Reflex neuromodulation of bladder function elicited by posterior tibial nerve stimulation in anesthetized rats

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Kovacevic M, Yoo PB. Reflex neuromodulation of bladder function elicited by posterior tibial nerve stimulation in anesthetized rats. Am J Physiol Renal Physiol 308: F320–F329, 2015. First published November 26, 2014; doi:10.1152/ajprenal.00212.2014.—Although posterior tibial nerve stimulation (PTNS) has been shown in both clinical and animal studies to elicit bladder-inhibitory reflexes, our understanding of the role of posterior tibial nerve (PTN) afferents that elicit these responses is significantly limited. To this end, we investigated the effects of frequency-dependant PTNS in urethane-anesthetized rats undergoing repeated urodynamic fills. Nerve stimulation trials (10 min) resulted in statistically significant inhibition of the urinary bladder, both during and after nerve stimulation ($P < 0.05$). PTNS applied at 5 Hz resulted in both acute and prolonged changes that corresponded to 38.0% and 34.1% reductions in the bladder contraction frequency, respectively. In contrast, PTNS applied at 10 Hz could only elicit an acute decrease (22.9%) in bladder activity. Subsequent electrical activation of individual PTN branches (lateral or medial plantar nerves) confirmed that these bladder reflexes are mediated by specific subsets of the PTN trunk. Both acute and prolonged inhibition of the bladder were achieved by electrical stimulation of the lateral plantar (10 and 20 Hz) and medial plantar (5 and 10 Hz) nerves. Finally, we report a bladder-excitatory reflex that is elicited by electrical activation of either the PTN trunk or lateral plantar nerve at 50 Hz. This study shows that multiple bladder reflexes are tuned to specific subsets of nerve afferents and stimulation frequencies, each of which provide novel insights into the physiological effects of PTNS.

As an alternative approach, posterior tibial nerve (PTN) stimulation (PTNS) has been examined as a means of modulating bladder function. Originally validated as a treatment for overactive bladder (OAB) (19), PTNS has been shown clinically to induce neural plastic changes in bladder function over long time periods (i.e., months) with repeated nerve stimulation (8, 9, 19, 25–27, 42, 43). The nature of this bladder-inhibitory reflex pathway has been studied in various animal models, where acute changes in bladder function have been described in response to finite-duration PTNS trials. In anesthetized cats, reflex inhibition of bladder function (e.g., increased bladder capacity) was achieved by both direct PTNS and transcutaneous hindfoot stimulation at frequencies of either 5 or 20 Hz (5, 36, 37, 38). More recently, Su et al. (33) reported robust bladder inhibition in anesthetized rats, where PTNS-evoked responses were reported as being tuned to very specific stimulation parameters: a frequency of 10 Hz and amplitude of 3T (where T is foot twitch threshold). Given the multitude of bladder reflexes that have been previously demonstrated by electrical nerve stimulation in rats (4, 13, 46, 52), the recent work investigating the effects of PTNS (17, 33) further validates the use of this animal as an appropriate model for investigating the mechanisms of bladder modulation.

However, there are significant limitations in our understanding of which specific stimulation parameter(s) is relevant to modulating bladder function by PTNS. Such variables include the diameter of the recruited axons, total number of activated fibers, specific anatomic subset(s) of fiber bundles (i.e., nerve fascicles), and the frequency at which these axons are pulsed. Current clinical data indicate that therapeutic effects can be achieved by 20-Hz stimulation (8, 25–28) and even 10-Hz stimulation (7) of the PTN trunk. That this bladder-inhibitory reflex also appears to work in cases of neurogenic bladder (e.g., multiple sclerosis and spinal cord injury) suggests PTNS therapy may possibly involve a spinal-mediated reflex pathway (1, 7, 11, 14). As demonstrated in previous work involving pudendal nerve stimulation (2, 47, 51), the characterization of stimulation parameters that can modulate bladder function is a key step in uncovering the currently unknown mechanisms of PTNS therapy.

