs within 15 minutes of intrathecal KYN, prior to forelimb paralysis which typically occurs between 20-30 minutes post injection. This observation is consistent with the results of the upper/lower thoracic injections that indicated that the MAP response is due to a spinal action, since diffusion of the injectate to the most rostral cord appears to occur after the maximal MAP response.

Perspectives

Our results further support to the idea that spinal glutamate is an important regulator of sympathetic nerve activity and MAP. To our knowledge, these are the first studies in conscious rats investigating the role of spinal glutamate in regulation of MAP during acute and chronic osmotic stress. A direct vasopressinergic pathway from the PVN to the spinal cord has been hypothesized to play a role in regulating MAP (Pyner 2009) (Riphagen and Pittman 1986) (Porter and Brody 1986) (Yang and Coote 2007) during osmotic stress (Riphagen and Pittman 1985; Gruber and Eskridge 1986; Antunes, Yao et al. 2006; Stocker, Osborn et al. 2008; Toney and Stocker 2010). However, our recent findings (Veitenheimer and Osborn 2011) combined with the present study suggests that spinal glutamate - but not vasopressin - receptors are required for the pressor responses to increased osmolality in conscious rats. Although this supports the hypothesis that the RVLM-spinal, rather than a PVN-spinal pathway is activated during osmotic stress, two additional explanations are evident. First, the data here could reflect antagonism of glutamate receptors on interneurons rather than SPNs. Second, there may be a role for a PVN-spinal that is glutamatergic (Yang, Wheatley et al. 2002), as

suggested by findings that all excitatory neurons that synapse on SPNs contain glutamate (Llewellyn-Smith, Minson et al. 1995). Whether other neuropeptides, besides vasopressin, interact with glutamate and are required in the pressor responses to osmotic stress remains to be determined.

Funding

This work was supported by a National Heart, Lung, and Blood Institute Grant (R01 HL-076312) awarded to the Neurogenic Cardiovascular Diseases Consortium. Britta Veitenheimer was supported by a Predoctoral Neuroscience Training Grant (NIH #T32 GM008471-17) and the University of Minnesota Doctoral Dissertation Fellowship.

Disclosures

J.W.O. is a consultant for Medtronic Cardiovascular.

Figure 1 – MAP and HR responses to Intrathecal KYN during Water Deprivation
A) Four-hour average MAP and HR values from the light and dark phases during baseline and at the end of 48 hours of water deprivation. B) Intrathecal kynurenate responses plotted as a change from 10-minute baseline during the water-replete baseline period and after 48 hours of water deprivation. # = $p < 0.05$ within baseline group. ^ = $p < 0.05$ within water-deprived group. * = $p < 0.05$ between baseline and water-deprived groups.

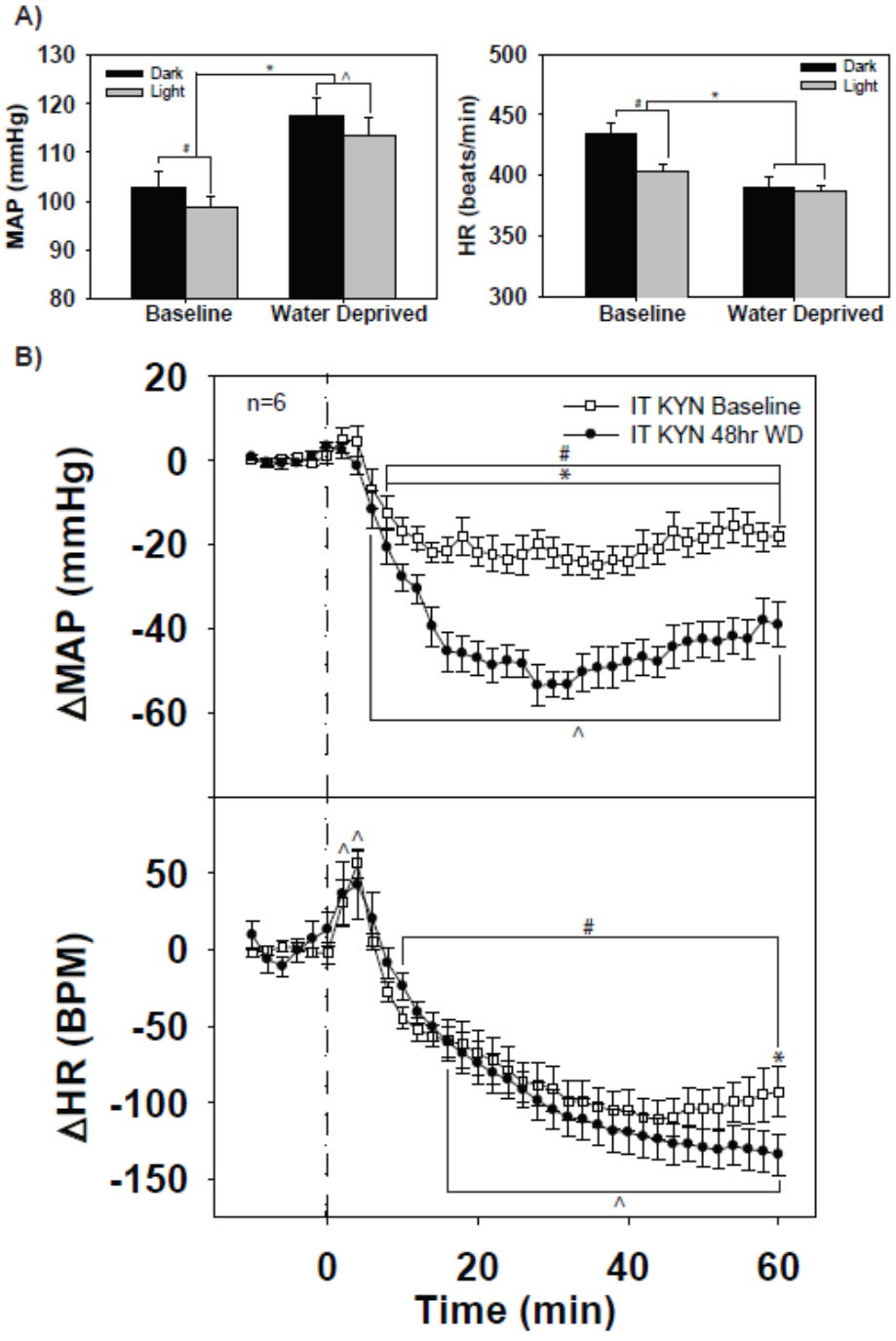


Figure 2 – MAP and HR responses to Intrathecal KYN during DOCA-salt Hypertension

A) 12-hour averages of day (light phase) and night (dark phase) values of MAP and HR in sham and DOCA-salt rats on Day 28 of treatment. B) Intrathecal kynurenate responses are plotted as a change from 10-minute baseline in sham rats and DOCA-salt rats. # = $p < 0.05$ within sham group. ^ = $p < 0.05$ within DOCA-salt group. * = $p < 0.05$ between sham and DOCA-salt rats.

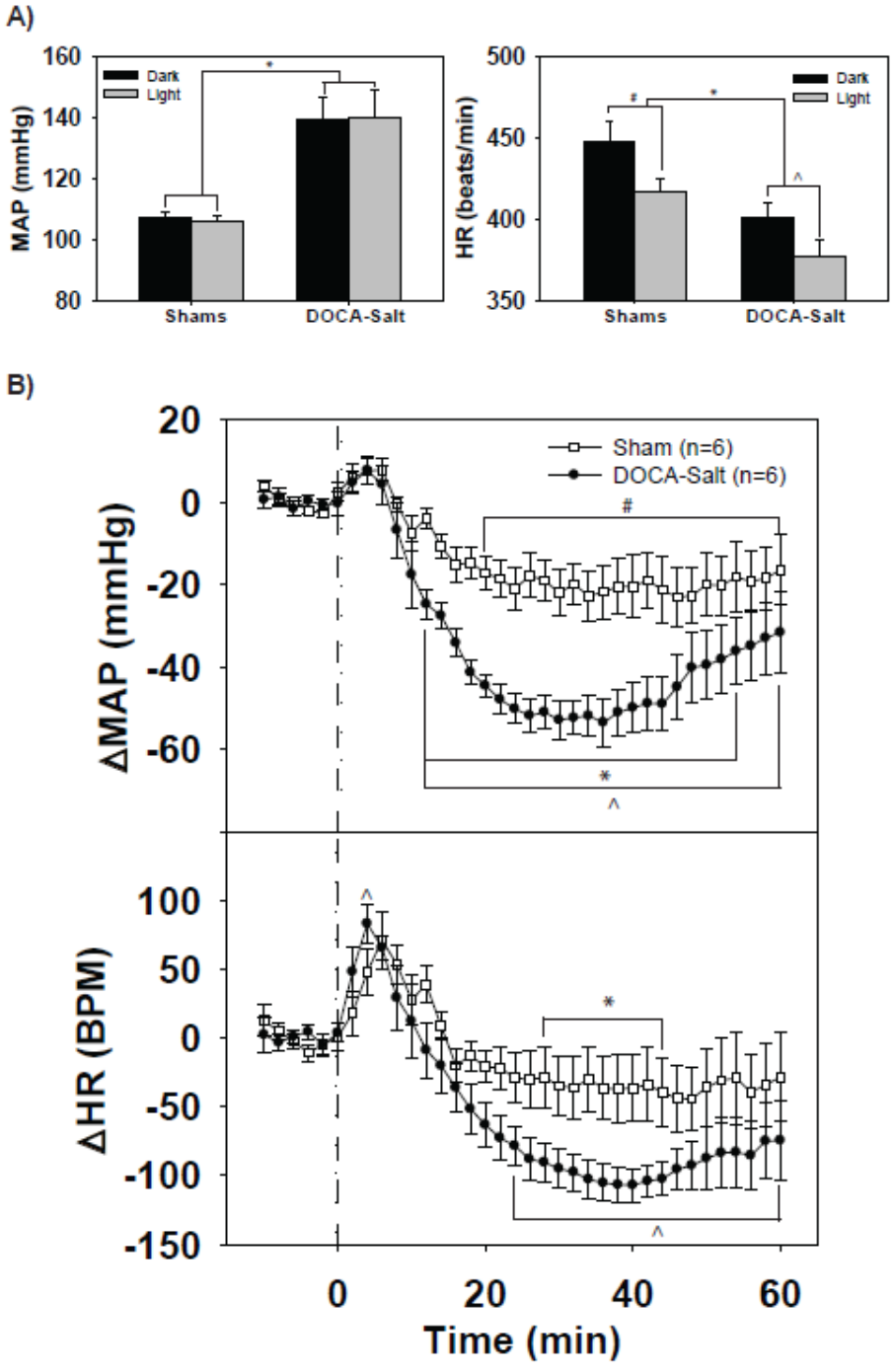


Figure 3 – MAP and HR responses to Intrathecal KYN Administered to Different Levels of the Thoracic Spinal Cord

Intrathecal kynurenate responses are plotted as a change from 10-minute baseline in normotensive anesthetized rats with intrathecal catheters placed in the Lower Thoracic (T10-T13) or Upper Thoracic (T1-T5). # = $p < 0.05$ within Lower Thoracic group vs. t = -6 min. ^ = $p < 0.05$ within Upper Thoracic group vs. t = -6 min. * = $p < 0.05$ between Upper Thoracic and Lower Thoracic rats.

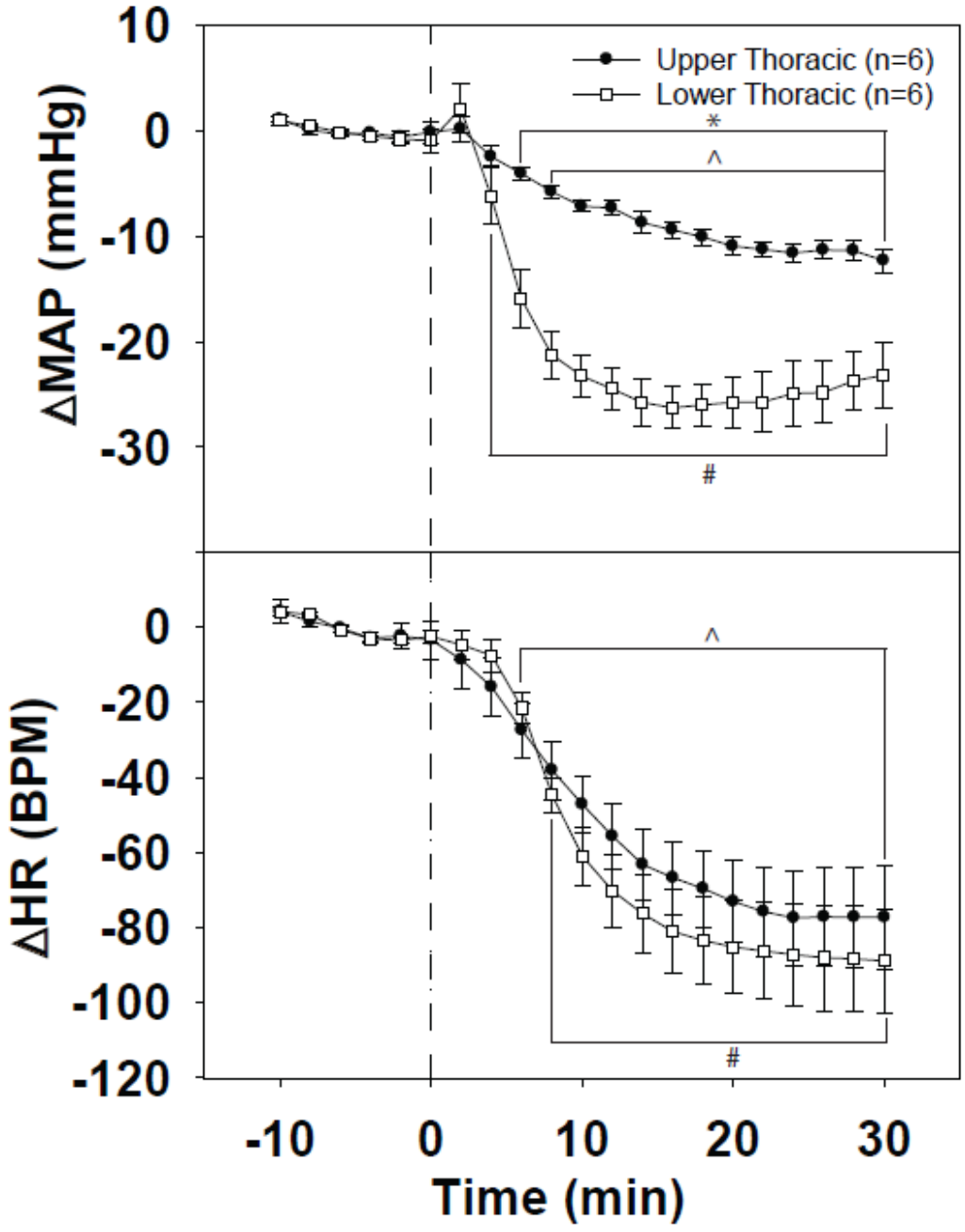
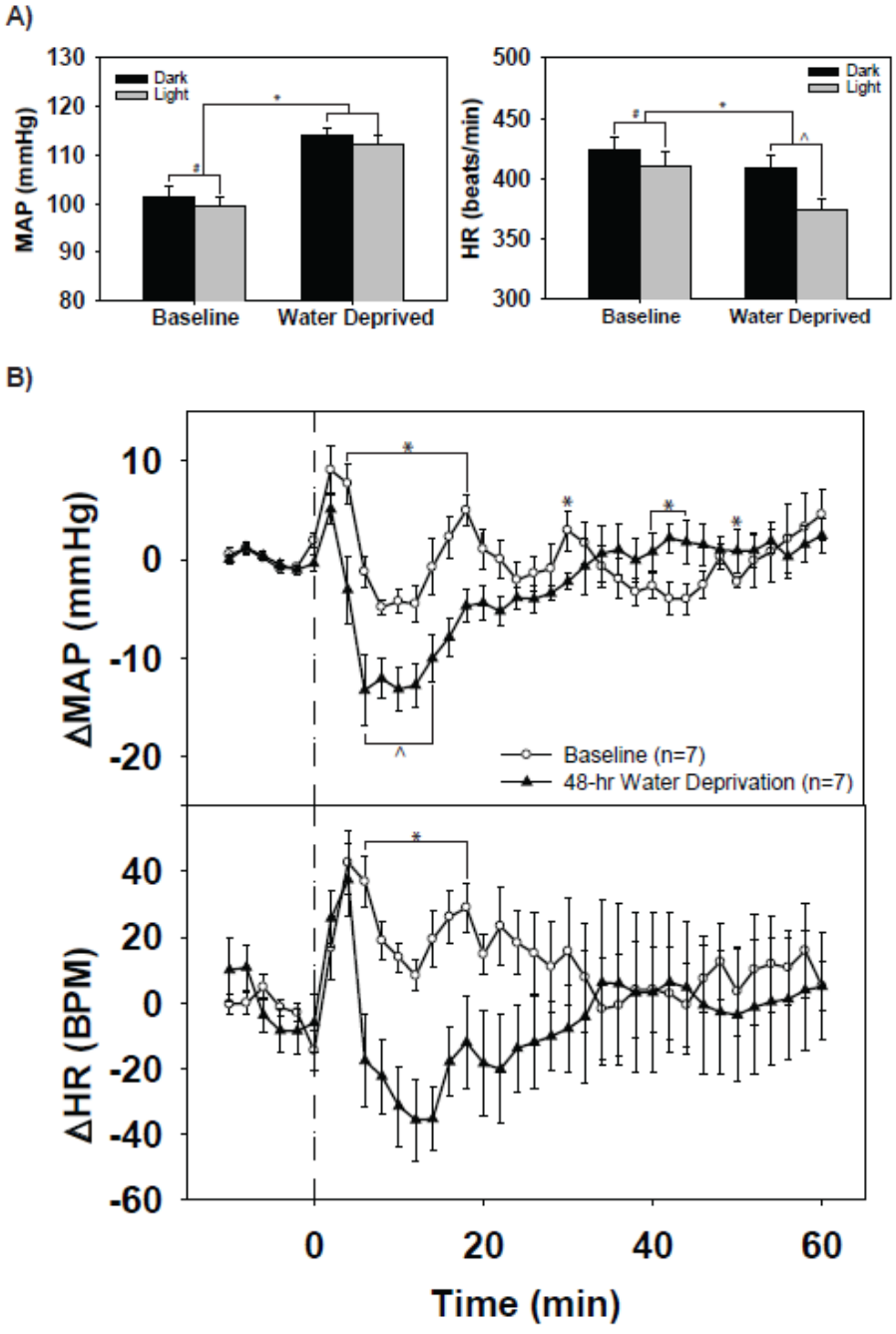


