Convergence and Cross-talk In Urogenital Neural Circuitries.

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Running Head:  Urogenital inputs to rostral medulla

Number of Pages: 32
Number of Figures: 8
Number of Tables: 1

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ABSTRACT

Despite common co-morbidity of sexual and urinary dysfunctions, the interrelationship between the neural control of these functions are poorly understood. The medullary reticular formation (MRF) contributes to both mating/arousal functions and micturition, making it a good site to test circuitry interactions. Urethane-anesthetized adult Wistar rats were used to examine the impact of electrically stimulating different nerve targets (dorsal nerve of the penis or clitoris - DNP / DNC; L6/S1 trunk) on responses of individual, extracellular-recorded MRF neurons. The effect of bladder filling on MRF neurons was also examined as was stimulation of DNP on bladder reflexes via cystometry. In total, 236 MRF neurons responded to neurostimulation: 102 to DNP stimulation (12 males), 64 to DNC stimulation (12 females), and 70 to L6/S1 trunk stimulation (12 males). Amplitude thresholds were significantly different at DNP (15.0 ± 0.6 µA), DNC (10.5 ± 0.7 µA) and L6/S1 trunk (54.2 ± 4.6 µA), whereas similar frequency responses were found (max responses near 30-40 Hz). In 5 males, filling/voiding cycles were lengthened with DNP stimulation (11.0 ± 0.9 µA), with a maximal effective frequency plateau beginning at 30 Hz. Bladder effects lasted around two minutes after DNP stimulus offset. Many MRF neurons receiving DNP/DNC input responded to bladder filling (35.0% and 68.3%, respectively), either just before (43%) or simultaneously with (57%) the voiding reflex. Taken together, MRF-evoked responses with neurostimulation of multiple nerve targets along with different responses to bladder infusion has implications for the role of MRF in multiple aspects of urogenital functions.

Keywords: pudendal nerve, medullary reticular formation, penis, clitoris, bladder
INTRODUCTION

Sexual dysfunction is a common public health concern that affects approximately a third of men and two-fifths of women (Dunn et al. 1998; Laumann et al. 1999). Sexual dysfunction takes on many forms with varying degrees of prevalence; for example, erectile dysfunction increases in prevalence with age (Beutel et al. 2006). Importantly, a substantial number of published studies have related comorbidity of sexual dysfunctions with other diseases/conditions, such as overactive bladder (Heidler et al. 2010), chronic kidney disease (Holley and Schmidt 2010), voiding symptoms and nocturia (Ponholzer et al. 2004), benign prostatic hyperplasia (Rosen et al. 2005), and other conditions, like diabetes, hypertension, stroke, and psychiatric disorders (Beutel et al. 2006). The purpose of the present study is to assess the interactions between neural circuitries mediating sexual and bladder function, as a therapy directed toward one of these organ systems could likely affect the other.

Studies targeting the interactions between male and female urogenital neural circuitries have been limited, despite the very high incidence of sexual disorders. The neural circuitries controlling ejaculation and voiding are very complex (Allard et al. 2005; Calabro et al. 2011). Ejaculation is dependent on myelinated penile afferents in the pudendal nerve (DNP – dorsal nerve of the penis) to the lumbosacral cord in rats (Hart and Leedy 1985; Johnson 2006; Wieder et al. 2000). Current views propose that a spinal center for ejaculation in the lumbar cord is somewhat capable of mediating the emission and expulsion of semen following acute (minutes to a few hours) but not chronic (30-60 days) mid-thoracic cord transection (Allard et al. 2005; Coolen et al. 2004; Truitt and Coolen 2002). The loss of inhibitory brainstem control of coordinated
pelvic striated muscle contractions via pudendal motoneurons results in loss of ejaculatory ability (Coolen et al. 2004; Giuliano and Rampin 2004; Johnson 2006).

Similarly, bladder function involves lumbar sympathetics, sacral parasympathetics and a somatic supply to the external sphincter (Fowler et al. 2008; Sengupta and Gebhart 1995). Bladder afferents travel to the spinal cord via the pelvic (primarily parasympathetic) and hypogastric (primarily sympathetic) nerves (Jancso and Maggi 1987; Pascual et al. 1993). Various techniques have characterized bladder afferents, spinal cord interneurons and intermediate zone receiving bladder input in rats (e.g., (Cadden and Morrison 1991; Hwang et al. 2005; Iijima et al. 2009; Wang et al. 1998)). The spino-bulbo-spinal control of micturition, involving the pontine micturition center, has also been characterized (Blok and Holstege 2000; Matsumoto et al. 1995; Nuding and Nadelhaft 1998).