In the present study, we investigated the input-output relationship between PTNS and corresponding changes in bladder function. Using a urodynamic bladder model in anesthetized rats, we varied the stimulation parameters over a broad range of amplitudes, frequencies, and neural targets. Our results showed that PTNS can elicit a complex set of bladder responses that were strongly dependant on the stimulation frequency and the specific neural subset (PTN branch) that was electrically activated. Stimulation-evoked responses were identified as either inhibitory or excitatory bladder reflexes and

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further denoted as acute or prolonged. Preliminary findings have been presented in abstract form (15).

MATERIALS AND METHODS

All surgeries and procedures were approved by the University of Toronto Animal Care Committee in accordance with the regulations of the Ontario Animal Research Act (Toronto, ON, Canada). Experiments were conducted on female Sprague-Dawley rats (n = 12) weighing 250–300 g. Inhaled isoflurane was used to induce (5%, induction chamber) and maintain (2–3%, gas mask, O2 flow rate: 0.1 l/min) anesthesia during the bladder catheterization and electrode implant procedures (duration: ~2 h). Before the nerve stimulation protocol began, the anesthesia was transitioned from isoflurane (2% to 0%, over 30 min) to urethane (2 half-dose intraperitoneal injections, 15 min apart, 1.2 g/kg). Heart rate (300–400 beats/min), blood O2 level (97–100%), and body temperature (37–39°C) were monitored throughout the experiment. If needed, supplemental doses of urethane (0.25–0.5 dose, 1.2g/kg) were administered. The animal was maintained on urethane for 8–10 h until the end of the experiment. At the end of the experiment, the animal was euthanized via an overdose of isoflurane (5% isoflurane, delivered over 10–15 min) or T61 injection (0.3 ml/kg).

Electrical stimulation of the PTN and PTN branches. A surgical incision was made on the medial aspect of the lower leg, cephalad to the ankle. This exposed the posterior tibial nerve and allowed for the implant of a custom-fabricated bipolar (interelectrode distance: 5 mm) platinum nerve cuff electrode. The electrode was connected to an isolated pulse stimulator (model 2100, A-M Systems, Carlsborg, WA), which delivered constant-current pulses (0.2 ms, square pulses) of varying amplitudes and frequencies. Selective electrical stimulation of the medial plantar nerve (MPN) and lateral plantar nerve (LPN) branches was achieved by carefully bisecting the PTN trunk at the incision site. The nerve electrode was then placed on either branch and secured via 6-0 sutures. The stimulation amplitude was slowly increased to confirm observable foot twitches. Stimulation-evoked twitches of the hallux and lateral digits indicated selective activation of the MPN and LPN branches, respectively.

Bladder pressure recording and saline infusion. After a midline abdominal incision to expose the bladder (Fig. 1B), a small cut was made at the dome of the bladder, through which a cannula (polyethylene-50) was inserted and secured with 6-0 sutures. The abdomen was closed in layers using 4-0 suture. Externally, the cannula was connected in series to a pressure transducer (Utah Med, Midvale, UT) and infusion pump (Harvard Apparatus, Holliston, MA). Bladder pressure signals were conditioned using a bridge amplifier (ETH256, iWorx, Dover, NH).

Foot electromyography and external urethral sphincter electromyography recordings. A pair of 35-gauge, PFA-coated, multi-stranded, stainless steel wire electrodes (A-M Systems) were used to record foot electromyography (fEMG) and external urethral sphincter electromyography (EUS EMG) activity. Recording of fEMG was achieved by inserting one of the electrodes between the hallux and long digit and the other injected medial to the hallux. PTN, posterior tibial nerve.

B: repeated BRCs were achieved by constant infusion of saline into the bladder. Polyethylene (PE)-50 tubing was used to connect the bladder dome with a pressure transducer and infusion pump. A pair of stainless steel wires were injected paraurethrally to measure external urethral sphincter (EUS) activity.

Data conditioning and acquisition. EMG electrode signals were filtered and amplified (EMG bandwidth: 100–3,000 Hz and gain: 1,000) through a low-noise voltage preamplifier (Stanford Research Systems, Sunnyvale, CA). All data were acquired using a PowerLab data acquisition system (AD Instruments, Colorado Springs, CO) that was integrated with the computer via LabChart software (AD Instruments) and sampled at a rate of 20,000 samples/s.

