Investigating Neuromodulation as a Treatment for Female Sexual Dysfunction

by

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Dedication

This thesis is dedicated to my family- my mother Martha, my father Michael, my stepfather Joe, my sister Michelle, and my brother Jaxson. I would not be here without their love and support.

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Abstract

Female sexual dysfunction (FSD) affects millions of women worldwide. FSD has a significant impact on quality of life and interpersonal relationships. The prevalence of at least one form of sexual dysfunction is 40-45% of adult women with 12% of women experiencing sexually related personal distress, yet there is no clear treatment option for a wide range of FSD deficits with high efficacy and low side effects.

Neuromodulation techniques using electrical stimulation of peripheral nerves have the potential to treat some forms of FSD. In clinical trials of sacral neuromodulation (SNM) and percutaneous tibial nerve stimulation (PTNS) for bladder dysfunction, women have reported that their sexual dysfunction symptoms improved as well. Even though this effect has been observed clinically, very little research has been done to examine the mechanisms or the optimal method of treatment specifically for women with FSD. This thesis aims to bridge that gap by investigating neuromodulation as a treatment for FSD through both preclinical and clinical studies.

The first aim of this thesis is to investigate a possible mechanism of the improvement to sexual functioning in response to tibial nerve stimulation by evaluating vaginal blood flow changes in rats. In 16 ketamine-anesthetized female rats, the tibial nerve was stimulated for 30 minutes while vaginal blood perfusion was recorded with laser Doppler flowmetry. A novel signal analysis and quantification metric was developed for this analysis. I found that tibial nerve stimulation could drive prolonged increases in vaginal blood perfusion, typically after 20-30

minutes of stimulation. These results suggest that clinical neuromodulation may be improving FSD symptoms by increasing genital blood flow.

One question yet to be investigated by neuromodulation studies is whether tibial nerve stimulation could be an on-demand treatment for FSD, such as Viagra is for men, or is more appropriate as a long-term treatment with improvements over time, such as PTNS for bladder dysfunction. In the second aim of this thesis I address this question by evaluating the sexual motivation and receptivity of female rats both immediately after a single stimulation session as well as after long-term, repeated stimulation sessions. I found that tibial nerve stimulation led to modest increases in sexual motivation in the short term, and larger increases in sexual receptivity in the long-term. These results suggest that long-term therapy may be required clinically.

Lastly, the third aim of this thesis evaluates a pilot clinical study of transcutaneous stimulation of the dorsal genital and posterior tibial nerves in nine women with FSD. The women received stimulation once a week for 12 weeks and their sexual functioning was measured using the Female Sexual Function Index (FSFI) at baseline, after 6 weeks of stimulation, after 12 weeks of stimulation, and at 18 weeks (6 weeks after the last stimulation session). The average total FSFI score across all subjects significantly increased from baseline to each of the time points in the study. Significant FSFI increases were seen in the sub-domains of lubrication, arousal, and orgasm, each of which is related to genital arousal.

This thesis provides evidence that peripheral neuromodulation can be an effective treatment for FSD. The stimulation is likely driving increases in genital blood flow, with greater effects observed when stimulation is repeatedly applied over time. This treatment has the potential to help millions of women worldwide.

Chapter 1 : Introduction

1.1 Female Sexual Function and Dysfunction

Sexuality plays an important role in individual lives and relationships, yet it is not always enjoyed equally. Sexual dysfunction affects women more than men, but women have far fewer treatment options than men.[1] This dissertation focuses on treatment for female sexual dysfunction. First it is important to understand healthy sexual responses.

1.1.1 Anatomy and Physiology of the Female Sexual Response

In a healthy female sexual response, there is both subjective and genital arousal. Sexual stimuli are processed in the limbic system which then cause genital vasocongestion.[2] Simultaneously, contextual sexual cues are cognitively processed and can potentially lead to subjective sexual arousal. Subjective arousal pertains to mental engagement and emotional response during sexual activity. Often, there can be disconnect between subjective and genital arousal, in which genital arousal is obtained without a woman recognizing it or experiencing subjective arousal.[3] Inversely, women may experience subjective arousal without experiencing genital engorgement or arousal. Genital arousal is controlled by both the sympathetic and parasympathetic nervous system. Sympathetic postganglionic neurons in the pelvis, the sympathetic hypogastric nerve, and parasympathetic nerves from sacral roots S_2-S_4 all mediate vasodilation, vulvar congestion, and smooth muscle relaxation.[4] Vasocongestion directly leads to vaginal lubrication and expansion while swelling the genitalia. Increasing blood flow to the vestibular bulbs in the labia during sexual excitement causes a 2-3x increase in the diameter of

the labia. Increased blood flow to the clitoris increases clitoral intracavernous pressure, which causes tumescense of the glans.[4] This vulval congestion can amplify enjoyment of genital contact, reinforcing the sexual stimuli. Vaginal vasocongestion is measured clinically using vaginal photoplethysmography, which uses an acrylic, tampon-shaped sensor that tracks vaginal pulse amplitude of blood flow.[5]

The lower urinary tract and the sexual and reproductive organs share common nerves. In relation to lower urinary tract organs, the parasympathetic pelvic nerve innervates the bladder and urethra and the sympathetic hypogastric nerve densely innervates the bladder near the ureters and bladder neck.[6,7] Also, the pelvic and hypogastric nerves both innervate the vagina, cervix, and uterus.[8] The somatic pudendal nerve, originating from the sacral plexus, innervates the pelvic striated perineal muscles and posterior labia.[9] The pudendal nerve also carries sensory information from the perineum, clitoris, and urethra.[10] Innervation of the pelvis is shown in Figure 1. [11]

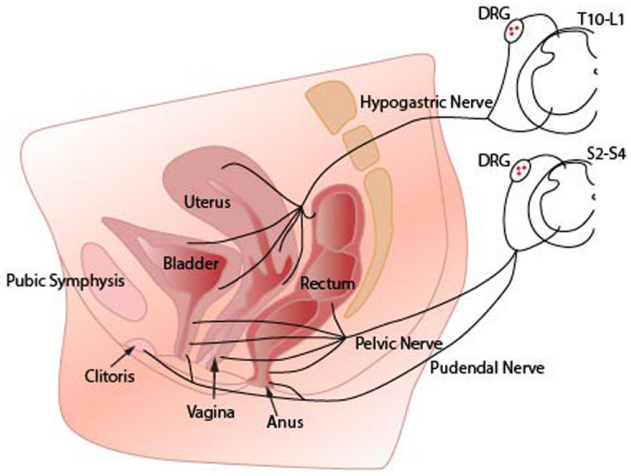

Figure 1. Innervation of the female pelvic organs.[11]

Hormones circulating in a female's body also mediate sexual responses. Estrogen promotes vasocongestion of the vagina and clitoris as well as maintains the vaginal epithelium, stromal cells, smith muscles of the vulva, thickness of the vaginal rugae, and vaginal lubrication.[4] Lack of proper estrogen levels due to aging, menopause or castration can lead to vaginal wall fibrosis, thinner vaginal walls, or a dry vaginal canal, each leading to sexual dysfunction. Testosterone and other androgens play a role in sexual arousal, libido, sexual

responsiveness, genital sensation, and orgasm, and insufficient androgen levels has been linked to sexual dysfunction in women.[4]

1.1.2 Female Sexual Dysfunction

Female sexual dysfunction (FSD) is significant disorder that affects millions of women worldwide. It is estimated that FSD affects 40-45% of adult women, with some studies estimating up to 63%.[1,12,13] Sexually related personal distress has been found in 12% of women.[12] The term FSD encompasses a variety of deficits. Within FSD, women may suffer from female sexual arousal disorder (FSAD), which includes either impaired genital response (lubrication/swelling) or persistent or recurrent lack of sexual excitement and pleasure during sexual activity,[14] or hypoactive sexual desire disorder (HSDD), which is defined as "persistent or recurrent deficiency or absence of sexual fantasies and desire for sexual activity that causes marked distress or interpersonal difficulty".[15] Recently, FSAD and HSDD have been lumped together as female sexual interest and arousal disorder (FSIAD).[16] Additional disorders included under FSD are female orgasmic disorder or anorgasmia, which is the delay, infrequency, or absence of orgasm, and genito-pelvic pain or penetration disorder, which is characterized by pain during penetrative sex.[17]

The Female Sexual Function Index (FSFI) is a validated 19-item questionnaire that evaluates sexual functioning within the realms of desire, arousal, lubrication, orgasm, satisfaction, and pain, and is used to diagnose FSD.[18] The cutoff score for diagnosing FSD is $\leq$26.55 points out of a maximum of 36.[19] There is difficulty in both diagnosis and treatment of FSD, as female sexual responses depend on both physiological and psychological inputs, as shown by the Basson model of the human sex response cycle in Figure 1.[20] Any deficit in emotional intimacy, sexual stimuli, sexual arousal, desire, or emotional and physical satisfaction

can have a negative impact on the entire sexual response.

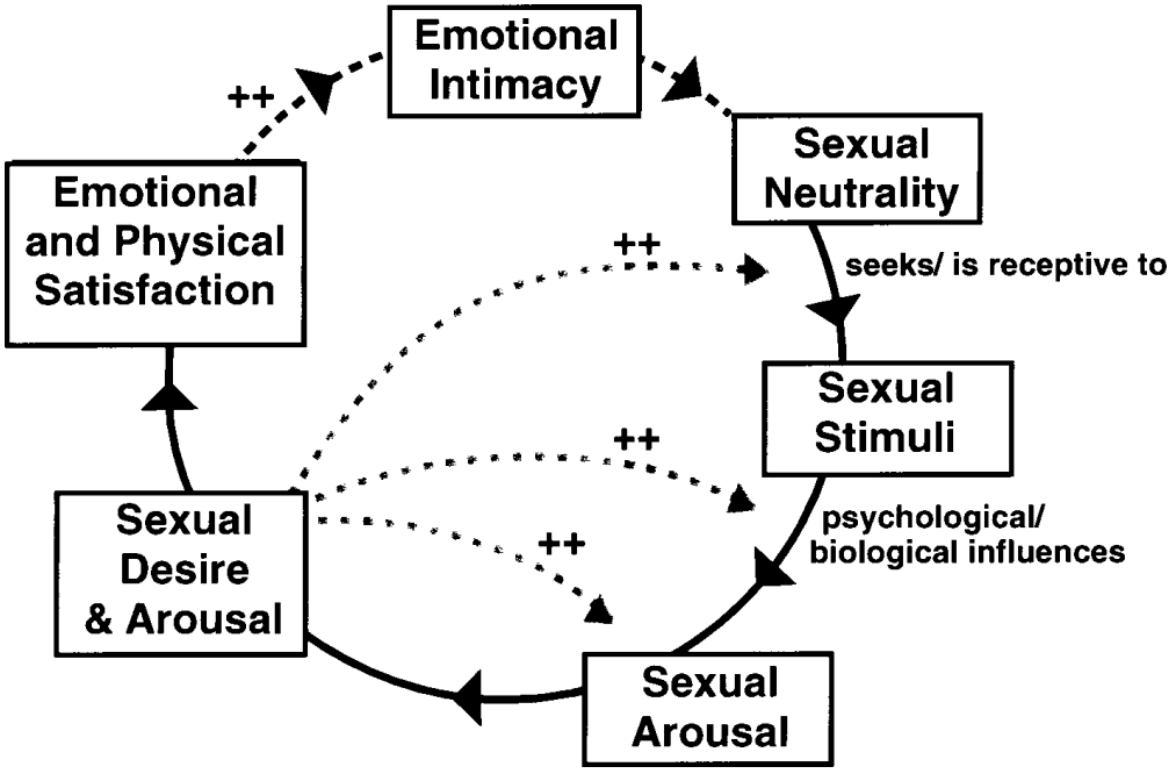

Figure 2. Human sex response cycle as described by Basson 2001.[20] An increase in sexual desire increases receptivity to sexual stimuli, likely leading to more intense arousal.

There is no single cause for female sexual dysfunction, and often times the exact cause is unknown. Cardiovascular disorders are associated with FSD. Hypertension and the use of hypertension medication have been linked to deficits in orgasm, lubrication, and desire.[21] Cardiovascular disease can cause decreased pelvic blood flow, leading to vaginal wall and clitoral smooth muscle fibrosis.[22] Diabetes mellitus has been indicated to adversely affect vaginal blood flow during sexual response.[23] Urinary incontinence has also been shown to affect all aspects of sexual functioning.[21] Neurogenic etiologies of FSD can include spinal cord injuries or disease of the nervous system.[4] Pharmaceuticals can be to blame as well. Serotonin reuptake inhibitors (SSRIs), typically prescribed for mental disorders such as anxiety and depression, have been shown to cause sexual dysfunction in 25-80% of patients taking the drug with symptoms

such as decreased libido, genital anesthesia, lubrication deficiency, and orgasm deficiency.[24,25] Due to the psychological inputs into the female sexual response, relationship issues, emotional well-being, self-esteem, and body image can all negatively impact sexual function.[4] General health and age are also factors, particularly menopause. Menopause is the result of reduced ovarian activity, which reduces circulating levels of oestrogen. The reduced levels of oestrogen lead to vulvovaginal atrophy and vaginal dryness that can cause sexual dysfunction in an estimated 50-60% of postmenopausal women.[1,26] Some women also experience decreased sexual desire during and after menopause.

1.1.3 Treatment of Female Sexual Dysfunction

There are limited treatment options available for women with FSD. One of the most widely used treatments is hormone therapy, especially among menopausal and perimenopausal women. Testosterone and other androgens can be used to treat HSDD with a variety of delivery methods, but can have inconsistent efficacy with an increased risk of cardiovascular disease.[27,28] Estrogen and progestin tablets are widely prescribed for menopausal symptoms such as hot flashes and vaginal symptoms, but are linked to increasing rates of cancer, strokes, coronary heart disease, and pulmonary embolism, making the risks outweigh the benefits.[29] Hormone therapy can be effective, but is not recommended for all patients.[30]

The most common medication to treat erectile dysfunction contain PDE5 inhibitors that aim to increase blood flow to the genitals.[30] These PDE5 inhibitors come in the form of sildenafil (Viagra), tadalafil (Cialis), and vardenafil (Levitra). Sildenafil has been administered to women as a potential treatment option, but has shown mixed results and frequently presents mild to moderate side effects[2,31,32] with a high likelihood of moderate adverse events, most commonly headaches.[33]

Two drugs have recently been approved by the FDA for treatment of FSD. Flibanserin (Addyi) is the first drug approved by the FDA to treat HSDD and has had positive results in increasing desire. The drug has been controversial for its risk/benefit ratio, as the drug has modest benefits and various side effects.[34] The daily pill led to an increase in one sexually satisfying event per month compared to placebo in a study.[15] Bremelanotide (Vyleesi) received FDA approval in June of 2019 for premenopausal women with HSDD. This treatment is on-demand and involves a subcutaneous injection ~45 minutes prior to sexual activity.[35] Bremelanotide was shown to significantly improve sexual desire, but very frequently came with a side effect of nausea in 40% of patients.[35] While both of these treatments have shown positive benefits to sexual desire, they have no impact on genital arousal.

More common, the typical treatment for FSD is psychosexual therapy. While psychological influences have a significant impact on sexual functioning in women, this type of therapy cannot treat physiological deficits.[30]

1.1.4 Rat Models for Female Sexual Functioning

Rats have proven to be a considerable representation of the pharmacology, neuroanatomy, and vasocongestive mechanisms involved in sexual function of women.[36] Physiological markers of sexual arousal have been modeled in anesthetized rats through pudendal, clitoral and pelvic nerve stimulation.[37–40] In these experiments, arousal was evaluated through the blood engorgement/perfusion of the vagina, measured with laser Doppler flowmetry (LDF). It has been shown that both clitoral and pelvic nerve stimulation can cause a brief, transient increase in vaginal blood perfusion. Vaginal luminal diameter has also been shown to increase at the onset of sexual arousal from short-duration nerve stimulation.[37] The increase in vaginal blood flow is very similar to the responses seen in genital arousal of women.[41] These

responses are dependent on the frequency of stimulation, and the responses are magnified by concurrent sildenafil administration.[38] Rats have been shown to be preferentially sexually receptive during the proestrus phase of their estrus cycle.[42] However, sexual motivation can be increased via hormone priming with progesterone and β-estradiol benzoate.[43,44]

There is evidence that the level of genital arousal in female rats can be evaluated through analysis of slow oscillations in vaginal blood flow.[41,45] In both numerical computer models as well as experiments using rat models, it has been shown that frequency domain analysis of LDF signals can be segmented into cardiac, respiration, myogenic, neurogenic and endothelial related metabolic activities.[46–48] The neurogenic frequency range for micro blood perfusion oscillations is between 0.076–0.2 Hz. This neurogenic frequency range allows for the isolation of changes to blood perfusion that are directly related to neurological inputs, such as modulations in autonomic control.

1.2 Neuromodulation

Neuromodulation is defined by the International Neuromodulation Society as "the alteration of nerve activity through targeted delivery of a stimulus, such as electrical stimulation or chemical agents, to specific neurological sites in the body". Neuromodulation via electrical stimulation has been investigated for centuries, as Luigi Galvani first started reanimating frog legs with electrical stimulation in 1786.[49] A common application of neuromodulation has been to treat bladder dysfunction. Through the use of electrical stimulation therapies for bladder dysfunction, it has been found that there can be significant improvements in sexual functioning for men and women. As the urological and sexual systems are controlled by similar nerve groups as described previously, the overlapping benefits are unsurprising. A large factor in this improvement could be due to the negative impact that lower urinary tract symptoms (LUTS) can

have on sexual function.[50,51] No studies have investigated this effect on exclusively FSD populations, so the results cannot easily be separated from a secondary effect from relieving bladder symptoms. The studies evaluating the changes to sexual functioning in women receiving treatment for bladder disorders are summarized here.

1.2.1 Sacral Neuromodulation

Sacral neuromodulation (SNM) is an established treatment for LUTS, typically using a Medtronic InterStim® system. It consists of a surgically implanted stimulation system with an electrode typically placed in the S3 sacral foramen and a pulse generator placed subcutaneously over the buttocks (Figure 3).[52] Stimulation is delivered constantly. The stimulation modulates bladder activity by altering afferent signaling. In clinical evaluations of SNM for LUTS, patients often spontaneously reported that their arousal and orgasms had improved, leading to more thorough investigations of the impact of SNM on sexual symptoms.[53–55]

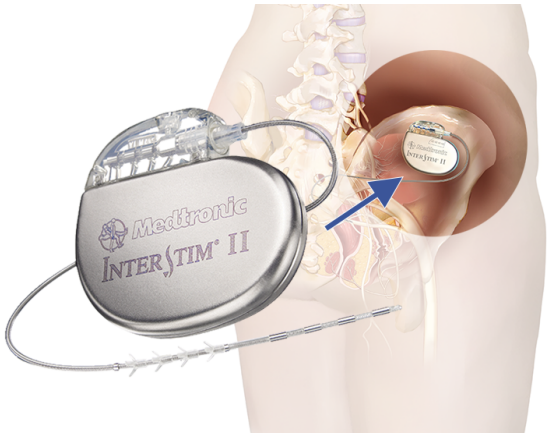

Figure 3. Sacral neuromodulation (SNM) implant with Medtronic InterSim® system.

Most studies on the effect of SNM on FSD find a significant improvement in total FSFI scores.[33,53,55–59] Improvements have been seen in the subdomains of desire, arousal, lubrication, orgasm, and satisfaction, with some studies noting an improvement in pain. Lombardi et al was the only study to separate idiopathic and neurogenic FSD patients, and found that satisfaction and total FSFI were significantly improved in both patient populations.[56]

As LUTS has been shown to negatively impact sexual functioning, some studies have found that improvements in the FSFI were significantly correlated to improvement in bladder

symptoms.[57] Patients often report that fear of incontinence restricts sexual activity [60]. However, in several studies, there was no significant relationship between improvements in urinary function and sexual functioning, implying that the impact on sexual functioning is not secondary.[53,60,61] Women could improve sexual symptoms even if there was not improvement in bladder symptoms. In a study evaluating vaginal blood flow responses via vaginal pulse amplitude (VPA) in patients before and after a SNM implant, women had higher VPA measurements post-implantation while stimulation was off, and even higher measurements during stimulation.[54] This provides evidence that the stimulation is having direct effect on the vasocongestion element of genital arousal, likely through activation of parasympathetic fibers, and that lasting changes in pelvic blood flow can ease symptoms of sexual dysfunction.

1.2.2 Posterior Tibial Nerve Stimulation

Posterior/percutaneous tibial nerve stimulation (PTNS) has been studied clinically for over 30 years for reducing symptoms of overactive bladder and incontinence.[62] Patients typically receive weekly 30-minute stimulation sessions for 12 weeks with periodic maintenance sessions thereafter.[63] The stimulation has a carry-over effect, causing lasting, positive changes long after the 30-minute stimulation.[64] Stimulation is delivered via a percutaneous needle placed at the tibial nerve near the ankle (Figure 4), but cutaneous stimulation with transcutaneous electrical nerve stimulation (TENS) electrodes have also shown efficacy in some studies.[65,66] The mechanisms of PTNS are not well understood, but the tibial nerve contains some of the sacral roots that innervate pelvic organs (Figure 5).[67] It also shares sacral roots with the target of SNM, which could explain the similar therapeutic

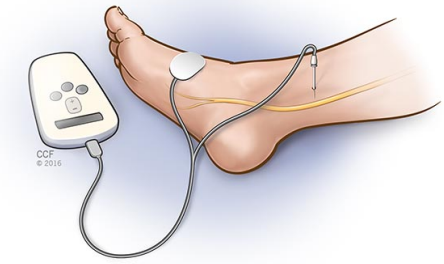

Figure 4. Percutaneous tibial nerve stimulation (PTNS) administration.

outcomes, even with very different treatment timings. It has been proposed that PTNS works through modulating signals to and from the bladder using retrograde afferent stimulation.[68] Similarly to SNM, women have spontaneously reported improvements in sexual functioning after receiving PTNS treatment for LUTS.

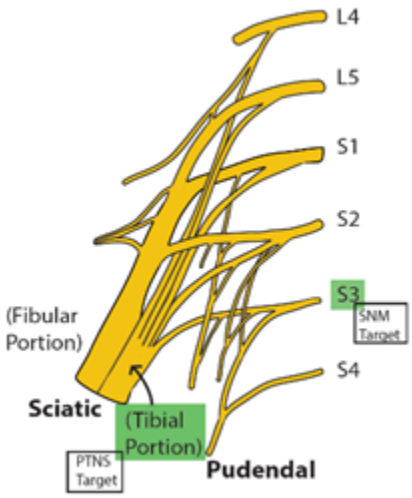

Figure 5. Sacral roots and related neuromodulation targets

Van Balken et al was the first study to make the link between PTNS and sexual functioning.[69] This study did not use the validated FSFI, but rather used the similar Dutch-language "Nine questions regarding Sexual Functioning" (NSF-9) to evaluate functioning. They found significant improvements in overall satisfaction, frequency of sexual activities, and libido. While no comparisons were made between bladder symptoms and sexual symptoms, the authors believe the sexual improvement to be directly related to the diminishing fear of incontinence during sexual activity. Gokyildiz et al utilized PTNS not for bladder dysfunction, but rather for chronic pelvic pain which has negative impacts on sexual functioning.[70] They were able to significantly reduce pelvic pain, but no other subdomains of the FSFI showed significant improvement.

Musco et al tested PTNS in 41 overactive bladder patients, using the FSFI to evaluate sexual functioning.[71] Twenty-one of the patients had FSD. In those patients, they found significant improvements in all subdomains. Even in women without FSD, there was a significant improvement in desire, satisfaction, and total FSFI. They also found that improvement in sexual symptoms were independent of urinary symptoms. Similar to SNM, this provides evidence that PTNS can have a direct effect on sexual functioning, and not secondary to

relieving bladder symptoms. Further studies need to be performed to validate PTNS as a treatment option for FSD.

1.2.3 Spinal Cord Stimulation

Epidural spinal cord stimulation (SCS) has been used to treat chronic pain for over 50 years.[72] In the past decade, SCS has also been used for the restoration of functions in patients with spinal cord injury.[73] SCS can either be delivered percutaneously or through surgical implantation of leads requiring a laminectomy. As with SNM and PTNS, an unintended but supplementary benefit of this therapy has been improvement in sexual function. Meloy et al investigated percutaneous SCS in 11 women with anorgasmia, which is the inability to achieve orgasm.[74] Electrodes were positioned near spinal level L1, placed depending on when the patients reported paresthesias in their genital region. Ten of the 11 subjects reported that the stimulation caused pleasurable genital stimulation. Orgasm during stimulation was achieved in 4 subjects. There were no validated questionnaires to evaluate sexual functioning, but subjects reported higher frequencies of sexual activity throughout the duration of the study. There are several patents for the use of epidural stimulation to treat sexual dysfunction, but limited clinical literature investigating the efficacy.

1.2.4 Potential Nerve Targets

There is reason to believe that stimulation of either the pudendal, dorsal genital, or vagus nerve either through transcutaneous, percutaneous, or implanted nerve cuff stimulation could be utilized to treat FSD. The pudendal nerve innervates the clitoris and is heavily involved in the sexual response. The pudendal nerve is also likely being modulated during SNM, as it derives from the same sacral levels. Pudendal nerve stimulation has been utilized to treat bladder dysfunctions, and a few studies have noted improvements in sexual dysfunction, but no studies

have deliberately tested pudendal stimulation on sexual function.[75] The dorsal genital nerve is a branch of the pudendal nerve that is involved in the sexual response, and is more easily accessible. Rather than requiring surgery like for pudendal stimulation, the dorsal genital nerve can be accessed percutaneously or transcutaneously. The vagus nerve as well presents a possibility of a neuromodulation target for FSD. In a study of women with complete spinal cord injury at T10 or higher, mechanical stimulation of the genitalia was able to activate regions of the brain (as seen on fMRI) that indicate an orgasm.[76] This lead researchers to believe that afferent signals related to sexual stimulation were bypassing the spinal cord via the vagus nerve. If that pathway could be properly modulated, it could be used to treat FSD.