Figure 4 – MAP and HR responses to Intrathecal AP5 during Water Deprivation
A) Four-hour average MAP and HR values from the light and dark phases during baseline and at the end of 48 hours of water deprivation for the rats in this experiment. B) Intrathecal AP5 responses are plotted as a change from 10-minute baseline during the water-replete baseline period and after 48 hours of water deprivation. # = $p < 0.05$ within baseline group vs. t = -6 min. ^ = $p < 0.05$ within water-deprived group vs. t = -6 min. * = $p < 0.05$ between baseline and water-deprived groups.



Chapter 4

Effect of global and regional sympathetic blockade on arterial pressure during water deprivation in conscious rats

Veitenheimer, B.J., W.C. Engeland, P.A. Guzman, G.D. Fink, J.W. Osborn (2012). Am J Physiol Heart Circ Physiol. (In press).

Forty-eight hours of water deprivation (WD) in conscious rats results in a paradoxical *increase* in mean arterial pressure (MAP). Previous studies suggest this may be due to increased sympathetic nerve activity (SNA). However, this remains to be investigated in conscious, freely behaving animals. The purpose of this study was to determine, in conscious rats, the role of the sympathetic nervous system (SNS) in mediating WD-induced increases in MAP and to identify which vascular beds are targeted by increased SNA. Each rat was chronically instrumented with a radiotelemetry transmitter to measure MAP and heart rate (HR) and an indwelling venous catheter for plasma sampling and/or drug delivery. MAP and HR were continuously measured during a 2 day baseline period followed by 48 hours of WD and then a recovery period. By the end of the WD period, MAP increased by ~15 mmHg in control groups whereas HR did not change significantly. Chronic blockade of α_1/β_1-adrenergic receptors significantly attenuated the WD-induced increase in MAP, suggesting a role for global activation of the SNS. However, the MAP response to WD was unaffected by selective denervations of the hindlimb, renal, or splanchnic vascular beds, or by adrenal demedullation. In contrast, complete adrenalectomy (with corticosterone and aldosterone replaced) significantly attenuated the MAP response to WD in the same time frame as α_1/β_1-adrenergic receptor blockade. These results suggest that, in conscious water-deprived rats, the sympathetic nervous system contributes to the MAP response and may be linked to release of adrenocortical hormones. Finally, this sympathetically mediated response is not dependent on increased SNA to one specific vascular bed.

Key words: osmolality, sympathetic nerve activity, water deprivation, denervation, adrenal cortex

Introduction

Water deprivation (WD) over long periods of time increases plasma osmolality and decreases blood volume. In spite of the hypovolemia induced by WD, it has been reported that mean arterial pressure (MAP) is not only maintained but actually *increases* in conscious rats (Brizzee, Harrison-Bernard et al. 1988; Russ, Brizzee et al. 1992; Blair, Woolf et al. 1997). The mechanisms mediating this paradoxical increase in MAP are not clear, but previous studies have shown that WD is accompanied by elevations in the renin-angiotensin-aldosterone system (Di Nicolantonio and Mendelsohn 1986; Blair, Woolf et al. 1997; Chatelain, Montel et al. 2003), plasma vasopressin and corticosterone (Dunn, Brennan et al. 1973; Kiss, Jezova et al. 1994; Chatelain, Montel et al. 2003), and sympathetic nerve activity (SNA) (Brooks, Huhtala et al. 1997; Scrogin, Grygielko et al. 1999; Stocker, Keith et al. 2004; Stocker, Hunwick et al. 2005). In this study, we investigated the sympathetic component of the MAP response to WD.

While it is generally accepted that the sympathetic nervous system is activated during WD to support MAP, the evidence supporting this is not entirely conclusive. Studies in which plasma norepinephrine was measured as an indicator of global sympathetic activity have been inconsistent, with some showing no change (Fejes-Toth, Naray-Fejes-Toth et al. 1985; Kiss, Jezova et al. 1994; Blair, Woolf et al. 1997) and others showing increased levels (Massett, Johnson et al. 1996) during WD. Furthermore, the role of the sympathetic nervous system has not been investigated in the context of *increasing* MAP during WD, particularly in conscious rats. Stocker et al. reported that ganglionic blockade caused a greater fall in MAP in anesthetized water-deprived rats than in euhydrated controls, suggesting a greater sympathetic contribution to MAP during WD (Stocker, Keith et al. 2004; Stocker, Hunwick et al. 2005). However, this has not been confirmed in conscious rats. Moreover, since the paradoxical increase in MAP during WD is not generally present in anesthetized rats (Massett, Johnson et al. 1996; Stocker, Keith et al. 2004; Stocker, Hunwick et al. 2005; Freeman and Brooks 2007), results of anesthetized experiments may not explain the mechanism of elevated MAP in conscious WD rats. To our knowledge, the contribution of global sympathetic contribution to the WD-induced increase in MAP in conscious rats has not been studied.

The question of whether WD results in sympathoexcitation is complicated by the fact that the sympathetic nervous system does not act as a single entity which is either increased or decreased. Rather, it is now clear that sympathetic activity is differentially controlled such that SNA may be increased to some vascular beds and decreased to others (Morrison 2001). Moreover, the precise pattern of SNA to various targets is state-specific in that different stimuli (e.g., hypotension or hypoglycemia) generate different "sympathetic signatures" (McAllen and May 1994; Morrison 2001; Osborn and Kuroki 2012). Although the sympathetic signature associated with WD has not been specifically investigated, direct recordings of SNA to specific vascular beds during WD have been reported. Sympathetic nerve recordings in anesthetized, water-deprived rats suggest that lumbar SNA, specifically, is increased. For example, lumbar SNA (lSNA), expressed as the percent of maximum baroreflex curve, is elevated during WD (Scrogin, Grygielko et al. 1999), but renal SNA (rSNA) is not (Scrogin, McKeogh et al. 2002). Another study in anesthetized rats reported that inhibition of the paraventricular nucleus (PVN) caused a greater fall in lSNA than in rSNA during WD, while PVN blockade in euhydrated rats had no effect on either lSNA or rSNA (Stocker, Hunwick et al. 2005). This suggests that PVN support of lSNA and rSNA is (differentially) enhanced during water deprivation. Additionally, Brooks et al. found that lSNA decreases when plasma osmolality is normalized in WD rats, suggesting that lSNA during WD is driven by osmotically sensitive brain regions (Scrogin, Grygielko et al. 1999; Brooks, Qi et al. 2005). Finally, the mesenteric vascular bed and the adrenal gland could also be important sympathetic targets. The splanchnic sympathetic nerves are known to exert a powerful influence on both the distribution of blood volume and total vascular resistance (King, Osborn et al. 2007); and adrenal hormones could affect the MAP response to water deprivation through central or peripheral adrenosteroid actions, for example (Kageyama and Bravo 1988; Gomez-Sanchez, Fort et al. 1990; Janiak, Lewis et al. 1990; Scheuer, Bechtold et al. 2004). Taken together, these studies suggest that differential sympathetic activation occurs during water deprivation and provide rationale for selectively investigating the roles of individual sympathetic targets in mediating the WD-induced pressor response.

The objective of the present study was to determine the role of global and regional sympathetic activity in mediating the increase in MAP induced by WD in *conscious* rats. MAP was measured continuously in unrestrained rats under control conditions for 2 days, followed by 48 hours of WD and then a recovery period. The contribution of global sympathetic activity to the MAP response during WD was established by chronic adrenoreceptor blockade. The contribution of organ/region-specific changes in SNA to WD-induced changes in MAP was determined by selective denervation of specific targets. Finally, we also investigated the effects of bilateral adrenal demedullation or complete adrenalectomies (with corticosterone and aldosterone replacement) on the control of MAP during WD.

Methods

Animals and General Procedures

Male Sprague Dawley rats from Charles River Laboratory (Wilmington, MA) were singly housed in a temperature-controlled animal room with a 12:12 hour light:dark cycle (lights on at 0800h). The rats ate normal rat chow (Lab Diet 5012) and drank deionized water. All protocols in this study were approved by the Institutional Animal Care and Use Committee.

Rats were implanted with a radiotelemetry transmitter (model TA11PA-C40, Data Sciences International, St. Paul, MN) for continuous monitoring of mean arterial pressure (MAP) and heart rate (HR) and an intravenous (i.v.) catheter for blood sampling and/or systemic drug delivery. The transmitter catheter was implanted into the descending aorta via the femoral artery. The i.v. catheter was advanced to the abdominal vena cava via the femoral vein, tunneled through a spring that was attached to the skin between the scapulae, and attached to a swivel above the cage. These procedures were described previously in greater detail (Veitenheimer and Osborn 2011).

General Experimental Protocol

Rats were conscious and freely moving in their home cages for the duration of the study. Each cage was placed on a receiver (model RPC1) that was connected to a computer via a Data Exchange Matrix (DSI; St. Paul, MN). MAP and HR data were collected at 500Hz over 10s every 4 min from the beginning of the protocol to the time of euthanasia.

All rats recovered from surgery for at least 5 days before beginning the experimental protocol; during the first 3 days of recovery, rats received amoxicillin in their drinking water (1.0 mg/ml). After the surgical recovery period, 2 days of baseline data were collected. The following day, water bottles were removed at 1200h for 48 hours. Five days after water deprivation, rats were euthanized with isoflurane. Water intake was monitored, and rats were weighed regularly throughout the protocol.

Plasma samples were obtained on Day 2 of Baseline and at 24 hours and 48 hours of Water Deprivation (WD). At 1200h, 0.2 ml of blood was collected into heparinized syringes and stored on ice. The sampled volume was replaced with 0.2 ml isotonic saline. The blood samples were centrifuged at 4°C at 5000 RPM for 10 minutes, the plasma was collected for measurement of osmolality in triplicate using a freezing-point depression micro-osmometer (Advanced Instruments model 3320; Norwood, MA).

In the denervation experiments, tissues were harvested at the time of euthanasia for measurement of norepinephrine (NE) content to assess completeness of region/organ-specific denervation. Select organs were flash frozen in liquid nitrogen and then stored at -80°C. Upon completion of the entire study tissues were sent to Core Assay Laboratory at Michigan State University for measurement of NE content using high-performance liquid chromatography (King, Osborn et al. 2007).

Specific Experimental Protocols

Experiment #1: Effect of Chronic Sympathetic Blockade on the Pressor Response to Water Deprivation

The purpose of this protocol was to verify that the increase in MAP during WD is associated with an increase in global sympathetic activity. On the day of surgery, rats (350-400g) were divided into two groups (vehicle and blocked) and were started on intravenous infusions of 0.9% saline vehicle or a combination of an α_1-adrenergic receptor antagonist (terazosin hydrochloride; 1.2 mg/24h, Sigma-Aldrich, St. Louis, MO) and β_1-adrenergic receptor antagonist (atenolol; 24 mg/24h, Sigma-Aldrich). Drugs and saline vehicle were continuously infused using Harvard syringe pumps with the speed adjusted to deliver ~4 ml/day. The efficacy of α_1/β_1 blockade was assessed by intravenous injections of the α_1-agonist, phenylephrine (3.5 μg, Baxter Healthcare Corporation, Deerfield, IL), and the β_1-agonist, isoproterenol hydrochloride (0.7 μg, Sigma-Aldrich) on Day 2 of Baseline and on the day prior to euthanasia. Agonists were dissolved in 0.35 ml of 0.9% saline, and injections were followed by a 0.2 ml saline flush. At least an hour was allowed between the two injections.

Effect of Organ/Regional Sympathectomy on the Pressor Response to Water Deprivation

The purpose of this protocol was to establish the role of regional sympathetic activity in mediating the increase in MAP induced by WD. In these experiments, bilateral regional denervation, or a sham denervation, was performed at the time of transmitter implantation (described above).

