Amplitude-dependent Modulation of Bladder Function with Tibial Nerve Stimulation in a Continuous-fill Anesthetized Rat Model

by

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A thesis submitted in conformity with the requirements for the degree of Master of Applied Science
Institute of Biomaterials and Biomedical Engineering
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Abstract

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Although percutaneous tibial nerve stimulation (PTNS) is considered a clinically-effective therapy for treating overactive bladder (OAB), its symptom suppression mechanism remains unclear. The goal of this study was to better understand the role of specific neural inputs on the bladder-inhibitory effects of tibial nerve stimulation (TNS). In 24 urethane-anesthetized rats, a suprapubic saline continuous-fill model was used to achieve repeated filling and emptying of the bladder. In each experiment, four TNS trials were applied in randomized order: no stimulation, Aβ fiber activation, Aδ fiber activation, and C fiber activation. Each trial was 30 minutes in duration, with a washout period of 60 to 90 minutes. Our findings showed that TNS evoked statistically significant post-stimulation changes in bladder function only at stimulation amplitudes that recruited unmyelinated C fibers. The bladder-inhibitory effects of TNS in a continuous-fill bladder model suggest that recruitment of small unmyelinated C fibers has important functional significance.
At this time, I would like to take this opportunity to first thank my supervisor Dr. Paul Yoo for his unwavering guidance and support. This was a truly enriching experience and I am grateful for the opportunity to have been a part of this lab. To my fellow lab mates, Parisa, Zainab, Karly, Eshani, and Silviu, thank you for your help, moral support, and company throughout the ups and downs of grad school. Even through the tough times, I am glad that we could always manage to find something to laugh about. I wish you all the best of luck! I would like to thank my committee members, Dr. Julie Audet, Dr. José Zariffa, and Dr. Lesley Carr, for their constructive feedback and willingness to help me progress through my thesis. It has been a distinct pleasure to have shared this research journey with all of you. Also, Dr. Audet, thank you for your generous offering of time spent to help me tame my flock of statistical demons. I would like to express my sincerest gratitude to Dr. Jonathan Carp and Dr. Phillip Smith for sharing your “trade secrets” regarding void collection and rodent bladder experimentation. Without your assistance, I cannot begin to imagine how my project would have progressed. Thanks to all of the IBBME staff for keeping the department running like a well-oiled machine! It was a pleasure to meet all of you! Also, a special shout-out to Nick for his company, late into long experiments. To my parents, thank you for your unconditional love and support through every minute of this process. Regardless of your knowledge of the subject matter, you always managed to keep me moving forward. Dad, thank you for your all of your time and effort spent helping me put together the void collection apparatus. Lastly, to my best friend. Steph, thank you for your love, support, and ability to keep me smiling. You always found time to lend an ear and encouraged me to be my best self. You rock, don’t ever change.
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List of Abbreviations

BC  Bladder capacity  
BP  Basal pressure  
CMG  Cystometrogram  
EMG  Electromyogram/Electromyography  
ENG  Electroneurogram/Electroneurography  
EUS  External urethral sphincter  
FC  Filling compliance  
HGN  Hypogastric nerve  
ICI  Inter-contraction interval  
ICV  Inter-contraction volume  
IUS  Internal urethral sphincter  
LUT  Lower urinary tract  
MP  Maximal pressure  
OAB  Overactive bladder  
PAG  Periaqueductal gray  
PDN  Pudendal nerve  
PMC  Pontine micturition center  
PTNS  Percutaneous tibial nerve stimulation  
PVN  Pelvic nerve  
RV  Residual volume  
SCI  Spinal cord injury  
SEM  Standard error of the mean  
$T_{EMG}$  Foot EMG activation threshold  
$T_{mot}$  Foot/toe motor threshold  
TN  Tibial nerve  
TNS  Tibial nerve stimulation  
TP  Threshold pressure  
VE  Voiding efficiency  
VV  Voided volume
Chapter 1

Introduction

1.1 Motivating Problem

Overactive bladder (OAB) has become an overarching term synonymous with the terms urgency and frequency. More specifically, OAB syndrome has been defined by the International Continence Society as “urinary urgency, usually accompanied by frequency and nocturia, with or without urgency urinary incontinence, in the absence of urinary tract infection or other obvious pathology” [1]. The prevalence of OAB has been estimated by many studies to be between 8-18% in both Canada and the United States with a pronounced increase in prevalence with age [2]–[5]. Financially, OAB has significantly contributed to an annual health care impact (direct plus indirect) on both the Canadian and US medical systems: $0.5B/year [4] and $66B/year [6], respectively. Aside from its notable financial burden, OAB significantly lowers quality of life due to its debilitating effect on an individual’s confidence, social life, and anxiety whereby a large majority of sufferers either forego primary care consultation out of embarrassment or if they do consult, many do not seek further treatment [7], [8]. Common neurogenic causes of OAB symptoms are Multiple Sclerosis [9], Parkinson’s Disease [10], and spinal cord injury (SCI) [11]. Often times, symptoms may present themselves due to mechanical causes, as a result of pregnancy or outlet obstruction of the urethra, but these are often excluded from OAB diagnoses [5]. In individuals without clear neural or mechanical deficits, the cause of OAB is typically defined as idiopathic [12].

At this point in time, there is no one-size fits all cure for OAB symptoms, but instead there are a set of first-, second-, and third-line treatments available, for indefinite periods of use. Typically, patients are recommended behavioural training and exercise to strengthen their pelvic floor in parallel with pharmacotherapy to suppress bladder overactivity. Unfortunately, patients often become refractory to drug therapy and feel burdened by the accompanying side-effects [13]. Neuromodulation using electrical stimulation has become a prominent third-line therapy for patients that are no longer interested in continuing with their drug therapy. Sacral neuromodulation is an effective FDA-approved therapy that targets the sacral nerves of the spinal cord to provide continence for OAB patients [14]. Although it has shown to be successful for OAB patients, its clear disadvantage lies in the requirement of surgical-implantation of the stimulator and electrode.

For patients deterred by drugs and surgery, percutaneous tibial nerve stimulation (PTNS) has become an attractive alternative neuromodulation treatment due to its minimal invasiveness and proven efficacy (>50% improvement in OAB symptoms) [15]–[19]. With its origin derived, in part, from Chinese...
acupuncture [20], PTNS is performed by electrically activating the tibial nerve at the level of the ankle with a needle electrode. The standard PTNS therapy protocol involves weekly 30-minute stimulation sessions administered by a clinician for a 12-week period [21], [22]. OAB patients administered with PTNS experience decreased urinary frequency, decreased nighttime voiding, and increased quality of life scores, when compared with pre-therapy values [18]. The results of PTNS have been shown to be comparable with drug therapy [23] and are not placebo [18], [19]. Following the 12-week protocol, patients are recommended to have maintenance PTNS sessions 1 to 2 times per month, to sustain bladder inhibition [24], [25].

Significant strides have been made both clinically and in animal models towards revealing the underlying mechanism of PTNS, but the picture is far from complete. Acute studies in OAB patients have shown that PTNS provided during filling of the bladder can delay the sensation of bladder fullness to larger volumes [26]. Consistent with this finding, pre-clinical studies in cats and rats have reliably observed that bladder capacity can be significantly increased by tibial nerve stimulation (TNS) with both acute and prolonged effects (1 to 2 hours) [27], [28]. Recent studies in cats have shown that modulation of bladder capacity by TNS is likely due to the suppression of ascending stretch signals from the bladder pelvic nerves [27], [29]–[31], but a consensus has not been reached as to whether prolonged suppression occurs at the level of the spinal cord or in the midbrain [32]. Further, it is largely suggested that afferent fibers within the tibial nerve are responsible for TNS-evoked bladder inhibition [27], [29], but it is not clear which fibers are most important. To this point, contrasting evidence has been reported in cat studies, stating that either large [33] or small diameter afferent tibial nerve fibers [34], [35] are most effective.

The primary goal of this study was to investigate the role of different tibial nerve fiber types that can elicit bladder inhibitory reflexes in anesthetized rats. Our results indicate that the bladder-inhibitory effects of TNS are strongly dependent on the type of nerve fiber that is recruited during electrical stimulation.

1.2 Organization of Thesis

This thesis contains six chapters. Chapter 1 (Introduction) outlines the motivating problem of overactive bladder, including its prevalence, monetary and social costs, current modes of therapy, and a brief summary of the current state of PTNS treatment. Chapter 2 (Background) provides an overview of the related anatomy and physiology of peripheral nerves, electrical stimulation, the tibial nerve, and the lower urinary tract. Further in the chapter, a review of commonly used models to test bladder function is presented as well as a summary of both clinical and pre-clinical study results, pertaining to TNS treatment. Chapter 2 concludes by highlighting gaps found in the current literature surrounding tibial nerve stimulation bladder neuromodulation and defines the hypotheses and objectives of this work. Chapter 3 (Methodology) describes the experimental protocols followed for this study along with the approaches used to acquire, process, and analyze the resulting data. Chapter 4 (Results) summarizes key observations, displays representative examples within the raw data, and presents statistical significance testing results. Chapter 5 (Discussion) gives context to the results of this work and aims to connect findings with previous related studies, concluding with a discussion of potential TNS mechanisms. Chapter 6 (Conclusions & Future Work) gives a high-level summary of the results of this work, its clinical implications, model improvements, and a discussion of potential areas to explore in future studies.
Chapter 2

Background

2.1 Anatomy & Physiology

2.1.1 Peripheral Nerves

Peripheral nerves connect the central nervous system to the periphery by conveying signals of sensation and motor movement. Within a given nerve, hundreds to thousands of nerve fibers are present, each of which can be classified based on their diameter, conduction velocity, and level of myelination. Myelin is an insulating layer surrounding nerve axons that increases signal speed via “saltatory conduction” [36]. Along the axon, gaps in the myelin sheath called “Nodes of Ranvier” are the sites for action potentials to occur. Action potentials are transmitted much faster in myelinated fibers than unmyelinated fibers because they only need to occur at the nodes to propagate, rather than needing to propagate within the entire length of fibers [36].

A representative cross-section of a peripheral nerve is shown in Fig. 2.1A, where the larger diameter fibers have a myelin sheath while the smaller diameter fibers do not. Conduction velocity has an approximately linear relationship to fiber diameter, where largest diameter fibers experience the fastest conduction rates [37]. Two conventions systems were developed for naming of fiber groups: the Erlanger and Gasser system [38], [39] for efferent and afferent fibers and the Lloyd system [40], [41] for afferent fibers (Fig. 2.1B).

In the case of afferent (sensory) fibers, action potentials propagate naturally from the sensory organs at the periphery to the spinal cord. Efferent (motor) fibers on the other hand, naturally transmit action potentials from the spinal cord to the effector muscles. When electrical stimulation is applied to a nerve, action potentials are generated in both the natural (orthodromic) and unnatural (antidromic) directions [42] (Fig. 2.2). In the bipolar electrode configuration, two electrodes are placed some distance apart along the direction of the nerve fibers. One contact is referred to as the cathode where it provides a negative polarity stimulus to the outer layer of the nervous tissue [43]. The other contact is known as the anode and it provides a return path for the electrical current. When a sufficient stimulus is applied, the nervous tissue below the cathode becomes depolarized and it generates two action potentials moving, in opposite directions, away from the site [43]. Simultaneously, the nervous tissue below the anode becomes hyperpolarized. If the tissue below the anode is significantly hyperpolarized, this could create a situation known as “anodal block” where the incoming action potential from the cathode is not able to proceed [43], [44].
Large diameter myelinated fibers have the lowest electrical activation thresholds while small diameter myelinated fibers require modest stimulation intensities to be recruited \[44, 47\]. Activation of the smallest diameter unmyelinated fibers necessitates the largest amount of electrical stimulation for activation \[44\]. Determination of fiber thresholds (T) elicited by electrical stimulation can be performed by placing one stimulation electrode at one end of the nerve and another electrode some distance away to record the evoked potentials. With supramaximal stimulation, compound action potentials are generated where waveforms for distinct fiber groups can be distinguishable based on their conduction delay and amplitude (Fig. 2.3A). Empirical studies have shown that nerve fiber thresholds have an inverse relationship with pulse duration, known as the “strength-duration curve” (Fig. 2.3B). Short pulse durations require high intensity stimulation to activate nerve fibers, while increasing the pulse duration lowers the intensity threshold \[44, 47\]. Table 2.1 contains a compiled list of nerve activation amplitudes determined for different fiber groups measured in cats and rats.

