The Pennsylvania State University

The Graduate School

Department of Cell and Molecular Physiology

CONTRIBUTION OF THE ORGANUM VASCULOSUM OF THE LAMINA TERMINALIS TO SALT-INDUCED HYPERTENSION

A Thesis in

Physiology

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

May 2013

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ABSTRACT

Increased dietary intake of salt raises plasma and cerebrospinal fluid (CSF) sodium concentrations and strongly correlates with an increase in arterial blood pressure (ABP). The pressor effect of increased sodium intake is mediated by central mechanisms and depends on increased sympathetic nerve activity (SNA). Both peripheral and central administration of hyperosmotic NaCl increases blood pressure through differential regulation of multiple sympathetic vascular beds.

A region of the forebrain lamina terminalis near the anterior ventral 3rd ventricle, known as the AV3V region, has been identified as an integral part of the brain involved in mediating the sympathoexcitatory and hypertensive response to increased sodium intake. This region is comprised of several brain centers known to contain osmosensitive neurons including the organum vasculosum lateral terminals (OVLT), subfornical organ (SFO), and the median pre-optic area (MePO). Multiple studies have shown that lesioning of the AV3V region can attenuate many of the responses to increased sodium intake including increased fluid intake, and vasopressin release as well as hypertension and sympathoexcitation.

The OVLT, a circumventricular organ with an incomplete blood brain barrier, has also been shown to be involved in regulating many of the responses to increased sodium intake. Specific ablation of the OVLT alone attenuates the increased drinking and vasopressin release in response to sodium load and has been implicated in the regulation of SNA in response to intracerebroventricularly (ICV) administered NaCl. However, the exact contribution of the OVLT and its effects on various sympathetic vascular beds in response to ICV sodium loading has not been fully characterized. Therefore, the purpose of this study was twofold: 1. to determine the effect of lesioning of the OVLT on the sympathoexcitatory and hypertensive

effects in response to increased central sodium levels and to compare this to the effect seen with the much larger AV3V ablation, and 2. to characterize the effects of OVLT ablation on the responses of the individual sympathetic vascular beds mediating the response to increased central sodium loads.

In chloralose anesthetized, vasopressin blocked rats, ICV infusion of 5ul of 1.0M NaCl over 10min. increased ABP, HR and lumbar SNA compared to baseline. Renal and splanchnic SNA initially decreased during NaCl infusion, but showed a delayed increase to above baseline values following cessation of the infusion. AV3V ablation resulted in almost complete attenuation of these effects as compared to control. Alternatively, OVLT ablation only partially attenuated the effects on ABP, heart rate (HR) and SNA as compared to AV3V ablation levels.

These results suggest that the AV3V region is integral in the body's ability to respond to increases in central sodium levels and the OVLT mediates, at least in part, the sympathoexcitatory and hypertensive effects of increased central sodium loading through changes in lumbar sympathetic activity. However, the fact that the OVLT ablation only partially attenuates lumbar SNA and ABP compared to that seen with AV3V ablation suggests brain regions other than the OVLT are also likely involved.

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Chapter 1

Introduction

As more of the world becomes increasingly industrialized, diets once dominated by agricultural based produce such as fresh fruits and vegetables have come to include a much higher percentage of processed packaged foods. These foods usually contain high levels of sodium since the most common additive used in preservation is salt. In turn, the price for increased convenience and a longer shelf life of our foods has become an ever-increasing level of sodium intake. Excessive dietary salt intake is strongly associated with an increased risk of developing multiple cardiovascular diseases including hypertension [1-5]. Recent statistics from the World Health Organization show that over 25% of the U.S. has been diagnosed with hypertension and cardiovascular mortality is the leading cause of death worldwide [6]. Current averages of sodium intake in many of the world's industrialized nations have been estimated to be to be as high as 9 to 12 grams/day[7]. These levels are in stark comparison to the current American Heart Association Guidelines for daily dietary sodium intake recommendation of <2.3 grams/day for the general public and <1.5 grams/day for individuals with one or more cardiovascular risk factors[8]. However, even with a preponderance of evidence linking increased sodium intake to increases in mortality and morbidity, few individuals have been able to successfully reduce their daily sodium intake to the suggested levels. In addition, the number of patients unable to achieve acceptable levels of blood pressure even after being placed on multiple antihypertensive medications continues to rise [9].

Variability of Sensitivity to Changes in Dietary Salt

One of the biggest hurdles in combating dietary salt hypertension is, for all the literature that exists defining the effects of increased sodium intake on the cardiovascular system, a detailed understanding of how salt functions to produce these effects continues to be lacking. Salt-induced hypertension has a complex pathology affecting multiple organ systems and involves genetic, hormonal and environmental factors that often interact to produce hypertension. Complicating these facts, individual responses to increases in sodium load vary significantly from person to person. Approximately 30% of individuals can be classified as "salt-sensitive" and respond to increased sodium load with an increase in blood pressure; the remainder of the population appear relatively protected from the hypertensive effects of sodium and are classified as "salt-resistant" [10-13]. Even though only about a third of the population may be salt-sensitive, up to 70% of essential hypertensive individuals are salt-sensitive and, although not currently hypertensive, salt-resistant individuals are still at increased risk of developing future hypertension[14], reiterating the importance of understanding the pathophysiology of salt-hypertension for the continued development of novel anti-hypertensive treatments and prevention strategies.

Connection Between Dietary Salt Intake and Increases in Blood Pressure

Moderating salt intake has been proposed as one of the most effective, modifiable factors in the prevention of hypertension [5, 15-18]. Increased dietary sodium intake has been shown to elevate both plasma and CSF sodium levels as well as ABP in salt sensitive individuals, however, the increases in plasma sodium levels reported have been modest at best leading some to question the link between dietary sodium levels and hypertension [19-24]. Although the changes in

plasma sodium are relatively small, total body sodium levels have been reported to increase significantly with increased dietary salt intake [25] and correlates strongly with blood pressure increases especially in salt sensitive individuals [25-27]. In order for the body to function properly, body fluid osmolarity has to be maintained in a narrow range. In fact, the body has a tremendous ability to maintain osmotic concentrations within a range of just a few millimolar and can detect and respond to changes of as little as 1-2%. In the normal individual, increased dietary salt intake increases the antihypertensive natriuretic and diuretic response, however, this response has been shown to be attenuated in the salt sensitive spontaneous hypertensive rat model [28] . Simply reducing the level of dietary salt consumption has been shown to improve cardiovascular risk [29, 30] and can effectively lower blood pressure in both human and animal models [31, 32]. However, given the low compliance rate to accepted dietary sodium intake guidelines and an increasing number of drug resistant hypertensive patients, novel approaches to the treatment of salt-induced hypertension continue to be needed to keep pace with the world-wide increases in dietary sodium intake.

Central Regulation of the Response to Increased Sodium Intake

In order to identify possible treatment targets, we first must know how and where increased levels of sodium are sensed in the body and how these processes differ between salt sensitive and resistant individuals. Several studies suggest the differences between salt sensitive and resistant individuals are mediated by central mechanisms. High salt intake has been shown to elevate plasma sodium levels similarly in both sensitive and resistant individuals[19], however, only salt sensitive models show an increase in central cerebrospinal fluid sodium concentrations[20, 24, 33]. Increased dietary sodium intake increases the activation of

osmoregulatory and cardiovascular brain centers [34-37] and this activation is elevated in salt-sensitive hypertensive models [38, 39]. Increases in dietary salt intake have been shown to strongly correlate with changes in several centrally mediated autonomic responses that affect cardiovascular hemodynamics such as increases in sympathetic nerve activity [40-49] and the baroreflex [50-55]. Increasing central sodium levels by injection of hypertonic saline into the cerebral blood supply via intracarotid artery (ICA) infusion [56-58] or directly into the ventricles of the brain increases blood pressure and SNA without affecting peripheral sodium levels [45, 59, 60]. Conversely, lowering central sodium concentration decreases renal SNA and blood pressure in barodenervated anesthetized cats [56]. Changes as small as 2% have been shown to be able to attenuate the hypertensive and lumbar sympathetic effects in water deprived [61] and DOCA-salt [62] rats. The effects of increased central sodium levels on sympathetic activity are thought to cause increases in blood pressure by producing vasoconstriction in peripheral sympathetic vascular beds, which in the environment of altered compensatory mechanisms such as the baroreflex, may allow for sustained hypertensive effects and the propagation of a chronic pathology.

Sympathetic Dependence of the Hypertensive Effects in Response to Increases in Sodium Levels

Functionally, the sympathetic dependence of salt-induced hypertension has been demonstrated in both humans and animal models through the use of various sympatholytic techniques. Blockade of sympathetic activity at the pre/postganglionic synapse via administration of ganglionic blockers [63-68] or at the receptor level by α/β blockers [69-71] produces a greater drop in blood pressure in salt-sensitive individuals on a high salt diet versus salt-resistant or salt sensitive on low salt diet [72]. In addition, blocking sympathetic activation of various peripheral

vascular beds by transection of the sympathetic nerves innervating them has also proven effecting in attenuating many forms of salt sensitive hypertension. Denervation of various sympathetic nerves such as renal [27, 73, 74], celiac ganglionectomy/splanchnic [75-77] or combined denervation [78] have all been shown to attenuate increased arterial blood pressure in both human and animal models of salt sensitive hypertension.