1.2.5 Mechanisms of Action

Few animal studies have attempted to manipulate the neural control of the genital arousal response. In these experiments, genital arousal was evaluated through the blood engorgement or perfusion of the vagina, measured with laser Doppler flowmetry (LDF). It has been shown that both dorsal genital and pelvic nerve electrical stimulation can cause a brief, transient increase in vaginal blood perfusion.[38,39,77] It is likely that these neuromodulation techniques are increasing or improving pelvic blood circulation which leads to a healthier genital arousal response. Proper vasocongestion of the vagina and vulva allows for better arousal, lubrication, ease of orgasm, and general satisfaction. Further studies on the timing or duration of these changes as well as other potential mechanisms are needed.

1.3 Thesis Work

As this chapter summarizes, FSD affects a significant number of women, but there are few treatment options available. Neuromodulation has shown clinical promise in treating FSD, but it remains understudied. Underlying mechanisms have yet to be investigated, and the women

receiving neuromodulation treatment clinically all have underlying bladder issues. In Chapter 2, I will investigate the effect of tibial nerve stimulation on the genital arousal of female rats. In Chapter 3, I will investigate both the short-term and long-term effect of tibial nerve stimulation on the sexual behavior of female rats. In Chapter 4, I will analyze the results from a clinical study of women with FSD receiving weekly transcutaneous stimulation at either the dorsal genital and posterior tibial nerve.

Through the research in this thesis, I will demonstrate that increases in genital blood flow is one likely mechanism of how tibial nerve stimulation improves sexual functioning, as vaginal blood perfusion increases in response to stimulation in Chapter 2. I will also suggest that long-term, weekly tibial nerve stimulation is a more effective than on-demand stimulation as a neuromodulation treatment for FSD, as there are stronger increases in sexual behavior when stimulation is delivered weekly in Chapter 3. In Chapter 4, I will demonstrate that transcutaneous stimulation at both the dorsal genital nerve and tibial nerve can improve the sexual dysfunction symptoms of women without there being underlying bladder dysfunction. The results of these studies lay the groundwork for how and why neuromodulation can be an effective treatment for FSD.

Chapter 2 : Tibial Nerve Stimulation to Drive Genital Sexual Arousal in an Anesthetized Female Rat

(Previously published in Journal of Sexual Medicine, February 2018[78])

2.1 Abstract

Background: There is clinical evidence that percutaneous tibial nerve stimulation (PTNS) can positively benefit women with female sexual interest/arousal disorder (FSIAD), yet no studies have explored the potential mechanisms further.

Aim: To investigate the effect of tibial nerve stimulation on vaginal blood perfusion (VBP) in an anesthetized rat model.

Methods: Sixteen ketamine-anesthetized rats were surgically implanted with a nerve cuff electrode on one tibial nerve. The tibial nerve was stimulated for 30 minutes, either continuously or non-continuously, at a frequency between 10-25 Hz.

Outcomes: VBP was measured with laser Doppler flowmetry (LDF) and analyzed using a wavelet transform of time-frequency representations with a focus on the neurogenic energy range (0.076-0.200 Hz).

Results: Twenty-five of thirty-three (75.8%) stimulation periods had at least a 500% increase in LDF neurogenic energy compared to baseline. This increase was most common within 20-35 minutes after the start of stimulation. There was no statistically significant difference between frequency used or estrous cycle stage.

Clinical Translation: The results of this study provide further support for PTNS as an alternative treatment option for women suffering from FSIAD.

Strengths & Limitations: This study successfully demonstrates the ability of tibial nerve stimulation to increase vaginal blood perfusion oscillations. However, further studies to determine parameter optimization and to illuminate neural mechanisms are needed. Further studies are also necessary to determine effects of repeated stimulation sessions.
Conclusion: Long-duration tibial stimulation was successful at driving increases in neurogenic oscillations in VBP, providing evidence that tibial stimulation could be used to treat genital arousal aspects of FSIAD by improving pelvic blood flow.

2.2 Background

Female sexual dysfunction (FSD) affects millions of women worldwide.[79] Dysfunction can arise from biological, sociocultural, and psychological factors. FSD has a significant impact on quality of life and interpersonal relationships.[80,81] The prevalence of at least one form of sexual dysfunction is 40-45% of adult women with 12% of women experiencing sexually related personal distress,[21,82] yet there is no clear treatment option for a wide range of FSD deficits with high efficacy and low side effects. Female sexual interest/arousal disorder (FSIAD) and female orgasmic disorder are associated with inadequate genital arousal, which can be caused by decreased genital blood flow.[17]

Flibanserin has had mixed but generally positive results in treating the sexual interest deficit in women with FSIAD,[15,34,83] but fails to treat the physiological genital arousal aspects. Sildenafil has been shown to improve clitoral and vaginal blood flow in women, with resulting improvements in sexual function.[31] However, there are conflicting reports of clinical benefits and efficacy[32,84] as well as a high likelihood of moderate adverse events.[33]

Percutaneous tibial nerve stimulation (PTNS), also referred to as posterior tibial nerve stimulation, has been studied clinically for over 30 years for reducing symptoms of overactive

bladder and incontinence.[62] Patients typically receive weekly 30-minute stimulation sessions for 12 weeks with periodic maintenance sessions thereafter.[63] The stimulation has a carry-over effect, causing lasting bladder improvements long after the 30-minute stimulation. The mechanisms of PTNS are not well understood, but the tibial nerve enters the spinal cord at some of the sacral roots that innervate pelvic organs.[67] It has been proposed that it works through modulating signals to and from the bladder via the sacral plexus using retrograde afferent stimulation.[68] One theory for the mechanism is that PTNS results in improved pelvic blood flow.[85] In studies of patients receiving PTNS for lower urinary tract dysfunction, some women noted significant improvements in sexual functioning, including arousal, desire, lubrication, and ease of orgasm.[69,71] As bladder dysfunction has been shown to cause decreases in sexual functioning,[50] it is possible that treating women for bladder dysfunction would improve sexual functioning as a result. However, these results were not correlated with improvement in bladder functioning, providing evidence that PTNS had direct impacts on sexual functioning. PTNS has also improved sexual functioning in women receiving treatment for chronic pelvic pain.[86] However, these results have not been entirely separated from a secondary effect of treating bladder dysfunction or pain, or studied further.

Rats are a standard animal model for the pharmacology, neuroanatomy, and vasocongestive mechanisms involved in sexual function of women.[87] Physiological markers of sexual arousal have been modeled in a few prior limited anesthetized rat studies through pudendal, clitoral and pelvic nerve stimulation.[38,40,77,88] In these experiments, stimulation-driven genital arousal was evaluated through the blood engorgement or perfusion of the vagina, measured with laser Doppler flowmetry (LDF). These experiments showed that short-duration pudendal, clitoral and pelvic nerve electrical stimulation can cause a brief, transient increase in

vaginal blood perfusion (VBP). This increase is very similar to responses seen in sexually aroused women.[89] These responses can be dependent on the frequency of stimulation.[38] Vaginal luminal diameter in anesthetized rats has also been shown to increase concurrently with blood flow during genital arousal driven by acute nerve stimulation.[90]

There is evidence that the level of genital arousal in female rats can be evaluated through analysis of slow oscillations in vaginal blood flow.[41,89] In both numerical computer models as well as experiments using rat models, it has been shown that frequency domain analysis of blood flow LDF signals can be segmented into cardiac, respiration, myogenic, neurogenic and endothelial related metabolic activities.[46,48] The neurogenic frequency range for micro blood perfusion oscillations is between 0.076–0.200 Hz. Neurogenic oscillations are associated with sympathetically-driven changes in microvascular perfusion, as would be the expected mechanism in sexual arousal.[91] An analysis of this blood flow parameter may yield further insights than prior use of LDF alone.

This exploratory study aims to investigate the ability to drive genital sexual arousal with tibial nerve electrical stimulation. This study is also intended to introduce a novel method of analyzing genital blood perfusion as an arousal response, through the evaluation of wavelet analysis of neurogenic LDF oscillations. We intend to use this wavelet analysis to determine the ability of long-duration tibial nerve stimulation to drive prolonged increases in VBP. The goal of this study is to further establish PTNS as a potential treatment option for women with sexual dysfunction while investigating the relationship between stimulation and VBP.

2.3 Methods

2.3.1 Animals

All procedures were approved by the local Institutional Animal Care and Use Committee in accordance with the National Institute of Health's guidelines for the care and use of laboratory animals. Animals were housed under standard conditions. Experiments were conducted in 16 nulliparous female Sprague-Dawley rats (Charles River Breeding Labs, Wilmington, MA, USA) weighing 210-310 g. The animals were anesthetized with a ketamine/xylazine/acepromazine (90mg/kg, 7.5 mg/kg, 1.5 mg/kg respectively) cocktail during surgery and maintained with ketamine (30 mg/kg every 30 minutes) during testing, as ketamine has been used in similar studies evaluating sexual arousal in sedated rats.[38,92,93] The temperature was maintained at 37°C with a heating pad and monitored with a rectal thermometer. Heart rate, respiration rate, and SpO_2 levels were monitored and recorded every 15 minutes. A vaginal lavage was performed after anesthesia induction prior to surgery to determine the estrous stage.[94]

The tibial nerve was accessed on the right hind limb above the ankle on the medial side. A bipolar nerve cuff with stranded stainless steel wire (0.016” diameter, Cooner Wire Co, Chatsworth, CA, USA) and silicone elastomer tubing (1 mm inner diameter, Dow Corning, Midland, MI, USA) was placed around the nerve and connected to an Isolated Pulse Generator (Model 2100, AM Systems, Carlsborg, WA, USA). In the last 13 experiments, the bladder was manually drained and a catheter (PE50) was inserted 3 cm through the urethra into the bladder and connected to pressure transducer (DPT-100, Utah Medical Products, Inc., Midvale, UT, USA) and a Grass amplifier (Model CP511 High Perforance AC Preamplifier, Astro-Med, Inc., West Warwick, RI, USA). The bladder was able to drain around the catheter throughout the procedure. Vaginal blood perfusion was assessed using a laser Doppler probe (MNP110XP,

ADInstruments, Colorado Springs, CO, USA) connected to a Blood FlowMeter (50 Hz sampling rate, INL191, ADInstruments). The Blood FlowMeter measures blood perfusion on a scale of 0-5000 arbitrary blood perfusion units (BPU). The probe was inserted 1-2 cm into the vagina and was angled laterally against the vaginal wall. Probe location was adjusted until the baseline LDF signal remained relatively stable. A baseline LDF signal was recorded for 30 s – 10 min (average 4.53 ± 2.84 min) before any stimulation was delivered. Vaginal diameter (VD) was measured from the external vaginal opening with digital calipers at the beginning and end of each stimulation experiment. Change in VD (ΔVD) was measured as the percent change between the final vaginal diameter and the starting diameter. After all experimental procedures had been completed, animals were euthanized with an intraperitoneal injection of sodium pentobarbital (300–400 mg/kg).

2.3.2 Stimulation Protocol

Stimulation of the tibial nerve was delivered through the nerve cuff electrode with biphasic, rectangular pulses (0.2 ms pulse width) at frequencies ranging from 10-25 Hz. This frequency range is similar to typical stimulation frequency used clinically for PTNS (20 Hz).[63,69] Stimulation was delivered at 2-4 times the minimum level that caused a distal toe twitch. To mimic PTNS treatment protocols, stimulation was delivered for 30 minutes continuously in the first nine animals.[63,69] In the final seven animals, stimulation was delivered non-continuously in one minute intervals, with one minute of stimulation followed by one minute of rest, for a total of 30 minutes of stimulation over the course of one hour. This alternate stimulation sequence was performed to see if short stimulation periods led to observable transients in LDF signals, due possibly to pelvic floor contractions and relaxations, and to evaluate whether rest periods led to

different measurements. In all animals two or three stimulation sequences were performed, with a 20-30 minute period of rest between sequences.

2.3.3 Data Analysis

All data was analyzed in MATLAB (Mathworks, Nantick, MA, USA). LDF signals were analyzed using time-frequency representations (TFRs), with a continuous wavelet transform (CWT) method.[95,96] The primary frequency content analyzed was the neurogenic band (0.076 – 0.200 Hz), as defined by Humeau and colleagues.[95,96] Scalogram energies were calculated to convert to a single continuous parameter in time, sampled every 10 seconds, in arbitrary units.[97] As the energy is relative, we determined the percent change in energy compared to the baseline period. Appropriate statistical tests were used depending on the data set being analyzed, as indicated in the Results section. A significance level of 0.05 was used. Where appropriate, values are given as mean ± standard deviation.

2.4 Results

2.4.1 Neurogenic Oscillations in LDF Response

Wavelet analysis was performed on a total of 33 stimulation trials across 14 rats. A 500% increase in the energy of the neurogenic LDF frequency band over the baseline level was used as a threshold for identifying stimulation trials with large increases in VBP.[98] The 500% threshold was chosen as it was above the mean coefficient of variance for the neurogenic frequency band during the baseline period of all trials (159.1%), as well as the maximum value (282.0%). Example trials using different stimulation patterns are shown in Figure 6.

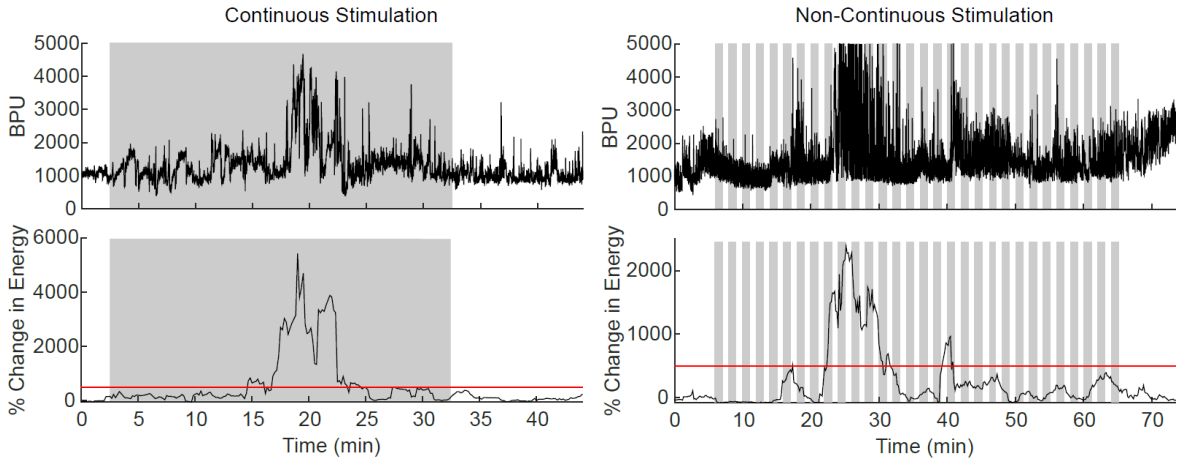

Figure 6. Example tibial nerve stimulation trials. Top left: Raw LDF measured in BPU for continuous stimulation at 20 Hz (grey box) after a 2.5 minute baseline period. Bottom left: Corresponding neurogenic energy. Threshold (horizontal line) represents a 500% increase from average baseline levels. A large increase in neurogenic energy occurs ~16 minutes into the trial, lasting for ~9 minutes. Top right: Raw LDF for non-continuous stimulation at 15 Hz (grey boxes), delivered in one-minute on/off intervals for a total of 30 minutes after a 6-minute baseline period. Bottom right: Corresponding neurogenic energy. A large increase in neurogenic energy occurs ~22 minutes into the trial, lasting for ~10 minutes.

The threshold was crossed in 75.8% (n = 25) of all stimulation trials (Table 1). There was no difference in the probability of trials crossing the threshold between the use of continuous (70%) and non-continuous (84.6%) stimulation ($p = 0.35$ for Chi-squared test between continuous and non-continuous trials). In the twenty-five trials that crossed the threshold, the average duration spent above the threshold was 10.73 ± 8.35 minutes. Although continuous stimulation had a longer average duration, the duration was not significantly different from non-continuous stimulation trials (Table 1; $p = 0.12$ for t-test). The average percentage increase for all trials that exceeded the threshold was 1737.8 ± 1874.9%, which also was not significantly different between continuous and non-continuous stimulation (Table 1; $p = 0.33$ for t-test). There was no difference in results between animals receiving a bladder catheter and animals that did not (Table 1), for proportion of trials crossing the threshold ($p = 0.64$), time above threshold ($p = 0.46$) and average percentage increase ($p = 0.47$).

Table 1. Summary of experimental measures. Statistical comparisons are given in the text.

			For trials that exceeded threshold	
Total trials	Type	Trials that cross threshold	Time above threshold (min)	Average % increase
33	All trials	25 (75.8%)	10.73 ± 8.35	1737.8 ± 1874.9
20	Continuous stimulation	14 (70.0%)	13.06 ± 9.52	2068.0 ± 2145.7
13	Non-continuous stimulation	11 (84.6%)	7.77 ± 5.70	1317.5 ± 1450.7
6	No bladder catheter	5 (83.3%)	13.13 ± 7.39	2720.1 ± 3424.7
27	Bladder catheter used	20 (74.1%)	10.13 ± 8.65	1492.2 ± 1286.0
4	Diestrus phase	3 (75%)	5.22 ± 4.84	815.5 ± 464.4
15	Proestrus phase	12 (80%)	10.14 ± 9.42	1550.5 ± 1570.2
9	Estrus phase	6 (66.7%)	12.47 ± 6.93	1530.0 ± 727.9
5	Inconclusive phase	4 (80%)	14.04 ± 9.20	3302.9 ± 3703.0
6	10 Hz stimulation	4 (66.7%)	10.67 ± 11.56	1239.2 ± 712.8
5	15 Hz stimulation	3 (60%)	9.50 ± 2.85	934.7 ± 364.8
18	20 Hz stimulation	14 (77.8%)	11.81 ± 8.69	2288.7 ± 2345.1
4	25 Hz stimulation	4 (100%)	7.96 ± 8.65	910.3 ± 624.0

Across experiments, there was a distribution of time points above the threshold. As Figure 7 shows, most threshold crossings were typically within 20 – 35 minutes after initiation of stimulation (mean = 29.1 min). An analysis of threshold crossings as a function of applied stimulation duration (accounting for stimulation off intervals in non-continuous stimulation trials) did not yield a clear trend.

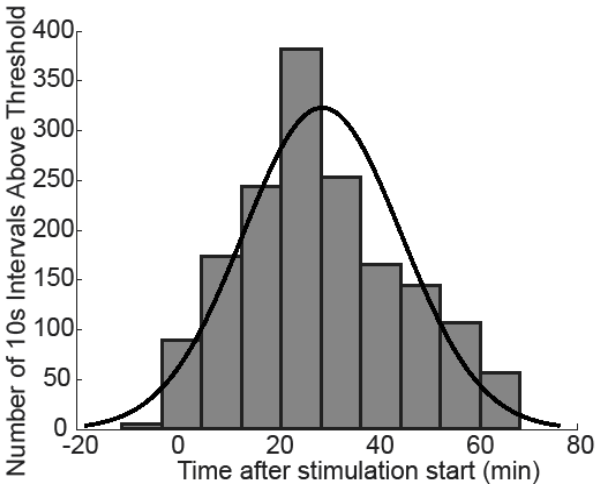

Figure 7. Histogram of neurogenic scalogram data points that crossed above the threshold in relation to the time after stimulation start. The curve represents a normal distribution fit (mean=29.1 min, standard deviation=15.8 min).

2.4.2 Change in Vaginal Diameter

VD was recorded before and after twenty stimulation trials. There was a positive ΔVD in 95% (19/20) of these trials. The average percent increase was 84.4 ± 79.4%. Using a linear fit model, the relationship between ΔVD and duration above the threshold was not significant ($R^2 < 0.001$, $p = 0.91$). The relationship between ΔVD and average increase in neurogenic oscillations was also not significant ($R^2 = 0.017$, $p = 0.63$). No pelvic floor contractions were observed during testing.

2.4.3 Frequency of Stimulation Effect on Genital Arousal

Stimulation was delivered at 10 Hz, 15 Hz, 20 Hz, and 25 Hz (Table 1). The stimulation frequency did not have a significant effect on the average percent increase in neurogenic energy

($F(3,32) = 0.91$, $p = 0.49$) or duration above threshold ($F(3,32) = 0.23$, $p = 0.87$) according to an ANOVA. That the stimulation frequency group sizes are dominated by the 20 Hz group may have affected this analysis.

2.4.4 Estrous Phase Effect on Genital Arousal

Across all trials with a conclusive vaginal lavage (n = 28 of which 21 crossed threshold), rats on average had a larger duration of time above the threshold and percent increase in neurogenic energy if they were in the proestrus or estrus phase compared to diestrus (Table 1). This trend was not statistically significant ($F(2,27) = 0.43$, $p = 0.66$ for duration above threshold; $F(2,27) = 0.39$, $p = 0.68$ for percentage increase) according to an ANOVA. Inclusion of the trials with inclusive readings as a separate treatment was still non-significant for both outcome measures ($F(3,32) = 0.51$, $p = 0.68$ for duration above threshold; $F(3,32) = 1.24$, $p = 0.31$ for percentage increase).

2.4.5 Bladder Pressure

Continuous, rhythmic non-voiding bladder contractions were consistently observed before, during, and after stimulation intervals with no relationship to the applied stimulation. The frequency of these contractions was generally centered on 0.06 Hz (Figure 8). Occasionally bladder pressure changes were visible in LDF recordings, but were lower than the frequency range of interest in our analysis. Bladder voiding contractions also occurred at lower frequencies, with a void typically every 4-8 minutes (0.0021-0.0042) Hz.

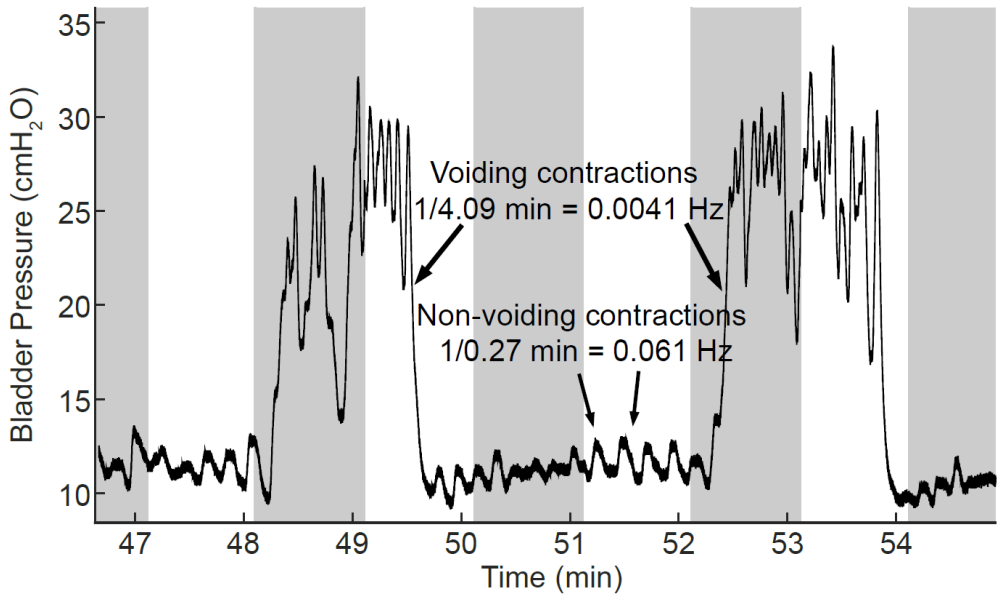

Figure 8. Example of typical bladder pressure recording taken from middle of a non-continuous stimulation trial at 15 Hz. Smaller, non-voiding contractions are seen with an approximate frequency of 0.061 Hz. Larger voiding contractions are seen with an approximate frequency of 0.0041 Hz.

2.5 Discussion

To our knowledge, this study is the first to explore tibial nerve stimulation to drive genital sexual arousal responses in a rat model. Previous clinical trials have made a connection between PTNS and improvement in sexual function, but this study is the first to model the effect preclinically. Our results suggest that long-duration tibial nerve stimulation can lead to increases in genital arousal. Poor blood perfusion to the genitals during sexual activity is a contributing factor to sexual dysfunction.[33,80] A treatment that improves perfusion may be beneficial for genital arousal deficits in women with FSIAD. These results also indicate that the clinical improvements in sexual functioning after PTNS treatment may be a direct result of the stimulation. The relationship between bladder function and sexual function in clinical stimulation studies remains unclear. The sexual benefits observed clinically may be related to improvements in pelvic blood flow.

During most (75.8%) stimulation trials the neurogenic frequency band of the VBP crossed our arbitrary 500% threshold increase over baseline (Figure 6, Table 1). Threshold

crossings were most common after 20-35 minutes from stimulation onset (Figure 7), a period similar to the typical clinical PTNS stimulation session duration. This suggests that long-duration stimulation (tens of minutes) is more effective than short-duration stimulation (seconds to few minutes) at increasing neurogenic oscillations in VBP. The delay in the onset of increases in blood perfusion (Figure 7) may be due to descending cortical inhibition.[99] The trend of rats in the proestrus or estrus phase having longer time intervals above the threshold (Table 1) may be related to previous behavioral studies that indicated rats are more sexually receptive during the proestrus phase.[42] The results of this study are similar to a study in anesthetized rats which showed that pudendal nerve stimulation for at least fifteen minutes led to significant increases in VBP.[98] In that study, peaks in maximal vaginal LDF responses also occurred near 30 minutes after stimulation start[98], suggesting that similar spinal circuits and blood flow dynamics may be activated in each approach. In that study, estrous phase had no effect on changes in VBP[98], suggesting non-significant differences in our study may be due to variability.

There was an increase in ΔVD in almost all experiments. As there was no clear relationship between neurogenic oscillations and ΔVD, the amount of change in VD does not appear to be a quantifiable indicator of degree of arousal. A measure of intravaginal pressure may be more accurate. The consistent positive increases in VD seem to eliminate a reflex pelvic floor contraction as a cause of LDF increases. Preclinical and clinical studies examining PTNS for bladder function have both shown a lack of pelvic floor tone during stimulation.[100,101] Responses to stimulation had inconsistencies across experiments, as shown by the variability in threshold crossings (Figures 6 & 7). As the tibial nerve does not directly innervate the sexual organs, tibial nerve stimulation can only be causing a sensory reflex response in the genitalia as opposed to a motor response. Stimulation-driven activation of sensory reflexes controlling pelvic

organs has been reported to be variable across experiments due to noise in the sensory system[40,98,102,103], which may have been a factor here also. Physiological differences between rats, variations in the depth of anesthesia, and differences in the LDF probe location may have also contributed to the evoked responses across experiments.