Stimulation amplitude settings. In each rat, the minimum stimulus amplitude that evoked any fEMG activity during PTN trunk stimulation, LPN branch stimulation (LPNS), or MPN branch stimulation (MPNS) was defined as Tm, Tm-1, and Tm-m, respectively. The tibial nerve was stimulated at 1, 2, 4, 6, 8 and 10 times T (biphasic, square, pulse width: 200 μs, 2 Hz, 10 s), which resulted in 20 evoked signals recorded by fEMG electrodes. At each amplitude (Fig. 2A), all 20 signals were averaged, and the area under the EMG curve (AUC) was calculated and normalized by the AUEC obtained by stimulation at 10T. The AUEC (percent activation) was plotted against amplitude to obtain an EMG recruitment curve (Fig. 2B). Based on these recruitment characteristics and preliminary experiments (15), the default amplitude of all stimulation trials (PTN, LPN, and MPN) was set to 6T.

Acquisition and interpretation of bladder rhythmic contraction data. Periodic bladder rhythmic contractions (BRCs) were achieved by continuous saline infusion (0.1–0.3 ml/min) via the suprapubic catheter. Bladder voiding was achieved by either sustained contractions or passive leakage through the urethral meatus. Increases in
bladder pressure with concomitant high-frequency bursts in EUS EMG activity were indicative of a single contraction. The experimental protocol involved an initial control period of BRC (no stimulation) lasting 10 min followed by a 10-min stimulation period during which the tibial nerve was stimulated at 6Tm and a given frequency. This process of alternating 10-min control and stimulation periods was repeated for 2, 5, 10, 20, and 50 Hz, applied in random fashion. In some cases, prolonged bladder inhibition was observed as passive leakage of the urinary bladder. These were characterized by a sustained and constant bladder pressure (20–30 cmH2O), during which large bladder contractions or characteristic high-frequency EUS EMG bursts were not observed. Although stimulation trials at 50 Hz could restore BRCs, these results were not factored into our data analysis.

The time between each successive bladder contraction was used to calculate the average bladder contraction frequency (aBCF) for both the 10-min “stimulation” and “poststimulation” periods. aBCF for each period was normalized using the control period preceding the stimulation trial and was expressed as the percent change in aBCF with respect to control (Fig. 3). In the present study, bladder inhibition or excitation, whether acute or prolonged, was defined as a 10% change in aBCF from control. All data were processed using LabChart (AD Instruments) software and was further analyzed using MatLab (MathWorks, Torrance, CA) and MS Excel (Microsoft, Redmond, WA). The entire process was repeated for MPN and LPN branches using stimulation amplitudes of 6Tm-m and 6Tm-l, respectively. The time required to complete the entire stimulation protocol was 8–10 h.

Statistical significance. To determine statistical significance, values were compared using a Mann-Whitney U-test, where P values of <0.05 were deemed to be statistically significant. All statistical comparisons were performed using the response of 2-Hz PTNS as our control stimulation parameter. Both our preliminary results (15) and a previous rat study (33) indicated that 2-Hz PTNS does not elicit any notable changes in bladder function. All summary data are expressed as means ± SE.

RESULTS

Experiments were conducted in 12 rats, where PTNS-evoked fEMG recordings were collected from every animal. Due to the extended duration each experiment (>9 h in total), fEMG recruitment and bladder modulation data generated by selective PTNS were obtained in only 8 of 12 rats.
Foot EMG recruitment by nerve stimulation. The threshold amplitude for eliciting a foot twitch by PTNS (T_m) was 20.8 ± 1.7 μA (range: 5–60 μA, n = 12). As shown in Fig. 2A, the evoked fEMG appears to approximate maximum recruitment at 4T_m. The overall pattern of nerve fiber activation is shown in Fig. 2B, where PTNS at 2T_m and 4T_m reached 66.1 ± 9.1% (range: 4.5–103.3%, n = 12) and 89.1 ± 4.4% (range: 45.5–104.9%, n = 12) of the fEMG activity elicited at 10T_m, respectively. The aggregate recruitment curve of PTNS (Fig. 2B) suggests that full activation of large myelinated (e.g., Aα) fibers is achieved by 4T_m and that smaller Aβ-fibers are recruited at 6T_m and 8T_m.