*Experiment #2: Lumbar Sympathetic Denervation (LDNx)***.** A midline incision was made and the intestines were retracted with gauze to expose the aorta below the level of the renal artery. The aorta and vena cava were gently retracted with suture to expose the lumbar sympathetic chain. For the LDNx group, the chain was dissected from L2 to the aortic bifurcation. Small nerve fibers on the surface of the aorta and vena cava were also removed. Sham surgery involved exposing, but not sectioning, the nerves.

Experiment #3: Renal Denervation (RDNx). A midline incision was made and the intestines were retracted with gauze to expose the renal artery and vein. In the RDNx group, all visible nerves from the aorta to the renal bifurcation were dissected from the artery using a microscope. The artery and vein were then brushed with a 10% phenol solution. Sham surgery involved exposing the renal vessels, but nerves were not sectioned and phenol was not applied.

Experiment #4: Celiac Ganglionectomy (CGx). CGx was performed via a midline abdominal incision to denervate the splanchnic vascular bed. The celiac plexus was removed, and all visible nerves were removed from the nearby aorta, celiac artery, and superior mesenteric artery (King, Osborn et al. 2007). For the sham procedure, the celiac plexus was visualized by making a midline abdominal incision and retracting the intestines, but nerves were not sectioned.

Experiment #5: Effect of Adrenal Demedullation (ADMx) on the Pressor Response to Water Deprivation

The aim of this experiment was to determine the contribution of adrenal medullary catecholamines to the regulation of MAP during WD. At the time of transmitter implantation (described above), the adrenals were exposed via a midline

abdominal incision. A tiny incision was made on the top of each adrenal using a #11 scalpel blade, and a forceps was used to gently extrude the adrenal medulla. For the sham procedure, the adrenal glands were visualized. Rats in this experiment were given 3 weeks to recover from surgery to allow the damaged adrenal cortex to regenerate (Ulrich-Lai and Engeland 2000).

Experiment #6: Effect of Total Adrenalectomy (ADx) on the Pressor Response to Water Deprivation

The purpose of this protocol, in conjunction with the adrenal demedullation study above, was to determine whether hormones secreted from the adrenal cortex, such as aldosterone or corticosterone, contribute to the pressor response to WD. On the day of surgery, rats were subjected to either ADx or sham surgery via a midline incision. To maintain steady, near-physiological hormone levels, ADx rats received subcutaneous aldosterone (8 μg/day; Steraloids, Newport, RI) at a constant rate via an osmotic minipump (#2002, Alzet, Palo Alto, CA) and cholesterol pellets (Sigma-Aldrich) containing 50% corticosterone (75 mg; Roussel Uclaf, Romaineville, France). Doses were based on a previous protocol which resulted in plasma concentrations in the physiological range (Wotus and Engeland 2003). Sham rats received vehicle (propylene glycol; Sigma-Aldrich) in the minipump and cholesterol-only pellets.

In this study, 0.4 ml of blood was obtained during the sampling at 1200h on Day 2 of Baseline and at 48 hours of Water Deprivation (WD). Since the volume of blood sampled was twice that of the other experiments in this study, the 24hr plasma sample was omitted in this experiment. In addition to the osmolality measurements, some of the plasma was aliquotted and frozen for later analysis of plasma corticosterone and adrenocorticotropic hormone (ACTH) (Jasper and Engeland 1991; Wotus and Engeland 2003). At the end of the experiment, body and thymus weights were also collected as physiological indicators of adequate corticosterone replacement (Akana, Cascio et al. 1985).

Data Analysis and Statistics

Telemetry data was acquired and analyzed with Dataquest A.R.T. 4.0 software (DSI; St. Paul, MN). Two-hour averages of MAP and HR were plotted as means $\pm$ standard errors. SigmaStat software (3.5, San Jose, CA) was used to identify statistical significance within experiments. The data plotted over time were analyzed using a two-way analysis of variance (ANOVA) for repeated measures over time, followed by the Holm-Sidak post hoc test. Sham groups were used as the control between groups, and the t=0 time point was used as the control within groups.

To summarize the effects of WD, changes in MAP and HR were grouped by lights-on/lights-off (light/dark) phases and plotted as bar graphs. Values were averaged over the last 4 hours of the dark phase (t= -8 to -5) and the first 4 hours of the light phase (t = -4 to -1) at the end of the control period, and these were compared to the last 4 hours of the dark phase (t= 40 to 43) and first 4 hours of the light phase (t=44 to 47) on the last day of water deprivation. These data were analyzed using a two-way ANOVA and the Holm-Sidak post hoc test, with the light phase and baseline period data set as the controls between and within groups, respectively.

One-way ANOVAs on ranks were used to detect changes in plasma osmolality during WD, and the Student's t-test was used to compare the norepinephrine data between sham and experimental groups. Results of the corticosterone and ACTH assays were analyzed using two-way ANOVAs and the Holm-Sidak post hoc test. For all comparisons, a p value of <0.05 was defined as statistically significant.

Results

General Responses of All Study Groups

Baseline plasma osmolality was similar between all groups ($p = 0.099$) averaging 298 $\pm$ 1 mOsm/kg (Table 1). None of the experimental groups showed statistically significant differences in plasma osmolality from their sham/vehicle control groups at any time point measured. Plasma osmolality values were significantly increased by an average of 7 $\pm$ 1 mOsm/kg after 24 hours of WD and by 13 $\pm$ 1 mOsm/kg by the end of 48 hours of water deprivation.

Water deprivation for 48 hours resulted in a 10-15% decrease in body weight. Sympathetically blocked rats lost the least amount of body weight (11 $\pm$ 1%), which was significantly less than the two groups with greatest weight loss (ADx: 14 $\pm$ 2% and sham ADx: 15 $\pm$ 1%). There were no differences between any other groups, and there were no differences in weight loss between the experimental groups and their control groups. Additionally, there were no between-group differences in water intake during the 2 days of control (44 $\pm$ 1 ml/day) or during the recovery from WD (51 $\pm$ 1 ml/day).

Effect of Chronic Sympathetic Blockade on the Pressor Response to Water Deprivation

Table 2 shows 4-hour averages of baseline MAP and HR during the light and dark phases (as described in Methods) in vehicle and α_1/β_1-blocked rats (Exp. #1). Chronic α_1/β_1 blockade significantly lowered baseline MAP and HR during both light and dark phases. Shown in Figure 1A are the changes from baseline for MAP and HR plotted as two-hour averages during the last 48 hours of baseline, 48 hours of WD, and 48 hours of recovery. In vehicle rats, MAP increased ~5 mmHg above baseline after 24 hours of WD and ~15 mmHg by the end of the 48 hr WD period. In contrast, HR tended to decrease during WD but did not achieve statistical significance. More importantly, although α_1/β_1 blockade had no effect on the MAP response during the first 24 hours of WD, by the second day of WD, the blocked rats had significantly lower MAP levels than vehicle rats. In fact, during the final light-phase hours of WD, MAP levels in α_1/β_1-blocked rats had returned to values similar to those of baseline (Fig. 1a). HR response to WD was unaffected by α_1/β_1 blockade.

Figure 1B summarizes the responses to WD in both groups. WD increased MAP in the vehicle group by 15 ± 1 and 17 ± 1 mmHg during the dark and light phases, respectively. The MAP response to WD was significantly less in α_1/β_1-blocked rats, with increases of 7 ± 3 (dark phase) and 5 ± 2 (light phase) mmHg. The HR response to WD was significantly different between light and dark phases in the vehicle group, but there were no difference in HR response between vehicle and blocked rats.

During both control and recovery periods, bolus i.v. injections of phenylephrine and isoproterenol were given to confirm successful α_1/β_1 blockade. The average MAP response to phenylephrine was 22 ± 3 mmHg in vehicle rats. This response was abolished in α_1/β_1-blocked rats (2 ± 3 mmHg). Similarly, the HR response to isoproterenol was markedly reduced in α_1/β_1-blocked rats (26 ± 7 beats/min) compared to vehicle rats (92 ± 12 beats/min). Because isoproterenol also caused a fall in MAP (-25 ± 6 mmHg in α_1/β_1-blocked rats and -23 ± 3 mmHg in vehicle rats), it is possible that the remaining HR response in α_1/β_1-blocked rats was due to vagal inhibition. Therefore, in several rats, we also examined the response to isoproterenol after vagal blockade. The attenuation of the HR response to isoproterenol in α_1/β_1-blocked rats was even greater with atropine pretreatment (14 ± 8 beats/min compared to 93 ± 11 beats/min in vehicle rats).

Effect of Organ/Regional Sympathectomy on the Pressor Response to Water Deprivation

Lumbar sympathectomy (LDNx) had no effect on baseline MAP or HR (Table 2, Exp. #2). It also had no effect on the MAP or HR response to WD (Fig. 2A). WD increased MAP in the sham group by 14 ± 2 mmHg during the dark phase and 15 ± 3 mmHg during the light phase, while the respective changes in the lumbar denervated rats were 18 ± 2 and 17 ± 2 mmHg (Fig. 2B). WD had no significant effect on HR in LDNx or sham rats (Figs 2A and 2B). Norepinephrine content was significantly decreased by 87% (left) and 68% (right) in the soleus muscles of LDNx rats compared to sham rats (Table 3, Exp. #2). LDNx had no effect on norepinephrine levels in the kidney or spleen (data not shown).

There was a statistically significant treatment effect of bilateral renal denervation (RDNx) on baseline MAP ($p = 0.0145$ for sham vs. RDNx), but this effect did not achieve significance when analyzed further between the dark and light phases, as reported in Table 2 (Exp. #3). More importantly, RDNx had no effect on the MAP or HR response to WD (Fig. 3A). WD increased MAP in the sham group by 14 ± 4 mmHg during the dark phase and 14 ± 3 mmHg during the light phase, while the respective changes in the RDNx rats were 16 ± 3 and 18 ± 3 mmHg (Fig. 3B). WD had no significant effect on HR in either group. Renal norepinephrine content was decreased by 97% (left) and 95% (right) in the kidneys of RDNx rats compared to those of sham rats (Table 3, Exp. #3).

Baseline MAP was significantly lower in rats subjected to celiac ganglionectomy (CGx) compared to sham rats (Table 2, Exp. #4). Although the CGx rats tended to have a slightly smaller increase in MAP than sham rats at some points during WD, these effects were not significant (Fig. 4A). There were, however, several points during the recovery from WD in which CGx rats had lower MAP compared to baseline than sham rats (Fig. 4A). Two days of WD caused an average increase in MAP of 20 ± 3 mmHg during the dark phase and 19 ± 2 mmHg during the light phase in the sham group, while the respective changes in the CGx rats were 17 ± 3 and 20 ± 2 mmHg (Fig. 4b). WD had no significant effect on HR in either group. Norepinephrine content was decreased in the liver (82%), spleen (99%), and duodenum (99%) compared to sham rats (Table 3, Exp. #4).

Effect of Adrenal Demedullation on the Pressor Response to Water Deprivation

Baseline MAP was significantly lower in adrenal demedullated (ADMx) rats compared to sham controls (Table 2, Exp. #5). However, it is important to note that the MAP values in Exp. #5 sham rats were greater than in other experiments, at least during the dark period. ADMx had no effect on the MAP or HR response to WD, with the exception of several points during the recovery period (Fig. 5A). The average increase in MAP during 48 hours of WD in the sham group was 15 ± 1 mmHg during the dark phase and 15 ± 1 mmHg during the light phase, while the respective changes in the

ADMx rats were 14 ± 1 and 15 ± 2 mmHg (Fig. 5B). WD had no significant effect on HR in either group.

Effect of Adrenalectomy on the Pressor Response to Water Deprivation

Bilateral adrenalectomy with exogenous corticosterone and aldosterone (ADx+Cort+Aldo) had no effect on baseline MAP or HR (Table 2, Exp. #6). Similarly, there were no significant differences in baseline plasma ACTH or corticosterone concentrations between ADx+Cort+Aldo and sham rats (Figure 6). WD increased plasma ACTH levels in both groups, but this increase was statistically significant only in ADx+Cort+Aldo rats (Figure 6A). Plasma corticosterone tended to increase in response to WD in sham rats, but this was not significant due to the variability of the response (Figure 6B). As expected, plasma corticosterone remained at baseline levels during water deprivation in ADx+Cort+Aldo rats (Figure 6B). Finally, thymus weights, normalized to body weight, were similar between sham (1.48 ± 0.06 mg/g) and ADx+Cort+Aldo rats (1.61 ± 0.08 mg/g).

The MAP response to WD was not affected during the first 24 hours of WD in the ADx+Cort+Aldo group; however, similar to the timing of the sympathetic blockade effects (Fig. 1), the pressor response was significantly attenuated beginning in the second night of WD (Fig. 7A). The average increase in MAP during WD in the sham group was 16 ± 2 mmHg during the dark phase and 18 ± 2 mmHg during the light phase, while the respective changes in the ADx+Cort+Aldo rats were 10 ± 2 and 11 ± 2 mmHg (Fig. 7B). Heart rate remained unchanged from baseline in both groups.

Discussion

During water deprivation, baroreceptor activation and increased plasma osmolality initiate neural and hormonal responses which maintain perfusion of vital tissues such as the heart and brain in the face of hypovolemia. Studies in conscious rats have revealed that arterial pressure is not merely maintained during water deprivation, but actually increases above baseline levels (Brizzee, Harrison-Bernard et al. 1988; Russ, Brizzee et al. 1992; Blair, Woolf et al. 1997). We hypothesize that this paradoxical hypertensive response may be mediated by sympathoexcitation secondary to activation of osmosensitive pathways in the brain (for reviews, see (Toney and Stocker ; Dampney, Horiuchi et al. 2003; Toney, Chen et al. 2003; Brooks, Haywood et al. 2005; Bourque 2008; Stocker, Osborn et al. 2008)). Indeed, it has been proposed that a detailed understanding of the mechanisms coupling plasma osmolality to sympathetic activity during water deprivation may translate to understanding mechanisms linking dietary salt and neurogenic hypertension (Brooks, Qi et al. 2005; Stocker, Osborn et al. 2008).

Therefore, the objective of the present study was to investigate the role of global and regional sympathetic activity in regulating arterial pressure in conscious water-deprived rats. The approach was similar to our recent characterization of the "sympathetic signature" of AngII-salt model of hypertension in the rat (Osborn and Kuroki 2012). Our results confirmed that the pressor response *on the second day* of 48-hour water deprivation is dependent on increased global sympathetic activity; however, the pressor response during the first 24-hour period of water deprivation appears to rely on non-sympathetic mechanisms. Additionally, unlike the AngII-salt model in which sympathetic activity to a single vascular bed (i.e., splanchnic) was predominant, we did not find such evidence for the pressor response to water deprivation. Finally, an unexpected finding of this study was that increased sympathetic activity during water deprivation in conscious rats may be linked to release of adrenal cortical hormones.

Role of Global Sympathetic Activity in the Regulation of Arterial Pressure During Water Deprivation

To examine global sympathetic nerve activity, we compared the arterial pressure and heart rate responses to 48 hours of water deprivation between control rats and rats chronically treated with the α_1- and β_1- adrenergic antagonists, terazosin and atenolol, respectively. In agreement with previous studies (Brizzee, Harrison-Bernard et al. 1988; Russ, Brizzee et al. 1992), 48 hours of water deprivation resulted in an arterial pressure increase of ~15 mmHg above baseline in the control group. Sympathetic blockade with α_1/β_1 antagonists significantly attenuated this pressor response to the extent that arterial pressure had returned to normal levels on the last day of water deprivation. This implies that the MAP response to water deprivation depends, in part, on sympathetic activity, particularly on the second day of water deprivation. This finding is in agreement with a previous study employing acute ganglionic blockade in anesthetized rats (Stocker, Keith et al. 2004); however, it is important to note that arterial pressure was not different between water-deprived and euhydrated rats in the presence of anesthesia in that study.

