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Investigations of potential mechanisms underlying spinal cord injury-induced polyuria.

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INVESTIGATION OF POTENTIAL MECHANISMS UNDERLYING SPINAL CORD INJURY-INDUCED POLYURIA

By

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B.S., Southern Illinois University, 2013
M.S., University of Louisville, 2019

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A Dissertation Approved on

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DEDICATION

This dissertation is dedicated to

My Mother, Ms. Cynthia Hagen-Gumbel,

who was the first person to introduce me to science and all its wonder,

and has been extremely supportive, understanding, and loving my whole life.

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I would first like to acknowledge and thank my mentor, Dr. Charles Hubscher for his support, guidance, and excellence in mentorship. His training and mentorship allowed me to achieve many goals, both academic and personal. I'm thankful to have had a mentor that was able to lead by example and teach me valuable lessons for the rest of my career. I like to thank my committee, Drs. Jennifer Brueckner-Collins, Cynthia Corbitt, David S. Magnuson, and Patrick J. Moore, who have been generous and helpful with their valuable time and input. I am grateful for their feedback and guidance which was essential for my success. I would also like to acknowledge the fellow members of the Hubscher lab, Mr. Jason Fell, Mr. Lucas Miozza, Dr. Cuibo Yang, Dr. Yun Zhou, Dr. Daniel Medina Aguinaga, and especially Mr. James Armstrong and Ms. Lindsey Zipperer. I would especially like to thank Dr. Casey Steadman and Ms. Natasha Wilkins—as fellow graduate students, their friendship and scientific assistance were vital to my sanity and success. Additionally, I would like to thank Christine Yarberry and Darlene Burke of the KSCIRC Core for their surgical and technical assistance. I would like to extend my gratitude to the Craig H Neilsen Foundation (#546893) for financial support of these research endeavors. I would like to thank my family for their love and support: Justin, Andrea, Ainsley, and Brenna Gumbel, Fred and Shirley Hagen, Kim, Christopher, and Andrew Hagen-Rapp. Lastly, thank you to Dr. Tyler B. Jones for his support, love, and endless entertainment.

ABSTRACT

INVESTIGATION OF POTENTIAL MECHANISMS UNDERLYING SPINAL CORD INJURY-INDUCED POLYURIA

Jason H. Gumbel

September 15, 2021

Spinal cord injury (SCI) results in neurological impairments including motor, sensory, and autonomic dysfunction. These neurological deficits result in a litany of complications apart from muscular paralysis, including bladder, bowel, cardiovascular, and sexual function. SCI-induced polyuria (the overproduction/passage of urine) remains understudied, and therefore mechanisms behind it are largely unknown and require extensive investigation for potential targeted therapies to improve quality of life.

The objective of this dissertation was to investigate potential mechanisms of SCI-induced polyuria and explore potential therapies to improve quality of life in the SCI population. Metabolic cages, Western blot, enzyme-linked immunoassay, and immunostaining were first used to determine the timing of fluctuations in biomarkers associated with SCI-induced polyuria, including arginine vasopressin (AVP), atrial natriuretic peptide (ANP), vasopressin 2 receptor (V2R), natriuretic peptide receptor A (NPRA), and epithelial sodium channel (ENaC). Next, to identify which neural substrates induce polyuria with a T9-level SCI, a higher level (T3) contusion above the local sympathetic supply to

the kidneys were also examined. Lastly, the effect of anantin (NPRA antagonist) on SCI-induced polyuria was explored, in addition to utilizing an established treadmill activity-based recovery training (ABRT) program.

There were significant alterations of multiple biomarkers after SCI, beginning at 7 days post injury (dpi), in addition to a lower number of AVP-labeled neurons in the hypothalamus. By 7 dpi, continuing through 6 weeks post-SCI, T3 contused rats showed a significant increase in 24-hour void volume as well as significant changes in ANP and AVP like the T9 injury. There was also a significant decrease in AVP-labelled cells in the suprachiasmatic nucleus post-T9 and T3 contusion relative to controls. A reduction in void volume was found for rats having ABRT but not anantin treatment. A significant decrease in mean arterial pressure was measured in all animal groups lasting chronically, and there was a significant increase in serum potassium at 14 dpi in addition to a significant decrease in serum sodium at the chronic time point. Together, these studies provide a detailed account of systemic responses to SCI that are associated with SCI-induced polyuria and fluid homeostasis.

TABLE OF CONTENTS

PAGE

LIST OF FIGURES

CHAPTER I

GENERAL INTRODUCTION

Hormonal events and spinal cord injury: a focus on vasopressin and natriuretic peptide

Spinal cord injury (SCI), which directly damages one system of the body (the nervous system), precipitates a whole-body disease, affecting all other major bodily systems including the circulatory, digestive, endocrine, immune, muscular, renal, reproductive, respiratory, and skeletal systems. Although the effects of SCI on musculoskeletal system deficits have historically received a lion's share of the attention with respect to pre-clinical and clinical research, studies focused on other bodily systems have been more limited, with relatively few considerations of cross-system interactions. The current chapter reviews the SCI literature to-date that has focused on the endocrine system, with a specific emphasis upon the dysregulation of two neurohormones, vasopressin and atrial natriuretic peptide. These hormones influence multiple systems (cardiovascular, renal and urinary), and resulting deficits impact both daily activities and quality of life.

Gumbel, J.H., Hubscher, C.H. (2021). Hormonal events and spinal cord injury: a focus on vasopressin and natriuretic peptide. *The Neuroscience of Spinal Cord Injury*. (in press).

Dysfunctions of the upper and lower urinary tracts that arise after SCI include detrusor-sphincter dyssynergia, incontinence, polyuria, urinary retention and loss of sensation. Bladder emptying methods include intermittent catheterization (self or by an attendant), indwelling catheters (transurethral or suprapubic), reflex triggering, straining, manual compression (Crede), and sacral anterior root stimulation. A poorly understood complication that develops after SCI which impacts the daily frequency of conducting these management techniques is polyuria, the overproduction and/or passage of urine, which has been documented in both human SCI and pre-clinical contusion models. [1-4] This excessive volume of urine requires more frequent catheterizations, increases the risk of bladder and urinary tract infections, and prompts the need for nightly awakenings for bladder emptying (nocturia) which is disruptive to sleep and receiving enough rest. Often times, SCI individuals will limit their fluid intake to avoid nocturia, which introduces further and potentially more serious confounding issues such as dehydration, which exasperates bowel management through constipation, and autonomic dysreflexia, a life-threatening sudden increase in arterial blood pressure that is elicited by noxious (e.g., pain) and/or innocuous stimuli (e.g., bladder filling) from below the level of injury. [5, 6]

The underlying mechanisms of polyuria/nocturia are unknown, but likely includes arginine vasopressin (AVP; also commonly referred to as anti-diuretic hormone), which regulates urine production and fluid homeostasis and has recently been shown to correlate with the incidence of SCI-induced polyuria. [7, 8] However, it remains undetermined what directly causes the decrease in AVP

after SCI, as there are likely multiple factors contributing to polyuria (Figure 1). These factors include the natriuretic peptides (NP), such as atrial natriuretic peptide (ANP), brain-derived natriuretic peptide (BNP), and C-type natriuretic peptide (CNP).

The focus of the current chapter review is upon SCI-induced changes in AVP and ANP as they play a major role in water/electrolyte balance. In addition, these neurohormones also impact the cardiovascular system. Substantial cardiovascular deficits present amongst the SCI population include orthostatic hypotension, bradycardia, daily fluctuations in blood pressure, and autonomic dysreflexia. [3, 9, 10] Given these multiple functional roles of neurohormones, any imbalance is likely to impact the overall well-being of the SCI population.

Function of vasopressin (AVP)

One of the bodies most important hormones required for the regulation of salt and water balance is AVP. The production of AVP takes place within neurons located in the supraoptic nucleus (SON), suprachiasmatic nucleus (SCN), and paraventricular nucleus (PVN) of the hypothalamus, regions of the brains limbic system having a central neuroendocrine function. [11, 12] These hypothalamic neurons terminate in the posterior lobe of the pituitary and release AVP in response to several different stimuli. First, under normal physiological circumstances, osmoreceptors located in the hypothalamus respond to an increase in blood osmolality (occurs with dehydration or a high sodium diet) [13, 14] through physical shrinkage which triggers a signaling cascade to release AVP from the pituitary. Within the kidney, vasopressin 2 receptors (V2R) respond to elevated AVP circulating in the blood by increasing reabsorption of solute-free water back into the circulatory system through the opening of aquaporin 2 (AQP2) channels, which results in a decrease in urine volume. As many as thirteen different types of aquaporins have been identified in mammals, of which at least seven are located within the kidney and function to transport water across membranes. [15] However, AQP2 is the only kidney aquaporin known to be regulated by AVP.

There are three main AVP receptors: V1, V2, and V3. Each of these receptors has specific functions within different regions of the body. The V1 receptor (V1R) is mainly located in vascular smooth muscle and platelets, but can be found in brain, testis, superior cervical ganglion, liver, blood vessels, and

kidney. [16] Functionally, the V1R is a G protein-coupled receptor and utilizes the activation of calcium influx, phospholipase A2, phospholipase C, and phospholipase D. The V1R functions mostly as a vasoconstrictor and in thrombosis, but has been shown to be associated with myocardial hypertrophy, glycogenolysis, and uterine contraction. [17] V1Rs can also be found in the kidney where it also functions to promote vasoconstriction within the efferent arterioles, which reduces glomerular filtration rate.

The V2 receptor (V2R) is primarily located within the kidney and promotes a strong antidiuretic effect. As illustrated in Figure 2, upon the binding of AVP to kidney V2 receptors, cAMP is activated which then triggers the fusion of AQP2 channels to the apical membrane of the collecting ducts. [18] The increase in AQP2 channels promotes water reabsorption. For this reason, V2R is a target of pharmacological therapies to decrease urine production in cases like diabetes insipidus [19] and SCI. [20, 21] V2R is also located in vascular endothelium and smooth muscle cells and has vasodilation properties.

The V3 receptor (V3R) is also a G protein-coupled receptor but is far less distributed than either V1R or V2R. Less is known about V3R, but it is overexpressed in adrenocorticotropic hormone (ACTH)-secreting tumors. Additionally, its function is dependent on the concentration of AVP. One function of V3R is to promote ACTH release from the pituitary, but at other concentrations of AVP, V3R can increase DNA and cAMP synthesis, which is seen in tumor growth.

Function of natriuretic peptides (NP)

NPs include three structurally and functionally related hormone factors: ANP, BNP, and CNP, although there is evidence of a fourth, *Dendroaspis natriuretic peptide (*DNP, isolated from the green mamba snake). The primary function of NPs is to promote natriuresis. [22] Natriuretic peptide receptor A (NPRA) is the primary receptor for all three NPs. Although primarily found in the kidney as its function is to promote diuresis and natriuresis, NPRA is also located in lung, vasculature, heart, adrenal, adipose, and brain tissues. [23-25] The ANP/NPRA interaction regulates blood pressure, and therefore is essential for fluid balance homeostasis.

The release of both ANP and BNP is triggered by an increase in blood volume within the heart. While ANP is released primarily within the atria, BNP is released within the ventricles. Although ANP and BNP have a similar function, BNP has an estimated 10-fold lower affinity than ANP, and therefore is considered a weaker diuretic. Clinically, serum ANP is used as a biomarker for cardiovascular disease, including myocardial infarction, stroke, coronary artery disease, and heart failure. [26-28] In clinical SCI research settings, ANP has been investigated as a therapeutic in ischemia/reperfusion injury, [29] as well as for its role in exercise therapy [30] and bladder distention. [30] Further work is necessary to elucidate the importance and extent to which the changes in these peptides after SCI affect urinary and cardiovascular health.

SCI-induced polyuria/nocturia

Nocturia is defined as the necessity to void one or more times at night, specifically while sleeping. [31] There are many potential causes of nocturia, including decreased bladder capacity, increase in fluid intake, and increased diuresis. [32] A common occurrence in the human SCI population is nocturnal polyuria, or an excess of urine production/passage at night. [8] This issue causes sleep disruptions, bladder overdistention, and an increased risk of acquiring lower urinary tract infection due to the necessity for additional intermittent catheterizations. Historically, SCI-induced polyuria was thought to be the effect of decreased vascular tone and pooling of fluid in the lower extremities, which upon fluid redistribution when supine at night-time causes “intravascular flooding” and subsequent diuresis. [33, 34] However, polyuria is present in animal SCI models [2, 4, 7, 35] which lack positional fluid redistribution, suggesting that other mechanisms are likely involved.

In chronic SCI patients, increased blood pressure at night versus day is associated with increased nighttime urine production. [36] Further, nocturnal polyuria, SCI-induced or otherwise, can be caused by decreased AVP circulation, increased ANP, and cardiovascular insufficiency. [37] A study by Denys et al. revealed that SCI patients lacked circadian control of renal function (clearance of creatinine, free water, and solutes). [8] Together, these measured changes in circadian control of renal function, blood pressure, AVP, and ANP after SCI are all likely contributing to the mechanisms driving SCI-induced polyuria.

AVP after SCI

One potential factor that contributes to SCI-induced polyuria is a decrease in levels of AVP. Under normal conditions, humans exhibit an increase in circulating AVP at night which results in decreased sleep-time urine production and thus volume, thereby controlling the need for toileting. Clinically, children with nocturnal polyuria experience disruptions in the diurnal variation of AVP, specifically a decrease in circulating AVP, causing an abnormal increase in night-time urine production which may induce bed-wetting. [38] Similarly, the SCI population commonly experience disrupted diurnal variation of AVP, [1, 39] leading to the need for bladder emptying one or more times a night. Additionally, individuals with SCI exhibit disrupted circadian control of renal creatinine clearance, water diuresis, and solute diuresis. [8] This decrease in night-time AVP is likely a major contributor to SCI-induced polyuria/nocturia. Furthermore, persons with injuries above T6 tend to demonstrate an increase in nocturnal sodium excretion, which would suggest that the mechanisms behind SCI-induced polyuria include other factors such as ANP/BNP. [8] However, polyuria has also been observed in individuals having varying severities and injury levels, trends consistent with metabolic cage results in the clinically relevant rat contusion model whereby polyuria was measured regardless of extent of injury. [2]

In order to further elucidate the potential mechanism of SCI-induced polyuria, pre-clinical experiments were conducted to investigate the changes in AVP and NP's in the rat SCI model. The reported data [7] are consistent with those described for the human SCI population. At two-weeks post SCI, male rats

demonstrated a significant decrease in serum AVP, [7] which continued at a chronic time point as well. [35] In addition to the sub-acute and chronic decrease in AVP, the key AVP receptor in the kidney, V2R, was significantly lower than uninjured surgical sham rats. [35] This result is somewhat surprising as V2R is a G-coupled protein receptor and under normal physiological conditions, becomes desensitized to an abundance of its ligand (in this case, AVP), and inversely becomes sensitized under low levels of its ligand. Moreover, as illustrated in Figure 2, protein levels of AQP2 channel and epithelial sodium channel (ENaC) within the kidney were also reduced along with V2R, further highlighting the importance of AVP in SCI-induced polyuria, as AQP2 channels are formed in response to the activation of V2R to promote water absorption, and ENaC functions to reabsorb sodium ions back into the blood stream. Taken together, the changes in these receptor densities after SCI, as shown in Figure 3 and 4, are in the direction expected to promote polyuria.

A significant increase in AVP has also been reported in female piglets 15 minutes after a cervical spinal transection, which is likely an acute response to cardiovascular changes to maintain homeostatic blood pressure. [40] This finding demonstrates that the hemodynamic changes seen after SCI are likely affecting the changes in AVP, as orthostatic hypotension is another common occurrence in the human SCI population. [9, 41, 42].

While desmopressin (a synthetic AVP analogue) is used to suppress bed wetting in children [43] and has yielded positive results in some cases of SCI-induced nocturnal polyuria, [21, 44] it’s use may be limited to those at lower risk

of cardiovascular disease. Additionally, desmopressin only targets one of the several potential factors that lead to polyuria and leaves other pathways unchecked, such as ANP/NPRA.

ANP after SCI

Mechanisms driving the chronic decrease in AVP/V2R after SCI are unknown. Although blood osmolality is one driving force behind AVP release, non-osmotic stimuli such as NPs, glucocorticoids, and norepinephrine may control AVP levels and urine production. [45, 46] Of these stimuli, NPs (ANP/BNP) and corticosterone (CORT; primary glucocorticoid in rats) have been further investigated in the rat SCI model (see Figure 1). While there is some evidence that SCI can induce changes in ANP in the clinical setting, [47] further pre-clinical and clinical experimental studies are necessary to further elucidate the impact of level and severity of injury.

Changes in urinary ANP and CORT have been documented as early as two weeks after a moderate contusion injury in male rats, together with serum AVP. [7] In a 2018 study of SCI rats with polyuria, both urinary ANP and CORT were significantly increased two weeks post-contusion, while surgical sham animals demonstrated no differences. Both ANP and CORT inhibit the function and release of AVP, therefore the significant changes in these hormones (increases in ANP and CORT and a decrease in AVP) are in the direction expected with respect to polyuria. [48, 49] It is important to note that urinary BNP levels have not been shown to change after SCI. However, BNP is a weaker diuretic compared to ANP. It cannot be ruled out that plasma BNP changes occur after SCI, as its half-life is relatively short and potentially difficult to capture.

It has also been demonstrated that the significant changes in AVP and ANP continue chronically in SCI male rats. [35] In a recent study, SCI animals

having polyuria were randomized into one of two groups receiving different forms of activity based recovery training (ABRT) on a treadmill or one of two groups having no therapeutic intervention (one group harnessed without any treadmill activity and one remaining in their home cage). [50] While all groups still demonstrated polyuria at the end of the study such that their 24-hour urine volumes remained significantly increased from baseline pre-injury levels, the ABRT animals showed a significantly lower 24-hour urine volume than the non-trained animals after the eight weeks of daily 60-minute training sessions. These findings were similar to those previously reported in animal [4, 51] and human studies [52] demonstrating multisystem benefits of locomotor training. In addition to changes in AVP, ANP and corticosterone, relative expression densities of NPRA was found to be significantly increased in the non-trained animals, but not the ABRT animal groups (see Figure 3). The inverse was found for V2R and AQP2 as their expression were significantly decreased in only the non-trained animal groups, while the ABRT animals were not significantly different from surgical sham animals. These findings, illustrated in Figure 3, suggest that one potential mechanism behind the improvement in polyuria after exercise therapy is the regulation of key receptors within the kidney. Note that although high-intensity exercise induces an acute increase in AVP in able-bodied subjects [53, 54] as well as ANP, [55] these effects may be short-lived and may differ under conditions such as SCI. More studies are needed to further elucidate underlying mechanisms.

Potential mechanisms causing changes in AVP and/or ANP after SCI

Although many variables lead to the release of AVP, two important physical changes include hypertonicity and/or hypovolemia, which are important because orthostatic hypotension is a common occurrence amongst the SCI population. [9] Another issue for the SCI population, specifically with higher level injuries, is autonomic dysreflexia (AD), a sudden onset of hypertension. [56] Repeated and frequent occurrences of AD may have an impact on AVP release and subsequent concentration of V2R in the kidney. Such changes in alpha adrenergic receptors (receptors for epinephrine and norepinephrine) have already been linked to AD. [57] AD has also been characterized following severe injuries in the rat model of SCI. [58]

One potential mechanism behind the observed increase in NPRA after SCI in rodents [35] is an increase in glucocorticoids. An increase in glucocorticoids can increase glomerular filtration rate, [48] decrease the level of plasma AVP, upregulate NPRA within the kidney, [59] and increase ANP release. [60] Corticosterone, the main glucocorticoid in rodents (equivalent to cortisol in humans), is elevated in both humans and rodents after SCI, which could relate to stress. [61, 62] It is also documented that glucocorticoids have the ability to alter immune activity, whereby a significant increase may lead to immunosuppression. [63-65] After SCI, specifically high-level injuries, elevated glucocorticoids are associated with a significant decrease in immune responses, which can lead to an increased risk of infections, including that of the bladder and kidney. [66]

It is noteworthy that the renin aldosterone angiotensin system (RAAS) also regulates cardiovascular homeostasis (blood pressure and volume) in addition to fluid and electrolyte balance (urine concentration and excretion). RAAS is an additional factor that can affect both AVP and ANP, and therefore needs further investigation in its potential role in SCI-induced polyuria. While there are studies in the clinical setting that indicate there are acute changes in renin and aldosterone after SCI, [67, 68] the focus of these studies relate to cardiovascular function.

Redistribution of body fluids in the SCI population at bedtime when shifting from a sitting to supine position produces cardiovascular fluctuations, which in turn affects the delicate hormone balance that controls homeostatic fluid balance. [69] This fluid redistribution is one potential mechanism of SCI-induced polyuria. Support comes from a study where use of compression stockings was shown to reduce leg edema during the day and subsequent urine production during the night. [69] However, animal models with SCI-induced polyuria that do not undergo fluid redistribution suggest other mechanisms are likely involved, such as hormones previously identified with respect to fluid balance (see Figure 1). As further research progresses, additional key hormones/receptors will be identified along with AVP and ANP, and their roles elucidated with respect to SCI-induced polyuria as well as cardiovascular health.

Applications to Other Areas of Neuroscience

Several associations have been made between altered AVP function and several neurological disorders besides SCI, including autism, depression, bipolar, and schizophrenia. [70] For example, altered binding of AVP and V1R in the CNS has been linked to autism disorders, [71] and elevated CSF levels of neurophysin II (hypothalamic carrier protein for AVP) were found in patients with schizophrenia. [72] Also, in multiple sclerosis, an autoimmune disorder that affects the myelin sheaths of nerves within the CNS, urinary tract dysfunctions include polyuria/nocturia. [73] The potential mechanisms behind multiple sclerosis associated polyuria overlap with SCI-induced polyuria, such as disrupted AVP secretion and nocturnal hypertension. [74]

Other neurological involvement related to NPs have been shown for studies involving the olfactory bulb, [75] Purkinje fibers of the cerebellum, [76] the retina, [77] and development. [78] There have also been studies indicating a potential neuroprotective role for NPs, likely through increasing cGMP levels. [78, 79] Together, it is clear that both NPs and AVP are two critical entities that should be considered in other settings in addition to SCI.

Experimental Directions

The objective of this dissertation is to investigate the mechanisms and explore potential therapeutic targets for SCI-induced polyuria using a pre-clinical animal model. Currently, it is unknown when the previously identified biomarkers (AVP, ANP, V2R, NPRA, AQP2, and ENaC) involved in SCI-induced polyuria are significantly altered. Therefore, the timeline of fluctuations in specific biomarkers associated with the incidence of SCI-induced polyuria was investigated by carrying out enzyme-linked immunosorbent assay (ELISA), Western blot, immunohistochemistry, and 24-hour metabolic cage experiments on selected tissue and samples derived from SCI animals on 3, 7, 14, 28, 42, and 84 days post injury (dpi) compared to surgical sham controls. It was hypothesized that SCI-induced polyuria-associated biomarkers in the kidney and hypothalamus (specifically AVP) would be altered post-SCI and remain imbalanced chronically. Secondly, the incidence and extent of polyuria, along with AVP and ANP levels after a T3 level injury was explored in order to determine if level of injury plays a role in SCI-induced polyuria, as a high thoracic injury (T3) will disrupt only supraspinal sympathetic input to the kidneys, rather than both supraspinal and preganglionic sympathetic fibers with a low thoracic injury. It was hypothesized that a T3 level SCI would result in a significant disruption of urinary function, including SCI-induced polyuria in conjunction with altered AVP/ANP levels. Lastly, the NPRA antagonist anantin was administered, with and without the addition of ABRT to determine if NPRA could be a potential target for pharmacological intervention in reducing the effect of SCI-induced polyuria.

Additionally, secondary outcomes measures, such as mean arterial pressure (MAP), serum sodium, and serum potassium were measured to record any potential effects of either anantin and/or ABRT in SCI rats. It was hypothesized that the combination of ABRT and anantin would decrease polyuria compared to the vehicle treated and non-trained animals, in addition to significant changes in MAP, sodium, and potassium levels. Together, these experiments provide further insight into understanding the mechanisms behind the incidence and maintenance of SCI-induced polyuria, in addition to providing details of systemic occurrences that contribute to urologic health.

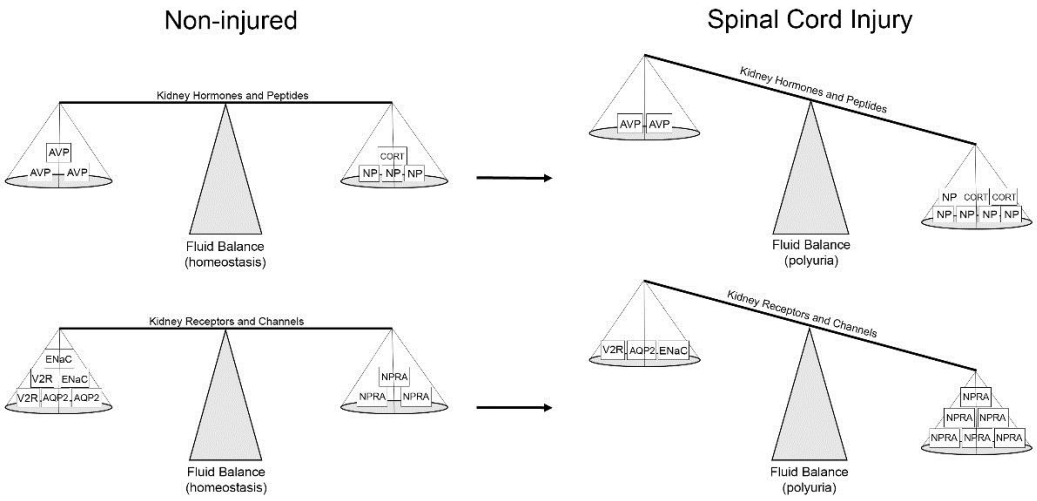

Figure 1:

Key hormone/receptors in fluid balance and how they are affected after SCI. Depiction of key hormones such as AVP, NPs, and CORT, along with paired receptors such as NPRA, V2R, AQP2, and ENaC, and how they contribute to fluid balance/homeostasis. In the non-injured state, these hormones and receptors work together to closely regulate fluid balance. After injury, dysregulation occurs leading to an overproduction of water (polyuria/nocturia). Changes in the opposite direction would cause a decrease in urine production (oliguria).