Selective electrical stimulation of MTN and LTN branches yielded fEMG thresholds of 19.4 ± 1.2 μA (T_m,m, range: 6–40 μA, n = 8) and 81.8 ± 16.6 μA (T_m,l, range: 5–400 μA, n = 7), respectively. As shown in Fig. 2B, full activation of both MTN and LTN branches occurred at 8T_m,m (99.4 ± 1.4% of 10T_m,m, range: 92.6–105.1%, n = 8) and 8T_m,l (95.8 ± 3.3% of 10T_m,l, range: 91.2–99.3%, n = 7), respectively. Although electrical recruitment of the MTN appears to occur at lower stimulation amplitudes, both branches approximate maximum activation of myelinated fibers by 6T stimulation.

Frequency-dependant bladder inhibition (PTNS). Our data show that electrical stimulation of the PTN trunk at 5 and 10 Hz resulted in a statistically significant reduction of ongoing bladder activity (decreased BRC; Fig. 3). As shown in Fig. 4A, PTNS trials applied at 10 Hz evoked robust bladder-inhibitory responses (38.0 ± 10.8% decrease in aBCF, range: −4.2% to 100%, n = 12, P = 0.002) during the stimulation period (acute inhibition), whereas there was a limited change in bladder function (7.6 ± 11.9% decrease in aBCF, range: −48.5% to 100%, n = 12, P = 0.544) during the poststimulation period (prolonged inhibition). The predominantly acute inhibitory effects of 10-Hz PTNS are reflected in the percentage of experiments that exhibited reflex bladder inhibition (as defined by a minimum 10% decrease in aBCF with respect to control), where acute and prolonged bladder responses were observed in 67% and 50% of experiments, respectively. The response rate of PTNS in rats is summarized below. In one experiment, 10-Hz PTNS resulted in a prolonged bladder-inhibitory response that lasted for ~1 h (Fig. 5B). During this period of bladder inactivity, both continuous saline infusion and externally applied abdominal pressure (labeled as “manual pressure” in Fig. 5B) failed to restore BRCs.

In contrast, PTNS applied at 5 Hz generated statistically significant decreases in aBCF (Fig. 3), both during (22.9 ± 9.5%, range: −30.9% to 100%, n = 11, P = 0.0178) and after (34.1 ± 12.0%, range: −30.9% to 100%, n = 11, P = 0.004) each stimulation trial. The probability of evoking a prolonged bladder response was markedly greater (9 of 11 experiments, compared with baseline BRCs (10 min before stimulation), 10-Hz PTNS evoked acute suppression of bladder activity that was followed by a rapid return of baseline-level BRCs (no poststimulation effect). Passive transurethral leakage of saline and absence of EUS activity (not shown here) during stimulation indicated the absence of bladder function. B: PTNS applied at 5 Hz resulted in a delayed suppression of BRCs (~8–9 min after the start of stimulation) that persisted beyond the stimulation period. Passive leakage of the bladder was observed throughout the 10-min poststimulation period. C: evidence that a PTNS-evoked bladder excitatory reflex was evoked by 50-Hz PTNS. Robust BRCs resumed within 10 min of stimulation. D: compared with baseline BRCs, 50-Hz PTNS immediately resulted in a marked increase in bladder activity that continued beyond the stimulation period (i.e., poststimulation effect). Time bar = 2 min.
82%) than that for acute bladder inhibition (6 of 11 of experiments, 55%). Similar to 10-Hz PTNS, extended bladder inhibition after PTNS at 5 Hz was observed in a limited number of experiments (Figs. 4 and 5).