In addition to clearly summarizing the peak MAP and HR responses to WD, the data were plotted as bar graphs in order to assess if the responses might be specific to one phase of the light/dark cycle. The MAP responses to WD were similar between dark and light phases for all groups in all experiments. However, the HR response to WD was significantly greater during the dark phase compared to the light phase in vehicle rats in Experiment #1. The same trend can be seen in the sham rats in Experiments #2, #4, and #6, although these data did not achieve significance. Taken together, the majority of the experiments suggest that HR decreases with water deprivation, and this bradycardia may be greater during the dark phase compared to the light phase. In other words, the typical increase in HR during the lights-off period may be blunted in water-deprived rats; however, the importance of this observation to overall homeostasis is unclear.

Effect of Organ/Region-Specific Denervation on the Pressor Response to Water Deprivation

Emerging evidence suggests that chronic activation of the sympathetic nervous system can occur in a disease-specific pattern or "sympathetic signature" (Kuroki, Guzman et al. 2012). For example, in the ovine pacing-induced model of heart failure,

sympathetic activity to the heart is increased, but renal SNA is normal (Ramchandra, Hood et al. 2012). This sympathetic signature appears to be similar in humans with heart failure (Esler 2010). The AngII-salt model of hypertension in the rat is characterized by increased SNA to the splanchnic vascular bed whereas SNA to the kidneys and hindlimb is decreased or unchanged, respectively (Yoshimoto, Miki et al. 2008; Osborn and Kuroki 2012). Additionally, the hypertension in this model is largely prevented by celiac ganglionectomy, whereas renal denervation or lumbar sympathectomy has no effect (King, Osborn et al. 2007). Clearly, understanding disease-specific sympathetic signatures is essential for the development of targeted sympathetic-ablation therapies, which may avoid unwanted side effects associated with global sympathetic blockade. The recent success of catheter based renal denervation to treat hypertension in humans provides convincing evidence of this approach as a novel therapeutic strategy (Schlaich, Sobotka et al. 2009).

In this study, we denervated individual vascular beds to investigate the "sympathetic signature" involved in the pressor response to water deprivation. The possible peripheral targets of sympathetic outflow that could mediate the water deprivation-induced increase in arterial pressure include the heart, kidneys, splanchnic vascular bed, skeletal muscle, and the adrenal glands. Based on our observation that heart rate did not increase during water deprivation we did not pursue selective denervation of the heart in these studies. Therefore, we focused on the effects of selective denervation of renal, splanchnic and hindlimb vascular beds - as well as adrenalectomy and adrenal demedullation - on the regulation of arterial pressure during water deprivation.

Previous studies have suggested that lumbar sympathetic nerve activity may increase during water deprivation (Scrogin, Grygielko et al. 1999; Brooks, Qi et al. 2005; Stocker, Hunwick et al. 2005), which led to the hypothesis that LDNx would attenuate the arterial pressure response. However, there were no differences between LDNx and sham groups for either the arterial pressure or heart rate responses to water deprivation. One possible explanation for this discrepancy is the method used to quantify lumbar sympathetic nerve activity (lSNA) in previous studies. Until recently, chronic recording

of SNA in conscious rats over periods long enough to measure within-animal responses was unachievable. As a result, various methods have been used to normalize nerve activity to correct for differences in electrode contact with multifiber nerves and permit between-animal comparisons. For example, since lSNA during water deprivation could not be compared to baseline, it was normalized to the plateau of the baroreflex curve (Scrogin, Grygielko et al. 1999). This method of normalization of SNA has been debated and is not resolved (Burke and Head 2003). Another possible explanation for the discrepancy between our study and those in which lSNA was measured is that previous studies were conducted within 24 hours after implantation of the nerve electrode (Scrogin, Grygielko et al. 1999; Brooks, Qi et al. 2005; Stocker, Hunwick et al. 2005), so the lSNA may have been elevated due to post-surgical stress. A final possible explanation is that lSNA may be increased during water deprivation as previous studies suggest, but the changes in lSNA may not quantitatively relate to arterial pressure. In other words, it is possible that the degree to which lSNA is increased during water deprivation is not sufficient to result in increased hindlimb vascular resistance and arterial pressure.

Similar to lumbar denervation, renal denervation had no effect on the arterial pressure or heart rate responses to water deprivation. This finding is in agreement with Scrogin et al. (Scrogin, McKeogh et al. 2002), who reported no elevation in renal SNA (rSNA), expressed as percent of the plateau of the baroreflex curve, during water deprivation. These findings differ from those of Blair et al. (Blair, Woolf et al. 1997), who reported a decrease in the arterial pressure response to water deprivation in renal denervated rats. In that study, rats were transferred to a recording cage and arterial pressure was recorded for a 10 minute period. As a result, their protocol did not allow within-group comparisons and the acute stress induced by the method for measuring arterial pressure may have influenced the results. Stocker et al. reported that inhibition of the paraventricular nucleus caused a fall in both rSNA and lSNA in dehydrated rats but not in euhydrated rats (Stocker, Keith et al. 2004; Stocker, Hunwick et al. 2005). One interpretation of these results is that both lSNA and rSNA may be elevated during water

deprivation. However, neither LDNx or RDNx affected the arterial pressure response to water deprivation in our study.

The splanchnic sympathetic nerves have a major influence on both total vascular resistance and venous capacitance (Gootman and Cohen 1970; Fink and Osborn 2010), and mesenteric blood flow has been reported to be decreased in water-deprived rats (Massett, Johnson et al. 1996). However, denervation of the splanchnic vascular bed by celiac ganglionectomy, similar to RDNx and LDNx, had no effect on the arterial pressure and heart rate responses to water deprivation compared to sham rats. To our knowledge, direct measurement of splanchnic SNA in water deprived animals has not been reported.

Role of the Adrenal Gland in the Regulation of Arterial Pressure During Water Deprivation

Catecholamine-producing chromaffin cells in the adrenal medulla receive input from preganglionic nerves in segments T5-T11 of the spinal cord. These neurons travel through the greater splanchnic nerve to the suprarenal ganglion, where they either synapse onto postganglionic cells that innervate the adrenal or continue on to innervate the medulla directly (Celler and Schramm 1981). Removal of the celiac ganglion in the CGx experiment likely did not affect adrenal innervation, since the celiac ganglion is "downstream" of the suprarenal ganglion. Therefore, we examined the effect of bilateral adrenal demedullation on the arterial pressure and heart rate responses to water deprivation. Similar to LDNx, RDNx and CGx, adrenal demedullation had no effect on the cardiovascular responses to water deprivation at any point in time during the protocol. Past studies have reported incongruent results in terms of catecholamine responses, which are in part dependent on adrenal catecholamines, during WD. Some studies found that water deprivation increases plasma catecholamines (Massett, Johnson et al. 1996; Chatelain, Montel et al. 2003) while many others found no significant effect (Fejes-Toth, Naray-Fejes-Toth et al. 1985; Kiss, Jezova et al. 1994; Blair, Woolf et al. 1997). However, as Brooks et al. point out, metabolism and reuptake affect the catecholamine availability and a simple measurement of the plasma levels may be insufficient (Brooks, Huhtala et al. 1997). Their study, which analyzed adrenal mRNA levels involved in

production and reuptake of norepinephrine, suggested that water deprivation increases adrenal activity, in part via the sympathetic nerves (Brooks, Huhtala et al. 1997).

Although our results suggest that adrenal medullary catecholamines do not mediate the cardiovascular responses during dehydration, we also tested the hypothesis that adrenal cortical hormones play a role by examining the effect of complete bilateral adrenalectomies on the cardiovascular responses to water deprivation. In the ADx rats, we replaced aldosterone and corticosterone at a constant rate according to past studies (Wotus and Engeland 2003). Since the ADx rats are unable to increase production of adrenocortical hormones during water deprivation, we reasoned that this would be an effective way to test their role in the cardiovascular response to water deprivation. Indeed, this intervention attenuated the arterial pressure response to water deprivation in the same time frame as chronic blockade of adrenoreceptors, suggesting a possible link between adrenocortical hormones and sympathetic activity during water deprivation. Some evidence suggests that the adrenal cortex is innervated by sympathetic nerves that affect the release of adrenal cortical hormones (Engeland 1998; Bornstein and Chrousos 1999), so the effect of sympathetic blockade on the arterial pressure response to water deprivation may be due to loss of neural control of the adrenal cortex. On the other hand, release of adrenal cortical hormones during water deprivation could also modulate activity of the sympathetic nervous system centrally and/or peripherally. The adrenocortical hormones corticosterone (Saruta 1996; Scheuer and Bechtold 2001; Scheuer, Bechtold et al. 2004; Yang and Zhang 2004; Scheuer 2010), aldosterone (Kageyama and Bravo 1988; Gomez-Sanchez, Fort et al. 1990; Janiak, Lewis et al. 1990; Geerling, Kawata et al. 2006; Abrams and Osborn 2008), and endogenous oubain (Ludens, Clark et al. 1992; Aileru, De Albuquerque et al. 2001; Nicholls, Lewis et al. 2009; Leenen 2010; Blaustein, Leenen et al. 2012) all have the ability to influence sympathetic nerve activity and affect arterial pressure. Additional studies are required to determine the role of these hormones in the regulation of arterial pressure during water deprivation.

Plasma corticosterone and ACTH were measured to assess the approximate amount of corticosterone replaced and verify that the levels were not unphysiological

(aldosterone measurements were omitted due to the large blood volume required). ADx rats had plasma ACTH and corticosterone levels within physiological ranges and thymus weights were similar to sham rats, suggesting appropriate corticosterone replacement. Additionally, these measurements were an indicator of corticosterone changes in sham rats with water deprivation. In agreement with previous studies, ACTH did not significantly increase with water deprivation in sham rats (Kiss, Jezova et al. 1994; Ulrich-Lai and Engeland 2002; Roberts, Pope et al. 2011). Although plasma corticosterone tended to increase during water deprivation in sham rats, we did not see a statistical effect, in contrast to previous studies (Kiss, Jezova et al. 1994; Ulrich-Lai and Engeland 2002; Roberts, Pope et al. 2011). This is likely due to the high variability among the plasma corticosterone measurements in our sham rats, which was likely a result of the rats being weighed 3 hours prior to blood sampling. As expected, in ADx rats, water deprivation did not affect corticosterone levels. However, plasma ACTH levels increased with water deprivation in ADx rats, suggesting an attempt to increase corticosterone levels higher than the level of corticosterone replaced.

These results indicate that our corticosterone replacement levels were not abnormally high or low; nevertheless, we acknowledge that we do not have enough information to determine whether the corticosterone/aldosterone replacements affected the typical plasma renin response to water deprivation in the present study. For example, if the dose of aldosterone was too high, basal levels of basal plasma renin activity may have been suppressed and the response of renin to water deprivation may have been affected. Follow-up studies are needed to examine in more detail the ways in which adrenocortical hormones affect the MAP response to water deprivation. However, as stated previously, the similar timing of the MAP response between ADx and α_1/β_1-blocked rats suggests a possible link to sympathetic activation.

Effect of Organ/Region-Specific Sympathetic Blockade on Basal Levels of Arterial Pressure

Although not the goal of this study, this is the first report in which the effects of organ/region-specific blockade on arterial pressure has been systematically studied in

conscious rats using continuous radiotelemetric recording. Using this approach, we have previously reported that renal denervation results in an immediate and sustained decrease in basal levels of arterial pressure of ~10 mmHg in normotensive Sprague Dawley rats (Jacob, Ariza et al. 2003). That finding, which is confirmed in the present study, was surprising at the time because of the redundancy of mechanisms for regulation of arterial pressure, including sympathetic activity to other vascular beds. We extend that finding in the present study in which we observed that celiac ganglionectomy and adrenal demedullation, but not lumbar denervation, also decreased basal levels of arterial pressure on the order of ~10 mm Hg. These results suggest that basal sympathetic tone to the kidneys, splanchnic circulation, and adrenal medulla may all play an important role in the long term control of arterial pressure under normal conditions.

Summary & Perspectives

This study demonstrates for the first time in conscious rats that increased arterial pressure during water deprivation is dependent on increased activity of the sympathetic nervous system. Our findings suggest that this sympathetically driven pressor response is not mediated by increased activity to a specific vascular bed; however, it may be linked to adrenal cortical hormones.

It remains unclear why organ/region-selective denervation is effective in attenuating hypertension in humans (Schlaich, Sobotka et al. 2009; Schlaich, Sobotka et al. 2009) and certain rat models of hypertension (King, Osborn et al. 2007; Kandlikar and Fink 2011), but no such effect was observed in water deprived rats. One explanation might be related to the physiological concept of redundancy - that there is more than one mechanism by which a certain outcome is achieved. This is consistent with the theory of a neural "set point" for arterial pressure that could be altered by the changes in plasma osmolality and circulating hormones induced by water deprivation (Osborn 2005; Osborn, Jacob et al. 2005). This theory would predict that loss of sympathetic activity to one vascular bed would result in a compensatory increase in activity to another target to defend the arterial pressure set point. It may be that the modest increase in arterial pressure during water deprivation relies on a more generalized increase in sympathetic

activity rather than an organ specific sympathetic signature seen in essential and experimental hypertension. Additional studies are needed to test this hypothesis.

Support & Funding[1]

This work was funded by a National Heart, Lung, and Blood Institute Grant (R01 HL-076312) awarded to the Neurogenic Cardiovascular Diseases Consortium. Britta Veitenheimer was supported by a Predoctoral Neuroscience Training Grant (NIH #T32 GM008471-17) and the University of Minnesota Doctoral Dissertation Fellowship. William Engeland and Marina Yoder were supported in part by funding from the National Science Foundation (IOS-1023199).

Disclosures

J.W.O. is a consultant for Medtronic Cardiovascular.

[1]We thank Marina Yoder for performing the radioimmunoassay measurements of plasma corticosterone and ACTH. We also thank Ninitha Julfiya for her instruction and advice regarding the lumbar denervation surgery, as well as Robert Burnett from the Michigan State Core Assay Laboratory for performing the norepinephrine analysis. Finally, we thank Marcos Kuroki and Jason Foss for their insight and instruction on the CGx and RDNx surgeries.

Table 1 - Plasma Osmolality Measurements during Baseline, 24-, and 48-hour Water Deprivation

Blood samples for plasma osmolality measurements were obtained at 1200h on day 2 of the baseline period and after 24 and 48 hours of water deprivation. The 24-hour sample was omitted in Experiment 6 due to the larger volume of blood sampled over the duration of that protocol. Data are reported as mean ± standard error (mOsm/kg). N's are reported because the plasma sample size in some groups was reduced due to inability to obtain blood sample from the i.v. catheter. * = $p < 0.05$ vs. vehicle/sham. ^ = $p < 0.05$ vs. light period within experimental groups. # = $p < 0.05$ vs. light period within vehicle/sham groups.

