TO PEE OR NOT TO PEE: A CHARACTERIZATION OF CANINE BLADDER PHYSIOLOGY FOLLOWING LONG-TERM LOWER SPINAL ROOT TRANSECTION AND SURGICAL REINNERVATION

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ABSTRACT

Bladder incontinence in patients who suffer from sacral spinal cord injury can wreak havoc on one's quality of life. A 2012 survey suggests that patients who sustain spinal cord injury prioritize the recovery of bladder function over other faculties. With about 12,000 new spinal cord injury cases reported in the United States each year, finding ways to combat the disabilities that result from lower spinal cord dysfunction should be of utmost importance to the scientific research community.

Prior to studying the effects of surgical reinnervation on the bladder after long-term decentralization, it was critical to understand the effects that decentralization had on the integrity of both smooth muscle and intramural nerves of the bladder, the function of which could determine the success of surgical reinnervation. Chapter 2 describes in vivo stimulation, ex vivo smooth muscle contractility studies, and immunohistochemical techniques that were used to assess the condition of the functional components of the bladder. Collective results showed that although pelvic plexus-induced stimulation decreased when decentralization included the bilateral transection of the L7 dorsal root, smooth muscle cells and intramural nerves maintained their function after long-term bladder decentralization. Thus, preservation of at least some nerve activity may allow for successful surgical reinnervation after long-term injury.

Following confirmation of smooth muscle and intramural nerve viability after decentralization, we sought to determine if nerve transfer after long-term decentralization restores bladder function in canines. In Chapter 3, we detail both decentralization and surgical reinnervation procedures used in our model. Briefly, decentralization of the bladder included bilateral transection of hypogastric nerves, as well as all spinal roots.
caudal to L7, with a subset of animals undergoing additional transection of the dorsal root
of L7. One year after decentralization, animals that showed consistent loss of sensory and
motor function underwent surgical reinnervation, which included the bilateral transfer of
part of the obturator nerve to the anterior vesical branch of the pelvic nerve and the
semimembranosus branch of the sciatic nerve to the pudendal nerve. Behavioral
observations, in vivo stimulation of transferred nerves, and retrograde tracing studies
were used to explore the efficacy of reinnervation on both sensory and motor components
of bladder function. Ultimately, results showed that the new neuronal pathways created
by nerve transfer can restore bladder sensation and possibly motor function in lower
motor neuron-lesioned canines.

Beyond the effects of surgical reinnervation on bladder function, we were
interested in taking a closer look at the mechanisms that dictate function after
decentralization and reinnervation (Chapter 3). Based on our previous work that found
that transfer of somatic nerves resulted in bladder smooth muscle expression of a
nicotinic receptor subunit thought to be expressed primarily in striated muscles, we were
interested in assessing changes in the profile of nicotinic receptors responsible for bladder
function. Ex vivo smooth muscle contractility studies showed that response to nicotinic
receptor agonists were not altered after decentralization or reinnervation. Furthermore,
the $\alpha_1$ nicotinic receptor subunit was expressed in bladder smooth muscle across all
surgical groups. Future studies are necessary to better elicit the physiological relevance of
these nicotinic receptors in the bladder.

Additionally, due to the complexity of surgical reinnervation, it was important to
understand all contributions to bladder innervation (Chapter 4). We previously identified
that cells in the ventral horns of spinal cord levels rostral to the sacral cord can directly innervate the bladder via retrograde tracing. Because these direct inputs were not in proximity of the spinal root transections made during decentralization, we wanted to know how decentralization and reinnervation impacted their effects on the bladder when stimulated. L2-mediated detrusor contractions were significantly decreased by transection of the hypogastric nerves, suggesting that many of the nerves originating from the L2 cord are sympathetic in nature; however, treatment with phentolamine did not completely eliminate the increase in pressure in response to L2 stimulation. Therefore, the remaining inputs likely act upon the bladder through a yet undefined pathway. The quantity of positively labelled cells did not change in sections of the L2 ventral horn across all surgical groups, suggesting no change in the contribution of direct inputs to bladder innervation.

Finally, anatomical feasibility of the obturator and semimembranosus branch of the sciatic nerve transfers has been assessed in an unembalmed cadaver, the results of which have not yet been published. Overall, this research gives us reason to believe that surgical reinnervation is a viable option for patients who develop lower neurogenic bladder after injury to the sacral cord, cauda equina, or peripheral nerves mediating bladder function.
DEDICATION

To my best friend, Louie, whose love and support sustain me.
ACKNOWLEDGEMENTS

I found home in the labs of Dr. Michael Ruggieri, Sr. and Dr. Mary Barbe, my co-
mentors over the past few years. Their support and generosity were the fuel necessary to complete the project presented in this dissertation.

Dr. Ruggieri taught me to how to critically think about the pharmacology implemented not only within my experiments, but also beyond the lab as I move on to clinical practice. His “see one, do one, teach one” philosophy required that I quickly embrace the challenges of surgery, which I grew to enjoy with each passing procedure. He also constantly reminded me to avoid making assumptions, but instead to find evidence that would strengthen my ideas, which would ultimately make me a better scientist and prospective physician. I also enjoyed his dad jokes more than I like to admit.

Dr. Barbe showed me the beauty in slowing down to appreciate what tissue had to say. Her expertise in anatomy, physiology, immunohistochemistry, and microscopy knows no bounds, which drove me to work harder and be better for myself and those around me. She also regularly encouraged me to participate in scientific writing and presentation, which ultimately helped me learn how to critically think about the words I put to paper. Dr. Barbe is a brilliant model for women in science and I cannot be more grateful to have had the privilege of being her mentee.

I will miss the kind, yet constructive feedback I consistently received from my thesis committee: Dr. T. Dianne Langford, Dr. Michel Lemay, and Dr. Scott Rawls. Committee meetings were always an enjoyable experience, despite the stress leading up to them. I additionally appreciate the time and effort put forth by my external reader, Dr. Michel Pontari.
A special thank you to all past and present lab members: Mamta Amin, Dr. Nagat Frara, Dr. Ekta Tiwari, Dr. Alan Braverman, Luke Hobson, Geneva Cruz, Dr. Brendan Hillard, Dr. Amanda White, Joseph Tarr, Michele Harris, Dr. Michael Mazzei, and Dr. Oneida Arosarena. It was a privilege to have such a wonderful community in which to work and think. I’ll miss the silly moments the most.

Furthermore, I’m grateful for my MD/PhD academic advisor, Dr. Dianne Soprano, and all of my classmates for their support and feedback over the past several years. The challenges of graduate school were made easier by guidance from students who preceded me, encouragement from my immediate classmates, and enthusiasm from those who follow in my footsteps.

I must also mention my family—my parents Allison and Richard, as well as my sisters Lauren, Melissa, and Alicia—whose unconditional patience, love, and encouragement made the most frustrating moments bearable. And to my friend Amanda Stahl, who provided undivided attention when I needed a fresh perspective.

Lastly, I could not have done any of this without my fiancé, Louie Porreca. He has been in my corner since my pre-med years and has seen me through all highs and lows. Thank you for helping me shoulder the weight of it all. We make the best team.
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<tr>
<td>ACh</td>
<td>acetylcholine</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>Ca/CaM</td>
<td>Ca(^{2+}) / calmodulin complex</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole, dihydrochloride</td>
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<tr>
<td>DSD</td>
<td>detrusor-sphincter dyssynergia</td>
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<td>EFS</td>
<td>electric field stimulation</td>
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<td>EUS</td>
<td>external urethral sphincter</td>
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<tr>
<td>FG</td>
<td>Fluorogold</td>
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<tr>
<td>KCl</td>
<td>potassium chloride</td>
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<tr>
<td>L</td>
<td>lumbar</td>
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<tr>
<td>LMN</td>
<td>lower motor neuron</td>
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<tr>
<td>LUT</td>
<td>lower urinary tract</td>
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<tr>
<td>MACHR</td>
<td>muscarinic cholinergic receptor</td>
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<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
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<tr>
<td>NACHR</td>
<td>nicotinic cholinergic receptor</td>
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<tr>
<td>NE</td>
<td>norepinephrine</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<td>ON</td>
<td>Onuf’s nucleus</td>
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<tr>
<td>PAG</td>
<td>periaqueductal grey</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PMC</td>
<td>pontine micturition center</td>
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<tr>
<td>PUSC</td>
<td>pontine urine storage center</td>
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<tr>
<td>S</td>
<td>sacral</td>
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<tr>
<td>SCI</td>
<td>spinal cord injury</td>
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<tr>
<td>SEM</td>
<td>standard error of mean</td>
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<td>SG</td>
<td>spinal ganglia</td>
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<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<td>T</td>
<td>thoracic</td>
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<td>TTX</td>
<td>tetrodotoxin</td>
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<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
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<tr>
<td>UI</td>
<td>urinary incontinence</td>
</tr>
<tr>
<td>UMN</td>
<td>upper motor neuron</td>
</tr>
<tr>
<td>UTI</td>
<td>urinary tract infection</td>
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<tr>
<td>VIP</td>
<td>vasoactive intestinal polypeptide</td>
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CHAPTER 1

INTRODUCTION

1.1 Motivation for Study

Urinary incontinence (UI) is one of many challenges faced by those who sustain cauda equina and spinal cord injury (SCI).\(^1\) Of the 12,000 new cases of SCI each year, up to 84% will result in neurogenic lower urinary tract dysfunction.\(^2,3\) The International Continence Society defines UI as the “involuntary loss of urine that is a social or hygienic problem.”\(^4\) In other words, UI can wreak havoc on one’s quality of life. Complications due to bladder dysfunction, one of the leading comorbidities in SCI patients,\(^5\) include septicemia following urinary tract infections and kidney failure.\(^6\) Furthermore, due to the social implications of UI such as embarrassment and dependence on others, patients often experience anxiety and depression.\(^3\) Thus, it is no surprise that patients who sustain SCI prioritize recovery of bladder function above most other bodily functions.\(^1\) UI also carries a national economic impact of $65.9 billion per year,\(^7\) a large portion of which stems from complications of neurogenic bladder.\(^8\) The scientific community should therefore emphasize the study of effective long-term treatments for UI after SCI.

Current treatment for SCI-induced UI (i.e. neurogenic bladder) include indwelling or intermittent catheterization\(^9\) and pharmacological intervention such as anticholinergics,\(^10\) both of which fail to target the neurogenic source of the pathology. To combat the current imprecise treatments of neurogenic bladder, the labs of Dr. Ruggieri and Dr. Barbe study the surgical reinnervation of the bladder following long-term lower spinal root injury in canines.
Over the past couple of decades, we have explored reinnervation using a variety of donor nerves. It was determined that following transection of sacral roots innervating the bladder, immediate reinnervation via transfer of coccygeal spinal roots to transected sacral roots or peripheral genitofemoral nerve transfer to the pelvic nerves can recover bladder function.\textsuperscript{11} To better represent the timeframe in which patients may seek surgical intervention for neurogenic bladder following SCI, it was shown that both genitofemoral nerve\textsuperscript{12} and femoral nerve transfer to the pelvic nerve of the bladder up to three months after sacral root transection can also recover bladder function.\textsuperscript{13,14} The labs now pursue a reinnervation model that includes bilateral transfer of the obturator nerve to the anterior vesical branch of the pelvic nerve and a branch of the sciatic nerve to the pudendal nerve with the goal of reinnervating both the bladder and the external urethral sphincter, functional components of the lower urinary tract.

As best described by one of my mentors, this pursuit is “as translational as it [biomedical science] gets.” We have the opportunity to work closely with peripheral neurosurgeons and urologists who would ultimately implement the developed surgical treatment in the clinic.

1.2 Specific Aims and Hypothesis

The following dissertation is one of many components that make up the efforts put forth by the labs of Dr. Ruggieri and Dr. Barbe to reinnervate the bladder after long-term spinal root injury. The current canine model under investigation includes long-term decentralization of the canine bladder via bilateral transection of all roots caudal to L7, the dorsal roots of L7, and the hypogastric nerves. This decentralization technique is a controlled, reproducible procedure that mimics lower motor neuron lesions that may
cause neurogenic bladder such as lower spinal cord/cauda equina injury, spina bifida, and multiple sclerosis. After one year, the bladder and external urethral sphincter (EUS) are surgically reinnervated via bilateral transfer of the obturator nerve to the anterior vesical branch of the pelvic nerve and a branch of the sciatic nerve to the pudendal nerve respectively.

1.2.1 Aim 1

Although our group and others have extensively explored the feasibility of surgical nerve transfer to treat neurogenic bladder, to date, nobody has assessed the function of individual bladder-nerve components following long-term lower spinal root injury and prior to intervention. We aim to determine the viability of both smooth muscle and intramural nerves following year-long bilateral transection of all sacral roots, the dorsal root of L7, and hypogastric nerves (i.e. decentralization), the health of which would determine the success of future bladder reinnervation.

We hypothesize that the integrity of bladder smooth muscle contractility and intramural nerve function will remain intact after long-term decentralization.

1.2.2 Aim 2

In prior studies, we confirmed motor reinnervation of the decentralized canine urinary bladder via observation of increased detrusor pressure after electrical stimulation of the transferred nerve, such as the genitofemoral or femoral nerve, which was sutured end-to-end to the anterior vesical branch of the pelvic nerve. Behavioral observations provide the most global assessment of whether or not the animals have been able to recover. To prove that surgical reinnervation is responsible for restoring sensory and
motor bladder function, it is necessary to demonstrate that these behaviors are not observed in decentralized animals that have not undergone nerve transfer surgeries in addition to evaluation via in vivo nerve stimulation and neuronal tracing techniques. Our aim here is to determine the success of bladder reinnervation via behavioral, electrophysiological, and immunohistochemical techniques. It is important to note that this aim is co-addressed in the dissertation of Ekta Tiwari, a graduate student of the College of Engineering at Temple University.

*We hypothesize that surgical reinnervation via bilateral transfer of the obturator nerve to the anterior vesical branch of the pelvic nerve and a branch of the sciatic nerve to the pudendal nerve can recover both sensory and motor function of the bladder following long-term decentralization.*

1.2.3 Aim 3

One of our recent studies discovered that succinylcholine, a depolarizing neuromuscular nicotinic receptor antagonist, blocked smooth muscle detrusor contraction despite stimulation of the transferred genitofemoral or femoral nerves, but failed to do so in sham-operated control animals. Immunohistochemistry for neuromuscular nicotinic receptor subunit α1, a subunit thought to only exist in neuromuscular nicotinic receptor subtypes, showed immunopositive puncta on the surface of smooth muscle fascicles, which had not been previously identified in bladder tissue. Phenotypic changes in the receptors responsible for bladder activity post reinnervation would require the reconsideration of drugs that physicians may use to maintain recovered function. We aim to determine changes in the profile of cholinergic nicotinic receptors as a result of long-
term lower spinal cord injury and surgical reinnervation via obturator-to-pelvic nerve and 
sciatic-to-pudendal nerve transfer.