Early attempts to directly measure the changes in sympathetic activity and correlate these to changes in blood pressure were often inconclusive and often contradictory. Using techniques such as plasma and urinary norepinephrine or dopamine levels as an assay of sympathetic activity, most studies were able to show a clear increase in sympathetic activation that correlated well with blood pressure levels in essential hypertensive patients[79-82] and in salt sensitive hypertension animal models[83], however, the results from normotensive or even borderline hypertensive patients were much more variable [80, 84, 85]. More recently, with the advent of newer techniques such as tissue specific norepinephrine spillover and direct nerve recordings, a much clearer picture of how individual vascular beds are regulated in the development of salt-sensitive hypertension has emerged. To date, a vast amount of literature has confirmed the fact that salt-induced sympathoexcitation is not a simple all-or-none mechanism whereby activity to all sympathetic vascular beds is increased uniformly, but rather it is has been shown that each nerve and thus the effect on the tissues it innervates can be regulated independent of the others. For example, intravenous administration of hypertonic NaCl has been shown to increase lumbar SNA [86] but decrease SNA to the kidney, and have little effect on splanchnic SNA[47, 87]. Alternatively, ICA hypertonic NaCl causes similar responses to IV NaCl in lumbar and splanchnic SNA but produces an increase in renal SNA [88] illustrating the variability in SNA responses of individual sympathetic vascular beds under specific experimental conditions.

Identification of Brain Centers Involved in the Response to Increases in Sodium Concentration

A significant amount of research has been aimed at uncovering what regions in the brain are involved in responding to changes in osmolarity. Early studies showed that salt-induced increases in fluid intake and vasopressin release could be elicited both by peripheral as well as central hyperosmotic infusions [89-92]. Andersson was the first to demonstrate that a region of the anterior medial hypothalamus was responsive to hyperosmolar stimuli at levels much lower than required via peripheral administration [93]. Jewell et. al. and Johnson et. al used various techniques such as ligation/ablation and ventricle blockage respectively to narrow the osmosensitive region the periventricular region of the anterior hypothalamus [94, 95]. Ablation of this region that lies at the anterior ventral edge of the 3rd ventricle and would come to be referred to simply as the AV3V region attenuates the drinking and vasopressin release response to intracranially administered hyperosmolar stimuli in the rat [91], goat[96], sheep[89, 97] and dog [98].

Anteroventral Third Ventricle

The AV3V region of the forebrain lamina terminalis is composed of several distinct brain centers including the circumventricular organs (CVO) of the SFO and the OVLT as well as the median preoptic nucleus (MePO). The CVOs are characterized by a lack of a complete blood-brain barrier and are usually located along the walls of ventricles in direct contact with the cerebrospinal fluid (CSF). This fact along with the unique characteristics of CVOs allows these brain centers the perfect position to sense and respond to blood borne peripheral signals. In order for the body to function properly, fluid osmolarity must be maintained in a relatively narrow range, and responses that influence behavior such as drinking, renal sodium handling, or hormone

secretion such as release of vasopressin have all been shown to be affected by even modest changes in sodium levels. The AV3V region has been shown to be integral in the body's ability to defend against changes in osmolarity of body fluids. Lesion of the AV3V region causes acute hypodipsia, and an increase in plasma Na+ concentration that abates over time, however, even with return of normal fluid intake and plasma osmolarity the animal remains unable to respond to challenges to body osmolarity [89, 94, 99-101]. Severe lesions have been shown to produce complete adipsia which can persist permanently leading to lethal dehydration in a few days unless the animal receives supportive fluid resuscitation. In the rat, lesion of the AV3V region has been shown to attenuate the drinking response [101-103] to central and peripheral hypertonic NaCl, angiotensin II, water deprivation and after administration of polyethylene glycol (cellular dehydration). AV3V lesion in the rat has also been shown to alter the hormonal response to osmotic challenge by attenuating the release of vasopressin following central NaCl [104] angiotensin II [102, 105] and water deprivation [104, 106]. Finally, lesion of the AV3V attenuates the pressor response to osmotic challenge following central and peripheral angiotensin II [101, 102] or central administration of NaCl [104] . Similar responses to AV3V lesion have been shown in other animal models as well. In the sheep, complete ablation of the lamina terminalis results in a loss of increased drinking following IV NaCl administration or angiotensin II [107].

Within the AV3V, increased activation of the OVLT, SFO and MePO has been demonstrated following both peripheral and central hyperosmolarity through the increased expression of c-Fos, an early activation gene marker used as a standard assay for generalized neuronal activation [108-113]. Additionally, in vivo and in vitro cell recordings as well as resonance imagining studies have identified cells within the OVLT [114-122], SFO [122-124] and MePO [125, 126] that appear to be intrinsically osmosensitive. Tract tracing studies have revealed both monosynaptic and polysynaptic connections between the OVLT, SFO and MePO

as well as with other cardiovascular regulatory centers such as the hypothalamic paraventricular nucleus (PVN) [127-133] and the rostral ventrolateral medulla (RVLM) [134-136], suggesting the presence of the underlying architecture that would be required for a system that could not only sense changes in osmolarity, but could also respond to these changes through regulation of peripheral tissues involved in maintaining a homeostatic fluid osmolarity.

The Organum Vasculosum of the Lamina Terminalis (OVLT)

The OVLT is located along the midline, anteroventral-most aspect of the lamina terminalis on the rostral wall of the third ventricle. In addition to extensive interconnections between the regions of the lamina terminalis, efferent projections from the OVLT have been identified innervating forebrain structures such as the bed nucleus of the stria terminalis, cingulate cortex, lateral and dorsal hypothalamus (anterior, preoptic lateral preoptic, ventromedial nucleus) midbrain (periaqueductal grey) and hindbrain (locus coeruleus) [132, 137]. Electrophysiological studies show the densest projections involved in osmosensation innervate SFO, MePO and PVN and SON [116-118, 138, 139]. The OVLT receives afferent input from the insular and cingulated cortex, amygdala, lateral septum, ventral subiculum, diagonal band as well as from hindbrain structures (raphe nucleus and nucleus of the solitary tract) [137, 140-143]. Given its extensive interconnectivity with brain centers involved in hormone production and release, not surprisingly, the OVLT expresses receptors for and responds to a vast number of humoral inputs. Of particular interest in regards to fluid and osmoregulation and vascular hemodynamics, renin- angiotensin II, mineralocorticoids, atrial naturetic peptide, vasopressin, somatostatin, biogenic amines, and amino acid receptors have all been identified in the OVLT [22, 144-149].

Changes in the activity of the OVLT produce a variety of osmoregulatory and cardiovascular effects. Electrical stimulation of the rat OVLT increases arterial pressure,

mesenteric and renal vascular resistance and these responses are attenuated by ganglionic blockade suggesting they are sympathetically mediated [150]. Electrolytic lesion of the OVLT attenuates the increases in fluid intake in response to hypertonic saline in rat [151], sheep [97, 99, 107] and dog [152]. OVLT lesion also attenuates the increase in vasopressin release following hypertonic NaCl in vitro in the rat [153] and in vivo in sheep[107] and dog[152] . Additionally, OVLT lesion also attenuates the sympathoexcitation following ICA hypertonic NaCl in rat [88]. Interestingly however, OVLT or anterior AV3V (equivalent to OVLT) lesion does not consistently alter ICA angiotensin II induced drinking in sheep [99] or intravenous angiotensin II pressor response in the dog [154]. These facts argue against the possibility that the OVLT mediates a general response to hyperosmotic stimuli and suggest the OVLT responds mainly to changes in sodium concentration specifically.

As evident from the discussion above, a major difficulty in making generalized conclusions on the role of the OVLT in response to osmotic changes is the variability in the division of functionality across animal models. According to the available literature, the dog appears to have the clearest division of the function of osmoreception ascribed to the OVLT. Specific ablation of the OVLT results in an almost complete loss of increased fluid intake and vasopressin release following osmotic challenge [152], while lesion of the SFO does not appear to affect these responses significantly [155]. Sheep and rat on the other hand appear to have a much more diffuse distribution of cells governing the response to changes in osmolarity. Individual lesions of the OVLT, SFO or MePO each have little to no effect on drinking and vasopressin responses, and a much larger lesion encompassing a greater extent of these brain centers is required to produce significant or a near complete attenuation in response to hypertonic NaCl [107, 151].

It is obvious that the OVLT is intimately involved in many aspects of response to changes in osmolarity, however, the exact role it plays is dependent on multiple factors including

the animal in question, the nature of the osmotic stimulus, and the manner in which the stimulus is presented to the body (i.e. route of administration). To understand the hyperosmotic responses contributing to pathology as complex as salt-induced hypertension, it is imperative to first understand exactly where and how changes in body fluid osmolarity are sensed, how the information from multiple systems is processed, and the manner in which response to this information is conveyed to the periphery in order to effect changes. Thus, a thorough and detailed characterization to a given osmotic challenge is essential.

Chapter 2

Objectives

Although extensive literature exists concerning the cardiovascular effects of increased dietary salt intake, the exact mechanisms mediating these effects still has yet to be fully understood. Therefore, the purpose of this study was twofold: (1) to determine the extent to which the OVLT contributes to the attenuation of the cardiovascular and sympathoexcitatory effects in response to central salt load seen in the AV3V ablations, and (2) to characterize the response of the individual sympathetic vascular beds mediating the response to increased central sodium loads.

Chapter 3

Methodology

Animals

All experimental procedures were approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee and conducted in accordance to the NIH *Guide for the Health and Use of Laboratory Animals*. Adult, male Sprague-Dawley rats (250-400g, Charles River Laboratories) were housed three per cage in a temperature-controlled room (22±1ºC) with a 12-hour light-dark cycle. Rats had ad libitum access to standard chow (Harlan Teklad Global Diet 2018) and deionized water prior to experimental procedures.