### 2.1.2 Tibial Nerve

The tibial nerve (TN) is a mixed nerve and the largest branch of the sciatic nerve. It provides motor innervation to the gastrocnemius, soleus, and flexor muscles of the back of the leg and foot as well as cutaneous connections to the sole and heel of the foot \[54, 55\]. In humans, the tibial nerve can be traced back to the L4-S3 roots of the spinal cord \[55, 56\]. In rats, neuronal tracing studies have found
that the tibial nerve has both sensory and motor connections to the L3-L6 roots \([57]-[60]\) of the spinal cord where 98-99% of sensory connections are at the L4-L5 roots \([60]\), while motor connections primarily occupy L4-L6 \([59]\). Fiber composition studies in rats have determined that of the roughly 14,000 fibers estimated within the tibial nerve, 40% are unmyelinated afferents and 25% are myelinated afferents with the remainder being efferent \([58]\). In cats, cadaveric dissection studies determined that the origin of the tibial nerve is at the L6-S1 spinal cord level \([61]\).

### 2.1.3 Lower Urinary Tract

At a high level, the lower urinary tract (LUT) can be thought of as a system involving a storage reservoir (bladder), a voiding outlet (urethra), and control values (sphincter muscles). Bladder function is controlled by a complex set of neural circuits that act upon four main anatomical features, namely, the detrusor muscle of the bladder, the urethra, the internal urethral sphincter (IUS), and the external urethral sphincter (EUS) \([62]\). Urine is transported to the bladder from the kidneys via the ureters.
Table 2.1: Collection of peripheral nerve fiber activation amplitudes in cats and rodents. T: threshold for evoking large-diameter fibers.

<table>
<thead>
<tr>
<th>Study</th>
<th>Animal</th>
<th>Nerve(s) (A(\alpha))</th>
<th>Group I (A(\alpha))</th>
<th>Group II (A(\beta))</th>
<th>Group III (A(\delta))</th>
<th>Group IV (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adelson et al [48]</td>
<td>rats</td>
<td>sciatic</td>
<td>-</td>
<td>1T</td>
<td>&gt;-4T</td>
<td>-</td>
</tr>
<tr>
<td>Harms et al [49]</td>
<td>rats</td>
<td>tibial</td>
<td>1T</td>
<td>5T</td>
<td>100T</td>
<td>-</td>
</tr>
<tr>
<td>Onda et al [50]</td>
<td>rats</td>
<td>pudendal</td>
<td>1T</td>
<td>2-3T</td>
<td>20-30T</td>
<td>-</td>
</tr>
<tr>
<td>Sato et al [34]</td>
<td>cats</td>
<td>misc. hindlimb</td>
<td>1T</td>
<td>4-10T</td>
<td>50-100T</td>
<td>-</td>
</tr>
<tr>
<td>Sato et al [35]</td>
<td>cats</td>
<td>misc. hindlimb</td>
<td>-</td>
<td>3-5T</td>
<td>67-100T</td>
<td>-</td>
</tr>
<tr>
<td>Schomburg et al [51]</td>
<td>mice</td>
<td>peroneal, sural, tibial</td>
<td>1T</td>
<td>&gt;10T</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Steffens et al [52]</td>
<td>mice</td>
<td>peroneal, sural, tibial</td>
<td>1T</td>
<td>7-8T</td>
<td>&gt;100T</td>
<td>-</td>
</tr>
<tr>
<td>Wu et al [53]</td>
<td>cats</td>
<td>saphenous</td>
<td>-</td>
<td>-</td>
<td>&gt;30T</td>
<td>-</td>
</tr>
</tbody>
</table>

The bladder acts as a reservoir to store urine until “ready” to urinate. This system is regulated by the parasympathetic, sympathetic, and somatic nervous systems, lending to its complexity. When operating normally, this system effectively occupies two different states: storage of urine and elimination of urine.

Operation of these two states is controlled by both spinal and supraspinal reflexes that involve three main peripheral nerves: the pelvic nerve (PVN, parasympathetic), hypogastric nerve (HGN, sympathetic), and pudendal nerve (PDN, somatic) [62]. The pelvic nerve is primarily responsible for relaying bladder wall distension through its afferent A\(\delta\) fibers and also delivering efferent commands to contract the bladder and relax the IUS. The hypogastric nerve provides innervation to relax the bladder wall and constrict the smooth IUS and bladder neck to facilitate filling. Finally, the pudendal nerve provides efferent input to the striated EUS (Fig. 2.4A). The complexity of this system is further enhanced by the formation of nerve plexi from the combination of sympathetic and parasympathetic nerve fibers [63].

In humans, spinal attachments of the LUT are as follows: T10-L2 (HGN) and S2-S4 (PVN and PDN) [55], [56], [63]. This innervation differs slightly in rats: L1-L2 (HGN) and L6-S1 (PVN and PDN) [64]–[66]. Differences in spinal location are also present in cats: L2-L4 (HGN) and S1-S3 (PVN and PDN) [67], [68].

![Figure 2.4](image-url)

Figure 2.4: Neural circuits that control micturition. (A) Representative female (human) anatomical structures of the LUT. (B) Bladder storage neural circuit. (C) Bladder voiding neural circuit. HGN: hypogastric nerve, PVN: pelvic nerve, PDN: pudendal nerve, SN: sciatic nerve, PAG: periaqueductal gray, PMC: pontine micturition center. [63]

During bladder filling, distension is sensed and transmitted by afferent pelvic nerve fibers which 1)
activate the hypogastric nerve to relax the bladder wall and constrict the IUS and 2) engage the pudendal nerve to constrict the EUS (Fig. 2.4B). There is some evidence that the rostral pons in the brain might also contribute to EUS constriction during filling, but this state is largely governed by spinal reflexes [63].

Elimination of urine (voiding) involves the spinobulbospinal micturition reflex that is triggered by a volume-pressure threshold being met in the bladder that instigates intense afferent firing of the pelvic nerve due to bladder wall distension (Fig. 2.4C). These afferent signals travel through the spinal cord up to the periaqueductal gray (PAG) relay station in the midbrain that relays commands to the pontine micturition center (PMC) in the brainstem. In response to the intense afferent firing, the PMC coordinates with higher brain regions, then transmits descending signals to the spinal cord to inhibit hypogastric nerve activity, promote pelvic nerve activity, and inhibit pudendal nerve activity. The effect of these changes result in relaxation of both sphincters as well as contraction of the bladder, allowing for urine to be expelled out of the urethra. To ensure efficient voiding, the bladder continues to contract (via secondary reflexes) until its contents are virtually empty with some residual remaining. Secondary reflexes use bladder wall distension and urine flow sensation in the urethra as a positive feedback loop to keep the bladder detrusor muscle contracting until it empties [63].

2.1.4 Species Comparison

For this body of work, it was necessary to compare and contrast common animal models with humans. There are slight differences in spinal nerve connections but the LUT system has been shown to have the same set of principles as described earlier. The main difference in urinary function observed in small rodents (rats and mice) is the action of the EUS during voiding. Unlike in humans and in some larger animals, the EUS of small rodents is not silent during voiding, and instead, contracts in bursts, on and off, with a rhythm of 3 to 10 Hz. It has been commonly accepted that this bursting action helps facilitate voiding and increase efficiency in these animals due to their small anatomy as voids are expelled as droplets and not as a continuous stream [69].

For our purposes, it was also of interest to generate a side-by-side comparison of the relevant neural connections of the tibial and bladder nerves to the spinal cord in these species and in humans (Fig. 2.5).

From this naive look at spinal connections, it is interesting to note that the hypogastric nerve in all species is distant from the rest and that the spinal segments to which the tibial nerve projects have direct overlap with the pelvic and pudendal nerves at the spinal cord. Consistent with these spinal arrangements, in cats it has been shown that electrical stimulation of tibial nerve afferent branches evokes suppression of activity by way of the pelvic nerve and not the hypogastric nerve [34]. It is not clear whether this prolonged inhibition of pelvic activity occurs primarily at the level of the spinal cord or in the micturition centers of the midbrain [32].

2.2 Models for Evaluating Bladder Function

Models used for investigating bladder function in humans and animals has been around since the mid-1800s where the primary variables are the bladder pressure and bladder volume [70]. Cystometry is a diagnostic procedure used (in animals and humans) to evaluate urodynamic function of the LUT by means of infusing fluid into the bladder and observing how it performs in collaboration with the urethra [71], [72]. This section details common bladder models used in pre-clinical research.
Chapter 2. Background

2.2.1 Isovolumetric Bladder

In this isovolumetric model, bladders are catheterized through the urethra with a tight seal to prevent leakage of fluid around the catheter. Fluid is infused into the bladder and held at a desired constant volume. Interestingly, bladders enter a state of rhythmicity where high amplitude contractions are observed with silent rest periods at baseline pressures in between 74, 75 (Fig. 2.6). When the bladder volume is increased, the baseline pressure within the bladder also increases, causing the frequency of large amplitude bladder contraction to increase 75. This model has shown to be reliable in producing regular and consistent bladder contractions 33. The two main parameters of interest from these are the maximal pressure (MP) and the inter-contraction interval (ICI, or its reciprocal, the bladder contraction frequency). Studies that use this model intend to either evoke or inhibit contractions with their treatment. One disadvantage of this model is that voiding information cannot be extracted because the urethral outlet is blocked. Irritation of the urethra has also been noted as another potential disadvantage 76.

2.2.2 Single-fill Cystometry

Application of the single-fill model can be performed either by catheterizing the bladder via the urethra (identical to the isovolumetric model) or the bladder dome 71. Infusion is performed on an empty bladder and stopped once the first large amplitude contraction occurs (Fig. 2.7). Catheterization via
Chapter 2. Background

Figure 2.7: Single-fill cystometry bladder pressure recording. MP: maximal pressure, TP: threshold pressure, FC: filling compliance ($Q_{pump} \cdot \Delta T/\Delta P$).

The dome allows for normal voiding to occur through the urethra. Most commonly, the parameters of interest are the threshold pressure (TP), bladder capacity BC, maximal pressure, filling compliance (FC), voided volume (VV), residual volume (RV), and voiding efficiency (VE). One disadvantage of this preparation is that prior to each fill, the bladder requires emptying via syringe which may disrupt the bladder by causing non-physiological pressure transients to occur \[77\].

2.2.3 Continuous-fill Cystometry

Figure 2.8: Continuous-fill cystometry bladder pressure and voided volume recordings. MP: maximal pressure, TP: threshold pressure, BP: basal pressure, VV: voided volume, ICI: inter-contraction interval.

Similar to the single-fill cystometry model, continuous-fill cystometry involves infusion of the bladder via the dome typically performed at supraphysiological rates \[71\]. Voided volumes are either collected and measured manually via syringe or gravimetrically using a balance or transducer. Also, electromyography activity of the EUS is often measured via percutaneous wire electrodes. The main difference from the single-fill model is that continuous-fill cystometry evokes repeated bladder contractions without emptying the bladder in between contractions (Fig. 2.8), mitigating any potential disturbance issues. A 30- to 120-minute baseline infusion period is typically employed to stabilize contractions prior to treatment testing \[71\]. Parameters of interest for this model include those stated for single-fill infusion but also the inter-contraction interval (or its reciprocal, the bladder contraction frequency), EUS bursting, and basal
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pressure. Since the bladder is not emptied during the trial, the bladder volume must be estimated using by using the voided volume, infused volume, and the final residual volume at the end of the trial. A thorough discussion of bladder volume estimation during continuous-fill cystometry has been described by Danziger and Grill [77].

2.2.4 Hyperactive Bladder

Physiological saline is commonly used for bladder studies, but other animal models have been developed to simulate human bladder conditions. The hyperactive bladder model simulates OAB by irritating the bladder with low-concentration acetic acid, causing an increased bladder contraction frequency (BCF), decreased BC, and decreased threshold pressure [78], [79]. This irritation operates through a spinal reflex by activating ‘silent’ unmyelinated nociceptive C fibers within the bladder that are sensitive to chemical irritation but not bladder distension. With increased firing of these fibers, the micturition reflex can be initiated much earlier than with just distension [81]. Interestingly, a host of pre-clinical evidence has shown that these ‘silent’ C fibers can undergo neuroplastic changes to respond to mechanical stimuli of the bladder in cases of chronic spinal cord injury (SCI) [82], [83].

2.3 Tibial Nerve Stimulation Therapy

This section provides a summary of key clinical and pre-clinical studies that tested the efficacy of tibial nerve stimulation as an effective bladder treatment as well as its physiological mechanism.

