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Research article

Spinal mechanisms of pudendal nerve stimulation-induced inhibition of bladder hypersensitivity in rats

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ABSTRACT

Bilateral electrical pudendal nerve stimulation (bPNS) reduces bladder hypersensitivity in rat models of bladder pain and anecdotally reduces pain in humans with pelvic pain of urologic origin. The spinal neurochemical mechanisms of this antinociception are unknown. In the present study, bladder hypersensitivity was produced by neonatal bladder inflammation in rat pups coupled with a second inflammatory insult as an adult. Visceromotor responses (VMRs; abdominal muscle contractions) to urinary bladder distension (UBD) were used as a nociceptive endpoint under urethane-isoflurane anesthesia. bPNS consisted of bilateral biphasic electrical stimulation of the mixed motor/sensory component of the pudendal nerves. Following determination of the inhibitory effect of bPNS on VMRs, pharmacological antagonists were administered via an intrathecal catheter onto the lumbosacral spinal cord and bPNS effects on VMRs redetermined. bPNS resulted in statistically significant inhibition of VMRs to UBD in hypersensitive rats that was statistically reduced by the intrathecal administration of methysergide, WAY100636, CGP35348 and strychnine but was unaffected by naloxone, bicuculline, phentolamine, ondansetron and normal saline. This study suggests that inhibitory effects of bPNS may include serotonergic, GABA-B-ergic and glycinergic mechanisms suggesting the potential for interaction of the neuromodulatory effect with concommitant drug therapies.

1. Introduction

Bilateral electrical pudendal nerve stimulation (bPNS) has been demonstrated to inhibit nociceptive responses to urinary bladder distension (UBD) in rats made hypersensitive to bladder stimuli [1]. Coupled with multiple anecdotal and case series reports in which the use of nerve stimulation resulted in improved pain control in people with the diagnosis of interstitial cystitis/bladder pain syndrome (IC/BPS) [2–4] these findings suggest the need for a controlled clinical trial examining this therapeutic modality. Previous studies related to the spinal mechanisms of pudendal nerve stimulation have suggested a link to multiple inhibitory neurotransmitters including opioids, serotonin, glycine and GABA [5–10]. However, these other studies were predominantly in feline models and/or studied systemic drug effects (rather than spinal) using cystometric measures as primary endpoints rather than models more commonly associated with nociception. Further study appears warranted as concomitant drug use which could alter these inhibitory systems, could also potentially alter clinical responses to the pain-relieving effects of this manipulation.

In our previous studies we identified optimal stimulation parameters and sites of electrical stimulation for the pudendal nerves (which arise from the same spinal segments as the lumbosacral nerves which contain afferents from the bladder) [1]. We studied these effects in a model of bladder hypersensitivity in which rats experience neonatal bladder inflammation and then receive a second bladder inflammatory challenge as adults [11]. It is thought that this model may be particularly relevant to the classic form of IC/BPS [12], in that it is associated with multiple features of IC/BPS including the presence of increased micturition rates, altered cystometry indicating a functionally small capacity, hypersensitive bladder, altered bladder neurochemistry, the presence of vascular fragility of submucosal tissues following prolonged hydrodistention, the presence of increased pelvic floor muscular

Abbreviations: 5HT, 5-hydroxytryptamine (serotonin); AUC, Area-Under-the-Curve; bPNS, bilateral pudendal nerve stimulation; GABA, gamma amino butyric acid; IC/BPS, interstitial cystitis/bladder pain syndrome; i.v., intravenous; s.c., subcutaneous; T, stimulation intensity threshold; UBD, urinary bladder distension; VMR, visceromotor response

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tone and increased responsiveness to intravesical cold and potassium-containing fluids [11,13–16]. Using a before-after design, the following study examined the role of known spinal inhibitory neurotransmitters in the effects of bPNS by intrathecally administering selective antagonists to receptors activated by these substances.

2. Materials and methods

2.1. Animal subjects/anesthesia

All studies were approved by the UAB Institutional Animal Care and Utilization Committee. Subjects were adult, female Sprague-Dawley rats, raised from birth; the maternal animal source was Harlan Laboratories (Sprattville, AL). For all surgical procedures or pretreatments, rats were deeply anesthetized using deep isoflurane (1–3%) inhaled anesthesia. In order to measure EMG responses, rats were maintained in a lightly anesthetized state (urethane 1.2 gm/kg s.c. with 0-0.5% isoflurane in oxygen delivered by tight-fitting mask within a ventilation hood.) In these experiments isoflurane anesthesia was lowered (typically to $\leq$0.2%) until flexion reflexes were present in the hind limbs, but spontaneous escape behaviors were absent.