Exp. #	Group	n	Baseline	24h WD	48h WD
1	Vehicle	6	296 ± 3	300 ± 1	306 ± 1#
1	Block	5	295 ± 1	302 ± 2^	308 ± 1^
2	Sham	3	298 ± 2	306 ± 2#	312 ± 1#
2	LDNx	5	299 ± 1	307 ± 1^	314 ± 2^
3	Sham	4	304 ± 1	307 ± 2	316 ± 2#
3	RDNx	5	301 ± 2	311 ± 1^	314 ± 2^
4	Sham	5	300 ± 1	308 ± 2#	314 ± 1#
4	CGx	5	299 ± 2	309 ± 1^	317 ± 2^
5	Sham	2	296 ± 2	301 ± 1#	304 ± 1#
5	ADMx	2	296 ± 1	304 ± 1^	308 ± 2^
6	Sham	9	298 ± 2	NA	312 ± 2#
6	ADx	8	298 ± 1	NA	312 ± 3^

Table 2 – Baseline MAP and HR values for Sham and Experimental Groups from Each Experiment

Four hours of baseline data from the dark and light phases were averaged as described in Methods. Data are reported as mean ± standard error. * = $p < 0.05$ vs. vehicle/sham. ^ = $p < 0.05$ vs. light period within experimental groups. # = $p < 0.05$ vs. light period within vehicle/sham groups.

Exp. #	Group	MAP dark	MAP light	HR dark	HR light
1	Vehicle	99 ± 3	97 ± 2	425 ± 9 #	385 ± 8
1	Block	90 ± 2 *	88 ± 2 *	330 ± 4 *	318 ± 4 *
2	Sham	103 ± 2	99 ± 1	448 ± 5 #	408 ± 5
2	LDNx	99 ± 2	97 ± 2	445 ± 9 ^	421 ± 7
3	Sham	103 ± 5	101 ± 3	425 ± 15	401 ± 4
3	RDNx	95 ± 2	92 ± 2	425 ± 11	396 ± 12
4	Sham	100 ± 3	98 ± 3	446 ± 12 #	403 ± 19
4	CGx	89 ± 4 *	85 ± 4 *	427 ± 13	396 ± 17
5	Sham	110 ± 5	103 ± 2	396 ± 6 #	337 ± 3
5	ADMx	100 ± 2 *^	93 ± 2 *	379 ± 11 ^	335 ± 10
6	Sham	103 ± 1	100 ± 1	445 ± 4 #	412 ± 6
6	ADx	103 ± 2	100 ± 2	447 ± 5 ^	425 ± 8

Table 3 – Tissue Norepinephrine Levels in Denervated Organs
Norepinephrine content (ng/g tissue) as an indicator of successful denervation. Tissue was analyzed from the soleus muscles in the hindlimb to assess lumbar denervation, the kidneys to assess renal denervation, and the spleen, liver, and duodenum to assess splanchnic denervation. * = $p < 0.05$ vs. sham group.

Exp. #	Denervation	Tissue	Group	NE (ng/g)
2	**Lumbar**	Left Soleus	Sham	66 ± 13
			LDNx	9 ± 2 *
		Right Soleus	Sham	65 ± 12
			LDNx	21 ± 8 *
3	**Renal**	Left Kidney	Sham	134 ± 12
			RDNx	4 ± 1 *
		Right Kidney	Sham	122 ± 14
			RDNx	6 ± 1 *
4	**Splanchnic**	Liver	Sham	33 ± 6
			CGx	6 ± 1 *
		Spleen	Sham	189 ± 34
			CGx	2 ± 0.1 *
		Duodenum	Sham	455 ± 42
			CGx	6 ± 2 *
5	**Adrenal Demedullation**	Left Adrenal	Sham	156,146 ± 44,059
			ADMx	868 ± 611 *
		Right Adrenal	Sham	116,090 ± 24051
			ADMx	359 ± 136 *

Figure 1 – Effect of Chronic Sympathetic Blockade on the Pressor Response to Water Deprivation

A) MAP and HR responses to 48 hours of water deprivation with continuous infusion of either saline vehicle or a combination of α_1- and β_1- adrenergic receptor antagonists. B) MAP and HR changes from baseline: 4 hour averages from the light and dark phases at the end of water deprivation compared to the equivalent baseline averages. * = $p < 0.05$ between groups. # = $p < 0.05$ within vehicle. ^ = p 0.05 within blocked.

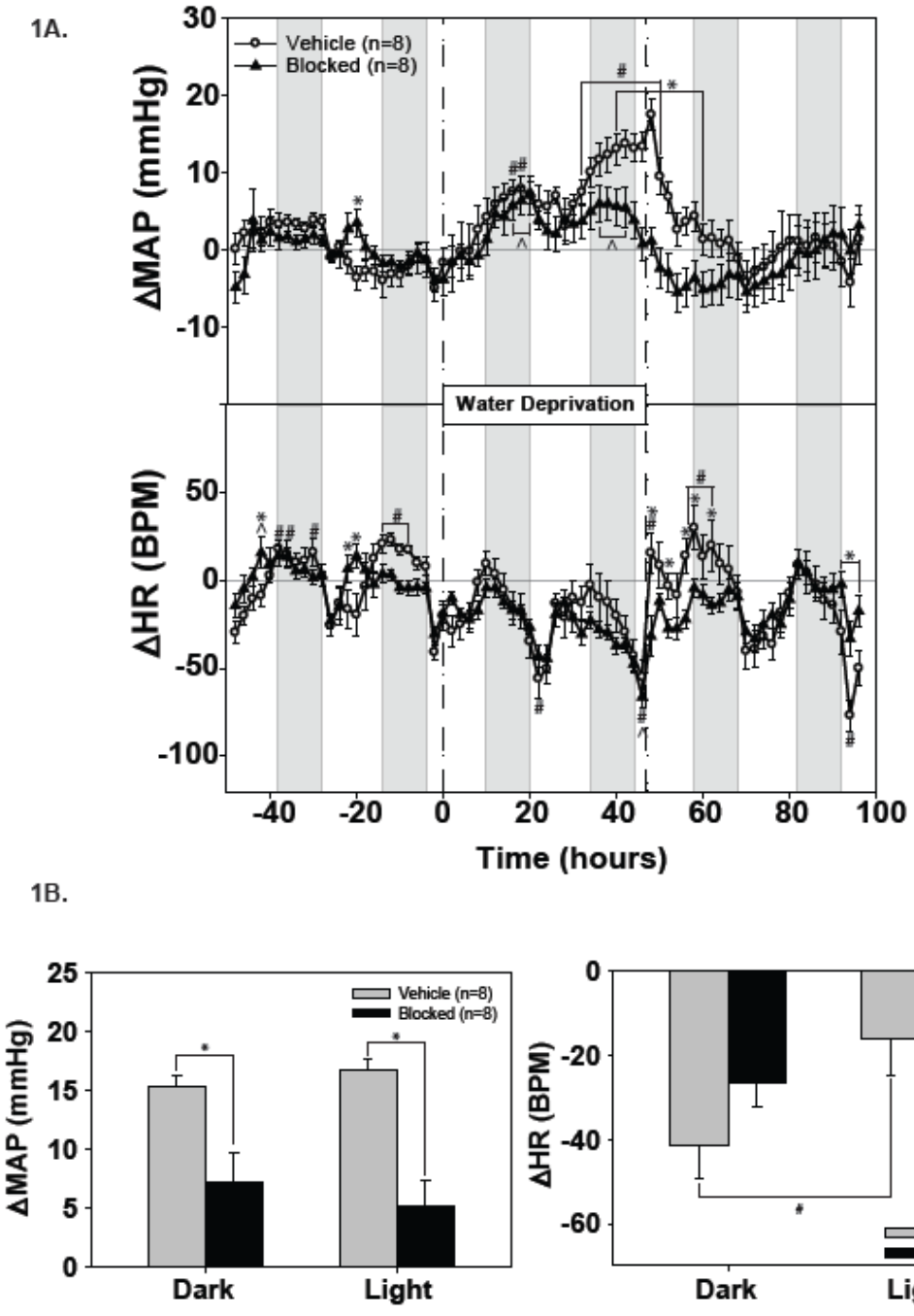


Figure 2 – Effect of Lumbar Denervation on the Pressor Response to Water Deprivation

A) MAP and HR responses to 48 hours of water deprivation in rats with (sham) or without (LDNx) lumbar nerves B) MAP and HR changes from baseline: 4 hour averages from the light and dark phases at the end of water deprivation compared to the equivalent baseline averages. * = $p < 0.05$ between groups. # = $p < 0.05$ within sham ^ = p 0.05 within LDNx.

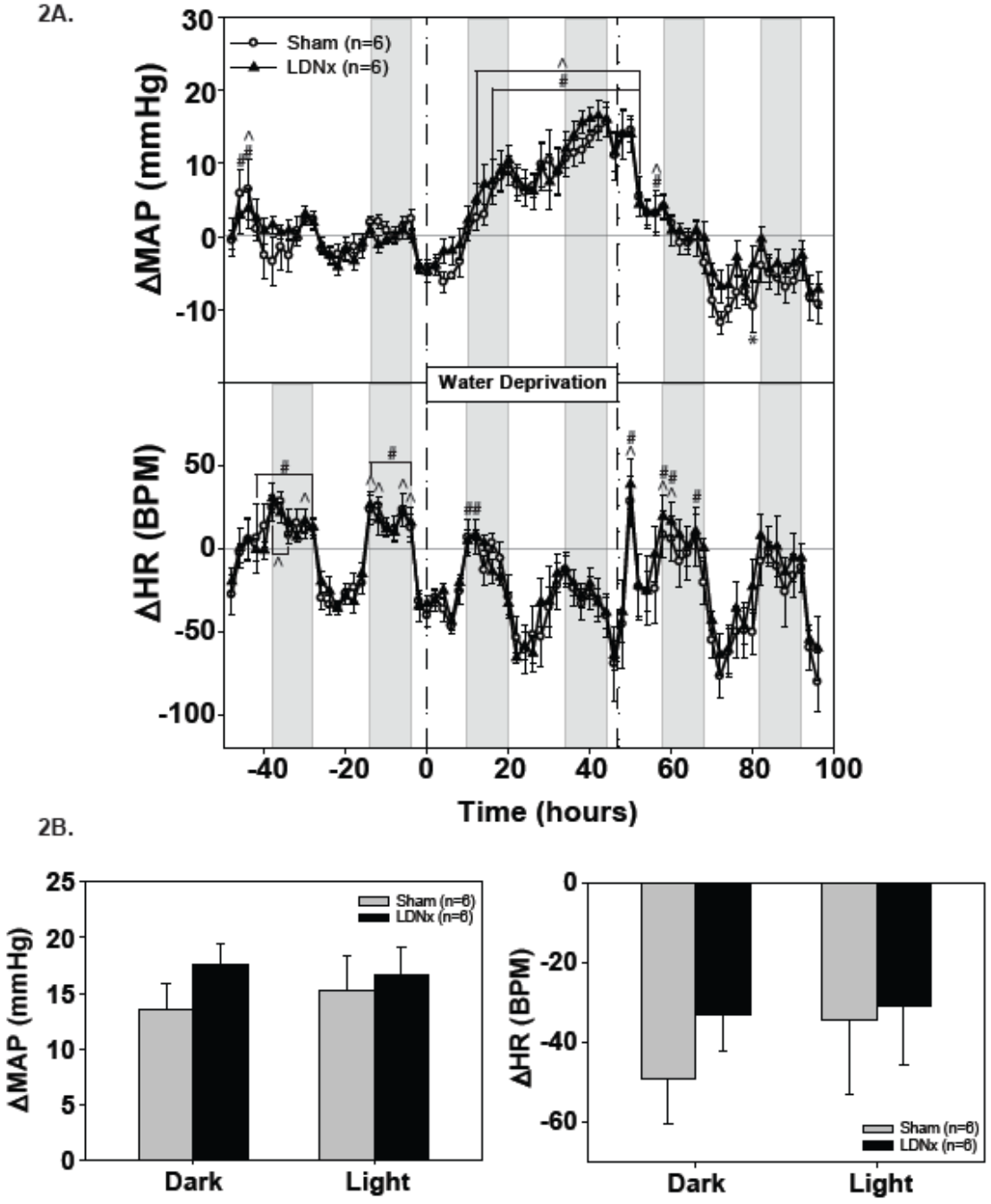


Figure 3 – Effect of Renal Denervation on the Pressor Response to Water Deprivation

A) MAP and HR responses to 48 hours of water deprivation in rats with (sham) or without (RDNx) renal nerves B) MAP and HR changes from baseline: 4 hour averages from the light and dark phases at the end of water deprivation compared to the equivalent baseline averages. * = $p < 0.05$ between groups. # = $p < 0.05$ within sham ^ = p 0.05 within RDNx.

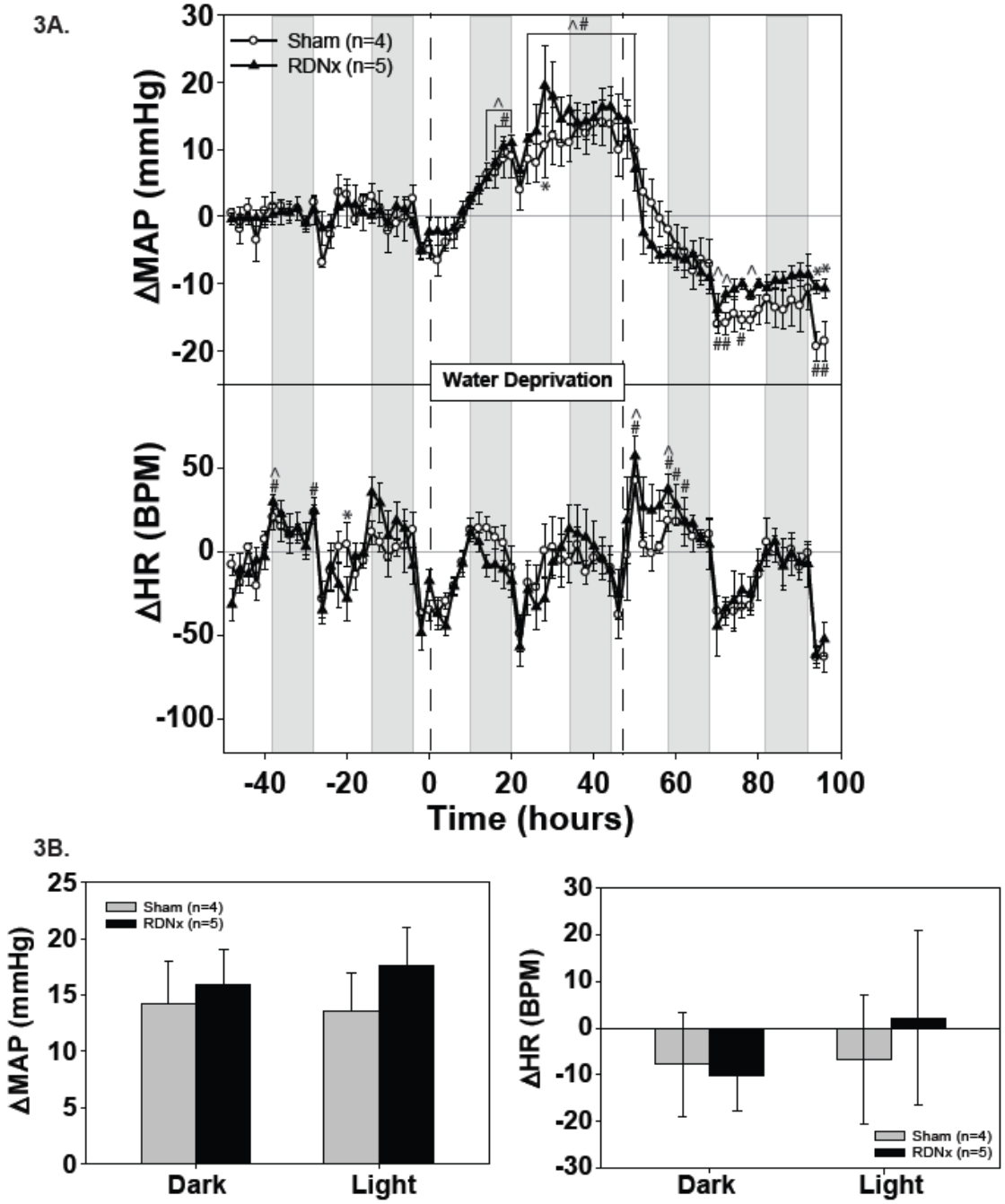


Figure 4 – Effect of Celiac Ganglionectomy on the Pressor Response to Water Deprivation

A) MAP and HR responses to 48 hours of water deprivation in rats with (sham) or without (CGx) splanchnic nerves B) MAP and HR changes from baseline: 4 hour averages from the light and dark phases at the end of water deprivation compared to the equivalent baseline averages. * = $p < 0.05$ between groups. # = $p < 0.05$ within sham ^ = p 0.05 within CGx.