General Surgical Procedures

Rats were initially anesthetized with isoflurane (2% in 100% O2) and maintained on 2% for the duration of surgical procedures excluding lesioning. An effective plane of anesthesia was assessed periodically by absence of withdrawal reflex in response to foot pinch and lack of corneal reflex. Arterial and venous access was obtained by placement of catheters into the femoral artery and vein for the purpose of mean arterial blood pressure (ABP) and heart rate (HR) measurement and to provide intra venous access for the infusion of post-surgical anesthesia respectively. ABP and HR were measured via connection of the arterial catheter to a small volume transducer. To maintain adequate levels of oxygen and CO_2 an endotrachial tube was placed and animals were artificially ventilated with 100% oxygen with end-tidal CO_2 maintained at 4.0-4.5% (MicroCapStar End-Tidal CO_2 Analyzer). Body temperature was maintained at 37±0.5ºC with a rectal Thermometer and circulating water heating pad.

Rats were prepared for sympathetic nerve recordings of lumbar and renal nerves as described previously by our laboratory and others [156, 157]. In addition, the splanchnic nerve was isolated through

the same retroperitoneal incision as the renal nerve and recordings were obtained using a similar procedure.

Rats were placed in a prone position and fixed in a stereotaxic head frame (Kopf instruments) with the head leveled between lambda and bregma. A midline incision was made in the skin overlying the dorsal skull and the connective tissue gently retracted to expose the bone. A small craniotomy was performed to remove the bone overlying the cortex to allow access to the lateral ventricle and anterior 3rd ventricle region. A small section of the dura was cut just prior to placement of pipettes or stimulating electrodes. Either a single barrel glass micropipette (0.86mm OD; 20-30µm tip diameter) or steel infusion cannula (22G) was placed in the right lateral ventricle using the following coordinates in reference to bregma: 1.2-1.5mm caudal, 1.6-1.7mm lateral and 4.6-4.7mm ventral. Intracerebroventricular infusions of 1.0M NaCl were performed using a syringe pump at a rate of 5ul over 10 minutes. Upon completion of all surgical procedures, animals were transferred from isoflurane to α-chloralose with an initial bolus (50mg/kg IV) and followed by maintenance infusion (25mg/kg/hr IV). Animals were allowed to stabilize for at least 60 minutes prior to beginning of any experimental procedures.

Experimental Protocols

Protocol 1: Contribution of the AV3V to the sympathoexcitatory and hypertensive effects of ICV NaCl in vasopressin blocked animals

To determine the contribution of the AV3V region to the blood pressure and sympathetic effects of specific vascular beds in response to central sodium load, animals were first pretreated with a vasopressin receptor blocker (Manning Compound-Sigma) 10µg/kg IV and allowed to restabilize for at least 15 minutes. Animals were randomly assigned to Control, Sham or AV3V lesion groups and baseline measurements were recorded for 15 minutes. 1.0M NaCl was infused ICV (5µl/10min) and

ABP, lumbar, renal, and splanchnic SNA were recorded for 40 minutes. In the AV3V lesion animals, a Teflon-coated tungsten electrode (250 μm tip) was lowered into the AV3V region at an angle of 4º in the coronal plane to avoid damaging the superior sagittal sinus and using coordinates in reference to bregma: 0.2-0.3 mm rostral, 0.3-0.5 mm lateral and 7.8.-7.9 mm ventral. DC current (0.5-1mA) was applied for 20-30 seconds. Current intensity and time were adjusted to produce lesions covering the extent of the AV3V region encompassing the OVLT and the majority of the MePO confirmed histologically. Sham animals underwent a similar procedure with the electrode placed just dorsal to the AV3V region and no current passed. Animals were allowed to restabilize and the 1.0M NaCl infusion was repeated.

Protocol 2: Contribution of the OVLT to the sympathoexcitatory and hypertensive effects of ICV NaCl in vasopressin blocked animals

Following completion of surgical procedures, the OVLT was localized using stimulus-triggered averaging of SNA. A stimulating electrode angled at 4º was lowered into the ventral forebrain using starting coordinates 500um rostral to the OVLT in reference to bregma: SNA responses to 30 single-pulse electrical stimuli (1ms, 0.5Hz, 500uA) were averaged. Successful stimulation of OVLT yielded a single peak with a latency of approximately 160ms. Moving caudally, current intensities were decreased until the optimal position yielding a consistent peak at a current ≤50uA was achieved. Mediolateral and dorsoventral position was also adjusted when needed using similar procedure until the optimal response was obtained. Final coordinates for localization of the OVLT in reference to bregma ranged from: 0.0-0.5mm rostral, 0.2-0.5 mm lateral and 7.8-8.0mm ventral. Histological analysis confirmed the location of optimal response using this procedure consistently placed the stimulating electrode in the OVLT. Following stimulus-triggered averaging localization, and after allowing at least one hour for stabilization, a control infusion of 1.0M NaCl was administered into the right lateral ventricle as described in protocol 1. The OVLT was then lesioned using DC current (100uA) for approximately 20-30sec. Successful OVLT lesion was confirmed histologically and defined as necrotic damage of the majority of the OVLT

without significant extension beyond its boundaries, with attention paid to exclude caudal/dorsal damage that impinged on the medial preoptic region. The ICV NaCl infusion was repeated and pre/post lesion responses compared.

Histology

Following completion of experimental procedures, a solution of Chicago Blue dye (1µl 2% in water) was infused through the lateral ventricle pipette or cannula. Animals were transcardially perfused with 4% paraformaldehyde (60ml) and the brain removed and post-fixed in 4% paraformaldehyde overnight. Brains were sectioned in100µm coronal slices using a vibratome and mounted on glass slides. Sections were counterstained with cresyl violet and analyzed by brightfield microscopy using a Nikon Eclipse 90i fluorescent microscope to confirm location.

Statistical Analysis

All data is expressed as mean±SEM. Raw nerve activity was rectified, integrated with a 1 second time constant and background noise was determined and subtracted using hexamethonium (30mg/kg) IV or by physically crushing the nerve. One minute sections were averaged and compared to baseline or control. Data was analyzed using 1- way ANOVA, with repeated measures when necessary. Post-hoc tests were performed using paired t-tests with a layered Bonferroni correction for multiple comparisons. Fisher LSD test was used for comparison of mean changes over extended time periods (Figure 3-5). $P<0.05$ was considered statistically significant.

Chapter 4

Results

Effects of Multiple Infusions on the Magnitude and Duration of the Sympathoexcitatory and Hypertensive Effects in Response to Central NaCl Load

To insure successive infusions of ICV NaCl (5ul/10min) did not differ in magnitude or duration of responses, two successive infusions separated by approximately 2-3 hrs. were conducted. Compared to baseline values, both Trial 1 and Trial 2 resulted in significant increases in ABP, HR, and lumbar SNA ($p \leq 0.05$), while renal and splanchnic SNA decreased initially during the NaCl infusion and showed a delayed increase to above baseline values following the end of infusion ($p \leq 0.05$). Summary Data is shown in Figure 1-1. Resting baseline values and response to NaCl did not differ significantly between Trial 1 and 2 ($p \geq 0.05$).

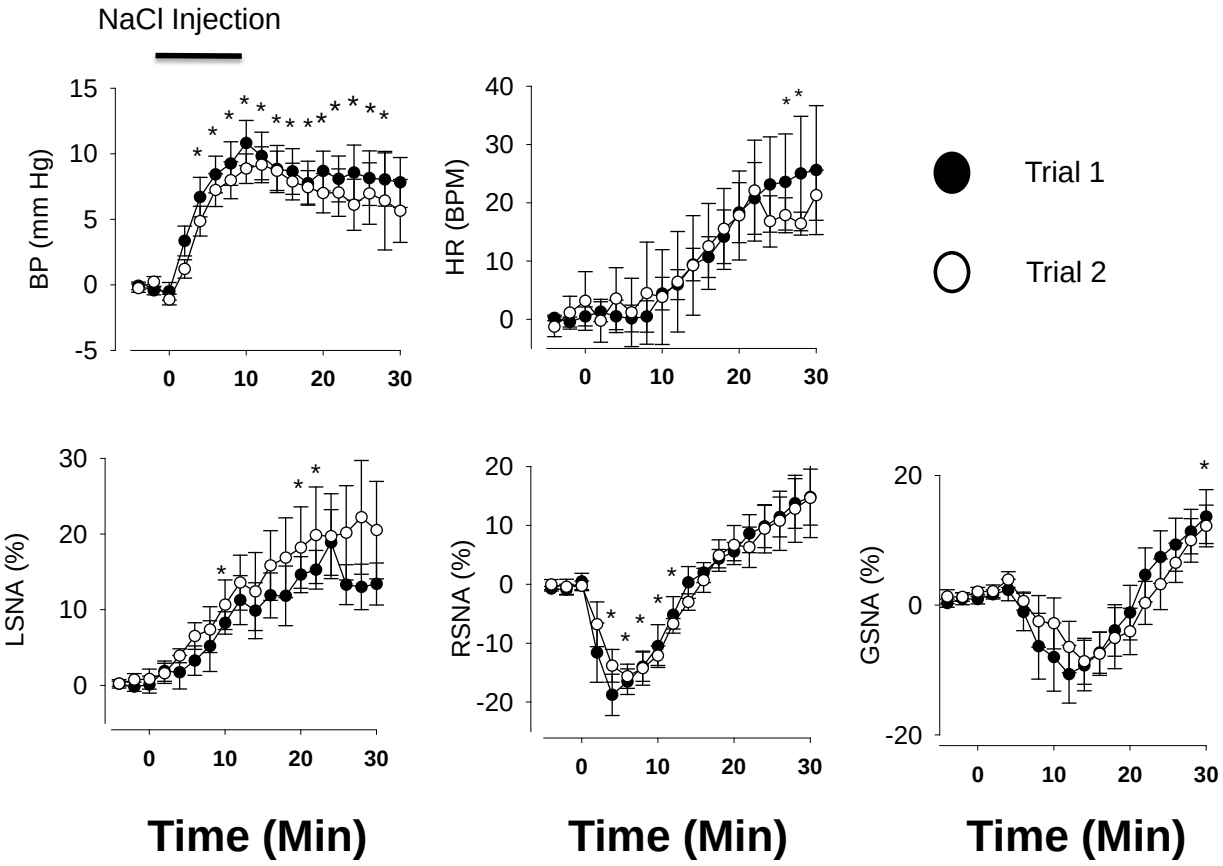

Figure 1-1. Summary Data For Multiple Injection Time Controls. Mean and pulsatile ABP, HR and integrated lumbar, renal and splanchnic SNA in response to centrally administered 1M NaCl (5ul/10min) for two consecutive trials. (* $p \leq 0.05$ vs. baseline n=6)