2.2. Pretreatments producing bladder hypersenstivity

Following a previously published protocol [11], rat pups, on three consecutive days (Postnatal Days P14-P16), were anesthetized with inhaled isoflurane, treated with s.c. ampicillin, swabbed with an iodine/povidone solution and had their urethras cannulated using a 24 gauge angiocatheter; intravesical zymosan (1% in saline, 0.1 ml) was then instilled for 30 min. They were kept warm on a heating pad and returned to their mothers following this treatment. As adults (12–15 weeks of age), these same rats received additional pretreatments one day prior to their terminal experiments. They were similarly anesthetized with inhaled isoflurane, ampicillin and iodine/povidone solution. They had their urethras cannulated using a 22 gauge angiocatheter and intravesical zymosan (1% in saline, 0.5 ml) was instilled for 30 min. They were allowed to recover from anesthesia and studied 24 h later for bPNS effects.

2.3. Pudendal nerve electrical stimulation

As previously reported [1], while deeply anesthetized with inhaled isoflurane, the mixed motor/sensory pudendal nerves were exposed immediately after they exited the lumbosacral plexus as they started to pass anteriorly into the pelvis. Pudendal nerves had double hook electrodes placed around each nerve which were held in place with polysiloxane gel. Grounding electrodes were placed dorsally for all rat groups. Electrical stimuli consisted of trains of biphasic pulses (100 μsec; 10 Hz) delivered at 1 T or 3 T (1 x or 3 x motor threshold, T; the minimal current needed to evoke any observable skeletal muscle contraction). Stimulation intensities to each side were adjusted independently and were typically in the range of 0.5–1.0 mA.

2.4. Measure of VMRs to UBD

The abdominal muscle electromyographic (EMG) activity to UBD has been widely used to evaluate bladder pain-related responses. While lightly anesthetized with a combination of injected urethane and inhaled isoflurane, VMRs remain remarkably stable over time [1,17]. For this measurement, electrodes (silver wire) were inserted into the left lower external oblique musculature immediately superior to the inguinal ligament. Contraction of the abdominal musculature, recorded as EMG activity, was measured via the electrodes using standard differential amplification and rectification and saved on a computer as quantified as previously described (Grass Inc, P511 AC amplifiers; 50x_amplification, 60 Hz clipping, low filter setting 10 Hz–high filter setting 3 kHz) [1]. Following surgery for nerve stimulation and intrathecal catheter placement (described below), a 22-gauge polytetrafluoroethylene angiocatheter was placed into the bladder via the urethra and held in place by a tight suture around the distal urethral orifice. Following surgery, UBDs for 20 s were produced using compressed air, and intravesical pressure was monitored using an in-line pressure transducer. Approximately 15 min after initial anesthesia induction, EMG activity to repeated presentations of 60 mm Hg UBD at 3-minute intervals was recorded until responses to UBD were stable (+/−20%).

2.5. Intrathecal drug protocol

Using the technique of Yaksh and Rudy [18] the atlanto-occipital membrane was incised allowing a 7.5 cm PE10 catheter to be inserted and advanced to the level of the lumbosacral spinal cord. Two rats demonstrating neurological deficits after this insertion were discarded from additional study. Since it would be difficult to observe IT drug effects on bPNS-induced inhibition unless such an inhibitory effect of bPNS were present and due to the complex nature of the experimental preparation and fragility of the pudendal nerves which can lead to technical failures of the preparation, a subset of rats (64 of 72 rats in the total sample) which demonstrated robust inhibitory effects of bPNS were selected for further pharmacological study. All of these rats were characterized for the effects of bPNS on VMRs evoked by graded UBD (10–60 mm Hg, 20 s with 1 min inter-trial periods). The following drugs were then administered intrathecally in a 10 μl volume followed by a 10 μl normal saline flush: naloxone hydrochloride (10 μg; a nonselective opioid receptor antagonist), bicuculline methiodide (0.5 μg; a GABA-A receptor antagonist), CGP35348 (30 μg; Tocris Pharmaceuticals, a GABA-B receptor antagonist), methysergide maleate (30 μg; a nonselective serotonergic antagonist), WAY100636 maleate (10 μg; a 5 H T1A receptor antagonist), ondansetron HCl (10 μg; a 5 H T3 receptor antagonist), phentolamine HCl (30 μg; a nonselective alpha adrenoceptor antagonist), strychnine HCl (1 μg; a glycinergic receptor antagonist) and normal saline (vehicle). The source of drugs utilized was Sigma/Aldrich (St. Louis, MO) unless otherwise noted. The doses employed were selected based upon published literature in rats [e.g.,17,18,19,20,21,22,23].