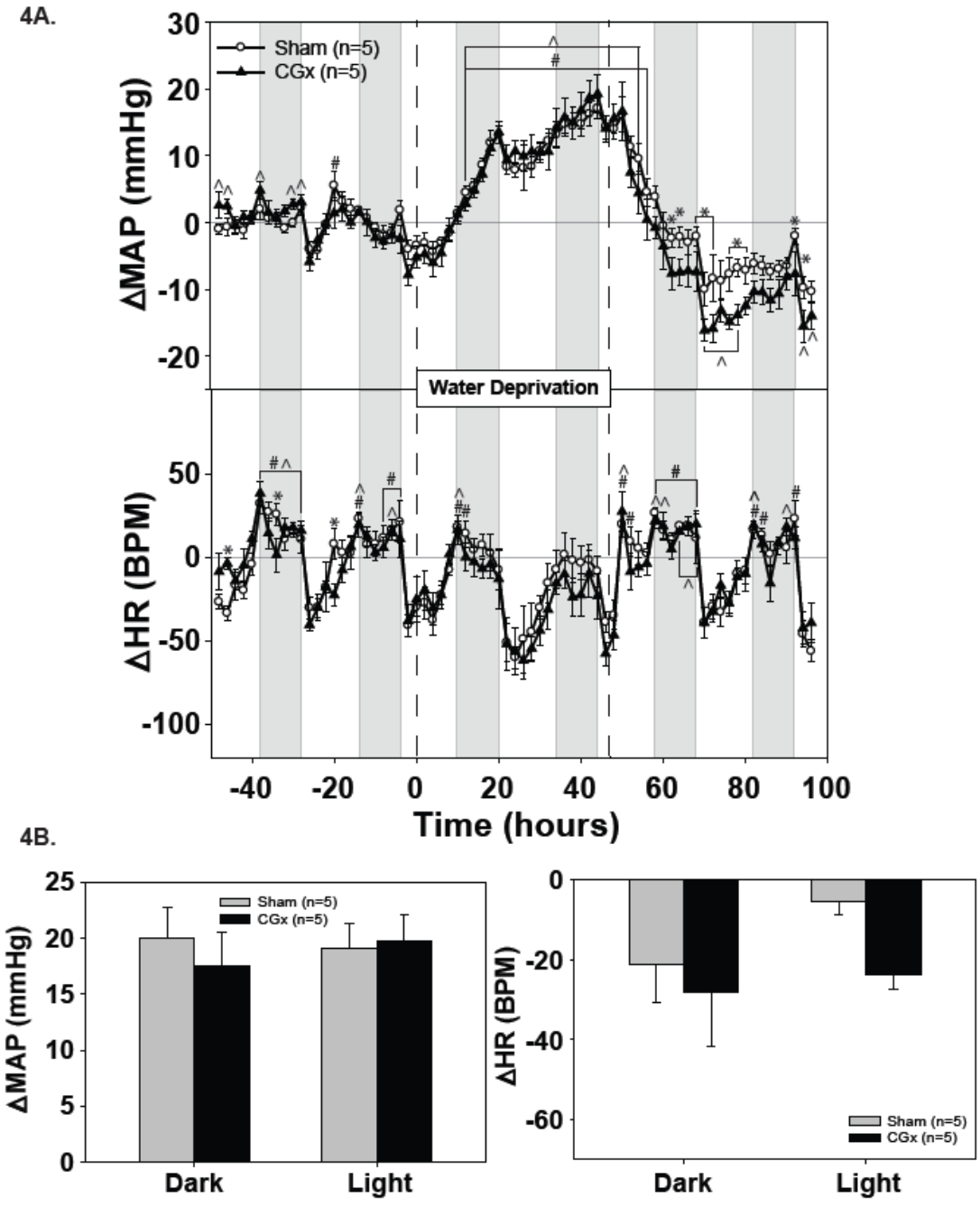


Figure 5 – Effect of Adrenal Demedullation on the Pressor Response to Water Deprivation

A) MAP and HR responses to 48 hours of water deprivation in rats with (sham) or without (ADMx) adrenal medullas B) MAP and HR changes from baseline: 4 hour averages from the light and dark phases at the end of water deprivation compared to the equivalent baseline averages. * = p < 0.05 between groups. # = p < 0.05 within sham ^ = p 0.05 within ADMx.

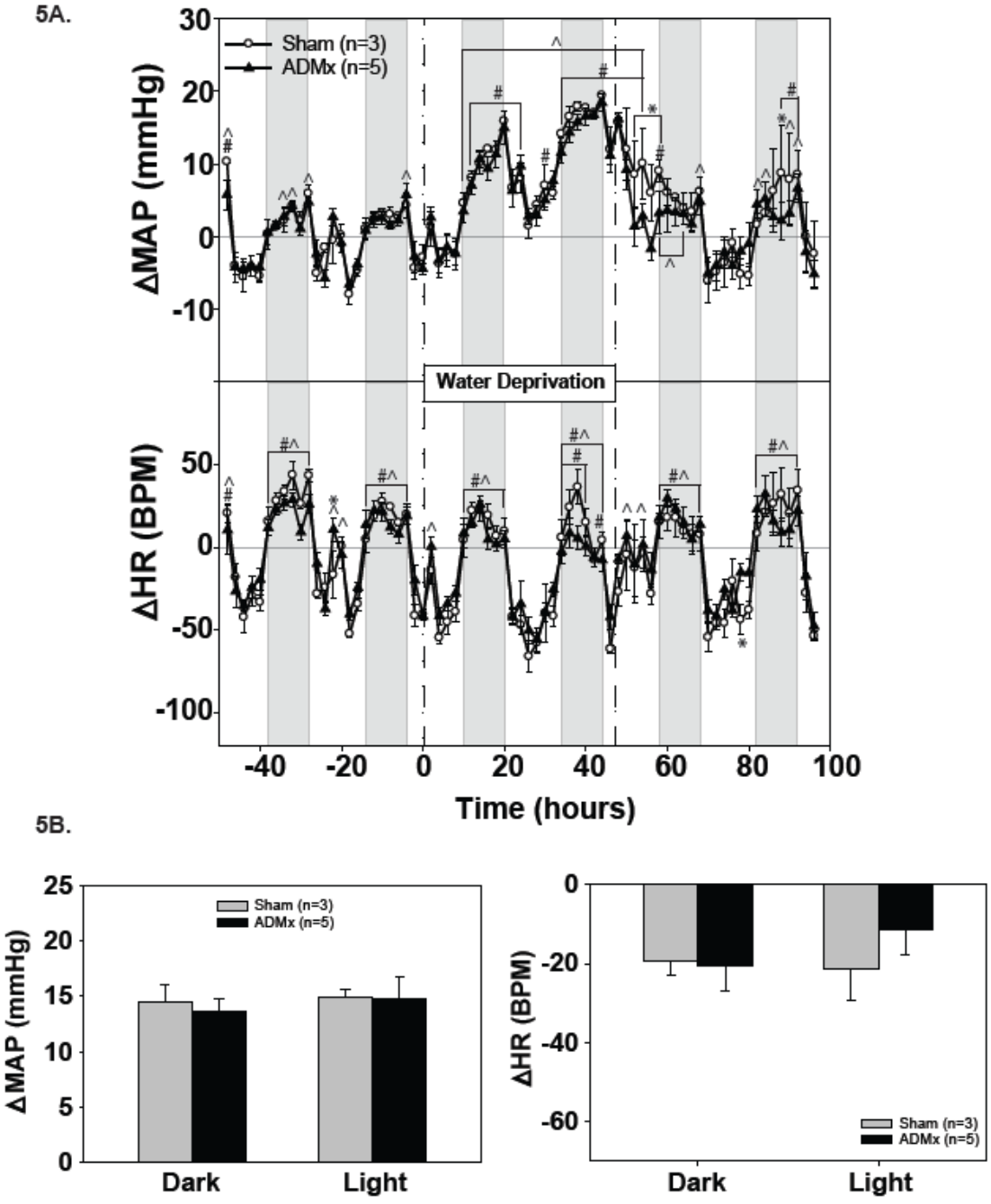
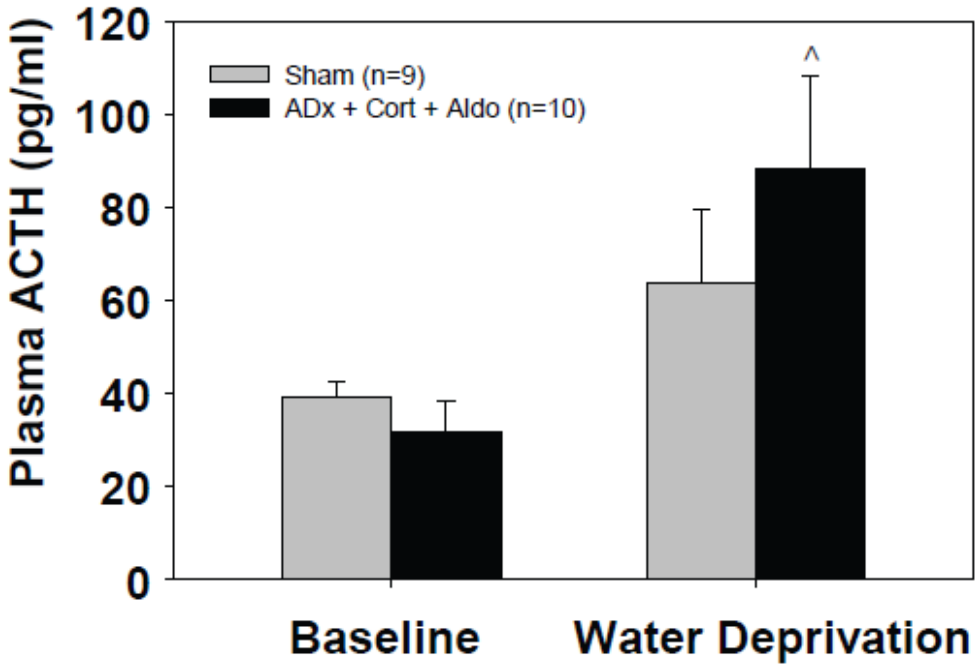


Figure 6 – Plasma ACTH and Corticosterone in Sham and ADx Rats
A) Plasma ACTH during baseline and water deprivation. B) Plasma corticosterone during baseline and water deprivation. ^ = p 0.05 within ADx.

6A.



6B.

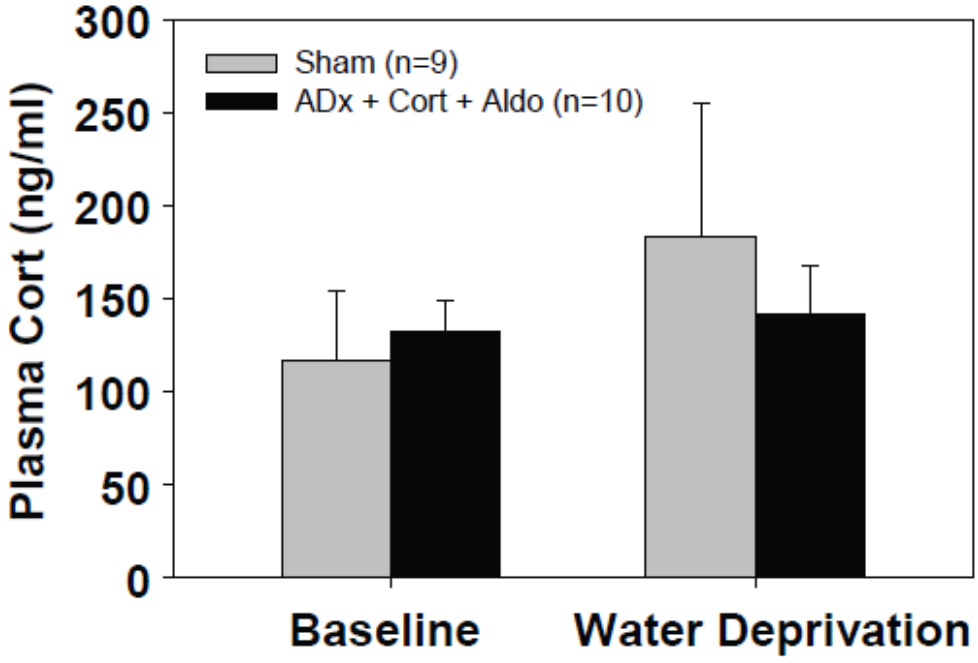
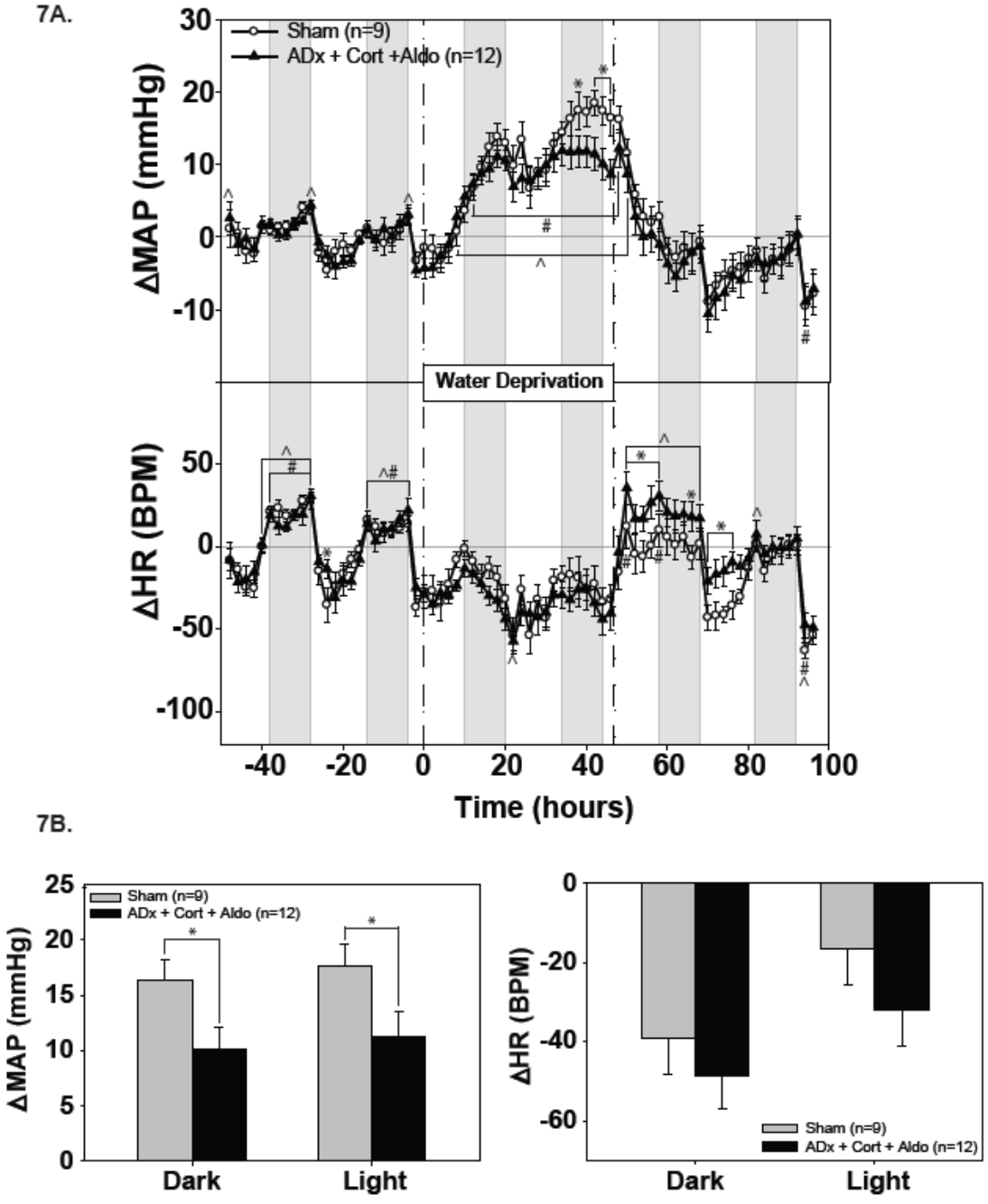