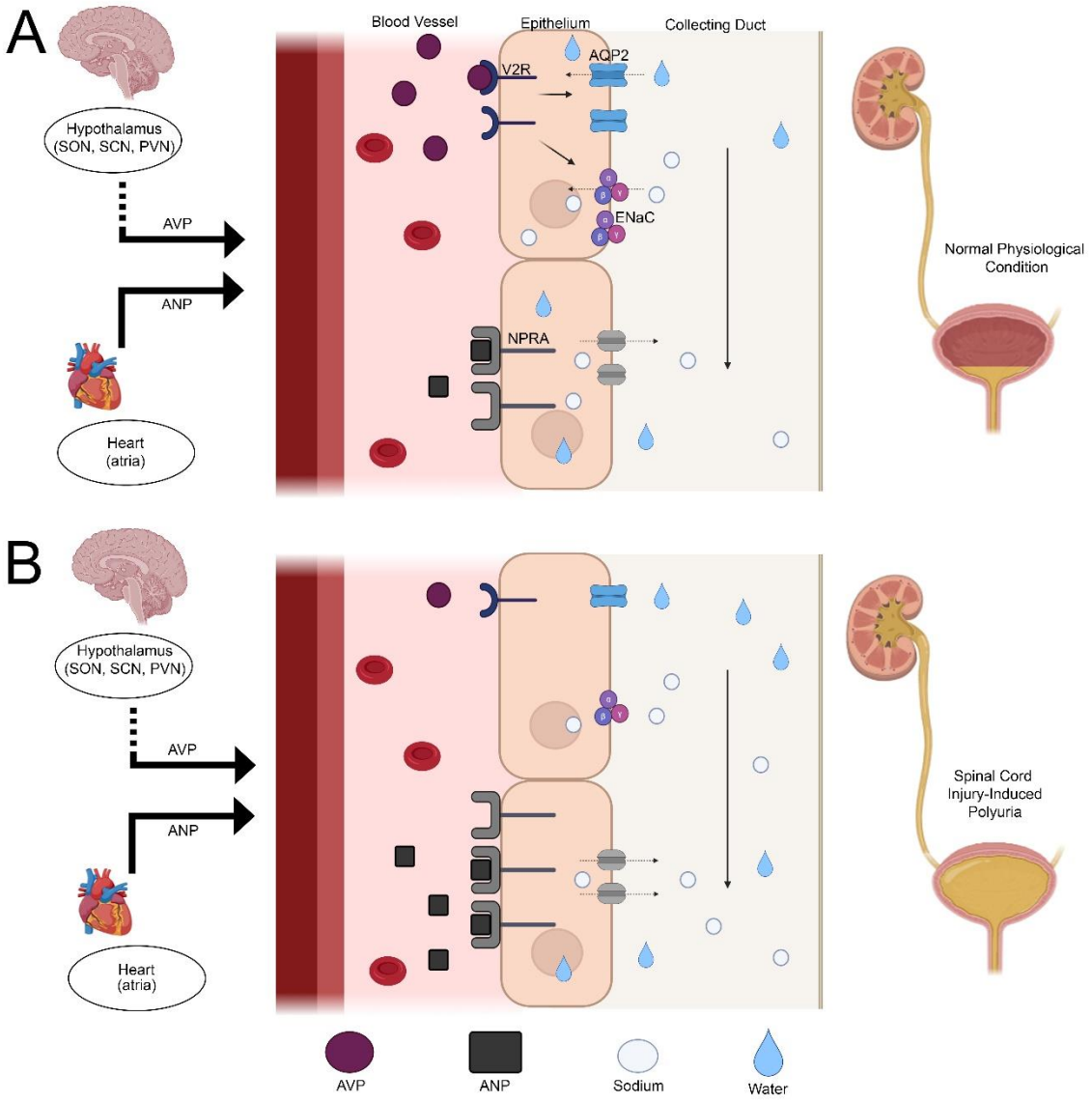

Figure 2:

Diagram of AVP/ANP and their receptors within the kidney under normal physiological conditions and after SCI. As part of normal physiological processes to maintain fluid homeostasis, AVP is released by the hypothalamus in response to conditions of hyperosmolality (A, upper portion). Once in the kidney, AVP binds to V2R, which triggers a response to open AQP2 channels to allow water flow from the kidney collecting duct back into the epithelium while ENaC allows

for sodium to be reabsorbed. However, under conditions of elevated blood volume, ANP is released from the heart (A, lower portion) and upon binding to NPRA within the kidney, a cascade is triggered to permit salt and subsequently water excretions. As illustrated in B, there is a decrease in AVP, V2R, and ENaC as well as an increase in ANP and NPRA after SCI, which together contribute to the injury-induced occurrence of polyuria/nocturia.

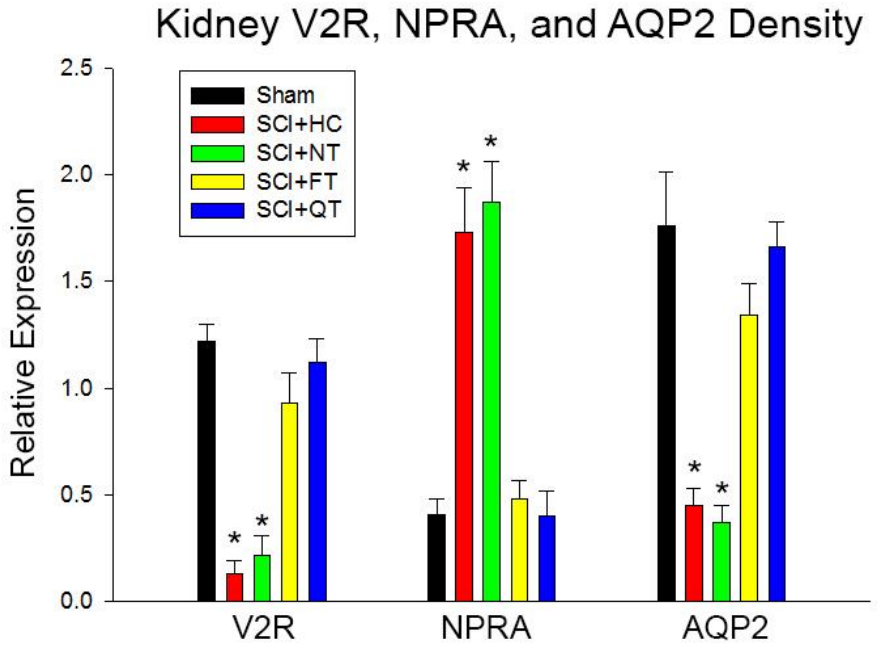

Figure 3:

Activity-Based Recovery Training reverses several key fluid balance receptors in the kidney after SCI. After chronic SCI, V2R, NPRA and AQP2 are significantly altered. Relative expression of proteins using Western blot reveals that V2R and AQP2 are significantly decreased while NPRA is significantly increased in groups of male Wistar rats that did not receive any therapeutic interventions (NT = non-trained; HC = home cage) after a moderate-severe contusion injury that yields approximately 11% white matter sparing at the T9 spinal level lesion epicenter. Animals that received ABRT involving quadrupedal or forelimb-only training (QT and FT, respectively) by stepping on a treadmill for one hour a day, 7 days a week for 8 weeks beginning two weeks post-injury were found to have similar levels of these receptor/channel densities to surgical sham animals (spinal laminectomy but no contusion).

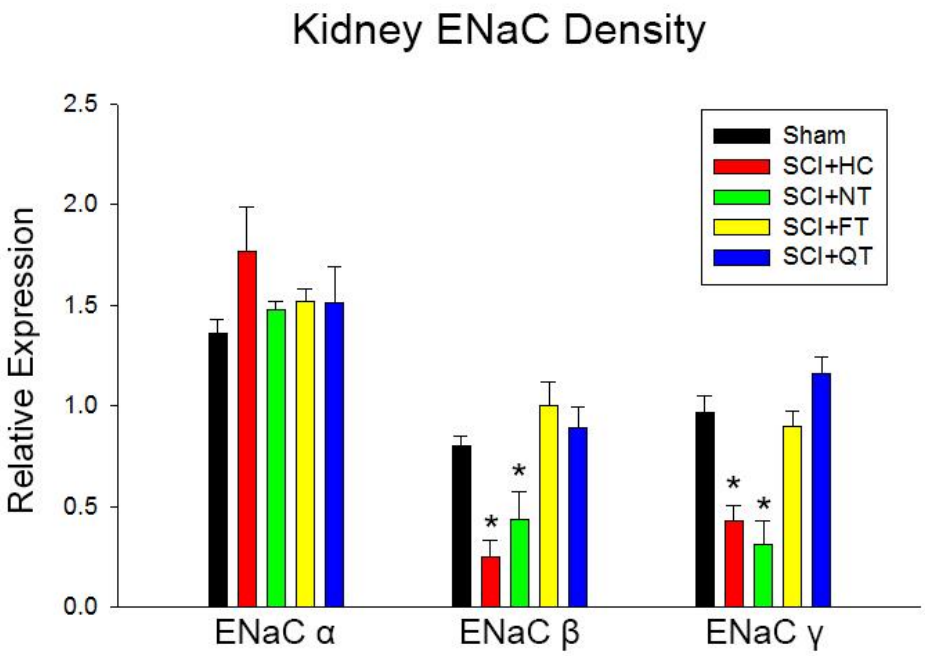

Figure 4:

Activity-Based Recovery Training reverses ENaC β and γ subunits deficit after SCI. After chronic SCI, both β and γ subunits of ENaC are significantly decreased. However, after ABRT (SCI+FT and SCI+QT), the relative density of both β and γ subunits were reversed to similar levels of surgical sham animals. Group abbreviations per Figure 3.

CHAPTER II

TIMELINE OF CHANGES IN BIOMARKERS ASSOCIATED WITH SPINAL CORD INJURY-INDUCED POLYURIA

Introduction

Spinal cord injury (SCI) results in deficits that are widespread across all bodily systems, including those involving motor, sensory, autonomic, endocrine, and immune functions. Urologic dysfunctions are rated by SCI individuals as one of the most important factors impacting quality of life. [80] However, the vast majority of pre-clinical and clinical studies targeting the urinary system have focused on the lower urinary tract (bladder, urethra, and external urethral sphincter), with relatively fewer studies addressing deficits related to the upper urinary tract (kidney) post-SCI. A common issue that arises after SCI that complicates urinary function is the excess production/passage of urine (polyuria/nocturia), [81] which has been reported clinically and in pre-clinical animal models. [2, 4, 8, 82] The frequency of daily bladder catheterizations, including during the night-time, is disruptive to daily activities and sleep while simultaneously increasing the risk of urinary tract and bladder infections. [83] However, many persons with SCI accommodate by limiting their intake and/or

Gumbel J.H., Yang C.B., and Hubscher C.H. (2021) Timeline of changes in key hormones and their receptors associated with spinal cord injury-induced polyuria after spinal cord injury. *Neurotrauma Reports.* (in press)

type of fluids, which complicates the estimation of polyuria prevalence and can lead to dehydration or other issues impacting health. [84]

Our group has previously shown using a pre-clinical animal incomplete contusion model that 1) polyuria occurs regardless of severity of SCI, [2] 2) alterations of hormones affecting diuresis are present at 14 days' post injury (dpi), [7] and 3) related receptor/channel densities are altered in the kidney at 10 weeks post-SCI. [35] Although several key hormones and their receptors in the kidney impacting fluid balance have been shown to contribute to the mechanisms underlying SCI-induced polyuria/nocturia, more information is needed for the optimization of therapeutic approaches. [7, 35]

Several studies have already been published to date demonstrating significant disruptions in arginine vasopressin (AVP, also commonly referred to as anti-diuretic hormone), atrial natriuretic peptide (ANP), and their associated receptors (V2R and NPRA, respectively) in the kidney after SCI. [1, 7, 35] We have also shown that kidney aquaporin 2 channels (AQP2) and epithelial sodium channels (ENaC) were significantly decreased following SCI in adult male rats. [35] Together, these fluctuations in AVP and ANP and their accompanying receptors/channels lead to an increase in urine production based on their physiologic function. These receptors also play major roles in salt and fluid balance within the body, as well as cardiovascular homeostasis. [85, 86] Clinically, desmopressin (synthetic AVP) may be used to treat nocturnal polyuria, but with limited results and potential deleterious side-effects. [20, 87] Additionally, desmopressin often impacts blood pressure, which may increase bouts of

autonomic dysreflexia—a phenomenon that occurs when blood pressure rises uncontrollably and cannot be corrected due to disruptions of descending sympathetic pathways in the spinal cord. [88, 89]

The purpose of the current study was to ascertain a timeline for the development and maintenance of SCI-induced polyuria, with a focus on contributions of key targets not only in the kidney, but within the hypothalamus as well. Neurons in the hypothalamus manufacture AVP in the supraoptic nucleus (SON), paraventricular nucleus (PVN) and, the suprachiasmatic nucleus (SCN). [90] Under normal physiological circumstances, AVP is released by the posterior pituitary in response to hypotension, and/or an increase in extracellular osmolarity. In the kidney, AVP activates V2R to increase AQP2 channels for the reabsorption of solute-free water back into the bloodstream. An increase in blood volume will trigger the release of ANP from the heart and within the kidney will activate NPRA in order to increase the excretion of salt and inhibit the reabsorption of water. [91, 92] Understanding the timeline of when these key biomarkers are significantly altered post-injury will provide a therapeutic window for intervention.

Methods

Animals

All animal experiments and procedure protocols were reviewed and approved by the University of Louisville Institutional Animal Use and Care Committee (IACUC) and carried out in accordance with the National Institutes of Health (NIH) guidelines. A total of 94 adult male Wistar rats were used for the entirety of these experiments (Table 1). We have opted to utilize only adult male rats for the current study since prevalence of SCI is predominantly male. Six timeline SCI groups included study endpoints at 3, 7, 14, 28, 42, and 84 days post-injury (dpi) as well as three surgical sham groups matched for endpoints 3, 14, and 42 dpi.

Spinal Cord Injury (SCI)

After baseline/pre-injury data was obtained (see below), animals were anesthetized with an intraperitoneal injection of a mixture of ketamine (80 mg/kg, Ketoset®; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (10mg/kg, AnaSed; Lloyd Laboratories, Shenandoah, IA). Both toe-pinch and orbital reflexes were monitored to assure a deep anesthetic plane was reached. The surgical site was shaved and cleansed with Chlorhexidine scrub 4% (Henry Schein) before placement on a heating pad and application of sterile ocular lubricant (OptixCare, Aventix). A T8 laminectomy was performed to expose the T9 spinal cord level. An Infinite Horizon (IH) impactor (Precision Systems and Instrumentation LLC; Fairfax Station, VA) was used to administer a 215-kilodyne

contusion with no dwell time at the T9 spinal level, which yields a moderate-severe extent of injury with white matter sparing averaging approximately 11%. [2, 93] The muscular layer was then sutured with 4-0 surgical suture (Ethicon; Somerville, NJ), and the skin closed with surgical wound clips. Surgical sham animals underwent the same procedure, but no contusion injury was administered. Both penicillin G (Penject; Henry Schein Animal Health, Dublin, OH) and meloxicam (Eloxiject; Henry Schein Animal Health) were injected subcutaneously per established post-operative care procedures. [35, 50, 94] Physiological saline was also administered post-operatively (5mL before contusion and 5mL after contusion). Bladder emptying via Crede maneuver was performed three times daily until individual animals reached reflexive bladder function (by 6 days [95, 96]). Residual volumes at 4 dpi were collected and measured for use as indicator of lesion severity, per our previous study showing peak amounts at this time point due to flaccid paralysis from spinal shock during the earliest phase post-injury. [2] Note that the injury procedure is available for online viewing in a published video journal from our lab. [50]

Metabolic Cage Data Collection

A six station Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH) was used to monitor 24-hour urine output volume and drink volume following established protocols. [2, 35, 97] Food and water were available *ad libitum*. As part of the acclimation procedure of animals to the CLAMS unit, pre-injury baseline data was collected twice in one week, but

only the second 24-hour period was used for analysis. Metabolic cage assessments were then carried out once weekly after SCI (number of times variable between time-course groups). [7, 35, 50]

Blood Sample Collections

For serum samples, blood was collected via the lateral tail vein at pre-injury and terminal time points following standard procedures. [7, 98] Animals were anesthetized with isoflurane before shaving the base of the tail to better visualize the lateral tail veins. Once the animals were placed on a heating pad, an 18g needle was used to puncture either of the lateral tail veins. Between 0.5-0.7 mL of venous blood was then collected into serum separator tubes (BD microcontainer, Becton, Dickinson and Co.). Light pressure was applied to the puncture site with 2”x2” gauze until bleeding stopped. When necessary, wound clotting was achieved using styptic powder with benzocaine (Kwik-Stop, ARC Laboratories). The blood samples were centrifuged at 14,000 rpm for 15 minutes and serum was collected and frozen at -20^{o}C.

Locomotor Assessment

The Basso-Beattie-Bresnahan (BBB) open field locomotor test [99] was performed weekly on each rat as well as the day prior to termination and subsequent tissue removal. Each single score per animal represents an average of the left and right hindlimb BBB score assigned by two experimenters blinded to

time point status. This assessment is used as an additional indicator of lesion severity and spontaneous recovery, per our previously published data. [2]

Tissue Collection and Histology

On the specified terminal date (3, 7, 14, 28, 42, 84 dpi/sham laminectomy), animals were perfused transcardially with a solution of heparinized saline (1mL heparin/100mL normal saline) followed by 4% paraformaldehyde. Although multiple tissues were retrieved, only the brain, spinal cord (lesion site), and the left kidney were removed and utilized in this study for immunohistochemistry, contusion epicenter reconstruction, and Western Blot analysis, respectively. Note that the kidney was retrieved prior to the 4% paraformaldehyde perfusion for Western Blot assessments. Each kidney was cut to ~0.1g sections (including both medulla and cortex) prior to storage at -80°C.

The brain and spinal cord were submerged in 4% paraformaldehyde and stored for at least 24h at 4°C. Tissue was then moved to a 30% sucrose solution and stored at 4°C until tissue has sunk to the bottom of the 15 mL conical tube (24-48h) and stored until it was sectioned using a cryostat (Leica CM 1850). Tissue was frozen in tissue freezing medium at -80°C to prevent freezing artifact. Once ready to section, tissue was acclimated to -20°C inside the cryostat for at least 30 min. Hypothalamus sections were collected at serial 25µm sections and transferred to glass slides. Slides were then stored at 4°C until tissue was ready to be stained.

Immunohistochemistry was performed on tissue sections containing the hypothalamus to visualize and quantify AVP-positive neurons. Fixed tissue was washed in phosphate buffered saline (PBS) before unmasking epitopes using antigen retrieval (IHC antigen retrieval reagent citrate, pH 6.0; Enzo Life Sciences). After antigen retrieval, tissue sections were washed and blocked (SuperBlock Blocking Buffer; ThermoFisher Scientific) before applying primary antibodies. Tissue sections were incubated with primary antibody anti-vasopressin (1:1,000 dilution; ab39363, Abcam) overnight at 4°C. Slides were then washed prior to incubation with fluorescent-conjugated goat anti-rabbit secondary antibody (Alexa Fluor 488; ThermoFisher Scientific). Slides were again washed before counterstaining with 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei.

To quantify the number of AVP-labeled neurons in the hypothalamus, the SON, SCN, and PVN were targeted based upon regional landmarks identified in the rat brain atlas. [100] A block of tissue from the collected brain was formed rostral to the optic chiasm and rostral to the cerebellum. Once frozen, tissue sections were cut at 25µm thickness and each slide stained contained three tissue sections ≥75 µm apart to ensure cells were not counted more than once. Slides were coded by an experimenter not directly involved in the study to blind group identity. Using ImageJ (NIH), the area of the SON, SCN, or PVN was outlined, and the number of AVP-positive cells with an intensity threshold at least 1.5 times above background level were counted to obtain the cells/area for

quantification. At least four different sections of nuclei per animal were averaged together for analyses.

The spinal lesion histology was carried out as previously described. [35, 97, 101] The spinal cord area containing the lesion (including ~2 levels above and below) was sectioned at 20 µm thickness and stained with Luxol fast blue and cresyl violet. The lesion epicenter and white matter sparing was captured and analyzed using Spot Advanced software (Diagnostic Instruments, Sterline Heights, MI) and Nikon E400 microscope. Spinal cord white mater was divided into dorsal columns, dorsolateral funiculus, ventrolateral funiculus, and the ventromedial funiculus, then separated into left and right sides. The percent white matter sparing (WMS) was calculated by dividing the intact white matter at the lesion epicenter by the average area of intact white matter present in more intact sections both rostral and caudal the injury site. An average of two areas within 2 mm rostral and caudal was used for intact white matter. [102]

ELISA

Urinary ANP and creatinine was measured as previously described [35] using an atrial natriuretic peptide Enzyme Immunoassay Kit (cat. No. K026-H1; Arbor Assays; Ann Arbor, MI). Urine samples were diluted 1:5 for ANP, 1:20 for creatinine and then plated in a 96-well plate in duplicate. Plates were read at 450nm OD using SoftMax Pro software (Molecular Devices). Urinary creatinine was measured using DetectX Urinary Creatinine Detection Kit (cat. No. K002-H5; Arbor Assays; Ann Arbor, MI). To control for differing urinary concentrations, the

urinary ANP levels were divided by urinary creatinine levels per ANP ELISA kit instructions.

Baseline and terminal levels of serum AVP were determined using an arginine vasopressin ELISA kit (cat. No. OKEH02585; Aviva Systems Biology). Stored serum samples (see above) were diluted at 1:5 and ELISA was carried out according to kit instructions.

Western Blot

Relative expression of AVP, NPR-A, AQP2, and ENaC (α, β, and γ subunits) were analyzed by Western blot analysis according to our previously published protocols. [7, 35] Kidney tissue was homogenized in ice-cold RIPA buffer (Sigma, R0278) and protease inhibitor (78425, Thermo Scientific). The concentration of protein was determined for each sample using Bradford protein assay reagent (5000201, BioRad) and a spectrophotometer (at 595-nm absorbance). Gels were then run by loading 50µg of protein per lane on a 4-15% gradient gel (456-1085, mini protean TGX gels, BioRad) at 100 V for 80 minutes in mini-protean gel tanks (running buffer was a 1X Tris-glycine-SDS buffer). The protein was transferred to PVDF membranes for 2 hours in 4°C at 80V and stained with Ponceau S. to visualize bands. The membranes were washed with 1X Tris-buffered saline with Tween 20 (TBST), then blocked in 5% non-fat dry milk TBST solution for 1 hour. Membranes were then incubated in the primary antibody at 4°C overnight. Antibodies used in this study included anti-NPRA (Abcam ab154280, Cambridge, MA; dilution 1:1000), anti-AVPR V2 (Abcam

ab108145, Cambridge, MA; dilution 1:750), anti-AQP2 (Abcam ab108065, Cambridge, MA; dilution 1:1000), anti-ENaC α subunit (SPC-403 StressMarq Biosciences; dilution 1:1000), anti-ENaC γ subunit (SPC-405, StressMarq Biosciences; dilution 1:1000), anti-ENaC β subunit (14134-1-P, Proteintech; dilution 1:750), and anti-β-actin (Sigma Aldrich #A5316, St. Louis, MO; dilution 1:5000). The membranes were washed three times with TBST prior to incubation with HRP-conjugated secondary antibody in blocking solution for 2 hours at 1:3000. Lastly, the membranes were washed three times with TBST and developed with ECL substrate before being imaged using BioRad Imaging System and analyzed using ImageJ software (version 1.8, National Institute of Health). Standardized values were obtained by normalizing experimental groups to β-actin.

Statistical analysis

Both 24-hour urine and drink volumes from CLAMS data were exported from Oxymax software to Microsoft Excel (Redmond, WA) for analysis. Each void event recorded by the sensor of ≥0.2g within the 24-hour timeframe was calculated for each animal. The total drink volume was recorded through the CLAMS volumetric drink monitor. One-way repeated measures ANOVA analyses were used across all SCI animal groups using SigmaStat v3.5 (Systat Software) where significance was determined for $p<0.05$.

Data files for AVP, ANP and creatinine were exported from SoftMax Pro to Microsoft Excel (Redmond, WA) for analysis. Samples and standards were

averaged to create a standard curve to determine protein concentration. Due to combined variability and sample size, terminal protein concentrations were compared to their normalized baseline level for statistical analysis. Signed-ranks tests were used to compare normalized baseline to terminal protein concentrations where $p<0.05$ was considered statistical significance. A two-way ANOVA was used for Western blot analysis to determine statistically significant interactions between antibodies used and terminal time points. Values were considered significant if $p<0.05$.