2.6. Study protocol

The upper panel of Fig. 1 is a diagrammatic description of the study. Rats which had experienced the bladder inflammatory pretreatments described above were prepared surgically and allowed to establish stable responses to UBD. Briefly, using the same protocol as previously published [1], the effects of bPNS on the VMRs to three repeated 60 mm Hg, 20 s UBDs (3 min intertrial interval) and graded (10–60 mm Hg, 20 s) UBD were assessed after 10 min of stimulation at 1 T or 3 T intensities of bPNS and after a 10-minute period of No Stimulation (0 T). Each stimulation period was followed by a 10-minute recovery period with no bPNS. The ordering of 0 T, 1 T and 3 T measurement sessions was randomized from rat to rat. Electrical stimulation was briefly stopped prior to each UBD to avoid artifact in the EMG signal and restarted immediately following each UBD until each set of VMRs was obtained. Following completion of Pre-Drug measures, the intrathecal drug was administered and 15 min later the protocol described above was repeated and Post-Drug Measures obtained. Panels A & B in Fig. 1 graphically present an individual example of a rat treated with intrathecal methysergide maleate.

2.7. Statistics and sample size calculations

All data represents mean ± SEM unless otherwise stated. An ANOVA with Tukey's HSD post hoc tests were used to characterize pre-drug responses to bPNS. Effects of drug administration were compared