Electrolytic Lesion of the AV3V Abolishes the Sympathoexcitatory and Hypertensive Response to Central NaCl Load

Raw traces for mean and pulsatile ABP, HR, and integrated lumbar, renal and splanchnic SNA for a single characteristic animal before and after AV3V ablation are shown in Figure 2-1. Baseline resting ABP did not vary significantly following either AV3V or OVLT ablation, however, HR did increase post-ablation with only AV3V ablation reaching significance ($p \leq 0.05$) (Table 1). Each trace shows a 10 minute baseline followed by the 10 minute 1M NaCl infusion and recorded for 20 additional minutes. Similar to the Time Control experiment, ICV infusion of 1M NaCl (5ul/10min) resulted in an increase in ABP, HR and lumbar SNA while renal and splanchnic SNA decreased initially followed by a delayed increase once the infusion was stopped.

AV3V ablation resulted in the complete attenuation of the increase in ABP, HR and lumbar SNA as well as the initial decrease and delayed increase in renal and splanchnic SNA; all measured variables did not differ significantly from baseline in response to central 1M NaCl infusion ($p \leq 0.05$). Summary data for control vs. AV3V lesion is shown in Figure 2-2. Sham lesion did not differ significantly from control infusion (data not shown).

Ablation location and extent was determined histologically with individual lesions circumscribed using signs of necrosis to determine extent of tissue damage. Figure 3-4 is a schematic illustration showing location of electrode tip sites and the summated extent of lesion damage produced by overlying corresponding coronal slices of individual animals and tracing the outer extent of lesion damage.

Table 1-1. Resting Baseline Blood Pressure and Heart Rate Before and After Lesioning

	Blood Pressure (mmHg)			**Heart Rate (BPM)**		
	Before	After	Sham	Before	After	Sham
AV3V	106.6±2.9 (n=16)	107.9±3.9 (n=7)	110.1±3.0 (n=5)	384.7±8.1	422.9±12.1* ($p \leq 0.05$)	387.3±8.9
OVLT	108.3±4.6 (n=7)	102.7±6.8 (n=6)		392.5±15.0	415.3±16.4	

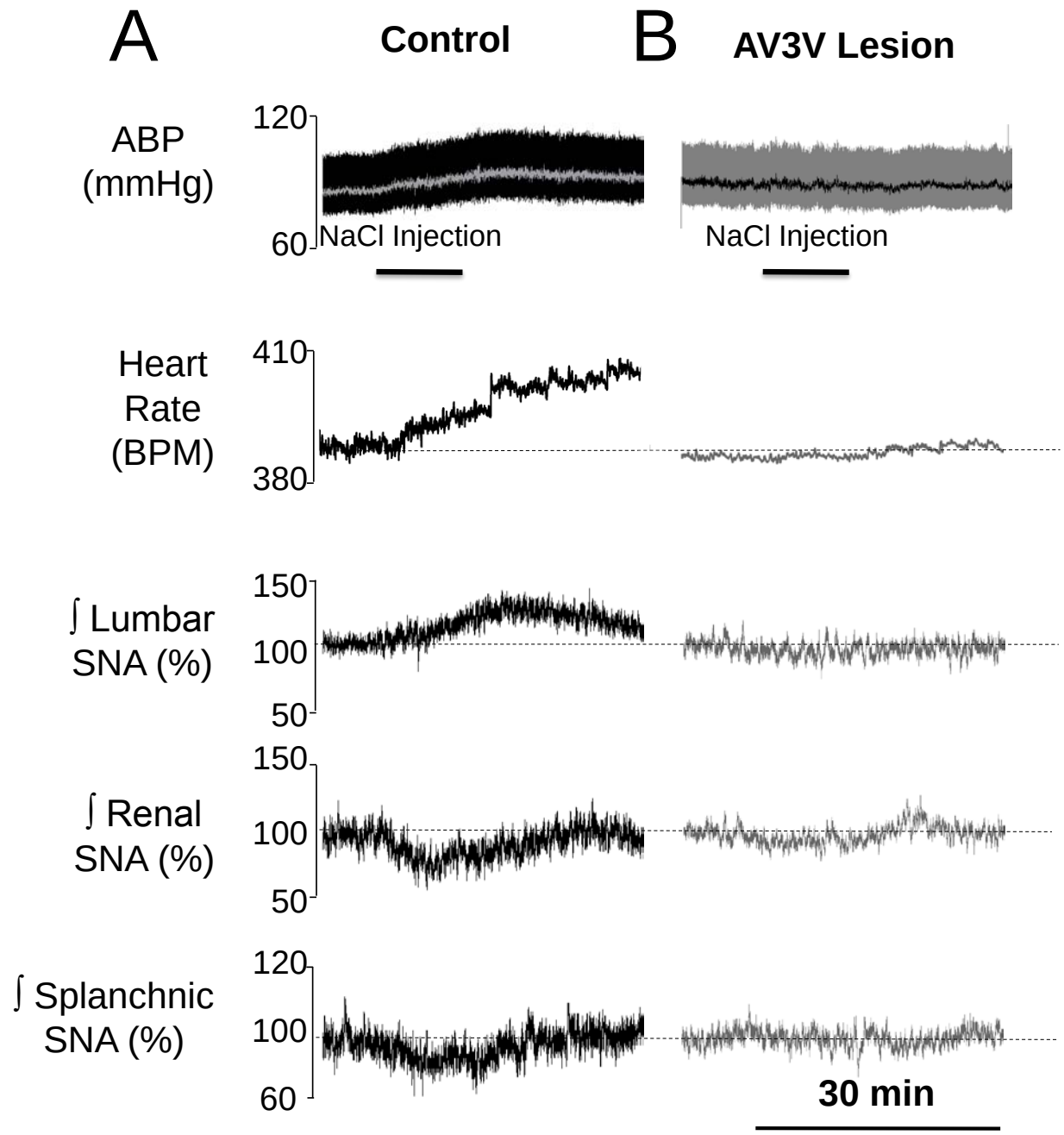

Figure 2-2. Raw Trace Showing a Characteristic Response to Central Infusion of 1M NaCl and the Attenuation from AV3V Ablation. Mean and pulsatile ABP, HR and integrated lumbar, renal and splanchnic SNA in response to centrally administered 1M NaCl (5ul/10min) A.) before and B.) after AV3V ablation.