The use of laser Doppler flowmetry as a measure of sexual arousal in rats is not well documented. It has been primarily used in experiments with short recording intervals.[38,40,77,88] LDF recordings are highly susceptible to artifacts from various sources, particularly physiological processes such as breathing and bladder contractions.[38] However, the bladder contractions we observed were typically around 0.065 contractions per second, (reports in literature have been recorded as typically between 0.012-0.076 contractions per second)[104–106], which is below the neurogenic range. The respiratory rate in our experiments, recorded from vitals monitoring as between 60-120 breaths/min (1-2 Hz), was above the neurogenic range. While these physiological rhythms did not affect our results, it is important to note that they have the potential for large artifacts on the raw signal. Other studies using this LDF measurement system to evaluate genital arousal did not measure bladder pressure, and did not account for breathing rate when discussing the spectral analysis of the blood perfusion recordings.[41,88] To the best of our knowledge, there have been no vaginal LDF studies that acknowledged these physiological rhythms in their spectral analysis.

This research would benefit from a follow-up study with a more sophisticated study design. As this was an exploratory study, an equal distribution of parameters and methods were not employed. Further research is needed to determine ideal stimulation parameters. There is limited research on long-duration LDF recordings of VBP, so the analysis methods were newly developed here. This new analysis method would benefit from comparisons to naturally-induced

arousal conditions. Additionally, neural recordings of nerves involved in the sexual response would further illuminate the mechanisms of blood perfusion increases.

The tibial nerve presents an ideal clinical use for therapeutic benefits due to its ease of location. The nerve can be easily accessed with a percutaneous needle, and potentially with transcutaneous stimulation. There are transcutaneous PTNS studies that have had success with bladder dysfunction patients.[107] The clinical benefit of PTNS typically has a cumulative effect after a regular regimen of 30-mintue stimulation sessions, as opposed to an instantaneous improvement. Treatment for FSD using PTNS would likely have a similar effect, with an improvement in pelvic organ blood flow over time. Further studies are needed to determine ideal stimulation parameters and intervals of treatment. To further explore the effect of tibial stimulation on sexual responses, studies are needed to assess the long-term impact of repeated stimulation sessions on organ function and behavior and to further examine the underlying mechanisms.

2.6 Conclusion

Tibial nerve stimulation can elicit changes in VBP. The most common time for blood flow response were after 20-35 minutes of stimulation, suggesting longer-duration stimulation is necessary for an effect. Our evaluation of LDF signals using TFRs with a wavelet transform reduces the impact of unrelated artifacts on signal analysis. This focus on the neurogenic range of blood flow oscillations provides insight into the neurological source of changes. Tibial nerve stimulation-driven changes in VBP may have direct effects on genital arousal, such as lubrication and ease of orgasm. Further studies are needed to investigate the physiological mechanisms and to assess the utility of PTNS as a potential treatment for the genital arousal components of FSIAD.

Chapter 3 : Immediate and Long-Term Effect of Tibial Nerve Stimulation on the Sexual Behavior of Female Rats

3.1 Abstract

Background: There are limited treatment options for female sexual dysfunction. Percutaneous tibial nerve stimulation has shown improvements in sexual dysfunction symptoms in neuromodulation clinical studies, but the direct effects of stimulation on sexual function are not understood.

Aim: Evaluate the immediate and long-term effects of percutaneous tibial nerve stimulation on the sexual motivation and receptivity of female rats.

Methods: In two experiments, after receiving treatment, ovariectomized female rats were placed in an operant chamber apparatus with two compartments: a tethered, sexually active male was on one side and the female's access to the male's side was controlled by nose poking according to a fixed interval 15 sec. There were five treatment conditions that involved (S+) or without (S-) percutaneous tibial nerve stimulation and no (H-), partial (H+), or full (H++) hormone priming. In Experiment 1, rats were rotated through each treatment condition with behavioral testing immediately following treatment for 10 weeks. In Experiment 2, rats were committed to one treatment condition for 6 weeks and sexual behavior was tracked over time.

Outcomes: Sexual motivation was quantified through number, latency, and frequency of nose pokes as well as completed intervals, and sexual receptivity was quantified through mounts, lordosis quotient, and time spent in chamber zones.

Results: In Experiment 1, there were non-significant trends of increased sexual motivation immediately following tibial nerve stimulation, but not receptivity. In Experiment 2, there were non-significant trends of increasing sexual receptivity and some sexual motivation metrics increased when tibial nerve stimulation was applied long-term.

Clinical Translation: This study further supports the use of tibial nerve stimulation as a treatment for female sexual dysfunction.

Strengths & Limitations: This study is the first to evaluate sexual behavior in response to nerve stimulation. The low sample sizes may have contributed to a lack of statistical significance for most measures.

Conclusion: Tibial nerve stimulation combined with hormone priming shows potential for increasing sexual motivation in the short-term and sexual receptivity in the long-term in rats, but further studies are needed.

3.2 Introduction

Female sexual dysfunction (FSD) affects a significant number of women. Up to 40-50% of women present some form of sexual dysfunction symptom, with low sexual desire and arousal being the most common complaints.[1,82,108] FSD can have a serious impact on women's quality of life.[108] Despite a significant patient population, there are limited treatment options available.[109] For several years, neuromodulation techniques that apply electrical stimulation to nerves to treat conditions have shown evidence of being able to treat female sexual dysfunction.[61] However, little research has been done on how best to utilize this treatment or to examine its mechanisms.

Percutaneous tibial nerve stimulation (PTNS) is a minimally invasive neuromodulation therapy that has been utilized as a treatment for lower urinary tract dysfunctions such as overactive bladder for several decades.[110] In this therapy, electrical stimulation is delivered to the tibial nerve near the

ankle with a percutaneous wire. PTNS is typically delivered in 30-minute stimulation sessions once a week for 12 weeks with periodic maintenance sessions thereafter. Positive benefits begin to present after several weeks of stimulation.[110] In some studies of PTNS for bladder dysfunction, a secondary outcome of improving sexual dysfunction symptoms has been observed.[111] Recently we conducted a pilot study of skin-surface PTNS and dorsal genital nerve stimulation (DGNS) in women with FSD but no bladder dysfunction to determine if improvements in sexual functioning can be achieved without concomitant improvement in bladder function.[112] In this study, the subjects receiving PTNS had significant increases in their sexual functioning, most significantly in the genital arousal subdomains of arousal, lubrication, and orgasm.[112] However, the mechanism of these improvements are not understood.

In a previous preclinical study, we investigated a possible mechanism of action by evaluating the effect of tibial nerve stimulation on the vaginal blood perfusion of anesthetized rats.[78] We showed that long durations (20-40 minutes) of tibial nerve stimulation at 20 Hz can lead to prolonged increases in vaginal blood perfusion, as seen by laser Doppler flowmetry. These increases in genital blood flow may explain why improvements were seen in the genital arousal components of women with FSD in the short term, but there have been no studies that evaluate how genital blood flow changes with repeated, long-term tibial nerve stimulation. Additionally, there have been no studies evaluating the effect of tibial nerve stimulation on the sexual behavior of rats, which may have implications for women.

Here, we investigate whether tibial nerve stimulation can lead to increases in sexual motivation and receptivity. It has been shown that tactile clitoral and vaginal stimulation can modulate sexual behavior[113,114], but it is unknown whether tibial nerve stimulation can have a similar effect. As ovariectomized rats have been shown to have reduced vaginal blood flow as well as reduced sexual

motivation[115,116], they are used as our model for sexual dysfunction. This model is an effective model of a post-menopausal woman. Ovariectomized rats have lower sexual motivation and receptivity than intact rats, but their sexual behavior can be restored through hormone priming.[43]

It is unclear if tibial nerve stimulation would be most beneficial in treating FSD as an on-demand treatment right before sexual activity or as a long term treatment that leads to improvements over time, similar to PTNS clinical use for bladder dysfunction. In this study, we performed two experiments: one that evaluated the sexual behavior of rats immediately after receiving percutaneous tibial nerve stimulation to investigate the short-term impact of stimulation, and a second that evaluated the sexual behavior of rats over time with repeated tibial nerve stimulation to evaluate the long-term impact of stimulation. The effects of stimulation were evaluated in animals in separate experimental conditions with and without hormone priming.

3.3 Methods

3.3.1 Animals and Preparation

A total of 28 adult female (200-300 g) and 12 adult male (300-400 g) rats (Charles River Laboratories, Wilmington, MA, USA) were used in this study. Animals were housed in same-sex pairs and maintained on a 14:10 light:dark cycle (lights off at 12:00 pm) with free access to chow and water. All female rats were bilaterally ovariectomized under isoflurane anesthesia and given carprofen for analgesia in the two days after surgery. Beginning 4 days after surgery, vaginal epithelium samples were taken via saline lavage daily for 10 days to confirm a complete ovariectomy. All procedures were conducted in accordance with the National Institutes of Health (NIH) guidelines on laboratory animal use and care, using a study protocol approved by the University of Michigan Committee on Use and Care of Animals.

3.3.2 Behavioral Apparatus and Training

The behavioral testing apparatus used in this study is described in Cummings et al 2012.[43] The apparatus consists of a dual-compartment Plexiglas operant chamber. Separating the compartments is a horizontal sliding door that is controlled by rodent nose poking. Animals are tracked using an overhead monochrome board camera (DMM 72BUC02-ML, The Imaging Source, Charlotte, NC, USA) connected to a computer running the ANY-maze program (Stoetling Co. Inc., Wood Dale, IL, USA), which tracks the animal, collects nose poke information, and controls the door. The smaller chamber is the female side, where the female is free to roam. There are two nose poke holes on the female side, one active and one inactive. The active hole has a corresponding light cue. Nose poking in the active hole in a manner fitting the operant schedule opens the door, giving the female access to the larger side, which has a tethered male who cannot reach into the female side.

All females were trained once a week for 4 weeks before the start of testing with hormone priming that induced sexual receptivity. Females were hormone-primed with 10 μg estradiol benzoate (EB) 48 and 24 hours prior and 500 μg progesterone (P) 4-5 hours before each training session. Females were trained to nose poke the active hole to open the sliding door on a fixed ratio (FR) and fixed interval (FI) schedule in 30-minute sessions. With each nose poke there is a light cue for 1s. Training females began at FR1, which requires a single nose poke to open the door, and then advanced to FR5, which requires 5 nose pokes to open the door. FR5 had a fixed interval of 15 seconds (FI15), in which an initial nose poke began a 15-second interval in which nose pokes are recorded but inconsequential.[117] Females were required to master each level of training before moving to the next level. The door would only open if the female poked during a 5s period after

the 15s interval, otherwise the interval would reset. FI15 was used as the schedule for experimental testing.

3.3.3 Testing Conditions

The conditions involved either stimulation (S+) or no stimulation (S-) with no hormones (H-), partial hormone (H+), or full hormone (H++) dosing. Proven breeder males were randomly rotated for each female to prevent mate preferences.

3.3.4 Hormones

Animals receiving hormones (S-H+,S+H+,S-H++) were administered 10 μg EB 48 and 24 hours prior to testing, and either 100 μg (partial hormone, H+) or 500 μg (full hormone, H++) P 4-5 hours prior to testing. It has been shown that the sexual receptivity in female rats can be scaled with P dose.[118] The 100 μg P (H+) dose was chosen to represent approximately half of the maximum receptivity a female can exhibit, and 500 μg (H++) represents maximum receptivity.[118] The H+ dosing was used to allow room for increases in sexual receptivity when tibial stimulation is applied. H- animals in a given testing week were administered injections of 0.1 mL peanut oil 48, 24, and 4-5 hours prior to testing as a control.

3.3.5 Stimulation

Electrical stimulation was delivered to the right posterior tibial nerve via a bipolar wire electrode (EMG hook electrode, Microprobes for Life Science, Gaithersburg, MD) connected to an Isolated High Power Stimulator (Model 4100, A-M Systems, Sequim, WA, USA). Each week, every animal (S- and S+) was anesthetized with 5% isoflurane for 10 minutes. In S+ animals the wire was placed percutaneously while under anesthesia in the right leg near the tibial nerve.[119] Surgical tape was wrapped around the leg where the wire exited the skin for reinforcement and to

prevent the rat from chewing the wire. S- animals received the same taping as a control but the wire was not placed. The motor threshold for electrical stimulation which elicited a toe twitch was found. Once the wire was secured and the threshold was found during the 10 min isoflurane interval, isoflurane was turned off and animals were placed in open-top cages to receive stimulation while awake. Stimulation was delivered at twice the motor threshold with biphasic, 20 Hz, 200 μs pulse-width stimulation for 30 minutes. These stimulation parameters were used to mimic parameters used in rat studies investigating the effect of tibial nerve stimulation on both sexual[78] and bladder functioning.[120,121]

3.3.6 Data Analysis

The number of nose pokes per test, nose pokes per interval, nose-poke latencies (time to first nose poke), and number of attempted and completed FI15s intervals were obtained from the ANY-maze software program. Videos were scored using Solomon Coder (version: beta 19.08.02, https://solomon.andraspeter.com) by two observers who were blinded to the experimental condition of the rat. The time spent in each chamber zone (female zone, male zone, and in doorway), counts of mounts and intromissions by the males, and lordosis expressions by the females were scored. When it was performed, lordosis was scored as partial (1-point or 2-point lordosis intensity) or full (3-point lordosis intensity).[122] Lordosis quotient (LQ) was calculated as the number of lordosis expressions (partial and full) divided by the number of mounts. The average value between the two observers was used for each measure. Data were analyzed and statistical analyses, as described below, were performed using MATLAB R2018a (Natick, MA).

3.3.7 Experiment 1

Experiment 1 was designed to test the immediate effect of stimulation on sexual behavior by performing testing immediately following tibial nerve stimulation. Eight Sprague Dawley

females (250-300 g) were used in this experiment. Four adult Sprague Dawley males who were proven breeders were used as stimulus males. Experiment 1 testing took place over 10 weeks. Rats were randomly assigned to undergo each of the five conditions twice across the study duration. S+ animals received one round of stimulation in a testing week. Stimulation was delivered from 35 minutes prior to behavioral testing until 5 minutes prior to testing to allow time to remove the wire and tape and move female rats to the testing chamber. Data is presented as the average and standard deviation for each condition across all animals. To determine statistical significance, linear mixed effects models were performed using R (Vienna, Austria). The mixed effects models used the treatment group and week as fixed effects and the female rat identifications as a random effect. Male rat identifications had no effect on the linear models. Comparisons were made between each condition group for each behavioral metric with significance set at $p < 0.05$.

3.3.8 Experiment 2

Experiment 2 was designed to test the long-term effects of stimulation by administering stimulation twice a week for six weeks for S+ animals. Twenty Sprague Dawley females (250-300 g) were used in this experiment. Eight adult Sprague Dawley males who were proven breeders were used as stimulus males. Experiment 2 testing took place over 6 weeks. Rats were assigned to one of the five conditions for the entire duration of the study. S+ animals received two rounds of stimulation in a testing week, at 48 hours and 24 hours prior to testing to synchronize with EB injections. Linear correlations were determined for each metric over time. To determine statistical significance, analysis of covariance (ANCOVA) tests were performed using R (Vienna, Austria). Comparisons were made between each condition group for each behavioral metric with significance set at $p < 0.05$.

3.4 Results

The main comparisons of interest between condition groups were between S+H- and S-H- rats and S+H+ and S-H+ rats. Both comparisons evaluate rats receiving the same hormone treatment (either H- or H+) with S+ rats also receiving stimulation. This comparison allows for an examination of the effects of tibial nerve stimulation on the sexual behavior of rats, depending on hormone condition.

3.4.1 Experiment 1

All eight rats completed each of the ten weeks of testing. The average amplitude of electrical stimulation administered was 1.18 ± 0.66 mA. Stimulation was typically well tolerated. Some rats lifted their leg or attempted to chew the wires in response to stimulation, indicating some level of stimulation perception or discomfort.

3.4.1.1 Sexual Motivation

Data for motivational metrics for Experiment 1 are presented in Table 2 and Figure 9. S+H+ rats on average had more nose pokes per test (Figure 9a) than S-H+ rats ($p = 0.42$), while S+H- rats had fewer nose pokes per test than S-H- rats ($p = 0.48$). S+H+ rats had the highest nose poke frequency, including over both S-H+ ($p = 0.23$) and S-H++ ($p = 0.85$).

S+H- rats had significantly fewer nose pokes per interval than S-H+ ($p = 0.04$) and S-H++ rats ($p = 0.02$), they also had fewer nose pokes per interval than S-H- rats ($p = 0.30$). S+H+ rats had the shortest initial latency (Figure 9b) and shortest inter-interval latency, over S-H- ($p = 0.33$, $p = 0.57$, respectively), S+H- ($p = 0.34$, $p = 0.77$, respectively), S-H+ ($p=0.32$, $p =0.38$, respectively), and S-H++ ($p = 0.13$, $p = 0.64$, respectively). S+H- rats had longer initial latencies ($p = 0.98$) and inter-interval latencies ($p = 0.74$) than S-H- rats. S+H+ had fewer completed intervals than S-H+ ($p = 0.67$) and S-H++ ($p = 0.52$) and more failed intervals than S-H+ ($p =$

0.48) and S-H++ ($p = 0.87$). S+H- rats had fewer completed intervals ($p = 0.55$) and more failed intervals ($p = 0.39$) than S-H- rats. Increases in nose pokes per test and interval, nose poke frequency, and completed intervals as well as decreases in initial and inter-interval latency are all indicative of increased sexual motivation.

Table 2. Sexual motivation metrics presented as average ± standard deviation for each treatment group. Bolded values with * represent conditions that had significantly different values from other conditions.

	S-H-	S+H-	S-H+	S+H+	S-H++
Nose Pokes per Test	30.06 ± 28.95	26.00 ± 24.52	30.69 ± 28.33	35.63 ± 32.75	37.38 ± 39.05
Nose Pokes per Interval	3.73 ± 2.54	**3.20* ± 2.17**	3.88 ± 2.97	3.88 ± 3.38	4.12 ± 3.65
Nose Poke Frequency	1.59 ± 1.75	1.38 ± 1.76	1.64 ± 1.52	2.12 ± 2.02	1.60 ± 1.86
Initial Latency	225.34 ± 418.60	236.81 ± 426.68	320.63 ± 481.90	133.74 ± 120.19	310.23 ± 583.03
Inter-Interval Latency	98.17 ± 217.47	99.93 ± 170.68	118.39 ± 278.92	89.41 ± 157.65	95.38 ± 194.38
Completed Intervals	2.19 ± 2.71	1.81 ± 2.29	3.06 ± 3.55	2.81 ± 3.12	3.25 ± 3.97
Failed Intervals	4.31 ± 3.94	5.25 ± 3.51	3.94 ± 3.70	4.69 ± 3.34	4.50 ± 3.06

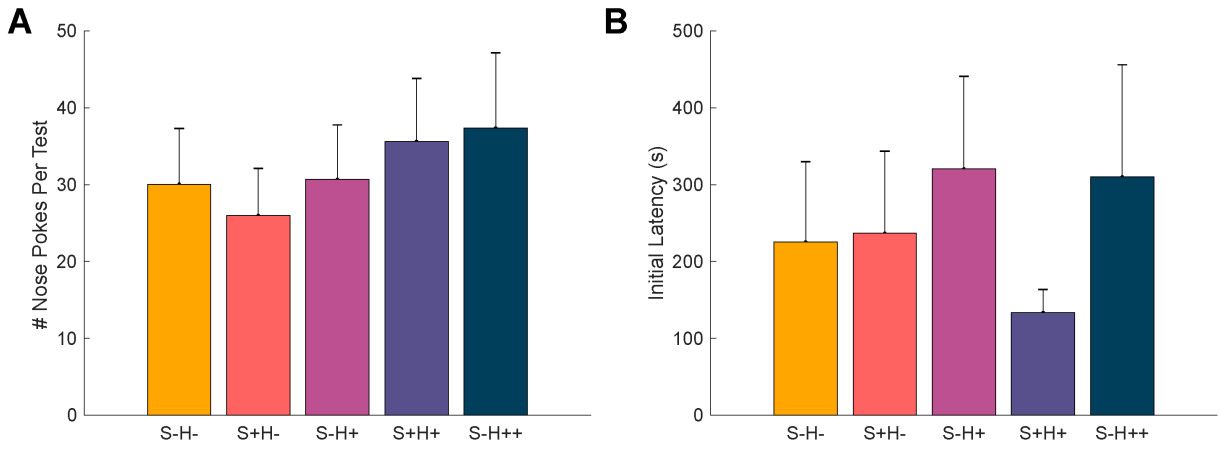

Figure 9. A: Average total nose pokes per test. B: Latency to first nose poke in test session. Error bars represent standard error.

3.4.1.2 Sexual Receptivity

Data for receptivity metrics for Experiment 1 are shown in Table 3 and Figure 10. S+H+ rats had fewer mounts, intromissions and lower LQs than S-H+ rats ($p = 0.91$, $p = 0.95$, $p = 0.84$, respectively) and S-H++ rats ($p = 0.52$, $p = 0.17$, $p = 0.14$, respectively), as shown in Figure 10a. S+H- rats had on average more mounts and intromissions than S-H- rats ($p = 0.80$, $p = 0.57$, respectively). S-H- had significantly fewer mounts than S-H+ ($p = 0.04$), S+H+ ($p = 0.05$) and S-H++ rats ($p = 0.01$). S-H- and S+H- rats both had significantly fewer intromissions than S-H++ rats ($p < 0.01$, $p = 0.02$ respectively). S+H+ rats on average spent the highest percentage of time with males, including more than S-H+ ($p = 0.66$) and S-H++ rats ($p=0.38$), as well as highest percentage of time in the doorway (Figure 10b), including significantly more than S-H++ ($p < 0.01$). S+H+ rats spent the least amount of time alone, significantly less than S-H++ rats ($p = 0.02$). Increases in mounts, intromissions, LQ, time with male, as well as decreases in time alone are all indicative of increased sexual receptivity.

Table 3. Average of each receptivity metric across each treatment group in Experiment 1.

	S-H-	S+H-	S-H+	S+H+	S-H++
Mounts	3.50 ± 4.90	4.50 ± 10.81	13.56 ± 17.75	12.75 ± 14.94	15.94 ± 20.35
Intromissions	1.47 ± 2.75	3.50 ± 9.64	7.91 ± 11.19	7.53 ± 10.52	12.78 ± 16.88
Lordosis Quotient	0.09 ± 0.20	0.09 ± 0.25	0.31 ± 0.34	0.29 ± 0.34	0.45 ± 0.45
% Time with Male	10.43 ± 19.24	10.26 ± 18.80	10.66 ± 15.66	11.96 ± 13.57	7.87 ± 10.61
% Time in Doorway	9.85 ± 11.03	5.87 ± 6.97	10.81 ± 11.34	15.14 ± 14.87	4.00 ± 5.47
% Time Alone	79.73 ± 26.39	83.87 ± 22.38	78.53 ± 24.21	72.89 ± 25.29	88.12 ± 5.47

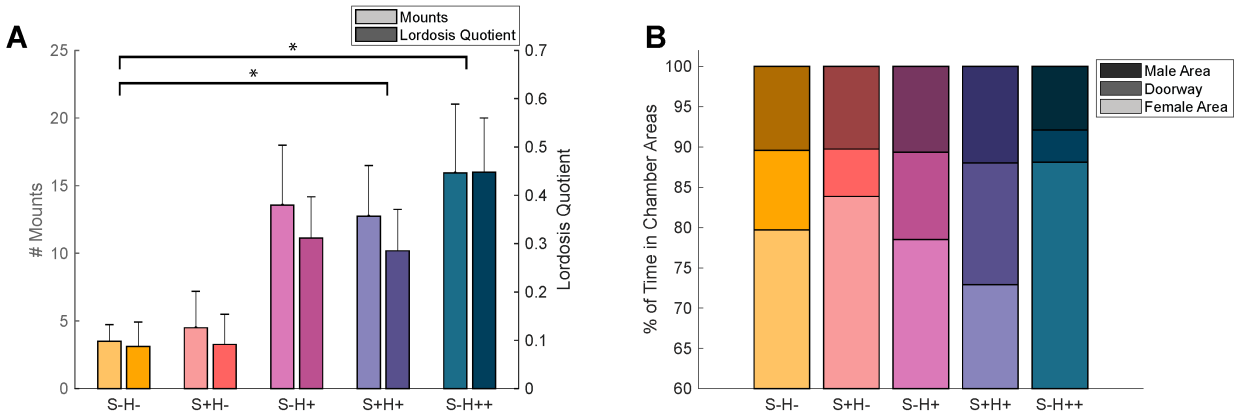

Figure 10. A: Average mounts and lordosis quotient across conditions. Error bars give standard error. Brackets with * represent significant difference between condition groups ($p < 0.05$) for both mounts and LQ. B: Average percentage of time spent in the male area, doorway, and female area across conditions.

3.4.2 Experiment 2

All twenty rats completed each of the six weeks of treatment and testing. The average amplitude of stimulation administered was 3.24 ± 2.46 mA. Stimulation was typically well tolerated. Some rats lifted their leg or attempted to chew the wires in response to stimulation, indicating some level of stimulation perception or discomfort. All data is presented as average ± standard deviation per treatment group per week in the Appendix.

3.4.2.1 Sexual Motivation

There was high variability across metrics. Therefore, trends over time for the condition groups are the focus of our analysis. Key sexual motivation metrics are shown in Figure 11. Linear correlation slope values for Experiment 2 are shown in Table 4. S+H+ rats had the highest slope for nose pokes per interval across testing sessions ($p = 0.27$), a lower slope than S-H+ for nose pokes per test (Figure 11a, $p = 0.93$) and completed intervals (Figure 11c, $p = 0.93$). S+H+ rats had a higher slope for nose poke frequency than S-H+ but lower than S-H++ ($p = 0.31$). S+H+ rats had the most negative slope for initial latency (Figure 11b, $p = 0.54$). Increases in nose pokes per test and interval, nose poke frequency, and completed intervals as well as decreases in initial latency are all indicative of increased sexual motivation.

3.4.2.2 Sexual Receptivity

Key sexual receptivity metrics are shown in Figure 12. Linear correlation slopes are shown in Table 4. S+H+ rats had the highest positive slope for mounts (Figure 12a, $p = 0.52$), intromissions (Figure 12b, $p = 0.17$), lordosis quotient (Figure 12c, $p = 0.16$), and time spent with male (Figure 12e, $p = 0.81$), as well as the most negative slope for time alone (Figure 12d, $p = 0.74$). Increases in mounts, LQ, intromissions, time with male, as well as decreases in time alone are all indicative of increased sexual receptivity.