2.3.1 Clinical Results

The first reported case of successful bladder inhibition in patients using tibial nerve stimulation was by McGuire et al in 1983 [20]. Transcutaneous electrodes were placed bilaterally over each tibial nerve (at the level of the ankle), and stimulation was applied at 2-10 Hz with an intensity set by the patient. The conception of the tibial nerve as a potential target for bladder therapy stemmed from observed results in Chinese acupuncture. This therapy was effective for patients with and without SCI by reducing the frequency of uncontrolled urgency episodes and increasing effective filling capacity. Of the 22 patients within the study, 12 (55%) were relieved of their symptoms, and 8 (36%) showed improvement.

The year of 1999 marked a significant turning point in tibial nerve stimulation therapy following the presentation of an abstract published by Stoller [15]. In this work, Stoller described “Stoller Afferent Nerve Stimulation” (SANS) technique for applying tibial nerve stimulation for bladder therapy, along with associated clinical results. Of the 90 patients in the study that had experienced pelvic floor dysfunction issues for many years, 80% showed at least a 50% improvement in voiding and pain scores due to weekly 20- to 30-minute PTNS sessions for 10 weeks.

SANS is performed by a clinician inserting a 34-gauge acupuncture-like needle into the leg approximately 5 cm cephalad from the medial malleolus (2-4 cm in depth) and an adhesive return electrode attached to the medial side of the heel. With the electrodes connected to a stimulation controller, electrical pulses (20 Hz, 200 μs) are applied with stimulation intensity gradually increased until flexion of the foot and toes is visible. The intensity is then reduced slightly to activate the tibial nerve without a motor response. With the intensity set, stimulation is provided for 30 minutes. [84]. The conception
of SANS was fundamental for the instantiation of PTNS treatment protocol standards as many groups began to use this technique.

With the introduction of SANS, two different groups based in the Netherlands [22] and the United States [21] set out to perform key multicenter trials to validate the effectiveness of PTNS over the years of 1999-2001. In contrast to the 10-week protocol used by Stoller [15], both groups chose to perform weekly PTNS sessions for 12 weeks. Following the 12 weekly sessions, Govier et al (USA) observed a 71% success rate of the 53 OAB patients enrolled, where success was defined as a minimum of 25% reduction in daytime and/or nighttime frequency. Van Balken et al (Netherlands) published months later with a 60% success rate of the 49 patients enrolled. In contrast to Govier et al, Van Balken et al defined success as the patient request further chronic treatment, which is inherently subjective. Nonetheless, the results of these two primary multicenter studies gave further credence to the PTNS cause.

In 2003, Vandoninck et al published results from two multicenter studies using the same 12-week protocol used by Govier et al and Van Balken et al. Results from the first trial showed, that of 35 patients that were enrolled 63% requested chronic PTNS treatment following the 12-week program, 70% had at least 50% less leakage episodes per day as compared to before the study, and 46% of patients were cured of all leakage episodes by the end of the trial [16]. In the second study, 90 patients were enrolled with identical success measures as the first study but with another goal of identifying predictive factors for better PTNS outcomes through cystometric analysis [17]. It was shown that 64% of patients requested further chronic treatment and 56% had objective improvements in leakage events per day. The study also concluded that detrusor instability could not be abolished by PTNS.

Since McGuire et al published their seminal work in 1983, most studies tended to focus on the cumulative long-term effects of PTNS, with little focus on the acute effects during stimulation sessions. In 2003, Amarenco et al performed transcutaneous PTNS at 10 Hz during cystometry fills on 44 OAB patients, with a variety of neural impairments [26]. Success was achieved by a 50% (or 100 mL) increase to either the first involuntary contraction volume (FICV) or to the maximum cystometric capacity (MCC) during filling. The study observed a 50% success rate with mean increases to FICV and MCC of 143% and 125% during filling as compared to pre-filling values, respectively.

With positive findings found under both acute and prolonged conditions, confidence in PTNS began to grow, and a shift towards parameter optimization began to form. In 2004, Finazzi-Agro compared the results of PTNS between patients that were provided with 30-minute stimulation 3 times per week versus the standard once per week [85]. Through bladder diaries, quality of life questionnaires, and cystometric capacity testing, it was concluded that stimulation 3 times per week was no better than once per week following 12-week PTNS therapy. It was noted that more stimulation sessions could attribute to a more rapid onset of relief during the program but had diminishing returns by the end.

In 2009, Peters et al performed the first randomized multicenter trial to compare the effects of tolterodine (an antimuscarinic) and PTNS therapy [23]. One hundred OAB patients were randomly assigned into the drug or PTNS group with 24-hour void diaries taken before and after the 12-week study. PTNS was administered once per week for 30 minutes, while the drug group ingested 4 mg of tolterodine daily. Their results showed that 80% of PTNS patients were cured or showed improvement in voiding diary symptoms while only 55% of the tolterodine group showed success. Although this study was not placebo-controlled, it showed that PTNS therapy could be a clinically significant alternative OAB therapy. To address the lack of control in PTNS trials, Peters et al developed an effective sham procedure to be used in future trials [86]. With the sham validated, Peters et al were able to gain level
1 scientific evidence during a multicenter, double-blinded, randomized, controlled PTNS trial, coined the SUmiT Trial [18]. Two hundred and twenty OAB patients were randomly assigned into a sham or PTNS treatment group. Following 12 weeks of treatment, baseline 3-day diary summaries were compared with 3-day diary summaries one week after treatment had ended. The PTNS group showed a 55% response rate to significant improvement in bladder symptoms while the sham group had a 21% rate of improvement. Using a different sham technique, a follow-up study by Finazzi-Agro et al showed a 71% response rate in the PTNS group with no responders in the sham group [19].

Following the SUmiT Trial, PTNS gained further attention and recognition of long-term efficacy during the STEP Study performed by Peters et al [25]. This study aimed to determine any potential long-term benefits of PTNS treatment in patients that were positive responders to PTNS therapy during the SUmiT Trial. Fifty patients were initially selected for the study and provided with a 14-week PTNS treatment plan that eventually tapered down to one treatment per month by its end. Symptom improvement scores were evaluated on a three-month cadence while voiding diaries were analyzed every 6 months. Following 3 years of observation, 29 patients had completed the study with a mean PTNS treatment cadence of 1.1 sessions per month after the 14-week tapering period. In summary, all patients maintained significant increases to quality of life scores as compared to pre-SUmiT baseline levels and mean voids per day decreased from 12.0 to 8.7.

2.3.2 Pre-clinical Results

The mechanism of TNS has been explored pre-clinically for many years, with the majority of its initial work performed in cats with more recent contributions from rat models. This section will outline some of the key findings from these studies, first in cats, then in rats.

In 1966, using an isovolumetric model, McPherson showed that stimulation of the tibial nerve slightly above threshold at 0.17-0.75 Hz could reliably inhibit rhythmic contractions in the anesthetized cat [33]. From nerve recordings at the dorsal roots, it was found that the corresponding action potentials to stimulation were likely due to large diameter afferent fibers as their conduction velocities exceeded 50 m/s. Conversely, this observed inhibition was not present in acute- or chronic-SCI anesthetized animals. Interestingly, stimulation in unanesthetized decerebrate cats further enhanced contractions, rather than providing inhibition. He concluded that a supraspinal pathway was necessary for TNS bladder inhibition.

In 1980, Sato et al performed hindlimb nerve stimulation studies in anesthetized cats under isovolumetric conditions [34]. Two major findings from their work were that 1) the level of distention of the bladder could modulate whether hindlimb nerve stimulation had excitatory or inhibitory effects and 2) recruitment of group III (Aδ) and IV (C) afferent fibers were required to elicit these excitatory and inhibitory effects. In anesthetized intact cats, hindlimb nerve stimulation applied under empty or near empty bladder conditions, evoked an immediate contraction of the bladder while the same stimulation applied to a full bladder inhibited rhythmic contractions during stimulation with some lasting effect after stimulation ended. Of particular note, stimulation of the nerves to the gastrocnemius and soleus muscles (both branches of the tibial nerve) at 2 to 10 Hz was effective in inhibiting rhythmic contractions. Recording of the pelvic and hypogastric nerves showed that both the excitatory and inhibitory effects of stimulation involved only the pelvic nerve branches. It was concluded that the excitatory effects on quiescent bladders were due to spinal reflexes because they were still present after cervical spinal transection (at the end of experiments) and the inhibitory effects on large bladders were due to supraspinal pathways to the brain. Three years later, Sato et al followed-up with a similar study but in anesthetized
chronic-SCI cats [35]. Similar to their findings in anesthetized intact animals, they found that hindlimb nerve stimulation, only at intensities able to activate group III and group IV afferents, evoked excitatory effects on the empty bladder and inhibition of rhythmic contractions of the full bladder. The inhibitory effects observed in anesthetized chronic-SCI cats by Sato et al were in direct contrast to the lack of effects noted by McPherson.

In 1993, a study by Walter et al enforced the possibility that anesthetized animals may complicate our understanding of stimulation mechanisms on the bladder [87]. Their study showed that stimulation of the tibial nerve in unanesthetized chronic-SCI cats at intensities below, equal to, and above threshold at frequencies ranging from 3.5 to 35 Hz all failed to inhibit rhythmic bladder contractions.

In recent times, a definitive shift in focus was made from strictly inhibition of isovolumetric contractions to modulating the capacity of the bladder during single-fill trials. Also, the hyperactive bladder model, using low-concentration acetic acid, was introduced and became a staple for simulating overactive bladder conditions during TNS studies. In 2010 and 2011, the results of two studies performed by Tai et al showed that TNS (nerve cuff) or plantar surface stimulation (sole of foot) applied at 2 to 4 times the toe or foot motor threshold ($T_{\text{mot}}$), at 5 to 30 Hz, could reliably inhibit rhythmic isovolumetric contractions and increase bladder capacity under normal (saline) and hyperactive (acetic acid) conditions [29], [88]. When stimulation was applied during single-fills, saline BC typically increased to 140-150% of saline control while the hyperactive bladder typically increased from 20% of saline control to 40-50% of saline control. Interestingly, when stimulation was applied during fills (typically 3 to 5 minutes) there were no lasting effects on BC following termination of stimulation. As a follow-up study, Tai et al had noted that repeated short duration TNS during isovolumetric contractions contributed to lasting increases in BC (around 180% of saline control) when single-fill trials were performed afterwards [27]. Interested in the possibility of prolonged inhibition, they were able to show that prolonged increases to BC (over 2 hours) could be achieved following 30 minutes of TNS at 2 to 4$T_{\text{mot}}$ (5 or 30 Hz). A similar study performed by Chen et al, also showed prolonged increase to BC following 30-minute surface plantar stimulation but this only lasted for 10 to 15 minutes after stimulation was ended [89].

A first step was taken by Tai et al into understanding the potential TNS mechanism by investigating the role of opioid receptors [90]. Using an opioid receptor blocker (naloxone), they were able to show that naloxone alone did not affect BC of the hyperactive bladder but it significantly reduced BC under normal (saline) conditions. When provided before or after stimulation, it was found that naloxone could eliminate acute TNS inhibition of the hyperactive bladder. In the case of the normal bladder, naloxone significantly reduced BC when applied before or after TNS, but this effect was no different than when naloxone was applied alone. From these results, they concluded that naloxone modulates normal bladder function but not hyperactive bladder function and that it eliminates the effect of TNS in the hyperactive but not the normal bladder [31]. Curious of the discovery of a potential opioid receptor mechanism for TNS, Zhang et al investigated the effects of an opioid receptor agonist (tramadol) on TNS and bladder hyperactivity [91]. Their findings showed that both tramadol and TNS (2-4$T_{\text{mot}}$, 5 Hz) could individually increase hyperactive BC from 20% to 40-60% of saline control but the combination of the two could fully restore BC. Further, high dose tramadol combined with TNS could increase hyperactive BC to 140-150% of saline control. This study was also able to show that combined tramadol and TNS treatment could unmask a prolonged increase to hyperactive bladders for 1-2 hours following stimulation. As a confirmation, naloxone treatment completely reversed the effects of tramadol and TNS. A similar study in the same year by Mally et al, confirmed this same prolonged increase to hyperactive BC with
combination treatment of tramadol and plantar surface stimulation [92]. The necessity of tramadol for evoking a prolonged BC increase to hyperactive bladders was reconfirmed by Ferroni et al when it was determined that 30-minute TNS could not elicit lasting increases to BC following stimulation [31]. A series of follow-up studies to investigate combinational drug and stimulation therapies were performed by Matsuta et al [93] and Schwen et al [94, 95]. Respectively, their findings concluded that an metabotropic glutamate receptor antagonist could suppress TNS inhibition of hyperactive bladders and that tolterodine (an antimuscarinic) combined with plantar stimulation could restore hyperactive bladders to control BC.