Excitatory bladder reflex evoked by PTNS. In addition to bladder-inhibitory responses elicited by PTNS (5 and 10 Hz), electrical stimulation of the PTN trunk at 50 Hz could also reflexively increase ongoing bladder activity (aBCF; Fig. 4D) and even restore BRCs that were suppressed by prolonged inhibitory reflexes (Figs. 4C and 5B). As shown in Fig. 3, this excitatory reflex was observed both during (29.9 ± 18.7% increase in aBCF, range: −33.1% to 142.6%, n = 10, P = 0.48) and after (32.4 ± 20.9% increase in aBCF, range: −27.7% to 123.5%, n = 10, P = 0.07) each 10-min PTNS trial. However, the overall effect of 50-Hz PTNS was not statistically significant, nor was it consistently observed across experiments [acute excitation: 4 of 10 experiments (40%) and prolonged excitation: 5 of 10 experiments (50%)].

Effects of selective MPNS. Selective MPNS at 10 Hz resulted in statistically significant decreases in aBCF (Fig. 6), both during (38.2 ± 5.5% decrease, range: 17.3–66.7%, n = 8, P = 0.001) and after (28.5 ± 10.5%, range: −25.0% to 68.1%, n = 8, P = 0.03) stimulation trials. In particular, 10-Hz MPNS evoked acute bladder inhibition in all eight of eight experiments (100%) and prolonged bladder inhibition in six of eight experiments (75%). However, unlike PTNS applied at 5 Hz (Fig. 3), selective 5-Hz MPNS failed to elicit acute bladder inhibition (Fig. 6). Instead, only prolonged bladder-inhibitory responses were elicited at this lower stimulation frequency (31.2 ± 13.3% decrease, range: −12.7% to 48.6%, n = 8, P = 0.03). This response was observed in six of eight experiments (75%). There were no statistically significant responses evoked by MPNS applied at 2, 20, or 50 Hz.

Effects of selective LPNS. Compared to MPNS, selective LPNS evoked bladder-inhibitory responses at slightly higher stimulation frequencies. As shown in Fig. 7, statistically significant decreases in aBCF were evoked by LPNS at both 10 and 20 Hz. Acute responses involved 14.4 ± 4.9% (range: −1.8% to 29.2%, n = 6, P = 0.02) and 21.9 ± 7.5% (range: 0.0–53.0%, n = 6, P = 0.02) decreases in bladder activity,
whereas prolonged responses exhibited $33.3 \pm 6.8\%$ (range: $14.8–58.6\%$, $n = 6$, $P = 0.002$) and $17.7 \pm 4.9\%$ (range: $-2.6\%$ to $34.1\%$, $n = 6$, $P = 0.008$) decreases in aBCF, respectively. Again, note that LPNS applied at 10 Hz resulted in prolonged bladder inhibition in all six of six experiments (100%), whereas the response rate of the other stimulation parameters ranged from 67% to 83%. Although LPNS applied at 2 and 5 Hz did not have any effect on the urinary bladder, selective 50-Hz LPNS evoked bladder-excitatory responses, similar to those achieved by 50-Hz PTNS (Fig. 3). The average magnitude of these responses was large (acute: $50.3 \pm 35.7\%$ and prolonged: $131.0 \pm 102.1\%$, Fig. 7) but not statistically significant ($P = 0.104$). Similar to PTNS at 50 Hz, these excitatory responses were observed in <50% of experiments (acute: 3 of 7 rats and prolonged: 4 of 6 rats).

**Response rate of PTNS in anesthetized rats.** The experimental data were further analyzed as the percentage of experiments that exhibited either an acute response or a prolonged response (i.e., minimum 10% reduction in bladder pressure) to electrical nerve stimulation. The results are shown in Figs. 8 and 9, respectively, where the response rates were categorized with respect to the target nerve (sections A–C), stimulation frequency (5–50 Hz), and magnitude of response (shaded and solid bars). Among the various response rates that were observed, it is interesting to point out that MPNS at 10 Hz (Fig. 8B) and LPNS at 10 Hz (Fig. 9B) both exhibited 100% response rates.

