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Title: Time-frequency analysis of increases in vaginal blood perfusion elicited by long-duration pudendal neuromodulation in anesthetized rats

Running Title: Neuromodulation-driven vaginal blood oscillations

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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

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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.

Keywords: pudendal nerve stimulation, sexual function, internal organ function, blood flow, peripheral nerve stimulation

Introduction

Female sexual dysfunction (FSD) is a widespread medical problem that may affect up to 45% of women [1], [2]. FSD has several subtypes, which may be concurrent [3]. 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 [4]. Currently, there are no effective treatment options specifically targeting the genital, rather than psychological, component of female sexual arousal [5]. 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 [6]. 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 [7]. Studies with sildenafil in women have correlated increases in clitoral and vaginal blood flow to improvements in sexual function [8], [9]. However, sildenafil administered to women has a high likelihood of moderate adverse events, most commonly headaches, [7] and has had unclear clinical benefits due to conflicting reports of efficacy [10], [11]. 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 [12], and plays an important role in physiological sexual arousal [13]. 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 [14]. A few studies have shown that some women treated with SNM for overactive bladder also have an improvement in sexual function [15]–[17], which were not correlated with improvements in bladder function [17]. 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 [18]–[20]. 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 [18]–[23]. 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 [24], [25]. Microvascular perfusion exhibits oscillations in activity based on underlying metabolic, neurogenic, and myogenic activity, as well as oscillations associated with respiration and heart rate [24]–[26]. The neurogenic oscillatory range of LDF is associated with sympathetically-driven changes in microvascular perfusion [27]. Several studies have indicated that increased sympathetic drive leads to increased genital arousal in women [28]–[31]. 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 [18], [21]–[23], 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.

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 [32], 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

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responses [23], [33], [34]. 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 [35]) 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 [24], [25] 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 [24], [25]. The scalogram energy (in arbitrary units) was calculated for each frequency range, to convert to a single continuous parameter in time [36]. 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 ($w_{Model-1}$, 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 ($w_{Model-2}$, equation 4) that is constant for 30 minutes and then decays with λ.

$$s_{eff} = w * s_{app} \tag{1}$$

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

$$w_{Model-1} = e^{-\lambda t} \tag{3}$$

$$\lambda = \frac{\log 0.001}{1800} \tag{4}$$

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

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 1 for a repeated short-duration stimulation experiment.

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.

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 ± 0.13, p < 0.01 for all experiments). Figure 2 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 3). 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 [37].

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 4 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. 2). 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 4 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.

Through our analysis of effective stimulation, we generally observed stronger correlations between the vaginal responses and s_{eff-1} or s_{eff-2} rather than s_{app}. Figures 5 and 6 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 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 ($r = 0.34 \pm 0.27$) 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 ($r = 0.35 \pm 0.22$) 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 ($r = 0.03 \pm 0.15$ for correlations with neurogenic energy, $r = 0.02 \pm 0.12$ for correlations with myogenic energy).

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.

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 2, 5, 6). 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 3). 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 [24], 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 4).

The results of our study indicate that there was a cumulative effect of stimulation, which eventually fades (Figures 4, 5, 6). 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 [13]. 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 [38]. 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 [18] and pelvic nerve stimulation [21]–[23] for changes in vaginal blood flow used stimulation durations within 5-30 seconds, with further examination focused on neural pathways [18] or the impact of various pharmacological interventions [23]. 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 [18], 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 [39]. 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 2 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 [18], [21]–[23], and thus artifacts may have contaminated their observations. Breathing and bladder contractions can lead to signal confounds [23], 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) [24], [25]. Bladder contractions under ketamine anesthesia while saline is also being infused have been reported at rates within 0.012-0.076 contractions per second [40]–[42], 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 [19] 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 [35], [43], as did a prior study assessing short pudendal nerve stimulation for changes in vaginal blood perfusion [18]. 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 [40], [44]. 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 [22], [23]. 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 [45], 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 [46]. 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 ≈ 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 [47] 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 [35], [43], 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 4), 10 Hz was also used in all but one of the mixed-frequency experiments. It is possible that variations in the stimulus pattern [43], 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.

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.

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Figure Legends

Figure 1. 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}.

Figure 2. 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 grey background indicate when the VBFT was exceeded.

Figure 3. 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 minutes, ** VBFT-time 5-10 minutes, *** VBFT-time > 10 minutes.

Figure 4. 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 minutes, 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).

Figure 5. 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 (grey). 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 grey). f-h)

Linear regression between blood perfusion (black) or percent change in energy (neurogenic =

dark grey, myogenic = light grey) and effective stimulation model indicated in (c-e). Shaded

regions indicate 95% confidence intervals on each mean value distribution.

Figure 6. Effective stimulation analysis for long-stimulation experiment (Experiment 9; 10 Hz

stimulation). Figure sub-parts as in Figure 5.

Time (min)

Neuromodulation Proof

Figure legend (as shown in plot):

All Trials

Stimulation Duration
······· Short
– – – Long

Stimulus Frequency
——— 10 Hz only
——— Mixed

Axes:
- Y-axis: Number of Data Points Above VBFT (0, 20, 40, 60, 80, 100)
- X-axis: Time After Stimulation Start (min) (0, 40, 80, 120, 160)