Starting in 2014, a further push was directed towards the mechanistic operations of TNS bladder neuromodulation in relation to the central nervous system. Similar to the findings of McPherson under normal conditions, Xiao et al showed that TNS could not inhibit rhythmic contractions or increase BC of acute-SCI anesthetized under hyperactive bladder conditions [96]. In 2015, Ferroni et al showed that application of naloxone intravenously or to the brain stem in decerebrate anesthetized cats completely eliminated acute TNS inhibition of hyperactive bladders - reinforcing the potential of opioid receptor mediation. Lyon et al were able to determine that pudendal nerve stimulation but not TNS could suppress efferent contractile signals evoked from electrical stimulation of the PMC in the brain [30]. This result was important because it began to solidify the notion that TNS inhibition acts on ascending bladder afferent signals and not descending efferent signals. To determine the critical spinal components of TNS neuromodulation, Bansal et al monitored the effects of TNS on hyperactive bladders after individual lumbosacral transections from L5 to S3 in cats. Although, transection of L5 and L6 dorsal roots had no effect and only a mild reduction in TNS inhibition, respectively, transection of L7 dorsal roots completely eliminated TNS inhibition [97]. A recent study by Yecies et al, showed that under constant pressure conditions, normal bladder afferent firing from stretch receptors was inhibited by TNS at the S2 level of the spinal cord [32]. This inhibition was not altered by intravenous naloxone, which is consistent with the findings of Tai et al [90].

Although the abundance of TNS literature is not as numerous for rats as for cats, there is a level of consistency within the observed effects of TNS, with a few notable differences. Under isovolumetric conditions, Su et al showed that bilateral stimulation of the tibial nerve produced acute inhibition of normal rhythmic contractions in urethane-anesthetized rats [98]. By varying stimulation parameters, this inhibition was found to be both amplitude- and frequency-dependent, with optimal inhibition observed with $3T_{mot}$ at 10 Hz. A follow-up study by the same group showed that there was no difference between bilateral or unilateral TNS and that inhibition of bladder contractions was short lasting, usually most effective within the first 5 minutes of treatment [99].

To assess the acute and prolonged effects of TNS, Matsuta et al performed single-fill trials on anesthetized rats [28]. In contrast to the results from anesthetized cats, TNS was unable to increase BC when applied during filling in anesthetized rats. Similar to cats, a prolonged increase of 140-150% from control BC was observed for about 50 minutes following 30-minute ($3T_{mot}$, 5 Hz) TNS under normal bladder conditions. TNS was not shown to significantly affect voiding pressure or voiding efficiency - in-line with the notion that it only affects ascending afferent neural pathways.

For the first time, the effects of TNS were assessed during continuous-fill cystometry in rats by Kovacevic and Yoo [100]. Using stimulation at 6 times the threshold for evoking foot electromyography (EMG) activity ($T_{EMG}$), they were able to show frequency-dependent decreases to the bladder contraction frequency with the most significant effects observed at 5 and 10 Hz.
To assess the effects of TNS on hyperactive bladder conditions in the rat, Choudhary et al performed 30-minute stimulation trials to elicit post-stimulation increases to BC \cite{101}. Unexpectedly, during the first arm of their study they were unable to show increases to BC (under saline conditions) following 30-minutes of TNS using the same stimulation parameters as Matsuta et al. They suspected that differences in stimulation configuration (tripolar versus bipolar) or rat type (Sprague-Dawley versus Wistar) may have given rise to the inconsistent results. Under hyperactive conditions, Choudhary et al were able to show increases to BC as well as a decrease in afferent bladder firing from the pelvic nerves.

Most recently, Chen et al performed foot stimulation in unanesthetized chronic-SCI rats (3 weeks post-SCI) \cite{102}. Unlike the results found by Walter et al in unanesthetized chronic-SCI cats and Matsuta et al in anesthetized rats, Chen et al showed that BC could be increased with stimulation provided during single-fills at 2-4T_{mot} (5 Hz).

Taken as a whole, these results can be summarized as follows:

- TNS can inhibit rhythmic isovolumetric contractions under normal and hyperactive conditions.
- TNS can significantly increase BC under normal and hyperactive conditions.
- TNS inhibition is optimal when applied at 2-6T_{mot} between 5-30 Hz.
- TNS applied continuously for 30 minutes can elicit prolonged increases to BC.
- TNS primarily affects ascending (capacity) and not descending (voiding) pathways of bladder function.
- TNS sites of action may be both spinal and supraspinal.
- Opioid receptors interactions may be involved in the TNS mechanism.
- The inhibiting effects of TNS are not identical across species and may be modulated by anesthesia.

### 2.3.3 Gaps in Literature

Although the bladder-inhibiting effects of TNS in cats and rats are not identical across all models, it is clear that TNS is able to inhibit rhythmic isovolumetric contractions and provide acute and prolonged bladder capacity increases without affecting voiding in single-fill models. Kovacevic and Yoo \cite{100} were successful in showing significant changes to bladder contraction frequency with TNS applied during continuous-fill cystometry, but how changes to this parameter relate to bladder capacity and voiding is not clear. Also, since the TNS trials were of 10-minute duration, only acute effects were observed, so it would be of interest to further expand this model by performing 30-minute TNS trials to compare the prolonged effects of TNS in single-fill models with a continuous-fill model. By a similar token, there has yet to be a study in rats to observe TNS effects on bladder function during stimulation for 30 minutes.

Stimulation amplitude and frequency for effective TNS bladder inhibition seem to have converged onto narrow ranges of 2 to 6T_{mot} and 5 to 30 Hz \cite{27, 29, 98, 99}, respectively. What is not clear from these studies, is the level of nerve fiber activation, as nerve conduction studies have not been performed. The foot or toe twitch response is simple and non-invasive method for determining nerve activation that is commonly used but it is qualitative and subjective. Kovacevic and Yoo \cite{100} addressed this concern by determining TN activation thresholds using evoked foot EMG instead of the visual motor twitches, to give a quantitative basis for this threshold. Recruitment curves determined that stimulation at 6
times this EMG threshold ($T_{\text{EMG}}$) was able to fully recruit efferent activity. Since it is largely agreed upon that afferent stimulation of the TN provides bladder neuromodulation [27], [29]–[31], afferent nerve fiber recruitment should dictate how nerve thresholds are determined. It is interesting to note, that the threshold range found by Kovacevic & Yoo [100] was one order of magnitude lower than those of Su et al [98], [99] and Matsuta et al [28] (Table 2.2).

In past cat studies, conflicting results have been reported regarding which nerve fibers in the TN are present during bladder inhibition. McPherson reported that inhibition of rhythmic isovolumetric contractions in cats occurred using 1 to 2 V stimulation (100 µs) and compound action potentials, measured at the dorsal root, had conduction velocities in excess of 50 m/s, implying large myelinated afferent fibers [33]. On the other hand, Sato et al showed that these contractions in cats were not inhibited by group I or II ($\alpha$ or $\beta$) afferents but rather high threshold group III ($\delta$) and group IV (C) afferents [34]. A similar study, by Onda et al, performed in rats, showed that pudendal nerve stimulation, at amplitudes that activated small diameter nociceptive fibers, was most effective at inhibiting bladder function [50].

Further disconnects regarding stimulation amplitude result from studies that use constant-voltage stimulation rather than constant-current, as nerve activation will depend on the electrode impedance. Recent studies have repeatedly presumed that stimulation at 2 to $6T_{\text{mot}}$ is below the threshold for activating small diameter nociceptive fibers [27], [29], [88], [92], [103]–[106], but this still has yet to be confirmed.

Table 2.2: Comparison of stimulation amplitudes and frequencies used in pre-clinical TNS studies. *Values not found in publication but sourced from direct communication with authors.

<table>
<thead>
<tr>
<th>Study</th>
<th>Animal</th>
<th>Threshold</th>
<th>Threshold Range</th>
<th>Trial Amplitude</th>
<th>Trial Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bansal et al [97]</td>
<td>cats</td>
<td>toe twitch</td>
<td>0.8-1.2 V</td>
<td>$4T_{\text{mot}}$</td>
<td>5 Hz</td>
</tr>
<tr>
<td>Choudhary et al [101]</td>
<td>rats</td>
<td>toe twitch</td>
<td>0.7-2 V*</td>
<td>$3T_{\text{mot}}$</td>
<td>5 Hz</td>
</tr>
<tr>
<td>Ferroni et al [31]</td>
<td>cats</td>
<td>toe twitch</td>
<td>0.22-3 V</td>
<td>$3T_{\text{mot}}$</td>
<td>5, 30 Hz</td>
</tr>
<tr>
<td>Fuller et al [105]</td>
<td>cats</td>
<td>toe twitch</td>
<td>0.6-3 V</td>
<td>$2, 4T_{\text{mot}}$</td>
<td>5 Hz</td>
</tr>
<tr>
<td>Kovacevic &amp; Yoo [100]</td>
<td>rats</td>
<td>foot EMG</td>
<td>5-60 µA</td>
<td>$6T_{\text{EMG}}$</td>
<td>2, 5, 10, 20, 50 Hz</td>
</tr>
<tr>
<td>Lyon et al [30]</td>
<td>cats</td>
<td>toe twitch</td>
<td>1-2 V</td>
<td>$2, 4T_{\text{mot}}$</td>
<td>5 Hz</td>
</tr>
<tr>
<td>Matsuta et al [93]</td>
<td>cats</td>
<td>toe twitch</td>
<td>0.35-2.4 V</td>
<td>$2, 4T_{\text{mot}}$</td>
<td>5 Hz</td>
</tr>
<tr>
<td>Matsuta et al [28]</td>
<td>rats</td>
<td>toe twitch</td>
<td>50-600 µA</td>
<td>$2-4T_{\text{mot}}$</td>
<td>5 Hz</td>
</tr>
<tr>
<td>Su et al [98]</td>
<td>rats</td>
<td>toe twitch</td>
<td>10-480 µA</td>
<td>$1-5T_{\text{mot}}$</td>
<td>1, 10, 20, 50 Hz</td>
</tr>
<tr>
<td>Su et al [91]</td>
<td>rats</td>
<td>toe twitch</td>
<td>10-600 µA</td>
<td>0.8-6$T_{\text{mot}}$</td>
<td>10 Hz</td>
</tr>
<tr>
<td>Tai et al [27]</td>
<td>cats</td>
<td>toe twitch</td>
<td>1.5 V</td>
<td>$2, 4T_{\text{mot}}$</td>
<td>5, 30 Hz</td>
</tr>
<tr>
<td>Tai et al [27]</td>
<td>cats</td>
<td>toe twitch</td>
<td>3-3.75 V</td>
<td>$2, 4T_{\text{mot}}$</td>
<td>5, 30 Hz</td>
</tr>
<tr>
<td>Tai et al [90]</td>
<td>cats</td>
<td>toe twitch</td>
<td>0.5-3 V</td>
<td>$4, 8T_{\text{mot}}$</td>
<td>5 Hz</td>
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<td>Xiao et al [76]</td>
<td>cats</td>
<td>toe twitch</td>
<td>1.2-3.5 V</td>
<td>$2, 4T_{\text{mot}}$</td>
<td>1, 5, 10, 20, 40 Hz</td>
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<tr>
<td>Yeceles et al [72]</td>
<td>cats</td>
<td>toe twitch</td>
<td>0.5-2.4 V</td>
<td>$2, 4T_{\text{mot}}$</td>
<td>5 Hz</td>
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<tr>
<td>Zhang et al [91]</td>
<td>cats</td>
<td>toe twitch</td>
<td>0.35-4.5 V</td>
<td>$2, 4T_{\text{mot}}$</td>
<td>5 Hz</td>
</tr>
</tbody>
</table>

In summary, there is a need to further connect the TNS continuous-fill model with previously performed isovolumetric and single-fill models, specifically to identify if prolonged BC increases are present. Secondly, it is important to relate stimulation amplitude to physiologically relevant information, namely, which afferent fibers in the TN are activated during stimulation.