Results

Nine animal groups in total consisted of six post-SCI recovery time points (3, 7, 14, 28, 42, and 84 dpi) and three post-sham laminectomy time points (3, 14, and 42 dpi). Prior to contusion injuries, pre-injury baseline data was collected including: 24-hour urine output and collection/drink volume, blood draws, and BBB assessments. All animals scored 19 or above on the BBB scale indicating that there were no prior or pre-existing motor deficits. *Post-hoc* analyses were carried out between the six SCI groups and revealed no significant differences for injury parameters (force, displacement), or 4-dpi residual urine volume emptied by Credé (Table 1). However, there were significant differences among BBB scores where both the 3 and 7 dpi animal groups received significantly lower scores at terminal time points compared to the 14, 28, 42, and 84 dpi animals ($p<0.05$), a finding consistent with the timing of previous locomotor outcome data showing some limited recovery during the first two weeks post-contusion. [2]

24-hour Urine Volume

Both urine production and drink volume were measured weekly across 24-hours to determine presence/absence of SCI-induced polyuria. Prior to contusion injuries, each animal's baseline was recorded as a further control (in addition to sham groups) for each time point group. SCI-induced polyuria was present in all SCI animal groups except for 3 dpi (Figure 5). By 7 dpi, each animal group had a statistically significant increase in 24-h urine volume ($p<0.05$). Neither of the time

point groups after 3 dpi (7, 14, 28, 42, 84 dpi) were significantly different from each other in urine output. Despite the significant increase in urine production among all SCI animal groups apart from 3 dpi, there were no significant increases in drink volume across any of the groups, a finding consistent with previous studies. [2, 7, 35]

ELISA for Urinary ANP and Serum AVP

The importance of ANP in water balance, specifically in relation to cardiovascular changes, is well established. [103-105] Thus, ELISA was used to determine when and if alterations in urinary ANP occurred after SCI as previously reported, [7, 35] using urine samples from the 24-h metabolic cage assessments. Urinary ANP levels were found to be significantly increased at both 14 dpi and 42 dpi relative to baseline levels (Figure 6). Because blood pressure and water balance are heavily affected by circulating AVP, ELISA was also performed to obtain serum concentrations of AVP at pre-injury and terminal time points. Serum AVP levels were significantly lower at two of the chronic time points relative to pre-injury baseline concentrations (Figure 6).

Western Blot of Kidney Tissues

Western Blot analysis was performed on kidney tissue from six randomly selected animals per group to visualize relative expression density of NPRA, V2R, AQP2, and ENaC (α, β, and γ subunits) (Figure 7). For analysis of the time point factor overall (regardless of receptor or channel), both sham and 3dpi were

significantly different from all other time points, except for the 84 dpi group which differed from 3 dpi but not shams. Relative expression of kidney V2R was significantly decreased by 7 dpi and remained depressed at each subsequent time point except for 84 dpi. The same pattern occurred for kidney AQP2 expression, which is directly related to V2R expression. For both V2R and AQP2, 7 dpi was significantly lower than several of the later time points. In contrast, the density of NPRA expression was significantly higher at 3, 7, and 42 dpi relative to only 14 dpi. In addition, since the α, β, and γ subunits form together to make ENaC, Western blot analysis was done on all three. Although the density of ENaC α expression was unchanged at all time points compared to time point-matched sham groups, both ENaC β and γ expression densities were decreased after SCI at several time points, beginning at 7 dpi. All pairwise multiple comparison procedures (Bonferroni t-test) revealed a significant effect for only the ENaC α subunit relative to the other 5 antibodies ($p<0.001$), reflecting the lack of a time point effect for relative expression of ENaC α.

AVP labeling in hypothalamus

The number of AVP-positive cells in the SON was examined in all groups and the only difference found was for the 14 dpi time point which were significantly fewer relative to shams (Figure 8). Although the SON was the main target of this study, both PVN and SCN neurons that were present within the same tissue slices were examined as well. However, due to their relative size and location relative to the SON, the overall cellular sample size was smaller.

Within the PVN, there were no significant decreases in observed AVP-positive cells at any time points compared to sham animals (data not shown). However, the average AVP-positive cells/area within the SCN were significantly decreased at 14 dpi and remained that way at all later time points (Figure 9).

White matter sparing

White matter sparing (WMS), an indicator of injury severity, was quantified for all SCI animal groups. As shown in Figure 10, the WMS for both 3 and 7 dpi were not different from each other. There was a statistically significant decrease in WMS at the 14 dpi time point and beyond compared to both 3 and 7 dpi. Also, WMS was further decreased by the 84 dpi time point, indicating continued progression of damage at the lesion epicenter beyond 6 weeks post-contusion.

Discussion

SCI-induced polyuria has been established as a major systemic issue that arises after SCI in both human and pre-clinical animal SCI models. [1, 2, 106] Previous data from our lab has shown that several key hormones and receptors including AVP, ANP, V2R, and NPRA are associated with mechanisms underlying SCI-induced polyuria. [7, 35] The current data reveal the timing of these changes within the kidney itself with some additional contribution by AVP-positive neurons in the hypothalamus. Together, the current results illustrate both peripheral and supra-spinal effects within one of many internal organ systems post-SCI and demonstrates the impact of disrupting the body's homeostatic mechanisms on functional outcome and ultimately quality of daily living.

The animals in the current study presented with polyuria by 7 dpi and lasted through the longest time point examined (84 dpi). This result differs from the emergence of deficits in locomotor function, which appear immediately due to initial spinal shock, but then partially recovers and plateaus after several weeks at a level that is known to correlate with the extent of injury [107, 108] (and as shown by the BBB scores in Table 1). The fact that polyuria onset is slightly delayed and maintains at a steady state despite continued progression of damage at the lesion epicenter (re WMS data), indicates that the underlying mechanisms are secondary to damage of the spinal cord itself. Noteworthy is that this increase in urine volume was not matched with an increase in drink volume, which likely means the rats were under-hydrated, but typical signs of dehydration were not present (skin turgidity, reduced urine volume, urine color

change reflecting higher concentration, porphyrin staining of the eyes and nose) as they were visually inspected and tended to daily. One important question surrounding polyuria involves the source of the additional urine being voided. Previous experiments investigating fecal water weight resulted in a difference in acute, but not chronic time points, suggesting water does not come from any changes in water content within the feces. [2] Serum osmolality was also previously tested and found to be significantly increased after SCI. [7] Increased osmolality was in the direction that should stimulate the release of more AVP, which does not occur, indicating an overall disruption of homeostatic mechanisms. Although animals may be under-hydrated, physical dehydration is unlikely the cause for SCI-induced polyuria, as many SCI patients purposefully restrict fluids to avoid nighttime catheterizations.

Urinary ANP and serum AVP levels for the various SCI animal time point groups were significantly altered in the direction that would produce polyuria at various 14 to 42 dpi time points, but did not match the onset (7 dpi) and duration of the overproduction of urine that was measured. These findings signify that changes in serum levels of ANP and AVP are variable and not static, which implicates other important factors such as corticosterone (increased urinary levels previously shown at 14 dpi) and/or key receptors/channels (V2R, NPRA, AQP2, and ENaC) in the kidney (altered levels previously shown at 70 dpi time point). [7, 35] Although AVP and ANP are key regulators of water and solute balance and likely play a major role in SCI-induced polyuria, they are not the only factors involved in urine production. The result that the significant alterations do

not match up with chronic polyuria is indicative that other mechanisms and biomarkers are involved.

In the current study, Western blot analysis of fresh kidney tissue at each time point after SCI revealed significant changes at many post-SCI but not sham laminectomy or 3 dpi time points for one or more of the receptors/channels examined. Both V2R and AQP2, which work together, were significantly decreased starting at 7 dpi but reverted to sham levels by 84 dpi. Since polyuria is still present at this time point, it is likely that these levels reflect constant variability within the homeostatic water balance system rather than recovery to baseline levels. Later time points beyond 84 dpi would be necessary to address this possibility.

For NPRA, significant changes were present in several time points relative to 14 dpi, but not sham and 3 dpi groups. The timing of the fluctuations, when considered with the ANP ELISA data, indicates that different compensatory mechanisms may be at play. The fluctuations of NPRA levels and ANP post-SCI could reflect its functional role on two different systems. NPRA levels could be a response to low blood volume that is often seen after SCI and/or due to disruption of urinary regulations. [109-112] Increased NPRA has been shown to be associated with the down-regulation of ANP. [85] SCI often results in hypotension, which may result in an increase in NPRA causing a significant decrease in blood pressure. [111] Low levels of ANP is associated with hypertension, and the inverse is true that an increase in ANP leads to hypotension. [113] Further research will be needed to better understand these

multi-system effects. Note that previously, these receptors/channels were shown to be positively impacted with activity-based recovery training. [35] However, training benefits multiple locomotor and non-locomotor systems, including functions of both the urinary tract and cardiovascular system. [4, 35, 101, 114, 115]

There was no variation in the relative density of the ENaC α subunit after SCI, whereas both ENaC β and γ were significantly decreased at several time points. Differences in the kidney expression levels of the various ENaC subunits post-SCI remains unclear. ENaC α is critical for ENaC pore formation, as it has the ability to form a functional channel on its own, whereas ENaC β and γ are responsible for membrane surface expression (functionality). [116] Channels formed only with the α subunit form less functional channels than with all three subunits together. Further, the β and γ subunits are more molecularly similar, making it clear there is a distinct difference between the β/γ versus α subunit. [117] Note that studies investigating the distinction between the β and γ subunit concluded that the γ is more important than the β subunit for cell membrane surface trafficking and expression, thus overall channel functionality. [118, 119]

Previously we found that both AVP and ANP, in addition to kidney V2R, NPRA, AQP2, and ENaC levels, were altered two weeks post-SCI and were significantly altered at 10 weeks. Interestingly, findings throughout this study include these same fluctuations in both AVP/ANP as well as several of the receptors, but several of the time points in between revert to sham levels. These results suggest that the system may be constantly trying to correct itself (as seen

by “normal” levels of AVP/ANP and receptor densities). However, polyuria is present at every time point starting at 7 dpi which further suggests that water balance homeostasis is never fully achieved after SCI and there are likely multiple systems involved, including others not examined here such as the renin angiotensin aldosterone system (RAAS) and/or corticosterone levels. [120] The RAAS is essential for fluid balance and cardiovascular health (i.e. blood pressure regulation) which are commonly impacted by SCI. [121, 122] The extent of how RASS is affected by SCI is currently unknown, and therefore should be examined further in future studies. [123] Note that the ENaC is regulated by aldosterone, [124, 125] a critical part of RAAS, [120] and both the ENaC β and γ subunits in kidney significantly decreased after SCI.

To further identify possible mechanisms contributing to SCI-induced AVP dysfunction, the density of AVP-positive cells in SON, SCN, and PVN of the hypothalamus was examined. The critical role of AVP in controlling water/salt balance is well-established. AVP is also known to escalate inflammation which further contributes to neuronal disruptions after traumatic brain injury, in addition to its association with SCI-induced polyuria. [7, 35] While SON analysis revealed a significant decrease in AVP-labeled cells at 14 dpi, there were no other time points that demonstrated significant differences compared to surgical sham animals. Previously, a decrease in serum AVP was found as early as two weeks post-contusion, whereas this study found significant decrease in AVP at 28 and 42 dpi, with a recovery at 84 dpi. However, Western blot analysis revealed that kidney V2R levels were decreased as early as 7 dpi. This finding suggests that

there is a significant change in V2R regardless of serum AVP levels, and further reveals the instability of this system after SCI. Under normal physiological conditions, the sympathetic nervous system works together with neuroendocrine and hormonal control in both central and peripheral homeostatic mechanisms. After SCI, this balance is disrupted and causes a cascade of dysfunction that involves hormonal and neuroendocrine changes which subsequently lead to physical symptoms such as orthostatic hypotension and SCI-induced polyuria, to name a couple.

In contrast to the SON, the number of AVP-labeled cells in the SCN was significantly lower in all SCI animal groups starting at 14 dpi. The SCN is closely tied with circadian control, which is very important for AVP release. [126] Recent studies have shown significant disruptions of circadian rhythmicity post-SCI, including activity, body temperature, clock gene expression, and corticosterone production. [127, 128] Clock genes, which are in part responsible for many biological functions such as sleep/wake cycles, the autonomic nervous system, body temperature, and gastrointestinal motility, [129-131] are also functions regulated by the SCN and are all disrupted post-SCI. [132-134] The contribution of SCN to SCI-induced polyuria likely relates to the diurnal variation of AVP production, as clinical studies have identified the loss of this pattern as being responsible for the emergence of nocturia after SCI. [1, 82, 135]

For the PVN, no significant differences in AVP-labeled cells were found post-SCI. Note that the entire PVN was not examined and based upon its location relative to the SON, fewer areas were analyzed, which reduces the

possibility of finding any differences that may exist. The PVN is only one of three regions where AVP is produced [90], and to a lesser extent than the SON, [136] but a full analysis of the entire region should be done in future experiments to determine definitively if SCI impacts this area of the hypothalamus. As the data taken from the PVN analysis is limited, it is pilot in nature and further studies are needed to make any conclusions. However, an increase in animals and PVN cell counts will likely not yield significant results as the SON yielded a significant decrease in AVP-positive cells at only one time point.

Taken together, the results from this study provide further evidence that SCI is a complex and progressive injury, which includes changes in various metabolic peptides (AVP, ANP, V2R, NPRA, ENaC) that contribute to the development and maintenance of SCI-induced polyuria. One study limitation is that the RAAS system was not examined. As mentioned above, RAAS plays a major role in homeostatic fluid balance and should be a target for future studies, not only for SCI-induced polyuria, but for cardiovascular health as well. Future studies that target individual elements alone or in combination including RAAS may elucidate each of their contributions toward SCI-induced polyuria and help identify potential therapeutic targets beyond desmopressin, which has limited efficacy (such as hyponatremia [87] and restricted use on patients ≥65 years of age [137]) and is likely, as the current data suggest, not the only contributing factor related to SCI-induced polyuria.

Table 1

SCI Impactor Parameters and Assessment Outcome Values

Group	*n*	*Injury force (kdyne)*	*Displacement (μm)*	*4-day urine*	*7 dpi BBB*	*Terminal BBB*
Sham	28^	-	-	0	20.1 ± 0.74	19.8 ± 0.83
3 dpi	12	223.4 ± 15.2	1367.4 ± 124.5	-	-	4.3 ± 2.63*
7 dpi	10	244.4 ± 34.5	1330.8 ± 311.7	3.95 ± 0.66	6.00 ± 0.78	6.0 ± 0.78*
14 dpi	11	222.7 ± 3.8	1378.5 ± 134.1	3.31 ± 1.07	7.6 ± 0.20	8.4 ± 1.57
28 dpi	11	224.3 ± 13.7	1427.8 ± 139.1	3.5 ± 1.07	7.4 ± 2.25	9.6 ± 1.57
42 dpi	10	238.5 ± 33.3	1501.7 ± 79.8	1.86 ± 1.16	5.2 ± 2.93	9.4 ± 1.35
84 dpi	12	237.3 ± 35.3	1494.4 ± 162.3	4.25 ± 1.11	6.4 ± 2.43	9.9 ± 1.24

Values indicated are mean ± standard deviation; all SCI groups are significantly different ($p<0.01$) from sham (4-day urine, 7 dpi and terminal BBB).

^ Data collected from 3 sham animal groups were combined as no significant differences were found.

* Both the 3 and 7 dpi injured groups received significantly lower scores than 14, 28, 42, and 84 dpi groups ($p<0.05$).

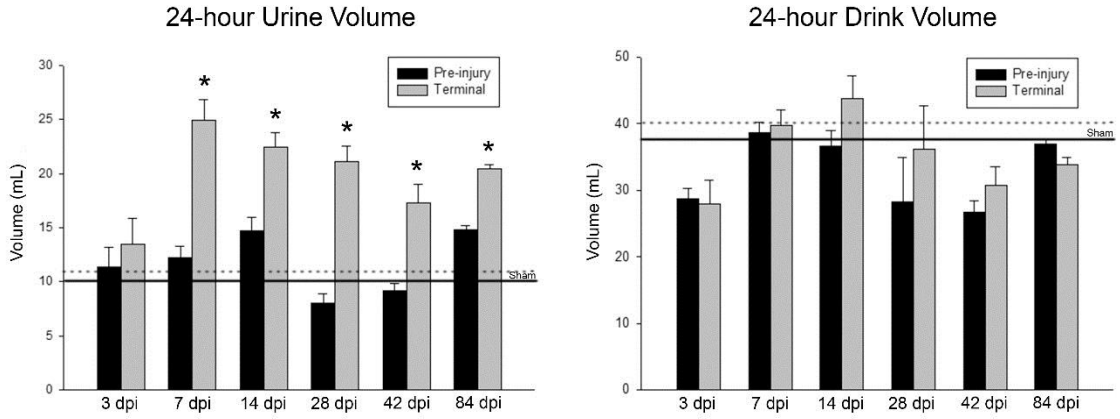

Figure 5:

Metabolic Cage Data Summary. Total 24-hour urine volume output (left plot) indicates a statistically significantly increase at the 7, 14, 28, 42, and 84 dpi time points but not at 3-dpi relative to their pre-injury baseline and shams. Total 24-hour drink volume (right plot) indicates the absence of any statistically significant differences over time relative to pre-injury levels as well as shams. Values are represented as means with standard error of means (SEM) bars included (*$p<0.05$). Data collected from 3 sham animal groups were combined as no significant differences were found (solid horizontal line represents pre-injury time point; dotted line post-injury terminal time point).

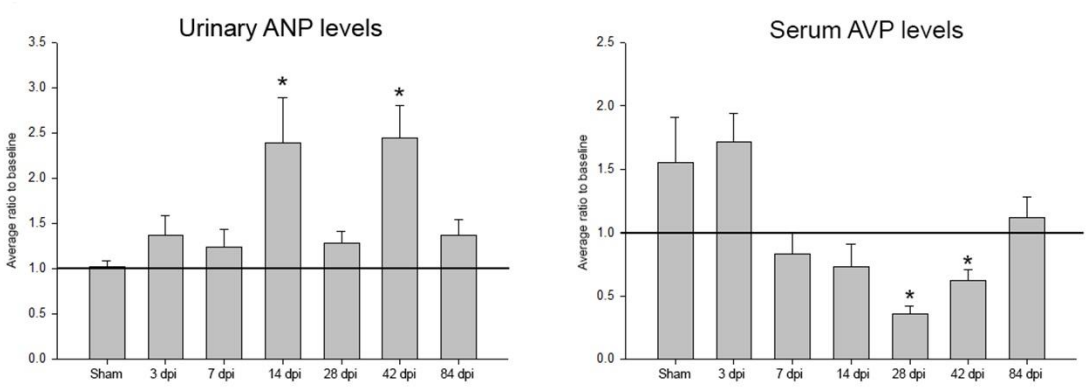

Figure 6:

Urinary ANP and serum AVP. Both urinary ANP and serum AVP levels were normalized to their pre-injury baseline concentrations. At 14 dpi and 42 dpi, the urinary ANP/creatinine levels were significantly increased compared to pre-injury baseline levels. However, sham, 3, 7, or 84 dpi groups did not demonstrate any statistically significant changes in urinary ANP/creatinine levels. Serum AVP concentrations were significantly decreased at 28 dpi and 42 dpi, but not 84 dpi. Error bars represent SEM *significant different from pre-injury baseline, $p<0.05$.

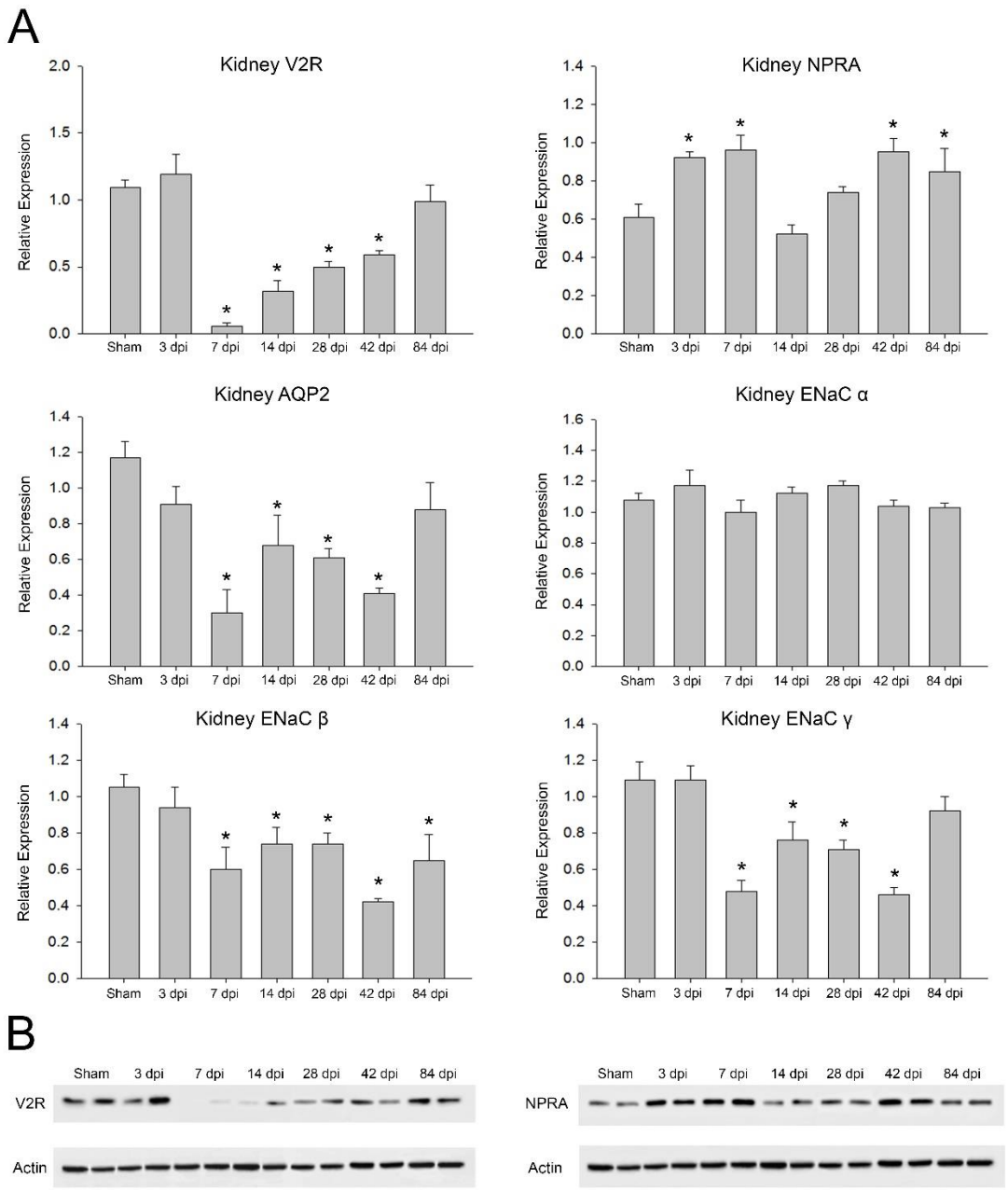

Figure 7:

Kidney tissue Western blot summary of results. As shown graphically in (A), relative expression of vasopressin 2 receptor (V2R), aquaporin 2 channel (AQP2) densities and epithelial sodium channels (ENaC β and γ subunits) from kidney tissue were significantly depressed at various time points compared to surgical

sham animals, as well as 3 dpi (*). In contrast, the relative expression of natriuretic peptide receptor A (NPRA) in the kidney was significantly lower at 14 dpi compared to 3, 7, and 42 dpi (#). Representative examples of Western blots showing expression levels is provided in (B) for V2R and NPRA.

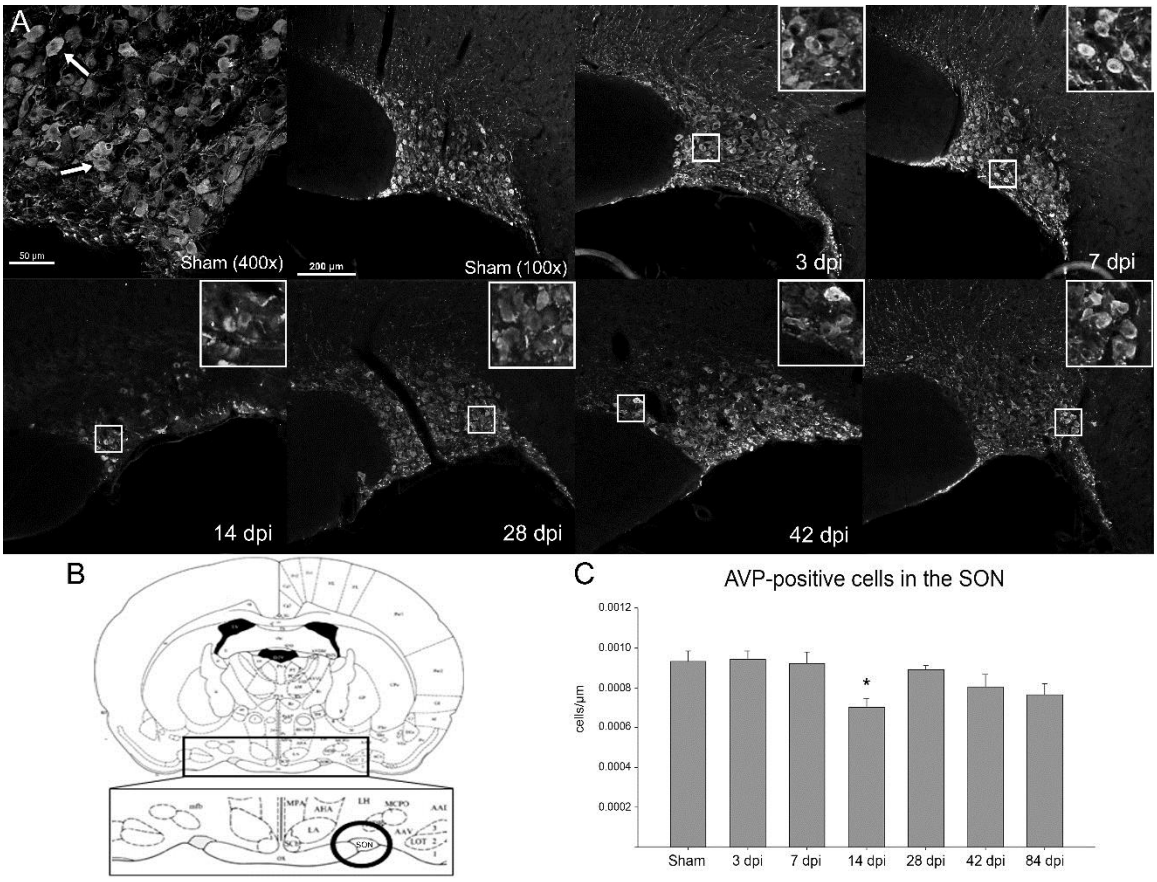

Figure 8:

The supraoptic nucleus (SON). A representative section showing AVP-labeled cells in SON at each time point group is provided in (A), including a higher magnification of the sham group example for visualization of labelled (arrow) and non-labelled (arrowhead) cells. In (B), a modified plate from the Rat Brain Atlas illustrates the location of the SON within the hypothalamus.[29] The average number of AVP-labeled cells per area in SON for each time point group is presented graphically in (C). The only time point after SCI that demonstrated a statistically significant different number of AVP-positive cells in the SON compared to the sham group was at 14 dpi (*$p<0.05$, error bars represent SEM).