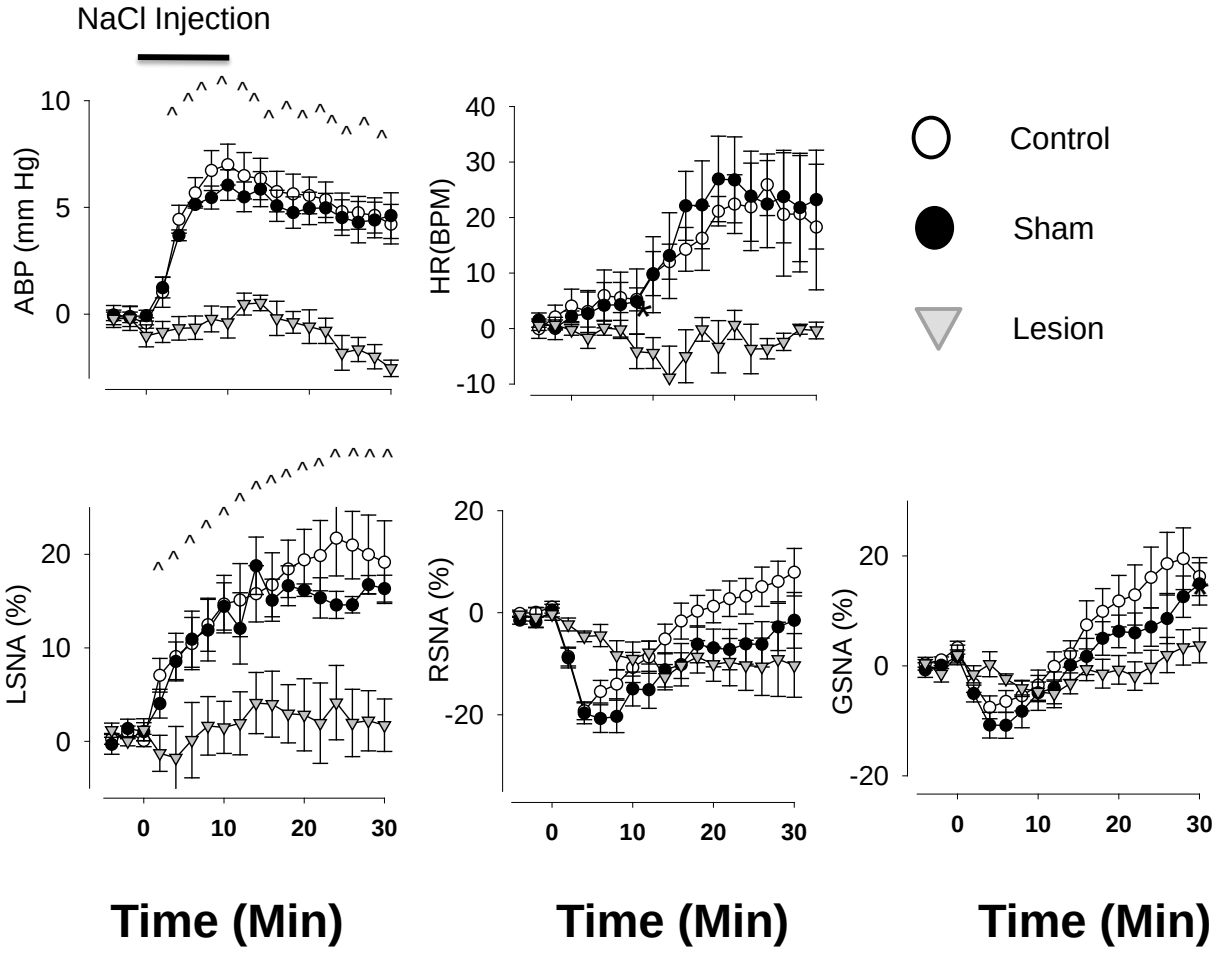

Figure 2-2. Summary Data for AV3V Lesion Group. Mean ± SEM ABP, HR and lumbar, renal and Splanchnic SNA for Control 1M NaCl infusion before (n=14-16) and after (n=5-7) AV3V ablation and Sham lesion (n=3-5) (^ $p \leq 0.05$ control vs. lesion)

Electrolytic Lesion of the OVLT Attenuates the Sympathoexcitatory and Hypertensive Response to Central NaCl Load

The small size of the OVLT, only a few hundred microns diameter at its widest point, and its location deep within the forebrain makes consistent localization through use of standard stereotaxic coordinates alone difficult. Therefore, stimulus-triggered averaging was used to improve localization of the OVLT prior to ablation. Figure 3-1 is a representation of the stimulus-triggered averaging technique along with the recorded responses of a single animal with OVLT localization confirmed histologically as well as the responses obtained approximately 200 um away from the OVLT in the rostral, dorsal and lateral directions. Notice successful placement of the electrode tip in the OVLT results in spikes in lumbar, renal and splanchnic SNA at a latency of approximately 160-170ms. Placement of the tip as little as 100um away from the OVLT shows little to no response at stimulation currents of 50uA and below.

Figure 3-2 shows representative raw traces of ABP, HR, and lumbar, renal and splanchnic SNA of a single animal in response to 1M NaCl ICV (5ul/10min) before and after OVLT specific lesion. Similar to experiment 1, compared to baseline 1M NaCl resulted in an increase in ABP, HR, and lumbar SNA while renal and splanchnic decreased initially during the NaCl infusion and then increased once the infusion had ended, however, splanchnic SNA changes did not reach significance ($p \leq 0.05$). Compared to control, lesion of the OVLT resulted in an attenuation of the increase in ABP and lumbar SNA as well as the initial decrease and delayed increase in renal and splanchnic SNA. All measured variables for OVLT lesion did not differ significantly from baseline ($p \leq 0.05$) shown in Figure 3-3.

Schematic representation of the summated lesion area and a characteristic example of a single animal for OVLT lesion is shown in Figure 3-4. Notice the overall size of the OVLT lesion is much smaller and unlike the AV3V lesion, the OVLT lesion damage does not extend caudally to the level of the MePO.

Figure 3-5 shows summary data for mean changes over the time period specified for control AV3V and OVLT lesion groups. Compared to control, AV3V lesion resulted in an attenuation of the increase in ABP, HR, lumbar SNA as well as the initial decrease and delayed increase in renal and splanchnic SNA, however, only changes in ABP and lumbar SNA reached significance ($p \leq 0.05$). OVLT lesion also resulted in an attenuation of the response to 1M NaCl compared to baseline, however, only the changes in ABP, lumbar SNA and the initial decrease in RSNA were significant ($p \leq 0.05$). In comparison to AV3V lesion, OVLT lesion resulted in only a partial attenuation of the ABP, HR, lumbar SNA and the delayed increase in renal and splanchnic SNA, however, these were not statistically significant ($p \leq 0.05$). Interestingly however, compared to AV3V lesion OVLT lesion resulted in a larger attenuation in the initial decrease in renal and splanchnic SNA in response to 1M NaCl infusion but only the renal SNA response was significant ($p \leq 0.05$).

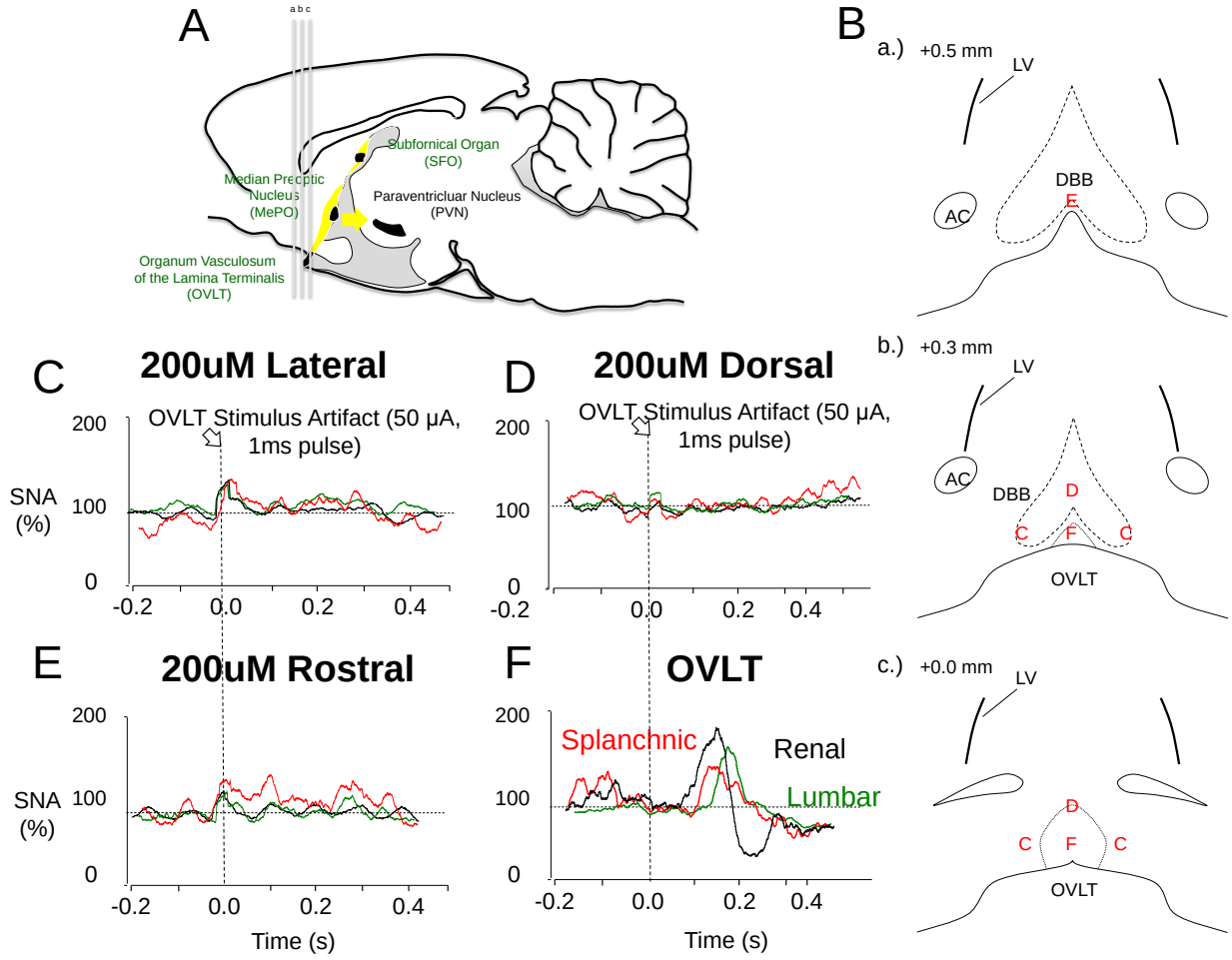

Figure 3-1. Representation of Stimulus-Triggered Averaging Technique and Recorded Responses from a Single Animal. A.) Illustration of a midsagittal section of the brain depicting the location of the OVLT in reference to the electrode tracts starting rostral to the OVLT and moving progressively caudal until optimal response to electrical stimulation is obtained. C-D.) Shows recorded responses to stimulation (50uA, 0.5Hz, 1ms duration) 200um outside of the OVLT and corresponding to the locations in B.) referenced by rostral-caudal distances from bregma illustrated in the upper left hand corner. F.) Recorded response to successful OVLT stimulation confirmed histologically. (abbrev. LV, lateral cerebral ventricle; DBB, diagonal band of Broca; AC, anterior commisure)