### 2.4 Research Objectives

The aim of this study was to investigate the effects of electrical activation of different TN fiber types on reflex bladder inhibition in anesthetized rats.
2.4.1 Hypotheses

We hypothesize that:

1. TNS applied at 5 Hz for 30 minutes will not significantly affect VV, but will significantly increase BC during stimulation (acute) with a prolonged increase following the termination of stimulation.

2. Increases to BC evoked by TNS will be dose-dependent with C fiber activation yielding the strongest response.

2.4.2 Objectives

To test these hypotheses this study aims to:

1. Determine TN fiber threshold activation through nerve conduction testing.

2. Utilize a ureter-transected continuous-fill rodent model with void collection to accurately measure voided volume and estimate bladder volume throughout each experiment.

3. Investigate the effects of TN fiber type activation (i.e. stimulation amplitude) on key bladder pressure and volume parameters.
Chapter 3

Methodology

All surgical and experimental procedures were approved by the University of Toronto Animal Care Committee in accordance with the regulations of the Ontario Animal Research Act (Toronto, ON, Canada).

3.1 Anesthetic Protocol

Twenty-four female Sprague-Dawley rats (298 ± 6 g, range: 240 to 332 g) were initially induced with 5% isoflurane (100% O₂, 1 L/min) and maintained with 1-2% isoflurane for the duration of surgery (1-2 hours). Following surgical procedures, the anesthesia was transitioned to urethane (1.6 g/kg in 1 mL saline, s.c.) over a period of 60 to 90 minutes (Fig. 3.1). As isoflurane was gradually titrated down to 0%, urethane was administered in two boluses (0.7 mL and 0.3 mL aliquots), 30 minutes apart. Oxygen flow was discontinued, and the animal was allowed to breathe room air for the duration of the experiment. Rectal temperature was maintained at 37°C with a feedback heating plate throughout the entire experiment (TCAT-2LV, Physitemp Instruments, Clifton, NJ, USA). Heart rate was monitored and recorded via three-lead electrocardiograph. At the end of each experiment, the animal was euthanized by intracardiac injection of T-61 (0.3 g/kg, Merck, Kenilworth, NJ, USA).

<table>
<thead>
<tr>
<th>Surgery</th>
<th>TNS Foot EMG Thresholds</th>
<th>Apparatus Set-up &amp; Calibration</th>
<th>Animal Stabilization (BT/HR/BR)</th>
<th>Bladder Data Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 hr</td>
<td>0.5 hr</td>
<td>0.5 hr</td>
<td>1.5 hr</td>
<td>8.5 to 9.5 hr</td>
</tr>
<tr>
<td>0 g/kg</td>
<td>3%</td>
<td></td>
<td>1.6 g/kg Urethane</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.1: Anesthesia protocol displaying the transition from isoflurane to urethane anesthesia over time.
3.2 Surgical Protocol

The abdomen, pubic region, and entire right lower limb were shaved clear of any hair. An incision along the animal’s midline was made to expose the abdominal cavity and a separator was used to maintain the visual field of view. The flared end of a suprapubic bladder catheter (PE-50) was inserted into the bladder dome and secured with a suture ligature. Both ureters were ligated distally, and the proximal ends were catheterized (PE-10) 2 to 3 cm caudal to the kidneys (Fig. 3.2). The bladder was then emptied of all contents and the abdomen was sutured closed in two layers with bladder and ureteral catheters externalized and secured to the skin with sutures. The EMG activity of the EUS was measured using a pair of stainless steel wires (791000, diameter = 0.003”, A-M Systems, Sequim, WA, USA) inserted suprapubically parallel to the urethra and secured to the skin. EUS EMG was conditioned (gain = 1000 V/V, bandwidth = 0.3 to 3,000 Hz) with a low-noise amplifier (SR560, Stanford Research Systems, Sunnyvale, CA, USA). A small incision was made cephalad to the medial malleolus followed by blunt dissection to expose the tibial nerve. A custom bipolar stimulating nerve cuff electrode (platinum contacts = 1 mm x 4 mm, inter-contact distance = 2 mm) was implanted on the nerve trunk. The risk of nerve drying, and electrode displacement was mitigated by applying a two-part silicone adhesive (KWIK-CAST, World Precision Instruments, Sarasota, FL, USA) over the electrode, nerve, and surrounding tissue. One pair of stainless steel wires was inserted into the mid-sole and between the hallux and second digit to capture efferent activity mediated by the tibial nerve. A reference ground needle electrode was placed in the left hind limb.

3.3 Setting the Stimulation Amplitude

The threshold amplitude for eliciting a foot EMG response ($T_{EMG}$) was determined by applying 5 second trains of biphasic electrical pulses (200 µs/phase, frequency = 2 Hz, cathode proximal) through the nerve cuff electrode with an isolated constant-current stimulator (2100, A-M Systems, Sequim, WA, USA). The amplitude was increased incrementally in 1 µA steps until threshold electrical muscle activity was observed. The stimulation amplitude was further increased to determine the motor threshold ($T_{mot}$), which was defined as the stimulation amplitude that evoked visible toe twitches corresponding to each electrical pulse.

At the end of the experiment in 4 animals, the electrical recruitment of different fiber types of the tibial nerve (e.g., Aβ, Aδ, and C fibers) was investigated by recording the evoked electroneurogram (ENG) at location proximal to the site of electrical stimulation. With the animal placed in a lateral position, the sciatic nerve was accessed by an incision and blunt dissection of the biceps femoris muscle. The tibial branch of the sciatic nerve was surgically isolated and a recording bipolar nerve cuff electrode (stainless steel, inner diameter = 1 mm, inter-contact distance = 1 mm, MicroProbes, Gaithersburg, MD, USA) was implanted around the nerve (4 to 5 cm proximal to the first stimulation nerve electrode). The measured ENG and EMG were both conditioned (gain = 1000 V/V, bandwidth = 30 to 30,000 Hz) with low-noise amplifiers (SR560, Stanford Research Systems, Sunnyvale, CA, USA). Foot EMG and tibial ENG were simultaneously measured as the stimulation amplitude of individual pulse trains (duration = 5 s, frequency = 2 Hz) was increased from 0.5 to 300 times $T_{EMG}$. Fiber classification of waveforms was determined by the expected threshold and conduction velocity from past pre-clinical studies in rodents [48], [49], [52].
1. Bladder catheter  4. TNS stimulation electrodes
2. Ureter catheters  5. Foot EMG electrodes
3. EUS EMG electrodes  6. GND electrode

Figure 3.2: Surgical setup including catheterization and electrode implantation.

3.4 Measurement of Voided Volume

Real-time measurement of the voided volume was achieved by placing the rat in a prone position on a custom-fabricated apparatus and aligning the pubic region with a slit perforation (1” wide) made through the top surface (Fig. 3.3). A force-displacement transducer (FT03C, Grass Instruments, Quincy, MA, USA) was placed directly below the perforation and urethral meatus for gravimetric voided volume measurement [107], [108]. The output of the transducer was amplified (range = ±10 mV) and filtered (bandwidth = DC to 2 Hz) with a bridge amplifier (FE220, ADInstruments, Colorado Springs, CO, USA). The accuracy of the voided volume was confirmed post-hoc by 1) manually collecting and measuring the cumulated voided volume with a syringe and 2) measuring the residual bladder volume at the end of the experiment. An infusion pump (Pump 11 Elite, Harvard Apparatus, Holliston, MA, USA) mounted with a 60 mL syringe was used to fill the bladder with saline (infusion rate = 0.1 mL/min). A pressure transducer (Deltran® 6069, Utah Medical, Midvale, UT, USA) was also connected in series with the syringe and positioned at the same vertical height as the animal’s bladder (0 cmH2O). Bladder pressure was amplified (range = ±5 mV) and filtered (DC to 300 Hz) using the bridge amplifier. In each experiment, calibration of both transducers was performed (see Appendix B), and bladder infusion was continuous (without stoppage) for 8.5 to 9.5 hours.
3.5 Experimental Protocol

Prior to bladder infusion, all physiological parameters (heart rate, breathing rate, and body temperature) were allowed to stabilize under urethane for a period of 90 minutes (Fig. 3.1). Data collection began with a baseline period, where the bladder was continuously infused for 150 minutes (Fig. 3.4). A total of 4 TN stimulation trials were applied, with constant duration (30 minutes), frequency (5 Hz), and biphasic pulses (200 µs/phase, cathode proximal). The stimulation frequency and pulse width were selected based on previous work [100]. There was a 60-minute washout period (i.e., no stimulation) following each stimulation trial that was aimed at 1) minimizing any carryover effects and 2) analyzing post-stimulation effects on bladder function. The choice of 60-minutes was based off of the 50-minutes of prolonged TNS inhibition observed by Matsuta et al [28]. It should be noted that wash-out had to be extended to 90 minutes for a subset of trials that observed prolonged overflow incontinence (passive bladder leakage without EUS bursting). Within a single experiment, each stimulation trial was applied at one of the 4 different stimulation amplitudes (0TN, 6TN, 15TN, and 100TN). The selected stimulation amplitudes were determined from past peripheral nerve conduction threshold studies (see Table 2.1 with the intention of activating 1) no TN fibers, 2) Aβ TN fibers, 3) Aβ and Aδ TN fibers, and 4) Aβ, Aδ, and C TN fibers, respectively. A total of 24 trial permutations were needed to ensure each animal was randomly assigned a unique four trial sequence. Each stimulation trial was initiated immediately following a voiding contraction.

3.6 Data Analysis

All data was acquired using PowerLab 16/35 with LabChart 8 Pro software (ADInstruments, Colorado Springs, CO, USA) and stored on a PC. Foot EMG and nerve recording data were sampled at 100,000 samples/s while bladder pressure, voided volume, and EUS EMG were sampled at 10,000 samples/s. EUS EMG was rectified, lowpass filtered (DC to 5 Hz), and downsampled to 10 samples/s to be presented...
for figures in this work. All data figures were generated in Excel 2016 (Microsoft, Redmond, WA, USA). Bladder pressure and voided volume data were downsampled to 10 samples/s and imported into JMP Pro 13 (SAS, Cary, NC, USA). Voiding events were identified as a sharp increase in bladder pressure (above 15 cmH₂O) with corresponding EUS EMG bursting and voided volume output. Basal pressure was measured as the lowest point of pressure immediately following each contraction. Voided volume for each contraction was calculated as the difference between the raw voided volume trace before and after the contraction. In some cases, there were delayed drops that occurred between contractions, these volumes were accounted for by adding their volumes to the VV of the previous contraction. Inter-contraction interval for each contraction was measured as the time between the current contractions maximal pressure peak and that of the previous contraction.

### 3.6.1 Bladder Parameter Equations

The next step was to determine the residual volume for each contraction using the following recursive mass balance equation (Eq. 3.1), as described by Danziger and Grill [77].

\[ RV[i] + VV[i] = UV[i] + ICV[i] + RV[i - 1], \quad i = 1, 2, 3, ... \]  

Where \( i \) is the contraction index, \( RV[i] \) is the residual volume of the current contraction, \( VV[i] \) is the voided volume of the current contraction, \( UV[i] \) is the contribution of urine between the current contraction and the previous contraction, \( UV[i] \) is the contribution of urine between the current contraction and the previous contraction, \( UV[i] \) is the contribution of urine between the current contraction and the previous contraction, \( ICV[i] \) is the infused inter-contraction volume, equal to \( ICI[i] \) times the pump rate, and \( RV[i - 1] \) is the residual volume of the previous contraction. In our model, \( RV[0] \) refers to the initial bladder volume at infusion start, which equals zero, as the bladder was initially empty. To further simplify this equation, we can remove \( UV[i] \) as there is no urine entering the bladder, since the ureters are externally catheterized. Since \( VV[i] \) and \( ICV[i] \) were previously determined for all
contractions and the pump rate is known, RV for all contractions could be calculated. With RV and VV determined for all contractions, bladder capacity and voiding efficiency could be calculated using Eqs. 3.2 and 3.3 respectively. Fig. 3.5 gives a pictorial representation of all key parameters within the raw data signals.

\[
BC[i] = VV[i] + RV[i], \quad i = 1, 2, 3, ...
\]  

(3.2)

\[
VE[i] = \left( \frac{VV[i]}{BC[i]} \right) \cdot 100\%, \quad i = 1, 2, 3, ...
\]  

(3.3)

ICI (or its inverse, bladder contraction frequency) is a common parameter used to evaluate bladder function in many urodynamic animal models as it is quite tangible when relating to clinical outcomes, but the relationship between changes to this parameter and changes to bladder physiology are not clear. This can be addressed by rearranging Eq. 3.1 removing \( UV[i] \), and substituting in Eq. 3.2.

\[
ICV[i] = RV[i] + VV[i] - RV[i - 1], \quad i = 1, 2, 3, ...
\]  

(3.4)

Figure 3.5: Repeated cystometrograms displaying key bladder parameters from raw data. Maximal pressure (MP), basal pressure (BP), inter-contraction interval (ICI), voided volume (VV), bladder capacity (BC), and residual volume (RV).
\[ ICV[i] = BC[i] - (BC[i - 1] - VV[i - 1]), \quad i = 1, 2, 3, ... \quad (3.5) \]

\[ ICV[i] = \Delta BC[i] + VV[i - 1], \quad i = 1, 2, 3, ... \quad (3.6) \]

From Eq. 3.6, we can see that ICV can be neatly unpacked as the summation of the change in bladder capacity of adjacent contractions and the voided volume from the previous contraction. Since bladder capacity changes and voiding changes are governed by different neural mechanisms in the CNS [63], we can realize ICV (or ICI) as a composite parameter that corresponds to changes in response to both processes.