SON: sham (n=9), 3 dpi (n=7), 7 dpi (n=7), 14 dpi (n=8), 28 dpi (n=6), 42 dpi (n=7), 84 dpi (n=6).

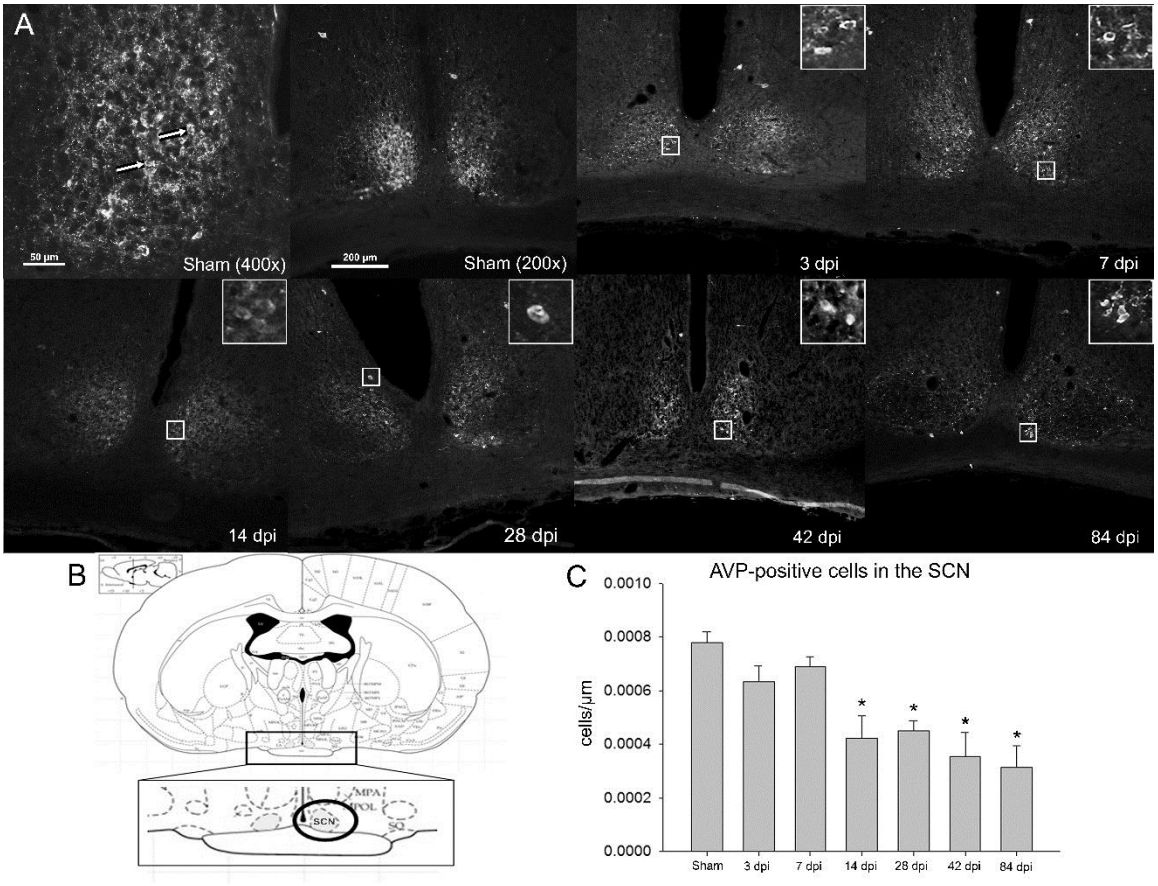

Figure 9:

The suprachiasmatic nucleus (SCN). A representative section showing AVP-labeled cells in SCN at each time point group is provided in (A), including a higher magnification of the sham group example for visualization of labelled (arrow) cells. In (B), a modified plate from the Rat Brain Atlas illustrates the location of the SCN within the hypothalamus.[29] The average number of AVP+ cells/µm in the SCN (shown in (C)) are significantly decreased at the 14, 28, 42, and 84 dpi time points, but not at 3 or 7 dpi (*$p<0.05$, error bars represent SEM). SCN: sham (n=3), 3 dpi (n=4), 7 dpi (n=3), 14 dpi (n=4), 28 dpi (n=3), 42 dpi (n=3), 84 dpi (n=5).

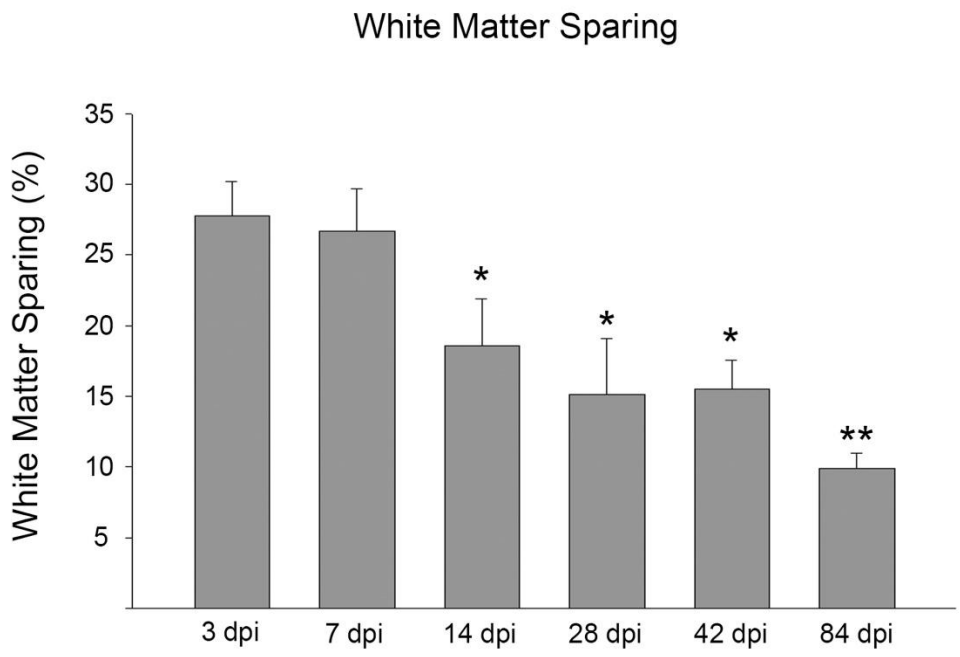

Figure 10:

White Matter Sparing. A significant decrease in white matter sparing (WMS) occurred from the 14 dpi time point onward. At 84 dpi, there was a further significant decrease in WMS relative to 42 dpi (* versus 3 and 7 dpi, $p<0.05$; ** versus 42 dpi, $p<0.05$). Bars represent means and error bars represent SEM.

CHAPTER III

EFFECT OF T3 SPINAL CONTUSION INJURY ON UPPER URINARY TRACT FUNCTION

Introduction

Spinal cord injury (SCI) is a progressive injury that significantly impacts multiple systems affecting quality of life, including motor, respiratory, cardiovascular, gastrointestinal, and bladder/urinary tract functions. [80, 110, 138-140] The presence of SCI-induced polyuria increases the number of catheterizations, especially at night (disrupting sleep), which raises the risk of developing genitourinary infections, a leading cause for hospitalizations in the SCI population. [141] SCI-induced polyuria has shown to be present in both the pre-clinical SCI animal model and clinically, [2, 35, 36, 142] regardless of severity [2] or completeness [143] of injury.

Under normal physiological conditions, systemic arginine vasopressin (AVP) and blood pressure decreases at nighttime, slowing the production of urine and allowing for uninterrupted sleep. Previous clinical findings have reported a significant lack in diurnal variation (fluctuations between day and night) for urine output and serum AVP levels in the SCI population. [144] Production of AVP occurs within the supraoptic nucleus (SON), paraventricular nucleus (PVN), and suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN, which is a main

regulator of circadian control, including the sleep/wake cycles, was recently shown in a T9-level contusion rat model to contain significantly fewer AVP-labelled cells beginning as early as 14 days post-injury (dpi). [145] However, significant up or down regulation at various time points, beginning at 7 days post injury, have been shown for kidney natriuretic peptide receptor-A (NPRA), kidney vasopressin-2 receptor (V2R), kidney aquaporin-2 channels (AQP2) and kidney epithelial sodium channels (ENaC, β and γ but not α subunits), suggesting both central and peripheral mechanisms are involved in the development and maintenance of polyuria. [7, 35, 145]

Autonomic dysregulation occurs after SCI due to disruption of supraspinal sympathetic pathways descending from various brain regions and/or local spinal networks and/or pre-ganglionic sympathetic outputs. The kidneys, which regulate and balance the body's water and metabolite content (filtration from blood as urine), receives sensory innervation from T9-L2 ipsilateral dorsal root ganglia and sympathetic supply from post-ganglionic neurons located within the celiac ganglion, [146] which has been traced to pre-ganglionic neurons at T4-T13 spinal levels. [147] Note that there is limited evidence for specific parasympathetic supply to the kidneys. [148, 149] Sympathetic preganglionic fibers receive input from both intraspinal and supraspinal neurons. [150] The descending supraspinal inputs to sympathetic preganglionic neurons arise from the rostral ventrolateral medulla, rostral ventromedial medulla, caudal raphe nuclei, the A5 region, and the paraventricular nucleus of the hypothalamus. [151, 152] Subsequent to the loss of supraspinal drive post-SCI, spinal autonomic interneurons undergo

plasticity and are key regulators of spinal sympathetic preganglionic circuitry. [153] Note also that the vagal supply of the viscera has been shown to undergo neurochemical plasticity post-SCI, a finding with implications for visceral homeostatic mechanisms and nociceptive signaling following chronic injury. [154]

Our pre-clinical research on SCI-induced polyuria to date has focused on a T9 level injury, [2, 35, 142] which disrupts both supraspinal and local preganglionic/interneuronal sympathetic spinal circuitries to the kidney and surrounding vasculature. The goal of the current investigation was to determine if loss of supraspinal sympathetic control to the kidney and/or disruption of local spinal circuitry surrounding a T9 level SCI mediate the development and maintenance of polyuria by examining outcomes after a T3-level spinal contusion, which is just above the sensory/interneuron/preganglionic supply to/from the kidneys. Of potential relevance are findings showing that cervical (C2-C8) level SCI in individuals generate higher urinary outputs than those having T1-L1 level injuries, specifically at night [36] in addition to being at greater risk for autonomic dysreflexia. [88]

Methods

Animals

All animal experimental procedures and protocols were reviewed and approved by the Institutional Animal Use and Care Committee (IACUC) at the University of Louisville School of Medicine and carried out according to National Institutes of Health (NIH) guidelines. For this study, 18 adult male Wistar rats (~250 g) were individually housed with a 12:12 light-dark cycle. Animals either received a T3 SCI (n=12) or sham surgery (laminectomy with no injury, n=6).

Spinal Cord Injury (SCI)

Following pre-injury baseline assessments (see below), animals were anesthetized with intraperitoneal injection of ketamine (80 mg/kg, Ketoset®; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (10mg/kg, AnaSed; Lloyd Laboratories, Shenandoah, IA). To assure a deep anesthetic plane, both toe pinch and orbital reflexes were monitored. The surgical area was shaved and cleansed with 4% chlorhexidine scrub (Henry Schein) and sterile ocular lubricant (OptixCare, Aventix) applied. A T2 laminectomy was performed to expose the T3 level of spinal cord. Contusions were produced at T3 using an Infinite Horizon (IH) impactor (Precision Systems and Instrumentation LLC; Fairfax Station, VA) per established T9 protocols (215-kilodyne force with no dwell time). [2, 155] The muscular layer and skin were closed with 4-0 surgical suture (Ethicon; Somerville, NJ), and surgical wound clips. Antibiotic (penicillin G, PenJect; Henry Schein Animal Health, Dublin, OH) and analgesic (meloxicam, Eloxiject; Henry

Schein Animal Health) were injected subcutaneously per established post-operative care procedures (0.1 mL, and 0.2 mL/animal, respectively). [35, 50, 94] Physiological saline was also administered at 5mL before contusion and 5mL after contusion. Manual bladder emptying was performed via Crede maneuver three times daily until individual animals reached reflexive bladder function (by 6 dpi [95, 96]). This procedure from our lab is available in video journal format online. [50]

Metabolic Cage Data Collection

For 24-hour metabolic cage data collection, animals were placed in a six station Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH) to monitor 24-hour urine output volume and drink volume according to established protocols. [2, 35, 97] To acclimate animals to the CLAMS unit, pre-injury baseline data was collected twice in one week, but only the second 24-hour period was used for analysis. Metabolic cage assessments were then carried out once weekly. [7, 35, 50] Calculation of 24-hour urine volumes was obtained by taking the sum of each void event ≥0.2g recorded by the sensor within the 24-hour timeframe. The total drink volume was recorded through the CLAMS volumetric drink monitor.

Blood and Urine Sample Collection

The lateral tail vein was used for blood/serum sample collection at pre-injury and end of the study (six weeks post-injury (wpi)) time points. Isoflurane

was used to anesthetize the animals. The base of the tail was shaved for better visualization of lateral tail veins. Animals were then placed on a heating pad where an 18g needle was used to puncture either of the lateral tail veins, and 0.5-0.7 mL of blood was collected into serum separator tubes (BD microcontainer, Becton, Dickinson and Co.). Bleeding was stopped by applying light pressure with 2”x2” gauze, or when necessary styptic powder with benzocaine (Kwik-Stop, ARC Laboratories). Blood samples were then centrifuged at 14,000 rpm for 15 minutes and the serum was collected and stored at -20^{o}C for future analysis. Urine samples were collected from 24-hour metabolic cages. Urine samples were then centrifuged at 14,000 rpm for 15 minutes. The urine was aliquoted into 2mL tubes and stored at -20^{o}C until used for analysis.

Locomotor Assessment

For locomotor assessment, the Basso-Beattie-Bresnahan (BBB) open field locomotor test [99] was performed weekly on each rat as well as the day prior to terminal time point. A single score per animal was obtained by averaging the left and right hindlimb BBB score assigned by two experimenters blinded to time point status. These scores are used as an additional indicator of lesion severity and spontaneous recovery, per our previously published data, where taken together with white matter sparing (WMS) can identify injury outliers. [2]

Tissue Collection and Histology

Animals underwent transcardial perfusions with heparinized saline (1mL heparin/100mL normal saline) followed by 4% paraformaldehyde at the end of the study (6 wpi). Multiple tissues were removed, but only the brain (for hypothalamus) and the spinal cord (lesion site) were used for this study. The brain and spinal cord were then submerged in 4% paraformaldehyde for storage at 4°C for at least 24 hours, then moved to a 30% sucrose solution and stored at 4°C until it was sectioned using a cryostat (Leica CM 1850).

For hypothalamus tissue, serial sections of 25 µm were cut, assuring that each glass slide contains three sections of ≥75 µm apart to avoid double counting of cells to be stained for analysis. Tissue was stained with anti-AVP primary antibody (ab39363, Abcam) to visualize and quantify the number of AVP-labelled cells in the SCN, a region involved in the diurnal variation of AVP. [156] Slides were washed in 1X phosphate buffered saline before and after antigen retrieval (Enzo Life Sciences), then incubated in 0.3% H_2O_2 for peroxide blocking. Blocking was performed using SuperBlock (Thermo Scientific) and primary antibody was diluted at 1:1000 in 4% normal goat serum, applied to slides, and incubated at 4°C overnight. Secondary antibody (fluorescent-conjugated goat anti-rabbit; Alexa Fluor 488, ThermoFisher Scientific) was diluted in 4% normal goat serum and incubated for 1 hour at room temperature. Slides were washed, incubated with 4′,6-diamidino-2-phenylindole (DAPI, Thermo Scientific), then cover slipped before imaging and analysis. The number of AVP-positive cells was quantified using ImageJ (NIH). First, the area of the SCN was outlined, and AVP-labelled cells with an intensity threshold at least 1.5

times above background level were counted to obtain the cells/area for quantification. At least four different sections of nuclei per animal were averaged together for analyses, per our published protocols. [145]

Histology of the spinal lesion site for WMS was carried out as previously described. [35, 97, 101] The spinal cord lesion site, including ~2 levels above and below, was sectioned at 20 µm thickness and stained with Luxol fast blue and cresyl violet. The lesion epicenter and WMS was captured and analyzed using Spot Advanced software (Diagnostic Instruments, Sterline Heights, MI) and Nikon E400 microscope. The percent WMS was calculated by dividing the intact white matter at the lesion epicenter by intact white matter rostral and caudal the injury site. An average of two areas ≥2 mm both rostral and caudal was used for intact white matter.

ELISA

Urinary atrial natriuretic peptide (ANP) was measured using an Enzyme Immunoassay Kit (cat. No. K026-H1; Arbor Assays; Ann Arbor, MI) and creatinine was measured using DetectX Urinary Creatinine Detection Kit (cat. No. K002-H5; Arbor Assays; Ann Arbor, MI) for pre-injury and 6 wpi time points. [35] Urine samples were diluted at 1:5 for ANP and 1:20 for creatinine, and then plated in a 96-well plate in duplicate. ANP and creatinine plates were read at 450nm OD using SoftMax Pro software (Molecular Devices). Urinary creatinine levels were used to control for differing urine concentrations per ANP ELISA kit

instructions. To obtain accurate urinary ANP levels, the ANP levels were divided by the creatinine levels.

Baseline and terminal levels of serum AVP were determined using an arginine vasopressin ELISA kit (cat. No. OKEH02585; Aviva Systems Biology). Stored serum samples (see above) were diluted at 1:5 and ELISA was carried out according to kit instructions.

Statistical analysis

Two-way ANOVA analyses were performed to compare T3 to sham animals for metabolic cage, ELISA, and AVP-labelled cell count data using SigmaStat v3.5 (Systat Software) where significance was determined for $p<0.05$.

For ELISA analysis, the data files for AVP, ANP and creatinine were exported from SoftMax Pro to Microsoft Excel (Redmond, WA). Averages of samples and standards were used to create a standard curve for the determination of protein concentration. Due to combined variability and sample size, terminal protein concentrations were compared to their normalized baseline level for statistical analysis. Signed-ranks tests were used to compare normalized baseline to terminal protein concentrations where $p<0.05$ was considered statistical significance.

Results

Data was obtained from a total of 17 adult male Wistar rats (T3 SCI n=11; surgical sham n=6; one animal died due to complications of injury). Prior to contusion injuries, pre-injury baseline data was collected, which included 24-hour metabolic cages, blood draws, and BBB assessments. The mean force and displacement was 218.8 ± 7.5 (standard deviation) kdyne and 1310.6 ± 175 μm, respectively. The 4-day residual volume (measured on day 4 post-injury using Crede) was 0.35 ± 0.21 mL. The 7 days post-injury (dpi) and 6 wpi BBB scores were 8.80 ± 1.6 and 11.0 ± 0, respectively. The 7 dpi and 6 wpi BBB scores for the surgical sham group was 20.0 ± 0 and 20.3 ± 0.82, respectively. *Post-hoc* analyses for outliers (Grubbs' Test) were done and none were found in either animal group.

24-hour Urine and Drink Volumes

To determine the presence of SCI-induced polyuria, metabolic cages were used to quantify 24-hour urine volumes. The data are presented in Figure 11. Compared to both sham and pre-injury baseline volumes, T3 animals revealed a statistically significant increase in 24-hour urine volumes starting at 1 wpi and was present at every other time point. Drink volumes were not significantly different between the T3 SCI animals and shams at any time point, nor were any time points statistically significantly different from each other.

ANP and AVP

Both urinary ANP and serum AVP were investigated using ELISA, as they are key regulators of cardiovascular and water/solute homeostasis. Serum levels of AVP were significantly lower in T3 SCI animals compared to normalized baseline levels at 6 weeks post injury (0.62 ± 0.08 fold change). Additionally, urinary ANP was significantly elevated (average 1.62 ± 0.07 fold change) at 6 wpi compared to baseline in T3 SCI animals.

AVP labeling in hypothalamus

The number of AVP producing cells in the SCN was quantified and revealed that the average AVP-positive cells/µm was significantly lower in the T3 SCI animals than the T3 shams (Figure 12). Note the integrity of the hypothalamus tissue was not ideal for quantification in several of the animals, yielding n=5 for T3 SCI and n=4 for T3 sham groups. It is important to note that tissue collection from all animals occurred within the same two-hour timeframe, as AVP production in the SCN is time dependent. [157]

White Matter Sparing

The mean percent WMS for the T3 SCI group was 21.2% ± 1.7%. As a further analysis, a Pearson correlation was conducted on WMS and terminal (6 wpi) 24-hour urine volumes. There was no significant correlation between WMS and 24-hour urine volume ($r=0.34$; $p>0.05$).

Discussion

The results of the current T3-level contusion SCI study demonstrate a significant increase in 24-hour urine volumes at 1 through 6 wpi. Since the spinal sympathetic supply to the kidney is mostly intact below a T3 level of injury, the development and maintenance of SCI-induced polyuria is likely due to loss of descending supraspinal circuitries, which likely disrupts fluid and metabolite homeostasis precipitating plasticity within the kidney itself (such as previously seen with fluctuations in the relative expression of receptors and channels[7, 35, 145] as well as central regions including those containing AVP-producing neurons within the hypothalamus. [145] Notably, the daily urine volumes in the T3 SCI animals was equivalent to T9 SCI animals of prior studies. [35, 145] Further, similar to previous findings with a T9 SCI, there were no significant changes in drink volumes at any of the time points. [2, 35] The stability in drink volume from pre-SCI through chronic SCI, taken together with an increase in urine production/passage, is indicative of the extent to which systemic body water/solute balance is severely disrupted after SCI. Further studies investigating sources of this disbalance are in progress.

Also consistent with prior findings in T9 contused rats was a decrease in serum AVP and an increase in urinary ANP after chronic T3 SCI. [35, 142] Both ANP and AVP are crucial for body water/solute homeostasis, and the change of their alteration observed in this and recent studies are in the direction that would result in polyuria. [35, 158] The similar findings with T3 SCI animals in relation to

T9 SCI animals previously reported [35, 145] suggest that these mechanisms are present regardless of level of injury.

An additional focus of this study was the SCN of the hypothalamus, as previous data from our lab suggests there are no consistent changes in numbers of AVP-labeled cells within the SON or PVN after chronic T9 SCI and clinically, diurnal variations of AVP are lost. As seen with T9 lesions, there were significantly fewer AVP-labeled cells in the SCN of the hypothalamus in T3 SCI rats. [145] The SCN is one of three nuclei in the hypothalamus where AVP is made, [159] and is heavily involved in circadian control of both humans and rodents. [160, 161] Recent studies have reported that circadian rhythmicity is significantly disrupted by SCI. [127, 144, 162] However, we have previously shown with a T9 level injury that significant polyuria is present in both the quiescent and active phase of the light-dark cycle in rats, [2] suggesting that persons with SCI may also experience polyuria at both day/night cycles as well. Thus, a prospective study with appropriate controls (age, weight, blood pressure, etc.) measuring both drink and urine volumes on an hourly basis may be beneficial to elucidate this effect. Clinically, investigations have found reduced sodium conservation in SCI individuals with higher levels of injury [163] and a greater imbalance in day/night urine flow rates among those with cervical region versus all other levels of SCI. [36] Further investigations are clearly needed to better understand the role of circadian rhythms in fluid homeostasis.

The mechanisms behind SCI-induced polyuria are not yet fully understood. However, recent studies have shown AVP, atrial natriuretic peptide

(ANP) and their associated receptors vasopressin 2 receptor (V2R) and natriuretic peptide receptor (NPRA), plus water channel aquaporin channel 2 (AQP2) and epithelial sodium channel (ENaC) are significantly altered after SCI in rats. [35, 142] The significant decrease in AVP, specifically at night, has also been shown in the clinical setting as well. [1, 144] Together, the changes in these hormone peptides/receptors are in the direction that would indicate an increase in urine production, and require further investigation for the elucidation of SCI-induced polyuria mechanisms.

Taken together, the findings reported here suggest that polyuria is induced from the loss of supraspinal inputs to preganglionic sympathetic neurons supplying the kidneys. This loss, as suggested by changes in a variety of biomarker of fluid homeostasis including ANP, is further complicated by alterations in cardiovascular dynamics with higher levels of SCI. The spacing between T3 and T9 is not likely wide enough to detect significant differences in 24-hour urine volumes, or ANP/AVP concentrations, as is evident clinically in the population with cervical level injuries. [163] Blood pressure and urine production work in conjunction to keep each other balanced. There are several mechanisms that work to maintain water/solute homeostasis by affecting blood pressure. For example, studies focusing on hypertensive models, both animal and clinical, have demonstrated that denervation of the sympathetic supply to the kidneys results in abolishment of hypertension. [164, 165] Thus, alterations of the neural control of the kidney is likely a contributing factor to orthostatic hypotension, a common occurrence post-SCI. [166] Multiple studies have suggested that a

higher level of injury may result in further impairment of cardiovascular and upper urinary dysfunction. [163, 167] Therefore, a future study investigating an even higher level of SCI (cervical) would likely be beneficial in elucidating the extent that level of injury has upon SCI-induced polyuria.