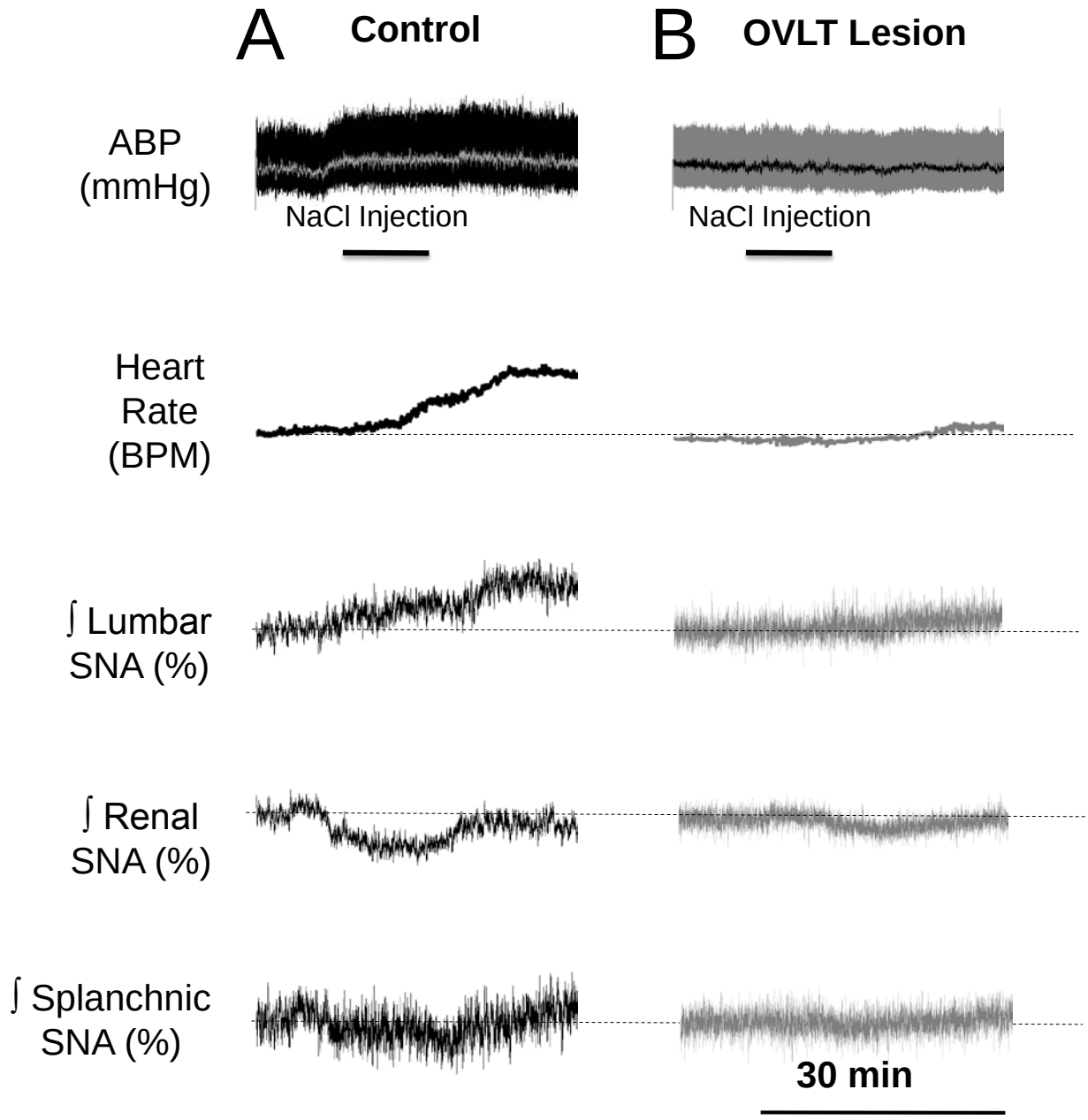

Figure 3-2. Raw Trace Showing a Characteristic Response to Central Infusion of 1M NaCl and the Attenuation from OVLT Ablation. Mean and pulsatile ABP, HR, and integrated lumbar, renal and splanchnic SNA in response to centrally administered 1M NaCl A.) before and B.) after OVLT ablation.

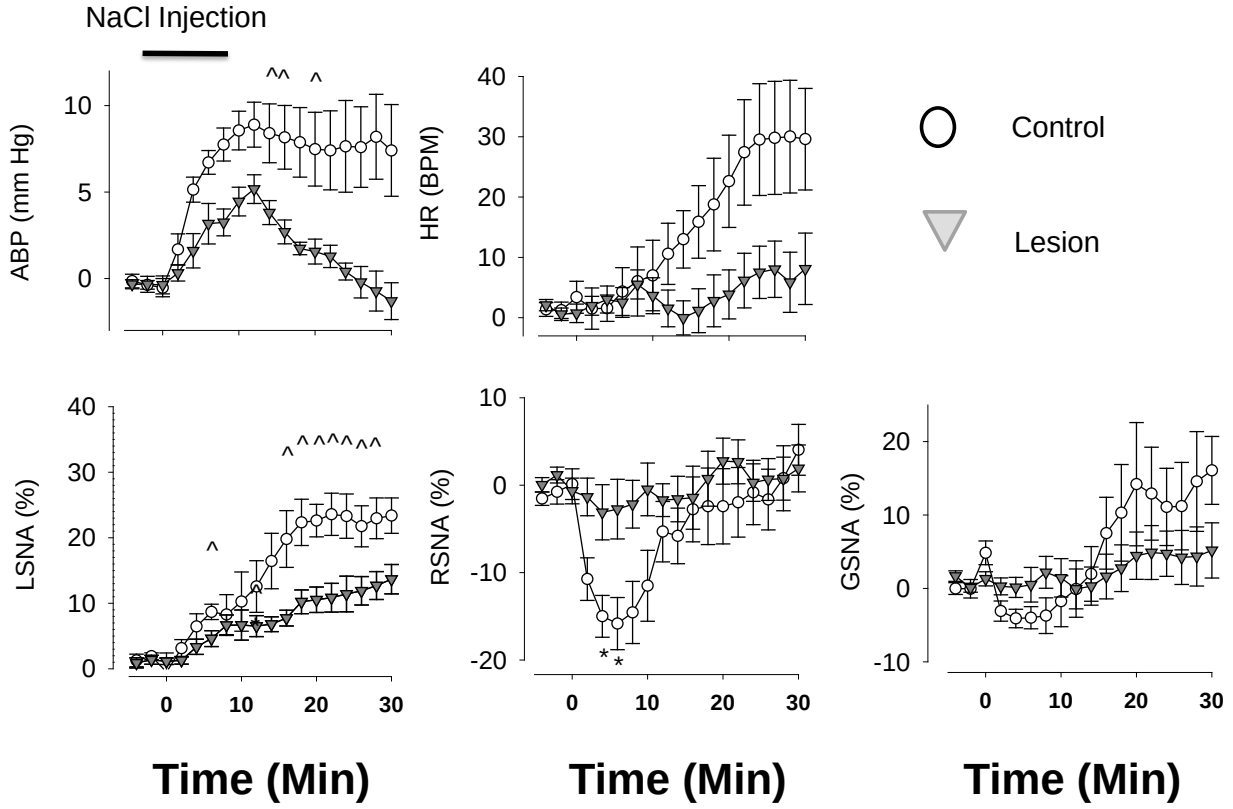

Figure 3-3. Summary Data for OVLT Lesion Group. Mean ± SEM ABP, HR, and lumbar, renal and splanchnic SNA in response to 1M NaCl infusion before (n=6-7) and after (n=6) OVLT ablation. (^ $p \leq 0.05$ control vs. lesion)

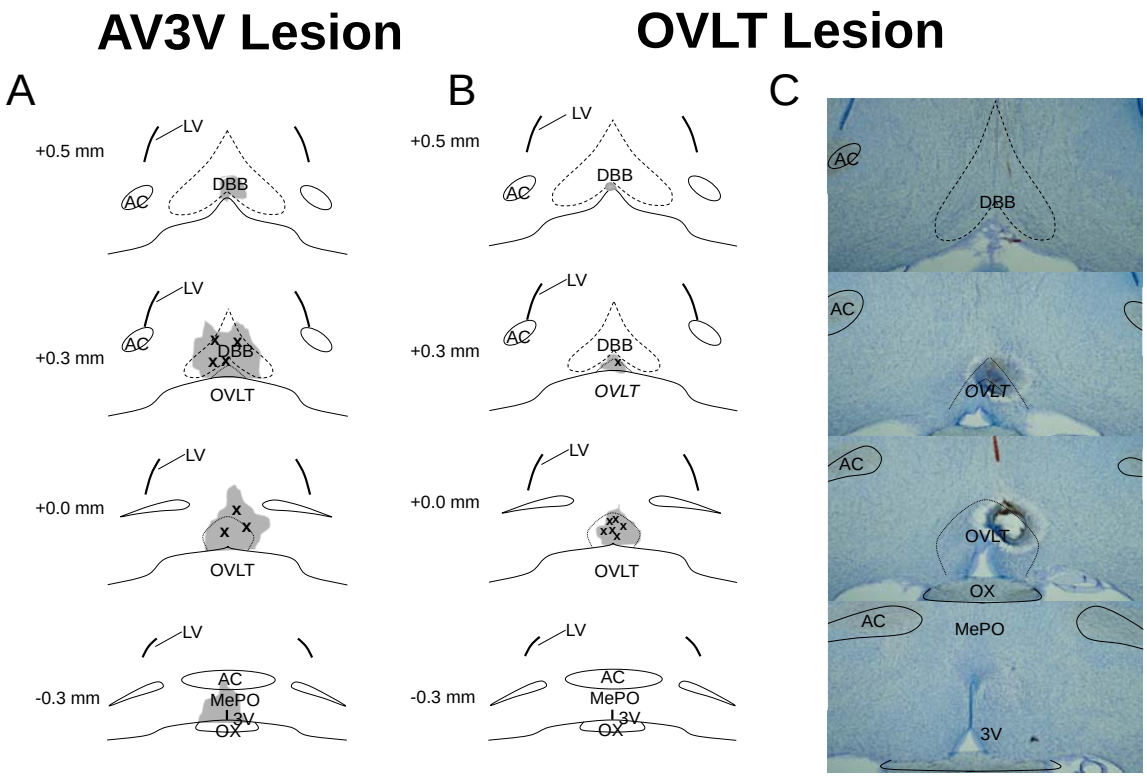

Figure 3-4. Histological Illustration of Lesion Location and Extent. Summated area of individual A.) AV3V and B.) OVLT lesions. Tip of electrode placement are denoted by X. C.) Representative example of single OVLT lesion animal. (abbrev. 3V, third ventricle; AC, anterior commisure; DBB, diagonal band of Broca; LV, lateral cerebral ventricle; MePO, median preoptic nucleus; OVLT, Organum Vasculosum of the lateral terminalis; OX, optic chiasm)