The medullary reticular formation (MRF) is involved in the control of sexual function in males and females (Daniels et al. 1999; Marson and McKenna 1990; Modianos and Pfaff 1979; Schwartz-Giblin et al. 1996; Tanaka and Arnold 1993). Using microstimulation, a discrete ventrolateral MRF area in the gigantocellularis pars alpha and lateral paragigantocellularis nuclei produces an inhibitory effect on pudendal motoneuron reflex discharges (Johnson and Hubscher 1998) and produces microstimulation-elicited field potentials in pudendal motor pools (Tanaka and Arnold 1993). Bilateral lesioning of this MRF area has been shown to affect ejaculation (Marson and McKenna 1990; Yells et al. 1992), and this area receives connections from the mPOA and PAG (Murphy and Hoffman 2001) and sends bulbospinal axons to the pudendal motor spinal circuitry (Hermann et al. 2003; Marson and McKenna 1996).
Neurons in this MRF region are labeled bilaterally after unilateral injection of the transsynaptic pseudorabies virus tracer into the bulbospongiousus muscle which contributes to erection, ejaculation and micturition (Johnson et al. 2011).

MRF neurons also show high convergence of a high variety of pelvic structures. Extracellular recordings of single MRF neurons have demonstrated widespread convergent somato-visceral ascending inputs, including the lower urinary tract and external genitalia (Hubscher and Johnson 1996; Hubscher et al. 2004; Kaddumi and Hubscher 2006). Differential inhibition and excitation may lead to coordinated responses, such as inhibition of voiding and defecation circuitries during ejaculation (Kaddumi and Hubscher 2006) and cross-talk between pelvic organ systems may aid normal visceral functioning. For example, distention of the urinary bladder leads to contractions of the external anal sphincter, preventing defecation and allowing for micturition (Basinski et al. 2003) and vice versa. The convergence of inputs from the lower urinary tract on MRF circuitries mediating arousal (Wu et al. 2007) could relate to the inhibition of micturition such as during low levels (sleep) and high levels (sex) of activity. Injury or trauma leading to disruption with the coordination of any of these mechanisms can cause the injured organ to affect the functionality of the non-injured one (Kaddumi and Hubscher 2006; Malykhina 2007; Malykhina et al. 2006; Pezzone et al. 2005). Both bladder-to-colon and colon-to-bladder cross-sensitization has been linked with painful sensations (Alagiri et al. 1997; Brumovsky and Gebhart 2010; Malykhina et al. 2013; Nickel et al. 2010; Pezzone et al. 2005; Qin et al. 2005; Theoharides et al. 2008; Ustinova et al. 2006; 2010).
Electrical stimulation of various nerves has been used to inhibit and activate bladder reflexes of animals (Snellings and Grill 2012; Su et al. 2012a; Woock et al. 2010) and as a therapy area that has been designed for use with both idiopathic and neurogenic bladder disorders (Chartier-Kastler 2008; Kennelly et al. 2011; Light 1993; Middleton and Keast 2004; Schmidt et al. 1999; Thompson et al. 2010). Electrical stimulation of the DNP or dorsal nerve of the clitoris (DNC) can also be used to activate MRF neurons to mimic sexual sensory inputs (Hubscher 2006; Hubscher and Johnson 1996). Given this high level of convergence, the present study examined amplitude and frequency responsive curves of individual MRF neurons to DNP/DNC stimulation using an in vivo urethane anesthetized rat preparation. These effects were compared to neurostimulation responses evoked by a more proximal stimulation site of the L6/S1 trunk. Lastly, the effects of DNP neurostimulation on voiding were determined followed by the examination of DNP/DNC-responsive MRF neuronal responsiveness to bladder distension with varying levels of neurostimulation. Further characterization of interactions between urogenital circuitries both peripherally and centrally would further our understanding of the underlying interactions which will help in the development of potential therapies for related disorders.
METHODS

Animals. A total of thirty-six Wistar rats (twenty-four males and twelve females, Harlan Sprague Dawley, Inc.) approximately 60-70 days old weighing 220-250 grams were used in this study. Each rat was housed individually in an animal room with a 12 hour light and dark cycle. All animal procedures were reviewed and approved by the Institutional Animal Use and Care Committee at the University of Louisville, School of Medicine. For the female rats, vaginal smears were taken just prior to the start of the experiments to insure that none were performed during proestrus as MRF neuronal response properties have been shown to vary during proestrus as a consequence of elevated serum levels of estradiol (Hubscher 2006). All rats were anesthetized with 50% urethane (1.2 g/kg i.p.). A catheter (PE-50) was placed in the jugular vein for anesthetic supplement (5% urethane: 0.1 ml of 0.05 g/ml urethane, as needed). The trachea was exposed and intubated for monitoring respiratory rate/end expired pCO₂ level (to monitor anesthesia depth in addition to standard reflex testing). Body temperature was monitored throughout the experiment by an esophageal probe connected to a thermometer and maintained at around 37 °C using a circulating water-heating pad.