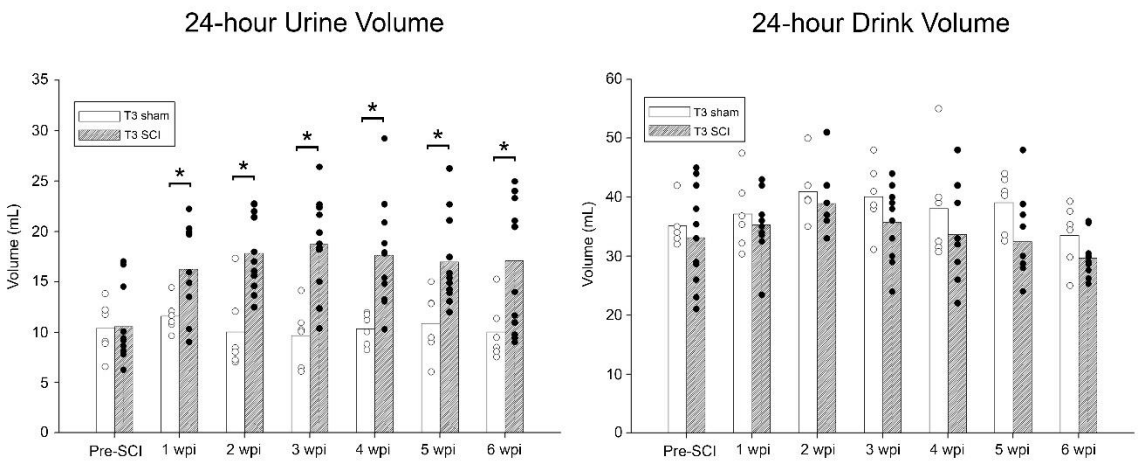

Figure 11:

Metabolic Cage Data Summary. Total 24-hour urine volume output (left graph) demonstrates a statistically significant increase in urine production/passage at 1 wpi, and lasting through 6 wpi compared to pre-injury baseline volume and sham volumes. The total 24-hour drink volume (right graph) indicates an absence of statistically significant changes in water intake across all time points relative to pre-injury and sham groups. (*$p<0.05$)

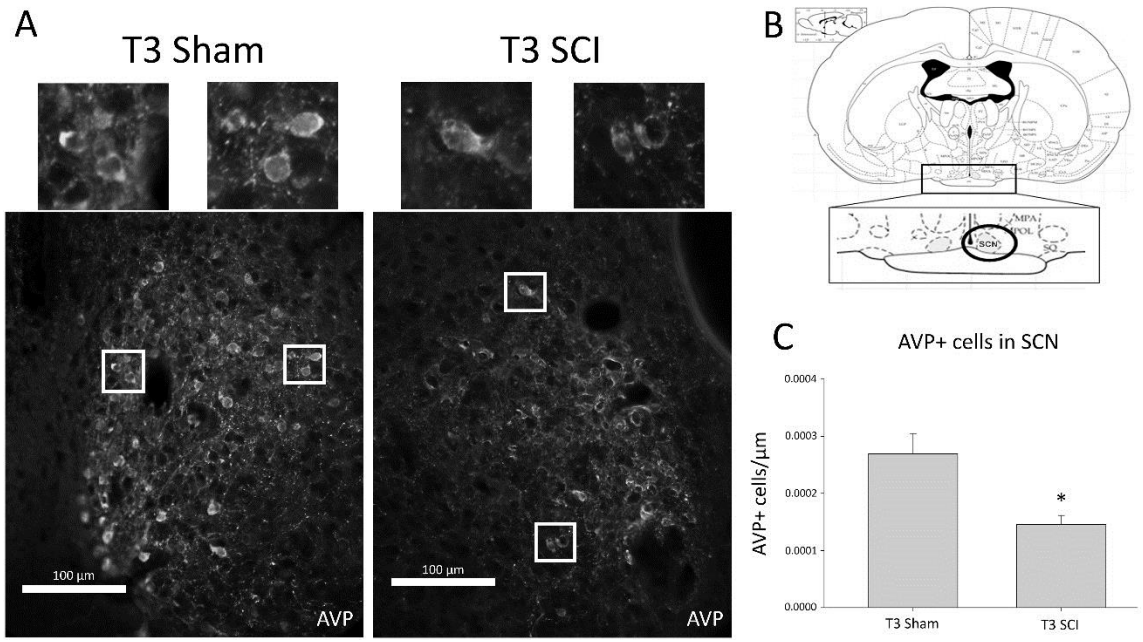

Figure 12:

Suprachiasmatic nucleus (SCN) immunohistochemistry. A representative section showing AVP-labeled cells at 400x in the SCN in both T3 sham and T3 SCI animals (A). In (B), a modified plate from the Rat Brain Atlas illustrates the location of the SCN within the hypothalamus. [168] The average quantified AVP-labeled cells/µm in the SCN was statistically significantly lower in the T3 SCI animals compared to sham controls (C; $p<0.05$; T3 SCI (n=5), T3 sham (n=4)). Values (shown in C) represent means; error bars represent standard error of means.

CHAPTER IV

EFFECT OF ANANTIN ON POLYURIA IN SPINAL CORD INJURED RATS

Introduction

Spinal cord injury (SCI) negatively impacts many systems, including sexual, bladder, bowel, and cardiovascular functions. [9, 80, 140] Polyuria (the overproduction/passage of urine), including nocturia (nighttime polyuria), is highly prevalent amongst the SCI population, especially those with high level injuries. [8, 169, 170] Although SCI-associated nocturia is thought to be precipitated in part by the redistribution of fluid at nighttime from repositioning of the lower limbs, pre-clinical models exhibit SCI-induced polyuria where redistribution of fluids is not a factor, suggesting additional biological mechanisms exist. Recently, several key biomarkers such as arginine vasopressin (AVP) and atrial natriuretic peptide (ANP), along with several of their receptors including vasopressin 2 receptor (V2R) and natriuretic peptide receptor A (NPRA), have shown to be associated with SCI-induced polyuria in rats. [35, 142, 145] Desmopressin, a synthetic analogue of vasopressin, has had limited results in the SCI population [171] and is restrictive to certain age groups as it can cause water retention and hyponatremia, [172] which is common amongst the SCI population. [163, 173] Therefore, other therapeutics targeting different associated mechanisms/receptors should be investigated.

Under normal physiological conditions, NPRA is located in the lungs, adipose tissue, heart/blood vessels, and the kidney and functions to promote vasodilation and diuresis to decrease blood pressure. [174-176] Specifically in the kidney, NPRA works to balance salt and water homeostasis by excreting salt into the collection ducts which controls solute concentration of both urine and body osmolality. Further, the ANP/NPRA system is directly related to the modulation of cardiac hypertrophy seen in cardiovascular failure, [177] in addition to other circulatory disorders seen in the SCI population. [178, 179] Recent research in pre-clinical SCI rats with polyuria has demonstrated that kidney levels of NPRA are significantly increased after SCI in addition to a significant increase in urinary ANP levels, [35] which together likely exacerbate SCI-induced polyuria.

The NPRA antagonist anantin is a peptide isolated from *Streptomyces coerulescens, [*180] and has been shown to acutely decrease urinary production/output in non-SCI settings. [181, 182] Several studies investigating effects of anantin have been either *in vitro* or acute (less than 24-hours). [183, 184] The increase in ANP and NPRA post-SCI [35] likely contributes to both low blood pressure and polyuria seen after SCI. In the current study, anantin was used as a potential treatment alone or in combination with activity-based recovery training (ABRT), as ABRT alone has previously been shown to significantly reduce the overproduction of urine, but not to pre-injury levels. [35] Because blocking NPRA could potentially trigger an increase of blood pressure, [111, 185] cardiovascular function was also examined. In addition, as inhibition of NPRA may also precipitate a sodium/solute imbalance, [186, 187] serum sodium

and potassium levels were measured at pre-SCI, post-injury/pre-ABRT, and end of study (post-ABRT) time points.

Methods

Animals

All animal experimental procedures were carried out in accordance with National Institutes of Health (NIH) guidelines and all protocols were approved by the University of Louisville School of Medicine Institutional Animal Care and Use committee. Adult male Wistar rats were used as the prevalence of SCI is predominantly male. [188, 189] All rats were housed individually in an animal room with a 12-hour light/dark cycle. After baseline data was collected and injuries performed, animals were randomly assigned to either anantin (A) or vehicle (veh) groups. Animals were further divided into forelimb trained (FT) or non-trained (NT) sub-groups prior to the start of ABRT at 3 weeks post-injury (wpi). Thus, four groups at the start of training were NT+A, NT+veh, FT+A, and FT+veh. Experimenters were blinded to which rat was given the drug or vehicle.

Spinal cord injuries

After one week of acclimation to the vivarium and a week of baseline data collection (metabolic cage data, blood draws, blood pressure recordings), all rats (n=78; includes 30 for the dose-response portion) were anesthetized with a mixture of ketamine (80 mg/kg, Ketoset®; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (10mg/kg, AnaSed; Lloyd Laboratories, Shenandoah, IA), administered intraperitoneally (ip), and underwent a moderate contusion injury at the T9 spinal level as previously described [35, 190] Briefly, once animals were fully anesthetized (surgical plane of anesthesia was confirmed using toe pinch

and ocular reflexes), the surgical site was shaved and disinfected using 4% chlorhexidine soap (Henry Schein). Animals were then placed on a heating pad and ocular lubricant (OptixCare, Aventix) was applied. Animals then underwent a T8 laminectomy for a T9 spinal level contusion using an IH impactor. [155] In order to achieve a moderate contusion injury, a 225 kilodyne force with no dwell time was used. The muscular layer was sutured, and skin closed with surgical wound clips. Antibiotic (penicillin G, PenJect; Henry Schein Animal Health, Dublin, OH) and analgesic (meloxicam, Eloxiject; Henry Schein Animal Health) were administered subcutaneously as part of our standard post-operative nursing care. [4, 97, 101, 190] Manual bladder expressions were performed using Credé's maneuver twice daily (early morning and late afternoon) until reflexive bladder function was restored (4-6 days post-injury).

Metabolic Cages

Animals were housed in a six station Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH) to monitor 24-hour urine output volume and drink volume following established protocols. [2, 4, 35] For pre-injury baseline measurements, animals were acclimated to the metabolic cages by housing them for two separate 24-hour time periods, but only the second measurement was recorded for analysis. Following injury, animals were placed in metabolic cages once weekly until the terminal time point (8 wpi). Food and water were available *ad libitum*.

Anantin treatment

The competitive antagonist of NPRA, anantin (United States Biological; Salem, MA), was used to determine if inhibiting the activation of NPRA affects polyuria after SCI. Previous studies using anantin as a method to decrease urine production have been in acute and non-SCI-induced settings, [181, 191] therefore we carried out a brief dose-response study in a small cohort of SCI rats to identify the lowest effective dose. Doses of 0 µg (vehicle only, n=12), 15 µg (n=3), 30 µg (n=3), and 60 µg (n=12) were administered intraperitoneally (i.p.) once daily for two weeks, as SCI-induced polyuria presents by 7 days [145] post-SCI. [2, 35, 142]

Based on the dose-response findings (see Results), aliquots of 60 µg/animal anantin dissolved in ddH2O (as saline may interfere with lyophilized anantin efficacy, per manufacturer) or vehicle only were prepared and stored at -20° C until the day of use. Dosing with either 60 µg or vehicle was initiated on the day of injury and continued throughout the study duration (8 wpi). The decision to give the same dose of anantin/animal was made based upon the absence of any correlation (Pearson test) between baseline weight and baseline 24-hour urine volume (correlation coefficient=0.28; p=0.06). Injections were administered within the same timeframe daily throughout the study (between 14:00-16:00 hours).

Treadmill training

ABRT on a treadmill (Exer-3R treadmill, Columbus Instruments, Columbus, OH) was adopted from well-established protocols. [35, 190] However,

only forelimb training (FT; n=24) and non-trained (NT; n=24) groups were used in this experiment as no significant differences in 24-hour urine volume was found previously between quadrupedal and forelimb only stepping groups. [35] Further, in lieu of daily ABRT treadmill training, five consecutive days a week with two days off on the weekend was used to reflect a more clinically relevant exercise regimen. [52, 192] FT began at the start of week 3 post-SCI to allow ample time for recovery. Animals were acclimated to the harness and treadmill. On day one, the trained animals were put into harnesses and acclimated to the treadmill for 10 minutes. From there, each day the animals continued training with increasing daily increments of 10 minutes until the full 58-minute protocol was achieved. For this study, each animal reached acclimation by day 5. Trained animals continued the 5 days/week protocol for 6 weeks, for a total of 30 sessions.

Blood pressure

Blood pressure was assessed using a non-invasive tail cuff method (Harvard apparatus, LE5001). [191] Heart rate, systolic, diastolic, and mean arterial pressure (MAP) were measured. To assure maximum comfort and accurate recordings, a heating pad at a low setting was placed under the rat tube holder to ensure proper vasodilation per manufacturer instruction. For pre-injury baseline, animals were first acclimated to a quiet testing room (in cages) for 15 minutes. Then, each animal was handled by the examiner for a minimum of 5 minutes prior to being placed in testing tubes (Harvard apparatus, LE5022), which were of a narrow design to limit movements. Animals were left in the tubes

for a 15-minute period, then placed back in cages. For data collection, animals were allotted 15 min for acclimation to the testing room each time, and 15 minutes for acclimation to tubes prior to recordings. The pressure cuff was placed mid-tail to assure that the same general area was consistently used for the weekly blood pressures recordings, which were timed to be taken prior to training sessions. For each weekly session, the goal was to obtain a minimum of three measurements but no more than five per rat for a weekly average reading.

Blood Sampling

Each animal underwent blood collections at three time points - pre-injury (baseline), 2 wpi (post-SCI with anantin or vehicle treatment/pre-ABRT), and 8 wpi (terminal post-ABRT/NT with either anantin or vehicle). Animals were briefly anesthetized with isoflurane (2%) for blood draws. Once ocular reflexes were tested to assure anesthetic plane was reached, the base of the tail was shaved for visualization of the lateral tail veins. Animals were placed on a heating pad, and using an 18g needle the lateral tail vein was punctured and 0.5-0.7 mL of blood was collected into serum separator tubes (BD microcontainer, Becton, Dickinson and Co.). [193] The bleeding was stopped using light pressure with sterile gauze, and if necessary styptic powder with benzocaine (Kwik-Stop, ARC Laboratories). Serum was collected from samples centrifuged at 14,000 rpm for 15 minutes, then stored at -20°C until analyses were performed.

Flame Photometry

Flame photometry was carried out to calculate serum sodium and potassium concentrations, as ANP/NPRA are key regulators of serum sodium concentration. Flame photometry is used to accurately calculate concentrations of solutes in liquid samples (e.g., serum, plasma). [194, 195] Standards were diluted and used for calibration prior to any data recording, per manufacturer instructions. After serum samples were acclimated to room temperature, each sample was diluted 1:150 in accordance with manufacturer recommendations. The flame photometer utilizes a nebulizer to aerosolize the diluted samples and based upon the light intensity of the flame color that has characteristic wavelengths for specific metal ions, a concentration is displayed.

White Matter Sparing

Histological analysis of the lesion site was carried out for calculation of white matter sparing (WMS) as previously reported. [35, 97, 101, 102] Briefly, the spinal injury epicenter, in addition to ~2 spinal levels above and below, was removed from animals' post-perfusion and sectioned at 20 µm thickness prior to staining with Luxol fast blue and cresyl violet. The lesion site and WMS were captured with a Nikon E400 microscope and analyzed using Spot Advanced software (Diagnostic Instruments, Sterline Heights, MI). The percent WMS was calculated by dividing intact white matter remaining at the lesion epicenter by the average intact white matter at areas rostral and caudal the injury site where white matter was more intact. The average of two areas ≥2 mm both rostral and caudal was used for intact white matter.

Statistical Analyses

One-way repeated measures ANOVA analyses were performed to compare the four treatment groups (NT+A, NT+veh, FT+A, FT+veh) for 24-hour urine volume, MAP, and serum sodium/potassium concentrations across specified time points. One-way ANOVA analyses were performed to compare BBB and WMS between groups. To reduce variability inherent with single time point measures of 24-hour void volume and blood pressure, pairs of consecutive recordings were averaged together to yield 5 different data bins: Pre-SCI baseline, 1-2 wpi (post-SCI, pre-training), 3-4 wpi, 5-6 wpi, and 7-8 wpi, where training occurred from 3-8 wpi. Pearson correlations studies were carried out to distinguish if any significant correlations between WMS, BBB, or weight and 24-hour urine volumes were present, in addition to serum sodium and MAP. All statistical analyses were carried out using SigmaStat v3.5 (Systat Software) where significance was determined for $p<0.05$ for all statistical tests utilized. All data was checked for outliers using the online GraphPad calculator (Grubb's test).

Results

Anantin Dose-Response Pilot

To determine the minimum effective dose of anantin on 24-hour urine volume, an initial pilot experiment was done comparing SCI rats treated with anantin, at doses of 15 µg (n=3), 30 µg (n=3), and 60 µg (n=12), with vehicle-only treated injured animals (n=12). As illustrated in Figure 13, after both one and two weeks of daily i.p. anantin or vehicle injections, all SCI groups demonstrated a significant increase in 24-hour urine volume compared to pre-SCI baseline levels. The 60 µg anantin treated animals, however, produced a significantly lower average urine volume relative to vehicle treated animals. Note that the 24-hour drink volumes were not significantly different between any of the groups or at any time point (data not shown).

Experimental Groups

Complete data sets were obtained for 44 out of 48 rats. Details about each group including injury parameters are summarized in Table 2. No significant differences were found between groups with respect to baseline body weight, impactor force and displacement, and two-week pre-ABRT BBB, indicating successful randomization of the rats. Although each group initially had an N of 12, several rats from different groups were eliminated from the study due to either complications of the injury itself or severity was too mild and yielded significant outliers for BBB (score of 19 or higher) and/or WMS (89% or higher).

Metabolic cage

The Day 4 residual urine volumes (Table 2) did not differ between anantin and vehicle treated groups. Each of the groups demonstrated significant increases in 24-hour urine volumes at 1 wpi, and each of the weeks following through 8 wpi (Figure 14). There was a significant decrease in the average change of void volume in the FT+A and FT+veh animal groups compared to NT+veh at 5-6 wpi and both the NT+A and NT+veh animal groups at 7-8 wpi. Of note, the drink volumes for each of the groups did not differ from each other, or at any of the time points (mean of 34.3 ml at baseline; data not shown).

Blood pressure

At-rest heart rate (measured in beats per minute (BPM)), systolic, diastolic, and mean arterial pressure (MAP) were all recorded from each animal once weekly using a non-invasive tail cuff apparatus. There was a significant decrease in blood pressure but not heart rate post-SCI, which lasted chronically (MAP data provided in Figure 15). Note that pre-training blood pressure was significantly lower for vehicle versus anantin treated rats (examined as two larger groups since the time point is pre-training). However, this effect was not observed at any of the remaining time points, regardless of training group (no significant differences in heart rate as well across any of the time points or between any of the animal groups).

Serum sodium and potassium

There was a significant decrease in relative serum sodium levels in all four animal groups at 8 wpi compared to baseline levels (Figure 16A). However, there was no significant change at 2 wpi in serum sodium compared to baseline. Further, neither anantin nor FT had any effect on serum sodium levels, as there were no significant differences between any of the groups at either 2 or 8 wpi. In contrast with sodium, serum potassium levels were significantly increased at 2 wpi in all four animal groups relative to baseline levels (Figure 16B). However, at 8 wpi, there was no significant difference in any of the groups compared to baseline. As with serum sodium levels, neither anantin nor FT had any effect on serum potassium levels.

White matter sparing

The average percent WMS was 13.8% ± 1.3%, 21.9% ± 2.12%, 17.5% ± 4.3%, and 23.5% ± 3.7% for NT+A, NT+veh, FT+A, and FT+veh, respectively. There were no significant differences in WMS between any of the groups. Further, Pearson correlations revealed that there were no significant correlations between WMS and 24-hour void volume change ($r=0.20$, $p=0.21$), 24-hour drink volume ($r=-0.18$, $p=0.24$), terminal body weight ($r=0.11$, $p=0.47$), 8 wpi MAP ($r=0.16$, $p=0.31$), sodium levels ($r=-0.10$, $p=0.52$) serum potassium ($r=0.21$, $p=0.19$).

Discussion

Current interventions for SCI-induced polyuria include fluid restriction (most common) and desmopressin (DDAVP), which has restricted use and limitations. [171, 172] An expansion of the small number of available treatment options needs consideration, as the frequent daily practice of restricting fluids can lead to a myriad of other problems, such as dehydration and autonomic dysreflexia by way of bowel impaction. [196] Although the complete mechanism behind SCI-induced polyuria is still unknown, several related biomarkers have previously been identified, [35, 142, 145] including NPRA, which was targeted with the receptor antagonist anantin in the current study both alone and in combination with ABRT.

Although our initial pilot study revealed animals treated with 60 µg of anantin given i.p. voided lower volumes than those treated with vehicle during the first two weeks after SCI, both groups had significant SCI-induced polyuria. The rationale of incorporating FT into this study was to determine the efficacy of a combinatorial therapy approach with treatment of anantin in conjunction with ABRT, which has shown to be effective in decreasing 24-hour void volumes, but not back to pre-injury baseline levels [35]. Although there was a significant effect of FT on void volume, no effect was found for anantin treatment in conjunction with FT. However, initially post-SCI, a small but statistically significant effect was found for anantin treatment on blood pressure.

There are several potential explanations as to why anantin did not have an effect on 24-hour void volumes. Firstly, the bioavailability (including half-life and time to reach kidney NPRA) of i.p. anantin is unknown, and therefore the dose given may not have had a lasting effect on urine production. Also, the dosage of 60µg/animal may have been too low, as this dosing was derived based upon published studies that utilized either intravenous or direct application of the drug. One study that used an anantin dosage of 100µg/kg body weight [197] did not report route of administration or void volumes. Additionally, NPRA may not be a significant driver for SCI-induced polyuria, as kidney NPRA levels have recently been shown to fluctuate after SCI, [145] suggesting that other factors are likely to be more prominent for maintaining the overproduction of urine volume.

Another key finding of this study was the significant chronic decrease in MAP. The presence of hypotension in awake rats at rest is clinically relevant, as hypotension is common in the SCI population. [9, 167] Several studies investigating MAP in rats after SCI that utilize radio telemetric pressure transducer devices with pressure probes placed directly in the abdominal aorta have yielded different results, which may be explained by outcome differences between central MAP versus peripheral MAP recordings. [198, 199] It is important to note that clinically, it is thought that central MAP is more accurate as it is more closely related to organ supply and health. However, SCI is more nuanced than typical cardiovascular issues, as demonstrated by the fact that persons with SCI (and likely SCI animals as well) [200] rely on the renin-angiotensin-aldosterone system (RAAS) more than able-bodied individuals. [200-

204] Moving forward, using a non-invasive tail cuff procedure to record at-rest blood pressure would be beneficial in monitoring cardiovascular health (both blood pressure and heart rate), similar to how BBB scores are commonly utilized in rodent SCI research.

Additional study results indicate that there was a chronic decrease in serum sodium, and significant acute increase in serum potassium. Previously, we have reported that the serum osmolality was increased acutely (2 wpi), [142] which suggests that there was an excess of serum levels of solutes, such as sodium or other electrolytes. Although no significant effect of anantin treatment on serum sodium levels was found in the current study, there was a significant decrease in serum sodium relative to pre-injury baseline chronically. A decrease in serum sodium is also found clinically, where hyponatremia is associated with hypotension after SCI. [205] Further, hyponatremia has been associated with disrupted descending renal sympathetic circuits, as seen in SCI-induced hypotension/cardiovascular dysfunction. [205] Thus, our data is likely indicative of the severe dysfunction in mechanisms regulating water/solute homeostasis, such as AVP/V2R, epithelial sodium channel (ENaC), [145] or potential disruptions in the RAAS after SCI. Although further research is necessary to decipher the extent that RASS is disrupted after SCI, it has already been demonstrated that the AVP/V2R and ENaC system is significantly disrupted after SCI. [35]

The flame photometry findings also indicate the presence of a significant increase in potassium at 2 wpi, but not at 8 wpi. The most common causes for

hyperkalemia are kidney disease, urinary tract/kidney infections, drug administration, cardiovascular ischemia, and significant muscle atrophy. [206] However, both anantin and vehicle animals demonstrated significant increases in potassium, therefore it is unlikely the increase in serum potassium was due to anantin administration. The only reported cases of hyperkalemia in the SCI population has been associated with succinylcholine administration, a paralytic drug commonly given for anesthesia, which our rats did not receive. [207] Thus, possible causes for the significant spike in serum potassium is muscle atrophy and/or kidney dysfunction. Muscle atrophy is not as likely due to the timing of the increase (during the acute/sub-acute stage post-SCI). Kidney dysfunction may occur via several different mechanisms. The increased potassium at 2 wpi may result from spinal shock which could relate to the presence of hematuria in rats shortly after SCI. [95] Although hematuria resolves within a week, there may be lasting damage to the kidney. Additionally, spinal shock after injury significantly disrupts bladder function, resulting in an initial state of detrusor areflexia and the need to empty using the Crede method, which could cause urine to flow retrogradely through the ureters and damage kidney tissue. Further investigation on the timeline of changes in serum (and urinary) potassium and sodium may be important in not only determining SCI-induced polyuria mechanisms, but also cardiovascular health, as solute/water balance is heavily regulated by the kidneys.