Prior to statistical testing, all bladder parameter data was summarized as the mean of 15-minute bins that were time-synchronized by the start of each 30-minute TNS trial. Bins of 15-minute duration were chosen to 1) balance the resolution of observing time trends and 2) ensure that all time bins contained a minimum of one single contraction event, as infusion rate was constant and bladder capacities were highly variable.

### 3.6.2 Handling of Extended Overflow Incontinence

In a few trials, there were instances of extended loss in bladder contractility (overflow incontinence), for over 15 minutes, as observed by an increase in bladder pressure, loss of EUS EMG bursting, and passive leakage through the urethral meatus [109]–[117]. To address this, we considered each passive droplet as a voiding event to be able to perform the calculations defined above, otherwise there would be no way to assess the bladder capacity during these passive states where the bladder is still under continuous-filling. Although these droplets were not caused by actual contractions, it enabled us to quantify changes in bladder parameters that were consistent with extremely low voiding efficiency associated with urinary retention. The duration of overflow incontinence was defined as the time from the first passive leakage event to the initiating of a bladder contraction with EUS EMG bursting.

### 3.7 Statistical Analysis

All summarized data in this study are expressed as the mean ± the standard error of the mean (SEM). All statistical testing was performed in JMP Pro 13 (SAS Institute, Cary, NC, USA). To test for factor significance, a mixed model was used with an AR(1) covariance matrix structure. Factors assigned within the model were: trial order, trial amplitude, trial time, trial amplitude * trial time, and a pre-stimulation parametric covariate. To determine significance (\( p < 0.05 \)) across trial amplitude * trial time, a Dunnett multiple comparison test was used, comparing parameter values for each trial amplitude * trial time to the 0-minute time point in the control (0T\(_{EMG}\)) trial.
Chapter 4

Results

4.1 EMG and ENG Nerve Recruitment

In all 24 experiments, the threshold amplitude for evoking foot EMG activity ($T_{EMG}$, 12.7 ± 1.3 µA, range: 4 to 27 µA) was consistently lower than that needed to elicit visible motor twitches ($T_{mot}$, 14.7 ± 1.4 µA, range: 5 to 30 µA). In 4 of these experiments, the electrical recruitment of TN fiber types was characterized by simultaneous measurement of foot EMG and the ENG activity of the proximal TN (Fig. 4.1).

Figure 4.1: TN ENG and foot EMG recordings at $T_{EMG}$. Example of tibial nerve ENG and foot EMG activity evoked by TNS applied at $T_{EMG}$ (vertical arrow). In this example, the distances between stimulating nerve cuff electrode and the recording ENG and EMG electrodes were 4 cm and 3 cm, respectively. The raw waveforms ($n = 11$ pulses) are plotted in gray, while the averaged signal is superimposed in black. [$T_{EMG} = 15$ µA, biphasic waveform, 200 µs/phase, frequency = 2 Hz].
The threshold amplitude for evoking large myelinated $A\beta$ fiber activity was always lower than $T_{EMG}$, at around $0.8T_{EMG}$. In these four recruitment experiments, stimulation amplitude was further increased to find that the threshold to evoke afferent $A\delta$ fiber activity was $2.5 \pm 0.3 \ T_{EMG}$ (range: 2 to 3 $T_{EMG}$), and C fiber activity was $52.5 \pm 4.8 \ T_{EMG}$ (range: 40 to 60 $T_{EMG}$). Fig. 4.2 depicts the acquired waveforms for each ENG fiber waveforms ($A\beta$, $A\delta$, C) at each of the trial amplitudes.

![Waveforms](image)

Figure 4.2: Sample data of TNS-evoked ENG signals corresponding to pulses applied at $1T_{EMG}$, $6T_{EMG}$, $15T_{EMG}$, and $100T_{EMG}$. Based on the different post-stimulus latencies, both the time and amplitude scales were modified to show the waveforms of $A\beta$, $A\delta$, and unmyelinated C fibers. The distance between stimulation electrode and recording electrode was 4 cm. The raw waveforms ($n = 11$ pulses) are plotted in gray, while the averaged signal is superimposed in black. [$T_{EMG} = 16 \mu A$, biphasic waveform, 200 $\mu s$/phase, frequency = 2 Hz].

### 4.2 Validation of Urodynamic Measurements

A baseline period of greater than 60 minutes ensured that urodynamic parameters stabilized with continuous bladder infusion under urethane anesthesia. As shown in Fig. 4.3A, the BP trended from 12 cmH$_2$O to 5 cmH$_2$O over the first 60 minutes of infusion, whereas, the VV and ICV increased from 0.2 mL to 0.3 mL. The accuracy of our VV measurement was validated by the fit with ICV (Fig. 4.3B), consistent with Eq. 3.6.
Chapter 4. Results

4.3 TNS-evoked Bladder Neuromodulation

TNS did not produce any statistically significant changes to any key bladder parameters during the 30-minute trial stimulation period ($p > 0.05$). Instances of clear post-stimulation inhibition within the bladder pressure and volume traces appeared in one of two forms: 1) increased bladder capacity without loss in voiding or EUS bursting (Fig. 4.4A) or 2) increased bladder capacity with overflow incontinence (Fig. 4.4B), i.e. loss of voiding and EUS bursting with passive leakage through urethral meatus.

Mean summary trends for all six key parameters are shown in Fig. 4.5. BC, VE, RV, and BP all showed amplitude-dependent changes with the largest effects observed in the 15-minute period immediately following 100T$_{EMG}$ stimulation. BC for the 0T$_{EMG}$ (control) trial at time zero (first 15-minute bin of stimulation) was 0.83 ± 0.07 mL and did not change over trial time ($p > 0.75$). BC for all other amplitude trials during 30-minute TNS did not significantly differ from the control at trial time zero ($p > 0.85$). The 15-minute period immediately following TNS produced the largest changes in BC for 6T$_{EMG}$, 15T$_{EMG}$, and 100T$_{EMG}$ trials. These changes (as a percentage of control), respectively were: 105% (0.87 ± 0.05 mL, $p = 0.77$), 117% (0.96 ± 0.08 mL, $p = 0.42$), and 150% (1.24 ± 0.07 mL, $p < 0.0001$). BC for all trial amplitudes returned to pre-stimulation levels over the 60-minute washout period. It is noted that 100T$_{EMG}$ had the most gradual of reversals, with the mean trend staying significantly elevated from control values for at least 45 minutes of the washout period consistent with the notion of prolonged inhibition. This same post-stimulation mean trend was present in 6T$_{EMG}$ and 15T$_{EMG}$ trials, but their values returned to pre-stimulation levels within 30 minutes. VV and ICV were not significantly altered by trial amplitude. BP was significantly affected immediately following 100T$_{EMG}$ by rising to 336% of control at trial time zero (13.0 ± 1.9 cmH$_2$O vs. 3.9 ± 1.6 cmH$_2$O, $p < 0.0001$). Non-significant increases to BP immediately following 6T$_{EMG}$ and 15T$_{EMG}$ trials were 147% (5.7 ± 1.0 cmH$_2$O, $p = 0.28$) and 153% (5.9 ± 0.9 cmH$_2$O, $p = 0.40$) of control at trial time zero, respectively. In the case of BC, VE, RV, and BP, trial amplitude, trial time, and trial amplitude*trial time were statistically significant factors ($p < 0.005$) in our mixed model analyses. Trial order was not statistically significant for any of the six key bladder parameters ($p > 0.10$). Detailed statistical results...
4.4 Incidence of Overflow Incontinence

Overflow incontinence was observed in 6 experiments, where both the bladder pressure and volume reached asymptotic values for finite periods of time (Fig. 4.6A). The incidence of the overflow incontinence periods appeared to depend on stimulation amplitude, where most occurrences (6 of 9 trials) appeared following 100T_{EMG} stimulation. Overflow incontinence duration also appeared to depend on stimulation amplitude, although sample size is small. Overflow incontinence due to 100T_{EMG} stimulation lasted for \( 52.6 \pm 7.4 \) min (range: 36.2 to 84.7 min) and interestingly were more prevalent in the latter two trials of an experiment (Fig. 4.6B). There were three instances of overflow incontinence that occurred following the lower amplitude trials of duration \( 21.8 \pm 2.1 \) min (range: 17.6 to 24.3 min). No instances of overflow incontinence were observed for any control trials.
Figure 4.4: Representative data from complete experiments that exhibited (A) TNS-evoked changes in bladder function and (B) an example of overflow incontinence. The key urinary measures were bladder pressure, bladder volume and the rectified EUS EMG. In both experiments, TNS resulted in significant increases in bladder capacity at $15T_{EMG}$ and $100T_{EMG}$. The $T_{EMG}$ values were 8 $\mu$A (panel A) and 13 $\mu$A (panel B).
Figure 4.5: Summary of key bladder parameters: bladder capacity, voided volume, residual bladder volume, voiding efficiency, inter-contraction volume, and basal bladder pressure. Each data point (mean ± SEM) represents the mean value within a 15-minute bin (n = 24) and was plotted with respect to the start time of TNS trials (t = 0 min). The stimulation amplitude was indicated by different symbols: 0T_{EMG} (white square), 6T_{EMG} (white triangle), 15T_{EMG} (black square), and 100T_{EMG} (black triangle). Significance was determined by Dunnett comparison with the 0-minute 0T_{EMG} (control) bin for each parameter, following mixed model analysis. *: p < 0.05, **: p < 0.001, ***: p < 0.0001. [gray area = 30-minute TNS trial period].

Figure 4.6: Summary of overflow incontinence data. (A) Raw bladder pressure traces from all experiments where TNS elicited overflow incontinence. This occurred only in response to TNS applied at 100T_{EMG}, T_{EMG} = 16.0 ± 2.7 µA (n = 6, range: 9 to 27 µA). (B) The number of experiments in which overflow incontinence was elicited by TNS was plotted as a function of trial order (1 through 4). [gray area = 30-minute TNS trial period].
Chapter 5

Discussion

The results of this present study show that 30-minute TNS applied at 5 Hz to anesthetized rats during continuous cystometry was able to elicit a prolonged post-stimulation increase in bladder capacity without significant changes to voided volume. This observed inhibition was stimulation amplitude-dependent, where the most significant inhibition occurred immediately following 100T_{EMG} trials, with a gradual reversion to pre-stimulation levels during the 60- to 90-minute washout period. TNS, applied at any amplitude, did not have any significant effects to bladder parameters during the 30-minute stimulation period. Results from our TN fiber conduction trials indicate that stimulation at 100T_{EMG} was capable of activating all afferent TN fibers (including unmyelinated C fibers) while both 6T_{EMG} and 15T_{EMG} only activated small and large myelinated fibers. These results provide further insight into the potential nerve fiber targets of TNS for activating inhibitory mechanisms that are optimal for bladder neuromodulation therapy.

5.1 Nerve Fiber Thresholds

Our observed foot EMG thresholds found prior to each experiment were consistent with the findings of Kovacevic and Yoo [100]. Following our nerve conduction trials performed in four rats, we found that A_β, A_δ, and C fiber thresholds were 0.8T_{EMG}, 2-3T_{EMG}, and 40-60T_{EMG}, respectively. These thresholds are consistent with peripheral nerve fiber thresholds determined by other groups (see Table 2.1). From these results, we can presume that 6T_{EMG} stimulation largely activated A_β fibers with some activation of A_δ fibers. Increasing stimulation to 15T_{EMG} largely activated both A_β and A_δ fibers but was well below C fiber threshold levels. Further increasing our stimulation to 100T_{EMG} ensured that all fibers were fully activated.