Surgical preparation. For the transvesical bladder catheter (Maggi et al. 1986), the bladder was exposed via a midline abdominal incision through skin and musculature. A purse-string suture (4-0 Ethilon) was placed in the urothelium of the bladder dome. The tip of the PE-60 tubing was heated to form a collar ~2 mm from the end. The catheter was inserted through the bladder dome within the suture limits, secured, and connected
to an infusion pump and pressure transducer. After closing the skin with wound clips, a
dorsal incision through the gluteus superficialis and biceps femoris muscles was made
in each animal to expose the DNP/DNC (males/females, respectively) bilaterally. The
nerve was separated from the connective tissues and placed on specially fabricated
bipolar electrodes (Hubscher and Johnson 1996). In a separate group of 12 male rats,
the L6/S1 trunk on one side was also exposed proximal to the DNP where the pelvic
and pudendal branches merge – see Figure 1 in (Pastelin et al. 2008). The rat was then
mounted onto a stereotaxic device. A dorsal incision was made to gain access to the
brainstem and the dorsal surface of rostral medulla was exposed by removing part of
the occipital bone and suctioning the caudal midline portion of the overlying cerebellum
(Hubscher and Johnson 1999b; 1996; Kaddumi and Hubscher 2006).

DNP/DNC and L6/S1 Trunk Neurostimulation. Using two Grass Photoelectric Stimulus
Isolation Units (Model PSIU6; one per nerve) and a Grass S88 stimulator (Astro-Med Inc., West Warwick, RI), the stimulus intensity was initially set at 30-50 μA, 0.1 msec
duration with trains of 14 pulses at 50 PPS, 100 msec train duration, 1 train/sec per our
previous studies (Hubscher 2006; Hubscher and Johnson 1999b; 1996; Hubscher et al.
2010; Kaddumi and Hubscher 2006). To test the effect of neurostimulation on voiding in
a subset of male rats prior to electrophysiological recordings (first 5 of the 12 used),
normal saline was infused into the bladder at a rate of 0.25 ml/min to evoke voiding
contractions (Maggi et al. 1986). Once the voiding cycles were consistent (at least five
consecutive voiding events with consistent time intervals in between), the effects of
bilateral DNP neurostimulation was assessed with varying intensities to determine
amplitude response threshold. Voiding and non-voiding events were easily visualized as the animal was mounted in a Kopf small animal spinal unit (Model 900 with 980 base) using hip pins with their tail elevated. Bladder pressure readings were displayed on a calibrated BP-1 Pressure Monitor from World Precision Instrument (Sarasota, FL). Pressures and time (Fisher Scientific timer) were logged into the experimenter notes every 15 seconds as well as whenever there was a pressure change greater than 2 mmHg. Voids were logged as well (stream, single drop, or multiple drops). Pressures were recorded with a VR-10B Digital Data Recorder from Instrutech Corp (Port Washington, NY) to videotape (Sony VCR) for offline playback with Datawave software (www.dwavetech.com). Voiding cycle length and pressure changes were calculated from the tabulated log entries.

MRF Recordings. A tungsten microelectrode with $\approx 7\pm 1$ MOhms impedance (Fredrich Haer and Co., Bowdoinham, ME), coated with fluorescent dye (DiI; Invitrogen, Carlsbad, CA) (Chadha and Hubscher 2008; Massey et al. 2006), was lowered from the dorsal surface of the brainstem with a motorized drive (Fredrich Haer and Co., Bowdoinham, ME) into the MRF. Stereotaxic coordinates were 2700-3300 µm rostral to obex and 300-1400 µm lateral to midline on both sides of the brainstem. The search area for each dorso-ventral track was 2800-3000 µm in length, which included the rostral part of the nucleus reticularis gigantocellularis (Gi), Gi pars alpha (GiA), and the medial part of the lateral paragigantocellular nucleus (LPGi) (Hubscher and Johnson 1999a).
Recording of a single neuron was established by monitoring the action potential on an oscilloscope with a spike-triggered analog delay module for discrimination of somato-dendritic neuron profiles from nerve fiber spikes as described previously (Hubscher and Johnson 2006; 1999b; 1996). Once a single neuron responsive to bilateral DNP or DNC was found, stimulus frequency and amplitude were varied to assess intensity threshold as well as threshold and optimal frequency. Each stimulus parameter was tested multiple successive times to account for any response variations due to potential wind-up. Responses to bladder distention were then tested (Kaddumi and Hubscher 2007; 2006), using the transvesical PE 60 catheter attached to a syringe (manual inflation at a steady rate of approximately 4 ml/min). In addition, for the 12 female rats, MRF responses to cervix pressure were also evaluated as previously described, using a glass probe assembly device connected to a pressure transducer and monitor (Hubscher 2006). In a separate group of 12 male rats, responses to L6/S1 trunk were also tested. MRF neuronal responses were recorded to videotape and analyzed offline using Data Wave software (www.dwavetech.com).