There is evidence that both hyponatremia and orthostatic hypotension is prevalent in the SCI population, which is supported by the findings presented

here. [9, 208] Although there was not a significant correlation between MAP and serum sodium levels in this study, using these methods for future studies could be useful in elucidating treatments for alleviating these potentially harmful conditions. Hyponatremia can cause attention deficit, gait instability, and osteoporosis. [209] Further, the results of this study indicate that ABRT does not alleviate hyponatremia, suggesting that other treatment options should be explored in this regard.

Together, the current results suggest that NPRA alone or in combination with ABRT is not an ideal candidate as a target for therapy to alleviate SCI-induced polyuria, as other complex mechanisms involving multiple systems are likely involved, which requires further investigation.

Table 2

SCI Impactor Parameters and Assessment Outcome Values

Group	n	Injury force (kdyne)	Displacement (μm)	Baseline weight	4-day residual vol	14 dpi BBB	Terminal BBB
NT+A	12	219 ± 3.80	1331 ± 229	337 ± 22	1.85 ± 0.59	9.8 ± 1.2	11.8 ± 3.8
NT+veh	11	234 ± 24.7	1417 ± 170	331 ± 32	2.13 ± 0.59	11 ± 0.97	11.7 ± 3.9
FT+A	12	224 ± 14.8	1387 ± 163	346 ± 23	2.7 ± 0.67	10.8 ± 0.62	10.5 ± 1.8
NT+veh	9	227.6 ± 16.9	1375 ± 210	342 ± 18	1.4 ± 0.44	11.8 ± 1.11	12.8 ± 3.6

No significant differences; $p>0.05$. Values are means ± standard deviation

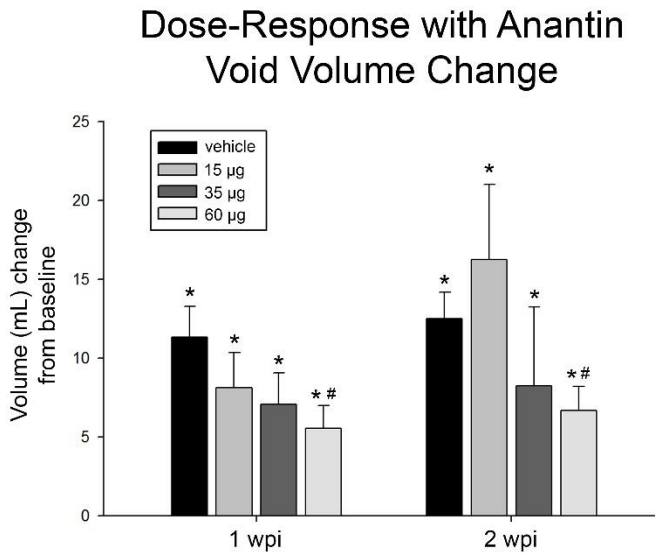

Figure 13:

Metabolic cage data from anantin pilot study. At 2 wpi, the change in 24-hour void volumes (average 10.47 mL) from baseline were significantly increased compared to baseline levels for both anantin and vehicle (veh) treated animals (*$p<0.05$). The testing of responses to different dosages of anantin revealed significantly lower void volumes with 60 µg than the veh treated animals at 2 wpi (#$p<0.05$). Values represent means of volume change with error bars representing standard error.

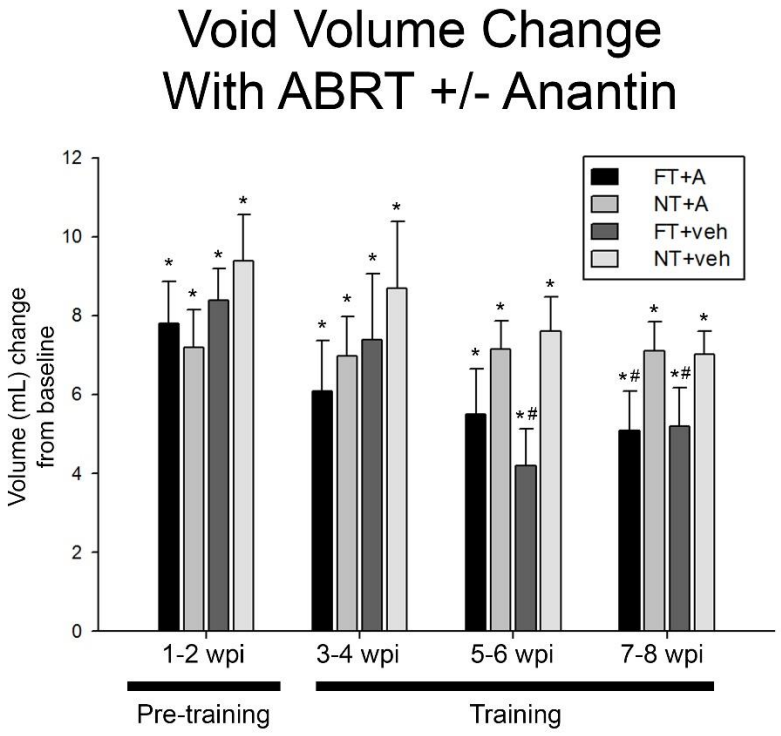

Figure 14:

Metabolic Cage Data Summary. The average change in 24-hour void volume output demonstrates a statistically significant increase in urine production/passage at 1 wpi-8 wpi compared to pre-injury baseline (average 8.01 mL) levels for all animal groups and at every time point post-SCI (*$p<0.05$). Comparisons between early, mid, and later training time points reveal a significantly lower void volume change for the FT+veh animal group mid-training and both FT animal groups by the end of training relative to the NT animal groups (#$p<0.05$). Values represent means of change in void volume from baseline with standard error.

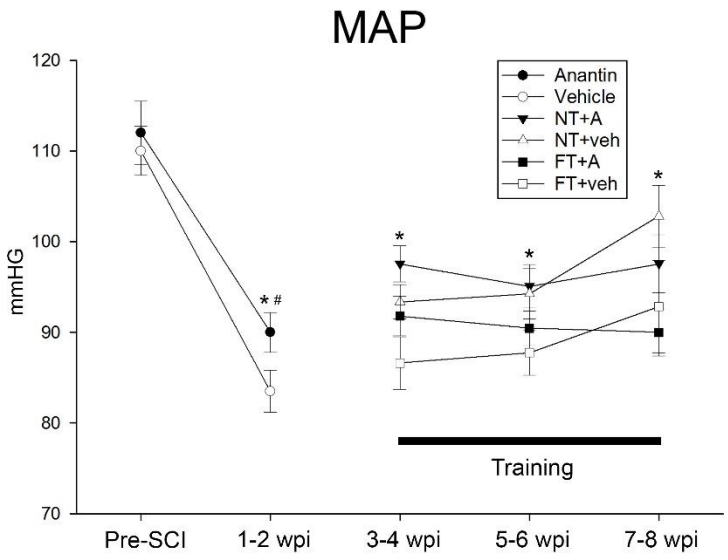

Figure 15:

Change in MAP after SCI. There is a significant decrease in MAP post-SCI in both anantin (A) and vehicle (veh) treated animals (*$p<0.05$; relative to Pre-SCI). Note that because training did not commence until 3 wpi, animals were grouped together based on anantin/veh treatment, then separated by group once ABRT began. The veh treated animal group demonstrated a significantly lower MAP than the anantin treated animal group post-injury, prior to ABRT (1-2 wpi time point; #$p<0.05$). Values represent means of MAP with standard error.

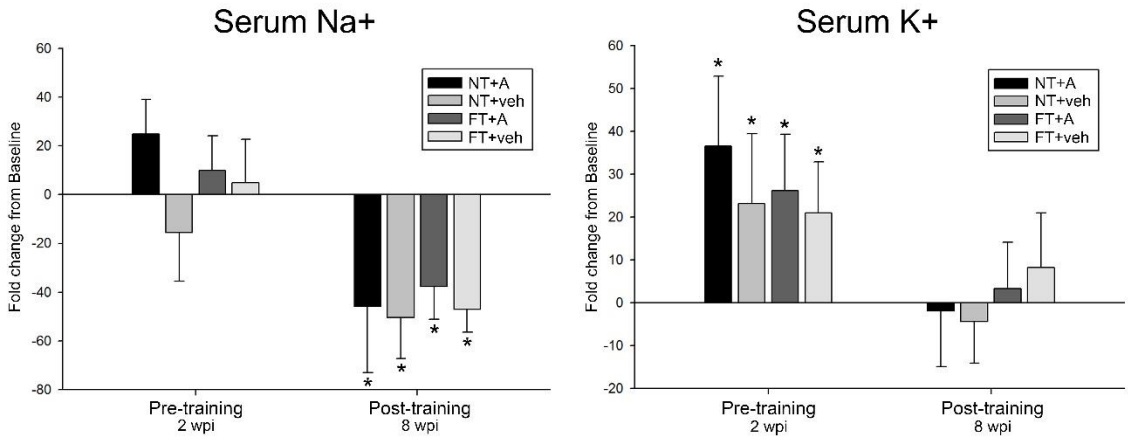

Figure 16:

Changes in serum sodium and potassium. The average change in relative serum sodium levels were statistically significantly lower at 8 wpi (post-training) compared to pre-injury baseline and 2 wpi (pre-training; left graph; *$p<0.05$). The average change in serum potassium levels were significantly increased at 2 wpi (pre-training) compared to pre-SCI baseline levels in both anantin (A) and vehicle (veh) groups, but there was no significant difference in potassium levels at 8 wpi compared to pre-SCI baseline levels for either group (right graph; *$p<0.05$), regardless of training (forelimb trained [FT] versus non-trained [NT]). Values represent means of groups with standard error.

CHAPTER V

GENERAL DISCUSSION AND FUTURE DIRECTIONS

The goal of this project was to further investigate the mechanisms behind SCI-induced polyuria, as the only information known clinically was that 1) there is a significant lack of diurnal variation of AVP observed in the SCI population and that 2) the use of compression socks throughout the day improved nocturia in one study. However, it is unknown what causes the disruption of AVP or what time point after injury it presents. Further, animals do not undergo the same amount of fluid redistribution that humans do, but still present with SCI-induced polyuria, suggesting that while compression stockings may partially reduce nocturia, it may not be improving physiological mechanisms associated with SCI-induced polyuria, such as AVP production. The results from this pre-clinical study identify multiple key biomarkers, including ANP, that could be investigated in human SCI as well. Further, the effect SCI has on hypothalamic nuclei requires further investigation. This study has demonstrated that SCI-induced polyuria likely involves several systems: hypothalamic/circadian, renal, sympathetic, and cardiovascular systems.

Previously, an increase in ANP and decrease in AVP were found at 2 wpi, and 10 wpi, [7, 35] while fluctuations in NPRA, V2R, AQP2, and ENaC were

found to be altered at 10 wpi. [35] A timeline in the current study showed changes in key biomarkers as early as 7 dpi, but not during the initial acute phase (3 dpi). Also, at 7 dpi through 42 dpi, V2R is significantly decreased. However, NPRA does not follow the same trend and is only significantly increased at 14 dpi and 42 dpi, suggesting that the changes in these too are not being controlled by each other, as ANP/NPRA activation inhibits AVP release. Further, the fluctuations in AQP2, ENaC, AVP, and ANP at the sub-acute time point support the hypothesis that the overproduction of urine is secondary to the injury itself, but continue chronically, further evidence of how SCI is a progressive and ongoing injury. Additionally, variability of biomarkers seen in the current and previous studies [7] suggest that the system is likely attempting to correct itself to achieve homeostasis. However, since SCI affects multiple system, a balanced system is not achieved and requires therapeutic/rehabilitative interventions, such as ABRT. [35]

Circadian disruption of AVP, specifically low levels of circulating AVP, has been well established in the SCI population. [1, 8, 39] Although the findings from this study that AVP is significantly decreased after SCI (both T3 and T9 contusion) is not a novel one, its importance should not be undervalued. Interestingly, a novel finding in both T9 and T3 animals was the significant decrease in AVP-labeled cells in the SCN, suggesting that there are further significant changes within the hypothalamus after SCI, which has been supported by several studies investigating circadian genes post-SCI. Further, these changes line up with the significant decrease in serum AVP, confirming that

hypothalamic changes are at least in part responsible for the disrupted diurnal variation seen in AVP production and urine production specifically at night. Although the synthetic analogue of AVP, desmopressin (DDAVP) exists and is used clinically, chronic use may not be beneficial, but instead harmful. Although the use of DDAVP is successfully used primarily in adolescent children who display chronic bed-wetting with success, SCI-induced polyuria has a more complex etiology. Utilization of DDAVP in the SCI population is different from its use in other settings, as SCI results in far more complications, such as cardiovascular and hormonal dysfunction. Further, the risk of hyponatremia is far greater in the SCI population, and therefore DDAVP should be used with extreme caution. Therefore, DDAVP is not an ideal pharmacological intervention to treat SCI-induced polyuria.

The significant findings from the T3 study validate that SCI-induced polyuria is still present when the local spinal circuitry of sympathetic fibers is left intact, and thus only descending supraspinal renal sympathetic pathways are disrupted. Further, the results indicate that similar mechanisms, such as ANP/AVP dysregulation are likely driving SCI-polyuria, regardless of injury level. It may be interesting for future studies to investigate a timeline of alterations in biomarkers and associated functional deficits similar to that of Chapter II and IV. For example, it is anticipated that blood pressure and related biomarkers of T3 level SCI animals would also be significantly impacted, lasting chronically. [210] Clinically, a higher incidence of SCI-induced polyuria, in addition to cardiovascular disorders, are reported for individuals having cervical levels of

SCI. The link to cardiovascular health and renal health are very closely related, suggesting that the drop in MAP seen in both pre-clinical animal and clinical settings is also associated in SCI-induced polyuria. The results from Chapter IV (anantin study) revealed that MAP is significantly and chronically reduced. However, the results from Chapter II revealed that there are fluctuations in most of these biomarkers as previously mention, suggesting that while cardiovascular health may be associated with SCI-induced polyuria, additional mechanisms are present as well. While the results presented here suggest a T3 injury does not increase the risk of developing SCI-induced polyuria, a study involving a cervical injury, as well as a lower T13 injury, and investigating their effects on aforementioned biomarkers would be beneficial to further identify the importance level of injury has on SCI-induced polyuria. Taking what has been reported clinically, it would be expected that a lower T13 injury, well below any sympathetic pathways to the kidney, compared to a C4 injury, which would not only effect descending sympathetic fibers to the kidney but also the sympathetic circuitry for the heart, in addition to respiratory impairment, would likely result in significant differences in both cardiovascular and upper urinary tract health. [36, 211, 212]

Of further relevance for inter-system interactions is that the significant decrease in MAP matches the decrease in AVP (found in Chapter II), which is a potent vasoconstrictor. However, NPRA levels within the kidney are not always upregulated, suggesting it may not be an ideal target for pharmacological therapy. This outcome also likely explains why there was no effect found with

anantin treatment compared to vehicle only. Although the bioavailability along with the dosing of anantin is a potential reason as to why an effect was not observed, taken with the results from Chapter II, it is more likely that targeting of NPRA alone is not sufficient. Additionally, it may also be that the significant increase of ANP is too great for anantin to make an impact on 24-hour void volume. However, as previously reported, ABRT has the ability to reverse the change of kidney receptors (NPRA, V2R, AQP2, and ENaC) seen after SCI, to sham levels. [35] Although kidney receptors were not investigated in the anantin study, it would be hypothesized that the ABRT animals demonstrated this same effect, which is why we saw a significant decrease in 24-hour void volume change compared to NT animals, but not back to pre-SCI baseline levels. Based upon these findings, it is likely that a combined treatment modality, such as ABRT in addition to pharmacological interventions is necessary to fully alleviate SCI-induced polyuria.

Although anantin treatment compared to vehicle treatment did not result in any significant findings, the use of flame photometry proved useful for future studies. As previously mentioned, there was a significant increase in serum potassium at 2 wpi, and a significant decrease in serum sodium at 8 wpi in all animal groups. Under normal physiological conditions, any excess of circulating potassium in the blood is excreted by the kidneys through urine production. Several factors can contribute to an increase in serum potassium, including muscle/tissue degradation, certain disorders (Addison’s disease), certain drugs (angiotensin-converting enzyme inhibitors), and acute/chronic kidney

failure/disease. [213] Although the major basis for the significant increase in serum potassium compared to pre-injury levels is likely the injury itself and subsequent muscle loss, this increase may contribute to kidney damage. The extent of kidney damage resulting from acute/sub-acute SCI is still unknown and requires further investigation. A future study that investigates the timing of when an increase in serum potassium begins post-SCI utilizing a temporary intravenous cannula for acute SCI, and daily blood draws for sub-acute samplings would be useful. Further, because the volume necessary for flame photometry is low, serum sodium should be investigated as well, as it has been reported that acute SCI causes a significant decrease in serum sodium. [173, 205] Although there was no significant decrease in serum sodium at 2 wpi in this study, there was a chronic decrease, which supports existing literature that hyponatremia is a major issue in the chronic SCI setting. [214, 215] Thus, it is likely that the increases in NPRA and ANP due to SCI are driving chronic hyponatremia, which is a novel finding. However, a future study investigating fluctuations in ANP, NPRA, and serum sodium are necessary to confirm this hypothesis.

Taken together, the results of this dissertation have further clarified several potential mechanisms and the timing of which they occur regarding to SCI-induced polyuria. Further, the data advance our current knowledge of SCI-induced polyuria that can be used in future pre-clinical animal models and in the clinical SCI setting as well. Although it is well-established that AVP levels are significantly decreased in the human SCI population, there have been no

published studies in the clinical setting on ANP levels (either urinary or serum-derived), which is a key biomarker for both cardiovascular and urological health. Further, ANP is antagonistic against the RAAS and sympathetic control. [216] It has been reported that the SCI population rely heavily on the RAAS for cardiovascular homeostasis, specifically regarding to postural changes. [67, 217, 218] The SCI research community would benefit from understanding if ANP is a significant driver for SCI-induced polyuria, or rather a symptom of a greater systemic disruption. Under normal physiological conditions, ANP is released only when cardiomyocytes within the atria of the heart are stretched in response to an increase in blood volume. [219] It is counter intuitive that ANP levels are increased after SCI, as low blood pressure is a major occurrence and concern after SCI. The underlying cause of the increase in ANP/NPRA seen in this and previous studies may be a further indication of a homeostatic system attempting to correct itself. Interestingly, this study also revealed that SCI rats also demonstrate hyponatremia at the chronic time point. In the future, both ANP/NPRA should be investigated in combination with serum/urinary sodium levels. The results from this study would suggest that the lack of supraspinal input to the cardiovascular system below the level of injury, including but not limited to the kidneys, causes a significant drop in MAP. However, all the studies reported here utilized an injury that would not directly impact the circuitry of the heart, suggesting that the heart is able to respond normally to blood pressure increases caused by mobility in rodents post-SCI. The acute increase in blood pressure releases ANP, thus causing the excretion of sodium and water. Further,

as orthostatic hypotension is well-established in the human SCI population, as it is established in this study as well, chronic low blood pressure results in an upregulation of NPRA in the kidney due to desensitization. This chronic disbalance is likely why fluctuations in NPRA and ANP were observed post-SCI. These data could explain why the use of anantin to block NPRA was not effective enough to significantly decrease void volumes. Therefore, further studies are needed to identify further biomarkers to target for pharmacological interventions.

Another important finding from the anantin study was the significant decrease in void volume change with ABRT. Similar to previous reports, there was a significant decrease in 24-hour void volumes after the administration of ABRT. The mechanisms behind how ABRT improves SCI-induced polyuria is still unclear, but it is likely that it helps the system regain homeostasis by regulating specific biomarkers as a previously published study has pointed out. [35] However, further mechanisms are likely involved as well and require further investigation to elucidate how exercise, and to what extent or types of exercise is needed to reduce SCI-induced polyuria. It is important to note that clinically, ABRT is administered for 70 sessions, rather than a set number of days per week. Because this study utilized a training routine of 30 sessions with significant results, it may be beneficial to investigate the effects of different training plans, such as two days/week for 70 sessions compared to once/day for 70 sessions. How long the training effect lasts if a stoppage occurs (re patient compliance to a particular exercise regimen) is another issue that needs to be addressed. Although the results of this study revealed that 30 sessions of ABRT (5

days/week) was not sufficient to improve MAP, it would be beneficial to continue monitoring at-rest peripheral blood pressure in conjunction with different amounts of ABRT as a primary outcome measure, as cardiovascular health is closely related to urologic health.

Another method that could be utilized in future studies to investigate important mechanisms and therapeutic targets for SCI-induced polyuria is the use of knockout (KO) mouse models. Although NPRA would appear to not be an ideal target for therapy based off the results from this study, there were limitations that were possibly confounding the results (such as the bioavailability of anantin and dosing). Studies report that an NPRA KO model produces a lower urine flow in response to ANP injections compared to wild type controls. [187] Because we have found that ANP is increased after SCI, it is expected that an NPRA KO would decrease the effect of SCI-induced polyuria. However, because there are other mechanisms and biomarkers at play, the 24-hour void volumes may not return to pre-injury baseline levels. Therefore, the use of other KO mouse models, such as V2R, or ENaC KO would further confirm their role in SCI-induced polyuria.

Further investigation outside of SCI may also illuminate potential mechanisms on SCI-induced polyuria. For instance, the incidence of nocturia in patients with multiple sclerosis (MS) is considered one of the most bothersome lower urinary tract symptom affecting quality of life, similar to the SCI population. [73, 74] Currently there are several animal models for MS, the main one being an experimental autoimmune encephalomyelitis (EAE), which can be achieved

through manipulation in either mouse or rat models. [220, 221] Therefore, it may be beneficial to study polyuria in these animal models, and investigate whether the biomarkers associated with SCI-induced polyuria overlap with those in the EAE model. Although MS and SCI are two very different and complicated whole system disorders, there are similarities between the two. Both MS and SCI affect primarily the central nervous system (CNS), which unlike the peripheral nervous system (PNS), is unable to functionally repair itself. Investigation of MS models compared to SCI models in areas such as cardiovascular function may also be beneficial to both fields.

Although AVP, ANP, V2R, NPRA, and ENaC are key biomarkers involved in both cardiovascular and urinary homeostatic control, the RAAS is another mechanism that helps regulate both blood pressure and urinary output. Upon normal physiological function, the RAAS is a hormonal system that is comprised mainly of renin, angiotensin II, and aldosterone and is regulated primarily by renal blood perfusion. Renin is released in the kidney in response to either decreased sodium, reduced perfusion pressure, or sympathetic stimulation of specific kidney areas (juxtaglomerular apparatus). As previously mentioned, the release of renin is inhibited by the release of ANP, which we have found to be significantly increased at various time points post-SCI. Angiotensin II comes from the cleavage of angiotensinogen by renin into angiotensin I, which is then converted to angiotensin II by angiotensin converting enzyme (ACE). Angiotensin II has multiple effects systemically, such as vasoconstriction of arterioles, sodium reabsorption within the kidney, release of noradrenaline, release of AVP by the

hypothalamus/pituitary gland, and the stimulation of aldosterone. Aldosterone mainly effects the kidney by increasing ENaC and promote sodium reabsorption, which may also result with a reduced level of potassium. Although there have been several studies investigating the separate components of RAAS after SCI, [202, 205, 222] there is yet to be an association demonstrated between RAAS and SCI-induced polyuria. Further, there is evidence that after SCI, liver function is significantly disrupted, potentially leading to metabolic disease. [223-225], which may also disrupt the RAAS further. It would be beneficial to determine the effect of chronic SCI on circulating renin, angiotensin II, aldosterone levels, along with associated angiotensin AT2 receptor, which has been shown to be neuroprotective after experimental SCI, and other RAAS-associated biomarkers. [226] Understanding these potential disruptions will not only further illuminate the mechanisms behind SCI-induced polyuria, but also provide other potential targets to improve quality of life for SCI individuals.

An additional potential therapy to alleviate SCI-induced polyuria is epidural stimulation, in conjunction with exercise therapy. Currently, there are many clinical and pre-clinical animal studies utilizing epidural stimulation as a rehabilitation therapy to improve multi-systems after SCI. [227-230] There is evidence that epidural stimulation improves cardiovascular health, including better regulation of MAP. [229] A prospective study including SCI patients that have reported nocturia and epidural stimulation as a treatment modality would potentially lessen the incidence of SCI-induced polyuria but could also help elucidate the extent cardiovascular health has on SCI-induced polyuria.

In conclusion, this dissertation research has provided further knowledge on mechanisms responsible for SCI-induced polyuria. Although seemingly simple, an overproduction of urine seen after SCI likely has multiple causes and is a symptom seen due to multi-systems failure, including hormonal imbalance, hypothalamic disruptions, cardiovascular dysregulation, significantly disrupted solute handling, and damaged supraspinal sympathetic pathways. Future studies in both clinical and pre-clinical animal settings investigating both cardiovascular and urinary function will benefit from the data presented from this work.

REFERENCES

1. Szollar, S.M., et al., *Nocturnal polyuria and antidiuretic hormone levels in spinal cord injury.* Archives of physical medicine and rehabilitation, 1997. **78**(5): p. 455-458.
2. Ward, P.J. and C.H. Hubscher, *Persistent polyuria in a rat spinal contusion model.* Journal of neurotrauma, 2012. **29**(15): p. 2490-2498.
3. Goh, M.Y., et al., *Diurnal blood pressure and urine production in acute spinal cord injury compared with controls.* Spinal Cord, 2017. **55**(1): p. 39-46.
4. Hubscher, C.H., et al., *Effects of exercise training on urinary tract function after spinal cord injury.* American Journal of Physiology-Renal Physiology, 2016. **310**(11): p. F1258-F1268.
5. Dolinak, D. and E. Balraj, *Autonomic dysreflexia and sudden death in people with traumatic spinal cord injury.* The American Journal of Forensic Medicine and Pathology, 2007. **28**(2): p. 95-98.
6. Wan, D. and A.V. Krassioukov, *Life-threatening outcomes associated with autonomic dysreflexia: a clinical review.* The journal of spinal cord medicine, 2014. **37**(1): p. 2-10.
7. Montgomery, L.R. and C.H. Hubscher, *Altered vasopressin and natriuretic peptide levels in a rat model of spinal cord injury: implications for the*

development of polyuria. American Journal of Physiology-Renal Physiology, 2017. **314**(1): p. F58-F66.