*We hypothesize that the bladder undergoes local and functional changes in 
nicotinic receptor expression after long-term spinal root injury and surgical 
reinnervation.*

### 1.2.4 Aim 4

Micturition and urine storage are complex activities primarily driven by 
parasympathetic innervation from the sacral spinal cord and sympathetic innervation 
from the lower thoracic and upper lumbar spinal cord respectively. Additionally, we 
previously found that the detrusor is also innervated by a small number of direct inputs 
originating in the lower thoracic and upper lumbar ventral horns, the function of which is 
not yet understood. It is critical to understand all contributors to the neuroanatomy that 
dictate bladder function so that we can better interpret the effects of surgical 
reinnervation on the decentralized canine bladder. We aim to study these inputs using in 
vivo nerve stimulation and retrograde neuronal tracing techniques.

*We hypothesize that the L2 root carries direct, non-sympathetic innervation to the 
bladder that remains undisturbed following bladder decentralization and surgical 
reinnervation.*

### 1.3 Review of Relevant Literature

#### 1.3.1 Lower Urinary Tract Anatomy

The lower urinary tract (LUT) has two functional components: the urinary bladder 
and the bladder outlet. The former is a smooth muscle organ that can store and expel
urine produced by the kidneys. The latter—made up of the bladder neck, urethra, and external urethral sphincter (EUS)—is the channel through which urine exits the body.\(^{15}\)

The wall of the bladder is made up of four layers. The inner mucosa, or urothelium, is a transitional epithelial layer of cells that maintains urine composition and serves as a barrier against infectious organisms.\(^{16}\) A submucosa separates the mucosa and the detrusor, a layer of interweaving bundles of smooth muscle responsible for bladder contraction (Figure 1.1).\(^{17,18}\) The smooth muscle layer contains three layers of smooth muscle, where the cells in the outer and inner layers are oriented longitudinally and those in the middle layer are oriented circularly.\(^{15}\) The outer bladder adventitia is the connective tissue that interfaces with other structures of the abdomen.

The bladder can be divided into three anatomical regions from top to bottom: the dome, the body, and the neck. The bladder trigone is located on the dorsal aspect of the bladder between the ureteral orifices and the neck of the bladder.\(^{19}\) The neck along with

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**Figure 1.1. Normal histology of the bladder wall.** A. Urothelium indicated with a dashed line. Deep to the urothelium is the submucosa, which contains numerous blood vessels and connective tissue. The muscularis mucosae contains an abundance of smooth muscle bundles in both longitudinal and circular orientation. B. Higher magnification to highlight individual smooth muscle cells (SM). Copyright (2012) Wiley. Modified and used with permission from (Cheng et al., Chapter 1. Normal Anatomy and Histology, *Bladder Pathology* and John Wiley & Sons, Inc.)
the surrounding EUS, a voluntarily controlled striated muscle, and the urethra are the caudal most components of the LUT.

1.3.2 Smooth Muscle Physiology

1.3.2.1 Functional Components of Bladder Smooth Muscle

Within the wall of the bladder, smooth muscle cells are arranged in bundles, or fascicles, and work in concert to efficiently contract the bladder. Each smooth muscle cell contains contractile proteins actin and myosin, which are structurally connected in a network by cytoplasmic dense bodies containing the protein α-actinin. Actin has a closely associated structural protein, tropomyosin, as well as the two regulatory proteins caldesmon and calponin. The smooth muscle cell also contains a dense sarcoplasmic reticulum (SR) that serves as a storage site of Ca²⁺.

1.3.2.2 Smooth Muscle Contraction

The interaction between actin and myosin is critical to the contractile property of the muscle. When the intracellular concentration of Ca²⁺ increases, either from the extracellular space through ion channels or from the intracellularly located SR, Ca²⁺ binds to calmodulin to form a complex (Ca/CaM). Ca/CaM then acts through two mechanisms: it inhibits calponin and caldesmon to allow movement of tropomyosin, which exposes the myosin phosphorylation site; and it activates myosin light chain kinase (MLCK), which phosphorylates myosin and stimulates the ATPase activity of myosin. Myosin can then hydrolyze attached ATP, which allows for interaction between myosin and actin, ultimately leading to smooth muscle contraction.
1.3.2.3 Smooth Muscle Relaxation

Relaxation of smooth muscle is mediated by myosin light chain phosphatase, which dephosphorylates myosin, thus blocking its ATPase activity to allow for relaxation.\textsuperscript{26} Ca/CaM also breaks down into Ca\textsuperscript{2+} and free calmodulin, which initiates the reactivation of caldesmon and calponin, thus moving tropomyosin into a position that blocks the phosphorylation site of myosin and, ultimately, any interaction between actin and myosin.\textsuperscript{24} Ca\textsuperscript{2+} also returns to the SR or leaves the cells through a variety of channels.\textsuperscript{26}

1.3.3 Receptor Signaling in the Bladder

Both urine storage and micturition are regulated by a variety of receptors that coordinate the contraction or relaxation of muscular components of the LUT.

1.3.3.1 Muscarinic Receptor Cholinergic Activity

Bladder emptying is generally dictated by the activities of the neurotransmitter acetylcholine (ACh), which can interact with a variety of cholinergic receptor subtypes. More specifically, bladder smooth muscle membranes are lined with G protein-coupled muscarinic ACh receptors (MACHR), which induce smooth muscle contraction when activated. While M\textsubscript{3} receptors are thought to be the primary receptor subtype expressed in the bladder,\textsuperscript{18,27} additional studies have provided evidence for the functional importance of M\textsubscript{2} receptors.\textsuperscript{28-30} Each subtype has a different mechanism of action: M\textsubscript{3} receptors triggers an increase in intracellular Ca\textsuperscript{2+} and thus direct smooth muscle contraction via activation of phospholipase C through the G\textsubscript{q} protein family;\textsuperscript{31} in contrast, the M\textsubscript{2} receptor selectively couples with and inhibits adenylyl cyclase through the G\textsubscript{i} protein.
family, which in turn inhibits sympathetic β-adrenoceptor-mediated relaxation of smooth muscle.\textsuperscript{32}

1.3.3.2 Purinergic Receptor Activity

While cholinergic signaling predominantly mediates bladder contraction, purinergic signaling can also induce contraction. Adenosine triphosphate (ATP) can act through P2X ligand-gated ion channels, which allows for the influx of Na\textsuperscript{+} and Ca\textsuperscript{2+}.\textsuperscript{33} It’s important to note that while purinergic signaling plays an important role in bladder contractility in many mammalian species,\textsuperscript{34} human bladders primarily contract via cholinergic signaling\textsuperscript{35} unless a pathology such as detrusor overactivity or interstitial cystitis is present.\textsuperscript{36,37}

1.3.3.3 Adrenergic Receptor Activity

Bladder relaxation is primarily mediated by norepinephrine (NE)-induced activation of β-adrenoceptors of the detrusor\textsuperscript{38} via the G\textsubscript{s} protein family. When G\textsubscript{s} interacts with adenylyl cyclase, cAMP levels increase and activate cAMP-dependent protein kinase A, which goes on to phosphorylate myosin light chain, thus blocking Ca\textsuperscript{2+}-mediated smooth muscle contraction.\textsuperscript{39} Studies have shown that the bladder contains β\textsubscript{1},\textsuperscript{40} β\textsubscript{2},\textsuperscript{41} and β\textsubscript{3} subtypes.\textsuperscript{40} It is important to note that sympathetic signaling does not exclusively mediate the relaxation of bladder smooth muscle. Contraction-mediating α-adrenoceptors have also been identified throughout the detrusor and trigone.\textsuperscript{42}

1.3.3.4 Nicotinic Cholinergic Receptor Activity

Nicotinic cholinergic receptors (NACChR) have been identified as regulators of synaptic transmission in bladder ganglia.\textsuperscript{43} These receptors are pentameric ion channels,
which are either homomeric or heteromeric, can be composed of a variety of subunits. Subunits identified in mammals include $\alpha_{1-7, 10}$, $\beta_{1-4}$, $\delta$, $\epsilon$, and $\gamma$. When activated, they permit the influx of $\text{Na}^+$ and $\text{Ca}^{2+}$. NACHR found in individual tissue types tend to express certain subunits. For example, neuromuscular NACHR are exclusively made up of two $\alpha_1$, one $\beta_1$, one $\delta$, and one $\epsilon$ subunits.\textsuperscript{44} Further breakdown of identified tissue-specific receptor subtypes are described in Figure 1.2. A previous study found that $\alpha_3$ and $\beta_4$ NACHR subunits are necessary for normal bladder function.\textsuperscript{43} The ganglionic nicotinic receptors are thought to enhance mechanosensitive bladder afferent nerve activity.\textsuperscript{45}

1.3.3.5 Additional Receptor Signaling

Nitric oxide (NO) is a gaseous neurotransmitter that also plays a role in bladder smooth muscle relaxation, although the mechanism of action is less clear.\textsuperscript{15}
Neuropeptides can also modulate bladder contractility. For example, vasoactive intestinal polypeptide (VIP) has been found to inhibit spontaneous contractile activity of the detrusor. In contrast, endothelins, tachykinins, and angiotensins can induce smooth muscle contraction can induce detrusor smooth muscle contractions to varying degrees. Another class of molecules, prostanoids, has been implicated in the maintenance of detrusor tone.

1.3.4 Relevant Neuroanatomy

The nervous system can be broken down into two anatomical components: the central nervous system, composed of the brain and spinal cord; and the peripheral nervous system, comprised of all nervous tissue outside of the central nervous system, including the dorsal and ventral roots that immediately emerge from the spinal cord. The dorsal roots carry sensory afferents into a dorsal root ganglion (or spinal ganglion in canines), while ventral roots carry motor output from the spinal cord to the rest of the body. They join to form the spinal nerve, where sensory and motor nerves will cross and form the ventral and dorsal rami to carry both sensory and motor fibers to their end organs.

It is important to note that canines have eight cervical, twelve thoracic, seven lumbar, and three sacral vertebral levels that have corresponding dorsal and ventral roots. The spinal cord terminates at the L6-L7 vertebral junction and begins to form the cauda equina, or sacral roots without corresponding spinal cord segments.
1.3.5 Functional Neuroanatomy of the Lower Urinary Tract

1.3.5.1 Peripheral Innervation of the Bladder

Beyond the immediate receptor signaling that takes place in the bladder, LUT function relies upon a network of neurocircuitry coordinated by the brain and spinal cord. Both autonomic and somatic innervation play critical roles in LUT physiology. During bladder filling, sympathetic input via hypogastric and splanchnic nerves regulates the relaxation of bladder smooth muscle in coordination with somatic nerve-mediated contraction of the EUS primarily through pudendal nerves, thus preventing urine from exiting the organ. Micturition occurs when parasympathetic activity through pelvic nerves induces simultaneous smooth muscle contraction and inhibition of EUS activity. While neuroanatomy can vary between individuals within a species, the origination of nerves that dictate bladder function are well documented. Preganglionic sympathetic cell bodies are found in the thoracic (T)11-lumbar (L)2 spinal segments while preganglionic parasympathetic neurons and somatic afferents to the EUS originate in the sacral (S)1-S3 spinal segments (Figure 1.3).

1.3.5.2 Central Nervous System Regulation of the Bladder

There are numerous pathways within the central nervous system that modulate the aforementioned peripheral nerves that carry action potentials to bladder components. The spinobulbospinal voiding reflex, the most rostral end of which is found in the periaqueductal grey (PAG) of the brain stem, regulates the switch between urine storage and voiding. As the bladder fills, the PAG sends signals to the pontine micturition center (PMC), which, when threshold is met, activates parasympathetic sacral spinal nuclei to initiate voiding via bladder smooth muscle contraction. PMC neurons
Figure 1.3. Diagram of typical sensory, sympathetic, parasympathetic and somatic motor innervation of the bladder and external urethral sphincter (EUS) in dogs. Sensory input (dotted black arrow) project to SG at L6-S2 of the spinal cord from the bladder and S1-S2 from the EUS. Preganglionic sympathetic axons (dashed red arrow) to lower urinary tract originate from preganglionic neurons in lumbar spinal cord segments and contribute to: 1) sympathetic trunk ganglia along the vertebral column; 2) lumbar splanchnic nerves to the caudal mesenteric ganglia via the hypogastric nerve. Postganglionic sympathetic axons to the bladder originate mostly from L7-S2 sympathetic ganglia and caudal mesenteric ganglion. Sensory and sympathetic axons hitchhike on other nerve bundles and arteries. Parasympathetic innervation (solid blue arrow) originates primarily from S1-S3 and often synapse on pelvic plexus ganglia adjacent to the bladder. The EUS is innervated by somatic motor inputs (solid green arrow) originating from the sacral cord. The function of direct inputs from the lower thoracic and upper lumbar spinal cord (solid grey arrow) to the bladder is currently unknown. EUS = external urethral sphincter; CMG = caudal mesenteric ganglion (referred to as the inferior mesenteric ganglion in human anatomy); PPG = Pelvic Plexus Ganglia (multiple) located in mesenteries of bladder. Figure used with permission from Barbe et al., 2018.
also project to GABAergic inhibitory interneurons that synapse on EUS-contracting motoneurons within the sacrally located Onuf’s nucleus (ON), thus causing relaxation.\textsuperscript{59} Additionally, the sympathetic storage reflex is triggered by sensory afferents from the bladder to the lumbar preganglionic sympathetic neurons, which synapse on the bladder and signal relaxation.\textsuperscript{60} ON motoneurons also exhibit tonic activity that increases as the bladder fills to keep the EUS contracted and the urethra closed.\textsuperscript{61} The pontine urine storage center (PUSC) also inhibits PMC activity and further stimulates ON motoneurons to ensure conditions required during bladder filling.\textsuperscript{62}

1.3.6 Neurogenic Bladder

Neurogenic bladder is made up of a constellation of lower urinary tract symptoms that arise following an injury to the brain, spinal cord, or peripheral innervation of the LUT. Due to the complexity of bladder innervation, there are a few types of neurogenic bladder, depending on where the lesion occurs. While this dissertation will emphasize treatment for the type that occurs when the lower spinal roots and cauda equina are injured, it is important to understand differences between each type.