5.2 Absence of Acute TNS Inhibition

TNS has shown to have acute effects during stimulation by inhibiting rhythmic isovolumetric contractions (in cats and rats) [27], [98], [99], increasing BC during filling (cats and SCI rats) [27], [102], and reducing the bladder contraction frequency during continuous-filling (in rats) [100]. Our results did not show any significant changes to bladder parameters (from control) at any trial amplitude. One primary reason for this may be due to differences in how the data was summarized. Our results were averages of 15-minute
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data, providing two data points per animal within each 30-minute stimulation trial. In contrast, Su et al and Kovacevic and Yoo used 5- and 10-minute intervals to summarize data. In the case of Su et al, TNS in anesthetized rats provided the most significant inhibition of rhythmic isovolumetric contractions (-31% of control) during the first 5 minutes of 15-minute stimulation [99]. If averaged over the entire 15-minute interval, this change would likely have become insignificant. When 5- and 10-minute binning schemes were applied to our data, we did not observe any differences in trends during or following 30-minute TNS (data not shown).

Kovacevic and Yoo performed repeated 10-minute stimulation trials during continuous-filling with 10-minute rest periods in between, using the bladder contraction frequency as their key parameter of interest [100]. They observed significant decreases in bladder contraction frequency during stimulation using 5 Hz (-38% of control) and 10 Hz (-22% of control) stimulation. Another potential reason for the lack of acute changes noted in our study may be due to how the data was normalized by Kovacevic and Yoo. In their protocol, the 10-minute periods of rest prior to 10-minutes of stimulation were used both as controls and as post-stimulation periods, and the infusion rate was not constant (0.1 to 0.3 mL/min). As noted in their discussion, carryover effects may have been present across trials and may have confounded the analysis.

5.3 Amplitude-dependent Post-TNS Inhibition

Consistent with the work of Matsuta et al in rats [28] and Tai et al in cats [27], we were able to elicit a significant prolonged post-stimulation increase to BC following 30 minutes of TNS at 5 Hz, under saline conditions. Of considerable interest is the magnitude of response with respect to the applied stimulation. In the case of Matsuta et al, stimulation applied at an intensity of 2-4T_{mot} evoked a 140% increase in BC from control lasting for 50 minutes following the cessation of stimulation. Similarly, in cats, Tai et al observed an increase in BC of 160% following 30-minute stimulation applied at 2-4T_{mot}, lasting for over 2 hours.

In our study, stimulation at 100T_{EMG} significantly increased mean post-stimulation BC to 150% of control with gradual a reversion back to control levels over the course of an hour. Stimulation at 6T_{EMG} or 15T_{EMG} provided non-significant mean increases of 105% and 117% of control, respectively, lasting for 30 minutes or less. From Eqns. 3.2 and 3.3, it is clear that RV and VE are both dependent on BC and VV. Our results showed that BC had a significant post-stimulation increase during 100T_{EMG} trials while VV did not, thus, RV and VE both had significantly changes for this trial as well. VV and ICV during 100T_{EMG} trials did show a slight non-significant decrease post-stimulation, but this was due to the limited cases of overflow incontinence where voiding was very poor. Matsuta et al had reported a 20 to 30% non-significant decrease in voiding efficiency, but did not explicitly report VV. This reduction in VE was likely due to the significant increase in BC (140%) that they had observed with minimal changes to VV, in-line with our findings.

It is interesting that for a comparable level of BC increase post-stimulation, we required a multiple of the threshold that was 25-50 times larger than that of Matsuta et al. One distinct difference in preparations is that our study used T_{EMG} as our threshold while Matsuta et al used the visually determined T_{mot}. In all of our experiments we acquired T_{mot}, but this was consistently only 1-2 µA (around 120%) larger than T_{EMG} and we do not believe this would have made a considerable difference during our trials. One stark difference noted between the present study and Matsuta et al is the range
of current intensities used. Matsuta et al did not provide specific information regarding $T_{mot}$ but did state a range of 0.1-2.4 mA used for 2-4$T_{mot}$ stimulation which is remarkably similar to the amplitudes that we used for 100$T_{EMG}$ stimulation (0.3-2.7 mA). This observed difference may be due to the fact that their experimental setup used a tripolar stimulation electrode configuration while ours was bipolar (cathode: proximal).

In contrast to the results of Matsuta et al, a recent study in anesthetized rats by Choudhary et al was unable to evoke significant changes to BC, under saline conditions, following 30 minutes of 5 Hz stimulation at 3$T_{mot}$ \[101\]. Similar to our study, a bipolar electrode configuration was used but raw threshold intensities were not presented. They concluded that the non-result under saline conditions may have been due to differences in rat type (Wistar versus Sprague-Dawley) or electrode configuration (bipolar versus tripolar).

It is quite possible that a difference in bladder models may have been a possible source of disconnect between the level of stimulation required to elicit similar increases to BC between our study and Matsuta et al. Our study used a continuous-fill model with filling at 0.1 mL/min while Matsuta et al performed repeated single-fills at 0.05 mL/min. It has been shown in rats that increasing cystometric filling rates can modulate afferent bladder firing from mechanoreceptors, which may be present in our preparation \[118\]. Also, the continuous-fill model may require higher a stimulus for bladder inhibition treatment than isovolumetric and single-fill preparations due to its repetitive nature and increased stimulus to void \[72\].

5.4 TNS-evoked Overflow Incontinence

Overflow incontinence is a well-documented phenomenon present in pharmacological treatment \[109\]–\[115\], neuromodulation \[117\], and pelvic nerve crush studies \[116\]. In animal studies, this condition is associated with high bladder pressure, bladder retention, lack of EUS bursting, and passive leakage of fluid from the urethral meatus \[109, 112, 114, 115, 119, 120\]. Overflow incontinence was not observed in all rats, but it was most prevalent immediately following the 100$T_{EMG}$ trials, particularly during trials 3 and 4 of an experiment. Although trial order was not found to be a significant factor in our statistical mixed model analysis, we cannot completely rule out the possibility of carryover effects of repeated stimulation trials during our experiment.

While in a distended overflow condition, the afferent bladder firing due to stretch receptors to the brain may have been lessened by a temporary increase in compliance \[121\]. Matsuta et al reported a significant increase in bladder compliance during the first two bladder fills following 30-minute TNS in rats with a reversion back to baseline after the third fill \[28\]. We suspect that the animals with overflow incontinence in our study likely had extreme compliance changes due to the significant increase in bladder capacity.

Since the infusion pump remained on for the entirety of each experiment, it is possible that overflow episodes may have contributed to plastic deformations of the bladder due to overdistention. Prolonged overdistention of the detrusor muscle has been shown to increase compliance, weaken contractile force, and reduce afferent stretch firing \[121\]. The combination of lessened firing due to bladder overdistention with the inhibiting effects of TNS to afferent bladder pathways is likely to have caused prolonged overflow. The departure of overflow incontinence may have been due to the inhibiting effects of TNS wearing off, allowing for more afferent stretch signals to reach the higher centers in the brain to trigger normal
5.5 Activation of Small Diameter Fibers

The stimulation amplitudes that were used for our study were physiologically relevant to the types of afferent fibers contained within the TN. Similar to studies by Sato et al and Onda et al, stimulation of tibial nerve branches or the pudendal nerve, respectively, at amplitudes that recruit small diameter myelinated and unmyelinated fibers produced the most significant inhibition of bladder function [34], [35], [50]. When electrically stimulated, small diameter nociceptive fibers release the neuropeptide “ Substance P” into the dorsal horn of the spinal cord [122]–[125]. The transmission of these signals have been shown to relay pain information to midbrain structures for further processing. The PAG is one of the first midbrain structures to receive this information, which is of key interest because it also happens to be the relay station for bladder afferent information [63]. It has been shown that descending neural pathways are present within animals that inhibit ascending nociceptive input by releasing endogenous opioids into the spinal cord. These opioids act on both pre-synaptic and post-synaptic terminals to inhibit the release and transmission of substance P [36]. It has been shown in both animals and humans that the level of endogenous opioid peptides can be increased within the CNS following electrical stimulation of somatic nociceptive nerve fibers [126] or transcutaneous surface stimulation [127]. The question of where the site of TNS action occurs and what transmitters mediate the process is still open as bladder inhibition has been observed in both intact [27], [28] and SCI animals [35], [102].

5.6 Effects of Anesthesia

At this time, the subject of TNS mechanisms remains difficult to encapsulate as many factors may confound the results. One such factor that has been acknowledged, is the confounding effects of anesthesia.

Urethane anesthesia is the gold-standard anesthesia for use in rats as it is able to spare the micturition reflex whilst providing a long stable plane of anesthesia [119], [128]. Although its use has been prevalent, it has been shown to have a dose-dependent depression on voiding efficiency [129]. This may have been why our control VE were much lower (50-60%) than that for awake rats (>95%) [109]. Urethane has also been shown to cause spontaneous sleep-like state changes within rats, similar to rapid-eye-movement (REM) states during normal sleep [120], [130], [131]. A recent study by Crook and Lovick was able to correlate these changes in rat brain states, under urethane, with significant changes to voiding threshold capacity and threshold pressure during continuous cystometry [120]. In our data, spontaneous events similar to those noted by Crook and Lovick were present. These effects of these events were likely minimized as the data was averaged out across the 24 animal experiments by 15-minute time bins.

Considering that much of mechanistic work on TNS has been performed in anesthetized animals, it is unclear how directly transferable the information is to a clinical setting. Conclusions drawn will always be limited to the model from which they originated.
Chapter 6

Conclusions & Future Work

Neuromodulation therapy has a clear role in the future of treating bladder dysfunction, as patients are becoming more open to non-pharmacological alternatives. As evident from a plethora of clinical and pre-clinical studies, electrical stimulation of the tibial nerve is an efficacious bladder therapy, although much is still to be understood regarding its exact mechanism. Comparative animal models will still remain as the key sources for unveiling this mechanism, but future work will require modifications to navigate past their shortcomings.

As demonstrated in this study and both others, stimulation intensity remains to be a key parameter for TN inhibition of bladder function. In this study, we built upon the framework developed by Kovacevic and Yoo [100] to further expand the continuous-fill rat model to compare prolonged TNS-evoked bladder inhibition with other bladder models. Based on our results, we concluded that 30-minute TNS applied at an intensity capable of activating unmyelinated C fibers was most effective at producing a prolonged increase in bladder capacity.

6.1 Clinical Implications

Patient tolerability to electrical stimulation during PTNS can vary considerably and this may affect treatment outcomes. The stimulation amplitude for PTNS is typically increased until a foot motor response is observed and reduced slightly, but in certain studies the amplitude was increased to the highest tolerable level. Since an uninsulated needle is used for PTNS, it is difficult to know which fibers of the tibial nerve are being activated. Also, many cutaneous afferents within the skin are likely activated during PTNS as well, which may affect pain tolerability to stimulation. Our study suggests that TNS applied at intensities to activate small diameter nociceptive (pain) fibers provides the most effective bladder inhibition responses in anesthetized rats. Conscious patients may not be able to endure this type of stimulus, especially for extended periods of time. To validate this, a recent study by Sharan et al reported that transcutaneous stimulation of the tibial nerve was tolerable up to twice the nerve activation threshold and four times the cutaneous afferent sensation threshold [132]. It is possible that the maximum tolerable stimulation amplitude may have activated small diameter afferents on the skin but it uncertain if this would have activated small diameter afferents of the tibial nerve. Further understanding of the translatability between stimulation intensities in animals and patients is required for identifying key nerve fiber targets during PTNS therapy.
6.2 Model Improvements

Our study, similar to many, made use of a urethane-anesthetized rat model. Comparative models have made great strides in identifying key physiological relationships, but their limitations must be acknowledged. In future works of this type, it would be recommended to use a decerebrate or awake preparation for bladder testing. Eliminating the potential confounding effects of urethane anesthesia with treatment would be the main advantage with these preparations. Another limitation of this model was the use of repeated trials within the same animal. The clear benefit of this approach was that the number of animals could be significantly reduced, as the large variability across subjects could be excluded. On the other hand, experiments were long in duration and the elimination of carryover effects between treatment trials within the same animal was not 100% guaranteed by the 60- to 90-minute washout periods. To circumvent these issues, only a single trial should be performed in each animal, and a dedicated group of control animals should be used.