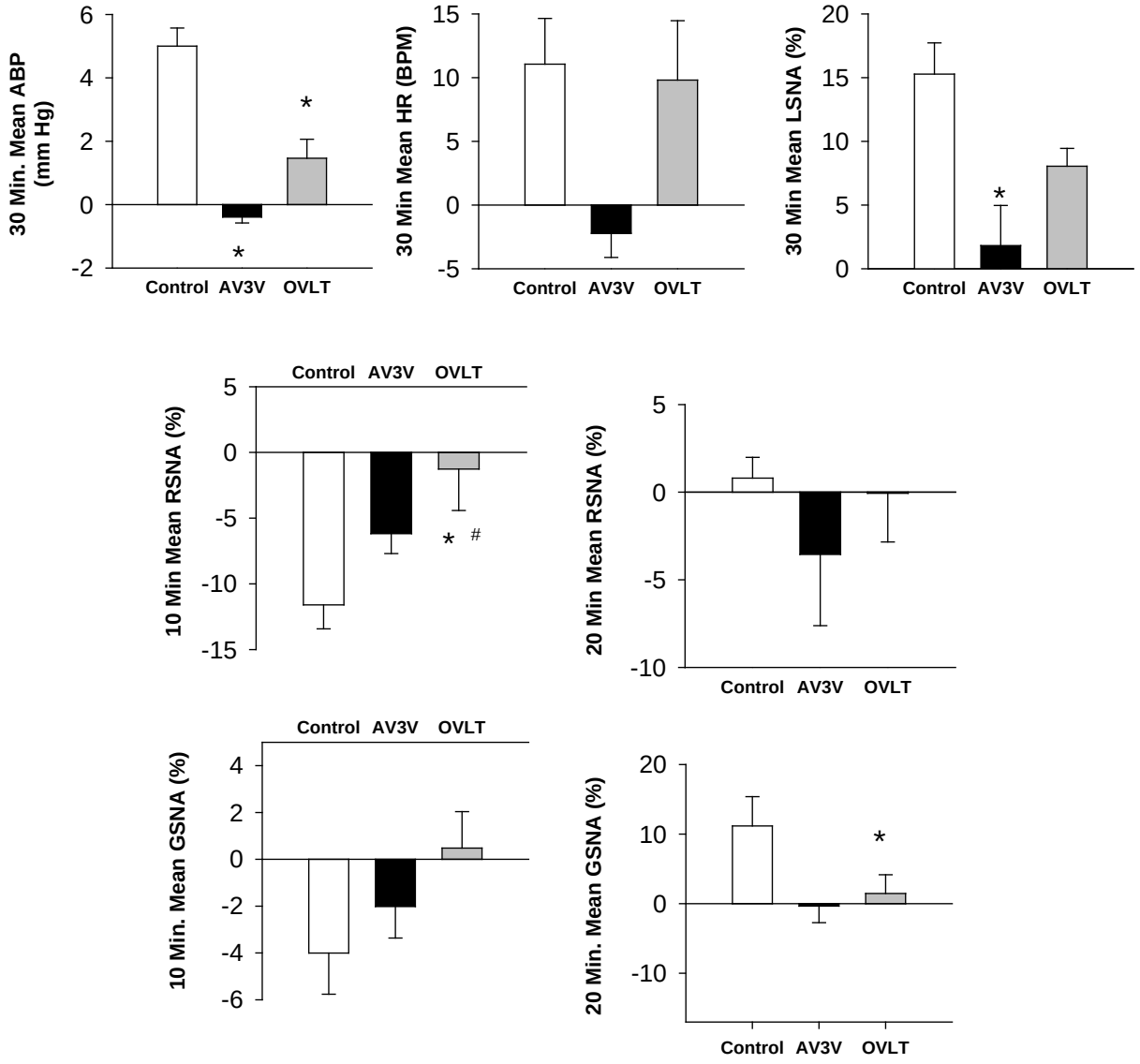

Figure 3-5. Comparison of AV3V to OVLT Lesion. The average of 1 min. bins for Mean ABP, HR, lumbar, renal and splanchnic for the times listed are shown for control and AV3V or OVLT lesion. (* $p \leq 0.05$ vs. control; # $p \leq 0.05$ vs. AV3V).

Chapter 5

Discussion

Taken as a whole, the data presented in this study strongly suggests a role for the OVLT, and specifically its effects on lumbar sympathetic activation, in mediating the hypertensive effects of increased central sodium loads. However, the fact that the OVLT ablations produced only a partial attenuation of the sympathoexcitatory and hypertensive effects in response to central sodium loading compared to the effects of the much larger AV3V lesion speaks to the complexity and redundancy of these mechanisms and indicates the likelihood that other brain regions are also involved. Besides the OVLT, the AV3V region contains several other candidate centers that may be involved in mediating the response to increases in sodium concentrations. Both the SFO [111, 123, 124, 158-160] and MePO [39, 161-167] region have been shown to contain osmosensitive neurons and project to many of the same cardiovascular regulatory regions as the OVLT. Lesioning studies have also shown that ablation of the SFO or MePO can attenuate the drinking behavior and vasopressin release following central and peripheral sodium loading in animals such as the rat[151, 168] and sheep[107, 169]. In addition, both the SFO and MePO have been shown to mediate the osmotically stimulated sympathoexcitatory and pressor effects of other salt-sensitive hypertensive models such as ANGII-salt[170] and the DOCA-salt[171, 172] rat models. Future studies will be needed to characterize the effects of increased sodium load mediated by other osmosensitive brain centers to gain a complete understanding of all the players involved and the relative contribution of each to salt-induced hypertension. It is likely that the SFO and/or the MePO play some part in the development or progression of salt-induced hypertension, however, several factors were taken into consideration in choosing to start with the OVLT. First, direct electrical stimulation of the

OVLT results in increased blood pressure and vascular resistance in the mesenteric and renal vasculature[150]. Stimulation of various locations in the MePO or periventricular preoptic nuclei produces decreases in arterial pressure, lumbar vasodialation and has little effect on mesenteric or renal vasculature resistance [150]. Additionally, in the dog ablation of the OVLT almost completely abolishes the drinking and vasopressin response to IV hypertonic saline while SFO ablation showed little effect on this response but was able to significantly attenuate the same responses to ANGII administration [152]. Third, experiments employing precise knife cuts to severe neural pathways originating from the SFO while maintaining the integrity of those from the OVLT showed no effect on drinking in response to subcutaneous or IV hypertonic saline in rats but again, the response following administration of ANGII was significantly attenuated [173]. These results suggest a significant role for the OVLT in the regulation of the response to increases in central sodium concentration, while the SFO appears to be more responsive to changes in other osmotic stimuli such as ANGII administration.

Although the data from this study suggest a role for OVLT-mediated increases in lumbar SNA in salt-induced hypertension, it does not speak to the possible cellular mechanisms governing this response. Various sodium channels have been proposed to possibly mediate responses to changes in osmotic concentrations; however, two channels have received the most attention in regards to centrally mediated processes: the epithelial sodium channel (ENaC) and the transient receptor potential channel vanilloid type (TRPV).

The ENaC channel is a non-voltage dependent cation channel that consists of three subunits (α, β, and γ) found mainly in the apical membrane of epithelial tissue throughout the body including the brain [174-182]. ENaC channels have been identified in multiple cardiovascular and osmoregulatory brain centers such as the paraventricular nucleus and supraoptic nucleus and have been implicated in several forms of salt-sensitive hypertension [31, 183-189]. Emerging evidence suggests that central ENaC channels are involved in the development of salt-sensitive hypertension. ICV infusion of the Amiloride analogue Benzamil, a non-voltage dependent sodium channel blocker, attenuates the sympathoexcitatory

and hypertensive effects of acute and chronic central sodium loading [190]. In salt-sensitive models of hypertension such as the Dahl salt-sensitive , DOCA-salt [191], and SHR rats , ICV Benzamil [190, 192] but not subcutaneous[193] or IV [190] at the same dose attenuates the sympathoexcitation and hypertensive response to increased central sodium but does not affect non-salt sensitive hypertension such as the renovascular hypertensive rat [190]. One caution in the interpretation of the Benzamil data must be added however, Benzamil although somewhat selective for ENaC due to its higher affinity for these channels has also been shown to be able to block several other Na+ channels at the doses used in most of these experiments including the Na+/Ca++ exchanger, Na+/H+ exchanger, the Na+ pump and the Ca++ pump[194]. Some evidence does exists to support the role of Na+ channels other than ENaC in the pathology of salt-induced hypertension based on renal effects, but the role of these channels in centrally mediated control in response to changes in osmolarity remains controversial [191, 195]

TRPV1 is a nonselective cation channel that was first identified and cloned using its specific activation by capsaicin derived from chili peppers [196]. TRPV1 receptors are expressed primarily on sensory primary afferent nerve fibers however, attempts to localize it within the OVLT have been inconsistent [143, 197, 198]. In vitro experiments using OVLT neurons from a mouse model lacking the TRPV1 gene showed significant attenuation of hypertonicity-induced responses and these responses were dependent on TRPV1 since they could also be blocked in wild type OVLT neurons by addition of ruthineum red, a TRPV channel blocker [121, 199]. However, results from in vivo studies have been more variable and somewhat inconsistent and more likely represent responses from peripheral sensory neurons expressing TRPV1 rather than neurons located in the OVLT [121, 198, 200].