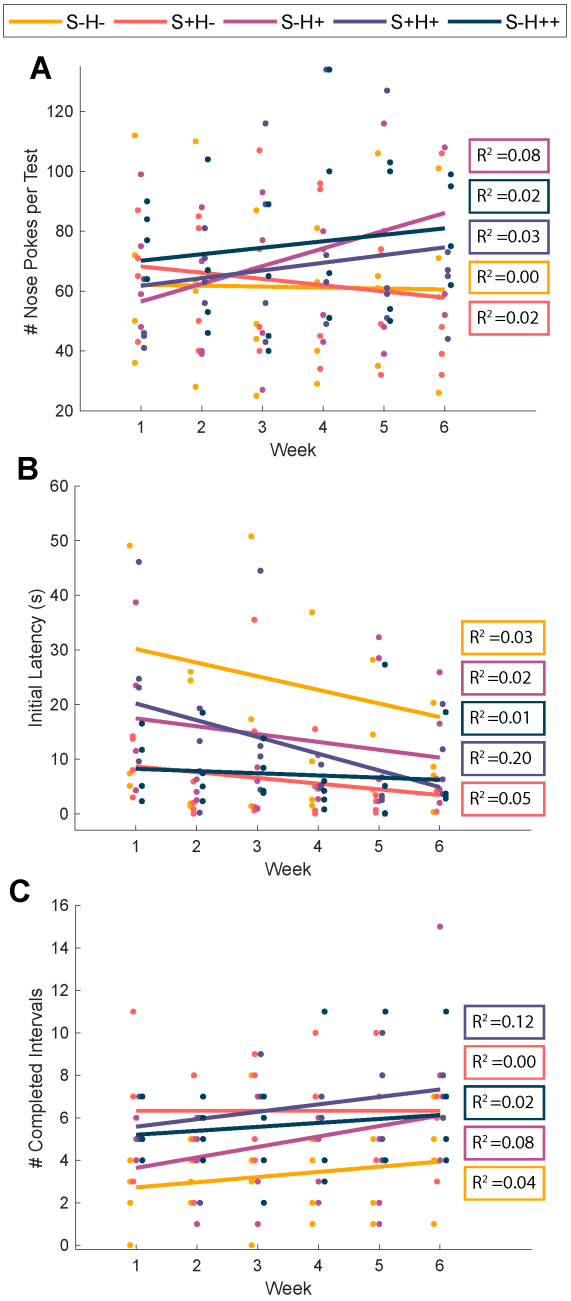

Figure 11. Motivation metrics for Experiment 2 across the 6-week duration. Linear correlation lines across 6 weeks presented with R^2 values. A: Nose pokes per test. B: Latency to first nose poke of test C: Completed intervals.

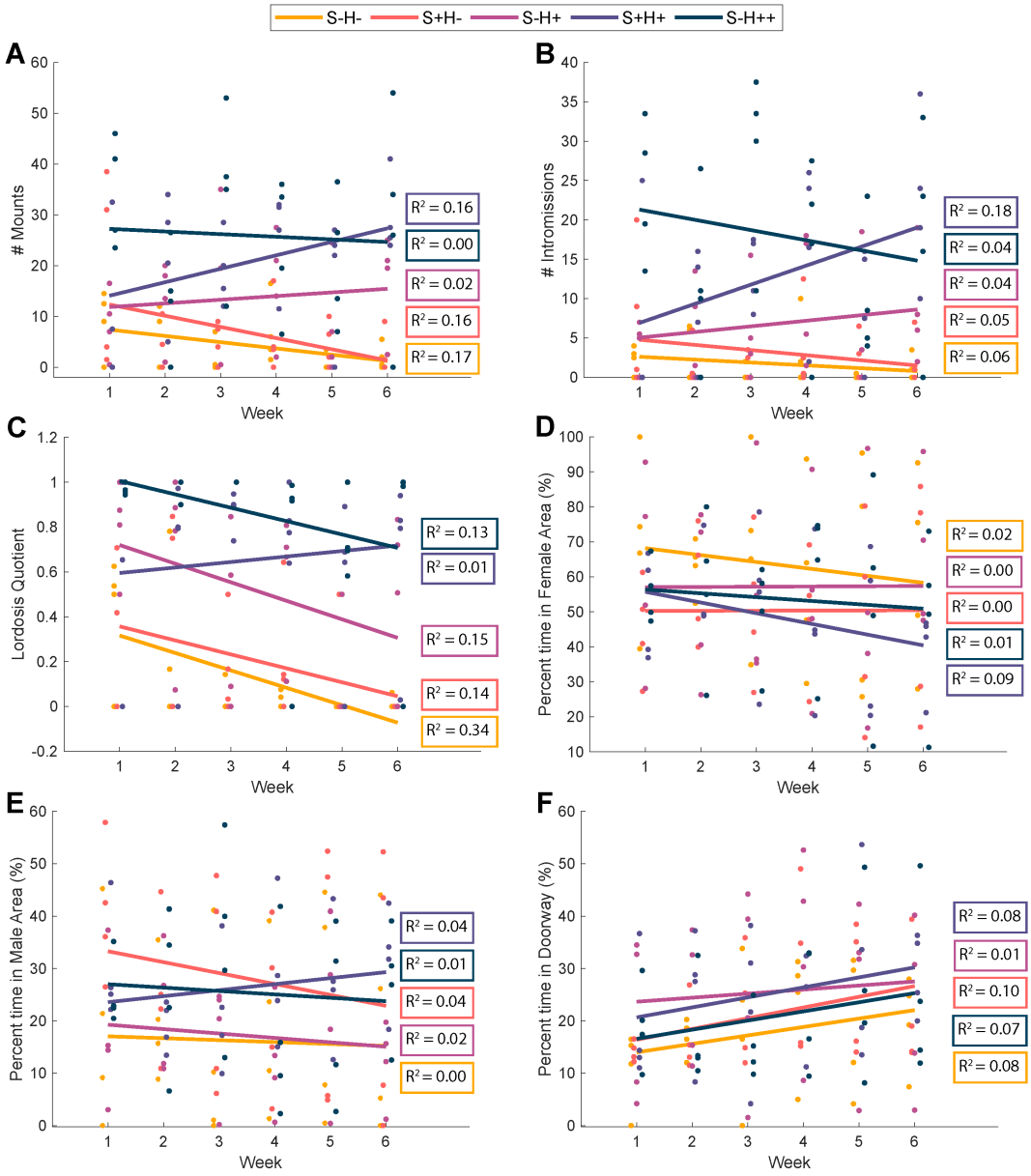

Figure 12. Sexual receptivity metrics for Experiment 2. Linear correlation across 6 weeks. R2 values presented. A. Mounts. B. Total lordosis quotient. C. Intromissions. D. Percentage time alone. E. Percentage of time with male. F. Percentage of time in doorway.

Table 4. Linear correlation slopes for Experiment 2 sexual motivation and receptivity metrics.

	Unit	S-H-	S+H-	S-H+	S+H+	S-H++
Motivation Metrics						
Nose Pokes per Test	Nose pokes	-0.314	-2.086	5.921	2.571	2.164
Nose Pokes per Interval	Nose pokes	-0.282	0.125	0.404	0.467	-0.196
Nose Poke Frequency	Nose pokes/min	0.294	-0.015	0.463	0.661	1.357
Initial Latency	Seconds	-1.232	-2.200	0.718	2.650	-0.518
Inter-Interval Latency	Seconds	-0.884	1.413	-0.581	-1.338	-1.864
Completed Intervals	Intervals	0.243	0.000	0.493	0.350	0.186
Failed Intervals	Intervals	-1.093	-0.921	-0.814	-1.336	-0.579
Receptivity Metrics						
Mounts	Mounts	-1.232	-2.200	0.718	2.650	-0.518
Lordosis Quotient	# Lordosis Expressions / # Mounts	-0.078	-0.062	-0.083	0.024	-0.060
Intromissions	Intromissions	-0.364	-0.654	0.714	2.425	-1.296
% Time with Male	% of 30 minute test	-0.366	-2.084	-0.841	1.148	-0.649
% Time in Doorway	% of 30 minute test	1.611	2.050	0.774	1.911	1.746
% Time Alone	% of 30 minute test	-1.977	0.034	0.067	-3.059	-1.098

3.5 Discussion

This study was the first investigate tibial neuromodulation in a preclinical sexual motivation and sexual behavior paradigm. To our knowledge, this study was also the first study to evaluate the combination of neuromodulation and hormone dosing as a potential sexual dysfunction treatment in either a clinical or preclinical setting. In Experiment 1, we found trends of increasing sexual motivation immediately following tibial nerve stimulation, but not receptivity. In Experiment 2, we found stronger trends of increasing sexual receptivity when tibial nerve stimulation was delivered long-term on a twice weekly basis.

Regarding the potential immediate effects of stimulation on sexual motivation, the trend of S+H+ rats having more nose pokes per test than S-H+ rats (Figure 9a), the highest nose poke

frequency, shortest initial latency (Figure 9b) and shortest inter-interval latency compared to other treatment groups suggests an increase in motivation to reach the male immediately after receiving stimulation. When stimulation was delivered long-term in Experiment 2, S+H+ rats had higher increases in some of the motivational metrics over time (decreased initial latency (Figure 11b), increased nose pokes per interval) but did not outperform on other metrics (nose pokes per test (Figure 11a), completed intervals (Figure 11c)) compared to S-H+ and S-H++ rats. While non-significant, these trends suggest a stronger increase in motivation immediately following stimulation compared to an effect over time with long-term stimulation.

No increases in sexual receptivity were seen immediately after stimulation in Experiment 1. S+H+ rats had a similar number of mounts and lordosis expressions as S-H+ rats. Lordosis has been shown to depend on oestrogens followed by progestins[123], so the lack of immediate impact of tibial stimulation on sexual receptivity is unsurprising. However, strong trends of increasing receptivity were seen when stimulation was applied long term in Experiment 2. S+H+ rats had the highest increases in mounts, lordosis quotient, intromissions, time with male, and decreasing time alone across the five conditions (Figure 12). It is unclear why these changes were seen for repeated stimulation in Experiment 2 but not immediately after single stimulation sessions in Experiment 1. Further studies repeating this paradigm with larger sample sizes or clinical studies comparing on-demand versus long-term tibial nerve stimulation are needed to confirm these results. These experiments suggest that long-term PTNS over several weeks may be a better treatment option for women with FSD than an on-demand stimulation treatment paradigm. If cumulative stimulation is necessary to cause increases in sexually receptive behavior, as seen in Experiment 2, it is possible that rotating the rats though the conditions in Experiment 1 was unpriming the rats for each testing session.

In both Experiments, S+H+ rats spent more time with the male and in the doorway and less time alone. This may suggest an increase in motivation to socialize with the male in response to stimulation.

As the trends described above are only seen in S+H+ and not S+H- rats, our results suggest that tibial nerve electrical stimulation alone does not alter sexual motivation or receptivity. Rather, a combination of stimulation and hormone priming may be an effective treatment for FSD. S+H- exhibited the least sexual motivation and receptivity out of all treatment groups for some metrics (nose pokes per test, nose pokes per interval, completed intervals). These results may indicate that stimulation alone may have an adverse effect on behavior, particularly immediately following stimulation. It is possible that the wire placement and electrical stimulation were disorienting if it was not combined with some hormones to motivate sexual behavior.

There are several possible explanations for these observations. Diminished genital blood flow is a common physiological factor in FSD[4] and is a side effect of ovariectomizing female rats.[116] Tibial nerve stimulation has been shown to increase genital blood flow in rats.[78] Stimulation delivered in sessions repeated across a long-term interval could be continually improving genital blood flow, leading to more positive sexual experiences for female rats. Electrical stimulation may strengthen the spinal circuit for sexual function or may lead to cortical changes. Cortically, the electrical stimulation could be leading to local synthesis of estradiol or activation of estrogen-concentrating regions such as the medial preoptic area, ventromedial hypothalamus, or the medial amygdala that are typically activated during sexual stimulation.[124] The increases in lordosis expressions are more likely to be driven by cortical changes than increases in genital blood flow, as lordosis is a cortically-controlled behavior.

This study had several limitations. In both experiments, there was high variability in the measurements. Some rats were occasionally unresponsive or had difficulty opening the door following the FI15 interval. Rats in these instances would nose poke several times in a clear attempt to open the door, but would miss the 5s latency period after the 15s interval in which a nose poke was required to open the door. When rats consistently failed in this manner, there were no interactions with the male or changes in apparatus zones. This lowered their metrics for sexual receptivity despite showing motivation. This issue could be related to inadequate training and could be remedied by a higher number of training sessions prior to testing. Future studies could use a simpler chamber to reduce variance.[125,126] Statistical significance was not found in many of the treatment groups, due in part to unresponsive sessions. Larger sample sizes may have led to clearer outcomes in the measures.

Another limitation of this study is that verification of consistent stimulation for an entire session was challenging with awake rats, as when the rats were ambulatory the stimulation-driven toe twitches in their hind limbs became imperceptible. The percutaneous wires were loosely anchored and had the possibility of shifting once inserted. It is possible that inconsistent tibial nerve stimulation occurred across the study. As this was the first study to test the effects of long-term tibial nerve stimulation on sexual behavior, there was not precedent for the duration of treatment. It is possible that further benefits would be seen if the stimulation was delivered for longer intervals or more sessions than the 6-week treatment period used here.

The average stimulation amplitude was higher in Experiment 2 than Experiment 1. Rats who received stimulation in Experiment 2 were being stimulated more frequently and more regularly than rats in Experiment 1. This increase in stimulation sessions could have led to an

increased inflammatory response or scar tissue buildup that necessitated a higher amplitude to achieve motor contractions due to higher tissue resistance.

Future research to replicate or extend this study with a higher number of animals could lead to more conclusive results. To ensure more consistent stimulation, chronic nerve cuffs could be surgically implanted on the tibial nerve.[127] Additionally, related studies could examine the effect of tibial nerve stimulation on circulating hormone levels to determine if stimulation is leading to an increase in estradiol or progesterone synthesis. An increase in hormone production in response to nerve stimulation at other locations has been documented[128,129] and could explain the increase in sexually receptive behavior over time that we observed here. The cortical effects of stimulation can also be studied by looking at c-fos activation in the estrogen-containing regions of the brain in response to tibial stimulation.[124]

3.6 Conclusion

In our study we observed that a combination of electrical stimulation of the tibial nerve and hormone priming led to a non-significant immediate effect of increasing sexual motivation but not receptivity. When this combined therapy was applied across weeks, there were increases in sexual motivation and stronger increases in sexual receptivity. These trends suggest that long-term tibial nerve stimulation could be a useful treatment modality for treating female sexual dysfunction, especially in post-menopausal populations receiving hormone replacement therapy.

3.7 Acknowledgements

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The authors thank Katie Yoest, Sara Bender-Bier, and Alex Ramer for their assistance in setting up this study.

3.8 Appendix

Table 5. Motivation metrics from Experiment 2 presented as average ± standard deviation per treatment group per week. %Δ W1:W6 represents the percent change from the average in week 1 to week 6 for that metric per condition.

	Condition	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	%Δ W1:W6
Nose Pokes per Test	S-H-	67.5 ± 33.16	65.25 ± 33.78	51.25 ± 25.98	53.25 ± 23.3	66.75 ± 29.35	64 ± 31.08	-5.2%
	S+H-	66.5 ± 18.21	64 ± 22.38	67.25 ± 30.21	67.25 ± 32.37	56.75 ± 20.02	56.25 ± 33.81	-15.4%
	S-H+	70.25 ± 22.14	59.25 ± 23.96	60.75 ± 29.78	62.25 ± 17.59	70.75 ± 34.92	104.5 ± 67.75	48.8%
	S+H+	49 ± 10.23	67.75 ± 10.75	76 ± 32.95	79.5 ± 37.55	74.5 ± 35.27	62.25 ± 12.63	27.0%
	S-H++	78.75 ± 11.18	67.5 ± 25.85	59.75 ± 22.29	87.75 ± 37.03	76.75 ± 28.65	82.75 ± 17.37	5.1%
Nose Pokes per Interval	S-H-	4.82 ± 3.25	3.84 ± 2.9	3.8 ± 3.12	4.18 ± 4.36	5.68 ± 5.98	5.69 ± 5.08	18.1%
	S+H-	3.86 ± 2.14	3.94 ± 2.19	4.27 ± 2.34	4.56 ± 2.93	4.45 ± 2.04	4.33 ± 2.46	12.2%
	S-H+	3.47 ± 2.07	3.95 ± 3.14	3.63 ± 2.99	3.95 ± 3.32	5.05 ± 5.3	5.57 ± 4.82	60.5%
	S+H+	3.27 ± 1.77	3.43 ± 2.26	4 ± 2.87	5.8 ± 4.07	4.73 ± 3.28	5.3 ± 2.8	62.1%
	S-H++	4.7 ± 3.13	4.03 ± 3.56	3.85 ± 2.44	5.32 ± 4.48	4.8 ± 5.33	5.34 ± 6.4	13.6%
Nose Poke Frequency	S-H-	3.99 ± 3.54	3.54 ± 2.29	2.93 ± 1.83	3.37 ± 1.73	5.71 ± 4.64	4.66 ± 3.4	16.8%
	S+H-	4.87 ± 0.63	3.89 ± 1.54	4.34 ± 1.33	4.67 ± 1.85	4.86 ± 1.95	4.11 ± 1.68	-15.6%
	S-H+	4.7 ± 3.16	4.72 ± 4.12	4.18 ± 2.64	5.42 ± 4.83	8.44 ± 9.72	5.46 ± 3.3	16.2%
	S+H+	3.22 ± 0.66	4.13 ± 1.16	4.95 ± 1.99	7.09 ± 4.35	6.52 ± 2.47	5.98 ± 3.55	85.7%
	S-H++	4.6 ± 1.03	4.35 ± 1.85	4.04 ± 1.01	6.06 ± 4.8	9.79 ± 12.41	10.43 ± 12.25	126.7%
Initial Latency	S-H-	36.35 ± 37.54	13.42 ± 13.61	33.53 ± 29.21	12.65 ± 16.56	36.8 ± 43.31	9.05 ± 8.32	-75.1%
	S+H-	9.73 ± 5.29	2.18 ± 2.62	13.13 ± 16.34	5.28 ± 7.17	1.65 ± 1.47	4.3 ± 2.81	-55.8%
	S-H+	19.5 ± 15.05	22.5 ± 36.5	4.1 ± 3.78	5.73 ± 3.46	17.35 ± 15.24	12.23 ± 11.1	-37.3%
	S+H+	25.88 ± 15.09	10.15 ± 8.13	17.95 ± 18.03	6.98 ± 2.35	3.8 ± 1.96	10.45 ± 7.28	-59.6%
	S-H++	8.9 ± 6.42	8.3 ± 7.11	7.55 ± 4.66	3.4 ± 2.22	8.13 ± 13	7.03 ± 7.73	-21.0%
Inter-Interval Latency	S-H-	82.67 ± 112.99	57.82 ± 94.95	81.36 ± 100.29	75.69 ± 101.68	63.23 ± 114.27	72.33 ± 94.15	-12.5%
	S+H-	46.48 ± 75.25	42.57 ± 78.69	51.71 ± 100.46	47.62 ± 104.73	66.81 ± 127.79	42.49 ± 82.48	-8.6%
	S-H+	39.68 ± 55.99	45.35 ± 91.68	52.14 ± 114.7	44.4 ± 70.02	51.04 ± 68.51	34.29 ± 41.3	-13.6%

	S+H+	56.12 ± 67.79	31.42 ± 55.24	35.64 ± 70.89	53.39 ± 79.6	42.19 ± 74.74	33.43 ± 39.73	-40.4%
	S-H++	51.34 ± 84.79	42.26 ± 51.71	39.31 ± 56.79	42.18 ± 54.52	53.45 ± 102.9	30.84 ± 51.88	-39.9%
Completed Intervals	S-H-	2.25 ± 1.71	3.5 ± 1.29	3.75 ± 3.3	3 ± 1.83	3.25 ± 2.06	4.25 ± 2.5	88.9%
	S+H-	7 ± 3.27	4.75 ± 2.5	6.5 ± 2.38	7.25 ± 2.06	7 ± 3.46	5.5 ± 1.73	-21.4%
	S-H+	5.25 ± 0.96	4 ± 2.16	3.5 ± 2.52	4 ± 1.83	3.75 ± 2.75	8.75 ± 4.57	66.7%
	S+H+	5.5 ± 1	5 ± 2	8 ± 1.15	6.25 ± 0.5	6.75 ± 2.75	7.25 ± 0.96	31.8%
	S-H++	5.75 ± 1.5	5.5 ± 1.29	4.75 ± 2.22	5.5 ± 3.7	5.75 ± 3.5	6.75 ± 3.1	17.4%
Failed Intervals	S-H-	11.5 ± 3.42	12.75 ± 6.99	9.25 ± 2.99	9.5 ± 3.11	8.25 ± 5.74	6.5 ± 3.51	-43.5%
	S+H-	9.75 ± 6.65	10.5 ± 4.2	9 ± 3.56	7 ± 4.24	5 ± 2.16	7 ± 7.53	-28.2%
	S-H+	14.75 ± 3.95	10.25 ± 4.43	12.75 ± 9.6	11.5 ± 6.86	9.5 ± 5.32	9.75 ± 7.54	-33.9%
	S+H+	9 ± 5.29	13.75 ± 6.8	10.75 ± 5.91	6.75 ± 4.35	8.25 ± 6.9	3.75 ± 1.5	-58.3%
	S-H++	11 ± 5.72	10.25 ± 6.4	9.75 ± 7.18	10.5 ± 4.8	9.5 ± 8.96	7.25 ± 6.29	-34.1%

Table 6. Receptivity Metrics from Experiment 2 presented as average ± standard deviation per treatment group per week. %Δ W1:W6 represents the percent change from the average in week 1 to week 6 for that metric per condition.

	Condition	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**	**%Δ W1:W6**
Mounts	S-H-	9 ± 6.42	5.25 ± 6.18	1.88 ± 3.42	6.88 ± 6.68	0.88 ± 1.75	2 ± 2.48	-77.8%
	S+H-	18.75 ± 18.75	3.75 ± 4.97	5.13 ± 4.01	6.13 ± 7.47	4.63 ± 4.5	2.63 ± 4.31	-86.0%
	S-H+	8.63 ± 6.69	13.13 ± 8.53	18.63 ± 14.12	16.13 ± 10.91	8.38 ± 11.15	17 ± 9.94	97.0%
	S+H+	10 ± 15.41	22 ± 12.62	19 ± 7.13	25.5 ± 9.6	18.25 ± 12.34	29.5 ± 7.8	195.0%
	S-H++	34.38 ± 10.83	13.63 ± 10.86	34.38 ± 16.91	23.88 ± 13.67	20.88 ± 13.2	28.5 ± 22.35	-17.1%
Lordosis Quotient	S-H-	0.42 ± 0.28	0.24 ± 0.37	0.04 ± 0.07	0.03 ± 0.04	0 ± 0	0.02 ± 0.03	-95.2%
	S+H-	0.28 ± 0.35	0.4 ± 0.46	0.17 ± 0.23	0.23 ± 0.28	0.13 ± 0.25	0 ± 0	-100.0%
	S-H+	0.8 ± 0.21	0.69 ± 0.42	0.38 ± 0.4	0.57 ± 0.31	0.13 ± 0.25	0.52 ± 0.37	-35.0%
	S+H+	0.41 ± 0.5	0.64 ± 0.44	0.87 ± 0.09	0.81 ± 0.15	0.56 ± 0.39	0.65 ± 0.42	58.5%
	S-H++	0.97 ± 0.02	0.98 ± 0.05	1 ± 0	0.71 ± 0.47	0.75 ± 0.18	0.74 ± 0.5	-23.7%
Intromissions	S-H-	2.38 ± 1.7	3.13 ± 3.61	0.5 ± 1	3 ± 4.76	0.13 ± 0.25	1.13 ± 1.65	-52.5%
	S+H-	7.5 ± 9.26	1.63 ± 2.93	1.5 ± 1.78	3.88 ± 5.85	2.38 ± 3.09	2 ± 3.37	-73.3%

	S-H+	4.38 ± 3.04	6 ± 6.36	5.88 ± 6.74	10.5 ± 8.26	5.5 ± 8.82	8.75 ± 7.27	99.8%
	S+H+	6.25 ± 12.5	9.25 ± 7.27	13.38 ± 4.64	17.13 ± 10.88	9.63 ± 7.45	22.25 ± 10.84	256.0%
	S-H++	23.75 ± 8.96	11.88 ± 10.94	28 ± 11.74	16.63 ± 11.88	10.13 ± 8.8	18 ± 13.88	-24.2%
% Time with Male	S-H-	18.94 ± 19.58	20.09 ± 11.29	13.09 ± 19.24	18.65 ± 16.45	22.65 ± 21.79	18.85 ± 20.22	-0.5%
	S+H-	40.75 ± 13.18	25.73 ± 14.01	26.42 ± 20.94	22.28 ± 16.55	27.61 ± 25.83	25.86 ± 25.85	-36.5%
	S-H+	17.53 ± 14.35	21.45 ± 12.28	17.33 ± 11.58	12.41 ± 10.77	22.14 ± 17.16	12.22 ± 7.95	-30.3%
	S+H+	30.02 ± 11.04	18.99 ± 4.67	21.99 ± 11.97	28.71 ± 13.57	27.35 ± 12.56	31.69 ± 9.98	5.6%
	S-H++	25.26 ± 6.7	26.26 ± 15.23	35.01 ± 18.61	17.39 ± 17.21	21.21 ± 16.91	27.27 ± 11.08	8.0%
% Time in Doorway	S-H-	10.91 ± 7.54	16.86 ± 3.57	18.6 ± 14.28	22.62 ± 11.98	19.38 ± 13.43	19.87 ± 9.05	82.1%
	S+H-	14.18 ± 1.92	16.72 ± 6.96	22.03 ± 10.88	28.73 ± 16.31	25.95 ± 12.63	21.65 ± 12.09	52.7%
	S-H+	19.95 ± 15.91	24.2 ± 12.75	26.45 ± 19.49	33.6 ± 18.97	27.51 ± 17.06	21.95 ± 16.71	10.0%
	S+H+	18.78 ± 12.03	25.49 ± 12.21	23.8 ± 14.72	25.66 ± 10.02	29.89 ± 17.98	29.15 ± 7.79	55.2%
	S-H++	19.23 ± 8.22	17.33 ± 10.2	15.53 ± 6.64	22.97 ± 11.87	25.72 ± 17.34	24.93 ± 17.21	29.6%
% Time Alone	S-H-	70.15 ± 24.91	63.05 ± 7.74	68.31 ± 26.81	58.73 ± 27.22	57.96 ± 35.03	61.28 ± 28.52	-12.6%
	S+H-	45.07 ± 14.47	57.54 ± 16.48	51.56 ± 21.23	48.99 ± 18.68	46.44 ± 29.42	52.48 ± 34.62	16.4%
	S-H+	62.52 ± 28.45	54.35 ± 24.92	56.22 ± 29.41	53.99 ± 28.77	50.35 ± 33.78	65.83 ± 22.53	5.3%
	S+H+	51.2 ± 15.31	55.52 ± 12.82	54.2 ± 22.77	45.63 ± 21.85	42.76 ± 24.62	39.15 ± 12.09	-23.5%
	S-H++	55.51 ± 8.98	56.41 ± 22.67	49.45 ± 15.52	59.64 ± 23.4	53.07 ± 32.29	47.8 ± 26.25	-13.9%

Chapter 4 : Transcutaneous Electrical Nerve Stimulation to Improve Female Sexual Dysfunction Symptoms: A Pilot Study

(Previously published in Neuromodulation: Technology at the Neural Interface, July 2018[112])

4.1 Abstract

Objectives: To perform a pilot study using transcutaneous electrical nerve stimulation (TENS) on the dorsal genital nerve and the posterior tibial nerve for improving symptoms of female sexual dysfunction in women without bladder problems. We hypothesize that this therapy will be effective at improving genital arousal deficits.