1.3.6.1 Uninhibited Bladder

Following a stroke or the development of a brain tumor above the pontine micturition center, a patient may experience uninhibited neurogenic bladder dysfunction in which there is a reduced awareness of bladder fullness and a decreased bladder capacity.\textsuperscript{63}
1.3.6.2 Upper Motor Neuron Neurogenic Bladder

A traumatic spinal cord injury or multiple sclerosis with plaques occurring between the PMC and the sacral cord can result in upper motor neuron (UMN) neurogenic bladder dysfunction,\(^\text{63}\) which is characterized by the presence of either intermittent or continuous detrusor-sphincter dyssynergia (DSD).\(^\text{64}\) Patients with DSD experience simultaneous detrusor and EUS contraction (rather than EUS relaxation, which is necessary for the passage of urine), and thus an increase in intravesical pressure and a consistent lack of complete voiding. Long-term UMN neurogenic bladder can lead to increased occurrence of urinary tract infections (UTI), lithiasis, and ultimately kidney damage.\(^\text{65}\)

1.3.6.3 Lower Motor Neuron Neurogenic Bladder

Injury to the lower spinal cord, cauda equina, or peripheral nerves that mediate bladder function may result in lower motor neuron (LMN) neurogenic bladder, also known as detrusor areflexia, due to the loss of parasympathetic input to the bladder while sympathetic input remains intact.\(^\text{63,66}\) The bladder capacity typically increases due to the loss of tone in the detrusor. As a result, patients often experience overflow incontinence and urinary tract infections.\(^\text{63}\)

1.3.7 Current Treatments for Neurogenic Bladder

1.3.7.1 Non-Pharmacological Treatments

Intermittent catheterization, performed independently or with aid, is the standard method by which patients with neurogenic bladder that includes partial or complete urinary retention manage bladder emptying.\(^\text{67}\) Patients may also be taught behavioral
bladder emptying techniques such as the Credé maneuver (physical pressure placed on the abdomen to aid expression), Valsalva maneuver, triggered reflex voiding (suprapubic tapping or cutaneous stimulation that initiates voiding in a subpopulation of patients).\textsuperscript{68} Fluid scheduling, which limits the amount of fluid a patient intakes during meals and throughout the day, becomes an important part of the daily routine to avoid bladder distention.\textsuperscript{63} Absorbent incontinence pads may also be used, but they increase the risk of skin infection if changed infrequently.

\textit{1.3.7.2 Pharmacological Treatments}

Anticholinergic drugs that block M\textsubscript{AChR} activity, the receptor primarily responsible for bladder contraction, are used to treat detrusor overactivity as a result of UMN neurogenic bladder. Although effective, antimuscarinics have a variety of undesirable side effects including dry mouth, blurred vision, tachycardia, hyperthermia, and mental confusion in the elderly.\textsuperscript{69} Such treatment is contraindicated in patients who sustain LMN injuries due to the loss of parasympathetic input to the bladder, and thus decreased M\textsubscript{AChR} activity.

Alpha-2 adrenergic agonists such as clonidine and tizanidine can be prescribed for patients with DSD after UMN injury to relax the internal urethral sphincter.\textsuperscript{63} This class of drugs causes reduced presynaptic release of NE release, which reduces the overall sympathetic input to the neck of the bladder, thus allowing for a decrease contraction.\textsuperscript{70} Side effects experienced by patients include fatigue, dizziness, lightheadedness, dry mouth, constipation, and occasionally cardiac dysrhythmias and depression.\textsuperscript{63}

Although rarely prescribed, botulinum toxin injections into the detrusor serve as another treatment for those who experience DSD. This effectively blocks the release of
ACh at neuromuscular junctions\textsuperscript{71} and attenuates activity at sensory nerve endings in the bladder.\textsuperscript{72}

\subsection*{1.3.7.3 Sacral Neuromodulation}

Patients with DSD and detrusor overactivity may also benefit from sacral neuromodulation, a treatment in which programmed electrodes are implanted typically within the S3 sacral foramina to modulate afferent and efferent signals propagating across the region.\textsuperscript{57,73} A recent systematic review suggested that further investigation is necessary to solidify sacral neuromodulation as a treatment for types of neurogenic bladder that result from UMN spinal cord injuries.\textsuperscript{74}

\subsection*{1.3.7.4 Limitations to Lower Motor Neuron Neurogenic Bladder Treatment}

Unlike detrusor overactivity, treatments for flaccid bladder following LMN spinal injury is under-investigated.\textsuperscript{75} Although treatment with parasympathomimetics such as bethanechol or carbachol is available, it has minimal efficacy\textsuperscript{76} with costly side effects such as bradycardia and pulmonary edema.\textsuperscript{77} Unfortunately, most patients are left with intermittent catheterization as their only treatment option.\textsuperscript{78}

\subsection*{1.3.8 Surgical Bladder Reinnervation Models}

Investigators have pursued surgical strategies of bladder reinnervation for many decades with varying success. Both homotopic and heterotopic repair, methods that involve either direct repair of the injury or the use of a surrogate nerve to circumvent the injury, have been explored. Additionally, peripheral nerve transfers have been pursued to reinnervate the bladder.
1.3.8.1 Homotopic Repair

One study explored homotopic restoration in the feline whereby the S1 and S2 ventral roots were transected bilaterally, and then immediately repaired via end-to-end realignment and implantation of a steel, mesh cylinder to keep repair intact.\textsuperscript{79} Similarly, another study included bilateral transection of S2-S4 ventral or dorsal roots followed by immediate end-to-end repair in the porcine model.\textsuperscript{80} In both studies, bladder function recovered approximately 4-7 months after the initial injury.\textsuperscript{79,80} Our lab also investigated homotopic repair in canines following bilateral transection of S1 and S2 dorsal and ventral roots. To determine the efficacy of nerve growth factors on recovery of function, one side received repair that included only suturing of the disrupted epineurium while the opposite side contained an additional silicone sheath that supplied brain-derived neurotrophic factor (BDNF) to the site of injury for two weeks after the repair. We concluded that the inclusion of BDNF hindered axonal regrowth due to the formation of neuromas.\textsuperscript{81}

1.3.8.2 Heterotopic Repair

Heterotrophic surgical techniques in a variety of animal models have been under investigation for over a century.\textsuperscript{82} Some of the first experiments explored the transfer of the proximal ends of ipsilateral spinal roots to the distal ends of transected roots innervating the bladder. Electrical stimulation of the proximal donor roots resulted in partial expulsion of urine\textsuperscript{83} A similar study in felines was later conducted where L6 and L7 ventral roots were transferred to S1 and S2 ventral roots bilaterally. Histological experiments revealed axons penetrating the distal transected roots, suggesting possible reinnervation.\textsuperscript{79} More recently, our lab has explored heterotopic repair via transfer of
coccygeal nerves that exclusively caused tail contractions upon stimulation to transected S1 and S2 ventral roots. Retrograde dye injections confirmed regrowth of coccygeal donors into the site of transection.11

1.3.8.3 Peripheral Nerve Transfer

Similar to heterotopic repair, bladder reinnervation via peripheral nerve transfer has been extensively studied by our group and others. An early canine study included unilateral transfer of the proximal end of the hypogastric nerve to the distal end of a pelvic splanchnic and, months later, transfer of the obturator nerve to the pelvic nerve on the opposite side. Both procedures resulted in newly established pathways that induced bladder contractions when stimulated.84 A later murine study repeated the study, but saw no recovery of function after hypogastric nerve transfer and some recovery after obturator nerve transfer.85

Our lab has explored a variety of somatic nerve transfers to the pelvic nerve over the past couple of decades. We used the genitofemoral nerve, a mixed somatosensory nerve that originates from the L1-L2 spinal levels, as a donor one month or three months after transection of all ventral and dorsal sacral roots. Although no decentralized controls were included in this study for comparison, pelvic nerve stimulation induced an increase in bladder pressure. Furthermore, positively labelled cells were identified in the L1-L2 lateral ventral horns and in the zona intermedia of upper lumbar spinal cord three weeks after the bladder was injected with retrograde dye, suggesting that the genitofemoral nerves reinnervated the bladder.12 We also explored the feasibility of EUS reinnervation by transfer of the femoral nerve, a somatic nerve that originates to from the L2-L4 spinal levels, the transected pudendal nerve. Stimulation of the transferred femoral nerve
increased EUS sphincter and positive labelling was found in L2-L4 spinal cord after injection of the EUS.\textsuperscript{13}

Our most recent published study explored the effectiveness of somatic nerve transfer to the anterior vesical branch of the pelvic nerve compared to mixed somatosensory nerve transfer, the femoral nerve compared to the genitofemoral nerve respectively. While both donor types elicited detrusor contraction upon stimulation, the strength of the femoral nerve stimulation-induced contraction was significantly higher than that of the genitofemoral nerve, suggesting that somatic nerves may be more suitable for transfer.\textsuperscript{14} These findings provide justification for using the primarily somatic obturator and sciatic nerves for transfer to the anterior vesical branch of the pelvic nerve and the pudendal nerve respectively.

\textit{1.3.8.4 Why Canines?}

We have chosen to develop our reinnervation technique in the canine model for a number of reasons: 1. Similar to human bladders,\textsuperscript{86} canine bladders contain numerous intramural ganglia,\textsuperscript{87} the activity of which modulates bladder function. Murine bladders, in contrast, do not contain intramural ganglia.\textsuperscript{88,89} Furthermore, transection of the major pelvic ganglia bilaterally in rats results in bladder denervation,\textsuperscript{90} a result not found in the human or canine model. 2. As detailed by the literature review of surgical bladder reinnervation models, our lab has had extensive experience with a variety of decentralization and reinnervation techniques in the canine and thus possesses the skills necessary to move forward with this project.
CHAPTER 2

DETERMINING INTEGRITY OF BLADDER INNERVATION AND SMOOTH MUSCLE FUNCTION ONE YEAR AFTER LOWER SPINAL ROOT TRANSECTION IN CANINES*

*Extracted from previously published work in which Salvadeo DM was the first author and aided with all surgeries, as well as smooth muscle contractility studies, and performed all immunohistochemical assays. Salvadeo DM, Tiwari E, Frara N, et al. Determining integrity of bladder innervation and smooth muscle function 1 year after lower spinal root transection in canines. Neurourology and Urodynamics. 2018; 37:2495–2501. https://doi.org/10.1002/nau.23765

Overview

Our aim was to assess bladder smooth muscle function and innervation after long-term lower spinal root transection in canines. Thirteen female mixed-breed hound dogs underwent bladder decentralization, which included transection of all sacral dorsal and ventral roots caudal to L7 and hypogastric nerves, bilaterally (n=3); all sacral roots and hypogastric nerves plus transection of L7 dorsal roots, bilaterally (n=4); or a sham operation (n=6). At a year after initial surgery, bladder function was assessed in vivo by stimulation of the pelvic plexus. The bladder was harvested for ex vivo smooth muscle contractility studies. Remaining bladder was evaluated for nerve morphology immunohistochemically using neuronal marker PGP9.5, apoptotic activity using terminal deoxynucleotidyl transferase dUTP nick end labeling, and histopathology using a hematoxylin and eosin stain. Sacral root decentralization did not reduce maximum strength of pelvic plexus stimulation-induced bladder contraction, although long-term sacral dorsal and ventral root plus L7 dorsal root transection significantly decreased contraction strength. Electric field stimulation-induced contractions of the detrusor from
all decentralized animals were preserved, compared to controls. Viable nerves and intramural ganglia were visualized in the bladder wall, regardless of group. There was no difference in amount of apoptosis in bladder smooth muscle between groups. Bladder smooth muscle cells maintain their function after long-term bladder decentralization. While pelvic plexus-induced bladder contractions were less robust at one year after lower spinal root transection, the absence of atrophy and preservation of at least some nerve activity may allow for successful surgical reinnervation after long-term injury.

2.1 Introduction

Neurogenic bladder is a disorder of the lower urinary tract caused by the disruption of the neurocircuitry that regulates bladder function.\textsuperscript{10} If left untreated, it can lead to complications such as bladder and kidney stones, chronic urinary tract infections, and depression due to the social consequences of incontinence.\textsuperscript{63} Urodynamic dysfunction frequently occurs in patients with spinal cord injury\textsuperscript{91} or neurological disorders, such as spina bifida\textsuperscript{92} and multiple sclerosis.\textsuperscript{93} A 2012 survey showed that patients who sustain spinal cord injuries prioritize recovery of bladder function over other faculties.\textsuperscript{1} The most common type of neurogenic bladder in patients with sacral spinal cord dysfunction is a lower motor neuron neurogenic bladder, which is characterized by detrusor areflexia and external urethral sphincter (EUS) denervation.\textsuperscript{63} As a consequence, patients often experience urine stasis that requires frequent clean intermittent catheterization.\textsuperscript{94} Given the undesirable ramifications of urinary incontinence, finding ways to combat the disabilities that result from lower spinal cord dysfunction should be of utmost importance to the scientific research community.
Our lab ultimately aims to develop an approach to surgically reinnervate the bladder for treatment of lower motor neuron lesion-induced bladder dysfunction in a canine model; however, we must first establish the impact that the induced long-term injury has on the integrity of the bladder. While skeletal muscle degeneration following nerve injury has been well investigated, comparably little is known about the effects of nerve root injury on smooth muscle. Confirmation of bladder smooth muscle function after long-term neuronal injury is critical to the success of surgical reinnervation due to the likelihood that patients would delay undergoing an invasive surgery until all non-surgical therapies are exhausted. In this study, we utilize a long-term lower spinal root injury canine model to study the impact of sacral decentralization, or a marked decrease in neuronal input, on bladder smooth muscle and intramural neuronal function.

2.2 Materials and Methods

2.2.1 Animals

Studies were conducted with Temple University IACUC approval and in compliance with NIH, USDA and AAALAC guidelines. Thirteen female mixed-breed hound dogs, acquired at 6-8 months of age weighing 18-25 kg (Marshall BioResources, North Rose, NY) were used in this study. Dogs were housed in groups of three and exposed to a 12-hr light/dark cycle. Animals were randomly assigned to a 12-month survival after surgical sacral root transection (n=3), 12-month survival after surgical sacral dorsal and ventral root plus L7 dorsal root transection (n=4), or a sham operation (n=6). Credé's maneuver was performed on all experimental animals twice daily.
2.2.2 Surgical Spinal Root Injury

Immediately prior to surgery, animals received 20 mg/kg IV dose of cefazolin with re-dosing every four hours until completion of the procedure. Antibiotic prophylaxis included 30 mg/kg cephallexin PO twice a day for five days following the surgery. Animals received 6 mg/kg IV of propofol to allow for placement of endotracheal tube before placement on 0.5-4% mean alveolar concentration of isoflurane with oxygen.

Following initial anesthesia, catheterization was performed by passing a double balloon Foley catheter through the urethra and into the bladder. External pressure transducers were interfaced with the bladder lumen port to monitor intravesical pressure and the distal balloon to monitor urethral sphincter pressure. An additional catheter with balloons was placed into the rectum and measured both rectal and anal sphincter pressure, the former serving as a substitution for abdominal pressure. Normal saline cystometrograms were recorded with an infusion rate of 30 mL/min.