6.3 Further Exploration

PTNS OAB therapy involves the use of repeated stimulation sessions over the course of 12 weeks, while pre-clinical TNS trials are generally non-survival, with effect durations on the order of several minutes to hours – not weeks. To further close the translational gap between animal and clinical studies, chronic animal studies should be employed to better understand the long-term effects of TNS. Stimulation sessions matching PTNS therapy may be used or even regimes with more frequent stimulation sessions (i.e. daily vs. weekly).

Another exploration path that has garnered much attention is to seek out alternative neural pathways for bladder neuromodulation. Recently, our lab has successfully demonstrated that electrical stimulation of the saphenous nerve could increase the time between contractions as well as bladder capacity in anesthetized rats. A recent simulation study showed that activation of saphenous nerve afferents was possible during clinical PTNS therapy with an uninsulated needle. Further efficacy of this treatment was observed during a follow-up clinical pilot study that showed significant decreases to OAB symptoms and increases to quality of life scores. One obvious potential benefit of this therapy, is that the saphenous nerve contains only afferent fibers, meaning that there would be no muscle twitches associated with its administration, allowing patients to go about their daily lives undisrupted. As with TNS, further pre-clinical studies need to be performed for saphenous nerve stimulation to uncover its mechanism.
Appendix A

Void Collection System Fabrication

Prior to performing experimental trials, there was a need for a system that could reliably facilitate void collection during rat continuous-fill experiments. As experiments were many hours in duration and involved hundreds of voiding events, this system would allow for automation of data collection, rather than manual measurements of individual voided volumes. Two key components needed to be fabricated: 1) a gravimetric void collection transducer compatible with our PowerLab/LabChart acquisition system and 2) a light-weight table-top apparatus that could elevate animals for gravimetric void collection and hold all necessary experiment equipment.

Although not essential, a third device was developed to allow for recording of infused volume from the infusion pump into our data acquisition system. This adapter enabled the estimation of bladder volume in real-time during experiments as well as a convenient means to record start and stop times of the pump.

A.1 Void Collection Transducer

Following a detailed literature search of void collection systems used for rodent cystometry, it was clear that the transducer of choice was the FT03 force-transducer by Grass Instruments [108], [136]–[140]. The transducer is no longer in production, but pre-owned units could be found at a very low cost. A custom cable had to be fabricated to connect the transducer to our bridge amplifier. The female connector (WK-6-21C, ITT Cannon, Irvine, CA, USA) that was required to be attached to the transducer also had to be sourced from vendors of pre-owned products. This connector had six contacts with five main sources: \(V_{\text{sig}+}, V_{\text{sig}-}, V_{\text{ex}+}, V_{\text{ex}-},\) and GND. Leads from a re-purposed male DIN-8 pressure transducer cable were used to attach to the female connector as show in Fig. A.1.

The excitation voltage was set by using a resistor between pins 5 and 8 of the DIN-8 connector. The equation relating \(R_{\text{set}}\) to excitation voltage is as follows.

\[
R_{\text{set}} = \left( \frac{20V}{V_{\text{ex}}} - 1 \right) \cdot 150 \, \text{k}\Omega \tag{A.1}
\]

For our purposes, 5 V \((\pm 2.5 \, \text{V})\) was sufficient, using E24 5% resistors the closest value was 470 k\(\Omega\), making our actual excitation voltage equal to 4.8 V \((\pm 2.4 \, \text{V})\). Wiring diagrams for the transducer and DIN-8 cable can be found in [141] and [142], respectively.
To elevate the transducer in the air, a magnetic base with a 9” vertical rod was used. A 90-degree 0.5” rod end connector was fitted onto the magnetic base rod to hold the transducer in a horizontal position.

The last step was to fabricate the collection cup to be fastened to the transducer. A method suggested by both Dr. Jonathan Carp and Dr. Phillip Smith was used, where a syringe was used. The syringe’s tip and plunger were removed and a 4-40 x 0.5” screw was placed through the tip opening and sealed on the inside with a thin layer of waterproof epoxy (MarineWeld, J-B Weld, Atlanta, GA, USA). The final product is shown in Fig. A.2 below.

### A.2 Cystometry Table

The main requirements for these tables were that they could fit on top of our surgical table, could be moved easily, and afforded enough space for all of our equipment. Acrylic (5/6” thick) was a low-cost choice for the table-top and aluminum legs with plastic feet made the tables light-weight. The shorter of the two tables was for the infusion pump to rest on. For this table, a stainless steel ruler was fixed vertically to allow for pressure calibration. The taller table was for the animal to rest on with enough room for connection wires to be fastened using clips as well as a slit in the table for voids to be collected on the transducer below. Engineering drawings of the tables are shown in Fig. A.3 below.

### A.3 Infused Volume Acquisition Adapter

The Harvard Apparatus Pump 11 Elite infusion pump is configured for serial communication using a USB virtual COM port connection. Using this method, command signals to operate the device can be sent and device status can be extracted. The requirements for this device were that 1) it should be standalone (not connected to a PC), 2) it continuously polls the infusion pump for infusion pump for infused volume, and 3) it outputs an analog voltage for our data acquisition to record.
To take advantage of the many firmware libraries available, the Arduino Uno Rev3 (A000066, Arduino, Somerville, MA, USA) was used along with a USB host shield (DEV-09947, SparkFun Electronics, Boulder, CO, USA) to interface with the infusion pump, and a 12-bit digital-to-analog converter (DAC, MCP4725, Microchip Technology, Chandler, AZ, USA) to output the analog voltage to record with our acquisition system. The abstract control module (ACM) example code from the open-source USB Host Shield 2.0 Arduino library \[143\] was modified to continuously poll the infusion pump for infused volume status, parse the extracted string for infused volume data, convert the value for the 12-bit DAC, and output the resulting analog voltage. The output voltage range of the DAC was 0 to 5 V (0 to 60 mL) which was then be calibrated back into milliliters using the LabChart Multipoint Calibration add-in. The device was then encased in a plastic container with alligator clip leads soldered to the board for connection to the PowerLab acquisition leads. The completed device is shown in Fig. A.4.
Figure A.3: Engineering drawings for acrylic cystometry table. Dimensions are all in inches.

Figure A.4: Completed infused volume acquisition adapter, composed of an Arduino Uno Rev3, USB host shield, and digital-to-analog converter.
Appendix B

Transducer Calibration

To ensure that both transducers were functioning correctly, calibration was performed the beginning of each experiment.

B.1 Void Collection Transducer

With the collection cup screwed into the transducer, the apparatus was set in place. A small 3” line level was used to ensure that the transducer was level prior to calibration. The transducer cable was attached to the bridge amplifier and the signal was zeroed in LabChart. Using a 1 mL syringe, eleven 1 mL volumes were sequentially inserted into the collection cup. Using the LabChart Multipoint Calibration add-in, a linear calibration fit was made of the 12 data points (0 to 11 mL). The fit-line for calibration always had an $R^2$ value exceeding 0.999 and calibration slope was $6.289 \pm 0.007$ mL/mV (range: 6.223 to 6.344 mL/mV, n = 24).

![Figure B.1: Calibration of voided volume in LabChart using the Multipoint Calibration add-in.](image)

One thing to be mindful of is that LabChart has a finite amount of data points (1,000,000) that can be selected at one time for the Multipoint Calibration add-in. In our case, we reduced the sampling rate...
from 10,000 samples/s to 1,000 samples/s so that our calibration selection window was increased from 100 s to 1000 s.

### B.2 Pressure Transducer

Pressure calibration was performed by attaching a 40 cm PE-50 catheter (fitted with a 23-gauge needle) to the pressure transducer, with the other end of the transducer attached to a 60 mL saline-filled syringe seated in the infusion pump. Using a piece of tape, the free end of the catheter was attached to the vertical ruler at the level of the transducer. This position was the same height as the animal’s bladder (0 cmH₂O). The infusion pump was set to 0.1 mL/min to clear the catheter of any air bubbles.

![Figure B.2: Calibration of bladder pressure in LabChart using the Multipoint Calibration add-in.](image)

The transducer cable was attached to the bridge amplifier and the signal was zeroed in LabChart. Three more calibration points were taken at 10 cm, 20 cm, and 30 cm above the initial placement. The catheter was then placed at the 0 cmH₂O level to confirm its signal level had not changed. Using the LabChart Multipoint Calibration add-in, a linear calibration fit was made of the 4 data points (0 to 30 cmH₂O). The fit-line for calibration always had an $R^2$ value exceeding 0.999 and calibration slope was $57.039 \pm 0.073$ cmH₂O/mV (range: 56.411 to 57.627 cmH₂O/mV, $n = 24$).
Appendix C

Sources of Error

The use of voided volume collection in this study provided the ability for further extraction of key bladder parameters, essential for understanding the underlying physiology. One further improvement to this model was the disconnection of the ureters from the bladder, giving complete control of bladder volume to the experimenter. This was necessary as urine production is an unknown variable that can create erroneous bladder volume estimates over time, as described by Danziger and Grill [77].

Two practical considerations that are often overlooked in rodent cystometry studies is the voided volume collection accuracy and infused volume accuracy. Due to the small volumes associated with rodent bladders, these may or may not be of concern to those performing cystometry. In this section, we quantified these two measurement errors based on the results of our 24 rat experiments.

C.1 Voided Volume Collection

Total voided volume was extracted via a 10 mL syringe, measured, and then emptied into a tray on a digital balance. Comparing the calculated voided volume from the calibrated transducer to the volume (syringe) and mass (digital balance) measurements gave the following results, respectively: 1.4 ± 0.1% (range: 0.6 to 2.1%, n = 24) and 1.9 ± 0.1% (range: 0.9 to 2.7%, n = 24). Given that the 10 mL syringe had 0.2 division, volumes could only reliably be estimated within ±0.1 mL (1% full scale), which is within the range we observed. The increased error in the digital balance measurement is likely due to the loss of some fluid from syringe to balance tray and density discrepancies of the saline. We concluded that our measurements of voided volume were within tolerances of our equipment and sufficient for our study.

C.2 Infused Volume

The infusion pump used for our study has a nominal ±0.5% accuracy, stated in the manual [144]. The manufacturer also recommends using a new syringe for each infusion and to input the measured diameter of each syringe for best accuracy. Our infusion rate was constant at 0.1 mL/min, so based on the nominal accuracy of this pump, we could expect our infusion to be in error by ±0.5 μL/min, which seems excellent. The devil is in the details, as our experiments involved continuous infusion for 8.5 to 9.5 hours, meaning that the infused volume for the entire experiment could be off by 0.26 to 0.29 mL.
Again, this seems like a trivial amount but what needs to be considered is the size of this error relative to the final RV of the rats. Our results showed that under control conditions, rats had a mean RV around 0.4 mL. What this means is that the pump error could cause the final residual volume measurements to potentially be off by $\pm 65\%$ to $\pm 73\%$, under nominal conditions.

In our case, we did not always use new syringes for each infusion and used the stock diameter for the infusion specifications. From our analysis, comparing our final extracted RV value with calculated (based on an ideal infusion pump rate of 0.1 mL/min), our pump accuracy was $-0.48 \pm 0.10\%$ (range: -1.4 to 0.4%, $n = 24$), which, on average was within the manufacturer’s acceptable range of nominal errors.
Appendix D

Detailed Statistical Analysis

Statistical analysis was performed using the Fit Model platform within JMP Pro 13 using a mixed model with AR(1) covariance matrix for repeated time points. Using the multi-comparison report, all parameter data points at each amplitude were compared with control ($0T_{EMG}$) values at trial time zero using post-hoc Dunnett’s test. Adjusted p-values were calculated to determine significant effects.
Figure D.1: Detailed mixed model statistical analysis for bladder capacity amplitude trial data.
Figure D.2: Detailed mixed model statistical analysis for voided volume amplitude trial data. Multi-comparison reports were not generated because the mixed model did not find amplitude, trial time, or amplitude*trial time significant.
Figure D.3: Detailed mixed model statistical analysis for residual volume amplitude trial data.
**Figure D.5:** Detailed mixed model statistical analysis for inter-contraction volume amplitude trial data. Multi-comparison reports were not generated because the mixed model did not find amplitude, trial time, or amplitude*trial time significant.
Appendix D. Detailed Statistical Analysis

Figure D.6: Detailed mixed model statistical analysis for basal pressure amplitude trial data.
Bibliography