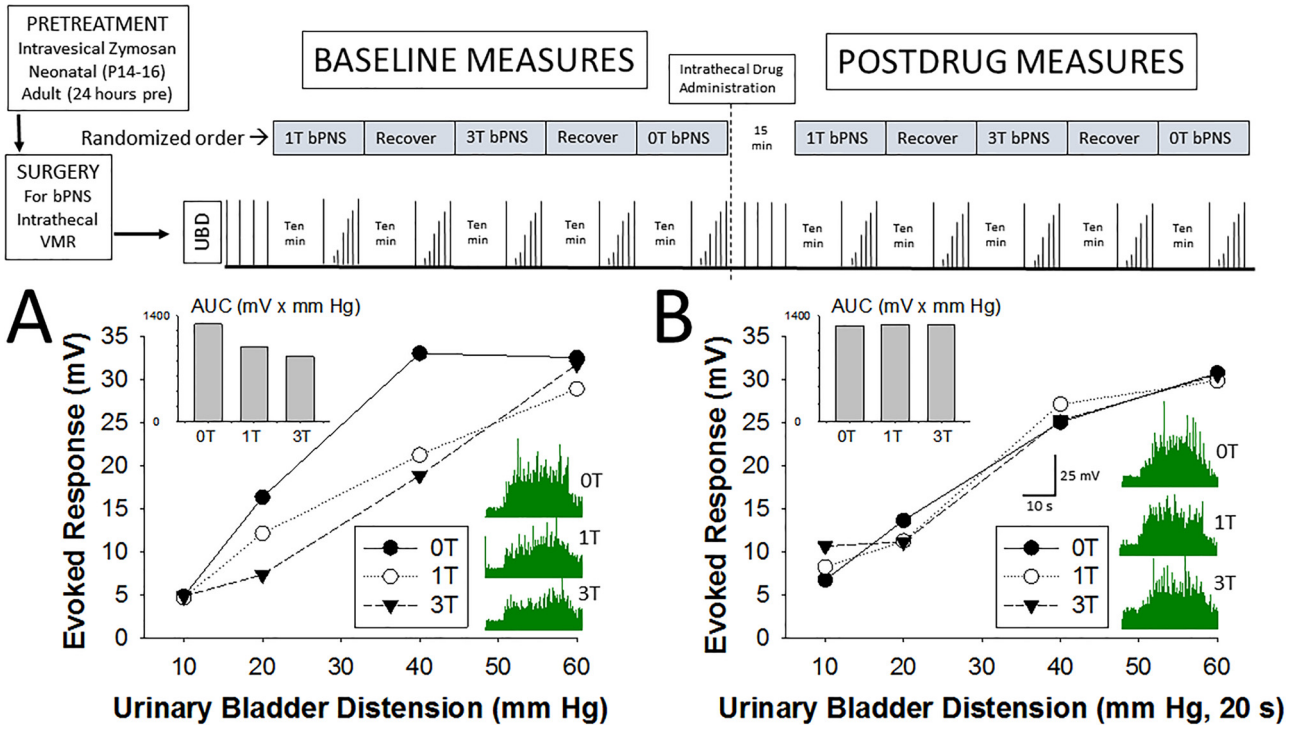

Fig. 1. Upper panel is a schematic diagram of experimental protocol. See text for greater description. Typical examples of visceromotor responses (VMRs) and the effect of bilateral pudendal nerve stimulation (bPNS) in one rat are given in panels A (before drug) and B (after drug). Drug administration was the intrathecal administration of 30 μg of methysergide maleate. Main panels are the stimulus-response functions describing the relation between urinary bladder distending pressure used a stimulus and the Evoked Visceromotor Response (increase in mean electromyographic {EMG} activity, measured in mV as the mean rectified EMG during bladder distension minus the mean rectified EMG measured in the 5 s period preceding the distending stimulus) in the absence (0 T) and presence (1 T & 3 T) of differing intensities of bPNS. Intensity of bPNS indicated as multiples of T – the minimal current needed to evoke a motor twitch). Insets of each panel describe the Area-Under-the-Curve (AUC) of these plotted stimulus response functions. At right in each panel are rectified EMG tracings related to the VMRs evoked by a 40 mm Hg, 20 s urinary bladder distension coupled with/without bPNS at the intensity indicated.

using paired Student's t-tests where appropriate and in some cases, analyzed as categorical using a sign test (Graph Pad Software, La Jolla, CA). Areas-under-the-Curve (AUCs) were calculated from stimulus-response functions to graded VMRs using the urinary bladder distending pressure as the independent variable and the evoked visceromotor response (quantified as the increase in mean rectified electromyographic voltage during the distension period when compared with the 5 s period prior to distension). These AUCs were then normalized as percentages of the 0 T (No Stim) responses from the same session.

3. Results

3.1. bPNS inhibits VMRs to UBD

Graded VMRs evoked by graded UBD were inhibited by bPNS in a reliable fashion in the 64 rats used to study intrathecal drug effects. Fig. 2 demonstrates the statistically significant effect of bPNS at multiple intensities of UBD. An overall ANOVA of such a large sample demonstrated statistical significance of the effect to $P < 0.0001$ with surprisingly, no intensity-dependent effect. This is reflected in the AUC data (inset of Fig. 2): when normalized to the VMR evoked without stimulation (No Stim) of the pudendal nerves, bPNS at 1 T and 3 T intensities had virtually identical effects. As a consequence, the mean of the two measurements was utilized in subsequent statistical analysis of the bPNS effect.

3.2. Effects of intrathecal drugs on baseline VMRs

Results are summarized in Table 1. Following phentolamine, WAY100636 and strychnine administration, the AUC measures of the