8. Denys, M.-A., et al., *Circadian rhythms in water and solute handling in adults with a spinal cord injury.* The Journal of urology, 2017. **197**(2): p. 445-451.
9. Claydon, V., J. Steeves, and A. Krassioukov, *Orthostatic hypotension following spinal cord injury: understanding clinical pathophysiology.* Spinal cord, 2006. **44**(6): p. 341-351.
10. Lindan, R., et al., *Incidence and clinical features of autonomic dysreflexia in patients with spinal cord injury.* Spinal cord, 1980. **18**(5): p. 285-292.
11. Swanson, L. and P.E. Sawchenko, *Hypothalamic integration: organization of the paraventricular and supraoptic nuclei.* Annual review of neuroscience, 1983. **6**(1): p. 269-324.
12. Van Leeuwen, F., D. Swaab, and C. De Raay, *Immunoelectronmicroscopic localization of vasopressin in the rat suprachiasmatic nucleus.* Cell and tissue research, 1978. **193**(1): p. 1-10.
13. Cowley Jr, A., et al., *Influence of daily sodium intake on vasopressin secretion and drinking in dogs.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 1983. **245**(6): p. R860-R872.
14. Kjeldsen, S.E., et al., *Dietary sodium intake increases vasopressin secretion in man.* J Clin Hypertens, 1985. **1**(2): p. 123-31.

15. Nielsen, S., et al., *Aquaporins in the kidney: from molecules to medicine.* Physiological reviews, 2002. **82**(1): p. 205-244.

16. Phillips, P.A., et al., *Localization of vasopressin binding sites in rat tissues using specific VI and V2 selective ligands.* Endocrinology, 1990. **126**(3): p. 1478-1484.

17. Holmes, C.L., D.W. Landry, and J.T. Granton, *Science review: vasopressin and the cardiovascular system part 1–receptor physiology.* Critical care, 2003. **7**(6): p. 427.

18. Harris, H., et al., *Characterization of purified endosomes containing the antidiuretic hormone-sensitive water channel from rat renal papilla.* Journal of Biological Chemistry, 1994. **269**(16): p. 11993-12000.

19. Bichet, D.G., et al., *Hemodynamic and coagulation responses to 1-desamino [8-D-arginine] vasopressin in patients with congenital nephrogenic diabetes insipidus.* New England Journal of Medicine, 1988. **318**(14): p. 881-887.

20. Zahariou, A., et al., *The use of desmopressin in the management of nocturnal enuresis in patients with spinal cord injury.* Europa medicophysica, 2007. **43**(3): p. 333-338.

21. Chancellor, M.B., D.A. Rivas, and W.E. Staas Jr, *DDAVP in the urological management of the difficult neurogenic bladder in spinal cord injury: preliminary report.* The Journal of the American Paraplegia Society, 1994. **17**(4): p. 165-167.

22. Potter, L.R., et al., *Natriuretic peptides: their structures, receptors, physiologic functions and therapeutic applications*, in *cGMP: Generators, Effectors and Therapeutic Implications*. 2009, Springer. p. 341-366.

23. Goy, M.F., et al., *Evidence for a novel natriuretic peptide receptor that prefers brain natriuretic peptide over atrial natriuretic peptide.* Biochemical Journal, 2001. **358**(2): p. 379-387.

24. Lowe, D.G., et al., *Human atrial natriuretic peptide receptor defines a new paradigm for second messenger signal transduction.* The EMBO journal, 1989. **8**(5): p. 1377-1384.

25. Nagase, M., et al., *Tissue distribution and localization of natriuretic peptide receptor subtypes in stroke-prone spontaneously hypertensive rats.* Journal of hypertension, 1997. **15**(11): p. 1235-1243.

26. Wang, T.J., et al., *Plasma natriuretic peptide levels and the risk of cardiovascular events and death.* New England Journal of Medicine, 2004. **350**(7): p. 655-663.

27. Sabatine, M.S., et al., *Evaluation of multiple biomarkers of cardiovascular stress for risk prediction and guiding medical therapy in patients with stable coronary disease.* Circulation, 2012. **125**(2): p. 233-240.

28. Barbato, E., et al., *NT-proANP circulating level is a prognostic marker in stable ischemic heart disease.* International journal of cardiology, 2012. **155**(2): p. 311-312.

29. Nakayama, T., et al., *Atrial natriuretic peptide reduces ischemia/reperfusion-induced spinal cord injury in rats by enhancing*

sensory neuron activation. Journal of Pharmacology and Experimental Therapeutics, 2007. **322**(2): p. 582-590.

30. Yamamoto, M., et al., *Static exercise—induced increase in blood pressure in individuals with cervical spinal cord injury.* Archives of physical medicine and rehabilitation, 1999. **80**(3): p. 288-293.
31. Van Kerrebroeck, P., et al., *The standardisation of terminology in nocturia: report from the Standardisation Sub-committee of the International Continence Society.* Neurourology and urodynamics, 2002. **21**(2): p. 179-183.
32. Oelke, M., et al., *A practical approach to the management of nocturia.* International journal of clinical practice, 2017. **71**(11): p. e13027.
33. Williams, H.H., et al., *Nonosmotic stimuli alter osmoregulation in patients with spinal cord injury.* The Journal of Clinical Endocrinology & Metabolism, 1990. **71**(6): p. 1536-1543.
34. Krum, H., et al., *Diurnal blood pressure variation in quadriplegic chronic spinal cord injury patients.* Clinical science, 1991. **80**(3): p. 271-276.
35. Gumbel, J.H., et al., *Activity-Based Training Reverses Spinal Cord Injury-Induced Changes in Kidney Receptor Densities and Membrane Proteins.* Journal of neurotrauma, 2020. **37**(3): p. 555-563.
36. Goh, M.Y., et al., *Comparison of diurnal blood pressure and urine production between people with and without chronic spinal cord injury.* Spinal cord, 2018. **56**(9): p. 847-855.

37. Oelke, M., et al., *Nocturia: state of the art and critical analysis of current assessment and treatment strategies*. 2014, Springer.

38. Rittig, S., et al., *Abnormal diurnal rhythm of plasma vasopressin and urinary output in patients with enuresis.* American Journal of Physiology-Renal Physiology, 1989. **256**(4): p. F664-F671.

39. Kilinc, S., et al., *Diurnal variation of antidiuretic hormone and urinary output in spinal cord injury.* Spinal Cord, 1999. **37**(5).

40. Zahra, M., et al., *Acute changes in systemic hemodynamics and serum vasopressin after complete cervical spinal cord injury in piglets.* Neurocritical care, 2010. **13**(1): p. 132-140.

41. Illman, A., K. Stiller, and M. Williams, *The prevalence of orthostatic hypotension during physiotherapy treatment in patients with an acute spinal cord injury.* Spinal Cord, 2000. **38**(12): p. 741-747.

42. Zerbe, R.L., D.P. Henry, and G.L. Robertson, *Vasopressin response to orthostatic hypotension: etiologic and clinical implications.* The American journal of medicine, 1983. **74**(2): p. 265-271.

43. Glazener, C.M. and J.H. Evans, *Desmopressin for nocturnal enuresis in children.* Cochrane Database of Systematic Reviews, 2002(3).

44. Zahariou, A., et al., *Maximal bladder capacity is a positive predictor of response to desmopressin treatment in patients with MS and nocturia.* International Urology and Nephrology, 2008. **40**(1): p. 65-69.

45. Dillingham, M.A. and R.J. Anderson, *Inhibition of vasopressin action by atrial natriuretic factor.* Science, 1986. **231**(4745): p. 1572-1573.

46. Schrier, R., T. Berl, and R. Anderson, *Osmotic and nonosmotic control of vasopressin release.* American Journal of Physiology-Renal Physiology, 1979. **236**(4): p. F321-F332.

47. Tajima, F., et al., *Cardiovascular, renal, and endocrine responses in male quadriplegics during head-out water immersion.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 1990. **258**(6): p. R1424-R1430.

48. Haack, D., et al., *Comparative study on development of corticosterone and DOCA hypertension in rats.* American Journal of Physiology-Renal Physiology, 1977. **233**(5): p. F403-F411.

49. Inoue, T., H. Nonoguchi, and K. Tomita, *Physiological effects of vasopressin and atrial natriuretic peptide in the collecting duct.* Cardiovascular research, 2001. **51**(3): p. 470-480.

50. Gumbel, J.H., et al., *Activity-based training on a treadmill with spinal cord injured Wistar rats.* JoVE (Journal of Visualized Experiments), 2019(143): p. e58983.

51. Ward, P.J., et al., *Novel multi-system functional gains via task specific training in spinal cord injured male rats.* Journal of neurotrauma, 2014. **31**(9): p. 819-833.

52. Hubscher, C.H., et al., *Improvements in bladder, bowel and sexual outcomes following task-specific locomotor training in human spinal cord injury.* PloS one, 2018. **13**(1): p. e0190998.

53. Convertino, V., et al., *Plasma volume, osmolality, vasopressin, and renin activity during graded exercise in man.* Journal of Applied Physiology, 1981. **50**(1): p. 123-128.

54. Inder, W., et al., *Prolonged exercise increases peripheral plasma ACTH, CRH, and AVP in male athletes.* Journal of Applied Physiology, 1998. **85**(3): p. 835-841.

55. Ohba, H., et al., *Effects of prolonged strenuous exercise on plasma levels of atrial natriuretic peptide and brain natriuretic peptide in healthy men.* American heart journal, 2001. **141**(5): p. 751-758.

56. Krassioukov, A.V. and L.C. Weaver, *Episodic hypertension due to autonomic dysreflexia in acute and chronic spinal cord-injured rats.* American Journal of Physiology-Heart and Circulatory Physiology, 1995. **268**(5): p. H2077-H2083.

57. Arnold, J., et al., *Autonomic dysreflexia in tetraplegic patients: evidence for α-adrenoceptor hyper-responsiveness.* Clinical Autonomic Research, 1995. **5**(5): p. 267-270.

58. Maiorov, D.N., M.G. Fehlings, and A.V. Krassioukov, *Relationship between severity of spinal cord injury and abnormalities in neurogenic cardiovascular control in conscious rats.* Journal of neurotrauma, 1998. **15**(5): p. 365-374.

59. Liu, C., et al., *Glucocorticoids improve renal responsiveness to atrial natriuretic peptide by up-regulating natriuretic peptide receptor-A expression in the renal inner medullary collecting duct in decompensated*

heart failure. Journal of Pharmacology and Experimental Therapeutics, 2011. **339**(1): p. 203-209.

60. Dananberg, J. and R.J. Grekin, *Corticoid regulation of atrial natriuretic factor secretion and gene expression.* American Journal of Physiology-Heart and Circulatory Physiology, 1992. **263**(5): p. H1377-H1381.
61. Lucin, K.M., et al., *Impaired antibody synthesis after spinal cord injury is level dependent and is due to sympathetic nervous system dysregulation.* Experimental neurology, 2007. **207**(1): p. 75-84.
62. Popovich, P.G., et al., *Alterations in immune cell phenotype and function after experimental spinal cord injury.* Journal of neurotrauma, 2001. **18**(9): p. 957-966.
63. DePasquale-Jardieu, P. and P.J. Fraker, *The role of corticosterone in the loss in immune function in the zinc-deficient A/J mouse.* The Journal of nutrition, 1979. **109**(11): p. 1847-1855.
64. Wiegers, G.J., et al., *Glucocorticoids regulate TCR-induced elevation of CD4: functional implications.* The Journal of Immunology, 2000. **164**(12): p. 6213-6220.
65. Coutinho, A.E. and K.E. Chapman, *The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights.* Molecular and cellular endocrinology, 2011. **335**(1): p. 2-13.

66. Lucin, K.M., V.M. Sanders, and P.G. Popovich, *Stress hormones collaborate to induce lymphocyte apoptosis after high level spinal cord injury.* Journal of neurochemistry, 2009. **110**(5): p. 1409-1421.

67. Mathias, C., et al., *Plasma catecholamines, plasma renin activity and plasma aldosterone in tetraplegic man, horizontal and tilted.* Clinical science and molecular medicine, 1975. **49**(4): p. 291-299.

68. Wall, B.M., et al., *Characteristics of vasopressin release during controlled reduction in arterial pressure.* The Journal of laboratory and clinical medicine, 1994. **124**(4): p. 554-563.

69. Viaene, A., et al., *Conservative treatment for leg oedema and the effect on nocturnal polyuria in patients with spinal cord injury.* BJU international, 2019. **123**(5A): p. E43-E50.

70. Iovino, M., et al., *The role of neurohypophyseal hormones vasopressin and oxytocin in neuropsychiatric disorders.* Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders), 2018. **18**(4): p. 341-347.

71. Wassink, T., et al., *Examination of AVPR1a as an autism susceptibility gene.* Molecular psychiatry, 2004. **9**(10): p. 968-972.

72. Linkowski, P., et al., *Cerebrospinal fluid neurophysins in affective illness and in schizophrenia.* European archives of psychiatry and neurological sciences, 1984. **234**(3): p. 162-165.

73. Panicker, J.N., C.J. Fowler, and T.M. Kessler, *Lower urinary tract dysfunction in the neurological patient: clinical assessment and management.* The Lancet Neurology, 2015. **14**(7): p. 720-732.

74. Peyronnet, B., et al., *Nocturia in Patients With Multiple Sclerosis.* Reviews in urology, 2019. **21**(2-3): p. 63.

75. Wildey, G. and C.C. Glembotski, *Cross-linking of atrial natriuretic peptide to binding sites in rat olfactory bulb membranes.* Journal of Neuroscience, 1986. **6**(12): p. 3767-3776.

76. McKenzie, J.C., et al., *Atrial natriuretic peptide-like immunoreactivity in neurons and astrocytes of human cerebellum and inferior olivary complex.* Journal of Histochemistry & Cytochemistry, 2001. **49**(11): p. 1453-1467.

77. Špiranec Spes, K., et al., *Natriuretic peptides attenuate retinal pathological neovascularization via cyclic guanosine monophosphate signaling in pericytes and astrocytes.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2020. **40**(1): p. 159-174.

78. Cao, L.-H. and X.-L. Yang, *Natriuretic peptides and their receptors in the central nervous system.* Progress in neurobiology, 2008. **84**(3): p. 234-248.

79. Fiscus, R.R., A.W. Tu, and S.B.C. Chew, *Natriuretic peptides inhibit apoptosis and prolong the survival of serum-deprived PC12 cells.* Neuroreport, 2001. **12**(2): p. 185-189.

80. Anderson, K.D., *Targeting recovery: priorities of the spinal cord-injured population.* Journal of neurotrauma, 2004. **21**(10): p. 1371-1383.

81. Everaert, K., et al., *Nocturia is more bothersome than daytime LUTS: Results from an Observational, Real-life Practice Database including 8659 European and American LUTS patients.* International journal of clinical practice, 2018. **72**(6): p. e13091.

82. Kilinc, S., et al., *Diurnal variation of antidiuretic hormone and urinary output in spinal cord injury.* Spinal Cord, 1999. **37**(5): p. 332-335.

83. Jamil, F., *Towards a catheter free status in neurogenic bladder dysfunction: a review of bladder management options in spinal cord injury (SCI).* Spinal cord, 2001. **39**(7): p. 355.

84. Vaidyanathan, S., et al., *What is the optimum fluid intake in male patients with spinal cord injury and neuropathic bladder?* Spinal Cord, 1999. **37**: p. 594-594.

85. Pandey, K.N., *Guanylyl cyclase/atrial natriuretic peptide receptor-A: role in the pathophysiology of cardiovascular regulation.* Canadian journal of physiology and pharmacology, 2011. **89**(8): p. 557-573.

86. Jard, S., *Vasopressin: mechanisms of receptor activation*, in *Progress in brain research*. 1983, Elsevier. p. 383-394.

87. Robson, W., J. Nørgaard, and A. Leung, *Hyponatremia in patients with nocturnal enuresis treated with DDAVP.* European journal of pediatrics, 1996. **155**(11): p. 959-962.

88. Curt, A., et al., *Assessment of autonomic dysreflexia in patients with spinal cord injury.* Journal of Neurology, Neurosurgery & Psychiatry, 1997. **62**(5): p. 473-477.

89. Krassioukov, A., *Autonomic dysreflexia: current evidence related to unstable arterial blood pressure control among athletes with spinal cord injury.* Clinical Journal of Sport Medicine, 2012. **22**(1): p. 39-45.

90. Swaab, D., F. Nijveldt, and C. Pool, *Distribution of oxytocin and vasopressin in the rat supraoptic and paraventricular nucleus.* Journal of Endocrinology, 1975. **67**(3): p. 461-462.

91. Potter, L.R., et al., *Natriuretic peptides: their structures, receptors, physiologic functions and therapeutic applications.* Handb Exp Pharmacol, 2009(191): p. 341-66.

92. Kerkelä, R., J. Ulvila, and J. Magga, *Natriuretic peptides in the regulation of cardiovascular physiology and metabolic events.* Journal of the American Heart Association, 2015. **4**(10): p. e002423.

93. Scheff, S.W., et al., *Experimental modeling of spinal cord injury: characterization of a force-defined injury device.* J Neurotrauma, 2003. **20**(2): p. 179-93.

94. Steadman, C.J., S.S. Vangoor, and C.H. Hubscher, *Kinematic analysis of penile reflexes in a rat model of spinal cord injury.* Asian journal of andrology, 2021. **23**(1): p. 30.

95. Ferrero, S.L., et al., *Effects of lateral funiculus sparing, spinal lesion level, and gender on recovery of bladder voiding reflexes and hematuria in rats.* Journal of neurotrauma, 2015. **32**(3): p. 200-208.

96. Holmes, G.M., et al., *Recommendations for evaluation of bladder and bowel function in pre-clinical spinal cord injury research.* The journal of spinal cord medicine, 2020. **43**(2): p. 165-176.

97. Ward, P.J., et al., *Novel multi-system functional gains via task specific training in spinal cord injured male rats.* J Neurotrauma, 2014. **31**(9): p. 819-33.

98. Brown, C., *Blood collection from the tail of a rat.* Lab Anim (NY), 2006. **35**(8): p. 24-5.

99. Basso, D.M., M.S. Beattie, and J.C. Bresnahan, *A sensitive and reliable locomotor rating scale for open field testing in rats.* Journal of neurotrauma, 1995. **12**(1): p. 1-21.

100. Paxinos, G. and C. Watson, *The Rat Brain in Stereotaxic Coordinates*. 1998: Academic Press.

101. Steadman, C.J., et al., *Activity-based training alters penile reflex responses in a rat model of spinal cord injury.* The journal of sexual medicine, 2019. **16**(8): p. 1143-1154.

102. Hall, B.J., et al., *Spinal cord injuries containing asymmetrical damage in the ventrolateral funiculus is associated with a higher incidence of at-level allodynia.* The Journal of Pain, 2010. **11**(9): p. 864-875.

103. Deliva, R.D. and U. Ackermann, *Atrial natriuretic peptide and mechanisms of cardiovascular control. Role of serotonergic receptors.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 1998. **274**(3): p. R711-R717.

104. Melo, L.G., et al., *Chronic regulation of arterial blood pressure in ANP transgenic and knockout mice: role of cardiovascular sympathetic tone.* Cardiovascular research, 1999. **43**(2): p. 437-444.

105. Roy, D.R., *Effect of synthetic ANP on renal and loop of Henle functions in the young rat.* American Journal of Physiology-Renal Physiology, 1986. **251**(2): p. F220-F225.

106. Haddad, R., et al., *Nocturia and nocturnal polyuria in neurological patients: from epidemiology to treatment. A systematic review of the literature.* European urology focus, 2020.

107. Cao, Q., et al., *Functional and electrophysiological changes after graded traumatic spinal cord injury in adult rat.* Experimental neurology, 2005. **191**: p. S3-S16.

108. Michele Basso, D., M.S. Beattie, and J.C. Bresnahan, *Descending systems contributing to locomotor recovery after mild or moderate spinal cord injury in rats: experimental evidence and a review of literature.* Restorative neurology and neuroscience, 2002. **20**(5): p. 189-218.

109. Hagen, E.M., et al., *Cardiovascular complications of spinal cord injury.* Tidsskrift for Den norske legeforening, 2012.

110. Krassioukov, A. and V.E. Claydon, *The clinical problems in cardiovascular control following spinal cord injury: an overview.* Progress in brain research, 2006. **152**: p. 223-229.

111. Oliver, P.M., et al., *Natriuretic peptide receptor 1 expression influences blood pressures of mice in a dose-dependent manner.* Proceedings of the National Academy of Sciences, 1998. **95**(5): p. 2547-2551.

112. Seifert, J., et al., *Microcirculation and blood volume in rats before and after spinal cord injury.* Spinal Cord, 1979. **17**(4): p. 436-440.

113. Steinhelper, M.E., K.L. Cochrane, and L.J. Field, *Hypotension in transgenic mice expressing atrial natriuretic factor fusion genes.* Hypertension, 1990. **16**(3): p. 301-307.

114. Hubscher, C.H., et al., *Improvements in bladder, bowel and sexual outcomes following task-specific locomotor training in human spinal cord injury.* PloS one, 2018. **13**(1).

115. Ward, P.J., et al., *Training-Induced Functional Gains following SCI.* Neural Plast, 2016. **2016**: p. 4307694.

116. Shehata, M.F., *The Epithelial Sodium Channel α subunit (α ENaC) alternatively spliced form" b" in Dahl rats: What's next?* International archives of medicine, 2010. **3**(1): p. 1-6.

117. Waldmann, R., et al., *Molecular cloning and functional expression of a novel amiloride-sensitive Na+ channel.* Journal of Biological Chemistry, 1995. **270**(46): p. 27411-27414.

118. Konstas, A.-A. and C. Korbmacher, *The γ-subunit of ENaC is more important for channel surface expression than the β-subunit.* American Journal of Physiology-Cell Physiology, 2003. **284**(2): p. C447-C456.

119. McNicholas, C.M. and C.M. Canessa, *Diversity of channels generated by different combinations of epithelial sodium channel subunits.* The Journal of general physiology, 1997. **109**(6): p. 681-692.

120. Masilamani, S., et al., *Aldosterone-mediated regulation of ENaC α, β, and γ subunit proteins in rat kidney.* The Journal of clinical investigation, 1999. **104**(7): p. R19-R23.

121. Wecht, J.M. and W.A. Bauman, *Implication of altered autonomic control for orthostatic tolerance in SCI.* Autonomic Neuroscience, 2018. **209**: p. 51-58.

122. Katzelnick, C.G., et al., *Impact of blood pressure, lesion level, and physical activity on aortic augmentation index in persons with spinal cord injury.* Journal of neurotrauma, 2017. **34**(24): p. 3407-3415.

123. Katzelnick, C.G., *Orthostatic Blood Pressure and Arterial Stiffness in Persons with Spinal Cord Injury: The Effect of the Renin Angiotensin Aldosterone System.* 2020.

124. Tsang, L.L., et al, *Enhanced epithelial Na+ channel (ENaC) activity in mouse endometrial epithelium by upregulation of γENaC subunit.* The Japanese journal of physiology, 2001. **51**(4): p. 539-543.

125. Garty, H. and L.G. Palmer, *Epithelial sodium channels: function, structure, and regulation.* Physiological reviews, 1997. **77**(2): p. 359-396.

126. Inouye, S.-I.T. and S. Shibata, *Neurochemical organization of circadian rhythm in the suprachiasmatic nucleus.* Neuroscience research, 1994. **20**(2): p. 109-130.

127. Gaudet, A.D., et al., *Spinal cord injury in rats disrupts the circadian system.* Eneuro, 2018. **5**(6).

128. Kostovski, E., et al., *Normalization of disrupted clock gene expression in males with tetraplegia: a crossover randomized placebo-controlled trial of melatonin supplementation.* Spinal cord, 2018. **56**(11): p. 1076-1083.

129. Hoogerwerf, W.A., *Role of clock genes in gastrointestinal motility.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2010. **299**(3): p. G549-G555.

130. Franken, P. and D.J. Dijk, *Circadian clock genes and sleep homeostasis.* European Journal of Neuroscience, 2009. **29**(9): p. 1820-1829.

131. Hastings, M.H. and E.D. Herzog, *Clock genes, oscillators, and cellular networks in the suprachiasmatic nuclei.* Journal of biological rhythms, 2004. **19**(5): p. 400-413.

132. Sankari, A., et al., *Sleep disordered breathing in chronic spinal cord injury.* Journal of Clinical Sleep Medicine, 2014. **10**(1): p. 65-72.

133. Khan, S., et al., *Hypothermia in patients with chronic spinal cord injury.* The journal of spinal cord medicine, 2007. **30**(1): p. 27-30.

134. Holmes, G.M. and E.N. Blanke, *Gastrointestinal dysfunction after spinal cord injury.* Experimental neurology, 2019. **320**: p. 113009.

135. Szollar, S., J. North, and J. Chung, *Antidiuretic hormone levels and polyuria in spinal cord injury. A preliminary report.* Spinal Cord, 1995. **33**(2): p. 94-97.

136. Rhodes, C., J. Morriell, and D. Pfaff, *Immunohistochemical analysis of magnocellular elements in rat hypothalamus: distribution and numbers of cells containing neurophysin, oxytocin, and vasopressin.* Journal of Comparative Neurology, 1981. **198**(1): p. 45-64.