Figure 7 – Effect of Adrenalectomy on the Pressor Response to Water Deprivation A) MAP and HR responses to 48 hours of water deprivation in rats with (sham) or without (ADx + Cort + Aldo) adrenals. Adrenalectomized rats received replacement corticosterone and aldosterone. B) MAP and HR changes from baseline: 4 hour averages from the light and dark phases at the end of water deprivation compared to the equivalent baseline averages. * = $p < 0.05$ between groups. # = $p < 0.05$ within sham ^ = p 0.05 within ADx + Cort + Aldo.



Chapter 5

Conclusions

This thesis has focused on the spinal regulation of arterial pressure during conditions of increased osmolality. Specifically, the goals were to investigate the roles of two possible spinal neurotransmitters and to identify the sympathetic signature that regulates arterial pressure responses to water deprivation. The two major findings of this thesis work were: 1) glutamate - not vasopressin - is the spinal neurotransmitter required for the pressor response to increased osmolality; 2) elevated MAP during water deprivation is not due to increased SNA to a specific vascular bed; however, it appears to be dependent on the adrenal cortex.

Intrathecal Approach for Spinal Drug Delivery

In chapters 2 and 3 of this thesis, chronic intrathecal catheterization was used to deliver drugs to the spinal cords of conscious rats. A method for chronic intrathecal catheterization was initially developed by Yaksh et al. (Yaksh and Rudy 1976); but most of the experiments in these studies used an approach more similar to that of Storkson et al. (Storkson, Kjorsvik et al. 1996), which involves inserting a catheter into the intrathecal space in the *lumbar* region of the spinal cord and advancing the catheter *rostrally*. The catheters were advanced ~7cm so that the tip ended adjacent to spinal segments T10-T13. To verify catheter placement post-mortem, the rat was perfused with saline, Chicago Sky Blue dye was injected intrathecally, and a laminectomy was performed to expose the spinal cord. Analysis of dye spread was used as criteria for discarding rats from the study; however, some interesting observations were made regarding the chronic catheterization technique and are worth mentioning here.

1) There are clear differences in post-mortem dye appearance between intrathecal, epidural, and intra-spinal catheter placement.

By far, the majority of catheters were placed correctly into the intrathecal space. These catheters were almost always found on the dorsal surface of the cord, and the dye filled the intrathecal space and diffused rostrally along the spinal cord (Figure 1A). Rarely, a catheter was located in the epidural space. These catheters were found on top of the dura, and the dye injected through these catheters was barely present (Figure 1B).

Occasionally, the catheter was found *in* the spinal cord. This was much more common in the study (in Chapter 2) in which the catheter was advanced from the atlanto-occipital membrane instead of from the lumbar region. The dye injected through these catheters was barely visible upon vertebrae removal but could be seen in the cross section when the spinal cord was cut (Figure 1C).

2) Two catheters can be implanted simultaneously in different parts of the spinal cord to assess range of intrathecal antagonist.

The intrathecal V1a antagonist injected (with the lumbar approach of intrathecal catheterization) in Chapter 2 had no effect on arterial pressure, but the possibility remained that the physiological site of action was in more rostral parts of the thoracic cord and the antagonist was not reaching those sites. To address this, we implanted one catheter in the typical way (lumbar approach to T10-T13) and a second catheter from the atlanto-occipital membrane, advanced caudally to T1. We delivered a V1a agonist through the atlanto-occipital catheter and were able to block the arterial pressure responses by pretreating with the V1a antagonist from the lumbar catheter. In other words, the lumbar catheter antagonist blocked the actions of the agonist from the atlanto-occipital catheter. To our knowledge, this is a novel approach to assess the extent of blockade of intrathecally delivered antagonists.

3) Varying location of catheter and careful analysis of dye spread can provide information about rostral-caudal site of action of injectate.

In Chapter 3, we used the lumbar approach to catheterize two groups of rats. In one group, the catheter was advanced in the typical way to T10-T13; in the second group, the catheter was advanced ~10cm to T1-T5. Significant differences were seen in the arterial pressure responses to intrathecal injection of the antagonist between the two groups. In this case, the data implied that most - if not all - of the response was due to blockade of receptors in the spinal cord, as opposed to the brainstem.

Dye spread is only an estimation of antagonist spread due to differences in injectate properties; however, post-mortem dye analysis showed distinct differences

between the two groups. When the intrathecal catheter tip was in the typical location (T10-T13), blue dye was visible throughout the entire thoracic spinal region (Figure 2A). In contrast, when the injection was administered at T1-T5, the dye was most concentrated in the upper thoracic and cervical segments (Figure 2B).

To summarize, intrathecal catheterization is a useful technique for pharmacological manipulations in the spinal cords of conscious animals, but the diffuseness of intrathecal injections makes interpretations challenging. It is impossible to know precisely where the injectate acts, both in the cross-section and along the longitudinal axis. However, experiments with varied catheter placement and careful post-mortem dye analysis can provide additional information about the injectate's site of action, at least along the rostral-caudal axis.

Revised Model of Osmotic-SNA pathways

As stated in the introduction, most of the literature cites two possible pathways involved in regulation of SNA and MAP during increased osmolality (Stocker, Osborn et al. 2008) – a direct, vasopressinergic projection from the PVN (Antunes, Yao et al. 2006) and a PVN-RVLM-IML pathway that potentially uses glutamate as the spinal neurotransmitter (Morrison 2003). Despite the limited amount of data to indicate involvement of the direct vasopressinergic projection from the PVN (Antunes, Yao et al. 2006), it is often cited in the literature (Riphagen and Pittman 1985; Gruber and Eskridge 1986; Stocker, Osborn et al. 2008) or drawn in diagrams (Toney, Chen et al. 2003) as the likely pathway mediating MAP responses to increased osmolality.

The data reported in this thesis indicate that the direct vasopressinergic pathway may not be as important as the literature suggests, at least during the arterial pressure response to IV hypertonic saline, 48-hours of water deprivation, or 4 weeks of DOCA-salt hypertension. Importantly, the most convincing evidence for this pathway involved the lumbar SNA response to IV hypertonic saline (Antunes, Yao et al. 2006), but that study was conducted in an *in situ* rat preparation in which it was impossible to measure arterial pressure. As a result of the findings here that spinal blockade of V1a receptors had no effect during any of the tested hyperosmotic conditions, strong reservations

should accompany the mention of the direct vasopressinergic PVN pathway as a primary driver of MAP in regards to increased osmolality.

In contrast to the negative results of spinal V1a receptor blockade, intrathecal kynurenate caused a dramatic fall in arterial pressure during both water deprivation and DOCA-salt hypertension. These results suggest that spinal glutamate, which is thought to be the primary neurotransmitter of the descending RVLM neurons (Morrison 2003), is essential for the maintenance of elevated MAP at the time points tested. This had not previously been shown in conscious, osmotically stressed rats.

To further explore the implications of these results, an important distinction needs to be addressed: the intrathecal technique used in these studies indiscriminately blocks receptors throughout the spinal cord. Therefore, although the rationale behind investigating vasopressin and glutamate was due to the fact that they were the most likely to act on sympathetic preganglionic neurons to drive SNA responses to increased osmolality, it is impossible to know if the effects were due to actions on the SPNs or on other neurons in the spinal cord. This is particularly important considering that spinal interneurons may receive glutamatergic input (Llewellyn-Smith, Martin et al. 2007) and may contribute to regulation of sympathetic nerve activity (Deuchars 2007).

It is also important to consider that this thesis only investigated the role of the two most likely neurotransmitters to regulate SNA during hyperosmolality, but there are *many* other potential candidates (McCall 1988). For example, in a recent review (Llewellyn-Smith 2009), Llewellyn-Smith et al. listed 20 neuropeptides that potentially provide input to SPNs in addition to excitatory amino acids and monoamines. It is possible that neuropeptides modulate the action of excitatory amino acids by influencing the level of neuronal excitability (McCall 1988), and this theory is not only compatible with the findings in this thesis but also suggests that much more work may be needed in order to fully understand the neurotransmitter regulation of SPNs. Nevertheless, the findings here are an important first step.

To summarize, the results of this thesis provide support for the PVN – RVLM – spinal pathway over the direct vasopressinergic PVN – spinal pathway in control of SNA and MAP during hyperosmotic conditions (Figure 3). However, we cannot claim that the

pathway responsible is the PVN-RVLM-spinal pathway, because the effects of intrathecal kynurenate were not necessarily due to blockade of SPN receptors receiving glutamatergic input from the RLVM. For example, i.t. KYN effects could be due to actions on interneurons (Llewellyn-Smith, Martin et al. 2007). We also cannot claim that the direct PVN-spinal projection is not primarily responsible; as it is possible the direct projection from the PVN could be glutamatergic (Llewellyn-Smith, Minson et al. 1995; Yang, Wheatley et al. 2002). However, the results here suggest it is unlikely that a direct vasopressinergic pathway from the PVN is the primary mediator of MAP during increased osmolality. If spinal vasopressin is involved, it is likely in a modulatory role that is unnecessary for the maintenance of blood pressure during osmotic stress.

Revised Sympathetic Signature of Water Deprivation

Previous studies suggested that differential regulation of sympathetic nerve activity occurs during water deprivation, with clear elevations in lumbar SNA and variable responses in renal SNA (Scrogin, Grygielko et al. 1999; Scrogin, McKeogh et al. 2002; Stocker, Hunwick et al. 2005). These observations, along with studies suggesting that specific 'sympathetic signatures' occur in various physiologic and pathologic states (McAllen and May 1994; Morrison 2001; Osborn and Kuroki 2012; Ramchandra, Hood et al. 2012) and that SNA to individual vascular beds can affect blood pressure (Jacob, Clark et al. 2005; King, Osborn et al. 2007), led to the goal of identifying the functional 'sympathetic signature' of water deprivation through selective sympathetic denervations. Our results demonstrated that while the pressor response after ~24 hours of water deprivation relies on global sympathetic activation, there was no evidence that increased SNA to any specific vascular bed was responsible for the pressor response.

In order to explore possible explanations for these results, it is helpful to examine the evidence behind the rationale of this study. First, an abundance of anatomical and physiological evidence suggests that autonomic outflow is differentially regulated (Morrison 2001). For example, selective stimulation of the RVLM can change muscle blood flow independently of renal nerve activity (McAllen and Dampney 1990), freezing behavior in rats is accompanied by differential responses in lumbar and renal SNA

(Yoshimoto, Nagata et al. 2010), and heart failure in sheep (May, Frithiof et al. 2010) and humans (Esler 2010) is characterized by selective elevations in cardiac SNA. However, the fact that differential regulation is possible does not necessarily mean that increased SNA to an individual vascular bed *causes* an increase in arterial pressure, which raises the question about selective denervation as a technique to investigate the 'sympathetic signature.' Nevertheless, selective denervations in the AngII-salt rat (King, Osborn et al. 2007) and DOCA-salt rat (Jacob, Clark et al. 2005) attenuated the hypertension in these models, suggesting a causative link between selective sympathetic activation and increased arterial pressure. Additionally, recent clinical trials have shown that renal denervation successfully lowers arterial pressure in hypertensive humans (Schlaich, Sobotka et al. 2009; Schlaich, Sobotka et al. 2009).

There are at least two possible reasons the arterial pressure response to water deprivation is unaffected by selective denervations, in contrast to the above-mentioned studies. First, water deprivation may simply involve global sympathetic outflow, while the above models may rely more on specific sympathetic activation. Water deprivation differs from the salt-sensitive rat models and human hypertension in several ways: the arterial pressure response during water deprivation is small by comparison and develops over a relatively short time-frame; it is accompanied by hypovolemia; and it involves increased motor activity and psychological stress due to thirst. The combination of all of these factors makes it difficult to predict if the sympathetic signature of water deprivation should resemble that of other hyperosmotic conditions. Nevertheless, as stated in the introduction, water deprivation is likely the only hyperosmotic condition in evolutionary history. Therefore, it seems possible that the mechanisms underlying the pressor response to water deprivation are similar to those involved in the development of salt-sensitive hypertension.

Another potential explanation may be that the denervations in previous studies may reflect the ‘sympathetic signatures’ of pathological conditions rather than specific patterns of centrally driven sympathetic outflow to increase arterial pressure. For example, it has been proposed that renal sympathetic nerve ablation lowers blood pressure in humans by inhibiting renal *afferent* nerves, which are otherwise activated by

renal damage and lead to elevations in central sympathetic outflow (DiBona and Esler 2010). Indeed, the renal denervation procedure lowered whole-body norepinephrine spillover and reduced SNA to skeletal muscle (Schlaich, Sobotka et al. 2009), suggesting that reductions in non-renal sympathetic activity may contribute to the antihypertensive effects of this treatment.