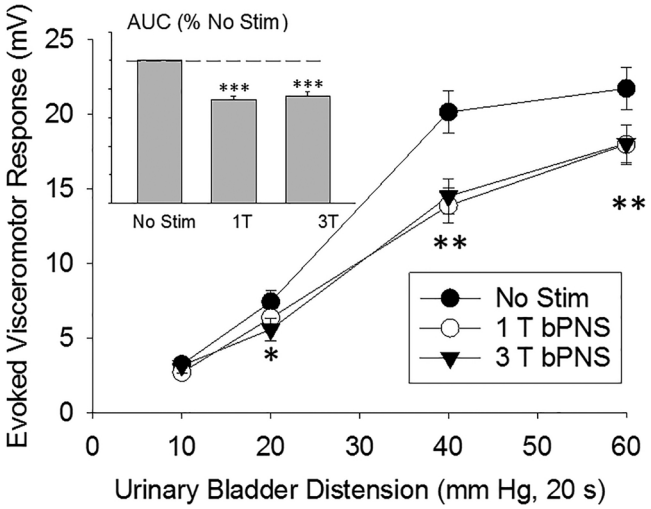

Fig. 2. Effect of bilateral pudendal nerve stimulation (bPNS; 10 Hz, 100 μsec biphasic pulses) at intensities 1 and 3 times the minimal current to evoke motor responses (1 T and 3 T respectively) on visceromotor responses to graded, phasic urinary bladder distension in rats. bPNS was briefly interrupted prior to each distension. Inset represents Area-Under-the-Curve (AUC) measures of the stimulus-response functions displayed in the larger graph normalized to the measures obtained in the absence of bPNS (No Stim; 0 T). *, ** and *** indicate significant changes from the No Stim measure with $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. N = 64. Notably, there were no intensity-dependent effects noted at these intensities.

Table 1
Effect of Intrathecal Drugs on AUC of VMRs to UBD.

DRUG (dose)	n	Post Drug VMR with No Stim (% PreDrug VMR)	Effect of bPNS on Pre Drug VMR (% Change from No Stim)	Effect bPNS on VMR Post Drug (% Change from No Stim)
Normal Saline (10 μl)	7	107 ± 13%	−17 ± 3%	−19 ± 3%
Naloxone (10 μg)	7	96 + 10%	−19 + 5%	−26 + 3%
Phentolamine (30 μg)	6	**74 + 11%***	−17 + 2%	−25 + 11%
Methysergide (30 μg)	7	90 + 13%	−21 + 4%	**+8 + 13%***
WAY100635 (10 μg)	7	**56 + 11%***	−30 + 4%	**−3 + 9%***
Ondansetron (10 μg)	7	82 + 8%	−25 + 3%	−20 + 5%
Bicuculline (0.5 μg)	6	**138 + 12%***	−28 + 6%	−26 + 4%
CGP35348 (30 μg)	9	79 + 9%	−43 + 8%	**+7 + 13%***
Strychnine (1 μg)	8	**69 + 8%***	−22 + 4%	**+22 + 12%***

See text for quantification of visceromotor responses (VMRs) and Area-Under-the Curve (AUC).
Data represents mean + SEM. Bold notation indicates significant drug effect.
* indicates data are statistically different from pre-Drug measure; $p < 0.05$; paired *t*-test or sign test.

0 T VMRs (obtained in the absence of bPNS) decreased significantly suggesting tonic excitatory effects involving alpha adrenergic, 5 H T1A-ergic and glycinergic mechanisms on this visceral nociceptive measure. Although not formally studied here, it was notable that rats otherwise appeared hyper-responsive to mechanical stimulation of the hindpaw or back skin with robust flexion reflexes that could be evoked by minimal mechanical stimuli in the strychnine-treated rats. In contrast, the AUC measures of the 0 T VMRs following bicuculline were significantly greater following drug administration suggesting the potential for a tonic GABA-A-ergic inhibitory effects at a spinal level. The hyper-excitable flexion reflexes noted in the strychnine-treated rats was also present in the bicuculline-treated rats, but not in any other treatment groups. Similar “allodynia” measures related to flexion reflexes have been reported by multiple other investigators [e.g. [20],].

3.3. Effects on inhibition produced by bPNS

Results are summarized in Table 1. Statistically significant inhibition of VMRs continued to be produced by bPNS in rats treated intrathecally with naloxone, phentolamine, ondansetron, bicuculline and normal saline control groups. However, treatment with intrathecal methysergide, WAY100636, CGP35348 and strychnine abolished effects of bPNS.

4. Discussion

The most important finding of the present study is that intrathecal serotonergic, GABA-B-ergic and glycinergic receptor blocking agents reduced the effects of bPNS on VMRs in rats with bladder hypersensitivity. In addition, tonic inhibitory effects of GABA-A mechanisms were noted and tonic excitatory effects of alpha adrenergic, serotonergic and glycinergic mechanisms were observed. These findings suggest the potential for drug interactions when concomitant medications are being utilized which could potentially confound the ability to study bPNS effectiveness for the treatment of pelvic pains of urological origin. Subsequent studies of specific drugs with mechanisms involving these systems that are used in the treatment of urological disorders may be needed prior to clinical trials.