Tissue Processing  After completion of the terminal electrophysiology experiment, each animal was euthanized with an anesthetic overdose of urethane. Perfusions were performed transcardially with 0.9% saline followed by 4% paraformaldehyde. The portion of brainstem tissue containing the recording sites was then removed, stored in a 10% formalin/30% sucrose solution, and subsequently cut on a vibratome in 50 µm sections. The dye traced electrode tracks were visualized and reconstructed as previously described (Chadha and Hubscher 2008; Massey et al. 2006).
Analysis. For the effects of neurostimulation on voiding, the data were averaged within each animal (for the different parameters tested) and then the mean values were calculated across all five rats. For the MRF recordings, group means for male and female rats were calculated based upon animal averages. Comparisons were made between groups using analysis of variance (ANOVA) with a significance level of p<.05. All values reported in the manuscript are mean ± standard error.

RESULTS

DNP-evoked MRF Responses

Single unit extracellular recordings were done in the rostral medulla under urethane anesthesia on 12 adult male Wistar rats. In total, 102 MRF neurons responded to DNP stimulation (n=12). DNP response threshold was significantly higher for neurons located within the nucleus reticularis gigantocellularus pars alpha (GiA) sub-region of the MRF (21.5 ± 2.5 µA) relative to the other MRF sub-regions (nucleus reticularis gigantocellularus [Gi]: 15.4 ± 0.8 µA; intermediate reticular nucleus: 11.7 ± 1.1 µA; dorsal para-Gi [DPGi]: 13.0 ± 1.1 µA). An example showing the response threshold for a typical Gi neuron is presented in Figure 1. Response latency was very short (< 20 msec) for 23.5% of the neurons tested, with no differences based on sub-region location (16.7% DPGi versus 26.0% in the Gi; no significant difference, p>0.05). Response latencies 20 msec and above were significantly longer for the DPGi sub-region of the MRF (292.2 ± 27.5 msec) relative to the other sub-regions (Gi: 179.2 ± 16.1 msec; GiA: 195.0 ± 33.0 msec). Neuronal location based on histological
reconstructions were made per previous studies (Hubscher 2006; Hubscher and
Johnson 1999a; 2002; 1999b; 2004; 1996; Hubscher et al. 2010; Kaddumi and
Hubscher 2006) using a standard rat atlas for sub-region specifications (Paxinos and
Watson 1998).

The majority of MRF neurons responsive to DNP neurostimulation were excited
with stimulation (64.7%), whereas 30.4% were inhibited. No significant differences were
found between MRF sub-regions. The remaining 5 (of 102) were characterized as
“mixed” (see Hubscher 2006). Two of the MRF neurons, for example, had no
background activity and were unresponsive to DNP stimulation. However, when excited
by stimulation of the face, the DNP inhibited the response (when stimulated
simultaneously). This was the case when other parts of the body, such as the
hindpaws, were stimulated (so effect not exclusive to DNP – may relate to a general
attention/orientation or arousal type of response). A total of 45.8% of the DNP-
responsive MRF neurons had spontaneous background activity. The mean activity of
those neurons was 10.9 ± 1.0 impulses per second. No significant differences were
found between the various MRF sub-regions.

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**DNC-evoked MRF Responses**

Single unit extracellular recordings were also done under urethane anesthesia on 12 adult female Wistar rats. A total of 64 MRF neurons responded to DNC stimulation. DNC response threshold was significantly higher for the Gi sub-region of the MRF (10.5 ± 0.7 µA) relative to DPGi sub-region (7.9 ± 0.8 µA), with an average threshold frequency of 20 Hz in both regions. Response latency was very short (< 20 msec) for 32.7% of the neurons tested, with a greater proportion located in the Gi (17.6% DPGi versus 45.5% in the Gi; significant difference, p<0.01). The longer latency responses had a mean latency of 186.0 ± 11.7 msec and did not differ between MRF sub regions. A typical example of a neuronal response within the Gi sub-region of female rats is provided in Figure 2. An example showing the lower DNC response threshold in DPGi is provided in Figure 3.