Regardless of *why* selective denervations have no effect on the pressure response to water deprivation, these results suggest that our initial goal of identifying a specific spinal target for anti-hypertensive therapies may be unrealistic. If osmotic stress during water deprivation activates the same pathways involved in salt-sensitive hypertension, the centrally driven sympathetic outflow may be more global than target-specific, making it more difficult to block with spinal treatments.

An Unexpected Player: The Adrenal Cortex

An unexpected finding of Chapter 4 was that complete adrenalectomy attenuated the pressor response to water deprivation in the same time course as global sympathetic blockade. This suggests that adrenocortical hormones may interact with the sympathetic nervous system to increase MAP during water deprivation. For example, sympathetic activation may trigger hormonal release, since some evidence suggests that the adrenal cortex receives sympathetic innervation (Engeland 1998; Bornstein and Chrousos 1999). Alternatively, adrenocortical hormones may influence sympathetic activity in a variety of ways (discussed below).

The adrenal cortex releases hormones that have been shown to modulate sympathetic nervous system activity, both centrally and peripherally. These include glucocorticoids (e.g. corticosterone in the rat) from the zona fasciculata, and aldosterone and endogenous ouabain (Laredo, Hamilton et al. 1995) from the zona glomerulosa.

It is well known that stress plays a role in human hypertension and cardiovascular disease (Girod and Brotman 2004; Esler, Eikelis et al. 2008; Esler 2009), and systemic corticosterone administration has been found to increase arterial pressure (Scheuer and Bechtold 2001). The mechanisms by which glucocorticoids influence arterial pressure are not clear; however, they are thought to involve both peripheral (Saruta 1996) and

central (Scheuer 2010) actions. For example, glucocorticoids act on endothelial and smooth muscle cells to enhance sympathetic vasoconstriction (Yang and Zhang 2004). Additionally, blockade of glucocorticoid receptors in the dorsal hindbrain region lowers arterial pressure in a model of hypertension induced by systemically administered glucocorticoids (Scheuer, Bechtold et al. 2004). Finally, it was recently reported that glucocorticoid receptor binding is required for sympathetically mediated activation of NaCl cotransporters that allow increased sodium reabsorption to occur in the distal nephron (Ellison and Brooks 2011; Mu, Shimosawa et al. 2011). Taken together, these studies support the hypothesis that glucocorticoids can modulate sympathetic regulation of arterial pressure. Additional studies are required to determine the role of a glucocorticoid-sympathetic interaction in the regulation of arterial pressure during water deprivation.

Aldosterone is another adrenocortical hormone that could affect SNA and contribute to the MAP response during water deprivation. Aldosterone is well characterized as a sodium-retaining mineralocorticoid that acts in the distal tubule and collecting duct of the kidney to increase the expression of ENaCs (Schild 2010). However, evidence suggests that it can also play an important role in the central nervous system to control sympathetic responses to increased plasma osmolality (Abrams and Osborn 2008). Although aldosterone does not cross the blood-brain-barrier (Pardridge and Mietus 1979), it is thought that circulating aldosterone is sensed by neurons in the circumventricular organs (CVOs) (Geerling, Kawata et al. 2006), which have a weakened blood-brain-barrier. It is hypothesized that increased systemic aldosterone can bind to mineralocorticoid receptors in CVO neurons and activate nuclear transcription of epithelial sodium channels (ENaCs), which are then transported to the membrane to increase sodium conductance (Abrams and Osborn 2008). Therefore, under conditions of increased osmolality, such as water deprivation, the CVO neurons would be more easily depolarized in response to sodium and potentially activate downstream pathways to increase sympathetic nerve activity. Indeed, introcerebroventricular (i.c.v.) infusion of aldosterone increases arterial pressure (Kageyama and Bravo 1988), and intracerebroventricular administration of mineralocorticoid receptor antagonists prevent

the hypertensive response to systemic aldosterone (Gomez-Sanchez, Fort et al. 1990; Janiak, Lewis et al. 1990). Furthermore, intracerebroventricular administration of drugs that block ENaCs, such as benzamil, blocks the acute pressor responses to increased cerebrospinal fluid sodium concentration (Abrams and Osborn 2008) as well as an aldosterone - salt model of hypertension (Abrams, Engeland et al. 2010). These studies support the hypothesis that sympathetic activity is increased under conditions in which both plasma osmolality and aldosterone are elevated, such as water deprivation. This is consistent with our results in which adrenalectomy attenuated the arterial pressure response to water deprivation in a similar time frame as sympathetic blockade.

Finally, a third adrenal cortical hormone that may be important in modulating sympathetic activity during water deprivation is endogenous ouabain. Ouabain is thought to be produced by the adrenal cortex (Ludens, Clark et al. 1992) and hypothalamus (Kawamura, Guo et al. 1999) and is implicated in human hypertension (Iwamoto and Kita 2006). Ouabain is a sodium pump inhibitor and, like aldosterone, is thought to act both peripherally and centrally to increase arterial pressure (Blaustein, Leenen et al. 2012). Indeed, it is proposed that it acts centrally *in conjunction with* aldosterone and ENaCs (Leenen 2010) to activate a brain renin-angiotensin system which enhances sympathoexcitatory pathways (Blaustein, Leenen et al. 2012). Moreover, in the periphery, ouabain can enhance sympathetic vasoconstriction by increasing norepinephrine release from sympathetic nerve terminals (Raina, Zhang et al. 2010) and amplify transmission at the sympathetic ganglia (Aileru, De Albuquerque et al. 2001). To our knowledge, the role of ouabain in cardiovascular regulation during water deprivation has not been investigated. However, it is also important to note that the evidence in support of endogenous ouabain as an important physiological regulator has been questioned (Nicholls, Lewis et al. 2009).

Conclusion and Perspectives

To summarize, this thesis has contributed several new findings to the field: 1) Spinally released glutamate, but *not vasopressin*, is required for the arterial pressor responses to increases in plasma osmolality; 2) increased arterial pressure during water

deprivation depends, in part, on global sympathetic activation but does not rely on specific sympathetic outflow to any individual vascular bed; and 3) hormones from the adrenal cortex may be involved in the pressor response to water deprivation and may be linked to sympathetic activation during dehydration. These findings are summarized in Figure 3. Much more research is required in order to fully understand how osmotically induced sympathetic outflow and arterial pressure are regulated at the level of the spinal cord, but the findings in this thesis have expanded what was known and provided several redirections of focus for future studies.

The initial motivation behind the studies in this thesis was to identify potential spinal targets for novel antihypertensive therapies. We speculated that a particular neurotransmitter may be responsible for increasing sympathetic nerve activity to a specific vascular bed (i.e. vasopressin activation of lumbar SNA), which would present a distinct therapeutic target in the spinal cord. In light of the findings here, it is unlikely that the solution to spinal antihypertensive therapies will be that simple. The spinal cord is the ultimate site of modulation of sympathetic outflow, suggesting potential importance as a therapeutic target. However, its size and complexity makes it difficult to study in conscious animals, and much about its regulation remain unknown. Sympathetic regulation at the spinal cord will likely need to be understood in much greater detail before a specific spinal target for antihypertensive therapies can be identified.

Figure 1

Examples of dye spread when the tip of the catheter was found in the A) intrathecal space, B) epidural space, or C) in the spinal cord.

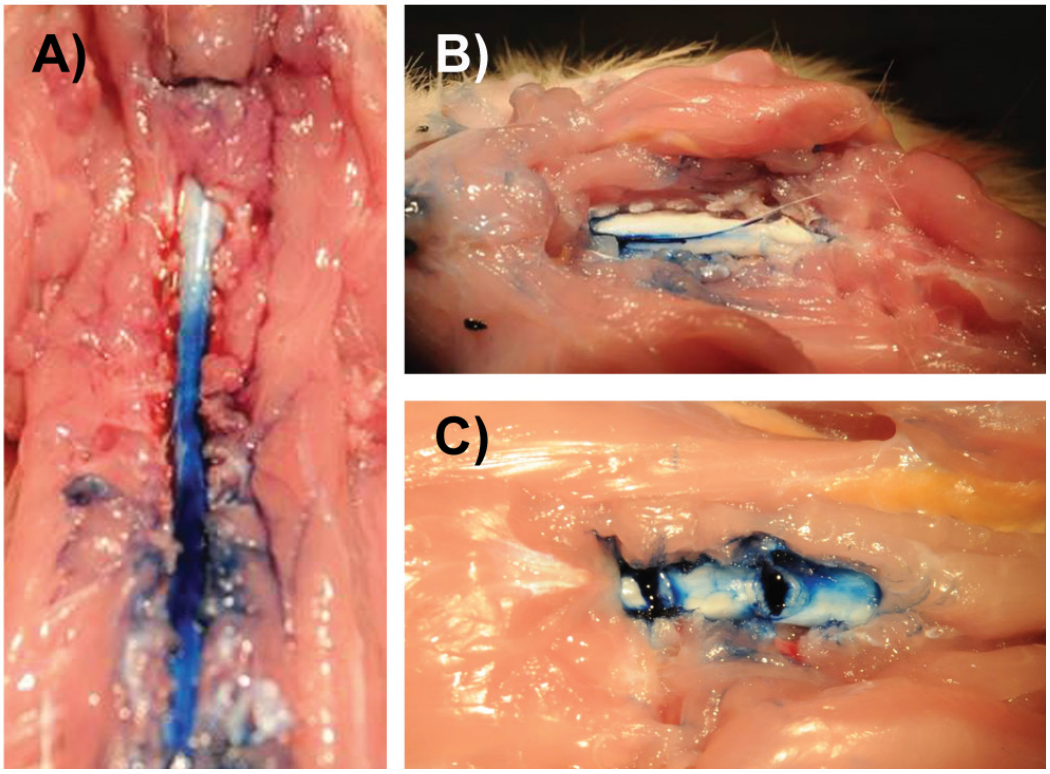


Figure 2

Examples of dye spread when the tip of the intrathecal catheter was placed in the A) caudal thoracic spinal cord or B) rostral thoracic spinal cord.

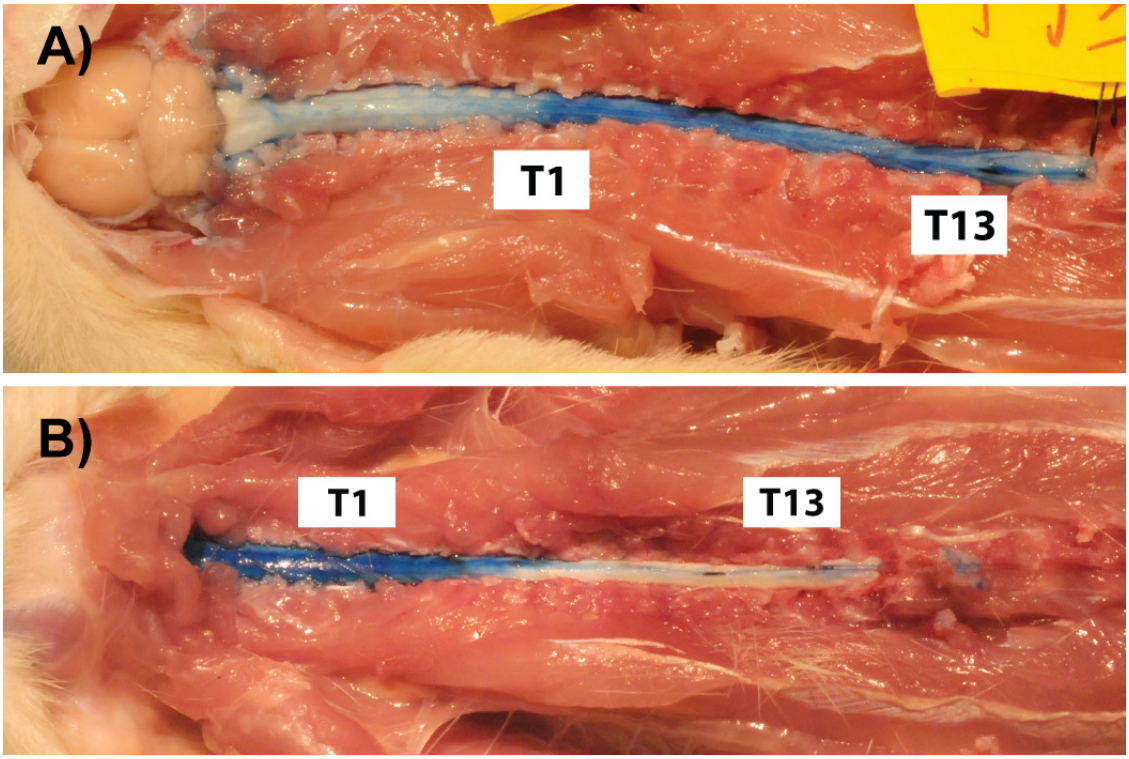
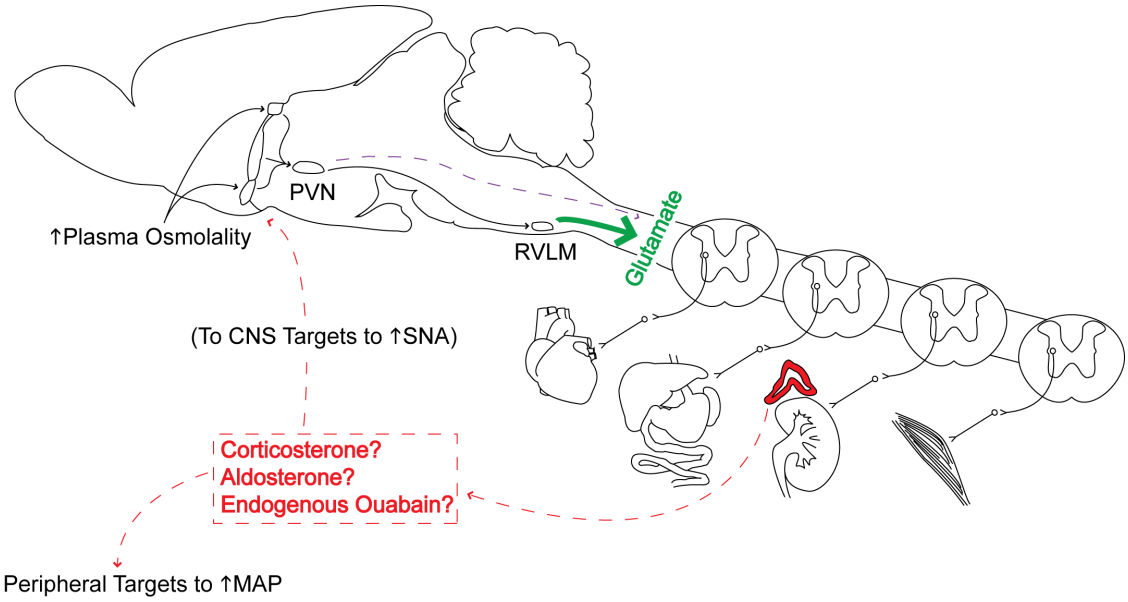


Figure 3

Revised schematic of osmosensitive neural pathways mediating the arterial pressure response during water deprivation. The data presented in this thesis suggest that the PVN-IML vasopressinergic pathway (purple dashed line) is not required for the pressor response to osmotic activation as previously hypothesized. Instead, the pressor response appears to be mediated by spinally released glutamate, likely from the RVLM (green arrow). The response relies on global sympathetic activation, as opposed to sympathetic activation to select vascular beds; and adrenocortical hormones may be involved and linked to sympathetic activation.



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