Materials and Methods: Nine women with female sexual dysfunction (FSD) completed the study. Subjects received 12 sessions of transcutaneous dorsal genital nerve stimulation (DGNS) (n=6) or posterior tibial nerve stimulation (PTNS) (n=3). Stimulation was delivered for 30 minutes at 20 Hz. Sexual functioning was evaluated with the Female Sexual Functioning Index (FSFI), and surveys were also given on general health, urological functioning, and the Patients' Global Impression of Change (PGIC) after treatment. Surveys were given before treatment (baseline), after 6 and 12 weeks of treatment, and 6 weeks after the completion of stimulation sessions.

Results: The average total FSFI score across all subjects significantly increased from 15.3 ± 4.8 at baseline to 20.3 ± 7.8 after 6 sessions, 21.7 ± 7.5 after 12 sessions, and 21.3 ± 7.1 at study completion. Significant FSFI increases were seen in the sub-domains of lubrication, arousal, and orgasm, each of which is related to genital arousal. Bladder and general health surveys did not change across the study. PGIC had a significant increase.

Conclusions: This study provides evidence that transcutaneous stimulation of peripheral nerves can be a valuable therapeutic tool for women with FSD, specifically those with genital arousal deficiencies.

4.2 Introduction

Female sexual dysfunction (FSD) affects 40-45% of adult women and is a difficult condition to diagnose and treat[1,12]. Low arousal and poor lubrication affects between 8-28% of women and orgasm difficulties affect 16-25% [12,21]. Hormone therapy can be effective, but is not recommended for all subjects and is typically not recommended for long-term treatment [30]. Flibanserin, a recently FDA-approved drug, has some success in increasing sexual desire but does not impact genital arousal [15,130]. Sildenafil has occasionally been reported to improve genital arousal [31], but results are inconsistent and frequently present with mild to moderate side effects [2]. There is a need for an effective treatment for women who have genital arousal deficiencies without concurrent side effects.

Peripheral neuromodulation therapies have been implemented for patients with bladder dysfunction for decades. Sacral neuromodulation (SNM) involves the surgical implantation of a stimulation system, with an electrode near the S3 sacral foramen delivering continuous stimulation [131]. Percutaneous tibial nerve stimulation (PTNS) is a treatment where patients receive 30 minutes of electrical stimulation a week for 12 weeks with periodic maintenance sessions thereafter [63,132], though benefits have been observed after as few as 6 sessions [133]. Stimulation is delivered via a percutaneous needle placed at the tibial nerve near the ankle, but cutaneous stimulation with transcutaneous electrical nerve stimulation (TENS) electrodes have also shown efficacy in some studies [65,66,134]. Dorsal genital nerve stimulation (DGNS) is typically delivered transcutaneously above the clitoris and lateral to the labia majora in women [135–137],

though percutaneous electrodes may also be used [138]. The dorsal genital nerve is a distal branch of the pudendal nerve, which is stimulated centrally with SNM.

In clinical studies in which patients received neuromodulation treatment for bladder dysfunction, significant improvements in sexual functioning as evaluated with the Female Sexual Function Index (FSFI) were noted in both SNM [56,58–60] and PTNS [69–71] therapies. While bladder dysfunction has a known negative effect on sexual function[50,51], improvements in sexual functioning were found to be independent from improvements in bladder functioning [60,71], indicating that the neuromodulation may have a direct impact on genital arousal. No studies have evaluated the effects of peripheral nerve stimulation specifically on patients with FSD, without an underlying urological condition.

The goal of this pilot study was to evaluate weekly skin-surface TENS of the dorsal genital nerve and the posterior tibial nerve for improving sexual function in women with FSD but no clinically-diagnosed bladder problems.

4.3 Methods

Approval for this study was obtained from the Michigan Medicine Institutional Review Board (IRB) prior to initiation (study number HUM00101713). Participants were recruited through Michigan Medicine sexual health practices, gynecology clinics, and an online University of Michigan health research portal (umhealthresearch.org). This study was registered at clinicaltrials.gov under identifier NCT02692417.

In a phone call with a study coordinator, subjects were screened for study eligibility. All subjects were 18 years or older cis-gender women, neurologically stable, and sexually active at least once a month. The short-form Female Sexual Function Index (FSFI-6) was used to screen for FSD, with scores below 19 required for inclusion [139]. Women who were pregnant or planning

pregnancy, had clinically diagnosed bladder dysfunction or pelvic pain, previous pelvic surgery, experience with electrical stimulation for bladder or sexual problems, recent use of TENS on their pelvis, back or legs, had an implanted pacemaker, defibrillator, spinal cord stimulator, or other nerve stimulator, or were taking any investigational drug were excluded from the study. All subjects provided written informed consent. A pregnancy test was also performed at the first session to confirm nongravidity if the subjects were premenopausal and had not had a hysterectomy.

At the first stimulation session, patients were randomized into one of two study groups, DGNS or PTNS. Randomization was accomplished using a random-number table and block size of two. Allocation assignment was performed using sequentially numbered, opaque sealed envelopes, which were opened in the presence of the subjects. Subjects received skin-surface stimulation with a transcutaneous electrical nerve stimulation (TENS) unit (Empi Select, DJO Global, Vista, CA). Electrodes were 1.25-inch round neurostimulation electrodes (ValuTrode Fabric CF3200, Axelgaard Manufacturing Co., Ltd., Fallbrook, CA). For DGNS participants, each electrode was placed on either lateral side of the clitoris [137]. For PTNS participants, electrodes were placed just above the medial malleolus and the ipsilateral calcaneus [66,134]. Stimulation was applied at 20 Hz, as is typical for PTNS [110]. Starting from a low amplitude, current was increased until the participant expressed discomfort or a maximal level of 60 mA was reached, as 60 mA was the maximum amplitude possible for the TENS unit. If the participants perceived stimulation, the current was lowered to a comfortable level. Subsequently, stimulation was applied using that amplitude for 30 minutes, at 20 Hz.

Participants completed a total of twelve stimulation sessions [63,110,131]. Our goal was to schedule sessions on consecutive weeks for the duration of participation. However, scheduling

conflicts, holidays, and other events led to variations in intra-session intervals across subjects. Participants were compensated for their time.

Patients completed a series of validated clinical surveys as outcome measures at baseline, after six stimulation sessions, after twelve stimulation sessions, and six weeks after the final session. At all survey intervals, participants completed the full Female Sexual Function Index (FSFI) [18], the short-form 36-question (SF-36) quality of life survey [140], and the 6-question American Urological Association Symptom Index (AUASI) bladder symptom index [141]. At the 6-week and later survey intervals participants also completed the one-question Patients' Global Impression of Change (PGIC) [142]. All surveys were completed and stored through a secure online portal (REDcap)[143].

Comparisons between FSFI, SF-36, AUASI, and PGIC scores at different time points were analyzed with related-samples Wilcoxon signed rank tests with a significance level of 0.05. Tests were run with DGNS and PTNS arms separately as well as pooled together. Where appropriate, values are presented as mean ± standard deviation.

4.4 Results

Sixteen subjects were enrolled in the study (Figure 13). Seven subjects dropped out of the study during intervention, due to scheduling conflicts (n=6) and an adverse event described below (n=1). Of the 9 subjects that completed the study, the average age was 46.2 ± 14.5, with a minimum age of 23 and maximum of 66 (Table 7). One subject who was enrolled, but did not receive stimulation, did not complete the demographics survey.

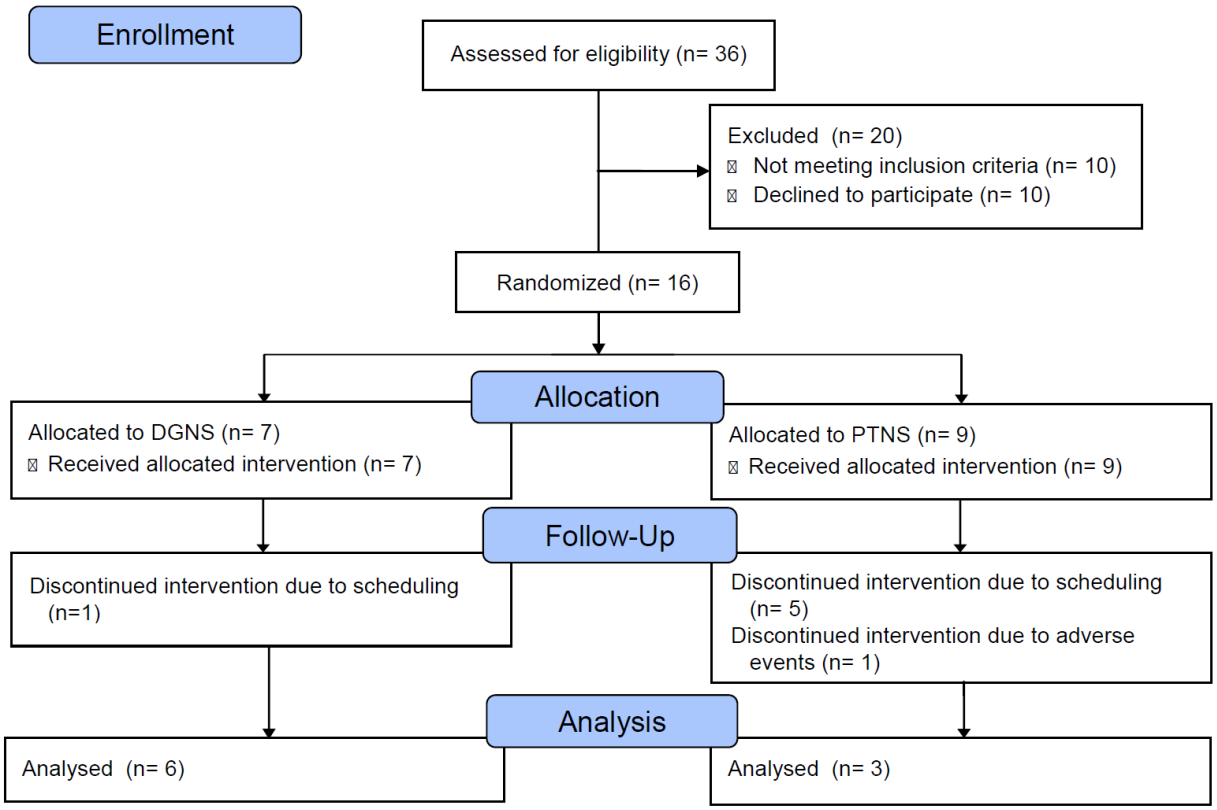

Figure 13. Study CONSORT diagram

Table 7. Patient demographics.

Category	All enrolled participants	PTNS completed	DGNS completed
Total	16	3	6
Age (years)	40.9 ± 15.0	37.3 ± 19.1	50.7 ± 11.0
BMI (kg/m^2)	26.8 ± 4.4	28.4 ± 5.0	26.3 ± 4.9
Race/Ethnicity			
White	10 (67%)	2 (67%)	6 (100%)
Black	1 (7%)	1 (33%)	0 (0%)
Asian or Pacific Islander	1 (7%)	0 (0%)	0 (0%)
Other	3 (20%)	0 (0%)	0 (0%)
Relationship status			
Single	2 (13%)	0 (0%)	1 (17%)
Non-married relationship	3 (20%)	1 (33%)	0 (0%)
Married	10 (67%)	2 (67%)	5 (83%)
On prescription antidepressant	6 (40%)	2 (67%)	3 (50%)
Baseline FSFI	17.1 ± 5.0	15.2 ± 5.3	15.5 ± 4.6
Baseline SF-36	83.1 ± 11.6	87.9 ± 4.4	80.5 ± 9.0
Baseline AUASI	6.4 ± 5.3	9.3 ± 6.4	4.5 ± 2.9

Due to varying scheduling conflicts, stimulation was not always delivered in exact 1-week intervals. The average days between sessions was 12.5 ± 10.3 days. The stimulation current amplitude that was delivered ranged from 2.5 mA to 60.0 mA. Stimulation was delivered at an average of 36.2 ± 22.7 mA for all subjects, 24.3 ± 18.6 mA for DGNS subjects, and 60.0 ± 0.0 mA for PTNS subjects.

All women began the study with an FSFI total score below the clinical cut-off for diagnosing FSD (26.55) [19], with an average initial score of 15.3 ± 4.8. Overall sexual function,

and arousal and orgasm FSFI sub-scores all showed significant improvement at 6 weeks, 12 weeks, and 18 weeks from baseline (Figure 14, Figure 15, Table 8). The lubrication sub-scores had a significant improvement at 12 weeks over baseline (Figure 15). Three of the 9 subjects (33.3%) reached an FSFI score above the clinical cut-off for FSD, and another participant scored just below the threshold (26.4). Four subjects (1 DGNS, 3 PTNS) increased their FSFI score by at least 50%.

Table 8. Average FSFI scores across all subjects as well as across two subject groups, with standard deviation in (). Significance as comparison between study time point and baseline scores with $p < 0.05$ indicated in bold with *.

	Desire	Arousal	Lubrication	Orgasm	Satisfaction	Pain	Total
All (n=9)							
Baseline	2.3 (1.0)	2.2 (0.9)	2.8 (1.5)	2.7 (1.7)	2.7 (1.1)	2.6 (2.1)	15.3 (4.8)
6 weeks	2.7 (1.3)	**3.3 (1.3)***	3.6 (2.1)	**3.8 (1.7)***	3.2 (1.9)	3.6 (2.5)	**20.3 (7.8)***
12 weeks	3.0 (1.2)	**3.6 (1.4)***	**3.7 (1.5)***	**3.78 (1.5)***	3.8 (1.7)	3.8 (2.6)	**21.7 (7.5)***
18 weeks	2.7 (1.3)	**4.0 (1.4)***	3.8 (1.5)	**4.4 (1.6)***	3.6 (2.0)	2.8 (2.7)	**21.3 (7.1)***
DGNS (n=6)							
Baseline	2.3 (1.0)	2.3 (1.0)	2.9 (1.6)	2.7 (2.0)	2.6 (1.4)	2.4 (2.5)	15.2 (5.3)
6 weeks	2.0 (1.0)	2.8 (1.0)	3.3 (2.1)	3.8 (1.6)	2.5 (1.9)	3.0 (2.9)	17.4 (6.6)
12 weeks	2.5 (1.2)	3.0 (1.1)	**3.6 (1.4)***	3.5 (1.6)	3.1 (1.7)	3.0 (2.9)	**18.7 (6.9)***
18 weeks	2.2 (0.8)	**3.7 (1.4)***	4.1 (1.2)	4.1 (1.7)	2.8 (1.8)	2.1 (2.8)	**18.8 (5.4)***
PTNS (n=3)							
Baseline	2.4 (1.2)	2.1 (0.9)	2.5 (1.5)	2.5 (1.2)	2.9 (0.2)	3.1 (1.3)	15.5 (4.6)
6 weeks	4.0 (0.7	4.4 (1.5)	4.4 (2.3)	3.9 (2.3)	4.7 (1.0)	4.7 (1.4)	26.0 (7.8)
12 weeks	4.0 (0.3)	4.9 (0.9)	4.1 (2.0)	4.3 (1.3)	5.2 (0.0)	5.3 (0.6)	27.8 (4.8)
18 weeks	3.8 (1.5)	4.6 (1.5)	3.4 (2.3)	4.9 (1.5)	5.3 (1.2)	4.1 (2.2)	26.2 (8.6)

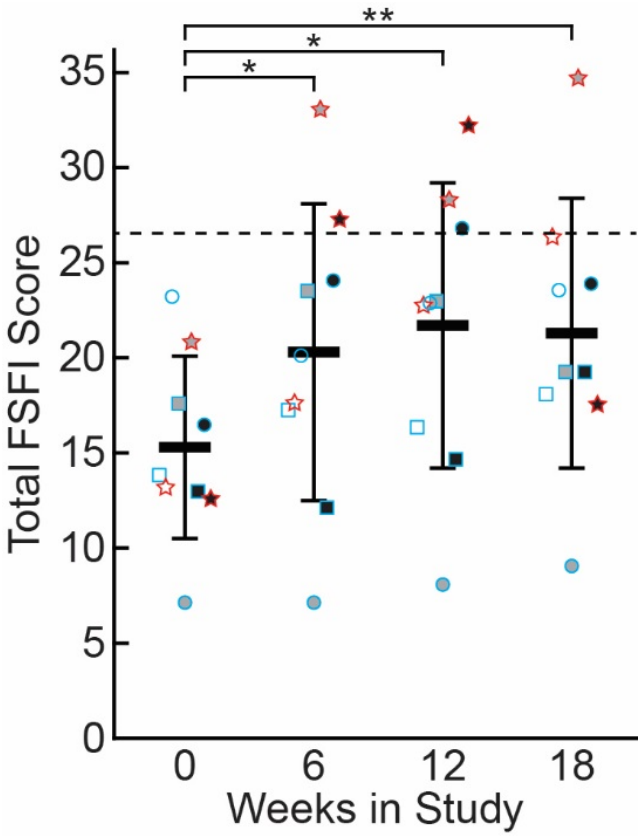

Figure 14. Average total FSFI score for all subjects (PTNS and DGNS) at each survey time point. Error bars give standard deviations. Significant improvement from baseline occurred at each time point. Individual icons are unique for each participant, with PTNS patients indicated with stars. Within each study week, icon order from left to right indicates study participation order. (*, $p < 0.05$; **, $p < 0.01$)

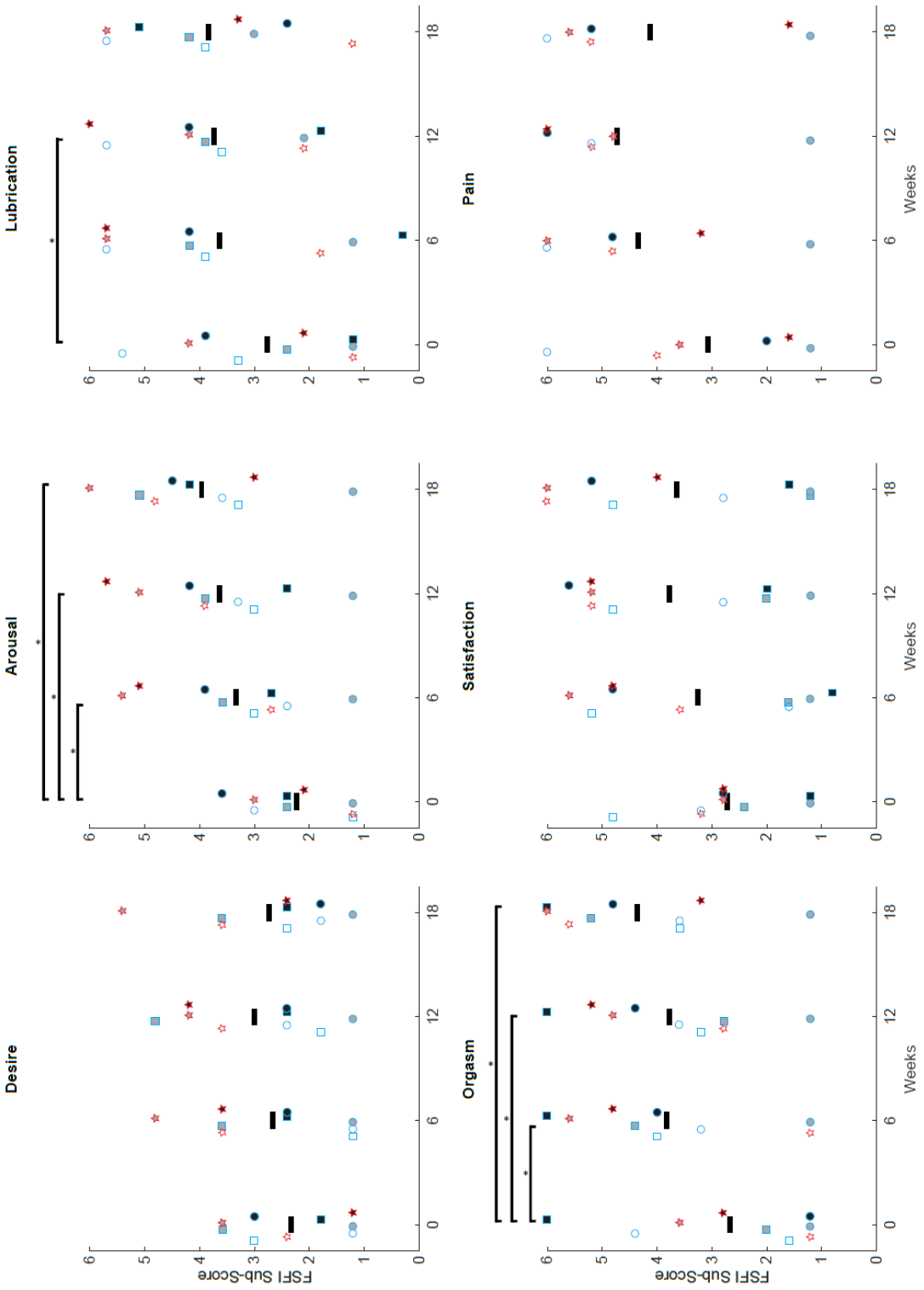

Figure 15. Individual FSFI sub-domain scores for all subjects. The pooled mean is given by the horizontal bar. Individual icons are unique for each participant, with PTNS patients indicated with stars. Within each study week, icon order from left to right indicates study participation order. (*, $p < 0.05$)

Changes in FSFI scores were not related to variations in the intervals between stimulation sessions. The FSFI percent increase had no relationship with average stimulation session intervals at 12 weeks ($y=-0.077x + 40.58$, $R^2= 0.0139$, $p=0.78$) or at 18 weeks ($y=--0.1309x + 57.97$, $R^2= 0.026$, $p=0.70$).

Overall, participants perceived an improvement in sexual function, as PGIC scores were 3.3 ± 2.0 at 6 weeks (3.0 = "a little better"), 4.0 ± 1.9 at 12 weeks (4.0 = "somewhat better"), and 4.1 ± 1.9 at 18 weeks. These scores are each significantly different from a PGIC score of 1.0 ($p<0.05$), which would indicate "no change or worse". The three women (2 PTNS, 1 DGNS) who achieved FSFI scores above the FSD clinical cutoff scored either a 5 ("moderately better") or 6 ("better") at each time point. Overall quality of health scores from the SF-36 remained generally stable across the study duration. The SF-36 category of role limitations due to physical health improved from 88.9% pre-treatment to 97.2% at the 18 week time point across all subjects. Also the SF-36 category emotional well-being showed a significant worsening from 80.0% pre-treatment to 74.2% at week 6 ($p=0.042$) for DGNS subjects. Participant's bladder functioning, as scored by the AUASI, did not show significant change across all subjects across the study time points, except for the domain of nocturia. There was a significant 25.0% reduction in nocturia symptoms ($p=0.046$) from baseline (1.78 ± 1.30) to the 18 week time-point (1.33 ± 1.22).

Subjects were given the opportunity to refrain from answering questions. The unanswered questions were scored as a 0, which affected FSFI scoring. Three DGNS subjects (blue/white square, blue/grey square, blue/black square in Figs. 11 & 12) refrained from answering questions about pain. Two of those subjects (blue/grey square, blue/black square) also refrained from answering questions about satisfaction. One of those subjects (blue/black square)

also refrained from answering questions about lubrication. One subject (red/black star) reported that between week 12 and week 18 surveys, she was diagnosed with a severe pelvic infection from E. coli. She indicated that this unrelated event would negatively impact her 18 week survey, as seen by declines in her scores from week 12 to week 18, particularly in pain (Fig. 12).

One participant receiving PTNS withdrew from the study after 3 sessions after feeling sciatic nerve pain during stimulation. The subject had a history of sciatic pain. Aggravation reemerged after both lowering the amplitude of current delivered and switching the stimulation location to the alternate leg.

4.5 Discussion

In this study we demonstrate the feasibility of transcutaneous stimulation as a treatment for genital arousal disorders in women. Significant improvements were achieved in the areas of arousal, lubrication, and orgasm (Fig. 12), leading to overall better sexual functioning (Fig. 11). These domains are each related to genital arousal. Subjects reported the highest sexual functioning at 12 weeks into the study, indicating that maintenance sessions may be beneficial, as is typical for bladder patients. The subjects in the PTNS arm had a greater improvement in sexual functioning (Table 8), but the imbalance of subjects in each arm makes it difficult to perform any statistical comparisons. Two PTNS subjects commented that they planned to purchase their own TENS equipment to continue treatment at home after study completion.