Of the DNC-responsive MRF neurons tested for input from the cervix (pressure), 74.5% responded (38 of 51) with a mean response pressure of 34.3 ± 2.7 mmHg (equivalent to 46.6 grams-force/centimeter²). This force is known to elevate mean arterial blood pressure by approximately 15 mmHg and heart rate by approximately 10 beats per minute in fully conscious rats (Catelli et al. 1987). The low response pressures are consistent with the rats’ estrous phase (vaginal smears taken just prior to the start of the electrophysiological recordings) (Hubscher 2006). Elevated threshold
during proestrus due to increased serum estradiol has been related to aspects of mating (see Discussion in (Hubscher 2006)).

Gender Differences in MRF Responses

When compared with MRF recordings in male rats (n=12), those in the females had significantly lower response thresholds for stimulation of the DNC versus the DNP in male rats (p<0.001), a significant difference that was found in both Gi and DPGi MRF sub-regions. Note that no DNC-responsive neurons were found in the Gi-pars alpha sub-region, versus 7% in males (DNP). In addition, maximal response frequency was similar for stimulation of the DNC in female and DNP in male rats (30 – 40 Hz). An example from each is provided in Figures 4 and 5, respectively. Although no overall significant differences in MRF response latencies were found for DNP versus DNC stimulation (p>0.05), the response latencies for the DPGi sub-region differed significantly from Gi but in different ways. In female rats, a smaller proportion of short latency DPGi neuronal responses were found relative to Gi, whereas in the DPGi of male rats, latencies above 20 msec were significantly longer relative to Gi.
Single unit extracellular recordings were done in the rostral medulla under urethane anesthesia on 12 additional adult male Wistar rats. In total, 70 MRF neurons responded to unilateral (left side) stimulation of the L6/S1 trunk (n=12). Mean response threshold to trunk stimulus was 54.2 ± 4.6 µA (range from 16 – 400 µA), which was significantly higher than the threshold for stimulation of the DNP (p<0.001). An example showing the response threshold for a typical MRF neuron for stimulation of the left L6/S1 trunk and left DNP in the same rat is presented in Figure 6. Similar to DNP/DNC, stimulation above threshold resulted in increased MRF neuronal firing, less wind-up, and longer after-discharges. Maximal frequency reached when the neuronal response (impulses per sec) would plateau was 30 Hz, although response thresholds occurred with settings as low as 10 Hz. MRF response latencies to L6/S1 trunk neurostimulation were very short (< 20 msec) for 23% of the neurons tested (no significant difference with respect to DNP, p>0.05, in terms of sub-region location). The longer latency responses (77% of those tested) had a mean latency of 229.0 ± 18.8 msec, with a range between 54 and 732 msec (no significant difference with respect to DNP, p>0.05). Similar to DNP neurostimulation (first group of 12 male rats), the majority of trunk-responsive MRF neurons were excitatory (73.0%), whereas 20.0% were inhibitory. The remaining 7% were characterized as "mixed". Also, a total of 48.6% of the trunk-responsive MRF neurons had background activity. The mean activity of those neurons was 11.1 ± 1.4 impulses per second. Because the L6/S1 trunk is a mixture of afferent and efferent projections, muscle contractions were observed with stimulation. The mean threshold for muscle contraction in the perineum/scrotum/hindquarter region with L6/S1 trunk
stimulation was 36.8 ± 6.6 µA (range from 6 to 80 µA; n=12), suggesting that stimulation intensity was rarely, if ever, greater than 2X motor threshold.

Effects of DNP stimulation on voiding

Voiding was evaluated with and without neurostimulation using 5 adult male urethane-anesthetized Wistar rats. The mean bladder pressure at onset of voiding was 25.4 ± 2.7 mmHg. Filling/voiding cycles were lengthened with DNP neurostimulation at amplitude of 11.0 ± 0.9 µA, with a maximal effective frequency of 30 Hz. During stimulation at or just above threshold, bladder pressures remained elevated (near void pressure) with slow increases then slight decreases with the release of one to two drops of urine. An example is provided in Figure 7. The fill/void cycle interval would then return to pre-stimulation patterns at about 2 to 3 minutes post-stimulus offset (at stimulus threshold; larger effects were seen with supra-threshold levels of stimulation, data not shown).
**MRF neuronal responses to bladder filling**

Centrally, many DNP/DNC-responsive MRF neurons responded with either excitation or inhibition to bladder filling (35.0% DNP and 68.3% DNC) at proportions consistent with our previous experiments in male rats (51.1%; not significantly different from either value) (Kaddumi and Hubscher 2007; 2006). A typical example from a male rat is provided in Figure 8. Evaluation of almost all neurons tested in 12 female rats (n=60) revealed that the MRF responses to bladder filling occurred either just before (43%) or right at (57%) the initiation of the voiding reflex. In addition, most bladder-responsive neurons in the DPGi sub-region (94%) responded at the time of voiding, whereas those in the Gi sub-region were a mix of just before or at void response times, adding to the complexity of the central neural circuitries for the control of micturition. Note that an example of a before void response is shown in Figure 2.