Animals underwent a laminectomy of L6-S3 vertebrae to expose the lower spinal cord and spinal roots. Roots were stimulated with a current of 1-3 mAmp, a frequency of 20-Hz, and a duration of 0.2 msec using a monopolar electrode to identify L7, S1, S2, and S3 roots prior to transection. As previously described,13,14 three animals underwent surgical transection of all dorsal and ventral sacral roots caudal to L7, bilaterally, and hypogastric nerves, bilaterally. The dorsal root of L7 was also transected, bilaterally, in addition to the sacral root and hypogastric nerve transections, in another a subset of animals (n=4). Five to ten mm were removed from each transected root or nerve to ensure complete separation. The loose ends of sacral roots were ligated with silk sutures. Hypogastric nerves were accessed via abdominal surgery. Sham-operated controls
underwent lumbosacral laminectomy, nerve root identification via electrical stimulation without root transection, and abdominal opening with identification of hypogastric nerves. All animals underwent tail amputation at the end of the procedure to prevent self-mutilation of the now decentralized tail.

2.2.3 In vivo Bladder Functional Electrical Stimulation

Immediately prior to euthanasia one year after transection, nerves within the pelvic plexus of the bladder were stimulated with either a monopolar or bipolar electrode with a current of 0.5 mAmp-10 mAmp, a frequency of 20-Hz, and a duration of 0.2 msec. Bladders were stimulated bilaterally and the side that produced the largest contraction was reported. Bladder capacity was determined during three successive filling cystometrograms using normal saline at 30 mL/minute. The bladder was then fully emptied and filled again to about half of the bladder capacity. Changes in pressures were continuously recorded with external pressure transducers interfaced with the PowerLab® multichannel data acquisition system and LabChart® software (ADInstruments, Colorado Springs, CO). Strength of nerve-evoked bladder contractions after pelvic plexus stimulation were derived from differences between the resting baseline pressure and the peak pressure obtained during continuous stimulation.

2.2.4 Ex vivo Nerve-evoked Stimulation in Bladder Smooth Muscle

The dorsal aspect of the bladder was harvested at euthanasia and placed in HTK organ preservation media, composed of 15 mM NaCl, 9 mM KCl, 1 mM potassium hydrogen 2-ketoglutarate, 4 mM MgCl₂, 18 mM histidine NaCl, 2 mM tryptophan, 30 mM mannitol, and 0.015 mM CaCl₂ and kept on ice overnight. Strips of smooth muscle
were isolated from bladders and denuded of the mucosal layers. They were then suspended in muscle baths between platinum plates located approximately 1 cm apart in 10 mL of oxygenated Tyrode’s solution, composed of 125 mM NaCl, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 23.8 mM NaHCO₃, and 5.6 mM glucose, kept at 37 °C, stretched to ~2 g tension, and allowed to accommodate to the bath for at least 30 min before testing. Strips were treated with iso-osmolar Tyrode’s solution with 120 mM potassium chloride (KCl) solution and the force of contraction that was generated was measured. Electric field stimulation (EFS) was delivered to the strips at 12 V, with a pulse duration of 1 ms, until a maximum contraction was obtained using varying frequencies (2 Hz, 5 Hz, 12 Hz, 20 Hz, and 30 Hz) using a Grass S88 stimulator (Natus Neurology Inc., Warwick, RI) interfaced with a Stimu-Splitter II (Med-Lab Instruments, Loveland, CO) power amplifier and LabChart® software (ADInstruments, Colorado Springs, CO). Frequency response curves were generated. Muscle strips were then treated with 1 µM tetrodotoxin (TTX) and allowed to incubate for 15 min before repeating EFS.

2.2.5 Tissue Collection, PGP9.5 Immunohistochemistry, and TUNEL Assay

At euthanasia, full-thickness bladder tissue was collected, fixed in 4% paraformaldehyde for 4 h, and equilibrated in 10% sucrose in phosphate buffer overnight, following by 30% sucrose in phosphate buffer for approximately 6 h. Tissues were embedded in OCT Compound (Scigen, Gardena, CA) and stored at -80°C until processing. Tissues were then cryosectioned into 14 µm sections. Subsets of sections were permeabilized with 0.1% Triton-X, blocked using 4% goat serum, and stained using 1° anti-PGP9.5 at 5 µg/ml (Abcam, Cambridge, MA) and 2° CyTM3 Goat anti-Mouse at
2.5 µg/ml (Jackson IR, West Grove, PA) to visualize nerves. Additional sections were processed using ApopTag® Fluorescein Direct In Situ Apoptosis Detection Kit (Millipore, Burlington, MA) according to the manufacturer’s directions, which detects apoptotic cells using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Slides were then stained with 0.1 µg/ml 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI) in PBS for 15 min before coverslipping with 80% glycerol in PBS. Slides were imaged using a Nikon Eclipse E1000 upright microscope (Nikon, Melville, NY) equipped with an EXi Aqua bio-imaging camera (QImaging, Surrey, BC, Canada). Adjacent sections were stained with hematoxylin and eosin and were grossly assessed in a blinded fashion for presence of histopathology. A minimum of three sections were examined per stain and per animal.

2.2.6 Data Analysis

Bladder capacities were measured using the inflection point of the generated cystometrograms. If an inflection point was not apparent due to root transection, capacities were determined using the volume that generated 60 cmH₂O, a criterion selected based on exhibited signs of discomfort during awake cystometry.

Data were analyzed using either two-tailed unpaired t-tests or ANOVA with Tukey’s post hoc analysis using GraphPad Prism 7.0 (La Jolla, CA). A p-value of < 0.05 was considered statistically significant. All data are expressed as mean and standard error of the mean (SEM).
2.3 Results

2.3.1 Behavioral Observations following Surgery

Although one of the surgical groups underwent an extensive decentralization that included the dorsal root of L7, bilaterally, all animals retained hind limb function. Animals that underwent sacral ventral and dorsal root plus dorsal root of L7 consistently showed evidence of overflow incontinence such as frequent urine dribbling. A culture-confirmed UTI was treated in one animal from the group that included L7 dorsal root transection.

2.3.2 In vivo Bladder Contractility

All animals had similar bladder capacities at initial surgery prior to decentralization of 61.15 ± 14.18 mL for sham animals, 55.20 ± 4.80 mL for animals that underwent transection of ventral and dorsal sacral roots and the hypogastric nerves, and 62.68 ± 6.96 mL for animals that underwent transection of sacral roots plus the dorsal roots of L7 and hypogastric nerves. There was no difference in the bladder capacity between sham animals (87.50 ± 29.62 mL), animals that received the sacral root transection (146.0 ± 66.85 mL; p = 0.5748) and animals that received sacral root plus L7 dorsal root transection (67.03 ± 14.97 mL; p = 0.6719). There was no significant difference between the resting bladder pressure of sham-operated animals (25.32 ± 15.32 cmH₂O), animals that received sacral decentralization (11.35 ± 3.249 cmH₂O; p=0.5774), and animals that received sacral and L7 dorsal root decentralization (18.16 ± 4.465 cmH₂O; p=0.6696). Maximum detrusor contraction after pelvic plexus stimulation was recorded during the terminal surgery. While year-long sacral root injury did not decrease the strength of pelvic plexus stimulation-induced bladder contractions in vivo (10.2 ± 2.8
cmH\textsubscript{2}O), compared to sham controls (23.3 ± 4.3 cmH\textsubscript{2}O), sacral decentralization with additional L7 dorsal root transection significantly decreased the induced contraction (5.1 ± 1.3 cmH\textsubscript{2}O; p<0.05; Figure 2.1). Despite the diminished strength of contraction, all animals exhibited a maximum strength of contraction > 0 cmH\textsubscript{2}O.

2.3.3 Ex vivo Bladder Contractility Experiment

Within 24 h of the terminal surgery, at one year after the root transection, bladder smooth muscle contractility and intramural nerve function was tested ex vivo. In contrast to the in vivo experiment, bladder smooth muscle strips isolated from animals that underwent long-term injury exhibited equally robust contractions to electric field stimulation.

![Graph](image)

**Figure 2.1. Bladder pressure via stimulation of the pelvic plexus decreases after long-term lower spinal root injury in vivo.** Each point represents the maximal contraction yielded from stimulation of either left or right pelvic plexus in a single animal, whichever produced the strongest bladder contraction. Animals that received sacral dorsal and ventral root transection (S1-S3; dark gray square, n=3) did not significantly decrease in maximal bladder contraction after pelvic plexus stimulation, compared to sham-operated controls (black circle, n=5). Animals that additionally received L7 dorsal root transection (S1-S3 ventral and dorsal + L7 dorsal; light gray triangle, n=4) showed a significant decrease. Despite the decrease, note that no animals exhibited a complete loss of pelvic plexus stimulation-induced bladder contractions. Data was analyzed using a one-way ANOVA followed by Tukey’s post hoc analysis.
stimulation at all tested frequencies compared to strips from sham control animals (Figure 2.2). Treatment with 1 µM TTX blocked EFS-induced contractions across groups (Figure 2.2).

### 2.3.4 PGP9.5 Immunohistochemistry

Nerve density appeared qualitatively similar across groups. Intramural ganglia were identified in both decentralized and sham groups (Figure 2.3). Intramural ganglia containing large rounded neuronal cell bodies were also identified within the bladder smooth muscle layers in each decentralized group and in the sham operated group (Figure 3). Hematoxylin and eosin staining showed no presence of pyknotic neuronal cell bodies.

![Figure 2.2](image)

Figure 2.2. *Ex vivo* nerve-evoked bladder smooth muscle contractility changes at one year after lower spinal root injury. Frequency-response curves were generated by exposing muscle strips to varying frequencies while keeping voltage (12 V) and pulse duration (1 ms) consistent. Solid curves represent pre-drug responses and dashed curves represent response to electric field stimulation after treatment with 1 µM tetrodotoxin (TTX). Sham data are represented by blue circles (pre-drug: N=6, n=125; TTX: N=6, n=24), sacral decentralization by red squares (pre-drug: N=3, n=48; TTX: N=3, n=6), and sacral + L7 dorsal root decentralization by green triangles (pre-drug: N=4, n=108; TTX: N=4, n=17). N = number of animals within treatment group; n = number of strips tested within treatment group. Mean ± SEM per group (generated by mean per dog within group) is shown.
2.3.5 Apoptosis Assay

No difference in apoptosis within smooth muscle of the bladder was observed after staining with TUNEL (0 smooth muscle cells/area for all groups). Apoptotic cells were observed in the bladder urothelium (the inner epithelial layer) of each transected group and control group. Positive staining in the urothelium served as an internal positive control for the assay.

Figure 2.3. Intramural ganglia and nerves are present within bladder wall at one year after sacral root + L7 dorsal root transection. Full thickness bladder tissue stained for PGP9.5 (neuronal marker) and DAPI. A. PGP9.5+ nerves within smooth muscle of the bladder in sham-operated animal. B. PGP9.5+ intramural ganglion in sham with HE of the same ganglion. C. PGP9.5+ nerves within smooth muscle of the bladder in sacral + L7 dorsal root transection animal. D. PGP9.5+ intramural ganglion in sacral + L7 dorsal root transection animal. Scale bar represents 100 microns.
2.3.6 H&E Assessment of Bladder Wall

The smooth muscle uniformly appeared intact without signs of cell death across all tissues. The urothelium frequently looked disturbed in samples from sacral dorsal and ventral + L7 dorsal root transection animals, with variations in size and quantity of epithelial cells (Figure 2.4). An increase in blood vessels in the suburothelial space was observed in animals that received both the sacral transection and the sacral dorsal and ventral + L7 dorsal root transection (Figure 2.4, noted by asterisk). While inflammatory infiltrates were visible in all samples, lymphocytic clumping was noted in two out of four animals that received sacral dorsal and + L7 dorsal root transection (not shown).

2.4 Discussion

While some studies have addressed urodynamic changes after spinal cord injury in patients\(^95\) and the short-term consequences of decentralization on bladder function in animal models,\(^96\) no study to date has evaluated the effects of long-term lower spinal root injury on bladder function in vivo or on the individual components that contribute bladder function within the same model. In this study, we combined in vivo urodynamic tests with \textit{ex vivo} assessment of isolated smooth muscle fascicles from the same animals to determine the extent to which smooth muscle dysfunction contributes to lower motor neuron neurogenic bladder in a canine model.

The extensive decentralization in this study, which included transection of all sacral roots plus the dorsal root of L7, as well as the hypogastric nerves bilaterally, was selected based on a previous study that identified the L7 dorsal root as one of the major sources of sensory neuron cell bodies innervating the bladder.\(^97\) Because these animals
are part of a larger reinnervation study, we needed to ensure that the bladder was
decentralized as much as possible to better evaluate the success of reinnervation.

Preservation of smooth muscle contractility may indicate that detrusor smooth
muscle integrity does not depend on external neuronal input. However, it is more likely
that other neuronal inputs (originating from sites other than the sacral spinal cord) may be
maintaining the detrusor smooth muscle, such as the network of intramural ganglia that
act upon smooth muscle. The robust response to electric field stimulation seen in the
smooth muscle strips regardless of treatment group indicates that the intramural neurocircuitry is still viable because the response to EFS is action potential-dependent, which was confirmed by the inhibition of contractions following treatment with sodium channel blocker TTX.

Nerve density between each group looks grossly similar, although density can vary from animal to animal as a result of variations in tissue processing. We conducted a TUNEL assay to evaluate apoptosis occurring within the bladder tissue. As expected, there was evidence of apoptosis in the urothelium (the inner epithelial layer) of the bladder due to the rate of cell turnover as an immunological response to environmental factors. The lack of TUNEL labeling in the smooth muscle of samples from the injury group confirms that the decentralization procedure did not induce cell death within the smooth muscle of the bladder. Future studies will address the effects of decentralization on urothelial integrity.

Pelvic plexus-induced bladder contractions significantly decreased by one year after decentralization that included the transection of all sacral roots, the dorsal roots of L7, and the hypogastric nerves, bilaterally, confirming a previous finding that the L7 dorsal root plays an important role in bladder sensation. Despite the significant decrease in the strength of bladder contraction, the preservation of smooth muscle function and at least some nerve activity may encourage successful surgical reinnervation after long-term lower spinal root injury.
CHAPTER 3

RESTORATION OF BLADDER AND URETHRAL FUNCTION VIA NERVE TRANSFER IN LOWER MOTOR NEURON-LESIONED CANINES*

*Extracted from recent submission to The Journal of Neurosurgery: Spine in which Salvadeo DM was co-first author with Tiwari E and aided with all surgeries, animal care, awake bladder filling experiments, and microscopy.