**DISCUSSION**

The results of the present study significantly expand the current knowledge of bladder-inhibitory reflexes evoked by PTNS. By examining both acute and prolonged types of responses in anesthetized rats, we describe multiple sets of PTNS parameters that can effectively inhibit bladder activity. Similar to previous work (17, 33, 34, 38), PTNS-evoked responses were strongly dependent on both the stimulation amplitude and frequency. In this study, we show, for the first time, that activation of these reflexes can be achieved by electrically stimulating specific PTN branches (i.e., the MTN and LTN). We further showed that both acute and prolonged types of bladder-inhibitory responses were preferentially tuned to 10-Hz MPNS and 10-Hz LPNS, respectively. In addition, we also uncovered novel evidence of a bladder-excitatory reflex mediated by 50-Hz PTNS, where selective PTN branch stimulation confirmed the LPN as the neuroanatomic origin of this response.

**Inhibitory effects of PTN afferents.** Previous studies in animals have shown that PTNS can elicit bladder-inhibitory effects at various stimulation frequencies. In cats, reflex bladder inhibition was demonstrated by PTNS applied 5, 20, and 30 Hz (37, 38), whereas in rats, similar responses were evoked by PTNS applied at 5 Hz (33) and 10 Hz (17). These results were obtained using experimental models based on isovolumetric bladder activity or independent urodynamic bladder fills. In contrast, the present study used a continuous bladder filling model (repeated urodynamic fills) that provided sensory input via pelvic and/or pudendal nerve afferents that persisted both
during and after each nerve stimulation trial. Although the present study did not consider 30-Hz stimulation (38), we were able to characterize bladder-inhibitory reflexes by systematically varying the stimulation frequency applied to each neural target: 5 Hz (PTNS and MPNS), 10 Hz (PTNS, MPNS, and LPNS), and 20 Hz (LPNS only).

Considerations of PTNS amplitude. In a recent study by Su et al. (33), bladder-inhibitory reflexes in anesthetized rats were evoked only in response to electrical pulses applied at 3T_m. This response pattern is particularly interesting given that all other studies (including this study) demonstrated changes in bladder function at stimulation amplitudes of 4T_m or greater (17, 36, 38). Among the various factors that can be attributed to this discrepancy, both the nerve electrode type (nerve cuff vs. insulated wires) and method used to determine foot twitch threshold are identified as important variables. In our study, T_m was defined by the fEMG measured with an oscilloscope, whereas other studies determined the threshold by visually observed foot twitches. As a consequence, our typical T_m value (0.02 mA) was much lower than that of the Su et al. study (0.16 mA), which translated to PTNS trials applied at markedly different amplitudes of 0.1 mA (6T_m) and 0.5 mA (3T_m), respectively. Given the much higher levels of electrical current delivered by percutaneous wire electrodes, it is difficult to predict the number and type of sensory fibers that were recruited during stimulation. This likely contributed to the in-

Fig. 8. Summary of the percentage of experiments that exhibited acute stimulation-evoked bladder inhibition (10% decrease in aBCR). A: PTNS. The response rate of animals that exhibited statistically significant bladder inhibition ranged from 55% (5 Hz, 6 of 11 rats) to 67% (10 Hz, 8 of 12 rats). Although 20-Hz PTNS caused bladder inhibition in 58% of experiments (7 of 12 rats), the overall reduction in aBCF was not significant. B: MPNS. The response rate of animals that exhibited statistically significant bladder inhibition was 100% (10 Hz, 8 of 8 rats). C: LPNS. The response rate of animals that exhibited statistically significant bladder inhibition was 66% (4 of 6 rats) at both 10 and 20 Hz.

Fig. 9. Summary of the percentage of experiments that exhibited prolonged stimulation-evoked bladder inhibition (10% decrease in aBCR). A: PTNS. The response rate of animals that exhibited statistically significant bladder inhibition was 81% (5 Hz, 9 of 11 rats). B: MPNS. The response rate of animals that exhibited statistically significant bladder inhibition was 75% (6 of 8 rats) at both 5 and 10 Hz. C: LPNS. The response rate of animals that exhibited statistically significant bladder inhibition ranged from 84% (20 Hz, 5 of 6 rats) to 100% (10 Hz, 6 of 6 rats).
consistent bladder responses observed across the different studies.