The simplest theoretical basis for the observed effects of bPNS are those of gate-control theory [24] in which same-segmental neuromodulatory inputs activated segmental inhibitory mechanisms. There is extensive evidence that GABA and glycine are the predominant inhibitory neurotransmitters altering segmental spinal reflexes. It is notable that there were effects of strychnine treatment on baseline VMRs suggesting potentially tonic excitatory effects of glycine in these hypersensitive rats. Gate control theory has required modification through the years to account for additional supraspinal modulatory mechanisms and similarly, it is necessary to propose that descending serotonergic mechanisms may be involved in the bPNS-induced inhibition given the effects of methysergide and WAY100636. It is notable, that not all serotonin receptor antagonists (e.g. ondansetron) were effective and the pharmacology associated with serotonergic mechanisms is complex (e.g., methysergide has 5 H T1A agonist actions, but produces 5 H T2B and 5 H T2C antagonism). Thus, the precise definition of serotonergic mechanisms has yet to be defined.

Previous studies have identified similar pudendal nerve stimulation-induced inhibition of bladder reflexes in cats [e.g. 25], although the pharmacological results differed somewhat from the present studies in rats. This can relate to afferent versus efferent effects/measures of the model systems and associated species differences as well as methodological differences such as the spinal delivery of these drugs (doesn’t address potential supraspinal effects) and the use of urethane/isoflurane for anesthesia in the present experiments which can alter many measures. Whereas Chen et al [8] noted that i.v. naloxone suppressed effects of pudendal nerve stimulation, the present study did not observe any effect of intrathecal naloxone on bPNS effects. Schwen et al [6] noted similar suppressive effects of i.v. ondansetron however, the present study did not observe similar effects of this drug given intrathecally in rats. In contrast, Fuller et al [9] demonstrated no effects of i.v. GABA-B receptor antagonists on pudendal nerve stimulation induced inhibition, yet, the present study observed robust suppression of the inhibitory effects of bPNS. McGee et al [25] observed robust effects of the GABA-A receptor antagonist, picrotoxin, on pudendal nerve stimulation-induced inhibition of bladder contractions in cats, as well as robust effects of alpha adrenergic receptor antagonists, but not naloxone. Findings comparable to the present study were observed in other feline studies employing strychnine [10,25] and methysergide [11]. Randich et al [21], in rats, observed tonic serotonergic and alpha-adrenergic influences on VMRs similar to those observed in the current study following acute inflammation of the bladder. Notably, in their study, they also noted effects of ondansetron which were not observed in the present study. In summary, when comparing the present study to those of others, many commonalities of mechanisms were identified, but differences in species, specific model system and endpoints all result in measures that are unique to each study. However, the important take home message from all of these studies are that additional drug therapies could impact on the efficacy of bPNS as an analgesic therapeutic.

The GABAergic mechanisms of antinociception have their own complex pharmacology: GABA-A receptors are inhibitory ion channels and GABA-B receptors are G-protein coupled receptors with tissue dependent intracellular effects (typically Gi-related). The observation of the present study that spinal GABA-B receptor antagonism blocks inhibitory effects of bPNS argues for the presence of GABA-B receptors that could have analgesic properties and suggest potential benefits from intrathecal GABA-B agonists, such as baclofen, as therapeutic interventions for pelvic pains of urological origin. Given that baclofen is one of the few drugs approved for intraspinal infusion makes this

observation of even greater clinical significance. Spinal GABA-B-linked mechanisms have been observed in multiple investigations of both segmental and heterosegmental effects, as well as those produced by spinal cord stimulation [e.g., 22,23]. These observed effects fit well within the theoretical framework of gate-control theory.

5. Conclusions

The present pharmacological investigation of mechanisms associated with bPNS and its inhibitory effect on bladder hypersensitivity further extends previous studies that systematically examined the parameters and sites of stimulation that can produce neuromodulatory effects in pelvic sensory systems. Given the involvement of numerous spinal neurotransmitter systems, it will be important to assess the effects of concomitant pharmacotherapy when attempting to treat painful bladder disorders. In particular, based on the present study, pharmacological agents which might have actions at GABAergic, serotonergic and/or glycinergic receptors would be expected to have a high potential to alter the efficacy of neuromodulatory treatments.

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