Overview

Background: Previous studies have shown that patients who sustain spinal cord injuries prioritize recovery of bladder function. Objective: We sought to determine if nerve transfer after long-term decentralization restores bladder function in canines. Methods: Twenty-four female canines underwent transection of sacral roots and hypogastric nerves (S Dec, n=6), or also bilateral transection of L7 dorsal roots (L7d+S Dec; n=7). Twelve months later, three L7d+S Dec animals underwent obturator-to-pelvic nerve and sciatic-to-pudendal nerve transfers (L7d+S Dec+Reinn). Eleven animals served as controls. Squat-and-void behaviors were tracked monthly before and after decentralization, after reinnervation, and following awake bladder filling procedures. Bladders were cystoscopically injected with Fluorogold three weeks before euthanasia. Immediately prior to euthanasia, transferred nerves were stimulated to evaluate motor function. Then, spinal ganglia were collected and assessed for retrogradely labelled neurons. Expected Outcomes: Transection of only sacral roots failed to reduce squat-and-void postures. L7 dorsal root transection was necessary for significant reduction. Three L7d+S Dec animals showing loss of postures post-decentralization were chosen for reinnervation surgeries.
L7d+S Dec+Reinn animals recovered squat-and-void postures at 4-6 months post reinnervation. Each showed obturator nerve stimulation-induced bladder contractions and sciatic nerve stimulation-induced anal sphincter contractions; one showed sciatic stimulation-induced external urethral sphincter contractions and voluntarily voided on two occasions following bladder filling. Reinnervation was confirmed by increased presence of retrogradely labelled cells in L2, L4-L6 spinal ganglia of L7d+S Dec+Reinn animals, compared to controls. **Discussion:** New neuronal pathways created by nerve transfer can restore bladder sensation and possibly motor function in lower motor neuron-lesioned canines.

### 3.1 Introduction

In canines, most sensory and motor axons innervating the bladder originate from the thoracolumbar (T10-L2) and sacral (S2-S4) regions of the spinal cord and travel via the hypogastric, pelvic, and pudendal nerves to the bladder and external urethral sphincter.\(^9^7\) Urine storage and voiding are controlled by proper coordination of these neural pathways.\(^1^8\)

Spinal cord injuries and other neurological disorders can disrupt the neurocircuitry that controls lower urinary tract function, resulting in neurogenic bladder.\(^9^9\) This dysfunction can lead to urinary retention, incontinence, and increased frequency of voiding, which can diminish one’s quality of life, self-esteem, and limit social contacts.\(^3\) Restoration of bladder function is consistently identified as a top recovery priority for people with spinal cord injury\(^1,1^0^0\) and therefore should be an emphasis of investigation.
The ability to restore motor function of the bladder in canines that underwent sacral root transection has previously been shown after surgical transfer of genitofemoral or femoral nerves to the anterior vesicle branch of the pelvic nerve. Either procedure resulted in increased detrusor pressure upon electrical stimulation of the transferred nerve proximal to the anastomosis site in 21 of 28 animals. A pilot study showed evidence of new sensory pathways based on neuronal tracing results and observation of squat-and-void postures in the reinnervated animals. However, in these animals, decentralization was achieved by bilateral transection of S1-S3 roots alone. No attempt was made to eliminate bladder innervation from the hypogastric nerves, or L7 dorsal root-mediated sensory innervation of the bladder as the latter was only recently identified. Therefore, it was not clear whether sensation was transmitted through sensory fibres in the newly reinnervated pathway, via fibres hitchhiking on hypogastric nerves, or through remaining L7 dorsal root afferents. Furthermore, behavioral analysis of squat-and-void postures is needed to confirm if sensation of bladder fullness can be restored by nerve transfer surgeries.

This study aims to determine if decentralization using more extensive spinal root transection strategies can eliminate bladder function, and then, if surgical reinnervation after a year of decentralization results in a return of bladder sensation and voluntary voiding in canines. This was addressed by behavioral observation of squat-and-void postures at regular intervals and measurement and characterization of voided volume (% squat-and-void volume or % leaked volume) following awake bladder filling. Functional electrical stimulation of transferred nerves was performed to evaluate motor function. Sensory innervation was also assessed using retrograde neurotracing techniques.
3.2 Methods

3.2.1 Animals

All animal studies were approved by the Institutional Animal Care and Use Committee and were compliant with the National Institutes of Health, United States Department of Agriculture, and American Association for Assessment of Laboratory Animal Care guidelines. Twenty-four female mixed-breed hounds, 6-8 months of age, weighing 20-25 Kg, from Covance Research Products Inc. (Cumberland, Virginia) or Marshall BioResources (North Rose, New York) were used. All canines had free access to food and water and were maintained on a 12:12 hour light-dark cycle.

3.2.2 Decentralization and Nerve Transfer Surgeries

Animals were anesthetized as previously described and randomized into 5 separate surgical groups (Fig. 3.1). Sacral root decentralized animals (S Dec, n=6) underwent extradural sacral decentralization in which all spinal roots below L7 were exposed and transected, bilaterally, in addition to bilateral transection of hypogastric nerves (Fig. 3.2A). Seven animals underwent similar sacral root and bilateral hypogastric nerve transections, plus bilateral transection of L7 dorsal roots (L7d+S Dec; Fig. 3.2B). Three L7d+S Dec animals showing few to no squat-and-void postures across the 12 months after decentralization were chosen for reinnervation surgeries (L7d+S Dec+Reinn, n=3) at 12 months post-decentralization. These 3 animals were anesthetized, and the obturator nerves were identified and divided into two fairly even longitudinal fascicles using a micro-scalpel. Half remained intact to retain innervation of hindlimb adductor muscles. The other half was transected, transferred, and sutured end-to-end to the transected anterior vesical branch of the pelvic nerve, bilaterally, as previously
These 3 animals were again anesthetized three weeks later, the pudendal nerve was identified, and bilateral transfer of the semimembranosus branch of the sciatic to the pudendal nerve was performed for potential reinnervation of urethral and anal sphincters. Controls included eight Sham-Operated (underwent lumbosacral laminectomy without root transection) and three unoperated animals.

### 3.2.3 Behavioral Observation of Squat-and-Void Postures

The frequency of squat-and-void postures, defined by the position exhibited by female canines as they urinate, was recorded for 24 hours at monthly intervals in Sham-Operated, S Dec, L7d+ S Dec, and L7d+S Dec+Reinn groups using video surveillance.
Figure 3.2. Decentralization and nerve transfer surgical approach. Transection included the removal of approximately 1 cm-length segment of the root while leaving approximately 2 mm of root attached to the spinal ganglion (dorsal root ganglion, D) intact. A. All roots caudal to L7 were transected bilaterally. B. All roots caudal to L7 in addition to L7 dorsal roots were transected, bilaterally. C. Nerve transfers (obturato-pelvic and sciatic-to-pudendal) were performed 12 months after bilateral transection of all roots caudal to L7 as well as bilateral L7 dorsal root transection. A-B. All decentralization procedures included bilateral hypogastric nerve transection (not shown in diagram).
cameras placed over housing cages. For each animal, at least one recording was performed during the acclimation period, prior to the first surgery, for pre-surgery voiding postural data. No video recording was performed for unoperated controls. The Credé maneuver was performed twice daily to empty the bladder as necessary during the 12-month recovery period post-decentralization or 9-month post-reinnervation recovery period.

3.2.4 Observation of Squat-and-Void Postures with Full Bladder

Conscious filling and the ability to empty a full bladder was assessed in L7d+S Dec, L7d+S Dec+Reinn, and unoperated control animals. These animals were placed in a sling 2-3 times/week, 10 minutes each session, for 1 week prior to the first awake bladder filling procedure for acclimation to the apparatus. Prior to filling, they were anesthetized with propofol (i.v., 6 mg/kg) for insertion of bladder, urethra, rectal, and anal sphincter catheters. After recovery from propofol, pressures were monitored with the fully awake animal in the sling and bladder filling curves were recorded using external pressure transducers interfaced with the PowerLab® multichannel data acquisition system and LabChart® software (ADInstruments, Colorado Springs, CO). The bladder was filled with 0.9% normal saline solution to cystometric capacity, defined as the infused volume inducing a marked increase in the slope of the volume-pressure curve. In the absence of an inflection point in the pressure-time curve (seen decentralized animals), the bladder was filled to 60 cm of water pressure. Following the recording of filling curves, animals were transferred to a transport cage, the Foley catheter was removed while the bladder remained full, and behaviors were video recorded for 10 minutes to assess bladder emptying via voluntary squat-and-void posture or leakage only. Any bladder contents
expelled during squat-and-void postures were collected, measured, compared to the
cystometric capacity volume previously infused into the bladder, and recorded as percent
recovered volume (% Squat-and-void volume). Leaked bladder volume from animals that
did not show any squat-and-void postures was also collected, measured, and compared to
the volume previously infused into the bladder (% Leaked volume). The residual volume
was calculated (% Calculated residual volume) by subtracting the squat-and-void volume
or leaked volume from the total infused volume.

3.2.5 Retrograde Dye Injection

Three weeks prior to euthanasia, animals were sedated and the bladder wall was
cystoscopically injected around the ureterovesical junction with Fluorogold (4-5% w/v in
0.9% saline, Fluorochrome, LLC, Denver, CO), as previously described. True Blue (2%
w/v in 0.9 % saline, Life Technologies Corporation, Grand Island, NY) was also injected
into the external urethral sphincter at four different sites. No dye injections were
performed in Unoperated Controls. One animal of the S Dec group died during the post-
surgery recovery period and did not undergo this procedure; two other S Dec animals
underwent different procedures after the 12-month decentralization period and were
excluded from this analysis.

3.2.6 In vivo Functional Electrical Stimulation

Immediately prior to euthanasia, animals were re-anesthetized as previously
described. Bladder, urethral sphincter, rectal, and anal sphincter pressures were
continuously monitored throughout the surgeries, as were vital signs. Three successive
filling cystometrograms were performed to determine bladder capacity using the
previously described setup. Transferred nerves (obturator-to-pelvic and sciatic-to-pudendal) were stimulated (3-10mA, 20Hz, 0.2 msec) using hand-held monopolar or bipolar electrodes. Changes in detrusor pressure were recorded. Strength of nerve-evoked bladder, external urethral sphincter, and anal sphincter contractions were derived from differences between the resting baseline pressure and the peak pressure obtained during continuous stimulation.

3.2.7 Euthanasia and Tissue Collection

Three weeks after dye injections, animals were euthanized by a terminal dose of Euthasol (pentobarbital sodium 86 mg/kg and phenytoin sodium 11 mg/kg i.v.). Tissues were collected, fixed, sectioned and counted as previously described. Briefly, T12 through S3 spinal ganglia (SG) were collected and fixed by immersion in 4% buffered paraformaldehyde for 4 hours. Ganglia were cryosectioned into 20 µm sections. Every third section of SG was mounted onto slides, coverslipped with 80% glycerol/PBS, and evaluated for retrogradely labelled neuronal cell bodies.

3.2.8 Statistical analysis

Means and standard error of means (SEM) are reported throughout. Retrograde labelling was analyzed using a two-way ANOVA with Tukey post-hoc multiple comparisons. A p<0.05 was considered statistically different.
3.3 Results

3.3.1 L7 dorsal root, sacral roots, plus hypogastric nerve transections are needed to reduce squat-and-void postures

Frequencies of squat-and-void postures are reported based on pre- and monthly post-operation behavior analysis over 24-hour periods in housing cages (Fig. 3.3-6). Squat-and-void postures were consistently observed in S Dec animals (Fig. 3.3), at similar frequencies as Sham-Operated animals (Fig. 3.6). Of the L7d+S Dec animals, #7 showed only one posture at 5 and 10 months (150 and 300 days), #8 showed no postures across the 12-month post decentralization period (350 days), while #9 showed increased frequency of <10/day only at 2 and 4 months post decentralization (60 and 120 days) (Fig. 3.4). Animals #9 and #10 showed unusual postures (intermediate between squat-

Sacral Decentralization (all roots caudal to L7)

![Graphs showing postures per day for different canines](image)

Figure 3.3. Squat-and-void postures observed in sacral decentralized animals during monthly 24-hour recording periods in home cages. Sacral Decentralization included bilateral transection of all roots caudal to L7 (i.e., S1-S3) and bilateral transection of hypogastric nerves. Symbols: Blue squares = squat-and-void postures per day.
Figure 3.4. Squat-and-void postures observed in L7d+S Dec animals during monthly 24-h recording periods in home cages. L7 Dorsal + Sacral Decentralization included bilateral transection of all roots caudal to L7 (i.e., S1-S3) and bilateral transection of hypogastric nerves, with additional bilateral transection of L7 dorsal roots. Symbols: Blue squares = squat-and-void postures per day; Green stars = unusual postures between micturition and defecation.
Figure 3.5. Squat-and-void postures observed in L7d+S Dec+Reinn animals during monthly 24-h recording periods in home cages. Three animals of the L7d+S Dec group that did not show voiding behaviors at one year post decentralization underwent obturator-to-pelvic and sciatic-to-pudendal nerve transfers, followed by further observation as shown. Symbols: Blue squares = squat-and-void postures per day; Green stars = unusual postures between micturition and defecation; Red squares = Voiding postures with full bladder after awake bladder filling.
and-void and defecation postures with longer duration) that coincided with culture-confirmed bacteriuria that disappeared with antimicrobial treatment (Fig. 3.4). One of these 4 animals (#10) was euthanized at the 11th post-operative month (330 days) due to kidney and bladder stones. The remaining (#11, #12, and #13) showed consistent squat and-void postures at monthly observation periods and were euthanized at 8-9 post-operative months (Fig. 3.4).

Nerve transfers surgeries were only performed in the 2 animals with few to no incidences of squat-and-void postures (#7 and #8) and in the one (#9) that showed intermediate postures only during culture confirmed bacteriuria (Fig. 3.4 and 3.5). Importantly, at 4-6 months (120-180 days) after the reinnervation surgery, squat-and-void postures were observed in all 3 reinnervated animals (Fig. 3.5). Additionally, during daily

![Graphs showing squat-and-void postures observed in Sham-operated animals over monthly 24-h recording periods in home cages.](image)

**Sham-Operated**

- Canine # 14
- Canine # 15
- Canine # 16
- Canine # 17
- Canine # 18
- Canine # 19

**Figure 3.6.** Squat-and-void postures observed in Sham-operated animals over monthly 24-h recording periods in home cages. Sham-Operated animals underwent lumbosacral laminectomies without root transection. Symbols: Blue squares = squat-and-void postures per day.
care, bladders were usually empty (66.7 ± 11.8% of the time over two-month span prior
to euthanasia, n=3) when performing the Credé maneuver during this post-reinnervation
period.