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**Histology**

Neurons from all 36 rats combined (236 in total) were distributed across various MRF sub-regions, the majority of which were located within the Gi (n=171; 72.5%). Significant differences (p<0.05) based on location were found related to gender for the DPGi sub-region and for GiA for DNP neurostimulation (Table 1), which could reflect differences in neurostimulation activation thresholds and/or differences in circuitries.
DISCUSSION

Summary

The results of this study demonstrate that many MRF neurons are responsive to electrical stimulation of several nerves innervating the pelvic region. This was true targeting a distal nerve branch in both males (DNP stimulation) and females (DNC stimulation) as well as a more proximal nerve target (L6/S1 trunk). At all stimulation sites, the majority of MRF neuronal responses were excitatory, though both inhibitory and ‘mixed’ cellular responses were also seen. Stimulation amplitudes to evoke MRF responses differed across nerve targets and gender (L6/S1 trunk >> DNP > DNC) and also showed some variation across different sub-regions within the MRF. Stimulation frequencies to induce MRF responses were similar at all sites and generally plateaued (e.g. maximum response) at 30-40 Hz. This frequency (30 Hz) also effectively increased voiding cycles during filling and many MRF neurons altered their firing near or at the time of voiding during bladder filling experiments.
MRF responses to neurostimulation at DNP, DNC and L6/S1

MRF neuronal response properties to DNP and L6/S1 trunk neurostimulation were excitatory (majority; firing at least twice the threshold relative to background), inhibitory (firing at a rate at least half of background) and mixed (responded with excitation or inhibition, depending on the convergent territory being stimulated).

Gender differences in the MRF responses to stimulation of the sensory branch of the pudendal nerve (DNP/DNC) were found, likely reflecting differences in their respective roles for mating and reproduction. There were several differences between male and female rat MRF responsiveness to neurostimulation, including the amplitude threshold, response latency, and cellular properties (ratio of excitatory to inhibitory to mixed), which likely reflects the composition of afferent types in the nerves relating to their roles in various aspects of urogenital functions. The lower amplitude threshold for DNC stimulation could also relate to the presence of ovarian steroids (low serum concentrations present as female rats were not tested if they were in or entering the proestrus stage of their cycle). In previous studies using female rats, variations in neuronal responses to pelvic reproductive organ stimulation was found to vary with stage of estrus as a consequence of levels of estradiol and not progesterone. These findings were demonstrated for neurons present at different regions of the neural axis, including the preoptic area of the hypothalamus and bed nucleus of the stria terminalis (Chadha and Hubscher 2008), the thalamus (Reed et al. 2009), and the medullary reticular formation (Hubscher 2006). Responses to cervix stimulation at low pressures were consistent with low and not high serum levels of ovarian hormones in the present study (Chadha and Hubscher 2008; Hubscher 2006; Reed et al. 2009).
The location of the electrodes revealed a significantly lower MRF response threshold for stimulation of the DNP relative to the L6/S1 trunk, a finding consistent with previous cat studies (Snellings and Grill 2012) and a likely consequence of directly stimulating the appropriate axons and also avoiding unwanted muscle contractions that would occur with the higher stimulus amplitudes that would be needed for activation of the L6/S1 trunk.

MRF responses to bladder filling and voiding

The present data revealed that many MRF neurons respond to bladder infusion. Two distinct responses were seen in MRF, one involving responses prior to voiding and the other at the time of void. These two types of responses could participate in multiple aspects of eliminative functions including continence and micturition. For example, the reciprocal activation and inhibition may result in the rhythmic activity such as fill and void cycle of the bladder. A previous study in cat demonstrated two ascending and two descending pathways for micturition, with direct projections between the pontine micturition center and spinal cord and indirect ones via the MRF (Sugaya et al. 2003). They demonstrated MRF neurons involved in storage and voiding of urine (Sugaya et al. 2003), findings consistent with the current study in rats as well as previous work which also demonstrated neurons in the ventromedial medulla responding before and during micturition (Baez et al. 2005). Those responses of MRF neurons just prior to voiding have been related to a preparatory role for micturition, as demonstrated with a variety of noxious stimuli that delay the onset of voiding (Baez et al. 2005). Given the
established role of MRF neurons in sexual function, it is possible that MRF neurons may also participate in the cross-inhibition of different behaviors (e.g. urination is inhibited during sexual activity).