137. Callréus, T., E. Ekman, and M. Andersen, *Hyponatremia in elderly patients treated with desmopressin for nocturia: a review of a case series.* European journal of clinical pharmacology, 2005. **61**(4): p. 281-284.

138. Anderson, K., et al., *The impact of spinal cord injury on sexual function: concerns of the general population.* Spinal cord, 2007. **45**(5): p. 328-337.

139. Cruz, C.D. and F. Cruz, *Spinal cord injury and bladder dysfunction: new ideas about an old problem.* TheScientificWorldJournal, 2011. **11**: p. 214-234.

140. Steadman, C.J. and C.H. Hubscher, *Sexual function after spinal cord injury: innervation, assessment, and treatment.* Current Sexual Health Reports, 2016. **8**(2): p. 106-115.

141. Cardenas, D.D., et al., *Etiology and incidence of rehospitalization after traumatic spinal cord injury: a multicenter analysis.* Archives of physical medicine and rehabilitation, 2004. **85**(11): p. 1757-1763.

142. Montgomery, L.R. and C.H. Hubscher, *Altered vasopressin and natriuretic peptide levels in a rat model of spinal cord injury: implications for the development of polyuria.* American Journal of Physiology-Renal Physiology, 2018. **314**(1): p. F58-F66.

143. Viaene, A., et al., *Evaluation of the occurrence and diagnose definitions for nocturnal polyuria in spinal cord injured patients during rehabilitation.* European journal of physical and rehabilitation medicine, 2017. **55**(1): p. 40-46.

144. Kilinç, S., et al., *Diurnal variation of antidiuretic hormone and urinary output in spinal cord injury.* Spinal Cord, 1999. **37**(5): p. 332-335.

145. Gumbel, J.H., C.B. Yang, and C.H. Hubscher, *Timeline of changes in biomarkers associated with spinal cord injury-induced polyuria.* Neurotrauma Reports, 2021: p. in press.

146. Gattone 2nd, V., C.F. Marfurt, and S. Dallie, *Extrinsic innervation of the rat kidney: a retrograde tracing study.* American Journal of Physiology-Renal Physiology, 1986. **250**(2): p. F189-F196.

147. Strack, A., et al., *Spinal origin of sympathetic preganglionic neurons in the rat.* Brain research, 1988. **455**(1): p. 187-191.

148. Maeda, S., et al., *Origin of efferent fibers of the renal plexus in the rat autonomic nervous system.* The Journal of veterinary medical science, 2014. **76**(5): p. 763-765.

149. Kirkpatrick, J.J., S. Foutz, and S.W. Leslie, *Anatomy, abdomen and pelvis, kidney nerves.* StatPearls [Internet], 2020.

150. Llewellyn-Smith, I.J., L.C. Weaver, and J.R. Keast, *Effects of spinal cord injury on synaptic inputs to sympathetic preganglionic neurons.* Progress in brain research, 2006. **152**: p. 11-26.

151. Strack, A., et al., *A general pattern of CNS innervation of the sympathetic outflow demonstrated by transneuronal pseudorabies viral infections.* Brain research, 1989. **491**(1): p. 156-162.

152. Sved, A.F., G. Cano, and J.P. Card, *Neuroanatomical specificity of the circuits controlling sympathetic outflow to different targets.* Clinical and experimental pharmacology & physiology, 2001. **28**(1-2): p. 115-119.

153. Schramm, L.P., *Spinal sympathetic interneurons: their identification and roles after spinal cord injury.* Progress in brain research, 2006. **152**: p. 27-37.

154. Herrity, A.N., et al., *The effect of spinal cord injury on the neurochemical properties of vagal sensory neurons.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2015. **308**(12): p. R1021-R1033.

155. Scheff, S.W., et al., *Experimental modeling of spinal cord injury: characterization of a force-defined injury device.* Journal of neurotrauma, 2003. **20**(2): p. 179-193.

156. Mieda, M., et al., *Cellular clocks in AVP neurons of the SCN are critical for interneuronal coupling regulating circadian behavior rhythm.* Neuron, 2015. **85**(5): p. 1103-1116.

157. Kalsbeek, A., et al., *In vivo measurement of a diurnal variation in vasopressin release in the rat suprachiasmatic nucleus.* Brain research, 1995. **682**(1-2): p. 75-82.

158. Zerbe, R.L. and G.L. Robertson, *A comparison of plasma vasopressin measurements with a standard indirect test in the differential diagnosis of polyuria.* New England Journal of Medicine, 1981. **305**(26): p. 1539-1546.

159. Sofroniew, M.V. and A. Weindl, *Projections from the parvocellular vasopressin-and neurophysin-containing neurons of the suprachiasmatic nucleus.* American Journal of Anatomy, 1978. **153**(3): p. 391-429.

160. Meijer, J. and W. Rietveld, *Neurophysiology of the suprachiasmatic circadian pacemaker in rodents.* Physiological Reviews, 1989. **69**(3): p. 671-707.

161. Hofman, M., J. Purba, and D. Swaab, *Annual variations in the vasopressin neuron population of the human suprachiasmatic nucleus.* Neuroscience, 1993. **53**(4): p. 1103-1112.

162. Baschieri, F., et al., *Circadian and state-dependent core body temperature in people with spinal cord injury.* Spinal Cord, 2021. **59**(5): p. 538-546.

163. Frisbie, J., *Salt wasting, hypotension, polydipsia, and hyponatremia and the level of spinal cord injury.* Spinal cord, 2007. **45**(8): p. 563-568.

164. Bello-Reuss, E., et al., *Effects of acute unilateral renal denervation in the rat.* The Journal of clinical investigation, 1975. **56**(1): p. 208-217.

165. Kannan, A., et al., *Renal sympathetic nervous system and the effects of denervation on renal arteries.* World journal of cardiology, 2014. **6**(8): p. 814-823.

166. Claydon, V.E., J.D. Steeves, and A. Krassioukov, *Orthostatic hypotension following spinal cord injury: understanding clinical pathophysiology.* Spinal Cord, 2006. **44**(6): p. 341-351.

167. Teasell, R.W., et al., *Cardiovascular consequences of loss of supraspinal control of the sympathetic nervous system after spinal cord injury.* Archives of physical medicine and rehabilitation, 2000. **81**(4): p. 506-516.

168. Paxinos, G. and C. Watson, *The rat brain in stereotaxic coordinates: hard cover edition*. 2006: Elsevier.

169. Goh, M., et al., *A retrospective review of the ambulatory blood pressure patterns and diurnal urine production in subgroups of spinal cord injured patients.* Spinal cord, 2015. **53**(1): p. 49-53.

170. Viaene, A., et al., *Evaluation of the occurrence and diagnose definitions for nocturnal polyuria in spinal cord injured patients during rehabilitation.* European journal of physical and rehabilitation medicine, 2019. **55**(1): p. 40-46.

171. Karlsson, A. and A. Krassioukov, *Hyponatremia-induced transient visual disturbances in acute spinal cord injury.* Spinal Cord, 2004. **42**(3): p. 204-207.

172. Rembratt, A., A. Riis, and J. Norgaard, *Desmopressin treatment in nocturia; an analysis of risk factors for hyponatremia.* Neurourology and Urodynamics: Official Journal of the International Continence Society, 2006. **25**(2): p. 105-109.

173. Peruzzi, W.T., et al., *Hyponatremia in acute spinal cord injury.* Critical care medicine, 1994. **22**(2): p. 252-258.

174. Levin, E.R., D.G. Gardner, and W.K. Samson, *Natriuretic peptides.* New England Journal of Medicine, 1998. **339**(5): p. 321-328.

175. Clerico, A., G. Iervasi, and G. Mariani, *Pathophysiologic relevance of measuring the plasma levels of cardiac natriuretic peptide hormones in humans.* Hormone and metabolic research, 1999. **31**(09): p. 487-498.

176. Yamane, T., et al., *Preliminary immunohistochemical study of natriuretic peptide receptor localization in canine and feline heart.* Journal of Veterinary Medical Science, 2010: p. 1010120361-1010120361.

177. Knowles, J.W., et al., *Pressure-independent enhancement of cardiac hypertrophy in natriuretic peptide receptor A–deficient mice.* The Journal of clinical investigation, 2001. **107**(8): p. 975-984.

178. Pettersson, A., J. Hedner, and T. Hedner, *Renal interaction between sympathetic activity and ANP in rats with chronic ischaemic heart failure.* Acta physiologica scandinavica, 1989. **135**(4): p. 487-492.

179. Groah, S., et al., *The relationship between neurological level of injury and symptomatic cardiovascular disease risk in the aging spinal injured.* Spinal Cord, 2001. **39**(6): p. 310-317.

180. Weber, W., et al., *Anantin-a peptide antagonist of the atrial natriuretic factor (ANF).* The Journal of antibiotics, 1991. **44**(2): p. 164-171.

181. El-Ayoubi, R., et al., *Urinary responses to acute moxonidine are inhibited by natriuretic peptide receptor antagonist.* British journal of pharmacology, 2005. **145**(1): p. 50-56.

182. Dobrowolski, L., et al., *Role of atrial natriuretic peptide in mediating the blood pressure-independent natriuresis elicited by systemic inhibition of nitric oxide.* Pflügers Archiv-European Journal of Physiology, 2015. **467**(4): p. 833-841.

183. Nachshon, S., et al., *Effects of ANP receptor antagonists on ANP secretion from adult rat cultured atrial myocytes.* American Journal of Physiology-Endocrinology And Metabolism, 1995. **268**(3): p. E428-E432.

184. Dodd-o, J.M., et al., *The role of natriuretic peptide receptor-A signaling in unilateral lung ischemia-reperfusion injury in the intact mouse.* American Journal of Physiology-Lung Cellular and Molecular Physiology, 2008. **294**(4): p. L714-L723.

185. Pandey, K.N., *Molecular and genetic aspects of guanylyl cyclase natriuretic peptide receptor-A in regulation of blood pressure and renal function.* Physiological genomics, 2018. **50**(11): p. 913-928.

186. Shi, S.-J., et al., *Natriuretic peptide receptor A mediates renal sodium excretory responses to blood volume expansion.* American Journal of Physiology-Renal Physiology, 2003. **285**(4): p. F694-F702.

187. Dietz, J.R., et al., *Effects of cardiac hormones on arterial pressure and sodium excretion in NPRA knockout mice.* Experimental Biology and Medicine, 2004. **229**(8): p. 813-818.

188. Organization, W.H. *Spinal Cord Injury*. 2013 [cited 2016.

189. Singh, A., et al., *Global prevalence and incidence of traumatic spinal cord injury.* Clinical epidemiology, 2014. **6**: p. 309.

190. Gumbel, J.H., et al., *Activity-based Training on a Treadmill with Spinal Cord Injured Wistar Rats.* J Vis Exp, 2019(143).

191. Alfany-Fernandez, I., et al., *Therapeutic targets in liver transplantation: angiotensin II in nonsteatotic grafts and angiotensin-(1–7) in steatotic grafts.* American Journal of Transplantation, 2009. **9**(3): p. 439-451.

192. Harkema, S.J., et al., *Balance and ambulation improvements in individuals with chronic incomplete spinal cord injury using locomotor training–based rehabilitation.* Archives of physical medicine and rehabilitation, 2012. **93**(9): p. 1508-1517.

193. Brown, C., *Blood collection from the tail of a rat.* Lab animal, 2006. **35**(8): p. 24-25.

194. Dryer, R., *Semimicro flame photometry of serum sodium and potassium.* Clinical chemistry, 1956. **2**: p. 112-116.

195. Garcia, R.A., et al., *Comparative analysis for strength serum sodium and potassium in three different methods: Flame photometry, ion-selective electrode (ISE) and colorimetric enzymatic.* Journal of clinical laboratory analysis, 2018. **32**(9): p. e22594.

196. Weaver, L.C., et al., *Central mechanisms for autonomic dysreflexia after spinal cord injury.* Progress in brain research, 2002. **137**: p. 83-95.

197. Kim, M., et al., *GLP-1 receptor activation and Epac2 link atrial natriuretic peptide secretion to control of blood pressure.* Nature medicine, 2013. **19**(5): p. 567-575.

198. Segers, P., et al., *Amplification of the pressure pulse in the upper limb in healthy, middle-aged men and women.* Hypertension, 2009. **54**(2): p. 414-420.

199. Kroeker, E.J. and E.H. Wood, *Comparison of simultaneously recorded central and peripheral arterial pressure pulses during rest, exercise and tilted position in man.* Circulation research, 1955. **3**(6): p. 623-632.

200. Al Dera, H. and J.A. Brock, *Spinal cord injury increases the reactivity of rat tail artery to angiotensin II.* Frontiers in neuroscience, 2015. **8**: p. 435.

201. Groothuis, J.T., et al., *Angiotensin II contributes to the increased baseline leg vascular resistance in spinal cord-injured individuals.* Journal of hypertension, 2010. **28**(10): p. 2094-2101.

202. Katzelnick, C.G., et al., *Increased pulse wave velocity in persons with spinal cord injury: the effect of the renin-angiotensin-aldosterone system.* American Journal of Physiology-Heart and Circulatory Physiology, 2021. **320**(1): p. H272-H280.

203. Wecht, J.M., et al., *Orthostatic effects of midodrine versus L-NAME on cerebral blood flow and the renin-angiotensin-aldosterone system in tetraplegia.* Archives of physical medicine and rehabilitation, 2011. **92**(11): p. 1789-1795.

204. Lee, A.H., A.A. Phillips, and A.V. Krassioukov, *Increased central arterial stiffness after spinal cord injury: contributing factors, implications, and possible interventions.* Journal of neurotrauma, 2017. **34**(6): p. 1129-1140.

205. Furlan, J.C. and M.G. Fehlings, *Hyponatremia in the acute stage after traumatic cervical spinal cord injury: clinical and neuroanatomic evidence for autonomic dysfunction.* Spine, 2009. **34**(5): p. 501-511.

206. Acker, C.G., et al., *Hyperkalemia in hospitalized patients: causes, adequacy of treatment, and results of an attempt to improve physician compliance with published therapy guidelines.* Archives of internal medicine, 1998. **158**(8): p. 917-924.

207. Thapa, S. and S.J. Brull, *Succinylcholine-induced hyperkalemia in patients with renal failure: an old question revisited.* Anesthesia & Analgesia, 2000. **91**(1): p. 237-241.

208. Soni, B., et al., *A retrospective study of hyponatremia in tetraplegic/paraplegic patients with a review of the literature.* Spinal Cord, 1994. **32**(9): p. 597-607.

209. Giuliani, C. and A. Peri, *Effects of hyponatremia on the brain.* Journal of clinical medicine, 2014. **3**(4): p. 1163-1177.

210. West, C.R., et al., *Characterizing the temporal development of cardiovascular dysfunction in response to spinal cord injury.* Journal of neurotrauma, 2015. **32**(12): p. 922-930.

211. Lane, M.A., et al., *Respiratory function following bilateral mid-cervical contusion injury in the adult rat.* Experimental neurology, 2012. **235**(1): p. 197-210.

212. Mathias, C.J., *Orthostatic hypotension and paroxysmal hypertension in humans with high spinal cord injury.* Progress in brain research, 2006. **152**: p. 231-243.

213. Mayo Clinic (n.d.). *High potassium (hyperkalemia)*. 2020; Available from: https://www.mayoclinic.org/symptoms/hyperkalemia/basics/causes/sym-20050776.

214. Sica, D.A., et al., *Hyponatremia in spinal cord injury.* The Journal of the American Paraplegia Society, 1990. **13**(4): p. 78-83.

215. Sweis, R. and J. Biller, *Systemic complications of spinal cord injury.* Current neurology and neuroscience reports, 2017. **17**(1): p. 1-8.

216. De Bold, A., et al., *A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats.* Life sciences, 1981. **28**(1): p. 89-94.

217. Johnson, R. and D. Park, *Effect of change of posture on blood pressure and plasma renin concentration in men with spinal transections.* Clinical Science and Molecular Medicine, 1973. **44**(6): p. 539-546.

218. Wecht, J., et al., *Partial angiotensin–converting enzyme inhibition during acute orthostatic stress in persons with tetraplegia.* The journal of spinal cord medicine, 2005. **28**(2): p. 103-108.

219. Edwards, B.S., et al., *Atrial stretch, not pressure, is the principal determinant controlling the acute release of atrial natriuretic factor.* Circulation research, 1988. **62**(2): p. 191-195.

220. Weissert, R., et al., *MHC haplotype-dependent regulation of MOG-induced EAE in rats.* The Journal of clinical investigation, 1998. **102**(6): p. 1265-1273.

221. Wekerle, H., *Lessons from multiple sclerosis: models, concepts, observations.* Annals of the rheumatic diseases, 2008. **67**(Suppl 3): p. iii56-iii60.

222. Popa, C., et al., *Vascular dysfunctions following spinal cord injury.* Journal of medicine and life, 2010. **3**(3): p. 275.

223. Sauerbeck, A.D., et al., *Spinal cord injury causes chronic liver pathology in rats.* Journal of neurotrauma, 2015. **32**(3): p. 159-169.

224. Liu, X.-H., et al., *Spinal Cord Injury Reduces Serum Levels of Fibroblast Growth Factor-21 and Impairs its Signaling Pathways in Liver and Adipose Tissue in Mice.* Frontiers in endocrinology, 2021. **12**: p. 522.

225. Barbonetti, A., et al., *Low testosterone and non-alcoholic fatty liver disease: Evidence for their independent association in men with chronic spinal cord injury.* The journal of spinal cord medicine, 2016. **39**(4): p. 443-449.

226. Namsolleck, P., et al., *AT2-receptor stimulation enhances axonal plasticity after spinal cord injury by upregulating BDNF expression.* Neurobiology of disease, 2013. **51**: p. 177-191.

227. Edgerton, V.R. and S. Harkema, *Epidural stimulation of the spinal cord in spinal cord injury: current status and future challenges.* Expert review of neurotherapeutics, 2011. **11**(10): p. 1351-1353.

228. Herrity, A., et al., *Lumbosacral spinal cord epidural stimulation improves voiding function after human spinal cord injury.* Scientific reports, 2018. **8**(1): p. 1-11.

229. Harkema, S.J., et al., *Normalization of blood pressure with spinal cord epidural stimulation after severe spinal cord injury.* Frontiers in human neuroscience, 2018. **12**: p. 83.

230. Gad, P., et al., *Development of a multi-electrode array for spinal cord epidural stimulation to facilitate stepping and standing after a complete spinal cord injury in adult rats.* Journal of neuroengineering and rehabilitation, 2013. **10**(1): p. 1-18.

CURRICULUM VITAE

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Education

PhD
Anatomical Sciences and Neurobiology
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September 2021

MS
Anatomical Sciences and Neurobiology
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Bachelor of Science
Biological Sciences, Minor in Chemistry
Southern Illinois University Carbondale, Carbondale, IL
December 2013

Work Experience

Research Technician
Washington University in St. Louis 2014-2016

Employed by Dr. Kenneth Carson, MD, PhD. Clinical research focus is multiple myeloma, non-Hodgkin lymphoma, and leukemia. Duties included data abstraction and entry, data analysis, administrative work, and manuscript/grant writing.

Teaching Assistant- Physiology 301: Gross Human Anatomy
Southern Illinois University School of Medicine 2013

Under instructor Lauren Macklin, MS. Duties included instructing/assisting students through cadaver dissections, setting up, administering, and grading practical examinations.

Undergraduate Research Assistant
Southern Illinois University School of Medicine 2013-2014

Employed with Dr. Joseph Cheatwood, PhD. Research focus is neuroanatomical changes underlying spontaneous and induced recovery of sensory, cognitive, and motor functions after central nervous system injury (i.e. stroke). Primary duties included: qPCR, IHC staining, training rats (bar-walk and reaching), and literature reviews of genes involved in neuronal protection, growth, or repair.

Phlebotomist
Southern Illinois Healthcare, Herrin Hospital, Herrin, IL 2013-2014

Duties include aseptic blood drawing procedures and techniques; determined best site for venipuncture, drawn blood, and labels; perform related clerical and support services within the laboratory.

Undergraduate Research Assistant
Southern Illinois University School of Medicine 2009-2013

Employed by Dr. Buffy Ellsworth, PhD. Research focus was studying the role of *Foxd1* in the development of the pituitary. Duties included: IHC staining, PCR, RT-PCR, histological staining, statistical analysis, and scientific writing.

Activities and Leadership

- Graduate Student Council Representative 2018-2020
- Science Policy and Outreach Group Executive Board (Secretary, President) 2017-2020
- Society for Neuroscience Local Chapter Kentucky Science Center Brain Days 2016-2020
- SIU Research Enriched Academic Challenge (REACH) grant award recipient- 2013
- Fund-raising Chair for Up 'til Dawn St. Jude Children's Hospital Fundraiser 2011-2012
- Program Chair for the SIU Leadership Conference 2012
- Student Researchers in Physiology and Anatomy (SRPA) 2011-2013

Publications

Gumbel J.H., Zipperer L.E., and Hubscher, C.H. (2021). Treatment of spinal cord injury-induced polyuria with NPRA antagonist anantin in chronic contused rats. (In preparation)

Gumbel, J.H., Hubscher, C.H (2021). Effect of T3 level spinal cord injury on upper urinary tract function. (In review)

Hubscher, C.H., **Gumbel, J.H.**, Armstrong, J.E., and Montgomery, L.R. (2021) Impact of activity-based recovery training and desmopressin on spinal cord injury induced polyuria in Wistar rats. (In review)

Gumbel J.H., Yang C.B., and Hubscher C.H. (2021) Timeline of changes in key biomarkers associated with spinal cord injury-induced polyuria after spinal cord injury. *Neurotrauma Reports.* (in press)

Gumbel, J.H., Hubscher, C.H. (2021). Hormonal events and spinal cord injury: a focus on vasopressin and natriuretic peptide. *The Neuroscience of Spinal Cord Injury*. (in press).

Gumbel, J. H., Montgomery, L. R., Yang, C. B., & Hubscher, C. H. (2020). Activity-Based Training Reverses Spinal Cord Injury-Induced Changes in Kidney Receptor Densities and Membrane Proteins. *Journal of neurotrauma*, *37*(3), 555-563.

Gumbel, J. H., Steadman, C. J., Hoey, R. F., Armstrong, J. E., Fell, J. D., Yang, C. B., Montgomery, L. R., Hubscher, C. H. Activity-based Training on a Treadmill with Spinal Cord Injured Wistar Rats. *J. Vis. Exp.* (143), e58983, doi:10.3791/58983 (2019).

Chang, S. H., **Gumbel, J. H.**, Thomas, T. S., Luo, S., Sanfilippo, K. M., Colditz, G. A., & Carson, K. R. (2017). Post-MGUS Diagnosis Serum M-Protein Velocity and the Risk of Progression of MGUS to Multiple Myeloma.

Blue, B. J., Luo, S., Sanfilippo, K. M., Ganti, A., **Gumbel, J**., O'brian, K., & Carson, K. R. (2017). Race-based differences in routine cytogenetic profiles of patients with multiple myeloma. *British journal of haematology*, *176*(2), 322-324.

Sanfilippo, K.M., Keller, J., Gage, B.F., Luo, S., Wang, T.F., Moskowitz, G., **Gumbel, J**., Blue, B., O'Brian, K. and Carson, K.R., (2016). Statins are

associated with reduced mortality in multiple myeloma. *Journal of Clinical Oncology*, *34*(33), p.4008.

Gumbel JH, Patterson EM, Owusu SA et al. "The forkhead transcription factor, Foxd1, is necessary for pituitary luteinizing hormone expression in mice." *PloS one* 7.12 (2012): e52156.

Posters

Gumbel, JH, CB Yang, Hubscher CH. (2019) *Urinary function after acute, sub-acute, and chronic spinal cord injury.* Poster: 50th SfN Annual Meeting, Chicago, IL

- Also presented at Graduate Student Council Research Symposium 2019
- Also presented at KSCIRC Research Symposium

Gumbel JH, Hubscher, CH. (2018). *Activity-based training effects on upper urinary tract function following spinal cord injury.* Poster: 49th SfN Annual Meeting, San Diego, CA

- Also presented at Graduate Student Council Research Symposium 2018
- Also presented at local chapter of SfN Research Meeting

Gumbel JH, Fell JD, Hubscher CH. (2017). *Task-specific training effects on at-level allodynia in a rat model of spinal cord injury.* Poster: 48th SfN Annual Meeting, Washington D.C.

Blue BJ, Sanfilippo KM, Ganti A, **Gumbel J**, O'Brian K**,** Luo S, Carson KC. (2014). *Race-Based Differences in Routine Cytogenetic Profiles of Patients with Multiple Myeloma.* 56th ASH Annual Meeting, San Francisco, CA

Gumbel J, Kabat BE, Hopkins T, Jung DO, Ellsworth BS. (2012). *Foxd1 is Important for Normal Expression of Luteinizing Hormone*. Abstract: 4th Illinois Symposium on Reproductive Sciences, Northwestern University, Chicago, IL

Gumbel J, Jung DO, Ellsworth BS. (2012). *Foxd1 is Important for Normal Expression of Luteinizing Hormone*. Abstract: 45th SSR Annual Meeting, Pennsylvania State University, PA.

Gumbel J, Jung DO, Ellsworth BS. (2011). *The Role of FOXD1 in Normal*

Reproductive Function. Abstract: 3rd Illinois Symposium on Reproductive Sciences,
UIUC, IL

Presentations

Gumbel, JH, (2020) *Impact of spinal cord injury on upper urinary tract function.* KSCIRC Seminar.

Gumbel, JH, (2019) *Urinary function after SCI in male rats: Preliminary findings***.** KSCIRC Seminar.

Gumbel JH, (2018) *Introduction to Spinal Cord Injury research/Research in urinary function after SCI in rats.* Louisville Science Pathway Seminar.