3.3.2 Observation of bladder fullness sensation and squat-and-void posture events
with full bladder

All 3 Unoperated Control animals showed squat-and-void postures over the 10-
minute video recording with full bladder. The measured % squat-and-void volume
was recorded as 71.1 ± 2.9% of their cystometric bladder capacity (Table 3.1). In contrast,
squat-and-void postures were absent in the L7d+S Dec animals after removal of the Foley
catheter in the transport cage (Table 3.1). The % leaked volume in L7d+S Dec animals
was measured as 29.5 ± 6.0% (Table 3.1). Interestingly, following the awake bladder
filling procedure, upon return to the transport cage, one of the L7d+S Dec+Reinn animals
assumed a squat-and-void posture and partially emptied its bladder voluntarily on two
occasions (#8; Fig. 5). Recovered % squat-and-void volume for this animal was measured
as 7.1 ± 4.3% of its capacity in the transport cage (Table 3.1). The other two L7d+S Dec+
Reinn animals (#7 and #9) did not show any squat-and-void postures after bladder filling
during the 10-minute video recording period after their return to the transport cage. In
these two reinnervated animals, % leaked volume was measured as 43.1 ± 5.5%. The %
calculated residual volume was 28.9 ± 2.9% in Unoperated controls, 70.5 ± 6.0% in
L7d+S Dec, 56.9 ± 5.5% in L7d+S Dec+Reinn with no squat-and-void postures (#7 &
#9) and 92.9 ± 4.3% in L7d+S Dec+Reinn animal (#8) with squat-and-void posture.
Table 3.1. Awake urodynamic results in L7d+S Dec, L7d+S Dec+Reinn, and Unoperated control animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Squat and void postures in 10 minutes</th>
<th>% Squat-and-voided volume</th>
<th>% Leaked volume</th>
<th>% Calculated residual volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unoperated controls (n=3)</td>
<td>Yes</td>
<td>71.1 ± 2.9</td>
<td>0.0</td>
<td>28.9 ± 2.9</td>
</tr>
<tr>
<td>L7d+S Dec (n=3)</td>
<td>No</td>
<td>0.0</td>
<td>29.5 ± 6.0</td>
<td>70.5 ± 6.0</td>
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<tr>
<td>L7d+S Dec+Reinn (n=2)</td>
<td>No</td>
<td>0.0</td>
<td>43.1 ± 5.5</td>
<td>56.9 ± 5.5</td>
</tr>
<tr>
<td>L7d+S Dec+Reinn (n=1)</td>
<td>Yes</td>
<td>7.1 ± 4.3</td>
<td>0.0</td>
<td>92.9 ± 4.3</td>
</tr>
</tbody>
</table>

3.3.3 Muscle contractions were induced after in-vivo electrical stimulation of the transferred nerves

Immediately prior to euthanasia, L7d+S Dec+Reinn animals were re-anesthetized, and bladder, urethral sphincter, rectal and anal sphincter pressures were continuously monitored during three successive filling cystometrograms. Obturator nerve stimulation induced detrusor muscle contractions up to 10 cmH₂O in animal #7 and #8 (Fig. 7A). However, only a slight increase in detrusor pressure (2 cmH₂O) was observed during obturator nerve stimulation of animal #9 (Fig. 7A). When the sciatic nerve was stimulated in these three reinnervated animals, only one (#8) showed external urethral sphincter contractions (15 cmH₂O; Fig. 7B), although high anal sphincter pressures were induced in all three animals (Fig. 7C). 3.3.4 Retrograde dye labelling of transferred nerves shows integrity of anastomosis sites Fluorogold labelling from the bladder was observed in the transferred obturator nerves immediately proximal to their site of
Figure 3. Representative traces of recordings from transferred nerve stimulations from L7t-Der-Recin animals. A. Obturator nerve stimulation induced bladder contractions. B. Sciatic nerve stimulation induced external urethral sphincter contractions. C. Sciatic nerve stimulation induced anal sphincter contractions. EUS - External Urethral Sphincter. Period of stimulation indicated by “On” and “Off”.
anastomosis to the pelvic nerve branch, at 6-9 months after nerve transfer (Fig. 8A).
Similarly, True Blue labelling from the urethra was observed in the transferred sciatic nerves immediately proximal to their site of anastomosis to the pudendal nerves (Fig. 8B).

3.3.5 Retrograde dye labelling in dorsal root ganglia confirms growth of sensory axons to the bladder in transferred nerves

Examples of retrogradely labelled neurons in spinal ganglia are shown in Fig. 9A-C for Sham-Operated Controls (Fig. 9A), L7d+S Dec (Fig. 9B) and L7d+S Dec + Reinn

Figure 3.8. Retrograde dye injected into bladder was observed in transferred obturator and sciatic nerves immediately proximal to site of anastomoses. A. Whole mount of mesenteries (left panel) containing the Fluorogold-labelled obturator nerve (right panel) immediately proximal to site of anastomosis to the anterior vesicle branch of the pelvic nerve. B. Histological sections containing True-blue labelled sciatic nerve semimembranosus branch, immediately proximal to site of anastomosis to the pudendal nerve; arrow indicates suture at the pudendal end near the EUS (external urethral sphincter).
Figure 3.9. Labelling of cells in spinal ganglia after cystoscopic injection of Fluorogold retrograde dye into bladder wall. A. Representative retrogradely labelled cells in L7 spinal ganglia of a Sham control. B. Retrogradely labelled cells in L7 DRG of L7d+S Dec animal. C. Retrogradely labelled cells in L5 DRG of L7d+S Dec+Reinn animal. D-G. Number of cells/mm² counted in spinal ganglia at spinal level of: D. Sham-Operated Controls; E. S Dec; F. L7d+S Dec; and G. L7d+S Dec+Reinn animals. * and **: p<0.05 and 0.01, respectively, compared to Sham-Operated Controls; & and &&, p<0.05 and 0.01, respectively, compared to L7 Dorsal + Sacral Decentralized animals.
animals (Fig. 9C). Quantification of these labelled neurons showed a decrease in S1-S2 spinal ganglia of S Dec (Fig. 9E) and L7d+S Dec animals (Fig. 9F), compared to Sham-Operated Controls (Fig 9D).

In contrast, there were increased numbers of labelled cell bodies in L5 and L6 spinal ganglia of L7d+S Dec+Reinn animals (Fig 9G), compared to L7d+S Dec animals (Fig. 9F), as well as increases in L2, L4-L6 spinal ganglia of L7d+S Dec+Reinn animals, compared to Sham-Operated Controls (Fig. 9D).

3.4 Discussion

Bilateral somatic nerve transfer to the anterior vesicle branch of the pelvic nerve and pudendal nerve reversed yearlong decentralization-induced loss of squat-and-void postures in the home cage 4-6 months after reinnervation in all three animals. One animal also clearly showed evidence of voluntary voiding after awake bladder filling. Stimulation of the surgically transferred obturator and sciatic nerves induced bladder and external urethral sphincter contractions respectively, confirming motor function of the newly established pathways.

While two L7d+S Dec+Reinn animals did not perform squat-and-void postures following awake bladder filling, they showed the behavior in their home cages. This difference may be due to lack of EUS reinnervation, as indicated by the absence of sciatic nerve stimulation-induced EUS contractions. Upon removal of the catheter, they immediately leaked the added volume, suggesting a deficiency of EUS tone. When the bladder is allowed to fill at a physiological rate, the smooth muscle of the bladder neck may provide enough tone for urine storage.
Two L7d+S Dec animals exhibited unusual postures and four exhibited increased frequency of squat-and-void postures concurrent with culture confirmed bacteriuria, which disappeared upon treatment with antibiotics, suggesting sensation of discomfort or pain in the bladder. Present squat-and-void behaviors despite decentralization indicates that they either regained or retained some bladder sensation following decentralization, perhaps due to sensory nerve sprouting or variations in bladder sensory innervation respectively.

We have previously reported that genitofemoral or femoral nerve transfer to the anterior vesical branch of the pelvic nerve results in increased Fluorogold-labelled neurons in ventral horn regions of lumbar spinal cord segments from which the donor nerves originated following retrograde dye injection of the detrusor around the ureterovesical junctions. The labelling was not observed in sham or unoperated control animals, confirming that distal ends of the transferred nerves sprouted into the bladder end organ. Furthermore, pelvic-plexus induced stimulation remained intact following femoral nerve transfer, suggesting that somatic nerve transfer can maintain motor function to the bladder.

It is important to note that the decentralization strategy previously implemented did not include transection of hypogastric nerves, which would reduce sympathetic and some sensory input, or L7 dorsal roots, which would reduce sensory input to the bladder. In this current study, squat-and-void postures were retained in animals that underwent hypogastric nerve and sacral root transections, while leaving the L7 dorsal roots intact (S Dec), with frequencies similar to that of Sham-Operated animals. In contrast, these postures were reduced or eliminated in four of the seven animals in which
L7 dorsal roots were transected in addition to the hypogastric nerve and sacral root transections. These findings provide further support that L7 dorsal roots also provide sensory innervation to the bladder and, therefore, must be transected for a more complete decentralization. Despite this extensive decentralization, 4 of the 7 animals continued to show squat-and-void postures after decentralization presumably due to either diversity in bladder sensory innervation or postoperative sensory nerve sprouting resulting in spontaneous bladder sensory reinnervation.

The increased Fluorogold-labelled cells in L5-L6 spinal ganglia of L7d+S Dec+Reinn animals, compared to L7d+S Dec animals, and in L4-L6 spinal ganglia, compared to Sham-Operated controls, indicate that axons of these sensory neurons innervate the bladder. Importantly, a future study will include age-matched decentralized animals for comparison to reinnervated animals to confirm that the increase in labelling is due solely to the reinnervation, rather than de novo sprouting. Although there were significantly decreased numbers of labelled cells in S1 and S2 spinal ganglia in S Dec and L7d+S Dec groups compared to controls, we still observed low levels of retrogradely labelled neurons were seen in spinal ganglia of roots that had been previously transected, which is likely due to the fact that the spinal ganglia were spared during the initial decentralization. If they had not resorbed by the time of collection, they were harvested for assessment and showed retrogradely labelled neurons. Future decentralization studies will also include removal of spinal ganglia in addition to transection of spinal roots.

3.5 Conclusion

All three animals that underwent reinnervation exhibited a disappearance or marked reduction of squat-and-void postures during the 12-month decentralization period
followed by a restoration of posture frequency 6-8 months after reinnervation, indicating recovery of sensory innervation of the bladder. They each showed strong bladder and anal sphincter contractions, while one showed urethral contractions during *in vivo* electrical stimulation of the transferred nerves, which also supports return of motor function. Most notably, one animal also showed voluntary voiding after awake bladder filling with saline. These findings indicate that nerve transfer can induce recovery of both sensation of bladder fullness and the ability to empty the bladder, and thus may be an appropriate surgical treatment for patients who sustain lower motor neuron lesion-induced bladder dysfunction.
CHAPTER 4
AN ASSESSMENT OF BLADDER-SPECIFIC NICOTINIC RECEPTOR
EXPRESSION AND FUNCTION FOLLOWING LOWER SPINAL ROOT
INJURY AND SURGICAL REINNERVATION

4.1 Introduction

A multitude of receptor signaling pathways dictate lower urinary tract (LUT) function. Muscarinic cholinergic receptors (MACHR) found on the plasma membrane of smooth muscle are G protein-coupled receptors that are activated by the neurotransmitter acetylcholine (ACh) to initiate contraction.\textsuperscript{18} We previously determined that the strength of smooth muscle contraction following decentralization remains undisturbed, although stimulation of peripheral pelvic nerves carrying signals to the bladder were less effective at inducing bladder contractions.\textsuperscript{103} These finding suggest that the MACHR that mediate emptying of the bladder are still present and functional.

While the role of MACHR in bladder contraction is well established, the importance of nicotinic cholinergic receptors (NACHR) in modulation of bladder contractility is still under investigation. NACHR are ion channels made up of five varying subunits that open when bound by ACh to initiate influx of Na\textsuperscript{+} and Ca\textsuperscript{2+} ions. The most prevalent receptor subtype is the ganglionic nicotinic receptor, typically made up of α\textsubscript{3} and β\textsubscript{4} subunits. The neuromuscular NACHR, made up of two α\textsubscript{1}, one β\textsubscript{1}, one δ, and one ε subunits,\textsuperscript{44} is not typically associated with bladder function.

We performed nerve re-routing surgery following long-term decentralization in canines with the goal of restoring bladder function. We previously found that the depolarizing neuromuscular NACHR blocker, succinylcholine, blocks transferred nerve
stimulation-induced bladder contraction \textit{in vivo} in reinnervated dogs, but not in sham operated dogs. Furthermore, immunohistochemistry identified $\alpha_1$ receptor subunits associated with bladder smooth muscle of reinnervated animals, which were absent in that of control animals\textsuperscript{104} This study further characterizes the $\alpha_1$-containing NACHR found in reinnervated bladders through \textit{ex vivo} smooth muscle strip experiments and ELISA.

4.2 Methods

4.2.1 Animals and Surgical Groups

All studies were approved by the Temple University IACUC and maintained compliance with NIH, USDA, and AAALAC guidelines. Female mixed-breed hound dogs (Marshall BioResources, North Rose, NY) were randomly assigned to one of the following groups: 1. 12-month survival after surgical sacral root transection (i.e. decentralization, S Dec, n=3); 2. 12-month survival after surgical sacral dorsal and ventral root plus L7 dorsal root decentralization (L7d+S Dec; n=4); 3. 12-month sacral decentralization followed by six-month reinnervation via bilateral transfer of the obturator nerve to the anterior vesical branch of the pelvic nerve and semimembranosus branch of the sciatic nerve to the pudendal nerve (S Dec+Reinn; n=2); 4. 12-month sacral plus L7 dorsal root decentralization followed by six-month reinnervation as described for Group 3 (L7d+S Dec+Reinn; n=3); or a sham operation (n=6).

4.2.3 Euthanasia and Tissue Collection

Prior to euthanasia, the bladder was harvested for processing. The ventral aspect of the bladder was collected for immunohistochemistry and ELISA experiments. A
portion full-thickness tissue from the body of the bladder was collected, fixed in 4% paraformaldehyde for 4 h, followed by incubation in 10% sucrose in phosphate buffer overnight and 30% sucrose in phosphate buffer for approximately 6 h thereafter. Tissues were embedded in OCT Compound (Scigen, Gardena, CA) and stored at -80°C until processing. Tissues were then cryosectioned into 14 µm sections. Additional pieces of tissue were flash frozen using liquid nitrogen and immediately stored at -80°C until homogenization. The dorsal aspect of the bladder was rinsed with Tyrode’s solution, composed of 125 mM NaCl, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 23.8 mM NaHCO₃, and 5.6 mM glucose, and stored in HTK organ preservation media, composed of 15 mM NaCl, 9 mM KCl, 1 mM potassium hydrogen 2-ketoglutarate, 4 mM MgCl₂, 18 mM histidine NaCl, 2 mM tryptophan, 30 mM mannitol, and 0.015 mM CaCl₂ and kept on ice overnight. Animals were then euthanized using a terminal dose of Euthasol (pentobarbital sodium 86 mg/kg and phenytoin sodium 11 mg/kg i.v.).