DNP stimulation effects on voiding

DNP stimulation increased the voiding cycle during filling at relatively low amplitudes and frequencies, with a lasting after-effect of two minutes on average post-stimulation. This finding is consistent with clinical neuromodulation studies showing that conditional and continuous DNP stimulation significantly increases bladder capacity (Kirkham et al. 2001). The stimulus parameters to evoke the response are also consistent with animal studies showing low frequency DNP stimulation inhibits bladder contractions (Su et al. 2012a; Woock et al. 2008). Studies in adult cats also indicate that optimal inhibition of bladder contractions have a wider effective range of frequencies and amplitudes via DNP stimulation than other targets (pudendal, S1) (Snellings and Grill 2012).

In contrast, stimulation of DNP afferents in cats has also been shown to promote bladder contractions. In multiple studies, stimulation frequencies in the 20-40 Hz range evoked bladder contractions, but even lower frequencies (10 Hz) could promote contractions, which was hypothesized to be related to stimulation of multiple pudendal branches and/or afferent fiber diameter (Boggs et al. 2005; Snellings and Grill 2012; Yoo and Grill 2007). Note that pudendal sensory afferent discharges with similar ranges of frequencies have been shown to be related to rate of fluid flow along the urethra.
The above data are also consistent with the low amplitude stimuli needed in cat experiments where direct lower lumbar dorsal root ganglion neuron stimulation in the 5 to 20 µA range evokes responses in the hind limb region of primary somatosensory cortex (Hokanson et al. 2011).

The DNP contains a mix of afferent fiber types (Johnson and Halata 1991) that innervate the skin of the glans penis and penile shaft (involved in sensory aspects related to erection, ejaculation and orgasm) and those innervating the urethra (involved in the expulsion of seminal fluid) and project to a functionally heterogeneous population of MRF neurons. DNP innervation of the urethra, shown in both rats and humans (Johnson and Halata 1991; Yang and Bradley 1998a; b) could be responsible for the inhibition of the release of urine during and immediately after stimulation in coordination with simultaneous activation of the circuitries mediating erection/ejaculation. It is conceivable that different MRF sub-regions receive inputs from different regions of the penis which are part of neural circuitries that mediate a variety functions related to urogenital function.

**Convergence of urinary and genital neural circuitries**

The study results demonstrate significant overlap in the neurostimulation parameters (amplitudes and frequencies) for producing both an increase in the interval of voiding during filling and for evoking or altering neuronal responses of MRF neurons in the rostral medulla, a site known to be involved in the control of sexual function (Marson and McKenna 1990). Previous studies examining neurostimulation for altering
urinary physiology have demonstrated a U-shaped inhibitory curve with maximal effects at frequencies near 10 Hz (Snellings and Grill 2012; Su et al. 2012a; b; Woock et al. 2008). Interestingly, the present results with neurostimulation effects on MRF neuronal responses showed a plateau, or sigmoidal, effect with respect to frequency. That is, while most MRF neuronal responses were maximal by 30-40 Hz, frequencies as high as 90 Hz continued to evoke maximal responses. These data suggest some ability to separate effects on different physiological systems based on the electrical stimulation parameters employed, but also show overlap potentially due to the convergence of urogenital inputs centrally.

Neural circuitries related to both bladder and sexual functions converge at the MRF. This convergence, along with different MRF neuronal responses to urinary and sexual inputs also creates the potential for cross-organ sensitization centrally. Cross-talk between organs is an area of research that has been receiving increasing attention as it likely explains why some individuals with irritation in one pelvic/visceral organ experience referred pain or altered sensations in unaffected viscera (Alagiri et al. 1997; Dmitrieva and Berkley 2002; Malykhina et al. 2006; Pezzone et al. 2005; Qin et al. 2005; Theoharides et al. 2008; Winnard et al. 2006). MRF neurons are well placed to be a potential site for central cross-organ sensitization. The MRF is also known to play a more general role in the neural circuitries mediating arousal and attention, which could also explain the high degree of somato-visceral and viscero-visceral convergence in this brain stem region (Mason 2001; Hubscher 2006; Hubscher and Johnson 1996; Kaddumi and Hubscher 2006).
Acknowledgements: The authors thank James Armstrong and Jason Fell for excellent technical assistance. This study was carried out at the University of Louisville and funded by a grant from Medtronic Inc.

FIGURE LEGENDS

Figure 1. Recording in the nucleus reticularis gigantocellularis illustrating the typical response threshold (above 14 µA but below 18 µA) for stimulation of the dorsal nerve of the penis. Each arrow represents the onset of a single stimulus train (100 msec duration). Note the wind-up effect for the response to the near threshold stimulus (overall mean 15 µA) relative to the supra-threshold stimulus (30 µA), which has a much longer after-discharge (last up to several minutes - (Hubscher and Johnson 1996)).