Across all subjects the average total FSFI score increased by 6.4. This is comparable to the BEGONIA trial investigating flibanserin for hypoactive sexual desire, in which the placebo group total FSFI score improved by 3.5 and the flibanserin treatment group improved by 5.3 [15]. Our results are also comparable to a study investigating bremelanotide (BMT) for female sexual dysfunctions, in which the placebo group total FSFI score increased by 1.9 and the most

effective BMT dose group increased by 4.4.[144] The improvements in sexual functioning are also on a similar scale to a clinical studies of neuromodulation, including one of PTNS in OAB patients, where total FSFI score increased by 6.5 after therapy [71], as well as one sacral neuromodulation study, where total FSFI increased by 4.3 [57].

These results provide further evidence that the improvements to sexual functioning seen in neuromodulation studies for bladder dysfunction are a direct result of the therapy, as opposed to a secondary result from treated bladder symptoms. Peripheral nerve stimulation could be used as a clinical tool to treat women with genital arousal deficiencies. A potential mechanism is an improvement or increase in pelvic blood flow, as has been modeled by preclinical studies investigating similar stimulation techniques [78,98], however more research is needed.

An important limitation in this study is the lack of a control. As the results are based on patient-reported outcomes, the impact of a placebo effect could be considerable. Neither the researchers nor the subjects were blinded. There were challenges in recruitment for the study, but more notably in retention. Two primary factors were a need for weekly stimulation sessions during normal business hours and the location of the clinical research center, which required a car or bus to reach. Six of the 7 subjects who discontinued the study were in the PTNS arm, leading to an unequal distribution of subjects. Once enrolled, it was also difficult to schedule subjects every week, so most did not complete the study in the expected 18 weeks. This was due to both patient scheduling conflicts as well as clinician availability. Although the stimulation session intervals often differed from standard PTNS clinical practice for bladder symptoms, no effect on our results was observed. Finally, skin-surface transcutaneous stimulation was utilized, and though it has been shown to be effective clinically [107,137,145], it is less specific than percutaneous needles.

Future studies with sham or placebo controls, as have been completed for bladder care, are necessary to confirm the efficacy of this treatment modality[63]. In addition, percutaneous stimulation could be used for more accurate recruitment of target nerves.

4.6 Conclusion

This pilot study demonstrates the feasibility of using transcutaneous neuromodulation of peripheral nerves to treat symptoms of female sexual dysfunction. Improvements were primarily seen in genital arousal components of sexual functioning, including lubrication, arousal, and orgasm. This study provides further evidence that improvements seen in the sexual functioning of women receiving neuromodulation treatment for bladder dysfunction were independent of improvements in bladder symptoms, and that stimulation has a direct impact on sexual arousal.

4.7 Acknowledgments

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Chapter 5 : Discussion

The research presented in this thesis is among the first studies to directly investigate the use of peripheral nerve stimulation as a treatment for female sexual dysfunction. These studies in this thesis looked at how tibial nerve stimulation increased vaginal blood perfusion and sexual behavior in female rats, as well as how overall sexual functioning improved in women with FSD receiving weekly transcutaneous stimulation of either the tibial or dorsal genital nerve. There is a substantial clinical need to address FSD, as there are limited treatment options available. Establishing an effective treatment for FSD could have a positive impact on millions of women.

In Chapter 2, I presented the first study to ever investigate the effect of tibial nerve stimulation on genital arousal in rats. In this study, I stimulated the tibial nerve in anesthetized rats and measured their vaginal blood perfusion as a proxy for genital arousal. To quantify this response, we established a new method for evaluating laser Doppler flowmetry signals. This method involved using wavelet analysis to isolate the neurogenic oscillations of signals and setting a threshold of a 500% increase from baseline. Vaginal blood perfusion crossed this threshold most often between 20-35 minutes after the start of stimulation, indicating that long durations of stimulations are needed to increase genital blood flow. There was also an increase in the vaginal luminal diameter in rats receiving tibial nerve stimulation.

This observed increase in vaginal blood perfusion in response to tibial nerve stimulation provides an explanation for a potential mechanism in how peripheral neuromodulation could be improving sexual dysfunction in women receiving PTNS.[111] Low genital blood flow is linked to

several issues in the female sexual response.[4] The increase in genital blood flow from tibial nerve stimulation is likely improving the female sexual response through increasing vulvar engorgement, vaginal lubrication, and genital sensitivity.

The results of this study are similar to our other study in anesthetized rats which showed that pudendal nerve stimulation using similar methodology for at least fifteen minutes led to significant increases in vaginal blood perfusion (Appendix A).[98] In that study, peaks in maximal vaginal laser Doppler flowmetry responses also occurred near 30 minutes after stimulation start, suggesting that similar spinal circuits and blood flow dynamics may be activated in each approach. Previous studies have shown transient increases in vaginal blood flow in response to pelvic nerve stimulation.[38] The pelvic nerve contains motor neurons that directly innervate the genitalia, so the increase in blood flow is likely a motor response. This would potentially make pelvic nerve stimulation an on-demand treatment to increase the genital arousal response before sexual activity. However, the pelvic nerve can only be stimulated with an implanted stimulator as there is no superficial access point for transcutaneous or percutaneous stimulation, presenting a greater challenge for clinical treatment.

There are many possible avenues to explore the genital arousal response to tibial nerve stimulation further. In the research presented in Chapter 2 and Appendix A, there were no comparisons to animals under similar conditions that did not receive stimulation. It is unclear how vaginal blood perfusions changes over long periods of time while under anesthesia. The animals used were also healthy with normal sexual functioning. Future studies could involve ovariectomized female rats to determine how hormone cycling or the lack thereof can impact genital arousal responses. These ovariectomized rats could also be hormone primed to control for estrus states. Neural mechanisms could also be investigated further by repeating a similar

experiment in rats with complete spinal cord injuries. In this model, all cortical processing and influences would be removed, leaving only the spinal reflex circuit to enact changes to genital blood flow. An alternative to tracking neurological mechanisms would be to place nerve cuffs on the nerves that facilitate the sexual response and record their electrophysiological changes in response to stimulation.

There are limitations to using laser Doppler flowmetry to evaluate the genital arousal response in these types of studies. LDF recordings are highly susceptible to artifacts from various sources, such as motor twitches, breathing and bladder contractions.[38] While these physiological rhythms did not affect our results, it is important to note that they have the potential for large artifacts on the raw signal. There is evidence that alternative blood flow recording devices, such as laser speckle imaging (Appendix B) or laser Doppler imaging, would more accurately quantify genital blood flow.[146]

In Chapter 3, I evaluated the impact of both short-term and long-term tibial nerve stimulation has on the sexual behavior of female rats. I discovered trends of increasing sexual motivation in rats immediately following tibial nerve stimulation when combined with hormone priming. When stimulation was applied long-term with hormone priming, I found some increases in sexual motivation over time, but also larger increases in sexual receptivity. These results, while not significant, suggest that tibial nerve stimulation may have greater impact on sexual functioning when applied as a continuous treatment instead of as an on-demand remedy. These increases in sexual behavior were not seen in rats that received tibial nerve stimulation but no hormone priming.

While there are no comparable studies looking at the effect of nerve stimulation on sexual behavior, comparisons can be made to studies looking at how tactile stimulation of the genitalia

alters sexual behavior. Vaginocervical stimulation of female rats has been shown to facilitate lordosis and pacing behaviors.[125] If tibial nerve stimulation is increasing vaginal blood flow as demonstrated in Chapter 2, it is possible that the vulvar engorgement can cause a similar effect as the vaginocervical stimulation.

The sample sizes in this study may have contributed to the lack of statistical significance found. Repeated studies of a similar nature with more animals may be beneficial. One hindering factor of this study was the difficulty of the operant-controlled chamber. Rats would frequently attempt to open the sliding door but would fail due to the timing of the FI15 intervals. A simpler chamber could be used that allowed for female pacing through barriers that only females can pass through, without the need for nose poking-triggered FI15 intervals.[125] This setup would remove sexual motivation data quantified through nose poking, but may lead to less variability among experiments. In addition, future studies could examine the cortical effects of stimulation by looking at c-fos activation in the estrogen-containing regions of the brain in response to tibial nerve stimulation.[124]

In Chapter 4, I reported on the results from the first-ever clinical study to evaluate transcutaneous nerve stimulation as a treatment for FSD without concurrent bladder dysfunction. In this study, women were either delivered dorsal genital nerve or tibial nerve stimulation for 30 minutes a week for 12 weeks. Their sexual functioning was tracked using the FSFI survey given at the 0, 6, 12, and 18-week time points in the study. In my analysis, I found that there was significant improvement in women's overall sexual functioning at 6, 12, and 18 weeks compared to their initial baseline measurements. These improvements were primarily seen in the domains of arousal, lubrication, and orgasm. These three subdomains of sexual arousal each pertain to genital arousal.

The results of the clinical study align with the results of Chapter 2 and Chapter 3. An improvement in genital blood flow, as proposed in Chapter 2, would likely lead to the improvements in genital arousal seen in the clinical study. In addition, Chapter 3 demonstrated that long-term stimulation is more effective at improving sexual functioning than on-demand stimulation. In the clinical study, the improvements were seen most strongly after 12 weeks of stimulation.

While this clinical study was not placebo or sham controlled, we can compare our results to other placebo controlled studies on treatments for FSD. In a review of 8 FSD treatment studies using the FSFI to evaluate improvements in sexual functioning, an average increase in total FSFI was found to be 5.35 for treatment groups and 3.62 for placebo groups.[147] In our pilot study, the average increase in total FSFI score was 6.4 at the 12-week time point, higher than both the average placebo and average treatment group.

Future clinical studies to confirm these results should have a placebo control. In addition, it would be interesting to link the experiments in Chapter 2 and Appendix A to the clinical trial by tracking women's vaginal blood flow in response to transcutaneous stimulation both during stimulation as well as tracking changes over time. This would typically be done using vaginal photoplethysmography.[148] This would add a stronger physiological measurement to the success of the treatment as opposed to patient-reported outcomes via the FSFI.

This thesis looks at the effect of peripheral neuromodulation as a treatment for FSD through a variety of perspectives. Each of these perspectives point to a similar conclusion: there is great potential for this treatment avenue. Chapter 2 demonstrated that improvements may come from increases in genital blood flow. Chapter 3 suggested that the treatment may be more effective when applied long-term, as opposed to an on-demand treatment. Chapter 4 showed that

when neuromodulation applied clinically, women suffering from FSD can have significant improvements in sexual functioning, primarily genital arousal.

Each of these studies has been the first or among the first of its kind, and there are many different directions that this research can be taken. There is still much to be done in terms of determining the optimal methodology in applying the peripheral nerve stimulation. There are very few treatment options currently available for women with FSD, and most of the approved pharmaceutical agents are intended to treat low libido. There is a significant clinical need for treatments that can address genital arousal dysfunction in women as well. A combination treatment of neuromodulation with pharmaceuticals or hormone therapies may be a very effective option.

These results provide further evidence for adopting neuromodulation as a clinical therapy for women with FSD. The most likely method to first be adopted would be the standard PTNS protocol that is used clinically for bladder dysfunction. There are several implanted tibial nerve stimulation devices currently being investigated, which require simple procedures to implant and could lead to better patient outcomes and higher retention than weekly PTNS treatment.[149,150] These new devices could be adopted for patients with FSD as well. In the future, wearable neuromodulation devices using transcutaneous stimulation would present a minimally-invasive, low-cost solution to bring benefits in sexual functioning to patients.

I believe that this technology has the potential to relieve the sexual distress of millions of women worldwide, and I hope that my research has pushed us closer to that goal.

Appendices

Appendix A: Time-Frequency Analysis of Increases in Vaginal Blood Perfusion Elicited by Long-Duration Pudendal Neuromodulation in Anesthetized Rats

(Previously published in Neuromodulation: Technology at the Neural Interface, September 2017[98]. Manuscript led by Indie Rice, I was second author and significant contributor.)

A.1 Abstract

Objectives: Female sexual dysfunction (FSD) affects a significant portion of the population. Although treatment options for FSD are limited, neuromodulation for bladder dysfunction has improved sexual function in some women. A few studies have investigated peripheral neuromodulation for eliciting changes in vaginal blood flow, as a proxy for modulating genital sexual arousal, however results are generally transient. Our central hypothesis is that repeated or extended-duration pudendal nerve stimulation can elicit maintained vaginal blood flow increases.

Materials and Methods: Under ketamine anesthesia, the pudendal nerve of 14 female rats was stimulated at varying frequencies (1-100 Hz) and durations (0.15-60 minutes). Vaginal blood perfusion was measured with a laser Doppler flowmetry probe. Changes in blood perfusion were determined through raw signal analysis and increases in the energy of neurogenic (0.076-0.200 Hz) and myogenic (0.200-0.740 Hz) frequency bands through wavelet analysis. Additionally, a convolution model was developed for a carry-over stimulation effect.

Results: Each experiment had significant increases in vaginal blood perfusion due to pudendal nerve stimulation. In addition, there were large concurrent increases in neurogenic and myogenic

frequency-band energy in 11/14 experiments, with an average maximal response at 31.3 minutes after stimulation initiation. An effective stimulation model with a 30-minute carry-over effect had a stronger correlation to blood perfusion than the stimulation period itself.
Conclusions: Repeated or extended-duration pudendal nerve stimulation can elicit maintained increases in vaginal blood perfusion. This work indicates the potential for pudendal neuromodulation as a method for increasing genital arousal as a potential treatment for FSD.

A.2 Introduction

Female sexual dysfunction (FSD) is a widespread medical problem that may affect up to 45% of women [21,79]. FSD has several subtypes, which may be concurrent [16]. Female orgasmic disorder is characterized by rare or absent orgasm (10-42% prevalence). Female sexual interest or arousal disorder (FSIAD) is characterized by significantly reduced psychological sexual interest, lack of physical arousal, pleasure, or both. Genito-pelvic pain or penetration disorder is characterized by vulvovaginal or pelvic pain due to intercourse or penetration (15% prevalence). In contrast to male sexual arousal, the physical and psychological aspects of female sexual arousal are highly dependent on one another. Flibanserin is a recent FDA-approved drug that exclusively treats FSIAD [151]. Currently, there are no effective treatment options specifically targeting the genital, rather than psychological, component of female sexual arousal [80]. In part, this is due to the challenge of studying the mechanisms of the physiological components of female arousal, an area of research that is still developing [152]. A treatment for genital arousal dysfunction may also affect other components of sexual dysfunction, and thus would have wide applicability across FSD subtypes.

Sildenafil, FDA-approved for male erectile dysfunction, has been shown to improve FSD symptoms in some women with sexual arousal disorder, including improvements in sensation,

lubrication, and orgasm [33]. Studies with sildenafil in women have correlated increases in clitoral and vaginal blood flow to improvements in sexual function [31,153]. However, sildenafil administered to women has a high likelihood of moderate adverse events, most commonly headaches, [33] and has had unclear clinical benefits due to conflicting reports of efficacy [32,84]. An optimal treatment would result in consistent improvements in sexual function while minimizing adverse events.

Preliminary studies in women have indicated a potential for spinal and peripheral neural stimulation to improve sexual functioning. The pudendal nerve innervates the pelvic region, including the vagina, labia, and clitoris [154], and plays an important role in physiological sexual arousal [99]. Sacral neuromodulation (SNM), in which stimulation is applied to spinal roots that include the pudendal nerve, has gained acceptance as a treatment option for bladder or bowel dysfunction [155]. A few studies have shown that some women treated with SNM for overactive bladder also have an improvement in sexual function [60,61,156], which were not correlated with improvements in bladder function [156]. While these studies indicate the promising potential of sexual neuromodulation, there has not been a thorough analysis of the mechanisms and effects of stimulation.

Vaginal blood perfusion can be used as a proxy for physiological sexual arousal as increased vaginal blood flow is associated with genital arousal [40,41,89]. Laser Doppler flowmetry (LDF) is a non-invasive method for measuring microvascular blood perfusion, and has been used to assess vaginal blood perfusion in rodent studies [38,40,41,77,88,89]. Raw LDF measurements have several limitations, including non-absolute values and a high sensitivity to motion artifacts. A time-frequency analysis method has been developed for various uses of LDF, that aims to mitigate these limitations [95,96]. Microvascular perfusion exhibits oscillations in activity based on

underlying metabolic, neurogenic, and myogenic activity, as well as oscillations associated with respiration and heart rate [95,96,157]. The neurogenic oscillatory range of LDF is associated with sympathetically-driven changes in microvascular perfusion [91]. Several studies have indicated that increased sympathetic drive leads to increased genital arousal in women [158–161]. Since the pudendal nerve carries sensory inputs to autonomic spinal circuits associated with arousal, we expect that pudendal nerve stimulation will have a similar effect as direct sympathetic stimulation of arousal circuits. Thus, we hypothesized that there would be increases in the neurogenic range of intravaginal LDF measurements due to pudendal nerve stimulation. In addition, the myogenic frequency range, associated with increased vascular diameter due to a rise in blood pressure, may also increase as the vaginal tissue becomes engorged.

This study specifically examines the potential for neuromodulation via pudendal nerve stimulation to increase vaginal blood perfusion, a preliminary step in the development of a neuromodulation treatment for FSD. While some rat studies have shown that peripheral nerve stimulation, including short-duration pudendal nerve stimulation, can cause transient changes in vaginal blood flow [38,40,77,88], none have shown a sustained arousal response lasting minutes or longer. In this study with anesthetized rats we used raw LDF and time-frequency analyses to assess vaginal blood perfusion changes induced by up to 30 minutes of stimulation, and developed a simple stimulation-carryover model.

A.3 Materials and Methods

All procedures were approved by the University of Michigan Institutional Animal Care and Use Committee, in accordance with the National Institutes of Health's guidelines for the care and use of laboratory animals. Female, mature, nulliparous Sprague-Dawley rats (n = 18; animals 1-4: Envigo, Haslett, MI, USA; animals 5-18: Charles River Breeding Labs, Wilmington, MA,

USA) weighing between 210-330 g (261.1 ± 33.4 g) were used. Anesthesia was induced prior to surgery by isoflurane (4-5%) followed by a ketamine/xylazine/acepromazine (90 mg/kg, 7.5 mg/kg, 1.5 mg/kg respectively) intraperitoneal cocktail. Since the rat estrous stage is known to have a significant effect on rat sexual receptivity [94], a vaginal lavage was performed after induction of anesthesia prior to surgery in order to determine the estrous stage. Anesthesia during surgery and experimentation was maintained with ketamine (30 mg/kg every 30 minutes; intraperitoneal), as it has been used in related studies examining sexual arousal-related responses [38,92,93]. Four experiments did not result in data collection due to surgery or electrode failure, resulting in 14 animals for data collection.

The left pudendal nerve was exposed via a posterior approach. Platinum-wire hook electrodes were secured to the nerve (n = 5) with silicone elastomer (Kwik-Cast, World Precision Instruments, Sarasota, FL, USA) or lab-made nerve cuffs (1-mm inner diameter silastic tubing, AS636 wire, Cooner Wire, Chatsworth, CA, USA) were placed around the nerve (n = 9). Stimulation was performed with varying frequencies (1-100 Hz) and amplitudes (0.5-3 V) with an isolated pulse stimulator (Model 2100, AM Systems, Carlsborg, WA, USA). Most stimulation periods used 10 Hz (applied in 13/14 experiments; previously shown to activate sympathetic pathways to pelvic organs [103]) and 2 V stimulation, which was ~2x the motor threshold for an anal twitch. Stimulation was current controlled in the first two experiments (156.9 ± 130.5 μA mean amplitude) and voltage-controlled stimulation in the remaining experiments (2.38 ± 0.95 V). The entire pudendal nerve (motor and sensory branches) was stimulated, as to better replicate the non-specific stimulation delivered in clinical SNM.

A LDF pencil probe (MNP110XP, ADInstruments, Colorado Springs, CO, USA) was inserted 1-2 cm into the vagina and angled against the anterior wall. Vaginal blood perfusion

with the LDF probe was measured with a Blood FlowMeter (50 Hz sampling rate, model INL191, ADInstruments), which measures blood perfusion on a scale of 0-5000 blood perfusion units (BPU). A Grapevine Neural Interface Processor (Ripple, Salt Lake City, UT, USA) and desktop PC were used as a data acquisition system, with a custom MATLAB (Mathworks, Nantick, MA, USA) interface created for real-time data viewing. Recordings were paused for maintenance anesthesia dosing or other animal adjustments that may have introduced motion artifacts into the blood perfusion signals. The vaginal lumen diameter (VLD) was measured with digital calipers at two time points in the last six experiments; before any stimulation was applied and at the end of the experiment after LDF recordings were completed. After completion of all experimental procedures, animals were euthanized with an intraperitoneal injection of euthasol (sodium pentobarbital, 300-400 mg/kg).

Two types of pudendal nerve stimulation experiments were performed. The first set of experiments (n = 6) were short stimulation duration experiments. In these experiments multiple stimulation periods (0.17-5.00 minutes, 0.97 ± 0.64 minutes) were delivered sequentially with short inter-trial breaks. Blood perfusion was measured before any stimulation was delivered (0.60-2.32 minutes, 0.99 ± 0.68 minutes), during stimulation (26.62-114.37 minutes; 55.73 ± 30.80 minutes, including time between stimulation periods), and after stimulation (1.09-3.04 minutes; 1.64 ± 0.78 minutes). The results of short stimulation duration experiments indicated that stimulation had a cumulative effect. A second set of experiments (n = 8) used long stimulation durations. In these experiments, long continuous stimulation periods (4.98-57.68 minutes, 27.53 ± 11.25 minutes) were used. Again, blood perfusion was measured before (1.11-5.95 minutes; 2.74 ± 1.94 minutes), during (31.12-141.15 minutes; 92.54 ± 33.47 minutes, including time between sequential long stimulation periods), and after (5.23-27.65 minutes;

15.64 ± 9.90 minutes) stimulation. Baseline periods are defined as the time before the start of the first stimulation.

All data were analyzed in MATLAB. Sequential stimulation recording periods in one experiment were combined into one data file for analysis. We assumed that changes in LDF signals during brief non-recording periods between data files, i.e. for ketamine re-dosing, were negligible. LDF baseline levels between trials and animals sometimes varied based on slight differences in probe position, so the minimum value of each trial was set to zero prior to any data file combination. Stimulation intervals with strong, efferent-driven muscle contractions, characterized by at least a 100% increase in blood perfusion within 1 second of stimulation onset, were not included in data analysis as they were assumed to be direct activation of pelvic floor muscles. In order to maintain consistent analysis between experiments, the time between trials was considered negligible.

LDF signals were also analyzed using time-frequency representations (TFRs), with a continuous wavelet transform (CWT) method [95,96] in MATLAB. Blood perfusion signals were segmented into two key frequency domains: neurogenic (0.076-0.200 Hz), and myogenic (0.200-0.740 Hz), as previously established by Humeau and colleagues [95,96]. The scalogram energy (in arbitrary units) was calculated for each frequency range, to convert to a single continuous parameter in time [97]. Scalogram energy is typically used as a relative measurement. As we were primarily interested in changes in scalogram energy throughout the experiment, we determined the percent change in each energy range as compared to the baseline period.

While there is no established threshold for sexual arousal in the framework of changes in vaginal blood flow, we sought to quantify the timing and duration of large changes in vaginal perfusion. We identified a combined threshold that was sufficiently effective at separating

periods of inactivity from periods with notable changes in vaginal blood flow dynamics by visual inspection of a subset of experiments. The vaginal blood flow threshold (VBFT) is defined by simultaneous increases in raw LDF blood perfusion (100% increase), neurogenic energy (500% increase), and myogenic energy (500% increase) as compared to the corresponding mean baseline levels for each.

Our results suggested that vaginal blood flow responses are not directly correlated with the onset and offset of pudendal nerve stimulation, but rather with an accumulation of applied stimulation over time. Thus, we investigated the potential relationship between vaginal blood perfusion and pudendal stimulation based on a cumulative amount of stimulation delivered. In order to assess potential time dependency of changes in vaginal blood perfusion to the applied stimulation, we assessed the distribution of data points that exceeded the VBFT over time. As discussed in the Results, the peak probability of the VBFT being exceeded was at 31.3 minutes after stimulation started followed by a decay to baseline. For the analysis here, we rounded this time parameter to 30 minutes. Using this information, we investigated two potential models of stimulation accumulation and duration of effect (s_{eff}, equation 1), in addition to a direct relationship to the applied stimulation (s_{app}, equation 2). For the s_{eff} of the first model (Model 1), we convolved s_{app} with an exponential function ($w_{Model\text{-}1}$, equation 3 where t is time in seconds) which has an empirically determined decay constant λ (equation 4). For the s_{eff} of the second model (Model 2), we convolved s_{app} with a function ($w_{Model\text{-}2}$, equation 4) that is constant for 30 minutes and then decays with λ.

$$s_{eff} = w * s_{app} \quad (1)$$

$$s_{app} = \begin{cases} 0, stimulation\ off \\ 1, stimulation\ on \end{cases} \quad (2)$$

$$w_{Model-1} = e^{-\lambda t} \quad (3)$$

$$\lambda = \frac{\log 0.001}{1800} \quad (4)$$

$$w_{Model-2} = \begin{cases} 1, t \leq 1800s \\ e^{-\lambda(t-1800)}, t > 1800s \end{cases} \quad (5)$$

For each model, s_{eff} was separately normalized across experiments so that the maximum stimulation equaled one. Each model resulted in a unique effective stimulation curve, as shown in Figure 16 for a repeated short-duration stimulation experiment.