4.2.4 Smooth Muscle Contractility Experiment

Bladder was denuded of mucosa, dissected along visible fascicle lines to separate into individual smooth muscle strips. Strips were then suspended in muscle baths containing 10 mL of oxygenated Tyrode’s solution, stretched to ~2 g tension, and allowed to accommodate to the bath for at least 30 min before testing. Bather were drained and filled with iso-osmolar Tyrode’s solution containing 120 mM potassium chloride (KCl) solution until the tissue was completely submerged. The generated smooth muscle contraction was measured using force transducers that were connected to a computer running LabChart® software (ADInstruments, Colorado Springs, CO).
Samples were then incubated with a NACChR antagonists such as neuromuscular blocker tubocurarine (1 μM), ganglionic nicotinic antagonists hexamethonium (100 μM) or mecamylamine (10μM), or nicotine (1 mM) for 20 min before addition of NACChR agonist epibatidine (10μM). Effects of NACChR agonist nicotine (1mM) on smooth muscle contraction was also determined. Strength of contractions are reported as a percent of the KCl-induced contraction. Due to the evolution of study design over time, _ex vivo_ contractility data are reported only for a subset of animals from the sham-operated group (N=3), L7d+S Dec (N=4), and L7d+S Dec+Reinn animals (N=3).

### 4.2.4 Tissue Homogenization and CHRNA1 ELISA

All tissue was thawed on ice prior to homogenization. Bladder samples from each animal were individually homogenized in phosphate buffer containing Thermo Scientific Pierce Protease Inhibitor (Thermo Fisher Scientific, Rockford, IL) using a mortar and pestle sitting on liquid nitrogen. Ground sample powder and buffer were incubated on a rotator for 1 h at 4°C before being centrifuged at 12,000 rpm for 15 min. Pellets were isolated and resuspended in M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Rockford, IL) containing a cocktail of protease inhibitors. Total protein was determined using a commercial BCA protein assay (Thermo Fisher Scientific, Rockford, IL). Tissue lysates were analyzed for CHRNA1 using a dog-specific CHRNA1 ELISA kit according to the manufacturer’s protocol (MyBioSource, San Diego, CA). Each sample was run in duplicate and data were normalized to pg total protein.
4.2.5 Data Analysis

Data were analyzed via one- or two-way ANOVA with Tukey’s post hoc analysis where appropriate using GraphPad Prism 7.0 (La Jolla, CA). Means and standard error of means (SEM) are reported throughout. A p<0.05 was considered statistically different.

4.3 Results

4.3.1 *Ex vivo* Smooth Muscle Response to Nicotine

There was no difference in average smooth muscle strip response to KCl in L7d+S Dec (1.8±0.3 g, N=4, n=32) or L7d+S Dec+Reinn (1.5±0.2 g, N=3, n=32) animals compared to sham-operated animals (2.1±0.3 g, N=3, n=32; Figure 4.1A). Thus, average response to KCl was used to normalize response to nicotine. L7d+S Dec (23.8±2.9%, N=4, n=4-5) and L7d+S Dec+Reinn (15.7±5.5%, N=3, n=4-5) had similar average responses to treatment with 1 mM nicotine compared to sham-operated animals (16.3±3.9%, N=3, n=4-5; Figure 4.1B).

![Figure 4.1 Ex vivo response to 1 mM nicotine treatment across experimental groups. A. Average tension generated in grams (g) after treatment with KCl. B. Average strength of contraction as a percentage of response to KCl-induced contraction; each symbol represents the average for an individual animal. Blue = Sham-Operated Control, Red = L7d+S Dec, Green = L7d+S Dec+Reinn, ns = no significance; N = number of animals, n = number of strips/animal.](image-url)
4.3.2 Effects of NACChR antagonists on 10 μM epibatidine-induced contractions in smooth muscle *ex vivo*

There was no change in the average strength of smooth muscle contraction generated by treatment with 10 μM epibatidine in L7d+S Dec (30.9±3.1%, N=4) and L7d+S Dec+Reinn (29.2±12.7%) compared to sham (35.4±3.2%, N=3). Furthermore, treatment with 1 μM tubocurare, 10 μM mecamylamine, 100 μM hexamethonium, or 1 mM nicotine completely blocked epibatidine-induced contractions across all three groups.

Figure 4.2 Nicotinic receptor antagonists block 10μM epibatidine-induced contractions. Black = treatment (tx) with water before introduction of epibatidine; Red checker = 100 μM hexamethonium tx; Orange stripe = 10 μM mecamylamine tx; Blue stripe = 1 μM tubocurare treatment; Green stripe = 1 mM nicotine treatment. N = number of animals, n = number of strips/animal; * = p<0.05
4.3.3 Quantification of CHRNA1 protein in bladder tissue using ELISA

CHRNA1 protein was identified in all samples of bladder tissue. Interestingly, levels of CHRNA1 were increased in L7d+S Dec (0.22±0.026 pg/μg; p<0.05) compared to sham-operated animals (0.11±0.013 pg/μg). In contrast, levels of CHRNA1 were not altered in S Dec (0.11±0.009 pg/μg), S Dec+Reinn (0.16±0.009), and L7d+Dec+Reinn (0.17±0.039 pg/μg) compared to sham-operated controls (Figure 4.3).

![Figure 4.3 Quantification of CHRNA1 concentrations using ELISA.](image)

**Figure 4.3 Quantification of CHRNA1 concentrations using ELISA.** CHRNA expression is increased in homogenized bladder smooth muscle samples of L7d+Sac D animals compared to Sham controls; mean ± standard error of the mean are shown; blue = Sham-Operated Control, solid red = S Dec, striped red = S Dec+Reinn, solid green = L7d+Dec, striped green = L7d+S Dec+Reinn. * = p<0.05.
4.4 Discussion

The strength of smooth muscle contractions generated from treatment with either nicotine or epibatidine did not change across groups, suggesting that the overall net effects of NAcR activation on bladder activity functionally did not change after decentralization or surgical reinnervation. It is important to note that pre-junctional NAcR facilitate the release of ACh and thus induce bladder contractions. Furthermore, both nicotine and epibatidine are indiscriminate NAcR agonists that act upon all receptor subtypes, including a variety of ganglionic nicotinic receptors found on the preganglionic terminals at intramural ganglia. To date, there are no known neuromuscular NAcR-specific agonists that could better elicit receptor function. Thus, it is difficult to isolate the function of individual subtypes of NAcR expressed in the bladder.

The nature of the studied NAcR across all surgical groups are unusual due to their sensitivity to antagonists that are thought to be selective for neuromuscular NAcR (tubocurare) and ganglionic nicotinic receptors (hexamethonium and mecamylamine). A previous study observed similar effects of hexamethonium and tubocurare on nicotine-induced contractions in normal guinea pig bladder, although no follow-up studies have been performed.

Additional experiments are necessary to determine the function, if any, of the $\alpha_1$ subunit-containing NAcR found in sham, decentralized, and reinnervated canine bladders. Numerous attempts were made to repeat immunohistochemistry previously performed with the hopes of achieving dual-labelling of these uncharacterized
NACHR; however, differences in tissue processing and antibody availability may have been responsible for unsuccessful experiments.

4.5 Conclusion

The neuromuscular NACHR is not thought to play a role in bladder physiology; however, we previously identified the $\alpha_1$ subunit found exclusively in neuromuscular NACHR in animals that received mixed somatosensory or somatic nerve transfer to the bladder, which were not seen in controls. This study showed that smooth muscle contractility studies are limited by the receptor specificity of available NACHR agonists and antagonists. Furthermore, ELISA data suggest that the $\alpha_1$ subunit is present in normal bladder tissue, although other experiments are necessary to determine its physiological relevance.
CHAPTER 5
ASSESSMENT OF DIRECT INNERVATION FROM THE LOWER THORACIC
AND UPPER LUMBAR SPINAL CORD TO THE BLADDER

5.1 Introduction

Bladder function is coordinated by an intricate network of neurocircuitry that integrates activity from both central (brain and spinal cord) and peripheral (sympathetic, parasympathetic, and somatic, and sensory fibres) components of the nervous system. It is well established that parasympathetic innervation from the sacral spinal cord and sympathetic innervation from the lower thoracic and upper lumbar spinal cord mediate bladder emptying and storage respectively.\textsuperscript{51} Norepinephrine (NE) can mediate detrusor relaxation and contraction through $\beta$-adrenoceptors and $\alpha$-adrenoceptors, respectively.\textsuperscript{38} Acetylcholine, in contrast, primarily drives bladder contraction through activation of cholinergic muscarinic receptors.\textsuperscript{27} It’s typically thought that both types of innervation act through two-neuron systems;\textsuperscript{109,110} however, the scientific field fails to address the function of additional direct inputs from the lower thoracic and upper lumbar ventral horns of the spinal cord, which were recently highlighted by our lab\textsuperscript{11,12,97} after being described by early investigators of bladder innervation.\textsuperscript{111,112}

The complexity of bladder innervation must be considered when developing a surgical procedure to reinnervate the bladder. While the gross neuroanatomy that facilitates bladder function has been under investigation for well over a century, subsets of neuronal contribution to the bladder remain underexplored. This study aims to define the function of direct upper lumbar contributions to the bladder and if bladder decentralization and surgical reinnervation alter their function using \textit{in vivo} functional
electrical stimulation and pharmacology experiments, *ex vivo* smooth muscle contractility studies, and Fluorogold (FG) retrograde labeling.

5.2 Methods

5.2.1 Animals

This study was conducted with the approval of the IACUC and was in compliance with the National Institutes of Health, United States Department of Agriculture, and American Association for Assessment of Laboratory Animal Care guidelines. All animals used were between 6-8 months of age and weighed 20-25 kg upon acquisition.

5.2.1.1 Acute Study

Three female mixed-breed hounds underwent an acute spinal root stimulation study. Animals were sacrificed at the end of procedure using a terminal dose of 86 mg/kg pentobarbital sodium and 11 mg/kg phenytoin sodium i.v.

5.2.1.2 Long-term Decentralization and Reinnervation

As described in Chapter 3, animals were randomly assigned to a surgical group. Five animals underwent twelve-month sacral decentralization in which all ventral and dorsal roots below L7 and hypogastric nerves were transected (S Dec).72 Seven animals underwent identical decentralization, plus bilateral transection of L7 dorsal roots (L7d+S Dec). After twelve months, two S Dec animals and three L7d+S Dec were chosen for reinnervation surgeries (S Dec+Reinn and L7d+S Dec+Reinn, respectively). Reinnervation included surgical transfer of the obturator nerve to the anterior vesical branch of the pelvic nerve and, three weeks later, transfer of the semimembranosus
branch of the sciatic nerve to the pudendal nerve via end-to-end coaptation, bilaterally, as previously described. Animals were studied for up to eight months after reinnervation.

Six Sham-Operated Controls underwent lumbosacral laminectomy without disruption of bladder innervation. After the final in vivo experiments, animals were sacrificed as described in the previous section.

5.2.2 In vivo Functional Electrical Stimulation Experiments

5.2.2.1 Acute Study

Prior to the start of the procedure, animals were anesthetized using 6 mg/kg propofol i.v. for intubation and were maintained using isoflurane at 2-3% maximum alveolar concentration. A double-ballooned Foley catheter connected to force transducers was placed in the bladder to allow for bladder filling and measurement of bladder and EUS pressure. An additional double-ballooned catheter was placed in the rectum to measure rectal and anal sphincter pressure, the former of which serving as a surrogate for abdominal pressure. A laminectomy from T10-S3 was performed to expose spinal cord and roots. Spinal roots were systematically stimulated from T12-S3 using handheld monopolar or bipolar probes set to 1-4 mAmp current. As part of another study, L2 was selected for placement of a cuff electrode to allow for remote stimulation. To ensure that any bladder pressure generated from stimulation was not due to current propagation, the sacral roots and spinal cord caudal to L5 were transected and the systematic stimulation was repeated. Then, to eliminate hypogastric-mediated bladder contractions, the hypogastric nerves were bilaterally transected and systematic stimulation was repeated. Additionally, the animal was treated with 1 mg/kg phentolamine i.v. to block α-adrenoceptor activity and systematic stimulation was repeated after 10 min incubation of
the drug. Finally, stimulation of the L2 spinal cord was repeated 10 min after administration of 25 mg/kg atracurium i.v.

5.2.2.2 Long-term Decentralization and Reinnervation

At the terminal procedure, animals were prepared as described in the previous section. Spinal roots from T12-S3 were stimulated to determine their motor contribution to bladder function.

5.2.3 Fluorogold Injection of the Bladder

Three weeks prior to euthanasia, all animals in the long-term study were anesthetized for cystoscopic injection of FG (4-5% w/v in 0.9% saline, Fluorochrome, LLC, Denver, CO) as previously detailed. Briefly, dye was injected into the detrusor at four locations (0.5 mL per site) around the ureteral openings, bilaterally.

5.2.4 Tissue Processing and Dye Quantification

Following euthanasia, the spinal cord was harvested by segment, immersion fixed in 4% paraformaldehyde overnight at 4°C, placed in 10% sucrose in phosphate buffered saline (PBS) for 6 h at 4°C, followed by 30% sucrose in PBS overnight at 4°C to equilibrate before embedding. Samples were then frozen in OCT on dry ice and stored at -80°C until sectioning. L2 spinal cord was sectioned at 20 μm, dried overnight at room temperature, and coverslipped with 80% glycerol in PBS before imaging.

FG-labelled cells in the L2 ventral horn were imaged and counted using Nikon E1000 fluorescence microscope interfaced with a bioquantitation software (BIOQUANT, Nashville, TN) and an X, Y motorized stage. A section from the spinal cord was randomly selected for assessment. Ventral horn gray matter of each section was traced at
1x to determine total area of the ventral horn. The entire area was systematically assessed for all FG-positive cells within the outlined region (Fig. 5.1).

5.2.5 Data Analysis

Means and standard errors of means are presented for all *in vivo* stimulation and cell counts. One-way analyses of variances (ANOVA) were used to assess differences in spinal cord or root stimulation-induced detrusor contractions at each spinal level across all groups. A two-way ANOVA was used to compare the mean number of Fluorogold-labeled neurons per surgical group (S Dec, L7d+S Dec, S Dec+Reinn, L7d+S Dec+Reinn, Sham-Operated Control). Tukey’s multiple comparisons were used to measure the differences between individual groups. Unpaired, one-tailed or two-tailed t-tests were used to compare differences of L2 stimulation-induced detrusor contractions after a variety of treatments in the acute study. Comparisons were made between each
treatment and the immediately preceding treatment. A value of \( p < 0.05 \) was considered statistically significant.