Alternatively, the TRPV4 isoform which has been characterized as a stretch-sensitive nonselective cation channel has also been implicated as a mediator of osmosensation. Increases in extracellular osmolarity causes water to move from the intracellular environment to the extracellular space leading to a decrease in cell volume and subsequent increase in intracellular osmolarity. TRPV4 has been shown to respond to mechanical stress involved in cell volume defense [201], however, the

evidence for the presence of TRPV4 in the brain, specifically in the OVLT and SFO is inconsistent [201, 202]. Deletion of the TRPV4 channel in mice increases resting plasma osmolarity and attenuates Fos activation in the OVLT in vitro and in vivo as well as vasopressin release in vivo following hypertonic challenge [203]. Activation of TRPV4 decreases blood pressure in rats on a normal salt diet and this antihypertensive effect is enhanced on a high salt diet [204]. In the Dahl salt-sensitive rat, activation of TRPV4 but not TRPV1 attenuates the salt induced increase in blood pressure while blockade of TRPV4 increases blood pressure only in resistant animals [205], however, similar to TRPV1 the in vivo responses to TRPV4 activation are likely mediated by peripheral sensory neurons rather those in the OVLT.

In addition to the question of the identity of receptors mediating central osmosensation, there remains a question as to where these receptors are located in respect to the blood-brain barrier as well as whether these receptors are responsive to changes in sodium concentration specifically or if they are responding to generalized changes in total osmolarity irrespective of individual solute concentrations. Gilman in 1937 was the first to show that peripheral infusion of hypertonic NaCl but not urea increased plasma sodium concentrations and caused an increase in the release of vasopressin and drinking in the dog [206] . Similar results regarding fluid intake and vasopressin were obtained by Wolf in 1950 utilizing injection of hypertonic saline directly into the CSF bypassing the question of movement across the blood-brain barrier and leading him to hypothesize that sodium which is relatively cell impermeable, acted directly on cells in the brain by causing a withdrawal of fluid from the cell and a subsequent decrease in cell volume leading to the observed effects on thirst and vasopressin release [207]. Urea on the other hand being cell permeable, was readily taken up into cells and was unable to cause cellular dehydration. These early results seemed to suggest the presence of a central sodium receptor located outside the blood-brain barrier. In this scenario, increased peripheral sodium levels would cause increased central sodium levels in areas lacking a complete blood-brain barrier (such as the CVOs) where sodium could interact directly with osmosensitive neurons to cause cellular dehydration, a decrease in cell volume and activation of the cell through mechanosensitive mechanisms. Interestingly however, several

studies using sodium salts other than NaCl such as $NaHCO_3$ or sodium acetate reported that infusion of these salts containing equimolar amounts of Na+ lead to variable effects [208-211] suggesting possible additive effects of other electrolytes in addition to Na+ and arguing against being solely mediated by a sodium specific receptor. Additional evidence against the sodium receptor came with the reports that solutes other than NaCl such as sucrose, which does not increase plasma sodium concentrations were able to produce similar responses in drinking and vasopressin release when infused IV or ICA [57, 89, 212, 213]. Unlike in Wolf's experiments, where solutes were injected directly into the CSF, solutes injected IV or ICA have to contend with crossing the blood-brain barrier. Sucrose, although unable to raise plasma sodium levels, does contribute to total plasma osmolarity and in brain regions with an intact blood-brain barrier causes withdrawal of water from the CSF or brain tissue due to creation of an osmotic gradient and thus increases sodium concentrations on the opposite side of the barrier as water movers into the intravascular space [57, 89]. Similar to Wolf's experiments, increased sodium concentrations in the CSF and extracellular space causes cellular dehydration and explains the effects seen with peripheral infusion of NaCl or Sucrose assuming the presence of a sodium receptor inside the blood-brain barrier, however, this model does not explain the lack of response observed with IV or ICA urea or glucose which have also has been shown to increase CSF sodium concentration [89, 206]. The data however, do not rule out the possibility of multiple receptor types located throughout the body. For instance, a sodium receptor located outside the blood-brain barrier could respond to central sodium changes as seen with NaCl infusion IV or ICA, while a peripheral osmoreceptor sensitive to sucrose could account for lack of response to urea or glucose. In fact, the presence of peripheral osmoreceptors that respond to hypertonic solutions injected directly into hepatic and splanchnic tissue have been reported and their activation has been shown to lead to increased vasopressin release [214, 215]. Additional studies will be required to definitively identify the location and type of receptor(s) mediating the response to changes in osmolarity.

Along with the questions discussed above that remain to be answered; there are also several limitations on the conclusions that can be drawn from the present study that must be addressed. First, the

fact that the hypertensive and lumbar sympathoexcitatory effects were attenuated by ablation of the OVLT does not confirm that the cells responsible for these responses reside within the OVLT. Electrolytic ablation destroys not only the cell bodies in the area surrounding the electrode, but it also destroys the axons of cells whose bodies may lie in regions other than the OVLT and whose axons traverse the ablated area. Destroying all cells and axons of passage in the OVLT rather than only those activated by increased sodium load and residing within the OVLT could lead to a misinterpretation of the data and produce erroneous conclusions. Additional studies utilizing more precise techniques that affect only the osmosensitive cells residing within the OVLT are required in order to definitively determine the role of the OVLT in salt-induced responses. Use of newer techniques such as optigenetics could allow for activation/inactivation of a specific cell population under precise temporal control. In addition, if used in conjunction with retrogradely transported viral vectors that have been used in tract tracing experiments for decades; it is possible not only to confine experimental manipulations to the osmosensitive OVLT cells of interest, but to also limit manipulations to those cells projecting to a specific organ/tissue of interest. For example, it would be possible to look at the effects of activation/inhibition of only those osmosensitive cells in the OVLT that project to the lumbar sympathetic vascular bed effectively removing the possible confounding effects on other nerves.

Secondly, the correlation between increased lumbar sympathetic activation in response to central sodium loading and the increase in ABP does not confirm a functional link. Results from this study show that central sodium loading causes an increase in lumbar SNA and it is hypothesized that this sympathoactivation leads to vasoconstriction of the lumbar vascular, resulting in an increase in blood pressure. However, the observed changes in lumbar SNA secondary to increased sodium load do not occur in the absence of additional effects of increased central sodium load on other tissues or the reflexive changes these effects may have on the system as a whole. For example, direct electrical stimulation of the lumbar sympathetic chain has been shown to cause vasodialation in hindlimb musculature in either the absence of changes in blood pressure or a slight decrease of arterial pressure [216]. We have shown here

however, that central sodium loading causes an increase in lumbar SNA and this correlates with an increase in blood pressure. In addition, we have also shown a decrease in renal and splanchnic SNA in response to central hyperosmotic challenge, a change that would be expected to correlate with vasodilation and a decrease in blood pressure. It is possible the effects seen in renal and/or splanchnic SNA are due to a baromediated inhibitory reflex in response to the observed increase in blood pressure following hypertonic saline infusion rather than a direct effect on the nerves themselves. Additionally, if the changes in lumbar sympathetic activity are responsible for the hypertensive effects of increased sodium load it would be hypothesized that blocking the neural input to the lumbar sympathetic vascular bed by transecting the lumbar nerve should prevent these effects. Additional studies characterizing the effects of activation of specific neural cell population innervating individual sympathetic vascular beds as well as the effects denervation of these beds has on the observed responses following central sodium loading are needed to definitively ascertain the contribution of each to the pathogenesis of salt-induced hypertension.

Third, acute central sodium loading in an anesthetized animal model does not necessarily translate into the effects seen in salt-sensitive human essential hypertensive patients. Although the level of sodium infusions used in this study is towards the lower end of what has been used in similar studies, it may not represent the physiological levels typical of hypertensive humans. Measurements of CSF sodium concentrations in the vicinity of the cells hypothesized to be sensing these changes are needed to determine the optimal concentrations and rate of infusion of central hypertonic saline to reproduce the physiological environment associated with the development and progression of salt-hypertension as closely as possible. In addition, although the anesthetized animal preparation allows for experimental manipulations that would not be possible in conscious animals or humans, the effect of the anesthesia on measured responses and the physiological differences between rats and humans remains unknown and extreme caution must be taken when extrapolating results obtained in anesthetized animal preparations to a clinical human population.

In summary, the results of this experiment suggest a role of the OVLT in the sympathoexcitatory and hypertensive response to increased central sodium loads through increases in lumbar SNA. However, much more work is needed to fully characterize the effects of increased osmolarity of various solutes, the brain centers involved in mediating these responses and the mechanism by which changes in osmolarity are sensed and conveyed to peripheral tissues.

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