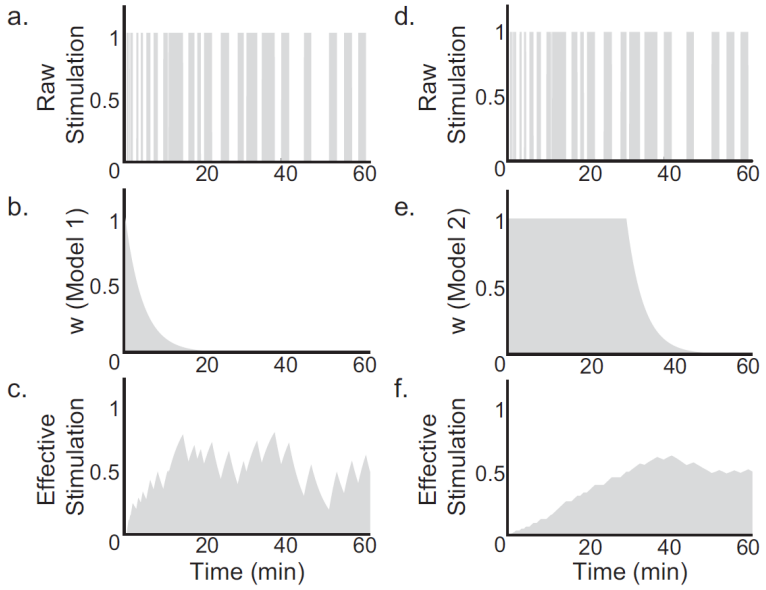

Figure 16. Models of effective stimulation for a repeated short-duration stimulation experiment (Experiment 5). a,d. Applied stimulation s_{app} for Experiment 5. b. Model 1 convolution function $w_{Model-1}$. c. Corresponding s_{eff-1}. e. Model 2 convolution function $w_{Model-2}$. f. Corresponding s_{eff-2}.

In order to determine if there was a significant change in vaginal blood flow from baseline, we performed a t-test (unpaired samples, unequal variance) between the LDF blood perfusion, neurogenic energy, and myogenic energy data sets for before and after the first stimulation period in each experiment. A Bonferroni correction was used to account for multiple comparisons. A significant difference was characterized by $\alpha < 0.01$ ($n = 3$, $p < 0.003$ with

Bonferroni correction). A one-way ANOVA was performed to determine if estrous state had a significant effect on the total time above VBFT for each experiment. To quantify the relationship between stimulation and the changes in vaginal blood flow in each experiment, we performed a linear regression between each stimulation curve (s_{app}, s_{eff-1}, s_{eff-2}) and each test variable (blood perfusion, percent change in neurogenic energy, percent change in myogenic energy). The relationship between neurogenic and myogenic energy was also investigated with a linear regression. The linear correlation coefficient r-value and corresponding p-value were determined for each linear regression. The linear regressions were considered significant if $p < 0.01$. Presented values are given as mean ± standard deviation, when appropriate.

A.4 Results

Most rats (n = 12) had significant increases in raw LDF blood perfusion as compared to the baseline period ($p < 0.003$). Increases in blood perfusion were frequently accompanied with increases in neurogenic and myogenic energy. Across experiments, neurogenic and myogenic energy were strongly correlated ($r = 0.79 \pm 0.13$, $p < 0.01$ for all experiments). Figure 17 shows blood perfusion and the corresponding time frequency analysis in an example experiment. Only rarely were the entire neurogenic and myogenic energy values significantly greater ($p< 0.003$) than baseline for the duration of an experiment (n = 1 for each neurogenic and myogenic). However, 11 of 14 experiments crossed the VBFT (Figure 18). The average total duration above VBFT was 11.7 ± 11.7 minutes across all 14 experiments (14.9 ± 11.2 minutes for 11 experiments that exceeded VBFT). All raw and analyzed data sets, as well as MATLAB analysis code, are accessible online [162].

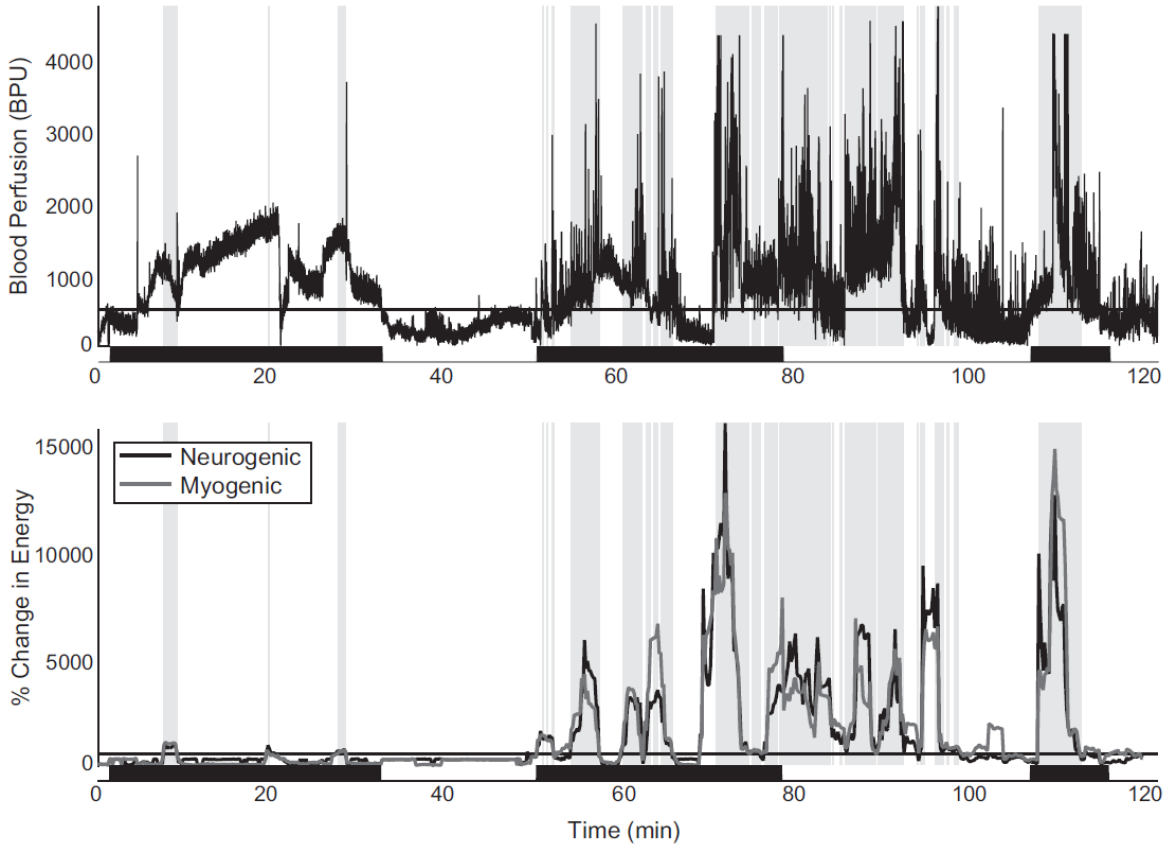

Figure 17. Example long-duration pudendal stimulation experiment showing increases in vaginal blood perfusion (Experiment 8). a. Vaginal blood perfusion. The horizontal line indicates the threshold for a 100% increase in raw blood perfusion from the baseline period. b. Percent change in neurogenic and myogenic energy. The horizontal line indicates a 500% increase in energy from the baseline period for both energy bands. Stimulation intervals (10 Hz) are indicated by black bars above the x-axis. Regions with gray background indicate when the VBFT was exceeded.

The increases in vaginal blood perfusion were not directly related to the onset of stimulation (except in cases of efferent muscle activation that were omitted from analysis), but rather exhibited a delayed or cumulative response. Figure 19 shows the distribution of time points across all experiments that were above VBFT. The peak probability of response was at 31.3 minutes after stimulation started, for analyzed trials. Subsequent peaks in the distribution are due to additional stimulation sequences throughout some experiments (e.g. Fig. 14). We used this information to inform our stimulation models, as described above. Model 1 assumes that the effect of stimulation decays exponentially over 30 minutes. Model 2 assumes that the effect of

stimulation is constant for 30 minutes, and then decays exponentially over 30 minutes. Figure 19 also gives distribution fits for experiment data split by stimulation duration or stimulus frequency. Each data division had a peak probability of response within 25 to 30 minutes after stimulation initiation. The 10 Hz-only experiments are six of the eight long-duration stimulation experiments, leading to a strong relationship between the two curves.

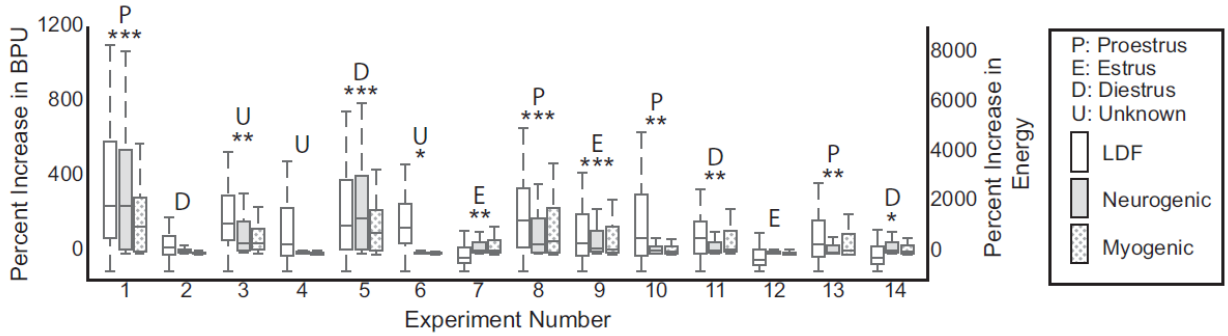

Figure 18. Distribution of vaginal blood perfusion changes across experiments. Box plots for percent increases in blood perfusion (left axis) and neurogenic energy and myogenic energy (right axis) for each experiment. *VBFT-time <5 min, **VBFT-time 5–10 min, ***VBFT-time >10 min.

Through our analysis of effective stimulation, we generally observed stronger correlations between the vaginal responses and $s_{eff\text{-}1}$ or $s_{eff\text{-}2}$ rather than s_{app}. Figures 20 and 21 show effective stimulation models for example short-duration and long-duration stimulation experiments, as well as the linear regression fit between each stimulation curve and the test

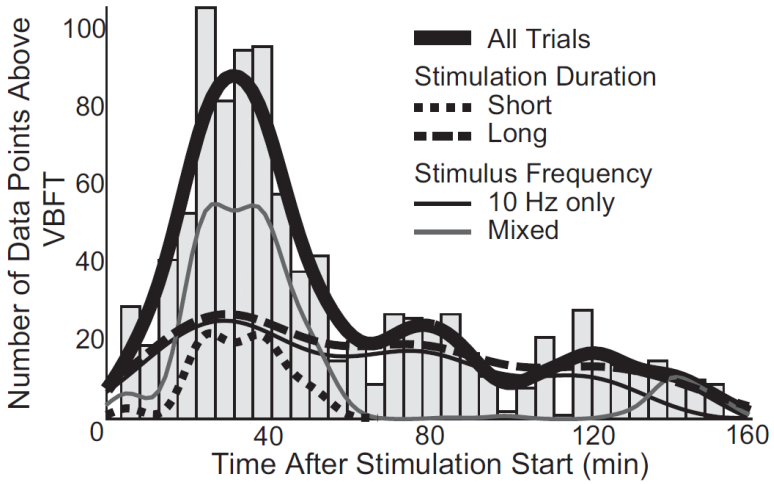

Figure 19. Distribution of experimental time points crossing VBFT across all experiments. The superimposed curve ("All Trials") is a non-parametric kernel-smoothing distribution, which provides a fit to histogram data with multiple peaks. This curve-fit yields a maximum at 31.3 min, with a strong fit to the data (r^2=0.911, p<0.001). Distribution-fit curves are also given for two separate data divisions. Dashed curves represent separate fits to short-stimulation duration experiments (n=6; peak at 25.7 min) and long-stimulation duration experiments (n=8; peak at 30.9 min). Thin, solid curves represent fits to experiments with only 10 Hz used (n=6; peak at 29.6 min) and experiments in which the applied stimulation frequencies were mixed (n=8; peak at 26.4 min).

variables. We found that in many experiments (n = 4 short-duration; n = 4 long-duration), blood perfusion was most positively correlated with Model 2 (r = 0.33 ± 0.13). Blood perfusion for two long-duration experiments was most positively correlated (r = 0.34, 0.36) with Model 1 and for four experiments (n = 2 short-duration; n = 2 long-duration) was most positively correlated (r = 0.25 ± 0.17) with s_{app}. For all experiments, the correlations between blood perfusion and each of the three models were significant ($p < 0.01$). The percent increase in neurogenic energy was most likely to be positively correlated (r = 0.34 ± 0.27) with Model 2 (n = 6 short-duration; n = 5 long-duration). The percent increase in neurogenic energy in one (n = 1) long-duration experiment was most positively correlated (r = 0.24) with Model 1 and, in two long-duration experiments (n = 2), was most positively correlated (r = 0.12, 0.29) with s_{app}. All correlations between percent increase in neurogenic energy and the most highly correlated model were significant ($p < 0.01$). The percent increase in myogenic energy was also most likely to be positively correlated (r = 0.35 ± 0.22) with Model 2 (n = 5 short-duration; n = 5 long-duration). For all of these experiments, the correlation with Model 2 was significant ($p < 0.01$). The percent increase in myogenic energy in two (n = 2) long-duration experiments was most positively correlated (r = 0.07, 0.26) with Model 1; one experiment was significantly correlated with Model 1 ($p < 0.01$) and one experiment showed a trend towards a correlation with Model 1 ($p = 0.06$). The percent increase in myogenic energy in two experiments (n = 1 short-duration; n = 1 long-duration) was most positively correlated (r = -0.12, 0.29) with s_{app}; one experiment was significantly correlated with the raw stimulation curve ($p < 0.01$) and one experiment showed a trend towards a correlation ($p = 0.11$). Since neurogenic and myogenic energy were strongly correlated, the correlation coefficients between each model and neurogenic/myogenic energy were similar. In 11 experiments, neurogenic and myogenic energy were most positively correlated with the same

model. In the experiments where the most positively correlated model differed between neurogenic and myogenic energy, the most positive correlations with the models were relatively weak (r = 0.03 ± 0.15 for correlations with neurogenic energy, r = 0.02 ± 0.12 for correlations with myogenic energy).

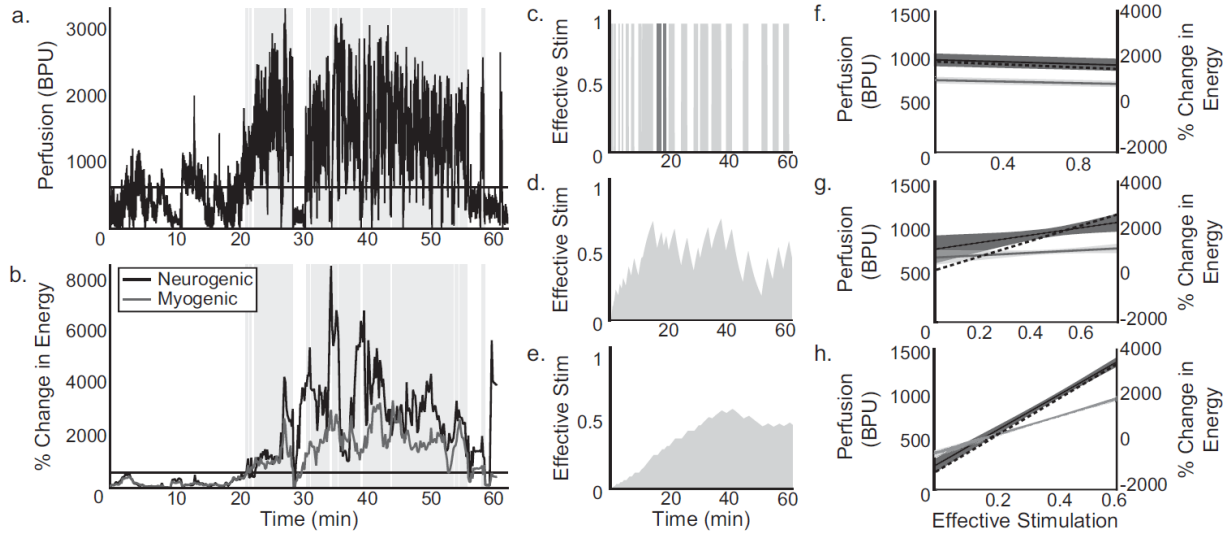

Figure 20. Effective stimulation analysis for repeated short-stimulation experiment (Experiment 5; 10 and 20 Hz stimulation). a. Blood perfusion. Horizontal line indicates 100% increase in perfusion compared to baseline. VBFT crossed in shaded regions. b. Percent change in neurogenic (black) and myogenic energy (gray). Horizontal line indicates 500% increase in energy for both energy bands. VBFT crossed in shaded regions. c–e. Effective stimulation curves (normalized to one, unitless) for no stimulation transformation (c), Model 1 (d), and Model 2 (e). Each stimulation interval was 10 Hz except for two 20 Hz intervals (dark gray). f–h. Linear regression between blood perfusion (black) or percent change in energy (neurogenic5dark gray, myogenic5light gray) and effective stimulation model indicated in (c–e). Shaded regions indicate 95% confidence intervals on each mean value distribution.

Four experiments occurred during the proestrus phase, three experiments occurred during the estrus phase, and four experiments occurred during the diestrus phase (Fig. 3). The remaining experiments had poor or unclear lavage samples. When comparing the total time that each experiment was above VBFT, experiments conducted during proestrus, diestrus, and estrus were not statistically different ($F(2,8)=1.44$, $p = 0.29$). However, there was a trend towards proestrus having the longest time above threshold (25.08 ± 20.84 min) compared to estrus (10.10 ± 10.49 min) and diestrus (17.05 ± 21.71 min). In the six experiments where VLD was measured, there was a mean increase in lumen diameter across the stimulation period of 50.8 ± 52.0%. Five of six

experiments had an increase in diameter, with only Experiment 11 decreasing (-5.1%). Across all experiments, we did not visually observe any pelvic floor contractions.

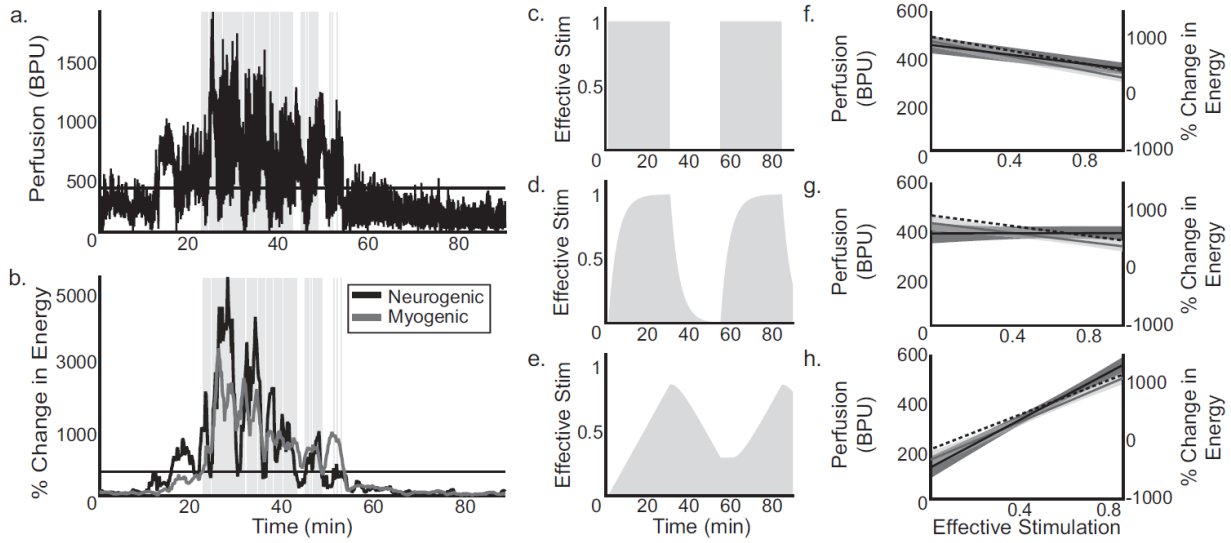

Figure 21. Effective stimulation analysis for long-stimulation experiment (Experiment 9; 10 Hz stimulation). Figure subparts as in Figure 17.

A.5 Discussion

In these experiments we demonstrated that repeated or extended-duration electrical stimulation on the pudendal nerve can lead to increases in vaginal blood perfusion, both in the raw LDF signal and the low frequency signal content within neurogenic and myogenic frequency bands (Figures 17, 20, 21). Although this was not a consistent response, a majority of our experiments (79%) had large concurrent increases in the raw LDF signal as well as TFRs in the two frequency bands (Figure 18). There was often a strong relationship between neurogenic and myogenic energy, which indicates a general increase in low frequency oscillations in blood perfusion due to pudendal nerve stimulation. As blood flow changes can be related to sympathetic-mediated contractions of smooth muscle in blood vessel walls [95], this relationship is expected. The concurrent crossing of three LDF signal parameters (raw signal increase, neurogenic frequency band increase, myogenic frequency band increase) is a potential novel

approach for detecting maximal blood flow changes. Interestingly, on average across experiments, most VBFT crossings occurred about 20-40 minutes after stimulation initiation (Figure 19).

The results of our study indicate that there was a cumulative effect of stimulation, which eventually fades (Figures 19, 20, 21). In our convolution model we estimated that stimulation has an effect for up to one hour, but that the effect of stimulation decays starting at approximately 30 minutes. This estimation of stimulation effect generally described the results more accurately than assuming there was a direct response to single stimulation periods or that the stimulation automatically decays. A cumulative stimulation effect may indicate increased activation of spinal circuits over time or is necessary to overcome descending inhibition [99]. Increased sympathetic activity due to arousal may also result in a positive feedback loop, which would increase the effective duration of stimulation. While a mechanism for sexual arousal involving sympathetic and parasympathetic feedback has been proposed, the dynamics are not well understood [163]. Our observed blood flow responses are much longer in duration than rat coitus. Similarly, the continuous stimulation applied by SNM does not relate to intercourse duration. It is possible that the benefits of neuromodulation for FSD include an improvement in genital organ blood flow and an increased ability to become aroused. Further preclinical and clinical studies are needed to investigate these relationships.

To our knowledge, this is the first work to evaluate the effect of long-duration (up to 30 minutes) pudendal nerve stimulation on vaginal blood perfusion. Prior studies evaluating pudendal [40] and pelvic nerve stimulation [38,77,88] for changes in vaginal blood flow used stimulation durations within 5-30 seconds, with further examination focused on neural pathways [40] or the impact of various pharmacological interventions [38]. The observed blood flow responses

in these studies were generally on the same time-duration order of magnitude (~15 seconds to 2 minutes in duration) as the applied stimulation. In those experiments, it is possible that pelvic floor contractions were occurring, with blood flow responses mirroring somatic muscle contraction. In our studies, the large blood perfusion responses that lasted for 5-10 minutes or longer show a longer-time course in the response, providing further support to autonomic nervous system modulation. In Cai et al., their 20-second pudendal nerve stimulation trials generally had LDF signal increases that were delayed after stimulation cessation [40], possibly obtaining a shorter response version of the results in our study. Our additional observation of increased VLD after stimulation, in five of six measured animals, suggests that the pelvic floor was not contracting for the duration of our applied stimulation. A future study including analyses of blood flow changes in other pelvic structures, such as the rectum, might indicate if our observed response is a local or multi-organ response. Interestingly, our use of (up to) 30-minute stimulation periods is similar to stimulation session durations of percutaneous tibial nerve stimulation (PTNS) for bladder function, which has also had benefits for some women with FSD [69]. Future work should also investigate this alternative pathway.

Our use of TFRs to provide additional analyses of LDF responses mitigates against signal contamination due to artifacts. For example, the LDF signal drift during the first stimulation period in Figure 17 was likely due to a slow settling of the LDF probe position. TFR analysis removed this artifact, and ultimately large perfusion changes were observed in the experiment. Prior nerve stimulation work generally focused on the raw LDF signal [38,40,77,88], and thus artifacts may have contaminated their observations. Breathing and bladder contractions can lead to signal confounds [38], however the typical rate of those activities are outside our specific frequency ranges of interest. Under anesthesia, our rats had breathing rates within 44-120 breaths per

minute, which aligns with a frequency range previously reported (0.74-2 Hz) [95,96]. Bladder contractions under ketamine anesthesia while saline is also being infused have been reported at rates within 0.012-0.076 contractions per second [104–106], below our neurogenic frequency range. One study which utilized TFRs in rat vaginal blood flow analyses evaluated combined frequency responses in a 0.013-0.6 Hz low frequency range and a 0.6-2.5 Hz high frequency range [41] without accounting for possible bladder or respiration contamination. Although we did not record bladder pressure in this work, our use of TFRs specifically in the neurogenic and myogenic ranges should have mitigated against their effect on our data.

The probability and timing of a LDF response to stimulation varied across experiments (e.g. Figs. 2, 5, 6). Studies investigating peripheral nerve stimulation for similar autonomic applications like bladder control, also have also reported inconsistent responses to the repeated stimulation parameters across experiments [102,103], as did a prior study assessing short pudendal nerve stimulation for changes in vaginal blood perfusion [40]. There are several potential explanations for these variations. Experiments were performed during the day, when rats are typically less active and thus handling may have caused stress. The anesthetic depth may have changed within or across animals, particularly as urethane is known to be a better anesthetic agent for studies of pelvic organ function than ketamine [104,164]. While we attempted to be consistent in LDF probe placement, the relative position may have varied within or across experiments. We did not perform a wide stimulation parameter evaluation, although our primary use of 10 and 20 Hz stimulation frequencies align with prior work that obtained maximal responses for these patterns in comparison to others [38,77]. It is also possible that surgical exposure and electrode placement may have led to nerve damage, though nerve functionality was confirmed at the start with observation of anal twitch responses. Additionally, there is the normal

presence of noise in the nervous system [165], which in our case may be accentuated by involving sensory, spinal, and motor pathways. Even with these potential factors, the large blood perfusion increases in a majority of experiments provide support for pudendal nerve stimulation for driving neural circuits that affect vaginal blood flow. Future studies will investigate the specific nerve pathways and spinal circuits involved in these responses.

Another potential cause of variation between animals is the estrous cycle. Female rats' hormones are determined by the estrous cycle, which is analogous to the human menstrual cycle. However, rat sexual receptivity is strongly affected by estrous stage. There are three main phases of the rat estrous cycle: proestrus, estrus, and diestrus. The estrus phase is generally considered sexually receptive stage, but recent studies have shown that sexual receptivity also occurs during proestrus and that sexual receptivity is low during the daylight hours of estrus [42]. Our results did not have a significant dependence on the estrous phase, which could indicate two possibilities. First, this could indicate that hormone levels do not affect genital arousal driven by the peripheral nervous system. Second, this could indicate that pudendal nerve stimulation is able to offset differences in baseline receptivity. The latter case could indicate the potential for pudendal nerve stimulation to offset low arousal and provide a treatment for aspects of female sexual dysfunction. A post-hoc power analysis of our results ($P = 0.09$) revealed that a larger scale study ($n \approx 160$) would be necessary to better determine the relationship between estrous phase and response to stimulation. In addition to larger scale studies, future work will include the use of ovariectomized and hormone-primed rats [43] to further investigate the impact of estrous cycle on pudendal nerve stimulation-driven vaginal arousal.