5.3 Results

5.3.1 *In vivo* Functional Electrical Stimulation of Spinal Cord or Roots in Acute Animals

Upon *in vivo* stimulation, S2 spinal roots or cord yielded the highest bladder contraction across all three animals (18.8±5.0 cmH\(_2\)O). Of the lower thoracic and upper lumbar spinal roots, L2 root stimulation produced the highest bladder contraction upon stimulation (9.6±2.0 cmH\(_2\)O, Figure 5.2A, treatment A in 5.2B) and thus was studied for the remainder of the study using a custom cuff electrode. Strength of detrusor contraction did not change after transection of all dorsal and ventral roots caudal to L5 (14.6±4.2 cmH\(_2\)O, treatment B). Bilateral transection of hypogastric nerves or hypogastric plexus significantly decreased strength of L2-mediated detrusor contractions (2.9±0.8 cmH\(_2\)O, treatment C, \( p < 0.05 \)) compared to post-treatment B stimulation-induced contraction. While there was no difference between detrusor pressure during L2 root stimulation post treatment C and after administration of 1 mg/kg phentolamine (5.6±0.8 cmH\(_2\)O, treatment D), strength of contraction after treatment with 25 mg/kg atracurium significantly decreased compared to stimulation after treatment D (0.3±0.2 cmH\(_2\)O, treatment E, \( p < 0.05 \)).
5.3.2 In vivo Functional Electrical Stimulation of Spinal Cord or Roots in Long-Term Animals.

There was no difference in strength of bladder contraction across all groups from spinal cord level T12 to L7, with the exception of a significant increase in T12-mediated contraction in L7d+S Dec animals (9.3±0.5 cmH₂O, N=4) compared to controls (1.9±1.1 cmH₂O, N=5). All groups exhibited a significant decrease in the strength of S1 stimulation-induced bladder contractions (S Dec = 2.1±2.1 cmH₂O, N=3; S Dec+Reinn = 2.4±2.4 cmH₂O, N=2; L7d+S Dec = 0.0±0.0 cmH₂O, N=4; L7d+S Dec+Reinn = 1.8±0.9 cmH₂O, N=3) with a complete elimination of S2 and S3-mediated bladder contractions, compared to controls (S1 = 14.8±4.2 cmH₂O, S2 = 33.2±2.3 cmH₂O, S3 = 5.6±2.8 cmH₂O; p<0.05).

Figure 5.2 Spinal cord or root stimulation-induced bladder contractions in acute animals. The blue arrow indicates L2 root stimulation before any treatment. A. The spinal roots and cord were systematically stimulated from T12 to S3 prior to any treatments. B. A cuff electrode was placed on the L2 spinal root to allow for stimulation. Treatment A – pre-treatment, treatment B – after transection of everything caudal to L5, treatment C – after bilateral transection of the hypogastric nerves, treatment D – following administration of 1 mg/kg phentolamine, treatment E – following administration of 25 mg/kg atracurium. *p<0.05
Figure 5.3 Spinal cord or spinal root stimulation-induced bladder contractions across all surgical groups. Spinal cord roots and cord were systematically stimulated from T12-S3 to determine effects on bladder contraction. Detrusor pressure reported as cmH$_2$O. A. Sham-operated control, blue box, N=4; B. Sacral decentralization (S Dec), red box, N=3; C. Sacral decentralization + reinnervation (S Dec+Reinn), red box, N=2; D. L7 dorsal root + sacral decentralization (L7d+S Dec), green box, N=4; E. L7 dorsal root + sacral root decentralization + reinnervation (L7d+S Dec+Reinn), green box, N=3; *p<0.05 compared to respective sham spinal cord level; ns = no significance.
5.3.3 Fluorogold Quantification in L2 Spinal Cord of Long-Term Decentralized and Reinnervated Animals

There was no difference in the number of FG-positive cells in cross sections of the ventral horn of the L2 spinal cord across treatment groups (Figure 5.4).

![Fluorogold-labelled cells in the L2 spinal cord](image)

**Figure 5.4** Fluorogold-labelled cells in the L2 spinal cord were quantified across all groups. A. Image of positively-labelled neurons in the ventral horn of the L2 spinal cord. Asterisk indicates example of FG-positive cell with magnified image in right corner. Scalebar = 100 um; B. Quantification of # cells/mm² in cross-section of L2 ventral horn across groups. blue = Sham-Operated Control, solid red = S Dec, striped red = S Dec+Reinn, solid green = L7d+Dec, striped green = L7d+S Dec+Reinn. There was no significant difference across groups.

5.4 Discussion

It is thought that lumbar-originating nerves that provide input to the bladder are strictly sympathetic in nature and travel through the two-neuron sympathetic and hypogastric plexuses to reach their end organ. Furthermore, the lower thoracic and upper lumbar sensory ganglia provide some sensory innervation to the bladder. This study shows that there are also some direct inputs from the ventral horn to the bladder in
addition to the well-established sympathetic innervation through \textit{in vivo} and retrograde tracing techniques. Furthermore, it indicates that the presence of direct L2 inputs remain intact following long-term decentralization of the bladder and surgical reinnervation.

Several \textit{in vivo} experiments were conducted in unoperated canines to determine the nature of L2-mediated bladder contractions in normal bladder physiology. It was first important to establish that stimulation at the L2 cord or roots was not propagating down the spinal cord through sacral parasympathetic pathways. There was no difference in strength of detrusor contraction after L2 stimulation before and after the transection of all roots caudal to L5, proving that the contraction was mediated by action potentials that traveled along L2-originating nerves.

It was initially unclear if the L2 stimulation-induced contractions were strictly mediated through sympathetic nerves that travel through the hypogastric plexus before synapsing at the bladder. There was a significant decrease in the strength of contraction following the bilateral transection of hypogastric nerves or plexus, indicating that a large portion of L2-mediated bladder contractions were a result of sympathetic activation; however, it is important to note that the treatment did not completely eliminate L2 root stimulation-induced bladder contractions. Furthermore, treatment with 1 mg/kg phentolamine, an \(\alpha\)-adrenoeceptor antagonist, did not change the strength of L2-mediated contractions compared to the contraction post hypogastric nerve transection, suggesting that any remaining nerves mediating the response did not rely upon sympathetic \(\alpha\)-adrenoeceptors known to mediate detrusor contractions.

We then sought to determine if the L2 stimulation-induced contractions were facilitated by atracurium-sensitive nicotinic receptors, a functional receptor subtype
thought to be exclusively expressed in striated muscle, due to a previous study in which we found that stimulation of transferred mixed somatosensory or somatic nerves to the anterior vesical branch of the pelvic nerve was blocked by the depolarizing neuromuscular nicotinic receptor antagonist succinylcholine. Interestingly, atracurium treatment completely blocked detrusor contraction in response to L2 root stimulation, suggesting that neuromuscular nicotinic receptors mediate the activities of direct inputs from the L2 spinal cord to the bladder. Further studies are necessary to determine where along the pathway from the L2 spinal cord to the bladder atracurium-sensitive receptors are expressed.

Following decentralization in both S Dec and L7d+S Dec animals, there was loss of sacral root-mediated bladder contractions, confirming that bladder decentralization was successfully achieved. Furthermore, the absence of sacral input to the bladder persisted following reinnervation. Upon systematic stimulation of the spinal cord and roots, we found that neither decentralization nor reinnervation interrupted effects of lower thoracic and upper lumbar-mediated bladder contractions.

The presence of Fluorogold-positive cells in the ventral horn of L2 across all surgical groups confirms the existence direct inputs from L2 to the bladder. Assessment of more sections per animal is necessary to confirm this finding. The redundancy in contraction-inducing innervation outside of the sacral spinal cord may be advantageous to recovery following a lower spinal root injury. Future studies should include a correlation between direct inputs from the lower thoracic and upper lumbar spinal cord and behavioral measures that indicate the loss of function after decentralization and the recovery of function following reinnervation.
5.5 Conclusion

It is critical to understand the complexity of bladder neuroanatomy when developing bladder reinnervation techniques. The presence of detrusor contractions after lower thoracic/upper lumbar root stimulation regardless of sacral root and hypogastric nerve transection suggests that the contractions are mediated by nerves other than the traditional sacral parasympathetic or hypogastric sympathetic innervation. A significant decrease in detrusor contraction after treatment with atracurium indicates that activity of this subpopulation of nerves is mediated by neuromuscular nicotinic receptors. Future studies should include further investigation of these direct inputs to the bladder and how they contribute to bladder function after lower motoneuron injury and reinnervation.
CHAPTER 6

CONCLUSION

6.1 Dissertation Summary

The overall goal of this dissertation was to explore the effects of long-term decentralization of the bladder via spinal root transection, a model of lower neurogenic bladder, as well as surgical reinnervation on bladder physiology. To do so, we address the following aims:

1. Determine the viability of both smooth muscle and intramural nerves following year-long bilateral transection of all sacral roots, the dorsal root of L7, and hypogastric nerves (i.e. decentralization)
2. Determine the success of bladder reinnervation via behavioral, electrophysiological, and immunohistochemical techniques
3. Assess changes in the profile of cholinergic nicotinic receptors as a result of long-term lower spinal cord injury and surgical reinnervation via obturator-to-pelvic nerve and sciatic-to-pudendal nerve transfer
4. Study previously uncharacterized direct inputs from the L2 spinal cord to the bladder using in vivo nerve stimulation and retrograde neuronal tracing techniques

In Chapter 1, we provide the background necessary to understand the impact that incontinence as a result of neurogenic bladder has on patients afflicted with the disorder. It describes the complex neurophysiology of the bladder and how different neuronal lesions alter bladder function. Ultimately, we support the need for research into surgical
reinnervation models and justify our use of canines with our past research experience and the similarities between this animal model and human physiology.

Chapter 2 addresses how decentralization via bilateral transection of the hypogastric nerves and all spinal roots caudal to L7, as well as the inclusion of L7 dorsal root transection in a subset of animals, affects the integrity of smooth muscle and intramural nerves that are critical to bladder function. We found that although the extensive decentralization that includes the L7 dorsal root significantly decreases pelvic plexus stimulation-induced bladder contractions (Figure 2.1), *ex vivo* EFS experiments revealed that both the intramural nerves and smooth muscle of the bladder were still responsive to stimulation (Figure 2.2). Furthermore, there was no evidence of apoptosis in the bladder smooth muscle and intramural nerves were visible using immunohistochemistry (Figure 2.3). Thus, we concluded that the functional components of the bladder remain intact after decentralization, which increases the likelihood of success after surgical reinnervation.

The following chapter details our bladder decentralization and surgical reinnervation model in canines. We use behavioral assessments, *in vivo* stimulation of transferred nerves, and retrograde labelling to assess the consequences that long-term decentralization of the bladder via transection of spinal roots and hypogastric nerves has on bladder function, and the effects of reinnervation via bilateral transfer of the obturator nerve to the anterior vesical branch of the pelvic nerve and the semimembranosus branch of the sciatic nerve to the pudendal nerve has on bladder sensation and motor function. It was determined that functional decentralization, which included the absence of the squat-and-void posture in the home cage and after bladder filling (Figure 3.4), could be
achieved only if the decentralization included the bilateral transection of the dorsal root of L7 in addition to the hypogastric nerves and all ventral and dorsal sacral roots. Animals that showed complete or near complete loss of behavior underwent reinnervation, which resulted in a return of squat-and-void behaviors in home cages 4-6 months after the reinnervation was performed in all three animals, as well as after awake bladder filling in one of the reinnervated animals (Figure 3.5). Stimulation of the transferred obturator nerves showed an increase in detrusor pressure in all three animals, while only one of the reinnervated animals showed return of pudendal nerve-mediated EUS contraction (Figure 3.7). We observed an increased number of Fluorogold-labelled cells in L4-L6 spinal ganglia, the origin of the obturator nerve in canines, of reinnervated animals compared to controls, indicating that axons of these sensory neurons innervate the bladder as a result of the nerve transfer. We’re confident that these results provide evidence for the viability nerve transfer as an appropriate surgical treatment for patients who sustain lower motor neuron lesion-induced bladder dysfunction.

Chapter 4 is a short report that explores changes in nicotinic receptor expression after long-term decentralization and reinnervation. While neuromuscular NACHR are not thought to exist in the bladder, we previously identified the $\alpha_1$ subunit in reinnervated animals of our previous study, which were not seen in controls. In this study, the sum effects of treatment with nicotinic receptor agonists did not change between surgical groups (Figure 4.2). Furthermore, $\alpha_1$ subunit is present in bladder tissue from normal, decentralized, and reinnervated animals (Figure 4.3). The function of these receptors is not yet understood.
Finally, we previously noticed that there was a dearth in literature about direct inputs from the lower thoracic and upper lumbar ventral horns to the bladder, despite their discovery in the late nineteenth century. Because the success of developing an effective surgical reinnervation depends upon our understanding of bladder neurophysiology, we decided to further investigate this subset of nerves. In focusing on the L2 root, which yielded the highest contraction outside of the sacral cord (Figure 5.2A), it was determined that root stimulation-induced contraction was primarily sympathetic in nature based on response to transection of hypogastric nerves (Figure 5.2B); however, treatment with an α-adrenoceptor blocker did not completely eliminate the contraction, suggesting that a subset of the nerves function through an alternative mechanism. Interestingly, the remaining L2-mediated contraction was inhibited by a neuromuscular NACHr receptor antagonist, indicating that the neuromuscular NACHr receptor subtype may facilitate the contraction. Future studies—including the use of other neuromuscular nicotinic receptor antagonists in vivo to confirm NACHr-mediated L2 stimulation-induced bladder activity—should consider the role that these direct inputs to the bladder have in bladder function and dysfunction.

6.2 Future Directions

The overall aim of this project is to develop a surgical approach to reinnervate the lower neurogenic bladder after a long-term neuronal injury. Following proof of concept in canines, the next step would involve translation to humans. To date, anatomical feasibility of the obturator and semimembranosus branch of the sciatic nerve transfers has been assessed in an unembalmed cadaver (not yet reported). In collaboration with both neurosurgeons and urologists, a patient population has been potentially identified as an
appropriate target for this procedure, namely patients who have developed a chordoma of
the sacral spinal cord.

A chordoma is a slow-growing malignant neoplasm that develops from the
notochordal remnant along the axial skeleton. Due to their poor response to standard
cancer therapies such as chemotherapy and radiation, patients often undergo surgical
removal of the tumor. Because the cancer, as well as the surgical intervention, often
involves disruption of surrounding structures, such as the sacral spinal cord and
associated roots, individuals who develop chordomas usually experience lower
neurogenic bladder, similar to the manifestation of bladder dysfunction in our canine
model. Thus, we have reason to believe that this patient population could benefit from
surgical bladder reinnervation. We could realize the ultimate goal of our efforts: seeing
our work benefit people who would otherwise suffer the physical and emotional
consequences of lower motoneuron injury-driven urinary incontinence.
REFERENCES


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