Figure 2. Single neuron recording in the nucleus reticularis gigantocellularis (Gi) illustrating a much lower DNC response threshold for neurostimulation of the equivalent nerve tested in male rats (DNP neurostimulation in Figure 1). This neuron responded to inserting a glass probe into the vaginal canal (equivalent to a mating stimulus) as well as gentle pressure on the cervix (noxious stimulus). This particular neuron responded to bladder distention as well.

Figure 3. Single neuron recording in the dorsal paragigantocellularis nucleus (DPGi) illustrating a lower DNC response threshold (6 µA) for neurostimulation relative to the Gi
(re Figure 2). This recording also provides an example of an inhibitory response to neurostimulation in MRF. Note that the inhibitory response to stimulation at 2X threshold (first 12 µA stimulus relative to 6 µA) and with wind-up (second 12 µA stimulus relative to first one) was more complete. This neuron also responded to gentle pressure on the cervix (noxious stimulus) but not to bladder distention.

**Figure 4.** Typical responses of a single nucleus reticularis gigantocellularis (Gi) neuron (illustrated in the recording provided in A; 2 second epoch) to varying frequencies of neurostimulation (illustrated in the histogram provided in B) - a supra-threshold level of stimulation of the dorsal nerve of the clitoris (20 µA) was used. Note that the frequency threshold is 20 Hz, with a maximal plateau being reached beginning at a frequency of 40 Hz. Each frequency was tested four times.

**Figure 5.** Responses of a single nucleus reticularis gigantocellularis (Gi) neuron (illustrated in the recording provided in A; 2 second epoch) to varying frequencies of neurostimulation (illustrated in the histogram provided in B) - a supra-threshold level of stimulation of the dorsal nerve of the penis (30 µA) was used. Note that the frequency threshold is 10 Hz, with a maximal plateau reached beginning at a frequency of 30 Hz, although only about 13% of the neurons respond to frequencies of 10 Hz (20 Hz more typical, as shown in the example obtained from a female rat in Figure 4). Each frequency was tested twice.

**Figure 6.** Recording of a single neuron in the nucleus reticularis gigantocellularis illustrating the response threshold difference between two different stimulation sites, the dorsal nerve of the penis and the L6-S1 trunk. Large stimulus artifacts can be seen in
both recordings. Note the slightly longer response latency for the L6-S1 trunk relative to stimulus onset and first action potential spike.

**Figure 7.** Bladder pressure recording (0.25 ml/min infusion rate; raw record shown) in a urethane-anesthetized male rat illustrating the effect of bilateral DNP neurostimulation on voiding. Pressures ranged from a baseline of 10 mmHg (13.6 cmH₂O) to a peak of approximately 35 mmHg (47.6 cmH₂O). Note the slight rise in pressure at the offset of the 3 min DNP stimulus. Single drops of urine would release every 20 to 30 seconds during the stimulation as well as after it until voiding resumed (between 2 to 3 min after stimulus offset for stimulation intensities at the response threshold). The interruption in the voiding cycle in the example shown lasted 38 min until voiding cycles resumed to the pre-stimulation pattern (supra-threshold intensity of 20 µA at a frequency of 30 Hz was used for the example shown).

**Figure 8.** Typical medullary reticular formation neuronal recording illustrating the onset of an excitatory response at the time of voiding. Note that the bladder was not emptied prior to infusion of saline (thus a relatively short response time).


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Medullary Reticular Formation Single Neuron Recording: 
bDNP stimulation at different intensities

14 μA

18 μA

30 μA
Medullary Reticular Formation Single Neuron Recording
During Infusion of Saline into the Urinary Bladder

Begin to fill  VOID  1 sec
Table 1: Summary of responsive MRF neurons by sub-region locations based upon electrode track reconstructions.

<table>
<thead>
<tr>
<th>Neurostimulation Site</th>
<th># of Units</th>
<th>Units per track</th>
<th>% DPGi</th>
<th>% Gi</th>
<th>% GiA</th>
<th>% IRt</th>
</tr>
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<tbody>
<tr>
<td>DNP</td>
<td>102</td>
<td>2.9</td>
<td>14.7</td>
<td>71.6</td>
<td>7.8*</td>
<td>5.9</td>
</tr>
<tr>
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<td>29.7*</td>
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<td>3.1</td>
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<tr>
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<td>15.7</td>
<td>78.6</td>
<td>0</td>
<td>5.7</td>
</tr>
</tbody>
</table>

*significantly different from other groups (p < 0.05)