As the stimulation pattern and parameters are important in activation of autonomic pathways for organ control [102,103], further studies need to be conducted to determine the optimal

stimulation paradigm to increase neurogenic vaginal blood perfusion oscillations. Due to variations in stimulation duration and some alternating of stimulus frequencies during short stimulation-duration experiments, a balanced, comprehensive statistical comparison of stimulation patterns was not possible for the experiments performed here. Qualitatively, across experiments there were no relationships to applied stimulation frequency or amplitude. Although 10-Hz only experiments had a different distribution of points above the VBFT than experiments with mixed frequencies (Figure 19), 10 Hz was also used in all but one of the mixed-frequency experiments. It is possible that variations in the stimulus pattern [102], as was used to some extent in the mixed-frequency experiments, may lead to a greater excitation. We plan to perform a more rigorous evaluation of the stimulation parameter space in future work. The methods presented in this paper may be used for such a study to provide valuable quantitative analysis.

A.6 Conclusions

This study provides further insight into electrical stimulation of peripheral nerves for eliciting changes in vaginal blood flow. Our use of repeated and long-duration stimuli are closer in relevance to stimulation applied with SNM and PTNS than prior short-duration stimulation studies. Clinical improvements in sexual function for women with SNM may be due, at least in part, to neural-mediated processes leading to changes such as increases in vaginal blood flow. A detailed analysis of both the physiological effects of stimulation as well as the dynamics of the response to stimulation will help to inform future work towards the development of neuromodulation treatments for FSD.

A.7 Acknowledgments

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Appendix B: Laser Speckle Imaging to Evaluate Genital Blood Perfusion in Response to Pudendal Nerve Stimulation

B.1 Background

Quantifying the genital sexual arousal of anesthetized rats is a difficult challenge, but is often necessary to determine the success of eliciting sexual responses. The most common method used for rats is laser Doppler flowmetry (LDF), which measures capillary blood flow.[39] The flowmetry probe can be inserted into the vagina, where a laser light is reflected off of moving red blood cells on to a photodetector, giving values proportional to the velocity of blood flow in the vagina.[89,166] However, these signals are highly prone to motion artifact. In our studies (Chapter 2, Appendix A), LDF signals were recorded with overlapping artifacts from breathing, bladder contractions, and whole body movements of the rats either from twitching or from manipulation by the researchers. We utilized novel analysis methods to isolate the important frequency ranges of signals to minimize the impact of artifacts, but it is still possible that artifacts affected our data.

Laser Doppler and speckle imaging are possible alternatives to LDF that may be less susceptible to artifacts. Laser Doppler imaging similarly uses frequency shifts from the Doppler effect to measure velocity.[167] Laser speckle imaging gives a grainy appearance to objects illuminated by laser light, and if the object contains individual moving scatterers, such as moving red blood cells, the speckle image fluctuates, providing information about the velocity of the blood cells.[167] Both methods look at superficial blood flow velocity in an full-field imaged area,

as opposed to a 0.5-1 mm diameter area as is done by LDF. By imaging an entire area, small fluctuations are less likely to cause artifacts. Laser speckle allows for instantaneous, real-time velocity recording while laser Doppler does not.

There is evidence that laser Doppler imaging is more accurate at predicting genital arousal clinically in women than the standard vaginal photoplethysmography.[146] Using the same device for evaluating both preclinical and clinical genital arousal responses would provide better translation for preclinical research. In this pilot experiment we tested the feasibility of using laser speckle imaging to evaluate genital arousal in an anesthetized rat for the first time.

B.2 Methods

One female Sprague-Dawley rat was used in this experiment. The rat was anesthetized with 90 mg/kg ketamine. The pudendal nerve was surgically accessed and a nerve hook was placed on the nerve, similar to the methods in Appendix A.3. In this experiment, pudendal nerve stimulation was delivered at 20 Hz for 35 minutes at an amplitude twice the motor threshold.

A laser speckle contrast imager (Moor Instruments, Wilmington, DE, USA) was placed above the rat, pointed at the genitalia. Three regions of interest (ROI) were selected for speckle evaluation: the clitoris (fuschia), external vagina (purple), and vaginal wall (green), shown in Figure 22a. Laser speckle real-time imaging began 2.5 minutes before pudendal nerve stimulation, during the 35 minutes of stimulation, and for 10 minutes after the end of stimulation. Results were analyzed in MATLAB (Mathworks, Nantick, MA, USA).

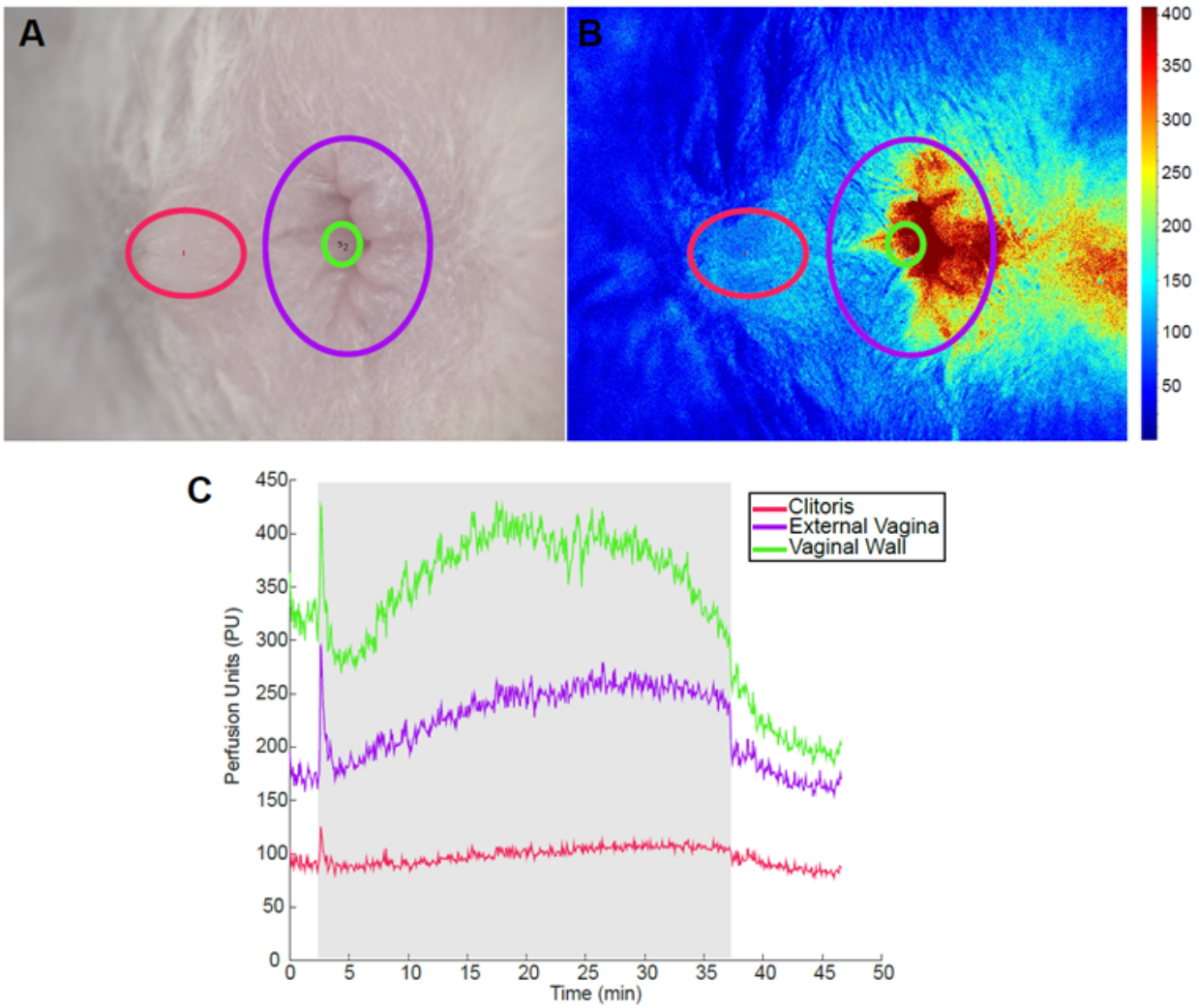

Figure 22. A: Picture of genitalia region being speckle imaged. Circles indicate regions of interest. Fuschia: clitoris. Purple: external vagina. Green: vaginal wall. B: Speckle image captured during pudendal nerve stimulation. Colors defined in legend correspond to blood velocity in arbitrary perfusion units. Circles indicate regions of interest. C: Perfusion units plotted over the course of the experiment in the clitoris, external vagina, and vaginal wall. Grey area denotes when pudendal stimulation was being delivered.

B.3 Results

Increases in blood perfusion were seen in each ROI. Figure 22b is a visualization of an instantaneous flux of blood perfusion taken during pudendal stimulation, and Figure 22c shows the average perfusion units (PUs) within each ROI over time. There is a sharp increase in all ROIs at the start of stimulation, which may be due to efferent activation of pelvic floor muscles from pudendal stimulation.

There was a slight increase in the clitoral blood perfusion over the course of pudendal stimulation (Figure 22c). The average PU for the clitoris ROI during baseline was 91.3 and then dropped 3.6% to 87.9 PU during the first 3 minutes after the initial spike when pudendal nerve stimulation began. During the last 3 minutes of stimulation, clitoral blood perfusion had increased 15.7% from baseline to an average of 105.6 PU. After stimulation stopped, the average clitoral perfusion dropped to 88.3 PU, a 3.2% decrease from baseline.

Blood perfusion in the external vagina steadily increased during the entire pudendal nerve stimulation (Figure 22c). The average PU for the external vagina ROI during baseline was 172.6 and then increased 6.8% to an average of 184.4 PU during the first 3 minutes after the initial spike when pudendal nerve stimulation began. During the last 3 minutes of stimulation, external vagina perfusion had increased 42.9% from baseline to an average of 246.6 PU. After stimulation stopped, the average clitoral perfusion dropped to 174.1 PU, a 0.9% increase from baseline.

Blood perfusion in the vaginal wall did not steadily increase during the 35 minutes of pudendal nerve stimulation, but rather quickly increased to a peak between 13-18 minutes after the start of stimulation, and then decreased again (Figure 22c). The baseline perfusion for the vaginal wall was 323.2 PU, and then it decreased 11.3% to an average of 286.6 PU at the start of stimulation for the first three minutes after the initial spike. After this initial decline, vaginal wall perfusion increased 26.0% to 407.3 PU between 14.5 and 17.5 minutes after the start of stimulation. After this peak, perfusion dropped to an average of 322.2, a 0.3% decrease from baseline, in the last 3 minutes of stimulation. Once stimulation was stopped, the average perfusion for the vaginal wall dropped to 214.4 PU, a 33.7% decrease from baseline.

B.4 Discussion

The increases in blood perfusion in the external vagina and vaginal wall demonstrate that pudendal nerve stimulation was successful in driving genital arousal by increasing vaginal blood perfusion. As this was only one animal, it is difficult to draw large conclusions. However, in this rat, it seems that main increases were limited to the vagina, while very moderate increases were seen in the clitoris. However, this may be due to the relative positioning of the clitoris in relation to the imager, or the that the membranous tissue of the vagina is more visible than the clitoris which is partially covered by hair. It is also interesting that the external vagina steadily increased throughout the duration of pudendal nerve stimulation, but the internal vaginal wall peaked in perfusion 13-18 minutes after the start of stimulation before decreasing below baseline values. This difference may be due to efferent, tetanic contraction of muscles in the pelvic floor. This contraction would be more apparent in the vaginal wall compared to the external vagina and clitoris. The decreasing blood flow could be due to muscles fatiguing during stimulation.

The stable perfusion measurements in this experiment were seemingly less disturbed by artifact compared to LDF.[78] This experiment showed potential for laser speckle imaging to quantify genital arousal in anesthetized female rats. Repeated experiments would further prove its utility and allow for studies of different experimental conditions.

Appendix C: Changes to Locomotor Activity During Tethering and Pudendal Nerve Stimulation

C.1 Background

Before the experiments in Chapter 3 were performed, we hypothesized that chronically implanting a nerve cuff on either of the pudendal or tibial nerves would be the optimal method of delivering stimulation for long-term neuromodulation studies. Very few studies have investigated the use of chronic pudendal or tibial nerve stimulation for urogenital treatment.[127,168] In these studies, there is no discussion about the behavioral or locomotor implications of delivering stimulation. If there are negative impacts on behavior, due to stimulation or implant discomfort, it could lead to decreased sexual behavior in motivational studies such as Chapter 3. In this exploratory study, we investigated the changes to locomotor activity for female rats in response to pudendal stimulation through an implanted nerve cuff, and we looked for signs of discomfort.

C.2 Methods

C.2.1 Animals and Surgery

Fifteen female Sprague Dawley rats weighing between 200-300g were used in this study. Surgeries were performed under isoflurane anesthesia using aseptic techniques. Nerve cuffs with a 0.5 mm diameter were implanted on the left pudendal nerve using a dorsal access point. Nerve cuffs were either made in-house using polyethylene tubing and platinum-iridium wires as

electrodes and leads (n=8), or manufactured by MicroProbes for Life Science (Gaithersburg, MD, USA) using platinum wires (n=6), or Micro-Leads (Somerville, MA, USA) with a 0.75 mm inner diameter and platinum-iridium electrodes and stretchable lead wires (n=1). The nerve cuff lead wires are connected to a connection port (assembled in-house) to be accessed externally. The connection port has six channels (MS363, P1 Technologies, Roanoke, VA, USA) with stainless steel electrodes embedded (363A/PKG, P1 Technologies) and secured with super glue. Nerve cuffs were sealed in place with Kwik-Sil (World Precision Instruments, Sarasota, FL, USA). In some surgeries, a suture was placed in surrounding tissue to anchor and secure the wire stemming from the nerve cuff to reduce strain on the nerve. The wire from the nerve cuff was tunneled rostrally under the skin along the spine.

Head cap implantation was performed using methods from Patel et al 2016.[169] A ~2 cm incision was made on the scalp after a subcutaneous lidocaine injection (4mg/kg). The skin flaps were retracted with hemostats and the skull surface was cleaned. Four holes were drilled into

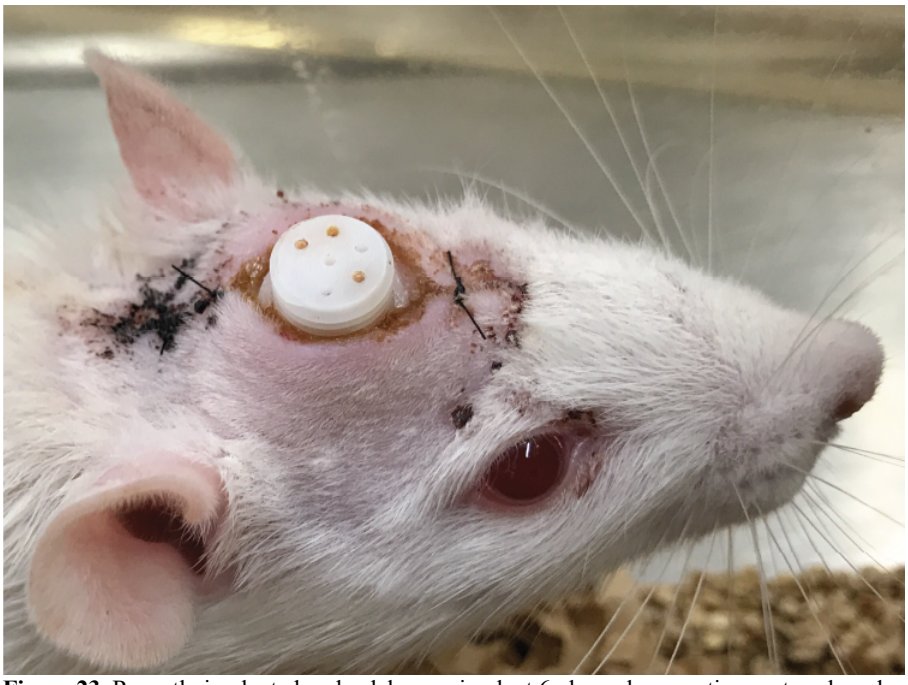

Figure 23. Recently implanted pudendal nerve implant 6-channel connection port anchored to the skull.

four peripheral corners of the skull using a burr bit (19008-07, Fine Science Tools, Foster City, CA) and four bone screws (19010-00, Fine Science Tools, Foster City, CA) were placed in the holes. The connection port for the nerve cuff was then anchored to the screws using dental acrylic. The skin flaps were pulled over the dental acrylic and sutured closed around the connection port, leaving the connection port accessible (Figure 23). Rats were given carprofen for 48 hours after surgery. One week after the surgery, rats were tested for pudendal nerve motor thresholds.

C.2.2 Open Field Chamber Testing

Locomotor and behavioral analysis took place in an open field chamber. The chamber was 24 x 24 inches with noise-dampening padding and limited light (Figure 24). A camera was placed at the top of the chamber facing downwards to track rat movement.

Figure 24. Tethered rat inside of the open field chamber.

Prior to surgery, rats were placed in the chamber for 30 minutes of open-field testing. The rats were free to move around with no stimuli. This testing was repeated after surgery while rats were tethered by the head cap connection port to a cable (363-363, P1 Technologies) leading to a

commutator (SL6C/SB, P1 Technologies) at the top of the chamber (Figure 24). Testing was repeated again while stimulation was delivered to the pudendal nerve. Stimulation was delivered for the entire 30 minutes of testing at 20 Hz at either 95% or 50% amplitude of the motor threshold (MT). If pudendal nerve cuff implant surgeries were unsuccessful, the baseline measurements for that rat were not used in the analysis.

C.2.3 Data Analysis

Animals were monitored for signs of pain or discomfort during tethering and pudendal nerve stimulation. Distance traveled by the rats was determined through video analysis of the testing sessions analyzed in MATLAB (Mathworks, Nantick, MA, USA) using software described in Tort et al 2006.[170]

C.3 Results

Fifteen female rats underwent pudendal nerve cuff implantation surgery. Two rats were successfully implanted, meaning that when electrical stimulation was applied through the head cap connection port one week after implantation, there was a resulting pudendal nerve motor response of anal sphincter contractions. However, one of the two successful rats lost the ability to be stimulated two weeks after surgery for unknown reasons. Only one rat was consistently stimulated for 3 months post-implantation. Both of these animals were implanted with Microprobes for Life Science nerve cuffs. The Micro-Leads cuff with an inner diameter of 0.75 mm proved to be too large for the pudendal nerve. There were several complications that resulted in 13 unsuccessful surgeries out of 15 (Table 9). The causes for failure were head cap breakage, current leakage near head cap or nerve cuff, rats chewing through their skin and then wires, or no response to stimulation. The most common failure was a lack of response to stimulation from

non-definitive causes. It is likely that the implant procedure or the nerve cuff damaged the pudendal nerve.

Table 9. Results and complications from 15 pudendal nerve implant procedures.

Result of Implant Surgery	**Number of Animals**
Head cap breakage	2
Current leakage near head cap	3
Current leakage near nerve cuff	1
Rat chewed through skin and wires	2
No response to stimulation	5
Successful pudendal nerve implant with pudendal motor response	2

The two rats with initially successful pudendal nerve implants were used for open field testing. The two were tested for a total of 5 baseline tests and 7 tethered tests. The sole rat that maintained successful stimulation of the pudendal nerve was used for two open field tests while receiving pudendal nerve stimulation. One stimulation session was delivered at an amplitude at 50% of the motor threshold, and one was delivered at 95% of motor threshold. The average total distances traveled in each testing condition are shown in Figure 25. Figure 26 shows the distances traveled in 5-minute intervals under different conditions. Rats traveled on average 1862.8 inches during baseline testing, and 1485.2 inches while they were tethered by a cable, a 20.3% decrease. During 95% MT stimulation, the rat traveled 842.4 inches, which is 54.8% less than baseline testing and 43.3% less than tethering. During 50% MT stimulation, the rat traveled 99.6 inches, which is 46.3% less than baseline testing and 32.7% less than tethering. The rat traveled 18% more distance when the amplitude was at 50% MT compared to 95%. However,

there is only one test in each of these conditions. Rats did not show any obvious signs of discomfort or pain during tethering or stimulation.

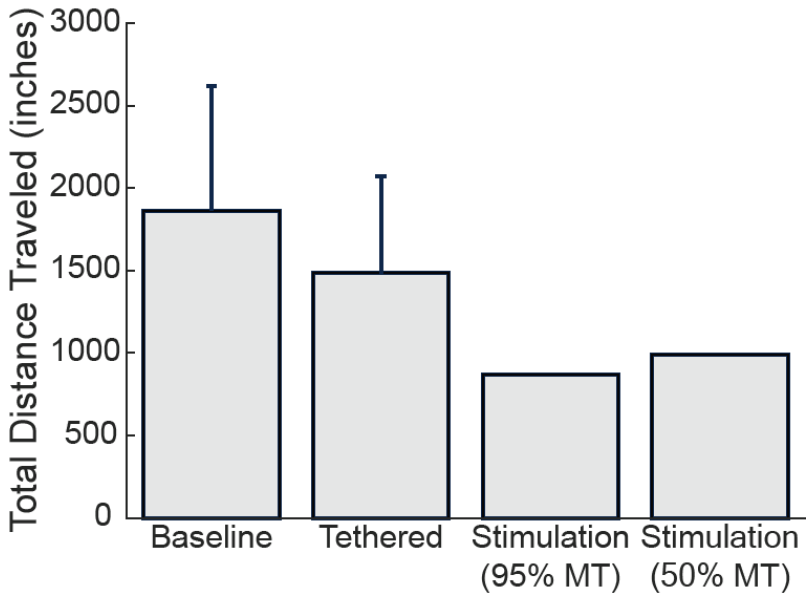

Figure 25. Total distances traveled in 30 minutes of an open field test under different conditions for one rat. (MT=Motor Threshold)

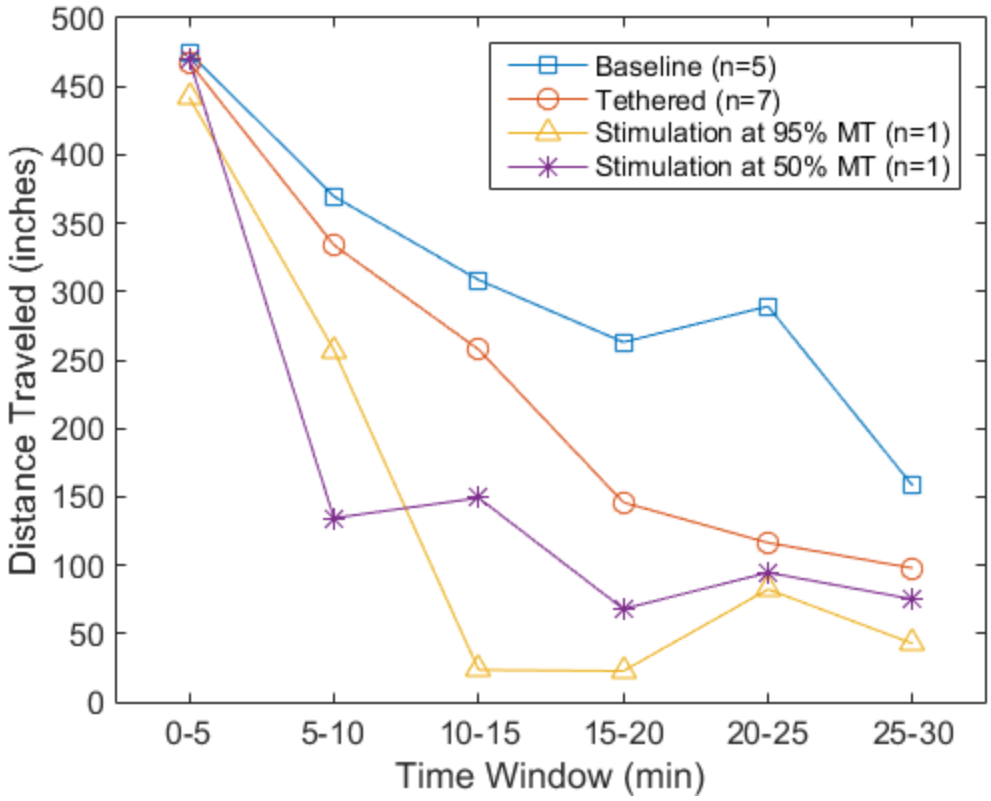

Figure 26. Mean distances traveled in 5-minute increments for one rat.

C.4 Discussion

These experiments demonstrated that pudendal nerve stimulation may have negative impacts on the locomotor activity of rats. Higher amplitudes of stimulation current also may be negatively correlated with locomotor behavior. While the number of tests were low, there was a noticeable decline in distance traveled. This may have implications for sexual behavior testing, as the stimulation may have further ramifications on behavior beyond distances traveled.

The larger issue with this study was the low success rate of the pudendal nerve cuff implants. Several of the initial issues were hardware malfunctions that were easily remedied, such as current leakage due to insufficient insulation of the wires and connections at the head cap. However, the lack of response to stimulation proved to be a greater problem. It is likely that the pudendal nerve was damaged either upon implantation or the result of tugging on the nerve

by the cuff after surgery. The nerve may have been too damaged to carry out a motor response in the period after surgery. Several changes to the surgery were made to alleviate this problem, such as reinforcement sutures and using nerve cuffs with stretchy, radial lead wires from MicroLeads, but with very little success. If the nerve was damaged, it is possible that waiting for a longer time after surgery for the nerve to heal may have allowed for functional testing.

As a result of these difficulties, we decided to use percutaneous stimulation of the tibial nerve for the experiments described in Chapter 3. Percutaneous stimulation does not carry the high risks for damaging the nerve of interest because the wires typically do not touch the nerve, but rather are just close to it. The tibial nerve was chosen for its ease of access for percutaneous stimulation. This also meant that rats could not be stimulated during behavioral testing, as the wire would likely be displaced. Behavioral testing took place immediately after stimulation in Experiment 1 of Chapter 3, which possibly avoided the negative impact of stimulation on locomotor activity.

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