**Functional role of different fiber types.** During initial PTNS trials applied at stimulation amplitudes below 6T$_{m}$, we recorded the electrical recruitment of large-diameter fibers but did not observe any changes in ongoing bladder or EUS activity (15). At amplitudes of 200T$_{m}$ and above, we observed evoked potentials that exhibited peak-to-peak amplitudes and poststimulation latencies that were consistent with that of C-fibers (data not shown). Our nerve fiber recruitment data were consistent with that reported by Sato et al. (29), where virtually identical stimulation parameters (bipolar, 0.2-ms pulse width) and stimulation-recording electrode configuration (interelectrode distance: 2 cm) were used to examine the recruitment of different nerve fiber types in the hindlimb of cats. The authors concluded that small-diameter Aδ-fibers (group III) were activated in the 4–10T$_{ENG}$ range, whereas C-fibers (group IV) were activated in the 100–200T$_{ENG}$ range, the latter of which was consistent with our C-fiber activation threshold (200T$_{m}$). Accordingly, these results suggest that PTNS-evoked bladder inhibition requires the electrical recruitment of small Aδ-fibers, with very limited need for activating unmyelinated C-fibers.

Interestingly, McPherson et al. (20) reported reflex bladder excitation in spinally intact anesthetized cats that was evoked by applying ice to the hindpaws. The resulting decrease in the interval between bladder contractions was attributed to unmyelinated C-fibers (tonic nociceptors) that were activated by this low-temperature input. Assuming a similar bladder-excitatory reflex, which is mediated by high-activation threshold C-fibers, also exists in rats, this may partially explain the diminished effectiveness of PTNS observed at much higher (above 3T$_{m}$) stimulation amplitudes (33).

**PTNS-mediated excitation of the bladder.** This study also presents evidence of a bladder-excitatory reflex that is elicited by higher-frequency (50 Hz) stimulation of myelinated PTN fibers, localized specifically within the LPN. Given the electrical recruitment properties of unmyelinated C-fibers in mammals (50), it is highly unlikely that this reflex was evoked by the mechanism reported by McPherson et al. (20). The frequency at which this excitative reflex was evoked is clearly higher than the typical values (10–30 Hz) used to elicit bladder inhibition by PTNS (34, 36). In fact, it is also higher than the broad range of frequencies (2–35 Hz) shown to excite stimulation-evoked bladder contractions mediated by the pudendal nerve trunk (3), dorsal genital nerve (47), or urethral sensory branch (51).

A potential factor contributing to this excitatory reflex involves the experimental setup. Unlike previous work that used isovolumetric conditions (5, 16, 30, 31, 33, 35–38), our study used continuous (i.e., urodynamic) bladder infusion that provided constant and/or periodic neural feedback via pelvic and pudendal afferents during each repeated bladder filling and voiding. We speculate that the pudendal afferents may have played a significant role in unmasking this otherwise quiescent 50-Hz excitatory reflex. This hypothesis is supported by previous work of Peng et al. (24), where significant reduction of bladder voiding efficiency in anesthetized rats was demonstrated after transection of the pudendal sensory branch. (24).

Anatomically, a potential interaction between PTN and pudendal afferents is also suggested by the work of Pacheco et al. (21, 22), where the authors showed that the lumbosacral trunk nerve (LSN) and L6-S1 nerve are common to both the pudendal (primarily L6-S1) and sciatic (primarily LSN) nerves. More specifically, in addition to the LSN consisting of contributions from L4-L6 and therefore sharing the L6 contribution with the L6-S1 nerve more distally, the LSN also contributes a branch to the pudendal nerve and its corresponding sensory branch before continuing on to become the sciatic nerve and eventually the tibial nerve. This suggests evidence for an anatomic mechanism through which pudendal afferent nerves can converge and interact with the stimulus arising from the PTN or its distal branches. Further investigation of the interaction between pudendal afferents and LPNS-evoked bladder reflexes could provide important insights into this reflex and potential clinical treatment of detrusor underactivity (40).

**Specific PTN afferents reflexively inhibit bladder function.** Among the multitude of bladder responses elicited by selective PTNS, as depicted by the percentage change in aBCF and the overall response rates, the overall results of this study indicate that there are two primary bladder-inhibitory pathways mediated by PTN afferents: acute bladder inhibition evoked by selective MPNS at 10 Hz and prolonged bladder inhibition evoked by selective LPNS at 10 Hz. Not only did both responses achieve statistical significance, but these inhibitory reflexes were also observed in every experiment that tested selective PTNS. The consistency with which these bladder-inhibitory responses were observed suggest that these involve “hard-wired” neural pathways, similar to those elicited by pudendal nerve (or dorsal genital nerve) stimulation (10, 39, 47).

When bladder responses evoked by PTNS to those of MPNS/LPNS at specific stimulation frequencies are compared, there are cases where a particular response can be attributed to one or both nerve branches. For example, prolonged bladder inhibition achieved by 5-Hz PTNS appears to be mediated by the MPN, and acute bladder inhibition evoked by 10-Hz PTNS appears to involve afferent inputs from both the MPN and LPN. However, there are inconsistencies that suggest the individual responses elicited by selective LPNS/MPNS do not necessarily sum in a linear fashion. This is illustrated by the prolonged bladder-inhibitory responses evoked by 10-Hz stimulation. Here, PTNS failed to elicit a postsimulus response (Fig. 3), yet both selective LPNS and MPNS resulted in robust bladder inhibition (Figs. 6 and 7). In a similar fashion, 5-Hz PTNS resulted in acute bladder inhibition, but neither 5-Hz LPNS nor MPNS managed to evoke a significant response. While the unpredictable variability of these responses might suggest a complex interaction of sensory inputs within the central nervous system, which is further reflected by the wide range of response rates (64–82%; Figs. 8 and 9), this may also be attributed to prolonged bladder responses affecting subsequent stimulation trials. Despite our efforts to minimize potential carryover effects, by both randomizing the stimulation parameters and limiting the duration of each trial to 10 min, the experimental design may not have completely accounted for this variable. Better characterization of the role of each afferent input will be important for investigating the central nervous mechanisms that regulate these bladder-modulatory pathways.

**Clinical implications.** The results of this study suggest that the current PTNS protocol used to treat patients with OAB may be significantly enhanced by modifying the stimulation param-
eters. This approach is similar to that implemented by Inter-
Stim therapy, where sacral nerve stimulation is adjusted over a 
range of frequencies (10–20 Hz) and amplitudes to optimize 
the therapeutic effects (23). Given the consistent evidence 
of poststimulus bladder-inhibitory responses reported both in this 
study (bladder inhibition lasting up to 0.5 h) and previous 
animal experiments (5, 17, 38), it appears that electrical pulses 
applied at 5 or 10 Hz may potentially achieve more effective 
long-term treatment of OAB symptoms.

However, all preclinical data, including those from this 
study, indicate that maximum inhibition of bladder function 
requires the stimulation amplitude to exceed the foot motor 
threshold. Our electrophysiological evidence shows that this 
involves very small-diameter (Aδ) fibers that are activated by 
stimulus amplitudes equal to or greater than three times this 
threshold amplitude. Unfortunately, these high levels of nerve 
activation exceed the stimulus intensity that is typically toler-
ated by patients. Further work is needed to determine whether 
this therapeutic limitation can be circumvented, for example, 
by lower stimulation frequencies, which may potentially pro-
vide a more tolerable sensation to patients undergoing PTNS 
therapy (18).

Clinical studies are also needed to elucidate the potential 
therapeutic effects of selective activation of individual 
branches of the PTN (e.g., the MTN and LTN). More specifi-
cally, given the high response rate (100%) achieved by 10-Hz 
LPNS and MPNS, we recommend these neural targets and 
frequencies as a starting point for clinical translational studies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.K. and P.B.Y. performed experiments; M.K. and 
P.B.Y. analyzed data; M.K. and P.B.Y. interpreted results of experiments; 
M.K. prepared figures; M.K. drafted manuscript; M.K. and P.B.Y. edited and 
edited the manuscript; M.K. and P.B.Y. approved final version of manuscript; 
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